



# Article Evaluating the Ecological Impact of Wastewater Discharges on Microbial and Contaminant Dynamics in Rivers

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Abstract: This study focused on assessing the impact of pollutants discharged from the Fuxin Meng Wastewater Treatment Plant (FMWWTP) on the microbial communities in the Xi River; specific comparison between upstream and downstream regions was made. Water samples were obtained by mixing morning, midday, and evening sampling at two points: one upstream and one downstream, each 500 m from the FMWWTP outfall. Utilizing metagenomic sequencing in conjunction with measured conventional physical and chemical properties, the research aimed to elucidate differences in microbial community structure, metabolic functions, potential biological contamination, and antibiotic resistance gene prevalence. The findings indicated a notable decrease in microbial diversity downstream compared to upstream; this was influenced primarily by the effluent from FMWWTP. This disparity in microbial diversity was evident at various taxonomic levels, with downstream samples showing higher diversity at the phylum level than at the genus level. Furthermore, downstream microbial populations demonstrated a broader range of metabolic and functional genetic diversity. Interestingly, the abundance of metabolic systems was generally greater downstream, with the notable exception of energy metabolism. This could be attributed to the stress imposed on downstream microorganisms by organic chemicals discharged from the treatment plant, and this prompts an enhanced metabolic decomposition function. The study also uncovered significant levels of potential biological contamination and antibiotic resistance gene pollution. This was more pronounced downstream of FMWWTP. In conclusion, discharge from FMWWTP has a substantial impact on the microbial communities of the Xi River, and this underscores the urgent need to optimize wastewater treatment processes to better comply with environmental quality standards.

**Keywords:** wastewater; water treatment plant impact; metagenomic contamination analysis; microbial community dynamics; antibiotic resistance genes

# 1. Introduction

Wastewater Treatment Plants (WWTPs) play a crucial role in processing urban domestic wastewater. They do this by effectively reducing harmful substances to minimize environmental impact. Despite their importance, effluents from WWTPs often fail to meet set standards [1,2]. Historically, the pollution of rivers by WWTP effluents may have been underestimated due to belief in the self-purifying capacity of rivers. However, as wastewater discharge and industrialization has increased, rivers have been put under growing stress, which diminishes their self-purification ability. The impact of WWTP discharge exceeds the inherent capacity of rivers, and this directly affects vital water resources and the ecological balance.

Studies have identified WWTP effluents as major sources of nitrogen and phosphorus in rivers. There is a positive correlation between the concentration of nitrogen [3], phospho-



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**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). rus, total nitrogen (TN), and chemical oxygen demand (COD) [4] in rivers, and the volume of wastewater discharged. This effluent adversely affects the aquatic ecosystem [5], altering microbial community structures and promoting the proliferation of difficult-to-remove resistance genes [6]. Consequently, recipient rivers may suffer from biological and chemical pollution, spread of resistance genes, and bioaccumulation of trace pollutants [7,8]. This significantly harms water quality and affects healthy ecosystems. A comprehensive analysis is needed to understand the extent of the damage to water quality and ecology in these rivers.

This study uses both chemical and biological indicators to comprehensively analyze the impact of FMWWTP discharge on the Xi River. This method differs from traditional single-factor index analyses. Traditional culturing techniques cannot fully identify the complex microbial communities [9], therefore, potential microbial mechanisms affected by the effluent are often overlooked [10]. Metagenomics was, therefore, chosen to analyze the comprehensive impact of FMWWTP on the Xi River. Metagenomics, which is a reliable technique for analyzing the DNA of all environmental microbes [11], helps in the study of the composition, function, diversity, interactions, and genetic aspects of microbial communities in various environmental processes [12]. This approach avoids PCR biases, and offers advantages over 16S amplicon sequencing [13]. This integrated analysis, which combines metagenomic technology with physicochemical property indicators, provides a more accurate reflection of the pollution in the river from the FMWWTP; in comparison to traditional single-factor indicators.

This research primarily focuses on water quality changes resulting from river discharge, which emphasizes the impact on microbial communities. Through gene abundance analysis, we aim to unravel the underlying mechanisms driving community succession, and to identify traits and responses from a vast gene pool. This study uniquely considers the unilateral effects of WWTP effluent, as no other effluent sources were identified between our sampling sites. By examining changes in water quality, microbial community structure, metabolic functions, and anti-pollution genes, we endeavor to present a comprehensive overview of the effects of sewage discharge on the Xi River.

## 2. Materials and Methods

### 2.1. Sample Collection

Water samples were collected from the Xi River, situated near Fuxin in the Liaoning Province of China. The exact sampling coordinates are 42.052334° N, 121.747798° E, as depicted in Figure 1. Two sampling sites were chosen along the river: one 500 m upstream (XDU) and another 500 m downstream (XDD) from the Fuxin Meng wastewater treatment plant's outflow into the Xi River. This sampling approach was designed to investigate the impact of the wastewater treatment plant on the quality of river water in these areas.

Samples were collected three times: once in the morning, once at midday, and one in the evening. Each sample amounted to 1 L. After collection, they were immediately placed in ice bags and stored at 4 °C until the next day. Water quality was tested according to GB3838-2002 standards [14]. Temperature and pH were measured at the sampling sites. BOD5, COD, TP, TN and ammonia nitrogen (AN) were analyzed in the laboratory immediately after collection, as per GB3838-2002 methods. The results are presented in Table 1 [15].

Table 1. Physical and chemical properties of Xi River samples.

Physical and	Temperature	рН	BOD <sub>5</sub>	COD	TP	TN	AN
Chemical Properties	(°C)		(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
Upstream	11.4	6.8	10	12.5	0.079	24.93	3.669
Downstream	10.9	6.9	43	100	0.242	25.13	0.524



Figure 1. Geographical sampling locations along the Xi River.

Studies have indicated that eutrophication is evident when phosphorus and nitrogen concentrations exceed 0.02 mg/L [16]. This suggests that the Xi River is in a eutrophic state. According to previous investigations, the composite water pollution index in the Xi River Basin has fluctuated between 0.2 and 0.4 over the past two decades. This indicates that there is a consistent pattern [17]. This index reflects the overall pollution level, including nutrients such as phosphorus and nitrogen, which contribute to eutrophication. Therefore, the data presented in this study reflect these broader environmental conditions.

### 2.2. DNA Extraction

Water samples were initially filtered through a 0.22  $\mu$ m pore size membrane (polycarbonate membrane) to capture precipitate on the membrane surface. This precipitate was then used for DNA extraction, while the supernatant was separated by centrifugation and stored at -4 °C. The DNA extraction was performed using a Tiangen Biotech Co., Ltd. (Beijing, China) DNA kit. This yielded 30  $\mu$ L of microbial DNA from each sample. The DNA samples were tested for quality using a NanoPhotometer (Implen, Munich, Germany), to ensure the DNA met stringent quality standards. High-quality DNA samples were subsequently packaged in ice bags and sent to Shanghai Major Biomedical Technology Co (Shanghai, China). for metagenomic sequencing. This is essential for analyzing microbial communities in the water samples.

## 2.3. Bioinformatics Processing of Metagenomic Sequences

We employed the Illumina sequencing platform for second-generation sequencing of the samples. To ensure data quality, fastp software (V 0.23.4) was utilized for quality control. Read assembly was performed using Megahit's single splicing strategy, and employing the succinct de Bruijn graph method. Cluster analysis was conducted using CD-HIT to identify similarities and differences between samples; cluster similarity and gene sequence clustering coverage parameters were set to greater than 90%. For gene abundance information, the SOAP aligner software (V 1.06) compared the non-redundant gene set. Gene abundance was calculated based on a similarity threshold equal to or higher than 0.95, with insertion fragment lengths between 500 and 300 bp. The non-redundant gene set was compared with the NR database, including bacteria, fungi, archaea and viruses, using DIAMOND (with parameters set to blastp and E-value  $\leq 1 \times 10^{-5}$ ) to obtain biological information from individual samples. This comprehensive approach provided insights into

the microbial community structure and potential functions present in the samples. The metagenomic sequencing data from this study have been submitted to the National Center for Biotechnology Information (NCBI) database; this is accessible under accession codes SRR18119958 and SRR18119957.

## 3. Result and Discussion

#### 3.1. Overall Microbial Community Structure

Microbial community analysis of the Xi River's upstream water samples revealed significant diversity. In total there were 132 phyla, 294 classes, 684 orders, 1252 families, and 3049 genera. Downstream samples, meanwhile, displayed 135 phyla, 297 classes, 680 orders, 1260 families, and 3134 genera. These findings underscore the substantial microbial diversity in both upstream and downstream areas, showing only minor differences in their taxonomic compositions. The detailed taxonomic data derived from DIAMOND analysis provides crucial insights into the diverse microbial communities in the Xi River, highlighting their potential ecological functions in this aquatic environment.

## 3.2. Alpha Diversity Analysis

We utilized alpha diversity statistical methods to analyze the genetic data obtained from sequencing samples from upstream and downstream of the Xi River. This analysis included calculating the Shannon index at the domain classification level to assess community diversity, and calculating the Shannon evenness index to evaluate community evenness. As illustrated in Figure 2, the results indicate that the microbial community in the upstream samples (XDU) exhibited higher diversity and evenness compared to the downstream samples (XDD) at the domain classification level. The increased diversity and evenness of the upstream microbial community suggest a more balanced and stable ecosystem in that region. These findings further corroborate previous observations that the overall water quality at the upstream sampling points is superior to that at the downstream points. The enhanced diversity and homogeneity of the upstream microbial community imply a healthier and less disturbed ecosystem, while the diversity and homogeneity of the microbial community downstream are reduced due to the influence of the wastewater treatment plant's effluents. A detailed analysis of diversity at finer taxonomic levels will be discussed later in the text.



**Figure 2.** Alpha diversity analysis in upstream (XDU) and downstream (XDD) samples. (**a**) Shannon index; (**b**) Shannon evenness index.

# 3.3. Taxonomic Analysis at the Phylum and Genus Levels

The metagenomic sampling results, when compared with the NR species database, revealed the abundance of dominant microorganisms. Figure 3a presents these data as a bar chart. The upstream samples (XDU) had six dominant phyla with  $\geq 1\%$  abundance at the phylum level. These included *Proteobacteria* (53.42%), *Bacteroidetes* (19.11%), *Bacillariophyta* 

(6.63%), unclassified\_d\_Eukaryota (5.06%), Cyanobacteria (3.37%), and Verrucomicrobia (1.38%). Conversely, the downstream (XDD) samples showed a broader diversity; there were 13 dominant phyla of  $\geq$ 1% abundance, highlighting *Proteobacteria* (53.92%), *Bacillariophyta* (2.34%), and others such as unclassified\_d\_Bacteria (2.10%), Nitrospirae (1.68%), Cyanobacteria (1.52%), unclassified\_d\_Eukaryota (1.36%), Planctomycetes (1.33%), and Gemmatimonadetes (1.27%).



**Figure 3.** Column chart of microbial communities in upstream (XDU) and downstream (XDD) samples at (**a**) phylum; and (**b**) genus level.

Figure 3b presents the genus-level microbial composition in the Xi River, where the upstream (XDU) sample revealed 3049 genera, 16 of which were predominant genera, i.e., each had over 1% abundance. Among these, *Flavobacterium* (7.12%), *Limnohabitans* (6.09%), and *Pseudomonas* (3.73%) are notable. *Thalassiosira* (3.43%), *Betaproteobacteria* (2.67%), *Hydrogenophaga* (2.59%), and *Rhodoferax* (2.45%) are also significant. Downstream (XDD) samples, however, showed 3134 genera, of which 14 genera were at  $\geq 1\%$  abundance. These included *Chloroflexi* (3.84%), *Methylotenera* (3.40%). *Flavobacterium* (2.80%), *Pseudomonas* (2.24%), *unclassified\_c\_Bacteria* (2.06%), *Thalassiosira* (1.66%), *Nitrospira* (1.60%), *unclassified\_d\_Viruses* (1.44%), *Limnohabitans* (1.31%), *Acidovorax* (1.26%), *Betaproteobacteria* (1.25%), *Hydrogenophaga* (1.12%), and *Nitrosomona* (1.08%). The metagenomic data thus provide a comprehensive overview of the microbial ecosystem's complexity and its ecological interplay within the river.

Figure 3 depicts the distribution of microbial taxa at both the phylum and genus levels. At the phylum level, downstream microbes exhibit a more uniform distribution; this is indicative of a diverse microbial presence. This suggests adaptation of various microbial phyla to the downstream environment. This is likely influenced by factors such as wastewater treatment plant discharge. In contrast, at the genus level upstream microbes show a higher degree of evenness, with certain genera dominating within their respective phyla. These dominant genera play key roles in the ecosystem, and contribute to the microbial diversity in the upstream Xi River. These findings highlight the importance of analyzing microbial data across different taxonomic levels, providing a deeper understanding of the ecological structures and the specific dynamics within taxa. Such detailed analysis

is essential for assessing the impacts of environmental changes on aquatic ecosystems' microbial communities.

The sequencing analysis in this study focused on the 50 most abundant species, with their relative abundances illustrated on a heatmap using average values. Figure 4 highlights that at the phylum level, downstream microbes have a higher overall relative abundance compared to upstream microbes. This suggests a diverse range of phyla in the downstream environment that are influenced by the wastewater treatment plant effluent and changes in water quality. At the genus level, however, upstream microbes show greater dominance, indicating that specific genera are more prevalent in the upstream region of the Xi River. These dominant genera are likely significant in shaping the ecosystem, and contribute to the observed higher evenness in the upstream microbial community at the genus level.



**Figure 4.** Heatmap of bacterial communities in upstream (XDU) and downstream (XDD) samples at **(a)** genus and **(b)** phylum level.

Observations from Figures 3 and 4 demonstrate the distinct impacts of wastewater treatment plants on the Xi River's microbial community at different taxonomic levels. Downstream, there is a notable increase in the relative abundance of microbial phyla; this indicates a shift toward a more diverse microbial community. In contrast, the upstream region showcases more evenness and dominance at the genus level, suggesting a stable and established microbial structure. This variation in response at different taxonomic levels highlights the complexities in microbial adaptation to environmental shifts and emphasizes the importance of multi-level taxonomic analysis for a comprehensive understanding of the microbial dynamics in the Xi River. Additionally, these findings stress the need for focused

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monitoring and management approaches to address the specific impacts of wastewater discharges on aquatic microbial communities.

## 3.4. Kyoto Encylopedia of Genes and Genomes (KEGG) Metabolic Analysis

Microbial populations in the Xi River play a pivotal role in metabolic processes, which are basically linked to the river's physicochemical dynamics. An in-depth examination of these biological functions is essential. The figure shows the classification of metabolic pathways as classified by the Kyoto Encyclopedia of Genes and Genomes (KEGG), focusing on secondary metabolism. This classification allows for a comparative analysis of metabolic variations; statistical tools such as Fisher's exact test and a two-tailed test are used, with a threshold of significance set at a *p*-value of 0.05.

Figure 5 illustrates that in the Xi River, the overall metabolic pathway's abundance values are higher downstream than upstream, except in most individual metabolic categories, where upstream values prevail. Notably, elevated Xenobiotics biodegradation and metabolism downstream suggest a higher concentration of chemicals, drugs, and organic pollutants in these waters [18]. Additionally, downstream replication and repair values significantly surpass upstream values, indicating more active DNA replication and repair processes downstream. This could signify a greater environmental shift downstream, as reflected by the microbial community's heightened sensitivity and adaptability [19]. Consequently, discharge from the FMWWPT has introduced more pollution, such as organic compounds into the Xi River, thereby imposing stress on downstream microbial populations. These microbes have consequently ramped up their catabolic metabolic functions to cope with the increased organic compound levels. Such adaptability mirrors the microbial response to environmental modifications induced by FMWWPT discharge. In contrast, metabolic aspects such as energy metabolism and translation exhibit higher abundance upstream. This is potentially due to a richer and more diverse microbial community in these regions.



**Figure 5.** Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic classification functional differential analysis plot ( $0.001 **, <math>p \le 0.001$  \*\*\*).

## 3.5. Potential Biological Pollution

Activated sludge plays a crucial role in the secondary treatment of wastewater treatment plants [20]. This process is meticulously designed to degrade a variety of organic pollutants [21,22]; this results in the emergence of a highly complex community structure [23,24]. This structure encompasses a diverse ecological network, including prokaryotes, eukaryotes, phages, and various other microorganisms [25]. Following this stage, tertiary treatment focuses on disinfecting and sterilizing the sewage that has undergone secondary treatment. An analysis of the changes in microbial abundance in the Xi River, particularly in relation to activated sludge, reveals the potential biological pollution of the Xi River caused by the FMWWTP. This detailed examination of the microbial abundance changes associated with activated sludge in the Xi River water body emphasizes the potential biological pollution that the FMWWTP might inflict on the Xi River ecosystem.

Among the numerous elements of activated sludge, Proteobacteria emerges as the most prevalent species [26]. Its significant role in the removal of nitrogen and phosphorus, along with the degradation of organic matter, is pivotal. The high abundance of Proteobacteria is crucial for the effective operation of the activated sludge system in wastewater treatment facilities. Moreover, in sewage treatment plants utilizing the AAO (anaerobic anoxic oxic) process, the dominant phyla include *Bacteroidetes, Actinobacteria, Acidobacteria, Chloroflexi, Nitrospirae*, and others [27]. Additionally, it has been observed that the dominant microbial genera in various activated sludge samples demonstrate notable differences in their distribution percentages. This variation underlines the diversity and complexity of microbial communities within activated sludge systems. In the AAO process, for instance, the dominant functional genera comprise *Comamonas, Rhodoferax, Nitrospira, Novosphingobium, Terrimonas, Thauera*, and others, including *Zoogloea, Dechloromonas, Acidovorax* [28–30].

Figure 6a demonstrates that, at the phylum level, apart from a decrease in the downstream abundance of *Bacteroidetes*, the abundances of *Proteobacteria*, *Actinobacteria*, *Nitrospirae*, *Acidobacteria*, and *Chloroflexi* are significantly higher in the downstream samples than in the upstream samples. This observation can be attributed to higher contents of BOD<sub>5</sub>, COD, TP, and TN in the downstream samples, which are more closely related to the physical and chemical properties of wastewater treatment plant effluents. Additionally, the presence of corresponding microorganisms in the downstream samples leads to a considerable increase in the abundance of *Bacteroidetes* may be due to its negative correlation with organophosphorus concentration in TP [31,32], suggesting that the FMWWTP discharge could contribute to elevated levels of organic phosphorus in the Xi River's waters.

In Figure 6b, it is evident that the abundance values of *Acidovorax* and *Rhodoferax* at the genus level are lower in the downstream samples compared to the upstream samples. This difference may be attributed to variations in the types of activated sludge employed. However, the downstream samples exhibit higher abundance values of most other bacteria, indicating higher overall microbial activity compared to the upstream samples. Notably, *Zoogloea*, a commonly used component of activated sludge, was observed to have proliferated in the downstream samples [33].

The metagenomic sequencing results revealed the presence of unique downstream microorganisms, including *Pinguiophyceae*, *Xenacoelomorpha*, *Poribacteria*, *Chaetognatha*, and *Nanoarchaeota*. Among these, *Pinguiophyceae* is known to play a significant role in sewage treatment systems [34]. It is speculated that the biological pollution caused by the FMWWTP discharge contributes to the increased abundance of *Pinguiophyceae* in the downstream region.

Analyzing the microbial community structure further shows an increase in the percentage abundance of bacteria and a decrease in the percentage abundance of fungi in the downstream compared to the upstream. Bacteria are commonly found to be the predominant flora in activated sludge [35]. This change is likely a consequence of the chemical contamination of the Xi River due to wastewater treatment plant discharge. These contaminants alter the water's original physicochemical properties, creating a more favorable environment for the growth and reproduction of pollutant-treating bacterial species. As a result, the dominance of bacterial populations increases.



**Figure 6.** Histograms of bacterial communities (%) in the upstream (XDU) and downstream (XDD) samples at (**a**) phylum and (**b**) genus.

These findings collectively indicate that the FMWWTP has indeed influenced the microbial community structure in the Xi River water body to a certain extent. Moreover, they suggest the potential presence of microbial pollution resulting from wastewater treatment plant discharge. These insights emphasize the need for continuous monitoring and effective management strategies to mitigate the impact of wastewater discharges on the ecological health of the Xi River.

## 3.6. Water Eutrophication River

Nitrogen and phosphorus are vital elements for the proper functioning of living systems and are essential for human life. However, poorly treated discharge of nitrogen and phosphorus can lead to significant damage to the ecological environment. According to widely accepted international standards, elevated levels of nitrogen and phosphorus in natural water bodies are the primary factors responsible for eutrophication [36]. Eutrophication refers to the excessive growth of algae and aquatic plants in water bodies, which can lead to a cascade of detrimental effects on the ecosystem, such as oxygen depletion, harmful algal blooms, and disruption of the aquatic food chain [37]. Therefore, proper management and treatment of nitrogen and phosphorus discharge from various sources, including wastewater treatment plants, are essential to protect and maintain the ecological balance of water bodies and ensure the sustainability of aquatic ecosystems.

#### 3.6.1. Nitrogen-Content Variations and Their Effects on Microorganisms

A microbial-related denitrification process in water involves three main nitrogen conversion processes: ammonification, nitrification, and denitrification. Nitrogen fixation can occur under both aerobic and anaerobic conditions, while nitrification is specific to aerobic conditions, and denitrification occurs in anoxic conditions [36]. Ammonification refers to the process in which microorganisms decompose organic nitrogen compounds to produce ammonia. Nitrification is the process by which nitrifying bacteria convert ammonia nitrogen in wastewater into nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ). Denitrification is the process where denitrifying bacteria reduce nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ). back to nitrogen gas [37].

As can be inferred from Figure 7, the nitrogen processing activities of microorganisms in the downstream (including ammonification, nitrification, and denitrification) are more active than those upstream. This observation, coupled with the fact that the total nitrogen (TN) content is comparatively lower upstream but the ammonium nitrogen (AN) content is significantly higher upstream than downstream, suggests that the TN levels in the Xi River have risen from upstream to downstream due to the discharge from the FMWWTP; and this subsequently increases the nitrogen processing activities of the microorganisms in the river. Notably, the heightened nitrification activity downstream indicates that during the river's flow, nitrification-related bacteria converted a substantial amount of AN into nitrate nitrogen, leading to a much higher AN content upstream than downstream, as shown in Table 1. Sequencing results also reveal higher typical abundance values of nitrifying bacterial genera, such as Nitrosococcus and Nitrosomonas, in the downstream samples. These findings collectively demonstrate the significant impact of microbial activity and community composition on the nitrogen transformation processes in the waters of the Xi River. The lower abundance values of the nitrate/nitrite transport system downstream, as shown in Figure 7, may be due to differences in the microbial communities between upstream and downstream, resulting in variations in the associated nitrogen metabolism processes.



Figure 7. Nitrogen metabolism process of Xi River samples.

3.6.2. Phosphorus-Content Variations and Their Effects on Microorganisms

Polyphosphate-accumulating organisms (PAO), also called phosphorus-absorbing bacteria, are bacteria that degrade large amounts of phosphorus in wastewater under certain

environmental factors. Polyphosphate-accumulating organisms are commonly found in ecological environments with high phosphorus content, such as in activated sludge in the enhanced biological phosphate removal (EBPR) system [38]. These organisms are typically found in high-phosphorus ecological environments, such as within the activated sludge of enhanced biological phosphate removal (EBPR) processes. Interestingly, phosphorus accumulating bacteria can also thrive in some natural water bodies, even those with lower phosphorus levels. In the context of the FM sewage treatment plant, the activated sludge in the AAO process includes phosphorus-accumulating bacteria that efficiently absorb excess phosphorus. The common taxonomic groups of these bacteria encompass *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, *Acidobacteria*, among others [39]. This highlights the diversity and adaptability of these bacteria in various environmental settings.

As indicated in Figure 8, among the common polyphosphate-accumulating organisms (PAO), the abundance of related bacteria in the downstream is higher than that in the upstream, with the exception of *Bacteroidetes*. The reduction in *Bacteroidetes* abundance can be associated with the discharge from the FMWWTP. This leads to increased phosphorus and organic phosphorus content in the Xi River. This indicates that the high organic phosphorus content of the discharge has a favorable impact on the survival environment for the majority of polyphosphoric bacteria in the river, thereby promoting their overall growth and reproduction. These shifts in microbial community composition highlight the potential influence of wastewater treatment plant discharge on the ecological dynamics of phosphorus-accumulating bacteria in the Xi River.



Figure 8. Phosphorus accumulating bacteria at the common phylum level.

#### 3.7. Resistance Gene Pollution

Since the latter half of the 20th century, there has been a substantial increase in the production and usage of antibiotics. Unfortunately, the incomplete absorption of these antibiotics by humans and animals results in significant amounts of antibiotic residues and metabolites being released into the natural environment via excreta. This release exerts selective pressure on microorganisms, and encourages the acquisition of antibiotic resistance genes through mutation and their dissemination through horizontal gene transfer. The enduring presence of these resistance genes in the environment is a significant hurdle in their eradication. Moreover, studies have indicated that resistance genes can transfer from transgenic plants to microorganisms, potentially expanding the spread of antibiotic resistance beyond microbial communities [40]. Elevated antibiotic levels in water environments can interrupt the nitrogen cycle, impeding denitrification and anaerobic ammonia oxidation processes. This disturbance leads to the emission of nitrous oxide, and contributes

to environmental problems such as eutrophication, the greenhouse effect, and ozone depletion [41]. Recent studies have highlighted that surface waters receiving wastewater treatment plant effluent show a significant increase in levels of antibiotic-resistant bacteria. This trend indicates that wastewater treatment plant effluents are likely a major source of such bacteria in natural water bodies [42]. The growing presence of antibiotic residues and the escalation of antibiotic-resistant bacteria in aquatic environments underline the critical need for effective waste management, the enhancement of wastewater treatment processes, and judicious use of antibiotics. These measures are vital to protect human health and the environment from the adverse consequences of antibiotic resistance.

As depicted in Figure 9, downstream samples from the Xi River show a heightened abundance compared to upstream samples of various antibiotic resistance gene categories such as sulfonamide, tetracycline, penicillin, streptomycin, and fluoroquinolone. Interestingly, the bacitracin resistance category is more prevalent in upstream samples. This increased presence of resistance genes downstream can be attributed to the influence of the FMWWTP effluent; this is reflected in the observed microbial metabolic profile. The variance in resistance gene abundance between upstream and downstream samples may stem from differences in the disinfection treatment procedures during water discharge and treatment. These procedures might result in varying levels of inactivation of microorganisms carrying these resistance genes [43].



Figure 9. Crossplots of resistance genes upstream (XDU) and downstream (XDD).

In general, the downstream of Xi River is more seriously contaminated with antibiotic resistance genes and needs to be treated effectively. There are various methods for removing antibiotic resistance genes from the river, such as AGS-SBR technology or anaerobic double membrane bioreactor (AnMBR) technology. This can be used in wastewater treatment plants for effective removal of antibiotic resistance genes [44,45]. A variety of modified biochar adsorption, among other methods, can be used for removal of antibiotic resistance genes [46,47]. Additionally, the practice of prudent antibiotic usage is essential to minimize the emergence and dissemination of antibiotic-resistant bacteria and genes.

# 4. Conclusions

This study comprehensively reveals the interaction between environmental factors and microbial communities in the waters of the Xi River that are impacted by the Fuxin Meng Wastewater Treatment Plant (FMWWTP) discharge. Through metagenomic sequencing and physicochemical property analysis, changes in microbial diversity, metabolic functions, and potential contamination above and below the FMWWTP discharge point were assessed. The results indicate that the microbial community structure of the Xi River is significantly impacted by the pollutants from FMWWTP. Downstream microbial diversity is notably reduced, and there are changes in the dominance of microorganisms or organic matter, as well as an increase in genetic diversity related to metabolic and functional capabilities. These changes may be adaptive responses to stress caused by organic pollutants. Most importantly, the study discovered an increase in the presence of potential biological contaminants and antibiotic resistance genes downstream. This signals risks to environmental quality and public health. The pollution status of the recipient water bodies necessitates improvements in various aspects, such as adopting new wastewater treatment technologies and facilities at the treatment plants, pre-treating non-domestic wastewater, and reducing the misuse of antibiotics in daily life.

The methods used in this study facilitate continuous monitoring of microbial community structure, metabolic functions, and pollution levels in similar environments. This emphasizes the necessity of routine comparisons between downstream and upstream areas. The study advocates for continuous improvements in wastewater treatment processes and adherence to strict environmental standards, and highlights the need for effective management and sustainable practices to protect the ecological integrity of the Xi River and other water bodies affected by human activities.

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# References

- Alharbi, O.A.; Jarvis, E.; Galani, A.; Thomaidis, N.S.; Nika, M.-C.; Chapman, D.V. Assessment of selected pharmaceuticals in Riyadh wastewater treatment plants, Saudi Arabia: Mass loadings, seasonal variations, removal efficiency and environmental risk. *Sci. Total Environ.* 2023, *882*, 163284. [CrossRef]
- Hernandez-Chover, V.; Castellet-Viciano, L.; Fuentes, R.; Hernandez-Sancho, F. Circular economy and efficiency to ensure the sustainability in the wastewater treatment plants. *J. Clean. Prod.* 2023, *384*, 135563. [CrossRef]
- Li, Y.; Han, Q.; Li, B. Engineering-scale application of sulfur-driven autotrophic denitrification wetland for advanced treatment of municipal tailwater. *Bioresour. Technol.* 2023, 379, 129035. [CrossRef] [PubMed]
- Li, J.; Tao, T.; Li, X.-B.; Li, J.-H. A pilot-scale experimental research on biological phosphorus removal in a modified SBR treating urban wastewater. In Proceedings of the 2nd International Conference on Chemical Engineering and Advanced Materials (CEAM 2012), Guangzhou, China, 13–15 July 2012; pp. 2329–2332.
- 5. Douxfils, J.; Mandiki, R.; Silvestre, F.; Bertrand, A.; Leroy, D.; Thome, J.-P.; Kestemont, P. Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? *Sci. Total Environ.* **2011**, *409*, 4139. [CrossRef]
- Zhu, T.-T.; Su, Z.-X.; Lai, W.-X.; Zhang, Y.-B.; Liu, Y.-W. Insights into the fate and removal of antibiotics and antibiotic resistance genes using biological wastewater treatment technology. *Sci. Total Environ.* 2021, 776, 145906. [CrossRef]
- Wang, J.; Chen, Y.; Cai, P.; Gao, Q.; Zhong, H.; Sun, W.; Chen, Q. Impacts of municipal wastewater treatment plant discharge on microbial community structure and function of the receiving river in Northwest Tibetan Plateau. *J. Hazard. Mater.* 2022, 423, 127170. [CrossRef]
- 8. Igere, B.E.; Okoh, A.I.; Nwodo, U.U. Wastewater treatment plants and release: The vase of Odin for emerging bacterial contaminants, resistance and determinant of environmental wellness. *Emerg. Contam.* **2020**, *6*, 212–224. [CrossRef]
- 9. Wommack, K.E.; Ravel, J. *Microbiome*, demystifying the role of microbial communities in the biosphere. *Microbiome* **2013**, *1*, 1. [CrossRef]

- 10. Shade, A. Diversity is the question, not the answer. ISME J. 2017, 11, 1–6. [CrossRef]
- 11. Yang, H.; Xu, M.; Wang, L.; Wang, X.; Jeppesen, E.; Zhang, W. Metagenomic analysis to determine the characteristics of antibiotic resistance genes in typical antibiotic-contaminated sediments. *J. Environ. Sci.* **2023**, *128*, 12–25. [CrossRef]
- 12. Li, M.; Zhao, S.; Zheng, N.; Wang, J. Advances in Microbial Macrogenomic Data Analysis Methods. J. Mirobiol. 2020, 40, 89–95.
- Chen, G.; Bai, R.; Zhang, Y.; Zhao, B.; Xiao, Y. Application of metagenomics to biological wastewater treatment. *Sci. Total Environ.* 2022, 807, 150737. [CrossRef]
- 14. *GB 3838-2002;* Environmental Quality Standards for Surface Water. Chinese Research Academy of Environmental Sciences: Beijing, China, 2002.
- 15. Zhu, H.; Li, B.; Ding, N.; Hua, Z.; Jiang, X. A Case Study on Microbial Diversity Impacts of a Wastewater Treatment Plant to the Receiving River. J. Geosci. Environ. Prot. 2021, 9, 206–220. [CrossRef]
- 16. Zhang, X.; Zhang, X.-Q.; Yu, H.-B.; Song, H.-L.; Du, M.-X. Phosphorus Removal from Wastewater by Electrocoagulation with Magnetized Iron Particle Anode. *Water Air Soil Pollut.* **2020**, 231, 502. [CrossRef]
- 17. Wang, Q.L.C. Quantitative assessment study of water pollution composite index in Fuxin Xi River Basin. *Heilongjiang Water Sci. Technol.* **2021**, *49*, 17–19+47. [CrossRef]
- Chao, Y.; Ma, L.; Yang, Y.; Ju, F.; Zhang, X.-X.; Wu, W.-M.; Zhang, T. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. *Sci. Rep.* 2013, *3*, 3550. [CrossRef]
- 19. Feng, M.; Lu, Y.; Yang, Y.; Zhang, M.; Xu, Y.-J.; Gao, H.-L.; Dong, L.; Xu, W.-P.; Yu, S.-H. Bioinspired greigite magnetic nanocrystals: Chemical synthesis and biomedicine applications. *Sci. Rep.* **2013**, *3*, 2994. [CrossRef]
- Bae, H.-K. A Comparative Analysis of Two Different Wastewater Treatment Processes in Actual Wastewater Treatment Plants. *Quant. Bio-Sci.* 2018, 37, 19–26. [CrossRef]
- Alkan, U.; Eleren, S.C.; Nalbur, B.E.; Odabas, E. Influence of the activated sludge system configuration on heavy metal toxicity reduction. World J. Microbiol. Biotechnol. 2008, 24, 1435–1443. [CrossRef]
- 22. Ouyang, J.; Li, C.; Wei, L.; Wei, D.; Zhao, M.; Zhao, Z.; Zhang, J.; Chang, C.-C. Activated sludge and other aerobic suspended culture processes. *Water Environ. Res.* 2020, *92*, 1717–1725. [CrossRef]
- 23. Ya, X.; Jingcai, L.; Lu, D.; Yuqiang, L.; Weishi, L.; Changxing, N.; Qifei, H. Buffering distance between hazardous waste landfill and water supply wells in a shallow aquifer. *J. Clean. Prod.* **2019**, *211*, 1180–1189. [CrossRef]
- Yu, K.; Zhang, T. Metagenomic and Metatranscriptomic Analysis of Microbial Community Structure and Gene Expression of Activated Sludge. PLoS ONE 2020, 15, e0243233. [CrossRef]
- 25. Wang, F.; Sun, X.; Li, J.; Ai, S.; Ren, Q.; Nie, Z.; Bian, D. Analysis of microbial characteristics and population difference between different compartments of the mixed system at low temperature. *Desalination Water Treat*. **2021**, 225, 53–62. [CrossRef]
- Zhang, L.; Shen, Z.; Fang, W.; Gao, G. Composition of bacterial communities in municipal wastewater treatment plant. *Sci. Total Environ.* 2019, 689, 1181–1191. [CrossRef]
- Ma, Q.-Q.; Yuan, L.-J.; Niu, Z.-D.; Zhao, J.; Huang, C. Microbial Community Structure of Activated Sludge and its Response to Environmental Factors. *Huan Jing Ke Xue Huanjing Kexue* 2021, 42, 3886–3893. [CrossRef]
- Lou, T.; Peng, Z.; Jiang, K.; Niu, N.; Wang, J.; Liu, A. Nitrogen removal characteristics of biofilms in each area of a full-scale AAO oxidation ditch process. *Chemosphere* 2022, 302, 134871. [CrossRef]
- Liu, F.; Qian, G.; Zhao, X.; Hu, X. Mechanism insights into the nitrogen removal of bio-augmented AAO: Specifically focusing on the nitrogen metabolic pathways and microbial taxa-functional genes associations. *J. Water Process Eng.* 2022, 50, 103245. [CrossRef]
- Wang, L.; Yuan, L.; Li, Z.-H.; Zhang, X.; Leung, K.M.Y.; Sheng, G.-P. Extracellular polymeric substances (EPS) associated extracellular antibiotic resistance genes in activated sludge along the AAO process: Distribution and microbial secretors. *Sci. Total Environ.* 2022, *816*, 151575. [CrossRef]
- 31. Yan, X.; He, M.; Zheng, J.; Zhu, T.; Zou, Z.; Tang, B.; Yu, Y.; Mai, B. Tris (1,3-dichloro-2-propyl) phosphate exposure disrupts the gut microbiome and its associated metabolites in mice. *Environ. Int.* **2021**, *146*, 106256. [CrossRef]
- 32. Wang, D.; Yan, S.; Yan, J.; Teng, M.; Meng, Z.; Li, R.; Zhou, Z.; Zhu, W. Effects of triphenyl phosphate exposure during fetal development on obesity and metabolic dysfunctions in adult mice: Impaired lipid metabolism and intestinal dysbiosis. *Environ. Pollut.* **2019**, *246*, 630–638. [CrossRef]
- An, W.; Guo, F.; Song, Y.; Gao, N.; Bai, S.; Dai, J.; Wei, H.; Zhang, L.; Yu, D.; Xia, M.; et al. Comparative genomics analyses on EPS biosynthesis genes required for floc formation of Zoogloea resiniphila and other activated sludge bacteria. *Water Res.* 2016, 102, 494–504. [CrossRef]
- McMahon, K.D.; Jenkins, D.; Keasling, J.D. Polyphosphate kinase genes from activated sludge carrying out enhanced biological phosphorus removal. *Water Sci. Technol.* 2002, 46, 155–162. [CrossRef]
- 35. Chen, Y.; Geng, N.; Hu, T.; Baeyens, J.; Wang, S.; Su, H. Adaptive regulation of activated sludge's core functional flora based on granular internal spatial microenvironment. *J. Environ. Manag.* **2022**, *319*, 115714. [CrossRef]
- 36. Ding, S.; Dan, S.F.; Liu, Y.; He, J.; Zhu, D.; Jiao, L. Importance of ammonia nitrogen potentially released from sediments to the development of eutrophication in a plateau lake. *Environ. Pollut.* **2022**, *305*, 119275. [CrossRef]
- 37. Smith, V.H.; Schindler, D.W. Eutrophication science: Where do we go from here? Trends Ecol. Evol. 2009, 24, 201–207. [CrossRef]
- He, S.; Gall, D.L.; McMahon, K.D. "Candidatus accumulibacter" population structure in enhanced biological phosphorus removal Sludges as revealed by polyphosphate kinase genes. *Appl. Environ. Microbiol.* 2007, 73, 5865–5874. [CrossRef]

- 39. Oehmen, A.; Lemos, P.C.; Carvalho, G.; Yuan, Z.; Keller, J.; Blackall, L.L.; Reis, M.A.M. Advances in enhanced biological phosphorus removal: From micro to macro scale. *Water Res.* 2007, *41*, 2271–2300. [CrossRef]
- Pontiroli, A.; Rizzi, A.; Simonet, P.; Daffonchio, D.; Vogel, T.M.; Monier, J.-M. Visual Evidence of Horizontal Gene Transfer between Plants and Bacteria in the Phytosphere of Transplastomic Tobacco. *Appl. Environ. Microbiol.* 2009, 75, 3314–3322. [CrossRef] [PubMed]
- Chee-Sanford, J.C.; Mackie, R.I.; Koike, S.; Krapac, I.G.; Lin, Y.-F.; Yannarell, A.C.; Maxwell, S.; Aminov, R.I. Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. *J. Environ. Qual.* 2009, 38, 1086–1108. [CrossRef] [PubMed]
- Galvin, S.; Boyle, F.; Hickey, P.; Vellinga, A.; Morris, D.; Cormican, M. Enumeration and Characterization of Antimicrobial-Resistant Escherichia coli Bacteria in Effluent from Municipal, Hospital, and Secondary Treatment Facility Sources. *Appl. Environ. Microbiol.* 2010, *76*, 4772–4779. [CrossRef] [PubMed]
- 43. Munir, M.; Wong, K.; Xagoraraki, I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 2011, 45, 681–693. [CrossRef] [PubMed]
- Perez-Bou, L.; Gonzalez-Martinez, A.; Gonzalez-Lopez, J.; Correa-Galeote, D. Promising bioprocesses for the efficient removal of antibiotics and antibiotic-resistance genes from urban and hospital wastewaters: Potentialities of aerobic granular systems. *Environ. Pollut.* 2024, 342, 123115. [CrossRef]
- Wang, K.; Zhou, L.; Meng, S.; Wang, Y.; Yu, B.; Wang, J. Anaerobic membrane bioreactor for real antibiotic pharmaceutical wastewater treatment: Positive effect of fouling layer on antibiotics and antibiotic resistance genes removals. *J. Clean. Prod.* 2023, 409, 137234. [CrossRef]
- 46. Ding, Z.; Hu, X.; Wan, Y.; Wang, S.; Gao, B. Removal of lead, copper, cadmium, zinc, and nickel from aqueous solutions by alkali-modified biochar: Batch and column tests. *J. Ind. Eng. Chem.* **2016**, *33*, 239–245. [CrossRef]
- 47. Zhou, Y.; Leong, S.Y.; Li, Q. Modified biochar for removal of antibiotics and antibiotic resistance genes in the aqueous environment: A review. J. Water Process Eng. 2023, 55, 104222. [CrossRef]

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