Glyphosate, AMPA and glufosinate in soils and earthworms in a French arable landscape.

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Abstract

Although Glyphosate-based herbicides are often marketed as environmentally friendly and easily biodegradable, its bioavailability and risks to wildlife raise significant concerns. Among non-target organisms, earthworms which live in close contact with the soil can be directly exposed to pesticides and harmed. We investigated soil contamination and the exposure of earthworms to glyphosate, its metabolite AMPA, and glufosinate in an arable landscape in France, both in treated (i.e. temporary grasslands and cereal fields under conventional farming), and nontreated habitats (i.e. hedgerows, permanent grasslands and cereal fields under organic farming) (n=120 sampling sites in total). Glyphosate, AMPA and glufosinate were detected in 88%, 58% and 35% of the soil samples, and in 74%, 38% and 12% of the earthworm samples, respectively. For both glyphosate and AMPA, concentrations in soils were at least 10 times lower than predicted environmental concentrations. However, the maximum glyphosate soil concentration measured (i.e., 0.598 mg kg^{-1}) was only 2 to 3 times lower than the concentrations revealed to affect earthworms (survival and avoidance) in the literature. These compounds were found both in conventional and organic farming fields, thus supporting a recent study, and for the first time they were detected in hedgerows and grasslands. However, glyphosate and AMPA were more frequently detected in soils from cereal fields and hedgerows than in grasslands, and median concentrations measured in soils from cereal fields were significantly higher than in the two other habitats. Bioaccumulation of glyphosate and AMPA in earthworms was higher than expected according to the properties of the molecules. Our findings raised issues about the high occurrence of glyphosate and AMPA in soils from cropped and more natural areas in arable landscapes. They also highlight the potential for transfer of these molecules in terrestrial food webs as earthworms are prey for numerous animals.

Keywords: habitats; agriculture; exposure; terrestrial invertebrates; accumulation

1. Introduction

Since its introduction on the market in 1974 (Richmond 2018), the global use of glyphosate (N-(phosphonomethyl)glycine) has increased (Székács and Darvas 2018), making it the most widely used herbicide in the world. Worldwide, more than 800,000 tons are applied each year and more than 750 commercial formulations containing the molecule are authorized (Benbrook 2016; Kniss 2017), environmental and health impacts of which have raised numerous questions this past decade (Mesnage et al., 2015; EPC EU 2017). Whereas the renewal of the marketing authorization for glyphosate (European Commission, 2017b) evaluated by the European Commission does not find a consensus, the evaluation reports emphasize the lack of knowledge and data on the concentrations of glyphosate and metabolites in environmental matrices (in particular animal matrices), and on the assessment of the risks to biodiversity. This lack of fundamental knowledge and field data on the ecotoxicology of glyphosate hamper decisions on its regulation and constitutes a limit to the objectives of assessing risks and impacts of this herbicide. The glufosinate, which is mostly used under the glufosinate-ammonium form, was considered by the European commission (2017a) as “one of the very few alternatives to glyphosate”. Regarding the similarities in the chemical structure and properties of the two herbicides (PPDB, 2021), we can expect a similar behavior of them in sols and earthworms.

Despite its theoretical short persistence in soil (half-life DT50 field of 6.45 days; Pesticide Properties Database (PPDB) 2021), glyphosate and its main degradation product, AMPA (aminomethylphosphonic acid) (DT50 field of 32 days; Simonsen et al., 2008), are the two most commonly found pesticides in rivers in France and other European countries (Carles et al. 2019; Medalie et al. 2020). In the USA and Argentina, glyphosate and AMPA are usually detected together, occuring widely in sediments, ditches and drains, precipitation, rivers and streams (Aparicio et al., 2013; Battaglin et al., 2014; Medalie et al., 2020). Data are however scarcer regarding their concentrations in other environmental compartments such as soil and even more rare in living organisms. The few studies that measured glyphosate and AMPA in natural soils reported that they were among the active substances the most frequently found and at the highest concentration in agricultural and urban soils (Karasali et al., 2019; Geissen et al., 2021; Silva et al., 2019). Geissen et al. (2021) found AMPA in 96 and 83% of the topsoil samples from conventional and organic fields, respectively. As far as we know, no data exist on the contamination of semi-natural landscape elements such as hedgerows or grasslands by glyphosate, AMPA and glufosinate. Yet, in farmland, these habitats are refuges for biodiversity (Geiger et al., 2010) and the occurrence of these compounds can be a threat for living organisms. Thus, assessing the contamination by glyphosate, its metabolite AMPA and glufosinate would bring knowledge on biodiversity exposure in these areas.

Among non-target organisms, animals living in close contact with the soil can be directly exposed to pesticides and harmed (Gill et al., 2018; Gunstone et al., 2021). Earthworms are key soil organisms for their role in soil structure, organic matter dynamics, productivity, and more generally for their contribution to a number of ecosystem services (Schon and Dominati, 2020). Numerous studies have shown negative impacts of glyphosate and glyphosate-based herbicides at recommended rates on earthworms, their activity and the soil functions to which they contribute, with potential implications on growth, yield and quality performance of plants (Owagboriaye et al., 2020a; Zaller et al., 2021). To date, only two studies were found on glyphosate bioaccumulation in earthworms, both under laboratory conditions, and both showing that earthworms bioaccumulated glyphosate proportionally to the contact period (bioaccumulation factor >1) (Andréa et al., 2004; Owagboriaye et al., 2020b). No data are available on the accumulation of glyphosate under natural conditions, or on AMPA and glufosinate concentration in earthworms, either in laboratory or under field conditions. Characterizing the exposure and potential bioaccumulation under natural conditions could help for a better understanding of the link between agricultural practices and impacts on soil non-target organisms as well as assess the potential for transfer of the molecules in food webs as earthworms are prey for numerous animals.

The aim of this study was to assess the concentrations of glyphosate, its metabolite AMPA and glufosinate in soils and earthworms in 120 pesticide-treated and nontreated habitats of a cereal plain. We hypothesized that the concentrations of glyphosate, AMPA, and glufosinate in soils and earthworms would be higher in treated habitats than in seminatural habitats and organic fields which are not directly targeted by pesticides. We also hypothesized, according to literature, that bioaccumulation of the molecules would occur in soil organisms. Based on the concentrations we measured in soils and earthworms sampled within the same habitats, bioaccumulation factors were calculated to assess the potential for accumulation in organisms and in food webs of the 3 compounds. Measured concentrations in soils were compared to predicted environmental concentrations of the studied pesticides in soils (i.e. PEC_s provided in risk assessment documents according to the European regulation) and to toxic thresholds for earthworm to assess potential environmental risk.

2. Materials and methods

2.1. Sampling area and design

Sampling was conducted during one week in March 2018, in the Long-Term Socio-Ecological Research Site (LTSER) “Zone Atelier Plaine & Val de Sèvre” (ZAPVS; Bretagnolle et al., 2018), where forty sampling squares of 1 km^2 were selected (Figure 1). Spring has been chosen as it is a period of great activity for earthworms (Bouché, 1972).

In the sampling area, glyphosate applications occurred at two periods during the cropping season 2017-2018, in autumn (early September 2017) before winter crops sowing, and in spring (April to mid-May 2018) before spring crops sowing. Glyphosate is never applied during the crop growing season, being rather mainly applied for weed control before sowing. Thus, it was not applied in all the sampled cereal fields. It was applied only in one of the sampled cereal fields as a pre-sowing treatment (i.e., Sampling location 18 C in Table S1). Based on a National survey conducted in agricultural areas all over France in 2018-2019, glyphosate in air in France was detected in every sample and with concentrations among the highest (0.05 to 0.2 ng m^{-3}) in May and June, while the less likely to be detected (17-43%) with concentrations being the lowest (around 0.01 ng m^{-3}) from November to February (LCSQA/Ineris, 2020). During the period March-April, the frequency of detection in air varied around 62 to 75% and the concentrations varied around ranged between 0.025 ng m^{-3} (LCSQA/Ineris, 2020).

2.2. Collection of soils and earthworms

In each of the forty sampling squares of 1 km^2, soils and earthworms were sampled in three habitats: arable fields sown with winter cereals, grasslands and hedgerows (as close as possible to the cereal field) (Figure 1), for a total of 120 sampling plot locations. Fifteen grassland soils were considered nontreated by pesticides, which came from either temporary grasslands under organic farming (*n* = 10) or permanent grasslands (*n* = 5). Eleven winter cereal soils under organic farming conditions were collected, and were thus considered nontreated. The farming practices in the organic cereal fields and grasslands respected the rules of the AB France label and were under organic farming for at least three years at the time of sampling.

At each sampling plot, three soil cores were sampled using a 5 cm Ø soil auger at a 0–5 cm depth. They were bulked to obtain one composite sample per plot. The 120 soil samples were frozen at -20°C before being analyzed (Table S1). Soil properties were measured at the Soil Analysis Laboratory of INRAE (LAS Arras – "Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement", France), which benefits from the COFRAC (French accreditation committee) accreditation of its analytical quality regarding soil characteristics. Briefly, soils were dried at room temperature and then disaggregated and homogenized before being sieved at 2 mm. The following soil characteristics were measured, according to international standard methods (for individual references for the cited standard methods please see AFNOR 2004): pH (water suspension, NF ISO 10390), organic matter and nitrogen contents (dry combustion in a CHN autoanalyzer Carlo Erba NA 1500, in g kg^{-1}), grain size distribution (NF X 31-107) (clay < 2 µm, silt 2-20 µm, and sand > 20 µm, in g kg^{-1}), total calcium carbonate $CaCO_3$ (in g kg^{-1}), and total phosphorus P_2O_5 (by ICP-MS spectrometry, in g kg^{-1}, NF ISO 22036, NF X31-147).

Between two and five earthworm individuals were also collected at each sampling plot. We chose the earthworm species *Allolobophora chlorotica* which is well represented in the different sampled habitats in the ZAPVS. Moreover, this endogeic species lives within the top centimeters of the soil. Because pesticides generally accumulate at the soil surface, species living in contact with the soil surface will potentially be more strongly exposed than those living deeper (Pelosi et al., 2013a). The earthworm individuals were sampled by superficially digging the soil. Before being weighed and frozen at -80°C, earthworms were individually placed in petri dishes on damp filter paper for at least 48 h to void their gut contents.

2.3. Analysis of the pesticide residues

The analysis of glyphosate, aminomethylphosphonic acid (AMPA), and glufosinate in both the 120 soils and 120 earthworm samples (*A. chlorotica*) was performed according to a new sensitive and selective method (Delhomme et al., 2021) (Table S1). Briefly, the samples (15 g for soils, and between one to three earthworm individuals pooled according to their mass to attain approximately 1 g) were extracted with a borate buffer and derivatized with 9-Fluorenylmethyl chloroformate (FMOC-Cl). The excess FMOC-Cl was removed by liquid-liquid extraction with diethyl ether. The purification of derivatized extracts was carried out by solid phase extraction (SPE) on Chromabond XLB cartridges (Macherey-Nagel, France) before internal standard quantification by liquid chromatography coupled to tandem mass spectrometry (LC/MSMS). The elution step was performed with acidic methanol (1% formic acid). The extraction and purification method followed by analysis of the two herbicides and AMPA in soils using LC/MSMS determined limits of detection (LOD) and quantification (LOQ) that are described in Table 1. Concentrations in soils are expressed in mg kg^{-1} dry weight (DW), and concentrations in earthworms are expressed in mg kg^{-1} fresh weight (FW). For quality control, soil and earthworm samples were spiked with two internal standards before the extraction and derivation phase. One of the two internal standards was used for the calibration and the second one was used to evaluate the effectiveness of the derivatization step (see Delhomme et al. (2021) for more details). Recoveries of glufosinate, glyphosate and AMPA obtained during the optimization steps are shown in Delhomme et al. (2021).

Glyphosate and glufosinate concentrations in soil samples were compared to the recommended field rate for application, converted in mg kg^{-1} soil. For that, we considered that the average glyphosate use rate ranged from 0.40 to 4.32 kg active ingredient a.i. ha^{-1} in cereal crops (Antier et al., 2020; EFSA, 2015). For most glufosinate-based herbicides, the upper limit of recommended application rate is 5 L ha^{-1} with an active concentration in commercial herbicide of 200 g L^{-1} (e.g., Dennis et al., 2018 for Basta®). Considering a penetration of 5 cm depth and a soil bulk density of 1.5 (EFSA, 2017), the recommended dose of glufosinate is considered as 1.33 mg kg^{-1} soil DW and it ranges from 0.53 to 5.76 mg kg^{-1} soil DW for glyphosate.

To assess pesticides' bioaccumulation in earthworms (i.e. the net uptake of a pesticide from the environment by all possible exposure routes, e.g., respiration, diet, dermal), a bioaccumulation factor

(BAF) was calculated as the ratio of the chemical concentration in earthworm (in mg kg^{-1})fresh weight (FW) according to OECD (2010)) to the soil concentration (in mg kg^{-1} DW). When the soil concentrations were < LOD, the value LOD/2 was attributed (in order to be able to calculate a ratio). The ratio with the concentration in earthworm dry weight (DW) was also calculated in order to be able to compare with the few available data of the literature. As earthworms are composed of 65 to 90% water depending on the state of hydration (Lee, 1985), we considered 80% and calculated the concentration in earthworm DW as concentration in earthworm FW/0.2.

2.4 Risk assessment for earthworms

The predicted environmental concentrations of glyphosate, AMPA and glufosinate in soils (PECs; concentrations at recommended application rates obtained from calculation, modelling and/or measured concentrations in trials), and acute (LC_{50}) or chronic (NOEC reproduction) toxicity thresholds for earthworms (*Eisenia fetida*) were collected from evaluation reports or reports of risk assessments (i.e. (European Commission, 2015; EFSA, 2005, 2015) and/or scientific publications. *E. fetida* is the species recommended in risk assessment procedures before pesticide marketing authorization at the European level (ISO 11268-1, 1993). Although it has been shown to be less sensitive to pesticides and metabolites than earthworm species present in cultivated fields, it was used in this study because data were available for the three studied chemicals. The risks for earthworms calculated with *E. fetida* can thus be underestimated.

In order to provide quantitative data about the general patterns of contamination with regards to expected levels in the environment, the measured concentrations in soils (MECs) in our study for glyphosate, AMPA and glufosinate were compared to the predicted environmental concentrations (i.e. PECs initial after treatment, long term PECs and maximum PECs calculated for winter wheat when available, or for other cereals). MECs are hypothesized to be equal to or lower than the maximum PECs. In order to evaluate the potential ecotoxicity of MECs to earthworms, MECs in soils were also compared to the values of LC_{50} or NOEC for earthworms. This was conducted following the classical risk assessment method for pesticide regulation defined by European legislation by assessing toxicity/exposure ratio for earthworms (i.e toxicological benchmarks divided by MECs) with trigger values of 10 for acute toxicity and of 5 for chronic toxicity set as risk limits (TERs above the trigger limits indicate a negligible risk probability) (European Commission, 2003; EPC EU, 2009).

2.5. Statistics

Except for the calculation of the BAF (see section 2.3), when a pesticide was not detected in a sample (value < LOD), the concentration value was set at 0 to perform statistical analyses. When a pesticide was detected at a level below the LOQ but above the LOD, the LOD value was attributed. ANOVA was used to compare the pesticide variables (i.e., number of molecules or concentrations) in earthworms or soils between the three habitats (i.e., cereal fields, grasslands, hedgerows). When assumptions regarding the normality and homoscedasticity of variances were not respected, we used Kruskal-Wallis tests. The paired samples t-test was used to compare earthworm and soil pesticide variables between the two modalities of pesticide use (treated/nontreated) at plot scale. When assumptions regarding the normality and homoscedasticity of variances were not respected, we used the Wilcoxon tests. For the differences between conventional and organic systems (t-test or Wilcoxon test), the data from the hedgerows were removed from the dataset, considering only cereal fields and grasslands in the analysis. All statistical analyses were performed in RStudio version 3.5.1 using the packages pgirmess (Giraudoux, 2018) and car (Fox et al., 2018).

3. Results and discussion

3.1. Occurrence and concentrations in soils

Glyphosate, AMPA and glufosinate were detected in 88%, 58% and 35% of the soil samples respectively Table 2a; Table S1). The high frequency of glyphosate and AMPA in the soils is probably due to the popularity of glyphosate-based herbicides in current agricultural practices (Gill et al., 2018). Moreover, although it is expected to be biodegradable (Kissane and Shephard, 2017) and non-persistent in soils, the DT90 lab and field of glyphosate are 297 and 170 days respectively (PPDB, 2021), suggesting that residues could be detected in soils for at least 6 to 10 months after a treatment. These findings on occurrence are slightly higher for glyphosate than in Geissen et al. (2021) who found a frequency of 78% in vegetables, orange, grape and potatoes cropping systems, while we detected AMPA less often than in the cited study, which reported a frequency of 83%. The frequencies of detection of both glyphosate and AMPA were higher here than in Silva et al (2019) who showed 21% and 42% of occurrence, respectively, in 317 EU agricultural topsoils (from conventional crops including cereals, orchards and vineyards, oilseed rape and sunflower, potatoes and sugar beet, other vegetables, and dry pulses, flowers and fodder crops such as temporary grasslands, alfalfa, clovers and strawberries). Despite differences between studies that can be due to cultivated crops, sampling date with respect to pesticide applications, differences in application rates, climate, depth of soils sampling, soil properties such as texture, pH, CEC (cation exchange capacity) or organic carbon (Dollinger et al., 2015; Nguyen et al., 2018), our results are in line with them by revealing residues of glyphosate and AMPA in a great proportion of arable soils. Moreover, limits of quantification were higher in Geissen et al. (2021) and Silva et al. (2019) (i.e., 0.05 mg kg^{-1} for both glyphosate and AMPA) than in our study (0.030 mg kg^{-1} and 0.025 mg kg^{-1} for glyphosate and AMPA, respectively), which can explain that these authors had lower detection frequencies.

All the 58% of the soil samples containing AMPA also contained glyphosate, and in the 12% of cases where no glyphosate was found, the AMPA was concomitantly undetected. The co-occurrence of glyphosate and AMPA and the significant correlation between the soil concentrations of glyphosate and AMPA (Pearson coefficient of 0.37, see Table S2) are coherent considering that AMPA is a glyphosate's transformation product, thus the parent and the by-product compounds are associated. However, glyphosate was detected without AMPA in 29% of the samples. AMPA is the main but not the sole metabolite as an alternative pathway may conduct to sarcosine and glycine (Singh et al., 2019), which were not investigated in this study. However, unlike glyphosate, AMPA has been classified as persistent in soils, with a typical half-life of 151 days, but varying from 76 to 240 days depending on field conditions (Lewis et al., 2016). Studying agricultural topsoils from six different crop types (i.e. cereals, root crops, non-permanent industrial crops, dry pulses and fodder crops, permanent crops, vegetables and others) from eleven countries across Europe, Silva et al. (2019) showed that AMPA was the prominent form, as it occurred in 42% of soils whereas glyphosate was detected in 21%, and both compounds were present in 18% of the soil samples. Differences with this study regarding AMPA could be explained by the fate of glyphosate which depends on the considered matrix (e.g., water, different types of soils), sampling date with respect to pesticide applications (higher glyphosate:AMPA ratio if samples were collected close to the time of application), the frequency and spatial extent of herbicide-treatments over the studied areas, as well as exogeneous inputs from wind and water transport (Dollinger et al., 2015; Silva et al., 2019). Moreover, AMPA may come from other sources than glyphosate degradation (e.g., adjuvants, detergents, plastics), which may have been less preponderant in the case of the ZAPVS cereal plain.

The maximum contents of glyphosate, AMPA and glufosinate in soils were 0.598 (in a grassland), 0.135 and 0.041 mg kg^{-1} (in cereal fields for both) respectively (Table 2.a). For glufosinate, this value is more than 30 times lower than the upper limit of recommended application rate (see section 2.3 for the calculation). For glyphosate, this value is very close to the calculated lowest value of the recommended dose (i.e., 0.53 mg kg^{-1} in cereal crops, see section 2.3). The measured maximum contents are much lower than those reported by Silva et al. (2019) (i.e. 2.05 and 1.92 mg kg^{-1} for glyphosate and AMPA respectively) or Laitanen et al. (2006) (i.e., 2.06 and 0.30 mg kg^{-1}, respectively). Similarly, median values were, in our study, 0.088, 0.007 (i.e. < LOQ) mg kg^{-1} and < LOD (0.006 mg kg^{-1}) for glyphosate, AMPA and glufosinate, respectively, whereas Silva et al. (2019) reported 0.14 and 0.15 mg kg^{-1} for glyphosate and AMPA, respectively. Some of the differences might be explained by the higher number of soils sampled investigated in Silva et al. (2019) in a larger geographical zone, involving a larger range of agricultural practices and climate (i.e., 317 samples, with southern parts of the EU showing the highest concentrations of glyphosate and AMPA in topsoils), or by the higher number of nontreated habitats included in our study (i.e. hedgerows, permanent grasslands and fields under organic farming, versus conventional crops only in Silva et al. 2019). Moreover, analytically speaking, extraction yields for glyphosate and AMPA were highly variable for two different soil types (Laitinen et al., 2006). Finally, this result could be partly due to the depth of the soil samples, being 0-5 cm in our study and 0-15/20 cm in Silva et al. (2019). Although glyphosate and AMPA were found to be mainly retained in the upper soil surface layer (in the first cm of soil according to Yang et al., 2019), residues can be also found down to 1 m soil depth (Lupi et al., 2019; Laitinen et al., 2006). For instance, Lupi et al. (2019) mainly found glyphosate and

AMPA in soil at a depth of 0-5 cm but they also reported smaller concentrations of residues at 5-9 cm and deeper (45-60 cm and 130-140 cm).

3.2. Occurrence and concentrations in earthworms

For earthworms, when considering all samples (i.e., from cereal fields, hedgerows and grasslands), the frequency of the three studied molecules were lower than in soils (Table 2.b, Table S1). Glyphosate, AMPA and glufosinate were detected in 74%, 38% and 12% of the samples, respectively (on average over the three habitats, see Table 2.b). In cereal fields, the frequency of glyphosate is the same for soils and earthworms (i.e., 95%, see Table 2). In 36% of the sampled earthworms, glyphosate and AMPA were detected together. In 23% of cases, no glyphosate and no AMPA were found. A significant correlation was found between the concentrations of glyphosate and AMPA in earthworms (Pearson coefficient of 0.47, see Table S2). AMPA was detected without glyphosate in 3% of the earthworm samples, whereas glyphosate was detected without AMPA in 38% of the samples. Thus, as for soil, AMPA was almost never found without the parent molecule in earthworms. Conversely, more than a third of samples contained detectable glyphosate but no its metabolite. This might be considered as surprising given the expected biodegradability of glyphosate, and might suggest a higher persistence and/or bioavailability of the parent molecule than anticipated from the literature as emphasized by Kissane and Shephard (2017).

The maximum concentrations of glyphosate, AMPA and glufosinate in earthworms were 0.395, 0.247 and 0.04 (< LOQ) mg kg^{-1} FW, respectively, being all from cereal fields (Table 2.b, Table 5). Median values in earthworms were 0.07 (<LOQ) mg kg^{-1} FW for glyphosate and < LOD for AMPA and glufosinate. To our knowledge, this is the first study which quantified the amount of these three chemicals in earthworms under natural conditions, making it impossible to compare with previous data.

The median value for the calculated BAF (FW/DW) for glyphosate was around 1 (maximum 15.56, measured four times in nontreated habitats: two organic cereal fields, one hedgerow and one organic temporary grassland), and around 5 on DW basis (maximum 78) (Table 3). This is in line with the results reported by Owagboriaye et al. (2020b) under laboratory conditions on two earthworm tropical species which were found to be bioaccumalators and biomagnifiers of glyphosate (BAF > 1 after 8th week post glyphosate application). Similarly, Contardo-Jara et al. (2009) reported that the glyphosate bioaccumulation factor for *Lumbriculus variegatus,* a sediment dwelling invertebrate, varied between 1.4 and 5.9 which was higher than estimated from chemical properties (i.e., log P_{ow}). For AMPA, in our study, the median BAF (FW/DW) varied between zero in grasslands and hedgerows to 1.62 in cereals (maximum 67.14 in a conventional cereal field), corresponding to median BAF DW/DW around 8 in cereals (maximum values from 93 in a hedgerow, 147 in a grassland and 336 in a conventional cereal plot) (Table 3). This meant that AMPA would be less often accumulated but at higher concentration than glyphosate and/or that it would be less metabolized by earthworms. The method developed by Delhomme et al. (2021) to measure glyphosate and AMPA in earthworms should help going further on toxicokinetics of glyphosate in earthworms.

We found in our study that bioaccumulation was higher than expected according to the low measured or expected bio-concentration factors (BCF, Table 4), low Partition Coefficient n-Octanol/Water (log K_{ow}, Table 4) and the high solubility of glyphosate in water. With a Partition Coefficient Octanol/Air log K_{oa} ranging from 7.26 to 8.40 (Table 4), glyphosate, AMPA and glufosinate fall into the category of high log K_{oa} (i.e., >6) compounds, which have a potential for bioaccumulation in air-breathing organisms and biomagnification in terrestrial food webs because of slow respiratory elimination rate (Fremlin et al., 2020; Kelly et al., 2007). Indeed, Kelly et al. (2007) evidenced that moderately lipophilic compounds having a low log K_{ow} (between 2 and 5) but a high K_{oa} can biomagnifiy in food webs containing air-breathing animals while they do not in aquatic food webs. Here we found that concentrations of glyphosate and AMPA in soils and earthworms were all highly correlated to each other (Table S2) and that glyphosate and its transformation products may bioaccumulate in earthworms, organisms that are a trophic resource for many invertebrates, birds and mammals. Altogether, this raises questions about the behaviour of glyphosate, AMPA and glufosinate in terrestrial food webs but, currently, models usable to assess both the trophic transfer and magnification potential for these types of polar ionic chemicals (i.e., hydrophilic, where lipid is a not the main storage compartment within the organism) are lacking (Gobas et al., 2016).

Glufosinate was found at relatively low frequency and concentrations in soils and earthworms. This is coherent with the literature which reports a quick degradation of this chemical in soils (DT50 of 7.4 days for glufosinate ammonium, PPDB 2021). Furthermore, glufosinate was much less used than glyphosate. The reported sales of glyphosate in the county “Deux-Sèvres” where the site is located reached 113 and 115 tons in 2017 and 2018, respectively, while the sales of glufosinate were reported as 0.2 and 0.1 tons, respectively (BNVD, 2020). Additional analytical development to investigate the occurrence and fate of transformation products of glufosinate would be needed to get further insights into the toxicokinetics and environmental impact of this herbicide.

3.3. Patterns of contamination according to habitats and agricultural management

Glyphosate was more frequently detected in soils from cereal fields and hedgerows (95 and 93%, respectively) than in grasslands (75% of the samples) (Table 2a). Moreover, median concentrations of glyphosate measured in soils from cereal fields were significantly higher than in the two other habitats (Table 5). When considering only cropped fields as it is done in the rare studies on glyphosate in soils, glyphosate was more frequently detected but at slightly lower concentrations compared to Geissen et al. (2021) or Silva et al. (2019). Pesticide use (i.e. treated/nontreated by pesticides) and cropping system (i.e. field under conventional/organic farming) did not influence the mean concentration of the three studied molecules (Table 5). This means that the concentration of glyphosate, AMPA and glufosinate in soils were not influenced by the cropping system, but by the type of habitat itself. This is in accordance with Geissen et al. (2021) who showed that AMPA was the most frequent residue found in both conventional and organic fields, with a frequency of 96 and 83%, respectively. Here, the maximum concentration of glyphosate in soils was reached in a temporary grassland, in which glyphosate can be used to destroy grass before switching to an annual crop. More surprisingly, the maximum concentration of AMPA was found in a cereal field under organic farming since 2009.

When calculable (i.e., glyphosate concentration > LOD), the median (min value; max value) of the concentration ratio AMPA:glyphosate was 0.28 (0; 1.01) in cereal fields, 0.09 (0; 8.33) in hedgerows and 0.00 (0; 0.78) in grasslands. This suggests that the degradation of glyphosate in grasslands is lower than in the other two habitats. Soil characteristics and notably exchangeable acidity (H^+ and

Al^{3+}), exchangeable Ca^{2+} ions and ammonium lactate extractable K were reported to be the key soil parameters governing glyphosate mineralization (Nguyen et al., 2018). These parameters were not measured in our soils but pH, which can inform on exchangeable acidity, was not different between the three habitats (mean of 8.1, 8.1 and 8.2 for cereal fields, hedgerows and grasslands, respectively, n=40). The organic matter content was 46, 88 and 53 g kg^{-1} in cereal fields, hedgerows and grasslands, respectively, thus not explaining neither the potential difference in glyphosate degradation between the three habitats. Moreover, we found no significant correlation between soil concentrations of glyphosate or AMPA and soil characteristics (Table S2), except a weak one (-0.19) between sand content and soil glyphosate concentration (Figure S1). As glyphosate was less frequently found in grasslands compared to the other habitats (Table 2), microorganisms could be less adapted to glyphosate degradation in this habitat (Schlatter et al., 2017).

In earthworms, glyphosate and AMPA were more frequently detected (Table 2b) and at higher concentrations (Table 5) in cereal fields than in the other two habitats, and the highest glyphosate and AMPA concentrations (i.e., 0.395 and 0.247 mg kg^{-1}, respectively) were measured in individuals sampled in two different conventional cereal fields. The maximum concentration measured for glyphosate in a cereal field is 50% higher than the maximum raised in hedgerows and grasslands (Table 2b). As for soils, cropping system (conventional/organic farming) did not influence the mean concentration of the three studied molecules found in earthworms (Table 5). However, a higher median concentration of glyphosate was measured in earthworms from treated habitats (0.15 mg kg^{-1} in temporary grasslands and fields under conventional farming) than from habitats where pesticides were not applied (<LOQ in hedgerows, permanent grasslands and fields under organic farming) (Table 5).No correlation was found between the concentrations of glyphosate or AMPA in earthworms and the soil characteristics (Table S2). The BAF for glyphosate did not differ between the three habitats (Kruskal-Wallis test, p = 0.480) but the BAF calculated for AMPA was the highest for earthworms sampled in cereal fields (BAF cereals > BAF grasslands, Kruskal-Wallis test $p < 0.001$; non-significant differences between hedgerows and others) (Table 3). This finding underlines potential issues related to the contamination of an important trophic resource for many terrestrial animals.

3.4. Risk to earthworms

Considering predicted environmental concentrations in soils (PEC$_s$), the maximum concentrations of glyphosate were about ten times lower than the maximum PEC$_s$ which were calculated at 5.974 mg kg^{-1} and 6.616 mg kg^{-1} in the worst case for annual and permanent crops, respectively (European Commission, 2015; EFSA, 2015). For AMPA, measured concentrations reached maximum values that were 30- to 60-fold lower than accumulated PEC$_s$ (3.0719 mg kg^{-1} and 6.1797 mg kg^{-1} for annual and permanent crops, respectively). Finally, concerning glufosinate, the PEC$_s$ provided in regulatory documents reached 2.0 mg kg^{-1} initially and 0.32 mg kg^{-1} a hundred days after maximum, which is again several order of magnitude greater than the measured values in the sampled soils (European Food Safety Authority (EFSA), 2005).

Information on glufosinate effects on earthworms are almost inexistent and the concentrations obtained in this study were low. In the pesticide risk assessment of glufosinate released in 2005 (EFSA, 2005), the LC$_{50}$ for earthworms was reported as higher than 1000 mg kg^{-1} dry soil for the active substance as well as for several commercial formulations and the transformation product 3-methyl-phosphinico-propionic acid (MPP). For the two other transformation products of glufosinate (i.e., 2-methylphosphinico-acetic acid (MPA) and N-acetyl-glufosinate (NAG)), LC$_{50}$ for earthworms are far from trace levels (> 760 and > 300 mg kg^{-1} dry soil, respectively) (EFSA, 2005).

One study provides a value allowing to calculate a risk of AMPA concentrations in soils to earthworms (Von Mérey et al., 2016). The reproductive no-observed-effect concentration (NOEC, after a 4-week adult exposure period) was 198.1 mg kg^{-1} dry soil, a value close to another previous NOEC for reproduction calculated at 131.9 mg kg^{-1} (European Commission, 2015; EFSA, 2015), which is 800 times higher than the highest AMPA concentrations measured in the sampled soils. This value was obtained through tests under laboratory conditions with *E. fetida* which is known to be less sensitive to pesticides than other earthworm species (Pelosi et al., 2013). However, our results, along with those of Von Mérey et al. (2016), suggested low likelihood of adverse effects of field concentrations of AMPA on the reproduction of earthworms.

The LC$_{50}$ for the earthworm *E. fetida* provided in regulatory documents for glyphosate was 5600 mg active substance kg^{-1} dry soil, and the NOEC was 473 mg active ingredient kg^{-1} dry soil (European

Commission, 2015; EFSA, 2015). These values are far higher than the measured concentrations in natural soils, ending up in a toxicity-exposure ratio greater than the trigger values, thus suggesting a low probability of unintentional effects based on the endpoints considered in the toxicity tests on soil fauna. However, as explained above, these values are obtained from regulatory documents with the model species *E. fetida* and considering endpoints that are not necessarily the most sensitive. For glyphosate, lower values have been reported to have detrimental effects on earthworm survival, reproduction, growth and activity. For instance, the lethal concentration LC_{50} for Grassate®, a non-selective glyphosate-based herbicide, averaged at 3.045 ± 0.08 mg kg^{-1} (i.e. mean 1.46 a.i. mg kg^{-1}) for the earthworm *Aporrectodea longa*, a common large species in temperate soils (Ogeleka et al., 2017). Casabé et al. (2007) found that *E. fetida* individuals avoided soils treated with glyphosate in formulation (Roundup FG®) at the manufacturers' recommended rate (i.e. 1.44 a.i. kg ha^{-1}, corresponding to 1.92 a.i. mg kg^{-1} considering a penetration of 5 cm depth, and a soil bulk density of 1.5 (EFSA, 2017)). This concentration of glyphosate also reduced the earthworm success of reproduction in the latter study. Thus, the maximum glyphosate soil concentration measured in our study was only 2 to 3 times higher than these effect concentrations found in the scientific literature. It is noteworthy that ecotoxicity of commercial formulations of glyphosate can also be due to adjuvants (Gill et al., 2018). Several other studies have revealed the toxicity of glyphosate and glyphosate-based herbicides for soil animals, in particular earthworms, and belowground interactions even at rate lower than recommended by the manufacturer (Martin, 1982; Springett and Gray, 1992; Gill et al., 2018; Zaller et al., 2014). Our results indicate a context of chronic and low-dose exposure, involving exposure to concentrations accumulated in tissues while concentrations in soils were no longer detectable, which inadequately matches with the protocols applied in laboratory standardized tests, especially in terms of duration. Moreover, links between chronic exposure to glyphosate and nervous, immune and endocrine systems have been demonstrated (Kissane and Shephard, 2017) and several studies suggested the predominance of endocrine disrupting mechanisms caused by environmentally relevant levels of exposure (Mesnage et al., 2015). Considering the scarce data on the exposure of earthworms to glyphosate and AMPA, the lack of data linking internal concentrations and ecotoxicological effects, the importance of earthworms in soil functioning, and the socio-economic and environmental issues related the use of glyphosate, further studies are needed on the exposure, bioaccumulation of glyphosate and AMPA, in relevant earthworm species, and related effects on populations, communities and related-ecosystem functions. Furthermore, recent finding obtained over the site studied here has revealed the general occurrence of mixtures of residues of currently used insecticides, fungicides and herbicides in both soils and earthworms (Pelosi et al., 2021). This highlights that earthworms may not be exposed to glyphosate and its transformation products only but to a broad spectrum of organic chemicals likely to interact in terms of toxicodynamics. This renders crucial the need to better assess and predict risks, to acquire more knowledge about both the characteristics of pesticide concentrations in the environment and biota, and the effects of exposure to such complex chemical mixtures on organisms.

4. Conclusion

The present study reports for the first-time glyphosate and AMPA concentrations in soils from semi-natural habitats (hedgerows, permanent grasslands) and in one species of earthworm. We here showed a high frequency of concentrations of glyphosate and AMPA residues in agricultural soils and in earthworms, that might be considered as low with regards towards predicted environmental concentrations and relatively low regarding no effect concentrations for earthworms. In both soils and earthworms, a ubiquity of the occurrence of residues was evidenced since the compounds were detected (more frequently and at higher concentrations) in conventional cereal fields but also in nontreated fields (i.e., under organic farming) and hedgerows. However, pesticide use (i.e. treated/nontreated by pesticides) and cropping system (i.e. field under conventional/organic farming) did not influence the mean soil concentration of the three studied molecules. Surprisingly, residues of glyphosate and/or AMPA were in some cases quantified in earthworms while not detectable in soils where they were sampled, which raises questions about the inputs and fate of glyphosate-based herbicides in terrestrial habitats, together with toxicokinetics in soil biota. The bioaccumulation factors calculated from this dataset, along with previous other studies, highlight the potential of glyphosate and AMPA to bioaccumulate in terrestrial organisms, and call for further research about the transfer and trophic magnification risk of these compounds in terrestrial food webs. The situation of glufosinate should be considered as well, since detected while slightly used over the area and sharing similar physico-chemical properties with glyphosate which could confer abilities for bioaccumulation in terrestrial food webs. Thus, attention should be paid to those key organisms that promote the soil functioning, to the fate of contaminants and more generally to the sustainability of agroecosystems. These concerns regarding glyphosate fate and effects can be extended to wildlife inhabiting cropland and, seven years later, we echo Battaglin et al. (2014) who concluded that effects of chronic low-level exposures to glyphosate, AMPA, associated adjuvants and mixtures on ecosystems remain to be determined.

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Author Contributions

C.P., C.B. and C.F. coordinated the research project. C.P., C.B., C.F., S.G. and V.B. designed the experiments and organized the sampling design. C.P. carried out the sampling. C.B. managed data curation and extraction of land use metrics from maps of ZAPVS in Geographic Information Systems. C.B. and C.F. prepared the databases. C.P. analyzed the data. C.P. wrote the first draft of the manuscript. C.F. performed the risk analyses. O.D and M.M. developed the analytical method for pesticide measurements and performed the analyses. S.N. and M.D. participated to the method development and supervised the chemical consistency of the interpretations. S.G. and V.B. supervised the ZAPVS sampling area and collected data on land use and farming practices. All authors contributed to the writing of the final version of the manuscript, which was revised for English by American Journal Experts (AJE®).

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Figure 1. A) Map of France showing B) the area where the forty sampling squares of 1 km2 were selected, and C) the three habitats where sis and earthworms were sampled in each square.

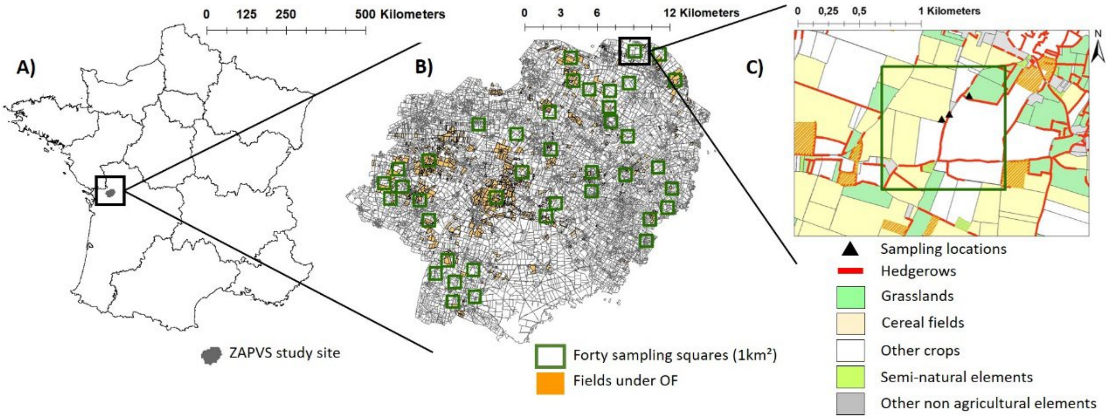

Table 1. Limits of detection (LOD) and quantification (LOQ) for glyphosate, AMPA and glufosinate in soil and earthworm matrices.

	Glyphosate		AMPA		Glufosinate	
	Soil	Earthworm	Soil	Earthworm	Soil	Earthworm
LOD	0.009	0.070	0.007	0.065	0.006	0.040
LOQ	0.030	0.230	0.025	0.200	0.020	0.120

Table 2. Residues of the three chemicals in a) the 120 soils and b) the 120 earthworms in the three studied habitats: cereal fields, whether under conventional (treated by pesticides) or organic (nontreated) farming, temporary (treated) or permanent (nontreated) grasslands, and hedgerows (nontreated). nd for not detected. < LOQ lower than the limit of quantification. Q1 (the first quartile concentrations, i.e. 25% of the values above LOQ), the maximum concentrations and the median concentrations are expressed in mg kg^{-1} dry weight for soil and fresh weight for earthworms. BAF for earthworms is the bioaccumulation factor with the standard deviation between brackets.

a)

	Glyphosate	AMPA	Glufosinate
Cereal fields			
Freq (%)	95	85	50
Q1	0.089	<LOQ	nd
Max	0.432	0.135	0.041
Median	0.142	0.038	<LOQ
Hedgerows			
Freq (%)	93	65	28
Q1	<LOQ	nd	nd
Max	0.322	0.075	<LOQ
Median	0.081	<LOQ	nd
Grasslands			
Freq (%)	75	25	28
Q1	<LOQ	nd	nd
Max	0.598	0.031	<LOQ
Median	<LOQ	nd	nd
All samples			
Freq (%)	88	58	35
Q1	<LOQ	nd	nd
Max	0.598	0.135	0.041
Median	0.088	<LOQ	nd

b)

	Glyphosate	AMPA	Glufosinate
Cereal fields			
Freq (%)	95	68	20
Q1	<LOQ	nd	nd
Max	0.395	0.247	<LOQ
Median	0.225	<LOQ	nd
Hedgerows			
Freq (%)	68	35	15
Q1	nd	nd	nd
Max	0.261	0.239	<LOQ
Median	<LOQ	nd	nd
Grasslands			
Freq (%)	60	13	0
Q1	nd	nd	nd
Max	0.269	0.206	nd
Median	<LOQ	nd	nd
All samples			
Freq (%)	74	38	12
Q1	nd	nd	nd
Max	0.395	0.247	<LOQ
Median	<LOQ	nd	nd

Table 3. Median and maximum of the bioaccumulation factor (BAF) of glyphosate and AMPA, calculated as the ratio of the chemical concentration in earthworms (in mg kg^{-1} fresh weight or dry weight) to the soil concentration (in mg kg^{-1} dry weight) in the three studied habitats: cereal fields, whether under conventional (treated by pesticides) or organic (nontreated) farming, temporary (treated) or permanent (nontreated) grasslands, and hedgerows (nontreated).

	Fresh weight			Dry weight		
Glyphosate	Q1	Median	Max	Q1	Median	Max
Cereal fields	0.78	1.09	15.56	3.90	5.44	77.78
Hedgerows	0	0.90	15.56	0	4.52	77.78
Grasslands	0	1.09	15.56	0	5.44	77.78
All habitats	0	1.00	15.56	0	5.01	77.78
AMPA						
Cereal fields	0	1.62	67.14	0	8.12	335.71
Hedgerows	0	0	18.57	0	0	92.86
Grasslands	0	0	29.43	0	0	147.14
All habitats	0	0	67.14	0	0	335.71

Table 4. Values of Partition Coefficient Octanol/Water (log K_{ow}), Partition Coefficient Octanol/Air (log K_{oa}), and Bio-concentration Factor (BCF) of glyphosate, AMPA and glufosinate reported in chemical databases.

Compound	**Log K_{ow}**	**Log K_{oa}**	**BCF**
Glyphosate	-3.12§	8.40§	0.5¤
AMPA	-2.42§	7.26§	NA¤
Glufosinate	-1.74§	8.32§	Considered as low (log $K_{ow} < 3$) ¤

§ refers to US EPA comptox database https://comptox.epa.gov/dashboard

¤ refers to PPDB database http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm

Table 5. Differences in patterns of contamination (median values and maximum between brackets) by the three chemicals according to habitats (i.e., cereal fields, whether under conventional (treated by pesticides) or organic (nontreated) farming, temporary (treated) or permanent (nontreated) grasslands, and hedgerows (nontreated)) and agricultural management (pesticide use and cropping system) in a) soils and b) earthworms. For cereal fields, n = 11 under organic farming (nontreated). For grasslands, n = 10 under organic farming, and n = 5 in permanent grasslands, for a total of 15 nontreated grasslands. Nonparametric Kruskal-Wallis tests were used for all variables. Different letters indicate significant differences at $p = 0.05$ between habitats (one analysis per chemical). For pesticide use and cropping system analyses, NS means not significant and $^{**}p < 0.01$ (Wilcoxon tests).

a)

							Pesticide use	Cropping system
Compound	Cereal fields (n=40)		Hedgerows (n=40)		Grasslands (n=40)		Treated/untreated	Organic/conventional
Glyphosate	0.142 (0.432)	a	0.080 (0.322)	b	<LOQ	b	NS	NS
AMPA	0.034 (0.135)	a	<LOQ	b	nd	c	NS	NS
Glufosinate	<LOQ	a	nd	a	nd	a	NS	NS

b)

							Pesticide use	Cropping system
Compound	Cereal fields (n=40)		Hedgerows (n=40)		Grasslands (n=40)		Treated/untreated	Organic/conventional
Glyphosate	0.225 (0.395)	a	<LOQ	b	<LOQ	b	**	NS
AMPA	<LOQ	a	nd	b	nd	b	NS	NS
Glufosinate	nd	a	nd	a	nd	a	NS	NS

Graphical abstract