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Characterization of organic phosphorus in leachate from a grassland soil

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Abstract

The degree of eutrophication in fresh water ecosystems may be influenced by the forms of phosphorus (P) leached from agricultural systems. Physico-chemical fractionation of P in leachate from a grassland soil carried out over a two year period indicated that the majority of the P loss from the Lismore soil occurred in unreactive particulate (55–76%) P forms. ^{31}P nuclear magnetic resonance analysis of a selected leachate sample indicated that unreactive P was mainly comprised of monoester and diester forms of organic P. The presence of phosphomonoesterase (20–200 μg *p* nitrophenol l^{-1} h^{-1}) and phosphodiesterase (68 μg *bis-p* nitrophenol l^{-1} h^{-1}) activity in leachate resulted in hydrolysis of 10–21% of total unreactive P (TUP), indicating that some of the monoesters and diesters can be eventually hydrolyzed into inorganic P forms during P transport. Enzyme hydrolysis showed that 23% of the TUP was present as labile monoester P (LMP), followed by 20% as inositol hexakisphosphate (IHP) and 14% as diesters (phospholipids and nucleic acids). The findings of this study suggest that LMP, IHP and diesters are an important component of organic P leaching from the grassland soil.

Keywords: Nuclear magnetic resonance; Phosphatases; Organic P forms; Leachate; Water quality

1. Introduction

Phosphorus (P) is considered a major limiting nutrient in freshwater ecosystems for algal growth (Delgado and Lapointe, 1994; Thingstad and Rassoulzadegan, 1995). Dissolved inorganic P is readily available to aquatic communities in the short-term. It comprises a large component of bioavailable P. However, a variable portion of particulate inorganic and organic P that is in equilibrium with dissolved P represents a secondary and long-term source of bioavailable P in water bodies (Sharpley et al., 1992).

Research over the last four decades has shown that organic P is mobile in the soil profile (Frossard et al., 1989; Hannapel et al., 1964a,b), and can represent a significant proportion of P present in leachate from grassland soils (Heathwaite and Dils, 2000). Other studies have indicated that organic forms of P are potentially available to organisms in aquatic ecosystems (Whitton et al., 1991). The identification of these organic P forms will improve our understanding of the mechanisms responsible for their release from soils, so that specific mitigation strategies can be implemented at the P source.

^{31}P nuclear magnetic resonance (NMR) spectroscopy has been used extensively over the past 20 years to investigate the chemical nature and dynamics of organic P in the soil-plant system (Condron et al., 1997; Magid et al., 1996; Makarov et al., 2002). However, ^{31}P NMR has not been used to examine P species in leachate and overland flow from agricultural land. There has been some success in using enzyme hydrolysis to characterize organic P in soil (Hayes et al., 2000; Shand and Smith, 1997; Turner et al., 2002) and manure extracts (He and Honeycutt, 2001). For example, Turner et al. (2002) found that orthophosphate diesters were the predominant form of organic P present in water extracts of grassland soils, while Pant et al. (1994) reported that up to 50% of the total P in soil solution was hydrolyzed by phytase.

The main objective of this study was to determine the chemical nature and potential bioavailability of organic P in

Abbreviations: P, phosphorus; TP, total P; TUP, total unreactive P; TRP, total reactive P; TDP, total dissolved P; DUP, dissolved unreactive P; PUP, particulate unreactive P; DRP, dissolved reactive P; LMP, labile monoester P; IHP, inositol hexakisphosphate; FDE, farm dairy effluent; PMEase, phosphomonoesterases; PDEase, phosphodiesterases; NMR, nuclear magnetic resonance.

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leachate collected from a series of grassland lysimeters using a combination of ^{31}P NMR and phosphatase enzyme hydrolysis.

2. Materials and methods

2.1. Lysimeter and leachate collection

The main study was designed to quantify P loss from irrigated grassland soil as influenced by application of a combination of treatments related to dairying. Intact soil monoliths (50 cm diameter, 70 cm depth) were collected from a free draining Lismore silt loam soil (Udic Haplustept), as described elsewhere (Cameron et al., 1992; Di and Cameron, 2002). The experiment was established in a complete randomized block design with four replicates. The lysimeters received different combinations of P fertilizer, farm dairy effluent (FDE), nitrogen (N) fertilizer (urea) and cow urine. Leachate was collected over a two year period and analyzed for different P fractions. For this part of the study, two treatments one with only mineral P fertilizer and the other with P fertilizer and FDE were selected, to compare the impact of dairying with normal field conditions on the P loss. The control treatment (P45) received P fertilizer at 45 kg P ha^{-1} y^{-1} (20% potash superphosphate) in November each year. The treatment with P45 and FDE200 received additional FDE at 200 kg N ha^{-1} in four split applications in February, May, August and November each year. Leachate collected from these two treatment lysimeters between February and May 2001 was subjected to the detailed analysis as outlined in the following sections.

2.2. Physico-chemical phosphorus fractions

Leachate collected after irrigation or a significant rainfall event was analyzed for dissolved reactive P (DRP) and total dissolved P (TDP) in a filtered (<0.45 μm) sample, and total reactive P (TRP) and total P (TP) in an unfiltered sample within 48 h of collection using malachite green colorimetry (Ohno and Zibilski, 1991; van Veldhoven and Mannaerts, 1987). Reactive P is thought to consist of orthophosphate, while unreactive P may contain organic and some condensed forms of P (Ron Vaz et al., 1993). The TDP and TP samples were digested according to Ebina et al. (1983) before analysis. The difference in P concentration between TP and TRP was taken to be the concentration of total unreactive P (TUP). Similarly, the other P fractions were calculated as: dissolved unreactive P (DUP) = TDP − DRP; particulate unreactive P (PUP) = TUP − DUP.

2.3. ^{31}P NMR spectroscopy

On one occasion (August 2000), 6 l of leachate was collected from FDE amended lysimeters 24 h after FDE application for ^{31}P NMR analysis. Leachate was freeze-dried before extracting 1 g of freeze-dried sample with 50 ml of 0.25 M NaOH + 0.05 M EDTA solution for 16 h (Cade-Menun and Preston, 1996; Turner et al., 2003). The extract was filtered through Whatman No. 41 filter paper. To prepare samples for NMR spectroscopy the lyophilized extract was weighed into a 50 ml plastic centrifuge tube with 2.6 ml of D_2O and 0.4 ml of 10 M NaOH. The NaOH was added to increase and standardize the pH, for optimal peak separation (Crouse et al., 2000). Samples were vortexed for two minutes and were then left to stand at room temperature for 2 h, with occasional vortexing. The samples were then centrifuged for 20 min at 5000 rpm, and decanted into 10 mm NMR tubes. The samples were prepared no more than one hour prior to analysis by NMR spectroscopy.

Phosphorus-31 NMR spectra were carried out at 202.45 MHz on a GE Omega-500 spectrometer equipped with a 10 mm broadband probe. Samples were maintained at 25 °C. The acquisition time was 0.68 s, with a pulse delay of 4.32 s and a pulse width of 66. All spectra were acquired over a 20,325.20 Hz spectral window centered at 0 ppm (referenced to the phosphate singlet in an external standard of phosphoric acid in D_2O). Each sample was run twice, with two acquisition times: 3 h (2000 scans) and 11 h (8000 scans). There were no observable differences between the short and long runs for any samples. The spectra presented here are from the 11-hour runs, and were processed with a line broadening of seven. Peak areas were determined by integration, and the assignment of peaks was based on Newman and Tate (1980) and Adams and Byrne (1989).

2.4. Enzyme activities

In order to assess the abundance of enzymic activity (phosphomonoesterase and phosphodiesterase) in the aquatic environments, artificial substrates are widely used (Hino, 1989; Jansson et al., 1988; Strickland and Solarenzo, 1966). As in the current study, a large proportion of the P in leachate was present in unreactive (organic) P forms, so the presence of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) activity might have a controlling influence on the P forms determined in leachate. The presence of enzymic activity in leachate also reflects the biological processes occurring in the soil, because phosphatase are released by organisms in response to a need for P (e.g. Tadano et al., 1993; Tarafdar and Claassen, 1988).

Phosphomonoesterase was assayed by a method adapted for soil PMEase (Tabatabai, 1982), based on the use of disodium *p*-nitrophenyl phosphate (*p*NPP) as an analogue substrate for PMEase. Similarly, phosphodiesterase activity was investigated using the diester substrate *bis-p*NPP, as described by Browman and Tabatabai (1978) and Tabatabai (1982). The principle of these techniques is that the hydrolytic action of the enzyme present in leachate acts

on the substrate, leaving free *p*-nitrophenol (*p*NP) in the solution that is colorimetrically measured.

Four ml of unfiltered leachate was added in a screw-cap glass tube together with 0.5 ml of 0.1 M Tris–HCl buffer (pH 6.5) for PMEase or 0.05 M Tris–H_2SO_4 (pH 8) for PDEase assay. The assay was initiated by the addition of 0.5 ml of 0.005 M *p*NPP (for PMEase) or *bis*-*p*NPP (for PDEase) and samples were incubated at 37 °C in an incubator. After 1 h, the reaction was terminated by the addition of 1 ml of 0.5 M NaOH for PMEase or 0.1 M Tris–NaOH at pH 12 for PDEase and absorbance was measured at 410 nm. Controls were performed for each sample to correct for colour not derived from *p*-nitrophenol released by enzyme activity. This involved using 4 ml of leachate and 0.5 ml of buffer, but *p*NPP or *bis*-*p*NPP substrate was added immediately after addition of NaOH or Tris–NaOH at the end of the incubation. Results were also corrected for small amounts of *p*NPP auto-hydrolysis, determined by incubating buffer and substrate with autoclave-sterilized deionized water.

2.5. Enzyme hydrolyzable phosphorus

Commercial phosphatase enzymes were used to assess their ability to hydrolyze different organic P compounds present in leachate. This was made possible by using alkaline phosphatase (Type III chromatographically purified from *Escherichia coli*), which is known to hydrolyze labile monoester P (LMP) (glucose-6-phosphate, mononucleotides), phosphodiesterase (Phosphodiesterase 1, Type IV from *Crotalus atrox*) which hydrolyzes diester P (nucleic acids, phospholipids), and phytase (3-phytase from *Aspergillus ficuum*), which hydrolyzes inositol hexakisphosphate (IHP). These enzymes were obtained from Sigma Chemicals. No inhibition tests were conducted as Turner et al. (2002) reported that the presence of enzymes at these concentrations did not interfere with the determination of reactive P in standard samples.

The enzyme mixtures were prepared in the appropriate buffer as follows: Alkaline phosphatase: 1 unit activity ml^{-1} (0.1 ml of enzyme in 20 ml of 0.1 M Tris–HCl at pH 8 buffer), Phosphodiesterase: 0.03 units activity ml^{-1} (20 mg of enzyme in 20 ml of 0.1 M Tris–HCl at pH 8.8 buffer), Phytase: 1 unit ml^{-1} (30 mg of enzyme in 80 ml of 0.1 M Glycine–HCl at pH 2.5 buffer and centrifuged for 10 min at 3000 rpm). All buffers contained 0.002 M $MgCl_2$, because Mg^{2+} ions are natural activators of most enzymes acting on phosphorylated compounds (Dixon and Webb, 1966).

The assay mixture consisted of 4.5 ml of unfiltered leachate, 0.25 ml of 0.1 M sodium azide (to prevent microbial interference during the assay time, Feuillade and Dorioz, 1992) and 0.25 ml of respective enzyme mixture (alkaline phosphatase or phosphodiesterase or phytase) in the appropriate buffer. The mixtures were incubated for 16 h at 37 °C in 15 ml glass tubes. Controls were performed for each leachate by adding sterilized deionized water instead of enzyme to study the hydrolyzation of organic P due to the inherent enzymic activity in leachate. The P concentrations in the mixtures were measured using the malachite green method as is outlined in the earlier section (see Section 2.2).

3. Results

3.1. Physico-chemical fractionation

From the extensive chemical analysis carried out on leachate samples over the two years, it was found that TUP was the predominant P fraction and comprised 85–88% of the TP concentration (Table 1). For the FDE amended treatments, PUP concentrations were three times higher than the DUP. However, for the P45 treatment, the relative proportions of DUP and PUP were similar.

3.2. ^{31}P NMR

Characterization of leachate P with NMR spectroscopy revealed that most of the P was present in organic forms (88%) and the remainder as inorganic orthophosphate (12%) (Fig. 1). The organic P in leachate was dominated by monoesters (67.4%) followed by diesters (20.2%). In this sample, the relative proportion of P as TRP and TUP was 11 and 89% of TP, respectively.

Table 1
Concentrations of unreactive P forms in leachate determined over the two years from different treatments (means of 51 drainage events between June 1999 and May 2001)

	TUP		DUP (<0.45 μm)		PUP (>0.45 μm)	
	μg l^{-1}	% of TP	μg l^{-1}	% of TUP	μg l^{-1}	% of TUP
P45	82 (9)[a]	88.2	37 (9)	45.1	45 (10)	54.9
P45/FDE200	221 (61)	84.7	55 (9)	24.9	166 (49)	75.1

P45, superphosphate applied at 45 kg P ha^{-1} y^{-1}; FDE200, farm dairy effluent applied at 200 kg N ha^{-1} y^{-1}; TUP, total unreactive P; DUP, dissolved unreactive P; PUP, particulate unreactive P.

[a] Data in parentheses are standard errors of the means.

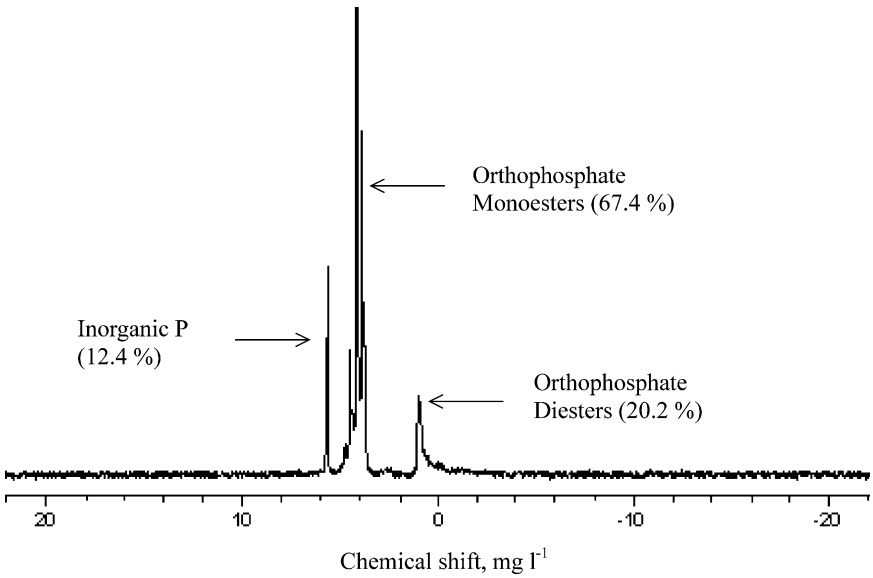

Fig. 1. ^{31}P NMR spectum of NaOH–EDTA extractable P from freeze dried leachate collected 24 h after FDE application.

3.3. Enzymic activity

Soils are known to be the reservoirs of different enzymes (Adams, 1992; Kiss et al., 1975; Tabatabai, 1982), and it is likely that some of these enzymes are subjected to movement into waterways. These enzymes may then act on the specific organic P forms and result in the hydrolysis of organic P into inorganic P. Fig. 2 shows that the mean PMEase activity in leachate was typically <200 μg *p*-nitrophenol (*p*NP) l^{-1} h^{-1}, except immediately following FDE application to P45/FDE200 treatment during February (726 μg *p*NP l^{-1} h^{-1}) and May (449 μg *p*NP l^{-1} h^{-1}). Phosphodiesterase activity was only detected following FDE application to P45/FDE200 treatment on 14 February (68 μg *bis-p*NP l^{-1} h^{-1}) (data not shown). In contrast, little enzyme activity in similar systems has been reported. Christmas (1998) observed PDEase activity only on one occasion for river waters in the UK. In this study, due to the presence of inherent enzymic activity in the leachate, 10–21% of TUP (9–336 μg P l^{-1}) was hydrolyzed into inorganic P when samples were incubated without any addition of commercial phosphatases (Table 2).

3.4. Enzyme hydrolyzable phosphorus

To identify the biodegradability of organic P forms in leachate, artificial phosphatases were used to catalyze the hydrolysis of specific organic P compounds with

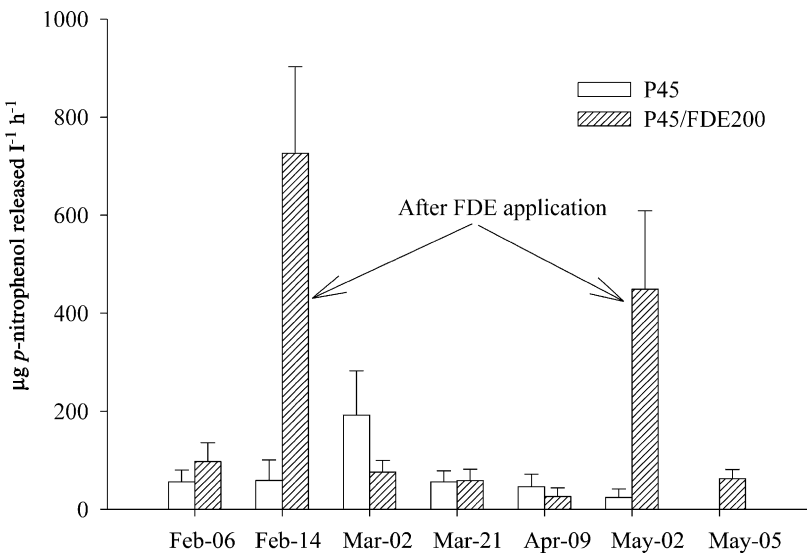

Fig. 2. Phosphomonoesterase activity (μg *p*-nitrophenol l^{-1} h^{-1}) determined in leachate collected from two treatments during February–May 2001 (standard errors of the means are shown by vertical bars).

Table 2
Changes in concentrations of phosphorus (μg P l^{-1}) in leachate incubated without phosphatases

Date	Treatments	μg P l^{-1}	% of TUP	% of TP
21/03/2001	P45	12 (2)[a]	10	9
	P45/FDE200	16 (3)	11	10
09/04/2001	P45	13 (2)	21	19
	P45/FDE200	12 (3)	15	13
02/05/2001	P45	nd	nd	nd
	P45/FDE200	336 (70)	16	13
05/05/2001	P45	9 (1)	14	10
	P45/FDE200	22 (2)	10	7

P45, superphosphate applied at 45 kg P ha^{-1} y^{-1}; FDE200, farm dairy effluent applied at 200 kg N ha^{-1} y^{-1}; nd, not detected; TUP, total unreactive P; TP, total P.
[a] Data in parentheses are standard errors of the means.

the subsequent release of inorganic P. Monoesters were classified into LMP and IHP (Table 3). Mean concentrations of LMP and IHP were 23 (commonly 5–48 μg P l^{-1}) and 20% (commonly 6–33 μg P l^{-1}) of TUP, respectively, together comprising approximately half of the organic P. For the P45/FDE200 treatment, mean concentrations of LMP were 111 μg l^{-1} followed by 69 μg P l^{-1} of IHP and 50 μg l^{-1} of diesters. However, for the P45 treatment concentrations of all these P species were <17 μg l^{-1}. Following FDE application to P45/FDE200 treatment on May 02, concentrations of LMP, IHP and diesters were 432 μg l^{-1} (20% of the TUP), 263 μg l^{-1} (12% of TUP) and 186 μg l^{-1} (9% of TUP), respectively. This suggests that FDE was a rich source of these organic P species, which were directly transported through the soil following its application. Using phosphatases, 16–99% of TUP was characterized during March–May 2001.

4. Discussion

Physico-chemical analysis revealed that a majority of P in leachate was present in the TUP form (85–88%). Previous workers (e.g. Ron Vaz et al., 1993) suggested that the unreactive P pool includes inorganic P species such as polyphosphates or condensed phosphates. Consequently, these workers suggested the use of the term 'unreactive' rather than 'organic'. However, the fact that ^{31}P NMR analysis of a leachate sample collected following FDE application showed that most of the P was present as organic P, tentatively suggests that unreactive P was mainly organic P. The organic P in the leachate consisted of monoesters (67.4%) and diesters (20.2%). The predominance of monoesters in the leachate is not surprising since monoester organic P has been shown to be the predominant form of organic P in most soils (Magid et al., 1996).

It was only possible to analyze one leachate sample using ^{31}P NMR due to the considerable time and effort involved. Selective hydrolysis using phosphatase enzymes was used as an alternative way of assessing organic P in leachate, which could be applied to a large number of samples. Phosphatase enzymes stimulate the hydrolysis of organic P in soil and are produced by roots and microorganisms according to their demand for P relative to the availability of inorganic P (Spier and Ross, 1978; Tadano et al., 1993; Tarafdar and Claassen, 1988). The use of phosphatase enzymes to determine bioavailable organic P was first reported by Strickland and Solarenzo (1966). Thereafter, this method has been used to investigate the cycling of organic P in aquatic environments and its contribution to biological P supply (Cooper et al., 1991; Francko and Heath, 1979; Hino, 1989). In this study, the detailed analysis of

Table 3
Concentrations (μg l^{-1}) of enzymatically hydrolyzed labile monoester P, inositol hexakisphosphate and diester P in leachate collected from two treatments during March–May 2001

		TRP (μg l^{-1})	TUP (μg l^{-1})	Labile monoester P		Inositol hexakisphosphate		Diester P		Total increase in P due to enzymes	
				μg l^{-1}	% of TUP	μg l^{-1}	% of TUP	μg l^{-1}	% of TUP	μg l^{-1}	% of TUP
02/03/2001	P45	16 (2.4)[a]	46 (4.0)	12 (4.5)	26	nd		12 (3.4)	26	24 (7.9)	52
	P45/FDE200	20 (3.2)	92 (28.7)	25 (9.2)	27	nd		21 (6.6)	23	46 (15.7)	51
21/03/2001	P45	10 (0.3)	123 (15.5)	20 (2.4)	16	21 (1.3)	17	12 (1.4)	10	53 (5.0)	43
	P45/FDE200	14 (3.2)	146 (13.7)	25 (2.8)	17	29 (3.5)	20	15 (1.8)	10	69 (8.1)	47
09/04/2001	P45	8 (1.2)	60 (2.4)	19 (1.8)	31	22 (1.7)	37	19 (4.5)	31	60 (8.0)	99
	P45/FDE200	12 (0.9)	81 (12.0)	23 (2.9)	29	21 (1.6)	27	13 (1.9)	17	58 (6.4)	72
02/05/2001	P45	23 (1.2)	74 (13.0)	5 (3.0)	7	6 (1.3)	8	1 (0.1)	2	12 (2.7)	16
	P45/FDE200	390 (57.2)	2165 (264.9)	432 (32.5)	20	263 (46.9)	12	186 (4.9)	9	881 (84.3)	41
05/05/2001	P45	28 (3.0)	65 (5.0)	23 (1.2)	36	12 (1.6)	18	4 (1.2)	6	39 (3.9)	60
	P45/FDE200	75 (38.1)	221 (43.6)	48 (8.4)	21	33 (5.6)	15	13 (9.2)	6	93 (23.2)	42
Mean											
	P45	17	74	16	23	12	20	10	15	38	54
	P45/FDE200	102	541	111	23	69	19	50	13	230	51

P45, superphosphate applied at 45 kg P ha^{-1} y^{-1}; FDE200, farm dairy effluent applied at 200 kg N ha^{-1} y^{-1}; TRP, total reactive P; TUP, total unreactive P; nd, not detected.
[a] Data in parentheses are standard errors of the means.

leachate using phosphatase revealed that monoesters were comprised of LMP and IHP. This finding has direct implications for increased eutrophication in water bodies as LMP and IHP species has been reported to be directly utilized by aquatic microorganisms (Whitton et al., 1991).

As organic P forms, determined in leachate, constituted 85–88% of TP and significant enzymic activities were detected in leachate, it is likely that a part of the organic P may be mineralized to inorganic P once favorable conditions (e.g. temperature) are approached. For example, 10–21% of the TUP was hydrolyzed when leachate was incubated at 37 °C without any addition of phosphatases due to the presence of inherent enzymic activity in leachate. These results show the potential for organic P to (a) be mineralized during the course of P transport to water bodies and (b) present a greater threat to the freshwater ecosystems resulting from their transfer.

Shan et al. (1994) suggested that monoesters constitute a variable proportion of organic P, ranging from negligible values to 70%, and bacteria and phytoplankton obtain much of their P requirement from DRP and a part from monoesters in the aquatic environment. In a temperate mesotrophic lake, Hernandez et al. (1997) suggested that monoesters supplied >40% of the total algal P demand. Whitton et al. (1991) reported that LMP, IHP and diesters are readily available to blue–green algae in watercourses further highlighting the importance of these organic P species. It is known that IHP is strongly sorbed to clays and the sesquioxides (Stewart and Tiessen, 1987), so the presence of IHP in leachate suggests that this form may have been associated with PUP, which represented 54–76% of TP in leachate (Table 1) as they are much less mobile than the LMP and diesters in soil (Cole et al., 1977; Magid et al., 1996). Conversely, LMP and diesters may be components of the DUP suggesting their greater mobility in the soil.

Using phosphatase enzymes, >50% of TUP was identified during March–May 2001. It is likely that this identified pool comprises only labile or potentially mobile organic P. A number of researchers have reported that a combination of phosphatases typically hydrolyzed <65% of unreactive P present in soil solution (Hayes et al., 2000; Hens and Merckx, 2001; Pant et al., 1994).

A large proportion of the remaining unreactive P is still biochemically unidentified and it probably represents insoluble complexes with clay minerals and organic matter (Condron et al., 1997; Magid et al., 1996; Stewart and Tiessen, 1987). The limited hydrolysis of unreactive P confirms the previous studies in soil solutions and soil water extracts that only a part of this pool is accessible to enzymes (Pant et al., 1994; Shand and Smith, 1997). The unidentified unreactive P may either contain P bonds which are resistant to enzyme hydrolysis or may consist of hydrolyzable P bonds which are protected from enzyme attack possibly through occlusion within colloidal particles (Hens and Merckx, 2001).

5. Conclusions

The results of this study demonstrate that monoesters and diesters are the main components of the organic P in leachate, together comprising up to 88% of TP. The major part (>50%) of the organic P was hydrolyzable with different phosphatases and among monoesters a distinction was achieved between LMP and IHP. The presence of inherent enzymic activity in leachate further hydrolyzed a significant proportion of TUP (10–21%) indicating that organic P can itself be mineralized in the water bodies once favorable conditions prevail.

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