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Response of Soil Microbes and Soil Enzymatic Activity to 20 Years of Fertilization

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Abstract: Fertilization is a worldwide agricultural practice used in agronomy to increase crop yields. Fertilizer application influences overall soil characteristics, including soil microbial community composition and metabolic processes mediated by microbial enzymatic activity. Changes in the structure of microbial communities and their metabolic activity after long-term fertilization were studied in this research. We hypothesized that the different types of fertilization regimes affect nutrient levels in the soil which subsequently influence the metabolic processes and microbial diversity and community structure. Manure (MF; 330 kg N/ha), sewage sludge at two application doses (SF; 330 kg N/ha and SF3x; 990 kg N/ha) and chemical (NPK; N-P-K nutrients in concentrations of 330-90-300 kg/ha) fertilizers have been applied regularly to an experimental field since 1996. The microbial diversity increased in all soils amended with both organic (MF, SF, SF3x) and chemical (NPK) fertilizers. The shifts in microbial communities were observed, which were mainly caused by less abundant genera that were mostly associated with one or more fertilization treatment(s). Fertilization also influenced soil chemistry and the activity of β -xylosidase, β -N-acetylglucosaminidase (NAG), acid phosphatase and FDA-hydrolases. Specifically, all fertilization treatments were associated with a higher activity of β xylosidase and lower NAG activity. Only the NPK treatment was associated with a higher activity of acid phosphatase.

Keywords: enzymatic activity; β-glucosidase; β-xylosidase; β-N-acetylglucosaminidase; acid phosphatase; fluorescein diacetate hydrolysis; microbial community structure

1. Introduction

High crop yield has always been the main goal of agriculture, hence it has also become the most important determinant of soil quality [1]. However, taking into account increased concerns about environmental safety, the concept of soil quality encompasses more factors nowadays. Plant, animal and human health, air and water sustainable quality have been included into the definition of high-quality soil [2]. Since fertilization, tillage and other typical agricultural practices manipulate soil properties, changes in soil quality need to be monitored [3]. Total organic carbon is the most frequently used indicator of soil quality; however, more sensitive and rapid indicators are needed to assess soil quality [4]. There is a long discussion about which particular biological parameters should



be determined for an efficient assessment of soil quality. Microbial diversity, composition of microbial communities and their enzymatic activity offer an effective and sensitive approach to monitoring soil quality, as they respond quickly to disturbances [2].

Soil is colonized by a wide range of diverse microbial populations. Their higher diversity is associated with better soil quality and stress resistance, since a broad spectrum of metabolic pathways is potentially employed [5]. The majority of the microbes live in the upper part of the soil profile, where the largest amount of organic deposits, air and water is available [6]. It was proved that any disturbances, including fertilizer application, crop rotation, tillage, or contamination, mainly influence this organic horizon and affect microbial communities therein [7–9]. As an example, fertilizer application leads to a cascade of several processes: the first alteration of physicochemical soil properties [10] is followed by changes in microbial diversity or/and microbial community structure [11], which is tightly associated with their metabolic activity and biological function in the soil [12]. An increasing number of studies have showed that the alteration of soil microbial communities has consequences on the soil ecosystem [13,14]. Interestingly, low-abundance species rather than the dominant taxa are the main drivers of soil multifunctionality [15]. For instance, *Desulfosporosinus* with its low abundance of 0.006% of the total prokaryotic community was detected as an important sulphate reducer in peatlands [16], and low-abundance (< 0.1% relative abundance) populations were the major drivers of the bacterial composition in the rhizosphere of 19 grassland plant species [17].

Enzymatic transformations play a crucial role in the nutrient cycling in soil. Soil enzymes catalyze the release of inorganic nutrients required for plant growth from organic matter [18] and may be of plant or animal origin, but the primary source are microorganisms [19]. Extracellular microbial enzymes are released into soil from either living or dead cells, and their activity is strongly associated with microbial biomass, community structure, substrate availability, the size of soil particles and environmental conditions [20,21].

While the microbial community structure reflects the functional potential of the soil microbes, their enzymatic activity may be a much more accurate reflection of the processes currently taking place in the soil. Hydrolytic enzymes decompose organic matter, and the rate of decomposition is likely to be influenced by agricultural management. Primary enzymes involved in the carbon cycle, such as β -glucosidase or β -xylosidase, are monitored due to their rapid response to changes in the management regime [22]. They exhibit higher activity with the application of sewage sludge and manure, due to their microbial growth-promoting content of cellulose, hemicellulose and lignin [23,24]. Other enzymes have also been reported to be influenced by fertilization, and are monitored in soils to determine the soil biological quality. β -N-acetylglucosaminidase (NAG), involved in the N cycle, catalyzes chitin breakdown to amino sugars and is a major source of mineralizable nitrogen in the soil [25]. NAG activity was reported to be higher after the application of sludge to soil, which was explained by an increased fungal biomass [26]. Hydrolases, such as lipases, proteases and esterases, are all able to hydrolyze fluorescein diacetate (FDA), which made the degradation of FDA a generally accepted method in environmental microbiology for measuring total microbial activity [27,28].

This study is a follow up to our previous work in which we described the response of endophytic microbial communities to the long-term fertilization of agricultural soils [29]. Since the community structure was significantly altered, in this study we focused on the metabolic processes happening in some of these fertilized soils. Samples for this study were collected a year and half after the previous study, hence the microbial communities were reanalyzed as well. The objective of this study was to align the changes in the microbial community structure that depended on the application of manure, sewage sludge or NPK with enzymatic activity in these fertilized soils. The activity of five enzymes: β -glucosidase, β -xylosidase, NAG, acid phosphatase and FDA hydrolases, was monitored to gain an insight into various pathways of degradation of organic matter in the soil. We hypothesized that organic amendments will be reflected predominantly by enzymes involved in the C-cycle, and the application of organic fertilizers will influence the microbial diversity and community structure partly because of a direct transmission of microbes to the soil. In contrast, NPK amendment was expected to

predominantly influence the activity of enzymes involved in the P and N-cycles and promote higher microbial diversity.

2. Materials and Methods

2.1. Experimental Design and Soil Sampling

The experimental field-plot is located in the Czech Republic (CZE; 49°33'16" N, 15°21'2" E) and has been regularly fertilized since 1996. Before this year, all field-plots were treated the same way. The experimental soil is characterized as cambisol, with a pH of 5.27 ± 0.5 , clay content of 5.84%, silt content of 43.55% and sand content of 50.61%. The experimental field-plot was fertilized with: (i) sewage sludge (330 kg N/ha, SF), (ii) sewage sludge (990 kg N/ha, SF3x), (iii) manure (330 kg N/ha, MF), (iv) NPK (N-P-K nutrients were 330-90-300 kg/ha, NPK). Non-fertilized soil was used as a control (CF). Potato (*Solanum tuberosum* L.), winter wheat (*Triticum aestivum* L.), and spring barley (*Hordeum vulgare* L.) respectively were planted at the field-plot in three-year rotations. An illustration of the experimental field plot is displayed in Figure S1. According to typical agronomical practice [30], sewage sludge and manure were applied once per three years before potato plowing, and NPK was applied regularly throughout the three-year rotation cycle. Therefore, the application rate of fertilizers was normalized based on the total nitrogen input over the whole three-year rotation period.

Ten months after the last fertilization, in September 2017 after potato harvesting, four bulk soil samples were taken from each fertilized variant (sub-plot, 60 m² each) from the 0- to 20-cm topsoil layer, homogenized, and three replicates were used for further analysis of microbial communities, enzymatic activities and physicochemical parameters.

2.2. Physicochemical Soil Analysis

Soil pH was determined in a 0.2 mol/L KCl 2:5 w/v solution according to the methodology of the Central Institute for Supervising and Testing in Agriculture, Czech Republic [31]. The total carbon and nitrogen in soils were determined by using a CHNS Vario MACRO cube (Elementar Analysensysteme GmbH, Langenselbold, Germany) analyzer equipped with a thermal conductivity detector. Inorganic–N forms (N-NH₄⁺ and N-NO₃⁻) were determined via a SKALAR San Plus System continuous-flow segmented analyzer (Skalar, Netherlands).

The Mehlich III extraction procedure was used to determine the available macro- and micronutrient contents in the soils as follows: 1 g of soil is extracted with 10 mL of the extraction mixture (0.2 mol/L of CH₃COOH + 0.25 mol/L of NH₄NO₃ + 0.013 mol/L of HNO₃ + 0.015 mol/L of NH₄F + 0.001 mol/L of EDTA) for 10 min [32]. Each extraction was done in three technical replicates, all the chemicals used were of analytical grade purity, and were purchased from Analytika and Lach-Ner (Neratovice, Czech Republic). For the centrifugation of the extracts, a Hettich Universal 30 RF (Tuttlingen, Germany) device was used. The reaction mixture was centrifuged at 3000 min⁻¹ (i.e., 460 g) for 10 min at the end of each extraction procedure, and the supernatants were kept at 6 °C prior to the measurement.

To determine the S, P, Zn, and Ca contents in the extracts, inductively coupled plasma-atomic emission spectrometry (ICP-OES) was used with an Agilent 720 (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a two-channel peristaltic pump, a Struman-Masters spray chamber, and a V-groove pneumatic nebulizer made of inert material (spectrometry parameters were: power: 1.2 kW; plasma flow: 15.0 L/min; auxiliary flow: 0.75 L/min; nebulizer flow: 0.9 L/min). Flame atomic absorption spectrometry (F-AAS, Varian 280FS, Varian, Australia; air flow of 13.5 L/min, acetylene flow of 2.2 L/min, burner height of 13.5 cm, nebulizer uptake rate of 5 mL/min) was used for K determination in the extracts.

2.3. Enzyme Assays

The activity of β -glucosidase, β -xylosidase, NAG, and acid phosphatase was measured fluorometrically using 4-methylumbelliferyl (MUB) substrates (Table 1, Sigma-Aldrich, St Louis, MO, USA). The total microbial activity was measured using fluorescein diacetate (FDA, Table 1, Sigma-Aldrich, St Louis, MO, USA). The procedure for measuring enzyme activities was adapted from [33]. Briefly, 2 grams of soil was mixed with 50 mL of acetate buffer (50 mM, pH 5) and placed on a horizontal shaker for 2 hours at 28 °C (180 rpm, incubated in dark). From each sample of soil-buffer slurry, three technical replicates (200 μ L) were transferred to a microtiter plate and mixed with 50 μ L of substrate. Seven concentrations of each substrate were always used: 2000, 1500, 1000, 500, 200, 100, and 10 µM. Slurry mixed with sterile distilled water and acetate buffer mixed with substrates (of each concentration) were used as negative controls. For better understanding, the reagent mixing procedure is summarized in Table 2. Then, the plate was shaken horizontally for 2 hours under the same conditions as previously. The reaction was stopped by adding 10 µL NaOH (1M) and incubated for 20 min. The fluorescence intensity was measured using Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA) with 360 nm excitation and 450 nm emission filters for MUB-linked substrates. When measuring FDA hydrolysis activity, the procedure was slightly different: (i) acetone was used as the solvent of the substrate, (ii) 2 grams of soil was mixed with phosphate buffer (100 mM, pH 5.8) and (iii) 485 nm excitation and 510 nm emission filters were used for fluorometric assays.

Table 1. MUB-linked substrates used for enzyme assays.

Substrate	Enzyme	Dissolvent
4-Methylumbelliferyl-β-D-glucopyranoside	β-glucosidase	water
4-Methylumbelliferyl-β-D-xylopyranoside	β-xylosidase	water
4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide	β-N-acetyl-hexosaminidase	water
Fluorescein diacetate	Total microbial activity	acetone
4-Methylumbelliferyl phosphate	Acid phosphatase	water

Table 2. Scheme of mixing procedure of soil-buffer slurry with substrate. The slurry of each soil replicate (A, B, C) was divided into three technical replicates (1, 2, and 3) and mixed with different concentrations of substrate.

		200 µl									
	Substrate	Technical Replicate 1			Technical Replicate 2			Technical Replicate 3			Acetate Buffer
50 μl	2000 µM	Α	В	С	Α	В	С	Α	В	С	
	1500 µM		Sample fluorescence							Negative control	
	1000 µM										of buffer
	500 µM										
	200 µM										
	100 µM										
	10 µM	Quench coefficient							Reference standard		
	Distilled Water			Neg	ative o	contro	l of sar	nple			Blank

Enzyme activities for each substrate concentration were calculated according to the equation adopted from [33], in which the weight of oven-dried soil determined after 24h at 105 °C was used. To compare the enzymatic activities of the five monitored enzymes across fertilization treatments, the calculated activities and substrate concentrations were inverted, and the Lineweaver-Burk reciprocal equation was used for the calculation of maximal velocity (V_{max}).

2.4. DNA Isolation and Molecular Analysis

Metagenomic DNA was isolated from soil samples using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) and purified with a Genomic DNA Clean and Concentrator kit (ZYMO Research, Irvine, CA, USA) according to the manufacturer's protocol. The concentration and purity of isolated DNA were measured in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The amplification of 16S rRNA genes was performed with two polymerase chain reactions (PCRs), the reaction mix and temperature program of which were adopted from [34]. Briefly, the 515 forward (5'-GTGYCAGCMGCNGCGG-3') and 926 reverse (5'-CCGYCAATTYMTTTRAGTTT-3') primers used were targeted to hypervariable regions V4-V5 of the 16S rRNA gene (adapted from [35]). In the first PCR, the 15 µL reactions contained: 0.02 U/µL KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), 0.3 µM of each primer (Sigma-Aldrich, St Louis, MO, USA), template DNA $(\sim 10 \text{ ng/}\mu\text{L})$ and water for molecular biology (Sigma-Aldrich, St Louis, MO, USA). The primers for the second PCR were modified with adaptors and internal barcodes [34] and were used at a $1-\mu M$ concentration. As the DNA template, 0.5 µL of the previous PCR product was used and together with KAPA HiFi HotStart ReadyMix (0.02 U/ μ L) and water for molecular biology was added into the 25 μ L final volume. The temperature program for the first PCR was set as follows: 5 min at 95 °C, 20 s at 98 °C, 28–30 cycles of 15 s at 56 °C, 15 s at 72 °C and a final extension of 5 min at 72 °C. The temperature program for the second PCR was the same with two modifications: (i) the annealing temperature was 50 °C, and (ii) 8–10 cycles were used for the amplification. The final PCR amplicons were purified with SPRIselect magnetic beads (Beckman Coulter, Miami, FL, USA) and sent in ice packs to the Core Facility for Nucleic Acid Analysis at the University of Alaska Fairbanks for Illumina Miseq platform sequencing, where the DNA concentration was normalized to $1-2 \text{ ng/}\mu\text{L}$ using a SequalPrep Kit (Thermo Fisher Scientific, Waltham, MA, USA) before the sequencing process.

Along with the amplicons of soil samples, amplicons of mock communities (artificial community of 12 bacterial strains) were also prepared under the same conditions to set the proper parameters for the subsequent sequence data processing. The mock communities were laboratory prepared according to [36].

2.5. Data Processing and Multivariate Statistical Analyses

In the program R [37], raw sequencing data were processed through a pipeline of DADA2 [38], which was modified based on the obtained sequences of the mock community. The sequences were filtered according to automatically calculated quality parameters (using *calculateTrunc* function), and the primers were trimmed off. After the dereplication step, amplicon sequence variants (ASVs) were inferred from input unique sequences after removing sequencing errors. The forward and reverse denoised reads were merged, and chimeric sequences were removed from the constructed sequence table. Sequences differing by one base were merged, and the more abundant one was kept as valid. The taxonomy was assigned to the average sequence variants (ASVs) using rdp_species_assignment_16 [39]. All sequence data were deposited into the NCBI Short Read Archive under the accession number PRJNA644622.

Further statistical analyses were performed in R, using the phyloseq [40] and vegan [41] packages. ASVs with no assigned taxonomy at the phylum level were removed (4% of all reads), and the dataset was rarefied to the smallest integer (2900). The broadly used alfa-diversity indices (Shannon and Simpson) were calculated, and the differences in the indices between organically (MF, SF, SF3x treatments together), chemically (NPK) and non-treated (CF) soil samples were tested using analysis of variance (ANOVA) and Tukey's HSD test. The ASVs community data were Hellinger transformed. The influence of fertilization treatments on microbial community structure was evaluated with permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distance. The association of prokaryotic communities clustered at the genus level with the fertilization regime was analyzed using canonical correspondence analysis (CCA) and an ANOVA-like permutation test based on CCA. The adjusted R^2 (R^2_{adi}) of explained variation was calculated with the *RsquareAdj*

function (*vegan* package). CCA ordinations with displayed samples were used for the visualization of differences between prokaryotic communities in treated soil. CCA ordination with displayed taxa was used to map the genera abundance profile across the treatments, and the genera abundance was divided into four levels based on the quartiles. To identify genera with significantly different abundance in fertilized sub-plots compared to control soils (CF), differential analysis was conducted using the DESeq2 package [42]. To determine statistical significance, the fold change threshold was set to 1.2 and the false discovery rate cutoff was set to 0.1.

Differences in the V_{max} and soil physicochemical parameters between fertilization regimes were tested using multiple ANOVAs, and the calculated *p*-values were adjusted with false discovery rate (*FDR*) correction [43]. The significantly influenced V_{max} or physicochemical parameters were then applied to Tukey's HSD test for multiple pairwise comparisons, and the results were visualized with a boxplot in which significant differences between two bars were denoted with letters. To determine the size of the effect of the fertilization treatment on V_{max} , an ANOVA-like permutation test for redundancy analysis was conducted and the obtained R^2 was adjusted with an *RsquareAdj* function (*vegan* package).

3. Results

3.1. Response of Soil Microorganisms to Fertilization

The influence of fertilization on soil microbial diversity was assessed by Shannon and Simpson diversity indices. The diversity of prokaryotes in soil was significantly higher after the application of organic (MF, SF and SF3x; p_{adj} -value ≤ 0.05) and chemical (NPK; p_{adj} -value ≤ 0.1) fertilizers compared to CF. No statistical difference was observed between the microbial diversity of organically and chemically treated soils.

The microbial community structure was significantly associated with the fertilization treatment (PERMANOVA, *p*-value ≤ 0.01). CCA ordination with displayed samples (Figure 1a) shows a clear separation of prokaryotic community structure based on different fertilization treatments (*p*-value ≤ 0.001). The communities in SF and SF3x-treated soils were the most similar to each another. Based on the R^2_{adj} , the application of fertilizers contributed to 15.7% of the variance in the prokaryotic community.



Figure 1. CCA ordinations of Hellinger-transformed sequence data of prokaryotic communities under different fertilization treatments: control (CF), manure (MF, 330 kg N/ha), NPK (NPK, 330-90-330 kg/ha), sewage sludge (SF, 330 kg N/ha), sewage sludge (SF3x, 990 kg N/ha). Ordination (**a**) shows sample separation in three-dimensional ordination space. Ordination (**b**) was constructed with the first two CCA axes, and shows bacterial genera divided into three groups according to their abundance.

CCA ordination in Figure 1b shows the distribution of genera and their abundance in the ordination space. The abundance was divided into four levels based on the quartiles—Q3 was 78.25, Q2 (median) was 17, and Q1 was 6.25. Together with the quartiles, the maximum (3201) and minimum number of reads (1) show an uneven abundance of prokaryotic genera. The genera with higher abundance (from 78.25 to 3,201) centered in the ordination space, while genera with low abundance (< 6.25) were mostly specific for one of the treatments or their combination.

Differential analysis was used to identify the genera with significantly (p_{adj} -value ≤ 0.1) different relative abundance between control (CF) and fertilized soils (Figure 2). Most of the identified genera had significantly higher relative abundance in SF and SF3x- treated soils compared to CF. In contrast to SF treatment, in which the relative abundance of *Pseudoxanthomonas*, *Rhodanobacter*, *Solirubrobacter* and *Nitrosospira* was significantly higher, the application of SF3x was also associated with a higher relative abundance of *Coprothremobacter*, *Romboutsia*, *Opitutus*, *Porphyrobacter*, *Chitinophaga*, *Actinoallomurus* and *Ktedonobacter*. Additionally, the relative abundance of *Solirubrobacter* was also higher in MF-treated soil, and the higher relative abundance of *Rhodanobacter* and *Porphyrobacter*, over 20-fold higher, was also observed with the NPK treatment.



Figure 2. Pairwise comparison (DeSeq analysis) showing prokaryotic genera with significantly $(p_{adj}$ -value ≤ 0.1) different relative abundance in fertilizer-treated soils compared to CF. Fertilizers used: control (CF), manure (MF, 330 kg N/ha), NPK (NPK, 330-90-330 kg/ha), sewage sludge (SF, 330 kg N/ha), NPK (NPK, 330-90-330 kg/ha), sewage sludge (SF3x, 990 kg N/ha). Negative "log2 Fold Change" values (x-axis) indicate higher abundance of genera in fertilized soils, and positive values indicate higher abundance in CF.

3.2. Enzymatic Activity in Fertilized Soil

The V_{max} values (Table S1) of β -xylosidase, NAG, acid phosphatase and FDA-hydrolases were significantly associated with fertilization treatments (ANOVA, p_{adj} -value ≤ 0.01), while the V_{max} value of β -glucosidase was not. NPK treatment was the only treatment for which the V_{max} was significantly altered compared to CF (Tukey's HSD test, p_{adj} -value ≤ 0.05) in all enzymes (Figure 3); specifically, the V_{max} of β -xylosidase and acid phosphatase was significantly higher, while those of NAG and FDA-hydrolases were significantly lower. Furthermore, the V_{max} of FDA-hydrolyses was significantly lower for both sewage sludge (SF, SF3x) treatments, the V_{max} of β -xylosidase was higher for all fertilized variants, and the V_{max} of NAG was lower for all fertilized variants compared to CF. To quantify the size of the effect of fertilization on V_{max} data variability, an ANOVA-like permutation test for RDA analysis was performed. Based on R^2_{adj} , fertilization explained 66% of V_{max} data variability.



Figure 3. V_{max} of monitored enzymes in soils amended with: (i) sewage sludge (SF; 330 kg N/ha), (ii) sewage sludge (SF3x; 990 kg N/ha), (iii) manure (MF; 330 kg N/ha), (iv) NPK (NPK; N-P-K nutrients were 330-90-300 kg/ha). Untreated soil was taken as a control (CF). Different letters indicate significant differences between treatments (*p*-value ≤ 0.05) and were assigned according to conducted Tukey HSD post hoc tests.

3.3. Physicochemical Properties of Soils

The monitored chemical parameters (Ca, S, Zn, total N, P, K, NO₃⁻, NH₄⁺) were found to be significantly associated with fertilization treatments (ANOVA, p_{adj} -value ≤ 0.05), and mostly differed in SF3x and MF treatments (Figure 4) (Tukey's HSD tests, p_{adj} -value ≤ 0.05). The levels of N, K, Zn, P, NO₃⁻ and NH₄⁺ significantly differed in fertilized soils compared to CF, while the levels of Ca and S differed only between NPK and SF3x (Figure 4). The concentration of the soil chemical parameters was the highest in SF3x except for K, the concentration of which was the highest in the MF treatment. The concentration of monitored chemical parameters is summarized in Table S2.



Figure 4. Concentration of N, P, K, S and Zn in soils amended with fertilizers: (i) sewage sludge (SF; 330 kg N/ha), (ii) sewage sludge (SF3x; 990 kg N/ha), (iii) manure (MF; 330 kg N/ha), (iv) NPK (NPK; N-P-K nutrients were 330-90-300 kg/ha). Untreated soil was taken as a control (CF). Different letters indicate significant differences between treatments (*p*-value ≤ 0.05).

4. Discussion

In this study, the overall examination of the response of the soil microbial community and their enzymatic activity to 20 years of periodical fertilizer application was evaluated. The long-term application of both organic (MF, SF, SF3x) and chemical (NPK) fertilizers led to a significant increase in prokaryotic diversity in soil compared to the CF, which is in agreement with other studies showing a significant influence of fertilization on soil microbial diversity [44–46]. Although the majority of the studies revealed an increased microbial diversity, especially after organic fertilization [46,47], there is also some evidence of a decline, probably due to the introduction of substances directly reducing richness and evenness, or altering pH and consequently reducing the diversity [44,48,49]. Higher microbial diversity generally promotes the decomposition of organic carbon [50]. This would be in agreement with the higher activity of β -xylosidase in all fertilized treatments (Figure 3), implying enhanced hydrolysis of xylan, which is a group of hemicelluloses and a very abundant biopolymer in nature. Simple sugars released by the activity of β -xylosidase are further used by soil microorganisms as a source of carbon [51]. Interestingly, the activity of β -glucosidase, which is usually referred to as the most sensitive indicator of soil quality and which has been reported to increase with both organic [52,53] and chemical [54] fertilizers, was not found to be affected by the fertilization treatment in this study.

Overall, the fertilization regime explained 66% of the variability in enzymatic data, showing the enzymes to have a higher sensitivity to soil disturbances than the microbial community structure, for which fertilization only explained 15.7% of its variability. In addition to the activity of β -xylosidase, the activity of NAG, acid phosphatase and FDA-hydrolases was significantly associated with fertilization regimes. The activity of NAG was significantly lower for all fertilization treatments compared to CF, with the lowest rates in SF and SF3x treatments. NAG was previously found to play an important role in nitrogen mineralization, and was suggested to be used as an index of the amount of mineralizable nitrogen in soil [55]; however, the NAG activity in our soils was not associated with the amount of total N, NO₃⁻ or NH₄⁺. In fact, although the regular fertilization led to significantly higher total nitrogen levels in SF-treated soil and significantly higher levels of NO₃⁻ and NH₄⁺ for the SF, SF3x and NPK treatments (Figure 4), the activity of NAG was significantly lower for these treatments than for CF. This inverse correlation in the presence of high levels of NO₃⁻ and NH₄⁺ was previously explained by the repression of the production of NAG enzyme by inorganic nitrogen [56], which might have taken place in our soils.

As the NPK application is supposed to increase the inorganic phosphorus concentration in soil, it is not surprising that the highest activity of acid phosphatase was detected in NPK-treated soil. Acid phosphatase is considered to be an index of soil quality; its higher activity is attributed to the stimulation of microbial growth, organic matter enrichment and improvement of the P cycle in soil [18]. From this point of view, the NPK treatment seems to stimulate the microbial activity in NPK-treated soils in order to hydrolyze organic phosphorus compounds followed by the release of inorganic P.

Sewage sludge and manure application had a lower influence on metabolic processes in the soil compared to NPK in terms of the number of enzymes with influenced activity (V_{max}), even though the application of organic fertilizers was associated with significantly higher levels of several soil chemical parameters. Specifically, the concentration of N, P, S, Zn, Ca, nitrate and ammonia was the highest in SF3x, while the K concentration was the highest in MF treatment. However, the soils in this experiment were sampled after the harvest of potatoes, and a single dose of organic fertilizers was applied before potato planting, whereas NPK was regularly applied every year. Thus, a decrease in the element contents in soils treated with organic fertilizers can be anticipated in the next two years, as observed in previous study [57] for sulfur. However, the FDA hydrolysis, which is performed by various hydrolytic enzymes [19], was significantly lower in all fertilization treatments, indicating a lower total soil microbial activity after fertilizer application. This finding is in contrast with our hypothesis that expected an increase in total microbial activity via fertilization, and also in contrast with other studies [58,59]. However, FDA hydrolysis decreases with time [60], suggesting that there

might have been increased FDA hydrolysis immediately after the fertilizer application, but it lowered over time.

Not only the diversity and enzymatic activity, but also the microbial community structure was significantly associated with fertilization treatments (Figure 1a). Taking into account the abundance profile of the genera (Figure 1b), it seems that the community shifts were more to do with the less abundant genera. The most abundant genera, which centered in the ordination space, indicating that they were similarly distributed across all the treatments, generally have a low influence on soil functioning and the ability to reflect soil disturbances [15]. The association of rare or low-abundance species with one of the fertilization treatments or combination of multiple treatments (Figure 1b) can be explained by several theories, such as a lack of competitiveness with other microbes, waiting for more favorable conditions, or a higher sensitivity to environmental fluctuations, but their important role in biogeochemical processes has been described [61]. While the majority of the community shared similar functions, rare or low abundant species are mainly associated with specific environmental disturbances and provide new or desired biogeochemical traits due to their ability to utilize a limited source of nutrients [61–63].

Most of the differently abundant genera had higher relative abundance in fertilized soils (Figure 2). *Opitutus, Chitinophaga* and *Solirubrobacter* had higher relative abundance in organically treated soils (MF, SF and/or SF3x), indicating that their presence might be associated with the higher organic and nutrient content in soil, which is in accordance with [64]. Furthermore, *Solirubrobacter* was primarily found to be enriched in healthy soils [65]. Other genera which were enriched in SF and SF3x-treated soils *Romboutsia, Rhodanobacter, Pseudoxanthomonas, Opitutus* and *Nitrosospira* were previously detected in sewage sludge [66–69], suggesting their possible direct transmission from the fertilizer. *Coprothermobacter*, the genus which was also enriched in SF3x, is associated with digestion processes in organic wastes treated at high temperatures [70,71]. The significantly increased relative abundance of ammonium-oxidizing *Nitrosospira* in SF3x-treated soil was in accordance with the significantly higher levels of ammonium (Figure 4), which is oxidized to nitrite by *Nitrosospira* activity [72]. The significant enrichment of such bacteria can indicate that the soil microbiota composition was altered in order to utilize the new input of different organic compounds that were introduced into the soil with the fertilizer application [73,74].

In summary, our data show that fertilization influenced the nutrient status of soil, which was clearly reflected by changes in microbial communities and their enzymatic activities. Both organic and chemical fertilizers promoted the decomposition of organic matter. Our further studies will aim to uncover the microbial community succession over time in these fertilized soils, and the possible transmission of antibiotic resistance genes via organic fertilization. Thus, we will obtain a comprehensive picture of this management practice, which could be beneficial for further decision-making strategies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1542/s1. Table S1 The V_{max} values of β -glucosidase (GLU), β -xylosidase (XYL), β -N-acetylglucosaminidase (NAG), acid phosphatase (PHO) and FDA-hydrolases (FDA) under different fertilization treatments: control (CF), manure (MF, 330 kg N/ha), NPK (NPK, 330-90-330 kg/ha), sewage sludge (SF, 330 kg N/ha), sewage sludge (SF3x, 990 kg N/ha). Table S2 Physicochemical parameters of soils under different fertilization treatments: control (CF), manure (MF, 330 kg N/ha), NPK (NPK, 330-90-330 kg/ha), sewage sludge (SF, 330 kg N/ha), sewage sludge (SF3x, 990 kg N/ha).

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