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Microbial Communities Along 2,3,7,8-tetrachlorodibenzodioxin Concentration Gradient in Soils Polluted with Agent Orange Based on Metagenomic Analyses

Huyen-Trang Tran[1] · Hung-Minh Nguyen[2] · Thi-Minh-Hue Nguyen[3] · Chieh Chang[4] · Wei-Ling Huang[4] · Chao-Li Huang[5] · Tzen-Yuh Chiang[4]

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Abstract

The 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a contaminant in Agent Orange released during the US–Vietnam War, led to a severe environmental crisis. Approximately, 50 years have passed since the end of this war, and vegetation has gradually recovered from the pollution. Soil bacterial communities were investigated by 16S metagenomics in habitats with different vegetation physiognomies in Central Vietnam, namely, forests (S0), barren land (S1), grassland (S2), and developing woods (S3). Vegetation complexity was negatively associated with TCDD concentrations, revealing the reasoning behind the utilization of vegetation physiognomy as an indicator for ecological succession along the gradient of pollutants. Stark changes in bacterial composition were detected between S0 and S1, with an increase in Firmicutes and a decrease in Acidobacteria and Bacteroidetes. Notably, dioxin digesters *Arthrobacter*, *Rhodococcus*, *Comamonadaceae*, and *Bacialles* were detected in highly contaminated soil (S1). Along the TCDD gradients, following the dioxin decay from S1 to S2, the abundance of Firmicutes and Actinobacteria decreased, while that of Acidobacteria increased; slight changes occurred at the phylum level from S2 to S3. Although metagenomics analyses disclosed a trend toward bacterial communities before contamination with vegetation recovery, non-metric multidimensional scaling analysis unveiled a new trajectory deviating from the native state. Recovery of the bacterial community may have been hindered, as indicated by lower bacterial diversity in S3 compared to S0 due to a significant loss of bacterial taxa and recruitment of fewer colonizers. The results indicate that dioxins significantly altered the soil microbiomes into a state of disorder with a deviating trajectory in restoration.

Keywords 16S-metagenomics · Bacterial assembly · Succession trajectory · Vegetation physiognomy · Agent Orange · US-Vietnam War

Introduction

Agent Orange, the colloquial name of the notorious defoliants sprayed in the US–Vietnam War (1961–1971), has led to serious environmental, societal, and health effects [1]. In

Huyen-Trang Tran, Chieh Chang and Wei-Ling Huang contributed equally to this work.

✉ Chao-Li Huang
clhuang65535@gmail.com

✉ Tzen-Yuh Chiang
tychiang@mail.ncku.edu.tw

[1] Department of Biology, Vinh University, Vinh, Nghe An 461010, Vietnam

[2] Center for responding to climate change, Department of Climate Change, Ministry of Natural Resources and Environment, Hanoi 125000, Vietnam

[3] Analytical laboratory for Environment, Dioxin and Toxins, Northern Center for Environmental Monitoring, Vietnam Environment Administration, Hanoi 115000, Vietnam

[4] Department of Life Sciences, National Cheng Kung University, Tainan 70101, Taiwan

[5] Institute of Tropical Plant Sciences and Microbiology, National Cheng Kung University, Tainan 70101, Taiwan

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Vietnam, thousands of tons of Agent Orange were sprayed by the US Army to remove forests, with the aim of revealing potential enemy guerrilla forces. Over 3,100,000 hectares of forests were defoliated [2]. Agent Orange, a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), contains the common contaminant 2,3,7,8-tetrachlorodibenzodioxin (TCDD) that poses long-term impacts for soil, sediment, and human health [3]. A half century after the US–Vietnam War, some polluted forest stands have gradually recovered, with small areas showing emerging weeds, shrubs, and tree seedlings.

Ecologically, soil functions as a microbial habitat. Bacteria are an essential component of the soil ecosystem and are linked closely with vegetation [4]. Thus, soil microbial community characterization is increasingly being performed to determine the responses of soil to various disturbances. Microbial diversity in soils impacts the survival and growth of plants. The soil microbes and dwelling plants interact as a whole, resulting in various vegetation types [5]. Vegetation physiognomy often reflects the succession status, e.g., Cerrado physiognomies ranging from savanna grasslands to forest formations along the soil types [5]. Likewise, in a landscape, bare land, scattering grasses/herbs, grassland with woods, and well-developed forests represent a series of ecological succession along a certain gradient. Considering its ecological function, the microbial community can, therefore, serve as an indicator of succession, a status due to their rapid response capability and contribution to carbon cycles [6].

The TCDD-contaminated lands in Central Vietnam cover a small ecological zone of tropical monsoon climate [7], polluted with dioxins as a result of storage and spray missions [1]. They provide an ideal site for inferring the dynamics of the microbiome following intense, long-term chemical disturbance. Here, herbicides used in the Vietnam War have created a mosaic landscape of rehabilitation plants in different stages of succession. After nearly 50 years, in the Da Nang Air Base, a dioxin hotspot for storage (up to 365,000 pg/g, 2005) [8], natural regeneration occurred slowly with minimal weed growth, or even no vegetation [9]. In Asho, a small dioxin hotspot for chemical transshipment, restoration of native plants has begun, with the development of grasslands with *Imperata* and *Pennisetum*, as well as a scattering of *Rhodomyrtus* [10]. In this region, high dioxin concentrations (up to 897.9 pg/g, 1996) remained [11]. In the Quang Tri mountain area, a tropical forest completely destroyed by spray missions, the natural restoration process has allowed for the emergence of a green forest. These sites dominated by various vegetation physiognomies represent different recovery statuses under the disturbance of residual dioxins.

Due to a well-known contamination history and the half-life of dioxins, Central Vietnam provides an excellent opportunity to investigate successional changes in the microbial community. This study aimed to gain insight into how soil bacterial diversity and community structure changed under long-term contamination with dioxins. We conducted 16S-metagenomics to uncover the microbial composition in soils of different vegetation physiognomies in Central Vietnam with the objectives to find the answers to following questions:

1. Were different vegetation physiognomies associated with TCDD concentrations in the soil?
2. Did high levels of TCDD play a limiting factor in shaping bacterial communities?
3. How did the bacterial community change along the vegetation physiognomies with increasing plant diversity?
4. Which bacterial groups were possibly involved in ecological restoration?

Material and Methods

State Descriptions

This study was conducted in the Corps Tactical Zone I (16°40′N to 16°01′N; 106°16,050′E to 108°019′E) in Central Vietnam [7], where Agent Orange campaigns were conducted from 1961 to 1971. On the basis of the half-life of dioxin in the top surface soil (9–15 years) [12], the history of Agent Orange/dioxin spraying [1, 8], the status of dioxins [8, 10], and vegetation at the surveyed sites [9, 11], four states with vegetation physiognomies were recognized: the S0 state with well-developed forests was found in Son Tra (ST), Ba Na (BN), and Hai Van (HV); the S1 state with barren lands or sporadic grasses was represented by the Da Nang Air Base (DN); the S2 state with partially restored grasslands of *Imperata* and *Pennisetum* and a scattering of *Rhodomyrtus* was found at the Asho-A Luoi Air Base (AL); and the S3 state with restored woods with bamboos, shrubs, and fast-growing trees was displayed in Quang Tri (QT) (Figs. 1 and 2). Except for the sites of S0 state, all the other sites were polluted with TCDD at different levels during the US-Vietnam War.

Soil Collection and Physico-chemical Analysis

Soil core samples were randomly collected from the surface (0–20 cm) at 6 sites in Central Vietnam (BN, HV, ST, DN, AL, and QT), with at least five biological replicates 200 m apart from each other. Each core sample, about 1.0–1.5 kg soil, was a homogenous mixture of five sub-samples from an area of 1 m × 1 m. In total, 31 core samples were collected ($n = 6$ for DN and $n = 5$ for all other sites). This sampling procedure followed those previously developed and

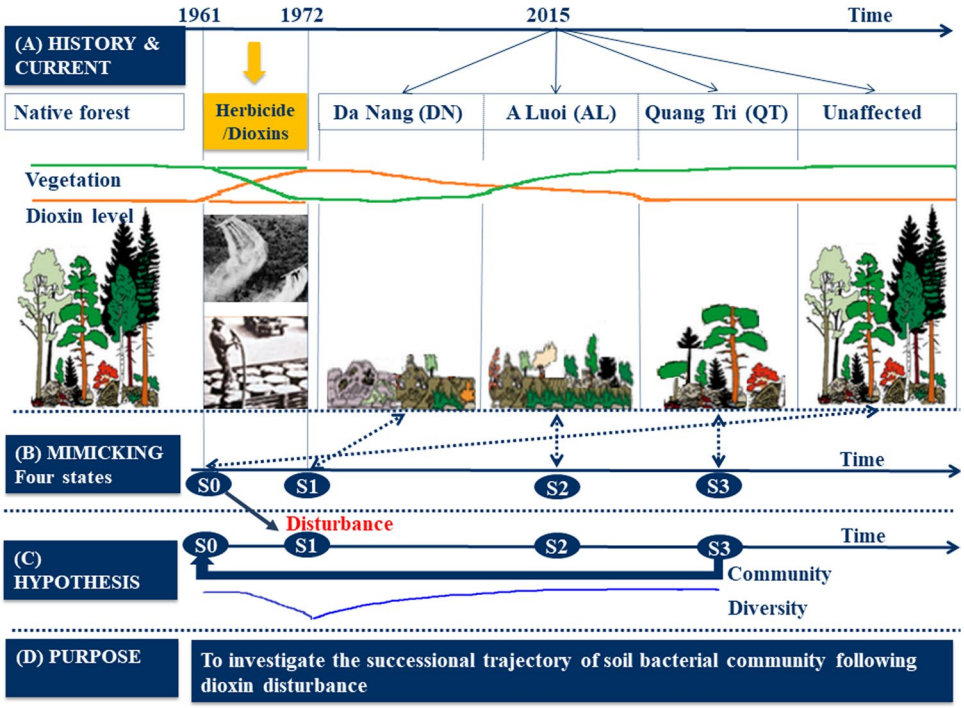


Fig. 1 Definition of the soil microbial communities in four vegetation physiognomies in central Vietnam. Diagram shows the history and current statuses, and vegetation physiognomies of forest (S0), barren land (S1), grassland (S2), and developing woods (S3)

applied by Hatfield, for Agent Orange assessment projects in Vietnam [8, 11]. Samples were then placed in plastic bags, covered by bin bags, and transported to the laboratory for DNA extraction. The DNA was then sent to the Center for Environmental Monitoring (CEM-Vietnam environment administration) to undergo soil physical–chemical analyses.

All physico-chemical properties of the soil, total toxic equivalent quantity (TEQ), water content, pH, texture, soil organic matter (SOM), total organic carbon (TOC), total nitrogen, and total phosphorus content (US EPA method 1613B; ISO 11465: 1993; ISO 10390:2005; ISO 11277:2009; ISO 10694:1995; EPA 9060B:2007; ISO 11261:1995; ISO 11263:1994; EPA 9030B:2007) were determined by the Center for Environmental Monitoring, Vietnam. For Da Nang Air Base hotspot (DN) samples, the remaining soil was only sufficient for TEQ measurements, as only a small amount of DNA was collected, for isolation only. For the other soil parameters, information from the Vietnam Ministry of Natural Resources and Environment [8] was extracted to provide estimations, whereby, the S1 sample is classified as an acidic soil (pH = 2.6–5.0), with low SOM contents (0.3–3.0%) and a poor capacity to retain water and organic materials (Fig. S1, Tables 1 and 2).

DNA Extraction, PCR, and Sequencing

The total DNA from the soil samples were extracted with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA). Multiple primers were used to minimize the amplification bias [13]. The V1–V3, V4–V6, and V5–V8 regions of the 16S rRNA gene were amplified with three pairs of universal primers, 27F/338R, 789F/1053R, and 926F/1392R, for bacteria. The primers were as follows: 27F-5′-AGRGTTYGATYMTGGCTCAG-3′ and 338R-5′TGCTGCCTCCCGTAGGAGT-3′, 789F-5′-TAGATACCCSSGTAGTCC-3′ and 1053R-5′- CTGACGRCRGCCATGC-3′, and 926F-5′-AAACTYAAAKGAATTGACGG-3′ and 1392 R-5′-ACGGGCGGTGTGTRC-3′ [14].

For each sample, PCR was performed in a 50 µL reaction following conditions of 95 °C for 5 min; 24 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 50 s, and a final extension at 72 °C for 10 min. The size of the PCR products was visualized using gel electrophoresis (agarose 1.2%) and eluted using the Gel/PCR DNA Extraction Kit (Geneaid, ISO 9001:2008 QMS). All purified PCR products were precipitated with isopropanol, dissolved by water, and quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Oregon, USA). The three amplicons for each sample were mixed with equal amounts [15] and sequenced using the Illumina MiSeq with 300 bp paired-end sequencing (Genetech Biotech Co., Ltd, Taiwan). The raw sequences were deposited in NCBI under the accession number PRJNA720488.

Generating OTUs and Combining Three Primers

Operational taxonomical units (OTUs) were identified by clustering haplotypes with OTUs from the Greengenes database (August 2013 version) at 97% sequence identity using the "pick_closed_reference_otu.py" function

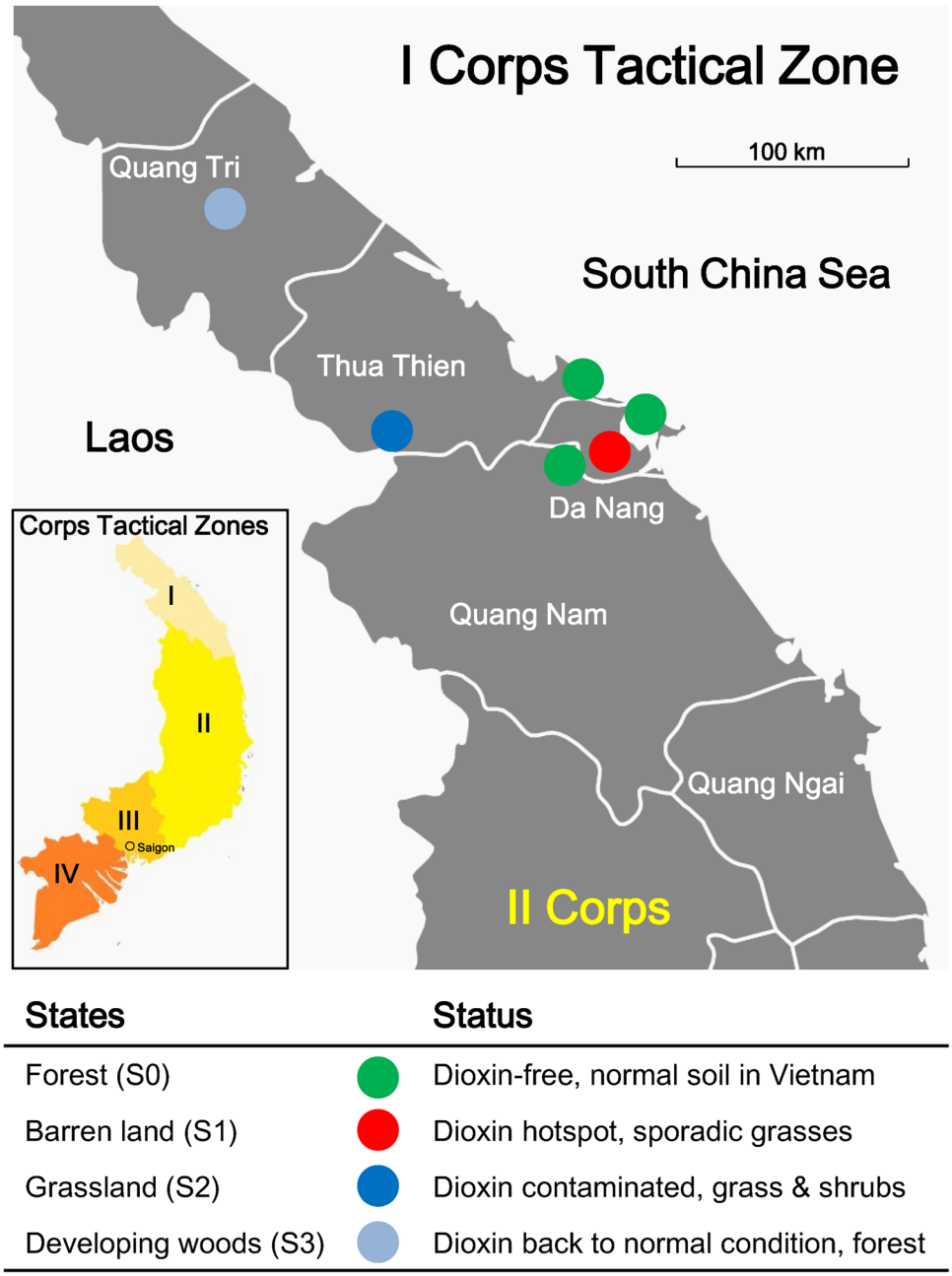


Fig. 2 Map of sampling locations across states of I corps tactical zone. Dots indicate the position of six sampling sites. Colors represent four states of vegetation physiognomies. The figure is modified from https://freevectormaps.com/vietnam/VN-EPS-01-0002?ref=atr

implemented in QIIME software packages 1.9.0 [16]. Mitochondrial, chloroplast, and singleton OTUs were excluded. As there were few reads belonging to archaea (1.2%), only bacteria were included in subsequent analyses. The function "rrarefy" in the *vegan* v.2.4–4 package [17] was used to rarefy all samples to 3735 reads (the lowest count with 926F/1392R in DN-001 sample) for all primers to normalize the differences of sequencing depths [18]. The abundance of OTUs was estimated by averaging the reads in the three amplicons after rarefaction. All community analyses were conducted based on the rarefied OTU table.

Diversity, Composition, and Statistical analysis

The *vegan* package was used to calculate species richness (Chao1 index) and species diversity (Shannon index). Boxplots were created by the ggplot2 package in R package v. 2.4.4. Significant differences in alpha diversity between states were evaluated by a Mann–Whitney test. Correlations between the abundance of taxa and the concentration of TCDD were estimated using the Spearman's method.

Two-dimension nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities was conducted using the vegan package. The physico-chemical

Table 1 Characteristics of the soil samples from succession states (S0–S3) of four vegetation physiognomies in Central Vietnam. Samples along a gradient based on historical and current statuses in Central Vietnam

Succession states	S0	S1	S2	S3
Vegetation	Evergreen closed forests	Barren land with scattering grasses	Wild grass and herbs with few shrubs	Woods with bamboo and fast-growing trees
Soil taxonomy[a]	Sandy loam	Sand	Sandy loam	Sandy loam
Climate[b]	Tropical monsoon climate	Tropical monsoon climate	Tropical monsoon climate	Tropical monsoon climate
Total number of sample in this study	**15**	**6**	**5**	**5**

[a]Based on the data of Center for Environmental Monitoring, Vietnam

[b]Based on the source: Climate-Data.org

properties were fitted on NMDS results by the "ordisurf" function of the vegan package. The significance of the correlations of these properties to the community structure was assessed by the "Adonis" function of the vegan package. Canonical correlation analysis (CCA) was performed to assess the significance of the environmental variables. Hierarchical cluster analysis (HCA) was conducted with Bray–Curtis dissimilarities of relative abundance of the OTUs. State distance analysis based on Bray–Curtis similarity was applied to test the association between decay rate of community similarity and state distance [19]. Linear discriminant analysis (LEfSe) was performed on the galaxy web server (http://huttenhower.sph.harvard.edu/galaxy/).

Co-occurrence networks were generated for each state to examine co-occurrence patterns of bacterial communities in each state and to identify the cut-off OTUs that play central roles in the network. First, we generated a correlation matrix with coefficients r between OTUs in the same state with Spearman's method [20] by removing the low-abundance OTUs (< 0.05%). Connections between the OTUs were defined if significant correlations were detected ($p < 0.05$) [20]. The "igraph" package was used to convert correlation matrixes to graph objects [21]. Additionally, a strong link was defined if abs(r) is greater than 0.5 [20]. We next used the "Intergraph" package to convert graph objects to network objects [22]. The "gtrans" function in the sna package was used to calculate transitivity (the ratio of closed triplet links to the total number of open and closed triplet links) between nodes. Finally, the Fruchterman–Reingold layout was used to visualize the networks [23].

Results

Physico-chemical Characteristics of Soils

The dioxin/furan concentrations of all states were measured as 0.8–2.3 pg/g for S0 and 234–14,757 pg/g for S1; they dropped to 8.2–8.3 pg/g at S2 and fell to the background

Table 2 Characteristics of the soil samples from succession states (S0-S3) of four vegetation physiognomies in Central Vietnam. Physico-chemical properties of soil samples of the vegetation physiognomies. Succession stages are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3)

No	Parameter of soil properties	Succession states			
		S0	S1	S2	S3
1	Total TEQ (pg/g)	1.53 ± 0.74	4058 ± 6059	8.26 ± 0.02	1.29 ± 0.29
2	2,3,7,8-TCDD (pg/g)	0.14 ± 0.08	3957 ± 6003	5.87 ± 0.76	0.06 ± 0.09
3	2,3,7,8-TCDD/TEQ (%)	10.88 ± 6.94	87.17 ± 18.4	71.06 ± 9.35	4.23 ± 5.98
4	Water content (%)	19.99 ± 4.35	-	21.48 ± 2.02	20.94 ± 0.56
5	pH	5.39 ± 0.36	-	4.51 ± 0.41	4.85 ± 0.07
6	Soil organic matter (SOM) (mg/kg)	25,001 ± 6606	-	20,358 ± 5516	19,483 ± 4258
7	Total organic carbon (TOC) (mg/kg)	9571 ± 2938	-	7830 ± 2305	7315 ± 1689
8	Total nitrogen (mg/kg)	1869 ± 573	-	875 ± 186.68	930 ± 93.34
9	Total phosphorus (mg/kg)	132 ± 14.93	-	187 ± 67.88	130 ± 33.94
10	Sulfur (mg/kg)	4.88 ± 1.16	-	3.27 ± 0.95	2.78 ± 1.15

Dashes indicate results not determined

level at S3 (Table 2), seemingly reflecting a trend of decreasing TCDD concentrations as vegetation complexity increased. Most physicochemical parameters of the soil (pH, SOM, TOC, nitrogen, and sulfur) tended to decrease gradually from S0 to S3 and reached the lowest point at S2. In contrast, water content and phosphorus tended to change in the opposite direction (Table 2 and Fig. S1). Two soil types were identified, namely, sandy loam (S0, S2, and S3) and sand (S1) (Table 1).

Bacterial Diversity in Habitats with Different Vegetation Physiognomies

Along the physiognomies, from barren land to well-developed forests, decreasing Shannon's indices of the bacterial community were detected, with significant differences in pairwise comparisons of S0–S2 ($p = 0.019$) and S0–S3 (Mann–Whitney's test, $p = 0.019$ and 0.0001), respectively (Fig. 3a, Tables 3, 4, and 5). In contrast, Chao1's index indicated increasing species richness from S0 to all states (Fig. 3b, Table 3, 4, and 5).

Bacterial Communities in Various Vegetation Physiognomies

The NMDS analysis for each state, by grouping all sites and replicates for each state, revealed distinct bacterial communities, except for two comparisons of S1 vs S2 and S0 vs S3 (Fig. 3c). Distance decay analysis revealed that the bacterial community similarities were negatively correlated with the state distances (Fig. 3d). Samples from the undisturbed state (BN, HV, and ST) were clustered in the HCA dendrogram (Fig. 3e). The bacterial communities of plant physiognomies of the grassland (AL) and developing wood (QT) were clustered together first, then linking to the S0 state, samples from barren lands (DN) were distinct from all other sites.

Regarding the taxonomic affiliations at the phylum level, Proteobacteria was the most abundant across all states, accounting for 36% on average, followed by Acidobacteria (25%) and Actinobacteria (14%) (Fig. 4). The state of barren lands (S1) was characterized by high proportions of Actinobacteria and Firmicutes ($28 \pm 19\%$ and $23 \pm 10\%$, respectively). The relative abundance of Acidobacteria decreased slightly in S1 ($9 \pm 4\%$), while it increased dramatically in S2 and S3 states ($32 \pm 1\%$ and $45 \pm 6\%$), reaching a much higher level than that in S0 ($13 \pm 5\%$). These results indicate a substantial shift in phylum composition along the vegetation physiognomies.

The LEfSe analysis identified discriminant taxa representing each vegetation type. Greater numbers of discriminant taxa from the S0 (16) and S1 (18) states were detected than those in S2 (3) and S3 (1) states (Fig. 5). Of these, seven taxa belonged to Acidobacteria, including orders of iii1-15, RB40, and RB41 for the S0 state, and Solibacteriales for the S2 state. The discriminant taxa of Bacteroidetes and Armatimonadetes exclusively existed at the S0 state, and those of Actinobacteria and Chloroflexi were enriched in the S1 state, including Micrococcaceae, Micromonosporaceae, Pseudonocardiaceae, and Anaerolineales.

Bacterial Communities Along Vegetation Physiognomies

CCA revealed that the level of TCDD showed significant correlations with the changes in bacterial communities ($p = 0.002$) (Fig. S2a). Of the abundant phyla (> 1%), four showed significant correlations with the log-transformed concentration of TCDD (log-TCDD). Firmicutes displayed a positive correlation (Spearman's $r = 0.62$, $p = 0.01$), while Acidobacteria, Planctomycetes, and Bacteroidetes showed negative correlations (Fig. S2b–e).

At the OTU level, 29 OTUs showed significant correlations with log-TCDD (Spearman's $r > 0.5$, $p < 0.05$) (Table S1), with 20 OTUs showing positive correlations, and nine OTUs showing negative correlations. Of the positively correlated OTUs, ten, seven, and three belonged to Actinobacteria, Proteobacteria, and Firmicutes, respectively (Table S1). Of the negatively correlated OTUs, four, three, and two were Acidobacteria, Proteobacteria, and Verrucomicrobia, respectively (Table S1). The Shannon index did not show a significant correlation with log-TCDD.

Co-occurrence Networks in Each State of Vegetation Physiognomy

The co-occurrence networks of bacterial OTU were distinct among four states (Fig. S3, Table S2). The numbers of nodes varied along the vegetation physiognomies, with the S3 state yielding the lowest number (2205 in S0, 2785 for S1, 2146 for S2, and 741 in S3). Notably, more than 80% of edges revealed strong links in each state (Table S2). The ratio of total edges to nodes was the lowest in S3 (5.15). In addition, co-occurrence networks of S1 and S2 had higher density and transitivity (tendency of the nodes to cluster together) than S0 and S3 (Table S2), suggesting that the OTUs in the S1 and S2 networks were connected more closely than S0 and S3.

We detected 14 cut-off OTUs connecting the network structure; one OTU belonged to Betaproteobacteria in S0; two OTUs of Actinobacteria, one of the Order Ellin6513 (Acidobacteria), and one of *Burkholderia* (Betaproteobacteria) in S1; two OTUs of *Clostridium* (Firmicutes), one of Ellin6513, and one of Oxalobacteraceae (Proteobacteria) in

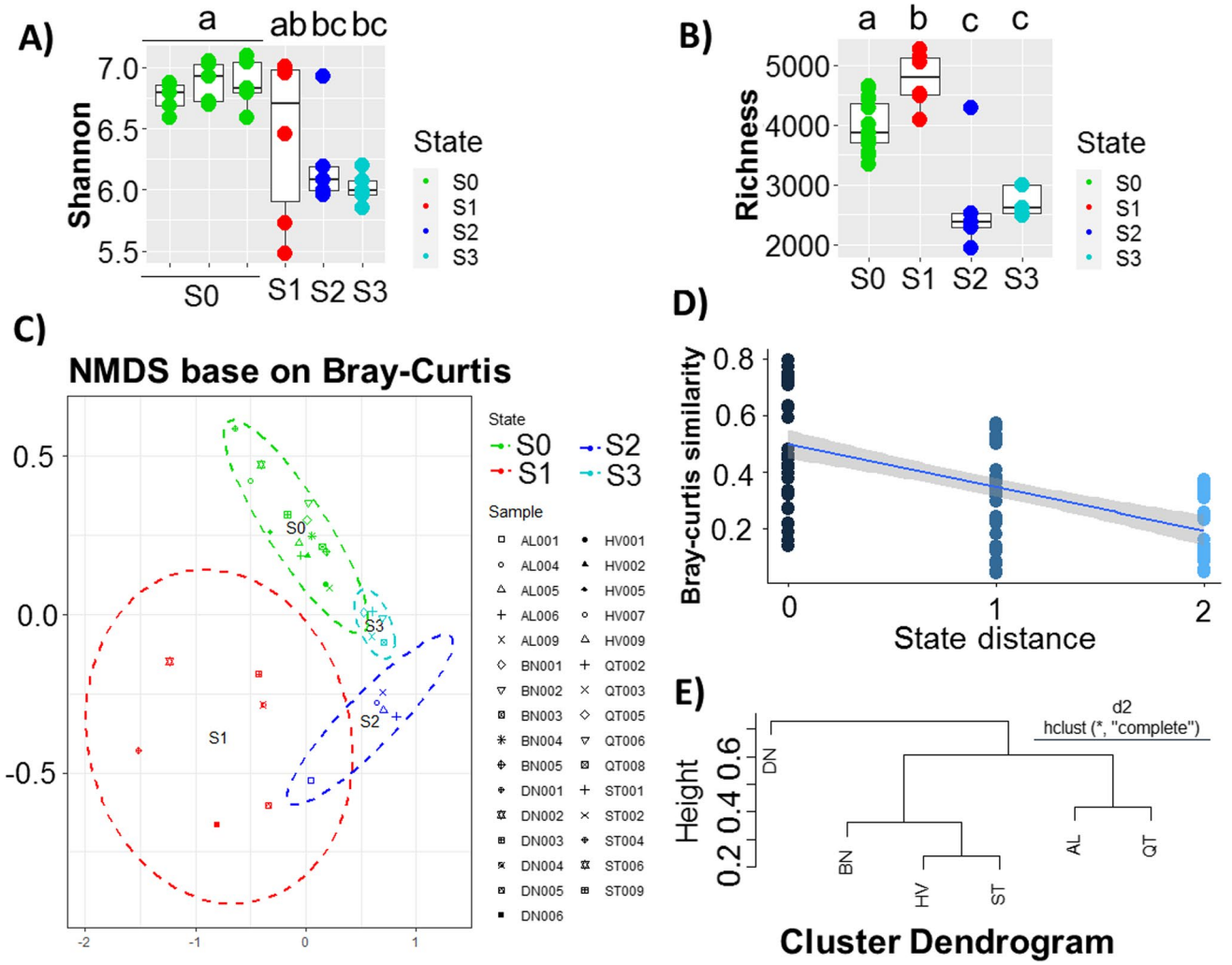


Fig. 3 Bacterial community structure in central Vietnam across vegetation physiognomies. **A** Shannon index of six sites. Letters indicate the statistical differences among sites. **B** OTU richness. **C** NMDS plot for 31 samples showing groups of bacterial communities in four vegetation physiognomies in Central Vietnam. **D** State distance analysis showing the correlation between similarity of bacterial communities and difference in states. Along the x-axis: "0" indicates the distance between samples belonging to the same state; "1" shows the distances of S1 vs. S2 and S2 vs. S3; "2" indicates the distance of S1 vs. S3. **E** Hierarchical cluster analysis based on the Pearson's correlation between the six sites. Vegetation physiognomies are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3)

S2; and two of *Methylocadum* and Oxalobacteriaceae (Proteobacteria), one of Firmicutes, one of Actinobacteria, and one of Nitrospira in S3 (Table S2b).

Table 3 Bacterial diversity of soils in four succession states. Succession stages are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3).Shannon and Chao indices

States	Average ± S.E	
	Shannon	Chao1
S0	6.8 ± 0.2	3975.1 ± 409. 8
S1	6.4 ± 0.6	4765.4 ± 422. 6
S2	6.2 ± 0.4	2683.2 ± 824.6
S3	6.0 ± 0.1	2724.9 ± 227.0

Discussion

Bacterial Communities Contaminated with TCDD

Agent Orange contamination in Central Vietnam can be dated back to the US–Vietnam War in 1961–1972 [1]. Chemical analyses revealed that residual dioxins remained in the S1 and S2 states, while the TCDD concentration in S3 state approximated that in undisturbed soil (Fig. S1b), supporting the assumption that vegetation physiognomies were highly associated with the concentrations of dioxins. This pattern, concordant with a previous finding [8], further supported the reasoning behind utilizing vegetation physiognomy as an indicator of the disturbance/recovery status,

Table 4 Bacterial diversity of soils in four succession states. Succession stages are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3).Diversity indices between soil states

Comparison pair	*P*-value	
	Shannon	Richness (S.chao1)
S0–S1	0.381	0.003***
S0–S2	0.019*	0.010*
S0–S3	0.000***	0.000***
S1–S2	0.537	0.009***
S1–S3	0.429	0.004***
S2–S3	0.421	0.310

Asterisks indicate significant pairwise differences (Mann–Whitney tests: * for $p < 0.05$ and *** for $p < 0.01$, respectively)

although temporal transitions among physiognomies were not observed directly. That is, vegetation physiognomies across the sites can be a wealth indicator of ecological succession. Accordingly, the states S1 to S3 tend to represent different recovery statuses. It is understood that various concentrations of Agent Orange were sprayed by the US Air Force across the landscape, leading to a mosaic-like recovery of vegetation ever since. Therefore, S0 tended to represent the undisturbed stage prior to the spraying of Agent Orange, S1 represented the barren land stage following dioxin spraying, while S2 and S3 stood for different developing stages, from grassland to woods (Fig. 1).

Table 5 Bacterial diversity of soils in four succession states. Succession stages are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3).Diversity indices between sample sites

Comparison pair	*p*-value	
	Shannon	Richness (S.chao1)
BN–DN	1.000	0.004***
HV–DN	0.329	0.082
ST–DN	0.329	0.052
BN–AL	0.151	0.151
HV–AL	0.056	0.056
ST–AL	0.056	0.056
BN–QT	0.008***	0.008***
HV–QT	0.008***	0.008***
ST–QT	0.008***	0.008***
DN–AL	0.537	0.009***
DN–QT	0.429	0.004***
AL–QT	0.421	0.310

Asterisks indicate significant pairwise differences (Mann–Whitney tests: * for $p < 0.05$ and *** for $p < 0.01$, respectively). *AL*, Asho-A Luoi Air Base; *BN*, Ba Na; *DN*, Da Nang Air Base; *HV*, Hai Van; *QT*, Quang Tri

According to official documents, the long-lasting pollution of Agent Orange altered the biophysical chemistry properties of the soil, e.g., low nutrients and a low pH, compared to undisturbed soil (Tables 1 and 2) [3, 8], leading to changes in carbon and nitrogen sources for microorganisms. Inevitably, these changes would largely affect the microbial community, as indicated by the differences between S0 and S1 states, which represent the bacterial community changes after contamination (Figs. 3b–c, 4, 5, and S3). Of the three dominant phyla, Firmicutes (4% vs 23%) and Actinobacteria (12% vs 28%) markedly increased in S1 (both $p < 0.01$), while Acidobacteria (9% vs 22%) decreased ($p = 0.001$) (Fig. 4). Of the top 10 phyla, Planctomycetes, Acidobacteria, and Bacteroidetes were found to be negatively correlated with TCDD, while only Firmicutes showed a positive correlation with TCDD (Fig. S2b). Soil types may also differentiate the microbiomes between S1 and the others.

At the generic level, the abundance of *Bacillus* and *Clostridium* (Firmicutes) increased largely from S0 to S1, with frequencies of 2–5% and 0.1–3%, respectively. Nearly 50% of the predominant OTUs (> 1% relative abundance) showed a significant correlation with the concentrations of TCDD ($p < 0.05$) (Table S1). Of these, 20 OTUs showed positive correlations (33%) whereas the remaining OTUs showed negative correlations (15%). Among positively correlated OTUs, 10 Actinobacteria (50%, including *Arthrobacter* and *Rhodococcus*), seven Proteobacteria (35%, Oxalobacteraceae, Comamonadaceae, and *Methylocaldum*), and three Firmicutes (15%, Bacillales) were identified (Table S1). Remarkably, dioxin digesters of *Arthrobacter*, *Rhodococcus*, *Commanodaceae*, and *Bacialles* were detected in S1 with high concentrations of TCDD [24]. In contrast, most negatively correlated OTUs belong to Proteobacteria, including many symbiotic bacteria with plant roots, and Acidobacteria, which are actively involved in plant-bacteria interactions [25–27] (Table S1). The decreasing trend indicated that the long-term residue of dioxins was likely the cause of the decline of microorganisms that actively interact with plants.

The existence of private and discriminant clades in LEfSe analysis (Fig. 5) as well as the co-occurrence network (Fig. S3) revealed that the disturbances of dioxins have shaped distinct communities between S0 and S1. Specifically, the co-occurrence connections between microorganisms were more intense in the state with a high level of TCDD (S1) (Fig. S3), suggesting a stronger coherence of bacterial communities in S1 than that in S0. These changes in composition and taxonomic diversity suggested that dioxins must have played a role in the re-establishment of microorganisms in the post-contamination period.

Given the harsh nature of TCDD, higher concentrations of dioxins would constrain the growth and survival of many

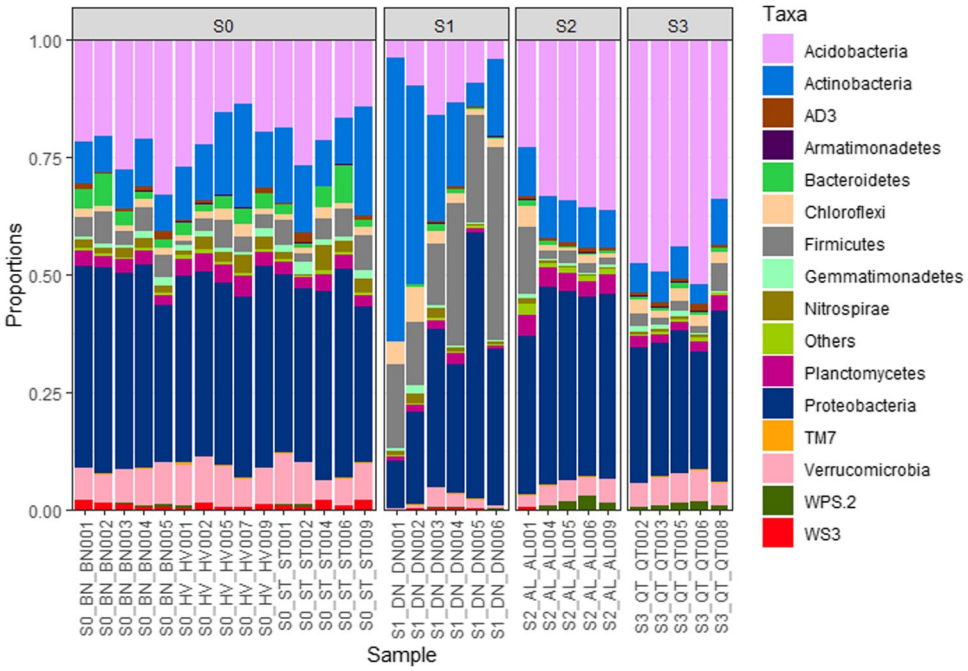


Fig. 4 Distribution of relative abundance of bacterial phyla across samples and states of different vegetation physiognomies. Vegetation physiognomies are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3)

bacteria, likely resulting in lower bacterial diversity. Nevertheless, there was no diversity reduction identified from S0 to S1 (Fig. 3a). Approximating loss and gain may explain such an unusual result. That is, the number of lost bacteria approximated that of colonizers in S1. A high concentration of dioxins is harmful and results in the elimination of some bacteria, yet these so-called "toxins," as energy sources, may also attract some other adaptive bacteria.

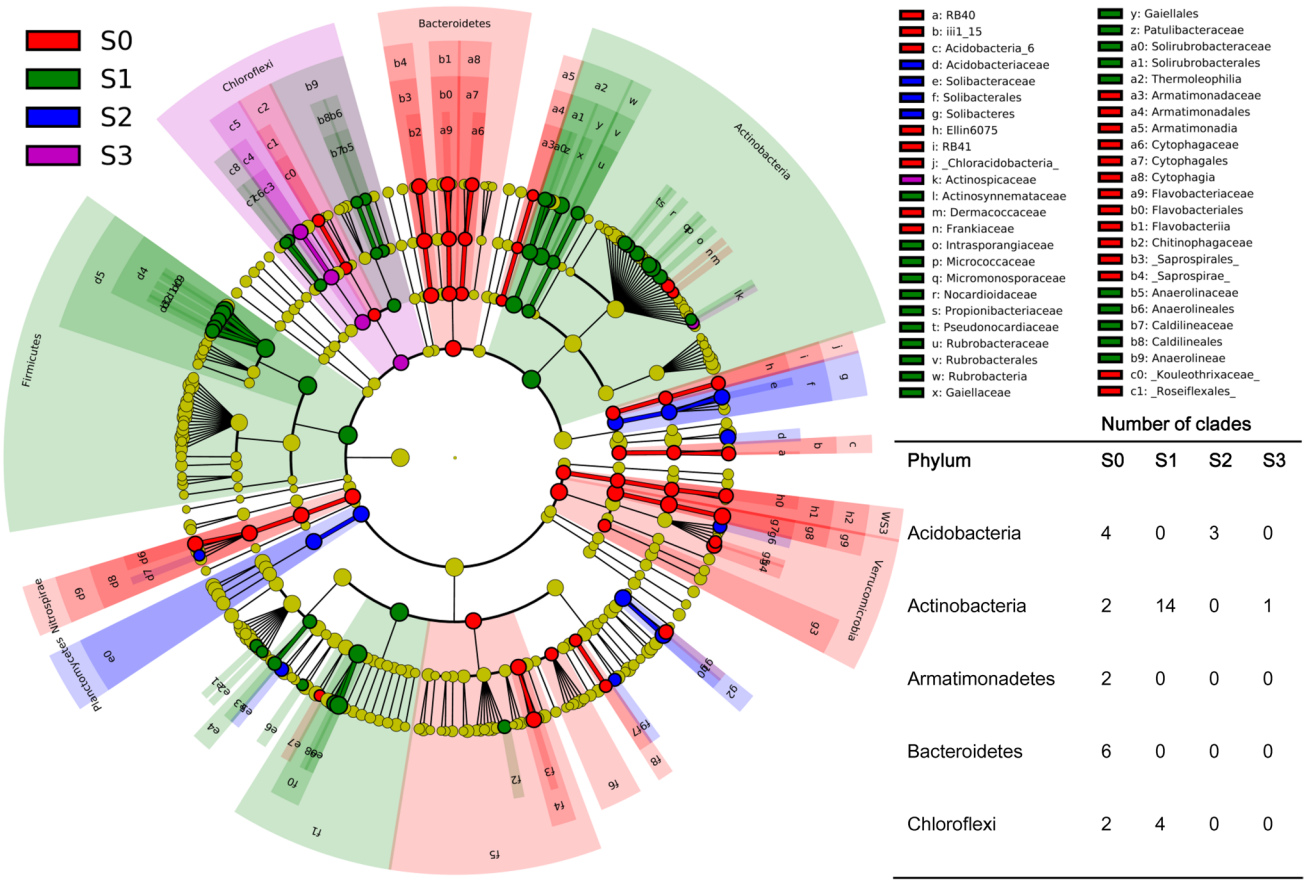
The top six unique OTUs abundant in S1 (each OTU > = 100 reads, summing up to 20% total reads) were all Actinobacteria. Among unique bacteria with lower abundances (top 20 with 25% total reads), twelve OTUs of Actinobacteria, three OTUs of Firmicutes, three OTUs of Proteobacteria, and two OTUs of Chloroflexi were identified. Of the bacteria related to biological treatments, *Arthrobacter* and *Rhodococcus* (Actinobacteria) are dioxin-degradation bacteria [28], Dehalococcoidales (Chloroflexi) are anaerobic bacteria relevant to halogen cycling and bioremediation [29], *Desulfosporosinus* (Firmicutes) are sulfate reducers thriving in terrestrial environments and able to degrade toluene [30], and Actinobacteria have considerable potentials for the biotransformation and biodegradation of pesticides with widely different chemical structures [28, 31]. Here, these bacteria distributed in the soil with high levels of TCDD represent organisms adapting to extreme environments.

In addition, bacterial communities in S1 displayed greater species richness (Fig. 3b), density, and co-occurrence rates (Table S2) than those in S0. The results indicate that microorganisms in a microbial community with stronger correlative relationships in S1 tended to survive better in environments with carbon and nitrogen sources derived from the breakdown of TCDD [32]. However, TCDD breakdown takes a long time and requires numerous biochemical steps that require the participation of various microorganisms. Products broken down by a group of microorganisms in a previous step can become food sources for bacteria in the next step [32], reflecting a symbiotic relationship. The process takes place until TCDD is entirely resolved. These actions in the bacterial community form mutually beneficial associations, which might promote the breakdown of dioxins/TCDD.

Back to Nature: Long Recovery from Dioxin Pollution

Over time, under the influence of natural factors such as sunlight, erosion, and organism activity, the process of dioxin degradation occurs [3, 8]. Photo-degradation is likely a main process, particularly given the intense sunlight in Central Vietnam. The reduction of dioxin concentrations at contaminated sites eventually lead to a return to its original state, a sequence as observed in sites at different phases [8, 9, 11]. After approximately 50 years, the succession of native plants negatively responded to different levels of dioxins contamination [8, 11]. These observations in a relatively small ecological zone (Central Vietnam) provided an excellent opportunity for studying the dynamics of bacteria in the long-term effect of dioxins/TCDD contamination, as the four states were established (S0, S1, S2, and S3).

We have noticed that the microbial community has changed dramatically in response to the extreme dioxins/TCDD contamination (S1). The vegetation physiognomies



Number of clades

Phylum	S0	S1	S2	S3
Acidobacteria	4	0	3	0
Actinobacteria	2	14	0	1
Armatimonadetes	2	0	0	0
Bacteroidetes	6	0	0	0
Chloroflexi	2	4	0	0

Fig. 5 Discriminant taxa identified by LEfSe analysis for the four states. Different colors of the cladogram represent discrimant taxa of different states. Vegetation physiognomies are defined as forest (S0), barren land (S1), grassland (S2), and developing woods (S3)

represent different succession states likely disturbed by dioxin contamination. A dramatic change from S1 to S2 vs. a gradual change from S2 to S3 was detected in the microbial community (Figs. 3a–e, 4, and S3), as indicated by a large decline in biodiversity indices from S1 to S2 (Fig. 3a–b, $p = 0.5$). Co-occurrence network (Fig. S3), Bray–Curtis similarity (Fig. 3d), and HCA (Fig. 3e) analyses also showed a sharp difference between S1 and S2 and high similarities between S2 and S3.

The decrease in the number of discriminant taxa from S1 (18 clades) to S2 (3 clades) and S3 (1 clade) was also evident for changes in the bacterial community along succession (Fig. 5), except for AL01, a sample of S2 sharing discriminant taxa with S1 and S2 as revealed by NMDS analysis. Seemingly, AL01 with higher concentrations of the TCDD displayed a delayed recovery and likely represented a transition between S1 and S2. A rational explanation for this phenomenon is that dioxins are determinants of microbial assembly as discussed in the S0 and S1 comparison. It means that the facilitated dioxin degradation in S2 and S3 (as suggested by the much lower level of TCDD than in S1) substantially changed the microorganism community composition. Accordingly, the exceptionally high concentrations of TCDD in S1 differentiated the microbes from those in S2 and S3. It is very probable that the much lower dioxin levels in the soil led to similar microbial compositions between S2 and S3.

Revegetation provides another opportunity for soil microbial community changes to emerge between S2 and S3, mostly via reciprocal causation, although such changes occur gradually compared to the drastic differences between barren lands and grassland (S1 vs. S2). This explanation is supported by the increasing abundance of *Acidobacteria* in S2 and S3, a taxon deemed to play a significant role in plant-microbial interactions in forest restoration [27].

Delay in Bacterial Recovery or Permanent Change

The microbial community dramatically changed after heavy contamination of dioxins and gradually recovered along with vegetation restoration. Comparing the bacterial communities between S3 and S0 revealed that the microbial composition tended to recover, based on microbial distribution analyses

(Fig. 4), as revealed by NMDS analysis (Fig. 3c) and shared bacterial co-network topology (Fig. S3) (cf. [32]). Nevertheless, bacterial community recovery may have been hindered, as indicated by the lower bacterial diversity in S3 compared to S0 (Fig. 3a, Table 3, 4, and 5, p-value < 0.001). This difference is likely attributable to the loss of bacterial taxa (up to 3898 OTUs) and the recruitment of fewer colonizers (265 OTUs).

The bacterial community in S3 showed a lower number of nodes, edges, and density than S0 in the co-occurrence network analysis (Table S2), suggesting that the long-term impact of dioxins has disrupted physiological correlations between bacterial species in the community. These differences in the co-occurrence network revealed a clear difference in the microbial communities between S3 and S0. After severe ecological disturbance, it is assumed that the soil microbes tend to recover at slower rates. Metagenomics analysis revealed that dioxins dramatically changed the soil bacteria and without a doubt posed deleterious effects on the environments.

Conclusion

This is the first comprehensive study on the microbial changes after long-term chemical disturbance by dioxins in Vietnam. Our results showed that the complexity of vegetation physiognomies was negatively associated with the level of residual dioxins. The long-term exposure of dioxins impacted the entire microbial community. Specifically, the abundance of gram-positive Firmicutes and Actinobacteria tended to increase with dioxin concentration, while Acidobacteria, Planctomycetes, and Bacteroidetes gradually recovered as the concentration of TCDD reduced. This indicated that the dioxin presence is a key factor regulating the re-establishment of microbial communities. In terms of the impacts of long-term dioxin-induced disturbance, the microbial community has gradually recovered owing to forest succession and biodegradation. High concentration of the TCDD not only attracted dioxin digesters but also recruited subsequent microbial members that lived on the digested compounds. This revealed a dynamic equilibrium with a birth-and-loss pattern characterized by microbial competition. In such a dynamic process, Acidobacteria appeared to play a key role in forest succession. Our results provide insight into the microbial fluctuations along the trajectory of vegetation recovery following dispersal of the Agent Orange during the US–Vietnam War.

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Author Contribution Huyen-Trang Tran: investigation, sampling, experiments, methodology, analysis of data, conceptualization, and writing—original draft and editing; Minh-Hung Nguyen: sampling; Nguyen-Thi-Minh-Hue: sampling; Chieh Chang, Wei-Ling Huang, and Chao-Li Huang: analysis data, data curation, methodology, conceptualization, review, and editing; Tzen-Yuh Chiang: conceptualization, supervision, writing—review and editing, and fund acquisition.

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Data Availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Code Availability All code generated or analyzed during this study are included in supplementary information files.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing interests The authors declare no competing interests.

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