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Nitrogen availability after repeated additions of raw and anaerobically digested 15N-labelled pig slurry

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Short-term nitrogen availability after one or repeated additions of raw and anaerobically digested ^{15}N labelled pig slurry

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Keywords

Animal manure; mineralization; immobilization; residual effects; CO_2 emissions

Highlights

- We studied slurry decomposition after single or repeated additions in two laboratory incubations
- We used unlabelled and ^{15}N labelled pig slurries to estimate N availability
- N potentially available for plants was close to that supplied in mineral form with slurries
- Slurry N availability increased with repeated additions but the increase was small for both slurries
- Net contribution to residual organic N and microbially-immobilized N was low

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Abstract

Quantifying short- and long-term availability of animal manure nitrogen (N) is of practical interest to ensure adequate crop yield, minimize N pollution, and reduce mineral-N fertilizer inputs. We measured short-term carbon (C) and N dynamics after one or six repeated additions to soil (every 56 days) of ammonium sulphate (AS), undigested (PS) and anaerobically digested (DPS) pig slurry in a laboratory incubation experiment. Soil CO_2 emissions, pH and mineral N (ammonium + nitrate) were measured in the period day 0–56 following fertilizer additions. An accompanying experiment was conducted with similar but ^{15}N labelled fertilizers to measure soil mineral N 56 and 112 days after one, three or six repeated additions, and to estimate the increase of slurry available N after repeated additions. Nitrogen potentially available for plants (77–91%, 44–58% and 57–66% of added N for AS, PS and DPS, respectively) was close to that supplied in mineral form with the fertilizer, indicating negligible net mineralization of slurry organic N, both after one and repeated additions. In fact, soil mineral N increased in most of the treatments because of repeated additions, but the increases were very small, on average 0.5–2.1% of added N per period of 56–112 days. Calculations of fertilizer N availability based on unlabelled N were equally precise compared to those estimated with ^{15}N, but trends over time were rather more variable. We conclude that many repeated additions (simulating a long manuring history) are needed to obtain marked increase of available slurry N, even under controlled conditions.

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Introduction

The incomplete retention of organic matter and nutrients by livestock causes relevant amounts of them to remain in faeces and urine, which are stored and matured as either solid farmyard manure or liquid slurry, or are subjected to anaerobic digestion (Webb *et al.*, 2013). Anaerobic digestion transforms part of slurry organic matter (and, possibly, of other co-digested substrates) into biogas. The by-product of anaerobic digestion is the digestate, which can be a good source of nutrients for crops (Loria *et al.*, 2007; Chantigny *et al.*, 2008; de Boer, 2008; Cavalli *et al.*, 2016a).

Uncertainty exists in estimating raw and digested slurries N dynamics in soil, and thus their value as fertilizers (Gutser *et al.*, 2005). Livestock diet, type of stable, duration of slurry storage, and presence of litter all have strong impacts on the physical-chemical properties of slurries, and in turn on their mineralization rate (Kyvsgaard *et al.*, 2000; Sørensen & Fernández, 2003; Powell *et al.*, 2006). In addition, slurry N availability is affected by anaerobic digestion that modifies the composition of raw slurry (Möller & Müller, 2012).

Manure N availability can be estimated with incubations of manure-soil mixtures under controlled soil temperature and water content conditions; these studies found C and N decomposition dynamics which reflected those observed in the field (Delin *et al.*, 2012; Gale *et al.*, 2006; Sørensen & Fernández, 2003), avoiding the confounding effects of soil and weather heterogeneity and soil-crop interactions (Delin *et al.*, 2012). Results of such laboratory experiments showed that short-term availability (0–3 months) of N from solid manures (Gale *et al.*, 2006; Calderón *et al.*, 2005), undigested slurries (Bechini & Marino, 2009; Morvan & Nicolardot, 2009; Sørensen & Fernández, 2003) and digestates (Kirchmann & Lundvall, 1993; Loria & Sawyer, 2005) often approximates

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the ammonium (NH_4-N) content of the manure. These results suggest that net mineralization (the balance between organic N mineralization and microbial N immobilization) of applied organic N is negligible, or that mineralized N compensated for N losses that, in close systems, can occur as NH_3 volatilization or N_2O/N_2 emissions. Moreover, significant microbial immobilization of N can occur soon after slurry addition to soil, due to fast microbial decomposition of labile N-poor compounds like volatile fatty acids (VFA) (Kirchmann & Lundvall, 1993). Net N immobilization can last for several weeks (Bechini & Marino, 2009; Morvan *et al.*, 2006), during decomposition of low-N fiber fractions (Morvan & Nicolardot, 2009; Peters & Jensen, 2011). Remineralization of this microbially immobilized N, together with the late mineralization of slurry organic N give rise to residual effects beyond the year of application (Schröder *et al.*, 2013). As a result, availability of slurry N, as estimated by crop N uptake, increases when slurries are repeatedly applied to the same field (Nevens & Reheul, 2005).

Estimations of residual N effects based on single or repeated manure additions are relatively scarce. These effects were studied in field trials involving pig (Sørensen & Amato, 2002; Sørensen & Thomsen, 2005) and cattle slurries (Schröder *et al.*, 2007; Cusick *et al.*, 2006). Measured residual N effects (i.e. mineralized N in the first 12 months, the second 12 months and the third 12 months after the year of application) decreased over time due to the progressive exhaustion of added organic matter, and averaged 13, 8 and 6% of applied organic N for the three years following addition of liquid and solid manures (Schröder *et al.*, 2013). Residual effects are indeed small, highlighting a rather low net mineralization of resistant manure organic fractions even in the long-term. In addition, experiments that used ^{15}N labelled fertilizers applied once,

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reported residual N effects of liquid slurries often close to that of mineral fertilizers, especially for the third year after fertilizer addition, showing that even immobilized N is slowly re-mineralized in subsequent years (Webb *et al.*, 2013). Since residual N effects are small, it follows that many years of repeated slurry additions are needed to measure consistent effects in the field. Therefore, to acquire new knowledge in the field on residual effects of digested and undigested pig slurries would require long-term experiments. On the other hand, attempts to study the effects of repeated applications on slurry turnover in the laboratory are rare (Cavalli *et al.*, 2014, 2016b). The analysis of the literature reveals that no attempts were made to study, at the laboratory scale, the effects of repeated applications to soil of raw and digested pig slurry. Because slurry decomposition is usually faster in the laboratory than in the field (due to higher temperatures and optimal soil water content), it is possible to apply manures more frequently in the laboratory compared to the field to obtain similar decomposition rates. However, it is important that, similarly to a field situation, the slurry is added when its easily decomposable fractions (causing high CO_2 emission rates and often net N immobilization) are almost depleted. Thus, only slurry resistant fractions and immobilized NH_4-N contribute to additional available N in subsequent applications, reflecting those fractions that, under field conditions, give rise to residual effects.

Availability of slurry N can be estimated with the difference and the direct method (Rao *et al.*, 1992; Muñoz *et al.*, 2004; Cusick *et al.*, 2006). The former method calculates slurry N availability as the difference between soil mineral N, for incubation experiments, or crop N uptake, for field experiments, measured in fertilized treatments and that measured in an unfertilized control treatment. This method is frequently adopted in field and incubation studies because it does not require labelled fertilizers. It

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129 assumes that soil native N turnover is the same in all treatments. However, the
130 occurrence of priming effect, either negative (e.g. preferential substrate utilization) or
131 positive (enhanced soil N turnover), produces errors in the estimates obtained with this
132 method (Rao *et al.*, 1992). Conversely, the direct method allows tracking the dynamics
133 of applied ^{15}N separately from that of soil native N. While isotopic fractionation at ^{15}N
134 enrichments higher than natural abundance does not result in relevant errors in the
135 estimation of ^{15}N availability, substitution of ^{15}N with unlabelled N leads to
136 underestimation of available slurry N (Rao *et al.*, 1992; Murphy *et al.*, 2003). It is
137 therefore necessary that added and native soil pools are in equilibrium in order to be
138 equally affected by microbial (immobilization, denitrification) and abiotic processes
139 (such as NH_4^+ clay fixation). While it seems interesting to apply both approaches to
140 study slurry (and fertilizer) N turnover in soil, experiments that employ both methods
141 are not frequent (Muñoz *et al.*, 2004; Cusick *et al.*, 2006).
142 The main objective of this work was to measure, in a laboratory incubation under
143 controlled conditions, the fate of N repeatedly added to soil with undigested pig slurry,
144 digested pig slurry, or ammonium sulphate. We expected that a higher fraction of the
145 added N was available (*i.e.* in mineral forms) after repeated fertilizer additions
146 compared to a single addition. A second objective was to compare net N mineralization
147 estimated with the difference and the direct method; for this reason, both unlabelled and
148 ^{15}N labelled fertilizers were used. We therefore wanted to test whether both methods
149 provided reliable and comparable estimates of fertilizer N availability.
150 The study was accompanied by measurements of CO_2 emissions. Detailed dynamics of
151 C mineralization enable to quantify microbial activity after fertilizer additions, by
152 identifying the time periods when decomposition of easily degradable and more

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153 resistant slurry fractions occurs. In addition, measurements of soil pH after each manure
154 application enable to understand whether a marked decrease of soil pH – as a result of
155 nitrification – reduced microbial activity.

156 Materials and methods

157 *Treatments and experimental set-up*

158 We carried out two incubation experiments involving unlabelled (experiment ULAB)
159 and ^{15}N labelled fertilizers (experiment LAB). Both considered the full factorial
160 combination of the two factors fertilizer type and number of cumulated fertilizer
161 applications. The fertilizer type included a raw (PS) and an anaerobically digested
162 (DPS) pig slurry, an unfertilized control (CON) and a mineral fertilizer control
163 (ammonium sulphate, AS). The factor number of applications ranged from one to six,
164 with an elapsed time of 56 days between any two applications (Figure 1). The
165 experiment ULAB compared a single application (Application 1) with six repeated
166 applications (Application 6), while experiment LAB had Application 1, 3 and 6. Thus,
167 the experimental design provided a total of 8 and 12 treatment combinations for the two
168 experiments, respectively, each replicated four times. We decided to apply fertilizers
169 every 56 days in order to deplete the fast-decomposing organic matter fraction of the
170 slurry (as confirmed by low CO_2 emission rates, close to those of CON, at the end of a
171 56-day period; Bechini and Marino, 2009). As a result, we expected to have mainly
172 slurry resistant fractions and immobilized NH_4-N contributing to N availability in
173 subsequent applications.
174

175 *Slurries and soil*

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Unlabelled and ^{15}N labelled PS were produced by two groups of two young pigs each. Pigs of about 25 kg were fed for 7 days a ration made of maize grain (900 g day^{-1}) and concentrate (300 g day^{-1}; 37% crude protein). After that period, one group continued to be fed with the ration, while the other group was fed with a similar ration made with ^{15}N labelled maize grain (6.078 atom% ^{15}N) and unlabelled concentrate. Starting from the third day, faeces and urine were collected, separately for the two groups, mixed, added with water to reach a N concentration of about 2.5 g N kg^{-1}, and then stored for 28 days under anaerobic conditions at 5° C in PVC containers.

Unlabelled and ^{15}N labelled DPS were produced from unlabelled and ^{15}N labelled PS, respectively, using 3-litres laboratory semi-batch reactors (one for each type of slurry). An initial amount of 4 g DM of thawed PS was added to 16 g DM of fresh inoculum consisting of unlabelled digesting pig slurry taken from a commercial digestion plant. Anaerobic digestion was conducted under mesophilic conditions (40°C) and lasted 70 days. During digestion, aliquots (on average 6 g) of thawed PS were added to the fermenting mixtures about every 9 days through a funnel, preserving anaerobiosis, as confirmed by biogas composition (CH_4 65–74%, CO_2 26–35%, O_2 < 3%). This procedure allowed obtaining DPS composed of organic matter at different fermentation stages, reproducing a common situation in farm plants.

After anaerobically storage (PS) or digestion (DPS), slurries were homogenized using an Ultra Turrax T-25 disperser (IKA Werke GmbH & Co. KG, Germany) and stored in plastic bottles at −20°C until the start of the experiments. The characteristics of unlabelled and ^{15}N labelled PS and DPS are given in Table 1. Reagent grade ^{15}N labelled AS (10.869 atom% excess ^{15}N) was taken from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

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The soil used in both experiments (Table 2) was sampled from the 0–30 cm profile of unfertilized plots located at the experimental farm "A. Servadei" of the University of Udine (Italy) (46° 02' N, 13° 13' E). The soil was air dried, gently hand-ground, and sieved (2 mm) to remove coarse fragments of roots and skeleton. Thereafter, it was re-moistened and pre-incubated at 25±1°C for 12 days.

Incubation procedure

After pre-incubation, fertilizers were applied to experimental units (microcosms consisting of 115 g of dry soil) at a rate of 71 mg N kg^{-1} of dry soil, except CON that received no N addition. Incubation experiments were carried out in the dark in a thermostatic room at 25±1°C and constant soil water content (WC_{-50kPa}, Table 2); their duration was 336 and 392 days in experiment ULAB and LAB, respectively (Figure 1). Soil water content was kept constant by individually weighting (every 1–3 days) each experimental unit and replenishing lost water using deionized water (maximum measured water loss was ±4% WC_{-50kPa}). To avoid excessive soil water content from fertilizer application, all experimental units belonging to Application 3 and 6 were partially air-dried during five days before subsequent applications.

In experiment ULAB, measurements of CO_2 emissions, NH_4-N and nitrate-N (NO_3-N) were taken on six dates: 1, 3, 7, 14, 28 and 56 days after Application 1 and 6. Soil NH_4-N and NO_3-N were also measured at day 0, more precisely two hours after fertilizer addition. Soil pH was measured at day 0 and day 56 after Application 1 and 6.

In experiment LAB, measurements of NH_4-N and NO_3-N were taken at day 0, 56 and 112 after Application 1, 3 and 6. At the same time, ^{15}N enrichment of NO_3-N was determined, while that of NH_4-N was determined solely at day 0, given the very low

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224 NH_4-N concentration at day 56 and 112 (< 0.5 mg kg^{-1}). In experiment LAB, total soil
225 N (organic N + SMN) and its ^{15}N enrichment were also measured at day 56 and 112
226 after Application 3 and 6.
227 In order to allow destructive measurements on different experimental units on each
228 sampling date, we prepared 224 (8 treatments × 7 sampling dates × 4 replicates) and
229 144 (12 treatments × 3 sampling dates × 4 replicates) experimental units, in experiment
230 ULAB and LAB, respectively, for which measurements were done only once.
231
232 ***Measurements during incubation***
233 At the beginning of each incubation interval, 16 experimental units were placed
234 separately in 3-litres sealed jars along with a beaker containing 20 ml of 0.5 M NaOH
235 and a plastic bottle containing 30 ml of water. At the end of each interval, CO_2 evolved
236 in each jar was measured by titrating residual NaOH in the trap, after carbonate
237 precipitation with $BaCl_2$. Soil pH was determined potentiometrically with a Crison GLP
238 21 + pH-meter (Crison S.A., Alella, Spain) on a soil-water mixture with a ratio of 1:2.5.
239 Soluble and exchangeable ammonium-N (NH_4-N) and nitrate-N (NO_3-N) were
240 extracted for 2 hours with a solution of 1M KCl (Cavalli *et al.*, 2016b), and determined
241 by flow injection analysis and detected with a spectrometer (FIAstar 5000 Analyzer,
242 Foss Tecator, Hillerød, Denmark) according to the ISO 11732 (1997) and 13395 (1996)
243 procedures, respectively.
244 Total N in soil samples was determined via dry combustion of air dried samples using a
245 EA/NA-1100 elemental analyzer (Carlo Erba, Milano, Italy). Prior to analysis, soil
246 samples were air dried and crushed to pass through a 0.5 mm sieve using a ZM100
247 ultracentrifuge mill (Retsch GmbH, Haan, Germany).

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Determination of $\delta^{15}N$ in solid samples was carried out using the EA/NA-1100 elemental analyzer coupled on-line through a helium flux to a Thermo Finnigan Delta Plus XP mass spectrometer (Thermo Finnigan, Bremen, Germany). Reproducibility and uncertainty of the method at different $\delta^{15}N$ are reported in Table S1.

Nitrogen in liquid samples was transferred on a solid support (glass filter trap) for determination of $\delta^{15}N$ following the procedure of Sørensen & Jensen (1991) for 1M KCl soil extracts and that of Stark & Hart (1996) for Kjeldahl extracts. Results of preliminary recovery test with diffused and non-diffused 1M KCl solutions are reported in Table S2.

Calculations

Net CO_2 emission rates of PS and DPS were calculated subtracting gross CO_2 rates (mg C kg^{-1}) measured in CON from those measured in the slurry-fertilized treatments; results were then expressed as percentage of added C.

Gross soil mineral nitrogen (SMN, mg N kg^{-1}) was calculated as the sum of 1M KCl extractable NH_4-N and NO_3-N. We did not measure non-exchangeable NH_4-N because preliminary test on this soil confirmed the lack of NH_4-N clay-fixation. Net SMN was calculated with the difference method, by subtracting gross SMN measured in CON from that measured in fertilized treatments; results were then expressed as percentage of total N applied with fertilizers (Muñoz *et al.*, 2004).

In addition, in experiment LAB, net SMN was calculated with the direct method, according to the following equation (Muñoz *et al.*, 2004):

$$Net\ SMN\ = \frac{\mathrm{SMN}_{FERT} \times (atom\%\ ^{15}N\ \mathrm{SMN}_{FERT} - atom\%\ ^{15}N\ SMN_{CON})}{\sum N_{FERT} \times \left(atom\%\ ^{15}N\ \mathrm{N}_{FERT} - atom\%\ ^{15}N\ \mathrm{N}_{FERT\_BG}\right)}$$

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where FERT and FERT_BG refer to labelled and unlabelled AS, PS or DPS, respectively. As stated above, at day 56 and 112, SMN was assumed to be equal to NO_3-N. The same equation was used to calculate the recovery of applied ^{15}N in soil, substituting total soil N for SMN.

In the experiment LAB, the increase of net SMN ($Delta_{SMN}$) on day 56 and 112 after one fertilizer application was calculated separately for each fertilizer type, according to the following equation:

$$Delta_{SMN} = \left(\frac{\text{Net SMN}_{A6} - \text{Net SMN}_{A1}}{5} + \frac{\text{Net SMN}_{A6} - \text{Net SMN}_{A3}}{3} + \frac{\text{Net SMN}_{A3} - \text{Net SMN}_{A1}}{2}\right)/3$$

where A1, A3 and A6 refer to application numbers.

Similarly, $Delta_{SMN}$ on day 56 was calculated in ULAB, using only Application 1 and 6.

Statistical analysis

In experiment ULAB, a three-way full factorial ANOVA model was used to assess the effects of fertilizer type (FER), number of fertilizer applications (APP), sampling date (DATE), and their interactions on gross and net CO_2 emission rates, soil pH, gross and net SMN.

Gross and net CO_2 emission rates were analysed as log-transformed variables (natural logarithm), in order to obtain a linear trend with time (also log-transformed).

Means were compared using planned contrasts. A set of polynomial contrasts was defined to test for the linear trend of log-transformed CO_2-C emission rates and of gross SMN during the 56 days, within each FER × APP combination.

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A second set of contrasts was built to identify differences in the dependent variables among treatments, within each level of FER or each level of APP, separately for the period day 0–7, day 14, 28 and 56.

In experiment LAB, the same three-way full factorial ANOVA model was used to assess the effects of FER, APP, DATE, and their interactions on net SMN calculated with both difference and direct methods, and on the recovery of applied ^{15}N in soil. A set of polynomial contrasts was defined to test for the linear trend of net SMN from Application 1 to Application 6, within each FER × DATE combination.

All ANOVAs were conducted with the GLM procedure of SPSS, Version 24.0.0 (IBM Inc., Armonk, New York); contrasts were calculated with the LMATRIX command. The *P* values were corrected for multiple comparisons adopting the Bonferroni procedure. Significant mean differences are reported when the corrected *P* value was below 0.05.

Results

CO_2 emissions

Gross daily CO_2 emission rates (Figure 2a) were significantly affected by fertilizer × application × sampling date interaction ($P < 0.05$). In all treatments respiration rates significantly decreased during the 56 day period following each addition, and from Application 1 to Application 6 ($P < 0.05$). After Application 1, 56-days accumulated CO_2 emissions were similar in CON and AS (Figure 2b), accounting for 2.4 (CON) and 2.2% (AS) of soil C. Conversely, after Application 6, C respiration rates were significantly and markedly lower in AS compared to CON ($P < 0.05$; Figure 2a).

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313 Indeed, accumulated CO_2 was 1.1 (CON) and 0.8% (AS) of soil C after 56 days,
314 respectively (Figure 2b).
315 During incubation, slurry-amended treatments had always significantly higher C
316 respiration rates compared to controls ($P < 0.05$; Figure 2a) and accumulated more CO_2
317 in both applications (Figure 2b). ANOVA showed that fertilizer, sampling date and
318 application × sampling date interaction significantly affected net daily CO_2 emission
319 rates (Table S3). Net CO_2 emission rates (Figure 2c) also significantly decreased over
320 time during each application period as observed for gross rates ($P < 0.05$). However,
321 they did not significantly change between applications (Table 3), with the exception of
322 DPS that respired slightly more C (+9 % of applied C) in the first week following
323 Application 1 compared to Application 6 (Figure 2d).
324 Net C respiration was higher following addition of PS compared to DPS, and, at the end
325 of each application period, accumulated CO_2 accounted for 52 and 56% of applied C,
326 for PS, and 42% and 33% of applied C for DPS, in Application 1 and 6, respectively
327 (Figure 2d). However, differences between slurries were significant only during the first
328 month after Application 6, and during the second month after Application 1 ($P < 0.05$).
329
330 ***Soil pH***
331 Soil pH (Table 4) was significantly affected by two-ways interactions among factors
332 fertilizer, number of applications and sampling date ($P < 0.05$). During the entire
333 incubation period, significantly ($P < 0.05$) higher pH was measured in CON compared
334 to the average of all fertilizers (+0.05–0.42 pH units), and in slurry-amended treatments
335 compared to AS (+0.13–0.48 pH units). Changes of soil pH from day 0 to day 56 within
336 each application period were very narrow for all treatments (Table 4), even if always

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337 significant ($P < 0.05$), with the exception of PS and DPS in Application 6 and 1,
338 respectively. Conversely, significant differences were registered between Application 1
339 and 6 at both day 0 and 56 in all treatments except CON (Table 4). Indeed, soil pH
340 decreased by 0.45, 0.17 and 0.17 units (averages between day 0 and 56) in AS, PS and
341 DPS, respectively.

342 ***Soil mineral N***

343 Measurements of NH_4-N and NO_3-N taken at day 0 and 56 in experiment ULAB were
344 in excellent agreement with those obtained in experiment LAB (Figure S1), confirming
345 the similarity of experimental conditions between the two incubation trials.

346 In the two experiments, day 0 recovery of fertilizer NH_4-N averaged 95% (data not
347 shown), confirming the lack of NH_4-N clay-fixation.

348 Gross SMN (Figure S2) was significantly affected by fertilizer × application × sampling
349 date interaction ($P < 0.05$). In all treatments, gross SMN significantly increased during
350 each 56-day application period, and from Application 1 to Application 6 ($P < 0.05$).
351 Accumulated SMN in CON, compared to the start of the experiment, corresponded to
352 1.3 and 4.4% of soil total N at day 56 after Application 1 and 6, respectively.

353 Added NH_4-N was quickly nitrified within one week (Figure S2); as a result of NO_3
354 accumulation, gross SMN was significantly higher in fertilized treatments compared to
355 CON ($P < 0.05$).

356 In both experiments, also net SMN (% applied N; Figure 3) was significantly affected
357 by the interaction of fertilizer × applications × sampling date (Tables S4–S6). Dynamics
358 of net SMN over time measured in experiment ULAB differed among treatments
359 (Figure 3). After Application 1, net SMN in AS averaged 89% of applied N and did not
360 significantly change over time ($P < 0.05$). Conversely, in PS net SMN significantly

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361 decreased in the period day 0–7 (–9% of applied N; $P < 0.05$), and significantly
362 increased thereafter (+4% of applied N). An intermediate pattern between AS and PS
363 was observed in DPS, where net SMN significantly ($P < 0.05$) decreased during the first
364 week (–4% of applied N), without any significant variation thereafter. Differently from
365 Application 1, after Application 6 net SMN remained constant in all treatments during
366 the day 0–56 period (Figure 3).
367 Despite differences in net SMN over time, in both Applications 1 and 6 net SMN was
368 significantly higher ($P < 0.05$) after addition of AS compared to slurries (+29–36% of
369 added N), and with the addition of DPS compared to PS (+7–19% of added N).
370 Differences in net SMN between applications occurred only in PS for the whole period
371 day 0–56, and in DPS for the period day 0–7, when net SMN was significantly higher
372 following Application 6 than Application 1 (Table 5).
373 Estimates of the increase of net SMN at day 56 from Application 1 to Application 6
374 obtained in experiment ULAB (Table 5) were very similar to those obtained in
375 experiment LAB with calculations based on the difference method (Table 6): small
376 (<2%) or non-significant increase in AS and DPS, and significant increase in PS (5.9%
377 and 4.7% in experiment ULAB and LAB, respectively). This increase in net SMN in PS
378 resulted in $Delta_{SMN}$ of 1.2% of applied N per application period (Table 7). Differently
379 from day 56, results of experiment LAB showed that the increase in net SMN at day
380 112 across applications (Figure 4) was sizable and significant in all treatments,
381 corresponding to 9.0, 8.0 and 5.3% of added N in AS, PS and DPS, respectively (Table
382 6). Thus, at day 112 $Delta_{SMN}$ calculated with the difference method was positive for all
383 treatments, in the range 1.7–2.9 % of applied N per application period (Table 7).

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Calculation based on ^{15}N measurements (direct method) confirmed results obtained considering unlabelled N, with the exception of DPS, that showed a significant increase in net SMN across applications also at day 56 (Table 6). In addition, estimated trend of net SMN based on ^{15}N measurements were usually higher (+0.3–5.2% of added N) at day 56, and lower (–1.6–4.1% of added N) at day 112, than those estimated with the difference method (Table 6). Calculated $Delta_{SMN}$ with the direct method were quite different from those obtained with the difference method (Table 7), especially for AS and DPS that showed contrasting results at day 112 and 56, respectively.

Recovery of applied ^{15}N

Recovery of applied ^{15}N in soil (Table 8) was significantly affected only by fertilizer ($P < 0.05$). However, differences among treatments were very narrow and occurred mostly between AS (on average 95% of applied ^{15}N) and slurries (on average 99% of applied ^{15}N).

Discussion

CO_2 emissions

During incubation, soil and slurry organic matter was progressively mineralized (Figure 2), as confirmed by decreasing CO_2 emission rates over time after each application (Morvan *et al.*, 2006; Sørensen & Fernández, 2003; Loria & Sawyer, 2005), and by lower gross C respiration after repeated slurry additions (Cavalli *et al.*, 2014).

In AS, lower emissions compared to CON after Application 6 (Figure 2b) could be related to the large decrease of soil pH in AS (Table 4) as a consequence of nitrification of added NH_4-N (Loria & Sawyer, 2005; Cavalli *et al.*, 2016b), that might have

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diminished soil organic matter decomposition compared to CON (Aciego Pietri & Brookes, 2008).

Accumulated C respiration in PS and DPS (54% and 33–42% of slurry C, respectively; Figure 2d) was similar to 46–63% and 34–43% reported by Kirchmann & Lundvall (1993) and Alburquerque *et al.* (2011) for undigested and digested pig slurries incubated in soil for the same duration . Sørensen and Fernández (2003) obtained rather lower 56-days emissions for PS (25–42% of slurry C), probably because their incubation was carried out at lower temperature (8°C vs. 25°C).

Higher respiration rates (Figure 2c), and thus higher cumulative CO_2 emissions in PS than in DPS (Figure 2d) (Kirchmann & Lundvall, 1993; Marcato *et al.*, 2009) could be attributed to the loss of degradable C compounds (48% of initial C was not recovered in DPS; Table 1) during anaerobic digestion (Möller & Müller, 2012), and to the accumulation of chemically recalcitrant organic matter in digestate (Malerba *et al.*, 2014).

It was expected that after repeated additions of PS and DPS slurry C decomposition would increase due to the accumulation of decomposable organic matter in soil. Indeed, repeated 84-days additions of cattle slurries to a sandy loam and a clay loam soil resulted in an increase in CO_2 emissions of 1.43% of slurry C per period of 84 days (Cavalli *et al.*, 2014). However, in this experiment, we applied only 450 and 260 mg C kg^{-1} with PS and DPS, respectively, an amount comparable to that of soil microbial biomass (about 337 mg C kg^{-1}, assuming that it represented 1.7% of soil organic C; Xu *et al.*, 2013).

For this reason, we make the hypothesis that slurry C was mainly used as an energy source and thus did not allow a relevant microbial biomass growth. Therefore, the lack

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of any measurable increase in net CO_2 emissions could have been due to a reduced accumulation of microbial residues, and to the persistence of highly resistant residual fractions of slurry organic matter.

Soil mineral N dynamics

Losses of N from the soil due to NH_3 volatilization or N_2O/N_2 emissions, were very low, as confirmed by day 0 recovery of applied NH_4-N (on average 96% for all fertilizers), and by the recovery of applied ^{15}N (on average 98%) at day 56 and 112 after Applications 3 and 6 (Table 8). In addition, the lack of NH_4-N clay-fixation suggests that any variation of SMN measured in both experiments was mainly due to the microbial immobilization-mineralization turnover.

During incubation, immobilization of added N in the AS treatment (i.e. N recovered as total soil ^{15}N minus that recovered as labelled SMN) did not change over time and was 5–11% of added N (Figure 3–4; Table 8), similar to 6–13% reported for AS-fertilized soil after 20 days of incubation by Trehan (1996). Higher immobilization percentage (19–29% of N added to soil as either AS or ammonium nitrate) was found after 1–3 years of fertilizers addition to cultivated fields (Sørensen & Amato, 2002; Sørensen & Thomsen, 2005), possibly due to retention of some fertilizer N by dead crop roots.

After slurry addition, net N immobilization occurred in the period day 0–7, even if it was statistically significant only after Application 1 (Figure 3). Net N immobilization reached a maximum 3 days after the first fertilizer addition (47% and 32% of slurry organic N for PS and DPS), and one week after Application 6 (9% and 14% of slurry organic N for PS and DPS). Net N immobilization in the week following slurry addition to soil was reported in the range 14–83% and 15–54% of slurry organic N for raw and

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456 digested pig slurry-amended soils, respectively (Alburquerque *et al.*, 2011; Kirchmann
457 & Lundvall, 1993; Morvan & Nicolardot, 2009; Sørensen and Fernández, 2003). Short-
458 term net N immobilization was likely due to fast decomposition of N-poor easily-
459 decomposable slurry organic matter like volatile fatty acids (Kirchmann & Lundvall,
460 1993) and to decomposition of slurry fibrous fraction (Morvan & Nicolardot, 2009;
461 Peters & Jensen, 2011). Moreover, digested slurries are likely to contain less VFAs than
462 undigested slurries (Finzi *et al.*, 2015) and less decomposable C (Figure 2) (Möller &
463 Müller, 2012; Malerba *et al.*, 2014); therefore they immobilized less N during
464 decomposition in soil (Figure 3) (Kirchmann & Lundvall, 1993; Loria & Sawyer, 2005).
465
466 After Application 1, in PS net SMN significantly increased from day 14 to day 56
467 (Figure 3). However, increase in SMN was not enough to compensate for initial N
468 immobilization and therefore after 56 days, net N immobilization compared to day 0
469 still occurred, accounting for 12–16% of slurry organic N (Figure 3–4).
470 Conversely, in DPS net SMN concentration did not change significantly after day 7, and
471 net N immobilization averaged 17% of slurry organic N at day 56, similarly to that
472 measured in PS (Figure 3–4). In our experiment it is unlikely that prolonged net N
473 immobilization in PS and DPS was due to extended decomposition of slurry fibrous
474 fractions (Morvan & Nicolardot, 2009; Peters & Jensen, 2011), given the ration used to
475 feed pigs (low fibre content). Therefore, net N immobilization at day 56, compared to
476 applied N, was the result of no net variation of SMN in DPS from day 14 to 56, and a
477 slow but positive mineralization in PS in the same period that did not balance
478 previously immobilized N.

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After Application 6, net SMN did not significantly change during time in all treatments (Figure 3–4): for both slurry-amended treatments N immobilization was low, or net N mineralization occurred for both slurry-amended treatments, ranging from –2 to 8% (PS) and from –10 to –5% (DPS) of slurry organic N at day 56 (Figure 3–4).

A lower net N immobilization after Application 6 compared to Application 1 (Table 5) suggests a reduced N requirement by soil microbial biomass after repeated slurry additions.

Increase of net soil mineral nitrogen

Results of experiments ULAB (Figure 3) and LAB (Figure 4) confirmed that fertilizer N availability at day 56 and 112 was proportional to the mineral N content of the fertilizer as indicated also by Gutser *et al.* (2005), in the order AS (77–91% of added N) > DPS (57–66% of added N) > PS (44–58% of added N). This means that during the relatively short duration of this experiment, the net contribution of slurry organic N to N availability was negligible (Bechini & Marino, 2009). Repeated additions of fertilizers resulted in a general increase in net SMN across applications, even if it was not always significant (Table 5–6). In particular, the trend of net SMN was more pronounced for PS than for AS and DPS, especially at day 56 (Table 5–6; Figure 4). The increase in fertilizer N availability indicated positive $Delta_{SMN}$ for most of the treatments at both day 56 and 112 (Table 7). Estimations based on the direct method were more in agreement compared to those based on the difference method with the hypothesis that $Delta_{SMN}$, as residual N effects in the field, originates from the long-term release of previously immobilized NH_4-N and the mineralization of residual organic N (Webb *et al.*, 2013). Indeed, net SMN calculated with the direct method clearly increased across

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applications in all treatments, and at both sampling dates (Figure 4; Table 6). Conversely, estimations based on unlabelled N were rather more variable, and the trend of net SMN over time was less clear (Figure 4). However, estimations obtained with both methods of calculation (difference and direct) were equally precise, with similar standard errors (Table 5–6). Instead, discrepancy between the results could be attributed to different accuracy of the difference and the direct method, the former relying on measurements taken in fertilized soil and in CON, the latter only on measurements taken in the fertilized treatments. Indeed, any occurrence of priming effect, either positive or negative, leads to a wrong estimation of fertilizer-N availability as calculated with the difference method (Rao *et al.*, 1992). Conversely, pool substitution would provide lower estimates with the direct method compared to the difference method. However, the lack of microbial biomass measurements does not allow clarifying the occurrence of one or both processes. Regardless of the method of calculation, estimated $Delta_{SMN}$ were low (on average 0.5–2.1% of added N per period of 56–112 days; Table 7), indicating that remineralization of immobilized N and mineralization of accumulated organic N occur at slow rates (Schröder *et al.*, 2013; Webb *et al.*, 2013), even under optimal temperature and water content for microbial activity in soil. In a similar experiment (Cavalli *et al.*, 2016b), we obtained sizable and slightly higher $Delta_{SMN}$ (1.5–4.0% of added N per period of 84 days) for a clay loam soil fertilized with AS, a heifer and dairy cow slurry, while in a sandy loam soil no clear trend of net SMN was observed over time. Smaller $Delta_{SMN}$ compared to our previous incubation experiment are in agreement with a coarser soil texture and smaller accumulation of residual N in soil due to lower addition of organic matter in both PS and DPS and a lower net N immobilization of slurry N.

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Conclusions

After repeated additions of ammonium sulphate, undigested and anaerobically digested pig slurry to a loam soil, available N approximated the mineral N content of the fertilizer, even after repeated additions, indicating that net mineralization of slurry organic N and re-mineralization of immobilized mineral N was negligible. Indeed, increase of available slurry N at both day 56 and 112 after fertilizer addition was positive but rather small, on average 0.5–2.1% of added N per period of 56–112 days. Values calculated with both the difference and the direct methods (based on unlabeled and ^{15}N labelled measurements, respectively) were equally precise, with similar standard errors. However, measurements of soil mineral ^{15}N followed a clear trend over time and across applications, in agreement with the hypothesis that the increase of soil mineral nitrogen, like residual effects in the field, originates from the long-term release of previously immobilized NH_4-N and the mineralization of residual organic N. Differently from N, no sizable effects of repeated additions was detected for CO_2 emissions, possibly due to the low amount of C added to soil with both slurries.

Supporting Information

The following supporting information is available in the online version of this article:

Figure S1. Linear regression between soil NH_4-N and NO_3-N measured in the two incubation experiments (ULAB and LAB) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry.

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Figure S2. Concentration of soil NH_4-N, NO_3-N and mineral nitrogen (SMN = NH_4-N + NO_3-N) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry. SE, standard error.

Table S1. Reproducibility and uncertainty of the analysis for $\delta^{15}N$ determination at different values of sample ^{15}N enrichment.

Table S2. Recovery of N and ^{15}N in filters (n = 3). Solutions of ammonium sulphate and 1M KCl were used for the test. Non-diffused samples were prepared by direct addition of solutions on acidified filters, while diffused samples were prepared according to Sørensen & Jensen (1991).

Table S3. Summary of the analysis of variance for net daily CO_2 emission rates (experiment ULAB). The dependent variable was log-transformed (natural logarithm) prior analysis.

Table S4. Summary of the analysis of variance for net SMN (% applied N) (experiment ULAB).

Table S5. Summary of the analysis of variance for net SMN (% applied N), calculated with the difference method (experiment LAB).

Table S6. Summary of the analysis of variance for net SMN (% applied N), calculated with the direct method (experiment LAB).

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~~Nitrogen~~Short-term nitrogen availability after one or repeated additions of raw and anaerobically digested ^{15}N labelled pig slurry

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Keywords

~~Manure~~Animal manure; mineralization; immobilization; residual effects; CO_2 ~~emission~~emissions

Highlights

- We studied slurry decomposition after single or repeated additions in two laboratory incubations
- We used unlabelled and ^{15}N labelled pig slurries to estimate N availability ~~and residual N effects~~
- N potentially available for plants was close to that supplied in mineral form with slurries
- Slurry N availability increased with repeated additions but ~~residual effects were~~the increase was small for both slurries
- Net contribution to residual ~~effects of~~ organic N and microbially-immobilized N was low

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Abstract

Quantifying short- and long-term availability of animal manure nitrogen (N) is of practical interest to ensure adequate crop yield, minimize N pollution, and reduce mineral-N fertilizer inputs. We measured short-term carbon (C) and N dynamics after one or six repeated additions to soil (every 56 days) of ammonium sulphate (AS), undigested (PS) and anaerobically digested (DPS) pig slurry in a laboratory incubation experiment. Soil CO_2 emissions, pH and mineral N (ammonium + nitrate) were measured in the period day 0–56 following fertilizer additions. An accompanying experiment was conducted with similar but ^{15}N labelled fertilizers to measure soil mineral N 56 and 112 days after one, three or six repeated additions, and to estimate the increase of slurry available N ~~residual effects.~~after repeated additions. Nitrogen potentially available for plants (77–91%, ~~57–66~~ 44–58% and ~~44–58~~57–66% of added N for AS, PS and DPS, respectively) was close to that supplied in mineral form with the fertilizer, indicating negligible net mineralization of slurry organic N, both after one and repeated additions. In fact, soil mineral N increased in most of the treatments because of repeated additions, but ~~residual effects~~the increases were very small, on average 0.5–2.1% of added N per period of 56–112 days. ~~Calculation~~Calculations of fertilizer N availability ~~and residual effects~~ based on unlabelled N were equally precise compared to those estimated with ^{15}N, but trends over time were rather more variable. We conclude that many repeated additions (simulating a long manuring history) are needed to obtain marked ~~residual effects~~increase of available slurry N, even under controlled conditions.

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Introduction

The incomplete ~~utilization of feed~~retention of organic matter and nutrients by livestock causes relevant amounts of ~~organic matter and nutrients~~them to remain in faeces and urine, which are stored and matured as either solid farmyard manure or liquid slurry, or are subjected to anaerobic digestion (Webb *et al.*, 2013). Anaerobic digestion transforms part of slurry organic matter (and, possibly, of other co-digested substrates) into biogas~~, which is then converted to power energy.~~. The by-product of anaerobic digestion is the digestate, which can be a good source of nutrients for crops (Loria *et al.*, 2007; Chantigny *et al.*, 2008; de Boer, 2008; Cavalli *et al.*, 2016a).

Uncertainty exists in estimating raw and digested slurries N dynamics in soil, and thus their value as fertilizers (Gutser *et al.*, 2005). Livestock diet, type of stable, duration of slurry storage, and presence of litter all have strong impacts on the physical-chemical properties of slurries, and in turn on their mineralization rate (Kyvsgaard *et al.*, 2000; Sørensen & Fernández, 2003; Powell *et al.*, 2006). In addition, slurry N availability is affected by anaerobic digestion that modifies the composition of raw slurry (Möller & Müller, 2012).

Manure N availability can be estimated with incubations of manure-soil mixtures under controlled soil temperature and water content conditions; these studies found C and N decomposition dynamics which reflected those observed in the field (Delin *et al.*, 2012; Gale *et al.*, 2006; Sørensen & Fernández, 2003), avoiding the confounding effects of soil and weather heterogeneity and soil-crop interactions (Delin *et al.*, 2012). Results of such laboratory experiments showed that short-term availability (0–3 months) of N from solid manures (Gale *et al.*, 2006; Calderón *et al.*, 2005), undigested slurries (Bechini & Marino, 2009; Morvan & Nicolardot, 2009; Sørensen & Fernández, 2003) and

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digestates (Kirchmann & Lundvall, 1993; Loria & Sawyer, 2005) often approximates the ammonium (NH_4-N) content of the manure~~, indicating~~. These results suggest that net mineralization (the balance between organic N mineralization and microbial N immobilization) of applied organic N is negligible~~.~~, or that mineralized N compensated for N losses that, in close systems, can occur as NH_3 volatilization or N_2O/N_2 emissions. Moreover, significant microbial immobilization of N can occur soon after slurry addition to soil, due to fast microbial decomposition of labile N-poor compounds like volatile fatty acids (VFA) (Kirchmann & Lundvall, 1993~~; Sørensen, 1998~~). Net N immobilization can last for several weeks (Bechini & Marino, 2009; Morvan *et al.*, 2006), during decomposition of low-N fiber fractions (Morvan & Nicolardot, 2009; Peters & Jensen, 2011). Remineralization of this microbially immobilized N, together with the late mineralization of slurry organic N give rise to residual effects beyond the year of application (Schröder *et al.*, 2013). As a result, availability of slurry N, as estimated by crop N uptake, increases when slurries are repeatedly applied to the same field (Nevens & Reheul, 2005).

Estimations of residual N effects based on single or repeated manure additions are relatively scarce. These effects were studied in field trials involving pig (Sørensen & Amato, 2002; Sørensen & Thomsen, 2005) and cattle slurries (Schröder *et al.*, 2007; Cusick *et al.*, 2006). Measured residual N effects (i.e. mineralized N in the first 12 months, the second 12 months and the third 12 months after the year of application) decreased over time due to the progressive exhaustion of added organic matter, and averaged 13, 8 and 6% of applied organic N for the three years following addition of liquid and solid manures (Schröder *et al.*, 2013). Residual effects are indeed small, highlighting a rather low net mineralization of resistant manure organic fractions even

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in the long-term. In addition, experiments that used ^{15}N labelled fertilizers applied once, reported residual N effects of liquid slurries often close to that of mineral fertilizers, especially for the third year after fertilizer addition, showing that even immobilized N is slowly re-mineralized in subsequent years (Webb *et al.*, 2013). Since residual N effects are small, it follows that many years of repeated slurry additions are needed to measure consistent effects in the field. Therefore, to acquire new knowledge in the field on residual effects of digested and undigested pig slurries would require long-term experiments. On the other hand, attempts to study the effects of repeated applications on slurry turnover in the laboratory are rare (Cavalli *et al.*, 2014, 2016b). The analysis of the literature reveals that no attempts were made to study, at the laboratory scale, the effects of repeated applications to soil of raw and digested pig slurry. Because slurry decomposition is usually faster in the laboratory than in the field (due to higher temperatures and optimal soil water content), it is possible to apply manures more frequently in the laboratory compared to the field to obtain similar decomposition rates. However, it is important that, similarly to a field situation, the slurry is added when its easily decomposable fractions (causing high CO_2 emission rates and often net N immobilization) are almost depleted. Thus, only slurry resistant fractions and immobilized NH_4-N contribute to additional available N in subsequent applications, reflecting those fractions that, under field conditions, give rise to residual effects.

~~Short term availability~~Availability of slurry N ~~and residual effects~~ can be estimated with the difference and the direct ~~methods~~method (Rao *et al.*, 1992; Muñoz *et al.*, 2004; Cusick *et al.*, 2006). The former method ~~calculate~~calculates slurry N availability as the difference between soil mineral N, for incubation experiments, or crop N uptake, for field experiments, measured in fertilized treatments and that measured in ~~a control~~an

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unfertilized control treatment. This method is frequently adopted in field and incubation studies because it does not require labelled fertilizers. ~~Conversely, incubation experiments that use ^{15}N labelled fertilizers allow tracking the dynamics of applied N separately from that of native soil N, and thus better follow the fate of applied N in different soil pools. However, experiments that employ both methods to estimate slurry N availability~~It assumes that soil native N turnover is the same in all treatments. However, the occurrence of priming effect, either negative (e.g. preferential substrate utilization) or positive (enhanced soil N turnover), produces errors in the estimates obtained with this method (Rao *et al.*, 1992). Conversely, the direct method allows tracking the dynamics of applied ^{15}N separately from that of soil native N. While isotopic fractionation at ^{15}N enrichments higher than natural abundance does not result in relevant errors in the estimation of ^{15}N availability, substitution of ^{15}N with unlabelled N leads to underestimation of available slurry N (Rao *et al.*, 1992; Murphy *et al.*, 2003). It is therefore necessary that added and native soil pools are in equilibrium in order to be equally affected by microbial (immobilization, denitrification) and abiotic processes (such as NH_4^+ clay fixation). While it seems interesting to apply both approaches to study slurry (and fertilizer) N turnover in soil, experiments that employ both methods are not frequent (Muñoz *et al.*, 2004; Cusick *et al.*, 2006).

The main objective of this work was to measure, in a laboratory incubation under controlled conditions, the fate of N repeatedly added to soil with undigested pig slurry, digested pig slurry, or ammonium sulphate. ~~An additional~~We expected that a higher fraction of the added N was available (*i.e.* in mineral forms) after repeated fertilizer additions compared to a single addition. A second objective was to compare net N mineralization estimated with the difference and the direct method; for this reason, both

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unlabelled and ^{15}N labelled fertilizers were used. We therefore wanted to test whether both methods provided reliable and comparable estimates of fertilizer N availability.

The study was accompanied by measurements of CO_2 emissions. Detailed dynamics of C mineralization enable to quantify microbial activity after fertilizer additions, by identifying the time periods when decomposition of easily degradable and more resistant slurry fractions occurs. In addition, measurements of soil pH after each manure application enable to understand whether a marked decrease of soil pH – as a result of nitrification – reduced microbial activity.

Materials and methods

Treatments and experimental set-up

We carried out two incubation experiments~~. The first one (ULAB) was defined to study mainly short-term N availability after one or repeated~~ involving unlabelled ~~fertilizer additions. The second (~~experiment ~~(LAB) was designed to study residual effects of added N,~~ ULAB) and ~~involved~~ ^{15}N labelled fertilizers~~. The two experiments~~ (experiment LAB). Both considered the full factorial combination of the two factors fertilizer type and number of cumulated fertilizer applications. The fertilizer type included a raw (PS) and an anaerobically digested (DPS) pig slurry, an unfertilized control (CON) and a mineral fertilizer control (ammonium sulphate, AS). The factor number of applications ranged from one to six, with an elapsed time of 56 days between any two applications (Figure 1). The experiment ULAB compared a single application (Application 1) with six repeated applications (Application 6), while experiment LAB had Application 1, 3 and 6. Thus, the experimental design provided a total of 8 and 12 treatment combinations for the two experiments, respectively, each replicated four

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times. We decided to apply fertilizers every 56 days in order to deplete the fast-decomposing organic matter fraction of the slurry (as confirmed by low CO_2 emission rates, close to those of CON, at the end of a 56-day period; Bechini and Marino, 2009). As a result, we expected to have mainly slurry resistant fractions and immobilized NH_4-N contributing to N availability in subsequent applications.

Slurries and soil

Unlabelled and ^{15}N labelled PS were produced by two groups of two young pigs each. Pigs of about 25 kg were fed for 7 days a ration made of maize grain (900 g day^{-1}) and concentrate (300 g day^{-1}; 37% crude protein). After that period, one group continued to be fed with the ration, while the other group was fed with a similar ration made with ^{15}N labelled maize grain (6.078 atom% ^{15}N) and unlabelled concentrate. Starting from the third day, faeces and urine were collected, separately for the two groups, mixed, added with water to reach a N concentration of about 2.5 g N kg^{-1}, and then stored for 28 days under anaerobic conditions at 5° C in PVC containers.

Unlabelled and ^{15}N labelled DPS were produced from unlabelled and ^{15}N labelled PS, respectively, using 3-litres laboratory semi-batch reactors (one for each type of slurry). An initial amount of 4 g DM of thawed PS was added to 16 g DM of fresh inoculum consisting of unlabelled digesting pig slurry taken from a commercial digestion plant. Anaerobic digestion was conducted under mesophilic conditions (40°C) and lasted 70 days. During digestion, ~~seven~~ aliquots ~~of~~(on average 6 g) of thawed PS were added to the fermenting mixtures about every 9 days through a funnel, preserving anaerobiosis, as confirmed by biogas composition (CH_4 65–74%, CO_2 26–35%, O_2 < 3%). This

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procedure allowed obtaining DPS composed of organic matter at different fermentation stages, reproducing a common situation in farm plants.

After anaerobically storage (PS) or digestion (DPS), slurries were homogenized using an Ultra Turrax T-25 disperser (IKA Werke GmbH & Co. KG, Germany) and stored in plastic bottles at −20°C until the start of the experiments. The characteristics of unlabelled and ^{15}N labelled PS and DPS are given in Table 1. Reagent- grade ^{15}N labelled AS (10.869 atom% excess ^{15}N) was taken from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO).

The soil used in both experiments (Table 2) was sampled from the 0–30 cm profile of unfertilized ~~lysimeters~~plots located at the experimental farm "A. Servadei" of the University of Udine (Italy) (46° 02' N, 13° 13' E). The soil was air dried, gently hand-ground, and sieved (2 mm) to remove coarse fragments of roots and skeleton. Thereafter, it was re-moistened and pre-incubated at 25±1°C for 12 days.

Incubation procedure

After pre-incubation, fertilizers were applied to experimental units (microcosms consisting of 115 g of dry soil) at a rate of 71 mg N kg^{-1} of dry soil, except CON that received no N addition. Incubation experiments were carried out in the dark in a thermostatic room at 25±1°C and constant soil water content (WC_{-50kPa}, Table 2); ~~they lasted~~their duration was 336 and 392 days in experiment ~~1~~ULAB and ~~2~~LAB, respectively (Figure 1). Soil water content was kept constant by individually weighting (every 1–3 days) each experimental unit and replenishing lost water using deionized water (maximum measured water loss was ±4% WC_{-50kPa}). To avoid excessive soil

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water content from fertilizer application, all experimental units belonging to Application 3 and 6 were partially air-dried during five days before subsequent applications.

In experiment ULAB, measurements of CO_2 emissions, NH_4-N and nitrate-N (NO_3-N) were taken on six dates: 1, 3, 7, 14, 28 and 56 days after Application 1 and 6. Soil NH_4-N and NO_3-N were also measured at day 0, more precisely two hours after fertilizer addition. Soil pH was measured at day 0 and day 56 after Application 1 and 6.

In experiment LAB, measurements of NH_4-N and NO_3-N were taken at day 0, 56 and 112 after Application 1, 3 and 6. At the same time, ^{15}N enrichment of NO_3-N was determined, while that of NH_4-N was determined solely at day 0, given the very low NH_4-N concentration at day 56 and 112 (< 0.5 mg kg^{-1}). In experiment LAB, total soil N (organic N + SMN) and its ^{15}N enrichment were also measured at day 56 and 112 after Application 3 and 6.

In order to allow destructive measurements on different experimental units on each sampling date, we prepared 224 (8 treatments × 7 sampling dates × 4 replicates) and 144 (12 treatments × 3 sampling dates × 4 replicates) experimental units, in experiment ~~1~~ULAB and ~~2~~LAB, respectively, for which measurements were done only once.

Measurements during incubation

At the beginning of each incubation interval, 16 experimental units were placed separately in 3-litres sealed jars along with a beaker containing 20 ml of 0.5 M NaOH and a plastic bottle containing 30 ml of water. At the end of each interval, CO_2 evolved in each jar was measured by titrating residual NaOH in the trap, after carbonate precipitation with $BaCl_2$. Soil pH was determined potentiometrically with a Crison GLP 21 + pH-meter (Crison S.A., Alella, Spain) on a soil-water mixture with a ratio of 1:2.5.

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Soluble and exchangeable ammonium-N (NH_4-N) and nitrate-N (NO_3-N) were extracted for 2 hours with a solution of 1M KCl (Cavalli *et al.*, 2016b), and determined by flow injection analysis and detected with a spectrometer (FIAstar 5000 Analyzer, Foss Tecator, Hillerød, Denmark) according to the ISO 11732 (1997) and 13395 (1996) procedures, respectively.

Total N in soil samples was determined via dry combustion of air dried samples using a EA/NA-1100 elemental analyzer (Carlo Erba, Milano, Italy). Prior to analysis, soil samples were air dried and crushed to pass through a 0.5 mm sieve using a ZM100 ultracentrifuge mill (Retsch GmbH, Haan, Germany).

Determination of $\delta^{15}N$ in solid samples was carried out using the EA/NA-1100 elemental analyzer coupled on-line through a helium flux to a Thermo Finnigan Delta Plus XP mass spectrometer (Thermo Finnigan, Bremen, Germany). Reproducibility and uncertainty of the method at different $\delta^{15}N$ are reported in Table S1.

Nitrogen in liquid samples was transferred on a solid support (glass filter trap) for determination of $\delta^{15}N$ following the procedure of Sørensen & Jensen (1991) for ~~of~~ 1M KCl soil extracts and that of Stark & Hart (1996) for Kjeldahl extracts. Results of preliminary recovery test with diffused and non-diffused 1M KCl solutions are reported in Table S2.

Calculations

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Net CO_2 emission rates of PS and DPS were calculated subtracting gross CO_2 rates (mg C kg^{-1}) measured in CON from those measured in the slurry-fertilized treatments; results were then expressed as percentage of added C.

Gross soil mineral nitrogen (SMN, mg N kg^{-1}) was calculated as the sum of 1M KCl extractable NH_4-N and NO_3-N. We did not measure non-exchangeable NH_4-N because preliminary test on this soil confirmed the lack of NH_4-N clay-fixation. Net SMN was calculated with the difference method, by subtracting gross SMN measured in CON from that measured in fertilized treatments; results were then expressed as percentage of total N applied with fertilizers (Muñoz *et al.*, 2004).

In addition, in experiment LAB, net SMN was calculated with the direct method, according to the following equation (Muñoz *et al.*, 2004):

$$Net\ SMN = \frac{\mathrm{SMN}_{FERT} \times (atom\%\ ^{15}N\ \mathrm{SMN}_{FERT} - atom\%\ ^{15}N\ SMN_{CON})}{\sum N_{FERT} \times \left(atom\%\ ^{15}N\ \mathrm{N}_{FERT} - atom\%\ ^{15}N\ \mathrm{N}_{FERT\_BG}\right)}$$

where FERT and FERT_BG refer to labelled and unlabelled AS, PS or DPS, respectively. As stated above, at day 56 and 112, SMN was assumed to be equal to NO_3-N. The same equation was used to calculate the recovery of applied ^{15}N in soil, substituting total soil N for SMN.

In the experiment LAB, ~~N residual effects (NRE~~the increase of net SMN ($Delta_{SMN}$) on day 56 and 112 after one fertilizer application ~~were~~was calculated separately for each fertilizer type, according to the following equation:

$$\textit{~~NRE~~}Delta_{SMN} = \left(\frac{\mathrm{Net\ SMN}_{A6} - \mathrm{Net\ SMN}_{A1}}{5} + \frac{\mathrm{Net\ SMN}_{A6} - \mathrm{Net\ SMN}_{A3}}{3} + \frac{\mathrm{Net\ SMN}_{A3} - \mathrm{Net\ SMN}_{A1}}{2}\right)/3$$

where A1, A3 and A6 refer to application numbers.

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Similarly, ~~NREs~~Delta$_{SMN}$ on day 56 ~~were~~was calculated in ULAB, using only Application 1 and 6.

Statistical analysis

In experiment ULAB, a three-way full factorial ANOVA model was used to assess the effects of fertilizer type (FER), number of fertilizer applications (APP), sampling date (DATE), and their interactions on gross and net CO_2 emission rates, soil pH, gross and net SMN.

Gross and net CO_2 emission rates were analysed as log-transformed variables (natural logarithm), in order to obtain a linear trend with time (also log-transformed).

Means were compared using planned contrasts. A set of polynomial contrasts was defined to test for the linear trend of log-transformed CO_2-C emission rates and of gross SMN during the 56 days, within each FER × APP combination.

A second set of contrasts was built to identify differences in the dependent variables among treatments, within each level of FER or each level of APP, separately for the period day 0–7, day 14, 28 and 56.

In experiment LAB, the same three-way full factorial ANOVA model was used to assess the effects of FER, APP, DATE, and their interactions on net SMN calculated with both difference and direct methods, and on the recovery of applied ^{15}N in soil. A set of polynomial contrasts was defined to test for the linear trend of net SMN from Application 1 to Application 6, within each FER × DATE combination.

All ANOVAs were conducted with the GLM procedure of SPSS, Version 24.0.0 (IBM Inc., Armonk, New York); contrasts were calculated with the LMATRIX command. The P values were corrected for multiple comparisons adopting the Bonferroni

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procedure. Significant mean differences are reported when the corrected *P* value was below 0.05.

Results

CO_2 emissions

Gross daily CO_2 emission rates (Figure 2a) were significantly affected by fertilizer × application × sampling date interaction (~~Table S3~~$P < 0.05$). In ~~both controls (CON and AS) and slurry amended~~all treatments ~~(PS and DPS)~~ respiration rates significantly decreased during the 56 day period following ~~Application 1 and 6 (Table S4),~~each addition, and from Application 1 to Application 6 (~~Table S5).~~

~~Addition of AS caused little differences in accumulated CO_2 emissions compared to CON after Application 1 (Table S6), when~~$P < 0.05$). After Application 1, 56-days accumulated ~~C respiration was 468 and 441 mg C kg^{-1}~~CO_2 emissions were similar in CON and AS (Figure 2b), ~~respectively,~~ accounting for 2.4 (CON) and 2.2% (AS) of soil C. Conversely, after Application 6, C respiration rates were significantly and markedly lower in AS compared to CON (~~Table S6).~~$P < 0.05$; Figure 2a). Indeed, ~~56 days~~ accumulated ~~C respiration~~CO_2 was ~~216 and 156 mg C kg^{-1} in CON and AS (Figure 2b), respectively, accounting for~~ 1.1 (CON) and 0.8% (AS) of soil C~~.~~ after 56 days, respectively (Figure 2b).

During incubation, slurry-amended treatments had always significantly higher C respiration rates compared to controls (~~Table S6~~$P < 0.05$; Figure 2a~~),~~) and accumulated ~~184 and 199 mg kg^{-1}~~ more CO_2 ~~C than controls during the 56 days following Applications 1 and 6, respectively~~ in both applications (Figure 2b).

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ANOVA showed that fertilizer, sampling date and application × sampling date interaction significantly affected net daily CO_2 emission rates (Table ~~S7). As observed for gross respiration rates, net~~S3). Net CO_2 emission rates (Figure 2c) also significantly decreased over time during each application period~~, in both PS and DPS (Table S8~~ as observed for gross rates ($P < 0.05$). However, they did not significantly change between applications (Table 3), with the exception of DPS that respired slightly more C (+9 % of applied C) in the first week following Application 1 compared to Application 6 (Figure 2d).

~~Manure net~~Net C respiration was higher following addition of PS compared to DPS, and, at the end of each application period, accumulated CO_2 accounted for ~~-~~52 and 56% of applied C, for PS, and 42% and 33% of applied C for DPS, in Application 1 and 6, respectively (Figure 2d). However, differences ~~in net C respiration rates~~ between slurries were significant only during the first month after Application 6, and during the second month after Application ~~6 (Table S9~~1 ($P < 0.05$).

Soil pH

Soil pH (Table 4) was significantly affected by two-ways interactions among factors fertilizer, number of applications and sampling date (~~Table S10). However, with the exception of AS, variation in soil pH was very narrow, both among fertilizers (<~~$P <$ 0.~~29 pH units), between applications (<~~05). During the entire incubation period, significantly ($P <$ 0.~~21~~05) higher pH was measured in CON compared to the average of all fertilizers (+0.05–0.42 pH units), and ~~between day 0 and~~ in slurry-amended treatments compared to AS (+0.13–0.48 pH units). Changes of soil pH from day 0 to day 56 ~~(< 0.13 pH units)~~within each application period were very narrow for all

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363 treatments (Table 4), even if always significant ($P < 0.05$), with the exception of PS and
364 DPS in Application 6 and 1, respectively. Conversely, ~~marked~~significant differences
365 were registered between Application 1 and 6 ~~for AS (on average 0.45 pH units),~~
366 ~~between AS and CON (range for both applications: 0.18–0.69 pH units),~~at both day 0
367 and ~~between AS~~56 in all treatments except CON (Table 4). Indeed, soil pH decreased
368 by 0.45, 0.17 and ~~slurries (range for both applications: 0.12–0.51 pH units).~~0.17 units
369 (averages between day 0 and 56) in AS, PS and DPS, respectively.
370

371 ***Soil mineral N***

372 Measurements of NH_4-N and NO_3-N taken at day 0 and 56 in experiment ULAB were
373 in excellent agreement with those obtained in experiment LAB (Figure S1), confirming
374 the similarity of experimental conditions between the two incubation trials. ~~We will~~
375 ~~therefore use results from experiment ULAB to describe short term (56 days) SMN~~
376 ~~dynamics after one or six fertilizer applications, and results of both experiments to~~
377 ~~estimate the expected increase in net SMN as a consequence of repeated fertilizer~~
378 ~~applications in the short term. In addition, results of experiment LAB were used to~~
379 ~~estimate medium term (day 112) variation of net SMN after repeated fertilizer~~
380 ~~applications.~~
381 In the two experiments, day 0 recovery of ~~applied~~fertilizer NH_4-N ~~with fertilizers~~
382 averaged 95% (data not shown), confirming the lack of NH_4-N clay-fixation. ~~Within~~
383 ~~one week after fertilizer addition, NH_4-N concentration in fertilized treatments reached~~
384 ~~values very similar to those measured in CON (<3 mg kg^{-1}); in the same period, NO_3-N~~
385 ~~started to accumulate in soil (Figure S2).~~

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386 Gross SMN (Figure S2) was significantly affected by fertilizer × application × sampling
387 date interaction (~~Table S11~~$P < 0.05$). In all treatments, gross SMN significantly
388 increased during each 56-day application period ~~(Table S12),~~ and from Application 1 to
389 Application 6 (~~Table S13~~$P < 0.05$). Accumulated SMN in CON, compared to the start
390 of the experiment, corresponded to 1.3 and 4.4% of soil total N at day 56 after
391 Application 1 and 6, respectively.
392 ~~Addition~~Added NH_4-N was quickly nitrified within one week (Figure S2); as a result of
393 ~~N fertilizers always resulted in~~ NO_3 accumulation, gross SMN was significantly ~~and~~
394 ~~markedly~~ higher ~~gross SMN concentration~~in fertilized treatments compared to CON
395 (~~Figure S2, Table S14~~$P < 0.05$).
396 In both experiments, also net SMN (% applied N; Figure 3) was significantly affected
397 by the interaction of fertilizer × applications × sampling date (Tables ~~S15–S17~~S4–S6).
398 Dynamics of net SMN over time measured in experiment ULAB differed among
399 treatments (Figure 3). After Application 1, net SMN in AS averaged 89% of applied N
400 and did not significantly change over time (~~Table S18~~$P < 0.05$). Conversely, in PS net
401 SMN significantly decreased in the period day 0–7 (–9% of applied N; ~~Table S18~~$P <$
402 0.05), and significantly increased thereafter (+4% of applied N~~; Table S18~~). An
403 intermediate pattern between AS and PS was observed in DPS, where net SMN
404 significantly ($P < 0.05$) decreased during the first week (–4% of applied N~~; Table S18~~),
405 without any significant variation thereafter ~~(Table S18),~~. Differently from Application
406 1, after Application 6~~,~~ net SMN remained constant in all treatments during the day 0–56
407 period (~~Table S18,~~ Figure 3).
408 Despite differences in net SMN over time, in both Applications 1 and 6~~,~~ net SMN was
409 significantly higher ($P < 0.05$) after addition of AS compared to slurries (+29–36% of

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added N~~; Table S19~~), and with the addition of DPS compared to PS (+7–19% of added N~~; Table S19~~).

Differences in net SMN between applications occurred only in PS for the whole period day 0–56, and in DPS for the period day 0–7, when net SMN was significantly higher following Application 6 than Application 1 (Table 5).

Estimates of the increase of net SMN at day 56 from Application 1 to Application 6 obtained in experiment ULAB (Table 5) were very similar to those obtained in experiment LAB with calculations based on the difference method (Table 6): small (<2%) or non-significant increase in AS and DPS, and significant increase in PS (5.9% and 4.7% in experiment ULAB and LAB, respectively). This increase in net SMN in PS resulted in ~~NRE~~$Delta_{SMN}$ of 1.2% of applied N per application period (Table 7). Differently from day 56, results of experiment LAB showed that the increase in net SMN at day 112 across applications (Figure 4) was sizable and significant in all treatments, corresponding to 9.0, 8.0 and 5.3% of added N in AS, PS and DPS, respectively (Table 6). Thus, at day 112 ~~NREs~~$Delta_{SMN}$ calculated with the difference method ~~were~~was positive for all treatments, in the range 1.7–2.9 % of applied N per application period (Table 7).

Calculation based on ^{15}N measurements (direct method) confirmed results obtained considering unlabelled N, with the exception of DPS, that showed a significant increase in net SMN across applications also at day 56 (Table 6). In addition, estimated trend of net SMN based on ^{15}N measurements were usually higher (+0.3–5.2% of added N) at day 56, and lower (–1.6–4.1% of added N) at day 112, than those estimated with the difference method (Table 6). ~~Nitrogen residual effects calculated~~Calculated $Delta_{SMN}$ with the direct method were quite different from those ~~calculated~~obtained with the

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difference method (Table 7), especially for AS and DPS that showed contrasting results at day 112 and 56, respectively.

Recovery of applied ^{15}N

Recovery of applied ^{15}N in soil (Table 8) was significantly affected only by fertilizer (~~Table S20~~$P < 0.05$). However, differences among treatments were very narrow and occurred mostly between AS (on average 95% of applied ^{15}N) and slurries (on average 99% of applied ^{15}N).

Discussion

CO_2 emissions

During incubation, soil and slurry organic matter was progressively mineralized (Figure 2), as confirmed by decreasing CO_2 emission rates over time after each application ~~(Table S4)~~ (Morvan *et al.*, 2006; Sørensen & Fernández, 2003; Loria & Sawyer, 2005), and by lower gross C respiration after ~~Application 6 than after Application 1 (Table S5)~~repeated slurry additions (Cavalli *et al.*, 2014).

In AS, lower emissions compared to CON after Application 6 (Figure 2b~~; Table S6~~) could be related to the large decrease of soil pH in AS (Table 4) as a consequence of nitrification of added NH_4-N (Loria & Sawyer, 2005; Cavalli *et al.*, 2016b), that might have diminished soil organic matter decomposition compared to CON (Aciego Pietri & Brookes, 2008).

Accumulated C respiration in PS and DPS (54% and 33–42% of slurry C, respectively; Figure 2d) was similar to 46–63% and 34–43% reported by Kirchmann & Lundvall (1993) and Alburquerque *et al.* (2011) for undigested and digested ~~PS~~pig slurries

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incubated in soil for the same duration ~~(Kirchmann & Lundvall, 1993; Alburquerque *et al.*, 2011).~~. Sørensen and Fernández (2003) obtained rather lower 56-days emissions for PS (25–42% of slurry C), probably because their incubation was carried out at lower temperature (8°C vs. 25°C).

Higher respiration rates (~~Table S9;~~ Figure 2c), and thus higher cumulative CO_2 emissions in PS than in DPS (Figure 2d) (Kirchmann & Lundvall, 1993; Marcato *et al.*, 2009) could be attributed to the loss of ~~easily~~ degradable C compounds (48% of initial C was not recovered in DPS; Table 1) during anaerobic digestion (Möller & Müller, 2012), and to the accumulation of chemically recalcitrant organic matter in digestate (Malerba *et al.*, 2014).

It was expected that after repeated additions of PS and DPS slurry C decomposition would increase due to the accumulation of decomposable organic matter in soil. Indeed, repeated 84-days additions of cattle slurries to a sandy loam and a clay loam soil resulted in ~~a residual effect~~an increase in CO_2 emissions of 1.43% of slurry C per period of 84 days (Cavalli *et al.*, 2014). However, in this experiment, we applied only 450 and 260 mg C kg^{-1} with PS and DPS, respectively, an amount comparable to that of soil microbial biomass (about 337 mg C kg^{-1}, assuming that it represented 1.7% of soil organic C; Xu *et al.*, 2013).

For this reason, we make the hypothesis that slurry C was mainly used as an energy source and thus did not allow a relevant microbial biomass growth. ~~This phenomenon could have been more relevant after Application 6 than after Application 1 because, as microbial biomass grew due to repeated slurry additions (Rochette *et al.*, 2000), the amount of applied C was progressively similar to the size of microbial biomass C. Conversely, Cavalli *et al.* (2014) applied more slurry C (860–1000 mg C kg^{-1}) that~~

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~~presumably induced microbial growth after each slurry application, with subsequent accumulation of microbial residues. In the present experiment~~Therefore, the lack of any measurable ~~C residual effect~~increase in net CO_2 emissions could ~~therefore~~ have been due to a reduced accumulation of microbial residues, and to the persistence of highly ~~recalcitrant~~resistant residual fractions of slurry organic matter.

Soil mineral N dynamics

Losses of N from the soil due to NH_3 volatilization or N_2O/N_2 emissions, were very low, as confirmed by day 0 recovery of applied NH_4-N (on average 96% for all fertilizers), and by the recovery of applied ^{15}N (on average 98%) at day 56 and 112 after Applications 3 and 6 (Table 8). In addition, the lack of NH_4-N clay-fixation suggests that any variation of SMN measured in both experiments was mainly due to the microbial immobilization-mineralization turnover.

During incubation, immobilization of added N in the AS treatment (i.e. N recovered as total soil ^{15}N minus that recovered as labelled SMN) did not change ~~during~~over time ~~(Table S18)~~ and was 5–11% of added N (Figure 3–4; Table 8), similar to 6–13% reported for AS-fertilized soil after 20 days of incubation ~~(~~by Trehan~~,~~ (1996). Higher immobilization percentage (19–29% of N added to soil as either AS or ammonium nitrate) was found after 1–3 years of fertilizers addition to cultivated fields (Sørensen & Amato, 2002; Sørensen & Thomsen, 2005), possibly due to retention of some fertilizer N by dead crop roots.

After slurry addition, net N immobilization occurred in the period day 0–7, even if it was statistically significant only after Application 1 (~~Table S18;~~ Figure 3). Net N immobilization reached a maximum 3 days after the first fertilizer addition (47% and

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32% of slurry organic N for PS and DPS), and one week after Application 6 (9% and 14% of slurry organic N for PS and DPS). Net N immobilization in the week following slurry addition to soil was reported in the range 14–83% and 15–54% of slurry organic N for ~~PS~~raw and ~~DPS~~ digested pig slurry-amended soils, respectively (Alburquerque *et al.*, 2011; Kirchmann & Lundvall, 1993; Morvan & Nicolardot, 2009; Sørensen and Fernández, 2003). Short-term net N immobilization was likely due to fast decomposition of N-poor easily-decomposable slurry organic matter like volatile fatty acids (Kirchmann & Lundvall, 1993~~; Sørensen, 1998~~) and to decomposition of slurry fibrous fraction (Morvan & Nicolardot, 2009; Peters & Jensen, 2011). Moreover, digested slurries are likely to contain less VFAs than undigested slurries (Finzi *et al.*, 2015) and less decomposable C (Figure 2~~; Table 1~~) (Möller & Müller, 2012; Malerba *et al.*, 2014); therefore they immobilized less N during decomposition in soil (Figure 3) (Kirchmann & Lundvall, 1993; Loria & Sawyer, 2005).

~~At the end of~~

After Application 1, in PS~~, despite~~ net SMN significantly increased from day 14 to day 56 (~~Table S18),~~Figure 3). However, increase in SMN was not enough to compensate for initial N immobilization and therefore after 56 days, net N immobilization~~,~~ compared to ~~applied N,~~day 0 still occurred, accounting for 12–16% of slurry organic N (Figure 3–4). Conversely, in DPS net SMN concentration did not change significantly after day 7, and net N immobilization averaged 17% of slurry organic N at day 56, similarly to that measured in PS (Figure 3–4). In our experiment it is unlikely that prolonged net N immobilization in PS and DPS was due to extended decomposition of slurry fibrous fractions (Morvan & Nicolardot, 2009; Peters & Jensen, 2011), given the ration used to feed pigs (low fibre content). Therefore, net N immobilization at day 56, compared to

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applied N, was the result of no net variation of SMN in DPS from day 14 to 56, and a slow but positive mineralization in PS in the same period that did not balance previously immobilized N.

After Application 6, net SMN did not significantly change during time in all treatments (Figure 3–4~~, Table S18~~): for both slurry-amended treatments N immobilization was low, or net N mineralization occurred for both slurry-amended treatments, ranging from –2 to 8% (PS) and from –10 to –5% (DPS) of slurry organic N at day 56 (Figure 3–4).

A lower net N immobilization after Application 6 compared to Application 1 (Table 5) ~~could be due to~~suggests a reduced N requirement by soil microbial biomass after repeated slurry additions. ~~In fact, as hypothesized above, it is more likely that microbial biomass grew following Application 1, inducing net N immobilization; conversely, if after Application 6 most of the decomposed slurry was used as an energy source (without a consistent associated growth), and previously immobilized N was recycled, extra N was not required by microbial biomass.~~

~~***N residual effects***~~

Increase of net soil mineral nitrogen

Results of experiments ULAB (~~Table S19;~~ Figure 3) and LAB (Figure 4) confirmed that fertilizer N availability at day 56 and 112 was proportional to the mineral N content of the fertilizer as indicated also by Gutser *et al.* (2005), in the order AS (77–91% of added N) > DPS (57–66% of added N) > PS (44–58% of added N). This means that during the relatively short duration of this experiment, the net contribution of slurry organic N to N availability was negligible (Bechini & Marino, 2009). Repeated additions of fertilizers resulted in a general increase in net SMN across applications, even if it was not always

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554 significant (Table 5–6). In particular, the trend of net SMN was more pronounced for
555 PS than for AS and DPS, especially at day 56 (Table 5–6; Figure 4). The increase in
556 fertilizer N availability indicated positive ~~residual effects~~Delta$_{SMN}$ for most of the
557 treatments at both day 56 and 112 (Table 7). Estimations based on the direct method
558 were more in agreement compared to those based on the difference method with the
559 hypothesis that ~~NRE~~Delta$_{SMN}$, as residual N effects in the field, originates from the
560 long-term release of previously immobilized NH_4-N and the mineralization of residual
561 organic N (Webb *et al.*, 2013). Indeed, net SMN calculated with the direct method
562 clearly increased across applications in all treatments, and at both sampling dates
563 (Figure 4; Table 6). Conversely, ~~estimation~~estimations based on unlabelled N were
564 rather more variable, and the trend of net SMN over time was less clear (Figure 4).
565 However, ~~estimation~~estimations obtained with both methods of calculation (difference
566 and direct) were equally precise, with similar standard errors (Table 5–6). Instead,
567 discrepancy between the results could be attributed to different accuracy of the
568 difference and the direct method, the former relying on measurements taken in fertilized
569 soil and in CON, the latter only on measurements taken in the fertilized treatments.
570 Indeed, any occurrence of priming effect, either positive or negative, leads to a wrong
571 estimation of fertilizer-N availability as calculated with the difference method (Rao *et*
572 *al.*, 1992). Conversely, pool substitution would provide lower estimates with the direct
573 method compared to the difference method. However, the lack of microbial biomass
574 measurements does not allow clarifying the occurrence of one or both processes.
575 Regardless of the method of calculation, estimated ~~NRE~~sDelta$_{SMN}$ were low (on average
576 0.5–2.1% of added N per period of 56–112 days; Table 7), indicating that
577 remineralization of immobilized N and mineralization of accumulated organic N occur

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at slow rates (Schröder *et al.*, 2013; Webb *et al.*, 2013), even under optimal temperature and water content for microbial activity in soil. In a similar experiment (Cavalli *et al.*, 2016b), we obtained sizable and slightly higher ~~NRES~~Delta$_{SMN}$ (1.5–4.0% of added N per period of 84 days) for a clay loam soil fertilized with AS, a heifer and dairy cow slurry, while in a sandy loam soil~~,~~ no clear trend of net SMN was observed over time. Smaller ~~NRES~~Delta$_{SMN}$ compared to our previous incubation experiment are in agreement with ~~low~~a coarser soil texture and smaller accumulation of residual N in soil due to lower addition of organic matter in both PS and DPS~~, and thus to a reduced microbial growth, especially after repeated~~ and a lower net N immobilization of slurry ~~additions, in addition to a coarser soil texture~~N.

Conclusions

After repeated additions of ammonium sulphate, undigested and anaerobically digested pig slurry to a loam ~~agricultural~~ soil, available N approximated the mineral N content of the fertilizer, even after repeated additions, indicating that net mineralization of slurry organic N and re-mineralization of immobilized mineral N was negligible. Indeed, ~~residual effects~~increase of available slurry N at both day 56 and 112 after fertilizer addition ~~were~~was positive but rather small, on average 0.5–2.1% of added N per period of 56–112 days. ~~Residual effects~~Values calculated with both the difference and the direct methods (based on unlabeled and ^{15}N labelled measurements, respectively) were equally precise, with similar standard errors. However, measurements of soil mineral ^{15}N followed a clear trend over time and across applications, in agreement with the hypothesis that the increase of soil mineral nitrogen, like residual effects in the field, originates from the long-term release of previously immobilized NH_4-N and the

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mineralization of residual organic N. Differently from N, no sizable ~~residual~~ effects of ~~fertilizers~~repeated additions was detected ~~related~~ for CO_2 emissions, possibly due to the low amount of C added to soil with both slurries.

Supporting Information

The following supporting information is available in the online version of this article:

Figure S1. Linear regression between soil NH_4-N and NO_3-N measured in the two incubation experiments (ULAB and LAB) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry.

Figure S2. Concentration of soil NH_4-N, NO_3-N and mineral nitrogen (SMN = NH_4-N + NO_3-N) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry. SE, standard error.

Table S1. Reproducibility and uncertainty of the analysis for $\delta^{15}N$ determination at different values of sample ^{15}N enrichment.

Table S2. Recovery of N and ^{15}N in filters (n = 3). Solutions of ammonium sulphate and 1M KCl were used for the test. Non-diffused samples were prepared by direct addition of solutions on acidified filters, while diffused samples were prepared according to Sørensen & Jensen (1991).

Table S3. ~~Summary of the analysis of variance for gross daily CO_2 emission rates measured in experiment ULAB. The dependent variable was log transformed (natural logarithm) prior analysis.~~

~~**Table S4.** Planned polynomial contrasts of linear effect of sampling date on gross daily CO_2 emission rates within fertilizer × application combinations (experiment ULAB).~~

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626 ~~**Table S5.** Planned contrasts of number of applications effect on gross daily CO_2~~
627 ~~emission rates within fertilizer × sampling date combinations (experiment ULAB).~~
628 ~~**Table S6.** Planned contrasts of fertilizer effect on gross daily CO_2 emission rates within~~
629 ~~number of applications × sampling date combinations (experiment ULAB).~~
630 ~~**Table S7.** ~~Summary of the analysis of variance for net daily CO_2 emission rates
631 (experiment ULAB). The dependent variable was log-transformed (natural logarithm)
632 prior analysis.
633 **Table** ~~**S8.** Planned polynomial contrasts of linear effect of sampling date on net daily~~
634 ~~CO_2 emission rates within fertilizer × application combinations~~**S4.** Summary of the
635 analysis of variance for net SMN (% applied N) (experiment ULAB).
636 **Table** ~~**S9.** Planned contrasts of fertilizer effect on net daily CO_2 emission rates within~~
637 ~~number of applications × sampling date combinations (experiment ULAB).~~
638 ~~**Table S10.** Summary of the analysis of variance for pH (experiment ULAB).~~
639 ~~**Table S11.** Summary of the analysis of variance for gross SMN (mg N kg 1)~~
640 ~~(experiment ULAB).~~
641 ~~**Table S12.** Planned polynomial contrasts of linear effect of sampling date on gross~~
642 ~~SMN within fertilizer × application combinations (experiment ULAB).~~
643 ~~**Table S13.** Planned contrasts of number of applications effect on gross SMN within~~
644 ~~fertilizer × sampling date combinations (experiment ULAB).~~
645 ~~**Table S14.** Planned contrasts of fertilizer effect on gross SMN within number of~~
646 ~~applications × sampling date combinations (experiment ULAB).~~
647 ~~**Table S15.** Summary of the analysis of variance for net SMN (% applied N)~~
648 ~~(experiment ULAB).~~

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649 ~~**Table S16**~~**S5.** Summary of the analysis of variance for net SMN (% applied N),
650 calculated with the difference method (experiment LAB).

651 **Table ~~S17~~S6.** Summary of the analysis of variance for net SMN (% applied N),
652 calculated with the direct method (experiment LAB).

653 ~~**Table S18.** Planned polynomial contrasts of linear effect of sampling date on net SMN~~
654 ~~within fertilizer × application combinations (experiment ULAB).~~

655 ~~**Table S19.** Planned contrasts of fertilizer effect on net SMN within number of~~
656 ~~applications × sampling date combinations (experiment ULAB).~~

657 ~~**Table S20.** Summary of the analysis of variance for recovery of applied ^{15}N (%) in~~
658 ~~experiment LAB.~~

659

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Table 1. Characteristics of the slurries used in the two incubation experiments.

Pig slurry	Type	pH	DM[a]	TKN[b]	NH_4-N	Org. N[c]	C	Atom% excess ^{15}N			C/N	C/Org. N	NH_4-N/TKN	Recovery after anaerobic digestion / % initial		
			/ %	/ % DM				TKN	NH_4-N	Org. N			/ %	C	TKN	NH_4-N
Raw	Unlabelled	7.9	2.9	6.2	3.5	2.7	39.6	—	—	—	6.4	14.7	56.5	—	—	—
(PS)	^{15}N labelled	7.4	4.3	6.2	3.5	2.8	42.2	2.092	1.872	2.368	6.8	15.3	55.7	—	—	—
Digested	Unlabelled	8.2	1.7	9.4	6.3	3.1	34.1	—	—	—	3.6	10.9	66.9	51	97	115
(DPS)	^{15}N labelled	8.2	1.9	9.9	6.5	3.4	37.1	1.643	1.725	1.484	3.7	10.9	65.8	53	100	116

[a]dry matter.

[b]total Kjeldahl N.

[c]organic N.

Table 2. Characteristics of the soil used in the two incubation experiments.

Variable / unit	Value
Sand / g kg^{-1}	340
Silt / g kg^{-1}	495
Clay / g kg^{-1}	165
Total C / g kg^{-1}	19.8
Total N / g kg^{-1}	1.7
$CaCO_3$ / g kg^{-1}	9.6
pH in water	7.9
Water content at –50 kPa (WC_{-50kPa}) / g H_2O kg^{-1}	217

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Table 3. Planned contrasts of number of applications effect on net daily CO_2 emission rates (experiment ULAB) within fertilizer × sampling date combinations.

Fertilizer[a]	Sampling date	Net CO_2 emission rate[b]		S.E.[c]	P[d]
		Application 1	Application 6		
PS	Day 1–7	1.81 (5.92)	1.66 (5.07)	0.10	1.000
	Day 14–28	–0.11 (0.69)	0.09 (0.90)	0.13	1.000
	Day 56	–1.02 (0.16)	–0.73 (0.28)	0.18	1.000
DPS	Day 1–7	1.64 (4.96)	1.29 (3.44)	0.11	0.016
	Day 14–28	–0.38 (0.49)	–0.44 (0.45)	0.13	1.000
	Day 56	–1.65 (–0.01)	–1.17 (0.11)	0.18	0.144

[a]PS, raw pig slurry; DPS, anaerobically digested pig slurry.

[b]Net daily CO_2 emission rates were log-transformed (natural logarithm) prior analysis. Untransformed values (% applied C day^{-1}) are reported in parenthesis.

[c]Standard error of the difference between log-transformed net daily CO_2 emission rates.

[d]*P* values adjusted for multiple comparison according to Bonferroni procedure.

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Table 4. Planned contrasts of number of applications effect on soil pH within fertilizer × sampling date combinations (experiment ULAB).

Fertilizer	Sampling date	Soil pH		S.E.	P^c
		Application 1	Application 6		
CON	Day 0	7.71	7.75	0.02	1.000
	Day 56	7.85	7.85	0.02	1.000
AS	Day 0	7.53	7.12	0.02	<0.001
	Day 56	7.64	7.16	0.02	<0.001
PS	Day 0	7.68	7.56	0.02	<0.001
	Day 56	7.77	7.56	0.02	<0.001
DPS	Day 0	7.77	7.63	0.02	<0.001
	Day 56	7.76	7.56	0.02	<0.001

[a]CON, control with wter; AS, control with ammonium sulphate; PS, raw pig slurry; DPS, anaerobically digested pig slurry.

[b]Standard error of the difference between soil pH.

[c]*P* values adjusted for multiple comparison according to Bonferroni procedure.

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Table 5. Planned contrasts of number of applications effect on net SMN within fertilizer × sampling date combinations (experiment ULAB).

Fertilizer[a]	Sampling date	Net SMN / % applied N		S.E.[b]	*P*[c]
		Application 1	Application 6		
AS	Day 0–7	89.5	88.5	0.7	1.000
	Day 14–28	88.6	89.5	1.0	1.000
	Day 56	87.1	90.7	1.4	0.462
PS	Day 0–7	45.4	54.4	0.7	<0.001
	Day 14–28	45.9	55.1	1.0	<0.001
	Day 56	50.7	56.6	1.4	<0.001
DPS	Day 0–7	61.0	64.1	0.7	<0.001
	Day 14–28	64.6	65.5	1.0	1.000
	Day 56	65.3	63.8	1.4	1.000

[a]AS, control with ammonium sulphate; PS, raw pig slurry; DPS, anaerobically digested pig slurry.

[b]Standard error of the difference between net SMN.

[c]*P* values adjusted for multiple comparison according to Bonferroni procedure.

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Table 6. Planned polynomial contrasts of linear effect of number of applications on net SMN within fertilizer × sampling date combinations (experiment LAB).

Fertilizer[a]	Sampling date	Difference method		Direct method	
		Trend in net SMN / % applied N[b]	*P*[c]	Trend in net SMN / % applied N	*P*
AS	Day 56	1.94±0.92	0.038	2.24±1.32	0.094
	Day 112	8.97±0.92	<0.001	4.90±1.24	<0.001
PS	Day 56	4.68±0.92	<0.001	6.91±1.23	<0.001
	Day 112	7.99±0.92	<0.001	6.16±1.24	<0.001
DPS	Day 56	0.44±0.92	0.632	5.60±1.24	<0.001
	Day 112	5.32±0.92	<0.001	3.72±1.23	0.003

[a]AS, control with ammonium sulphate; PS, raw pig slurry; DPS, anaerobically digested pig slurry.

[b]Estimated increase or decrease in net SMN (% applied N) from Application 1 to Application 6.

[c]*P* values adjusted for multiple comparison according to Bonferroni procedure.

The fitted trend in net SMN is given for each contrast within each Application 1–6 period (±standard error).

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Table 7. Estimated increase of net soil mineral N ($Delta_{SMN}$, % applied N per application period ± standard error) at day 56 and 112 after addition of fertilizers.

Fertilizer	Day	Experiment ULAB	Experiment LAB	
			Difference method	Direct method
Ammonium	56	0.7±0.3	0.2±0.3	0.7±0.2
sulphate (AS)	112	—	2.9±0.1	0.9±0.9
Raw pig	56	1.2±0.2	1.2±0.2	2.0±0.1
slurry (PS)	112	—	2.3±0.2	1.8±0.1
Digested pig	56	–0.3±0.1	0.0±0.1	1.7±0.1
slurry (DPS)	112	—	1.7±0.2	1.1±0.2

Table 8. Recovery of applied ^{15}N in soil (%) after three or six applications of fertilizers in experiment LAB. S.E., standard error.

Fertilizer	Number of applications	Sampling day		S.E.
		Day 56	Day 112	
Ammonium sulphate (AS)	3	95.2	96.6	1.2
	6	94.1	94.4	1.2
Raw pig slurry (PS)	3	98.1	98.3	1.2
	6	99.9	100.2	1.2
Digested pig slurry (DPS)	3	101.1	99.5	1.2
	6	100.7	96.8	1.2

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Figure 1. The experiment ULAB evaluated the application of unlabelled fertilizers (water, ammonium sulphate, raw and anaerobically digested pig slurry) for one or six times. The experiment LAB evaluated the application of ^{15}N labelled fertilizers for one, three or six times. After applications, measurements were made during 56 (experiment ULAB) or 112 (experiment LAB) days. Gray points represent fertilizer applications. Ticks on horizontal bars represent measurement dates.

Figure 2. Daily CO_2 emission rates (a, c) and accumulated CO_2 emissions (b, d) measured during 56 days following one or six repeated additions of water or fertilizers to soil in the ULAB experiment. CON, control with water; AS, control with ammonium sulphate; PS, raw slurry; DPS, anaerobically digested pig slurry. SE, standard error.

Figure 3. Net soluble plus exchangeable mineral N concentration measured during 56 days following one or six repeated additions of water or fertilizers to soil in the ULAB experiment. AS, control with ammonium sulphate; PS, raw slurry; DPS, anaerobically digested pig slurry. SE, standard error. Dashed horizontal gray lines indicate the amount of inorganic N added with fertilizers.

Figure 4. Net soluble plus exchangeable mineral N concentration measured 56 and 112 days following one, three or six repeated additions of water or fertilizers to soil in the LAB experiment. AS, control with ammonium sulphate; PS, raw slurry; DPS, anaerobically digested pig slurry. Net soil mineral N was calculated with the difference and direct methods. SE, standard error. Dashed horizontal gray lines indicate the amount of inorganic N added with fertilizers.

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Figure 1

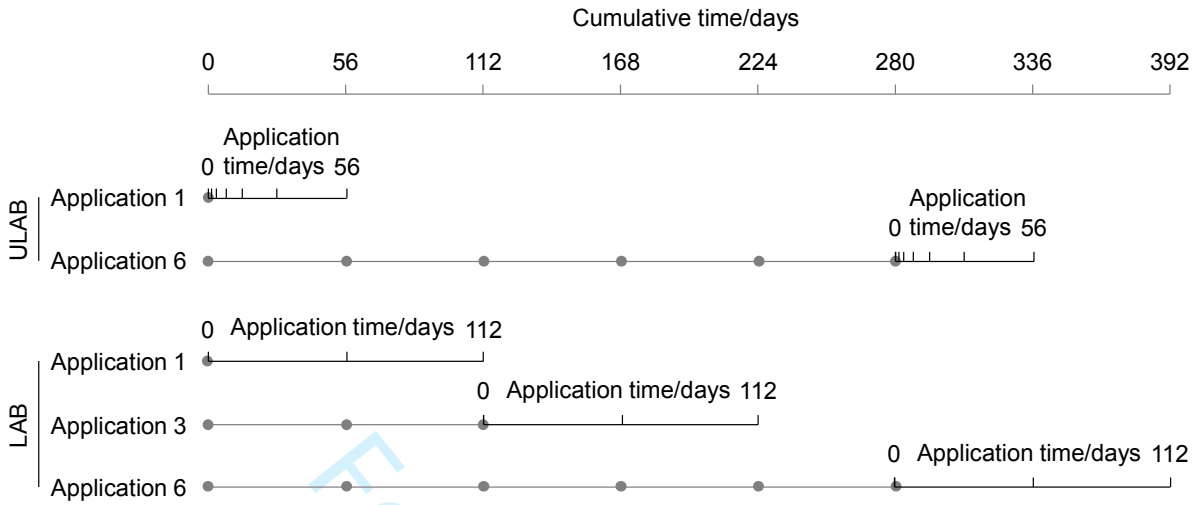

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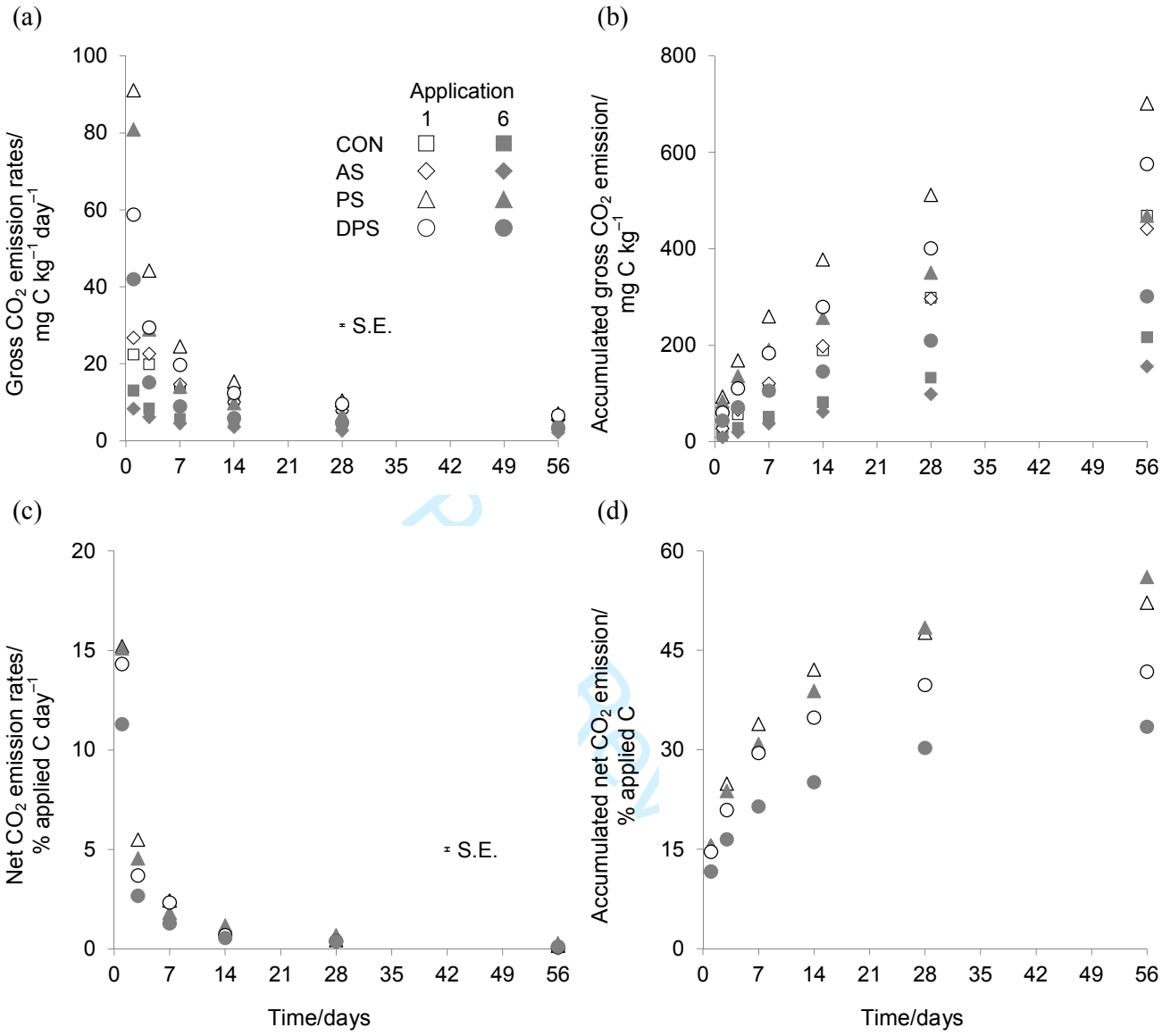
Figure 2


(a)
(b)
(c)
(d)
Application
1
6
CON
AS
PS
DPS
± S.E.
Gross CO2 emission rates/ mg C kg-1 day-1
Accumulated gross CO2 emission/ mg C kg-1
Net CO2 emission rates/ % applied C day-1
Accumulated net CO2 emission/ % applied C
Time/days
Time/days

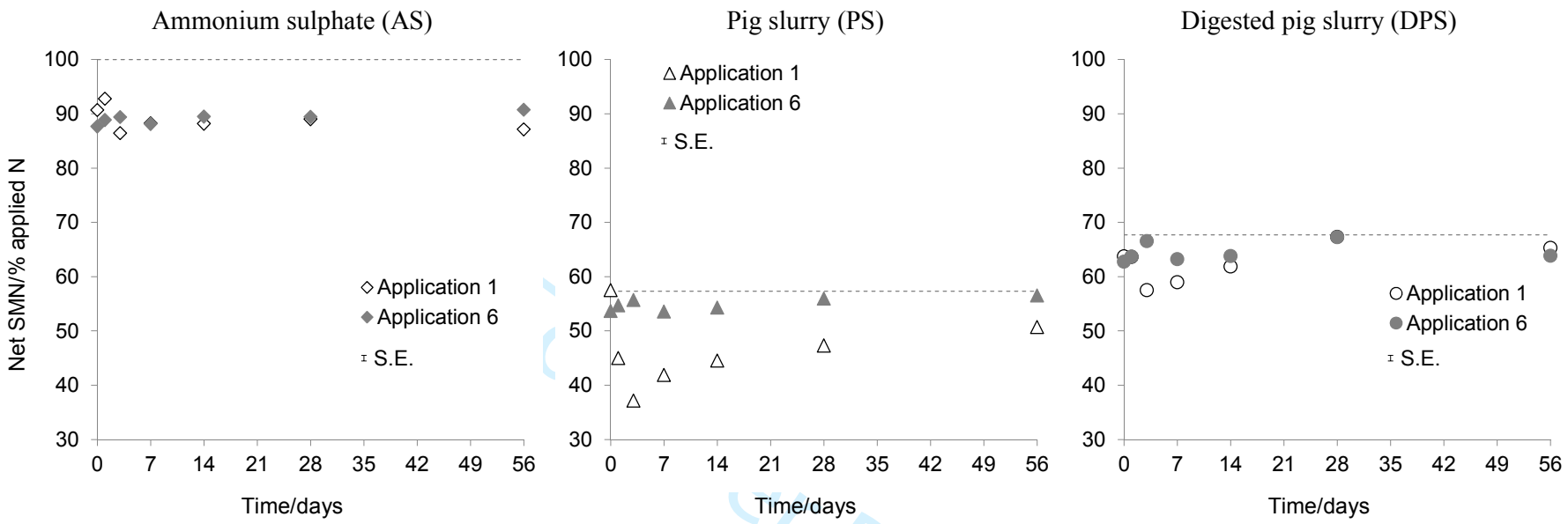

Ammonium sulphate (AS)
Pig slurry (PS)
Digested pig slurry (DPS)
Net SMN/% applied N
Time/days
Application 1
Application 6
S.E.
100
90
80
70
60
50
40
30
0
7
14
21
28
35
42
49
56

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Figure 4

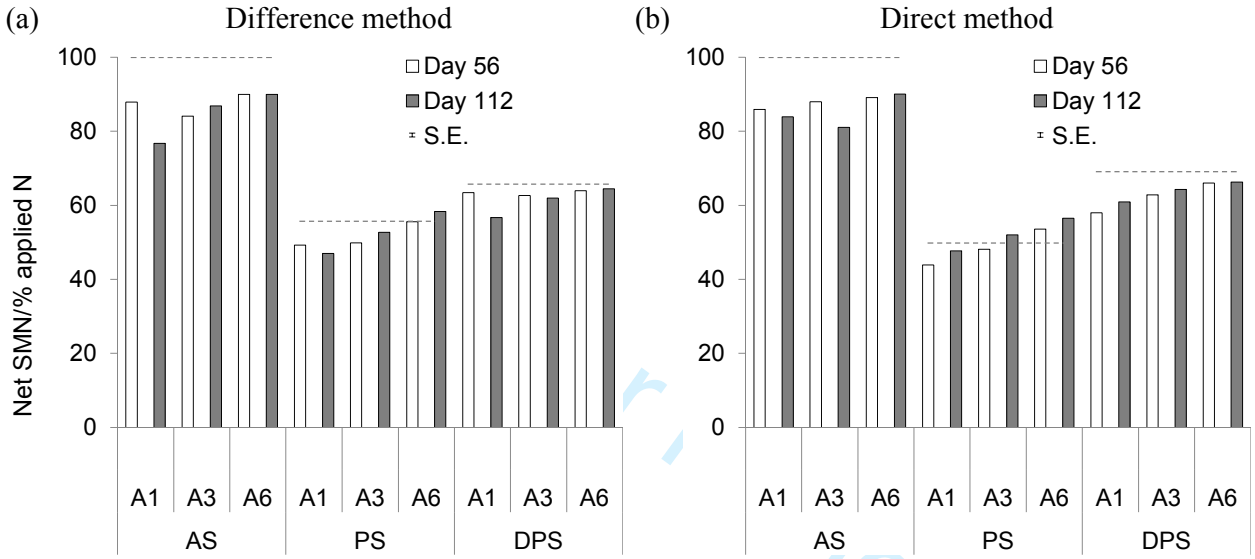
(a)
Difference method
(b)
Direct method
Net SMN/% applied N
100
80
60
40
20
0
Day 56
Day 112
S.E.
A1
A3
A6
AS
PS
DPS

Figure S1. Linear regression between soil NH_4-N and NO_3-N measured in the two incubation experiments (ULAB and LAB) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry.

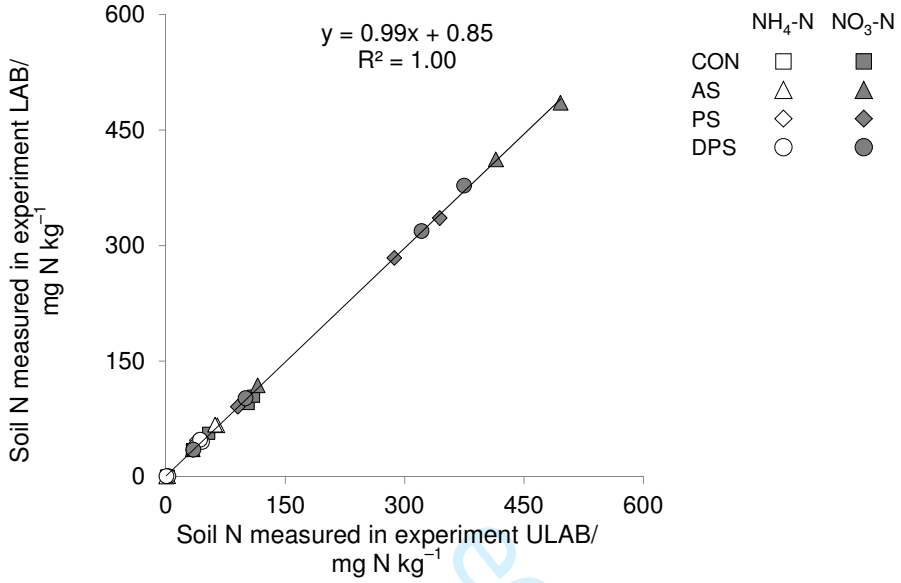

Figure S2. Concentration of soil NH_4-N, NO_3-N and mineral nitrogen (SMN = NH_4-N + NO_3-N) following one or six repeated additions of water (CON), ammonium sulphate (AS), raw (PS) and anaerobically digested (DPS) pig slurry. SE, standard error.

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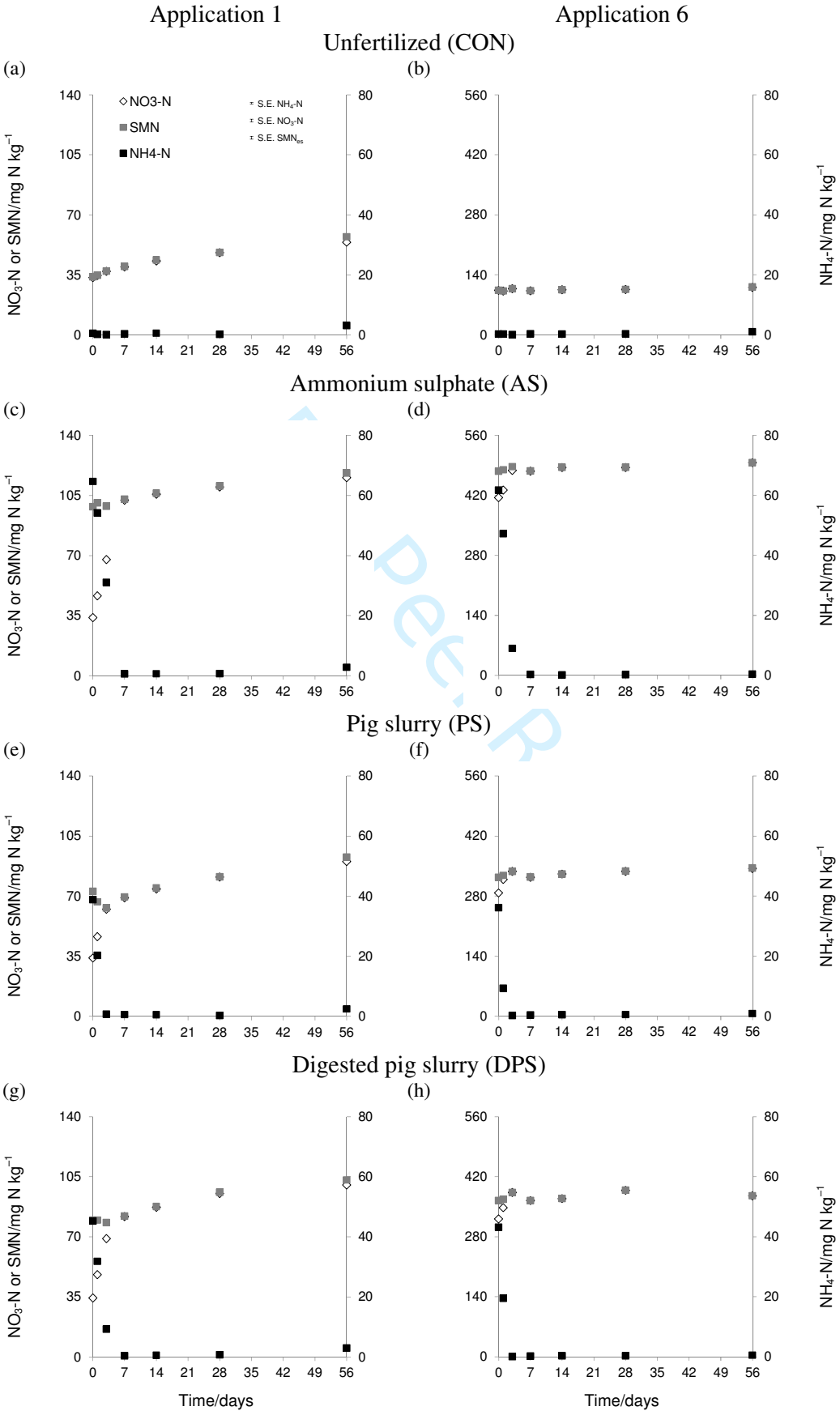
Application 1
Application 6
Unfertilized (CON)
(a)
(b)
NO3-N
SMN
NH4-N
S.E. NH4-N
S.E. NO3-N
S.E. SMNes
Ammonium sulphate (AS)
(c)
(d)
Pig slurry (PS)
(e)
(f)
Digested pig slurry (DPS)
(g)
(h)
NO3-N or SMN/mg N kg-1
NH4-N/mg N kg-1
Time/days

Table S1. Reproducibility and uncertainty of the analysis for $\delta^{15}N$ determination at different values of sample ^{15}N enrichment.

$\delta^{15}N$ / ‰	Reproducibility / ‰	Uncertainty / ‰
500	± 2	± 9
1500	± 5	± 29
3000	± 19	± 66
7000	± 27	± 137

Table S2. Recovery of N and ^{15}N in filters (n = 3). Solutions of ammonium sulphate and 1M KCl were used for the test. Non-diffused samples were prepared by direct addition of solutions on acidified filters, while diffused samples were prepared according to Sørensen & Jensen (1991).

Total N on filter / µg	Atom% ^{15}N	Non-diffused samples			Diffused samples		
		Recovery of N on filter / %		Estimated atom% ^{15}N	Recovery of N on filter / %		Estimated atom% ^{15}N
		Total N	^{15}N		Total N	^{15}N	
30	0.933	97±1	95±1	0.919±0.004	97±0	97±0	0.927±0.003
	1.498	97±2	95±1	1.472±0.014	—	—	—
	3.191	98±1	95±1	3.109±0.011	98±0	97±2	3.180±0.049
120	0.933	93±2	94±2	0.934±0.001	98±2	99±2	0.940±0.000
	1.498	95±1	95±1	1.507±0.002	—	—	—
	3.191	94±1	93±1	3.170±0.009	98±0	96±0	3.113±0.006

Table S3. Summary of the analysis of variance for net daily CO_2 emission rates (experiment ULAB). The dependent variable was log-transformed (natural logarithm) prior analysis.

Model	Degrees of freedom	Mean square	F	P
Fertilizer	1	3.010	48.05	<0.001
Application	1	0.031	0.49	0.485
Sampling date	5	28.635	457.07	<0.001
Fertilizer × application	1	0.147	2.35	0.130
Fertilizer × sampling date	5	0.099	1.58	0.177
Application × sampling date	5	0.321	5.12	<0.001
Fertilizer × application × sampling date	5	0.031	0.50	0.774
Error	71	0.063		

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Table S4. Summary of the analysis of variance for net SMN (% applied N) (experiment ULAB).

Model	Degrees of freedom	Mean square	*F*	*P*
Fertilizer	2	21388.13	5136.44	<0.001
Application	1	526.84	126.52	<0.001
Sampling date	6	68.09	16.35	<0.001
Fertilizer × application	2	280.04	67.25	<0.001
Fertilizer × sampling date	12	27.25	6.55	<0.001
Application × sampling date	6	89.27	21.44	<0.001
Fertilizer × application × sampling date	12	22.71	5.45	<0.001
Error	126	4.16		

Table S5. Summary of the analysis of variance for net SMN (% applied N), calculated with the difference method (experiment LAB).

Model	Degrees of freedom	Mean square	*F*	*P*
Fertilizer	2	11793.79	3486.37	<0.001
Application	2	195.74	57.86	<0.001
Sampling date	2	86.48	25.57	<0.001
Fertilizer × application	4	8.51	2.51	0.048
Fertilizer × sampling date	4	47.71	14.10	<0.001
Application × sampling date	4	171.74	50.77	<0.001
Fertilizer × application × sampling date	8	14.96	4.42	<0.001
Error	81	3.38		

Table S6. Summary of the analysis of variance for net SMN (% applied N), calculated with the direct method (experiment LAB).

Model	Degrees of freedom	Mean square	*F*	*P*
Fertilizer	2	12296.25	2020.96	<0.001
Application	2	343.50	56.46	<0.001
Sampling date	2	174.05	28.61	<0.001
Fertilizer × application	4	20.94	3.44	0.012
Fertilizer × sampling date	4	46.92	7.71	<0.001
Application × sampling date	4	15.05	2.47	0.051
Fertilizer × application × sampling date	8	14.72	2.42	0.022
Error	76	6.08		

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