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1 **Graphical abstract**

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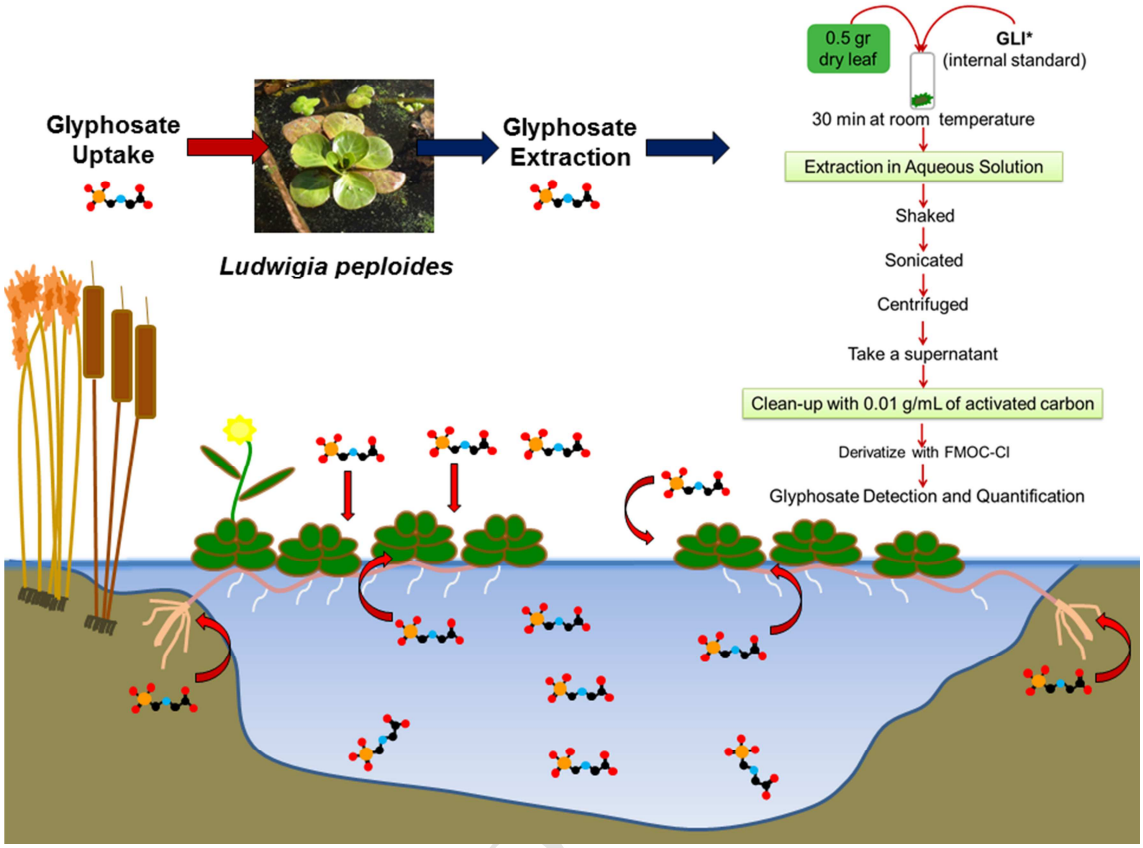

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Can an aquatic macrophyte bioaccumulate glyphosate? Development of a new method of glyphosate extraction in *Ludwigia peploides* and watershed scale validation

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Abstract

Glyphosate is intensively used in agricultural fields and it is frequently detected in non-target wetland ecosystems. The floating hydrophyte *Ludwigia peploides* is widely distributed in American streams and it is an abundant species. Therefore, our objectives were (1) to establish and validate an extraction and quantification methodology for glyphosate in *L.peploides* and (2) to evaluate the role of this species as a potential glyphosate biomonitor in an agricultural watershed. We developed a new method of glyphosate extraction from leaves of *L.peploides.* The method recovery was 117 ± 20% and the matrix effect 20%. To validate the method using environmental samples, plants of *L.peploides* were collected in March 2016 from eight monitoring sites of El Crespo stream. Surface water and sediment samples were collected at the same time to measure glyphosate and to calculate bioconcentration factors (BCFs) and biota-sediment accumulation factors (BSAFs). Glyphosate was detected in 94.11% in leaves, the concentrations ranging between 4 – 108 µg/Kg. Glyphosate was detected in surface water and sediments at 75% and 100% of the samples, at concentrations that varied between 0 – 1.7 µg/L and 5-10.50 µg/Kg dry weight, respectively. The mean BCFs and BSAFs were 88.10 L/Kg and 7.61, respectively. These results indicate that *L. peploides* bioaccumulates glyphosate mainly bioavailable in the surface water. In this sense, *L.peploides* could be used as a biomonitor organism to evaluate glyphosate levels in freshwater aquatic ecosystems because, in addition to its capacity to bioconcentrate glyphosate, it is easy to sample and it has a restricted mobility.

Keywords: *Ludwigia peploides,* hydrophyte, glyphosate extraction, bioconcentration factor, biota-sediment accumulation factor, agricultural watershed.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] is an effective and non-selective post-emergence herbicide used worldwide for the control of many grasses, broadleaf weeds, aquatic grasses and brush (Zhang et al., 2011). Due to its massive application within agro-ecosystems it is frequently detected, as well as its metabolite aminomethylphosphonic acid (AMPA). The reported concentrations of glyphosate and AMPA in USA surface waters range between 0.08 and 450 µg/L (Coupé et al., 2012; Battaglin et al., 2014), while the concentrations in sediments reach 470 µg/Kg (Battaglin et al., 2014). In Switzerland, the reported glyphosate concentrations range from 0.024 to 3.3 µg/L (Hanke et al., 2010); while in Argentina, levels in surface water are within 0.5 - 7.6 µg/L and from 5 to 200 µg/Kg in sediments (Aparicio et al., 2013).

In particular, in El Crespo watershed, which is focus the of the present study, the glyphosate and AMPA levels in surface water ranged from 2.00 to 2.90 µg/L, and in sediments from 18.50 to 47.50 µg/Kg (Pérez et al., 2017). The spatial variations are mainly dependent on the proximity of the agricultural fields, in the upper basin, where there are extensive crops, glyphosate and AMPA levels increase in surface water and in the lower basin, where the main farming activity is the extensive livestock, the levels decrease (Pérez et al., 2017).

Floating, submerged and emergent macrophytes can be used as *in situ* bioindicators of water quality because of their ability to accumulate agrochemicals, and

because wetlands and agricultural fields are strongly associated (Lewis, 1995; Carvahlo et al., 2007; Turgut, 2005; Pérez et al., 2013). They comprise an important component of benthic primary production in wetlands that must be protected from adverse chemical effects in order to maintain ecosystem structures and functions. Macrophytes fulfil several critical functions in aquatic ecosystems such as the conversion of solar energy and carbon dioxide into organic matter, oxygen providers, nutrient cycling, sediment stabilization, and habitat and shelter for aquatic life (Freemark and Boutin, 1994; Arts et al., 2010). Also, they provide natural habitats for pollinators and beneficial insects that can act as biological pests control in nearby agricultural fields. However, these plant resources may be at significant ecotoxicological risk from herbicides applied in crop fields.

The genus *Ludwigia* (Fam. Onagraceae) has been extensively studied because it belongs to a native aquatic group of macrophytes of North and South America (Bedoya and Madriñán, 2014). Nowadays, this genus has become important due to its expansion as an alien species in some European countries (Dandelot et al., 2005; Bou Manobens and Font Garcia, 2016).

Ludwigia peploides (H.B.K.) or floating primrose willow is a native perennial dicotyledonous hydrophyte, extensively distributed from USA and Mexico to South America (Lahitte and Hurell, 1997). *L. peploides* commonly grows in natural wetlands and fresh marshes (Lahitte and Hurell, 1997), and it is frequently found in Austral Pampas streams (Menone et al., 2015). This riparian hydrophyte is a postrate amphibious plant anchored in water-logged soils (Ellmore, 1981). It commonly grows forming abundant clumps of large floating shoots, which are easy to sample from the wetlands. In addition, *L. peploides* has been demonstrated to be a biomonitor of

organochlorine pesticide residues in Argentinean streams (Gonzalez et al., 2013). Therefore, we have chosen *L. peploides* as a potential aquatic macrophyte biomonitor.

Over the past decades, the use of persistent highly lipophilic organic pesticides has resulted in a wide range of adverse ecological effects due to their high bioaccumulation capacity. For this reason, nowadays there is an increase in the use of less persistent and more water-soluble (hydrophilic) pesticides, which generally have low bioconcentration factors (Alvarez et al., 2008). The physicochemical properties of glyphosate, such as its high water solubility (Log Kow = -3.57) and high adsorption to different soil/sediment components, as organic matter and clay minerals (Okada et al., 2016), suggest that this compound would have low bioconcentration (BCFs) and biota-sediment accumulation factors (BSAFs) in the aquatic biota. However, the environmental fate of glyphosate in plant tissues of aquatic macrophytes is a topic scarcely studied.

There are several extraction protocols for glyphosate extraction in plant tissues (Koskinen et al., 2016). Due to the complex and diverse composition of this type of material, in relation to photosynthetic pigments, lipids and proteins, there is not a consensus about the use of a standardized protocol. In this sense, it is necessary to determine an optimal glyphosate extraction protocol for the plant model to be used.

The objectives of this study were (1) to establish and to validate a methodology of glyphosate extraction and quantification in the hydrophyte *Ludwigia peploides* and (2) to evaluate the role of this species as a potential glyphosate biomonitor in aquatic ecosystems.

2. Materials and Methods

2.1. Study area

El Crespo is a third-order stream located in the southeast of Buenos Aires Province - Argentina with the catchment area of 489.42 Km^2 and flows from south-west to north-east through 65 Km (Fig. 1A) and with a mean discharge of 0.85 m^3/s (Pérez et al., 2017). The headwaters are located in the Tandilia hills System in the southern upper part; while the mouth is located at the northern end into the floodplains (Fig. 1A). This watershed is only influenced by farming activities without urban or industrial impact; also without significant inputs from other streams or surface water channels, being an optimal site to study processes like pollution, transport and dynamic of pesticides. The watershed can be divided in two areas: the southern upper basin mainly composed of agricultural lands and the northern lower basin, with native grassland coverage, used only for extensive livestock, without history of pesticide applications (Fig. 1B). The sampling sites were enumerated from the headwater (S1) to the mouth (S8), which have been previously characterized concerning glyphosate pollution. Sites S1-S7 are surrounded by agricultural lands, mainly transgenic crops, as soybean and maize, where the occurrence and input of glyphosate is increased, and S8 belongs to an area of natural grassland without agricultural activities, where the levels of glyphosate are lower than the upper sampling sites (Pérez et al., 2017).

2.2. Sample collection

2.2.1. Plant material

Plants of *Ludwigia peploides* were collected at the eight sampling sites of El Crespo stream (Fig.1) in the same week on March, 2016. In its natural habitat, *Ludwigia peploides* forms abundant clumps of large emergent floating shoots. Figure 2 shows clumps of *L.peploides* at some of the sampling sites of El Crespo stream, and a close up of the leaves and flower. Taking into account that *L.peploides* flourishes in spring and

summer (September to February) (Lahitte and Hurrell, 1997), only young specimens without reproductive structures were collected (n = 5 shoots per site). Upon arrival to the laboratory, plants were rinsed three times with tap water to remove possible glyphosate deposited on the surface of the plants, in order to determine only the accumulated glyphosate inside the leaves. The leaves were placed in paper bags and dried at constant temperature in an oven at 60°C until constant weight and then were milled. The samples were preserved in dry chambers with silica gel until their analysis.

2.2.2. Surface water and sediments

Surface water and sediments were sampled in the same sites as the plant material. Water samples were collected using 0.5 L polypropylene bottles. Immediately, pH and conductivity were measured. After that, water samples were filtered through a 0.45 µm nylon membrane and stored at -20°C until analysis. Sediment samples were collected using a cylinder core of 5 cm diameter and 20 cm of length. The upper 5 cm of sediments were used for the analysis. Samples were dried at constant temperature in an oven at 30°C for 3 days. They were milled and sieved through 0.5 or 2 mm for total organic carbon (TOC) and particle size distribution (PSD) determination, respectively, following the loss of ignition method (Schulte and Hopkins, 1996) and the pipette method for estimate three sizes: clay (< 2 µm), silt (2 – 50 µm) and sand (50 µm – 2000 µm) (Gee and Bauder, 1996). The pH and electrical conductivity were measured in 1:2.5 w/v sediment:water.

2.3. Glyphosate and AMPA determination and analytical methodology

2.3.1. Plant samples

Different protocols were used to setup the glyphosate and AMPA extraction in leaves of *L. peploides*. For all protocols subsamples of 0.5 g of plant material were used for analysis. All the samples were fortified with a stock solution of 1µg/mL [1,2-^{13}C,^{15}N] glyphosate ([1,2-^{13}C,^{15}N]-Gly) to determine matrix effect and recovery. What follows is a description of each of the protocols assayed:

- *Method 1:* Extraction was done following the standard extraction protocol for glyphosate and AMPA in soil samples (Aparicio et al., 2013) by adding 25 mL of an alkaline buffer solution (100 mM $Na_2B_4O_7 \cdot 10H_2O$/100 mM K_3PO_4, pH= 9). Samples were then sonicated three times for 15 min, and finally centrifuged at 3000 rpm. An aliquot of 2 mL of each sample was taken from the supernatant.

- *Method 2:* The extraction was done by adding 20 mL of ultrapure water to each sample and then shaking for 60 min at 250 rpm. Samples were then centrifuged at 3000 rpm. An aliquot of 2 mL was taken from the supernatant and added to 1 mL of buffer solution (100 mM $Na_2B_4O_7 \cdot 10H_2O$/100 mM K_3PO_4, pH= 9).

- *Method 3:* The extraction was done by adding 20 mL of ultrapure water to each sample. After that, they were shaken during 60 min at 250 rpm, sonicated twice for 15 min and then centrifuged at 3000 rpm. An aliquot of 5 mL was taken from the supernatant and treated with 5 mL of hexane and kept in the darkness overnight. An aliquot of 2 mL of each sample was taken from the supernatant and added to 1 mL of buffer solution (100 mM $Na_2B_4O_7 \cdot 10H_2O$/100 mM K_3PO_4, pH= 9).

- *Method 4:* Samples were extracted with 20 mL of ultrapure water. Then they were shaken during 60 min at 250 rpm and sonicated twice during 15 min and centrifuged at 3000 rpm. An aliquot of 5 mL was taken from the supernatant and treated with 0.01 g/mL (Method 4A) or 0.02 g/mL (Method 4B) of activated carbon and kept in darkness

overnight. After, an aliquot of 2 mL of each extract was filtrated through a 0.22 µm nylon filter to remove the activated carbon. Then, 1 mL of buffer solution (100 mM $Na_2B_4O_7 \cdot 10H_2O$/100 mM K_3PO_4, pH: 9) was added to the samples.

Control samples to evaluate the matrix effect were performed for each method, which consisted of plant samples treated in the same way as described in each method, but the [1,2-^{13}C,^{15}N]-Gly aliquot was added to the final extract obtained after the extraction. A standard curve for glyphosate and AMPA with six concentrations (0.5, 1, 10, 20, 50 and 100 µg/L) was prepared for the evaluation of each method. Each point of the standard curve had an equivalent amount of [1,2-^{13}C,^{15}N]-Gly to that of the final concentration of the samples. After the extraction steps mentioned above, all samples and the solutions of the standard curve were derivatized with 2 mL of a solution of 1 mg/mL of 9-fluorenylmethylchloroformate (FMOC-Cl) in acetonitrile in darkness during 24 h. After that, the samples and the standard curve were shaken for 3 min with 5 mL of dichloromethane and centrifuged at 3000 rpm. The hydrophilic phase in all cases was filtrated through a 0.22 µm nylon filter and disposed into a 1 mL vial for UHPLC-MS/MS determination. Analyses were performed by injecting 20 µL of the final extract in the UHPLC-MS/MS system (Waters® Acquity) calibrated for positive detection, using a Waters® Acquity® UPLC column (C18, 1.7 µm, 50 x 2.1 mm). The mobile phase consisted of a gradient of water-methanol [5mM $NH_4(CH_3COO)$].

The limit of detection (LOD), defined as the minimum concentration at which the analyte signal differs from noise, was obtained with the lowest concentration which signal/noise ratio was 3. The limit of quantification (LOQ) was established as the minimum concentration validated by the method using fortified samples with satisfactory recovery (between 70% and 120%) and accuracy (Relative Standard Deviation, RSD $\leq$ 20) (Ibañez et al., 2005).

223

224 *2.3.2. Surface water and sediment samples*

225 A subsample of 2 mL of surface water and 5 g of sediments were used for
226 analysis. The surface water and sediment samples were fortified with 10 µL and 50 µL
227 of 1 µg/mL stock solution of [1,2-^{13}C,^{15}N]-Gly, respectively, to determine matrix effects
228 and recovery. After 30 min, the liquid and solid samples were extracted with 1 mL and
229 25 mL of buffer solution (100 mM $Na_2B_4O_7 \cdot 10H_2O$/100 mM K_3PO_4, pH= 9),
230 respectively. After that, the sediment samples were sonicated three times for 15 min and
231 centrifuged at 3000 rpm. An aliquot of 2 mL was taken from the supernatant. A
232 standard curve with six points, 0.5, 1, 10, 20, 50 and 100 µg/L of glyphosate and AMPA
233 was prepared with each set of surface water and sediment samples, with an equivalent
234 amount of [1,2-^{13}C,^{15}N]-Gly in each point of the curve. After that, surface water and
235 sediment samples and the standard curve solutions were derivatized with 2 mL of a
236 solution of 1 mg/mL of FMOC-Cl in acetonitrile in darkness during 24 h. Then, samples
237 and the standard curve solutions were shaken for 3 min with 5 mL of dichloromethane
238 to end the clean-up step. The samples were centrifuged at 3000 rpm, the hydrophilic
239 phase obtained was filtrated through a 0.22 µm nylon filter and disposed into a 1 mL
240 vial for UHPLC–MS/MS determination. Analyses were performed by injecting 20 µL
241 of the final extract in the UHPLC–MS/MS system as described in the Plant samples
242 section. The LOD and LOQ for both glyphosate and AMPA were 0.1 and 0.5 µg/L in
243 surface water and 0.5 and 3 µg/Kg in sediments.

244

245 *2.4. Data analysis*

246 *2.4.1. Glyphosate and AMPA levels in Ludwigia peploides, surface water and*
247 *sediments*

In leaves of *Ludwigia peploides* the mean concentration of glyphosate and AMPA were calculated using all data. In samples where the glyphosate and AMPA levels were below the LOD, values were set to zero. When the concentration of the compound was below the LOQ, the concentration was set to the LOD value (censored value).

In surface water and sediments samples, when the levels of glyphosate and AMPA were below the LOQ, censored values were used, as the same criteria of the leaves samples.

2.4.2. Relation between glyphosate levels in surface water or sediment and Ludwigia peploides leaves

A linear regression was used to evaluate the relation between glyphosate concentration in surface water or sediment (as independent variables), and glyphosate concentration in leaves of *Ludwigia peploides* (as dependent variable). The analyses were done with a significance level of 0.05.

2.4.3. Bioconcentration Factors (BCFs) and Biota-Sediment Accumulation Factors (BASFs) determination

The BCF and BSAF were determinate for each sampling site. They were calculated as the ratio between the average glyphosate or AMPA concentrations in the leaves divided by the glyphosate or AMPA concentrations in surface water (BCF) or sediment samples (BASF).

3. Results and Discussion

3.1. Surface water and sediments physico-chemical properties

Table 1 shows the physico-chemical properties of surface water and sediment obtained from all sampling sites. Surface water was slightly alkaline to alkaline, with a range of pH= 7.70 – 9.65, and with low electrical conductivities near to 1 mS/cm. These values are in agreed with other data obtained at the same stream (Pérez et al., 2017) and to other streams of the southeast Pampas (Romanelli et al., 2011). Sediments at both sites were slightly alkaline with a range of pH= 7.63 - 8.41 and with conductivities of 0.28 - 0.48 mS/cm. Sediments were characterized by high total organic carbon (TOC) content, ranging between 1.70 - 6.20%, and as sandy loam and sandy silt loam, with a distribution particle size about 32.19 – 67.29% of sand, 19.94 – 35.60 % of silt and 12.29 – 28.69% of clay (Table 1).

3.2. Glyphosate and AMPA determination and analytical methodology

Due to the lack of a consensus methodology on analysis of glyphosate and its metabolite AMPA in plants, different methods were used for glyphosate extraction. Method 1, based on an alkaline extraction buffer, had a low recovery rate < 20 % and high matrix interference (70%) (Table 2). In Method 2, based in an aqueous extraction, the matrix interference was reduced at 50%, however the recovery of spiked samples with [1,2-^{13}C,^{15}N]-Gly was similar to Method 1. This aqueous extraction increased its recovery when the extracts were treated with hexane (Method 3) however the interferences were not reduced significantly (Table 2). The method based in an aqueous solution extraction and a clean-up with 0.01 g/mL of activated carbon was optimal (Method 4). This method was found to be precise, with matrix effect < 20% and accurate, with satisfactory recoveries for spiked samples higher than 110 ± 23 % (Table 2). The LOD and LOQ of glyphosate and AMPA in *L.peploides* were set in 4 μg/Kg and 12 μg/Kg dry weight, respectively.

These results are in agreement with other studies using similar methods developed for some terrestrial crop plants (Hernández et al., 2000; Goscinny et al, 2012), in which the extraction was made using an aqueous phase. The main problem of the extraction with aqueous solution is the presence of other water-soluble component that will interfere in the analysis. To reduce these interferences from the matrix a clean-up step is necessary. It is possible to clean-up with organic solvents, e.g. for tissues with high content of proteins and lipids (Goscinny et al., 2012), or with sorbents, e.g. activated carbon is used to remove chlorophyll and other pigments, because chlorophyll has strong affinity for activated carbon (Agilent, 2016).

In the present study, the best result for the extraction of glyphosate and AMPA was obtained with activated carbon at 0.01 g/mL as sorbent of photosynthetic pigments. However, a high concentration of this sorbent (0.02 g/mL, Method 2B) can interfere in the analysis, increasing the matrix effect (Table 2). The excess of activated carbon can interact with glyphosate molecules, increased the matrix effect (Table 2) Therefore, Method 4A was used for the further analysis of glyphosate and AMPA in environmental samples of *L. peploides*.

3.3. Glyphosate and AMPA in environmental samples of Ludwigia peploides, surface water and sediment

In the present study, both glyphosate and AMPA were detected in 75% of the surface water samples and in 100% of the sediment samples. The glyphosate concentrations in surface water varied between 0 – 1.70 µg/L, and AMPA levels varied between 0 – 0.10 µg/L (Table 3). In sediments, the glyphosate and AMPA levels varied between 3.00 – 10.50 µg/Kg and 3.50 – 93.50 µg/Kg dry weight, respectively (Table 3). The occurrence of glyphosate and AMPA in surface water and sediments from El

Crespo stream are in agreement with its massive use in the watershed. In fact, this compound was the main herbicide used in El Crespo watershed in the past campaign 2014 – 2015 (SIIA, 2016). The commercial formulations with 54% of active ingredient were the most commonly applied by the farmers (Pérez et al., 2017). The area of El Crespo upper basin sowed with glyphosate-resistant crops (i.e. soybean and maize) was approximately 147.44 Km^2 (SIIA, 2016). In general, there are three application periods of glyphosate: one during the fallow period in winter-spring, the second before sowing, and the third during the growth stage of maize and soyben, reaching the total annual application dose of 5 L/ha on these transgenic crops (Pérez et al., 2017).

Glyphosate detection frequency in leaves of *L. peploides* was 94.12% while AMPA residues were not detected in the leaves (Table 3). Glyphosate levels varied from 4.00 -108.00 µg/Kg dry weight in leaves (Table 3). Glyphosate concentration in leaf tissue was directly related to glyphosate concentration in surface water (R^2 = 0.591, $p < 0.001$) (Fig. 3A), while there was no relation with glyphosate concentration in sediments (R^2 = 0.013, p = 0.689) (Fig. 3B). These results indicate that the higher the glyphosate levels in water, the higher the glyphosate concentration in the leaves.

Glyphosate was detected in all sampling sites where *L.peploides* was collected, including S8 where it was never applied. However, glyphosate residues have been detected at site S8 in surface water and sediments by a previous study as a result of downstream transport from the upstream agricultural fields (Perez et al., 2017). The levels of glyphosate in the leaves did not show a defined pattern in relation with the land uses in the watershed. At site S8, the levels were similar or lower than in sampling sites located near croplands. Site S5 had the highest concentration of glyphosate in leaves (100.00 ± 11.31 µg/Kg) and in surface water (1.7 µg/L) (Table 3). In the rest of the sampling sites different uptake routes could have contributed to the accumulation of

348 glyphosate in *L. peploides*. In S1 and S2, where glyphosate in water was under the
349 detection limit, incorporation from the sediment in roots or the deposition from spray
350 drift in the floating leaves could have occurred.

351 The plants were healthy at macroscopic level; they did not present chlorosis
352 symptoms which could be related to the wilting of the leaves that produce the contact to
353 herbicides. However, it is possible that the presence of glyphosate residues could induce
354 sublethal effects at different suborganism levels, as it was reported in others species
355 (Boutin et al., 2014) or hormetic effects, such as the increase of biomass growth that has
356 been studied in terrestrial species (Cedergreen et al., 2007; Cedergreen, 2008). The
357 effects of herbicides in non-target plants is emerging as one of the central issues in
358 biodiversity conservation. Therefore the evaluation of adverse and hormetic effects of
359 glyphosate in aquatic macrophytes will be the focus of future risk assessment studies.

360 Koskinen et al. (2016) reported that there are some studies about the
361 determination of glyphosate occurrence and levels in plants material, however to our
362 knowledge, there are no *in situ* studies about the bioaccumulation of this herbicide in an
363 aquatic macrophyte. In the present study, we evaluated the glyphosate and AMPA
364 bioconcentration and bioaccumulation in leaves of *L. peploides*, through the BCFs and
365 BSAFs. The BCFs and BSAFs of glyphosate showed wide variations, while both
366 parameters for AMPA were impossible to calculate, because AMPA residues were no
367 detectable in the leaf tissue (Table 3). In S1 and S2 there was no detection of glyphosate
368 in surface water, therefore, it was not possible to calculate the corresponding factors.
369 Anyway, BCFs values obtained in S3, S4, S5, S6, S7 and S8 were higher than the
370 BSAFs from the same sites (Table 3), indicating that glyphosate bioavailability is
371 provided by the molecules dissolved in the surface water more than in the sediments. It
372 is important to note that the commercial glyphosate formulations contain surfactants

(e.g. polyoxyethylene tallow amine, also known as POEA) to enhance foliar uptake. Residues of POEA has been detected in agricultural soils of USA (Tush and Meyer, 2016). Therefore, it is possible that there are surfactants present in the agricultural stream that may also favor glyphosate uptake into leaves of aquatic hydrophytes.

Glyphosate uptake in *L.peploides* could be through the floating leaves or through the submerged floating roots. The anatomy of *L. peploides* also contributes to glyphosate uptake because it is characterized by the absence of cuticle and amphistomatic leaves (Bedoya and Madriñán, 2014) that increase the surface exchange with water. The submerged floating root uptake is facilitated by the presence of pneumatophores that could be involved in the exchange of substances and ions dissolved in water (Ellmore, 1981; Bedoya and Madriñán, 2014). The low BSAFs for glyphosate in *L.peploides* could be explained by the strong adsorption of the molecules to the organic matter fraction of the sediment (Table 1). In this sense, the glyphosate bioavailability from sediments to roots anchored to the substrate was low (Table 3).

Residues of AMPA were detected in surface water at S1, S3, S4, S5 and S7; and in sediments at all sampling sites (Table 3). Also, AMPA concentrations in surface water were the same to glyphosate at S3, S4 and S6 (0.10 μg/L). However, AMPA residues were not detected in leaf tissues at any sampling sites (Table 3). The absent of AMPA in leaves could be due to small differences in the molecular structure between glyphosate and AMPA. In this sense, these structural differences could reduce plant uptake of AMPA, in comparison to the parental compound. Also, the absence of AMPA in leaf tissues indicates that the bioconcentrate glyphosate does not metabolize inside the plants at the AMPA pathway.

In fact, until recently, the metabolic degradation of glyphosate by plants was neither well documented nor accepted (Duke and Powles, 2008). González-Torralva et

al. (2012) compared the glyphosate metabolism in two biotypes of the terrestrial species horseweed *Conyza canadensis*. They found a complete disappearance of glyphosate from the resistant biotype by conversion into glyoxylate, sarcosine and AMPA within 96 hours after treatment. However, in the susceptible biotype only glyoxylate was detected. Other studies also describe AMPA detection in glyphosate-resistant or glyphosate-tolerant plants (Cruz- Hipolito et al., 2011; Rojano- Delgado et al., 2012). However, information of biotransformation of glyphosate in susceptible wild aquatic plants is not available in the literature so far. Further studies are needed to elucidate AMPA uptake and degradation in aquatic plants as *L.peploides*. Also, studies about the possible release of glyphosate or its metabolites, including AMPA, from the leaves to an aqueous media would help to clarify its metabolism or excretion in this species.

The capacity of *L. peploides* to accumulate contaminants has been demonstrated for organochlorine pesticides (Gonzalez et al., 2013), indicating together with our study, the importance of this specie as a biomonitor to evaluate pesticide levels in a wetland ecosystem. Indeed, due to its broad geographic distribution in the Americas, this hydrophyte can be proposed for biomonitoring programs. Despite its utility as a biomonitor species, it is also important to highlight that the capacity to bioconcentrate and bioaccumulate pesticides can also be potentially adverse for the plant itself, increasing the relevance of monitoring studies at catchment scale.

4. Conclusions

Concluding, a straightforward and accurate methodology to extract glyphosate from the hydrophyte *Ludwigia peploides* was established. This new method allowed the extraction and quantification of glyphosate and AMPA in leaf tissues of *L. peploides* and it was validated using environmental samples. *L. peploides* accumulated glyphosate

in its leaves, mainly through bioconcentration from surface water. Finally, we propose the use of this widely distributed species as a glyphosate biomonitor in freshwater ecosystems affected by glyphosate inputs.

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Figure 1: Study area of El Crespo watershed and sampling sites, S1 – S7: Agricultural lands; S8: Natural grassland with extensive livestock (e.g. S1, S6, S7 and S8). Double arrow indicates soybean crops fields. Geographic coordinates of sampling sites: **S1:** 37° 53´ 12.01" S; 58° 27´ 35.96" W; **S2:** 37° 52´ 40.16" S; 58° 26´ 50.91" W; **S3:** 37° 51´ 13.47" S; 58° 24´ 05.72" W; **S4:** 37° 50´ 29.74" S; 58° 25´ 08.60" W; **S5:** 37° 48´ 50.40" S; 58° 27´ 27.51" W; **S6:** 37° 45´ 51.64" S; 58° 22´ 01.83" W; **S7:** 37° 44´ 16.65" S; 58° 21´ 03.64" W; **S8:** 37° 34´ 04.35" S; 58° 02´43.63" W.

Figure 2: *Ludwigia peploides* in its natural habitat at some of the sampling sites of El Crespo stream. Clumps of emergent floating shoots (A – C), floating leaves (D), flower (E).

Figure 3: Linear regression between glyphosate levels in leaves of *Ludwigia peploides* and surface water (A) and sediments (B).

Table 1: Physico-chemical properties of surface water and sediments.

Sampling Site	*Surface water*		*Sediments*					
	pH	EC	pH	EC	TOC (%)	Silt (%)	Clay (%)	Sand (%)
S1	8.27	1.19	7.98	0.33	2.20	28.62	16.39	54.99
S2	8.01	0.96	7.89	0.29	3.00	28.07	15.16	56.77
S3	8.71	1.26	7.63	0.48	6.20	39.12	28.69	32.19
S4	7.70	1.50	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
S5	8.48	1.15	8.15	0.35	2.60	24.00	23.59	52.41
S6	9.65	0.94	8.41	0.28	1.90	19.94	12.76	67.29
S7	8.56	1.12	8.20	0.34	2.30	31.69	15.39	52.92
S8	8.56	1.12	8.20	0.38	1.70	35.60	13.73	50.66

EC: Electrical Conductivity (mS/cm)

TOC: Total Organic Carbon

n.a.: not analyzed

Table 2: Glyphosate recovery and matrix effect for the different extraction methods from leaves of *Ludwigia peploides*.

Extraction Method [a]	*Recovery (%)*	*Matrix effect (%)*
Method 1	< 20	70
Method 2	< 20	50
Method 3	40	50
Method 4A	117 ± 20	20
Method 4B	110 ± 23	40

[a] Extraction Method; Method 1: extraction in alkaline buffer solution; Method 2: extraction in aqueous solution; Method 3: extraction in aqueous solution and clean-up step with hexane; Method 4: extraction in aqueous solution and clean-up step with 0.01 g/mL (A) or 0.02 g/mL (B) of activated carbon.

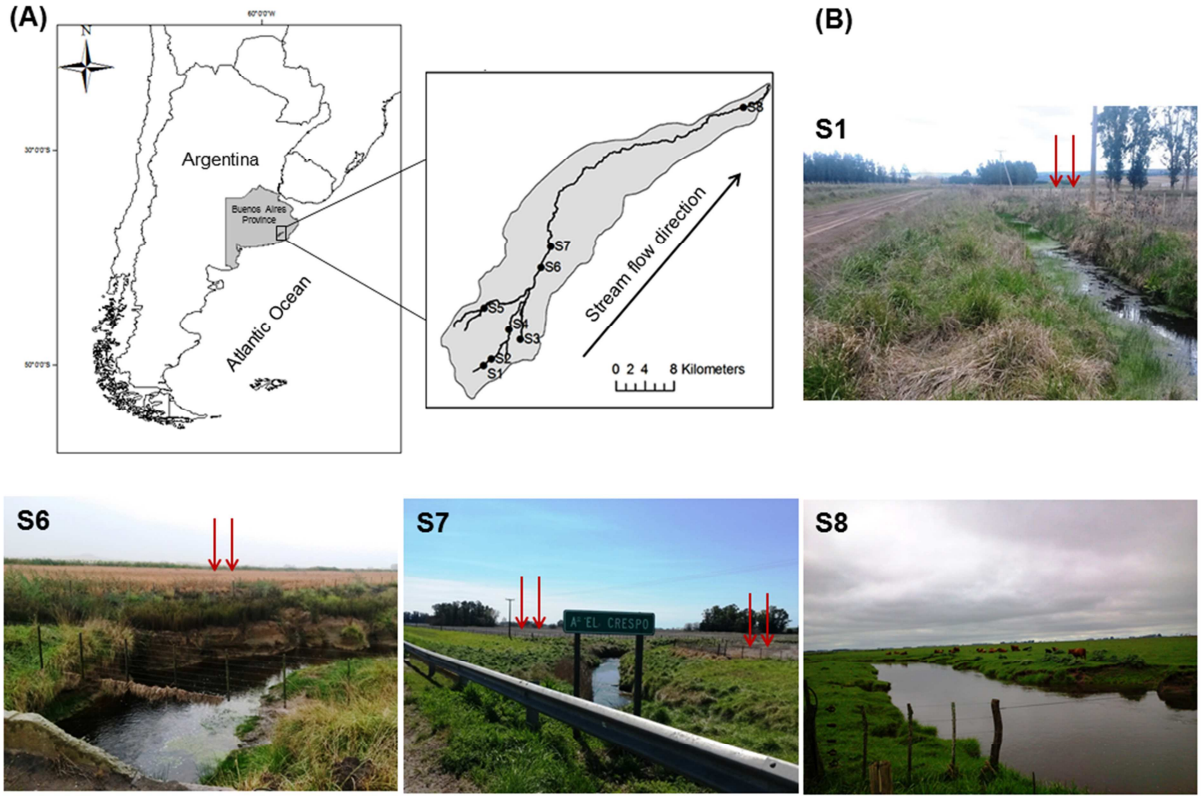
Table 3: Glyphosate and AMPA levels in *Ludwigia peploides* (mean ± SE), surface water and sediments, and bioconcentration factors (BCFs) (mean ± SE) and biota-sediment accumulation factors (BSAFs) (mean ± SE).

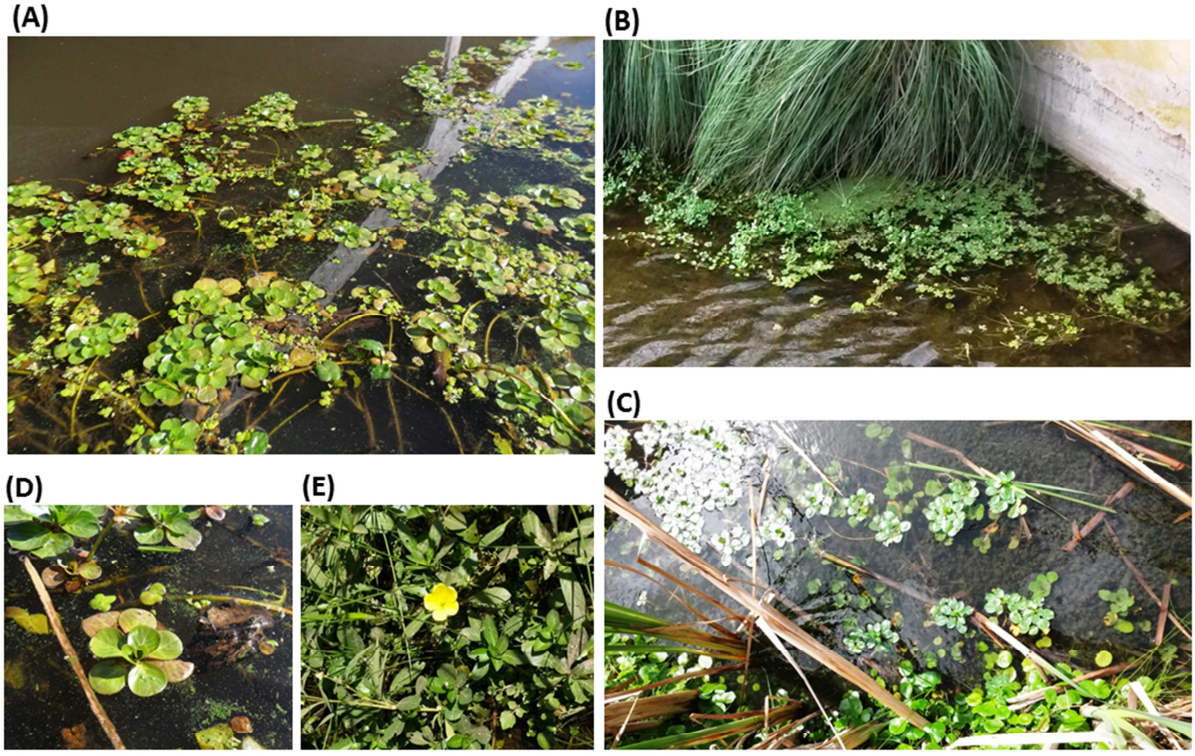
Sampling Site	*Glyphosate*					*AMPA*		
	Ludwigia peploides (μg/Kg)	Surface water *(μg/L)*	Sediment *(μg/Kg)*	BCFs *(L/g)*	BSAFs[a]	*Ludwigia peploides (μg/Kg)*	Surface water *(μg/L)*	Sediment *(μg/Kg)*
S1	24.00 ± 8.00	n.d.	9.50	n.c.	2.52 ± 0.84	n.d.	0.10	15.00
S2	56.00 ± 5.65	n.d.	10.41	n.c.	5.37 ± 0.54	n.d.	n.d.	19.30
S3	22.00 ± 8.48	0.10	5.00	220.00 ± 60.0	4.40 ± 1.70	n.d.	0.10	4.50
S4	12.00 ± 11.31	0.10	5.50	120.00 ± 80.0	2.20 ± 2.10	n.d.	0.10	9.00
S5	100.00 ± 11.31	1.70	3.00	58.80 ± 4.70	33.30 ± 3.80	n.d.	0.10	11.00
S6	2.00 ± 2.82	0.10	10.50	20.00 ± 20.00	0.20 ± 0.27	n.d.	0.10	93.50
S7	26.00 ± 8.48	0.70	3.00	37.10 ± 8.50	8.66 ± 2.82	n.d.	0.10	4.00
S8	34.00 ± 2.82	0.50	5.00	68.00 ± 4.00	6.80 ± 0.56	n.d.	n.d.	4.00

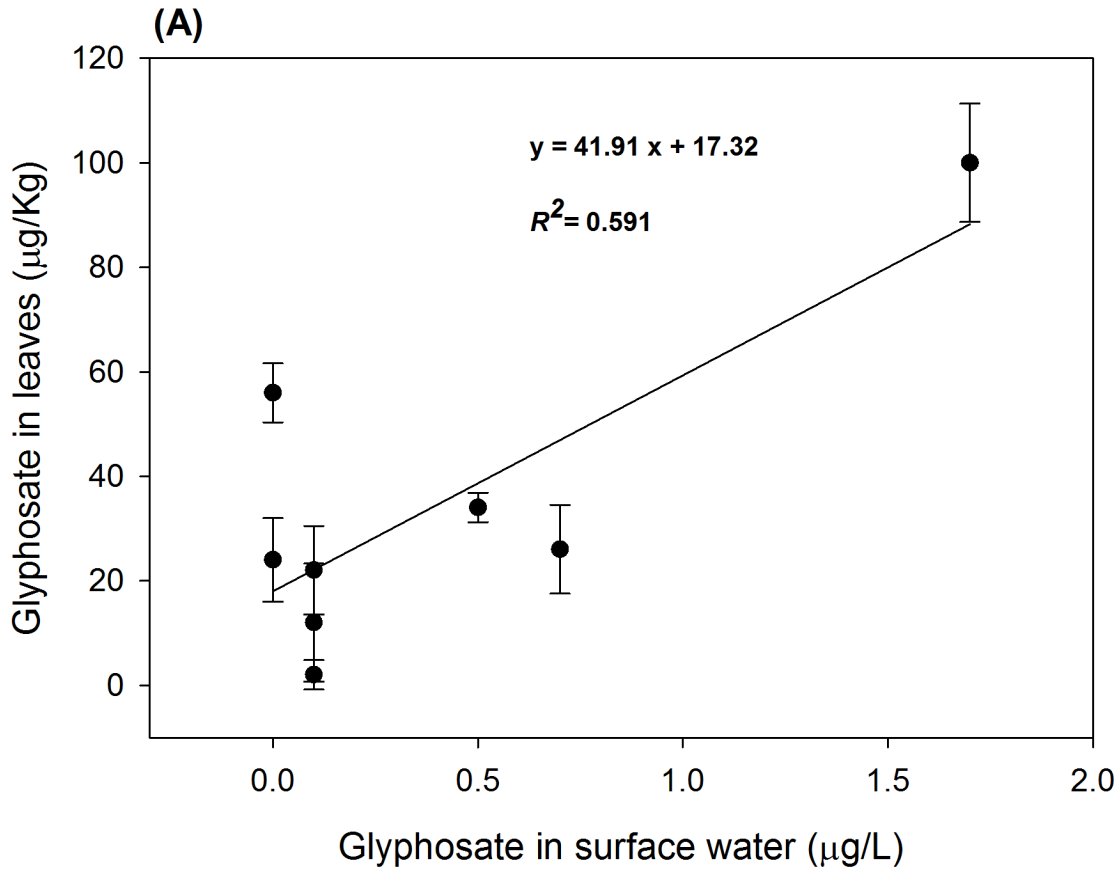
[a]: BSAFs without units

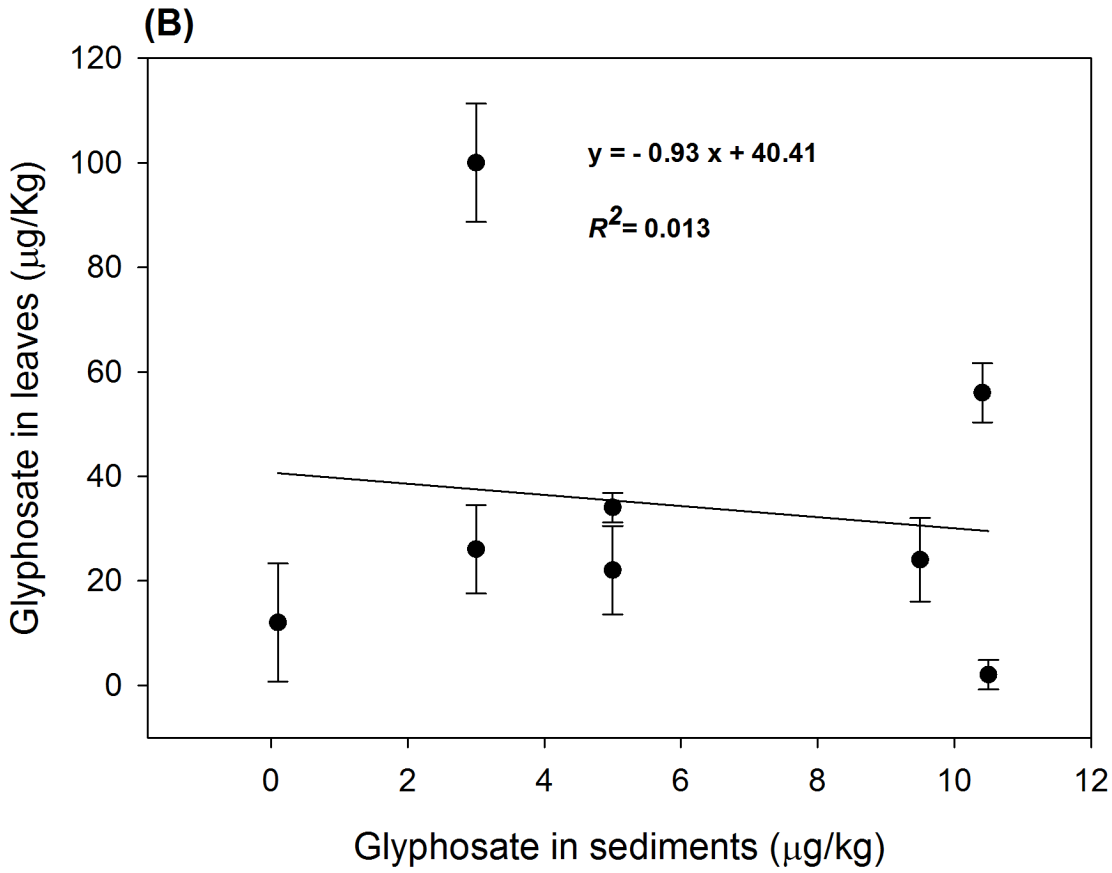
n.d.: not detected

n.c.: not calculated






(A)
y = 41.91 x + 17.32
R^2= 0.591
Glyphosate in leaves (µg/Kg)
Glyphosate in surface water (µg/L)
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0


(B)
y = - 0.93 x + 40.41
R2= 0.013
Glyphosate in leaves (µg/Kg)
Glyphosate in sediments (µg/kg)
0
20
40
60
80
100
120
0
2
4
6
8
10
12

Highlights

- A glyphosate extraction method in the hydrophyte *Ludwigia peploides* was developed.
- Environmental levels of glyphosate in *Ludwigia peploides* were measured.
- Glyphosate bioconcentration and bioaccumulation in *Ludwigia peploides* was calculated.
- *Ludwigia peploides* accumulates glyphosate in its leaves mainly from surface water.
- *Ludwigia peploides* can be used as a biomonitor of glyphosate levels in stream water.