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Lotic Ecosystem Sediment Microbial Communities' Resilience to the Impact of Wastewater Effluents in a Polluted European Hotspot—Mureș Basin (Transylvania, Romania)

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Abstract: The aim of this study was to assess the impact of WWTP effluents on the sediment microbial communities throughout the Mureș River. This study shows the existence of an ecological equilibrium between the WWTP effluent disruptors and the resilience of the Mureș River sediment microbiomes, a fact that suggests the river's stable/balanced ecological status in this regard, partly due to the microbial communities' resilience to the local impact of WWTP effluents. High-throughput 16S bacterial metabarcoding was used to evaluate the bacterial communities in the sediment. Due to the lotic system's sediment microbial communities' sensitivity to environmental changes, we assumed the dependency of these community structures and functions on environmental abiotic and abiotic parameters. The study results show that, although bacterial communities are equally diverse in the three locations (upstream WWTP, WWTP effluents, and downstream WWTP), there is a difference in community structure between the upstream samples and the WWTP samples, while the downstream samples contain a mixture of the upstream and WWTP effluent communities. Just downstream of the WWTP sediment, microbial communities are influenced by the specific input from the WWTP effluents; nevertheless, the river sediment microbiome is resilient and able to further recover its natural microbial composition, as evidenced by the similarity in bacterial community structures at all upstream river locations. This study demonstrates the ecological equilibrium between the WWTP effluent disruptors and the resilience capacity of the Mureș River sediment microbiomes, a fact that indicates the river's stable/balanced ecological status, in part due to the microbial communities' resilience to the local impact of WWTP effluents. Based on these findings, a monitoring system should be implemented here in the future.

Keywords: pollution hotspot; wastewater treatment plants; sediment contamination impact; microbial communities' sensitivity and resilience

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Citation: Boeraș, I.; Burcea, A.; Bănăduc, D.; Florea, D.-I.; Curtean-Bănăduc, A. Lotic Ecosystem Sediment Microbial Communities' Resilience to the Impact of Wastewater Effluents in a Polluted European Hotspot—Mureș Basin (Transylvania, Romania). *Water* **2024**, *16*, 402. https://doi.org/10.3390/w16030402

Academic Editor: Abasiofiok Mark Ibekwe

Received: 2 December 2023
Revised: 18 January 2024
Accepted: 20 January 2024
Published: 25 January 2024

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1. Introduction

Most ecosystems are highly dynamic in terms of both their species composition and abundances and their functioning [1]. At the top, lotic systems are excellent, diverse, dynamic, nonlinear, and natural systems created by the long-term interactions between natural abiotic and biotic elements and forces [2–4].

Since the Anthropocene began, a wide variety of human-induced stressors have significantly impacted these ecosystems [5]. In systems impacted by humans, elements of environmental flows are a key tool for the sustainable management of water [6,7]. As such, there is a need for in-depth knowledge of the compartments of the entire lotic ecosystem.

The abiotic and biotic elements of rivers and stream watersheds are described as having pronounced ecological unpredictability, particularly since the Industrial Age began [8–11].

Problems related to human impact can be found all over the earth, and the Danube Basin is no exception [12–28], experiencing problems in terms of complexity, resilience, homeostasis, autopoiesis, etc.

The studied Mureș River is, regrettably, not excluded from this context; from this perspective, it is considered a European hotspot [29–34], having been impacted by humans from the Stone Age onwards [35–39]. It is the major tributary of the Tisza, situated between a longitude of 20°11′ E and 25°44′ E and a latitude of 45°14′ and 47°08′, with a length of 761 km and a basin surface area of 28,319 km^2. It passes through 25% mountainous relief, 55% lower highlands, 15% valleys and meadows, and 5% plains [40,41].

The Mureș Basin was selected for this study for several reasons, including its large surface and rank within the Danube Basin [42], its large human population [40,43], and the related human activities and impact [29,33,34,37–39,44] that disturb the river basin's ecological state.

The effects of some pollutants have rarely been evaluated in relation to the Mureş River's taxonomical categories, including bacteria [45–48], which have been studied only with a focus on particular groups, such as fecal streptococci and coliforms [49–52], despite the fact that bacterial microbiomes are important both for the environment's ecological status and for human health [53,54].

Alongside human evolution, various environmental interactions have exerted a profound influence on shaping symbiotic relationships with a multitude of species. Far from being a solitary entity, the human body hosts about 3.8×10^{13} bacterial cells—more than the latest human cell count, estimated at 3.0×10^{13} [55]. Weighing only 0.2 kg, the human microbiome has at least 100-fold more unique genes than the human host genome, with many of them directly influencing human health and inducing many illnesses [55–71].

The diversity and structure of microbial communities are inevitably impacted by environmental stress; however, our knowledge of its effects is relatively limited [72]. Generally, insufficient research has been undertaken on the bacterial communities in Romania's rivers and streams, with only one large-scale study focused on the Danube [73]. Research that focuses on the Mureș River basin has ordinarily had constricted aims, for instance, accounting for few pathogenic bacteria, coliforms, antibiotic-resistant bacteria, and bacteria and sediment relations [74–76]. This research reveals some of the pollution-associated risks to the health of the studied lotic ecosystem and the dynamics of the bacterial microbiota community, as well as its capacity for resilience under the impact of stress from wastewater plant effluent.

Aquatic ecosystems are built and function in a complex and multi-dimensional spatiotemporal manner [77–83]. Such self-adaptive ecological systems demonstrate variability, with a high number of co-dependent self-governing elements organized into complex systems that have the ability to self-adjust and learn from the past [84].

The trend of ecological decline observed in lotic systems around the world, comprising those that are large in size [45,85–87] and are anticipated to have good levels of resilience, is a cause of significant concern regarding the challenges to their sustainable management [88]. Bacteria are the most primitive and adaptive cellular life forms on Earth and constitute its primary biomass, having made complex evolutionary achievements [89]. Historically, the field of bacterial microbiome research emerged from environmental microbiome research (microbial ecology), providing an interdisciplinary platform and a useful approach for many fields of interest [90].

Regarding microbial ecology, if significant advancements were made in finding answers to the question of "Who is on this long list?", then researchers would face more complex challenges in terms of addressing questions such as "What and how are they doing" [91].

Although, in general, we can identify highly rich phylogenetic types of microbial associations, less is known about their communities' structures and integrated means of their complex functioning. The main factors affecting river ecosystems include rainwater, drainage from the land along the riverbed, sediments and substances that are carried

downstream, and, last but not least, sewage and polluted water that are released into the lotic systems.

Microbial communities in the sediment of lotic systems are among the most diverse communities described to date [92–94]; they constitute an essential component of the ecosystem, being implicated in all steps of the carbon cycle, for example, from primary producers all the way to decomposers [95–97]. Due to their plastic metabolism, microorganisms are involved in water decontamination processes and are able to metabolize a wide range of substances [98–101]. Rivers are under constant pressure from anthropogenic sources that also put pressure on the microbial communities in this type of ecosystem [102]. Due to their diversity and plasticity, microorganisms should be able to adapt to and counter most anthropogenic stressors but not all of them and not in all circumstances. This raises the question of what will happen to sediment microbial communities that are under pressure from wastewater treatment plants: will they be resilient and able to overcome the stressors, or will they change their structure to better survive in changing conditions?

In this context, sediments fulfil a vital role in the ecology of streams and rivers [103–105]. Some of the most essential ecological processes in rivers and streams, such as biogeochemical and nutrient cycling, are primarily undertaken by microbial communities that live in sediments [95,97,106] and that are responsible for over 90% of respiration [96,107–111]. The relatively good general ability of rivers and streams to sustain natural biodegradation and bioremediation processes is connected to the microbial communities' diversity, flexibility, and health in the lowest levels of sediments [112]. Even though the microbiomes of sediments in lotic systems are much more varied than the microbiomes of water [92–94], they have been less thoroughly researched [113,114]. Microbial communities are sensitive to fluctuations and alterations in the environment's physicochemical parameters [115–118] and can be used as reliable indicators of an environment's ecological status [119]. Rather than focusing on the indication value of microbial biomes, we intend to identify their potential resilience to stress factors related to effluents from wastewater treatment plants (WWTP); this is an important quality that supports the resilience of ecosystems because this characteristic has positive significance for biogeochemical and nutrient cycling processes, which support other taxonomic groups/communities with lower levels of resilience [30].

WWTPs are major stressors on river ecosystems. Water released from wastewater treatment plants affects the microbial communities in the natural downstream waters in three ways: by changing the nutrient content of the water [120]; by introducing different chemical substances [121–128]; and by introducing new microbial strains [129]. The role of the WWTP is to clean the wastewater and make it safe to return to the natural environment. Conventional wastewater treatment plants (WWTPs) are designed to remove pathogens and coliforms and to reduce the amount of carbon, nitrogen, and phosphorus in the wastewater [60,128,130,131]. However, the treatments and processes in the wastewater treatment plant are not usually able to remove all of the chemicals present in wastewater; for this reason, some are released into natural waters [128,131]. Although WWTPs focus on removing pathogenic bacteria such as coliforms, microorganisms in wastewater are very abundant, to the order of 10^9, and some of them persist and are released into natural waters [125,132,133]. Among the microorganisms that have been found to be released from WWTPs are signature human fecal bacteria belonging to orders including *Bacteroidales*, *Clostridiales*, and *Bifidobacteriales* [134]. Another major concern is the release and transfer of antibiotic resistance genes and other genetic elements [124–126].

Previous scientific studies have considered the impact of WWTP effluent on the diversity of the downstream microbial community; in some cases, they have found an increase in diversity [135,136], in other cases, a decrease in diversity [137], and in some instances, no change in levels of diversity [138]. Many factors have been assessed to determine their impact on the microbial communities' structure. For planktonic bacteria, it has been shown that the bacterial community profiles downstream of WWTPs are a mixture of those found upstream of the WWTP and those found in the WWTP effluent

due to the mixing of the two water flows [139,140]. Even though intensive studies have been conducted on the microbial community's composition and function in hyporheic zones, far less attention has been paid to the impact of WWTP effluent on the microbial ecology of riverbed sediments [136,137,141]. To date, there is no consensus on how WWTP effluents impact microbial communities in the receiving river, and many factors have been shown to influence how the microbes are affected. The impact of a WWTP on the downstream sediment microbiota depends on different factors, such as the type of WWTP and hence the composition of the effluent, the population size of the riverine area, the climate and geography of the region, the size and flow rate of the river, and the buffering and self-purification capacity of the stream sediments under the impact of stresses [141].

Many studies have focused mainly on the immediate effect downstream of the WWTP; however, this research took a comparatively holistic approach to test how the input from a WWTP affects a river in its entirety. The main objective was to assess the impact of WWTP effluents on the microbial communities in sediments throughout the Mureș River.

2. Materials and Methods

2.1. Study Area and Sample Collection

This river was chosen as it is the second longest river in Romania, after the Danube, and it traverses a significant portion of the country from east to west, passing some major cities including Târgu Mureș, Alba Iulia, Deva, Arad, etc. [33]. The samples were collected from 10 locations/cities with 3 sampling sites per location (on the river 200 m upstream of the WWTP effluent, on the river downstream of the WWTP effluent, and the WWTP effluent) and 3 extractions per sampling site, resulting in a total of 90 samples.

High-throughput 16S bacterial metabarcoding was used to evaluate the bacterial communities in the sediment samples. The bacterial communities upstream, downstream, and in the WWTP effluent from 10 different sites along the river were evaluated. River sediment was collected from 10 WWTP sites along the Mureș River watershed in the fall and winter of 2018–2019. At each WWTP site, three different samples were collected: sediment from the river upstream of the WWTP, sediment from the river downstream of the WWTP, and sediment from the WWTP effluent. A map of the sampling sites is shown in Figure 1. The samples were transported in a portable refrigerator at 4 °C and then kept in a freezer at −50 °C until they were analyzed.

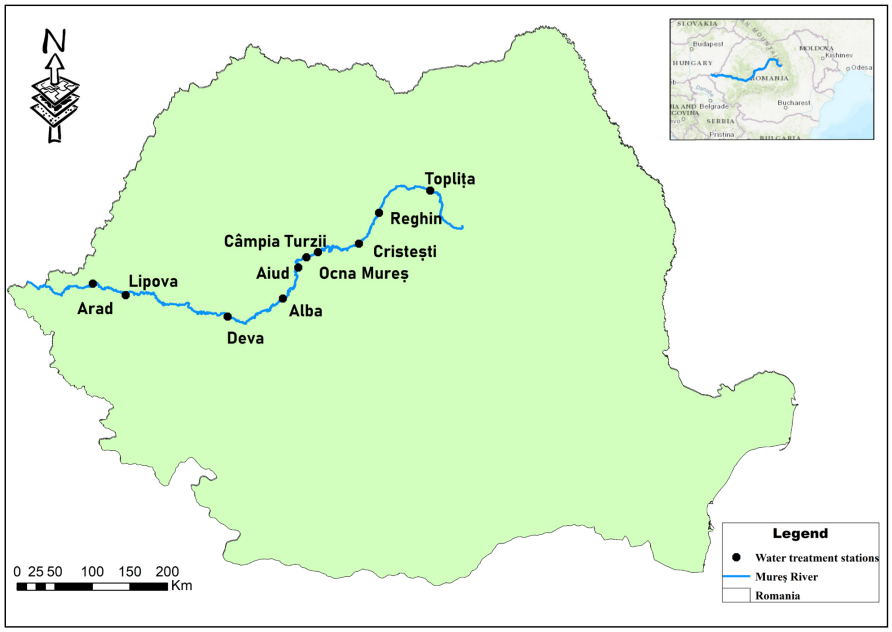


Figure 1. Map depicting the 10 wastewater treatment stations along the Mureș River where the samples were collected.

2.2. DNA Extraction and Quantification

DNA was extracted from each sediment sample in triplicate. It was extracted from 500 milligrams of the sampled sediment using the Quick-DNA Fecal/Soil Microbe Miniprep kit from Zymo Research (Irvine, CA, USA) following the manufacturer's instructions. Briefly, the sampled sediment was weighted and added to a tube containing bashing beads and 750 µL bashing bead buffer. These tubes were vortexed at a maximum speed for 20 min and then centrifuged for 1 min at 10,000× *g*. The supernatant was filtered and then lysed using a genomic lysis buffer. The lysate was loaded onto a spin column that retained the genomic DNA. Once the DNA was bound to the column, it was washed once with pre-wash buffer and once with g-DNA wash buffer. After the washes, DNA was eluted from the column using a DNA elution buffer. As a last step, the DNA was cleared of humic acid by being filtered through another column. After the last cleaning step, the DNA's quality and quantity were checked in preparation for the PCR and sequencing. The amount of DNA was quantified using a Specord 210 plus spectrophotometer (AnalyticJena, Jena, Germany), and the DNA quality was assessed via electrophoresis on 0.8% agarose gel. The DNA was then packed and sent to the LGC Group (Berlin, Germany) for next-generation sequencing on the Illumina MiSeq platform (San Diego, CA, USA).

2.3. 16S Metabarcoding Analysis

In this research, the gene for 16S ribosomal RNA was amplified using primers targeting the V3–V4 region [142]. Adapters were added to the PCR products, and a library was then created. The adapters also contained a barcode for multiplexing during sequencing. The 16S libraries were sequenced using the Illumina MiSeq platform at the LGC Group. The libraries were demutiplexed using Illumina bcl2fastq 2.17.1.14 conversion software [143]. When the barcode distances between all libraries on the lane permitted, one or two mismatches or Ns were allowed in the barcode read. Reads with missing barcodes, one-sided barcodes, or conflicting barcode pairs were discarded. Additionally, reads shorter than 100 nucleotides were discarded. After sorting, the barcode and adapter sequences were clipped. The next steps were performed using the QIIME 2 workflow [144,145]. First, sequence quality control and feature table construction were undertaken using the DADA2 plugin, which corrects amplicon sequence data and filters phiX reads and chimeric sequences [146]. During this step, nucleotides corresponding to the primer sequences were deleted, forward sequences were trimmed to a maximum length of 280 nucleotides, and reverse sequences were trimmed to 240 nucleotides based on the quality of the reads. This analysis generated two curated files, one containing the frequency and the other the sequence, of all the amplicon sequence variants (ASVs) obtained from the sequencing analysis. The sequences were aligned with MAFFT [147]; then, a phylogenetic tree was created using FastTree 2 [140]. Replicate sequences at each sampling site were grouped using the mean ceiling, and this was followed by rarefaction [148] to a maximum depth of 16,294. We trained a classifier using a naïve Bayes method [149] with the used primer pair [142] and the Greengenes 13.8 database [150] at 99% identity. The generated classifier was then used to reference our sequences with the sklearn plugin [149]. The sequence data generated in this study were deposited in the GenBank/NCBI Sequence Read Archive (SRI)/ under the following accession number: PRJNA1015452 [151].

2.4. Statistical Analysis

Most of the statistical analysis was conducted using the QIIME2 workflow. Several alpha diversity indices were obtained by computing a user-specified alpha diversity metric for all samples in a feature table: ACE [152], Chao1 [153], observed OTUs [150], Pielou Evenness [154], Shannon [155], and Simpson [156]. The command core–metrics–phylogenetics was used in this study to compute the alpha diversity index, Faith's Phylogenetic Diversity, and beta unweighted UniFrac [157], generalized UniFrac [158], weighted normalized UniFrac, weighted unnormalized UniFrac [157], and the Bray–Curtis [159], Euclidean [160], and Jaccard [161] diversity metrics.

Within-group differences were assessed using group significance based on the Faith PD and evenness alpha diversity metrics, while between-group differences were assessed using PERMANOVA (permutational multivariate ANOVA) to test the pairwise unweighted UniFrac-type significance using QIIME 2 commands.

Data were graphed in QIIME 2 using the EMPeror tool software [162], which generated principal coordinate analysis (PCoA) plots for each of the beta diversity metrics. Graphs depicting the bacterial community structures were generated in excel. The constrained RDA analysis of the data was realized in Canoco 5 [163]. The map depicting the sample location was generated using QGIS version 3.22.7.

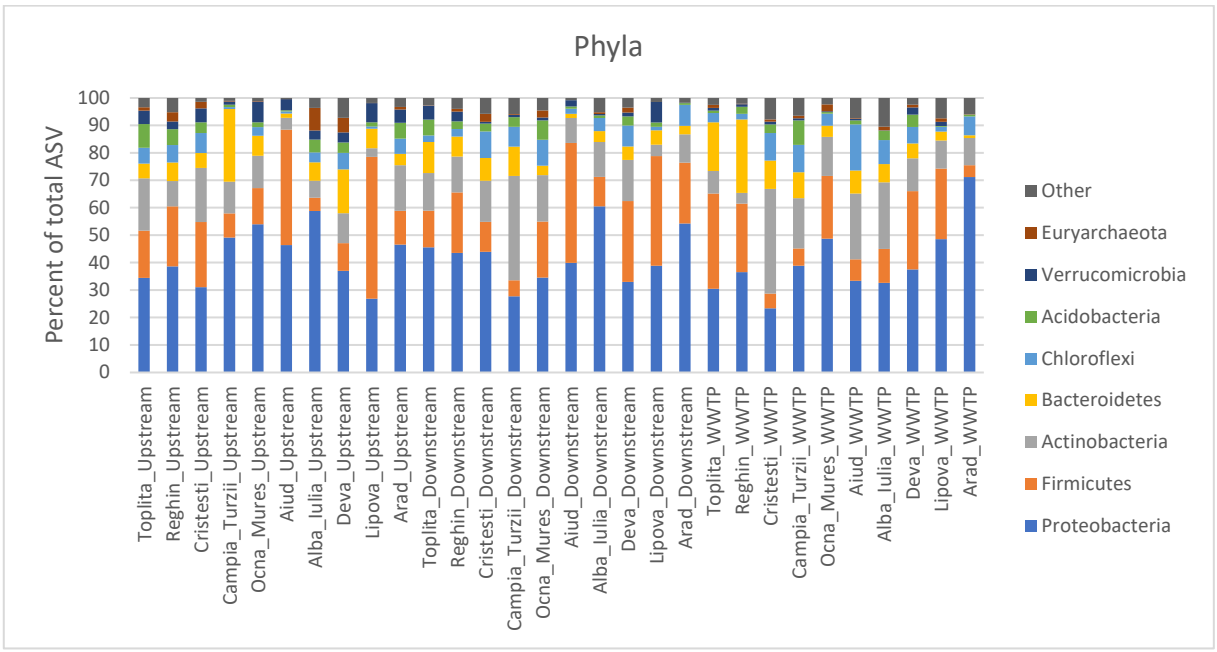
3. Results and Discussions

Ten wastewater treatment plants (WWTP) serving the main cities along the Mureș River were selected for analysis (Figure 1). At each WWTP, sediment samples were collected from the portions of the river upstream and downstream of the WWTP and from the WWTP effluent. DNA was extracted in triplicate from each sediment sample and subjected to 16S rRNA sequencing. The primary reads obtained after sequencing were analyzed using the QIIME 2 workflow. A total of 2,352,102 high-quality reads were obtained after sequencing, with an average of 63,570 reads per sample replicate. The taxonomical classification of the sequences was performed against the GreenGenes database [150]. Sequences from other domains of life, except bacteria and archaea, were removed. Samples were rarefied to the lowest sample total of 16,294 reads.

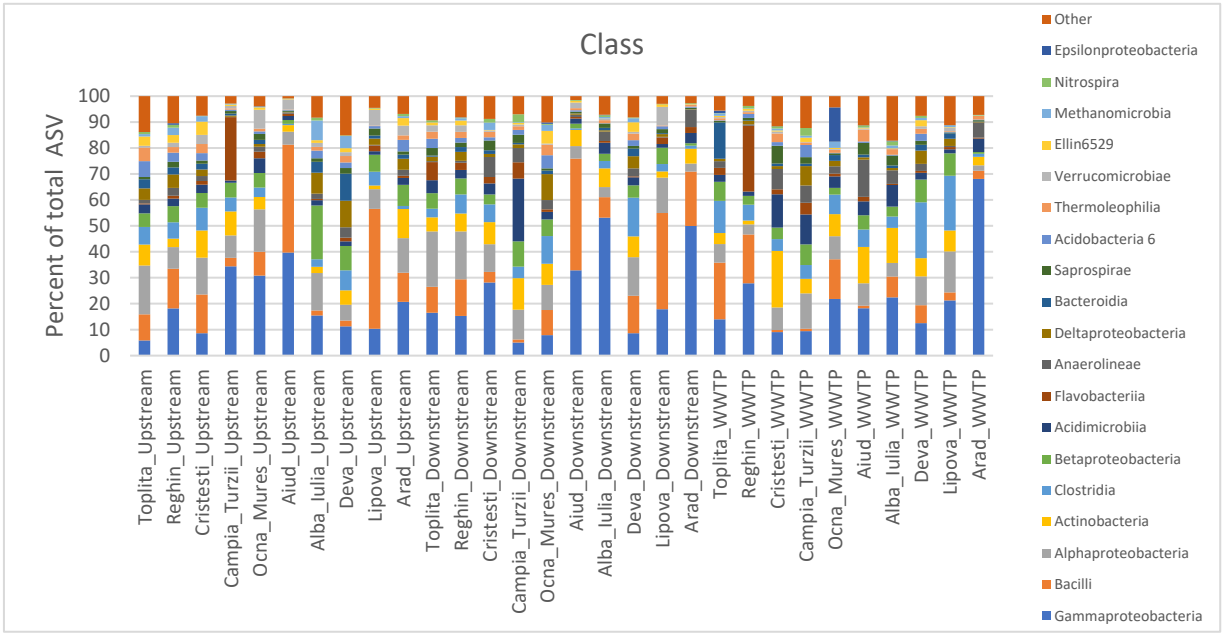
The bacterial community structure in the sediment samples was analyzed at the phylum and class levels. At each of these two phylogenetic levels, the abundance of bacteria and archaea was calculated for each sample as the percentage relative to the total number of taxons/ASV (Figure 2). At the phylum level, we found Proteobacteria to be the best represented, with an average abundance of 41%. The highest abundance of Proteobacteria was in the WWTP effluent in the Arad locality (71%), while the lowest was in the WWTP effluent of the Cristești locality (23%). This result is in accordance with the literature, where Proteobacteria was also found to be the most abundant phylum in the river sediment as well as in WWTP microbial communities [137,138,141,164]. The second-best-represented phylum in our study was Firmicutes (19.9%), followed by Actinobacteria (14%) (Figure 2A). Here, we do not see the same predominance as with Proteobacteria, and there are instances where Actinobacteria is more abundant than Firmicutes. In the literature, however, Bacteroidetes seems to be more frequently in second place, before Firmicutes or Actinobacteria [137,138,164,165]. In our study, Bacteroidetes was the fourth most abundant phylum. At the class level, the best-represented class was Gammaproteobacteria (21%), followed by Bacilli (13%), Alphaproteobacteria (10%), and Actinobacteria (7%) (Figure 2B). In other studies, Alphaproteobacteria was found to be the most abundant class, followed by Gamaproteobacteria [165]. Huo et al. [138] also found Alphaproteobacteria to be the most abundant when they undertook sampling in the spring; however, when they did so in the fall, they saw a shift towards more Gamaproteobacteria [138]. Since we sampled in the fall, this could explain why we also found a higher abundance of Gamaproteobacteria in comparison to other studies.

Phyla and class are not uniformly distributed among the samples, and there are instances where a phylum or class is more abundant in one sample compared to other samples. This difference in the bacterial distribution is probably due to sample properties; therefore, we considered whether it was possible to identify a distinct preference for a certain phylum or class in the samples taken upstream, downstream, or from the WWTP effluent. Therefore, we averaged the results based on these three sampling sites. When we averaged the results of the samples taken upstream, downstream, and from the WWTP effluent at the 10 sampling sites, we found no obvious difference between the three in the relative abundance at the phylum or class levels (Figure 3A,B). Although we did not detect a difference in the abundance of bacteria at the phylum and class levels between the upstream, downstream, and WWTP sampling sites, this does not mean there are no

differences between their bacterial communities. However, these differences most likely manifest at the family and genus levels, as Chu et al. show [164].

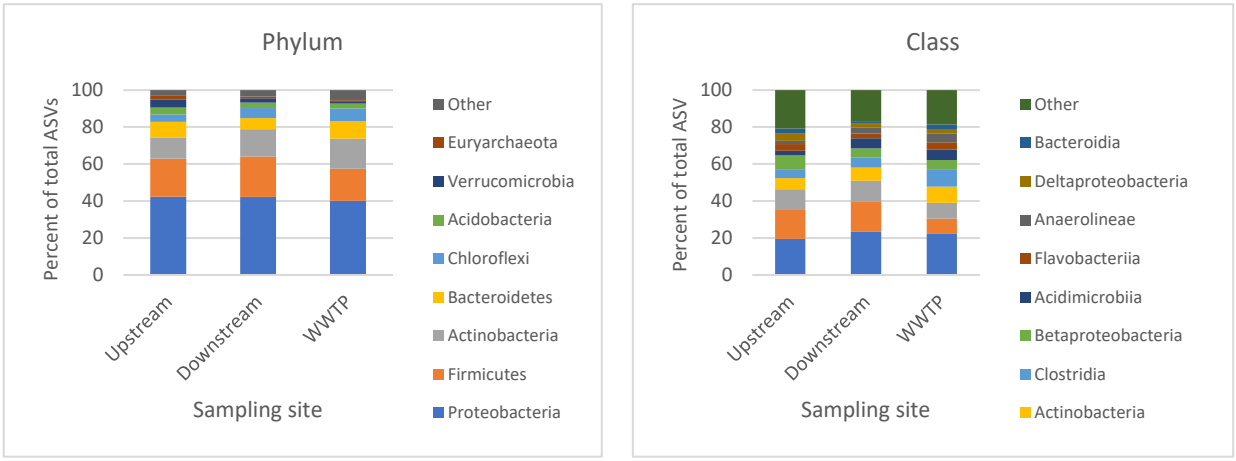


(**A**)



(**B**)

Figure 2. Bar plots representing the relative abundance of bacteria at the (**A**) phyla and (**B**) class levels at each sampling site.



(**A**) (**B**)

Figure 3. Bar plots showing the average ASV for each (**A**) phyla and (**B**) class at the three different sampling sites: upstream, downstream, and WWTP.

We next assessed the alpha diversity found in the sediment samples by measuring the following indices for each sampling site: ACE, Chao1, observed OTUs, Pielou evenness, Shannon, and Simpson (Table 1). Kruskal–Wallis pairwise analysis showed that there was no significant difference in alpha diversity, based on Faith's phylogenetic diversity or evenness, between the groups composed of samples taken upstream, downstream, or from the WWTP effluent (Table 2). As effluents from wastewater treatment plants bring nutrients, pollutants, and a mixture of microorganisms into the receiving river, they are expected to have an impact on the river's microbial communities. Several studies have shown that this impact results in a reduction in the alpha diversity in sites immediately downstream of WWTPs [129,137], while others have shown a change in the balance between autotrophic and heterotrophic bacteria [166]. Other studies detect no change in alpha diversity before and after the WWTP [167–169]. The apparent lack of impact on alpha diversity might be explained by the fact that, while micropollutants can negatively affect the abundance of certain taxa, nutrients can favor the growth of others [166]. The result would be microbial communities that are distinct, as far as beta-diversity is concerned, but which have similar alpha diversity indices [170].

Therefore, to determine whether the microbial communities upstream, downstream, and in the WWTP effluent were similar or not, we needed to measure the beta-diversity. To make comparisons between the samples, we assessed the beta diversity by measuring the Bray–Curtis, Jaccard, and unweighted UniFrac distances. To visualize the results, principal coordinate analysis was applied to the beta-diversity results for the three types of distance. The three emperor plots obtained using the program QIIME 2 and the Bray–Curtis, Jaccard, and unweighted UniFrac distances show the samples taken upstream of the WWTP separately from those taken from the WWTP effluent (Figure 4A–C); meanwhile, the samples taken downstream of the WWTP overlap with both the upstream and WWTP samples (Figure 4A–C). Moreover, when we measured the dissimilarity between the samples from the three different groups (upstream, downstream, and WWTP) according to the unweighted UniFrac beta-diversity, we found a significant difference between samples taken upstream of the WWTP and samples taken from the WWTP effluent (Permanova $p < 0.002$). Samples taken downstream of the WWTP were not significantly different from either the upstream or WWTP samples, suggesting that these samples are influenced by both microbial sources. In conclusion, the microbial communities from samples taken upstream of the WWTP are distinct from those taken from the WWTP effluent, while the communities in the studied river downstream of the WWTP are a mixture of the upstream and WWTP communities.

Table 1. Alpha diversity indices.

	Upstream						Downstream						WWTP					
	ace	**chao1**	**Observed _otus**	**pielou_e**	**shannon**	**simpson**	**ace**	**chao1**	**Observed _otus**	**pielou_e**	**shannon**	**simpson**	**ace**	**chao1**	**Observed _otus**	**pielou_e**	**shannon**	**simpson**
Toplita	1825.451	1851.101	1716	0.887405	9.535022	0.994856	1762.361	1763.651	1636	0.869219	9.27975	0.994416	1648.392	1621.023	1438	0.796373	8.353833	0.985776
Reghin	2452.286	2438.87	2114	0.836419	9.238887	0.987506	2321.928	2303.753	1919	0.848317	9.25186	0.995004	1263.291	1267.406	1113	0.696515	7.048895	0.962853
Cristesti	1656.773	1649.671	1470	0.814555	8.570418	0.986669	1102.947	1098.951	1010	0.820356	8.187268	0.988087	1487.327	1480.721	1285	0.827887	8.550042	0.992252
Campia Turzii	1372.223	1334.133	1153	0.711435	7.236132	0.963658	1466.169	1441.463	1313	0.786708	8.149237	0.978618	1687.261	1685.844	1553	0.8701	9.223789	0.994038
Ocna Mures	1395.385	1398.017	1244	0.816336	8.392566	0.986875	2207.08	2174.717	1954	0.861217	9.41501	0.994969	927.7893	937.7248	851	0.743373	7.235265	0.973194
Aiud	949.3681	926.8194	758	0.612522	5.859419	0.949195	1017.532	1002.938	851	0.617382	6.008988	0.943233	1440.128	1446.745	1219	0.821399	8.420559	0.992683
Alba Iulia	1917.09	1923.907	1731	0.887616	9.548435	0.996946	1071.286	1060.75	954	0.742997	7.354069	0.972143	1190.859	1189.164	1066	0.821933	8.266997	0.991616
Deva	2335.379	2318.371	2118	0.904717	9.995758	0.997948	1529.433	1533.409	1405	0.850043	8.888353	0.991452	1731.43	1724.012	1594	0.84453	8.984481	0.989953
Lipova	1544.162	1524.377	1275	0.729054	7.521126	0.967971	973.5606	982.9364	897	0.751448	7.370925	0.970607	1258.432	1272.625	1053	0.816498	8.19788	0.990158
Arad	2364.827	2307.446	1961	0.834409	9.126244	0.992989	820.2182	801.4219	697	0.636724	6.013872	0.944808	694.131	669.6726	576	0.543884	4.987373	0.877648

Table 2. Group significance based on faith pd and evenness alpha diversity metrics.

Method	Group 1	Group 2	H	*p*-Value	q-Value
Faith-pd	Downstream (n = 10)	Upstream (n = 10)	0.28	0.596701	0.705457
	Downstream (n = 10)	WWTP (n = 10)	0.142857	0.705457	0.705457
	Upstream (n = 10)	WWTP (n = 10)	0.205714	0.650147	0.705457
Evenness	Downstream (n = 10)	Upstream (n = 10)	0.205714	0.650147	0.879829
	Downstream (n = 10)	WWTP (n = 10)	0.022857	0.879829	0.879829
	Upstream (n = 10)	WWTP (n = 10)	0.462857	0.496292	0.879829

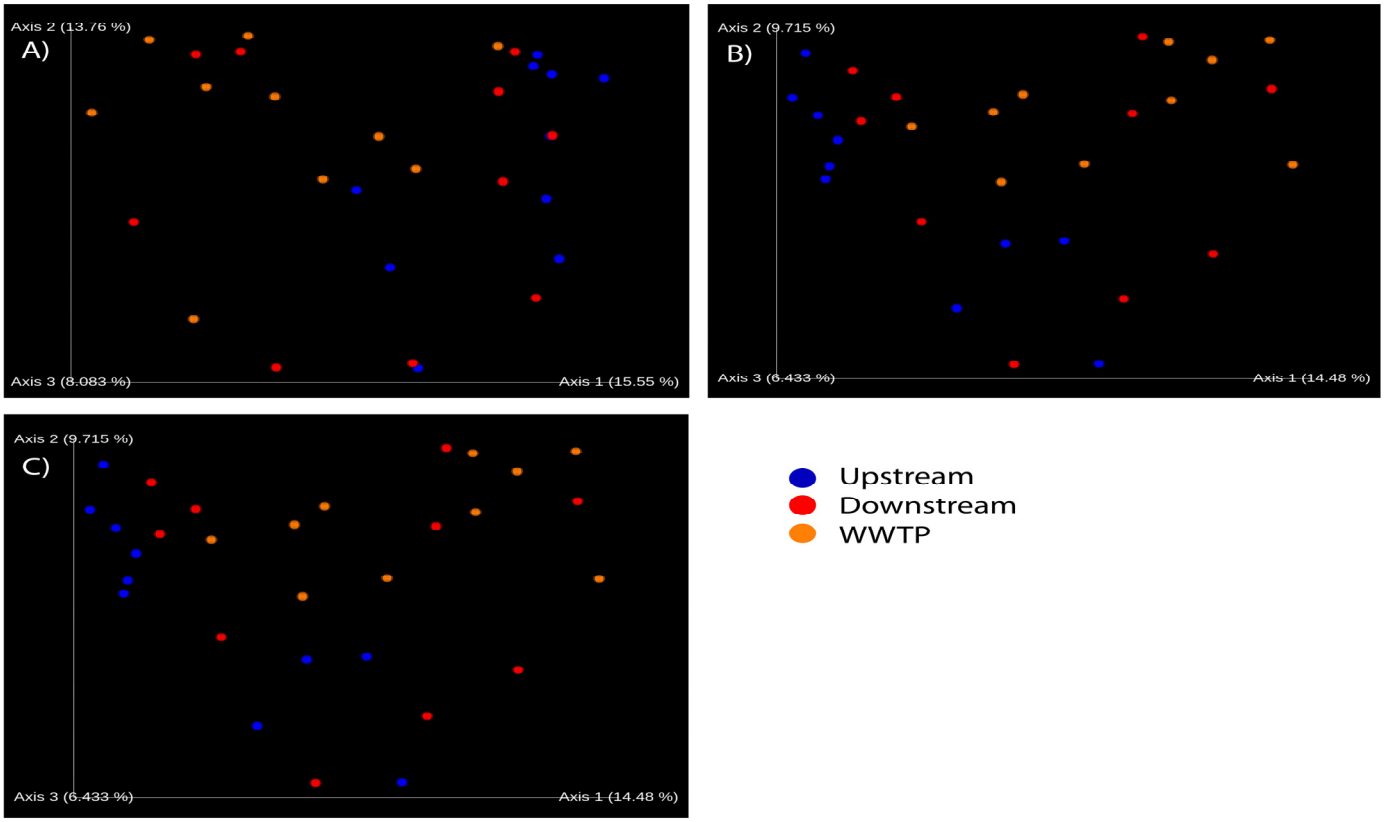


Figure 4. Principal coordinate analyses based on (**A**) Bray–Curtis, (**B**) Jaccard, and (**C**) unweighted UniFrac distances. Each dot represents a sample. Based on the sampling site, the samples are color coded as blue—upstream; red—downstream; and orange—WWTP.

To further confirm this finding, we performed multivariate analysis (RDA) using Canoco 5 on the bacterial community composition at the species level and the sampling sites. The results clearly show specific bacterial species in the upstream and WWTP sites, with the downstream standing in the middle (Figure 5A). The results also show the upstream sites being grouped among themselves, with WWTP on the opposite end of the line, while the downstream samples lay in the middle between the two (Figure 5B). These results further confirm the presence of distinct microbial communities in the river sediment and WWTP effluent. Moreover, they show the impact of WWTP effluent on downstream microbial communities, which have shifted from the microbial structure found in the upstream sites to the microbial structure of the WWTP effluent. This result was expected, as other studies have also found WWTP effluent to have an impact on downstream microbial communities [93,136,139,169,171]. However, unlike our study, these studies only looked immediately downstream of the WWTP and not at the whole river. In this study, we sampled the length of the whole river; as such, we achieved findings that went unnoticed in previous studies. In the first instance, the river microbial community downstream of

the WWTP only partially shifted towards WWTP bacteria and remained similar to the upstream microbial community. The results showed no significant difference between downstream and either WWTP or upstream. Moreover, because we looked at multiple sites along the same river, we were able to determine that the river's microbial community structure recovers from the impact of the WWTP, as all the upstream sites have similar microbial communities. The resilience of the microbial community's structure has been shown before, and our study confirms it [172,173].

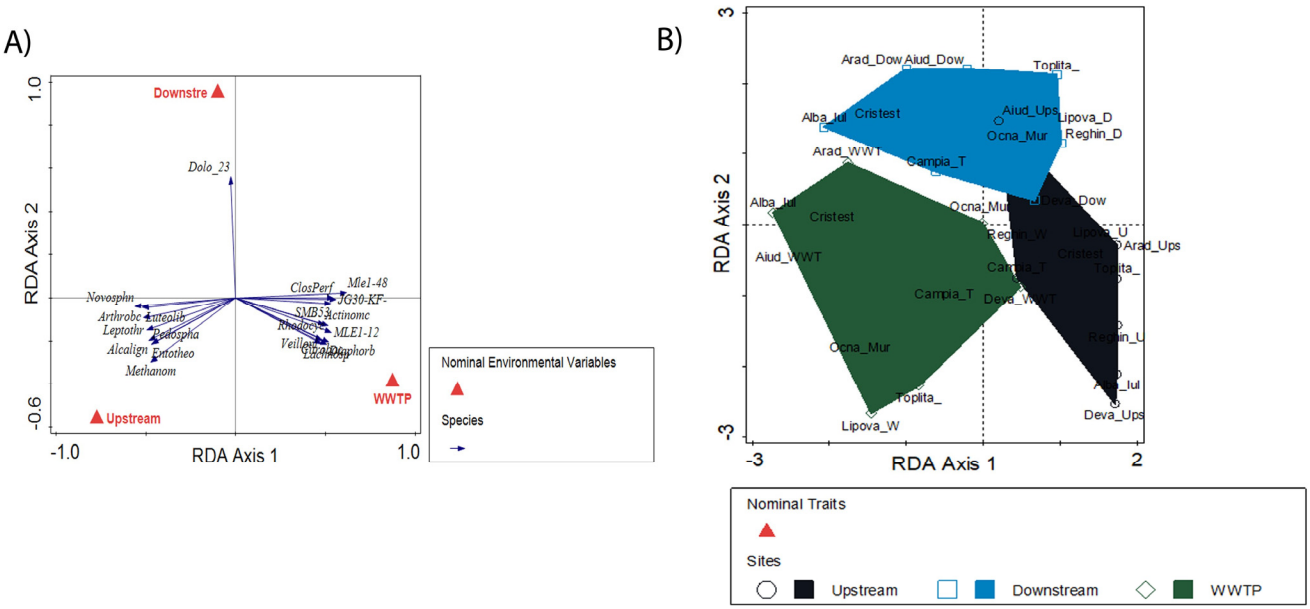


Figure 5. Constrained RDA analysis depicting the distribution of (**A**) species and (**B**) sampling stations.

To answer our question regarding what happens when sediment microbial communities come under pressure from wastewater treatment plants, we can certainly say that the communities exhibit the plastic resistance needed to change, as well as the capacity to withstand the pollution induced by the WWTPs, at least in certain circumstances and up to a specific level of human impact.

Microbial communities in sediments play a key role in aquatic ecosystems. Identifying the structure and functional dynamics of these ecosystems' sediment microbial communities will help us to assess the impact of humans on the environment and, moreover, to predict whether and how these ecosystems will transform and change [174–178].

4. Conclusions

Although the bacterial communities were found to be equally diverse in the three locations (upstream of the WWTP, WWTP effluents, and downstream of the WWTP), there is a difference in community structure between the upstream samples and the WWTP samples; meanwhile, the downstream samples contain a mixture of the upstream and WWTP effluent communities, even though, downstream of the WWTP, the sediment microbial communities are influenced by the WWTP effluents. The main finding of this study is that despite this type of impact, the river sediment microbiome is resilient and able to recover its natural microbial composition, at least to a certain level of impact; furthermore, this is evidenced by the similarity in the bacterial community structures at all upstream river locations. The overall results indicate the ongoing existence of an ecological equilibrium between the WWTP effluent disruptors and the resilience of the Mureș River sediment microbiomes. From this perspective, the Mureș River has a stable/balanced ecological status, partly due to the microbial communities' resilience to the local impact of WWTP effluents. This study is significant because it presents the first dataset referring

to microbial communities' ecological status; it can be used as a reference in necessary monitoring activities in the future.

Author Contributions: Conceptualization, I.B. and A.C.-B.; data curation, I.B. and A.C.-B.; formal analysis, I.B. and A.C.-B.; funding acquisition, I.B. and A.C.-B.; methodology, I.B., A.B. and A.C.-B.; project administration A.C.-B. and I.B.; resources, A.C.-B. and I.B.; software, I.B. and A.B.; supervision, I.B. and A.C.-B.; validation, A.C.-B. and D.B.; visualization, I.B., D.B. and A.C.-B.; writing—original draft I.B., A.B., D.B., D.-I.F. and A.C.-B. writing—review and editing, I.B. and D.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the RO04 Programme—Reduction of Hazardous Substances, through the EEA Grants 2009–2014 financial mechanism ("SIDPOP-Support instrument for decision making in POPS management. Case study: Mureş Catchment Area"). Supplementarily, this research project was financed by Lucian Blaga University of Sibiu through the research grant LBUS-IRG-2022-08, awarded to Ioana Boeraș.

Data Availability Statement: Supplementary data related to the ones presented in this study are not published and are not part of repositories, but can be available on request from the corresponding authors.

Acknowledgments: The authors thank the funding entities which supported this work.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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