MANURE PHOSPHORUS SPECIATION AND TRANSFORMATION IN AN ACIDIC ENVIRONMENT

by

YEBIN ZHAO

(Under the Direction of Miguel Cabrera)

ABSTRACT

Phosphorus (P) is an important nutrient for crops and livestock and is commonly present in animal manures. Consequently, animal manures have been broadly applied to agricultural lands as fertilizer. However, long-term manure application to soil may lead to P losses through surface runoff with potential impairment of surface waters. The objectives of this dissertation were 1) to develop an animal manure P extraction method that simulates P release in acidic soils, 2) to identify P compounds extracted from broiler litter by various extractants at different pH levels, and 3) to evaluate the effects of temperature and water content on release of broiler litter P during storage.

Broiler litter, layer manure, and dairy slurry samples were extracted with water or MES buffer (2-(N-morpholino) ethanesulfonic acid at pH 6) at three manure:extractant ratios (10:1, 100:1, or 200:1) and three extraction times (1, 4, or 24 h). In most cases, extraction with MES buffer removed larger amounts of Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Bioavailable P (BAP) than extraction with water. Likewise, widening the water to manure ratio (10:1 to 200:1) as well as extending the extraction time from 1 to 4 or 24 h usually increased the amount of TDP, DRP, and BAP extracted.
A P fractionation study of broiler litter and layer manure indicated that the major P fraction was MES-P, and that the P species present were highly associated with Ca and Mg cations. Results from $^{31}$P Nuclear Magnetic Resonance (NMR) analysis found that significant amounts of orthophosphate and phytate were extracted from broiler litter and layer manure.

A laboratory incubation of broiler litter at three temperatures (10, 20, or 30°C) and three water contents (400, 800, or 1200 g kg$^{-1}$) showed that incubation brought about an increase of TDP in the MES fraction across all water contents and temperatures. This change resulted from a transfer of TDP from the NaHCO$_3$, NaOH, and Residual P (RP) fractions to the MES fraction. Microbial activity played an important role in P transformation during the incubation, and in most cases a shorter storage term resulted in a smaller release of soluble P than a longer storage.

INDEX WORDS: Total dissolved phosphorus, animal manure, extraction, incubation, storage, fractionation, speciation, $^{31}$P NMR
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>2</td>
</tr>
<tr>
<td>Total Dissolved Phosphorus and Bioavailable Phosphorus</td>
<td>2</td>
</tr>
<tr>
<td>Fractionation and Speciation of Phosphorus in Animal Manure</td>
<td>9</td>
</tr>
<tr>
<td>Manure Phosphorus Transformation during Storage</td>
<td>14</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>16</td>
</tr>
<tr>
<td>2 EXTRACTION METHOD AFFECTS TOTAL DISSOLVED AND BIOAVAILABLE PHOSPHORUS IN BROILER LITTER</td>
<td>28</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>29</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>30</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>33</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>36</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>41</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>42</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1: Selected properties of three broiler litter samples used in the study ............................46

Table 2.2: Analysis of variance for Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from three broiler litter samples ..........................................................................................................................46

Table 2.3: Extracting conditions for maximum removal of Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) from three broiler litter samples. ..............................................................................................................47

Table 2.4: Total Dissolved P (TDP), Dissolved Reactive P (DRP), Total Bioavailable P (TBAP), Soluble Bioavailable P1 (SBAP1), and Soluble Bioavailable P2 (SBAP2) extracted from three broiler litter samples at a ratio of 200:1 with MES buffer at pH 6 for 24 h........................................................................................................................................47

Table 3.1: Selected properties of four layer manure samples and three dairy slurry samples used in the study ................................................................................................................................................72

Table 3.2: Analysis of variance for Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from all layer manure and dairy slurry samples..............................................................................................................72
Table 3.3: Total Dissolved P (TDP), Dissolved Reactive P (DRP), Total Bioavailable P (TBAP), Soluble Bioavailable P1 (SBAP1), and Soluble Bioavailable P2 (SBAP2) extracted from all layer manure and dairy slurry samples at a ratio of 200:1 with MES buffer at pH 6 for 24 h .................................................................73

Table 4.1: Selected characteristics of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2).................................................................................................................................105

Table 4.2: Molar composition in sequentially-extracted fractions (starting with DI water) of one broiler litter (BL), and two layer manure samples (LM #1 and LM #2)..................................................105

Table 4.3: Molar composition in sequentially-extracted fractions (starting with 2-(N-morpholino) ethanesulfonic acid - MES) of one broiler litter (BL) and two layer manure samples LM #1 and LM #2).................................................................................................................106

Table 4.4: Extracted P species detected by P-31 Nuclear Magnetic Resonance (NMR). The species were extracted from one broiler litter (BL) and two layer manure samples (LM #1 and LM #2) with DI water, 2-(N-morpholino) ethanesulfonic acid (MES), or NaOH + EDTA .................................................................................................................106

Table 4.5: Chemical composition of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2). Fractions were extracted with DI water, 2-(N-morpholino) ethanesulfonic acid (MES), or NaOH + EDTA.................................................................107

Table 4.6: The concentration of P species measured by P-31 Nuclear Magnetic Resonance (NMR) in fractions of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2) extracted with DI water, MES buffer, or NaOH + EDTA .................................................................108

Table 5.1: Selected composition of the broiler litter used for the incubation study .................132
Table 5.2: Analysis of variance for Total Dissolved P (TDP) and Dissolved Reactive P (DRP) extracted from broiler litter by MES, NaHCO$_3$, and NaOH ..............................................132

Table 5.3: Significance of pairwise comparisons using Fisher’s LSD of mean Total Dissolved P (TDP) in the MES fraction, and mean sum of TDP in MES + NaHCO$_3$ + NaOH at four incubation times and four water contents ....................................................................................133

Table 5.4: Significance of pairwise comparisons using Fisher’s LSD of mean Total Dissolved P (TDP) in the MES fraction, and mean sum of TDP in MES + NaHCO$_3$ + NaOH at four incubation times and three temperatures ..........................................................................................133
LIST OF FIGURES

Page

Fig. 2.1: The effects of two extractants and (a) extraction ratio and (b) extraction time on Total Dissolved P (TDP) extracted from broiler litter samples #1, #2, and #3. .......................48

Fig. 2.2: The effects of (a) extraction ratio and extraction time, (b) extractant and extraction time, and (c) extractant, extraction ratio, and extraction time on DRP extracted from broiler litter samples #1, #2, and #3..........................................................................................49

Fig. 2.3: The effects of extractant and extraction time on Soluble Bioavailable P1 (SBAP1) tested by Murphy and Riley (1962) and extracted from broiler litter samples #1, #2, and #3

Fig. 2.4: The effects of (a) extractant and extraction ratio, and (b) extraction ratio and extraction time on Total Bioavailable P (TBAP) extracted from broiler litter samples #1, #2, and #3..................................................................................................................................51

Fig. 3.1: The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #1...........................................................................................................................................74
Fig. 3.2: The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #2.

Fig. 3.3: The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #3.

Fig. 3.4: The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #4.

Fig. 3.5: The effects of extraction ratio and extraction time on average amounts of Total Dissolved P (TDP) extracted from all layer manures.

Fig. 3.6: The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #1.

Fig. 3.7: The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #2.
Fig. 3.8: The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #3.

Fig. 4.1: Phosphorus distribution in sequentially-extracted fractions of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2). Significant differences for Total Dissolved P (TDP) in each fraction between DI water-, and MES- leading fractionation schemes are represented as capital and lower case letters, respectively (P<0.05).

Fig. 4.2: Solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectra of broiler litter P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry litter), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45º pulse, 1.6-s acquisition, 5-s relaxation, for 5000 scans (about 9 h).

Fig. 4.3: Solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectra of layer manure #1 P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry manure), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45º pulse, 1.6-s acquisition, 5-s relaxation, for 256 scans (29 min).

Fig. 4.4: Solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectra of layer manure #2 P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry manure), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45º pulse, 1.6-s acquisition, 5-s relaxation, for 256 scans (29 min).
Fig. 4.5: Relationships between concentrations of orthophosphate (mg kg$^{-1}$ dry animal waste) determined by $^{31}$P Nuclear Magnetic Resonance (NMR) and Dissolved Reactive P (DRP) determined by the molybdate colorimetric method in three extracts from three animal wastes. The regression line describes the model: Orthophosphate by NMR = 1852 + 0.56 (orthophosphate by molybdate colorimetric method), P <0.0001, $R^2$ = 91.3%.

Fig. 5.1: Dissolved Reactive P (DRP), and Dissolved Unreactive P (DUP) extracted from broiler litter by MES, NaHCO$_3$, and NaOH as affected by water content x time (M: MES, B: NaHCO$_3$, S: NaOH)...

Fig. 5.2: Cumulative C-CO$_2$ release from incubated broiler litter as affected by water content x time...

Fig. 5.3: Dissolved Reactive P (DRP), and Dissolved Unreactive P (DUP) extracted from broiler litter by MES, NaHCO$_3$ and NaOH as affected by temperature x time (M: MES, B: NaHCO$_3$, S: NaOH)...

Fig. 5.4: Cumulative C-CO$_2$ release from incubated broiler litter as affected by temperature x time...

Fig. 5.5: Total Dissolved P (TDP), and Dissolved Reactive P (DRP) extracted by NaOH as affected by temperature x water content x time...
CHAPTER 1
INTRODUCTION

In 2006, Georgia was the largest broiler litter-producing state in the United States, with a production of approximately 1.3 billion broilers (Georgia Agricultural Statistics Service, 2007) and an estimated generation of 2 million Mg of broiler litter (bedding material and excreta). Because most broiler litter is surface applied to grasslands as fertilizer, surface runoff interacting with the litter may solubilize phosphorus (P) and transport it to surface waters, where it may accelerate eutrophication (Sharpley, 1995; Sims et al., 1998).

Previous work has shown that P in runoff is mainly controlled by the amount of Total Dissolved P (TDP) extracted with water from soil and applied manures (DeLaune et al., 2004; Haggard et al., 2005; Kleinman et al., 2006; McDowell and Sharpley, 2001). However, the release of TDP from animal manures may vary with factors such as 1) nature of manure and distribution of P in different pools or fractions, 2) extracting conditions such as water:manure ratio, shaking time, and pH of extracting solution, and 3) manure storage conditions, such as water content and temperature. The research described here used laboratory experiments to evaluate the effects of these factors on TDP release from broiler litter, layer manure, and dairy slurry. The following diagram illustrates all P fractions considered in this work, as well as their abbreviations and relationships (Boström et al., 1982; Sharpley and Smith, 1993).
LITERATURE REVIEW

1. Total Dissolved Phosphorus and Bioavailable Phosphorus

1.1 Extraction and Determination of Total Dissolved Phosphorus and Dissolved Reactive Phosphorus

Total P dissolved by water from animal manures (<0.45 µm) is considered a key indicator of soluble P that may contaminate surface runoff because extraction by water may simulate Total Dissolved Phosphorus (TDP) extraction by rainfall and runoff water (Sharpley, 2000). Kleinman et al. (2002a) applied dairy slurry, poultry manure and swine manure to the surface of three highly weathered soils. They found that TDP concentration of these three manures was highly correlated with Dissolved Reactive P (DRP) losses in runoff, because manure application to soils results in large, temporary increases in TDP at the soil surface, where it serves as a source of P for runoff. Although manure TDP is important for environmental interpretation of manure management, there is no general consensus on accepted methods for its measurement.

Traditionally, manure TDP has been estimated by suspending manure in water and shaking the suspension for a certain time (Self-Davis and Moore, 2000; Sharpley and Moyer, 2000). The basic mechanism is simple, but effects of many factors have not been clarified such as the moisture of the manure, ratio of water to manure, extraction time, and extraction pH of the manure suspension. Chapuis-Lardy et al. (2004) evaluated the effect of manure moisture by measuring DRP in 40 fresh and oven-dried (65°C) dairy manures at an extraction ratio of 333:1 (water: dairy slurry). They observed that concentration of DRP extracted from dried-ground sample was lower than that from wet fresh samples. However, Vadas et al. (2006) found that oven-dried (90°C) swine manure increased in both DRP and TDP relative to fresh swine manure at extraction ratios greater than 16:1 (water: manure). Ajiboye et al. (2004) extracted swine manure at a ratio of 100:1
by water, and found that oven-drying (105°C) increased DRP relative to fresh manure by breaking down Dissolved Unreactive P (DUP) (mainly phytic acid) into DRP.

The extraction ratio and time for manure TDP extraction were first recommended by Self-Davis and Moore (2000) and Sharpley and Moyer (2000). Self-Davis and Moore used an extraction ratio of 10:1 (water to poultry manure) and 2-h extraction time, whereas Sharpley and Moyer extracted 1 g (dry weight equivalent) of fresh manure with 200 mL of water for 1 h. Different manure to water ratios and extraction times may remove different concentrations of TDP from the same type of manure. For instance, Kleiman et al. (2002b) extracted fresh dairy manure, layer manure, and swine slurry at water to manure (dry matter based) ratios of 10:1, 20:1, 40:1, and 200:1 for 1 to 1440 min. They found the TDP concentration increased with the water to manure ratios due to dissolution of calcium phosphates by dilution. They also found that the maximum amount of DRP was extracted with a 24-h extraction, although >70% of the maximum P was released within 60 min. Also, the strength of the relationship between TDP in runoff and TDP in manure increased from 1 to 60 min and decreased after 60 min. Kleinman et al. (2002b) concluded that the optimum extraction time for TDP from animal manure was between 30 and 120 min. Similarly, Haggard et al. (2005) extracted six different types of fresh poultry litter with water to litter ratios (dry matter based) of 10:1, 20:1, 50:1, 100:1, and 200:1 for 2 h. They reported that manure TDP increased with the amount of water used during the extraction. Recently, Kleiman et al. (2007) extracted 20 different types of animal wastes using three water to waste ratios (10:1, 100:1, 200:1) and an extraction time of 1 h. They finally concluded that a final extraction ratio of 100:1 for 1 h could serve as a consistent indicator of runoff P loss potential for a wide range of land-applied manures.
The studies referenced above extracted TDP at the original manure pH. As an example, the pH of all three manure samples used by Kleiman et al. (2002b) ranged from 7.3 to 8.9. The pH of manure and water mixtures is usually alkaline because of the presence of NH$_3$ as well as phosphates of Ca, Na, and Mg (Griffiths, 1998). This elevated pH supports the stability of Ca and Mg phosphates (Lindsay et al., 1989). When manure is applied to acidic soils, however, its pH may decrease rapidly to equal that of the soil. For example, Tasistro et al. (2004) reported that the pH of broiler litter decreased from an initial value of 8.1 to 6.7 within 30 days after surface application to a pasture. The acidification of manure could result in an increase of TDP by solubilization of orthophosphates (Gerritse and Vriesema, 1984), which are mainly present as Ca, Mg, and K salts (Champagne, 1988; Cooperband and Good, 2002). Consequently, measuring manure TDP at the original manure pH could result in an underestimation of the TDP released from manure applied to acidic soils (Tasistro et al., 2004). To better predict TDP loading to the acidic soil environments, Tasistro et al. (2007) selected an extraction pH of 6, as this is a typical surface pH for grasslands in Georgia, and investigated the effect of different buffers on DRP and DUP extraction. They found that extracting broiler litter or layer manure for 4 h with a 0.1 M buffer (pH = 6) solution of 2-(N-morpholino) ethanesulfonic acid (MES) at an extracting ratio of 200:1 (solution:dry manure) released similar amounts of DRP and DUP as acidifying the sample to pH 6 with HCl. These results suggested that the MES buffer was not forming metal complexes and thus was not increasing the solubility of inorganic or organic P associated with metals in the manure. Furthermore, Tasistro et al. (2007) showed that the extraction with MES buffer at pH 6 removed more TDP than extraction with water. Therefore, the MES buffer may be a good extractant to measure animal manure TDP at pH 6 in an effort to better estimate the amount of TDP that may be released from animal manure after it is applied to soil.
As recommended by Self-Davis and Moore (2000), Inductively Coupled Plasma-Optical Emission Spectrograph has been commonly used in determining manure TDP, whereas colorimetric determination by Murphy and Riley (1962) has been the preferred method for DRP determination. However, many analytical variables may impact TDP and DRP measurement, such as separation processes (filtering and centrifuging), manure sample holding time, manure extract storage procedure (acidification or no acidification). Self-Davis and Moore (2000) and Sharpley and Moyer (2000) recommended a 0.45-µm filter for TDP filtering, but Kleinman et al. (2002b) found a significant decrease in TDP concentration in dairy and poultry manures when samples were filtered through 0.45-µm filter instead of filter paper (Whatman no. 1, pore size 11-µm ). Wolf et al. (2005) found that holding poultry manure samples for up to 22 days at 4°C did not affect TDP extraction. Self-Davis and Moore (2000) recommended acidifying TDP extracts to avoid precipitation of calcium phosphate before TDP measurement. Wolf et al. (2005) reported that manure extract should be acidified, refrigerated and measured within 18 days after extraction. Previous articles discussing the colorimetric determination of DRP have noted that hydrolysis of condensed phosphates can occur when the solution is acidified or in contact with acid for extended periods of time (Lee et al., 1965). Also, acidification might bring about the flocculation of organics (Self-Davis and Moore, 2000).

1.2 Extraction and Determination of Bioavailable Phosphorus

Eutrophication management in lakes and streams always focuses on P inputs to surface water bodies (Carpenter et al., 1998). Phosphorus in surface water can be divided into TDP and particulate P (PP). Although DRP present in TDP is the main fraction readily available to bacteria and algae in surface water bodies (Hatch et al., 1999), some fraction of PP may be available to algae because there is a dynamic equilibrium between TDP and PP during transport.
from manure/soil to runoff (Ellison and Brett, 2006). The sum of immediately available P and P that can be transformed from PP into an available species by naturally occurring process is defined as Bioavailable P (BAP) (Boström et al., 1982; Sharpley and Smith, 1993). Previous research has shown that BAP rather than TDP provides the most accurate assessment of water quality conditions in lake and streams (Gerdes and Kunst, 1998). During the last several decades, numerous procedures have been developed to extract and estimate BAP (plant-available P) in soils. Early on, BAP in soil was mainly evaluated by solubilization of calcium, iron, and aluminum phosphates by dilute acids such as sulfuric acid (Truog, 1930), hydrochloric acid (Bray and Kurtz, 1945), or a mixture of these two acids (Nelson et al., 1953) by hydrolysis of cation-binding P (Olsen et al., 1954) and by desorption of phosphates from surface of calcium carbonate or hydroxides of iron and aluminum by introducing anions (Bray and Kurtz, 1945). Although a good correlation was shown between the amount of P extracted from soils with these methods and plant growth, these methods, including Bray 1 (Bray and Kurtz, 1945), Bray 2 (Bray and Kurtz, 1945), Olsen (Olsen et al., 1954), and Resin (Amer et al., 1955) extract some P species that may not be available for plant use (Menon et al., 1990b). Sissingh (1983) first evaluated soil BAP by shaking a soil solution with iron-oxide filter paper. This method rapidly gained popularity as a soil-P test for estimating soil BAP (Chardon et al., 1996), and lately it has been widely used for testing both soil and water BAP. In this method, iron-oxide paper acts as a strong P sink preferentially adsorbing P ions over other anions from solution onto its surface. The adsorbed P is then removed for P analysis. Unlike extracting solutions used in traditional soil-P tests, such as water, dilute un-buffered salt solutions, or dilute solutions of weak acid, the iron-oxide paper acts as a sink for P entering the soil solution. A low P concentration still exists in solution, therefore solution P levels are not increased to levels that restrict further P release.
from the soil (Indiati and Rossi, 2002). Menon et al. (1989a) tested BAP in four soil samples with iron-oxide paper strips and the traditional methods mentioned above. By correlating the P uptake and maize dry matter yield, they found that plant available P in soils determined by iron-oxide paper strips was a potential tool for predicting of P fertilizer requirement in soils. Similar results were also reported by Menon et al (1990a) when plant available P was tested using 18 soils with widely differing properties. Sharpley (1991) correlated P removed by iron-oxide paper with different P fractions in 203 surface soil, and suggested that iron-oxide paper may extract P closely related to plant available P for soils with widely ranging properties. The iron-oxide paper method was also applied to evaluate BAP in water. Sharpkey (1993a) found the method to be convenient and interference-free while estimating the BAP content of runoff from 20 agricultural watersheds during a 3-yr period. Furthermore, Shapley (1993) found that the iron-oxide paper method simulated P uptake by algae in sediment-water samples. Dils and Heathwaite (1998) compared BAP from runoff water fractionated by traditional physico-chemical methods and by iron-oxide paper, and found that the iron-oxide paper method provided better recovery of BAP. Compared to traditional BAP analysis method, such as the algal cultural bioassays (Fitzgera.G P, 1970) or chemical extraction (Golterman et al., 1978), the iron-oxide paper method is rapid and convenient because filter paper can be sent to the field and the adsorbed BAP from runoff samples then returned to the lab for analysis (Shapley et al., 1994).

A drawback of the iron-oxide paper method is that the paper may be contaminated by fine soil particles derived from soil or runoff samples (Chardon et al., 1996; Myers et al., 1995). For instance, Usitalo and Yli-Halla (1999) reported that soil contamination of the iron-oxide paper with sediment particles may over-estimate desorbable P in turbid runoff water. Myers et al. (1995) recommended preparing the iron-oxide papers with filter paper containing pore openings
<5.0 µm and held fixed between polyethylene screens during shaking to reduce soil adhesion to the paper. Furthermore, different pore size and paper size may adsorb different amounts of P. Lin et al. (1991) studied BAP adsorption capacity of eight different pore size filter papers and found the amounts of phosphate extracted by iron coated Whatman no. 50 (2.7-µm openings) from 39 soils of major agricultural areas were well correlated with those extracted by young wheat seedlings or other chemical extractants commonly used for soil testing. Originally, the used filter paper was cut in 2- by 10- cm strips from 15-cm circles of filter paper coated with iron-oxide (Menon et al., 1990a; Robinson et al., 1994). Later, Myers et al. (1995) and Myers et al. (1997) used 5.5-cm circles of Whatman no. 50 filter paper. The immersion period of filter paper in FeCl$_3$ is another factor that may affect P adsorption. However, in the original procedure of Sissingh (1983) the duration of the period of immersion in FeCl$_3$ was not mentioned. Lin et al. (1991) evaluated the effect of the immersing period in FeCl$_3$ and found extending the immersing period from 0.5 to 2.3 h increased the final amount of P adsorbed by 8%; longer periods gave no further increase.

Currently, there is no information available on manure BAP testing by iron-oxide paper. Similar to manure TDP analysis, the effects of factors such as extractant to manure ratio, extraction time, and solution pH should be considered in manure BAP analysis by the iron-oxide paper method. For example, Menon (1992b) found the quantity of P adsorbed to the filter paper tended to increase when the extractant: soil ratio increased from 5:1 to 60:1. Sissingh (1983) found the amount of soil P adsorbed by iron-oxide paper increased with contact time up to 64 h. Menon et al. (1990a) found that a 16-h extraction time gave the best correlation between P adsorbed on iron-oxide paper and P uptake by maize grown on acidic, alkaline, and calcareous soils. Busselli (1994) reported that sorption of P onto iron-oxide paper decreased above pH 6.5
because the amount of positive charges on the iron-oxide paper decreased above this pH level. Thus, it is not recommended to compare BAP data from acidic soils with neutral or calcareous soils (Chardon et al., 1996).

Bioavailable P was commonly removed from iron-oxide paper by dissolution of iron-oxide in 40 to 50 mL of 0.1 M or 0.2 M of H$_2$SO$_4$ for 1 h (Lin et al., 1991; Myers et al., 1995; Van der Zee SEATM et al., 1987) followed by measurement of the dissolved P by a colorimetric method (Murphy and Riley, 1962). To obtain better removal of adsorbed P, Pierzynski et al (1994) used 0.4 M H$_2$SO$_4$. However, Perrot and Wise (1993) compared different strength of H$_2$SO$_4$ (0.1 M and 0.5 M) and found no differences in the amount of P removed from the paper during 1-h dissolution, because P mainly adsorbs on the surface of the iron-oxide coating. Due to the large binding capacity of the iron-oxide paper, organic P species were also found bound to iron-oxide paper and treatment with higher strength of H$_2$SO$_4$ may promote the hydrolysis of this organic P (Robinson and Sharpley, 1994). Consequently, Chardon et al. (1996) recommended removing adsorbed P by shaking with 40 mL 0.1 M H$_2$SO$_4$ for 1 h.

2. Fractionation and Speciation of Phosphorus in Animal Manure

Edwards and Daniel (1993) found that P in runoff from pastures receiving animal manure was mainly in dissolved inorganic P species, and was about 2000 times greater than the critical concentration associated with accelerated eutrophication (0.01 mg P L$^{-1}$). Many researchers also found a significant amount of organic P in soil leachate from manure-amended soils (Chardon et al., 1997; McDowell and Koopmans, 2006; Toor et al., 2003). This is because swine (Sus scrofa domesticus) and poultry (Gallus gallus) lack the phytase enzymes to break down inositol 6-phosphate (IP6) present in feed grains, and as a result excrete most of this species of organic P (Harper et al., 1997; Zyla et al., 2000). For example, Gerritse and Vriesema (1984) found 40% of
total P (TP) in poultry manure was organic P, and Peperzak et al. (1959) reported that 2 to 60% of total organic P in poultry manure was IP6. The retention of organic P in soils is determined by the nature of P compounds and animal manure. Celi et al. (2000) found that IP6 was tightly bound by soils whereas nucleotides and glucose phosphate were more mobile. Iyamruemye et al. (1996) found that the organic ligands in applied manure lead to an increase in the concentration of soluble P in runoff by complexing with Fe and Al in soils, which decreases precipitation of P with these metals, and compete with P for sorption sites.

2.1 Manure Phosphorus Sequential Fractionation

The fate of P from animal manures in the environment is in part determined by the P fractions present in the manure. Sequential fractionation strategy was developed by Chang and Jackson (1957) and has been widely used in characterizing soil P (Hedley et al., 1982; Sharpley and Moyer, 2000; Sui et al., 1999). This method is based on the assumption that chemical extractants selectively dissolve different groups of P compounds (He et al., 2003). These P fractions are usually identified by the extractant used, such as resin- or H$_2$O-P, NaHCO$_3$-P, NaOH-P, and H$_2$SO$_4$-P or HCl-P. Resin- or H$_2$O-P, and NaHCO$_3$- extractable P are labile P fractions readily available for plant and algae; the NaOH extractable P fraction is considered moderately labile P (Fe-, Al-associated P), and the P extracted by HCl is regarded as moderately stable P (Ca-associated P) (Williams et al., 1980; Zhang et al., 2004). It is believed that P species associated with these fractions decrease in vulnerability to environmental loss in the order of H$_2$O-, NaHCO$_3$-, NaOH-, and HCl-extracted P as well as residual P (Dou et al., 2003; He and Honeycutt, 2001; Sharpley and Moyer, 2000). This fractionation method may not be suitable for manures (He et al., 2003) because soils have both mineral and organic matter phases, which requires caustic chemical treatment to release the different soil P species associated with soil
surfaces and existing as insoluble phosphates such as insoluble Ca, Al, and Fe compounds in various stages of crystallization (Chang and Jackson, 1958; Lindsay, 1979; Stumm, 1986). In contrast, animal manures are composed of partly digested feed, which is primarily organic in nature (Dao et al., 2006). However, many studies have applied this sequential fractionation method on manure P composition analysis (Barnett, 1994; Dou et al., 2000; Leinweber et al., 1997; Peperzak et al., 1959), and have found that P distribution varied with the nature of animal manure. For example, Sharpley and Moyer (2000), Leinweber et al. (1997), and Dou et al. (2000) sequentially fractionated P from hog, dairy, and poultry manures with deionized water (DI water), 0.5 M NaHCO$_3$, 0.1 M NaOH, and 0.1 M HCl. The percentages of the total P in broiler litter present in these fractions were 28, 35, 7, and 29% for Sharpley and Moyer (2000), and 49, 19, 5, and 25% for Dou et al. (2000). Leinweber et al. (1997) reported that 24, 27, 10, and 39% of the total P in hog manure was DI water-, NaHCO$_3$-, NaOH-, and acid-extractable P, respectively. They also concluded that the large percentage (>50% of total P) of P in water and NaHCO$_3$ fractions demonstrated a weak binding energy of P, which increased the potential for runoff P.

Most of the previously published research used DI water as the first extractant to fractionate labile or plant available P pool in the sequential extraction (Dou et al., 2000; Leinweber et al., 1997; Sharpley and Moyer, 2000). However, the pH of a suspension of manure in DI water is generally alkaline due to the presence of NH$_3$ and Ca, Na, and Mg phosphates (Griffiths, 1998). Also, Tasistro et al. (2004) found that the pH of surface-applied broiler litter decreased from initially 8.1 to 6.7 within 30 days after application to the surface of a pasture. Acidification of manure could result in greater release of TDP by solubilization of orthophosphates (Gerritse and Vriesema, 1984), since most inorganic P in manure is present as Ca, Mg, and K salts (Cooperband and Good, 2002). Also, Stumm and Morgan (1981) and
Champagne (1988) found that the solubility of metal-phytate complexes increased in acidic environments. Thus, DI water-leading fractionation may under estimate labile P from animal waste applied to acidic soils. Instead, the MES buffer used by Tasistro et al. (2007) may more closely estimate manure labile P when manure is in an acidic soil environment.

2.2 Manure Phosphorus Speciation

Although techniques such as fractionation yield information on the relative proportions of inorganic and organic P, they can not identify specific P species. It was reported that some organic P species are more labile than others (Makarov et al., 2002), and hence information on P species in animal manures is necessary to help understand manure P transformation in the field. Commonly used chromatographic technique, such as High Performance Liquid Chromatography (HPLC) is not able to provide detailed information on P speciation because of poor column retention and matrix interference with standards (Kemme et al., 1999; Turner et al., 2002). Instead, solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectroscopy has become a widely accepted technique for identifying P species in environmental samples because it allows the simultaneous identification of multiple P compounds in complex matrices with minimal sample handling (Condron et al., 1997), and because $^{31}$P is the only naturally-occurring P isotope (100% natural abundance) with a large gyromagnetic ratio. By monitoring the response of the electronic environment around various P nuclei to external magnetic field, qualitative and quantitative detection of a variety of P species by $^{31}$P NMR can be achieved.

Phosphorus NMR spectroscopy has been successfully used in the characterization of P in soil and animal manures. Using $^{31}$P NMR, Hansen et al. (2004) investigated P extracted from soils amended with dairy manure by NaOH + EDTA, and found that the majority of organic P in soils is phytic acid. Leinweber et al. (1997) identified orthophosphate monoesters and diesters in
NaOH extracts of swine slurry. Turner (2004) studied three different animal manures to optimize the $^{31}$P NMR technique in manure P speciation, and found organic P in manures was in phosphate monoesters and phosphate diester species.

Many studies have shown that identifying an appropriate extractant for environmental samples is the key to obtaining a satisfactory $^{31}$P NMR spectra (Turner and Leytem, 2004; Turner et al., 2003c). However, the solubilized P species may vary with the extractant and the nature of animal wastes. Currently, there is no agreement on the most suitable extractant for extracting P from soils and environmental samples, although NaOH + EDTA has been widely used as an extractant for soil and manure in $^{31}$P NMR analysis. Bowman and Moir (1993) evaluated basic EDTA as an extractant for P in soils that were low in organic C content and with a mineralogy dominated by crystalline minerals. After extracting 10 soils from different regions of the United States, they found that NaOH + EDTA extracted 32% more organic P than wet sequential extraction, and that 0.25 M NaOH + 0.05 M EDTA extracted the most organic P from 10 soils. Some studies have used stronger alkaline solutions (0.5 M NaOH) to extract soil samples for $^{31}$P NMR analysis (Newman and Tate, 1980; Robinson et al., 1998), although hydrolysis of P compounds could happen during extraction and concentration with strong base (Condron et al., 1990). Leinweber et al (1997) applied various concentrations of NaOH to extract manure and soils, and found that $^{31}$P NMR spectra of 0.5 M NaOH extracts from pig manure, poultry manure, and some soils produced greater signal intensities for orthophosphate and monoester P than 0.1 M NaOH extracts. Mixtures of NaOH and EDTA have also been used for manure extraction. Turner (2004) studied extractants for dairy slurry, swine manure, and poultry litter by analyzing P recovered from extracting manure with various concentrations of EDTA and
NaOH. He found that swine manure extracts with 0.5 M NaOH + 50 mM EDTA produced the best spectra resolution.

Although NaOH + EDTA extraction provides a simple process for P analysis in soil and animal manure samples, this alkaline extractant may not provide an accurate estimate of P species that may be extracted by rainfall or runoff in acidic soil environments. For example, Tasistro et al. (2004) found that extracting manure organic P at the original manure pH (~pH 8) may underestimate TDP, because the pH of broiler litter applied on the surface of a pasture was initially 8.1 and decreased to 6.7 in 30 days. As a result, the DUP measured in soils and thatch was twice as much as that measured at the original manure pH. Their laboratory study also found that acidifying litter suspensions to pH 6 increased TDP by 34 to 72% and DRP by 24 to 69%. Similarly, extraction of manure with a solution buffered to the pH of the soil may more closely resemble the P species that may be soluble during rainfall and runoff. Considering the potential contribution of manure P to the P fertility of soils (Oehl et al., 2001) and the potential for accelerating eutrophication due to the transfer of P to surface waters (Haygarth and Jarvis, 1999), it is critical to understand the P speciation and composition in animal manure at various pH levels.

3. Manure Phosphorus Transformation during Storage

Because most broiler litter is surface applied to grasslands as fertilizer, surface runoff interacting with the litter may solubilize P and transport it to surface waters, where it may accelerate eutrophication (Sharpley, 1995; Sims et al., 1998). Therefore, the concentration of TDP in broiler litter is an important characteristic that may affect the potential for surface runoff contamination. Broiler litter collected from broiler houses may be directly applied to grasslands or temporarily stored. Litter storage prior to land application allows flexible applications to
coincide with maximum crop uptake and minimum runoff potential, which would reduce the potential for adverse impacts on water quality (Brodiek and Carr, 1988; Moore Jr. et al., 1995). However, there is limited information on P transformation during storage of broiler litter. Vadas et al. (2004) reported that TDP increased about 52% in raw manure that went through freeze-thaw cycles. McGrath et al. (2005) found a large increase in orthophosphate concentration and a decrease in phytic acid concentration in broiler litter stored at a water content of 370 g kg\(^{-1}\) when compared to broiler litter stored at a water content of 210 g kg\(^{-1}\). The authors concluded that microbial activity played an important role in transferring non-labile P to labile P during storage at the greater water content. Maguire et al. (2006) found that TDP in broiler litter stored with a water content of 600 g kg\(^{-1}\) increased with storage time. We have observed that the water content of broiler litter removed from broiler houses can range from low (about 200 g kg\(^{-1}\)) to very high (> 1000 g kg\(^{-1}\)). Information on P transformations as affected by such a wide range of water content is currently not available. Also, temperature during storage can vary depending on environmental conditions, and information on the effect of temperature on P transformations during storage is currently limited.
REFERENCES


CHAPTER 2

EXTRACTION METHOD AFFECTS TOTAL DISSOLVED AND BIOAVAILABLE PHOSPHORUS IN BROILER LITTER

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ABSTRACT

Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Bioavailable P (BAP) from broiler litter are important contaminants in surface runoff from boiler litter-amended fields. Thus, a standard method of estimating TDP, DRP and BAP in broiler litter is needed to interpret the environmental impact of broiler litter phosphorus. We evaluated three broiler litter samples to determine the effect of extractant (water or 2-(N-morpholino) ethanesulfonic acid - (MES) buffer at pH 6), extractant:litter ratio (10:1, 100:1, or 200:1) and extraction time (1, 4, or 24 h) on TDP, DRP, and BAP. Widening water to manure ratio (10:1 to 200:1) increased manure TDP, DRP, and BAP in most cases, probably because of dissolution of calcium phosphates. In most cases, decreasing the extraction pH from the original broiler litter pH to pH 6 by means of a buffer increased the amount of TDP, DRP, and BAP, likely because acidification solubilized calcium phosphates and phytates (the main organic phosphate species) in broiler litter. Likewise, extending extraction time from 1 to 4 or 24 h increased the amount of TDP, DRP, and BAP. This study also showed that the iron-oxide paper method might underestimate manure BAP because negatively charged organic matter in broiler litter competes with DRP for adsorption sites on iron-oxide paper.
INTRODUCTION

Because broiler litter has considerable amounts of Total Dissolved Phosphorus (TDP; Kleinman et al. 2005), its surface application to grasslands can contaminate surface waters with P, with the potential risk of accelerating eutrophication (United States Geological Survey, 1999). Total dissolved P is defined as P that passes through a 0.45-µm filter, and can be divided into Dissolved Reactive P (DRP) and Dissolved un-reactive P (DUP), depending on whether or not it can be determined with the molybdate-blue method of Murphy and Riley (1962). In water bodies, DRP is the main fraction that can be readily utilized by bacteria and algae (Hatch et al., 1999; Haygarth and Jarvis, 1999). Phosphorus that does not pass through a 0.45-µm filter is usually called particulate P (PP). Bioavailable P (BAP) is made up of DRP as well as DUP and PP that can be transformed into available forms by natural processes (Boström et al., 1982). Recently, BAP has received considerable attention due to its direct effects on algal growth in aquatic systems (Fang et al., 2005; Sharpley et al., 1995).

Although TDP and BAP derived from broiler litter may be important contaminants of runoff water, there is limited information on factors that affect their determination. Self-Davis and Moore (2000) recommended studying TDP by extracting 20 g chicken litter (wet weight) with 200 mL water for 2 h. This method was originally developed for soil analysis and adapted to manure. Sharpley and Moyer (2000) originally extracted 1 g (dry weight equivalent) fresh manure with 200 mL water for 1 h. Later, Maguire et al. (2005) used 10:1 and 200:1 water:litter ratios to extract TDP from poultry litter, and found that a greater amount of TDP was extracted at 200:1 than at 10:1. Haggard et al. (2005) extracted various types of poultry manure using water to manure ratios of 10:1, 20:1, 50:1, 100:1, and 200:1. Manure TDP increased proportionally with the amount of water used in the extraction.
In the studies reported above, manure TDP was determined at the original pH of the water–manure mixture, which is generally alkaline (Griffiths, 1998). However, this pH level may not be suitable for estimating the amount of TDP released once manures equilibrate with soil. For example, Tasistro et al. (2004) found that broiler litter pH changed from 8.1 to 6.7 within 30 days after its application to the surface of a pasture. The total DUP in manure that had equilibrated with the surface soil for 30 days was twice as much as that extracted initially at the original manure pH (at a ratio of 200:1). Stumm and Morgan (1981) and Champagne (1988) stated that the solubility of inorganic phosphates and metal-phytate complexes increased in acidic environments. Therefore, measuring manure TDP at an alkaline pH could lead to underestimation of the amounts of TDP that would be released in an acidic soil (Tasistro et al., 2004).

To better predict the phosphorus loading to the environment, it is important to investigate a suitable pH level for TDP extraction from manure applied to acidic soils. Tasistro et al. (2007) selected an extraction pH of 6, as this is the recommended pH for grasslands in Georgia, and investigated the effect of different buffers on DRP and DUP extraction. Extracting broiler litter or layer manure for 4 h with a 0.1 M buffer (pH = 6) solution of 2-(N-morpholino) ethanesulfonic acid (MES) at an extracting ratio of 200:1 (solution: dry manure) released similar amounts of DRP and DUP as acidifying the sample to pH 6 with HCl. These results suggested that the MES buffer was not forming metal complexes and thus was not increasing the solubility of inorganic or organic P associated with metals in the manure. Furthermore, extracting broiler litter or layer manure with MES buffer at pH 6 removed more DRP and DUP than extracting with water. Consequently, to better estimate the amount of TDP that may be released from manure after it is applied to soil, the MES buffer may be a good extractant to measure DRP and
DUP at pH 6. In a recent laboratory incubation study, we evaluated the amount of TDP released from surface applied broiler litter that had been previously extracted with water or MES buffer. We found that the amount of TDP released in 14 days at 25°C was five times larger from broiler litter that had been previously extracted with water than from broiler litter that had been previously extracted with MES buffer at pH 6 (Picone et al., unpublished results). These results indicated that extracting with MES buffer at pH 6 was able to extract more of the broiler litter P that would be released as TDP when applied to soil than extraction with water.

As in the case of TDP, there is no widely accepted BAP testing method for broiler litter. Because BAP may contain some PP fraction, in addition to TDP, it would not be accurate to assess the manure potential for eutrophication based solely on the TDP fraction. Instead, BAP measurements should include some portion of PP. Previous research work has shown that BAP, not TDP, provides the most accurate assessment of water quality conditions for surface water (Gerdes and Kunst, 1998). Bioavailable P testing for soil (Menon et al., 1990a; Menon et al., 1997; Menon et al., 1989b; Menon et al., 1990b; Myers et al., 1997; Myers et al., 2005; Sissingh, 1983) and runoff samples (Dils and Heathwaite, 1998; Sharpley, 1993a) has been conducted using the iron-oxide filter paper methodology. Limited research has been published on the effect of factors such as extractant to soil ratio, extraction time, and solution pH on BAP extraction. Menon et al. (1990a) and Myers et al. (1995) both used an extractant:soil ratio of 40:1 to measure soil BAP. Menon (1992b) found the quantity of P adsorbed to the filter paper tended to increase when the extractant: soil ratio increased from 5:1 to 60:1. As to extraction time, Sissingh (1983) found the amount of P adsorbed by iron-oxide paper increased with time up to 64 h. Menon et al. (1990a) found that a 16-h extraction time gave the best correlation between P adsorbed on iron-oxide paper and P uptake by maize grown on acidic, alkaline, and calcareous
soils. Sisingh (1983) found that a 16-h extraction time gave the best correlation with uptake of P in a pot experiment. Busselli (1994) reported that sorption of P onto iron-oxide paper decreased above pH 6.5 because the amount of positive charges on the iron-oxide paper decreased above this pH level. Thus, it is not recommended to compare BAP data from acidic soils with neutral or calcareous soils (Chardon et al., 1996).

We could not find any published studies in which the iron-oxide paper method was used to measure broiler litter BAP. Clearly, standardized methods for TDP and BAP in broiler litter are needed to better estimate the environmental impact of P in broiler litter. The objective of this study was to extend the work of Tasistro et al. (2007) by evaluating the effects of extractant, solution to manure ratio, and extraction time on the amounts of TDP, DRP, and BAP extracted from broiler litter samples. The main goal was to identify the level of each of these factors at which maximum extraction of TDP, DRP, and BAP from broiler litter is achieved.

**MATERIALS AND METHODS**

**Broiler Litter**

Three samples of fresh broiler litter with a wide range of total P, Ca, and Mg concentrations were selected for this study (Table 2.1). The samples were oven-dried at 65°C, ground to pass a 2-mm sieve, and stored in sealed plastic containers at 4°C until used. Total P, Ca, and Mg were measured by dry ash digestion (USEPA, 1995) followed by determination with an Inductively Coupled Plasma-Optical Emission Spectrograph (ICP-OES).

**Total Dissolved P (TDP) and Bioavailable P (BAP) Analysis**

A factorial combination of two extracting solutions (0.1 M MES buffer at pH 6 and DI water), three extracting ratios (10:1, 100:1, and 200:1; solution:dry broiler litter), and three extraction times (1, 4, and 24 h) to generate a 18 treatments for extraction of TDP, DRP, Soluble
Bioavailable P (SBAP), and Total Bioavailable P (TBAP) from broiler litter. For TDP and DRP extraction, the solution–litter mixtures were shaken horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature (~23°C). After extraction, the samples were centrifuged for 20 min at 1840 x g and then filtered through a 0.45-μm filter. The filtered extracts were analyzed for DRP by Murphy and Riley (1962) and for TDP by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). Each treatment was carried out in triplicate.

For BAP extraction, iron-oxide filter paper (Whatman no. 50; 5.5-cm diameter) was prepared as described by Myers et al. (1997). The amount of iron-oxide coating was determined by weighing filter paper before and after iron-oxide coating. The amount of iron-oxide coated on filter paper ranged from 20 to 40 mg. Phosphorus adsorption capacity, and the P/Fe ratio (in moles) of a single iron-oxide filter paper was evaluated by mixing newly made iron-oxide paper with solutions containing different amounts of P (0.6 to 9 mg P as KH₂PO₄) in centrifuge tubes, and shaking horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature for 24 h. The amount of P adsorbed by the paper was calculated as the difference between the initial and final amount of P in solution. The amount of P left in solution after shaking was determined by Murphy and Riley (1962), with each treatment carried out in triplicate. The maximum amount of P adsorbed on one iron-oxide filter paper was 3.05 mg P, and the P/Fe ratio (in moles) was approximately 1/5.

The number of iron-oxide filters needed to adsorb all BAP from each treatment was determined in a preliminary study with each of the extractants and ratios in which the manure suspensions were shaken continuously for 96 h with an iron-oxide filter paper being replaced with a fresh one every 24 h. The P adsorbed by each iron-oxide paper was removed with 40 mL
of 0.2 M H$_2$SO$_4$ (Myers et al., 1997). The number of filter papers for each extractant and ratio was determined to be the number of filter papers used before the P removed from the last added paper was below the detection limit of ICP-AES ($<0.06$ mg L$^{-1}$ or 2.4 μg P). This preliminary study showed that the number of iron-oxide filter papers needed was three for a ratio of 10:1 (1 g broiler litter), and only one for ratios of 100:1 (0.1 g broiler litter) and 200:1 (0.05 g broiler litter).

Soluble Bioavailable P was determined using a separate set of extractions (without the iron-oxide paper) at the same extracting conditions used for TDP. After the extraction, the supernatants were filtered through a 0.45-μm filter. The filtrates were then shaken with iron-oxide filter paper horizontally on an end-to-end shaker at 120 oscillations per minute for 24 h. The P adsorbed by the filter paper was desorbed as previously described and measured by Murphy and Riley (1962) (SBAP1) and by ICP-AES (SBAP2). The Murphy and Riley method determines inorganic P while the ICP-AES measures the inorganic and organic P in the testing solutions. Each treatment was carried out in triplicate.

Total bioavailable P was extracted by shaking the samples with iron-oxide paper with the same extractants, extraction ratios, and extraction times used for TDP analysis. The P adsorbed by the iron-oxide filter paper was removed with 40 mL of 0.2 M H$_2$SO$_4$ (Myers et al., 1997) and the desorbed P concentration was determined by the molybdate-blue method of Murphy and Riley (1962).

**Statistical Analysis**

An analysis of variance for each extracted fraction was carried out with PROC GLM in SAS, V.9.0 (SAS Institute Inc., 2002). The statistical model considered the main effects of extractant, extraction ratio, extracting time, and broiler litter sample, as well as all two-way, three-way, and four-way interactions in a randomized complete block design with three
replications. Fisher’s protected Least Significant Difference (LSD) was used to separate means, and a 5% confidence level was used to conclude significant effects.

RESULTS AND DISCUSSION

Total Dissolved P

There were significant Sample x Ratio x Extractant, and Sample x Extractant x Time interactions for TDP (Table 2.2). The variable Sample refers to the individual broiler litters used in the study.

Sample x Ratio x Extractant

For the three broiler litters, the amount of TDP extracted increased as the extraction ratio changed from 10:1 to 100:1, both with DI water (original litter pH) and MES buffer (pH 6) (Fig. 2.1a). This was an expected effect because a larger solution:litter ratio would lead to a lower solution P concentration during extraction, thereby facilitating P desorption or dissolution from broiler litter. The increased amount of TDP extracted as the ratio changed from 10:1 to 100:1 varied among litters, with Sample #2 having the smallest amount and Sample #3 the largest amount. The smallest increase with Sample #2 may have been due to its small P concentration, whereas the largest increase with Sample #3 may have been related to its relatively high original pH (8.1). Increasing the extraction ratio from 100:1 to 200:1 did not affect TDP in most cases, with the exception of Sample #2 in which there was a decrease in TDP when extracted with DI water.

For Samples #1 and #2, a greater amount of TDP was extracted at all three ratios with MES (pH 6) than with DI water (original pH). In contrast, for Sample #3 only the 10:1 extraction ratio showed differences in TDP between extractions with MES and DI water. The reason for the
different behavior of Sample #3 is unclear. In all cases, however, an extraction with MES at pH 6 with a ratio of 100:1 or 200:1 resulted in the greatest amount of TDP extracted.

**Sample x Extractant x Time**

For all three broiler litter samples, TDP increased as extraction time with MES buffer increased from 1 h to 24 h (Fig. 2.1b), but the increase differed among litter samples. As in the response to extracting ratio, the smallest response to extraction time was obtained with Sample #2 and the largest response with Sample #3. In general, there was no difference between 4 and 24-h extractions with MES.

For extractions with DI water, only Sample #3 showed a positive response as extraction time increased from 4 to 24 h. The percentage of TDP extracted in 1 h with respect to the amount extracted in 24 h was 102% for Sample #1, 73% for Sample #2, and 85% for Sample #3. These results are similar to those of Kleinman et al. (2002b) who found that the amount of TDP extracted at a 200:1 ratio in 1 h corresponded to more than 70% of the total amount extracted in 24 h.

For all three broiler litter samples, extraction with MES for 4 or 24 h resulted in the maximum amounts of TDP. In summary, an extraction ratio of 100:1 or 200:1 with MES for 4 or 24 h removed the maximum amount of TDP from all three tested broiler litter samples (Table 2.3).

**Dissolved Reactive P**

There were significant Sample x Ratio x Time, Sample x Extractant x Time, and Ratio x Extractant x Time interactions for DRP (Table 2.2).
Sample x Ratio x Time

Increasing the ratio from 10:1 to 200:1 at an extraction time of 24 h caused larger
increases in DRP with Sample #1 than with Sample #2, and had no effect with Sample #3 (Fig.
2.2a). For all litter samples, the maximum extraction of DRP was obtained with 24 h at a ratio of
100:1 or 200:1.

Sample x Extractant x Time

For all broiler litters, increasing the extraction time from 1 to 24 h increased the amount
of DRP extracted with MES (Fig. 2.2b). The effect of extraction time was not as marked for
extractions with DI water, especially for Sample #1 and #2. For all samples, the maximum
amount of DRP was obtained with MES extractions for 24 h.

Ratio x Extractant x Time

For extractions with MES, the effect of increasing extraction time from 1 to 24 h was
more marked at ratios of 100:1 and 200:1 than at 10:1 (Fig. 2.2c). For extractions with DI water,
increasing the extraction time from 1 to 24 h either had no effect or decreased the amount of
DRP extracted at all extraction ratios. Consequently, an extraction ratio of 100:1 for 1 h removed
the maximum amount of DRP with DI water. A similar finding was reported by Dou et al. (2000)
who studied DRP release from poultry manure and dairy slurry as affected by extraction time at a
water:manure ratio of 100:1. They observed that DRP increased by a small magnitude when
extending extraction time from 1 to 16 h, and recommended using 1-h extraction as the basis to
develop methods for manure DRP study. In summary, the maximum amount of DRP extracted
from broiler litter was obtained with MES and ratios of 100:1 or 200:1 for 24 h (Table 2.3).
Soluble Bioavailable P

There was a significant Ratio effect and a significant Sample x Extractant x Time interaction for SBAP1 (Table 2.2).

**Ratio Effect**

Increasing the extraction ratio from 10:1 to 100:1 increased the average amount of SBAP1 extracted from 1,700 to 4,700 mg P kg\(^{-1}\), but there was no difference between 100:1 and 200:1 (4,600 mg P kg\(^{-1}\)).

**Sample x Extractant x time**

Increasing extraction time from 1 to 24 h did not increase SBAP1 in Samples #1 and #2 with either extractant (Fig. 2.3). However, increasing extraction time from 1 to 24 h increased SBAP1 in Sample #3 when extracting with MES. In general, there was no difference between extraction times of 4 and 24 h. In summary, the maximum removal of SBAP1 for all broiler litter samples was obtained with MES at an extraction ratio of 100:1 or 200:1 for 4 or 24 h (Table 2.3).

Total Bioavailable P

There were significant Ratio x Extractant and Sample x Ratio x Time interactions for TBAP (Table 2.2) extraction.

**Ratio x Extractant**

For both extractants, the amount of TBAP extracted increased with increasing ratio, but a larger increase was obtained with MES than with DI water (Fig. 2.4a). The larger increase observed with MES may have been due in part to the lower extraction pH, which would have led to more positive charges on the iron oxide papers.
Sample x Ratio x Time

Increasing extraction time from 1 to 24 h had little or no effect on TBAP extraction from all litters at a ratio of 10:1 (Fig. 2.4b). At a ratio of 200:1, increasing extraction time from 1 to 24 h did not affect TBAP extraction from Sample #1, but caused a large increase in TBAP extraction from Samples #2 and #3.

In summary, the maximum removal of TBAP from all broiler litters was obtained with MES at an extraction ratio of 200:1 for 24 h (Table 2.3).

Extraction with MES Buffer at pH 6

Average values for extraction of TDP, DRP, SBAP, and TBAP with MES buffer (pH 6) at a ratio of 200:1 for 24 h are presented in Table 2.4. These results show that TBAP values were larger than TDP values. For the three broiler litter samples, TBAP as percentage of total P ranged from 71 to 92%, whereas TDP ranged from 54 to 63%. Therefore, measuring TBAP with MES buffer in broiler litter may provide a better estimate of the potential for release of BAP than simply measuring TDP.

It should be pointed out that SBAP values measured by Murphy and Riley (1962) (SBAP1) and by ICP-AES (SBAP2) were in most cases smaller than DRP values (Table 2.4), when they were expected to be at least equal to DRP. Some of these unexpected results may have been due to competition by water-soluble, negatively charged organic compounds (released from broiler litter) for adsorption sites on the iron-oxide paper. Evidence for this is provided by the fact that SBAP values determined by ICP-AES (SBAP2) were greater than SBAP1 values determined by Murphy and Riley (1962) (SBAP2-SBAP1, Table 2.4). These results suggest that organic compounds containing P had been sorbed onto the iron-oxide papers, possibly competing with inorganic P for adsorption sites. This effect is noticeable for Samples #2 and #3, in which
the amount of organic P (SBAP2-SBAP1 in Table 2.4) removed by the iron-oxide paper is comparable to the amount of DRP that was not adsorbed by the paper (DRP-SBAP1, Table 2.4).

CONCLUSIONS

In general, extracting broiler litter with MES buffer at pH 6 resulted in greater values of TDP, DRP, SBAP, and TBAP than extracting broiler litter with DI water. Maximum amounts of these variables were extracted with an extractant:broiler litter ratio of 100:1 or 200:1 and extraction times of 4 or 24 hours (Table 2.4). Additional research should be conducted to determine whether extraction with MES buffer under these conditions can provide a better estimate than extraction with DI water of the amount of TDP and BAP that can be released from broiler litter after application to soil.
REFERENCES


Table 2.1. Selected properties of three broiler litter samples used in the study.

<table>
<thead>
<tr>
<th>Broiler litter sample</th>
<th>pH†</th>
<th>Total P</th>
<th>Total C</th>
<th>Total N</th>
<th>Total Ca</th>
<th>Total Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>7.4</td>
<td>19,667</td>
<td>333,033</td>
<td>36,787</td>
<td>29,140</td>
<td>6,379</td>
</tr>
<tr>
<td>#2</td>
<td>7.0</td>
<td>12,604</td>
<td>354,433</td>
<td>35,637</td>
<td>16,767</td>
<td>4,948</td>
</tr>
<tr>
<td>#3</td>
<td>8.1</td>
<td>21,880</td>
<td>382,500</td>
<td>36,277</td>
<td>30,730</td>
<td>9,650</td>
</tr>
</tbody>
</table>

† pH was measured at a ratio of 10:1 (water:broiler litter).

Table 2.2. Analysis of variance for Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from three broiler litter samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>TDP</th>
<th>DRP</th>
<th>SBAP1</th>
<th>TBAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>--P</td>
<td>--P</td>
<td>--P</td>
<td>--P</td>
</tr>
<tr>
<td>Sample</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>Ratio</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sample*Ratio</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Extractant</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sample*Extractant</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio*Extractant</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Sample<em>Ratio</em>Extractant</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Time</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>Sample*Time</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Ratio*Time</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Sample<em>Ratio</em>Time</td>
<td>NS</td>
<td>**</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Extractant*Time</td>
<td>**</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sample<em>Extractant</em>Time</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio<em>Extractant</em>Time</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sample<em>Ratio</em>Extractant*Time</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Statistical significance at the 0.05 probability level, ** Statistical significance at the 0.01 probability level, NS: Non significant
Table 2.3. Extracting conditions for maximum removal of Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) from three broiler litter samples.

<table>
<thead>
<tr>
<th>P fraction</th>
<th>Extractant</th>
<th>Extraction time (h)</th>
<th>Extractant:litter ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MES</td>
<td>DI Water</td>
<td>1</td>
</tr>
<tr>
<td>TDP</td>
<td>†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRP</td>
<td>†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBAP1</td>
<td>†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBAP</td>
<td>†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Recommended level for each factor

Table 2.4. Total Dissolved P (TDP), Dissolved Reactive P (DRP), Total Bioavailable P (TBAP), Soluble Bioavailable P1 (SBAP1), and Soluble Bioavailable P2 (SBAP2) extracted from three broiler litters at a ratio of 200:1 with MES buffer at pH 6 for 24 h.

<table>
<thead>
<tr>
<th>Broiler litter sample</th>
<th>TDP</th>
<th>DRP</th>
<th>SBAP1†</th>
<th>SBAP2‡</th>
<th>TBAP</th>
<th>DRP-SBAP1</th>
<th>SBAP2-SBAP1</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>11,961</td>
<td>9,620</td>
<td>6,456</td>
<td>7,835</td>
<td>13,973</td>
<td>3,164</td>
<td>1,379</td>
</tr>
<tr>
<td>#2</td>
<td>7,889</td>
<td>7,150</td>
<td>4,184</td>
<td>6,237</td>
<td>11,529</td>
<td>2,966</td>
<td>2,053</td>
</tr>
<tr>
<td>#3</td>
<td>11,864</td>
<td>8,837</td>
<td>6,621</td>
<td>9,248</td>
<td>18,613</td>
<td>2,215</td>
<td>2,627</td>
</tr>
</tbody>
</table>

SBAP1†: SBAP determined by ascorbic acid method, Murphy and Riley (1962)
SBAP2‡: SBAP determined by ICP-AES
Fig. 2.1. The effects of two extractants and (a) extraction ratio and (b) extraction time on Total Dissolved P (TDP) extracted from broiler litter samples #1, #2, and #3.
Fig. 2.2. The effects of (a) extraction ratio and extraction time, (b) extractant and extraction time, and (c) extractant, extraction ratio, and extraction time on DRP extracted from broiler litter samples #1, #2, and #3.
Fig. 2.3. The effects of extractant and extraction time on Soluble Bioavailable P1 (SBAP1) tested by Murphy and Riley (1962) and extracted from broiler litter samples #1, #2, and #3.
Fig. 2.4. The effects of (a) extractant and extraction ratio, and (b) extraction ratio and extraction time on Total Bioavailable P (TBAP) extracted from broiler litter samples #1, #2, and #3.
CHAPTER 3

EXTRACTING SOLUTIONS, RATIOS, AND TIMES AFFECT SOLUBLE P FRACTIONS FROM LAYER MANURE AND DAIRY SLURRY

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ABSTRACT

Previous research has reported that phosphorus (P) released from layer manure and dairy slurry may be important contaminants in surface runoff from manure-applied soils. Current methods extract Total Dissolved P (TDP), Dissolved Reactive P (DRP) and Bioavailable P (BAP) from manures with DI water. The extraction is at the original manure pH, which is commonly alkaline. Because manure pH equilibrates to the pH of the soil, which is usually acidic in soils from the southeastern USA, extraction of TDP, DRP, and BAP with a solution buffered to a common soil pH (such as 6) may yield more realistic results in terms of the amounts of TDP and BAP released. We evaluated the effects of extractant pH (water or 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 6), extractant:manure ratio (10:1, 100:1, or 200:1), and extraction time (1, 4, or 24 h) on TDP, DRP and BAP in layer manures and dairy slurries. In most cases, widening water to manure ratio (10:1 to 200:1) increased manure TDP, DRP, and BAP, which probably because of the dissolution of calcium phosphates present in manure. In most cases, extracting with MES buffer at pH 6 increased the amount of TDP, DRP, and BAP, when compared to extraction with DI water. To get the maximum recovery of all P fractions, MES buffer at a ratio of 200:1 was recommended to extract layer manure. But whereas a 1-h shaking was sufficient for TDP and DRP, a shaking time of 24 h was needed for BAP.
INTRODUCTION

Land-applied livestock manures have led to an accumulation of phosphorus in many soils (Lander et al., 1998; Lanyon, 2000), with a consequent increase in the potential for P enrichment of surface waters via surface runoff (Sharpley, 1993b; Sims et al., 1998). In response to impaired water resources, many states are developing regulations for applying animal waste (USEPA, 2004). There is limited information on extracting conditions to analyze animal manures for their potential to contaminate surface waters with P. Kleinman et al. (2002b), and Moore et al. (2000) found a strong correlation between Total Dissolved P (TDP) in animal manures and soluble P in surface runoff, and concluded that TDP is an effective indicator of P loss from manure application. More recent studies confirm that manure TDP is an effective indicator of TDP loss in runoff from surface-applied manure (Kleinman and Sharpley, 2003; Kleinman et al., 2002a; Maguire et al., 2005). Likewise, the contribution of animal manure to Bioavailable P (BAP) in surface waters has received considerable attention due to its direct effects on algal growth in aquatic systems (Fang et al., 2005; Sharpley et al., 1995). Manure BAP is made up of manure Dissolved Reactive P (DRP) as well as some dissolved un-reactive P (DUP) and particulate P (PP) that can be transformed into available forms by naturally occurring processes (Boström et al., 1982).

The first TDP protocols for animal manure were published by Self-Davis and Moore (2000) and Sharpley and Moyer (2000) which involved extracting 20 g manure (wet weight) with 200 mL water for 2 h. This method was originally developed for soil analysis and adapted to poultry manure. Sharpley and Moyer (2000) originally extracted 1 g (dry weight equivalent) fresh manure with 200 mL water for 1 h. Maguire et al. (2005) used 10:1 and 200:1 water: litter ratios to extract TDP from poultry litter, and found that greater amounts of TDP was extracted at
200:1 than at 10:1. Haggard et al. (2005) extracted various types of poultry manure using water to manure ratios of 10:1, 20:1, 50:1, 100:1, and 200:1. They reported that manure TDP increased proportionally with the amount of water used in the extraction. By studying an array of manure and biosolids samples across 10 labs in the United States and Canada, Kleinman et al., (2007) recommended to extract manure or biosolids with water at a ratio of 100:1 (solution:dry matter) for 1 h.

In the studies reported above, TDP was determined at the original pH of the water–manure mixture, which is generally alkaline (Griffiths, 1998) and therefore could support Ca phosphate and Mg phosphate stability (Lindsay et al., 1989). However, this pH level may not be suitable for estimating the amount of TDP released once manures equilibrate with soil. For example, Tasistro et al. (2004) found that broiler litter pH changed from 8.1 to 6.7 within 30 days after its application to the surface of a pasture. They also observed that total DUP in manure that had equilibrated with the surface soil for 30 days was twice as much as that extracted initially at the original pH of the manure (at a ratio of 200:1). Stumm and Morgan (1981) and Champagne (1988) found that the solubility of inorganic phosphates (Ca phosphates, and Mg phosphates) and metal-phytate complexes increased in acidic environments. Therefore, measuring manure TDP at an alkaline pH could lead to an underestimation of the amounts of TDP that would be released in an acidic soil (Tasistro et al., 2004). To better predict the phosphorus loading to the environment, it is important to investigate a suitable pH level for TDP extraction from manure/litter applied to acidic soils. Tasistro et al. (2007) selected an extraction pH of 6, as this is the recommended pH for grasslands in Georgia, and investigated the effect of different buffers on DRP and DUP extraction. They found that extracting broiler litter or layer manure for 4 h with a 0.1 M buffer (pH = 6) solution of 2-(N-morpholino) ethanesulfonic acid
(MES) at an extracting ratio of 200:1 (solution: dry manure) released similar amounts of DRP and DUP as acidifying the sample to pH 6 with HCl. These results suggested that the MES buffer was not forming metal complexes and thus was not increasing the solubility of inorganic or organic P associated with metals in the manure and litter. Furthermore, Tasistro et al. (2007) showed that the extraction with MES buffer at pH 6 removed more DRP and DUP than extraction with DI water. Consequently, the MES buffer may be a good extractant to measure DRP and DUP at pH 6 in an effort to better estimate the amount of TDP that may be released from manure/litter after applied to soil. For example, in a recent laboratory incubation study, we evaluated the amount of TDP released from surface applied broiler litter that had been previously extracted with DI water or MES buffer. We found that the amount of TDP released in 14 days at 25ºC was five times larger from broiler litter that had been previously extracted with water than from broiler litter that had been previously extracted with MES buffer at pH 6 (Picone et al., unpublished results). These results suggested that MES buffer at pH 6 was extracting more of the manure P that would be released as TDP (when applied to soil) than DI water.

Similarly to TDP, there is no widely accepted BAP testing method for animal manures. Because BAP may contain some PP fractions, in addition to TDP, it would be inaccurate to assess the manure/broiler litter potential for eutrophication based solely on the TDP fraction. Instead, BAP measurements should include PP. Previous research work has shown that BAP rather than TDP provides the most accurate assessment of water quality conditions for surface water (Gerdes and Kunst, 1998). Within the past three decades, BAP testing for soil (Menon et al., 1990a; Menon et al., 1997; Menon et al., 1989b; Menon et al., 1990b; Myers et al., 1997; Myers et al., 2005; Sissingh, 1983) and runoff samples (Dils and Heathwaite, 1998; Sharpley, 1993a) has been conducted using the iron-oxide filter paper methodology.
Limited research has been published on the effect of factors such as extractant to soil ratio, extraction time and solution pH on BAP extraction. Menon et al. (1990a) and Myers et al. (1995) both used an extractant: soil ratio of 40:1 to measure soil BAP. Menon (1992a) found the quantity of P adsorbed to the filter paper tended to increase when the extractant:soil ratio increased from 5:1 to 60:1. As to extraction time, Sissingh (1983) found the amount of P adsorbed by iron-oxide paper increased with time up to 64 h. Menon et al. (1990a) found that a 16-h extraction time gave the best correlation between P adsorbed on iron-oxide paper and P uptake by maize grown on acidic, alkaline and calcareous soils. Buselli (1994) reported that sorption of P onto iron-oxide paper decreased above pH 6.5 because the amount of positive charges on the iron-oxide paper decreased above this pH level. Thus, it is not recommended to compare BAP data from acidic soils with neutral or calcareous soils (Chardon et al., 1996).

Previous work with broiler litter indicated that maximum extraction of TDP, DRP, and BAP was obtained with MES buffer at ratios of 100:1 to 200:1 and 4 to 24 h of extraction (Zhao et al., unpublished results). However, the content of metal elements differ between layer manure and broiler litter. For example, layer manure usually has greater amount of Ca- and Mg-phosphate than broiler litter (Kleinman et al., 2005) because layer diets contain a substantial amount of Ca to promote egg production. As these Ca and Mg phosphates control manure P solubility, the extraction procedure for maximum TDP removal may be different for broiler litter and layer manure. A similar situation may be encountered for dairy slurry when compared with broiler litter. Consequently, the objective of this study was to evaluate the effect of extractant, extraction ratio, and extraction time on TDP, DRP and BAP in layer manure and dairy slurry samples. Our main goal was to identify the conditions for maximum extraction of TDP, DRP, and BAP from layer manure and dairy slurry.
MATERIALS AND METHODS

Layer Manures and Dairy Slurries

Four samples of fresh layer manure and three samples of dairy slurry with a wide range of total P, Ca, and Mg concentrations were selected for this study (Table 3.1). Fresh layer manure was oven-dried at 65°C, ground to pass a 2-mm sieve, and stored in sealed plastic containers at 4°C until used. Dairy slurries were kept refrigerated (4°C) until used. The liquid to solid ratio for the three samples of dairy slurry was determined by lyophilization, and ranged from 20:1 to 97:1. The total P, Ca, and Mg in layer manure and dairy slurry samples was measured by dry ash digestion (USEPA, 1995) followed by determination with an Inductively Coupled Plasma-Optical Emission Spectrograph (ICP-OES).

Total Dissolved P and Bioavailable P Analysis

A factorial combination of two extracting solutions (MES buffer at pH 6 or DI water), three extracting ratios (10:1, 100:1, 200:1; solution: dry manure), and three extraction times (1, 4, 24 h) was used to generate 18 treatments for extraction of TDP, DRP, Total Bioavailable P (TBAP), and Soluble Bioavailable P (SBAP) from layer manure. Extraction of the same fractions from dairy slurry was evaluated with 12 treatments generated by combining two extracting solutions (MES buffer at pH 6 or DI water), two extracting ratios (100:1 or 200:1), and three extraction times (1, 4, 24 h). An extraction ratio of 10:1 was not possible with dairy slurries because the original liquid: solid ratio was greater than 10:1. In all cases, the treatments were arranged in a completely randomized design with three replications.

For TDP and DRP extraction, the solution-manure/dairy slurry mixtures were shaken horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature (~23°C). Subsequently, the samples were centrifuged for 20 min at 1841 x g and filtered through a 0.45-
μm filter. Each filtered extract was analyzed for DRP by Murphy and Riley (1962) and for TDP by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES).

For BAP extraction, iron-oxide filter paper (Whatman no. 50; 5.5-cm diameter) was prepared as described by Myers et al. (1997). The amount of iron-oxide coating was determined by weighing filter paper before and after iron-oxide coating. The amount of iron-oxide coated on filter paper ranged from 20 to 40 mg. Phosphorus adsorption capacity, and the P/Fe ratio (in mole) of a single iron-oxide filter paper was evaluated by mixing newly made iron-oxide paper with solutions containing different amounts of P (0.6 to 9 mg P as \( \text{KH}_2\text{PO}_4 \)) in centrifuge tubes, and shaking horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature for 24 h. The amount of P adsorbed by the paper was calculated as the difference between the initial and final amount of P in solution. The amount of P left in solution after shaking was determined by Murphy and Riley (1962), with each treatment carried out in triplicate. The maximum amount of P adsorbed on iron-oxide filter paper was 3.05 mg P, and the P:Fe ratio (in mole) was averaged to be 1:5.

The number of iron-oxide filters needed to adsorb all BAP from each treatment was determined in a preliminary study with each of the extractants and ratios in which the manure suspensions were shaken continuously for 96 h with a fresh iron-oxide filter paper being replaced every 24 h. The P adsorbed by the iron-oxide paper was removed with 40 mL of 0.2 M \( \text{H}_2\text{SO}_4 \) (Myers et al., 1997) and analyzed by ICP-AES. The number of filter papers to be used for each extractant and ratio was the number of filter papers that were used before the P removed from the last added paper was below the detection limit of ICP-AES (<0.06 mg L\(^{-1}\) or 2.4 μg P in the paper). This preliminary study showed that the number of iron-oxide filter papers needed was
three for a ratio of 10:1 (1 g layer manure), and one for ratios of 100:1 (0.1 g layer manure/dairy slurry) and 200:1 (0.05 g layer manure/dairy slurry).

Total bioavailable P was extracted by shaking the layer manure and dairy slurry samples with iron-oxide paper at the same extraction times, solution to layer manure ratios, and pH values used for the TDP analysis. The P adsorbed by the iron-oxide filter paper was removed with 40 mL of 0.2 M H$_2$SO$_4$ (Myers et al., 1997) and the desorbed P concentration was determined by the molybdate-blue method of Murphy and Riley (1962).

Soluble bioavailable P was determined using a separate set of extractions (without the iron-oxide paper) at the same extraction conditions used for TDP. After the extraction, the supernatants were filtered through a 0.45-μm filter. The filtrates were then shaken with iron-oxide filter paper horizontally on an end-to-end shaker at 120 oscillations per minute for 24 h. The P adsorbed by the filter paper was desorbed as described above and measured by the method of Murphy and Riley (1962) (SBAP1) and by ICP-AES (SBAP2). The Murphy and Riley method determines inorganic P while the ICP-AES measures the inorganic and organic P in the testing solutions. Each treatment was carried out in triplicate.

**Statistical Analysis**

An analysis of variance for each extracted fraction was carried out with PROC GLM in SAS, V.9.0 (SAS Institute Inc., 2002). The statistical model used considered the main effects of manure sample, extractant, extraction ratio, and shaking time, as well as all two-way, three-way, and four-way interactions in a completely randomized design with three replications. Fisher’s protected Least Significant Difference (LSD) was used to separate means, and a 5% confidence level was used to conclude significant effects.
RESULTS AND DISCUSSION

Layer Manure

For layer manure, there was a significant four-way interaction on TBAP extraction, and there were many significant three-way interactions for the different fractions (Table 3.2). The three-way interaction of Sample x Ratio x Extractant was significant for TDP, DRP, and SBAP, whereas the interaction of Sample x Extractant x Time was significant for TDP and DRP only. These interactions with manure sample were probably caused by differences in manure characteristics (Table 3.1). Consequently, significant effects are discussed by manure sample.

Layer Manure #1

The concentrations of TDP, DRP and SBAP increased significantly as the extraction ratio increased from 10:1 to 100:1 with both MES and DI water extraction (Fig. 3.1a). This increase was probably caused by the 100:1 ratio promoting dissolution of sparingly soluble Ca and Mg phosphates. However, when the ratio was increased from 100:1 to 200:1, only TDP and SBAP extracted with MES increased significantly (Fig. 3.1a). This resulted in an increase in DUP (DUP = TDP - DRP) because DRP did not show a concurrent increase when the ratio was increased from 100:1 to 200:1. In summary, the maximum extraction of TDP, DRP, and SBAP from layer manure #1 was obtained with MES at a ratio of 200:1.

The concentrations of TDP and DRP did not change with any of the two extractants as extraction time increased from 1 to 24 h (Fig. 3.1b). This result is different from that of Kleinman et al. (2002b) who reported that layer manure TDP concentration was positively related to shaking time, although 1-h shaking extracted over 70% of TDP released with a 24-h extraction. The difference could be due to the nature of the samples used in this study, which
were dry and ground, whereas in Kleinman’s study the manure samples were wet. The agitation may have broken down wet manure aggregates over time, thereby increasing the release of TDP.

In contrast to TDP and DRP, TBAP extracted by either DI water or MES increased when extraction time increased from 1 to 24 h, but the increase was greater at 200:1 than at 10:1 (Fig. 3.1c). At all extraction ratios, TBAP was greater with MES than with DI water.

In summary, TDP, DRP, SBAP and TBAP concentrations were greater when layer manure #1 was extracted at a ratio of 200:1 with MES than when extracted with DI water. Also, the maximum extraction of TDP and DRP was obtained with 1-h extraction, whereas a 4-h extraction was required for maximum removal of TBAP.

**Layer Manure #2**

The concentrations of TDP, DRP, and SBAP tended to increase as extraction ratio changed from 10:1 to 100:1 and 200:1 (Fig. 3.2a). As in the case of layer manure #1, TDP and DRP did not respond to an increase in extraction time (Fig. 3.2b), and all values were larger for extraction with MES than for extraction with DI water.

With the exception of MES extraction at 10:1, TBAP increased as extraction time increased from 1 to 24 h, both with MES and DI water, and the increase was larger at larger ratios (Fig. 3.2c). In summary, a 1-h extraction of manure #2 with MES at a ratio of 200:1 resulted in maximum extraction of TDP and DRP, whereas a 4-h extraction with MES at the same ratio was required for TBAP.

**Layer Manure #3**

The extraction of TDP, DRP and SBAP from layer manure #3 behaved similarly to that from manure #1 in that increases were observed with MES and DI water as the ratio changed from 10:1 to 100:1, but only with MES as the ratio increased from 100:1 to 200:1 (Fig. 3.3a).
Also, as with manures #1 and #2, TDP, DRP and SBAP were greater with MES extraction than with DI water extraction (Fig. 3.3a). Maximum concentrations of all variables were obtained with MES at a 200:1 ratio.

As with manures #1 and #2, TDP and DRP did not respond to increases in extraction time (Fig 3.3b). Thus, extraction with MES at a ratio of 200:1 for 1 h was needed to obtain maximum removal of TDP and DRP fractions from manure #3.

The extraction of TBAP from layer manure #3 responded similarly to that from layer manure #1 in that increases were observed with MES and DI water as the extraction time increased from 1 to 24 h, and as the ratio changed from 10:1 to 200:1 (Fig. 3.3c).

In summary, extraction with MES at a ratio of 200:1 for 1 h was sufficient to extract maximum amounts of TDP and DRP from layer manure #3. In contrast to manures #1 and #2, a 24-h extraction was needed for maximum removal of TBAP at a ratio of 200:1.

**Layer Manure #4**

Manure #4 was different from the others in that the increase in DRP observed with MES when compared to DI water was small (Fig. 3.4a). This effect may have been due to the large concentration of Ca in the manure (Table 3.1), which could have resulted in the formation of calcium phosphates with low solubility (Kleiman et al. 2005). In contrast, TDP and SBAP responded to extraction ratio and extractant similarly to the other layer manures (Fig. 3.4a).

Similarly to the other layer manures, TDP and DRP did not respond to extraction time (Fig. 3.4b). So, a ratio of 200:1 and an extraction time of 1 h with MES extracted the maximum amounts of TDP and DRP from manure #4. The extraction of TBAP behaved similarly to that of manures #1 and #2 in that a 4-h extraction was sufficient for maximum removal (Fig. 3.4c).
All Layer Manures

For all layer manures there was a significant main effect of extraction time on SBAP extraction (Table 3.2). The average amounts of SBAP extracted were 3,329 mg P kg\(^{-1}\) for 1 h, 3,533 mg P kg\(^{-1}\) for 4 h, and 4,114 mg P kg\(^{-1}\) for 24 h.

Also, for all layer manures, there was a significant extraction ratio x extraction time interaction for TDP (Fig. 3.5). This interaction was caused by the fact that increasing extraction time tended to decrease TDP at a ratio of 10:1, but tended to increase TDP at ratios of 100:1 and 200:1.

In conclusion, maximum amounts of TDP and DRP from all layer manures were extracted with MES at a ratio of 200:1. No difference was found between 1-h and 24-h extraction for TDP and DRP. In contrast, an extraction time of 24 h achieved maximum removal of SBAP and TBAP with MES at 200:1. In some cases there were no differences in the amounts of SBAP and TBAP extracted with 4 and 24 h.

Dairy Slurry

For dairy slurry, there was a significant Sample x Extractant interaction for TDP, DRP, SBAP, and TBAP (Table 3.2). Extraction ratio and shaking time did not affect any of the studied variables. For all fractions, extractions with MES yielded greater values than extraction with DI water, but the magnitude of the differences varied with dairy slurry (Figs. 3.6, 3.7, and 3.8).

Extraction with MES Buffer at pH 6

Average values for extraction of TDP, DRP, SBAP, and TBAP with MES buffer (pH 6) at a ratio of 200:1 for 24 h are presented in Table 3.3. These results show that TBAP values were larger than TDP values. TBAP as percentage of total P ranged from 59 to 78%, whereas TDP ranged from 35 to 61% (Table 3.3). The TBAP recovered from dairy slurry was greater (dairy
slurry #2) or equal (dairy slurry #1 and #3) to TDP. This variability could be caused by the property of dairy slurry. Therefore, measuring TBAP with MES buffer in layer manure may provide a better estimate of the potential for release of BAP than simply measuring TDP.

Soluble bioavailable P from layers manure measured by Murphy and Riley (SBAP1) and by ICP-AES (SBAP2) was in some cases smaller than the DRP value (Table 3.3), when they were expected to be larger or at least equal to DRP. Some of these unexpected results may have been due to competition by water-soluble, negatively charged organic compounds (released from layer manure) for adsorption sites on the iron-oxide paper. Evidence for this was provided by the fact that SBAP values determined by ICP-AES (SBAP2) were greater than SBAP values determined by Murphy and Riley (1962) (SBAP2-SBAP1, Table 3.3). These results suggest that organic compounds containing P had been sorbed onto the iron-oxide papers, possibly competing with inorganic P for adsorption sites. This effect is very noticeable for layer manures #1, #2 and #3, in which the amount of organic P (SBAP2-SBAP1 in Table 3.3) removed by the iron-oxide paper is comparable to the amount of DRP that was not adsorbed by the paper (DRP-SBAP1, Table 3.3).

**CONCLUSIONS**

Although for some layer manures, an extraction with MES at a 100:1 ratio for 1 h was sufficient to extract the maximum amounts of TDP and DRP, for all layer manures an extraction ratio of 200:1 for 1 h removed the maximum amounts of TDP and DRP. Maximum amounts of SBAP and TBAP were extracted from all layer manures with MES at a ratio of 200:1 for 24 h. For dairy slurries, an extraction with MES at a ratio of 100:1 for 1 h was sufficient to remove the maximum amounts of TDP, DRP, SBAP, and TBAP. Additional research should be conducted to determine if the amounts of TDP, DRP, SBAP, and TBAP extracted with MES under the
conditions described above are well correlated with the amounts of these variables found in surface runoff from fields receiving applications of layer manure or dairy slurry.
REFERENCES


Table 3.1. Selected properties of four layer manures and three dairy slurry samples used in the study.

<table>
<thead>
<tr>
<th>Manure Type</th>
<th>Sample</th>
<th>pH†</th>
<th>Total P ---mg kg⁻¹---</th>
<th>Total C</th>
<th>Total N</th>
<th>Total Ca</th>
<th>Total Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer</td>
<td>#1</td>
<td>9.6</td>
<td>21,232</td>
<td>248,500</td>
<td>29,057</td>
<td>163,107</td>
<td>6,281</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>9.4</td>
<td>27,320</td>
<td>235,000</td>
<td>26,737</td>
<td>151,187</td>
<td>8,544</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>8.7</td>
<td>24,659</td>
<td>264,667</td>
<td>31,140</td>
<td>150,733</td>
<td>6,916</td>
</tr>
<tr>
<td></td>
<td>#4</td>
<td>8.1</td>
<td>23,980</td>
<td>246,433</td>
<td>29,990</td>
<td>147,300</td>
<td>9,468</td>
</tr>
<tr>
<td>Dairy slurry</td>
<td>#1</td>
<td>7.4</td>
<td>13,267</td>
<td>334,867</td>
<td>36,570</td>
<td>29,434</td>
<td>11,384</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>7.5</td>
<td>5,615</td>
<td>425,567</td>
<td>29,173</td>
<td>15,812</td>
<td>4,277</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>7.8</td>
<td>13,425</td>
<td>339,267</td>
<td>33,737</td>
<td>27,670</td>
<td>10,890</td>
</tr>
</tbody>
</table>

†pH was measured at ratios of 10:1 (water:manure) for layer manure, and 100:1 (water:dry manure) for dairy slurry.

Table 3.2. Analysis of variance for Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from all layer manure and dairy slurry samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Layer manure</th>
<th>Dairy slurry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Ratio</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sample x Ratio</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Extractant</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sample x Extractant</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Ratio x Extractant</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sample x Ratio x Extractant</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Time</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sample x Time</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Ratio x Time</td>
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<tr>
<td>Sample x Ratio x Time</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Extractant x Time</td>
<td>**</td>
<td>*</td>
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<tr>
<td>Sample x Extractant x Time</td>
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<td>*</td>
</tr>
<tr>
<td>Ratio x Extractant x Time</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sample x ratio x Extractant x Time</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* statistical significance at the 0.05 probability level, ** statistical significance at the 0.01 probability level NS: non significant
SBAP1: SBAP determined by ascorbic acid method, Murphy and Riley (1962)
Table 3.3. Total Dissolved P (TDP), Dissolved Reactive P (DRP), Total Bioavailable P (TBAP), Soluble Bioavailable P1 (SBAP1), and Soluble Bioavailable P2 (SBAP2) extracted from four layer manures and three dairy slurries at a ratio of 200:1 with MES buffer at pH 6 for 24 h

<table>
<thead>
<tr>
<th>Manure type</th>
<th>Sample</th>
<th>TDP</th>
<th>DRP</th>
<th>TBAP</th>
<th>SBAP1</th>
<th>SBAP2</th>
<th>DRP-SBAP1</th>
<th>SBAP2-SBAP1</th>
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</thead>
<tbody>
<tr>
<td>Layer Manure</td>
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<td>10,129</td>
<td>8,067</td>
<td>14,476</td>
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<td>8,691</td>
<td>1,315</td>
<td>1,939</td>
</tr>
<tr>
<td></td>
<td>#2</td>
<td>9,612</td>
<td>9,011</td>
<td>16,000</td>
<td>6,320</td>
<td>8,544</td>
<td>2,691</td>
<td>2,224</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>15,040</td>
<td>12,100</td>
<td>17,848</td>
<td>9,672</td>
<td>11,939</td>
<td>2,428</td>
<td>2,267</td>
</tr>
<tr>
<td></td>
<td>#4</td>
<td>8,856</td>
<td>3,571</td>
<td>18,640</td>
<td>5,552</td>
<td>7,975</td>
<td>-1,801</td>
<td>2,423</td>
</tr>
<tr>
<td>Dairy Slurry</td>
<td>#1</td>
<td>6,599</td>
<td>5,368</td>
<td>6,307</td>
<td>5,972</td>
<td>6,590</td>
<td>-604</td>
<td>618</td>
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<tr>
<td></td>
<td>#2</td>
<td>2,727</td>
<td>2,603</td>
<td>5,117</td>
<td>4,859</td>
<td>5,240</td>
<td>-2,256</td>
<td>381</td>
</tr>
<tr>
<td></td>
<td>#3</td>
<td>7,359</td>
<td>2,039</td>
<td>7,340</td>
<td>3,229</td>
<td>3,789</td>
<td>-1,190</td>
<td>560</td>
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</tbody>
</table>

SBAP1: SBAP determined by ascorbic acid method, Murphy and Riley (1962)
SBAP2: SBAP determined by ICP-AES method
Fig. 3.1. The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #1.
Fig. 3.2. The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #2.
Fig. 3.3. The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #3.
Fig. 3.4. The effects of (a) extractant and extraction ratio on Total Dissolved P (TDP), Dissolved Reactive P (DRP), and Soluble Bioavailable P1 (SBAP1), (b) the effects of extractant and extraction time on TDP, and DRP, and (c) the effects of extraction ratio, extractant and extraction time on Total Bioavailable P (TBAP) extracted from layer manure sample #4.
Fig. 3.5. The effects of extraction ratio and extraction time on average amounts of Total Dissolved P (TDP) extracted from all layer manures.

Fig. 3.6. The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #1.
Fig. 3.7. The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #2.

Fig. 3.8. The effects of extractant on Total Dissolved P (TDP), Dissolved Reactive P (DRP), Soluble Bioavailable P1 (SBAP1), and Total Bioavailable P (TBAP) extracted from dairy slurry sample #3.
CHAPTER 4

FRACTIONATION AND CHARACTERIZATION OF PHOSPHORUS IN BROILER LITTER AND LAYER MANURE

To be submitted to Journal of Environmental Quality
ABSTRACT

Fractionation and characterization of manure phosphorus (P) may be useful in understanding its reactions in soil. This study compared two fractionation schemes, one beginning with deionized water (DI water), and one leading with buffer at pH 6 (2-(N-morpholino) ethanesulfonic acid - MES) in one broiler litter and two layer manures. After the initial extraction, both schemes used sequential extraction of residues with NaHCO$_3$ and NaOH. The largest amount of labile P was extracted with MES at pH 6, and that the increased P extracted by MES, when compared to DI water, was mainly from residual P or NaHCO$_3$-P. The P species extracted by MES appeared to be highly associated with Ca and Mg cations. In contrast, NaOH-extractable P was in the form of Fe-, Al-bound P and Ca-, Mg-associated P, and was relatively stable in both DI water- and MES- fractionation schemes. To characterize the extracted P, P-31 Nuclear Magnetic Resonance (NMR) spectroscopy was used to qualify and identify manure P extracted with DI water, MES at pH 6, or NaOH + EDTA. While most of the P extracted by DI water and MES was in the form of inorganic P, significant amounts of phytate were extracted from broiler litter with DI water and from layer manure with MES. Although for all three manures, NaOH + EDTA extracted the largest amount of P. However, as the alkaline pH level provided by this extractant, it may not provide an accurate estimate of P species that may be extracted by rainfall or runoff in an acidic soil environment.
INTRODUCTION

Long-term animal manure application to agricultural soils may lead to soil P accumulation which has the potential to accelerate P transfer to water bodies through surface runoff. This process often contributes to water quality impairment. Edwards and Daniel (1993) found that P in runoff from pastures receiving poultry litter was mainly in dissolved inorganic form, and was about 2000 times greater than the critical concentration associated with accelerated eutrophication (0.01 mg P L⁻¹). Many researchers also found a significant amount of organic P in soil leachate from manure-amended soils (Chardon et al., 1997; McDowell and Koopmans, 2006; Toor et al., 2003). This is because swine (*Sus scrofa domesticus*) and poultry (*Gallus gallus*) lack the phytase enzymes to break down inositol six-phosphate (IP6) present in feed grains, and as a result excrete most of this form of organic P (Harper et al., 1997; Zyla et al., 2000). For example, Gerritse and Vriesema (1984) found 40% of Total P (TP) in poultry manure was organic P, and Peperzak et al (1959) reported that 2 to 60% of total organic P in poultry manure was IP6. The retention of organic P in soils is determined by the nature of P compounds and animal wastes. Celi et al. (2000) found that IP6 was tightly bound by soils whereas nucleotides and glucose phosphate were more mobile. Iyamruemye et al. (1996) found that the organic ligands in applied manure lead to an increase of concentration of soluble P in runoff by complexing with Fe and Al in soils, which decreases precipitation of P with these metals, and by competing with P for sorption sites.

The fate of P from animal waste in the environment is, in part, determined by the P fractions present in the manure. The sequential fractionation method has been applied on manure P pool distribution analysis (Barnett, 1994; Dou et al., 2000; Leinweber et al., 1997; Peperzak et al., 1959), and is based on the assumption that chemical extractants selectively dissolve different
P species (He et al., 2003). The P fractions have been defined according to extractants, such as Resin- or H$_2$O-P, NaHCO$_3$-P, NaOH-P, and H$_2$SO$_4$-P or HCl-P. Resin-or H$_2$O-P, and NaHCO$_3$-extractable P are liable P fractions that are readily available for plant and algae; the NaOH-extractable P fraction is considered moderately labile P (Fe-, Al-associated P), and the P extracted by HCl is regarded as moderately stable P (Ca-associated P) (Williams et al., 1980; Zhang et al., 2004). It is believed that P species associated with these fractions decrease in vulnerability to environmental loss in an order of H$_2$O-, NaHCO$_3$-, NaOH-, and HCl-extracted P as well as residual P (Dou et al., 2003; He and Honeycutt, 2001; Sharpley and Moyer, 2000). However, P distribution varies with the nature of animal manure. For example, Sharpley and Moyer (2000), Leinweber et al. (1997), and Dou et al. (2000) fractionated hog manure, dairy manure and poultry manure P using sequential extraction reagents including deionized water (DI water), 0.5 M NaHCO$_3$, 0.1 M NaOH, and 0.1 M HCl; the percentages of the total P in broiler litter present in these fractions were 28, 35, 7, and 29% for Sharpley and Moyer (2000), and 49, 19, 5, and 25% for Dou et al. (2000), respectively. Leinweber et al. (1997) reported that 24, 27, 10, and 39% of the TP in hog manure was DI water-, NaHCO$_3$-, NaOH-, and acid-extractable P, respectively. They also concluded that the large percentage (>50% of TP) of P in the water and NaHCO$_3$ fractions demonstrated a weak binding energy of P, which increased the potential for runoff P. Most of the previously published research applied DI water as the first extractant to fractionate labile or plant available P pool in the sequential extraction (Dou et al., 2000; Leinweber et al., 1997; Sharpley and Moyer, 2000). However, it was reported that the pH of a suspension of manure in DI water is generally alkaline due to the presence of NH$_3$ and the phosphate of Ca, Na, and Mg (Griffiths, 1998). Also, Tasistro et al. (2004) found that the pH of surface-applied broiler litter may decrease from initially 8.1 to 6.7 within 30 days by contacting
with acidic soil. Acidification of manure could result in greater release of Total Dissolved P (TDP) by solubilization of orthophosphates (Gerritse and Vriesema, 1984), since most inorganic P in manure is present as Ca, Mg, and K salts (Cooperband and Good, 2002). Also, Stumm and Morgan (1981) and Champagne (1988) reported that the solubility of metal-phytate complexes increased in acidic environments. Thus, DI water-leading fractionation may under-estimate labile P from animal waste applied to acidic soils. A selected buffer 2-(N-morpholino) ethanesulfonic acid (MES) (Tasistro et al., 2007) instead may closely estimate the manure labile P when manure is in acidic soil environment.

Although techniques such as fractionation yield information on the relative proportions of inorganic and organic P, they cannot identify specific P forms. It was reported that some forms of organic P are more labile than others (Makarov et al., 2002), and hence information on specific P forms is necessary to help understand manure P transformation in the field. Commonly used chromatographic techniques, such as High Performance Liquid Chromatography (HPLC), are not able to provide detailed information on P speciation because of poor column retention and matrix interference with standards (Kemme et al., 1999; Turner et al., 2002). Solution $^{31}\text{P}$ Nuclear Magnetic Resonance (NMR) spectroscopy has become a widely accepted technique for identifying P species in environmental samples because it allows the simultaneous identification of multiple P compounds in the complex matrices with minimal sample handling (Condron et al., 1997), and because $^{31}\text{P}$ is the only naturally occurring P isotope (100% natural abundance) with a large gyromagnetic ratio. By monitoring the response of the electronic environment around various P nuclei to external magnetic field, qualitative and quantitative detection of a variety of P species by $^{31}\text{P}$ NMR can be achieved.
Phosphorus NMR spectroscopy has been successfully used in characterization of P in soil and animal wastes. Using $^{31}$P NMR, Hansen et al. (2004) investigated P extracted from soils amended with dairy manure by NaOH + EDTA, and found that the majority of organic P in soils is phytic acid. Leinweber et al. (1997) identified orthophosphate monoesters and diesters in NaOH extracts of swine slurry. Turner (2004) studied three different animal manure to optimize the $^{31}$P NMR technique in manure P speciation, and found organic P in manures was in phosphate monoesters and phosphate diester forms.

Many studies have shown that identifying an appropriate extractant for environmental samples is the key to obtaining a satisfactory $^{31}$P NMR spectra (Turner and Leytem, 2004; Turner et al., 2003c). However, the solubilized P species may vary with the extractant, and the nature of animal wastes. Currently, NaOH + EDTA has been widely used as an extractant for soil and manure when conducting $^{31}$P NMR analysis. Bowman and Moir (1993) evaluated basic EDTA as an extractant for P in soils that were low in organic C content and with a mineralogy dominated by crystalline minerals. After extracting 10 soils from different parts of the USA, they found that NaOH + EDTA extracted 32% more organic P than wet sequential extraction, and that 0.25 M NaOH + 0.05 M EDTA extracted the most organic P from 10 soils. Some studies have used stronger alkaline solutions (0.5 M NaOH) to extract soil samples for $^{31}$P NMR analysis (Newman and Tate, 1980; Robinson et al., 1998), although hydrolysis of P compounds could occur during extraction with strong base (Condron et al., 1990). Mixture of NaOH and EDTA has also been used for manure extraction. Leinweber et al (1997) applied various concentrations of NaOH to extract manure and soils, and found that $^{31}$P NMR spectra of 0.5 M NaOH extracts from pig manure, poultry manure, and some soils produced greater signal intensities for orthophosphate and monoester P than 0.1M NaOH extracts. Turner (2004) studied extractants for
dairy, swine manure, and poultry litter by analyzing P recovered from extracting manure with various concentrations of EDTA and NaOH. He found that swine manure extracts with 0.5 M NaOH + 0.05 M EDTA produced the best spectra resolution.

Although NaOH + EDTA extraction provides a simple process for P analysis in soil and animal manure samples, this alkaline extractant may not provide an accurate estimate of P species that may be extracted by rainfall or runoff in acidic soil environment. For example, Tasistro et al. (2004) found that extracting manure organic P with original manure pH (~pH 8) may underestimate TDP, because the pH of broiler litter applied on the surface of a pasture was initially 8.1 and decreased to 6.7 in 30 days. As a result, the Dissolved Un-reactive P (DUP) measured in soils and thatch was twice as much as that measured at the original manure pH. Their laboratory study also found that acidification of litter suspensions to pH = 6 increased TDP by 34 to 72% and Dissolved Reactive P (DRP) by 24 to 69%. Similarly, extraction of manure with a solution buffered to the pH of the soil may more closely resemble the P species that may be soluble during rainfall and runoff.

Considering the potential contribution of manure P to the P fertility of soils (Oehl et al., 2001), and the potential for accelerating eutrophication due to the transfer of organic P to surface waters (Haygarth and Jarvis, 1999), it is critical to understand the P speciation and composition in animal manure at various pH levels. The objectives of this study were to: 1) to evaluate P pools in manure by two sequential fractionations, one leading with DI water, one leading with MES; 2) to characterize manure organic P species extracted by DI water, MES, and by NaOH + EDTA, and to compare P extracted by DI water, MES, and by widely used extractant NaOH + EDTA using $^{31}$P NMR spectroscopy.
MATERIALS AND METHODS

Broiler Litter and Layer Manure

One fresh broiler litter sample and two layer manure samples were collected from three different poultry farms in Georgia. The samples were dried at 65°C, ground, sieved through a 2-mm screen, and stored at 4°C until used. The TP, Ca, Mg, Fe, and Al (Table 4.1) in broiler litter and layer manure was measured by dry ash digestion (USEPA, 1995) followed by determination with an Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Total C and N were determined by dry combustion (University of Wisconsin Cooperative Extension Publishing, 2003).

Sequential Fractionation

Dried and ground samples were extracted with two sequential fractionation schemes. One scheme used DI water as the first extractant, followed by 0.5 M NaHCO$_3$ (pH = 8.5), and 0.1 M NaOH (pH = 12.5) according to procedures described by Sui et al. (1999). The other scheme started with 0.1 M MES (pH = 6), and was followed by 0.5 M NaHCO$_3$ (pH = 8.5), and 0.1 M NaOH (pH = 12.5). In each fractionation scheme, the solution:manure mixtures (200:1) were shaken horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature ($\approx$23°C) for 24 h. A preliminary study showed that a ratio of 100:1 or 200:1 extracted the maximum amount of soluble P from broiler litter and layer manure. After shaking, the mixture was centrifuged for 20 min at 1841 x g, and was filtered through a 0.45-μm filter. The filtrate was analyzed for TP by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). The residue from MES or DI water extraction was sequentially extracted by the following extractants with the same procedure described above. The residual P was calculated by the difference between TP and extracted P. This residual P included HCl extracted P (Ca-P) defined
by Sui et al. (1999). Because our preliminary work found MES also extracted a large amount of Ca-related P, HCl related P can not be strictly defined as Ca-related P since the chemical composition of poultry manure is different from that of soils. Each fractionation was carried out in three replicates.

**Extraction of Broiler Litter and Layer Manure for Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy Analysis**

Each 10-g of dried animal waste sample was extracted with 1 L of deionized water (original manure pH ~8), 0.5 M NaOH + 0.05 M Na\textsubscript{4}EDTA (pH = 12.5), or 0.1 M MES (pH = 6) with a solution:dry manure ratio of 100:1. The solution–manure mixtures were shaken horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature (\(\approx23^\circ\text{C}\)). The extracts were centrifuged for 20 min at 1841 x \(g\) and were sequentially filtered through a 0.45-\(\mu\)m filter. To get better NMR signals, the filtrate (\(\approx800\) ml) was concentrated by freeze-drier.

Each 1-g of the same sample was mixed with 100 mL of DI water, 0.5 M NaOH + 0.05 M Na\textsubscript{4}EDTA (pH = 12.5), or 0.1 M MES (pH = 6) for TDP and DRP analysis. The solution–manure mixtures were shaken horizontally on an end-to-end shaker at 120 oscillations per minute at room temperature (\(\approx23^\circ\text{C}\)), centrifuged, and filtered as described above. A 10-mL aliquot of the filtrate was analyzed for DRP by Murphy and Riley (1962) and for TP by ICP-AES. Every treatment was replicated three times.

**Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy**

The freeze-dried extracts were weighed and ground. Each 1-g of ground sample was re-dissolved into 1 M NaOH solution (with the pH of the re-dissolved samples adjusted to 12.5–13.5), and 0.2 mL of deuterium oxide (D\textsubscript{2}O) was added. The sample stood for 2 h with occasional vortex mixing, filtered through a 0.45-\(\mu\)m filter before being transferred to NMR
tubes, and stored at 4°C before analysis within 24 h. Solution $^{31}\text{P}$ NMR spectra were acquired on Varian Unity INOVA 500 MHz spectrometer (Varian NMR Systems, CA, USA) using a 45° pulse, 1.6-s acquisitions, and 5-s relaxation. Acquisition temperature was 25°C for each sample. The number of scans required to give an acceptable signal-to-noise ratio varied with TP concentration in the sample. The NMR experiment for each broiler litter extract lasted about 9 h, with 5000 scans, and the NMR experiments for each layer manure extract lasted 29 min with 256 scans.

Chemical shifts of signals from manure extracts were determined in parts per million (ppm) relative to 85% H$_3$PO$_4$ and assigned to individual P species or functional groups based on published results by Cade-Menun (2005) and Turner (2003a). The spectra were processed with MestRe-C software (Gómez and López, 2004).

Signal areas were calculated by integration, and the concentration of individual P species such as orthophosphate was calculated by multiplying the proportion of a spectral area assigned to a specific signal by the TDP concentration (mg P kg$^{-1}$ dry manure) measured by ICP-AES in original manure extract (Turner, 2004). The concentration of phytic acid was determined by summing the areas of the four signals at approximately 5.9, 5.0, 4.6 and 4.5 ppm with a signal ratio of 1:2:2:1, and multiplying the proportion of the summed areas by TDP measured by ICP-AES in original manure extract (Turner, 2004). The concentration of DRP in each sample was determined by Murphy and Riley (1962).

**Statistical Analysis**

An analysis of variance for each extracted fraction in sequential extraction study was carried out with PROC GLM in SAS, V.9.0 (SAS Institute Inc., 2002). Fisher’s protected Least
Significant Difference (LSD) was used to separate means, and a 5% confidence level was used to conclude significant effects.

RESULTS AND DISCUSSION

**Manure Phosphorus Fractionation**

**Phosphorus Distribution in Sequentially Extracted Fractions**

Among the three animal wastes, TP varied from 18,117 to 21,232 mg kg\(^{-1}\) dry manure (Table 4.1). The concentration of Ca, the major element related to phosphate solubility in animal manure, was 29,140 mg kg\(^{-1}\) dry litter in broiler litter, and about 163,000 mg kg\(^{-1}\) dry manure in layer manures. The large Ca concentration in layer manure is caused by layer diets containing a substantial amount of Ca to promote egg production. This is consistent with the observation by Kleinman et al. (2005), who reported that layer manure had a greater amount of Ca- and Mg-related phosphate than broiler litter.

The distribution of TDP in each fraction was compared between DI water- and MES-leading fractionation (Fig. 4.1). A large portion of TP was MES/DI water and NaHCO\(_3\) associated P (35 to 55% of TP). The amount of TDP extracted by MES (37 to 50.1% of TP) was always larger (P<0.05) than that extracted by DI water (16.7 to 30.2% of TP). The NaOH extracted P ranged from 3.2 to 12.6% of TP (686 to 2,279 mg kg\(^{-1}\) dry animal waste). However, TDP in the NaOH fraction was not significantly different between MES- and DI water-leading fractionation from each animal waste. Phosphorus left in the residual P faction was between 40 and 59% of TP depending on manure type.

For broiler litter, TDP in MES fraction was greater than TDP in DI water fraction probably because MES solubilized a larger amount of residual P than DI water. Residual P in MES-leading fractionation (45% of TP) was less than that in DI water-leading fractionation
(62.8% of TP), while no changes were found in the NaHCO₃, and NaOH fractions (Fig. 4.1). However, the increase of TDP in the MES fraction from layer manure #1 was mainly from the NaHCO₃ fraction (physically absorbed P) because only the NaHCO₃ extracted TDP was found lower in MES-leading fractionation than that in water-leading fractionation (Fig. 4.1). Thus, acidification with MES only caused a transfer of TDP between MES and NaHCO₃ fractions, which are categorized as labile P (Dou et al., 2000; Leinweber et al., 1997). These results imply that NaOH extractable P and residual P from layer manure #1 were relatively stable even in an acidic environment, because most of the NaOH extractable P was Fe-, and Al- phytate, which is soluble at pH >8 (Jackson and Black, 1951). In contrast, the increase in TDP in the MES fraction compared to the DI water fraction in layer manure #2 was from both NaHCO₃-P and residual P, because P in these fractions was less in MES-leading fractionation than that in DI water-leading fractionation.

In summary, the studied samples contained a large proportion of labile P (35 to 55% of TP). MES-leading fractionation always recovered a larger amount of labile P than DI water-leading fractionation. The greater amount of labile P extracted from MES-leading fractionation was either from residual P or NaHCO₃-P, whereas NaOH-P was stable in both fractionation schemes.

**Elemental Distribution in Sequentially Extracted Fractions**

To further explore the phosphate speciation in these three animal wastes, the concentrations of four major metal ions, Ca, Mg, Fe and Al, in DI water- and MES- leading sequentially extracted fractions were determined by ICP-AES (Table 4.2 and 4.3). The molar concentrations of Ca and Mg are much greater than those of Fe and Al in DI water (Table 4.2), MES (Table 4.3), and NaHCO₃ (Table 4.2 and 4.3) fractions of the three samples. Thus, it is
likely that labile P (DI water/MES-, and NaHCO$_3$-associated P) is mainly Ca- and Mg-related P, and that Al and Fe played only limited roles in the P extractability of these fractions. These P fractions likely include both Ca-, and Mg- related phosphate and phytate because it was reported that Ca-, and Mg-phosphate are soluble at low pH levels (Lindsay et al., 1989), Ca- phosphate below pH 6, and Mg-phosphate below pH 9.7 (Jackson and Black, 1951). In the NaOH fraction from both DI water- and MES-leading fractionation, the ratio of Ca + Mg to P was smaller than or similar to that of Fe + Al to P (Table 2 and 3), and the molar concentration of Fe + Al is greater than that of Ca + Mg. It indicates that TDP in the NaOH fraction includes a large amount of Fe-, Al-associated P. These P species are likely Fe phytate or Al phytate because, Jackson and Black (1951) found that Fe phytate was only soluble at pH>8, and Al phytate at pH>9, with their solubility increasing as pH increased. This agrees with results published by He et al. (2003), who found that large amounts of Al, with some Fe and Ca was extracted in 0.1 M NaOH solutions by a sequential fractionation of swine manure. They concluded that NaOH-extractable P could not be strictly assigned to Al- or Fe-bound P.

In summary, the results showed that the largest amount of labile P was extracted with MES at pH 6, and that the increased P extracted by MES, when compared to DI water, was mainly from residual P or NaHCO$_3$-P. These P species extracted by MES appeared to be highly associated with Ca and Mg cations. NaOH extractable P was in the form of Fe-, Al-associated P, and it was relatively stable in DI water- and MES-fractionation systems.
Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Assignment of Phosphorus Species

Broiler Litter

Figure 4.2a, b, and c display the spectra of three broiler litter extracts by DI water, 0.1 M MES and 0.5 M NaOH + 0.05M Na₄EDTA. Signals with similar chemical shifts patterns were detected in the NMR spectrum of the three extracts. The strongest signal appearing at 6.4 and 6.5 was assigned to inorganic orthophosphate. This is similar to the region (6.17 to 6.28 ppm) assigned to orthophosphate by Turner (2004), and to the region (6.54 to 6.63 ppm) assigned to orthophosphate by McDowell and Stewart (2005). Similar to previously published observations, the phosphate monoester occurs between 4 and 6 ppm (Fig. 4.2a, b and c). Many individual signals were detected in the orthophosphate monoester region. However, only the spectrum of NaOH + EDTA extract showed well-resolved signals for myo-inositol hexakisphosphate (phytic acid) occurring at 5.86, 4.86, 4.58, and 4.45 ppm in the ratio of 1:2:2:1. This is similar to observations by Turner et al. (2003a) who reported signals appearing at 5.85, 4.92, 4.55, and 4.43 ppm as phytic acid in spectrum of NaOH + EDTA extract from soils. The weak signals between 1.33 and 1.97 ppm were assigned to phospholipids, similar to that assigned to phosphatidyl ethanolamine (1.8 ppm) by Turner (2004). A small signal appearing at -0.36 ppm in spectrum of MES extract was assigned to deoxyribonucleic acid (DNA) (-0.3 ppm by Turner 2004).

Layer Manure #1

The ³¹P NMR spectra for layer manure #1 extracted by DI water, MES, and NaOH + EDTA are given in Fig. 4.3a, b, and c. Among all three spectra, the strong orthophosphate signals were between 6.29 and 6.52 ppm. The orthophosphate monoesters were assigned to peaks between 4 and 6 ppm. In all three spectra, the phytic acid peaks were well-resolved, appearing at
5.9, 4.9, 4.6, and 4.5 ppm. The chemical shifts of the phytic acid signals were slightly upfield than those published by Turner et al. (2003b), due to the differences in ionic strength in our extracts compared with those of Turner et al (2003b). Our manure extracts contained greater concentrations of Ca, Mg, but smaller concentration of paramagnetic ions Fe and Mn compared with soil extracts studied by Turner et al (2003b). In the spectra of water and NaOH + EDTA extract, weak signals of a few of other organic phosphate compounds were also detected. For instance, a small signal of phospholipids was detected at chemical shift 1.62 ppm in the spectrum of water extract (Fig. 4.3a), and a specific inorganic pyrophosphate peak was found at -3.4 ppm (Koopmans et al., 2007) in the spectrum of NaOH + EDTA extract (Fig. 4.3c). No other organic P species were detected from the spectrum of MES extract (Fig. 4.3b).

**Layer Manure #2**

The spectra of layer manure #2 for three extractants indicated similar signal patterns to those found from layer manure #1. A strong signal detected on all three spectra between 6.4 and 6.5 ppm was assigned to orthophosphate (Fig. 4.4a, b, and c). The phosphate monoester region was located between 4 and 6 ppm. Phytic acid, which appeared at approximately 5.9, 5.0, 4.6 and 4.5 ppm, was detected in this region for all three extracts. Similarly to layer manure #1, in the spectrum of water extract, a number of other individual signals were also detected within this region (Fig. 4.3a, 4.4a), which were not found in the spectra of MES or NaOH + EDTA extracts (Fig. 4.3b, c, 4.4b,c). Weak signals of phosphatidyl ethanolamine (1.70 ppm), and polyphosphate (-3.9 ppm) (Turner, 2004; Turner et al., 2003b) were detected in the spectrum of water extract (Fig. 4.4a). A weak peak appearing at -3.64 ppm in the spectrum of NaOH + EDTA extract was assigned to polyphosphate (McDowell and Stewart, 2005).
Difference of Phosphorus Composition

The recovered P species identified by NMR are listed in Table 4.4. The concentrations of TDP, DRP and related elements in the original extract of three samples are shown in Table 4.5. The concentrations of identified P species calculated by $^{31}$P NMR peak integration are displayed in Table 4.6. To evaluate the accuracy of peak integration on quantifying organic P species by NMR, the concentrations of DRP determined by Murphy and Riley (1962) and by NMR peak integration were compared (Fig. 4.5). In DI water and MES fraction, the concentration of orthophosphate from three samples determined by $^{31}$P NMR spectroscopy well matched those determined by colorimetric method. However, the concentration of orthophosphate in NaOH + EDTA fraction from three samples determined by $^{31}$P NMR spectroscopy was lower than those determined by colorimetric method. This is also opposite to that reported by Turner et al. (2003a), who found the concentration of orthophosphate determined by $^{31}$P NMR in soil NaOH + EDTA fraction was greater than that measured with colorimetric method. Our results may have been caused by acid-labile organic P and polyphosphate species (Table 4.4) recovered in NaOH + EDTA being degraded by the strong acid used to neutralize the NaOH + EDTA extract before colorimetric analysis, and by the strong acid used in the reagent of the colorimetric method (Turner et al., 2003a).

The efficiency of three extractants on manure TDP extraction measured in terms of percentage of TP was in the order of NaOH + EDTA > MES > DI water (Table 4.5). Similarly to published results by Turner (2004) in which >90% of TP was recovered by NaOH + EDTA from broiler littler, cattle manure, and swine manure, we found that larger than 78% of TP was recovered by NaOH + EDTA. At the same time, MES extracted between 32 and 49% of TP, whereas DI water extracted from 17 to 37% of TP from the three samples. To further explore
differences in recovered P species from animal wastes, the P compositions of the different manures was compared by extractant.

**DI water Extraction**

The concentration of TDP detected in water extracts from broiler litter (37% of TP) was significantly greater than that from layer manures (17-21% of TP). Orthophosphate, phytic acid, and phospholipids were detected in DI water extracts of all three samples, and a trace amount of polyphosphate was observed in layer manure #2 only. Orthophosphate dominated in DI water fraction (75 to 84% of TDP was found as DRP), and phytic acid was the major organic P in water extracts of all samples. Broiler litter contained a larger amount of orthophosphate and phytic acid than the two layer manures.

**MES Extraction**

As in the DI water extract, orthophosphate and phytic acid were all found in the MES extract in the three manures (Table 4.4). However, phospholipids and DNA were only detected in broiler litter. No polyphosphate was found in the MES extracts for all three manure samples. Compared to DI water extraction, MES extraction recovered a larger amount of TDP from all three samples (Table 4.5). Interestingly, an increase of orthophosphate along with a decrease of organic P was observed in the MES fraction of broiler litter (Table 4.6). This is likely because acidification promoted solubilization of precipitates of Ca- and Mg-related P, and hydrolysis of organic phosphate.

The major organic P detected in layer manure extractions was phytic acid. The concentrations of both orthophosphate and phytic acid in MES extraction from the two layer manures were greater than those in the DI water fraction (Table 4.6), implying that acidification promoted solubility of these P compounds in layer manures. The observed phospholipids group
in the DI water fraction was not detected in the MES fraction for the two layer manures. These phosphate diesters are more labile than phytic acid and inorganic P species such as polyphosphate in soils (Condron et al., 1990), and can be easily converted into stable phosphate monoesters (Hinedi et al., 1988). Thus, the disappearance of phosphate diester in the spectrum of layer manure extracts by MES may result from the rapid degradation of these phosphate diesters. Besides, the concentrations of Ca and Mg are much greater than those of P, Fe and Al, which indicates the solubilized P compounds in layer manures by MES are mainly Ca- and Mg-associated.

**NaOH + EDTA Extraction**

Similar to MES extraction, P compounds identified in the NaOH + EDTA extracts included both orthophosphate and phytic acid in the three samples. Phospholipids were only detected in broiler litter extraction, and polyphosphate was found in the extraction of both layer manures. No DNA was observed in any sample.

NaOH + EDTA removed the highest amount of TDP among the three extractants. Similarly, the concentrations of orthophosphate (42.6-56.5% of TP) and phytic acid (18.9-32.4% of TP) detected in NaOH + EDTA extractions were greater than those found in the other two extractants (Table 4.6). The larger P extraction by NaOH + EDTA is likely due to the chelation between EDTA and Ca, and some Fe and Al (Cade-Menun and Preston, 1996; Turner, 2004), and hence the increased P recovery. Similarly to MES extraction, the increased P recovery from all three animal wastes seemed mainly from P associated with Ca and Mg cations, because the concentrations of Ca and Mg are much greater than the concentrations of Fe and Al (Table 4.5).

In summary, the major P species in the three animal wastes studied was orthophosphate (52-91% of TP) and its importance varied with manure and extractant. The major organic P
compound in the three samples was phytic acid, with its concentration ranging between 88 and 6,875 mg P kg$^{-1}$ dry animal waste.

**CONCLUSIONS**

The amount of P recovered from animal wastes varied with extractant and the nature of animal wastes. Results from the fractionation study showed that the major P fraction was MES-extractable P (pH 6), and that P species extracted by MES was highly associated with Ca and Mg cations. NaOH extractable P was in mainly Fe-, Al- associated P, and was relatively stable in both DI water- and MES- fractionation schemes. Results from $^{31}$P NMR analysis confirmed that most of the P extracted by DI water and MES was in the form of inorganic P, and that significant amounts of phytate were extracted from broiler litter with DI water and from layer manure with MES.
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# TABLES

Table 4.1. Selected characteristics of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2).

<table>
<thead>
<tr>
<th>Manure Type</th>
<th>Total P</th>
<th>Total C</th>
<th>Total N</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Al</th>
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</tr>
<tr>
<td>BL</td>
<td>19,667</td>
<td>333,033</td>
<td>36,787</td>
<td>29,140</td>
<td>6,379</td>
<td>1,647</td>
<td>3,028</td>
</tr>
<tr>
<td>LM #1</td>
<td>21,232</td>
<td>248,500</td>
<td>29,057</td>
<td>163,107</td>
<td>6,281</td>
<td>1,275</td>
<td>954</td>
</tr>
<tr>
<td>LM #2</td>
<td>18,117</td>
<td>246,433</td>
<td>29,990</td>
<td>163,480</td>
<td>7,225</td>
<td>1,098</td>
<td>880</td>
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</tbody>
</table>

Table 4.2. Molar composition in sequentially-extracted fractions (starting with DI water) of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2).

<table>
<thead>
<tr>
<th>Total dissolved P</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Al</th>
<th>(Ca+Mg):P</th>
<th>(Fe+Al):P</th>
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<tr>
<td>DI water fraction</td>
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</tr>
<tr>
<td>BL</td>
<td>256.9±59.1†</td>
<td>7.4±3.0</td>
<td>63.7±14.7</td>
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<tr>
<td>LM #1</td>
<td>191.5±40.4</td>
<td>54.5±22.4</td>
<td>95.6±27.8</td>
<td>1.0±0.3</td>
<td>&lt;DL‡</td>
<td>0.8</td>
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<tr>
<td>LM #2</td>
<td>97.6±16.4</td>
<td>90.2±16.4</td>
<td>72.2±13.4</td>
<td>0.4</td>
<td>&lt;DL</td>
<td>1.7</td>
</tr>
<tr>
<td>0.5 M NaHCO₃ fraction</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>157.6±6.0</td>
<td>39.3±2.2</td>
<td>144.6±15.6</td>
<td>1.0±0.1</td>
<td>&lt;DL</td>
<td>1.2</td>
</tr>
<tr>
<td>LM #1</td>
<td>32.7±10.5</td>
<td>62.3±6.7</td>
<td>43.8±9.8</td>
<td>0.7±0.2</td>
<td>&lt;DL</td>
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<tr>
<td>LM #2</td>
<td>121.8±11.6</td>
<td>76.7±13.1</td>
<td>36.6±4.3</td>
<td>1.4±0.1</td>
<td>&lt;DL</td>
<td>0.9</td>
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<tr>
<td>0.1 M NaOH fraction</td>
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<tr>
<td>BL</td>
<td>6.4±0.7</td>
<td>2.3±0.4</td>
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<td>1.1±0.3</td>
<td>11.4±2.0</td>
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<tr>
<td>LM #1</td>
<td>35.1±10.0</td>
<td>10.5±3.5</td>
<td>1.8±1.0</td>
<td>0.9±0.4</td>
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<tr>
<td>LM #2</td>
<td>64.2±1.0</td>
<td>5.6±2.2</td>
<td>0.3±0.1</td>
<td>0.7±0.1</td>
<td>9.6±3.8</td>
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</table>

† Mean ± standard deviation. ‡ Lower than detection limit
Table 4.3. Molar composition in sequentially-extracted fractions (starting with 2-(N-morpholino)ethanesulfonic acid - MES) of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2).

<table>
<thead>
<tr>
<th></th>
<th>Total dissolved P</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Al</th>
<th>(Ca+Mg):P</th>
<th>(Fe+Al):P</th>
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<tr>
<td><strong>MES fraction</strong></td>
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<tr>
<td>BL</td>
<td>274.3±21.8†</td>
<td>444.1±28.9</td>
<td>196.5±15.3</td>
<td>0.3±0.0</td>
<td>&lt;DL‡</td>
<td>2.3</td>
<td>&lt;0.01</td>
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<tr>
<td>LM #1</td>
<td>317.7±82.5</td>
<td>186.9±52.7</td>
<td>162.9±43.4</td>
<td>0.8±0.2</td>
<td>0.2</td>
<td>1.1</td>
<td>&lt;0.01</td>
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<td>LM #2</td>
<td>216.5±11.5</td>
<td>536.9±41.6</td>
<td>117.3±8.3</td>
<td>0.3±0.0</td>
<td>&lt;DL‡</td>
<td>3.0</td>
<td>&lt;0.01</td>
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<td><strong>0.5 M NaHCO₃ fraction</strong></td>
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<tr>
<td>BL</td>
<td>134.7±38.2</td>
<td>39.4±9.3</td>
<td>19.7±6.1</td>
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<tr>
<td>LM #1</td>
<td>9.6±0.7</td>
<td>59.8±4.8</td>
<td>16.2±3.1</td>
<td>0.5±0.1</td>
<td>0.2</td>
<td>7.9</td>
<td>0.05</td>
</tr>
<tr>
<td>LM #2</td>
<td>58.2±7.5</td>
<td>68.0±13.8</td>
<td>13.2±2.1</td>
<td>1.8±0.3</td>
<td>1.7</td>
<td>1.4</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>0.1 M NaOH fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>10.6±4.9</td>
<td>3.5±1.6</td>
<td>0.7±0.4</td>
<td>1.8±0.8</td>
<td>21.8±9.2</td>
<td>0.4</td>
<td>2.23</td>
</tr>
<tr>
<td>LM #1</td>
<td>21.8±2.3</td>
<td>8.9±0.4</td>
<td>0.5±0.1</td>
<td>0.6±0.1</td>
<td>8.2±0.8</td>
<td>0.4</td>
<td>0.40</td>
</tr>
<tr>
<td>LM #2</td>
<td>73.5±4.6</td>
<td>11.9±0.7</td>
<td>0.2±0.0</td>
<td>1.7±0.1</td>
<td>16.4±0.7</td>
<td>0.2</td>
<td>0.25</td>
</tr>
</tbody>
</table>

† Mean ± standard deviation. ‡ DL: detection limit

Table 4.4. Extracted P species detected by P-31 Nuclear Magnetic Resonance (NMR). The species were extracted from one broiler litter (BL) and two layer manure samples (LM #1 and LM #2) with DI water, 2-(N-morpholino)ethanesulfonic acid (MES), or NaOH + EDTA.

<table>
<thead>
<tr>
<th>P species</th>
<th>Chemical shift</th>
<th>DI water</th>
<th>MES</th>
<th>NaOH + EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BL</td>
<td>LM</td>
<td>BL</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>6.29-6.5</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Phytic acid</td>
<td>4-6</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.33-1.97</td>
<td>†</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>DNA</td>
<td>-0.36</td>
<td>†</td>
<td>†</td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>-3.4</td>
<td></td>
<td></td>
<td>†</td>
</tr>
<tr>
<td>Polyphosphate</td>
<td>-3.6, -3.9</td>
<td>†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5. Chemical composition of one broiler litter (BL) and two layer manures (LM #1 and LM #2). Fractions were extracted with DI water, 2-(N-morpholino) ethanesulfonic acid (MES), or NaOH + EDTA.

<table>
<thead>
<tr>
<th></th>
<th>Total dissolved P</th>
<th>Dissolved reactive P</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Al</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>------ mg kg(^{-1}) dry animal waste------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI water</td>
<td>BL</td>
<td>7,275±308</td>
<td>6,268±195</td>
<td>1,862±75</td>
<td>2,935±172</td>
<td>109±2</td>
<td>8±2</td>
</tr>
<tr>
<td></td>
<td>LM #1</td>
<td>3,683±65</td>
<td>2,599±335</td>
<td>4,239±203</td>
<td>2,099±35</td>
<td>44±2</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>LM #2</td>
<td>3,956±255</td>
<td>3,064±245</td>
<td>4,197±320</td>
<td>2,300±107</td>
<td>42±9</td>
<td>24±30</td>
</tr>
<tr>
<td>MES</td>
<td>BL</td>
<td>9,578±626</td>
<td>8,597±569</td>
<td>6,461±240</td>
<td>3,304±193</td>
<td>76±3</td>
<td>5±0.3</td>
</tr>
<tr>
<td></td>
<td>LM #1</td>
<td>5,871±184</td>
<td>5,165±161</td>
<td>18,627±493</td>
<td>2,474±120</td>
<td>36±4</td>
<td>&lt;DL</td>
</tr>
<tr>
<td></td>
<td>LM #2</td>
<td>6,371±177</td>
<td>5,536±124</td>
<td>19,913±343</td>
<td>2,668±137</td>
<td>31±5</td>
<td>3</td>
</tr>
<tr>
<td>NaOH + EDTA</td>
<td>BL</td>
<td>16,080±301</td>
<td>13,032±155</td>
<td>24,325±814</td>
<td>3,688±86</td>
<td>117±58</td>
<td>428±93</td>
</tr>
<tr>
<td></td>
<td>LM #1</td>
<td>16,735±1,204</td>
<td>13,442±423</td>
<td>110,800±7,917</td>
<td>2,487±233</td>
<td>20±3</td>
<td>171±59</td>
</tr>
<tr>
<td></td>
<td>LM #2</td>
<td>15,243±758</td>
<td>14,982±499</td>
<td>122,085±8,755</td>
<td>2,039±242</td>
<td>15±0.4</td>
<td>97</td>
</tr>
</tbody>
</table>

DL: detection limit
† Values are means of three replicate extracts. Samples were extracted for 24 h under a ratio of 100:1 (extractant solution:animal manure).
‡ Values are mean pH of three replicate extracts, determined in extraction solution after 24-h extraction with a ratio of 100:1 (extractant solution:animal manure).
Table 4.6. The concentration of P species measured by P-31 Nuclear Magnetic Resonance (NMR) in fractions of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2) extracted by DI water, MES buffer, or NaOH + EDTA.

<table>
<thead>
<tr>
<th></th>
<th>Total dissolved P†</th>
<th>Orthophosphate‡</th>
<th>Phytate‡</th>
<th>Other P species‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-------</td>
<td>----------------</td>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>DI water</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>7,275 (36.9)</td>
<td>5,470 (27.8)</td>
<td>1,532 (7.8)</td>
<td>274 (1.3)</td>
</tr>
<tr>
<td>LM #1</td>
<td>3,683 (17.3)</td>
<td>3,069 (14.5)</td>
<td>276 (1.3)</td>
<td>338 (1.5)</td>
</tr>
<tr>
<td>LM #2</td>
<td>3,956 (21.9)</td>
<td>3,243 (17.9)</td>
<td>324 (1.8)</td>
<td>389 (2.2)</td>
</tr>
<tr>
<td><strong>MES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>9,578 (48.7)</td>
<td>8,707 (44.3)</td>
<td>88 (0.4)</td>
<td>783 (4)</td>
</tr>
<tr>
<td>LM #1</td>
<td>5,871 (32.4)</td>
<td>4,285 (23.7)</td>
<td>1,586 (8.8)</td>
<td>0</td>
</tr>
<tr>
<td>LM #2</td>
<td>6,371 (35.2)</td>
<td>4,863 (26.8)</td>
<td>1,582 (8.3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>NaOH + EDTA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>16,080 (81.8)</td>
<td>8,418 (42.8)</td>
<td>3,711 (18.9)</td>
<td>3,950 (20.1)</td>
</tr>
<tr>
<td>LM #1</td>
<td>16,735 (78.8)</td>
<td>9,046 (42.6)</td>
<td>6,875 (32.4)</td>
<td>1,255 (3.9)</td>
</tr>
<tr>
<td>LM #2</td>
<td>15,243 (84.1)</td>
<td>10,230 (56.5)</td>
<td>4,479 (24.8)</td>
<td>534 (2.9)</td>
</tr>
</tbody>
</table>

† Determined by ICP-AES
‡ Calculated by multiplying the proportion of the total spectral area assigned to a specific signal by the TDP concentration.
Values in parentheses are percentages of total P
Fig. 4.1. Phosphorus distribution in sequentially-extracted fractions of one broiler litter (BL) and two layer manure samples (LM #1 and LM #2). Significant differences for Total Dissolved P (TDP) in each fraction between DI water-, and MES- leading fractionation schemes are represented as capital and lower case letters, respectively (P<0.05).
Fig. 4.2. Solution $^{31}\text{P}$ Nuclear Magnetic Resonance (NMR) spectra of broiler litter P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry litter), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45° pulse, 1.6-s acquisition, 5-s relaxation, for 5000 scans (about 9 h).
Fig. 4.3. Solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectra of layer manure #1 P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry manure), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45° pulse, 1.6-s acquisition, 5-s relaxation, for 256 scans (29 min).
Fig. 4.4 Solution $^{31}$P Nuclear Magnetic Resonance (NMR) spectra of layer manure #2 P extracted with (a) DI water, (b) MES, or (c) NaOH + EDTA at a ratio of 100:1 (extractant:dry manure), for 24 h. The spectrum was produced on a Varian Unity INOVA 500 MHz spectrometer using a 45° pulse, 1.6-s acquisition, 5-s relaxation, for 256 scans (29 min).
Fig. 4.5. Relationships between concentrations of orthophosphate (mg kg\(^{-1}\) dry animal waste) determined by \(^{31}\)P Nuclear Magnetic Resonance (NMR) and Dissolved Reactive P (DRP) determined by the molybdate colorimetric method in three extracts from three animal wastes. The regression line describes the model: Orthophosphate by NMR = 1852 + 0.56 (orthophosphate by molybdate colorimetric method), P < 0.0001, \(R^2 = 91.3\%\).
CHAPTER 5

TRANSFORMATION OF BROILER LITTER PHOSPHORUS DURING STORAGE:
EFFECTS OF WATER CONTENT AND TEMPERATURE

To be submitted to Journal of Environmental Quality
ABSTRACT

In areas with intensive broiler production, broiler litter storage prior to land application allows flexible land applications at times of maximum nutrient demand by crops and minimum runoff potential. Limited information is available, however, on P transformation during broiler litter storage. We evaluated the effects of water content and temperature on transformation of P during storage. Freshly collected broiler litter was incubated at four water contents (400, 800, 1200, or 1600 g kg\(^{-1}\)) and three temperatures (10, 20, and 30°C) for 60 days. A modified sequential fractionation method with 2-(N-morpholino) ethanesulfonic acid (MES), NaHCO\(_3\), and NaOH was applied on incubated samples to investigate P distribution. Incubation brought about an increase in Total Dissolved P (TDP) in the MES fraction across all water contents and temperatures. This change resulted from a transfer of TDP from the NaHCO\(_3\), NaOH, and Residual P (RP) fractions to the MES fraction. Dissolved Reactive P (DRP) in the MES fraction along with Dissolved Unreactive P (DUP) in the NaOH fraction usually contributed to the increase of the sum of TDP from the three extractants. The general agreement between CO\(_2\) production during incubation and the sum of TDP in three extractants indicated that microbial activity played an important role in P transformation during storage. In general, a larger increase in TDP in the three extractants was observed with an extension of incubation time than with an increase in water content or temperature.
INTRODUCTION

In 2006, Georgia was the largest broiler litter-producing state in the United States, with a production of approximately 1.3 billion broilers (Georgia Agricultural Statistics Service, 2007) and an estimated generation of 2 million Mg of broiler litter (mainly bedding material and excreta). Because most broiler litter is surface applied to grasslands as fertilizer, surface runoff interacting with the litter may solubilize P and transport it to surface waters, where it may accelerate eutrophication (Sharpley, 1995; Sims et al., 1998). Therefore, the concentration of Total Dissolved P (TDP) in broiler litter is an important characteristic that may affect the potential for surface runoff contamination.

Broiler litter collected from broiler houses may be directly applied to grasslands or stored temporarily. Litter storage prior to land application allows flexible applications to coincide with maximum crop uptake and minimum runoff potential, which would reduce the potential for adverse impact on water quality (Brodie and Carr, 1988; Moore Jr. et al., 1995). However, there is limited information on P transformation during broiler litter storage. Vadas et al. (2004) reported that TDP increased about 52% in raw manure that went through freeze-thaw cycles. McGrath et al. (2005) found a large increase in orthophosphate concentration and a decrease in phytic acid concentration in broiler litter stored at a water content of 370 g kg\(^{-1}\) when compared to a water content of 210 g kg\(^{-1}\). The authors concluded that microbial activity played an important role in transferring non-labile P to labile P during the storage at the greater water content. Maguire et al. (2006) also found that TDP in broiler litter stored with a water content of 600 g kg\(^{-1}\) increased with storage time.

The sequential fractionation method has been applied to evaluate P transformations in broiler litter-amended soils (Adeli et al., 2005), and manure P composition (Barnett, 1994; Doug...
et al., 2000; Leinweber et al., 1997; Peperzak et al., 1959). This method is based on the assumption that chemical extractants selectively dissolve different P fractions (He et al., 2003). These P fractions are commonly defined according to extractants, such as resin- or H$_2$O-P, NaHCO$_3$-P, NaOH-P, and H$_2$SO$_4$-P or HCl-P. Resin-or H$_2$O-P, and NaHCO$_3$- extractable P are labile P fractions readily available for plant and algae; the NaOH extractable P fraction is considered to be moderately labile P (Fe-, Al-associated P), whereas P extracted by HCl is regarded as moderately stable P (Ca-associated P) (Williams et al., 1980; Zhang et al., 2004). It is believed that P species associated with these fractions decrease in vulnerability to environmental loss in the order of H$_2$O-, NaHCO$_3$-, NaOH-, and HCl-extracted P as well as Residual P (RP, P not extracted by sequential extraction) (Dou et al., 2003; He and Honeycutt, 2001; Sharpley and Moyer, 2000).

We have observed that the water content of broiler litter removed from broiler houses can range from low (about 200 g kg$^{-1}$) to very high (> 1000 g kg$^{-1}$). Information on P transformations as affected by such a wide range of water content is currently not available. Also, temperature during storage can vary depending on environmental conditions, and information on its effect on P transformations during storage is currently limited. Consequently, this laboratory study used a modified fractionation method with MES as the first extractant to evaluate the effects of water content and temperature during storage on P transformation and distribution among P pools in broiler litter.

**MATERIALS AND METHODS**

**Broiler Litter**

Fresh broiler litter (mixture of broiler litter and wood shavings) was collected from a broiler house and was stored at 4°C until used. Total C and N were determined by dry
combustion (University of Wisconsin Cooperative Extension Publishing, 2003), whereas total P, Ca, and Mg contents were measured by dry ash digestion (USEPA, 1995) followed by determination with an Inductively Coupled Plasma-Optical Emission Spectrograph (ICP-OES) (Table 5.1).

**Laboratory Incubation**

Four water contents (original (x) at 400 g kg\(^{-1}\)), 2x, 3x, and 4x) and three temperatures (10, 20, and 30°C) were factorially combined to generate 12 treatments that were replicated three times. The selected temperatures are well below values required for thermophilic microorganisms, and hence no composting should have occurred during the study (Zibilske, 1997). For each treatment, 5 g of fresh broiler litter was placed in a 100-mL beaker to be incubated for 60 days, with subsamples (0.2 g) removed at 2, 10, and 60 days for fractionation analysis. Each beaker was placed inside a 1-L mason jar which was closed with a lid and aerated regularly to ensure aerobic conditions.

**Sample Fractionation**

Non-incubated (control), fresh broiler litter (stored at 4°C) with a water content of 400 g kg\(^{-1}\) as well as broiler litter subsampled from incubated samples were sequentially extracted using a modification of the fractionation method developed by Sui et al. (1999). Instead of using water as the first extractant at original broiler litter pH (~8), as in Sui et al. (1999), we used 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6. It was reported by Tasistro et al. (2007) that a decrease in pH led to an increase in TDP in broiler litter because of an increase in the solubility of Ca and Mg phosphates, as well as organic P compounds. The authors also showed that extracting broiler litter with MES buffered at pH 6 (recommended pH for grasslands in Georgia) extracted a similar amount of P as that obtained by lowering the pH of the extract with
HCl. This indicated that MES was not causing any unwanted complexation effects on the solubilization of P. Therefore, a sequential fractionation scheme that uses MES instead of water as the first extractant may provide a better insight into transformations that lead to TDP in broiler litter. MES extraction was followed by 0.5 M NaHCO$_3$ at pH 8.5, and 0.1 M NaOH at pH 12.5. All extractions were conducted at a solution:dry matter ratio of 200:1. Residual P was calculated as the difference between total P and the sum of extracted P by all extractants. During extraction, samples were shaken horizontally on an end-to-end shaker at 120 oscillations per minute for 24 h at room temperature ($\approx$23$^\circ$C). After extraction, the samples were centrifuged for 20 min at 1841 x g and filtered through a 0.45-μm filter. The filtrates were analyzed for Dissolved Reactive P (DRP) by Murphy and Riley (1962) and for TDP by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). Dissolved Unreactive P (DUP) was calculated as the difference between TDP and DRP.

Measurement of Carbon Dioxide Evolution

The evolved CO$_2$ from each incubation jar was trapped with a process described by Anderson (1982) to obtain an estimate of microbial activity. In each jar, a vial with 10 mL of 2 N NaOH was used for absorption of evolved CO$_2$. Then, the NaOH solution was mixed with 10 mL of 3 N BaCl$_2$ to precipitate the produced sodium carbonate as insoluble BaCO$_3$. The solution was titrated to pH 7 with 2 N HCl using a Schott TW Alpha titrator (Schott-Geräte, Hofheim a. Ts., Germany).

The amount of CO$_2$ evolved during the incubation period was calculated from the following equation:

$$C\text{-CO}_2 \ (\text{g} \ \text{kg}^{-1} \ \text{dry broiler litter}) = (B - V) \ \text{N E/m} \ * \ 1000$$
B = the volume (ml) of HCl needed to titrate the NaOH in the jars from control jar to the end point (pH = 7)

V = the volume (ml) of HCl needed to titrate the NaOH in the jars exposed to broiler litter samples

N = normality of HCl, E = equivalent weight (E = 6), m = amount (g) of incubated broiler litter samples

**Water Potential Determination**

The water potential of broiler litter was measured with a WP4 Dewpoint Potential Meter (Decagon Devices, Pullman, WA, USA). Approximately 2 g of fresh broiler litter was mixed with various amounts of water (from original water content to saturated water content), allowed to equilibrate, and then placed in measurement cups. The measurement cups were sealed under constant room temperature (≈23°C) to equilibrate for at least 30 min, and then the water potential was read in Dewpoint Potential Meter. Samples in the measurement cups were subsequently transferred to aluminum pans, and oven dried at 65°C for 48 h to determine water content.

**Statistical Analysis**

An analysis of variance for each extracted P fraction was carried out with PROC MIXED, and PROG GLM in SAS, V.9.0 (SAS Institute Inc., 2002). The statistical model used considered the main effects of water content, temperature, time, and extractant, as well as all two-way, three-way interactions between those factors in a repeated measures analysis. Fisher’s protected Least Significant Difference (LSD) was used to separate means, and a 5% confidence level was used to conclude significant effects.
RESULTS AND DISCUSSION

Phosphorus Distribution in Initial Sample (0 day), and in Incubated Samples

In the initial sample, RP was the largest P fraction (49% of total P; Fig 5.1), whereas MES, NaHCO₃, and NaOH fractions represented 35.5, 9.4, and 6% of total P, respectively.

During incubation, considerable changes were observed in the different P pools. These changes were mainly affected by two-way and some three-way interactions (Table 5.2). In order to compare P transformation and distribution among extractants, the DRP, DUP and RP as percentages of total P in each fraction are displayed in Fig. 5.1 (water content x time effect) and Fig. 5.3 (temperature x time effect), and discussed separately.

The Effects of Water Content over Incubation Time

Compared to initial samples, samples incubated for 10 and 60 days had a larger (P<0.1) amount of TDP (sum of TDP from three extractants) at all water contents (Fig. 5.1, Table 5.3). Greater water contents and longer incubation time produced a larger amount of CO₂, implying greater biological activity (Fig. 5.2). However, the increase in TDP was more affected by incubation time than by water content, because a greater increase was observed when extending incubation time at constant water content than when increasing water content at the same incubation time (Fig. 5.1). To further compare the effect of these two factors, TDP changes are discussed separately by water content.

Original Broiler Litter Water Content (400 g kg⁻¹)

When expressed as percentage of total P, the sum of TDP in three fractions considered together increased between 12 and 13% over time, as compared to the initial sample (Fig. 5.1a). However, while TDP increased in the MES fraction (18 to 22% of total P), it decreased in the NaHCO₃ (4 to 8%) and NaOH fractions (0.8 to 2%). Residual P also decreased by 12 to 16%,....
relative to the initial sample. This TDP changing pattern indicated that the increase in MES-extractable P was derived from NaHCO₃, NaOH, and RP fractions.

The amount of DRP was greatest at 2 days (mainly from the MES fraction) and was followed by a decrease with time (Fig. 5.1a). These results suggest a larger rate of mineralization of DUP to DRP during the first 2 days, followed by a dominant immobilization process in later incubation periods.

The MES-extractable P is considered to be P released to soils from broiler litter when attached to acidic soils (Tasistro et al., 2007). The highest concentration of DRP (54.8% of total P) in the MES fraction was found at 2 days of incubation. This rapid release of large amounts of bioavailable P (Sharpley et al., 1995) can be potentially detrimental to surface water quality when provisions for P sinks are not readily available. Our results also show that a longer storage time (60 days) immobilized some DRP found at 2 days, and could reduce the amount of labile P released from broiler litter stored at a water content of 400 g kg⁻¹. Immobilization of P may have been carried out by fungi that can subsist under a relative low water content and low water potential. Our measurements indicates that the water potential of broiler litter at 400 g kg⁻¹ was about -18.3 MPa (at 23°C).

**Water Content of 800 g kg⁻¹**

At this water content, the sum of TDP in the three extractants at 10 and 60 days increased by 37 to 83%, respectively, when compared to the initial sample (Fig. 5.1b). Total dissolved P in the MES fraction at 2, 10, and 60 days increased between 67 and 115% depending on incubation time. This larger increase of TDP in the MES fraction, when compared to broiler litter incubated at 400 g kg⁻¹ was mainly caused by an increase in DUP (Fig. 5.1b) probably derived from microorganisms. In a parallel ³¹P Nuclear Magnetic Resonance study (unpublished results) we
found that DUP in an MES extract from broiler litter contained small concentrations of phosphate monoesters (including some phytic acid), some DNA, and phospholipids (Zhao’s unpublished results). Although increasing with incubation time, the amount of TDP in NaHCO$_3$ and NaOH fractions, and the amount of P in the RP fraction were smaller than in the initial sample. Since DUP extracted by NaOH is mainly in phytic acid form (Turner and Leytem, 2004), the observed increase in DUP with time is likely related to solubilization of phytate by microbial activity (Jones, 1998). The measured water potential of broiler litter at 800 g kg$^{-1}$ was greater than that at 400 g kg$^{-1}$ (-7.1 MPa vs -18.3 MPa at 23ºC) and therefore greater microbial activity should have been present, as evidenced by CO$_2$ evolution (Fig. 5.2). Although a similar amount of DRP in the MES fraction was found at the three incubation times (2, 10, and 60 days), the increased DUP observed at 60 days resulted in a significant (P<0.01) increase in TDP relative to the amount of TDP found at 2 days (Table 5.3).

**Water Content of 1200 and 1600 g kg$^{-1}$**

Broiler litter stored at water contents of 1200 and 1600 g kg$^{-1}$ underwent similar P changing patterns during the incubation, probably because their water potentials were similar (-4.0 and -3.2 MPa at 23ºC). As shown in Fig. 5.1c and 5.1d, the main trends for TDP concentrations were large increases in the MES fraction (Table 5.3), and slight increases in the NaHCO$_3$ and NaOH fractions, with large concomitant decreases in RP. When expressed as a percentage of total P, the sum of TDP from the three fractions considered together increased between 37 and 60% over time, as compared to the initial sample, for extractions on days 10 and 60. This increase was mainly caused by a rapid increase of DRP in the MES fraction (Fig. 5.1c and 5.1d). Relative to the initial sample, DRP in the MES fraction increased between 33 and 138%, whereas DRP in the NaHCO$_3$ fraction decreased between 72 and 82%, and DRP in the
NaOH fraction decreased between 68 and 83%. Similarly, DUP in the NaOH fraction increased from 115 to 214%, which contributed to the observed increase in the sum of TDP from all three fractions. This P distribution pattern indicated that incubation resulted in a DRP shift from NaHCO₃, NaOH, and RP fractions to the MES fraction over time. Therefore, a short storage time would appear to be better than a long storage time with respect to the potential for release of soluble P from broiler litter with elevated water contents.

**Summary of Effect of Water Content**

In summary, incubation resulted in an increase of TDP in the MES fraction across all water contents, and promoted DRP shift from NaHCO₃, NaOH, and RP fraction to the MES fraction. This transformation increased with water content and time, and a larger increase was always observed with an extension of incubation time than with an increase in water content. Therefore, shorter broiler litter storage may help to reduce the potential adverse impact brought about by P release from broiler litter with elevated water contents. But, a longer incubation time for broiler litter with low water content may reduce DRP by immobilization. The cumulative CO₂ release coincided with TDP changes with water content and incubation time, and indicated that microbial activity played an important role in P transformation during the incubation.

**The Effects of Incubation Temperature over Incubation Time**

The effect of incubation time on TDP in MES, NaHCO₃, and NaOH fractions varied with temperature (Fig. 5.3, Table 5.4). In most cases, these changes were brought about mainly by TDP changes in the MES fraction, which contained 53 to 71% of the total P. To further evaluate the effects of time and temperature, TDP changes are discussed separately by temperature.
**Incubation at 10°C**

Compared to the initial sample, incubation at 10°C, resulted in a significant (P<0.01) increase in TDP in the MES fraction across incubation times (Table 5.4 and Fig. 5.3a). However, changes in the sum of TDP from the three fractions at 2- and 10-day incubation, relative to the initial sample, were not significant (Table 5.4). The lack of significance was caused by a TDP decrease in the NaHCO$_3$ and NaOH fractions at 2- and 10-day incubations. Whereas TDP in the MES fraction increased by about 50%, it decreased by 50 to 76% in the NaHCO$_3$ fraction, and by 10 to 31% in the NaOH fraction. No difference was found in TDP in the MES fraction and the sum of TDP in the three fractions between 2- and 10-day incubation, but there was a significant (P<0.01) increase in the sum of TDP at 60 days. This increase was mainly caused by the transfer of RP (13% of total P), which contributed to the increase of DRP in the MES fraction (Fig. 5.3a). Total dissolved P changes during the incubation at 10°C showed that long-term incubation (60 days) promoted TDP release from broiler litter, whereas short-term incubation did not increase TDP (P>0.05), but resulted in an increase in MES-TDP, which has been regarded as potentially labile P in broiler litter (Tasistro et al., 2007).

**Incubation at 20 and 30°C**

Incubation at these two temperatures responded similarly to time. At both temperatures, TDP in the MES fraction was greater in incubated samples than in the initial sample (Table 5.4, Fig. 5.3b and 5.3c). This increase was caused by an increase of DRP in the MES fraction (127%), which occurred with a concomitant 50% decrease in DUP in the MES fraction. In contrast to results obtained at 10°C, at 20°C the sum of TDP in the three fractions was 25 to 52% greater than in the initial sample at both 10- and 60-day incubation, while there was no difference between 10 and 60 days (Table 5.4). This increase was caused mainly by a DRP increase in the
MES fraction and in part by a DUP increase in NaOH fraction, which accounted for 13 to 24% of the increase at these two incubation periods. An explanation for this increase is that a higher temperature enhanced microbial activity, which promoted the solubility of phytate, the main organic P compound extracted by NaOH (Turner and Leytem, 2004). At 30°C, TDP in the MES fraction was greater than in the initial sample by 61 to 100% at 10 and 60 days. For the same incubation times, the sum of TDP in three fractions was 41 to 72% greater than in the initial sample. The sum of TDP in the three fractions was greatest at 60 days at 30°C. This larger amount of TDP at 60-day incubation compared to TDP at 2 and 10 days was mainly from an increase in the DUP in the three fractions (Fig. 5.3c). It is likely that microbial activity under these conditions further promoted the mineralization and solubility of organic P, which is consistent with the cumulative CO$_2$ evolved (Fig. 5.4).

**Phosphorus Transformation in the NaOH Fraction**

There was a temperature x water content x time interaction for TDP and DRP in the NaOH fraction (Table 5.2). The general trend was for TDP to increase at 60 days compared to the initial sample, whereas DRP tended to decrease in that time period (Fig. 5.5). The magnitude of these changes varied depending on temperature and water content. Because DRP decreased, the increase in TDP is explained by an increase in DUP, which represented 48 to 75% of TDP across incubation time, temperatures, and water contents. The main compound in the NaOH fraction was reported by Turner and Leytem (2004) as phytic acid.

**CONCLUSIONS**

Our results indicated that incubation resulted in an increase in the sum of TDP in the three fractions across all water contents and temperatures, and also promoted TDP and DRP shift from NaHCO$_3$, NaOH, and RP fractions to the MES fraction over time. The increase of the sum
of TDP in the three fractions was mainly caused by an increase of DRP in the MES fraction, and in part by an increase in DUP in the NaOH fraction due to microbial activity. This transformation increased with water content, time, and temperature, and a larger increase was usually observed with an extension of incubation time than with an increase in water content or temperature.
REFERENCES


University of Wisconsin Cooperative Extension Publishing. 2003. Recommended methods of manure analysis (A3769), Madison, WI.


### Table 5.1. Selected composition of the broiler litter used for the incubation Study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Broiler litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry mater, g kg⁻¹</td>
<td>691</td>
</tr>
<tr>
<td>pH†</td>
<td>8.6</td>
</tr>
<tr>
<td>Total P, g kg⁻¹</td>
<td>19.7</td>
</tr>
<tr>
<td>Total N, g kg⁻¹</td>
<td>36.79</td>
</tr>
<tr>
<td>Total C, g kg⁻¹</td>
<td>333.03</td>
</tr>
<tr>
<td>Al, g kg⁻¹</td>
<td>3.02</td>
</tr>
<tr>
<td>Ca, g kg⁻¹</td>
<td>29.14</td>
</tr>
<tr>
<td>Fe, g kg⁻¹</td>
<td>1.62</td>
</tr>
<tr>
<td>K, g kg⁻¹</td>
<td>28.16</td>
</tr>
<tr>
<td>Mg, g kg⁻¹</td>
<td>6.38</td>
</tr>
</tbody>
</table>

† pH was measured by mixing dry broiler litter with water in 100:1 (water:manure) ratio.

### Table 5.2. Analysis of variance for Total Dissolved P (TDP) and Dissolved Reactive P (DRP) extracted from broiler litter by MES, NaHCO₃, and NaOH.

<table>
<thead>
<tr>
<th>Source</th>
<th>TDP</th>
<th>DRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MES</td>
<td>NaHCO₃</td>
</tr>
<tr>
<td>Time</td>
<td>0.001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Water content</td>
<td>0.028</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Water content x time</td>
<td>0.021</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.165</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Temperature x time</td>
<td>0.093</td>
<td>0.012</td>
</tr>
<tr>
<td>Temperature x water content</td>
<td>0.652</td>
<td>0.202</td>
</tr>
<tr>
<td>Temperature x water content x time</td>
<td>0.473</td>
<td>0.182</td>
</tr>
</tbody>
</table>
Table 5.3. Significance of pairwise comparisons using Fisher's LSD of mean Total Dissolved P (TDP) in the MES fraction, and mean sum of TDP in MES + NaHCO$_3$ + NaOH at four incubation times, and four water contents.

<table>
<thead>
<tr>
<th>WC = 400 g kg$^{-1}$</th>
<th>WC = 800 g kg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (d)</strong></td>
<td>0†</td>
</tr>
<tr>
<td>MES-TDP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Sum TDP‡</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>WC = 1200 g kg$^{-1}$</th>
<th>WC = 1600 g kg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (d)</strong></td>
<td>0†</td>
</tr>
<tr>
<td>MES-TDP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Sum TDP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

† Time 0 stands for initial sample, ‡ the sum of TDP extracted in the MES, NaHCO$_3$, and NaOH fraction NS: non significant, * statistical significance at the 0.05 probability level, ** statistical significance at the 0.01 probability level.

Table 5.4. Significance of pairwise comparisons using Fisher’s LSD of mean Total Dissolved P (TDP) in the MES fraction, and mean sum of TDP in MES + NaHCO$_3$ + NaOH at four incubation times and three temperatures.

<table>
<thead>
<tr>
<th>Temperature 10ºC</th>
<th>Temperature 20ºC</th>
<th>Temperature 30ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (d)</strong></td>
<td>0†</td>
<td>2</td>
</tr>
<tr>
<td>MES-TDP</td>
<td>0</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Sum TDP‡</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>NS</td>
</tr>
</tbody>
</table>

† Time 0 stands for initial sample, ‡ the sum of TDP extracted in the MES, NaHCO$_3$, and NaOH fraction NS: non significant, * statistical significance at the 0.05 probability level, ** statistical significance at the 0.01 probability level.
Fig. 5.1. Dissolved Reactive P (DRP), and Dissolved Unreactive P (DUP) extracted from broiler litter by MES, \( \text{NaHCO}_3 \) and \( \text{NaOH} \) as affected by water content x time. (M: MES, B: \( \text{NaHCO}_3 \), S: \( \text{NaOH} \)).
Fig. 5.2. Cumulative C-CO$_2$ release from incubated broiler litter as affected by water content x time.
Fig. 5.3. Dissolved Reactive P (DRP), and Dissolved Unreactive P (DUP) extracted from broiler litter by MES, NaHCO$_3$ and NaOH as affected by temperature x time. (M: MES, B: NaHCO$_3$, S: NaOH).
Fig. 5.4. Cumulative C-CO$_2$ release from incubated broiler litter as affected by temperature x time.
Fig. 5.5. Total Dissolved P (TDP), and Dissolved Reactive P (DRP) extracted by NaOH as affected by temperature x water content x time.
CHAPTER 6

SUMMARY AND CONCLUSIONS

Manure surface application to grasslands may lead to contamination of surface waters with P, with potential risk of accelerating eutrophication. Manure Total Dissolved P (TDP) has been considered as a key indicator of the soluble P that may contaminate surface runoff from surface-applied manures. The research presented in this dissertation evaluated the critical factors that may affect manure P release when applied to acidic soils, the composition and speciation of manure soluble P, and the transformation of manure P during storage.

In chapter 2 and 3, experiments clearly demonstrated the role of extraction pH, time, and extraction ratio on P extraction from broiler litter, layer manure and dairy slurry. In general, extracting broiler litter with 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 6 resulted in greater values of TDP, Dissolved Reactive P (DRP), Soluble Bioavailable P (SBAP), and Total Bioavailable P (TBAP) than extracting broiler litter with DI water. Maximum amounts of these variables were extracted with an extractant:broiler litter ratio of 100:1 or 200:1 and extraction times of 4 or 24 hours. Although for some layer manures, an extraction with MES at a 100:1 ratio for 1 h was sufficient to extract the maximum amounts of TD and DRP, for most of layer manures an extraction ratio of 200:1 for 1 h removed the maximum amounts of TDP and DRP. Maximum amounts of SBAP and TBAP were extracted from all layer manures with MES at a ratio of 200:1 for 24 h. For dairy slurries, an extraction with MES at a ratio of 100:1 for 1 h was sufficient to remove the maximum amounts of TDP, DRP, SBAP, and TBAP.
The fate of P from animal waste in the environment is in part determined by its chemical composition. The sequential fractionation method has been commonly applied on manure P composition analysis, and \(^{31}\)P Nuclear Magnetic Resonance (NMR) is widely used on manure P speciation. Results in Chapter 4 indicated that the amount of P recovered from animal wastes varied with extractant and the nature of animal wastes. Results from the sequential fractionation (DI water- and MES-leading fractionation) study showed that the major P fraction was MES-extractable P (pH 6), and that P species extracted by MES were highly related to Ca and Mg cations. NaOH extractable P was in both Fe-, Al- bound P and Ca-, Mg-associated P, and was relatively stable in both DI water- and MES- leading fractionation schemes. \(^{31}\)P NMR analysis confirmed that the most of the P extracted by DI water and MES was in the form of inorganic P, and that significant amounts of phytate were extracted from broiler litter with DI water and from layer manure with MES.

Manure storage prior to land application allows flexible applications to coincide with maximum crop uptake and minimum runoff potential, which would reduce the potential for adverse impact on water quality. However, manure P loss in the field is partly decided by its storage conditions, such as storage moisture, temperature. Our results in Chapter 5 found that incubation resulted in an increase in the sum of TDP in the three sequential fractions (MES, \(\text{NaHCO}_3\) and \(\text{NaOH}\) fractions) across all water contents and temperatures. Storage also promoted TDP and DRP shift from \(\text{NaHCO}_3\), NaOH, and Residual P (RP) fractions to the MES fraction over time. The increase of the sum of TDP in the three fractions was mainly caused by an increase of DRP in the MES fraction, and partially by an increase in DUP in the NaOH fraction due to microbial activity. This transformation increased with water content, time, and temperature, and a larger increase was usually observed with an extension of incubation time.
than with an increase in water content or temperature. Therefore, in most cases a shorter broiler litter storage term may help to reduce the potential release of soluble P from broiler litter.

Overall, the work presented in this dissertation resulted in results that should be helpful in understanding leaching of P from animal manures. However, because these results were found under laboratory conditions, further work should be conducted to determine if they apply during field conditions.