Pesticides drive risk of micropollutants in wastewater-impacted streams during low flow conditions

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Abstract

Micropollutants enter surface waters through various pathways, of which wastewater treatment plants (WWTPs) are a major source. The large diversity of micropollutants and their many modes of toxic action pose a challenge for assessing environmental risks. In this study, we investigated the potential impact of WWTPs on receiving ecosystems by describing concentration patterns of micropollutants, predicting acute risks for aquatic organisms and validating these results with macroinvertebrate biomonitoring data. Grab samples were taken upstream, downstream and at the effluent of 24 Swiss WWTPs during low flow conditions across independent catchments with different land uses. Using liquid chromatography high resolution tandem mass spectrometry, a comprehensive target screening of almost 400 organic substances, focusing mainly on pesticides and pharmaceuticals, was conducted at two time points, and complemented with the analysis of a priority mixture of 57 substances over eight time points. Acute toxic pressure was predicted using the risk assessment approach of the multi-substance potentially affected fraction, first applying concentration addition for substances with the same toxic mode of action and subsequently response addition for the calculation of the risk of the total mixture. This toxic pressure was compared to macroinvertebrate sensitivity to pesticides upstream and downstream of the WWTPs (SPEAR index). The concentrations were, as expected, especially for pharmaceuticals and other household chemicals higher downstream than upstream, with the detection frequency of plant protection products upstream correlating with the fraction of arable land in the catchments. While the concentration sums downstream were clearly dominated by pharmaceuticals or other household chemicals, the acute toxic pressure was mainly driven by pesticides, often caused by the episodic occurrence of these compounds even during low flow conditions. In general, five single substances explained much of the total risk, with diclofenac, diazinon and clothianidin as the main drivers. Despite the low predicted acute risk of 0% to 2.1% for affected species, a significant positive correlation with macroinvertebrate sensitivity to pesticides was observed. However, more effect data for pharmaceuticals and a better quantification of episodic pesticide pollution events are needed for a more comprehensive risk assessment.

Keywords: pesticides, pharmaceuticals, wastewater treatment plants, mixture toxicity, multi-substance potentially affected fraction (msPAF), risk assessment

1 Introduction

Thousands of chemicals are in use daily for a wide range of purposes (Schwarzenbach *et al.*, 2006) and hence enter the environment from various sources, such as agriculture, households and industry. This leads to the ubiquitous detections of such organic micropollutants in the ng/L to µg/L concentration range in surface waters (Loos *et al.*, 2013; Schäfer *et al.*, 2011). Conventional wastewater treatment plants (WWTPs), mainly designed for the removal of nutrients, only eliminate micropollutants to a limited extent, thus representing a major input source of micropollutants in the environment (Eggen *et al.*, 2014). It has been shown that WWTP effluents contain a wide variety of substances, including not only pharmaceuticals, biocides and/or other typical household chemicals, but also many plant protection products (PPP) used in agriculture or for material protection in urban areas (Gerecke *et al.*, 2002; Müller *et al.*, 2002; Neumann *et al.*, 2002; Wittmer *et al.*, 2010). Furthermore, in most catchments in developed areas, urban and agricultural land use types co-occur (Couperus *et al.*, 2016; Englert *et al.*, 2015; Fairbairn *et al.*, 2016; Gómez *et al.*, 2012; Triebskorn *et al.*, 2013; Wittmer *et al.*, 2010). Therefore, for a holistic view of the risk it is important to include different substance types from various sources to link chemical exposure to observed effects in these aquatic ecosystems (Barber *et al.*, 2013; Fairbairn *et al.*, 2016; Triebskorn *et al.*, 2013). However, as it is often not possible to monitor hundreds of compounds regularly, focusing on priority mixtures whose compositions are representative for specific sources or sites is seen as a practical alternative (Altenburger *et al.*, 2015). Moreover, regarding sampling strategies, further compromises may have to be made. Most studies have either collected temporally highly resolved samples at one or few sites (e.g. Wittmer *et al.*, 2010) or included multiple sites but relied on single grab sampling given the logistical tradeoffs (Fairbairn et al., 2016; Gómez et al., 2012). Still, both sampling approaches are powerful tools for the understanding of pathway dynamics and apportioning sources and to help identify hotspots regarding risks towards the aquatic ecosystem.

The variety of organic micropollutants also poses challenges to properly quantify the ecotoxicological risk. Especially substances designed to be bioactive, such as pharmaceuticals or pesticides, are known to affect aquatic organisms and impact ecological systems at the trace-level concentrations detected in the environment (Malaj *et al.*, 2014; Stalter *et al.*, 2013). Different approaches for risk assessment have been used to estimate adverse effects on aquatic organisms. The European Water Framework Directive integrates environmental quality standards (EQSs) for priority substances as

protective thresholds to detect whether the environmental concentration poses a risk to any species (EC, 2011). However, as EQSs are not established for many substances, one of the most common risk assessment approaches is the use of toxic units (TUs). TUs are calculated using the ratio between the environmental concentration of the substance and an effect concentration (e.g. EC_{50}, NOEC). In contrast to EQSs where assessment factors are used, TUs reflect the toxicity to single species and cannot be regarded as a protective threshold, because the possibility of more sensitive speciesis not taken into account.

Moreover, aquatic communities, comprised of many different species, are exposed to mixtures of a wide range of compounds, which generally induce toxic effects greater than the effects of the single substances alone (Backhaus & Karlsson, 2014; Kortenkamp *et al.*, 2009). A valuable tool that includes multiple species in the risk assessment is the species sensitivity distribution (SSD), which integrates the effect of a given substance on several species. Based on SSDs of single substances and their observed environmental concentrations, potentially affected fractions (PAF) can be calculated.

Regarding mixture toxicity, the two following concepts have been established: concentration addition (CA; Loewe & Muischnek, 1926) and response addition (RA; Bliss, 1939), which are mainly used for similarly and dissimilarly acting substances, respectively. It has been shown that the concept of CA is a reasonable worst-case estimation for single species tests (Junghans *et al.*, 2006), but this assumption does not hold true for flat concentration-response curves. SSDs are often flatter than concentration-response curves, meaning this can be an issue when applying CA on SSD (Gregorio *et al.*, 2013).

In real environmental mixtures both similarly and dissimilarly acting substances are present, thus justifying the combination of the two approaches. De Zwart and Posthuma (2005) introduced a two-step, mixed-model approach where mixture toxicity for substances of the same toxic mode of action (TMoA) is evaluated using the CA model, and the total mixture toxicity is then evaluated by combining the toxicities of the TMoA groups by the RA model. In combination with the concept of SSD, the “multi-substance potentially affected fraction” (msPAF), which describes the risk for whole species assemblages, can be predicted (De Zwart & Posthuma, 2005). Changes in species abundances from field biomonitoring data repeatedly correlated with effects of mixtures predicted using the msPAF, highlighting the potential of this approach (Posthuma & De Zwart, 2006, 2012).

92 In this study, we investigated concentration patterns of a large number of micropollutants in 24
93 wastewater-impacted streams, as well as in the wastewater effluents, across independent catchments
94 with different land uses. Our hypothesis was that the potential risk to aquatic organisms of these
95 mixtures of micropollutants, using the risk assessment approach of the msPAF, correlates with
96 biomonitoring data collected from the same sites. Specifically, we wanted to determine whether the
97 risk for aquatic organisms in wastewater-impacted streams during low flow conditions is mainly driven
98 by the higher concentrations of pharmaceuticals and other typical wastewater contaminants or if
99 pesticides are a larger driver of risk. Furthermore, we aimed to assess how well priority mixtures
100 describe environmental risks compared to extensive screening when tested across 24 sites.
101
102 **2 Methods**
103 *2.1 Sites and sampling strategy*
104 In total, 24 WWTPs distributed over the Swiss Plateau, the Jura mountains and the Prealps were
105 selected, and were located in catchments with different land use patterns (Figure 1, Table S1.1). The
106 sites were selected such that there was no WWTP upstream, a minimum of 20% wastewater
107 downstream during low flow conditions, and no substantial changes in morphology or riparian
108 vegetation between up- and downstream locations (Stamm *et al.*, 2016). All WWTPs were operated as
109 activated sludge treatments, except for Colombier which uses a trickling filter system. We defined an
110 upstream and downstream location at each site, as described in detail in Burdon *et al.* (2016). Briefly,
111 the downstream location was defined where the effluent was completely mixed across the channel; the
112 upstream point was selected some 20 m upstream from the effluent discharge point. During the study
113 period, the site Val-de-Ruz was found to have an additional underground input from a WWTP located
114 upstream of the upstream site. This WWTP with ~110 connected inhabitants is comparatively small to
115 the ~11000 connected inhabitants of the WWTP Val-de-Ruz. However, an influence on the chemical
116 composition cannot be excluded and results from this site were interpreted with care.
117 Grab samples were taken over two consecutive years, including eight time points during low flow
118 conditions. The field study was divided into two parts. In the first part, a sampling campaign was
119 conducted in June 2013 at 12 sites and repeated in February 2014 at the same sites. Based on the
120 results of the first campaigns, the temporal resolution was increased for the second part for the
121 following 12 sites, where 6 bi-monthly campaigns were conducted from March 2014 to January 2015
122 (the exact sampling dates are reported in Table S1.2). For the analysis of organic micropollutants we

collected water samples of 1 L upstream, downstream and at the effluent of the WWTPs (except for June 2013, when no effluent was sampled). The samples were cooled and stored at -20°C until analysis. For the second part, additional samples for heavy metals were collected, which were directly acidified and filtered on-site.

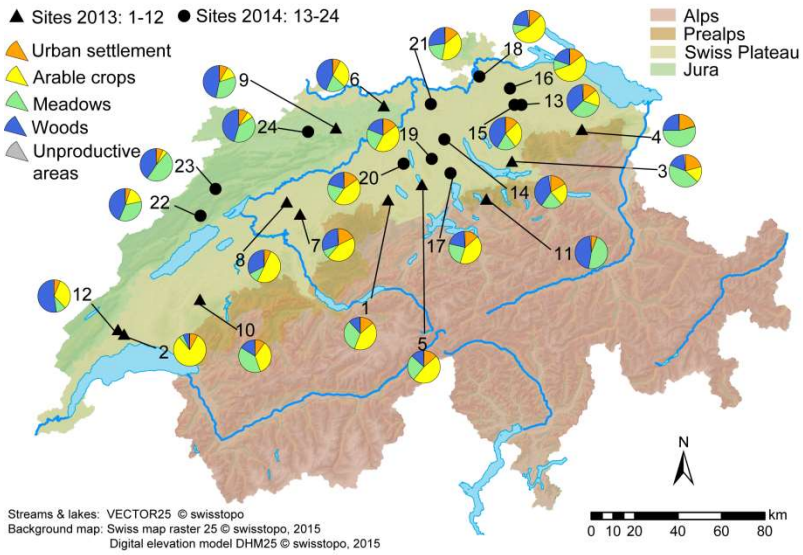


Figure 1: Location of the sampled wastewater treatment plants in Switzerland with the land use categories for each catchment shown in the pie charts (BFS, 2014). The aggregated land use categories and site names are listed in Table S1.1 of the supporting information.

2.2 Substances analyzed

For the upstream and downstream samples of June 2013 and May 2014 an extended screening of 389 organic micropollutants was conducted (Tables S1.2, S2.1). For all the other sites and time points a priority mixture of 57 substances was quantified. We prioritized the 57 substances to be relevant for Swiss streams using the following criteria: i) ubiquitous detections and effluent concentrations above 250 ng/L in municipal wastewater (Schymanski *et al.*, 2014), ii) inclusion of different classes of pesticides (herbicides, fungicides, insecticides) with frequent detections (Moschet *et al.*, 2014), iii) pesticides known to have high toxicity and/or a common mode of action (e.g. photosynthesis inhibitor herbicides), and iv) toxic pharmaceuticals with chronic EQS values below 1 µg/L (Ecotox Centre Eawag-EPFL, 2016). The compiled list of ~100 substances fulfilling at least one criteria was reduced by additional expert judgement (e.g. including different chemical structures) and analytical constraints (e.g. low recovery or high carryover during LC-MS analysis). The final priority mixture consisted of 28 pesticides (7 herbicides, 9 fungicides, 12 insecticides), 3 biocides, 22 pharmaceuticals/personal care

145 products as well as 2 corrosion inhibitors, the sweetener sucralose, and caffeine as a tracer for
146 releases of untreated wastewater. In this study, pesticides refer to PPP which are or were listed in the
147 Swiss Ordinance on Plant Protection Products (OPPP, 2010) and to biocides, which are substances
148 listed under the Ordinance on Biocidal Products (OBP, 2005). Substances approved as both PPP and
149 biocide were categorized regarding their main usage in Switzerland (e.g. isoproturon as plant
150 protection product and diuron as biocide) based on expert knowledge and former studies (e.g. Wittmer
151 *et al*., 2010, Moschet *et al*., 2014). . Dissolved heavy metal concentrations were determined for Ag,
152 Cd, Cr, Co, Cu, Fe, , Mn, Ni, Pb and Zn during the March 2014 to January 2015 sampling period.
153
154 *2.3 Sample preparation and chemical analysis*
155 For the analysis of organic micropollutants, the samples of the June 2013 campaign were enriched
156 with offline solid phase extraction (SPE) as described in Kern *et al.* (2009), whereas all the other
157 samples were enriched with an automated online SPE similar to Huntscha *et al.* (2012). A liquid
158 chromatography (LC) high resolution tandem mass spectrometry (HRMS/MS) setup was used for the
159 analysis. The heavy metals were analyzed using high resolution inductively coupled plasma mass
160 spectrometry (HR-ICP-MS). Further details are described in Section S2.1 of the supporting information
161 (SI). Tables S2.1 and S2.2 list the LOQ of all organic substances analyzed.
162
163 *2.4 Data analysis*
164 To compare the variation of the concentrations of the different substances at the sampling locations
165 upstream and effluent, a principal component analysis (PCA) was carried out with the R package
166 “prcomp” (R Development Core Team, 2008). To assure equal contribution of each variable to the total
167 variance the values were zero centered and scaled to have unit variance. Values below the LOQ were
168 considered as zero. On the upstream data a 1/8-power transformation was performed because of the
169 left-shifted distribution of the data set (many low concentration values). Only the 57 substances of the
170 priority mixture were included.
171
172 For a land use analysis two different types of catchments were considered: i) the WWTP catchment for
173 the correlation with effluent concentrations, and ii) the hydrological catchment for the correlation with
174 upstream concentrations. The boundaries of the WWTP catchments correspond approximately to the
175 boundaries of the communes discharging their wastewater into the respective WWTP (Herlyn &

176 Maurer, 2007). The hydrological catchments correspond to the catchments of the points of discharge
177 of the WWTP effluents into the streams, as defined in the Swiss data set GAB-EZGG-CH (BAFU,
178 2013). For the two catchment types, we calculated the different areal fractions of the land uses using
179 the Swiss land use statistics at the aggregation level of 27 different land use types (BFS, 2014). Areal
180 fractions had to account for at least 2.5% in the given catchments to be considered in the further
181 analysis. For comparison with our chemical data and to account for the high number of values below
182 LOQ, correlations between the average detection frequency of single substance groups (e.g.
183 pesticides) with the fraction of a specific land use type at the different sites (e.g. arable land) were
184 performed. Only substances of the priority mixture were considered. For the land use analysis ArcGIS
185 v10.3 was used.
186
187 *2.5 Risk assessment*
188 For the risk assessment we used the approach of the multi-substance potentially affected fraction
189 (msPAF), which is described in detail in De Zwart and Posthuma (2005). Toxicity data for more than
190 1000 substances were gathered in a database (De Zwart, 2002). Only log-transformed acute EC50
191 values were used if possible, because repeatedly and empirically it was found that the msPAF based
192 on acute median toxicity (EC50) comes the closest to the ecological effects that may be observed in
193 the field (e.g. De Zwart *et al.*, 2009; Posthuma & De Zwart, 2006; Posthuma & de Zwart, 2012). The
194 detailed procedure on the treatment of the toxicity data is described in Section S3.1 of the SI. All
195 substances were categorized based on their major TMoA, which was retrieved from literature and
196 based on expert judgment. If the TMoA was not known, the substances were kept as single
197 substances and not grouped with others. Finally, toxicity data for the calculation of the msPAF was
198 available for 165 and 46 of the 389 and 57 analyzed substances in the extended screening and priority
199 mixture data set, respectively. For isobaric substances analyzed as sums, only one of the two
200 substances was considered. If toxicity data was available only for one, this value was taken for the
201 substance pair. If toxicity data was available for both substances, the higher quality value was taken,
202 or if of equal quality the more toxic value was taken. The definition of high quality data is described in
203 Section S3.1 of the SI. Toxicity data used are listed in Table S3.1 in the SI. Values below LOQ were
204 treated as zero if not stated differently.
205

In a first step, the msPAF was calculated with the CA approach for all substances with the same TMoA using the cumulative density function of the normal distribution by Taylor-series approximation. This can be done using the Excel function *$msPAF_{CA}$ = NORM.DIST(log(∑c_i/$EC50_i$), mean=0, σ_i, cumulative = TRUE)*, with c_i as the environmental concentration of compound i of a specific TMoA group and σ_i as the standard deviation of the SSD.

In a second step, the groups of TMoA were integrated based on the RA approach given by

$$\mathrm{msPAF_{RA}} = 1 - \prod_1^n (1 - \mathrm{msPAF_{CA}}) \quad (1)$$

with 1 to n groups of different TMoA. In the following $msPAF_{RA}$ will be simply stated as msPAF.

To put our results into a regulatory context a comparison with risk quotients (RQs) based on acute MAC-EQSs (maximal allowable concentration) were calculated:

$$RQ_{mix} = \sum \frac{c_i}{EQS_i} \quad (2)$$

For this, published MAC-EQSs values from the centre for applied ecotoxicology in Switzerland (Ecotox Centre Eawag-EPFL, 2016) complemented with values from the EU Water Framework Directive (EU, 2013) were used, resulting in 60 and 36 MAC-EQSs for the screening and priority mixture data sets, respectively (Table S3.2).

2.6 Macroinvertebrates

Macroinvertebrates were collected in March/April 2013 (sites 1-12) and 2014 (sites 13-24) at the upstream and downstream locations following standard protocols for macroinvertebrate biomonitoring in Switzerland (Stucki, 2010). Eight kick-net samples per location were collected, pooled and stored in 80% ethanol prior to identification. Further details on the sampling procedure are described in Burdon *et al.* (2016). The data were used to calculate the trait-based Species At Risk (SPEAR) pesticide index (hereafter SPEAR index; Table S4.1), which is indicative of organic and especially pesticide contamination based on the presence or absence of taxa sensitive to pesticides (Liess & Von Der Ohe, 2005). For the SPEAR calculation, the SPEAR Calculator v0.9.0 was used (SPEAR Calculator, 2016).

The correlation between the msPAF and SPEAR index was tested using three linear mixed-effects (LME) models. Further details on the model structure are described in Section S4.1 of the SI.

3 Results and Discussion

3.1 Exposure

3.1.1 General exposure to organic micropollutants in wastewater-impacted streams

Of the 389 organic substances analyzed, 257 substances were measured at least once above the LOQ. All detected substances were found downstream, except for 3 pesticides and one biocide with only one detection upstream at concentrations close to the LOQ. In total 173 substances were detected upstream and 253 downstream. At all individual sites, more substances were detected at the downstream locations (60-167 substances) compared to the upstream locations (20-98 substances).

The mean detection frequency of the organic substances measured above the LOQ was around 30%, ranging from 0.7% (2 detections over 276 samples) for the two insecticides chlorpyrifos and methyl-chlorpyrifos, to 96% (264 detections over 276 samples) for the corrosion inhibitor benzotriazole. The concentration ranges of the detected substances are shown in Figure 2 for the different substance groups for the upstream and downstream locations. Substances with highest maximum concentrations measured in the streams were terbutylazine for PPPs (3000 ng/L, Jun ’13; median = 4.3 ng/L), iopromide and gabapentin for pharmaceuticals (both 2000 ng/L, Jan ’15, Jul & Sep’14, median= 0 ng/L), diuron for biocides (220 ng/L, Jul ’14, median = 4.4 ng/L) and benzotriazole for corrosion inhibitors (3500 ng/L, Sep ’14, median = 65 ng/L). 95% of the detected substances consisted to equal parts of pesticides and pharmaceuticals/narcotics. In agreement with several other studies (Fairbairn *et al.*, 2016; Triebskorn *et al.*, 2013), Figure 2 shows that the median values for PPPs remain in the same order of magnitude at the upstream and downstream locations. In contrast, the median of pharmaceuticals, and others even more, increases by almost one order of magnitude downstream of the WWTPs. The upstream concentrations of pharmaceuticals and household chemicals can be caused by releases through combined sewer overflows during rain events occurring before the sampling days, pipe leakages or wrong sewer connections (Joss *et al.*, 2008). Single concentration values for all organic substances are reported in the SI (Table S2.3).

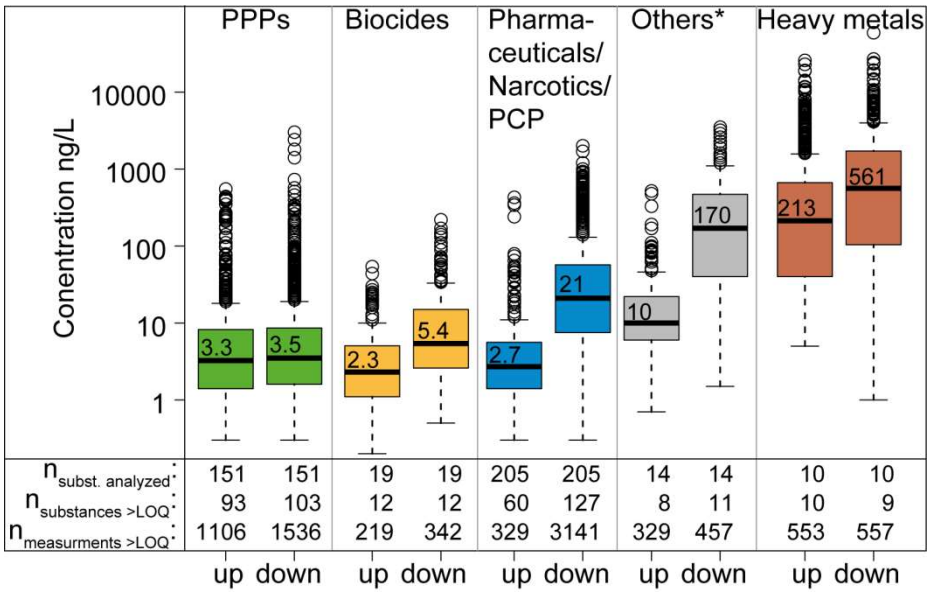


Figure 2: Concentration levels of detected substances upstream (up) and downstream (down) of WWTPs. Organic micropollutants were measured at sites 1 to 24 over 8 time points (2 time points with extended target screening). Heavy metals were only measured at sites 13 to 24 in the 6 bi-monthly samples from March 2014 to January 2015. LOQ = limit of quantification. *Others: corrosion inhibitors, food additives, caffeine, industrial chemicals. Numbers in boxplot indicate median value.

All 57 substances of the priority mixture were found at least once upstream and/or downstream and at least once in the effluent. Almost 50% (28 substances) were detected at all sites (upstream and/or downstream) and in all effluents indicating their proper selection as exposure indicators. The average sum concentrations of the priority mixture ranged between 0.01-1.1 µg/L, 0.2-17 µg/L and 2.2-33 µg/L over the upstream, downstream and effluent locations, respectively.

3.1.2 Influence of input pathways and land use on concentration pattern

The results of the PCA show that the substances divide clearly into two groups with orthogonal loading directions: pesticides (or substances with mainly diffuse input, e.g. agriculture) and pharmaceuticals (or mainly wastewater originating substances) (Figure S2.1). This points towards an independence of the input pathways and sources of these substance groups, which is in accordance with expectations. Interestingly, this pattern is seen upstream as well as in the effluent, two independent sampling locations, indicating similar behavior of the concentration dynamics of the different substance groups for the two sampling locations.

When considering the different substance groups in the context of land use (Figure 1, Figure S2.2), PPPs showed a significant positive correlation with arable land for the upstream values, but not for the

287 effluent (Figure S2.3). A slightly negative correlation was found with meadows and woods for the
288 upstream concentrations. For any other substance group no significant correlations were found for the
289 different land use types.
290
291 *3.1.3 Seasonality of micropollutant loads and concentration patterns*
292 Effluent loads were calculated using the average daily discharge of the WWTPs on the sampling
293 dates, which were provided by the cantonal authorities and/or WWTP operators. The pharmaceutical
294 loads in the effluent showed overall a decrease in summer (Figure 3), which could be due to higher
295 temperatures and consequently higher degradation in the WWTP (Matamoros *et al.*, 2016; Sun *et al.*,
296 2014; Vieno *et al.*, 2005). Additionally, a lower consumption rate of some pharmaceuticals such as
297 antibiotics in summer could have an influence on the lower loads measured (Castiglioni *et al.*, 2006;
298 Petrie *et al.*, 2015). For pesticides in general, no clear seasonality was observed (Figure S2.4).
299 However, single substances, such as for example terbutylazine, showed a seasonal concentration
300 pattern following the application period, indicating an input from agricultural land use also in the
301 WWTP (Figure 3a). Regarding the heavy metals, no seasonal pattern was observed in the effluent. In
302 addition, no considerable differences were observed between upstream, downstream and the effluent,
303 with concentrations levels generally in the same order of magnitude (Table S2.4). The detection
304 frequency for heavy metals was above 80%, except for Ag and Cd with 1% and 28%, respectively.
305

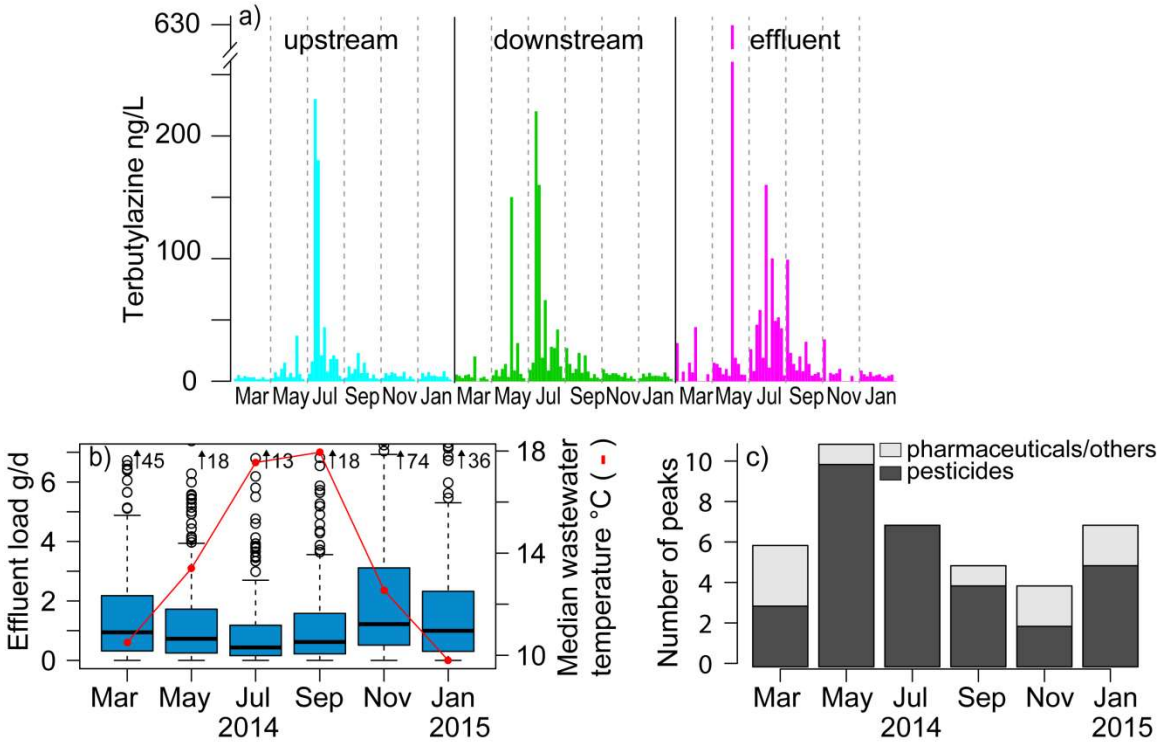


Figure 3: Seasonal patterns of a) terbutylazine concentration, plotted from site 13 to 24 for each month from March 2014 to January 2015, b) effluent loads of pharmaceuticals and personal care products (n=21; boxplots; numbers in plot indicate maxima) with the median wastewater temperature (red line), and c) total number of concentration peaks (10 times higher than the average concentration), summed over upstream and downstream locations, for substances with at least 20% detection frequency. For this evaluation only substances of the priority mixture at the sites 13-24 were considered. No data of the wastewater discharge and temperature was available for the WWTP Val-de-Ruz. Val-de-Ruz was excluded from the peak analysis, because of the wastewater input upstream.

3.1.4 Concentration peaks during low flow conditions

The highly dynamic pesticide concentrations, which are episodic and mainly occur during rain events, are only covered by chance when using grab samples. However, our sampling strategy targeted dry weather conditions, so that high concentration peaks can be attributed to unintentional spills rather than stormwater runoff. The occurrence of such elevated pesticide concentrations in effluent from WWTPs has been regularly reported in the literature (Gerecke *et al.*, 2002, Müller *et al.*, 2002, Wittmer *et al.*, 2010) and also confirmed by measurements of Swiss cantonal authorities (U. Ochsenbein, pers. communication). In fact, looking at the concentration patterns of single substances (e.g. Figure 3a, Figure S2.4), single concentration peaks are still detectable despite the relatively few grab samples

collected. To understand how often such high concentrations occur during low flow conditions and whether they may have an influence on the total risk, we defined concentrations 10 times higher than the average as peaks. However, peaks, as defined in our study, should not be regarded as equal to maximum concentrations, which can only be detected using highly resolved temporal concentration data. For the analysis, only substances with at least 20% detections were considered, and values below the LOQ were replaced with half the LOQ-values (Table S2.1).

In total, at over 80% of the sites (20 sites), 69 peaks of 40 substances were detected (including the extended screening data set). For 23 and 14 substances of the priority mixture, we observed 31 and 22 peaks downstream and upstream, respectively, with most of them detected in spring (Figure 3c). 70% of the 31 downstream peaks (22 peaks of 20 substances) showed higher concentrations downstream than upstream, which indicates an input from the WWTP (Table 1). With the extended target screening, three additional downstream peaks from the WWTP of single pesticides were observed. These findings are interesting especially regarding PPPs which constituted two-thirds of the peak substances, pointing towards a relevance of their release over the WWTP even during low flow conditions.

Due to the constraint of using only substances with 20% detection frequency, many insecticides were excluded because of their generally low concentrations close to the LOQ. However, dimethoate, pirimicarb and thiacloprid were all substances that showed high single inputs from the WWTP with peaks downstream of up to 100 ng/L while no or lower detections were observed upstream (Figure S2.4).

Table 1: Substances with peaks detected downstream (10-fold higher than the average downstream concentration) which had a detection frequency of at least 20%. Priority mixture analyzed over eight time points above dashed lines and the additional substances measured for June 2013 and May 2014 below dashed lines.

Substance	**Sub-stance group[a]**	**#peaks$_{down}$**	**#peaks$_{down}$ where $c_{down}>c_{up}$**	**Conc. peak$_{down}$ where $c_{down}>c_{up}$ (ng/L)**	**PAF of peak$_{down}$ where $c_{down}>c_{up}$[b] (%)**	**Total msPAF (%)**	**Site[c]**	**Date**
Atrazine	ppp (h)	1	1	99	**0.03**	0.05	17	Mar‘14
Azoxystrobin	ppp (f)	2	1	73	<0.0001	0.05	6	Jun‘13

Chlortoluron	ppp (h)	2	0					
Cyproconazole	ppp (f)	1	1	17	<0.0001	0.8	21	May‘14
Cyprodinil	ppp (f)	1	1	8.9	<0.0001	0.8	21	May‘14
Diazinon	ppp (i)	1	1	130	**0.890**	0.9	8	Jun‘13
Epoxyconazole	ppp (f)	1	0					
Isoproturon	ppp (h)	1	1	59	**0.0025**	0.3	19	Jul‘14
Metribuzin	ppp (h)	2	1	37	0.0001	0.2	13	Nov‘14
Penconazole	ppp (f)	1	1	730	<0.0001	0.2	4	Jun‘13
Propamocarb	ppp (f)	2	1	510	<0.0001	0.9	8	Jun‘13
Propiconazole	ppp (f)	3	0					
Simazine	ppp (h)	1	1	39	<0.0001	0.1	20	Sep‘14
Tebuconazole	ppp (f)	1	1	1800	0.0001	0.2	19	Mar‘14
Terbutylazine	ppp (h)	1	1	3000	**0.39**	0.5	1	Jun‘13
Bentazon	ppp (h)	1	0					
Dimethenamid	ppp (h)	1	0					
Linuron	ppp (h)	1	1	110	**0.012**	0.50	6	Jun‘13
Metamitron	ppp (h)	1	1	210	-	0.80	21	May‘14
Metamitron-Desamino	ppp (h)	1	0					
Metolachlor	ppp (h)	1	1	570	0.0005	1.60	19	May‘14
Prosulfocarb	ppp (h)	1	0					
All PPPs		*28*	*15*					
Carbendazim	b (f)	2	2	99; 89	0.0001;<0.0001	0.2;0.06	13	Sep’14,Jan‘15
Diuron	b (h)	1	1	220	**0.035**	0.1	21	Jul‘14
Terbutryn	b (h)	1	1	110	**0.13**	0.5	13	Mar‘14
All Biocides		*4*	*4*					
Amisulprid	ph	1	1	310	-	0.1	15	Nov‘14
Iopromid	ph	2	2	2000; 1700	-	0.02, 0.03	17; 20	Jan’15,Nov‘14
Torasemid	ph	1	1	160	-	0.2	19	Mar‘14
Vildagliptin	ph	1	1	120	-	0.2	19	Mar‘14
Sulfamethazine	ph	1	0					
All Pharmaceuticals		*6*	*5*					
Caffeine	t	1	1	490	<0.0001	0.002	24	Jan‘15
All Substances		***38***	***25***					

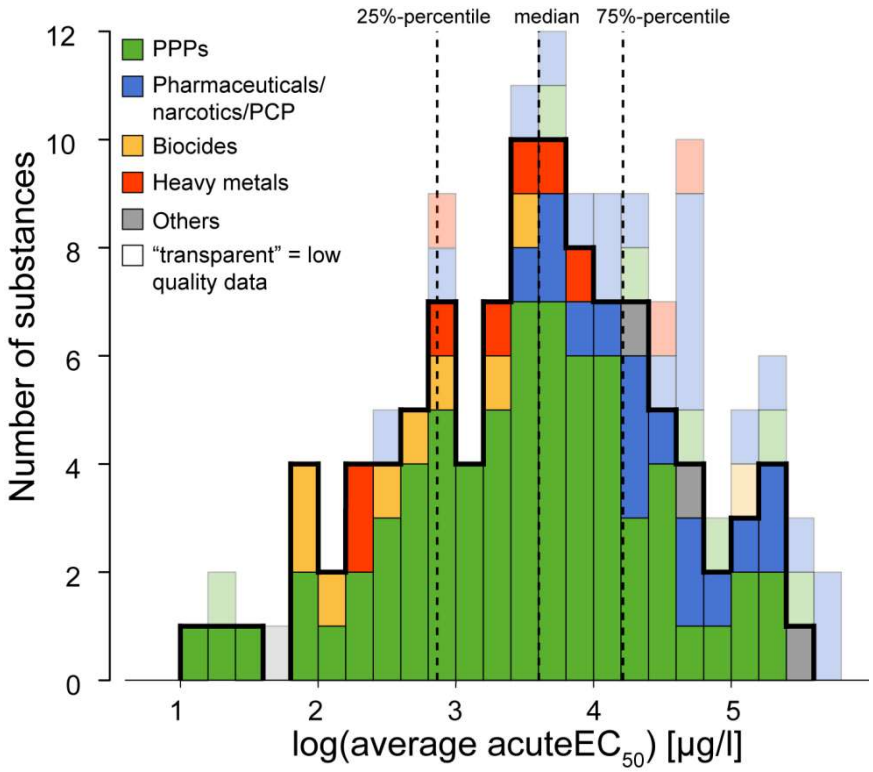
[a]ppp: plant protection product, ph: pharmaceutical, b: biocide, t: tracer; h: herbicide, f: fungicide, i: insecticide
[b]**bold**: peak is in Top5 of the msPAF at the specific site and date, - no toxicity data available
[c]Site code as in Table S1.1 of the SI

3.2 Toxic pressure on aquatic organisms

3.2.1 Toxicity data set

In total, 165 organic substances had available toxicity data for the construction of a SSD. The low-quality data (n=41) was excluded because of greater uncertainties, resulting in 124 substances used for the msPAF calculations. Of the 124 organic substances, 94 were measured at least once above

361 the LOQ. For all heavy metals toxicity data was available; three (Co, Fe, Mn) were categorized as low
362 quality data. Very little toxicity data was available for pharmaceuticals, and that which was available
363 was mostly of low quality, limiting the interpretation regarding their risk. Nevertheless, the toxicity
364 distribution of the available data shows that the majority of the pharmaceuticals are less toxic
365 compared to the pesticides (Figure 4). For the 94 detected substances 55 different TMoA were
366 assigned. Regarding the priority mixture, 36 of the 46 substances with available toxicity data were of
367 good quality, which were categorized into 23 different TMoA. The toxicity distribution of the priority
368 mixture covered a similar range as the whole data set (±0.2 µg/L of the percentiles, see Figure 4).



369
370 Figure 4: Distribution of toxicity data for substances measured at least once above the limit of
371 quantification (n=101, 94 organic substances + 7 heavy metals). Percentiles are calculated excluding
372 low quality data. PPPs: Plant Protection Products, PCP: Personal Care Products, Others: Corrosion
373 inhibitors, caffeine, sucralose.

374

375 *3.2.2 Acute toxic pressure: comparison of msPAF and EQS*

376 Over the 24 sites, the acute toxic pressure on aquatic organisms (msPAF) is relatively low, in the
377 range of 0% to 2.1% (Figure 5), and thus below the generally accepted threshold for acceptable risk of
378 5% of the species (Posthuma *et al.*, 2002; Smetanová *et al.*, 2014). A study investigating the risk of
379 ~300 organic substances in Dutch rivers and effluents also found very low toxic pressures, with a

380 maximum of 0.3%, which highlights that low acute toxic pressures in rivers may be a future trend
381 because of the better removal processes and regulating measures taken at the source (Hamers *et al.*,
382 2016). In contrast, the RQ_{mix} using MAC-EQSs was higher than 1 for 35% and 2% of the downstream
383 and upstream samples, respectively (Figure 5). As the RQ_{mix} has a precautionary purpose,
384 exceedances of 1 indicate that negative impacts on aquatic organisms cannot be excluded.
385
386 The highest toxic pressures were predicted at downstream sites, except for the maximal toxic pressure
387 which was found at Messen upstream in June 2013 using the extended target screening. The high
388 acute toxic pressure there (2.1% of affected species) is mainly due to the herbicide triflusulfuron-
389 methyl, which at 41 ng/L had already a great impact on the risk due to its high toxicity. In this particular
390 case, the msPAF would be reduced to 0.14% when considering only the priority mixture. All msPAF
391 values are reported in Tables S3.3 and S3.4 of the SI.
392
393 The discrepancy between the rather low toxic pressure predicted with the msPAF and the large
394 exceedances of the MAC-EQSs are expected because of the different purposes of the two
395 approaches (estimation of observable effects in the field vs. precautionary risk threshold).
396 Nevertheless, other factors can contribute to these differences. Due to the use of acute toxicity values
397 for the msPAF, which are more robust than chronic data, the sub-lethal (chronic) effects on aquatic
398 organisms may not be fully detected (Carafa *et al.*, 2011). It has been shown that the acute msPAF
399 may therefore underestimate the observations in the field (Smetanová *et al.*, 2014). This depends as
400 well on the sampling strategy, as the grab samples used may not reflect the actual levels of
401 contamination.
402

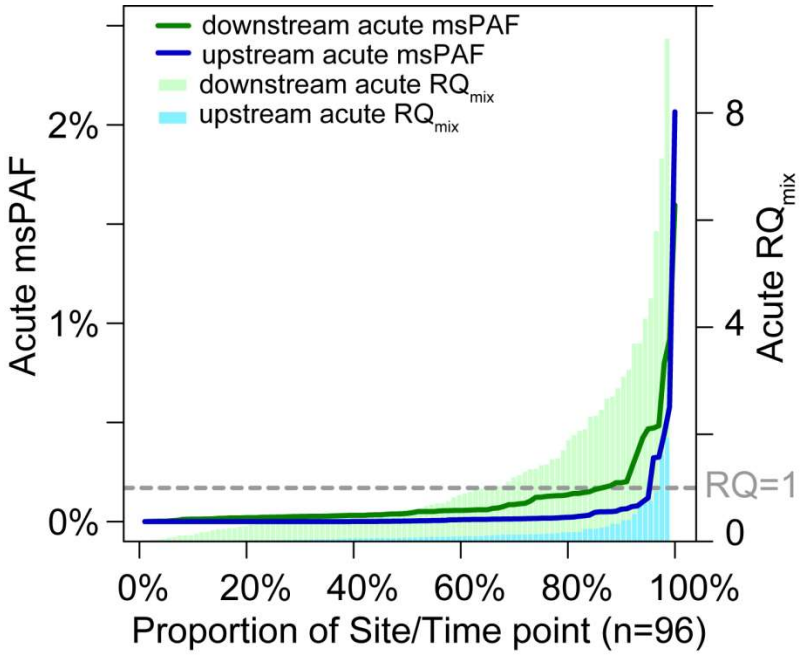


Figure 5: Acute msPAFs and RQs plotted against the proportion of site/time point (=sample).

3.2.3 Risk driving substances

In general, only a few single substances were the main drivers of the acute risk (Figure 6), which was also observed in other studies (e.g. Backhaus & Karlsson, 2014). In most cases the Top5 substances of the msPAFs for each site and time point explained already the total predicted risk. They were defined for each site and time point as the five substances with the highest PAF, under the condition that the PAF was greater or equal to 0.0001%. Three substances – diclofenac, diazinon and clothianidin – were clearly the most substantial drivers of the risk: 50% to 90% of the concentration values contributed to the Top5 of the total msPAFs over the different sites and time points. By removing these three substances from the data set, the acute toxic pressure decreased by more than 60% for almost 80% of all samples when only msPAF values greater than 0.001% were considered (Figure S3.1, S3.2). For substances with at least 20% overall detections, additional calculations were performed using half the LOQ-values for values measured below the LOQ. No large differences were observed in the msPAF results (Figure S3.1), underlining the importance of the influence of single substances.

Overall, it seems that the msPAF is more driven by substances with above average toxicity than by substances with above average concentrations. Half of the 26 Top5 substances are part of the 25% most toxic substances in the data set (Figure S3.1). This also highlights the importance of pesticides

424 and especially insecticides, which are generally much more toxic to aquatic organisms than
425 pharmaceuticals. Underscoring this point, 19 of the 26 Top5 substances are plant protection products.
426 The study on the ecotoxicological risk in Spanish rivers supports the importance of pesticides as major
427 contributors to the overall risk (Kuzmanović *et al.*, 2015). However, we used acute toxicity values and
428 it is known that several pharmaceuticals (e.g. diclofenac) have high acute to chronic ratios, thus
429 reducing the calculated risk of pharmaceuticals when only using acute values. Despite this, some
430 pharmaceuticals also appeared as relevant effect contributors, such as diclofenac, clarithromycin or
431 naproxen, although a definite statement of the real impact of pharmaceuticals is limited due to the
432 restricted availability of effect data. However, the analysis of the RQ_{mix} (based on MAC-EQSs) also
433 showed that the risk was mainly driven by a few single substances, with pesticides, such as for
434 example diazinon and diuron, having the greatest influence. Unfortunately, no MAC-EQSs are
435 available for diclofenac and clothianidin, so as to compare with the most influential drivers of the
436 msPAF (see EC, 2011).
437
438 In some cases high concentrations also substantially contributed to the total risk. Of the 69
439 concentration peaks detected overall, 52 were from substances with available toxicity data (27
440 substances) of which 25% (13 peaks) appeared in the Top5 of the msPAF. Seven of the downstream
441 peaks had higher concentrations downstream than upstream, pointing towards the WWTP as source
442 (Table 1). As more of these peaks in higher concentrations have to be expected than covered with our
443 sampling strategy, an increase of the predicted risk due to high concentrations is expected. This is
444 supported by considering also substances with less than 20% detection frequency, which were
445 excluded for the peak analysis: for example, the aforementioned elevated concentrations of the
446 insecticides dimethoate, pirimicarb and thiacloprid detected downstream appear all in the Top5 of the
447 msPAF.
448
449 For matter of completeness, the msPAF was also calculated including the analyzed heavy metals as
450 previous studies described their potential influence on the risk (Carafa *et al.*, 2011; Kuzmanović *et al.*,
451 2015). In our case, the heavy metals played only a minor role: Zn, at seven downstream and one
452 upstream site, and Ni at only one upstream site, were part of the Top5 substances and contributed
453 with fractions of 0.01-0.05% to the total msPAF (Figure S3.1).
454

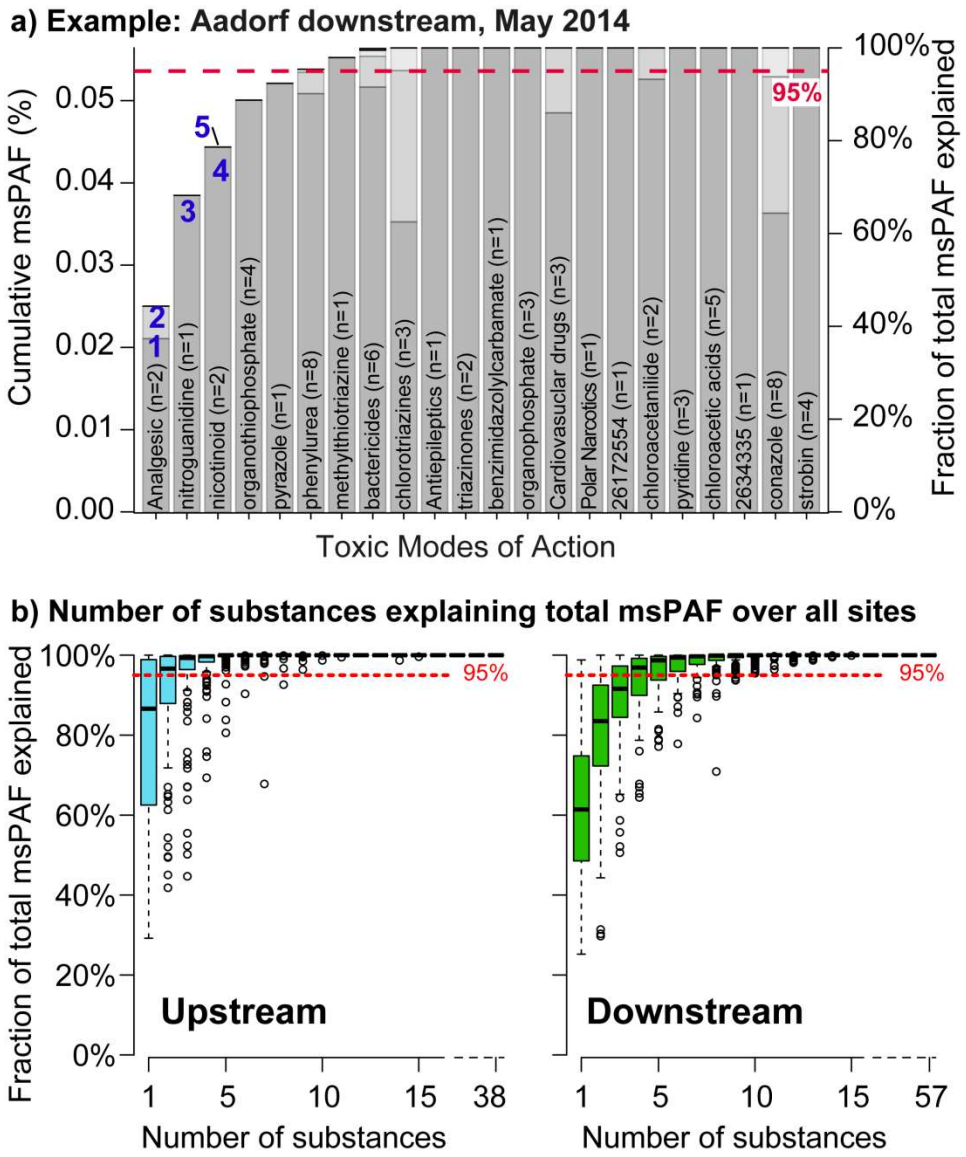
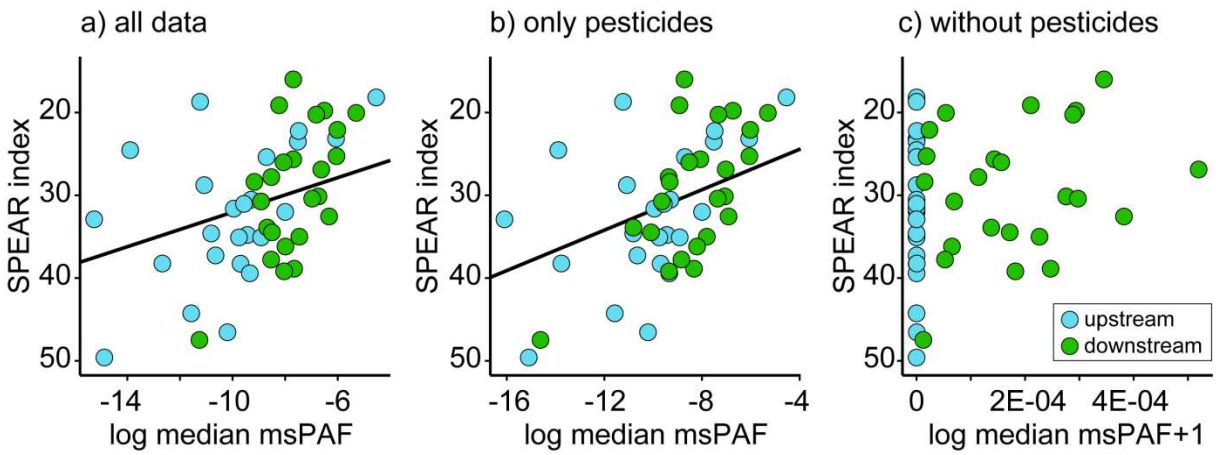


Figure 6: Exemplary cumulative distribution of a) the msPAF for Aadorf downstream in May 2014, and b) the cumulative fraction of the total msPAF explained by single substances at all sites for upstream and downstream. (a): the single bars correspond to the msPAF calculated using concentration addition for each TMoA (the stacked bars indicate the single substances within one TMoA). Iterative calculation of the total msPAF was achieved using response addition. Division of single substances within each TMoA was done by using the proportions of the hazard units (effect concentration over measured concentration). Number of substances of each TMoA in brackets. (b): The plot was constructed using the cumulative distributions of the single TMoA for each sample (see plot a)). Therefore, for each sample the $msPAF_{CA}$ of the single TMoA was calculated using concentration addition and ranked in decreasing order. Within each TMoA the substances were sorted in decreasing order by their hazard unit. The whole substance list was then ranked, from 1 to n substances contributing to the total

467 msPAF, as it is shown in (a) for the first 5 substances: diclofenac, naproxen, clothianidin, imidacloprid,
468 flonicamid (blue numbers).
469
470 *3.2.4 Extended screening vs. priority mixture*
471 The msPAF showed very similar results for the extended screening data set and the priority mixture,
472 where 94 and 36 detected substances were included, respectively (Figure S3.3). Moschet *et al.* (2014)
473 showed that a well-defined substance selection for pesticides predicts almost the same risk level as
474 would be predicted using the whole list of registered pesticides in areas dominated by agricultural land
475 uses. In our study, a total of 26 substances appeared in the Top5 of the msPAF of which only three
476 substances, triflusulfuron-methyl, naproxen, and irgarol, are not part of the priority mixture. Overall,
477 regarding the substances with the greatest influence on the msPAF, no major differences could be
478 detected using the screening or the priority mixture. These findings clearly highlight the substances
479 used in the priority mixture not only being good indicators for exposure but also for risk.
480
481 Including all the available toxicity data (with low quality data; see Section S3.1 of the SI), the msPAF
482 was substantially increased when the extended data set was used as opposed to the priority mixture
483 (Figure S3.4). However, two substances, furosemide and desethyl-atrazine, were responsible for this
484 difference. Furosemide shows a moderate toxicity but a relatively high standard deviation of the SSD.
485 Substances with high standard deviations can have a large impact on the risk even at low
486 concentrations, thus often leading to an overestimation of the risk (Gregorio *et al.*, 2013). Overall, this
487 highlights on one hand the selection of toxicity data as a critical factor, and on the other hand
488 demonstrates the potential influence of single substances in driving the total risk.
489
490 *3.2.5 Effects on macroinvertebrates*
491 The risk indicators described above were compared to macroinvertebrate data collected at the same
492 sites. Results from the 2013 sites showed that the macroinvertebrate community downstream seemed
493 to be influenced by discharged wastewater. A larger fraction of wastewater at the downstream
494 locations was correlated with a larger decrease from upstream to downstream in taxa sensitive to
495 pesticides as indicated by the SPEAR index (Burdon *et al.*, 2016). Because the SPEAR index is an
496 indicator of sensitivity to pesticides (Liess & Von Der Ohe, 2005), this correlation suggested the
497 possible widespread influence of these substances in treated wastewater (Burdon *et al.*, 2016).

Furthermore, at upstream sampling locations, the SPEAR index was strongly negatively correlated with the fraction of arable land in the catchment (Burdon *et al.*, 2016; Stamm *et al.*, 2016). Thus, quantifying risk factors through analysis of chemical data and linking with observed influences on stream macroinvertebrates is a logical step in determining the scale of this environmental problem.

Our risk assessment suggests an overall good agreement with the observed effects on stream macroinvertebrates, where pesticides seemed to be the main drivers of the risk at both upstream and downstream locations. This was shown by the significant correlation of the msPAF values with the SPEAR index across all 24 sites ($t_{23} = -3.759$, $p < 0.001$; Figure 7; Table S4.2). A similar correlation was observed when considering only pesticides for the msPAF calculation ($t_{23}=-3.778$, $p<0.001$), while no significant correlation was seen for the rest of the substances ($t_{22}=-0.349$, $p=0.730$). The significant relationship between pesticide risk and macroinvertebrate sensitivity to pesticides was observed despite household chemicals constituting the highest loads at downstream locations, suggesting that toxicity and not total concentrations is a key determinant of micropollutant impacts in mixed land use environments. Similar results were found for Spanish rivers, where an increase in toxic pressure was linked to observed losses of sensitive macroinvertebrate species (Kuzmanović *et al.*, 2015). Our findings and those of Kuzmanović *et al.* (2015) clearly highlight the validity of approaches linking chemical data and risk predictions in detecting realized toxic effects that are observed in the field. Whilst the use of both methods is optimal, the correlation between risk factors and pesticide sensitivity suggests either approach is useful for detecting pesticide effects in the environment.



521 Figure 7: Relationship between the SPEAR index and the msPAF a) for all data (t_{23} = -3.759,
522 p<0.001), b) only for pesticides (t_{23}=-3.778, p<0.001), and c) without (t_{22}=-0.349 , p=0.730), at all 24
523 sites. Relationships were tested using linear mixed-effects models where the fixed effects included
524 "msPAF" and "Location" (only c)), and the random effect was "Site". The axes of the SPEAR index
525 have been reversed to aid interpretability.
526
527 However, as the toxicity data for pharmaceuticals is very limited compared to the pesticide data, our
528 interpretation is restricted as pharmaceuticals from the WWTP might also have an impact on aquatic
529 organisms, especially fish (Ankley & Villeneuve, 2015; Brodin *et al.*, 2013). Behavioral changes, such
530 as feeding rate, mating success and parental care have been reported for fish and it has been shown
531 that for example the psychiatric drug oxazepam elicited effects towards perch but not towards
532 damselflies (Brodin *et al.*, 2014). This highlights on the one hand the need for more studies on effects
533 of pharmaceuticals and on the other hand the difficulty of the proper use and interpretation of the
534 effect data.
535
536 **4 Conclusions**
537 • We showed that a multitude of micropollutants are regularly released to the environment from
538 WWTPs during low flow conditions, with occasional concentration peaks resulting mainly from
539 pesticides. Analysis of the different substance groups showed a differentiation of input sources
540 and pathways (e.g. agriculture and households) for pesticides and pharmaceuticals, as well as
541 a correlation of arable land use with the concentrations upstream of plant protection products.
542 • While household chemicals constituted the highest loads and were regularly released from the
543 WWTPs, pesticides were discharged only during episodic events, meaning concentration
544 peaks are poorly represented when using only grab samples. Nevertheless, the predicted risk
545 seemed to be clearly dominated by pesticides (apart from diclofenac), although interpretation
546 regarding pharmaceuticals is limited due to a paucity of toxicity data.
547 • The risk was clearly driven by a few single substances, independent of the data set used
548 (extended screening or priority mixture). Thus, the priority mixture was found to be both a
549 good exposure and risk indicator for wastewater-impacted streams during low flow conditions.
550 • Despite the overall very low predicted acute risk (msPAF<5%), observed effects on
551 macroinvertebrates from field biomonitoring (SPEAR index) showed a significant positive

correlation. The findings clearly support the importance of pesticides in wastewater-impacted streams also during low flow conditions.

- The importance of pesticides regarding risk towards aquatic organisms also during low flow conditions is expected to be typical for catchments where urban and agricultural land use types co-occur as it is the case for many European countries.

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Appendix A. Supplementary information

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