グリホサートカリウム塩

要旨及び評価結果

（環境動態）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 7.5
Report author	De Polo, A. *et al.*
Report year	2019
Report title	From the traces in the wells of the urban aqueduct network to the subsequent prohibition of the use of glyphosate: the case of an area of high-intensity wine production in the province of Treviso, Veneto. Original Title: Dai residui nei pozzi della rete acquedottistica urbana al successivo divieto di utilizzo del glifosate: il caso di un'area ad alta intensità vitivinicola in provincia di Treviso, Veneto.
Document No	Igiene e sanità pubblica, (2019) Vol. 75, No. 6, pp. 451-460
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

In 2016, extraordinary samplings of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were carried out in 12 wells of the water network intended for domestic consumption in the territory of the Ulss2 (*Unità Locale Socio-Sanitaria*, Local Health Unit) 2 - District of Pieve di Soligo, Province of Treviso, Veneto region, Italy. The area includes 13 municipalities at high-intensity "Prosecco d.o.c.g." wine production. Traces of glyphosate (maximum reached 0.08 µg/L) and AMPA (maximum reached 0.25 µg/L) were detected in 2 wells supplying an urban area. Following these findings, an inter-municipal order to suspend the use of glyphosate was introduced and then entered definitively in the rural police regulation concerning all the municipalities in the Prosecco d.o.c.g. area, which led to the elimination of glyphosate and AMPA also in the initially contaminated wells. The case shows that high-consumption herbicides can reach the drinking water network of a city surrounded by territories with high agricultural activity. Moreover, the combined intervention of the institutions was fundamental to eliminate a "probable carcinogen" from the urban drinking water and to promote the abandonment of potentially harmful agricultural practices in favour of solutions with reduced environmental and health impact.

Materials and methods

The monitoring plan was developed in accordance with the 2014-2018 Regional Prevention Plan of Veneto "Plant protection products and health protection: raising awareness of compliance with correct sales conditions and the adoption of good usage practices". In 2016, the Prevention Department of the Ulss2 - *Marca Trevigiana* of Veneto prepared a monitoring plan for glyphosate and AMPA. This involved twelve drinking water sampling works in the territory (ex Ulss7) of the district of *Pieve di Soligo* (Treviso), a territory (of about 215,000 inhabitants) including 13 of the 15 municipalities forming the area of high wine business intensity for the production of Prosecco d.o.c.g. (Figure 1).

It should be pointed out that the two molecules, sampled by USGS Techniques and Methods 5-A10:2009, are subject to the same limits:

- The lower detection rate, intrinsic to the precision of the instrument, which at the beginning of the monitoring period was 0.05 µg/L, then lowered to 0.02 µg/L from 01/05/2017 thanks to technical improvements.
- The higher limit, defined by law as 0.1 µg/L (as per Legislative Decree 31/01).

Figure 1: **Location of the geographical area of interest. Province of Treviso, Ulss2 *Marca Trevigiana*, District of *Pieve di Soligo*.**

Results

As reported in the Table 1, ten of the twelve sampled sites showed no traceable glyphosate or AMPA residues. The other two, belonging to the water network of the city of *Conegliano* (Treviso), reported significant traces of glyphosate while the AMPA level reached the limit value imposed by law.

Table 1: **List of 12 drinking water collection works sampled in the *Pieve di Soligo* district by the Ulss2 Prevention Department - *Marca Trevigiana del Veneto* (Food Hygiene and Nutrition Service), in the period March-July 2016**

ID	Aqueduct	Sample date	Glyphosate [µg/L]	AMPA [µg/L]
1	*Conegliano* (*Scomigo*)	30/03/2016	0.05	0.10
2	*Vittorio Veneto*	27/04/2016	<0.05	<0.05
3	*Moriago della Battaglia*	11/05/2016	<0.05	<0.05
4	*Conegliano* (*Colnù*)	25/05/2016	0.08	<0.05
5	*Susegana*	11/07/2016	<0.05	<0.05
6	*San Pietro di Feletto*	15/06/2016	<0.05	<0.05
7	*Santa Lucia di Piave*	22/06/2016	<0.05	<0.05
8	*Tarzo*	29/09/2016	<0.05	<0.05
9	*Conegliano*	06/07/2016	<0.05	<0.05
10	*Farra di Soligo*	13/07/2016	<0.05	<0.05
11	*Farra di Soligo*	20/07/2016	<0.05	<0.05
12	*Conegliano*	27/07/2016	<0.05	<0.05

The two wells that tested positive at the first sampling have a depth of 22 m (*Scomigo*) and 30 m (*Colnù*) respectively. Both were then monitored approximately every month from March 2016 to February 2017. Traces of glyphosate and AMPA were detected in numerous subsequent samplings, exceeding the legal limit in three of them (Figures 2 and 3).

Following these findings, the municipality of *Conegliano* and four of its neighbouring municipalities (*Colle Umberto*, *San Pietro di Feletto*, *Tarzo* and *Vittorio Veneto*) have adopted, starting from 2 March 2017, a mayoral decree to suspend the use of glyphosate-based herbicides on the respective municipal soils, in compliance with the measures proposed by the Ulss2 Public Health and Hygiene Service. Following this ordinance, the monthly checks on the two wells in the city of *Conegliano*, which were positive at the first sampling, gave a stable negative result for all the following 12 months (Figures 4 and 5). It should be noted that, also as a result of other problems, the *Scomigo - Cal dell'Ebreo* well was excluded from the *Conegliano* drinking water network following the first evidence of contamination. However, a further check carried out on a public fountain in the urban centre of *Conegliano* (sampled monthly starting from 2017) reported traces of glyphosate (0.04 µg/L) on 14/06/2017 (Figure 6). Although this value is well below the legal limit and even lower than the traceability limit of the previously used instrument (until 01/05/2017), it confirmed the persistence of sporadic residual uses of glyphosate, as well as the ease with which the groundwater is subject to contamination.

Based on these considerations, the mayoral decree prohibiting the use of glyphosate was initially reiterated in all five municipalities also for the year 2018; it was then included in the inter-municipal regulation of the rural police, effective from the 1 January 2019 and concerning all 15 municipalities of the Prosecco d.o.c.g wine production area. This legislation banned the use of herbicides containing glyphosate, as well as all other herbicides, with the exception of those of natural and/or organic origin, on all crops, herbaceous, arboreal, arable land and orchards. An exception is made for orchards and vineyards that are young within three years of life or located on slopes where mechanical weeding is not feasible, for which the use of herbicides is allowed as long as they do not contain glyphosate, hazard warnings or risk phrases for human health.

Figure 2: **Levels of glyphosate and AMPA in sequential sampling at the well located in the hamlet of *Scomigo* in the municipality of *Conegliano* in the period March 2016 to February 2017**

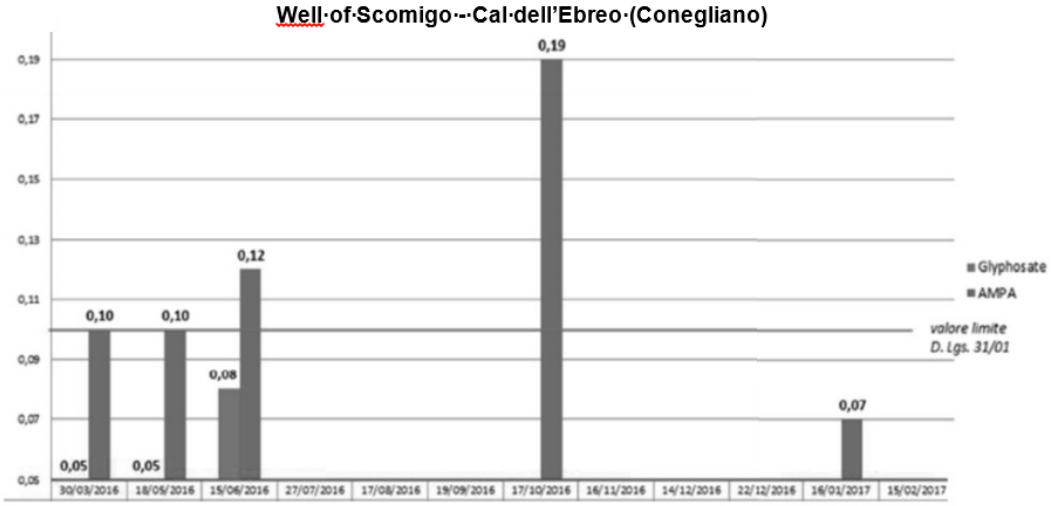


Figure 3: **Levels of glyphosate and AMPA in sequential sampling at the well located in *Colnù* in the municipality of *Conegliano* in the period May 2016 to February 2017**

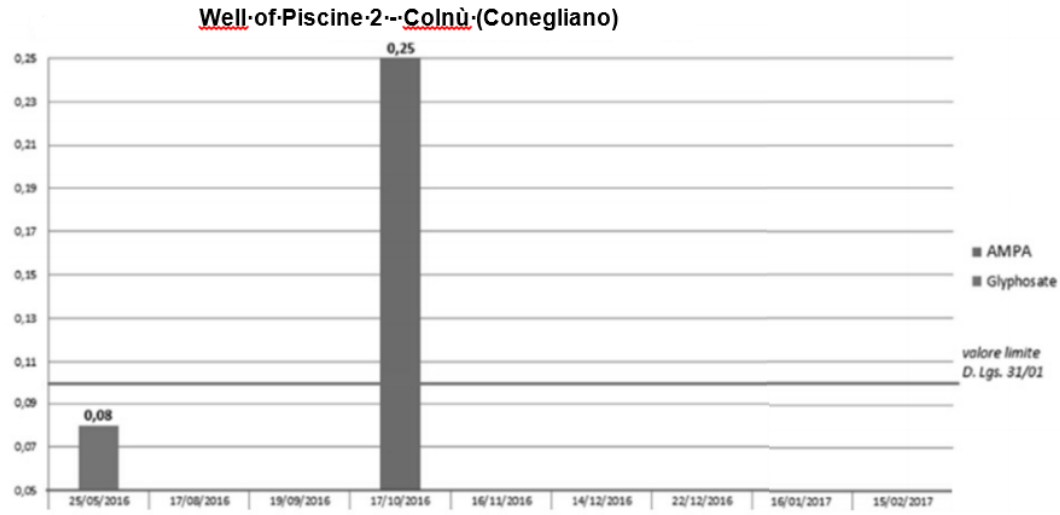


Figure 4: **Levels of glyphosate and AMPA in sequential sampling at the well located in the hamlet of *Scomigo* in the municipality of *Conegliano* in the period March 2017 to February 2018**

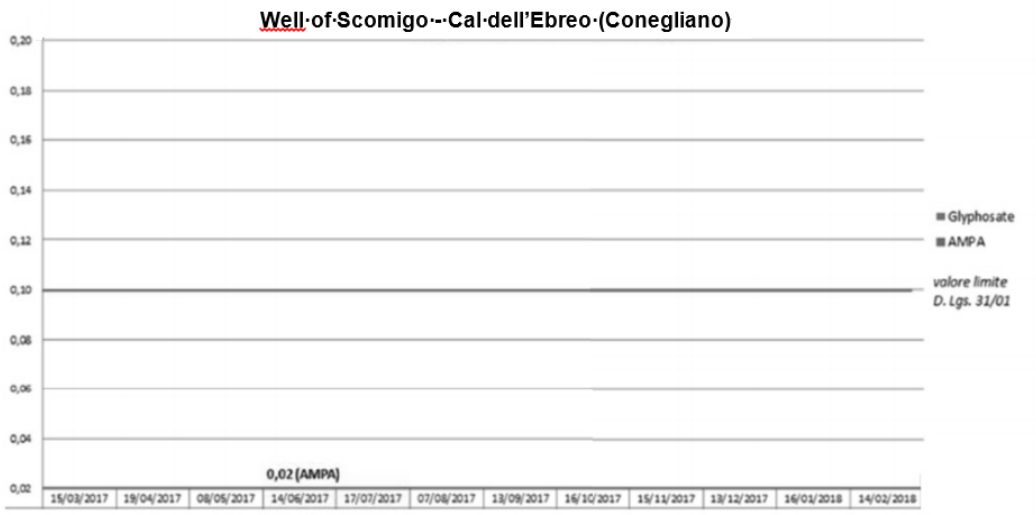


Figure 5: **Levels of glyphosate and AMPA in sequential sampling at the well located in the *Colnù* locality of the municipality of *Conegliano* in the period March 2017 to February 2018**

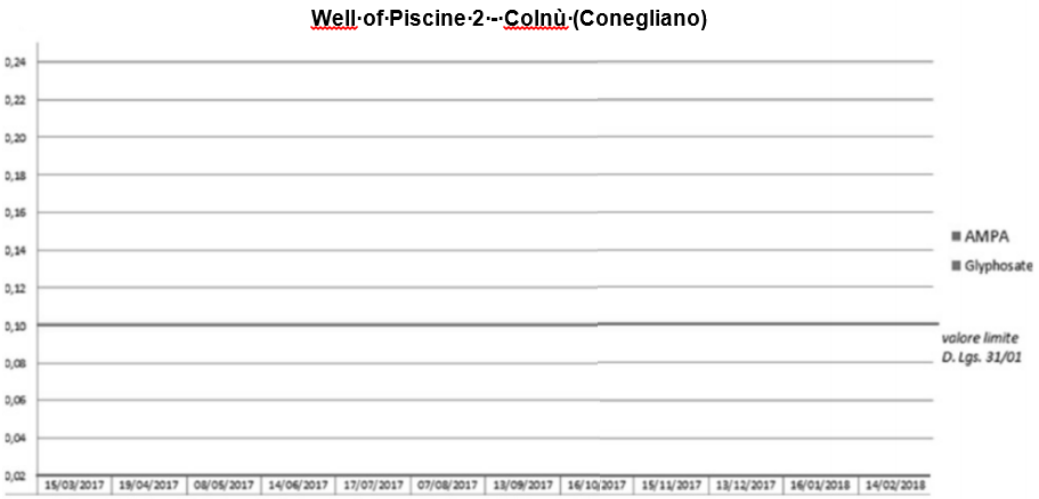
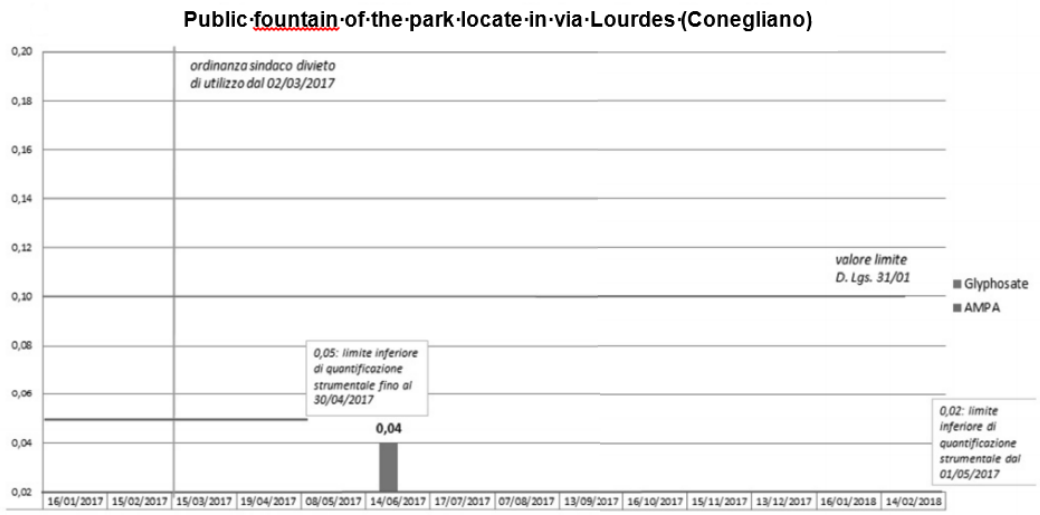


Figure 6: **Levels of glyphosate and AMPA in sequential sampling at the fountain of the public garden in *via Lourdes*, *Conegliano* in the period January 2017 to February 2018**



Conclusions

High-consumption herbicidal products such as glyphosate, and consequently its metabolite AMPA, can reach the drinking water network of an urban settlement surrounded by areas with high agricultural and wine-growing activity. Although the issue about the toxicity of glyphosate for humans is still widely debated, the experience of the Venetian Ulss2 demonstrates how the joint intervention of the institutions (in this case, the local Hygiene and Public Health Service and the municipal administrations) can contribute to eliminating the presence of a "probable carcinogen" in the city's drinking water. Furthermore, such interventions can make it mandatory to abandon potentially harmful agricultural practices, in favour of alternative solutions characterized by reduced impact on the environment and the health of local populations.

3. Assessment and conclusion

Assessmentand conclusion by applicant:
The article reports concentrations of glyphosate and AMPA found in 12 wells used for drinking water consumption in Northern Italy, situated within a wine growing area. Traces of glyphosate (maximum reached 0.08 µg/L) and AMPA (maximum reached 0.25 µg/L) were detected in 2 wells supplying an urban area. The article is therefore considered as reliable with restrictions. Sampling and analytical methods are not described. Nature of groundwater wells not described, point sources could be possible. No information on precipitation is reported. No description of monitoring sites other than very rough map of area and aqueduct names.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 7.1.4.2
Report author	Gros, P. *et al.*
Report year	2020
Report title	Leaching and degradation of $^{13}C_2$-^{15}N-glyphosate in field lysimeters
Document No	Environmental monitoring and assessment, (2020) Vol. 192, No. 2, pp. 127 DOI 10.1007/s10661-019-8045-4
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate (GLYP) may have effects in various compartments of the environment such as soil and water. Although laboratory studies showed fast microbial degradation and a low leaching potential, it is often detected in various environmental compartments, but pathways are unknown. Therefore, the objective was to study GLYP leaching and transformations in a lysimeter field experiment over a study period of one hydrological year using non-radioactive $^{13}C_2$-^{5}N-GLYP labelling and maize cultivation. ^{15}N and ^{13}C were selectively measured using isotopic ratio mass spectrometry (IR-MS) in leachates, soil, and plant material. Additionally, HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) was used for quantitation of GLYP and its main degradation product aminomethylphosphonic acid (AMPA) in different environmental compartments (leachates and soil). Results show low recoveries for GLYP (<3 %) and AMPA (<level of detection) in soil after the study period, whereas recoveries of ^{15}N (11–19 %) and ^{13}C (23–54 %) were higher. Time independent enrichment of ^{15}N and ^{13}C and the absence of GLYP and AMPA in leachates indicated further degradation. ^{15}N was enriched in all compartments of maize plants (roots, shoots, and cobs). ^{13}C was only enriched in roots. Results confirmed rapid degradation to further degradation products, e.g., $^{15}NH_4^+$, which plausibly was taken up as nutrient by plants. Due to the discrepancy of low GLYP and AMPA concentrations in soil, but higher values for ^{15}N and ^{13}C after the study period, it cannot be excluded that non-extractable residues of GLYP remained and accumulated in soil.

Materials and methods

The leaching experiment was set up in two field lysimeters (non-weighing zero tension), which were installed in the Lysimeter Station at the Helmholtz Centre for Environmental Research-UFZ (Falkenberg, Germany; 52°51′ N, 11°48′ E). These lysimeters were constructed in 1981 in sheet steel vessels with cuboid shape of 1 × 1 m surface area and 1.25 m depth. The lysimeters were filled with sandy loam (0–30 cm topsoil: 74 % sand, 14 % silt, 12 % clay, pH 4.8, organic C = 1.1 %; 30-100 cm subsoil: 75 % sand, 17 % silt, 8 % clay, pH 5.6, organic C = 0.2 %) and an additional 25 cm-drainage layer composed of three sublayers (sand, gravel, and coarse gravel) at the bottom. The soil texture is representative for the river Elbe valley in the Federal State Saxony-Anhalt. Conventional agricultural management was oriented according to best management practice. In 2017, maize was planted which was embedded in a regionally typical crop rotation of sugar beets-winter wheat-potatoes-winter barley-maize. The present study investigated a period of one hydrological year starting in the hydrological summer semester in May 2017. Any weeds were removed mechanically, followed by $^{13}C_2$-^{15}N-GLYP (GLYP*i*) application (2017/24/04) via spraying as a worst-case scenario. Application rate was equivalent to maximum allowed annual for Germany (3.6 kg/ha/a) with practical concentration of GLYP formulations (480 g/kg) (360 mL GLYP*i*, dissolved in 750 mL H_2O). Drift by air flow was prevented by temporally fencing the application area with a ring of steel (1 m in height). Three days after GLYP*i* application, 5 L of the conservative KBr tracer solution was

applied at a rate corresponding to 40 kg/KBr/ha to each of the lysimeters to provide information on the movement of water through the soil column. Lysimeters were cultivated with maize (9 plants per lysimeter, equally spaced). No fertilizers or treatments for weeding were executed during the study period.

Sampling of leachates, soil, and plant material
Lysimeter soils were sampled from 0 to 5 cm depth (5 spots equally spaced in each lysimeter) at 4 dates over the study period (before and directly after application, 165 and 360 days after application). Soil sampling before application characterizes the basic level of GLYP*i* concentration, whereas the sample directly after application represents 100 % of initial GLYP*i*. To keep the soil column intact, samples from the whole topsoil (0-30 cm) and the subsoil (30-60 cm) were taken only at the end (day 360 after application) of the study period. Soil samples were air dried and sieved (2 mm). Subsamples of the sieved soils were finely ground for further measurement with IR-MS. Residues of GLYP*i* and AMPA*i* were extracted from 5 g of the sieved soil in 40 mL of a 1 M KOH solution (shaking overnight and centrifugation for 10 min at 1558 g) and stored at -20 °C until quantitation via HPLC coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS/MS).

Leachates were collected weekly in polyethylene canisters and volumes were recorded. Subsamples of 150 mL were taken and stored in a freezer at -20 °C in 3 × 50 mL centrifuge tubes for further measurements with ion chromatography (IC) and HPLC-ESI-MS/MS. A total of 50 mL of each sample were lyophilized to dryness (-50 °C, 0.025 mbar; Christ Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, D-37250 Osterode, Germany) and solid residue amounts were weighed back and stored for measurements with isotopic ratio mass spectrometry (IR-MS).

Mature maize plants (roots, shoots, and cobs) were harvested in September 2017 from the two treated lysimeters and one untreated neighbouring plot as reference. Subsamples of 3 plants per lysimeter were harvested for further measurements of plant biomass. Moist weight was determined followed by drying at 60 °C and measuring of dry matter weight. Plant compartment samples (root, shoot, and cobs) were shredded and subsequently finely ground separately and stored until further measurements with IR-MS.

Sample analyses
Conservative tracer and isotope ratio analyses
Br^- tracer analysis in the leachate was performed using ion chromatography (column: Metrosep A SUPP 5150 × 4.0 mm, pre-column: Metrosep A SUPP 4/5 Guard, eluent: 0.3 mM Na_2CO_3 and 1.0 mM $NaHCO_3$, flow: 0.7 mL/min, separation mode: isocratic; Metrohm, D-70794 Filderstadt, Germany).

Isotopic ratios for $^{15}N/^{14}N$ and $^{13}C/^{12}C$ in soil, plant compartments, and lyophilized leachate samples were measured through the elemental analyser (Eurovector EA, Via F.lli Cuzio 42, 27100 PAVIA, Italy; IR-MS GVIsoprome, Elementar Analysensysteme GmbH, Elementar-Straße 1, 63505 Langenselbold, Germany) in the Institute for Nutritional Sciences, University of Gießen, Germany. For this purpose, finely ground soil and plant samples from the two treated sites and one untreated site (reference) were measured in triplicates. Lyophilized leachate samples from lysimeter leachates were measured in duplicates. Equations 1 and 2 show the calculation of $\delta^{15}N$ and $\delta^{13}C$ derived from isotopic ratios of the sample in relation to defined standard isotopic ratios from air for N and Pee Dee Belemnite (PDB) for C; values are generally given in ‰.

$$\delta^{13}C = \left(\frac{\left(^{13}C/^{12}C\right)_{\text{sample}}}{\left(^{13}C/^{12}C\right)_{\text{PDB}}} - 1 \right) \qquad (1)$$

$$\delta^{15}N = \left(\frac{\left(^{15}N/^{14}N\right)_{\text{sample}}}{\left(^{15}N/^{14}N\right)_{\text{air}}} - 1 \right). \qquad (2)$$

GLYP*i* and AMPA*i* analyses

Soil extracts and leachate samples were analysed for GLYP*i* and AMPA*i* with HPLC-ESI-MS/MS after derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl), as described in Wirth *et al.* (2019). The utilized system was composed of an LC-2040C Nexerai and a triple quadrupole mass spectrometer LCMS8060 (Shimadzu, Duisburg, Germany) equipped with a heated ESI-source. The FMOC derivatives were separated on a Gemini 3 µm NX-C_{18} column (Column 1: 150 × 2 mm, Aschaffenburg, Phenomenex, Germany).

Non-isotope-labelled GLYP (LGC Standards, Wesel, Germany) was used as internal standard for GLYP*i* (Sigma Aldrich, Taufkirchen, Germany) quantitation. Since AMPA*i* is not commercially available as a standard substance, no HPLC-ESI-MS/MS-optimization and, thus, no calibration could be carried out for this compound. Therefore, AMPA*i* was determined only qualitatively. Analytes were detected in the multiple reaction monitoring (MRM) mode. The MRM transitions were determined and optimized utilizing standard compounds (Table 1). However, as AMPA*i* is not commercially available, instrumental MRM optimization for AMPA*i*-FMOC could not be performed. Therefore, the settings for the MRM transitions for this compound were chosen as follows: optimization was carried out for ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC (LGC Standards, Wesel, Germany) and their fragmentation patterns were utilized to derive the expected masses of the precursor and product ions for ^{15}N-AMPA-FMOC (AMPA*i*-FMOC). Further parameters of the MRM transitions were set by averaging values for ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC (Table 1).

To further verify that the targeted and detected compound was the ^{15}N-AMPA-FMOC, a selection of samples was additionally separated on a different LC-column (Column 2: Kinetex 2.6 µm EVO C18 100 Å, 150 × 2.1 mm, Phenomenex, Aschaffenburg, Germany). The proposed AMPA*i*-FMOC was eluted from both columns at similar retention times as AMPA-FMOC (Table 1) which confirms its presence. Due to the lack of an AMPA*i*-FMOC calibration, these data could be evaluated only semi-quantitatively. Quantitation of GLYP*i* was carried out through weighting with the glyphosate internal standard signal.

Table 1 **Measurement modes for identification and quantitation of $^{13}C_2$-^{15}N-glyphosate and ^{15}N-aminomethylphosphonic acid using high performance liquid chromatography tandem mass spectrometry (HPLC-ESI-MS/MS)**

Component	Measurement mode	Precursor m/z	Product m/z	Collision energy	Retention time column 1(min)	Retention time column 2 (min)
$^{13}C_2$-^{15}N-Glyphosate-FMOC (GLP*i*)	-	392.10	170.15	14	9.10	8.77
			152.20	24		
			63.10	48		
Glyphosate-FMOC	-	390.00	168.15	14	9.10	8.76
			150.20	23		
			63.05	49		
^{15}N-AMPA-FMOC* (AMPA*i*)	+	335.20	179.05	−23	9.47	9.18
			178.15	−48		
			157.05	−10		
			113.05	−15		
AMPA-FMOC	+	334.20	179.05	−23	9.47	9.19
			178.15	−46		
			156.00	−10		
			112.05	−15		
^{13}C-^{15}N-AMPA-FMOC	+	336.20	179.05	−22	9.46	n.a.
			178.10	−50		
			158.15	−10		
			114.10	−15		

*derived from the optimized MRM transitions of ^{13}C-^{15}N-AMPA-FMOC and AMPA-FMOC

Results

Precipitation and leachate analysis

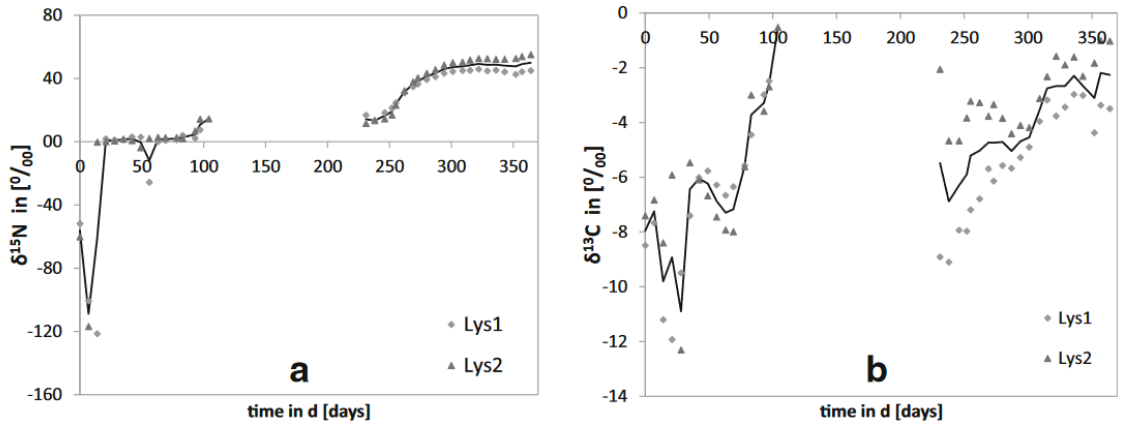
The study period from May 2017 to April 2018 was characterized by overall high amounts of precipitation that exceeded the monthly 30-year mean values (1981-2010) for this region, except for the months May, September, and February. Especially, June and July were characterized by heavy rainfall events that summed up to 123 and 125 mm per month precipitation, greatly exceeding the mean values of 57 ±22 mm (June) and 61 ±32 mm (July). These events resulted in large amounts of leachate in July 2017 (60.4 and 66.3 L). Weekly leachate amounts, collected from May 2017 until July 2017 to December 2017 until April 2018, had a mean volume of 5.1 L per week. For the period from August 2017 to November 2017, no leachates were received although precipitation occurred, most likely because of transpiration and water uptake by plants. Total volumes of leachates for the two lysimeters were 203 and 215 L over the study period. The Br^--breakthrough started in week 10 after application, where 35 and 37 L of leachate were received in the two tested lysimeters. Residues from the conservative tracer KBr were detected later on in all leachates. Due to the occurrence of Br^- in the leachates after 10 weeks and its slowly increasing concentrations over the following weeks along with continually received leachates, the main transport mechanism through the soil column can be assumed as matrix flow for the studied period.

For the natural ^{15}N background representing the ratio of $^{15}N/^{14}N$ of the air nitrogen, the $\delta^{15}N$ has been set to 0 (Fig. 1a). Discrepancies towards higher values indicate an enrichment of ^{15}N. In the first 2 weeks after application, a strong decrease of leachate $\delta^{15}N$ to negative values was detected, indicating an enrichment of ^{14}N. In the following weeks 3 to 10, the $\delta^{15}N$ in the leachate was constant between 0 and 1.7 ‰, and it increased over time from week 11 after GLYP*i* application. After the period with no leachates, the trend of $\delta^{15}N$ had a sigmoidal shape with an assumed maximum limit of about 50 ‰ for the last 10 weeks of the experimental period. This maximum level corresponds to a mass rate of about 10 µg ^{15}N/week of leached GLYP*i* active ingredient equivalent or its N-containing degradation products.

Values for $\delta^{13}C$ started at about -8 ‰ and fluctuated between -12 and -6 ‰ for the first 10 weeks before they strongly increased and reached values of -2.6 and -0.5 ‰ in the two lysimeters (Fig. 1b). After the period with no leachates, the $\delta^{13}C$ started at lower levels of -8.9 and -2.1 in lysimeters 1 and 2, respectively. The trend of increasing $\delta^{13}C$ values starting at -5.5 ‰ went on and ended at -2.3 ‰ for the remaining 20 weeks of the study, although with a less steep slope than in the first experimental phase.

Trends of ^{13}C and ^{15}N originating from GLYP*i* in the leachate (Fig. 1) did not correlate, which may be an indication for an independent movement of these isotopes through the soil column. Also, IR-MS cannot distinguish between GLYP*i* and its degradation products, but simultaneous occurrence and parallel trend would be an indication for a displacement of intact GLYP*i*, which appears unlikely from these data. The analyses for GLYP*i* and AMPA*i* using HPLC-ESI-MS/MS in the leachates showed no occurrence of residues of these compounds above detection limits (0.1 µg/L). Therefore, the leached ^{15}N and ^{13}C residues are most likely no constituents of intact GLYP*i* or AMPA*i* but originated from further degradation products.

Fig. 1 **^{15}N (a) and $\delta^{13}C$ (b) values for lyophilized leachates over the one-year study period in lysimeter 1 (Lys1) and lysimeter 2 (Lys2) and mean values (continuous line)**

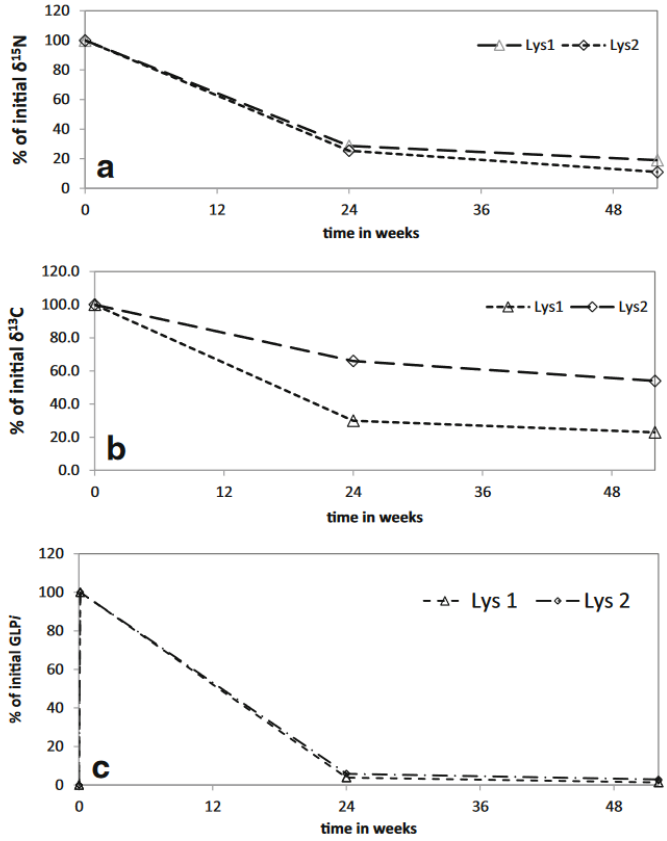


Soil analyses

The concentrations of GLYP*i*, ^{15}N and ^{13}C in the lysimeter soils derived from HPLC-ESI-MS/MS and IR-MS, respectively, were normalized and set to 100 % since GLYP*i* was not detectable in soil extracts sampled before GLYP*i* application (data not shown) (Fig. 2a). The $\delta^{15}N$ decreased within 165 days after application to 24 and 29 % of the initial value and decreased further to 11 and 19 % until the end of the study period. This indicates that amounts of the added artificial ^{15}N isotopes in soil decreased over time. The same was true for $\delta^{13}C$ which decreased down to 30 and 66 % compared with the initial value and ended at 23 and 54 % in the two lysimeters (Fig. 2b).

Measurement of the GLYP*i* residues through HPLC-ESI-MS/MS showed that about 4 and 6 % of the initial GLYP*i* concentration remained in the soil after 165 days and the recovery decreased further down to 1 and 3 % in the two lysimeters until the end of the study (Fig. 2c). AMPA*i* was detected in the topsoil extracts of all samples after application, and GLYP*i* and AMPA*i* were not detected in the subsoil (results not shown). This indicates that AMPA*i* had not been formed, and GLYP*i* was already decomposed by microorganisms or scarcely displaced from surface into subsoil. Therefore, leaching of GLYP*i* or AMPA*i* can be considered as insignificant in this experiment and rapid degradation to further products most likely happened. Nevertheless, it is still possible that strongly bound non-extractable, and therefore non-detected residues of GLYP*i* or AMPA*i* could have remained in soil too, partly explaining the higher amounts of ^{13}C and ^{15}N after 165 and 360 days (Fig. 2a and b).

Fig. 2 **Development of $\delta^{15}N$ (a), $\delta^{13}C$ (b), and $^{13}C_2$-^{15}N-glyphosate (c) in topsoil samples compared with initial values (set to 100 %) over the studied period in lysimeter 1 (Lys1) and lysimeter2 (Lys2)**



Plant material analyses

^{15}N was enriched highly significantly ($p < 0.01$) in all sampled plant compartments (root, 39 ±10 ‰ and 54 ±16; shoot, 28 ±13 ‰ and 51 ±16 ‰; cob, 34 ±12 ‰ and 51 ±14 ‰) compared with reference plant parts from a lysimeter that was not treated with GLYP*i* (root, 2.5 ±1.6 ‰; shoot, 2.0 ±0.9 ‰; cob, 4.0 ±1.9 ‰) (Fig. 3). By comparison, ^{13}C was highly significantly enriched only in the plant roots from the two lysimeters treated with GLYP*i* (-12.75 ±0.07 ‰ and -12.84 ±0.06 ‰) compared with maize roots from the lysimeter with no herbicide treatment (-13.03 ±0.08 ‰). In contrast, ^{13}C was significantly depleted ($p < 0.01$) in the cob material of plants from lysimeters with GLYP*i* treatment (-23.24 ±0.18 ‰ and -24.06 ±0.96 ‰) compared with those with no treatment (-22.84 ±0.25 ‰). There was not a significant difference in the shoots between the treated (-13.69 ±0.06 ‰ and -13.58 ±0.05 ‰) and non-treated lysimeters (-13.56 ±0.45 ‰).

The enrichment of ^{15}N in roots, shoots, and cobs can result only from uptake from the soil and distribution through the plant. Since ^{15}N is bound in GLYP*i* or its ^{15}N containing degradation products (Fig. 4), those degradation products must have acted as plant nutrients. Furthermore, as plants do not take up organic substances like GLYP*i* or AMPA*i* over the root system, the occurrence of ^{15}N can be plausibly explained only by an uptake of mineral ^{15}N ($^{15}NH_4^+$ and/ or $^{15}NO_3^-$) as mineralized degradation products from GLYP*i*, which are formed by microbial degradation in the rhizosphere (Duke *et al.* 2012).

The enrichment of ^{13}C in the roots compared with plants from the non-treated lysimeter may be explained by attachment, possibly due to mycorrhizal fungi associated with the maize roots (Bott *et al.* 2011) that utilize organic substances as nutrients for growth.

In summary, since (i) ^{15}N has been taken up by the maize roots and distributed into all plant compartments and (ii) ^{13}C is only associated with the plant roots, the interaction of these labelled atoms with the plants most plausibly resulted from the independent interaction of the inorganic degradation products of GLYP*i*.

$^{13}CO_2$ and $^{15}NH_3$ as the inorganic end-products of the degradation process can be emitted via the air path. This was shown for ^{14}C labelled GLYP (Grundmann *et al.* 2008). But since (i) $^{15}NH_3$ is water-soluble and forms $^{15}NH_4^+$ in soil solution and (ii) ^{15}N was taken up by plants, it is rather unlikely for inorganic N to be emitted into the air.

Fig. 3 **$\delta^{15}N$ and $\delta^{13}C$ mean values in plant material of roots, shoots, and cobs of maize plants of the tested lysimeter 1 (Lys1), lysimeter 2 (Lys2), and a reference lysimeter (LysRef)**

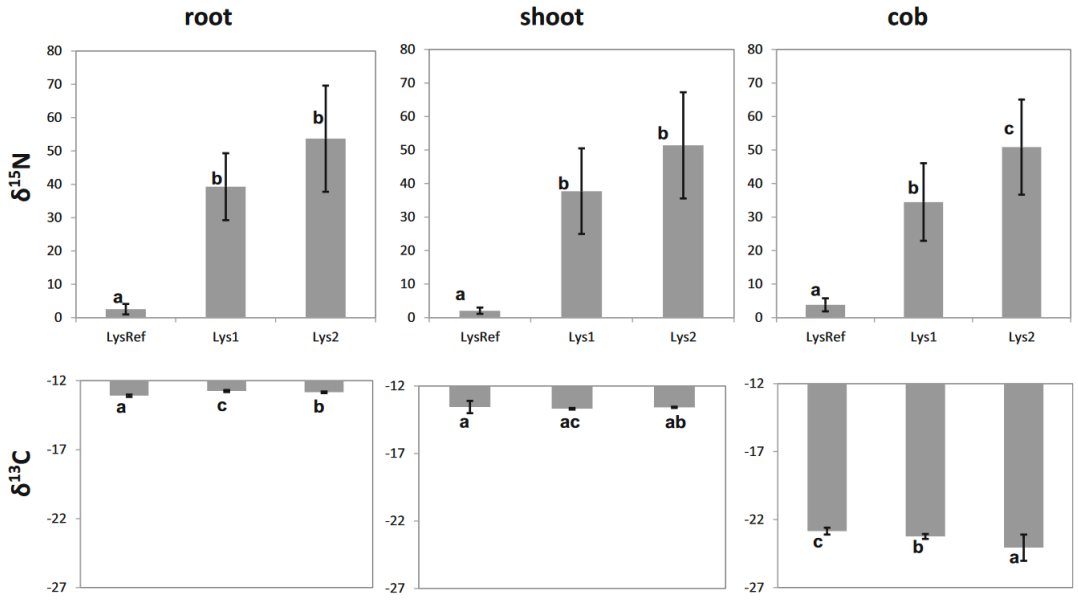
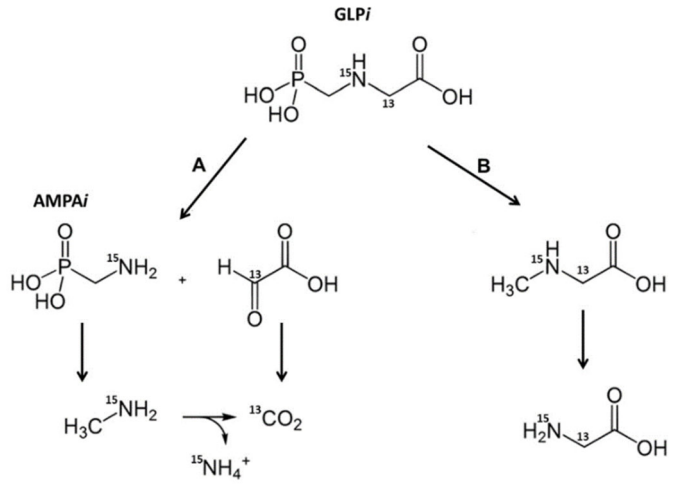


Fig. 4 **Degradation pathways of isotopic labelled $^{13}C_2$-^{15}N-glyphosate (GLP*i*) and its main degradation product ^{15}N-aminomethylphosphonic acid (AMPA*i*) with indicated positions of labelling (modified from Giesy *et al.* 2000)**



Discussion

The present study was designed in detail so that field conditions are reflected and also all relevant compartments are considered.

In the present study, concentrations of extracted GLYP*i*-residues were low in soil at the end of the study period compared with the initial concentrations at the beginning. But fractions of ^{15}N and ^{13}C above extracted GLYP*i*-residues indicate that either non-extractable GLYP*i* is still left and/ or further degradation products accumulated in soil.

The ^{13}C and ^{15}N are signals of leachates (Fig. 1), but absence or low concentrated (<LOD) residues of GLYP*i* and AMPA*i* indicate that further degradation products have been leached through the soil column. The noncorrelated appearance of ^{13}C and ^{15}N signals in leachates (Fig. 1) makes the degradation pathway B in Fig. 4 rather unlikely. Instead, pathway A in Fig. 4 is supported by the noncorrelated appearance of ^{13}C and ^{15}N signals in leachates, among which ^{13}C can originate from glyoxylic acid and ^{15}N from detected AMPA*i* or further degradation products, such as methylamine and ammonium ions (Fig. 4). Along with small concentrations of extracted GLYP*i* (Fig. 2c) under practically optimal leaching conditions in the very wet hydrological year 2017/ 2018, the present findings indicate that rapid degradation most likely is the best explanation for the absence of concentrations above LOD of GLYP*i* and AMPA*i* in leachates.

Conclusions

Isotopic ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and resulting changes of $\delta^{13}C$ and $\delta^{15}N$ from isotopically labelled glyphosate (GLYP*i*) and its degradation products were successfully quantified using isotopic ratio mass spectrometry (IR-MS) in different compartments (leachates, soil, and plant material) of a field lysimeter. Therefore, this experimental approach was well suited to trace GLYP*i* under practice-near experimental conditions.

Since (i) the great decline of GLYP*i* content down to <3 % of initial amounts in soil during the one-year study period and (ii) a lower decline of ^{13}C (<60 %) and ^{15}N (<20 %), we conclude that either further degradation products had been formed and/ or non-extractable and, therefore, strongly bound GLYP*i* remained in soil and accumulated. The disparate increase of $\delta^{13}C$ and $\delta^{15}N$ values in leachates and plant material is explained plausibly by (i) rapid degradation of GLYP*i* within one vegetation period and, also (ii) the selective uptake of mineralized ^{15}N species from degraded GLYP*i* as plant nutrient, most likely NH_4^+ or NO_3^-. These findings from a wet hydrological year support the assumption that the risk of leaching of applied GLYP to other waterbodies can be considered to be low under central European climatic conditions. Accumulation in soil may enhance the risk of further distribution in the environment by soil erosion.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The article reports results of a lysimeter experiment with $^{13}C_2$-^{5}N-glyphosate in Germany. Besides analysis of lysimeter leachate, also soil and plant (maize) samples were analyzed. Although, the methods and results are well described, no endpoint can be derived due to some deviations from the relevant guideline (OECD 22). For example, it is not clear whether an undisturbed soil monolith has been used, and the origin and storage of soil is not reported. Amounts of precipitation are not reported in sufficient detail (only for 2 months) and amounts of leachate are only given as overall sum and weekly averages. glyphosate, the sensitivity of the analytical method is not reported (AMPA was analyzed only qualitatively), and the stability of analytes in leachates and extracts during frozen storage was not shown. Results of leachate analysis were not reported in µg/L (only as $\delta^{13}C$ and $\delta^{15}N$), and results of soil analysis were only given in % of initial concentration.
The article is therefore considered as reliable with restrictions.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（生活環境動植物及び家畜に対する毒性）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 8.6.2
Report author	Rogacz D. *et al.*
Report year	2020
Report title	Ecotoxicological effects of new C-substituted derivatives of *N*-phosphonomethylglycine (glyphosate) and their preliminary evaluation towards herbicidal application in agriculture.
Document No	Ecotoxicology and environmental safety, (2020) Vol. 194, Art. No. 110331.
Guidelines followed in study	OECD 208 (for NTTPs)
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable

2. Full summary of the study according to OECD format

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE methodology (OECD 2006). The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

Materials and methods

Glyphosate (≥ 98.0 %) was purchased from Aldrich, Poznań, Poland.

Plant growth test

The plant growth test of glyphosate was performed in laboratory conditions following the Organisation for Economic Co-operation and Development standard Guideline Terrestrial Plants Growth Test (OECD 2006). According to the OECD 208 standard, the plant growth test was carried out in sandy soil of the following parameters: granulometric composition of soil: 77% sand, 16% dust and loam, organic carbon content of approx. 1.7%, pH (KCl) = 6.5.

Tests were carried out in polypropylene pots (diameter of 90 mm and capacity of 300 cm^3), which were filled with the control soil or with the soil mixed with the tested compounds added at following concentrations: 100, 200, 400, 800 and 1000 mg/kg of soil dry weight (s.d.w.). Each concentration was done in triplicate (3 pots for oat, 3 pots for common radish).

Twenty seeds of each of the selected plant species were sown into the soil. Oat (*Avena sativa*) and radish (*Raphanus sativus*) were selected as the representing species of monocotyledonous and dicotyledonous plants respectively. Seeds of each species originated from the same source. Plants were grown for 14 days under controlled conditions: a constant humidity content at the level required for the plants (70% field water capacity), temperature (22 ± 2 °C), constant light intensity (7000 lux), were maintained in the system of 16h/day and 8 h/night.

The evaluation of phytotoxicity at applied concentrations was made by comparing the germination, dry weight of control plants sprouts (seedlings) with germination and of dry and fresh plants sprouts grown in the soil with an admixture of given amounts of the tested compounds. Inhibition of fresh mass, root and shoot of plants was measured as described in the work by Lewkowski et al. 2016a, 2016b; Rogacz et al. 2018, Lewkowski et al. 2017a, 2017b, Matusiak et al. 2013.

Pigment assay

Photosynthetic pigments content was determined according to a method reported by Oren et al. 1995. Fresh leaves (200 mg) were thoroughly homogenized in 20 mL of 80% acetone in a cooled mortar and centrifuged afterward. The content of chlorophyll A, chlorophyll B and carotenoids was calculated based on the absorbance at wavelength 470, 647 and 664 nm. The content of photosynthetic pigments was expressed in mg/g of dry weight.

Statistics

One-way analysis of variance (ANOVA) has been used for comparison of obtained results related to phytotoxicity. Tukey's test with $p < 0.05$ (STATISTICA 13.3) was used to determine the significance of reported differences. The data presented are expressed as the mean with standard deviation obtained from 3 measurement replicates.

Results

Seedling emergence and growth test on oat and radish

The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure to glyphosate are summarized in the table below.

Table 1: Effects of glyphosate on the shoot height, the root length and the fresh matter of oat and radish seedlings plant compared to control (mean ± SD, n = 15).

Compound concentration [mg a.s./kg s.d.w.]	Mean shoot height inhibition ± SD [%]	Mean root length inhibition ± SD [%]	Mean inhibition to fresh matter ± SD [%]
	Oat		
100	7.2 ± 0.1	18.6 ± 1.1	10.7 ± 0.1
200	18.2 ± 0.4	25.1 ± 1.0	19.6 ± 0.4
400	53.4 ± 1.2	33.1 ± 0.6	43.8 ± 0.5
800	84.2 ± 2.0	41.2 ± 0.3	85.4 ± 0.7
1000	94.0 ± 0.9	90.4 ± 0.3	88.2 ± 0.1
	Radish		
100	7.3 ± 0.5	19.2 ± 0.3	11.1 ± 0.1
200	18.3 ± 0.7	26.7 ± 1.1	21.4 ± 0.1
400	57.1 ± 0.6	88.0 ± 0.8	60.7 ± 0.1
800	85.4 ± 0.3	91.8 ± 0.1	87.1 ± 0.1
1000	95.6 ± 0.1	94.2 ± 0.1	94.1 ± 0.1

Inhibition of shoots, roots and fresh matter in both plants (oat/radish) have been observed at all glyphosate concentrations tested (100, 200, 400, 800 and 1000 mg a.s./kg of soil dry weight).

Germination

The percentage germination of the tested plants treated with glyphosate is shown in the table below.

Table 2: Average changes (mean of three replicates) of germination of oat and radish treated with glyphosate.

Compound concentration [mg a.s./kg s.d.w.]	Number of Emerged Seedlings		Germination [%]	
	Oat	Radish	Oat	Radish
Control	20	19	100	100
100	19	17	98	88
200	19	16	98	86
400	18	15	93	77
800	18	13	92	70
1000	17	12	86	65
% Germination refers to number of emerged plants expressed as a percent of control plants.				

The higher the concentration of glyphosate in the soil, the stronger adverse effect was observed. At the highest concentration (1000 mg/kg), percentage germination was lower for radish (65%) when compared to oat (86%).

Dry matter

Dry matter of both treated plants oat/radish increased with growing concentration of glyphosate in the soil.

NOEC, LOEC and EC_{50} values

Changes of dry matter level caused by glyphosate are in accordance LOEC and NOEC values for oat and radish seedlings.

Calculated values of NOEC and LOEC of glyphosate for oat/radish were **100 and 200 mg a.s./kg of s.d.w.**, respectively.

The EC_{50} values of shoots was **373.7 mg a.s./kg s.d.w.** for oat, and **357.8 mg a.s./kg s.d.w.** for radish.

The EC_{50} values of roots was **269.3 mg a.s./kg s.d.w.** for radish and **556.9 mg a.s./kg s.d.w.** for oat.

The EC_{50} values of fresh weight was **333.2 mg a.s./kg s.d.w.** for radish and **418 mg a.s./kg s.d.w.** for oat.

Changes of pigment levels

The chlorophyll content measured in the radish/oat leaves decreased upon exposure to glyphosate. Glyphosate caused accumulation of carotenoids in leaves of both tested plants.

Conclusion

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE methodology (OECD 2006). The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study investigated the effects of glyphosate on the seedling emergence and growth of non-target terrestrial plants (oat and radish) based on OECD 208 guideline. Plants were exposed to glyphosate mixed into sandy soil at 5 concentrations between 100 and 100 mg a.s./kg of soil dry weight with 3 replicates each. There were 20 seeds of each plant species per test concentration sown into the soil. Evaluations were based on fresh mass, root length and shoot height of plants after 14 days of exposure.

The test design was adequately described, but the application of the test item into the soil is not specified. The seedling emergence was acceptable as recommended in the guideline (100 and 95 % in the control for oat and radish, respectively). However, the phytotoxic effects and the survival of the control plants during the study is not reported.
Reliable endpoints for the risk assessment of NTTPs can be obtained for glyphosate: EC_{50} value of 373.7 mg a.s./kg s.d.w. for oat based on shoot height and an EC_{50} value of 357.8 mg a.s./kg s.d.w. for radish based on shoot height.

The article is classified as reliable for NTTPs.

Assessment and conclusion by RMS:

1. Information on the study

Data point:	CA 8.1.4
Report author	Turhan D. Ö *et al.*
Report year	2020
Report title	Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles
Document No	Bulletin of Environmental Contamination and Toxicology, (2020) Vol. 104, No. 2, pp. 173-179
Guidelines followed in study	ASTM (2003) American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-*Xenopus* (FETAX), E1439-98
Deviations from current test guideline	Jelly coats should be removed from developing embryos mid blastula stage embryos – ca. stage 8, prior to 96 hour exposure well plates. The removal is achieved using 2% cysteine solution followed by rinsing. No reference is provided in the article to see whether this step was completed (it is a relevant as it informs on ion-exchange and impacts from concentrated solution / osmotic and diffuse pressures on the embryos in the increasing concentrations of test media.) Test guideline followed for the late development stage 46 (96 hr test) exposure test was not stated. The FETAX assay runs until 80-90% of the control tadpoles reach developmental stage 47. This cannot be confirmed. Water quality at test start appears to be within specification, although after 96 hrs now water quality data appeared to have been determined.
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

[Information in the publication relating to a potassium salt based glyphosate formulation has been excluded from this summary as it cannot be related to the representative formulation MON 52276].

Materials and methods

Glyphosate was purchased from Sigma-Aldrich (PESTANAL®, analytic grade, 45521).

The embryos and tadpoles used in the tests were obtained from male and female frog pairs from an adult *X. laevis* colony in the Author's laboratory. *X. laevis* breeding and acquisition of embryos were performed according to ASTM-E1439-98 (ASTM 2003). Embryos and tadpoles were maintained in a standard Frog Embryo Teratogenesis Assay Xenopus (FETAX) test medium (ASTM 2003) with the following composition: 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \times 2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per liter of distilled water.

The exposure solutions of glyphosate were prepared fresh daily in the standard FETAX test medium. The pH of glyphosate solutions was adjusted to 7.9, as recommended for FETAX tests, using NaOH. Embryos and tadpoles were exposed to test solutions under semi-static test conditions with a 12:12 h light:dark photo-period at 23 °C (± 1 °C).
Before starting the FETAX test, glyphosate levels in the test medium were measured using high-performance liquid chromatography (HPLC) (1100 system, Agilent Technologies, Santa Clara, CA, USA). The measured glyphosate concentrations in exposure media were determined to be at least 92% of the nominal concentrations.

For the FETAX test, stage 8 embryos were exposed to different glyphosate concentrations for 96 h. Four embryos were randomly selected, and placed with 2 mL of test medium into each well of 24-well plates, serving as one replicate per treatment. All concentrations were tested with eight replicates and thus a total of 32 embryos. In the FETAX test, seven concentrations of glyphosate (282–500 mg/L) were tested plus controls. The test medium was changed every 24 h. Dead tadpoles were removed and incidences were recorded. The median lethal concentrations (LC_{50}) were determined after 24, 48, 72, and 96 h of exposure. At termination of the bioassays, surviving embryos were euthanized and observed for developmental abnormalities.

For the tadpole-toxicity bioassays, stage 46 tadpoles were exposed to different glyphosate concentrations for 96 h. Five randomly selected tadpoles were placed in each well of 12-well plates containing 3 mL of test solution. All concentrations were tested with 6 replicates resulting in a total of 30 tadpoles per concentration. In the tadpole-toxicity tests, seven concentrations of glyphosate (250–403 mg/L) were tested excluding control groups.

For biochemical assays, stage 46 tadpoles were exposed to three glyphosate concentrations (50-250 mg/L) for an exposure period of 96 h. Fifteen randomly selected tadpoles and 10 mL of test solution were placed into 25 mL polycarbonate containers. All concentrations were tested with five replicates resulting in a total of 75 tadpoles per treatment. At the end of the exposure period, surviving tadpoles were euthanized and their enzyme activities were measured (glutathione S-transferase (GST), glutathione reductase (GR), carboxylesterase (CaE), acetylcholinesterase (AChE), superoxide dis-mutase (SOD)).

Graphpad Prism software (Version 5, USA) was used to calculate the average lethal concentration (LC_{50}) and 95% confidence intervals (CI) and for other statistical analyses. A log(dose)-normalized response curve ($Y = 100/ (1 + 10^{(logEC50-x)} \times hillslope$) to fit mortality data. For statistical analysis of biomarkers, data were tested initially for homogeneity of variances and normality distributions by the Bartlett and Kolmogorov-Smirnov tests, respectively. Nonparametric data were analyzed using Kruskal-Wallis test followed by pairwise comparisons of groups using Mann-Whitney U tests. Parametric data were analyzed using the One-way Analysis of Variance (ANOVA) followed by the unpaired t test. A Bonferroni correction was applied ($0.05/3 = 0.016$). In order to determine growth inhibition, the head-to-tail lengths were measured and the lengths were compared using ANOVA (Dunnett's post hoc test, $p < 0.05$).

Results

Even the highest glyphosate concentrations did not cause a lethality higher than 17% for both *X. laevis* embryos and tadpoles in this study.
Moreover, the highest glyphosate concentrations (403 and 500 mg/L) caused no growth inhibition in embryos or tadpoles (see table below).

Table 1: Length of stage 8 embryos and stage 46 tadpoles of *Xenopus laevis* after 96-h exposure to different concentrations of glyphosate.

Glyphosate					
Conc. (mg/L)	***n***	**Length (mm)[a)]**	**Conc. (mg/L)**	***n***	**Length (mm)[a)]**
8th stage			**46th stage**		
Control	32	7.14 ± 0.07	Control[b]	30	10.13 ± 0.10
282	31	7.15 ± 0.08	250	25	10.52 ± 0.07
310	31	7.14 ± 0.07	275	29	10.36 ± 0.08
342	26	7.04 ± 0.08	303	26	10.44 ± 0.09
376	32	7.22 ± 0.06	333	29	10.28 ± 0.04
413	32	7.21 ± 0.08	367	26	10.23 ± 0.08
455	30	7.38 ± 0.08	403	25	10.48 ± 0.07
500	29	7.04 ± 0.10	---	---	---

[a)] Lengths are expressed as mean± standard errors. These values were obtained from the lengths of the surviving individuals (n).
[b)] The average length of tadpoles at the beginning of this test is 7.30 ±0.10 mm (*n* = 32).

In addition, the selected biochemical markers in tadpoles exposed to glyphosate did not show any statistically significant changes (see table below).

Table 2: The enzyme activities of stage 46 tadpoles after 96-h exposure to different concentrations of glyphosate.

		Glyphosate				
Concentration (mg/L)	***n***	**GST[a)]**	**GR[a)]**	**CaE[a)]**	**AChE[a)]**	**SOD[b)]**
Control	5	130 ± 3.9	11.2 ± 0.43	167 ± 4.4	142 ± 3.2	0.65 ± 0.05
50	5	134 ± 4.9	11.3 ± 0.38	155 ± 3.9	128 ± 6.9	0.60 ± 0.02
100	5	131 ± 1.0	9.7 ± 0.47	154 ± 2.5	126 ± 5.4	0.60 ± 0.05
250	5	141 ± 3.5	10.7 ± 0.28	170 ± 3.1	132 ± 6.5	0.64 ± 0.03

[a)] The enzyme activity was expressed as nmol/min × mg protein ± standard error.
[b)] The enzyme activity was expressed as U/mg protein ± standard error.

Conclusion

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Effects of pure glyphosate and a glyphosate-based product Roundup® Star (containing glyphosate in a form of a potassium salt and including 6% surfactant as ethoxylated alkylamine based, were evaluated comparatively using two embryonic development stages of the amphibian *Xenopus laevis* as model system. As the glyphosate-based product Roundup® Star is not the representative formulation for the European renewal of glyphosate, the summary only provides information for pure glyphosate.

However, this publication confirms a general trend that toxic effects caused by glyphosate-based products, compared to pure glyphosate, are increased mainly due to additives present in glyphosate formulations and that it may be a result of synergistic effects between glyphosate and adjuvant in the formulations.

In this study, no lethality >17 % or developmental effects (growth inhibition) were observed in embryos or tadpoles with pure glyphosate at any glyphosate concentration tested (282-500 mg/L for stage 8 embryos) and (250-403 mg/L stage 46 tadpoles)
In addition, no effect was observed with regards to enzymatic activity of stage 46 tadpoles at any glyphosate concentration tested (50-250 mg/L).

The article is classified as reliable with restrictions for the following reason : The specific purity of the test item was not reported. No OECD guidance has been followed. The American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-Xenopus (FETAX test), E1439-98 has been followed with some deviations to the recognised approach (see deviations above). The FETAX assay is a developmental toxicity screening test, which for the most part has been superseded by amphibian metamorphosis and developmental toxicity assays using *Xenopus laevis* (OECD 231 and OECD 241). Studies performed according to both of these recognised test guidelines were submitted with the Annex I dossier (M-CA Section 8.2.3/002 and M-CA Section 8.2.3/003). Whilst the FETAX assay is not directly recognised at the EU level, elements of the FETAX assay are considered in the conduct of the OECD 231 test guideline.
Control mortalities were not reported (only LC_{50} final results). Analytical verifications of the concentrations in the test medium were reported only before starting the test, but exposure medium was changed every 24 h to maintain the desired concentrations.

Assessment and conclusion by RMS:

グリホサートカリウム塩

要旨及び評価結果

（農作物及び畜産物への残留）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 6.9
Report author	Panseri, S. *et al.*
Report year	2020
Report title	Occurrence of perchlorate, chlorate and polar herbicides in different baby food commodities
Document No	Food chemistry, (2020) Vol. 330, Art. No. 127205 DOI 10.1016/j.foodchem.2020.127205
Guidelines followed in study	SANTE/11813/2017: Method validation & quality control procedures for pesticide residues analysis in food & feed US FDA FVM (2015): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data within the Office of Foods and Veterinary Medicine. US FDA FVM (2019): FDA Foods and Veterinary Medicine Science and Research Steering Committee: Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition. (Analytical methods)
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable

2. Full summary of the study according to OECD format

The incidence of glyphosate, its metabolite aminomethylphosphonic acid (AMPA) and other substances was estimated in baby food commodities (meat, fish, cheese, vegetable and fruit). Ion chromatography coupled to high resolution mass spectrometry analysis of the 105 samples did not show traces of glyphosate or its metabolite AMPA.

Materials and methods

Chemicals and reagents
Glyphosate and aminomethylphosphonic acid (AMPA) (certificated standards) and the internal standard (IS) *N*-Acetyl-D3-glufosinate were purchased from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Formic acid (98-100 %) was from Riedel-de Haën (Sigma-Aldrich). Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany).

Standard solutions
The principal standard solutions of each compound (1 mg/mL) were prepared in water and stored as recommended by the EU Reference Laboratory for pesticides (Anastassiades *et al.*, 2015). The working solution was kept in 4 °C plastic flasks to avoid pesticide interaction with glass-surfaces.

Sample collection
A total of 105 baby food samples were collected; all details are specified in Table 1. They were from different commercial brands, present in international markets, and bought in different Italian supermarkets (94 samples) and in some Serbian supermarkets (11 samples). In particular, all products are commercialized as homogenized food, packaged in sterile conditions (jar of 80 g) made from vegetable, fruits, meats, fish, cheese or combining different of these matrices, directly ready-for-eat. No sample processing (rehydration/mixing) is necessary before consumption.

Table 1 **Sample collection details according to the different matrix typology**

Meat / meat+vegetables n=43[#]	**Fish / fish+vegetable n=13**	**Fruit / vegetables n=42**	**Cheese / cheese+ham n=7**
veal	plaice	apple	cheese (bovine milk)
swine	hake	plum	cheese and ham**
horse	plaice and potatoes*	pear	
lamb	trout and vegetables*	pear and blueberry	
rabbit	bream and vegetables*	apple and blueberry	
chicken	bream and potatoes*	apple and banana	
turkey	bass and vegetables*	apple and peach	
veal and ham	cod and potatoes*	apple and apricot	
chicken and carrots*	cod and vegetables*	banana and kiwi	
chicken with green beans and zucchini*	salmon and vegetables*	zucchini mix fruit	
turkey, corn and potatoes*		carrot and apple	
veal and carrots*		legumes	
veal and potatoes*		sweet potato and carrots	
veal, potatoes and mushrooms*		broccoli	
veal, broccoli and carrots*		carrots, potatoes and zucchini	
veal and vegetables*		mixed vegetables	
		tomato and vegetables	
		peas and spinach	

[#]- number of samples per category
*for mixed categories, meat and fish represented the major component as declared in the label
**for mixed categories, cheese is the dominant component as declared in the label

Sample extraction
The samples (1 g) were extracted as designated by Chiesa, Nobile, Panseri, and Arioli (2019). The only changes were the internal standard (IS), *N*-Acetyl-D3-glufosinate, and its concentration (0.1 µg/g) for each matrix category. Briefly, 1 g, representative of each single purchased sample, was spiked with the IS, extracted with a mixture of 3 mL of methanol and 7 mL of 1 % formic acidified water, vortexed and sonicated for 15 min, after centrifugation (4 °C, 10 min, 2500 ×g), 1 mL of the supernatant was filtered in a vial, ready for the analysis.

IC-HRMS Orbitrap parameters
Ion chromatography high resolution mass spectrometry (IC-HRMS) instrumentations, parameters and software are described in Chiesa *et al.* (2019). Briefly, the instrumental analysis was performed by an Ion Chromatography Dionex ICS-5000 + system (Sunnyvale, CA, USA) made up of Dual Pump, a Conductivity Detector and an Autosampler. The column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm) kept at 30 °C. The column was chosen on the basis of preliminary trials. In fact, the Thermo Scientific Dionex IonPac AS11 column provided similar results, as evaluated also by Rajski, Díaz Galiano, Cutillas, and Fernández-Alba (2018).

The KOH eluent was converted to water by a Dionex AERS 144 500, 2 mm suppressor (Thermo Scientific). The eluent flow rate (0.3 mL/min), chromatographic run duration (30 min) gradient and injection volume were the same as described by Chiesa *et al.* (2019). Chromatographical separation started with an isocratic 15 mM $KOH_{(aq)}$ elution for the first 8 min, than increased linearly (from 8 to 20 min) up to 55 mM $KOH_{(aq)}$, and was held in these conditions for next 4 min. The initial KOH concentration was brought back at 24.1 min, which was followed by 6 min equilibrium time. The injection volume was 50 µL.

Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), high resolution mass spectrometer equipped with heated electrospray ionization (HESI) source operating in negative mode was used for characterisation of compounds / anions of interest with the same operative conditions described in our original method (Chiesa *et al.*, 2019). Briefly, capillary temperature and vaporizer temperature were set at 330 °C and 280 °C, while the HESI voltage was 3.50 kV. Sheath and auxiliary gas were adjusted at 35 and 15 arbitrary units, with S lens RF level of 60. Instrument calibration was done every analytical session with a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The Full Scan (FS, resolution – 70,000 FWHM) was accompanied by a Data-Independent Acquisition (DIA resolution – 35,000 FWHM). DIA method recorded the MS/MS fragmentation events for all compounds/ anions enrolled in this study. On the basis of our compound list, a scan range of *m*/*z* 50–250 was chosen in FS with the automatic gain control (AGC) of 1×10^{-6}, while maximum injection time was 100 ms. For the DIA segment the AGC target was set to 5×10^{-4}, with an auto regulated maximum injection time. The precursor ions were filtered by the quadrupole which operated at an isolation window of 1 *m*/*z*. Fragmentation of precursors was optimised as three-stepped normalized collision energy (NCE) (10, 25 and 50 eV). Detection of analytes was achieved by comparing the retention time at which negative molecular ions with exact *m*/*z* value appeared, accompanied with two specific characteristic fragments. The chemical formulas, retention times, the theoretical mass of the precursors and the corresponding diagnostic fragments / isotopic ratios applied for confirmation purposes are reported in Table 2.

Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while XcaliburTM 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) to control the HRMS system, the exact mass of the compounds, record and elaborate data.

Method validation

Validation was assessed according to the European Commission (2017) SANTE/11813/2017 Guidance document on method validation & quality control procedures for pesticide residue analysis in food & feed, as described in our recently published papers (Chiesa *et al.*, 2019). Moreover, the method follows the US Food and Drug Administration Foods Veterinary Medicine Research Steering Committee (US FDA FVM) recommendations that regard the validation of chemical methods (US FDA FVM, 2019) and criteria for confirmation of identity of chemical residues using Exact Mass Data (US FDA FVM, 2015). The method was validated for four different blank samples (baby food based on meat, fish, vegetables and cheese) to assess selectivity / specificity, linearity, recovery, precision (as coefficient of variation, CV %) and limit of quantification (LOQ). Besides quantitative validation aspects, also the identification parameters were assessed e.g. ion ratio and retention time. A number of 6 replicates were analysed to check the recovery and precision at two concentration levels (LOQ and 50 ng/g). The LOQ was the lowest validated spiked level meeting the requirements of recovery within the range of 70–120 % and an RSD ≤20 %. The matrix effect was also evaluated by comparing the response produced from the analyte in a solvent solution with that obtained from the same quantity of analyte in the sample extract, expressed as percentage. The matrix-matched calibration curves were made of 5 calibration points in triplicate at 5, 10, 20, 50 and 100 ng/g for all analytes to cover the high concentrations found in the samples.

Table 2 **IC-HRMS data of the studied compounds acquisitioned in negative ionisation mode: formulas, retention time (RT) exact mass, of parent pseudo-molecular anion confirmation fragments and isotopic pattern**

Compound	Formula	R_t (min)	Precursor (*m/z*)	Main products (*m/z*)		
AMPA	CH_6NO_3P	14.15	110.00125	62.96417	78.95904	80.97468
Glyphosate	$C_3H_8NO_5P$	23.66	168.00673	62.96417	124.01687	149.99612
N-Acetyl-glufosinate-D3 (IS)	$C_7D_3H_{11}NO_5P$	13.98	225.07251	62.96413	137.05953	181.08277

Results

The method, already described in a previous study (Chiesa *et al.*, 2019), confirmed the satisfactory validation parameters for all compounds analysed, in Table 3. Briefly, the validated method for different baby food typology showed high specificity, without any interference close to the retention time of selected analytes; good selectivity with a S/N ≥3 and ions with mass accuracy ≤5 ppm since the lowest concentration. Our LOQs were 5 ng/g, recoveries were from 90 to 102 %, linearities demonstrated a good fit with a R^2 >0.99 and precisions expressed as CVs % were lower than 11 %, for all the matrices investigated. The ion ratio was always compliant with the validation guidelines within ±30 % and the matrix effect was always within ±20 % signal suppression or enhancement, as recommended. The internal standard glyphosate-2-^{13}C, ^{15}N used in the previous work (Chiesa *et al.*, 2019), was substituted by *N*-acetyl-glufosinate-D3, more stable and consistent with an absolute recovery ranging from 98 to 102 % in the four different investigated matrices. The proposed method, in comparison to the original QuPPE one (Anastassiades *et al.*, 2015) shows better analytical limits and the protocol is the identical for matrices, not only for vegetables but also for those of animal origin.

The European Union has always paid attention to baby food components and contaminants, glyphosate and its metabolites, as shown by the directives and the regulations on the subject. Regulation (EU) No. 609/2013 (European Regulation, 2013) defines a 'baby food' as a "food intended to fulfil the particular requirements of infants in good health while they are being weaned, and of young children in good health as a supplement to their diet and/ or for their progressive adaptation to ordinary food". The same document specifies that follow-up on the use of pesticides in baby food shall be updated regularly and suggests limiting the use of pesticides as much as possible. The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. The absence of these analytes is therefore an important result, given the worldwide alert and debates on the glyphosate issue, as well as decisions that will be taken in coming years. The lack of data in literature on screening that covers all types of baby food matrices and the encouraging results of this study, also considering the different origin context of our samples, show that the products of the most common brands present on the Italian and international market are safe for infants. Maybe the attention or the presence of glyphosate is mostly linked to soy-based products or cereals, in particular from American products, differently regulated, as can also be seen from the results of Rodrigues and de Souza (2018).

Table 3 **Validation parameters about all selected compounds in the four different baby food analysed by IC-HRMS**

Vegetable baby food	LOQ (ng g^{-1})	Matrix effects % at LOQ	CV % (at 2 Levels*)	Recovery % (at 2 Levels*)	Linearity R^2
AMPA	5	92	7, 5	95, 97	0.9906
Glyphosate	5	95	5, 4	97, 100	0.9915
Cheese baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	90	9, 8	90, 93	0.9996
Glyphosate	5	94	5, 3	94, 97	0.9984
Fish baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	95	9, 8	96, 98	0.9931
Glyphosate	5	93	5, 3	93, 95	0.9965
Meat baby food	**LOQ (ng g^{-1})**	**Matrix effects % at LOQ**	**CV % (at 2 Levels*)**	**Recovery % (at 2 Levels*)**	**Linearity R^2**
AMPA	5	99	10, 9	97, 99	0.9936
Glyphosate	5	107	6, 4	102, 100	0.9973

*The 2 concentration levels were LOQ and 50 ng/g.

Conclusions

The 105 baby food samples analysed for this research, divided on the bases of the matrix origin, did not show traces of glyphosate or its metabolite AMPA. Generally summarising, the levels reported for glyphosate in exanimated baby food commodities indicate compliance with existing / forthcoming legislation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The article describes a monitoring of residues of glyphosate and AMPA in 105 commercially available baby food samples. The article is well described, the samples were analysed using validated analytical methods and the methodology is considered as reliable. The publications shows clearly absence of residues of glyphosate and AMPA in all tested commercially available baby food. This finding indicates that infants and toddlers that are mainly fed with ready-to-eat baby food are not exposed to significant levels of glyphosate or AMPA residues. However the finding cannot be directly related to the supported representative uses of glyphosate for renewal.

Assessment and conclusion by RMS: