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Use of *Lolium multiflorum* for Remediation of Phosphorus from Poultry-Litter-Contaminated Media.

A Thesis for the University Honors Program

Western Kentucky University

Daniel Lee Starnes

Spring 2006

Approved By

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There are many people that if it were not for their help and contributions this endeavor would have been a fruitless one. I would like to thank my research advisor Dr. Shivendra Sahi, for his financial and technical assistance, and more importantly giving me the freedom to conduct this research project. I would also like to thank Dr. Nilesh Sharma for his many hours of working with me on this project and double checking all of my calculations and keeping me on track. Also I would like to thank Dr. Byron Sleugh for serving as my second reader and offering his expertise in forages and offering ideas and incite that helped to further my project. I owe a great deal of thanks to Dr. John Andersland for his multitude of skills and talents that were instrumental in the completion of this research project.

I would also like to thank all of the members of the Honor's Society as they are the real reason that I have had the opportunity to complete such a momentous task. I would like to personally thank Mr. Walker Rutledge for finding all of my comma splices and putting up with my way with words. I would like to thank the Honor's Director Dr. Craig Cobane for his vision and bold leadership of the Honors Program.

Last but not least, I would like to thank my father, Larry Starnes, my mother Linda Starnes, and my brother John Starnes, for their support and encouragement.

ABSTRACT

With the number of intensive livestock operations having increased dramatically in recent years, the concentration of large-scale operations has caused a sharp increase in the amounts of animal manure being produced. Farmers have utilized these manures as a cheap and effective source of fertilizer. However, over time the levels of phosphorus (P) have increased well beyond the nutritional requirements of the plants being grown. This imbalance could have potential ecological and economical impacts. *Lolium multiflorum* is a cool-season annual ryegrass that is grown throughout the world as a mainstay of pasture land and forage feed. It has been reported that two cultivars of *Lolium multiflorum*, Gulf and Marshall, have the ability to remove up to 20 g of phosphorus per kg of dry tissue from aqueous orthophosphate. The hypothesis for this research was that these two cultivars could have potential application in the removal of phosphorus from poultry-litter-enriched media. We report that both grasses have the ability to remove 10 g of phosphorus per kg of dry tissue from aqueous poultry-litter solutions. The addition of chelators to aqueous media had no increase in phosphorus uptake, but did have a dramatic increase in biomass, in some cases increasing the biomass 185% over the control. We also report that in the presence of soil amended with poultry litter that Gulf and Marshall could remove around 7 and 6 g P/kg tissue, respectively.

INTRODUCTION

Intensive farming practices are fast becoming the mainstay of sustainable agriculture. There has been a shift from large numbers of small-scale agricultural enterprises to a relatively small number of large-scale concentrated agricultural enterprises[1]. The number of livestock operations has decreased dramatically while the number of animals produced has increased exponentially. Specialization and concentration of livestock operations have streamlined the meat industry, thereby decreasing cost and increasing profits. This concentration of livestock operations has led to increased amounts of manure, which is one of the least expensive and most productive fertilizers available to the farmer. On the other hand, extensive use of animal manure has caused a sharp increase in phosphorus levels in the soil that exceed the requirements of crops[1,2]. Of the major livestock manures, poultry manure is among the highest in phosphorus content, with phosphorus levels ranging from 3.6 to 5.1 kg per ton[3]. Due to leaching and runoff, these high levels of phosphorus are leading to non-point pollution of lakes, rivers, and other bodies of water in the United States and other regions of the world[4,5]. The field application of water treatment plant residues such as aluminum chloride, calcium salts, and iron salts has been shown to effectively reduce the amount of phosphate runoff [6,7]. This method of phosphorus immobilization has been shown to be stable for long periods of time, reaching up to ten years.

However, water-treatment residue application is not a viable solution to solving phosphorus levels in soil, as the phosphorus is not removed from the soil. The residues only immobilize the phosphorus[8]. Additionally, the phosphorus that is bound by the chemical treatments becomes unavailable to plants and potentially could have effects on soil properties.

Using plants to extract phosphorus is currently being studied as an alternative to chemical immobilization. Mining of soil phosphorus by harvesting crops that have been grown in phosphorus-rich soils has been suggested as a potential management strategy[8-12,16-19]. Phytoremediation is rapidly becoming a preferred technique as it is nonintrusive, economical, and has been shown to be effective[13]. The capacity of plants used in the extraction of soil phosphate still is largely unknown. The majority of plants accumulate 0.1 to 0.6% phosphorus (dry weight basis)[8]. One major problem with phosphorus (P) content of soil is that the majority of lands with animal manure applications are used in the production of row crops. Row crops are low accumulators of phosphorus (0.6 kg P Mg^{-1} tissue), whereas costal Bermuda grass can accumulate higher levels of phosphorus (3.2 kg P Mg^{-1}), as reviewed by Novak and Chan[8].

Annual ryegrass (*Lolium multiflorum*) is a closely related species to perennial ryegrass (*Lolium perenne*), and both are grown in many regions across the world as a key forage crop for animal production industries[14]. It has been reported that *L.multiflorum* can accumulate up to 2% P (dry weight basis) in hydroponic conditions[17] and more than 1% P from soil supplemented with potassium phosphate in greenhouse studies[18]. This plant exhibits luxuriant growth and produces large amounts of above-ground biomass. Both grasses are considered to be highly digestible and palatable[14]. Delorme et. al

suggested two potential ways of reducing soil phosphorus: one is finding plants that have a high phosphorus requirement and/or finding plants that produce large amounts of above-ground biomass[9].

Our laboratory is working with two species of the cucurbits which have removal capacity exceeding 20 g phosphorus per kg of tissue (DW)[16]. Also we found that the sunflower has removal capacity around 12 g/kg. These three crops are significant because of their large biomass production, and they also produce secondary products that could be utilized elsewhere (fruit, seed oil, etc.). Additionally these crops have a ratio of shoot-to-root phosphorus levels in excess of 1.0. Recently it was shown that transferring a purple acid phsophatase gene from *M. truncatula* into *Arabidopsis* increased the ability of *Arabidopsis* to break down organic phosphorus in liquid culture, improved the biomass of the plant, and increased the amount of phosphorus taken up by the plant[15].

The goal of this study was to quantify the capacity of Gulf and Marshall Ryegrass, two cultivars of *L. multiflorum*, to remove phosphate from soils augmented with different concentrations of poultry litter.

MATERIALS AND METHODS

Seed Germination.

Seeds of Gulf and Marshall ryegrass (*L. multiflorum*) were provided by the USDA-ARS Lab in Starkville, Mississippi. The seeds were sterilized in mercuric chloride (0.1% v/v) and thoroughly rinsed in sterilized deionized water. Seeds were then transferred to a water-agar (0.8%) medium in Magenta boxes for germination. The boxes were maintained at 25°C ± 2°C under 12-hour light and dark periods. Seeds were allowed to germinate and grown for 11 days before plants were transferred to various treatments.

Poultry Litter Treatment and Growth of Seedlings.

Poultry litter was provided by Poteet and Son Farms in south-central Kentucky. The litter was collected after the poultry barn had been cleared of poultry and was collected from random places throughout the house. The poultry litter was dehydrated at the farm by air drying, and ground to a powder in the lab using a mill and sieved through a #40 mesh screen. The powdered litter was used in the following studies:

Hydroponics Study

Seedlings were grown *in vitro* in the culture vessels (15x2.5 cm), each containing 10 ml poultry litter solutions. Poultry litter solutions were prepared by dispensing 0-100 grams poultry litter per liter of deionized water. Ten seedlings of ryegrass were

transferred to each culture vessel aseptically. Ten replicates were maintained for each treatment. Seedlings were grown in a Percival growth chamber at 25°C ± 2°C under 16/8 light/dark (1800-2000 µmol $m^{-2}s^{-1}$ of cool fluorescent light) photoperiod. Seedlings were harvested after a period of 15 days.

Soil Study

Soil used in this experiment was from a Pembroke series and was of a Mollic epipedon dark brown silt loam that was neutral to slightly alkaline. The soil was collected from the Western Kentucky University's Agricultural Farm and Dairy. The top soil was mixed with sand at a ratio of 3 soil to 1 sand (w/w) in order to promote drainage of the soil. The dried, ground poultry litter and the soil were separately autoclaved (123°C, 20 min dry cycle). Fifty grams of litter were mixed thoroughly with one kg of soil, and distributed into pots at the rate of 1.5 kg each pot. Following this, five clumps of grass seedlings were transplanted in each pot (each clump consisted of 10-12 seedlings). Seedlings were grown into a greenhouse maintained at 25°C ± 2°C in 16/8 light/dark photoperiod. Plants were watered 3 times per week or as needed, and fertilized with modified 1/4 strength Hoagland's solution (without phosphorus) [16-18]. After 5 weeks of growth, plants were harvested and washed thoroughly. The biomass was measured, and P content in tissues was analyzed as described below.

Treatment of Chelators

Four chelators were selected for their use in the hydroponic experiment: EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), HEDTA (carboxymethyl(N-2) ethylenediaminetetraacetic acid), and Citric Acid. Poultry litter solution contained 10 g poultry litter per liter of deionized water. Chelators

were applied at the rate of 0-1.0 mM per liter of poultry litter solution (Poultry litter 10g/L). Ten seedlings per culture vessel were grown *in vitro* as described above. Ten replicates were maintained for each treatment for a period of 15 days.

Biomass studies

Plants harvested from experiments were washed and rinsed thrice with deionized water. Then plants were blotted dry with paper towels. Fresh weight (FW) of plants was recorded from each treatment.

Analysis of phosphorus in Plant Tissue.

Following the treatments, plants were harvested and washed thoroughly and rinsed with deionized water. Plants from each treatment were divided into root-and-shoot and dried in an oven at 70°C for three days, after which the dry weights were recorded. Fifty mg of dry tissue were placed in a 15 ml Teflon beaker. Three ml of concentrated HNO_3 were added to each beaker, which was heated to 100°C and left overnight. Samples were cooled to room temperature and gravimetrically made to a volume of 20 ml with deionized water. Phosphorus content in the samples was analyzed using Inductively Coupled Plasma Atomic Emission Spectroscopy at the Materials Characterization Laboratory of Western Kentucky University.

RESULTS

P accumulation of Ryegrass grown in aqueous poultry litter.

Figures 1 shows the phosphorus uptake in g/kg of tissue by Gulf ryegrass grown in aqueous poultry litter. The phosphorus accumulation in seedlings grown in different concentrations of poultry litter was significantly higher than the control, reaching the highest uptake greater than 10 g P/kg (>1% DW) in shoots at 10 g poultry litter/L of media. As the concentration of poultry litter increased beyond 10 g/L, the uptake of phosphorus decreased but remained significantly higher than that of the control. Figure 2 shows the phosphorus uptake by Marshall ryegrass grown in aqueous poultry litter. Seedlings grown in different concentrations of poultry litter had significantly higher phosphorus accumulation than that of the control; however, maximum uptake was recorded at the 10 g/L concentration, with accumulation reaching just over 10 g P/kg tissue (1 % dw). As the concentration increased beyond 10g/L, there was a significant reduction in the phosphorous accumulation, but all the experimental groups still had higher P levels than the control. A similar trend of P accumulation was also recorded for root tissues in both Gulf and Marshall ryegrass (Figure 1 and 2). Figure 3 shows the average biomass (Fresh weight) of Gulf and Marshall ryegrass grown in aqueous poultry litter. A trend was noticed that Marshall had better biomass production than Gulf,

although not significantly different. In both grasses maximum biomass was recorded at the concentration of 10 g poultry litter per liter of media.

Growth and P accumulation by Ryegrass assisted with chelators.

Seedlings of Gulf and Marshall ryegrasses were grown in aqueous poultry litter (10 g/L) containing different concentrations and types of chelators to study the phosphorus uptake potential of grasses. Figures 4 and 5 show accumulation of P by Gulf ryegrass grown in the above-described conditions. Results show that in the presence of all four chelators the accumulation of P either in root or shoot was equal to or lesser than the control group (no chelator treatment) in the case of Gulf ryegrass. The maximum P accumulated (10 g P/kg tissue) in the presence of 0.3 mM DTPA in roots (Figure 4). The concentration of 0.5 mM Citric acid had maximum P accumulation (~10 g P/kg tissue) in shoots (Figure 4). EDTA and HEDTA had lower P accumulation than control at any concentration (Figure 5). Figures 6 and 7 show accumulation of P by Marshall ryegrass grown in the above-described conditions. Results show that in the presence of all four chelators the accumulation of P was equal to or greater than the control group (no chelator treatment). The maximum P accumulation (> 9 g P/kg tissue) in shoots occurred in the presence of 0.3 mM EDTA, 1.0 mM Citric Acid, and all concentrations of DTPA (Figure 6 and 7). HEDTA (0.3 mM) had maximum P accumulation (7 g P/kg tissue) in roots (Figure 7).

Figures 8 and 9 show the growth pattern of seedlings of Gulf and Marshall ryegrasses grown in aqueous poultry litter (10 g/L) supplemented with different types and concentrations of chelators. Seedlings grew very well and had biomass increase more than 100% of the control group at any chelator and any concentration. The highest

increase in biomass in Gulf occurred when DTPA (0.3 mM) was added to the poultry-litter solution (Figure 8). The highest increase in biomass in Marshall occurred when EDTA (0.3 mM) was added to the poultry-litter solution (Figure 8). The percent increase was calculated over the control group.

P accumulation by Ryegrass grown in soil mixed with Poultry litter

Figures 10 and 11 show the phosphorus accumulation of both ryegrasses grown in soil amended with poultry litter for 5 weeks. Gulf and Marshall achieved a maximum phosphorus removal when the concentration of poultry litter was 50 g/kg of soil. In shoots Gulf removed 7 grams of phosphorus, while Marshall removed 6 grams of phosphorus per Kg of dry matter. This represented a significant increase in P removal than the control. There was no statistical difference in the P accumulation in roots or shoots of Gulf and Marshall ryegrass at any concentration of poultry litter amended soil. Figure 12 shows the biomasses of both grasses grown in soil supplemented with different amounts of poultry litter. The maximum growth occurred at soil (1 kg^{-1}) supplemented with 10 g of poultry litter. A decrease in the biomass of both grasses was observed when poultry litter concentration was increased beyond 10 g/Kg of soil. Table 1 shows the ratio of shoot-to-root phosphorous for both grasses grown in soil supplemented with different concentrations of poultry litter. Gulf had a ratio of 0.8 or higher, and Marshall had a ratio higher that 1.0 in most concentrations.

DISCUSSION

This study was to establish the capacity of annual ryegrass to remove phosphorus from media that had been supplemented with poultry litter. Poultry litter is high in phosphorus; however, the phosphorus is primarily in the form of organic phosphorus and is less utilizable by plants. So the uptake potential for ryegrass under these conditions was not expected to reach the levels reported earier[17,18]. As presented in figures 1 and 2; both Gulf and Marshall ryegrass had maximum uptake slightly higher than 10 g/kg of tissue (1.1% DW) when grown in aqueous poultry litter. Although it was half of the highest recorded uptake by ryegrass in hydroponic orthophosphate, it was still in the realm of the suggested range for hyper-accumulation, as suggested by Novak and Chan[8]. Previous studies established that Gulf ryegrass has better phosphorus accumulation than Marshall[17,18]. In the aqueous-variable-poultry-litter-concentration experiment (Figures 1 and 2) there was no statistical difference between the phosphorus accumulations at 10 and 100 g/L poultry litter in the shoot tissue. As the concentration increased above 10g/L poultry litter, the accumulation of phosphorus decreased significantly. Also the biomass of both grasses was negatively impacted if the concentration of the poultry litter exceeded 10 g/L (Figure 3). Poultry litter contains many plant-toxic substances, including antibiotics, micro-organisms, and high levels of urea[3]. Thus, reduction in biomass at high concentrations of poultry litter is caused by the toxic substances associated with the litter.

The concentration of 10g of poultry litter per liter of DI water was considered the optimum, and used in the remaining hydroponics experiments.

In the chelator-assisted experiment it was initially hoped that the chelators would be able to increase the phosphorus accumulation, but this was not observed. Dao et al. found that using chelators could help in removing phosphorus from soil[19]. In fact, it was noted that there seemed to be a slight inhibitory effect associated with the addition of any chelator at any concentration. Again no trend was observed that showed phosphorus accumulation was higher in one grass type. An unexpected phenomenon did occur in the presence of the chelators. In all chelators and in all concentrations the biomass of the grass was vastly affected (Figures 8 and 9). In each case there was an increase in biomass exceeding 100% over the control groups. Previous studies in our laboratory have showed that Marshall grass grew better than did Gulf grass[17,18]. This trend was not observed in our experiments. There was no overriding difference in the biomasses between the two grasses. It was seen that Gulf had the highest increase in biomass in the presence of 0.3 mM EDTA (Figure 8), and Marshall grass benefited the most from 0.3 mM DTPAH (Figure 7).

Overall, the growth patterns favored Marshall, but P uptake was favored in Gulf ryegrass. The pattern seen here was similar to observations made by Sharma in 2004 and 2005. It was initially hoped that the addition of chelators would increase the assimilation of P into the plants. This was not observed; in fact, the opposite was true for Gulf ryegrass (Figure 4 and 5). As for Marshall, the P accumulation was equal to or slightly greater than the control (Figure 6 and 7). The biomasses reported here are the percent increase in biomass between the initial weight of the plants and the final weight of the

plants, on a per-test tube basis. The biomasses of the plants were vastly affected. In both Gulf and Marshall there were increases in biomasses exceeding 185% over control when DTPA was added to the poultry-litter solution (Figure 8). It was also noticed that there was an overall inverse relationship to the percent increase in biomass and the concentration of the chelator. In every chelator and in all concentrations there was a significant boost to the plants growth. This increase was speculated to be the result of the chelating agents assisting the plants in acquiring micro and trace elements more efficiently[20]. The amount of phosphorus removed by plants through the increased biomass did not offset the decrease in phosphorus acquisition when the chelators were added. As a result, it was determined that the application of chelators is not a viable management strategy for decreasing the levels of phosphorus in the soil.

Figures 11 and 12, which show the phosphorus acquisition from poultry-litter-amended soil, indicate that a maximum uptake occurred when the concentration was at 50 grams. The levels for both grasses fell below the suggested levels for hyper-accumulation (7 g P/Kg Gulf, 6 g P/Kg Marshall), but it should be noted that the intra-soil interactions may have further incapacitated the available phosphorus. Soil native to the areas around Western Kentucky University tend to be high in iron (personal communication, Dr. Becky Gilfillen), and studies have been done which show that iron can immobilize phosphorus. Also the concentration of poultry litter mixed in the soil was low when compared to the volume of soil (1:30). The volume of soil was also a problem; the plant roots tended to mesh and grow up against the edge of the container, decreasing their exposure to the poultry litter in the center of the pot. Although the uptake was not as high as expected, the percent increase in phosphorus removal for Gulf ryegrass was

approximately 30% in the 50 g poultry litter per Kg soil and 20% in the 10 g poultry litter per Kg soil. In Gulf grass we observed that as the concentration of poultry litter increased, the ratio of shoot-to-root phosphorus approached 1.0. In Marshall grass this ratio was higher than 1.0 in all categories except the 10g poultry litter per Kg soil (Table 1). Higher accumulation of P in shoots than roots could be considered advantageous for removal of P contaminated sites.

CONCLUSIONS

We report that both grass types can accumulate as high as 1.1% (11 g P/Kg tissue) in their shoot tissues. The addition of chelators did not enhance the uptake potential of either grass type; it did, however, enhance the phosphorus-removal potential through the increase in biomass. The addition of chelators fit part of Delorme's postulate that more phosphorus could be removed via increases in biomass, but the decrease in phosphorus uptake exceeded the amount removed through the biomass. We observed that both grass types could tolerate up to 2.5% (25 g poultry litter/L) in aqueous solution, and that 1% (10 g poultry litter/L) was the optimum concentration in hydroponic systems.

Our studies favorably showed the application of *Lolium multiflorum* as a potential bioremediator of soil phosphorus. However, all of our experiments were conducted in the controlled environment of a laboratory. The potential uptake by *L. multiflorum* in the field setting remains to be determined. One area yet to be studied is multiple harvests of the grass from the poultry-litter-enriched soils. In a good year 3-5 harvests of the grass could be collected from the same plot, and the over-time removal capacity could increase the amount of soil phosphorus removed from the site. Another potential variable that needs to be investigated is adding chemicals that would aid the plant by converting the organic phosphorus into orthophosphate. Also

the grasses have the benefit of having shoot-to-root ratios around 1.0, making them more ideal for harvesting the above-ground biomass.

There is a great deal of potential for the use of *Lolium multiflorum* as a means of reducing soil phosphorus loads. Protocols need to be developed so that the phosphorus-removal capacity of the plants can be optimized either by modifying the genes that are utilized for phosphorus uptake, or by increasing the above-ground biomass of the plants.

GRAPHS AND FIGURES

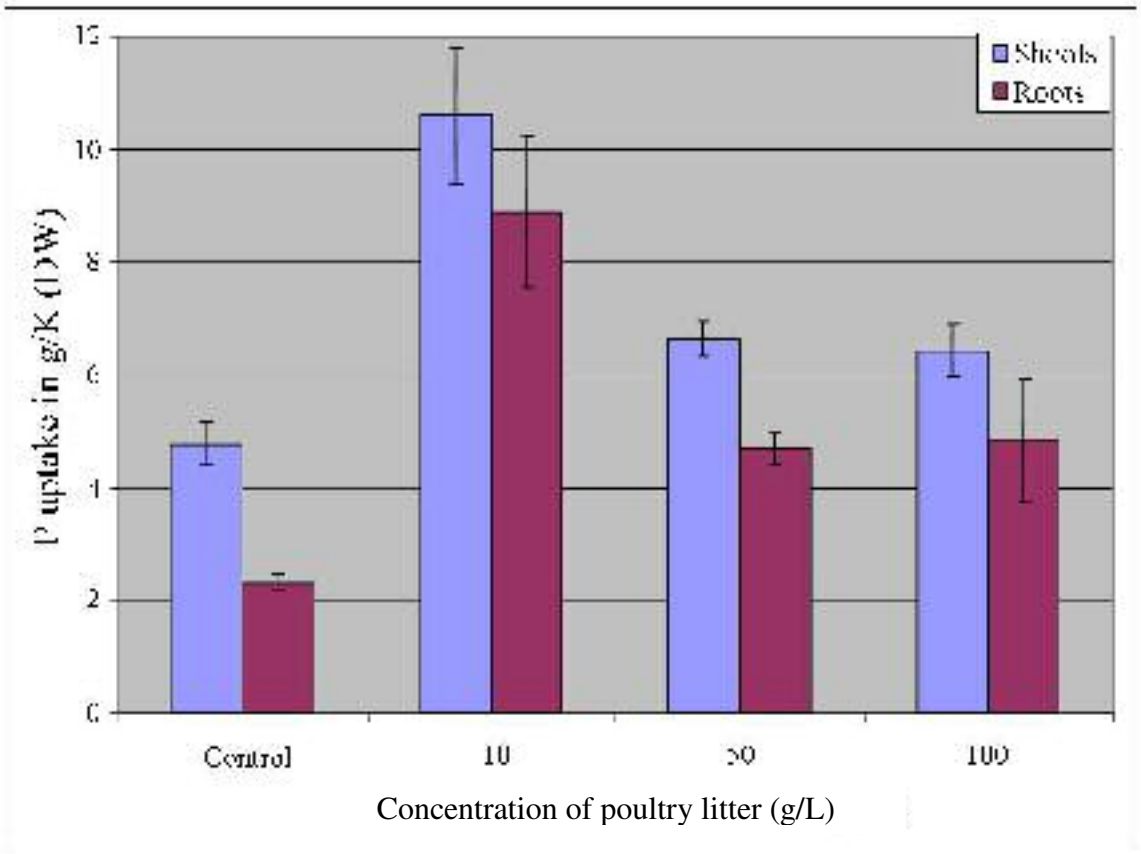

Figure 1. Accumulation of Phosphorus by Gulf Ryegrass grown in aqueous poultry litter (0-100 g/L) for 15 days. Values represent four replicates ± standard error of the mean.

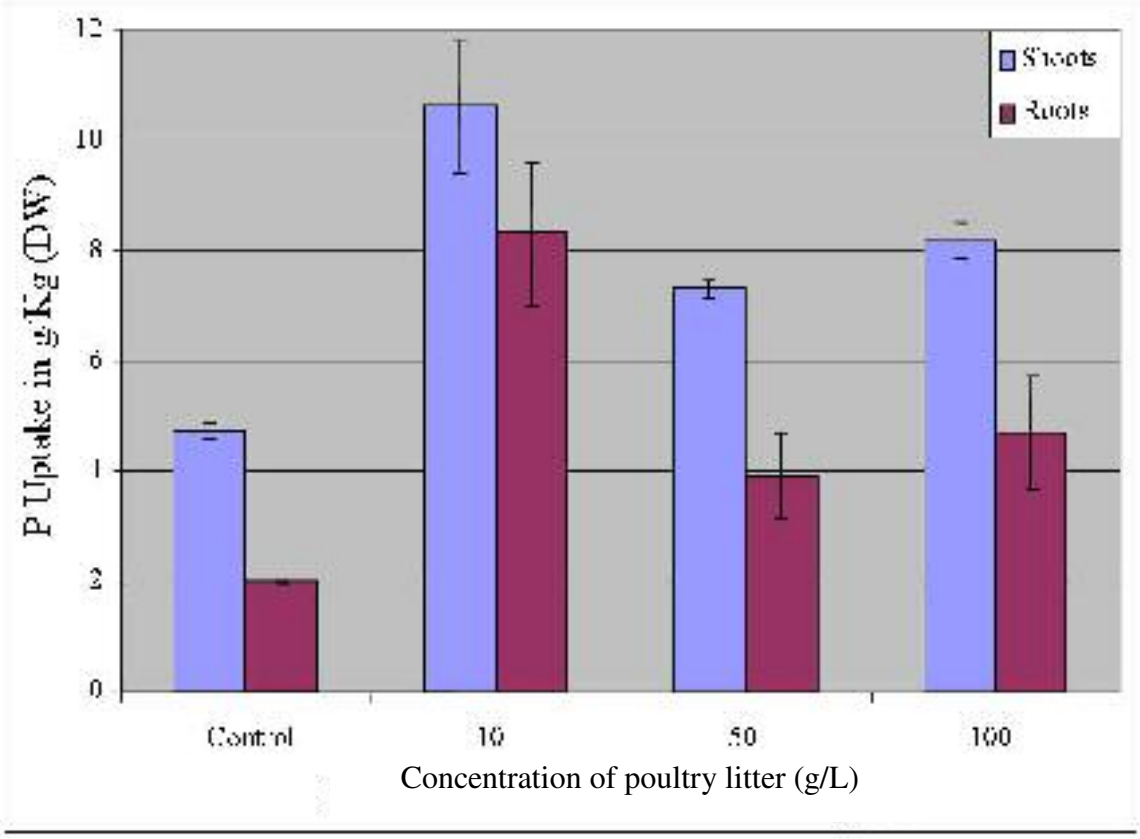

Figure 2. Accumulation of Phosphorus by Marshall Ryegrass grown in aqueous poultry litter (0-100 g/L) for 15 days. Values represent four replicates ± standard error of the mean.

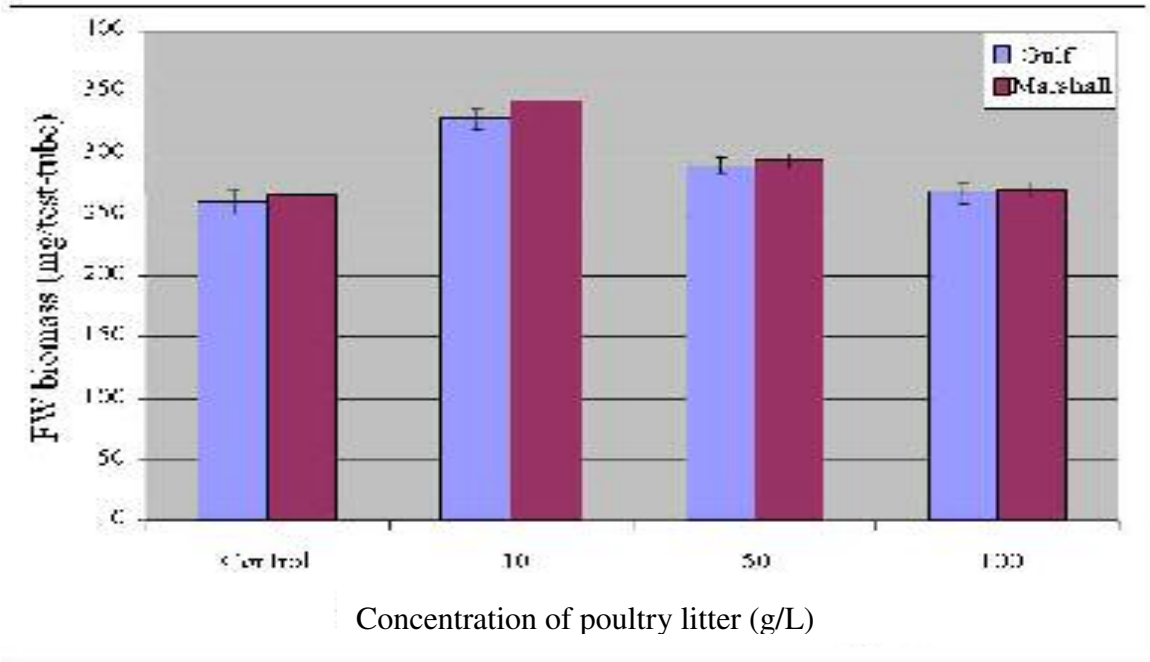

Figure 3. Fresh-weight biomass of Gulf and Marshall Ryegrass grown in aqueous poultry litter (0-100 g/L) for 15 days. Values represent four replicates ± standard error of the mean.

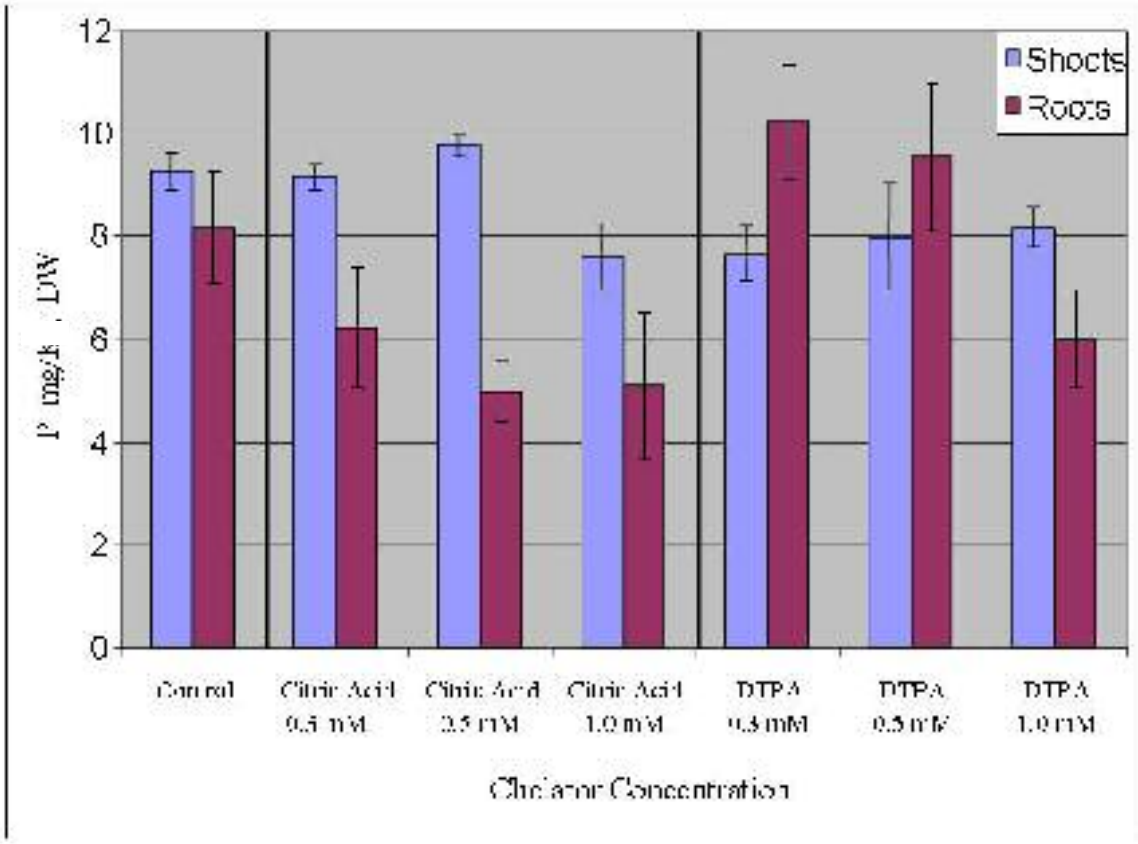

Figure 4. Accumulation of Phosphorus by Gulf Ryegrass grown in aqueous poultry litter (10 g/L), plus different chelators (0.3 mM to 1.0 mM), for 15 days. Values represent four replicates ± standard error of the mean.

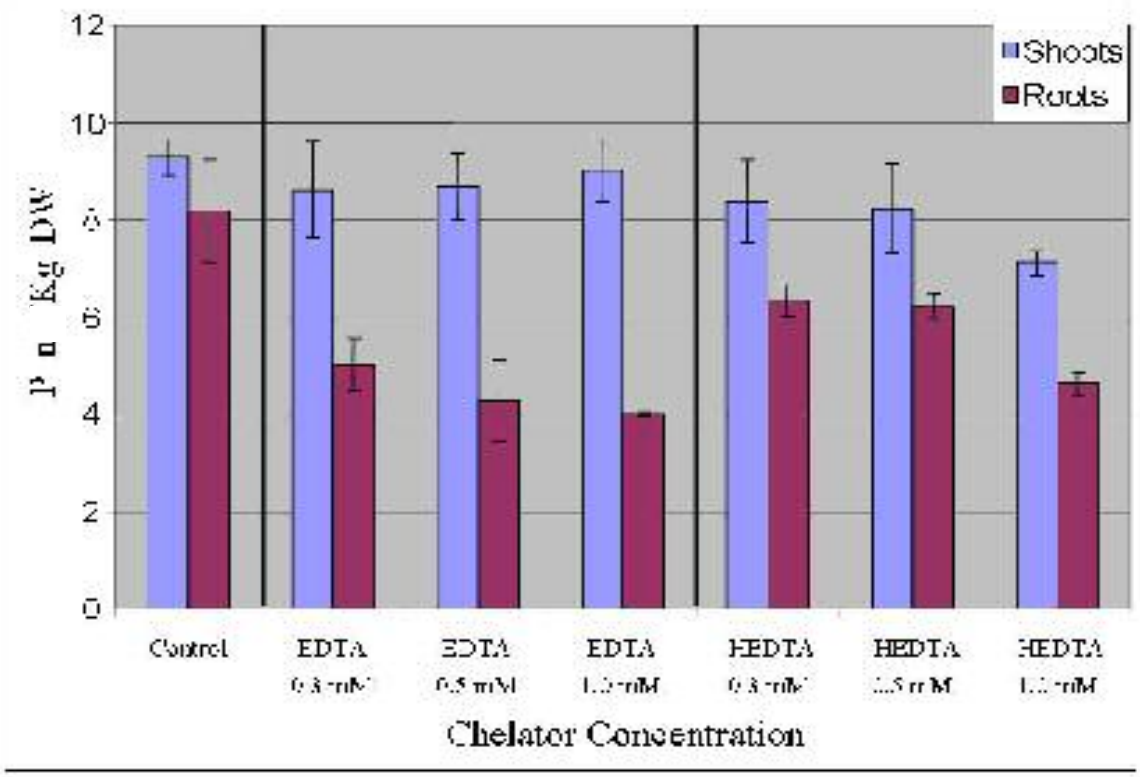

Figure 5. Accumulation of Phosphorus by Gulf Ryegrass grown in aqueous poultry litter (10 g/L) for 15 days, plus different Chelators (0.3 to 1.0 mM). Values represent four replicates ± standard error of the mean.

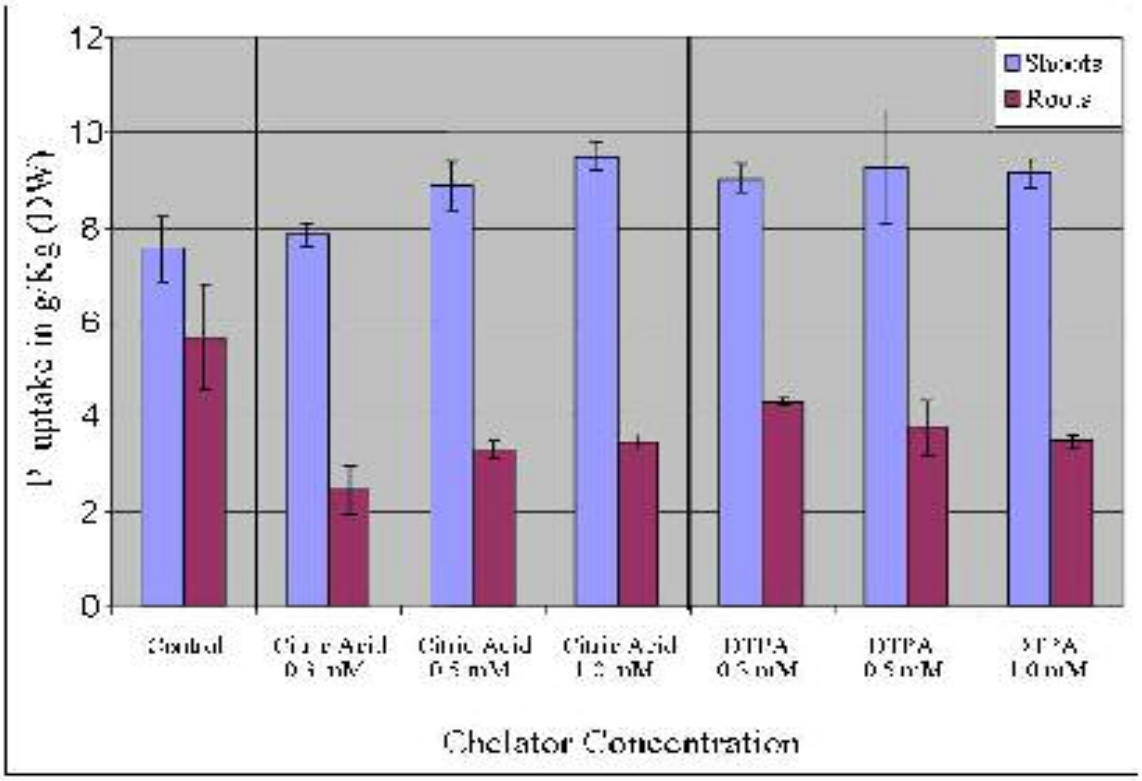

Figure 6. Accumulation of Phosphorus by Marshall Ryegrass from media containing aqueous poultry litter (10 g/L) for 15 days, plus different Chelators (0.3 to 1.0 mM). Values represent four replicates ± standard error of the mean.

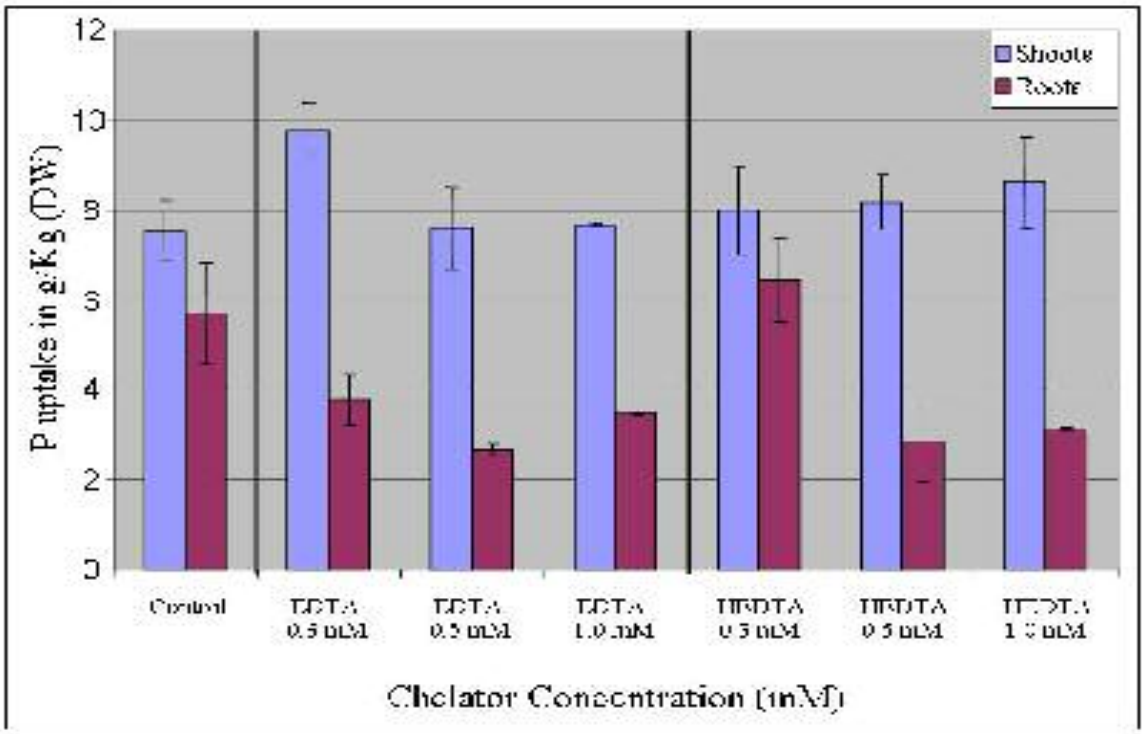

Figure 7. Accumulation of phosphorus by Marshall Ryegrass from media containing aqueous poultry litter (10 g/L) for 15 days, plus different chelators (0.3 to 1.0 mM). Values represent four replicates ± standard error of the mean.

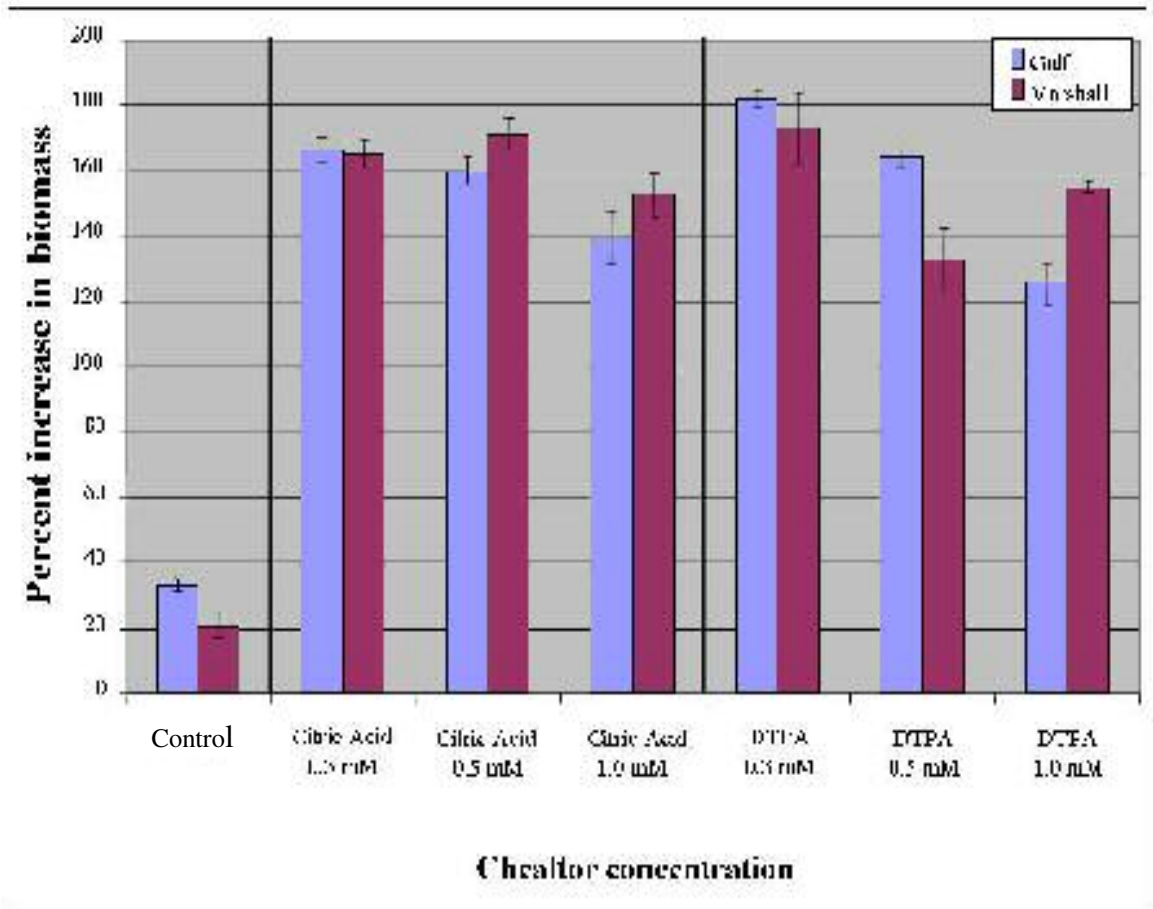

Figure 8. Percent increase in biomass of Gulf (blue bars) and Marshall (Red bars) Ryegrasses grown in aqueous poultry litter (10 g/L) for 15 days, plus different Chelators (0.3 to 1.0 mM). Values represent four replicates ± standard error of the mean.

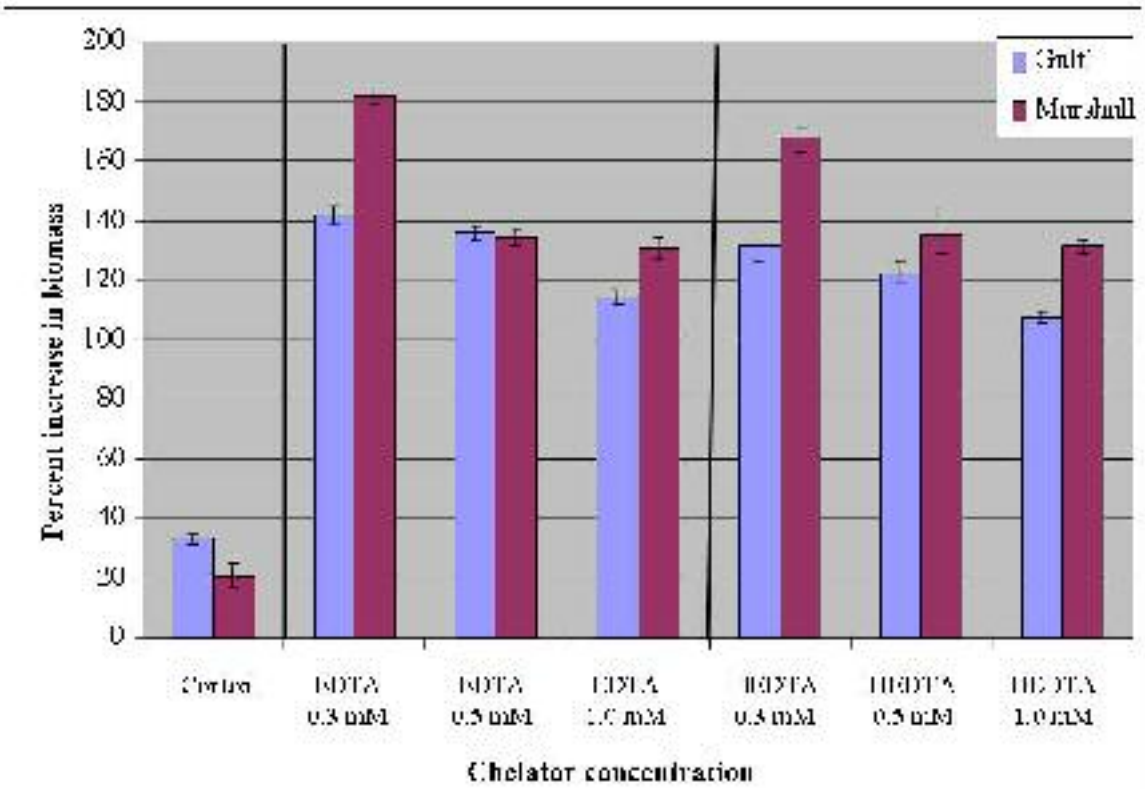

Figure 9. Percent increase in biomass of Gulf (blue bars) and Marshall (Red bars) Ryegrasses grown in aqueous poultry litter (10 g/L) for 15 days, plus different Chelators (0.3 to 1.0 mM). Values represent four replicates ± standard error of the mean.

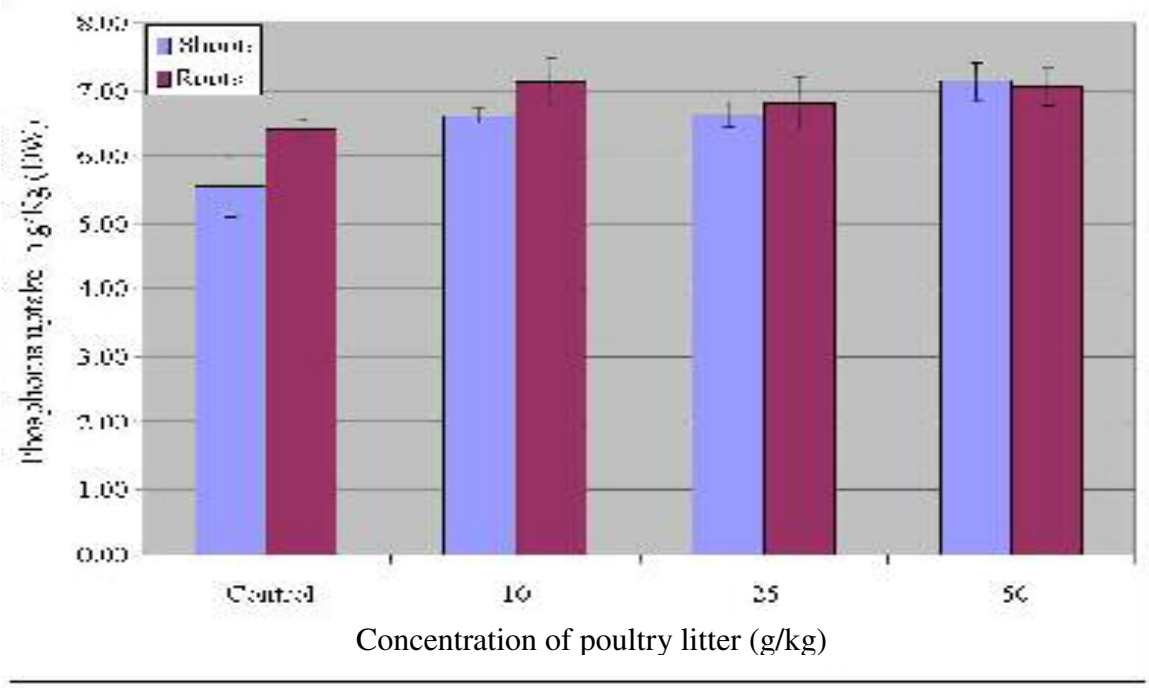

Figure 10. Accumulation of Phosphorus by Gulf Ryegrass grown in soil amended with poultry litter (0-50 g/Kg) for 5 weeks. Values represent four replicates ± standard error of the mean.

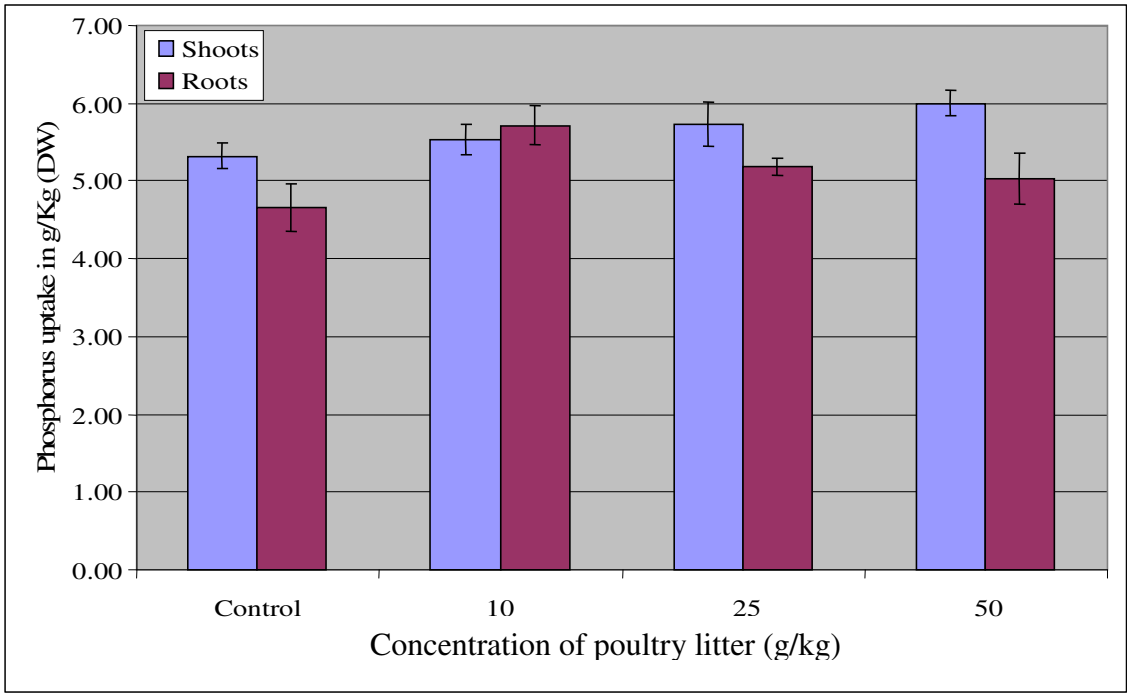

Figure 11. Accumulation of Phosphorus by Marshall Ryegrass grown in soil amended with poultry litter (0-50 g/Kg) for 5 weeks. Values represent four replicates ± standard error of the mean.

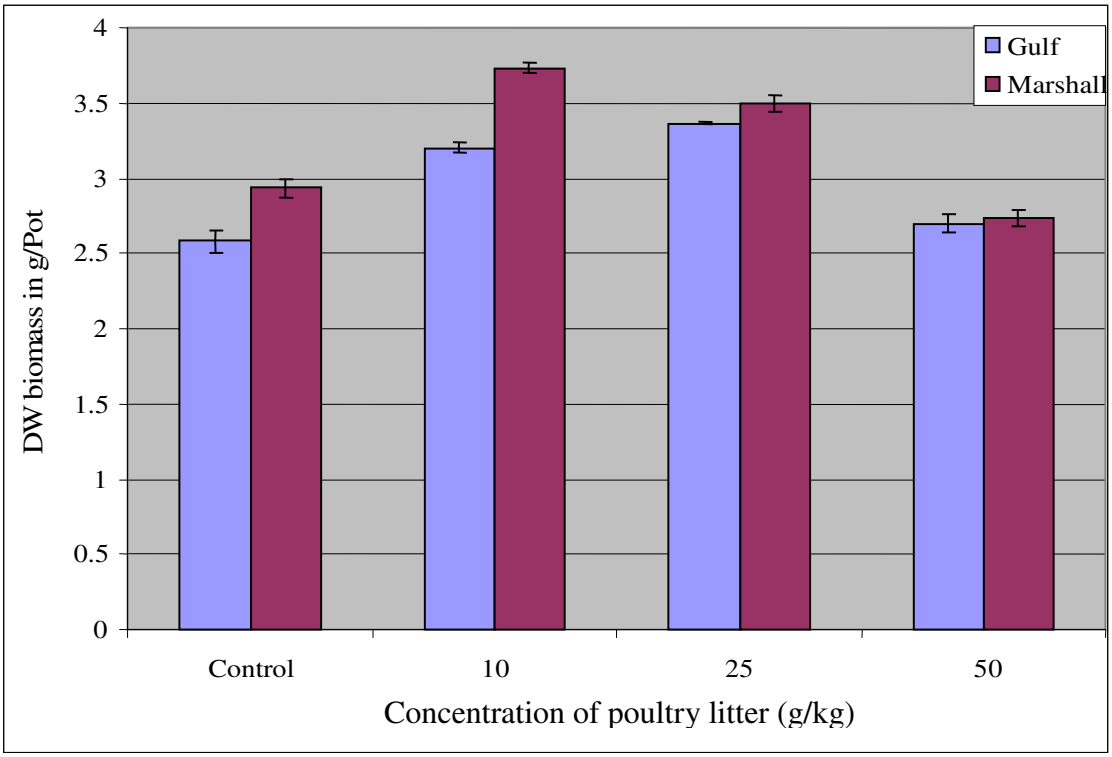

Figure 12. Dry weight biomass Ryegrass grown in soil amended with poultry litter (0-50 g/Kg) for 5 weeks. Values represent four replicates ± standard error of the mean.

Table 1. Phosphorus accumulation in root and shoot by Gulf and Marshall Ryegrasses grown in soil with different concentrations of poultry litter to determine phytoremediation potential.

Concentration of Poultry Litter (g/Kg)	Shoot P	Root P	Shoot/Root P
	----------g/kg dry weight (± S.E.)----------		
Gulf			
Control	5.55±0.44	6.49±0.17	0.87
10 g/kg	6.61±0.11	7.12±0.38	0.93
25 g/kg	6.64±0.28	6.81±0.38	0.98
50 g/kg	7.14±0.28	7.05±0.28	1.1
Marshall			
Control	5.32±0.16	4.66±0.29	1.2
10 g/kg	5.53±0.18	5.72±0.24	0.97
25 g/kg	5.74±0.28	5.19±0.11	1.12

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