Internal concentrations in gammarids reveal increased risk of organic micropollutants in wastewater-impacted streams

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TOC:

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Abstract

Internal concentrations link external exposure to the potential effect, as they reflect what the organisms actually take up and experience physiologically. In this study, we investigated whether frequently detected risk-driving substances in water were found in the exposed organisms and if they are classified the same based on the whole body internal concentrations. Field gammarids were collected upstream and downstream of ten wastewater treatment plants in mixed land use catchments. The sampling was conducted in autumn and winter, during low flow conditions when diffuse agricultural input was reduced. The field study was complemented with laboratory and flume experiments to determine the bioaccumulation potentials of selected substances. For 32 substances, apparent bioaccumulation factors in gammarids were determined for the first time. With a sensitive multi-residue method based on online-solid phase extraction followed by liquid chromatography coupled to high resolution mass spectrometry, we detected 63 (semi-) polar organic substances in the field gammarids, showing higher concentrations downstream than upstream. Interestingly, neonicotinoids, which are particularly toxic towards invertebrates, were frequently detected and were further determined as major contributors to the toxic pressure based on the toxic unit approach integrating internal concentration and toxic potency. The total toxic pressure based on internal concentrations was substantially higher compared to when external concentrations were used. Thus, internal concentrations may add more value to the current environmental risk assessment that is typically based solely on external exposure.

Introduction

Thousands of chemicals are regularly released into the environment from various sources (e.g. households, agriculture) and can enter streams through different pathways (e.g. wastewater effluent, urban and agricultural runoff). Consequently, numerous compounds are detected in freshwater ecosystems as complex mixtures, mostly at trace concentrations in the ng/L to µg/L range.¹ Some of these compounds are designed to be biologically active (i.e., pesticides, pharmaceuticals) and when taken up, they may adversely affect non-target organisms.²⁻⁴

While environmental risk assessment at present is typically based on exposure levels in water, the inclusion of the internal concentrations of chemicals is increasingly regarded as indispensable for the

effects assessment of chemicals in aquatic organisms.^{2, 5-6} Compared to water concentrations, internal concentrations do not only assess what the aquatic organisms “see”, but also reflect what they actually take up and could therefore provide indications of potential effects. This is especially true if only short-term sampling of water is conducted. While grab samples represent snapshots of the exposure, internal concentrations integrate the concentrations over time. Sometimes, passive sampling is applied to biomimic integrative sampling of organisms⁷, but processes such as active uptake and metabolism cannot be represented with passive samplers. In biomonitoring studies, fish are often considered as most appropriate organisms because they are not only relevant for the environment but also for humans as a food source. However, besides ethical considerations, one of their limitations is their relatively high mobility. In contrast, invertebrates (e.g. gammarids) have a much lower mobility and can serve as good site-specific bioindicators.⁴ Gammarids are important keystone species for detritus processing (shredders) and form an important link to higher trophic level organisms as they are preyed on by fish.⁸⁻⁹ These amphipods are highly sensitive towards a range of different stressors and are thus often used as model organisms in ecotoxicological studies.⁸⁻¹¹ Because of their high abundance in freshwater systems^{8, 10}, they are suitable for investigations of internal exposures in field biomonitoring studies.

However, information on bioaccumulation of organic compounds in aquatic organisms other than fish is rather limited, especially for chemicals with many polar functional groups wherein prediction of bioaccumulation is difficult.¹² Although non-polar compounds have a higher bioaccumulation potential, polar compounds are usually present in surface water at much higher bioavailable concentrations and thus they can be considered as equally relevant for toxicity.¹³ Studies investigating the bioaccumulation of selected polar substances in gammarids under controlled laboratory conditions showed that many polar compounds are taken up and can contribute to possible adverse effects.¹⁴⁻¹⁹ In only a few field studies, the presence of polar organic substances in gammarids was observed in experiments with caged organisms²⁰⁻²¹ and gammarids directly collected from streams²²⁻²⁴.

The goal of this study was to determine the chemical risk of polar and semi-polar organic micropollutants, which are frequently analyzed and detected in water, based on whole body internal concentrations (hereafter named internal concentrations) in gammarids collected in the field. In a previous study on the external exposure of micropollutants we identified pesticides as main drivers of the risk.²⁵ Thus, we wanted to investigate whether i) the identified risk drivers in water are actually

detected in gammarids collected at the same time, and ii) if yes, whether they would also be classified as risk drivers based on the internal exposure. Laboratory experiments and artificial flume experiments supplemented the field data for a more comprehensive understanding of the bioaccumulation potential under controlled and semi-realistic exposure conditions. Gammarids were collected at ten sites in mixed land use catchments upstream and downstream of wastewater treatment plants (WWTP), where our previous study on the external exposure was also conducted.²⁵ The potential mixture toxic pressure on invertebrates was estimated based on the internal concentrations and the laboratory determined bioaccumulation potentials as well as EC50 values for invertebrates, and was then compared to the toxic pressure calculated with the external water concentrations.

Material & Methods

Field samples (gammarids and water)

Gammarids (mainly *G.fossarum*) were collected at nine sites upstream and downstream of WWTPs (Aadorf, Elgg, Ellikon, Knonau, Marthalen, Unterehrendingen, Val-de-Ruz, Villeret, Zullwil in September 2014 and January 2015 and at the site Ellikon also in October 2015 (Figure S1)). Herisau WWTP underwent a major process upgrade in 2015 with the addition of an advanced treatment based on sorption to powdered activated carbon for the enhanced elimination of micropollutants. Hence, additional gammarids collection was completed in May 2015 and June 2016 to assess the impact of treatment upgrades. Water grab samples were previously taken at the same sites and time points using the methodology described in Munz et al. (2017)²⁵. As controls, uncontaminated gammarids were collected at a pristine site near Zurich, Switzerland.¹⁹ At each site, approximately 100 individuals, without visible eggs, were collected using kick-nets. They were transferred to the lab in buckets filled with stream water and leaves. In the lab, gammarids were transferred to Falcon tubes, frozen at -20 °C and then stored at -80 °C until analysis. Replicate samples were analyzed for each site whenever possible. To determine recovery, thawed gammarids from two sites (upstream and downstream) were spiked with each target substance at a level of 250 ng/L; the area (for absolute recovery) or concentration (for relative recovery) was subtracted by the value of the unspiked sample and divided by the value of the corresponding calibration standard. Number, weight and major species of gammarids are provided in Table S1.

Laboratory exposure experiment

Gammarids (*G. pulex*) were collected at the pristine site mentioned above and acclimatized to the laboratory conditions (12 °C, 12 h light/12 h dark cycle) in an aquarium using artificial pond water (APW) and fed with alder leaves collected in the creek. Details on the preparation of APW are provided in Rösch et al. (2016).¹⁹ Gammarids were exposed to i) APW spiked with a mixture of 52 (semi-) polar organic substances (e.g. pesticides, pharmaceuticals) at nominal concentrations of 5 µg/L each, and ii) to wastewater diluted in APW (30%, 60%, 90% of wastewater). The list of substances was selected based on the priority mixture described in Munz et al. (2017)²⁵. For details on the substance selection and the wastewater sample see Section S1. Gammarids without visible eggs (13 - 16 organisms) were added to 600 mL-glass beakers filled in triplicate with 500 mL of the exposure media and 5 alder leaves, and were incubated for 48 h at 12 °C. An incubation period of 48 h was regarded as sufficient since other studies have shown that steady state was reached within this time frame for chemicals with comparable logK_{ow} values.^{15, 17, 19, 26} Controls i) without chemicals or wastewater (triplicates), ii) without organisms (duplicates), and iii) without leaves and organisms (duplicates) were performed. The exposure media was sampled at the beginning (t₀), after 24 hours (t₂₄) and at the end (t₄₈) of the experiment. Gammarids were also collected directly from the aquarium to determine recoveries and background concentrations. All samples were stored at -20 °C until analysis. Number and weight of gammarids per replicate are listed in Table S2.

Flume experiments

A description of the setup of the flume experiments can be found in Stamm et al. (2016)²⁷. Briefly, 16 channels arranged in four blocks (four replicates of each treatment) were setup directly at the WWTP Bachwis (Fällanden, Switzerland). They were fed with upstream water of the Glatt River and treated wastewater (experiment 1) or spiked chemicals in river water (experiment 3) (experiment numbering according to Stamm et al. (2016)²⁷). The gammarids were placed in cages in the channels during four (experiment 3, *G. fossarum*) to five weeks (experiment 1, *G. pulex*). The experiments were conducted in August 2014 (experiment 1) and June 2015 (experiment 3). The gammarids were collected at the end of the experiments and directly frozen at -20°C and stored at -80°C until analysis. To obtain a sufficient mass of gammarids (~500 mg) for bioanalysis, different replicates from the four blocks were pooled, if necessary. Further details on the two flume experiments are described in Section S2. Number and weight of gammarids per channel are listed in Table S3. Water samples of the flumes

were taken at different time points of the experiment. The analysis of 57 organic micropollutants in the flume water was done using online solid phase extraction (SPE) followed by liquid chromatography coupled to high resolution tandem mass spectrometry (LC-HRMS/MS) as previously described in Munz et al. (2017)²⁵.

Sample preparation, enrichment and clean-up

Thawed gammarids were quickly rinsed with nanopure water, blotted dry with tissue and weighed into a 2-mL microcentrifuge tube to a final weight of approximately 500 mg (on average 40 organisms depending on size; in the exposure experiment all exposed gammarids were used). After the addition of 80 µL internal standard (1 mg/L) they were stored overnight at 4°C. The remaining solvent was shortly evaporated with a gentle stream of nitrogen, then 500 mg of 1 mm zirconia/silica beads (BioSpec Products, Inc., U.S.A.), 500 µL of acetonitrile (ACN) and 500 µL of nanopure water were added. Extraction and homogenization were carried out using a Fast Prep bead beater (MP Biomedicals, Switzerland) in two cycles of 15 s at 6 m/s with cooling on ice in between. Afterwards, samples were centrifuged (6 min, 10 000 rpm, 20 °C) and 800 µL of the supernatant was transferred into a tube containing 500 mg QuEChERS salts (4:1, MgSO₄:NaCl, Agilent Technologies), immediately vortexed and then centrifuged again (6 min, 10 000 rpm, 20 °C). ACN (500 µL) was added to the first homogenate with the already used QuEChERS salts and all the steps were repeated to increase recoveries. For a further clean-up step, especially for the elimination of lipids, the combined supernatant (approx. 800 µL) was transferred to a tube containing heptane (500 µL), vortexed and centrifuged (6 min, 10 000 rpm, 20 °C). Heptane (400 µL) was removed and a second heptane extraction (500 µL) was carried out. Finally, 700 µL of the ACN phase (bottom layer) was transferred to a clean HPLC glass vial and was filled with methanol to a final volume of 2 mL. The extracts were stored at 4 °C until analysis.

Chemical analysis of gammarid extracts

All gammarid extracts were analyzed using the online SPE LC-HRMS/MS setup similar to the water samples described in Munz et al. (2017)²⁵ (see Section S3). For the gammarid extracts, 200 µL of the extract was spiked into an online vial filled with 20 mL nanopure water. Quantification of up to 84 target substances was done with internal standard calibration using the software TraceFinder

v3.2/v4.1 (Thermo Scientific; Table S4). In addition to the selected substances in the lab experiment (n=52), the field gammarids were screened for further target substances (spiked in the calibration standards) using CompoundDiscoverer 2.1 (Thermo Scientific). A mass list of the additional 395 target substances spiked in the calibration standards (Table S5) was processed over the five most polluted samples (≥ 10 detected substances). Only substances analyzed in positive ionization mode were considered in this step because of higher sensitivity and larger number of analytes. After filtering out the background, only targets which showed an acceptable signal (i.e., peak shape, retention time) in the calibration curve and in at least one of the selected samples were considered for further quantification in TraceFinder.

For the reported internal concentration, the average of the concentrations in the replicates was calculated. If the concentration of one replicate was $>LOQ$ and the concentration of the other replicate was $<LOQ$ and $>LOD$, half the LOQ value was used. If the concentration of one replicate was $>LOQ$ and the concentration of the other replicate was $<LOD$, zero was taken for the value $<LOD$. If the concentrations of both replicates were between LOQ and LOD, the substance was counted as detected ($<LOQ$), but not quantified. Otherwise, the substance was listed as not detected.

Determination of water and lipid content in gammarids

For selected sites, enough sample was available to determine the water and lipid content of the gammarids. The water content was determined gravimetrically after freeze-drying of the gammarids. Lipids of the freeze dried gammarids were extracted with a mixture of isopropanol-cyclohexane-water (4:5:5.5, v:v:v) based on the method described in Kretschmann et al. (2011)²⁸. The lipid content was determined gravimetrically by weighing of the lipid extracts. Further details are described in the Section S4 and Table S6.

Partition coefficients

Experimental octanol-water partition coefficients ($\log K_{ow}$) were initially searched in different databases (Footprint pesticide properties database²⁹, EPA CompTox Chemistry Dashboard³⁰, drugbank³¹) and when no data were found, predicted values (EPA CompTox Chemistry Dashboard³⁰) were taken (Table 1, Table S4). To account for speciation of ionizable substances, the $\log D_{ow}$ (pH-dependent $\log K_{ow}$) was calculated at pH7.9 (pH conditions during the lab experiment and also representing typical conditions found in many Swiss streams) for anionic and cationic substances (Section S5).

196 *Bioaccumulation factors (BAFs)*

197 The apparent bioaccumulation factor (BAF) [L/kg] was calculated as the ratio of the internal
 198 concentration ($C_{internal}$) [ng/kg wet weight (w.w.)] and the exposure concentration ($C_{exposure}$) [ng/L] at
 199 steady state (Eq.1). If not otherwise stated, the term BAF refers to the apparent BAF.

$$200 \quad BAF_{exp} = \frac{C_{internal}}{C_{exposure}} \quad (1)$$

201 To see whether the results from the software tool EPISuite designed for fish could be transferred to
 202 gammarids, we compared our apparent BAFs with the predicted BAFs for lower trophic level fish³². To
 203 account for differences in lipid content we corrected the BAFs with the defined lipid contents in
 204 EPISuite (5.98%) and the determined mean lipid content of 2.7% in gammarids (Table S6),
 205 respectively.

206

207 *Calculation of toxic pressure*

208 The toxic pressure was predicted using the approach of toxic units (TUs) focusing on invertebrates
 209 only. In order to calculate the TUs for the internal concentrations, we transformed the EC50 values,
 210 which are based on water concentrations, to internal EC50 values using the apparent BAFs
 211 determined in the lab experiment (Eq. 2). TUs for each substance i were calculated by dividing the
 212 internal concentration ($C_{internal,i}$) by the geometric mean of acute internal EC50 values for invertebrates
 213 ($EC50_{internal,i}$, Eq.3). The geometric mean (Table S7) over all available data on invertebrates (data
 214 collected in de Zwart (2002)³³) was used since bioaccumulation varies between species^{16, 18, 34-35}, and
 215 EC50 values for gammarids were not available for all substances. For single sites and time points, the
 216 mixture risk was calculated according to the concept of concentration addition by summing up the
 217 single TUs (Eq. 4).

$$218 \quad EC50_{internal,i} = EC50_i \cdot BAF_i \quad (2)$$

$$219 \quad TU_{internal,i} = \frac{C_{internal,i}}{EC50_{internal,i}} \quad (3)$$

$$220 \quad sumTU = \sum_i^n TU_{internal,i} \quad (4)$$

221

Results & Discussion

Analytical performance

The method for the extraction of gammarids was successfully applied in the field study and laboratory experiment for the quantification of 84 substances (Table S4). The absolute recoveries were on average only around 30%, ranging from 9 to 70%. Mecoprop and sucralose had absolute recoveries below 5% and their results should be interpreted with caution. However, the relative recoveries using 57 corresponding isotope labelled internal standards were in an acceptable range for quantification (70 – 130%) and low LOQs, ranging from 0.1 to 9 ng/g w.w., were achieved for all substances (except for sucralose having a LOQ of 380 ng/g w.w.). Overall, the LOQs were comparable to other studies.^{20, 23-24} Berlioz-Barbiez et al. (2014)²⁰ who used a miniaturized setup with nanoLC, reported similar LOQs in the range of 0.5 to 15.4 ng/g w.w, while Inostroza et al. (2016)²³ reached slightly lower LOQs for some single substances, ranging from 0.01 to 2.1 ng/g w.w.. They performed the LC-MS/MS analysis on a triple quadrupole instrument, which usually allows for higher sensitivity compared to fullscan HRMS analysis³⁶⁻³⁷ but does not allow such a broad screening as performed in our study. Miller et al. (2015)²⁴ achieved LOQs ranging from 4 to 61 ng/g dry weight (d.w.), which expressed as wet weight (assuming ~75% of water content, Table S6), are as well comparable to the values of this study. Further details on the method performance are described in Section S3.

Detected substances and concentration patterns in the field gammarids

Overall, 63 of the 84 analyzed substances were detected at least once over all sites and time points (Table 1, Table S4). To our knowledge, this is the highest total number of substances reported in gammarids collected in the field. In addition to the 32 detected target compounds, 31 further substances were detected with the rough screening of 395 positive ionizing compounds, of which 25 were above LOQs. All internal concentrations at the different sites and time points are listed in Table S8.

249 Table 1: Detected substances in the field gammarids, with substance information, LOQs and relative
250 recoveries, concentrations ranges, number of detections and calculated BAFs from the lab
251 experiment. Bold: substance was quantified with structurally identical isotope labelled standard. B:
252 Biocide, Corr: Corrosion inhibitor, PCP: Personal Care Product, Ph: Pharmaceutical, PPP: Plant
253 Protection Product, n: neutral, a: anionic, c: cationic, z: zwitterionic, Up: upstream, Down: downstream.

Substance	CAS No.	Group	Speci- ation pH7.9	logK _{ow}	logD _{ow} pH7.9 ^f	BAF [L/kg] ^g	Rel. rec. [%]	Concentrations [ng/g w.w.]				# Detections		
								LO Q	Max- imum	95%- perc.	Med- ian	Up	Down	Tota l
4-Acetamido- antipyrin	83-15-8	Ph	n	0.13 ^b	0.1		182	0.3	1.2	1.1	0.5	1	13	14
5-Methyl- Benzotriazole (5- MZB)	136-85-6	Corr	n	1.52 ^b	1.5	3.9 ± 0.04	103	0.9	4.2	3.8	1.3	3	15	18
Acetamiprid	160430- 64-8	B	n	0.8 ^a	0.8		90	0.3	0.7	0.6	0.6	1	1	2
Alfuzosin	81403- 80-7	Ph	n	1.4 ^d	1.4		163	0.3	1.2	1.2	0.7	3	3	6
Aliskiren	173334- 57-1	Ph	c	3.3 ^d	1.6		109	1.2	6.2	5.4	1.8	1	9	10
Amisulpride (AMS)	71675- 85-9	Ph	n	1.06 ^d	1.1	2.8 ± 0.6	99	0.8	1.4	1.2	0.8	0	9	9
Atenolol (ATN)	29122- 68-7	Ph	c	0.16 ^d	-1.6	1 ± 0.2	100	0.3	<LOQ			0	1	1
Atrazine (ATZ)	1912-24- 9	PPP	n	2.7 ^a	2.7	3.2 ± 0.4	91	0.3	<LOQ			2	0	2
Azoxystrobin (AZS)	131860- 33-8	PPP	n	2.5 ^a	2.5	31 ± 1.8	95	0.1	0.7	0.6	0.3	2	4	6
Benzophenon 3 Benzotriazole (BZT)	131-57-7 95-14-7	PCP Corr	a n	3.38 ^c 1.44 ^c	2.5 1.4	2.9 ± 0.2	127 93	4 2.2	28 19	26 13	8.3 3.2	4 4	17 21	21 25
Bezafibrate	41859- 67-0	Ph	a	3.35 ^b	-0.7		98	4	7.6	7.4	3.9	4	4	8
Bupirimate	41483- 43-6	PPP	n	3.68 ^a	3.7		104	0.8	<LOQ			2	0	2
Carbamazepine (CMP)	298-46-4	Ph	n	2.45 ^d	2.4	4.1 ± 0.1	94	0.2	1.0	1.0	0.3	0	15	15
Carbendazim (CBD)	10605- 21-7	B	n	1.48 ^a	1.5	2.7 ± 0.1	99	0.4	<LOQ			2	0	2
Celiprolol	57470- 78-7	Ph	c	1.92 ^c	0.2		119	0.5	0.7	0.7	0.6	1	1	2
Chlortoluron (CTL)	15545- 48-9	PPP	n	2.5 ^a	2.5	5 ± 0.3	105	0.9	<LOQ			0	1	1
Citalopram (CTP)	59729- 33-8	Ph	c	3.5 ^d	1.6	16 ± 1.7	102	0.9	7.3	4.5	2.2	1	20	21
Clarithromycin (CRT)	81103- 11-9	Ph	c	3.16 ^d	2.6	1.2 ± 0.2	94	1.3	1.0	1.0	1.0	0	4	4
Climbazol (CBZ)	38083- 17-9	PCP	n	3.76 ^a	3.8	18 ± 0.6	102	0.4	0.7	0.6	0.4	1	6	7
Clothianidin (CTN)	210880- 92-5	PPP	n	0.905 ^a	0.9	0.7 ± 0.3	97	2.02	<LOQ			1	0	1
Crotamiton	483-63-6	Ph	n	2.9 ^d	2.9		75	0.1	0.2	0.2	0.2	0	2	2
Cycloxydim	101205- 02-1	PPP	n	1.36 ^a	1.4		126	1.2	<LOQ			0	1	1
Cyprodinil (CPD)	121552- 61-2	PPP	n	4 ^a	4	120 ± 11	120	0.6	0.8	0.8	0.8	1	2	3
Diazinon	333-41-5	PPP	n	3.69 ^a	3.7	15 ^e	97	0.6	0.8	0.8	0.8	0	3	3
Diclofenac (DCF)	15307- 86-5	Ph	a	4.51 ^d	0.6	16 ± 2.2	94	0.7	5.4	5.0	1.1	1	13	14
Diffubenzuron	35367- 38-5	PPP	n	3.89 ^a	3.9		82	3.2	8.9	8.9	8.9	0	1	1
Dimefuron	34205- 21-5	PPP	n	2.51 ^a	2.5		67	0.6	<LOQ			6	5	11
Diphenhydramine	58-73-1	Ph	c	3.27 ^c	2.3		100	0.6	9.0	7.2	1.9	5	5	10
Diuron (DRN)	330-54-1	B	n	2.87 ^a	2.9	10 ± 0.6	103	0.6	1.4	1.4	1.3	0	2	2
Efavirenz	154598- 52-4	Ph	n	4.6 ^d	4.6		98	2.2	2.6	2.6	2.6	0	4	4
Etodolac	41340- 25-4	Ph	a	2.5 ^d	-0.7		125	0.6	1.2	1.2	1.2	0	1	1
Fexofenadine (FXN)	83799- 24-0	Ph	z	5.6 ^d	2.9	0.8 ± 0.2	97	0.4	1.1	1.1	1.1	0	1	1
Flecainid	54143- 55-4	Ph	c	3.78 ^c	2.1		100	0.3	0.3	0.3	0.3	0	1	1
Flufenamic acid	530-78-9	Ph	a	5.25 ^{c,d}	1.2		43	0.9	8.9	8.5	5.1	0	2	2
Flusilazole	85509- 19-9	PPP	n	3.87 ^a	3.9		83	0.7	2.6	2.5	2.0	1	1	2
Hydrochlorothiazid e (HCT)	58-93-5	Ph	n	-0.07 ^d	-0.1	0.7 ± 0.2	97	0.2	1.8	1.6	0.6	1	10	11

Substance	CAS No.	Group	Speci- ation pH7.9	logK _{ow}	logD _{ow} pH7.9 ^f	BAF [L/kg] ^g	Rel. rec. [%]	Concentrations [ng/g w.w.]				# Detections		
								LO Q	Max- imum	95%- perc.	Med- ian	Up	Down	Tota l
Imidacloprid	138261-41-3	PPP	n	0.57 ^a	0.6	7 ^e	100	0.9	3.3	3.2	1.5	2	7	9
Irbesartan (IRS)	138402-11-6	Ph	a	6 ^d	3.9	0.4 ± 0.1	95	0.5	1.7	1.6	1.2	0	4	4
Irgarol	28159-98-0	B	n	3.95 ^a	3.9		105	0.6	0.6	0.6	0.6	0	1	1
Irgarol- descyclopropyl	30125-65-6	B	n	2.14 ^b	2.1		76	0.9	<LOQ			0	1	1
Lamotrigine (LMT)	84057-84-1	Ph	n	2.5 ^d	2.5	3.5 ± 0.4	97	0.4	3.5	3.2	0.5	0	5	5
Lidocaine	137-58-6	Ph	n	2.44 ^{c,d}	2.4		99	0.2	4.0	3.8	0.6	4	4	8
Metaxalone	1665-48-1	Ph	n	2.3 ^d	2.3		61	2.1	<LOQ			2	8	10
Metoprolol (MTP)	37350-58-6	Ph	c	1.88 ^d	0.1	1.3 ± 0.3	97	0.3	1.2	1.1	1.0	0	4	4
Myclobutanil	88671-89-0	PPP	n	2.89 ^a	2.9		95	3.9	14	11	2.4	4	4	8
Napropamide	15299-99-7	PPP	n	3.3 ^a	3.3		117	0.1	1.7	1.6	0.9	1	2	3
N-Desvenlafaxine	149289-30-5	Ph	c	2.82 ^b	0.9		103	3.8	<LOQ			0	2	2
NN- Dimethyldecylamin N-oxide	2605-79-0	B	n	3.13 ^b	3.1		132	0.3	5.5	5.5	5.5	0	2	2
Orbencarb	34622-58-7	PPP	n	4.17 ^a	4.2		97	0.6	4.2	4.2	4.2	0	1	1
Oxazepam (OXZ)	604-75-1	Ph	n	2.24 ^d	2.2	10 ± 0.4	100	1.8	4.6	4.6	4.6	0	8	8
Penconazole (PNZ)	66246-88-6	PPP	n	3.72 ^a	3.7	66 ± 4.8	85	0.8	1.3	1.3	1.1	0	2	2
Propamocarb (PMC)	24579-73-5	PPP	c	0.84 ^a	-0.6	0.9 ± 0.1	118	1	<LOQ			2	2	4
Propanolol	525-66-6	Ph	c	3.48 ^d	1.7		122	1.3	1.4	1.4	1.4	0	3	3
Rivastigmin	123441-03-2	Ph	c	2.3 ^d	1.3		115	0.1	0.2	0.2	0.2	0	2	2
Sitagliptin	486460-32-6	Ph	c	1.5 ^d	0.6		114	2.2	2.3	2.3	2.3	0	1	1
Tebuconazole (TBZ)	107534-96-3	PPP	n	3.7 ^a	3.7	32 ± 2.2	102	0.6	7.5	7.4	6.5	1	2	3
Telmisartan	144701-48-4	Ph	a	7.7 ^d	3.4		145	2.2	<LOQ			0	4	4
Terbutryn (TBR)	886-50-0	B	n	3.66 ^a	3.7	45 ± 5.2	98	0.3	<LOQ			0	2	2
Thiacloprid (TCP)	111988-49-9	PPP	n	1.3 ^a	1.3	8.2 ± 0.3	100	1.2	21	18	4.9	9	11	20
Valsartan	137862-53-4	Ph	a	5.8 ^d	2.3		104	2.9	4.0	3.9	3.1	0	4	4
Venlafaxine (VLX)	93413-69-5	Ph	c	2.38 ^b	1.3	3.2 ± 0.1	104	0.8	3.1	3.0	0.9	0	6	6
Venlafaxine-O- desmethyl	93413-62-8	Ph	c	2.16 ^b	1.1		116	0.1	1.9	1.7	0.3	0	10	10

^aFootprint (exp)²⁹, ^bComptox EPA (est)³⁰, ^cComptox EPA (exp)³⁰, ^ddrugbank (exp)³¹, ^eNyman, et al. ¹⁸, ^fLogD_{ow} was calculated according to Eq.S1, ^gapparent BAFs from the laboratory experiment with the defined spiked substance mixture ± standard deviation (3 replicates)

254

255 As expected, the observed concentration patterns showed higher concentrations and often larger
256 number of detected substances downstream than upstream (Figure 1). This finding is also consistent
257 with the measured water concentrations at the same sites and time points.²⁵ The large differences in
258 the downstream concentrations at the site Val-de-Ruz with significantly higher concentrations in
259 September than January were also reflected in the water concentrations at these time points. This
260 might be an indication for a very dry period in September with less dilution and thus higher fraction of
261 treated wastewater downstream. Overall, the internal concentrations were also in a similar range
262 compared to values reported in other studies investigating field gammarids.^{20, 22-24, 38} For some
263 substances, a similar or higher number of detections was found upstream compared to downstream,
264 indicating a diffuse input upstream of the WWTPs. While these detections were mainly observed for

pesticides, also few pharmaceuticals were detected upstream, some of which are also known being used for veterinary purposes (e.g. lidocaine). Additional releases through combined sewer overflows or man-made hydraulic shortcuts (such as road storm drains or manholes of drainage systems) during rain events occurring shortly before the sampling days, pipe leakages or wrong sewer connections could lead to detections of pharmaceuticals and household chemicals upstream of the WWTPs.³⁹⁻⁴⁰ On average 14 and 4 substances were detected at downstream and upstream sites, respectively, with a maximal number of 37 substances detected at the site Val-de-Ruz downstream. For this site, an additional wastewater input upstream had been identified²⁵, but no substantial contribution to the total upstream detections was observed compared to the other sites. The sum internal concentrations ranged from <LOQ to 16 ng/g w.w. at upstream sites and <LOQ to 86 ng/g w.w. at downstream sites, with generally lower concentrations and fewer detections in January 2015 compared to September 2014. Substances with highest detection frequencies (>20 detections), were the neonicotinoid thiacloprid, the antidepressant citalopram, the UV-filter benzophenon and the corrosion inhibitor benzotriazole.

For Herisau, we analyzed samples before and after the upgrade of the WWTP with powdered activated carbon. The internal concentrations and the number of detected substances clearly decreased at the downstream site after the upgrade whereas the upstream site remained similar (Figure 1). This is consistent with the lower water concentrations after the upgrade (Table S9) and nicely demonstrates the enhanced elimination of organic micropollutants through WWTP upgrade and the resulting improved environmental quality.⁴¹

Figure 1. Total internal concentrations (ng/g w.w.) of detected substances in gammarids collected in the field. Concentrations are summed up by substance group. In brackets: number of quantified substances / number of detected substances. For Herisau the time point before and after the upgrade of the WWTP with the advanced treatment step based on sorption to powdered activated carbon is indicated.

High detection frequency of neonicotinoids

The neonicotinoid thiacloprid was the most detected plant protection product (PPP) and showed also the highest maximum concentration of 21 ng/g w.w. for PPPs. In addition, imidacloprid was also present in the gammarid samples with frequent detections at nine sites. Acetamiprid was detected twice, clothianidin once with concentrations below the LOQ, and thiamethoxam was not detected in any sample (Table 1; the other two neonicotinoids, dinotefuran and nitenpyram, were not analyzed as they are not registered as PPP in Switzerland). Thiacloprid was already detected in field gammarids collected in the Danube²³ as one of 17 detected organic substances of total 74 analyzed substances.

In a smaller stream in Germany, the Holtemme River, imidacloprid and thiacloprid were the only insecticides detected in gammarids collected in autumn.²² Just recently, frequent detections of thiacloprid and imidacloprid were observed in anthropogenically impacted streams of the River Bode catchment in Germany.⁴²

This high detection frequency of neonicotinoids was unexpected, considering that these substances are very polar ($\log D_{ow}$ 0.6 - 1.3) and only few detections were found in the water samples at the same sites with maximum concentration of 24 ng/L for thiacloprid.²⁵ Further, a recent report on the uptake of pesticides applying the toxicokinetic-toxicodynamic model developed by Jager et al. (2011)⁴³ predicts no delayed release of imidacloprid indicating a fast elimination of this substance in gammarids.⁴⁴

While the high frequency of neonicotinoid detections in this study during autumn and winter is concerning, even higher concentrations in summer and spring during the main pesticide application period have to be expected. In fact, a temporally highly resolved screening of pesticides during spring and summer in five small streams in Switzerland has shown water concentrations of individual neonicotinoids in half-day composite samples of up to 1000 ng/L.⁴⁵ Recent studies further found seasonal differences in sensitivities of invertebrates to insecticides, with generally increased sensitivities in spring and summer.⁴⁶⁻⁴⁷ Gammarids have been shown to be one to two orders of magnitude more sensitive than *D. magna* (similar sensitivity is expected for other organic micropollutants), and crustaceans showed delayed effects after exposure to neonicotinoids, with these substances significantly contributing to the overall toxic effects.⁴⁸⁻⁵⁰

Interestingly, a recent study on the accumulation of neonicotinoids in gammarids suggests a high relevance of dietary exposure as accumulation pathway.⁵¹ They showed that higher effects are expected when gammarids are exposed simultaneously to the contaminated water phase and contaminated leaves compared to water exposure only. This supports the rather unexpected high detection frequency of thiacloprid and imidacloprid in field gammarids in our study. The relevance of dietary exposure, however, should be investigated further, as earlier studies with gammarids suggested negligible uptake through the diet (<1%) for 15 organic micropollutants ($\log K_{ow}$ from 0.33 to 5.18), including imidacloprid.²⁶

Experimental BAFs and comparison with predictions

To support our findings from the field study, we conducted laboratory exposure experiments and flume experiments to determine the bioaccumulation potential of selected substances under controlled and semi-realistic conditions. The BAFs were calculated based on internal concentrations after 48 h and average exposure concentrations over the whole exposure period (see Tables S10-S14, Figure S2 and Section S6 for details on results and performance of the lab and flume experiments). For 42 substances with $\log D_{ow}$ from -1.6 to 3.8 spiked in the lab experiment, BAFs could be determined and ranged from 0.4 to 120 L/kg w.w. (Table S10), and are well below the REACH criteria for bioaccumulative compounds with a defined BAF of > 2000 L/kg.⁵² BAFs of ten substances could be compared and were consistent with the BAFs in gammarids reported in other studies (Table S15).¹⁵⁻¹⁹ To our knowledge, the BAFs of the 32 remaining substances were determined for the first time.

For most substances, the BAFs of the wastewater-exposed gammarids in the lab experiments and in the flume experiments were in good agreement with the BAFs from the laboratory exposure with the defined spiked substance mixture (in most cases within a factor of 5, Figure S3). Exceptions were single substances such as citalopram, clarithromycin and climbazole (showing ~10-fold higher BAFs) potentially caused by measurement uncertainties due to the internal concentrations close to the LOQ. Overall, this generally supports the theory that bioaccumulation is a concentration and matrix independent process.⁵³⁻⁵⁴ In contrast, recent field studies report an inverse relationship between exposure concentration and BAF in fish.⁵⁵⁻⁵⁶ However, this was based on grab samples only, which represent just snapshots of the real exposure conditions and lack the integration of the uptake over time. Similarly, if we calculated field BAFs for our study using the corresponding grab samples, which were taken concurrently to the collection of the gammarids, we got significantly higher BAFs compared to the lab experiments (Mann-Whitney-U test $p < 0.001$, Figure S5).

In general, extrapolation from lab to field conditions regarding the bioaccumulation potential is not trivial as food sources, other chemical parameters and physiological state of the organism differ. This was thoroughly discussed among experts for hydrophobic substances⁵⁷ and Muir et al. (2017)⁵⁶ concluded that the water composition/chemistry (e.g. pH, DOC, temperature) affects the uptake in fish more than the physiological impact due to higher exposure concentrations. This finding potentially applies also to this study, as the wastewater effluent was not filtered before exposure to gammarids,

leaving the possibility of the substances, especially with higher logD_{ow} values, to be present in a particle-bound form, compared to the exposure with the defined spiked substances mixture in APW.

When correlating our apparent BAFs from the exposure experiment to the hydrophobicity represented as logK_{ow}, we saw a significant but only weak relationship for the neutral substances ($R^2 = 0.54$, $p < 0.001$). Considering all substances, i.e. including the ionic substances, the correlation becomes as expected weaker indicating a different partitioning behavior of the ionic substances (Figure 2a). For example, only irbesartan of the three investigated anionic substances of the sartan class (candesartan, irbesartan, valsartan) was detected in gammarids. With a logK_{ow} >5, sartans are expected to highly accumulate in biological tissues based on hydrophobicity only. However, at pH 7.9 (= exposure conditions) they are negatively charged suggesting a slower uptake through the membranes.⁵³ Jeon et al. (2013)¹⁵ determined a BAF of 2.2 for valsartan at higher exposure concentrations (100 µg/L) and reported minor biotransformation, which is in agreement with our findings. Accounting for the speciation of the ionic substances using the pH corrected partition coefficient logD_{ow}, the correlation slightly improved for all substances, but it remained still weaker than when considering neutral substances only (Figure S4). Poor correlations between logD_{ow} and the bioaccumulation potential for polar substances, especially for ionized molecules, have already been observed in other studies.^{17, 58}

Bioaccumulation cannot always be assessed experimentally for all the substances of interest, and thus values are often retrieved from prediction tools that are mainly based on hydrophobicity. To our knowledge, there is no software tool for the prediction of the bioaccumulation potential of invertebrates. We therefore estimated the BAFs for lower trophic level fish in EPISuite³² (which considers also mechanistic processes) and compared them to our apparent BAFs from the exposure experiment with the defined spiked substances to see how well the predictions apply to gammarids. The model in EPISuite is not recommended for chemicals that ionize, but surprisingly the predictions for almost all substances were within a factor of 10 of the apparent BAFs, and for more than 40% of the substances (18 substances) the values were even within a factor of 2 (Figure 2b). There is no clear trend of any systematic over- or underestimation of the model. Consequently, this suggests that for many substances, this model can be a good first approximation on the range of the bioaccumulation potential in gammarids. Furthermore, the correction with the lipid fraction clearly improved the match of the BAFs, although other tissues, e.g. proteins⁵⁹⁻⁶⁰ or the exoskeleton of

invertebrates^{17, 35, 53}, may act as significant storage site for polar compounds. Miller et al (2016)¹⁷ observed accumulation of pharmaceuticals in the exoskeleton of up to 24%. This observation can of course bias the actual level of internal concentration as well as the potential of bioaccumulation resulting in effects.

Figure 2. a) $\log K_{ow}$ versus apparent BAFs [L/kg w.w.] of the exposure experiment with the defined spiked substance mixture. Correlation (black solid line, R-squared and p-value) was done considering neutral substances at pH 7.9, dashed lines indicate 95% confidence interval. The grey line (and R-squared) indicates correlation considering all substances, for comparison, b) comparison of the lipid content corrected experimental BAFs [L/kg l.w.] with the lipid content corrected BAFs [L/kg l.w.] predicted with EPISuite for the lower trophic level fish (l.w. = lipid weight). Correlation (black solid line; only neutral substances at pH 7.9 considered, as the EPISuite model is not recommended for ionic substances), 1:1-line (grey solid line), 1:2 and 1:10-lines (grey dashed lines). Error bars correspond to the standard deviation (n=3). See Table 1 for abbreviations of the substance names (substances not included in Table 1: BOS = boscalid, CPZ = cyproconazole, DXM = dexamethason, EPC = epoxyconazole, FXC = fenoxycarb, IPC = iprovalicarb, ISP = isoproturon, MTF = methoxyfenozid, MTB = metribuzin, PPZ = propiconazole, SMZ = simazine, TMX = thiamethoxam, VDG = vildagliptin).

Potential toxic pressure based on internal concentrations

In order to determine the toxic pressure based on the internal concentrations measured in field gammarids, the EC50 values in literature, which are based on water concentrations, were transformed to internal EC50 values using the BAFs from the lab experiment with the defined spiked substance mixture (n=42, see Eq.2). We consider the use of the lab BAFs as reasonable for the interpretation of the internal concentrations of the field gammarids, as the lab and flume experiments showed overall consistent results (see section above).

The calculated mixture risk, expressed as sumTU, was higher when based on the internal concentrations than when based on the measured water concentrations reported in Munz et al. (2017)²⁵ (Figure 3a). Interestingly, the frequently detected neonicotinoids, thiacloprid and imidacloprid, substantially influenced the total toxic pressure (Figure 3b). A similar relevance of neonicotinoids was observed by Inostroza et al. (2016)²² where instead of determined BAFs, predicted K_{ows} (corrected with an assumed lipid fraction of 1.34% for gammarids) were used to calculate freely dissolved concentrations from measured internal concentrations. Only the neonicotinoids exhibited a logTU higher than -3, which is a value above which chronic effects can be expected.⁶¹ In our study, the TUs calculated with the internal concentrations exceeded as well this threshold for thiacloprid, imidacloprid and diazinon, while the TUs calculated with the measured water concentrations were always below this threshold. These results support the previous findings that suggest the relevance of pesticides on driving the toxic pressure in wastewater-impacted streams during low flow conditions (based on the water concentrations).²⁵ It is important to note that other substances than pesticides should not be neglected. Figure 3b, where the substances are sorted in decreasing order based on their toxicity (geometric mean of EC50), shows that a few substances contribute to the total toxic pressure because of their presence in high concentrations, rather than their toxicity. These are mainly household chemicals, such as diclofenac, carbamazepine, 5-methylbenzotriazole and lamotrigine.

Finally, we compared the sumTU with macroinvertebrate abundance data from the field expressed as SPEAR index (invertebrate sensitivity to pesticides, see Munz et al. (2017)²⁵ for data on SPEAR), as a significant correlation was reported between the toxic pressure based on the water concentrations and the SPEAR index.²⁵ In contrast to the results observed for the water concentrations, no correlation was observed between the SPEAR and the sumTU based on internal concentrations (Figure S6).

However, as only few data points are available for the internal concentrations, no definite statement can be made.

Figure 3: a) Sum of toxic units (sumTU) calculated with the measured external water concentrations²⁵ and the internal concentrations using the average experimental BAF with defined spiked substance mixture. This is shown for all substances (“All”) and different substance groups (PPP: plant protection products, B: biocides, Ph: pharmaceuticals, PCP: personal care products, Corr: corrosion inhibitors). Numbers in plot indicate maximum values, b) logTU for single substances based on the measured water concentrations (above) and the internal concentrations (below). Note that the variances of the sumTU and single TU considering the standard deviation of the BAFs were minimal and are therefore not shown in the figures.

Environmental relevance

The analysis of internal concentrations in field organisms can bridge the uncertainty surrounding the bioaccumulation potential and link the exposure closer to the effect. The results show that polar to semi-polar compounds, with a broad range of logK_{ow}, are ubiquitously present in gammarids and have

the potential to induce toxic effects, especially with frequently detected neonicotinoids that are known to be highly toxic towards non-target aquatic invertebrates. The findings further support our previous study that pesticides are important contributors to toxic pressure in wastewater-impacted streams.²⁵ However, the lack of effect data was a limiting factor, especially with regard to household chemicals (e.g. pharmaceuticals). Further, for a proper assessment, effect data based on internal concentrations are needed. In that way, also the variability of the bioaccumulation processes in lab and field would not influence the assessment of the toxic pressure. Nevertheless, the fact that we collected the gammarids in autumn and winter is concerning, as higher and more detections of these substances are expected during the main application period of pesticides (spring/summer). In general, the identification of sources or use activities of pesticides entering the streams through the WWTPs needs to be addressed to be able to take appropriate measures to reduce these inputs. Fortunately, the upgrade of WWTPs by activated carbon treatment or ozonation has proven to substantially reduce the input of those pesticides as well as their bioaccumulation into organisms as shown for one WWTP. Up to 37 compounds constituting a total concentration of 90 ng/g could be detected in the gammarids at one site indicating a high body burden in wastewater-impacted streams. Still, additional compounds not amenable to LC-HRMS such as highly toxic pyrethroids as well as metabolites formed in the organisms for which no standards are available might add further to the toxic pressure. Together with other stressors such as temperature or pathogens which are also relevant for wastewater discharge this chemical stress might increase the impact on the organisms' health and abundance.⁶²

Finally, it is important to note that the detection of a substance in an organism does not directly imply the induction of an adverse effect.¹⁸ Especially for crustaceans such as gammarids, the sorption on the exoskeleton can bias the true internal concentration and thus the amount of substance available to cause an effect.^{17, 35, 53} Organ specific analysis might be a way to get an even closer link to the effects. This has been carried out in lab experiments for single compounds in aquatic invertebrates¹⁸, but application to field organisms and to a large set of substances at low concentrations might be challenging. Overall, we believe that whole body internal concentrations can support environmental risk assessment. For better interpretation, reliable environmental quality standards in biota, similar to that for mercury or highly hydrophobic organic compounds (e.g. PCB, PBDE),⁶³ or equivalent quality criteria (e.g. critical body burden)⁶⁴ are needed.

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Supporting Information

Details on the performance of analytical methods, lab experiments, flume experiments, internal concentrations in field gammarids. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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