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PII: S0304-3894(20)31996-8

DOI: <https://doi.org/10.1016/j.jhazmat.2020.124006>

Reference: HAZMAT124006

To appear in: *Journal of Hazardous Materials*

Received date: 29 June 2020

Revised date: 1 September 2020

Accepted date: 13 September 2020

Please cite this article as: Hui-Juan Xu, Jing Bai, Wenyan Li, J. Colin Murrell, Yulong Zhang, Jinjin Wang, Chunling Luo and Yongtao Li, Mechanisms of the enhanced DDT removal from soils by earthworms: identification of DDT degraders in drilosphere and non-drilosphere matrices, *Journal of Hazardous Materials*, (2020) doi:<https://doi.org/10.1016/j.jhazmat.2020.124006>

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Mechanisms of the enhanced DDT removal from soils by earthworms: identification of DDT degraders in drilosphere and non-drilosphere matrices

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Abbreviations

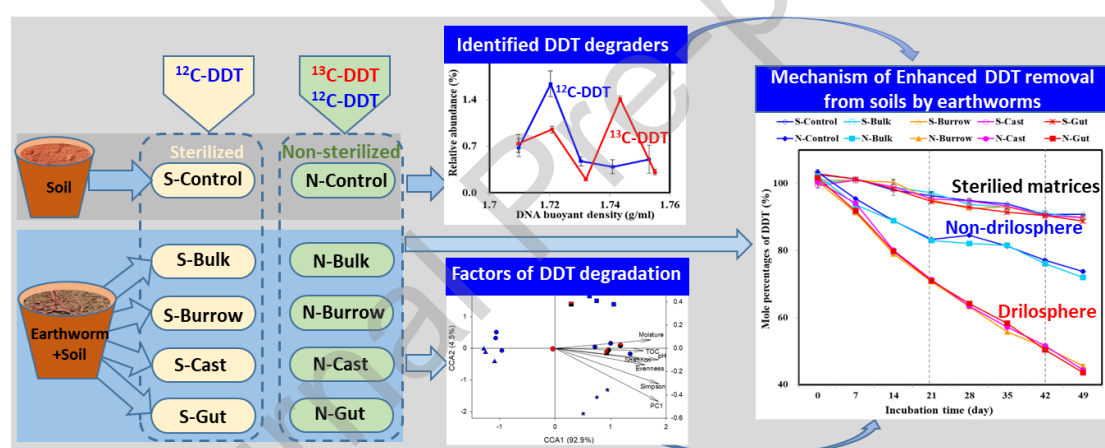
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
DDD	1,1-dichloro-2,2-bis(4-chlorophenyl)ethane
DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene
DDMU	1-chloro-2,2-bis(4-chlorophenyl)ethylene
DBP	4,4-dichlorobenzophenone
DDTs	DDT and its metabolites
Dicofol	2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol
TOC	Total organic carbon
CCA	Canonical correspondence analysis
PCA	Principal component analysis

Abstract

The remediation of soil contaminated by 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) remains an important issue in environmental research. Although our previous studies demonstrated that earthworms could enhance the degradation of DDT in soils, the underlying mechanisms and microorganisms involved in these transformation processes are still not clear. Here we studied the transformation of DDT in sterilized/non-sterilized drilosphere and non-drilosphere matrices and identified DDT degraders using the technique of

DNA-stable isotope probing. The results show that DDT degradation in non-sterilized drilosphere was quicker than that in their non-drilosphere counterparts. Earthworms enhance DDT removal mainly by improving soil properties, thus stimulating indigenous microorganisms rather than abiotic degradation or tissue accumulating. Ten new genera, including *Streptomyces*, *Streptacidiphilus*, *Dermacoccus*, *Brevibacterium*, *Bacillus*, *Virgibacillus*, were identified as DDT ring cleavage degrading bacteria in the five matrices tested. *Bacillus* and *Dermacoccus* may also play vital roles in the dechlorination of DDTs as they were highly enriched during the incubations. The results of this study provide robust evidence for the application of earthworms in remediating soils polluted with DDT and highlight the importance of using combinations of cultivation-independent techniques together with process-based measurements to examine the function of microbes degrading organic pollutants in drilosphere matrices.

Graphical abstract



Keywords: Degradation, dechlorination, ring cleavage, DNA-SIP, remediation

1. Introduction

Despite having been banned in most countries for about 40 years, the residues of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) are still widely distributed in a number of different soils (Chen et al., 2005, Li et al., 2006, Yu et al., 2014), sediment (Gao et al., 2015), water (Yu et al., 2014) and air (Qiu et al., 2004) due to its wide use in the 20th century, persistent stability (Jiang et al., 2009) and long-range transport (Sheng et al., 2013). Nowadays, DDT is still being used to control vector-borne diseases (e.g. malaria) in some developing countries. In addition, dicofol and antifouling agents have also added fresh inputs of DDT to the environment (Li et al., 2006, Qiu et al., 2005, Tao et al., 2020, Yu et al., 2020). Considering the current DDT contamination in soil, its high toxicity and bioaccumulation, the remediation of DDT in contaminated soil remains a key research priority for environmental scientists (Kang et al., 2019).

To eliminate soil DDT, many methods have been explored, including biodegradation (Xu et al., 2019), photochemical reactions (Quan et al., 2005), electrochemical processes (Sudharshan et al., 2012), high-temperature calcinations (Yan et al., 2014), zero-valent metals (Kang et al., 2018). Of these methods, biodegradation, especially *in situ* microbial degradation by indigenous microorganisms, is regarded as one of the most promising detoxifying technologies because of its low cost, environment-friendly effects and low secondary pollution (Abbes et al., 2018, Purnomo et al., 2011). However, natural biodegradation of DDT in soils has been found to be very slow (Mansouri et al., 2017). Therefore, to improve the bioremediation efficiency of DDT contamination in soil, researchers have focused efforts on the enhancement of *in situ* degradation of DDT in soil. Our previous studies clearly showed that earthworms could enhance the removal of DDT from soils (Lin et al., 2012, Xu et al., 2019). Although it has been proposed that earthworms can accelerate soil DDT degradation, both by intestinal digestion and stimulating the microbial degradation of DDT by indigenous microbes (Xu et al., 2019), the removal efficiency is sub-optimal and the underlying mechanisms involved in these processes are still not well known.

In soils, the complete mineralization of DDT to CO₂ or CH₄ requires two processes: dechlorination and ring cleavage. Ring cleavage has been regarded as the key process that limits the complete degradation of DDT. Most previous studies on soil DDT degradation have concentrated on the dechlorination processes. DDT could be reductively dechlorinated to 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD) under anaerobic conditions, or transformed to 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE) through biotic dehydrochlorination or photochemical degradation under aerobic environments (Sudharshan et al., 2012). DDD and DDE could be degraded to

1-chloro-2,2-bis(4-chlorophenyl)ethylene (DDMU) via dehydrochlorination and reductive dechlorination, respectively, which are further transformed into DBP by a series of dehydrochlorination, reductive dechlorination and oxidation-reduction reactions (Sudharshan et al., 2012). The main pathways of DDT degradation are shown in Fig. S1 and Fig. S2. However, the dechlorinated degradation products are still toxic, easily bio-accumulated (Guo et al., 2013), and may be more resistant to degradation (Sudharshan et al., 2012). For example, very few bacterial or fungal cultures have been reported to be capable of fully degrading DDE to CO₂ and the reported cultures can only degrade DDE using co-metabolism of biphenyl to obtain the enzymes (biophenyl dioxygenase) required to degrade DDE, while not all microorganisms that produce biophenyl dioxygenase could degrade DDE (Thomas et al., 2008). DDE has been found to be more persistent than DDT in many soils (Boul et al., 1994, Spencer et al., 1996). Therefore, the ring-opening degradation of DDT and underlying mechanism needs further study, which will provide robust data necessary for developing efficient remediation techniques of DDT-contaminated soils. Previous studies on DDT ring-opening have largely focused on use of a few pure cultures, e.g., *Ralstonia eutropha* strain A5 (*Alcaligenes eutrophus* A5), *Pseudomonas* sp. and *Pseudomonas acidovorans* M3GY, which are capable of opening the ring of DDT, DDE and DDD (Hay and Focht 1998, Hay and Focht 2000, Nadeau et al., 1998). Few reports have focused on identifying functional microorganisms involved in DDT ring cleavage in such a complex environment like soil. Our previous studies have shown that the degradation pathways of soil DDT in drilosphere and non-drilosphere matrices were different and that the microbial community composition and activities varied considerably between matrices (Lin et al., 2012, Xu et al., 2019). However, the functional microorganisms involved in the ring fission of DDT in each matrix, the shift of bacterial community composition during the process of DDT degradation, and the microbial mechanisms of enhanced DDT removal by earthworms are still far from clear. A more detailed mechanistic understanding of these microbial processes are essential for developing more effective combinations of techniques to remediate soils contaminated by DDT.

As most microorganisms in soils are difficult to cultivate in the laboratory, the traditional culture-dependent approaches are unable to capture all the active functional microorganisms in a specific environment. This has hindered the development of soil bioremediation techniques. DNA stable isotope probing (DNA-SIP) is a useful method to identify the functional microorganisms *in situ* using stable isotope labelled compounds, and can link microbial identity to ecosystem function (Dumont and Murrell 2005, Neufeld et al., 2007, Song et al., 2015). In the previous research on organic pollutant degradation by bacteria, DNA-SIP has been mainly combined with 16S rRNA gene profiling using T-RFLP, DGGE and clone library analysis (Cheng et

al., 2013, Noguchi et al., 2014, Song et al., 2015). However, due to the limitations of these molecular techniques in cloning and experimental accuracy, there are deviations in the identification of functional microorganisms. In recent years, studies have combined DNA-SIP and high throughput sequencing to overcome these obstacles and show great potential in identifying functional microbes (Li et al., 2019, Lv et al., 2018, Thomas et al., 2019). In order to explore the microbial community of DDT degrading microorganisms in more detail, DNA-SIP combined with Illumina sequencing has been applied in this study to identify DDT degraders in drilosphere and non-drilosphere matrices. The main objectives of this study were to: 1) explore the transformation of DDT in drilosphere and non-drilosphere matrices; 2) identify functional bacteria involved in DDT degradation in different matrices; 3) figure out the further mechanisms of enhanced DDT removal from soils by earthworms.

2. Materials and methods

2.1. Experimental soils and earthworms

The experimental soil was collected from the Arboretum (23°9'33"N, 113°21'22"E) in South China Agricultural University in Guangzhou, China. It was identified as Plinthudult, with a pH of 4.32, organic matter of 29.3 g/kg, total nitrogen of 2.32 g/kg, total phosphorus of 0.46 g/kg and total potassium of 11.4 g/kg. The soil was classified as clay loam based on its mechanical composition (sand 38.7%, silt 33.4% and clay 27.9%) according to the United States Department of Agriculture soil texture triangle standard.

The experimental earthworms *Amyntas robustus* E.Perrier (*A. robustus*) were also collected from the same sites where the soil samples were collected.

2.2. Drilosphere and non-drilosphere matrix collection

The fresh soil was sieved through a 2-mm mesh and stored in a 4°C refrigerator for one week. 2 kg soil was placed into each pot (25 cm high, 14 cm up radius, 10 cm low radius), sealed with black plastic bags with small pinholes, and inoculated with *A. robustus* after incubated for 24 h of 12 h/12 h in light/dark condition. Soil without *A. robustus* (Control, blank soil) was used as a control. Each pot was inoculated with 15 earthworms (total weight of about 8.4 g), and incubated in the dark at 15°C for 30 days. Pots were ventilated every 3 days and sterilized water was added to maintain a water content of 21% (w/w). Worm cast was collected every 3 days when earthworm excrement could be seen on the soil surface. Destructive sampling was conducted after a month. The blank soil (Control) is the soil without earthworms, and the control soil (Bulk) is the soil in the pot with *A. robustus* without intestinal contents, earthworm cast or burrow lining matrices. Drilosphere matrices, including gut (Gut),

cast (Cast) and burrow lining (Burrow), were obtained using methods described in our previous study (Xu et al., 2019). A portion of the collected matrix samples were stored at - 20°C before extracting DNA, while the remainder was stored at 4°C before physico-chemical properties were determined and for subsequent experiments.

2.3. Experimental design

Half of the five collected matrices were sterilized, and half were not sterilized (remained natural). Thus, we obtained ten matrices, including sterilized Control (S-Control), sterilized Bulk (S-Bulk), sterilized Burrow (S-Burrow), Sterilized Cast (S-Cast), Sterilized Gut (S-Gut), non-sterilized Control (N-Control), non-sterilized Bulk (N-Bulk), non-sterilized Burrow (N-Burrow), non-sterilized Cast (N-Cast) and non-sterilized Gut (N-Gut). The details of the experiment are as follows: 1 g of each matrix was put into a 100-mL serum bottle. Then, 10 µL DDT solution of 1 g L⁻¹ was sprayed and mixed thoroughly into the matrices in the serum bottles. The opened bottles were kept on the bench for 6 h to volatilize n-hexane. Basic mineral salt solution (BMSS, 1 g/L (NH₄)₂SO₄, 2.7 g/L KH₂PO₄, 10.955 g/L Na₂HPO₄•12H₂O, 0.03 g/L Ca(NO₃)₂, 0.01 g/L FeSO₄, 0.2 g/L MgSO₄) was added and mixed with the matrices. The mixture were incubated at dark at 25°C, with shaking at 150 rpm. The serum bottles were aerated for 20 min every two days. Destructive sampling was conducted every 7 days to determine soil DDT residues. To identify the functional microorganisms responsible for DDT degradation, treatments with [¹³C]-DDT and non-sterilized matrices were established at the same time (Table 1). When the concentration of DDT decreased to 75% and 50% of the initial added DDT concentration, total DNA was extracted from the matrices to study the overall bacterial community composition and identify the functional microorganisms by combining ultrahigh-speed density gradient centrifugation and high-throughput sequencing.

2.4. Determination of soil DDT and metabolites

Soil DDT and its metabolites (DDTs) were extracted using an ultrasound extracting method with a mixed solution of dichloromethane: hexane (2:1, v/v). The extracted DDTs were then determined by a gas chromatography (GC, Agilent 7890A, USA) equipped with a 63Ni electron-capture detector (ECD) and a HP-5 capillary column (30 m × 320 µm × 0.25 µm, Agilent, USA). The furnace, had an initial temperature of 60°C, which was increased to 140°C at a rate of 30°C/min, which was maintained for 4 min, then increased to 280°C at a rate of 20°C/min and then maintained for 4 min. The inlet and detector temperature was 220°C and 280°C, respectively. The carrier gas was high-purity nitrogen (99.999%). The method recovery of DDT and its metabolites was estimated at approximately 94.6-110.5%.

2.5. DNA extraction and ultra-high-speed centrifugation

Total DNA from the matrices were extracted using the Mobio PowerMax DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. DNA concentration was determined using a ND-1000 spectrophotometer (Nanodrop Technologies-ThermoFisher Scientific, Wilmington, DE). Up to 10,000 ng DNA, in Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)-cesium chloride (CsCl) solution, was put into Quick-Seal polyallomer tubes (13 mm × 51 mm, 5.1 mL; Beckman Coulter), adjusted to a buoyant density (BD) of $\sim 1.77 \text{ g mL}^{-1}$, sealed and centrifuged at $178,000 \times g$ (20°C), for 48 h using an ultracentrifuge (Beckman Optima XPN-100, Beckman Coulter, USA) (Song et al., 2015). Then the centrifuged DNA was fractionated into 22 fractions. Each fraction of the DNA was purified using the Omega MicroElute DNA Clean-Up Kit (Omega Bio-Tek, Norcross, GA, USA).

2.6. PCR and high-throughput sequencing of 16S rRNA genes

The V4-V5 region of the 16S rRNA gene was amplified from all DNA samples using bacterial primers 515F (5'-GTGCCAGCMGCCGCGGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). Forward- and reverse-primers were added with barcodes of six bases at the 5' end to distinguish different samples for subsequent 16S rRNA gene sequence analysis.

Reaction conditions of PCR amplification: 95°C pre-denaturation for 3 min, followed by 35 cycles of 94°C denaturation for 30 s, 50°C annealing for 1 min, 72°C extension for 1 min, and a final 72°C extension for 10 min. The PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek) according to the manufacturer's instructions. The purified PCR product was verified by electrophoresis on a 1.5% (w/v) agarose gel. The concentration of the PCR product was quantified by Synergy H1 (Biotek USA) using a PicoGreen quantitative kit.

The purified PCR products were mixed in equal molar amounts and purified again as follows: adding 5 M NaCl solution (4°C precooling) to be a final concentration of 0.2 M, gently mixing; adding 2.5 volumes of anhydrous ethanol; mixing and precipitating overnight; centrifuging for 1 h at 4°C, $15,000 \times g$; discarding the supernatant, and adding 1 mL 70% (v/v) ethanol, centrifuging for 15 min at 4°C at $15,000 \times g$; discarding supernatant again; drying the DNA pellet; dissolving the DNA in 70 μL ultra-pure water. The mixed and purified samples were then sent to Novogene company for high-throughput sequencing on Illumina HiSeq 2500 platform.

2.7. Statistical analysis

One-way ANOVA ($p < 0.05$) and Independent sample T test ($p < 0.05$) were conducted using PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA). Principal Component Analysis (PCA), Canonical Correspondence Analysis (CCA) and Diversity Index analysis was performed using R software (Team 2019) and the community ecological package-Vegan (Oksanen et al., 2013). Figures were mainly plotted using Microsoft Excel 2013 (Microsoft Corporation, USA) and Sigmaplot 14.0 (Systat Software Inc., San Jose, California, USA). A heatmap was completed using HemI software (Heatmap Illustrator, version 1.0) (Deng et al., 2014).

HTS sequencing (Illumina) results were processed and analyzed using QIIME 2.0 (Quantitative Insights Into Microbial Ecology, <http://qiime.org/>) platform (Caporaso et al., 2010). The analysis process was as follows: (1) removing the low-quality sequences; (2) dividing the operation classification units according to 97% similarity degree to generate OTU tables; (3) removing the chimeras and single sequences in OTU tables, comparing with the sequences in the database, and classifying OTU species according to 97% confidence. The relative abundance of each taxon was calculated as the ratio of the number of sequences aligned to the taxon to the total number of sequences obtained from the sample.

3. Results and Discussion

3.1. Earthworms improve soil properties by forming the drilosphere

Drilosphere matrices had higher ($p < 0.05$) pH, moisture and organic carbon than their non-drilosphere counterparts (Table S1). Gut had the highest pH (5.18 ± 0.04 , average \pm standard deviation, hereafter the same), followed by Cast (4.57 ± 0.04) and Burrow (4.44 ± 0.01). Water content in the matrices presented the following: Gut > Burrow > Cast > Control > Bulk. Organic carbon in Gut was much higher than in Cast and Burrow ($p < 0.05$). In addition, Control had higher pH and moisture relative to Bulk ($p < 0.05$). These results demonstrate that earthworms can improve soil properties by raising the pH of acidic soil, increasing soil organic carbon and changing the water capacity of soils by forming drilosphere.

3.2. Earthworms enhance DDT removal by accelerating biotic degradation rather than abiotic degradation of DDT in soil

DDT residues in different matrices are presented in the form of mole percentages of the initial DDT concentration, which was 10 mg/kg (Fig. 1). The DDT residues in the sterilized group (S-Control, S-Bulk, S-Burrow, S-Cast and S-Gut) were significantly higher ($p < 0.05$) than those in the non-sterilized group (N-Control,

N-Bulk, N-Burrow, N-Cast and N-Gut), and there was no statistical differences between the sterilized matrices (Fig. 1). At the end of the incubation, the DDT content decreased to $90.8 \pm 0.1\%$, $89.9 \pm 0.9\%$, $90.0 \pm 0.5\%$, $89.8 \pm 1.4\%$ and $88.8 \pm 0.6\%$ of the initial concentration (10 mg/kg) in S-Control, S-Bulk, S-Burrow, S-Cast and S-Gut respectively, while only $73.8 \pm 0.8\%$, $72.0 \pm 0.4\%$, $45.7 \pm 0.7\%$, $44.4 \pm 0.8\%$ and $43.6 \pm 0.4\%$ were left in N-Control, N-Bulk, N-Burrow, N-Cast and N-Gut, respectively (Fig. 1). In the non-sterilized group, DDT residues in drilosphere matrices (N-Burrow, N-Cast and N-Gut) were about 26%~30% lower than those in non-drilosphere matrices (N-Control and N-Bulk) after incubation.

The much lower DDT residues in the non-sterilized group compared to those in the sterilized group (Fig. 1) demonstrated the effectiveness of the sterilization and further confirmed our previous results that biotic transformation was the dominant process in DDT reduction in soils and drilosphere matrices (Xu et al., 2019). The abiotic degradation rate of soil DDT was very slow as only about 9.2~11.2% of the initial DDT disappeared after 49 days. The degradation of DDT in the sterilized matrices could be attributed to abiotic degradation of DDT (Sudharshan et al., 2012). As the experiments were conducted in the dark, photolysis may contribute very little to DDT degradation. Therefore, hydrolysis and oxidation-reduction are probably the dominant processes in sterilized drilosphere matrices. The insignificant differences in DDT residues between the sterilized matrices proved that earthworms had no significant influence on the abiotic degradation rates of soil DDT.

In our previous study with living earthworm treatments, although we proposed that earthworms could enhance DDT removal from soils by promoting the indigenous microbial degradation of DDT and intestinal digesting (Xu et al., 2019), we had no direct proof that earthworm did not accelerate the abiotic degradation rates of DDT due to the living earthworms bringing microorganisms into the sterilized soil. In this study, biotic degradation contributed 17.0~19.3% to DDT degradation compared to the contribution of abiotic degradation of 10.3~11.6% in non-drilosphere matrices, while in drilosphere matrices biotic and abiotic degradation contributed 43.3~46.2% and 9.7~13.8%, respectively. These results provide solid and direct proof that earthworms enhance DDT removal by stimulating the biotic transformation of DDT but not the abiotic degradation of DDT.

3.3. Earthworms influence the transforming pathways of soil DDT

The formation of DDT degradation products (metabolites) is also presented in the form of mole percentages of the initial DDT concentration (10 mg/kg), which was calculated based on the concentration and molecular mass of DDTs (Fig. 2). Contrary to the trend of DDT residues in the matrices, the contents of DDD (Fig. 2a), DDE

(Fig. 2b), DDMU (Fig. 2c) and DBP (Fig. 2d) in the matrices from the non-sterilized group were higher than those from the sterilized group throughout the incubation. No DBP was detected in S-Control and S-Bulk, and only a small amount of DBP (0.10 mg/kg) was detected in S-Burrow on the 35th day of incubation (Fig. 2d).

After incubation, mole percentages of DDD in S-Control and S-bulk were $3.4\% \pm 0.8\%$ (0.31 ± 0.07 mg/kg), significantly higher than mole percentages of DDE, which were $2.4 \pm 0.4\%$ (0.22 ± 0.04 mg/kg) and $2.7 \pm 0.3\%$ (0.24 ± 0.03 mg/kg). However, in the sterilized drilosphere matrices, DDE was more abundant than DDD, with DDE at $5.0 \pm 0.2\%$, $3.9 \pm 0.2\%$ and $4.1 \pm 0.1\%$ and DDD at $3.3 \pm 0.4\%$, $2.7 \pm 0.2\%$ and $3.7 \pm 0.4\%$ in S-Burrow, S-Cast and S-Gut respectively. This infers that earthworms may exert an influence on the abiotic DDT transformation pathway, despite showing no difference in DDT abiotic degradation rates (Fig. 1). The fact that more DDT was transformed to DDE than to DDD in sterilized drilosphere matrices may be because earthworms increased soil pH, which could influence the abiotic hydrolysis of DDT or it could be due to the higher organic matter in drilosphere matrices which changed the oxidation-reduction environment (Sudharshan et al., 2012). The abiotic hydrolysis of DDT is strongly pH dependent. It has been reported that increase in pH enhanced the conversion of DDT to DDE at the expense of DDD (Nash et al., 1973). Organic compounds usually contain reduced forms of carbon, favouring the oxidation reaction of DDT, and thus influencing the oxidation-reduction environment.

In the non-sterilized group, the contents of DDD, DDE and DDMU in drilosphere matrices (N-Burrow, N-Cast and N-Gut) were higher than those in their non-drilosphere counterparts (N-Control and N-Bulk). DDD was the dominant product in N-Control, N-Bulk, N-Cast and N-Gut throughout the incubation period, while DDE was the most abundant product in N-Burrow during the 28th to 35th days of the incubation. After incubation, about $12.9 \pm 0.1\%$ (equals 1.16 ± 0.01 mg/kg DDE) of the initial DDT was transformed into DDE in N-Burrow, which was significantly higher than those in other matrices. The concentrations of DDMU in N-Gut and N-Cast were higher than that in the N-Burrow (Fig. 2c). The concentrations of DBP in N-Burrow, N-Cast and N-Gut reached $3.6 \pm 0.3\%$ (0.25 ± 0.02 mg/kg), $3.7 \pm 0.7\%$ (0.26 ± 0.05 mg/kg) and $5.2 \pm 0.3\%$ (0.37 ± 0.02 mg/kg), respectively at the end of the incubation, which were much higher than those in the non-drilosphere matrices (Fig. 2d).

The degradation of DDT and its metabolites was rather slow. In the sterilized matrices, over 96% of the initial DDT presented in the form of DDT, DDD and DDE (Fig. 3a), and over 97.5% presented in the form of DDT, DDD, DDE, DDMU and DBP (Fig. 3b), while in the non-sterilized matrices, over 72% of the initial DDT presented in the form of DDT, DDD and DDE (Fig. 3a), and over 85% presented in

the form of DDT, DDD, DDE, DDMU and DBP (Fig. 3b) at the end of the incubation. The total amounts of DDT, DDD, DDE, DDMU and DBP fluctuated during the incubation as there were probably other DDT intermediate metabolites that were not detected in this study.

All the results described above show that earthworms not only enhanced DDT degradation, but also shifted its transformation pathways and affected the further degradation of its products. DDD have been reported to be the dominant products of DDT in soils (Aislabie et al., 1997, Xu et al., 2019). In this current study, the higher percentage of DDE than that of DDD in N-Burrow shows that earthworms could change the dominant reductive dechlorination of DDT in soils to the aerobic dehydrochlorination, which would be helpful for the thorough mineralization of DDT (Sudharshan et al., 2012). This could be attributed to the improved soil structure induced by earthworms in burrows (Devliegher and Verstraete 1997), which provides more of an aerobic environment for DDT degradation. In addition, although there were no significant differences in DDT contents between the non-sterilized drilosphere matrices, N-Cast have significant lower total amounts of DDT, DDD and DDE at the 35th and 49th day of the incubation (Fig. 3a), and much lower total amounts of DDT, DDD, DDE, DDMU and DBP than N-Burrow and N-Gut (Fig. 3b). This means that the cast provides more appropriate condition for the further degradation of DDT products. Therefore, cast from earthworm producing factories could be very useful material to stimulate the bioremediation of soil contaminated by DDT.

3.4. Response of bacterial community structure to DDT stress

To further explore the microbial mechanisms of the effect of earthworms on DDT degradation, soil bacterial composition were studied. Based on the measurements of DDT residues in the matrices, the total DNA of N-Control, N-Bulk, N-Burrow, N-Cast and N-Gut was extracted on day 0, day 21 and day 42 during the incubation, 16S rRNA gene sequences were obtained by PCR and sequenced on an Illumina platform (Hiseq 2500) to study the shifts in bacterial community composition in matrices during the incubation.

Shannon index, Pielou's evenness index, Species richness index and Simpson index were calculated using the relative abundance of each taxa at the genus level as shown in Fig. 4. The diversity index, evenness index, richness index and dominance index of N-Control, N-Bulk, N-Burrow and N-Cast decreased, while these indices of N-Gut increased with the incubation time. Before incubation, all the α -diversity indices of N-Gut were significantly lower than those of N-Control, N-Bulk, N-Burrow and N-Cast, while after incubation, all the indices of N-Gut were significantly higher

than those of the other matrices. Relative to non-drilosphere matrices, drilosphere matrices had higher Shannon index, Pielou's evenness index and Simpson index after incubation.

Principal component analysis was conducted to study the β -diversity of the bacterial composition during the incubation (Fig. S3). The incubation with DDT shifted soil bacterial community composition dramatically as samples from day 0, day 21 and day 42 scattered distantly along the first component axis, which explained 57.2% of the total variance (Fig. S3a). Before incubation, the bacterial composition in Gut was quite different from the other matrices, and the bacterial composition in Control was similar with that in Bulk, while Cast shared more in common with Burrow (Fig. S3b). After incubation, the samples of Control, Bulk and Burrow clustered together, distant from both Cast and Gut (Fig. S3d).

To find out the possible functional bacteria involved in DDT degradation, 35 top abundant genera, whose relative abundances accounted for >62% in 0d-matrices, >92% in 21d-matrices and >99% in 42d-matrices except 42d-Gut (53.7%), were selected to make heatmaps (Fig. 5). Before incubation, dominant bacteria were mainly from the phyla of Proteobacteria, Acidobacteria, Bacteroidetes and Chloroflexi. More abundant genera in 0d-Control were from Acidobacteria, while more abundant genera in 0d-Burrow and 0d-Cast were from Proteobacteria. N-Burrow and N-Cast shared more genera, while N-Bulk shared many abundant bacteria both with N-Control and N-Burrow. The dominant taxon in N-Gut was Unclassified *Enterobacteriaceae*, with a relative abundance of $96.9\% \pm 0.5\%$. After incubated for 21 days, the dominant taxa in all the five matrices shifted dramatically compared with those on day 0, mainly falling into the phyla of Firmicutes and Actinobacteria. *Dermaococcus*, *Streptomyces*, *Bacillus*, Unclassified *Sporolactobacillaceae* and Unclassified *Bacilliales* were enriched in all matrices during the incubation, among which Unclassified *Sporolactobacillaceae* was the most enriched, with relative abundances of $81.8\% \pm 5.1\%$, $48.3\% \pm 2.5\%$, $50.1\% \pm 1.2\%$, $36.8\% \pm 3.9\%$ and $64.5\% \pm 0.4\%$ in 21d-Control, 21d-Bulk, 21d-Burrow, 21d-Cast and 21d-Gut, respectively. The dominant taxa in the matrices at the 42nd day of the incubation were similar with those at the 21st day of the incubation with slight changes. Unclassified *Sporolactobacillaceae* was highly enriched in 42d-Control ($90.2 \pm 4.4\%$), 42d-Bulk ($83.5 \pm 8.1\%$), 42d-Burrow ($77.8 \pm 3.1\%$) and 42d-Cast ($38.1 \pm 2.7\%$), but not in 42d-Gut ($1.5 \pm 1.3\%$) anymore. After incubation, many more genera were abundant in 21d-Gut and 42d-Gut than in the other four matrices, an observation which was supported by the increased α -diversity, richness and evenness of N-Gut after incubation (Fig. 4). This could be attributed to the ample and diverse carbon in the earthworm gut that might provide a variety of carbon sources for more bacteria.

3.5 Bacteria involved in the ring cleavage of DDT

In order to identify the bacteria involved in opening the aromatic ring of DDT, matrices were collected from treatments of ^{13}C labelled DDT and ^{12}C -DDT control for DNA extraction when the DDT concentration in the drilosphere matrices was 75% of their initial concentration (21 d). Then DNA was fractionated by ultra-high-speed centrifuging with CsCl and PCR amplicons of 16S rRNA genes present in these DNA samples were sequenced on an Illumina platform (Hiseq 2500). The relative abundance of genera in ^{12}C -DDT and ^{13}C -DDT treatments (as estimated by relative abundance of 16S rRNA genes present in each sample) were plotted as a function of DNA buoyant density. Genera that have obvious differences between ^{12}C -DDT and ^{13}C -DDT treatments or which had a much higher relative abundance in the heavier fraction of ^{13}C -DDT treatment, were considered to be bacteria that could potentially assimilate ^{13}C -DDT by opening the ring of DDT (Fig. S4 to Fig. S8). Ten genera from three orders of Actinomycetales, Bacillales and Xanthomonadales were identified as potential ring-fission DDT degraders from the five matrices (Table 2), among which, *Dermacoccus* and *Unclassified Bacillales* were identified in four and three matrices, respectively.

Four genera (*Streptomyces*, *Streptacidiphilus*, *Dermacoccus* and *Brevibacterium*) from the order of Actinomycetales were identified as possible DDT ring cleavage degraders (Table 2 and Fig. S4 to Fig. S8). Many pure culture studies have shown that *Streptomyces* can degrade DDT (Benimeli et al., 2003), catalyze the oxidative degradation of dichlorophenol by producing extracellular lignin peroxidase (Yee and Wood 1997), degrade HCH by the production of dechlorinase (Cuozzo et al., 2009). However, it has not been documented whether *Streptomyces* are capable of opening the ring of DDT. It is known that the ring cleavage of DDT, DDD, DDE and DBP all needs to be catalyzed by a dioxygenase enzyme. Studies have shown that *Streptomyces* carries a gene *bphA1*(2072) encoding a biphenyl dioxygenase, which can hydroxylate flavanone to 2,3-dihydroxyflavanone (Chun et al., 2003). This is similar to the hydroxylation to 2, 3-dihydroxy-DDT catalyzed by biphenyl dioxygenase of *Pseudomonas*, followed by the meta-cleavage of DDT (Kamanavalli and Ninnekar 2004). Moreover, some studies have shown that *Streptomyces* contains genes encoding a catechol 1,2-dioxygenase, which can degrade phenol and benzoic acid through ortho-cleavage (An et al., 2001). Therefore, *Streptomyces* may have the ability to participate in the whole degradation process of DDT dechlorination and ring fission, and could well be an important indigenous DDT degrader in soils.

Streptacidiphilus and *Streptomyces* belong to the same family, Streptomycetaceae. Although no research has yet shown that *Streptacidiphilus* can participate in DDT degradation, it could be concluded from its phylogenetic

relationship with *Streptomyces* and these results in this study that it might be involved in degradation of DDT by ring opening.

Dermacoccus is an important putative ring cleavage bacterium in Bulk, Burrow Cast and Gut, and had very high relative abundance in the total bacterial community present in these experiments (Fig. 5). Although it is widespread in the environment, the only reported study we are aware of on the degradation of organic pollutants by *Dermacoccus* is decolorization of a food azo dye. There is no information about its ring-cleavage function (Lang et al., 2014). *Dermacoccus* is a gram-positive aerobic bacterium that can produce catalase and is a representative genus in the family Dermacocaceae of the order Micrococcales (Erko Stackebrandt 2014). *Kytococcus sedentarius*, a member of the Dermacocaceae, can split and hydroxylate the phenyl of diphenyl-arsenic acid, and then open the ortho position of the ring of the phenyl group to *cis, cis*-muconate (Nakamiya et al., 2007). This degradation process is consistent with the degradation process of 4-CBA and 3-CBA (DDT metabolites) by *Rhodococcus* through catechol 1,2-dioxygenase (encoded by *cata*) (Bajaj et al., 2014). Another phylogenetically close genus, *Terrabacter* from the same order with *Dermacoccus*, can degrade DDE via meta-cleavage catalyzed by dioxygenases (Aislabie et al., 1999). In addition, the relative abundance of *Dermacoccus* was enriched in all matrices during incubations with DDT (Fig. 5). Therefore, *Dermacoccus* might well participate in the ring opening degradation of DDT and its metabolites through a dioxygenase, which would be of great significance for the complete mineralization of DDT.

Brevibacterium was only enriched in burrow matrix (Fig. 3b and 3c) and identified as a possible DDT ring cleavage degrader (Table 2 and Fig. S6). It has not been documented before that *Brevibacterium* can degrade DDT. However, *Brevibacterium* has been found to have catechol 1,2-dioxygenases (Nakagawa et al., 1963), protocatechuate 3,4-dioxygenases (Whittaker et al., 1984) and homoprotocatechuate 2,3-dioxygenases (Miller and Lipscomb 1996), which can participate in the ring cleavage of C-C bonds, such as in the degradation of dibenzofuran (Strubel et al., 1991). It has also been found that it has the ability to degrade PCBs by ring opening (Dudasova et al., 2014, Liz et al., 2009). Therefore, *Brevibacterium* was probably one dominant DDT degrader in burrow matrix and this warrants further investigation in the future.

Five genera from the order of Bacillales were identified as possible ring cleavage degraders of DDT (Table 2 and Fig. S4 to Fig. S8), among which four genera are from the family Bacillaceae and *Paenibacillus* belongs to the family Paenibacillaceae. A previous study has shown that *Bacillus* can dechlorinate DDT and degrade it to DDA after oxidation (Patil et al., 1970). In addition, *Bacillus* has

3,4-dihydroxyphenylacetate 2,3-dioxygenase (Que et al., 1981), which can degrade 3,4-dihydroxybenzoic acid via meta cleavage (Crawford 1975). Based on our results and the above discussion, it can be concluded that *Bacillus* may well participate in the dechlorination and ring opening degradation of DDT, which is more beneficial to the thorough degradation of DDT. Studies have shown that *Paenibacillus* can significantly promote the dechlorination and degradation of dichlorobiphenyls (Sakai et al., 2005), and has a *bphA* gene-encoding biphenyl dioxygenase and was the dominant ring cleavage degrader of polychlorinated biphenyls (PCBs), as revealed by stable isotope probing (Uhlik et al., 2009). It has also been found that *Paenibacillus* contains a *cadA* gene-encoding oxygenase, which can open the hydroxylated benzene ring through dioxygenase and degrades 2-methyl-chlorophenoxyacetic acid (Liu et al., 2013). However, the degradation of DDT by *Paenibacillus* has not been reported before.

Some previously reported pure cultures of DDT degraders (Bajaj et al., 2014) were not identified as ring cleavage bacteria. This could be attributed to the complexity of the soil environment and the lower competitiveness of these bacteria relative to others. Different groups of putative DDT-degrading bacteria were identified in different matrices. This could be due to the specific properties of the matrices (Table S1) favouring more specialised bacteria (Fig. 3).

3.6. Links between DDT degradation, properties of matrices and bacterial composition

Although incubated under the same conditions, the concentration of added DDT in the non-sterilized drilosphere matrices was reduced much more quickly than in their non-drilosphere counterparts (Fig. 1). That means that without the effect of living earthworms, the bacteria in drilosphere matrices could still accelerate DDT degradation. This could be attributed to two possible reasons. Firstly, drilosphere matrices had more abundant DDT degrading bacteria. Secondly, the higher organic carbon and pH provided more suitable environment for these bacteria. However, based on the bacterial community composition (Fig. S3 and Fig. 5), the relative abundances of some putative DDT degraders in drilosphere matrices were even lower than those in non-drilosphere matrices. Therefore, it can be concluded that the more rapid DDT degradation in drilosphere matrices is mainly due to the special properties of the drilosphere matrices (Table S1), which favors the activity and growth of a wide range of bacteria. This is supported by the higher bacterial α -diversities observed in drilosphere matrices after incubation (Fig. 4) and the significant correlation between matrix pH, TOC, moisture and bacterial α -diversity (Table S2).

The half-life of DDT in drilosphere matrices (10 mg/kg) is around 7 weeks (Fig. 1), which is 1 week shorter than soil inoculated with living earthworms with an initial DDT concentration of 4 mg/kg (Xu et al., 2019). This means that without the activity of living earthworms, the degradation of DDT in drilosphere matrices was even quicker. This result demonstrates the dominant role of the stimulated degradation of DDT by indigenous microorganisms in soil DDT removal.

Canonical Correspondence Analysis (CCA) were conducted to figure out the factors contributing to the enhanced DDT removal from soils by earthworms and to compare with our previous study (Xu et al., 2019). At day 21, soil pH, TOC, moisture and Simpson index were tested to have significant contributions to the composition of DDTs using ANOVA analysis and Envfit function filtration (Fig. 6a). At 42 d, Shannon, Evenness and bacterial composition (first component of the PCA analysis based on bacterial community composition at the genus level) also contributed significantly to the matrix DDTs composition besides Simpson index, soil pH, TOC and moisture (Fig. 5b). The CCA results further show that the higher the soil pH, TOC, moisture and bacterial α -diversity, the faster the transformation of DDT to DDD, DDE and DDMU. The significant higher soil pH, TOC and moisture in drilosphere matrices (Table 2) leads to the faster DDT degradation relative to non-drilosphere matrices. This provide further evidence that earthworms enhance DDT removal by improving soil properties, thus stimulating indigenous microorganisms to degrade DDT. However, although the pH, TOC and moisture of Gut was significantly higher than that of Cast and Burrow, there were no differences in DDT residues between Gut, Cast and Burrow. This may be because some other factors were limiting the degradation of DDT when soil organic matter and pH was among the appropriate range for DDT degradation

The results of this study show that earthworms enhance DDT removal mainly by improving soil properties, especially pH and soil organic matter, thus stimulating indigenous microorganisms rather than intestinal digesting or tissue accumulating (Xu et al., 2019).

4. Conclusion

The natural degradation of DDT and its metabolites in soil is rather slow. Earthworms could improve DDT removal from soils by enhancing biotic degradation of DDT rather than accelerating its abiotic degradation. The improved soil properties,

sufficient carbon source and the subsequent stimulation of bacteria were the main factors driving the degradation of DDTs in drilosphere. Earthworms could also accelerate the further degradation of DDT products. The cast matrix was the most efficient for the thorough mineralization of DDTs. These result could help the development of bioremediation technique by reusing the wastes from the earthworm farming industry. This study also provides further knowledge which can be used to design future processes using earthworms to enhance the growth and activity of bacteria that carry out ring-cleavage degradation of DDT and assist in remediating DDT-contaminated soils.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (No. 41877063, No. 41171210, and No. 41907037), the National Key Research and Development Program of China (No. 2017YFD0800704).

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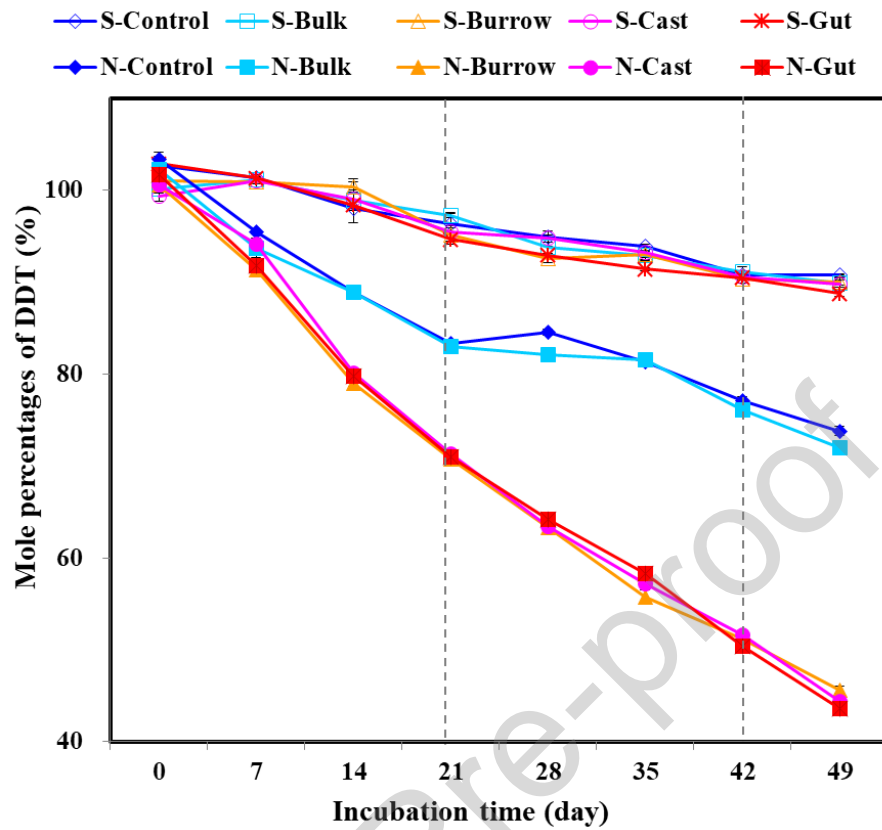


Fig. 1 Mole percentages of DDT residues to the initial DDT contents in sterilized and non-sterilized soil and drilosphere matrices during the microcosm incubation. The initial DDT concentration is 10 mg/kg. Error bars represent standard errors of three replicates. Dotted lines indicate the time points of DNA extraction.

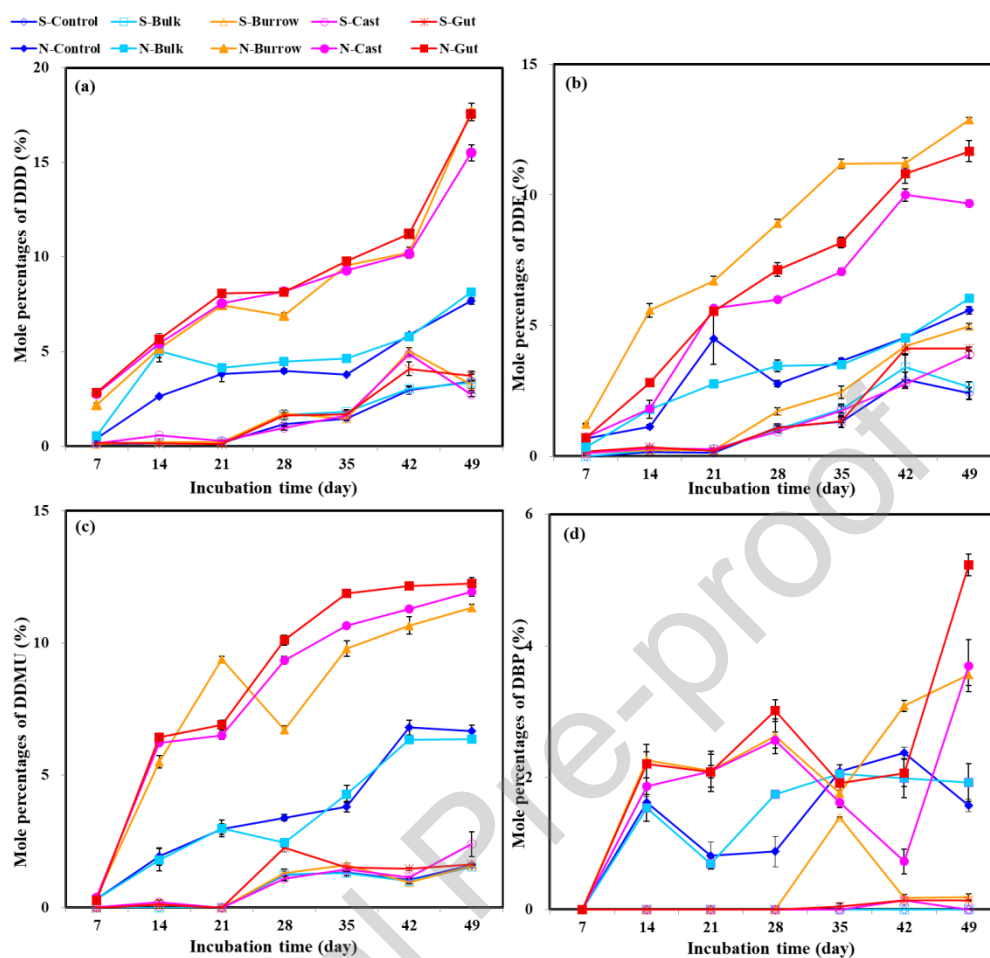


Fig. 2 Mole percentages of the metabolites DDD (b), DDE (c), DDMU (d) and DBP (e) to the initial DDT concentration (10 mg/kg) in sterilized and non-sterilized soil and drilosphere matrices during the microcosm incubation. Error bars represent standard errors of three replicates.

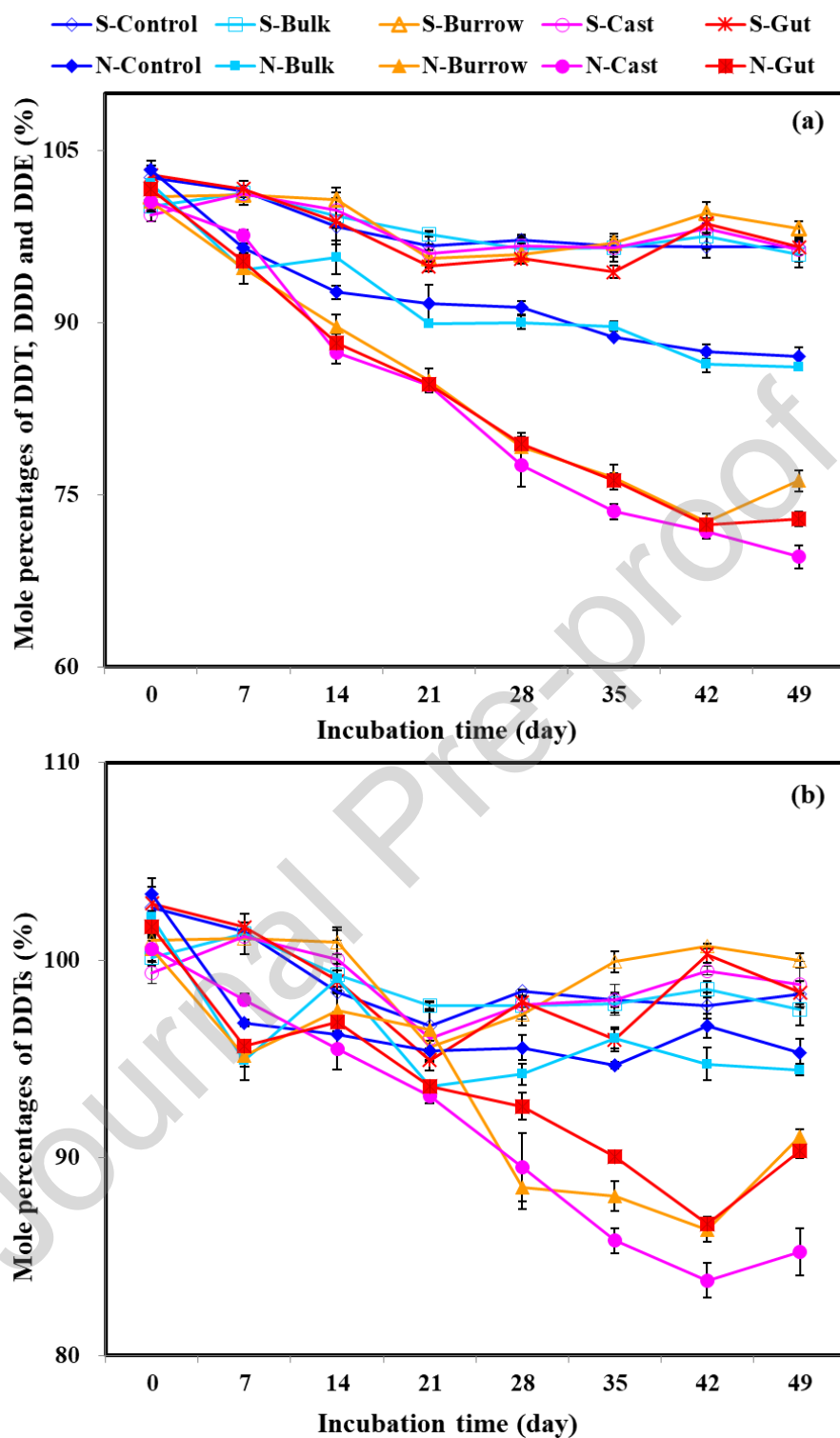


Fig. 3 Total amounts of DDT, DDD and DDE (a) and Total amounts of DDTs (b) including DDT, DDD, DDE, DDMU and DBP in sterilized and non-sterilized soil and drilosphere matrices during the microcosm incubation. Error bars represent standard errors of three replicates.

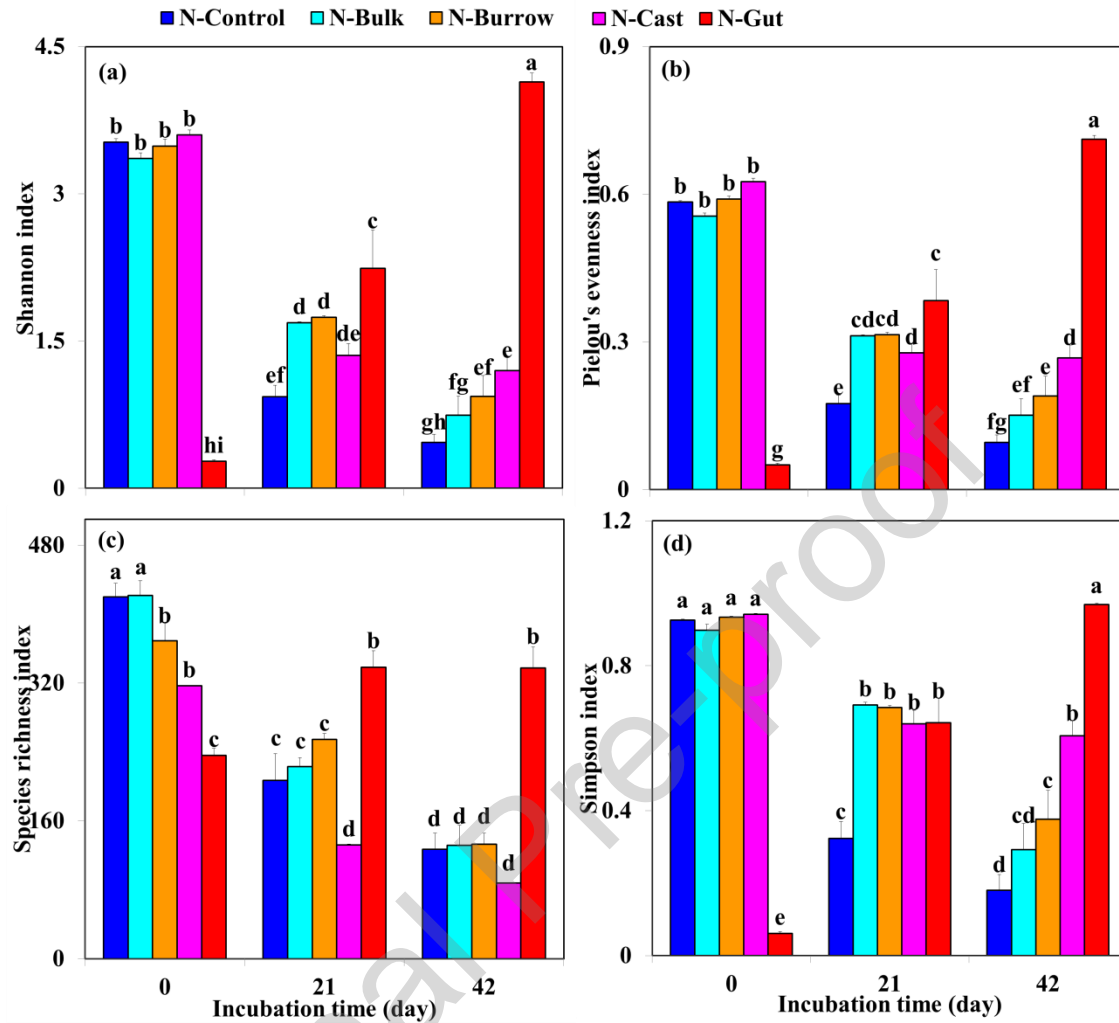


Fig. 4 Diversity indices of bacteria based on 16S rRNA sequences in non-drilosphere and drilosphere matrices at the 0th day, 21st day and 42nd day of the microcosm incubation. Fig. 2 (a), (b), (c), (d) and (e) represent the Shannon index, Pielou's evenness index, Species richness index and Simpson index of bacterial community in matrices, respectively. Error bars represent standard errors of three replicates. Different lowercase letters indicate significant differences between different matrices at the sampling date (ANOVA, $n=3$, Duncan's test, $p < 0.05$).

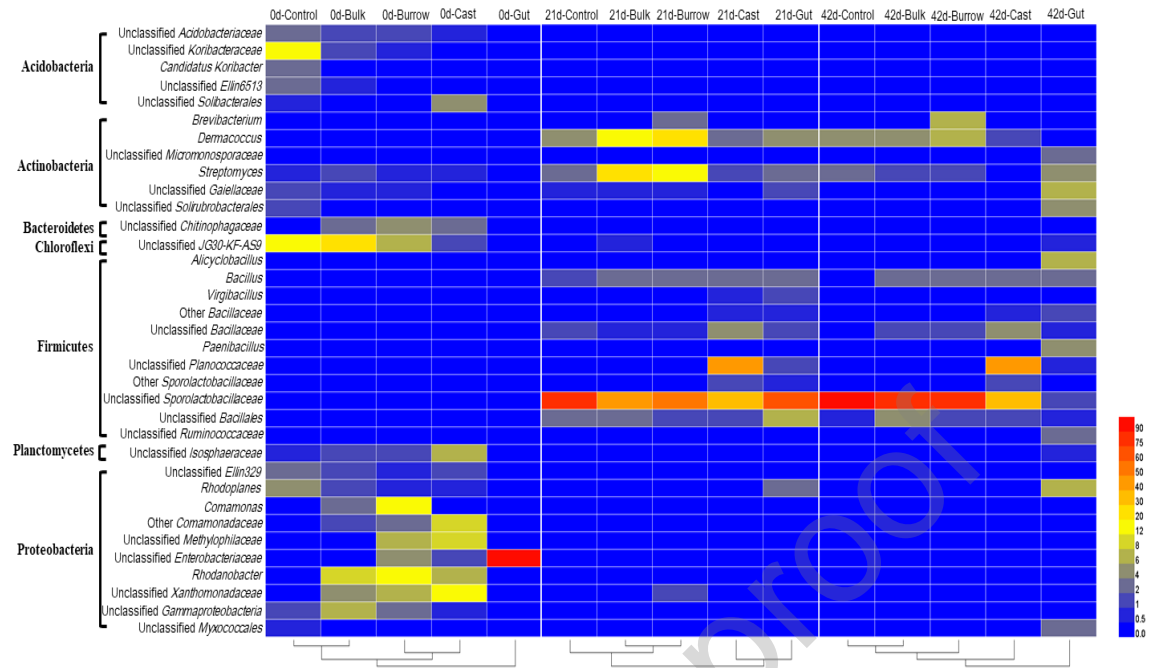


Fig. 5 Heatmaps of most abundant genera in non-drilosphere and drilosphere matrices before the incubation (a), on the 21st day of the incubation (b) and the 42nd day of the incubation (c). Relative abundance of matrixes were the mean of three replicates (n=3).

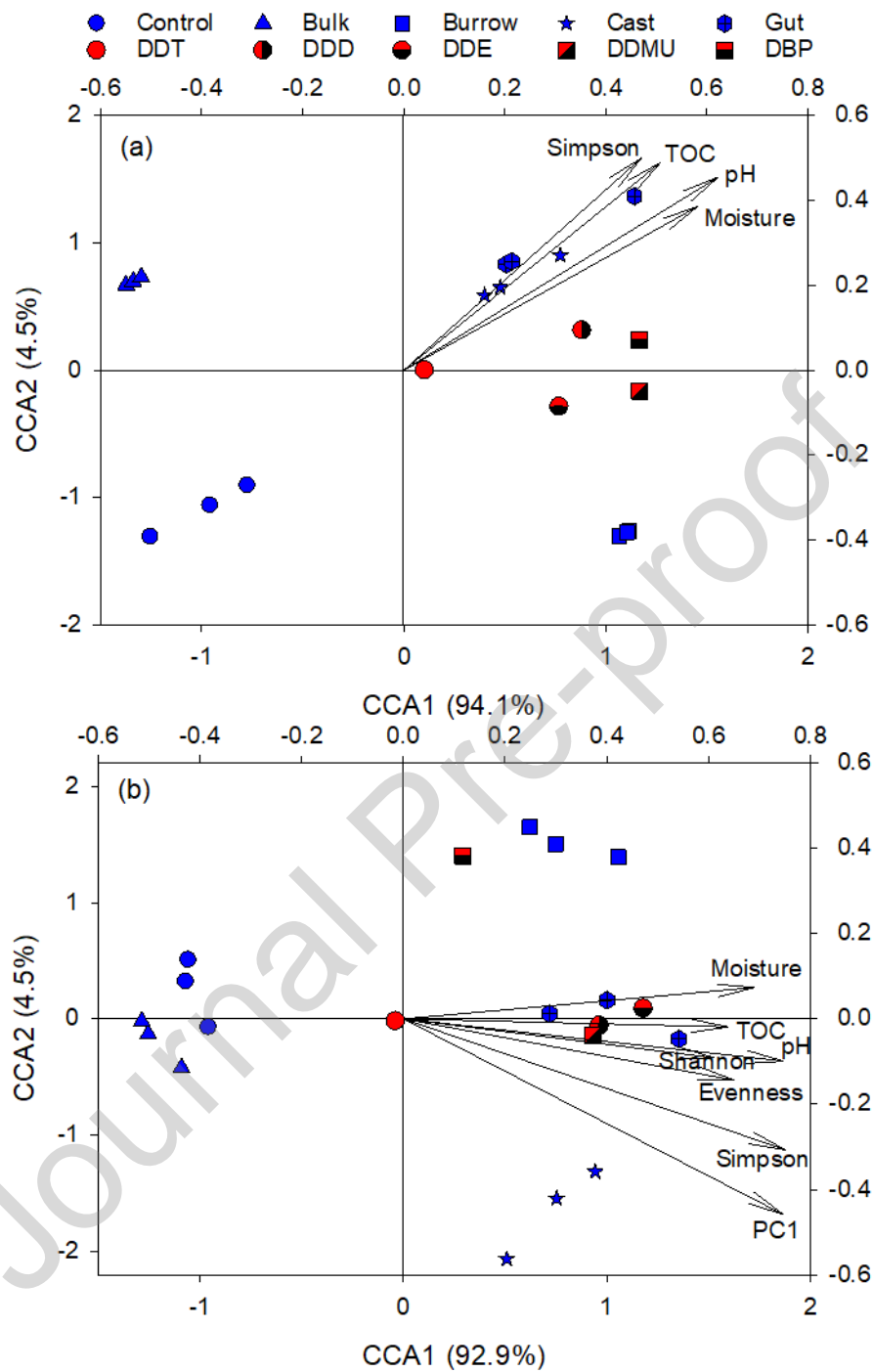


Fig. 6 CCA analyses exploring the factors shaping the DDTs composition at the 21st (a) and 42nd (b) day of the incubation in non-drilosphere and drilosphere matrices. The arrows represent the filtered variables contributing to the DDTs composition significantly. PC1: the first component of principal analysis of bacterial community composition at genus level; TOC: soil total organic carbon; Simpson, Shannon and Evenness: α -diversity indices of soil bacterial community at genus level.

Table 1 Treatment details of the experiment

Treatment	Matrix (g)	[¹²C]-DDT (mg/kg)	[¹³C]-DDT (mg/kg)	BMSS^a (μL)	Replicates
S-Control	1	10	-	4	3
N-Control	1	10	-	4	3
S-Bulk	1	10	-	4	3
N-Bulk	1	10	-	4	3
S-Burrow	1	10	-	4	3
N-Burrow	1	10	-	4	3
S-Cast	1	10	-	4	3
N-Cast	1	10	-	4	3
S-Gut	1	10	-	4	3
N-Gut	1	10	-	4	3
¹³ C-Control	1	-	10	4	3
¹³ C-Bulk	1	-	10	4	3
¹³ C-Burrow	1	-	10	4	3

¹³ C-Cast	1	-	10	4	3
¹³ C-Gut	1	-	10	4	3

Note: ^a BMSS: basic mineral salt solution, 1 g/L (NH₄)₂SO₄, 2.7 g/L KH₂PO₄, 10.955 g/L Na₂HPO₄•12H₂O, 0.03 g/L Ca(NO₃)₂, 0.01 g/L FeSO₄, 0.2 g/L MgSO₄.

Table 2 Identified functional bacteria in different matrices

Phylum / Class / Order	Family	Genus	Contr ol	Bul k	Burro w	Cas t	Gu t
Actinobacteria / Actinobacteria / Actinomycetales	Streptomycetace ae	<i>Streptomyces</i>	+	-	-	+	-
		<i>Streptacidiphil us</i>	+	-	-	-	+
	Dermacoccaceae	<i>Dermacoccus</i>	-	+	+	+	+
	Brevibacteriaceae	<i>Brevibacteriu m</i>	-	-	+	-	-
Firmicutes / Bacilli / Bacillales	Bacillaceae	<i>Bacillus</i>	+	-	+	-	-
	Bacillaceae	<i>Virgibacillus</i>	+	+	-	-	-
	Bacillaceae	<i>Other</i>	-	+	-	-	-
	Unclassified	<i>Unclassified</i>	-	+	+	+	-
	Paenibacillaceae	<i>Paenibacillus</i>	-	-	+	-	-

Proteobacteria /								
Gammaproteobacte	Xanthomonadac	<i>Unclassified</i>	-	-	-	+	-	
ria /	eae							
Xanthomonadales								

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Credit Authors Statement

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Wenyan Li: Conceptualization

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Chunling Luo: Data Curation

Yongtao Li: Supervision, Project administration, Funding acquisition

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Earthworms enhanced DDT removal from soils by accelerating its biotic degradation;
- Earthworm cast facilitates more complete degradation of DDT than its burrow and gut;
- Drilosphere soil enhanced the growth and activity of bacteria;
- Earthworms enhanced DDT removal by improving soil properties, thus stimulating bacteria;
- Using DNA-SIP techniques, ten DDT degrading bacteria were identified.