DETERMINATION OF THE SEDIMENT PHOSPHATE COMPOSITION BY THE EDTA METHOD OF FRACTIONATION

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ABSTRACT

The bioavailability of phosphate in sediments is a central issue in the study of the eutrophication of aquatic systems. The determination of the P-fractional composition is an essential tool to separate inorganic and organic P-pools in sediments. Sediments from temporary ponds of the Doiiana National Park (SW, Spain) are rich in organic matter and total P concentrations are relatively high. These ranged from 200 to 464 mg kg^{-1} dw. at different sites in a temporary pond in Doiiana National Park in samples taken in March and September 1999. The EDTA method (Golterman, 1996) has been shown to be a suitable P-extraction procedure for this kind of sediments. Additionally, this procedure allows the determination of the chemical structure of some organic-P substrates by enzymatic methods. With this method it appeared that the sediment phosphate composition was dominated by P-organic fractions (64-92%). The concentration of phytic acid in the sediment ranged from 9 to 31 mg kg^{-1} dw. and was determined by the use of phytase (at pH 2.5). The presence of o-P in the fulvic acids extracted with 1-butanol from the P-fraction bound to $CaCO_3$, suggested that the org-P compounds extracted by the Na_2-EDTA may be partly hydrolysed during the Murphy & Riley reaction in these sediments.

Keywords: P-fractionation, bioavailability, phytate, temporary pond, Doiiana National Park

RESUMEN

La biodisponibilidad del fosfato en los sedimentos es el principal objetivo en el estudio de la eutrofización de los sistemas acuaticos. La determinación de la composición de las fracciones del fósforo es una herramienta esencial para separar los grupos de fósforo inorgánico y orgánico de los sedimentos. Los sedimentos de lagunas temporales del Parque Nacional de Doiiuna (SW de España) son ricos en materia orgánica y las concentruciones de P total son relativamente altas. Estas oscilan entre 200 y 464 mg kg^{-1} ps. en diferentes lugares de una laguna temporal del Parque Nucional de Doñana en muestras recogidas en Marzo y Setiembre de 1999. El me'todo EDTA (Golterman, 1996) ha mostrado ser un procedimiento adecuado para la extruccidn de fósforo para esta clase de sedimentos. De forma adicionul, este procedimiento permite la determinacidn de la estructura quimica de algunos substratos de P-orgánico por métodos enzimáticos. Con este me'todo se ha observado que la composicidn de fósforo en el sedimento estabu dominado por jracciones de P-orgánico (64-92%). La concentración de ácido fítico en el sedimento oscilaba entre 9 y 31 mg kg^{-1} ps. Y fue determinudo rnediante el uso de fitasa (a pH 2.5). La presencia de P-orgánico en los ácidos fúlvicos extraídos con 1-butanol de la fracción de P asociada a $CaCO_3$, sugiere que el P-orgánico extraído con Nu,-EDTA puede ser parcialmente hidrolizado durante la reación de Murphy & Riley en estos sedimentos.

Palalabras clave: Fraccionamiento de P, biodisponibilidad, fitato, laguna temporal, Parque Nacional de Doñana

INTRODUCTION

Despite the great importance of sediments for the bioavailability of nutrients in shallow aquatic systems, the chemistry of the sediment-water exchange is poorly understood (Golterman, 1995). The organic P-fractions have often been underestimated in sediment chemistry (Golterman & Booman, 1988). De Groot & Golterman (1990) and De Groot (1990) showed that a general underestimation of organic P-fractions occurred because of hydrolysis of organic P-compounds by aggressive non-specific extractants, such as NaOH, used in the extraction of inorganic P-fractions from the sediment. In contrast, an extraction procedure of the inorganic P-fractions with chelating agents, such as EDTA, carried out at a pH near to that of the sediment avoids alterations in the sediment P-composition (Golterman & Booman, 1988; Golterman, 1996). Although NaOH is often used as an extractant for the fractionation of sediment-bound phosphate, Golterman *et al.* (1998) showed that cold NaOH does not only extract iron-bound phosphate but phytate phosphate and humic phosphate as well. Phytate (or phytic acid) is an organic phosphate that is widely spread in nature. It has been found in soils all over the world and is recognised to be the largest organic P-fraction, identified in soils (Stevenson, 1982 in De Groot & Golterman, 1993).

The characterisation of the organic P-compounds is difficult. Specific enzymatic essays to elaborate the chemical structure of organic P-substrates are seldom used. However, several compounds such as phytic acid, nucleic acids and adenosine-phosphate have been detected in sediments by means of enzymatic activity (Feuillade & Dorion, 1992; De Groot & Golterman, 1993). Besides, an uncertain portion of the organic P-pool is often attributed to the presence of unspecific complexes of humic substances with iron (Paludan & Jensen, 1995). However, the nature and bioavailability of such complexes remain to be elucidated.

The aim of this study is to present the EDTA method (Golterman, 1996) applied to the fractionation of sediments from a temporary pond in the Doñana National Park. Additionally, some complementary analyses were performed to shed some light on the nature of the organic P-compounds in this kind of sediments. The presence of phytate is revealed by the use of an enzymatic analysis. A further extraction with 1-butanol is used to examine the presence of an organic P-fraction in the supernatant extracted by Na_2-EDTA.

Abbreviations used:

o-P= ortho-phosphate
Tot-P= total phosphorus
Fe(OOH)~P= phosphate bound onto Fe(OOH)
$CaCO_3$~P= calcium-bound phosphate
org-P_{alkali}= organic P-fraction soluble in alkali
org-P_{acid}= organic P-fraction soluble in acid
ROP= residual organic phosphate
Fe(OOH)~org-P_{EDTA}= organic P-fraction dissolved by the Ca-EDTA/dithionite
$CaCO_3$~org-P_{EDTA}= organic P-fraction dissolved by the Na_2-EDTA

STUDY AREA

The Doñana National Park is located on the Atlantic coast of Southwest Spain. The area is dominated by a Mediterranean climate with Atlantic influence, generally classified as dry subhumid. An average year includes a dry and a wet season with annual average rainfall of 580 mm. The average wet season (from October to April) includes 80% of this, but rainfall is highly irregular from one year to another. Summers are very dry and hot, while winters are short and mild (Ménanteau, 1982).

Doñana ponds are fed by a combination of groundwater and rainfall (Sacks *et al.,* 1992). The ponds are very shallow temporary freshwater bodies that only receive surface drainage during floods. Their basins were formed by eolian sands stabilised in the Holocene period (Vanney & Ménanteau, 1985). Due to its young age and lack

of differentiation, the substrate is classified as an entisol with a different aquic moisture regime depending on flooding conditions (Siljeström & Clemente, 1990). The percentage of sand in pond sediments ranges from 44% to 99% (Jaúregui & Toja, 1993). Doñana sands, being of quartzitic nature, are relatively poor in calcium. Water in the ponds is rich in NaCl and the alkalinity is high and dominated by sodium; water pH usually ranges between 7 and 9. Detailed information on the limnology of these ponds is given elsewhere (López et al., 1991; Toja et al., 1991; Serrano & Toja, 1995).

MATERIAL AND METHODS

Top sediment samples (2 cm depth) were collected in March and September 1999 from three different sites in Las Verdes pond: the centre (or lowest point of the pond basin), the littoral and the floodplain. On those dates all sampling sites were dry except the centre site on March 1999 which registered a water depth of 20 cm. Dry sediments were collected with a small garden shovel, while the wet sediment was collected with a hand gauge.

In the laboratory, dry sediments were sieved through 0.1 mm and wet sediments were wet-sieved through a nytal mesh of 0.1 mm pore size. Sediment suspensions were made within 24 h after collection with 80-100 g of dry weight (dw.) in 1 litre of distilled H_2O. Redox potential and pH were measured in these sediment suspensions. The concentration of o-P was measured following the method of Murphy & Riley (1962). The Tot-P concentration in the samples was measured after digestion with 0.5 M H_2SO_4 and $K_2S_2O_8$ (1-2 **g**) at 120 °C. The percentage of organic matter in the sediment was estimated in four replicates by loss on ignition (450 °C, 5 h).

The P-fractionation of the fine sediment portions was performed in two replicates by sequential extraction with chelating compounds (Golterman, 1996). In this method the inorganic P-fraction of Fe(OOH)~P is first extracted with Ca-EDTA/dithionite, followed by an extraction

of the inorganic P-fraction of $CaCO_3$~P with Na_2-EDTA, after which acid and alkaline soluble pools are extracted. The fractional composition was determined on approximately 0.6-0.7 g dw. of sediment. This procedure was slightly modified to adapt it to the Doñana sediments, rich in organic matter i.e. Tot-P from the Ca-EDTA/dithionite and the Na_2-EDTA extracts were also measured in order to quantify the pool of organic P-compounds present in these extracts (Diaz-Espejo et al., 1999).

In order to detect phytate by means of enzymatic analysis, the undigested phosphate in the sediment extracts was acidified with 1 M H_2SO_4. A set of aliquots (5 ml) was incubated for 24 h with (and without) 0.5 ml of phytase (60 mg l^{-1}) from Aspergillus ficuum crude extract (Sigma). The concentration of o-P was then simultaneously measured in the aliquots, with and without enzyme, and the concentration of o-P liberated by the enzyme was then calculated by subtraction. Phytic acid from rice (Sigma) was used as a standard solution. The range of the enzyme dilution (2.4-30 mg ml^{-1}) and the variation of the enzymatic activity with time (0-140 h) were investigated prior to the analyses.

A further extraction with 1-butanol (HPLC Grade) was performed on the Na_2-EDTA extract by adding 10 ml of 1-butanol and 10 ml of 1 M H_2SO_4 to 10 ml of the sample. The mixture was shaken vigorously and let to separate for 20 h. The Na_2-EDTA extract was then separated into two phases: a top layer of humic acids dissolved in 1-butanol and below, the fulvic portion dissolved in the acid solution. Next, a volume of 5 ml of the fulvic phase was carefully taken with a pipette to measure the concentration of o-P. Another volume of 5 ml was digested in order to measure the concentration of Tot-P.

RESULTS AND DISCUSSION

The Tot-P concentration of the fine sediment (<0.1 mm) of Las Verdes pond ranged from 200 to 464 mg kg^{-1} dw. and was lowest at the littoral site (Table 1). Similarly, the content of Tot-P in

Table 1. Mean concentration ± SD of o-P in the sediment fractions of the littoral, centre and floodplain sites during March and September 1999. Between brackets: mean concentration ± SD of o-P liberated after the incubation with phytase. *Concentraciones medius ± SD de o-P en las frucciones del sedimento litoral, centro y de la llanura aluvial en Marzo y Septiembre de 1999. Entre paréntesis: concentraciones medius ± SD de o-P liberado después de la incubacidn con fitasa.*

P-fractions (mg kg^{-1} dw.)	littoral		centre		floodplain	
	March	Sept.	March	Sept.	March	Sept.
Fe(OOH)~P	41.0±0.5 (ND)	27.3±1.5	87.8±2.7 (ND)	59.7±0.5	38.5±0.7 (9.2±1.1)	13.4±0.3
Ca$_2$CO$_3$~P	22.2±0.3 (ND)	10.7±2.3	45.2±4.2 (ND)	55.5±5.5	46.8±3.4 (4.6±1.0)	15.5±0.7
Fe(OOH)~orgPEDTA	5.3±1.0	40.9±4.4	18.4±2.0	41.2±2.2	13.1±0.9	41.5±7.5
Ca$_2$CO$_3$~orgPEDTA	1.2±0.7	46.7±6.8	12.4±2.5	41.9k1.3	102.021.0	114.7±0.7
org-Pacid	17.7±0.0 (4.0±0.4)	12.021.5	25.7±1.2 (5.1±0.4)	9.2±1.2	21.6±0.8 (6.3±0.8)	15.5±0.6
org-Palkali	110.22 (5.1)	108.7±15.6	172.0±0.3 (7.1±0.1)	121.9±1.7	225.0±17.6 (11.3±0.3)	154.8±5.3
ROP	9.4±0.3	10.6±0.1	13.5±0.4	12.3±0.4	18.2±1.5	8.2±0.2
Σ fractions	200.7±3.7 (9.1±0.4)	268.1±15.3	374.9±2.5 (12.2±0.3)	346.3±1.1	465.0k16.9(31.4±1.3)	363.3±12.2

ND= not detectable

the sediments of other small temporary ponds of Doñana ranged from 56 to 531 mg kg^{-1} dw. (Jauregui & Toja, 1993) and from 3382 to 495 mg kg^{-1} dw. (Diaz-Espejo *et al.,* 1999). However, higher values were detected in the larger Dulce pond, which also showed a wider range of tot-P concentrations between the littoral and the centre sites: from 27 to 3887 mg kg^{-1} dw. (Jauregui & Toja, 1993).

Given the low pH of the sediment samples from this pond (Table 2), calcium in the sediment cold not be in the form of CaCO$_3$. The 'apparent P-fraction bound to CaCO$_3$' (the fraction extracted by Na$_2$-EDTA) is, in our sediments, most likely to be related to Ca-humic complexes. In particular, a fulvic subfraction of the Na$_2$-EDTA extract was easily separated using 1-butanol. The concentration of o-P in this fulvic subfraction ranged from 7 to 26 mg kg^{-1} dw. and the Tot-P concentration ranged from 33 to 49 mg kg^{-1} dw. (Table 3). It is likely that the fulvic acids of the sediment were brought into solution during the extraction with Na$_2$-EDTA at pH 5 and that some organic P-compounds were partly hydrolysed during the Murphy & Riley reaction, yielding a low concentration of o-P that was wrongly considered as an "inorganic P-fraction". However it is more likely that the previous extraction with Ca-EDTA/dithionite was not completed and part of the Fe(OOH)~P was still present in the sedi-

Table 2. pH, redox potential (mV) and concentration of OM (%) in sediment samples from the littoral, centre and floodplain sites in March and September 1999. *pH, potenciul redox (mV) y concentracidn de OM (%) en muestras de estaciones del litoral, centro y llanura aluvial en Marzo y Septiembre de 1999.*

	March			September		
	pH	mV	%OM	pH	mV	%OM
littoral	5.8	+344	9	6.2	+224	13
centre	5.6	+94	15	6.2	+236	17
floodplain	5.6	+205	25	6.4	+230	22

Table 3. Mean concentrations ± SD of o-P and Tot-P (mg kg^{-1} dw.) in the fulvic acids of the Na$_2$-EDTA fractions extracted with 1-butanol in the sediment samples of the littoral, centre and floodplain sites. *Concentraciones medias ± SD de P y Tot-P (mg kg-1 ps) en los ácidos fúlvicos de las frucciones Nu,-EDTA extraidus con 1-butanol en muestras de estaciones del sedimento del litoral, centro y llanura aluvial.*

	fulvic acids	
	[o-P]	[Tot-P]
littoral	7.30k0.3	31.6±2.0
centre	25.6±2.4	48.9±0.6
floodplain	12.0k0.2	46.1±3.4

ment and was, therefore, extracted in the following fraction by the Na$_2$-EDTA (unpublished data). This inorganic P-fraction was then soluble in the acid phase that also included the fulvic

acids. The Tot-P concentration was higher than the concentration of o-P in these fulvic acids indicating that they contained a larger portion of organic P-compounds (Table 3).

The sediment organic P-pool at the different sites within Las Verdes pond comprised 64-92 % of the sum of the P-fractions (Table 1). In all sites, the highest phosphate concentration was found in the org-P_{alkali} fraction, whereas it was lowest in the org-P_{acid} and ROP fractions. A similar phosphate composition was found in other small temporary ponds where the organic P-pool ranged from 68 to 79% of the sum of all P-fractions (Diaz-Espejo et al., 1999).

Two results characterised the sediments from the floodplain with respect to the other sites: they were poor in Fe(OOH)~P but rich in organic P-fractions, especially the fraction of $CaCO_3$~org-P_{EDTA} (Table 1). It is likely that the growth of grass *(Agrostis stolonifera, Cynodon dactylon)* that covered the floodplain produced a decrease of the inorganic P-fractions and an increase of the organic P-fractions due to decomposition, leaching and root activity. In contrast, the proportions of the P-fractions in the sediment of the centre and littoral sites were rather similar though the phosphate concentration of each fraction was usually higher in the sediment of the centre site.

The pond gradually dried out towards the summer. In March 1999, only the sediment of the centre site was wet, but in September 1999 the pond was completely dry. The littoral site remained dry and mostly bare during the study period while the sediment of the centre site experienced a drastic change as the pond water level dropped from 20 cm above the ground to 15 cm below it in September. Hence, the redox potential of the sediment increased from +94 mV in March to +236 mV in September in the centre site (Table 2). Besides, the floodplain sediment was likely affected by the growth of grass during spring and summer. As a result, the phosphate fractional composition of the sediment samples changed in all sites (Table 1). The concentration of phosphate in the fraction of Fe(OOH)~P decreased significantly in sediments from all sites (paired t-Student test, P<0.05). The fraction of Fe(OOH)~org-P_{EDTA} increased in all sites (Table 1) though this change was significant only in sediments of the centre and littoral sites (P<0.05). In September, the phosphate fractional composition of the org-P_{alkali} and org-P_{acid} fractions decreased in the sediments of all sites, but this change was significant (P<0.01) in the floodplain and open-water only. No significant change (P<0.05) was found in the ROP fraction of sediments from all sites. This fact reinforces the non-available nature of the ROP fraction as found in other similar studies (Díaz-Espejo et al, 1999; De Groot & Fabre, 1993).

The changes in the sediment phosphate composition of the different sites can give some information on the bioavailability of each P-fraction. Both inorganic P-fractions of Fe(OOH)~P and $CaCO_3$~P appeared to be a source of bioavailable P for phytoplankton growth (De Graaf Bierbrauwer-Wurtz & Golterman, 1989 in Golterman, 1995). During the study period, the concentration of phosphate in the fraction of the Fe(OOH)~P experienced a two-fold decrease in the sediment of the floodplain site, compared to the other sites (Table 1). The 'apparent inorganic P-fraction" of $CaCO_3$~P also decreased significantly only in the floodplain sediment (P<0.05). Therefore, it seems that these inorganic P-fractions were readily available for plant growth on the floodplain site. The o-P concentrations of other organic P-fractions also decreased significantly in some sediments (Table 1). This suggests that the organic P-pool is not a mere deposit of refractory humic phosphate, but contains some organic P-compounds, such as phytate, that may be readily available for the growth of phytoplankton and plants.

Although phytate is common in soils and sediments, it is relatively stable as it can be strongly adsorbed onto iron hydroxide and multivalent cations (De Groot & Golterman, 1993). However, extracellular phytase activity is very common in soil microorganisms and plants (Dvoráková, 1998). The concentration of phytate in the sediment ranged from 9 to **31** mg kg^{-1} dw. (Table 1), but this result is surely an underestimation of the total concentration of this compound in sediments, because the enzymatic determination of this com-

pound required a pH of 2.5. At this pH, in most extracts a precipitate occurs that prevents an unknown portion of phosphate sediment from being analysed, especially in the alkaline fractions such as the org-P$_{alkali}$. Furthermore, phytase activity can be inhibited by the presence of large amounts of fulvic acids (De Groot & Golterman, 1993). Therefore, a small volume of substrate was analysed to minimise this inhibition. Nevertheless, phytate was detected in all the undigested sediment fractions from the floodplain site, being higher in the org-P$_{alkali}$ fraction (Table 1). Extractions of sediment phosphate with cold NaOH can easily liberate phytate and humic phosphate into the supposedly iron-bound phosphate fraction (Golterman *et al.,* 1998). Therefore, the selection of a suitable extraction method is critical for the study of the bioavailability of P in the sediment, especially in organic rich sediments.

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