Micropollutant-induced tolerance of *in situ* periphyton: establishing causality in wastewater-impacted streams

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This document is the accepted manuscript version of the following article:
Tlili, A., Hollender, J., Kienle, C., & Behra, R. (2017). Micropollutant-induced tolerance of in situ periphyton: establishing causality in wastewater-impacted streams. Water Research, 111, 185-194. https://doi.org/10.1016/j.watres.2017.01.016

Abstract

The overarching aim of this field study was to examine causal links between *in-situ* exposure to complex mixtures of micropollutants from wastewater treatment plants and effects on freshwater microbial communities in the receiving streams. To reach this goal, we assessed the toxicity of serial dilutions of micropollutant mixtures, extracted from deployed passive samplers at the discharge sites of four Swiss wastewater treatment plants, to *in situ* periphyton from upstream and downstream of the effluents. On the one hand, comparison of the sensitivities of upstream and downstream periphyton to the micropollutant mixtures indicated that algal and bacterial communities composing the periphyton displayed higher tolerance towards these micropollutants downstream than upstream. On the other hand, molecular analyses of the algal and bacterial structure showed a clear separation between upstream and downstream periphyton across the sites. This finding provides an additional line of evidence that micropollutants from the wastewater discharges were directly responsible for the change in the community structure at the sampling sites by eliminating the micropollutant-sensitive species and favouring the tolerant ones. What is more, the fold increase of algal and bacterial tolerance from upstream to downstream locations was variable among sampling sites and was strongly correlated to the intensity of contamination by micropollutants at the respective sites. Overall, our study highlights the sensitivity of the proposed approach to disentangle effects of micropollutant mixtures from other environmental factors occurring in the field and, thus, establishing a causal link between exposure and the observed ecological effects on freshwater microbial communities.

Keywords: Pollution-induced community tolerance; passive samplers; wastewater treatment plants; causality; micropollutant mixture; biofilm

1. Introduction

Fresh waters are among the most threatened ecosystems in terms of species extinctions and losses in ecosystem services, and micropollutants, such as pharmaceuticals or pesticides, are considered as one of the major threats (Bernhardt et al. 2016, Gessner and Tlili 2016, Stehle and Schulz 2015, Vörösmarty et al. 2010). Because wastewater treatment plants (WWTPs) were primarily designed to retain organic matter, nutrients and microbes, micropollutants are not completely removed by common treatment steps (Joss et al. 2008, Rodriguez-Mozaz et al. 2015). Consequently, effluents represent a point source for complex mixtures of micropollutants into fresh waters, potentially leading to the degradation of the chemical and ecological status of the receiving ecosystems. Furthermore, input of micropollutants from wastewater discharges into fresh waters is usually associated with other stressors such as an increase of temperature or high loads of nutrients and organic matter (Petrie et al. 2015). In such a complex situation, one of the key challenges is to diagnose specific micropollutant effects and their contribution to the general degradation of the ecosystem status (Bundschuh 2014, Stamm et al. 2016).

Current methods and tools applied in regulatory contexts do not pinpoint the specific *in situ* impacts of micropollutant mixtures on ecosystem status (Bundschuh 2014). On the one hand, assessment of chemical status is based on comparing measured concentrations of selected compounds to concentrations considered safe for the environment (environmental quality standards, EQS). This approach allows for quantification of specific compounds within the mixture of micropollutants but does not reflect the dynamic composition of pollutants in the environment or their *in situ* effects on the biota. EQS are derived from single-species bioassays that measure the sensitivity of a single organism to micropollutants by observing parameters such as survival, growth or photosynthesis. On the other hand, assessment of the ecological status of ecosystems relies on diverse bioindicators, focusing on species taxonomy or abundance, which

are designed to provide an overall picture of the aquatic system status but cannot ascribe a change to a specific effect of micropollutants or other stressors (Coste et al. 2009, Feio et al. 2007).

Periphyton, a consortium of microorganisms composed by algae, bacteria, fungi and protozoa that grow on submerged substrata surfaces, has been recognised as an important biological indicator to classify water bodies according to their pH, salinity, or saprobic state, as well as increasingly to examine micropollutant effects (Battin et al. 2016, Sabater et al. 2007). In streams of small to moderate size, periphyton plays a crucial ecological role as a basis of the food web; and it is sensitive to environmental stressors. Therefore, a large set of functional and structural descriptors using periphyton has been developed to evaluate the risks posed by environmental stressors in fresh waters (Sabater et al. 2007). Here again, if solely applied to periphyton in the field, these descriptors do not allow for distinction between the contribution of micropollutants and other stressors to the effects observed in the environment.

A shift in community composition that results from the replacement of sensitive species by tolerant ones upon chronic exposures of communities to micropollutants is a common response in contaminated ecosystems and is considered as a reliable indicator of *in situ* micropollutant impacts (Amiard-Triquet 2011). The difference in sensitivity to micropollutants among species forms the basis of the pollution-induced community tolerance (PICT) concept (Blanck et al. 1988). The rationale of the concept is that disappearance of sensitive species and dominance of tolerant ones is an expected outcome of chronic exposure. Thereby a community that was previously affected through exposure to micropollutants is anticipated to display a higher tolerance to those micropollutants than a reference community that has never been exposed.

Increased community tolerance following exposure to micropollutants has been demonstrated with periphyton (Blanck 2002) but PICT studies with periphyton in the field remain rare. One continued difficulty is to disentangle micropollutant from other stressor effects.

To overcome this difficulty, an assessment of tolerance to passive sampler extracts obtained from the studied sites has recently been proposed (Kim Tiam et al. 2016, Tlili et al. 2016). Passive samplers accumulate chemicals by diffusion and sorption, mimicking the bioaccumulation of these chemicals within biological matrices. They can provide an integrative picture of pollutants over a period of time, allowing for quantification of average concentrations to which organisms have been exposed (Alvarez et al. 2008). Indeed, some studies have used this approach with periphyton grown in indoor microcosms, which were chronically exposed to extracts from passive samplers that had been deployed in streams contaminated with pesticides (Foulquier et al. 2015, Kim Tiam et al. 2015, Morin et al. 2012). Furthermore, Pesce et al. (2011) examined the tolerance to passive sampler extracts of *in situ* periphyton from a stream with a gradient of agricultural pollution. Although these studies focused only on the phototrophic but not the heterotrophic component of periphyton, they showed that upon chronic exposure to micropollutant algal communities developed tolerance to the passive sampler extracts.

Given this background, the overarching goal of our study was to determine the contribution of complex mixtures of micropollutants from wastewater discharges to the ecological status of *in situ* microbial communities in fresh waters, using periphyton as a biological model. The specific aims were (i) to examine phototrophic and heterotrophic periphyton tolerance from upstream and downstream of WWTPs towards micropollutants extracted from passive samplers deployed in the wastewater discharges, (ii) to link the changes of diversity in the phototrophic and heterotrophic communities composing the periphyton to the micropollutants released from the WWTPs and (iii) to compare the PICT approach with traditionally used approaches such as single species bioassays and functional and structural bioindicators of periphyton. We hypothesized that even at low concentrations of individual chemical compounds, the continuous input of complex mixtures of micropollutants will alter the chemical and ecological status of the receiving streams, affecting

the diversity of downstream periphyton and leading to increased tolerance towards these micropollutants.

2. Material and methods

2.1. Experimental design

Periphyton was grown on artificial glass substrates upstream and downstream of four WWTPs located in north-eastern Switzerland. During the colonisation period, Chemcatcher® passive samplers were deployed at each discharge site in the receiving streams to accumulate polar to semi-polar organic micropollutants. After 6 weeks, periphyton and passive samplers were retrieved from the field and transported to the laboratory. Micropollutants accumulated in the passive samplers were extracted and extracts were used to assess tolerance to micropollutants. For this, upstream and downstream periphyton from each site were exposed to a series of increasing dilutions of the corresponding extract during 12 hours. Afterward, inhibition of various functional endpoints, specifically targeting the heterotrophic and phototrophic components of periphyton, was assessed. Toxicity of the extracts to a single green alga, *Pseudokirchneriella subcapitata*, was also evaluated to specifically inform on the activity of photosynthesis inhibitors. Additionally, various functional descriptors as well as the structure of the upstream and downstream periphyton were examined. Finally, during the colonisation period, two litres of grab water samples were collected every two weeks at each sampling location to measure 8 water quality parameters.

2.2. Study sites

Four WWTPs, located in Switzerland and named Steinach, Herisau, Buttisholz and Hochdorf, were selected as study sites (Fig. 1). Buttisholz, Herisau and Hochdorf were selected based on the surveys conducted by Munz et al. (2016) in 2013 and 2014 to investigate

139 concentration patterns of 57 to 400 micropollutants in 24 wastewater-impacted streams in
140 Switzerland as well as in the wastewater effluents. Their results showed that concentrations of the
141 majority of the measured micropollutants were significantly higher downstream than upstream of
142 the wastewater discharges (Table S2). Similarly, the Steinach site was also selected because the
143 analyses of 57 selected compounds in grab water samples showed higher micropollutant
144 concentrations downstream than upstream at this site (Table S2). Another criterion for the sites
145 selection is that the WWTPs contributed by a minimum of 20 % of wastewater to the total stream
146 flow at the downstream locations. All sites corresponded to small to moderately sized streams. At
147 each study site, one upstream and one downstream location was selected as reference and
148 impacted site, respectively. Upstream and downstream sites were chosen to be as similar as
149 possible with regard to stream morphology, riparian land use and vegetation. Furthermore,
150 downstream sites were selected so that water from the effluent was completely mixed with the
151 stream water during low flow conditions (Burdon et al. 2016, Munz et al. 2016, Stamm et al.
152 2016).

153 2.3. Periphyton colonisation and sampling

154 This study was conducted from the 15th of March to the 30th of April 2014. Three large
155 glass slides (380 cm^2 per slide) were fixed vertically in perforated plastic boxes (60×40×14.5 cm)
156 and used as artificial substrata to allow biofilm colonisation. At each sampling location, three
157 boxes were installed at the centre of the stream and considered as biological replicates. After six
158 weeks of colonisation, the glass slides were retrieved, placed individually in plastic bags
159 containing stream water from the corresponding sampling location and transported to the
160 laboratory in cooling boxes within five hours after sampling. Immediately after arrival at the
161 laboratory, periphyton growing on the three glass slides from the same box was carefully scraped
162 using a polypropylene spatula and suspended in 250 mL of Evian mineral water. In order to allow

comparison among sampling sites, Evian mineral water was used as medium to avoid the influence of different stream water compositions on the bioavailability of micropollutants, which can affect the biological responses of periphyton during the assays. Evian water is a commonly used medium in microbiological and ecotoxicological studies because of its uniform quality.

2.4. Passive samplers

In order to accumulate the polar to semi-polar micropollutants which are released from the studied WWTPs, Chemcatcher® passive samplers – styrenedivinylbenzene (SDB) discs – (47 mm diameter, Sigma Aldrich, Switzerland) were chosen for our study (Vermeirssen et al. 2009). During the six weeks of periphyton colonisation, Chemcatchers® were placed at the discharge site in the receiving stream of each WWTP. In order to be in the linear uptake phase, two passive samplers were deployed during two weeks before being replaced by two new ones, leading to a total of six passive samplers per sampling site. The retrieved SDB discs were put in 6 mL of acetone, brought to the lab and stored at -20 °C until use. After extraction of the accumulated micropollutants in the SDB discs as described in Vermeirssen et al. (2013), the extracts from the six passive samplers deployed at the same site were pooled. Afterward, the solvents were completely evaporated under a gentle nitrogen stream and the extracts were suspended in 7 ml of Evian mineral water for the PICT assays, the algae bioassay and the micropollutant analyses.

2.5. Physicochemical measurements and micropollutant analyses

During the study, water temperature, pH, conductivity, oxygen concentrations (WTW Meters, Germany) and water velocity (FlowTracker Handheld ADV, YSI, Inc., USA) were measured every two weeks at each sampling location. Additionally, two-litres water samples were collected in glass bottles for measurements of dissolved organic carbon (DOC), dissolved nitrogen (DN), orthophosphates, and silica using standard methods as described for the Swiss

National River Monitoring and Survey Programme (NADUF; www.bafu.admin.ch/wasser/13462/14737/15108/15109).

In total 56 polar to semi-polar micropollutants were measured and quantified in grab water samples from upstream and downstream of each sampling site and in the passive samplers extracts (Tables S2-3). The compounds were selected based on their frequent detection, higher concentration, toxicity as well representation of different substance classes and exposure pathways (Munz et al. 2016). Thirty-one of these compounds were pesticides including nine herbicides, twelve insecticides and ten fungicides. Twenty compounds were pharmaceuticals, including antibiotics, analgesics, antidiabetics, anti-inflammatory, antihypertensive drugs, β-blockers, diuretics, histamine analogues and psychiatric drugs. The five remaining compounds were household chemicals and included two anticorrosion compounds, one tracer for human excretion contamination in wastewater, one food additive and a personal care product. The water samples and passive sampler extracts were enriched with an online solid phase extraction similar to Huntscha et al. (2012) and subsequently analysed with a liquid chromatography high resolution tandem mass spectrometry method as described in Munz et al. (2016).

2.6. Periphyton characterisation

2.6.1. Total, algal and bacterial biomass

The total periphyton biomass was evaluated by calculating the ash-free dry weight (AFDW) in three subsamples of each periphyton suspension (2 mL) as described in Tlili et al. (2008). Results are expressed as g m^{-2}.

Chlorophyll-a content in 5 mL from each periphyton suspension was used as a proxy for algal biomass and quantified by high-performance liquid chromatography using an external calibration with purified chlorophyll-a ($C_{55}H_{72}MgN_4O_5$, Carl Roth GmBH & Co) as described in Tlili et al. (2008). Final concentrations are given as µg g^{-1} AFDW.

Bacterial biomass was estimated according to Frossard et al. (2012) with few modifications. Briefly, 5 mL from each periphyton suspension were put in 5 mL of phosphate-buffered formalin (2% final concentration). After an ultrasonic treatment for 1 min (Branson Digital Sonifier 250, Germany), periphyton suspensions were centrifuged at 2000 g for 15 min. One mL of the supernatant containing bacterial cells was stained with 0.1µL mL^{-1} of SYBER® Green I (Promega, Switzerland) in anhydrous dimethylsulfoxide and incubated during 15 min in the dark. Fluorescent beads (Flowcount flurospheres, Beckman Coulter, Switzerland) with a known concentration were spiked to the samples as a standard to determine the cell concentration. Samples were analysed using a Gallios flow cytometer (Beckman Coulter, Switzerland). Bacterial numbers were converted to bacterial biomass considering a mean bacterial biomass of 20 fg $cell^{-1}$ (Norland 1993) and data expressed as µg g^{-1} AFDW.

2.6.2. Functional analyses

Photosynthetic efficiency, which is based on measurement of the effective quantum yield of algae, was assessed using an Imaging-PAM (pulse amplitude-modulated) fluorimeter (Heinz Walz GmbH, Germany). Chlorophyll-a fluorescence from each periphyton suspension was measured at 665 nm after applying a single saturation pulse to calculate the effective quantum yield (ϕ') as: $\phi' = (F'_m - F'_0) / F'_m$; where F'_m is the maximum fluorescence after the saturation pulse and F'_0 is the steady-state fluorescence.

Algal production was measured by ^{14}C-carbonate incorporation rate as described in Dorigo and Leboulanger (2001). Briefly, a 2-mL aliquot from each periphyton suspension was put into a 20-mL glass scintillation vial containing 25 µL of $NaH^{14}CO_3$ (2.09 GBq $mmol^{-1}$, Hartmann Analytic GmbH, Germany) and incubated under the light at 16°C during 2 hours. The reaction was stopped by adding formaldehyde (final concentration of 3.7%), followed by 100 µL of glacial acetic acid to remove the inorganic carbon. Periphyton suspensions were dried

234 overnight at 60°C before adding 1 mL of DMSO and incubating for one hour at 60°C to dissolve
235 the labelled organic matter. Ten mL of scintillation cocktail (Ultima Gold LLT, GmbH,
236 Germany) were added, and radioactivity was measured in a Tri-Carb 2810 TR liquid scintillation
237 counter (PerkinElmer GmbH, Germany) with quench correction. Results are expressed as µg C g^{-1}
238 AFDW day^{-1}.

239 Bacterial production was measured by ^{14}C-leucine incorporation into protein according to
240 Buesing and Gessner (2003) with few modifications. Briefly, 2.9 mL from each periphyton
241 suspension were put into a 20-mL glass scintillation vial and incubated at 16°C for 30 min with
242 4.5 µM ^{14}C-leucine (12.32 GBq mmol^{-1}; Hartmann Analytic GmbH, Germany) and 2.5 mM of
243 non-radioactive leucine. Incubations were stopped by adding trichloroacetic acid (TCA) to a final
244 concentration of 5%. After ultrasonic treatment for 1 min (Branson Digital Sonifier 250,
245 Germany), samples were centrifuged at 4000 g for 30 min. Afterward, the pellets were
246 consecutively washed with 5% TCA, 40 mM cold leucine, 80% ethanol and sterile ultrapure
247 water. After the last washing, the pellets were resuspended in 1.5 mL of 0.3% SDS, 75 mM
248 EDTA and 1.5 M NaOH and heated for 1 h at 90 °C to dissolve proteins. After cooling to
249 ambient temperature, the tubes were centrifuged for 10 min at 14.000 x *g* and 500 µL of the
250 supernatant was transferred to a scintillation vial containing 5 mL Hionic Fluor scintillation
251 cocktail (PerkinElmer GmbH, Germany). The radioactivity incorporated into the dissolved
252 proteins was measured in a Tri-Carb 2810 TR liquid scintillation counter (PerkinElmer GmbH,
253 Germany) with quench correction. Results are expressed as µg C g^{-1} AFDW day^{-1}.

254 Microbial substrate-induced respiration (SIR) of the heterotrophic periphyton component
255 was measured using the MicroResp^{TM} technique and glucose as carbon source following the
256 procedure described in Tlili et al. (2011c). The system consists of two 96-well microplates placed
257 face-to-face. One is a 1.2 mL deep-well microplate in which each well contains 500 µL of the

periphyton suspension and 30 µL of D-glucose (6.2 mg of C per well, pH = 7). The second microplate contained the detection gel. The two microplates were joined with a silicone seal, with interconnecting holes between the corresponding wells. The assembly was clamped together and the system was incubated in the dark at 16 °C for 15 h. Absorbance of the detection gel was measured at 572 nm (Tecan Infinite 200 PRO microplate reader, Tecan Trading AG, Switzerland) immediately before sealing to the deep-well plate and after incubation. Quantities of the produced CO_2 by the microbial communities were calculated using a calibration curve of absorbance values *versus* CO_2 quantity measured by gas chromatography (MTI 200 thermal conductivity detector). Results were then expressed as µg CO_2.mg^{-1} of AFDW.h^{-1}.

Potential activities of the three extracellular hydrolytic 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), which are involved in the degradation of polysaccharide compounds, organic phosphorus compounds and peptides respectively, were measured following the methodology described by Romani et al. (2004). Because they reflect the ability of organisms to decompose and assimilate nutrients, enzyme activities have been used to assess changes in ecosystem functioning due water quality alterations. BG (4-methylumbelliferyl-β-D-glucopyranoside) and AP (4-methylumbelliferyl-phosphate) activities were measured using MUF (methylumbelliferyl) fluorescent-linked substrates while LAP (L- Leucine-7-amido-4-methylcoumarin hydro-chloride) activity was measured using AMC (aminomethyl-coumarin) fluorescent-linked substrate. Briefly, one mL from each periphyton suspension was incubated with MUF- or AMC-substrates at saturating substrate concentrations (i.e. 1 mM) in the dark during one hour at 16 °C. After incubation, 1 ml of glycine buffer (pH 10.4) was added to the samples in order to stop microbial metabolism. Then, fluorescence was measured at 365-455 nm (excitation-emission wavelengths, respectively) for MUF-substrates and at 364-445 nm for AMC-substrate (Tecan Infinite 200 PRO

microplate reader, Tecan Trading AG, Switzerland). Enzyme activity was expressed as µmole MUF or AMC g^{-1} AFDW h^{-1}.

2.6.3. Diversity analyses

Molecular fingerprints of the periphyton community were obtained by using denaturing gradient gel electrophoresis (DGGE). Subsamples of 2 mL from each periphyton suspension were centrifuged at 14,000 ×g for 30 min at 4 °C. Then, the supernatants were removed and the pellets kept at -80 °C. Nucleic acid extraction was performed on the periphyton pellets using the PowerBiofilm DNA Isolation Kit (MO BIO Laboratories, CA) following the manufacturer's instructions.

PCR amplification of algal 18S rRNA gene fragments and bacterial 16S rRNA gene fragments and their DGGE analysis were performed according to Tlili et al. (2008). Briefly, 60 ng of template DNA and the Euk1Af and Euk516r-GC primers were used to amplify the algal 18S rRNA gene fragment. PCR amplification of the bacterial 16S rRNA gene fragment was done with 30 ng of template DNA, and the primers 341f, to which a GC-rich fragment was attached, and 907rM. DGGE analyses were performed as described in Dorigo et al. (2007). Images were processed with the software ImageJ in which nucleic acid bands, corresponding to taxonomic operational units (OTUs), were identified and their presence or absence at a given height in each lane was scored as 1 or 0, respectively.

2.6.4. PICT measurements

In order to measure induced tolerance to micropollutants from WWTPs effluents, upstream and downstream periphyton from each sampling site were subjected to short-term exposure assays with serial dilutions of the passive sampler extracts from the corresponding effluent. Semi-logarithmic series of concentrations of the micropollutant extracts (six increasing concentrations) were freshly prepared, with a multiplication factor of $10^{0.5}$, by serial dilutions of

306 the stock solutions in Evian mineral water. Periphyton suspension aliquots (9 mL) from upstream
307 and downstream locations were exposed in glass Erlenmeyer to 1 mL of the six dilutions of the
308 corresponding micropollutant extract, in addition to one control in which 1 mL of mineral water
309 was added and one abiotic control with 1 mL of formaldehyde (three biological replicates per
310 sampling location). Based on previous tests to determine the optimal bioassays duration (data not
311 shown), samples were incubated during 12 hours at 15 °C under artificial light and gentle
312 shaking. Afterward, subsamples from the periphyton suspensions were taken from each
313 Erlenmeyer for photosynthetic efficiency, primary production and secondary production
314 measurements as described previously in section 2.6.2.

315 2.7. Algal bioassay

316 In order to compare between the toxicity of photosynthesis inhibitors contained in the
317 passive sampler extracts at the single-species and the community levels a combined algae assay
318 with the green alga *Pseudokirchneriella subcapitata* was performed according to Escher et al.
319 (2008). *P. subcapitata* is among the most widely used and recommended species for freshwater
320 toxicity testing (Katsumata et al. 2006, Pavlić et al. 2005), for which standard guidelines have
321 already been established (OECD 1984, USEPA 1994). In brief, a culture of *P. subcapitata* was
322 added to a 96-well plate containing increasing dilutions (1:2 dilution series) of the passive
323 samplers extracts and of diuron as reference substance. The effective quantum yield of
324 photosynthesis (see section 2.6.2) was measured after 2 and 24 h of exposure using an Imaging-
325 PAM.

326 2.8. Data analyses

327 Student's *t*-tests were performed to compare physicochemical (n = 4) and functional (n =
328 3) data between upstream and downstream locations in each sampling site. The significance level
329 was set at 5%. In order to assess induced tolerance in periphyton as well as the sensitivity of the

green alga *P. subcapitata* to micropollutants, concentration-effect curves were plotted as a function of decreasing passive sampler dilutions and fitted to a four-parameter logistic equation based on the Hill model, which allowed calculating EC_{50} values. The x-axis of the concentration-effect curves was expressed in unitless relative dilution factor (RDF) of the passive sampler extract and therefore also the EC_{50} values are expressed in RDF. Bootstrap-Monte-Carlo simulations were used to calculate 95% confidence intervals (n = 21). All tests were performed with GraphPad Prism5 software (GraphPad Software, California USA).

Presence and absence matrices of OTUs obtained from the DGGE analysis were used to determine algal and bacterial diversity differences in the sampling sites. Data were submitted to detrended correspondence analysis (DCA). Maximum gradient length was < 3 SDs for all measured parameters, indicating that linear methods were appropriate. Consequently, principal component analysis (PCA) was carried out. PCA allows the ordination of the samples, making it possible to visually assess similarities and differences between samples and determine whether they can be grouped. The closer are the samples to each other along a given axis the more similar they are, and vice versa. DCA and PCA were performed using the CANOCO version 4.5 software.

3. Results and discussion

3.1. Physicochemical measurements and micropollutant analyses

General chemical parameters showed a clear impact of the wastewater discharges on the receiving waters. Indeed, when compared to the corresponding upstream, all downstream locations were characterised by higher conductivity and nutrient loads represented by DOC, dissolved nitrogen, $P\text{-}PO_4$ and SiO_2 (Table S1). The highest differences between upstream and downstream locations were measured at Herisau and Steinach for DOC and dissolved nitrogen,

353 respectively. Additionally, P-PO_4 concentrations markedly increased downstream of Steinach,
354 Herisau and Buttisholz and to a lesser extent at Hochdorf. Micropollutants increased as well from
355 upstream to downstream at all sampling sites, especially for pharmaceuticals and the household
356 chemicals (Table S2, Fig. 2). Similarly to nutrients, the composition and relative concentrations
357 of micropollutants differed among the sampling sites. Downstream of Steinach and Herisau had
358 the highest concentrations of micropollutants for all categories (up to 1 µg L^{-1}) and Buttisholz
359 had the lowest ones except for pesticides that were dominated by the herbicide terbuthilazine (3
360 µg L^{-1}) (Table S2). Altogether, the chemical data indicate that there were significant differences
361 among sampling sites in how WWTP effluents influenced chemical status of the receiving
362 streams. This is due to differences in the land use pattern in the catchments as well as to the
363 treatment technologies that removed differently the nutrients and micropollutants (Burdon et al.
364 2016, Munz et al. 2016).

365 The measured concentrations of micropollutants in the passive sampler extracts from the
366 effluents sites indicated also a dominance of pharmaceuticals and household chemicals within the
367 analysed mixtures followed by herbicides (Fig. 3, Table S3). Interestingly, the herbicidal
368 compounds found in the effluents of Steinach and Herisau are not of agricultural origin but are
369 dominated by two biocides (i.e. diuron and terbutryn) amongst others used to protect facades
370 against algae. Since enrichment in the samplers is varying for different compounds and samplers
371 were exposed to the WWTP effluents, measured concentrations in the extracts cannot directly be
372 compared with the water concentrations downstream (Vermeirssen et al. 2010). However, the
373 detection frequency of the compounds in both samples indicates that passive sampling provides a
374 practical tool for strong enrichment of the real micropollutant mixture of roughly thousand,
375 justifying its use in biological assays as applied also in other studies (Creusot et al. 2014,
376 Vermeirssen et al. 2010).

377 3.2. Effects of wastewater discharges on periphyton

378 *3.2.1. Algal and bacterial tolerance as a specific indicator for micropollutant mixture effects*

379 Increased community tolerance following exposure to micropollutants, as a basis of the PICT
380 concept, is an expected outcome in contaminated ecosystems. As a consequence, it is considered
381 as a reliable indicator to disentangle impacts of organic micropollutants from potential effects of
382 other stressors (Blanck and Wängberg 1988, Guasch et al. 2007, Millward and Klerks 2002, Tlili
383 et al. 2010, Tlili et al. 2016). In the present study, the results show clearly that all downstream
384 periphyton displayed higher tolerance towards the extracted micropollutants from the passive
385 samplers than the communities upstream (Table 1; Fig. S1). Based on photosynthetic yield and
386 algal primary production measurements, calculated EC_{50} values were higher downstream than
387 upstream, reflecting an increased tolerance of the phototrophic communities to the micropollutant
388 mixtures. These results were in agreement with the chemical analyses of the micropollutants.
389 Indeed, compounds targeting phototrophs such as herbicides, were present in the effluents of all
390 assessed WWTPs and their concentrations were higher downstream than upstream. What is more,
391 when tolerance measurements were based on photosynthesis efficiency, fold increase of the EC_{50}
392 values was variable among sites, ranging from 2 to 152, with the highest increase being observed
393 at Buttisholz, followed by Herisau, Steinach and Hochdorf (Table 1). Most striking, differences
394 in herbicide concentrations between upstream and downstream locations followed the exact same
395 order (Table S2, Fig. 2), which clearly reflects the strong correlation between community
396 tolerance and the intensity of contamination by micropollutants.

397 Besides phototrophs, heterotrophs such as bacteria are an important component of periphyton
398 and direct targets for a high number of micropollutants released by WWTPs. Based on bacterial
399 production measurements, the results showed that, except at Herisau for which EC_{50} was higher
400 downstream than upstream location but statistically not significant, EC_{50} values for periphyton

401 from downstream of Steinach, Buttisholz and Hochdorf were significantly higher than EC_{50} for
402 upstream periphyton (Table 1, Fig. S1), reflecting again a significant increased tolerance to the
403 passive sampler extracts, this time for bacteria.

404 As for algae, the fold increase of the calculated EC_{50} values for bacteria differed among sites.
405 Compared to upstream locations, bacterial tolerance in downstream periphyton increased by 86
406 times at Hochdorf, 22 times at Buttisholz and twice at Steinach. These findings concur with the
407 chemical analyses that indicated the presence at higher concentrations downstream of compounds
408 effective on bacteria, such as the antibiotics clarithromycin and sulfamethoxazole, the anti-
409 inflammatory drug diclofenac and the β-blockers atenolol and metoprolol (Table S2). One
410 possible explanation of the low fold increase of bacterial tolerance at Steinach is the lower
411 difference between upstream and downstream concentrations of bactericides at this site compared
412 to Buttisholz and Hochdorf (Table S2). Furthermore, the concentrations of these compounds and
413 especially antibiotics where higher downstream of Hochdorf than downstream of Buttisholz
414 which might explain the 86-fold increase of EC_{50} value at Hochdorf while at Buttisholz it
415 increased by 22 times. The differences among sites for the concentrations of these compounds
416 was confirmed by further measurements presented in Munz et al. 2016. These findings suggest
417 that different fold increase in tolerance reflects different composition and relative concentrations
418 of compounds driving the effects within the micropollutant mixtures.

419 *3.2.2. Shifts in algal and bacterial community structure*

420 A greater community tolerance can result from the replacement of sensitive species by
421 tolerant ones under toxic exposure (termed “toxicant-induced succession” (Blanck 2002)), which
422 can be observed as a change in community structure. In the present study, the principal
423 component analysis applied to the DGGE data (Fig. 4), showed that for bacteria and algae axes
424 PC1 and PC2 together explained more than 63% and 54 % of the total variability, respectively.

PC1 in both cases was clearly related to the sampling location and separated Steinach and Herisau from Hochdorf and Buttisholz (Fig. 1). Such results underline the fact that a combination of local, regional and biogeographic factors might influence the natural variation of stream community composition, as conceptualized by the context-dependency concept (Clements et al. 2016).

Most importantly, either for algae or bacteria, PC2 were clearly correlated to the influence of the wastewater discharge and separated all upstream from downstream locations. In a similar vein, earlier studies have shown changes of the microbial structure in periphyton upon chronic exposures to organic micropollutants (Corcoll et al. 2014, Dorigo et al. 2007). For instance, a shift in bacterial (Dorigo et al. 2010) and algal (Pesce et al. 2011) structure has been observed in periphyton along a contamination gradient by agricultural runoff. Similarly, Corcoll et al. (2014) showed that WWTP effluents altered the phototrophic diversity in periphyton with an increase of the relative abundance of cyanobacteria and a decrease of the relative abundance of diatoms. Clearly, the restructuring of the microbial communities composing periphyton, as observed in our study, cannot be strictly related to the released micropollutants from the effluents. However, the measured increase of algal and bacterial tolerance provides an additional line of evidence that micropollutants were directly responsible for the change in the community structure in the sampling sites by eliminating the micropollutant-sensitive species and favouring the tolerant ones.

3.2.3. Traditional descriptors of periphyton as water quality indicators

Unlike for tolerance measurements, most of the traditional periphyton descriptors such as biomass, photosynthesis or algal and bacterial production did not respond consistently between upstream and downstream locations across the sampling sites (Table 2). Indeed, no significant correlation has been found between the periphyton descriptors and physicochemical

449 characteristics of the stream water from all sites (Spearman's correlation; data not shown). For
450 instance, algal and bacterial productions increased from upstream to downstream of Steinach
451 while it remained unchanged at the other sites. Microbial respiration (SIR) responded also
452 differently among sites and was lower downstream than upstream of Herisau and similar between
453 upstream and downstream of the three other sites. Because of a consistent increase of
454 pharmaceutical and herbicide concentrations downstream of all sites, negative effects on algal
455 and bacterial communities, as target organisms, should be expected across the four sites. At low
456 levels, however, environmental factors such as nutrients, organic matter and increased
457 temperature are beneficial for autotrophic and heterotrophic organisms (Aristi et al. 2015). As a
458 consequence, they could mitigate negative micropollutant effects as it has been conceptualized in
459 the subsidy-stress concept (Wagenhoff et al. 2011). Clearly, such results underscore that
460 traditional descriptors for periphyton are relevant to reflect general alterations of the water quality
461 in streams, but not to disentangle specific impacts of micropollutants from the other
462 environmental factors on freshwater biota and ecosystems.

463 3.3. Sensitivity to photosynthesis inhibitors of periphyton *versus* the green alga *P. subcapitata*

464 Another interesting finding in our study is that based on photosynthesis efficiency
465 measurements, all upstream periphyton were significantly more sensitive to the passive sampler
466 extracts than the single alga *P. subcapitata* (Table 3; Fig. S2). The higher sensitivity of
467 periphyton was even observed at shorter exposure duration than for the alga; i.e. 12 hours and 24
468 hours for periphyton and *P. subcapitata*, respectively. Assessment of the environmental risks
469 posed by micropollutants typically relies on effects on single-species, with a few model
470 organisms, which are extrapolated to ecosystems using safety margins. Although sensitivity of
471 only one algal species has been measured in the present study, results indicate that micropollutant
472 effects could be exacerbated when assays are performed at the community level. Along this line,

McClellan et al. (2008) measured the sensitivity to diuron of freshwater periphyton, using photosynthesis efficiency as descriptor, and showed that effects were not predictable from single-species tests in which species interactions are not considered. Clearly, considering species interactions at the community level is an important aspect to strengthen the assessment of micropollutant impacts in fresh waters.

4. Conclusions

- PICT measurements combined with the use of extracts from passive samplers appear to be very sensitive and efficient to assess the effects of micropollutant mixtures from WWTPs effluents on *in situ* periphyton. This approach greatly increases the environmental relevance of the assessment and allows the establishment of a causality between exposure to complex mixtures of micropollutants and community-level effects.
- PICT field studies can provide a diagnostic tool for retrospective risk assessment of micropollutants from wastewater effluents. Their integration within regulatory frameworks to link between the ecological and chemical status of aquatic ecosystems can lead to more ecological relevance and ecotoxicological specificity in the currently used battery of bioindicators.
- Given the robust link between the proportional increase in tolerance and the intensity of exposure to micropollutants, PICT can be used as an effect-based tool, in combination with passive samplers, to monitor the recovery of impacted streams following for instance the upgrading or the removal of the WWTPs.

Acknowledgment

We thank the AUA Lab and N. Munz (Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland) for the general water chemistry and micropollutant analyses, respectively. We are also thankful to A. Schifferli (Swiss Centre for Applied Ecotoxicology Eawag-EPFL) for the performance of the algal assay, as well as to B. Wagner and R. Britt (Eawag, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland) for the help in the Lab with the periphyton samples and the elaboration of the sampling sites map by GIS, respectively. We also acknowledge the support by all operators of the WWTPs of Steinach, Herisau, Buttisholz and Hochdorf. K. Schirmer, Ch. Stamm and R. Eggen provided valuable comments on an earlier draft of the manuscript, which we greatly appreciate. This work was supported by the Swiss Federal Office for the Environment, FOEN [grant number M202-0817]; and EU FP7 project SOLUTIONS [grant number 603437].

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Table 1. EC_{50} and tolerance ratios (R) for photosynthetic efficiency, primary production and secondary production for upstream (Up) and downstream (Dn) periphyton from the four sampling sites. Higher EC_{50} downstream than upstream means increased tolerance to the micropollutant mixture. The x-axis of the concentration-effect curves was expressed in unitless relative dilution factor (RDF) and therefore also the EC_{50} values are expressed in RDF. Values in parentheses are 95% confidence intervals (n = 21). Ratio EC_{50} was calculated for each site by dividing the EC_{50} Dn by the corresponding EC_{50} Up (R = 1 = no induced tolerance; R > 1 = induced tolerance).

		Photosynthetic efficiency	**Primary production**	**Secondary production**
Steinach	EC_{50} Up	1.9 (1.6 - 2.2)	31 (24 - 37)	28 (14 - 42)
	EC_{50} Dn	6.5 (3.5 - 11.7)	101 (70 - 132)	73 (34 - 112)
	R	3.5	3	2
Herisau	EC_{50} Up	1.5 (0.8 - 2.4)	29 (23 - 36)	118 (11 - 224)
	EC_{50} Dn	6.5 (2.8 -14.5)	98 (37 - 159)	147 (115 - 178)
	R	4.5	3	1
Buttisholz	EC_{50} Up	0.5 (0.3 - 0.8)	89 (75 - 103)	0.4 (0.2 - 0.6)
	EC_{50} Dn	76 (65.5 - 88)	187 (131-242)	9 (2 - 16)
	R	152	2	22
Hochdorf	EC_{50} Up	2.6 (1.3 - 5)	8 (0.4 - 17)	0.3 (0.01 - 0.6)
	EC_{50} Dn	5.7 (3 - 11)	47 (31 - 63)	26 (2 - 50)
	R	2	6	86

Table 2. Measured descriptors of upstream (Up) and downstream (Dn) periphyton from the four sampling sites. Data are means ± standard deviation (n = 3). Asterisks indicate significant differences between Up and Dn samples at each sampling site according to the Student's *t*-test (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

	Steinach		Herisau		Buttisholz		Hochdorf	
	Up	Dn	Up	Dn	Up	Dn	Up	Dn
AFDW ($g\ cm^{-2}$)	1.56 ± 0.55	1.32 ± 0.03	1.20 ± 0.14	1.46 ± 0.53	1.78 ± 0.61	2.05 ± 0.48	2.55 ± 0.20	2.39 ± 0.40
Chlorophyll-a ($mg\ g^{-1}$ AFDW)	9.44 ± 2.30	19.25** ± 1.19	4.53 ± 1.22	13.93** ± 1.82	8.76 ± 1.37	17.53** ± 1.85	6.96 ± 0.26	10.76** ± 1.05
Bacterial biomass ($\mu g\ g^{-1}$ AFDW)	1.04 ± 0.44	2.03 ± 0.90	1.95 ± 0.79	1.62 ± 0.16	0.96 ± 0.57	0.73 ± 0.43	0.85 ± 0.38	0.64 ± 0.06
Quantum yield (ϕ')	0.21 ± 0.01	0.21 ± 0.08	0.23 ± 0.04	0.21 ± 0.01	0.15 ± 0.07	0.16 ± 0.04	0.22 ± 0.03	0.24 ± 0.06
Algal Production ($\mu g\ C\ g^{-1}$ AFDW day^{-1})	10.90 ± 0.91	21.23*** ± 1.41	12.18 ± 2.43	14.13 ± 2.98	11.32 ± 6.10	12.58 ± 7.30	11.66 ± 4.21	12.61 ± 7.46
Bacterial Production ($\mu g\ C\ g^{-1}$ AFDW day^{-1})	2.63 ± 1.42	6.77* ± 1.18	8.29 ± 1.49	17.78 ± 8.56	2.92 ± 0.42	3.79 ± 1.42	12.88 ± 4.20	3.62* ± 0.71
Substrate induced respiration ($\mu g\ CO_2\ g^{-1}$ AFDW day^{-1})	265.15 ± 71.35	323.71 ± 2.62	324.73 ± 17.87	193.61** ± 20.48	317.00 ± 68.64	323.99 ± 66.30	259.94 ± 48.85	267.90 ± 30.29
β-glucosidase ($\mu mole\ MUF\ g^{-1}$ AFDW h^{-1})	20.72 ± 11.79	88.12** ± 17.06	0.59 ± 0.23	16.70* ± 9.26	34.67 ± 15.12	16.37 ± 8.35	15.00 ± 2.90	18.68 ± 3.38
Alkaline phosphatase ($\mu mole\ MUF\ g^{-1}$ AFDW h^{-1})	38.96 ± 15.00	92.94* ± 27.09	81.02 ± 18.92	75.57 ± 12.57	101.90 ± 46.01	40.25 ± 11.21	47.17 ± 7.53	55.88 ± 11.20
Leucine amino-peptidase ($\mu mole\ AMC\ g^{-1}$ AFDW h^{-1})	85.39 ± 23.41	122.60 ± 20.94	121.23 ± 20.06	57.11** ± 9.49	67.97 ± 17.24	38.14 ± 11.48	46.00 ± 6.35	34.27 ± 12.08

Table 3. EC_{50} values based on photosynthetic efficiency for upstream periphyton exposed to a dilution series of the passive samplers extract from the corresponding site during 12 hours and for the green alga *Pseudokirchneriella subcapitata* exposed to the same dilution series of the extract during 2 and 24 hours. The x-axis of the concentration-effect curves was expressed in unitless relative dilution factor (RDF) of the passive sampler extract and therefore also the EC_{50} values are expressed in RDF. Values in parentheses are 95% confidence intervals (n = 21 and 24 for periphyton and *P. subcapitata*, respectively)

	EC_{50} (RDF)		
Passive sampler extract	**Periphyton 12h**	***P. subcapitata* 2h**	***P. subcapitata* 24h**
Steinach	1.9 (1.6 - 2.2)	55.8 (52.1 - 59.8)	46.2 (43.1 - 49.5)
Herisau	1.5 (0.8 - 2.4)	12.8 (12.2 - 13.4)	11.6 (11.1 -12.1)
Buttisholz	0.5 (0.3 - 0.8)	137.8 (131.2 - 144.8)	102.2 (94.7 - 110.4)
Hochdorf	2.6 (1.3 - 5)	11.5 (11.2 - 11.8)	5.6 (5.4 - 5.9)

717 **Figure captions**

718 Fig. 1 Upstream (Up) and downstream (Dn) locations for each of the four studied sites in
719 Switzerland. Values in parentheses are distances in Km between the upstream or downstream
720 location and the corresponding wastewater effluent

721 Fig. 2. Concentrations in ng L^{-1} of the 56 analysed organic micropollutants grouped into four
722 categories (herbicides, pesticides other than herbicides, household chemicals and
723 pharmaceuticals) in the grab water samples from upstream (Up) and downstream (Dn) of the
724 sampling sites.

725 Fig. 3. Concentrations in µg L^{-1} of the 56 analysed organic micropollutants grouped into four
726 categories (herbicides, pesticides other than herbicides, household chemicals and
727 pharmaceuticals) in the passive samplers extracts

728 Fig. 4. Principal component analysis ordination of the bacterial and algal structure in periphyton
729 (n = 3) sampled from upstream (Up) and downstream (Dn) locations of the four sampling site

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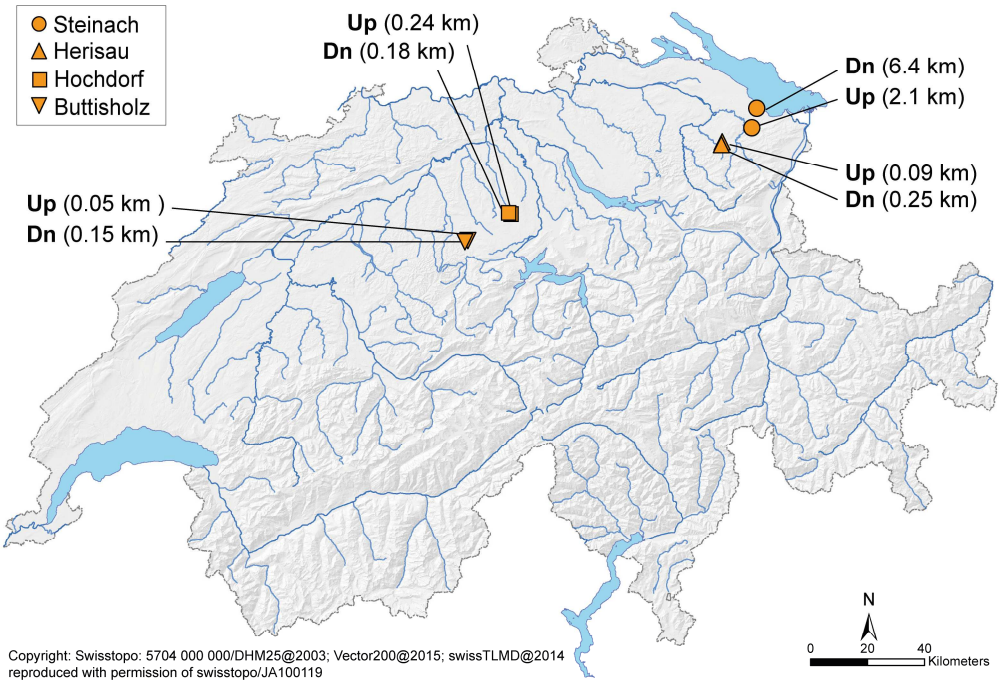

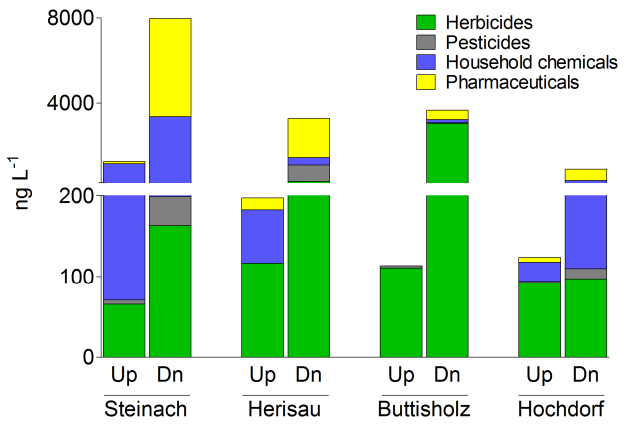

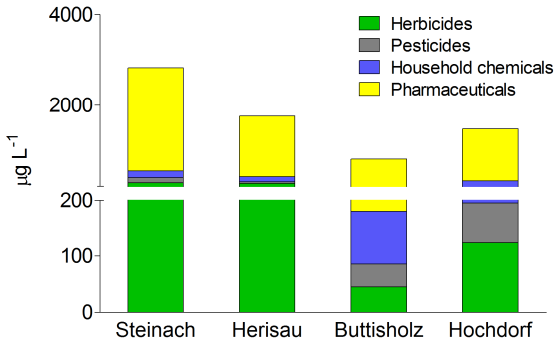

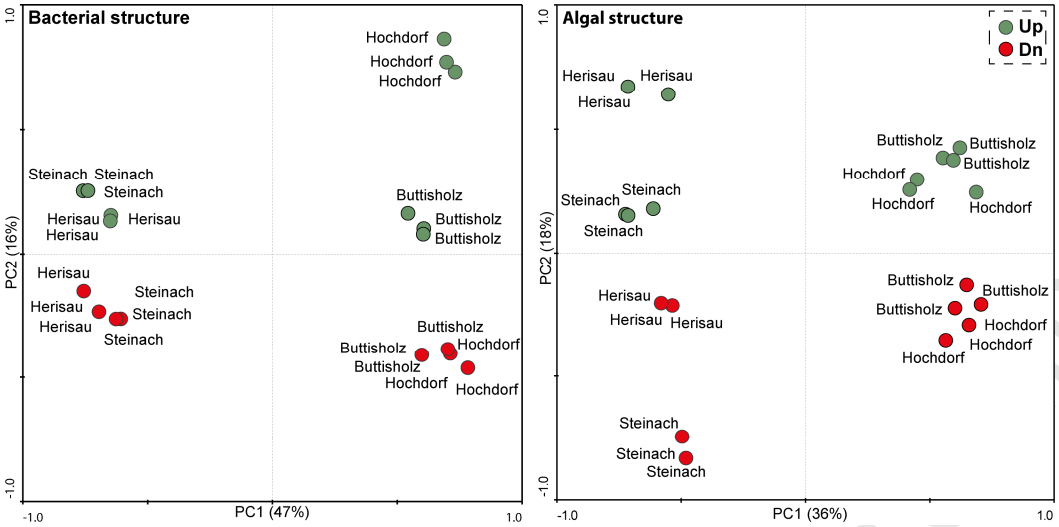

- *In situ* periphyton developed tolerance to micropollutants
- Increased tolerance to micropollutants correlates to the *in situ* contamination
- Microbial restructuring reflects selection of tolerant species to micropollutants