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High throughput method for extracting polyphosphates from diatoms

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Polyphosphates (polyP) are widely distributed in living organisms and have crucial cellular functions. Little is known about the physiological roles of polyP in algae, especially in diatoms. Therefore, we have developed a standardized method for polyP extraction and quantification from diatoms, using the freshwater model organism *A. minutissimum* and further marine diatoms to validate the method. Investigating the efficiency of polyP extraction methods, we found that a commercial DNA isolation kit yielded best results. The method is based on separation of polyP from lower molecular weight compounds using gel filtration spin columns. Moreover, we demonstrate that self-made gel filtration spin columns and extraction buffers can also extract polyP from *A. minutissimum* efficiently. Under defined conditions, samples spiked with polyP of chain-lengths between 45 to 700 P_i moieties consistently resulted in a recovery of more than 70% of the initially loaded polyP. PolyP was quantified with an ascorbate-antimony-molybdate assay that detects phosphates (P_i) by hydrolysis of a specific exopolyphosphatase (PPX). The method detects as little as 3 µM polyP in high throughput analyses using 96-well plates. This sensitive, rapid and straightforward method is ideal to characterize the physiological role of polyP in diatoms.

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Introduction

Polyphosphates (polyP) are a naturally occurring polymers consisting of three to several hundreds of phosphate units (P_i) condensed by high-energy phosphoanhydride bonds. In eukaryotic microalgae and cyanobacteria, polyP play an important role in a number of physiological processes, including acclimation to nutrient deprivation (Falkner and Falkner 2011), DNA synthesis (Murata et al. 2016), heat stress (Barcytė et al. 2020), metal ion sequestration (Sicko-Goad and Lazinsky 1986), and osmotic stress (Leitão et al. 1995). PolyP recently received attention because of their implication for biogeochemical cycling in marine ecosystems (Orchard et al. 2010; Martin et al. 2018; Martin et al. 2014).

Here, we describe an efficient extraction of polyP from the diatom *Achnanthidium minutissimum* (Round 2004; Hlúbiková et al. 2011; Potapova and Hamilton 2007), and compare it to published methods for polyP extraction. Available protocols for polyP analyses in biological samples follow in principle similar workflows, comprising extraction and quantification. PolyP quantification based on the fluorescence of 4',6-diamidino-2-phenylindole (DAPI)-polyP interaction was developed by Aschar-Sobbi et al. (2008), and revised by Martin and Van Mooy (2013). The latter protocol includes a brief boiling step of the samples in order to inactivate enzymes, followed by protein digestion with proteinase K. This method has often been used for extraction and quantification of polyP from cyanobacteria and marine environmental samples (Li et al. 2019; Martin et al. 2014; Zhang et al. 2015). Although being sensitive (detection limit < 1 µM), it requires multiple extractions, and interference of DAPI with nucleic acids may be problematic (Omelon et al. 2016). Other quantification procedures have been designed for polyP analyses in *Saccharomyces cerevisiae* (Bru et al. 2016; Christ and Blank 2018a) and bacteria (Pokhrel et al. 2019), including enzymatic hydrolysis of polyP with the exopolyphosphatase enzyme (PPX), followed by colorimetric quantification of the released P_i. In this study, we developed a straightforward high throughput method for polyP quantification from diatoms combining and optimizing existing polyP quantification steps.

Materials and Methods

Cultivation conditions

The freshwater diatom *A. minutissimum* (Kützing) Czarnecki (strain MW1), was isolated from photoautotrophic, epilithic biofilms in Lake Constance (47°41´N; 9°11`E, Germany) and purified from associated bacteria using the antibiotic imipenem (Windler et al. 2012). The diatoms were cultivated at 20°C, under a 16:8 light:dark cycle with a light intensity of 70 µmol m^{-2} s^{-1} and with constant shaking at 130 rpm. Diatoms were cultivated in "*Achnanthidium* medium" ((AM, (Windler 2014)), a modified, 'basal' version of "Bacillariophycean medium" (BM), (Schlösser 1994) but without soil extract. Vitamins, silica/selenate, and trace metal ion solution were supplemented as described for F/2 medium (Guillard 1975). *A. minutissimum* was cultivated in P_i-replete AM (55 µM P_i as K_2HPO_4). Marine diatoms were cultivated in artificial seawater medium (ASW) (Tropic Marin), supplemented with Guillard's F/2 (Sigma-Aldrich) with modifications (Guillard 1975). *Phaeodactylum tricornutum* (CCMP 2561) was cultivated in half salt ASW (16 g/l NaCl), without silica and Tris, and adjusted to pH 7.0. *Thalassossira pseudonana* (CCMP 1335) was cultivated in full salt ASW (33 g/l NaCl) with addition of silica ($Na_2Si_iO_3 \cdot 9H_2O$) but without Tris, and adjusted to pH 7.0. The two marine diatoms were incubated at 20°C with a 12:12 h light/dark regime, with a light intensity of 70 µmol $m^{-2}s^{-1}$ and with constant shaking at 130 rpm.

Extraction and purification of polyp

A. minutissimum was cultivated in 2 l AM and the cultures were weekly diluted with fresh AM to keep the culture in the log phase (ca. $1 \cdot 10^6$ cells/ml). Marine diatoms were cultivated in 100 ml of the respective medium, and kept in log phase. For harvesting, aliquots of 30 ml were centrifuged (5000 g, 5 min), and the supernatant was discarded. Cells were subsequently resuspended with 1 ml of fresh AM and transferred to a 2 ml tube. After pelleting the cells (5000 g, 2 min), the supernatant was discarded, and the pellets were frozen in liquid nitrogen. Each tube contained ca. $3 \cdot 10^7$ cells, and was stored at -80°C.

All chemicals used were brought to room temperature prior to use unless stated otherwise. For the five different polyP extraction protocols, four replicates as well as the corresponding negative controls (blanks containing only the corresponding buffers) were utilized for each method (except for Method 4 where only three replicates were used).

***Method 1*: Extraction and purification of polyP from *A. minutissimum* with Nexttec DNA isolation kit**

The samples were resuspended in the lysis buffer containing 140 µl of buffer G and 10 µl of proteinase K, as described by the manufacturer (Biozym, Hessisch Oldendorf, Germany). Samples were incubated for 1 h at 56°C at 1200 rpm. After centrifugation (5 min, 18000 g), the supernatant was transferred to a pre-conditioned column provided in the kit, and left for 3 min at room temperature for elution. After centrifugation of the column (700 g, 1 min), the eluate containing polyP was stored at -80°C until polyP quantification.

***Method 2*: Extraction and purification of polyP from *A. minutissimum* with phenol/chloroform extraction and ethanol precipitation (Bru et al. 2016)**

Samples were resuspended in 400 µl AE buffer (50 mM sodium acetate, 10 mM Na_2EDTA, pH 5.3) at 4°C. Samples were mixed with 300 µl phenol and 40 µl 10% SDS four times by inversion, vortexed for 5 s, and incubated at 65°C for 5 min in a thermomixer. After cooling the samples to room temperature, 300 µl of chloroform were added and the sample mixed and vortexed as before. After centrifugation (13000 g, 2 min), the aqueous phase was transferred into a new tube containing 350 µl chloroform. After mixing and vortexing, the samples were centrifuged again, and the resulting upper aqueous phase transferred into a new tube. 2 µl of 10 mg/ml RNAse (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and DNAse (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were added, and the samples were incubated for 1 h at 37°C. The mixture was transferred to pre-cooled tubes (-20°C) containing 1 ml of absolute ethanol and 40 µl of 3 M sodium acetate (pH 5.3), and incubated for 3 h at -20°C to precipitate the extracted polyP. After centrifugation (13000 g, 20 min, 4°C), the supernatant was discarded and 500 µl of 70% ethanol were added. Samples were centrifuged for 5 min, and the supernatant discarded. After a final centrifugation step for 1 min at 13000 g, the last drops of ethanol in the tube were removed with a pipette. The pellet was left for 10 min under a gentle air flow to evaporate the residual ethanol. The resulting white pellet (polyP) was dissolved in 400 µl of ddH_2O water and stored at -80°C until polyP quantification.

***Method 3*: Extraction and purification of polyP from *A. minutissimum* with phenol/chloroform but without ethanol precipitation (Christ and Blank 2018a)**

Samples were resuspended in 400 µl of sterile filtered (0.2 µm) ME-buffer (25 mM MOPS, 2.5 mM Na_2EDTA, pH 7.0). After 20 s of vortexing, the resuspended cells were briefly centrifuged (5 s, 1000 g) to remove drops of sample from the lid and 300 µl of phenol (Carl Roth GmbH, Karlsruhe, Germany) were added. After 20 s of vortexing, samples were incubated (45°C, 10 min). After a brief centrifugation (1000 g, 5 s), 1 ml of chloroform were added to each sample followed by 20 s of vortexing. The tubes were briefly centrifuged, and their lids were filled with autoclaved silicone vacuum grease (Beckman instruments, Inc. Palo Alto, USA), which acts as an aqueous/organic phase barrier. After centrifugation (18000 g, 5 min), the aqueous phase above the silicone vacuum grease containing polyP was

entirely transferred to another tube and kept at -80°C until polyP quantification.

***Method 4*: Extraction and purification of polyP from *A. minutissimum* with boiling and nuclease/proteinase K treatment, modified from Martin and Van Mooy (2013)**

Samples were sonicated for 15 s in 500 µl of Tris buffer (20 mM, pH 7.0), boiled for 20 min in a thermomixer (100°C, no shaking) and incubated for 2 h with 40 µl of 20 mg/ml of proteinase K (37°C, 300 rpm). Because we did not quantify the extracted polyP by DAPI staining, we omitted nuclease treatment to reduce the fluorescence of DAPI as recommended by the authors. Samples were centrifuged (16100 g, 1 min), and the supernatant was collected and stored at -80°C until polyP quantification.

***Method 5*: Extraction and purification of polyP from *A. minutissimum* with guanidine isothiocyanate followed by column purification (Pokhrel et al. 2019)**

Samples were resuspended in 250 µl GITC (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) (guanidine isothiocyanate, chaotropic salt) lysis buffer (4 M GITC, 50 mM Tris-HCl, pH 7.0) and incubated for 10 min in 95°C. After cooling to room temperature, 250 µl of 95% ethanol were added, and the samples were vortexed for 10 s. The resulting mixture was transferred into a NucleoSpin silica column (Macherey-Nagel, Düren, Germany), and centrifuged (30 s, 16100 g). After discarding the flow-through, the column was rinsed with 750 µl of a mixture containing Tris-HCl (5 mM, pH 7.5), NaCl (50 mM), EDTA (5 mM), 50% ethanol, and centrifuged (30 s, 16100 g). The flow-through was discarded and 150 µl of Tris-HCl (50 mM, pH 8.0) was added in the column. After five min of incubation the column was centrifuged (2 min, 8000 g) and the eluate was kept at -80°C until polyP quantification.

PolyP quantification

PolyP quantification was performed in a 96-well plate following the protocol developed by Christ and Blank (2018b) with modifications. The purified polyP samples and blanks were diluted with ddH_2O prior to quantification to yield a final polyP concentration in the well ranging between 3 to 100 µM P_i (see below). Quantification was performed by enzymatic hydrolysis of polyP with the recombinant *Escherichia coli* exopolyphosphatase (PPX, MyBioSource, Inc., San Diego, USA). As the polyP chains in diatoms did not contain short chain-length of two to four P_i moieties (polyP-2 to polyP-4), we omitted the use of the pyrophosphatase described in Christ and Blank (2018b). Briefly, 100 µl of a diluted polyP extract were pipetted into two different wells of a 96-well plate. In the first well, a volume of 50 µl of enzyme reaction buffer (magnesium acetate 15 mM, Tris 60 mM, ammonium acetate 150 mM, pH 7.7) was added. In the second well, 49 µl of enzyme reaction buffer containing 1 µl PPX (1 mg/ml) of PPX was pipetted. Elsewhere in the microplate, 100 µl Pi standard (K_2HPO_4) (0 to 100 µM) and 50 µl of enzyme reaction buffer were added in triplicate. The microplate was incubated at 37°C for 1 h in a plate reader (Infinite® 200 PRO, TECAN). After incubation, P_i concentrations were measured using the ascorbate-antimony-molybdate assay described by Christ and Blank (2018b). Briefly, 50 µl of P_i detecting reagent containing ammonium heptamolybdate (2.4 mM), H_2SO_4 (600 mM), antimony potassium tartrate (0.6 mM) and ascorbic acid (88 mM) were added to the wells. After 2 min of incubation at room temperature and gentle shaking of the microplate, the absorbance of the samples was determined at 882 nm in the microplate reader. The lowest amount of polyP that could be determined with suitable precision and accuracy was given by the limit of quantitation (LOQ) and was calculated according to the Equation 1 (σ is the standard deviation of the 0 µM P_i standard value given in µM, and S the value of the slope of the P_i standard curve where an example is given in Figure S1) (ICH Guideline, 1996):

$$LOQ = (10 * \sigma)/S \qquad (1)$$

For every microplate measured, a calibration curve was performed as indicated above, and the calculations given in Equation 1 lead to an LOQ value of ca. 3 µM P_i. Therefore, we only considered polyP concentrations detected between LOQ and the upper limit of detection (100 µM P_i). The polyP content in each cell was calculated using Equation 2:

$$PolyP\ amount\ (fg\ Pi.cell^{-1}) = \frac{polyP\ sample\ (M) * dilutions * volume\ cell\ extract\ (L) * M_{Pi}\ (g.mol^{-1}) * 10^{15}}{\text{cell number}} \qquad (2)$$

The absorbance of each well was corrected by subtracting the value by the mean value of the blank wells containing 0 µM P_i standard. For each replicate, the polyP content was calculated by subtracting the amount of P_i determined in the sample treated with PPX by the amount of P_i determined in the well without PPX and referred to as "polyP sample" in Equation 2. PolyP was specified as P_i equivalent with a molecular weight of: MP_i 94.97 g mol^{-1}. For each different diatom culture a pre-assay was performed using only one sample to determine the correct dilution to apply on the cell extract, in order to achieve a final P_i concentration between 3 to 100 µM. Naturally, this dilution step was omitted for sample with P_i concentrations < 6 µM, which would result in a concentration lower than the LOQ after the two-fold dilution occurring in the well. For example, a polyP extract was first diluted five-fold with ddH_2O. It was subsequently diluted by two-fold in the wells at the end of the assay and the resulting value in "dilutions" in Equation 2 was 10.

Determination of polyP recovery

The efficiency of polyP extraction was determined for defined amounts (8 and 30 nmol) of standard polyP differing

in their number of phosphate units, (polyP-6, polyP-45, Sigma-Aldrich), (polyP-14, polyP-60, polyP-130, RegeneTiss Incorporated) and (polyP-700, Kerafast). PolyP standards were dissolved in ddH_2O. For each polyP standard of either 8 or 30 nmol, diatom samples were resuspended in 200 µl ddH_2O and divided into two subsamples of 100 µl each. One subsample was spiked with 50 µl of polyP standard to reach a final concentration of either 8 or 30 nmol and was called spiked sample. The other subsample was diluted with the same volume of ddH_2O and was called unspiked sample. PolyP in spiked and unspiked samples were identically extracted and purified using Method 1 (see Material and Methods), and the recovery of each standard was quantified after 1 h of incubation with PPX using the Equation 3 (C is the polyP concentration of the different samples in fg P_i/cell calculated using Equation 2):

$$PolyP\ recovery\ (\%) = ((C_{spiked\ sample} - C_{un-spiked\ sample}) * 100))/C_{added} \quad (3)$$

Common lysis buffers and self-made gel filtration columns preparation

The composition of the lysis buffer of the Nexttec kit and the nature of its spin column resin is not publicly available. Thus, we tried different size exclusion resins and prepared two lysis buffers containing two different common detergents and proteinase K. The first buffer contained sodium dodecyl sulfate (SDS) (SDS 0.02% w/v, proteinase K, 0.4 mg/ml, 30 mM Tris-HCl, pH 8.0), while the second contained Triton X-100 (Triton X-100 0.001% w/v, proteinase K 0.4 mg/ml, 30 mM Tris-HCl, pH 8.0). The concentrations of the detergents were equal to the tenth of their critical micellar concentration (CMC) (Singh and Tyagi, 2015), to avoid any micelles to pass through the gel filtration column. We observed that micelles produced by higher concentrations of detergents could interfere with PPX at concentration > CMC (data not shown). To prepare the size exclusion columns, we disassembled all the components (collection tube, plastic ring, membrane discs) (Figure S2B) of commercial spin columns (GENECLEAN® Turbo Cartridges, MP Biomedicals Germany GmbH) (Figure S2A), leaving only the lower filter disc to support the gel filtration matrix (Figure S2C). The column and its collection tube were washed in 0.1 M NaOH for 5 min, three times with sterile ddH_2O, and dried under a gentle air flow. We prepared three different columns using Bio-Gel P-6 (Biorad), LH-20 (Cytiva), and Sephadex® G-50 (Merck) size exclusion resins. 500 µl of an overnight swollen matrix in Tris-HCl buffer (30 mM, pH 8.0) were transferred into the cleaned spin columns and the excess buffer was removed by centrifugation (350 g, 1 min). The process was repeated several times until the bed height reached 1 cm, which corresponded to ca. 0.4 g of the swollen resins after removing the excess buffer (Figure S2D). The spin columns were preconditioned with 350 µl of Tris-HCl buffer (30 mM, pH 8.0) and stored at 4°C until further use. For polyP extraction, 150 µl of the two different lysis buffers containing SDS and Triton X-100 were used to resuspend the diatom samples. In parallel, other diatom samples were resuspended with the Nexttec DNA isolation kit lysis buffer following Method 1 (see Material and Methods). All the samples were incubated for cell lysis in the same manner as for Method 1 (1 h, 56°C 1200 rpm) (see Materials and Methods). Subsequently, lysed cells were centrifuged (5 min, 18000 g), each supernatant containing the cell extract was transferred in another tube, and stored at 4°C before use. Before applying the cell extracts onto the different columns, a Nexttec DNA isolation kit column was conditioned as described in Method 1. In parallel, the different self-made gel filtration columns were centrifuged (1 min, 350 g) and the eluate was discarded. Carefully, 100 µl of the extracts obtained after lysis of cells using SDS buffer were applied into the middle of each column. After 3 min of incubation, the columns were centrifuged (700 g, 1 min) and the eluate of each column containing the polyP was transferred into a 1.5 ml tube and kept at -80°C until quantification. This process was repeated for the other extracts obtained after using Triton X-100 and the Nexttec DNA isolation kit lysis buffers. Before adding the samples, the four different columns were carefully washed three times with 350 µl of 0.1 M NaOH and then equilibrated three times with 350 µl of Tris-HCl buffer (30 mM, pH 8.0).

Statistical analysis and diagrams

Statistical analyses were performed using the analyzing tools in GraphPad Prism software. Diagrams were drawn using Biorender.com

Results

Comparison of different polyP extraction methods in *A. minutissimum*

In order to develop a polyP quantification protocol optimized for diatoms, we compared several published polyP extraction protocols (Bru et al. 2016; Martin and Van Mooy 2013; Christ and Blank 2018a; Pokhrel et al. 2019) and a new approach using a commercial DNA isolation kit based on size exclusion. PolyP extracted from *A. minutissimum* using the different methods were all hydrolyzed by PPX, and the released P_i was quantified by the same colorimetric assay (Figure 1).

Extractions using the DNA kit as well as the phenol/chloroform approach with ethanol precipitation yielded the highest amounts of polyP corresponding to 531 ± 59 and 479 ± 47 fg P_i/cell, respectively. Although the Nexttec DNA isolation kit extracted 10% more polyP than the phenol/chloroform extraction from Bru et al. (2016), there was no significant difference between the two extraction steps ($p > 0.05$). The subsequent purification of the extract by ethanol precipitation seems to be critical for extracting polyP from the diatom, as the phenol/chloroform extraction without ethanol precipitation by Christ and Blank

(2018a) extracted five times less polyP than the method from Bru et al. (2016) with 103 ± 18 fg P_i/cell. Regarding improvement of the protocol by Martin and Van Mooy (2013), we tested, whether only one extraction using Tris-boiling may be sufficient to obtain the entire polyP from the cells if boiling time, proteinase K concentration and incubation time were increased. However, the respective yield in this case was only slightly higher (192 ± 9 fg P_i/cell), but 40% less efficient than the size exclusion separation using DNA kit spin columns. Finally, extractions using GITC with silica spin column yielded the lowest amount of polyP corresponding to 89 ± 22 fg P_i/cell.

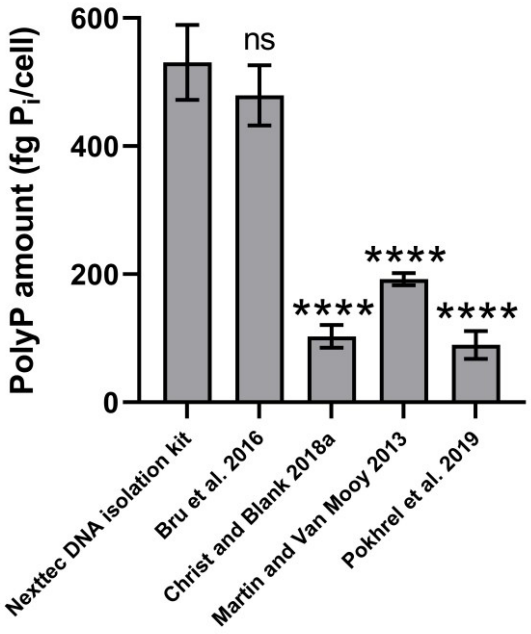

Figure 1: Comparison of the polyP extraction efficiencies from *A. minutissimum* using the following methods: Lysis buffer with proteinase K and purification by gel filtration spin column using the Nexttec DNA isolation kit, phenol/chloroform extraction and purification by ethanol precipitation (Bru et al. 2016), phenol/chloroform extraction without ethanol precipitation (Christ and Blank 2018a), boiling extraction and proteinase K treatment (Martin and Van Mooy 2013), extraction using guanidine isothiocyanate followed by silica spin column purification (Pohkrel et al. 2019). The amounts of polyP extracted from *A. minutissimum* are given in fg P_i/cell after enzymatic hydrolysis. Data represent means, and standard deviations are given as error bar of n = 4 replicates except for Martin and Van Mooy (2013) where n = 3. Asterisks indicate significant differences (one-way ANOVA, ****$p < 0.0001$) and ns indicates no significant difference of polyP levels.

PolyP extraction using common lysis buffers and self-made gel filtration columns

The Nexttec DNA isolation kit requires two steps, cell lysis using a specific lysis buffer (Nexttec buffer), followed by polyP purification using a gel filtration spin column (Nexttec column).

Therefore, we tested whether these steps are reproducible using commonly used lysis buffers and self-made gel filtration spin columns. We examined the efficiency of two lysis buffers combined with three different gel filtration columns for extracting polyP from *A. minutissimum*, and used the results obtained with the Nexttec DNA isolation kit approach as a reference (Figure 2). Using Triton X-100, SDS, or Nexttec buffers combined with the G-50 column resulted in 23, 38, and 42% lower polyP yields than obtained with the DNA isolation kit. However, a combination of Triton X-100 with either a P-6 or LH-20 column led to an increase of polyP yield by 25 and 22% respectively compared to the Nexttec DNA isolation kit. Yields were even about 31 and 34% higher when SDS was applied in combination with the same columns. These results confirm that quantification of polyP from the diatom works well with self-made gel filtration spin columns and buffers.

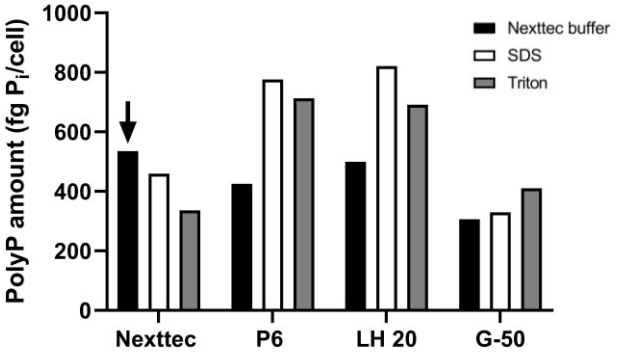

Figure 2: PolyP extraction efficiencies from *A. minutissimum* using a combination of different lysis buffers ((Nexttec DNA isolation kit (Nexttec buffer), 0.01% (w/v) sodium dodecyl sulfate (SDS) and 0.002% (w/v) Triton X-100 (Triton)) with three different self-made gel filtration columns (P-6, LH-20 and G-50) and one pre-packed column provided in the Nexttec DNA isolation kit (Nexttec). The black arrow represents the amount of polyP yielded after extracting and purifying a sample following the DNA kit isolation protocol. The amounts of polyP extracted from *A. minutissimum* are given as fg P_i/cell after enzymatic hydrolysis. Each bar represents the polyP amounts from one sample (n = 1) subjected to an extraction and purification using one of the three lysis buffers combined with one of the four columns.

Characterization of the polyP extraction and quantification parameters using the Nexttec DNA isolation kit approach

For the further optimization of the polyP analysis, we addressed the reproducibility of the Nexttec DNA isolation kit (Method 1). We first tested whether increasing the extraction time may influence polyP yields. *A. minutissimum* samples were subjected to both regular 1 h extractions as well as extended overnight extractions (15 h) (Figure 3A).

The 15 h extraction yielded less polyP (0.3 fg P_i/cell) compared to the 1 h extraction (Figure 3A), suggesting that a prolonged time at 56°C may promote polyP degradation.

Additionally, we tested whether further extractions of the cell pellet could enhance the extraction efficiency, however, after a second round of extraction, no additional polyP was detected (Figure 3B). In addition, an increase of the incubation time with PPX did not yield more P_i, indicating that incubation for 1 h with the described enzyme concentration is sufficient (Figure 3C). Moreover, with the aim to exclude other potential sources of hydrolyzed P_i, we tested whether potentially co-isolated adenosine 5'-triphosphate (ATP) or DNA could also be hydrolyzed by PPX. A direct incubation of 8 nmol ATP in presence of PPX resulted in very minor ATP hydrolysis, releasing 1.4 ± 0.1 nmol P_i within 1 h (Figure 3D). When ATP was subjected to Nexttec spin column purification, no P_i was released after its subsequent incubation with PPX, suggesting that ATP was retained within the column. Similarly, we tested whether 8 nmol of a DNA PCR fragment obtained from *A. minutissimum* could be hydrolyzed by PPX but no P_i was detected with or without column purification (data not shown).

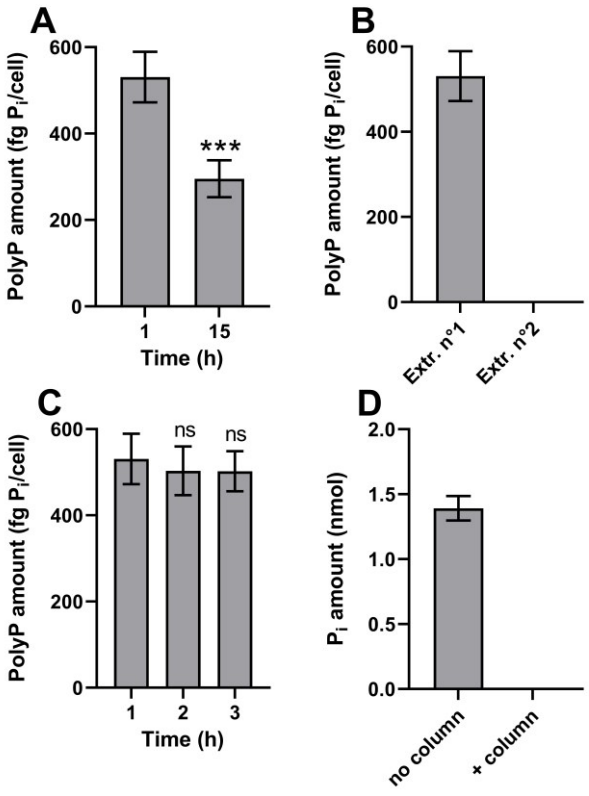

Figure 3: Characterization of the polyP extraction, purification, and quantification parameters using the Nexttec DNA isolation kit. The amounts of polyP extracted from *A. minutissimum* are given in fg P_i/cell after enzymatic hydrolysis. (A) Comparison of P_i yields after 1 or 15 h of incubation with the lysis buffer. (B) Yields after a first extraction of 1 h of the sample followed by a second extraction of 1 h using fresh lysis buffer. (C) Yields after 1 h of extraction, but with different times of enzymatic treatment. (D) Enzymatic hydrolysis of 8 nmol of adenosine 5'-triphosphate (ATP) without or with column purification. Data represent means, and standard deviations are given as error bar of n = 4 replicates. Asterisks indicate significant difference of polyP amounts compared to the reference yield indicated in the first column of A, B and C (unpaired t-test, ***$p < 0.001$; C, one-way ANOVA, ns: not significant).

Recovery of polyP after extraction and purification using the optimized Nexttec DNA isolation kit protocol

Because the Nexttec isolation method showed convincing results after about one hour of polyP extraction and purification and an additional hour of PPX treatment, we further challenged the accuracy of the extraction by supplementing the diatom samples with known amounts of polyP standards of different P_i unit lengths, preceding the extraction (Figure 4). After extraction and purification, we observed that 8 nmol of added standard consisting of polyP containing 130 and 700 P_i units (polyP-130 and polyP-700 respectively), were recovered at 67 ± 9% and 78 ± 4%, respectively, while the shorter standards polyP-6 and polyP-14 were recovered much less efficiently: 21 ± 7% and 55 ± 2%, respectively (Figure 4A). Chains of more than 14 P_i residues yield a minimum recovery of 60%. Moreover, the recovery depends on the initial amount of polyP. Increasing the concentration of polyP standard to 30 nmol, which corresponds to ca. 20% of the polyP found in diatom samples, resulted in different recovery rates of standards. PolyP ranging from 45 to 700 P_i units were recovered best with an average recovery of 77 ± 4%, while shorter standards like polyP-14 were recovered less well with 63 ± 2% recovery. The polyP-6 standard was recovered with only 27 ± 2% recovery (Figure 4B).

Scope of the newly established polyP extraction and purification method

Finally, we tested the optimized method on other representative diatoms. We chose two marine species, *P. tricornutum* and *T. pseudonana,* which are considered as model laboratory strains (Bowler et al. 2008; Armbrust et al. 2004). PolyP was found in all diatoms tested. *P. tricornutum* and *T. pseudonana* yielded 16 ± 5 and 70 ± 10 fg P_i/cell, respectively (Figure 5). With 531 ± 59 fg P_i/cell, polyP amounts in *A. minutissimum* were 33 times higher than in *P. tricornutum*.

Discussion

We developed a straightforward method for the extraction of polyP from diatoms. Several methods for polyP extraction and purification exist, in particular for microorganisms (Bru et al. 2016; Martin and Van Mooy 2013; Pokhrel et al. 2019). When studying the diatom *A. minutissimum*, comparison of different approaches indicated that extraction using either the Nexttec DNA isolation kit consisting of a lysis buffer containing proteinase K and gel filtration columns, as well as a phenol/chloroform extraction followed by ethanol precipitation gave the best yields of polyP. However, when comparing both approaches, polyP can be extracted five times faster using the Nexttec DNA isolation kit, thanks to less handling steps, and without

the use of hazardous solvents, such as phenol (Table. S1). Hence, this method is ideal for routine measurements of polyP.

A practical advantage of the polyP purification by gel filtration is its single elution step, which simplifies considerably the time and the handling process. In comparison, polyP purification by silica spin columns requires several elution steps (Ault-Riche et al. 1998; Lee et al. 2018), and polyP purification by ethanol precipitation takes at least 3 h to recover polyP (Bru et al. 2016). Gel filtration columns have the drawback to retain small molecular weight compounds such as short polyP, such as polyP-6, while long fragments ranging from polyP-14 to polyP-700 are directly eluted. However, because polyP from *A. minutissimum* range mostly between ca. 130 to 500 P_i units (Lapointe et al. unpublished), this is not problematic here. In contrast, silica matrices were reported to fail recovering polyP shorter than 60 P_i units (Ogawa et al. 2000), hindering a comprehensive analysis of organisms producing polyP ≤ 60 P_i units. Accordingly, gel filtration is the method of choice for recovering a wide range of polyP chains in a single step (Ohtomo and Saito 2005; Ohtomo and Saito 2008). Furthermore, we demonstrated that self-made spin columns (P-6 or LH-20 resin) in combination with

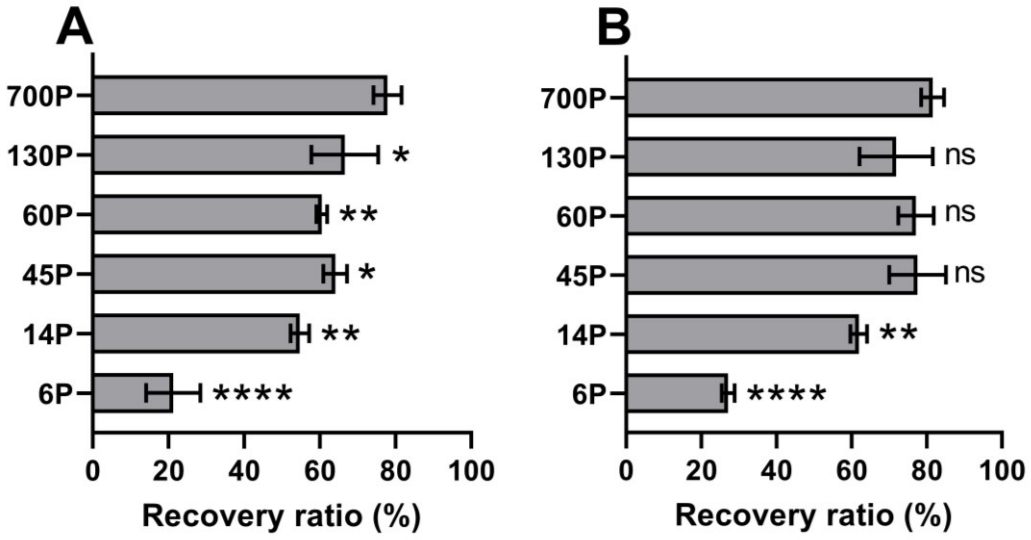

Figure 4: Recovery of polyP standards of different sizes (6-700 Pi units) using the Nexttec DNA isolation kit. The amounts of polyP extracted from *A. minutissimum* are given as fg P_i/cell after enzymatic hydrolysis. Prior to extraction, each diatom sample was split in two subsamples (equal volume). One subsample was supplemented with (A) 8 nmol (B) or 30 nmol of a polyp standard containing 6, 14, 45, 60, 130 or 700 P_i units. The other subsample was supplemented with ddH_2O. Both subsamples were extracted and purified using the Nexttec DNA isolation kit, and their polyP concentrations were determined. The recovery of the polyP standards was calculated using equation 3. Data represent means, and standard deviations are given as error bar of n = 4 replicates. Asterisks indicate a significant difference of polyP amounts compared to the amounts recovered for the standard of 700 P_i units. A and B, one-way ANOVA, *p < 0.05; **p < 0.01; ***p < 0.001; ns: not significant.

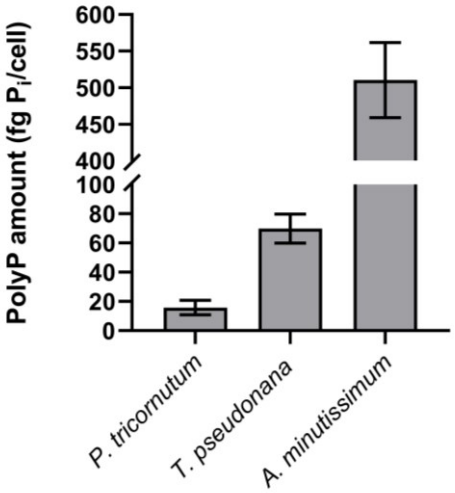

Figure 5: PolyP quantification using the optimized Nexttec DNA isolation kit protocol and performed on the marine algae *P. tricornutum*, *T. pseudonana*, as well as on the freshwater diatom *A. minutissimum*. Data represent means, standard deviations are given as error bar of n = 3 replicates.

common detergents, such as Triton X-100 or SDS lead to very good polyP extraction, indicating that polyP could be purified without using a commercial kit (Figure 6). As the purification by gel filtration removes low molecular weight compounds like salts or pigments, the colorless eluate can be used for spectroscopic analysis like the ascorbate-antimony-molybdate assay. In addition, the high purity of the sample is critical for polyP enzyme-based quantifications. Ohtomo and Saito (2005) used gel filtration resins (BioGel P-2 and P-6) with fractionation range of about polyP-20 to remove the urea contained in the extraction buffer from arbuscular mycorrhizal fungi polyP samples, allowing a comprehensive polyP quantification when using the polyphosphate kinase (PPK) (Ault-Riché et al. 1998).

Because gel filtration elutes other macromolecules along with polyP, quantification requires enzyme assays that are specific for polyP. While the DAPI staining has been found to interact with other molecules (Omelon et al. 2016), PPX (Werner et al. 2005; Bru et al. 2016) and PPK (Ault-Riché et al. 1998; Ohtomo and Saito 2005) assays allow a specific quantification of polyP. The polyP quantification using PPK converts polyP to ATP that is subsequently quantified by a luciferase assay (Ault-Riché et al. 1998). Although this PPK assay is very sensitive (picomolar range), the enzyme can only detect polyP with > 38 P_i units (Ault-Riché et al. 1998). Alternatively, PPX is well suited for short-chain polyP quantification, although it is less sensitive (> 3 µM). It cleaves down to polyP-3 (with ca. 50% efficiency) (Ohtomo and Saito 2008), enabling the quantification of a wider polyp range than PPK. Using our current column gel filtration, PPX can only detect chain-length ≥ 14 P_i units, which is still better than the PPK chain-length detection limit. Although there is a functional interaction between linear DNA and PPX during quantification (Bru et al. 2016), using excess PPX (1 µg/well) overcomes this issue (Bru et al. 2016; Pokhrel et al. 2019). Finally, the use of PPX in the micro-assay developed by Christ and Blank (2018b) is well suited for specific and fast quantification of polyP in high throughput.

In summary, our straightforward method for polyP quantification will be a valuable tool to reveal the physiological role of polyP in diatoms in different growth situations.

Conclusion

The described method allows to quantify a wide range of polyP, ranging from polyP-14 to polyP-700, in diatoms. The approach is fast, specific, and can be performed in high throughput.

Acknowledgments

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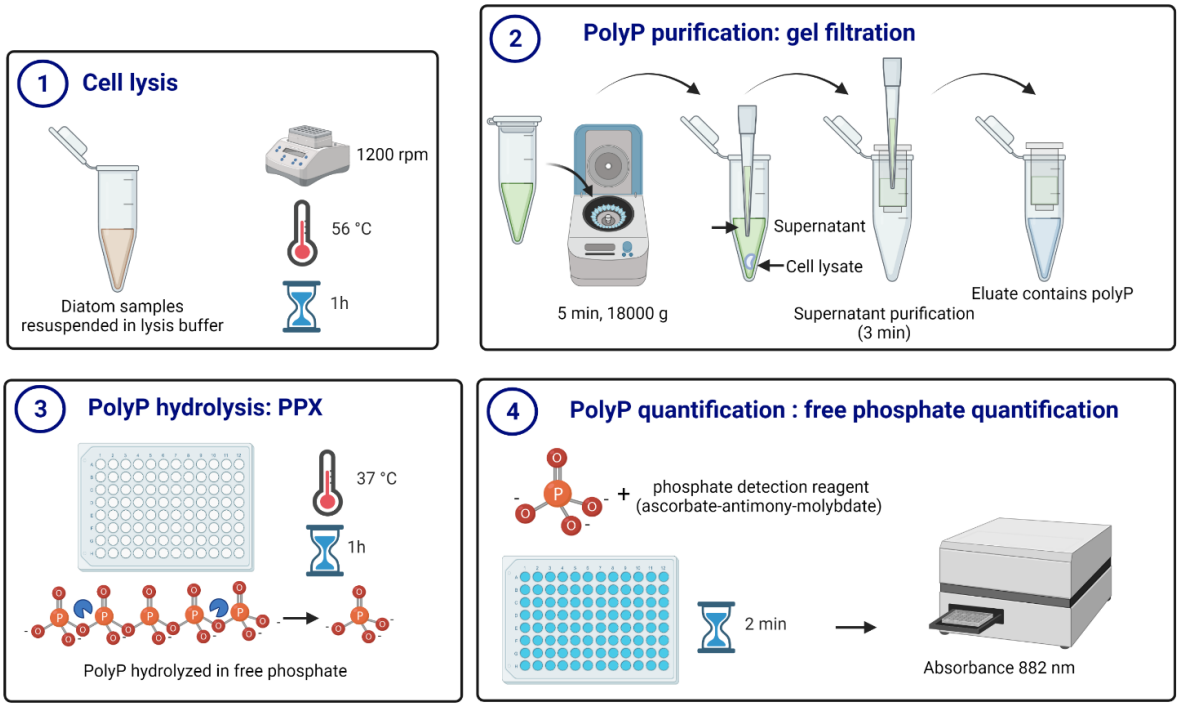

Figure 6: Scheme of the workflow of the protocol for extraction, purification and quantification of polyP in diatoms.

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Supporting information

Table S1: Comparison of the polyP determination methods: List of chemicals and organisms used as well as the number of steps and the duration (min) for extracting polyP using different approaches.

PolyP extraction protocol	Chemicals used for extraction	Organism used in protocol	Number of steps	Duration of extraction and purification
Method 1 Nexttec DNA isolation 1-Step™	Lysis buffer Proteinase k	Tissues and cells	2	ca. 60
Method 2 (Bru et al. 2016)	Phenol Chloroform Nucleases	Yeast	5	ca. 300
Method 3 (Christ and Blank 2018a)	Phenol Chloroform	Yeast	1	ca. 20
Method 4 Adapted from Martin and Van Mooy (2013)	Tris Proteinase K	Environmental plankton	2	ca. 140
Method 5 (Pokhrel et al. 2020)	GITC	Bacteria	3	ca. 15

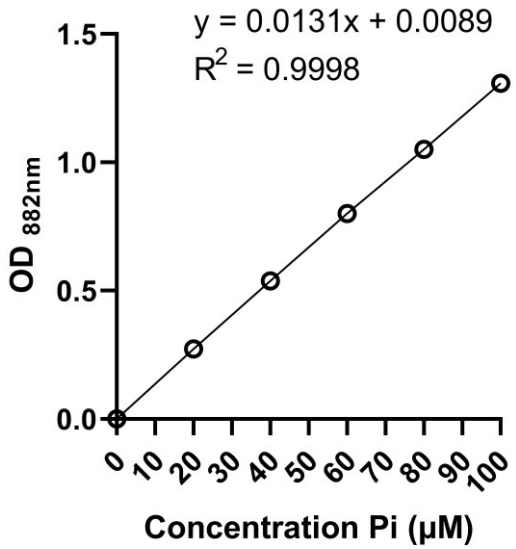

Figure S1: Standard curve of the OD 882 nm vs the concentration of phosphate (P_i). Data represent means, and standard deviations are given as error bar of n = 3 replicates. The error bars are not visible due to too small standard deviation (≤ 0.01).

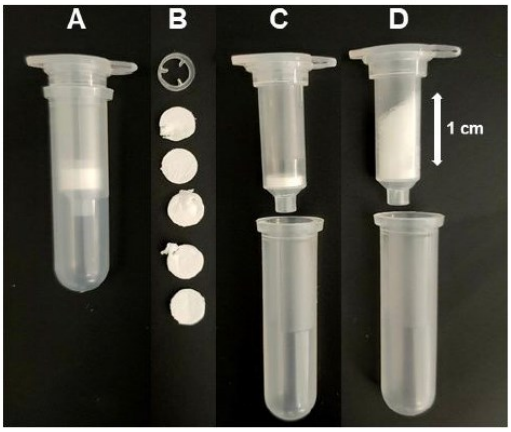

Figure S2: Preparation of self-made gel filtration columns using empty commercial spin columns. (A) Used commercial spin column from GENECLEAN®. (B) The upper plastic ring stopper and the membrane discs are removed using tweezers. (C) The remaining lower filter disc is left for supporting the gel filtration resin. (D) Gel filtration resin is added until reaching a height of 1 cm.