INDOOR SIMULATION OF REMEDIATION OF 2, 2', 4, 4'-TETRABROMODIPHENYL ETHER CONTAMINATED SOIL BY *BACILLUS LATEROSPORA*

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(Received 13th Jul 2019; accepted 25th Nov 2019)

Abstract. High-throughput sequencing and traditional microbial culturing were used to analyze the effects of the addition of free or immobilized degrading bacteria on the soil physicochemical properties and microbial community structure by simulating the pollution of 2, 2', 4, 4'-tetrabromo diphenyl ether (BDE-47), and to measure the degradation rates. The results showed that (1) The soil physicochemical properties including pH, organic matter, total nitrogen, total phosphorus and total potassium were not significantly affected by the addition of BDE-47, free or immobilized bacteria; (2) *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the dominant phyla in the simulation systems, of which the relative abundance of *Proteobacteria* and *Acidobacteria* decreased whereas that of *Actinobacteria* increased through the simulation. *Firmicutes* was dominant in the simulation system B. Seven phyla, including *Bacteroidetes*, *Gemmatimonadetes*, and *Nitrospirae*, were shared by all simulation systems. The free degrading strain adapted well to the simulation system, and the cell count increased from $(5.0 \pm 0.1) \times 10^4$ Colony-forming unit (CFU) /g to $(1.0 \pm 0.2) \times 10^5$ CFU/g at the end of the simulation; (3) The degradation of BDE-47 occurred in the original soil used for the simulation, while the addition of the free degrading strain promoted the degradation of BDE-47 at a rate of 39.77%. The addition of the immobilized strain also promoted the degradation of BDE-47, but only at a rate of 36.94%, slightly lower than that of the free strain.
Keywords: *BDE-47, microbial degradation, immobilization, high-throughput sequencing*

Introduction

As persistent organic pollutants, polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) widely used in the electronics industry, electrical appliances, household appliances, textile, oil and construction materials (Dewit, 2002). The general chemical formula of PBDEs is $C_{12}H_{(0-9)}Br_{(1-10)}O$, and there are 209 homologs with different numbers of bromine atoms (Costa et al., 2008). As of 2001, the market demand for the flame retardants PBDEs has exceeded 67,440 metric tons and keeps rising (Arias, 1992; Dewit, 2002). Due to a lack of chemical bonds, PBDEs added to products can easily enter their environment through volatilization and leaching, and with the migration to the atmosphere and water bodies, they may extensively pollute the atmosphere, water, sediments, soil, and biosphere. PBDEs are highly lipophilic,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1801_10011011

chemically stable, and resistant to degradation. Numerous studies have found that PBDEs have endocrine disrupting toxicity (Walter et al., 2017), developmental neurotoxicity (Chen et al., 2014), and immunotoxicity (Talsness, 2008), all of which can be enriched, amplified along food chains (Zhou et al., 2016) and transferred to the next generation through breast milk, placenta, and cord blood (Chen et al., 2014; Xu et al., 2017), posing a potential threat to human and environmental health. Therefore, at the 4th Meeting of the Conference of the Parties (COP-4) of the Stockholm Convention on Persistent Organic Pollutants (POPs) in May 2009, tetrabromo and pentabromo diphenyl ethers in commercial pentabromide products, and the hexabromo and heptabromo diphenyl ethers in commercial octabromide products were added to the Annex A of the Convention as new POPs.

As the primary commercial, pentabromo diphenyl ether (Penta-BDE) monomer 2, 2', 4, 4'-tetrabromo diphenyl ether (BDE-47) is very toxic and can cause harm even at very low doses. Soil is one of the final destinations of BDE-47 and plays a very important role in the spatiotemporal distribution and geochemical cycling of BDE-47. The micro-ecological environment of the polluted soil is complex, where microbes play the central part and the abiotic and biotic factors interact with each other. As an essential biological component of the soil environment, microbes play a pivotal role in the migration and transformation of BDE-47. At present, physical, chemical and biological methods are mainly used for remediation of contaminated soil. Microbial remediation, as the main means of bioremediation, is widely used because of its economic and non-secondary pollution characteristics. One of the widely applied bioremediation techniques is microbial enhancement technology. It improves the pollutant degradation ability of soil or a system by adding bacterial strains screened from a natural source to the soil or biological treatment system for quickly removing the pollutants. As a microbial enhancement technology, microbial immobilization basically confines or localizes free cells or microorganisms to a defined spatial region by using chemical or physical methods to keep them active and reusable. The advantages of this technique include high microbial density, fast reaction rate, strong resistance to toxicity, and low microbial loss.

In the present study, the trends of the dominant bacteria in the simulation systems were dynamically tracked using high-throughput sequencing technology. Chen et al. (2006) reviewed the methods for determining the structural and functional diversities of microbial communities in polluted soils at China and abroad in recent years, and observed that the analysis and research on the microbial diversity of polluted soils were still not robust and conclusive. One of the reasons for this was the fact that the methods and techniques for determining microbial species had not yet considerably developed for comprehensive and accurate analysis of the soil microbial diversity, while the other was the high heterogeneity of the environment. The local spatial-temporal environment kept changing all the time (Vanelsas et al., 1997). In recent years, with the development of high-throughput sequencing technologies, more studies on the microbial structure changes in polluted soils are being conducted. Wang (2016) used high-throughput sequencing to analyze the response of various farmland soil microbial communities to oil spill pollution and planted black locusts. Likewise, the characteristics of the microbial

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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community structure and distribution in petroleum-contaminated soil were analyzed by Liang using high-throughput sequencing (Yang et al., 2013).

In this study, indoor simulation of BDE-47 contaminated soil environment was carried out; an immobilized degrading strain was used to simulate soil remediation, and the changes in microbial community structural during the restoration was analyzed. Our study provides a theoretical basis and technical support for the microbial remediation of BDE-47 polluted soil.

Materials and Methods

Immobilization of the degrading strain

A strain with a good aerobic degradation ability for BDE-47 was isolated and purified from the soil of an electronic waste dismantling site in Taizhou City, Zhejiang Province, China. The strain was identified as *Bacillus laterospora* of the genus *Bacillus* and is deposited in the China General Microbiological Culture Collection Center (deposition number CGMCC No. 8616). The optimum growth temperature for the strain is 30-37°C, with reddish brown colonies on the bacterial solid medium, having moist surface and uneven edges.

In this study, sodium alginate was used as the immobilization carrier for the bacteria, and the cells were embedded inside according to the embedding method discussed. In brief, before simulation, the degrading strain was acclimated in an inorganic liquid culture medium containing 500 μg/L BDE-47, for 10 d acclimation period and a total of four periods. The acclimated strain was enriched in liquid culture and the cells were collected by centrifugation and washed three times with sterilized water to prepare the bacterial suspension (OD_{600} = 2.5) for immobilization. The bacterial suspension was added to the sodium alginate sol, thoroughly stirred and mixed. The mixed solution was dropped into a calcium chloride solution using a syringe, to form beads which were fixed at 4°C. Then the beads were washed with 0.85% sterilized physiological saline and filtered. After drying on a sterilized filter paper, they were stored at 4°C. According to the comprehensive properties of the immobilized beads, the final immobilization conditions were set as bacteria (2 g/L), sodium alginate (4% w/v), calcium chloride (3% w/v), and fixation for 4 h.

Construction of the simulation systems

Taizhou, Zhejiang is a typical e-waste dismantling site in China. To explore the effect of the preliminary screened free strain and its immobilized form on the polluted soil, the soil sample for simulation in this study was collected from the surface (0-20 cm) at the Taizhou Park with no BDE-47 pollution. During the collection, the debris such as plant residues were removed. The soil sample was sealed in a sampling bag and transported to the laboratory in a sampling box with an ice pack. One part (about 15 kg) of the soil sample was directly filtered through a 10 mm sieve for indoor simulation experiments, the other part (about 2 kg) was ground and air-dried, filtered through a 60 mm sieve for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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preparing the polluted mother soil, and the remaining part was stored at respective 4°C (about 1 kg) and -20°C (about 200 g) for further soil physicochemical analyses and high-throughput sequencing, respectively.

Construction of microbial remediation simulation system for BDE-47 contaminated soil: The vases of height 18 cm and an opening diameter of 21 cm were selected as the simulation containers. In system A the simulated soil and pollutant BDE-47 were added. In system B the simulated soil, pollutant BDE-47, and the free degrading bacteria were added, while the degrading bacteria were in the form of a bacterial suspension. In system C, the simulated soil, pollutant BDE-47 and the immobilized degrading bacteria, in the form of 100 g/kg immobilized beads were added. The soils were turned once every 30 days. The concentration of BDE-47 in the simulation systems were approximately 1,500 ng/g. A certain amount of sterilized water was added to the simulation systems regularly to maintain the water content of the tested soil. Each simulation system had two replicates and the simulation remediation time was 90 days.

Preparation of bacterial suspension: Before the simulation, the degrading strain was acclimated in the inorganic liquid culture medium containing 500 μg/L BDE-47, for 10 d as an acclimation period and four periods totally. The acclimated strain was enriched in the Luria-Bertani (LB) liquid medium, collected by centrifugation and washed three times with sterilized water to avoid the interference of nutrients in the bacterial liquid medium. Then the bacterial suspension was prepared for further simulation experiment with free degrading bacteria.

LB liquid medium: peptone, 10 g; yeast extract, 5 g; and NaCl, 10 g were added to 1 L water. The pH was adjusted to 7 and the medium was sterilized at 121°C for 20 min.

Determination of physicochemical properties of the soil sample

The soil pH, organic matter (OM), total nitrogen (TN), total phosphorus (TP) and total potassium (TK) were determined by the potentiometric method (NY/T1377-2007), potassium dichromate was determined by external heating method (NY/T1121.6-2006) and semi-micro Kjeldahl method (NY/T1121.24-2012), sulfuric acid was determined by perchloric acid digestion (NY/T88-1988) and flame photometric method (NY/T87-1988) (Bao et al., 2008).

Determination of microbial count and community structure

Determination of microbial count: Soil sample (10 g) was aseptically transferred to an Erlenmeyer flask containing 90 mL of sterilized water with an appropriate amount of glass beads in the flask, shaken for 2 h at 37°C and 120 rpm. Then, 1:10 dilution was prepared, which was further diluted 1:10 and a ten-fold serial dilution was made as described above. For each dilution, 1 mL of each dilution was plated on a sterilized LB plate, with three replicates for each dilution. The plates were cultured at 37°C for 36 h, and those with 30-300 total colonies were considered for determining the microbial count.

Determination of microbial community structure: Microbial community structure test analysis process as shown in the literature (Edgar et al., 2011; Knight et al., 2018).

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Determination of BDE-47 content in the soil

The soil of each simulation system was collected, dried, ground, and filtered through a 100-mesh sieve. The soil sample (10 g) was added with an appropriate amount of diatomaceous earth, mixed, and extracted with an accelerated solvent extractor. The extraction tank was 34 mL, the solvent used was hexane/dichloromethane (1:1), and the static extraction was performed at 100°C and 1.03 kpa for five minutes and two cycles. The extract was transferred to a 100 mL rotary evaporator flask for rotary evaporation at 30°C to 1-2 mL, then 10 mL hexane was added to it and rotary evaporated to 1-2 mL again. The prepared sample was loaded on a silica gel alumina column and eluted with 75 mL hexane/dichloromethane (1:1) for purification. The eluent was collected and again rotary evaporated at 30°C to 1-2 mL, then 5 mL acetonitrile was added and concentrated again to approximately 1 mL. Next, it was transferred to a 2 mL sample vial, concentrated under nitrogen, fixed with 1 mL with acetonitrile, and filtered through a 0.22 μm membrane. Finally, it was loaded onto the Waters ACQUITY UPLC C18 column, sized 2.1 mm × 50 mm × 1.7 μm, with 100% acetonitrile as the mobile phase. The flow rate was set to 0.2 mL/min, the column temperature was 30°C, and the absorbance was measured at 226 nm.

Results and discussion

Changes in soil physicochemical properties

Compared with the original, natural soil, there was no significant change in the soil pH, OM, TN, TP or TK in the simulation systems, indicating that in the simulation processes, the addition of BDE-47, free or immobilized bacteria, had no significant effect on the soil physicochemical properties (*Table 1*). Liu et al. (2011) showed that the OM in soil could maintain soil water content and water availability, and thus, can be used as a routine indicator to measure soil water retention and availability. The increase of soil OM content may cause changes in soil aggregate structure to loosen the texture and increase the water permeability and water holding capacity (Guo, 2007). The nitrogen in the soil exists primarily in the surface soil and is closely related to the organic matter content. Nearly 80~97% of the total nitrogen in the surface soil comes from the decomposition of organic matter (Ros et al., 2004). Therefore, the changes in OM content change the soil nitrogen content (Huangshan, 1998). Total phosphorus is an index measuring the sum of various forms of phosphorus in soil. Its value is greatly impacted by the soil parent material and soil formation, and it is also related to soil texture and organic matter (Ding et al., 2010). While the changes in soil physicochemical properties directly affect the microbial community structure, various physicochemical properties synergistically affect the microbial community. As a result, none of the tested simulation systems demonstrated significant changes in physicochemical properties, thus, indicating that the simulation process does not significantly impact the microbial community structure.

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DOI: http://dx.doi.org/10.15666/aeer/1801_10011011

Table 1. *Soil Physical and Chemical Properties in Simulated System*

Properties	Original soil	A-E	B-E	C-E
pH	7.2	7.3	7.2	7.1
OM (g/kg)	21.7±0.66a	21.80±0.757a	22.04±0.76a	20.4±0.7a
TN (g/kg)	2.18±0.023a	2.19±0.02a	2.23±0.07a	2.18±0.06a
TP (g/kg)	0.58±0.008a	0.58±0.008a	0.59±0.012a	0.61±0.002a
TK (g/kg)	10.53±0.55a	10.54±0.7a	10.80±0.2a	10.82±0.11a

A, B, and C represent the three simulation systems, and E represents the end of the simulation

Analysis of microbial structure changes

Changes in microbial community structure

As mentioned in *Fig. 1*, *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were the dominant phyla in the simulation systems. During the initial stage of the simulation, *Proteobacteria* accounted for 41.58%, 34.47% and 40.97% in systems A, B, and C, respectively. Along the simulation process, its percentages depleted, and accounted for 31.53%, 27.33%, and 29.35%, respectively, at the end of the simulation. The changes in *Acidobacteria* population was similar to that of *Proteobacteria*. The percentage of *Actinobacteria* went up along the simulation, changing from initial 18.62%, 12.27%, and 19.81% to the final 42.02%, 37.66%, and 43.34%, respectively, for the systems A, B, and C. *Firmicutes* were the dominant phylum in system B, and its percentages before and after simulation were 18.80% and 13.89%, respectively. The phyla *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Verrucomicrobia*, *Nitrospirae*, *Planctomycetes*, and *Candidate_division_WS3* were common in all simulation systems, while the other bacteria were rarely found.

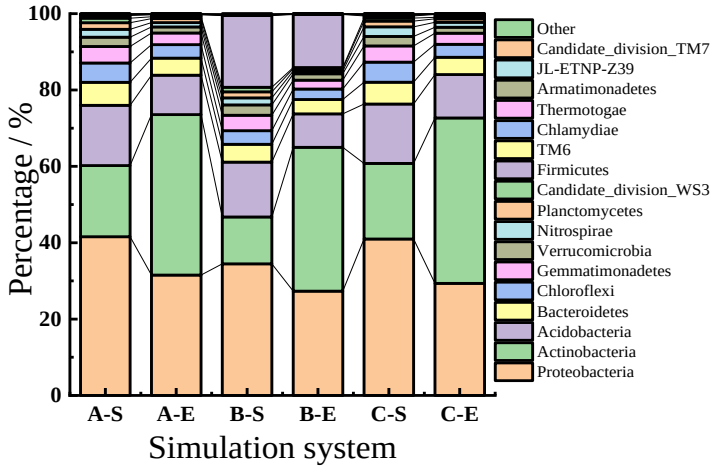

Figure 1. *Analysis of microbial community structure at Phylum level start and end each simulation system. A: Soil + BDE-47; B: Soil + BDE-47 + free degradation bacteria; C: Soil + BDE-47 + immobilized strains; S: Simulation start; E: Simulation end*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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In system A and C, as the simulation progressed, the proportions and trends of the bacterial phyla were consistent, indicating that the addition of the immobilized degrading strain did not affect the microbial community structure in the soil significantly. Although, the addition of the free degrading strain actually impacted the original microbial community structure to some extent. For system B, the dominant role of *Firmicutes* was observed because the added free degrading bacteria belonged to this phylum. In summary, for the simulation systems, the change of the microbial community structure in the soil was related more closely to the addition of the degrading bacteria in the simulation process. The degrading strain was screened and isolated from the contaminated soil in Zhejiang and was more adaptable to the soil ecological environment in that province. Hence, when it was applied to remediate the soil polluted by BDE-47 in Zhejiang, it had less impact on the soil microbial community structure.

Analysis of the trend of the degrading strain

As per the high-throughput sequencing results (*Figure 2*), the dynamic tracking of the degrading strain which belonged to *Bacillus* showed that the relative abundance of *Bacillus* in system A dropped from 0.0062 at the initial stage to 0.0041 at the later stage. Whereas in system B, the addition of the degrading *Bacillus* strain directly caused its relative abundance to increase to 0.1857 at the initial stage, and decrease later to 0.13813, although it was still the dominant species. In the system C, the relative abundance of *Bacillus* at the initial stage was 0.00553, and with the simulated remediation, its relative abundance increased by 0.16% to the final 0.00714. The analysis of the free degrading strain count by the traditional microbial culturing technique in system B showed an initial count of $(5.0 \pm 0.1) \times 10^4$ CFU/g, which increased along the simulation to a final count of $(1.0 \pm 0.2) \times 10^5$ CFU/g (*Fig. 3*).

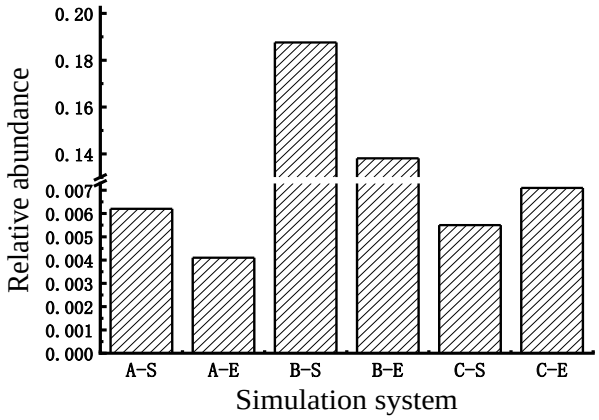

Figure 2. *Analysis of relative abundance variation of Bacillus sp in each simulation system*

The two approaches revealed that the free degrading strain adapted well to the simulated soil environment and that the decrease in *Bacillus* may be due to cell death and DNA degradation during the simulation process, resulting in a decline in final relative

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DOI: http://dx.doi.org/10.15666/aeer/1801_10011011

abundance. At present, only about a dozen strains have been found capable of aerobic degradation of PBDEs, and the reported ones include *Sphingomonas* sp. (Schmidt et al., 1992, 1993; Hundt et al., 1999), *Rhodococcus* sp. RR1, *Seudonocardia dioxanivorans* CB1190 (Robrock et al., 2009), *Lysinibacillus fusiformis* strain DB-1 (Deng et al., 2011), *Bacillus cereus* JP12, *Acinetobacter*, *Pseudomonas*, and *Staphylococcus* (Wang et al., 2016). Currently, there are very limited reports on the application of free strains for environmental remediation.

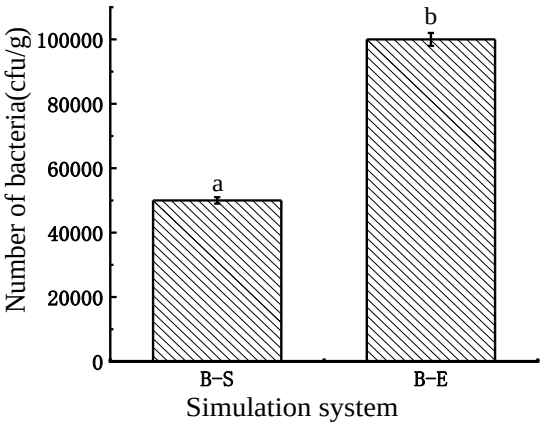

Figure 3. *Change in the number of free bacteria in B simulation system*

BDE-47 degradation analysis

As shown in *Table 2*, at the start of simulation, the BDE-47 concentrations were 1,504 ng/g in system A, 1,775 ng/g in system B, and 1,654 ng/g in system C. After 90 days of simulation remediation, the remaining BDE-47 concentrations were 1,135 ng/g in system A, 1,069 ng/g in system B, and 1,043 ng/g in system C. The degradation rate of BDE-47 in system A was 24.53%, demonstrating existence of BDE-47 degrading strain in the simulated original soil. The degradation rate in system B was 39.77%, indicating that the added free degrading strain potentially promoted the degradation of BDE-47. The degradation rate in system C was 36.94%, indicating that while the added immobilized strain could promote the degradation of BDE-47 as well, but it was inferior to the free strain. The micro-ecological environment of the polluted soil consists mostly of microorganisms, which are important biotic components of the soil environment. The soil used for simulations was collected from Taizhou, Zhejiang and the added degrading strain was also screened from a contaminated site in Taizhou, Zhejiang. Therefore, the free strain could adapt well to this soil environment, promoting the degradation of the pollutant BDE-47. Although studies show that the immobilization of degrading bacteria can increase the concentration of microorganisms, keep the strains highly active and repeatedly usable, and greatly enhance the environmental adaptability of the immobilized microbes and their ability to degrade organic matter (Karamanev et al., 1998; Su et al., 2006, 2008; Lu et al., 2015). In this present study, although the

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DOI: http://dx.doi.org/10.15666/aeer/1801_10011011

immobilization increased the concentration of bacteria inside the immobilized beads, the bacteria could not diffuse. Therefore, it was not conducive to the degradation of pollutant in the soil.

Table 2. *BDE-47 concentration chromatographic results*

System	Simulation time (day)	Peak time (min)	Peak area (μA/s)	Peak height (μA)	BDE-47 concentration (ng/g)
A	0	1.106	264969	135094	1504
	90	1.104	204801	106061	1135
B	0	1.106	308973	154296	1775
	90	1.105	194046	100537	1069
C	0	1.104	289360	147979	1654
	90	1.104	189882	94948	1043

Conclusions

a) In all simulation systems, the addition of pollutant BDE-47, and the free or immobilized strain had little effect on the soil physicochemical properties such as pH, OM, TN, TP, and TK.

b) The dominant phyla in the simulation systems were *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*. A total of seven phyla including *Bacteroidetes*, *Gemmatimonadetes*, and *Nitrospirae* were common bacteria shared by all three simulation systems. The *Firmicutes* became the dominant bacterial phylum because the added strain of free bacteria belonged to this phylum. The free degrading strain adapted well to the simulation system, and its count was increased in the later stages.

c) The original soil used for the simulation contained the BDE-47 degrading strain, and the addition of the free degrading strain could promote the degradation of BDE-47 at a rate of 39.77%. The addition of the immobilized strain also promoted the degradation of BDE-47, although the degradation rate was 36.94%, slightly below that of the free strain. The next research plan focuses on the degradation pathway of BDE-47, which lays the foundation for regulating the degradation process of BDE-47.

Acknowledgments. This work supported by PhD Research Foundation Project (MNUB201709) and Heilongjiang Provincial Department of Education Basic Research Project (1353MSYYB026).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1801_10011011