

Communication

# Long-Term Exposure to Azo Dyes from Textile Wastewater Causes the Abundance of *Saccharibacteria* Population

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**Abstract:** Discharge of untreated wastewater is one of the major problems in various countries. The use of azo dyes in textile industries are one of the key xenobiotic compounds which effect both soil and water ecosystems and result in drastic effect on the microbial communities. Orathupalayam dam, which is constructed over Noyyal river in Tamil Nadu, India has become a sink of wastewater from the nearby textile industries. The present study had aimed to characterize the bacterial diversity and community profiles of soil collected from the vicinity of the dam (DS) and allied agricultural field (ALS) nearby the catchment area. The soil dehydrogenase and cellulase activities were significantly lower in DS compared to ALS. Additionally, the long-term exposure to azo dye compounds resulted in higher relative abundance of *Saccharibacteria* (36.4%) which are important for degradation of azo dyes. On the other hand, the relative abundance of *Proteobacteria* (25.4%) were higher in ALS. Interestingly, the abundance of *Saccharibacteria* (15.2%) were also prominent in ALS suggesting that the azo compounds might have deposited in the agricultural field through irrigation. Hence, this study revealed the potential bacterial phyla which can be key drivers for designing viable technologies for degradation of xenobiotic dyes.

**Keywords:** azo dye; textile; wastewater; diversity; xenobiotics; pollutant

## 1. Introduction

Soil is a biologically balanced system and any variations in the soil micro-environment results in the changes in native microbial community profiles. Similarly, the accumulation of pollutants such as polycyclic aromatic hydrocarbons (PAHs) [1], petroleum compounds [2] and heavy metals [3] have drastic effect on the bacterial diversity. The driving factors which determine the abundance of the microbial community in a particular contaminated soil are the genetic variation which results in altered metabolic pathways or the selective enrichment of microbes which are able to transform the particular pollutant [4].

Rapid advent of industrialization has led to deleterious effect on the environment, mostly due to the improper discharge of industrial waste. The textile industries use a

large amount of synthetic dyes which results in discharge of colored wastewater [5,6]. Azo dyes account for 70% of all the commercial dyes used in textile industries across the globe [7]. These dyes have been proven to be harmful for the aquatic life forms as they result in increasing the biological oxidation demand (BOD) [8]. Additionally, these azo compounds are also reported to be highly toxic and carcinogenic in nature [7]. It is estimated that around 50% of the synthetic dyes used in the textile industries does not bind to the fabric and result in getting discharged into the environment [9]. The compounds can leach into the groundwater and the use of those water sources for farming results in contamination of agricultural fields [10]. The bioremediation of contaminant by isolation and characterization of indigenous microbial community have been studied in various cases [11,12]. The classical enrichment technique does not give the broader idea about the various microbial activities occurring in the soil. Metagenomic analysis by using high-throughput sequencing helps to overcome the limitations of conventional methods of studying microbial community dynamics in soil. Researchers have concentrated on designing numerous technologies [13,14] for treatment of textile wastewater and also studied the changes in microbial community and diversity in those reactor systems which are used for remediating such wastewater [15]. However, there have been no studies on high-throughput detection of microbial community profiles of environmental samples such as water or soil which have been severely affected by long-term exposure to improper discharge of textile wastewater rich in azo dye compounds.

Orathupalayam dam is constructed on the Noyyal river in Tamil Nadu, India. The dam has become a reservoir of azo dye compounds due to the continuous discharge of effluents from the textile industries located in the nearby area of Tiruppur. In a previous study, bacterial and fungal species were isolated from the soil samples collected from the nearby catchment area and allied agricultural fields of this dam and characterized based on their ability to degrade azo dyes in a batch scale [16]. Whereas, in this study, we aimed to characterize the bacterial community profiles of soils collected from the nearby area of Orathupalayam dam and allied agricultural fields.

## 2. Materials and Methods

### 2.1. Collection of Soil Sample

Soil samples were collected from the vicinity of Orathupalayam dam and its allied agricultural lands. The dam is located (11°06'31.56" N; 77°32'26.33" E) in Tiruppur District, Tamil Nadu, India (Figure 1). A total of 6 composite soil samples were collected from the vicinity of the dam and 4 composite soil samples were collected from the agricultural lands situated within 2 km radius. Samples were collected by leaving at least 40 m distance between samples and 5–20 cm depth. Both the samples were made into a composite one which resulted in two soil samples: Dam Soil (DS) and Agricultural Soil (ALS).

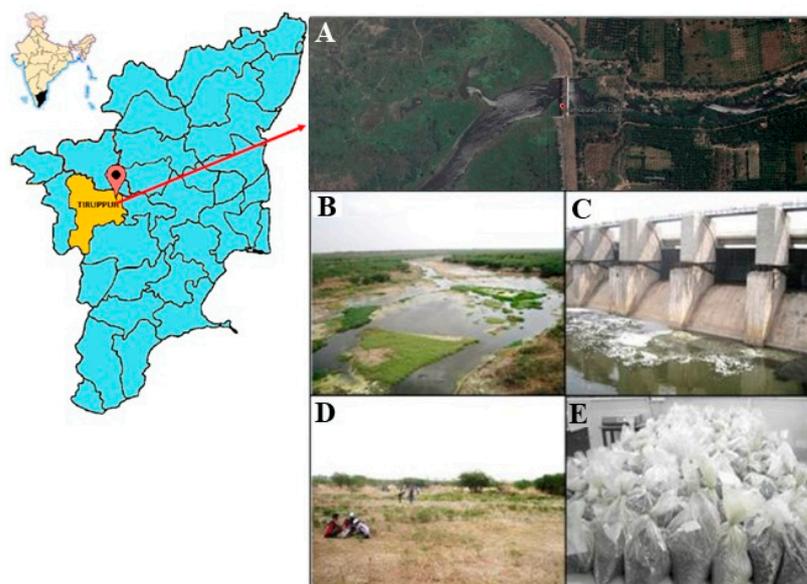
### 2.2. Soil Enzyme Activities

Dehydrogenase was measured by assaying 2,3,5-triphenyl tetrazolium chloride (TTC) as substrate and defined as production of triphenylformazan (TPF)  $\text{g}^{-1} \text{soil h}^{-1}$  at 37 °C [17]. Urease activity was measured based on the amount of ammonium released after incubation of soil samples with urea solution for 2 h at 37 °C and determined calorimetrically by indophenol reaction at 690 nm [18]. Cellulase activity was measured by determining the breakdown of carboxymethylcellulose (CMC) to glucose at 30 °C for 24 h [19]. Acid phosphomonoesterase activity was determined by measuring the concentration of *p*-nitrophenol (*p*-NP) released after incubation of soil with *p*-NP-linked substrates for 1 h at 37 °C [20].

### 2.3. Soil DNA Extraction

Total soil DNA was extracted from 0.5 g of fresh soil sample by using HiPurA Soil DNA Purification Kit (Hi Media, Mumbai, India) following the manufacturer's protocol. Quality and quantity of the extracted DNA samples were checked using agarose

gel electrophoresis as well as Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, NC, USA).



**Figure 1.** Overview of the soil sampling site and soil collection. (A) Google earth image of Orathupalayam dam constructed over Noyyal river in Tamil Nadu, India, (B) Orathupalayam dam inlet, (C) Orathupalayam dam outlet, (D) Soil sampling site, (E) Collected soil samples.

#### 2.4. PCR Amplification and Phylogenetic Marker Library Preparation

Library construction involved two PCR reactions (Kapa HiFi Hot start, Kapa Biosystems, MA, US): amplicon PCR and indexing PCR. In amplicon PCR, the 16S rRNA gene was amplified using PCR with primers 341F, 5'-CCTACGGGAGGCAGCAG-3' and 518R, 5'-ATTACCGGGCTGCTGG-3' [21–23] targeting the V3-V4 region for construction of bacterial phylogenetic marker library. Subsequently, the Illumina sequencing adapters and dual indexing barcodes were added using indexing PCR. The library of final products was cleaned using HighPrep PCR (Magbio, Gaithersburg, MD, USA) magnetic beads and quality was evaluated on a Bioanalyzer, using a DNA1000 lab chip (Agilent, USA). The raw reads were deposited in SRA archives and can be accessed by BioProject number PRJNA522349.

#### 2.5. Pyrosequencing and Pre-Processing of Sequence Reads

Purified PCR products (library) were pooled in equimolar ratios and paired-end reads were generated on an Illumina GAIIx sequencer. Image analysis and base calling were done using Illumina Analysis pipeline (Version 2.2). High quality reads with more than 70% of bases with Phred Score greater than 20 were considered for subsequent analysis. Reads with adapter sequences, primer sequences, the barcode and the degenerate bases were removed using an automated Perl code generating processed reads. Duplicates and chimeras were removed using CD-HIT DUP [24].

#### 2.6. Data Analysis

The resulting dataset was pre-screened using uclust for a minimum of 70% identity to ribosomal sequences and then clustered at 97% identity against the Silva (SSU115) 16S rRNA database using in MG-RAST [25]. Taxonomic assignment from phylum level to strain level was assigned based on the hits. Abundance graphs were plotted based on the number of hits. Rarefaction curves were plotted using MG-RAST [25]. Diversity index was calculated using Mothur [26]. Differences in the relative abundances of microbial community between groups were evaluated with an unequal variances t-test (Welch's *t*-test) considering unequal variance in taxonomic groups.

### 3. Results and Discussion

#### 3.1. Soil Enzyme Activities

Soil quality can be determined by measuring the enzyme activities [27]. Among the various soil enzymes, dehydrogenase is considered as one of the most abundantly used parameter to determine the soil quality and reflects the rate of nutrient transformations occurring in soil. [28]. Dehydrogenase activity is affected with respect to the presence of contaminant in soil [29]. The DS had shown significantly lower dehydrogenase activity compared to ALS (Table 1). The presence of high concentration of azo compounds in DS [16] might have resulted in decrease in microbial population and subsequently reducing the enzyme activity. Results of this study match with the results of Kaczyńska et al. [28], where the authors found reduced dehydrogenase activity due to soil contamination. On the other hand, the cellulase activity is higher in soils which are rich in plant biomass [30], and it corroborates to our study where the cellulase activity has been observed to be highly significant in ALS. It can be assumed that the DS contains low amount of organic plant biomasses due to azo dye impact on plant development than that of ALS. However, urease and acid phosphomonoesterase activities did not show any significant difference among the soil samples. Urease and acid phosphomonoesterase activities are used for indirect estimation of soil nitrogen and phosphorus transformation, respectively [20].

**Table 1.** Soil enzyme activities of DS and ALS.

Soil Samples	Dehydrogenase (ng TPF g <sup>-1</sup> Soil h <sup>-1</sup> )	Urease (mg NH <sub>4</sub> <sup>+</sup> -N g <sup>-1</sup> Soil h <sup>-1</sup> )	Cellulase (µg Glucose g <sup>-1</sup> Soil h <sup>-1</sup> )	Acid Phospho- monoesterase (µg Glucose g <sup>-1</sup> Soil h <sup>-1</sup> )
DS	15.37 ± 1.96	3.36 ± 0.47 <sup>a</sup>	27.28 ± 1.41	54.92 ± 9.16 <sup>a</sup>
ALS	22.35 ± 2.68	5.11 ± 0.63 <sup>a</sup>	40.75 ± 4.55	55.71 ± 11.35 <sup>a</sup>
LSD ( <i>p</i> ≤ 0.05)	9.17	1.99	10.56	37.53

<sup>a</sup> The enzyme activities are not significantly different from each other at 5% threshold (LSD). Values are mean ± SE (standard error) of three independent determinations.

#### 3.2. Diversity Indices

The microbial diversity is affected due to the presence of high amount of pollutants in the soil [31]. It has been observed that the azo dye contaminated soils have changes in microbial community structure based on the phospholipid fatty acid profiles [5]. Similarly, the total number of bacterial OTUs were observed to be 8302 and 12,162 for DS and ALS respectively (Table 2), after clustering at 3% cutoff level. Significant variation observed in OUT of DS and ALS, illustrated the negative impact of azo dye on soil microbial population and diversity. Similarly, the negative impact of individual azo dyes (Direct Red 81, Reactive Black 5 and Acid Yellow) on soil bacterial and fungal diversity was reported by Imran et al. [5]. In addition, the authors reported that azo dyes affect plant beneficial bacteria habitats in soil. The rarefaction curve confirmed the adequacy of sampling effort in the current microbial diversity analysis (Figure S1). The diversity indices measured in terms of Shanon and Inv-simpson indices, and the richness measured in terms of Chao1 index were higher in ALS compared to DS (Table 2). The diversity indices give the overview of richness and evenness of a community. Hence, the result of the diversity indices infers that the textile wastewater decreased the  $\alpha$ -diversity of the microbial community and also decreased the richness and evenness of the microbial community in DS [32]. The major drawback of the study is that a single replicate was used for studying the bacterial diversity. However, it can be used as a preliminary study for understanding the effect of azo dyes on bacterial community profiles.

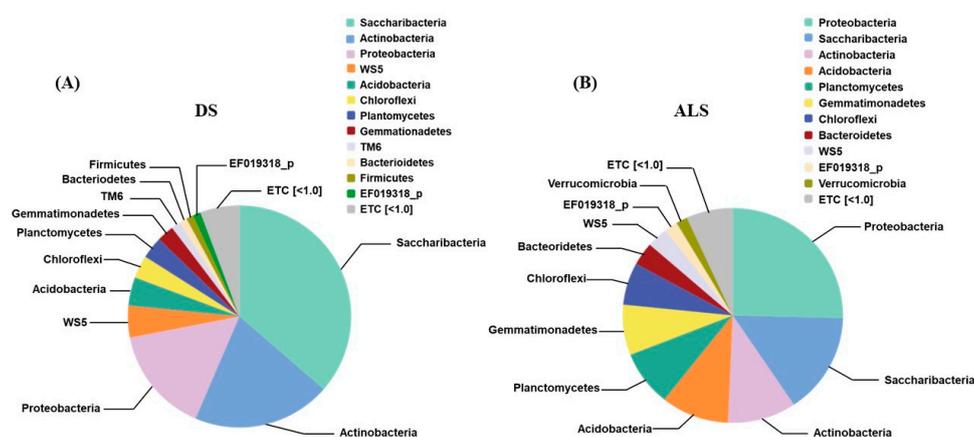
**Table 2.** Microbial diversity estimates of soil samples.

Soil Samples	Number of OTUs	Chao 1 (Richness)	Shanon (Diversity)	Inv-Simpson (Diversity)
DS	8302	1303.71	5.54	51.82
ALS	12162	1472.93	6.25	158.78

### 3.3. Bacterial Diversity and Community Profiles

Soil contaminated with myriad of pollutants has become a serious problem across the world. Studies which concentrate on deciphering the microbial community profiles of contaminated soil helps us to design strategies to remediate such sites by targeting the key microbial players in degradation of the particular pollutant [33]. There have been numerous studies in designing reactors for treating textile wastewater which are rich in azo compounds for decreasing the levels of effluents in the environment [13,14].

In this study, a total of 50 and 54 phyla were detected for DS and ALS respectively (Figure 2). The phyla profiles were different for both DS and ALS. *Saccharibacteria* (36.4%) were observed to be the most abundant in DS (Figure 2a) whereas *Proteobacteria* (25.4%) were the abundant ones in ALS (Figure 2b). Negative impact of azo dyes on soil *Proteobacteria* were previously reported [5]. Reactive Black 5 Direct Red 81 used by Imran et al. [5] found to reduce the *Proteobacteria* population significantly in soil. The other phyla dominating DS are *Actinobacteria* (20.02%), *Proteobacteria* (15.49%), WS5 (4.63%), *Acidobacteria* (4.16%) and *Chloroflexi* (3.31%). Similarly, the other phyla dominating ALS are *Saccharibacteria* (15.24%), *Actinobacteria* (10.03%), *Acidobacteria* (9.94%), *Planctomycetes* (8.4%), *Gemmatimonadetes* (7.59%), *Chloroflexi* (6.34%) and *Bacteroidetes* (3.48%). Different genera of *Proteobacteria* were isolated from the textile effluents wastewater treatment plants [34], and these *Proteobacteria* might have been involved in degradation of azo dyes. *Saccharibacteria* have been observed to be highly active in sludge reduction and dye wastewater treatment plants [14,35]. Furthermore, *Saccharibacteria* are regarded as one of the active microbial group in anoxic carbon-based fluidized bed reactor treating coal pyrolysis wastewater which contains abundant phenolic compounds [36]. In another study, it has been reported that *Actinobacterial* extract [37] and its related enzymes [38] are important in degradation of azo dyes. Hence, the abundance of *Saccharibacteria* and *Actinobacteria* in this study can be attributed to the fact that these phyla are responsible for remediating/decolorizing azo dye compounds present in the textile wastewater. *Saccharibacteria* and *Actinobacteria* present in Azo dye contaminated soil, indicates their important in degradation of soil pollutants. Since the results obtained by pyrosequencing techniques, many of them might be unculturable. In future, selective culturing of *Saccharibacteria* and *Actinobacteria* from this azo dye contaminated soils could be the opportunity to obtain efficient strains for remediation of azo dye polluted soils.

**Figure 2.** Relative abundance of bacterial lineages of (A) DS and (B) ALS in phylum level.

On the other hand, the abundance of *Proteobacteria* in agricultural soil also corroborates to previous studies [32]. As the agricultural sector uses mostly inorganic NPK fertilizers, which has resulted in increase in the relative abundance of *Proteobacteria* in agricultural fields [39]. The abundance of *Saccharibacteria* in agricultural soil can be correlated to the evidence that the dam water used for irrigation of the agricultural soil has resulted in contamination of the field [13,34]. The abundance of *Actinobacteria* in ALS can be explained by the fact that these bacterial group consists of plethora of plant growth promoters which are predominantly found in agricultural soils [18]. The genus and species composition of these two soil groups had revealed the presence of a number of rare bacterial species (Figure 3). There have been approximately 75% and 78% of rare bacterial species in DS and ALS, respectively. Exploiting these bacterial species can be helpful in designing efficient technologies in remediating/ decolorization of azo dyes.

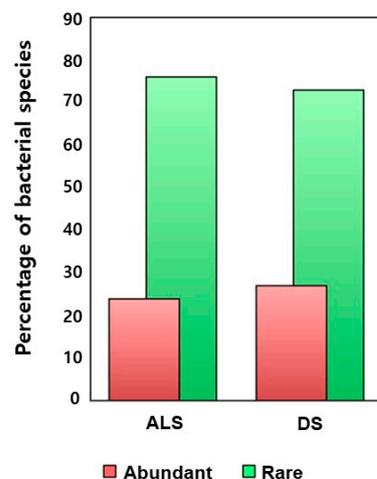


Figure 3. Percentage of abundant and rare bacterial species in ALS and DS.

#### 4. Conclusions

The long-term exposure of wastewater from textile industries has resulted in changes in soil enzyme activities and bacterial diversity. The azo-dye contamination caused the *Saccharibacteria* population to proliferate in higher abundance compared to other bacterial phyla in DS. Moreover, ALS had shown relatively high abundance of *Saccharibacteria* which might be due to the use of polluted water from the dam for irrigation purposes. Further studies concentrating on elucidating the fungal community dynamics will help to detect the major microbial drivers important for degradation of azo dyes. The isolation and enrichment of these specific microbes (*Saccharibacteria* and *Actinobacteria*) can help to design/propose novel technologies in remediation of textile wastewater for reducing the contaminant footprint in the environment.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2076-3417/11/1/379/s1>, Figure S1: Rarefaction curve of bacterial communities.

**Author Contributions:** R.A. and T.S.: conceptualization of the study; R.K. and A.R.C.: designed experiments, performed experiments and analysis of sequencing data; P.A.J., K.S., M.S., J.P. and N.O.G.: assisted in soil sampling and experiments; J.C. and K.K.: assisted in data analysis and discussion; R.K., A.R.C.: wrote the manuscript; R.A. and T.S.: critical reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data and analyses from the current study are available from the corresponding authors upon reasonable request. The raw reads were deposited in SRA archives and can be accessed by BioProject no. PRJNA522349.

**Conflicts of Interest:** The authors declare no conflict of interest.

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