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Influence of Added Nitrogen Interactions in Estimating Recovery Efficiency of Labeled Nitrogen

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ABSTRACT

The addition of N fertilizer to soil has been shown to stimulate the uptake of native soil N via a priming effect recently termed *added nitrogen interaction* (ANI). This ANI, due to pool substitution, can substantially affect the N-recovery efficiency (NRE) by plants as calculated by the ^{15}N isotopic dilution technique. We evaluated NRE in a pot study using 'Edwall' spring wheat (*Triticum aestivum* L.) with three soils (Palouse, a fine-silty, mixed, mesic Pachic Ultic Haploxeroll; Ritzville, a coarse-silty, mixed, mesic Calciorthidic Haploxeroll; and Shano, a coarse-silty, mixed mesic Xerollic Camborthid) of varying organic C (OC) levels and five levels of ^{15}N-labeled fertilizer. Data obtained at 60 d after emergence (DAE) showed that NRE generally increased with fertilizer additions, was lowest in the soil having the greatest OC, and ranged from 57 to 79%. The NRE values estimated by the isotopic method averaged 20% lower than those estimated by the difference method, although the two estimates were strongly related. Although the magnitude of ANI showed no direct relationship to OC levels, it was strongly related to N rates, OC, soil C/N ratio, and N lost, which together explained 73% of the relationship. The magnitude of occurrence of ANI appears to be influenced by several factors and thus may be soil specific. Regression of NRE with soil properties showed that NRE was influenced primarily by the same factors that influenced ANI. This study suggests that, for accurate comparisons of nitrogen recovery efficiency between soils or treatments using the isotopic method or for comparing methods of determining N-recovery efficiency, the role of added N interaction must be considered.

After several decades of extensive work on N-fertilizer use, there are still uncertainties in determining the NRE (Bock, 1984) by plants of the applied fertilizer N. The most common method for determining NRE is the difference method, in which the amount of fertilizer N taken up by the crop is calculated as the difference between fertilized and unfertilized plots. The assumption is that immobilization–mineralization and other N transformations during the course of the experiment are the same for both treated and control plots. It is known that this method may be vitiated by the priming effect, which results in increased uptake of N from soil organic matter in fertilized plots, causing an overestimation of NRE (Hauck and Bremner, 1976). The increased availability of soil N in fertilized plots has been attributed to: (i) stimulation of microbial activity by addition of N fertilizer (Westerman and Kurtz, 1973); (ii) nitrification of NH_4 and amide fertilizers, causing acid hydrolysis of soil organic substances (Turchin, 1964); (iii) changes in the plant's physiological processes induced by fertilizer N (Sapozhnikov et al., 1968); (iv) osmotic effects (Broadbent and Nakashima, 1971); and (v) increased root growth in fertilized plots (Olson and Swallow, 1984).

The isotopic dilution technique is used by many workers to determine the NRE by direct measurement of ^{15}N-labeled fertilizer taken up by the plant. However, this method is influenced by pool substitution of ^{15}N for ^{14}N, which can result in erroneous NRE estimations when substitution is not accounted for quantitatively. The labeled N acts as a substitute for unlabeled soil N that otherwise would have been abstracted from the pool during processes such as immobilization and denitrification (Jenkinson et al., 1985). This substitution leaves less ^{15}N available for plant uptake and so N recoveries estimated by this method may be low.

All such interactions (e.g., priming, pool substitution) that occur when fertilizer N is added to the soil and changes the soil N content in a given pool constitute ANI (Jenkinson et al., 1985). Quantitatively, ANI is measured as the difference in the uptake of soil-derived N between fertilized and control treatments.

Although several hypotheses have been proposed to explain the discrepancies between the two methods of estimating NRE, no quantitative relationship has been established between the soil and fertilizer factors and the NRE estimations by the isotopic method. Hence, it has been an enigmatic question as to the conditions under which the isotopic method is more or less accurate than the difference method. It is hypothesized that ANI is responsible for the discrepancy between NRE estimations by the difference and isotopic methods. Several factors may contribute to ANI and thus influence NRE estimations by either method. The most important factors are possibly soil OC, soil C/N ratio, and microbial biomass, which will affect pool substitution via immobilization, denitrification, and mineralization. The objectives of this study were to: (i) assess the existence and importance of ANI in estimating NRE by the isotopic method in soils varying in OC levels and at different stages of crop growth and (ii) to examine some factors influencing ANI, using ^{15}N-labeled fertilizer. To study these phenomena, an experiment under controlled conditions is most likely to yield explicit results and hence a pot study with spring wheat was conducted.

MATERIALS AND METHODS

Soils

Three benchmark agricultural soils from eastern Washington state widely differing in total C and N were used in this study. The soils were collected from the upper 15 cm of the profile, air dried, and sieved through a 2-mm screen. Before air drying, each soil was analyzed for total and inorganic N. Moisture curves for different tensions, water content of the

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Abbreviations: ANI, added-nitrogen interaction; NRE, nitrogen-recovery efficiency; OC, organic C; DAE, days after emergence; ANOVA, analysis of variance.

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Table 1. Selected properties of the three soils used in this study.

Soil	Classification	pH	Organic C	Total N	Inorganic N	Clay content
			mg kg^{-1}			%
Shano silt loam	Xerollic Camborthid	7.2	4000	490	21.8	6
Ritzville silt loam	Calciorthidic Haploxeroll	6.3	6200	630	12.2	10
Palouse silt loam	Pachic Ultic Haploxeroll	5.5	16000	1450	14.3	20

soils at the time of weighing into pots, particle-size analysis by the hydrometer method (Gee and Bauder, 1986), pH in 1:1 soil/water (Peech, 1965), and OC by the wet oxidation method (Snyder and Trofymow, 1984) were also determined. Selected soil properties are given in Table 1.

Experimental Procedure

A pot experiment with spring wheat was conducted during August to October 1989, with treatments consisting of the three soils, five levels of applied fertilizer N (0, 62, 124, 186, and 248 mg N pot^{-1}), and three harvests (20, 40,and 60 DAE of seedlings). Before sowing, soil for each treatment (2.8 kg pot^{-1}) was rotary mixed with 1 g each of $Ca(H_2PO_4)_2 \cdot H_2O$ and K_2SO_4. The N was mixed into the soil batch in enough solution to bring the soil water content to 45% of the water content at −0.03 MPa. The N form was $(NH_4)_2SO_4$ containing 10.0 atom % ^{15}N. The treated soil was packed uniformly into plastic pots (15 cm diam. by 17 cm) lined with plastic. Eight seeds of spring wheat were sown in each pot and thinned to six plants after emergence. There were four replicates per treatment. The pots were kept in the open except for rainy days when they were moved indoors. The pots were watered on a daily basis as necessary (by weighing) to maintain the soil water content near field capacity.

At 20, 40, and 60 DAE, whole plants were separated from the soil by hand and remaining root fragments were sieved from the soil. The plant material was washed and then dried at 60 °C for 48 h, after which the shoots and roots were weighed separately. Soil samples were taken at each harvest and stored at 5 °C for microbial-biomass estimation using the chloroform fumigation incubation method (Jenkinson and Powlson, 1976). Soil samples for inorganic N were processed immediately. and samples for ^{15}N analysis were air dried.

Plant and soil samples were finely ground (<75 μm) in acid-washed glass jars, containing four to six stainless steel bars, on a roller mill for 12 to 15 h (Smith and Um, 1990). For the two later harvests, the entire mass of shoot or roots was first ground in a high-speed rotary mill before a subsample was finely ground on the roller mill.

Chemical Analysis

Inorganic N in the soil was determined by KCl extraction (Keeney and Nelson, 1982) and autoflow injection analysis (U.S. Environmental Protection Agency, 1983). Total N and ^{15}N contents were determined by direct combustion isotope ratio mass spectrometry, using a Roboprep Tracer mass spectrometer interfaced to an automatic N analyzer (Europa Scientific, Crewe, England). Duplicate samples of shoot, root, and soil were analyzed for each harvest.

Calculations

The ^{15}N calculations for fertilizer-N recovery in plant shoots and roots and in the soil are given by the ratio of atom % excess ^{15}N in the pool to the atom % excess ^{15}N in the fertilizer (Cabrera and Kissel, 1989). The NRE was calculated by four methods:

1. difference method (total N uptake in fertilized minus total N uptake in the control);
2. unlabeled regression (total uptake of N by the plants vs. N rates applied);
3. isotopic method (^{15}N recovered in the plants); and
4. labeled regression (^{15}N recovered in the plants vs. N rates applied). The intercept is considered theoretically as zero.

Table 2. Response to applied N fertilizer of dry-matter at 60 days after emergence.†

N rate	Palouse shoot	Palouse root	Ritzville shoot	Ritzville root	Shano shoot	Shano root
mg N pot^{-1}	g pot^{-1}					
0	9.09	3.79	7.68	3.62	7.43	3.61
62	11.71	4.54	10.52	4.56	11.04	4.55
124	11.73	4.57	12.43	4.55	12.55	5.14
186	12.99	4.82	13.97	5.86	13.78	5.80
248	12.20	4.93	16.06	6.22	13.81	5.73

F-value significance

Main effects	Shoot	Root	N levels	Shoot
Soils	NS	*	Palouse soil	
N levels	***	***	linear	***
Soil × N levels	***	NS	quadratic	**
			cubic	NS
			quartic	*
N levels across three soils			Ritzville soil	
linear		***	linear	***
quadratic		NS	quadratic	NS
cubic		NS	cubic	NS
quartic		NS	quartic	NS
LSD (0.05) for N levels				
Across soils		0.50	Shano soil	
Palouse	1.17		linear	***
Ritzville	1.80		quadratic	***
Shano	1.29		cubic	NS
			quartic	NS

*,**,*** Significant at the 0.05, 0.01, and 0.001 levels of probability, respectively.

† Averages of four replicates.

The data set was statistically analyzed (ANOVA) using SAS (SAS Institute, 1985) for significance of N application levels, soils, and time on the measured parameters. The orthogonal polynomial contrast procedure was adopted in identifying the significant functions relating N levels and the measured variables. Stepwise regression was used to identify the important factors affecting NRE and ANI.

RESULTS AND DISCUSSION

Plant Production and Nitrogen Uptake

The statistical analysis of the entire data set in a three-way completely randomized design showed typical separation of the time and fertilizer factors on measured plant parameters. Fertilizer rates influenced total dry-matter production in all soils across all N rates (Table 2). There was a linear increase in total N uptake with fertilizer additions at 60 DAE. Other parameters such as N content, total N uptake, and ^{15}N uptake increased as fertilizer rates increased (Table 3). Since these parameters increased with time, the three time periods were significantly different from each other (data not shown).

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Table 3. Nitrogen balance and recovery of labeled fertilizer by spring wheat at 60 days after emergence.

N applied	Plant N		Plant N Uptake				^{15}N Immobilized in soil	N-Min†	Total ^{15}N recovered
			Fertilizer N		Soil N				
	Shoot	Roots	Shoot	Roots	Shoot	Roots			
mg N pot^{-1}	%		mg N pot^{-1}						%
					Palouse soil				
0	1.08	0.59	–	–	97.3	22.1	–	76.9	–
62	1.25	0.67	30.1	5.4	115.7	25.1	21.1	77.3	91.2
124	1.59	0.90	60.4	11.7	124.9	29.0	33.3	75.8	85.0
186	1.82	0.88	105.7	17.1	130.7	25.0	44.7	66.1	90.1
248	2.23	1.01	132.9	21.1	137.7	27.4	52.7	67.6	83.4
LSD (0.05)‡	0.27	0.06	5.5	2.2	11.7	3.7	7.3	NS	–
					Ritzville soil				
0	0.91	0.55	–	–	70.0	19.9	–	50.9	–
62	0.98	0.66	29.5	7.6	73.7	22.5	16.9	43.0	86.8
124	1.33	0.83	71.8	14.8	91.7	23.0	31.9	43.7	95.6
186	1.37	0.92	106.2	26.0	85.4	27.6	50.7	24.5	98.3
248	1.41	1.00	137.2	32.3	87.5	29.5	64.8	10.8	94.5
LSD (0.05)	0.15	0.06	5.5	1.7	7.4	2.9	7.3	10.7	–
					Shano soil				
0	0.91	0.63	–	–	67.4	22.2	–	23.7	–
62	1.06	0.78	32.6	7.9	82.9	27.2	18.3	26.1	94.8
124	1.22	0.85	68.2	16.5	84.5	27.0	31.4	11.6	93.6
186	1.65	0.95	121.4	25.1	104.9	29.8	38.2	32.2	99.2
248	1.73	1.17	138.9	31.3	100.2	35.7	66.3	5.2	95.4
LSD (0.05)	0.15	0.06	5.5	3.2	6.3	5.2	11.3	12.4	–
Main effects									
Soil	***	***	**	***	***	***	NS	***	
N levels	***	***	***	***	***	***	***	***	
Soil × N levels	***	NS	NS	***	***	**	*	***	
N levels									
linear		***	***						
quadratic		NS	**						

For the remaining parameters, only linear functions were significant for all three soils, with the exception of soil N in the Ritzville soil where the quadratic function was also significant.

*,**,*** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.
† N mineralized = (total N uptake + N lost) − (initial soil N + N applied + seed N).
‡ Least significant difference at 0.05 level of probability.

Nitrogen-15 Recovery

The N balance and recovery of ^{15}N at 60 DAE for each soil type is presented in Table 3. There was a linear increase in N content in the shoots and roots with increasing fertilizer rates for all soils. Also, as the fertilizer-N level decreased, a higher proportion of the plant N was soil derived. At the highest rate of N application, N uptake from fertilizer N was higher than from soil N in Ritzville and Shano soils; however, uptake from both sources was similar in the Palouse soil. The proportion of plant N that was derived from the applied fertilizer increased from 28, 27, and 20% at the low N rates to 59, 56, and 48% at the high N rates for the Ritzville, Shano, and Palouse soils, respectively.

The total percentage recovery of ^{15}N fertilizer applied is given in Table 3. The ^{15}N recovery is the sum total of the fertilizer-N uptake by the plant and the fertilizer N immobilized in the soil at the end of the experiment (Table 3). The unaccounted-for ^{15}N fertilizer at 60 DAE ranged from 1 to 17%, with the greatest average loss from the Palouse soil (12.6%), followed by Ritzville (6.2%) and Shano (4.2%) (Table 3). The losses were possibly due to denitrification and volatilization from the plant leaves,although the latter effect is usually significant only during plant senescence (Hooker et al., 1980). The Palouse soil has a higher clay content and denitrification potential than either Ritzville or Shano (Smith and Kennedy, 1989), which may have caused a lower recovery in this soil.

Added-Nitrogen Interaction and Nitrogen-Recovery Efficiency

The ANI defined earlier can be a REAL ANI if, for example, the fertilizer N causes an increase in root growth and a subsequent increase in soil N uptake, or if it causes increased N mineralization. The effect is an APPARENT ANI if the fertilizer N undergoes pool substitution due to displacement reactions, immobilization, or denitrification (Jenkinson et al., 1985). An ANI can be negative if the fertilized treatment takes up less soil N than the control. The most frequent occurrence is a positive APPARENT ANI, which is related to microbial immobilization.

The ANI can be calculated from ^{15}N studies that include a 0-N treatment, since the amount of soil-derived N that is taken up can be estimated. Table 3 shows the total N uptake from fertilizer and soil for each soil and treatment. The ANI for the Palouse soil 248 mg pot^{-1} treatment is simply the soil-N uptake of the shoot and roots (165.1 mg N) minus the N uptake of the control (119.4 mg N), which totals 45.7 mg N.

Table 4 shows the calculated ANI for the three soils

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Table 4. Added N interaction (ANI) at different stages of spring wheat growth, measured as days after emergence (DAE).

N rate	Palouse soil			Ritzville soil			Shano soil		
	20 DAE	40 DAE	60 DAE	20 DAE	40 DAE	60 DAE	20 DAE	40 DAE	60 DAE
	mg N pot^{-1}								
62	−2.6	7.7	21.4	−5.0	7.3	6.0	−12.7	13.1	20.6
124	−12.0	9.4	34.5	−11.2	17.5	24.7	−19.1	19.0	19.3
186	−12.4	1.7	36.3	−8.8	20.8	23.0	−24.3	21.0	45.2
248	−18.9	21.1	45.7	−19.5	23.1	26.9	−27.4	14.5	46.4

Effect of factors on soil-N uptake†

Factors	Significance	At 20 DAE	At 40 DAE	At 60 DAE
N levels	***	***	***	***
Soil	***	***	***	***
Time	***			
N level × soil	NS	NS	NS	***
N level × time	***			
Soil × time	***			
N level × soil × time	**			
LSD (0.05) level for N levels		6.7	7.2	Palouse = 11.3***
for soils		5.2	5.6	Ritzville = 8.9***
				Shano = 7.6***

*,**,*** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.
† The LSD values are valid for identifying the significant differences in ANI between N levels.

and sampling dates and fertilizer treatments. The ANI for 20 DAE is negative for all soils and N rates. This occurs because the inorganic-N pool was dominated by fertilizer N in the treated pots and thus the plants at this early stage had taken up less soil N than the plants in the control pots. In addition, the exchange and immobilization reactions had not depleted the fertilizer pool. In general, ANI increased with fertilizer rate and with time. At 60 DAE, the highest fertilizer rates showed the largest ANI, with Shano and Palouse having similar values and Ritzville a much lower ANI.

To determine if this positive ANI is REAL or APPARENT, the calculated net N mineralization in each treatment was scrutinized for increases due to the applied N fertilizer (Hart et al., 1986). Table 3 shows that only the Shano soil had increased N mineralization over the control, i.e., in the 62 and 186 mg N pot^{-1} treatments, though they are not statistically significant. Earlier reports indicate either no change (Harmsen and Kolenbrander, 1965) or depression (Jansson, 1958) in N mineralization due to applied fertilizer. Although there was increased root dry matter with N additions, there could be no increase in soil-N uptake without excess soil-N mineralization due to N fertilizer. Fertilizer additions are unlikely to increase root exploration or uptake efficiency, which would cause a REAL ANI (Jenkinson et al., 1985); thus, the observed increase in soil-N uptake is probably due to pool substitution. Since the added fertilizer N did not increase soil-N mineralization or soil-N uptake, there is no REAL ANI and the calculated ANI of all the three soils is 100% APPARENT. In general, the amount of fertilizer N immobilized in the soil (Table 3) is greater than the ANI, suggesting that pool substitution by microbial immobilization is the dominating factor causing ANI in this study. Hart et al. (1986) also observed the occurrence of pool substitution in pot experiments, but not in the field.

A positive APPARENT ANI may lead to erroneous results when calculating NRE using the isotopic-^{15}N method because pool substitution results in a portion of the applied labeled N being not accessible to the plant, and leads to a lower measured fertilizer recovery in the plant. The difference method, by definition, ignores whether the plant N comes from fertilizer or soil sources. Table 5 depicts the NRE calculated from the 60 DAE data across all fertilizer treatments using four estimation methods. The difference method gave higher recovery of added fertilizer than did the isotopic-^{15}N method for all three soils. Similar results have been reported by Moraghan et al. (1984) and Bloom et al. (1988). The difference method showed average recoveries of 81 to 93% and the isotopic method showed 61 to 70%, with the values calculated by these two methods being strongly correlated ($r^2 = 0.94$). The regression method for NRE is simply the slope of the relationship between total N uptake (by labeled or un-

Table 5. Fertilizer N-recovery efficiency of spring wheat calculated by different methods at 60 days after emergence.

N applied	Difference method	Isotopic technique	Regression† of total N	Regression‡ of ^{15}N
mg N pot^{-1}	%			
	Palouse soil			
62	91.8 ± 6.4§	57.2 ± 1.8	80.9	65.5
124	86.0 ± 7.2	58.2 ± 1.5	80.9	65.5
186	85.5 ± 4.9	66.0 ± 2.8	80.9	65.5
248	80.6 ± 8.1	62.1 ± 4.9	80.9	65.5
	Ritzville soil			
62	69.8 ± 4.3	59.7 ± 1.4	81.5	71.1
124	89.7 ± 3.9	69.8 ± 2.0	81.5	71.1
186	83.4 ± 6.3	71.1 ± 2.5	81.5	71.1
248	79.2 ± 5.9	71.8 ± 4.4	81.5	71.1
	Shano soil			
62	98.7 ± 6.2	65.3 ± 3.2	90.9	72.7
124	83.9 ± 3.2	68.3 ± 2.3	90.9	72.7
186	103.0 ± 5.9	78.7 ± 3.1	90.9	72.7
248	87.4 ± 3.3	68.6 ± 2.9	90.9	72.7

*** Significant at the 0.001 probability level.
† Palouse: $Y = 123.53 + 0.809$ N rate $R^2 = 0.97$***
Ritzville: $Y = 90.07 + 0.815$ N rate $R^2 = 0.98$***
Shano: $Y = 91.45 + 0.909$ N rate $R^2 = 0.97$***
‡ Palouse: $Y = -5.47 + 0.655$ N rate $R^2 = 0.97$***
Ritzville: $Y = -3.89 + 0.711$ N rate $R^2 = 0.98$***
Shano: $Y = -2.26 + 0.727$ N rate $R^2 = 0.97$***
§ Mean followed by standard deviation of four replicates.

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labeled calculation) and applied-N rates. The results were similar to the comparison between the isotopic and difference methods although there was closer agreement of the calculated NRE between the regressions.

The influence of ANI on NRE calculated by the isotopic method is evident from Table 5, which shows that pool substitution caused lower recoveries across all fertilizer treatments. The magnitude of ANI and NRE have been found to be significantly correlated ($P = 0.01$), although the correlation coefficient was not high (0.37). The lack of higher correlation suggests that different types and rates of pool substitution are occurring among the soils, in addition to other factors influencing NRE. Perhaps it could also be suggestive of a need for a better (direct and representative) way of quantifying the process of ANI than the one that was adopted (Jenkinson et al., 1985). In this study, the discrepancy in calculated NRE between the isotopic and difference methods is totally due to APPARENT ANI. On an average, the isotopic method estimated an NRE 20% lower than the difference method, which is of concern for studies utilizing ^{15}N-labeled fertilizer.

Factors Affecting Added-Nitrogen Interaction

It is not possible in every ^{15}N study to measure ANI or increased N mineralization due to fertilizer application, and thus it is problematic to determine which method is giving accurate NRE values. If a REAL ANI occurs, the NRE determined by the difference method will be in error or, if an APPARENT ANI occurs, the NRE calculated by the isotopic method will be in error (Jenkinson et al., 1985). However, the interpretations would be difficult in situations where both REAL and APPARENT ANI exist for the same treatment. In all mineralization processes, there is a component of immobilization and, hence, pool substitution is bound to occur to some degree. Factors such as OC and microbial-biomass concentration should relate directly to the degree of APPARENT ANI and these relationships could be used to estimate the potential for ANI to occur and to explain observed differences between soils in a ^{15}N experiment.

Using stepwise regression, we analyzed the available data from this experiment for factors that contribute to ANI. Testing the initial hypothesis that OC would influence the magnitude of ANI showed that this parameter only contributed to 11% of the ANI effect at 20 DAE, decreasing to 9 and 5% at 40 and 60 DAE, respectively. Similarly, individual parameters such as microbial biomass, total N, and clay content showed little influence on the magnitude of ANI. At 20 DAE, the dominant interacting factors that contributed to ANI were fertilizer rate, OC, and clay content. At 40 DAE, highest correlations with ANI were root weight and total N uptake; at 60 DAE, fertilizer-N levels, OC, soil C/N ratio, and N lost were highly correlated with ANI, explaining 73% of the variation in the predicted ANI.

Regression of NRE on fertilizer rates and OC content at 60 DAE could explain only 50% of the variation in NRE. The inclusion in the regression of N lost increased the fit of the model to 68%, with all the factors being significant at the 0.05 level. The significance of these factors in explaining the variation in NRE further supports the view that ANI due to pool substitution caused by immobilization and denitrification were dominant processes. The lower recoveries of applied N in soils with high OC contents have been observed in previous ^{15}N studies (Hart et al., 1986). Soils in this study ranged in OC from 4000 to 16 000 mg kg^{-1}, enough of a range to show an influence on NRE and, to some degree, on the magnitude of ANI.

CONCLUSIONS

The ANI calculated from this data set was entirely APPARENT ANI and was negative at 20 DAE and positive at 40 and 60 DAE. The NRE estimations by the isotopic method averaged 20% lower than by the difference method, although the two estimations were strongly related. Isotopic NRE was influenced by the same factors that influenced ANI, i.e., N rate, OC, and N lost, and there was significant correlation between ANI and NRE. Thereby, the significance of the phenomena of ANI in NRE was evident. There was no consistency in the soil and other factors that influence the magnitude of ANI at different crop-growth stages, thus making it more important to address this phenomena (ANI) while calculating NRE by the isotopic method.

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