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Evaluating the Impact of Wastewater Effluent on Microbial Communities in the Panke, an Urban River

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Abstract: Pharmaceuticals are consumed in high amounts and can enter as emerging organic compounds in surface waters as they are only partially retained in wastewater treatment plants (WWTPs). Receiving pharmaceuticals may burden the aquatic environment, as they are designed to be bioactive even at low concentrations. Sediment biofilm populations were analyzed in river sediments due to the exposure of an inflow of WWTP effluents. Illumina MiSeq 16S rRNA gene amplicon sequencing was performed of 108 sediment samples, which were taken from multiple cores within three sampling locations in the Panke River, with one sampling site located downstream of the inflow. Sequencing data were processed to infer microbial community structure in samples concerning the environmental variables, such as micropollutants and physicochemical parameters measured for each core. More than 25 different micropollutants were measured in pore water samples, in which bezafibrate, clofibric acid, carbamazepine, and diclofenac were detected at high concentrations. Bacterial 16S rRNA gene amplicons revealed *Nitrospirae*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Ignavibacteriae* as the most abundant groups in the samples. Differences in microbial community composition were observed with respect to micropollutants. However, our findings revealed that the composition of the microbial community was not only governed by the effluent. The significant changes in the alpha- and beta-diversity were explained by phenobarbital and SO_4^{2-}, which did not originate from the WWTP indicating that more unobserved factors are also likely to play a role in affecting the biofilm community's composition.

Keywords: micropollutants; wastewater treatment plant discharge; sediments; microbial communities; 16S rRNA gene amplicon sequencing

1. Introduction

Urban aquatic ecosystems in cities and areas with a high population density can be strongly affected by human activities, resulting in significant impacts on downstream aquatic ecosystems and water users [1]. Wastewater treatment plants (WWTPs) are the primary source of anthropogenic effluents into aquatic ecosystems, and the effluents can represent a significant proportion of the total flow of the receiving water body [2]. Effluents can contain a variety of pharmaceuticals since some of these cannot be completely removed from WWTPs. Discharges can also arise from non-point sources, such as diffuse runoff from urban and agricultural areas [3]. Pharmaceuticals are detected as micropollutants (MPs) at trace concentrations in the μg L^{-1} to ng L^{-1} range. Emerging organic micropollutants, such as pharmaceuticals, may cause harm to aquatic organisms in aquatic ecosystems, due to additive or synergistic effects [4,5].

Pharmaceuticals are distinct from other compounds, due to their potential ecotoxicological effects, their specific targeting of biochemical processes, and their bioactivity at low concentrations [6].

The ecotoxicology of compounds is tested under controlled laboratory conditions, and generally involves determining the acute toxicity of a single compound to a specific indicator species. In these investigations, biotic environmental factors are excluded, and only abiotic factors are controlled [7]. In particular, most studies have predominantly focused on eukaryotic organisms. Although, environmental factors such as temperature can exert physiological background stress and, therefore, influence the toxic effects of pollutants [8]. Due to the complexity of micropollutants' mixtures and various drivers in ecosystems, conclusions concerning risks to the aquatic ecosystem are not clear [9]. However, Gao et al. [10] documented a significant link between mixtures of micropollutants and microbial community structures in aquatic systems. Furthermore, Lawrence et al. [11] point out that microorganisms are stationary in ecosystems while forming biofilms on the surface layer in sediments, and thus are relevant as ubiquitous factors and are integral to the ecosystem's function. Biofilm populations can thus serve as indicators to assess the condition of an ecosystem, as they are known as primary drivers of essential ecosystem processes due to their biochemical cycling [12].

As a consequence, biofilm populations can be considered as a promising indicator for evaluating the impact of WWTPs on the aquatic environment. Since micropollutants are frequently found in wastewater and treated effluents, and are received by surface waters, their influence on river biofilm structure and function have been intensively studied [13–17]. Several studies have coalesced structural changes in sediment biofilm communities in response to the release of WWTP effluents [18–21]. It has further been shown by Yergeau et al. [22] that different pharmaceuticals in low concentrations were inducing transcriptional changes in a complex microbial community. Furthermore, a significant decrease in microbial diversity was detected in sediments downstream of the WWTP effluent compared to unaffected natural sediments upstream [23]. Atashgahi et al. [19] observed an adaptation of the microbial community in the sediment to nutrient-poor and oxic conditions as the operation of a WWTP promotes low-nutrient conditions in the longer term. To the best of our knowledge, only a few studies have investigated the effect of micropollutants on sediment biofilms [10,18].

The overall objectives of the present study were (1) to analyze micropollutant concentrations and physicochemical parameters in the River Panke at three sampling sites, (2) to determine the microbial community composition in urban river sediments by Illumina MiSeq 16S rRNA gene amplicon sequencing, (3) to evaluate the coherence between micropollutants and the microbial communtiy structure by using biostatistical analyses, and (4) to infer whether micropollutants and environmental factors affect the microbial community structure.

2. Materials and Methods

2.1. Study Site

The Panke River in Berlin was selected to represent a highly urbanized river, which arises in Bernau on the Barnim and releases into the river Spree. Seventeen kilometers and seven hundred meters of the river stretch is located in Berlin's urban region [24], and the three sampling sites were located in the north-eastern suburban area, Berlin-Karow. Since the end of 2015 the Panke has received treated effluent (1000 L s^{-1}) from the Schönerlinde WWTP (52°39′39′′ N, 13°24′54′′ E) via the Bucholzer Graben ditch (Figure 1). The PuBG sampling site (PuBG = 'Panke unterhalb Buchholzer Graben') (52°36′41′′ N, 13°27′06′′ E) is instantly affected by the discharge of the Buchholzer Graben (52°36′44′′ N, 13°27′10′′ E). The PoBG sampling site (PoBG = 'Panke oberhalb Buchholzer Graben') (52°36′58′′ N, 13°27′34′′ E) is located 600 m before the discharge upstream. About 1.9 km upstream of the discharge inflow is located the third sampling site PvLg (PvLg = 'Panke vor Lietzengraben') (52°37′26′′ N, 13°28′24′′ E), and was not affected by any ditches; just rainwater effluent nearby lead rainwater by massive storm events into the Panke. An overall overview is given in Figure 1, and the sediment characteristics are presented in Table S1 SI (Supplementary Information).

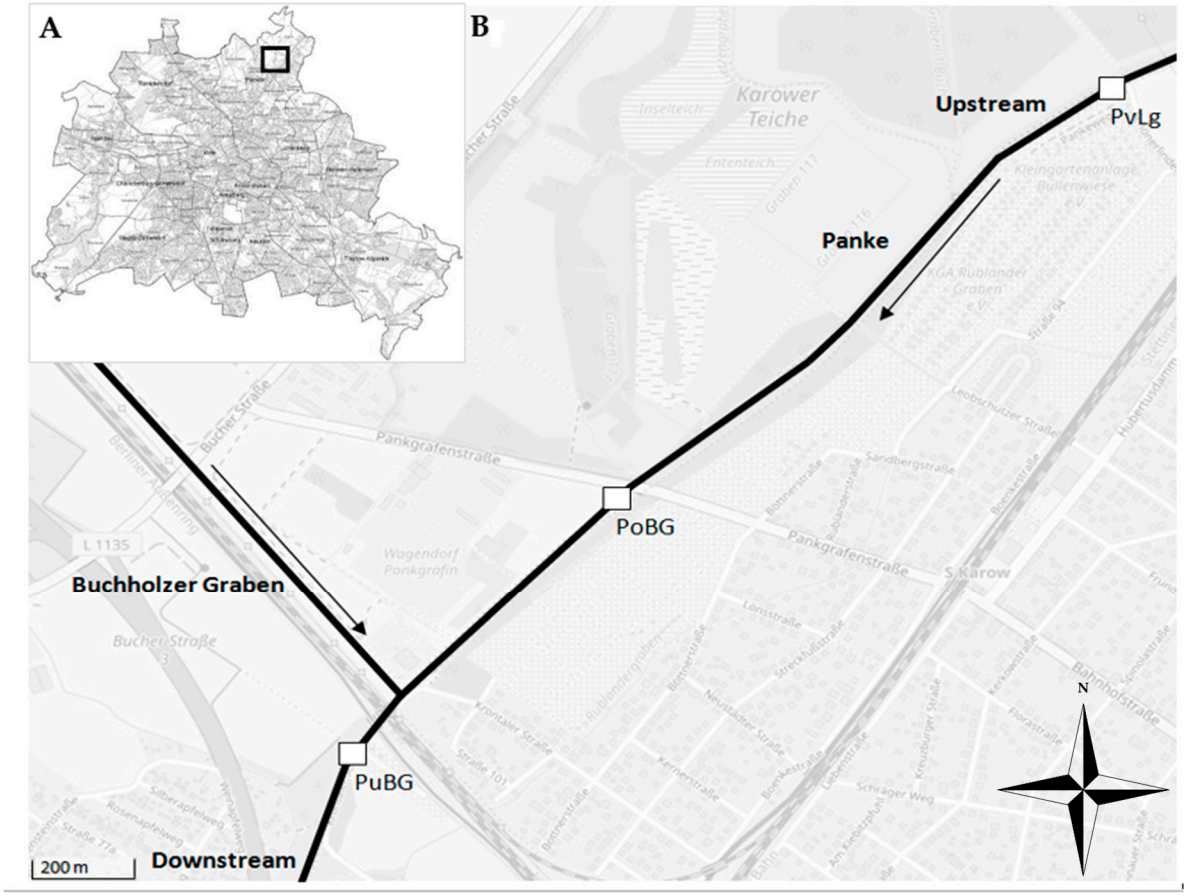

Figure 1. (**A**) The Berlin map shows the sampling area noted by a square. (**B**) Open squares highlight sampling sites. The clear water from the Schönerlinde WWTP enters in the Panke through the Buchholzer Graben. Map data copyrighted OpenStreetMap contributors (2018) and are available from http://www.openstreetmap.org.

2.2. Water Sampling and Water Analysis

Physicochemical parameters were measured (Table 1 and SI Table S2) using a portable conductivity meter, dissolved oxygen meter, pH/mV meter (LF 340, Oxi 330, pH 330 from WTW, Weilheim, Germany) and appropriate electrodes (TetraCon 325, CellOx 325 and Sen Tix 41, WTW, Weilheim, Germany, respectively). Sulfate and nitrate concentrations were measured in grab water samples with a colorimetric assay kit (Visocolor® HE, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The pore water samples and water samples were taken to determine micropollutant concentrations and dissolved organic carbon (DOC). The pore water samples were taken with a syringe (20 mL, sediment depth around 2 cm) and were collected in 100 mL glass bottles and stored at 4 °C in the dark. The analysis of pharmaceuticals was performed at the Berlin Water Company by using a Xevo TQ-S LC tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with a Z-Spray electrospray ionization source. For DOC measurements, ten mL of every sample were filtered through a Whatman cellulose nitrate membrane filter (pore size 0.45 µm). Before use, membrane filters were rinsed with 200 mL of demineralized water, and the filtered DOC samples were stored at 4 °C until further analysis (storage time < 1 week). Before the analysis, every sample was pretreated with 10% hydrochloric acid (HCl) and was measured in triplicate by using a Vario TOC cube from Elementar, Langenselbold, Germany.

Table 1. Physicochemical parameters of water samples were obtained at three sampling sites (PuBG, PoBG, and PvLg) during two seasons (autumn 2016 and summer 2017). Shown are means of six replicates ± standard deviation. *p*-values were adjusted with one-way analysis of variance (ANOVA) (passed Shapiro–Wilk test) and Holm–Sidak test. *Significant difference ($p < 0.05$) in comparison with PvLg site. Significance was tested for both seasons separately. All samples were measured in surface water; exclude DOC values were measured in pore water.

Sampling Sites Date Sample Number	PuBG 12 October 16 n = 6	PoBG 12 October 16 n = 6	PvLg 13 October 16 n = 6	PuBG[1] 26 June 17 n = 6	PoBG[1] 27 June 17 n = 6	PvLg[1] 26 June 17 n = 6
Dissolved oxygen [a]	4.7 ± 0.2	8.3 ± 0.3	8.1 ± 0.1	4.9 ± 1.0	7.5 ± 0.1	6.5 ± 0.3
DOC [a]	6.6 ± 1.6*	3.8 ± 0.6	2.0 ± 1.5	8.82 ± 1.1*	7.4 ± 0.2*	4.4 ± 0.5
Nitrate [a]	38.3 ± 11.8	3.0 ± 0	2.7 ± 0.5	30.0 ± 0	3.3 ± 0.5	1.3 ± 0.5
pH	7.7 ± 0.1	8.0 ± 0.1	8.0 ± 0	7.3 ± 0	7.9 ± 0.1	7.7 ± 0
Sulfate [a]	116.7 ± 7.5	85 ± 15.3	100 ± 15.3	83.3 ± 14.9	101.7 ± 3.7	38.3 ± 5.5
Water temperature [b]	14.8 ± 0	9.7 ± 0.1	9.7± 0.1	19.5 ± 0.4	15 ± 0	16.8 ± 0.1

[a] mgL^{-1}, [b] °C,[1] heavy rainfall took place just shortly before the sampling campaign in June 2017 (<3 days).

2.3. Sediment Sampling, DNA and RNA Extraction

Sediment cores were collected from the top 5-cm layer of the riverbed sediment in October 2016 and June 2017, at the sampling sites PuBG, PoBG and PvLg (Figure 1) using stainless steel soil sample rings (53 × 50, Eijkelkamp, Giesbeek, The Netherlands). In total, six sediment cores were taken as replicates from each sampling site. For RNA extraction, three subsamples of each core were taken, immediately transferred in liquid nitrogen and stored at −80 °C until RNA extraction. The RNA extraction was done by the third-party provider Microsynch AG, Zurich, Switzerland, using the Qiagen RNAeasy-Plus Kit. For cDNA synthesis from total RNA, the QuantiTect Reverse Transcription Kit from Qiagen was utilized. Six subsamples of 0.5 g wet sediment were taken for the DNA extraction from each sediment core. The sediment was transferred into Lysing Matrix E, tubes from the FastDNA™ Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and was kept at −20 °C until further DNA extraction. The DNA extraction proceeded according to the manufacturer's recommendations. In total, 108 genomic DNA samples were extracted, and successful DNA isolation was confirmed by agarose gel electrophoresis (1% Agarose, 1X TAE buffer (40 mM TRIS, 1 mM EDTA)). A negative extraction control was included to check for any cross-contamination, and this control was evaluated with the Qubit™ dsDNA HS Assay kit on the Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The final DNA samples were eluted into a final volume of 50 µL.

2.4. 16S rRNA Gene Amplicon Sequencing

Bacterial 16S rRNA gene amplicon sequencing of DNA and cDNA was performed using primers targeting the V3–V4 variable region adopted from Kozich et al. [25]. Amplicon library PCR was performed using 1:100 diluted DNA templates due to humic substances within the samples to avoid polymerization problems. The diluted DNA (1 µL) was used as a template for the PCR reaction. Each reaction was performed in a final volume of 18 µL containing 277 nM of each dNTP, 0.027 U/µL Phusion Hot Start II DNA Polymerase, and 1.3X Phusion HF Buffer (Thermo Fisher Scientific, Waltham, MA, USA). V3-V4 primers: 357F 5′-CCTACGGGAGGCAGCAG-3′ and 806r 5′-GGACTACHVGGGTWTCTAAT-3′ were used. Each primer was fused with unique diversity inline barcodes (MID) for paired-end sequencing. Amplification was done using the following PCR program: Initial denaturation at 98 °C for 30 s, 30 cycles of 98 °C for 9 s, 55 °C for 1 min, 72 °C for 90 s, and final elongation at 72 °C for 10 min. All PCR reactions were run in duplicate and were pooled afterward. The produced amplicons were processed further as previously described by Graspeuntner et al. [26]. Subsequently, resulting PCR products were purified using the Qiagen MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The 16S rRNA V3-V4 region PCR products were quantified by the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit™ dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples from both

sampling campaigns (October 2016 and June 2017), were pooled in equimolar concentrations, and each library pool was sequenced on a MiSeq platform (Illumina, USA) at the Max Planck Institute for Evolutionary Biology in Plön, Germany using the MiSeq Reagent Kit v3 (600 cycles). The sequencing procedures proceeded as described previously by Kozich et al. [25], with the exception of 10% PhiX control in the library (Illumina, USA) and a final library concentration of 20 pM.

All sequenced sample libraries were subsampled to 30,000 raw reads, and the quality of demultiplexed reads was checked with FastQC (v0.11.5, Babraham Bioinformatics, Babraham, UK) [27]. The quality filtering and pre-processing, including trimming of bases and bad reads, removal of adapter and primer sequences, was performed with BBDuk of the BBTools package (v37.54, DOEJoint Genome Institute, Walnut Creek, CA, USA) [28]. Only reads surviving quality filtering as pairs entered the downstream analysis. Filtered reads were further processed using mothur (v1.39.5, University of Michigan, Ann Arbor, MI, USA) [25,29]. Filtered 16S rRNA gene contigs were aligned to an in-house curated reference alignment built from 128,118 bacterial 16S rRNA gene sequences, which had been trimmed to variable regions of V3–V4 and dereplicated. Contigs that did not correctly align with the reference sequences were eliminated. Preclustering was performed to diminish PCR and sequencing artifacts [30], and chimeric sequences were removed by using UCHIME (v4.2.40, drive5.com, Sonoma, CA, USA) [31]. The taxonomic assignment of contigs was then performed using the Wang method [32] at a bootstrap threshold of 60%, and contigs were binned into Operational Taxonomic Units (OTUs) by 97% sequence similarity using the 'OptiClust' method [33]. Binning resulted in a total OTU count of 85,475. A 16S rRNA gene copy number information from rrnDB (v5.5, University of Michigan, Ann Arbor, MI, USA) was used to determine a copy number normalization (CNN) factor for each filtered OTU with an in-house R script [34]. 16S rRNA gene counts were normalized by this factor, keeping the total count per sample constant. OTUs were retained with at least 0.5% relative abundance in at least one sample. This procedure resulted in a filtered OTU count of 1567 for combined normalized 16S rRNA gene and rRNA data.

The obtained raw sequence data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB30051.

2.4. Statistical Analyses

2.4.1. Physicochemical Parameters and Micropollutant Concentrations

All statistical analyses of physicochemical parameters and micropollutant concentrations were run using SigmaPlot (Systat Software, San Jose, CA, USA). All data were analyzed for normality and homoscedasticity using the Shapiro–Wilk test and Brown–Forsythe test. All values of physicochemical parameters are averages of at least six replicates, and all values of micropollutants are averages of at least three replicates, if not stated otherwise.

2.4.2. Statistics on the Alpha Diversity of Microbial Communities

Multivariate statistics and data analysis from the Illumina® MiSeq sequencing were performed in R (v3.5.1, r-project.org, Vienna, Austria) [35]. For further statistical downstream analysis environmental variables were evaluated with the Pearson correlation across all samples, and these selected environmental variables (see SI Figure S1) were used as explanatory variables to statistically model the effective richness, ^{1}D with the R package nlme (v3.1.137, r-project.org, Vienna, Austria) [36]. The calculations of the alpha diversity of DNA samples were performed with R package vegan (v2.5.2 r-project.org, Vienna, Austria) [37]. The analyses were based on a linear regression model with the second- and third-order interaction effects of location and season with environmental variables and were represented with the following full-model formula:

$$^1\text{D} \sim \text{Location} \times \text{Season} \times (\text{Phenobarbital} + \text{SO}_4^{2-}) + \text{Season} \times (\text{Clofibric\_acid} + \text{Bezafibrate})$$

The optimal variance structure of the full model was chosen by using Akaike's Information Criterion (AIC) [38]. A generalized least square model (GLS) with different variance was estimated for individual sediment cores at each location combined with a 'constant plus power' variance structure for bezafibrate resulting in the lowest (i.e., most favorable) AIC. Residuals of the full model were visually examined for homogeneity of variance, normality, and absence of systematic patterns about explanatory variables. Backward selection simplified the model, and Wald's Chi-Square Test determined the significance of terms in the final model with type II sums of squares (SI Table S5). The effect sizes of environmental variables within significant interaction terms proceeded, and *p*-values were adjusted for multiple testing with the Holm–Bonferroni method.

2.4.3. Statistics on the Beta Diversity of Microbial Communities

The extent of change in OTU abundances across DNA samples by the selected environmental variables was explored with the R package DESeq2 (v1.22.1, r-project.org, Vienna, Austria) [39]. The OTU counts were normalized by library size and transformed using the regularized logarithm (*rlog*). Redundancy Analysis (RDA) was implemented in R package vegan and performed on *rlog*-transformed normalized OTU counts (OTU_{rlog}) to capture the effects of environmental variables on the microbial community. Model selection started with a full-model formula analogous to that applied in alpha diversity analysis:

$$OTU_{rlog} \sim \text{Location} \times \text{Season} \times (\text{Phenobarbital} + SO_4^{2-}) + \text{Season} \times (\text{Clofibric\_acid} + \text{Bezafibrate})$$

By backward selection, the full RDA model was simplified using permutation tests. Further investigations of the effect sizes proceeded as mentioned in Section 2.4.2.

2.4.4. Statistics of Significant Environmental Variables on Individual OTUs

The effects of significant environmental variables (in the overall RDA model) on individual OTUs were tested for statistical significance in DESeq2 v1.22.1 [39] applying the Wald test. OTU vector lengths in corresponding RDA models were used as independent information to reduce the number of tests. All OTUs with above-mean vector lengths were selected for testing in DESeq2.

3. Results

3.1. Physicochemical Characterization of the Panke

To determine changes in the Panke River due to the discharge of wastewater, physicochemical parameters and water quality parameters were investigated at three sampling sites in two sampling campaigns (October 2016 and June 2017). In both campaigns, DOC values were significantly higher at the PuBG site compared to the PvLg site ($p < 0.05$) (Table 1). DOC showed increased concentrations of 7.4 ± 0.2 mg L^{-1} at the PoBG site in the summer of 2017, and was significantly different from the PvLg site ($p < 0.05$). The pH values in the Panke River remained constant and did not show significant differences (Table 1). As a consequence of different seasons, water temperature differed between both sampling campaigns (Table 1). The water temperature showed differences at PvLg site with 9.7 °C and 14.8 °C at PuBG site in October 2016, but was not significantly different. Furthermore, there were differences in water temperature of about 4.85 ± 0.22 °C between the PuBG site and PoBG sites in both seasons. At the same time, no significant differences in pH, dissolved oxygen, nitrate, and sulfate (SO_4^{2-}) concentrations were detected. However, lower O_2 concentrations were detected at PuBG, whereas nitrate concentrations increased to 38.33 ± 11.79 mg L^{-1} and 30.0 ± 0 mg L^{-1}, respectively (Table 1). Further results are given in SI Table S2.

3.2. Determination of Micropollutants

A total of 29 micropollutants were analyzed at the PuBG, PoBG, and PvLg sampling sites in pore water samples (Figure 2, SI Tables S3 and S4). The pharmaceutical compounds belonged

to the acidic group. Selected results indicated high concentrations of gabapentin (1600 ng L^{-1}), diclofenac (1300 ng L^{-1}), clofibric acid (250 ng L^{-1}), and bezafibrate (100 ng L^{-1}). However, the highest concentrations were measured at the PuBG site (Figure 2). Some compounds like valsartan (3170 ng L^{-1}) and acesulfame (850 ng L^{-1}) were also measured in higher concentrations at the PoBG site (SI Tables S3 and S4). Decreasing concentrations of micropollutants were observed at the upstream sites (PoBG and PvLg) in both campaigns, except diclofenac which was detected at the PvLg site with a concentration of 200 ng L^{-1}) in June 2017 (Figure 2). Phenobarbital concentrations up to 50 ng L^{-1} were continuously detected at each sampling site (SI Table S3). The measurements revealed that micropollutant concentrations were detected overall with higher concentrations at the PuBG site, which indicated an impact by the wastewater effluent from the Schönerlinde WWTP. All other results of the micropollutant concentrations (µg L^{-1}) are shown in SI Tables S3 and S4.

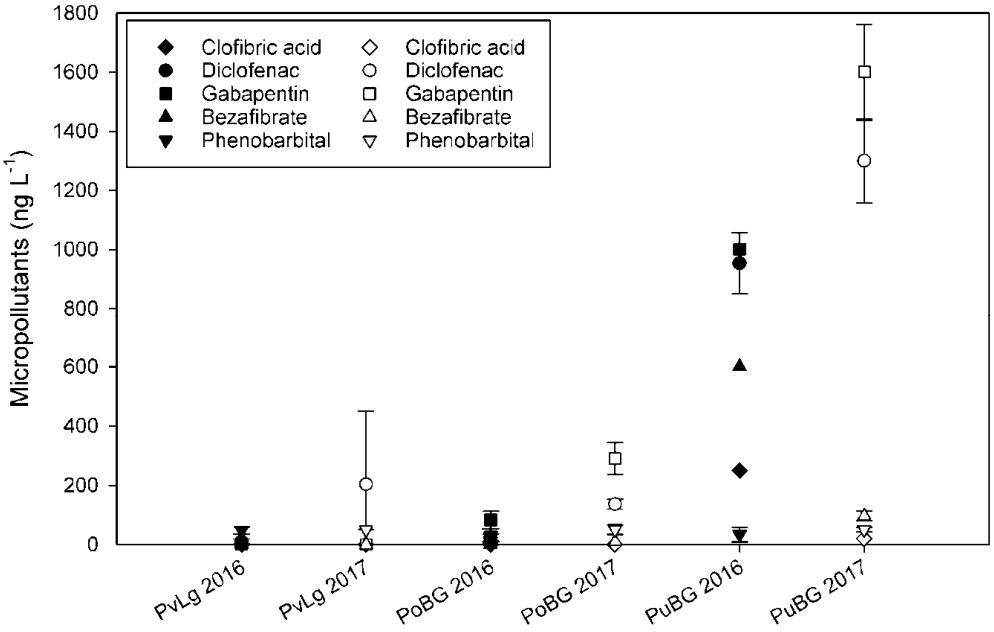

Figure 2. Micropollutant concentrations (ng L^{-1}) in pore water samples at the three sampling sites. The campaign in October 2016 is displayed by closed symbols, whereas the campaign in June 2017 by open symbols.

3.3. Alpha and Beta Diversity of the Microbial Community

Statistical analysis revealed a significant interaction of SO_4^{2-} on the alpha diversity of the microbial communities within the location and season PoBG: S1 and PoBG:S2 (Table 2). There was a correlation obtained (slope = 0.87) at the PoBG site in October 2016 (PoBG: S1, $p = 0.001$), while a steeper slope (9.10) followed at the PoBG site in June 2017 (PoBG:S2, $p < 0.001$), and represented a stronger impact on alpha diversity. As a result, increasing SO_4^{2-} concentrations implied increasing effective richness (^{1}D) within a particular sample. Conversely, negative slopes at PvLg in October 2016 (PvLg: S1, $p = 0.013$, slope = −0.50) and PvLg in June 2017 (PvLg: S2, $p < 0.001$, slope = −2.45) indicated an expected decreasing rate in ^{1}D with increasing SO_4^{2-} values. A strong positive correlation of clofibric acid was shown within the seasons S1 (slope = 149.76) and S2 (slope = 2333.71), inferring that an increase of one unit of clofibric acid could lead to a significant change in ^{1}D. A positive correlation was followed between phenobarbital and ^{1}D at the PoBG site ($p < 0.001$, slope = 1550.77), whereas it was interpreted that ^{1}D decrease with increasing values of phenobarbital at the PuBG and the PvLg site (slope = −708.96, $p \leq 0.001$; slope = −626.11, $p \leq 0.001$). These results suggest that a substantial change of phenobarbital concentrations would cause significant changes in the effective richness.

The global effects of environmental variables on beta diversity were performed with redundancy analysis (RDA). The final RDA model explained 34.6% of the total variance in OTU_{rlog} (adjusted R_2).

It consisted of 16 constrained dimensions, with the first three dimensions representing 80.3% of the total explained variance. Variables included in the RDA model are shown in SI, Table S6. The effect size and significance of SO_4^{2-} and phenobarbital were compared within DNA samples and the extent of change in OTU abundances was estimated (Table 3).

Table 2. Outputs of the effect size and significance of sulfate, clofibric acid, and phenobarbital on alpha diversity within location and season, season, or location. The effect size is represented as the slope. *p*-values were adjusted for multiple testing with the Holm–Bonferroni test, (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; . not statistically significant).

Condition	Slope	Standard Error	DF	Wald χ^2	*p*-Value	Adjusted *p*-Value	Significance
				Sulfate			
PoBG:S1 [1]	0.87	0.25	1	12.08	0.001	0.002	**
PoBG:S2 [2]	9.10	0.52	1	302.96	<0.001	<0.001	***
PuBG:S1	−0.11	0.72	1	0.02	0.883	0.883	
PuBG:S2	0.56	0.26	1	4.51	0.034	0.068	.
PvLg:S1	−0.50	0.20	1	6.16	0.013	0.039	*
PvLg:S2	−2.45	0.44	1	31.71	<0.001	<0.001	***
				Clofibric acid			
S1	149.76	40.90	1	13.41	<0.001	<0.001	***
S2	2333.71	358.03	1	42.49	<0.001	<0.001	***
				Phenobarbital			
PoBG	1550.77	150.14	1	106.68	<0.001	<0.001	***
PuBG	−708.96	195.57	1	13.92	<0.001	<0.001	***
PvLg	−626.11	132.99	1	22.17	<0.001	<0.001	***

Abbreviations: [1] S1: season 1 (October 2016); [2] S2: season 2 (June 2017).

Table 3. Effect size and significance of sulfate on beta diversity within the location and season, and phenobarbital within the location. *p*-values were adjusted for multiple testing with the Holm–Bonferroni test, (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; . not statistically significant).

Condition	Degrees of Freedom	F Statistic	*p*-Value	Adjusted *p*-Value	Significance
		Sulfate			
PoBG:S1 [1]	1	2.37	0.017	0.034	*
PoBG:S2 [2]	1	6.27	<0.001	0.001	***
PuBG:S1	1	3.86	<0.001	0.001	***
PuBG:S2	1	1.92	0.021	0.034	*
PvLg:S1	1	1.48	<0.001	0.001	***
PvLg:S2	1	7.39	<0.001	0.001	***
		Phenobarbital			
PoBG	1	5.37	<0.001	<0.001	***
PuBG	1	1.13	0.331	0.331	.
PvLg	1	4.05	<0.001	<0.001	***

Abbreviations: [1] S1: season 1 (October 2016); [2] S2: season 2 (June 2017).

The effect size of SO_4^{2-} on beta diversity in the microbial community was statistically significant within every location and season (Table 3; $p < 0.001$ and $p < 0.05$). Phenobarbital also showed significant interaction within the locations PoBG and PvLg ($p < 0.001$). These results suggest that the change in OTU abundance is significantly dependent of SO_4^{2-} concentrations within location and season, where as phenobarbital concentrations cause significant changes in beta diversity at the PoBG and PvLg sites.

3.4. Effect on Individual OTUs

The effects of SO_4^{2-}, bezafibrate, and phenobarbital on individual OTUs for the most significant OTUs are shown in Table 4, and only OTUs with the highest base mean are represented. Individual OTUs showed a correlation with SO_4^{2-} and phenobarbital that belonged mostly to the class of

Proteobacteria, Actinobacteria, *Acidimicrobiia,* and *Nitrospira.* The statistical analysis revealed a significant higher influence of bezafibrate on individual OTUs has been observed for only one OTU in season 1 (OTU_341, S1) (Table 4). However, the statistical correlation is negligible and implies a weak correlation and a low impact on the changes of the OTU abundance. A weak correlation of SO_4^{2-} (log_2(fold-change) = 0.1171) with OTU_12 *Actinobacteria* (base mean = 30.1) has been detected for PoBG:S2 and the abundance of OTU_12 would increase if SO_4^{2-} increased by one unit. Other *Actinobacteria* OTUs (OTU_6 and OTU_13) showed a similar correlation (Table 4). For OTU_3 *Nitrospira*, one of the most relevant log_2(fold-change) was determined for SO_4^{2-} (log_2(fold-change) = −0.2920). Although a weak correlation, indicated by low log_2(fold-change) values, for OTU_4 *Betaproteobacteria* with SO_4^{2-} and phenobarbital was detected at the PvLg site and the PoBG site, for both compounds an increase in one unit would increase the abundance of the OTU_4 (Table 4). Higher log_2(fold-change) values were only observed for rare detected OTUs (SI Table S7 and Table S8).

Table 4. Effect size and significance of sulfate within location and season, and phenobarbital within the location for individual OTUs. The condition is shown where levels for variables were grouped and were significant. The Identifier (ID) of the OTU and the class of bacteria is given; base mean: mean of the occurred OTUs in the library; log_2(fold-change): size-factor normalized OTU counts; lfcSE: log_2(fold-change) standard error; *p*-value: adjusted *p*-value; padj: false discovery rate (Benjamini–Hochberg adjusted *p*-value).

Condition	OTU	Class of Bacteria	Base Mean	Log_2(fold-change)	lfcSE	*p*-Value	Padj
Sulfate							
PoBG:S2	1	*Nitrospira*	91.8	−0.2758	6.49×10^{-2}	2.12×10^{-5}	4.80×10^{-4}
	3	*Nitrospira*	79.8	−0.2920	6.30×10^{-2}	3.57×10^{-6}	1.41×10^{-4}
	4	*Betaproteo-bacteria*	45.2	0.1362	3.58×10^{-2}	1.41×10^{-4}	2.07×10^{-3}
	6	*Actinobacteria*	60.7	0.1881	3.87×10^{-2}	1.12×10^{-6}	6.79×10^{-5}
	12	*Actinobacteria*	30.1	0.1171	2.97×10^{-2}	8.13×10^{-5}	1.43×10^{-3}
	13	*Actinobacteria*	38.2	0.1363	3.33×10^{-2}	4.20×10^{-5}	8.17×10^{-4}
	56	*Deltaproteo-bacteria*	23.9	0.2551	3.60×10^{-2}	1.46×10^{-12}	3.97×10^{-10}
PuBG:S1	303	*Bacteroidia*	43.8	0.1233	3.39×10^{-2}	2.80×10^{-4}	8.89×10^{-3}
	489	*Nitrospira*	42.9	−0.1112	2.80×10^{-2}	7.03×10^{-5}	4.43×10^{-3}
	509	*Opitutae*	44.5	0.2438	5.24×10^{-2}	3.21×10^{-6}	6.07×10^{-4}
PvLg:S2	4	*Betaproteobacteria*	21.3	0.1381	2.47×10^{-2}	2.36×10^{-8}	6.40×10^{-7}
	7	*Betaproteobacteria*	23.9	−0.1333	3.76×10^{-2}	3.90×10^{-4}	2.33×10^{-3}
	10	*Acidimicrobiia*	92.7	0.2175	2.92×10^{-2}	9.45×10^{-14}	1.62×10^{-11}
	13	*Actinobacteria*	24.1	0.1107	2.77×10^{-2}	6.52×10^{-5}	5.47×10^{-4}
	15	*Gammaproteo-bacteria*	45.9	0.1814	2.23×10^{-2}	4.40×10^{-16}	1.13×10^{-13}
	23	*Alphaproteo-bacteria*	23.0	−0.1441	2.74×10^{-2}	1.40×10^{-7}	2.79×10^{-6}
	27	*Gammaproteo-bacteria*	33.0	0.2329	2.44×10^{-2}	1.15×10^{-21}	5.93×10^{-19}
Bezafibrate							
S1	341	*Thermoleophilia*	20.1	−0.1661	4.24×10^{-2}	8.86×10^{-5}	1.29×10^{-2}
Phenobarbital							
PoBG	12	*Actinobacteria*	39.8	0.1044	2.89×10^{-2}	3.05×10^{-4}	1.14×10^{-2}
	13	*Actinobacteria*	45.4	0.1292	3.05×10^{-2}	2.22×10^{-5}	2.10×10^{-3}
	56	*Deltaproteo-bacteria*	19.7	0.2039	5.01×10^{-2}	4.76×10^{-5}	3.75×10^{-3}
PvLg	5	*Betaproteo-bacteria*	25.1	0.0891	2.44×10^{-2}	2.61×10^{-4}	3.47×10^{-3}
	10	*Acidimicrobiia*	50.1	0.1744	3.43×10^{-2}	3.56×10^{-7}	1.33×10^{-5}
	15	*Gammaproteo-bacteria*	33.0	0.1670	2.20×10^{-2}	3.18×10^{-14}	3.17×10^{-12}
	23	*Alphaproteo-bacteria*	28.3	−0.1230	3.01×10^{-2}	1.55×10^{-5}	4.22×10^{-4}
	27	*Gammaproteo -bacteria*	21.7	0.2081	2.80×10^{-2}	1.14×10^{-13}	9.74×10^{-12}

3.5. Composition and Distribution of the Microbial Communities

Nitrospirae, *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Ignavibacteriae* were the most abundant phyla in the samples, presented by phylum and species level (Figure 3). In both campaigns, the *Nitrospirae*phylum was most frequently detected at the PuBG and PvLg sampling sites, specifically in June 2017 by representing an amount of $\frac{3}{4}$ of the total detected OTUs (Figure 3, S2 DNA). However, the PoBG site is located upstream of the ditch Buchholzer Graben and showed a different pattern, dominated by *Chloroflexi* and *Actinobacteria* and different classes of *Proteobacteria* (Beta-, Delta-, and Gamma-proteobacteria; Figure 3).

Results of the cDNA-sequenced data revealed that *Actinobacteria*, *Nitrospirae, Proteobacteria*, *Bacteroidetes,* and *Acidobacteria* were the five most abundant groups at the phylum level (Figure 3). The *Actinobacteria* was the most abundant phylum in all sampling sites, followed by the *Nitrospirae* phylum at the PuBG and the PvLg site. Compared to the obtained sequences from extracted DNA, the low abundance of the *Chloroflexi* phylum was detected in cDNA samples. Contrary to the community composition obtained using DNA from sediment samples, a higher distribution of *Proteobacteria* phylum was demonstrated in the cDNA samples showing a different pattern of the microbial community.

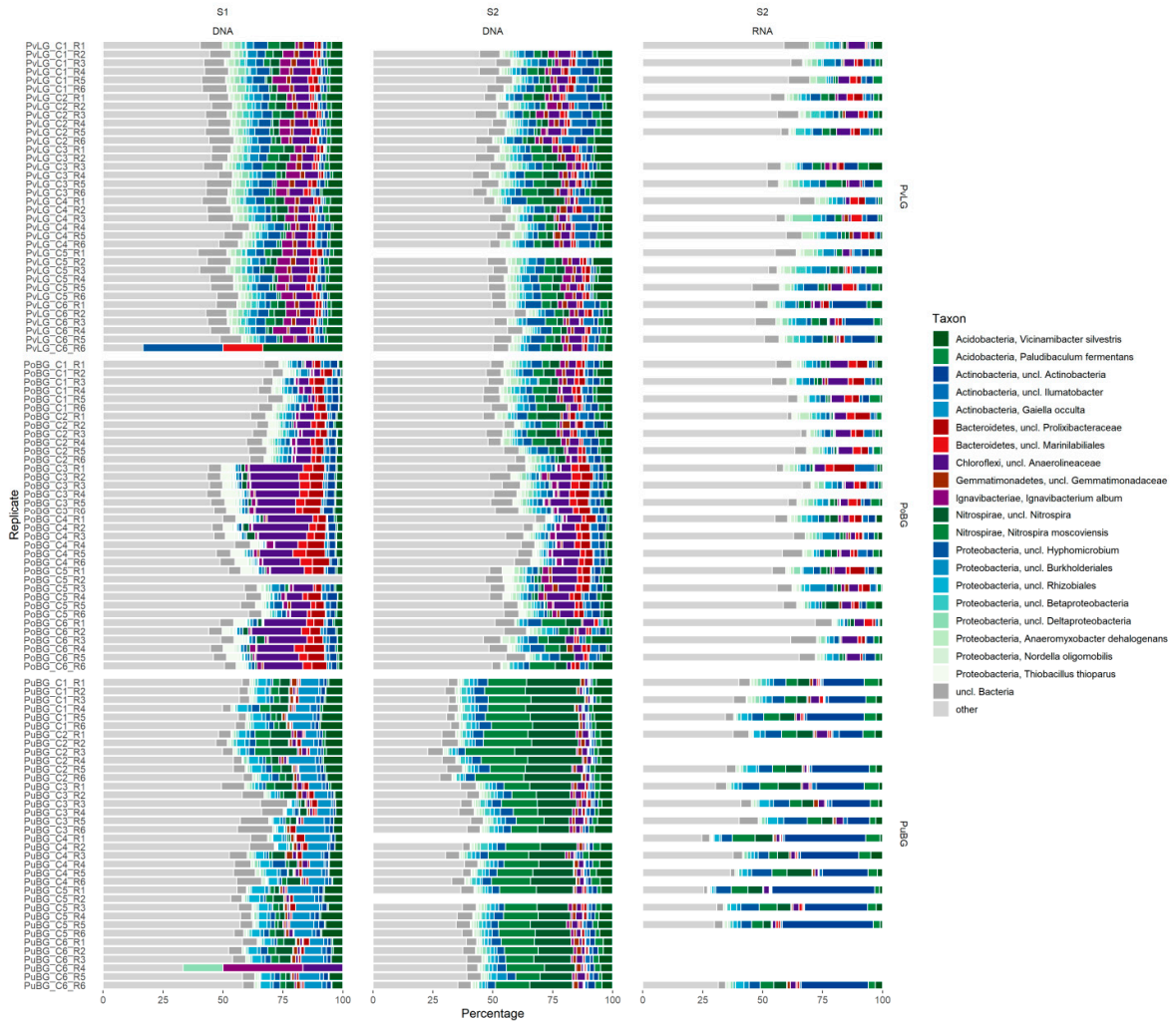

Figure 3. Bar graphs depicting taxon frequencies summarized by phylum and species level based on V3-V4 rRNA gene amplicon sequencing of extracted DNA from sediment samples associated to the three sampling sites in the Panke during two different seasons (S1 DNA and S2 DNA). Bar graphs of extracted RNA associated with the three sampling sites in the Panke during the summer season in 2017 (S2 RNA). S1: season 1 (October 2016) and S2: season 2 (June 2017).

4. Discussion

The objective of this study was to estimate the impact of treated wastewater effluents containing persistent micropollutants on sediment bacterial communities. One sampling site directly affected by the WWTP effluent and two sampling sites upstream were sampled along a river section of 1.9 km. Hence, large datasets of DNA and cDNA sequences were used in statistical analyses to evaluate specific correlations between micropollutants, physicochemical parameters, and the microbial communities.

The DOC in the Panke river was in accordance with the results of Aristi et al. [40], who found higher DOC values near a WWTP which decreased with increasing distance to the plant, and a several-fold increase in the DOC concentration caused by a WWTP effluent was also observed by Ruggiero et al. [41]. In our study, higher DOC concentrations were found mainly at the PuBG and the PoBG site, within the summer campaign 2017. This could be caused by massive rain events and possible subsequent runoffs into the Panke River, which are commonly recognized phenomena during storm events [42].

Furthermore, micropollutant concentrations up to μg L^{-1} were identified at the PuBG site, downstream of the Buchholzer Graben, which receives effluent from the WWTP. Higher concentrations in June 2017 could have been caused by heavy rain events and resulted in backwater effects, which have also been described for this site by Lange et al. [24] (SI Table S3). Bezafibrate, clofibric acid, carbamazepine, and diclofenac were detected up to 100, 250, 460, and 1300 ng L^{-1} (SI Tables S3 and S4) in pore water samples, and were in accordance to former studies of Ternes [43] who detected comparable concentrations in pore water and surface water samples.

The microbial community composition of the three investigated sampling sites revealed significant differences at the 16S rRNA level, which were analyzed using Illumina MiSeq amplicon sequencing. No significant differences in the microbial community composition were observed at the PvLg site between the sampling campaigns. The PoBG and PuBG sites revealed a significant difference in the microbial community patterns. The PoBG sampling site had unraveled a different composition of the microbial community, showing a significant occurrence of the *Chloroflexi* phylum. In contrast, the microbial communities at the PuBG sampling site, downstream of the WWTP effluent were dominated by *Nitrospirae* and *Proteobacteria*. The results at the PuBG site are consistent with the results of previous studies of Atashgahi et al. [19] and Drury et al. [23], who also found a high abundance of *Nitrospira* and *Proteobacteria* phyla in river sediment downstream of WWTP effluent. The present Illumina MiSeq sequencing results showed no significant differences in the microbial community composition at the PvLg site, located 1.9 km upstream from the Buchholzer Graben and thus unaffected by WWTP effluents. As 16S rRNA gene sequencing from extracted DNA revealed differences between the microbial community patterns of the different sampling sites in the first campaign, sequencing of 16S rRNA with synthesized cDNA samples from extracted total RNA as well as from extracted DNA was performed in the second campaign. The community composition based on cDNA samples showed a different composition compared to the DNA samples, especially at the PuBG sampling site (Figure 3). An increased proportion of SSU rRNA molecules of SSU rRNA genes per cell can be identified in highly metabolically active cells [44]. This means that highly active taxa with low cell counts may be underrepresented or not detected in DNA-based analyses, but may be overrepresented in RNA-based analyses [45,46].

Furthermore, 16S rRNA sequencing revealed the presence of sulfate-reducing bacteria (SRB) in the sediments, which belonged to the order *Desulfobacterales*, *Desulfuromonadales*, and *Syntrophobacterales.* The highest counts of OTUs belonging to the SRB group were found at the PoBG sampling site (SI Table S8). Statistical analysis of the next-generation sequencing (NGS) data showed a positive correlation of SO_4^{2-} and alpha diversity at the PoBG site (PoBG:S1 and PoBG:S2), and hence a significant increase in effective richness, while PvLg:S1 and PvLg:S2 indicate a slight decrease in effective richness with increasing SO_4^{2-} concentration at the PvLg site. With respect to effective richness (^{1}D) and SO_4^{2-}, no correlation has been observed for the PuBG site. Thus, the diversity of OTUs, especially at the PoBG

site, is influenced by SO_4^{2-} and may explain the higher abundance of the SRBs, without having to be the cause of higher SRB abundance.

However, it must also be taken into account that SO_4^{2-} was measured in surface water of the Panke River. Studies which investigated SO_4^{2-} fluxes into lake sediments, pointed out that increasing SO_4^{2-} concentrations in lake waters may not necessarily affect rates of sulfate reduction [47]. The primary sources of dissolved SO_4^{2-} in river waters may be derived by mineralization of organically-bound sulfur in the soil, from rain-water, and from agricultural and industrial origin such as fertilizers and industrial effluents [48,49], or conceivably also from surface runoffs in urban areas. Rivers can sometimes contain riparian zones in which reducing conditions prevail throughout most of the year, facilitating the occurrence of bacterial (dissimilatory) SO_4^{2-} reduction [50]. Hence, the backwater effects at the PoBG site [24], may even support the ability of SO_4^{2-} to diffuse into the sediment and to affect the microbial community in the sediment. Moreover, the mean porosity of the sediments was mostly high and indicated coarse and medium sand (SI Table S2). Therefore, it is likely that diffusion of SO_4^{2-} into the sediment could take place. Anoxic conditions at the PoBG site may have developed due to the backwater effects from the inflow of the Buchholzer Graben. Despite the higher oxygen amount of the surface water (Table 1), the high abundance of SRB at the PoBG site may indicate anoxic condition within the sediment, where sulfate acts as an electron acceptor.

Regarding the pharmaceuticals, clofibric acid was found at low concentrations (20–250 ng L^{-1}, SI Table S3) at the PuBG site. Former studies have detected clofibric acid concentrations up to 450 ng L^{-1} in Berlin surface water [51,52]. To our knowledge, since 2002 no studies have reported further measurements of clofibric acid in surface- and groundwater in Europe. The concentration detected in our study are consistent with the results of previous studies [52,53] and showed no significant change in clofibric acid concentrations. The results of the biostatistical analyses implied an effect of clofibric acid on the community composition and showed the effective richness (^1D) would theoretically increase with higher clofibric acid concentrations in both seasons. However, the natural range of variation of clofibric acid in the sample must be taken into account, because the statistical analyses are defining the changes of the environmental variable per unit of 1. Therefore, the effect affects by an environmental variable with a value <1 would decrease the real effect size on the microbial community.

Phenobarbital was detected up to 50 ng L^{-1} (SI Table S3) at each sampling site. Due to the persistence of phenobarbital, the occurrence could be traced back to industrial contamination or old contaminated land sites, which has been shown for different barbiturates in several German rivers by Peschka et al. [54]. The results showed that the effective richness would decrease with increasing phenobarbital concentration in Location PuBG and PvLg and would increase with increasing phenobarbital concentration in Location PoBG, albeit with large variance.

Our study showed that although the concentrations of phenobarbital and SO_4^{2-} were very low, the bacterial diversity was strongly correlated with phenobarbital so that the latter was an explanatory variable for disturbing diversity. This also counts to SO_4^{2-}, whereby, the coefficient of variation for phenobarbital was about twice as large as for SO_4^{2-}. These results suggest that SO_4^{2-} and phenobarbital influence bacterial diversity. In addition to the location and (in the case of SO_4^{2-}) the seasonal factor. As the variable "location" summarizes all environmental conditions, it can be assumed that there may be other influencing environmental factors/variables behind, which have not been explicitly measured in this study.

Considering that micropollutants and other organic compounds affect the microbial communities in aquatic systems. Ager et al. [55] have already shown in microcosms experiments that the microbial community reflects the impact of anthropogenic disturbance in aquatic systems as the occurrence of antibiotics promotes the development of antibiotic-resistant bacteria and antibiotic-resistance genes. Furthermore, the effects of micropollutant mixtures in aquatic systems are of rising concern, as some micropollutants do not elicit significant toxic effects when acting singly, but the combination of different micropollutants can enhance significant ecotoxicity [56]. In previous studies, the combination of

carbamazepine and clofibric acid showed stronger effects on *Daphnia magna*, compared to compounds exposed individually [57].

Most published studies have examined the effect on different fish species or phytoplankton. However, studies on riverine microbial communities are rare. Nevertheless, Lawrence et al. [11] showed that levels of pharmaceuticals at ten µg L^{-1} had toxic effects on microbial communities. Schreiber and Szewzyk [58] have investigated the initial adhesion of bacteria in batch cultures and showed that pharmaceuticals at trace level concentrations influenced bacterial adherence.

These previous findings revealed that pharmaceuticals, even in minimal doses, can affect microorganisms and need to be considered as critical factors. So far, laboratory experiments have provided valuable insights into the influence of pharmaceuticals on microbial communities or pure cultures. In this study, biostatistical analyses were used to assess whether causal relationships between microbial communities and micropollutants, as well as environmental variables, can be depicted under environmental conditions. The analyses based on linear regression models revealed an influence of SO_4^{2-} and phenobarbital on alpha- and beta-diversity, while clofibric acid had only an impact on alpha diversity. Furthermore, applying DESeq2 and the Wald test only a weak correlation and negligible effect of SO_4^{2-} or phenobarbital on the most significant OTUs could be proven.

5. Conclusions

In summary, this study presented a comprehensive profile of the bacterial sediment community of an urban River by providing suitable information regarding alterations of the bacterial population due to the impact of WWTP effluents. However, variations in the bacterial community structure were observed at the different sampling sites; the present findings revealed the composition of the bacterial community not governed only by the effluent.

Phenobarbital and SO_4^{2-}, which did not originate from the WWTP, explained the highest proportions of statistical significance and are therefore suggested to be the defining factors (depending on location) that affected the bacterial community structure and composition most. Nevertheless, their dependency on location indicates that also unobserved factors are likely to play a role than just SO_4^{2-} and phenobarbital.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/11/5/888/s1, Figure S1: Pearson correlation of measured environmental variables, Table S1: Sediment characteristics of the sampling sites PuBG and PoBG in the Panke, Table S2: Water characteristics in the Panke, Table S3: Micropollutant concentrations (µg L^{-1}) in the Panke obtained from three different sampling sites during two seasons (autumn 2016 and summer 2017). All water quality data represent mean standard deviation values. If not different stated all means represent values from triplicates, Table S4: Micropollutant concentrations (µg L^{-1}) in the Panke obtained from three different sampling sites during one season (autumn 2016 or summer 2017). All water quality data represent mean ± standard deviation values, Table S5: Terms of the overall alpha diversity GLS model, Table S6: The highest interaction terms of variables in the final RDA model of the beta diversity on the DNA level, Table S7: Effect size and significance of SO_4^{2-} within location and season and phenobarbital within the location for individual OTUs with high log_2(fold-change). Conditions are shown levels for variables were grouped, and missing levels did not yield significant results. The ID of the OTU and the class of bacteria is given; base mean: mean of the occurred OTUs in the library; log_2(fold-change): size-factor normalized OTU counts; lfcSE: log_2(fold-change) standard error; *p*-value: adjusted p-value; padj: false discovery rate (Benjamini–Hochberg adjusted *p*-value), Table S8: Individual OTUs belong to the order *Syntrophobacteriales*, *Desulfuromonadales,* and *Desulfobacterales.*

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