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Degradation of glyphosate and bioavailability of phosphorus derived from glyphosate in a soil-water system

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ABSTRACT

Glyphosate, the most commonly used herbicide in the world, can be degraded into more toxic and persistent products such as aminomethylphosphonic acid (AMPA) or non-toxic products such as sarcosine and glycine. In this study, we used liquid chromatography mass spectrometry (LC-MS) and electrospray ionization (ESI) source Q Extractive Orbitrap mass spectrometry (ESI-Orbitrap MS) to identify glyphosate degradation products and combined with sequential extraction and stable isotopes to investigate the degradation of glyphosate and transformation of phosphorous (P) product in a soil-water system. The LC-MS and ESI-Orbitrap MS results showed that glycine formed during the early stage but was rapidly utilized by soil microorganisms. AMPA started to accumulate at the late stage and was found to be 3–6 times more resistant than glyphosate against degradation; while no sarcosine was formed. The ^{18}O labeling and phosphate oxygen isotope results allowed a clear distinction of the fraction of inorganic P (P_i) derived from glyphosate, about half of which was then rapidly taken up and recycled by soil microorganisms. Our results provide the first evidence of the preferential utilization of glyphosate-derived P_i by microorganisms in the soil-water system. The rapid cycling of P_i derived from this disregarded source has important implications on nutrient management as well as water quality.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] is an effective broad-spectrum herbicide. Its application has dramatically increased since the introduction of genetically engineered herbicide-tolerant crops in 1996 (Dill et al., 2010). It has been the most heavily used pesticide in the agricultural sector in recent years with an estimated 130 million kilograms in the U.S. in 2016 (USGS, 2016). Glyphosate degradation is accomplished mainly by various soil microorganisms (Rueppel et al., 1977; Sviridov et al., 2011; Zhan et al., 2018). The degradation occurs either through C–N bond cleavage, which forms aminomethylphosphonic acid (AMPA), a more toxic and resistant metabolite than glyphosate; or through C–P bond cleavage, which yields much safer products, sarcosine and then glycine. Unlike AMPA, sarcosine is barely detected in the environment (Wang et al., 2016) aside from pure culture experiments (Zhan et al., 2018). The reason for this discrepancy is still unclear, but it might be due to the rapid oxidation of sarcosine, inefficient extraction from soil matrix, or present below the limit of detection. Although early assessments suggested low acute toxicity of glyphosate and AMPA, their possible chronic health effects on animals and humans have been reported to be higher in recent studies (Balbuena et al., 2015; Van Bruggen et al., 2018). Moreover, the report from the International Agency for Research on Cancer (IARC), which classified glyphosate as a probable carcinogen to humans (IARC, 2015), has triggered fierce controversy in the public and within regulatory bodies (USEPA, 2017). Strong interaction of glyphosate and AMPA with soils clays and organic matter bring a concern of potentially contaminating drinking water (USEPA, 2015). This highlights the need to study glyphosate and its metabolites transformation and the preference of the degradation pathways in the system that are relevant to natural water-soil environment.

Degradation products from glyphosate eventually generate inorganic forms of carbon, nitrogen, and phosphorus. Isolated bacterial and fungal strains have been reported to utilize one or more nutritional elements (C, N, and P) in glyphosate (see review by Zhan et al., 2018). Little information, however, has been reported in terms of glyphosate utilization in soil or under other natural environment (Borggaard and Gimsing, 2008; Wang et al., 2016),

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and the fate of P derived from glyphosate in P-rich agricultural soils are unknown. Given that a very small increase in P concentration, due to its low requirement compared to other major nutrients (106C: 16N: 1P; Redfield, 1958), can cause severe impacts on water quality such as promoting hypoxia and eutrophication. Therefore, the role of glyphosate-derived P loading cannot be ignored.

The sorption of P onto soil organic matter/minerals strongly affects its mobilization and bioavailability. The Hedley sequential extraction method and modification thereof (Hedley et al., 1982; Tiessen et al., 1984) have been used to separate and characterize different soil P pools and their availability as well as to estimate need for fertilizer on crops. Most recently, phosphate oxygen isotope has emerged as novel research tool and is combined with sequential extraction methods to investigate the bioavailability, turnover, and transformation of P in soil and other environment (Gooddy et al., 2016; Jaisi et al. 2017; Joshi et al., 2016; Pistocchi et al., 2017). The stable isotope ratios of O in P_i ($\delta^{18}O_P$) serves as a tracer of P due to its unique isotope effects: i) temperature-dependent equilibrium isotope effects due to the rapid O-isotope exchange between dissolved P_i and water catalyzed by pyrophosphatase enzyme commonly present in organisms (Longinelli and Nuti, 1973; Chang and Blake, 2015); and ii) kinetic isotope effects catalyzed by other phosphatase enzymes during the degradation of organic P compounds, which partially inherit isotope signatures from the parent molecules (Liang and Blake, 2009; Sun et al., 2017; von Sperber et al., 2015). The current state of basic and applied research in isotope and molecular separation methods provides a platform for studying the isotope exchange and microbial turnover of P during glyphosate biodegradation in soils and discriminating the particular source of P undergoing a specific pathway of cycling in the environment.

The major aim of this research was to study glyphosate degradation and the fate of glyphosate-derived P in the water-soil system, especially on its uptake and cycling by soil microorganisms. To do so, we developed a liquid chromatography mass spectrometry (LC-MS) method to detect and quantify glyphosate and its degradation products and then calculate their half-lives in the soil-water environment. Distribution of different P pools in soils was analyzed using the Hedley sequential extraction method. A refined isotope method was implemented to identify P availability and microbial turnover during glyphosate degradation in the soil incubated with ^{18}O-labeled water. Overall, these methodological advances in metabolite identification, P distribution, and isotopic signature tracking have brought forward a new dimension to better understand glyphosate degradation in the natural environment and cycling of P derived from glyphosate.

2. Materials and methods

2.1. Reagent and chemicals

Glyphohsate (≥96%), (Aminomethyl) phosphonic acid (≥98%) and 9-Fluorenyl-methoxycarbonyl chloride (FMOC-Cl) (≥97%) were obtained from Sigma-Aldrich. Isotope labeled compounds including glyphosate-2-^{13}C,^{15}N, glycine-d_5 and sarcosine-d_3 (methyl-d_3) were purchased from Sigma-Aldrich. Other chemicals including glycine (≥99%) and sarcosine (≥98%) were purchased either from Acros Organics or Fisher Scientific. All the reagents were of analytical grade and stock solution were prepared with DI water.

2.2. Soil collection and incubation

A typical silt loam soil (0–15 cm depth) from the Agricultural Experiment Station research farm at the University of Delaware was used in this study. The detailed information about the soil characterization has been reported in a previous publication (Joshi et al., 2016). After removing any plant residues and granular rock particles, the soil samples were air-dried, homogenized, passed through a 2 mm sieve, and stored until analyses.

A flowchart of the experimental and analytical approach used is shown in Fig. 1. The first degradation experiment was run to identify glyphosate and its degradation products in soil as well as to determine the degradation kinetics and half-lives of major products. The soil was incubated with 1 μmol/g unlabeled glyphosate at 20 °C in the dark with 60% water content for 175 d. A separate experiment with dual isotope (^{13}C and ^{15}N) labeled glyphosate (1 μmol/g) spiked in soil was performed for 35 d to accurately identify degradation products. The control experiment was performed under the same condition but without glyphosate. The natural soil incubation included both biotic and abiotic degradations. Identical experiment run with autoclaved water and soil served as abiotic degradation. At selected time points, 5 g subsamples were collected into 50 mL centrifuge tubes and stored at −20 °C until further analysis. All experiments were run in duplicate under the same condition.

In order to identify P distribution and bioavailability during glyphosate degradation, the second set of experiments was performed in two ^{18}O-labeled waters ($\delta^{18}O_{H2O} = -6.51$ and $+18.27$‰). To collect sufficient P for isotope analyses, 5 μmol/g unlabeled glyphosate was spiked into 300 g soil, and incubated with 600 mL ^{18}O-labeled water at 20 °C in the dark for 161 d. The spiked glyphosate concentration is much higher than application dose in agriculture (about 1 kg/ha), but is required to obtain reliable phosphate isotopic analyses. The experimental containers were tightly capped to avoid any water evaporation that compromises the water oxygen isotopes. The containers were shaken every day for ~15 min to homogenize the system and then briefly ventilated to replenish ambient oxygen and to preserve the oxic condition. The control experiments were run under the same condition but in the absence of glyphosate. Subsampling and processing followed a similar procedure as described above.

2.3. Extraction and analyses of glyphosate, AMPA, glycine, and sarcosine

The extraction of glyphosate, AMPA, glycine, and sarcosine was based on the published method (Ibáñez et al., 2005). Briefly, 1 g lyophilized soil samples degradation experiments were extracted with 5 mL 0.6 M KOH for 1 h by shaking at 140 rpm, then centrifuged at 2755×*g* for 30 min. One mL of supernatant was removed and neutralized by HCl and then 0.12 mL of borate buffer (pH = 9) and 0.12 mL FMOC-Cl (12 g/l) were added and shaken for 1 min on a vortex mixer. After an overnight reaction at room temperature, the mixture was filtered with a 0.45 μm syringe filter for LC-MS analysis.

Glyphosate, AMPA, glycine, and sarcosine standards were prepared to develop the separation method by using an Acclaim 120, C18 column (2.1 × 250 mm) under a gradient eluent program. After testing and running several programs, the optimized gradient was identified to be effective with a mixture of two mobile phases with a flow rate of 0.35 mL/min with (A) acetonitrile and (B) 5 mmol/L HAc/NH_4Ac: 0–6 min, 20–40% A, 80–60% B; 6–9 min, 40–75% A, 60–25% B; 9–10.2 min, 75–100% A, 65–0% B; 10.2–12 min, 100% A, 0% B; 12–12.1 min, 100–20% A, 0–80% B; 12.1–14 min, 20% A, 80% B. The chromatographic separation for each sample required 14 min.

Glyphosate and its degradation products were identified and quantified by a Waters single quadrupole LC-MS equipped with PDA and SQ detector. The optimized MS parameters are as follows: ESI positive mode, capillary voltage 3 kV, cone voltage 40 V,

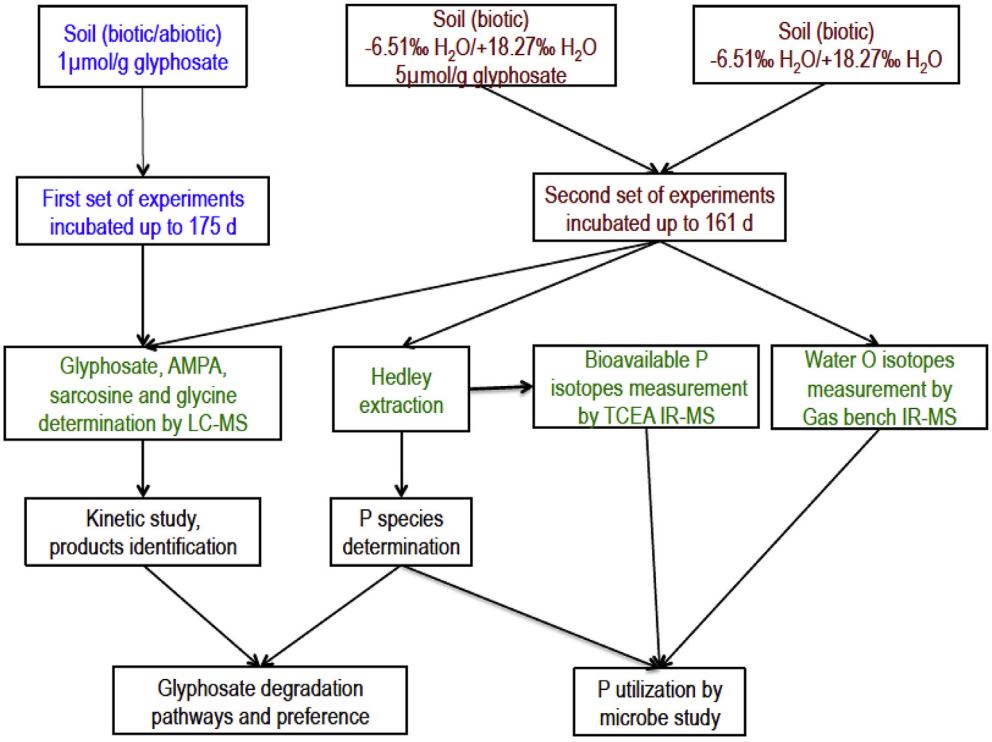

Fig. 1. Flowchart outlining glyphosate degradation experiments in the water-soil system.

desolvation temperature 200 °C, desolvation gas flow 650 L/hr, and full mass scan from 100 to 500 m/z. The unlabeled glyphosate and labeled sarcosine were quantified with labeled glyphosate and unlabeled sarcosine as internal standards. Similarly, labeled glycine was quantified by labeled glycine as an external standard to avoid any interference from glycine already present in soil. AMPA was determined by the soil spiked external standards. Labeled glyphosate degradation samples were analyzed with a high resolution mass spectrometry—Q Extractive Orbitrap Mass Spectrometry (Thermo, Germany) at the University of Delaware. Orbitrap MS data were acquired under the positive mode with scan range from 100 to 1000 m/z. Glycine formation during labeled glyphosate degradation were determined by external standard prepared by spiking labeled glycine in soil to avoid the interference of soil original glycine.

The extraction and derivatization methods for glyphosate, AMPA, glycine, and sarcosine were validated by spiking the known amounts of these compounds in soil. The recovery ranged from 85 to 107% for glyphosate, 79—93% for AMPA, 74—88% for glycine, and 80—97% for sarcosine with RSD below 20%, which is considered satisfactory. The limit of quantification (LOQ) for glyphosate and AMPA is 10 nmol/g soil and for glycine and sarcosine is 50 nmol/g in single quadrupole LC-MS, while it was largely improved by using Orbitrap (0.5 nmol/g).

2.4. Distribution of P derived from glyphosate into soil P pools

To differentiate and quantify the distribution of glyphosate-derived P in soil, samples from both control and glyphosate spiked soils (from the second set of experiments) were analyzed. A 0.3 g lyophilized soil was weighed and extracted with 30 mL DI water for 2 h using the modified Hedley et al. (1982) sequential extraction method (Tiessen et al., 1984). The supernatant was collected as H_2O extractable P_i (most labile P_i), and residual soil was extracted with 30 mL of 0.5 M $NaHCO_3$ for 16 h to collect labile and weakly adsorbed P_i. Inorganic P from those two pools represents microbially available P_i (Tiessen and Moir, 1993). The soil was further extracted for 16 h first with 30 mL of 0.1 M NaOH and then with 1 M HCl to obtain the NaOH extractable P_i (strongly sorbed P, fixed by Fe and Al oxides) and HCl extractable P_i (strongly fixed Ca—P), respectively. The concentration of P_i in each pool was measured by using the phosphomolybdate blue method (Murphy and Riley, 1962). The residual P in the soils after the completion of sequential extraction was quantified using ICP-MS.

2.5. Measurement of oxygen isotope ratios

Soil samples from control and glyphosate spiked (5 μmol/g) experiments with two ^{18}O-labeled waters were centrifuged first to extract waters to measure water oxygen isotopes ($\delta^{18}O_W$) by CO_2 equilibration method (Cohn and Urey, 1938). The measurement was done in a Finnigan GasBench II coupled with an isotope ratio mass spectrometer (IRMS; Thermo, Darmstadt, Germany) in the Environmental Biogeochemistry Laboratory at the University of Delaware.

To understand the P bioavailability, the H_2O- and $NaHCO_3$-extracted P_i pools were combined and processed for the measurement of phosphate oxygen isotope ratios ($\delta^{18}O_P$). Five grams of lyophilized soil samples from the second set of degradation experiments were processed following the Joshi et al (2018) method to purify and finally convert P_i into silver phosphate. The O-isotope ratios were measured by a thermochemolysis/elemental analyzer (TC/EA) couples with IRMS. All isotopes from samples and standards were run at least in triplicate.

The measured $\delta^{18}O_P$ values of P_i were calibrated against two silver phosphate standards (YR 1aR-2 and YR 3-2, with the $\delta^{18}O_P$ values of −5.49 and + 33.63‰, respectively). Similarly, the $\delta^{18}O_W$ values of porewater were calibrated with two USGS water standards ($\delta^{18}O_W$ values of −1.97 and −9.25‰, respectively). All isotope values are reported in per mil (‰) relative to the Vienna Standard

Mean Ocean Water (VSMOW).

3. Results and discussions

3.1. Degradation kinetics of glyphosate and its metabolites

The typical chromatography spectra of glyphosate, AMPA, sarcosine, and glycine are shown in Fig. 2. Based on the LC-MS results, the concentrations of the compounds were calculated and are shown in Fig. 3. Glyphosate gradually degraded over time and the extent of degradation reached >80% by 35 d of incubation but traces of residual glyphosate were still detected until 175 d.

AMPA, the major metabolite of glyphosate, appear after several days and accumulated during incubation and reached its maximum concentration at 35 and 56 d in the experiment with 1 μmol/g and 5 μmol/g glyphosate, respectively. Afterwards, its degradation dominated over accumulation. Neither the degradation of glyphosate nor the formation of AMPA was observed in the sterilized soil incubation (abiotic only experiment), indicating microorganisms play a crucial role in degrading glyphosate in soils.

The degradation of glyphosate with time is often described according to first-order kinetics (Beulke and Brown, 2001):

$$\ln(C/C_0) = -kt \quad (1)$$

$$t_{1/2} = \ln 2/k \quad (2)$$

where C_0 is the initial concentration, C is the concentration at time t, and k is the degradation rate constant. The maximum accumulated concentration of AMPA is used as its initial concentration since more than 80% of glyphosate was degraded at the time. The results show that both glyphosate and AMPA degradation follow first order kinetics with a strong correlation coefficient ($R^2 > 0.85$). The calculated half-lives of glyphosate under two sets of experiments are 28.9 and 31.5 d, respectively, consistent with the published results (Al-Rajab and Schiavon, 2010). A calculation based on the maximum amount of AMPA accumulated in the soil shows that the AMPA accounts for 48–68% of the products from glyphosate degradation. It shows much longer half-lives (138.6 and 173.3 d), which highlights the high risk because of its toxicity and persistence in the environment.

Glycine is a common amino acid and commonly present in soil and other environment. The isotope labeled glyphosate provides the reliability of detection because the labeled element is present in glycine as well. Labeled glycine appeared only after few days, accumulated, and reached the highest concentration after 5 d and then decreased but was still detectable after 35 d incubation (Fig. 3a). The concentration of labeled glycine is low, probably due to glycine derived from glyphosate was readily incorporated into microbial biomass son after it formed. Results from a separate labeled glycine incubation experiment showed a rapid decline of soil-spiked glycine (1 μmol/g) with half-life of 0.89 d (Fig. 4). Abiotic experiment showed no significant decline in glycine concentration in sterilized soil, validating methodology as well as indicating that soil microorganisms play a major role in glycine transformation. A recent study of labeled glyphosate reported the distribution of ^{13}C and ^{15}N into several amino acids including glycine (Wang et al., 2016) which our results corroborate. These findings, together, confirm that glyphosate derived glycine in the experiments should have rapidly utilized and metabolized by soil microorganisms.

Sarcosine is a commonly recognized precursor to glycine during glyphosate degradation primarily on pure cultures that include bacteria isolated from soils (Ermakova et al., 2017; Moore et al., 1983; Zhan et al., 2018), but rarely from the natural or simulated environments (Wang et al., 2016). In this study, sarcosine was not detected in any soil treatments including labeled glyphosate and high glyphosate (5 μmol/g) incubations. There might be three possibilities for the observed results: inefficient extraction from soil, fast oxidation of sarcosine, or presence below the detection limits of the analytical method. However, the recovery test performed by artificially spiking sarcosine in the same soil revealed that the method used could efficiently extract and accurately quantify sarcosine (yield 80–97%). The individual incubation experiment showed that sarcosine could be degraded fast in the

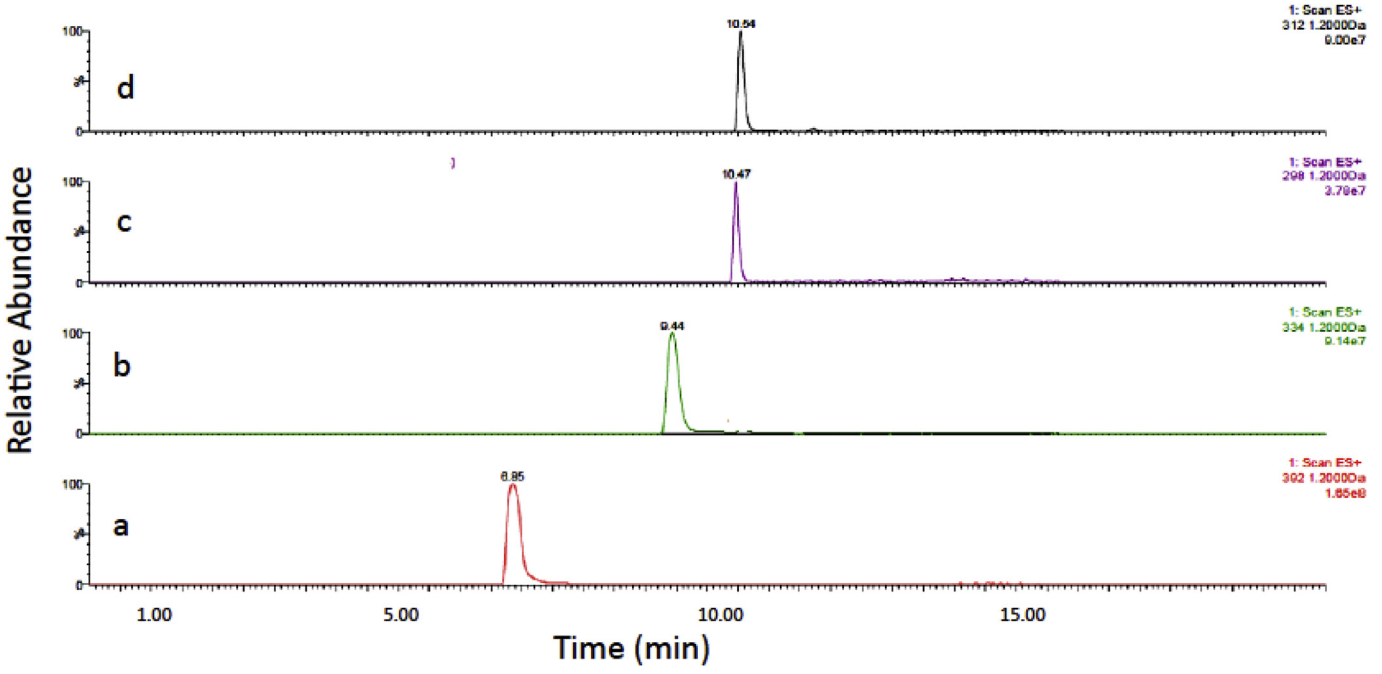

Fig. 2. Typical spectrum of glyphosate, AMPA, glycine and sarcosine analyzed by LC-MS (soil spiked with 1 μmol/g standards). a) glyphosate, b) AMPA, c) glycine, and d) sarcosine.

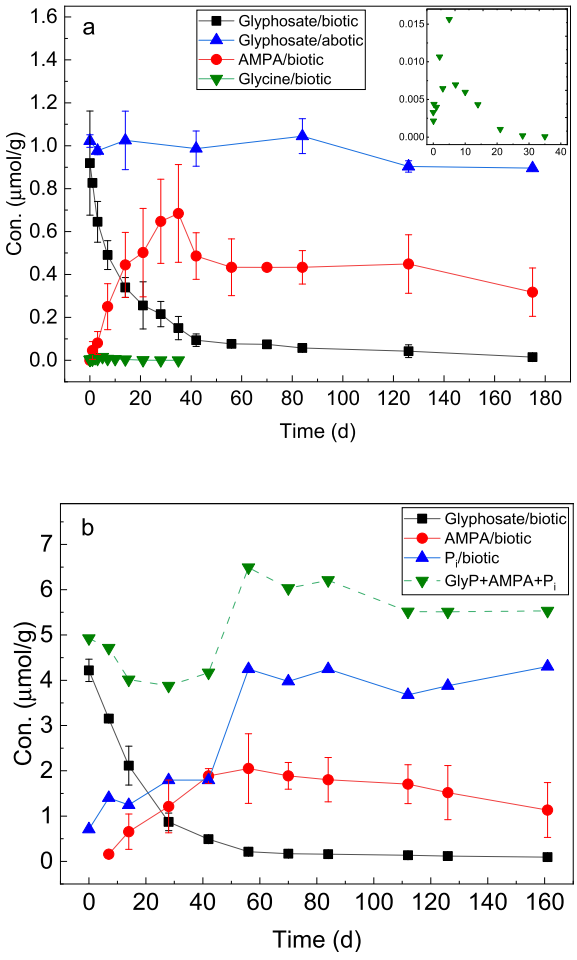

Fig. 3. Kinetics of glyphosate biotic (natural soil) and abiotic (sterilized soil) degradation and its products. a) incubated with 1 μmol/g glyphosate, and b) incubated with 5 μmol/g glyphosate. Please note that the natural soil incubation includes both bitoic and abiotic components of degradation.

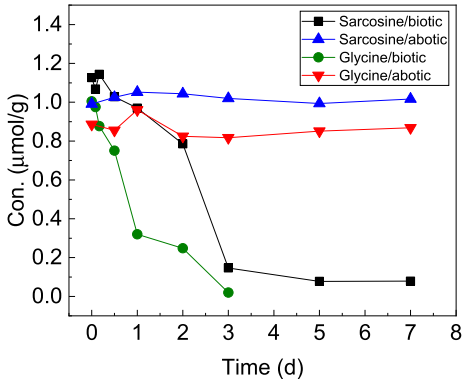

Fig. 4. Biotic (natural soil) and abiotic (sterilized soil) degradation of glycine and sarcosine in soil with spiked concentration of 1 μmol/g of each. Please note that the natural soil incubation includes both bitoic and abiotic components of degradation.

biotic experiment (with half-life of 0.99 d) but no significant decline in sterilized soil, which indicates degradation possible only by soil microorganisms. These lines of evidences suggest analytical method is not the reason, particularly since the high resolution Obitrap MS (detection limit of 0.5 nmol/g of sarcosine and glycine) was used. In the labeled glyphosate degradation experiments, soil samples were collected in several time points (0, 1, 2, 4 h, …until 35 d). The analytical method used successfully monitored the glycine formation and accumulation under extremely low concentration. If sarcosine was actually formed as a precursor to glycine, it should have detected by Obitrap MS since both sarcosine and glycine have similar half-lives. In a recent study, sarcosine was not detected in the abiotic degradation of glyphosate catalyzed by Mn minerals (Li et al., 2018). These authors used advanced analytical methods including NMR, HPLC, and density functional theory (DFT) based electronic structure calculations and concluded that sarcosine was not a necessary intermediate product. Overall, the reliable extraction and analytical methods and intensive time point sampling verified that sarcosine was not formed during glyphosate degradation by soil microorganisms in this study.

3.2. Distribution of glyphosate-derived phosphorous in soil

Concentrations of four soil P_i pools in the control and glyphosate-spiked soils during the second set of incubations are shown in Fig. 5. The experiments performed in two ^{18}O-labeled waters are considered duplicates because the difference in water oxygen isotopes does not impact the kinetics and extent of glyphosate degradation. Clearly, the control soil without glyphosate already contains high P_i and concentrations of P_i in different pools vary. It is noticeable, however, that the concentrations of P_i in these four pools remained essentially constant during the long-term incubation, with H_2O-P_i (1.01 ± 0.08 μmol/g), $NaHCO_3$-P_i (4.21 ± 0.23 μmol/g), NaOH-P_i (10.08 ± 0.91 μmol/g), and HCl-P_i (0.52 ± 0.06 μmol/g). This means that no significant transfer of P pools and organic-inorganic transformation occurred during the long-term incubation. The NaOH-P_i pool was the largest, indicating that Fe and Al minerals associated P is the major P sink in this soil, which is consistent with several other soils (Guo et al., 2000; Tiessen et al., 1984).

The results from the experiment in which glyphosate was spiked show that P_i derived from glyphosate transferred into different pools, resulting in an increase of corresponding pool size. The maximum concentration of H_2O-P_i was 2.11 μmol/g at 70 d of incubation. The difference between control (soil without glyphosate) and glyphosate spiked soil shows that there was 1.06 μmol/g glyphosate-derived P_i transferred into this pool. Similarly, a significant net increase of P_i was observed in $NaHCO_3$-P_i (1.40 μmol/g), NaOH-P_i (1.93 μmol/g), and HCl-P_i (0.23 μmol/g) pools, with the highest P_i concentrations measured around 56–126 d of incubation. It is interesting that the order of P pool was the same as that in the original (control) soil: NaOH-P_i > $NaHCO_3$-P_i > H_2O-P_i > HCl-P_i. Calculated P mass balance shows that the total increase in P_i among 4 pools was 4.30 μmol/g at the end of incubation, which accounts for ~86% of spiked glyphosate (5 μmol/g). The residual P in the control and glyphosate spiked soils were similar (7.99 ± 0.69 and 7.67 ± 0.69 μmol/g, respectively), indicating that there was no significant incorporation of glyphosate-derived P in the residual P pool. It also means that the Hedley extraction could efficiently extract almost all P and account P derived from biodegradation of glyphosate.

In terms of distribution P_i derived from glyphosate (Fig. 6), the H_2O- and $NaHCO_3$-P_i pools, which are considered readily available P_i for uptake by microorganisms and plant roots, received almost half (44%) of it. Meanwhile, around 33–38% of glyphosate P transformed into the NaOH-P_i pool, an unavailable or moderately bioavailable P pool depending on the soil P conditions and plant efficiency and time (Helfenstein et al., 2018). This means that this conditionally unavailable P pool might be further transported into open water systems by leaching or soil erosion and could increase the risk of polluting waters. The HCl-P_i, which is not directly

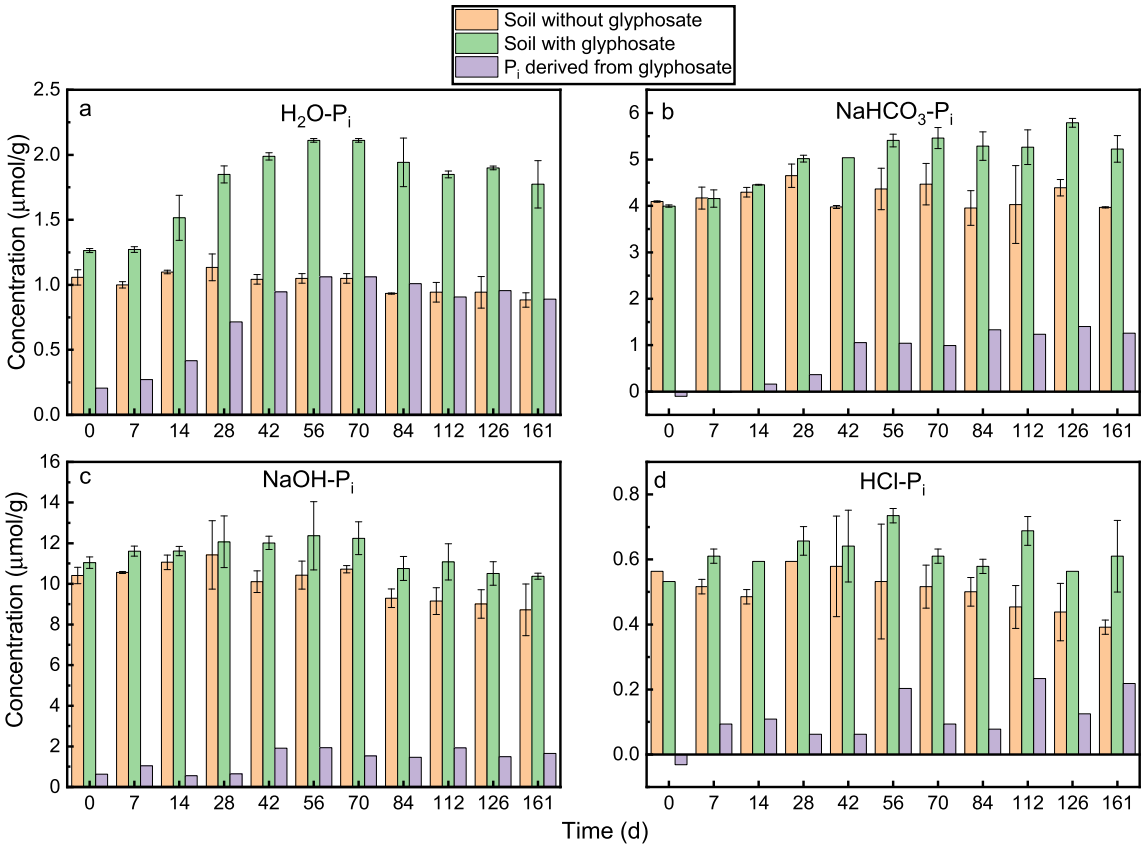

Fig. 5. Concentrations of P in different pools in original soil and glyphosate incubated soil during biotic degradation. H_2O and $NaHCO_3$ extracted P pools are considered bioavailable P in soil. Soil was spiked with 5 μmol/g glyphosate. Glyphosate derived P was calculated as the different between soil with and without glyphosate.

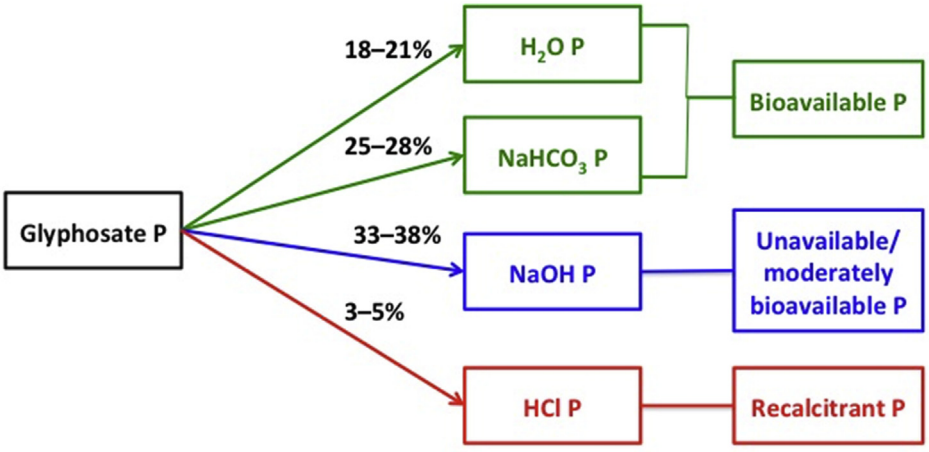

Fig. 6. Distribution of glyphosate-derived P to different P pools during its biodegradation in the soil-water system. Soil incubated with 5 μmol/g glyphosate.

utilized by plants and microorganisms and normally remains as an unavailable P pool in agricultural soil, only received 3–5% of P derived from glyphosate. These results highlight the fact that P load derived from a large amount of glyphosate application (with estimated 130 million kg used in the U.S.) (USGS, 2016) cannot be ignored.

Given that the P_i derived from glyphosate is steady means that it was gradually released as the degradation continues and distributed more into the bioavailable pool, and it may be a better P source for plants. Phosphorus fertilizer is the major P supply for plants with estimated 4 billion kg used in the U.S in 2014 (USEPA, 2018) with 50–70% use efficiency (Roberts and Johnston, 2015). However, its fast P release kinetics do not match the dynamic needs of different crop growth stages well (Liu et al., 2014) and this offset causes nutrient loss from soil to aquatic systems. Given the slow but steady P release from glyphosate degradation, it might be slightly more synchronous than commercial fertilizers, but still too fast than plant needs. Furthermore, multiple sprays of glyphosate during the crop lifetime (average of 1.6 times per crop year) (USGS, 2016) support the possibility of fractionating more into

bioavailable P that plants can readily take up. This demands reconsidering glyphosate not only use as a herbicide but a bonus P source to crops and should be included in estimations of crop P needs to improve the P efficiency of plant uptake as well reducing the P loss from agricultural soils.

3.3. Bioavailability of glyphosate-derived phosphorus

Once inside the cell, P_i is involved in several metabolic reactions catalyzed by enzymes including incorporation into cell biomass and ATP-ADP conversion. One of the unique enzymes is pyrophosphatase (PPase), which is highly conserved across all three domains of life, catalyzes the hydrolysis of pyrophosphate into P_i. This is a reversible reaction (Cohn, 1953) and leads to exchange of all four O atoms in P_i with O in ambient water and thus achieves O-isotopic equilibrium between phosphate and water. The equilibrium isotope depends on the temperature and water oxygen isotope value (Chang and Blake, 2015).

To further test the bioavailability and rate of microbial utilization of glyphosate-derived P_i, phosphate oxygen isotopes ($\delta^{18}O_P$) of P_i in the soil incubated with and without glyphosate were measured and compared with the equilibrium isotope values calculated from the temperature and oxygen isotopes of water ($\delta^{18}O_W$) in the experiments. The $\delta^{18}O_W$ values remained constant at −6.51 ± 0.30‰ and +18.27 ± 0.12‰ for two ^{18}O-labeled water experiments in the long-term incubation except at the end of the experiment (161 d), when an inadvertent evaporation resulted in slight enrichment of isotopes (−4.90‰ and +20.71‰, respectively). The expected isotopic equilibrium value ($\delta^{18}O_{P\text{-}eq}$) was calculated based on the (Chang and Blake, 2015) equation as:

$$\delta^{18}O_{p-eq} = e^{\left(\frac{14.43}{T} - 0.0265\right)} \times (\delta^{18}O_w + 1000) - 1000 \quad (3)$$

The $\delta^{18}O_{P\text{-}eq}$ values in the experiments incubated with −6.51‰ and +18.27‰ water are +15.83 ± 0.31‰ and +41.16 ± 0.12‰ (Fig. 7), respectively, and remained constant during the incubation period (except at 161d, in which water mass was not conserved). The starting isotope values of extracted P_i were consistent in all treatments: 20.77 ± 0.26‰, 21.02 ± 0.10‰, 21.38 ± 0.42‰ and 21.21 ± 0.16‰ in two controls (soil without glyphosate) and two glyphosate spiked experiments with −6.51‰ and +18.27‰ ^{18}O-labeled waters, respectively. It means that there are no different O sources or contaminants that might have impacted isotope values during the incubation period, besides the degradation of glyphosate.

The measured $\delta^{18}O_P$ values in the bioavailable P in ^{18}O spiked (+18.27‰) water became gradually heavier (Fig. 7a), shifting towards the equilibrium values (+41.16‰) and reached 32.04‰ at the end of incubation. This result reveals the rapid uptake of the available P by soil microorganisms and the release of cycled P back to the soil. At the early stage, $\delta^{18}O_P$ values of P_i in the soil spiked with glyphosate were consistent with those in original control experiments. However, they became lighter after 14 d and remained 1.2–2.5‰ lighter for a long period. This is due to the contribution from a much lighter isotope value of P_i (~4–9‰) derived from glyphosate (Li et al., 2016). The newly derived P_i from glyphosate degradation mixed with soil P_i pool and turned them into isotopic lighter and away from the equilibrium value (around +41.2‰). This result is consistent with P_i distribution that P_i was heavily released from glyphosate from 14 d to 84 d (Fig. 5) and preserving isotope record of the lighter glyphosate derived P_i in the system (see Fig. 5). However, the difference in isotope values between those two treatments gradually narrowed and eventually erased at 161 d, indicating that the soil microorganisms were efficient to uptake and cycle almost all of bioavailable P in the soil both from originally present soil and from glyphosate derived P_i.

The isotope trend in the experiments performed in −6.51‰ water (Fig. 7b) is comparable to heavy water, but with a minor difference. For example, the $\delta^{18}O_P$ values in glyphosate spiked soil became much lighter and reached the equilibrium value sooner than those from control soil (without glyphosate). The reason is that the P_i derived from glyphosate carries much lighter $\delta^{18}O_P$ values (as explained above), which brings the isotope values close to equilibrium (which is lighter: +15.83 ± 0.31‰, due to the lighter water oxygen isotopes). The gap between the two treatments was 0.8‰, and then increased to 2.3‰ due to the large contribution of lighter isotopes of glyphosate derived P_i, but with the enhancement of microbe turnover, it decreased again but still 1.6‰ off at the end of the incubation.

The observed results explained above provide several new insights on degradation of glyphosate and its metabolites and recycling of glyphosate derived-P and together have several implications on the fate and impact of glyphosate in soils. First, it proves that the isotope signature of glyphosate degradation can be detected in the experiments mimicking environmental systems. Second, it indicates that the degradation of glyphosate is faster than the microbial uptake and turn-over of P, so that the unique signature could be measured at the early to middle stage of the reaction. Third, if the $\delta^{18}O_P$ values of the P derived from organic compounds are farther/closer to the equilibrium range compared to those

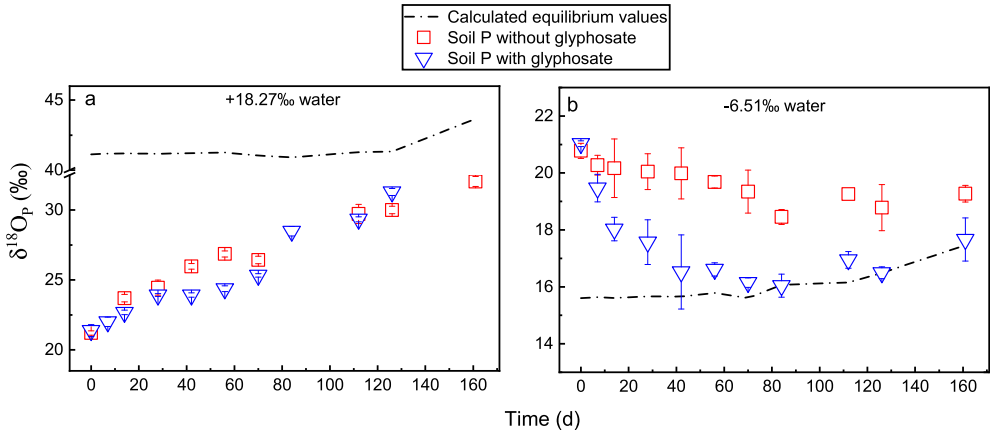

Fig. 7. Changes in phosphate oxygen isotopes during glyphosate biodegradation in the water-soil system. The calculated equilibrium values assumes all P is completely recycled by microorganisms. The closer the isotope values toward the equilibrium values, the higher the extent of P cycling.

present in-situ, they could easily shift/overprint bulk isotope value (due to mixing), leading to the inacurate estimation of the biological activities.

3.4. Microbial turnover of P in the soil-water system

To evaluate the extent of P taken up and recycled by soil microorganisms, the P turnover was calculated from the starting $\delta^{18}O_P$ values ($\delta^{18}O_{P\text{-}t0}$) at 0 h, measured values at time t ($\delta^{18}O_{P\text{-}t}$) and the equilibrium values ($\delta^{18}O_{P\text{-}eq}$):

$$\%P\ \ turnover = \frac{(\delta^{18}O_{p-t} - \delta^{18}O_{p-to})}{(\delta^{18}O_{p-eq} - \delta^{18}O_{p-t})} \times 100 \quad (4)$$

As the equation shows, the closer the values of $\delta^{18}O_{P\text{-}t0}$ to $\delta^{18}O_{P\text{-}eq}$, the higher the microbial turnover efficiency. The results show that P_i in the control experiment was rapidly exchanged by soil microorganisms and driven closer to the equilibrium values, with the turnover efficiency of 22–28% at 56 d and 45–48‰ at 161 d in two ^{18}O-labeled waters (Fig. 8). As expected, the efficiency of P turnover was similar irrespective of the starting isotopic values of ^{18}O-labeled water (−6.51‰ or +18.27‰).

In the glyphosate spiked experiments, the $\delta^{18}O_P$ value at time t ($\delta^{18}O_{P\text{-}t/spike}$) is the sum of glyphosate derived P_i ($\delta^{18}O_{P\text{-}t/gly}$) and the original P_i from control soil ($\delta^{18}O_{P\text{-}t/con}$), which can be calcualted from a simple mass balance equation as follows:

$$\delta^{18}O_{p-t/spike} = x\delta^{18}O_{p-t/gly} + (1-X)\delta^{18}O_{p-t/con} \quad (5)$$

where x is the fraction of P_i derived from glyphosate in the spiked samples. We calculated the starting isotope values of glyphosate derived P_i in two ^{18}O-labeled water systems at 0 h using previous results (Li et al., 2016), which are +6.92‰ and ±12.14‰ in −6.51‰ and +18.27‰ waters, respectively. Based on the starting values of glyphosate-derived P_i, its microbial turnover was calculated using equation (4). As shown in Fig. 8, the trend of P turnover in the soils receiving glyphosate-derived P_i was similar to that of control soil (without glyphosate), but the recycling efficiency was higher (67–75‰). Overall, phosphate oxygen isotopes allowed discrimination of sources and variable recycling efficiency of soil P vs glyphosate-derived P_i.

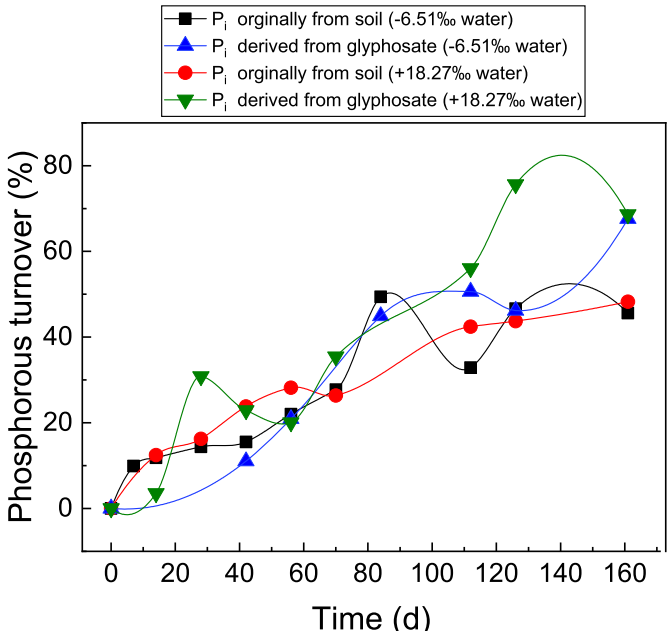

Fig. 8. Microbial turnover efficiency of soil P and glyphosate-derived P.

3.5. Glyphosate degradation pathways in soil

To understand the degradation pathways and specific preferences in the soil system studied, the released P_i extractable from four pools were combined together. The total P mass from glyphosate source was also calculated by adding glyphosate, AMPA, and released P_i and are shown in Fig. 3b. The released P_i steadily increased and reached the peak concentration around 56 d. AMPA remained at the accumulation stage and started to degraded at that time only when more than 80% of glyphosate was already degraded. There was slight decrease in total P (from original concentration of 4.92 μmol/g) at the early stage of degradation, and then remained almost constant during the incubation period. Consider the efficient extraction of the glyphosate-derived P_i, it implies that there might be some other non-detected P speciation during the early stage of glyphosate degradation besides glyphosate, AMPA, and inorganic P. A potential P compound could be methylphosphonic acid, which can be generated synchronously if glycine forms directly from glyphosate. Based on the data generated in this study and foregoing assumptions and published results (Jaisi et al., 2016; Zelenkova and Vinokurova, 2008), revised pathways and temporal preference of glyphosate degradation in the soil-water system is proposed and shown in Fig. 9. Under the action of soil microorganisms, at the early stage of degradation, glyphosate is cleaved at C(3)-N position to form glycine and methylphosphonic acid, the latter one is further degraded to form P_i, which accumulates in the system. Another bond cleavage occurs at C(2)-N position and form AMPA and glyoxylic acid. AMPA accumulates at the late stage of degradation. No sarcosine was generated in the soil-water system in this study, so it is not the required intermediate metabolite to form glycine.

4. Conclusion and implications

In this study, we studied degradation glyphosate and its metabolites and successfully utilized phosphate oxygen isotopes to confirm the biological availability of glyphosate-derived P in the simulated soil-water system. The broader conclusions derived from this study and the implications thereof are as follows:

1) A satisfactory method of extraction and separation of glyphosate and its major metabolites in soil was developed, which could be used to identify the fate of glyphosate in a variety of environments. The absence of degradation in sterilized soil showed the soil microorganisms play the essential role on the degradation of glyphosate. Temporal presence of glycine and AMPA varied as well as their microbial uptake and degradation. AMPA was found to be 3–6 times resistant than glyphosate against degradation, which brings a higher concern to the safety of environment.
2) The distribution of glyphosate-derived P_i in a soil was investigated. About half of the glyphosate-derived P_i transferred into the readily bioavailable P pool. A slow but steady release of P_i from the degradation of glyphosate could mean that its supply could be slightly more synchronous with plant P demand during plant growth especially becuase it is applied more than one time during a crop cycle. This means that a higher proportion of glyphosate-derived P, than P from commercial fertilizers which release P all at once, could be taken up by plants.
3) Glyphosate-derived P_i has a distinct isotopic signature and can aid in identification of its source. The natural environment,

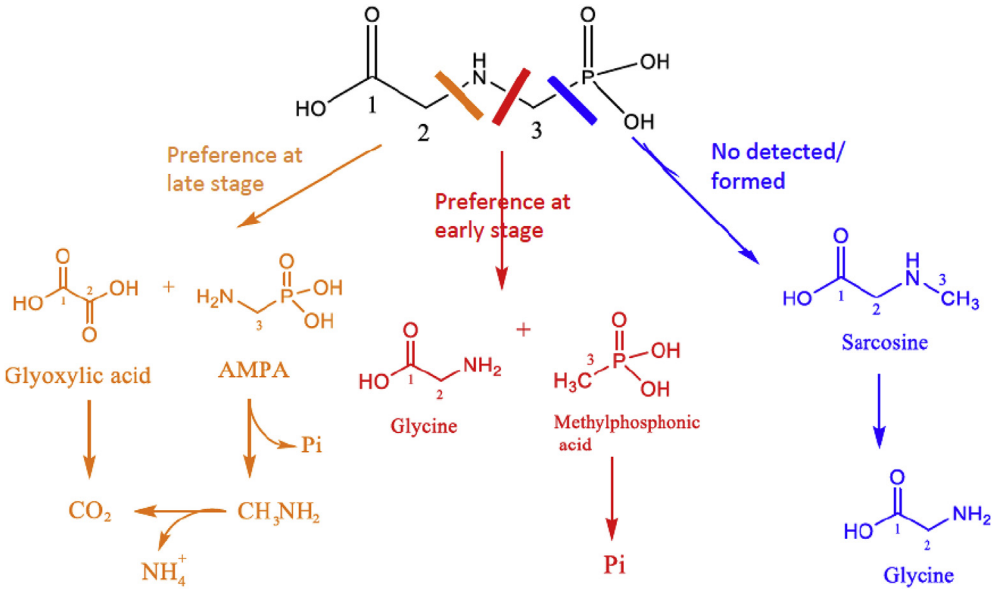

Fig. 9. (Bio)degradation pathways of glyphosate and preference of degradation in the water-soil system used in this study.

however, is complex and could pose additional challenges, most likely due to the low content of glyphosate and inappropriate sampling time could miss to detect significant offset of isotope values. This is because isotope signature could be erased or overprinted due to biological cycling of glyphosate-derived P.

4) ^{18}O-labeling in water and application of phosphate oxygen isotope method allowed explicit understanding of microbial uptake and extent of biological turnover of glyphosate derived-P. The microbial turnover of original P in soil and glyphosate-derived P was comparable, but it was found that the microorganisms were more efficient to utilize and recycle glyphosate-derived P. The research tool developed could be further used to investigate the extent of microbial activities in soils and other natural environments.

Declaration of interest statement

None.

Author agreement

This manuscript has not been published, is not currently submitted for publication elsewhere, wholly or in part, nor will it be submitted elsewhere while in the review process in Water Resources.

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