Comparison of Laboratory Methodologies to Determine Soil Nitrogen Mineralization from Organic Residues

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Recycling organic waste for use as fertilizer requires prior knowledge of mineral nitrogen (N) availability for crops. Estimation of soil N release or potentially mineralizable N is an important tool for the design of fertilization strategies that aim to minimize the use of N fertilizer. The aerobic incubation method is considered a standard technique to measure soil potential to mineralize N. In this study, alternative methods of aerobic incubation were evaluated to help overcome its limitations (long time and equipment). In this regard, biological methods (anaerobic incubation at 7 and 14 days) and chemical extraction (hot KCl) procedures were examined. To determine potentially mineralizable N, a silty clay loam soil was fertilized with spent mushroom substrates and anaerobic digestates from different origins (C/N ratio of 4 to 38). Based on the results, chemical extraction emerges as a reliable alternative to the aerobic incubation method, particularly when the C/N ratio of the organic residues ranges from 12 to 15. Moreover, its implementation in routine soil laboratories is straightforward and faster, and it does not require any special equipment.

Keywords: Aerobic incubation; Anaerobic incubation; KCl hot; Organic fertilizers; Anaerobic digestate; Spent mushroom substrates

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INTRODUCTION

In an effort to reduce waste, the reuse of waste materials is one of the key principles of the EU action plan for the Circular Economy (European Commission 2015). In this context, it is important to consider the agricultural benefits of reusing organic and nutrient-rich waste as an alternative to their disposal in landfill sites or incineration. This reuse would allow nutrient recycling as a new resource (the so-called end-of-waste criteria; European Commission 2008). Recycling organic waste on agricultural land can enhance soil quality and reduce the need for mineral fertilizers by adding macro- and micro-nutrients (Möller and Müller 2012; Tuhy *et al.* 2015). This approach also can increase the reserves of organic carbon in soil (Diacono and Montemurro 2010). However, the use of these organic resources as fertilizers calls for prior understanding of their capacity to supply essential nitrogen (N) to crops.

The estimation of potentially mineralizable nitrogen (PMN) could improve predictions of N availability to crops. Nitrogen is the main limiting nutrient for crop yield and is widely supplied by fertilizer, manures, and other sources. The estimation of N is a useful tool when designing fertilization strategies.

Mineralization can contribute 20% to 100% of the yearly crop N requirement, while the remainder should be supplied by other sources, such as synthetic fertilizers (Broadbent and Hauck 1984; Yost *et al.* 2012) and organic fertilizers with a high mineral N content such as anaerobic digestates (Walsh *et al.* 2012).

The availability (to plants) of N from organic waste is dependent on the rate of mineralization of organic N to inorganic N [nitrate NO_3^--N and ammonium NH_4^+-N] forms. Mineralization and immobilization occurs simultaneously in soil, and the relative magnitudes determine whether the overall effect is net mineralization or net immobilization (Cabrera *et al.* 2005). Fertilization with organic residue incorporates nutrients as N, organic matter and microorganisms that will affect to N cycle of the soil. The net mineralization potential of N varies associated to the type of organic residues applied to soil (Masunga *et al.* 2016), which can change soil organic matter turnover and release nutrients. This effect is known as the "priming effect" (Fontaine *et al.* 2003; Zhan *et al.* 2018).

Management practices that optimize the transformation and recycling of manure-derived N for crops are critical under field conditions to achieve the efficient use of N in cropping systems (Honeycutt 1999). However, it is difficult to predict how much N from organic residues becomes available for plant development.

Although different methodologies have been realized with the aim of estimating N availability for the crops (Martínez *et al.* 2018), no one method has yet been approval (Luce *et al.* 2011). Moreover, there are few studies that have incorporated new organic residues applied as fertilizer (*e.g.* liquid anaerobic digestates and spent mushroom substrates) for the study of N mineralization with different methodologies. The mineralization of N is directly related to microbial activity and biomass input, which are affected by biotic and abiotic factors, the latter including temperature and soil moisture. In this regard, a biological approach, namely aerobic incubation, was the reference methodology to evaluate PMN (Stanford and Smith 1972). This method uses optimal soil temperature and moisture conditions to favor N mineralization. However, this method has several drawbacks, including tedious handling and the need for incubation equipment. Furthermore, it is time-consuming and does not fit into the batch-analysis techniques of soil-testing laboratories (Sharifi *et al.* 2007).

To find alternative methods for estimating PMN that improve the reference method, alternative biological methods were considered that are faster, easy to apply, and that do not need special equipment for anaerobic incubation. To reduce the time required for assessments, the chemical method also was evaluated using extractants that simulate microbial activity. Fertilization strategies can affect PMN estimation through various methods (Wyngaard *et al.* 2018). The organic content and C:N ratio of organic residues when applied to soil influences their decomposition. These properties affect net mineralization of N and must be considered when assessing alternative methods of determining this parameter. For liquid products, the C:N ratio cannot be used to predict the PMN (Lazicki *et al.* 2020).

The use of organic residues from agri-food industries and farms (spent mushroom substrates, anaerobic digestates) as fertilizer could affect N mineralization and PMN estimation. To test this hypothesis, two alternative methods were evaluated for the aerobic incubation (reference method), namely anaerobic incubation and chemical extraction, when applying organic residues from different sources to soil.

EXPERIMENTAL

Materials

Origin of soil and organic residues

Soil samples were taken from the superficial layer (0 to 0.30 m) of a silty clay loam (31% clay, 5% sand, and 64% silt) located in Alcalá de Henares (Madrid, Spain). Prior to the start of the experiment, soil was sieved through a 2-mm mesh screen.

The organic treatments were chosen to assay a range of C:N ratios (4 to 38), because this ratio affects N mineralization. Five organic treatments using agricultural waste were tested. Mineral treatment using ammonium nitrate (MIN) and a control without fertilization (CO) were also included. Two of the organic residues were obtained from an anaerobic digestate (AD), which was produced from a mixture of tobacco powder, cereal powder, and pig slurry from a fattening farm. The anaerobic digestate was filtered through a 750-µm screen, and the liquid fraction (LF) and solid fraction (SF) were used for the assay.

The other two organic residues, characterized by lignocellulosic components, were spent mushroom substrates (SMSs) of *Agaricus bisporus* (SMS-A) and *Pleurotus ostreatus* (SMS-P). For the cultivation of *Agaricus*, wheat straw was mixed with broiler manure, gypsum, and urea and then watered. For *Pleurotus*, wheat straw was mixed with gypsum and watered. Both substrates were left in aerobic conditions until they attained around 65% to 70% humidity. They were then pasteurized and mycelium was added to each substrate. When mushroom production was depleted, the spent substrates remained (SMS-A and SMS-P). A sample treatment combining SMS-P and LF (MIX) at a C:N ratio of 15 was included in the assay.

In the SMSs and ADs from commercial plants, the origin and the mixing compositions for preparing the substrates, and digestate rates were always the same, thereby ensuring the relative stability of the composition of each product over time.

The dosages applied as fresh samples (FS) were 26.8, 38.4, 23.5, and 38.0 t ha^{-1} for SMS-A, SMS-P, SF, and LF, respectively. The treatment mixture (MIX) contained 86.4 and 38.7 t ha^{-1} of SMS-P and LF, respectively and this mixture was applied at a rate of 51.2 t ha^{-1}.

Soil and organic residue characterization

The main soil characteristics were pH (1:2.5, w:v): 8.5; organic matter (Walkey-Black; OM): 14.2 g kg^{-1}; electric conductivity (EC; 1:5, w:v): 0.11 dS m^{-1}; N (Kjeldahl): 0.09%; available P (Olsen): 29 mg kg^{-1}; and carbonates (calcimeter Bernard): 3.2%.

The chemical parameters evaluated for organic residue characterization are shown in Table 1. The pH and EC of fresh samples were determined in a saturated paste, solid residue, and liquid residue. Total-N was assessed using the Kjeldahl method in fresh samples. The dry matter (DM) content was obtained using an oven at 105 °C, and OM content by calcination at 580 °C. The NH_4^+-N, ureic-N, NO_3^--N, total-P, and P contents of organic residues were obtained after extraction with water and citrate following AOAC methods (2010).

Methods

Aerobic incubation as reference method

The soil treatments were subjected to aerobic incubation (N_0) in darkness at 25 °C for 14 d, following the method described by Stanford and Smith (1972). In brief, 80 g soil was placed in a plastic pot and mixed with distilled water to reach 60% water holding

capacity for each treatment. To minimize moisture loss while allowing oxygen exchange, each pot was covered with a lid that had seven 5-mm diameter holes. Moisture content was maintained by spraying distilled water onto the samples. Destructive sampling was performed by removing pots in triplicate for each treatment after organic residues incorporation to soil and mixed (time 0) and after 14 d of incubation (time 14). Inorganic N (NO_3^--N and NH_4^+-N) was measured.

Anaerobic incubation

An anaerobic incubation (N_{an}) experiment was conducted in a heater (at 40 ºC) in triplicate on each soil treatment. The time to determine N was determined as described by Waring and Bremmer (1964). Briefly, at the beginning of the experiment, 80 g of soil was placed in 100 mL pots for each treatment. Distilled water was then added in excess to saturate the sample (and to remove the bubbles) and to guarantee anaerobic conditions during incubation for 7 and 14 d ($N_{an\text{-}7}$ and $N_{an\text{-}14}$). Inorganic N (NH_4^+-N) was also measured.

Chemical extraction

Chemical extraction (N_q) was carried out adding 50 mL of a 2 M KCl (1:2.5; w:v) solution to 20 g soil in a stopped tube block digester at 100 °C for 4 h as described by Gianello and Bremmer (1986). After extraction, the concentrations of NO_3^--N and NH_4^+-N ions were measured at room temperature.

Determination of ammonium and nitrate ions

The soil from each method was extracted with a 2 M KCl solution as described by Maynard *et al.* (2006). The N concentration in ammonium and nitrate ions in the extracts was determined by SMARTCHEM200 (AMS Alliance, Roma, Italy) as per UNE-EN ISO 13395 (1997) and ISO/TS 14256-2 (2005) standards. The concentration of inorganic N was expressed in dry-weight soil (105 °C).

Statistical Analysis

Linear regression analysis was performed between mineral-N content determined by each of two alternative methods (independent variable) and by aerobic incubation (dependent variable). The significance levels are indicated using the following probability levels: * $0.05 \leq P < 0.01$; ** $0.01 \leq P < 0.001$, *** $0.001 \leq P < 0.0001$; and NS (not significant) $P > 0.05$ (R Core Team Software, R Foundation for Statistical Computing, version 3.6.1, 2019, Vienna, Austria).

Concordance between mineral-N content by the alternative methods (N_q and N_{an}) and reference aerobic incubation (N_0) was evaluated by mean absolute error (MAE; Eq. 1) and using a modeling efficiency factor statistic (MEF; Eq. 2),

$$MAE = \left[\frac{\sum_{i=1}^{n} |P_i - O_i|}{n} \right] \quad (1)$$

$$MEF = 1 - \left[\frac{\sum_{i=1}^{n} (P_i - O_i)^2}{\sum_{i=1}^{n} (O_i')^2} \right] \quad (2)$$

where *n* is the number of observed values, and *O*i and *P*i are observed and predicted values for the data pair *P*i´= *P*i-$\hat{O}$ (average of the observed) and *O*i´= *O*i-$\hat{O}$.

In a perfect fit, the MEF would be equal to one. If MFE is less than zero, the predicted values using the model are worse than the observed mean (Loague and Green 1991). The MEF statistic is a good indicator of goodness of fit (Mayer and Butler 1993).
In the paired sample T-test, the statistical significance is determined by the P-value, which indicates the probability of observing the test results under the null hypothesis. The lower the P-value (≤ 0.05), the lower the probability of obtaining a result like that observed when the null hypothesis is true. In this case, differences between alternative methods as compared to the reference aerobic incubation method were observed.

RESULTS AND DISCUSSION

In general, the pH values of the organic residues ranged between 6.7 and 8.4 (neutral to basic). The EC varied among treatments, with the higher values being observed in LF and SMS-A. Nutrient content showed differences between residues, as did the C/N ratio, whose values ranged from 4 (LF) to 38 (SMS-P) (Table 1). The values obtained for LF and SF were consistent with those reported by Möller and Müller (2012). In relation to SMS-A and SMS-P, the experimental parameters were in the range of values found by Paredes *et al.* (2016) for this type of material. Once the waste has been incorporated into the soil (time 0), the observed changes to NH_4^+-N and NO_3^--N in comparison those produced after 14 days of incubation (time 14) confirm that the used conditions were adequate for N mineralization.

The NH_4^+-N ion was the main mineral form of N present in the materials, accounting for 13% to 70% of total-N (Table 1). This form is available for crops and is susceptible to emission losses (Webb *et al*. 2013).

All treatments indicated a total N application dosage of 210 kg·Nha^{-1} from each organic residue, which is the permitted application dosage for organic sources in non-vulnerable zones according to nitrates Directive (European Commission, 1991), except for the control treatment. In this case, the values indicate the behavior of native N mineralization in a non-amended soil.

A strong linear relationship was observed between chemical extraction and aerobic incubation (N_0 = 1.20 N_q - 8.91; and R^2 = 0.95), where N_0 and N_q are expressed in mg N kg^{-1} dry soil ($P < 0.001$). The fitted regression equation had an intercept that did not differ from zero and a slope that did not differ from one. Furthermore, the paired sample T-test found no significant differences ($P > 0.05$) between chemical extraction and aerobic incubation methods. Therefore, the former could be considered as a potential alternative method to the latter. The MAE was 7.1 mg N kg^{-1} dry soil, and MEF was 0.93, which were considered as acceptable values.

Table 1. Mean Values of the Chemical Characteristics of the Organic Residues

Parameters	Digestate Liquid Fraction	Digestate Solid Fraction	Spent Mushroom *Agaricus bisporus*	Spent Mushroom *Pleurotus ostreatus*
pH Saturated Paste	8.1	8.4	7.5	6.7
Electrical Conductivity Saturated Paste (dS m^{-1})	26.1	4.0	18.0	4.0
Dry Matter (%)	6.2	22.2	29.4	22.7
Organic Matter (%)	58.3	62.4	58.0	72.8
Total-N (%)	8.9	4.0	2.7	1.1
Organic-N (%)	2.6	1.6	2.15	0.94
NH_4^+-N (%)	6.3	1.4	0.51	0.13
NO_3^--N (%)	0.01	0.001	0.001	ND
Ureic-N (%)	ND	ND	ND	ND
C/N	4	12	13	38
Mineral-N /Total-N	0.71	0.53	0.19	0.13
Organic-N /Total-N	0.29	0.47	0.80	0.86
Total-P (%)	1.2	2.2	0.77	ND
Soluble-P (%)	1.1	1.7	0.68	ND
Soluble-P/Total-P	0.92	0.77	88.3	-

Results expressed on dry matter (DM) except ratio, pH, and EC in LF were measured on raw material. Organic-N was calculated as difference between total-N and inorganic-N forms (sum of NH_4^+-N and NO_3^--N); ND: not detectable

The accumulation of NH_4^+-N and the absence of NO_3^--N after 7 and 14 days of incubation confirm anaerobic conditions during the process. The anaerobic incubation after 7 and 14 d showed a linear relationship with aerobic incubation ($N_0 = 1.74\ N_{an\text{-}7} - 7.90$; $R^2 = 0.85$; $P < 0.001$; and $N_0 = 1.57\ N_{an\text{-}14} - 6.64$; $R^2 = 0.94$; and $P < 0.001$, where N_0 and N_{an} are in mg N kg^{-1} dry soil), except for the mineral fertilizer treatment. The fitted regression equation had an intercept that did not differ from zero and a slope that differed from one. The paired-test revealed differences between anaerobic incubation (7 and 14 d) and aerobic incubation, revealing that the former is not a suitable alternative to the latter. The MAE was 24.2 and 21.6 mg N kg^{-1} dry soil, and MEF was 0.26 and 0.31 for $N_{an\text{-}7}$ and $N_{an\text{-}14}$, respectively. A possible limitation of the N_{an} method, as a predictor of PMN could be that NH_4^+ ion accumulation is underestimated as a result of either the suppression of mineralization due to high NH_4^+ ion concentrations or NH_4^+ fixation by abundant clay particles present in fine-textured soils (Clark *et al.* 2019), such as those in the current study's conditions, with fine textured soil and a high NH_4^+ content from the organic residues.

The N_{an} method calls for previous calibration to ascertain the reliability of the values obtained. However, this involves a step that requires an additional validation to use MEF, which probably differs for each type of soil texture, and residue. The N_{an} method is not recommended when nitrate ion sources are applied to soil since it would cause denitrification of this compound. Although this method considers the nitrate ion content at time 0 and adds the values obtained to NH_4^+-N after anaerobic incubation, the result does not show sufficient accuracy with respect to potentially available N.

The results from this study suggest that the method using hot KCl solution (the N_q method) is a promising tool to evaluate the availability of mineral-N (in the soil) to crops. This finding is consistent with those of other studies (Gianello and Bremner 1986; Smith

and Li 1993; Velthof and Oema 2010). The results for each organic residues showed reliable values for N_q *vs.* N_0 in residues with C:N ratios between 12 to 15 (SMS-A, SF, and MIX). However, in treatments with low C:N ratio, such as in LF, the N_q method underestimated PMN by around 15%, and the PMN was overestimated by 2.5% (9.6 *vs.* 23.3 mg mineral-N kg^{-1} soil by N_o and N_q, respectively) for SMS-P (Fig. 1). These observations are most likely associated with the speed of the chemical extraction, which did not permit detection or simulation of the processes that occur in SMS-P and LF substrates because of immobilization and priming effects occurring in short incubation periods.

In aerobic incubation, NH_4^+ ions undergo immediate nitrification at the same time as LF is introduced into the soil. This finding indicates not only a high rate of nitrification but also mineralization of organic matter. Consequently, the capacity of the residue to supply mineral-N from liquid digestate is comparable with that from urea (Tambone and Adani 2017). Some studies also report that the digestate application stimulates the decomposition of soil organic matter, in what is called the "priming effect" (Fontaine *et al.* 2003). As a result, the soil gains an additional pool of mineral-N readily available to plants (Manson-Jones *et al.* 2018). These "extra" pools of mineral-N could explain the underestimated values of LF observed in the N_q compared to N_0 method.

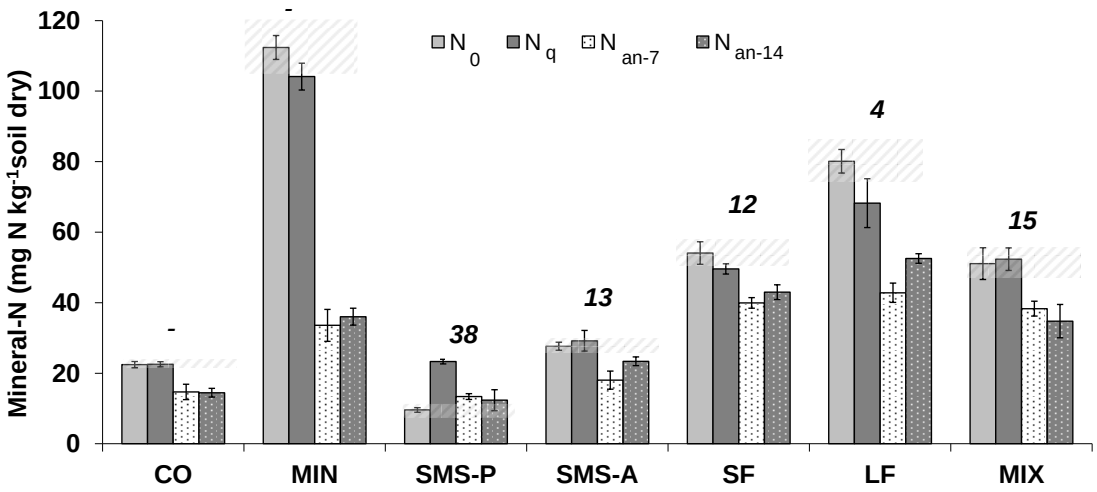

Fig. 1. Average value of each fertilizer treatment: Vertical bars indicate standard deviation; the cross shaded areas indicate 7% error of N_0 as assumable, numbers in bold italic indicate the C:N ratio. N_0: aerobic incubation at 14 days; N_q: chemical extraction; N_{an-7}: anaerobic incubation at 7 days, N_{an-14}: anaerobic incubation at 14 days.

Immobilization is the opposite process to mineralization. Soil microorganisms compete with crops for N. In this regard, mineral-N is taken up by soil organisms and therefore becomes unavailable to crops. The incorporation of organic materials with a high C:N ratio in aerobic incubation, such as SMS-P (in which the main contributor is wheat straw), increases biological activity, causing a greater demand for N and resulting in N immobilization in soil. This immobilization temporarily locks up N, and this nutrient becomes available again when the microorganisms die and their organic-N content is converted to nitrate form by nitrification. This fact was found by Dar *et al* (2009), who showed a lack of rice crop response to SMS-P, in comparison with SMS-A. In this case,

the microorganisms decomposed the SMS-P, using native N because of its higher C:N ratio (C:N: 15.2 *vs.* 37.6 for SMS-A and SMS-P, respectively) and preventing N availability during crop development. When the immobilization occurred after fertilization with SMS-P, the alternative methods for estimating PMN were not applicable.

CONCLUSIONS

1. The chemical extraction method with a 2 M KCl (1:2.5; w:v) solution at 100 ºC during 4 h extraction period emerges as a reliable alternative approach to the standard aerobic incubation method, as it is easy to implement in a routine soil laboratory, it is less time-consuming, and it does not require special equipment.
2. The results from this study indicated that chemical extraction is a useful method to obtain a rapid estimation of mineralizable N when the added organic residues have an optimum C:N ratio (12 to 15). Chemical extraction was found to be equivalent to the reference method for the evaluation of potentially mineralizable N (14 d in an aerobic incubation). It can be considered an alternative method for organic residues with a low C:N ratio (4). However, it underestimates N mineralization (approximately 15%) but offers a starting point for such residues.
3. The "priming effect" occur when labile organic matter addition to soil could induce short-term changes in the turnover of soil organic matter and release N in soil on the a very short time. To improve estimation of the "priming effect", further research using different organic residues and soil types (*i.e.*, soil textures, pH, and/or calcium contents), as well as different extractants and extraction times, is required to gain a better understanding of N mineralization, especially for residues with a low C:N ratio (< 12).

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