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Article

Impact of Non-Metallic Organic Tanning Agents with a Double-Triazine Structure on the Microbial Community Structure in Wastewater

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Abstract: This study examined the correlation between non-metallic organic tanning agents (NOTAs) and wastewater containing double-triazine (bistriazine). Specifically, high-throughput sequencing was used to analyze how the physical and chemical properties of the bistriazine-containing wastewater affected the microbial community. In total, 9995 operating taxonomy units (OTUs) were detected at a similarity level of 97%. According to the taxonomy results and relative bacterial richness, the non-metallic organic tanning agent (NOTA) wastewater communities showed significant differences. *Clostridium sensu stricto* and *Turicibacter* bacteria were dominant in NOTA T2, T3, and the blank control (KB) wastewater; *Trichococcus* and *Aeromonas* were dominant in NOTA T4; and *Pseudomonas* was dominant in T2, T4, and the blank control (KB). Thus, the addition of bistriazine ring derivatives altered the composition of the bacterial community. Furthermore, Spearman's correlation analysis revealed a substantial correlation in the composition of the communities and the physicochemical properties of the wastewater. *Acidocella* and *norank c Cyanobacteria* correlated positively with Total Phosphorus (TP) ($p \leq 0.001$) and *Mycobacterium* showed a positively held correlation with pH ($p \leq 0.05$). Conversely, *norank_f__Elev-16S-1332* indicated a negative correlation with TP, Total Nitrogen (TN), and Total Organic Carbon (TOC) ($p \leq 0.05$), whereas *Zoogloea* correlated negatively with NH_3–N, TOC, and TN ($p \leq 0.05$). Regarding water quality, a significant correlation between microbial communities and the physicochemical properties of bistriazine wastewater was also observed ($p \leq 0.05$). We concluded that *Trichococcus*, *Clostridium sensu stricto*, *Turicibacter*, *Pseudomonas*, *Intestinibacter*, *Acidocella*, *Bacillus*, and *Tolumonas* showed a high tolerance for bistriazine wastewater, which could offer insight into its bioremediation.

Keywords: microbial community; tanning agent; activated sludge; tannery wastewater

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1. Introduction

Tanning agents commonly used in the leather industry are generally categorized into two types: non-metallic organic tanning agents (NOTAs) with a double-triazine structure and metallic tanning agents [1]. The metallic tanning agents are the most widely used owing to their excellent tanning properties. However, the highly carcinogenic properties of hexavalent chromium are a significant environmental hazard. Therefore, to match the tanning properties of most of the chrome tanning agents while preventing potential toxic element (PTE) pollution, the NOTAs have attracted considerable worldwide research interest in recent decades [2]. Moreover, bistriazine tanning agents come under the NOTA category and exhibit excellent tanning properties [3,4]. Agents that are not fully used up in tanning are discharged into the wastewater, which means that the environmental effect of azine-based compounds, chrome-containing tanning agents of explosives [5], and pesticides [6] on the bacterial composition of tannery wastewater remains a concern.

The negative impact on the diversity of microbial communities from azine-based organics and chromium-containing wastewater in explosives and pesticides has been reported [5–7]. This diversity was also negatively affected by microbial community structural alterations and the abundance of bistriazine tanning agents. However, the current study is focused on the adaptation of microorganisms in toxic environments and the stable, effective operation of a tannery wastewater treatment system [8,9]. The eco-friendly performance and application prospects of bistriazine tanning agents in the tannery industry were also studied.

High-throughput sequencing technology deploys bacterial 16S rRNA amplification, which can detect most species without the cultivation of samples [10] and completely analyze microbial communities and their corresponding richness [11] in a river [12] and on land [13].

This work focused on using high-throughput sequencing to analyze the bacterial community composition in wastewater treatment setups containing bistriazine as a non-metallic organic tanning agent. The Canonical Correspondence Analysis (CCA) statistical approach helped us to connect the changes in microbial species with the physicochemical properties of the bistriazine-containing wastewater, thereby elucidating the post-degradational effects on the microbial community structure. Furthermore, changes in the dominant genera structure in bistriazine-containing wastewater were analyzed. The results provide insight into the ecological assessment of bistriazine as a non-metallic organic tanning agent.

2. Materials and Methods

2.1. Collection of Samples

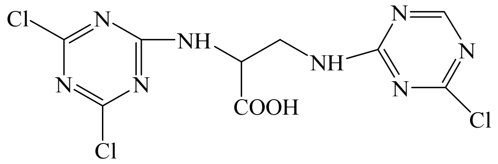
The structures of three NOTAs (T2, T3, and T4) are illustrated in Figure 1. Their degradation rates and properties are listed in Table 1. Wastewater sample collection was done in aeration tanks of the Shaanxi University of Science and Technology. According to the NOTA ratio of 1.5%, one of the 3 NOTAs, which was created in a laboratory, was added to a water sample for five continuous days, aerated for 12 h every day, and then stopped for 12 h. To the water sample was added glucose (0.4 g/L); NH_4Cl (0.153 g/L); and K_2HPO_4 (0.129 g/L). Three water samples of the T2, T3, and T4 tanning agents were set in parallel, and water samples without tanning agents were used as blank controls. The numbers were as follows: T2-1, T2-2, T2-3, T3-1, T3-2, T3-3, T4-1, T4-2, T4-3, KB-1, KB-2, KB-3 (KB = blank control. Three replicas of each water sample were prepared in a 10 mL sealed tube and then preserved in dry ice [14]. The samples were stored at −80 °C until the extraction of DNA.

Table 1. Basic physicochemical parameters of non-metallic organic tanning agents (NOTAs).

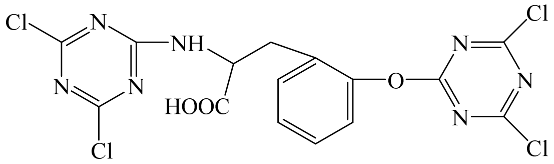
Sample	pH	(Chemical Oxygen Demand) COD(mg/L)
T2	1.74	3.43×10^4
T3	6.27	3.69×10^4
T4	2.12	2.99×10^4

2.2. Analysis of Physicochemical Properties of Bistriazine Ring Derivative Wastewater

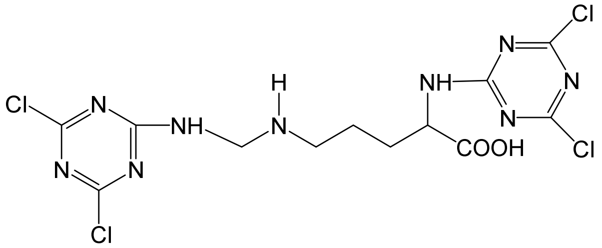
The values of Total Nitrogen (TN), Ammonia Nitrogen (NH_3–N), Nitrate (NO_3–N), Total Phosphorus (TP), and Total Organic Carbon (TOC) in wastewater with added bistriazine ring derivatives were determined using national standardized methods [15]. TN, NH_3–N, NO_3–N, and TP were determined by a UV spectrophotometer (UV-1800), TOC was determined by a total organic carbon analyzer (LiquiTOCII, Elementar Trading (Shanghai) Co., Ltd., Shanghai, China), and a PHS-3E pH meter (Sartorious, Gottingen, Germany) was used to compute the pH of corresponding samples.



(**T2**)



(**T3**)



(**T4**)

Figure 1. Basic structure of non-metallic organic tanning agents (NOTAs).

2.3. Extraction of DNA, Library Preparation, and Sequencing

2.3.1. Extraction of DNA

The prepared water samples were placed on dry ice and sent to Shanghai Meiji Biology (Shanghai, China) for DNA extraction and sequencing. Extraction was performed under the guiding principles of the E.Z.N.A.® soil kit (Omega Bio-tek, Norcross, GA, USA). Nanodrop2000 was employed to detect the purified DNA and its concentration, while the DNA's quality was determined through 1% agarose gel electrophoresis.

2.3.2. PCR Amplification

Variable regions (V3–V4) were amplified through PCR by using 338F (5′-ACTCCTACG GGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers [11]. The amplification was carried out in the following steps: pre-denaturation for 3 min at a particular temperature (95 °C); 27 cycles, including 30 s of denaturation at 95 °C; annealing at 55 °C for another 30 s; extension at 72 °C for 30 s; and another extension at 72 °C for 10 min (PCR equipment: ABI GeneAmp® 9700). The amplifying system consisted of 20 μL and 4 μL of 5*FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of primer (5 μM), 0.4 μL of FastPfu polymerase, and a DNA template of 10 ng.

2.3.3. Illumina Miseq Sequencing

Two percent agarose-containing gel was applied to recover the PCR product and an AxyPrep DNA Gel extraction kit (Axygen Biosciences, Union City, CA, USA) was used for purification. For elution, Tris–HCl was used followed by a 2% agarose electrophoretic process for final detection. With the help of QuantiFluor™-ST (Promega, Madison, Wisconsin, USA), the PCR product was detected and quantified. The standardized operational procedure of the Illumina MiSeq platform (Illumina, San Diego, CA, USA) was used to construct amplified and purified fragments to prepare the PE2 × 300 library. For sequencing, Illumina's MiseqPE300 platform was used (Shanghai Meiji Biomedical Technology Co., Ltd., Shanghai, China).

2.4. Analysis of Alpha and Beta Diversity

To further characterize the bacterial community structure, a bacterial database was constructed from 16S rRNAs amplified in the V3–V4 region from individually pooled DNA samples. Typical operating taxonomy units (OTUs) sequences were obtained by removing the chimera during clustering based on a 97% similarity level. For this analysis, 97% similarity of OTUs or other taxonomy levels were chosen. To analyze the alpha diversity index among dissimilar and irregular samples, Mothur software was used, whereas the illustrations were generated by R language [16]. To study the similar and different relationships of the composition of sample species, and to construct a sample-level clustering tree, clustering analysis was performed on the sample distance matrix. To compare the differences in the wastewater bacterial communities, the corresponding distance matrix prior to beta diversity was calculated regarding each sample's species richness. The Bray–Curtis procedure was used to measure sample distances. The larger the distance between samples, the greater the difference in bacterial community. Based on the distance matrix, a type clustering analysis was performed. Beta diversity was calculated according to the hierarchical clustering analysis of the beta diversity distance matrix by using Quantitative Insights Into Microbial Ecology (Qiime software (v 1.9.1)) (Ann Arbor, MI, USA) [17]. Subsequently, the tree was drawn using R language.

2.5. Data Processing

The trimmomatic software Fast Length Adjustment of SHort reads (FLASH, Baltimore, MD, USA) was used to control for indigenous sequences and perform splicing. First, a window of about 50 base pairs (bp) was set as a quality control. If a window's quality was lower than 20, the bp's back-end sequences were cut from the window's front end, followed by removal. Second, the splicing of both tails of the sequences was performed according to overlapping bases. Among the overlaps, 0.2 was highest mismatching rate, and the length had to exceed 10 bp.

Third, the splicing of a sequence into each sample was performed with barcodes and primers that were present at each corresponding sequence ending. The barcode needed to match exactly. The primer allowed 2 bases to be mismatched and we removed sequences with fuzzy bases. UPARSE software (Pomona, CA, USA) was used to perform sequence clustering of OTUs at a similarity level of 97% and eliminate solo sequences as well as chimeras throughout clustering. The Silva database (SSU123) was aligned to a 70% threshold to perform species classification and annotate each sequence by using the Ribosomal Database Project (RDP) classifier [18].

2.6. Canonical Correspondence Analysis (CCA) Method

The CCA method was proposed in order to estimate how much of the variation in species data can be accounted for by environmental data, which are highly robust when the presumption of no unimodal response holds [19,20]. This method displays a nonlinear relationship among environmental factors and species and selects better weights for environmental variables. We used CCA with Canonical Community Ordination (CANOCO) for the Windows 5.0 package to measure the correlation between the structure of microbial species and the physicochemical properties of wastewater containing different bistriazine tanning agents. The Monte Carlo unrestricted permutation test was used to assess each variable's statistical significance, which involved 499 enumerated permutations [21]. The biplot from the CCA analysis signified an overlapping relationship between the microbial communities and the combined physicochemical properties (Table 1). Environmental variants are represented by arrows and their maximum values are on the corresponding arrow tip.

3. Discussion and Results

3.1. Physicochemical Properties of Bistriazine Ring Derivative Wastewater after Degradation

The post-degradation pH, TN, TP, NH_3–N, NO_3–N, and TOC of the bistriazine ring derivative wastewater are listed in Table 2. The TN, NH_3–N, NO_3–N, and TOC levels were remarkably higher ($p < 0.05$) compared with the KB. This might have been due to the addition of C and N. The pH of T3 and the KB was significantly higher ($p < 0.05$) than that of T2 and T4 because the T3 tanning agent was neutral, whereas the T2 agent was acidic. The TN, NH_3–N, NO_3–N, and TOC levels in T2 were substantially higher ($p < 0.05$) compared with the other wastewater samples, which could be ascribed to the introduction of the heterocyclic compound N to the tanning agents and the huge quantity of organic matter (COD). The TP content of the T4 wastewater was significantly lower ($p < 0.05$) than that of T2 or T3.

Table 2. Physicochemical properties of bistriazine ring derivative wastewater after degradation.

Index	T2	T3	T4	KB
TN/mg·L^{-1}	0.171 ± 0.000 a	0.109 ± 0.001 b	0.168 ± 0.002 a	0.066 ± 0.001 c
TP/mg·L^{-1}	0.019 ± 0.000 a	0.020 ± 0.001 a	0.011 ± 0.000 b	0.018 ± 0.000 a
NH_3–N/mg·L^{-1}	52.400 ± 0.444 a	45.420 ± 0.414 b	51.650 ± 0.628 a	39.680 ± 0.100 c
NO_3–N/mg·L^{-1}	0.237 ± 0.007 a	0.083 ± 0.003 c	0.157 ± 0.003 b	0.003 ± 0.003 d
pH	1.810 ± 0.006 d	6.020 ± 0.009 a	2.250 ± 0.006 c	5.860 ± 0.010 b
TOC/mg·L^{-1}	721.000 ± 0.577 a	358.000 ± 1.732 c	640.000 ± 1.155 b	203.000 ± 1.732 d

Note: Values are displayed as Mean ± Standard Deviation (SD, $n = 3$). Significant differences among wastewater samples are displayed by different alphabet letters ($p < 0.05$). TN, Total Nitrogen; TP, Total Phosphorus; NH_3–N, Ammonia–Nitrogen; NO_3–N, Nitrate–Nitrogen; TOC, Total Organic Carbon.

Industrial wastewater has been reported to severely affect water quality due to the large amount of nitrogen and phosphorus [22,23]. As depicted in Table 1, the bistriazine-containing wastewater increased the phosphorus (P) and nitrogen (N) content, which was attributed to the high concentration of COD and NH_3.

3.2. Analysis of the Structure of the Bacterial Community in the Bistriazine Ring Derivative Wastewater

3.2.1. The Alpha Diversity Analysis of Bacteria

The results of the alpha diversity analysis of the bistriazine ring derivative and KB samples are presented in Table 3. High-quality bacterial sequences with a notable sum of 1,986,686 were obtained. UPARSE software was used to trim the sequences, with a maximum of 58,522 and a minimum of 41,495 sequences in each sample, while the average sequence number was 50,009. All the high-quality readings were categorized by taxonomy (from door to genus) by default. Alpha diversity analysis showed that all the coverages of the tested samples were above 0.992, indicating that the sequencing coverage of the samples was high. Furthermore, an estimate of the bacterial community diversity was made by the Shannon and Simpson indexes, whereas the Chao index was employed to reflect the bacterial community richness, and the number of bacterial OTUs varied from 692 to 923. The Shannon bacterial community diversity index ranged from 2.757 to 3.835, whereas the Simpson and Chao bacterial community diversity indexes ranged from 0.121 to 0.233 and from 826.161 to 1154.254, respectively.

Table 3. Bacterial Alpha Diversity Index and Sequence Length Table.

Sample	Shannon	Simpson	Chao	Coverage	Seq_Num	OTUs
T2	2.757	0.233	826.161	0.995	41,495	692
T3	3.208	0.153	1119.642	0.992	43,229	878
T4	3.835	0.153	1102.665	0.994	58,522	923
KB	3.580	0.121	1154.254	0.993	53,440	840

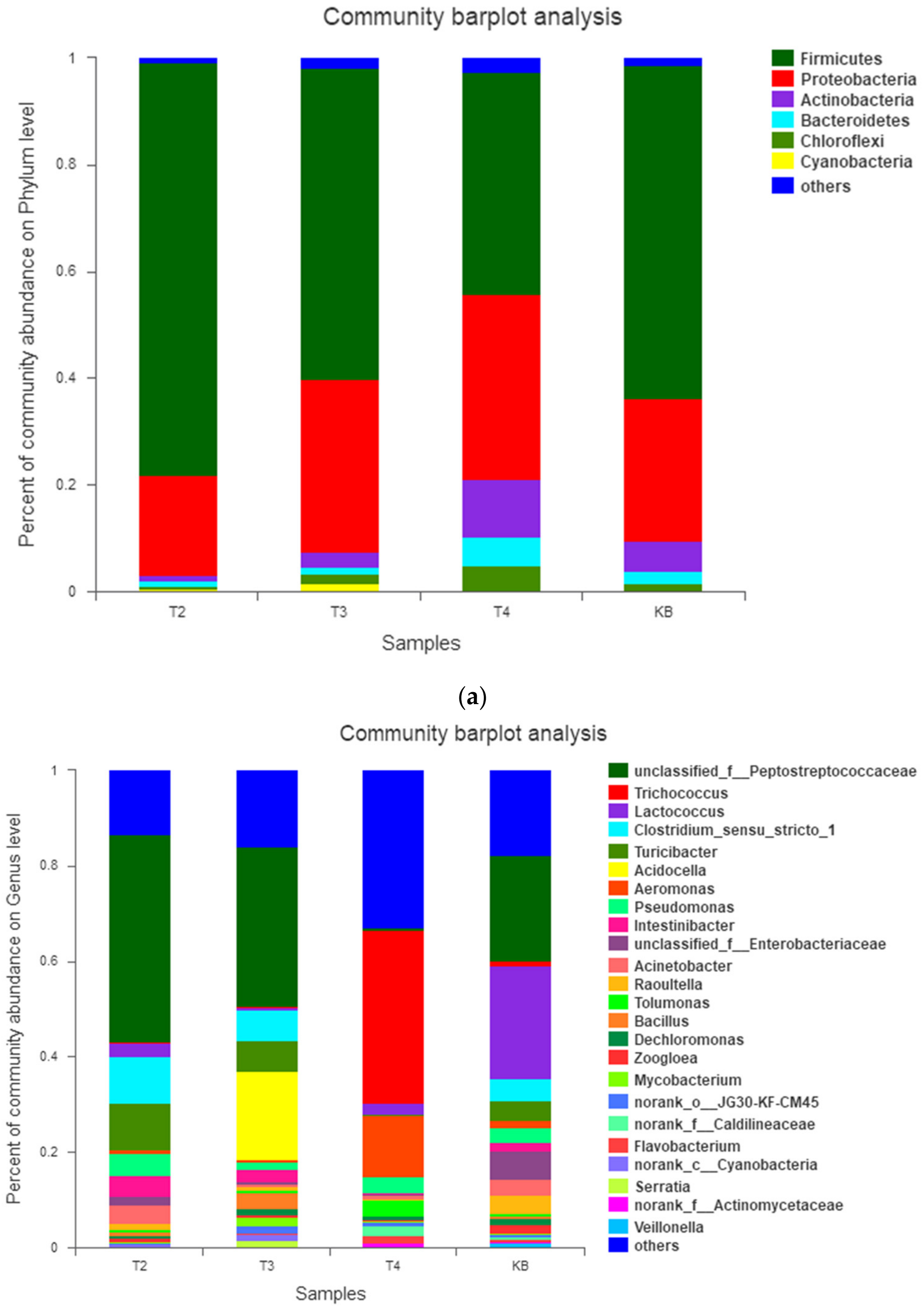
The Shannon index of the T4 wastewater was high, while the Simpson index was relatively low [24], indicating that the T4 wastewater had a high diversity of community species. It was reported in our previous study that microorganisms use non-metallic tanning agents as their carbon source, which leads to an increase in species diversity [16]. As shown in Table 1, the T4 bistriazine ring derivatives had a lower COD concentration and were more suitable for microbial metabolism and growth. When these derivatives were discharged into the wastewater, after a period of adaptation, the microorganisms might have metabolized the organic matter, resulting in an increase in the community species diversity. The index was the highest [24] in the KB as no exogenous pollutants and no bistriazine ring derivatives were introduced. Therefore, the impact on the microbial community structure was small. Although the species richness was higher, most of the microbial species showed a lower abundance due to the lack of nutrients.

3.2.2. Effect of Derivatives Containing Different Bistriazine Rings on the Bacterial Microbial Community in the Wastewater

Figure 2a,b illustrate the relative abundance of bistriazine-containing wastewater bacterial communities with respect to phylum and genera. Figure 2a shows the phylum only with abundance rates higher than 0.01%. A total of six phyla were identified and described: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi,* and *Cyanobacteria*. The dominance of *Firmicutes* and *Proteobacteria* was witnessed in T2, T3, T4, and KB wastewater with an average relative abundance of 77.57, 18.58, 58.47, and 32.50% and 41.69, 34.52, 62.30, and 26.76%, respectively. Previous research on the bacterial community composition in wastewater reported the dominance of *Firmicutes* and *Proteobacteria*. Xu [12] investigated six sewage treatment plants and studied their bacterial communities. This research proved that the main phyla present in the sewage water were *Cyanobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Proteobacteria*, *Planctomycetes,* and *Firmicutes*. Wang [25] found *Proteobacteria*, *Firmicutes*, *Bacteroidetes,* and *Actinobacteria* to be the main bacteria in a leather wastewater treatment plant. The abundance of *Actinobacteria* was low in T2 and T3 but higher in T4. *Cyanobacteria* was almost non-existent in T4, indicating a notable difference in the microbial community composition among the bistriazine ring derivative wastewater samples. Previous reports explained that microorganisms might consume organic matter for growth and reproduction, which can repress the growth of other bacteria [16,26] reported that the presence of *Pseudomonas* might repress the growth of other bacteria. These results indicate that the addition of bistriazine affects the bacterial community structure in wastewater.

Bacterial genera with a higher abundance (<0.01%) are shown in Figure 2b. In all, 24 genera were identified, and the type and quantity changed according to the different types of bistriazine ring derivatives. Notably, T4 had virtually no unclassified species, whereas the T2, T3, and KB populations ranged from 22.29 to 43.46%.

In T2, the dominant genera were *Clostridium sensu stricto* (9.93%), *Turicibacter* (9.59%), *Pseudomonas* (4.66%), *Intestinibacter* (4.43%), and *Acinetobacter* (3.93%). In T3, the abundance of *Turicibacter* and *Clostridium sensu stricto* decreased significantly, while that of *Acidocella* and *Bacillus* increased. The dominant bacterial genera were *Acidocella* (18.33%), *Turicibacter* (6.48%), *Clostridium sensu stricto* (6.45%), and *Bacillus* (3.37%). In T4, the dominant genera changed to *Trichococcus* (36.26%), *Aeromonas* (13.02%), *Pseudomonas* (3.38%), and *Tolumonas* (3.37%). In KB, *Lactococcus* (23.58%), *Clostridium sensu stricto* (4.64%), *Turicibacter* (4.14%), *Raoultella* (3.81%), *Acinetobacter* (3.31%), and *Pseudomonas* (3.21%) were dominant.



(**a**)

(**b**)

Figure 2. (**a**). Diagram of the bacterial community structure at the phylum level. (**b**). Genus-level bacterial community structure.

The presence of *Clostridium*, *Pseudomonas*, and *Acidocella* had a denitrification effect, whereas the presence of *Aeromonas* and *Raoultella* dephosphorized the bistriazine wastewater [16,27–30]. *Lactococcus* removed ammonia–nitrogen more effectively, indicating that the bacteria responsible for denitrification were predominant, whereas the bacteria responsible for dephosphorization and the removal of ammonia–nitrogen were dominant in the blank control wastewater. From the above analysis, we concluded that the bacterial community composition of the bistriazine ring derivative-containing wastewater systems was different from that of the blank control, and that the bacterial community structure changed dramatically. Jayamani [31] studied hexahydro-1,3,5-trinitro-1,3,5-triazine (generally called RDX) pollutants that affect the groundwater bacterial community structure and found *Pseudomonadaceae* and *Acinetobacter* to be the dominant bacteria. Livermore [32] similarly studied the effects of RDX pollutants and found *Pseudomonas* to be the dominant bacterial genus.

Figure 3 illustrates the sensitive and tolerant species found in the bistriazine ring derivative wastewater relative to the KB control. The abundance of *Lactococcus*, *Acinetobacter*, *Raoultella*, *Dechloromonas*, and *Zoogloea* was higher, whereas that of *Trichococcus*, *Clostridium sensu stricto*, *Turicibacter*, *Pseudodomonas*, *Acidocella*, *Intestinibacter*, *Tolumonas*, and *Bacillus* was lower. This indicates that the wastewater was rich in tolerant bacteria that provided suitable survival conditions and promoted bacterial reproduction and growth to maximize bistriazine degradation. For example, Jayakumar [33] reported that *Pseudomonas* is an effective bacterium for remediating tannery wastewater, and a study by Kapahi [34] reported that *Bacillus* removed heavy metals. A unique microorganism, *Acidocella*, also caused degradation. Such sensitive and tolerant bacteria must be investigated further to uncover more bioremediation options.

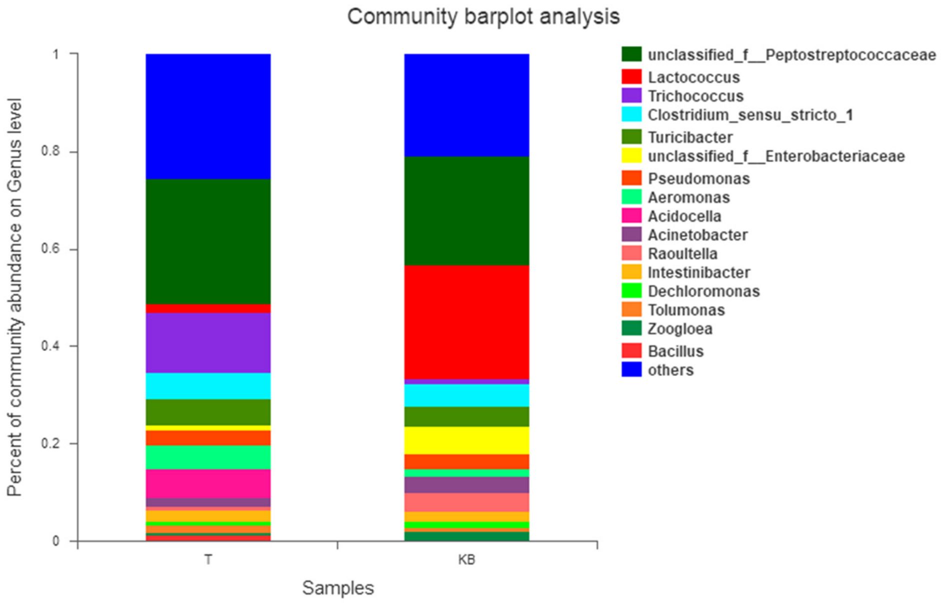


Figure 3. Bacterial community structure of bistriazine ring derivative wastewater and blank control wastewater.

3.3. *β*-Diversity Analysis

The results of PCoA, illustrated in Figure 4, show an obvious difference between the bacterial community in the bistriazine ring derivative wastewater and that in the blank control wastewater. The distance between T2 and T3 was less, indicating that these two sample communities had a similar composition, and T4 had a specific microbial community. Isazadeh [35] studied eight activated sludge wastewater treatment plants and specific changes in beta diversity were noted in each wastewater sample.

As shown in Table 1, the COD value of T4 was relatively small, so no significant difference between the high concentrations of organic pollutants (COD) found in T2 and T3 was observed.

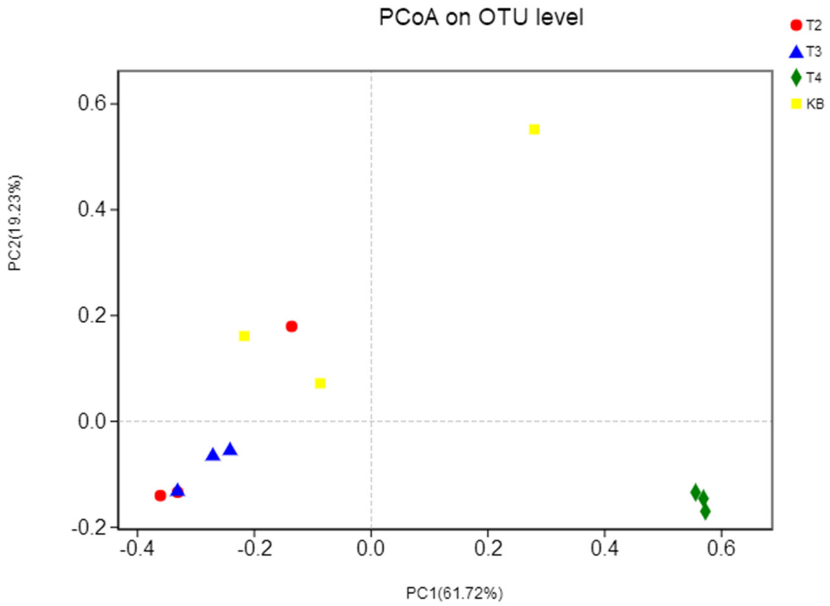


Figure 4. Principal component analysis (PCoA) diagram of the 16S rRNA gene sequencing data based on bistriazine ring derivative wastewater.

3.4. Correlation between Bacterial Community Composition and Physicochemical Properties of Bistriazine Ring Derivative Wastewater

The PcOA results show that the bacterial communities from different wastewater samples clustered according to their type (Figure 4). In Figure 5, the CCA shows a clear correlation between the bacterial community composition and physicochemical properties, the extent and direction of which influenced the degree and significance of the correlation. Previous studies reported how pH, TOC, TN, and TP affected the overall diversity and composition of the microbial community [36,37]. The correlation between the cataloging composition at the OTU level and the physicochemical properties of the bistriazine ring derivative wastewater (pH, TN, TP, NH_3–N, NO_3–N, and TOC) was determined by CCA (Figure 5). About 55.77% of the cataloging information was explained by the two axes. It was revealed by the CCA that the T4 wastewater samples showed a positive correlation for TN and NH_3–N, whereas T2 and T3 showed a positive correlation for NO_3–N, pH, and TP. A negative correlation was seen between the KB wastewater samples and TN, NH_3–N, and NO_3–N. These results are consistent with the changes in the microbiological classification structures. Li [16] reported that, based on a RDA analysis, the physicochemical properties had a significant correlation with the bacterial community structure in wastewater containing tanning agents. This indicates a similar microbial community composition in the same area, confirming that these properties were mainly responsible for affecting the bacterial community composition.

Based on the previous discussion, the primary elements that affected the microbial activity in water were pH, TOC, TN, TP, NO_3–N, and NH_3–N because of their effect on the overall diversity and bacterial communal structure [36–40] observed the effect of adding ferric nitrate at different levels of pH on the stability and bacterial communal system of sewage sludge. The results obtained by Illumina high-throughput sequencing showed that at an optimal level of pH, this group showed a significant increase in the abundance of the *Firmicutes* phylum (61.0–96.6%), while an increase in pH decreased it. Huang [41] and others analyzed the dynamics of microbial communities in pig farm wastewater treatment systems and found a negative correlation between *Clostridium sensu stricto* and NH_3–N and TN, whereas a positive correlation was found for *Acinetobacter*. Yang [42] explored the relationship between bacterial diversity in rain-cellar water and major water quality factors, confirming a positive correlation between *Acinetobacter* and pH, TN, TP, NO_3–N, and NH_3–N. Similarly, Zhang [43] studied the relation between environmental factors and microbes in farm wastewater and, using a redundancy analysis (RDA), revealed a correlation. In addition, the NH_3–N and TN content had the greatest impact on the environment. *Pseudomonas*, *Bacillus*, and *Aeromonas* correlated positively with pH but negatively with NH_3–N and TN. Shi [44] studied how different pH values affected the bacterial communities in river sediments, and the results from Illumina high-throughput sequencing revealed an increase in the comparative richness of the predominant *Acidocella* with an increase in the pH value from 3 to 5. Carvalho [45] considered the effects of pH on bacterial communities in textile wastewater and the Illumina high-throughput sequencing results indicated that the pH significantly affected the relative abundance of *Trichococcus*. García-Ruíz [46] considered the effects of pH on the bacterial community structure in a black glue fermentation. The results showed a positive correlation between *Intestinibacter* and pH; therefore, we concluded that physicochemical properties, such as pH, TOC, NH_3–N, NO_3–N, TP, and TN, had a strong influence on the bacterial community structure in wastewater containing bistriazine.

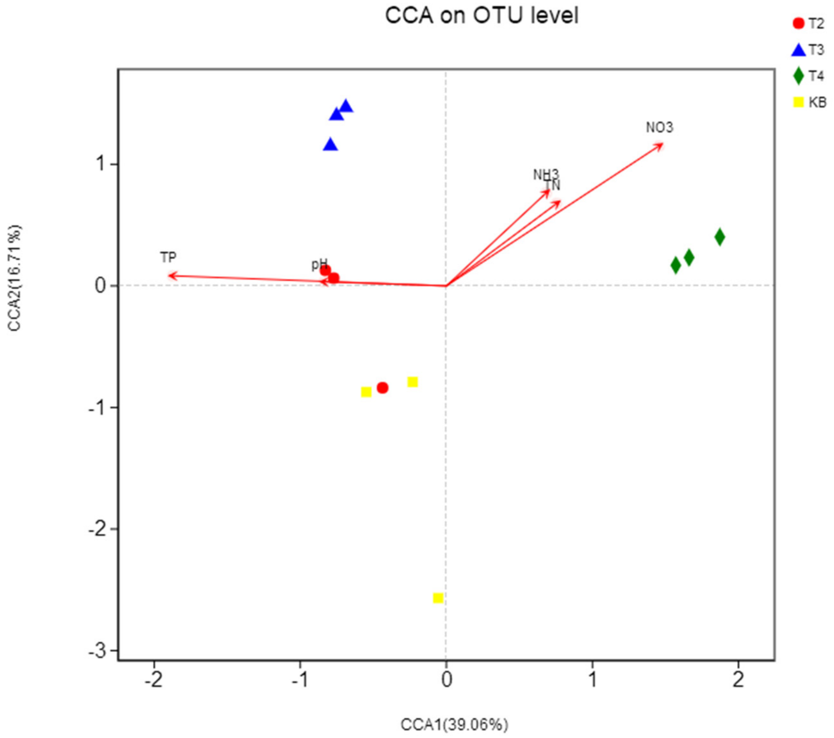


Figure 5. Correspondence Analysis (CCA) of the 16SrRNA gene sequencing data and physicochemical properties of bistriazine ring derivative wastewater. The arrow direction and size show the extent of the influence, positive and negative, on the microbial community composition.

3.5. Relationship between Bacterial Richness and Physicochemical Properties of Bistriazine Ring Derivative Wastewater

Spearman's rank correlation analysis was employed to analyze the correlation between microbial richness and the wastewater's physicochemical properties (Figure 6). The abundance of *Serratia*, *Lactobacillus*, *Proteus*, *Clostridium sensu stricto*, *unclassified_f__Peptostreptococcaceae*, *Turicibacter*, *Ruminococcus_1*, and *Terrisporobacter* positively correlated with the TP content of the bistriazine ring derivative wastewater ($p \leq 0.05$), whereas a positive correlation between the abundance of *Escherichia–Shigella*, *Blautia*, *Bacillus*, and *Ruminococcus* 2 and TP ($0.01 < p \leq 0.05$) was also observed. Similarly, there was a positive correlation between *Acidocella* and *norank c Cyanobacteria* and TP ($p \leq 0.001$), while the pH content showed a positive correlation with *Mycobacterium* and *norank_o__JG30-KF-CM45* abundance ($p \leq 0.05$). The NO_3–N content showed a positive correlation with *Norank_o__JG30-KF-CM45* and *unclassified_k__norank* abundance ($p \leq 0.05$). Conversely, *norank_f__Elev-16S-1332* abundance displayed a negative correlation with TP, TN, and TOC ($p \leq 0.05$). The community richness of *Aeromonas*, *norank_p__Saccharibacteria*, *Macellibacteroides*, *Tolumonas*, *Chryseobacterium*, and *Rhodobacter* showed a negative correlation with TP ($p \leq 0.05$). The abundance of *norank_f__Anaerolineaceae*, *norank_o__Acidimicrobiales*, *norank_o__Pem15*, *Candidatus* microthrix, *Flavobacterium*, and *Simplicispira* negatively correlated with TP ($0.01 < p \leq 0.05$). Moreover, the abundance of *norank_f__Caldilineaceae*, *Comamonas*, *Trichococcus*, *Tessaracoccus*, *norank_f__Actinomycetaceae*, and *Propioniciclava* correlated negatively with TP ($p \leq 0.001$). A negative correlation between *Zoogloea* abundance and NH_3–N, TN, and TOC was observed ($p \leq 0.05$). *Raoultella* abundance exhibited a negative correlation with NO_3–N ($p \leq 0.05$). The correlation-based heat map analysis showed that the physicochemical properties of the bistriazine ring derivative wastewater had both a favorable and an unfavorable effect on the bacterial community, causing a corresponding effect on its composition.

Five components of wastewater (TN, TP, TOC, NH_3–N, and NO_3–N) were studied to determine their environmental effect on their bacterial communities [47–49]. An increase in TP promoted the growth of *Serratia*, *Lactobacillus*, *Proteus*, *Clostridium sensu stricto*, *unclassified_f__Peptostreptococcaceae*, *Turicibacter*, *Ruminococcus* 1, *Terrisporobacter*, *Escherichia-Shigella*, *Blautia*, *Bacillus*, *Ruminococcus* 2, *Acidocella*, and *norank_c__Cyanobacteria*. Similarly, an increase in NO_3–N promoted the comparative abundance of *unclassified_k__norank* and *norank_o__JG30-KF-CM45*. These results confirm that TN, TP, TOC, NH_3–N, and NO_3–N, along with pH, induced changes in the bacterial community composition. The relative abundance of various sensitive species might also have been affected by these factors, thereby changing the entire bacterial community composition [49].

NOTAs were found to be more eco-friendly than chromium tanning agents [50,51]. The above analysis revealed that the changes in the bacterial community structure were attributable to the physical and chemical properties of the bistriazine wastewater. Thus, removing the nitrogen and phosphorus and enhancing the growth of active and tolerant bacteria by providing a suitable living environment may constitute an effective wastewater treatment. Further investigation into tolerant bacteria is required to enhance the treatment efficiency.

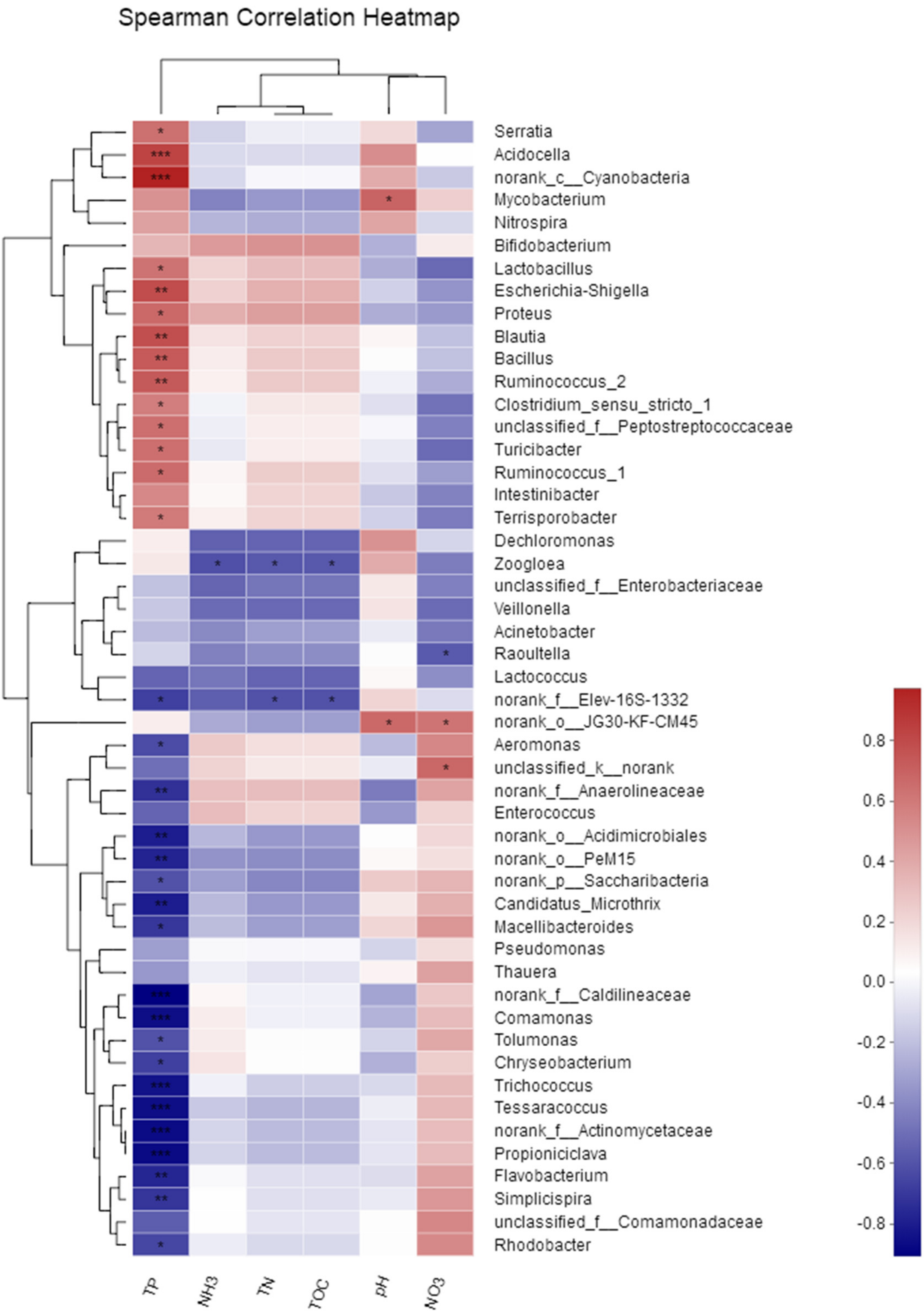


Figure 6. Spearman's rank correlation studies on physicochemical properties and bacterial species abundances. The correlation coefficient is the heat map's corresponding value, where the value of "r" ranges from −1 to 1; $r < 0$ indicates a negative correlation, while $r > 0$ indicates a positive correlation. * means the test was significant at $0.01 < p \leq 0.05$, ** means the test was significant at $0.001 < p \leq 0.01$, *** means the test was significant at $p \leq 0.001$.

4. Conclusions

This study investigated the structural changes in bacterial communities in wastewater samples caused by bistriazine non-metallic organically synthesized tanning agents via high-throughput sequencing. Subsequently, the composition of microbial communities was studied. Prominent changes were observed in the structure of the bacterial communities in the wastewater containing various bistriazine NOTAs. Several conclusions can be drawn based on the above discussion and results.

The wastewater's physicochemical properties (pH, TN, TP, TOC, NH_3–N, and NO_3–N) significantly affected the microbial community structure. Therefore, with regard to the treatment of tanning wastewater containing bistriazine NOTAs, efficiency might be improved by providing a reasonable living environment to promote microbial growth and reproduction, particularly of active functional bacteria.

The bistriazine-containing wastewater was found to have a relatively high microbial abundance and diversity. *Trichococcus*, *Clostridium sensu stricto*, *Turicibacter*, *Pseudomonas*, *Intestinibacter*, *Acidocella*, *Tolumonas,* and *Bacillus* all displayed a high tolerance for the bistriazine NOTAs and can remove nitrogen.

Such resistant microbes should be explored intensively for the bioremediation of bistriazine-containing wastewater.

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