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

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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

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

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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 $\mu g\ ml^{-1}$) and proteinase K (200 $\mu g\ ml^{-1}$). 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.

Table 2
Parameters tested in the optimization of cell lysis procedure.

Temperature [°C]	Enzymes	Mechanical force	SDS [%]	Extraction time [h]
25, 37, 49, 65	No enzymes, Lysozyme/Proteinase K	No force, Vigorous shaking with glass beads	1, 5	0.5, 1, 2, 5, 8, 24

Sample size and composition of the extraction buffer were the same for all methods.

isoamyl alcohol and twice with an equal volume of a mixture of chloroform and isoamyl alcohol [20]. This was achieved by shaking the sample with the organic mixture until an emulsion was formed. A subsequent 5 min centrifugation step at 6000*g* separated the lower organic phase from the upper, aqueous phase, containing DNA, by an interphase containing impurities. A 50 μl aliquot of the purified sample together with 50 μl MQ-water were loaded onto a spin filter and centrifuged at 6000*g* for 15 min, retaining DNA on top of the filter membrane. The sample was then washed by adding 100 μl of MQ water to the spin filter and centrifuging at 6000*g* for 15 min. The entire filter with the sample collected onto it was processed further (see Section 2.7).

2.5. Extraction of phospholipids

Extraction of PLs was performed using a scaled down version of a method described by Frostegård et al. [16]. Samples (0.2 g soil, 0.05 g sediment or compost, dry weight) were mixed with 1.5 ml of a single-phase mixture of chloroform, methanol and 0.15 M citrate buffer, adjusted to pH=4.0 (v:v:v 1:2:0.8) and extracted twice at room temperature for 20 min. After each extraction the samples were centrifuged at 5000*g* for 10 min and the supernatants were pooled. A two-phase mixture was created by adding 0.8 ml chloroform and 0.8 ml citrate buffer to the extract. The mixture was vortexed for 1 min and left for the phases to separate for 90 min at ambient room temperature. After a 10 min centrifugation step at 5000*g* a portion of the lower organic phase was pipetted out and 200 μl aliquots were transferred into glass vials and the organic solvent was evaporated under a stream of nitrogen. The PL material was immediately processed further (see Section 2.7).

2.6. Spiking experiments

Recovery, assessment of losses and matrix effects associated with the two extraction procedures were evaluated with DNA and PL standard solutions. Standard recovery (%) was determined by comparing the obtained P concentration of the standard samples (C_{st}) processed by the DNA and PL extraction protocols versus non-processed standard reference samples (C_{ref}) using

$$\text{Standardrecovery} \quad (\%) = (C_{st}/C_{ref})100\% \tag{1}$$

The effect of matrices on recovery was evaluated by spiking the different samples with 200 μl aliquots of DNA and PL standard solutions prior to extraction. Reference standard solutions ($C_{spike\ (DNA)}=0.5\ \text{mg ml}^{-1}$, $C_{spike\ (PL)}=0.1\ \text{mg ml}^{-1}$) were combined with samples after they had been suspended in the respective extraction buffer. The spiked samples were then extracted and analyzed in the usual manner. Recovery was determined by comparing the P concentration in the sample matrix without the addition of standard (C_{mat}), with the spiked sample (C_{spike}) and non-processed standard reference sample (C_{ref}) using

$$\text{Recoveryfromsample} \quad (\%) = [(C_{spike}-C_{mat})/C_{ref}]100\% \tag{2}$$

The experiment was carried out in duplicate or triplicate and mean values for C_{st}, C_{ref}, C_{mat} and C_{spike} were used in the calculations. Precision of the final results was evaluated through random error propagation [21].

2.7. Digestion

Phosphate in the DNA material was released through digestion with potassium persulfate, $K_2S_2O_8$. This was done by adding 4 ml of MQ water and 1 ml saturated persulfate solution (5%) to the vials containing the spin filters with the nucleic acid material. Phosphate in the PL material was released through dry ashing at 550 °C for 4 h and subsequent dissolution of the sample in 1 ml 1 M hydrochloric acid. All samples and standard solutions were autoclaved for 1 h at 120 °C. After cooling, acidic samples were neutralized with an aliquot of 1 M sodium hydroxide.

2.8. Characteristics of the determination method

The P concentration was determined using the molybdenum blue method [22] using ascorbic acid as a reducing agent and a detection wavelength of $\lambda=882$ nm. The method was evaluated for linearity, accuracy, and precision. The detection limit, defined as the concentration equivalent to the signal of the reagent blank ± 3 standard deviations (SD), was 0.003 μg P ml^{-1}. The analysis was performed on 3–5 replicates for each sample. The lowest measured concentration in the sample was always at least 10 times the detection limit. Precision was evaluated by calculating relative standard deviation (RSD) as a percentage from the ratio of the standard deviation and the average in mg P/g DW_{sample}. Precision based on triplicate measurements was always $<10\%$ RSD.

3. Results and discussion

3.1. Screening experiments

The key factor in maximizing the amount of the extracted DNA is cell lysis, which is dependent on the sample properties. Soils, especially those with high clay content, have been shown to adsorb charged molecules [23] making DNA extractions more difficult, compared to the relatively more homogeneous sediments. The isolation of DNA from animal cells suggests that cell walls are digested enzymatically and cell membrane solubilized with the help of detergents [20], however it has been shown that cell lysis could be improved by combining physical, chemical and enzymatic methods [24,25]. While enzymatic methods have been proven to improve cell lysis efficiency in DNA extraction from compost [26], a combination of methods gave highest yield in soil [24] and sediment [25]. Using matrix specific lysis methods were considered impractical in this work, and therefore a combination of lysis methods was used for all samples.

This work combined the direct in situ lysis extraction method, the most common surfactant, SDS, one of the frequently used extraction buffer solutions, based on Tris and EDTA, and two common enzymes, lysozyme and proteinase K.

Results of DNA extractions from yeast matrices [27] have pointed to possible interactions between the different parameters of the extraction protocol, which has led to a set of screening tests focusing on extraction parameter interactions. The most commonly used chemicals and extraction buffer components found in literature, as well as the effects of temperature and mechanical

Table 3
Experimental design, factorial fit, significant variables and interactions in screening experiments evaluating cell lysis.

Screening	Variable	Effect	Significant
1	Temperature	Positive	Yes
	Lysozyme	Negative	No
	Proteinase K	Negative	No
	EDTA	Negative	Yes
	Temperature*lysozyme	Negative	No
	Temperature*proteinase K	Positive	No
	Temperature*EDTA	Negative	No
	Lysozyme*proteinase K	Positive	Yes
	Lysozyme*EDTA	Negative	No
	Proteinase K*EDTA	Negative	No
2	Lysozyme	Negative	No
	Proteinase K	Negative	No
	SDS	Positive	Yes
	Incubation time	Positive	No
	Lysozyme*Proteinase K	Positive	No
	Lysozyme*SDS	Positive	Yes
	Lysozyme*Time	Negative	No
	Proteinase K*SDS	Positive	No
	Proteinase K*Time	Negative	No
	SDS*Time	Positive	No

force were tested. The results of the screening experiments are presented in Table 3.

The first screening experiment defined temperature and EDTA content of the buffer to be the significant single variables. Furthermore, there was an indication of a possible interaction between the two enzymes, which prompted their inclusion in the second screening experiment. An increase in temperature resulted in increased amount of nucleic acid extracted. EDTA concentration had an inverse effect on the extracted amount. High EDTA content decreased the extracted amount. However, the importance of including EDTA in the extraction buffer cannot be overlooked. The chelating properties of EDTA ensure the inactivation of nucleases, however, Krsek and Wellington showed that increased concentrations of EDTA had an adverse effect on the extraction by shearing DNA and decreasing its purity [24]. The second screening experiment showed no interaction between the different parameters and indicated SDS content as the only significant single variable.

The significant parameters from the screening experiments were included in the protocol together with three cell lysis treatments, alone or in combination with vigorous shaking with glass beads and complete results are presented in Supporting data (Table S1). Without the use of mechanical lysis, the increase in temperature and SDS concentration resulted in the increased DNA-P amount. The addition of lysozyme and proteinase K did not change the DNA-P amount. However, the addition of glass beads and shaking the sample proved superior to the effects achieved by temperature increase and the addition of enzymes separately.

One of the significant parameters explored in more detail was extraction time. The goal of the method was not only to extract and quantify DNA-P, but also to create an efficient and reproducible extraction procedure which effectively increased the DNA-P amount and minimized extraction time. In Fig. 1 it can be seen that for sediment and compost samples, DNA-P increased proportionately with time in the first 3 h of the extraction, but leveled off with prolonged extraction. The amount gain in the subsequent 3 h was marginal for sediment samples (2.5%), slightly higher for composted manure samples (7.5%); therefore a 3 h extraction was adopted for the final procedure. The soil samples exhibited different trends, where prolonged shaking actually increased the yield more than marginally (29%), although it is important to note that the extracted amount was very small even with the longest extraction time (0.039 mg P/g DW). It may be of consequence to consider that very long extraction times indicate the high affinity of particular forms of DNA to strongly adsorb to clay, hence impeding the bioavailability of that fraction.

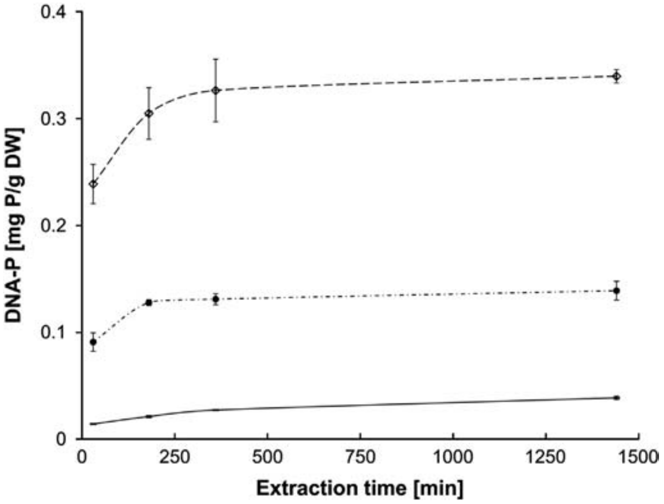

Fig. 1. Total extracted DNA-P amount (in mg P/g DW) as a function of time (min) using optimal extraction conditions for compost (diamonds), sediment (circles) and soil (dashes).

3.2. DNA

The initial assumption behind this methodology was that all nucleic acids could be extracted with the proposed protocol. Although there is undoubtedly at least trace amounts of RNA present in the samples, it does not appear to withstand the entire extraction procedure. Screening experiments with RNA standards showed a rapid degradation of the added RNA during the ultrafiltration step. Up to 80% of the standard passed through the spin filter when the final centrifugation step of the extraction procedure was evaluated. Recovery of RNA standards spiked into the compost sample matrix was 0% prompting the abandonment of RNA-P inclusion in the method development.

Sample size was reduced from several grams to a few hundred milligrams for all matrices, making processing of multiple samples more convenient. Choosing the appropriate amount of sample for the extraction procedure was based exclusively on the water content and total P (TP) content of the samples. It was desirable to achieve P concentrations in the same range, so that one calibration curve could be used for all samples. Additionally, the number of centrifugation and wash steps in the final protocol has been minimized to reduce losses.

The ultrafiltration step employed in the presented method thus contributes to the selectivity and has several advantages compared with other methods. It provides a simple way of concentrating large P containing compounds present in the sample extract i.e. separates the desired molecules by size exclusion and an effective clean-up of the sample through washing of salts and impurities. The cut-off size of 30 kDa assures that other abundant P compounds found in the sample, such as inorganic phosphates, or other compounds, such as inositol phosphates, which could have been co-extracted, are separated from DNA, which is retained on the spin filter membrane. The viability of this assumption was tested on DNA standards mixed with phosphate standards and extraction buffer (Table S2). The common way to use ultrafiltration devices is to concentrate samples after which the sample is either pipetted out or the filter is turned upside down and the sample collected into a new vial after centrifugation. Early screening tests with DNA standards showed that in addition to a 5% loss during filtration, up to 20% of DNA may be adsorbed to tube walls. In order to decrease these losses, extraction and wash steps were followed by processing of the entire spin filter together with the DNA fraction collected onto it, i.e. the sample together with the ultrafiltration device was subjected to digestion. Removal of smaller P containing molecules (< 30 kDa) from the DNA samples was demonstrated by colorimetric measurements performed on the filtrate solution collected after the ultrafiltration step. Phosphate removal efficiency from the spin filter (99.8%) as well as inositol phosphate removal efficiency (> 99%) was evaluated with MQ water spiked with inorganic phosphate and $InsP_6$ respectively.

The digestion procedure employed in the method is a critical step which, if incomplete, would underestimate the DNA-P pool. Potassium persulfate proved effective in digesting the extracted DNA fraction without any adverse effects of increasing sample size. This was verified by spiking the samples with DNA standards (Table S3 in Supporting data).

3.3. Phospholipids

The extraction of the lipid fraction was inspired by work done by Kowalenko and McKercher [28] who compared different methods for measurement of soil PLs in the 1970s. A starting point for the present project was a procedure for PL analysis described by Findlay et al. [29] and further developed by Frostegård et al. [16]. Difficulties associated with the accurate determination of the extracted PLs were due to the effectiveness of the digestion procedure. Conventionally digestions utilized rather time consuming protocols with the use of either perchloric acid [15] or potassium persulfate [29] with digestion times lasting from several hours to days. To minimize safety hazards, methods with heated perchloric acid were gradually replaced with persulfate, however, complete phosphate recovery was achieved only after a digestion for several days. Frostegård et al. [16] evaluated both methods and found the persulfate digestion less effective, though reproducible. The digestion employed 1 ml extract mixed with 1.8 ml saturated persulfate solution. Complete digestion could not be achieved unless the procedure was performed for 7 days, in particular for samples coming from an organic soil.

To address this problem, sample size in our method was scaled down from several grams to 50–200 mg and only a fraction (0.2 ml) of the extract was subjected to digestion with 1 ml oxidizing agent, giving a higher oxidizing agent to sample ratio. The amount of organic material processed was thus reduced. Additionally three different digestion procedures were tested, hydrochloric acid digestion, persulfate digestion and dry ashing, followed by hydrochloric acid digestion. Even though the organic solvent from the PL fraction was evaporated and replaced, there seemed to be a competition in the digestion procedure. Samples that had both lower organic content and lower PL content were digested completely with all three procedures, while samples with higher organic content were digested only after dry ashing. Preliminary results indicated that there was a correlation between the amount of organic matter in the sample and the effectiveness of the digestion. Reducing sample size and decreasing the sample to persulfate ratio in the persulfate digestion procedure also gave reproducible results, however it was concluded that adjusting this ratio for a new matrix would be an equally laborious procedure. Incomplete digestion of the organic matter in the extract would result in the underestimation of the P content in such samples. It has been established that a fast and accurate way of achieving complete digestion was through dry ashing, regardless of organic content, which was adopted in the final protocol.

Reducing sample size proved to have other advantages as well, e.g. work with organic solvents was minimized and made sample handling more practical. Also beneficial to sample handling was the lyophilization procedure prior to PL analysis as it was critical for the extraction to keep the ratio of organic solvent to aqueous buffer constant. It would need to be carefully calculated for each sample matrix, especially if it contained a large percentage of water. By freeze-drying the samples, the water was removed, and the same amount of extraction buffer could be used for all samples.

3.4. Method performance

The selectivity of the method lies in the selective isolation of the compound of interest, while colorimetric measurements offer sensitivity. Problems in P analysis with more complex instrumentation, such as solution P nuclear magnetic resonance spectroscopy (^{31}P –NMR) or mass spectrometry (MS), are often associated with high detection limits, which make it necessary to concentrate the samples at least by a factor of ten. In contrast, UV/vis spectrometry allows performing measurements on diluted samples in the μg kg^{-1} range. In common molecular analysis it is hard, if not impossible, to quantitatively analyze crude or partially purified samples containing nucleic acids because many organic compounds, such as humic acids, interfere with the traditional quantification methods, e.g. ethidium bromide staining or Hoechst 33258 fluorometry [13]. All purification methods are associated with extensive losses, but in the presented method the quantification is handled through indirect measurement, using the existence of P in the compounds studied, circumventing the problems associated with impurities and co-extracted organic compounds. Organic material is digested prior to analysis, converting organic P to orthophosphate.

Previous research has indicated that orthophosphate diesters can account for a substantial part of the organic P in soils and sediments [18]. With the described extraction procedure it was shown that DNA-P comprised 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 (Table 4). The values for PL-P were 0.5, 6.0 and 1.7% for soil, sediment and compost, respectively. The variability of organic P content in different environmental samples makes it hard to generalize about DNA-P and PL-P yields from a particular sample matrix. However, results of other studies were comparable to the presented study. Kowalenko and McKercher examined a variety of soils using a number of different extraction solvents combined with colorimetric estimations of P and found PL concentrations in the range from 0.6 to 14.5 μg P/g soil in Saskatchewan surface mineral soils [30].

Reliable reference values for DNA-P and PL-P extracted from environmental samples are limited. However, recovery experiments indicate good performance of the proposed method. When not integrated into the sample matrix most orthophosphate diesters are rapidly degraded. It was therefore important to assess DNA and PL stability during the extraction procedure. The methods were evaluated by measuring recovery on standards, prepared to match in concentration to that of the sample matrices. Extractions performed on DNA standard solutions had recovery of 90–95%, while extractions performed on PL standard solutions had recovery of 77–84%. Lower recovery rates for PLs were

Table 4
DNA and phospholipid (PL) phosphorus (P) content of soil, sediment and compost samples (mean ± SD, $n=3$), RSD < 10%.

Sample	DNA-P		PL-P	
	[mg P/g DW]	% of tot P	[mg P/g DW]	% of tot P
Soil	0.030 ± 0.001	3.1	0.005 ± 0.0001	0.5
Sediment	0.150 ± 0.003	10.4	0.086 ± 0.0008	6.0
Composted manure	0.381 ± 0.006	8.4	0.075 ± 0.0029	1.7

Table 5
DNA and phospholipid (PL) recovery (%) from soil, sediment and compost samples assessed by standard extraction and analysis methods (random error) [21].

Sample matrix	Recovery (%)	
	DNA	PL
Soil	103 (4)	98 (18)
Sediment	96 (5)	105 (19)
Composted manure	99 (7)	100 (14)

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expected as it has been shown that they degrade to phosphate monoesters during extraction [31,32], which also leads to their possible underestimation. The effect of sample matrices on recovery was small (Table 5). In fact, recoveries were higher in the spiked samples compared to standards alone, implying that the matrix may protect the diesters from degradation.

Estimates of organic P fractions from similar matrices support the findings of the present study. Ahlgren et al. used other extraction methods in combination with ^{31}P-NMR to identify seven P-containing groups in the sediment of Lake Erken [33], the same lake from which the sediment samples in the present study were collected. Three different compounds within the orthophosphate diester chemical shift region, including DNA-P and PL-P, were identified and quantified. It was concluded that the signals were most likely caused by DNA-P, PL-P and teichoic acid-P, but could also be of another form. The content of the individual P compounds was nevertheless in the same range as in the present study.

The main advantage of ^{31}P-NMR is the ability to detect multiple species of P simultaneously. However, it has been argued that alkali-labile phosphate diesters degrade during sample preparation due to the implementation of harsh extraction solvents, such as sodium hydroxide. It has been acknowledged that extractions aiming at extracting all forms of P may alter some of these forms, for example by hydrolysis and that additional information could be gained by using specific extraction techniques [34]. Despite the fact that ^{31}P-NMR is currently the most common technique used for the characterization of organic P, there are no standard procedures used by different laboratories. Both extraction and quantification practices differ which makes the comparison of results from different studies difficult. The most accurate way to compare samples today is through the use of standardized soil tests or well-established sequential extraction schemes that divide P into fractions based on chemical solubility [10,35]. Interpreting such results can be challenging because specific compounds may be found in more than one fraction and the same fraction can include both organic and inorganic P. In order to enhance our understanding of organic P bioavailability there is clearly a need for the development of new speciation methods that can be used to complement the tools used today.

The biological significance of the organic P pool may be much greater than previously believed and requires a more detailed examination. The method presented in this study can be viewed as a stepping stone toward a new standardized approach for P fractionation. It intentionally focuses on P derived from DNA and PLs, and overcomes the methodological difficulties attributed to previous protocols by offering greater compound selectivity in the extraction procedure. The experimental Sections 2.4, 2.5 and 2.7 offer a ready-to-use protocol which can be implemented to study the degradation and mineralization of DNA-P and PL-P. The method can also be used to facilitate comparative studies, especially in common characterization techniques which may underestimate these types of compounds. It offers a quick, straightforward technique to determine both the pool size and accumulation rates of DNA-P and PL-P, thus allowing assessment of a part of the organic P turnover in the ecosystem.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.042.

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