Supplementary Information

Occurrence and fate of nitrification and urease inhibitors in the aquatic environment

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Text S1: Method development and validation

LC-ESI-MS/MS analysis with electrospray ionization in positive ionization mode was applied for the detection of nitrification and urease inhibitors (NUIs).

General interface parameters were: ion spray voltage: 5.5 kV; heater temperature: 600 °C; collision gas: medium; ion source gas 1/2: 60/70 psi and curtain gas: 40 psi. Analyst 1.6.1 software was used to record and evaluate the obtained chromatographic data. Liquid chromatography (LC) analysis was carried out using a 1290 HPLC system from Agilent Technologies (Waldbronn, Germany) equipped with a solvent cabinet, a micro vacuum degasser, a binary pump, a high performance autosampler with two 54 vial plates and a temperature-controlled column compartment.

Several reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) columns were tested. However, some representatives of the analyzed NUIs are very polar compounds, which couldn´t be retained by reversed phase liquid chromatography columns under the applied conditions. Promising results were obtained for 1*H*-1,2,4-triazole and DCD with hydrophilic interaction liquid chromatography (HILIC), but the technique was only suitable for the most polar analytes, produced rather broad peaks and needed long re-equilibration time. Furthermore, for sufficient retention, HILIC technique requires are high percentage of organic solvent (acetonitrile) in the injected sample to prevent peak broadening. Such a solvent exchange is common when applying solid phase extraction (SPE), but results in higher limits of quantification due to the dilution with acetonitrile when a direct injection of water samples is applied (see below). As a consequence the above mentioned separation with a Hypercarb (Thermo Scientific, Waltham, USA) column was chosen. With this method the first target analyte 1*H*-1,2,4-triazole elutes after 4,8 min. This is at least one minute after the void volume of the column, which was proven by the injection of thiourea.

After establishing a chromatographic method several SPE cartridges were tested for sample clean-up and pre-concentration of the analytes. For all tested materials (C18, styrene-divinylbenzene (SDB), activated carbon) the obtained recoveries

were insufficient, especially for 1*H*-1,2,4-triazole and DCD. To differentiate between break-through of the analytes and insufficient elution, the filtrates were collected and measured. The comparison of peak areas obtained from the eluate and from an external standard with the same concentration showed, that SPE sorbens material with hydrophobic interactions were not able to extract the compounds adequately. For 1*H*-1,2,4-triazole and DCD a break-through of more than 80% was observed. Schermerhorn et al. (2005) used a mixed-mode cationic exchange material for the extraction of 1*H*-1,2,4-triazol from water samples at neutral pH, although the compound is present uncharged at pH 7.[1, 2] A relative recovery of 94%, after correction with an isotopically labeled internal standard was reported, which allows no conclusion about the real extraction efficiency (absolute recovery). Applying this SPE protocol an absolute recovery of 38% was obtained for 1*H*-1,2,4-triazole and only 2% for DCD.

Based on unsatisfactory results of the SPE experiments, it was decided to directly inject the aqueous samples into the LC-MS/MS system.

To protect the LC auto-sampler and the analytical column samples from suspended solids, water samples were filtered using 0.45 µm-PTFE-membrane filters (Millipore, Billerica, U.S.A.) and 1 mL BD Plastipack™ syringes (Becton Dickinson, Madrid, Spain) were used. Losses during filtration due to sorption were negligible.

For the assessment of compound specific limits of quantification (LOQs) two different approaches were applied for quality assurance. This procedure was chosen as qualifier transitions of 1*H*-1,2,4-triazol and DCD have poor signal intensities and can be used for confirmation purposes only at relatively high concentrations. First, LOQs were determined according to the calibration curve procedure according to German industry standard DIN 32 645 (level of confidence 95%) with an equidistant 10 level calibration. Secondly, the LOQ calculated according to DIN 32 645 was disallowed when the signal-to-noise ratio was <10. In that case, the lowest calibration point fulfilling this criterion was chosen as LOQ (Table S1).

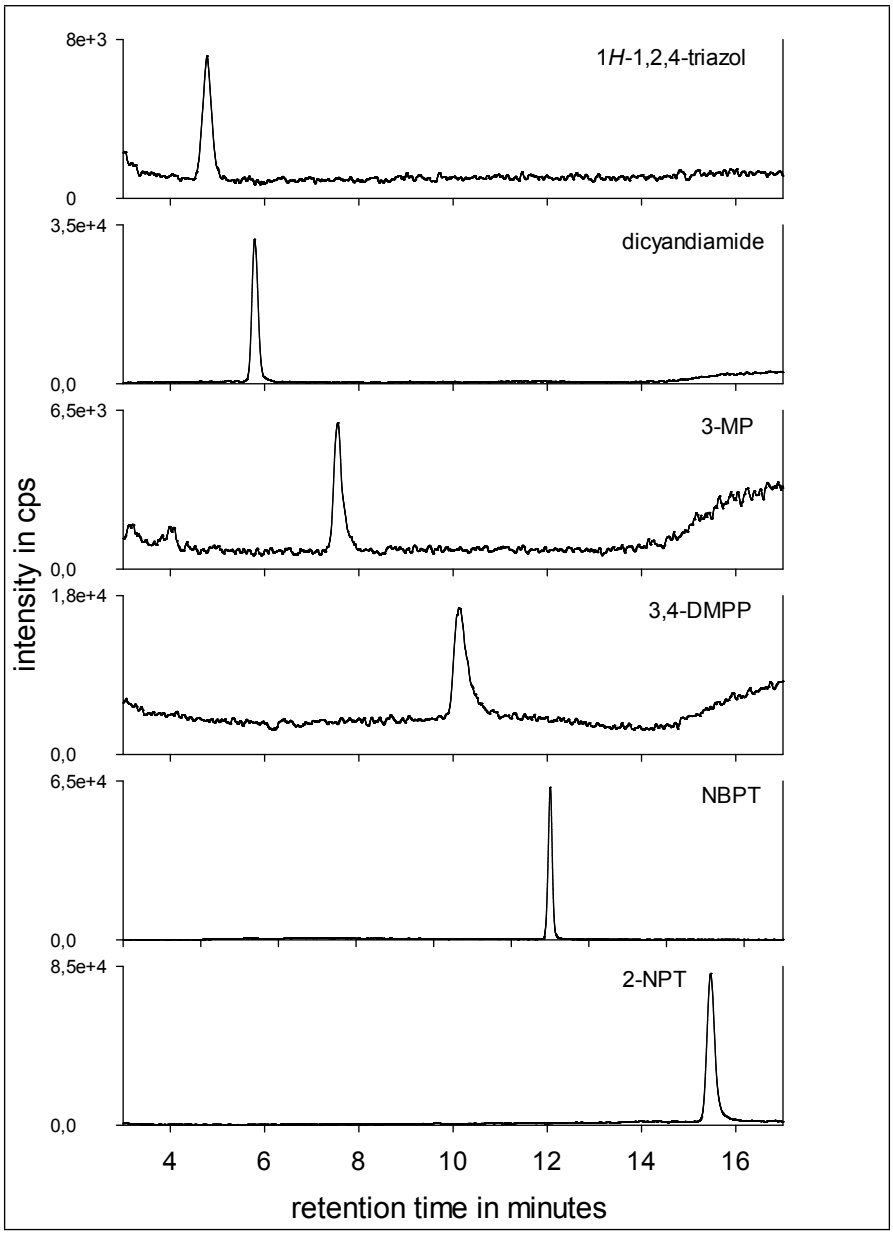


Figure S1: Extracted chromatograms of 50 ng/L standards in ultra-pure water (direct injection).

Table S1: Limits of quantification obtained by German industry standard DIN 32 645 and signal-to-noise ratio.

values in µg/L	1*H*-1,2,4-Triazol	DCD	3,4-DMPP	3-MP	NBPT	2-NPT
LOQ according to DIN 32645	0.03	0.02	0.01	0.01	0.01	0.01
S/N > 10 at	0.1	< 0.01	0.25	0.1	0.03	0.05

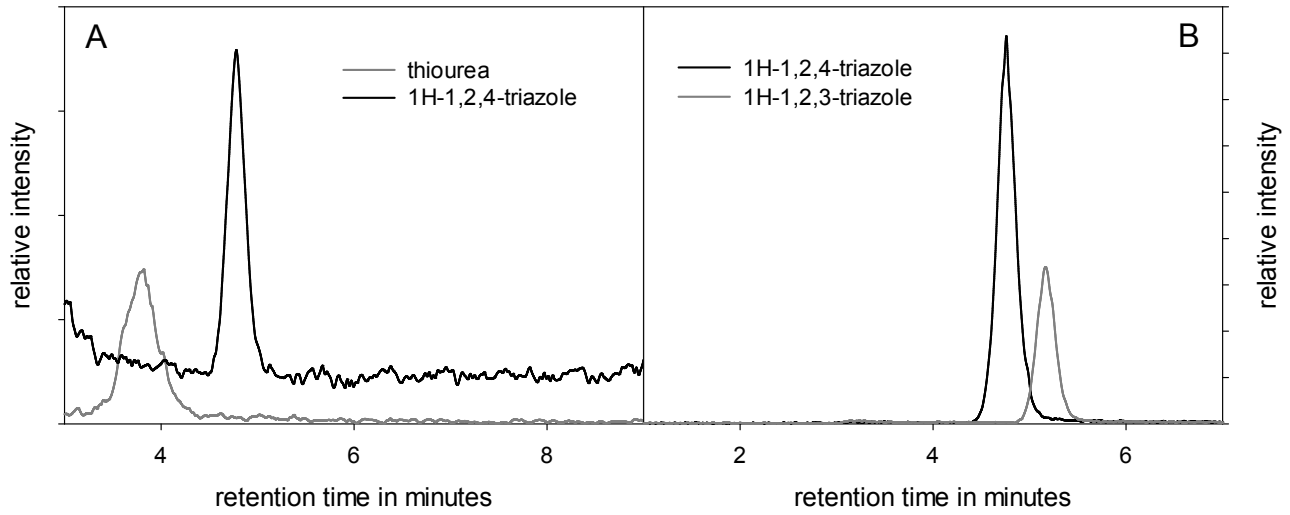
Matrix effect were assessed by comparing spiked potable water and surface water with standards of the same concentration in ultra-pure water. Absolute recoveries for most compounds were in range of 80% and 110%, except for 2-NPT.

MS/MS parameters for individual compounds were obtained by direct infusion into the MS interface. The two most intensive MS/MS transitions between precursor ion and product ions were used for identification and quantification in multiple reaction monitoring (MRM) mode. Precursor and product ions, declustering potential, collision energy, and cell exit potential derived from optimization of compound specific MS settings as well as general interface parameters are displayed in Table S2.

Table S2: MS/MS parameters and limits of quantification in µg/L (in brackets) of target analytes and internal standards, MS-system SCIEX Triple Quad™ 5500.

Analyte	Precursor ion	Product ions	DP [a]	CE [b]	CXP [c]
1*H*-1,2,4-triazole (0.1)	70.0	43.0 28.1	150 150	27 41	8 10
DCD (0.02)	85.0	68.0 42.9	66 66	27 21	6 20
3,4-dimethylpyrazol (0.25)	97.0	56.1 42.0	116 116	23 37	10 18
3-methylpyrazol (0.1)	83.0	42.0 56.1	156 156	25 23	8 10
NBPT (0.03)	168.1	95.0 74.0	56 56	25 15	10 10
2-NPT (0.05)	217.0	199.9 121.0	51 51	13 23	16 18
$^{15}N_4$-DCD	89.0	71.0	86	25	12
1*H*-1,2,4-triazole-$^{13}C_2$, ^{15}N	73.0	44.0	16	13	14

[a] DP = declustering potential in volt, [b] CE = collision energy in electron volt, [c] CXP = cell exit potential in volt



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163 **Figure S2**: Quality measures during method development. Extracted chromatograms of thiourea
164 and 1*H*-1,2,4-triazole proving retention of the latter (A) and extracted 1*H*-1,2,4-triazole and 1*H*-
165 1,2,3-triazole proving their different retention times (B).

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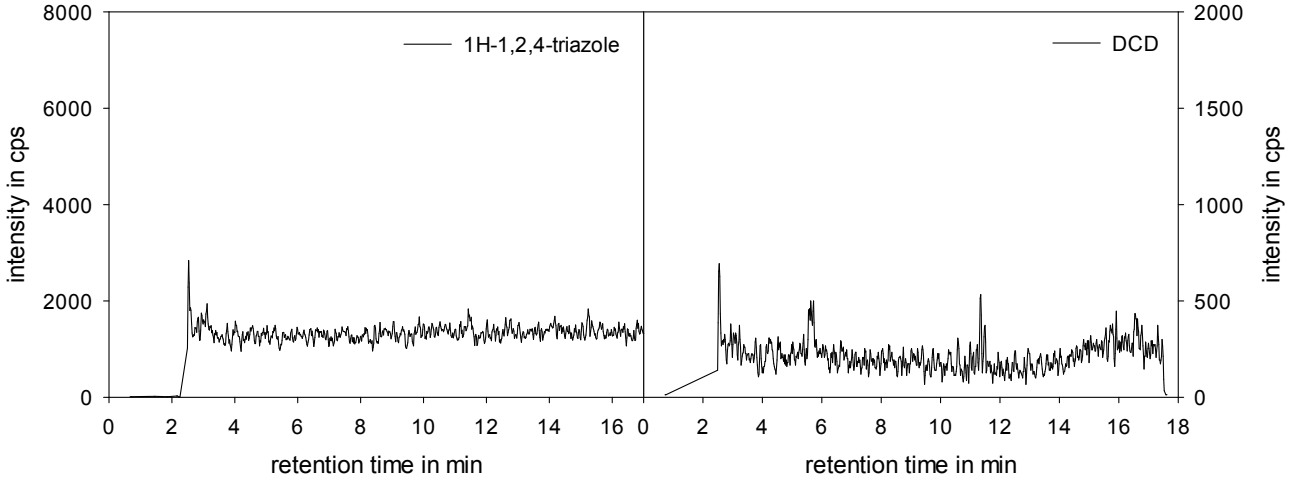

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169 **Figure S3**: Extracted chromatograms of 1*H*-1,2,4-triazole (left) and DCD (right) for a blank
170 sample. Note, the different scaling when comparing it with 50 ng/L standard in Figure S1.

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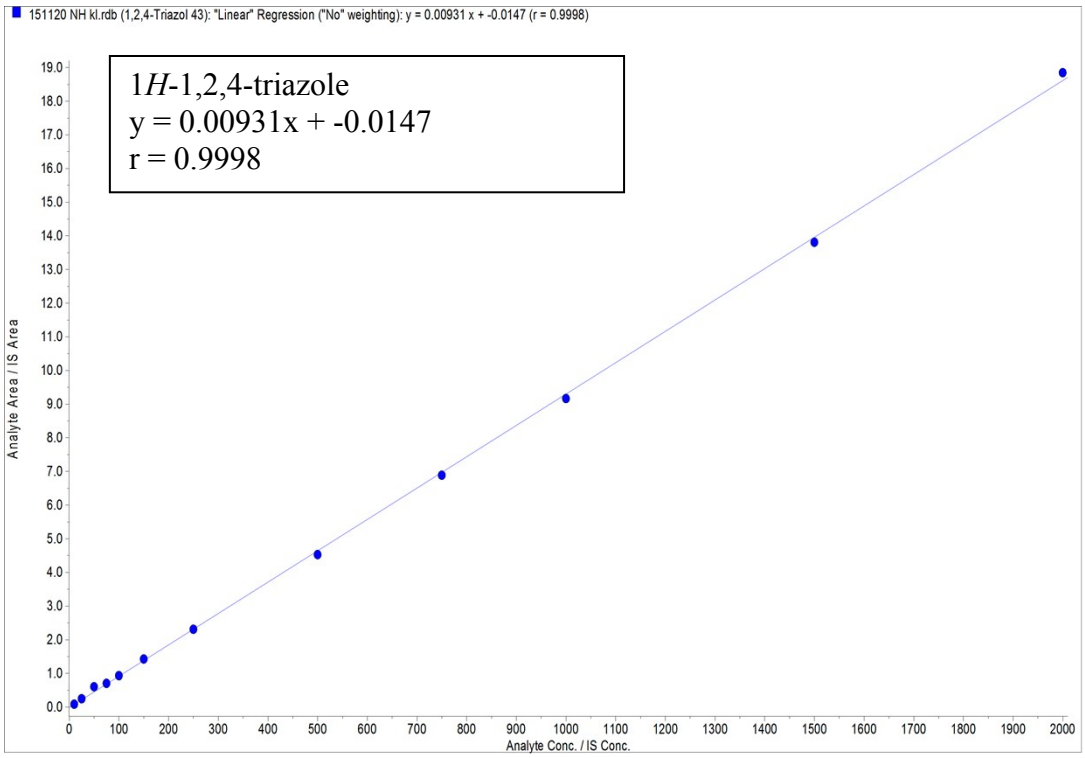

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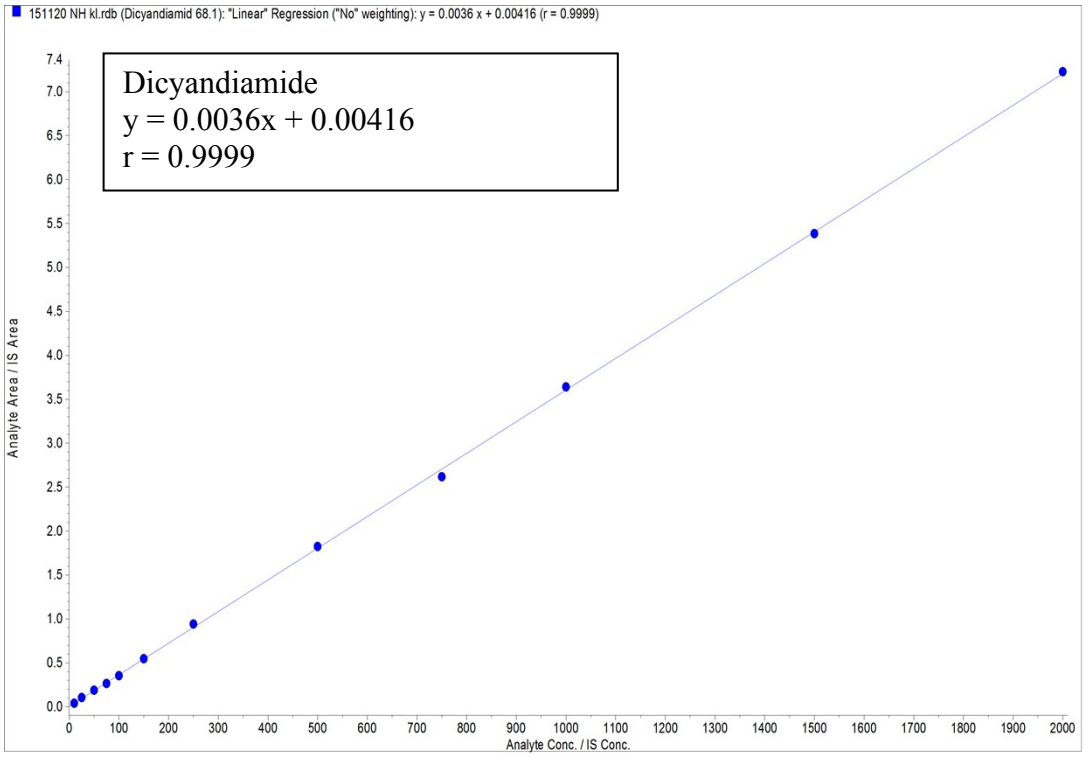

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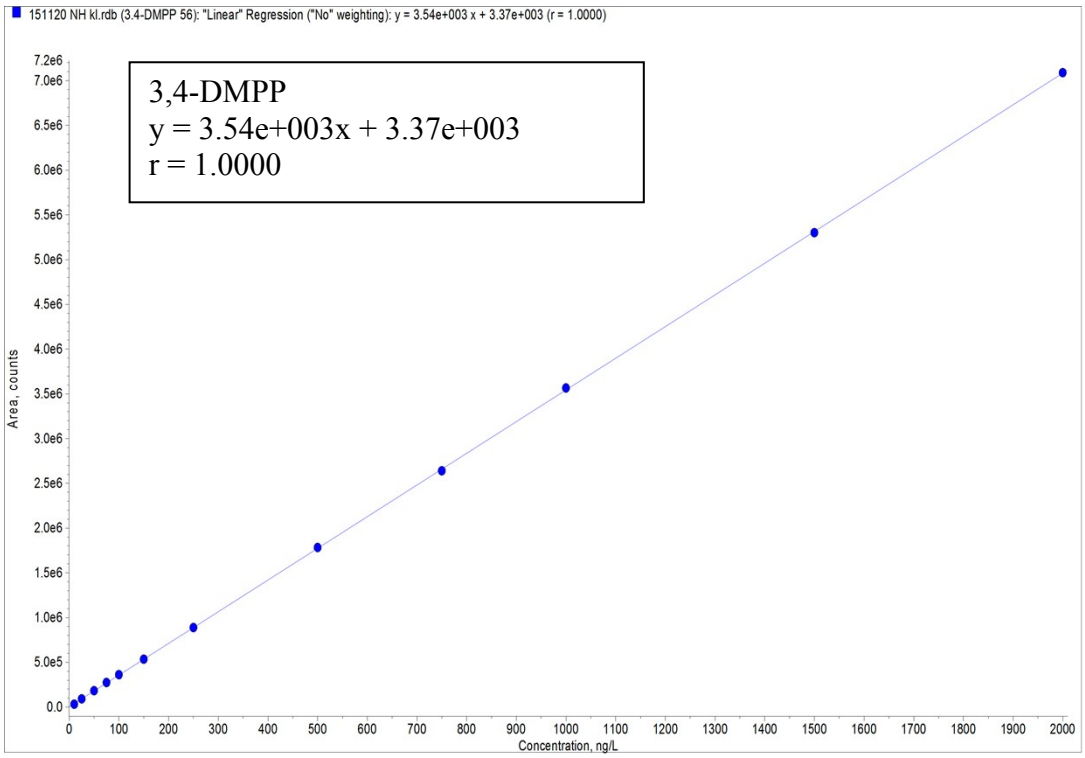
174 **Figure S4**: Calibration curves, linear equations and regression coefficients for all NUIs under
175 investigation.



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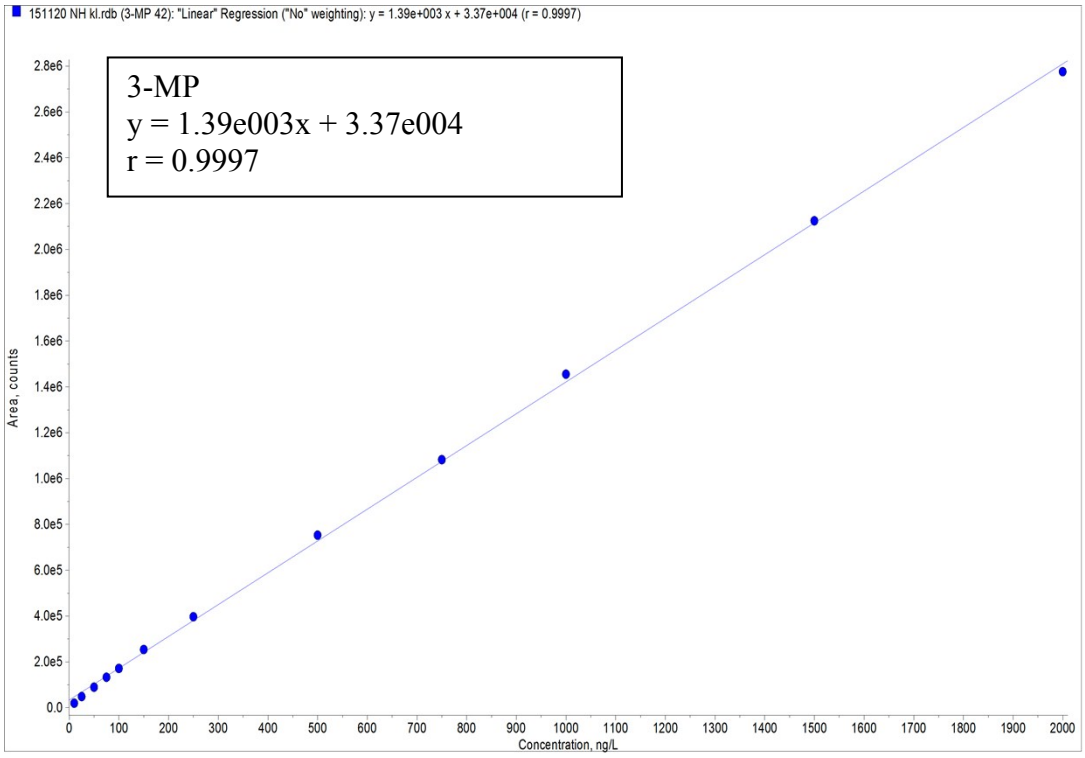

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178 **Figure S4**: continued

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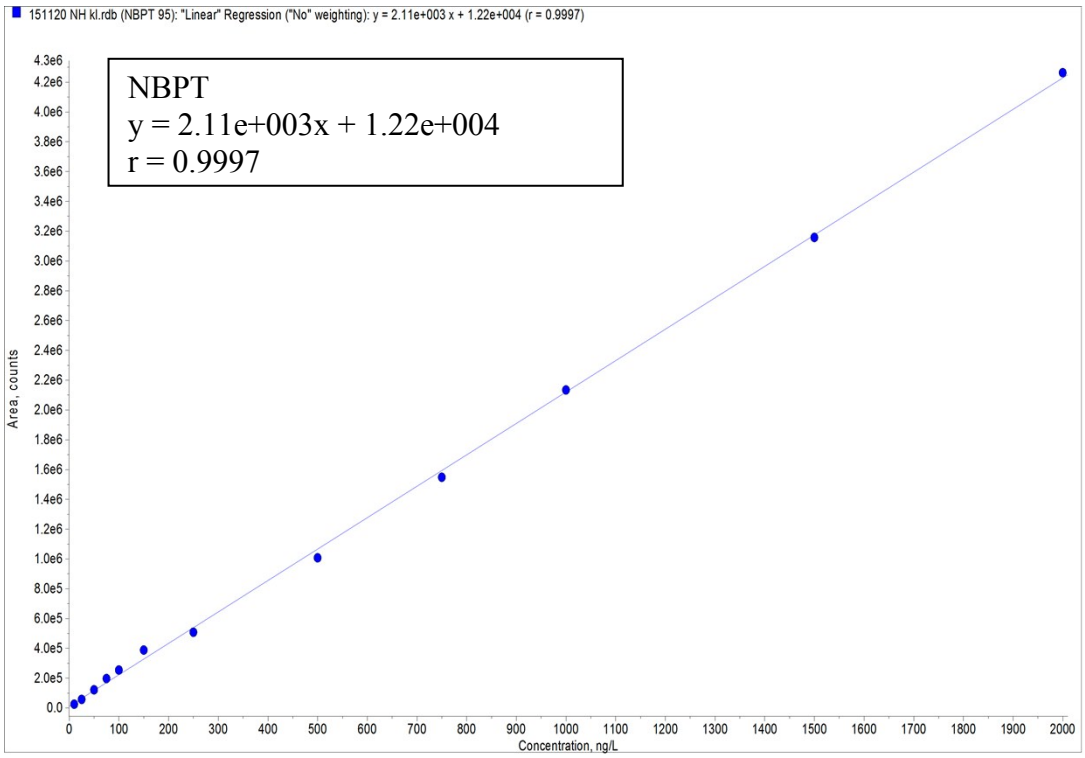

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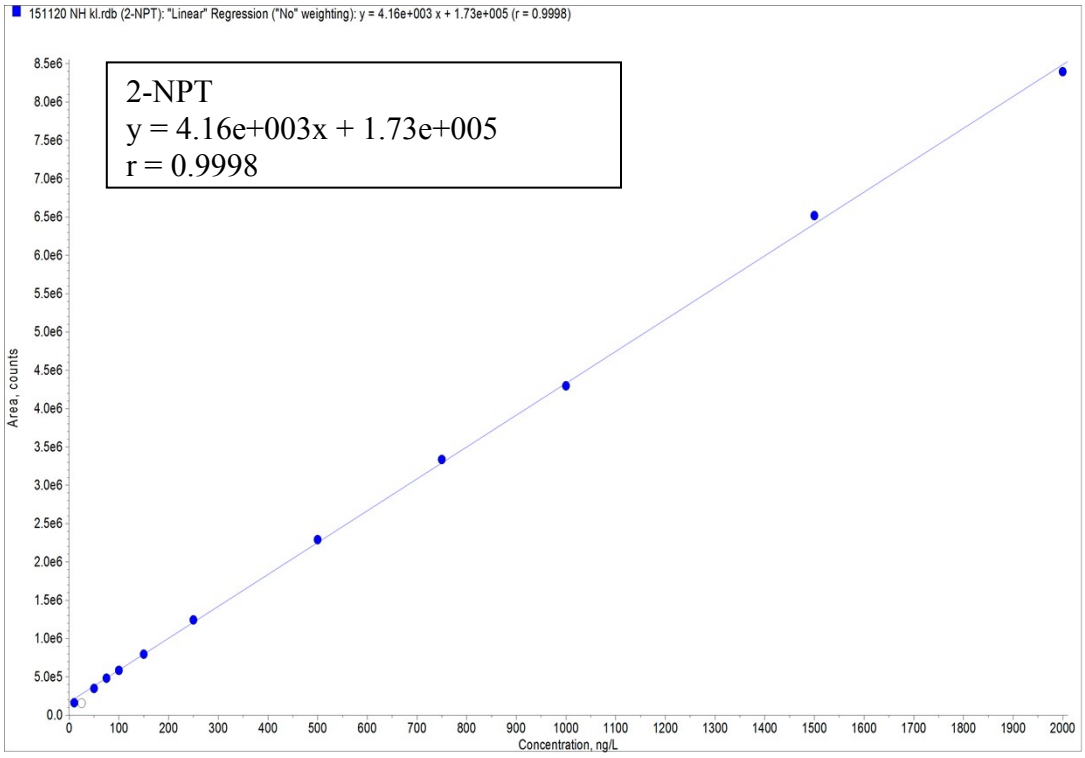

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182 **Figure S4**: continued

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Table S3: Proof of repeatability of the method by ten injection of an analytical standard.

	1H-1,2,4-triazol	DCD	3.4-DMPP	3-MP	NBPT	2-NPT
	peak area					
500 ng/L, injection 1	6,13E+05	3,22E+06	2,44E+06	7,13E+05	4,56E+06	1,65E+07
500 ng/L, injection 2	5,83E+05	3,25E+06	2,31E+06	7,27E+05	3,00E+06	1,51E+07
500 ng/L, injection 3	5,82E+05	3,23E+06	2,31E+06	8,68E+05	3,05E+06	1,52E+07
500 ng/L, injection 4	5,92E+05	3,12E+06	2,37E+06	7,09E+05	3,09E+06	1,50E+07
500 ng/L, injection 5	5,75E+05	3,23E+06	2,35E+06	7,26E+05	4,04E+06	1,51E+07
500 ng/L, injection 6	5,66E+05	3,00E+06	2,28E+06	7,02E+05	4,06E+06	1,46E+07
500 ng/L, injection 7	5,53E+05	3,10E+06	2,35E+06	8,48E+05	3,93E+06	1,45E+07
500 ng/L, injection 8	5,49E+05	3,03E+06	2,35E+06	7,03E+05	4,11E+06	1,40E+07
500 ng/L, injection 9	5,54E+05	3,24E+06	2,33E+06	7,56E+05	3,14E+06	1,38E+07
500 ng/L, injection 10	6,34E+05	3,22E+06	2,42E+06	7,22E+05	3,18E+06	1,41E+07
mean value	5,80E+05	3,16E+06	2,35E+06	7,47E+05	3,62E+06	1,48E+07
standard deviation	2,60E+04	8,89E+04	4,68E+04	5,74E+04	5,48E+05	7,44E+05
standard deviation in %	4.5	2.8	2.0	7.7	15.2	5.0

Text S2: Analysis of other micropollutants within monitoring programs 3 and 4.

Artificial sweeteners acesulfame and sucralose were analyzed according to Scheurer et al., (2009).[3] Briefly, 50 mL of the respective water sample were enriched at pH 3 with a styrol-divinylbenzene cartridges (Bakerbond SDB 1, 200 mg/6 mL from J.T. Baker, Deventer, The Netherlands). After drying of the sorbent material, analytes were eluted with 3 x 3 mL of methanol. The eluate was blown down to dryness and reconstituted with 500 µL buffer A and B (A: ultra-pure water, B: methanol, both with 2 mM ammonium acetate). Chromatographic separation was achieved with a Zorbax Eclipse XDB-C8 column (2.1 mm x 150 mm, 3.5 µm; Agilent Technologies, Santa Clara, CA, USA). Initial conditions of the gradient program were 90% buffer A, which was held for 2 min, then decreased to 25% within 4 min, held for 5 min and then increased again to the initial conditions within 1 min. After each sample run the column was re-equilibrated for 7 min. Liquid chromatography was carried out using a model 1290 HPLC system from Agilent Technologies. The HPLC system was connected to an API 5500 triple-quadrupole mass spectrometer (Applied Biosystems/ MDS Sciex Instruments, Concord, ON, Canada) with an electrospray interface operated in negative ionization mode. Sucralose-d_6 and acesulfame-d_4 were used for internal standard calibration.

Carbamazepine, gabapentin, primidone, metformin, valsartan acid, diatrizoate, hydrochlorothiazide, and oxypurinol were analyzed by direct injection of 40 µL via a MPS-DualHead-WorkStation (Gerstel, Mülheim a. d. Ruhr, Germany) onto the column. Chromatographic separation was achieved with a Kinetex EVO C18 column (2.1 mm x 150 mm, 5 µm; Phenomenex, Aschaffenburg, Germany) using ultra-pure water (A) and methanol (B), both containing 1 mM ammonium acetate, as eluents. Initial conditions of the gradient program were 98% eluent A, which was held for 5 min, then decreased to 25% within 20 min, held for 9 min and then increased again to the initial conditions within 1 min. After each sample run the column was re-equilibrated for 7 min. The HPLC system was connected to an API 5500 triple-quadrupole mass spectrometer

Carbamazepine-d_{10}, gabapentin-d_4, metformin-d_6, diatrizoate-d_6, hydrochlorothiazide-$^{13}C,d_2$ and oxypurinol-$^{13}C,^{15}N_2$ were used for internal standard calibration. Primidone and valsartan acid were analyzed without internal standards.

Analysis of ethylenediaminetetraacetic acid (EDTA) was perfomed according to DIN EN ISO 16588:2004-02.[4] Heptadecanoic acid nitrile was used as an internal standard for gas chromatography. 1,2-Diaminopropane-*N*,*N*,*N*′,*N*′-tetraacetic acid was used as an internal standatd for the whole analytical protocol.

Table S4: Characterization of standard soils LUFA 2.1 and LUFA 2.4 used for sorption study.

standard soil	**2.1**	**2.4**
sampling date	12.06.2013	10.06.2013
content of organic carbon in % C	0.66 ± 0.10	2.21 ± 0.46
pH in 0,01 M $CaCl_2$	5.2 ± 0.3	7.2 ± 0.2
cation exchange capacity in meq/100 g	4.1 ± 0.6	32.2 ± 4.4
particle size distribution in % according to USDA		
<0.002 mm	2.4 ± 0.4	26.5 ± 1.9
0.002 – 0.006 mm	1.6 ± 0.4	8.1 ± 1.0
0.006 – 0.02 mm	3.6 ± 0.4	14.8 ± 1.1
0.2 – 0.063 mm	7.0 ± 0.5	23.0 ± 1.0
0.063 – 0.2 mm	27.2 ± 0.5	19.0 ± 0.3
0.2 – 0.63 mm	55.7 ± 1.5	6.9 ± 2.2
0.63 – 2.0 mm	2.5 ± 0.4	1.7 ± 0.2
soil type	sand	loam

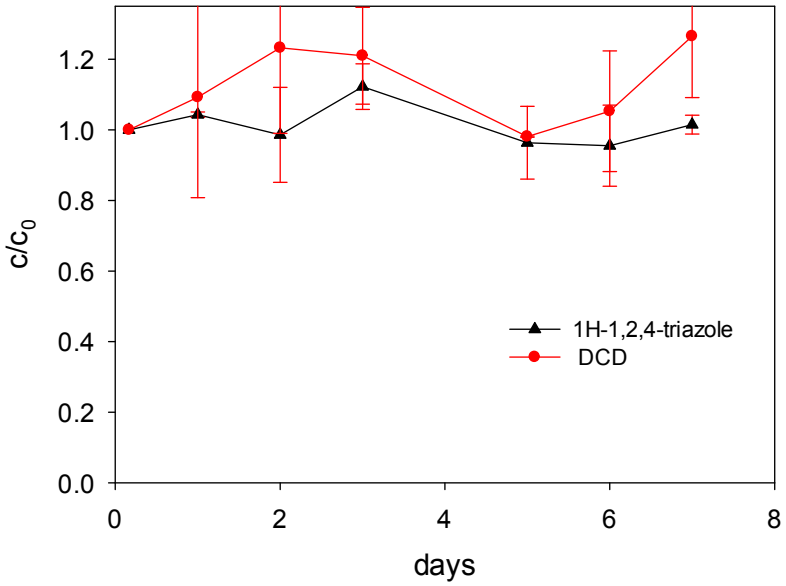
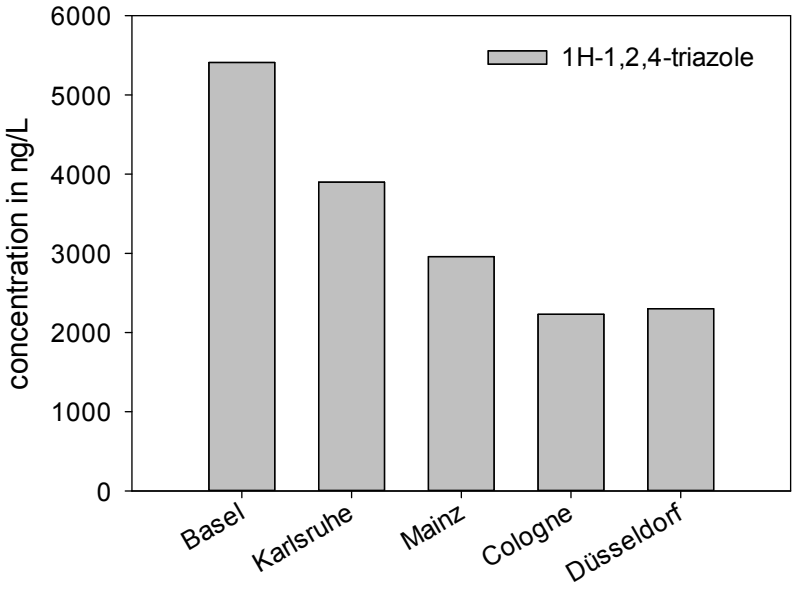


Figure S5: Behavior of 1*H*-1,2,4-triazole und DCD in batch tests with activated sludge (modified Zahn-Wellens test), test duration 7 d.



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243 **Figure S6:** Concentration of 1*H*-1,2,4-triazole in the River Rhine, samples taken at the same day in
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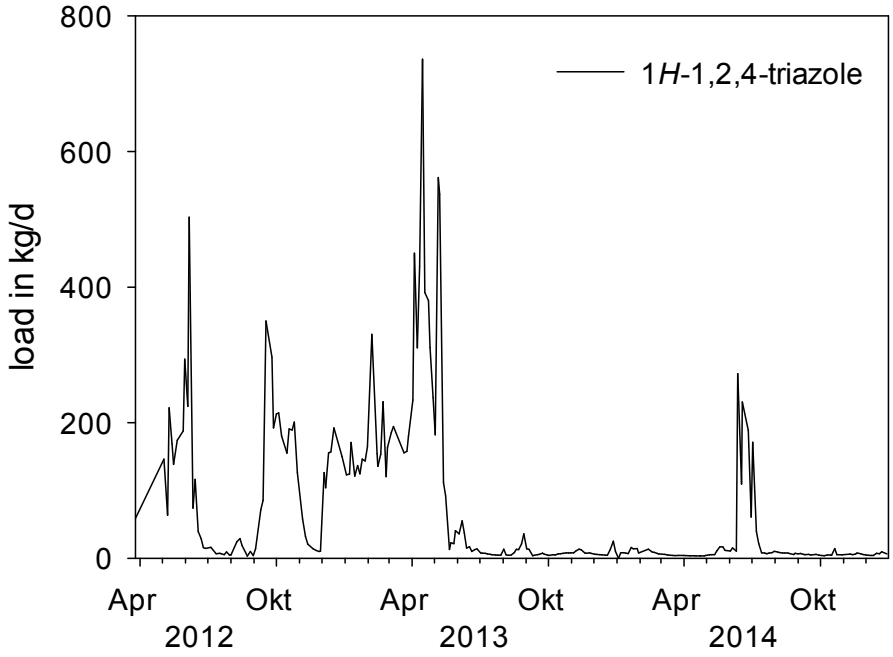

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246 **Figure S7:** Mass load of 1*H*-1,2,4-triazole in the River Rhine at the sampling point in Cologne
247 based on river discharge and measured concentration; samples taken between March 2012 and
248 December 2014.

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Text S3: HRMS measurement for confirmation of high DCD concentrations

For further confirmation purposes of high DCD concentrations within monitoring program 2, LC high resolution mass spectrometry measurements (HRMS) were performed with a Q-TOF 6540 (Agilent Technologies, Waldbronn, Germany). MS/MS spectra of a surface water sample close to the potential discharger was compared with that of a reference standard of DCD. Further confirmation of was given by the Federal Institute of Hydrology (BfG, Koblenz, Germany) by applying a LC/HRMS method with a different chromatography and MS system. The MS/MS spectra which were obtained at the retention time of DCD were similar and showed the same product ions as used in the quantitation method (Figure S8). The spectra corresponds also well with the one available from the National Institute of Standards and Technology (NIST) database, (http://webbook.nist.gov/cgi/cbook.cgi?ID=C461585&Mask=200#Mass-Spec) although a different fragmentation mechanism is used. According to Schymanski et al., 2014 an identification level 1, can be assigned to the observation of DCD in the surface water sample.[5]

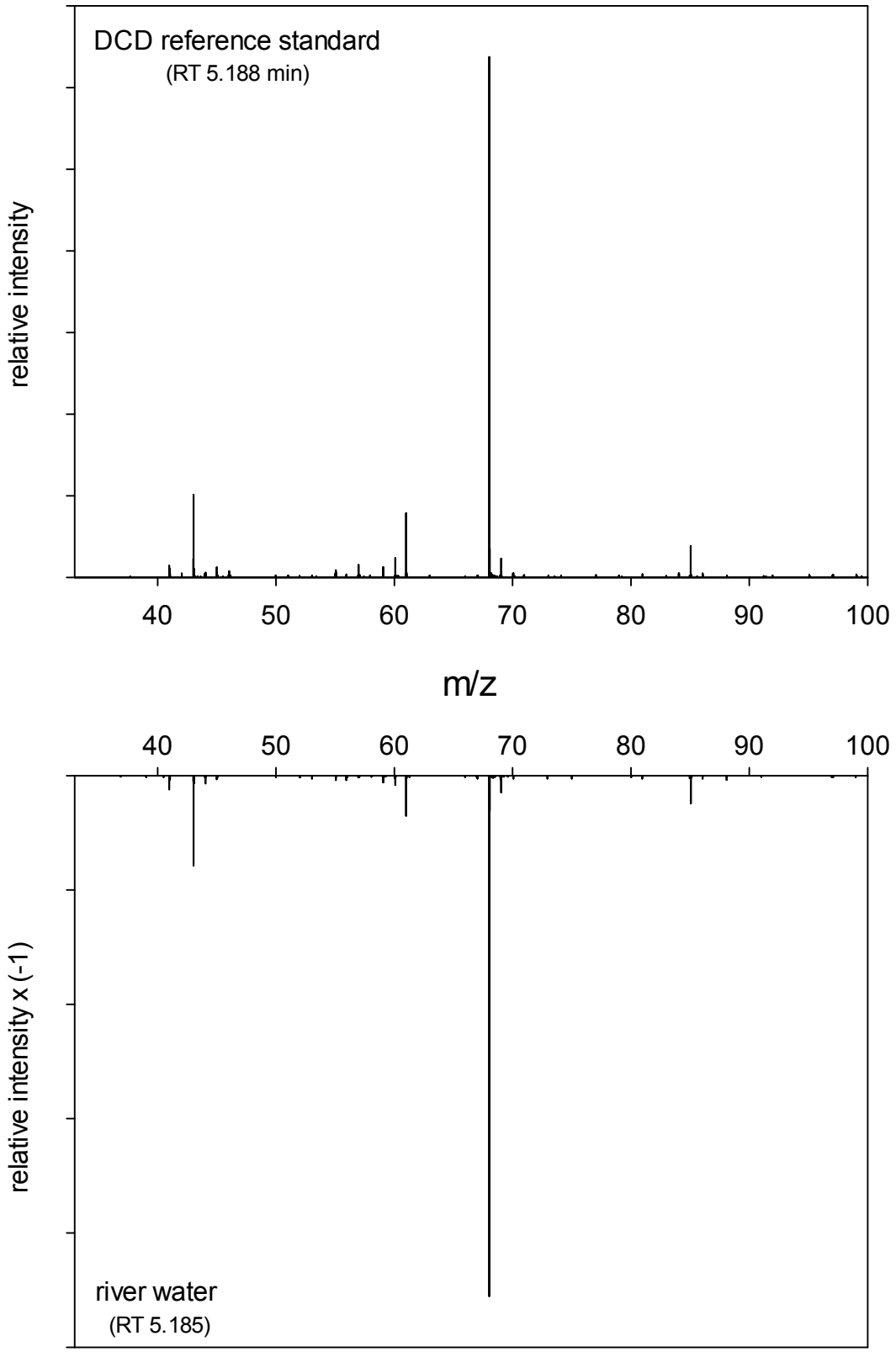

Figure S8: High resolution MS/MS mass spectra of a DCD reference standard and a river water sample close to the point of discharge (monitoring program 2) obtained with a Agilent Q-TOF 6540. Additional confirmation by BfG Koblenz, see Text S3.

Table S5A: Correlation coefficients of micropollutants considering all sampling points of monitoring program 3 (River Main and its tributaries). Numbers in bold indicate three micropollutants with the highest sum of correlation coefficients.

	CBZ	GAB	PRIM	MET	VAC	DCD	EDTA	SUC	ACE	DTZ	OXY	sum
CBZ	X	0.82	0.72	0.70	0.83	0.21	0.15	0.75	0.65	0.49	0.57	5.89
GAB	0.82	X	0.66	0.76	0.70	0.17	0.23	0.77	0.66	0.38	0.54	5.69
PRIM	0.72	0.66	X	0.52	0.82	0.40	0.33	0.73	0.60	0.67	0.79	**6.24**
MET	0.70	0.76	0.52	X	0.55	0.30	0.3	0.59	0.70	0.26	0.55	5.23
VAC	0.83	0.70	0.82	0.55	X	0.39	0.20	0.89	0.53	0.73	0.78	**6.42**
DCD	0.21	0.17	0.40	0.30	0.39	X	0.62	0.41	0.10	0.42	0.70	3.72
EDTA	0.15	0.23	0.33	0.30	0.20	0.62	X	0.28	0.31	0.36	0.52	3.30
SUC	0.75	0.77	0.73	0.59	0.89	0.41	0.28	X	0.41	0.61	0.73	6.17
ACE	0.65	0.66	0.60	0.70	0.53	0.10	0.31	0.41	X	0.31	0.42	4.69
DTZ	0.49	0.38	0.67	0.26	0.73	0.42	0.36	0.61	0.31	X	0.75	4.98
OXY	0.57	0.54	0.79	0.55	0.78	0.70	0.52	0.73	0.42	0.75	X	**6.35**

Table S5B: Correlation coefficients of micropollutants considering sampling points of monitoring program 3 (River Main only, tributaries were excluded). Numbers in bold indicate three micropollutants with the highest sum of correlation coefficients.

	CBZ	GAB	PRIM	MET	VAC	DCD	EDTA	SUC	ACE	DTZ	OXY	sum
CBZ	X	0.13	0.50	0.30	0.53	0.02	0.04	0.03	0.51	0.12	0.50	2.68
GAB	0.13	X	0.21	0.07	0.33	0.05	0	0.43	0.28	0	0.30	1.80
PRIM	0.50	0.21	X	0.06	0.75	0	0.02	0.05	0.65	0.10	0.40	2.74
MET	0.30	0.07	0.06	X	0.16	0.21	0.03	0.32	0.03	0.16	0.33	1.67
VAC	0.53	0.33	0.75	0.16	X	0.04	0	0.14	0.52	0.07	0.67	**3.21**
DCD	0.02	0.05	0	0.21	0.04	X	0.08	0.13	0.12	0.15	0.05	0.85
EDTA	0.04	0	0.02	0.03	0	0.08	X	0.06	0	0.01	0.03	0.27
SUC	0.03	0.43	0.05	0.32	0.14	0.13	0.06	X	0	0.23	0.13	1.52
ACE	0.51	0.28	0.65	0.03	0.52	0.12	0	0	X	0.33	0.37	**2.81**
DTZ	0.12	0,00	0.10	0.16	0.07	0.15	0.01	0.23	0.33	X	0	1.17
OXY	0.50	0.30	0.40	0.33	0.67	0.05	0.03	0.13	0.37	0	X	**2.78**

Table S6: Concentrations of 1*H*-1,2,4-triazole and DCD in several WWTP effluents as well as population equivalents of the WWTPs.

WWTP	**population equivalents**	**1H-1,2,4-triazole**	**DCD**
	(treatment capacity)	concentration in µg/L	
WWTP 1	55,000	< 0.5	0.37
WWTP 2	120,000	< 0.5	0.45
WWTP 3	250,000	< 0.5	2.9
WWTP 4	7,500	< 0.5 / <0.5	< 0.1 / < 0.1
WWTP 5	725,000	0.84	1.0
WWTP 6	14,300	< 0.5	0.18
WWTP 7	15,000	< 0.5	< 0.1
WWTP 8	15,000	< 0.5	0.27

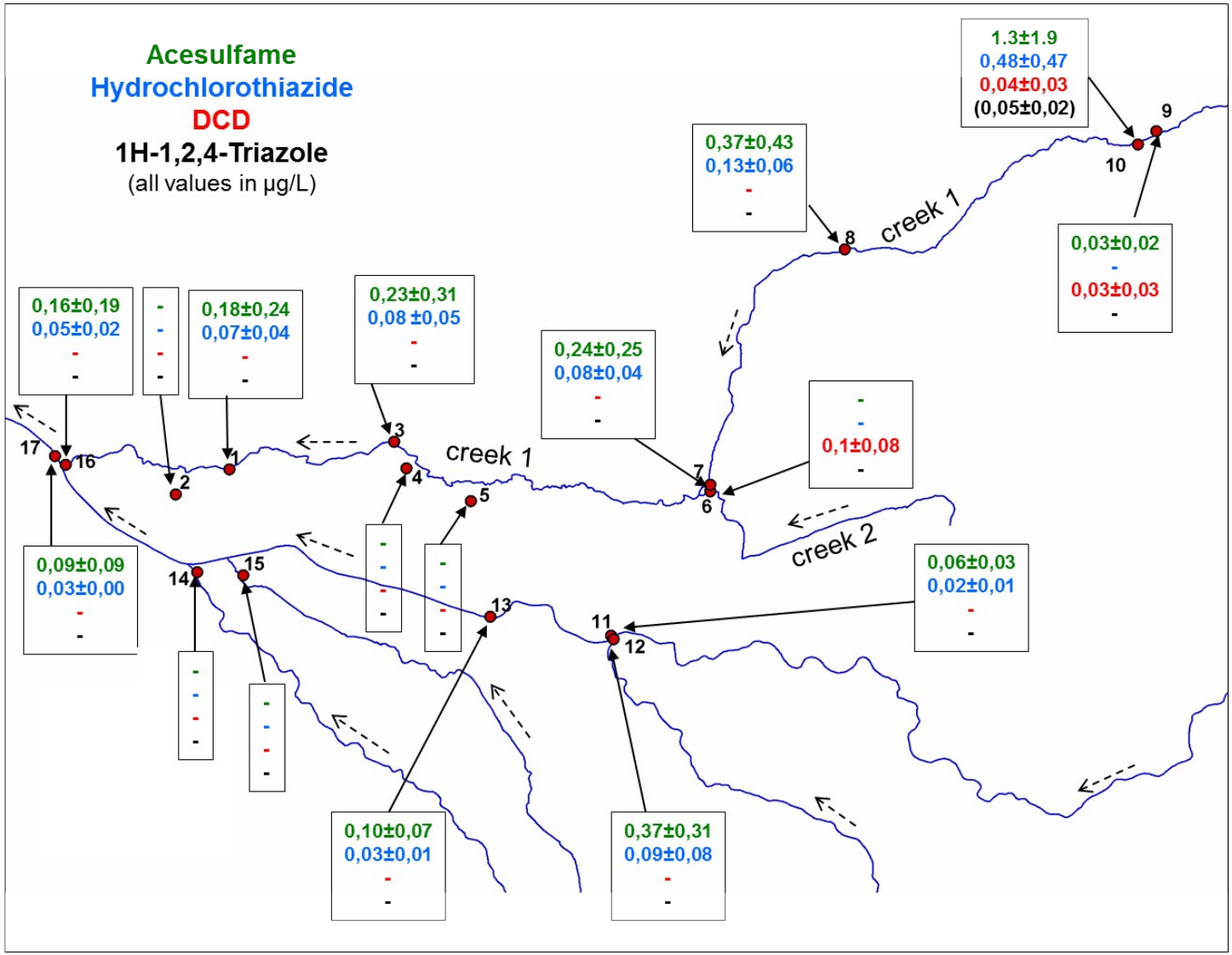

Figure S9: Concentrations of acesulfame, hydrochlorothiazide, DCD, and 1*H*-1,2,4-triazole measured in the catchment of a water utility (monitoring program 4). Mean values with standard deviation are given if more than 50% of the results were >LOQ. Dashed arrows indicate flow directions.

References

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