Supporting Information A (SI-A) to:

New Relevant Pesticide Transformation Products in Groundwater Detected Using Target and Suspect Screening for Agricultural and Urban Micropollutants with LC-HRMS

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SI-A 1: Analytical Methods

Spike solutions: Reference material was dissolved in ethanol, methanol, acetonitrile, ethanol/water mix, methanol/water mix, dimethyl sulfoxide, ethyl acetate, toluene, acetone, water, ethanol + 0.1 M HCl, or methanol + 0.1 M HCl at concentrations ranging from 100 to 1000 mg/L, depending on solubility and stability. Then, mix solutions were prepared in ethanol or acetonitrile at 10 mg/L which were combined for the final spike solutions (0.0001, 0.001, 0.01, 0.1 mg/L).

Sample preparation: The groundwater samples were thawed (pH ~7). The Büchi vials were rinsed with the sample, then, filled with 60.0 g of the sample. The sample was spiked with 60 µL isotope labelled internal standard (ILIS) solution (0.1 mg/L) at 100 ng/L. The sample was evaporated to 1-5 mL in the Syncore Analyst using the back-flush unit at 20 mbar and 45 °C within ~3.5 h. To reduce analyte losses, the Büchi vials were rinsed with 1.5 mL methanol:water (15:85). Then, the sample was evaporated to 0.3 mL, and transferred to a 0.5 mL vial. The sample volume was adjusted to 0.4 mL using ultrapure water. After centrifuging (10 000 rpm, 5 min; Eppendorf centrifuge 5415D) the supernatent was transferred to the measurement vial.

For quantification and quality control, 22 calibration standards at eleven concentration levels (0.1, 0.5, 1, 5, 10, 25, 50, 100, 250, 500, 1000 ng/L) prepared in ultrapure water, nine blank samples (ultrapure water, either freshly withdrawn from the purifier station or frozen for several weeks in the laboratory glass bottles) and six groundwater samples spiked at 10 or 100 ng/L were processed analogously to the groundwater samples. X-ray contrast media were spiked 5x higher due to the lower sensitivity. Perfluorinated carbons (PFCs) were calibrated from 0.1 to 50 ng/L. PFC-ILIS were spiked at 10 ng/L.

Büchi vials were cleaned with diluted HCl, hot tap water, deionised water, ultrapure water and finally methanol.

LC-HRMS/MS: To cover late-eluting perfluorocarbons (PFCs), the isocratic phase (95% eluent B) was prolonged from 10 to 12 min in negative ionization. In addition, a black carbon cartridge was installed directly after the pump to remove PFCs released from the pump.

Table SI-A 1: ESI-HRMS/MS settings

Parameter	
Spray voltage (kV)	4 / -3
Capillary temperature (°C)	320
Sheath gas (AU)	40
Auxiliary gas (AU)	10
S-lens RF level (AU)	50
Automatic gain control (AGC) target MS1	10^6
Maximum injection time MS1 (ms)	100
Scan range MS1 (m/z)	100 - 1000
Resolution MS1 (at m/z 200)	140 000
Data-dependent trigger	Ions of target compounds; if idle pick most intense
Isolation window (m/z)	1
Number of dd-MS/MS	Top 5
Resolution MS2 (at m/z 200)	17 500
Automatic gain control (AGC) target MS/MS	2 x 10^5
Maximum injection time MS/MS (ms)	80
Dynamic exclusion time (s)	8

SI-A 2: Target Quantification

ILIS Selection: Quantification was based on the peak area ratio of analyte and ILIS. If a structurally identical ILIS was not available, ILIS selection was supported by an internal R script using the R functions published at https://github.com/dutchjes/TFAnalyzeR/blob/master/RelativeRecoveryCalculation.R. First, the TraceFinder 4.1 export was imported to R (R Core Team 2016) and all ILIS co-eluting with the analyte within the given RT window (generally ±1.5 min) were selected (function selectISTDs()). Then, a linear calibration model was calculated for each combination of analyte and ILIS (function calibrationCalc()), and finally, sample concentrations were determined based on each calibration model (function predictConc()). Using the concentration *c* in the spiked / not spiked samples and the theoretical spike level, relative recoveries as defined in equation 1 were calculated,

$$Relative\ Recovery = \frac{\left(c_{spiked\ sample} - c_{not\ spiked\ sample}\right)}{Theoretical\ Spike\ Level} \quad (1)$$

if the following equation was true (function recoveryCalc()):

$$c_{not\ spiked\ sample} < \left(c_{spiked\ sample} - c_{not\ spiked\ sample}\right) \cdot 1.7 \quad (2)$$

This check ensured that relative recoveries were only determined if the concentration difference in the spiked and not spiked samples was large enough, to avoid cases where the relative recoveries were dominated by measurement uncertainty, and therefore, misleading. Finally, an ILIS was selected for which the mean relative recovery was close to 100% and the standard deviation of the relative

recoveries across the spiked samples was low. Final analyte concentrations were corrected by the relative recovery, if a structurally identical ILIS was not available.

Limit of Quantification (LOQ): The LOQ in ultrapure water ($LOQ_{Ultrapure}$) was defined as the lowest calibration standard with at least five data points along the chromatographic peak (MS1 full scan mode) and a peak area ratio (analyte vs. ILIS) of at least twice the peak area ratio in all blank samples. To estimate the LOQ in matrix (LOQ_{Matrix}), the $LOQ_{Ultrapure}$ was divided by the absolute recovery:

$$LOQ_{Matrix} = \frac{LOQ_{Ultrapure}}{Absolute\ Recovery} \tag{3}$$

If the sample concentration was in the range of the LOQ_{Matrix}, the so-defined LOQ_{Matrix} was lowered if the chromatographic peaks in the samples were defined by at least five data points.

Absolute recoveries were determined for each analyte by comparing the peak area in the matrix to the peak area in ultrapure water, as described in the following. If a structurally identical ILIS was available, the peak area of the ILIS in the matrix (environmental samples) was divided by the peak area of the ILIS in ultrapure water (median of all enriched calibration standards) according to equation 4:

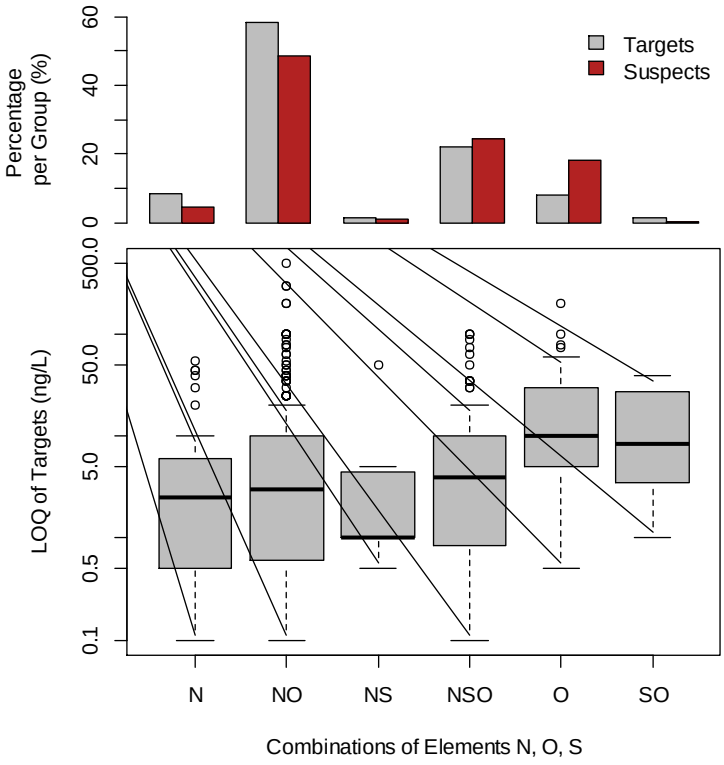
$$Absolute\ Recovery_{\substack{Identical \\ ILIS}} = Median \frac{Peak\ Area\ ILIS_{Matrix}}{Median\ (Peak\ Area\ ILIS_{Ultrapure})} \tag{4}$$

If a structurally identical ILIS was not available, the peak area of the analyte in the spiked sample (after subtracting the peak area in the not spiked sample) was compared to the peak area of the analyte in the calibration standard that corresponded to the spike level:

$$Absolute\ Recovery_{\substack{No\ Identical \\ ILIS}} = \frac{Peak\ Area_{\substack{Spiked \\ Sample}} - Peak\ Area_{\substack{Not\ Spiked \\ Sample}}}{Peak\ Area_{\substack{Calibration \\ Standard}}} \tag{5}$$

SI-A 3: Suspect Screening

a



b

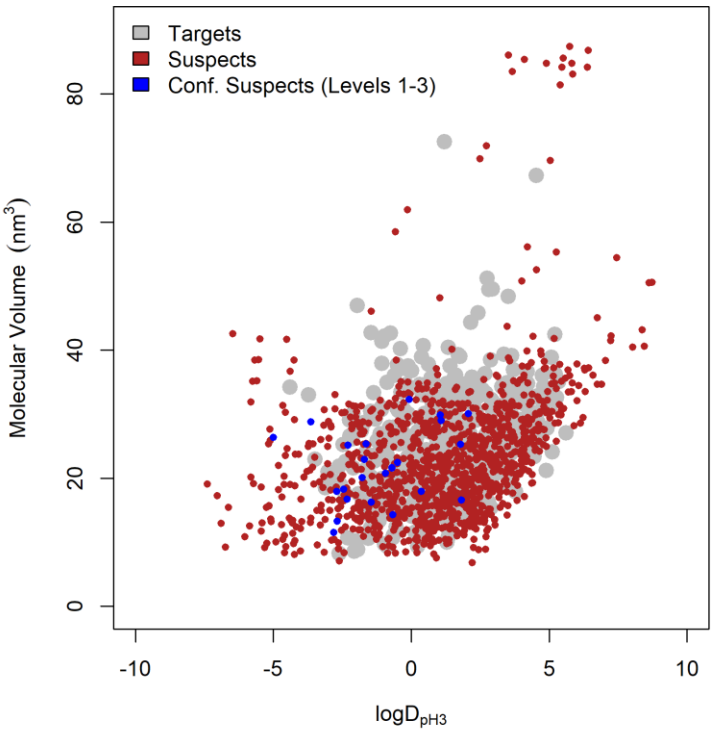


Figure SI-A 1: a) Bar plots: distribution of heteroatoms in targets and suspects; boxplots: LOQ of targets depending on heteroatoms; 0.5% of suspects do not contain any heteroatoms (not shown). b) Molecular volume vs. $logD_{pH3}$ of the targets (grey), suspects (red) and confirmed suspects (blue). The theoretical pH of the LC eluent was 3.

Automated Screening

The suspect screening with CD 2.1 was performed separately for positive and negative electrospray ionization. To optimize parameter settings, mass accuracy and RT shift of ILIS were analysed. The accurate mass of the ILIS deviated less than 5 ppm from their exact mass. Furthermore, ILIS shifted not more than 1.5 min within the measurement sequence (except for metolachlor-esa-d11: more than 15 min, Figure SI-A 2). Table SI-A 2 lists the used nodes and most important parameter settings for CD 2.1.

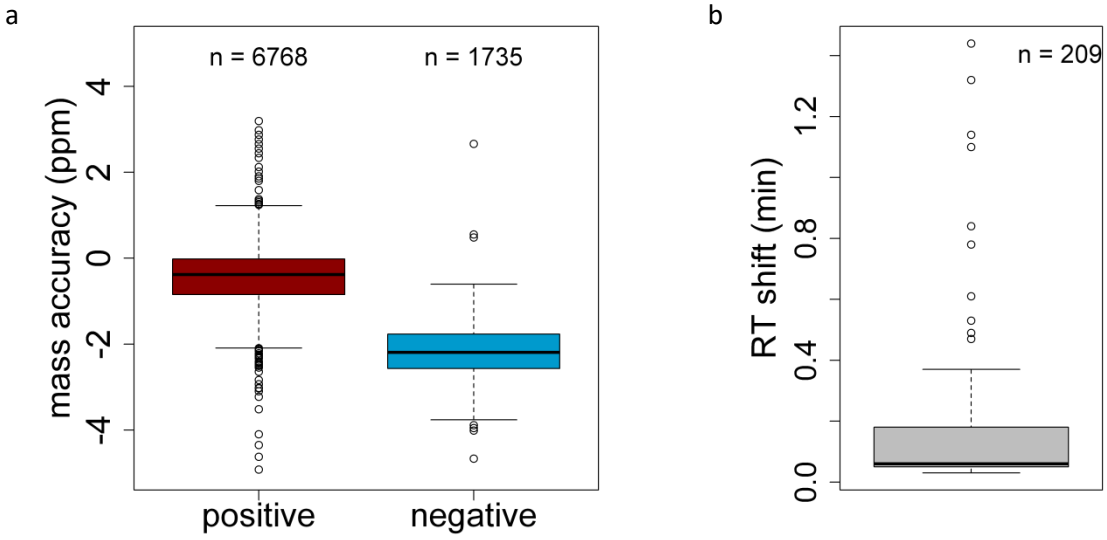


Figure SI-A 2: a) Mass accuracy of ILIS in positive and negative ionization mode; b) maximum RT shift of ILIS within measurement sequence.

Table SI-A 2: Nodes and parameter settings used for the CD 2.1 workflow.

Node	**Settings**
Input Files	
Select Spectra	• Precursor mass 100 – 1000 Da • Unrecognized mass analyser: FTMS • Unrecognized activation: HCD
RT Alignment	• Maximum shift 0.75 min • Mass tolerance 3 ppm (pos) / 5 ppm (neg)
Detect unknown compounds	• 3 ppm (pos); 5 ppm (neg) • 30% intensity tolerance • S/N threshold 3 • Min peak intensity 1000 (pos) and 500 (neg) Ions ○ Positive: [M+H]+1, [M+K]+1, [M+Na]+1, [M+NH4]+1, [2M+H]+1, [M+2H]+2, [M-e]+1

	○ Negative: [M+FA-H]-1, [2M+FA-H]-1, [M-H]-1, [M-2H]-2, [2M-H]-1 • Base Ions ○ [M+H]+1, [M+NH4]+1 (pos) / [M-H]-1 (neg) • Filter Peaks: True • Max. Peak Width: 0.8 min • Remove Singlets: **False** • Min Scans per Peak: 5 • Min. Isotopes: **1** • Max. element counts: C90 H190 Br3 Cl4 F6 K2 N10 Na2 O18 P3 S5
Merge Features	• 3 ppm (pos), 5 ppm (neg) • RT Tolerance 0.75 min
Group Unknown compounds	• 3 ppm (pos), 5 ppm (neg) • 0.75 min RT Tolerance • Preferred Ions [M+H]+1, [M+K]+1, [M+Na]+1, [M+NH4]+1 (pos) / [M-H]-1 (neg)
Search Mass Lists	• Lists with targets, ILIS and suspects • RT TRUE • RT Tolerance 2 min • Mass Tolerance 3 ppm (pos), 5 ppm (neg)
Assign Compound Annotations	• 3 ppm (pos), 5 ppm (neg)
Mark Background Compounds if…	• Max. Sample/Blank 3 • Hide Background: False
Predict Compositions	• Mass tolerance 3 ppm (pos), 5 ppm (neg) • S/N Threshold 3 • Mass tolerance for fragments matching: 10 ppm

RT Prediction

Each suspect hit was evaluated regarding the plausibility of the RT using a simple RT prediction model based on the logD. The logD values were predicted at pH 3 (theoretical pH of LC eluents) with JChem for Excel (Version 17.1.2300.1455; ChemAxon Ltd.) for 615 targets and ILIS. Then, a linear model was fitted (Figure SI-A 3) and suspect RTs were predicted from their $logD_{pH3}$ using R (R Core Team 2016). Suspect hits were considered as unlikely, and therefore, excluded, if measured and predicted RT differed more than 10 min.

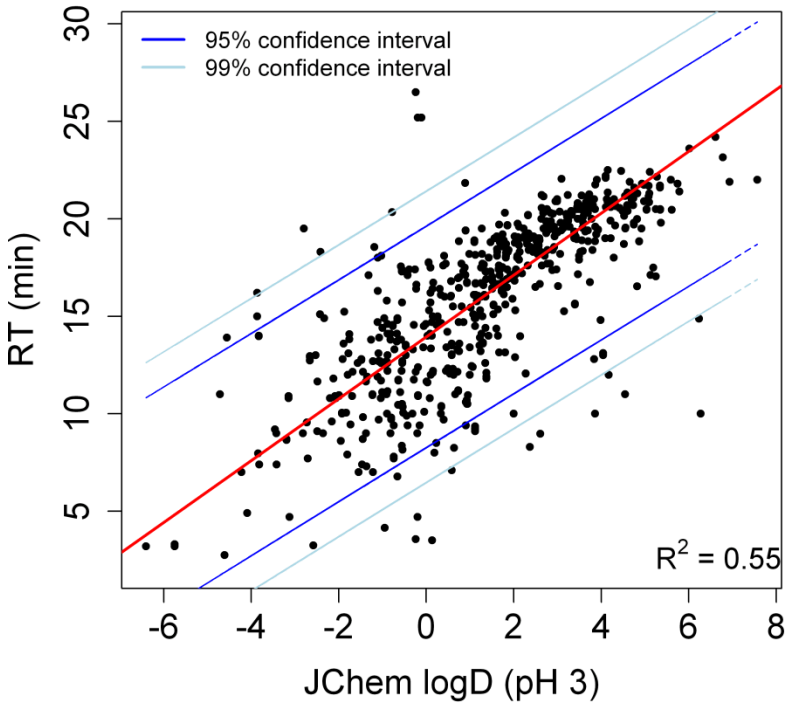


Figure SI-A 3: Linear RT prediction model based on logD values of 615 target compounds.

In silico Fragmentation with MetFrag

The in silico fragmenter MetFrag was used to test if the annotated suspect structures could explain measured MS/MS fragments. Using an internal R script, MetFrag CL 2.4.2 was run in batch mode. First, the MS/MS spectra were extracted from .mzXML files for each suspect hit using the RMassBank package (Stravs et al. 2013). For this, the m/z of the [M+H]+ / [M-H]- were calculated from the annotated suspect formula (RChemMass package, https://github.com/schymane/RChemMass/), and then, the most intense MS/MS scan triggered by the calculated m/z and acquired in the sample with the largest MS1 intensity was written to a .txt file. To reduce noise signals, peaks with intensity less than 1% relative to the base peak were removed. Next, MetFrag retrieved all structures from ChemSpider and the suspect list matching the measured m/z within 3 or 5 ppm (positive / negative mode). Salts and stereoisomers were removed using the unconnected compound and InChIKey filter. FragmenterScore, RetentionTimeScore, ChemSpider data (ChemSpiderDataSourceCount, ChemspiderReferenceCount, ChemSpiderPubMedCount, ChemSpiderRSCCount, ChemSpiderExternalReferenceCount), and OfflineMetFusionScore were weighted equally. MetFrag results are summarized in SI-B 3 comprising fragmenter score of the suspect, number and formulae of explained peaks by the suspect, number of ChemSpider hits, the compound from Chemspider that explains most fragments, that has highest fragmenter score, and the one that has the highest total score.

Suspect Hits not Further Investigated

143 suspects were not further investigated using reference material. Some suspects will remain unclear, e.g. for 21 suspects reference material could not be obtained or was not pursued as they were considered to be of lower priority. Eight suspects were not further investigated because they were only detected in one sample. Some suspects were disregarded as they showed a noisy EIC (35 suspects) or low intensity (57 suspects) hampering confirmation (area $<5x10^4$). 22 suspects were excluded as they were relatively unlikely to represent a positive hit: One suspect hit, annotated both as TP of various sulfurons and as terbuthylazine TP, was identified as terbuthylazine TP; in the case of three suspects the measured fragments did not match the main fragments reported by Reemtsma et al. (2013); 14 hits showed low mass accuracy (averaged over isotopologues/adducts: >2.5 ppm; for four suspects, the maximum fragmenter score of the ChemSpider candidates was more than three times larger than the fragmenter score of the suspect and therefore the suspects were deemed as unlikely to be the correct structure.

SI-A 4: Suspect Confirmation and Quantification

Seven samples comprising all suspects (sample aliquots which were not thawed previously), three spiked samples (10, 100, 1000 ng/L) and two blanks were enriched and measured as described in SI-A 1 with the following slight modifications. Calibration levels were 0.1, 0.5, 1, 5, 10, 25, 50, 100, 500, 1000, 2000 and 3000 ng/L. The dynamic exclusion time and number of dd-MS/MS experiments were reduced to 3 s and top 3, respectively, to increase the number of MS/MS scans along a chromatographic peak.

Suspects were confirmed based on retention time and matching MS/MS spectra in standard and sample with the following method. Using the R package MSnbase (Gatto and Lilley 2012), the EICs of the most intense adduct in standard, sample and spiked sample were extracted (mass window 5 ppm) and plotted to check the retention time. Then, the most intense fragments in the standard were determined, and the EICs of these fragments (in standard and samples) were plotted. Head to tail plots were created with the R package MSMSsim (https://github.com/dutchjes/MSMSsim). In addition, retention time was checked on a second chromatographic system using a reverse phase biphenyl column (Raptor Biphenyl, 2.7 µm, 100x3.0 mm; Restek, Bellefonte, U.S.). The gradient started with 100% eluent A (water + 0.1% concentrated formic acid + 2.5 mM ammonium formate) for 1.5 min, then eluent B (90% / 10% acetonitrile / water + 0.1% concentrated formic acid + 2.5 mM ammonium formate) was increased to 100% within 25 min, and held for 2 min. The column was re-equilibrated for 4 min.

Suspect concentrations were determined in the 31 samples by applying the calibration model determined later with the same LC-HRMS system. For this, seven samples were analysed twice, once in the first analysis, and once in the same measurement sequence as the calibration standards used for quantification. For this second sample preparation and measurement, the same ILIS spike solution was used as for the first sample preparation and measurement. The determined concentrations of both analyses matched within measurement accuracy.

SI-A 5: Results of Target and Suspect Screening

Table SI-A 3: Micropollutants (MPs) detected at least once with concentrations >100 ng/L in the 31 groundwater samples. MPs identified in suspect screening: italic. Median (c_{median}), 90th percentile (c_{90th}) and maximum (c_{max}) of concentrations in 31 samples.

MP	**MP Class**	**logD_{pH7}**	**LOQ (ng/L)**	**No. Of Detec-tions**	**C_{median} (ng/L)**	**C_{90th} (ng/L)**	**C_{max} (ng/L)**
Atrazine	Pesticide	2.2	0.5	25	7.1	37	180
Bentazone	Pesticide	-0.2	0.1	18	0.5	23	260
Atrazine-desethyl	Pesticide TP	1.5	0.5	29	11	59	150
Atrazine-desethyl-desisopropyl	Pesticide TP	0.5	0.3	30	17	78	120
Chloridazon-desphenyl	Pesticide TP	-0.7	1	28	120	1200	1800
Chloridazon-methyl-desphenyl	Pesticide TP	-0.6	0.5	22	32	220	670
Chlorothalonil TP R417888	Pesticide TP	-0.7	1	28	33	470	1300
Chlorothalonil TP Isomer of R417888, ***Level 3***	Pesticide TP	-0.7	no standar d	19	~21	~39	~120
Chlorothalonil TP R471811	Pesticide TP	-1.7	3	31	300	1100	2700
Chlorothalonil TP SYN507900	Pesticide TP	0.4	1.3	13	<1.3	33	150
Dimethachlor-ESA	Pesticide TP	-1.1	5	9	<5		120
Fipronil-TP RPA 106681, ***Level 2b***	Pesticide TP	1.0	no standar d	11	~1.9	14	~120
Fludioxonil TP CGA 192155	Pesticide TP	-0.7	3	2	-	-	200
Metolachlor TP CGA 368208 (=Acetochlor sulfonic acid)	Pesticide TP	-0.5	1	20	3.2	46	150
Metolachlor TP NOA413173	Pesticide TP	-3.4	1.7	22	7	130	430
Metolachlor-ESA*	Pesticide TP	-0.3	35	9*	69*	642*	970*
Terbuthylazine TP CSCD648241	Pesticide TP	-2.5		29	9.5	54	190
N-N-dimethylsulfamide	Biocide TP	-1.5	5	18	7.8	67	>200
Benzotriazole	Corrosion inhibitor	1.3	5	13	-	59	210
Melamine	Industrial chemical	-2.0	5	4	-	32	770
Diatrizoate	Pharmaceuti cal	-0.6	15	4	-	64	340
Acesulfame	Sweetener	-1.5	0.5	26	37	120	260
Sucralose	Sweetener	-0.5	10	7	-	93	520

* Metolachlor-ESA could only be analysed in 13 samples due to shifting RT.

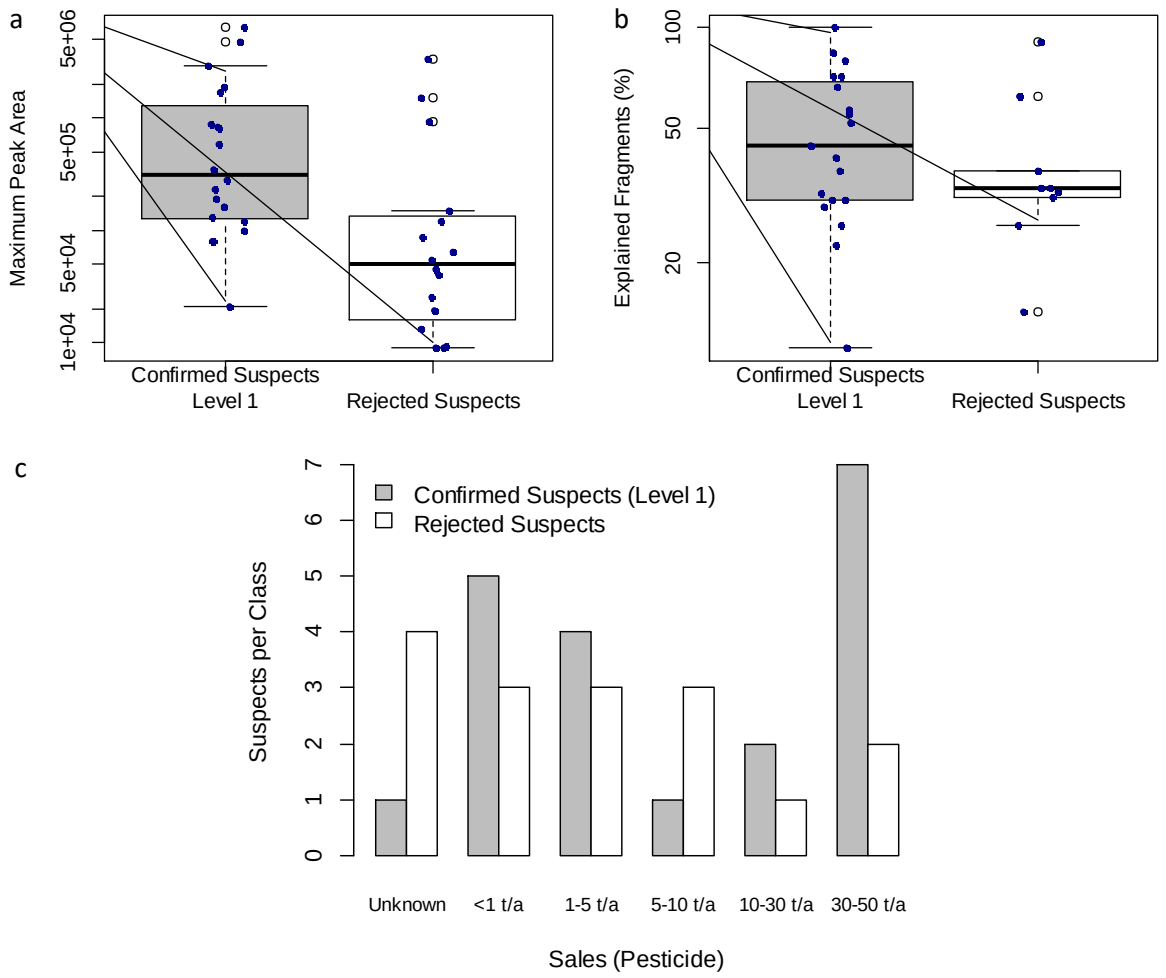


Figure SI-A 4: Comparison of maximum peak area (a), explained fragments from MetFrag (b) and sale volumes (c) of suspects which were confirmed or rejected using reference material.

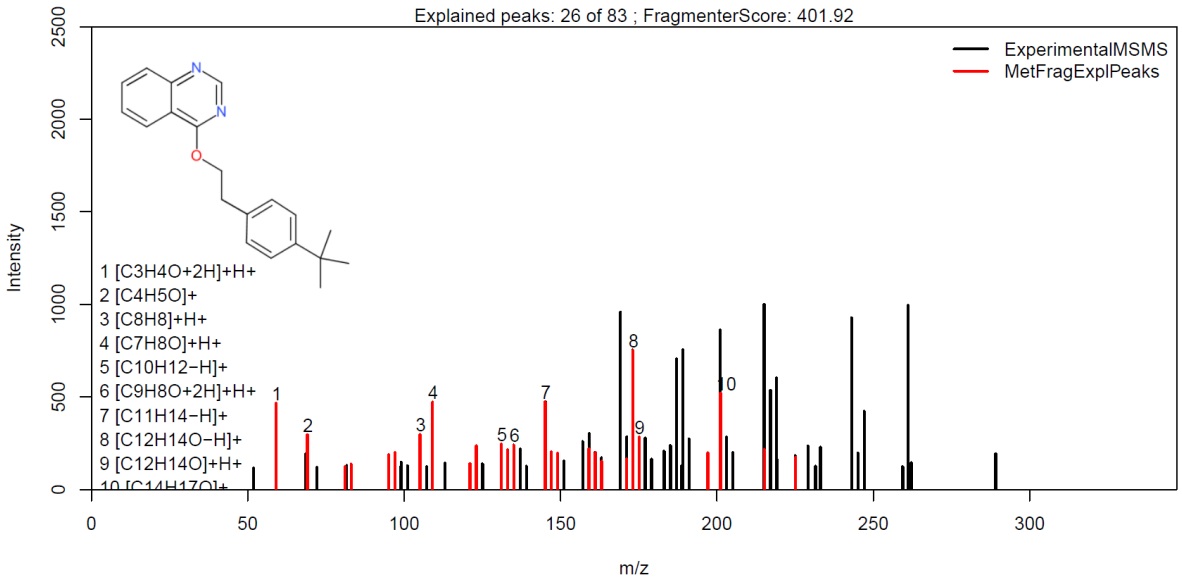


Figure SI-A 5: HRMS/MS spectrum of the suspect hit fenazaquin with peaks which could be explained by the structure marked in red; fenazaquin was rejected using reference material due to different RT.

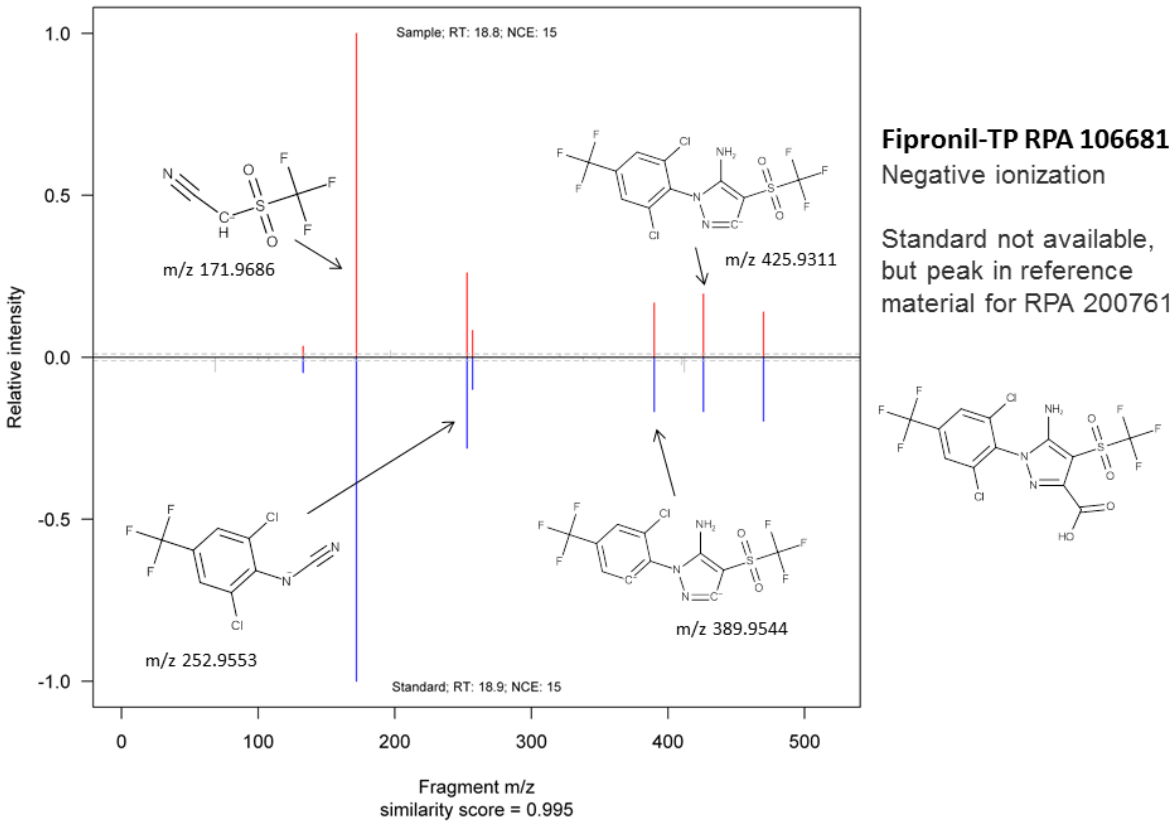


Figure SI-A 6: HRMS/MS spectra of fipronil TP RPA 106681 in sample (above, red) and reference material (below, blue) with structure proposals for four fragments. For RPA 106681, reference material was not available, but the obtained reference material for RPA 200761 revealed a peak at the precursor m/z of RPA 106681 ($[M+H]^+$ & $[M-H]^-$). RT matches with peaks in sample.

SI-A 6: Impact of Land Use on Groundwater Quality

Land use drives not only total concentrations of MP classes, as discussed in section 3.4, but explains also the presence of site-specific MPs. One sample was noticeable for the high contamination with the industrial chemical melamine (770 ng/L), which explained 47% of total MP concentration. Melamine is a transformation product (TP) of the biocide cyromazine but also a high production volume chemical used for the fabrication of a wide range of materials, such as laminate, paper, textiles, or glues (Europe: 250000 t/a). Melamine is well-degradable in wastewater treatment plants with adapted microbial community but relatively persistent in the environment (OECD 1999). We expect that the high amounts of melamine enter the aquifer via seepage from nearby landfills or from bank filtrate from the Rhone River, which acts as a receiving water for numerous municipal and, most importantly, industrial wastewater treatment plants. This explanation was supported by the detection of typical wastewater tracers such as benzotriazole (210 ng/L) and acesulfame (70 ng/L) at the same monitoring site. Seitz and Winzenbacher (2017) reported for melamine median concentrations of 360 ng/L in groundwater and 610 ng/L in stream water in Germany due to seepage from a nearby landfill and the discharge of treated wastewater, respectively.

The highest MP concentration (9000 ng/L) was detected in a spring draining intensively cultivated arable land. TPs of chlorothalonil, chloridazon and metolachlor explained 90% of total MP concentration. In contrast, the sample with the lowest MP concentration (60 ng/L) was dominated by the herbicide asulam (54 ng/L). Asulam concentration was more than five times higher than in all other groundwater wells. The herbicide has not been approved in the EU since 2012 (European Commission 2011), but is still used in Switzerland to combat dock and fern species, e.g. on pastures (*Rumex obtusifolius, Rumex alpinus, Pteridium aquilinum, Dryopteris filix-mas*). The catchment of the spring is dominated by grassland / pastures (63%) and forest (30%); wastewater impacted streams do not influence groundwater recharge. Consequently, the catchment explained both the generally low MP and the high asulam contamination.

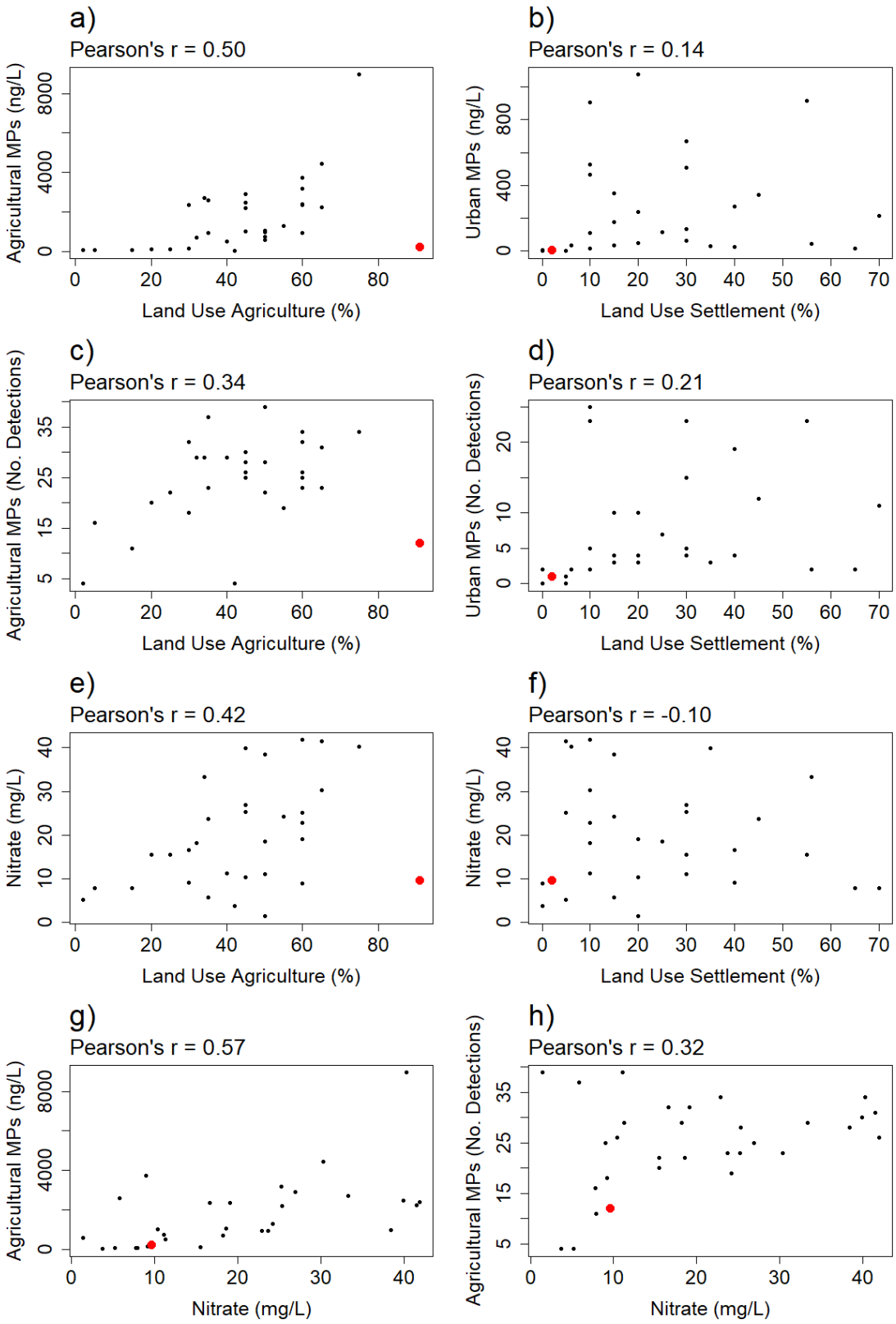


Figure SI-A 7: a, c, e: Correlation between total agricultural MP concentration / agricultural MP detections / nitrate concentration and percentage of agricultural land use in catchment (cropland, orchards, vineyards); b, d, f: correlation between total urban MP concentration / urban MP detections / nitrate concentration and percentage of settlement in catchment; g, h: correlation between total agricultural MP concentration / agricultural MP detections and nitrate concentration; "pesticides" = pesticides and TPs. Red dots: groundwater monitoring site with catchment dominated by agriculture, but low MP and nitrate contamination (section 3.4).

References

European Commission, 2011. Commission Implementing Regulation (EU) No 1045/2011 of 19 October 2011 concerning the non-approval of the active substance asulam, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending Commission Decision 2008/934/EC. Official Journal of the European Union 275.

Gatto, L. and Lilley, K.S., 2012. MSnbase - an R/Bioconductor package for isobaric tagged mass spectrometry data visualization, processing and quantitation. Bioinformatics (28), 288-289.

OECD, 1999. Screening Information Dataset (SIDS) Initial Assessment Report (SIAR): Melamine CAS N°:108-78-1. UNEP Publications, 110-198.

R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Reemtsma, T., Alder, L. and Banasiak, U., 2013. A multimethod for the determination of 150 pesticide metabolites in surface water and groundwater using direct injection liquid chromatography-mass spectrometry. Journal of chromatography A 1271(1), 95-104.

Seitz, W. and Winzenbacher, R., 2017. A survey on trace organic chemicals in a German water protection area and the proposal of relevant indicators for anthropogenic influences. Environmental Monitoring Assessment 189, 1-17.

Stravs, M.A., Schymanski, E.L., Singer, H.P. and Hollender, J., 2013. Automatic recalibration and processing of tandem mass spectra using formula annotation. J Mass Spectrom 48(1), 89-99.