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Soil Microbial Communities Involved in Proteolysis and Sulfate-Ester Hydrolysis Are More Influenced by Interannual Variability than by Crop Sequence

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‡ This work is dedicated to the memory of our dear colleague and friend Bernard Amiaud, Professor at the Université de Lorraine (1969–2018).

Abstract: Proteases, catalysing protein hydrolysis, and arylsulfatases, catalysing sulfate-ester hydrolysis, are key microbial enzymes for N and S mineralization in soil. However, knowledge gaps remain regarding the effect of crop successions and seasonal and interannual meteorological variations on microbial communities responsible for those activities. Here, we compared the effect of six cropping sequences on the abundance and activity of microbial communities involved in proteolysis and sulfate-ester hydrolysis in northern France over four years, with two sampling dates per year. Crop sequences impacted soil microbial communities involved in proteolysis but not those involved in sulfate-ester hydrolysis. Oilseed rape following wheat presented a higher abundance of fungal 18S rDNA, culturable bacteria and alkaline metalloprotease genes and higher protease activity than other crop sequences (wheat following oilseed rape or pea, barley following wheat and pea following barley). Net N and S mineralization was not impacted by the cropping sequence. However, interannual variability of microbial parameters was large, and largely overcame the effect of crop sequences. Precipitation variability between years was the likely cause of this effect. In conclusion, the interaction between current crop, previous crops and yearly meteorology can strongly impact the soil microbial communities in agroecosystems.

Keywords: arylsulfatase; subtilisin; alkaline metalloprotease; interannual variation; agroecology

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1. Introduction

Soil microorganisms' enzymatic activities are responsible for more than 80% of soil organic matter decomposition [1,2]. They are relevant to crop production as they control soil nutrients furniture to plants for major nutrients, including nitrogen (N) and sulfur (S). While N is the major limiting nutrient for all plants, S can be limiting for crops with high S needs, such as Brassicaceae and Fabaceae. Furthermore, S limitation can reduce plant N assimilation [3,4], modify plant resistance to pathogens [5] and impact the quality of harvested products [5,6].

Proteases (Enzyme Commission number EC 3.4), catalyzing protein hydrolysis to amino acids, are important enzymes for N mineralization as about 40% of soil organic N is under protein form [7]. Proteolysis is considered a limiting step for N mineralization [8], and protease activity is positively correlated with gross N mineralization [9–11]. Proteases in soil are predominantly of bacterial origin [12,13], and mostly belong to the classes' alkaline metalloprotease, neutral metalloprotease and subtilisin [14].

Arylsulfatases (EC 3.1.6.1), catalyzing sulfate-ester hydrolysis to sulfate, are important for plant S nutrition as most of S found in soil is in the form of sulfate-ester [15] while sulfate

is the only form available to plants [16]. Arylsulfatase activity is positively correlated with microbial gross S immobilization [17–19] and plant S uptake [20,21]. In soils, arylsulfatases are of both bacterial and fungal origin [22,23].

Protease and arylsulfatase activities, as other soil enzymes, are influenced by a wealth of biotic and abiotic factors, including crop plants and meteorology. During their life cycle, crop plants modify the abundance, composition and enzymatic activity of soil microbial communities [24–30] and this effect may last after the harvest (i.e., legacy effect) [31–35].

The effects of crops on soil microbial parameters depend on the crop species and especially on the botanical families to which these species belong, as they vary in characteristics that can impact soil microbial communities. Such characteristics include the length of the development cycle, N and S requirements, nature and quantities of rhizodeposits, shoot and root biomass, and residue characteristics [24,36–39]. For example, Brassicaceae such as oilseed rape (*Brassica napus*) have a long cycle, high requirements for S and N and produce high quantities of biomass and rhizodeposits. Poaceae such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) have a slightly shorter cycle, high requirements for N but low requirements for S and produce high amounts of residues and rhizodeposits. Fabaceae such as spring pea (*Pisum sativum*) have a short cycle, intermediate requirements for S and low requirements for N due to N_2 symbiotic fixation and produce a low amount of biomass and rhizodeposits with a high N content [40–46].

Higher arylsulfatase activity and abundance of bacteria producing this enzyme have been found in the rhizosphere of Brassicaceae compared to Poaceae [17,22,28], which has been attributed to both the higher rhizodeposition and the higher S uptake of Brassicaceae. However, the opposite results were found by [47], and attributed to a higher biomass production by wheat than by oilseed rape. In one of the rare studies investigating the impact of different crop species on protease activity, Kwiatkowski et al. [48] found higher protease activity under sugar beet and red clover than under Poaceae, which was attributed to higher root biomass production. In general, protease activity is positively correlated to several soil parameters that are affected by crops, such as microbial biomass and abundance [49–51], protein content [52] and nitrogen availability [53]. Nevertheless, knowledge gaps remain regarding the effect of crop identity and crop succession on soil microbial communities involved in N and S cycling and the resulting net mineralization fluxes. Filling those gaps is necessary to design cropping systems in which soil fertility management is improved and adapted to the pedoclimatic context [54,55].

Seasonal and interannual variability in temperature and precipitation can also strongly influence soil microbial communities' abundance, structure and activity [56–60]. The soil water content is positively correlated to the microbial biomass [61] and influences the bacterial community composition [62]. Positive effects of soil moisture and temperature have been observed on protease [63,64] and arylsulfatase activity [65], even if the mechanisms responsible for it remain unclear [66]. Soil water content controls the diffusion rate and the concentration of enzymes' substrates and products, thus modifying the enzymatic reactions' rates and the perception of the environment by the microorganisms [67–69]. Temperature similarly impacts enzyme's reaction rates and organisms' growth rates [68]. Indirect effects can also occur through increased nematode grazing in wetter soils [70] and the modification of plant growth and rhizodeposition by climatic conditions [71–73]. For example, Lauber et al. [74] hypothesized that seasonal variations in the bacterial community compositions in agricultural soils could be related to changes in plant C inputs to the soil (i.e., root exudates and plant litter) caused by changes in soil temperature and moisture over the season.

Field experiments are complicated by the high seasonal and interannual variability of microbial variables. The relative importance of climate and crop effects on microbial communities, and their interactions [75], remains largely unresolved and, to our knowledge, no study compared the influence of climatic conditions and different crop species on microbial communities implicated in protease and arylsulfatase activity. Pluriannual experiments are necessary to achieve this goal.

Here, we used a pluriannual dataset (four years) to compare the effect of six crop sequences on the abundance and activity of soil microbial communities involved in proteolysis and ester-sulfate hydrolysis, as well as the resulting mineralization fluxes, in two different seasons.

We hypothesized that (1) crops with higher N or S demand would favor the abundance and activity of microbes involved in protease and arylsulfatase activities, respectively, and would have legacy effects on the microbial community's abundance and activity in succeeding crops; (2) intra- and inter-annual variabilities in meteorology would more strongly influence microbial abundance and activity than the crop sequence; and (3) changes in microbial abundance and activity would modify net mineralization fluxes.

2. Material and Methods

2.1. Experimental Site Description

The experimental trial was set up in a field in north-eastern France (Champenoux, 48°73′ N, 6°31′ E), which has a temperate oceanic climate. Over the 2009–2013 period, the mean annual rainfall was 719 mm and the mean annual air temperature was 9.8 °C. Daily rainfall (mm) and temperature (°C) data were obtained from a meteorological station located 1.5 km away from the experimental farm (48°44′ N, 6°21′ E). Detailed seasonal meteorological fluctuations are presented in Table S1. The soil is a Vertic (Clayic) Cambisol [76]. The soil's physico-chemical properties are detailed in Table S2.

The field, which was previously cultivated with a maize–maize–spring barley succession (2005–2008), was divided into 4 experimental blocks in 2008. Each block (100 × 72 m) was divided into 8 plots of 50 × 18 m (Figure S1A). Each plot was cultivated with a 3- or 5-year crop rotation in a randomized "phase differences" design [77], allowing each crop of each rotation to be present each year in each block (Figure S1B). The 3-year crop rotation was a typical winter crop succession for northern France: winter oilseed rape (*Brassica napus* L.)–winter wheat (*Triticum aestivum* L.)–winter barley (*Hordeum vulgare* L.). The 5-year crop rotation system was characterized by the introduction of spring pea and was composed of winter oilseed rape–winter wheat–winter barley–spring pea (*Pisum sativum* L.)–winter wheat. Winter wheat sown in fall 2011 was destroyed by intense frost and replaced by spring wheat sown in March 2012. Detailed cropping practices are presented in Tables S3 and S4. All plots were deep-ploughed every year before crop establishment. Winter oilseed rape was sown in August, winter wheat and winter barley were sown in October, and spring pea was sown in March. Management practices were in accordance with the standard recommendations for fertilization and are representative of local practices. All plots received 42 T ha^{-1} bovine manure (equivalent to 160 kg N ha^{-1}) in August 2009 and 95 kg P-Ca(H_2PO_4)$_2$ and 63 kg K-KCl ha^{-1} in March 2011. Mineral nitrogen (N) and sulfur (S) fertilization was achieved with ammonium nitrate, urea or ammonium sulfonitrate. Oilseed rape was systematically fertilized with mineral N and S, wheat and barley were systematically fertilized with N, and pea plots did not receive N or S fertilization (Table S3). Cereal straw was exported, while oilseed rape and pea straw were retained. No irrigation was used. Pesticides were applied when necessary, according to the regional alert thresholds (Table S4).

2.2. Soil Sampling

Soil samples were collected from the top of the plough layer (0–10 cm), sieved at 5 mm, thoroughly homogenized and immediately used for enzymatic activity measurement, culturable bacteria quantification and mineralization kinetics. A subsample of soil was frozen at −20 °C for molecular analysis.

Soil samples were collected in every plot two times a year (May and July) from 2010 to 2013. In May, the crop phenological stages were flowering, stem elongation and leaf initiation for oilseed rape, cereals and pea, respectively. In July, all crops were close to maturity. Soil samples were collected on the same days for all crops despite phenological

differences, as we considered those differences an integral part of the plant effect, and as we expect the meteorology of the sampling season to strongly impact microbial communities.

Sampled crop sequences were defined as follows: wheat after oilseed rape (WaO), wheat after pea (WaP), oilseed rape after wheat (OaW), oilseed rape after barley (OaB), barley after wheat (BaW) and pea after barley (hereafter PaB).

The sampling area was identified by GPS coordinates to limit the sampling spatial variability between sampling times. A total of 32 soil samples were collected at each sampling time, resulting in 256 samples collected over the course of the experiment.

2.3. DNA Extraction and Quantification of Microbial Genes

Genomic DNA was extracted from each soil sample using a Fast DNA® SPIN Kit for Soil (MP Biomedical, Santa Anna, CA, USA) according to the manufacturer's instructions. The abundance of the total bacterial and fungal communities was quantified using qPCR on the 16S rDNA and 18S rDNA, respectively. The abundance of protease-carrying bacteria was evaluated using qPCR analysis of *apr* and *sub* genes, coding, respectively, the alkaline metalloprotease and the subtilisin [78]. The *npr* gene, coding the neutral metalloprotease, was also assessed using the methodology of Bach et al. [78], but was not detected in our soil.

All qPCR assays were carried out in a StepOne Plus™ thermocycler (Applied Biosystems, Foster City, CA, USA). Cycling, data collection and calculations were performed with StepOne™ Software v.2.1 (Applied Biosystems, Foster City, CA, USA). All reaction mixes contained 10 ng of template DNA and 1x Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The quantification of the 16S rDNA gene was performed in a 10 µL reaction volume containing 1 µM each of the 341F and 534R primers [79] and 250 ng T4Gene 32 (MP Biomedicals, Strasbourg, France). The PCR conditions were 95 °C for 10 min and then 35 cycles of 15 s at 95 °C (denaturation), 30 s at 60 °C (annealing) and 30 s at 72 °C (extension). The 18S rDNA gene was quantified in a 20 µL reaction volume containing 1.25 µM each of the FR1 and FF390 primers [80] and 100 ng T4Gene 32. The PCR conditions were 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C (denaturation), 30 s at 50 °C (annealing) and 60 s at 72 °C (extension). The *apr* and *sub* genes were quantified in a 20 µL reaction volume containing 1.75 µM each of the *apr* I and *apr* II primers or the *sub* I and *sub* II primers [81], respectively, and 150 ng T4Gene 32. The PCR conditions were 95 °C for 10 min followed by 40 cycles of 20 s at 95 °C, 30 s at 53 °C and 60 s at 72 °C [26]. For each sample, three replicates were performed on different PCR plates. Standard curves were obtained using 7 concentrations (10^2 to 10^8 copies per well) of linearized PCR8®/GW/TOPO® plasmids (Life Technologies, Carlsbad, CA, USA) containing the targeted DNA sequences obtained from soil bacterial or fungal strains. The specificity of the amplified products was checked by melt curve analysis and electrophoresis of the qPCR products on a 1.5% (*w/v*) agarose gel.

2.4. Quantification of Culturable Bacteria

The abundance of total, proteolytic and arylsulfatase-active culturable bacteria was determined and considered a measure of the active copiotrophic bacterial community [82]. For the total and proteolytic bacteria, we considered it a complementary information to molecular abundances [83]. For bacteria with arylsulfatase activity, cultivation is the only way to assess their abundance in the absence of specific primers for quantitative PCR. A soil suspension was prepared by mixing 10 g of soil with 100 mL of phosphate buffer (8 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 L^{-1}, pH 7.2) on an orbital shaker at 120 rpm for 30 min. The suspension was subsequently serially diluted before plating. The number of total culturable bacteria was determined by spread plating the dilutions (10^{-3} to 10^{-5}) onto tryptone soy agar 10% (Biokar Diagnostics, Pantin, France) in three replicates per soil sample. Colonies were counted after incubation in the dark at 28 °C for 10 days.

The abundance of culturable proteolytic bacteria was determined by plating the soil suspension (dilution 10^{-3}) on calcium caseinate-agar 30.7 g L^{-1} solid growth medium

(Sigma–Aldrich, Saint-Louis, MO, USA). Colonies surrounded by a clear halo, expressing protease activity, were counted after 10 days of incubation at 28 °C.

The abundance of arylsulfatase-active bacteria was estimated by plating the soil suspension (dilutions 10^{-3} to 10^{-4}) on M9-X-sulf medium [22]. X-sulf is a chromogenic substrate that develops a blue color when the bacterial hydrolysis of sulfate-ester occurs [84]. The blue colonies were counted after 21 days of incubation at 28 °C.

All bacterial abundance was expressed as colony forming units (CFUs) per gram of dry soil. While total and arylsulfatase activity bacteria were quantified at all sampling times, proteolytic bacteria were quantified only from July 2011 onwards.

2.5. Soil Protease and Arylsulfatase Activity

Arylsulfatase activity was quantified at all sampling dates from 2010 to 2013, while protease activity was quantified only from July 2011 onwards.

Potential protease activity was determined according to Ladd and Butler [85]. Briefly, 1 g of fresh soil was incubated with 2.5 mL of 0.1 M Tris-HCl buffer (pH 8) and 2.5 mL 2% sodium caseinate solution for 4 h at 45 °C. The addition of 5 mL 10% trichloroacetic acid terminated the enzymatic reaction. For each sample, a control assay was performed with 1 g of fresh soil incubated for 4 h at 45 °C in 2.5 mL of 0.1 M Tris-HCl buffer (pH 8). Then, 5 mL 10% trichloroacetic acid was added, followed by substrate (sodium caseinate) addition. After centrifugation at 16,000× *g* for 2 min, 0.5 mL of supernatant was mixed with 0.75 mL 1.4 M sodium carbonate and 0.25 mL 16.5% Folin–Ciocalteu reagent for 5 min. The concentration of released tyrosine was determined colorimetrically at 680 nm using a Cary® 300 spectrophotometer (Varian, Palo Alto, CA, USA). Protease activity was expressed as the amount of tyrosine produced per hour per gram of dry soil.

Potential arylsulfatase activity was determined according to Tabatabai and Bremner [86]. Briefly, 1 g of fresh soil was incubated with 4 mL of 0.5 M sodium acetate buffer (pH 5.8), 0.25 mL toluene and 1 mL of 25 mM p-nitrophenyl sulfate for 1 h at 37 °C. The addition of 1 mL 0.5 M $CaCl_2$ and 4 mL 0.5 M NaOH terminated the enzymatic reaction. For each sample, a control assay was performed with 1 g of fresh soil incubated for one hour at 37 °C in 4 mL of 0.5 M acetate buffer (pH 5.8) and 0.25 mL toluene. After filtration, 5 mL of NaOH 0.5 M and the substrate (p-nitrophenyl sulfate) were sequentially added. After filtration on Whatman 2 V filter paper (Whatman, Maidstone, UK), the concentration of p-nitrophenol released was determined colorimetrically at 400 nm using a Cary® 300 spectrophotometer (Varian, Palo Alto, CA, USA). Arylsulfatase activity was expressed as the amount of p-nitrophenol produced per hour per gram of dry soil.

2.6. Net Sulfur and Nitrogen Mineralization

Net mineralization was determined only for soil samples collected in July. Ten grams of equivalent dry soil was incubated in the dark at 20 °C for 28 days. The soil was moistened to 80% of the water-holding capacity (WHC). Microbial communities were placed under nonlimiting conditions for N and S by the addition of 0.7 mg N-KNO_3 and 0.2 mg S-Na_2SO_4. After the end of incubation, the soil was shaken with 25 mL 0.016 M KH_2PO_4 in an end-over-end shaker for 30 min [87]. The suspension was filtered on a Whatman 2 V and filtered again through a 0.45 µm cellulose nitrate filter (Millipore). KH_2PO_4 extracts were frozen at −20 °C until the determination of the soil mineral S (SO_4^{2-}) and N (NO_3^-) contents by ionic chromatography (IC 25, Dionex, Salt Lake City, UT, USA).

2.7. Data Analysis

The effects of crop sequences, sampling month and year and their interactions on the microbial abundance, enzymatic activity and net mineralization were tested independently for each response variable using either linear models or generalized linear models (GLMs) with a negative binomial distribution, followed by type II ANOVA and Tukey's post hoc tests. Multicollinearity was assessed by the variance inflation factor (VIF) using the *vif* function of the *car* library [88], and it was always less than 5. When necessary, the dependent

variable was square-root transformed to satisfy normality and homoscedasticity assumptions. We successively used the sum of squares given by ANOVA for each explicative variable to evaluate their respective contribution to the dependent variable variation [89]. The relationship between net mineralization and enzymatic activity and the relationship between enzymatic activity and microbial abundance were investigated using linear models with log-transformation of the abundance to ensure linearity. Relationships between meteorological variables and microbial parameters were investigated using Spearman's rank correlations. All analyses were conducted with R software 4.0.4 [90].

3. Results

3.1. Effect of Crop Sequences on the Microbial Abundance, Enzymatic Activity and Net N and S Mineralization

We investigated the effect of six crop sequences on the abundance of total microbial communities (fungi, bacteria and culturable bacteria), the abundance of microbial functional groups involved in proteolysis and sulfate-ester hydrolysis (bacteria with protease genes, with protease activity or with arylsulfatase activity), the microbial enzymatic activity (protease and arylsulfatase activity) and net N and S mineralization. Significant effects of crop sequences were observed on the abundance of culturable bacteria, the copy number of 18S rDNA, the relative abundance of *apr* genes and the activity of protease (Table 1). The abundance of culturable bacteria was slightly higher under OaW (mean ± SE $1.91 \times 10^8 \pm 4.12 \times 10^7$ CFU g^{-1} dry soil) compared with WaO ($1.21 \times 10^8 \pm 1.62 \times 10^7$ CFU g^{-1} dry soil), WaP ($1.21 \times 10^8 \pm 2.22 \times 10^7$ CFU g^{-1} dry soil) and PaB ($1.19 \times 10^8 \pm 1.92 \times 10^7$ CFU g^{-1} dry soil) (Figure 1). The trend was similar for the abundance of 18S rDNA, with the highest value under OaW ($2.91 \times 10^7 \pm 4.30 \times 10^6$ copy g^{-1} dry soil) and the lowest under WaO ($1.31 \times 10^7 \pm 1.26 \times 10^6$ copy g^{-1} dry soil) and PaB ($1.32 \times 10^7 \pm 2.29 \times 10^6$ copy g^{-1} dry soil). The relative abundance of *apr* was the highest under OaW (7.6 ± 2.0%) and lowest under WaP (5.8 ± 1.9%). The protease activity was the highest under OaW (151 ± 17.8 µg Tyr h^{-1} g^{-1} dry soil) and lowest under WaO (108 ± 11.2 µg Tyr h^{-1} g^{-1} dry soil) (Figure 2). No effect was observed on arylsulfatase activity, the density of culturable bacteria possessing this activity, or the net N and S mineralization (Figure 2).

Table 1. F values from the two-way ANOVA with interaction, with crop sequence, year and month as factors. Bold indicates a significant effect. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. LM: linear model, GLM.nb: generalized linear model with negative binomial error distribution. Functional gene relative abundance is expressed relative to the eubacterial abundance, and functional groups of culturable bacteria are expressed relative to the total culturable eubacterial abundance.

	Model (Transformation)	Crop Sequence	Month	Year	Crop Sequence X Month	Crop Sequence X Year
Microbial abundance						
16S rDNA	GLM.nb	1.77	**23.15** ***	**26.17** ***	1.10	0.92
18S rDNA	GLM.nb	**8.44** ***	**10.89** **	**48.48** ***	**3.10** **	**1.78** *
apr:16S rDNA	GLM.nb	**2.40** *	**5.65** *	**304.23** ***	0.38	0.80
sub:16S rDNA	GLM.nb	2.20	0.11	**37.45** ***	0.22	**2.23** **
Culturable bacteria	GLM.nb	**2.92** *	0.89	**64.46** ***	1.14	0.91
Proteolytic: culturable bacteria	GLM.nb	0.99	**27.47** ***	**26.67** ***	0.47	0.69
Arylsulfatase: culturable bacteria	GLM.nb	0.90	3.08	**517.79** ***	0.59	**1.79** *
Enzymatic activity						
Protease activity	LM (square root)	**2.53** *	**18.07** ***	**67.39** ***	0.97	1.72
Arylsulfatase activity	LM (square root)	0.18	**225.63** ***	**6.97** ***	0.11	0.23

Table 1. *Cont.*

	Model (Transformation)	Crop Sequence	Month	Year	Crop Sequence X Month	Crop Sequence X Year
Net mineralization						
N mineralization	GLM.nb	1.57	–	**20.69** ***	–	0.71
S mineralization	LM	0.31	–	**62.19** ***	–	0.34

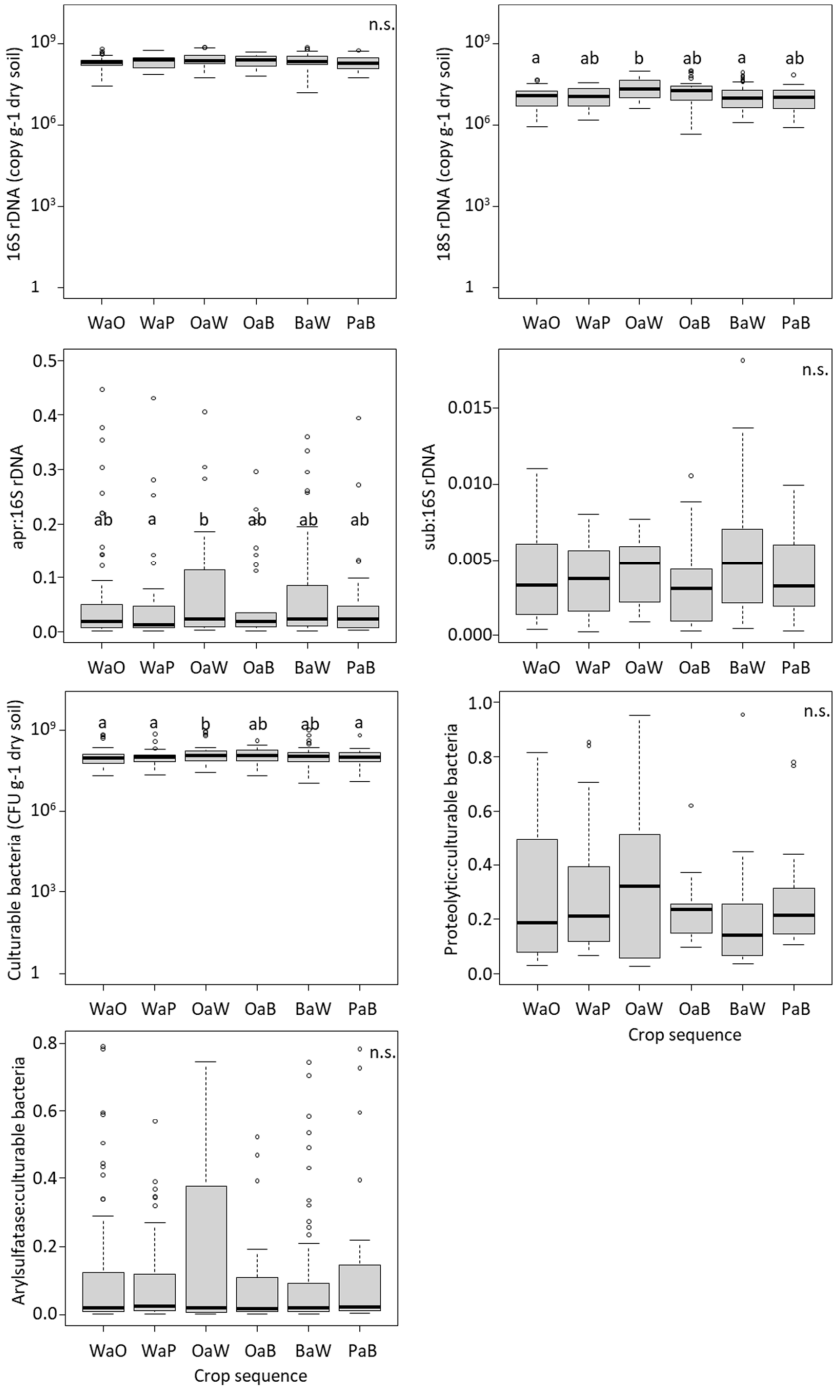

Figure 1. Microbial groups abundances in soil under different crop sequences. Boxplots with different letters indicate significant differences between species ($p < 0.05$, n.s.—not significant, Tukey post-hoc test). WaO, wheat after oilseed rape; WaP, wheat after pea; OaW, oilseed rape after wheat; OaB, oilseed rape after barley; BaW, barley after wheat; PaB, pea after barley.

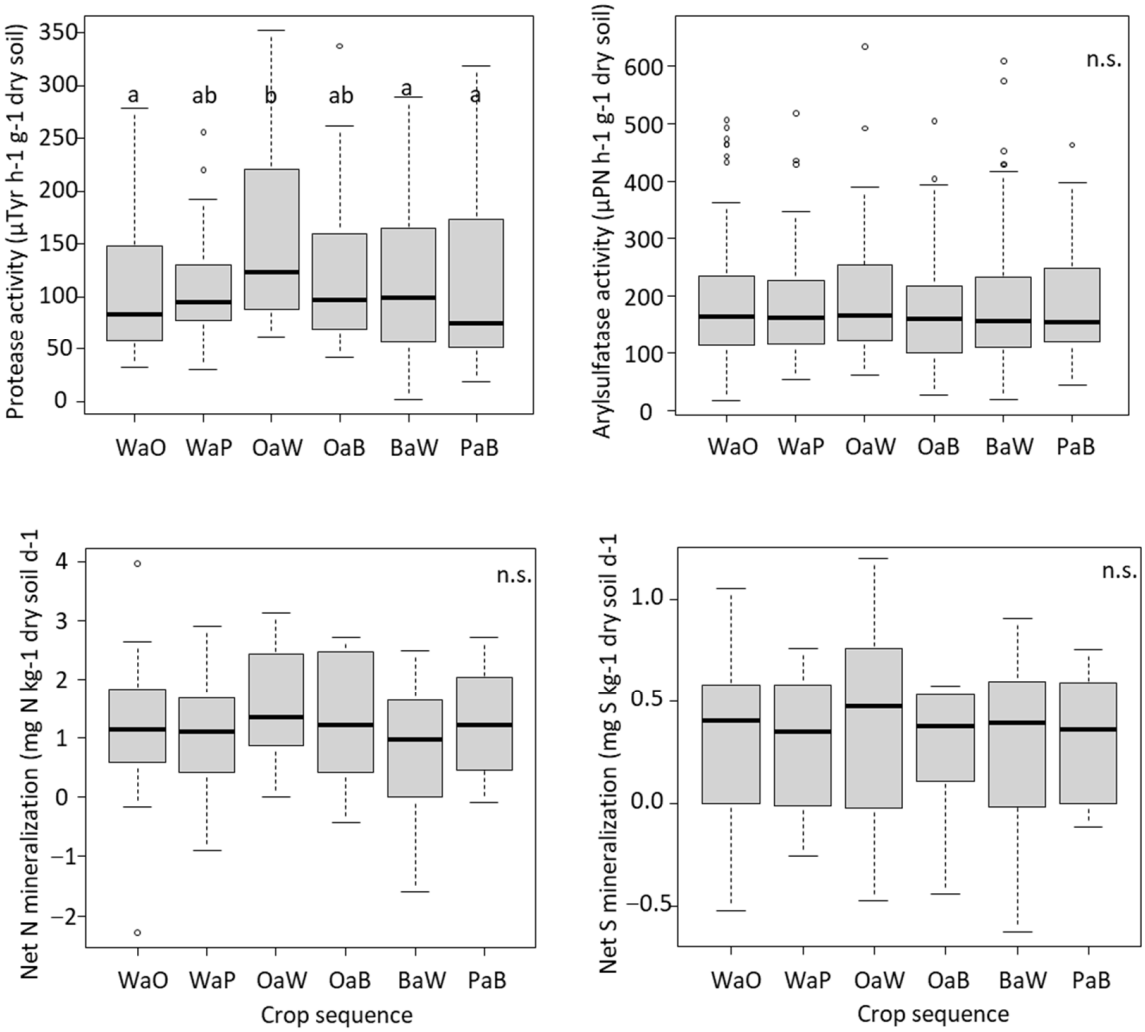

Figure 2. Enzymatic activities and net mineralization in soil under different crop sequences. Boxplots with different letters indicate significant differences between species ($p < 0.05$, n.s.—not significant, Tukey post-hoc test). WaO, wheat after oilseed rape; WaP, wheat after pea; OaW, oilseed rape after wheat; OaB, oilseed rape after barley; BaW, barley after wheat; PaB, pea after barley.

3.2. Relative Influence of Crop Sequence, Month and Year of Sampling

We used linear models to assess the relative importance of crop sequence, year and month, as well as their interactions, on the set of microbial variables (Figure 3). The explanatory power of the models varied, according to the variable considered, from 38% (16S rDNA) to 88% (arysulfatase:culturable bacteria) of variations explained. Overall, variations in microbial abundance (23 to 86%), protease activity (42%) and net mineralization (31 to 60%) were mostly explained by the year, suggesting an effect of interannual meteorological variations.

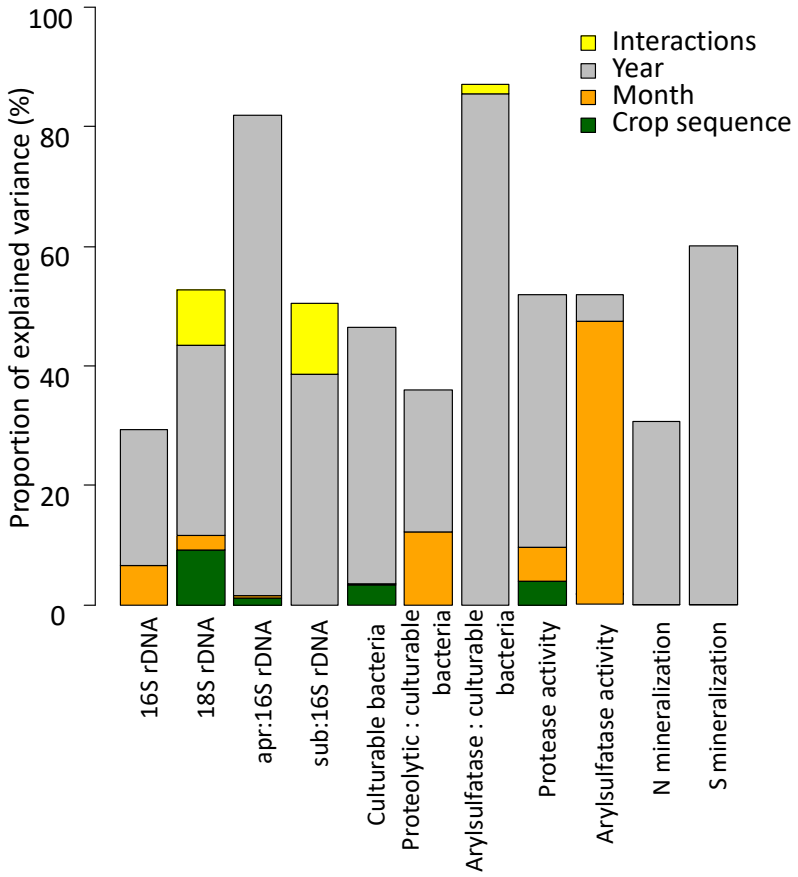

Figure 3. Percentage of explained variance by crop sequence, year, month and their interactions on microbial abundances and activities.

Overall, considering a three-month period prior to soil sampling, the total microbial abundance was negatively correlated with the sum of precipitation ($\rho = -0.25$ for 16S rDNA, -0.39 for 18S rDNA and -0.46 for culturable bacteria, $p < 0.001$ for all three variables) (Table S5). Functional bacterial abundances related to N, except proteolytic:culturable bacteria, were positively correlated with precipitation ($\rho = 0.52$, $p < 0.001$ for *apr*:16S rDNA and $\rho = 0.25$, $p < 0.05$ for *sub*:16S rDNA), whereas protease activity was not (Table S5). Concerning S cycle, arylsulfatase:culturable bacteria and arylsulfatase activity were positively correlated to both temperature ($\rho = 0.27$, $p < 0.001$ and 0.73, $p < 0.001$, respectively) and precipitation ($\rho = 0.43$, $p < 0.001$ and 0.50, $p < 0.001$, respectively) over the three months prior to soil sampling.

The month explained only a small part of the variance, with the exception of the protease:culturable bacteria relative abundance (12%) and arylsulfatase activity, for which the sampling month explained the greatest variation (47%). For the former variable, the activity was almost 2.5 times higher in July than in May (Table S6). Finally, the crop sequence explained only a small part of the variation, ranging from 0% (arysulfatase:culturable bacteria abundance and arylsulfatase activity) to 9% (18S rDNA abundance).

Interactions between crop sequence and sampling year were significant only for 18S rDNA abundance, *sub*:16S rDNA and arylsulfatase:culturable bacterial abundance (Table 1). It is interesting to note that for *sub*:16S rDNA and arylsulfatase:culturable bacteria, the interaction between the crop sequence and the year was significant, while the effect of the crop sequence was not, suggesting a differential influence of the year depending on the crop sequence. In the case of 18S rDNA, the interaction between the crop sequence and the month was also significant. 18S rDNA was more abundant in May than in July under all crops (Table S6) except pea, where it was more abundant in July ($1.35 \times 10^7 \pm 2.23 \times 10^6$ copy g^{-1} dry soil) than in May ($1.28 \times 10^7 \pm 4.09 \times 10^6$ copy g^{-1} dry soil).

We successively analysed the effect of the month and the year independently for each crop sequence to investigate whether the sampling date effect was stronger under specific crop sequences (Figure 4). The effect of the year was strong under all crop sequences and varied slightly between crop sequences. No single pattern of variation of the relative importance of the year and the month between crop sequences was observed. Instead, variations specific to each microbial group were observed. For example, 18S rDNA was the most impacted by year under OaW and the least impacted under PaB, while the reverse situation was observed for the arylsulfatase:culturable bacteria. The only exception might be OaB, for which the effect of the year and the month was strong for all three microbial variables.

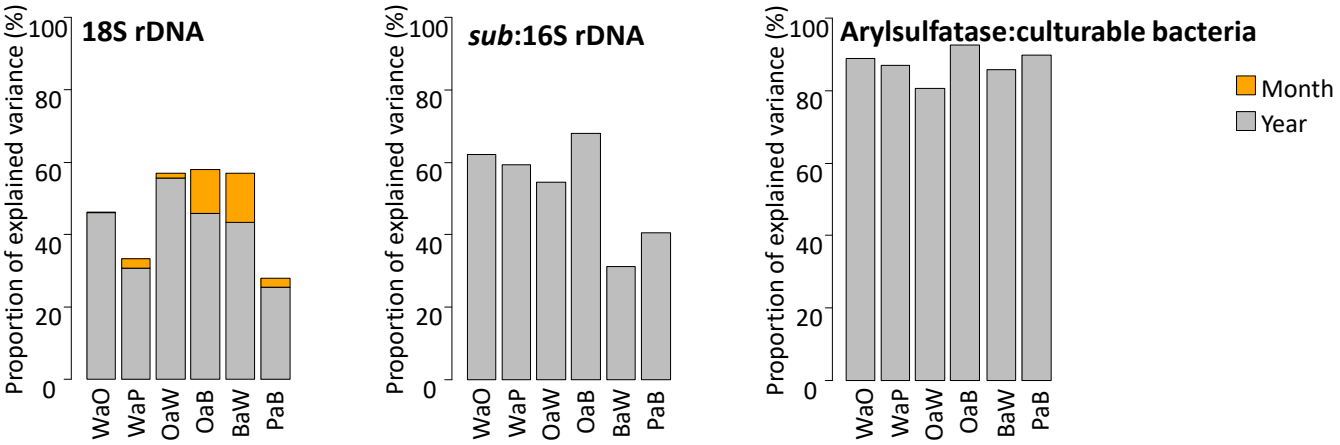

Figure 4. Percentage of explained variance by year and month depending on crop sequence for microbial variables with significant interaction between crop sequence and sampling date. WaO, wheat after oilseed rape; WaP, wheat after pea; OaW, oilseed rape after wheat; OaB, oilseed rape after barley; BaW, barley after wheat; PaB, pea after barley.

3.3. Relationships among Microbial Abundance, Enzymatic Activity and Net Mineralization

Crops and sampling dates impacted both microbial abundance and activity in our experiment. To identify the possible effects of abundance on activity, we studied the relationship between microbial parameters and enzymatic activity (for May and July) or net N and S mineralization (July only). Protease activity was positively correlated with the abundance of 18S rDNA and culturable bacteria without any relation with functional parameters relative to the N cycle, such as the abundance of protease genes or proteolytic culturable bacteria (Table 2A). Arylsulfatase activity was not explained by the abundance of any of the microbes. Finally, considering the net flux, net N mineralization increased with the abundance of 16S rDNA, protease activity and *sub*:16S rDNA, while it decreased with proteolytic:culturable bacteria (Table 2B). Net S mineralization decreased with arylsulfatase activity, while it increased with arylsulfatase:culturable bacteria.

Table 2. Linear model outputs for (A) protease and arylsulfatase activity and (B) net N and S mineralization. Covariates in the linear models for enzymatic activity were total bacterial and fungal abundance and relative abundance of bacterial functional groups with protease or arylsulfatase activity. Linear models for net mineralization included the same variables in addition to protease or arylsulfatase activity. Absolute abundance was log-transformed to ensure linearity. Estimates for selected variables are standardized and qualified by their standard errors. – not used in the model; n.s. nonsignificant; * $p < 0.05$; ** $p < 0.001$; *** $p \leq 0.001$.

A		
	Protease Activity	**Arylsulfatase Activity**
Transformation of the Response Variable	**Square Root**	**Square Root**
Log10(16S rDNA)	n.s.	n.s.
Log10(18S rDNA)	0.298 ± 0.168 **	n.s.
Log10(Culturable bacteria)	0.336 ± 0.137 *	n.s.
apr:16S rDNA	n.s.	–
sub:16S rDNA	n.s.	–
Proteolytic: culturable bacteria	n.s.	–
Arylsulfatase: culturable bacteria	–	n.s.
F value	8.20	2.50
r^2	0.29 ***	0.02 n.s.
B		
	Net N Mineralization	**Net S Mineralization**
Transformation of the Response Variable	**Square Root**	**None**
Log10(16S rDNA)	0.417 ± 0.146 ***	n.s.
Log10(18S rDNA)	n.s.	n.s.
Log10(Culturable bacteria)	n.s.	n.s.
apr:16S rDNA	n.s.	–
sub:16S rDNA	0.265 ± 0.098 ***	–
Proteolytic: culturable bacteria	−0.325 ± 0.115 **	
Arylsulfatase: culturable bacteria	–	0.382 ± 0.100 ***
Protease activity	0.366 ± 0.117 **	–
Arysulfatase activity	–	−0.508 ± 0.065 ***
F value	10.18	17.94
r^2	0.53 ***	0.50 ***

4. Discussion

4.1. Microbial Abundances Control N and S Mineralization and Protease Activity but Not Arylsulfatase Activity

As hypothesized, the net N and S mineralization fluxes were controlled by microbial activity and abundance. Protease activity was positively correlated with the abundance of fungi and culturable bacteria, while arylsulfatase activity was not linked to microbial abundance. Positive correlations between protease activity and microbial abundance are regularly observed [49,91,92] and can be explained by constitutive protease synthesis by certain bacteria and fungi. Culturable bacteria represent only a small proportion of soil

bacteria, however they are likely active and fast-growing [82], which might explain why they explained better protease activity than the proteolytic functional groups measured in this study. Furthermore, molecular approaches quantified different groups of proteolytic bacteria, with each one of them explaining probably a small amount of the total activity.

The lack of correlation between arylsulfatase activity and microbial abundance could be explained by a control of arylsulfatase synthesis by the availability of its substrates (sulfate-esters) and products (sulfates) [93–95]. Because a fraction of arylsulfatases is extracellular [96], it could also be stabilized by soil constituents [97].

Net N mineralization was positively correlated with protease activity, in accordance with other studies [9,11,98]. Net S mineralization was negatively correlated with arylsulfatase activity, which is inconsistent with studies that observed positive correlations between both [99,100]. However, some studies also found correlations with gross S immobilization [17–19,94]. Because arylsulfatase is synthesized in response to the need for S, it is possible that an increase in arylsulfatase activity is linked to an increase in microbial demand, resulting in the immobilization of S and in a decrease in net S mineralization.

4.2. Cropping Oilseed Rape after Wheat Increased the Microbial Abundance and Activity

Plant successions impacted the abundance and activity of soil microbial communities, as previously reported [22,101–103]. In particular, we observed that the OaW succession differs from the successions WaO, WaP, BaW and PaO, with a higher abundance of fungi and culturable bacteria, a higher relative abundance of alkaline metalloprotease genes and higher proteolytic activity. OaB did not clearly differentiate between the two groups. However, differences in microbial abundance and activity did not result in changes in net N and S mineralization.

This finding was contrary to our initial hypothesis that crops with higher N or S demand would favor microbes involved in proteolysis and sulfate-ester hydrolysis, respectively. It is also inconsistent with previous studies that have shown that the plant N uptake rate impacts on the abundance of other N-cycling functional groups, such as nitrifiers and denitrifiers [38,104,105]. However, different steps of the N cycle can be influenced differently by plant growth [106]. Other studies also found higher arylsulfatase activity in the rhizosphere of oilseed rape, a crop with high S requirements, compared with the rhizosphere of cereals [17,22,28,45]. However, those studies were conducted on soil types (i.e., calcareous and loess) that might have reduced the stability of extracellular enzymes [97] or weakened S mineralization fluxes due to soil organic matter protection. Contrary to other studies, here we studied the whole soil (i.e., bulk + rhizosphere soil), which might impact the results as microbial activities can be greatly enhanced in the rhizosphere compared with bulk soil [28]. Other differences between studies comes from differences in fertilization regimes. In particular, organic fertilization is known to increase microbial abundance [107], protease [51] and arylsulfatase activities [28], when compared with mineral fertilization.

The higher abundance of microorganisms and higher proteolytic activity under OaW could be caused by oilseed rape producing a higher amount of biomass, and thus higher amounts of root exudates and root litter compared with cereals and legumes [40,44,108–110]. This input of easily accessible organic matter to the soil could have fueled microbial communities, increasing their growth and activity. This might particularly be the case for protease activity which is upregulated by protein inputs [111,112].

It is not clear why OaW, but not OaB, differentiated from the other crop sequences. Two hypotheses could explain these differences between OaW and OaB. First, the growth of oilseed rape could have been affected by the previous crop, resulting in different nutrient uptake and rhizodeposition levels between the two sequences. Second, differences in the residue's quantity and quality between wheat and barley could have persisted under the following crop, despite the approximately eight months separating the residues burial and the first sampling date. Such a difference in oilseed rape growth following wheat or barley was observed by Sieling and Christen [113]. They postulated that a reduced yield following wheat was caused by the higher amount of straw produced by wheat and by its

lower decomposability, which could have reduced N availability. A similar mechanism might have occurred in our experiment and could have impacted microbial community abundance and protease activity. This result stresses the importance of considering both the effects of actual and previous crops on microbial communities because they may interact.

4.3. Yearly Meteorology More Strongly Impact Microbial Communities than Crops

As hypothesized, the sampling year had a major effect on microbial abundance and activity, which largely overcame the effects of crop sequences and seasonal variability. This effect was likely due to interannual meteorological variability. While the strong influence of climate on microbial abundance has already been demonstrated along spatial climatic gradients [61,114,115], our study emphasizes the existence of this phenomenon across time. Despite a limited dataset (four years), we observed a strong impact of precipitations on most of the measured microbial parameters and a more limited impact of temperature. Contrary to most other studies (e.g., [61,116,117]) we observed a negative correlation between microbial abundances and precipitations. This might be due to anoxia [118] caused by water saturation in our soil which had 60% clay content, or by increased nematode grazing at a high moisture content [70]. However, not all bacterial groups were affected in the same way by meteorological conditions as precipitations impacted the relative abundance of the studied microbial functional groups. A positive effect of soil moisture on the abundance of bacteria with arylsulfatase activity was already observed in the same soil [119] but the reason for this differential response remains elusive.

One particular case was arylsulfatase activity, for which the sampling month explained half the variation. Because bacterial abundance did not explain arylsulfatase activity in this study, monthly variations were likely caused by changes in arylsulfatase expression. The mechanisms responsible for such a high seasonal change in arylsulfatase expression could be linked to plant phenology. Arylsulfatase activity is known to increase during plant development [22,120]. This could result from changes in plant rhizodeposition and S uptake as arylsulfatase is synthesized in response to the limitation of S and to the presence of sulfate-ester [93–95]. However, different crop species were at different phenological stages in May and no interactions were observed between the crop sequence and arylsulfatase activity, which we interpret as an absence of phenological stage effect. The positive correlations between arylsulfatase activity and both temperature and precipitation point to increased enzyme synthesis caused by the increased substrate diffusion rate or microbial sulfate uptake.

For most microbial variables, the month and year effects were additive to the crop sequence effect. However, for three microbial parameters, namely, the abundance of fungi, the relative abundance of subtilisin and the relative abundance of culturable bacteria with arylsulfatase activity, the year and month parameters interacted with the crop sequence, which might mean that year and month effects are stronger under specific crops. This could be due to crop species responding in different ways to meteorological conditions, for parameters such as phenology, carbon allocation [121], rhizodeposition [74], fine root distribution patterns [122] or nutrient uptake [71–73,123]. This was particularly true for the abundance of fungi which was higher in summer than in spring under pea, a pattern opposite to all other crops.

Further investigations, with a longer time series, would be necessary to disentangle those interactions. This is particularly true for the effect of precipitation which display a high temporal variability. Precipitations vary in frequency and intensity within a year, displays pluri-annual cycles of variations and presents long-term trends caused by climate change (e.g., [124]). As such, precipitations might be responsible for high temporal variations of soil microbial communities and warrant further investigations. Unfortunately, agronomic and ecological experiments are rarely replicated across years, despite treatment by year interactions being frequently observed [125]. Our results stress the importance of integrating interannual meteorological variability when investigating plant and cropping practice impacts on microbial communities.

5. Conclusions

In this study, we found that cropping sequences impacted soil microbial communities involved in proteolysis but not those involved in sulfate-ester hydrolysis. In particular, oilseed rape following wheat presented a higher relative abundance of alkaline metalloprotease genes and higher protease activity than other cropping sequences (wheat following oilseed rape or pea, barley following wheat and pea following barley), while oilseed rape following barley had intermediate values between those two groups. These results evidence that interactions between actual and previous crops can strongly impact microbial communities.

However, we found that the sampling year impacted soil microbial communities more strongly than cropping sequences. Such an effect is probably due to interannual meteorological variability, especially changes in precipitation rates. The mechanisms by which meteorology impacts soil microbial communities are still poorly understood. The constitution of long pluriannual datasets should allow for the identification of meteorological variables that impact microbial communities. Then, their interactions with cropping practices could be identified. The obtained knowledge could be combined with seasonal climate forecasting tools, which are currently developed for agriculture [126,127], to predict cropping practice outcomes based on meteorological conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010180/s1, Figure S1: scheme of the field experimental set-up; Table S1: average monthly temperature and monthly precipitations amounts during the four cropping cycles; Table S2: soil physico-chemical properties per experimental block; Table S3: cropping practices per crop; Table S4: pesticides application per crop; Table S5: Spearman's rank correlation coefficient between microbial abundances, enzymatic activities, net mineralization and meteorological variables; Table S6: average ± standard error of microbial variables which are significantly different between May and July.

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