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Tolerance patterns in stream biofilms link complex chemical pollution to ecological impacts

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ABSTRACT. Preventing and remedying fresh waters from chemical pollution is a fundamental societal and scientific challenge. With other non-chemical stressors potentially co-occurring, assessing the ecological consequences of reducing chemical loads in the environment is arduous. In this case study, we comparatively assessed community structure, functions and tolerance of stream biofilms to micropollutant mixtures, extracted from deployed passive samplers at wastewater treatment plant effluents. These biofilms were growing up- and downstream of one upgraded and two non-upgraded wastewater treatment plants, before being sampled for analyses. Our results showed a substantial decrease in micropollutant concentrations by 85%, as the result of upgrading the wastewater treatment plant at one of the sampling sites with activated carbon filtration. This decrease was positively correlated with a loss of community tolerance to micropollutants and the recovery of community structure downstream of the effluent. On the other hand, downstream biofilms at the non-upgraded

sites displayed higher tolerance to the extracts than upstream biofilms. The observed higher tolerance was positively linked to micropollutant levels both in stream water and in biofilm samples, and to shifts in community structure. Although more investigations of upgraded sites are needed, our findings point toward the suitability of using community tolerance for the retrospective assessment of the risks posed by micropollutants, to assess community recovery, and to relate effects to causes in complex environmental conditions.

INTRODUCTION

Establishing a causal relationship between complex contaminant exposures in the field and observed effects in impacted ecosystems is a major challenge for the environmental risk assessment of micropollutants.[1-3] Additional stressors such as high concentrations of nutrients and organic matter are often associated with the release of micropollutants into the receiving aquatic environments,[4-6] thereby limiting the power of *in-situ* field studies to diagnose ecotoxicological effects of micropollutants.[7] Such complex situations highlight the need for approaches that do not only allow to establish causalities when impacts of micropollutants are assessed in the field,[1] but also consider the chemical and biological complexity of natural ecosystems.[7] In response, a number of specific community-level and ecosystem-level metrics, such as SPEcies At Risk (SPEAR)[8] and pollution-induced community tolerance (PICT),[9-11] have been developed to provide insights into the relationship between in-situ exposure to chemicals and effects on communities.

PICT relies on the fact that differences in the tolerance of species within a community to micropollutant lead to shifts in the community structure under chronic exposures, which in turn depends on the initial community composition and the micropollutant mode of action. Consequently, a community that has been or is still affected by exposure to micropollutants will be less sensitive to those micropollutants than a reference community that has never been exposed. Community tolerance to micropollutants is quantified by assessing physiological endpoints in short-term bioassays, and subsequently comparing the responses of the reference community and the chronically pre-exposed ones.[11]

Field studies examining the tolerance of stream biofilms to micropollutant mixtures, and of microbial communities in general, remain rare,[11-13] despite the recognized importance of biofilms in aquatic ecosystem functioning.[14] Indeed, stream biofilms, a consortium of microorganisms that grow on submerged substrata surfaces, contribute to crucial processes in aquatic ecosystems. They play a key role in primary production, ecosystem respiration and element cycling, and thus, form the basis of aquatic food-webs. The high microbial diversity and varying sensitivities to environmental change, suggest that biofilms are a suitable model to assess contaminant effects on complex communities. In a recent study, Tlili et al.[15] assessed biofilm tolerance to micropollutant mixtures in wastewater-impacted streams. Results showed an increase of tolerance from upstream to downstream of the wastewater discharges, which was statistically correlated to the intensity of contamination at the respective sites. However, statistical correlations do not necessarily imply causation. The question therefore remains as to how the potential influence of other environmental factors, in addition to micropollutant mixtures, on microbial tolerance can be excluded experimentally.

In Switzerland, the modified water protection act entered into force in March 2014. As a result, many wastewater treatment plants (WWTP) are currently upgraded with additional treatment steps, such as powder-activated carbon filtration, to reduce the input of micropollutants into aquatic ecosystems. [3, 16] While these upgrades decrease micropollutant loads into the receiving streams, it has little impact on other wastewater constituents, such as organic matter and nutrient concentrations.[17-19] Therefore, this initiative offers the unique opportunity to study the specific ecological and ecotoxicological consequences of reducing micropollutant loads from WWTP effluents in the real world. The overarching goal of this case study was therefore to examine tolerance of *in-situ* biofilms from three wastewater-impacted streams to micropollutant mixtures, extracted from passive samplers that were deployed at the wastewater discharge sites (Figure 1). These sampling sites were previously investigated in a study in 2014 where the *in-situ* effects of micropollutant mixtures from the wastewater discharges on biofilms were examined.[15] Here we could show an increased tolerance to micropollutants in all downstream biofilms compared to upstream ones. In the meantime, the WWTP at one site (Herisau) was upgraded in May 2015 with powder-activated carbon filtration, while the

WWTPs at the two other sites, Buttisholz and Hochdorf, were not modernized. The present case study was therefore implemented to examine the efficiency of activated carbon in removing micropollutants from the wastewater effluents and the subsequent effects on downstream biofilm communities. We hypothesized that, as a consequence of the WWTP upgrade, the previously measured micropollutant-tolerance of downstream biofilms[15] would be substantially reduced, while the tolerance difference between upstream and downstream communities sampled at the non-upgraded sites would remain largely stable.

MATERIAL AND METHODS

Experimental design

This study was carried out from the 15th of March to the 30th of April 2016, upstream and downstream of three WWTPs located in north-eastern Switzerland, named Herisau, Buttisholz and Hochdorf (Figure 1, Table S1). The sampling sites were identical to those investigated in a previous survey, conducted from the 15th of March to the 30th of April 2014.[15] The WWTP at Herisau was upgraded with powder-activated carbon filtration in June 2015, whereas no modifications occurred at Buttisholz and Hochdorf. This situation offered the opportunity to comparatively assess the ecological consequences of the WWTP upgrade. At the sampling sites, general physicochemical parameters and 57 micropollutants were measured in grab water samples from up- and downstream of the wastewater effluents.

Biofilms were grown for six weeks, upstream and downstream of each WWTP effluent, on glass slides (380 cm^2 per slide) fixed vertically in perforated plastic boxes (three slides per box, three boxes per sampling location) and installed in the center of each stream. The biofilm growth locations were selected in a way to minimize the influence of other environmental factors than those related to wastewater effluents. Therefore, light intensity, flow velocity, as well as water turbidity were similar between the upstream and downstream at each site (SI *Appendix*, Table S2). In parallel, Chemcatcher® passive samplers – styrenedivinylbenzene-reverse phase sulfonate (SDB-RPS) discs – (47 mm diameter, Sigma Aldrich, Switzerland) were deployed at each discharge site (i.e. effluent) of

101 the WWTP to accumulate polar organic micropollutants. Two passive samplers were installed for two
102 weeks, recollected and replaced by two new ones over the six weeks of the survey, which led to six
103 passive samplers in total per sampling site.

104 After the colonization period, biofilms and passive samplers were transported to the laboratory.
105 Immediately after arrival, biofilms colonizing the glass slides from the same box were scraped and
106 suspended together in 250 mL of Evian mineral water. Accumulated micropollutants in the retrieved
107 SDB discs were extracted,[20] and the extracts from the six passive samplers from the same site were
108 pooled. Subsequently, the solvents used for the extraction (i.e. acetone and methanol) were completely
109 evaporated under a gentle nitrogen stream and the extracts were redissolved in 7 ml of Evian mineral
110 water.

111 Extracts from the passive samplers were used for chemical analyses and biofilm tolerance
112 measurements by exposing upstream and downstream biofilms to a dilution series of the extract from
113 the corresponding sampling site. In addition, biofilms were also exposed to a dilution series of extracts
114 that have been obtained from the same sampling sites during the study performed in 2014.[15] This
115 allowed for an additional comparison of the toxicity to the biofilms of two extracts obtained in
116 different years from the same site. After 12 hours of exposure, inhibition of the algal and bacterial
117 production in biofilms was measured and results were plotted as concentrations-response curves. A set
118 of functional endpoints as well as the community composition of the biofilms were examined. In
119 addition, the 57 micropollutants were measured in the biofilms.

120 *Physicochemical and micropollutant analyses*

121 Water temperature, pH, conductivity (WTW Meters, Germany) and stream water velocity
122 (FlowTracker Handheld ADV, YSI, Inc., USA) were measured at each sampling location. In parallel,
123 2-L water samples were collected to measure dissolved organic carbon (DOC), dissolved nitrogen
124 (DN), orthophosphates, and silica, using standard methods as described for the Swiss National River
125 Monitoring and Survey Programme (NADUF;
126 www.bafu.admin.ch/wasser/13462/14737/15108/15109).

Fifty-seven micropollutants (Table S4 for the detailed list) were quantified in grab water samples and biofilms from upstream and downstream of each site, and in the passive sampler extracts, using liquid chromatography coupled to high-resolution tandem mass spectrometry. Prior to the analyzes, the samples were enriched with an online solid phase extraction. Biofilms were extracted by the QuEChERS-based method for gammarids, following Munz et al. with some modifications. [21] Briefly, 100 mg of freeze-dried sample were homogenized with 500 mg of 1 mm zirconia/silica beads (Biospec Products, Inc., U.S.A.) in 500 µL of acetonitrile (ACN) and 500 µL of water followed by extraction/purification with 300 mg of QuEChERS salts (4:1, $MgSO_4$:NaCl, Agilent Technologies). A last clean-up was performed in order to remove the lipids by adding 500 µL of heptane to the final vial containing approximately 800 µL of ACN. Final ACN extracts were analyzed by liquid chromatography high resolution tandem mass spectrometry (Q-Exactive-plus, ThermoScientific) through direct injection after evaporation and resuspension in methanol:water (50:50;v:v).[5] Due to technical issues, micropollutant analysis was not performed for the grab water samples from Buttisholz in 2016.

Total biofilm and algal biomass

Total biofilm biomass was measured by determining the ash-free dry weight (AFDW) in three subsamples of each biofilm suspension (2 mL).[22] Algal biomass was expressed as chlorophyll-a concentrations, measured in 5 mL of biofilm suspension by high-performance liquid chromatography.[22]

Functional measurements in the field grown biofilms

Photosynthetic efficiency based on chlorophyll-a fluorescence was measured with an Imaging-PAM (pulse amplitude-modulated) fluorimeter (Heinz Walz GmbH, Germany) in 4-mL biofilm suspension at 665 nm following a single saturation light pulse to calculate the maximal PSII quantum yield (ϕ_{PSII}).

Algal production was measured as ^{14}C-carbonate assimilation during photosynthesis.[23] Briefly, a 2-mL aliquot from each biofilm suspension were incubated during 2 hours with 25 µL of $NaH^{14}CO_3$

153 (2.09 GBq $mmol^{-1}$, Hartmann Analytic GmbH, Germany), under light at 16°C. Abiotic controls with
154 formaldehyde (3.7 % final concentration) were run during each assay. To stop the reaction,
155 formaldehyde (final concentration of 3.7%) was added followed by 100 µL of glacial acetic acid to
156 remove the inorganic carbon. Biofilm suspensions were then incubated at 60°C until complete dryness
157 before adding 1 mL of DMSO, and incubated for one hour at 60°C. Ten mL of scintillation cocktail
158 (Ultima Gold LLT, GmbH, Germany) were added to the samples, before measuring the radioactivity
159 in a Tri-Carb 2810 TR liquid scintillation counter (PerkinElmer GmbH, Germany) with quench
160 correction.

161 Bacterial production was measured as the incorporation of ^{14}C-leucine into proteins as described in
162 Buesing and Gessner [24] with few modifications. 2.9 mL of each biofilm suspension were incubated in
163 the dark during 30 min at 16°C with 4.5 µM ^{14}C-leucine (12.32 GBq $mmol^{-1}$; Hartmann Analytic
164 GmbH, Germany) and 2.5 mM of non-radioactive leucine. Abiotic controls with trichloroacetic acid
165 (TCA) (5 % final concentration) were run in parallel during each assay. Incubations were stopped with
166 TCA to a final concentration of 5%. Afterwards, samples were sonicated during 1 min (Branson
167 Digital Sonifier 250, Germany), and centrifuged at 4000 g for 30 min. Three successive washes with
168 5% TCA, 40 mM cold leucine, 80% ethanol, and sterile ultrapure water were then applied to the
169 pellets, before being suspended in 1.5 mL of 0.3% SDS, 75 mM EDTA, and 1.5 M NaOH and heated
170 for 1 h at 90 °C to dissolve proteins. The samples were then cooled to room temperature before being
171 centrifuged for 10 min at 14.000 g. 500 µL of the supernatant were transferred to a scintillation vial
172 with 5 mL Hionic Fluor scintillation cocktail (PerkinElmer GmbH, Germany). The radioactivity was
173 measured in a Tri-Carb 2810 TR liquid scintillation counter (PerkinElmer GmbH, Germany) with
174 quench correction.

175 Microbial respiration of heterotrophs in the biofilms was measured by using the MicroResp™
176 technique,[25] a spectrophotometric method based on CO_2 production in a sealed microplate system.
177 Briefly, 500 µL from each biofilm suspension and 30 µL of D-glucose (6.2 mg of C per well, pH = 7)
178 were distributed in a deep-well microplate. Then, a second microplate, containing a pH indicator
179 embedded in agar was placed on top of the first one. Both plates were then joined with a silicone seal

with interconnecting holes between the corresponding wells and clamped together. Absorbance of the CO_2-detection gel was measured at 572 nm (Tecan Infinite 200 PRO microplate reader, Tecan Trading AG, Switzerland), immediately before sealing the microplates and after 15 h of incubation in the dark at 16°C. The CO_2 produced was calculated based on a calibration curve of absorbance values versus CO_2 measured by gas chromatography (MTI 200 thermal conductivity detector).

Three extracellular enzymes, β-glucosidase (BG; EC 3.2.1.21), alkaline phosphatase (AP; EC 3.1.3.1-2), and leucine-aminopeptidase (LAP; EC 3.4.11.1), were measured in one mL of each biofilm suspension as indicators for polysaccharide degradation, and phosphorus and nitrogen acquisition by microorganisms, respectively.[26] Substrate analogues linked to methylumbelliferyl (MUF) or aminomethyl-coumarin (AMC) were used to measure BG (4-MUF-β-D-glucopyranoside), AP (4-MUF-phosphate) and LAP (L-leucine-AMC). All assays were performed for one hour at 16 °C under gentle shaking in the dark at a saturating substrate concentration of one mM. Abiotic controls receiving formaldehyde (3.7 % final concentration) were run separately for each assay. One mL of glycine buffer (pH 10.4) was added to the samples to stop the incubations before measuring fluorescence (Tecan Infinite 200 PRO microplate reader, Tecan Trading AG, Switzerland). The excitation and emission wavelengths were 365 and 455 nm (MUF-substrates), and 364 and 445 nm (AMC-substrate).

Biofilm community composition

Molecular genetic composition of the bacterial and algal communities in the biofilms was assessed by denaturing gradient gel electrophoresis (DGGE). Genomic DNA was extracted from the pellets of each biofilm suspension (2 mL) with the PowerBiofilm DNA Isolation Kit (MO BIO Laboratories, CA), following the manufacturer's instructions. The algal 18S rRNA fragment was amplified in 60 ng of extracted DNA with the Euk1Af and Euk516r-GC primers, and the bacterial 16S rRNA gene fragment s was amplified in 30 ng of extracted DNA with the primers 341f and 907rM. Algal and bacterial DGGE profiles were analysed by using the software ImageJ in which nucleic acid bands,

corresponding to operational taxonomic units (OTUs), were identified and their presence or absence at a given position was scored as 1 or 0, respectively.

Short-term bioassays for PICT measurements

Tolerance of upstream and downstream biofilms to micropollutants, released with the WWTP effluents, was assessed in short-term bioassays in which biofilms were exposed to serial dilutions of the passive sampler extracts from the corresponding effluent. For an additional line of comparison with the survey conducted in 2014,[15] the biofilms sampled in 2016 were also exposed to serial dilutions of passive sampler extracts collected in 2014. A semi-logarithmic dilution series (six dilutions with a dilution factor of 3.2) of the extracts was freshly prepared for each experiment in Evian mineral water. 4.5-mL aliquots of each biofilm suspension of comparable biomass (OD_{700nm} = 0.4) were exposed in glass vials to 0.45 mL of the six dilutions of the corresponding passive sampler extract. In parallel, one control with 0.45 mL added mineral water and one abiotic control with 4% formaldehyde were run. No solvent control was included because of the complete evaporation of the solvents used for the micropollutant extraction from the passive samplers. After an incubation for 12 hours at 16 °C under light, sub-aliquots were taken from each vial to measure algal and bacterial production as described above.

Data analyses

Significant differences in the physicochemical data and total micropollutant concentrations among sampling sites and years were assessed by Repeated-Measure one-way ANOVA, followed by Tukey's post'hoc tests when an effect was significant in the ANOVA. Additionally, effect size was measured based on the calculation of Cohen's d:

$$d = [m1 - m2]/\sqrt{\frac{[SD1^2 + SD2^2]}{2}}$$

Where m and SD are the mean and standard deviation of each group, respectively.

Student t-tests were performed to compare total and algal biomass, algal and bacterial production, SIR, and the enzymatic activities data between the upstream and downstream locations of each

sampling site. The significance level was set at 5% for all statistical tests. Normality of the residuals and homogeneity of variances were checked by Shapiro-Wilk and F tests, respectively. In order to assess the tolerance levels of the biofilms, passive sample extract concentrations causing a 50% effect, relative to the controls (EC_{50}), in the PICT assays were determined by nonlinear regression of sigmoidal dose–response curves using the Hill slope equation (GraphPad Prism version 7.00 for Windows, San Diego, CA, USA):

$$\text{Activity} = \text{min} + [100\text{-min}]/[1+10^{(X\text{-LogEC50})}]$$

Where min is the minimally inhibited activity, and X is the passive sampler concentration in the assays. Values of the estimated min and $LogEC_{50}$ are provided in SI Appendix, Table S7. Significant difference among EC_{50}s was based on calculated 95% confidence intervals via bootstrap Monte Carlo simulations.

DGGE data were used to compare algal and bacterial molecular community composition among sampling sites. Each DGGE band was considered as an operational taxonomic unit (OTU). DGGE gels were aligned and matrices based on the presence and absence of OTUs in each lane of the gel were established, and a principar component analyses was performed (CANOCO software for Windows, version 4.5).

RESULTS AND DISCUSSION

Physicochemical and micropollutant analyses

Based on the measured physicochemical parameters, wastewater discharges clearly influenced the receiving waters across sites and sampling years (i.e. 2014 and 2016: one year before and after the upgrade of Herisau, respectively) (Figure 2, Table S3). Indeed, all downstream locations were characterized by significantly higher conductivity, dissolved organic carbon, nitrate, chloride, and sulfate concentrations than the corresponding upstream locations (ANOVA; $P<0.05$). Furthermore, phosphate concentrations significantly increased from upstream to downstream of Herisau and Buttisholz (Tukey; $P<0.05$), while pH significantly decreased downstream of the discharge site at

255 Herisau (Tukey; $P<0.05$). Cohen's effect size values for these parameters ranged from 0.8 to 3.5,
256 suggesting also a high to very high significance.

257 Quantification of the micropollutants in the grab water samples and biofilms sampled in 2016
258 indicated that their composition and relative concentrations differed among the sampling sites (SI
259 *Appendix,* Tables S4 and S5). Such differences are most likely related to variable land use at the
260 catchment level and different treatment technologies.[5, 27] Nonetheless, total concentrations of
261 micropollutants in 2014 at Herisau, Hochdorf, and Buttisholz were significantly higher downstream
262 than upstream of the wastewater discharge sites in the water samples (Tukey; P=0.003, 0.01 and 0.02,
263 respectively) and in the biofilms (Tukey; P=0.002, 0.005 and 0.03, respectively) (Figure 3). These
264 significant differences were also corroborated by the Cohen's d values that ranged from 0.4 to 1,
265 indicating a medium to large significance based on size effects. Similar significant differences were
266 also observed in both water and biofilm samples from Hochdorf (Tukey; P=0.01) and in the water
267 samples from Buttisholz (Tukey; P=0.01 and 0.04, respectively) in the year 2016. In contrast to the
268 other sites and to the year 2014, total concentrations of the micropollutants in upstream and
269 downstream water and biofilm samples from Herisau in 2016 were not significantly different (Tukey;
270 P=0.1 and 0.9, respectively), with small Cohen's d values of 0.2 and 0.4, respectively. These findings,
271 recorded one year after the upgrade of the WWTP in 2015 with powder-activated carbon filtration
272 treatment, clearly demonstrate the improved removal by 85 % of micropollutants from the effluent.
273 Indeed, total concentrations of pharmaceuticals, pesticides and household chemicals decreased
274 substantially downstream of Herisau between 2014 and 2016 (from 1600 ± 380 to 290 ± 90 ng L^{-1},
275 from 410 ± 50 to 210 ± 100 ng L^{-1}, and from 1600 ± 160 to 50 ± 10 ng L^{-1}, respectively).

276 Concentrations of the micropollutants in extracts from the passive samplers that were installed at the
277 discharge sites indicated that pharmaceuticals and household chemicals were dominant within the
278 analyzed mixtures, except at Herisau, where pesticides were dominant (SI *Appendix*, Table S6),
279 similarly to the water samples. In comparison to the extracts from 2014, total micropollutant
280 concentrations in the extracts from 2016 were significantly lower for Herisau, but not different for
281 Hochdorf and higher for Buttisholz (*t*-test, P=0.002, 0.4 and 0.0008, respectively) (Figure 4). Direct

comparison between measured concentrations in the passive-sampler extracts and in the water samples is, however, not possible. This is mainly due to the compound specific enrichment in the samplers, and to the fact that the samplers were deployed directly at the WWTP effluents while the grab water samples were taken upstream and downstream of the effluents.[28] Nevertheless, the similar detection frequency of the compounds in both passive and grab samples indicates that passive samplers are a suitable tool to highly enrich environmental micropollutant mixtures, which justifies their use in toxicity testing as it has been done in other studies.[28, 29]

Biomass and functional descriptors of field-grown biofilms as indicators for general water quality

Most of the traditional biofilm descriptors measured in 2016 did not respond consistently across and within sampling sites (Table 1). Moreover, differences between upstream and downstream sites were mainly observed at Herisau, and only to a lesser degree at Buttisholz and Hochdorf, where only one descriptor was significantly impacted by the wastewater discharge. Indeed, in comparison to upstream biofilms, downstream biofilms at Herisau were characterized by significantly lower chlorophyll-*a* content and alkaline phosphatase activity and a higher induced respiration (t-test; $P<0.01$, 0.01, and 0.05, respectively). Bacterial production significantly decreased also from upstream to downstream of Buttisholz and algal production significantly increased from upstream to downstream of Hochdorf (t-test; $P<0.01$ and 0.001, respectively), whereas no significant changes occurred for the other descriptors (t-test; $P>0.05$). As micropollutant concentrations consistently increased downstream of the WWTP effluents at the two non-upgraded sites, toxic effects on target organisms such as algae and bacteria in the biofilms are expected. However, environmental factors such as increased temperature and nutrient concentrations can be beneficial for microorganisms,[6, 26, 30] which could mitigate the negative effects of the present micropollutants. [31] This is in line with our results, which show that changes in the traditional descriptors of biofilms are a pertinent indicator of the general changes in the stream water quality , but do not allow to specifically distinguish the ecological impacts of micropollutants from those of other environmental factors.

Shifts in algal and bacterial community composition

Analyses of community composition of the biofilms sampled in 2016 showed that for bacterial and algal communities, both axes PC1 and PC2 of the principal component analysis explained more than 54 % and 61% of the total variability, respectively (Figure 5). For both bacteria and algae, PC1 is related to the sampling location, separating Herisau from Buttisholz and Hochdorf. These results reflect the fact that natural variation of stream community composition is context-dependent, and can be influenced by various local, regional and biogeographic factors.[32] Indeed, Herisau belongs to a different catchment than Hochdorf and Buttisholz, and each catchment is characterized by a different land use,[27] explaining their separation along PC1.

Most importantly, for algae as well as for bacteria, PC2 is clearly correlated with the impact of the wastewater discharges on microbial community structure. Indeed, composition of algal and bacterial communities in biofilms was clearly different between upstream and downstream of Buttisholz and Hochdorf, respectively; a pattern that was also observed by previous studies at the same sites[15] and elsewhere.[6, 33] Nevertheless, despite the clear impacts of the wastewater discharges on microbial communities at the non-upgraded sites, restructuring of the algal and bacterial communities cannot only be attributed to the micropollutants in the effluents. Indeed, other wastewater constituents, such as nutrients, organic matters or microorganisms can play a role in shaping the microbial diversity at the receiving streams.[32] In contrast to Buttisholz and Hochdorf, the dissimilarity between the upstream and downstream community composition at Herisau was less pronounced for both bacteria and algae. This was the case despite the continuous influence of the wastewater discharge on the physicochemical characteristics of the receiving water, such as nutrient concentrations and temperature. Furthermore, when biofilm composition was examined one year before the WWTP upgrade at Herisau, a clear separation between upstream and downstream sites was observed.[15] Taken together, these results provide a first indication that micropollutant mixtures could be a major factor that shape the composition of microbial communities downstream of wastewater discharges, unless modern technologies such as activated carbon filtration are employed to decrease micropollutant concentrations. Further investigations to identify algae and bacteria specifically occurring in upstream

and downstream biofilms could point to taxa that are particularly sensitive or tolerant to micropollutants in wastewaters.

Microbial tolerance as a specific indicator for micropollutant effects

To further unravel the combined effects of general wastewater degradation from micropollutant contamination, the PICT concept was applied. Short-term exposure of biofilms collected in 2016 upstream and downstream of the Herisau WWTP to passive sampler extracts obtained in 2014 or 2016 resulted in non-significantly different EC_{50} values (Table 2). This indicates that in 2016 the chronic exposure to the micropollutants released in the WWTP effluent at this site has not caused a tolerance development in the biofilms. In contrast, in the study conducted in 2014 at Herisau[15], i.e., one year before the upgrade, downstream biofilms were still more tolerant to the passive sampler extract than upstream biofilm. Such differences in tolerance measurements are in agreement with the micropollutant concentrations measured in the grab water samples and biofilms at Herisau, which increased significantly from upstream to downstream before, but not after the upgrade. These results underline the potential relationship between tolerance measurements and the intensity of contamination by micropollutants in the environment. They also reflect the high resilience of biofilm communities in term of their sensitivity to micropollutants. It should be noted that other critical physicochemical factors such as nutrient and organic matter concentrations continued to be higher downstream than upstream of the effluent at Herisau in 2016 (Figure 2) and can therefore be ruled out as a factor causing the disappearing tolerance between 2014 to 2016. Moreover, because the differences in the physicochemical characteristics of the stream water between upstream and downstream remained similar over the years, the increased tolerance at Herisau measured before the WWTP upgrade [15] was most likely driven by the micropollutants emitted from the WWTP. Nonetheless, in order to rule out completely the role of these factors in tolerance development, further investigation focusing on their quality and not only on bulk concentrations should be performed.

In sharp contrast to the tolerance measurements at Herisau, our results show that biofilm tolerance at the non-upgraded sites (i.e. Buttisholz and Hochdorf) still display the same pattern as found in the

2014 survey.[15] Indeed, in both studies bacterial and algal communities that make up the downstream biofilms displayed significantly higher tolerance to both extracts than upstream biofilms, as reflected by the higher EC_{50} values (Table 2). This might be supported by the chemical analyses that identified higher micropollutant concentrations downstream than upstream of the wastewater discharges. These findings reflect that induced community tolerance might occur as long as the selection pressure by micropollutants is maintained, leading to toxicant induced succession by the elimination of pollutant-sensitive species and favoring the development of tolerant ones within the exposed communities termed "toxicant induced succession.[10]

The results obtained in our study provide a first basis to illustrate the potential relationship between the levels of contamination by micropollutant mixtures in streams, shifts in microbial community structure and the resulting development of tolerance to these micropollutants. This was confirmed by the loss biofilms tolerance after the reduction of micropollutant concentrations at the upgraded site, and also by the reproducible results obtained at the non-upgraded sites with an interval of two years. Although further surveys with more upgraded WWTPs are needed, our findings converge on the sensitivity and efficiency of the PICT approach combined with the use of extracts from passive samplers to establish causality between *in situ* exposure to complex micropollutant mixtures and community-level effects. Importantly, they provide first line of evidence on the relevance of using this approach to monitor the success of remediation measures following the removal of micropollutants from wastewater effluents via the implementation of activated carbon treatments.

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389 **Table 1.** Biomass and functional descriptors of upstream (Up) and downstream (Down) biofilms sampled in 2016 from the sites Herisau, Buttisholz,
390 and Hochdorf. Data are means ± standard deviation (n = 3). Significant differences between Up and Down biofilms at each sampling site are indicated
391 by an asterisk (Student's *t*-test; $p < 0.05$).

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	Herisau		**Buttisholz**		**Hochdorf**	
	Up	Down	Up	Down	Up	Down
AFDW (g cm^{-2})	1.7 ± 0.3	2.2 ± 0.4	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
Chlorophyll-*a* (mg g^{-1} AFDW)	3.5 ± 0.5	1.6 ± 0.2*	1.7 ± 0.7	1.6 ± 0.7	0.6 ± 0.1	0.7 ± 0.2
PSII Quantum Yield (ϕ)	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
Algal Production (µg C g^{-1} AFDW day^{-1})	1.2 ± 0.4	1.6 ± 0.8	22.6 ± 5.4	39.6 ± 29.3	40.7 ± 15.0	101.7 ± 16.7*
Bacterial Production (µg C g^{-1} AFDW day^{-1})	0.2 ± 0.1	0.1 ± 0.1	10.3 ± 0.6	5.8 ± 0.1*	4.8 ± 1.3	6.08 ± 0.8
Substrate-induced Respiration (µg CO_2 g^{-1} AFDW day^{-1})	16.3 ± 2.9	37.1 ± 5.8*	113.7 ± 23.4	121.1 ± 10.6	71.8 ± 16.8	81.4 ± 15.2
β-glucosidase (µmol MUF g^{-1} AFDW h^{-1})	1.3 ± 0.3	1.5 ± 0.2	2.6 ± 0.7	2.2 ± 0.1	2.00 ± 0.6	1.9 ± 0.3
Alkaline Phosphatase (µmol MUF g^{-1} AFDW h^{-1})	6.8 ± 1.0	2.8 ± 0.9*	3.9 ± 0.2	2.9 ± 0.2	4.7 ± 1.5	4.8 ± 1.6
Leucine Amino-peptidase (µmol AMC g^{-1} AFDW h^{-1})	5.1 ± 0.9	6.5 ± 1.3	14.8 ± 1.9	17.0 ± 0.4	11.4 ± 2.3	12.7 ± 0.7

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Table 2. EC_{50} values for algal and bacterial production of upstream (Up) and downstream (Down) biofilms sampled in 2016 and exposed to a dilution series of the passive sampler extracts collected in 2014 and 2016. Significantly increased EC_{50} values at the downstream sampling sites indicate increased tolerance levels of the biofilms (non-overlapping confidence intervals of the EC_{50} estimates, printed in bold). Concentrations are expressed in unitless relative dilution factor (RDF) of the passive sampler extracts. Values in parentheses are 95% confidence intervals (n = 21).

		Algal production		**Bacterial production**	
		Extract 2014	Extract 2016	Extract 2014	Extract 2016
Herisau	Up	6 (4 - 9)	21 (11 - 41)	0.5 (0.3 – 0.8)	1.3 (0.7 – 2)
	Down	9 (5 - 15)	26 (13- 50)	0.3 (0.1 – 0.9)	1.4 (0.9 – 2)
Buttisholz	Up	**11** (5 - 20)	**16** (9 - 29)	**1.6** (1 - 2.4)	**1.4** (0.8 - 2)
	Down	**54** (29 - 100)	**97** (67 - 141)	**10** (6 - 17)	**53** (19 - 147)
Hochdorf	Up	**4** (2 - 6)	**18** (14 - 24)	**0.9** (0.6 – 1.2)	**2** (1.4 - 3)
	Down	**21** (7 - 62)	**42** (24 - 74)	**5** (3 - 8)	**7** (4 - 13)

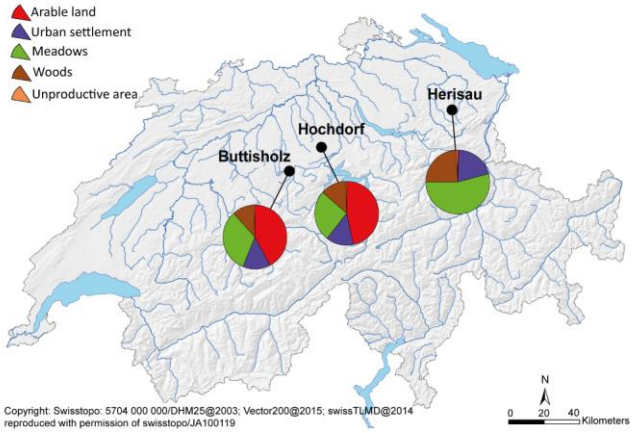


Figure 1. Location of the three sampled wastewater treatment plants in Switzerland with the land use pattern for each catchment shown in the pie charts (BFS, 2014). The aggregated land use categories and site names as well as their geographic coordinates are listed in Table S1 of the supporting information. Permission, swisstopo/JA 100119.

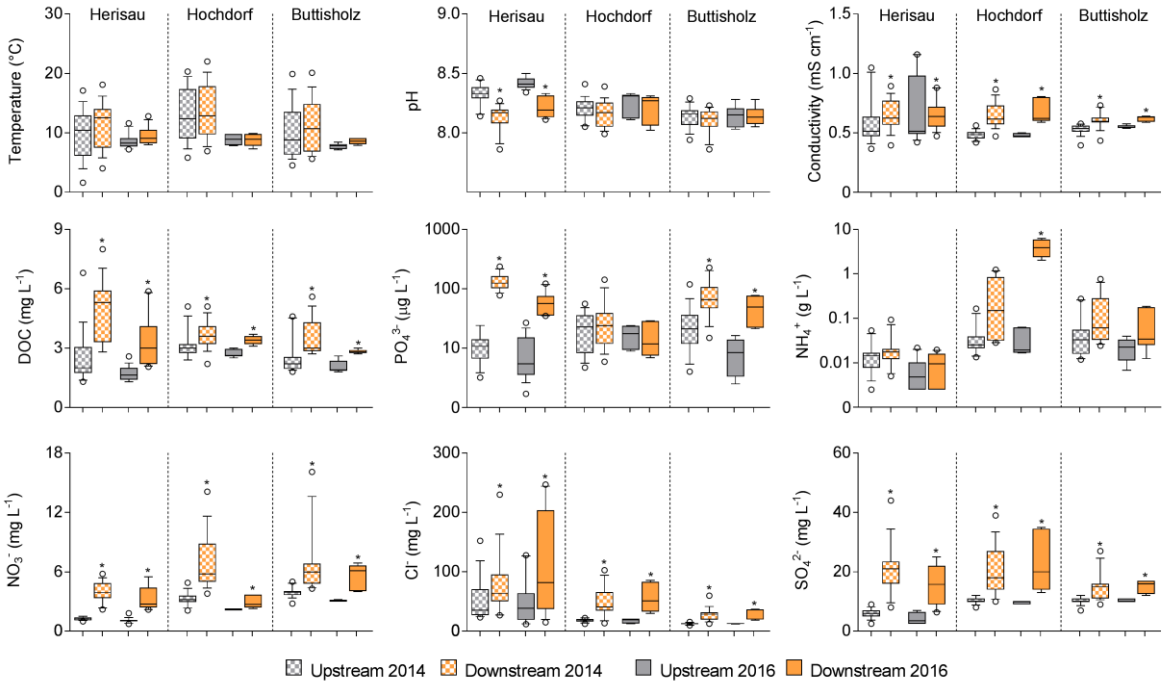
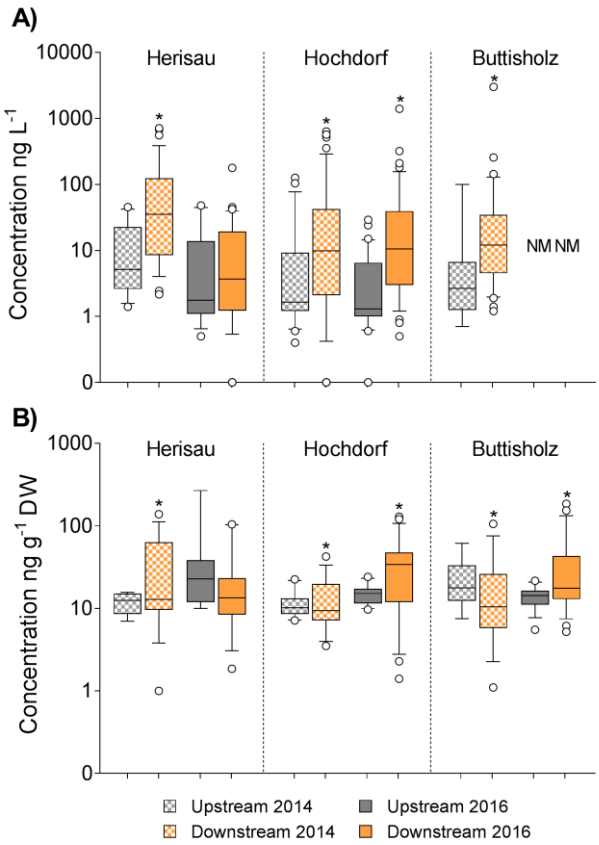


Figure 2. Box plots of the measured physicochemical parameters in the grab water samples from upstream and downstream of the sampling sites, one year before (2014) and after (2016) the upgrade of Herisau in2015. Sampling was performed monthly from January to May 2014 and from March to June 2016. The horizontal lines within the boxes indicate medians, boundaries of the boxes indicate the 10th and 90th percentiles, and the whiskers indicate the minimum and maximum values, excluding outliers (white circles) (n = 17 and 16 in 2014 and 2016, respectively). Asterisks above the boxes denote a significant difference between up- and downstream locations per sampling site and year ($P < 0.05$; Tukey's test following ANOVA).



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437 Figure 3. Box plots of the median concentrations of the 57 and 41 analyzed organic micropollutants in
438 the grab water samples (ng L-1) (A) and in the biofilms (ng g-1 biofilm dry weight) (B), respectively,
439 from upstream and downstream of the sampling sites, one year before and after the upgrade of Herisau
440 in 2015 (2014 and 2016, respectively). Due to technical issues, quantification in the water samples
441 from Buttisholz was not performed in 2016 (NM: not measured). The horizontal lines within the boxes
442 indicate medians, boundaries of the boxes indicate the 10th and 90th percentiles, and the whiskers
443 indicate the minimum and maximum values, excluding outliers (white circles). Asterisks above the
444 boxes denote a significant difference between up- and downstream locations per sampling site and
445 year ($P < 0.05$; Tukey's test following ANOVA).

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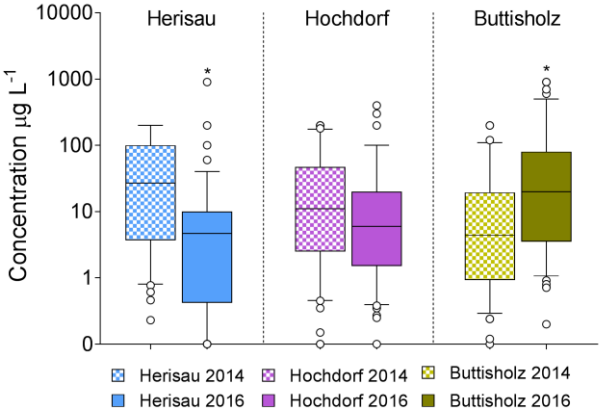

Figure 4. Box plots of the median concentrations of the 57 analyzed organic micropollutants in passive sampler extracts deployed in 2014 and 2016 at each wastewater effluent of the sampling sites. The horizontal lines within the boxes indicate medians, boundaries of the boxes indicate the 10th and 90th percentiles, and the whiskers indicate the minimum and maximum values, excluding outliers (white circles). Asterisks above the boxes denote a significant difference between up- and downstream locations per sampling site and year ($P < 0.05$; Tukey's test following ANOVA).

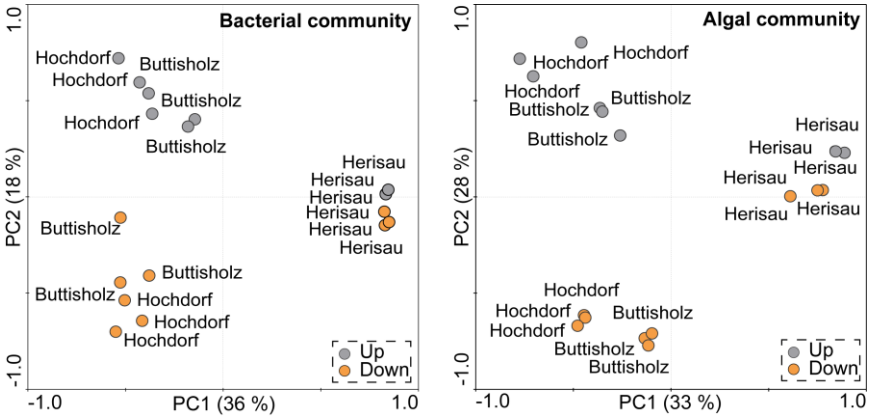

Figure 5. Principal component analysis ordination of the bacterial and algal community composition in the biofilms (n = 3) sampled in 2016 from upstream (Up) and downstream (Down) locations of the sampling sites Herisau, Hochdorf, and Buttisholz.

ASSOCIATED CONTENT

Supporting Information

Additional figures and tables as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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