

Article

Improving Nitrogen Fertilization Recommendations in Temperate Agricultural Systems: A Study on Walloon Soils Using Anaerobic Incubation and POxC

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Abstract

Crops nitrogen supply through the in situ mineralization of soil organic matter is a critical process for plant nutrition. However, accurately estimating the contribution of mineralization remains challenging. The complexity of biological, chemical, and physical processes in the soil, influenced by environmental conditions, makes it difficult to precisely quantify the amount of nitrogen available for crops. In this study, we created a database by collecting results from 121 mineralization monitoring experiments carried out between 2015 and 2021 on different experimental plots across Wallonia, Southern Belgium, and assessed the efficiency of predictive mineralization methods. The most impactful analytical parameters on in situ mineralization (ISM), determined using LIXIM program, appeared to be potentially mineralizable nitrogen (PMN) ($r = 0.79$). PMN, estimated by anaerobic soil incubation, also allowed the effective consideration of the after-effects of grassland termination and manure inputs. A multiple linear regression (MLR) combining PMN, POxC, pH, TOC:N, and TOC:clay significantly improved the prediction of soil nitrogen mineralization available for crops, achieving $r = 0.87$ (vs. $r = 0.58$ for the current method), while reducing dispersion by 41% (RMSE 56.35 $\rightarrow$ 33.13 kg N·ha⁻¹). The use of a more flexible Bootstrap Forest model (BFM) further enhanced performance, reaching $r = 0.92$ and a 50.8% reduction in dispersion compared to the current method (RMSE 56.35 $\rightarrow$ 27.76 kg N·ha⁻¹), i.e., about 16% lower RMSE than the MLR. Those models provided practical and efficient tools to better manage nitrogen resources in temperate agricultural systems.

Keywords: anaerobic incubation; soil mineralization; labile organic nitrogen; labile carbon; oxidizable carbon



Academic Editor: Francisco J. Corpas

Received: 4 August 2025

Revised: 2 September 2025

Accepted: 22 September 2025

Published: 1 October 2025

Citation: Cugnon, T.; De Toffoli, M.; Mahillon, J.; Lambert, R. Improving Nitrogen Fertilization Recommendations in Temperate Agricultural Systems: A Study on Walloon Soils Using Anaerobic Incubation and POxC. *Nitrogen* **2025**, *6*, 91. <https://doi.org/10.3390/nitrogen6040091>

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1. Introduction

Agriculture has undergone substantial transformations in recent decades, driven by increasing global food demand, rising costs of nitrogen fertilizers, and implementation of new environmental guidelines. These regulations aim to reduce the ecological footprint of agricultural practices while simultaneously improving nitrogen use efficiency. In this evolving context, farmers are expected to adopt innovative strategies that not only reduce fertilizer application rates but also maintain soil quality, preserve biodiversity, and ensure sustained productivity growth.

Designing reliable fertilization recommendations requires an accurate estimation of soil nitrogen mineralization—the process by which nitrogen is released from soil organic

matter. A major challenge lies in moving away from generalized estimates and toward site-specific, data-driven decisions that reflect the intricate interactions among soil properties, microbial activity, and nutrient dynamics. These interactions are influenced by a wide range of environmental, biological, and human factors, making it tough to build reliable prediction models [1–4]. Numerous approaches have been developed to estimate nitrogen mineralization, ranging from laboratory incubations [5–7], to modeling frameworks such as STICS or APSIM, as well as empirical estimators based on total organic carbon or nitrogen content [8–10], or even remote sensing and digital agriculture frameworks [11]. Decades of methodological development did not solve the problem of the variability and poor predictive performance of nitrogen mineralization estimators under field conditions. Process-based models calibrated on-site or zone-scale can help explain nitrogen dynamics, but still under- or overestimate responses depending on soil texture and other local factors. These models have limited operational precision as they lack intensive calibration and in-season data assimilation [12]. Field measurements of net mineralization across croplands also reveal large spatial and temporal variability, highlighting the challenge of transposing laboratory and model predictions to practice [13,14]. Spectroscopic approaches (vis–NIR/MIR) are promising for rapid screening, but model accuracy varies considerably across fields and texture groups due to a lack of context-specific calibrations [15]. Finally, multi-model comparisons show substantial uncertainty in simulating net mineralization and related N fluxes at farm-rotation scale, even with well-established platforms (APSIM, STICS, DAISY, etc.), highlighting the influence of weather, soil, and management interactions on the predictions' accuracy [16].

In Wallonia, southern Belgium, a network of laboratories called REQUASUD bases its fertilization recommendations on a model derived from the nitrogen mass balance approach [17]. This framework incorporates multiple parameters including soil type, crop nitrogen uptake, soil mineral nitrogen content at sowing, humus mineralization, direct and residual effects of organic fertilizers, contributions from cover crops, residues from previous crops, and springtime soil analyses to estimate the initial mineral nitrogen pool [18]. While some of these parameters are well-defined and measurable, others—particularly the mineralization of humus—remain difficult to precisely quantify, due to their dependence on complex and dynamic biological processes.

Soil incubation techniques that mimic natural soil conditions under controlled laboratory environments turned out to be valuable tools to better understand and predict nitrogen availability [19–21]. By combining the results of incubation experiments with detailed physicochemical soil analyses and measurements of organic matter fractions, the current study proposes a refined estimation model. This model aims to simplify the prediction of soil nitrogen mineralization by integrating key indicators that capture the biological processes underlying nitrogen cycling.

Beyond the immediate goal of improving nitrogen fertilization recommendations, this study contributes to the broader objective of reconciling agricultural productivity with environmental stewardship. By introducing a set of simple, robust biological indicators, our approach offers laboratories a practical framework for nitrogen management in soils. This work lays the foundations for a new methodology with deep implications for sustainable agriculture in temperate regions.

2. Material and Methods

2.1. Study Site and Experimental Design

A comprehensive dataset was compiled from nitrogen mineralization monitoring experiments conducted between 2015 and 2021 across 10 experimental sites in Wallonia, southern Belgium (Figure 1). These plots span a variety of soil types and climatic conditions. A summary of the agro-pedological characteristics and local weather data for each site is

presented in Table 1. Daily meteorological data were obtained from the Agromet network of agro-meteorological stations (CRA-W/Agromet.be).

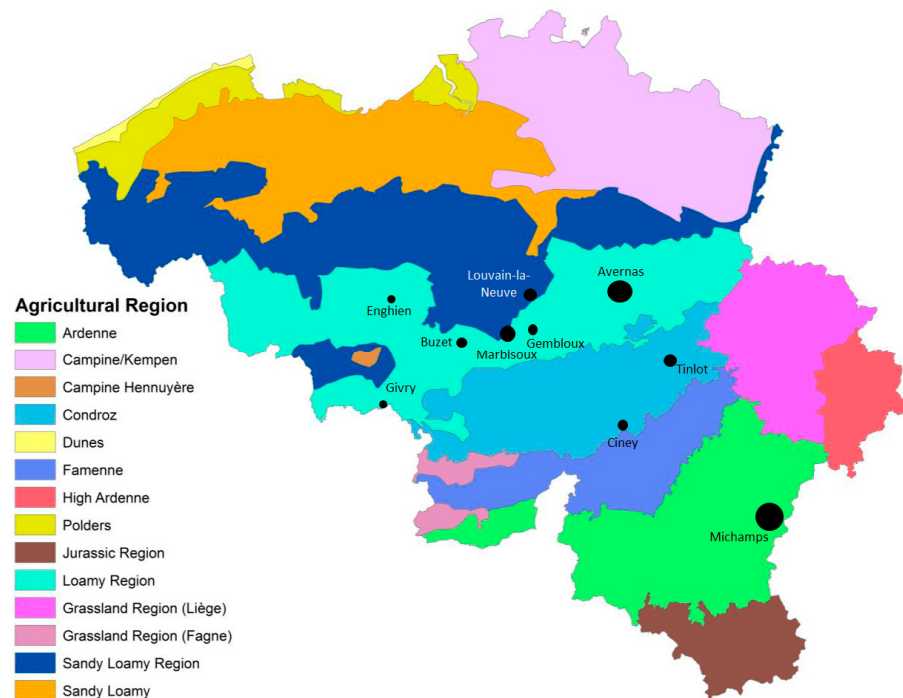


Figure 1. Geographical location of the experimental sites in the different agricultural regions of Belgium. The size of the points is proportional to the number of experimental plots monitored on each site.

Table 1. Agricultural regions, number of experimental plots (xp plots) and weather conditions (mean annual temperature and precipitations) across the 10 locations of the experimental plots. Standard deviation for temperature and precipitation on a location is only indicated if monitoring covered several years. Note that summer 2021 was especially rainy, resulting in a higher standard deviation on the precipitations measurement for two locations, Marbisoux and Louvain.

Location	Agricultural Region	Xp Plots	T °C	Rain (mm/y)	Years
Avernas	Loamy Region	30	11.0 ± 0.5	632.6 ± 25.2	2016–2018
Buzet	Loamy Region	8	11.7	668.4	2018
Ciney	Condroz	5	10.0	709.0	2017
Enghien	Loamy Region	2	11.0	660.8	2017
Gembloux	Loamy Region	4	10.8	603.7	2017
Givry	Loamy Region	4	11.7	641.0	2020
Louvain	Sandy Loamy Region	9	10.7 ± 0.4	713.8 ± 153.2	2017; 2021
Marbisoux	Loamy Region	15	10.4 ± 0.6	751.9 ± 142.5	2019; 2021
Michamps	Ardenne	34	9.0 ± 0.4	750.9 ± 41.0	2015–2018
Tinlot	Condroz	10	11.3	615.0	2017

To assess in situ nitrogen mineralization, each of the 121 test plots were kept free of vegetation throughout the monitoring period. The estimation was performed using the LIXIM model, developed by INRAE (the French National Research Institute for Agriculture, Food and Environment). LIXIM is a layer-based, daily time-step functional model that integrates frequent measurements of soil mineral nitrogen and water content, standard meteorological variables, and soil properties [22]. Nitrate transport is simulated using a mixing-cell approach. The model accounts for the effects of temperature and moisture on mineralization rates by computing a “normalized time,” defined as the number of days under standard temperature and moisture conditions in the mineralizing soil layer.

The robustness of LIXIM has been validated in arable cropping systems in France and in grasslands in the Ardennes region, even under limited monitoring datasets [10,23].

Anaerobic incubations were conducted in early spring at each plot. The resulting potentially mineralizable nitrogen (PMN) values were compared to nitrogen mineralization estimates obtained using the current REQUASUD method, which relies on total organic carbon (TOC) and total nitrogen (Nt) measurements. For a subset of 72 samples (plots monitored since 2017), additional measurements included the labile fraction of organic nitrogen (NI) and permanganate oxidizable carbon (POxC). These parameters were compared to determine their effectiveness as predictors of nitrogen mineralization.

2.2. Soil Analyses

At each plot, soil samples were collected before tillage. Gravimetric water content was determined in accordance with ISO 11465 [24]. Mineral nitrogen (NO_3^- -N and NH_4^+ -N) was analyzed within three soil layers (0–30 cm, 30–60 cm, and 60–90 cm) in accordance with ISO 14256-2 [25]. Total organic carbon (TOC) was measured using the Walkley–Black method [26], while total nitrogen (Nt) was assessed by the Kjeldahl method [27]. Soil pH (in 1N KCl) was determined using ISO 10390 [28]. Clay content data were derived from the Walloon soils reference dataset [29].

PMN was estimated using the *in vitro* anaerobic incubation method described by Keeney and Bremner [5], which consists of, in brief, incubating 5 g of soil in a closed test tube with 12.5 mL of distilled water under waterlogged conditions at 40 °C for 7 days. The concentration of NH_4^+ -N was determined before and after incubation via Kjeldahl distillation using 2 mol·L^{−1} KCl, followed by titration with 0.01 N HCl. Eventually, the mineralization potential of each sample was calculated as being the net increase in NH_4^+ -N. This *in vitro* anaerobic incubation appeared to be a promising method, as it yielded reliable results in a quite limited time and was easy to carry out in routine laboratories, compared to aerobic incubations methods, which generally require 28 to 90 days [10].

Labile nitrogen (NI) was included to represent a fraction of organic nitrogen that is hydrolyzable in an acidic environment, easy to quantify and, according to the work of Matrinez and Galantini [30], allows for the monitoring of moderately labile nitrogen reserves and mineralizable nitrogen. NI was quantified using a partial digestion method with 0.5 mol·L^{−1} sulfuric acid: 1.000 g of soil, which was mixed with 12 mL of H_2SO_4 solution and heated for 2 h at 95 °C. The extract was then subjected to Kjeldahl distillation and titrated with 0.005 N HCl.

Permanganate oxidizable carbon (POxC) was quantified using the method introduced by Weil [31] et al. (2003): A 2.5 g soil sample was mixed with 18 mL deionized water and 2 mL of 0.2 M KMnO_4 (prepared in 0.1 mol·L^{−1} CaCl_2). The mixture was shaken at 240 oscillations per minute for 2 min and allowed to settle for 10 min. A 0.5 mL aliquot of the supernatant was then diluted in 49.5 mL of deionized water. Absorbance of a 3 mL sub-sample was measured at 550 nm using a Shimadzu 500 spectrophotometer. Strict adherence to shaking and settling times is critical to ensure repeatability. POxC indicates changes in soil organic matter due to management activities; it is a commonly used indicator for assessing the quality of agricultural soils. It was initially considered as an indicator of the biologically active carbon pool in soils [31–34], but recent literature refutes this as it is poorly understood which compounds KMnO_4 is actually oxidizing [35]. Despite no longer being considered as a direct indicator of bioavailable C, POxC still shows a consistent and strong correlation with many common soil physicochemical and biological properties, including labile C, and it therefore continues to be endorsed as a key indicator of soil health [34].

In parallel, the REQUASUD method for estimating soil nitrogen mineralization was also applied. This balance-based approach integrates measured or estimated values of TOC, Nt, clay content, and other parameters. When Nt is not analyzed in routine, it should be estimated from the TOC value using regional TOC to Nt ratios. This generated two variants of mineralization estimates: the first one based on Nt (Soil N-N) and the second one based on TOC (Soil N-C). Additional factors related to agricultural practices—such as crop residues, cover crops, grassland turnover, and fertilizer inputs—were incorporated. Residues and cover crops contributed between -30 and $+45$ kg N·ha⁻¹, grassland turnover contributed $+10$ to $+140$ kg N·ha⁻¹, and long-term manure applications were factored in based on frequency and timing [18].

2.3. In Situ Nitrogen Mineralization

In situ nitrogen mineralization was estimated from repeated measurements of soil NO₃⁻-N and NH₄⁺-N carried out throughout the growing season (between three and eight sampling dates, depending on site and year). These data were used as an input into the LIXIM model along with:

Daily meteorological data (temperature, rainfall, evapotranspiration), soil physical properties including bulk density and water retention characteristics, mineralization depth, set to 25 cm in Ardenne soils and 30 cm elsewhere, standardized temperature (15 °C), and moisture (field capacity) values for normalization.

2.4. Statistical Analyses and Modeling Approach

All statistical analyses were performed using 2025 JMP® 17.2.0. JMP Statistical Discovery LLC, Cary, NC, USA. Descriptive statistics (mean, standard deviation, and range) were first computed for all soil properties and nitrogen mineralization indicators across the 121 experimental plots. To explore pairwise relationships among variables, Pearson correlation coefficients were calculated between in situ nitrogen mineralization (ISM), anaerobic nitrogen mineralization potential (PMN), and the other soil indicators. The Pearson method was chosen based on the continuous nature of the variables, their approximate normality and the assumption of linear relationships. These assumptions were validated through visual diagnostics and normality checks. This analysis allowed us to identify potentially relevant predictors for nitrogen mineralization, in line with established agronomic research [7,10,13,36].

Principal component analysis (PCA) was used to explore the multivariate structure of the dataset and identify the main axes of variability across soil properties, biological indicators, and agronomic practices. The PCA included indicators of nitrogen mineralization (ISM and PMN), soil characteristics (pH, TOC, Nt, C/N, NI, POxC, clay), regional averages, and agricultural practices (use of manure, cover crops, grassland turnover). Only components with eigenvalues >1 were retained for subsequent regression modeling.

In addition to stepwise multiple linear regression (MLR) approach, we implemented a bootstrap forest (random forest) to compare and determine the best predictive model to estimate ISM. Explanatory variables were introduced sequentially in the MLR based on their contribution to model accuracy. Model performance was evaluated using the coefficient of determination (r^2), bias (mean difference), root mean squared error (RMSE), and Akaike Information Criterion (AIC) to limit over-parameterization. Out-of-bag (OOB) validation was used to evaluate Bootstrap Forest model (BFM) robustness and to avoid overfitting. This non-parametric approach complemented traditional methods by providing additional insight into variable selection and model performance under more flexible conditions. The BFM was constructed using the Save Tolerant Prediction Formula. The prediction formula tolerates missing values by randomly allocating response values for missing predictors to a split.

All statistical procedures were conducted with a significance threshold set at $p < 0.05$.

3. Results

3.1. Soil Diversity

The diversity of soil types and climatic conditions across the study sites led to a wide range of soil characteristics (Table 2). Total nitrogen (Nt) values ranged from $1.30 \pm 0.14\%$ to $2.88 \pm 0.57\%$, while total organic carbon (TOC) varied from $0.98 \pm 0.11\%$ to $2.51 \pm 0.66\%$. Clay content ranged between 11.4% and 16.6%, and labile nitrogen (NL) concentrations varied from $194 \pm 16 \text{ mg}\cdot\text{kg}^{-1}$ to $496 \pm 63 \text{ mg}\cdot\text{kg}^{-1}$. Similarly, POxC ranged from $260 \pm 37 \text{ mg}\cdot\text{kg}^{-1}$ to $632 \pm 118 \text{ mg}\cdot\text{kg}^{-1}$. Soil pH also exhibited a wide range, from acidic (pH 5.49 ± 0.42) to slightly alkaline (pH 7.14 ± 0.63). Land use influenced this variability, with 24% of the experimental plots under grassland and the remainder under arable crops.

Table 2. Description of key soil characteristics (mean $\pm$ standard deviation) across the 10 study locations: total nitrogen (Nt), total organic carbon (TOC), clay content, labile nitrogen (NL), permanganate-oxidizable carbon (POxC), and pH.

Location	Xp Plots	Nt (%)	TOC (%)	Clay (G·KG ^{−1})	NL (MG·KG ^{−1})	POXC (MG·KG ^{−1})	PH
Avernas	30	1.37 ± 0.15 cd *	1.24 ± 0.15 c	114.0 ± 0.0 e	218 ± 24 de	380 ± 33 bc	7.11 ± 0.33 a
Buzet	8	1.30 ± 0.05 cd	0.98 ± 0.11 c	138.0 ± 0.0 bc	209 ± 31 e	293 ± 31 c	6.35 ± 0.05 b
Ciney	5	1.63 ± 0.17 bcd	1.60 ± 0.10 bc	144.0 ± 0.0 b	430 ± 43 ab	319 ± 18 bc	5.81 ± 0.26 bc
Enghien	2	1.35 ± 0.07 bcd	1.55 ± 0.07 bc	114.0 ± 0.0 cde	209 ± 6 de	241 ± 0 bc	6.35 ± 0.07 b
Gembloux	4	1.91 ± 0.37 bc	2.03 ± 0.44 ab	114.0 ± 0.0 de	365 ± 31 bc	632 ± 118 a	7.14 ± 0.63 a
Givry	4	2.00 ± 0.08 b	1.61 ± 0.16 bc	138.0 ± 0.0 bcd	248 ± 10 de	518 ± 30 ab	6.11 ± 0.24 b
Louvain	9	1.21 ± 0.10 d	1.01 ± 0.10 c	146.0 ± 38.1 b	221 ± 24 de	260 ± 37 c	7.13 ± 0.52 a
Marbisoux	15	1.34 ± 0.14 cd	1.25 ± 0.17 c	151.1 ± 9.8 b	194 ± 16 e	304 ± 10 bc	5.74 ± 0.14 bc
Michamps	34	2.88 ± 0.57 a	2.51 ± 0.66 a	165.8 ± 4.3 a	496 ± 63 a	435 ± 148 bc	5.49 ± 0.42 c
Tinlot	10	1.56 ± 0.38 bcd	1.58 ± 0.37 bc	138.0 ± 0.0 bc	303 ± 45 cd	368 ± 115 bc	6.13 ± 0.38 b

* Different letters indicate significant differences between sites for variables as determined by the HSD Tukey test ($p < 0.05$).

This variability of parameters resulted in a wide dispersion of mineralization values. In situ soil mineralization (ISM), estimated using the LIXIM model, ranged from $102 \pm 28 \text{ kg N}\cdot\text{ha}^{-1}$ to $341 \pm 82 \text{ kg N}\cdot\text{ha}^{-1}$ (Table 3). PMN values ranged from $116 \pm 21 \text{ kg N}\cdot\text{ha}^{-1}$ to $243 \pm 11 \text{ kg N}\cdot\text{ha}^{-1}$. Estimates of soil mineralization using the REQUASUD method (based on either TOC or Nt content) ranged from $78 \pm 5 \text{ kg N}\cdot\text{ha}^{-1}$ to $236 \pm 32 \text{ kg N}\cdot\text{ha}^{-1}$. Across the various locations, in situ mineralization values (ISM) differed, sometimes significantly, from the estimates obtained using all three indirect methods (PMN, Soil N-C, and Soil N-N). PMN showed the closest alignment with ISM, with statistically significant differences observed in only two locations (Givry and Enghien). In contrast, estimates based on the REQUASUD methodology (Soil N-C and Soil N-N) differed significantly from ISM in four locations each. While PMN was not significantly

different from Soil N-C or Soil N-N across locations, except in Ciney, its estimates more consistently approximated the values of ISM.

Table 3. Estimates of in situ nitrogen mineralization expressed in $\text{kg N}\cdot\text{ha}^{-1}$: simulated values from the LIXIM model (ISM), anaerobic incubation (PMN), and values estimated according to the REQUASUD methodology using either total nitrogen (Soil N-N) or total organic carbon (Soil N-C).

Location	ISM	PMN	Soil N-C	Soil N-N
Avernas	151 $\pm$ 27 a *	142 $\pm$ 22 a	163 $\pm$ 23 a	174 $\pm$ 27 a
Buzet	139 $\pm$ 62 ab	116 $\pm$ 21 b	126 $\pm$ 11 ab	163 $\pm$ 14 a
Ciney	151 $\pm$ 9 a	134 $\pm$ 16 a	78 $\pm$ 5 b	80 $\pm$ 8 b
Enghien	187 $\pm$ 1 a	151 $\pm$ 12 b	141 $\pm$ 6 b	134 $\pm$ 6 b
Gembloux	341 $\pm$ 82 a	243 $\pm$ 11 ab	233 $\pm$ 36 b	236 $\pm$ 32 b
Givry	102 $\pm$ 28 c	154 $\pm$ 12 ab	140 $\pm$ 14 b	178 $\pm$ 7 a
Louvain	138 $\pm$ 21 ab	149 $\pm$ 29 a	110 $\pm$ 16 ab	125 $\pm$ 17 b
Marbisoux	211 $\pm$ 61 a	183 $\pm$ 65 a	175 $\pm$ 35 a	187 $\pm$ 35 a
Michamps	159 $\pm$ 81 a	158 $\pm$ 79 a	149 $\pm$ 41 a	173 $\pm$ 41 a
Tinlot	131 $\pm$ 44 a	151 $\pm$ 31 a	127 $\pm$ 27 a	130 $\pm$ 28 a

* Different letters indicate significant differences between ISM, PMN, Soil N-C, and Soil N-N for each site as determined by the HSD Tukey test ($p < 0.05$). The sites were not compared to each other.

Three agricultural practices were implemented in different plots of the monitoring: grassland tillage, cover cropping, and manure spreading. The effects of these practices have been taken into account in the estimation of SOIL N-C and Soil N-N according to the REQUASUD method for estimating soil nitrogen mineralization as summarized in Table 4.

Table 4. Frequency (n) and magnitude of management practices in the experimental dataset ($n = 121$). Reported values are mean effect, SD, MIN, and MAX of the effect according to REQUASUD method. Plot-level effects were bounded by MIN-MAX values determined by (i) grassland effect (GE): age of the plowed grassland and termination date; (ii) cover cropping effect (CC): species mixture, termination timing, and residue incorporation; (iii) manure effect (ME): manure type, application rate, and nitrogen concentration.

Practice	n	Mean Effect ($\text{kg N}\cdot\text{ha}^{-1}$)	SD ($\text{kg N}\cdot\text{ha}^{-1}$)	Min ($\text{kg N}\cdot\text{ha}^{-1}$)	Max ($\text{kg N}\cdot\text{ha}^{-1}$)
Grassland effect (GE)	29	42.7	32.2	10	140
Cover cropping (CC)	53	27.0	6.1	15	45
Manure effect (ME)	30	65.6	27.9	27.4	131.6

3.2. Factors Influencing Soil Nitrogen Mineralization

Table 5 presents the correlation matrix between the different measured variables. Among all parameters, PMN exhibited the strongest correlation with ISM ($r = 0.79$). The use of normalized mineralization rates, such as V_p (rate per normalized day), did not improve this relationship; the correlation between PMN and V_p reached only $r = 0.55$. Although the PMN/ND ratio (PMN per normalized day) showed a slightly higher correlation with V_p ($r = 0.66$), none of the normalized variables performed better than the direct correlation between ISM and PMN.

Correlations of Soil N-N and Soil N-C with ISM, aLIX (LIXIM slope), and V_p were relatively similar, yet systematically lower than the ISM–PMN correlation. Notably, TOC showed no correlation with ISM ($r = 0.03$), nor with Soil N-C. Similarly, Nt was not correlated with Soil N-N ($r = 0.13$). Furthermore, TOC and Nt were not correlated with PMN ($r = 0.06$ and 0.00 , respectively).

The two additional biological indicators studied—NI (labile nitrogen) and POxC (permanganate oxidizable carbon)—exhibited clearly divergent behaviors. NI was closely correlated with TOC and Nt, as well as with clay content; however, it showed no significant

correlation with ISM. In contrast, POxC did not correlate with TOC or Nt, nor with clay content or the TOC/N ratio, but was significantly correlated with ISM ($r = 0.53$).

Table 5. Pearson correlation matrix (r) for the relationships among the following variables: in situ mineralization simulated by LIXIM (ISM), LIXIM slope parameter (aLIX), normalized mineralization rate (Vp), anaerobic nitrogen mineralization potential (PMN), ratio of PMN to normalized days from LIXIM (PMN/ND), total organic carbon (TOC), total nitrogen (Nt), total organic carbon to total nitrogen ratio (TOC/N), clay content, carbon-to-clay ratio (TOC/Clay), estimated mineralization using TOC (Soil N-C) and Nt (Soil N-N) according REQUASUD methodology, labile nitrogen (NI) ($n = 74$), and permanganate-oxidizable carbon (POxC) ($n = 74$).

	ISM	aLIX	Vp	PMN	PMN/ND	TOC (%)	NT (‰)	TOC/N	Clay	TOC/Clay	Soil N-C	Soil N-N	NI
aLIX	0.73 *												
Vp	0.77 *	0.94 *											
PMN	0.79 *	0.49 *	0.55 *										
PMN/ND	0.36 *	0.59 *	0.66 *	0.61 *									
TOC (%)	0.03	0.11	0.27 *	0.06	0.29 *								
NT (‰)	−0.06	0.06	0.21 *	0.00	0.27 *	0.95 *							
TOC/N	0.26 *	0.16	0.19 *	0.20 *	0.06	0.19 *	−0.13						
Clay	−0.01	−0.30 *	−0.17	0.12	−0.02	0.53 *	0.59 *	−0.12					
TOC/Clay	0.08	0.33 *	0.44 *	0.06	0.38 *	0.92 *	0.82 *	0.32 *	0.18 *				
Soil N-C	0.58 *	0.47 *	0.45 *	0.51 *	0.26 *	0.14	0.04	0.30 *	−0.11	0.26 *			
Soil N-N	0.49 *	0.45 *	0.41 *	0.43 *	0.27 *	0.08	0.13	−0.19 *	−0.01	0.13	0.86 *		
NI	0.12	−0.01	0.10	0.09	0.00	0.82 *	0.82 *	0.26 *	0.80 *	0.70 *	0.07	−0.05	
POxC	0.53 *	0.46 *	0.41 *	0.46 *	0.25 *	0.26 *	0.24 *	0.16	0.18	0.35 *	0.57 *	0.50 *	0.32 *

* Significant at $p < 0.05$.

Regarding agricultural practices as presented on Table 4, a MANOVA revealed that the grassland post-tillage effect had a statistically significant impact on nitrogen mineralization ($p < 0.01$), while no significant effect was found for cover crops or manure when considered individually—likely due to variability in soil properties across plots. Nevertheless, both POxC and NI were significantly affected by grassland tillage ($p < 0.001$) and by the combined application of manure and cover crops ($p = 0.04$). The effects of these two factors taken independently, however, were not statistically significant.

To further investigate the influence of management practices, correlations between ISM, Vp, PMN, and POxC were analyzed within subsets of plots grouped by treatment (Table 6). In plots that received manure, the correlations between PMN and Vp, and between POxC and ISM, were no longer significant. In contrast, when no manure was applied, correlations between ISM and PMN ($r = 0.78$), and between POxC and ISM ($r = 0.68$), remained strong. Similar patterns were observed for cover cropping: in the absence of cover crops, all correlations—particularly ISM–PMN ($r = 0.85$) and ISM–POxC ($r = 0.63$)—were stronger than when cover crops were used. The exception was the PMN–POxC correlation, which was higher in the presence of cover crops ($r = 0.72$).

Table 6. Pearson correlation coefficients (r) between ISM, Vp, PMN, and POxC, according to management practices: grassland effect (GE), cover crop effect (CC), and manure effect (ME). * Significant at $p < 0.05$.

	Manure Effect (ME)		Cover Crop Effect (CC)		Grassland Effect (GE)	
	Yes ($n = 30$)	NO ($n = 91$)	Yes ($n = 53$)	No ($n = 68$)	Yes ($n = 29$)	No ($n = 92$)
ISM-Vp	0.44 *	0.81 *	0.70 *	0.84 *	0.87 *	0.60 *
ISM-PMN	0.79 *	0.78 *	0.55 *	0.85 *	0.87 *	0.62 *
ISM-POxC	−0.19	0.68 *	0.46	0.63 *	0.96 *	0.40 *
PMN-Vp	0.22	0.61 *	0.18	0.67 *	0.55 *	0.32 *
PMN-POxC	0.37	0.65 *	0.75 *	0.56 *	0.82 *	0.38 *
POxC-Vp	−0.06	0.44 *	0.18	0.46 *	0.89 *	0.11

Grassland tillage had a particularly strong impact on the correlation between POxC and Vp ($r = 0.89$). PMN remained well correlated with ISM regardless of the presence or absence of a grassland effect ($r = 0.87$ and $r = 0.62$, respectively).

Principal Component Analysis (PCA) revealed that the first three components accounted for 85% of the total variance. The first component was primarily driven by soil physicochemical properties—positively by TOC, Nt, NI, and clay content, and negatively by regional average temperature and pH (Figure 2).

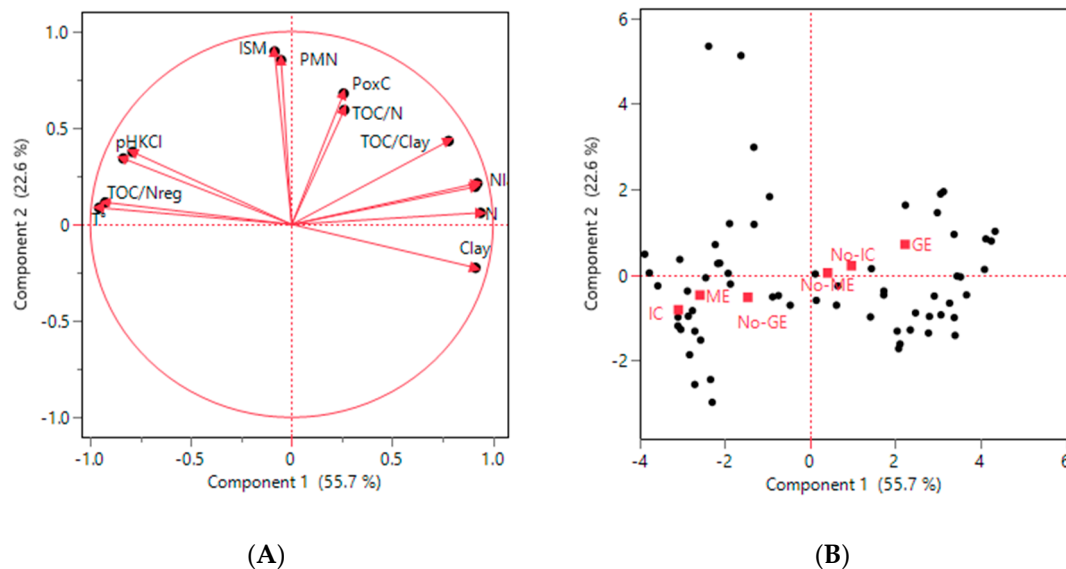


Figure 2. Principal component analysis (PCA) of soil and management indicators. (A) Correlation circle on PC1-PC2; (B) score plot on PC1-PC2 with centroids for management categories (GE/No-GE = grassland effect; ME/No-ME = manure; IC/No-IC = cover crop). Variables include nitrogen-mineralization indicators (ISM, PMN), soil properties (pHKCl, TOC, Nt, TOC/N, NI, POxC, Clay), and regional averages (T° , TOC/Nreg) used in the current fertilization method. Percent variance explained is shown on axes; variables were centered and scaled to unit variance, and management categories were projected as supplementary variables.

The second component was dominated by ISM (contributing 30.5%) and PMN (25.1%), followed by POxC (20.1%) and, to a lesser extent, the observed TOC/N ratio (13.9%). Interestingly, the regional TOC/N ratio, used in the current recommendation method, loaded onto a different component than the TOC/N ratio measured in the field. When management practices were added to the PCA (i.e., manure use, grassland tillage, cover crop presence), they appeared to be mainly correlated to the first component, even if the grassland effect—and to a lesser extent, manure use—slightly contributed to the second component. When the grassland and cover crop effects were integrated as individual data points (rather than supplementary effects), the grassland effect again appeared as a significant contributor (11%) to the second principal component, whereas cover crop presence slightly contributed to the first two components (<6%) (not illustrated).

Both the Bootstrap Forest model (BFM) and the multiple linear regression (MLR) ranked PMN as the primary predictor of ISM (Table 7). In the BFM, PMN showed the largest main effect and a sizable interaction component (Total–Main $\approx 31\%$), whereas POxC contributed as a consistent, interaction-free main effect. PH-KCl provided a minor but significant improvement in both models, while TOC/N and TOC/Clay had limited and non-significant effects once PMN and POxC were included in MLR.

Table 7. Relative importance of predictors from the Bootstrap Forest model (main, total effects and weights where the number of + represent the number of times that an observation occurs in the bootstrap sample) and the multiple linear regression (LogWorth, p).

Term	BFM			MLR	
	Main Effect	Total Effect	Weights	Logworth	p -Value
PMN	0.440	0.641	+++++++	7.180	0.0000
POxC	0.215	0.215	++	5.066	0.0001
TOC/N	0.126	0.126	+	0.175	0.6686
pHKCl	0.120	0.120	+	2.437	0.0037
TOC/Clay	0.098	0.098	+	0.692	0.2032

3.3. Improving the Prediction of Soil Nitrogen Mineralization

As illustrated on Figure 3, the current REQUASUD method based on TOC (Soil N-C model) showed the weakest correlation with ISM ($r = 0.58$) and the highest dispersion (RMSE = 56.35).

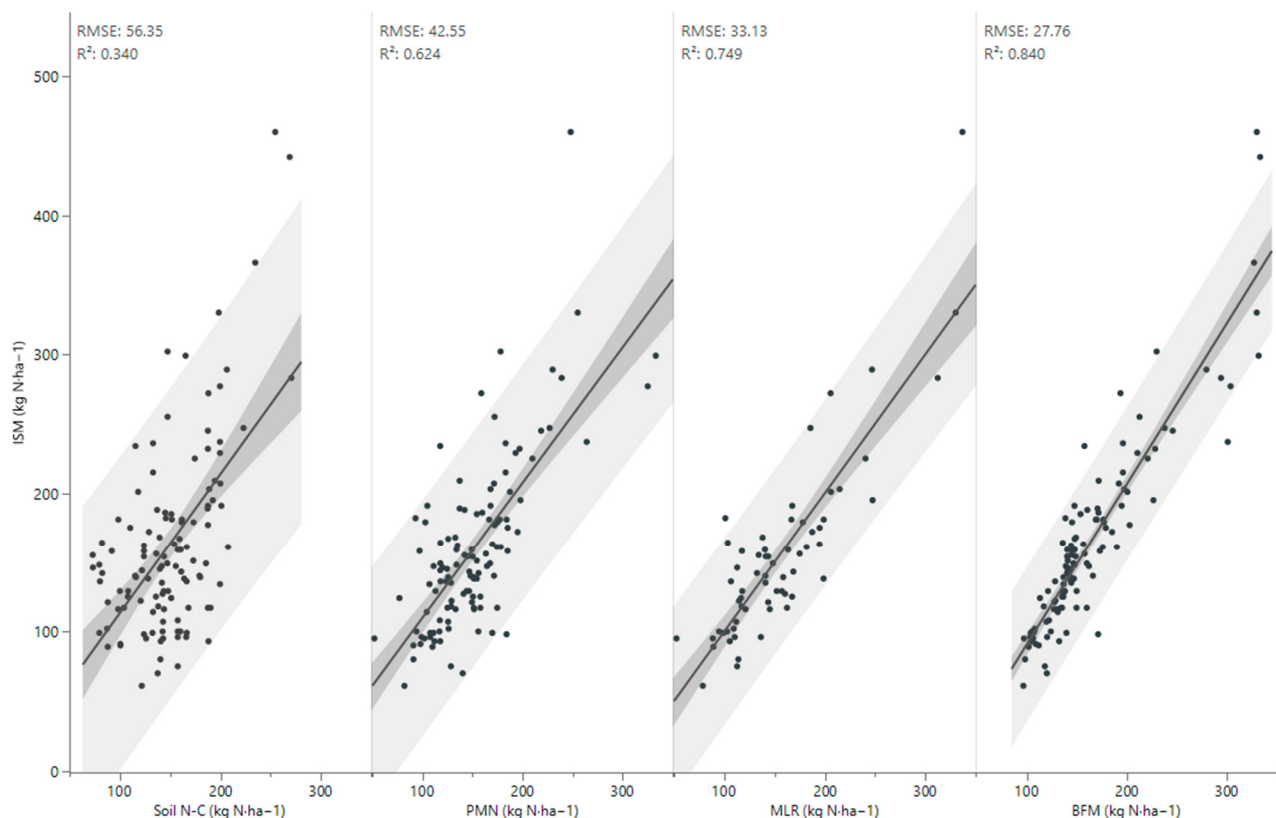


Figure 3. Stepwise improvement in the estimation of in situ mineralization (ISM), based on: (i) the current REQUASUD method (Soil N-C); (ii) the use of PMN alone; (iii) the MLR model integrating PMN, POxC, pH, TOC/N, and TOC/Clay ($n = 72$); and (iv) the BFM generated by machine learning. Dark grey region is the confidence region of the fitted line. Light grey region represent the confidence region for individual predicted values.

As previously emphasized, PMN is highly correlated to ISM and using this parameter instead of the SOIL N-C model would highly increase the correlation ($r = 0.79$) and reduce the dispersion by 24.5% (RMSE = 42.55).

The MLR helped to improve this relationship by combining PMN, POxC, pH KCl, TOC/N, and TOC/Clay. TOC/N and TOC/clay were not significant in MLR, but these data, commonly available in laboratories, slightly improved the performance of the model.

More complex functions or transformations than linear regression were tested for each parameter but did not improve the model. The best resulting MLR equation is as follows:

$$\text{ISM} = -182.311 + 0.814 \times \text{PMN} + 23.447 \times \text{pHKCl} - 1.878 \text{ TOC/N} + 0.177 \text{ POxC} + 17.943 \text{ TOC/Clay},$$

The MLR model achieved higher correlation ($r = 0.87$) and reduced the dispersion around attended values by 41% compared to current Soil N-C model ($\text{RMSE} = 33.13$). This model was run using a subset of 74 plots for which POxC data were available.

The BFM achieved the highest significant correlation ($r = 0.92$) and the biggest reduction in dispersion compared to SOIL N-C, lowering it by 50.8% ($\text{RMSE} = 27.76$), i.e., about 16% lower RMSE than the MLR.

4. Discussion

4.1. Time Normalization

In this study, the LIXIM model was used as a reference tool to assess in situ net nitrogen mineralization. As LIXIM expresses mineralization in terms of normalized days—and given that the monitoring duration varied across experimental plots—the question of standardizing incubation results arises. Such standardization would allow direct comparisons with values obtained from other studies, particularly those using laboratory incubations, and would also support the validation of our predictive model against external datasets.

Some authors have proposed equivalence functions for aerobic incubations, based on temperature and moisture adjustments, to calculate standardized mineralization days. However, our experiments used anaerobic incubation, for which such conversions are less established; although both temperature (40°C) and moisture (saturation) were kept constant during incubation, the underlying microbial processes differ substantially from those in aerobic conditions. Anaerobic conditions at 40°C likely induce extensive microbial cell lysis. As a result, ammonium production mainly arises from the ammonification of low molecular weight organic compounds such as amino acids, amino sugars, nucleotides, or urea. Among these, amino acids are particularly important due to their water solubility and accessibility to microorganisms [37,38]. Ammonification can occur via both extracellular and intracellular pathways, though the role of extracellular enzymes—aside from well-characterized examples such as urease or amino acid oxidases—remains poorly documented [38,39].

This biological context suggests that ammonification during anaerobic incubation is unlikely to follow a linear pattern. It more plausibly follows a logarithmic trend, with a rapid phase of mineralization occurring in the early stage of incubation. This would imply that PMN values should not be normalized by time but rather used in their raw form. This hypothesis is supported by our data: The correlation between PMN and raw ISM values was stronger than that between PMN and V_p , the normalized mineralization rate. Furthermore, most previous studies using LIXIM were conducted on conventional agricultural soils, whereas our work includes both grassland soils and a broader range of pedological conditions—potentially influencing the linearity assumptions embedded in the LIXIM model. In situ mineralization varied from 61 to $460 \text{ kg N}\cdot\text{ha}^{-1}$ (average = 163), V_p varied from 0.57 to $4.47 \text{ kg N}\cdot\text{ha}^{-1} \cdot \text{d}^{-1}$, and normalized time calculated by LIXIM ranged from 38 to 199 with an average of 129 N Days. Other studies based on LIXIM reported PMN ranging from 3 to $307 \text{ kg N}\cdot\text{ha}^{-1}$, V_p from 0.17 to $1.67 \text{ kg N}\cdot\text{ha}^{-1} \cdot \text{d}^{-1}$, and normalized time up to 550 N Days [23,40]. These studies also emphasized that the exponential model gave a slightly better fit than the linear model. However, the linear model is usually preferred for its simplicity and for the fact that the two parameters of the exponential model (N_0 and k) are strongly correlated and cannot be determined accurately individually, but only as their product. Valé [23] concluded that the linear model was

the simplest and most robust one for describing the N mineralization kinetics calculated in situ by LIXIM, but that its use could lead to a bias for plots with curvilinear kinetics. Our dataset revealed several mineralization curves that, when plotted in normalized time, displayed non-linear behavior (Figure 4). It is also worth noting that our field monitoring campaigns typically concluded in October, corresponding to the end of the crop growth cycle. Taken together, these considerations validate the comparison between PMN and total ISM, without requiring transformation into normalized mineralization rates.

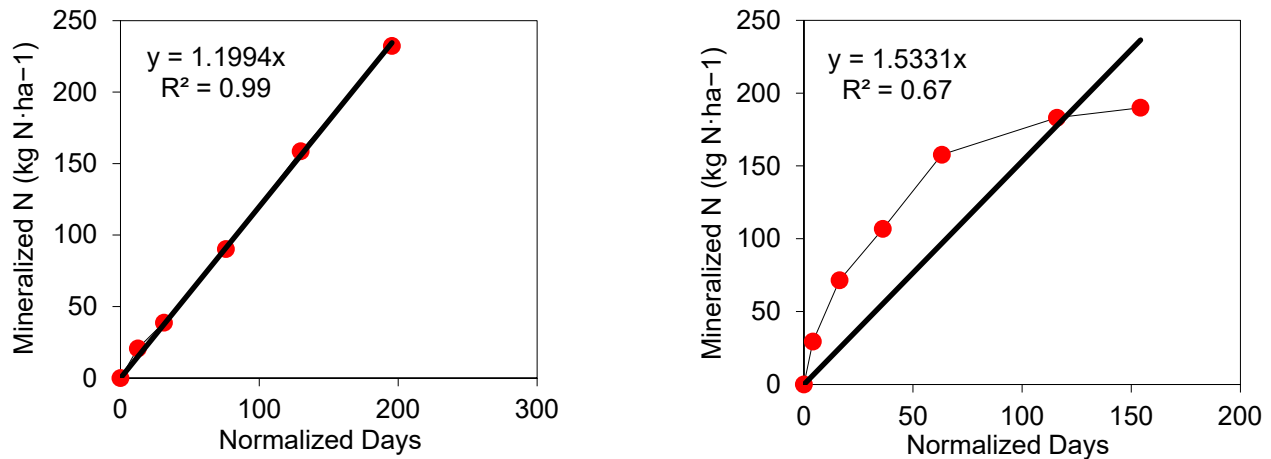


Figure 4. Nitrogen mineralization kinetics expressed in normalized days for two contrasting situations in 2021: a linear response in Marbisoux 4th plot (**left**) and a curvilinear response in Obourcy 1st plot (**right**) compared to the linear models (dark lines).

4.2. Major Influencing Factors

Across the dataset, PMN showed the strongest correlation with ISM, confirming that short anaerobic incubation captures a large part of the field-measured mineralization. This holds across contrasting soils and years and remains particularly clear in plots affected by a grassland effect (GE), where the PMN-ISM relationship was exceptionally strong (see correlations in Tables 5 and 6 and the comparison with LIXIM outputs in Figure 3). As previously demonstrated by Cugnon et al. [21], the LIXIM model performs adequately in simulating nitrogen mineralization following grassland plowing. In the present study, PMN also proved effective in accounting for the enhanced mineralization resulting from grassland turnover. Although a distinct grassland effect was detected by the principal component analysis (PCA), introducing this factor into the MLR or BFM did not improve prediction performance when PMN was already included. This confirms that anaerobic incubation adequately captures the post-grassland mineralization potential and supports using PMN as the primary biological indicator of soil N supply in fertilization recommendations. However, when manure was applied, the correlation between PMN and V_p (normalized mineralization rate) was no longer significant (Table 6). This observation makes sense, given that liquid manure and digestate quickly release nitrogen in forms that are not captured by the anaerobic incubation's slower dynamics. Conversely, solid manure—especially when mixed with straw—may cause temporary nitrogen immobilization as microbial communities break down high-C residues.

The absence of a statistically significant relationship between TOC and Soil N-C, and between Nt and Soil N-N, indicates that these variables—although central to the current REQUASUD estimation method—do not constitute the most relevant drivers of nitrogen mineralization under field conditions. This observation is reinforced by their low correlations with in situ mineralization (ISM) and with PMN, both in absolute and normalized terms. These results clearly show that TOC and Nt alone are insufficient to

reliably predict soil nitrogen mineralization. These findings contrast with those of several previous studies that reported significant correlations between soil total nitrogen (Nt) and nitrogen mineralization potential (PMN) ranging from $r = 0.36$ to $r = 0.65$ [10,26,36]. Our use of LIXIM as a modeling framework, combined with high-frequency mineral nitrogen measurements under field conditions, may explain the divergence from traditional laboratory-based results.

POxC, despite a modest correlation ($r = 0.53$), improved ISM prediction accuracy when added to PMN (and pH), behaving as a management-responsive carbon proxy rather than a substitute for PMN. In the correlation screening (Tables 5 and 6), POxC was positively associated with several mineralization indicators but effect sizes highly varied depending on management practices. Under manure inputs, the POxC–ISM correlation was no longer significant, probably because the amendments used (mainly liquid cattle manure and biodigestate) provided little carbon but a substantial amount of readily mineralizable N, thus modifying N dynamics without affecting the labile-C pool captured by POxC. This interpretation aligns with Culman et al. [32] and Hurisso et al. [41], who emphasized that POxC reflects a transformed and relatively stabilized fraction of organic matter rather than very fresh and highly degradable inputs. In line with our results, POxC therefore acts as a useful supplement, particularly when recent C inputs are moderate and management influences the active C pool, while PMN remains the primary indicator across management scenarios. In the modeling step (Table 7), POxC showed a stable main effect in the Bootstrap Forest model and a significant but smaller coefficient than PMN in the MLR, indicating additive information—most likely easily oxidizable C that feeds the microbial nitrogen cycle—without supplanting the central role of PMN. Management practices modulated this signal. Cover crops did not show any significant correlation with ISM, or in our PCA, or regression analysis, or machine learning, which is somewhat unexpected given their commonly acknowledged role in enhancing soil fertility and nitrogen supply [42–44]. Several hypotheses may explain this result. First, the LIXIM model is not designed to simulate the mineralization of crop residues, including those from cover crops [22]. Despite this limitation, our LIXIM simulations did not exhibit clear differences between plots with or without cover crops. Secondly, and more importantly, the variability in cover crop types, biomass production, incorporation timing, and management practices likely obscures any consistent effect on nitrogen mineralization. As reported in the literature, nitrogen release from cover crop residues depends on several interacting variables:

The nitrogen status of the soil: Under low-N conditions, cover crops may increase nitrogen immobilization by soil microbes [44,45].

The chemical composition of the residues, particularly their C/N ratio: Residues with high C/N ratios decompose more slowly and can delay nitrogen availability [46].

The spatial positioning of residues: When residues are incorporated into the soil rather than left on the surface, they are more accessible to microbial decomposers, influencing mineralization dynamics [47,48].

In our study, cover crops included a wide range of species and functional groups—such as mustard, oats, rye, ryegrass, vetch, and clover—used in different combinations and producing between 0.7 and 7 t·ha^{−1} of biomass. Some were incorporated into the soil before winter, others after, and some not at all. This agronomic diversity likely masked any clear trend in nitrogen dynamics that could have been attributed solely to the presence of cover crops. In Wallonia, southern Belgium, it has been shown that cover crops are able to assimilate up to 130 kg N·ha^{−1} depending on the type of cover crop used (associations performing best) and the vegetation period (up to 70 days) [42]. However, the restitution kinetics of this absorbed nitrogen after canopy destruction remains unclear. These observations are in line with recent studies emphasizing the complexity of nitrogen restitution by cover crops. For

instance, Constantin et al. [49], using the MERCI database in France, reported that nitrogen restitution to the following crop could range from -20 to $+80 \text{ kg N}\cdot\text{ha}^{-1}$, depending on the cover crop species, biomass, and decomposition conditions. This variability underscores the need for more detailed studies to understand and model the diverse kinetics of cover crop residue mineralization. Moreover, it becomes increasingly clear that assuming a rapid and complete nitrogen release from cover crop residues at the beginning of the growing season—as is currently practiced in the Walloon fertilization advisory system—does not reflect field reality. Such assumptions may lead to over- or underestimations of the amount of available nitrogen, depending on the type and fate of the residues.

Labile nitrogen (NI), measured using partial acid digestion of the soil with $0.5 \text{ mol}\cdot\text{L}^{-1} \text{ H}_2\text{SO}_4$, does not appear to be a reliable or useful indicator for the purpose of fertilization recommendations. In our dataset, NI did not correlate significantly with in situ mineralization (ISM), nor with PMN or other key biological indicators. This suggests that, as a proxy, NI fails to adequately reflect the biologically active nitrogen fraction that is mineralized and becomes plant-available during the growing season. Nonetheless, our analysis revealed a strong and statistically significant relationship between NI and soil clay content. This new finding suggests that, rather than representing a dynamic or functionally relevant nitrogen pool, the NI fraction may be largely determined by the soil's physical properties and its capacity to retain organic compounds. This observation aligns with the findings of Castellano et al. [50], who demonstrated in their meta-analysis of forest soils that clay content explains 35% and 53% of the coefficient of variation (CV) of nitrate (NO_3^-) and dissolved organic nitrogen (DON) concentrations, respectively. The form of nitrogen extracted by our acid digestion method may be comparable to the DON fraction described, in that it likely includes soluble organic compounds released from humified or partially decomposed organic matter, adsorbed onto mineral surfaces or retained within microaggregates. If so, the strong correlation between NI and clay content may indicate that this pool is stabilized through physicochemical mechanisms rather than being immediately accessible to microbial mineralization. Taken together, these results suggest that while NI may offer insights into long-term soil organic matter stabilization processes, it lacks the specificity and responsiveness required for operational use in nitrogen fertilization guidance. Future research may further explore its potential as a descriptor of soil buffering capacity or resilience, but it is unlikely to serve as a direct predictor of short-term nitrogen availability for crops.

4.3. Current and Further Improvement

Manure and grassland turnover effects are adequately accounted for through the PMN approach based on anaerobic incubation. This is a key advantage over other methods, which suffer from a frequent lack of precision in estimating the exact amount of organic nitrogen applied in the field—particularly in the case of solid manures. Indeed, solid manure is highly variable in its physical properties (e.g., water content, bulk density), and its field application is often carried out using non-calibrated equipment, which can lead to substantial inaccuracies in the declared doses. In this context, the use of anaerobic incubation to assess the net mineralization potential of soils offers a valuable way to integrate the cumulative impact of organic inputs—whether recent or residual—on soil nitrogen supply. PMN thus acts as a corrective and integrative measure, reflecting both the manure history and soil responsiveness to organic matter decomposition. Regarding cover crops, our results, as well as recent literature, suggest that the current methods ignore potential nitrogen immobilization—occurring in the weeks following residue incorporation—and it also underestimates the variability in nitrogen restitution to the subsequent crop as previously discussed. This limitation is reduced if MLR or BFMs are used, but it highlights

the need to better differentiate the N released from CC depending on the composition, the termination period, and type of the cover.

A major limit of the anaerobic incubation method is its duration. Despite being faster than aerobic methods, the 7-day anaerobic incubation can increase analysis turnaround times for the laboratory in spring, when farmers are waiting for quick fertilization advice. To speed up processing, visible–near-infrared (vis–NIR) and in some cases mid-infrared (MIR) spectroscopy may be used in the future. These tests are quick and need no reagents, but they require strong, local calibrations to be reliable. In practice, we recommend building local calibrations (e.g., on Walloon arable soils), stratifying sampling by texture and management, and performing independent external validation, reporting both R^2 and RPD. In the short term, evidence suggests that POxC is the most ready setting for routine spectral use, while PMN remains a promising parameter, but it requires larger-scale testing to confirm broad applicability [51–54].

Another factor worth considering in future modeling efforts is the potential influence of soil carbonate content. High levels of calcium carbonate (CaCO_3) in soils could interfere with the accuracy of mineralization predictions [36]. In our dataset, however, this issue did not arise, as none of the soils contained significant amounts of carbonates. Nonetheless, this potential limitation should be kept in mind when applying the model to calcareous soils. If used in such contexts, the model would need to be adjusted to account for CaCO_3 interference, which can alter organic matter stabilization, pH buffering, and nitrogen transformation processes.

Finally, weather conditions are likely to have influenced nitrogen dynamics through leaching processes. The year 2021 was exceptionally wet during our monitoring: In one experimental plot (Marbisoux 4), the LIXIM model simulated the loss of approximately $160 \text{ kg N} \cdot \text{ha}^{-1}$ via leaching between July 23 and the end of October (Figure 5). Regional rainfall records showed more than 300 mm of precipitation between June 29 and August 29 (CRA-W/Agromet.be). These heavy rainfall events inevitably disrupted the nitrogen balance and altered nitrogen availability for crops in place. Although LIXIM accounted for such exceptional conditions in its simulations, these events are difficult to anticipate at the time when fertilization decisions are made. Their increasing frequency due to climate change highlights the importance of integrating climatic risk into nitrogen management strategies. As nutrient losses through leaching become more common, the need for adaptive and resilient fertilization recommendations becomes urgent.

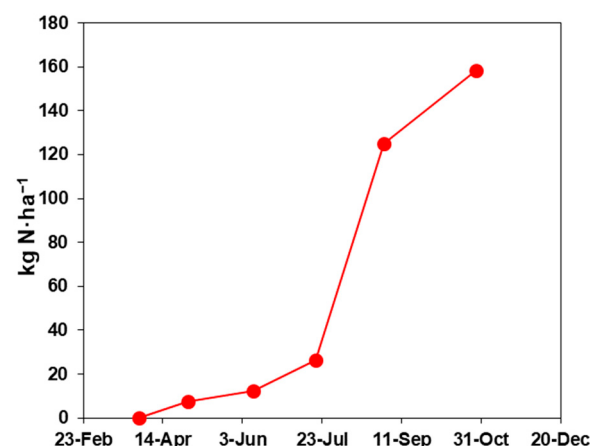


Figure 5. Simulation of nitrate leaching using the LIXIM model for the Marbisoux 4th plot in 2021.

5. Conclusions

This study, conducted over a six-year multi-site soil monitoring in temperate agricultural systems, confirmed that the anaerobic incubation method (PMN) provides a robust and relevant estimation of soil nitrogen mineralization. PMN measurements were consistently more closely correlated with in situ mineralization values (ISM, estimated by the LIXIM model) than the current indicators used in the Walloon Region (Soil N-C and Soil N-N), which are based on total organic carbon (TOC) and total nitrogen (Nt).

The use of PMN as a predictive variable significantly improved mineralization estimates. Additional variables—such as POxC, pH, and TOC to clay content—also contributed positively to prediction accuracy, although to a lesser extent than PMN. POxC, in particular, showed potential as a complementary indicator of biological activity.

Manure application and grassland turnover effects were both properly accounted for by PMN, confirming the relevance of the incubation method under a wide range of management conditions. On the other hand, cover crops did not show a consistent effect, likely due to the high variability in species, biomass, and management across plots. These observations highlight the need to improve the way cover crops are integrated into fertilization advice, particularly with respect to the timing and extent of nitrogen restitution.

While TOC and Nt remain useful descriptors of soil organic matter content, their predictive power to determine nitrogen mineralization turned out to be limited. Biological indicators such as PMN and POxC offer a more dynamic and accurate alternative. The currently used balance method would greatly benefit from the integration of such robust biological indicators to better capture the complexity of soil organic matter dynamics and crop nitrogen availability. This is especially relevant in systems receiving organic amendments or integrating agroecological practices like cover cropping and grassland rotations.

Author Contributions: Conceptualization, T.C. and R.L.; methodology, T.C., M.D.T. and R.L.; software, T.C.; validation, T.C., M.D.T. and R.L.; formal analysis, T.C.; investigation, T.C.; resources, T.C. and M.D.T.; data curation, T.C.; writing—original draft preparation, T.C.; writing—review and editing, T.C., R.L. and J.M.; visualization, T.C.; supervision, R.L.; project administration, T.C. and R.L.; funding acquisition, R.L. and J.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Walloon Government through the REQUASUD framework agreement. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding authors.

Acknowledgments: During the preparation of this manuscript, the authors used ChatGPT (OpenAI; GPT-4o) for limited writing assistance (clarity, grammar and phrasing) and for preliminary identification of candidate references. All AI-assisted text was reviewed and edited by the authors, and every citation was independently verified. No AI tools were used for data analysis or for generating figures/tables. The authors take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

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