Advances in techniques for phosphorus analysis in biological sources
Nehreen Majed\textsuperscript{1,2}, Yueyun Li\textsuperscript{1} and April Z Gu\textsuperscript{1}

In general, conventional P analysis methods suffer from not only the fastidious extraction and pre-treatment procedures required but also the generally low specificity and poor resolution regarding the P composition and its temporal and spatial dynamics. More powerful yet feasible P analysis tools are in demand to help elucidating the biochemistry nature, roles and dynamics of various phosphorus-containing molecules \textit{in vitro} and \textit{in vivo}. Recent advances in analytical chemistry, especially in molecular and atomic spectrometry such as NMR, Raman and X-ray techniques, have enabled unique capability of P analysis relevant to submicron scale biochemical processes in individual cell and in natural samples without introducing too complex and invasive pretreatment steps. Great potential still remains to be explored in wider and more combined and integrated requests of these techniques to allow for new possibilities and more powerful P analysis in biological systems. This review provides a comprehensive summary of the available methods and recent developments in analytical techniques and their applications for characterization and quantification of various forms of phosphorus, particularly polyphosphate, in different biological sources.

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\begin{wraptable}{r}{0.5\textwidth}
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\textbf{Current Opinion in Biotechnology} 2012, 23:852–859
\begin{itemize}
\item This review comes from a themed issue on \textit{Phosphorus biotechnology}
\item Edited by Andrew N Shilton and Lars M Blank
\item For a complete overview see the \textit{Issue} and the \textit{Editorial}
\item Available online 12th July 2012
\item 0958-1669/$ – \textsuperscript{c} 2012 Elsevier Ltd. All rights reserved.
\item \url{http://dx.doi.org/10.1016/j.copbio.2012.06.002}
\end{itemize}
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\textbf{Introduction}
Phosphorus (P), in its various forms and species, plays an essential role in cellular functions and its importance to biochemistry, biology and ecology has long been recognized. In biological systems, phosphorus is present mainly as free phosphate ions, inorganic polymeric phosphates, or organic phosphorus (OP). OP includes nucleic acids, phospholipids, inositol phosphates, phosphoamides, phosphoproteins, sugar phosphates, amino phosphoric acids and organic condensed P species. Inorganic polyphosphate (poly-P), an ubiquitous biopolymer found in every cell type in nature, is of particular interest because of the growing recognition of numerous cellular functions it performs: phosphate and energy storage, sequestration and storage of cations, pH buffer, formation of membrane channels for active biomolecule uptake, cell envelope formation and function, gene activity control, regulation of enzyme activities, stress response and survival and stationary phase adaptation [1,2]. Owing to its properties such as, inexpensiveness, non-toxicity and biodegradability, poly-Ps are widely used in industrial, agricultural, environmental and medical applications [3]. However, the mechanisms that determine the intracellular states of poly-P and its multiple metabolic functions remain obscure. The rising interests in P recovery from biological sources such as sludge from wastewater treatment presents another area where the understanding of the various forms of biologically or chemically bound phosphorus is of need [4]. Progress in our comprehension of the biochemistry nature and cellular function of various phosphorus-containing molecules rely on the availability of sensitive, definitive, and facile methods for P analysis. This paper reviews the techniques available for detecting and analyzing various forms of phosphorus in cellular components and biological sources, which are summarized in Table 1 and Figure 1.

\textbf{Methods for analyzing phosphorus in biological sources}
\textbf{Separation of P species and poly-P fractions}
Quantification and characterization of different forms of P usually requires a chemical separation and cleanup step to concentrate the selected P fractions of interest. Most extraction procedures must be regarded as a compromise between sufficient extraction efficiency and the protection of P compounds. Efficient and specific poly-P extraction is often difficult since poly-P can be associated with other cellular components and its solubility depends on the chain length. A biopolymer extraction and solubilization step, involving either strong acid or alkali treatment, is generally required before poly-P analysis. The extraction procedure has been reported often to lead to an underestimation of the levels of poly-P in samples owing to poly-P loss and composition changes [5–10]. The most effective and commonly used method for separation of poly-P of different chain lengths is gel electrophoresis, in which the percentage of polyacrylamide or agarose gel used dictates the resolution of separation lengths [11]. Chromatographic and filtration methods serve to separate
extracts of poly-P into more defined fractions or to remove other P compounds for purification [8,12]. By means of thin-layer chromatography, chains of condensed phosphate up to a length of eight can be separated. In two dimensions, this technique even allows for the separation of linear P from cyclic P compounds [13].

Methods have also been developed to distinguish between different intracellular P compounds as well as between chemically precipitated and biologically stored P in activated sludge systems [5,14]. A method consisting of sequential extractions using cold distilled water, bicarbonate–dithionite, NaOH and HCl was applied to differentiate the fractions of P such as adsorbed forms of P, metal bound P, P complexes, soluble P, organic P, CaCO₃, MgCO₃ and apatite bound P in activated sludge [8,15,16]. To discern different intracellular P fractions (i.e. nucleic acids, phospholipids and poly-P), ethanol extraction was applied for phospholipids collection and, activated carbon adsorption or cetyltrimethylammoniumbromide precipitation was employed to separate nucleic acids from poly-P in the cell extract [5,17,18]. Inositol phosphates can be precipitated with iron and barium salts after selective oxidation of dissolved organic P with hypobromite [19]. Despite the improvement in extraction methods in recent years, there still lacks standardized procedures and consented results among laboratories.

### Chemical analysis

Conventional chemical analysis-based quantification of intracellular P consists of biomass digestion to convert the poly-P and organic P to dissolved orthophosphate (ortho-P), then followed by ortho-P analysis via different colorimetric methods such as ion chromatography (IC) or inductively coupled plasma (ICP) atomic emission spectrometry (AES) [20,22–23]. Organic P is separated from poly-P through increasingly rigorous digestion procedure (i.e. higher temperature, higher acidity and/or more oxidation). Since the chemical analysis of P fractions depends on the accurate measurement of final orthophosphate concentration in the digested extracts, high background orthophosphate level and the susceptibility of ortho-P detection methods to interferences from inorganic colloidal P or soluble organic phosphate are of concern [27]. Organic P is rather refractory to both colorimetric and chromatography analysis, but some colloidal P can lead to an overestimation of the orthophosphate as soluble reactive P [63].

High-performance liquid chromatography (HPLC) has been used for sensitive separation and determination of

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linear poly-P up to a chain length of 35 [29]. A modified UV-assisted procedure was employed for the fractionation of total dissolved P in seawater that allowed differentiation of inorganic phosphate from photodecomposable organic P [50,52]. The high selectivity of electron ionization mass spectrometry (ESI-MS) allows the detection of different poly-P species without prior separation by ion chromatography or capillary electrophoresis [54], thus avoiding any underestimation associated with pre-extraction steps. Fractionation of soluble P forms via molecular weight cut-off (MWCO) ultrafiltration have been explored for characterizing biological wastewater effluent P [64]. Quite recently, the diffusive gradient in thin films technique (DGT) was introduced with its ability to concentrate diffusible P in situ at ambient ionic strength and pH without sample storage, so the changes in P speciation were minimized [27**,28]. Although traditional chemical analysis-based P quantification methods are labor-intensive and time-consuming, they are still widely applied in environmental studies owing to their cost-effectiveness and less demands on advanced instruments.

**Colorimetric method**

Metachromatic reaction of basic dyes such as Toluidine blue O (TBO) with poly-P presents an alternative method for polyphosphate quantification, but the chain length of poly-P must be higher than 10 [8]. The main advantage of the TBO assay is its rapidity, low-cost and easy operation; however, it suffers from limitations such as compatibility with other polyanions such as nucleic acids [30*].

**Electron microscopy**

Electron microscopy (EM) has been frequently used to visualize and locate intracellular poly-P granules in poly-P accumulating cells [8,23**,31]. EM is commonly applied with various staining methods for poly-P polymer detection including Neisser stain [65,66], Loeffler’s Methylene Blue [67] and more recently, DAPI staining (4’6-diamidino-2-phenylindole) [32–34]. Although electron microscopy or staining techniques alone cannot necessarily quantify the poly-P pool directly, they provide rather quick and easy identification and quantification of poly-P containing organisms (PAOs) in environmental samples such as activated sludge [68–70].
Cryoelectron tomography and spectroscopy imaging
Using the combination of cryoelectron tomography and spectroscopy imaging techniques, characterization of intact subcellular molecules such as P-rich or carbon-rich bodies in bacterial cells was demonstrated by Comolli et al. [53]. This technique allowed both chemical composition identification and the study of the biogenesis and morphology of these intracellular bodies at resolution greater than 10 nm.

DAPI fluorometric method
Fluorometric reaction of DAPI with polyphosphate allows quantitative detection of poly-P in sample extractions or in live cells such as PAOs, which is the key agent in enhanced biological phosphorus removal (EBPR) systems [23**,33,34]. Using excitation at 415 nm, fluorescence of the DAPI-poly-P complex can be detected at a higher wavelength of 550 nm for as little as 25 ng/ml P [22,33,35*,36]. Diaz and Ingall [35*] characterized the effect of polyphosphate chain length and showed that the fluorescence yielded by the DAPI-poly-P complex standards exhibit no chain length dependence across polyphosphates ranging from 15 to 130 Pi units in size. Poly-P of less than five phosphorus units in length contributes little to no signal in this approach [35*]. Kulakova et al. presented a direct DAPI-based protocol for quantification of intracellular poly-P without the requirement for poly-P extraction, but a simpler pretreatment (e.g. snap freezing in liquid nitrogen or at −20°C) is still needed to facilitate poly-P release from its bound state with variety of cellular compounds (e.g. proteins and nucleic acids) [37]. However, this direct DAPI protocol was found unsuitable for poly-P quantification in intact mammalian mitochondria owing to the high background fluorescence in such samples. At higher concentrations, DAPI dye reacts with both DNA and poly-P with poly-P–DAPI complex fluorescing as bright yellow and the DNA–DAPI complex fluorescing as sky blue. This feature has been employed to perform simultaneous observation and quantification of the relative abundance PAO as of total cells in a sample [32,69,70].

Enzymatic methods
Enzymatic methods involving exopolyphosphatase (PPX) and polyphosphate kinase (PPK) have been used to quantify polyphosphate with high specificity in bacterial samples [6,38]. PPX catalyze poly-P hydrolysis and the liberated phosphate is then quantified by the malachite green method. In PPK-based enzymatic assays, poly-P is quantitatively converted to ATP by PPK in the presence of a 10-fold excess of ADP. The ATP generated is then quantified using the luciferase system. A comparative study of different enzymatic methods for polyphosphate quantification was conducted by Ohtomo et al. [30*]. The PPK method was considered the most sensitive and specific for longer chain poly-P (>20 Pi units), whereas, the PPX method seemed to be the best suited for short-chain poly-P quantification. Other enzymes such as alkaline phosphatase (phosphomonesterase), phosphodiesterase, phospholipase and phytase have been employed for fractionation and quantification of organic P molecules [56,57*,58]. The advantage of enzymatic hydrolysis method is its ability to classify different types of P molecules and structures owing to the high specificity of these enzymes. But enzymatic techniques have had limited applications to heterogeneous environmental samples because of the difficulties in extracting highly pure polyphosphate analyte, potential interferences from metals and natural organic matters, as well as the unknown and complex composition of the P pools [8,57*].

Protein affinity labeling in vivo
Visualization of poly-P at the ultra-structural level in yeast was achieved by a novel technique based on enzymatic affinity and immunocytochemistry, in which specimens were labeled with epitopetagged PPBD, followed by the detection of the epitope tag by an indirect immunocytochemical method that uses the affinity of a recombinant poly-P-binding domain (PPBD) of E. coli PPX [55]. The method enabled direct visualization of the localization of poly-P at electron microscopic level with relatively high specificity and resolution.

Nuclear magnetic resonance spectroscopy
Nuclear Magnetic Resonance (NMR) is a powerful non-invasive technique for the investigation of different forms and species of phosphorus in both liquid and solid phases such as intact cells [25*,59]. The 31P NMR spectroscopic technique provides a detailed description of P-containing molecules with very high sensitivity to the chemical environment of the nucleus [8]. The main advantage of 31P NMR is that all P species, including poly-P, orthophosphate, pyrophosphate, phosphate monoesters, phosphate diesters and phosphonates are all visible in 31P NMR spectra and therefore they can be simultaneously characterized without the need of any complex cleanup and pre-fractionation procedures. Drawbacks of the method, however, include relatively higher detection limits and its vulnerability to the interferences resulted from the heterogeneous physical and chemical properties of the samples and the natural association of P with paramagnetic ions such as iron and manganese [8,59,60**]. Because 31P NMR can only detect phosphorus-containing molecules on the basis of bond class, the method is not ideally suited to distinguish between inorganic polyphosphate and other molecules that also contain phosphateanhydride bonds, such as nucleotides. 31P NMR is typically applied to indicate the relative abundance of polyphosphate in comparison to other P bond classes such as P esters and phosphonates, rather than to provide a direct measurement of the poly-P concentration [35*]. The 31P NMR spectroscopic technique was used for the detection and study of poly-P in different organism, poly-P storage in activated sludge and
phytoplankton samples [16,71]. $^{31}$P NMR measurements can be carried out with solid-state samples in vivo, which provide simultaneous detection of poly-P signals and other P metabolites making the technique an extremely powerful and least disruptive tool for on-line detection of poly-P or for phosphometabolomic profiling under various experimental conditions [39,72]. In addition to applying NMR for survey of phosphorus forms in environmental samples as has mostly been performed; the greater potential of NMR is yet to be explored for revealing phosphorus transformation, structures or reaction kinetics.

**Raman spectromicroscopy and near infrared reflectance (NIR) spectrometry**

Raman spectroscopy is based on inelastic light scattering events between a photon and a molecule that results in the excitation of molecular vibrations and it provides information about chemical composition and structure [40]. Recently, a Raman spectromicroscopy method was developed and applied in activated sludge from EBPR system for simultaneous identification and quantification of polyphosphate and other intracellular polymers in individual bacterial cell, making the investigation of metabolic states and heterogeneity of microbial populations in a biological system possible [41**,42,70]. Unique Raman spectra were identified to distinguish ortho-P, trimer, hexamer and longer poly-P present in PAOs. The ability of Raman spectroscopy for discriminating poly-P of varying chain lengths is yet to be investigated, but it has been suggested that this may be impossible owing to the broad appearance of the P-O-P bands in the same spectral region for polyphosphate of different chain lengths [26]. A notable advantage of this method is the minimal sample preparation required. In addition, it will be of interest to develop technique that combines Raman with other molecular tools for linking the intracellular polymers storage with the phylogenetic identity of the cells [41**]. Near infrared reflectance (NIR) spectrometry has been used to characterize OP compounds while minimizing the damage to cells, although its application is still rare [61].

**X-ray spectrometry and X-ray fluorescence spectromicroscopy**

When energy dispersive X-ray spectrometers (EDX) is applied in conjunction with scanning electron microscopy (SEM) and transmission electron microscopy (TEM) operating in the scanning transmission mode (STEM), it is abbreviated as EM-EDX [43]. EM-EDX or STEM-EDX allows direct quantification of dry mass and elemental contents of individual cells and their inclusions such as poly-P granules [43,44]. By using the EM-EDX technique, first evidence of intracellular poly-P granules and other forms of P enrichments have been provided for cultivated bacteria [45,46] and for activated sludge [8,47]. However, excessive preparation techniques should be avoided for X-ray analysis as those might cause unpredictable losses of P and other elements from cells [44]. In addition, quantification of the microbial poly-P content in environmental samples (i.e. sediment sample) by electron microscopy remains rather difficult if possible at all.

X-ray fluorescence spectromicroscopy (combined X-ray spectroscopy and X-ray microscopy) has been shown to be uniquely capable of elucidating the spatial concentration and speciation of elements at submicron scale in minimally prepared particulate samples including individual cells [48]. Several researchers have demonstrated the application of this method to unfold phosphorus chemistry and associated metals in individual microorganisms, particularly those that are difficult to culture or less prevalent in the sample [49**,73]. In comparison with electron microscopy techniques that offer compositional information at similar or higher spatial resolution, X-ray spectromicroscopy can analyze thicker samples (~10 μm) and has less restrictive requirements for sample preparation. In addition, this method typically results in far less radiation damage to soft specimens and therefore is well suited to the study of sensitive biological samples [48]. The main obstacles facing the expansion of X-ray spectromicroscopy as a prevalent technique are: the limited availability of suitable X-ray light sources with a broad range of X-ray energies (synchrotron facilities), precautions regarding sample mounting and, needs to establish standard spectral library [49**]. Furthermore, this technique only allows mapping of cells larger than 3 μm and smaller organisms may not be sufficiently resolved in X-ray micrographs at the moment [49**]. Soft X-ray fluorescence spectrometry in the form of X-ray absorption near edge structure spectrometry (XANES) allows the determination of OP containing species [62].

**Combination of P analysis with other molecular techniques**

Combining poly-P detection techniques with other molecular tools for phylogenetic identification of PAOs and for correlating specific PAOs populations with their metabolic functions have been demonstrated. For example, combination of DAPI–poly-P staining with Fluorescence In Situ Hybridization (FISH) for the detection and quantification of PAOs of different phylogenetic identities have been reported for EBPR systems [74]. This allows for the quantification of the relative abundances of PAOs subgroups in relative to the total PAOs populations in the activated sludge. Combined flow cytometry with fluorescent staining technique based on the complexation between the antibiotic tetracycline and divalent cations present in poly-P structure has been applied to sort active PAO populations. The phylogenetic affiliation of the PAOs population was further revealed by terminal restriction fragment length polymorphism (T-RFLP) profiling of 16S rRNA genes and sequencing [74,75**].
Conclusion remarks

There are a suite of phosphorus quantification and characterization techniques that have been developed for quantification and characterization of various forms of phosphorus in biological matrix. Most of the conventional, as well as some new analytical methods, require rather extensive pre-treatment and pre-fractionation procedures before analysis, which often introduce variations and errors in the results. There is still a lack of standardized extraction procedures and consented results reported among laboratories, making it difficult to compare results of different studies. P analysis based on sample extracts yield information on the mixed P pools and it misses the spatial or cellular resolution. Recently developed and more advanced analytical techniques, such as NMR, Raman and X-ray techniques, enable more comprehensive P analysis with higher molecular and spatial resolution at submicron scale in individual cells and in natural samples, yet with much less demands in sample pretreatment and preparation. These advances present great potential for their widespread applications in environmental and biological research. Selection of individual or combination of P analysis techniques should consider the particular P pools of interest, the sensitivity and resolution required, and the cost and accessibility to the instruments. Further research should focus on more frequent implementation of the non-invasive techniques, in environmental and biological sources, individually or in combinations, and identify approaches to improve their resolution, sensitivity and reliability.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

● of special interest
■ of outstanding interest

This paper reviews and discusses the current available methods for detection of key functionally-relevant intracellular polymers in poly-P accumulating microorganisms present in enhanced biological phosphorus removal systems.
This paper studied the application of 31P NMR in evaluating the changes in relative polyphosphate content, estimated as the intensity ratio of core polyphosphate signal and intracellular inorganic phosphate signal from 31P NMR spectra, during the growth of an algal fungi.

This paper compared four methods to measure P in solution: ion chromatography (IC), the malachite green colorimetric method (CM), the diffusive gradient in thin films technique (DGT) and, for totally dissolved P, optical inductively coupled plasma (ICP).


This paper evaluated the poly-P chain length specificity among three polyphosphate analysis methods and indicated that PPX methods is most appropriate to detect short-chain poly-P.


The authors describe a new approach for the direct quantification of inorganic polyphosphate in complex natural samples using fluorochrome reaction of 4',6-diamidino-2-phenylindole (DAPI) with dissolved polyphosphate.


This paper described a Raman spectromicroscopy method for detecting and quantifying poly-P inclusions inside individual cell.


The authors give detailed description of the protocol for applying X-ray fluorescence spectromicroscopy for revealing the poly-P inclusions and associated metal cations associated with poly-P in algal cells.


This study developed a new protocol to assess the enzymatically available fraction of dissolved organic phosphorus in waters under environmentally relevant conditions. The method was applied to sediment porewaters and sewage treatment plant liquors.


This paper provides a critical review of various methods for determination of organic phosphorus (OP) and organic nitrogen (ON) in aquatic systems. Methods widely used for quantification and characterization of organic phosphorus is described and discussed.


This paper demonstrated the application of a novel fluorescent staining technique using flow cytometry for the quantitative detection of polyphosphate granules on cellular level and enabled the detection of most active polyphosphate accumulating individuals/subcommunities.