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Impacts of Cropping Systems on Glyphosate and Aminomethylphosphonic Acid Contents and Microbial Community in Field Crop Soils in Quebec (Canada)

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Abstract: Glyphosate-based herbicide (GBH) usage is ubiquitous in Quebec field crops, apart from organic management. As glyphosate generally degrades rapidly in agricultural soils, aminomethylphosphonic acid (AMPA) is produced and persists longer than glyphosate. Repeated GBH applications year after year raise questions about glyphosate and AMPA pseudo-persistence in soils and its possible impacts on the soil microbial community. This research aims at understanding the influence of cropping systems and edaphic properties on glyphosate and AMPA contents and on the diversity and composition of the soil microbial community across nine field crop fields located in Southern Quebec (Canada) during 2019 and 2020. Average glyphosate soil contents (0.16 ± 0.15 $\mu g \cdot g^{-1}$ dry soil) were lower than average AMPA soil contents (0.37 ± 0.24 $\mu g \cdot g^{-1}$ dry soil). Glyphosate and AMPA contents were significantly lower at sites cultivated under organic management than conventional management. For conventional sites, cumulative GBH doses had a significant effect on glyphosate soil contents measured at the end of the growing season, but not on AMPA soil contents. Sites with higher GBH applications appear to accumulate glyphosate over time in the 0–40 cm soil horizon. Glyphosate and AMPA soil contents are inversely proportional to soil pH. Soil prokaryotic and fungal communities' alpha-diversity, beta-diversity, and functional potential were not impacted by cumulative GBH doses, but rather by soil chemical properties, soil texture, crop rotation, and manure inputs.

Keywords: glyphosate pseudo-persistence; AMPA; high-throughput sequencing; glyphosate-based herbicides; soil microbial community

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1. Introduction

The commercialization of glyphosate-resistant crops in the mid-1990s resulted in a major increase in the use of glyphosate-based herbicide (GBH) [1]. GBH can be applied as pre- and post-emergence applications on glyphosate-resistant crops [2]. In 2021, GBH was the most used herbicide representing close to 50% of all herbicides sold in Quebec, Canada [3]. Glyphosate has an affinity to be adsorbed to soil particles, although it may be transported to lower soil profiles depending on weather conditions and precipitation following GBH application [4,5]. Glyphosate adsorption ability on soil particles is influenced by cation exchange capacity (CEC), soil texture, P and Al contents, soil organic carbon quantity, and pH [6–8]. Spayed glyphosate molecules that do not adsorb on plant tissues or soil particles will migrate within soil interstitial waters, where glyphosate is prone to degradation [4]. Glyphosate degradation is mostly biological, although abiotic pathways such as photolysis and thermolysis can degrade glyphosate, producing a variety of metabolites, including aminomethylphosphonic acid (AMPA) [9,10]. Glyphosate biodegradation involves three

major metabolites, AMPA, sarcosine, and acetyl-glyphosate [11]. The pathway that produces AMPA is generally favored over the two other pathways in soils, with about 90% of glyphosate metabolites being AMPA molecules [4]. Glyphosate half-life is reported to range between 1 and 197 days, while AMPA half-life is between 23 and 958 days in temperate agricultural soils [12]. Once in the soil, AMPA is more resistant to biodegradation and has a higher affinity to soil particles compared to glyphosate, which increases its half-life in the soil [13,14]. Nonetheless, both molecules have similar physico-chemical properties with high water solubility, low lipophilicity, and very low volatilization potential [15,16]. The main difference between both molecules is their molecular weight (169.07 g·mol^{-1} for glyphosate and 111·04 g.mol^{-1} for AMPA) [17].

Conservation agriculture is gaining popularity among farmers and crop advisors to maintain high crop productivity while maintaining soil-beneficial microbial community and ecosystemic functions, reducing GHG, and increasing carbon sequestration and climate change resilience [18]. According to the Food and Agriculture Organization: "Conservation Agriculture is a farming system that promotes minimum soil disturbance (i.e., no tillage), maintenance of a permanent soil cover, and diversification of plant species. It enhances biodiversity and natural biological processes above and below the ground surface, contributing to increased water and nutrient use efficiency and to improved and sustained crop production" [19]. Quebec's field crop production, such as grain corn, usually involves either mechanical or chemical weeding strategies [20]. For producers adopting Conservation Agriculture principles such as no-till and winter cover crops, mechanical weed control is more restrictive or not recommended, resulting in a dependency on higher herbicide application doses to control problematic weeds such as perennial ones [21]. The use of higher GBH doses could be of concern for potential glyphosate and AMPA accumulation in soils or dissipation into waterways. This is of high relevance as glyphosate and AMPA were, respectively, detected in 98.9% and 93.3% of water samples from agricultural streams in Quebec between 2018 and 2020 [22]. Glyphosate and AMPA were detected in 42% and 70%, respectively, of 45 Québec agricultural soils in 2014 [23].

High-throughput sequencing (HTS), also known as next-generation sequencing, has revolutionized the study of soil microbial communities. Compared to techniques such as culture-based isolation, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, single-strand conformation polymorphism, and DNA amplification fingerprinting, HTS is high-throughput, less expensive, and less labor-intensive [24]. With HTS, taxonomic identification is performed based on databases for specific portions of genes, for instance, the V4 region of the *rRNA 16S* [25], *ITS1* [26], and *rRNA 18S* [27] for prokaryotic, fungal, and eukaryotic communities, respectively. Additionally, quantitative polymerase chain reaction (qPCR) of specific regions of the rRNA genes could be used to estimate microbial biomass, while qPCR of specific microbial genes could also be used to quantify biological pathways such as carbon fixation [28], nitrification [29] or pesticide degradation [30], which can be labor-intensive when analyzing multiple genes. The advent of databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) allows for the direct attribution of functional potential to microbial communities identified through HTS [31]. Nonetheless, HTS has limitations, based on the quality of the database used for taxonomic and functional potential identifications. For instance, fungal taxonomic identification is limited due to intraspecific variability in *ITS1* sequences [32].

Previous investigations of the soil microbial community following GBH application showed no effect on alpha-diversity or on overall composition [33–36]. On the other hand, cropping techniques used in conservation agriculture, such as reduced tillage or cover crop utilization, are known to significantly increase soil microbial alpha-diversity and have a profound effect on microbial community composition [37–39]. The interacting effects of GBH applications and cropping system strategies on soil microbial communities have received little attention so far [33,36]. Studies have so far focused on differences in previous years' GBH applications [36] or differences in tillage regime (no-till vs. chisel tillage) [33]; however, these studies used experimental plots with controlled cropping systems, focusing

on one parameter at a time. To our knowledge, there is no investigation in commercial producers' fields where these factors (herbicide application history, tillage regime, cover crop utilization) all vary between producers.

To have a better understanding of the interacting effects of management variables that define cropping systems such as crop rotations, manure applications, tillage, and GBH application doses on glyphosate and AMPA contents and on the diversity of microbial communities in soils, we sampled soils on nine field crop fields during two consecutive years. We hypothesized that glyphosate and AMPA contents would be linked to GBH doses applied over the previous years and to soil properties such as CEC, pH, and phosphorus content. Additionally, we hypothesized that the diversity (richness and composition) and functional diversity of the soil microbial community would be influenced by the combination of GBH applications, soil properties, and cropping system management practices.

2. Materials and Methods

2.1. Sites selection and Description

Nine fields with a rotation, including corn in 2019 and soybean in 2020, were selected in Southern Quebec (Canada). The Montérégie-Est and Centre-du-Québec regions represent, respectively, 62% and 15% of field crop areas in the province (Figure 1). Two fields (sites H and I) are managed by the same producer. Field selection aimed at establishing a gradient of cropping systems ranging from organic systems with no GBH application and conventional tillage to no-till systems with variable doses of GBH applied.

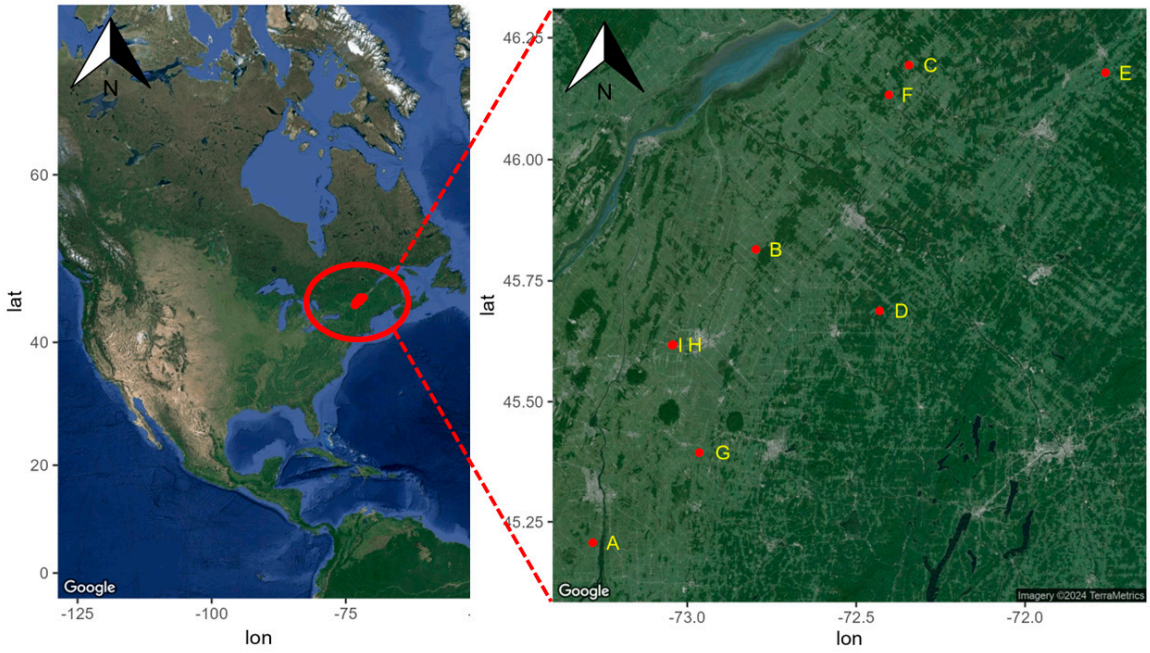

Figure 1. Location of the study sites (n = 9) in the province of Quebec, Canada. A red circle and a yellow letter denote each site.

Following site selection, a survey was sent to the farmers owning the nine sites. The survey included questions about soil tillage frequency and intensity, organic and mineral fertilization strategy, pesticide applications, seeding and harvest of field crops' information, and cover crop usage. From the information collected in the surveys, the nine sites were divided into different groups based on crop rotation used, frequency of manure application, tillage intensity, and cumulative amounts of GBH applied over the past 4 years (Figure 2). Crop rotations were divided into four categories; two rotations were corn alternated with soybean, one under organic management and the other under conventional management. Another rotation included corn and soybean as main crops, along with cereal rye as a cover crop between harvest and seeding. The fourth rotation included corn, soybean,

and wheat as main crops; a cover crop mixture was sown after wheat harvest. Manure frequency was based on the applications made between 2017 and 2020. Two applications or more were categorized as frequent, one as infrequent, and no application as never. For GBH applications, the number of grams of glyphosate sprayed per hectare between 2017 and 2020 was compiled (Table 1) and classified into two groups (>5400 g·ha^{-1} and <5400 g·ha^{-1}). Table 2 shows key physico-chemical properties from the nine sites.

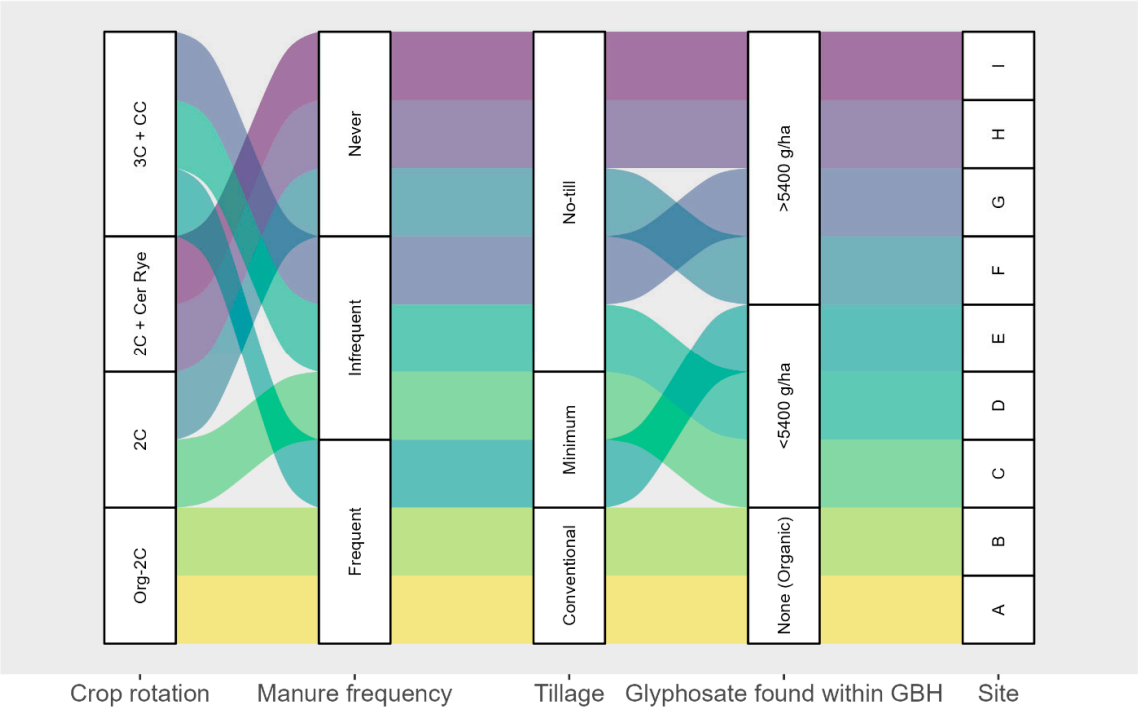

Figure 2. Classification of the nine sites based on crop rotation, frequency of manure application, soil tillage and cumulative GBH application between 2017 and 2020. Acronyms: Org-2c: organic system with maize and soybean (M-S); 2C: conventional system M-S; 3C + CC: conventional system M-S-Wheat and cover crops (cereal, radish, peas) after wheat harvest; 2C + Cer Rye: conventional system with M-S with cover crop (cereal rye) after every crop harvest.

Table 1. Information on GBH applications at the nine sites.

Sites	GBH Group: Cumulative Applications 2017–2020 (g·ha^{-1})	GBH 2019 Application (g·ha^{-1})	Cumulative GBH Applications 2017–2019 (g·ha^{-1})	GBH 2020 Application (g·ha^{-1})	Cumulative GBH Applications 2018–2020 (g·ha^{-1})
A	None	0	0	0	0
B	None	0	0	0	0
C	<5400	432	793.8	1350	2144
D	<5400	378	1998	1836	2916
E	<5400	999	2673	2160	3159
F	>5400	1080	4590	810	2430
G	>5400	1950	5010	1500	6510
H	>5400	2700	7182	2430	6912
I	>5400	2700	8100	2430	7830

Table 2. Physico-chemical properties of each site for the 0–20 cm soil profile. Mean values are shown for 2019 and 2020 samples. *p*-values comes from an analysis of variance for each variable; letters come from a post-hoc pair-wise comparison using Tukey adjustment.

Sites	Sand %	Silt %	Clay %	OM %	pH	ISP (%)	CEC (meq/100 g)	Na (ppm)
A	3.50 ± 2.67	50.25 ± 2.31 ^{ab}	46.25 ± 2.31	4.72 ± 0.45 ^{ab}	6.71 ± 0.11 ^{a}	4.10 ± 0.94 ^{c}	18.94 ± 1.24 ^{a}	23.74 ± 3.92 ^{a}

Table 2. *Cont.*

Sites	Sand %	Silt %	Clay %	OM %	pH	ISP (%)	CEC (meq/100 g)	Na (ppm)
B	36.25 ± 9.08	20.75 ± 4.3 ^{ab}	43.00 ± 6.8	4.68 ± 0.58 ^{ab}	6.50 ± 0.16 ^{ab}	6.46 ± 2.48 ^{c}	15.63 ± 1.84 ^{b}	26.28 ± 9.97 ^{a}
C	69.75 ± 6.73	18.50 ± 4.69 ^{b}	11.75 ± 2.05	3.45 ± 0.62 ^{cd}	6.19 ± 0.19 ^{bc}	20.62 ± 3.69 ^{a}	8.99 ± 1.30 ^{d}	3.96 ± 0.9 ^{c}
D	63.00 ± 6.96	23.75 ± 4.56 ^{ab}	13.25 ± 3.58	5.02 ± 0.73 ^{a}	6.50 ± 0.35 ^{ab}	14.39 ± 6.04 ^{b}	10.13 ± 1.57 ^{d}	4.37 ± 1.02 ^{c}
E	57.75 ± 2.87	29.75 ± 3.73 ^{a}	12.50 ± 0.93	4.81 ± 0.31 ^{a}	5.99 ± 0.33 ^{c}	5.30 ± 1.49 ^{c}	12.19 ± 0.90 ^{c}	9.20 ± 2.48 ^{bc}
F	42.25 ± 6.61	38.50 ± 5.26 ^{ab}	19.25 ± 2.19	3.96 ± 0.28 ^{bc}	6.75 ± 0.44 ^{a}	7.48 ± 2.63 ^{c}	9.67 ± 1.50 ^{d}	4.39 ± 0.63 ^{c}
G	3.25 ± 2.19	45.50 ± 0.53 ^{a}	51.25 ± 2.06	4.30 ± 0.22 ^{ab}	6.87 ± 0.24 ^{a}	5.04 ± 1.46 ^{c}	18.12 ± 1.39 ^{a}	14.44 ± 2.22 ^{b}
H	33.75 ± 9.77	36.25 ± 4.56 ^{ab}	36.50 ± 3.42	4.58 ± 0.51 ^{ab}	6.23 ± 0.24 ^{bc}	4.90 ± 1.00 ^{c}	14.75 ± 1.61 ^{bc}	11.45 ± 1.63 ^{b}
I	40.25 ± 5.63	28.25 ± 3.81 ^{ab}	31.50 ± 4.04	2.97 ± 0.26 ^{d}	6.53 ± 0.13 ^{ab}	7.60 ± 2.73 ^{c}	9.58 ± 0.69 ^{d}	10.08 ± 1.28 ^{bc}
p-value	0.313	0.01	0.162	<0.01	<0.01	<0.01	<0.01	<0.01

2.2. Soil Sampling

During September 2019 and 2020, each site was sampled at the same four georeferenced locations. These locations represented the extremity of a square, with its side measuring 50 m. At each location, soil cores were collected at two depths (0–20 cm and 20–40 cm) using an 8 cm diameter manual soil corer. Each sample consisted of a composite of four soil cores (total of ±500 g) collected at the extremity of a square with a length of one meter per side. Following sampling, all soils were homogenized, immediately put on ice, and transferred to −20 °C until further processing.

2.3. Analysis of Glyphosate and AMPA Contents

Glyphosate and AMPA extractions were performed according to the approach described by Samson-Brais, et al. [40]. Soils were freeze-dried and crushed using a pillar and mortar. Five grams of soil passed through a 2-mm sieve were mixed with 40 mL of an extraction solution composed of 34.5 mL of NH_4OH (28–30%) (Fisher Scientific, Ottawa, ON, Canada) with 13.6 g of KH_2PO_4 (Fisher Scientific, Ottawa, ON, Canada) in a total volume of 1 L [41]. The solution was mixed on a rotating wheel at 300 rpm for 45 min followed by centrifugation at 3500 rpm for 20 min. Then, 40 µL of the supernatant was transferred and evaporated to dryness under nitrogen flow. Samples were then derivatized using 1 mL of trifluoroacetic anhydride (TFAA) (Sigma-Aldrich, Saint-Louis, MO, USA) and 500 µL of trifluoroethanol (TFE) (Sigma-Aldrich, Saint-Louis, MO, USA), followed by heating for one hour at 100 °C. After heating, samples were cooled to room temperature, evaporated to dryness under nitrogen flow and then resuspended with 1 mL of ethyl acetate before injection (0.5 µL). A Varian CP 3800 gas chromatograph coupled with an electron capture detector and equipped with a Rxi-5Sil MS column (Restek, Bellefonte, PA, USA) (30 m × 0.25 mm × 0.25 µm) was used for glyphosate and AMPA quantification. The injector and detector were held at 280 °C and 300 °C, respectively. Hydrogen was used as the carrier gas with a column flow of 1.4 mL·min^{-1}. The oven temperature program began at an initial temperature of 70 °C, which was held for 1 min, followed by a 1 °C·min^{-1} increase up to 84 °C, followed by a 4 °C·min^{-1} increase up to 120 °C, and then followed by an 80 °C·min^{-1} increase up to 250 °C held for 7 min, for a total run time of 32.63 min.

The calculated limits of detection (LOD) and quantification (LOQ) are, respectively, 0.03 and 0.09 µg·g^{-1} dry soil for AMPA and 0.02 and 0.05 µg·g^{-1} dry soil for glyphosate [23]. Samples with values lower than the LOD are adjusted to 0.02 µg·g^{-1} dry soil for AMPA and 0.01 µg·g^{-1} dry soil for glyphosate, while samples with values between the LOD and the LOQ are adjusted to 0.06 µg·g^{-1} dry soil for AMPA and 0.03 µg·g^{-1} dry soil for glyphosate. To quantify glyphosate and AMPA, a calibration curve was made with a sample matrix including a blank and five different standards for each batch of samples (0, 0.1, 0.2, 0.3, 0.4, and 0.6 µg·g^{-1}, and 0, 0.2, 0.4, 0.6, 0.8, and 1.2 µg·g^{-1} for glyphosate and AMPA, respectively). Calibration curves showed good linearity ($r^2 > 0.95$; $p < 0.0001$) within the expected concentration range.

2.4. Soil Physico-Chemical Properties Analysis

Physical (granulometry, texture) and chemical (total C and N, OM, pH, CEC, Mehlich-3 elements) properties (Table 2) were all determined at the IRDA's laboratory of agroenviron-

mental analysis. The soil moisture content was determined by oven-drying at 105 °C for 24 h. Composite soil samples were air-dried, homogenized, and sieved to <2 mm. Total C and total N were determined on finely ground (100 mesh) subsamples by dry combustion using a LECO-CN828 analyzer. The percentage of organic matter was determined by loss ignition at 375 °C [42]. Soil pH was determined using a soil:water 1:1 suspension [43]. A similar procedure was used with a soil:SMP solution 1:1 suspension to determine soil buffer pH [44]. The concentrations of P, K, Ca, Mg, Al, B, Cu, Fe, Mn, Zn, and Na were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer Optima 4300DV, Shelton, CT, USA) after Mehlich 3 extraction [45]. The CEC was obtained by calculating base and acid cations using extractable Mehlich-3 K, Ca, Mg, and Na results and soil buffer pH [46]. The phosphorus saturation ratio (ISP) was calculated by dividing extractable Mehlich-3 P by extractable Mehlich-3 Al [47].

2.5. DNA Extraction, Sequencing and Quantitative PCR of Bacterial and Fungal DNA

DNA extractions were performed using a FastDNA Spin Kit for Soil (MPBio, Irvine, CA, USA). Soil samples were added to tubes containing 1 mL of the lysis buffer and 1.4 g of the bead matrix E supplied with the kit. DNA extraction was performed according to the manufacturer's instructions. The resulting DNA pellet was suspended in 100 µL of sterile molecular-grade water.

DNA extract quantity and quality were evaluated by spectrophotometry using a Biophotometer (Eppendorf, Mississauga, ON, Canada) with a G1.0 µCuvette (Eppendorf, Mississauga, ON, Canada) with readings at 260, 280, 230, and 320 nm. The V4 region of the prokaryota (archaea and bacteria) *rRNA 16S* gene was amplified using 515FB and 806RB primers [25,48]. For the fungi, the eukaryotic (fungal) *ITS1* gene was amplified using BITS-ITS1 and B58S3 primers [26]. For the eukaryotic communities, the eukaryotic *rRNA 18S* gene was amplified using E572F and B-E1009R primers [27]. All genes were amplified in a two-step dual-indexed approach PCR designed for Illumina instruments by Plateforme d'analyses génomiques (IBIS, Université Laval, Quebec City, QC, Canada).

DNA sequencing was performed by IBIS on an Illumina MiSeq platform, following the methods of Jeanne et al. [49]. The procedures used for fungal DNA amplification and sequencing were similar to the procedures used for prokaryotic DNA amplification. Obtained sequences were demultiplexed based on the tag used. Sequence quality control and features table construction were performed using QIIME2 [50] and the DADA2 plugin [51]. Reference databases SILVA 138 [52] (prokaryotic and eukaryotic communities) and UNITE version 8 [53] (fungal community) were used for taxonomic identification of amplicon sequence variants [54].

Total bacteria and total fungi amounts were quantified following the approach mentioned in [55]. Briefly, the primer pairs Eub-338/Eub518 and FF390/FR1 were used for total bacteria and total fungi, respectively. From the average CT values of samples, amplification units were derived using linear regressions designed by the Microbial Ecology Laboratory of the Institut de Recherche et Développement en Agroenvironnement (IRDA, Québec, QC, Canada) as described in [56].

To assess the functional potential of the prokaryotic community in soil samples, Picrust2 [57] metabolic inference approaches were used with an updated KEGG PATHWAY database (July 2022) [31] and Enzyme Classification numbers from MetaCyc (EC) [58]. Enzymes and metabolic pathways related to glyphosate included thiO (EC:1.4.3.19) [59], phnP (EC:3.1.4.55),phnN (EC:2.7.4.23), phnM (EC:3.6.1.63), phnJ (EC:4.7.1.1) and phnIGHL (EC:2.7.8.37) [60], and shikimate pathway (M00022), which are all related to glyphosate degradation in the soil and also the shikimate pathway known to be affected by glyphosate. Functional potentials of general pathways such as sulfur metabolism (M00176; M00596; M00595), phosphonate metabolism (Ko00440), nitrification (M00175; M00528; M00530; M00531; M00804), denitrification (M00529; M00973), and carbon fixation (M00165 to M00173; M00374 to M00377; M00579; M00620) were computed. Representative amplicon sequence variants (ASV) were used from QIIME2 analysis without filtration, followed

by the default pipeline with input gene family abundances unstratified by contributing organisms. These values were normalized by subtracting the sample mean value of the pathway and dividing by the sample standard deviation.

2.6. Downstream Data Analysis and Statistical Analysis

All statistical analyses were carried out in R 4.2.2 [61]. Glyphosate and AMPA quantifications were visualized in the ggplot2 package. Glyphosate and AMPA contents were log-transformed for statistical analysis. Impacts of sampling year and depth and GBH applications on glyphosate and AMPA contents were first assessed using an ANOVA, followed by a post-hoc pairwise comparison using the TukeyHSD function from the stats package. The differences between glyphosate contents in 2019 and 2020 for each sampling location were used to investigate the dissipation of the molecule and relate it to changes in GBH applications between 2019 and 2020. A similar approach was used to compare the dissipation of AMPA to the change in GBH application between 2019 and 2020. The glyphosate and AMPA soil contents in 2014 were collected from Maccario et al. [23]. An ANOVA followed by the TukeyHSD function was performed on log-transformed values to assess the effect of years on glyphosate and AMPA contents. Multiple regressions were used to evaluate the effect of cumulative GBH applications between 2017 and 2020, soil pH, CEC, clay content, and ISP ratio on glyphosate and AMPA content. A first set of multiple regressions was made with organic and conventional sites, while a second set of multiple regressions was made with only the conventional sites.

For soil prokaryotic, fungal, and eukaryotic communities, the number of reads was normalized at 12,500, 13,000, and 3900 reads, respectively, using the rarefy-even-depth function from the phyloseq R package [62]. Alpha-diversity was measured by Shannon and Chao1 indices with the function estimate_richness from the phyloseq package [63]. The impact of cumulative GBH applications and cropping systems on microbial diversity indices and quantification was analyzed using a mixed model with multiple regressions model, including sampling year and depth as fixed covariates and site as a random effect. Beta-diversity was assessed with principal coordinate analysis (PCoA) with Bray–Curtis dissimilarity for unsupervised ordination of the soil microbial community [64]. Distance-based redundancy analyses (db-RDA) were used to assess the marginal effect of all soil physico-chemical properties on microbial communities' composition. Soil properties with significant marginal effects were included in another db-RDA with soil management variables.

3. Results

3.1. Glyphosate and AMPA Soil Contents

Soil glyphosate contents range between below the LOD and up to 0.72 $\mu g \cdot g^{-1}$ dry soil, while soil AMPA contents range between below the LOD and up to 1.22 $\mu g \cdot g^{-1}$ dry soil (Table 3). Overall, 16% and 28% of soil samples present glyphosate contents below the LOD and between the LOD and the LOQ, respectively. Then, 10% and 28% of soil samples have AMPA contents below the LOD and between the LOD and the LOQ, respectively.

Table 3. Average soil glyphosate and AMPA contents in 2019 and 2020 with respect to cumulative GBH application categories between 2017 and 2020.

Cumulative GBH Applications 2017–2020	Soil Profile	Glyphosate ($\mu g \cdot g^{-1}$ Dry Soil)			AMPA ($\mu g \cdot g^{-1}$ Dry Soil)		
		Average	Max	Detection %	Average	Max	Detection %
None	0–20 cm	0.04 ± 0.03	0.11	81	0.10 ± 0.06	0.25	94
	20–40 cm	0.03 ± 0.02	0.06	50	0.05 ± 0.02	0.06	69
<5400 $g \cdot ha^{-1}$	0–20 cm	0.14 ± 0.13	0.58	100	0.45 ± 0.26	1.16	100
	20–40 cm	0.06 ± 0.03	0.13	83	0.20 ± 0.21	0.73	79
>5400 $g \cdot ha^{-1}$	0–20 cm	0.23 ± 0.18	0.69	100	0.45 ± 0.27	1.22	97
	20–40 cm	0.23 ± 0.18	0.72	97	0.15 ± 0.12	0.69	88

3.1.1. Impacts of Sampling Year and Sampling Depth

Glyphosate and AMPA soil contents are significantly related to sampling depth, sampling year, and cumulative GBH applications ($p < 0.01$). The interaction between sampling year and depth is significant for glyphosate content ($p < 0.01$), while it is not significant for AMPA ($p = 0.66$) (Table 3).

Glyphosate soil contents in 2020 at 20–40 cm depth are significantly lower ($p < 0.01$) than those at 0–20 cm depth for both years and at 20–40 cm depth in 2019. No other significant difference is observed between sampling depth and year. Glyphosate soil contents in conventional sites are significantly higher than those in organic sites ($p < 0.01$). More specifically, glyphosate soil contents in 2019 at farms that sprayed more than 5400 g·ha^{-1} between 2017 and 2020 are higher than those at farms that sprayed less than 5400 g·ha^{-1} (p <0.01).

AMPA soil contents are significantly higher in 2019 compared to 2020 ($p < 0.01$). AMPA contents are higher in the 0–20 cm depth compared to the 20–40 cm depth ($p < 0.01$). There is significantly more AMPA in soils at conventional sites compared to organic sites ($p < 0.01$). However, the difference in AMPA contents between farms that sprayed more than 5400 g·ha^{-1} and farms that sprayed less than 5400 g·ha^{-1} is not significant ($p = 0.95$).

Sites A, B, D, G, H&I are managed by producers who had their fields already sampled in 2014 by Maccario et al. [23]. Comparison between 2014, 2019, and 2020 glyphosate and AMPA content was performed (Figure 3). Organic sites (A and B) exhibit no increase or decrease in glyphosate and AMPA contents. AMPA is not significantly different between all years for all conventional sites, except between 2014 and 2019 for sites H and I. Glyphosate content is significantly higher for sites G and H&I, but not significantly higher for site D. Hence, sites with higher GBH applications between 2017 and 2020 appear to exhibit a significant increase in glyphosate contents between 2014 and 2019–2020.

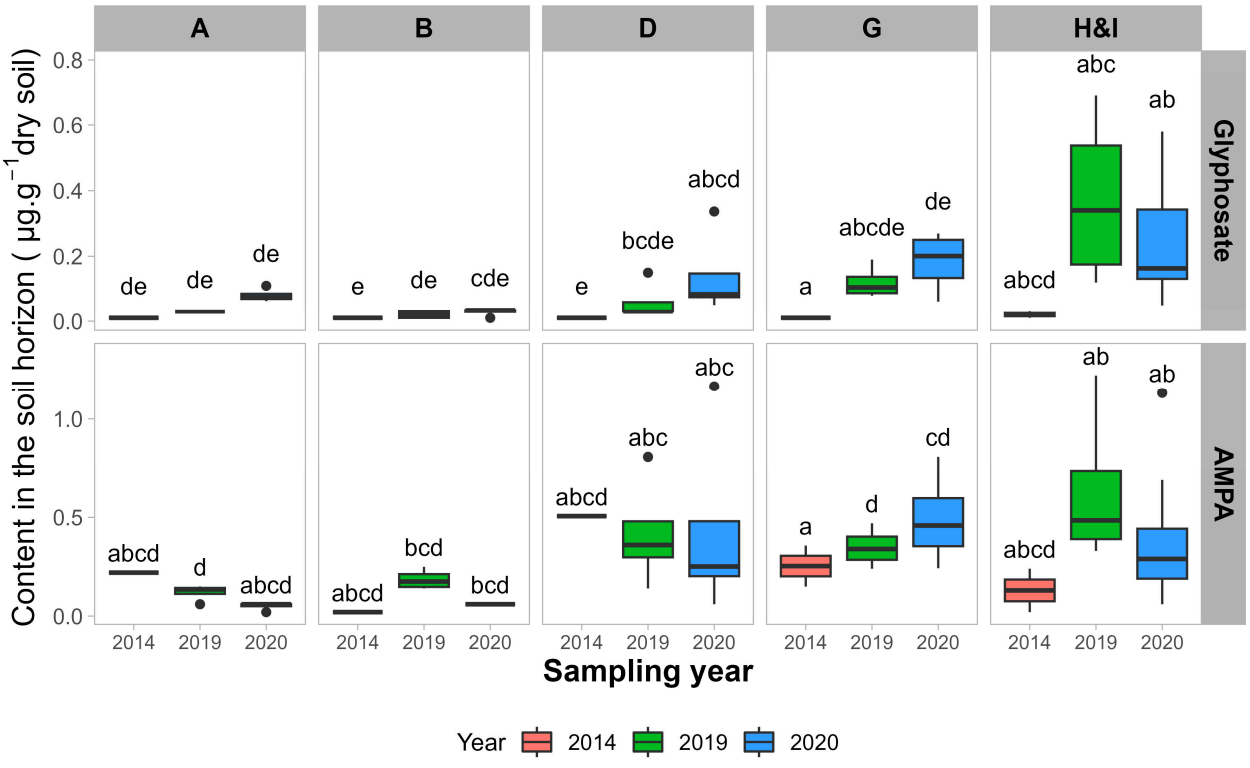

Figure 3. Glyphosate and AMPA soil contents at producers' fields that were part of both this experiment in 2019 and 2020 and of that of Maccario et al. [23]'s study in 2014. Soils were sampled at 0–20 cm depth. Letters represent post-hoc analysis for each molecule using Tukey adjustment.

Glyphosate soil content changes between 2019 and 2020 in the 0–20 cm layer are correlated to changes in GBH applications between the average of 2017–2019 and 2020 (Figure S1). However, this correlation is not observed for glyphosate in the 20–40 cm horizon and AMPA in the 0–20 cm horizon. AMPA contents in the 20–40 cm horizon present a negative correlation with GBH application (Figure S1).

3.1.2. Impacts of Management Practices and Soil Properties

A first set of multiple regressions including organic and conventional sites shows a significant correlation between GBH applications on glyphosate ($p < 0.01$) and AMPA ($p < 0.01$) contents in the 0–20 cm soil horizon. In contrast, all soil physical and chemical properties except soil pH do not have a significant correlation (Figure S2). This correlation of GBH applications is driven by the organic sites that had no GBH applications between 2017 and 2020, along with low glyphosate and AMPA contents. After excluding organic sites from the multiple regressions, the effect of GBH applications is significant for glyphosate ($p = 0.02$) but non-significant for AMPA ($p = 0.85$) contents (Figure 4).

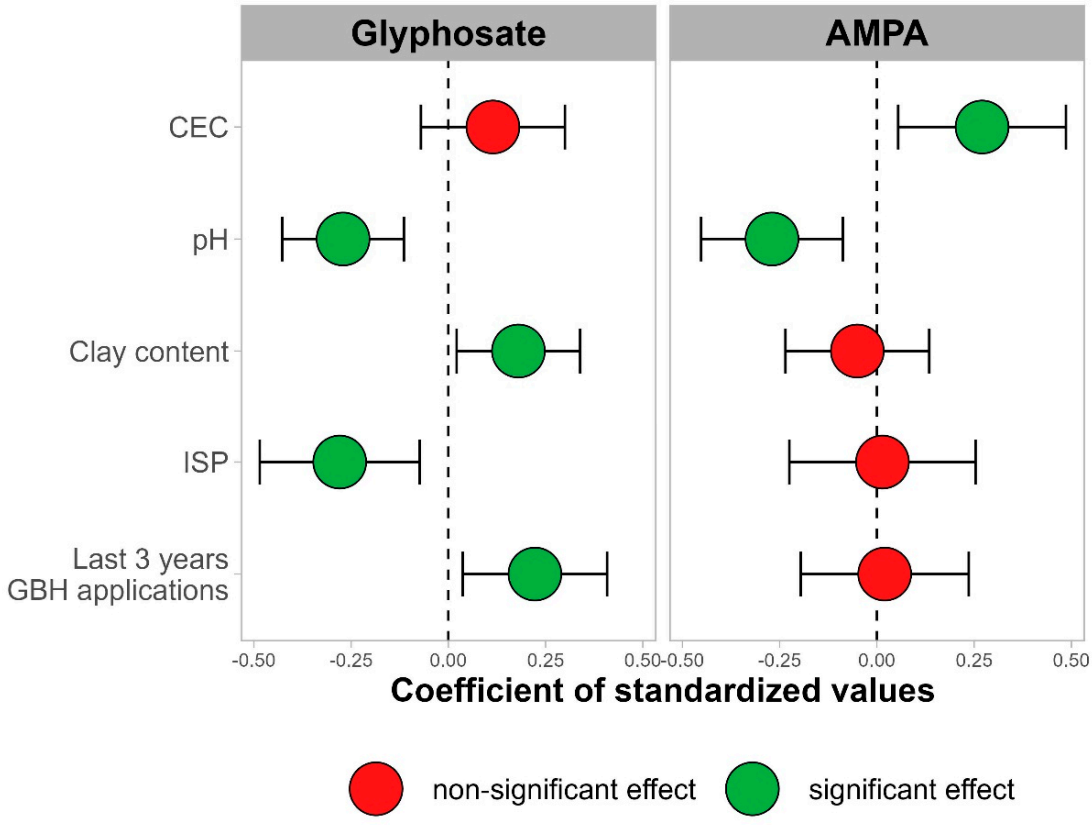

Figure 4. Coefficients values along with their 95% confidence interval for all independent variables in multiple regressions for AMPA (**right**) and glyphosate (**left**) content for 0–20 cm soil horizon of conventional sites.

When excluding organic sites from the multiple regressions, the adjusted R-squared values of the regressions are 50% for glyphosate and 19% for AMPA. Soil pH shows a significant negative effect on glyphosate ($p < 0.01$) and AMPA ($p < 0.01$) contents. Soil clay contents show a positive effect on glyphosate contents ($p = 0.03$) but not on AMPA contents ($p = 0.59$). Soil CEC has no significant effect on glyphosate ($p = 0.22$), but a significant positive effect on AMPA contents ($p = 0.02$). Soil ISP has a significant negative effect on glyphosate contents ($p = 0.01$) but not on AMPA contents ($p = 0.91$).

3.2. Soil Microbial Community

Amplicon sequencing on MiSeq of the three microbial groups yielded, after filtration, an average of 27,788 (±4818); 28,740 (±7809); and 4819 (±1464) sequences per sample for prokaryotes, fungi, and eukaryotes, respectively. When combining all samples, the prokaryotic community contained 17,400 unique ASVs, the fungal community contained 17,500 unique ASVs, and the eukaryotic community contained 6000 unique ASVs.

3.2.1. Alpha-Diversity and Abundance

The analysis of alpha-diversity metrics shows a high correlation between Shannon, Chao1, observed ASVs, and evenness metrics for each microbial community. Hence, the Shannon diversity index was used to assess the alpha-diversity of microbial communities, while quantification results assessed the total abundance of prokaryotes and fungi (Figure 5). The Shannon index for fungal and eukaryotic communities is not significantly impacted by sampling depth ($p > 0.05$) or year ($p > 0.10$). On the other hand, the prokaryotic Shannon index is significantly impacted by sampling year ($p < 0.001$) with higher diversity in 2020 compared to 2019, but no significant effect of sampling depth ($p = 0.19$). Quantification of prokaryotic and fungal communities is significantly impacted by both sampling year ($p < 0.001$) and sampling depth ($p < 0.001$). For both microbial communities, total quantities are higher in the 0–20 cm layer. For prokaryotic communities, quantification is higher in 2019 at sites A, B, C, D, G, and H, while it is higher for fungal communities in 2020 at sites C, D, F, G, H, I.

Cumulative GBH applications and cropping management have a small effect on the diversity and richness of microbial communities. Prokaryotic and fungal community Shannon diversity is not significantly impacted by cumulative GBH applications ($p > 0.27$) and crop rotations ($p > 0.05$). GBH applications significantly impact eukaryotic diversity ($p = 0.02$), with higher eukaryotic diversity in fields receiving GBH applications. Cumulative GBH applications and crop rotations do not impact prokaryotic and fungal quantification.

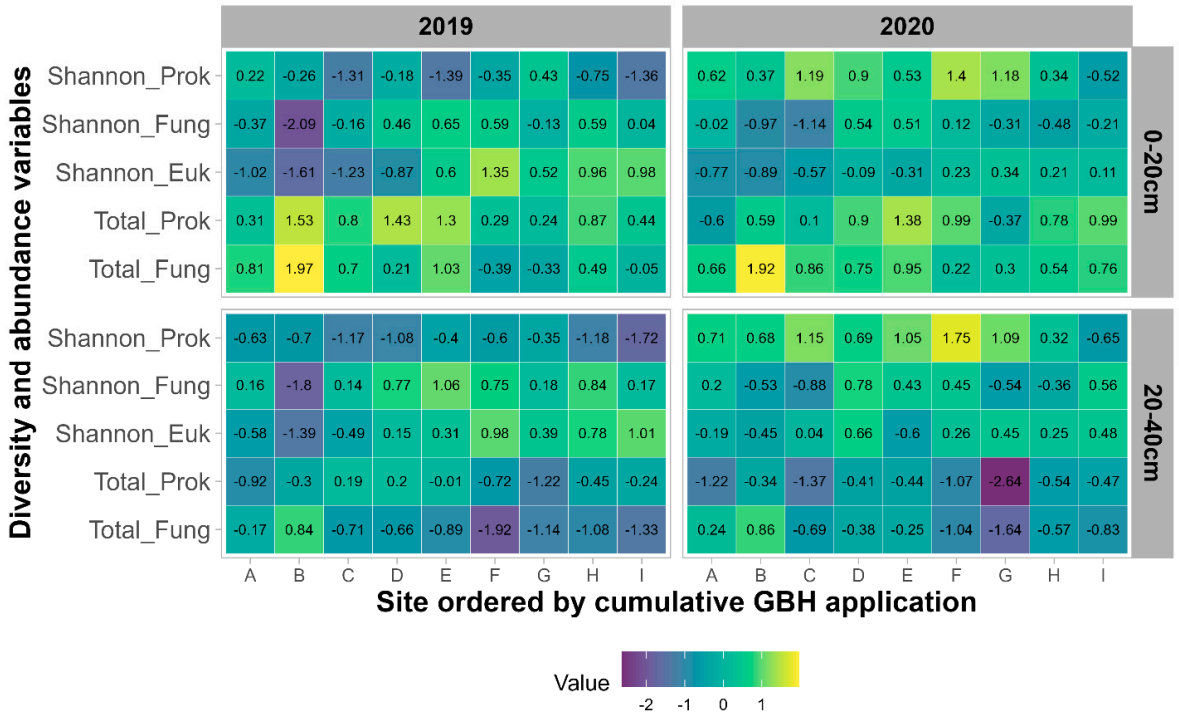

Figure 5. Normalized value for Shannon diversity index of prokaryotic (Shannon_Prok), fungal (Shannon_Fung), and eukaryotic (Shannon_Euk) communities along with total bacteria (Total_Prok) and total fungi (Total_Fung) quantification for each site for years 2019 (corn) and 2020 (soybean) and both horizons (0–20 cm and 20–40 cm).

3.2.2. Beta-Diversity

The composition of microbial communities, especially prokaryotic ones, is strongly influenced by soil texture (Figure 6). PCoA analysis shows that microbial communities found in coarse-texture soils tend to differ from those of fine-texture soils, with marked differences for prokaryotic communities and to a lesser degree for fungal and eukaryotic communities. Interestingly, fine-textured soil fungal communities cover a large span of the ordination plot, indicating that fungal communities in fine-textured soil can be quite diverse compared to prokaryotic communities, which are more similar in fine-textured soil (Figure 6).

Using db-RDAs, a set of soil properties variables (OM, Ca, Na, clay content, and sand content) were identified as having a significant effect on all three microbial communities' composition (Supplemental Table S1). The marginal effect of these selected soil properties is compared to the marginal effect of management practices (Table 4). Overall, sodium concentration has the highest marginal effect for all microbial communities, followed by clay and sand content, then crop rotation, manure inputs, and calcium content. While organic matter content has a significant marginal effect when considering only environmental variables (Supplemental Table S1), the effect is not significant in a model including management variables (Table 4). GBH application doses do not have a significant marginal effect on soil microbial communities.

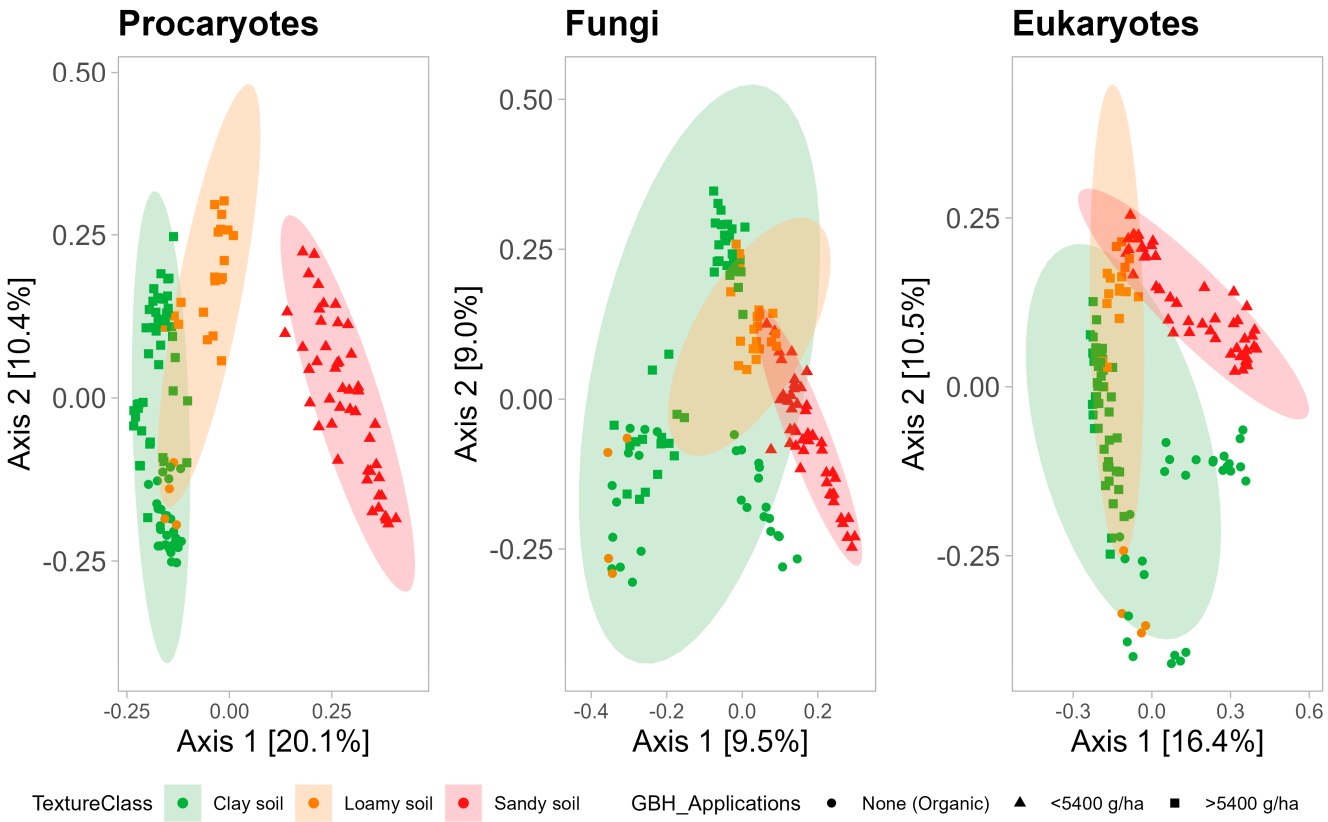

Figure 6. Principal coordinate analysis of prokaryotic (**left**), fungal (**middle**), and eukaryotic (**right**) communities. Point colors represent the groups based on GBH cumulative applications between 2017 and 2020. Point shapes and ellipses colors represent soil texture. Ellipses signification threshold was 0.05.

Table 4. Marginal effect from distance-based redundancy analysis of each environmental and management variables on soil microbial communities.

Type of Variable	Variable	d.f.	Prokaryotic Community		Fungal Community		Eukaryotic Community	
			Pseudo-F Value	*p*-Value	Pseudo-F Value	*p*-Value	Pseudo-F Value	*p*-Value
Environmental variables	Organic matter	1	0.882	0.574	1.11	0.262	0.98	0.408
	Ca	1	1.20	0.211	1.65	0.023	1.87	0.023
	Na	1	2.32	0.012	1.75	0.009	2.05	0.012
	Clay content	1	2.08	0.015	2.09	0.003	2.05	0.013
	Sand content	1	1.78	0.033	1.79	0.009	1.68	0.032
Management variables	Cumulative GBH application	1	0.853	0.585	1.18	0.198	0.878	0.55
	Crop rotation	3	1.41	0.045	1.46	0.004	1.69	0.005
	Manure inputs	2	2.05	0.005	2.06	0.001	2.04	0.002

3.2.3. Functional Potential

Tools such as Picrust2 allow for inferring different functional potentials, such as genes involved in glyphosate degradation, or general pathways that are important soil processes from microbial communities observed in our experiment (Figure 7). Overall, sampling year has a highly significant effect ($p < 0.001$) on all genes and pathways of interest, with higher values in 2020 compared to 2019. Soil horizon does not have a significant effect for all genes, with the exception of the genes related to the phn operon (phnIGHL, phnJ, phnM, phnN, and phnP), for which the potential was higher in the 0–20 cm horizon compared to the 20–40 cm horizon.

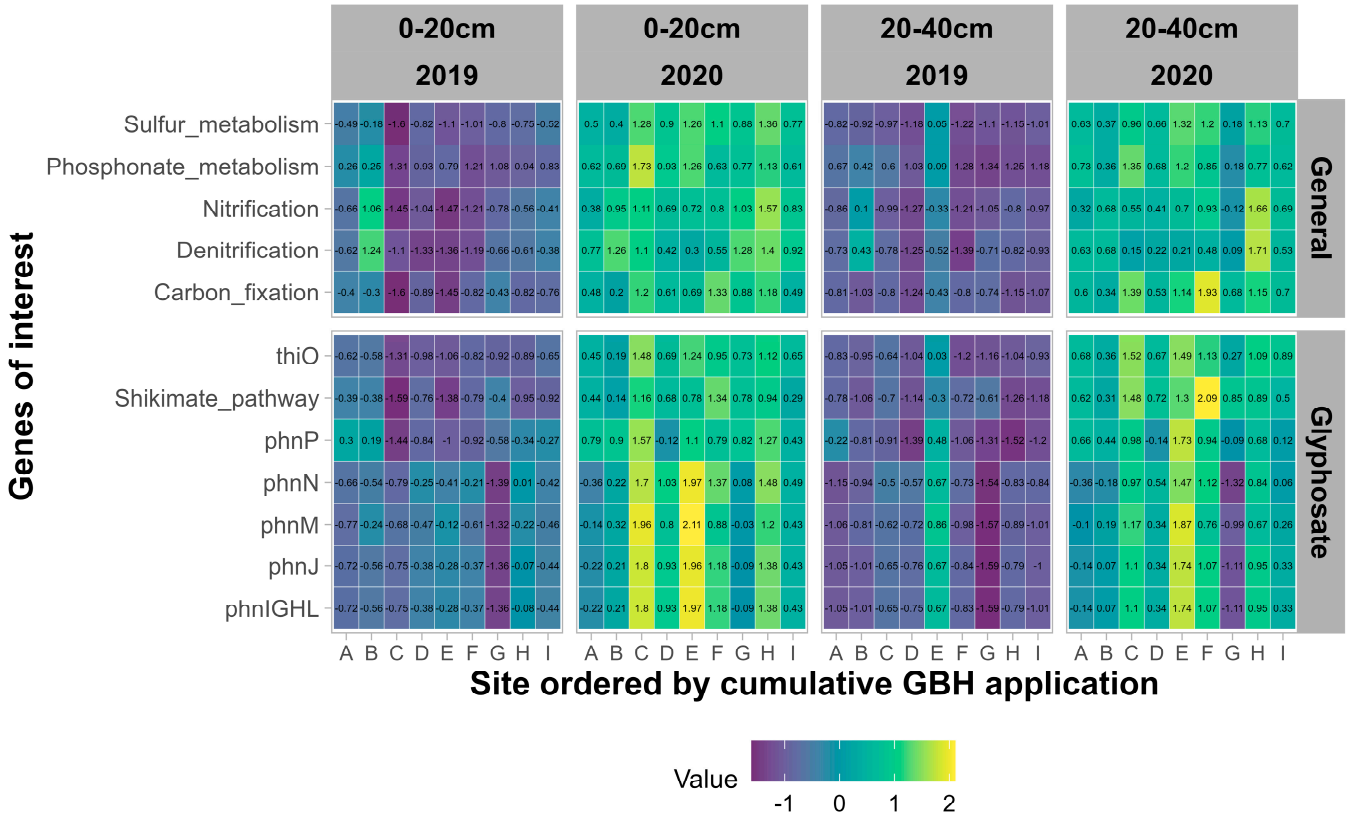

Figure 7. Normalized functional potential of prokaryotic communities for genes related to general metabolism (**top**) and genes related to glyphosate metabolism (**bottom**) for both years (2019 and 2020) and both soil horizons (0–20 cm and 20–40 cm).

Soil texture, expressed as sand and clay contents, has the most effects on both general functions and glyphosate-related functions. Crop rotations and manure inputs show a significant effect on the phn operon, with the exception of phnP. Frequent manure input has a significantly higher ($p = 0.044$) potential for phnM compared to infrequent manure inputs. Cumulative GBH applications do not have a significant effect on any functions of interest.

4. Discussion

4.1. Pseudo-Persistence of Glyphosate and AMPA in Soils

The pseudo-persistence of pollutants is defined by the constant addition of new molecules that replenish the molecules that are being removed [65]. As GBHs are applied nearly every year on soils that are not under organic management, both glyphosate and AMPA could be seen as pseudo-persistent pollutants in these soils [66]. Glyphosate and AMPA pseudo-persistence in analyzed sampled soils is almost ubiquitous, with 84% of samples containing detectable levels of glyphosate and 90% for AMPA. Sites with no GBH application for over a decade present detectable levels of glyphosate and AMPA in their soils. Traces of glyphosate and AMPA in organic sites can be explained by the frequent application of manure on organic fields. As the manure does not need to come from livestock that is organically managed, it can contain glyphosate and AMPA residues that come from conventional animal feed [67]. Fuchs et al. [67] applied poultry manure to a field that never received GBH. With an extremely high manure application rate (36 MT·ha^{-1}), Fuchs et al. were able to detect 1.7 µg·g^{-1} dry soil of glyphosate in an organic soil. This glyphosate content is much higher than any measurements reported here in organic and conventional fields, showcasing the ability of manure to import glyphosate and AMPA to organically managed fields.

Previous experiments that focused on field crop soils report slightly lower or similar levels of detection of glyphosate and AMPA in soils from Quebec (42% and 70%), Argentina (100% for both), and Brazil (94% and 100%), respectively [23,66,68]. The lower detection rate recorded in Quebec was observed in soil samples collected in 2014 from soybean fields [23]. All these studies were carried out on soils with frequent application of GBH in corn and soybean production, explaining the ubiquity of glyphosate and AMPA. Although detection rates are similar across regions of the world, our results suggest that Quebec's non-organic field crop soils that were sampled in this study have slightly higher glyphosate (0.17 µg·g^{-1} dry soil ±0.15) and AMPA (0.37 µg·g^{-1} dry soil ±0.24) contents compared to Brazilian (0.08 µg·g^{-1} dry soil ±0.09 for glyphosate and 0.17 µg·g^{-1} dry soil ±0.16 for AMPA) and European soils (0.11 µg·g^{-1} dry soil ±0.13 and 0.13 µg·g^{-1} dry soil ±0.14) [68,69] although smaller than Argentinian soil (2.30 µg·g^{-1} dry soil ±0.48 and 4.20 µg·g^{-1} dry soil ±2.26) [66]. Glyphosate and AMPA contents measured in the 0–20 cm horizon in 2019–2020 are generally higher than Maccario's study conducted in 2014 (0.06 µg·g^{-1} dry soil ±0.10 and 0.29 µg·g^{-1} dry soil ±0.21) [23]. Our analysis shows that AMPA contents are not significantly different between 2014 and 2020 for five producers. On the other hand, there is a significant difference in glyphosate content between 2014 and 2020 for the two producers with GBH applications higher than 5400 g·ha^{-1} between 2017 and 2020. This finding agrees with Primost et al. [66] who reported that glyphosate content was best correlated with cumulative GBH applications over several years compared to the last spraying dose. Glyphosate accumulation in soil seems to occur at a rate lower than predicted by Primost et al. [66]. It was suggested that an increase in glyphosate content of 1 µg·g^{-1} dry soil would happen every 5 GBH application [66]. However, in the course of over 6 years with yearly applications of GBH, we observed an average increase of 0.20 µg·g^{-1} dry soil for three fields (sites G, H, I) and no significant increase for one field (site D).

4.2. Impacts of Soil Properties and Cropping Systems on Glyphosate and AMPA Contents

Our results demonstrate that organic management results in a significant difference in glyphosate and AMPA content in soils, which is intuitive as GBH application is prohibited in this type of cropping system (Figure S2). When excluding organic sites from our analysis, cumulative GBH applications three years prior to sampling significantly influence glyphosate soil content but not AMPA soil content (Figure 4). This difference between the behavior of glyphosate and AMPA soil contents could be explained by the pseudo-persistence of glyphosate related to frequent inputs of glyphosate that are higher than dissipation rates [66] and by the use of crop residues as soil cover for no-till sites [70]. Even with a rapid glyphosate dissipation rate in soils [5,71], relatively high glyphosate inputs (>1350 $g \cdot ha^{-1} \cdot year^{-1}$) in agricultural soils could result in a partial accumulation of glyphosate that does not degrade in AMPA and further leaching in deeper soil horizons, especially following a heavy rain event [72]. For soil covered with plant biomass (i.e., living plants or crop residues) at the moment of GBH application, a portion of applied GBH is intercepted by plant biomass instead of onto the soil, which modifies glyphosate dissipation [70,73–75]. Glyphosate intercepted by plant biomass will generally slow glyphosate dissipation compared to application directly on the soil, as a greater portion of glyphosate is incorporated or adsorbed onto plant biomass compared to soil particles [70,74]. Plant biomass needs to be degraded for adsorbed and incorporated glyphosate to dissipate into the soil [70,73]. Plant biomass that is already partially degraded will adsorb glyphosate more strongly than fresh plant biomass [73] and a larger portion of glyphosate will remain as non-extractible residues found within plant biomass [75]. Usage of tillage to incorporate crop residues will accelerate plant biomass degradation by mixing it with the soil [70]. Hence, sites with no-till practices and frequent cover crop usage (Sites G, H, and I) could result in a slower glyphosate dissipation compared to sites with tillage and lower soil cover by crop residues (Site D). Additionally, repeated GBH applications can lead to lower soil microbial activity and soil respiration [76,77], potentially inhibiting glyphosate biodegradation into AMPA [40]. Hence higher GBH applications could not directly lead to higher AMPA contents in the soil, but rather to an accumulation of glyphosate. Such interpretations could explain the higher glyphosate content in deeper soil profiles for sites with >5400 $g \cdot ha^{-1}$ of GBH applied between 2017 and 2020 (Table 3) and the increase with time of glyphosate in the same sites sampled in 2014 and in 2020 (Figure 3).

Soil pH, ISP, and clay content have a significant effect on glyphosate and AMPA contents in soil, similar to previous experiments [7,8]. Our observations of a general negative relationship between glyphosate and soil pH and a positive relationship between glyphosate and clay content agree with the literature [78–81]. A study realized in Argentina showed that glyphosate exhibits a higher affinity to soil surfaces at a certain pH, which varies between soil types but is generally around pH 6 [82], which corresponds to the lower soil pH values recorded in our experiments. In the same study, the presence of phosphate was shown to reduce glyphosate adsorption to soil particles [82], which agrees with our finding of a negative relationship between glyphosate content and soil ISP value (Figure 4). Clay content and ISP do not present a significant correlation with AMPA contents, while soil CEC has a positive relationship with AMPA contents. Maccario et al. [23] also found that soil texture did not have an impact on AMPA content. This could be explained by the soil's higher affinity to AMPA compared to glyphosate [14,83].

4.3. Impacts of Cropping Systems and Soil Properties on Soil Microbial Community

The alpha-diversity and total biomass of prokaryotic and fungal communities are not affected by crop rotations in our study. Only eukaryotic alpha-diversity is significantly impacted by cumulative GBH applications. High cumulative GBH applications in this study are linked to other management choices such as no-till. Hence, the positive relationship between GBH application and eukaryotic communities could be explained by the adoption of no-till along with high GBH application. Indeed, Kepler et al. [33] showed that the impact of GBH applications on soil microbial communities was negligible compared to

tillage practices. Although a meta-analysis has shown no significant impact on fungal diversity from no-till practices [84], other studies have shown the negative effect of tillage on soil eukaryotic community [85–88], which supports our hypothesis.

Soil microbial community composition and functional potential are influenced by both soil properties and cropping systems in our study. Sodium contents have the most important effect on the composition of microbial communities, which is hard to explain. Soil sodium can have important negative effects at elevated contents (1000 ppm) [89,90]; however, in this study, all soil samples present Na contents below 40 ppm with values as low as 2.7 ppm. There is no report of an important effect of Na on soil microbial communities at the concentrations observed in our study. Soil texture has an important effect on the composition of microbial communities and their functional potential. Numerous studies have confirmed the driving effect of soil texture on shaping the composition and functional potential of microbial communities [91–93]. In terms of cropping systems, crop rotation and manure application frequency are often cited as having a significant effect on soil microbial communities [94–96]. Guo et al. [95] showed that fertilizer application, especially manure, had a stronger role than crop rotation and crop growth stage on soil microbial communities. Another study has shown that all microbial communities are not impacted similarly by crop rotation, with fungal communities being more responsive to the number of plant species in a crop rotation compared to prokaryotic communities [97]. In the present study, no significant effects from GBH application on soil microbial community structure and functional potential were observed, which is consistent with other reports in the literature using DNA approaches to assess microbial communities [33,77,98,99]. This suggests that other management practices, such as crop rotation, manure inputs, and soil tillage, have a greater impact on soil microbial community composition and functional potential compared to GBH applications.

5. Conclusions

Glyphosate and AMPA were detected in soils at all sites investigated, including organic sites. The cumulative doses of GBH are not correlated to AMPA soil contents in conventional sites. However, glyphosate soil contents were positively correlated to GBH cumulative doses, and there seems to have been an increase in glyphosate soil contents between 2014 and 2020 for sites with the heaviest GBH applications. Low soil pH and high clay content corresponded to higher glyphosate and AMPA pseudo-persistence in soil. Cumulative amounts of GBH applications did not seem to have an impact on soil microbial communities, while crop rotation and manure inputs had a significant impact. Hence, GBH applications seem to have minimal impact on soil microbial communities and on AMPA soil contents. On the other hand, GBH applications seem to increase glyphosate soil contents and should be monitored to ensure the accumulation of glyphosate does not lead to detrimental effects on soil microbial communities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040686/s1, Figure S1: Correlation between difference in glyphosate (top) and AMPA (bottom) contents between 2019 and 2020 and the difference in GBH application in 2020 and the yearly average GBH application between 2017 and 2019. Figure S2: Coefficients values along with their 95% confidence interval for all independent variables in multiple regressions for AMPA (right) and glyphosate (left) content for 0–20 cm soil profile of all sites. Figure S3: Chromatographs of a six-point calibration curve for AMPA (A) and a five-point calibration curve for glyphosate (B) on a soil matrix. Supplemental Table S1: Marginal effect of soil properties on microbial communities' composition for the 0–20 soil horizon.

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