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Improving quantification of particulate phosphorus

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Abstract

Total particulate phosphorus (TPP) is often determined using the high-temperature dry combustion (HTDC) method followed by hydrolysis of the ash and then molybdenum colorimetry. Here, we show that a higher than traditionally used combustion temperature, 800°C vs. 450–550°C, improves phosphorus recovery from several phosphorus standard compounds, marine phytoplankton cultures, and particulate samples from the field. The ashing auxiliary $MgSO_4$ further improves P recovery by improving decomposition, reducing volatilization during combustion, and increasing the efficiency of hydrolysis. A 0.2 M HCl hydrolysis, under 90°C for 30 min yields a higher P recovery compared with hydrolysis at room temperature or 60°C. In aggregate, these improvements to the method double the P recovery from phospholipids to 97%. TPP recovery from laboratory phytoplankton cultures and field samples increased an average of 11%, primarily due to the improvements in P recovery from phospholipids, polyphosphate, and nucleic acids. We refer to this new method as the eXtra high temperature dry combustion (X-HTDC) Ash/Hydrol method and recommend its application for measuring particulate phosphorus from organic compounds in aquatic systems.

Phosphorus (P) is required for the storage and expression of hereditary information (nucleic acids), cellular energy transduction (nucleotides), cell membranes (phospholipids), and metabolic regulatory functions. As an element that often limits primary production on land, in freshwaters, and in the ocean (Karl 2000; Elser et al. 2007), phosphorus is one of the primary determinants of total primary production and atmospheric carbon dioxide and oxygen concentrations over very long timescales (Anderson et al. 2001). Unlike particulate organic nitrogen (PON) and particulate organic carbon (POC), which can be analyzed by well-established and standardized methods using an Elemental Analyzer (Verardo et al. 1990), phosphorus is quantified by a number of methods with many variants (Saunders and Williams 1955; Solórzano and Sharp 1980; Karl et al. 1991; Fu et al. 2005; Lomas et al. 2010). To better understand, monitor, and predict the biogeochemistry of phosphorus in response to environmental and climatic change, standardized measurements of inorganic and organic phosphorus pools (including phytoplankton P) are critical (Kwiatkowski et al. 2018; Lomas et al. 2022).

In aquatic systems, particulate phosphorus is traditionally sampled by filtering water onto glass-fiber filter (GFF) with a nominal pore size of 0.7 μm (Sheldon 1972; Karl et al. 1991). These samples are composed of both particulate inorganic P (PIP) and particulate organic P (POP) materials. PIP includes P in minerals, inorganic P adsorbed to particles (biotic and abiotic), and cellular storage products (i.e., orthophosphate, pyrophosphate and polyphosphate) (Yoshimura et al. 2007). POP from aquatic samples is primarily composed of microbial biomass and detrital material in the form of phosphomonoesters (such as phosphosugars and phospholipids), phosphodiesters (RNA and DNA), nucleotides, di-phosphates, tri-phosphates, and phosphonates (Labry et al. 2013; Dyhrman 2016). Several chemical rinses have been developed (Tovar-Sanchez et al. 2003; Sañudo-Wilhelmy et al. 2004) to remove the adsorbed P from the particles, which improves the estimation of total organic phosphorus associated with microbial cells (cellular P, P_c). The filter with the total particulate phosphorus (TPP) or P_c sample is either high-temperature dry combusted (HTDC) or chemically wet oxidized (CWO) (Ormaza-González and Statham 1996; Suzumura 2008). The resulting product is hydrolyzed by acid to orthophosphate (PO_4^{3-}), which is then measured by the phosphomolybdenum blue colorimetric reaction (Briggs 1924; Murphy and Riley 1962; Strickland and Parsons 1972; Aspila et al. 1976). The combination of HTDC and acid hydrolysis is referred to as the Ash/Hydrol method

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(Monaghan and Ruttenberg 1999). The CWO method was developed by Menzel and Corwin (1965) to measure dissolved phosphorus, which is a simpler and less time-consuming procedure than the HTDC Ash/Hydrol method, especially for dissolved organic phosphorus measurements, but P recovery of organic phosphorus compounds is relatively poor (Ormaza-González and Statham 1996; Worsfold et al. 2005); for example, the P recovery from L-α-phosphatidylethanolamine and 2-aminoethylphosphonic acid is 55.7% and 73.5% using CWO compared with 103.5% and 95.4% using the HTDC Ash/Hydrol method with the addition of $MgSO_4$ as an ashing auxiliary (Monaghan and Ruttenberg 1999).

The HTDC Ash/Hydrol method is currently the best available method to estimate particulate phosphorus and is commonly used as the standard to evaluate new methods (Pujo-Pay and Raimbault 1994). Across studies, there is variability in several steps in the method, including (1) the temperature of combustion; (2) the use and type of ashing auxiliaries in the combustion step; (3) the hydrolysis temperature, duration, and acid molarity; and (4) the color development of phosphomolybdenum blue. For example, combustion temperatures of 450–500°C are most used (Solórzano and Sharp 1980; Karl et al. 1991; Ormaza-González and Statham 1996; Monaghan and Ruttenberg 1999; Zhou et al. 2003; Fu et al. 2005; Suzumura 2008; Labry et al. 2013; Lomas et al. 2022), but temperatures of 500–550°C have been recommended by Aspila et al. (1976), Lampman et al. (2001), Zhou et al. (2003), and Labry et al. (2013) while Zhang (2012) argues that even higher combustion temperatures should be applied. Thermal stability studies suggest that several organic molecules in culture samples may require temperatures higher than 500°C to be fully decomposed (Padmavathy et al. 2003; Sukarni 2020), including ATP (Hammami et al. 2016), DNA (Nizioł et al. 2019), and lipids (Sukarni et al. 2018). Therefore, oxidation at 500°C may not completely break the C—O—P and P—O—P bonds in phosphorus compounds. There are limited data on how combustion temperature influences the oxidation of various P standards such as ATP, DNA, RNA, phospholipids, polyphosphate, and phytoplankton.

$MgSO_4$ is the most commonly added ashing auxiliary used in the HTDC Ash/Hydrol method (Solórzano and Sharp 1980) but a variety of other ashing auxiliaries have been used including $MgCl_2$ and $CaCl_2$ and the strong oxidant $Mg(NO_3)_2$ (Aspila et al. 1976; Cembella et al. 1986; Ormaza-González and Statham 1996; Suzumura 2008). There is evidence that $Mg(NO_3)_2$ does not improve P recovery relative to $MgSO_4$ (Monaghan and Ruttenberg 1999). Using $Mg(NO_3)_2$, <90% of ATP, trimetaphosphate, and guanosine-5-diphosphate was recovered and only about 70% of 4-nitrophenyl phosphate was recovered (Ormaza-González and Statham 1996). There is no clear theoretical framework to explain how the ashing auxiliaries work, but there is some indication that they may reduce volatilization of P during combustion, or aid in thermal decomposition. Bloch-Frankenthal (1953) has reported that magnesium can catalyze the hydrolysis of inorganic pyrophosphate.

The ashed samples are hydrolyzed to convert pyrophosphate to orthophosphate and then measured using the phosphomolybdenum blue colorimetric method (Briggs 1924; Murphy and Riley 1962; Strickland and Parsons 1972; Aspila et al. 1976). While most studies use hydrochloric acid (HCl), some older studies used sulphuric acid (H_2SO_4) (Saunders and Williams 1955). The molarity of HCl used in the hydrolysis procedures to digest inorganic phosphorus to orthophosphate varies across studies; the most common used HCl molarity varies from 0.1 to 0.2 M (Ormaza-González and Statham 1996; Labry et al. 2013; Lomas et al. 2022), 0.5 M (Karl et al. 1991) and 0.75 to 1 M (Solórzano and Sharp 1980; Ruttenberg 1992; Monaghan and Ruttenberg 1999; Anderson et al. 2001; Labry et al. 2013). There is no clear evidence that the molarity of the acid alters P recovery; however, the use of HCl at molarity between 0.75 and 1 M requires dilution for downstream analysis, which potentially increases error. Furthermore, the duration of hydrolysis ranges from 30 min to 18 h and the temperature of the hydrolysis ranges from room temperature to boiling (Andersen 1976). To address these uncertainties in the HTDC Ash/Hydrol method on estimates of TPP here we test how higher combustion temperatures and other minor variations in the method can alter P recovery from common organic phosphorus standards, polyphosphate, phytoplankton, and marine particulate samples.

Materials and procedures

We apply and compare phosphorus recovery using an extra high temperature dry combustion Ash/Hydrol (X-HTDC Ash/Hydrol) method to the traditional HTDC Ash/Hydrol method on the phosphorus standards that are common components in phytoplankton, including (1) pure compounds: ATP, DNA, RNA, and polyphosphate (polyP-45); (2) mixtures: phospholipids (*Escherichia coli* total extract lipids) and Apple leaves (NIST1515); and (3) laboratory phytoplankton species and particulate seawater samples.

The main steps of our method are (1) cleaning procedures to prepare glassware and filters for sample collection, (2) sample collection and an oxalate wash step to remove adsorbed phosphorus that can be applied to microbial cultures and field samples, (3) high-temperature combustion of standard P compounds, laboratory or field samples, (4) hydrolysis of combusted standards or natural samples, (5) colorimetric measurement of orthophosphate, and (6) calculation of P recovery.

We assess the effectiveness of several methodological variations, including the temperature and duration of combustion and the addition of ashing auxiliaries ($MgSO_4$, $MgCl_2$, $CaCl_2$) in the combustion step, the molarity of acid (HCl) and duration of exposure and temperature used for sample hydrolysis,

the benefit of adding $MgSO_4$ in the hydrolysis step, and the duration of color development in the colorimetric measurement.

Phosphorus standards examined

All reagents were prepared in Milli-Q water (Millipore Direct 16). Adenosine-5-triphosphate (ATP, ATP007) was obtained from Bioshop. Potassium dihydrogen orthophosphate (P-4550) was obtained from ACP chemical. Magnesium sulfate anhydrous (M65500) was obtained from Fisher Scientific. Ammonium molybdate tetrahydrate (09878), Apple leaves (NIST1515), L-ascorbic acid (A5960), deoxyribonucleic acid sodium from salmon testes (DNA, D1626), sodium phosphate glass type 45 (polyP-45, S4379), ribonucleic acid from yeast (RNA, 10109223001), and *E. coli* total extract lipids (100500P: phosphatidylethanolamine 57.5%, phosphatidylglycerol 15.1%, and cardiolipin 9.8%) were all obtained from Sigma-Aldrich. Other chemicals were analytical reagent grade.

Instruments

The muffle furnace (F30428C) and the microplate reader (Varioskan LUX with SkanIt Software for Microplate Readers RE, ver. 6.0.1.6) were from Thermo Scientific, the shaking incubator (LSE 6753) was from Corning, the 96-well microplates (655101) were obtained from Greiner. Microtubes used for the colorimetric reaction were made of polypropylene. Porcelain crucibles (Fisherbrand™ J120) and crucible covers (Fisherbrand™ J128) were from Fisher Scientific.

Cleaning procedures

All glassware was cleaned with phosphate-free detergent then with 5% v/v HCl and thoroughly rinsed with reverse osmosis water. Porcelain crucibles were cleaned with phosphate-free detergent and then rinsed with reverse osmosis water. Crucibles were filled with 0.2 M HCl (about 80% full), covered with lids, and heated in the oven at 90°C for 30 min. After the acid was removed from the crucibles, crucibles were rinsed with reverse osmosis water and completely dried. Crucibles were heated up to 500°C at a ramp-rate of 150°C h^{-1} and then combusted at 500°C for 6 h. Some crucibles shattered before the oven reached 500°C, but the remainder were good for the combustion of samples at 800°C. Crucibles were numbered with pencil at the bottom of the crucible cups.

Microalgal and particulate matter sample collection

Phytoplankton samples were obtained from nine laboratory cultures (*Crocosphaera watsonii* WH0005, WH8501; *Synechococcus* CCMP2515, MITS1220; *Pelagomonas calceolata* CCMP1682; *Minutocellus polymorphus* CCMP501; *Chloroparvula pacifica* RCC4656; *Thalassiosira pseudonana* CCMP1355; *Prorocentrum triestinum* RCC6447) and two natural communities from the Bedford Basin (NS, Canada). Culturing and sampling conditions are summarized in Supporting Information Table S1. The polycarbonate (PC) filter without sample was used as a blank for field TPP samples. The PC filter with phytoplankton media was used as a blank for laboratory culture TPP samples. To estimate phosphorus associated with organic cellular material (P_c), adsorbed phosphate was removed by oxalate reagent (Tovar-Sanchez et al. 2003). Five milliliters of oxalate reagent were added onto the collected samples. After 5-min reaction, the oxalate reagent was removed by filtration (130 mmHg), and then the collected sample was rinsed with filtered artificial seawater (with no phosphate added). For the phytoplankton P_c samples the blank was phytoplankton media filtered through PC filters, treated with oxalate reagent for 5 min, and subsequently rinsed with filtered artificial seawater. For the field P_c samples, the blank was a PC filter treated with oxalate reagent for 5 min that was subsequently rinsed with filtered artificial seawater. All samples were collected with five replicates, and blanks were collected in duplicate.

Preparation of reagents and standards

Phosphate standards

About 0.2 μmol P was obtained from 80 μL 0.5 mg mL^{-1} ATP solution, 160 μL 0.50 mg mL^{-1} DNA solution, 150 μL 0.17 mg mL^{-1} sodium phosphate glass type 45 solution (polyP), $\sim$ 200 μg *E. coli* lipids extract, $\sim$100 μg RNA and $\sim$ 5 mg Apple leaves (NIST1515). All standards were prepared in triplicate. Final P content in the colorimetric measurement was around 50 μM.

Phosphate standard solutions were prepared following Karl et al. (1991). About 1 g of KH_2PO_4 was transferred into an acid-washed glass beaker. The beaker was covered with foil and dried at 110°C for at least 2 h. KH_2PO_4 was cooled to room temperature in a vacuum desiccator. A primary standard of 1000 μM was prepared by dissolving 0.136 g dried KH_2PO_4 in 1 L Milli-Q water. The primary standard was stored in the fridge without adding chloroform. The primary standard was further diluted into working standard solutions to obtain final concentrations from 0 to 200 μM.

Preparation of molybdate reagents for colorimetric P measurement

Colorimetric determination followed Chen et al. (1956). The molybdate reagent was a mixture of 2 parts Milli-Q water, 1 part 6 N sulfuric acid (1 mL 18 M H_2SO_4 and 5 mL Milli-Q), 1 part 2.5% ammonium molybdate (0.25 g ammonium molybdate topped to 10 g with Milli-Q in a 50-mL Falcon tube) and 1 part 10% ascorbic acid (1 g ascorbic acid topped to 10 g with Milli-Q in a 50-mL Falcon tube). Since ascorbic acid is sensitive to light, all solutions were prepared right before the measurement. Measurement of phosphate was scaled down to microtiter plate format; a batch of 40 samples required about 25 mL reagent.

The X-HTDC Ash/Hydrol method

Extra high-temperature dry combustion

Samples on PC filters and P standards were dried with 0.2 mL of 0.17 M $MgSO_4$ in capped porcelain crucibles at 90°C. Combustion temperature increased at 150°C h^{-1}, and then was maintained for 9 h at 800°C. The resulting ash was cooled slowly to room temperature in the muffle furnace.

Ash hydrolysis by HCl

After the combusted samples and standards were cooled, 5 mL 0.2 M HCl was added into the ash in the crucibles, capped, and incubated at 90°C for 30 min in the oven.

Colorimetric phosphate measurement

Working standard solutions, hydrolysate of blanks and samples (500 μL) were transferred to 2-mL microtubes followed by the addition of 500 μL molybdate reagent to each tube. The resulting reactants were then continuously shaken at 200 rpm (37°C) for 3 h. Duplicate 250 μL samples were loaded from the microtubes into the microtiter plate. The plate was continuously shaken for 5 s at 600 rpm and then the absorbance was recorded at 820 nm under room temperature, where the apparent light pathlength was about 5 mm.

Testing variants of the X-HTDC Ash/Hydrol method

Determining the optimal combustion temperature, duration regime, and addition of ashing auxiliaries

We tested how three different commonly used ashing auxiliaries, $MgSO_4$, $MgCl_2$, $CaCl_2$, influenced P recovery from DNA, RNA, phospholipids, polyP, ATP, and Apple leaves (NIST1515). The P standard compounds were dried with 0.2 mL 0.17 M $MgSO_4$, $MgCl_2$, or $CaCl_2$ in porcelain crucibles at 90°C. As soon as the content was completely dried, crucibles were combusted in a muffle furnace at a ramp rate of 150°C h^{-1}. Combustion temperature and duration was varied from 500°C to 800°C for 3–9 h. Ash was cooled slowly to room temperature in the muffle furnace. The resulting ash was hydrolyzed by 0.2 M HCl at 90°C for 30 min, followed by the colorimetric measurement.

Hydrolysis by HCl

ATP and Apple leaves (NIST1515), two common organic P standards used in the literature (Lomas et al. 2010), were combusted with $MgSO_4$ at 500°C for 6 h in 20-mL borosilicate scintillation vials. To determine the optimal molarity, temperature, and duration for HCl hydrolysis, the ash was exposed to (1) 5 mL of 0.2 M and 0.5 M HCl at 90°C for 30 min; and (2) 5 mL 0.2 M HCl at 90°C for 30, 90, 150, and 300 min, and 24 h. To determine the benefit of adding $MgSO_4$ in the hydrolysis step, ATP and Apple leaves (NIST1515) were combusted at 500°C for 6 h without $MgSO_4$. In the ash, 0.17 M $MgSO_4$ was added followed by 0.2 M HCl, and then incubated at 90°C for 30 and 90 min. To determine the optimal temperature for hydrolysis, DNA and Apple leaves (NIST1515) were combusted with $MgSO_4$ at 800°C for 9 h in the crucibles, after cooling 5 mL 0.2 M HCl was added, and then incubated at room temperature, 60°C or 90°C for 30 min.

Colorimetric method

To determine the optimal duration for a complete color development of phosphomolybdenum, 500 μL working standard solutions with 500 μL molybdate reagent was continuously shaken at 200 rpm under 37°C for 1–4 h.

Calculation of P recovery

For each tested method, a normalized recovery was determined by taking the purity and hydroscopic nature of the organic P standards into account (Solórzano and Sharp 1980). P extracted from Apple leaves (NIST1515) was estimated as 0.1661% of total mass (certified P content: 0.1526–0.1661%). Water content in DNA was estimated to be 7% (Sebastiani et al. 2014), and the purity was estimated to be 100%. Standard deviation was calculated over three replicates. The P content from the standards was calculated as:

$$(\mathrm{P/sample})_{\mu\mathrm{mol}} = (\mathrm{orthophosphate})_{\mu\mathrm{M}} \times (\mathrm{V_{HCl}})_{\mathrm{mL}} \times (0.001).$$

Data analysis

Linear regression and ANOVA were performed using the statistical software R (R version 4.1.2). One standard deviation is reported in the text, figures, and tables, unless otherwise noted.

Assessment

Treatment of crucibles

We have found that crucibles may lose their temperature resistance after acid-washing or long soaks in alkaline detergent. Crucibles tended to shatter in the oven during the initial increase from room temperature to 500°C, even when the ramp rate was carefully controlled at 150°C h^{-1}. We recommend not soaking crucibles in acid but instead we suggest the crucibles be filled with 0.2 M HCl and then incubated at 90°C for 30 min as the acid-washing step. It is necessary to inspect the temperature resistance of newly acquired crucibles by combusting them at 500°C for 6 h (ramp rate: 150°C h^{-1}) after acid-washing. We found that crucibles that pass this inspection do not usually shatter when heated to 800°C.

Filters for sample collection

TPP is traditionally collected on GFF. The maximum operation temperature of GFF is no higher than 550°C; this limits their use in our X-HTDC method, which requires 800°C as combustion temperature. Considering that the aquatic samples for biogenic silica measurement are collected on PC filters (Brzezinski and Nelson 1995), we propose using PC filters to

collect particulate phosphorus samples for the X-HTDC Ash/Hydrol method. Sample blanks from GFF (25 mm) combusted at 500°C, acid washed PC filters (25 mm, 0.2 μm) and non-acid-washed PC filters (25 mm, 0.2 μm) combusted at 800°C have 1.45 ± 0.32, 3.55 ± 0.097 and 1.91 ± 0.65 nmol P, respectively. The results indicate that PC filters do not need to be acid washed prior to sample collection. Note a fume hood or vent is required to exhaust toxic gas released from the PC filters during combustion.

Phosphomolybdenum-ascorbic reduction

Milli-Q water was used as calibration blank in the measurement of orthophosphate. The reagent blank was determined by combusting $MgSO_4$, $MgCl_2$, or $CaCl_2$ (0.2 mL 0.17 M), hydrolyzing the resulting ash with 0.2 M HCl, and then mixing the hydrolysate with molybdate reagent. The combination of $MgSO_4$, $MgCl_2$, or $CaCl_2$ with ACS grade HCl and molybdate reagent generally introduces 2.16 ± 1.52, 4.84 ± 0.32, or 4.84 ± 0.097 nmol P, respectively, into the measurement.

Orthophosphate was quantified by measuring the optical densities of phosphomolybdenum at 820 nm in a 96-well microtiter plate. Absorbance of phosphomolybdenum (5–100 μM) after incubation at 37°C was monitored every hour for 4 h. The sensitivity from 1 to 4 h is 4 h $\cong$ 3 h > 2 h > 1 h (Table 1). Sensitivity increased 5.9% in the 2nd hour, and then 1% in the 3rd hour. No significant change ($p > 0.05$) in sensitivity was observed from the 3rd to the 4th hour. Therefore, we recommend a 3-h incubation for color development for the highest sensitivity.

We observed a linear increase in absorbance from zero to 500 μM P, consistent with the Bouguer–Beer–Lambert law. The pathlength in the microplate is about 5 mm compared with traditional 10 mm cuvettes, but the sensitivity allows a limit of detection (Armbruster and Pry 2008) of 6.13 nmol phosphate, which is practical for TPP samples collected from low-biomass regions. The colorimetric method is expected to have a linear response in the range of P concentrations over which 12-MPA ($[H_4PMo_{12}O_{40}]^{3-}$) formation and reduction occur to the same degree. This balance requires sufficient Mo (VI) to stabilize 12-MPA and sufficient ascorbic acid reductant (Nagul et al. 2015). Increasing phosphate concentrations above a threshold, of about 500 μM, leads to no further increase in absorbance.

Acid hydrolysis: Temperature, duration, and molarity

Apple leaves (NIST1515) and DNA standards were combusted with $MgSO_4$ in scintillation vials at 500°C for 6 h. Under tightly capped conditions to avoid evaporation, the resulting ash was hydrolyzed by 0.2 M HCl for 30 min at room temperature, 60°C and 90°C to determine the optimal temperature for hydrolysis (Table 2). The rank recovery of phosphorus from Apple leaves (NIST1515) and DNA from different hydrolysis temperature is 90°C > 60°C > room temperature.

Apple leaves (NIST1515) and ATP were combusted with $MgSO_4$ in scintillation vials at 500°C for 6 h. Under tightly capped conditions to avoid evaporation, the resulting ash was either (1) hydrolyzed by 0.2 and 0.5 M HCl at 90°C for 30 min; or (2) hydrolyzed by 0.2 M HCl at 90°C for 30, 90, 150, 300 min and 24 h. The recovery of P from Apple leaves (NIST1515) and ATP hydrolyzed by 0.2 M and 0.5 M HCl was not significantly different ($p > 0.05$). Hydrolysis duration has no significant impact on P recovery from both Apple leaves (NIST1515) and ATP ($p > 0.05$). We recommend hydrolyzing ash with 0.2 M HCl at 90°C for 30 min, which is less acidic to the crucibles.

High-temperature combustion

Combustion of PC filters and samples at 500°C left a colored acid-insoluble residue that required removal via filtration or centrifugation before colorimetric determination, but it was not present when 800°C was used for combustion. It seems

Table 2. %P recovery (one SD) from apple leaves (NIST1515) and DNA with 0.2 M HCl hydrolysis under room temperature, 60°C, or 90°C for 30 min. Means from the same standard with no letter in common are significantly different (*t*-test; $p < 0.05$), where the highest value is denoted by letter a.

	Room temperature	60°C	90°C
Apple leaves	91.57 ^{c} (0.35)	94.94 ^{b} (0.67)	100.63 ^{a} (2.03)
DNA	89.99 ^{c} (0.35)	92.51 ^{b} (0.72)	100.91 ^{a} (2.02)

Table 1. Impact of duration (h) on color development as quantified by the slope of absorbance of phosphomolybdenum blue vs. P concentration (μM). Means with no letter in common are significantly different (*t*-test; $p < 0.05$), where the highest value is denoted by letter a.

Duration (h)	Slope	*SD*	R^2	*p*-value
1	8.50×10^{-3} ^{c}	8.50×10^{-5}	0.9997	1.59×10^{-4}
2	9.00×10^{-3} ^{b}	7.35×10^{-5}	0.9998	1.07×10^{-4}
3	9.09×10^{-3} ^{a}	5.25×10^{-5}	0.9999	4.19×10^{-5}
4	9.09×10^{-3} ^{a}	5.25×10^{-5}	0.9999	7.14×10^{-5}

Table 3. Comparison of % P recovery (one SD) from P standards when using different combustion temperatures (500°C, 700°C, and 800°C) and ashing auxiliaries (no auxiliary, $CaCl_2$, $MgCl_2$, and $MgSO_4$). Means within a single standard with no letter in common are significantly different (*t*-test; $p < 0.05$), where the highest value is denoted by letter a.

Standards	500	500+ $MgCl_2$	500+ $CaCl_2$	500+ $MgSO_4$	700+ $MgSO_4$	800	800+ $MgSO_4$
Apple leaves (NIST1515)	83.81^{c}	86.45^{b}	83.86^{c}	86.97^{b}	86.94^{b}	102.37^{a}	100.63^{a}
	(0.42)	(0.99)	(0.96)	(1.15)	(0.55)	(0.49)	(2.13)
Phospholipids	48.17^{e}	71.52^{cd}	65.50^{d}	49.25^{e}	80.74^{b}	76.58^{bc}	97.30^{a}
	(5.04)	(2.44)	(3.96)	(5.71)	(4.16)	(6.76)	(3.68)
Polyphosphate	36.93^{e}	90.32^{c}	76.88^{d}	93.29^{bc}	89.47^{c}	95.60^{ab}	98.51^{a}
	(2.93)	(0.64)	(0.66)	(0.38)	(3.63)	(2.46)	(0.63)
RNA	41.78^{e}	82.28^{c}	72.64^{d}	86.17^{c}	92.05^{b}	92.52^{b}	100.22^{a}
	(2.75)	(4.56)	(3.39)	(4.08)	(0.97)	(0.30)	(1.17)
DNA	74.75^{d}	82.12^{c}	76.37^{d}	89.60^{b}	97.28^{a}	70.49^{e}	100.91^{a}
	(0.91)	(0.68)	(0.63)	(1.72)	(1.17)	(0.94)	(2.03)
ATP	41.54^{c}	92.66^{b}	94.25^{b}	92.13^{b}	92.33 ^{b}	93.10^{b}	100.02^{a}
	(0.36)	(0.37)	(2.27)	(1.74)	(0.99)	(1.92)	(1.33)

Table 4. Percentage of P recovery (one SD) from apple leaves (NIST1515) and ATP after combustion at 500°C and hydrolysis, with and without $MgSO_4$ as an ashing auxiliary, two different hydrolysis durations, and with and without $MgSO_4$ in the hydrolysis. Means within a standard with no letter in common are significantly different (*t*-test; $p < 0.05$), where the highest value is denoted by letter a.

Ashing auxiliary ($MgSO_4$)	Hydrolysis (min)	$MgSO_4$ in hydrolysis	Apple leaves % recovery	ATP % recovery
No	30	No	79.85 ^{b} (0.42)	41.54 ^{c} (0.36)
No	30	Yes	86.88 ^{a} (0.79)	51.35 ^{b} (5.95)
No	90	Yes	86.72 ^{a} (0.88)	90.75 ^{a} (1.38)
Yes	30	No	86.97 ^{a} (1.15)	92.13 ^{a} (1.74)

likely that the formation of this residue contributes to incomplete recovery of phosphorus. Without the addition of an ashing auxiliary, combustion at 800°C compared with 500°C increases P recovery from all P compounds except for DNA (Table 3); however, P recovery from most of the P standards was still less than 100% (e.g., P recovery from the DNA and the phospholipid standard was only 70% and 77%, respectively).

Impact of ashing auxiliaries

$MgSO_4$, $MgCl_2$, or $CaCl_2$ (0.2 mL of 0.17 M) was added into the P standards, dried at 90°C, and then combusted at 500°C, 700°C, or 800°C for 9 h. The resulting ash was hydrolyzed by 0.2 M HCl at 90°C for 30 min followed by the measurement of phosphomolybdenum absorbance. The addition of $MgSO_4$ gave the highest P recovery from DNA compared with $MgCl_2$ and $CaCl_2$. $MgSO_4$ and $MgCl_2$ gave higher P recovery from Apple leaves (NIST1515), polyphosphate and RNA compared with $CaCl_2$. The rank order of ashing auxiliaries on P recovery from phospholipids is $MgCl_2 \cong CaCl_2 > MgSO_4$. All ashing auxiliaries increased P recovery 2.24-fold from ATP. With the addition of $MgSO_4$ a higher P recovery was obtained from phospholipids, RNA and DNA with the increase of the combustion temperature from 500°C to 700°C and from all P standards except for DNA with the increase of the combustion temperature from 700°C to 800°C. In summary, high P recovery from all P standards can be obtained from an 800°C combustion with the addition of $MgSO_4$. The highest P recovery for Apple leaves (NIST1515) and polyP was obtained following combustion at 800°C with or without $MgSO_4$. DNA and Apple leaves (NIST1515) were combusted with $MgSO_4$ at 800°C for 3–9 h, and then hydrolyzed by 0.2 M HCl at 90°C for 30 min. No significant difference in P recovery was found between the 3- and the 6-h combustion, but the 9-h combustion increased P recovery from DNA and Apple leaves (NIST1515) from 95.2% ± 0.4% and 92.6% ± 0.8% to 101% ± 2% and 101% ± 2%, respectively. Therefore, we recommend a 9-h combustion.

We also assessed if the addition of $MgSO_4$ into the hydrolysis step improved P recovery from ATP and Apple leaves (NIST1515) combusted without $MgSO_4$ at 500°C for 6 h (Table 4). Without the addition of $MgSO_4$ in either the combustion or hydrolysis step, P recovery was 80% and 42% from Apple leaves (NIST1515) and ATP, respectively. The addition of $MgSO_4$ into the ash and then hydrolyzed by 0.2 M HCl at 90°C for 30 min increased P-recovery from Apple leaves (NIST1515) to 87%; 90 min was required to increase P-recovery from ATP to 91%.

P recovery from phytoplankton samples

Phytoplankton samples were dried with 0.2 mL 0.17 M $MgSO_4$ at 90°C and then combusted at 500°C or 800°C for 9 h. In addition, *C. pacifica* and *T. pseudonana* were combusted at 800°C for 9 h without $MgSO_4$. The ash was hydrolyzed with 0.2 M HCl at 90°C for 30 min. Residue from the hydrolysate using 500°C combustion was removed by centrifugation. Hydrolysates and calibration standards were added to a 2 mL microtube followed by the addition of the molybdate reagent. The reactants were then continuously shaken at 200 rpm (37°C) for 3 h. Reactants were loaded into a microtiter plate (250 μL, duplicate). The absorbance was recorded at 820 nm.

With the addition of $MgSO_4$, increasing combustion temperature from 500°C to 800°C increased TPP and P_c recovery from phytoplankton and field samples between 6% and 18% and between 7% and 28%, respectively (Table 5). The absence of $MgSO_4$ in the combustion at 800°C decreased P_c in *T. pseudonana* and *C. pacifica* from 13.96 ± 0.46 to 8.04 ± 2.94 fmol cell^{-1} and 2.69 ± 0.10 to 1.91 ± 0.04 fmol cell^{-1}, respectively; TPP in *T. pseudonana* decreased from 15.87 ± 0.40 to 11.14 ± 0.81 fmol cell^{-1}, but there was no significant impact ($p > 0.05$) on TPP recovery from *C. pacifica*. Adsorbed P (P_{ADS}, the difference between TPP and P_c) was not significantly affected by increasing combustion temperature from 500°C to 800°C when $MgSO_4$ was added as an ashing auxiliary.

Discussion

Here, we revisit the conventional HTDC/Hydrol method to determine the optimal temperature and duration to fully decompose common organic P compounds and polyphosphate, the role of ashing auxiliaries in the promotion of P-recovery, the molarity, the temperature and duration for the HCl hydrolysis step to optimize P-recovery, and the duration of molybdenum color development that provides the highest sensitivity. We find that the addition of ashing auxiliaries significantly increases P recovery, likely through a combination of reduced P volatilization and increased organic matter decomposition. We also find that the addition of $MgSO_4$ to the ash can increase hydrolysis efficiency. We then

Table 5. Recovery of total particulate P (TPP), cellular P (rinsed with oxalate to remove adsorbed P, P_c), adsorbed P (P_{ADS}) of phytoplankton (fmol cell^{-1}), field samples (μM) combusted at 500°C with $MgSO_4$ (500+ $MgSO_4$) and combusted at 800°C with $MgSO_4$ (800+ $MgSO_4$), and the ratio of P recovered at 800°C relative to 500°C (800/500). Values in parentheses indicate one standard deviation.

	500+ $MgSO_4$			800+ $MgSO_4$			800/500		
Species	**TPP**	**P_c**	**P_{ADS}**	**TPP**	**P_c**	**P_{ADS}**	**TPP**	**P_c**	**P_{ADS}**
Synechococcus	0.19	0.20	−0.01	0.20	0.22	−0.02	1.06	1.07	2.00
(MITS1220)	(0.01)	(0.01)	(0.01)	(0.01)	(0.002)	(0.01)	(0.06)	(0.03)	(2.24)
Synechococcus	0.24	0.25	−0.01	0.27	0.27	−0.01	1.11	1.07	1
(CCMP2515)	(0.003)	(0.003)	(0.004)	(0.01)	(0.001)	(0.01)	(0.04)	(0.01)	(1.08)
Synechococcus	0.55	0.25	0.30	0.59	0.27	0.32	1.10	1.07	1.07
(MITS1220, N^-)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.02)	(0.04)	(0.07)
Pelagomonas calceolata	1.18	0.72	0.46	1.25	0.88	0.38	1.06	1.22	0.82
(CCMP1682)	(0.10)	(0.02)	(0.10)	(0.01)	(0.02)	(0.02)	(0.09)	(0.05)	(0.20)
Chloroparvula pacifica	2.55	2.52	0.03	2.76	2.69	0.07	1.08	1.07	2.42
(RCC4656)	(0.06)	(0.03)	(0.07)	(0.06)	(0.10)	(0.11)	(0.03)	(0.04)	(6.58)
Crocosphaera watsonii	17.76	17.21	0.55	20.35	19.46	0.89	1.15	1.13	1.61
(WH8501)	(0.26)	(0.56)	(0.62)	(0.36)	(0.17)	(0.40)	(0.03)	(0.04)	(1.94)
C. watsonii	9.52	9.09	0.43	11.02	9.93	1.09	1.16	1.09	2.53
(WH0005)	(0.33)	(0.30)	(0.45)	(0.46)	(0.31)	(0.55)	(0.06)	(0.05)	(2.91)
Minutocellus polymorphus	7.42	5.23	2.19	7.99	6.01	1.97	1.08	1.15	0.90
(CCMP501)	(0.10)	(0.19)	(0.21)	(0.18)	(0.29)	(0.55)	(0.03)	(0.07)	(0.18)
Thalassiosira pseudonana	14.88	13.08	1.81	15.87	13.96	1.91	1.11	1.07	1.62
(CCMP1355)	(0.38)	(0.24)	(0.44)	(0.40)	(0.46)	(0.61)	(0.04)	(0.04)	(1.68)
Prorocentrum triestinum	NT	237.66	NT	NT	264.71	NT	NT	1.11	NT
(RCC6447)		(5.24)			(7.29)			(0.04)	
Bedford Basin	0.29	0.18	0.11	0.32	0.23	0.08	1.08	1.28	0.76
(March)	(0.03)	(0.01)	(0.03)	(0.02)	(0.002)	(0.02)	(0.11)	(0.09)	(0.25)
Bedford Basin	0.54	NT	NT	0.64	NT	NT	1.18	NT	NT
(June)	(0.01)			(0.04)			(0.08)		

NT, not tested.

tested our improved X-HTDC-Ash/Hydrol method on phytoplankton cultures and particulate matter sampled from Bedford Basin seawater. We find that relative to the standard HTDC method, the combination of 9-h 800°C combustion with the ashing auxiliary $MgSO_4$ and the optimized hydrolysis/colorimetric measurement increases P recovery by 98% ± 24% from phospholipids, 16% ± 5.7% from RNA, 12.6% ± 3.1% from DNA, 8.6% ± 2.5% from ATP, 5.6% ± 0.8% from polyphosphate, 15.7% ± 2.4% from Apple leaves (NIST1515) (Table 3), and increases TPP 11% ± 4% from field samples and a taxonomically diverse range of phytoplankton cultures (Table 5). Most of the improvement in P recovery by the new method is due to the more complete decomposition of organic P-containing compounds under the higher combustion temperature.

The impact of temperature and ashing auxiliaries on P-recovery

The HTDC method has traditionally used combustion temperatures of 450–550°C. Inorganic compounds in total particulate P compounds, such as KH_2PO_4 and Na_2HPO_4, are converted to pyrophosphate between 400°C and 500°C (Banach and Makara 2011) but organic molecules with P—O—P, C—O—P, or C—P bonds can require temperatures in excess of 500°C to be completely converted to pyrophosphate. For example, thermogravimetric analysis of DNA indicates the mass loss of DNA at 500°C is ~ 52% (Nizioł et al. 2019); RNA loses 30% of its mass at 350°C (Chollakup and Smitthipong 2012); the methylene group in lipids does not decompose below 673°C (Sukarni et al. 2018), and temperatures >800°C are required for the full thermal degradation of *Spirulina platensis* (Sukarni et al. 2018). Here, we find an 800°C vs. 500°C combustion temperature significantly increases P-recovery from six common phosphorus standards (Table 3). For example, an increase in combustion temperature from 500°C to 800°C (with no ashing auxiliary) increases P-recovery from 83% to 102% from Apple leaves (NIST1515) and from 37% to 96% from polyphosphate. By contrast, P-recovery from DNA decreases from 75% to 71% with the increase in combustion temperature without ashing auxiliary. The addition of the ashing auxiliary $MgSO_4$ and 800°C yields 99–100% P recovery from all the phosphorus standards examined. These results highlight that both an increased combustion temperature and addition of an ashing auxiliary can significantly improve P-recovery from different organic molecules.

Ashing auxiliaries, such as $MgSO_4$, may impact both oxidation and volatilization of some molecules during combustion. François et al. (2016) found that organics trapped in $MgSO_4$ can undergo oxidation and sulphuration during pyrolysis and suggested that sulfates can protect organics trapped inside their crystal lattice from volatilization. Extending this idea, differences in the crystal and chemical structure of different ashing auxiliaries may explain their differential impact on phosphorus recovery from different organic standards. Specifically, we hypothesize the higher P-recovery from phospholipids with the addition of $MgCl_2$ relative to $CaCl_2$ and $MgSO_4$ is due to the rhombohedral crystal structure of $MgCl_2$ (a = 0.3846 nm, c = 1.7479 nm) that more efficiently traps phospholipids chain molecules and accelerates thermal decomposition relative to the orthorhombic crystal structure of $CaCl_2$ (a = 0.6259 nm, b = 0.6444 nm, c = 0.417 nm) and $MgSO_4$ (a = 0.5182 nm, b = 0.7893 nm, c = 0.6506 nm). With a 500°C combustion step the Mg-containing ashing auxiliaries significantly increased P-recovery from all the organic P standards tested except for Apple leaves (NIST1515). The addition of an ashing auxiliary may be unnecessary for Apple leaves (NIST1515) since they naturally contain Mg (2710 mg kg^{-1}). In particular, P recovery from phospholipids and DNA is relatively poor without the addition of $MgSO_4$, 77% and 71%, respectively, indicating that the addition of $MgSO_4$ may reduce volatilization of P during combustion. In addition to reducing volatilization in the combustion step, ashing auxiliaries may catalyze hydrolysis of some organic compounds, improving phosphorus recovery. Based on experimental observations by Bloch-Frankenthal (1953), we propose the addition of Mg^{2+} may activate the hydrolysis, likely by forming a complex with phosphorus $(MgP_20_7)^{2-}$. Here, we find that the addition of $MgSO_4$ in the 30-min 0.2 M HCl hydrolysis step, not the combustion step, can increase P-recovery from 80% to 87% from Apple leaves (NIST1515) and from 42% to 51% from ATP; and if the hydrolysis is extended to 90 min, P-recovery from ATP reaches 91% (Table 4). However, whether $MgSO_4$ is added before or after combustion, P recovery is limited by the efficiency of thermal decomposition, which is temperature dependent.

Although microalgae naturally contain magnesium, we still recommend the addition of $MgSO_4$ into the combustion step for improved phosphorus recovery. The concentration of magnesium in algae varies across species (Ho et al. 2003) and may be reduced by the oxalate rinse. If concentration of magnesium is too low, DNA and phospholipids will be susceptible to volatilization under high-temperature combustion. For example, in *C. pacifica*, the same TPP was obtained with and without $MgSO_4$ in the 800°C combustion, but P_c decreased 40.6% ± 5.6% when $MgSO_4$ was not added in the 800°C combustion, likely due to the loss of adsorbed Mg^{2+} (Ayed et al. 2015) by the oxalate treatment. In addition, theory and physical evidence suggests biogenic silicon may reduce P-recovery if $MgSO_4$ is not used in the combustion. For example, in the diatom *T. pseudonana*, measured TPP increased from 11.14 ± 0.81 to 15.87 ± 0.40 fmol $cell^{-1}$ (a 42% increase) when $MgSO_4$ was added to the 800°C combustion step. We occasionally observed the formation of dark-red crystals after diatom samples and field samples (June 2021) were combusted at 800°C with $MgSO_4$. Biogenic amorphous Si is transformed to an amorphous glassy state at 800°C and then hardens after cooling down. We speculate that this amorphous Si captures phosphorus, making it less susceptible to hydrolysis, and

thereby reduces P recovery. The addition of $MgSO_4$ can precipitate silicate (Rashid et al. 2011) and prevent the formation of glass droplets during the 800°C combustion. Based on the likely elemental content in algal ash-oxides (de Souza et al. 2020), we propose that the addition of $MgSO_4$ prevents the sequestration of P in viscous Si polymers, and that the dark-red crystals we have observed after the 800°C combustion of some diatom and field samples might be $(Mg,Co)SiO_3$ (which has a high melting point of 900°C). Considering that silicon can be found in a wide diversity of aquatic microbes across many environmental regimes (Finkel 2016) and the beneficial role of $MgSO_4$ in both preventing volatilization of various P compounds under the 800°C combustion and catalyzing hydrolysis, we strongly recommend adding $MgSO_4$ to all microalgae samples when using the X-HTDC method.

Compared to the traditional HTDC, our X-HTDC Ash/Hydrol method increased TPP recovery 6–16% across a diverse set of phytoplankton species in culture. Similarly, our X-HTDC Ash/Hydrol method increased TPP estimation 8–18% for particulate samples from the Bedford Basin in March and June, respectively. TPP provided by our X-HTDC method is not significantly higher for two of the phytoplankton cultures and one of the field samples, likely in part because sampling plus measurement errors can obscure the $\sim$10% increase (Table 5). Qualitative observation under a light microscope indicated samples from June 2021 were dominated by diatoms and dinoflagellates, but samples from March 2022 were dominated by smaller nanoplankton and picoplankton (consistent with Robicheau et al. 2022). There are large differences in the relative proportion of DNA, RNA, ATP, phospholipids, polyphosphate, and absorbed P across species and within species exposed to different environmental conditions (Zimmerman et al. 2014; Ebenezer et al. 2022) that will influence the magnitude of improvement provided by the X-HTDC method. The X-HTDC method will not provide much improvement for samples with relatively high concentrations of inorganic P (Sañudo-Wilhelmy et al. 2004), for example, when adsorbed P content is a large proportion of total TPP, because inorganic P does not require temperatures greater than 500°C to be converted to pyrophosphate. Consistent with this assertion, our estimates of adsorbed P (P_{ADS}, subtracting P_c from TPP) from phytoplankton cultures and field samples were not significantly different comparing HTDC with X-HTDC methods but P_c values from all tested samples using X-HTDC were significantly higher than the values from HTDC method.

Comments and recommendations

Here, we present an X-HTDC Ash/Hydrol method for the measurement of phosphorus from particulate aquatic samples. We propose that particulate phosphorus samples be collected on PC filters, that $MgSO_4$ be added as an ashing auxiliary, and that the samples be combusted at 800°C for 9 h to completely decompose phosphorus compounds. TPP is traditionally collected on GFF, but the GFF is transformed into an amorphous state under the high temperature in our method and when cooled the viscous glass traps P compounds preventing acid hydrolysis and TPP quantification. Using GFF may overestimate TPP compared with PC filter due to adsorption of dissolved organic phosphate to the filter (Lomas et al. 2010). If particulate C, N, and P are all to be measured, it may be desirable to measure TPP on GFF by using traditional HTDC method to match the filter usually used for POC and PON; but retaining our improvements in hydrolysis and colorimetric measurement. Alternatively, using a PC filter with a comparable pore size and filtration efficiency should permit a reasonable comparison with samples taken on GFF. We recommend hydrolysis of the resulting ash in 0.2 M HCl for 30 min at 90°C, and a three-hour incubation at 37°C for a complete color development of phosphomolybdenum complex. This method should be suitable for laboratory and diverse aquatic particulate samples. We did not investigate the application of our X-HTDC method to P recovery from soil samples, but we anticipate an improvement in this application as well. If the combustion converts ash to β-tricalcium phosphate, the acid solubility of apatite could be affected, and the recovery of P decreased. This is unlikely to be a concern as long as the combustion temperature remains below 900°C (Gee and Deitz 1955). The method has been developed for microtiter plate format for high throughput and the "miniaturizing" of assay reagent volumes. The lower limit of detection on this microtiter plate format is 6.13 nmol phosphorus. A detailed protocol for our method is available at protocols.io (Hu and Finkel 2022).

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