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Advances in techniques for phosphorus analysis in biological sources

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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 *in vitro* and *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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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, nontoxicity 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.

Methods for analyzing phosphorus in biological sources

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 solublization 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

Table 1

Summary of analytical methods for phosphorus analysis in biological systems

Forms of P	Analytical methods	References
Orthophosphate (ortho-P)	Colorimetry	[20,21,22,23••]
	Ion chromatography (IC)	[22]
	Inductively coupled plasma atomic emission spectroscopy (ICP-AES)	[24]
	Nuclear magnetic resonance spectroscopy (^{31}P NMR)	[8,25•]
	Raman spectrometry	[26]
	Diffusive gradient in thin films (DGT) technique	[27••,28]
Polyphosphate (poly-P)	Chemical digestion followed by ortho-P analysis	[22,23••]
	High performance liquid chromatography (HPLC)	[29]
	Gel electrophoresis	[11]
	Thin layer chromatography	[13]
	Metachromatic colorimetry (Toluidine blue O (TBO))	[8,30•]
	Electron microscopy (EM)	[8,23••,31]
	DAPI-staining	[23••,32–37]
	Enzymatic methods	[6,30•,38]
	^{31}P NMR	[8,25•,39]
	Raman spectromicroscopy	[26,40–42]
	Energy dispersive X-ray spectrometry	[8,43–47]
	X-ray fluorescence spectromicroscopy	[48,49••]
	UV-assisted procedure	[50–52]
	Cryoelectron tomography and spectroscopy imaging (CTSI)	[53]
	Electron ionization mass spectrometry (EI-MS)	[54]
	Protein affinity labeling *in vivo*	[55]
Organic phosphorus (OP)	Chemical digestion followed by ortho-P analysis	[22]
	Enzymatic methods	[56,57•,58]
	^{31}P NMR	[8,59,60••]
	Near infrared reflectance (NIR) spectrometry	[61]
	X-ray absorption near edge structure spectrometry (XANES)	[62]
	UV-assisted procedure	[50–52]
	Molecular weight cut-off ultrafiltration (MWCO)	[64]

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].

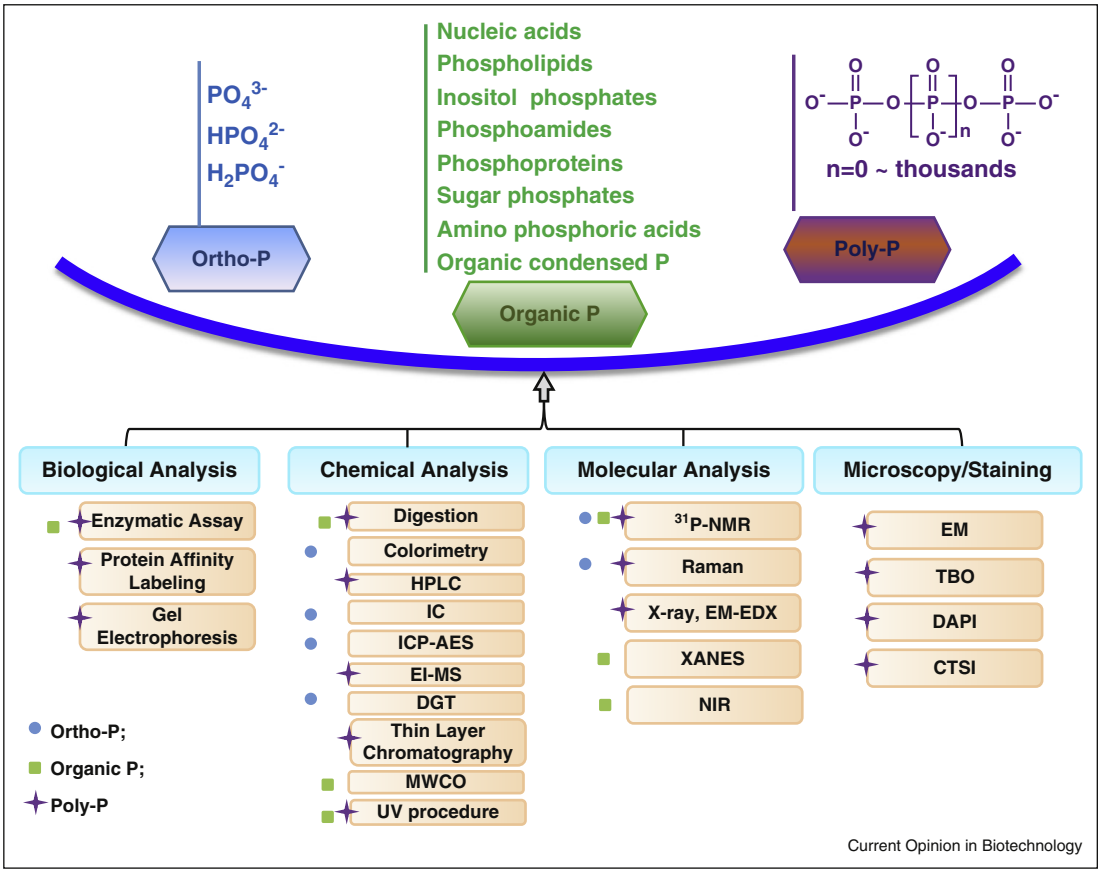
Methods have also been developed to distinguish between different intracellular P compounds as well as between chemically precipitated and biologically stored P in activated sludge systems [5,14]. A method consisting of sequential extractions using cold distilled water, bicarbonate–dithionite, NaOH and HCl was applied to differentiate the fractions of P such as adsorbed forms of P, metal bound P, P complexes, soluble P, organic P, $CaCO_3$, $MgCO_3$ 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••,24]. 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

Figure 1



Suites of analytical methods for detection and characterization of various forms of phosphorus present in biological sources. The forms of P that each method can analyze are indicated by symbols.

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 photo-decomposable 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* PPX1 [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 ^{31}P 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 ^{31}P NMR is that all P species, including poly-P, orthophosphate, pyrophosphate, phosphate monoesters, phosphate diesters and phosphonates are all visible in ^{31}P 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 ^{31}P 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 phosphoanhydride bonds, such as nucleotides. ^{31}P 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 ^{31}P 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 phosphorous 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 microorganism cells, 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

1. Rao NN, Gómez-García MR, Kornberg A: **Inorganic polyphosphate: essential for growth and survival**. *Annu Rev Biochem* 2009, **78**:605-647.
2. Kornberg A, Rao NN, Ault-Riché D: **Inorganic polyphosphate: a molecule of many functions**. *Annu Rev Biochem* 1999, **68**:89-125.
3. Kulakovskaya TV, Vagabov VM, Kulaev IS: **Inorganic polyphosphate in industry, agriculture and medicine: modern state and outlook**. *Process Biochem* 2012, **47**:1-10.
4. Levlin E, Hultman B: **Phosphorus recovery from phosphate rich side-streams in wastewater treatment plants**. *Polish Swedish Seminar*; *Gdansk, March 23–25, Polish-Swedish Report, No. 10: 2003:46-47*.
5. Mino T, Kawakami T, Matsuo T: **Location of phosphorus in activated sludge and function of intracellular polyphosphates in biological phosphorus removal process**. *Water Sci Technol* 1985, **17**:93-106.
6. Clark JE, Beegen H, Wood HG: **Isolation of intact chains of polyphosphate from *Propionibacterium shermanii* grown on glucose or lactate**. *J Bacteriol* 1986, **168**:1212-1219.
7. Lockwood GL, Lindrea KC, Seviour RJ: **The distribution of phosphorus and ions in the biological phosphorus removal process**. In *In Proceedings of the First Australian Conference on Biological Nutrient Removal from Wastewater*; *Bendigo: 1990:195-204*.
8. Hupfer M, Glöss S, Schmieder P, Grossart H-P: **Methods for detection and quantification of polyphosphate and polyphosphate accumulating microorganisms in aquatic sediments**. *Int Rev Hydrobiol* 2008, **93**:1-30.
9. Bonting CF, Kortstee GJ, Zehnder AJ: **Properties of polyphosphate: AMP phosphotransferase of Acinetobacter strain 210A**. *J Bacteriol* 1991, **173**:6484-6488.
10. Ahlgren J, Brabandere HD, Reitzel K, Rydin E, Gogoll A, Waldebäck M: **Sediment phosphorus extractants for phosphorus-^{31}P nuclear magnetic resonance analysis: a quantitative evaluation**. *J Environ Qual* 2007, **36**:892-898.
11. Clark JE, Wood HG: **Preparation of standards and determination of sizes of long-chain polyphosphates by gel electrophoresis**. *Anal Biochem* 1987, **161**:280-290.
12. Lorenz B, Schröder HC: **Methods for investigation of inorganic polyphosphates and polyphosphate-metabolising enzymes**. *Prog Mol Subcell Biol* 1999, **23**:217-239.
13. Tanzer JM, Krichevsky MI, Chassy B: **Separation of polyphosphates by anion exchange thin layer chromatography**. *J Chromatogr* 1968, **38**:526-531.
14. De Haas DW, Wentzel MC, Ekama GA: **The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting biological excess phosphate removal. Part 1: literature review**. *Water S A* 2000, **26**:439-452.
15. Uhlmann D, Röske I, Hupfer M, Ohms G: **A simple method to distinguish between polyphosphate and other phosphate fractions of activated-sludge**. *Water Res* 1990, **24**:1355-1360.
16. Röske I, Schönborn C: **Interactions between chemical and advanced biological phosphorus elimination**. *Water Res* 1994, **28**:1103-1109.
17. Lötter LH: **The role of bacterial phosphate metabolism in enhanced phosphorus removal from the activated sludge process**. *Water Sci Technol* 1985, **17**:127-138.
18. Karl DM, Bailiff MD: **The measurement and distribution of dissolved nucleic acids in aquatic environments**. *Limnol Oceanogr* 1989, **34**:543-558.
19. Cosgrove DJ: *Inositol Phosphates: Their Chemistry, Biochemistry and Physiology*. 1980.
20. Pierzynski GM (Ed): *Methods of phosphorus analysis for soils, sediment, residuals, and waters*. 2000, **39**:31-34.
21. Lindrea K, Lockwood G, Majone M: **The distribution and movement of polyphosphate and associated cations in sludges from NDEBPR plants in different configurations at pilot scale**. *Water Sci Technol* 1998, **37**:555-562.
22. APHA: *Standard Methods for the Examination of Water and Wastewater*. Washington, DC: American Public Health Association; 1998.
23. •• Serafim LS, Lemos PC, Levantesi C, Tandoi V, Santos H, Reis MAM: **Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms**. *J Microbiol Methods* 2002, **51**:1-18.

This paper reviews and discusses the current available methods for detection of key functionally-relevant intracellualr polymers in poly-P accumulating mciroorganisms present in enhanced biological phosphorus removal systems.

24. Coulibeuf C LJ: **A fast elemental determination of environmental samples with ICP-AES**. *ICP Application Report* 1998.
25. • Živić M, Zakrzewska J, Žižić M, Bačić G: **^{31}PNMR study of polyphosphate levels during different growth phases of Phycomyces blakesleeanus**. *Antonie Van Leeuwenhoek* 2007, **91**:169-177.

This paper studied the application of ^{31}P NMR in evaluating the changes in relative polyphosphate content, estimated as the intensity ratio of core polyphosphate signal and intracellular inorganic phosphate signal from ^{31}P NMR spectra, during the growth of an algal fungi.

26. Jager HJD, Heyns AM: **Study of the hydrolysis of sodium polyphosphate in water using Raman spectroscopy**. *Appl Spectrosc* 1998, **52**:808-814.

27. •• Moorleghem CV, Six L, Degryse F, Smolders E, Merckx R: **Effect of organic P forms and P present in inorganic colloids on the determination of dissolved P in environmental samples by the diffusive gradient in thin films technique, ion chromatography, and colorimetry**. *Anal Chem* 2011, **83**:5317-5323.
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 total dissolved P, optical inductively coupled plasma (ICP).

28. Ding S, Di X, Qin S, Hongbin Y, Chaosheng Z: **Measurement of dissolved reactive phosphorus using the diffusive gradients in thin films technique with a high-capacity binding phase**. *Environ Sci Technol* 2010, **44**:8169-8174.

29. Baba Y, Yoza N, Ohashi S: **Simultaneous determination of phosphate and phosphonate by flow injection analysis and high-performance liquid chromatography with a series detection system**. *J Chromatogr* 1985, **318**:319-324.

30. • Ohtomo R, Sekiguchi Y, Kojima T, Saito M: **Different chain length specificity among three polyphosphate quantification methods**. *Anal Biochem* 2008, **383**:210-216.
This paper evaluted the poly-P chain length specificity among three polyphophate analysis methods and indicated that PPX methods is most appropriate to detect short-chain poly-P.

31. Bond PL, Rees GN: **Microbiological aspects of phosphorus removal in activated sludge systems**. In *The Microbiology of Activated Sludge*. Edited by Seviour RJ, Blackall LL. London, UK: Kluwer Academic Publishers; 1999:227-256.

32. Streichan M, Golecki JR, Schön G: **Polyphosphate-accumulating bacteria from sewage plants with different proceses for biological phosphorus removal**. *FEMS Microbiol Lett* 1990, **73**:113-124.

33. Aschar-Sobbi R, Abramov AY, Diao C, Kargacin ME, Kargacin GJ, French RJ, Pavlov E: **High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach**. *J Fluorescence* 2008, **18**:859-866.

34. Eixler S, Selig U, Karsten U: **Extraction and detection methods for polyphosphate storage in autotrophic planktonic organisms**. *Hydrobiologia* 2005, **533**:135-143.

35. • Diaz JM, Ingall ED: **Fluorometric quantification of natural inorganic polyphosphate**. *Environ Sci Technol* 2010, **44**:4665-4671.
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.

36. Tijssen JP, Beekes HW, Van Steveninck J: **Localization of polyphosphates in *Saccharomyces fragilis*, as revealed by 4′,6-diamidino-2-phenylindole fluorescence**. *Biochim Biophys Acta* 1982, **721**:394-398.

37. Kulakova AN, Hobbs D, Simthen M, Pavlov E, Gilbert JA, Quinn JP, McGrath JW: **Direct quantification of inorganic polyphosphate in microbial cells using 4′-6-diamidino-2-phenylindole (DAPI)**. *Environ Sci Technol* 2011, **45**:7799-7803.

38. Ault-Riché D, Fraley CD, Tzeng CM, Kornberg A: **Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli***. *J Bacteriol* 1998, **180**:1841-1847.

39. Sannigrahi P, Ingall E: **Polyphosphate as a source of enhanced P fluxes in marine sediments overlain by anoxic waters: evidence from ^{31}PNMR**. *Geochem Trans* 2005, **6**:52-59.

40. Schuster K, Urlaub E, Gapes J: **Single-cell analysis of bacteria by Raman microscopy: spectral information on the chemical composition of cells and on the heterogeneity in a culture**. *J Microbiol Methods* 2000, **42**:29-38.

41. •• Majed N, Matthaus C, Diem M, Gu AZ: **Evaluation of intracellular polyphosphate dynamics in enhanced biological phosphorus removal process using Raman microscopy**. *Environ Sci Technol* 2009, **43**:5436-5442.
This paper described a Raman spectromicroscopy method for detecting and quantifying poly-P inclusions inside individual cell.

42. Majed N, Gu AZ: **Application of Raman microscopy for simultaneous and quantitative evaluation of multiple intracellular polymers dynamics functionally relevant to enhanced biological phosphorus removal processes**. *Environ Sci Technol* 2010, **44**:8601-8608.

43. Goldberg J, Gonzalez H, Jensen TE, Corpe WA: **Quantitative analysis of the elemental composition and the mass of bacterial polyphosphate bodies using STEM EDX**. *Microbios* 2001, **106**:177-188.

44. Heldal M: **Measurement of elemental content and dry weight of single cells: X-ray microanalysis**. *Handbook of Methods in Aquatic Microbial Ecology*. Boca Raton, Florida: Lewis Publishers; 1993:. 387–394.

45. Baxter M, Jensen T: **A study of methods for in situ X-ray energy dispersive analysis of polyphosphate bodies in Plectonema boryanum**. *Arch Microbiol* 1980, **126**:213-215.

46. Sicko-Goad LM, Crang RE, Jensen TE: **Phosphate metabolism in blue-green algae. IV. In situ analysis of polyphosphate bodies by X-ray energy dispersive analysis**. *Cytobiologie* 1975, **11**:430-437.

47. Buchan L: **The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal**. *Water S A* 1981, **7**:1-7.

48. Diaz J, Ingall E, Benitez-Nelson C, Paterson D, de Jonge MD, McNulty I, Brandes JA: **Marine polyphosphate: a key player in geologic phosphorus sequestration**. *Science* 2008, **320**:652-655.

49. •• Diaz J, Ingall E, Vogt S, de Jonge MD, Paterson D, Rau C, Brandes JA: **Characterization of phosphorus, calcium, iron, and other elements in organisms at sub-micron resolution using X-ray fluorescence spectromicroscopy**. *Limnol Oceanogr Methods* 2009, **7**:42-51.
The authors give detailed description of the protocol for applying X-ray fluoresence spectromicroscopy for revealing the poly-P inclusions and associated metal cations associated with poly-P in algal cells.

50. Yanagi K, Yasuda M, Fukui F: **Reexamination of the fractionation of total dissolved phosphorus in seawater using a modified UV-irradiation procedure, and its application to samples from Suruga Bay and Antarctic Ocean**. *J Oceanogr* 1992, **48**:267-281.

51. Armstrong FAJ, Tibbitts S: **Photochemical combustion of organic matter in sea water, for nitrogen, phosphorus and carbon determination**. *J Mar Biol Assoc U K* 1968, **48**:143-152.

52. Goossen JT, Kloosterboer JG: **Determination of phosphates in natural and waste waters after photochemical decomposition and acid hydrolysis of organic phosphorus compounds**. *Anal Chem* 1978, **50**:707-711.

53. Comolli LR, Kundmann M, Downing KH: **Characterization of intact subcellular bodies in whole bacteria by cryo-electron tomography and spectroscopic imaging**. *J Microsc* 2006, **223**:40-52.

54. Choi BK, Hercules DM, Houalla M: **Characterization of polyphosphates by electrospray mass spectrometry**. *Anal Chem* 2000, **72**:5087-5091.

55. Saito K, Ohtomo R, Kuga-Uetake Y, Aono T, Saito M: **Direct labeling of polyphosphate at the ultrastructural level in Saccharomyces cerevisiae by using the affinity of the polyphosphate binding domain of *Escherichia coli* exopolyphosphatase**. *Appl Environ Microbiol* 2005, **71**:5692-5701.

56. Turner BL, McKelvie ID, Haygarth PM: **Characterization of water-extractable soil organic phosphorus by phosphatase hydrolysis**. *Soil Biol Biochem* 2002, **34**:27-35.

57. • Monbet P, McKelvie ID, Saefumillah A, Worsfold PJ: **A protocol to assess the enzymatic release of dissolved organic phosphorus species in waters under environmentally relevant conditions**. *Environ Sci Technol* 2007, **41**:7479-7485.
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.

58. Monbet P, McKelvie ID, Worsfold PJ: **Dissolved organic phosphorus speciation in the waters of the Tamar estuary (SW England)**. *Geochim Cosmochim Acta* 2009, **73**:1027-1038.

59. Cade-Menun BJ: **Using Nuclear Magnetic Resonance Spectroscopy to characterize organic phosphorus in environmental samples**. In *Organic Phosphorus in the Environment.* Edited by Turner BL, Frossard E, Baldwin D. CABI publishing; 2004.

60. •• Worsfold PJ, Monbet P, Tapin AD, Fitzsimons MF, Stiles DA, McKelvie ID: **Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: a review**. *Anal Chim Acta* 2008, **624**:37-58.
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 quantifiaction and characterization of organic phosphorus is described and discussed.

61. Malley DF, Lockhart L, Wilkinson P, Houser B: **Determination of carbon, carbonate, nitrogen and phosphorus in freshwater sediments by near-infrared reflectance spectroscopy: rapid analysis and check on conventional analytical methods**. *J Paleolimnol* 2000, **24**:415-425.

62. Brandes JA, Ingall E, Paterson D: **Characterization of minerals and organic phosphorus species in marine sediments using soft X-ray fluorescence spectromicroscopy**. *Mar Chem* 2007, **103**:250-265.

63. Gu AZ, Liu L, Neethling JB, Stensel HD, Murthy S: **Treatability and fate of various phosphorus fractions in different wastewater treatment processes**. *Water Sci Technol* 2011, **63**:804-810.

64. Gu AZ, Liu L, Onnis-Hayden A, Smith S, Gray H, Houweling D, Takacs Imre: **Phosphorus Fractionation and Removal in Wastewater Treatment- Implications for Minimizing Effluent Phosphorus**. *Water Environment Research Foundation (WERF) and International Water Association (IWA) Publishing*, 2012.

65. Eikelboom DH, Van Buijsen HJJ: *Microscopic Sludge Investigation Manual*. edn 2. 1983.

66. Jenkins D, Richards MG, Daigger GT: *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*. edn 2. 1993.

67. Murray RGE, Doetsch RN, Robinow CF: **Determinative and cytological light microscopy**. In *Methods for General and Molecular Bacteriology.* Edited by Gehardt P, Murray RGE, Wood WA, Krieg NR. 1994.

68. Gu AZ, Saunders A, Neethling JB, Stensel HD, Blackall LL: **Functionally relevant microorganisms to enhanced biological phosphorus removal performance at full-scale wastewater treatment plants in the United States**. *Water Environ Res* 2008, **80**:688-698.

69. He S, Gu AZ, McMahon KD: **Progress toward understanding the distribution of Accumulibacter among full-scale enhanced biological phosphorus removal systems**. *Microb Ecol* 2008, **55**:229-236.

70. Majed N, Chernenko T, Diem M, Gu AZ: **Identification of functionally relevant populations in enhanced biological phosphorus removal processes based on intracellular polymers profiles and insights into the metabolic diversity and heterogeneity**. *Environ Sci Technol* 2012, **46**:5010-5017.

71. Feuillade J, Bielicki G, Renou JP: **^{31}P-NMR study of natural phytoplankton samples**. *Hydrobiologia* 1995, **300**:391-398.

72. Nemutlu E, Zhang S, Gupta A, Juranic NO, Macura SI, Terzic A, Jahangir A, Dzeja P: **Dynamic phosphometabolomic profiling of human tissues and transgenic models by 18O-assisted ^{31}P NMR and mass spectrometry**. *Physiol Genomics* 2012, **44**:386-402.

73. Krivtsov V, Bellinger E, Sigee D: **Elemental composition of Microcystis aeruginosa under conditions of lake nutrient depletion**. *Aquat Ecol* 2005, **39**:123-134.

74. Kawaharasaki M, Tanaka H, Kanagawa T, Nakamura K: **In situ identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4′,6-diamidino-2phenylindol (DAPI) at a polyphosphate-probing concentration**. *Water Res* 1999, **33**:257-265.

75. •• Günther S, Trutnau M, Kleinsteuber S, Hause G, Bley T, Röske I, Harms H, Müller S: **Dynamics of polyphosphate-accumulating bacteria in wastewater treatment plant microbial communities detected via DAPI (4′,6′-diamidino-2-phenylindole) and tetracycline labeling**. *Appl Environ Microbiol* 2009, **75**:2111.
This paper demonstrated the application of a novel fluorescent staining technique using flow cytometry for the quantitative detection of polyphosphate granules on cellular level that enabled the detection of most active polyphosphate accumulatingindividuals/subcommunities.