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RESEARCH ARTICLE

Microbial flora analysis for the degradation of beta-cypermethrin

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Abstract In the Xinjiang region of Eurasia, sustained long-term and continuous cropping of cotton over a wide expanse of land is practiced, which requires application of high levels of pyrethroid and other classes of pesticides—resulting in high levels of pesticide residues in the soil. In this study, soil samples were collected from areas of long-term continuous cotton crops with the aim of obtaining microbial resources applicable for remediation of pyrethroid pesticide contamination suitable for the soil type and climate of that area. Soil samples were first used to culture microbial flora capable of degrading beta-cypermethrin using an enrichment culture method. Structural changes and ultimate microbial floral composition during enrichment were analyzed by high-throughput sequencing. Four strains capable of degrading beta-cypermethrin were isolated and preliminarily classified. Finally, comparative rates and speeds of degradation of beta-cypermethrin between relevant microbial flora and single strains were determined. After continuous subculture for 3 weeks, soil sample microbial flora formed a new type of microbial flora by rapid succession, which showed stable growth by utilizing beta-cypermethrin as the sole carbon source (GXzq). This microbial flora mainly consisted of *Pseudomonas*, *Hyphomicrobium*, *Dokdonella*, and *Methyloversatilis*. Analysis of the microbial flora also permitted separation of four additional strains; i.e., GXZQ4, GXZQ6, GXZQ7, and GXZQ13 that, respectively, belonged to *Streptomyces*, *Enterobacter*, *Streptomyces*, and *Pseudomonas*. Under culture conditions of 37 °C and 180 rpm, the degradation rate of beta-cypermethrin by GXzq was as high as 89.84% within 96 h, which exceeded that achieved by the single strains GXZQ4, GXZQ6, GXZQ7, and GXZQ13 and their derived microbial flora GXh.

Keywords Beta-cypermethrin · Microbial floral structure · High-throughput sequencing · Isolation and identification · Degradation rate

Introduction

Pyrethroid pesticides have many advantages including a high level of safety, a low required dosage to achieve the desired effects, a high efficacy, and a broad insecticidal spectrum. Moreover, its insecticidal activity is increased by as much as 10–100-fold as compared older generations of insecticides. Pyrethroid pesticides are now one of the major pillar products of agricultural health insecticides (Van Wijngaarden et al., 2005) and have been widely used as household agricultural insecticides as an important tool to protect crops from pests (Dubey and Fulekar, 2012). However, they are also characterized by stable chemical properties, an inherent difficulty to undergo photolysis and long residual periods and so forth, wherein the residual issues and hazards have become increasingly more severe, causing adverse harm to ecosystems and human health alike. Since application of pyrethroid pesticides occurred much later than that of organophosphorus and organochlorine pesticides—compounds that also display higher toxicity than their pyrethroid counterparts, degradation of pyrethroid microbial residues has not been studied as intensely and only relatively recently (Lin et al. 2011; Xiao et al. 2015; Soderlund et al. 2002). Currently, the major microorganism that has been isolated from the natural environment, which is

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capable of degrading pyrethroid pesticides, belongs to the Pseudomonas species of bacteria (Grant et al. 2002), However, others have also been isolated including the fungal species *Aspergillus niger* (Chen et al. 2011a, b). In addition, studies predominantly focus on screening, isolation, identification, and understanding the degrading characteristics of various strains (Zhang et al. 2011; Chen et al. 2011a, b; Cycoń et al. 2014). In terms of understanding the application of biodegrading strains, the international scientific community has focused on screening and culture of single dominant strains. However, single dominant strains have several challenges including the difficulty in maintaining original strain characteristics during the actual application, and the knowledge that such strains are easily eliminated by other bacteria in the environment, which greatly affects their application (Arora. 2012).

Applying microbial flora or mixed strains of bacteria with the intent of eliminating pollutants has obvious advantages, foremost among which, are the elimination of several sources of pollution, and more thorough degradation of some refractory pollutants. However, the acquisition and structure of stable microbial flora that can quickly degrade a selection of targeted single pollutants has been poorly studied (Yen et al. 2009). Xinjiang is located in the hinterland of Eurasia, has dry weather and prominent soil salinization, which is the only long-staple cotton planting base in that region. A number of studies have reported that there are many important microbial types in that area, and the long-term continuous large area cropping of cotton has resulted in serious and persistent pollution of the environment by pyrethroids and other pesticides found in the soil (Zhang et al. 2013). In this study, microbial flora that could stably grow by utilizing beta-cypermethrin as the only carbon source was obtained through enrichment culture and domestication of the soil that was collected from that area. Also, this microbial flora was found to show a much faster degradation rate as well as more prominent degradative efficiency as compared with single strains and their composite microbial flora. In addition, analysis of the structural composition of the microbial flora assisted our ability to improve the enrichment method for microbial flora with particular important functions as well as enable our capacity to conduct in-depth studies on the degradation mechanism.

Materials and methods

Sampling time, method, and location

On June 5, 2015, soil was collected from the cotton area of Awat County, Xinjiang, China, with 10 years of continuous cropping of cotton using a five-point sampling method. Five locations were separately selected from three sampling fields, where the soil was drilled vertically using an earth borer with an inner diameter of 6 cm. Next, 1–20 cm of soil was collected and combined to a pooled sample, which was numbered AW10. The sampling range was (E 79°45′81″- 46′55″N 39°31′47″-45′50″). Ultimately, the collected sample was stored at −4 °C and transported to the laboratory for use within 1 week.

Culture medium and method

The baseline salt medium was formulated with NH_4NO_3 1.0 g/L, KH_2PO_4 0.5 g/L, KH_2PO_4 1.5 g/L, NaCl 0.5 g/L, $7H_2O{\cdot}FeSO_4$ 0.5 g/L, and $(NH_4)_2SO_4$ 0.5 g/L, pH 8.0, which was solubilized in distilled water to a final volume of 1 L, which was followed by high temperature sterilization. Pesticides of beta-cypermethrin (97%) were purchased from Guangxi Nanning Guangtai Chemical Industry Co., LTD, China. Culture method: In this procedure, 50 mL of baseline salt medium containing 100 mg/L beta-cypermethrin was decanted into a 250 mL Erlenmeyer flask, and then 2.5 g of soil sample (i.e., a 5% inoculation amount) was added, followed by incubation at 37 °C and 180 rpm shaking in the dark for 7 days. In this procedure, three replicates were prepared for each sample. The newly cultured substance was inoculated (2% inoculation amount) into a new baseline salt medium and cultured by the same procedure with the exception that the beta-cypermethrin concentration was increased by 50 mg/L. In the same manner, the soil sample was successively inoculated for 7 weeks, and the cultures were named GX1 to GX7 according to the respective culture weeks. Every week after the inoculation, part of the cultured sample was collected for DNA extraction.

Isolation and preliminary identification of degrading strains

At week 7, enrichment medium was collected, from which rapidly grown microbial flora were repeatedly screened on solid inorganic salt medium containing 100 mg/L beta-cypermethrin using the gradient dilution method and then isolated under the same conditions. Next, four strains with significantly different morphology were selected and numbered as GXZQ4, GXZQ6, GXZQ7, and GXZQ13 separately. Subsequently, total DNA was extracted (TIANamp Bacteria DNA Kit, Tiangen Biotech Co., Ltd., China) from each strain. By contrast, 16S rDNA was obtained by PCR amplification using the universal primer 27F/1492R (5′-AGAG TTTGATCCTGGCTCAG-3/5′-GGYTACCTTGTTAC GACTT-3′) (Heuer et al. 1997; Lane. 1991). The PCR cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1.25 min, with the last cycle followed by a 10-min extension at 72 °C. The PCR products were cloned into the pMD20-T

vector (TaKaRa Biotechnology Co. Ltd., China) and transformed into *Escherichia coli* clone DH5α cells. Clones were sequenced by Shanghai Yingjun Technology Co. Ltd., China, after purification. The resulting sequence (GenBank accession no. KU921426-29) was compared with the sequences in the GenBank database by BLAST searching strategies. Multiple sequence alignments were conducted with the selection of high homology sequences using CLUSTALX 1.8.1. The phylogenetic tree was analyzed and constructed with the neighbor-joining method using the MEGA version 4.0 software package (Tamura et al. 2007).

Total DNA extraction and high-throughput sequencing

Total DNA was extracted from the soil sample AW10 using the FastDNA® Spin kit (MP bio, Santa Ana, CA, USA) according to the kit instructions. Meanwhile, the continuously cultured microbial flora GX1 to GX7 was centrifuged and the bacteria were collected respectively, from which DNA was also extracted by the same method, which included three replicates for each sample. Then, total DNA of all replicates were pooled and stored at −20 °C for subsequent use. The 341F (CCTAYGGGRBGCASCAG)/806R (GGACTACNNGGGTATCTAAT) primer was used to amplify the 16S rRNA V3+4 area from total DNA of each sample. The 30 μL PCR system consisted of 15 μL Phusion Master Mix (2×), 1.5 μL upstream and downstream primers (2 μM) each, 10 μL DNA sample (1 ng/μL) of each sample and 2 μL double-distilled water. PCR was performed using a Bio-Rad T100 gradient PCR instrument with the following set procedures: 98 °C for 1 min, 98 °C for 10 s, 50 °C for 30 s and 72 °C for 30s, for a total of 30 cycles, followed by 72 °C for 5 min and stabilized at 4 °C. Subsequently, a UV spectrophotometer (NanoDrop-1000, USA) was used to accurately quantify the 16S rRNA gene V3+4 fragments of each amplified sample. Samples were fully mixed with an equivalent density, which was followed by 2% agarose gel electrophoresis in a 1×TAE buffer. The resolved 400–450 bp primary bands were recovered using a GeneJET gel recovery kit (Thermo Scientific). A library for the above samples was constructed using NEB Next® Ultra™ DNA Library Prep Kit for the Illumina system (New England Biolabs). Then, the library was certified by Qubit quantification and testing, which was subsequently sequenced using the Illumina HiSeq platform (Novogene, Beijing, China). The registration number of sequences obtained from this study in the NCBI SRA database was SRX2030640.

Bioinformatics and statistical analysis

Barcode and primer sequences were truncated using the Novogene script. For each sample, there were characteristic sequences of 6 bp at the front end of the upper primer sequence that were used to distinguish different samples, and there were at least 40, 000 sequences for accurate analysis after optimization (Magoč and Salzberg, 2011). Then, the UPARSE pipeline (v7.0.1001) was used to analyze the OTU and other relevant bioinformatics data for the sample sequences, wherein the degree of similarity was set at 97%. Species annotation of the representative sequences of OTUs was performed using the RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp-classifier/) and the GreenGene database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), which was set with a threshold of 0.8–1, while the community composition of each sample was analyzed statistically at each classification level.

The degree of similarity was compared among samples using Qiime software (Version 1.7.0). Alpha diversity of each sample was analyzed, while observed species and other indices were calculated, of which alpha diversity was compared among different samples using the R statistical software program (Version 2.15.3) (Caporaso et al. 2010). In terms of beta-diversity analysis, Qiime software (Version 1.7.0) was used to calculate distances as well as to construct the UPGMA clustering tree. Also, the R language vegan packages "vegdist" and "hclust" were used to cluster the degree of similarity of samples. The R statistical software package (Version 2.15.3) was used to plot PCA, etc. In addition, PCA analysis was performed using the ade4 package and the ggplot2 package of the R software (Wang et al. 2007).

Determination of degradation rate

We compared the degradation rates of four strains (i.e., GXZQ4, GXZQ6, GXZQ7, and GXZQ13) and their associated microbial flora GXh and GXzq on beta-cypermethrin. The strains were processed as follows: Single strains were cultured in nutrient broth that contained 100 mg/L beta-cypermethrin, which was conducted under the conditions described in the "Culture medium and method" section, followed by centrifugation at 4600 rpm for 1 min, from which bacteria were collected and diluted using sterile water to obtain an OD_{600} value of 1.0. Each strain was cultured with an inoculation of 2% and its degradation rate to beta-cypermethrin was determined, wherein the inoculation levels of the four strains in microbial flora GXh were, respectively, 0.5%. In terms of microbial flora GXzq, the bacteria could be directly collected from the subculture after centrifugation, followed by dilution with sterile water to obtain an OD_{600} value of 1.0, wherein the inoculation amount was also 2%. By contrast, the culture method and conditions were the same as those stated above. In addition, flasks that were not inoculated were considered as controls, and in these procedures three replicates for each sample were prepared. Beta-cypermethrin residue was determined by HPLC analysis (Akbar et al. 2015).

Results

Degradative microbial florae and strains

One specific microbial flora (i.e., GXzq) that was continuously cultured by its ability to metabolize beta-cypermethrin as the only carbon source was obtained. From this microbial flora, four strains of bacteria that displayed fast growth and large colony were separated and purified using the same culture methods and were given the identifying reference numbers: GXZQ4, GXZQ6, GXZQ7, and GXZQ13 with the aligned Genbank accession numbers of KU921426-29. Next, 16S rRNA gene similarity was compared, and the results showed that the 16S rRNA gene similarities between GXZQ4 and *Streptomyces*, GXZQ6 and *Enterobacter*, GXZQ7 and *Streptomyces*, as well as GXZQ13 and *Pseudomonas* were 99, 98, 98, and 99%, respectively. The phylogenetic tree is shown in Fig. 1.

Structural composition of beta-cypermethrin-degrading microbial flora GXzq

Analysis of sequencing numbers and possible species found in the high-throughput sequencing data of soil sample AW10 and differential culture of the microbial flora GX1 to GX7 revealed that the probability of the observed species approached a plateau when the sequencing number of sample AW10 reached 10,000. Moreover, the probability of observed species also approached a plateau when the sequencing number of sample GX1 to GX7 was only 5000 or less; however, the observed species number was gradually reduced with increasing passage number, with a value that was less than 1/8 of sample AW10 (Fig. 2).

In samples that received enrichment culture (GX1 to GX7), the most abundant ten genera were: *Methyloversatilis*, *Pseudomonas*, *Hyphomicrobium*, *Dokdonella*, *Methylophaga*, *Bdellovibrio*, *Pseudoxanthomonas*, *Lysobacter*, *Pontibacter*, and *Diaphorobacter*. Sequence numbers of these 10 genera accounted for more than 95% of the total number, of which *Pseudoxanthomonas* accounted for the largest proportion. In sample AW10, the number of sequences that could not be classified exceeded 98.3%, and the four genera that included *Methyloversatilis* among the above mentioned 10 genera were hardly detected in the enrichment culture sample (Fig. 3). In addition, in all samples, samples AW10 and GX1 had a larger number of unique tags and OTUs, while samples GX2 to GX7 had a smaller number of unique tags and OTUs, of which the numbers of unique tags and OTUs were, respectively, 0 and 115 in sample GX3 (Fig. 4).

From the phylum classification level, similarity degree-based clustering revealed that eight samples could be directly classified into two categories, of which AW10 was a single category and the remaining samples were clustered into a very similar group. The top 10 phyla accounting for the largest proportion and their compositions in

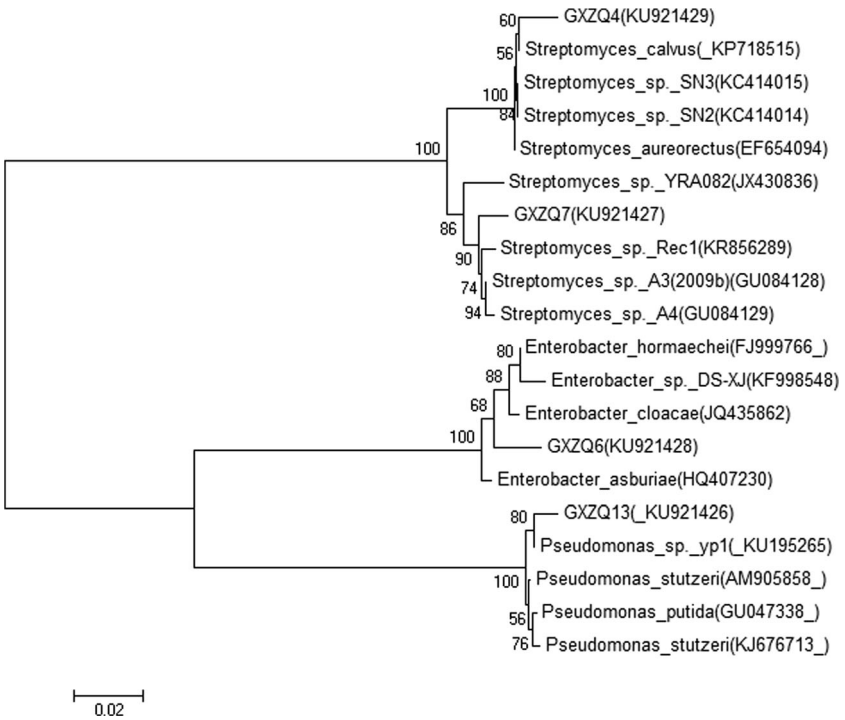

Fig. 1 A neighbor-joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strains XZQ4, GXZQ6, GXZQ7, and GXZQ13 and their closest relatives. Genbank accession numbers are given in parentheses. Numbers at the nodes indicate bootstrap values from the neighbor-joining analysis of 1000 resampled datasets. The *scale bar* above represents the sequence divergence

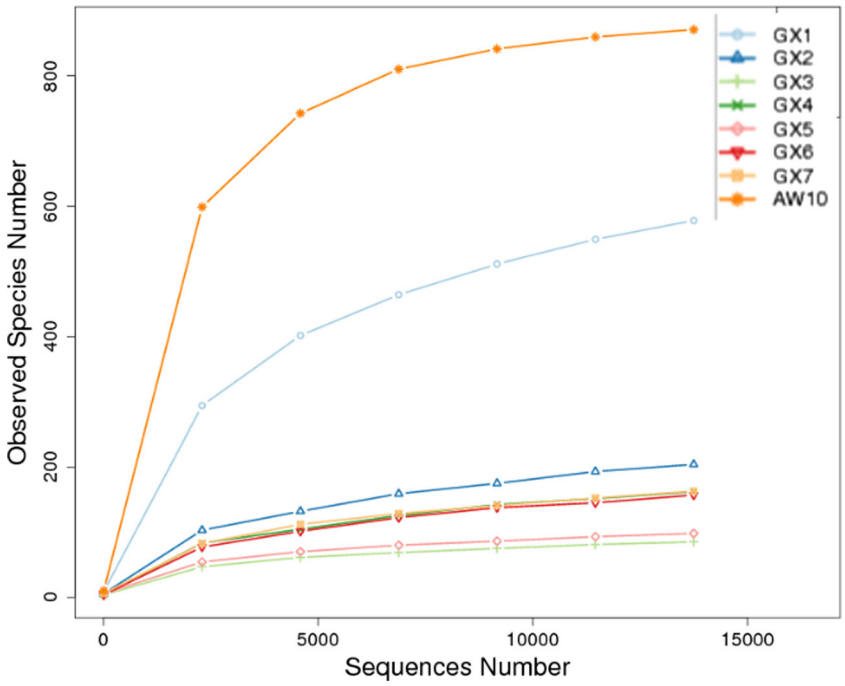

Fig. 2 Rarefaction curves of the observed species number. Sample AW10 with a history of 10-year succession cropping, and GX1-7, were named with different batch numbers from 1 to 7, respectively

samples AW10 and GX1 showed the largest differences from those seen in other samples (Fig. 5). Principal component analysis (PCA) showed that AW10 and GX1 were located at the farthest site of the two coordinates, respectively, while remaining samples were located near the origin and were positioned quite close to each other (Fig. 6).

Comparison of degradation rates of beta-cypermethrin in various strains and microbial flora

On detecting the degradation of 100 mg/L beta-cypermethrin, it was revealed that the degradation rate was highest in the single strain GXZQ7, wherein the final residual concentration of beta-cypermethrin was as low as 53.47 μg/mL. The residual concentrations in single strains GXZQ4, GXZQ6, and GXZQ13 were, respectively, 61.44, 78.29, and 66.34 μg/mL. Meanwhile, the residual concentrations in microbial flora GXh and GXzq were lower than those found in single strains, which were, respectively, 29.43 and 10.13 μg/mL (Fig. 7). In addition, as could be seen from Fig. 7, the degradation speed was also fastest in microbial flora GXzq, where more than half of the beta-cypermethrin was degraded by 60 h. Moreover, the degradation rate was lowest and the degradation speed was slowest in the single strain GXZQ6. Finally, 4.87% of the beta-cypermethrin was naturally degraded in the control group.

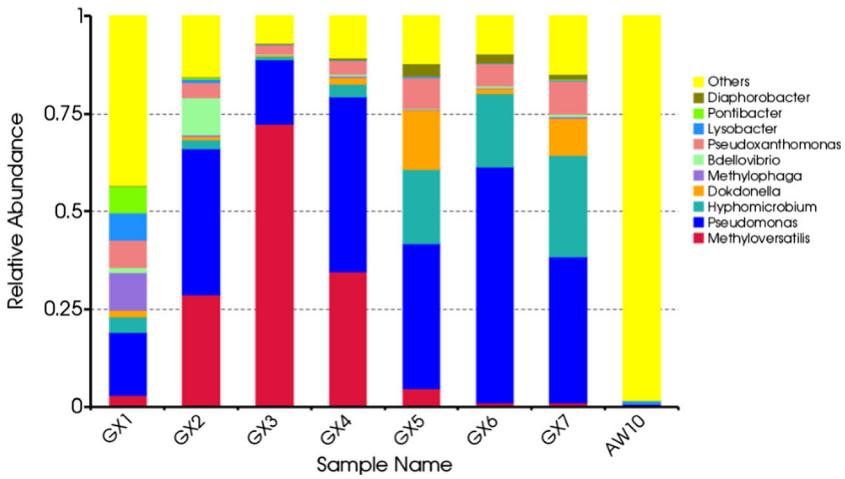

Fig. 3 Relative abundance of different bacterial genera in the samples of GX1-7 and AW10. The abundance is presented as a percentage of the total effective bacterial sequences in each sample. The top 10 abundant taxa are shown

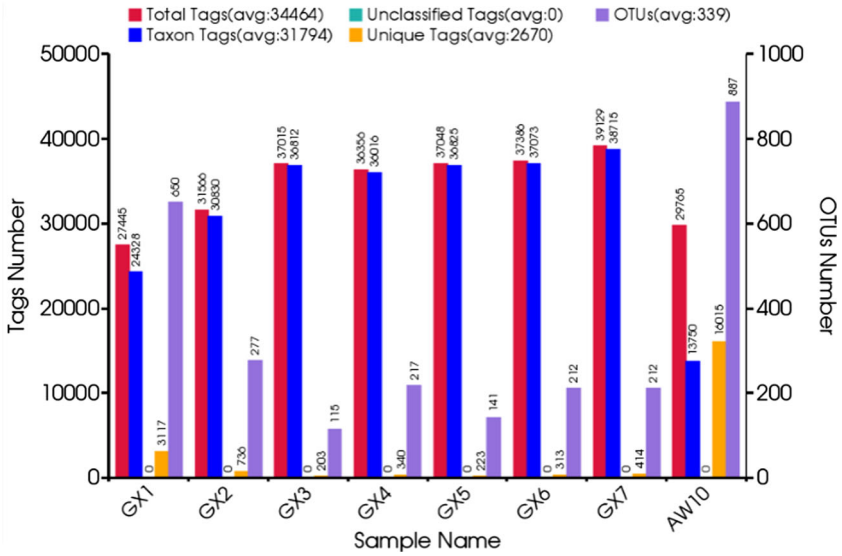

Fig. 4 Number of tags and OTUs in samples GX1-7 and AW10. Each *bar* represents the total tags, taxon tags, unclassified tags, unique tags, and OTUs of the eight samples

Discussion

Acquisition of degrading microbial flora and their stability

For unknown microbial flora with specific degrading functions, two aspects attracting most concern include understanding whether they can be stably passaged for a long time and determining their structural composition. This study found that the bacterial structure was stabilized in soil with a 10-year successive cropping of cotton and application of more than three annual treatments of beta-cypermethrin. For the microbial flora in the soil sample, only the microbial biomass that used beta-cypermethrin as the sole carbon source was increased after a 7-week successive passage. In addition, other microorganisms were continuously diluted and omitted, which could be confirmed by comparing the high-throughput data derived from each passage. High-throughput data also indicated that the microbial structure of the soil samples was extremely complex and the microbial diversity is decreased by the passage time. Meanwhile, after three passages, the microorganisms involved in the degradation of beta-cypermethrin formed the main body of microbial flora GXzq in the context of its abundance. Subsequently, the microbial flora GXzq was passaged once a week in basal salt medium containing 100 μg/mL beta-cypermethrin, during which the high-throughput data revealed that the structural composition of the microbial flora GXzq was very stable, since there was no significant change in the composition of the microbial flora and there was only a minimal change in the proportion of rare genera. In microbial flora GXzq, *Pseudomonas*, *Hyphomicrobium*, *Dokdonella*, and *Methyloversatilis* were consistently the main structural components. Even now, this microbial flora remains in continuous culture. Furthermore, during passage, the degrading ability of GXzq was continuously increased and the growth cycle was significantly shortened. The microbial biomass in the culture medium was optimal by 96 h, and the residual beta-

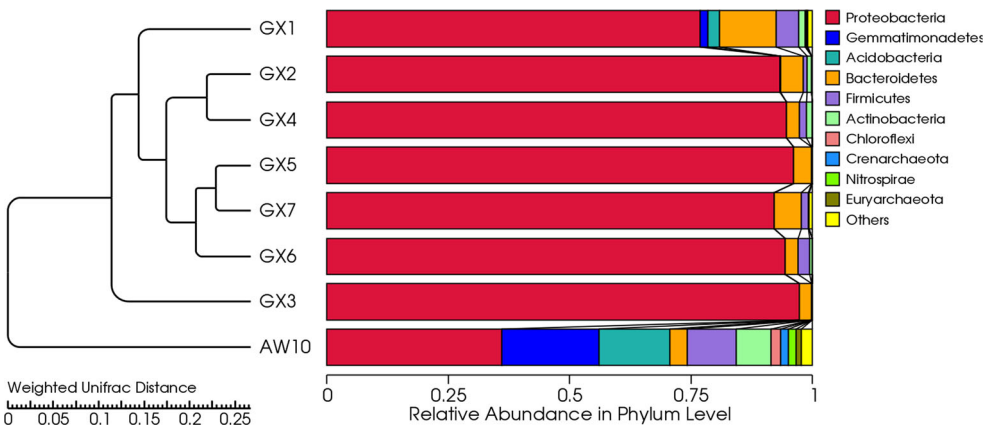

Fig. 5 Analysis of similarity of samples GX1-7 and AW10 using the weighted UniFrac approach and abundance of different bacterial phyla

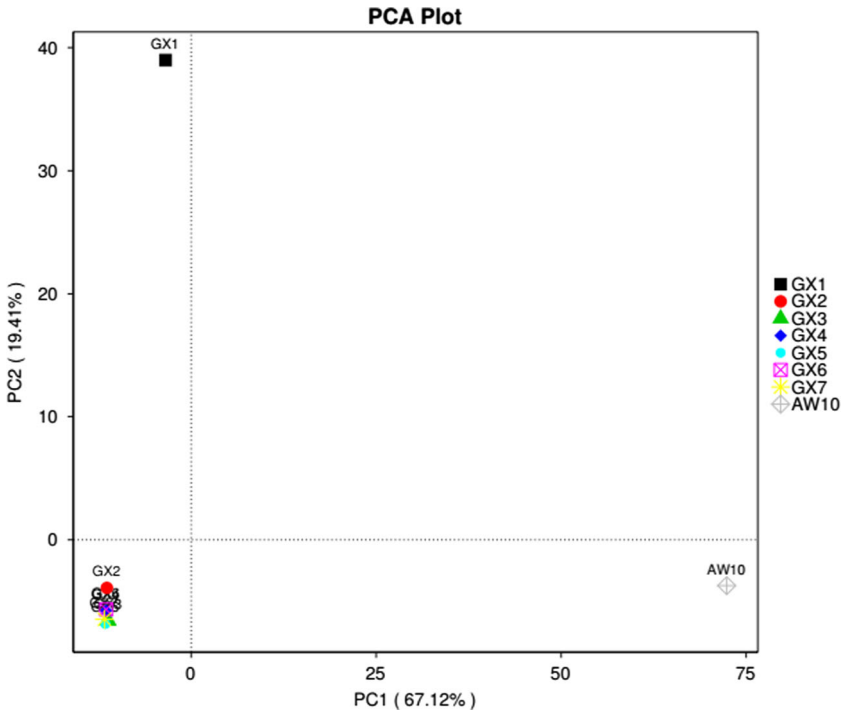

Fig. 6 Principal component analysis (PCA) of samples GX1-7 and AW10 calculated with QIIME. PCA plots were based on weighted UniFrac metrics for all samples

cypermethrin in the bacterial solution was at its lowest value. Therefore, the culture cycle was chosen to be 96 h to detect the degradation rate.

Formation process and structural composition of microbial flora GXzq

First, the structural composition of a new microbial flora is mainly determined by the culture conditions. Basal salt medium is mainly used to isolate the bacteria (Zengler et al. 2002; Janssen et al. 2002). Since the pH value is one of the major factors determining the structure of a new microbial flora (Xiong et al. 2012), the purpose of selecting pH 8 in this study was primarily to preserve consistency within the soil sampling environment. High-throughput sequencing data revealed that the structure of the microbial flora in soil sample AW10 was very complicated, wherein the Shannon and Simpson indices were as high as 8.621 and 0.995, respectively, and sequences that were unable to be grouped accounted for more than 98.3% at the genus classification level. Meanwhile, under the growth conditions of only providing beta-cypermethrin as the sole carbon source, the Shannon and Simpson indices were rapidly reduced to 1.663 and 0.449, respectively, during the first 3 weeks of culture, while the

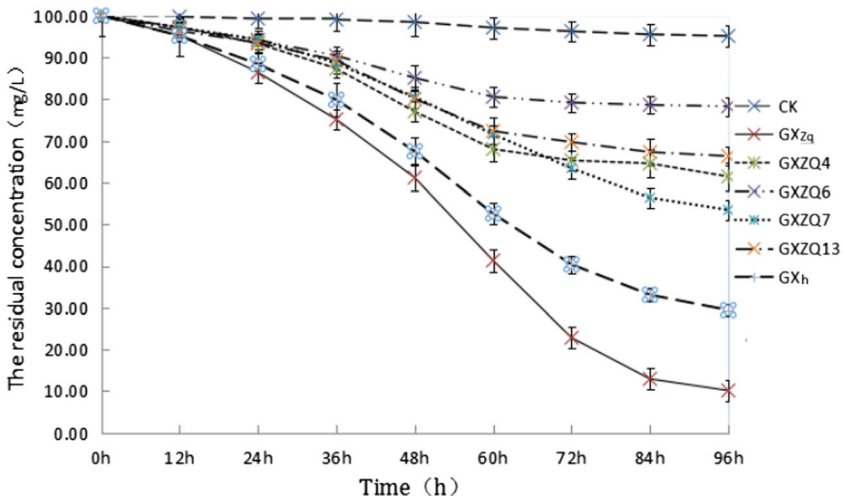

Fig. 7 Biodegradation of beta-cypermethrin with strain GXZQ4, GXZQ6, GXZQ7, GXZQ13, and the flora GXzq, and GXh. The initial concentration of beta-cypermethrin was 100 mg/L. Symbols represent the means of three replicates, and *error bars* represent the standard deviation

sequences that were unable to be grouped only accounted for less than 6.9%. Thus, the first three passages are the most critical period for adjusting microbial floral structure, which means that microbial floral structure tends to be simplified and the abundance of a dominant species is rapidly increased. In addition, in terms of the proportion of the categorized sequences, their composition and changes in the number of OTU in the microbial flora, the high-throughput sequencing data indicated that after four successive passages, the degrading microbial flora (GXzq) was formed and stabilized, and consisted mainly of four genera: *Pseudomonas*, *Hyphomicrobium*, *Dokdonella*, and *Pseudomonas*. It can be seen that under fixed culture conditions, microbial flora with a relatively stable structure (GXzq) can be formed spontaneously. Secondly, the newly formed degrading microbial flora (GXzq) can be passaged for a long time and has a stable degrading function. In subsequent successive passages, the growth cycle of the degrading microbial flora (GXzq) was significantly shortened. Furthermore, detecting the residual beta-cypermethrin and biomass in the culture medium showed that the microbial flora reached a stable stage within 96 h. The reason for the shortened growth cycle might be associated with the microbial flora structure, which tends to stabilize. Additionally, the microbial floral function is continuously improved. Furthermore, this degrading microbial flora (GXzq) has been passaged to the present and displays a very stable function. Third, at the level of genus classification, the degrading microbial flora (GXzq) contains most of the genera that have been reported to be able to degrade beta-cypermethrin (Zhang et al. 2010). However, this microbial flora also contains a very low level of mycobacteria that have not yet been reported due to the sensitivity of high-throughput sequencing. Whether these genera are directly or indirectly involved in the degradation of beta-cypermethrin is subject to further study. In the microbial flora GXzq, the proportion of unclassified parts was about 8%, which was presumed to be closely related to the more efficient degradation of beta-cypermethrin in this microbial flora as compared with those in single strains and combined microbial flora. Indeed, we not only found information of archaea in comparing the sequences but also identified fungal species in screening and subsequent culture analysis.

Degradation of beta-cypermethrin by microbial flora GXzq

In this study, the screened microbial flora GXzq showed a much higher degradation rate but also showed a much faster degradation speed of beta-cypermethrin as compared with other single strains and their combined microbial flora (GXh). Similar reports have mostly focused on culturing and purifying single degrading strains under optimal culture conditions to improve the degradation rate of pollutants or degrading key enzymes in the degradative pathway. By contrast, reports have been published of combining separate strains with differential degrading functions into microbial flora that can degrade complex pollutants (Laffin et al. 2010; Zhang et al. 2010). The main characteristics of microbial flora GXzq include the knowledge that it is cultivated in simple basal salt medium, and shows very strong growth characteristics as well as showing growth of a large amount of flocculent coenobium in the bacterial liquid. A large biomass allows it to be used directly for inoculation rather than undergoing enrichment culture to obtain numerous bacteria prior to its use for degradation studies, which is very similar to the general single degrading strains, and is very important in terms of practical applications. Also, the degradation rate of microbial flora GXzq is calculated from the entire peak areas of four isomers of beta-cypermethrin. This microbial flora not only has a high degradation rate, but also lacks specificity in degrading isomers of its substrate. Lastly, preliminary studies have found that microbial flora GXzq can thoroughly degrade beta-cypermethrin, during which each strain tends to spontaneously adjust its abundance. It is presumed that the appearance of different intermediate metabolites in the degradation process also changes the abundance of various bacteria that are capable of utilizing these intermediate metabolites. Thus, this microbial flora can be used for in-depth studies of its composition, and can be used for further relevant combined metabolic and genomics studies (Cunliffe and Kertesz, 2006; Li et al. 2007).

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