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Glyphosate in German adults – Time trend (2001 to 2015) of human exposure to a widely used herbicide

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Highlights:

- Time trend in the background exposure of the German population to glyphosate was analyzed.
- Data mirror increasing glyphosate application and suggest possible exposure reduction after 2012.
- Results warrant following up the time trend and investigating reasons for differences in exposure.

Abstract:

The broadband herbicide glyphosate (N-[phosphonomethyl]-glycine) and its main metabolite aminomethylphosphonic acid (AMPA) were analyzed by GC-MS-MS in 24 h-urine samples cryo-archived by the German Environmental Specimen Bank (ESB). Samples collected in 2001, 2003, 2005, 2007, 2009, 2011, 2012, 2013, 2014, and 2015 were chosen for this retrospective analysis. All urine samples had been provided by 20 to 29 years old individuals living in Greifswald, a city in north-eastern Germany. Out of the 399 analyzed urine samples, 127 (= 31.8 %) contained glyphosate concentrations at or above the limit of quantification (LOQ) of 0.1 µg/L. For AMPA this was the case for 160 (= 40.1 %) samples. The fraction of glyphosate levels at or above LOQ peaked in 2012 (57.5 %) and 2013 (56.4 %) after having discontinuously increased from 10.0 % in 2001. Quantification rates were lower again in 2014 and 2015 with 32.5 % and 40.0 %, respectively. The overall trend for quantifiable AMPA levels was similar. Glyphosate and AMPA concentrations in urine were statistically significantly correlated (spearman rank correlations coefficient = 0.506, $p \leq 0.001$). Urinary glyphosate and AMPA levels tended to be higher in males. The possible reduction in exposure since 2013 indicated by ESB data may be due to changes in glyphosate application in agricultural practice. The ESB will continue monitoring internal exposures to glyphosate and AMPA for following up the time trend, elucidating inter-individual differences, and contributing to the ongoing debate on the further regulation of glyphosate-based pesticides

Key words: Human biomonitoring; Time trends; Glyphosate; AMPA; Urine; Environmental Specimen Bank, Bio banking

1 Introduction and background

The broadband herbicide glyphosate (N-[phosphonomethyl]-glycine) was introduced in the 1970s into agricultural practice and has nowadays become the worldwide most widely used active substance for weed control (Dill et al., 2010; Duke and Powles, 2008; Jaworski, 1972). Glyphosate's main biodegradation product aminomethylphosphonic acid (AMPA) (Borggaard and Gimsing, 2008) is a known residue in crops and other plants treated with glyphosate and can be assumed to have a comparable toxicity profile (EFSA, 2015; FAO, 1998; UBA, 2015a, b; WHO, 2005).

In Germany, the sales volume of glyphosate (active substance) for the domestic market were approx. 3,300 tons in 2000. After having increased to approx. 6,300 tons in 2007, and slightly more than 7,600 tons in 2008, volumes dropped to approx. 4,000 tons in 2009. From 2010 to 2014 domestic sales fluctuated between 5,000 and 6,000 tons (German Parliament, 2015; Seng, 2016). Regarding the active substance with the highest domestic sales amount in 2014, glyphosate ranks second after the inert gas carbon dioxide in professional uses, while it ranks first in non-professional uses (BVL, 2015).

The German Environmental Specimen Bank (ESB) documents time trends of pollutant concentrations in humans and the environment. Starting in the late 1970s, human and environmental samples are cryo-preserved for retrospective analyses of potentially harmful substances, since the 1980s according to a standardized protocol (Kolossa-Gehring et al., 2012; Schröter-Kermani et al., 2016).

As the health impact of the general population's exposure to glyphosate and AMPA is currently controversially discussed (Cressey, 2015; EFSA, 2015; Guyton et al., 2015; Myers et al., 2016; Niemann et al., 2015), the German Environment Agency initiated a retrospective analysis of these compounds in ESB 24 h-urine samples. This analysis was carried out in 2015.

The main goal of this study was to elucidate the internal exposure of the general German population to glyphosate and AMPA and its change over time. The analyzed ESB urine samples from year 2001 up to year 2015 were provided by young adults, who had not been specifically exposed to glyphosate, i. a., because of non-existing occupational contact.

In addition to the temporal trends in glyphosate and AMPA concentrations in 24 h-urine samples, possible associations between substance concentrations and other parameters (e. g. sex, weight, and creatinine level) were investigated. In order to detect first indications of possible dietary effects on internal exposure of young adults to glyphosate and AMPA, samples of ESB participants who reported a vegetarian or vegan diet were exemplary analyzed for the years 2007 and 2015. Moreover, a second comparative sub-population of participants at another ESB sampling location (city of Muenster) was included in the study.

2 Methods

2.1 Sampling and study group

This retrospective monitoring study was based on 24 h-urine specimen collected in the annual sampling of the German ESB. To reduce the risk of contamination, all containers needed for sampling and aliquoting were carefully cleaned before use according to standard operating procedures (Eckard et al., 2011; Lermen et al., 2015). All samples have been provided by young adults (predominantly students) aged 20 to 29 years. To follow the time trend of human exposure to glyphosate and AMPA, cryo-preserved urine samples collected in 2001, 2003, 2005, 2007, 2009, 2011, 2012, 2013, 2014, and 2015 were analyzed. All urine samples were collected from individuals living in Greifswald, a city in north-eastern Germany. Annual ESB sampling in Greifswald is regularly carried out in the period of March and April.

From each of the ten study years, 24 h-urine samples donated by 20 male and 20 female participants were randomly selected for analyses. The only inclusion criterion for this random main study sample was that no specifically restricted diet – mainly vegetarian or vegan – had been reported by the sample provider in the self-administered ESB questionnaire. In 2001 the questionnaire item on dietary restrictions had not yet been implemented. Therefore, some samples from 2001 may have been provided by vegetarians or vegans. The fraction of vegetarian or vegan ESB participants, however, remained roughly between 2 and 14 % from 2002 to 2014 followed by fractions up to 18 % in 2015. Therefore, it can be assumed that, if any, only very few participants with restricted diets might have erroneously been included in the 2001 sample. One 2013 measurement had to be excluded from the main study sample, as the participant was later identified not to fulfill the inclusion criterion.

Hence, the main sample of this study consisted of 399 participants living in the ESB sampling location Greifswald (Wiesmüller et al., 2007) with virtually equal sample sizes and sex ratios in each study year (cf. Table 1). The average body mass index (BMI) of the study population was 23.1 kg/m². The volume of the acquired 24 h-urine samples varied substantially from 490 to 3,438 mL, with an arithmetic mean of 1,874 mL. On average, the creatinine concentration was 0.91 g per liter urine. Creatinine levels ranged from 0.18 to 3.73 g/L. Male ESB participants tended to have higher BMI values and urinary creatinine levels than females.

To investigate possible regional/seasonal differences of glyphosate and AMPA levels 40 urine samples collected in January 2005 and 2015 at the ESB sampling location Muenster (city in north-western Germany) have additionally been analyzed. Moreover, 20 urine samples from vegetarian or vegan participants have been analyzed as well, to investigate differences due to diet. These samples were collected in Greifswald in the years 2007 (10 females) and 2015 (5 males and 5 females) and represent all available samples from vegetarian or vegan participants. A description of the two additionally analyzed comparative ESB sub-populations (cf. section 3.2) is provided in Table 2. Participants in Muenster tended to have slightly lower BMI values. The other sub-population of self-reported vegetarians/vegans also exhibited lower average BMI values as well as higher 24 h-urine sample volumes and lower urinary creatinine concentrations.

2.2 Analytical procedure

The chemical analysis was based on the method by Alferness and Iwata (1994) initially developed for trace analysis of Glyphosate and AMPA in food which uses gas chromatography (GC) coupled with a single quadrupole mass selective detector (MSD). The newly developed method applied in the present study used GC with tandem mass spectrometry (GC-MS-MS) to reach a low limit of quantification (LOQ) in human urine along with high selectivity. Isotope labeled internal standards have been used for further increasing the method's performance.

2.2.1 Standards and reagents

All chemicals were of analytical grade unless stated otherwise. Reference compounds (glyphosate and AMPA) and internal standards (1,2-¹³C₂-¹⁵N-glyphosate and ¹³C-¹⁵N-AMPA) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) as solutions in water (10 µg/mL

each). 2,2,2-trifluoroethanol (99 %), trifluoroacetic anhydride (99 %) and acetonitrile were purchased from Sigma-Aldrich (Seelze, Germany). Methanol was obtained from Merck (Darmstadt, Germany). Water was purified by an ultra-water purification system from ELGA (Ransbach-Baumbach, Germany).

2.2.2 Sample preparation

50 µL of urine sample and 25 µL of the internal standard (IS) solution (containing 4 ng/mL of each IS) were transferred to 10 mL screw-capped glass tubes containing 1 mL of acetonitrile. After evaporation to dryness in a vacuum centrifuge, 0.5 mL of 2,2,2-trifluoroethanol and 1 mL of freezing cold (-40 °C) trifluoroacetic anhydride were added cautiously to the residue. The derivatization of the analytes was started by heating the closed tubes to 85 °C for 1 h in a heating block. After cooling down to room temperature the mixture was cautiously evaporated to dryness. The oily residue was then dissolved in 100 µL of methanol and transferred into a microvial. This final solution was used for GC-MS-MS analysis.

Mixed glyphosate and AMPA calibration solutions were prepared by serial dilution of a stock solution (each 5 ng/mL) in solutions of 50 µL water in 1 mL acetonitrile containing 25 µL of the IS-solution. These solutions were processed in the same way as described for human urine samples and represent sample concentrations from 0.1 to 10 µg/L.

2.2.3 GC-MS-MS analysis

The derivatized analytes were separated by gas chromatography using a GC system 7890 equipped with a split/split less injector (Agilent) and a MPS2 autosampler (Gerstel). The GC column was a HP INNOWAX, 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness (Agilent). 1 µL of the sample solution was injected split-less. To improve the chromatographic performance, highly deactivated SKY™ liner (Restek) were used. The injector temperature was 255 °C. The oven temperature was held at 75 °C for 0.5 min, then ramped to 170 °C at 20 °C per min, and was held for 5 min. Afterwards, a 3.5 min bake-out at 260 °C was carried out to elute high-boiling compounds. Helium 4.5 was used as carrier gas with a constant flow rate of 1.2 mL/min.

Quantification was performed by an Agilent 7000 mass spectrometer (MS-MS) operated in negative ion mode. The mass spectrometric parameter and ion transitions used are summarized in Table 3. While the primary transitions are well suitable for quantification of glyphosate and AMPA at low environmental internal exposure levels, the secondary

transitions of glyphosate and AMPA only worked well at urine concentrations beyond approx. 20 µg/L to confirm the identity of analytes. As the method is clearly focused on reaching the lowest quantification limits in human urine, the secondary transitions were not considered. The high specificity of the primary ion transition of was evaluated during the validation of analytical method (cf. section 2.2.4).

2.2.4 Validation and quality assurance measures of analytical method

For evaluation of the method performance the requirement of SANCO guideline 825 (European Commission, 2010) were considered which are mandatory for analytical methods in the context of pesticide registration and monitoring. We investigated specificity, linearity, working range, accuracy, precision and LOQ for evaluation of the method.

The specificity of the analytical method was checked by the chromatography of unfortified human urine samples which showed no other interfering peaks besides the analytes. Further, the sample solutions of 44 unfortified human urine samples containing residues of glyphosate were analyzed in parallel using separation columns with phases of different selectivity (HP INNOWAX vs. ZEBRON 5). Analyzed concentrations of glyphosate ($n = 44 > \text{LOQ}$) and AMPA ($n = 25 > \text{LOQ}$) ranged from 0.2 to 5 µg/L on both columns and correlated well: The respective slopes of the linear regression lines were close to unity (1.03 for glyphosate and 1.12 for AMPA) and the coefficients of determination (R^2) reached satisfactory values (0.9968 for glyphosate and 0.9893 for AMPA). Summarizing, it can be concluded that the primary transition is very selective and allows a reliable quantification of glyphosate and AMPA.

Basic calibration was performed by the measurement of 8 calibration solutions with concentrations ranging from 0.1 to 10 µg/L. A linear relationship between concentration and the ratio of the peak area of glyphosate and AMPA and its internal standards was observed. All calibration curve points were within 15 % of their respective theoretical value. The linear correlation coefficients were typically > 0.99 . Calibration curves for glyphosate and AMPA based on water and pooled human urine were both linear (each $R^2 > 0.99$) and ran parallel. The slopes differed only by approx. 2 %. This indicates that possible matrix effects are well compensated by the internal standards and matrix matched calibration solutions are not required for accurate determination of glyphosate and AMPA.

The LOQ for glyphosate and AMPA was determined by fortification of human urine samples. The lower level at 0.1 µg/L demonstrated sufficient recovery (86 to 115 %) and precision (8.9 to 9.1 %) for both analytes. This concentration was set as the LOQ of the GC-MS-MS method.

The urine samples were analyzed in a randomized order. Blank values (urine substituted by water) were measured during the analysis of urine samples regularly every 15th sample. All blank values were below the LOQ of 0.1 µg/L.

Evaluation of the accuracy and precision of the method was performed through recovery experiments. Pooled human urine samples with no detectable amount of glyphosate and AMPA (each < 0.1 µg/L) were fortified at 0.5, 1.0, 2.5, and 5 µg/L on 8 replicates each level. The recovery values ranged from 81 to 106 % with a relative standard deviation (RSD) below 8.3 %.

Further, we performed recovery experiments using individual human urine samples to check for possible matrix effects caused by variations in the composition of the samples. Ten individual urine samples free of glyphosate and AMPA (each < 0.1 µg/L) were spiked at 0.5, 2.5 and 5 µg/L and were analyzed in triplicate. The recoveries ranged from 87 to 110% proving that possible matrix effects were compensated by the internal standards ¹³C₂-¹⁵N-glyphosate and ¹³C-¹⁵N-AMPA.

In addition, the performance of the method was checked by measuring of control samples spiked at 0.5 and 2.5 µg/L during the analysis of the samples from this study (about every 33rd sample). A summary of the results of the control samples is provided in Table 4.

2.3 Statistical analysis

Glyphosate and AMPA concentrations below the LOQ were set to LOQ/2 prior to statistical evaluation. All data analyses were carried out in SPSS Statistics Version 20 (IBM Corporation, 2011). Differences between frequencies were tested with Pearson's Chi² test of independence after cross tabulation. Correlations between variables were quantified by Spearman's rank correlation coefficients, as concentration and other data mostly contained few extreme values. Box-plots were created in R Version 3.2.3 (R Core Team, 2015) displaying the 25th, median and 75th percentile as a box. The whiskers were set to extend to the minimum and maximum value, due to considerable skewness and obvious non-normality of the data. All p-values of 0.05 or lower were considered to indicate statistical significance.

3 Results and discussion

3.1 Urinary concentrations of glyphosate and AMPA in the main study sample

3.1.1 Frequency of quantifiable concentrations

Out of the 399 analyzed urine samples, 127 (= 31.8 %) contained glyphosate concentrations that reached or exceeded the LOQ of 0.1 µg/L. For AMPA this was the case for 160 (= 40.1 %) of all samples. The fraction of samples at or above LOQ varied significantly over the years investigated, both for glyphosate ($p \leq 0.001$) and AMPA ($p = 0.005$). As displayed in Table 5 and Figure 1, years with the highest quantification rates were 2012 (57.5 %) and 2013 (56.4 %) after rates having discontinuously increased from 10.0 % in 2001. Fractions of at least 0.1 µg/L were lower again in 2014 and 2015 with 32.5 % and 40.0 %, respectively. The overall trend for quantifiable AMPA levels was quite similar. The highest fraction of samples reaching or exceeding the LOQ was observed for samples taken in 2012 (60.0 %). The fractions of quantifiable levels of glyphosate and AMPA per year were generally higher in males. Especially for glyphosate, the principally increasing trend in urine concentrations was mainly due to samples provided by males (cf. Table 5). Fractions of quantifiable glyphosate levels in samples from females were particularly high only in 2012 (55.0 %) and 2013 (47.4 %). The same difference between males and females was also apparent – yet less pronounced – for AMPA.

Glyphosate sales in Germany have increased substantially from approx. 3,300 tons in 2000 to approx. 5,400 tons in 2014. The interim peak of approx. 7,600 tons in 2008 might be interrelated with the abolishment of EU set-aside requirements announced in 2007 (BBC News, 2007; European Commission, 2008). Against the background of these data, the increase in quantifiable glyphosate and AMPA concentrations in analyzed ESB urine samples were in agreement with expectations. Although the internal exposure to glyphosate and AMPA seems to have decreased again since 2013, there was a clear increase in comparison to 2001. The possible reduction in exposure since 2013 indicated by ESB data may be due to changes in application of glyphosate in agriculture: Austria, for example, banned the pre-harvest use of glyphosate in 2013 (GTF, 2014). Also in Germany, intended glyphosate uses as pre-harvest treatment have been restricted (e. g. to partial applications instead of whole field treatments) from 2014 onwards. Currently, no German sales data are available for the year 2015.

3.1.2 Distribution of concentrations

The 50th, 75th, and 95th percentiles and maximal values for glyphosate and AMPA levels by sex and study year are provided in Table 5. Only in 2012 and 2013 the median concentration of glyphosate was slightly above the LOQ of 0.1 µg/L. The 75th percentile exceeded the LOQ in all study years after 2007, reaching highest values in 2012 and 2013. The 95th percentiles of glyphosate concentrations in 24 h-urine were substantially higher in 2013 (1.12 µg/L) and 2014 (0.80 µg/L) compared to all other years. Also the maximum concentrations of glyphosate peaked in these two years (2013: 2.80 µg/L, 2014: 1.78 µg/L).

The median urinary AMPA concentration only slightly exceeded the LOQ in 2012. With the exception of the first year of the study, 2001, all 75th percentiles exceeded the LOQ with the highest level observed in 2013. The 95th percentiles of AMPA levels peaked in 2013. The two highest AMPA concentrations were observed in samples from 2013 (1.88 µg/L and 1.54 µg/L).

The observed urinary glyphosate and AMPA concentrations are in good agreement with findings from other studies (Hoppe, 2013; Krüger et al., 2014; LANUV, 2016; Niemann et al., 2015). In view of these results, ESB data can be assumed to provide a reliable indication of the background exposure to glyphosate and AMPA in Germany and its change from 2001 to 2015. Comparability with other studies is of course limited i. a. due to differences in the study population and in the type of urine samples.

As displayed in Figure 2 and Figure 3 glyphosate and AMPA concentrations were generally higher in samples from male ESB participants compared to samples from female participants. From 2011 onwards, median levels and 75th percentiles for glyphosate were higher in males. Box-plots for AMPA concentrations exhibit the same pattern. The maximum values for glyphosate and AMPA concentrations in urine, however, were observed in samples from female ESB participants.

Up to now, there is no satisfactory explanation for the differing urinary glyphosate and AMPA levels in males and females. The differences in urinary glyphosate might be due to differences in exposure patterns between males and females or to sex-related differences in physiological determinants of glyphosate and AMPA in urine. To further elucidate these aspects, additional research is necessary.

3.1.3 Correlations between glyphosate, AMPA and physiological parameters

Spearman rank correlations between glyphosate and AMPA levels in urine and physiological parameters observed in the main study sample are summarized in Table 6. Glyphosate and AMPA concentrations in urine were statistically correlated (spearman rank correlations coefficient $r_s = 0.506$, $p \leq 0.001$). When calculating coefficients of rank correlation separately for each study year, glyphosate and AMPA levels correlated statistically significantly in all years except for the first two, 2001 and 2003. For the following eight years of the study, r_s ranged between 0.360 and 0.616 (all p -values ≤ 0.05). A statistically significant association between glyphosate and AMPA concentrations in urine was also observed when cross tabulating all quantifiable and non-quantifiable levels for both analytes as well as when calculating the Pearson product-moment correlation coefficient (data not shown). There were, however, urine samples with comparatively high glyphosate and quite low AMPA concentrations, and vice versa.

The coefficients of correlation of glyphosate and AMPA with BMI were comparatively low and statistically significant only for glyphosate. Correlations between BMI and glyphosate concentrations in urine were only statistically significant at the 5 % level in 2011 ($r_s = 0.344$) and 2015 ($r_s = 0.365$). For AMPA, only the correlation of the concentrations in urine with the participants' BMI in 2015 reached statistical significance ($r_s = 0.346$).

Glyphosate and AMPA concentrations in urine were consistently negatively correlated with the urine sample volume ($r_s = -0.278$ and -0.327) and positively correlated with urinary creatinine levels ($r_s = 0.347$ and 0.373). All these coefficients of correlation were statistically significant ($p \leq 0.001$). The BMI was positively correlated with the creatinine concentration in 24 h-urine samples ($r_s = 0.252$, $p \leq 0.001$). Glyphosate and AMPA concentrations in urine were positively associated with urinary creatinine in all study years. The coefficients of correlation were statistically significant at the 5 % level in almost all study years. Examined for the individual years of the study, the r_s of urine sample volume and glyphosate as well as AMPA levels were consistently negative. The correlation, however, was often not statistically significant.

These results warrant a further discussion on options for a combined consideration of glyphosate and AMPA in exposure assessment. The quite low – yet significant – correlation

between BMI and glyphosate deserves attention when further investigating glyphosate exposure via food consumption.

The negative association of glyphosate and AMPA concentrations with 24 h-urine sample volumes and positive association with urinary creatinine concentrations are in line with expectations, as both parameters reflect the individual urinary diluteness. 24 h-creatinine excretion is usually higher in males (Forni Ognà et al., 2015). Hence, this result is of interest for the further analysis of sex-related differences in urinary glyphosate and AMPA.

3.2 Comparison with other ESB sub-populations

To get a first insight into differences in exposures due to the place of residence and season of sampling, 40 urine samples collected in 2005 and 2015 at the ESB sampling location Muenster were also analyzed for glyphosate and AMPA. In contrast to samples being taken in April/May in Greifswald, the annual Muenster sampling is carried out in January. The summary statistics for glyphosate and AMPA in this sub-population are given in Table 7. In 2005 and 2015 the percentage of quantifiable glyphosate levels was significantly higher in the main study sample (Greifswald) than in Muenster (2005: 30.0 % vs. 5.0 %, $p = 0.003$ and 2015: 40.0 % vs. 15.0 %, $p = 0.012$). For AMPA no statistically significant differences between Greifswald and Muenster samples were observed in 2005 (40.0% vs. 27.5 %, $p = 0.24$) and 2015 (42.5 % vs. 35.0 %, $p = 0.49$). Also the 75th and 95th percentile of urinary glyphosate concentrations in the main study sample are higher than in samples collected in Muenster. For AMPA these percentiles are quite similar for both populations.

A second comparative subsample analyzed for glyphosate and AMPA consists of 10 samples provided in 2007 and 2015 by self-reported vegetarians/vegans taking part in Greifswald (cf. Table 7). There was virtually no difference between self-reported vegetarians/vegans and the main study sample concerning quantifiable percentages of glyphosate in 2007 and 2015. For AMPA the fractions of samples with levels of at least 0.1 µg/L tended to be lower for vegetarians/vegans (2007: 0.0 % vs. 30.0 %, $p = 0.047$ and 2015: 30.0 % vs. 42.5 %, $p = 0.47$), being statistically significant only in 2007. In that year, all self-reported vegans/vegetarians who participated in Greifswald were female. When limiting the comparison to samples collected from women, the difference observed in 2007 was less pronounced and not statistically significant anymore (0.0 % vs. 25.0 %, $p = 0.083$).

Glyphosate concentrations in urine seem slightly higher in the main study sample in comparison to the Muenster sub-population. Although there are virtually no differences in urinary AMPA, this result hints to the possibility of regional or seasonal differences in exposure. Against expectations, the results of this study do not advocate urinary glyphosate and AMPA levels being higher in vegetarian/vegan participants. Unfortunately, no equal sex distribution could be achieved for the sub-population of self-reported vegetarians/vegans, due to a low participation rate of male vegetarians/vegans. This might especially reduce comparability of this sub-population, as males tend to exhibit higher glyphosate and AMPA concentrations in urine. Another limitation of this comparison is that vegetarian/vegan participants exhibit on average higher 24 h-urine sample volumes than in the main study sample without self-reported specifically restricted diet. In general, the sample sizes of the two sub-populations analyzed for comparison are possibly too small to draw general conclusions on seasonal or regional effects and on effects of dietary preferences. Therefore, larger scaled populations studies are warranted for further elucidate reasons for differences in internal glyphosate and AMPA exposure.

3.3 Strengths and limitations of the study

According to the authors' knowledge, this study provides the most comprehensive data on the time trend of glyphosate and AMPA in urine of the non-specifically exposed general population without occupational exposure. The standardized sampling procedures applied in the German ESB are a main strength of this study, as they warrant the comparability of exposure data over time. The consistency of data is further improved by cryo-preservation of samples and randomized analysis of all samples by means of up-to-date laboratory techniques. For some potentially important parameters, such as dietary preference, data was not available for all participants of this retrospective analysis, due to a later addition of these items to the ESB questionnaire. This causes some limitation of the study's explanatory power.

As only 30 to 40 % of glyphosate and AMPA concentrations in urine reached or exceeded the LOQ, the statistical analysis had to focus on differences in the fraction of quantifiable levels. A more sensitive method for quantifying glyphosate and AMPA appears necessary to overcome this limitation in future studies. Considering actual concentrations as the

explained variables in statistical analysis promises to provide additional insight especially into differences in glyphosate and AMPA exposure between sub-populations.

The analysis of 24 h-urine samples is another important strength of the study, as it can be assumed to reflect the exposure to pesticides during one day more reliably than spot urine or first-morning-void sample (Scher et al., 2006). The narrow age range of the study population supports the identification of changes in internal exposure over time. Adults aged 20 to 29 years, however, can represent the overall German population only to a limited extent.

3.4 Health-relevance of observed internal exposure

The acceptable daily intake (ADI) for glyphosate derived by the European Food Safety Authority (EFSA) is 0.5 mg/kg/d (EFSA, 2015). Assuming a bodyweight of 60 kg, an oral absorption of 20 % with fast elimination via urine, and a daily urine excretion of 1,500 to 2,000 mL, the concentration in 24 h-urine associated with this ADI results in 3,000 to 4,000 µg/L. This concentration is higher than the maximum concentration observed in this study (2.8 µg/L) by a factor of 1,000. Considering EFSA's risk assessment, no glyphosate concentration measured in ESB samples is problematic for human health. The International Agency for Research on Cancer (IARC), however, classified glyphosate in Group 2A ("probably carcinogenic to humans"). Taking this assessment into account, especially the increasing trend in internal glyphosate exposure documented by ESB samples deserves attention with regard to human health.

4 Outlook

Building on the results presented in this paper, the German ESB will continue to follow the time trend of urinary concentrations of glyphosate and AMPA in young adults, for which occupational exposure can be excluded. This is necessary to verify whether the exposure has further decreased since 2015. In case of a further regulation of glyphosate as active substance in plant protection products in the European Union or voluntary reductions in application, updated ESB data promise to support the evaluation of the effectiveness and efficiency of these actions.

Multivariate analysis of glyphosate and AMPA concentration data – taking into account at least sex, urinary creatinine, urine sample volume, and BMI – is likely to provide further

insight into reasons for differences in human exposure to these compounds. ESB questionnaire data on food consumption frequencies may further increase the explanatory power of such statistical models.

For further elucidating the variation in the population's exposure, the German Environment Agency currently is analyzing morning urine samples acquired in the cross-sectionally designed population-representative German Environmental Survey for Children and Adolescents (GerES 2014-2017) (Kolossa-Gehring et al., 2012) for glyphosate and AMPA.

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7 Figure captions

Figure 1 Temporal trend of glyphosate and AMPA in human 24 h-urine (fraction of samples at or above limit of quantification of 0.1 µg/L, ESB sampling location Greifswald, no self-reported specific dietary restriction)

Figure 2 Box-plots of glyphosate concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers extend to minimum and maximum value)

Figure 3 Box-plots of AMPA concentrations in 24 h-urine samples by study year and sex (ESB sampling location Greifswald, no self-reported specific dietary restriction, concentrations below LOQ of 0.1 µg/L set to LOQ/2 = horizontal solid line, box displays 25th, median and 75th percentile, whiskers extend to minimum and maximum value).

Figure 1

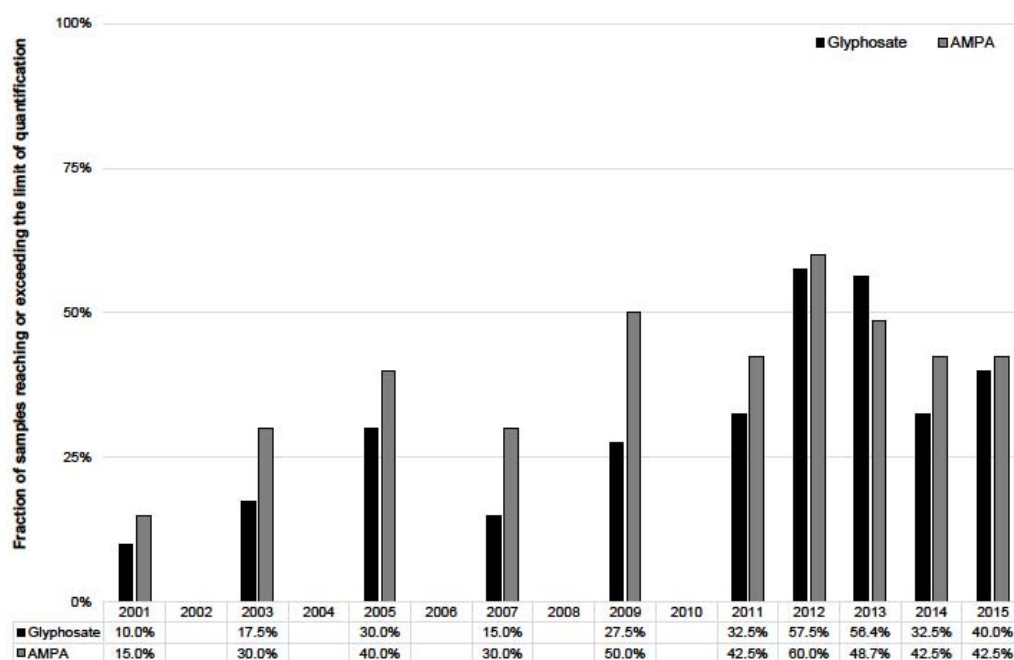


Figure 2

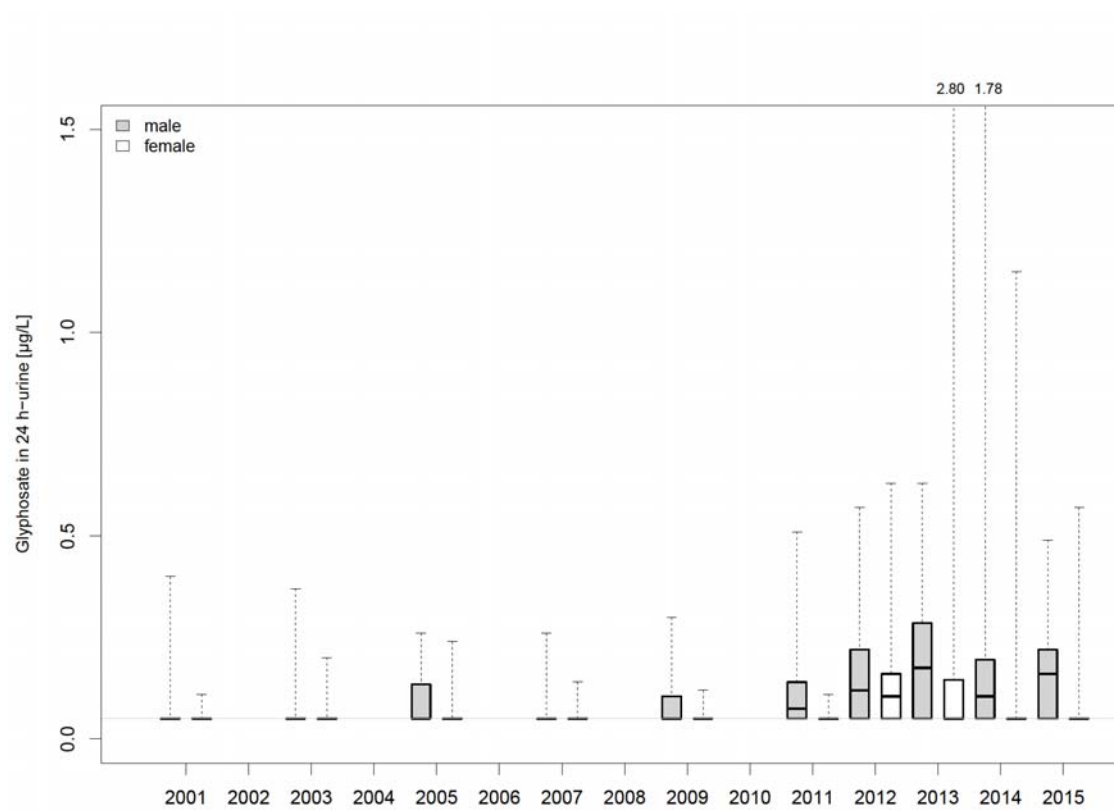
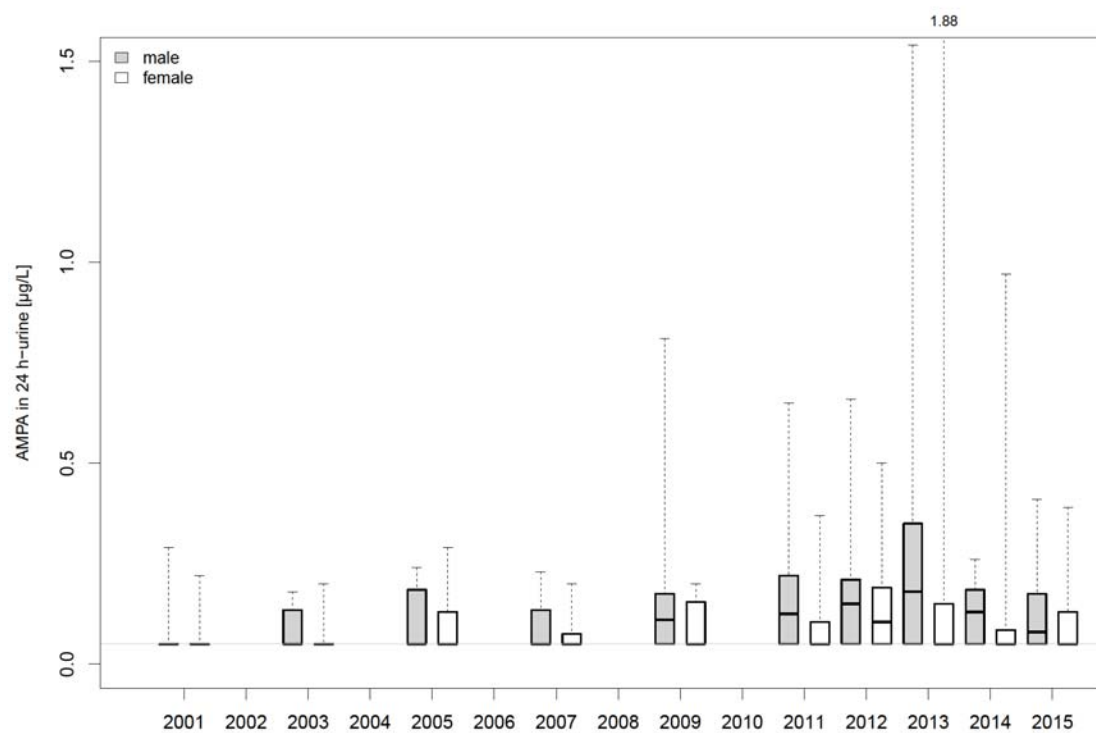


Figure 3



Tables 1-7

Table 1 Description of sample composition (ESB participants from Greifswald analyzed for glyphosate and AMPA concentration in 24 h-urine (no self-reported specific dietary restrictions))

Year	Sample size (male/female)	Age [years] AM (range)	24 h-urine volume [mL] AM (range)	Creatinine in urine [g/L] AM (range)	BMI [kg/m ²] AM (range)
2001	40 (20/20)	23.2 (20-28)	1757 (490-3000)	0.98 (0.38-3.73)	22.3 (17.8-27.4)
2003	40 (20/20)	24.6 (20-28)	1793 (770-2850)	1.06 (0.42-2.11)	23.4 (17.3-31.2)
2005	40 (20/20)	23.1 (20-29)	1910 (895-2841)	0.90 (0.29-2.38)	22.6 (18.1-33.2)
2007	40 (20/20)	23.8 (20-28)	1937 (771-3047)	0.96 (0.36-2.92)	23.3 (18.0-34.6)
2009	40 (20/20)	24.3 (20-29)	1959 (701-3438)	0.85 (0.37-2.39)	22.7 (17.8-34.6)
2011	40 (20/20)	24.3 (20-29)	1893 (783-3045)	0.87 (0.26-2.17)	23.6 (18.0-39.1)
2012	40 (20/20)	24.4 (20-29)	1802 (768-3076)	0.97 (0.32-2.27)	22.8 (17.5-29.8)
2013	39 (20/19)	24.7 (20-29)	1973 (924-3081)	0.75 (0.20-1.60)	23.9 (19.3-40.9)
2014	40 (20/20)	24.1 (20-28)	1958 (760-3069)	0.82 (0.28-2.07)	23.2 (17.9-44.8)
2015	40 (20/20)	24.3 (20-28)	1759 (588-2956)	0.93 (0.18-2.02)	23.0 (17.9-36.8)
Total	399	24.1 (20-29)	1874 (490-3438)	0.91 (0.18-3.73)	23.1 (17.3-44.8)
Male	200	24.4 (20-29)	1881 (490-3076)	1.09 (0.20-3.73)	24.1 (17.4-36.8)
Female	199	23.8 (20-29)	1866 (600-3438)	0.73 (0.18-2.02)	22.0 (17.3-44.8)

Notes: AM = arithmetic mean, BMI = body mass index

Table 2 Description of two sub-populations from Muenster (no self-reported specific dietary restrictions) and Greifswald (self-reported vegetarians/vegans) analyzed for comparison with the main study sample.

Year	Sample size (male/female)	Age [years] AM (range)	24 h-urine volume [mL] AM (range)	Creatinine in urine [g/L] AM (range)	BMI [kg/m ²] AM (range)
ESB sampling location Muenster (no self-reported specific dietary restrictions)					
2005	40 (20/20)	23.6 (20-28)	1790 (691-2962)	1.03 (0.19-2.41)	22.2 (17.4-29.3)
2015	40 (20/20)	23.6 (20-28)	1991 (271-4601)	0.75 (0.35-1.73)	22.1 (18.3-28.6)
Total	80 (40/40)	23.6 (20-28)	1891 (271-4601)	0.89 (0.19-2.41)	22.1 (17.4-29.3)
Male	40	24.0 (20-28)	1934 (797-2952)	0.98 (0.19-2.41)	23.1 (19.6-28.6)
Female	40	23.3 (20-28)	1847 (271-4601)	0.80 (0.35-1.65)	21.1 (17.4-29.3)
Self-reported vegetarians/vegans (ESB sampling location Greifswald)					
2007	10 (0/10)	24.5 (23-28)	2293 (457-3011)	0.67 (0.21-2.51)	23.0 (19.9-28.1)
2015	10 (5/5)	24.3 (20-28)	1831 (773-2993)	0.72 (0.24-1.4)	22.1 (17.7-25.1)
Total	20 (5/15)	24.4 (20-28)	2062 (457-3011)	0.69 (0.21-2.51)	22.5 (17.7-28.1)
Male	5	25.4 (24-27)	1915 (1135-2813)	0.80 (0.31-1.36)	22.6 (21.3-23.9)
Female	15	24.1 (20-28)	2111 (457-3011)	0.66 (0.21-2.51)	22.5 (17.7-28.1)

Notes: AM = arithmetic mean, BMI = body mass index

Table 3 Mass spectrometric parameter and ion transitions used in glyphosate and AMPA analyses

Mass spectrometric parameters				
Instrument	Agilent 7000			
Ion source temperature	150 °C			
Ionization type	Negative chemical ionization (NCI)			
Chemical ionization gas	Methane 4.5			
Collision gas	Argon 5.0			
Electron multiplier	Relative +200 V			
Mass transfers of analytes and internal standards				
Transition	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Use
Glyphosate 1 st transition	370	245	10	Analyte quantifier
Glyphosate 2 nd transition	351	268	5	Of limited suitability
AMPA 1 st transition	351	268	5	Analyte quantifier
AMPA 2 nd transition	271	188	5	Of limited suitability
1,2- ¹³ C ₂ - ¹⁵ N-glyphosate	371	246	10	Internal standard
¹³ C- ¹⁵ N-AMPA	353	270	5	Internal standard

Notes: The secondary transitions of glyphosate and AMPA are listed for sake of completeness only. As they provide reliable confirming information only at concentrations level beyond 20 µg/L they have not been used in this study.

Table 4 Results of control samples concurrently analyzed with the study samples

Analyte	Spiking level (µg/L)	Mean Recovery (%)	Range (%)	RSD (%)	Number of samples
Glyphosate	0.5	103	84.4 - 113.3	8.6	15
	2.5	102	94.2 - 111.3	5.1	15
AMPA	0.5	102.1	82.4 - 112.4	9.4	15
	2.5	101.3	91.2 - 111.0	5.7	15

Table 5 Summary statistics for glyphosate and AMPA concentrations in 24 h-urine samples by sex and year of sampling at ESB sampling location Greifswald (no self-reported specific dietary restrictions)

			Glyphosate					AMPA				
		N	% ≥ LOQ	P 50	P 75	P 95	Max.	% ≥ LOQ	P 50	P 75	P 95	Max.
2001	Male	20	15.0	< LOQ	< LOQ	0.26	0.40	15.0	< LOQ	< LOQ	0.25	0.29
	Female	20	5.0	< LOQ	< LOQ	< LOQ	0.11	15.0	< LOQ	< LOQ	0.21	0.22
	Total	40	10.0	< LOQ	< LOQ	0.12	0.40	15.0	< LOQ	< LOQ	0.22	0.29
2003	Male	20	20.0	< LOQ	< LOQ	0.25	0.37	40.0	< LOQ	0.14	0.18	0.18
	Female	20	15.0	< LOQ	< LOQ	0.16	0.20	20.0	< LOQ	< LOQ	0.19	0.20
	Total	40	17.5	< LOQ	< LOQ	0.16	0.37	30.0	< LOQ	0.13	0.18	0.20
2005	Male	20	40.0	< LOQ	0.14	0.26	0.26	45.0	< LOQ	0.19	0.24	0.24
	Female	20	20.0	< LOQ	< LOQ	0.19	0.24	35.0	< LOQ	0.13	0.26	0.29
	Total	40	30.0	< LOQ	0.11	0.25	0.26	40.0	< LOQ	0.17	0.24	0.29
2007	Male	20	10.0	< LOQ	< LOQ	0.20	0.26	35.0	< LOQ	0.14	0.23	0.23
	Female	20	20.0	< LOQ	< LOQ	0.14	0.14	25.0	< LOQ	< LOQ	0.19	0.20
	Total	40	15.0	< LOQ	< LOQ	0.14	0.26	30.0	< LOQ	0.13	0.21	0.23
2009	Male	20	40.0	< LOQ	0.11	0.22	0.30	55.0	0.11	0.18	0.55	0.81
	Female	20	15.0	< LOQ	< LOQ	0.12	0.12	45.0	< LOQ	0.16	0.20	0.20
	Total	40	27.5	< LOQ	0.10	0.13	0.30	50.0	< LOQ	0.17	0.26	0.81
2011	Male	20	50.0	< LOQ	0.14	0.38	0.51	60.0	0.13	0.22	0.48	0.65
	Female	20	15.0	< LOQ	< LOQ	0.11	0.11	25.0	< LOQ	0.11	0.32	0.37
	Total	40	32.5	< LOQ	0.11	0.25	0.51	42.5	< LOQ	0.18	0.34	0.65
2012	Male	20	60.0	0.12	0.22	0.48	0.57	65.0	0.15	0.21	0.61	0.66
	Female	20	55.0	0.11	0.16	0.44	0.63	55.0	0.11	0.19	0.46	0.50
	Total	40	57.5	0.11	0.20	0.48	0.63	60.0	0.12	0.21	0.56	0.66
2013	Male	20	65.0	0.18	0.29	0.55	0.63	60.0	0.18	0.35	1.03	1.54
	Female	19	47.4	< LOQ	0.16	2.80	2.80	36.8	< LOQ	0.16	1.88	1.88
	Total	39	56.4	0.11	0.27	1.25	2.80	48.7	< LOQ	0.29	1.54	1.88
2014	Male	20	55.0	0.11	0.20	1.12	1.78	60.0	0.13	0.19	0.25	0.26
	Female	20	10.0	< LOQ	< LOQ	0.63	1.15	25.0	< LOQ	< LOQ	0.60	0.97
	Total	40	32.5	< LOQ	0.11	0.80	1.78	42.5	< LOQ	0.16	0.25	0.97
2015	Male	20	70.0	0.16	0.22	0.45	0.49	50.0	< LOQ	0.18	0.38	0.41
	Female	20	10.0	< LOQ	< LOQ	0.37	0.57	35.0	< LOQ	0.13	0.38	0.39
	Total	40	40.0	< LOQ	0.16	0.45	0.57	42.5	< LOQ	0.16	0.38	0.41

Notes: N = sample size, LOQ = limit of quantification, P = percentiles, Max. = maximum value

Table 6 Spearman rank correlation coefficients between glyphosate and AMPA concentrations in 24 h-urine and physiological parameters

		AMPA in 24 h-urine [µg/L]	Body mass index [kg/m ²]	Volume of 24 h-urine sample [mL]	Creatinine in 24 h-urine [g/L]
Glyphosate in 24 h-urine [µg/L]	Correlation coeff.	0.506	0.161	-0.278	0.347
	P-value	≤ 0.001	0.001	≤ 0.001	≤ 0.001
	N	399	399	398	398
AMPA in 24 h-urine [µg/L]	Correlation coeff.		0.079	-0.327	0.373
	P-value		0.114	≤ 0.001	≤ 0.001
	N		399	398	398
Body mass index [kg/m ²]	Correlation coeff.			0.020	0.252
	P-value			0.692	≤ 0.001
	N			398	398
Volume of 24 h-urine sample [mL]	Correlation coeff.				-0.760
	P-value				≤ 0.001
	N				397

Notes: N = sample size, statistically significant correlation coefficients ($p \leq 0.05$) highlighted in bold

Table 7 Summary statistics for glyphosate and AMPA concentration in 24 h-urine by sex and year of sampling in two sub-populations from Muenster (no self-reported specific dietary restrictions) and Greifswald (self-reported vegetarians/vegans) analyzed for comparison with the main study sample.

			Glyphosate					AMPA				
N			% ≥ LOQ	P 50	P 75	P 95	Max.	% ≥ LOQ	P 50	P 75	P 95	Max.
ESB sampling location Muenster (no self-reported specific dietary restrictions)												
2005	Male	20	0.0	< LOQ	< LOQ	< LOQ	< LOQ	30.0	< LOQ	0.12	0.20	0.22
	Female	20	10.0	< LOQ	< LOQ	0.34	0.54	25.0	< LOQ	< LOQ	0.28	0.30
	Total	40	5.0	< LOQ	< LOQ	< LOQ	0.54	27.5	< LOQ	0.11	0.24	0.30
2015	Male	20	15.0	< LOQ	< LOQ	0.23	0.31	30.0	< LOQ	0.11	0.34	0.45
	Female	20	15.0	< LOQ	< LOQ	0.17	0.17	40.0	< LOQ	0.17	0.28	0.31
	Total	40	15.0	< LOQ	< LOQ	0.17	0.31	35.0	< LOQ	0.15	0.28	0.45
Self-reported vegetarians/vegans (ESB sampling location Greifswald)												
2007	Male	0										
	Female	10	10.0	< LOQ			0.14	0.0	< LOQ			< LOQ
	Total	10	10.0	< LOQ			0.14	0.0	< LOQ			< LOQ
2015	Male	5	60.0	0.26			0.61	40.0	< LOQ			0.33
	Female	5	20.0	< LOQ			0.53	20.0	< LOQ			0.43
	Total	10	40.0	< LOQ			0.61	30.0	< LOQ			0.43

Notes: N = sample size, LOQ = limit of quantification, P = percentiles, Max. = maximum value