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De-inking Sludge and Phosphorus Effects on Growth and Symbiotic Dinitrogen Fixation in Forage Legumes

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Abstract: The de-inking process produces a waste by-product, called de-inking paper sludge (DS), that contains paper fibers, clay particles and inks and high carbon (C) concentrations combined with low nitrogen (N) and phosphorus (P) concentrations. The use of high rates of DS to increase the soil organic matter thus requires provision of high rates of N and P for adequate plant growth. Using dinitrogen (N_2)-fixing forage legumes is an alternative to N fertilization under such circumstances. In a greenhouse study, DS rates of 0, 50 or 100 Mg ha^{-1} and five rates of P (40, 80, 120, 160, or 200 kg P_2O_5 ha^{-1}) were applied on two soil types, a clay loam (Pintendre) and a silty clay loam (St-Augustin). Nitrogen uptake and symbiotic N_2 fixation (SNF) were estimated in alfalfa (*Medicago sativa* L.), sweetclover (*Melilotus officinalis* L.) and red clover (*Trifolium pratense* L.); Bromegrass (*Bromus inermis* L.) and alfalfa ineffective for N_2 fixation were used as the reference (non-N_2 fixing) crops. Atmospheric N_2 fixation was estimated by natural abundance of ^{15}N ($\delta^{15}N$). Under controlled conditions, high rates of DS substantially reduced $\delta^{15}N$ values, particularly with high rates of P. In addition, N uptake of legumes generally increased with increased P concentrations and it peaked with 120 or 160 kg P_2O_5 ha^{-1}. Correlated with the trends observed with $\delta^{15}N$ values and it peaked with 120 or 160 kg P_2O_5 ha^{-1}. Present results showed that under high rates of application of DS and adequate P supply, forage legumes fixed more atmospheric N_2. $\delta^{15}N$ can be a good indicator of SNF under the above-mentioned conditions.

Key words: De-inking sludge, symbiotic fixation, forage legumes, phosphorus availability

INTRODUCTION

Novel, economically feasible and environmentally sound solutions are required to optimize land use, because of the many demands placed on the world by an ever increasing food requirement (Barrett-Lennard *et al*., 1986). Applying industrial wastes, such as pulp and paper sludge on agricultural lands, has been found to increase the chemical and physical properties of soil (Sauerbeck, 1987; Araújo *et al*., 2007). The de-inking process produces a waste by-product, De-inking Sludge (DS), that contains mainly paper fibers, clay particles and inks, which may be very beneficial as potential organic soil amendments and conditioners (Barclay, 1991; NCASI, 1991; Quebec Ministry of the Environment, 1984). The application of DS on fields has to be adjusted according to the exact composition of the sludge, the types of soil present and the type of crop being grown. The chemical composition of the sludge can vary considerably due to the origin of the recycled paper and different processes used by the paper industry (NCASI, 1984). Generally, the concentration of Ca in DS is high and macronutrient concentrations are relatively low, ranging from 0.14% to 4.1% N and 0.001-2.54% P, on dry-mass basis (NCASI, 1984; Beauchamp *et al*., 1998).

Therefore, a potential limitation for the use of fresh DS as soil amendment is the possibility of N and P deficiencies for adequate plant nutrition and growth, according to its relatively high C:N and C:P ratios which cause N and P immobilization by microorganisms (Tisdale *et al*., 1993; Fierro *et al*., 1999; García *et al*., 2003). The role and requirements of phosphorus with regards to maintaining healthy plants are well documented. Marschner (1995) observed a greater increase in nodule dry weight relative to shoot or root dry weight by increasing phosphorus availability. In dinitrogen-fixing plants, inadequate phosphorus supplies have been linked with nitrogen deficiencies (Dadson and Acquaah, 1984; Fabre and Planchon, 2000). In a greenhouse study (Fierro *et al*., 1997), all legume species, grown on soil-DS mixtures, showed an increase in shoot DM as P concentration increased.

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Under greenhouse conditions, medic (*Medicago truncatula*) plants assimilated 80% more soil N than wheat at a high rate of P (150 kg P_2O_5 ha^{-1}) and soil residues incorporation (Elabbadi *et al*., 1996). In their study, phosphorus treatments increased significantly the amount of nitrogen derived from atmosphere.

Although several studies have examined paper sludge mixtures in crop production (Chong *et al*., 1987; Chong, 1993; Chong and Cline, 1993; Fierro *et al*., 1997), the information is scarce on the effect of P rates on growth and symbiotic dinitrogen fixation of forage legume crops under high rates of application of DS without supplemental N, which was the first objective of the present study (Hansen and Vinther, 2001). On the other hand, the lack of fast and precise analytical techniques limited the use of the natural abundance of ^{15}N and it has so far only been used in a limited number of experiments (Bergersen *et al*., 1990; Ledgard and Peoples, 1988; Peoples *et al*., 1991; Sanford *et al*., 1994; Shearer and Kohl, 1986; Hogh-Jensen and Schjoerring, 1994; Wanek and Arndt, 2002). Therefore, the second aim of the present study was to determine the contribution of dinitrogen fixation in forage legumes by the natural abundance of ^{15}N under high rates of application of DS and greenhouse conditions.

MATERIALS AND METHODS

Experimental sites: This study was performed at greenhouse of Laval University, Québec (QC), Canada. The altitude, longitude and latitude, at which this greenhouse experiment was done, are 73 m, 71° 48′ 56′′ W and 46° 38′ 09′′ N, respectively.

Soil fertilizer and sowing application: In November 1995, soils were collected from the surface layer (0-25 cm) of two experimental fields from a freely drained, Silty Clay Loam Soil (SCLS), Typic Dystrochrept relatively low in organic matter (ca. 19.4 g C kg^{-1}) and from an imperfectly drained Clay-loam Soil (CLS), Typic Humaquept with an average of 27.0 g C kg^{-1}. The de-inking sludge was obtained from Les Produits Forestiers Daishowa Ltée' located in Quebec City. Samples of soil and sludge were oven-dried (at 60°C for 72 h) to determine the total and available mineral nutrients (Table 1). Prior to mixing, the samples were oven-dried (at 105°C for 48 h) to determine their moisture concentration.

The soils from the two sites were sieved (5 mm) prior to mixing with paper sludge. Then, de-inking sludge was passed through a Wiley Mill without screen prior to mixing with soils to homogenize particle size. Shredded sludge was mixed thoroughly with the soil at the rates of 0, 260 or 520 cm^3 per pot [equivalent to 0, 50 or 100 Mg DS (DM) ha^{-1}] (Table 2).

Table 1: Some physical and chemical characteristics of de-inking sludge and soils used in this study. Values are means of three determinations±standard error of means

Characteristics	De-inking sludge	Clay loam	Silty clay loam
pH	7.6±0.1	6.3±0.1	5.9±0.1
EC† (Fmho cm^{-1})	110±3	215±4	116±4
Bulk density (g cm^{-3})	0.14±0.01	1.47±0.11	1.47±0.09
water content (%)	50±2	-	-
Available elements (Fg g^{-1})			
P	35±1.3	10±1	62±2
K	52±1	76±1	171±3
Ca	7152±45	2287±62	1960±39
Mg	285±3	158±3	66±2
Total elements (Fg g^{-1})			
P	589±12	978±8	813±11
K	372±5	6897±32	10739±164
Ca	22894±543	4000±38	2160±34
Mg	2904±43	7320±85	9212±72
Al	28415±853	33078±670	34647±722
Cu	105.5±4.6	5.7±0.4	7.1±0.5
Zn	72.1±3.1	37.9±2.6	36.6±1.9
Fe	1230±36	27201±862	25784±1100
Total C (%)	36.4±0.4	1.7±0.1	1.8±0.1

†, Electrical conductivity

Table 2: The different levels of the sludge-soil mixture in pot experiment

Material (cm^3)	DS† (Mg dry matter ha^{-1}) DS_0	DS_{50}	DS_{100}
DS	0	240	400
Perlite	280	280	280
Soil	1120	880	710
Total	1400	1400	1400

†DS, de-inking sludge

Different rates of phosphorus (P) were supplied by applying KH_2PO_4 before sowing (60% of total) and one month after planting (40% of total) at rates equivalent to 40, 80, 120, 160 or 200 kg P_2O_5 ha^{-1}. Potassium chloride (KCl) was applied at varying rates to maintain potassium (K) concentration similar in all treatments. Top irrigation with tap water was effected to allow only minor leaching that was contained in the saucer; therefore, no mineral nutrients were lost by leaching.

Prior to the preparation of substrate mixtures, a composite sample of each of the basic substrate components was made to determine the available P, K, Ca and Mg (Mehlich III, CPVQ; 1993); totals of P, K, Ca, Mg, aluminum (Al), Fe, Zn and Cu (acid digestion, Olsen and Sommers, 1982) and totals of C and N by dry combustion (CNS-1000 analyzer, LECO Co., St. Joseph, MI). Soil pH and electrical conductivity were determined in a 3:1 (v:v) water:substrate slurry (Sparks *et al*., 1996) data shown in Table 1.

The forage legumes *Medicago sativa* (L.) cv. Saranac (alfalfa), *Trifolium pratense* (L.) cv. Florex (red clover), *Melilotus officinalis* (L.) cv. Norgold (sweetclover) and the non-fixing reference crops *Bromus inermis* (L.) cv. Saratoga (bromegrass) and *Medicago sativa* (L.) cv. Saranac (ineffective alfalfa) (Barnes *et al*., 1990) were selected for this study.

Legumes were inoculated before sowing with either *Sinorhizobium meliloti* for sweetclover and alfalfa (De Lajudie *et al*., 1994) and *Rhizobium leguminosarum* biovar *trifolii* for red clover (Jordan, 1984).

Then they were seeded at an equivalent rate of 600 seeds m^{-2} [11 seeds pot^{-1} (12 kg ha^{-1})] in 1400 cm^3 plastic pots (14.5 cm diameter by 14.5 cm depth) with saucer, filled with: one of two soil types (silty clay loam or clay loam), mixed with one of three rates of DS equivalent to 0 (DS_0), 50 (DS_{50}), or 100 (DS_{100}) Mg dry matter ha^{-1} and receiving one of 5 P rates equivalent to 40 (P_{40}), 80 (P_{80}), 120 (P_{120}), 160 (P_{160}), or 200 (P_{200}) kg P_2O_5 ha^{-1}. The substrate mixtures as well as some physical and chemical characteristics of DS and basic components of substrate mixtures are shown in Table 1 and 2, respectively.

Sampling and preparation of plant material: Natural photoperiod at the beginning and at the end of this experiment were 10.1 and 13.7 h, respectively. A photoperiod of 16 h was provided with high pressure sodium lamps (PPFD = 200 μmol m^{-2} sec^{-1}; PL Light System Canada Inc., Beamsville, Ontario, Canada) and day/night temperatures of 26/18°C were maintained in the greenhouse during 70 days.

At the end of experiment (i.e., 70 days after planting), all above ground plant material was clipped in each pot and dried in a forced-air oven at 60°C until constant weight. The plant samples were finely ground with a Retsch Centrifugal Mill Model ZM-1 (Brinkmann Instruments Canada Ltd., Rexdale, Ontario, Canada) fitted with a 0.5 mm ring sieve. About 10 g of representative subsamples were further ground with a ball mill (Retsch Mixer Mill Model MM-2; Brinkmann Instruments Canada Ltd., Rexdale, Ontario, Canada) to obtain the powder sample and analyzed for percent ^{15}N. The preparation of ^{15}N samples was done with the precautions necessary when measurements of small differences in the abundance of ^{15}N are to follow (Peoples *et al*., 1991; Eriksen and Hogh-Jensen, 1998).

Determination of dinitrogen fixation: Values of natural abundance of ^{15}N ($\delta^{15}N$; Eriksen and Hogh-Jensen, 1998) of above-ground plant material was also estimated to determine their trends on N_2 fixation dependency in the presence of DS.

Measurement of ^{15}N-natural abundance: There are small variations in the natural abundance of ^{15}N and ^{15}N-natural abundance values for the majority of biological systems are within the range 0.3630-0.3700 atom percent ^{15}N (Letolle, 1980).

The relevant conversion formula is:

$$\delta^{15}N(\%) = \left(\frac{\%\ ^{15}N\ \text{atom sample} - \%\ ^{15}N\ \text{atom air}}{\%\ ^{15}N\ \text{atom air}}\right) \times 1000$$

The following formula is used to calculate the proportion of plant nitrogen derived from atmosphere (Ndfa) by using natural variations in ^{15}N:

$$Ndfa\% = \left(\frac{\delta^{15}N\,nfs - \delta^{15}N\,fs}{\delta^{15}N\,nfs - B}\right) \times 100$$

where nfs refers to a non-N_2 fixing plant selected to match closely the studied legume in terms of uptake of soil sources of N, fs refers to N_2-fixing system (nodulating legume) and factor B refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking combined N (Unkovich *et al*., 1994). In this experiment, bromegrass and ineffective alfalfa were used as the reference crops.

Due to the experimental conditions, i.e., short duration of experiment under greenhouse conditions, low quantity of soil (and thus, soil N) in pots, coherent estimates of Ndfa % could not be obtained by ^{15}N-natural abundance method. Thus $\delta^{15}N$ values of plant shoots were used as indicator of relative dependency on N_2 fixation for the various treatment combinations.

Determine B values of red clover, alfalfa and sweetclover: Effects of host plant on $\delta^{15}N$ of mentioned species solely dependent on fixed N_2 (B value) were assessed in minus-N silica (80% silica and 20% perlite) culture conducted in a growth chamber, each plant species comprising four 7 inch pots of 8 plants (inoculated with the appropriate rhizobia for each of two growth-periods (45 or 60 days). The nutrient solution contained macronutrients and trace elements (Chalifour and Nelson, 1987).

At 45 or 60 days after planting, shoots were harvested, bulked on a per pot basis, dried, ground and analyzed for $\delta^{15}N$ as described previously.

Temperature regimes in the growth chamber involved nighttime (8 h) minima of 16°C and daytime (16 h) maxima of 25°C.

The average B values obtained from plant materials clipped 45 or 60 days after planting, were 0.33% for sweetclover, -0.24‰ for alfalfa and 0.15‰ for red clover. B values for each plant species did not change with time of harvest.

Statistical analyses: The experiment was a three-factor factorial in a Randomized Complete Block Design (RCBD)

with four replicates. The treatments consisted of three rates of de-inking sludge (for each soil type) and five rates of P (for forage species: red clover, alfalfa, sweetclover, bromegrass and IN alfalfa). For determining the percentages of N derived from atmosphere, only the three specified N_2-fixing species were considered. The General Linear Models Procedure (GLM) of the SAS statistical package (Release 6.12, Statistical Analysis System Institute Inc., 1996) was used to test the significance of the associations between each dependent variable and the treatments. Interpretation of statistical analyses was done on interactions, when these were significant. Scatter plots of the residuals from the respective statistical models as well as Bartlett's test (Steel and Torrie, 1980) were used to test homogeneity of the experimental error variances and to determine if data transformations were required. Due to heterogeneity of pooled error variances between the soils of the two sites for nearly all of dependent variables (Gomez and Gomez, 1984), statistical analyses were effected and presented separately for each soil type. Differences among treatments were determined by simple, first-order and second-Order class and trend contrasts (Little and Hills, 1978). F-values were considered significant at the 10% level as described by Steel and Torrie (1980) for the small experiments.

RESULTS

Effect of DS and P rates on N uptake of forage species: On both soil types, forage species showed differential responses in N uptake with DS application (DS × Species, Table 3 and 4) which were mainly due to the differences between N_2-fixing and non-N_2 fixing species and among N_2-fixing crops. Moreover, on the CLS, the effects of P rates on N uptake were not similar among forage crops (Species × P, Table 3 and 4), which were mainly due to the different effects between N_2-fixing and non-N_2 fixing crops and also between alfalfa and sweetclover.

For both soil types, N uptake of non-N_2 fixing plants decreased strongly with DS application (DS × Species, Table 3 and 4). On the CLS, N uptake of bromegrass and IN alfalfa were reduced by DS. Bromegrass N uptake was affected similarly by DS_{50} and DS_{100}, whereas N uptake of IN alfalfa continued to decrease at DS_{100} (DS_Q x Bromus vs IN Alfalfa, Table 3 and 4). There was a slight increase in N uptake of bromegrass with P addition, at DS_0 and DS_{50} particularly, while no such increase was observed for IN alfalfa (Bromus vs IN Alfalfa x P_L, Table 3 and 4). On the other hand, on the SCLS, N uptake of bromegrass and IN alfalfa were reduced similarly by DS (Table 3 and 4). On the CLS, N uptake of N_2-fixing plants were not so strongly reduced compared to non-N_2 fixing plants (Table 3 and 4).

Table 3: Analyses of variance for nitrogen uptake of forage species as affected by different rates of de-inking sludge and phosphorus on clay loam (Pintendre) and silty clay loam (St-Augustin) soils

		N uptake (mg pot^{-1}) probability	
Source of variation	df†	Clay loam	Silty clay loam
Block	3	ns‡	ns
De-inking sludge (DS)	2	ns	0.0001
DS_L §	1	ns	0.0001
DS_Q ¶	1	ns	0.05
Species	4	0.0001	0.0001
Bromus vs IN alfalfa††	1	0.05	0.0009
Reference vs fixing crop	1	0.0001	0.0001
Alfalfa vs sweetclover	1	0.0001	0.0001
Alfalfa vs red clover	1	0.0001	0.0001
Phosphorus (P)	4	0.0001	ns
P_L ‡‡	1	0.0001	ns
P_Q §§	1	0.0001	ns
DS X Species	8	0.03	0.0001
DS_L × Bromus vs IN alfalfa	1	ns	ns
DS_L × Reference vs fixing crop	1	0.008	0.0001
DS_L × Alfalfa vs sweetclover	1	ns	0.03
DS_L × Alfalfa vs red clover	1	ns	ns
DS_Q × Bromus vs IN alfalfa	1	0.07	ns
DS_Q × Reference vs fixing crop	1	0.001	0.01
DS_Q × Alfalfa vs sweetclover	1	0.002	0.07
DS_Q × Alfalfa vs red clover	1	ns	ns
DS X P	8	ns	ns
DS_L × P_L	1	ns	ns
DS_L × P_Q	1	ns	ns
DS_Q × P_L	1	ns	ns
DS_Q × P_Q	1	ns	ns
Species × P	16	0.0001	ns
Bromus vs IN alfalfa × P_L	1	0.001	ns
Reference vs fixing crop × P_L	1	0.0001	ns
Alfalfa vs sweetclover × P_L	1	0.0001	ns
Alfalfa vs red clover × P_L	1	0.005	ns
Bromus vs IN alfalfa × P_Q	1	ns	ns
Reference vs fixing crop × P_Q	1	ns	ns
Alfalfa vs sweetclover × P_Q	1	ns	ns
Alfalfa vs red clover × P_Q	1	ns	ns
DS × Species × P	32	ns	ns
Error	222		
CV (%) ¶¶		27.9	22.3

† df degree of freedom, ‡ ns, not significant, § DS_L, Linear effect of de-inking sludge, ¶ DS_Q, Quadratic effect of de-inking sludge, ††IN alfalfa, ineffective alfalfa, ‡‡P_L, Linear effect of phosphorus, §§P_Q, Quadratic effect of phosphorus, ¶¶CV, Coefficient of variation

De-inking sludge affected N uptake of alfalfa and red clover similarly, while sweetclover was the least affected (DS_Q × Alfalfa vs Red clover: non significant, DS_Q × Alfalfa vs Sweetclover, Table 3 and 4).

On the CLS, while P had very slight or no effect on N uptake of non-N_2 fixing species, P increased N uptake of N_2-fixing species and alleviated the inhibitory effect of DS on N uptake Table 3 and 4). The enhancement of N uptake by P was higher in red clover than in alfalfa (Alfalfa vs Red clover × P_L, Tables 3 and 4). Because N uptake of sweetclover was slightly or not reduced by DS, the response to P addition was smaller than for alfalfa or red clover (Alfalfa vs Sweetclover × P_L, Table 3 and 4). On the other hand, on the SCLS, N uptake did not vary with P addition.

Table 4: Nitrogen uptake *Medicago sativa* L. (alfalfa), *Trifolium pratense* L. (red clover), *Melilotus officinalis* L. (sweetclover), *Bromus inermis* L. (bromegrass) and *Medicago sativa* (IN) (ineffective alfalfa) as affected by different rates of de-inking sludge and phosphorus on clay loam (Pintendre) and silty clay loam (St-Augustin) soils. Values are means of four determinations±standard error of means

			N uptake (mg pot^{-1})				
			Plant species				
Soil type	DS† (mg ha^{-1})	Phosphorus (kg ha^{-1})	*M. sativa*	*T. pratense*	*M. officinalis*	*B. inermis*	*M. sativa* (IN)
	0	40	102±12	151±88	222±32	10.6±1.6	18.3±3.4
		80	134±42	239±76	267±54	8.8±0.9	15.5±2.9
		120	129±6	380±27	268±37	9.5±1.1	16.0±1.7
		160	141±31	388±88	303±42	8.8±0.8	17.5±1.6
		200	186±34	348±130	265±61	8.7±0.6	41.8±4.3
Clay loam	50	40	51±13	91±20	290±10	1.0±0.2	0.4±0.1
		80	54±18	188±66	281±51	1.2±0.2	0.8±0.1
		120	150±23	248±100	337±67	2.0±0.2	0.4±0.1
		160	137±37	407±25	295±88	1.2±0.2	0.6±0.1
		200	120±6	420±76	357±114	2.2±0.1	1.0±0.1
	100	40	39±6	119±27	134±34	0.7±0.1	0.4±0.1
		80	81±22	219±41	240±43	0.3±0.1	0.5±0.1
		120	127±29	391±76	329±88	0.4±0.1	0.5±0.1
		160	158±15	360±149	283±61	1.0±0.2	0.4±0.1
		200	152±46	432±241	329±58	0.4±0.1	0.3±0.1
	0	40	82±22	226±47	183±29	12.6±1.8	18.9±2.4
		80	83±17	281±69	118±30	9.5±0.9	13.6±1.7
		120	111±13	300±29	162±10	15.9±4.2	11.4±2.1
		160	60±9	308±99	121±12	18.1±1.6	15.6±9.5
		200	107±44	366±67	148±41	14.0±1.3	14.2±1.3
Silty clay loam	50	40	48±4	126±62	182±46	2.0±0.4	0.6±0.2
		80	78±8	310±99	222±28	2.6±1.1	0.6±0.1
		120	79±69	292±70	264±27	3.4±2.4	0.4±0.8
		160	121±10	296±52	331±82	2.5±0.6	-‡
		200	120±95	351±74	308±44	1.2±1.1	0.5±0.7
	100	40	84±36	117±75	176±84	1.3±0.8	-
		80	70±49	258±121	257±69	1.4±0.2	-
		120	74±42	331±55	229±35	1.1±0.4	-
		160	97±48	344±177	303±29	1.4±0.4	-
		200	150±28	303±212	294±76	1.2±0.4	0.2±0.1

†DS, De-inking sludge, ‡Not enough sample for nitrogen analysis

Symbiotic N_2 fixation: Natural abundance of ^{15}N ($\delta^{15}N$ values) of above-ground biomass (shoots) was also estimated, to determine trends in the N_2 fixation dependency of forage legumes in the presence of DS.

Effect of DS and P rates on natural abundance of ^{15}N of forage species: On the SCLS, high rates of P generally decreased $\delta^{15}N$ of N_2-fixing plants, but mostly at DS_0 and not at DS_{50} or DS_{100}; the decreases generally peaked at P_{120} or P_{160} (Table 5 and 6). The decreases in $\delta^{15}N$ values upon P addition indicate an enhancement of N_2 fixation by P. Furthermore, N_2 fixation was more strongly enhanced by the application of DS than by P addition (Table 5 and 6). In alfalfa, $\delta^{15}N$ values generally decreased strongly at P_{160} and with increasing DS rates compared with the other P rates, while in red clover, the reducing effect peaked at P_{160} and DS_0 and peaked at P_{120} and DS_{100}. In sweetclover, $\delta^{15}N$ values decreased and peaked at P_{120} and in the presence of DS_{50} and DS_{100}.

For both soil types, DS affected the $\delta^{15}N$ of forage species differently, mainly according to the different effects between N_2-fixing species and reference crops (DS x Species, Table 5 and 6). The presence of DS led to stronger decreases in $\delta^{15}N$ values in reference crops than in N_2-fixing species for both soils [DS_L x Reference vs Fixing crop (both soils) and DS_Q x Reference vs Fixing crop (CLS), Table 5 and 6]. With reference crops, the presence of DS caused stronger decreases in $\delta^{15}N$ values of IN alfalfa than those of bromegrass [DS_L × Bromus vs IN alfalfa (both soils) and DS_Q × Bromus vs IN alfalfa (SCLS), Table 5].

On the SCLS, the responses of $\delta^{15}N$ to P addition were dependent on DS rate (DS x P, Table 5 and 6). This could be described by differential responses between N_2-fixing crops and reference crops. In general, decreasing effect of P on $\delta^{15}N$ values of N_2-fixing species peaked at P_{160} (with DS_0 and DS_{50}) or P_{120} (with DS_{100}), whereas such responses were not observed in reference crops. Also, on the SCLS, the responses of $\delta^{15}N$ varied among species and P addition (Species × P, Table 5 and 6), which were mostly due to different responses of bromegrass and IN alfalfa to P addition on the SCLS (Bromus vs IN Alfalfa × P_L, Table 5) with stronger decreases in $\delta^{15}N$ values of IN alfalfa than those in bromegrass.

Table 5: Analyses of variance for ^{15}N-natural abundance ($\delta^{15}N$) of above-ground biomass of forage species as affected by different rates of de-inking sludge and phosphorus on clay loam (Pintendre) and silty clay loam (St-Augustin) soils

	Probability		
	Clay loam		Silty clay loam
Source of variation	df†	$\delta^{15}N$	$\delta^{15}N$
Block	3	0.002	0.007
De-inking Sludge (DS)	2	0.0001	0.0001
DS_L‡	1	0.0001	0.0001
DS_Q§	1	0.0001	ns
Species	4	0.0001	0.0001
Bromus vs IN Alfalfa¶	1	0.08	0.0001
Reference vs Fixing crop	1	0.0001	0.0001
Alfalfa vs Sweetclover	1	0.0001	0.004
Alfalfa vs Red clover	1	0.001	0.02
Phosphorus (P)	4	ns	0.001
P_L††	1	ns	0.0001
P_Q‡‡	1	0.06	ns
DS × Species	8	0.0001	0.0001
DS_L × Bromus vs IN Alfalfa	1	0.05	0.05
DS_L × Reference vs Fixing crop	1	0.0001	0.0001
DS_L × Alfalfa vs Sweetclover	1	ns	ns
DS_L × Alfalfa vs Red clover	1	ns	ns
DS_Q × Bromus vs IN Alfalfa	1	ns	0.0001
DS_Q × Reference vs Fixing crop	1	0.008	ns
DS_Q × Alfalfa vs Sweetclover	1	ns	ns
DS_Q × Alfalfa vs Red clover	1	ns	ns
DS × P	8	ns	0.0001
DS_L × P_L	1	ns	0.02
DS_L × P_Q	1	ns	ns
DS_Q × P_L	1	ns	0.04
DS_Q × P_Q	1	ns	0.003
Species × P	16	ns	0.008
Bromus vs IN Alfalfa X P_L	1	ns	0.0001
Reference vs Fixing crop X P_L	1	ns	ns
Alfalfa vs Sweetclover X P_L	1	ns	ns
Alfalfa vs Red clover X P_L	1	ns	ns
Bromus vs IN Alfalfa X P_Q	1	ns	ns
Reference vs Fixing crop X P_Q	1	ns	ns
Alfalfa vs Sweetclover X P_Q	1	ns	ns
Alfalfa vs Red clover X P_Q	1	ns	ns
Species × P	32	ns	0.0001
Error	222		
CV (%)§§		22.9	27.9

†df, Degree of freedom, $\delta^{15}N$, Natural abundance of ^{15}N, ‡DS_L, Linear effect of de-inking sludge, ¶IN alfalfa, ineffective alfalfa, §§DS_Q, Quadratic effect of de-inking sludge, ‡‡P_Q, Quadratic effect of phosphorus. §§CV, Coefficient of variation, ††P_L, Linear effect of phosphorus; ns, not significant

Table 6: Totals of ^{15}N-natural abundance of above-ground biomass of *Medicago sativa* L. (alfalfa), *Melilotus officinalis*. (sweetclover), *Trifolium pratense* L. (red clover), *Medicago sativa* (IN) (ineffective alfalfa) and *Bromus inermis* L. bromegrass) as affected by different rates of de-inking sludge and phosphorus on clay loam (Pintendre) and silty clay loam (St-Augustin) soils. Values are means of four determinations±standard error of means

		Species									
		M. sativa		*M. officinalis*		*T. pratense*		*M. sativa* (IN)		*B. inermis*	
		Soil type									
DS (mg ha^{-1})	Phosphorus (kg ha^{-1})	Clay loam	Silty clay loam	Clay loam	Silty clay loam	Clay loam	Silty clay loam	Clay loam	Silty clay loam	Clay loam	Silty clay loam
		$\delta^{15}N$ (%)									
0	40	0.14±0.40	-1.14±0.00	0.23±0.05	-0.49±0.78	0.24±1.07	-0.17±0.41	4.03±0.00	5.78±1.98	10.37±6.92	9.25±0.99
	80	-0.23±0.57	-0.49±0.88	0.19±0.11	0.38±0.49	-0.40±0.74	0.07±0.14	15.84±10.1	7.85±3.20	9.52±7.50	10.39±2.86
	120	-0.18±0.13	-0.84±0.28	-0.25±0.04	-0.39±0.72	-0.80±1.17	-0.30±0.04	11.52±3.62	5.15±3.03	9.46±7.71	9.10±2.75
	160	-0.56±0.78	-2.02±0.52	-0.09±0.36	-0.19±0.42	-0.17±0.24	-0.61±0.35	5.70±9.26	-1.56±1.78	9.13±6.40	1.37±0.47
	200	-0.54±0.04	-0.77±0.27	-0.66±0.54	0.56±0.49	-0.23±0.97	-0.24±0.21	6.33±0.00	4.48±2.50	3.21±3.49	11.52±2.57
50	40	-0.73±0.69	-1.38±0.99	-0.34±0.00	-0.31±0.00	-0.48±0.49	-0.47±0.37	-0.14±0.00	0.57±1.07	2.44±0.00	0.73±0.80
	80	-1.46±0.90	-0.84±0.16	-0.44±0.10	-0.06±0.26	-0.49±0.62	-0.21±0.14	0.04±1.88	2.05±1.49	2.24±1.24	6.99±1.67
	120	-1.08±0.11	-0.67±0.60	-0.80±0.04	-0.86±0.00	-0.68±0.50	-0.46±0.10	0.69±0.00	-1.72±0.33	2.27±0.00	10.36±1.94
	160	-1.57±0.51	-1.32±0.01	-0.55±0.59	-0.72±0.01	-0.59±0.62	-0.51±0.19	-1.72±0.00	-0.15±0.17	1.99±0.06	6.29±0.76
	200	-1.09±0.14	-1.01±0.14	-0.29±0.04	-0.35±0.27	-0.54±0.00	-0.59±0.44	1.01±0.00	-1.33±0.21	2.28±0.08	8.72±1.24

Table 6: Continued

DS (mg ha^{-1})	Phosphorus (kg ha^{-1})	Species / Soil type: *M. sativa* Clay loam	*M. sativa* Silty clay loam	*M. officinalis* Clay loam	*M. officinalis* Silty clay loam	*T. pratense* Clay loam	*T. pratense* Silty clay loam	*M. sativa* (IN) Clay loam	*M. sativa* (IN) Silty clay loam	*B. inermis* Clay loam	*B. inermis* Silty clay loam
		δ^{15}N (%)									
100	40	-1.90±0.82	-1.07±0.28	-0.62±0.21	-0.36±0.37	-0.65±0.06	-0.27±0.18	-1.51±0.00	-2.11±0.00	3.37±0.78	0.52±1.67
	80	-1.24±0.39	-1.01±0.17	-0.74±0.25	-0.15±0.11	-0.76±0.00	-0.70±0.00	-8.00±0.00	-1.22±0.17	3.14±0.83	1.29±0.00
	120	-1.54±0.42	-1.11±0.38	-0.39±0.13	-0.41±0.15	-0.41±0.13	-1.23±0.42	-	-1.41±0.37	1.95±1.48	1.18±0.00
	160	-1.51±0.43	-1.26±0.65	-0.04±0.18	-0.33±0.20	-0.43±0.27	-0.48±0.13	0.75±0.00	-1.18±0.00	3.30±0.21	2.11±0.00
	200	-1.23±0.13	-1.07±0.20	-0.34±0.35	-0.31±0.25	-0.55±0.20	-0.41±0.35	1.37±0.00	-0.84±0.83	3.48±0.00	1.98±0.00

DISCUSSION

Effect of DS and P rates on N uptake of forage crops

Non-N_2 fixing species: On the CLS, N uptake of bromegrass and IN alfalfa were reduced by DS. The N uptake of bromegrass increased with P treatments and P application alleviated the inhibitory effect of DS on N uptake. On both soil types, decreasing effect of DS on N uptake by reference crops could be mainly due to the high C:N ratio of DS, low N availability and therefore, N deficiency with high rates of application of DS in non-N_2 fixing species. Similar trends were found by Feagley *et al.* (1994), who reported that N uptake by bermudagrass (*Cyanodon dactylon* L.) decreased with increases in papermill sludge rate.

N_2-fixing species: For both soils, N uptake of N_2-fixing species did not decrease strongly compared with reference crops. On the other hand, on the CLS, P application increased N uptake of these species and again, P alleviated the inhibitory effect of DS on N uptake by N_2-fixing species and it peaked at P_{120} or P_{160}. These results compare well with those of a greenhouse experiment with medic (*Medicago truncatula*) and wheat (*Triticum turgidum*) using different rates of P on the soil in which wheat residues (45.5 g plant material pot^{-1}) were incorporated (Elabbadi *et al.*, 1996; García *et al.*, 2003). In their experiment, P treatments increased uptake of soil N for both species.

In contrast, the results of a greenhouse study (Fierro *et al.*, 1997) on the effect of different rates of de-inking sludge and P on the growth of the legumes *Galega orientalis*, *Medicago lupulina* and *Melilotus officinalis* indicated no significant differences in shoot N concentrations, including unamended soil.

Symbiotic N_2 fixation

Effect of P and DS rates on δ^{15}N: On the SCLS, high rates of P decreased δ^{15}N of N_2-fixing species, but mostly at DS_0 and the decreases generally peaked at P_{120} or P_{160}. The negative effect of P on the δ^{15}N of N_2-fixing species indicated the enhancement effect of P on N_2 fixation. On the CLS, different effects of DS were observed between N_2-fixing species and the reference crops and DS led to stronger decreases in δ^{15}N values in reference crops compared with those in N_2-fixing species. Moreover, DS caused higher decreases in δ^{15}N values in bromegrass than those in alfalfa.

Reducing effect of DS and P treatments on the δ^{15}N values of N_2-fixing species could be mainly due to low soil N (non-availability of N with high rates of application of DS) and high dependency of these plants on symbiotic N_2 fixation (Haynes, 1980; Hogh-Jensen and Kristensen, 1995; Araújo *et al.*, 2007) and external P. Similarly, in a greenhouse study (Heckman and Kluchinski, 1995), soybean grown on tree leaf- or crop residue-amended soil (20 g residue kg^{-1} soil; C:N ratio of residues ranged between 17 and 75) exhibited temporary N deficiency until nodulation. Also, they reported that nonnodulating plants were severely N deficient and stunted as a consequence of N immobilization. In their study, nodulating soybean plants grown on leaf or crop residue-amended soil were more dependent on symbiotically fixed N and had lower dry matter yields than the control (unamended) soil. When leaves were composted, the problem of N immobilization was avoided and dry matter yield was not reduced. Also, the results of a greenhouse study (Croteau and Zibilske, 1998) on the effect of papermill sludge (at 25 g sludge kg^{-1} soil) on the growth of snap bean (*Phaseolus vulgaris*) showed the immobilization of N with sludge application.

De-inking sludge may increase the pH of the soils, due to the high concentrations of Ca and also pH of DS, which is alkaline (Trépanier *et al.*, 1996; Fierro *et al.*, 1999). These factors may be very effective in increasing nodule number, similar to liming, in common bean (Buerkert *et al.*, 1990) or alfalfa (Pijnenberg and Lie, 1990) and may have enhancing effects on N_2 fixation (Hansen and Vinther, 2001; Buerkert *et al.*, 1990; Pijnenberg and Lie, 1990).

In the present study, on the SCLS, high rates of P generally decreased $\delta^{15}N$ of N_2-fixing plants at DS_0 only; the decreases generally peaked at P_{120} or P_{160}. Moreover, application of P and DS may increase percentages of Ndfa and N uptake in forage legumes. Similarly, in a field experiment, McDonagh *et al.* (1995) found that the application of P and K fertilizers approximately doubled residue yields, with the combined addition of lime more than doubling these yields again, resulting in 88% of Ndfa in the legumes cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogaea*). In their experiment, the highest legume DM yield and N uptake were in the treatments where lime and P and K were applied, where DM yield was almost four times that of the unfertilized control treatment. McDonagh *et al.* (1995) indicated that the response when lime and P and K were applied was far in excess of the sum of individual responses. This could be attributed to effects of lime on the availability of P for plant uptake and the large response to lime when added together with P was probably due to the alleviation of P deficiency, resulting in a greater demand for Ca.

Suitability of the methods for determination of symbiotic N_2 fixation: Lack of inorganic N in soils can increase atmospheric N_2 fixation in legumes (Wanek and Arndt, 2002; Haynes, 1980; Hogh-Jensen and Schjoerring, 1994). Present results showed and confirmed the increasing effect of DS on N_2 fixation by forage legumes.

The interesting finding of this study is that, despite the fact that estimates of the percentages of Ndfa could not be obtained by the ^{15}N-natural abundance method, the trends in $\delta^{15}N$ values nevertheless allowed discrimination among P rates and DS rates (Broadbent *et al.*, 1982; Danso, 1986).

As proposed by McNeill *et al.* (1996) the natural abundance technique for estimating symbiotic N_2 fixation under field conditions is suggested as a potentially useful alternative to the ^{15}N-enrichment method or the TNDM. Based on the results of the present study, the ^{15}N-natural abundance method needs to be evaluated under field conditions and with application of an agronomic amendment such as DS.

CONCLUSIONS

The composition of DS varies considerably due to the origin of the recycled paper and to the making processes. The macronutrient concentrations, i.e., P and N, in de-inking sludge are generally low. Therefore, a potential limitation for the use of fresh DS as a soil amendment is the possibility of N and P deficiencies for adequate plant nutrition and growth.

The addition of P led to significant decreases in $\delta^{15}N$ values with both soils. High rates of P generally decreased $\delta^{15}N$ of N_2-fixing plants. Furthermore, N_2 fixation was generally more strongly enhanced by the application of DS than by P addition. In addition, high rates of DS substantially reduced $\delta^{15}N$ of forage legumes, due to N deficiency (non-availability of N with high rates of application of DS) and high dependency of these plants on symbiotic N_2 fixation and external P.

Finally, the present study revealed that fresh DS is a suitable soil amendment; for N_2-fixing legumes in the presence of high rates of DS and without supplemental nitrogen and according to P concentration in DS composition, P_{120} seems to be an optimum P rate for all DS rates.

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