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Phosphorus Compounds in Sequential Extracts of Animal Manures: Chemical Speciation and a Novel Fractionation Procedure

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Pollution of water bodies by phosphorus in runoff from soil amended with animal manures is one of the greatest threats to water quality in developed countries. The environmental fate of manure phosphorus is determined in part by its chemical composition, yet extraction procedures to assess this are poorly developed and provide no structural information. We used solution ^{31}P NMR spectroscopy to quantify phosphorus compounds in sequential extracts of three contrasting manures (broiler litter, beef-cattle manure, swine manure). Using a procedure originally developed for soils, but commonly applied to manures, phosphorus was extracted sequentially with deionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH, and 0.5 M HCl. Water and $NaHCO_3$ extracted readily soluble compounds, including phosphate, phospholipids, DNA, and simple phosphate monoesters, which are mobile in soil and biologically available. In contrast, NaOH and HCl extracted poorly soluble compounds, including phytic acid (*myo*-inositol hexakisphosphate). The latter is immobile in soil and of limited biological availability. Based on these results, we developed a simplified two-step fractionation procedure involving extraction of readily soluble phosphorus in 0.5 M $NaHCO_3$ followed by extraction of stable phosphorus in a solution containing 0.5 M NaOH and 50 mM EDTA. This revised procedure separates manure phosphorus into structurally defined fractions with environmental relevance and will facilitate research on this important aspect of environmental science.

Introduction

Long-term manure application to agricultural land leads to soil phosphorus accumulation and an acceleration of phosphorus transfer in runoff to water bodies (*1*). This can contribute to eutrophication in freshwater ecosystems, and numerous examples of water quality impairment associated with phosphorus pollution from animal operations now exist (*2*–*4*). To address this, strategies involving dietary manipulation are being adopted to reduce manure phosphorus concentrations. For monogastric animals, which cannot digest phytic acid, such strategies include the isolation of mutant grains with low phytic acid concentrations (*5*) and supplementation of diets with microbial phytase to hydrolyze phytic acid in the gut (*6*). Such dietary manipulations alter the phosphorus composition of manure and influence its fate in the environment. Phytic acid is immobile in soils, because it sorbs strongly to clays and reacts with metal oxides to form insoluble compounds (*7, 8*). Other organic phosphates, such as phosphate diesters and simple phosphate monoesters, are only weakly retained in soil and can escape in leachate (*9, 10*). Phosphate is relatively soluble in manures, and concentrations are strongly correlated with those in surface runoff following recent manure application (*11*). However, it can be strongly retained in soil if drainage occurs downward through the profile (*9*).

Understanding the environmental fate of manure phosphorus requires robust procedures for determining its composition and solubility. Sequential fractionation involves extraction of phosphorus from manure with increasingly strong chemical solutions to quantify pools of phosphorus with varying degrees of solubility. The technique is commonly used because it provides information on manure phosphorus using standard laboratory procedures, which is important given that most laboratories to do not have access to the advanced analytical equipment required for detailed chemical speciation. McAuliffe and Peech (*12*) described the first fractionation procedure for phosphorus in manures, which was subsequently used by Peperzak and colleagues (*13*) to analyze manures from a wide variety of animals. Phytic acid, measured as acid-soluble phosphorus, was the major component of the organic phosphorus in most manures, with only small proportions of phospholipids. Comparable results were obtained subsequently using similar procedures (*14, 15*).

Interest in the environmental fate of manure phosphorus led recent studies to adopt the Hedley fractionation (*16*–*19*). This procedure was originally developed to assess phosphorus solubility in soil (*20*) and involves sequential extraction with water, $NaHCO_3$, NaOH, and HCl. Phosphorus extracted in water and $NaHCO_3$ is considered readily soluble, while that extracted in NaOH (assumed to be associated with amorphous iron/aluminum and organic matter) and HCl (assumed to be calcium phosphates) is considered poorly soluble. However, several problems compromise the suitability of the Hedley fractionation for manures. Phosphorus chemistry differs markedly between soils and manures, being controlled commonly by iron and aluminum oxides in soils (*20*), but by association with calcium and magnesium in manures (*21*). In addition, there is little information on phosphorus compounds in sequential extracts, so it is impossible to assign environmental significance to the extracted fractions. He and Honeycutt (*22*) used phosphatase enzymes to determine functional classes of hydrolyzable phosphorus in sequential fractions of swine and dairy manures, including simple phosphate monoesters, phytate-like phosphorus, and nucleotide-like phosphorus. However, a large proportion of the organic phosphorus was not identified, and no information was obtained on organic phosphorus in HCl extracts.

Nuclear magnetic resonance (NMR) spectroscopy can provide compound-specific information on manure phosphorus. Solid-state ^{31}P NMR spectroscopy was used recently to investigate phosphorus speciation in poultry litter (*23*) but is relatively insensitive and is unable to identify organic phosphates. In contrast, solution ^{31}P NMR spectroscopy offers the most convenient way to speciate phosphorus in manure extracts, because multiple phosphorus compounds can be

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TABLE 1. Total Elements in Three Animal Manures Determined by Microwave Digestion in Concentrated HNO_3 and H_2O_2 and Detection by ICP–OES

	mg kg^{-1} dry wt		
	broiler litter	cattle manure	swine manure
aluminum	473[a]	1534	215
calcium	20643	15940	11860
iron	860	1145	1131
magnesium	6472	6851	5387
phosphorus	15953	4943	14617

[a] Values are means of three replicate digests.

quantified simultaneously with minimal sample preparation and handling (*24*). Using this technique, Leinweber and colleagues (*16*) identified phosphate monoesters and diesters in NaOH extracts of swine slurry, while Crouse and colleagues (*25*) characterized functional phosphorus groups in NaOH-EDTA extracts of turkey litter. These studies suffered from poor resolution in the phosphate monoester region of the spectra, which precluded quantification of phytic acid, the dominant organic phosphate in manure from grain-fed animals (*13, 15, 24*). However this was overcome recently using a stronger NaOH solution and including EDTA (ethylenediaminetetraacetate), which markedly improved spectral resolution for extracts of swine manure and broiler litter (*24*).

Given the current widespread interest in the environmental fate of manure phosphorus, a straightforward procedure is urgently required that can provide information on its solubility and chemical composition without the need for advanced analytical equipment. Our aim was to characterize phosphorus compounds in sequential extracts of animal manures using solution ^{31}P NMR spectroscopy. Based on these results, we developed a simplified procedure that yields structurally defined phosphorus fractions with environmental relevance.

Materials and Methods

Manure Properties. Three manures were obtained: a swine manure (grain fed) and a beef-cattle manure (pasture-fed) from farms in southern Idaho and a broiler litter (mixture of broiler manure and sawdust bedding) from a farm in Delaware. Dry matter contents were 25% for the swine manure, 14% for the cattle manure, and 84% for the broiler litter. Samples were frozen at −80 °C, lyophilized, and ground to pass a 500 μm sieve. Total elements (Table 1) were determined by microwave digestion in concentrated HNO_3 and H_2O_2 (*26*) with detection by inductively coupled plasma optical-emission spectrometry (ICP–OES). The manures were analyzed in a previous study investigating optimum extraction conditions for solution ^{31}P NMR spectroscopy (*24*)

Sequential Fractionation. Manures were sequentially extracted by three procedures: a modified version of the procedure developed by Hedley and colleagues (*20*) for analysis of soil (but commonly applied to manure) and two revised procedures based on the results of the Hedley fractionation. For the Hedley fractionation, phosphorus was extracted sequentially with deionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH, and 1.0 M HCl. Each extraction was performed in a 1:60 manure-to-solution ratio for 1 h.

The revised procedures both involved initial extraction of manure in 0.5 M $NaHCO_3$ for 4 h. This was followed by extraction overnight (16 h) in either 1.0 M HCl or a solution containing 0.5 M NaOH and 50 mM EDTA (*24*). Based on conventional procedures, the manure-to-solution ratio was 1:60 for the $NaHCO_3$ and HCl extracts (*20*) and 1:20 for the NaOH-EDTA extracts (*24*). For all procedures, extracts were performed in triplicate at 20 °C, centrifuged at 10 000 × *g* for 30 min, and then suction filtered through 0.45 μm cellulose-nitrate membranes (Millipore, Billerica, NY). An aliquot of each replicate was diluted and analyzed separately for total elements by ICP–OES. The remaining solution in each set of replicate extracts was combined, frozen rapidly at −80 °C, lyophilized, and lightly ground. Prior to ICP–OES analysis and lyophilization, $NaHCO_3$ extracts were acidified with dilute HCl to approximately pH 3 to dissolve carbonates. All HCl extracts were neutralized with 1 M NaOH prior to lyophilization. Statistical analysis of differences between the three procedures (Hedley, $NaHCO_3$/HCl, $NaHCO_3$/NaOH-EDTA) for recovery of (i) total phosphorus and (ii) readily soluble phosphorus fractions (initial water extraction, initial $NaHCO_3$ extraction, sequential water plus $NaHCO_3$ extraction) was performed in SAS Version 8.0 using analysis of variance with Duncan's multiple range test ($P<0.05$) for means separation.

Solution ^{31}P NMR Spectroscopy. Immediately prior to NMR spectroscopy, approximately 100 mg of each freeze-dried extract was redissolved in 0.9 mL of a solution containing 1 M NaOH and 0.1 M EDTA. Deuterium oxide (0.1 mL) was added for signal lock, and the solution was transferred to a 5-mm NMR tube. The inclusion of NaOH ensures consistent chemical shifts at pH >13, while EDTA reduces line broadening by chelating paramagnetic ions (*27, 28*).

Solution ^{31}P NMR spectra were obtained using a Bruker Avance DRX 500 MHz spectrometer operating at 202.456 MHz for ^{31}P and 500.134 MHz for ^{1}H. All samples were analyzed using a 5 μs pulse (45°), a delay time of 2.0 s, an acquisition time of 0.4 s, and broadband proton decoupling. Between 13 000 and 33 000 scans were acquired depending on phosphorus concentration. Temperature was regulated at 20 °C to minimize degradation of phosphorus compounds and ensure consistent signal intensities (*29, 30*). All spectra were plotted with 1 Hz line broadening to preserve resolution, although spectra of water extracts were also plotted with 2 Hz line broadening to show fine detail in the phosphate monoester region.

Chemical shifts of signals were determined in parts per million (ppm) relative to 85% H_3PO_4 and assigned to individual phosphorus compounds or functional groups based on literature reports (*29*). Signal areas were calculated by integration and phosphorus concentrations calculated by multiplying the proportion of the spectral area assigned to a specific signal by the total phosphorus concentration (mg P kg^{-1} dry manure) in the original extract. Phytic acid was quantified in well-resolved spectra by summing the areas of the four signals at approximately 5.95, 5.06, 4.70, and 4.56 ppm occurring in the ratio 1:2:2:1 (*29*). In spectra where these signals overlapped with those from other phosphate monoesters, phytic acid was quantified by multiplying the signal from the phosphate group in the C-2 position on the inositol ring (occurring at approximately 5.95 ppm) by six (*24*). It is difficult to estimate the error in NMR spectroscopy without acquiring replicate spectra, but for manure and feed samples, analytical error has been estimated to be approximately 5% for larger signals and 10% for smaller signals (*16, 31*).

Results

Hedley Fractionation. Total phosphorus recovery by the Hedley fractionation procedure was 94% for broiler litter, 79% for cattle manure, and 92% for swine manure. There were marked differences among the three manures in total phosphorus recovery in the various sequential extracts (Table 2). For the broiler litter, most of the phosphorus was recovered in the water (29%) and HCl (48%) extracts. In contrast, only 11% of the phosphorus in cattle manure was recovered in the water extract, with most being recovered in $NaHCO_3$ (43%). A greater proportion of phosphorus was recovered in

TABLE 2. Concentrations of Phosphorus Compounds in Sequential Extracts of Animal Manures from the Hedley Fractionation Procedure Determined by Solution ^{31}P NMR Spectroscopy and ICP–OES Spectrometry

	mg P kg^{-1} dry wt					
	total phosphorus[a]	phosphate[b]	phosphate monoesters[b]	phospholipids[b]	DNA[b]	pyrophosphate[b]
Broiler Litter						
water	4547 ± 103 (29)	4036 (89)	291 (6)	138 (3)	82 (2)	ND[c]
$NaHCO_3$	826 ± 13 (5)	679 (82)	147 (18)	ND[c]	ND[c]	ND[c]
NaOH	1854 ± 27 (12)	266 (14)	1588 (86)[d]	ND[c]	ND[c]	Tr[e]
HCl	7734 ± 198 (48)	1114 (14)	6620 (86)[d]	ND[c]	ND[c]	ND[c]
sum of fractions	14961 ± 225 (94)	6095 (41)	8647 (58)	138 (<1)	82 (<1)	Tr[e]
Cattle Manure						
water	537 ± 18 (11)	228 (42)	200 (37)	ND[c]	109 (20)	ND[c]
$NaHCO_3$	2116 ± 17 (43)	2077 (98)	Tr[e]	ND[c]	ND[c]	39 (2)
NaOH	952 ± 33 (19)	492 (52)	350 (37)	ND[c]	ND[c]	110 (12)
HCl	311 ± 4 (6)	311 (100)	ND[c]	ND[c]	ND[c]	ND[c]
sum of fractions	3916 ± 41 (79)	3107 (79)	550 (14)	ND[c]	109 (3)	150 (4)
Swine Manure						
water	7992 ± 139 (55)	7644 (96)	278 (3)	70 (<1)	Tr[e]	Tr[e]
$NaHCO_3$	3419 ± 110 (23)	3308 (97)	111 (3)	ND[c]	ND[c]	ND[c]
NaOH	846 ± 36 (6)	498 (59)	325 (38)[f]	ND[c]	ND[c]	24 (3)
HCl	1252 ± 87 (9)	830 (66)	422 (34)[g]	ND[c]	ND[c]	ND[c]
sum of fractions	13508 ± 200 (92)	12279 (91)	1136 (8)	70 (<1)	Tr[e]	24 (<1)

[a] Data were determined by ICP–OES and are mean ± standard deviation of three replicate extracts. Values in parentheses are the recovery (%) of the total manure phosphorus in each extract. [b] Determined by solution ^{31}P NMR spectroscopy. Values in parentheses are the proportion (%) of the total phosphorus in each extract. [c] ND, not detected. [d] All phosphate monoesters were phytic acid (calculated by sum of signals). [e] Tr, trace. [f] Phytic acid concentration (C2*6) was 118 mg P kg^{-1} dry wt (14% of the extracted phosphorus). [g] Phytic acid concentration (sum of signals) was 401 mg P kg^{-1} dry wt (32% of the extracted phosphorus).

NaOH from the cattle manure (19%) than the other two manures. Most phosphorus in swine manure was recovered in the water and $NaHCO_3$ extracts (55 and 23%, respectively), with only small proportions in the NaOH and HCl extracts (6 and 9%, respectively).

Solution ^{31}P NMR spectra of sequential extracts from the Hedley fractionation are shown in Figure 1. The strong signal in all extracts at approximately 6.1 ppm was assigned to phosphate, although this signal occurred slightly downfield at 6.2 ppm or greater in $NaHCO_3$ and HCl extracts. A signal close to −4.4 ppm was assigned to pyrophosphate. This was the only complex inorganic phosphate detected in the manures, although long-chain polyphosphate was detected in NaOH-EDTA extracts of the cattle and swine manures in a previous study (*24*). Signals between 3.4 and 6.0 ppm were assigned to phosphate monoesters, with those at approximately 5.95, 5.06, 4.70, and 4.56 ppm in the ratio 1:2:2:1 assigned to phytic acid. Signals at 4.89 and 5.23 ppm were assigned to β-glycerophosphate and phosphatidic acid, respectively. These are hydrolysis products of phospholipids, such as phosphatidyl choline, in alkaline solution (*29*). Other signals in the phosphate monoester region (e.g., 4.82, 4.50, 4.41, 4.36 ppm in the water extract of the cattle manure; Figure 1) probably represented lower-order inositol phosphates or mononucleotides originating from the hydrolysis of RNA in alkaline solution (*29*). A strong signal close to 0 ppm was assigned to DNA, while signals between 0.5 and 2.0 ppm were assigned to phospholipids. Signals from phosphonates occur around 20 ppm but were not detected in any extract.

In the broiler litter, extractable phosphorus was dominated by phosphate (41%) and phosphate monoesters (58%), with the latter present almost entirely as phytic acid (Table 2). Water and $NaHCO_3$ extracts were both dominated by phosphate (82–89%; Table 2). The water extract also contained small concentrations of phosphate monoesters (including some phytic acid), DNA, and phospholipids (Figure 1). Three phospholipid signals were detected at 1.71, 1.39, and 1.08 ppm. The two unassigned compounds upfield of DNA were probably microbial in origin, because similar signals were detected in alkaline extracts of bacterial cultures (*32*). The water extract also contained a signal at 3.45 ppm assigned to glucose 1-phosphate plus unassigned signals slightly downfield of phosphate at 6.27 and 6.39 ppm that possibly represented inositol phosphates (*28*). The $NaHCO_3$ extract contained a smaller concentration of phosphate monoesters than the water extract plus a trace of phospholipids. The NaOH and HCl extracts of the broiler litter were dominated by phytic acid. This represented 86% of the total phosphorus in both extracts, although the concentration was much greater in the HCl extract. The NaOH and HCl extracts also contained phosphate (14%) and traces of other phosphate monoesters, including signals downfield of phosphate in the NaOH extract.

In the cattle manure, extractable phosphorus was dominated by phosphate (79%), with smaller proportions of phosphate monoesters (14%), DNA (3%), and pyrophosphate (4%). Indeed, phosphate accounted for 98% of the phosphorus in the $NaHCO_3$ extract and 100% in the HCl extract (Table 2), although it is possible that poor resolution prevented detection of compounds other than phosphate in the HCl extract (Figure 1). The water and NaOH extracts contained considerable proportions of phosphate monoesters (37% of the extracted phosphorus), although these were mostly breakdown products of alkali-labile phosphate diesters, with no evidence for the presence of phytic acid. DNA constituted 20% of the phosphorus in the water extract, and a trace of glucose 1-phosphate was also detected. Pyrophosphate was present only in the $NaHCO_3$ and NaOH extracts (2 and 12% of the extracted phosphorus, respectively).

In the swine manure, water, and $NaHCO_3$ extracts were dominated by phosphate (>96% of the extracted phosphorus; Table 2). All extracts contained phosphate monoesters, although they were a large proportion of the extracted phosphorus only in the NaOH and HCl extracts (38 and 34% of the extracted phosphorus, respectively), mainly in the form of phytic acid (Table 2). Phospholipids, DNA, and glucose 1-phosphate were detected only in the water extract. Phosphate monoesters in the $NaHCO_3$ extract were mainly hydrolysis products of alkali-labile phosphate diesters, while pyrophosphate was detected in a measurable concentration only in the NaOH extract.

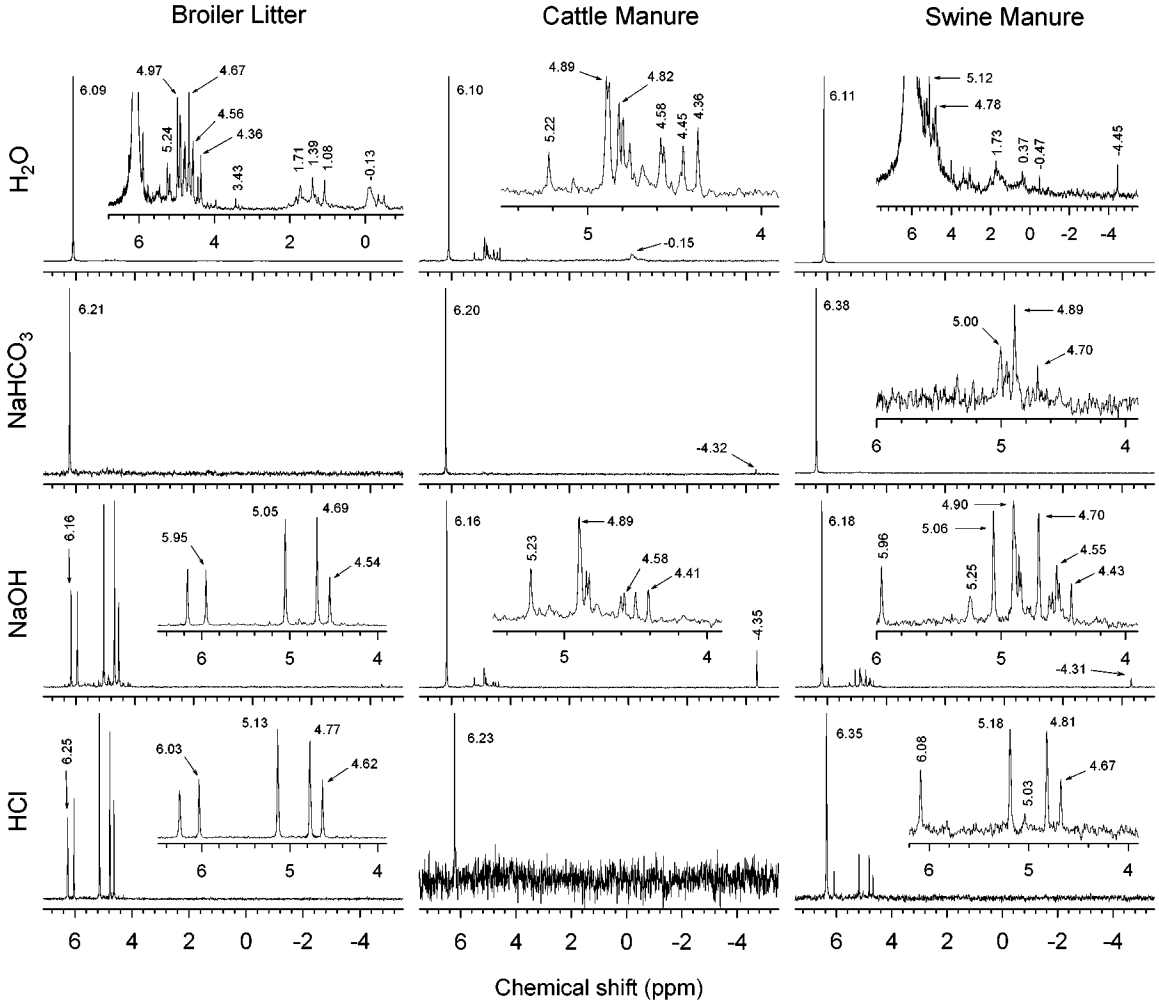

FIGURE 1. Solution ^{31}P NMR spectra of sequential extracts of a broiler litter, a cattle manure, and a swine manure, using a revised Hedley fractionation procedure (*20*). Manures were extracted sequentially with deionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH, and 1.0 M HCl. The main spectra are scaled to the full height of the phosphate signal at approximately 6.1 ppm, while inset spectra are scaled to the height of the largest phosphate monoester signal. All spectra are plotted with 1 Hz line broadening except the inset spectrum of the water extract (2 Hz).

Revised Fractionation Procedures. Total phosphorus recovery by the $NaHCO_3$/NaOH-EDTA procedure was 99% for the broiler litter, 83% for the cattle manure, and 94% for the swine manure (Table 3). These were greater than the Hedley and $NaHCO_3$/HCl procedures for all manures, although the difference was not statistically significant for the swine manure ($P>0.05$). The $NaHCO_3$/HCl procedure recovered less phosphorus than the Hedley procedure for all manures, but the difference was only statistically significant for the cattle manure ($P<0.05$). In terms of the readily soluble fraction, an initial $NaHCO_3$ extraction recovered more total phosphorus from all manures than an initial water extraction ($P<0.05$) but less than the combined water plus $NaHCO_3$ extraction of the Hedley procedure ($P<0.05$; Table 3).

Solution ^{31}P NMR spectra of extracts of the revised fractionation procedures are shown in Figure 2. Chemical shifts were similar to those for the Hedley fractionation (see above). Initial extraction with $NaHCO_3$ recovered more phosphate from all manures than initial water extraction but less than sequential water and $NaHCO_3$ extracts (Table 3). However, $NaHCO_3$ extraction recovered more phosphate monoesters than sequential water and $NaHCO_3$ extracts from the broiler litter and cattle manure. Neither phospholipids nor DNA were detected in initial $NaHCO_3$ extracts, despite at least one of these groups being detected in water extracts.

The $NaHCO_3$/NaOH-EDTA procedure recovered more phytic acid from the broiler litter and swine manure than the Hedley fractionation or the $NaHCO_3$/HCl procedure (Table 3; Figure 2). No phytic acid was detected in the initial $NaHCO_3$ extract of any manure (Table 3). Extraction in NaOH-EDTA was also effective for the cattle manure, recovering more phosphate monoesters, DNA, and pyrophosphate than the other procedures. Smaller phosphate concentrations were recovered from the cattle manure and broiler litter by the $NaHCO_3$/NaOH-EDTA procedure compared to the other procedures, suggesting that hydrolysis of acid-labile organic and condensed phosphates occurred during HCl extraction. Degradation products of phospholipids were clearly present in the NaOH-EDTA extracts of the cattle and swine manures. Long-chain polyphosphates were not detected in extracts of the revised procedures, which was expected because polyphosphate degrades in HCl and was not recovered in NaOH-EDTA extracts using 0.5 M NaOH, possibly due to coprecipitation with metals (*24*).

Element Recovery in Sequential Extracts. The Hedley fractionation recovered 75% or more of the total calcium and magnesium from all manures (Table 4). Most was recovered in the HCl extract, although water and $NaHCO_3$ also recovered large concentrations. For example, 31% of the calcium in the cattle manure was recovered in water,

TABLE 3. Concentrations of Phosphorus Compounds in Sequential Extracts of Animal Manures from Two Revised Fractionation Procedures Determined by Solution ^{31}P NMR Spectroscopy and ICP–OES Spectrometry

	mg P kg^{-1} dry wt					
	total phosphorus[a]	phosphate[b]	phosphate monoesters[b]	phospholipids[b]	DNA[b]	pyrophosphate[b]
Broiler Litter						
$NaHCO_3$	4826 ± 154 (30)	4222 (87)	604 (13)	ND[c]	ND[c]	Tr[d]
HCl	9808 ± 153 (61)	1385 (14)	8423 (86)[e]	ND[c]	ND[c]	Tr[d]
NaOH-EDTA	10964 ± 178 (69)	1336 (12)	9628 (88)[e]	ND[c]	ND[c]	Tr[d]
sum ($NaHCO_3$/HCl)	14634 ± 217 (92)	5607 (38)	9027 (62)	ND[c]	ND[c]	Tr[d]
sum ($NaHCO_3$/NaOH-EDTA)	15790 ± 235 (99)	5558 (35)	10232 (65)	ND[c]	ND[c]	Tr[d]
Cattle Manure						
$NaHCO_3$	2475 ± 43 (50)	2025 (82)	360 (15)	ND[c]	ND[c]	90 (4)
HCl	1318 ± 43 (27)	1180 (90)	66 (5)	ND[c]	ND[c]	72 (5)
NaOH-EDTA	1620 ± 21 (33)	620 (38)	648 (40)	Tr[d]	188 (12)	165 (10)
sum ($NaHCO_3$/HCl)	3793 ± 61 (77)	3205 (84)	426 (11)	ND[c]	ND[c]	162 (4)
sum ($NaHCO_3$/NaOH-EDTA)	4095 ± 48 (83)	2645 (65)	1009 (25)	Tr[d]	188 (5)	254 (6)
Swine Manure						
$NaHCO_3$	8636 ± 84 (59)	8385 (97)	251 (3)	ND[c]	ND[c]	ND[c]
HCl	4829 ± 458 (33)	3974 (82)	856 (18)[f]	ND[c]	ND[c]	ND[c]
NaOH-EDTA	5110 ± 530 (35)	3932 (77)	1134 (22)[g]	ND[c]	ND[c]	44 (<1)
sum ($NaHCO_3$/HCl)	13465 ± 465 (92)	12359 (92)	1106 (8)	ND[c]	ND[c]	ND[c]
sum ($NaHCO_3$/NaOH-EDTA)	13746 ± 565 (94)	12317 (90)	1385 (10)	ND[c]	ND[c]	44 (<1)

[a] Data were determined by ICP–OES and are mean ± standard deviation of three replicate extracts. Values in parentheses are the recovery (%) of the total manure phosphorus in each extract. [b] Determined by solution ^{31}P NMR spectroscopy. Values in parentheses are the proportion (%) of the total phosphorus in each extract. [c] ND, not detected. [d] Tr, trace. [e] All phosphate monoesters were phytic acid (calculated by sum of signals). [f] Phytic acid concentration (sum of signals) was 730 mg P kg^{-1} dry wt (15% of the extracted phosphorus). [g] Phytic acid concentration (sum of signals) was 869 mg P kg^{-1} dry wt (17% of the extracted phosphorus).

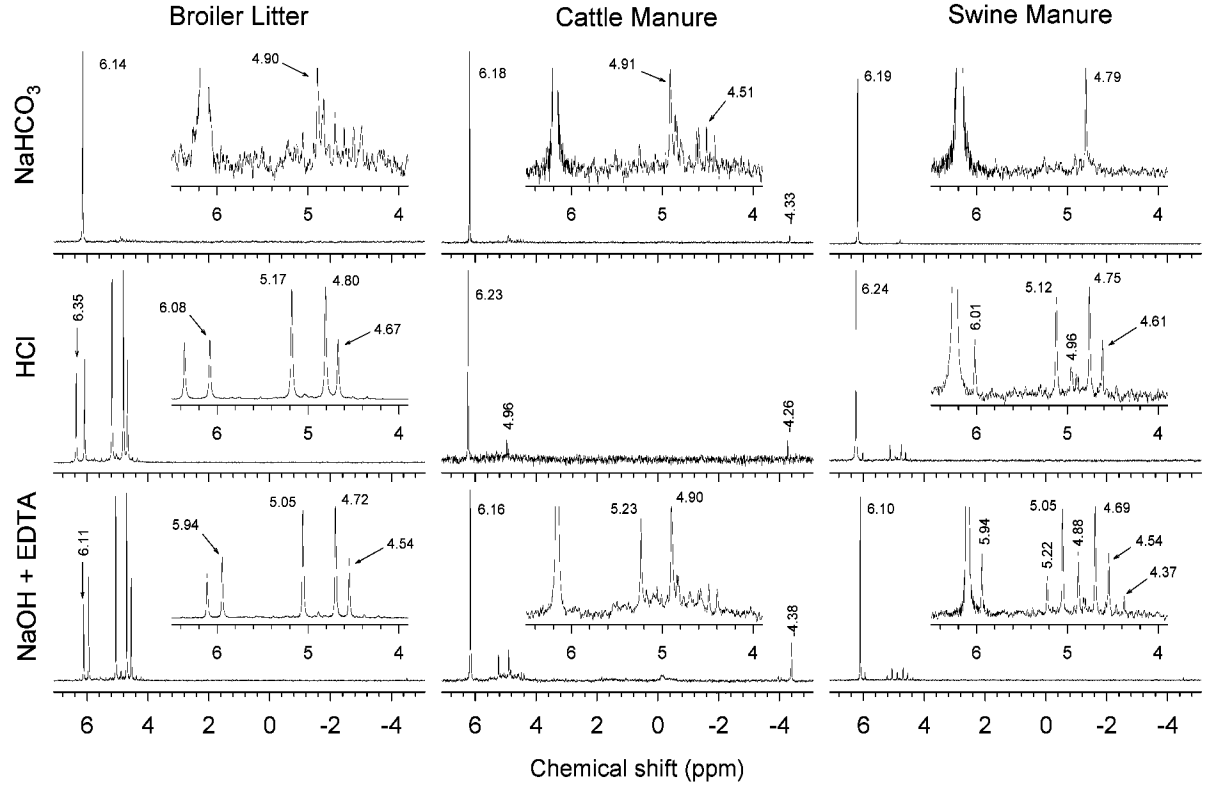

FIGURE 2. Solution ^{31}P NMR spectra of sequential extracts of a broiler litter, a cattle manure, and a swine manure, using revised fractionation procedures involving initial extraction in 0.5 M $NaHCO_3$ followed by a second extraction with either 1.0 M HCl or a solution containing 0.5 M NaOH and 50 mM EDTA. The main spectra are scaled to the full height of the phosphate signal at approximately 6.1 ppm, while inset spectra are scaled to the height of the largest phosphate monoester signal. All spectra are plotted with 1 Hz line broadening.

while most of the magnesium in cattle and swine manure was recovered in the water and $NaHCO_3$ extracts. A large proportion of magnesium in the broiler litter was extracted by water, although the greatest proportion was recovered in the HCl extract. Less than 1% of the magnesium was recovered in NaOH extracts. Recoveries of aluminum and iron were smaller and more variable than those of calcium and magnesium. For example, aluminum recovery was 63% from the broiler litter, 30% from the swine manure, and 9% from the cattle manure. In all manures, the greatest recovery of aluminum and iron was in the HCl extract.

The $NaHCO_3$/HCl procedure recovered significantly more of the metals than the Hedley or $NaHCO_3$/NaOH-EDTA procedures for the broiler litter and cattle manure ($P<0.05$; Table 4). Exceptions were iron and aluminum recovery from broiler litter, while for the swine manure only calcium and

TABLE 4. Total Element Concentrations in Sequential Extracts of Three Animal Manures from the Hedley Fractionation and Two Revised Fractionation Procedures[a]

	mg kg^{-1} dry wt			
	aluminum	calcium	iron	magnesium
		Broiler Litter		
water	16 ± 1 (3)	359 ± 9 (2)	90 ± 2 (10)	1051 ± 24 (16)
$NaHCO_3$	22 ± 1 (5)	2061 ± 39 (10)	37 ± 1 (4)	1464 ± 11 (23)
NaOH	42 ± 1 (9)	565 ± 13 (3)	18 ± 1 (2)	95 ± 4 (<1)
HCl	215 ± 5 (46)	15401 ± 288 (75)	393 ± 8 (46)	2216 ± 44 (34)
sum of fractions				
Hedley	296 ± 5 (63)[a]	18386 ± 291 (89)[b]	538 ± 9 (63)[a]	4826 ± 51 (75)[b]
$NaHCO_3$/HCl	272 ± 6 (58)[b]	20347 ± 502 (99)[a]	552 ± 7 (64)[a]	6180 ± 104 (95)[a]
$NaHCO_3$/NaOH-EDTA	199 ± 9 (42)c	18497 ± 980 (90)[b]	208 ± 20 (24)[b]	4822 ± 87 (75)[b]
		Cattle Manure		
water	1 ± 1 (<1)	4949 ± 31 (31)	19 ± <1 (2)	3513 ± 35 (51)
$NaHCO_3$	4 ± 6 (<1)	2170 ± 20 (14)	19 ± 3 (2)	1095 ± 18 (16)
NaOH	20 ± 2 (1)	215 ± 311 (1)	30 ± 3 (3)	35 ± 4 (<1)
HCl	115 ± 1 (8)	6716 ± 96 (42)	171 ± 1 (15)	641 ± 8 (9)
sum of fractions				
Hedley	141 ± 6 (9)[b]	14051 ± 147 (88)[b]	239 ± 5 (21)[b]	5285 ± 40 (77)[c]
$NaHCO_3$/HCl	166 ± 9 (11)[a]	14781 ± 319 (93)[a]	344 ± 10 (30)[a]	6103 ± 69 (89)[a]
$NaHCO_3$/NaOH-EDTA	133 ± 7 (9)[b]	13104 ± 226 (82)[c]	119 ± 7 (10)[c]	5554 ± 52 (81)[b]
		Swine Manure		
water	1 ± <1 (<1)	1276 ± 19 (11)	27 ± <1 (2)	3642 ± 55 (68)
$NaHCO_3$	0 ± 1 (<1)	3370 ± 78 (28)	8 ± 1 (<1)	586 ± 25 (11)
NaOH	21 ± 1 (10)	179 ± 22 (2)	28 ± 2 (2)	16 ± 2 (<1)
HCl	42 ± 1 (20)	4155 ± 175 (35)	277 ± 10 (24)	256 ± 8 (5)
sum of fractions				
Hedley	64 ± 2 (30)[a]	8980 ± 194 (76)[b]	340 ± 10 (30)[b]	4500 ± 61 (84)[a]
$NaHCO_3$/HCl	66 ± 14 (31)[a]	10829 ± 690 (91)[a]	522 ± 29 (46)[a]	4775 ± 242 (89)[a]
$NaHCO_3$/NaOH-EDTA	55 ± 9 (25)[a]	9306 ± 642 (78) [b]	97 ± 20 (9)c	3689 ± 500 (68)[b]

[a] Data are mean ± standard deviation of three replicate extracts, with the proportion (%) of the total element extracted in parentheses. For the sum of fractions, values with the same letter for each element and for each manure type are not significantly different (P>0.05).

iron recoveries were significantly greater. Recovery of calcium and magnesium tended to be similar for the Hedley and $NaHCO_3$/NaOH-EDTA procedures. However, recovery of iron was significantly lower in the $NaHCO_3$/NaOH-EDTA procedure for all manures (Table 4), almost certainly due to precipitation at high pH.

Discussion

Our results provide the first direct identification of phosphorus compounds in sequential extracts of animal manures. Two main groups of phosphorus compounds were extracted by the Hedley fractionation procedure, a readily soluble fraction extracted by water and $NaHCO_3$, and a stable fraction extracted by NaOH and HCl. Organic phosphorus in the readily soluble fraction included DNA, phospholipids, and simple phosphate monoesters. These compounds are sorbed only weakly in soil, so are mobile in the profile and have environmental significance even if present in relatively small concentrations (*10*). For example, Chardon and colleagues (*33*) found that leachate from a sandy soil that had received large amounts of swine slurry was almost entirely present in organic forms. Similarly, Toor and colleagues (*9*) reported that although organic phosphorus was only a small component of the total phosphorus in dairy shed effluent, it dominated the phosphorus in leachate following land application and irrigation. This was almost certainly due to the retention of phosphate in the soil profile, highlighting that soluble phosphate in manure is not necessarily mobile in the environment. Clearly, hydrological factors, including the pathway taken by runoff as it leaves the field, must be considered when assigning the risk of phosphorus transfer from recently applied manure (*34*).

Organic phosphorus extracted by NaOH and HCl is considered poorly soluble. In extracts of the swine manure and poultry litter this was almost all phytic acid. Phytic acid is immobile in soils, because it sorbs strongly to clays and reacts with metals to form insoluble precipitates (*7, 8*). It is also difficult for organisms to access phytic acid once it is stabilized in soil (*7*). Organic phosphorus in the NaOH and HCl fractions can therefore be considered stable in the environment. Phytic acid is present in manure from grain-fed cattle (*15*), but organic phosphorus in alkaline extracts of the pasture-fed cattle manure analyzed here was almost entirely degradation products of phospholipids and RNA. This was unsurprising given the grass-based diet. Some of these compounds may have originated from microbes, which can constitute a considerable proportion of the phosphorus in some manures (*17*). The presence of phytic acid in HCl extracts of the broiler litter and swine manure is significant, because few studies determine organic phosphorus in the HCl extract of the Hedley fractionation procedure (*22*). This is an important oversight and almost certainly accounts for the small proportion of organic phosphorus reported in poultry litter in some studies (*17*)

The revised $NaHCO_3$/NaOH-EDTA procedure simplified the assessment of manure phosphorus by separating readily soluble and poorly soluble phosphorus into two convenient extracts. The use of NaOH-EDTA rather than HCl as the second extract is important, because alkaline solution improves organic phosphorus recovery from cattle manure and facilitates subsequent NMR analysis if additional structural information is required (*24*). Further, extraction in HCl appeared to hydrolyze some organic and condensed phosphates, which compromises subsequent quantification of phosphate by molybdate colorimetry. There is some hydrolysis of phospholipids and RNA in NaOH-EDTA, but these compounds degrade to phosphate monoesters rather than free phosphate. This precludes error in the spectrophotometric estimation of organic and inorganic phosphorus in NaOH-EDTA extracts.

In the revised procedures an initial $NaHCO_3$ extract was used to approximate the readily soluble phosphorus fraction. Poor spectral resolution in solution ^{31}P NMR spectroscopy prevented characterization of the low concentrations of phosphate diesters in these extracts, which was probably due to the low concentrations relative to those of phosphate. We also note the much lower phosphorus recovery from the cattle manure in water compared to $NaHCO_3$, although the reason for this is unclear. Extraction in either water or $NaHCO_3$ could be used to quantify the readily soluble phosphorus pool, and the common use of water extraction may mean that this is more appropriate. However, $NaHCO_3$ extraction more closely approximates the whole pool of readily soluble phosphorus (i.e., the sum of water and $NaHCO_3$ extractable phosphorus in the Hedley procedure), and we recommend it unless there is a specific need to characterize labile organic phosphates by NMR spectroscopy.

Based on elemental concentrations in the various manure extracts, phosphorus appeared to be associated with calcium and magnesium, which agrees with previous studies. For example, Cooperband and Ward Good (*21*) used scanning electron microscopy and energy-dispersive X-ray spectroscopy of poultry manure to detect a range of sparingly soluble calcium phosphate minerals, including dicalcium phosphate, octocalcium phosphate, and amorphous calcium phosphate. Similarly, Fordham and Schwertmann (*35*) used X-ray diffraction of dairy manure to detect struvite (a magnesium phosphate mineral), with the presence of other calcium and magnesium phosphates, including trimagnesium phosphate, octocalcium phosphate, and dicalcium phosphate, inferred from solubility studies. Our data also support speculation that calcium and magnesium phytates are quantitatively important in at least some manures (*21*).

Results presented here for the Hedley fractionation are broadly comparable to those from other studies using this procedure. However, one of the most striking aspects of these studies is the variation in results for similar manures, notably in terms of the proportion of water soluble phosphate. These differences seem too great to be explained by diet alone, because phosphorus composition varies little in manures for some species despite widely different diets. As an example, swine fed mutant barley varieties containing a range of phytic acid concentrations produced manures with remarkably similar phosphorus composition (*36*), although this was not the case for poultry litters from birds fed diets with a range of phosphorus concentrations and phytase supplements (*37*). At least some of the differences may be explained by analytical methodology, because extractants, sample pretreatment, extraction time, and the number of replicate extractions for each solution all vary among studies (*16*–*18*). Importantly, there is no consensus on the optimum pretreatment of manures, which may be analyzed fresh, lyophilized, air-dried, or oven dried at elevated temperature. However, this is likely to influence phosphorus solubility. For example, Sharpley and Moyer (*17*) reported that most of the phosphate in a range of fresh manures stored at 4 °C was recovered in the NaOH fraction, whereas Dou and colleagues (*18*) recovered most of the phosphate from a dairy manure dried at 65 °C in the initial water extract, although this was performed exhaustively. Leinweber and colleagues (*16*) freeze-dried a liquid swine manure and a chicken manure prior to sequential fractionation but recovered only 60% of the phosphorus. We used lyophilization for two reasons. First, samples could be frozen immediately after collection, effectively halting microbial or enzymatic processes that might lead to changes in the manure. Second, lyophilization is a relatively mild form of drying, especially in comparison with oven drying, although we recognize the practicality of the latter treatment for routine analysis. Clearly, the pretreatment of manure prior to phosphorus analysis requires careful assessment.

In summary, phosphorus in animal manures can be conveniently fractionated on the basis of solubility by a simple two-step extraction procedure. Initial extraction in $NaHCO_3$ recovers readily soluble phosphorus, while a second extraction in NaOH-EDTA recovers poorly soluble phosphorus. The soluble fraction includes phosphate and small concentrations of phospholipids, nucleic acids, and simple phosphate monoesters that have environmental relevance due to their mobility in soil and biological availability in terrestrial and aquatic ecosystems. The poorly soluble fraction includes poorly soluble phosphate, plus phytic acid in manures from grain-fed animals. In manure from pasture-fed cattle the poorly soluble organic phosphorus fraction includes a range of phosphate monoesters and diesters. The inorganic and organic phosphorus pools can be estimated in the fractions by molybdate colorimetry, although the organic fraction may include inorganic pyrophosphate. The $NaHCO_3$/NaOH-EDTA procedure is simpler than the Hedley fractionation and separates structurally defined fractions with environmental relevance. Importantly, it is based on the nature of phosphorus in manures, rather than soils. The procedure requires validation for a range of manures, which should include standardization of pretreatment and evaluation of the relationship between the readily soluble fraction and phosphorus concentrations in runoff. This will allow detailed comparison of manures from a wide range of animals and diets and will ultimately contribute to a greater understanding of manure phosphorus in the environment.

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Literature Cited

(1) Sims, J. T.; Edwards, A. C.; Schoumans, O. F.; Simard, R. R. *J. Environ. Qual.* **2000**, *29*, 60–71.
(2) Boesch, D. F.; Brinsfield, R. B.; Magnien, R. E. *J. Environ. Qual.* **2001**, *30*, 303–320.
(3) Burkholder, J. A.; Glasgow Jr, H. B. *Limnol. Oceanogr.* **1997**, *42*, 1052–1075.
(4) *The Quality of our Nation's Waters: Nutrients and Pesticides;* U.S. Geological Survey, USGS Information Services: Denver, Colorado, 1999; 82 pp.
(5) Raboy, V.; Gerbasi, P. F.; Young, K. A.; Stoneberg, S. D.; Pickett, S. G.; Bauman, A. T.; Murthy, P. P. N.; Sheridan, W. F.; Ertl, D. S. *Plant Physiol.* **2000**, *124*, 355–368.
(6) Simons, P. C. M.; Versteegh, H. A. J.; Jongbloed, A. W.; Kemme, P. A.; Slump, P.; Bos, K. D.; Wolters, M. G. E.; Beudeker, R. F.; Verschoor, G. J. *Br. J. Nutr.* **1990**, *64*, 525–540.
(7) Turner, B. L.; Papházy, M.; Haygarth, P. M.; McKelvie, I. D. *Philos. Trans. R. Soc. London Ser. B* **2002**, *357*, 449–469.
(8) Celi, L.; Barbaris, E. In *Organic Phosphorus in the Environment*; Turner, B. L., Frossard, E., Baldwin, D. S., Eds.; CAB International: Wallingford, 2004; pp 113–132.
(9) Toor, G. S.; Condron, L. M.; Di, H. J.; Cameron, K. C.; Cade-Menun, B. J. *Soil Biol. Biochem.* **2003**, *35*, 1317–1323.
(10) Turner, B. L. In *Organic Phosphorus in the Environment*; Turner, B. L., Frossard, E., Baldwin, D. S., Eds.; CAB International: Wallingford, 2004; pp 269–294.
(11) Kleinman, P. J. A.; Sharpley, A. N.; Moyer, B. G.; Elwinger, G. F. *J. Environ. Qual.* **2002**, *31*, 2026–2033.
(12) McAuliffe, C.; Peech, M. *Soil Sci.* **1949**, *68*, 179–184.
(13) Peperzak, P.; Caldwell, A. G.; Hunziker, R. R.; Black, C. A. *Soil Sci.* **1959**, *87*, 293–302.
(14) Gerritse, R. G.; Vriesma, R. *J. Agric. Sci.* **1984**, *102*, 159–161.
(15) Barnett, G. M. *Bioresour. Technol.* **1994**, *49*, 139–147.
(16) Leinweber, P.; Haumaier, L.; Zech, W. *Biol. Fertil. Soils* **1997**, *25*, 89–94.
(17) Sharpley, A. N.; Moyer, B. *J. Environ. Qual.* **2000**, *29*, 1462–1469.
(18) Dou, Z.; Toth, J. D.; Galligan, D. T.; Ramberg, C. F. J.; Ferguson, J. D. *J. Environ. Qual.* **2000**, *29*, 508–514.
(19) Weinhold, B. J.; Miller, P. S. *J. Environ. Qual.* **2004**, *33*, 389–393.

(20) Hedley, M. J.; Stewart, J. W. B.; Chauhan, B. S. *Soil Sci. Soc. Am. J.* **1982**, *46*, 970−976.
(21) Cooperband, L. R.; Ward Good, L. *Environ. Sci. Technol.* **2002**, *36*, 5075−5082.
(22) He, Z.; Honeycutt, C. W. *J. Environ. Qual.* **2001**, *30*, 1685−1692.
(23) Hunger, S.; Cho, H.; Sims, J. T.; Sparks, D. L. *Environ. Sci. Technol.* **2004**, *38*, 674−681.
(24) Turner, B. L. *J. Environ. Qual.* **2004**, *33*, 757−766.
(25) Crouse, D. A.; Sierzputowska-Gracz, H.; Mikkelsen, R. L. *Commun. Soil Sci. Plant Anal.* **2000**, *31*, 229−240.
(26) *EPA Method 3052: Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices*; U.S. Environmental Protection Agency: Washington, DC, 1996.
(27) Nanny, M. A.; Minear, R. A. In *Nuclear Magnetic Resonance Spectroscopy in Environmental Chemistry*; Nanny, M. A., Minear, R. A., Leenheer, J. A., Eds.; Oxford University Press: New York, 1997; pp 221−246.
(28) Turner, B. L.; Richardson, A. E. *Soil Sci. Soc. Am. J.* **2004**, *68*, 802−808.
(29) Turner, B. L.; Mahieu, N.; Condron, L. M. *Soil Sci. Soc. Am. J.* **2003**, *67*, 497−510.
(30) Cade-Menun, B. J.; Liu, C. W.; Nunlist, R.; McColl, J. G. *J. Environ. Qual.* **2002**, *31*, 457−465.
(31) Kemme, P. A.; Lommen, A.; De Jonge, L. H.; van der Klis, J. D.; Jongbloed, A. W.; Mroz, Z.; Beynen, A. C. *J. Agric. Food Chem.* **1999**, *47*, 5116−5121.
(32) Makarov, M. I.; Haumaier, L.; Zech, W. *Soil Biol. Biochem.* **2002**, *34*, 1467−1477.
(33) Chardon, W. J.; Oenema, O.; del Castilho, P.; Vriesema, R.; Japenga, J.; Blaauw, D. *J. Environ. Qual.* **1997**, *26*, 372−378.
(34) Haygarth, P. M.; Jarvis, S. C. *Adv. Agron.* **1999**, *66*, 195−249.
(35) Fordham, A. W.; Schwertmann, U. *J. Environ. Qual.* **1977**, *6*, 133−136.
(36) Leytem, A. B.; Turner, B. L.; Thacker, P. A. *J. Environ. Qual.* **2004**, *33*, in press.
(37) Maguire, R. O.; Sims, J. T.; Saylor, W. W.; Turner, B. L.; Angel, R.; Applegate, T. J. *J. Environ. Qual.* **2004**, *33*, in press.

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