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Impact of wastewater on the microbial diversity of periphyton and its tolerance to micropollutants in an engineered flow-through channel system

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ABSTRACT

Wastewater treatment plants (WWTPs) play an important role in retaining organic matter and nutrients but to a lesser extent micropollutants. Therefore, treated wastewater is recognized as a major source of multiple stressors, including complex mixtures of micropollutants. These can potentially affect microbial communities in the receiving water bodies and the ecological functions they provide. In this study, we evaluated in flow-through channels the consequences of an exposure to a mixture of stream water and different percentages of urban WWTP effluent, ranging from 0% to 80%, on the microbial diversity and function of periphyton communities. Assuming that micropollutants exert a selective pressure for tolerant microorganisms within communities, we further examined the periphyton sensitivity to a micropollutant mixture extracted from passive samplers that were immersed in the wastewater effluent. As well, micropollutants in water and in periphyton were comprehensively quantified. Our results show that micropollutants detected in periphyton differed from those found in water, both in term of concentration and composition. Especially photosystem II inhibitors accumulated in periphyton more than other pesticides. Although effects of other substances cannot be excluded, this accumulation may have contributed to the observed higher tolerance of phototrophic communities to micropollutants upon exposure to 30% and 80% of wastewater. On the contrary, no difference in tolerance was observed for heterotrophic communities. Exposure to the gradient of wastewater led to structural differences in both prokaryotic and eukaryotic communities. For instance, the relative abundance of cyanobacteria was higher with increasing percentage of wastewater effluent, whereas the opposite was observed for diatoms. Such results could indicate that differences in community structure do not necessarily lead to higher tolerance. This highlights the need to consider other wastewater constituents such as nutrients and wastewater-derived microorganisms that can modulate community structure and tolerance. By using engineered flow-through channels that mimic to some extent the required field conditions for the development of tolerance in periphyton, our study constitutes a base to investigate the mechanisms underlying the increased tolerance, such as the potential role of microorganisms originating from wastewater effluents, and different treatment options to reduce the micropollutant load in effluents.

1. Introduction

Wastewater treatment plants (WWTPs) are point sources for complex mixtures of micropollutants, such as pharmaceuticals, pesticides and industrial chemicals (Stamm et al., 2016). Due to the large variety of their chemical structures and modes of biological action, these micropollutants can alter the function and structure of aquatic communities, which may lead to the downgrading of the ecological status of the receiving waterbodies (Gessner and Tlili, 2016; Vörösmarty et al., 2010). A further layer of complexity is added because wastewater discharge is accompanied by an increase of temperature, nutrient loads, organic matter (Petrie et al., 2015) and microorganisms from the

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effluent (Mansfeldt et al., 2020; Mußmann et al., 2013; Pascual-Benito et al., 2020). Disentangling the specific effects of micropollutants from those of the other environmental stressors therefore requires study designs that embrace the chemical and biological complexity within ecosystems, while providing means for controlled interventions. To accomplish such a study design, we constructed a flow-through channel system, which allows for exposure to increasing proportion of treated wastewater and used periphytic communities as a biological model.

Periphyton, also known as benthic biofilm, is dominated by complex and dynamic assemblages of phototrophic and heterotrophic microorganisms, as well as micro- and meiofauna, embedded in an extracellular matrix of organic detritus. Phototrophic microorganisms includes unicellular eukaryotes, such as diatoms and green algae, and prokaryotes, such as cyanobacteria, interacting with heterotrophic bacteria (prokaryotes) (Battin et al., 2016; Biggs et al., 1998). Periphytic communities are ubiquitous in streams of small to moderate sizes in which they play a central role as the basis of aquatic food webs. They contribute to primary production, ecosystem respiration, and element cycling (Battin et al., 2016). With their high microbial diversity, they exhibit a broad range of sensitivities to environmental stressors, including micropollutants. These features and ecological roles support the use of stream periphyton communities as a model to assess micropollutant effects in complex ecological systems (Montuelle et al., 2010; Sabater et al., 2007).

Several studies have demonstrated that exposure to wastewater leads to changes in the taxonomic diversity of periphyton communities (Chonova et al., 2018; Price et al., 2018; Romero et al., 2019; Subirats et al., 2017). Although these studies focused on the bacterial and not on the algal composition in periphyton, they showed that shifts in microbial communities were accompanied by the alteration of important ecological functions, such as algal primary production, heterotrophic respiration or organic matter decomposition. Notwithstanding these findings, such structural and functional changes by themselves cannot be ascribed to specific effects of micropollutants or other stressors that are associated with wastewater discharges (Carr et al., 2005; Lebkuecher et al., 2018). The concept of pollution-induced community tolerance (PICT) has been suggested by Blanck et al. (1988) as a tool to establish such a specific link between exposure to micropollutants and effects at the community level. It rests on the fact that a common response in contaminated ecosystems consists in changes in community composition that result from the replacement of sensitive species by tolerant ones upon chronic exposures of communities to chemical stress (Millward and Klerks, 2002). As a consequence, increased tolerance of a community to micropollutants may be used as an indicator of previous exposures (Tlili et al., 2016). Increased community tolerance to micropollutant mixtures has been demonstrated with stream periphyton communities in the field (Pesce et al., 2011) and in microcosm studies (Foulquier et al., 2015; Kim Tiam et al., 2016; Morin et al., 2012; Rotter et al., 2013).

In recent field surveys, Tlili et al. (2017, 2020) reported on an increased tolerance to micropollutant mixtures of phototrophic and heterotrophic communities in periphyton downstream compared to upstream of several WWTPs. The results showed that the fold increase in tolerance was positively correlated with the level of contamination with micropollutants at the respective sampling sites. Most importantly, a substantial decrease in micropollutant concentrations by 85%, as the result of upgrading the WWTP at one of the sampling sites with activated carbon filtration, led to the loss of the previously measured community tolerance (Tlili et al., 2020). However, these studies did not include phylogenetic data in the analysis of microbial composition and hence did not allow identifying specific taxa that were positively or negatively impacted by wastewater effluents.

Given this background, the overarching goal of this study was to describe the differences in periphytic community composition and diversity that might be associated with the higher tolerance of periphyton exposed to a gradient of treated wastewater. To reach this goal, periphyton was grown in flow-through channels that were continuously alimented with a mixture of stream water and different percentages of an urban WWTP effluent. According to the PICT concept, we then determined the tolerance of periphyton communities towards a micropollutant mixture that was extracted from passive samplers deployed in the wastewater effluent. Moreover, we performed a detailed analysis of the prokaryotic and eukaryotic communities' structure by means of genomic high-throughput sequencing, and a comprehensive quantification of micropollutant concentrations in the water, in passive sampler extracts and in periphyton samples. We hypothesised that periphyton communities will change across the gradient of wastewater constituents, such as nutrients, micropollutants, microorganisms and metals, leading to higher tolerance of the whole community to micropollutants. This increased tolerance was measured specifically via bioassays according to the PICT concept.

2. Material and methods

2.1. Experimental system and design

2.1.1. Channel system, treatments and periphyton colonization

The channel system used in our study corresponds to an indoor version of the "Maiandros" system previously used outdoor by Burdon et al. (2020) (see Supporting Information SI I. for a detailed description). Briefly, this system allows controlled mixing of different waters for comparative experiments in 16 independent flow-through channels (total length of 2.6 m, 0.15 m width and 0.1 m water depth), which are continuously fed with water by means of influent pumps, buffer tanks and mixing units. An additional lightening system that reproduces the sunlight spectrum (Philips Master LED tube HF 1200 mm) was installed to ensure a photoperiod of 12h light: 12h dark. The two influent pumps fed water into the two buffer tanks, one holding stream water and the other holding treated wastewater (see 2.1.2. below), both equipped with a stirrer to avoid settling of particulates. Stream water and wastewater were continuously and fully mixed in the mixing units equipped with static mixers and flow splitters at the respective ratio needed to obtain the targeted nominal percentage of wastewater in our study. The inflow to each channel was 120 L h^{-1}, resulting in a hydraulic residence time of 10 minutes. Each channel was equipped with a paddle wheel providing 0.2 m s^{-1} horizontal flow speed, which corresponds to the lower end of flow velocity observed in the adjacent stream. The 16 channels were randomly assigned to four treatments, corresponding to a nominal proportion of 0, 10, 30 and 80% treated wastewater (N = four replicate channels per treatment) (SI II.1.). Periphyton was grown on clean glass slides (210 × 75 × 4 mm) that were directly placed vertically in the channels (40 glass slides per channel). After 28 days, colonized glass slides were retrieved and immediately transported to the laboratory for biological analyses (see 2.2.2 below)

2.1.2. Water sources

The channels were continuously fed with a mixture of stream water that was directly pumped from Chriesbach, a small peri-urban stream (47°24′16.7"N 8°36′41.4"E; Dübendorf, Switzerland) and treated wastewater, thereafter called "wastewater". The untreated wastewater originated from a local municipal sewer in the catchment of Dübendorf, on a branch serving 20'000 persons plus some food processing industry. Untreated wastewater followed a standard activated sludge treatment for nitrification and denitrification, without chemical phosphorus precipitation. The treatment plant was pilot size for ca. 100 person equivalent, equipped with 5 mm grit removal, sand trap, primary clarifier and sequencing batch reactor treatment operated with pre-denitrification. Physicochemical characteristics of the treated wastewater are given in SI Tables 1 and 2.

2.2. Sampling

2.2.1. Sampling of water for analysis of physicochemical properties and micropollutants

The pH, temperature, conductivity and oxygen concentration were measured daily in the 2 buffer tanks and in the 16 channels by using a multi-parameter portable meter (WTW Meters, Germany). Additionally, water samples were taken every week in the two buffer tanks (stream water and wastewater) and in the 16 channels for the measurement of 20 other water quality parameters (see SI Table 2 for detailed list of parameters), using standard methods as described by the Swiss National River Monitoring and Survey Programme (FOEN, 2020).

A total of 51 organic micropollutants, consisting of 21 pesticides, 24 pharmaceuticals, 3 artificial sweeteners, 2 corrosion inhibitors and caffeine (a tracer of sewage effluent in natural waters) were analysed in grab and in 24-hour composite water samples, as well as in extracts from passive samplers (SI II.1. and SI Table 3). The substances were originally selected by Munz et al. (2017), who established a list of priority substances based on a large survey in 24 Swiss streams that are impacted by wastewater effluents. The selection criteria included detection frequency, concentrations in municipal wastewater, toxicity, analytical restrictions and substance classes. This mixture was also studied by Tlili et al. (2017) to investigate the tolerance of periphyton upstream and downstream of five Swiss WWTPs.

The channel system guaranteed a homogenous mixture of stream water and wastewater in each channel. Therefore, 1.4 mL of the grab water samples can be considered representative for the analysis of water soluble compounds. These samples were taken weekly in each channel and stored in 1.5 mL clear glass vials (short thread with screw caps septa silicone/PTFE, BGB, Switzerland) at -20°C until analysis. Preliminary results showed negligible variability among replicates, thus, only one channel has been selected for micropollutant analyses. The composite samples were also taken weekly from the stream water and wastewater during 24-hours with an automated water sampler (Maxx, TP5 C Aktiv, Germany). The water was automatically sampled (50 mL every 30 minutes), pooled together and 1.4 mL of each 24-hour sample was stored in 1.5 mL clear glass vials at -20°C until chemical analysis. Composite samples from a dry weather and a rain weather period were selected for each week. In parallel, two AttractSPE®Disks SDB-RPS (47 mm diameter, Affinisep, France) with Supor® polyethersulfone (PES) membrane disc filters (47 mm diameter, 0.45 µm pore size, VWR, Switzerland) were installed in two channels of each treatment to sample polar to semi-polar organic micropollutants according to Moschet et al. (2015). Two passive samplers from two different channels were selected from each sampling period for micropollutant analyses for each treatment (n=4). The same type of passive samplers but without PES membrane were also deployed in the wastewater buffer tank and the accumulated micropollutants were extracted and used for the PICT bioassays (Tlili et al., 2017). Prior to their deployment, all passive samplers were conditioned with methanol and then with nanopure water for 30 min each on a rotary shaker. Then, the SDB disks were placed on a steel plate, covered or not by the PES membrane, closed by a cover plate, and stored in nanopure water at room temperature. In order to remain in the linear adsorption range (Moschet et al., 2015), the passive samplers were deployed during twelve days and renewed for a second twelve day period.

2.2.2. Sampling for biological analyses

Periphyton growing on five glass slides from the same channel was scraped, pooled and suspended in 200 mL of Evian natural water (SI II.2.). Stock suspension was kept for periphyton characterization and PICT bioassays; the remaining volume was lyophilized before micropollutant analysis and Carbon:Nitrogen:Phosphorus (C:N:P) ratio determination. The tolerance of periphyton to micropollutants accumulated in the passive samplers deployed in the wastewater buffer tank was assessed according to the PICT concept, by measuring the inhibition of algal primary production, photosynthetic efficiency and bacterial secondary production (see 2.3.2. and 2.3.3.).

2.3. Chemical and biological analyses

2.3.1. Micropollutants in water and in periphyton

Passive samplers from the channels were prepared and extracted according to Moschet et al. (2015) with few modifications (SI III.1.). Micropollutant concentrations in the passive sampler extracts were then used to derive theoretical concentrations in the water by using the sampling rates (R_S) established by Moschet et al. (2015). Briefly, the actual micropollutant concentration in 1 mL extract (ng L^{-1}) was first converted to the extractable amount of micropollutant (ng) per passive sampler. Then, this value was further multiplied by the corresponding R_S value (L day^{-1}) and the deployment period of 12 days, resulting in a theoretical concentration in water (ng L^{-1}). Micropollutants were also analysed in the passive sampler extract used for the PICT bioassays (see Tlili et al., 2017) for the detailed description of the extraction). Concentrations of micropollutants accumulated in periphyton (44 of the 51 micropollutants analysed were quantifiable) were measured at the end of the colonization period of 28 days for each channel replicates after extraction by a Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method as described by Munz et al. (2018) with some modifications (SI III.2.). Micropollutant analysis in all samples was performed by HPLC-MS/MS (SI III.3.).

2.3.2. Periphyton characterization

The detailed description of each procedure used to characterise periphyton is provided in SI III.4. Total biomass was determined as ash free dry weight (AFDW) as described in Tlili et al. (2008). Chlorophyll-a content was additionally used as a proxy for algal biomass (Sartory and Grobbelaar, 1984). Bacterial biomass was estimated according to Frossard et al. (2012) with few modifications (SI III.4.1.). Freeze-dried periphyton samples were analysed for total carbon and total nitrogen by using an elemental analyser (HEKAtech Euro Elemental Analyzer; HEKAtech GmbH, Wegberg, Germany). Total phosphorus in freeze-dried periphyton was determined by colorimetry of the reduced phospho-molybdate blue complex (Murphy and Riley, 1962) on a San Plus SKALAR System analyser after an additional digestion step. This consisted of mixing each periphyton sample with 10 mL of digestive reagent (10 g L^{-1} potassium persulfate, 1.5 g L^{-1} sodium hydroxide) and heating at 121°C for 2 h.

Photosynthetic efficiency was assessed by 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 light saturation pulse to calculate the effective quantum yield (ϕ') as:

$$\phi' = \frac{F'm - F'0}{F'm} \quad (1)$$

where F'_m is the maximum fluorescence after the saturation pulse and $F'0$ is the steady-state fluorescence. Primary algal production was measured via ^{14}C-carbonate incorporation rate as described in Dorigo and Leboulanger (2001) with few modifications (SI III.4.2.). Secondary bacterial production was measured via ^{14}C-leucine incorporation into protein according to Buesing and Gessner (2003) with few modifications (SI III.4.2.). Microbial substrate-induced respiration (SIR) of the heterotrophic periphyton component was measured by using the MicroResp™ technique and glucose as carbon source following the procedure described in Tlili et al. (2011) and detailed in SI III.4.2.

2.3.3. PICT assays

Tolerance to micropollutants from the wastewater was determined in periphyton from each treatment via short-term exposure assays with serial dilutions of extracts of the passive samplers that had been deployed in the wastewater buffer tank (SI II.2). A logarithmic series of

six dilutions of the pure micropollutant extract was freshly prepared from the stock solution in Evian mineral water, using a dilution factor of 3.16, resulting in the following relative dilution factor (RDF) of the micropollutant extract: 1000 (pure extract), 313, 98, 31, 10 and 3. Fifty mL periphyton suspensions were prepared from the stock periphyton suspension by adjusting the optical density at 685 nm to 0.4. An aliquot of 4.5 mL of each suspension was then exposed in 20 mL glass vials (Econo glass vials with Foil-Lined Urea Screw Cap, PerkinElmer, Switzerland) to 0.5 mL of each of the six dilutions of the extract for 4 h. In addition, two controls were prepared. One consisted of the periphyton suspension and 0.5 mL of mineral water (chemical-free control) and the second of periphyton and 0.5 mL of 37% formaldehyde (i.e., formaldehyde control), the latter being used to determine the background activity. Subsamples from each vial were taken for algal primary production, bacterial secondary production and photosynthetic efficiency measurements, respectively, as described in 2.3.2.

2.3.4. Next generation sequencing for prokaryotic and eukaryotic community compositions

2.3.4.1. DNA extraction, library construction and sequencing. In order to compare the diversity of prokaryotes (i.e. bacteria, including cyanobacteria) and eukaryotes (e.g. diatoms and green algae) in periphyton and in wastewater, total genomic DNA was extracted from an aliquot of 2 mL from each periphyton suspension and from 100 mL wastewater samples taken regularly (3 times per week) during the experiment. The samples were centrifuged at 14'000 *g* for 30 min at 4°C and the pellets stored at -80°C until their analyses. DNA extraction was performed by using the Power-Biofilm DNA Isolation Kit (MO BIO Laboratories, CA) following the manufacturer's instructions. Total DNA was then quantified with a Qubit (1.0) fluorimeter following the recommended protocol for the dsDNA HS Assay (Life Technologies, Carlsbad, CA, USA). An extraction negative control was also included by using PCR-grade water as a starting material.

Library construction consisted in a two-step PCR process (SI III.5). The first PCR amplified the V3-V4 region of the 16S rRNA gene for prokaryotes and the V4-V5 region of the 18S rRNA gene for eukaryotes, using two different primer sets with overhang adapters from Herlemann et al. (2011) and Hugerth et al. (2014), respectively (SI Table 4). The first PCR was performed in triplicate for each DNA sample, negative PCR controls, as well as positive PCR controls for 16S rRNA and 18S rRNA, consisting of mock communities (SI Table 5). The second PCR, consisting in a limited-cycle amplification, was carried out to add multiplexing indices and Illumina sequencing adapters. The libraries were then normalized and pooled to achieve a 1.86 nM concentration. Paired end (2 × 300 nt) sequencing was performed on an Illumina MiSeq (MiSeq Reagent kit v3, 300 cycles) following the manufacture's run protocols (Illumina, Inc.). The MiSeq Control Software Version 2.2, including MiSeq Reporter 2.2, was used for the primary analysis and the de-multiplexing of the raw reads. All raw sequences are available at the National Center for Biotechnology Information (NCBI) under the SRA accession ID PRJNA699298.

2.3.4.2. Sequencing data processing, amplicon sequence variants binning and taxonomic assignment. The reads were checked for quality and end-trimmed by using FastQC v0.11.2 (Andrews, 2010) and seqtk (https://github.com/lh3/seqtk), respectively. For 16S rRNA, the reads were merged using FLASH v1.2.11 (minimum and maximum overlap of 15 and 300 bp, respectively; maximum mismatch density of 0.25) (Magoč and Salzberg, 2011) while only reads obtained with the forward primer were considered for 18S rRNA. The primers were trimmed by using cutadapt v1.12 (wildcards allowed; full-length overlap; error rate 0.01) (Martin, 2011). Quality filtering was performed with PRINSEQ-lite v0.20.4 (minimum quality mean 20; no ambiguous nucleotides; dust low-complexity filter with a threshold of 30) with a subsequent size and GC selection step (size selection range 330–600 bp; GC selection range 30–70%) (Schmieder and Edwards, 2011). The reads were processed with an Amplicon Sequencing Variants (ASV) analysis (Callahan et al., 2017). The sample reads were first denoised into ASVs with UNOISE3 in the USEARCH software v.11.0.667. The final predicted taxonomic assignments were performed with the SILVA v128 (16S rRNA) and the NCBI v200131 based 18S sequence databases (SI III.6) by using SINTAX in the USEARCH software v.11.0.667 (Edgar, 2016). The total reads obtained at each step of bioinformatic filtration are reported in SI Table 6.

2.4. Data analyses

In order to assess tolerance in periphyton induced by wastewater, concentration-activity curves of the four biological replicates (N = 4 channels) of each treatment were plotted as a function of decreasing passive sampler extract dilutions. The background measured in the formaldehyde controls was subtracted from each activity value. The final value was normalised by the activity measured in the micropollutant-free control. For each treatment, the data corresponding to four biological replicates and 6 passive sampler dilutions ($20 < n < 24$) were then fitted with the DoseResp function of the OriginPro 2016 software (Origin Lab Corporation, USA) characterized by the following equation:

$$E(x) = \frac{100}{1 + 10^{(EC50 - x) * p}} \quad (2)$$

where x is the relative dilution factor (RDF) of the micropollutant extract, $E(x)$ the normalized activity measured for each endpoint, and the two parameters fitted are $EC50$, the half maximal effective concentration leading to a 50% decrease of the activity, and p, the Hill slope. An arbitrary value of RDF = 1000 was set for the pure passive sampler extract. EC_{20} was also derived from each dose-response curve as an effective concentration for sub-lethal effects.

Significant differences among the treatments for the periphyton descriptors (i.e., AFDW, chlorophyll-a content, bacterial biomass, quantum yield, primary production, secondary production, SIR, C:N:P molar ratios and taxonomic abundance) and water physicochemical parameters were assessed using one-way ANOVA followed by separate post hoc comparisons (Tukey's test, $\alpha = 0.05$). The tested factor was the treatment (four modalities: 0%, 10%, 30% and 80% wastewater). Normality and homogeneity of variance were checked prior to ANOVA analysis (Kolmogorov-Smirnov's and Levene's tests, respectively, $\alpha = 0.05$). Data that were not normally distributed were transformed using logarithmic or Box-Cox functions. Statistical analyses were carried out in R 3.6.1 by using RStudio (Version 1.2.5001).

Sequencing data analyses were performed with the R package Phyloseq version 1.32.0 (McMurdie and Holmes, 2013). After rarefaction, 16S and 18S rRNA datasets were composed of samples containing the same number of reads (98,089 and 97,001 reads, respectively). Alpha diversity (i.e., richness and evenness of a given periphyton community) was evaluated for each wastewater proportion via Shannon diversity index and Chao1 species richness with the package Phyloseq. The analysis of beta-diversity (i.e., measuring the structural differences among several communities) was based on weighted unifrac distances, which use the phylogenetic distances between taxa and their relative abundances. Permutational Multivariate Analysis of Variance (PERMANOVA) tests were carried out on the weighted unifrac distances matrix of prokaryotic and eukaryotic communities using the R package "vegan". After testing homogeneity in prokaryotic and eukaryotic datasets dispersion, the adonis function was used to test the null hypothesis to see if experimental treatments shared similar centroids. Additional pairwise comparisons were carried out by using the pairwise.adonis function (Martinez Arbizu, 2020). Graphical representations were generated with the R package "ggplot2".

3. Results

3.1. General water physicochemical parameters

Physicochemical parameters differed between stream water and wastewater (SI Tables 1 and 2). The large majority of these parameters (i.e., temperature, conductivity, alkalinity, Na^+, K^+, Cl^-, SO_4^{2-}, NH_4^+, NO_2^-, *ortho*-phosphate, total phosphorus, silicic acid, dissolved organic carbon, total organic carbon, total nitrogen and total inorganic carbon) were significantly higher in wastewater than in stream water while the opposite was observed for pH and oxygen (ANOVA, $P < 0.001$). The Principal Component Analysis (PCA) of the entire physicochemical dataset clearly distinguishes the four groups corresponding to 0, 10, 30 and 80% of wastewater from the stream and wastewater buffer tanks at each sampling time (Fig. 1). This distinction was seen throughout the four weeks of experimentation despite the fact that the physicochemical parameters fluctuated over time (SI Tables 1 and 2), most likely due to rain events.

3.2. Micropollutants in water and periphyton

Stream water and wastewater were continuously sampled during the 4-week colonisation period for micropollutant analysis in composite samples. Most of the target substances were found in both water sources (SI Fig. 1 and SI Table 7). The total concentrations of each substance group was higher in wastewater than in stream water. For instance, pharmaceuticals, artificial sweeteners (i.e., acesulfame, cyclamate and sucralose) and corrosion inhibitors (i.e., benzotriazole and 5-methylbenzotriazole) were, on average, 20 times higher in the wastewater (SI Fig. 1 and SI Table 7).

Micropollutant concentrations in the channels were determined either in grab water samples (SI Table 8) or in the passive sampler extracts before back-calculating average concentrations in the water as described by Moschet et al. (2015) (SI Tables 9A and 9B). Micropollutant concentrations overall correlated between grab and passive samples (Pearson's $r = 0.88$, $P < 0.001$) except for eight pharmaceuticals and eight pesticides, including DEET and pirimicarb (only quantified in passive sampler extracts), and the pharmaceutical gabapentin (only quantified in grab samples) (SI Tables 8 and 9B). Total concentration of micropollutants in the channels increased with the wastewater proportion for all substance groups (Fig. 2). For instance, the concentrations of the two Photosystem II (PSII) inhibitors, diuron and isoproturon, seemed to increase by a factor of eight and ten, respectively, from 0 to 80% wastewater treatments (SI Table 9B).

The total micropollutant concentration in periphyton was as well increased with increasing wastewater proportion (Fig. 2), indicating that periphyton was able to accumulate micropollutants from the water phase. For instance, although only five out of the 21 targeted pesticides were detectable in periphyton, the concentrations of three of them, diuron, isoproturon and terbutryn, were approximately 1.5 to 3 times higher in the 80% wastewater treatment compared to the control (SI Table 10). These three compounds displayed the highest BCFs of all the compounds analysed (Table 1). However, the relative proportion of each substance group (Fig. 2) and detection frequency of each substance in periphyton (SI Table 10) differed strongly from those found in the water and the passive samplers (SI Table 9B). An example are artificial sweeteners whose relative proportion decreased in periphyton, whereas the opposite was observed for pesticides. For artificial sweeteners, the absence of matrix effect was checked by assigning the value of the limit of quantification (LOQ) of each compound when its concentration was <LOQ.

3.3. Periphyton characterization–biomass, nutrient stoichiometry and functions

Despite the measured increase in nutrient concentrations in the channels with increasing wastewater proportion, periphyton biomass (total, algal, bacterial) did not differ among the treatments (Table 2, ANOVA, $P = 0.0632$, $P = 0.1662$ and $P = 0.2099$, respectively). Importantly, the increase of available nutrients in the water phase did not impact the stoichiometry of nutrients in periphyton, as shown by the lack of significant differences among the treatments in the elemental ratios C:N, C:P and N:P (Table 2, ANOVA, $P = 0.4392$, $P = 0.265$ and $P = 0.2962$, respectively). Furthermore, the C:N:P ratios were about 126:18:1 and therefore closer to the one described for algae [106:16:1, Redfield et al. (1963)] than for bacteria [45:9:1, Goldman et al. (1987)].

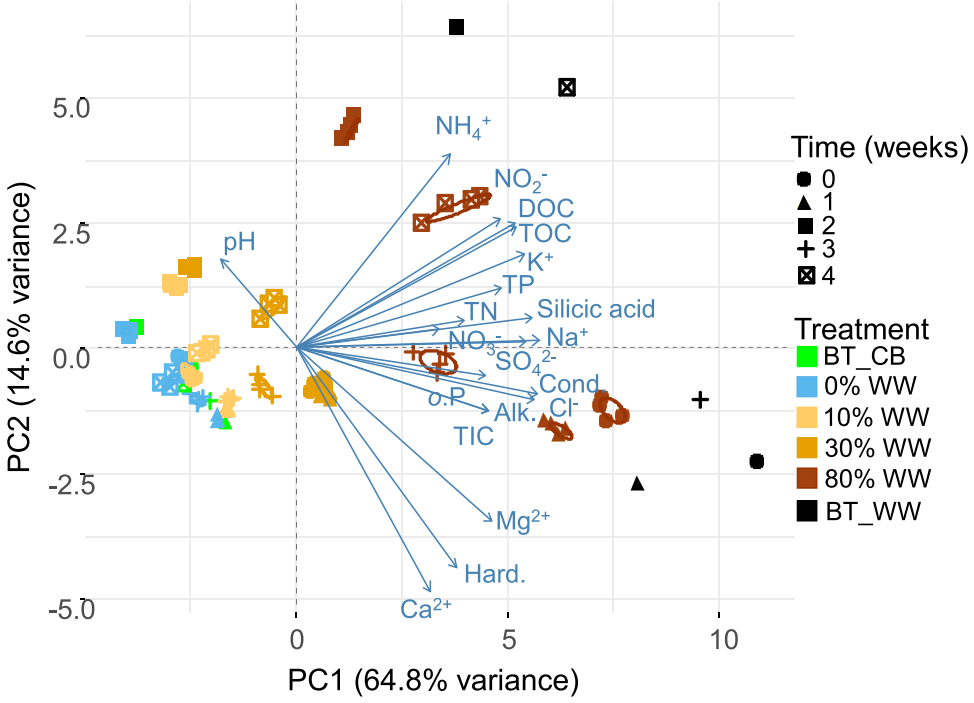

Fig. 1. Principal component analysis of water physicochemical parameters, measured every week in the channels and the two buffer tanks (stream and wastewater). A total of 21 variables were accounted for the analysis, including DOC (Dissolved Organic Carbon), TOC (Total Organic Carbon), TIC (Total Inorganic Carbon), TN (Total Nitrogen), TP (Total Phosphorus), *o*-P (*ortho*-Phosphate), Cond. (Conductivity), Alk. (Alkalinity) and Hard. (Hardness). The variables were normalized by the function PCA() of the R package FactoMineR. BT_CB: Chriesbach (stream) buffer tank; BT_WW: wastewater buffer tank. The other treatments correspond to the channels alimented with 0 (control), 10, 30 and 80% wastewater (WW), respectively. The 95 % confidence ellipse was added for each treatment at a given sampling time (weeks). Non-overlapping ellipses indicate significantly different treatments.

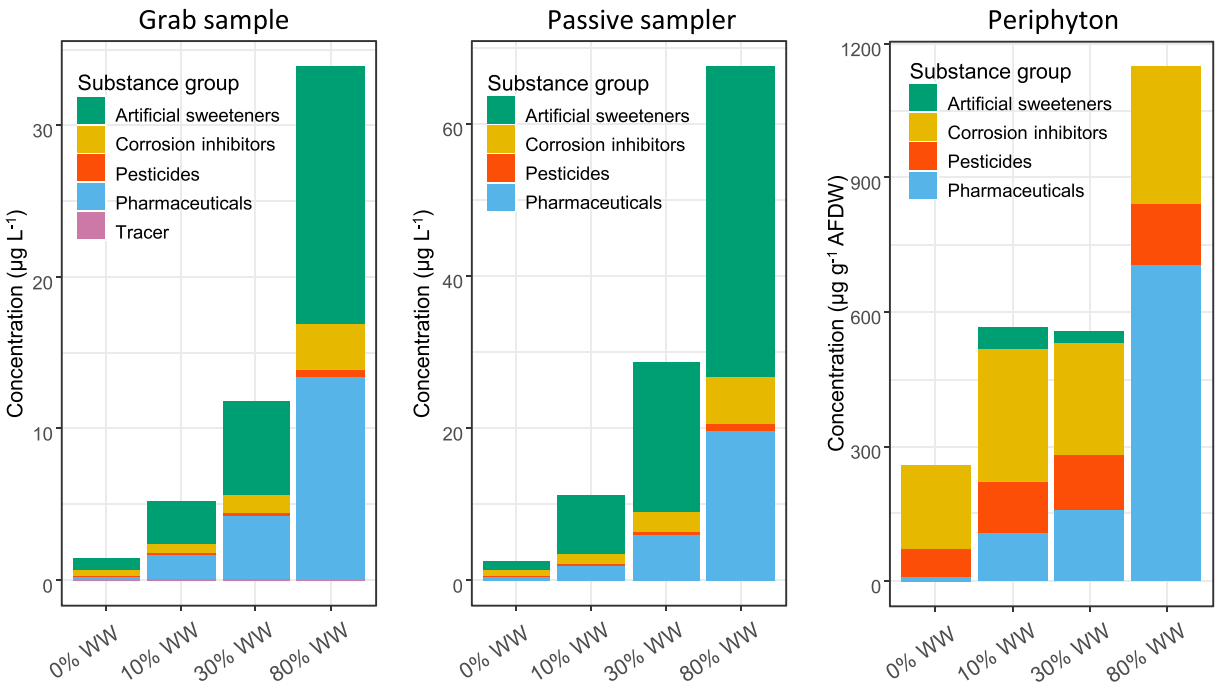

Fig. 2. Mean concentrations of the micropollutants analysed in the water (grab water samples and passive sampler extracts) and periphyton from the artificial streams. The total concentration of each substance group was calculated for each wastewater proportion (0%, 10%, 30% and 80% WW). Fifty-one substances were quantifiable in the water grab samples: 3 artificial sweeteners, 2 corrosion inhibitors, 21 pesticides, 24 pharmaceuticals and one tracer. Forty-seven substances were quantifiable in the passive sampler extracts: one artificial sweeteners, 2 corrosion inhibitors, 20 pesticides and 24 pharmaceuticals. Forty-four substances were quantifiable in periphyton: 3 artificial sweeteners, 2 corrosion inhibitors, 17 pesticides and 22 pharmaceuticals. For the passive sampler extract, micropollutant concentrations in water were calculated from SI Table 9A with the sampling rates, R_S, provided in Moschet et al. (2015). $N_{(grab\ samples)}$ = one replicate per week and treatment, $n_{(passive\ samplers)} = 2$ replicates per sampling period and treatment and $n_{(periphyton\ samples)} = 4$ replicates per treatment. See SI Tables 8, 9B and 10 for the standard deviations.

Among the measured functional endpoints, only bacterial secondary production significantly differed upon exposure to 30% wastewater compared to the control (0% wastewater) while photosynthetic activity differed between 30 and 80% wastewater (Table 2).

3.4. Community tolerance to micropollutants

Tolerance of periphyton was assessed via short-term assays with a micropollutant mixture extracted from the passive samplers that had been deployed in the buffer tank holding the wastewater. A higher tolerance of phototrophs was found for periphyton exposed to wastewater (Table 3 and SI Fig. 2). Specifically, the EC_{20} and EC_{50} values for photosynthetic efficiency were higher for the periphyton from 30% and 80% treatment than for the control (0% wastewater). For algal primary production, EC_{20} and EC_{50} values were significantly higher in the 80% wastewater treatment thanin the control, corroborating an increased tolerance of phototrophs. In contrast, secondary production, which is associated with heterotrophs, did not indicate any wastewater related tolerance, even with the highest tested micropollutant concentration (SI Fig. 2).

3.5. Diversity and taxonomic abundance of periphyton communities

In order to investigate the impact of wastewater on community composition, 18S and 16S rRNA gene sequencing was used, focussing on the richness, diversity and composition of eukaryotes (e.g. diatoms and green algae) and prokaryotes (e.g. cyanobacteria), respectively. Alpha diversity, i.e. the taxonomic richness and Shannon diversity index, of prokaryotes was higher in the 30% wastewater (Tukey's test, $P < 0.05$) whereas it did not differ from the control for the 80% wastewater (Fig. 3A and B). For eukaryotes, a similar trend was observed for taxonomic richness but not for diversity (Fig. 3C and D). Except for the eukaryotic Shannon index (Fig. 3D), alpha diversity in the community isolated from the wastewater buffer tank was similar to that of periphyton from the 80% wastewater treatment.

Wastewater also led to a separation in the beta-diversity of prokaryotic and eukaryotic communities (PERMANOVA, $P < 0.001$, Fig. 4). This separation increased with wastewater proportion, as attested by the significant differences between each periphyton group (pairwise PERMANOVA, $P < 0.05$).

In terms of abundance of taxa, a total of 36 prokaryotic and 34 eukaryotic phyla were identified in periphyton and the community sampled from the wastewater buffer tank (SI Figs. 3 and 4). The notable proportion of taxa without affiliation (NA) for eukaryotes reflects the lack of information in the databases in comparison to those for prokaryotes. The relative abundance of the ten most abundant prokaryotic and eukaryotic phyla in periphyton and wastewater communities is shown in Fig. 5.

Wastewater effluent differentially influenced the relative abundance of prokaryotic phyla in periphyton (Fig. 5A). Among the six phyla that were higher with increasing wastewater proportion (Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria and Parcubacteria), cyanobacteria (i.e. phototrophic prokaryotes) stood out in particular because their proportion was higher in periphyton with wastewater despite the fact that this phylum was present at low abundance in the wastewater buffer tank (Fig. 5A). A detailed analysis at the class level of Proteobacteria in the bacterial community shows that the relative abundance of Alpha- and Beta-proteobacteria decreases with wastewater proportion while the opposite is observed for Delta- and Epsilon-proteobacteria (SI Fig. 5).

Several eukaryotic phyla (Annelida, Platyhelminthes and Zoopagomycota) were also higher with wastewater proportion even though, similarly to cyanobacteria, their abundance in the wastewater buffer tank was relatively low (Fig. 5B). In contrast, Cercozoa and Discocea

Table 1
Ratios of micropollutant concentrations in periphyton and water, together with the LogKow (Log(octanol/water partition coefficient)) of each compound. For each compound, the bioconcentration factor (BCF) was calculated as the ratio between the average concentration in periphyton (in ng g^{-1} AFDW) and the average concentration in water (in ng L^{-1}) from the 80% wastewater channels. * compound detected only in periphyton but not in water (concentration in water <LOQ).

Substance group	Compound	BCF	LogKow
Artificial sweeteners	Acesulfame	0	-1.33
	Cyclamate	0	-1.61
	Sucralose-FA	0	-1
Corrosion inhibitors	4/5-Methylbenzotriazole	0.03	1.89
	Benzotriazole	0.15	1.44
Pesticides	2-6-Dichlorbenzamide	0	0.77
	Carbendazim (Azole)	0.09	1.52
	Chloridazone-methyl-desphenyl	0	-1.37
	Chlortoluron	0	2.41
	Diazinon	0	3.81
	Dimethenamid	0	2.15
	Dimethoate	0	0.78
	Diuron	0.63	2.68
	Epoxiconazole	0	3.3
	Fipronil	0	4
	Isoproturon	0.4	2.87
	Mecoprop	0	3.13
	Metamitron	0	0.83
	Pirimicarb	0	1.7
	Propiconazole	0*	3.72
	Tebuconazole	0	3.7
	Terbutryn	3.62	3.74
Pharmaceuticals	4-Acetamidoantipyrine	0.05	0.15
	Amisulprid	0.14	1.06
	Atenolol	0	0.16
	Candesartan	0.09	4.79
	Carbamazepine	0.04	2.45
	Cetirizine	0.05	0.89
	Clarithromycin	0.2	3.16
	Diclofenac	0.08	4.51
	Gabapentin	0	-1.1
	Hydrochlorothiazide	0.07	-0.07
	Lamotrigine	0.06	2.57
	Lidocaine (Diocaine)	0.26	2.26
	Mefenamic acid	0.12	5.12
	Metoprolol	0.31	1.88
	Naproxen	0	3.18
	Oxazepam	0.08	2.24
	Sitagliptin	0.08	1.39
	Sotalol	0.07	0.24
	Sulfamethoxazole	0	0.89
	Sulfapyridine	0	0.35
	Trimethoprim	0.07	0.91
	Venlafaxine	0.33	3.2

were dominant in the wastewater community but low in abundance in any of the artificial streams. Bacillariophyta (diatoms) and Chlorophyta (green algae) negatively correlated with the wastewater proportion (Fig. 5B). However, a detailed analysis at the genus level showed that responses to the wastewater exposure differed within each of these two phyla (SI Figs. 6 and 7). For instance, while several diatoms (e.g. *Cocconeis* and *Nitzschia*) and green algae (e.g. *Chlorochytrium* and *Scenedesmus*) genera were not impacted, the relative abundance of the diatom, *Ulnaria,* and the green algae, *Mychonastes,* were significantly higher in periphyton with the increase of wastewater proportion (SI Figs. 6 and 7).

To shed light on the partitioning of taxa among periphyton and the community isolated from the wastewater buffer tank, Venn diagrams were constructed (Fig. 6). In terms of prokaryotes (Fig. 6A), periphyton-specific taxa dominated in all treatments by at least 64% relative abundance. The taxa shared with the community from the wastewater buffer tank increased in relative abundance from 19% (0% wastewater) to 34% (80% wastewater). In terms of eukaryotes (Fig. 6B), periphyton-specific taxa again dominated in relative abundance for all treatments but the proportion shared with that of the community in the wastewater buffer tank was higher than for prokaryotes, reaching up to 53%.

4. Discussion

4.1. Validation of the experimental system

By mixing treated wastewater and stream water proportionally, the pre-set percentages of wastewater were approximately reached. This was confirmed by conductivity measurements: experimental proportions of 11± 8% and 23 ± 9% wastewater were calculated for the intended 10 and 30% wastewater proportions, respectively (SI Fig. 8). The general water chemistry data also indicate that our experimental system allowed exposing periphyton to distinct proportions of wastewater in the channels. Overall, these results show that the experimental system provides for a purposeful control of mixing stream and wastewater, while integrating the natural variations as would be typical in field situations with changes in environmental conditions (e.g. rain events). The slight deviations from the intended mixture values resulted from technical issues, such as accumulation of solid particulates in the flow rotameters. Indeed, particulate accumulation required daily cleaning and re-adjustment of the mixture according to conductivity values. This problem will be addressed in the future by setting up an online monitoring system for constant, automatic fine-tuning of the target mixture value.

Table 2
Descriptors of periphyton from the four experimental treatments. Data are means ± standard deviation from four replicate channels per treatment (N = 4). Significant differences between treatments are indicated by lower case letters (a < b, Tukey's test, P < 0.05). Significant difference between wastewater treatments and the control (0% wastewater) is marked in bold. The treatments correspond to periphyton grown in the presence of 0% (control), 10%, 30% and 80% wastewater (WW), respectively.

		0% WW	10% WW	30% WW	80% WW
Biomass					
	Ash-free dry weight (mg cm^{-2})	0.4 ± 0.02 (a)	0.3 ± 0.06 (a)	0.4 ± 0.02 (a)	0.3 ± 0.04 (a)
	Chlorophyll-a (mg g^{-1} AFDW)	25.6 ± 1.8 (a)	24.8 ± 4.0 (a)	24 ± 1.4 (a)	20.1 ± 5.2 (a)
	Bacterial biomass (µg C g^{-1} AFDW)	0.4 ± 0.06 (a)	0.5 ± 0.2 (a)	0.5 ± 0.2 (a)	0.5 ± 0.06 (a)
Nutrient ratio					
	Carbon:Nitrogen molar ratio	7 ± 0.4 (a)	7 ± 0.6 (a)	6.4 ± 0.4 (a)	7 ± 2 (a)
	Carbon:Phosphorus molar ratio	134 ± 45 (a)	140 ± 28 (a)	118 ± 14 (a)	115 ± 31 (a)
	Nitrogen:Phosphorus molar ratio	19 ± 6 (a)	19 ± 2 (a)	19 ± 1.4 (a)	17 ± 2 (a)
Functional endpoints					
	Photosynthetic efficiency (quantum yield ϕ')	0.4 ± 0.04 (ab)	0.3 ± 0.04 (ab)	0.4 ± 0.02 (b)	0.3 ± 0.014 (a)
	Primary production (µg C g^{-1} AFDW day^{-1})	68.1 ± 17.4 (a)	49.9 ± 9.6 (a)	56.3 ± 6.2 (a)	44.6 ± 15.8 (a)
	Secondary production (µg C g^{-1} AFDW day^{-1})	23.0 ± 4.6 (a)	27.2 ± 5.4 (a)	**38.9 ± 4.8 (b)**	22.0 ± 2.8 (a)
	Substrate-induced respiration (mg CO_2 g^{-1} AFDW day^{-1})	15.3 ± 1.3 (a)	18.8 ± 3.6 (a)	15.4 ± 1.4 (a)	17.6 ± 2 (a)

Table 3
EC_{20} and EC_{50} for photosynthetic efficiency and primary production for periphyton from the four experimental treatments. Four replicate channels per treatment were used in the fitting model (N = 4). Higher values for each wastewater treatment than the control means higher tolerance to the micropollutant mixture. The x-axis of the concentration-effect curves was expressed as unit-less relative dilution factor (RDF) and therefore the EC_{20} and EC_{50} values are also expressed in RDF. Values in parentheses provide the 95% confidence interval. The treatments correspond to periphyton grown in the presence of 0% (control), 10%, 30% and 80% wastewater (WW), respectively. Significant differences between control periphyton and each treatment are marked in bold. Ratios of EC_{20} and EC_{50} were calculated for each endpoint by dividing the mean EC of 10% WW, 30% WW and 80% WW by the corresponding EC of the control. (R ≤ 1 indicates no induced tolerance; R > 1 induced tolerance); n.d. means not determined due to the absence of inhibition.

	Photosynthetic efficiency				Primary production				Secondary production	
Treatment	EC_{20}	R (EC_{20})	EC_{50}	R (EC_{50})	EC_{20}	R (EC_{20})	EC_{50}	R (EC_{50})	EC_{20}	EC_{50}
0% WW	54.4 (26.9 - 81.9)		205.2 (153.2 - 274.9)		116 (56.6 - 175.3)		446 (331.9 - 599.2)		n.d.	n.d.
10% WW	80 (35.3 - 124.7)	1.5	238.6 (179.1 - 317.7)	1.2	101.8 (25.8 - 177.9)	0.9	369.1 (246.8 - 552)	0.8	n.d.	n.d.
30% WW	**160.4 (82.6 - 238.2)**	2.9	**366 (289.2 - 463.1)**	1.8	100.5 (38.6 - 162.5)	0.9	359.1 (260.5 - 495.1)	0.8	n.d.	n.d.
80% WW	**180.2 (118.9 - 241.4)**	3.3	**293.8 (268.2 - 321.9)**	1.4	**450.2 (169.6 - 730.8)**	3.9	**819.7 (636.9 - 1054.9)**	1.8	n.d.	n.d.

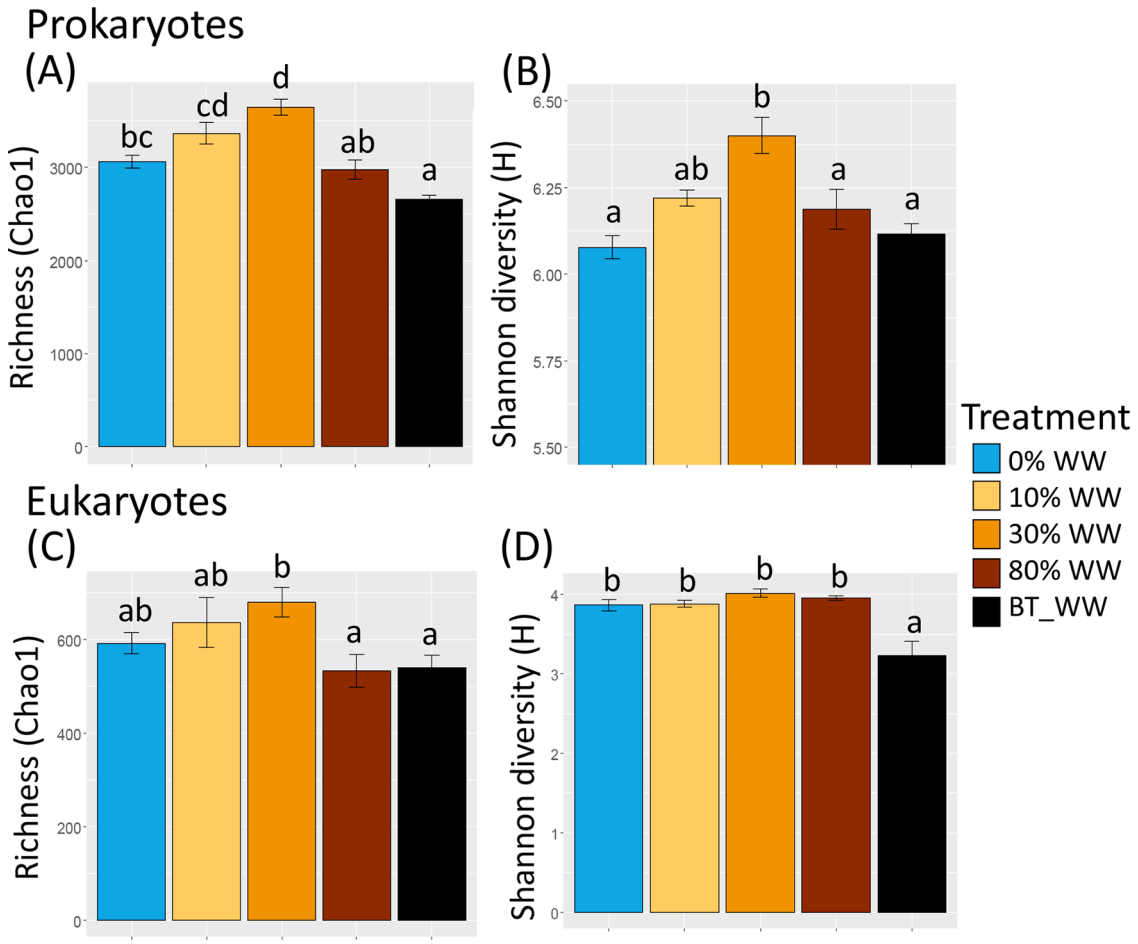

Fig. 3. Alpha diversity of prokaryotic (A, B) and eukaryotic (C, D) periphyton communities. The values of total ASV richness Chao1 (A, C) and Shannon's diversity index H′ (B, D) are reported as the mean ± SE of four replicate channels (N = 4). Significant differences are indicated by lowercase letters, a < b < c < d (Tukey's test, $P < 0.05$). The treatments correspond to periphyton grown in the presence of 0% (control), 10, 30 and 80% wastewater (WW), respectively. BT_WW: community from wastewater in the buffer tank.

4.2. Exposure to, and accumulation of, micropollutants in periphyton

Overall, the grab water samples and passive sampler extracts were comparable for the majority of the analysed substances, except for eight pesticides and eight pharmaceuticals. These substances were either only quantified via passive sampling or occasionally in few of the grab water samples, which indicates that they were not present all the time or present at concentrations below detection limits during grab sampling. Such results underline the fact that, in contrast to grab sampling, passive samplers better reflect the time-integrated in-situ exposure of periphyton to these compounds (Moschet et al., 2015).

The relative proportion of each substance group in periphyton differed from that found in the water, with lower and higher proportions of artificial sweeteners and pesticides, respectively, in periphyton. This shows that micropollutant composition and concentrations in periphyton also need to be characterized, as grab water or passive sampling cannot reflect their association with periphyton. Indeed, several factors may explain these differences. The physicochemical properties of each

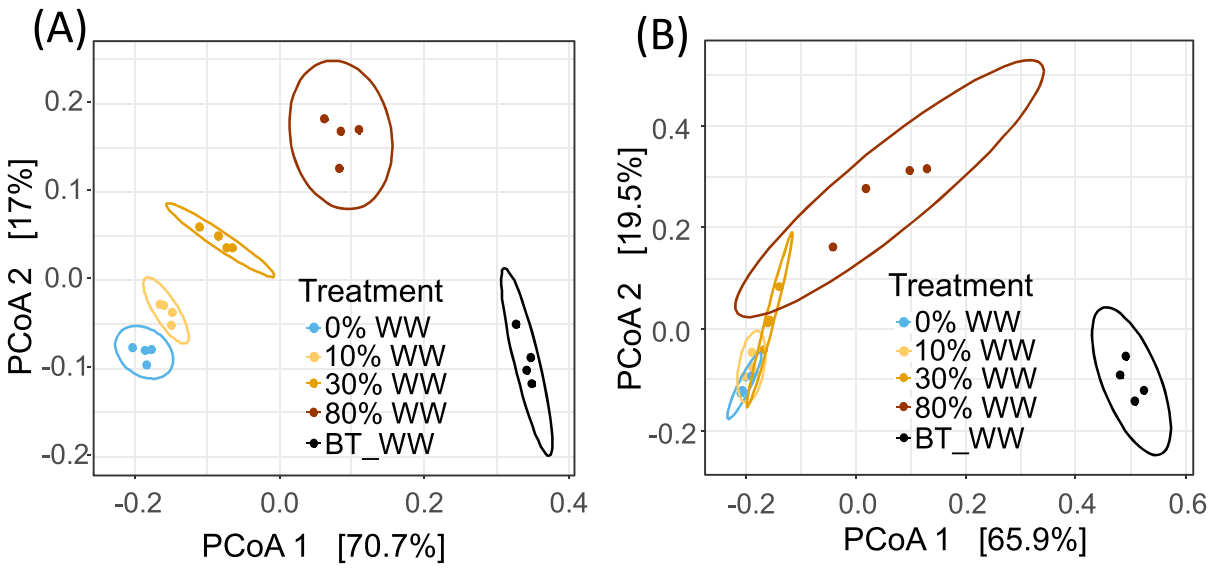

Fig. 4. Principal Coordinate Analysis (PCoA) of prokaryotic (A) and eukaryotic (B) communities based on weighted UniFrac distances. The treatments correspond to periphyton grown in the presence of 0 (control), 10, 30 and 80% wastewater (WW), respectively. BT_WW: community from wastewater in the buffer tank. The 95 % confidence ellipse was added for each treatment. Non-overlapping ellipses indicate significantly different treatments.

substance, such as hydrophobicity, reflected by the octanol/water partition coefficient (Kow), can influence bioaccumulation. For instance, the three quantified artificial sweeteners are highly hydrophilic (Log Kow $\leq$ -1) and therefore less prone to accumulate in (phospho)lipid structures according to Log Kow. However, hydrophobicity alone cannot fully explain our results since no significant correlation between Log Kow values and the ratios of micropollutant concentrations in periphyton and water was found when all the compounds (n = 44 micropollutants) were taken into account (Pearson's r = 0.23, $P = 0.14$, SI Table 11). Micropollutants can also undergo biotransformation processes within microbial cells, reducing parent compound concentrations, as it has been shown for the three artificial sweeteners, cyclamate, saccharine and acesulfame, in periphyton communities downstream of a WWTP (Desiante et al., 2021). Presence within cells of specific molecular binding sites may as well influence bioaccumulation of micropollutants, such as for the herbicides, diuron, terbutryn and isoproturon (Morin et al., 2018; Tlili et al., 2011b), thus explaining the high BCFs for these compounds compared to the other analysed micropollutants. These are PSII inhibitors that bind specifically to the protein D1 of the photosynthetic apparatus of phototrophic organisms, such as diatoms, green algae and cyanobacteria (Allen et al., 1983). The physicochemical properties of the extracellular matrix of periphyton itself, such as the presence of polysaccharide substances, can also interfere with micropollutant uptake within periphyton (Bonnineau et al., 2020).

4.3. *General descriptors of periphyton versus tolerance to micropollutants*

Traditional descriptors, such as biomass, respiration and photosynthetic activity, did not show a clear pattern in relation to the wastewater gradient, as also previously shown in mesocosms (Pereda et al., 2019) and field surveys (Lebkuecher et al., 2018; Tlili et al., 2017). Therefore, these descriptors are not useful as specific indicators for wastewater-borne stressors on periphyton. Yet, exposure to passive sampler extracts derived from the wastewater buffer tank revealed that the phototrophic fraction of the biofilm had become tolerant upon exposure to wastewater. Indeed, phototrophs responded less sensitively in terms of primary production and even less in terms of photosynthetic activity to the micropollutant mixture than the non-exposed control communities did. This outcome is in line with the reports on tolerance development in natural streams of periphyton exposed to wastewater extracts (Tlili et al., 2017, 2020). Hence, our engineered channel set-up allowed reproducing to some extent the field conditions that are necessary for the development of tolerance upon exposure to wastewater effluents. What is more, the extract used for tolerance measurements was representative of the micropollutant mixture in wastewater (SI Table 11), justifying its use in toxicity assays.

Photosynthetic efficiency was more affected, i.e. becoming more tolerant, than primary production. While both endpoints are proxies for photosynthesis, their underlying processes are distinct. Specifically, photosynthetic efficiency corresponds to light-dependent reactions (i.e. absorption of light energy by chlorophyll) while primary production refers to light-independent reactions (i.e. assembly of sugar molecule from carbon dioxide) (Yahia et al., 2019). Hence it is conceivable that their susceptibility to tolerance development may differ. Photosystem II inhibitors, i.e. those that were also found to preferentially accumulate in periphyton, target photosynthetic efficiency specifically. Primary production, on the other hand, corresponds to the incorporation of carbon into biomass and may be affected by different classes of micropollutants with various modes of action. This may therefore prevent the selection of tolerant taxa within the community when exposure levels are relatively low as in the 10% and 30% wastewater treatment.

In sharp contrast to phototrophs, heterotrophs were not affected by the exposure to the passive sampler extract as shown with secondary production, despite the presence of potential secondary production inhibitors within the mixture, such as the antibiotics sulfamethoxazole and trimethoprim. Bacterial secondary production has previously been used to assess the increased tolerance of heterotrophic communities in periphyton from downstream of WWTPs (Tlili et al., 2017, 2020). In these studies, bacterial secondary production was inhibited by micropollutant extracts from passive samplers deployed in wastewater effluents. The reasons for the differences among studies are not yet clear but could be caused by a high level of baseline tolerance in the bacterial component of periphyton grown in the channels during the 4-week colonisation period, including in the control communities (without wastewater). Such results underline the importance of selecting reference communities with a relatively low baseline tolerance in PICT studies.

4.4. *Effect of wastewater on the structure of periphyton communities*

Community structure was evaluated based on commonly used descriptors, specifically beta-diversity (i.e. structural differences among

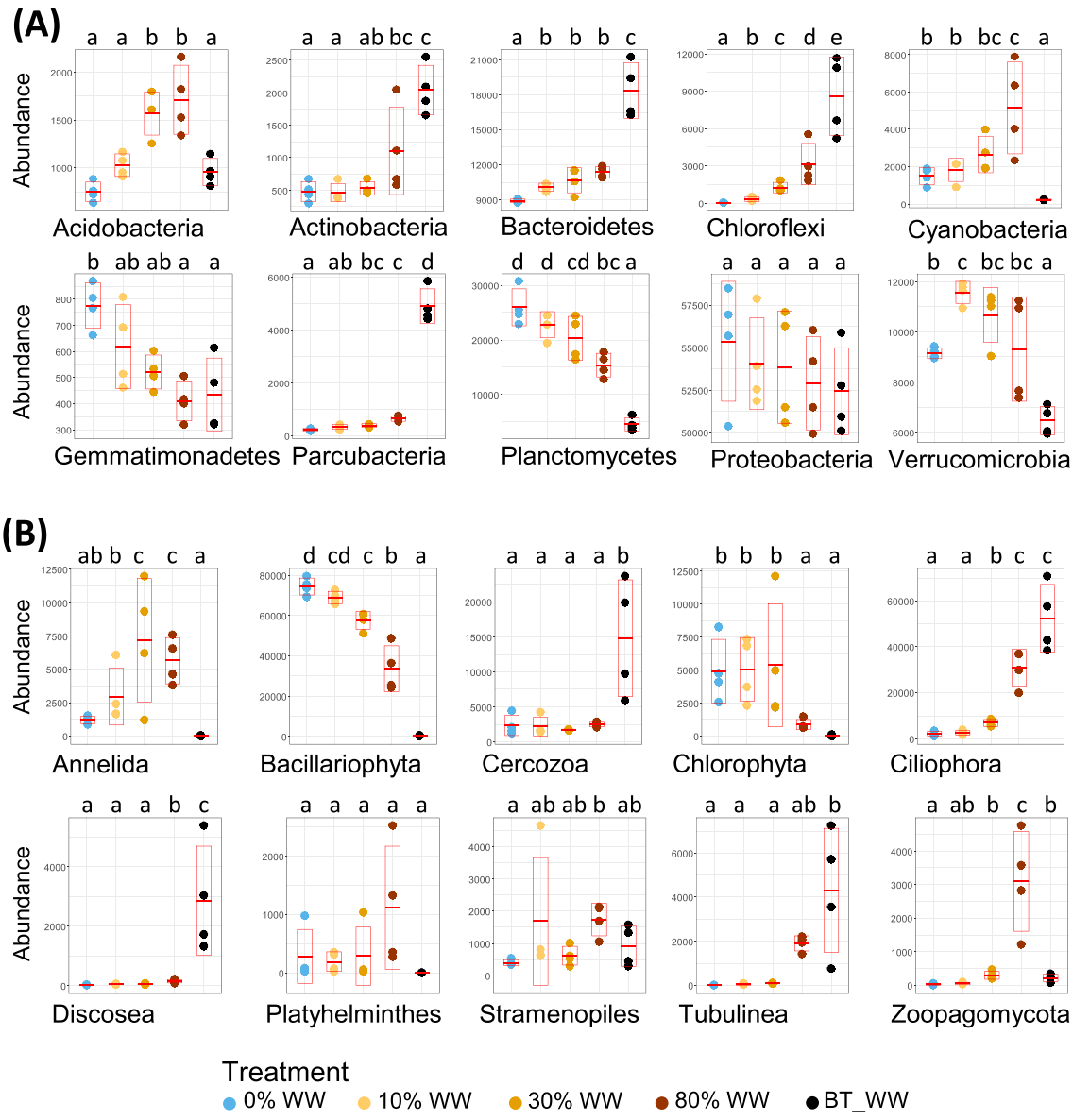

Fig. 5. Abundance of the top-ten prokaryotic (A) and eukaryotic (B) phyla in periphyton and wastewater. Amplicon Sequence Variants (ASVs) abundance for each channel replicate (n = 4). Significant differences are indicated by lowercase letters, a < b < c < d < e (Tukey's test, $P < 0.05$). The treatment corresponds to periphyton grown in the presence of 0% (control), 10%, 30% and 80% wastewater (WW), respectively. BT_WW: community from wastewater in the buffer tank. Horizontal lines in the red boxes correspond to the average values and the lower and higher limits of the standard deviation.

several communities), alpha-diversity (i.e. richness and Shannon diversity index) and relative abundance of taxa. Analysis of beta diversity revealed a differences of community structure with increasing wastewater proportion, starting as low as 10% wastewater. Several field studies also reported on the impact of wastewater on the structure of periphyton communities from downstream of urban (Aubertheau et al., 2017; Lebkuecher et al., 2018; Romero et al., 2019) and hospital (Chonova et al., 2019, 2018) effluents. In contrast, differences of alpha-diversity indices did not strictly follow the wastewater gradient. Similar contrasting results between alpha- and beta diversity indices have previously been reported in the field by comparing two upstream and two downstream sites of a WWTP (Lebkuecher et al., 2018) and may be explained by several wastewater-linked factors, such as nutrients, micropollutants, microorganisms and metals. Nutrients may favour the growth of certain taxa while micropollutants may negatively influence the abundance of others (Van Horn et al., 2011). On the opposite, antibiotics can also induce changes in the bacterial community structure of stream biofilms by favouring antibiotic-resistant bacteria (Proia et al., 2013). These changes may have consequences in terms of loss of biodiversity and alteration of biogeochemical cycles with potentially negative consequences for ecosystem functioning. Microorganisms originating from WWTPs are also able to colonize downstream periphyton (Chonova et al., 2019; Mußmann et al., 2013), thus likely influencing the structure of microbial community. This may lead to two distinct communities with regard to beta-diversity (i.e., measuring the structural differences among several microbial communities) being indistinguishable via alpha-diversity indices (i.e., richness and evenness of a given community).

The observed higher tolerance of the phototrophic component of periphyton to micropollutants may result from a direct effect of the wastewater on the abundance of phototrophs such as diatoms, green algae and cyanobacteria. For instance, despite the fact that several diatom genera, the dominating eukaryotic communities in periphyton (Battin et al., 2016), were negatively impacted by the exposure to wastewater, others were not impacted (*Cocconeis* and *Nitzschia*), or even stimulated (*Ulnaria*) by the presence of wastewater, and thus potentially

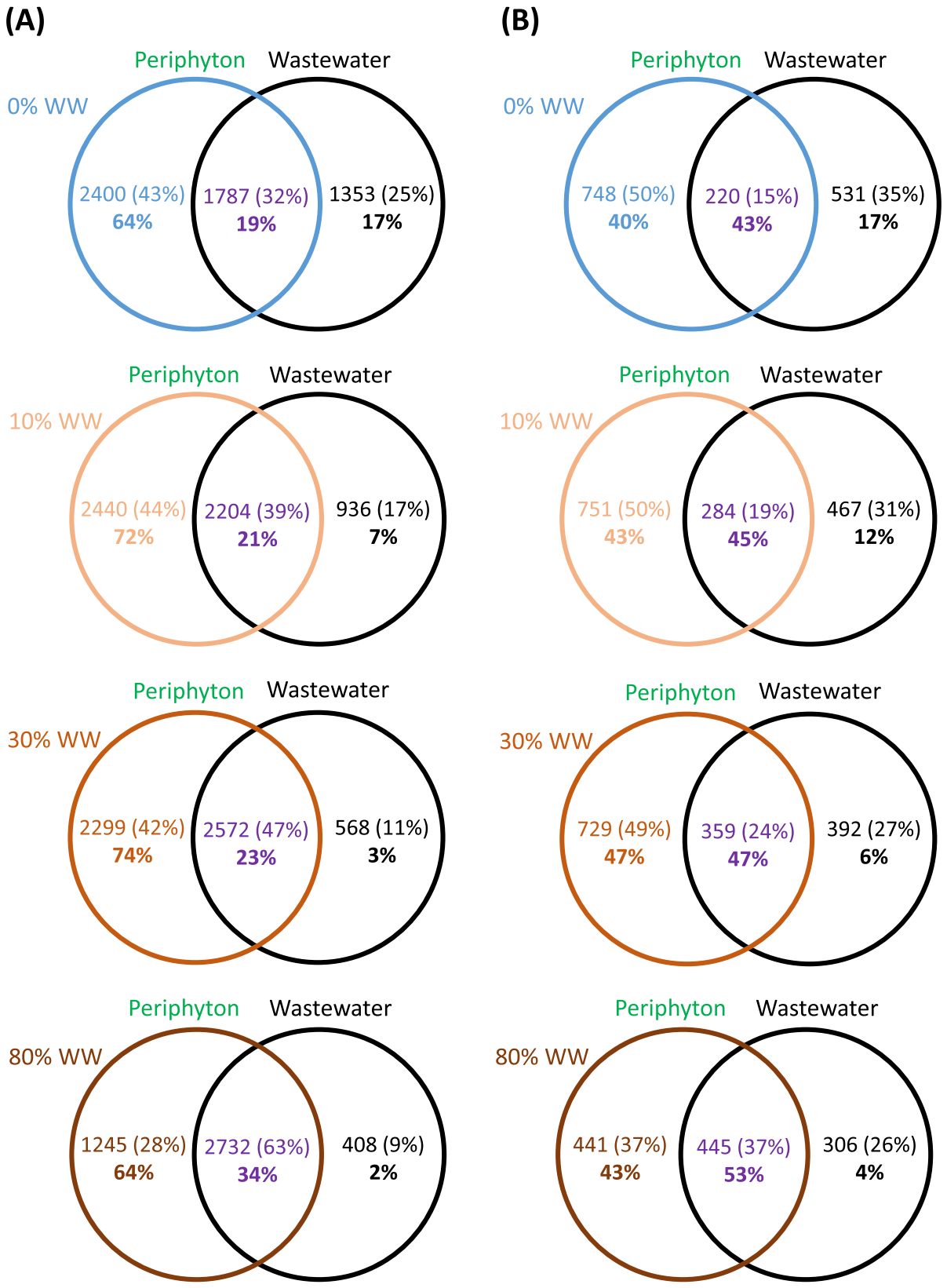

Fig. 6. Venn diagrams representing the repartition of prokaryotic (A) and eukaryotic (B) taxa among periphyton and the community isolated from wastewater in the buffer tank. The repartition of ASVs is described for each treatment between periphyton grown in the presence of 0, 10, 30 and 80% WW and wastewater samples. The results are expressed as ASV counts (relative proportion in % of ASV counts given in brackets) and relative overall abundance (%).

contribute to the higher tolerance observed. The higher abundance of cyanobacteria observed with increasing wastewater may also indicate potential contribution to the observed tolerance. These phototrophic bacteria have already been shown to increase in the presence of wastewater in natural stream (Corcoll et al., 2014) and microcosm (Carles and Artigas, 2020; Romero et al., 2019) studies. Indeed, cyanobacteria can benefit from the protective environment given by periphyton and are known to be particularly tolerant to several micropollutants, including herbicides (Forlani et al., 2008; Singh et al., 2013). Further experiments are needed to specifically investigate the direct link between PICT and microbial community composition, for instance by using simplified (synthetic) communities and sequential addition of each constituent of the wastewater effluent.

Besides the direct impacts of wastewater on the phototrophic communities, indirect effects may also occur through interspecific interactions and thereby contribute to the higher tolerance observed. For instance, micrograzers contribute significantly to the functioning of periphyton, notably by modulating microbial populations via their grazing activity (Weitere et al., 2018). In our study, Ciliophora abundance was higher in periphyton with increasing wastewater proportion, potentially adding grazing pressure already at early stages of biofilm development (Böhme et al., 2009; Peng et al., 2018). Little is known to date about the role of micrograzers in periphyton tolerance to micropollutants and effects of wastewater on periphyton communities in general. Indeed, most of the studies have focused on macro grazers feeding on periphyton. For instance, Guasch et al. (2016) have reported on synergistic effects of snails (*Radix ovata*, Lymnaeidae) and the biocide triclosan on periphyton, with a reduced capacity of detoxification and removal of dissolved nutrients. Complementary investigations are needed to specifically address this issue.

Despite the fact that periphyton and wastewater communities correspond to two different life styles (benthic vs. planktonic) and originate from distinct environments with different light conditions, they share a non-negligible proportion of their relative taxonomic abundance (20% and 43% for prokaryotes and eukaryotes, respectively). Together with the observed increase of shared taxa between periphyton and wastewater, and the decrease of taxa specific to wastewater, this finding provides a first indication that tolerance may indeed arise, at least in part, from colonisation of periphyton by microorganisms from the wastewater. This could be explained by a direct (i.e. colonisation of periphyton by micropollutant-tolerant taxa) or an indirect effect of wastewater microorganisms on the structure of periphyton community. The most plausible explanation is an indirect effect since phototrophs (diatoms, green algae and cyanobacteria), for which the increased tolerance was observed, were already present in control periphyton and almost not detected in wastewater. Several field surveys have shown that downstream bacterial (i.e. prokaryotic) community profiles in the water column were a mixture between the upstream and the effluent (Mansfeldt et al., 2020; Pascual-Benito et al., 2020; Price et al., 2018), with an exception for cyanobacteria (Mansfeldt et al. 2020). Much less is known for periphyton communities. One natural field study reported that several bacterial taxa considered to be indicators of wastewater effluents were also found in downstream periphyton, even though they represented less than 5% of the total number of taxa in periphyton (Chonova et al., 2019). Mußmann et al. (2013) have also shown that, among all identified nitrifiers from WWTPs, only one taxon colonized in downstream periphyton. Overall, a detailed characterization of the diversity profile of microorganisms from wastewater effluents that actively colonize downstream biofilms could help in identifying potential key players of the higher periphyton tolerance observed.

5. Conclusions

The present study aimed at describing the differences in microbial community composition and diversity associated with the higher tolerance observed for periphyton exposed to a gradient of wastewater effluent. We were able to grow periphyton in engineered flow-through channels that allowed us to closely mimic natural conditions while being able to control flow and stream vs. wastewater proportions. The comprehensive set of biological and chemical analyses used led us to the following main conclusions:

- The analysis of micropollutants in water and periphyton provided a comprehensive picture of exposure and bioavailability. The relative proportion of each substance group in periphyton differed from that found in the water, highlighting the need to consider bioaccumulation of micropollutants in periphyton in order to accurately link exposure to effects at the community level and the consequences for the ecosystem.
- The exposure of periphyton to wastewater led to a higher tolerance of phototrophs to the mixture of micropollutants extracted from passive samplers compared to the control. However, we could not observe any inhibition of bacterial secondary production and consequently no increased-tolerance for heterotrophs by the same micropollutant extract. Therefore, the potential impact of micropollutants from the wastewater on periphyton was likely higher for phototrophs and may impact essential ecosystem functions provided by periphyton, such as primary production and nutrient cycles
- Wastewater induced significant differences in the structure of both eukaryotic and prokaryotic communities. Several wastewater constituents (not only micropollutants, but also nutrients, microorganisms and metals) may lead to these differences. For instance, our study provides additional indication of a transfer of microorganisms from wastewater to periphyton communities. Therefore, the colonisation of periphyton by micropollutant-tolerant microorganisms coming from the wastewater may contribute to the increased tolerance of periphyton downstream of WWTP. Future studies are needed to look specifically at the relative contribution of wastewater-derived microorganisms in the establishment of periphyton tolerance to micropollutants. For example, this can be achieved by removing microorganisms from the effluent before measuring periphyton tolerance.

Author contributions

LC, AJ, R.I.L.E, KS, NS, CS and AT conceived and designed the study. LC, SW and AJ set up the experimental system. LC and SW performed the experiment and ran the sample and statistical analyses. LC and AT drafted the first version of the manuscript. All co-authors contributed to the data interpretation and subsequent revisions of the manuscript, and they approved the final submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117486.

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