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Microbial diversity, transformation and toxicity of azo dye biodegradation using thermo-alkaliphilic microbial consortia

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ABSTRACT

In this research, the transformation and toxicity of Reactive Red 141 and 239 biodegraded under anaerobic-aerobic conditions as well as metagenomic analysis of Reactive Red 239 degrading microbial consortia collected from Shala Hot spring were investigated. Toxicity of dyes before treatment and after treatment on three plants, fish and microorganisms were done. A halotolerant and thermo-alkaliphilic bacterial consortia decolorizing azo dyes (>98% RR 141 and > 96% RR 239 in 7 h) under optimum conditions of salt concentration (0.5%), temperature (55 °C) and pH (9), were used. Toxicity effect of untreated dyes and treated dyes in Tomato > Beetroot > Cabbage plants, while the effect was *Leuconostoc mesenteroides* > *Lactobacillus plantarum* > *Escherichia coli* in microorganisms. Among fishes, the toxicity effect was highest in *Oreochromis niloticus* followed by *Cyprinus carpio* and *Clarias gariepinus*. The three most dominant phyla that could be in charge of decolorizing RR 239 under anaerobic-aerobic systems were Bacteroidota (22.6–29.0%), Proteobacteria (13.5–29.0%), and Chloroflexi (8.8–23.5%). At class level microbial community structure determination, Bacteroidia (18.9–27.2%), Gammaproteobacteria (11.0–15.8%), Alphaproteobacteria (2.5–5.0%) and Anaerolineae (17.0–21.9%) were dominant classes. The transformation of RR 141 and RR 239 into amine compounds were proposed via high performance liquid chromatography-mass spectroscopy (HPLC/MS) and fourier transform infrared spectroscopy (FT-IR). Overall, dye containing wastewaters treated under anaerobic-aerobic systems using thermo-alkaliphilic microbial consortia were found to be safe to agricultural (fishes and vegetables) purposes.

1. Introduction

High BOD/COD, alkalinity, salinity, and high color in textile wastewaters have an impact on both human and environmental health [1–4]. Due to its composition, colored wastewater resulted from dye application is very difficult to remove its color [1,5]. Azo dyes can be hazardous in various ways depending on their reactivity, structural complexity, and substitution groups. According to earlier

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studies, the majority of aromatic amines and their parent azo dyes are carcinogenic, poisonous, and mutagenic to higher species dwelling in terrestrial and aquatic settings [6,7]. Ferraz et al. [8] reported that dyes were mutagenic to Salmonella fish, which raises the possibility that they may negatively affect the activity and makeup of microorganisms vulnerable to these toxins.

To remove dye contaminated wastewater, physical, chemical or biological treatment techniques may be used [3,9]. Despite the fact that the physico-chemical treatments effectively decolorize azo dyes and remove other environmental contaminants from textile wastewaters, they come with costs, energy demand, and secondary pollutant creation as downsides [2,10,11]. To overcome the drawbacks of physico-chemical textile wastewater treatment technologies, biological treatment is a viable option [4]. Due to its efficiency as a method of removing color, microbial decolorization of dyes has recently attracted attention [12]. On the other hand, mesophilic, neutrophilic, and non-halophilic bacteria are less able to biodecolorize textile wastewater due to its high pH (7–11), high temperatures (50–80 °C), and high salinity (15–20%) [10,13,14]. The capacity of particular microorganisms to adapt to and function in accordance with the nature of such harsh environmental conditions of textile wastewater is a major determinant of microbial decolorization's efficiency [4,15]. Consequently, the greatest candidates for azo dye degradation in harsh conditions of the textile wastewater are extremophiles, which can adapt and are active in these extreme conditions [9,16,17].

Furthermore, using mixed microbial cultures (consortia) is recently preferred over single strains (pure culture). Individual bacteria in a consortium may make use of metabolites produced by other strains already present or may assault the dye molecule from various positions to cause further degradation. Khehra et al. [18] mentioned that the application of microbial consortia in azo dye biodegradation has a substantial advantage over that of pure culture since the consortia has a complementary catabolic diversity and synergetic effect in eliminating a wide variety of contaminants from wastewater. Jadhav et al. [19] also reported that bacterial consortium for its enhanced effect due to their coordinated metabolic interactions on the textile wastewater and they have higher decolorization efficiency than that of individual bacterial strains. Moreover, Guo et al. [9] reported complementary catabolic diversity of the halo-thermophilic bacterial consortium as the main reason for their efficiency in dye decolorization than pure culture. For effective dye removal, a number of microbial consortia collected from different environments have been reported [4,9].

Biodecolorization occurs in aerobic, anaerobic, anoxic, or combined anaerobic-aerobic treatment conditions [20,21], and most dye decolorizing bacteria can do so in static and anaerobic circumstances [3,19]. Due to the fact that aerobic biological process is ineffective in removing colors, it has been hypothesized that aerobic bacteria could effectively stabilize dye metabolites and minimize COD in wastewater [10,22]. The availability of oxygen suppresses the azo bond reduction because aerobic respiration predominate NADH utilization, which prevents electron transfer from NADH to azo bonds. Under anaerobic circumstances, the N=N bond from azo dyes is broken, resulting in colorless, toxic, mutagenic and carcinogenic aromatic amines' formation [23]. In aerobic circumstances, these aromatic amines are totally mineralized and eliminated [21].

Understanding the composition and diversity of the bacterial community is essential to understanding how aerobic and anaerobic dye wastewater treatment reactors function. Because culture-dependent bacterial identification may only make up 1% of the total microbial community [24], environmental microbiology is increasingly and widely using culture-independent molecular techniques like sequencing of conserved genes like rRNA, fluorescent *in situ* hybridization, and high-through pyrosequencing analysis [25]. Moreover, culture-dependent methods are limited in their ability to reveal the variety of complex microbial communities, which means that many of the component species may go undetected. On the other hand, the adoption of culture-independent molecular approaches has revolutionized the identification of microbial communities from various natural environments and wastewater treatment plants [26].

Thermophilic microflora [16]; halo-thermophilic bacterial consortium [9], halophilic thermo-alkaliphilic bacterial consortium [13], and halotolerant bacterial consortium [14] have all been mentioned as effective azo dye decolorization tools. Although there have been numerous other studies done to use extremophiles to treat different types of wastewater (salinity, alkaline, uranium, recalcitrant, and hydrocarbons), most of them have considered a single [2,3,27] and few of them were considered two extreme conditions [9,13] during their enrichment. In this research three extreme conditions equivalent to the actual textile wastewater including high pH, temperature and salinity were considered during enrichment.

This study was aimed to evaluate microbial diversity, transformation and toxicity of azo dye biodegradation using thermo-alkaliphilic microbial consortia. The specific objectives were to: (i) analyze diversity of bacteria decolorizing Reactive Red 239; (ii) evaluate whether the application of wastewater for irrigation is significantly affected by the treatment methods (aerobic and anaerobic); and (iii) propose the transformation of RR 141 and RR 239 into amine compounds using high performance liquid chromatography-mass spectroscopy (HPLC/MS) and fourier transform infrared spectroscopy (FT-IR) analysis. We hope the result of this study insight into the application of thermo-alkaliphilic bacterial consortia for efficient textile wastewater treatment that can be used for safe disposal and agricultural purpose.

2. Materials and methods

2.1. Dyes

The two model dyes (Fig. S1) which are Reactive Red 141 (RR 141) and Reactive Red 239 (RR 239) were donated by Ayka Addis Textile Investment Group in Addis Ababa, Ethiopia. These two model dyes were selected by considering that they were most frequently used in textile industries for production of various fabric colors. The molecular formula of RR 141 is $C_{52}H_{26}Cl_2N_{14}Na_8O_{26}S_8$, its common name is Procion Red HE7B, molecular weight is 1774.19 g/mol and its maximum wavelength (λ_{max}) is 544 nm, while the molecular formula of RR 239 is $C_{31}H_{19}ClN_7Na_5O_{19}S_6$, its common name is Everzol Red 3BS, molecular weight is 1136.32 g/mol and its λ_{max} is 542 nm.

2.2. Wastewater source

Synthetic textile wastewaters of RR 141 [4] and RR 239 (Table S1) were prepared and treated. As explained by Tizazu et al. [4], microbial acclimatization was done by gradually exposing the consortia to increasing dye concentrations from 100 to 1000 mg/L. Using anaerobically adapted microbial consortia and the azo dyes (RR 141 and RR 239), the optimization of several (temperature, pH, nutrients, and salinity) parameters and removal efficiencies were assessed. Under aerobic conditions, further wastewater pollutant removal efficiency was also investigated. The aerobic reactor was incubated on a shaking (adjusted 150 rpm) in water bath for 48 h after being inoculated with 10% (v/v) aerobically acclimatized culture. The aerobically and anaerobically treated wastewaters of both dyes (RR 141 and RR 239) were incubated in refrigerator at 4 °C until used for toxicity tests and transformation analysis, while the samples for metagenomic analysis were kept at −80 °C.

2.3. Seeds and test organisms

Seeds of Beetroot (*Beta vulgaris*), Tomato (*Solanum lycopersicum*) and Cabbage (*Brasssica oleracea* var. *capitata*) with 85% germination capabilities (Fig. S2) were obtained from Pop Vriend Seeds (PV) Agriculture materials shop in Arba Minch, Ethiopia. The test microorganisms used in this study were also obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. Larvae of Tilapia (*Oreochromis niloticus*), was obtained from Arba Minch Fish Fingerling Production Center, Arba Minch, Ethiopia, while larvae of African cat fish (*Clarias gariepinus*), and Carp (*Cyprinus carpio*) were obtained from National Fish and other Aquatic Lives Research Center (NFLARC), Ethiopia.

2.4. Bacterial diversity analysis

Thermo-alkaliphilic microbial consortia used as inoculum was collected from Shala Hot spring located in Rift Valley Region, Ethiopia. The sediment samples were acclimatized under anaerobic and aerobic conditions with dye containing media. Although two model dyes were used, in this research, metagenomic analysis of RR 239 degrading bacteria was addressed, while the metagenomic analysis of RR 141 was investigated in our previous work [4]. Using the Rapid Spin Kit for DNA (MP Biomedicals, Santa Ana, California, USA), metagenomic DNA from aerobically and anaerobically treated wastewater was extracted in accordance with the manufacturer's instructions. A 1% agarose gel was used to examine the DNA extract, and using the Nanodrop 2000 UV–vis spectrophotometer the DNA concentration and purity were measured (Thermo Scientific, Wilmington, USA). Hyper variable region V3–V4 of 16S rRNA was amplified using the primers 515F (5′-TGTGTAGCGGTGAAATGCG-3′) and 806R (5′-CATCGTTTACGGCGTGGAC-3′) in a polymerase chain reaction (PCR) thermocycler (ABI GeneAmp®9700 USA). PCR enrichment conditions were carried out with our prior technique [4]. Initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturing for 30 s, annealing for 30 s, extension for 40 s, and a single extension for 10 min at 72 °C, with the final step occurring at 4 °C. A PCR mixture of 25 μL was utilized, which contained 4 μL of master mix, 2 μL of 2.5 mM dNTPs, 0.8 μL each of 5 μM forward and reverse primers, 0.4 μL of DNA polymerase, 2 μL of template DNA, and 15 μL of distilled water. All PCR reactions were carried out in sets of three, including the control reaction without a DNA template. The extracted and purified DNA was sequenced using Miseq throughput sequencing with Illumina (Shanghai Majorbio Biotechnology Co. Ltd., China). The Majorbio Cloud's free online platform was used to evaluate the 16S rRNA gene sequences at high throughput. Using 97% similarity cut values, sequences were grouped into operational taxonomic units (OTUs). Bacterial diversity indexes such as Simpson, Shannon, coverage, and Chao1 were computed.

2.5. Transformation analysis

Metabolites obtained after RR 141 and RR 239 decolorization was exposed to transformation product analysis via fourier-transform infrared spectroscopy (FT-IR) using FT-IR model 6600, JASCO, Japan. The dried sample was blended in a mortar for 5 min with spectroscopically pure KBr powder at 5:95 ratios. The mixture was pelletized by using mechanical presser in order to ready for the analysis. Then the pelleted sample was inserted into the sample compartment of the instrument. At different wavelengths, the changes in transmittance percentage were observed. The FT-IR analysis was done in the 500-4500 cm^{-1} mid-IR region [28] with 16 scan speed using FT-IR spectroscopy. One mL of the sample was centrifuged for 15 min at 3000 rpm and was dissolved in an HPLC-grade methanol and passed through 0.22 μm membrane filters before being subjected to high performance liquid chromatography/mass spectroscopy (HPLC/MS) analysis. The filtrate was collected and subjected to 50 cycles of sonication at 4 °C to remove gas. A C-18 Column (250 mm × 4.6 mm) version (Agilent 1260, LS/MSD G6125B, USA) outfitted with a C-18 guard column was used for reverse phase HPLC (InfinityLab, USA) analysis. After being degassed 50 times with a sonicator at 4 °C and operating in isocratic mode, a mobile phase made up of 65% acetonitrile and 35% ultrapure water was allowed to operate. After injecting the sample, which had a 20 mL volume, dye products were allowed to separate for 15 min at a flow rate of 1 mL min^{-1}. The temperature was maintained at 28 °C for the separation column. The UV–visible detector was operated in dual wavelength mode at 275 nm to track the purification profiles of the degraded metabolite. In order to examine the peaks, Origin 2018 was used.

2.6. Toxicity assessment

2.6.1. Phytotoxicity tests

To evaluate the effects of the untreated and treated wastewater three vegetables phytotoxicity tests were conducted using untreated

and treated dye containing textile wastewater. Seeds of Beetroot, Tomato and Cabbage were evaluated for germination and plant length (plumule and radicle) growth (Fig. S2a). In testing germination, 10 seeds of each experimental plant including Tomato, Cabbage, and Beet root were placed in 90 mm sterilized Petri dishes having two filter paper discs (Fig. S2b). Then, in 24 h intervals 5 mL of distilled water for control and 5 mL of untreated and treated (anaerobically and aerobically) wastewaters were added to the filter discs and incubated at room temperature. A seedling that was 7 days old was picked from each set for the purpose of measuring seedling growth, and the length of the root and shoot. Germination in each experimental set was recorded and germination percentage, plumule and radicle length were computed.

2.6.2. Microbial toxicity tests

Toxicity of the dyes (RR 141 and RR 239) and their transformation products was studied using *Lactobacillus plantarum* (*L. plantarum*), *Escherichia coli* (*E. coli*), and *Leuconostoc mesenteroides* (*L. mesenteroides*) strains. The influence of textile wastewater before and after treatment on growth of the bacterial strains was determined by cultivating them on ATCC media 697 broth as a control. The wastewaters were mixed (1:1 ratio) with ATCC 697 broth for each treatment, while the broth without wastewater was used as a control. By recording optical density (OD) measurements at 600 nm every 2 h for 10 h, the rate of bacterial growth was evaluated.

The growth inhibition (GI %) was determined as described by Capasso et al. [29] with some modifications following the formula: GI (%) = 100 – (100 × ODs/ODc), where GI (%) is growth inhibition percent, ODs is the sample's OD, ODc is the control's OD.

2.6.3. Fish larvae toxicity test

In a glass Petri dish with a 90 mm diameter and 40 mm depth, 10 fish larvae were exposed for 24 h to 20 mL of untreated and treated (anaerobically and aerobically) wastewater (Fig. S3). Food was not provided and distilled water was used as a control, and the test was conducted at a temperature of 25 °C. Dead larvae were collected at intervals of 6 h, and the number of dead individuals was recorded to calculate the mortality percentage [30].

2.7. Statistical analysis

The result of each experiment was presented as mean standard error, with each experiment being performed in triplicate. Analysis of variance (ANOVA) with the Tukey post hoc test was used to assess the statistical significance of differences between mean values. The difference was considered significant if $p \leq 0.05$.

3. Results and discussion

3.1. Bacterial diversity analysis

High-throughput sequencing was employed to assess diversity of the microbial consortia in anaerobic and aerobic reactors. All samples have coverage more than 0.986 and a total of 3226 counts of OTUs at the 97% similarity cutoff value. The initial sample had the largest OTU number (2179) when compared to that of the aerobic (n = 2117) and anaerobic (n = 2062) culturing conditions (Table 1), indicating that the initial bacterial community from the sediment sample was impacted by nature of the wastewater as well as the acclimatization conditions. Chao1 index of microbial structural richness showed that anaerobic reactor (n = 2772) harbor relatively higher microbial numbers than aerobic reactor (n = 2713) and initial (n = 2698) sample.

According to the Shannon index values, the diversity of initial sample (6.5) > aerobic (5.9) > anaerobic (5.4) treatments, which suggests that higher diversities are maintained after treatment. Indeed, textile wastewater is known with higher (50–80 °C) temperature, higher (7–11) pH and higher salinity (15–20%) natures, which is in line with the Shala hot spring from where thermo-alkaliphilic (pH = 7–11, temperature = 50–80 °C) samples were collected and used as inoculum source. So far, many works were done using neutrophilic microorganisms [31,32], which is challenged by higher alkaline, temperature and saline textile wastewater conditions. However, the rationale behind using higher alkalinity, temperature and saline conditions in the current study is that microorganisms which grew under thermo-alkaliphilic environment can easily adapt and perform well in the extreme conditions of actual textile wastewater. This could be the reason why Shala Hot Spring microorganisms were more diverse in their adaptation to the dye-contaminated environment when grown under aerobic conditions than anaerobic ones. The acclimatization and treatment settings resulted in the reduction of several microbial species in the initial sample; notably, compared to aerobic settings, anaerobic conditions significantly reduced the number of bacteria in the initial sample. This outcome is in line with earlier Shannon diversity index reports

Table 1
Microbial diversity index.

Sample	Tag number	OTU number	Index at 97%			Coverage
			Shannon	Simpson	Chao1	
Initial	54,021	2179	6.5	0.014708	2698	0.988912
An-RR239	51,064	2062	5.4	0.024538	2772	0.986958
Ar-RR239	53,648	2117	5.9	0.014848	2713	0.988313

An = Anaerobic, Ar = Aerobic, RR=Reactive Red.

investigated in our previous work on biodegradation of RR 141 [4]. A wider variety of metabolic activities are also reported in another study [33] due to the coexistence of a more diversified microbial population in the treatments, which may be the basis for effective wastewater treatment.

Microbial community structure in phylum and class levels were displayed in Fig. 1 for aerobic, anaerobic, and untreated samples. Seventeen phyla of bacterial covered 87.4%, while others (45 phyla) accounted only 12.6% (Fig. 1a) of all the sequences. Particularly, 10 bacterial phyla dominated (84.4%) the anaerobic reactor, while 12 (91.8%) and 11 (93.0%) phyla dominated the aerobic and initial samples, respectively. The first, second, and third most dominant phylum were found to be Bacteroidota (22.6–29.0%), Proteobacteria (recently referred to as Zetaproteobacteria) (13.5–29.0%), and Chloroflexi (8.8–23.5%), which is also in line with other previous studies [34–40]. Proteobacteria, which is responsible for removing various contaminants (ammonia, carbohydrate and sugar containing compounds) and biofilm formation in wastewater [34], was found to be the most dominant (29.2%) for the initial (untreated) sample, while Bacteroidota (29.0%) and Chloroflexi (23.5%) phyla, both of which involved in degradation of carbohydrates from biological wastewater, were dominating the anaerobic and aerobic treatments, respectively (Fig. 1a). This shows that the various treatment options influenced how the microbial communities are organized [4].

Cao et al. [41] mentioned that Proteobacteria, Bacteroidetes and Chloroflexi as the three most prevalent phyla of bacteria in their study employing the azo dye X-3B as part of co-substrate. Similarly, the 16S rRNA gene sequence results by Tizazu et al. [4] showed that Bacteroidetes (25.3%), Proteobacteria (21.0%), and Chloroflexi (18.5%) were dominant phyla in charge of biodegrading RR 141. Patel et al. [40], conducted a research at wetland to treat wastewater from textile dyeing process and mentioned that Bacteroidota (31.1%), Proteobacteria (9.6%), and Chloroflexi (7.7%) as the dominant bacterial phyla. According to Zhao et al. [35], Proteobacteria,

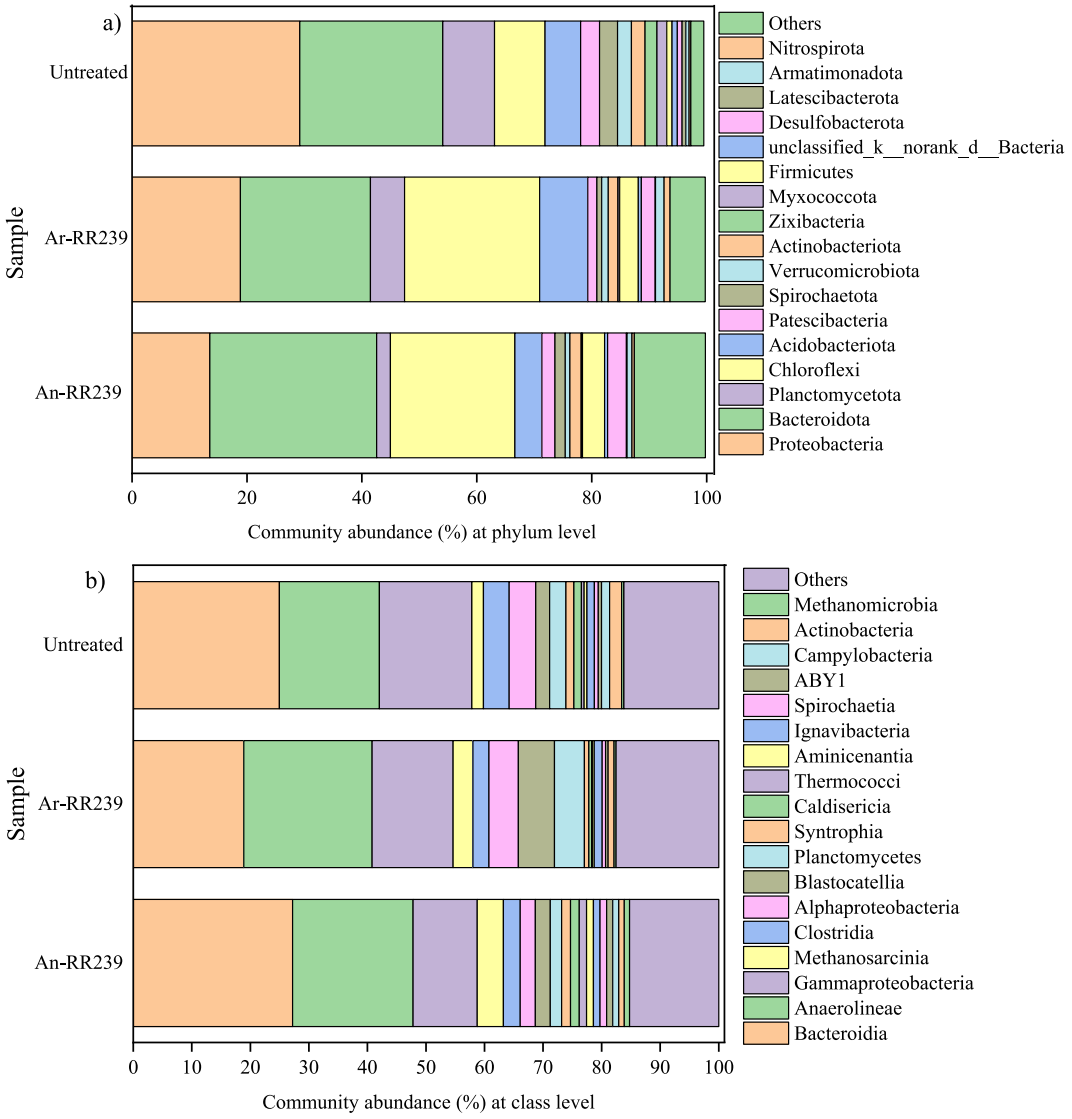

Fig. 1. Microbial community structure (a) phylum level, and (b) class level.

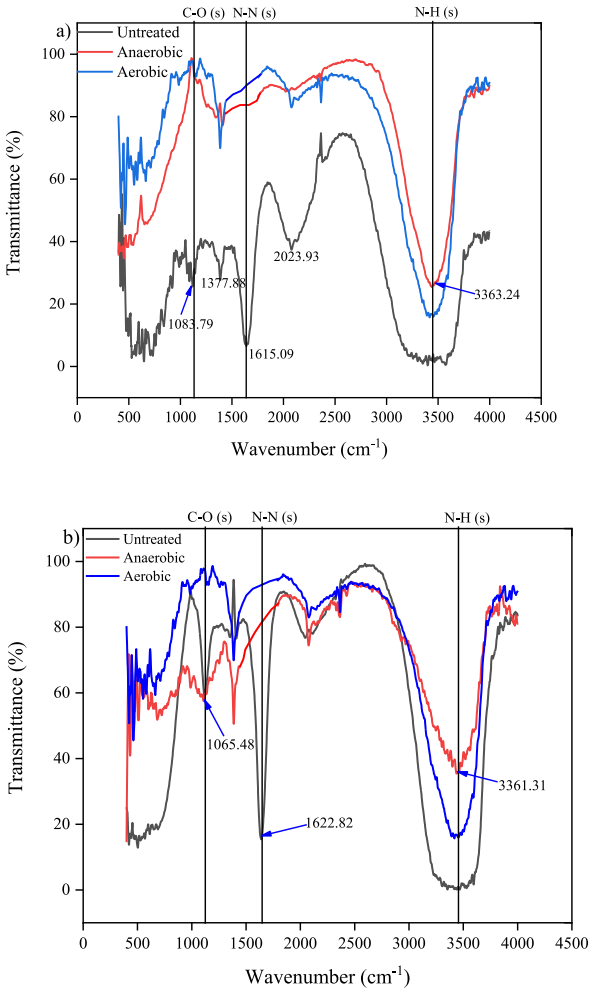

Fig. 2. The FT-IR analysis of untreated and treated samples (a) RR 141, and (b) RR 239.

Chloroflexi, and Bacteroidota have abundance ranges of 12.3–58.5, 2.8–37.7, and 0.7–19.2%, respectively, in numerous wastewater treatment facilities. Furthermore, bacterial phyla of Chloroflexi, Proteobacteria, and Bacteroidota were found to be the three most dominant in wastewater with pyridine treated in anaerobic, aerobic, and microaerobic environments [42]. According to Hu et al. [36], Proteobacteria was discovered to be the dominating phylum in some samples, while in other samples from various wastewater treatment plants, Bacteroidetes was the dominant phylum. Similarly, Sponza et al. [43], noted that the first and third most prevalent phyla in dye wastewater of anaerobic sequencing batch reactors were Bacteroidetes (36.3%) and Proteobacteria (19.6%), respectively.

At class level microbial community structure determination, Bacteroidia was discovered to be the most prevalent (18.9–27.2%) in all the treatments, while at family level, the Bacteroidetes vadinHA17 family (9.5–17.3%) was the most prevalent (Fig. S3). Anaerobic reactors (17.3%) have a higher prevalence of this family of bacteria than aerobic reactors (9.4%). Bacteroidetes was identified as a significant bacterial phylum in the biodegradation of raw textile influent under microaerophilic condition by the cooperative action of bacteria [44]. A prior description of the Bacteroidetes vadinHA17 identified it as an anaerobic protein and complex sugar polymer degrader [37,39,40]. Additionally, in the developed microbial fuel cell system for the treatment of dyestuff effluent, Bacteroidetes were identified as one of the predominant bacterial groups [45]. Furthermore, Bacteroidetes was also discovered to be a prominent bacterial phylum in the bacterial community structure, according to Sun et al. [46]. Mei et al. [39] found that populations of the bacterium Bacteroidetes vadinHA17 were the main degraders of proteolytic amino acids in anaerobic digesters. Zhao et al. [35] claimed that the degradation of proteinaceous substances is associated with the function of the Bacteroidetes vadinHA17 family in wastewater treatment. In this work, it is hypothesized that Bacteroidota significantly contribute to the COD and nitrogen removal processes from the effluent. According to Patel et al. [40], Bacteroidota were essential to the bioremediation of wastewater containing dyes. In our previous study [4], Bacteroidetes vadinHA17 also has been found the dominant bacteria suggested to degrade RR 141 dye containing wastewater.

Proteobacteria (17.0–25.0%) was the second-most prevalent phylum in both anaerobic and aerobic treatments. The two dominant classes in this phylum were Gammaproteobacteria (11.0–15.8%) and Alphaproteobacteria (2.5–5.0%) (Fig. 1b). Proteobacteria was

identified as a significant bacterial phylum in the raw textile effluent biodegradation by the cooperative action of bacteria [44]. Other previous research works also discovered Proteobacteria as the dominant bacterial phylum for the treatment of dyestuff effluent [45, 46]. Similarly, Proteobacteria were found to be the third most common phylum in dye wastewater from anaerobic sequencing batch reactors, according to Zhang et al. [25]. In another study by Cao et al. [41], Proteobacteria had relative abundances of 35.3% and 46.8% in both the biofilm electrode reactor and microbial fuel cells units, respectively. Because, they eliminate excess ammonia, some Proteobacterial species are known as ammonia-oxidizing Proteobacteria and are essential for the treatment of wastewater. As a result, this particular bacterial group in this research may also be in charge of nitrogen removal task [33]. Xin et al. [34], also mentioned that Proteobacteria as crucial for flocculation and biodegradation in biological wastewater treatments. Guadie et al. [42] also stated that ammonia increment in pyridine containing wastewater reactor were suggested to be due to Gammaproteobacteria. Similar finding was reported in our previous work on RR 141 [4].

With proportions of 21.7%, 23.5%, and 8.8% for anaerobic, aerobic, and untreated samples, respectively, Chloroflexi was the major phylum ranking third (Fig. 1a). Under Phylum Chloroflexi, Anaerolineae was determined to be the dominating class (Fig. 2b). In anaerobic, aerobic, and untreated samples, the proportion of Anaerolineae was 20.5, 21.9, and 17.0%, respectively. When all samples were taken into account, Anaerolineaceae was the second-most dominant family (Fig. S4) and when only phylum Chloroflexi was taken into account, it was the most dominant family (9.4–15.4%). Chloroflexi was also shown to be one of the dominating bacterial groups in the microbial fuel cell system for the treatment of dyestuff effluent [45]. According to Cao et al. [41], the third most prevalent bacterial phyla in their work using the azo dye X-3B as part of co-substrate was Chloroflexi. As mentioned by Hu et al. [36], the environmental role of Chloroflexi on the breakdown of biological components and carbohydrates is significant. The ability of Anaerolineaceae to remove nitrogen from wastewaters has been documented [38]. The third prominent family in this study, Caldilineaceae, which also belongs to the phylum Chloroflexi, was shown to be thermophilic and capable of flourishing in both aerobic and anaerobic conditions (Fig. S4). They can all grow on various forms of protein and carbohydrate containing materials since they are all chemoheterotrophs. They play a role in wastewater treatment systems to reduce COD and get rid of ammonia and other

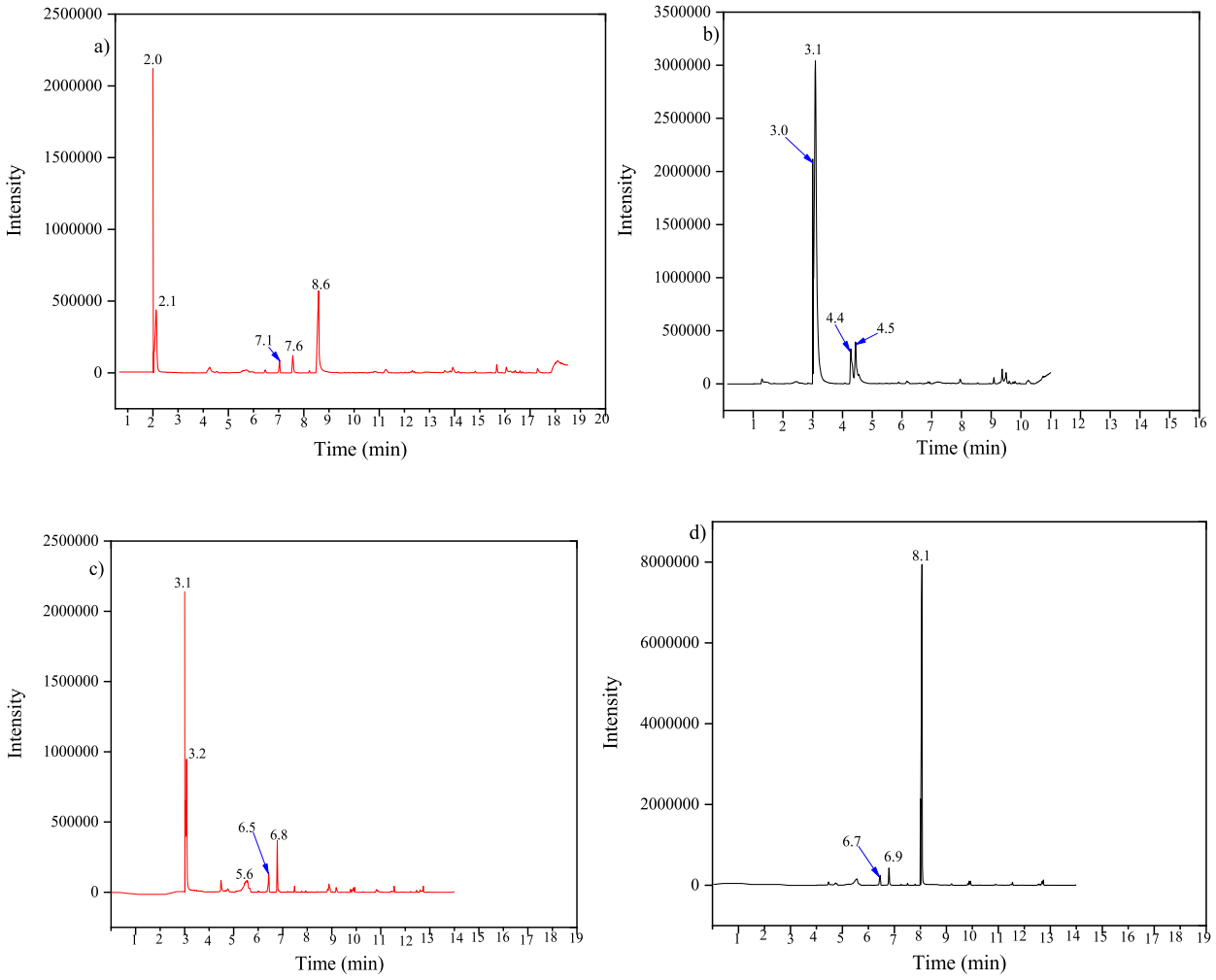

Fig. 3. The HPLC analysis of RR 141 (a and b), and RR 239, (c and d).

nitrogen-containing compounds [4,42]. Our culture condition (optimum at 55 °C) demonstrates that in thermophilic settings, such as hot springs, the Caldilineaceae family was identified. Caldilineaceae was identified by Ajibade et al. [33] as the primary (0.7–8.6%) family in secondary wastewater effluent.

3.2. Dye transformation analysis

The functional groups in the dyes (RR 141 and RR 239) and their degradation products were assessed using FT-IR. The FT-IR spectrum of RR 141 and RR 239 showed that the presence of peaks at 1065.48, 1083.79, 1377.88, 1615.09, 1622.82, 2023.93, 3361.31 and 3363.24 (Fig. 2). The peak at 1065.48 and 1083.79 indicates C—O stretching, and 1615.09 and 1622.82 indicates N—N stretching of RR 141 and RR 239, respectively. The reduction of the azo bond was confirmed by the presence of the characteristic azo group (—N═N—) at 1615.09 (RR 141) and 1622.82 cm^{-1} (RR 239) before treatment, which disappeared after the dyes were degraded by the bacterial consortia. The N—H peak detected in the decolorized (anaerobically treated) product at 3361.31 (RR 239) and 3363.24 cm^{-1} (RR 141) confirmed that the dye was degraded into compounds with —NH and —NH_2 groups. Similar findings from earlier studies indicated that bacterial consortiums degraded textile dyes into compounds containing —NH and —NH_2 groups [13,14]. When compared to the dye spectra before treatment, the FT-IR spectrum of the biodegraded products revealed a considerable shift in the position of peaks and the disappearance of some peaks. Chaieb et al. [47] reported that the peak positions in the untreated dye spectra were significantly shifted from the FT-IR spectrum of the biodegraded products.

High performance liquid chromatography analysis results of raw dyes (RR 141 and RR 239) and their biodegradation product metabolites by the bacterial consortia were shown in Fig. 3. The parent dye compounds were discovered with a high intensity retention time at 2.0 min for RR 141 and 3.1 min for RR 239. They were also identified with a low intensity retention time of 7.1, 7.6 and 8.6 min for RR 141 and 5.6, 6.5 and 6.8 min for RR 239 (Fig. 3a and c). These peaks seen in the untreated dyes vanished after treatment, and new peaks were created from the biodegraded dye metabolites. At the retention times of 3.0, 3.1, 4.4 and 4.5 min, the four new peaks were seen for RR 141 after aerobic biodegradation (Fig. 3b). Similarly, three new peaks were seen with retention times of 6.7, 6.9, and 8.1 min for RR 239 as biodegradation end product metabolites (Fig. 3d). The biodegradation of RR 141 and RR 239 has likely been carried out by the halotolerant and thermo-alkaliphilic bacterial consortia, as evidenced by the formation of new peaks in the dyes' biodegradation products and the elimination of the primary peak in the untreated dye. In agreement with these findings, Raj et al. [48] reported that a new peak developed and two peaks vanished during the treatment by the bacterial consortia, indicating either dye degradation or biotransformation. Similar to this, various prior researchers concluded degradation of textile dyes from the emergence of new peaks and/or the elimination of previous peaks [2,49,43].

Although proposing the biodegradation pathway is not a simple task which requires the application of different techniques and technologies, we tried to suggest and proposed RR 141 (Fig. 4) and RR 239 (Fig. S5) biodegradation pathways following FT-IR, HPLC-MS, dye removal information, spectroscopic analysis of contaminants (COD and ammonia), and literature review. Most often, a reductive cleavage of the azo link, which results in the formation of amines, triggers the bacterial degradation of azo dyes. Thus, the overall biodegradation of RR 141 and RR 239 involves a reductive cleavage of the dyes followed by desulfonation reaction. Biodegradation patterns of reductive cleavage corresponding to RR 141 transformation were 2-nitroso Naphthol; *p*-dinitrobenzene;

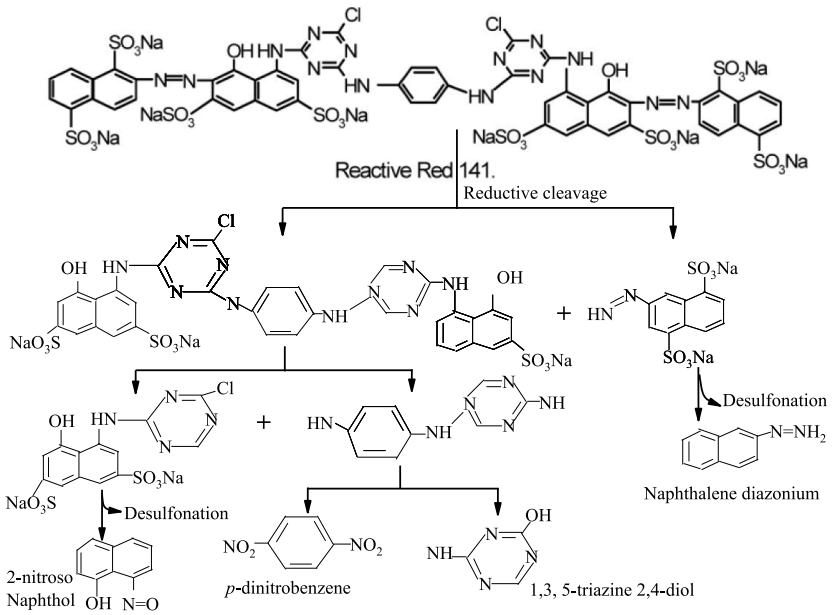

Fig. 4. Proposed biodegradation pathway of RR 141.

1,3, 5-triazine 2,4-diol; and Naphthalene diazonium (Fig. 4), while for RR 239 it consists of Aniline; 5-Amino-1-naphthol; and 1,3,5-triazine 2,4-diol (Fig. S5). Telke et al. [50] reported naphthalene diazonium, 1, 3, 5-triazine 2, 4-diol, and p-dinitrobenzene as degradation products of RR 141, while Aniline and 1,3,5-triazine were reported by Dias et al. [51] as degradation products of RR 239. Similarly, Sompark et al. [52] performed GC-Ms analysis to propose RR 141 transformation pathways and reported four fragmentation patterns as 3-diazenylnaphthalene-1,5-disulfonate, sodium naphthalene-2-sufonate, 4-chloro-1,3,5-triazin-2-amine and N1-(1,3, 5-triazin-2-yl) benzene-1, 4-diamine. Furthermore, Carliell et al. [53] proposed the transformation of RR 141 into 1, 3, 5-triazine, 2, 4, 6-trioxo, *p*-diaminobenzene, 2-aminonaphthalene-1,5-disulfonic acid and 1,7-diamino-8-naphthol-3,6-disulfonic acid. The degradation products reported in this research were also mentioned in various previous researches as degradation products of textile dyes [16, 54]. Aniline formed as a result of the biodegradation of Direct Black G textile dye by a newly discovered thermophilic bacteria was identified by Chen et al. [16]. Srinivasan & Sadasivam [54] reported 1,3,5-triazine-2,4,6-triol and 1,3,5-triazine-2,4 (1H,3H)-dione as degradation products (metabolites) of Drimaren Red CL-5B by A. *hydrophila* MTCC 1739 and *L. sphaericus* MTCC 9523, respectively.

Although not included in this study, we analyzed COD and ammonia concentration [4] after RR 141 dye biodegradation using microbial consortia and results have been found increasing compared with the initial sample suggesting that the parent dye (i.e, RR 141) was further degraded and mineralized.

3.3. Toxicity assessments

3.3.1. Phytotoxicity tests

To evaluate the toxicity of the untreated dye wastewaters (RR 141 and RR 239) and their biodegradation products, phytotoxicity test experiments were carried out using seeds of three plant species including tomato, beetroot, and cabbage were sterilized with 1% sodium hypochlorite solution for 15 min. Germination of 36.7 ± 5.7, 53.3 ± 6, 84.7 ± 7 and 85.3 ± 7% for Beetroot; 40.0 ± 5.0, 55.0 ± 6.1, 84.3 ± 7.0 and 90.0 ± 5.0% for Cabbage; and 33.3 ± 4.0, 40.0 ± 5.0, 84.0 ± 7.1 and 86.0 ± 5.0% for Tomato were recorded with untreated, anaerobically treated, aerobically treated and control, respectively (Table 2). The fact that all of the seeds were labeled as 85.0% germination accounts for the lower percentage of germination even with distilled water. In germination experiments, distilled water (control) and aerobically treated wastewater did not differ in a statistically meaningful way ($p > 0.05$) for all the three plant species (Table 2 and Table S2). This result suggests that the aerobically treated wastewater is equivalent to that of distilled water in germinating seeds of the tested vegetables. Compared to the control and the degradation products, RR 141 and RR 239 inhibited the three vegetable seed germination (Table 2 and Table S2). According to Priac et al. [55], as the content of Co1S1 raw discharge waters increased, the percentage of lettuce germination reduced. In the previous study [16], less rice and black bean seeds germinated after being treated with Direct Black G than after being treated with distilled water and its decolorization metabolites. According to Guo et al. [14], the seeds were also germinated 100% with distilled water and it was reduced with the wastewater. As shown in Table 2, Cabbage is resistant to the toxic effect of the wastewaters than that of Beetroot and Tomato. Similarly, Cabbage plants showed greater tolerance to copper toxicity than Beetroot plants, according to Schmitt et al. [56].

Shoot length were 2.74 ± 0.05, 4.23 ± 0.18, 4.64 ± 0.05 and 5.70 ± 0.04 cm for Beetroot; 0.80 ± 0.10, 1.30 ± 0.20, 1.90 ± 0.10 and 2.70 ± 0.10 cm for Cabbage; and 1.50 ± 0.20, 2.20 ± 0.10, 2.60 ± 0.10 and 3.20 ± 0.10 cm for tomato irrigated with untreated, anaerobically treated, aerobically treated and the control, respectively (Table 2). The untreated wastewater significantly inhibited shoot length of all the three vegetable plants. In similar manner, Chen et al. [16] studied the rice and black bean seeds treated with Direct Black G resulted in reduced shoot lengths (1.55 and 0 cm). According to Guo et al. [14], the plumule and radicle of the germinated seeds demonstrated that the toxicity was reduced with the treatment process, but the metabolites' toxicity was still greater than that of distilled water, which was anticipated to be explained by the remaining intermediates. According to Guo et al. [9], in the presence of 100 mg/L Metanil Yellow G, *O. sativa* and *C. sativus* seeds exhibited 63% and 50% germination rates, respectively. According to Guo et al. [9], *C. sativus* and *O. sativa* had longer plumule lengths (4.7 and 3.6 cm) and radicle lengths (3.8 and 2.6 cm), respectively after being exposed to the degradation products, suggesting that the dye could be detoxified by bacterial consortia into products with low toxicity.

Root length of 1.3 ± 0.10, 2.0 ± 0.10, 2.2 ± 0.10 and 2.9 ± 0.10 cm for Beetroot; 0.5 ± 0.10, 1.1 ± 0.10, 1.3 ± 0.10 and 1.5 ± 0.10 cm for Cabbage; and 1.1 ± 0.10, 1.7 ± 0.10, 1.6 ± 0.10 and 1.7 ± 0.10 cm for Tomato irrigated with untreated (RR141), anaerobically treated, aerobically treated and the control, respectively (Table 2). Similarly, compared to distilled water and the

Table 2
Phytotoxicity studies of Reactive Red 141 and its biodegradation products.

Plant species	Parameter	T-Un	T-An	T-Ar	Control
Beetroot (*Beta vulgaris*)	Germination (%)	36.7 ± 5.7^{a}	53.3 ± 6.0^{b}	85.3 ± 7.0^{c}	85.3 ± 6.0^{c}
	S. Length (cm)	2.7 ± 0.1^{a}	4.2 ± 0.2^{b}	4.6 ± 0.1^{b}	5.7 ± 0.1^{c}
	R. Length (cm)	1.3 ± 0.1^{a}	2.0 ± 0.1^{b}	2.2 ± 0.1^{b}	2.9 ± 0.1^{c}
Cabbage (*Brassica oleracea*)	Germination (%)	40.0 ± 5.0^{a}	55.3 ± 6.1^{b}	84.2 ± 7^{c}	90.0 ± 7.0^{c}
	S. Length (cm)	0.8 ± 0.1^{a}	1.3 ± 0.2^{b}	1.9 ± 0.1^{c}	2.7 ± 0.1^{c}
	R. Length (cm)	0.5 ± 0.1^{a}	1.1 ± 0.1^{b}	1.3 ± 0.1^{c}	1.5 ± 0.1^{c}
Tomato (*S. lycopersicum*)	Germination (%)	33.3 ± 4.0^{a}	40.0 ± 5.0^{b}	84.0 ± 7.1^{c}	86.0 ± 5.0^{c}
	S. Length (cm)	1.5 ± 0.2^{a}	2.2 ± 0.1^{b}	2.6 ± 0.1^{c}	3.2 ± 0.1^{c}
	R. Length (cm)	1.1 ± 0.1^{a}	1.6 ± 0.1^{b}	1.7 ± 0.1^{c}	1.8 ± 0.1^{c}

T-Un = Untreated wastewater, T-An = Anaerobically treated wastewater, T-Ar = Aerobically treated wastewater, R=Root, S=Shoot.

degradation products, RR 141 and RR 239 significantly inhibited the plumule (shoot) and radicle (root) length of Tomato, Beetroot and Cabbage (Table 2 and Table S2). Chen et al. [16] also reported that the rice and black bean seeds watered with Direct Black G resulted in shorter shoot (1.55 and 0 cm) and root (0 and 2.6 cm) lengths, respectively. Guo et al. [14] also reported the plumule and radicle length of the germination-proven seeds (*Cucumis sativus* and *Oryza sativa*) demonstrated that the decolorization process reduced the toxicity. In general, the least inhibition in germination, root and shoot length of all the test plant species was recorded in aerobically treated wastewater, confirming that the aerobically treated wastewater can be safely used for irrigation. For all three plant species, the difference was not statistically significant ($p > 0.05$) in shoot and root length between distilled water (control) and water that had undergone aerobic treatment. According to this finding, the aerobically treated wastewater is effective in enhancing the shoot and root growth (length) of plants.

To better understand the safety of using the treated wastewater for irrigation, this study examined treated and untreated wastewater effects on the percent of germination, plumule length (cm), and radicle length (cm) of vegetable plants (Table 2 and Table S2) and compared it to other previous research results (Table 3). The treated wastewater in this study resulted in 70–85% germination for the three plant species tested. Compared to Chen et al. [16]; Guo et al. [13]; Guo et al. [14]; and Guo et al. [9] this result was found to be poor, but this results from the seeds used for the study which were labeled as 85% germination. Thus, in germination experiment of this research, 85% germination is equivalent to 100% germination in other previous studies. Therefore, the present study result was equivalent to the above mentioned previous studies in germinating vegetable plants. Plumule and radicle length inhibition (%) of this research was 3–12% and 2–10%, respectively. This result was found to be much better than similar previous research results that were found to be 3–25% and 3–24% for plumule and radicle length, respectively [9,13,14,16].

3.3.2. Microbial toxicity assessment

In 10 h incubation period, the growth inhibition percent (GI%) of 82 ± 4, 15 ± 1.3 and 10 ± 1.1 for *Escherichia coli*; GI% of 87 ± 2, 21 ± 1.5 and 14 ± 1.6 for *Lactobacillus plantarum;* and GI% of 92 ± 1, 27 ± 1.5 and 21 ± 1 for *Leuconostoc mesenteroides* were recorded with untreated (RR141), anaerobically treated and aerobically treated wastewaters, respectively (Table 4). Growth inhibition percent of untreated wastewater (RR 141 and RR 239) is greater than that of the anaerobically treated wastewater and the anaerobic in turn is greater than that of the aerobically treated wastewater (Table 4). The least growth inhibition percent was recorded in aerobically treated wastewater. Growth inhibition of zero percent was recorded for control (ATCC 697 broth without dye) in all measurement periods. The toxicity tests on *Pseudomonas luteola*, according to Sponza & Isik [43], demonstrated that intermediate metabolites of Reactive Black 5 are hazardous, and a prolonged persistence of intermediary metabolites in anaerobic environments may further increase toxicity to bacterial populations. In comparison to *E. coli* the growth inhibition percent of *L. plantarum* and *L. mesenteroides* were large (Table 4). This is due to the fact that the *E. coli* is gram negative bacteria, while the latter two are gram positive. Due to the presence of the outer membrane, gram-negative bacteria are typically more resistant to toxicity than gram-positive bacteria [29]. For instance, *E. coli* builds up large amounts of three membrane phospholipids [59]. On the other hand, there are several other possible factors contributing for the toxicity resistance difference among microorganisms. The presence of innate or acquired toxicity resistant elements is one of these potential causes of the difference in toxicity resistance among bacteria and additionally, if the species was isolated from a multispecies consortia, horizontal gene transfers and modifications to an organism's metabolic profile may have significant effects on that organism's susceptibility to a toxic substance [60].

3.3.3. Toxicity test on fish larvae

Mortality percent of 65 ± 4, 41 ± 2 and 16 ± 1 for *Oreochromis niloticu*; 49 ± 3, 23 ± 2 and 7 ± 1 for *Clarias gariepinus*; and 61 ± 5, 36 ± 2 and 13 ± 1 for *Cyprinus carpio* were recorded with untreated, anaerobically treated and aerobically treated wastewater of RR 141, respectively (Fig. 5a). Similarly, mortality percent of 61 ± 4, 34 ± 3 and 13 ± 1 for *Oreochromis niloticu*; 47 ± 3, 14 ± 1 and 6 ± 1

Table 3
Comparison of phytotoxicity test results of this research to previous researches.

Plant species	Contaminated dye/WW type	Treatment type	Ger (%)	Len-inhib (%)		Reference
				Plumule	Radicle	
Beta vulgaris, Brassica oleracea & S. lycopersicum	RRs 141 & 239	An-Aer	70–85	3–12	2–10	This study
Cucumis sativus & Oryza sativa	MYG	Anaerobic	53–100	11–33	11–24	[13]
Phaseolus vulgaris & Oryza sativa	DBG	Microaerobic	100	12–14	9–13	[16]
Cucumis sativus & Oryza sativa	AYG	Anaerobic	48–100	19–25	13–22	[14]
Cucumis sativus & Oryza sativa	MYG	Anaerobic	96–100	3–15	3–11	[9]
Sorghum vulgare & Phaseolus mungo	Red BLI	Anoxic	60–90	21–80	11–79	[57]
Vigna radiate	Tex-Eff	An-Aer	48–85	–	–	[58]
Sorghum vulgare & Phaseolus mungo	RB 172	Microaerobic	30–90	2–54	5–53	[49]
Triticum aestivum & Phaseolus mungo	RO 16	Anoxic	0–80	52–100	35–100	[19]
Lactuca sativa	IWW	Aerobic	94–99	–	8–19	[55]
Vigna radiata	RR 141	Anaerobic	63–75	18–22	4–6	[52]

– = No information, WW=Wastewater, Ger = Germination, Len-inhib = Length inhibition, RR=Reactive Red, An-Aer = Anaerobic-Aerobic, MYG = Metanil Yellow G, DBG = Direct Black G, AYG = Acid Yellow Gold, Tex-Eff = Textile effluent, RB=Reactive Blue, RO=Reactive Orange, IWW=Industrial wastewater.

Table 4
Microbial toxicity test on RR 141 and RR 239.

Microbial species	Time (h)	Growth inhibition percent (GI (%))					
		Untreated		Anaerobic		Aerobic	
		RR 141	RR 239	RR 141	RR 239	RR 141	RR 239
Escherichia coli	2	70 ± 2.0^{a}	67 ± 2.0^{a}	68 ± 2.0^{a}	66 ± 2.0^{a}	58 ± 2.8^{c}	56 ± 2.6^{c}
	4	75 ± 3.0^{a}	71 ± 2.1^{a}	47 ± 2.0^{b}	45 ± 1.9^{b}	36 ± 2.0^{c}	35 ± 2.3^{c}
	6	77 ± 3.0^{a}	73 ± 2.3^{a}	36 ± 1.8^{a}	33 ± 1.5^{a}	27 ± 1.8^{b}	24 ± 2.0^{b}
	8	79 ± 4.0^{a}	76 ± 2.6^{a}	26 ± 1.5^{b}	24 ± 1.2^{b}	19 ± 1.9^{c}	16 ± 1.8^{c}
	10	82 ± 4.0^{a}	80 ± 3.0^{a}	15 ± 1.3^{b}	13 ± 1.0^{b}	10 ± 1.6^{c}	8 ± 1.4^{c}
Lactobacillus plantarum	2	75 ± 2.9^{a}	73 ± 2.8^{a}	69 ± 3.1^{b}	67 ± 3.0^{b}	63 ± 3.1^{c}	62 ± 3.0^{c}
	4	79 ± 3.1^{a}	77 ± 3.0^{a}	53 ± 3.0^{a}	51 ± 2.9^{a}	42 ± 2.9^{c}	40 ± 2.7^{c}
	6	81 ± 3.5^{a}	80 ± 3.3^{a}	41 ± 2.8^{a}	39 ± 2.7^{a}	31 ± 2.7^{c}	30 ± 2.5^{c}
	8	83 ± 3.7^{a}	82 ± 3.5^{a}	32 ± 2.1^{b}	29 ± 2.0^{b}	22 ± 2.2^{c}	19 ± 2.0^{c}
	10	87 ± 3.9^{a}	85 ± 4.0^{a}	21 ± 1.7^{b}	18 ± 1.5^{b}	14 ± 1.7^{c}	13 ± 1.6^{c}
Leuconostoc mesenteroides	2	81 ± 1.2^{a}	79 ± 1.0^{a}	75 ± 2.6^{b}	73 ± 3.0^{b}	69 ± 3.1^{b}	67 ± 3.0^{b}
	4	85 ± 2.3^{a}	83 ± 2.0^{a}	58 ± 2.2^{b}	56 ± 2.5^{b}	48 ± 2.6^{c}	47 ± 2.4^{c}
	6	87 ± 2.5^{a}	85 ± 2.0^{a}	47 ± 2.0^{b}	45 ± 2.0^{b}	38 ± 2.1^{c}	35 ± 2.0^{c}
	8	89 ± 2.9^{a}	86 ± 2.7^{a}	38 ± 1.8^{b}	36 ± 2.0^{b}	30 ± 1.9^{c}	27 ± 1.5^{c}
	10	92 ± 3.2^{a}	89 ± 3.0^{a}	27 ± 1.6^{b}	26 ± 1.5^{b}	21 ± 1.2^{b}	20 ± 1.0^{b}

for *Clarias gariepinus*; and 58 ± 4, 28 ± 2 and 7 ± 1 for *Cyprinus carpio* were recorded with untreated, anaerobically treated and aerobically treated wastewater of RR 239, respectively (Fig. 5b). Mortality percent was the highest with the untreated and anaerobically treated wastewater and least with the aerobically treated and distilled water for all the fish species (Fig. 5). For any of the three fish species, the difference was not statistically significant ($p > 0.05$) in mortality (%) between untreated and anaerobically treated water (Fig. 5), indicating that anaerobically treated wastewater is still toxic to animals due to the formation of aromatic amines, which are known to be harmful substances. No fish species was died with distilled water and thus, mortality percent of zero was recorded with distilled water for all the fish species. Similar to the current study, *Daphnia magna* toxicity studies performed by Sponza & Isik [43] revealed that samples from aerobic effluents did not demonstrate any inhibition, and the metabolites produced by the anaerobic breakdown of azo dyes were hazardous to the test organisms, and they were concluded that after aerobic treatment, toxicity was removed. Liu et al. [30] also mentioned that untreated wastewater in their work is more toxic to the test organisms than treated effluent. Kassaye [61] also reported absence of mortality in control and 10% (v/v) outlet effluent concentration and 100% (v/v) inlet effluent concentration. The highest percentage mortality was noted at 40% (v/v) inlet effluent concentration and 100% (v/v) outlet effluent concentration on Nile tilapia (*Oreochromis niloticus*). In another previous study by Ferraz et al. [8], Salmonella fish are mutagenic to azo dyes Disperse Red 1 and Disperse Red 13. In general, the present study on both of the dyes, least mortality percents were recorded with *Clarias gariepinus* and this fish species was found to be more resistant to toxicity than that of *Oreochromis niloticus* and *Cyprinus carpio*. Similarly, other previous research by Ezeonyejiaku et al. [62] mentioned that Catfish (*Clarias gariepinus*) was resistant to toxicity than that of tilapia. Furthermore, according to Kassaye [61], *Oreochromis niloticus* is less resistant to the toxicity of textile effluent than *Clarias gariepinus* which is consistent with the current study.

4. Conclusion

Bacteroidota, Proteobacteria, and Chloroflexi were the three most abundant phyla responsible for biodegrading RR 239. In the FT-IR analysis results the characteristic azo groups (—N=N—) were present at 1615.09 (RR141) and 1622.82 cm^{-1} (RR239), which were disappeared after the degradation of the dyes by the bacterial consortia suggesting that the azo bond was reduced. The N—H peak detected in the decolorized product at 3361.31 (RR239) and 3363.24 cm^{-1} (RR141) confirmed that the dye was degraded into compounds with —NH and $—NH_2$ groups, which are believed to be aromatic amines. Also from HPLC analysis result, the formation of new peaks in the dyes' biodegradation products and the disappearance of the primary peak in the untreated dyes are an indication that the halotolerant and thermo-alkaliphilic bacterial consortia likely carried out the biodegradation of RR 141 and RR 239. Compared to distilled water and the degradation products, RR 141 and RR 239 inhibited the seed germination of Tomato, Cabbage and Beetroot. GI % on *E. coli*, *L. plantarum*, and *L. mesenteroides* were significantly reduced after treatment of the wastewater. The results of the studies on RR 141's and RR 239's toxicity revealed that they became less harmful after biodegradation, and the wastewaters that were treated by the halotolerant and alkali-thermophilic bacterial consortia could be used safely for irrigation. Mortality percent was the highest with the untreated wastewater and least with the distilled water (the control) for all the fishes (*Cyprinus carpio, Oreochromis niloticus*, and *Clarias gariepinus*).

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

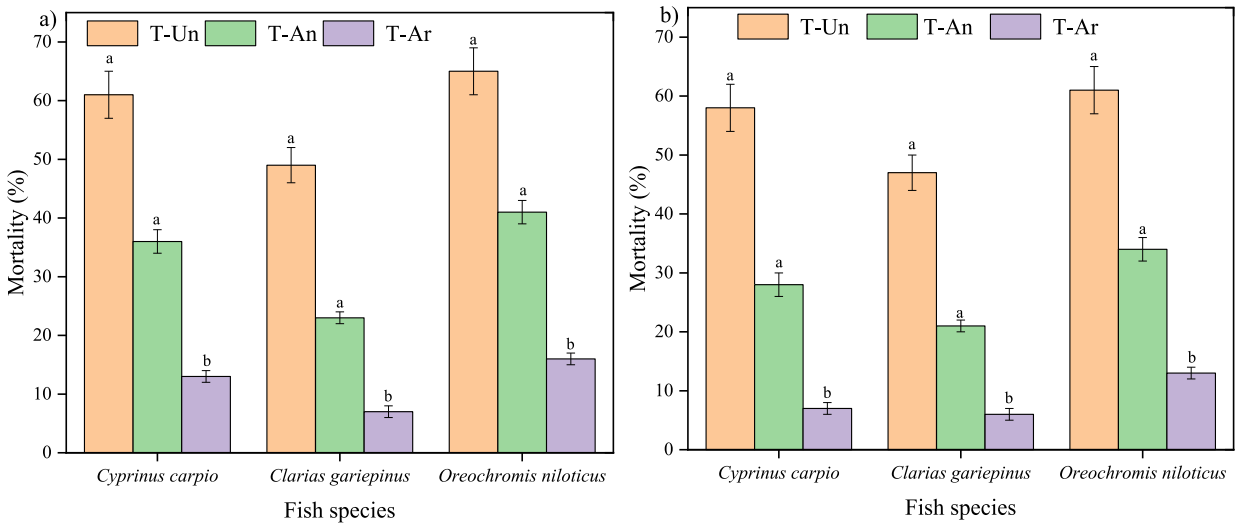

Fig. 5. Toxicity test on fish larvae (a) RR 141, and (b) RR 239.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e16857.

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