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Removal of High Concentrations Decachlorobiphenyl of Earthworm *Eisenia fetida* and its Symbiotic Bacteria in a Vermicomposting System

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Abstract The persistent organic pollutants decachlorobiphenyl (DCB) are a group of synthetic substances of high risk to human and environmental health. This study was aimed to evaluate the potential removal of DCB by earthworm *Eisenia fetida* and its symbiotic bacteria in a vermicomposting system for a period of 72 days using a complete randomized design. The results showed that the vermicomposting system was able to significantly remove high concentrations decachlorobiphenyl (DCB) from the polluted substrate. The addition of a concentration of 1000 mg L^{-1} during vermicomposting were a removal of 230.28 mg L^{-1} DCB and the results obtained from adding a concentration of 1500 mg L^{-1} DCB were 424.11 mg L^{-1}. The earthworms bioaccumulated less than 5 mg L^{-1} of DCB without an apparent toxic effect. The earthworm weight decreased during vermicomposting and DCB concentration compared to the control (non-polluting); however, earthworms survived until the end of experiment. Phylogenetic analysis of 16S rDNA gene sequences of *Eisenia fetida* gut strains grown in the presence of 1500 mg L^{-1} DCB were identified as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Solibacillus*, and *Staphyloccocus* at zero time (0-days). At 7 days of culture, the genera, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Klebsiella*, and *Staphylococcus* were identified, and at 72 days, the symbiotic bacteria isolated were classified into the genera, *Bacillus*, *Enterobacter*, *Klebsiella*, and *Staphylococcus*. The strains *Pseudomonas extremaustralis* ADA-5 and *Staphylococcus sciuri* ADA-12 showed higher potential of removal from the DCB (219.7 and 162.74 mg L^{-1}, respectively) at an initial concentration of 1500 mg L^{-1}. Both vermicomposting system and degrading bacteria from *Eisenia fetida* worms are useful to remove high concentrations of decachlorobiphenyl from contaminated soils.

Keywords Decachlorobiphenyl · *Eisenia fetida* · Symbiotic bacteria · Vermicompost

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1 Introduction

Polychlorinated biphenyls (PCBs) are compounds of toxic and highly persistent organic mixtures that comprise 209 congeners differing in the number and position of chlorine atoms bound to the two coupled-biphenyl rings (Polak et al. 2016). Within these compounds stands out the decachlorobiphenyl (DCB). Decachlorobiphenyl is completely chlorinated congener in the PCB class (2,2-3,3-4,4-5,5-6,6-DCB), it is also characterized by their lipophilicity and persistence and their biomagnification in food chains (Han et al. 2009).

DCB is found worldwide, including some very remote areas, for example, in the arctic, and even in breast milk or in human populations (She et al. 2007; Perez-Gonzalez et al. 2017). The congeners of PCB exhibit different coplanar levels according to the degree of substitution of chlorines, the PCB's mono-*ortho*-coplanares are easy to remove; however, the congeners with substitutions of cloros di-*ortho* in the DCB correspond to non-coplanar congeners and exhibit toxicological potential different from coplanar congeners, also increasing their elimination difficulty (Alonso et al. 2008; Han et al. 2009). This compound is still considered as a risk to both wildlife and humans. Health effects associated with exposure to DCB include carcinogenicity, genotoxicity, neurotoxicity, and reproductive toxicity (Qiu et al. 2016). Due to their high thermodynamic stability, metabolic degradation is generally very slow (Costabeber et al. 2006). Different methods like chemical and physical have been used for the destruction of PCBs in the environment (Zhao et al. 2012; Huang et al. 2014). The incineration has been used and approved as the standard method for the destruction of the DCB, in soil and sediment (Hatamian-Zarmi et al. 2009). However, this method has disadvantages that limit their full-scale applications and the contaminants can decompose partially (Tharakan et al. 2006).

Earthworms participate in the decomposition, transformation, and mineralization of organic matter by way of processes that take place in their digestive system (Šmídová and Hofman 2014). Earthworms can speed up the removal of contaminants from soil. The presence of earthworms in contaminated soil indicates that they can survive a wide range of different organic contaminants (Hernández-Castellanos et al. 2013; Rodriguez-Campos et al. 2014). For this reason, these have been employed as alternatives for bioremediation of PCBs due to the fact that they can tolerate a toxic chemical environment (Yadav and Garg 2011; Lin et al. 2016). However, little is known about the removal of the highly recalcitrant compound decachlorobiphenyl using vermicomposting and endosymbiotic bacteria of *E. foetida* as a suitable system due to the fact that these studies have used less chlorinated compounds such as Aroclor.

The microbial degradation has been reported as one of the most effective methods for removing chemical contaminants from the environment (Field and Sierra-Alvarez 2008; Mrozik and Piotrowska-Seget 2010; Rodriguez-Campos et al. 2014; Garrido-Sanz et al. 2018; Horváthová et al. 2018). Several species of bacteria have been secluded from the intestinal tract of the earthworm *Eisenia fetida* with high ability to biodegrade various types of toxic chemical contaminants (Hatamian-Zarmi et al. 2009; Asgharnia et al. 2014). Lin et al. (2016) identified the bacteria associated with earthworms; the bacteria found were *Alcaligenes*, *Pseudomonas*, and *Sphingomonas* which are known to degrade hydrocarbons and other organic compounds. However, little is known about DCB degrading bacteria associated with *Eisenia fetida* in vermicomposting process. Therefore, the aims of this study were as follows: (1) to evaluate the efficiency of a vermicomposting system using the earthworm *Eisenia fetida* for the removal of high concentrations of decachlorobiphenyl and (2) to determine the ability of the symbiotic bacteria isolated from earthworm intestine to degrade high concentrations of decachlorobiphenyl.

2 Materials and Methods

2.1 Reagents and Materials

Decachlorobiphenyl was 99.1% pure (Sigma-Aldrich®, USA). The substrate was formulated with rabbit manure and peat moss. Rabbit manure was collected from a rabbit farm located in Tuxtla Gutiérrez, Chiapas (Mexico), and the peat moss was a commercialized product acquired from Promix® Canadian Sphagnum (Quebec, Canada). Both substrates were milled to particle diameter (0.144 mm) and used as support for the earthworms.

2.2 Earthworm Used

Earthworms (*Eisenia fetida*) were cultivated in support based on rabbit manure and peat moss for 6 months for adaptation and growth. After, adult earthworms with developed clitellum (sexually mature) and with an average weight of 0.5 ± 0.1 g were selected for the experiment and protected from sunlight to avoid premature death.

2.3 Physicochemical Analyses from Vermicompost

Physicochemical characterization from vermicompost contaminated with a high concentration of DCB (1500 mg L^{-1}) and without the contaminant (negative control) during 0, 7 and 72 days of vermicomposting was analyzed according to the Association of Official Analytic Chemist (AOAC 1996). The pH and electric conductivity (EC) were measured using a digital pH

meter Mettler Toledo® Model S220 (New York, USA) in 1:10 (weight/volume) aqueous solution. Total nitrogen was measured by Kjeldhal method (Bremner 1996). The organic carbon content and C/N ratio and were analyzed according to AOAC methods (1996).

2.4 Experimental Design

The removal of decachlorobiphenyl (DCB) was evaluated using three concentrations at 0, 1000 and 1500 mg L^{-1} during 0, 7, 14, 28, 56, and 72 days, which generated 18 different combinations. Three replicas were used per treatment (concentration × sample day) and these were arranged in a completely randomized design, with a total of 54 experimental units that were evaluated. Each experimental unit consisted of a 1-L flask containing 30 g of total substrate of peat moss and rabbit manure 85:15 (weight/weight) sterilized three times for 30 min with an interval of a day with pressurized steam at 121 °C in autoclave. The total substrate was mixed and adjusted to a moisture of 75% by adding distilled sterilized water. Each experimental unit was inoculated with three adult earthworms as recommended by Villalobos-Maldonado et al. (2015). The flasks were stoppered with cloth to avoid earthworms from leaving and to establish aerobic conditions; the flasks were incubated at 25 °C, and earthworm behavior was monitored at the beginning of and over the 72-day period, moisture was monitored every 7 days and adjusted to 75% with distilled sterilized water. The average weight of earthworms and DCB concentration were evaluated.

2.5 Quantification of Decachlorobiphenyl in the Earthworms and Vermicompost

Earthworms were separated carefully from each experimental flask and their weight was recorded. The earthworms were placed in sterile glass vials for 24 h to remove traces of organic matter. After, earthworms were triturated to a fine powder with liquid nitrogen and adding anhydrous sodium sulfate to 1.5 times the weight of the worm. In the case of vermicompost, it was dried for 24 h at 50 °C and 10 g of it was weighed and transferred to 50-mL Falcon tubes. Samples corresponding to the earthworms or vermicompost were analyzed separately, as follows: 20 mL of pentane HPLC grade (Sigma-Aldrich®, USA) were added to the sample and, mixed on a vortex for 5 min, and subsequently sonicated for 40 min and centrifuged at 4000 rpm for 15 min. Next, the supernatant was placed in a 50-mL Falcon tube, and the procedure was repeated twice until the concentrate attained a volume of 1 mL. Finally, the accumulated supernatant was allowed to evaporate for 24 h using a chamber of evaporation. Then, the dry sample was suspended in 5 mL pentane HPLC grade. The decachlorobiphenyl in the sample (Earthworms or Vermicompost) was determined in an Agilent Technologies 7890 chromatograph coupled with a mass spectrometry MSD VL 5975 C (Wilmington, USA) using the 8270D method (USEPA 2007). The equipment operating conditions were as follows: capillary column PE-XLB measures 30 m × 0.25 mm × 0.25 μm, helium carrier gas at a pressure of 16 psig, injection temperature 110 °C, detector temperature 150 °C, quantified initial temperature 110 °C × 0.5 min, temperature program of 110 to 300 °C at 15 °C min^{-1} and 300 to 320 °C for 5 min. Flow 1.4 mL min^{-1} and final temperature 320 °C (Villalobos-Maldonado et al. 2015).

2.6 Diversity and DCB Removal Potential of *E. fetida* Gut Bacteria from Vermicomposting System

Based on removal kinetics of 1500 mg L^{-1} DCB (Fig. 1); days 7 and 72 were selected to study the bacterial diversity of *E. fetida* gut in vermicomposting system. For this initial process, the intestinal content of three sexually mature earthworms (approximately 1.0 g) were placed in 9.0 mL of Brain Heart Infusion (BHI) broth obtaining a base solution. Then serial dilutions were done 10^{-1} to 10^{-6}. Then, 5 mL of each bacterial dilution was striated on BHI medium (Hyun-Jung et al. 2004). Plates were incubated at 30 °C for 5 days. Pure cultures were preserved at 4 °C in 65% glycerol-BHI broth for temporary storage. Total genomic DNA of each strain was extracted using the DNA Isolation Kit for Cells and Tissues (ROCHE®, Basel, Switzerland) according to the manufacturer specifications. After, PCR was performed with bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCC-3′) for 16S rDNA, which amplify products of approximately 1500 bases (Weisburg et al. 1991). The PCR-16S rDNA products were digested with the *Rsa*I restriction enzyme (Thermo Scientific®) using the Amplified rDNA Restriction Analysis (ARDRA) technique and then the genomic profiles were analyzed and the Shannon-Weaver index related to the diversity and abundance of the bacterial species associated with the earthworm *E. fetida* were determined (Pereira et al. 2009). PCR products of the strains selected

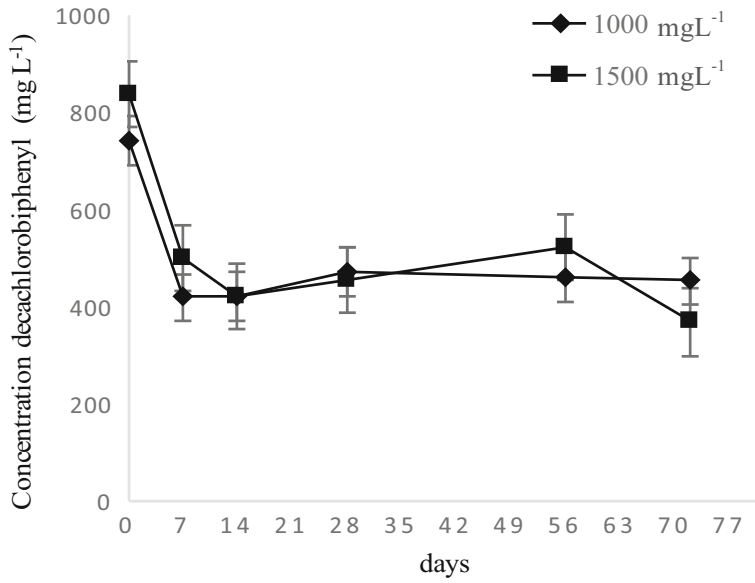

Fig. 1 Removal of decachlorobiphenyl (DCB) during the sampling days in a vermicomposting system by *Eisenia fetida*

by ARDRA were purified using the PCR Product Purification System Kit from Roche® and sequenced (Macrogen®, Korea). All sequences were compared using BLAST (Altschul et al. 1990) and were aligned by the CLUSTAL X (2.0) software (Larkin et al. 2007). Phylogenetic and molecular evolutionary analyses were done with MEGA v5.2 software. The phylogenetic tree was constructed by Neighbor-Joining model (Tamura et al. 2011). 16S rDNA gene sequences were deposited in the GenBank database under the accession numbers KY110406 to KY110420.

Representative strain of each bacterial genus, identified by 16S rDNA gene sequence analysis, was used to determine DCB removal potential. Flasks containing 25 mL of BHI broth were amended with 1500 mg L^{-1} of DCB. Each of the strains were individually subject to a DCB containing medium during 120 h, the time corresponding to the greatest removal potential observed in vermicomposting process (T-7 days). Samples of removed DCB were extracted at the final time (120 h), and simultaneously, extraction of control flasks (uninoculated broth with DCB). To measure the amount of DCB remaining in the medium at final time, 5 mL of broth were taken and added with pentane in a 1:1 ratio (volume/volume). The mixture was maintained for 20 min in vigorous shaking and 30 min under a sonicator. The supernatant obtained on organic phase was separated and concentrated in a solution of 1 mL, the process was repeated three times. The remaining 20 mL were used to separate biomass produced by each one of the evaluated bacteria. Samples were centrifuged at 4000 rpm for 5 min to form a biomass pellet and then were treated with the cellular breakdown according to the procedure recommended by Valenzuela-Encinas et al. (2009) with modifications that consist on adding 15 mL of pentane and sonicating for 30 min after heat shock. Afterwards, the supernatant obtained on organic phase was separated and concentrated in a solution of 1 mL, this method was repeated three times. Samples from broth and cellular biomass were injected into a gas chromatograph coupled to mass spectroscopy and then DCB concentration was determined.

2.7 Statistical Analysis

The variables, average earthworm weight and, DCB concentration were subjected to a one-way analysis of variance (ANOVA) using the Statgraphics Plus v.XV.5 for windows (1999), and mean difference significance was tested with the Tukey test ($P < 0.05$) and also by the *t* student's statistics.

3 Results

3.1 Characteristics of Vermicompost Contaminated with Decachlorobiphenyl

The physicochemical characteristics of the uncontaminated vermicompost and contaminated with

1500 mg L^{-1} decachlorobiphenyl at 0, 7, and 72 days are shown in Table 1. The initial alkaline pH decreases significantly ($P < 0.05$) through the vermicomposting process. The electrical conductivity (EC), which is an indirect measure of salinity, decreased drastically after 7 days in the vermicomposting system with 1500 mg L^{-1} DCB, and remained stable until 72 days, while the EC had no difference significant statistics in vermicompost without contaminants. The organic carbon content increased significantly ($P < 0.05$) along the composting process, while the total nitrogen showed a significant increase ($P < 0.05$) up after 7 days and remained stable until 72 days. The C/N relation increased significantly ($P < 0.05$) with respect to the time of vermicomposting, indicating the increase of organic nutrients in the system.

3.2 Weight of Earthworms during Vermicomposting

In the vermicomposting system amended with DCB, the *E. fetida* earthworms showed a significant decrease in weight ($P < 0.05$) during sampling days (Table 2). During the first 14 days, the earthworms registered the greatest weight in the control treatment (without DCB). However, the weight of earthworms at a concentration of DCB 1000 mg L^{-1} decreased significantly during the 72-day period ($P < 0.05$). The weight of the earthworms under the influence of the contaminant at 1500 mg L^{-1} showed a similar behavior. At the end of the experiment (72 days), the earthworms that grew at a concentration of 1500 mg L^{-1} of DCB had a higher weight compared to the other treatments evaluated ($P < 0.05$).

3.3 Removal of Decachlorobiphenyl in a Vermicomposting System

The decrease of DCB in the treatments at 1000 mg L^{-1} in the vermicomposting was observed in the first 7 days, while removal of DCB for treatment at 1500 mg L^{-1} occurred at 14 days of vermicomposting (Fig. 1), keeping these values constant until 72 days. It was observed that in the two treatments there was a rapid adaptation to the toxicity of the earthworms accelerating the process of removal in the first days of vermicomposting. In relation to the removal of DCB in the vermicomposting system over 72 days, the mass balance allowed to determine that the highest concentration removal of DCB was 230.28 mg L^{-1} with 1000 mg L^{-1} and with 1500 mg L^{-1}, the amount removal was 424.11 mg L^{-1} (Table 3). Therefore, these results allowed to determine the removal efficiency of the vermicomposting system which was the highest in the highest DCB concentration 1500 mg L^{-1} ($P < 0.008$). Also, the mass balance enabled to determine the remaining final concentration of DCB in the vermicompost for 1000 mg L^{-1} units was 450.87 mg L^{-1} and 366.38 mg L^{-1} for units with 1500 mg L^{-1} of DCB. Regarding the final concentration

Table 1 Comparison of physicochemical characteristic during the sampling days between the contaminated vermicompost 1500 mg L^{-1} DCB and uncontaminated

Physicochemical characteristic						
DCB concentration (mg L^{-1})	Sampling days	pH	EC (dS m-1)	Organic carbon (%)	Total nitrogen (%)	C/N ratio
0 (uncontaminated)	0	8.4[a] A[b]	5.26 A	32.0 B	2.2 A	14.4 C
	7	7.5 B	5.16 A	37.3 B	1.8 B	20.7 B
	72	6.5 C	5.06 A	47.1 A	1.4 C	32.1 A
	HSD	0.8475	0.5601	7.977	0.2769	5.8883
1500	0	8.16 A	5.3 A	16.6 C	2.33 A	14.3 C
	7	5.83 B	2.83 B	35.9 B	1.56 B	21.1 B
	72	5.26 C	2.0 B	45.8 A	1.36 B	33.3 A
	HSD =	0.2045	0.9185	1.1172	0.5411	1.6929

[a] Mean values of three replicates

[b] The means followed by the same letter do not show any significant differences according to Tukey's test, ($P < 0.05$)

HSD honest significant difference of Tukey's studentized range

Table 2 Average weight (g) of earthworms exposed at different decachlorobiphenyl (DCB) concentrations during sampling days in a vermicomposting system

DCB concentration (mg L^{-1})	Sample days							
	0	7	14	28	56	72	*P* value	HSD ($P < 0.05$)
0	1.22[a)] aA[b)]	1.12 bA	0.82 cA	0.65 dA	0.52 eA	0.42 fA	0.000	0.0529
1000	1.11 aB	0.74 abB	0.68 abB	0.46 bA	0.49 abA	0.37 bA	0.018	0.6236
1500	1.13 aB	1.0 abAB	0.70 bcB	0.73 abcA	0.63 bcA	0.44 cA	0.002	0.4354
P value	0.000	0.015	0.139	0.210	0.765	0.844		
HSD ($P < 0.05$)	0.044	0.279	0.194 0.194	0.422	0.631	0.524		

[a)] Average weight (g) of three earthworms that correspond to three replicates

[b)] Capital letters denote that there is a statistically significant difference in the weight of earthworms ($P < 0.05$). Lowercase letters denote that there is a statistically significant difference ($P < 0.05$)

HSD honest significant difference of Tukey's studentized range

of bioaccumulated DCB by the earthworms, the Student's test ($P < 0.05$) determined significant differences between the final concentrations contained in the earthworm. When the earthworms were treated with 1000 mg L^{-1}, the highest bioaccumulation was recorded at 59.29 mg L^{-1}.

3.4 Bacterial Diversity Associated with Earthworm *E. fetida* in Vermicomposting System

A total of 150 bacterial strains were isolated from the intestine of *E. fetida* at different cultivated times (0, 7, and 72 days) in a vermicomposting system with the highest concentration (1500 mg L^{-1} DCB). The isolates were grouped mainly within the class ɣ-proteobacteria and bacilli. Bacterial strains were identified by 16S rDNA gene sequences (Table 4). At the initial day, bacterial genera were identified, such as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Solibacillus*, and *Staphylococcus* and as the time of vermicomposting advanced, it was observed that the number of genera diminished. Thus on day 7, there were new bacteria identified as *Acinetobacter*, *Bacillus*, *Klebsiella*, and *Staphylococcus*, while at day 72, the genera found were *Staphylococcus*, *Bacillus*, *Klebsiella*, and *Enterobacter*, with *Bacillus* and *Staphylococcus* found at all three times of vermicomposting (0, 7, and 72 days). Corresponding to the diversity and abundance of bacterial species that are associated with *E. fetida*, the ARDRA allowed to determine the genomic fingerprinting of the different times of samples (Table 5). On day 0 (zero), the bacterial strains were grouped into seven groups with different genomic profiles. At 7 days, four groups were obtained and at 72 days, only five ARDRA groups with specific profiles were determined. The Shannon-Weaver index determined a high abundance or richness of bacterial species ($d = 4.72$) mainly on day 0. The abundance of the species was decreasing according to the sampling time. In the case of the bacterial diversity (H) associated with *E. fetida*, it was low in the three sampling times.

Table 3 Mass balance of decachlorobiphenyl (DCB) over 72 days in a vermicomposting system

DCB initial concentration in the matrix (mg L^{-1})	Extraction efficiency on day 0 (mg L^{-1})	Final concentration of DCB in the substrate (mg L^{-1})	Final concentration of DCB in the earthworms (mg L^{-1})	Estimated amount removed during assay (mg L^{-1})
1000 ppm	740.44 [a)] B	450.87 A[b)]	59.29 A	230.28 B
1500 ppm	834.37 A	366.38 B	43.88 B	424.11 A
(*P* value)	(0.003)	(0.025)	(0.019)	(0.008)

[a)] Mean values of three replicates

[b)] The means followed by the same capital letter do not show any significant differences (Student's *t* test, $P < 0.05$)

Table 4 Taxonomic affiliation of the symbiotic bacteria isolated from intestine of *Eisenia fetida* expose to 1500 mg L^{-1} DCB in vermicomposting system at time 0, 7, and 72 days

Isolate name	Accession number	Closest NCBI match	Species identity BLAST (%)	Class [a]
Time, 0 day				
ADA-1	KY110406	*Pseudomonas alcaligenes* PSA18 (KC622051	99.0	ɣ-proteobacteria
ADA-2	KY110409	*Bacillus subtilis* RD11 (KX268128)	99.0	Bacilli
ADA-3	KY110411	*Paenibacillus tundrae* A10bT (EU558284)	98.2	Bacilli
ADA-5	KY110419	*Pseudomonas extremaustralis* CT14-3T (AJ583501)	98.2	ɣ-proteobacteria
ADA-6	KY110412	*Solibacillus silvestris* HR3-23T (AJ006086)	99.1	Bacilli
ADA-8	KY110408	*Bacillus oceanisediminis* H2T (GQ292772)	98.1	Bacilli
ADA-9	KY110413	*Staphylococcus hominis* DSM 20328T (X66101)	98.6	Bacilli
Time, 7 days				
ADA-11	KY110415	*Klebsiella pneumoniae* ATCC13884T (Y17657)	98.1	ɣ-proteobacteria
ADA-12	KY110416	*Staphylococcus sciuri* DSM 20345T (AJ421446)	99.7	Bacilli
ADA-13	KY110407	*Bacillus cereus* LP10-S06 (KM350268)	100.0	Bacilli
ADA-15	KY110414	*Acinetobacter schindleri* LUH5832T (AJ278311)	99.4	ɣ-proteobacteria
Time, 72 days				
ADA-17	KY110420	*Staphylococcus cohnii* DRLL14 (KU550180)	100.0	Bacilli
ADA-18	KY110417	*Bacillus cereus* ATHH39 (KX344721)	98.0	Bacilli
ADA-19	KY110418	*Klebsiella pneumoniae* BCG12 (KT156817)	99.0	ɣ-proteobacteria
ADA-20	KY110410	*Enterobacter xiangfangensis* BC3 (KR534610)	99.0	ɣ-proteobacteria

[a] Bergey's manual 2004: www.bergeys.org

3.5 Removal Potential of Decachlorobiphenyl by Bacteria Associated with the Earthworm *E. fetida*

In this experiment, all the bacterial strains evaluated had the capacity to remove DCB in a vermicomposting system (Table 6). The strain *Pseudomonas extremaustralis* ADA-5 removed the highest concentration of DCB (326.49 mg L^{-1}) from an initial concentration of 1500 mg L^{-1}, which corresponded to 98.78% of DCB removal, determining that 66.47% was accumulated by the bacterial cellular biomass in comparison with the other strains evaluated in the experiment ($P < 0.05$). In contrast, the lowest amount of DCB removed (54.65 mg L^{-1}) was recorded by the strain *Acinetobacter schindleri* ADA-15 ($P < 0.05$).

4 Discussion

The vermicomposting that use the earthworm *Eisenia fetida* (vermi-remediation) have shown a high efficiency in the removal of different toxic contaminants, taking into consideration the metabolic capacity of this organism (Rodriguez-Campos et al. 2014). In this work, the

Table 5 Diversity and abundance of bacterial species associated with *Eisenia fetida* results during sampling days in the vermicompost exposed to 1500 mg L^{-1} decachlorobiphenyl

			Shannon-Weaver index[b]	
Sampling days	No. isolates	No. groups ARDRA[a] profiles	Richness (d)	Diversity (H)
0	21	7	4.72	1.29
7	20	6	3.91	1.17
72	18	5	3.18	1.07

[a] ARDRA profiles, amplified rDNA restriction analysis obtained with *Rsa*I restriction enzyme (GT^AC)

[b] Shannon-Weaver Index. It was estimated using method reported by Pereira et al. (2009)

Table 6 The potential removal of DCB in a high concentration of bacteria isolated from *Eisenia fetida* after within 72 days of growth in a culture medium

Bacterial strain	Final concentration of DCB in medium (mg L^{-1})	Final concentration of DCB in cell biomass (mg L^{-1})	Final concentration removed during sampling (mg L^{-1})	% of DCB removed or reduced	% of DCB bioaccumulated in cell biomass
Pseudomonas extremaustralis ADA-5	4.01 D[a)]	219.7 A	326.49 A	98.78 A	66.47 A
Staphylococcus sciuri ADA-12	6.12 D	162.74 B	324.38 A	98.14 A	49.24 B
Acinetobacter schindleri ADA-15	275.85 A	57.3 CD	54.65 D	16.53 D	17.33 CD
Bacillus cereus ADA-18	231.65 AB	73.13 C	98.85 CD	29.90 CD	22.12 C
Klebsiella pneumoniae ADA-19	201.07 B	65.51 CD	129.43 C	39.16 C	19.82 CD
Enterobacter xiangfangensis ADA-20	131.19 C	47.14 D	199.31 B	60.30 B	14.26 D
P value	0.000	0.000	0.000	0.000	0.000
HSD ($P < 0.05$)	50.127	17.453	42.365	15.790	7.003

[a)] The means followed by the same capital letter do not show any significant differences (Tukey's test, $P < 0.05$)

HSD, honest significant difference (Tukey's test, $P < 0.05$). DCB initial concentration in the culture medium,1500 mg L^{-1}

potential for removal of DCB by *E. fetida* and its symbiotic bacteria was evaluated in a vermicomposting system. Initially, the substrate formulated with rabbit manure and peat moss was composted for 72 days using *E. fetida* in the presence of high concentrations of 1500 mg L^{-1} DCB. At the end of the process, the vermicompost showed changes in its physicochemical properties (Table 1). The pH and electrical conductivity (EC) decreased significantly. The decrease in pH can be an important factor in the retention of nitrogen, since this element is lost in form of volatile ammonia at a higher pH (Hartenstein and Hartenstein 1981). The content of organic C and total N, and P content in the vermicompost showed a significant increase ($P < 0.05$). This phenomenon can be attributed to the release of humic acids, mucus, nitrogen compounds (urine), and other organic substances that contain high concentrations of C, P, and N from the worm's metabolism (Curry and Schmidt 2007). Also, this may be due to the nitrogen mineralization process in the vermicompost, where commonly the organic N transformed to the nitrate (Atiyeh et al. 2000), while the increase of organic carbon content over time may be due to the accumulation or assimilation of an external carbon source such as DCB in the vermicomposting system. This result which had been confirmed when the content of organic C incremented in the vermicomposting with other types recalcitrant pollutants (Contreras-Ramos et al. 2006).

The survival of *E. fetida* in the presence of high concentrations of DCB during a period of 72 days is shown in Table 2. Although the worms showed a significant decrease in their weight ($P < 0.05$) during the vermicomposting due to natural senescence and high concentrations of DCB, they showed a 100% survival and succeeded in removing the DCB contaminant without showing any morphological alteration, such as surface lesions and mid-segmental swellings or general ulcerated areas on the surface, as stipulated by the standards methods for toxicity (OECD 2000). In the same way, Villalobos-Maldonado et al. (2015) reported that the weight of earthworms increased with concentrations of DCB at 100, 150, and 200 mg L^{-1} in the first 7 days. However, the weight of earthworms began to decrease during the process of vermicomposting due to exhaustion of the nutrients (mainly the carbon source) and the contaminant in the vermicompost. Also, this same phenomenon was reported by Xie et al. (2013), when they analyzed the effect of ether decabromodiphenyl (BDE-209) on the survival, growth,

and reproduction of the earthworm (*Eisenia fetida*). Matscheko et al. (2002) reported at 5% decrease in *E. fetida* weight that was exposed to a soil that was contaminated with 16 different PAHs for 19 days. Earthworms have a complex interrelationship with microorganisms, which serve as their major source of nutrients, as well as earthworms that promote microbial activity by fragmentation and inoculation of decaying organic matter with microorganisms (Li et al. 2002).

The results obtained in this study have shown that in the vermicomposting system, there was high removal of concentration DCB during 72 days. Then, in this study, it was found that the highest DCB removal occurred with the highest initial concentration (1500 mg L^{-1}) during the first 7 days, stirring 424.11 mg L^{-1} of which 43.88 mg L^{-1} were retained in the matrix of the earthworm, which could be due to the lipophilicity of the cellular components of their tissues and also by the chemical structure of the congener DCB and its chlorine atoms, which is characterized by having greater lipophilicity, resistance, and absorption strength (Arbeli 2009) (Table 3). Similar results were obtained by Villalobos-Maldonado et al. (2015) reporting high levels of bioaccumulation in the matrix of the earthworm in treatments evaluated with 200 mg L^{-1} DCB in a vermicomposting system. Also, in this study, high levels were obtained in the two treatments, the residual concentrations in the vermicompost obtained were 366.38 mg L^{-1} at the highest concentration (1500 mg L^{-1}) (Table 3), caused by the minimal availability of substrate and the high levels of DCB that led to the contaminant being consumed as carbon source and also to the chemical characteristics of the DCB, since the congeners with substitutions of protons in the *meta* and *para* positions have been reported with greater susceptibility to biological removal processes (Llyas et al. 2013).

Diverse studies that are related with the potential removal of contaminants chemicals recalcitrants of the *E. fetida* were reported by (Tharakan et al. 2006; Contreras-Ramos et al. 2006; Lin et al. 2016). However, little is known about the removal capacity and biodegradation of the DCB of *E. fetida* and its symbiotic native bacteria. In this study, we managed to isolate and identify bacteria in the intestine of *E. fetida* that grew during 0, 7, and 12 days of vermicompost contaminant with high concentrations of DCB (Table 4). On day zero, there were seven different bacterial species that corresponded to ɣ-proteobacteria and bacilli. The sequences of 16S rDNA gen were similar to *Pseudomonas alcaligenes* and *P. extremaustralis* strains. Species of *Pseudomonas*, like *P. aeruginosa* TMU56 that were isolated in contaminant soil and with electrical transformer fluid (Askarel) had a capacity to degrade 73.3% of PBCs during a 4-day period of incubation (Hatamian-Zarmi et al. 2009). The strains ADA-2 and ADA-8 corresponded to *Bacillus*. Based on the sequence of the 16S rDNA gene, strain ADA-2 showed > 99.0% similarity with the sequence of *Bacillus subtilis* RD11 and the ADA-8 strain had 98.1% with the *B. oceanisediminis* H2 strain. Some strains, like *Bacillus* sp. JF8 showed degradation of PCB congeners including tetra- and pentachlorobiphenyl (Mukerjee-Dhar et al. 2005) and the *Bacillus cereus* JP12 that had the capacity to degrade decabromodiphenyl ether (Mang et al. 2013). ADA-3 showed > 98.2% similarities with the *Paenibacillus tundrea* A10b. ADA-6 strain had 99.15% similarity with the *Solibacillus silvestris* strain. ADA-9 strain was 98.65% to the *Staphylococcus hominis* DSM20328 (Table 4).

From day 7, the bacteria were identified and clustered within *Klebsiella*, *Staphylococcus*, *Bacillus*, and *Acinetobacter*. Based on the sequence of the 16S rDNA gene, strain ADA-11 showed > 98.1% similarity with the sequence of *Klebsiella pneumoniae* ATCC13884. ADA-12 strain had 99.7% genetic similarity to the *Staphylococcus sciuri* DSM20345. The ADA-13 and *Bacillus cereus* LP10-S6 strain were 100.0% similar to each other, and ADA-15 strain and *Acinetobacter schindleri* LUH5832 had 99.4% similarities to one another.

On day 72, *Staphylococcus*, *Bacillus*, *Klebsiella*, and *Enterobacter* were found. Noting that the isolates that were grouped in the genus *Bacillus* and *Staphylococcus* were found three times in vermicomposting (0, 7, and 72 days). *Klebsiella* and *Staphylococcus* are noticeable for their capacities to degrade different congeners of PCBs (Jianjun et al. 2016). As the case of *Staphylococcus xylosus* had the ability to degrade PCB to degrade PCBs during 168 h of incubation in liquid media (Leaes et al. 2006). The ADA-20 is the only bacterial species that had been found during this vermicomposting process and had 99.0% similarities to *Enterobacter xiangfangensis* BC3 (Ya-Ming et al. 2011).

Corresponding to the diversity and abundance of bacterial species are associated with *E. fetida* (Table 5). On day 0 (zero), the bacterial strains were grouped into seven groups with different genomic profiles. At 7 days, four groups were obtained and at

72 days, only five ARDRA profiles were determined. The Shannon-Weaver index determined a high abundance of bacterial species ($H = 4.72$) mainly on day 0. The abundance of the species was decreasing according to the sampling time. In the case of the bacterial diversity (d) associated with *E. fetida*, it was low in the three sampling times. The high concentration of DCB in the vermicomposting system acts as a chemical factor that limits the diversity and abundance of the species in the bacterial community that reside in the *Eisenia fetida* intestine. At the end of the vermicomposting process, only the bacterial species endowed with the enzymatic machinery capable of degrading this recalcitrant contaminant survive.

The maximum bioaccumulation of DCB was obtained with the bacterium *Pseudomonas extremaustralis* with 219.7 mg L^{-1} accumulated in its biomass while 98.78% de DCB was removed from the culture medium (Table 6), this bacterial genus has been reported to have a high potential for the degradation of organochlorine compounds. Komancová et al. (2003) reported that *Pseudomonas* sp. was able to eliminate congeners with lower chlorine content (*tri* and *tetra*-clorobiphenyl) with interference in the biphenyl structure with 48% of elimination. On the other hand, Hatamian-Zarmi et al. (2009) showed that *Pseudomonas aeruginosa* had the ability to eliminate up to 89% of hexachlorinated congeners, tolerating up to 200 mg L^{-1} of initial PCBs in the culture, which indicates that these results may be due to the obstruction and bioaccumulation observed in *P. extremaustralis* corresponds to an important initial process in the removal and possibly bacterial degradation of congener DCB (Alonso et al. 2008) where the lipids of its membrane and the lipophilicity of this congener are important characteristics to carry out the process of removal. The genera *Acinetobacter*, *Klebsiella*, and *Bacillus* have been reported as genera with potential for degradation of different persistent organic pollutants (Pieper 2005; Vasilyeva and Strijakova 2007). For instance, Borja et al. (2005) reported that these genera were isolated in the intestine of the earthworm and tolerated importantly the DCB, where the tolerance and aerobic degradation of these bacterial genera to different PCB congeners with lower chlorine content was evaluated. Also, in this study, the presence of 1,2-benzenedicarboxylic acid was detected qualitatively as an intermediate in the degradation pathway of PCBs in *Pseudomonas* such as was reported by Koubek et al. (2013).

5 Conclusions

In this study, we demonstrated for the first time that the earthworm *E. fetida* and its symbiotic bacteria in a vermicomposting system have a great potential to remove high concentrations of decachlorobiphenyl. In relation to the diversity and abundance of the bacterial species that inhabitant in the digestive tract, decrease significantly during the process of removal is due to the pressure given by contaminant DCB. Thus, the removal and bioaccumulation of DCB by *Pseudomonas extremaustralis* ADA-5 and *Staphylococcus sciuri* ADA-12 was greater at 1500 mg L^{-1} compared with other isolates. Therefore, it is important in the future to evaluate the presence of the biphenyl dioxygenase (*bph*) genes in the genome of the isolated bacteria to identify the potential of degradation of PCBs.

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