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Extraction and quantification of phosphorus derived from DNA and lipids in environmental samples

Julia V. Paraskova, Emil Rydin, Per J.R. Sjöberg *

Department of Chemistry – BMC, Uppsala University, Uppsala, Sweden

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ABSTRACT

Understanding the flux and turnover of phosphorus (P) in the environment is important due to the key role P plays in eutrophication and in the ambition to find cost-effective measures to mitigate it. Orthophosphate diesters, including DNA and phospholipids (PLs), represent a potentially degradable P pool that could support future primary production and eutrophication. In this study, extraction techniques were optimized and combined with colorimetric determination of extracted P to provide a selective quantification method for DNA-P and PL-P in agricultural soil, sediment and composted manure. The proposed method is rapid and reproducible with an RSD of $<10\%$. Recovery, evaluated by spiking the sample matrices with DNA and PL standards, was over 95% for both DNA and PLs. The method can be used for the determination of the pool size of the two organic P fractions. Results show that DNA-P comprises 3.0% by weight of the total P (TP) content in the studied soil, 10.4% in the sediment and 8.4% in the compost samples. The values for PL-P are 0.5%, 6.0% and 1.7% for soil, sediment and compost, respectively.

1. Introduction

Phosphorus (P) and its ecological significance have been studied for a long time. It was linked to marine productivity already in the 1940s [1] and decades later, recognized as one of the key factors causing eutrophication [2,3]. Organic P may be a significant contributor to eutrophication, since it has been shown that it can be converted into bioavailable orthophosphate through different processes, for example enzymatic hydrolysis [4], under increased salinity conditions [5] or under periods of P stress, i.e. when easily utilized inorganic phosphate becomes scarce [6]. Organic P encompasses numerous compounds, including orthophosphate monoesters, such as inositol phosphates, orthophosphate diesters, such as nucleic acids and phospholipids (PLs), phosphoproteins and others [7], all with potential different turnover time in the environment. We need tools to identify and quantify these specific organic P forms in order to fully understand their role in supporting ecosystem productivity.

Studying organic P in the environment has been a challenge due to difficulties in extraction and identification, and therefore there is a substantial gap in our current knowledge about its ecological role [8]. Sequential fractionation of P into pools based on solubility or biological relevance focuses on altering such parameters as ion strength, level of pH and redox potential of the extraction buffers [9,10]. However, the two main drawbacks of such methods are firstly that they are not compound specific, but rather operationally defined, i.e. acid-extracted P can include both organic and inorganic P, and secondly that they are hard to experimentally validate, especially for different matrices. A way to circumvent these drawbacks is by focusing on extracting and quantifying specific organic P compounds. The interest in the orthophosphate diester pool is prompted by the fact that nucleic acids, in particular DNA, and PLs come from living organisms. They are present in the ecosystem as live and dead matter and therefore offer a continuously replenishing pool of P available for uptake for plants and other organisms. It has been hypothesized that since orthophosphate diesters are more labile then monoesters, they are also more readily mineralized [11]. Richardson et al. has proposed that sugar phosphates and phosphate diesters may be of greater significance in P cycling than the more abundant fractions, such as inositol phosphates, because of their likely higher turnover rates [12], indicating that the diester compounds play a vital role in regulating the turnover of organic P in the ecosystem.

The isolation and quantification of DNA and PLs have been studied extensively because of their scientific relevance in many different fields, including biochemistry and microbiology. Some difficulties have been experienced in isolating DNA from environmental samples, especially soil and compost, because of the fact

Abbreviations: (PL), phospholipid; (TP), total phosphorus; (SDS), sodium dodecyl sulfate; ($InsP_6$), *myo*-Inositol hexakis (dihydrogenphosphate); (NMR), nuclear magnetic resonance; (MS), mass spectrometry.

* Correspondence to: P.O. Box 599, Husargatan 3, S-751 24 Uppsala, Sweden. Tel.: +46 18 471 3685; fax: +46 18 471 3692.
E-mail address: per.sjoberg@kemi.uu.se (P.J.R. Sjöberg).

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that many organic substances often co-extract during the procedure and interfere with DNA quantification methods [13,14]. An overview of DNA extraction strategies from soil reached the conclusion that regardless of the method, there will be always a compromise between expected DNA quantity and purity required in molecular analysis [14]. Given that the object of this work was to quantify the P in the DNA fraction and not the DNA itself, purity of the samples was not an issue. The strategy was therefore to increase the DNA-P yield using a minimal number of purification steps. Lipid extraction and fatty acid analysis have been used routinely in microbiology for the assessment of microbial biomass [15–17]. Although not difficult, the procedures can be time-consuming since they aim at fractionating the lipids into different groups, often based on polarity. Such fractionation was unnecessary in this work as the focus was on the PLs in the total lipid pool and the selectivity was achieved through targeting of P.

In this study existing knowledge in DNA and PL research is used to create a reliable analytical procedure for the extraction and quantification of P derived from DNA and PLs in environmental samples. The origins or fate of these compounds in the environment, which have been reviewed elsewhere [18,19], are not addressed. The proposed method is designed to act as a fast and reproducible quantification tool for P found in DNA and PLs in three common types of environmental samples: soil, sediment and compost. In this case, the isolation of a very specific class of compounds and their digestion prior to phosphate determination offers selectivity in the quantification, although an indirect colorimetric P determination method is used.

2. Materials and methods

2.1. Samples

Experiments were performed on agricultural soil, lake sediment and composted manure. Detailed sample characteristics are given in Table 1. Soil samples (silty clay loam, clay content 30%) were collected from a field in Bjertorp, Sweden in October 2008. The soil has been fertilized yearly with 40 kg P/ha, applied as superphosphate. Soil samples have been air-dried, sieved through a 2 mm sieve and stored at ambient room temperature. Sediment cores were collected with a gravity core sampler at a depth of 30 m in a typical mesotrophic lake, Lake Erken, Sweden in September 2011. The top 3 cm layer of the core was pooled and kept at 4 °C. Composted horse manure samples were collected from a local horse farm, Valsätra hästgård in Uppsala, Sweden in June 2010. The compost was homogenized by a rotary mixer (Philips, Germany) and after coarse matter was removed by hand, the samples were stored at 4 °C. Prior to PL extraction sediment and compost samples were lyophilized in a ScanVac CoolSafe freeze-dryer.

2.2. Chemicals and instrumentation

All chemicals were of analysis grade (Sigma-Aldrich, Germany), unless otherwise specified. Dilution was performed with Milli-Q (MQ) water, (Millipore, Bedford, MA). A vortex mixer (Vortex-Genie® 2, Scientific Industries, Inc.) with an attachment (MoBio Laboratories, Inc.) allowing for horizontal positioning of the vials was used for mechanical cell disruption. Purification of DNA was done with a mixture of phenol, chloroform and isoamyl alcohol (v:v:v 25:24:1) and a mixture of chloroform and isoamyl alcohol (v:v 24:1). All plasticwares and glass beads were purchased from VWR, Sweden. Ultrafiltration was achieved by centrifugation with Nanosep centrifugal devices 30 K (PALL Life Sciences), referred in the text as spin filters. P content was quantified by UV/vis-spectrometry with UNICAM 5626 UV/vis spectrometer (Unicam Limited, Cambridge, UK) with a 4 cm flow-through cuvette. Calibration was done with solutions prepared from single-element P stock (Spectrascan, Teknolab AB, Kungsbacka, Sweden) by dilution with MQ water. DNA recovery was evaluated with standards prepared from stock solution of deoxyribonucleic acid from calf thymus. PL recovery was evaluated with standards prepared from stock solution of phosphatidylserine. Spin filter efficacy was assessed with solutions of phosphate, DNA, ribonucleic acid (RNA) type VI from Torula yeast and the dipotassium salt of *myo*-Inositol hexakis (dihydrogenphosphate) ($InsP_6$).

2.3. Screening experiments

Two different four-factor analyses with 5% probability were designed in Minitab 15 to screen for possible interactions between the parameters influencing cell lysis, a prerequisite for the DNA extraction. The procedure was optimized based on the most commonly used methods and chemicals, the concentration of EDTA in the extraction buffer (50, 200 mM), incubation temperature (25, 49 °C) and the presence of two enzymes, lysozyme (50 μg ml^{-1}) and proteinase K (200 μg ml^{-1}). A follow up experiment examined the significance and interaction between sodium dodecyl sulfate (SDS) concentration (1, 5%), incubation time (1, 16 h) and the two enzymes. Each experiment was done in two blocks and in random order.

The significant parameters from the initial screening experiments were then tested together with three cell lysis treatments, alone or in combination with vigorous shaking with glass beads (Table 2).

2.4. Extraction of DNA

The samples (0.15 g soil, 0.12 g compost, 0.70 g sediment, wet weight) were mixed with 1.0 ml extraction buffer (50 mM NaCl, 50 mM EDTA, 50 mM Tris-HCl, pH=8.0), 50 μl lysozyme (100 mg ml^{-1}) and 0.5 ml glass beads (0.25–0.5 mm). Samples were shaken on the highest setting of a vortex mixer for 90 min at ambient room temperature. After the addition of 0.1 ml proteinase K (1 mg ml^{-1}) and 0.1 ml SDS (10% v-v) the samples were shaken for an additional 90 min. The samples were then centrifuged at 6000*g* for 5 min and the supernatant decanted into a fresh tube. The nucleic acid suspension was purified three times, once with an equal volume of a mixture of phenol, chloroform and

Table 1
Sample properties.

Sample type	Location	Land use	Organic content [%]	pH (H_2O)	TP [mg P/g DW*]
Soil	Bjertorp, Sweden	Arable	5.2	6.6	0.98 ± 0.05
Sediment	Erken, Sweden	Lake	21.7	7.5	1.44 ± 0.03
Composted manure	Uppsala, Sweden		77.0	7.5	4.51 ± 0.02

Soil pH was measured in water at a soil:water (v:v) ratio of 1:5. Organic content was determined by dry ashing (550 °C for 4 h). Total phosphorus (TP) content was determined by ICP-AES after dry ashing and hydrochloric acid digestion.

* DW-dry weight.