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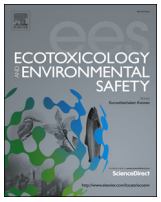

Influence of the herbicide haloxyfop-R-methyl on bacterial diversity in rhizosphere soil of *Spartina alterniflora*

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ABSTRACT

Haloxyfop-R-methyl (haloxyfop) can efficiently control *Spartina alterniflora* in coastal ecosystems, but its effect on soil microbial communities is not known. In the present study, the impact of the haloxyfop on rhizosphere soil bacterial communities of *S. alterniflora* over the dissipation process of the herbicide has been studied in a coastal wetland. The response of the bacterial community in the rhizoplane (iron plaque) of *S. alterniflora* subjected to haloxyfop treatment was also investigated. Results showed that the persistence of haloxyfop in the rhizosphere soil followed an exponential decay with a half-life of 2.6–4.9 days, and almost all of the haloxyfop dissipated on Day 30. The diversity of rhizosphere soil bacteria was decreased at the early stages (Days 1, 3 & 7) and recovered at late stages (Days 15 & 30) of the haloxyfop treatment. Application of haloxyfop treatment increased the relative abundance of the genera *Pseudomonas*, *Acinetobacter*, *Pontibacter*, *Shewanella* and *Aeromonas*. Strains isolated from these genera can degrade herbicides efficiently, which possibly played a role in the degradation of haloxyfop. The rhizoplane bacterial diversity was reduced on Day 15 while being vastly enhanced on Day 30. Soil variables, including the electric conductivity, redox potential, and soil moisture, along with the soil haloxyfop residue, jointly shape the bacterial community in rhizosphere soil.

1. Introduction

Spartina alterniflora Loisel is native to the Atlantic and Gulf coasts of North America, which was introduced into China in the 1980s to trap sediments and encourage saltmarsh accretion (Wang et al., 2008; Li et al., 2009; Riddin et al., 2016). Because of its rapid propagation ability and the relative higher adaptability to salinity compared to the native plants such as *Phragmites australis* and *Scirpus mariqueter*, *S. alterniflora* has become one of the most severe invasive plants in the estuarine and coastal ecosystems of China (Li et al., 2009; Yuan et al., 2011). As an invasive species, *S. alterniflora* has imposed substantial adverse effects on native ecosystems and caused substantial economic costs and social impacts in the invaded regions (Tang et al., 2009). Effective management and control of *S. alterniflora* can provide a suitable habitat for birds and benthos, maintain and improve the biodiversity and ecological service function of estuarine wetland. The ecological control of *S. alterniflora* in China has drawn full attention from biologists and ecologists (Wang et al., 2008; Tang et al., 2009; Yuan et al., 2011; Strong and Ayres, 2016). Both physical (Tang et al., 2009; Yuan et al., 2011; Strong and Ayres, 2016) and chemical (Patten, 2003; Walter et al., 2003; Carrie et al., 2013; Liu et al., 2018) approaches have been proposed to control *S. alterniflora* during the past two decades. Physical methods, include the various combinations of plant removal, mowing, burning, and flooding, have provided acceptable levels of control for *S. alterniflora* (Carrie et al., 2013; Riddin et al., 2016). Yuan et al. (2011) suggested that cutting plus a duration of 3 months waterlogging is efficient to control *S. alterniflora* in the Yangtze River Estuary of China. The chemical controls is mainly to control or eliminate the S. alterniflora by using some chemical herbicides, such as glyphosate (Walter et al., 2003; Carrie et al., 2013; Strong and Ayres, 2016), imazapyr (Patten, 2003; Carrie et al., 2013), Glufosinate and Fusilade Forte (Shimeta et al., 2016), and haloxyfop (Sheng et al., 2014; Wang et al., 2017). Using herbicides has been suggested to be a reliable method to eliminate *S. alterniflora* in natural environments (Carrie et al., 2013).

Soil microflora is crucial components of the pedosphere, and they are responsible for many functions, such as the promotion of plant growth, the cycling of macro-elements and the degradation of pollutants including herbicides (Saha et al., 2012; Wu et al., 2014; Romdhane et al., 2019). Due to the small size and high surface area to volume ratio, soil microorganisms are susceptible to external pollutants and may rapidly alter their diversity and activity (Schloter et al., 2003;

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Imfeld and Vuilleumier, 2012). The diversity and composition of microbial communities in the soil may serve as useful indicators of the impact of external disturbances (including pesticide application) on soil health (Wu et al., 2014). The existing researches on the control of *S. alterniflora* mainly focus on the effectiveness of the control, while the study on the ecological impact of these control methods on the changes of soil bacterial communities is relatively rare. According to previous studies, the herbicides in the soil may inhibit, promote, or have no effect on the diversity and function of soil microbial communities. The specific impact depends on the concentration of herbicide, the time of treatment and the type of soil (Singh et al., 2016; Du et al., 2018; Rose et al., 2018; Romdhane et al., 2019). Some researchers suggest that the herbicides only have minor or transient effects on the soil bacterial community when they are applied at the recommended doses (Imfeld and Vuilleumier, 2012; Bai et al., 2013; Rose et al., 2016, 2018). However, some other studies found the application of herbicide can alter the diversity and structure of the bacterial community. García-Delgado et al. (2018) found that the simultaneous application of two herbicides, triasulfuron, and prosulfocarb, significantly increased the soil dehydrogenase activity, and the abundance of Gram-positive bacteria, Gram-negative bacteria and Actinobacteria were also increased in the soil. Du et al. (2018) revealed that the soil microbial community structure could be affected by mesotrione at all experimental doses, and a high dose of mesotrione could induce negative impacts on soil microbes. Romdhane et al. (2019) found that 8 days of leptospermone exposure caused more significant changes in the diversity and structure of the soil bacterial community than sulcotrione and the diversity of the soil bacterial community was not completely recovered by the end of the experiment (45 days). These inconsistent results have generated great scientific interest in determining the effects of herbicides on soil bacterial communities (García-Delgado et al., 2018).

Haloxyfop-R-methyl (hereafter as haloxyfop) is a foliar-applied aryloxyphenoxy-propionate (AOPP) herbicide for the selective control of annual and perennial grasses in broadleaf crops (Zhou et al., 2018). This herbicide has been widely used in China to control *S. alterniflora* and achieved excellent results (Sheng et al., 2014; Wang et al., 2017). To the best of our knowledge, there is no relevant research has been conducted to investigate the impact of haloxyfop on rhizosphere soil bacterial communities under field conditions. In this study, a field experiment has been done in a coastal wetland to investigate the effect of the haloxyfop application on rhizosphere soil bacterial communities of *S. alterniflora* over the dissipation process of haloxyfop. The response of the bacterial community in the iron (Fe) plaque of *S. alterniflora* subjected to haloxyfop treatment was also investigated, as Fe plaque strongly links soil and plant and plays various functions in the nutrient acquisition and stresses resistance (Hu et al., 2015). The study of the epiphytic bacteria is crucial for a better understand of the plant-microbe interactions under the treatment of haloxyfop. We hypothesize that the treatment of haloxyfop can alter the composition and diversity of the bacterial community that presented in the rhizosphere soil, and the effect may vary with the treatment time. The next-generation sequencing method was applied in the present study, as this metagenomic sequencing technology allows a better representation of the bacterial diversity in soil (De Mandal et al., 2015).

2. Materials and methods

2.1. Experimental setup and samples collection

The field experimental site was conducted in a coastal wetland of Chongming Island in Shanghai, China (120°49′E~31°36′) (Fig. S1). In order to test the effect of different concentrations of haloxyfop on *S. alterniflora* and its effect on rhizosphere soil microbial diversity, we used polyvinylchloride (PVC) tubes to plant seedlings of *S. alterniflora* in the tidal flat and applied different concentrations of haloxyfop. A total of 12 PVC tubes were prepared. Each tube, with a dimension of 30 cm (height) * 25 cm (diameter), was transplanted with 10 seedlings of *S. alterniflora* (about 30 cm in height) in situ with their surrounding soil (Fig. S1). These tubes were parallelly buried in the middle to high levels of tide flat at an interval of 3 m apart from each other. The upper side of the PVC tube was made 3 cm higher than the tidal flat to reduce the straight flush of tidal water on the rhizosphere soil. After 15 days of rejuvenation, the PVC tubes were randomly divided into four groups, each in triplicate. Three groups received different levels (low, medium and high) of haloxyfop treatment. The different levels of haloxyfop treatment solution were prepared by dissolving 2, 5 and 10 ml of haloxyfop (10.8%, Dow AgroScience LLC, USA) in 500 ml distilled water, respectively. For the haloxyfop treatment, different levels of haloxyfop treatment solution were cautiously sprayed on the plant leaves in PVC tubes of the corresponding treatment. One group was sprayed with distilled water (without haloxyfop) and was served as control. The chosen of the treatment concentrations of haloxyfop was based on a pre-experiment, in which we found that haloxyfop at a medium dose (i.e., 5 ml 10.8% haloxyfop in 500 ml distilled water) can cause plant death in a short period of time and exert significant impacts on the soil microbial structure. Previous studies have shown that herbicides are greatly affected by tides in tidal flat environment, and the dissipation rate of herbicides in soil also has a certain relationship with the application concentration of herbicides (Patten, 2003; Romdhane et al., 2019). In the coastal wetlands around Shanghai, haloxyfop is used repeatedly every year to control the regrowth of *S. alterniflora*. In the above context, we are interested to know how the dissipation rate of herbicide itself is different under the treatment of herbicide below or above the medium concentration and how the herbicide will affect the soil microorganism under the condition of different dose of application. The rhizosphere soil of all the treatments were collected from each treatment group before (0 day) and after (1, 3, 7, 15, and 30 days) the application of haloxyfop treatment. Samples of plant roots were also collected on Days 15 & 30 to investigate the influences of haloxyfop treatment on the composition of the bacterial community in rhizoplane. Photos of each treatment were also taken at each sampling time. The collected soil and plant samples were brought back to the laboratory in ice chest and then stored at −80 °C until further analysis.

2.2. Measurement

2.2.1. Soil physiochemical characters

The soil humidity (WET), electrical conductivity (EC), and soil temperature (Temp) were measured in situ at each sampling time by a portable soil moisture/temperature/salinity meter (WE-2, Delta-T, England). The redox potential (Eh) of the soil was measured in situ by a portable redox potentiometer (FJA-6, Nanjing Chuandi Instrument Equipment Co., Ltd.).

2.2.2. Determination of haloxyfop-R-methyl in the soil

The haloxyfop in soils were extracted according to the method of García-Delgado et al. (2019) and Sun et al. (2015) with modifications. In brief, an aliquot of 8 g (fresh weight, FW) soil was weighed into a centrifugation tube. Then, 10 ml of methanol (Chromatographic grade) was added and extracted under ultrasonic for 1 h. Then the centrifugation tubes were shaken on a shaking table at 450 rpm for 24 h. After that, 0.5 g of sodium sulfate anhydrous was added into each tube to remove the water and then centrifugated at 9000 rpm for 5 min. The centrifuged supernatant was transferred into a new centrifugation tube, dried with nitrogen gas at 25 °C. The residue was dissolved in 0.5 ml of methanol: water (4:1, v:v), filtered through a 0.45 μm membrane filter (Waters Corporation) to remove particles > 0.45 μm, and finally transferred to a glass vial for high-performance liquid chromatography (HPLC) analysis. Haloxyfop standard (Beijing Manhage Biotechnology, China) was prepared in the same extract solution for soils, in concentrations ranging from 0.01 mg L^{-1} to 100 mg L^{-1}, and the standard curve was made every time before the samples were run. The precision

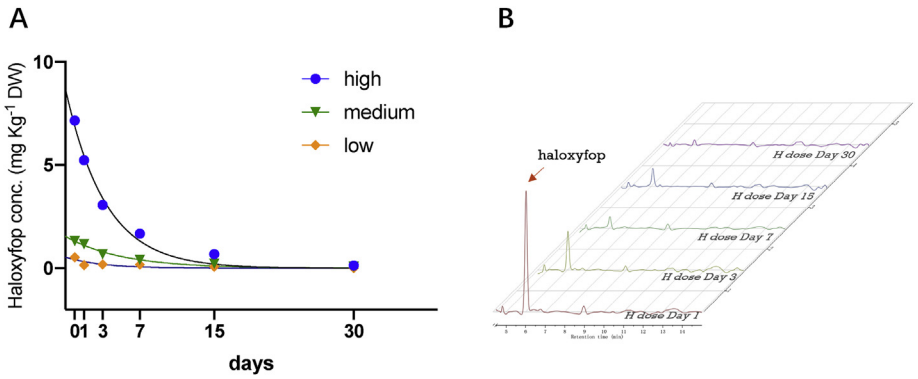

Fig. 1. (A) Changes of haloxyfop concentration in the rhizosphere soil of different treatments at different time; (B) HPLC chromatogram of haloxyfop of the high dose treatment at different time.

of the method was determined in terms of the recovery of spiked haloxyfop standards in soil samples at 1 mg kg^{-1} (Low), 10 mg kg^{-1} (Medium) and 100 mg kg^{-1} (High) FW and performing the extraction procedure as described above. The average recoveries and standard deviations of haloxyfop in the low, medium and high haloxyfop spiked soil samples were 142.6 ± 18.0%, 127.6 ± 10.5% and 97.3 ± 16.9%, respectively. The extractions of samples and standards were injected into an Agilent 1260 HPLC System (Agilent Technologies SpectraLab Scientific Inc., USA), using a Welch Ultimate XB-C18 column (5 μm, 150 × 4.6 mm) purchased from Welch Inc. The mobile phase was methanol:water (4:1, v:v, chromatographic grade). The injection volume was 20 μl, the flow rate was 0.6 ml min^{-1}, and the monitoring wavelength was 200 nm. The retention time of haloxyfop was 6.1 min in HPLC chromatogram (Fig. 1B). Calibration curves were adjusted using a weighted least squares regression, considering satisfactory linearity when $r^2 \geq 0.99$. The limit of detection was 0.1 mg kg^{-1} dry weight (DW) for haloxyfop in the soils.

2.2.3. DNA extraction and sequencing

The epiphytic bacteria present in the Fe plaque of root was extracted according to the method by Hu et al. (2015). In brief, the lateral root of *S. alterniflora* was washed three times with sterile water to remove the adhered soil. The iron plaque on the roots was extracted by the classic dithionite–citrate–bicarbonate (DCB) method (Taylor and Crowder, 1983). About 1 g of the fresh lateral root of *S. alterniflora* was agitated in 10 ml of pre-cold DCB solution (0.3 mol L^{-1} sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$), 1 mol L^{-1} sodium bicarbonate ($NaHCO_3$), and 60 g L^{-1} of sodium dithionite ($Na_2S_2O_4$)) at 25 °C for 3 h. The DCB extract was combined and centrifuged at 16,000 × g for 10 min, the supernatant was discarded, and the precipitates were used for DNA extraction for the microorganisms present on the plaque.

DNA in the rhizosphere soil and the Fe plaque was extracted using PowerSoil® DNA Extraction kit (MoBio, USA) for the corresponding sample. The concentration and purity were measured using the NanoDrop One (Thermo Fisher Scientific, MA, USA). 16S rRNA genes of distinct regions (V3–V4) were amplified used specific primer, 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), with 12 bp barcode. PCR reactions, containing 25 μl 2x Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 μl each primer (10 μM) and 3 μl DNA (20 ng μl^{-1}) template in a volume of 50 μl, were amplified by thermocycling: 5 min at 94 °C for initialization; 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 52 °C, and 30 s extension at 72 °C; followed by 10 min final elongation at 72 °C. The PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA, USA). PCR products were mixed in equidensity ratios according to the GeneTools Analysis Software (Version 4.03.05.0, SynGene). Then, the mixed PCR products were purified with the EZNA Gel Extraction Kit (Omega, USA). Sequencing libraries were generated using NEBNext® UltraTM DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) following the manufacturer's instructions and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Waldbron, Germany). At last, the library was sequenced on an Illumina Hiseq2500 platform and 250 bp paired-end reads were generated (Majorbio Co., Ltd, Shanghai, China).

2.3. Data analysis

Five indices of the alpha diversity, including Shannon index, richness (Chao), Shannon based evenness (Shannoneven), were calculated at the OTU level with QIIME (V1.9.1). The differences in Shannon index between the control and different haloxyfop treatments on Days 1–7 and Days 15–30 were analyzed separately by Student's t-test with R software (v.3.6.1). The shifts in the relative abundance of the bacteria on the phylum and genus levels were displayed by heatmaps with the Vegan package in R software. Bray-Curtis, weighted and unweighted unifrac beta diversity indexes were calculated by QIIME software. Principal Coordinate Analysis (PCoA) was performed to explore different treatments at different times that might explain the groupings of similar communities at the OUT level. A distance matrix of weighted or unweighted unifrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by the first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by the ggplot2 package in R software. Using the Vegan package for R software, we performed a redundancy analysis (RDA) on the genus levels to identify the effects of soil physicochemical variables (including haloxyfop residue) on soil bacterial community composition. The Spearman correlation coefficient of the top 30 abundant microbial genera and soil variables were calculated using the Vegan package and displayed on the heatmap with R software.

3. Results

3.1. Changes in soil physicochemical characteristics and plant growth

The soil physicochemical characteristics, including Eh, EC, Temp, and WET, changed synchronously along the different times after the treatment of haloxyfop (Fig. S2). The soil Eh, EC and temperature all peaked on Day 15, on when the soil moisture declined to the lowest level. The variation of these soil characteristics seems to be related to the tide change during the experiment (Fig. S3), the tidal level was the lowest on Day 15 of the trials, compared to the other times of sampling. Chlorosis & necrosis of the plant leaf started from Day 3, irrespective of the haloxyfop dose (Fig. S4). On Day 15 of haloxyfop treatment, the aboveground part of the plants began to wither. At the end of the treatment, the aboveground part of all the plants had withered. However, when sampling the roots of plants, we found that the roots of

plants are still alive, and there were some new tillering nodes that appeared on the roots.

3.2. The dissipations of haloxyfop in sediments

The persistence of haloxyfop in soil followed an exponential decay during the field experiment. The concentration of haloxyfop in rhizosphere soil decreased with time (Fig. 1A). The dissipation rate was proportional to the dose of haloxyfop. The higher the haloxyfop treatment, the faster the dissipation of haloxyfop in the soil. The concentration of haloxyfop in the rhizosphere soil on Day 15 were 0.079, 0.22 and 0.69 mg kg^{-1} for the low, medium and high level of haloxyfop treatment, respectively. At 30 days after herbicide application, the remaining percentages of haloxyfop were 1.8%, 6.1% of the initial concentrations, for the high and medium treatments, respectively. The haloxyfop in the soil of the low-level treatment was not detected due to the detection limit (0.1 mg kg^{-1} DW). The half-life values of haloxyfop, which was calculated from the best fit line of the logarithm of the residual haloxyfop concentration versus treatment time, were 2.61 days and 4.91 days for the medium and high levels of treatment, respectively.

3.3. Bacterial diversity changes in rhizosphere soil

After the treatment of haloxyfop, the diversity of the bacterial community (as indicated by the Shannon index) was decreased by 11.6%–25.6% on Days 3 and 7compared to the control group on Day 1 (Table 1). Similar changes were also recorded on Days 3 and 7, and the α-diversity of the bacterial community in the soil that received high levels of haloxyfop treatment decreased about 16.2% and 17.8%, for Days 3 and 7, respectively. On Days 15 & 30 of the exposure, the difference in the soil bacterial diversity between the highest level of haloxyfop treatment and the control were narrowed. A dose-dependent decrease in Shannon index of the soil bacteria was observed at the early stages of the haloxyfop treatment (Days 1, 3 & 7) but not at late stages (Days 15 & 30) (Fig. 2A). Principal Coordinate Analyses (PCoA) was performed to investigate the β-diversity of the soil bacterial (OTU levels) that receiving different levels of haloxyfop at different sampling times (Fig. 2B). According to the PCoA analyses, the bacterial community composition significantly differed among different sampling times. Samples collected at the early stages of different treatments (Days 0, 1, 3 & 7) were clearly separated from that obtained at the late stages (Days 15 & 30) along PC1, which explained up to 33.72% of the variation. The PC2 explained the remaining 15.44% dissimilarity. These results are consistent with the observed changes in α-diversity at different sampling times (Table 1).

Table 1
Major alpha diversity indices of the bacterial communities in the rhizosphere soil under the treatment of haloxyfop at different sampling time.

Samples	Shannon	Chao	Shannoneven
Day 0_Control	1.93	38.33	0.53
Day 0_L	1.78	33.00	0.51
Day 0_M	1.90	40.50	0.52
Day 0_H	1.99	44.00	0.53
Day 1_control	1.99	40.00	0.54
Day 1_L	1.76	35.00	0.50
Day 1_M	1.48	48.50	0.40
Day 1_H	1.66	37.50	0.46
Day 3_control	1.97	38.20	0.54
Day 3_L	1.88	37.00	0.52
Day 3_M	1.83	47.00	0.49
Day 3_H	1.65	40.00	0.45
Day 7_control	1.80	45.00	0.51
Day 7_L	1.77	36.00	0.49
Day 7_M	1.85	40.00	0.51
Day 7_H	1.48	48.00	0.40
Day 15_control	1.50	34.33	0.44
Day 15_L	1.45	32.33	0.42
Day 15_M	1.67	35.75	0.47
Day 15_H	1.33	35.00	0.38
Day 30_control	1.43	29.00	0.43
Day 30_L	1.66	33.00	0.48
Day 30_M	1.56	45.20	0.42
Day 30_H	1.46	35.17	0.41

3.4. Bacterial community composition and diversity in the rhizosphere soil

The bacterial community compositions (phylum and genus levels) of the rhizosphere soils from all the treatments at different times are presented in Fig. 3. On the phylum level, a total of 45 phyla were obtained, and the majority of the sequences (37.65–65.22%) belong to Proteobacteria, indicating that the sequences affiliated with Proteobacteria contributed to a higher percentage of the community DNA. Chloroflexi (7.8–28.9%), Bacteroidetes (2.74–18.05%), Actinobacteria (2.44–21.47%), Acidobacteria (2.53–11.81%), Firmicutes (1.28–11.39%), Patescibacteria (0.62–4.43%), Gemmatimonadetes (0.68–2.75%), Planctomycetes (0.19–2.15%), Verrucomicrobia (0.04–1.19%), Epsilonbacteraeota (0.09–1.51%), Nitrospirae (0.08–1.28%) were also the dominant phyla in the samples (Fig. 3A).

On the genus level, a total of 1180 genera were obtained, and the most representative genera (top30) are shown in Fig. 3B. The dominant genera were *Erythrobacter*, *Pseudomonas, Planococcus*, *Desulfuromonas*, *Flavobacterium, Acinetobacter*, *Pontibacter* and some other *norank genera*. One day after the application of haloxyfop treatment, the relative abundances of the genera *Pseudomonas* (phylum Proteobacteria), *Acinetobacter* (phylum Proteobacteria), *Pontibacter* (phylum Bacteroidetes), *Shewanella* (phylum Proteobacteria) and *Aeromonas* (phylum Proteobacteria) in the haloxyfop treated soil was about 6, 23, 37, 37 and 30 times of those in control group, respectively. The relative abundances of *Shewanella* and *Aeromonas* on Days 3 and 7 were also much higher than those in the control group. The relative abundance of *Hoppeia* was about 40% of that of control in the early stage of haloxyfop treatment and was greatly improved in the later stage of treatment (Days 15 & 30). Erythrobacter was the dominant genus in the late stage (Days 15 & 30), but the relative abundance of this genus was very low in the early stage of haloxyfop treatment.

3.5. Relationships between the soil environmental variables with microbial communities

As the soil microbial communities clustered firmly based on the time after the haloxyfop treatment, we performed two separate redundant analysis to identify the influence of the soil physicochemical factors on the rhizosphere bacterial community (genus level) at the early (Days 1, 3 & 7) and late stages (Days 15 & 30) (Fig. 4). The soil physicochemical factors, such as EC, Eh, Temp, WET and haloxyfop, explained a total of 49.16% and 68.52% of the variance of major bacterial groups for the early and late stages, respectively (Fig. 4). At the early stages (Days 1, 3 & 7), the soil EC ($p = 0.004$) and the concentrations of haloxyfop ($p = 0.012$) showed significant correlations with the first two RDA axes (Table S1), and the relative abundance of the dominant bacterial genera, such as *Pseudomonas*, *Erythrobacter*, *Microbulbifer*, *Acinetobacter*, *Flavobacterium,* and *Planococcus*, showed positive correlations with the haloxyfop levels in the soil. The genera *Marmoricola* and *Fusibacter* showed positive correlations with the soil EC. The genera *Ilumatobacter*, and *Desulfuromonas* showed a negative correlation with these two factors. At the late stages of the treatment, the concentration of haloxyfop in soil was no longer the significant factor that shapes the composition of bacterial community ($p = 0.856$). Instead, WET ($p = 0.021$) and EC ($p = 0.011$) showed significant correlations with the first two RDA axes (Table S1). Spearman correlation analysis showed that the genera *Bacillus, Vibrio* and *Shewanella* showed extremely significantly positive correlations with the soil

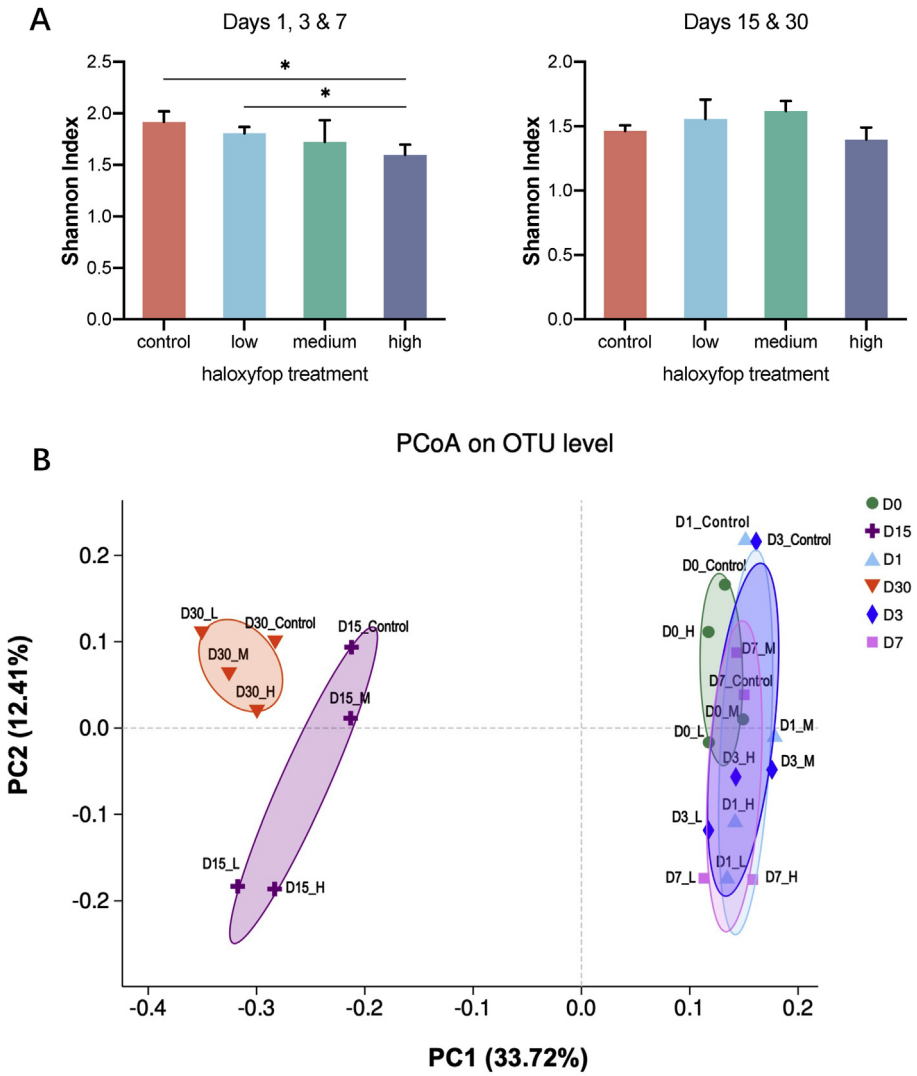

Fig. 2. (A) Changes of the averaged Shannon index on Days 1, 3 & 7 and Days 15 & 30 of haloxyfop treatments; (B) The principal coordinate analysis of bacterial communities based on Bray-Curtis dissimilarity matrices across different haloxyfop treatments at different time.

haloxyfop levels ($p < 0.001$). Other genera, such as *Pseudomonas*, *Marmoricola*, *Planococcus*, *Acinetobacter*, *Fusibacter*, *Paracoccus*, *Woeseia*, *Aeromonas*, *Exiguobacterium* and *Hyphomicrobium* also showed significant positive correlations with the soil haloxyfop level. *Hoppeia*, on the contrary, showed significant negative correlations with the soil haloxyfop level (Fig. 5).

3.6. Plant root microbial communities

The microbial communities in the rhizoplane (iron plaque) of *S. alterniflora* were investigated on Days 15 & 30 of the haloxyfop treatment. According to the results of rank-abundance curves (OUT level), the bacterial diversity decreased on Day 15 of the haloxyfop treatment (Fig. S5). A range of 36.1%–46.3% reduction in α-diversity (Shannon Index) was recorded from low to high levels of haloxyfop treatment. On Day 30, the bacterial diversity in rhizoplane was vastly enhanced, compared to that on Day 15 (Fig. S5).

A total of 2906 OTUs (921 genera and 43 phyla) was obtained in the rhizoplane of *S. alterniflora*, which was far less than that obtained in the rhizosphere soil (6678 OTUs, 1180 genera, and 45 phyla). Proteobacteria was also the dominant phylum, which contributed about 46.9–86.0% of the community DNA. Other phyla, such as Bacteroidetes (7.8–22.2%), Firmicutes (0.53–7.8%), Chloroflexi (0.16–6.2%), Epsilonbacteraeota (0.4–5.5%), Actinobacteria (0.4–3.8%), Acidobacteria (0.001–0.89%), Patescibacteria (0.4–1.6%) and Spirochaetes (0.2–2.6%) were also the dominant phyla in the samples (Fig. 6A). On Day 15, the treatment of haloxyfop increased the relative abundance of Epsilonbacteraeota, while that of Firmicutes was greatly decreased (Fig. 6A). The community heatmap showed that the relative abundance of most of the phyla increased on Day 30, compared to Day 15 (Fig. 6B).

The PCoA analysis at the OTU level showed that the soil microbial communities clustered strongly based on time. Rhizoplane soil microbial communities on Day 15 were clearly separated from those collected on Day 30 along PC1, which explained up to 54.53% of total variation (Fig. S6A). RDA analysis showed that the soil EC, Eh, TEMP, WET and haloxyfop explained a total of 92.11% of the variance of major bacterial groups in rhizoplane from Day 15–30 (Fig. S6B). The soil WET ($p = 0.017$), Temp ($p = 0.037$) and EC ($p = 0.008$) showed significant correlations with the first two RDA axes. As the most abundant phylum across all the treatment, the relative abundance of Proteobacteria was positively correlated with the soil EC, Eh, TEMP, while it was negatively correlated with the soil WET. The rhizoplane microbial composition was mainly controlled by the soil factors, including EC, Eh, TEMP, and haloxyfop on Day 15. However, the soil WET turned to be the major influencing factors on Day 30.

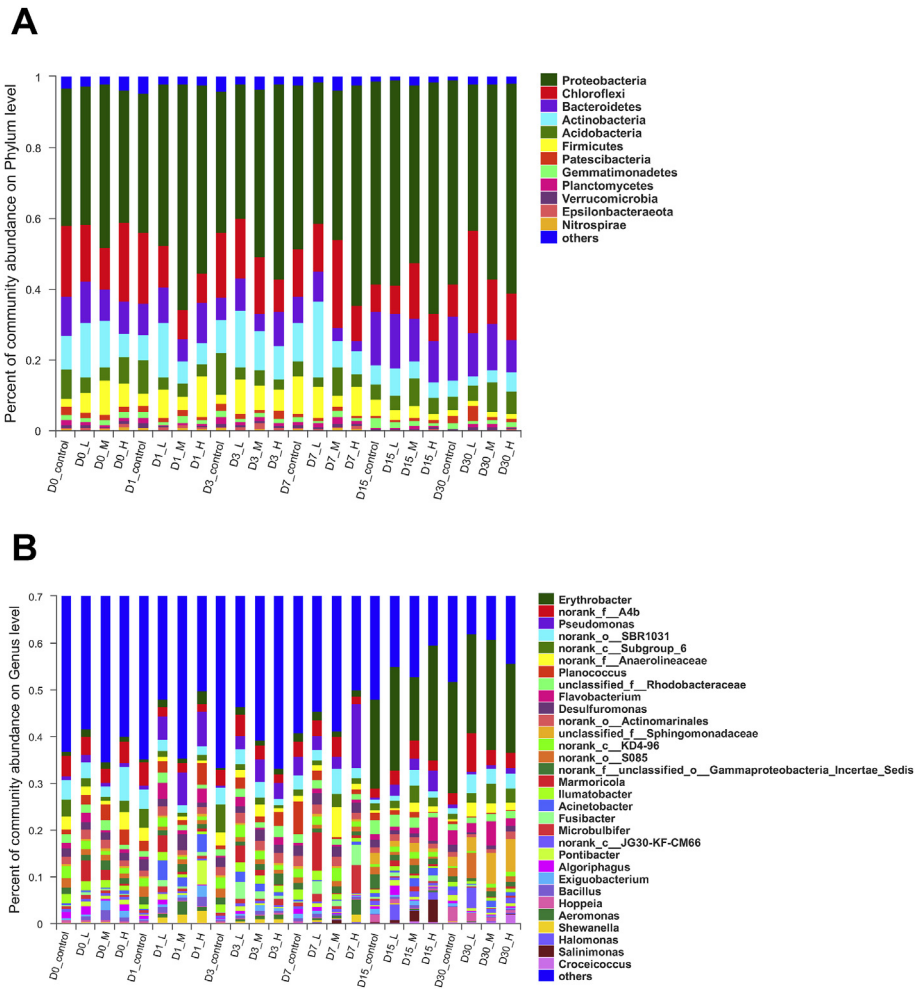

Fig. 3. Relative abundance of the dominant bacterial taxa on (A) phylum and (B) genus level among different haloxyfop treatment at different time.

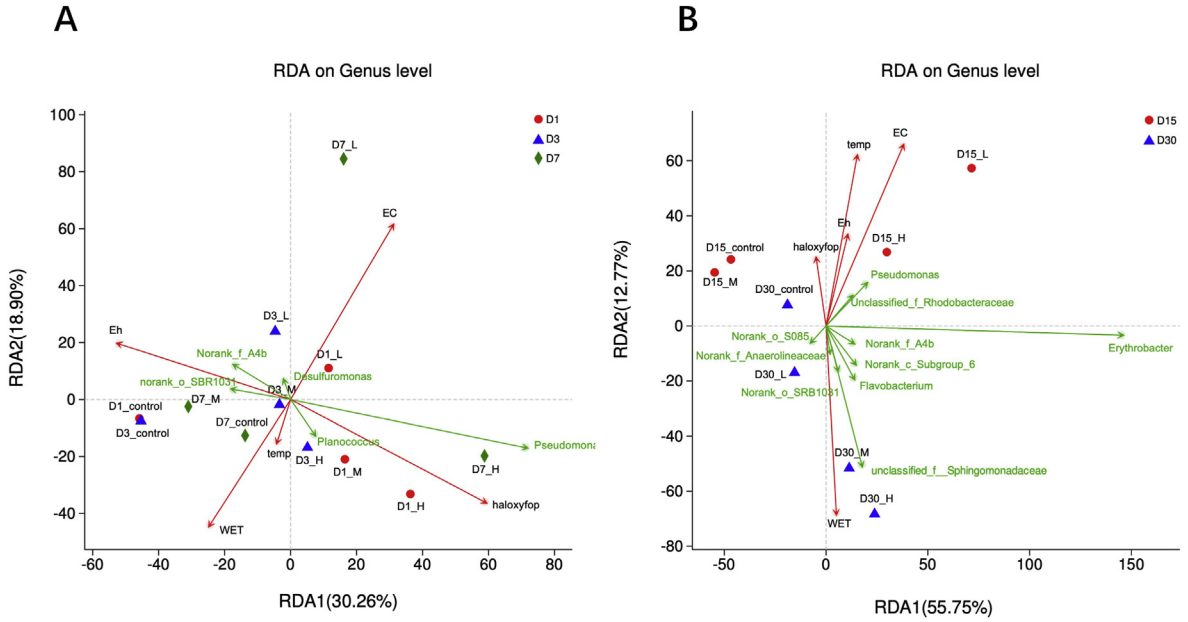

Fig. 4. Redundancy analysis showing the relationships among the major microbial genera and the soil physiochemical variables at (A) Days 1, 3 & 7 and (B) Days 15 & 30 of haloxyfop treatments. The points with different colors or shapes in the graph represent the sample groups under different haloxyfop treatment at different time. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

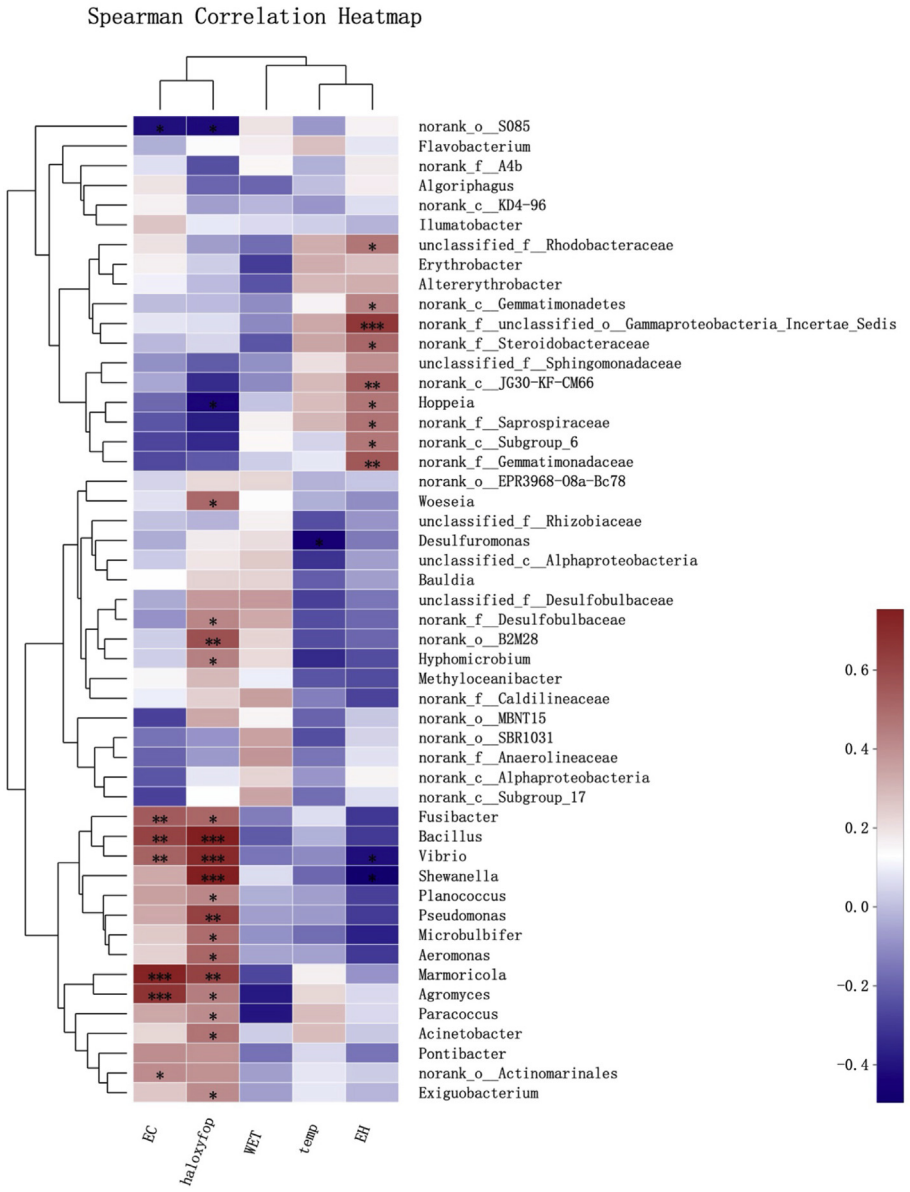

Fig. 5. Spearman correlation heatmap showing the correlation between different bacterial genera (top 30) and soil physiochemical variables. The x-axis and y-axis are soil variables and genera respectively. The R value is displayed in different colors in the figure, and the legend in the right side indicates the range of different R values. *, ** and *** indicate the correlation are significant at $p < 0.05$, 0.01 and 0.001, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In the present study, the dissipation rate of haloxyfop was found to be proportional to the application dosage of the herbicide. The higher the haloxyfop dosage, the faster the dissipation of haloxyfop. This result was consistant with previous studies on the dissipation of herbicides, such as triketones, sulcotrione and leptospermone in laboratory microcosm studies (Romdhane et al., 2016, 2019). The dissipation of haloxyfop in the wetland soil is much faster than other herbicides, such as leptospermone (Romdhane et al., 2016, 2019), triasulfuron (TSF) and prosulfocarb (García-Delgado et al., 2018) in the non-wetland soils. The half-life of haloxyfop in the present studies was 2.67–4.91 days for high and medium doses of haloxyfop treatments. This result was consistant with that measured in the soybean field soil by Wu et al. (2010), in which the half-life of haloxyfop in the soil of the soybean field was 4.06 days. In another study by Liu et al. (2018), as high as 8.1–12 days of the half-life of haloxyfop were recorded in the soil of the rape field. Tidal water dilution seemed to play a significant role in the fast dissipation of herbicides in the wetland soils. The persistence of the herbicide, imazapyr, was also found dissipated quickly in a coastal wetland, and its concentrations in the sediment approached the zero at 40 and 400 h after the application (Patten, 2003). Other soil processes, such as the sorption of herbicides on organic matter from plant roots and activity of herbicides degrading microbial community may also account for the fast dissipation of haloxyfop (Sun et al., 2015).

Microorganisms in the contaminated soils are usually less diverse compared with the uncontaminated environments due to the selective pressure from the toxic substance presented in the soil and the competition for niches and resources (Sasse et al., 2018). On Day 1, after the treatment of haloxyfop-R-methyl, the α-diversity of the bacterial community were decreased by 11.6%–25.6% compared to the control group on Day 1, and the most significant drop in α-diversity was observed under the treatment of middle to high levels of haloxyfop (Table 1). Similar patterns were also recorded on Days 3 and 7. The higher the

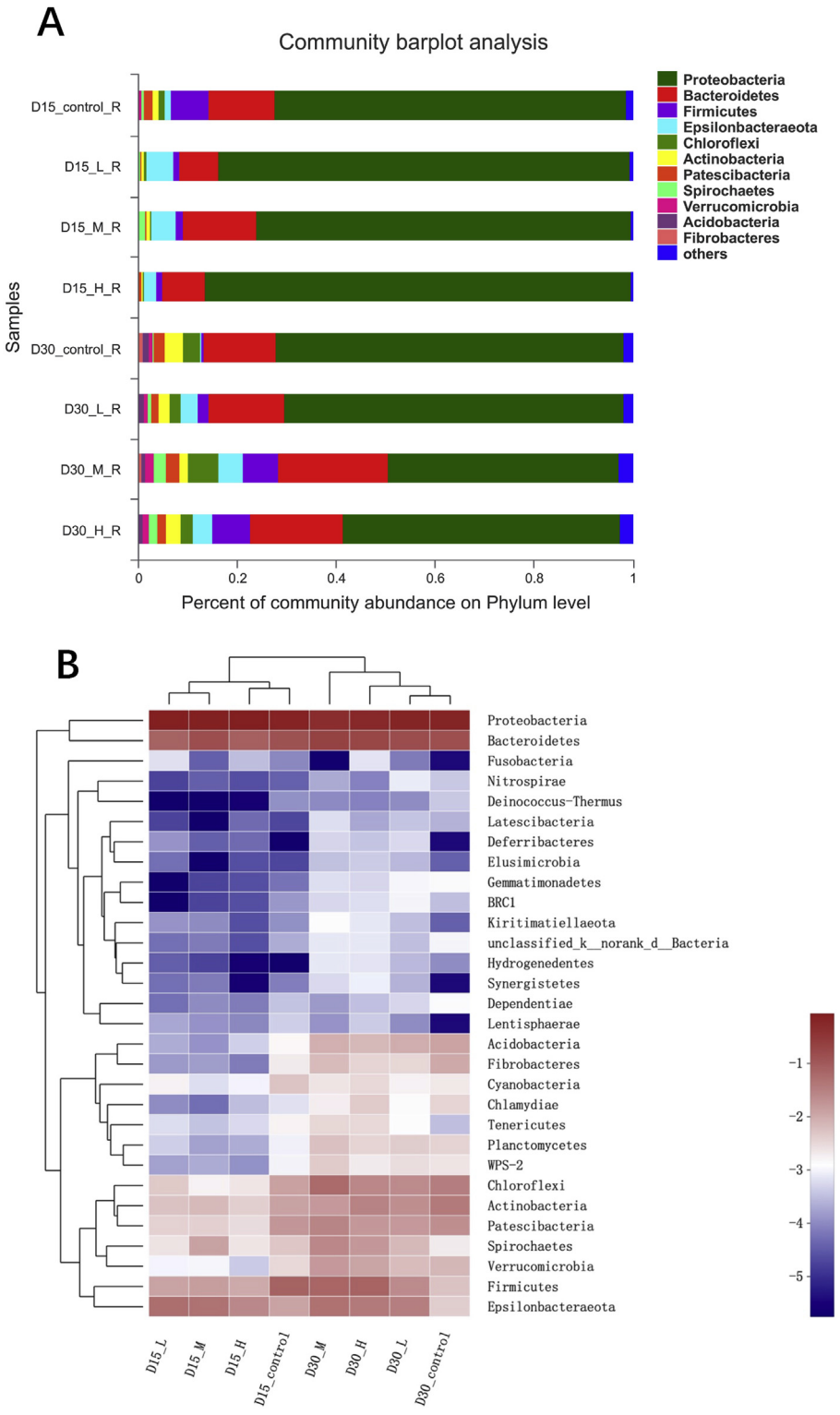

Fig. 6. (A) Relative abundance of the dominant bacterial taxa on (A) phylum level and (B) genus level in rhizoplane of *S. alterniflora* on Days 15 & 30 of the haloxyfop treatment.

haloxyfop dose, the lower the α- and β-diversities in the rhizospheric soil. These results suggest the haloxyfop induced substantial modifications of soil bacterial diversity. Diversity is a measurement of species richness combined with evenness. In the present study, the richness (as indicated by the Chao index) and evenness (as indicated by the Shannoneven) both decreased on Days 1, 3 & 7 (Table 1) under the medium to high levels of haloxyfop treatment. This results indicates the decrease of the bacterial diversity attributed to the less evenly distributed bacteria species in the topsoil after the treatment of haloxyfop. The shift in bacterial community structure could be associated with the application of the haloxyfop treatment, as the RDA analysis showed that the soil concentrations of haloxyfop had significant correlations ($p = 0.012$) with the first two RDA axes at the early stages (Days 1, 3 & 7). The reduction in soil bacterial diversity upon the exposure of herbicide has

also been reported in previous studies. Du et al. (2018) found that the exposure of herbicide mesotrione could reduce the Shannon index of the soil bacterial community.

Previous studies suggested that the microbial communities showed an obvious dose-dependent response with the application of herbicide, different time of herbicide treatment (short- or long-term) also induced different responses in the composition of soil microorganisms. On Days 15 & 30, the rhizosphere bacterial diversity under the medium to high levels of haloxyfop treatment were restored to the control level. The restoration in the bacterial diversity may partly due to the fact that the residue of haloxyfop in the soil was below the threshold of the herbicide that may generate a significant impact on the soil bacterial community. However, previous studies implied that the changes in the composition of soil bacterial community might not be solely influenced by residue of herbicide in the soil. In a microcosm incubation study by Romdhane et al. (2019), the herbicide leptospermone significantly modified the diversity and structure of the soil bacterial community on Day 8 of the exposure. Although the herbicide entirely dissipated over the 45 days incubation period, the diversity of the soil bacterial community was still not completely recovered by the end of the experiment. The discrepancy in the results further suggested that the responses in the soil bacterial community to herbicides treatment varied with the type of the herbicide and might be governed by other soil processes other than the herbicide residues in the soil. Short-term response in the soil microorganism to herbicides is mainly governed by an unspecific stress response of soil microorganisms in some cellular oxidation-reduction processes, while at long-term exposures, the changes in microbial community structure are likely due to the growth of resistant populations of microorganisms using a readily available source of carbon (dead biomass) from sensitive microorganisms impaired by the herbicide (Crouzet et al., 2010).

The relative abundance of some genera, such as *Pseudomonas*, *Acinetobacter*, *Shewanella* and *Aeromonas* in the phylum of *Proteobacteria*, and *Pontibacter* in the phylum of Bacteroidetes all increased significantly (5.6-fold–37.0-fold) compare to the control group at the early stage of haloxyfop treatment (Days 1, 3 & 7). Soil bacteria play a significant role in the degradation of many herbicides (including AOPP herbicides) in soils (Nie et al., 2011; Xu et al., 2019; Zhou et al., 2018). Bacterial strains isolated from *Pseudomonas* and *Acinetobacter* can withstand intense anthropogenic pressure, and many strains found in this genus can efficiently degrade herbicides, including haloxyfop-P-methyl (Nie et al., 2011; Abo-Amer, 2012; Kumar et al., 2012; Vásquez and Reyes, 2002; Ortiz-Hernández et al., 2013; Patyka et al., 2016; Xu et al., 2019). *Pseudomonas* sp. were found to have a great ability to degrade the herbicide Aroclor 1242 (Vásquez and Reyes, 2002) and the AOPP herbicide Cyhalofop-butyl (Nie et al., 2011). *Acinetobacter* sp. was found to be able to degrade the AOPP herbicide fenoxaprop-P-ethyl (FE) by hydrolyzing the ester bond (Dong et al., 2015). Ye et al. (2016) reported a strain that belongs to the genera *Shewanella*, can efficiently degrade the herbicide atrazine to cyanuric acid entirely within 36 h. Strains from *Aeromonas* spp. were also reported to be able to degrade paraquat (Viriyawattana and Surachat, 2014) and clodinafop propargyl (Kumar et al., 2014). Increases of the relative abundance of the phylum *Bacteroidetes* were also observed in response to the glyphosate application, and the addition of glyphosate disturbed bacterial interdependence and reduced the number of interactions at the family level (Guijarro et al., 2018). In this study, the increase in the relative abundance of those above herbicide degradable genera showed significant positive correlations with the soil haloxyfop ($p < 0.001$) at the early stages of treatment (Days 1, 3 & 7) (Fig. 4 and Table S1). The RDA analysis also showed the soil haloxyfop exerts extremely positive influences on the relative abundance of the genus *Pseudomonas* in the rhizosphere soil. These results suggested that these bacterial genera were involved in the degradation of the soil haloxyfop and might attribute to the fast dissipation of haloxyfop at the early stages of treatment.

In addition to haloxyfop, the other two soil variables, EC and WET, were also found to impose significant influences on the composition of the rhizosphere soil bacterial communities according to the RDA analysis. The soil EC showed significant correlations with the first two RDA axes at both the early ($p = 0.004$) and late stages ($p = 0.011$) of the haloxyfop treatment (Table S1). These results were consitent with many previous studies, that EC is a major soil factor that controlling microbial abundance, diversity, composition, and functions (Hu et al., 2014; Li et al., 2019). Soil salinization is usually associated with the increase of EC, and soil salinity was suggested to be the most dominant environmental factor that affects the soil microbial community in the Yangtze River estuary (Hu et al., 2014). The variation of soil salinity may change the abundances of soil bacterial communities and affected their function in saline coastal ecosystems (Li et al., 2019; Wang et al., 2019; Zhang et al., 2019). In this study, soil EC and WET show a negative correlation to some extent, which may be induced by periodic tidal fluctuation in the coastal wetland (Fig. S3).

Roots of plants can release nutrients, exudates, and oxygen into soils, which support a higher diversity of microorganisms in the rhizosphere soil than bulk soils (Edwards et al., 2015; Sasse et al., 2018). In this study, the variation of the bacterial community in the bulk soils was not followed, while the composition of bacteria in the rhizoplane (Fe plaque) was tracked on Days 15 & 30 of the herbicide applications. A total of 2906 OTUs (belong to 921 genera and 43 phyla) was obtained in the rhizoplane of *S. alterniflora*, which was far less than that obtained in the rhizosphere soil (6678 OTUs, 1180 genera, and 45 phyla). The diversity of the bacterial community in rhizoplane was also less than that in the rhizosphere soil, accompanied by a more dominant phylum of Proteobacteria (46.9–86.0% of the total community DNA). These results suggest that the rhizoplane of *S. alterniflora* could selectively attract certain bacteria species, which increased the dominance of some bacteria and decreased bacterial diversity. These results are in accordance to the previous study by Edwards et al. (2015) that the majority of the OTUs enriched in the rhizosphere were found to be simultaneously enriched in the rhizoplane of rice roots, and only a subset of the microbes that are attracted to the rhizosphere could bind the rhizoplane of rice. Our results showed that the bacterial diversity in rhizoplane was reduced on Day 15 while vastly enhanced on Day 30, and the relative abundance of most phyla also increased on Day 30. Although the dissipation of haloxyfop was very fast in the rhizosphere soil, there was still a considerable amount of haloxyfop residue in the soil on Day 15 (0.079, 0.22 and 0.69 mg kg^{-1} DW for the low, medium and high level of haloxyfop treatment, respectively). The correlations analysis showed that haloxyfop still had a significant negative impact on the relative abundance of some phyla, such as Cloacimonetes, Acidobacteria, and Chlamydiae. These results suggested that haloxyfop possibly still exert influences on the bacterial community in rhizoplane on Day 15. The release rate of root exudates is usually proportional to the plant growth and photosynthesis rate (Zhai et al., 2013). On Day 15, most of the aboveground parts of the plant withered under the treatment of haloxyfop, the weakened photosynthesis of the aboveground parts might reduce the root exudates and hence the diminished bacterial communities colonized in rhizoplane. It must be noted that on the soil physicochemical characteristics, such as Eh, EC and soil moisture, varied considerably from Day 15 to Day 30, which were possibly influenced by the periodic tide in the tidal flat. The variation in these soil characteristics, along with the haloxyfop, might jointly shape the bacterial community in both rhizosphere soil and the rhizoplane.

5. Conclusions

The present study revealed that haloxyfop can dissipate very quickly in the tidal flat soil of coastal wetlands. The haloxyfop treatment altered the composition of the rhizospheric bacteria community and reduced bacterial diversity in short-term (1–7 days), and the reduced bacterial diversity was accompanied by the increase in the relative abundance of

some bacteria genera, such as *Pseudomonas*, *Acinetobacter* and *Shewanella*, which are possibly involved in the degradation of haloxyfop in the rhizosphere soil. With the dissipation of haloxyfop, the bacterial diversity in the rhizosphere soil seemed to be rehabilitated, while the bacterial community in the rhizoplane still suffered direct or indirect influences from the haloxyfop on Day 15. Other soil variables in the tidal flat, such as EC, Eh and soil moisture were also the main drivers that shape the soil bacterial community structure. The results of this study confirm the previous view that the effect of herbicides on soil microbial structure is minor or transient. Nevertheless, it must be noted that haloxyfop is reused annually in coastal wetlands of China to control *S. alterniflora*. Although haloxyfop dissipates quickly in wetland soil, under the condition of long-term continuous use of the herbicide, the residue of haloxyfop in wetland soil and its comprehensive impact on other soil microorganisms still need to be studied in long-term to provide deeper insight for herbicide risk assessment.

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 to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110366.

Credit author statement

Qiuyao Liang: Investigation, Resources, Visualization, Formal analysis. Zhongzheng Yan: Conceptualization, Writing- Original draft preparation, Supervision, Writing- Reviewing and Editing. Xiuzhen Li: Supervision, Funding acquisition.

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