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Wet Chemical and Phosphorus-31 Nuclear Magnetic Resonance Analysis of Phosphorus Speciation in a Sandy Soil Receiving Long-Term Fertilizer or Animal Manure Applications

G. F. Koopmans,* W. J. Chardon, J. Dolfing, O. Oenema, P. van der Meer, and W. H. van Riemsdijk

ABSTRACT

In areas under intensive livestock farming and with high application rates of animal manure, inorganic and organic phosphorus (P) may be leached from soils. Since the contribution of these P compounds to P leaching may differ, it is important to determine the speciation of P in these soils. We determined the effect of various fertilization regimes on the P speciation in NaOH-Na₂EDTA (ethylenediamine-tetraacetic acid) and water extracts of acidic sandy soil samples from the top 5 cm of grassland with wet chemical analysis and ³¹P nuclear magnetic resonance (NMR) spectroscopy. These soils had been treated for a period of 11 years with no fertilizer (control), N (no P application), N-P-K, or different animal manures. Inorganic P was highly elevated in the NaOH-Na₂EDTA extracts of the soils amended with N-P-K or animal manures, while organic P increased only in the soil treated with pig slurry. Water-extractable P showed a similar trend. As indicated by ³¹P NMR, orthophosphate monoesters were the main organic P compounds in all soils. Our results suggest that long-term applications of large amounts of P fertilizer and animal manures caused an accumulation of inorganic P, resulting in an increase of the potential risk related to mobilization of inorganic P in the top 5 cm of these soils.

IN AREAS under intensive livestock farming, soil phosphorus (P) content has increased due to high application rates of P fertilizer and animal manure for decades, often exceeding the rate necessary to maintain optimal soil fertility for crop production (e.g., Breeuwsma et al., 1995). In the Netherlands, intensive livestock farming is mainly found on sandy soils in the east and south of the country. Sandy soils are generally characterized by a low sorption capacity for P, and as a result, P can leach, especially in flat areas with a high ground water level (e.g., Sims et al., 1998).

In animal manure, P is composed of both inorganic and organic P fractions, the latter varying from 5 to 25% of total P (Gerritse, 1981; Dou et al., 2000; Sharpley and Moyer, 2000). Dissolved organic P compounds in liquid pig slurry (a mixture of feces, urine, and cleaning water), representing 1% of total P, were found to be of high molecular weight and related to deoxyribonucleic acids (DNA), that is, orthophosphate diesters (Gerritse and Eksteen, 1978). In sandy soils amended with large amounts of pig and cattle slurry, the P leaching through the soil profile was found to be mainly organic P (Ger-

ritse, 1981; Chardon et al., 1997). In the study of Gerritse (1981), where pig slurry was used as an amendment, the organic P leached had molecular weight characteristics similar to dissolved organic P compounds in the liquid fraction of pig slurry found by Gerritse and Eksteen (1978). Therefore, some organic P compounds in animal manure may be more mobile in soil than inorganic P (Gerritse, 1981). In soils treated with large amounts of animal manure, increased inorganic and organic P contents can be expected. Since the contribution of these P compounds to P leaching may differ, it is necessary to determine the P speciation in these soils.

Various sequential extraction methods have been used to characterize P pools of different availability in relation to plant uptake of P (e.g., Tiessen and Moir, 1993). However, extraction methods do not give direct information on the structural composition of the various compounds of P in soil (Guggenberger et al., 1996). Liquid-state ³¹P nuclear magnetic resonance (NMR) spectroscopy, a relatively simple and direct technique, has been used to characterize P in soil extracts (e.g., Tate and Newman, 1982; Hawkes et al., 1984; Dai et al., 1996). Characterization of P in soil extracts with ³¹P NMR has indicated the presence of inorganic P compounds such as orthophosphate, pyrophosphate, and polyphosphate, and organic P compounds such as phosphonate, orthophosphate monoesters (e.g., inositol phosphates), and orthophosphate diesters (e.g., phospholipids and DNA) (Cade-Menun and Preston, 1996).

The method used for extracting P from soil may affect the results obtained by ³¹P NMR (Cade-Menun and Preston, 1996). In most ³¹P NMR studies, P has been extracted by alkaline extracts, such as 0.5 M NaOH (e.g., Tate and Newman, 1982; Hawkes et al., 1984; Bedrock et al., 1994). However, 0.5 M NaOH usually extracts <50% of the total P content from soil, whereas an ideal method should extract all P from soil without altering the P compounds in soil (Cade-Menun and Preston, 1996). The use of 0.25 M NaOH-0.05 M Na₂EDTA extracts more P from soil with minimal alkaline hydrolysis of organic P to inorganic P (Bowman and Moir, 1993; Cade-Menun and Preston, 1996; Dai et al., 1996). Alkaline hydrolysis of orthophosphate diesters may occur, especially at higher NaOH concentrations (Leinweber et al., 1997).

Very little information exists on changes of inorganic and organic P fractions and the distribution of P among the various P compounds within these fractions in soils exposed to long-term application with animal manure.

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Abbreviations: DPS, degree of phosphorus saturation; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; P_w, water-extractable phosphorus.

We therefore selected soil samples from the top 5 cm of grassland exposed to different fertilization regimes in a long-term field experiment, started in 1970 and ending in 1980 (van der Veen, 1985). These soil samples are part of an extensive archive for technical information and soil samples (TAGA) containing the results of about 20 000 field experiments, performed in the period between 1879 and 1998 in the Netherlands, and about 250 000 soil samples from selected experiments (de Willigen et al., 2001). Our first objective was to determine the P speciation in 0.25 M NaOH–0.05 M Na₂EDTA extracts of soils exposed to no fertilizer (control), or applications of N (no P application), N–P–K, or different animal manures. Phosphorus in the NaOH–Na₂EDTA extracts was characterized with wet chemical analysis and ³¹P NMR. Our second objective was to characterize P in water extracts at a soil to solution ratio of 1:5 (w/v), representing the fraction of P in soil relevant for potential mobilization of P to surface waters (e.g., McDowell and Sharpley, 2001), again with wet chemical analysis and ³¹P NMR.

MATERIALS AND METHODS

Field Experiment

We used soil samples from a long-term field experiment (1970–1980) on an acidic sandy soil (van der Veen, 1985). Samples were taken in 1981 from the top 5 cm of eight grassland sites, located in a single field, exhibiting a size of 2 by 2 m and separated by 5-cm-wide and 3-cm-deep trenches. The sites had been treated with no fertilizer (control), N (no P application), N–P–K, or different animal manures. Table 1 shows the treatments and the amounts applied for each application. All soils were treated annually since 1970, except for the pig slurry and the N–P–K treatments, which were applied from 1971 onward. In 1970, the animal manures were applied once, but from 1971 onward, applications were twice each year: at the start of the growing season and after the second harvest of the grass. From 1971 up to and including 1973, the N and N–P–K fertilizers were applied twice each year. From 1974 onward, N was applied four times each year: 50 kg of N ha⁻¹ was applied after the first, second, third, and fourth harvest of the grass, while the P and K fertilizers were applied once each year (before the start of the growing season). Fertilizers and animal manures were applied on the soil surface

without mechanical incorporation. No tillage was applied during the field experiment. After sampling, soils were dried at 40°C, sieved (2 mm), and stored in closed cardboard boxes at constant temperature and humidity in the archive TAGA, as described before (de Willigen et al., 2001).

Chemical Analyses

For the soil samples, we used the following results from van der Veen (1985): pH (KCl), organic matter (estimated from loss on ignition), P_w (water-extractable P at a soil to solution ratio of 1:60 [v/v]), total soil P content, and the P balance of the field experiment. The method of Sissingh (1971) was used to determine P_w. In the Netherlands, P_w is used for P fertilizer recommendations for arable land. Normally, P_w is expressed in mg P₂O₅ L⁻¹ of soil, but in this study in mg P kg⁻¹. For converting P_w, we calculated the density of the soil samples on the basis of the organic matter content. Other methods used are described in Verveijzer et al. (1979). Van der Veen (1985) calculated the cumulative P balance as the difference between the P applied and P removed with the harvested grass. For calculating the P balance, the total P content of the grass and animal manure and the yield of the harvested grass were determined. We determined the organic P content of the soil according to the ignition method of Kuo (1996). The method of Schwertmann (1964) was used for determining oxalate-extractable P, Al, and Fe. Concentrations of P, Al, and Fe were measured by inductively coupled plasma (ICP) spectroscopy. The degree of phosphorus saturation (DPS) was calculated according to Schoumans and Groenen-dijk (2000):

$$DPS = \frac{P_{ox}}{0.5 \times [Al + Fe]_{ox}} \times 100 \quad [1]$$

where DPS is expressed as a percentage, P_{ox} and [Al + Fe]_{ox} are expressed in mmol kg⁻¹, and the value 0.5 denotes the saturation factor of Al- and Fe-(hydr)oxides for P sorption in acidic sandy soils.

NaOH–Na₂EDTA Extracts

A slight modification of the single-step extraction method (0.25 M NaOH–0.05 M Na₂EDTA) of Bowman and Moir (1993) was used to extract P from soil. It is a simple method needing minimal analytical prowess and extracts a large part of total P from soil with minimal alkaline hydrolysis of organic P (Bowman and Moir, 1993; Cade-Menun and Preston, 1996). The NaOH solubilizes the organic material and EDTA in-

Table 1. Treatments, amounts applied for each application, cumulative P balance, and selected characteristics of the soils (0–5 cm) from the field experiment.

Treatment	Application	P balance†	pH (KCl)	Organic matter	P _w ‡	Total P	Organic P§	[Al + Fe] _{ox} §	DPS¶
		kg P ha ⁻¹		%	— mg P kg ⁻¹ —		mg P kg ⁻¹ (%)#	mmol kg ⁻¹	%
Initial soil			4.4	5.4	2.1	393	240 (61)	56	34
No fertilizer (control)		–158	4.0	5.9	3.0	436	269 (62)	54	39
N fertilizer	200 kg N ha ⁻¹	–219	4.0	6.8	1.4	349	286 (82)	45	35
N–P–K fertilizer	200 kg N, 52 kg P, and 199 kg K ha ⁻¹	236	4.1	6.3	13.8	742	288 (39)	56	66
Solid poultry manure	25 Mg ha ⁻¹	2486	6.5	6.5	29.0	2400	529 (22)	57	194
Idem mixed with litter	25 Mg ha ⁻¹	3542	6.3	6.8	39.8	2007	470 (23)	53	208
Poultry slurry	30 Mg ha ⁻¹	1690	6.3	6.0	22.7	1658	477 (29)	50	155
Calf slurry	25 Mg ha ⁻¹	1066	5.7	5.7	26.5	1134	328 (29)	56	111
Pig slurry	25 Mg ha ⁻¹	1000	5.4	6.7	22.0	1222	441 (36)	60	107

† The cumulative P balance was calculated as the difference between the P applied and P removed with the harvested grass.

‡ Water-extractable P at a soil to solution ratio of 1:60 (v/v) (Sissingh, 1971).

§ Organic soil P and [Al + Fe]_{ox} were determined in this study; other data were taken from van der Veen (1985).

¶ Degree of phosphorus saturation (DPS) was calculated according to Eq. [1].

Organic P as a fraction of total P is shown in parentheses.

creases the effectiveness of organic P extraction by breaking P containing Al and Fe complexes from soil. Three grams of soil were suspended in 60 mL of 0.25 M NaOH–0.05 M Na_2EDTA in a centrifuge tube and shaken reciprocally for 16 h at 85 strokes min^{-1} and 20°C. The suspension was centrifuged at $2100 \times g$ for 10 min and filtered through a Schleicher & Schuell (Dassel, Germany) 589/5 filter (pore size 2 to 4 μm). An aliquot of 30 mL of filtrate was freeze-dried and stored at -18°C . Before ^{31}P NMR analysis, the freeze-dried extract was dissolved in D_2O , vortexed for 2 min, and kept at room temperature for 2 h. The remaining filtrate was used to determine inorganic P and total P, Fe, and Mn, and the pH. Inorganic P measurements were made colorimetrically (Murphy and Riley, 1962) after removal of a large fraction of the solubilized organic matter through acidification and centrifugation (Tiessen and Moir, 1993). Total P, Fe, and Mn concentrations were measured by ICP. Organic P was calculated as the difference between total and inorganic P. The pH was measured by pH indicator strips (pH of approximately 13). The ^{31}P NMR spectra were obtained on a Bruker (Rheinstetten, Germany) DPX 300 spectrometer operating at 121.49 MHz and room temperature. Conditions used for ^{31}P analysis were a pulse angle of 90° , a pulse delay of 2 s, and an acquisition time of 0.67 s. For each sample, 512 scans were used to obtain an acceptable signal to background noise ratio. Chemical shifts (δ) of the peaks were measured according to an external 0.98 mM methylenediphosphonic acid trisodium salt tetrahydrate (MDP; 98%) standard, contained in a capillary tube measured simultaneously with each sample. In most other ^{31}P NMR studies, orthophosphoric acid is used as a standard. For comparison of our results with these studies, MDP was set at $\delta = 18.1$ ppm. Interpretation of the ^{31}P NMR spectra was based on literature assignments (Bedrock et al., 1994; Cade-Menun and Preston, 1996; Amelung et al., 2001). Peak areas were calculated by instrumental integration. The ^{31}P NMR spectra were evaluated semiquantitatively, that is, the fractions of the different P compounds were calculated by relating the respective peak areas to the total area.

Water Extracts

In addition to the P_w results of van der Veen (1985), we measured water-extractable P at a soil to solution ratio of 1:5 (w/v). Twelve grams of soil were suspended in 60 mL of water and shaken reciprocally for 16 h at 85 strokes min^{-1} and 20°C. The suspension was centrifuged at $2100 \times g$ for 10 min and filtered through a Schleicher & Schuell 589/5 filter. An aliquot of 40 mL of filtrate was freeze-dried and stored at -18°C . Before ^{31}P NMR analysis, the freeze-dried extract was dissolved in D_2O , vortexed for 2 min, and kept at room temperature for 2 h. The ^{31}P NMR spectra of the water extracts were obtained with the same ^{31}P NMR parameters as for the NaOH– Na_2EDTA extracts. Interpretation of the spectra was based on our experience with ^{31}P NMR analysis of solutions exhibiting a slightly acidic pH. The remaining filtrate was used to determine inorganic and organic P and total P, Fe, and Mn.

Test for Alkaline Hydrolysis of Organic Phosphorus

Alkaline hydrolysis of organic P to inorganic P was tested by measuring inorganic P before and after the addition of a volume of 0.25 M NaOH to a soil water extract. Five grams of soil were suspended in 25 mL of water and shaken reciprocally for 16 h at 85 strokes min^{-1} and 20°C. The suspension was centrifuged at $2100 \times g$ for 10 min and filtered through a Schleicher & Schuell 589/5 filter, and inorganic P and the pH were measured. On average, the pH was 5.34 ± 1.06 .

Three milliliters of 0.25 M NaOH were added to an aliquot of 10 mL of filtrate to increase the pH to approximately 13. The filtrate was shaken again for 16 h at 85 strokes min^{-1} and 20°C. Afterwards, inorganic P and the pH were measured ($\text{pH} = 12.90 \pm 0.03$).

RESULTS AND DISCUSSION

Soils

Table 1 shows the selected characteristics of the soil samples and the P balance of the field experiment. After 11 yr of treatment, soil characteristics such as pH and organic matter were considerably affected. The pH increased in the soils amended with animal manures, while the organic matter content increased in all soils. Mugwira (1976) also found an increase in soil pH and organic matter after 3 yr of dairy manure application at rates varying from 22 to 267 $\text{Mg ha}^{-1} \text{yr}^{-1}$. A higher pH decreases sorption of P in sandy soils, increasing the soil potential for P mobilization, and favors the microbial mineralization of organic P (Beek and van Riemsdijk, 1982).

The P_w (plant-available P) and total P content of the soils amended with N–P–K or animal manures were 6.6 to 19.0 and 1.9 to 6.1 times higher than the corresponding values in the initial soil, respectively, due to the positive P balance. Interestingly, the relative increase of P available for plant uptake was much higher than the increase of total P. The DPS showed a large increase in the N–P–K- and animal manure-amended soils (Table 1). The sum of oxalate-extractable Al and Fe remained relatively constant. As soils become increasingly saturated with P, any additional soluble P applied through P fertilizer or animal manure remains as (readily) available P in soil, causing a large increase of P_w . In the soils treated with animal manures, the DPS is, however, (much) higher than expected on basis of the theoretical maximum ($\text{DPS} = 100\%$) predicted by the sum of oxalate-extractable Al and Fe. This may indicate the buildup of a pool of Ca–P compounds in soil originating from the animal manures applied. In acidic to neutral soils, heavily enriched with P, Ca–P compounds may exist as metastable solid phases. De Haan and van Riemsdijk (1986) found indications for the existence of brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) in heavily pig manured sandy soils. The P_w value and total P content of the soil treated with N fertilizer (no P application) slightly decreased, due to the negative P balance. In contrast, P_w and total P of the control soil slightly increased. In the soils treated with animal manures, organic P was 1.4 to 2.2 times higher than in the initial soil, due to the accumulation of organic P resulting from animal manure application. In the control and N- and N–P–K-amended soils, organic P was 1.1 to 1.2 times higher. This slight increase may be explained by accumulation of organic P from plant residues. Since the increase of the total P content was much higher than the increase of organic P in the soils treated with N–P–K or animal manures, P had mainly accumulated in soil as inorganic P (results not shown). Similar results have been found in soils treated with large amounts of cattle feedlot manure over several

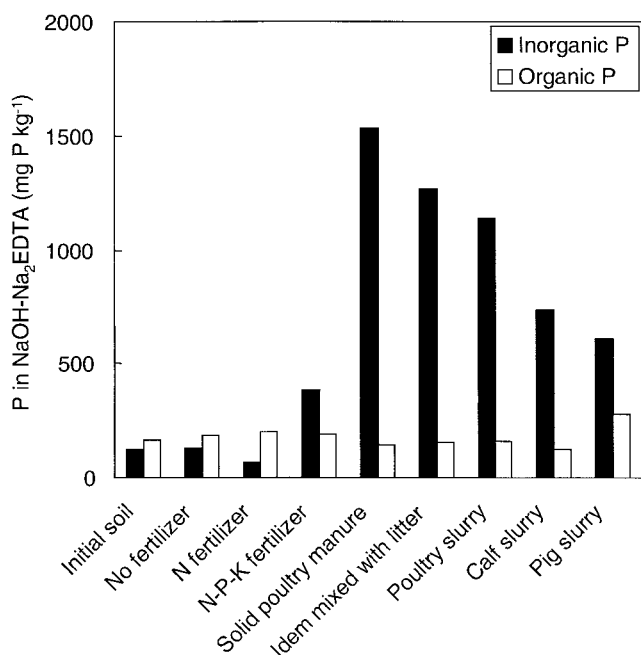


Fig. 1. Inorganic and organic P for the different fertilizer regimes, measured in NaOH-Na₂EDTA extracts by wet chemical analysis.

years; this was attributed to the high inorganic P fraction (78%) found in the cattle feedlot manure (Sharpley et al., 1984).

Wet Chemical Analysis of NaOH-Na₂EDTA-Extractable Phosphorus

Figure 1 and Table 2 show inorganic and organic P measured in the NaOH-Na₂EDTA extracts. Total P in the NaOH-Na₂EDTA extracts represented 70 to 78% of the total P content in soil (Table 2), implying that it is a reasonably good extractant for total P in our sandy soil samples, although the method was originally developed for extracting organic soil P (Bowman and Moir, 1993). Our results correspond well with recoveries of total P found in other studies where 0.25 M NaOH–0.05 M Na₂EDTA was used as an extractant: Cade-Menun and Preston (1996) and Dai et al. (1996) extracted 71 to 91% and 39 to 99% of total P from forest soils, respectively. The P remaining in soil after extraction

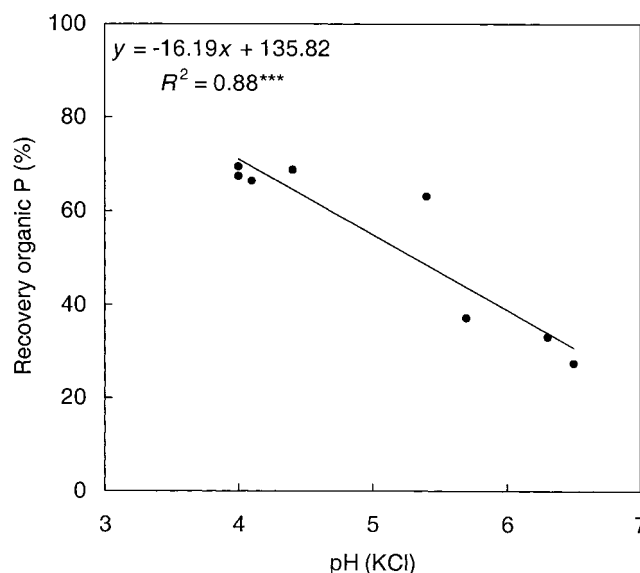


Fig. 2. Recovery of extracted organic P by NaOH-Na₂EDTA relative to organic soil P plotted against the soil pH (KCl).

with NaOH-Na₂EDTA may represent some insoluble inorganic P compounds (Dai et al., 1996). Organic P in our NaOH-Na₂EDTA extracts represented 27 to 70% of organic soil P (Table 2). These results agree with the 22 to 103% extracted by Dai et al. (1996). Interestingly, the recovery of extracted organic P relative to organic soil P decreased with increasing soil pH (Fig. 2). In the soils with pH values close to neutral, only 27 to 37% of organic soil P was extracted (Table 1 and 2; the solid poultry manure, solid poultry manure mixed with litter, and poultry and calf slurry treatments). Because of the higher soil pH of the latter treatments, the microbial mineralization rate may have been higher than in the soils of the other treatments; the remaining organic soil P may therefore have a lower extractability in NaOH-Na₂EDTA. Another explanation includes a lower extractability of organic P originating from solid poultry manure, solid poultry manure mixed with litter, and poultry and calf slurry. According to the sequential extraction method of Tiessen and Moir (1993), 0.1 M NaOH represents less available inorganic and organic P compounds. Since we did not use any other extraction

Table 2. Total, inorganic, and organic P measured in the NaOH-Na₂EDTA extracts by wet chemical analysis, and organic P as a fraction of total P, measured by wet chemical analysis or ³¹P nuclear magnetic resonance (NMR).

Treatment	Wet chemical analysis				³¹ P NMR
	Total P	Inorganic P	Organic P	Organic P/total P	Organic P/total P
	mg P kg ⁻¹ (%)†	mg P kg ⁻¹	mg P kg ⁻¹ (%)‡	%	% of total area§
Initial soil	285 (73)	120	165 (69)	58	52
No fertilizer (control)	310 (71)	128	182 (68)	59	54
N fertilizer	268 (77)	69	199 (70)	74	66
N-P-K fertilizer	574 (77)	383	191 (66)	33	29
Solid poultry manure	1681 (70)	1536	145 (27)	9	17
Idem mixed with litter	1424 (71)	1269	156 (33)	11	21
Poultry slurry	1298 (78)	1141	158 (33)	12	17
Calf slurry	857 (76)	736	122 (37)	14	24
Pig slurry	889 (73)	610	279 (63)	31	33

† Total P as a fraction of total soil P is shown in parentheses.

‡ Organic P as a fraction of organic soil P is shown in parentheses.

§ Sum of peak areas of organic P compounds as a fraction of total area.

prior to the NaOH- Na_2EDTA extraction, it thus contains both available and less available inorganic and organic P compounds, made available for characterization by ^{31}P NMR. Total P measured in NaOH- Na_2EDTA was 32 to 188 times higher than the corresponding P_w value (results not shown).

Inorganic P in the NaOH- Na_2EDTA extracts of soils amended with N-P-K or animal manures was 3.2 to 12.8 times higher than in the initial soil (Fig. 1 and Table 2). In contrast, removal of P from soil by cropping of grass decreased inorganic P in the N fertilizer treatment. Organic P remained relatively constant in all treatments except in the soil treated with pig slurry. Our results are further illustrated by organic P as a fraction of total P. The organic P fraction decreased from 58% in the initial soil to 9 to 33% in the soils amended with N-P-K or animal manures (Table 2). The high levels of inorganic P found in the NaOH- Na_2EDTA extracts of the soils treated with animal manures can be attributed to the large inorganic P fraction generally found in animal manures (Gerritse, 1981; Dou et al., 2000; Sharpley and Moyer, 2000). Long-term field experiments indicate a tendency for intensively managed agricultural soils to contain a higher fraction of inorganic P rather than organic P, reflecting the long-term application of P fertilizer and animal manure (Hawkes et al., 1984; Sharpley et al., 1984). In contrast with the N-P-K- and animal manure-amended soils, the organic P fraction in the N-treated soil showed a large increase (74%), probably resulting from uptake of inorganic P by the grass and some accumulation of organic P from plant residues, but remained relatively constant in the control soil (59%). In nonagricultural soils without P input, organic P becomes more important (Condon et al., 1990; Cade-Menun and Preston, 1996; Dai et al., 1996).

Test for Alkaline Hydrolysis of Organic Phosphorus

With any extraction method, soil P compounds can be chemically altered during or after extraction (Cade-Menun and Preston, 1996). Hydrolysis of organic P in alkaline extracts has been reported in literature (e.g., Leinweber et al., 1997). Alkaline hydrolysis was tested by increasing the pH of the water extracts from 5.34 ± 1.06 to 12.90 ± 0.03 . After shaking for 16 h, inorganic P in the water extracts increased to a small extent (on average $6.5 \pm 5.4\%$), probably due to alkaline hydrolysis of chemically labile organic P compounds into inorganic P. Thus, the possibility of some transformation of organic P to inorganic P during our NaOH- Na_2EDTA extraction cannot be excluded.

Phosphorus-31 Nuclear Magnetic Resonance Analysis of NaOH- Na_2EDTA -Extractable Phosphorus

Figure 3 shows representative ^{31}P NMR spectra of the NaOH- Na_2EDTA extracts from the soils either amended with solid poultry manure mixed with litter or amended with calf slurry. Four distinct P compounds were detected: orthophosphate ($\delta = 7.0\text{--}7.1$ ppm), or-

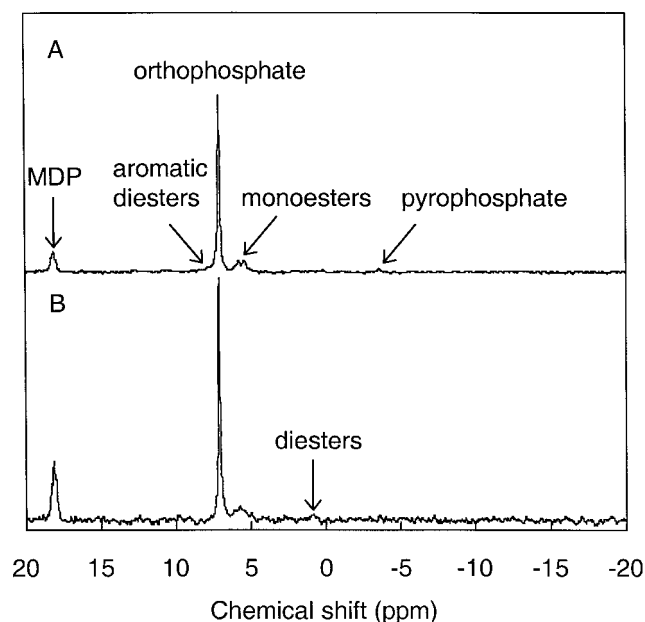


Fig. 3. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra of NaOH- Na_2EDTA extracts from soils either amended with solid poultry manure mixed with litter (A) or amended with calf slurry (B). MDP denotes the P standard.

thophosphate monoesters ($\delta = 4.7\text{--}5.8$ ppm), orthophosphate diesters ($\delta = 0.6\text{--}0.8$ ppm), and pyrophosphate ($\delta = -3.6$ to -3.7 ppm) (Cade-Menun and Preston, 1996). A peak appearing at $\delta = 7.6$ to 7.9 ppm may reflect the presence of aromatic orthophosphate diesters (Bedrock et al., 1994; Amelung et al., 2001). The quality of our ^{31}P NMR spectra was good, with a good separation of the orthophosphate and orthophosphate monoester peaks. This contrasts with the ^{31}P NMR spectra obtained by Cade-Menun and Preston (1996), with a relatively poor separation of the orthophosphate and orthophosphate monoester peaks. This was attributed to the complexing of Fe and Mn by EDTA. Extraction of paramagnetic ions, such as Fe and Mn, causes line broadening and distortion of the ^{31}P NMR spectra (Hawkes et al., 1984). In our study, however, Fe and Mn concentrations after extraction were on average only 45 and 3% of those of Cade-Menun and Preston (1996), respectively.

Table 2 shows, for the different fertilizer regimes, organic P as a fraction of total P measured by wet chemical analysis or ^{31}P NMR (% of total area). Differences between both organic P fractions were relatively small ($<11\%$) and agree well with results reported elsewhere in literature (e.g., Condon et al., 1985; Dai et al., 1996). Thus, both methods provided similar results in separating organic P from inorganic P and apparently no significant alkaline hydrolysis of organic P compounds occurred during concentration of the NaOH- Na_2EDTA extract and ^{31}P NMR analysis (Zhang et al., 1999). Differences between the two methods have been attributed to errors arising from the indirect wet chemical method used for estimating organic P (i.e., difference between total P and inorganic P) and to background noise in the ^{31}P NMR spectra (Dai et al., 1996).

Table 3. The P compounds as a fraction of total P measured by ^{31}P nuclear magnetic resonance (NMR) in the NaOH- Na_2EDTA extracts.

Treatment	Aromatic diesters	Orthophosphate	% of total area†		
			Monoesters	Diesters	Pyrophosphate
Initial soil	ND‡	48.3	51.7	ND	ND
No fertilizer (control)	1.9	45.9	52.2	ND	ND
N fertilizer	4.8	34.4	60.8	ND	ND
N-P-K fertilizer	ND	71.3	28.7	ND	ND
Solid poultry manure	0.9	83.1	15.6	0.2	0.2
Idem mixed with litter	3.6	77.0	17.9	ND	1.5
Poultry slurry	3.1	81.6	13.5	ND	1.8
Calf slurry	1.7	75.9	19.2	3.2	ND
Pig slurry	6.4	66.3	26.6	ND	0.7

† Peak areas of the respective P compounds as a fraction of total area.

‡ Not detected.

Table 3 shows the P compounds as a fraction of total P (% of total area) measured by ^{31}P NMR. In all treatments, inorganic P was mainly found as orthophosphate, although some very small fractions of pyrophosphate were found in the solid poultry manure-, solid poultry manure mixed with litter-, and poultry- and pig slurry-treated soils. Pyrophosphate can be a product of biological cycling of P in soil, but it can also be formed by hydrolyzation of organic esters during or after the extraction by NaOH- Na_2EDTA (Condrón et al., 1985). Pyrophosphate is a short-chain polyphosphate ($n = 2$), which is used as a microbial storage product appearing from microbial activity (Condrón et al., 1985; Cade-Menun et al., 2000a,b). The presence of pyrophosphate in the solid poultry manure-, solid poultry manure mixed with litter-, and poultry slurry-amended soils, with a pH close to neutral, further supports the idea of a higher microbial mineralization rate in these soils. Organic P was mainly found as orthophosphate monoesters. This agrees with results reported in other studies (e.g., Tate and Newman, 1982; Hawkes et al., 1984; Condrón et al., 1985, 1990; Bedrock et al., 1994). Orthophosphate diesters were detected in the solid poultry manure- and calf slurry-amended soils. Orthophosphate monoesters and diesters in soil can be derived from a combination of plant and microbial residues (Anderson, 1967; Bedrock et al., 1994; Guggenberger et al., 1996) and animal manure application. Using ^{31}P NMR, Leinweber et al. (1997) detected these P compounds in poultry and pig manure. Orthophosphate monoesters consist mainly of inositol phosphates, amounting to >50% of total organic P in soil (Anderson, 1967). Due to the high charge density of inositol phosphates, these P compounds are strongly sorbed by (hydr)oxides of Al and Fe in soil (Turrión et al., 2001), protecting inositol phosphates from mineralization. Orthophosphate diesters comprise P compounds such as phospholipids, ribonucleic acids (RNA), and DNA. Because of their lower charge density, orthophosphate diesters are only slightly retained in soil, and are more mobile and more accessible for microbial degradation than monoesters (Gerritse, 1981; Turrión et al., 2001). Under favorable soil conditions, such as an optimal pH and moisture content for microbial activity, orthophosphate diesters can be transformed into monoesters (Hinedi et al., 1988). In intensively managed grassland soils with favorable conditions for mineralization, such as in our soils, orthophosphate diesters can be absent (Condrón et al., 1990). In contrast,

in nonagricultural soils where conditions favored a lower rate of mineralization (low pH and high moisture content), significant amounts of orthophosphate diesters have been found (Condrón et al., 1990; Bedrock et al., 1994; Cade-Menun et al., 2000a). Moreover, alkaline hydrolysis of orthophosphate diesters into monoesters may have occurred to a small extent. Especially at higher NaOH concentrations (0.5 M NaOH), alkaline hydrolysis of orthophosphate diesters can occur (Leinweber et al., 1997). We used a lower NaOH concentration (0.25 M NaOH), possibly decreasing the potential of alkaline hydrolysis.

Summarizing, inorganic P was highly elevated in soils amended with N-P-K or animal manures, while organic P increased only in the soil treated with pig slurry. However, liquid-state ^{31}P NMR cannot be used to characterize inorganic P, since different inorganic P compounds (e.g., P associated with Al, Fe, or Ca) dissolved in the NaOH- Na_2EDTA extracts appear as one orthophosphate peak in the ^{31}P NMR spectra. Our results can therefore not be used to interpret whether long-term P applications resulted in accumulations of different inorganic P compounds. For this purpose, sequential extraction methods in combination with solid-state ^{31}P magic angle spinning (MAS)-NMR can be useful. Using the latter technique in acidic sandy soils amended with large amounts of animal manure, inorganic P was shown to be associated with Al and Ca; one of the Ca-P compounds was more soluble than the other P compounds (Lookman et al., 1997). However, solid state ^{31}P MAS-NMR has some limitations: paramagnetic ions, such as Fe and Mn, cause line broadening and large spinning-side bands complicating the interpretation of ^{31}P NMR spectra and the detection of P associated with these ions in soil (Hinedi et al., 1989; Lookman et al., 1997).

Wet Chemical and Phosphorus-31 Nuclear Magnetic Resonance Analysis of Water-Extractable Phosphorus

Characterization of P in NaOH- Na_2EDTA extracts gives limited information on the soil potential for P mobilization, since both available and less-available inorganic and organic P compounds are extracted. For a better understanding of the potential mobility of P in our soils, water- or 0.01 M CaCl_2 -extractable P needs to be characterized, since these extractants represent the P fraction in soil relevant for potential P loss to

Table 4. Total, inorganic, and organic P measured in the 1:5 (w/v) soil water extracts by wet chemical analysis, and organic P as a fraction of total P, measured by wet chemical analysis or ^{31}P nuclear magnetic resonance (NMR).

Treatment	Wet chemical analysis				^{31}P NMR
	Total P	Inorganic P	Organic P	Organic P/total P	Organic P/total P
	mg P kg ⁻¹ (%)†	mg P kg ⁻¹		%	% of total area‡
Initial soil	6.7 (1.7)	2.2	4.5	67	ND§
No fertilizer (control)	7.5 (1.7)	2.7	4.8	64	21
N fertilizer	8.4 (2.4)	2.6	5.8	69	12
N-P-K fertilizer	24.7 (3.3)	16.7	8.0	32	ND
Solid poultry manure	35.5 (1.5)	28.5	6.9	20	12
Idem mixed with litter	69.4 (3.5)	55.1	14.3	21	ND
Poultry slurry	27.4 (1.7)	20.5	6.9	25	8
Calf slurry	41.5 (3.7)	31.6	9.9	24	ND
Pig slurry	43.8 (3.6)	33.5	10.3	24	4

† Total P as a fraction of total soil P is shown in parentheses.

‡ Peak area of orthophosphate monoesters as a fraction of total area.

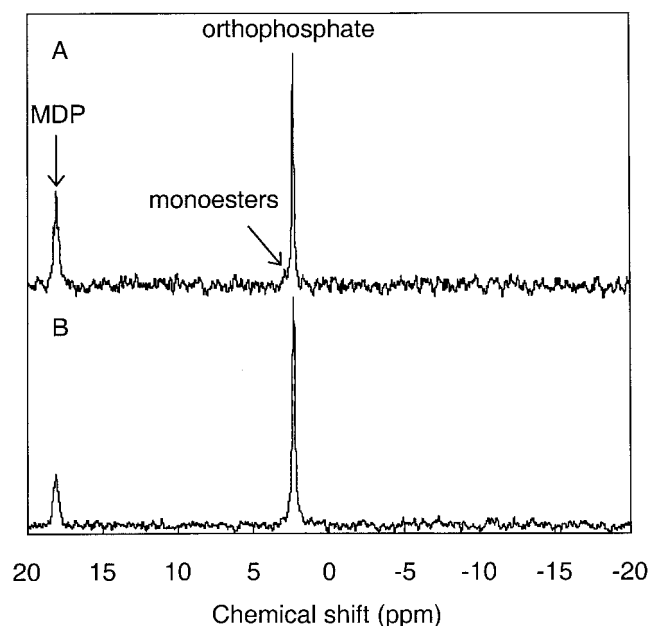
§ Not detected.

flowing water (e.g., McDowell and Sharpley, 2001). In addition to results of P_w , which has a low soil to solution ratio (1:60 [v/v]), taken from van der Veen (1985) (Table 1), we measured water-extractable P at a soil to solution ratio of 1:5 (w/v). Because of the higher soil to solution ratio of these water extracts, these results may be more indicative for the leaching of soil solution through the soil profile where a relatively large amount of soil comes into contact with a relatively limited volume of solution. Table 4 shows total, inorganic, and organic P measured in the water extracts by wet chemical analysis. Water extracted 1.5 to 3.7% of the total P content from soil (Table 4). The trend shown by inorganic P is similar to the one in the NaOH-Na₂EDTA extracts. Inorganic P increased by 7.5 to 24.8 times in the N-P-K- and animal manure-amended soils. In contrast, organic P increased only by 1.5 to 3.2 times. These results are further illustrated by organic P as a fraction of total P. The organic P fraction decreased from 67% in the initial soil to 20 to 32% in the soils amended with N-P-K or animal manures, but remained relatively constant in the control and N-amended soils (Table 4).

Figure 4 shows representative ^{31}P NMR spectra of the water extracts from the soils either amended with poultry or pig slurry. Two distinct P compounds were detected: orthophosphate ($\delta = 2.2$ –2.4 ppm) and orthophosphate monoesters ($\delta = 2.8$ –3.0 ppm). The chemical shifts of orthophosphate and orthophosphate monoesters in the water extracts differed from those in the NaOH-Na₂EDTA extracts. Since we did not adjust the pH of the solution containing the freeze-dried water extract prior to ^{31}P NMR analysis, the pH must have been lower than the pH of the solution with the freeze-dried NaOH-Na₂EDTA extract. As demonstrated by Crouse et al. (2000), the pH significantly affects the chemical shifts of the various P compounds in the ^{31}P NMR spectra: changing the pH causes a change in the chemical environment surrounding the P nuclei and as pH changes so do the chemical shifts. Thus, the differences between the chemical shifts of orthophosphate and orthophosphate monoesters in our NaOH-Na₂EDTA and water extracts can be attributed to a change in pH.

Orthophosphate was detected in all treatments except in the initial soil. In contrast to the results of wet chemi-

cal analysis (Table 4), organic P was found (as orthophosphate monoesters) only in the control soil and the N-, solid poultry manure-, and poultry- and pig slurry-amended soils. Since orthophosphate monoesters are strongly retained in soil (Turrión et al., 2001), these P compounds may have been mobilized in water because of dispersion of colloidal soil particles. Table 4 shows organic P as a fraction of total P measured by ^{31}P NMR (% of total area). Organic P fractions measured by ^{31}P NMR were much smaller than those obtained by wet chemical analysis, showing that some organic P was not detected by ^{31}P NMR. McDowell et al. (1998) reported ^{31}P NMR results of P in 1:5 (w/v) 0.01 M CaCl₂ extracts from four P-rich agricultural soils. They detected mainly orthophosphate and orthophosphate monoesters, and moreover, some small amounts of phosphonate, aromatic phosphate diesters, orthophosphate diesters, and pyrophosphate, indicating the potential mobility of these P compounds in soil. Most of these P compounds were

**Fig. 4.** Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra of water extracts from soils either amended with poultry slurry (A) or amended with pig slurry (B). MDP denotes the P standard.

also detected in the NaOH–Na₂EDTA extracts of our soils (Table 3), but apart from orthophosphate and orthophosphate monoesters, not in our water extracts. Concentrations of these P compounds may possibly have been below the detection limit of ³¹P NMR. In the future, we will work on the development of an improved method for extracting potentially mobile P compounds for characterization with ³¹P NMR. The choice of the extractant is important. Calcium chloride may affect results obtained by ³¹P NMR, since CaCl₂ induces precipitation of dissolved organic matter and associated P, especially of high molecular weight compounds (Dolfing et al., 1999; Oste et al., 2002).

CONCLUSIONS

The speciation of P in the top 5 cm of an acidic sandy grassland soil exposed to different fertilization regimes was studied in NaOH–Na₂EDTA and water extracts with wet chemical analysis and ³¹P NMR spectroscopy. After 11 yr of treatment with N–P–K or different animal manures, inorganic P was highly elevated in the NaOH–Na₂EDTA extracts, while accumulation of organic P was found only in the soil amended with pig slurry. In contrast, inorganic P remained relatively constant in the control soil (no fertilizer), while removal of P from soil by cropping of grass in the N-treated soil decreased inorganic P. In both soils, organic P remained relatively constant. Hence, in soils without P input, organic P becomes more important. In all soils, orthophosphate monoesters were the main organic P compounds. In the water extracts, the same trend was found: inorganic P was highly elevated in soils treated with N–P–K fertilizer or animal manures, while organic P showed only a small increase. Orthophosphate monoesters were the only organic P compounds detected in the water extracts. Long-term applications of large amounts of N–P–K or animal manures resulted mainly in an accumulation of inorganic P in the soils as measured in both the NaOH–Na₂EDTA and water extracts. Since water-extractable P is considered to represent potentially mobile P in soil, risk of P loss to water in the top 5 cm of these soils was mainly related to inorganic P.

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