PHTHALATE ESTER BIODEGRADATION IN AGRICULTURAL SOIL USING EXOGENOUS BACTERIAL CONSORTIUM UNDER FERTILIZER ADDITION

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การย่อยสลายทางชีวภาพของพทาเลทเอสเทอร์ในดินเกษตรโดยกลุ่มแบคทีเรียต่างถิ่นภายใต้ภาวะที่มีการเติมปุ๋ย

น.ส.ธีโอโดรา เมกะ ปุตรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PHTHALATE ESTER BIODEGRADATION IN AGRICULTURAL SOIL USING EXOGENOUS BACTERIAL CONSORTIUM UNDER FERTILIZER ADDITION
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Field of Study	Microbiology and Microbial Technology
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ธีโอโดรา เมกะ ปุตรี : การย่อยสลายทางชีวภาพของพทาเลทเอสเทอร์ในดินเกษตรโดยกลุ่มแบคทีเรียต่างถิ่นภายใต้ภาวะที่มีการเติมปุ๋ย. (PHTHALATE ESTER BIODEGRADATION IN AGRICULTURAL SOIL USING EXOGENOUS BACTERIAL CONSORTIUM UNDER FERTILIZER ADDITION) อ.ที่ปรึกษาหลัก : รศ. ดร.อรฤทัย ภิญญาคง

พทาเลทเอสเทอร์ (Phthalate esters; PAEs) เป็นสารมลพิษที่สำคัญเนื่องจากสมบัติการบกวนการทำงานของต่อมไร้ท่อ และการปนเปื้อนอย่างแพร่หลายในสิ่งแวดล้อม งานวิจัยก่อนหน้าได้คัดแยกกลุ่มแบคทีเรียย่อยสลาย PAEs จากดินหลุมฝังกลบขยะ งานวิจัยนี้จึงสนใจเลือกกลุ่มแบคทีเรียที่มีประสิทธิภาพย่อยสลาย PAEs เพื่อศึกษาจลนศาสตร์การย่อยสลาย การเปลี่ยนแปลงประชากร การรักษาประสิทธิภาพการย่อยสลายของกลุ่มแบคทีเรีย และความเป็นไปได้ในการนำไปใช้บำบัดสิ่งแวดล้อมในรูปแบบการเติมจุลินทรีย์ในระบบนิเวศจำลองดิน โดยกลุ่มแบคทีเรีย LF-NK-DEHP ย่อยสลายสารผสม Dibutyl phthalate (DBP) และ Di(2-ethylhexyl) phthalate (DEHP) ชนิดละ 100 มิลลิกรัมต่อลิตร ได้ 92.9 และ 63.4 เปอร์เซ็นต์ ตามลำดับ ใน 7 วัน จลนศาสตร์การย่อยสลาย DBP 500 มิลลิกรัมต่อลิตร สอดคล้องกับ modified Gompertz model (R^2 = 0.92, $t_{1/2}$ = 1.49 วัน) และ DEHP 500 มิลลิกรัมต่อลิตร สอดคล้องกับ zero-order kinetic model (R^2 = 0.94, $t_{1/2}$ = 7.23 วัน) การย่อยสลาย DBP มีความสัมพันธ์กับ *Microbacterium* และ DEHP สัมพันธ์กับ *Rhodococcus* ในขณะที่ *Pigmentiphaga* สัมพันธ์กับการย่อยสลาย PAEs ทั้ง 2 ชนิด การเติมแบคทีเรียเพียงอย่างเดียวไม่สามารถส่งเสริมการย่อยสลาย PAEs ในระบบนิเวศจำลองดินได้ แต่การเติมแบคทีเรียควบคู่กับปุ๋ยสามารถเพิ่มประสิทธิภาพได้ โดยย่อยสลาย DEHP 200 มิลลิกรัมต่อลิตร ได้ 75.87 เปอร์เซ็นต์ ใน 25 วัน และกำจัด DBP 200 มิลลิกรัมต่อลิตรได้สมบูรณ์ใน 3 วัน นอกจากนี้ยังเพิ่มจำนวนแบคทีเรียย่อยสลาย PAEs ในดิน รวมถึงลดความเป็นพิษของ PAEs ต่อพืชได้ งานวิจัยนี้ทำให้ได้กลุ่มแบคทีเรียกลุ่มใหม่ที่ย่อยสลาย PAEs และวิธีการคงประสิทธิภาพของกลุ่มแบคทีเรีย รวมถึงแนวทางการใช้สำหรับส่งเสริมการย่อยสลาย PAEs ในดิน

สาขาวิชา	จุลชีววิทยาและเทคโนโลยีจุลินทรีย์	ลายมือชื่อนิสิต .....
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Phthalate esters (PAEs) have been classified as a priority contaminant due to their endocrine-disrupting properties and abundance in the environment. Previously, several PAE-degrading consortia were enriched from landfill soil. This study aimed to initially select a consortium with efficient degradation activity, to characterize based on degradation kinetics and community dynamics, and to evaluate bioaugmentation feasibility while maintaining activity through continuous transfer. It was obtained based on enriched consortium selection that LF-NK-DEHP could simultaneously degrade 92.9% 100 mg l^{-1} DBP and 63.4% 100 mg l^{-1} DEHP within seven days. Kinetic degradation revealed DBP degradation by LF-NK-DEHP to fit the modified Gompertz model (R^2 = 0.92, $t_{1/2}$ =1.49 days, 500 mg l^{-1} DBP). On the other hand, DEHP degradation kinetics fit a zero-order kinetic model (R^2 = 0.94, $t_{1/2}$ =7.23 days, 500 mg l^{-1} DEHP). Community dynamics study revealed a significant correlation between the genus *Microbacterium* to DBP degradation and the genus *Rhodococcus* to DEHP degradation. Genus *Pigmentiphaga* was reported to correlate with both PAEs. In addition, continuous transfer of LF-NK-DEHP could maintain PAE degradation activity. Bioaugmentation of enriched consortium LF-NK-DEHP revealed insignificant degradation rate compared to natural attenuation. However, fertilizer addition was observed to improve the bioaugmentation performance. Bioaugmentation added with fertilizer could degrade 200 mg kg^{-1} DEHP up to 75.87% within 25 days while removing 200 mg kg^{-1} DBP altogether within three days. This was further confirmed through alleviation of PAE intermediates phytotoxicity. Upon completion of this research, new information was acquired, including methods to maintain enriched consortia degradation activity, potential novel PAE-degraders, and feasibility of bioaugmentation with exogenous enriched consortium for PAE bioremediation.

Field of Study:	Microbiology and Microbial Technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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CHAPTER I
INTRODUCTION

1.1. State of Problem

Environmental plastic pollution has become main concern in many countries, due to the high production and consumption of plastic-based products. It is estimated that worldwide annual plastic production have reach over 380 million tons with increasing rate 4% per year (Rosenboom et al., 2022). During plastic processing, chemicals are added to improve the physical properties of plastic polymer. Among these chemicals are plasticizer, which typically made up 10- 70% of plastic weight (Barrick et al., 2021). Plasticizers are added to increase flexibility, extensibility, and workability of plastic polymer. Increasing plastic production contributes to increasing production and application of plasticizers.

Phthalate esters (PAEs) are the most commonly used plasticizers. PAE constitutes for nearly 90% of global production of plasticizer (Sohn et al., 2022). Apart from application in plastic production, PAE are also widely used in plastics, coatings, and cosmetics (Chen et al., 2007). PAE as plasticizer was added through mixing and loosely bound to plastic polymer (Mondal et al., 2022). Therefore, when plasticized plastic comes into contact with other materials, PAE may migrate into the contact material, which varies depend on the nature of contact material. Resistence of PAE migration from plastic polymer are reduced with increasing molecular size and linearity of the esters. Due to this properties, PAE are detected in various environment, including water, soil, air and dust systems (Staples et al., 1997).

In addition, PAE are identified as endocrine-disrupting chemicals (Hlisníková et al., 2020), which further extend its potential as carcinogenic and teratogenic compound (Erkekoglu & Kocer-Gumusel, 2016). US EPA (United States Environmental Protection Agency) has listed six PAEs including butyl benzyl phthalate, dibutyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), diethyl phthalate, dimethyl phthalate, and di-n-octyl phthalate, as priority pollutants. DBP and DEHP are the most commonly detected PAE, as DEHP commonly used in PVC polymer and DBP in liquid-based chemicals (Hu et al., 2021).

Food is the major route for human exposure to PAE, according to previous study conducted in several country (Cao et al., 2016; Giovanoulis et al., 2018; Ji et al., 2014). While mainly related to plastic food packaging, human body is also exposed through perpetual consumption of PAE accumulated in vegetables and other agricultural products (Zhou et al., 2021). Agricultural practices, including use of plastic mulch, PVC water pipe, addition of biosolids and wastewater, use of sprayed chemical fertilizer and pesticides have contributed to PAE accumulation in vegetables (He et al., 2014). Furthermore, since in general have low water solubility, PAE tend to be accumulated within soil matrices and continuously exposed to plant bodies (Russell & McDuffie, 1986).

Another important problem related to PAE contamination in agricultural soil is its effect to the crop's quality. The effect of PAE accumulation varies according to the plant, although in general DBP was observed to inhibit root elongation, seedling growth and biomass of higher plant species (T. Ma et al., 2014). In other plant, such as peppers (*Capsicum* spp.), PAE accumulation decrease the content of vitamin C in the fruit (Yin et al., 2003). This observation increases the importance of PAE bioremediation in agricultural soil.

In the environment, PAE are degraded both through abiotic and biotic process, though abiotic degradation of PAE occur at much slower rate (Benjamin et al., 2015). Biodegradation is the predominant route for PAE degradation and there are several microorganisms (predominantly bacteria) reported to have PAE degradation activity (Hu et al., 2021). Bioaugmentation or the addition of exogenous bacteria known to have activities against target pollutants is proven efficient in enhancing the naturally occurring biodegradation process of PAE in soil. Initial stage in application of bioaugmentation strategy is to obtain microbes with PAE degradation activity. Such microbes can be obtained from heavily polluted environment in which indigenous microbes have potentially adapted to high PAE concentration, for example landfill soil. Landfill soil is a potential source for mining efficient PAE-degrading bacteria (Li et al., 2006).

Another emerging issue is the use of enriched consortia over pure culture for bioaugmentation purpose. The use of bacterial consortia containing several bacteria with variety of metabolic potential instead of pure culture is preferred since it can ensure complete mineralization of PAE (Bai et al., 2020). At present, community-based perspective in bioremediation have been rigorously studied for various types of target

pollutants. To obtain this specific community with their specific metabolic network, an enrichment strategy can be applied instead of conventional isolation protocol. This will further which contribute to top-down approach of designing and constructing synthetic bacterial community for PAE degradation (Hu et al., 2021).

Previously, four enriched consortia were obtained from landfill soil of Nongkham Landfill Site, Bangkok, Thailand (Meyawee Satiraphan, 2018). Each consortium was enriched from same soil sample and supplemented with different PAEs. Enrichment using four different PAE resulted in four different consortia with known activity against corresponding PAE congeners. However, it is unknown whether obtained consortia retain similar activities under exposure of mixed PAE substrate, which happened more often in the environment. Furthermore, activity of biological agent tends to be reduced in soil environment in comparison to activity in liquid medium. In this study, selected consortia were evaluated for their activity in mixture of two commonly detected PAE in soil system, DBP and DEHP (Q. Zhu et al., 2022).

There are several possible outcomes of bioaugmentation process. Exogenously added consortium may cause disruption of indigenous soil microbiome structure or unable to survive in the new environment due to competition with indigenous bacteria (Nwankwegu et al., 2022). Therefore, it is important to conduct initial characterization of obtained consortia and evaluation of its activity in microcosm scale, while also maintaining the stability of consortium through continuous transfer.

Additionally, previous study also showed that PAE biodegradation in soil is affected by soil properties, mainly soil pH and organic carbon content (Cheng et al., 2019). Fertilizer addition is one of commonly practiced agricultural technique in order to improve soil productivity. Fertilizer addition affects soil physicochemical properties, and consequently can affect the whole biodegradation process. In this study, the effects of fertilizer addition were investigated on how it would affect bioaugmentation performance, in comparison to treatment without fertilizer addition.

1.2. Objective

1. To select enriched consortia with efficient PAE mixture degradation activity.
2. To characterize selected enriched consortia based on community dynamics and degradation kinetic.
3. To evaluate biodegradation capacity of exogenous PAE-enriched consortia in agricultural soil.
4. To investigate the effect of fertilizer addition to the biodegradation in agricultural soil.

1.3. Scope of this study

This research was divided into three phases.

1.3.1. Selection and characterization of enriched consortia

Previously obtained enriched consortia were selected based on degradation efficiency on mixture of DBP and DEHP. Selected enriched consortia then characterized based on community dynamics during degradation and kinetics of degradation. Throughout following experiment, selected enriched consortia activity was maintained by continuous transfer and degradation activity evaluation.

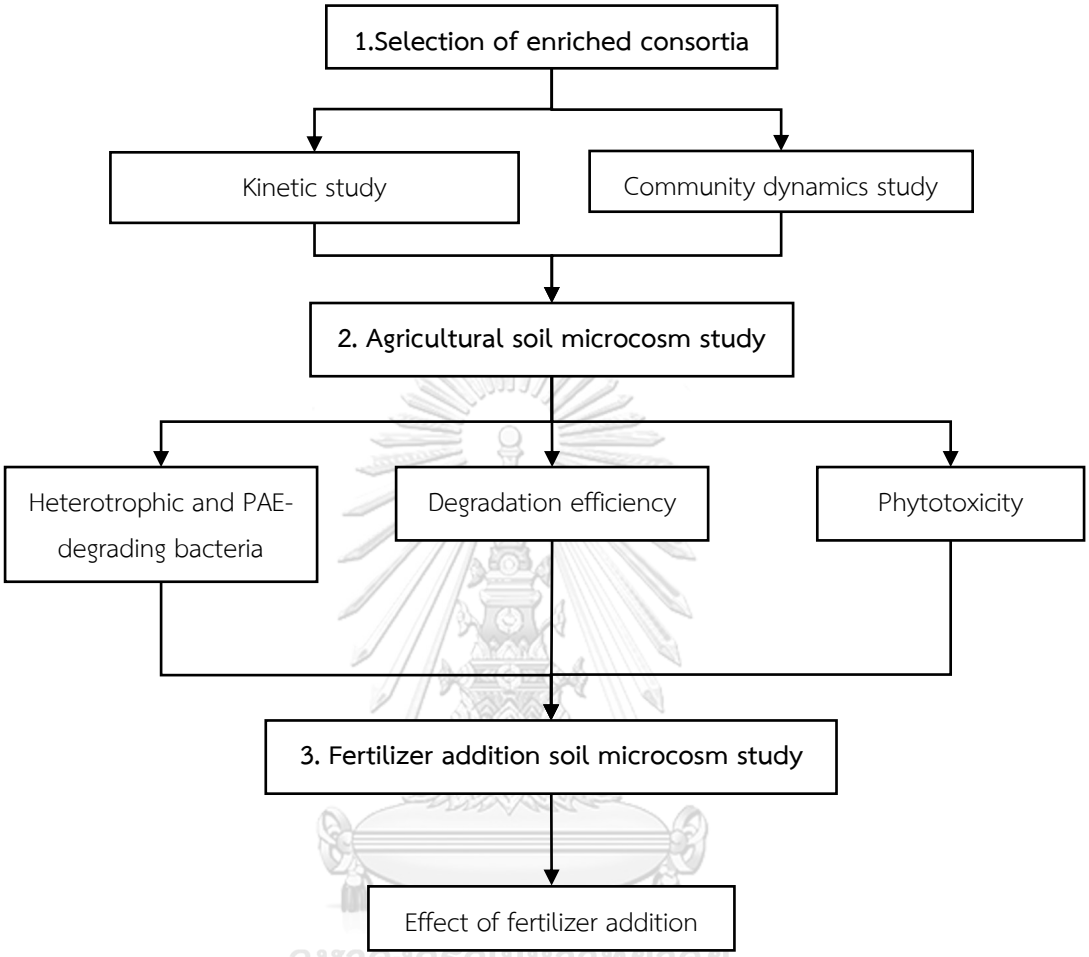
1.3.2. Agricultural soil microcosm study

Soil microcosm study was conducted with selected enriched consortia for 30 days incubation. Throughout incubation, residual PAE, bacterial count and phytotoxicity was compared for each treatment. Treatments variation including soil added with PAE only (natural attenuation), soil added with PAE, and enriched consortia (bioaugmentation) and sterilized soil added with enriched consortia and PAE.

1.3.3. Fertilizer addition soil microcosm study

Similarly, fertilizer addition soil microcosm study was conducted with selected enriched consortia. PAE degradation efficiency, bacteria count and phytotoxicity of soil sample were observed for 40 days incubation. Treatments variation including natural attenuation (with and without fertilizer) and bioaugmentation (with and without fertilizer).

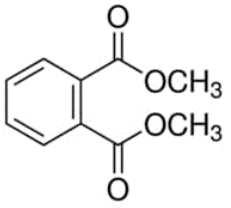
1.4. Flowchart of this study



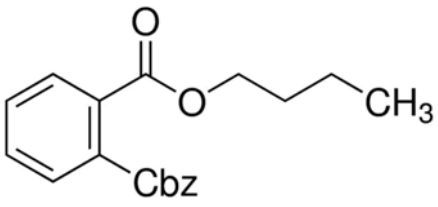
CHAPTER II
LITERATURE REVIEW

2.1. Phthalate esters (PAEs)

Phthalate esters (PAEs) or the esters of 1,2-benzene dicarboxylic acid are group of compounds which broadly applied in various chemical industry. Based on length of alkyl chain, PAE can be identified as high-molecular-weight PAEs (C8-C13 esters) and low-molecular-weight PAEs (C1-C7 esters). PAEs with lower molecular weight are commonly used as plasticizer for acrylics and urethane. Aside from plasticizer, short-alkyl PAE like dimethyl phthalate is used as diluent of organic peroxides, while diethyl phthalate is added as fixative for perfumes. Meanwhile high molecular weight PAEs including di-(2-ethylhexyl) phthalate, diisononyl phthalate and diisodecyl phthalate are commonly used as plasticizer for vinyl resins (Stanley et al., 2003). As plasticizer, typical concentrations of PAE added to plastic resins are between 10-70% weight fractions (Barrick et al., 2021). Highest additions are usually in polyvinyl chloride resins, and PAE addition enable molding at room temperature. Among these variations of PAEs, six commonly utilized PAEs have been listed as priority contaminants by United States Environmental Protection Agency (US EPA). Chemical structure and molecular weight of these PAEs are listed in Figure 1.



Dimethyl phthalate (DMP)
M = 194.2 g.mol^{-1}



Benzyl butyl phthalate (BBP)
M = 312.4 g.mol^{-1}

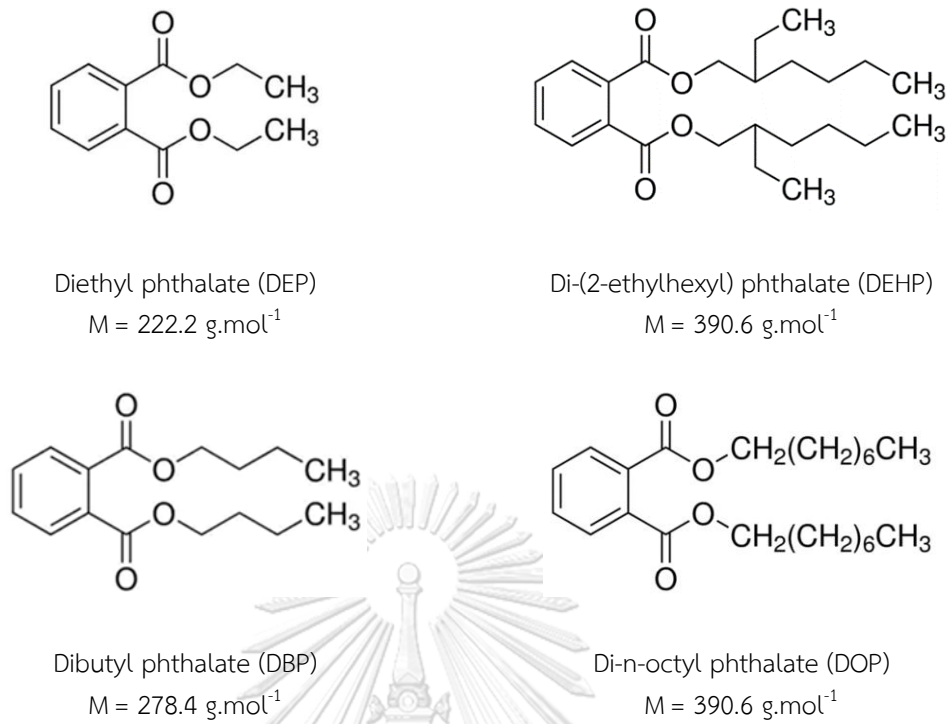


Figure 1 Chemical structure and molar mass of six PAEs listed by US EPA

PAE interact with vinyl resins to increase extensibility and flexibility, by reducing the glass transition temperature to be below room temperature. PAE do not form chemical bonds with the plastic resins; therefore, it can leach out. Contact with materials like water, blood, oil, air, soil cause migration of PAE into the attracting media. Diffusion of PAE into contacting materials depends upon PAE molecular weight, viscosity, compatibility with plastic resins, and the affinity of environment (Graham, 1973).

PAEs are manufactured through esterification of phthalic anhydride in the presence of acid catalyst. PAEs are mainly anthropogenic compounds, but PAEs have been frequently discovered in plant and microorganisms' sources, indicating its potential to be biosynthesized in nature as well. PAEs were previously detected in plant-derived essential oils, root exudates, and several group of bacteria, fungi, and algae. Main purpose of PAE biosynthesis is yet to be investigated, but it is hypothesized that PAE may function as allelopathic/ phytotoxic, anti-microbial, and insecticidal compound (Huang et al., 2021).

2.1.1. Physical-chemical properties of phthalate esters

PAEs are typically in liquid state at room temperature. Since melting points lie between 5.5°C and -58°C, at low environmental temperature phthalates may present in solid state (Staples et al., 1997). Some important physical-chemical properties of PAE are shown in Table 1.

Table 1 Calculated physical-chemical properties of PAEs at 25°C

Phthalate esters	**Melting point (°C)**	**C^S_{WL} [a] (mg l^{-1})**	**P_L [b] (Pa)**	**Log K_{OW} [c]**	**Log K_{OA} [d]**	***H* [e] (Pa mol m^{-3})**
DMP	5.5	5220	0.263	1.61	7.01	9.78×10^{-3}
DEP	-40	591	6.48×10^{-2}	2.54	7.55	2.44×10^{-2}
DBP	-35	9.9	4.73×10^{-3}	4.27	8.54	0.133
BBP	-35	3.8	2.49×10^{-3}	4.70	8.78	0.205
DEHP	-46	2.49×10^{-3}	2.52×10^{-5}	7.73	10.53	3.95
DOP	N/A	2.49×10^{-3}	2.52×10^{-5}	7.73	10.53	3.95

[a] C^S_{WL} is the solubility of liquid phthalate in water

[b] P_L is the liquid vapor pressure

[c] K_{OW} is the octanol-water partition coefficient

[d] K_{OA} is the octanol-air partition coefficient

[e] *H* is the Henry's law constant

In Table 1, it is shown that with increasing molecular weight, melting point, solubility in water, and liquid vapor pressure are declining, while log K_{OW}, log K_{OA}, and Henry's constant are increasing. K_{OW} or octanol-water partition coefficient is often used to predict partitioning in environment between water and organic matter like sediment/soil. K_{OA} or octanol-air partition coefficient on the other hand is used to describe partitioning of PAE in air and organic matter like plants or soil as well. *H* is the measurement of substrate equilibrium distribution between air and water.

Based on physical and chemical properties of PAEs, it can be predicted that low molecular weight PAE like DMP and DEP tend to have higher partition in water, then in soil and least distributed in air. High molecular weight PAE such as DEHP and DOP tend to be more distributed in soil, then in sediment, with very low distribution in air and water. Previously, fate of PAEs on evaluative environments were modelled using Equilibrium Criterion or EQC model based on calculated physical-chemical properties in Table 1 (Cousins et al., 2003). Through EQC Level I simulations, it was obtained that 92.2% DBP load to the environment was distributed in soil, 5.6% in water, 2.1% in sediments (soil-water system interface) and 0.2% in air. Meanwhile for DEHP, 97.8% partition was distributed in soil, 2.2% in sediments, and less than 0.1% in water and air (Cousins et al., 2003). In general, soil is the main reservoir of hydrophobic organic pollutants like PAE, in terrestrial environment.

2.1.2. Health hazard and phytotoxicity of phthalate esters

PAEs are one of the priority contaminants listed by US EPA due to their high toxicity. Many studies have consistently reported the effect of PAE exposure to human health, which includes decrease in sperm quality and attention deficit hyperactivity disorder (ADHD) (Chang et al., 2021). Other health effects that was reported but still need further confirmation are cardiovascular disease (Jaimes et al., 2019), thyroid disorders (Morgenstern et al., 2017), diabetes (Kim et al., 2013), obesity (Xia et al., 2018), respiratory diseases (Hoppin et al., 2013) and neurological disorders (Kim et al., 2018).

Previously, value of reference dose (RfD) and carcinogenic assessment of several PAE had been reviewed and collected in Integrated Risk Information System (IRIS) database of US EPA. RfD is the maximum acceptable oral dose of a toxic substance, established by US EPA, which is used as standard for regulation, so that people are not exposed to chemicals like PAE in amounts that exceed RfD. Meanwhile, carcinogenic assessment is the classification of toxic carcinogenic effect, which is based on experiments on other animals, like rats, mice, etc. Both of the values for each PAEs are listed in Table 2.

Table 2 Health risk assessment of PAEs based on Integrated Risk Information System. (EPA, 1990)

Type of PAEs	Non-cancer Assessment			Cancer Assessment	
	Critical system	RfD (Oral exposure)	Rfc (Inhalation exposure)	WOE (Weight of evidence) characterization	Basis
Benzyl butyl phthalate (BBP)	Hepatic	2×10^{-1} mg.kg^{-1}-day	Not assessed	C (Possible human carcinogen)	Based on statistically significant increase in mononuclear cell leukaemia in female rats (only).
Dibutyl phthalate (DBP)	Other	1×10^{-1} mg kg^{-1}/day	Not estimated	D (Not classifiable as to human carcinogen)	Pertinent data regarding carcinogenicity was not located in the available literature.
Diethyl phthalate (DEP)	Other	8×10^{-1} mg kg^{-1}/day	Not estimated	D (Not classifiable as to human carcinogen)	Pertinent data regarding carcinogenicity was not located in the available literature.
Di (2-ethylhexyl) phthalate (DEHP)	Hepatic	2×10^{-2} mg kg^{-1}/day	Not assessed	B2 (Probable human carcinogen-sufficient evidence of carcinogenicity in animals)	Orally administered DEHP produce significant dose-related increases in liver tumour responses in rats and mice of both sexes.

Dimethyl phthalate (DMP)	-	Not assessed	Not estimated	D (Not classifiable as to human carcinogen)	Pertinent data regarding carcinogenicity was not located in the available literature.

There are several researches that has reported various phytotoxic effect of PAE. One study based on DBP and corn plants (*Zea mays*) obtained that DBP contamination at concentration higher than 2000 mg kg^{-1} would decrease heights and fresh shoot weight of corn plants (Shea et al., 1982). Another experiment using water spinach (*Ipomoea aquatica* Forsk) and lettuce (*Lactuca sativa* L var. *ramose* Hort), obtained that chlorophyll *a* and carotenoid contents of plants were decreased when grown in soil contaminated with DBP (Ma et al., 2019; Ma et al., 2018).

DBP as well were shown to have higher phytotoxicity than DEHP (Ma et al., 2018). In mung bean (*Vigna radiata*) seedlings, DBP contamination at concentration 500 mg/kg inhibited root elongation and biomass fresh weight, while DEHP only slightly inhibited shoot elongation (T.-T. Ma et al., 2014). In the experiment using seven higher plants species, including wheat (*Triticum aestivum* L.), alfalfa (*Medicago sativa* L.), perennial ryegrass (*Lolium perenne*), radish (*Raphanus sativus* L.), cucumber (*Cucumis sativus* L.), oat (*Avena sativa*) and onion (*Allium cepa* L.), DBP inhibited root elongation and reduced fresh biomass weight, in contrast to DEHP which displayed no apparent effect (T. Ma et al., 2014). Moreover, DBP exposure cause chlorosis in radish plant (*Raphanus sativus* L.), due to inhibition of electron transport rate in thylakoid (Hannay & Millar, 1986).

Other than physical properties like inhibition of root or shoot elongation, and reduction of biomass, or disruption of photosynthetic organ, PAE also induced activity of antioxidant enzymes. In barley plants (*Horderum vulgare*), DBP contamination cause reduction in superoxide dismutase, ascorbate peroxidase, glutathione reductase activity, while on the other hand increase guaiacol peroxidase and catalase activity (Kumari & Kaur, 2020).

Study on the uptake of PAEs by edible plants including lettuce, carrot and strawberry observed that bioconcentration factor (BCF) varies with plant types. However, both DBP and DEHP have poor translocation from root to leaves and thus tend to be concentrated in roots. This was correlated with common observation of DBP effect to root elongation inhibition, instead of shoot, across variation of plant species. Moreover, mono-ethylhexyl phthalate ester (MEHP) and monobutyl phthalate ester (MBP) were detected in plant biomass, which indicated PAE transformation during uptake by plant. Therefore, the metabolites should be considered when assessing human exposure to PAE via dietary intake (Sun et al., 2015)

Another important finding is the effect of nanoplastics in increasing the phytotoxicity of PAE. Cooccurrence of amino-functionalized polystyrene nanoplastics (PSNP-NH_2) increased the foliar uptake of PAE by corn plant (Sun et al., 2022). Similarly, microplastic polyethylene increased DBP-induced phytotoxic effect to lettuce plant, which was significant in comparison to single DBP contamination (Gao et al., 2019). This observation indicates that in real environment, where both micro- or nano- plastics and PAE coexist, the phytotoxicity of PAE tend to be elevated in comparison to reported study. This is also correlated with PAE uptake by plant and the risk of human exposure. Therefore, PAE contamination in agricultural soil is an important problem which require urgent solution.

2.1.3. Phthalate ester occurrence

As previously mentioned, PAE as plasticizer can migrate from plastic resin to the environment since it is not chemically bound to the polymer. Therefore, PAE have been detected in various environment. Some research have reported PAE occurrence in house dust (Muenhor et al., 2018; Promtes et al., 2019), surface water (Kingsley & Witthayawirasak, 2020), tap and bottle water (C. Wang et al., 2021), even in pickled vegetables and juice we consumed daily (Alp & Yerlikaya, 2019; Arfaeinia et al., 2020; Cheshmazar et al., 2021) . Several PAE occurrence studies that were conducted in Thailand are listed in Table 3.

Table 3 PAE reported occurrence in Thailand

Sample and location	Concentration range	Reference
Sediment from canals in Bangpoo Industrial Estate, Samut Prakan	PAE, DEHP, DMP: ~1 mg kg^{-1}	(Brigden et al., 2003)
Sediment from Chao Phraya River, near plastic and chemical PVC facility, Samut Prakan	DBP, DEHP, DiNP: ~1 mg kg^{-1}	(Brigden et al., 2003)
Sediment and water of Chao Phraya River	DEP: 0.64-2.59 µg l^{-1} DEHP: 1.58-27.55 µg l^{-1}	(Sirivithayapakorn et al., 2014)
Floor and road dust from manual e-waste dismantling facility and nearby communities, Phatthalung Province, Southern Thailand	Total PAE in facility: 86-790 µg g^{-1} Total PAE in communities: 44-2700 µg g^{-1} Total PAE are including of DMP, DEP, PAE, DEHP, BBP and DOP.	(Muenhor et al., 2018)
House dust in Bangkok	Total PAE: 753- 13810 µg g^{-1} DEHP: 467- 8172 µg g^{-1} DiNP: 15.2-11052 µg g^{-1}	(Promtes et al., 2019)
Sediment and water samples from eastern coast of Thailand (Chanthaburi, Rayong and Chonburi)	PAE: 0.23-0.77 µg l^{-1} DEHP: 0.31-0.91 µg l^{-1}	(Malem et al., 2019)
Surface water of U-Tapao Canal, Southern Thailand	Total PAE: 1.44-12.08 µg l^{-1} Total PAE are including DEHP and DiNP	(Kingsley & Witthayawirasak, 2020)

In agricultural soils, the main anthropogenic sources of PAEs originate from agricultural films (J. Wang et al., 2013) , pesticides (X. Wang et al., 2013), and application of wastewater for irrigation and biosolids for soil amendment (Cai et al., 2007). PAEs from these sources are further distributed in the environment through various biogeochemical cycling processes supported by the soil. Besides degradation by microorganisms and uptake by plants, the PAEs in soil can also enter to the atmosphere

through evaporation and migrate into the groundwater and surface water by rain. Sources of PAE contamination in agricultural soil are summarized in Figure 2.

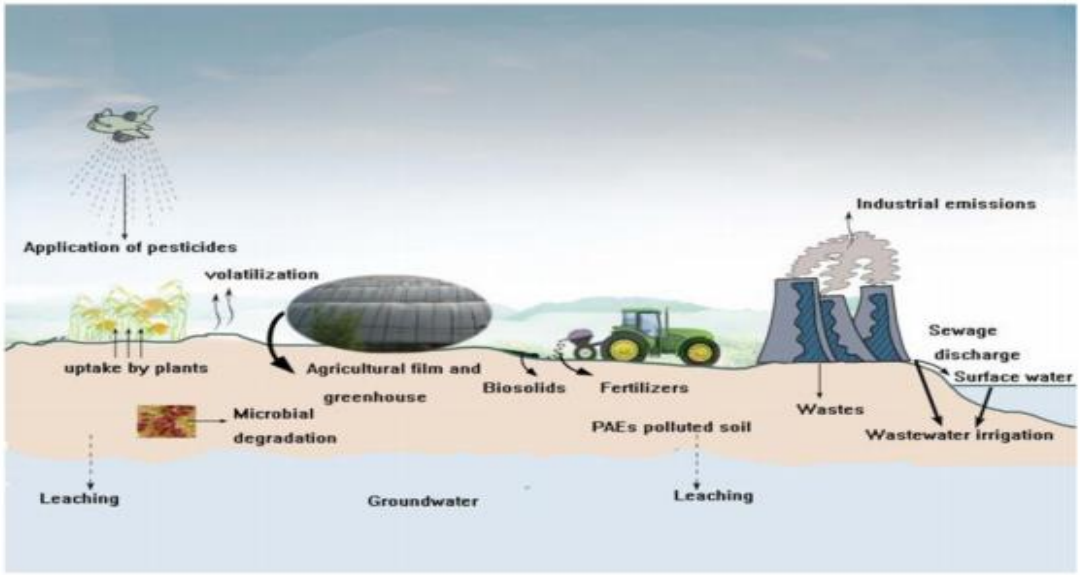


Figure 2 Sources of PAE in agricultural soils and their environmental fate (He et al., 2014)

Plastic mulch contribution on PAE pollution in agricultural soil was based on observation that the concentration of DBP and DEHP in plastic greenhouse soil were 2.5-3 times higher in comparison to soil which was not covered by plastic greenhouses (Wang et al., 2015). Furthermore, it was observed that black plastic mulch can absorb heat more readily than light-colored plastic mulch, resulting in the elevation of the temperature of the plastic. Higher polymer temperature then would decrease the retention strength between PAEs and vinyl resins which further induce PAE migration to the soil (Chen et al., 2012).

Wastewater reuse in agriculture is a common practice in developing countries (Thangarajan et al., 2012). Irrigation of sewage effluent to the agricultural land, yielded more crops, because sewage also supplied nitrogen, phosphorus, potassium and organic carbon to the soil. However, land application of untreated/ treated wastewater introduce PAEs in trace concentration which over time result in accumulation of PAEs in the receiving soil (Zhang et al., 2015).

Municipal biosolids are treated sewage sludge in solidified form. It is usually added to agricultural land to supply nutrients and organic matter, for similar purpose as wastewater irrigation (Zuloaga et al., 2012). However, PAE also tend to concentrate in biosolids due to the high affinity to solid/ organic matter and low solubility in wastewater. The effect of biosolids will need to be investigated further, and biosolids application rates will need to be controlled in order to manage potential pollution risk (He et al., 2014).

There are various pathways in which PAE can be redistributed in the soil. These pathways are related to transverse, vertical and seasonal changes in PAE distribution. Transverse distribution is usually site-specific and generally inversely proportional to the distance from industrial and commercial sources (Zeng et al., 2008). Vertical distribution of PAEs in soil occurred in the top 20 cm of the soil, and their concentration generally decreased with soil depth (Gao & Zhou, 2013). Leaching of PAEs depends on the soil type, seasonal water movement, and local water conditions (He et al., 2014). Some reports regarding PAE occurrence in agricultural soil in other countries are summarized in Table 4.

Table 4 Reported PAE contamination in agricultural soils and agricultural product

Location	**Sample**	**PAE concentration and predominant PAE**	**Reference**
Guangzhou city, southern China	Agricultural soil	**Σ**16 PAEs = 0.195 – 33.6 mg kg^{-1} Predominant PAE: DBP and DEHP	(Zeng et al., 2009)
Shandong Peninsula, eastern China (Qingdao, Weihai, Weifang, Yantai city)	Agricultural soil	**Σ**16 PAEs = 1.374-18.810 mg.kg^{-1} (average = 6.470 mg kg^{-1}) Predominant PAE: DBP	(Li et al., 2016)
Shanghai municipality and Jiangsu Province, eastern China	Agricultural soil	**Σ**6 PAEs = 0.109 – 5.56 mg kg^{-1} (average = 0.946 mg kg^{-1}) Predominant PAE: DEHP	(Sun et al., 2018)
	Vegetables	**Σ**6 PAEs = 0.06 – 2.39 mg kg^{-1} (average = 0.601 mg kg^{-1}) Predominant PAE: DEHP	

Location	Sample	PAE concentration and predominant PAE	Reference
Asalouyeh town, Persian Gulf	Agricultural soil	**Σ**16 PAEs = 60.04 ± 3.40 mg kg^{-1} Predominant PAE: DEHP	(Arfaeinia et al., 2019)
Guiyu region, south China	Agricultural soil and residential soil	**Σ**16 PAEs = 2.95 – 67.154 mg kg^{-1} (average = 13.28 mg kg^{-1}) Predominant PAE: BBP, DBP and DBEP	(Zhang et al., 2019)
Beijing municipality, China	Agricultural soil (greenhouses soil)	**Σ**6 PAEs = 0.73 – 9.48 mg kg^{-1} Predominant PAE: DBP and DEHP	(Li et al., 2020)
	Vegetables	**Σ**6 PAEs = 1.89 – 6.35 mg kg^{-1} Predominant PAE: DBP and DEHP	
Yinchuan city, northwest China	Agricultural soil	**Σ**16 PAEs = 0.391 – 11.924 mg kg^{-1} (average = 4.427 mg.kg^{-1}) Predominant PAE: DMP	(Tao et al., 2020)
Yangtze River Delta of China (Shanghai municipality, Jiangsu Province, Anhui Province, Zhejiang Province)	Agricultural soil	**Σ**6 PAEs = 0.054 – 1.58 mg.kg^{-1} (average = 0.197 mg kg^{-1}) Predominant PAE: DEHP	(Wei et al., 2020)
	Vegetables	**Σ**7 PAEs = 0.109 – 16.4 mg kg^{-1} (average = 0.536 mg kg^{-1}) Predominant PAE: DEHP	
Rongchang District, west Chongqing municipality, southwest China	Agricultural soil (greenhouses soil)	**Σ**6 PAEs (spring) = 2.26 ± 0.45 mg kg^{-1} **Σ**6 PAEs (autumn) = 0.35 ± 0.11 mg kg^{-1} Predominant PAE: DiBP and DEHP	(Li et al., 2021)
Huang-Huai-Hai region of China	Agricultural soil	**Σ**16 PAEs = 0.052 – 3.569 mg kg^{-1} (average = 0.903 mg kg^{-1}) Predominant PAE: DBP	(Zhou et al., 2021)
Coastal region of South China	Agricultural soil	**Σ**15 PAEs = 0.445 – 4.437 mg kg^{-1} (average = 1.582 ± 0.937 mg kg^{-1}) Predominant PAE: DBP and DEHP	(Xing et al., 2022)
	Plant	**Σ**15 PAEs = 2.176 - 30.276 mg kg^{-1}	

Location	Sample	PAE concentration and predominant PAE	Reference
		(average = 8.7± 5.84 mg kg^{-1}) Predominant PAE: DBP and DEHP	

Based on Table 4, most of the reports showed that DEHP and DBP were predominant contaminant compared to other PAE molecules. Besides, most of this reported value of PAE concentration in vegetables and soils were much higher than the RfD value for PAE by US EPA.

2.2. Bacteria-driven phthalate ester degradation in soil

Biodegradation is the major routes of PAE removal from the environment, and bacteria is the main microbes capable of PAE degradation activity. PAE-degrading bacteria with its degrading capacity, along with metabolic pathway of DBP and DEHP degradation by soil-borne bacteria will be summarized in this section. Additionally, several approaches that have been applied in order to improve PAE degradation efficiency by bacteria will be covered as well.

2.2.1. Phthalate ester degradation susceptibility in soil

PAE degradation rate in real environment varies depends on the media where it contaminates, and soil as the main reservoir of PAE relatively slower in comparison to water body and air. In soil, biological degradation is the main degradation mechanism for PAE (Staples et al., 1997), and bacteria is the main group of microorganism involve in PAE biodegradation (Hu et al., 2021). Each phthalate congeners as well have variety of biodegradability, which is likely due to the steric effect of phthalate ester side chains. Longer alkyl chains inhibit hydrolytic enzymes from binding to the phthalates (Liang et al., 2008).

Soil inherent physicochemical properties, for example moisture content, pH, nitrogen content, dissolved organic matter, and many others, in addition to environmental condition variation like temperature, affect the rate of PAE biodegradation

in soil. In investigation of biodegradation rate of PAE in soil, sterilized soil is used as abiotic control. One research reported decrease in extractable PAEs in sterilized soil after 30-days incubation, indicating sequestration of PAE in soil particle. Sequestration is the phenomenon of PAE strong adsorption to soil particles, which tend to be elevated with PAE of longer alkyl chain as well (Rüdel et al., 1993). Therefore, both adsorption and degradation occur simultaneously in soil, with negligible rate of desorption.

Sequestration risk become more problematic since it can reduce PAE bioavailability to the bacteria. Therefore, some research on biodegradation of PAE based on natural attenuation approach report much lower rate of PAE biodegradation in soil, with increasing alkyl ester chain (Peterson & Staples, 2003). Addition of PAE degrader into the soil can accelerate the naturally occurring degradation process and thus able to overcome sequestration problem. Based on environmental modelling of PAE sequestration in soil, it is recommended that biodegradation rate of low molecular weight PAE (including DMP, DEP, DBP and BBP) have to be at range of 0.1-0.4 day^{-1} and half-life at range of 1.7 - 6.9 days. Meanwhile for high molecular weight PAE (C6 alcohol side-chains and above), recommended range of degradation rate is 0.01-0.1 day^{-1} and half-life at 7- 69 days (Peterson & Staples, 2003).

2.2.2. Metabolic pathway of PAE degradation and reported bacterial isolates with PAE degradation activity

There are several elucidated pathways for PAE degradation. In general, PAE degraded in two stages, in which the first stage is conversion of PAE into phthalic acid, followed by phthalic acid mineralization in second stage. PAE can be converted to phthalic acid through three mechanisms, including de-esterification and β-oxidation, which is shown in Figure 3.

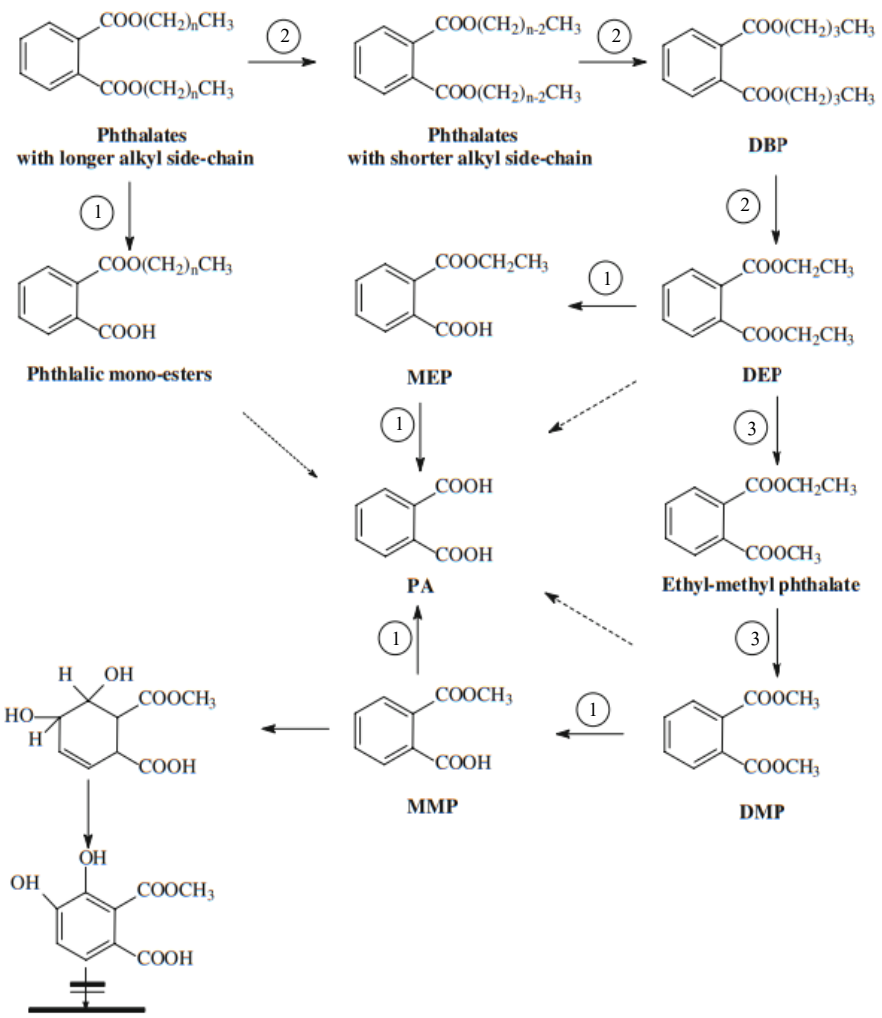


Figure 3 Compilation of reported PAE upper degradation pathway. (1): De-esterification; (2): β-oxidation; (3): trans-esterification. (Liang et al., 2008)

Through de-esterification, alkyl esters bind to phthalate ring will be serially hydrolyzed, forming mono-alkyl phthalate and phthalic acid as intermediate. Meanwhile for β-oxidation, PAE with alkyl longer than DEP will be converted to DEP by removing one ethyl group for each time. DEP then further converted to phthalic acid through de-esterification or *trans*-esterification. If de-esterification simply removes the whole alkyl chain, in *trans*-esterification, ethyl group will be first replaced with methyl group, producing ethyl-methyl phthalate and dimethyl phthalate.

Phthalic acid from upper degradation pathway is then further converted into several metabolites before mineralization with CO_2 as side product. Lower degradation pathway

is different in aerobic and anaerobic environment, in which aerobic conversion produce main metabolite protocatechuate, while anaerobic degradation converts phthalic acid to benzoic acid. Benzoic acid then can be subsequently converted to protocatechuate or directly mineralized with acetate and hydrogen as final mineralization product. On the other hand, protocatechuate will undergo *ortho-* or *meta-* cleavage before assimilation in Krebs cycle in the form of oxaloacetate and pyruvate. Lower metabolic pathway of PAE biodegradation is shown in Figure 4.

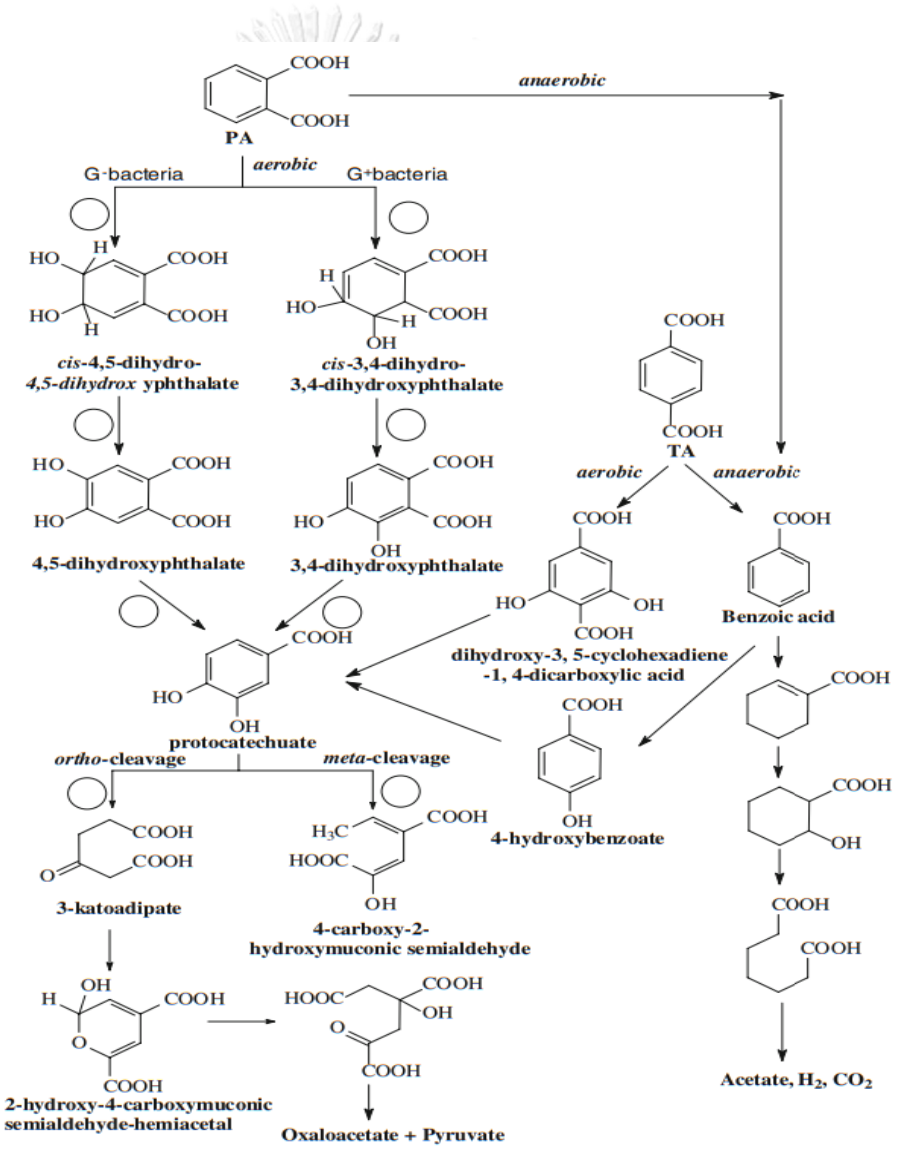


Figure 4 Compilation of reported PAE lower degradation pathway (Liang et al., 2008)

From phylogenetic perspective, PAE-degrading traits in bacteria belong to broad range of bacterial phyla, in which from current reports consist of Proteobacteria (45% of total reported bacterial isolates), Actinobacteria (40%), Firmicute (12%), Bacteroidetes (1.5%) and Deinococcus-Thermus (1.5%). Some of the commonly reported genera with PAE-degradation capacity are including *Gordonia*, *Rhodococcus*, *Microbacterium*, *Acinetobacter*, *Arthrobacter,* and *Pseudomonas* (Hu et al., 2021). Some of the reported bacterial isolates and enriched consortia are summarized in Table 5.

Table 5 Isolated PAE-degraders with degradation activity in liquid medium, optimal condition, and unique properties

Substrate	Microbes	Source	Optimal growth condition	Degradation percentage (Initial concentration)	Strategy to enhance stability and degradation efficiency	Microbes' unique feature	References
DBP	*Acinetobacter* sp. strain 33F	Municipal solid waste leachate	T = 30°C pH = 7 (6.8-7)	90% in 8 days (2000 mg l^{-1})	-	Substrate inhibition if DBP> 1000 mg/kg in agricultural soil.	(Sharma et al., 2021)
DBP	*B. subtilis* strain HB-T2	Sorrel plant root	T = 30°C - 40°C pH = 8 Inoculum size = 5% (v/v)	99% in 6 hours (20 mg l^{-1})	-	Endophytic bacteria, originally from plant tissue	(Xu et al., 2020)
DBP	*Pseudomonas* sp. strain YJB6	Contaminated soil from plastic film greenhouse	T = 31.4°C pH = 7.6 Inoculum size (OD600) = 0.6	97.5% in 3 days (200 mg l^{-1})	Immobilization with SA, PVA, SA-PVA SA → Sodium Alginate; PVA → Polyvinyl alcohol	-	(Feng et al., 2021)
DBP	*Enterobacter* sp. DNB-S2	Mollisol area (soils of grassland ecosystem)	T = 35°C (35-50°C) pH = 8 (7-10) DBP = 500 mg/l	99.95% in 7 days (500 mg l^{-1})	-	Very high heat tolerance (up to 50°C)	(Sun et al., 2019)
DBP	*Bacillus megaterium* strain YJB3	*Canna indica* root tissue	T = 34.2°C Acetate = 1.2 g/l Inoculum size = 1.8% pH = 7	82.5% in 5 days (200 mg l^{-1})	Addition of acetate to enhance biodegradation	Endophytic bacteria, originally from plant tissue	(Feng et al., 2018)
DBP	*B. subtilis* strain HB-T2	Sorrel plant root	T = 30°C - 40°C pH = 8	99% in 6 hours (20 mg l^{-1})	-	Endophytic bacteria, originally from plant tissue	(Xu et al., 2020)

Substrate	Microbes	Source	Optimal growth condition	Degradation percentage (Initial concentration)	Strategy to enhance stability and degradation efficiency	Microbes' unique feature	References
			Inoculum size = 5% (v/v)				
DEHP	*Achromobacter* sp. strain RX	Activated sludge	T = 30°C pH = 7 Nitrogen source = NO_3^-	94% in 100 hours (300 mg l^{-1})	Addition of biosurfactant and co-substrate MPSP (masson pine seed powder)	-	(P. Wang et al., 2021)
DEHP	*Gordonia terrae* strain RL-JC02	Contaminated red soil from plastic mulch	T = 30°C (20-40°C) pH = 6 (5-7) Salinity = 2-6%	100% in 72 hours (500 mg l^{-1})	-	Acid-tolerant (86.6% degradation under pH 5)	(Zhang et al., 2020)
DEHP	*Microbacterium* sp. strain J-1	Landfill soil	pH = 8.3 T = 32°C Inoculum size (OD600) = 0.8	96% in 5 days (200 mg l^{-1})	-	Capable for colonization of rhizosphere	(Zhao et al., 2017)
DEHP	*Enterobacter* spp. strain YC-IL1	Contaminated soil	pH =7 (5-7) T = 30°C (30-35°C) Salinity = 0% DEHP = 10-400 mg/l	100% in 7 days (100 mg l^{-1})	-	Acid-tolerant (remain stable up to pH 5)	(Lamraoui et al., 2020)
DEHP	*Providencia* sp. strain 2D	Manure compost	pH = 8.3 (6-10) T = 32.4°C (20-40°C) Inoculum size (OD600) = 0.6	100% in 3 days (200 mg l^{-1})	Addition of compost as co-substrate to stimulate microbial activity	Wide range of pH and temperature adaptation	(Zhao et al., 2015)

Substrate	Microbes	Source	Optimal growth condition	Degradation percentage (Initial concentration)	Strategy to enhance stability and degradation efficiency	Microbes' unique feature	References
DEHP	*Rhodococcus ruber* strain YC-YT1	Marine plastic debris in coastal saline seawater	pH = 7 T = 30°C DEHP = 10-900 mg/l	100% in 3 days (100 mg l^{-1})	-	Very high salt tolerance (up to 12% salinity)	(T. Yang et al., 2018)
DEHP	*Stenotrophomonas acidaminiphila* OR13	Marine sediment	-	83.7% in 8 days (100 mg l^{-1})	-	-	(Ningthoujam et al., 2023)
DBP	B1 (Consortium)	Municipal sewage sludge	T = 30°C pH = 5.5-8.5	92% in 3 days (500 mg l^{-1})	-	Wide range of pH adaptation	(J. Yang et al., 2018)
DEHP	CM9 (Consortium)	Contaminated farmland soil	-	94.85% in 24 hours (1000 mg l^{-1})	Biochar addition during bioaugmentation in soil	-	(Bai et al., 2020)
DEHP	LF (Consortium)	Activated sludge	T = 30°C pH = 6 Inoculum size = 5% (v/v)	93.84% in 48 hours (1000 mg l^{-1})	-	Halotolerant (91% degradation with salt up to 3%)	(Li et al., 2018)
DEHP	An6 (Consortium)	Wetland sediment	T = 30°C	97% in 3 days (500 mg l^{-1})	Water saturation	-	(Shariati et al., 2021)

In Table 5, we can see that some study applies pure culture while some other utilize consortia for biodegradation of PAE. These consortia can either be an artificial or natural consortium, which can also be either defined or undefined based on whether the exact species within community is known or not known. The idea of applying consortia instead of pure culture had been encouraged in the field of bioremediation, due to the fact that most bacteria depend on other bacteria to metabolize certain chemicals. As described in Figures 3 and 4, degradation of pollutants involves chains of chemical reactions which require enzymes to eventuate. Some bacteria can have metabolic resources within its genome for complete degradation (Sarkar et al., 2013), however some other depend on biochemical cooperation of several bacterial isolates (Wu et al., 2010). Aside from metabolic dependency, some cooperation can be established when one bacterium depends on other bacteria to relieve environmental stress, which can further accelerate degradation activity (X. Wang et al., 2021).

In the construction of consortia, there are two approaches, which are top-down and bottom-up approach. In top-down approach, the goal is to exploit naturally established bacterial consortium. Through enrichment and isolation of potentially involved bacteria from sample of interest, member of consortium can be identified and interaction between members can be elucidated. Information on members and interaction then can be engineered to fulfil the purpose of consortium construction. Meanwhile in bottom-up approach, several bacterial isolates with known metabolic capacities are combined to form synthetic consortia. Background information on bacterial isolates metabolic capacity will be utilized to construct codependency in a consortium (Hu et al., 2021). The common approach combines both top-down and bottom-up approach, in which naturally established consortia will be first obtained through enrichment of environmental sample, followed with isolation, and constructing synthetic consortia based on bacterial isolates' metabolic capabilities. Such work has been done for biodegradation of pyrene (Wanapaisan et al., 2018), petroleum oil (Dechsakulwatana et al., 2022) and polylactic acid (Mistry et al., 2023). Additionally, some computational tools like network analysis have been applied to find out naturally established cooperative relationship in environmental sample and enrichment culture. The predicted cooperativity from network analysis then was used as guidance for subsequent isolation (Ningthoujam et al., 2023).

Current issue however lies on how to capture as much as possible actual interaction of bacteria from environmental sample. Since bacteria in soil is dense and conventional approach of isolation cause bulk partitioning, chance of missing out some potential interaction become highly possible. Furthermore, isolation of potential degraders from enrichment culture still relies on the use of selective media and hence potential yet unculturable bacteria cannot be obtained. Currently some efforts have been made to advance culturomics technology. One example of the emerging platform for exploring soil microbiome is microfluidic technology, which still require some development due to some practical limitation (X. Zhu et al., 2022).

2.3. Bioaugmentation for PAE remediation in soil

Bioremediation is a process of destroying and rendering various contaminants using natural biological activity. Based on whether the elimination of contaminants located on the contamination sites or outside the contamination sites, there are two types of bioremediations:

- *In situ* bioremediation which is providing treatment on the actual site of contamination. Some of the most important *in situ* bioremediation are bioventing, bio-sparging and bioaugmentation.
- *Ex situ* bioremediation which involves the excavation or removal of contaminated soil from ground. Some of the most important *in situ* bioremediation are landfarming, composting and bioreactors (Nwankwegu et al., 2022).

Bioaugmentation is type of *in situ* bioremediation, where biological degrading agent is directly introduced to the site of contamination. Biological agent can be microorganisms including bacteria, as pure culture, or consortia, in free-cell form or in immobilized form. Previously there had been several studies conducted to assess bioaugmentation potential of isolated or enriched PAE degraders. Some of these studies are summarized in Table 6.

Table 6 Summarized PAE-degrading bacteria activity during bioaugmentation in soil

Substrate	**Microbes (Single strain/ Consortium)**	**Source**	**Degradation rate**	**References**
DBP	*Gordonia* sp. strain QH-11	Activated sludge	100% in soil for 10 days (400 mg kg^{-1})	(Kong et al., 2019)
DBP	*Acinetobacter* sp. strain 33F	Municipal solid waste leachate	50% in 6 days (1000 mg kg^{-1})	(Sharma et al., 2021)
DEHP	*Achromobacter* sp. strain RX	Activated sludge	86.4%-91.7% in 96 hours (100 mg kg^{-1})	(P. Wang et al., 2021)
DEHP	*Gordonia terrae* strain RL-JC02	Contaminated red soil from plastic mulch	91.8% in 30 days (50 mg kg^{-1})	(Zhang et al., 2020)
DEHP	*Providencia* sp. strain 2D	Manure compost	70.8- 87.6% in 10 days (100 mg kg^{-1})	(Zhao et al., 2015)
DEHP	*Rhodococcus ruber* strain YC-YT1	Marine plastic debris in coastal saline seawater	79.7% in 7 days (100 mg kg^{-1})	(T. Yang et al., 2018)
DEHP	*Gordonia* sp. Lff	River sludge	91.4% in 3 days (100 mg kg^{-1})	(Wang et al., 2019)
DEHP	*Rhodococcus* sp. 2G	Activated sludge	80% in 35 days (50 mg kg^{-1})	(Zhao et al., 2019)
DEHP	*Rhodococcus pyridinivorans* XB	Activated sludge	78.45% in 50 days (100 mg kg^{-1})	(Zhao et al., 2018)
DBP	B1 (Consortium)	Municipal sewage sludge	84% in 10 days (100 mg kg^{-1})	(J. Yang et al., 2018)
DEHP	CM9 (Consortium)	Contaminated farmland soil	87.53% in soil for 42 days (100 mg kg^{-1})	(Bai et al., 2020)

Substrate	**Microbes (Single strain/ Consortium)**	**Source**	**Degradation rate**	**References**
DEHP	*Rhizobium*-1 and *Ensifer*-1 (Consortium)	Contaminated soil from farmland with plastic mulch	80% in 6 days (10 mg kg^{-1})	(Song et al., 2019)

2.3.1. Consideration for bioaugmentation in soil

Bioaugmentation is the addition of biological agent to the site of contamination. Biological agent can be microorganisms including bacteria, as pure culture, or consortia, in free-cell form or in immobilized form. Bioaugmentation can utilize biological agent indigenous (autochthonous microorganism) to the site of contamination, or cultures isolated from different source (allochthonous microorganism). The latter is the commonly applied one. Factors that would affect the efficiency of bioaugmentation, or bioremediation in general, are the existence of microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients). In bioaugmentation, existence of degrading agent is challenged through competition to indigenous microbes.

Consideration in choosing autochthonous or allochthonous microorganism can be based on the period of pollution. In fresh spill, contaminated site lacks the enzymes and metabolic pathways that would prevent disruption. Therefore, use of allochthonous microorganism is preferable. Meanwhile for sites with prolong contamination, when microbes likely to form adaptation yet further cleaning still needed, autochthonous microorganism is preferred (Nwankwegu et al., 2022). Possible low bioaugmentation performance in soil can be due to inadequate amount of bacteria added, or not enough nutrition, aeration and moistening on the soil (Bakina et al., 2021).

Another possible scenario that might happen under bioaugmentation is the possible perturbation of soil ecological state. Ways to measure the degree of soil perturbation under bioaugmentation is through bacterial community analysis of contaminated site, before and after bioaugmentation. Another important factor to measure soil health is through observation of vegetation growth in the soil. As described in the work by Bakina et al. (2021), out of five bacterial preparation, one had positive effect on ecological state of soil and oil biodegradation, two others did not accelerate biodegradation, while two others had significant negative impact. Positive and negative impact was measured through soil respiration after bioaugmentation (CO_2 application) and biomass of vegetation (raw grasses) within four years following application. In this study, each bacterial preparation consists of oil-degrading bacteria, which during preparation varied in inoculum size and ratio of different members in community. This indicates the importance of bacterial preparation (consortium members and ratio of inoculation) along with assessment on how it would impact soil health, aside from evaluation on degradation performance only (Bakina et al., 2021).

2.3.2. Fertilizer addition and its effect to bioaugmentation

As summarized in Table 5, isolation of PAE-degrading bacteria and investigation of its degradation characteristics have been done extensively for the last several years. The conventional approach of this work involved collection of samples from contaminated site, enrichment from environmental sample, isolation from enrichment culture using selective medium, and characterization of pure bacterial isolates. Following issues would be how to apply the obtained bacterial isolates in the real environment, which is summarized in Table 6. The common trend for bioaugmentation is the decrease of bacterial isolates activity in real environment (or soil in particular) in comparison to activity displayed in laboratory condition. Therefore, some strategies were applied to improve the survivability of degraders.

One of the commonly applied strategies are soil amendment. Soil amendment is addition of materials to improve soil properties. Soil amendment is commonly practiced in agriculture since enhancement of soil positively linked to soil productivity. An example of soil amendment is the addition of fertilizer, which can be chemical or produced through composting. For bioremediation purpose, soil amendment also referred as

biostimulation, in which soil was added with nutrients that can improve degraders activity in the contaminated side.

In the study about DBP fate in agricultural soil conducted by Cheng et al. (2019), they found that soil pH, microbial biomass carbon (C_{mic}), clay content and organic carbon (OC) are the main affecting factors. There are two processes occur during DBP contamination, which are adsorption by soil particles and biodegradation by indigenous microbes. Each of this process is affected by different soil physicochemical factors. Soil adsorption particularly affected by OC, pH and clay, with prominent effect from OC content. On the other hand, biodegradation is affected by C_{mic}, pH and OC, with C_{mic} as the most affecting factors.

Fertilizer addition, particularly commercial fertilizer, will increase both Cmic and OC load to the soil. Meanwhile, adsorption and biodegradation are two antagonistic processes since adsorption can reduce bioavailability of pollutant for biodegradation. There are various reports on effect of fertilizer for bioaugmentation. In bioaugmentation of novel bacteria *Providencia* sp. 2D, it was reported that bacteria was stimulated in compost-amended soil (Zhao et al., 2015). Organic amendments also revealed through network analysis to improve soil microbiome functional abundance (Ling et al., 2016). However, real-life application of fertilizer in agriculture field do not solely based on organic fertilizer. Commercial fertilizer contains both chemical and natural nutrients, and some research reported the increase of PAE in soil during chemical fertilizer application (Mo et al., 2008). Apart from chemical fertilizer, DBP load was reported to be significantly higher in field fertilized with manure (Zorníková et al., 2014). In the observation of microbiome network, chemical fertilizer addition was shown to reduce connection within network when compared to organic amendment (Ling et al., 2016). This variation of possible outcome under fertilization for bioaugmentation of PAE thus require more investigation.

CHAPTER III
METHODOLOGY

3.1. Types of equipment

1. Autoclave, Kakusan, Japan.
2. Centrifuge model 1920, Kubota, Japan.
3. Benchtop centrifuge model Allegra X-30R, Beckman Coulter, USA.
4. Deep freezer -20°C model MDF-U332, Sanyo Electric, Japan.
5. Erlenmeyer flask 125 ml, 250 ml, 500 ml; Pyrex, USA.
6. Glass jar with lid (8 × 12.5 cm and 5 × 8.8 cm), JJGlass, Thailand.
7. Test tube, 18 ml; Pyrex, USA.
8. Gas Chromatography- Flame Ionization Detector (GC-FID) model 6890N equipped with 0.25 mm × 30 m HP5 column coated with 5% of phenyl methyl ciloxane (0.25 µm), Agilent Technologies, USA.
9. Hot air oven model D06063, Memmert, Germany.
10. ISSCO laminar flow model HT-122.5, International Scientific Supply, USA.
11. pH meter model 240, Corning, USA.
12. PTFE filters 0.2 µm, Chrom Tech, USA.
13. Spectrophotometer, Thermo Spectronic, USA.
14. Vortex mixer, model Genie 2, Scientific Industries, USA.
15. Balance, model P2002-S and AG285, Mettler Toledo, Switzerland.

3.2. Chemicals

1. Ammonium nitrate (NH_4NO_3), Alfa Aesar, USA.
2. Di-potassium hydrogen phosphate (KH_2PO_4), KemAus, Australia.
3. Di-sodium phosphate (Na_2HPO_4), Merck, Germany.
4. Magnesium sulfate heptahydrate ($MgSO_4$. $7H_2O$), KemAus, Australia.
5. Ferric chloride hexahydrate ($FeCl_3.6H_2O$), QReC, New Zealand.
6. Calcium chloride dihydrate ($CaCl_2.2H_2O$), Merck, Germany.
7. Tryptic soy broth, HiMedia, India.

8. Methanol, Merck, Germany.
9. Dichloromethane, Loba Chemie, India.
10. Hexane, Avantor, USA.
11. Di-2(ethylhexyl) phthalate ester (DEHP), TCI, Japan.
12. Dibutyl phthalate (DBP), TCI, Japan.
13. Sodium chloride (NaCl), KemAus, Australia.
14. Glycerol, Ajax FInechem, New Zealand.
15. Resazurin, Merck, Germany.
16. GenUP^{TM} Bacteria gDNA Kit, Biotechrabbit, Germany.
17. Calcium carbonate ($CaCO_3$), Merck, Germany.

3.3. Procedure

3.3.1. Acclimatization of deposited enriched consortia

Previously, four consortia were enriched from waste dumping site soil sample in Nongkham landfill, Nongkham, Bangkok. Each of these consortia were enriched with different PAE substrate. Two of the consortia, including DBP-enriched consortium and DEHP-enriched consortium, were selected for further screening of their activity under mixture of PAEs. Both enriched consortia were deposited in the culture collection of Department of Microbiology, Faculty of Science, Chulalongkorn University, under codes MSCU_1089 for DBP-enriched consortium and MSCU_1088 for DEHP-enriched consortium.

For acclimatization, the enriched consortia were cultured in 125 ml flask containing 45 ml 0.25X Tryptic soy broth (0.25X TSB) supplemented with 25 mg l^{-1} DBP and 25 mg l^{-1} DEHP. Inoculated medium then was incubated in room temperature with speed 200 rpm for two days. After incubation in tryptic soy broth, 5 ml of bacterial inoculum then was sub-cultured to 45 ml Carbon-free-mineral-medium (CFMM) (Appendix A) added with 25 mg l^{-1} DBP and 25 mg l^{-1} DEHP and incubated for three days. Acclimatization in CFMM was repeated for two times before inoculum preparation.

3.3.2. Enriched bacterial consortium selection

In this experiment, two of the acclimatized consortia will be selected based on its degradation efficiency in single PAE and mixed PAE substrate.

3.3.2.1. Bacterial inoculum preparation

Acclimatized culture from procedure in Section 3.3.1 was sub-cultured into 45 ml 0.25X TSB (10% v/v inoculum) for two days. After incubation period, bacterial cell pellets were collected through centrifugation at 8000 rpm and 4°C. Collected cells then were washed twice with 30 ml 0.85 % (w/v) NaCl solution and resuspended with the same solution to be prepared as resting cell suspension. Cell density of resting cell suspension was prepared to be approximately 10^8 CFU ml^{-1} or OD600 = 1. The resting cell suspension was prepared at allow cell to use remaining nutrient and be ready to degrade in new substrate. Resting cell suspension was incubated overnight at room temperature and rotary shaker at speed 200 rpm.

3.3.2.2. Phthalate ester degradation experiment

For selection based on degradation efficiency, 0.5 ml resting cell suspension of each consortium was sub-cultured to 4.5 ml CFMM supplemented with 100 mg l^{-1} DBP, 100 mg l^{-1} DEHP and mixture of 100 mg l^{-1} DBP and 100 mg l^{-1} DEHP, each in triplicates. All test tubes containing bacterial suspension and CFMM-PAE then were incubated for 7 days, 200 rpm, in room temperature (28-30°C). Previously, triplicate uninoculated tubes for abiotic control degradation were also prepared for all substrate variation. Viable cells were counted for bacterial suspension prior to incubation (Day 0) and inoculated bacteria after incubation (Day 7) by using triplicate spread plate method in diluted TSB agar media (0.25X TSB and 15 g l^{-1} agar).

3.3.2.3. Extraction and detection of residual phthalate ester

After 7 days of incubation, extraction was performed to measure residual PAE concentration. Dichloromethane (DCM) was used as solvent and were added at 1:1 volumes of DCM:medium. Medium and solvent were mixed with vortex mixer at maximum speed for 1 min and solvent phase at bottom layer was collected using Pasteur pipette. Extraction was repeated for three times and solvent fractions were pooled before evaporation at room temperature in fume hood. After approximately seven days evaporation, extracts were resuspended in 5 ml DCM and filtered with PTFE filters 0.2 µm prior to quantification in gas chromatography-flame ionization detector (GC-FID). In GC-FID, the samples were injected in split mode, with injection volume 1 µl and the detector temperature was set at 290°C. The oven temperature was programmed to increase from 50 °C (hold time: 1 min) to 280 °C at a rate of 30 °C min^{-1} and then to 310 °C (hold time: 6 min) at a rate of 15 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 16.4 mL min^{-1}.

Residual PAE concentration were obtained by converting peak areas of DBP and DEHP to concentration unit (mg l^{-1}). Degradation percentage was calculated using following formula.

$$Percent\ degradation\ (\%) = \frac{C_0 - C_t}{C_0} \times 100$$

where C_0 is the concentration at time 0 and C_t is the residual concentration at time t. Enriched consortium was selected based on percent degradation for each substrate.

3.3.2.4. Continuous transfer for activity maintenance

Activity of enriched consortium was maintained through continuous transfer. Resting cell suspension which was prepared for degradation study in Section 3.3.2.2 then was inoculated to 45 ml 0.25X TSB medium with inoculation volume of 10% v/v. Inoculated flask then was incubated for two days in room temperature and 200 rpm. Cells in 0.25X TSB medium then was subsequently transferred to 45 ml CFMM supplemented with 25 mg l^{-1} DBP and 25 mg l^{-1} DEHP and incubated for three days in room temperature and 200 rpm. Sub-culturing to CFMM was repeated for three times two times before enriched consortium was inoculated again in 0.25X TSB and investigated for its degradation

activity. Procedure for inoculum preparation and degradation experiment for continuous culture sample was described in previous Section 3.3.2.1 and 3.3.2.2.

3.3.3. Characterization of selected enriched consortium

The purpose of this experiment was to characterize the selected bacterial consortium, based on its composition and degradation kinetics. Degradation kinetics can be used as information about consortium degradation capacities within certain range of contaminant concentration. Meanwhile, bacterial consortium composition can be used to identify key degraders within enriched consortium community.

3.3.3.1. Phthalate ester degradation kinetics experiment

Degradation kinetic of selected enriched consortium was investigated by varying initial concentration of DBP and DEHP mixture. Variation of initial concentrations were 500, 400, 200, 100 and 50 mg l^{-1} for each of DBP and DEHP. Bacterial suspension was prepared as described in Section 3.3.2.1 and enriched consortium that underwent continuous transfer was used as the inoculated bacteria. 0.5 ml bacterial suspension then was added into 4.5 ml CFMM in tubes supplemented with PAE mixtures. Tubes were incubated in 200 rpm, room temperature and triplicate tubes were collected daily along with uninoculated tubes as abiotic control, until Day 7 of incubation. Extraction and quantification of residual PAE was conducted with procedure describe in Section 3.3.2.3. Residual DBP and DEHP concentration then were analyzed and fitted with degradation models using Curve Fitting app in MATLAB R2020b.

3.3.3.2. Community dynamics analysis

Selected enriched consortium composition was identified using 16S rRNA gene amplicon sequencing analysis. Composition of enriched consortia can change throughout degradation time and vary between initial substrate concentration. Therefore, consortium composition analysis was conducted in line with kinetic analysis using the same acclimatized culture. In this study, 5 ml of resting cell suspension was inoculated

in 45 ml CFMM supplemented with mixture of DBP and DEHP, with concentration of each PAE 50, 200 and 500 mg l^{-1}. Inoculated flasks then were incubated at room temperature and 200 rpm. On day 0, 3, 5 and 7, triplicate flasks were collected, and cells were harvested via centrifugation at 8000 rpm for 10 min at 4°C. DNA was extracted from enriched consortium cell pellets using GenUP™ Bacteria gDNA kit. Extraction was performed in triplicate and extracted DNA was pooled. Following extraction, 16S rRNA gene were amplified using 341F and 805R primers, targeting the V3-V4 regions of 16S rRNA and high-throughput 16S rRNA gene amplicon sequencing was performed (Muangchinda et al., 2018).

Cluster generation and 250-bp paired-end read sequencing were performed on Illumina MiSeq platform (Illumina, USA) at Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). Sequence read was initially processed with QIIME 2 version 2020.8 (Bolyen et al., 2018) followed with ASV (amplicon sequence variant) analysis which include assigning taxonomic and building phylogenetic tree. Result from OTU analysis then was used for diversity analysis which includes estimation of alpha-diversity metrics (observed OTUs and Faith's phylogenetic diversity (Faith, 1992)), beta diversity metrics (weighted UniFrac (Lozupone et al., 2007), unweighted UniFrac (Lozupone & Knight, 2005), Jaccard distance, and Bray-Curtis dissimilarity), principal coordinate analysis (PCoA), and taxonomic profiling. Taxonomy was assigned using the q2-feature-classifier (Bokulich et al., 2018) against the SILVA ribosomal RNA gene database (Quast et al., 2012).

Relative abundance results obtained from 16S rRNA amplicon sequencing then were obtained and paired with corresponding DBP and DEHP residual concentration data from kinetic study. Correlation analyses was performed for both parametric (Pearson's coefficient) and non-parametric analysis (Kendall's tau and Spearman's rho) in SPSS Statistics 22.0.

3.3.4. Agricultural soil microcosm experiment

In this experiment, selected enriched consortium were investigated for activity in soil microcosm, in comparison to previous activity in liquid medium. Soil sample used for microcosm experiment was collected from Durian Farm in Rayong Province, Thailand

(12°43'46.2"N 101°33'46.1"E). Collected agricultural soil sample is stored in 4°C prior to microcosm experiment. Prior to experiment, soil was characterized for its physicochemical properties including pH, organic matter (OM), lime requirement (LR), texture, available phosphate, exchangeable K, exchangeable Ca, exchangeable Mg, C/N ratio, electrical conductivity (EC 1:5), total N, total P, total K, and available water capacity (AWC) value. Physicochemical properties of soil were checked by 'Soil, Fertilizer, Environment Academic Service Project' of Department of Soil Science, Faculty of Agriculture, Kasetsart University.

3.3.4.1. Microcosm experiment setup

There were four variations of treatments in this experiment. Initially soil was divided into autoclaved (S) and non-autoclaved soil. Then both soils were separated into soil inoculated with exogenous bacteria (B) and uninoculated soil. Therefore, the four treatments were including:

(1) B (Bioaugmentation)

(2) S + B (sterilized then bioaugmented soil)

(3) NA (non-sterilized and non-inoculated soil or natural attenuation)

(4) C (sterilized and non-inoculated soil or the abiotic control)

Soils in all treatments were polluted with 300 mg kg^{-1} DBP and 300 mg kg^{-1} DEHP. All experiments were conducted in five replicates glass jar (diameter 8 cm, height 12.5 cm) containing 60 g soil. All soil prior to experiment was grinded and sieved with 10-mesh sieve. Initially for treatments with PAE supplementation, a quarter of soil (15 g) was separated from jar. Separated soil then was added with PAE diluted in hexane, to reach final concentration of 300 mg kg^{-1} for each of DBP and DEHP in 60 g soil. Additionally, remaining soil from all treatments was air-dried. After three days evaporation, spiked soil was mixed thoroughly with the rest of soil in the jar. Treatments which require soil sterilization then was autoclaved three times in 30 minutes with interval 1 day for each sterilization steps.

On the other hand, enriched consortium to be inoculated into the soil was prepared with the procedure described in Section 3.3.2.1., in which the inoculum was prepared from continuously transferred enriched consortium. Bacterial suspension was added to achieve bacterial density of 10^7 cells g^{-1} soil and water content 60% WHC (water holding capacity). Similarly, treatments without inoculation of exogenous bacteria were added with sterilized distilled water of the same volume. After setting up treatment variation, all glass jars were incubated in room temperature (25-33°C) and natural lighting. Samples were collected on Day 0, 2, 4, 6, 10, 20, and 30. For each sampling, 2 g soil sample was collected residual PAE extraction, 1 g for heterotrophic soil bacterial enumeration and 1 g for PAE-degrading bacterial enumeration. Heterotrophic soil bacterial and PAE-degrading bacterial enumeration were determined using MPN method.

3.3.4.2. Extraction and detection of residual phthalate ester from soil

PAE residual concentration was determined using extraction with DCM. During extraction process, 2 grams of soil sample was submerged with 10 ml of DCM in 18 ml-test tubes and sonicated for 30 minutes. Extract in solvent phase was separated from the tubes, and extraction was repeated thrice, yielding approximately 30 ml PAE extract. After evaporation for approximately 4 days, extract was resuspended in 3 ml DCM and filtered with PTFE filters, then transferred to GC vials for quantification in GC-FID.

3.3.4.3. Total heterotrophic bacteria enumeration

Previously, 1 g soil sample was added with 9 ml 0.85% (w/v) NaCl and mixed with vortex mixer. Meanwhile 96-well-plate were added with 180 µl 0.25X TSB in sterile condition. Serial dilution was performed by initially adding 20 µl of soil suspension in saline solution to the first row of 96-well-plate, followed with subsequent transfer of 20 µl from the first row to the second row. Tips were discarded so that each rows until the 10^{th} row of 96-well-plate have serially descending concentration of microbes. Plates were measured for absorbance in Day 0 at 540 nm (OD540), then incubated for another 2 days. After incubation, absorbance was measured in the same wavelength, and

increase of optical density was used to estimate total heterotrophic bacteria count per g soil.

3.3.4.4. Phthalate ester degrading bacteria enumeration

Similarly, 1 g soil sample was added with 9 ml 0.85% (w/v) NaCl and mixed with vortex mixer. Meanwhile 96-well-plate were added with 180 µl CFMM in sterile condition. Plates containing CFMM then supplemented with DBP 100 ppm and DEHP 100 ppm in hexane, including abiotic control plates. After evaporation for approximately one-hour, serial dilution was performed by initially adding 20 µl of soil suspension in saline solution to the first row of 96-well-plate, followed with subsequent transfer of 20 µl from the first row to the second row and so on until the 10^{th} row of 96-well-plate. Plates were incubated in room temperature 7 days. On last day of incubation period, resazurin solution was prepared by dissolving 270 mg resazurin in 40 ml sterile distilled water. Then, 10 µl of resazurin solution was added into each plate, including abiotic control wells. Following indicator solution addition, plates were incubated in 37°C for 18 hours (Sarker et al., 2007) . Incubated plates then observed for positive growth through change in color from blue to purple or pink.

3.3.4.5. Phthalate ester phytotoxicity experiment

For phytotoxicity experiment, protocol was adapted from Bandini et al. (2020) with several modification. Soil sample which was collected throughout experiment was pooled and mixed with sterile distilled water (ratio 1:4 w/w). Solution then was diluted 10 and 100 times and pH was measured for all mixtures. Seeds used was mung bean. All seeds were initially soaked in NaOCl 0.5% (v/v) solution for ten minutes and rinsed thoroughly with sterilized distilled water. After that, ten seeds were placed in petri dish (90 mm diameter) with filter paper moistened with 5 ml of three different solutions. Seeds moistened with distilled water used as control. Seeds were incubated for 3 days at room temperature in the dark. At the end of toxicity test, germinated seeds were observed for root length, shoot length, fresh biomass weight, and percent germination index (%GI). Percent germination index can be calculated with following equation.

$$GI = \frac{\sum root\ lengths}{n}$$

$$\%GI = \frac{GI_{si}}{GI_c} \times 100$$

where n is the number of germinated seeds, GI_c is the germination index of control and GI_{si} is the germination index of treatment petri dish.

3.3.5. Fertilizer addition soil microcosm study

Similar with experiment in Section 3.3.4, the effect of bioaugmentation of selected enriched consortium in agricultural soil was further observed by adding new variable to treatment variation, which is fertilizer addition.

3.3.5.1. Microcosm experiment setup

For fertilizer addition microcosm experiment, variations were separated based on soil sterilization, exogenous consortium inoculation and fertilizer addition. In short there were six treatments including:

(1) B (non-sterilized and bioaugmented soil)

(2) B + F (non-sterilized and bioaugmented soil added with fertilizer)

(3) NA (natural attenuation)

(4) NA + F (natural attenuation added with fertilizer)

(5) S + B + F (sterilized and bioaugmented soil added with fertilizer)

(6) C (abiotic control)

In all treatments, 200 mg kg^{-1} DBP and 200 mg kg^{-1} DEHP were added into the soil. All experiments were conducted in triplicate glass jar (diameter 5 cm, height 8.8 cm) containing 13 g soil for treated jar and 5 g soil for abiotic control. For each treatments, soils were separated in different jar for every sampling day. Samples were taken on Day 0, 3, 7, 15, 25. Similarly with previous microcosm experiment, all soils prior to experiment were grinded and sieved with 10-mesh sieve.

For PAE addition, 5 g soil was separated from jar. Separated soil then was added with PAE diluted in hexane, to reach final concentration of 200 mg kg^{-1} for each of DBP and DEHP. After three days evaporation, spiked soil was mixed with the rest of soil in the jar and mixed thoroughly. For treatment with fertilizer addition, fertilizer used was purchased from farmer shop under the brand "เขาเพชร" with fertilizer number 6-3-3 (Organic matter 10%, N 6%, P_2O_5 3%, K_2O 3%). Fertilizer was added based on product application suggestion for vegetable/ fruit farms, which is 50 -100 kg/rai (highest dose was applied). Addition of fertilizer was observed to reduce soil pH, which can be detrimental to the survival of inoculated consortium. In this experiment, pH soil was adjusted by adding $CaCO_3$ with proportion 0.95 mg $CaCO_3$ per g soil, based on $CaCO_3$ requirement from physicochemical analysis (Appendix C). Soil pH before and after $CaCO_3$ addition were recorded. Soil for abiotic control and exogenous consortia was sterilized with the same protocol described in Section 3.3.4.1

Enriched consortia to be inoculated into the soil was also prepared with the procedure described in Section 3.3.2.1. Similarly, added consortia was prepared from continuously transferred culture. Bacterial suspension will be added to achieve bacterial density of 10^8 cells g^{-1} soil and water content 80% WHC (water holding capacity). Treatments without inoculation of exogenous bacteria were added with sterilized distilled water to adjust the moisture content. All jars were incubated in room temperature (25-33°C) and natural lighting.

For each sampling, 10 g soil sample was collected residual PAE extraction, 1 g for heterotrophic soil bacterial enumeration, 1 g for PAE-degrading bacteria enumeration, and 1 g for phytotoxicity experiment. Procedure for bacteria enumeration was previously described in Section 3.3.4.3 and 3.3.4.4.

3.3.5.2. Extraction and detection of residual phthalate ester from fertilizer-added soil

In comparison to previous agricultural microcosm experiment extraction protocol, in this experiment, extraction was conducted with two different solvent. Initially, methanol was used for extraction by addition of 20 ml methanol to the jar. After continuous shaking for 15 minutes in room temperature and 200 rpm, methanol was separated from

soil particles through centrifugation for 5 min, 4000 rpm, 8°C. Methanol extract was evaporated for approximately seven days in fume hood at room temperature. On the other hand, remaining soil was extracted again with sonication using 30 ml DCM as solvent for 30 minutes. Solubilized extract in DCM was separated again from soil particles and evaporated for approximately two days in fume hood at room temperature. Extracts from different solvent was analyzed separately in GC-FID, after resuspension in 3 ml DCM and filtering with PTFE 0.2 µm.

3.3.5.3. Phthalate ester phytotoxicity experiment

In comparison to previous phytotoxicity study, seeds utilized for fertilizer-addition phytotoxicity experiments consisted of Chinese convolvulus (*Ipomoea aquatica*) and corn (*Zea mays*) seeds. Similarly, protocol for phytotoxicity study was described in Section 3.3.4.5.

CHAPTER IV
RESULT AND DISCUSSION

4.1. Enriched consortia selection

Phthalate esters (PAEs) are considered priority contaminants due to their widespread occurrence and toxicity. There are several PAE remediation strategies including physical, chemical and biological remediation. Bioremediation utilizing bacteria has been considered a favorable strategy among several PAE remediation strategies. Bacteria-driven PAE remediation is generally regarded as safer method, which also can ensure complete mineralization of PAE.

Publications on PAE occurrence reported that samples from wastewater influent, sewage sludge and landfill leachate contain the highest PAE concentration (Hu et al., 2021). Based on this information, it is likely that some bacteria were adapted to higher PAE concentrations and developed metabolic capacities to degrade PAE within these environments. Therefore, enrichment from these environmental samples can be applied to obtain these bacteria.

Previously, two bacterial consortia were obtained from landfill soil sample of Nongkham waste dumping site, Bangkok. The consortium enriched with DBP was LF-NK-DBP; meanwhile, LF-NK-DEHP was obtained from enrichment with DEHP. This experiment aims to determine enriched consortia used for bioaugmentation. Experiments from the previous study showed that LF-NK-DEHP could degrade 98.7% of initial DEHP 100 mg l^{-1} in 2 days; meanwhile, LF-NK-DBP was capable of 100 mg l^{-1} DBP degradation up to 71.0% in 5 days (Meyawee Satiraphan, 2018). In this experiment, both LF-NK-DBP and LF-NK-DEHP were evaluated for their degradation activity under 100 mg l^{-1} DBP, 100 mg l^{-1} DEHP and a mixture of 100 mg l^{-1} DBP and 100 mg l^{-1} DEHP. Results of degradation study for enriched consortia selection are shown in Figure 5.

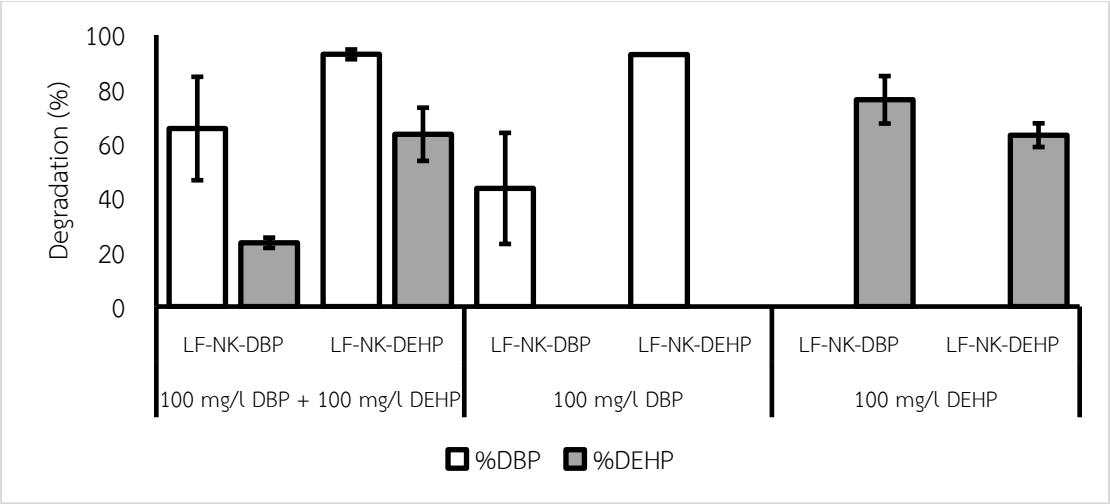


Figure 5 Degradation percentage of enriched consortia in single and mixed PAE

Based on Figure 5, LF-NK-DEHP has higher DBP degradation activity than LF-NK-DBP despite initial DEHP enrichment. LF-NK-DEHP degraded 92.9% DBP in the mixed substrate and 92.8% DBP in individual substrate addition. Both consortiums degraded DBP faster than DEHP due to the difference in molecular size of the two PAEs. DBP has shorter alkyl, which has a higher susceptibility to biotic and abiotic degradation (Cousins et al., 2003). DEHP is relatively more persistent than DBP, and both enriched consortia could not completely degrade DEHP despite the previous report from the enrichment procedure (Meyawee Satiraphan, 2018).

In this study, LF-NK-DBP performed higher DEHP degradation than LF-NK-DEHP in DEHP as single substrate (76± 8.7%). However, in mixed substrate, LF-NK-DBP was unable to maintain the same rate (22.5± 1.9%). LF-NK-DEHP, on the other hand, maintained similar degradation percentage for DEHP as single substrate (63± 4.4%) and DEHP in a mixture with DBP (63± 9.8%). LF-NK-DEHP could maintain almost similar degradation activity in DBP and DEHP as mixed substrate and single substrate. Therefore, this experiment selected LF-NK-DEHP as enriched consortia for exogenous consortia for bioaugmentation in agricultural soil.

There are two possibilities in the pattern of PAE biodegradation by LF-NK-DEHP. First, DEHP degraded after the initial degradation of DBP due to the preference of degrading enzymes for lower steric hindrance molecules. Second, both PAE congeners were degraded simultaneously but at different rates. Further characterization in microbial

degradation kinetics and community dynamics can give some insight into the interaction of varying substrates in the consortium of bacteria.

Additionally, in Figure 5, we can see that despite initial enrichment in DEHP, enriched consortium LF-NK-DEHP preferred DBP over DEHP. In general, bacteria tend to degrade short-alkyl PAE faster (Cousins et al., 2003). An enriched consortium required the specific enzyme to have efficient DEHP degradation, and there was a report on bacteria with a particular degradation of long alkyl PAE (Zhang et al., 2018). This observation shows that complex bacteria communities in enriched consortia may lose their initial degradation characteristic during storage. It is difficult to monitor these specific bacteria based on bacterial enumeration alone. We resolved this issue through activity-based monitoring, which we explained in the following section.

4.2. Activity maintenance through continuous transfer

In general, natural bacterial consortia gives higher degradation performance than synthetic consortia. However, the disadvantage of using enriched consortia is it is hard to maintain the activity. In addition, since natural enriched consortia consist of a complex community of bacteria, it is hard to point out which bacteria within the community are mainly responsible for PAE degradation. Therefore, the natural enriched consortium may lose activity during storage and transfer due to the reduced abundance of active PAE-degraders within the consortium. In contrast, the reduction of PAE-degraders can be monitored in synthetic consortia because there are a small number of bacteria within the consortium. Furthermore, each of the isolates in synthetic consortia was previously identified. Therefore, such method, for example, PCR, can detect the bacteria's presence or loss (Massot et al., 2022).

It is likely that during storage, LF-NK-DEHP degradation activity reduced. In order to improve the degradation activity, consortium LF-NK-DEHP was continuously sub-cultured as described in Chapter 3. Activity during transfer is shown in Figure 6.

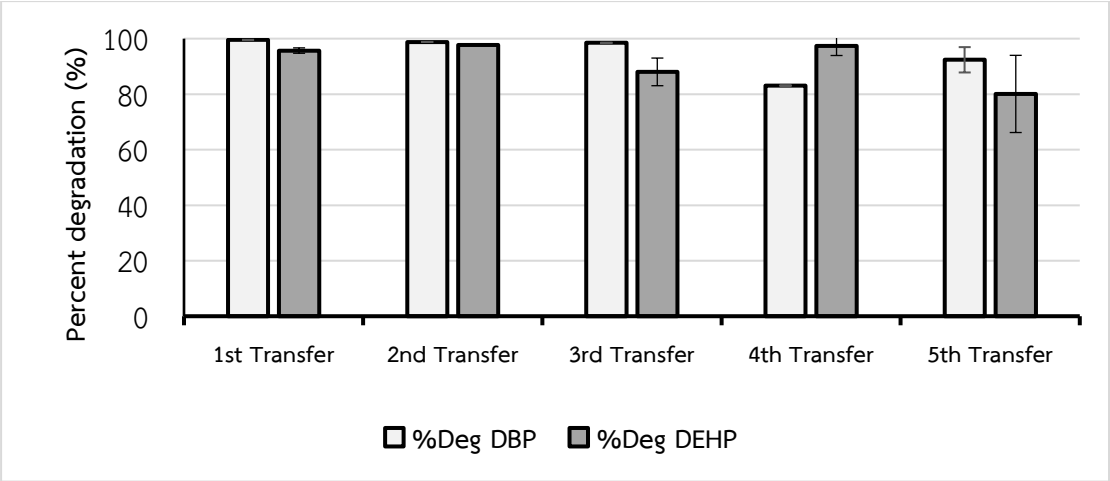


Figure 6 Degradation percentage of enriched consortia throughout continuous transfer

Through the maintenance of degradation activity, LF-NK-DEHP maintained DBP degradation at an average of 92% and DEHP degradation at 82%. On transfer 5, the DEHP degradation activity of LF-NK-DEHP slightly decreased, with 92% degradation of 100 mg l^{-1} DBP and 80% degradation of 100 mg l^{-1} DEHP after five days of incubation. However, in general, LF-NK-DEHP exhibited higher degradation activity in comparison to degradation performance in Figure 5. LF-NK-DEHP maintain a relatively high degradation activity of DBP and DEHP, as previously investigated in initial enrichment.

4.3. Selected enriched consortium characterization

4.3.1. Kinetic of PAE mixture degradation

After exogenous enriched consortia selection, the following part of the research was exogenous enriched consortia characterization. PAE degradation kinetic was one of the important characteristics required for a better understanding of LF-NK-DEHP. The kinetic study provides information on degradation rates and insight into the degradation mechanism, which is linked to performance in bioaugmentation later on.

This study investigated LF-NK-DEHP, which was previously observed to have higher DBP and DEHP biodegradation, for its degradation kinetics. Following are the residual concentration and degradation percentage of DBP in Figure 7 and 8, and DEHP in Figure 9 and 10.

By comparing Figures 7, 8, 9 and 10, we can see that DBP degraded faster than DEHP throughout the initial substrate addition. For example, LF-NK-DEHP could completely degraded DBP within three days when the substrate added lower than 400 mg l^{-1}. On the other hand, DEHP did not fully degrade up a higher concentration (500, 400, 200 mg l^{-1}) to seven days of incubation. This study determined the kinetic degradation model for DBP and DEHP through linear and non-linear regression analysis. Summary of kinetic study is shown in Table 7.

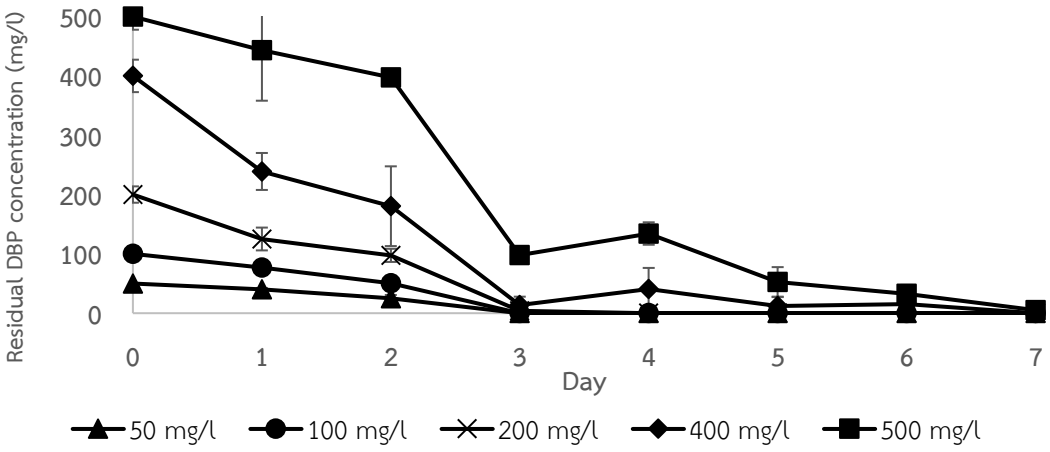


Figure 7 Residual DBP concentration for kinetic study

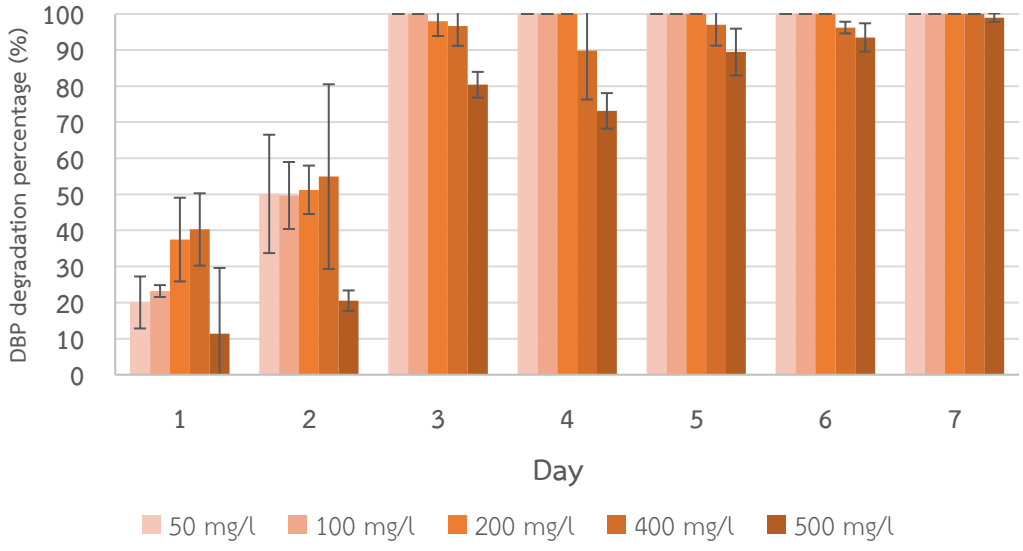


Figure 8 Degradation percentage of DBP for kinetic study

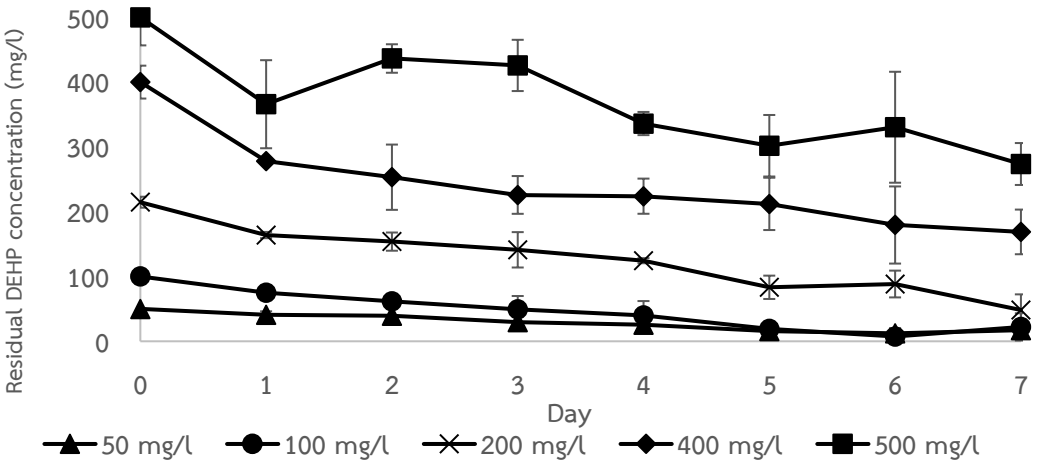


Figure 9 Residual DEHP concentration for kinetic study

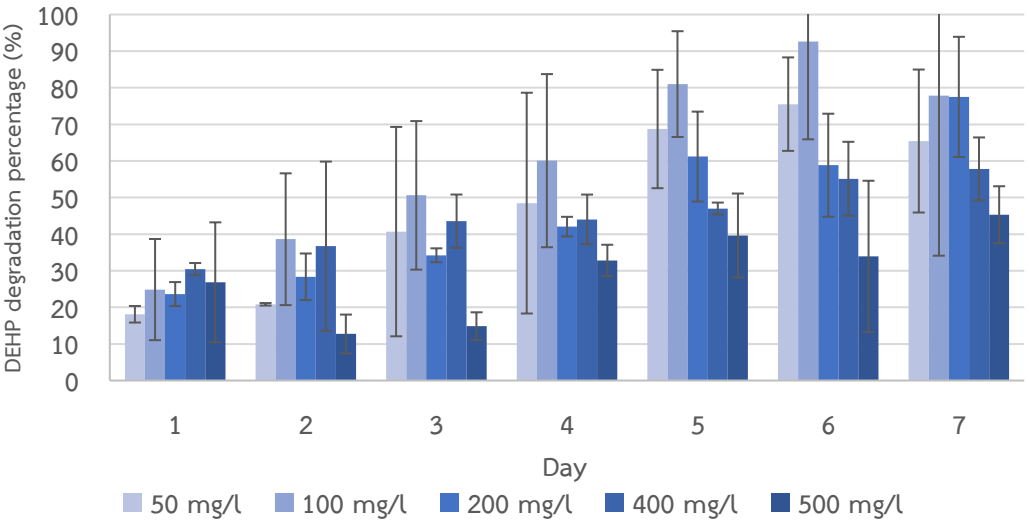


Figure 10 Residual concentration and degradation percentage of DEHP for kinetic study

Table 7 Kinetic model of DBP and DEHP degradation by enriched consortia LF-NK-DEHP

Substrate	Initial substrate	Kinetic equation	Calculated parameters	R^2
DBP	50 mg l^{-1}	Modified Gompertz	R_m = 1.04 mg. $l^{-1}.h^{-1}$ λ = 18.17 h	0.954
	100 mg l^{-1}	Modified Gompertz	R_m = 1.96 mg. $l^{-1}.h^{-1}$ λ = 39.86 h	0.939
	200 mg l^{-1}	Modified Gompertz	R_m = 3.14 mg. $l^{-1}.h^{-1}$ λ = 3.98 h	0.904
	400 mg l^{-1}	Modified Gompertz	R_m = 5.33 mg. $l^{-1}.h^{-1}$ λ = 20.54 h	0.902
	500 mg l^{-1}	Modified Gompertz	R_m = 6.65 mg. $l^{-1}.h^{-1}$ λ = 5.88 h	0.917
DEHP	50 mg l^{-1}	Zero-order	k_1 = 0.22 mg. $l^{-1}.h^{-1}$	0.904
	100 mg l^{-1}	Zero-order	k_1 = 0.59 mg. $l^{-1}.h^{-1}$	0.963
	200 mg l^{-1}	Zero-order	k_1 = 0.88 mg. $l^{-1}.h^{-1}$	0.946
	400 mg l^{-1}	Zero-order	k_1 = 0.73 mg. $l^{-1}.h^{-1}$	0.965
	500 mg l^{-1}	Zero-order	k_1 = 1.47 mg. $l^{-1}.h^{-1}$	0.938

Based on linear regression, DEHP degradation fitted the zero-order kinetic model, which indicates the degradation rate is independent of substrate concentration. Zero-order kinetic models indicated by linear disappearance curve for degraded chemicals or linear substrate disappearance with time. Usually, the degradation of pollutants follows a zero-order model in a condition where the uptake system of the cells is saturated or if the initial concentration of chemicals is insufficient to support a significant increase in the active bacteria population (Battersby, 1990).

On the other hand, DBP residual plot experimental data fitted with a nonlinear modified Gompertz kinetic model. Initially, the Gompertz model was used to describe microbial growth and bio-product formation kinetic. The following modified Gompertz

model was explicitly used to describe biodegradation's kinetics, shown in the following equation (Li et al., 2005).

$$S = S_0\left\{1 - \exp\left\{-\exp\left[\frac{R_m e}{S_0}(\lambda - t) + 1\right]\right\}\right\}$$

S indicates the chemical of interest (or, in this case, polluting chemical) concentration at time *t*, *S*0 is the initial concentration of pollutant, *e* is constant (2.71828128), R_m value indicates the maximum substrate consumption rate, and $\boldsymbol{\lambda}$ indicates the calculated period for lag phase. Compared to the zero-order model, two additional parameters exist in the modified Gompertz model. In Table 7, with increasing concentration of initial DBP, the transformation rate also becomes higher. On the other hand, the lag phase period does not correspond with higher or lower DBP initial concentration.

Similar to the modified Gompertz model for DBP, the rate constant for DEHP degradation was increased for substrate at concentration range from 50 mg l^{-1} to 200 mg l^{-1}. Slight decrease was observed at concentration 200 to 400 mg l^{-1}, which then increase again at supplementation of 500 mg l^{-1} DEHP. R_m of DBP modified Gompertz model and k_1 of zero-order model have the same unit; therefore, both values are comparable. In general, DEHP have lower value of rate constants across variation of initial substrate compared to DBP. In Table 8, LF-NK-DEHP kinetic is listed along with previous report on enriched consortium kinetic.

Table 8 References on enriched consortia degradation kinetics

Consortium	Substrate	Kinetic model	$t_{1/2}$	Concentration	References
Consortium B1	DBP	First-order	2.14 d	100 mg kg^{-1}	(J. Yang et al., 2018)
Consortium LF	DEHP	First-order	4.36 d	2000 mg l^{-1}	(Li et al., 2018)
Consortium CM9	DEHP	Non-linear	0.88 h	1000 mg l^{-1}	(Bai et al., 2020)
Consortium LF-NK-DEHP	DBP	Non-linear	1.49 d	500 mg l^{-1}	This study
	DEHP	Zero-order	7.23 d	500 mg l^{-1}	This study

This study was the first to evaluate enriched consortia's kinetic of mixed PAE degradation. LF-NK-DEHP showed high DBP degradation and slow DEHP degradation compared to other enriched consortia. The enrichment culture in Table 8, including consortium CM9 and LF, was capable of higher DEHP degradation within a shorter time. However, these reference studies did not evaluate the rate of DEHP degradation in the presence of shorter-alkyl PAE.

The modified Gompertz model was used as the kinetic model for enriched consortia CM9 in Table 8. Previously, this kinetic model was used to fit with PAE degradation by pure isolates. Table 9 lists some PAE-degrading bacteria with degradation kinetic best described with modified Gompertz. Compared to pure isolates in Table 9, LF-NK-DEHP gave higher DBP degradation despite being initially added with DEHP. Overall, the kinetic study revealed the unique degradation characteristic of the enriched consortium, which is likely to have hundreds or more different strains of bacteria towards a mixture of substrates. We obtained this knowledge through this study, which is expected to be observed in the soil microcosm study.

Table 9 References on modified Gompertz for PAEs degradation kinetics

Bacteria/ Consortium	**Substrate**	**Calculated parameters and R^2**	**References**
Rhodococcus rubber Sa	Dimethyl isophthalate (DMI) (Mix conc. 80 mg l^{-1})	R_m = 6.82 mg. l^{-1} $hour^{-1}$ λ = 1.66 hours R^2 = 0.999	(Li et al., 2005)
	Dimethyl terephthalate (DMT)	R_m = 1.91 mg. l^{-1} $hour^{-1}$ λ = 47.7 hours R^2 = 0.994	
	DMP (Dimethyl phthalate)	R_m = 1.1 mg. l^{-1} $hour^{-1}$ λ = 105.96 hours R^2 = 0.993	
Gordonia sp. strain QH-12	DBP 750 mg l^{-1}	R_m = 66.37 mg. l^{-1} $hour^{-1}$ λ = 11.43 hours R^2 = 0.994	(Jin et al., 2016)

Bacteria/ Consortium	Substrate	Calculated parameters and R^2	References
Gordonia sp. strain QH-11	DBP 750 mg l^{-1}	R_m = 35.87 mg. l^{-1} $hour^{-1}$ λ = 17.51 hours R^2 = 0.990	(Jin et al., 2012)
Burkholderia cepacian DA2	DMP 800 mg l^{-1}	R_m = 808.35 mg. l^{-1} day^{-1} λ = 11.55 days R^2 = 0.993	(Wang et al., 2008)
Consortium LF-NK-DEHP	DBP 500 mg l^{-1}	R_m = 6.65 mg. l^{-1} h^{-1} λ = 5.88 hours R^2 = 0.917	This study

4.3.2. Consortia community dynamics

For consortia community characterization, variations of initial concentration were 50, 200 and 500 mg l^{-1} for each DBP and DEHP. Collected cells were then extracted for DNA and sequenced for 16S rRNA gene for community analyses. Analyses include alpha and beta diversity, along with taxa community study. Table 10 lists the alpha diversity index, including the Shannon index and Faith's Phylogenetic Diversity (Faith's PD) of the LF-NK-DEHP consortium during incubation.

Table 10 Alpha diversity index (Shannon and Faith's PD) for community dynamic study

Day	Initial substrate	Shannon index	Faith's PD
Day – 0	-	2.055	2.081
Day – 3	50 mg l^{-1} DBP + 50 mg l^{-1} DEHP	2.502	2.193
	200 mg l^{-1} DBP + 200 mg l^{-1} DEHP	2.649	2.183
	500 mg l^{-1} DBP + 500 mg l^{-1} DEHP	2.68	2.141
Day – 5	50 mg l^{-1} DBP + 50 mg l^{-1} DEHP	2.655	2.13
	200 mg l^{-1} DBP + 200 mg l^{-1} DEHP	2.896	2.199
	500 mg l^{-1} DBP + 500 mg l^{-1} DEHP	2.772	2.2
Day – 7	50 mg l^{-1} DBP + 50 mg l^{-1} DEHP	2.527	2.193
	200 mg l^{-1} DBP + 200 mg l^{-1} DEHP	2.707	2.193
	500 mg l^{-1} DBP + 500 mg l^{-1} DEHP	2.651	2.198

In Table 10, alpha-diversity, or the measure to indicate how many different members within a bacterial community, was slightly increased from Day 0 to the following incubation time. However, there was no significant difference between increasing PAE substrate and longer incubation time.

On the other hand, beta-diversity analyses, shown in Figure 11, indicated the difference in community composition. Variation of PAE supplementation and incubation time did not give clustering pattern in PCoA plot. This observation indicates that community composition was also independent of these two variables.

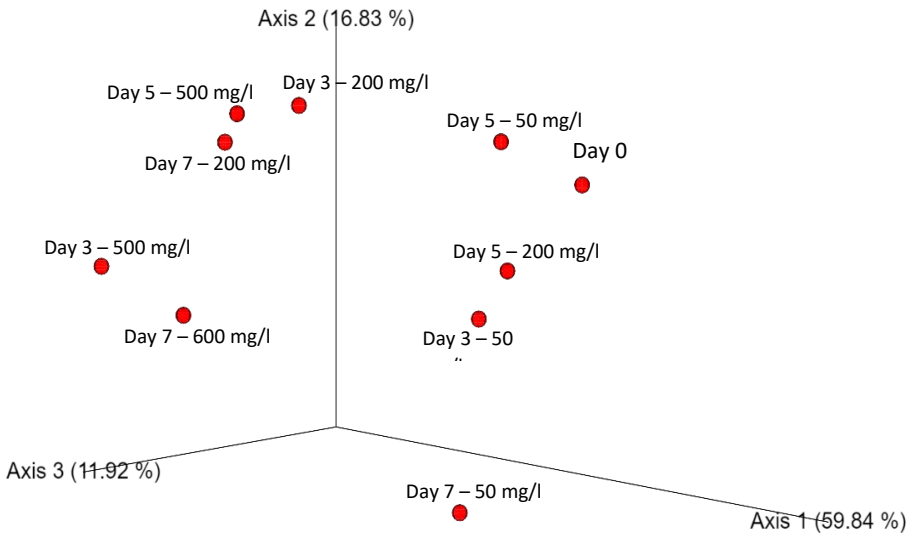


Figure 11 Principal coordinate analysis (PCoA) plots of beta diversity calculated using Bray-Curtis distance

PAE, in general, have a toxic effect on the bacterial community. The enrichment process reduced the number of bacteria in the environmental sample and the alpha diversity index. This change in alpha diversity will also affect the beta diversity of the community from the environmental sample and community of enrichment culture.

Visualization in the PCoA plot tends to separate a cluster of enrichment communities from the original sample community (Ningthoujam et al., 2023).

Meanwhile, in this experiment, alpha diversity slightly increased, and the community of resting cells was in close range with the community of the PAE-added sample (Figure 11). Enrichment and continuous transfer hypothesized to already reduce the number of bacteria from environmental sample. It is likely that some consortium members had low abundance in initial resting cell suspension, and 16S rRNA gene amplicon sequencing could not detect these groups. However, PAE supplementation and incubation enable some members' populations to increase. These members then could be detected and identified as different consortium member. This observation was also reflected in the relative abundance bar plot of LF-NK-DEHP for the taxonomic level genus shown in Figure 12.

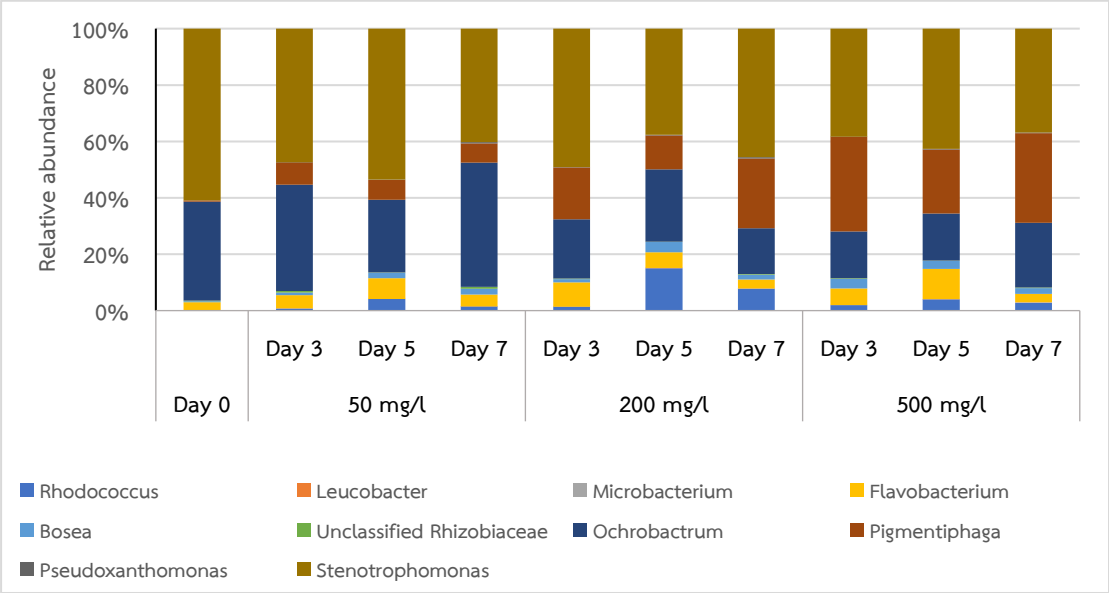


Figure 12 Relative abundance bar plot for taxonomic level genus

Figure 12 shows that genus *Ochrobactrum*, *Stenotrophomonas* and *Pigmentiphaga* dominated the LF-NK-DEHP community. Genus *Pigmentiphaga* especially, was not observed at sample taken from resting cell, or community before PAE addition (Day 0). Kinetic experiments and the study of the community were conducted simultaneously

to determine the community's dynamic during the degradation of DBP and DEHP. Correlation analyses were performed using data on the residual concentration of DBP and DEHP in the kinetic study and the relative abundance of each genus in the community study. The analysis was based on the hypothesis that PAE supplementation and incubation enable some members' populations to increase. In addition, these members are considered to be the main degraders within LF-NK-DEHP. Correlation analyses were performed, and the results are shown in Table 11.

Table 11 Parametric and non-parametric correlation analyses of taxa relative abundance and PAE removal

Substrate	Phylum	Pearson's Correlation	Sig. (2-tailed)	Kendall's tau	Sig. (2-tailed)	Spearman's rho	Sig. (2-tailed)
DBP	*Rhodococcus*	0.111	0.759	0.396	0.121	0.517	0.126
	Leucobacter	0.333	0.348	0.193	0.459	0.330	0.351
	Microbacterium	0.733*	0.016	0.582*	0.023	0.689*	0.027
	Flavobacterium	0.275	0.442	0.210	0.412	0.234	0.515
	Bosea	0.548	0.101	0.442	0.083	0.622	0.055
	Unclassified Rhizobiaceae	-0.487	0.154	-0.303	0.236	-0.326	0.358
	Ochrobactrum	-0.691*	0.027	-0.489	0.056	-0.714*	0.020
	Pigmentiphaga	0.882**	0.001	0.768**	0.003	0.899**	0.000
	Pseudoxanthomonas	0.134	0.711	0.256	0.316	0.357	0.311
	Stenotrophomonas	-0.658*	0.039	-0.629*	0.014	-0.757*	0.011
DEHP	*Rhodococcus*	0.643*	0.045	0.584*	0.020	0.736*	0.015
	Leucobacter	0.449	0.193	0.209	0.412	0.388	0.268
	Microbacterium	0.415	0.233	0.225	0.369	0.340	0.336
	Flavobacterium	-0.671*	0.949	0.090	0.719	0.067	0.854
	Bosea	0.437	0.207	0.360	0.151	0.456	0.185
	Unclassified Rhizobiaceae	-0.436	0.207	-0.090	0.719	-0.152	0.675
	Ochrobactrum	-0.671*	0.034	-0.494*	0.048	-0.717*	0.020
	Pigmentiphaga	0.648*	0.043	0.539*	0.031	0.729*	0.017
	Pseudoxanthomonas	0.564	0.089	0.405	0.106	0.590	0.073
	Stenotrophomonas	-0.558	0.094	-0.494*	0.048	-0.608	0.062

***. Correlation is significant at the 0.01 level (2-tailed).*

**. Correlation is significant at the 0.05 level (2-tailed).*

Parametric correlation analysis is based on the linear assumption that increasing or decreasing relative abundance correlates linearly with higher or lower DBP and DEHP removal. Meanwhile, the non-parametric correlation analysis by Kendall and Spearman is not based on linear assumptions.

From Table 11, correlation analysis obtained that *Microbacterium* significantly positively correlated with DBP degradation, while *Rhodococcus* significantly associated with DEHP degradation. Meanwhile, *Pigmentiphaga* positively correlates with the removal of both DBP and DEHP. On the other hand, several community members negatively correlate with PAE removal. For example, *Ochrobactrum* has a negative correlation for DBP and DEHP and *Stenotrophomonas* for DBP only. Furthermore, according to Pearson's correlation, *Flavobacterium* has a significant negative correlation with DEHP and *Stenotrophomonas* according to Kendall's tau.

This observation highlights that *Pigmentiphaga*, *Microbacterium* and *Rhodococcus* are likely to be the genus for PAE-degrading bacteria within LF-NK-DEHP. Furthermore, there is diversification where *Rhodococcus* only significantly correlated with DEHP and *Microbacterium* only correlated with DBP. *Pigmentiphaga* was significant at the 0.01 level for DBP degradation and 0.05 level for DEHP degradation.

Several publications have reported genus *Rhodococcus* for its degradation activity against DEHP (Kamaraj et al., 2021; T. Yang et al., 2018; Zhao et al., 2019; Zhao et al., 2018). *Microbacterium* also has been reported as an efficient degrader of DBP (Lu et al., 2020; J. Yang et al., 2018; Zhao et al., 2021). Another publication also reported the activity of *Rhodococcus* sp. strain F4 and *Microbacterium* sp. strain F8, which constantly dominated sludge-amended soil polluted with 100 mg/kg DBP and 100 mg/kg DEHP (Yuan et al., 2011). On the other hand, *Pigmentiphaga* is rarely reported to have activity towards PAE. Previously, one publication reported *Pigmentiphaga* as a core member of consortia enriched from contaminated farmland soil. However, during bioaugmentation, the author observed that *Pigmentiphaga* showed a sharp decrease (Bai et al., 2020). Through multi-omics analyses, it was also revealed that *Pigmentiphaga* sp. D-2 have gene cluster or contig with potential DEHP-degrading hydrolase, which displayed >70% amino acid identity to DEHP/MEHP hydrolase of DEHP degrader, *Acidovorax* sp. strain 210-6 (Wei et al., 2021).

4.4. Agricultural soil microcosm experiment

4.4.1. Phthalate ester degradation by exogenous consortia in agricultural soil

In the second research phase, LF-NK-DEHP were tested for bioaugmentation performance in agricultural soil microcosm. To compare the exogenous bacteria activity with indigenous soil bacteria, soil without inoculation was prepared (NA or natural attenuation). In addition, to observe exogenous bacteria activity in absence of natural microflora, soil was sterilized and inoculated with LF-NK-DEHP subsequently (S+B or sterilized soil and bioaugmentation). In total there were three treatments, and PAE degradation performance was monitored for 30 days. Result for DBP residual concentration degradation is shown in Figure 13.

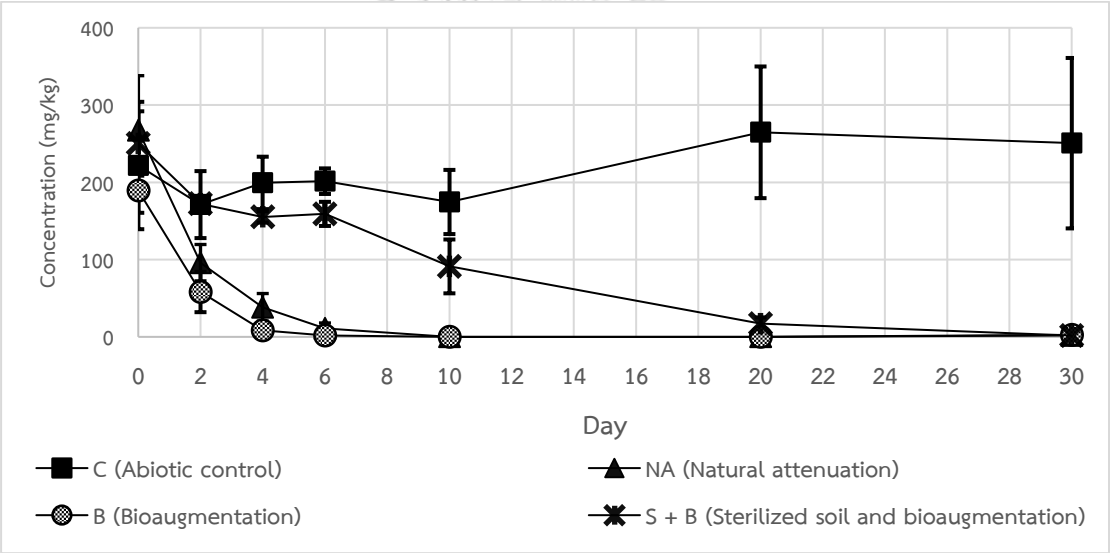


Figure 13 Residual DBP plot in agricultural soil microcosm

Based on Figure 13, DBP was completely degraded within four days in NA and B treatment, while it took 20 days for complete degradation in S+B treatment. A one-way ANOVA was performed to compare the effect of treatment variations (NA, B and S + B) on degradation percentage of 300 mg/kg DBP. A one-way ANOVA revealed that there was statistically significant difference in mean DBP degradation rate throughout sampling time between at least two groups ($F(2,86) = [19.57]$, $p<0.000$). Tukey's HSD Test for multiple comparisons found that the mean value of DBP degradation percentage was

significantly different between NA and S + B (p <0.000, 95% C.I = [20.53, 55.57]) and between B and S + B (p <0.000, 95% C.I = [23.53, 58.28]). There was no statistically significant difference in mean DBP degradation rate between B and NA (p= 0.920). DBP degradation percentage bar plot and results for statistical analysis for each sampling day is shown in Figure 14.

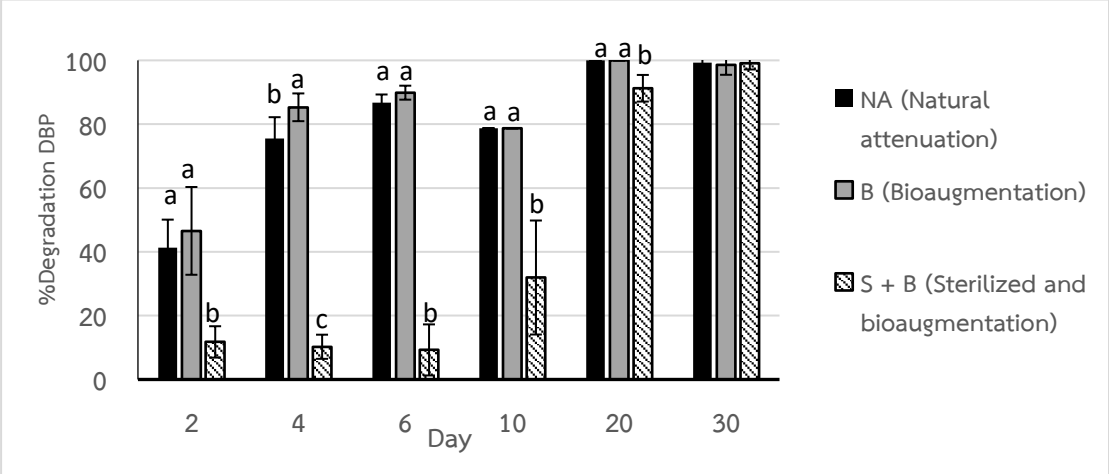


Figure 14 DBP degradation percentage in agricultural soil microcosm

DBP degradation on NA soil shown in Figure 13 and 14 indicates that indigenous bacteria of agricultural soil used in this study could degrade DBP. The soil was obtained from Durian Farm in Rayong Province with a pesticide application record. Chemicals used included glufosinate, cypermethrin, lambda-cyhalothrin, imidazole and glyphosate, with application frequency of up to four times a year. A report on plastic product applications such as PVC pipe or plastic mulch in the agricultural soil source was not provided. However, DBP, besides its application as a plasticizer, is commonly used as a fixative and lubricant (Stanley et al., 2003). One publication also reported the coexistence of PAEs and organochlorine pesticides (OCPs) in agricultural soil and both of these chemical groups are identified as endocrine disruptor compounds (EDCs) (Sun et al., 2016). Therefore, the indigenous soil community likely contains PAE-degraders due to prior exposure to PAEs.

However, the DBP degradation rate was insignificant in bioaugmentation treatment and natural attenuation. Instead, a significant reduction in the DBP degradation

rate was observed in sterilized soil where the indigenous community was expected to be absent. This observation contradicted the hypothesis that upon bioaugmentation with efficient PAE-degrading bacterial consortium, DBP degradation and PAE in general would be accelerated.

At the same time, DEHP was degraded at a slower rate and not completely removed during the soil microcosm study, as shown in the residual plot in Figure 15 and the bar plot for degradation percentage in Figure 16. Based on the average degradation percentage, DEHP was degraded at a higher percentage in NS+B, followed by S+B and NA. A one-way ANOVA was performed to compare the effect of treatment variations in DEHP degradation, revealing no statistically significant difference between the three groups.

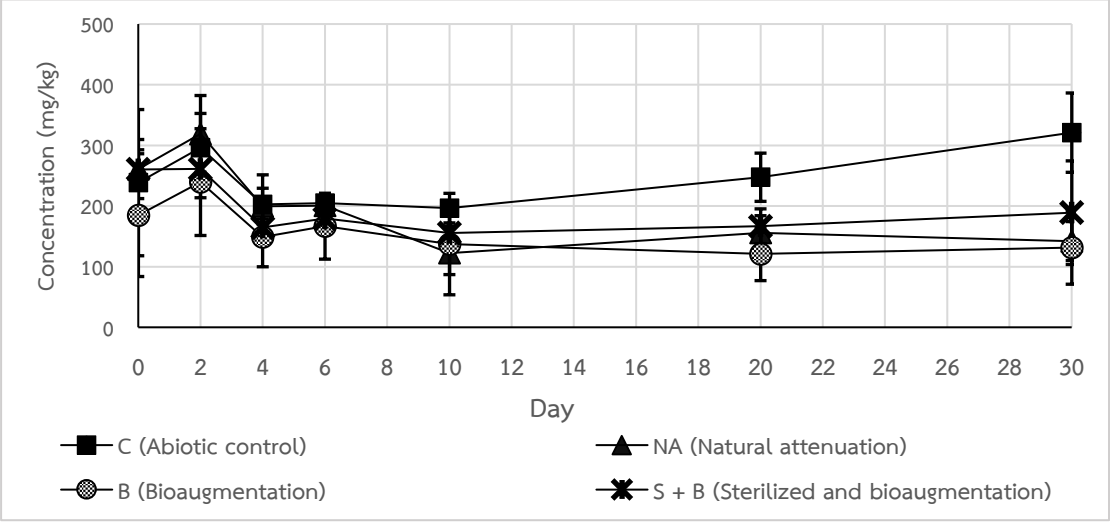


Figure 15 Residual DEHP plot in agricultural soil microcosm

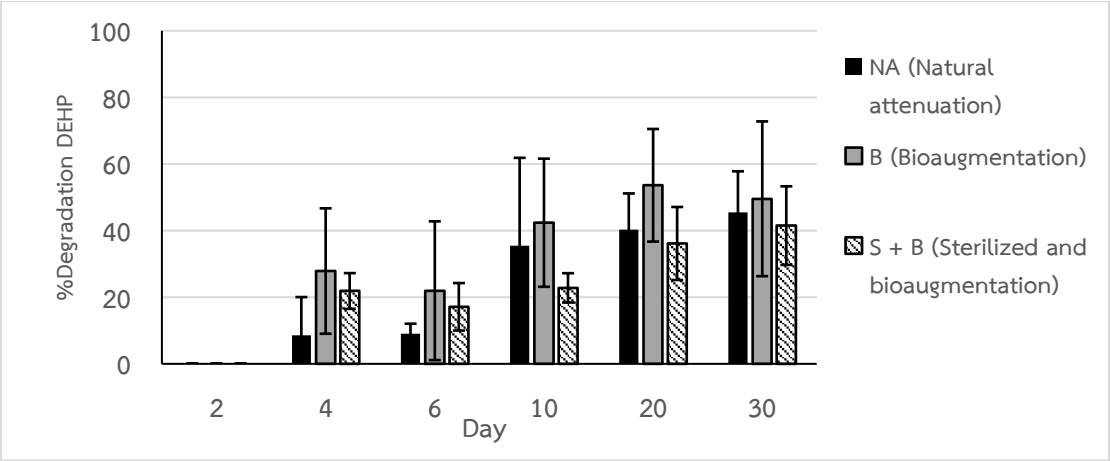


Figure 16 DEHP degradation percentage in agricultural soil microcosm

In addition to residual PAE, total heterotrophic and PAE-degrading bacteria were enumerated for each treatment in every sampling day using most-probable number methodology. Result on PAE-degrading bacteria enumeration is shown in Figure 17.

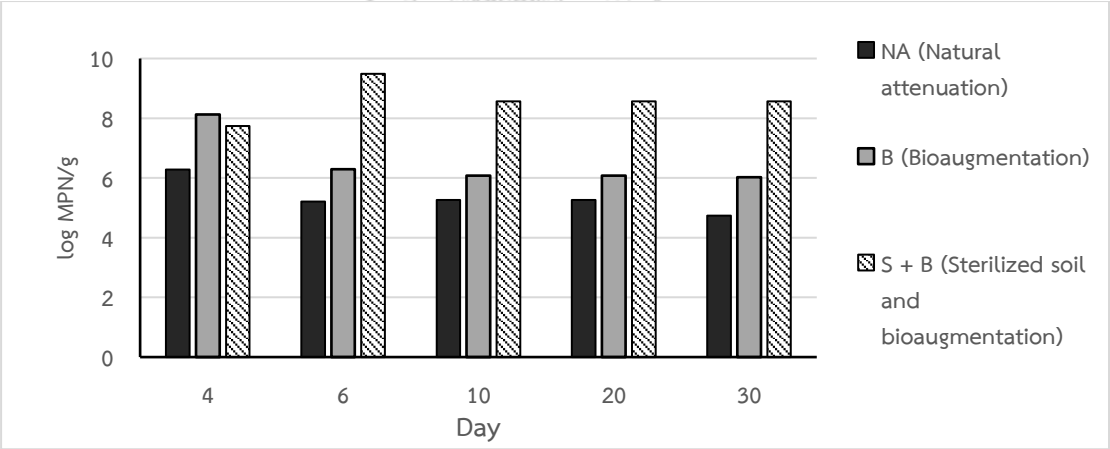
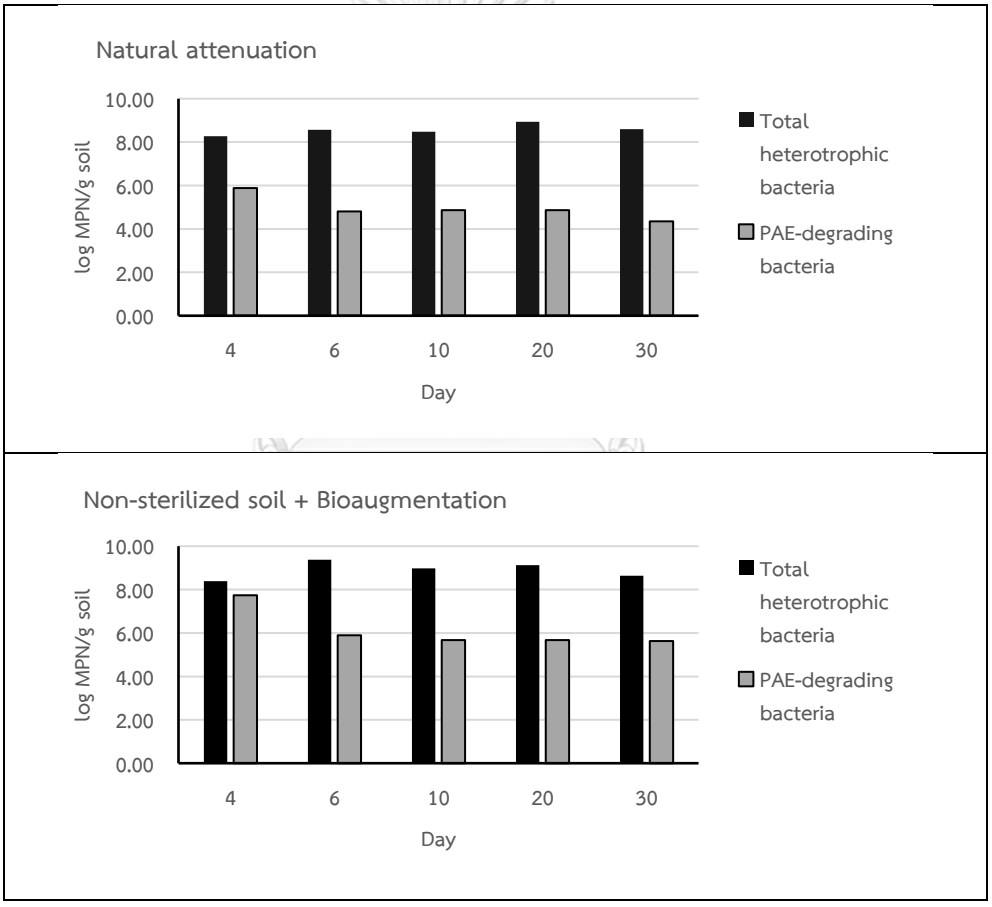


Figure 17 Enumerated PAE-degrading bacteria in agricultural soil microcosm study

In brief, a higher proportion of PAE-degrading bacteria was observed in sterilized soil inoculated with natural consortia LF-NK-DEHP than in non-sterilized soil without consortia addition along with natural attenuation soil. A one-way ANOVA was conducted, and there was a statistically significant difference in mean PAE-degrading bacteria between at least two groups ($F(2,12)=[26.42]$, $p<0.000$). Tukey's HSD Test for multiple

comparisons found that the mean value of enumerated PAE-degrading bacteria was significantly different between NA and S +B ($p < 0.000$, 95% C.I = [-4.43, -2.03]); and between B and S+B ($p = 0.002$, 95% C.I = [-3.26,-0.86]). There was no statistically significant difference between B and NA ($p = 0.057$). A one-way ANOVA also revealed no significant difference in mean total heterotrophic bacteria between all treatments ($p=0.136$). The bar plot of each pair of enumerated PAE-degrading bacteria and total heterotrophic bacteria on different sampling days for each treatment is shown in Figure 18.



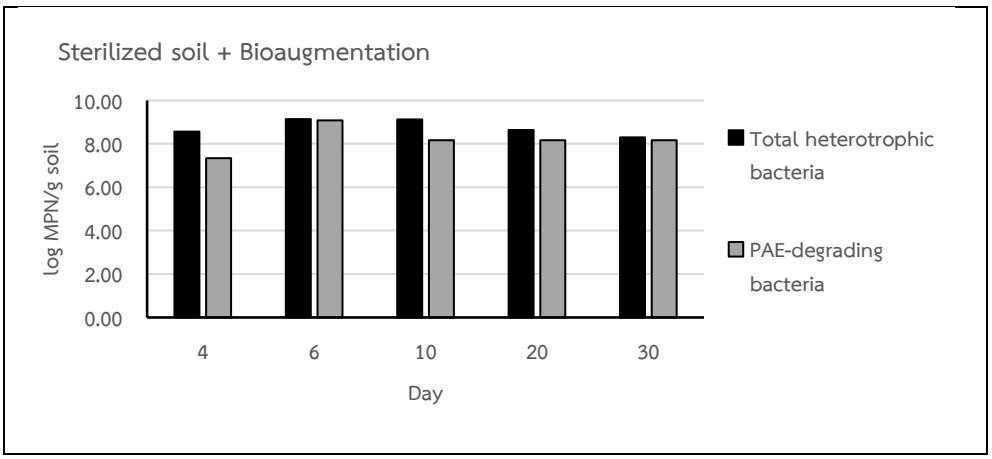


Figure 18 Proportion of total heterotrophic bacteria and PAE-degrading bacteria in each treatment throughout incubation period of soil microcosm

This experiment aimed to evaluate the biodegradation capacity of exogenous consortia in agricultural soil on a microcosm scale. An exogenous consortium from landfill soil was selected because landfill is one of the most highly PAE-polluted environments (Hu et al., 2021). Exogenous bacterial consortia enriched from landfill soil are likely to have specific and efficient catabolic activities for PAE biodegradation in comparison to native agricultural soil microbial community, where PAE occur at a lower concentration. On the other hand, there are several drawbacks to enriched consortia application. Apart from the problem with activity maintenance, applying naturally enriched consortia may risk exposure to pathogenic bacteria, which can harm the environment. Aside from pathogenicity risk, enriched consortia have one advantage over pure isolates when utilized in bioaugmentation. The enriched bacterial consortium is more robust than pure isolates. This property is important since the survivability of introduced bacteria generally correlates with efficient bioaugmentation performance.

In this experiment, the soil was sterilized before inoculation to study the exogenous consortium activity in the absence of indigenous microbes. The sterilization procedure chosen was moist heat sterilization or autoclave. Meanwhile, autoclave sterilization can alter soil physicochemical properties, affecting bioaugmentation performance. One of the noteworthy changes is the increase in extractable Mn levels from autoclaving soil. Other nutrients include increased extractable N (particularly NH_4-N and NO_3-N) P, S, and organic matter. Moist heat sterilization does not usually affect cation exchange capacity,

surface area and pH (Wolf & Skipper, 1994). However, increased micronutrient solubility in autoclaved soil, can be toxic for microbes. It was reported that Mn toxicity frequently occurs on autoclaved acid soil (Boyd, 1971).

Changes in soil chemical properties likely to affect bacteria activity in treatment with sterilization and exogenous consortia addition. However, these changes did not necessarily reduce LF-NK-DEHP colonization since enumerated PAE-degrading bacteria were observed in higher proportions in sterilized soil (Figure 17). Instead, higher micronutrients inhibited metabolic activity like PAE degradation, and lagging DBP degradation was observed in sterilized soil during initial 10 days of incubation.

On non-sterilized soil, changes in soil chemical properties did not occur, and an indigenous microbial community was present. It is yet to be investigated whether there is a competition between indigenous and exogenous PAE-degrading bacteria since the enumeration method used in this study cannot differentiate the two groups of bacteria. However, it should be noted that despite being statistically insignificant, there was a slightly higher DEHP degradation percentage in non-sterilized bioaugmentation soil. There was also a higher proportion of PAE-degraders in bioaugmentation soil on the fourth day of incubation (Figure 17 and Figure 18) and a slight decrease in the following sampling time. This observation indicated a slight reduction in the exogenous bacteria population in the non-sterilized soil. Nonetheless, the remaining population of LF-NK-DEHP is still active and degraded DEHP and indigenous PAE-degraders. Furthermore, exogenous bacteria could persist in the soil system during 30 days of incubation, both in soil with and without sterilization. This observation indicated the robustness of the enriched consortium.

Additionally, prior to soil microcosm experiment, soil physicochemical properties analysis was conducted. The analyzed physicochemical properties of soil used in this study is shown in Table 12.

Table 12 Agricultural soil physicochemical properties

Physicochemical properties		**Value**
pH[1]	pH value	5.64
	pH level	Slightly acid
$CaCO_3$ requirement[2]	kg $CaCO_3$/ Rai	403
Soil particles (%)[3]	Sand	65
	Silt	22
	Clay	13
Soil texture		Sandy loam
Organic matter[4]	g/kg	27
	level	Moderate
Phosphorus[5]	mg/kg	248
	level	Very high
Potassium[6]	mg/kg	96
	level	High
Calcium[7]	mg/kg	517
	level	Moderate
Magnesium[7]	mg/kg	76
	level	Moderate
EC 1:5	dS/m	0.07
OC	%	1.56
Total N	g/kg	0.91
Total P	g/kg	0.29
Total K	g/kg	0.40
AWCA	%	8.0

Based on soil properties analysis, soil used in the microcosm study was considered as slightly acid soil (pH 5.64). Generally, alkaline, or neutral soil pH enhances biodegradation, while acidic environments pose limitations to biodegradation. Usually, pH values between 6.5 to 8.0 are considered optimum for oil degradation. This was associated with highest bacterial population (Neina, 2019). In addition to soil pH information, information of calcium carbonate requirement to neutralize soil pH was also provided. Using this information, $CaCO_3$ was added accordingly in the following soil microcosm experiment.

4.4.2. Phytotoxicity of phthalate ester

In this study, phytotoxicity test was conducted after microcosm experiment. The objective was to observe the effect of bioaugmentation, in contrast to natural attenuation, on reducing PAE toxicity to selected plant (T.-T. Ma et al., 2014). It was hypothesized that removing PAE from soil would alleviate toxicity of PAE to germinating seeds. Observed variables included root length, shoot length, fresh biomass weight and germination index of germinating mung bean seedlings.

One-way ANOVA was conducted for four variables observed in phytotoxicity study (fresh weight, shoot and root length and germination idea), to compare the effect of three treatments. In addition, two controls (positive control, sterile distilled water and negative control, abiotic control soil in microcosm experiment). A one-way ANOVA revealed that there was a statistically significant difference in fresh biomass weight ($F(4,111) = [7.117]$, $p<0.000$), shoot length ($F(4,111) = [9.077]$, $p<0.000$) and germination index ($F(4,111) = [29.223]$, $p<0.000$), between at least four groups. The summarization of Post-hoc tests results is shown in Table 13.

*Table 13 Phytotoxicity test on mung bean seed using agricultural soil post-microcosm study**

Treatment	Fresh weight (g)	Shoot length (cm)	Germination index
Positive control (DIW sterile)	$0.329^{a} \pm 0.07$	$5.450^{a} \pm 1.58$	1^{a}
Negative control (Abiotic control)	$0.305^{a,b} \pm 0.02$	$4.671^{a,b} \pm 0.77$	$1.20^{a} \pm 0.19$
Natural attenuation	$0.257^{b} \pm 0.01$	$3.5^{b,c} \pm 0.64$	$0.755^{b} \pm 0.3$
Bioaugmentation	$0.258^{b} \pm 0.02$	$3.237^{c} \pm 1.20$	$0.665^{b} \pm 0.14$
Sterilized + Bioaugmentation	$0.322^{a} \pm 0.02$	$5.322^{a} \pm 0.67$	$1.205^{a} \pm 0.05$

*Alphabets indicated significant difference at 95% confidence interval

In Table 13, average fresh weight, shoot length and germination index of mung bean in natural attenuation and bioaugmentation were lower than positive control. Based on this observation, it can be concluded that natural attenuation and bioaugmentation increase toxicity of PAE, instead of reducing PAE toxicity.

Previously, the toxic effect of DBP and DEHP was investigated on germinating rape (*Brassica chinensis* L.) seeds (Ma et al., 2013). Comparing PAE individual toxicity showed that reduction in germination parameters was more apparent in DBP. In general, shorter-alkyl PAEs was observed to be more phytotoxic than PAE with long alkyl ester (C8-C13) because shorter alkyl ester enables plant for a higher uptake of PAE and subsequent distribution to other parts of the plant.

One important characteristic of biotic degradation is the chain of enzymatic reactions that convert pollutants of interest into metabolites, which are further mineralized into carbon dioxide and water. Similar to PAE, several intermediates of PAE catabolism induced phytotoxicity in the plant. Among these metabolites are the mono-alkyl phthalate ester and phthalic acid (Sun et al., 2015). The phytotoxicity of PAE metabolites tends to be more apparent since these molecules are much lighter than PAE molecules.

The phenomenon that occurs both in natural attenuation and bioaugmentation is biotic degradation. Through biodegradation by either indigenous or exogenous PAE-degraders, shorter alkyl ester PAEs, mono-alkyl PAEs, and phthalic acid were added into the soil. Accumulation of the toxic effect of phthalate metabolites likely gives higher phytotoxicity than individual PAE in abiotic control. However, in this study, metabolites of PAE were not extracted and analyzed. Therefore, further investigation is required to prove this hypothesis.

Lower phytotoxicity was also observed in sterilized soil inoculated with enriched consortia. Although PAE biodegradation also occurred in this treatment, labile macronutrients and micronutrients were in higher concentration due to initial soil sterilization. Thus, it is likely that readily available nutrients alleviated the toxicity of PAE and PAE metabolites. However, this assumption also requires further investigation, which can be done by initially investigating the effect of soil sterilization on the germination of the selected plant model.

4.5. Fertilizer addition soil microcosm experiment

4.5.1. Phthalate ester degradation under fertilizer addition

Following the first soil microcosm experiment, the second soil microcosm experiment was conducted to observe the effect of fertilizer addition in the bioaugmentation of exogenous enriched consortia. Similarly, observed variables included DBP and DEHP degradation, total heterotrophic and PAE-degrading bacteria, and phytotoxicity. In this study, along with fertilizer, calcium carbonate was added to the soil to increase soil pH. In addition, the author reduced the concentration of PAE used in this study to 200 mg/kg. Based on the first microcosm study, bioaugmentation did not wholly remove DEHP in all treatments. We suspected that DEHP supplementation was considered high for both soil bacteria and added consortium, and there was low degradation. Lower DEHP concentration was expected to induce degradation instead of inhibiting.

As a control to fertilizer addition, treatment without fertilizer addition was prepared for both natural attenuation and bioaugmentation. However, for treatment with initial soil sterilization, treatment without fertilizer addition was not prepared because the result in the first microcosm study revealed that the bacterial consortium exhibited a lag phase during the incubation period. In total, there were five treatments, excluding abiotic control. DBP degradation plot is shown in Figure 19.

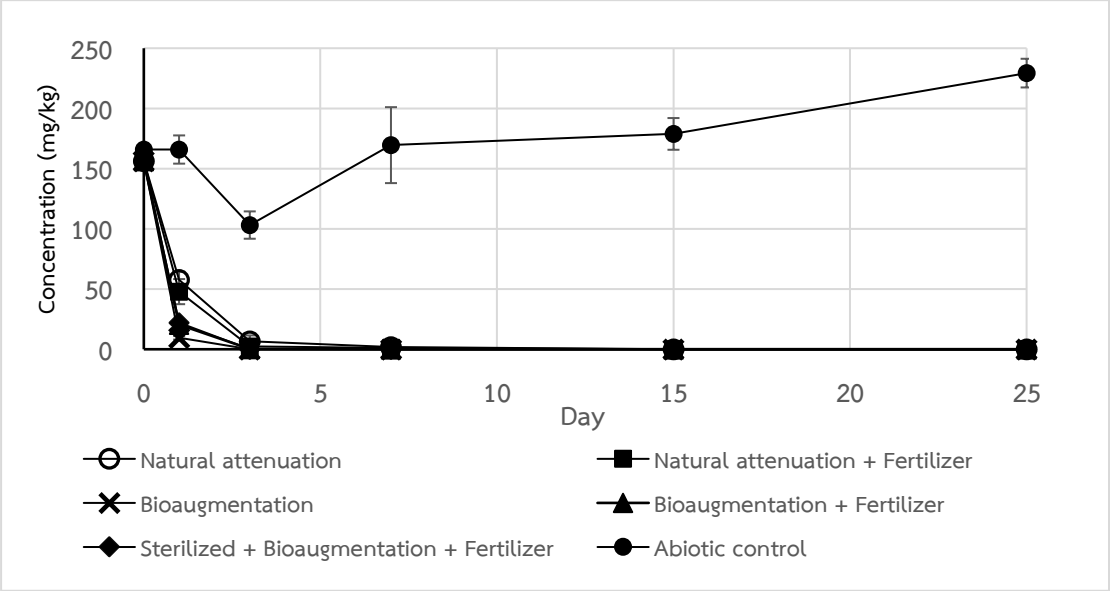


Figure 19 Residual DBP plot in fertilizer addition soil microcosm experiment

Based on degradation plot in Figure 19, DBP was completely degraded in all treatments within three days. To further analyze the difference between treatment variation, ANOVA One-way statistical analysis and Post-hoc Tukey's test was conducted on degradation percentage data. A one-way ANOVA revealed that there was no statistically significant difference in mean DBP degradation rate ($p = 0.746$) for all treatment across sampling day variation. Only during initial sampling on Day 1, there were significant mean difference which is summarized in Figure 20.

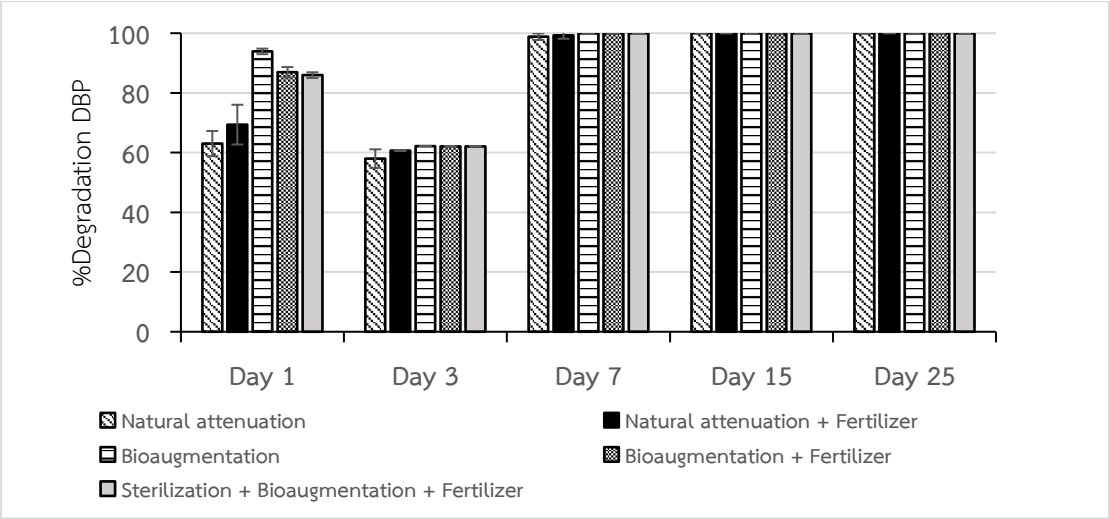


Figure 20 DBP degradation percentage in fertilizer addition soil microcosm experiment

Based on Figure 20, microbes in all treatments could degrade more than half of the initial DBP addition within one day of incubation. A higher mean of DBP degradation was observed in treatment with inoculation of exogenous consortia. However, extracted DBP in abiotic control for Day 3 was significantly reduced; therefore, abiotic DBP removal affected the biotic degradation percentage in all treatments.

Compared to the first soil microcosm study (Figure 13), a notable difference was observed in sterilized soil treatment. With fertilizer addition, the exogenous bacterial consortium degraded DBP at the same rate with bioaugmentation treatment, both with and without fertilizer addition. Meanwhile, in the first microcosm study (Figure 14), rapid DBP degradation was observed on Day 20 for exogenous consortia.

Similar with agricultural soil microcosm study, DEHP degradation was slower than DBP and could not be completely removed within 25 days. However, more DEHP was degraded in the fertilizer addition microcosm study compared to previous study. Residual DEHP plot is shown in Figure 21.

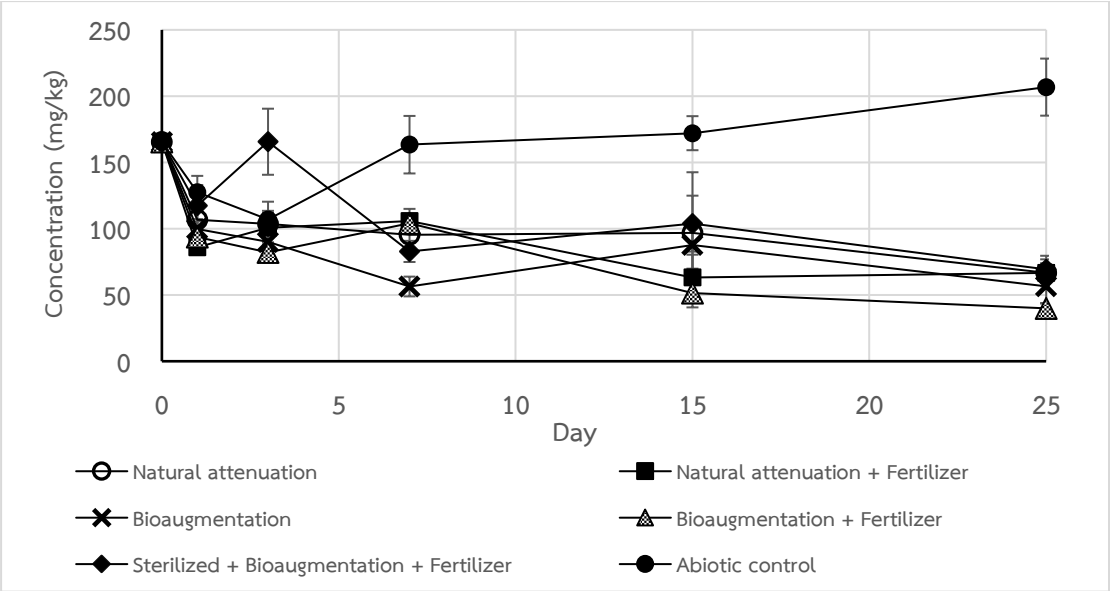


Figure 21 Residual DEHP plot in fertilizer addition soil microcosm experiment

A one-way ANOVA also revealed that there was statistically significant difference in mean DEHP degradation rate ($p = 0.287$) on Day 1, Day 7, and Day 25. Result for Post-hoc test and degradation percentage in Figure 22.

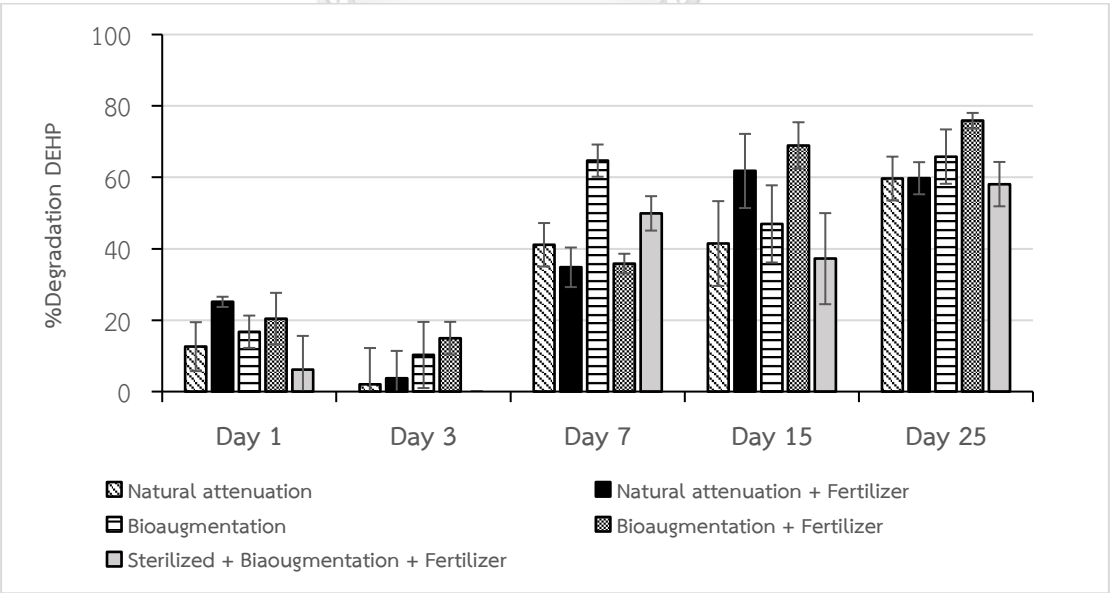


Figure 22 DEHP degradation percentage in fertilizer addition soil microcosm experiment

For DEHP degradation, the treatment with the highest degradation varies for every sampling day. Initially, natural attenuation added with fertilizer showed high degradation, followed by bioaugmentation on Day 7 and finally, higher degradation on the last sampling day by bioaugmentation added with fertilizer. However, in bioaugmentation on non-sterilized soil added with fertilizer, an incremental increase in DEHP removal was observed throughout incubation. Eventually, the DEHP degradation percentage reached 75.9 ± 2.17% for an initial 200 mg/kg concentration. The rate of DEHP degradation was significantly higher than the natural attenuation treatment, both with and without fertilizer addition.

In this study, growth of biodegradation agent was monitored through enumeration with most-probable number method, using PAE-supplemented growth medium. The proportion of PAE-degrading bacteria between treatment with and without fertilizer addition is shown in Figure 23.

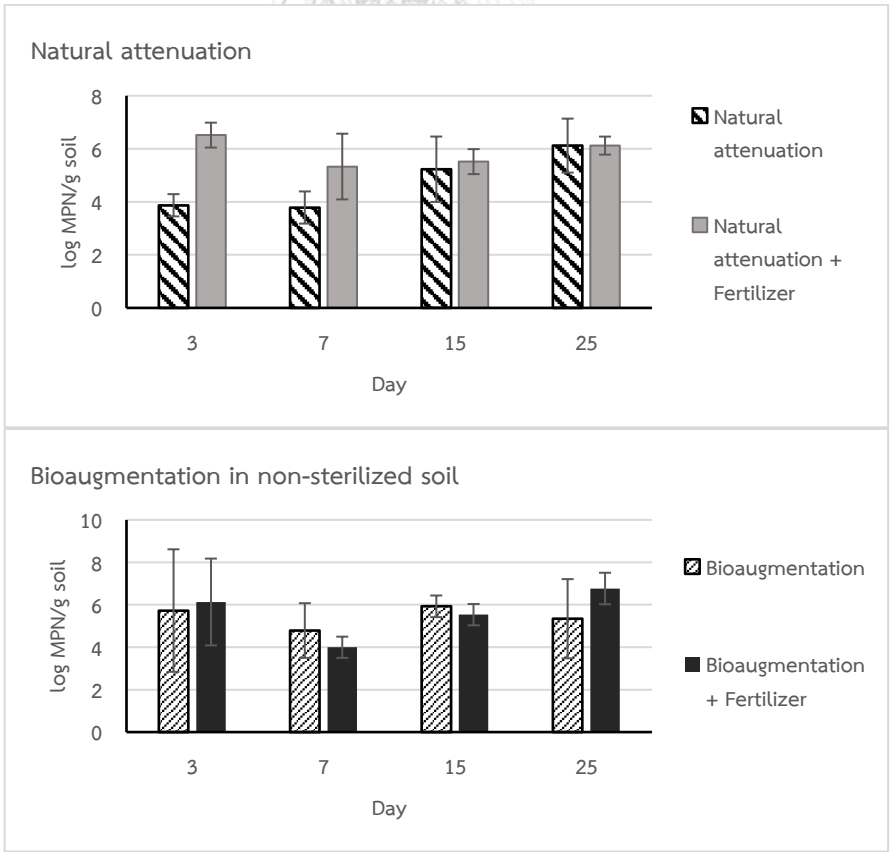


Figure 23 PAE-degrading bacteria in soil amended with fertilizer and without fertilizer

Based on Figure 23, the number of PAE-degrading bacteria fluctuated throughout sampling time without increasing or decreasing the trend corresponding to PAE residual concentration. However, an increasing pattern was observed from Day 7 to Day 25 in natural attenuation added with fertilizer treatment and bioaugmentation in non-sterilized soil added with fertilizer treatment. A significant increase was observed mainly on Day 25 for Bioaugmentation added with fertilizer, where the number of enumerated PAE degraders was up to 6.77 log MPN/g soil (at the highest DEHP degradation rate, 75.87 ± 2.2%).

How fertilizer addition positively affects the bioaugmentation performance of exogenous consortia might be linked to the provision of organic carbon and nutrients in the fertilizer. Since the fertilizer used in this experiment consists of organic and inorganic materials, additional C sources can enhance the growth and degradation ability of consortia, which was the case for strain 2D in compost-amended soil (Zhao et al., 2015).

Based on the observation in the previous microcosm study, moist heat sterilization or autoclave can increase the concentration of nutrients in the soil since heat can increase the solubility of heat-labile micro- and macronutrients. Sterilization made the additional nutrient became more abundant in soil amended with fertilizer. Therefore, in treatment where the soil was initially added with fertilizer and sterilized, then inoculated with exogenous consortia, we observed significant improvement in DBP degradation (Figure 13). Eventually, the biodegradation rate was similar to the rate of bioaugmentation in the presence of indigenous microbes. Based on this observation, we can conclude that the exogenous bacterial consortium used in this study displayed a lag phase due to its incapability to directly utilize macronutrients in the soil system and hence, incapable of carrying out the PAE metabolism.

4.5.2. Phytotoxicity of phthalate ester with fertilizer addition

Similar to the previous phytotoxicity study in the first soil microcosm experiment, the effect of fertilizer addition to phthalate ester phytotoxicity was also investigated. While fertilizer, in general, was applied to induce plant growth, it is interesting to gain information on how the presence of fertilizer in PAE-contaminated soil affects PAE's inherent toxic effect. This study used two plants as monocot and dicot seed

representatives, including Chinese convolvulus (*Ipomoea aquatica*) and corn (*Zea mays*). One-way ANOVA analysis revealed that significant differences based on one-way ANOVA analysis were observed only on convolvulus' root length (F (6,12) = [4.619], p=0.012).

Tukey's HSD Test for multiple comparisons found that the mean value of root length was significantly different between water control and natural attenuation (p=0.006, 95% C.I = [0.6618, 4.3216]). Furthermore, the mean root length of water control was also significantly different with natural attenuation added with fertilizer (p=0.001, 95% C.I = [1.2590, 4.9188]); and lastly, between water control and bioaugmentation to the soil added with fertilizer (p=0.018, 95% C.I = [0.311, 3.971]). The summary of Post-hoc test is shown in Table 14.

*Table 14 Phytotoxicity test on convolvulus seed using soil post- fertilizer addition microcosm study**

Treatment	**Root length (cm)**
Positive control (DIW sterile)	$4.6^{a} \pm 0.56$
Negative control (Abiotic control)	$3^{ab} \pm 0.36$
Natural attenuation	$2.108^{b} \pm 0.38$
Natural attenuation + Fertilizer	$1.511^{b} \pm 0.73$
Bioaugmentation	$1.85^{b} \pm 0.85$
Bioaugmentation + Fertilizer	$3.117^{ab} \pm 0.47$
Sterilized + Bioaugmentation + Fertilizer	$2.459^{b} \pm 0.42$

*Alphabets indicated significant difference at 95% confidence interval

Table 14 shows that all treatments other than bioaugmentation added with fertilizer and abiotic control showed a reduction in root length compared to the positive control. On the other hand, corn seed did not show inhibited germination, which may indicate the plant's robustness toward PAE phytotoxicity. In a previous report, phytotoxicity of PAE in corn plants became observable at the DBP range of 2000 mg kg-1 (Shea et al.,

1982). However, this robustness becomes problematic because despite showing average environmental growth, PAE uptake and storage by crop plants still occur.

Previously in the first microcosm study, three parameters were significantly reduced in natural attenuation and bioaugmentation, including fresh biomass weight, shoot length and germination index. In the second experiment, only one parameter was affected, root length. Reduction in root length as the effect of PAE toxicity is commonly observed since part of the plant first in contact with PAE is the root (Deng et al., 2017). In the first phytotoxicity study, it was argued that biological degradation in natural attenuation and bioaugmentation treatment produced intermediates which also have a phytotoxic effect. Since intermediates including shorter alkyl PAE, mono-alkyl PAE, and phthalic acid are lighter, these molecules are easily absorbed and transmitted in the plant body and, henceforth, give higher, more apparent toxicity in comparison to long-alkyl esters PAE like DEHP.

Similarly, in the second microcosm study, all treatments other than bioaugmentation with fertilizer addition and abiotic control had lower mean root length values than sterilized DIW. It was uniquely observed in natural attenuation soil (Natural Attenuation + Fertilizer) and sterilized soil added with bacteria (Sterilization + Bioaugmentation + Fertilizer). Fertilizer was supposed to affect plant growth positively, starting from the germination phase. However, it is likely that soil from these two treatments still contained the remaining PAE and its metabolites. Therefore, the phytotoxic effect is expected to become more apparent than fertilizer's positive effect.

Based on the PAE degradation plot in Figure 19 and Figure 21, we observed that biotic degradation occurred in all treatments. Furthermore, both indigenous microbial communities in natural attenuation or exogenous consortium LF-NK-DEHP in bioaugmentation could completely degrade DBP and, in general, half of DEHP. Therefore, PAE intermediates were produced in all treatments and had the potential to exhibit phytotoxicity in the following study.

However, an exception was observed in bioaugmentation added with fertilizer treatment. DBP was degraded in bioaugmentation with fertilizer addition, while DEHP

degraded by up to 75.9%, the highest percentage among all treatments. Through this observation, we concluded that fertilizer addition in unsterilized soil inoculated with exogenous bacteria ensured the degradation of phthalate and its intermediates, which alleviated the phytotoxicity of PAE. Fertilizer addition likely provides additional nutrients which enable bacteria, both indigenous and exogenous, to degrade PAE intermediates. With a reduction in PAE and PAE's intermediates' toxic effect, the positive impact of fertilizer added to the seed's germination can be observed, as seen in Table 14.

CHAPTER V
CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Phthalate esters (PAEs) are group of chemicals that have become priority pollutants due to its high abundance and toxicity. Reports on PAE occurrence in agricultural soil have become one of concerning issue, since PAE exposure to agricultural product increase risk of PAE exposure to human body. This research proposed bioaugmentation of exogenous bacterial consortium, originated from landfill soil, as solution for this emerging issue.

Initially, the enriched consortia were characterized for its initial activity in mixed PAE, kinetics of PAE mixture biodegradation and community dynamics. LF-NK-DEHP, previously enriched in DEHP, was observed to retain a high degradation capacity for DBP and DEHP as a mixture and single substrate. LF-NK-DEHP could simultaneously degrade 92.9±1.8% 100 mg l^{-1} DBP and 63.4±9.8% 100 mg l^{-1} DEHP within seven days.

Based on kinetic study, it was obtained that DBP degradation kinetic by LF-NK-DEHP was fitted with a modified Gompertz model (R^2 = 0.92, $t_{1/2}$ = 1.49 days, initial concentration 500 mg l^{-1} DBP), while DEHP degradation fit with the zero-order kinetic model (R^2 = 0.94, $t_{1/2}$ = 7.23 days, initial concentration 500 mg l^{-1} DEHP). Using relative abundance data paired with residual PAE concentration, correlation analyses predicted that *Rhodococcus, Microbacterium* and *Pigmentiphaga* as key degraders within consortium LF-NK-DEHP. Furthermore*, Rhodococcus* was predicted to be involved in DEHP degradation and *Microbacterium* for DBP degradation. *Pigmentiphaga* was significantly correlated with both DBP and DEHP degradation.

In the second phase of this work, bacterial consortium LF-NK-DEHP was evaluated for its activity in agricultural soil microcosm. Based on the soil microcosm study, LF-NK-DEHP inoculation in soil did not significantly accelerate the rate of PAE mixture removal. Furthermore, in treatment with initial soil sterilization followed by exogenous consortium inoculation, DBP was degraded at a slower rate. Hence, the initial soil microcosm study did not recommend the direct application of the bacteria.

Furthermore, phytotoxicity study revealed that PAE intermediates gives higher toxicity than PAE itself. Therefore, incomplete PAE mineralization give more apparent toxic effect to plant, specifically on germination phase.

In the third research phase, soil amendment with commercial fertilizers became an additional variable in studying LF-NK-DEHP bioaugmentation performance. Fertilizer addition improved the rate of PAE biodegradation by the exogenous consortium, especially for DEHP degradation. The highest DEHP degradation percentage was observed in bioaugmentation with fertilizer addition. Therefore, fertilizer improved the biodegradation performance of the exogenous consortium by supplying additional nutrients. Fertilizer amendment could supply additional nutrients required by the exogenous consortium to mineralize PAE metabolism intermediates; hence, fertilizer addition alleviated the phytotoxic effect of PAE.

Through the result in the third phase of the microcosm study, it can also be concluded that there were PAE-degraders within the indigenous microbial community of agriculture. However, PAE-degraders did not ensure a community of microbes capable of complete PAE mineralization. The absence of several microbes with a metabolic capacity to degrade PAE intermediates in native microflora of microcosm soil used in this study was observed in the natural attenuation and fertilizer treatment. Fertilizer addition did not significantly improve the rate of PAE degradation in natural attenuation nor reduce the phytotoxicity. Therefore, optimal degradation can be achieved by combining the activities of the exogenous bacterial consortium, indigenous microbes, and fertilizer.

Based on this study, we recommended using naturally enriched consortium as exogenous microbes for bioaugmentation. Enrichment cultures have robust and complex communities that can ensure the complete mineralization of PAE. This study indicates that obtaining a community capable of complete PAE mineralization is relatively more urgent than finding isolates with high PAE degradation efficiency yet incapable of intermediates metabolism. However, this study described several disadvantages of enriched consortium application, including possible loss of activity

during storage. Therefore, in this study, a protocol of continuous transfer was described to maintain the activity of the enriched consortium. Additionally, an issue on viable pathogenic bacteria within the consortium can be detected through the initial screening of pathogenic bacteria, either through culture-dependent or culture-independent methods. Therefore, further investigation on LF-NK-DEHP pathogenicity is highly required to determine this consortium's applicability.

Moreover, LF-NK-DEHP possessed the potential to be explored further in order to better understand the mechanism of PAE degradation by bacteria. *Pigmentiphaga*, in particular, was rarely reported to have PAE degradation activity and was predicted as the main degrader in this study. A future study might reveal novel PAE-degraders from the genus *Pigmentiphaga*.

5.2. Recommendations

In this research, a kinetic study was conducted as part of enriched consortium characterization before the soil microcosm experiment. Future research on bioaugmentation performance evaluation should refer to kinetic study results for designing bioaugmentation experiments. Therefore, based on the kinetic study in this project, it is advisable to increase the input of DBP and DEHP up to 500 mg kg-1 for the rate of natural attenuation and bioaugmentation distinguishable. In this study, DBP supplementation is low enough for quick degradation by indigenous and exogenous bacteria.

In this study, fertilizer addition improved the bioaugmentation performance of exogenous bacteria. Fertilizer addition was based on a recommendation for the vegetable farm, in which formulation was optimized for improving soil productivity or plant growth. However, for bioremediation of phthalate ester, an adequate amount of fertilizer can be optimized based on the C:N:P ratio. A common approach for this has revolved around supplying adequate inorganic nitrogen and phosphorus, adjusting soil water content, and providing soil mixing for aeration. However, in a bigger scale of bioaugmentation, all of these aspects (fertilizer addition, water content, soil mixing,

microbial inoculum preparation) can be optimized for better bioaugmentation performance.

A phytotoxicity study revealed that the biotic degradation of PAE has to remove phthalate intermediates for the phytotoxicity effect altogether not to occur. Therefore, monitoring metabolite concentration throughout bioaugmentation performance is recommended in future studies. Time-bound metabolite profiles can be initially investigated in a liquid system, and a similar approach utilized in this study can detect key bacteria members involved in each pathway.

Moreover, the soil sample used in this study can be used as an environmental sample to obtain novel bacteria with PAE degradation capacity. Upon enrichment and subsequent isolation, this bacteria can also be used as an identifier to monitor the dynamics of indigenous and exogenous interaction during bioaugmentation of phthalate ester. Furthermore, fertilizer can be added as a variable in perspective. Therefore, future studies can rely on identified indigenous and exogenous degraders to study how bioaugmentation was improved through fertilizer addition in this study.

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APPENDIX A

MEDIA AND SOLUTION

Carbon-Free Mineral Medium (CFMM)

NH_4NO_3	3 g
$Na_2HPO_4.12H_2O$	5.6 g
KH_2PO_4	0.8 g

Dissolve all initial three components in 1000 ml distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C, 15 minutes.

$MgSO_4.7H_2O$	0.1 g
$FeCl_3.6H_2O$	0.05 g
$CaCl_2.2H_2O$	0.05 g

Dissolve separately as stock solution with higher concentration (50 g l^{-1} $MgSO_4.7H_2O$, 25 g l^{-1} $FeCl_3.6H_2O$, 25 g l^{-1} $CaCl_2.2H_2O$) in distilled water, then sterilized by filtration. Added to the autoclaved solution accordingly prior to use.

PAE stock solution 10,000 mg l^{-1}

PAE	10 mg
Dichloromethane	990 µl

Dissolve PAE in Dichloromethane then filtrate through 0.2 µm PTFE filter and stored in -20°C.

0.25X Tryptone Soy Broth

Tryptone soy broth	7.5 g

Dissolve in 1000 ml distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C, 15 minutes.

0.25X Tryptone Soy Agar

Tryptone soy broth	7.5 g
Agar	150 g

Dissolve in 1000 ml distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C, 15 minutes.

Resazurin solution

Resazurin	270 mg

Dissolve in 40 ml autoclaved distilled water, and prepared fresh before use.

NaCl 0.85% (w/v) solution

NaCl	8.5 g

Dissolve in 1000 ml distilled water, then sterilized by autoclave at 15 pound per square inch, 121°C, 15 minutes.

APPENDIX B

PHASE I SUPPLEMENTARY DATA

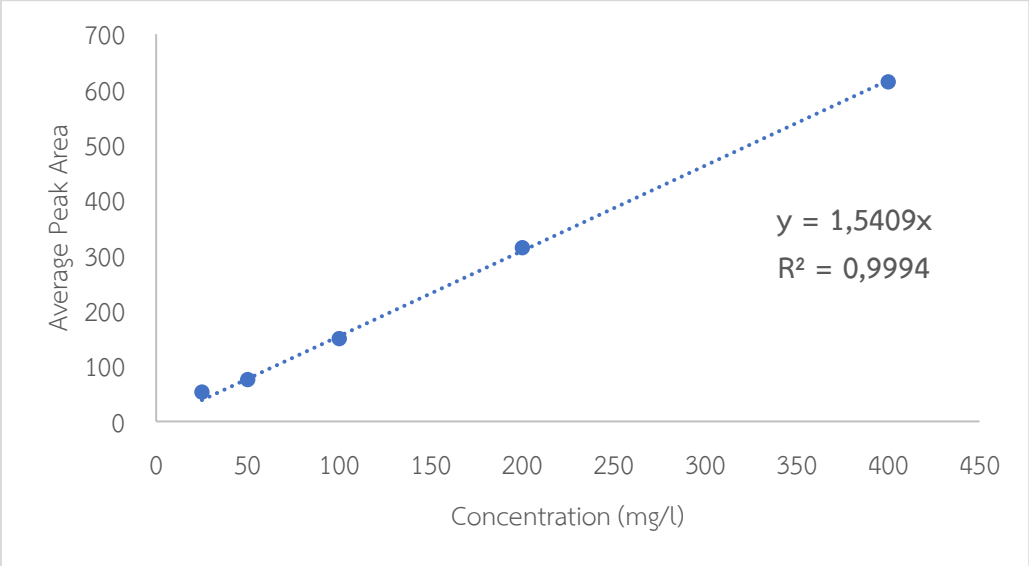

Figure 24 DEHP standard curve

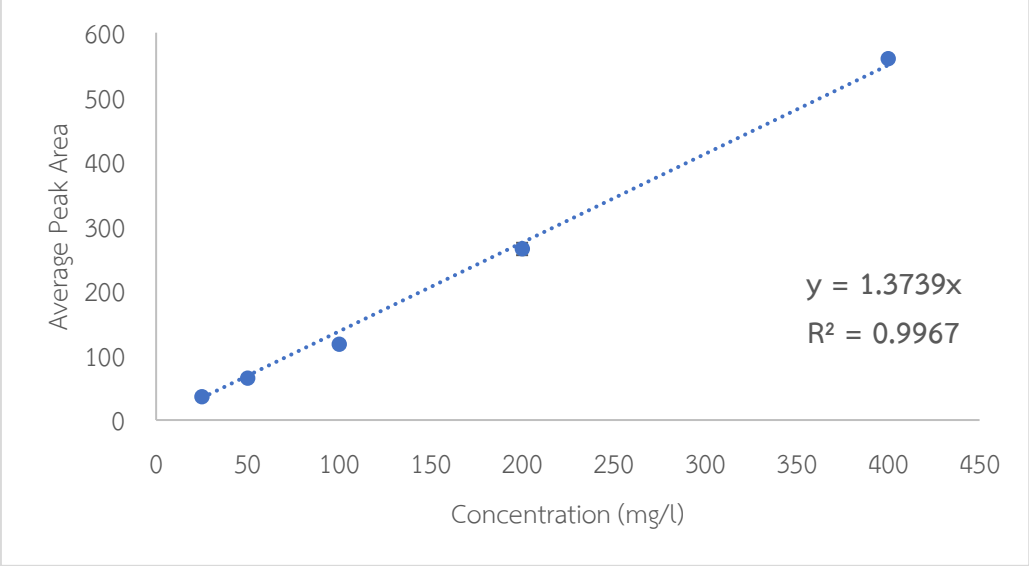

Figure 25 DBP standard curve

Table 15 Extraction recovery from liquid medium

Substrate	Initial concentration (mg l^{-1})	Extract concentration (mg l^{-1})	Average %Recovery (%)	Standard deviation
DBP	100	101.463	103.792	3.96
		107.378		
		101.536		
DEHP	100	107.924	99.66	8.37
		99.877		
		91.180		

Table 16 Residual PAE for selection of enriched consortia experiment

Substrate	Consortia	Residual concentration (mg l^{-1})	%Degradation	Average ± SD
100 mg l^{-1} DBP (single substrate)	LF-NK-DBP	83.267	2.653	29.9 ± 20.4
		58.883	29.052	
		32.171	57.972	
100 mg l^{-1} DEHP (single substrate)	LF-NK-DBP	20.962	69.958	54.1 ± 38.7
		10.189	82.312	
		73.269	9.973	
100 mg l^{-1} DBP (mixed substrate)	LF-NK-DBP	9.972	86.554	65.6 ± 19
		40.833	60.758	
		54.444	49.381	
100 mg l^{-1} DEHP (mixed substrate)	LF-NK-DBP	64.897	25.378	23.5 ± 1.9
		66.714	23.510	
		68.596	21.575	
100 mg l^{-1} DBP (single substrate)	LF-NK-DEHP	0.000	92.803	92.8 ± 0
		0.000	92.803	
		0.000	92.803	
	LF-NK-DEHP	28.230	61.622	63.1 ± 4.4
		29.918	59.687	

Substrate	Consortia	Residual concentration (mg l^{-1})	%Degradation	Average ± SD
100 mg l^{-1} DEHP (single substrate)		22.649	68.023	
100 mg l^{-1} DBP (mixed substrate)	LF-NK-DEHP	3.203	92.213	92.9 ± 1.8
		0.000	94.889	
		4.076	91.482	
100 mg l^{-1} DEHP (mixed substrate)	LF-NK-DEHP	20.637	70.885	63.4 ± 9.8
		24.336	67.082	
		38.679	52.335	

Table 17 Viable bacteria count for enriched consortia selection experiment

Consortia	Substrate	D0 bacteria count (log CFU ml^{-1})	D7 bacteria count (log CFU ml^{-1})
LF-NK-DBP	100 mg l^{-1} DBP	6.70 ± 0.14	7.06± 0.14
	100 mg l^{-1} DEHP		6.76± 0.22
	100 mg l^{-1} DBP + 100 mg l^{-1} DEHP		7.01± 0.09
LF-NK-DEHP	100 mg l^{-1} DBP	7.00 ± 0.11	7.18± 0.04
	100 mg l^{-1} DEHP		6.89± 0.02
	100 mg l^{-1} DBP + 100 mg l^{-1} DEHP		7.29± 0.10

Table 18 Degradation percentage of exogenous consortia throughout continuous transfer

Transfer	Sample	DBP (mg l^{-1})	%Degradation DBP	DEHP (mg l^{-1})	%Degradation DEHP
1st	Ctrl D7 (1)	107.868	0.447	179.830	0
	Ctrl D7 (2)	109.469		189.954	
	Ctrl D7 (3)	107.140		184.827	
	Ctrl D0 (1)	109.324		194.691	
	Ctrl D0 (2)	108.669		174.249	
	Ctrl D0 (3)	107.941		185.671	
	D7 (1)	0.000	99.553	8.631	95.331
	D7 (2)	0.000	99.553	9.280	94.98
	D7 (3)	0.000	99.553	5.906	96.806
2nd	Ctrl D7 (1)	84.286	1.188	88.065	2.327
	Ctrl D7 (2)	107.650		102.278	
	Ctrl D7 (3)	92.510		84.821	
	Ctrl D0 (1)	91.200		86.443	
	Ctrl D0 (2)	89.745		94.360	
	Ctrl D0 (3)	106.922		100.915	
	D7 (1)	0.000	98.812	0.000	97.673
	D7 (2)	0.000	98.812	0.000	97.673
	D7 (3)	0.000	98.812	0.000	97.673
3rd	Ctrl D7 (1)	97.533	1.506	179.830	3.64
	Ctrl D7 (2)	109.469		189.954	
	Ctrl D7 (3)	107.140		164.579	
	Ctrl D0 (1)	106.704		183.464	
	Ctrl D0 (2)	112.381		186.190	
	Ctrl D0 (3)	99.862		184.892	
	D7 (1)	0.000	98.494	25.310	82.668
	D7 (2)	0.000	98.494	7.139	92.449
	D7 (3)	0.000	98.494	13.758	88.917
4th	Ctrl D7 (1)	129.704	16.910	96.827	0.714

Transfer	Sample	DBP (mg l^{-1})	%Degradation DBP	DEHP (mg l^{-1})	%Degradation DEHP
	Ctrl D7 (2)	102.191		110.066	
	Ctrl D7 (3)	107.140		144.980	
	Ctrl D0 (1)	141.495		125.316	
	Ctrl D0 (2)	131.159		105.393	
	Ctrl D0 (3)	135.381		123.694	
	D7 (1)	0.000	83.09	6.944	93.408
	D7 (2)	0.000	83.09	0.000	99.286
	D7 (3)	0.000	83.09	0.000	99.286
5^{th}	Ctrl D7 (1)	84.504		104.225	
	Ctrl D7 (2)	90.764		110.001	
	Ctrl D7 (3)	89.963		94.750	
	Ctrl D0 (1)	87.634	2.93	110.260	5.734
	Ctrl D0 (2)	93.675		106.756	
	Ctrl D0 (3)	91.928		109.676	
	D7 (1)	0.000	97.07	0.00	94.266
	D7 (2)	8.298	87.96	28.620	66.478
	D7 (3)	4.513	92.115	15.186	79.521

Table 19 Kinetic study residual concentration and OD600 data

Initial addition	Day	DBP Residual (mg l^{-1})	DEHP Residual (mg l^{-1})	OD600
50 mg l^{-1}	1	39.98 ± 2.52	40.95 ± 5.30	0.311 ± 0.026
	2	24.94 ± 5.74	39.59 ± 5.30	0.244 ± 0.036
	3	0	29.66 ±11.25	0.265 ± 0.051
	4	0	25.76 ± 11.86	0.333 ± 0.048
	5	0	15.64 ± 6.36	0.488 ± 0.129
	6	0	12.24 ± 5.02	0.508 ± 0.057
	7	0	17.28 ±7.68	0.381 ± 0.019
100 mg l^{-1}	1	76.81 ± 1.14	75.15 ± 6.86	0.405 ± 0.023
	2	50.32 ± 6.41	61.39 ± 6.66	0.356 ± 0.027
	3	0	49.41 ± 20.44	0.482 ± 0.109
	4	0	39.93 ± 22.16	0.410 ± 0.014
	5	0	19.01 ± 6.96	0.525 ± 0.049
	6	0	7.42 ± 12.85	0.417 ± 0.101
	7	0	22.17 ± 21.05	0.399 ± 0.038
200 mg l^{-1}	1	125.07 ± 19.23	163.93 ± 4.81	0.479 ± 0.242
	2	97.48 ± 11.12	153.81 ± 4.81	0.574 ± 0.269
	3	3.95 ± 6.85	141.17 ± 27.31	0.581 ± 0.035
	4	0	124.39 ± 3.93	0.682 ± 0.118
	5	0	83.31 ± 18.02	0.567 ± 0.128
	6	0	88.39 ± 20.65	0.421 ± 0.054
	7	0	48.31 ± 24.09	0.454 ± 0.066
400 mg l^{-1}	1	238.93 ± 31.18	223.92 ± 3.64	0.425 ± 0.154
	2	180.34 ± 67.2	168.80 ± 3.65	0.7 ± 0.172
	3	13.56 ± 14.28	225.86 ± 29.14	0.851 ± 0.108
	4	40.86 ± 35.45	179.46 ± 27.13	0.841 ± 0.2
	5	11.89 ± 15.27	253.21 ± 40.36	1.124 ± 0.211
	6	15.12 ± 4.27	278.25 ± 59.52	0.575 ± 0.025
	7	0	212.04 ± 34.46	0.684 ± 0.034
500 mg l^{-1}	1	443.22 ± 85.05	365.87 ± 67.96	0.206 ± 0.033

Initial addition	Day	DBP Residual (mg l^{-1})	DEHP Residual (mg l^{-1})	OD600
	2	397.31 ± 10.88	436.41 ± 68.98	0.537 ± 0.151
	3	98.15 ± 13.74	425.73 ± 39.56	1.193 ± 0.107
	4	134.29 ± 18.93	336.06 ± 17.87	0.892 ± 0.097
	5	52.77 ± 24.90	301.79 ± 47.48	1.394 ± 0.08
	6	32.56 ± 15.10	330.33 ± 85.61	0.744 ± 0.023
	7	5.12 ± 4.50	273.61 ± 32.22	0.84 ± 0.068

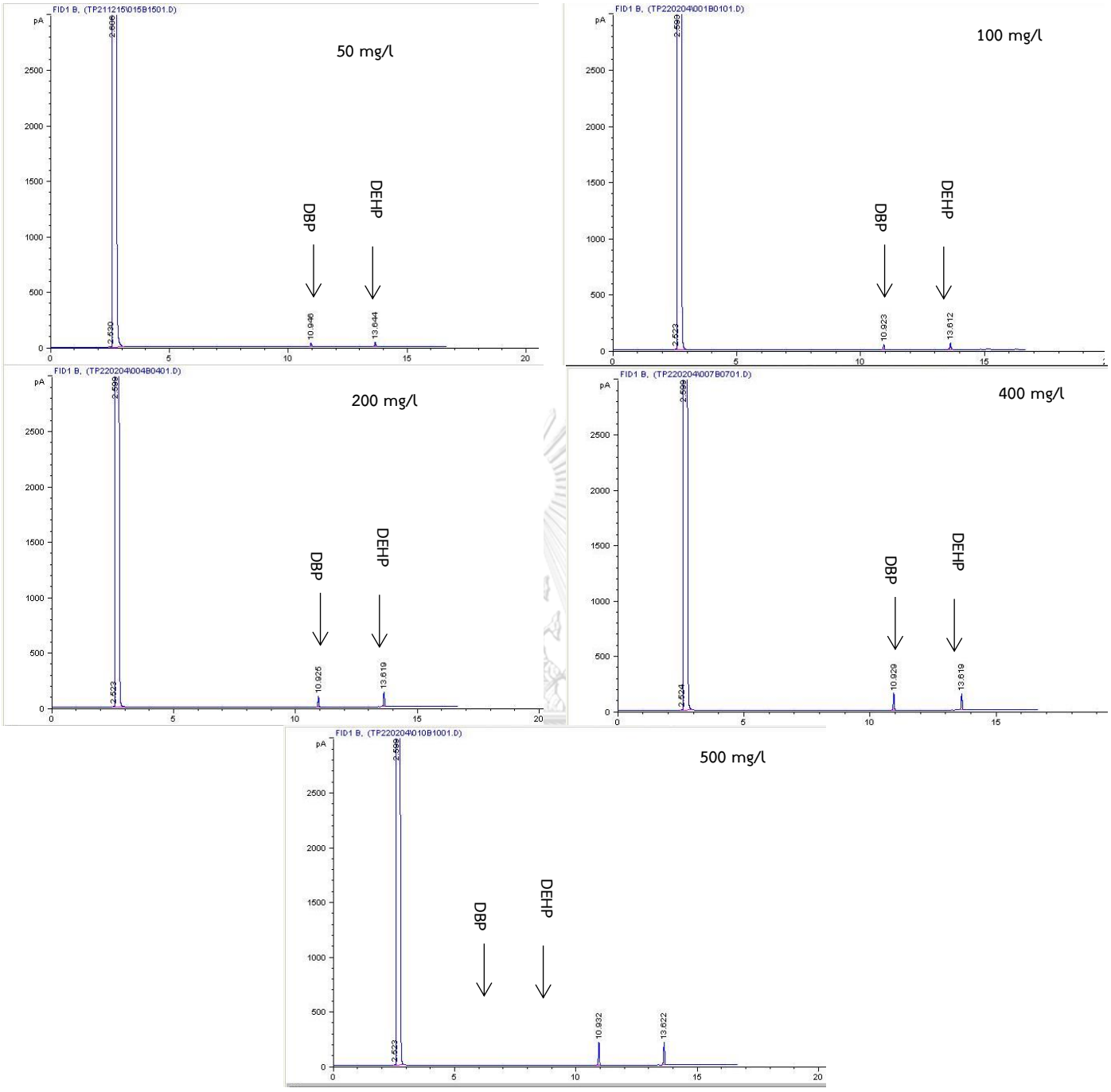

Figure 26 Chromatogram of kinetic study sample Day 0

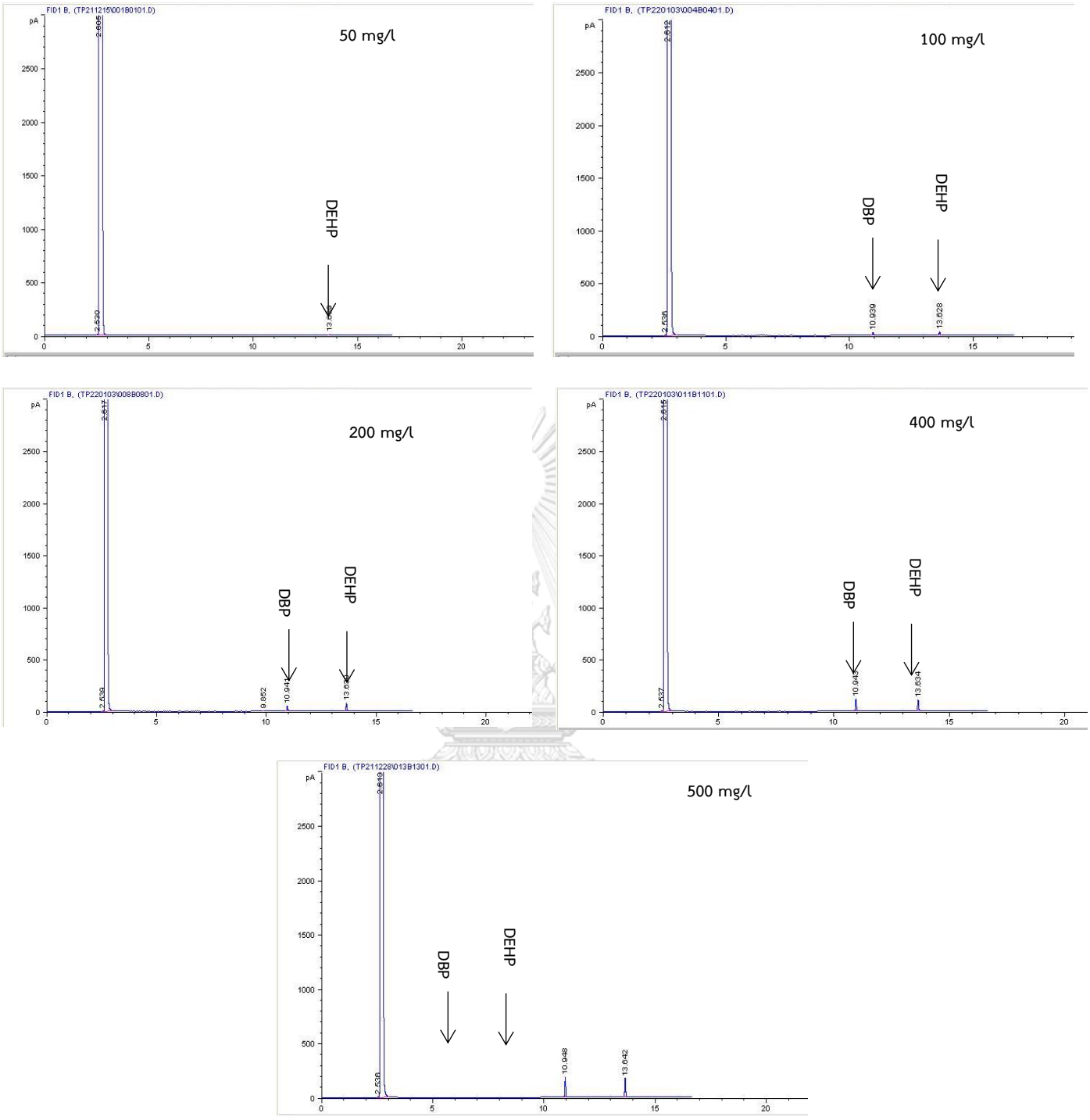

Figure 27 Chromatogram of kinetic study sample Day 1

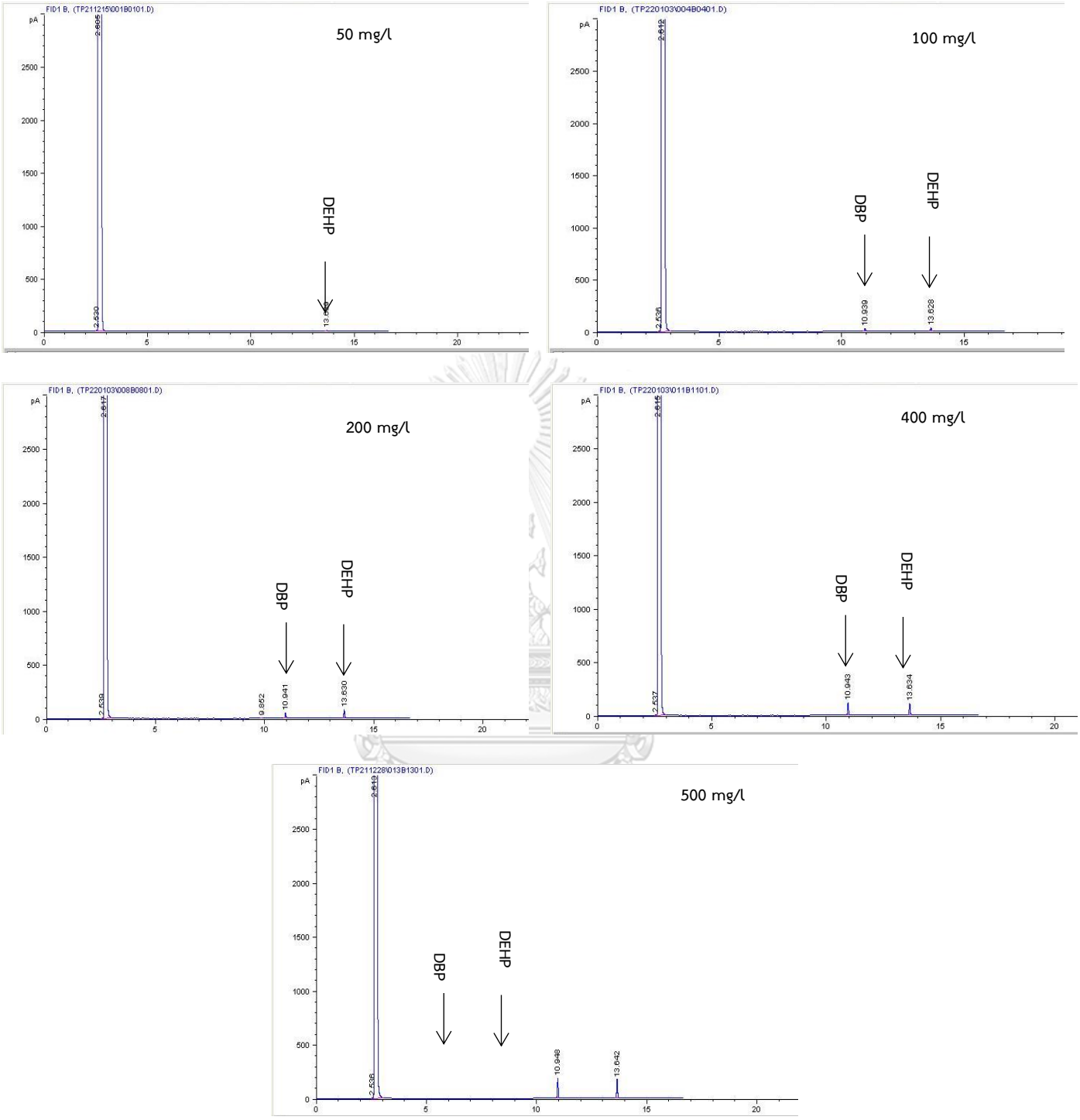

Figure 28 Chromatogram of kinetic study sample Day 2

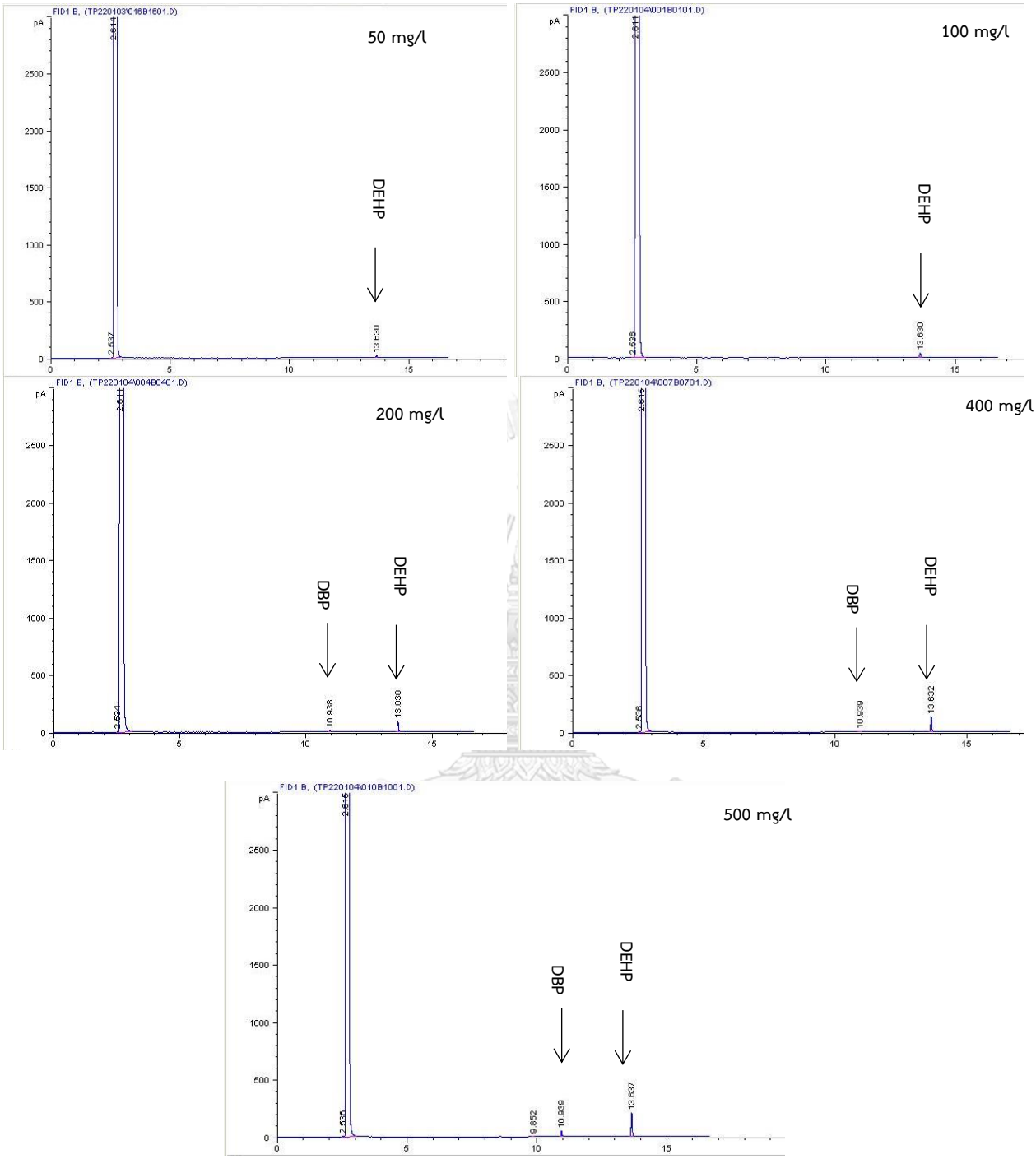

Figure 29 Chromatogram of kinetic study sample Day 3

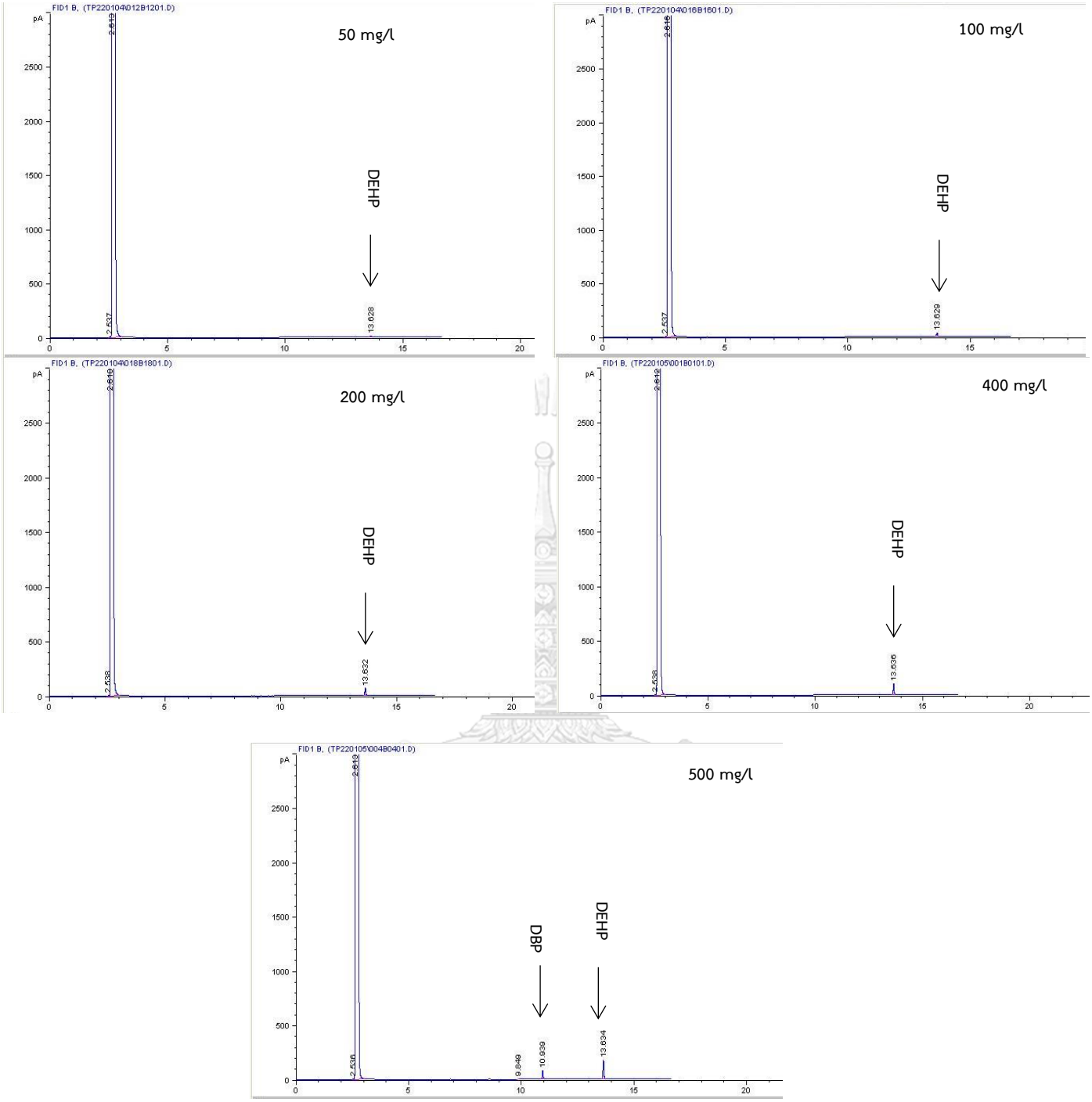

Figure 30 Chromatogram of kinetic study sample Day 4

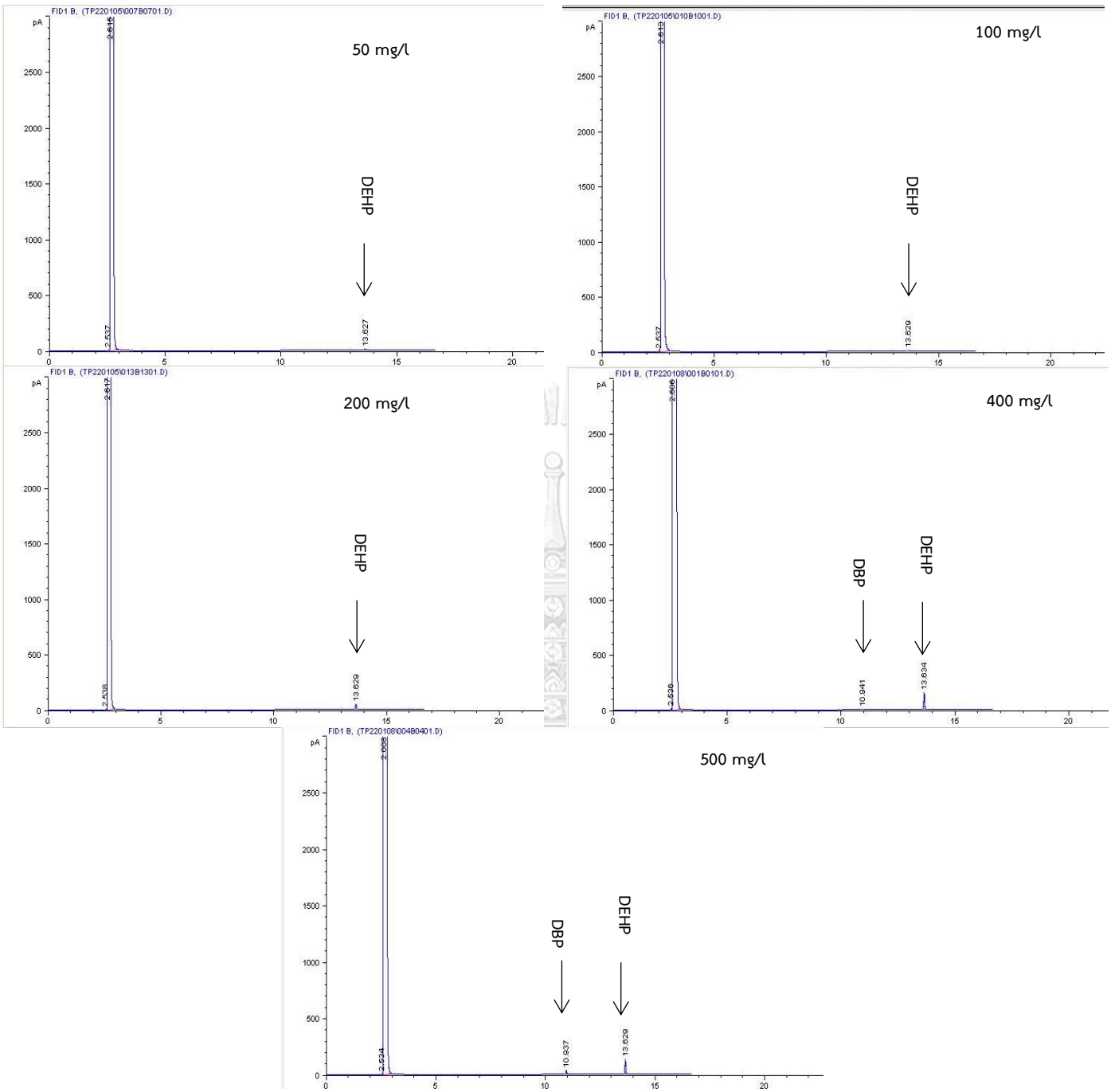

Figure 31 Chromatogram of kinetic study sample Day 5

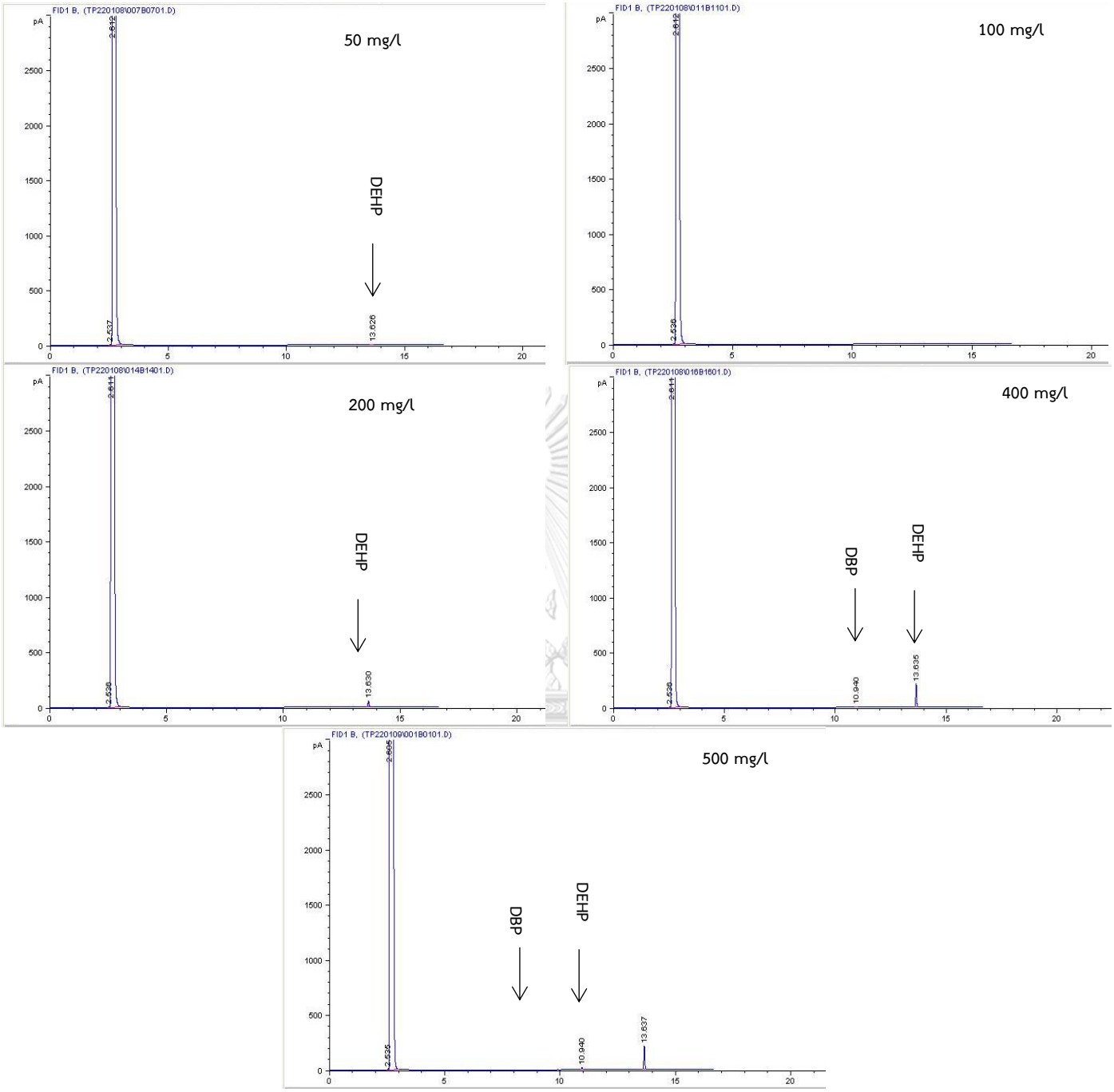

Figure 32 Chromatogram of kinetic study sample Day 6

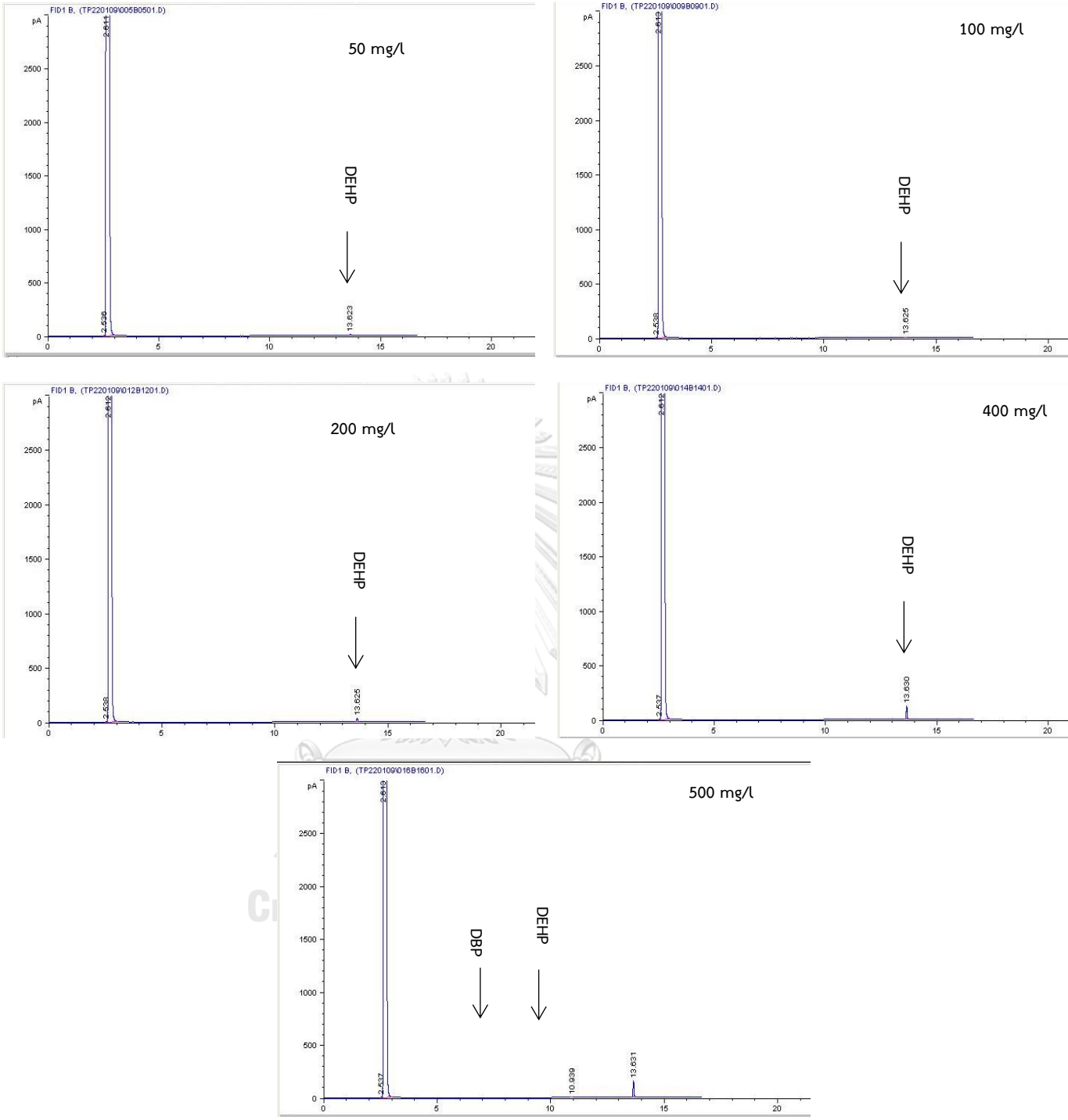

Figure 33 Chromatogram of kinetic study sample Day 7

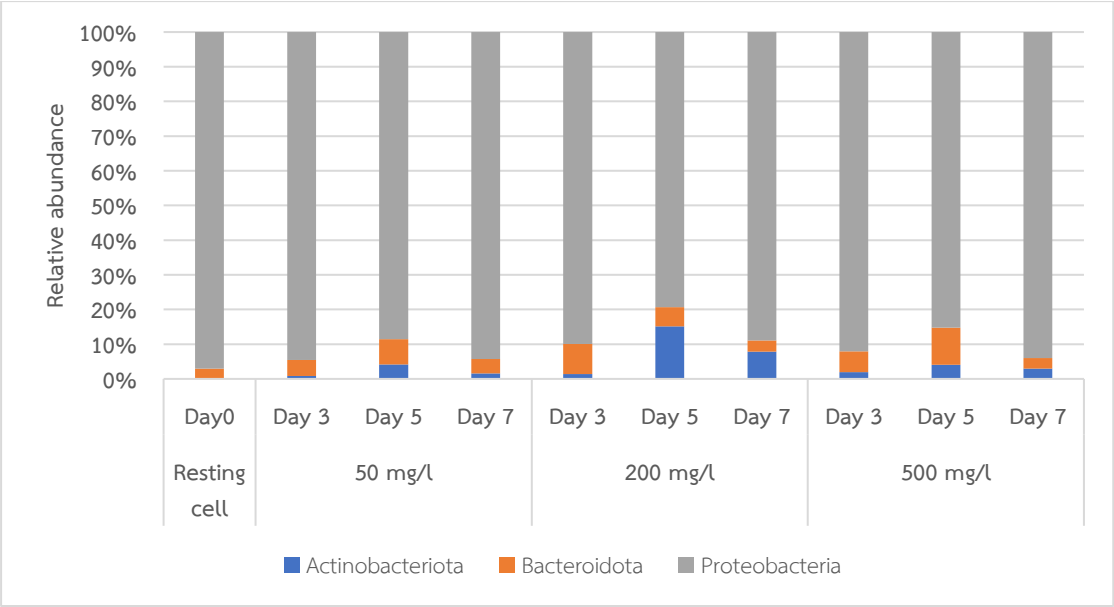

Figure 34 Relative abundance plot taxonomy level Phylum

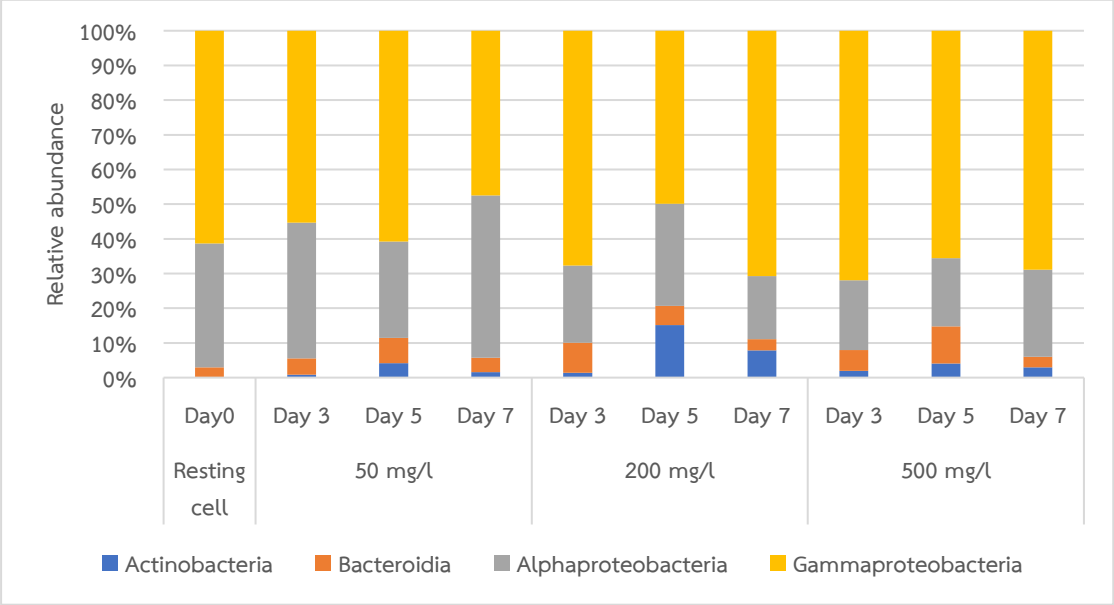

Figure 35 Relative abundance plot taxonomy level Class

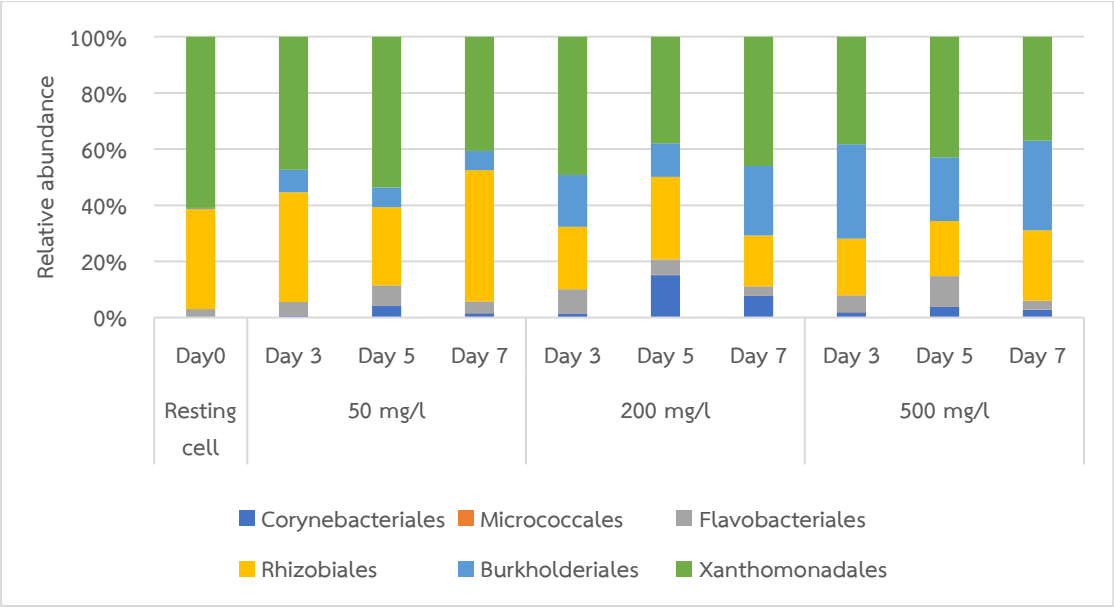

Figure 36 Relative abundance plot taxonomy level Order

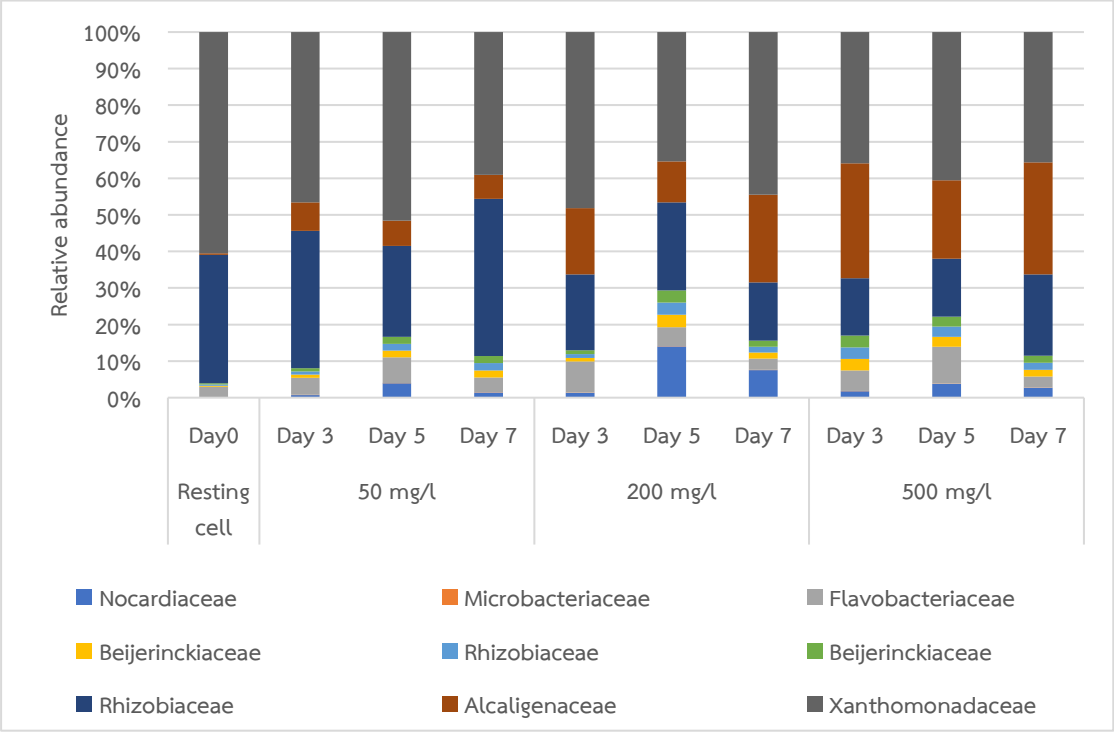

Figure 37 Relative abundance plot taxonomy level Family

APPENDIX C

PHASE II AND III SUPPLEMENTARY DATA

Table 20 DBP and DEHP extraction recovery from soil

DBP or DEHP (mg kg^{-1})	Extracted DBP (mg kg^{-1})	%Recovery DBP	Extracted DEHP (mg kg^{-1})	%Recovery DEHP
100	63.73	65.42±3.18	85.93	77.16±7.92
100	63.43		70.53	
100	69.09		75.03	
200	83.69	91.76±11.42	84.67	86.97±3.25
200	99.84		89.26	
400	107.92	104.72±4.53	85.91	86.49±30.83
400	101.52		87.08	

Table 21 Agricultural soil microcosm study result

Treatment	Day	DBP Residual (mg kg^{-1})	%Deg DBP (%)	DEHP Residual (mg kg^{-1})	%Deg DEHP (%)
Abiotic control	0	221.76±82.43	-	238.5±120.54	-
	2	171.19±43.34	22.81±19.54	296.04±56.54	N/A
	4	199.25±33.92	10.15±15.29	202.55±48.84	15.07±20.48
	6	201.49±16.56	9.14±7.47	204.74±5.77	14.16±2.42
	10	174.55±41.52	21.29±18.72	196.5±24.35	17.61±10.21
	20	264.71±85.21	N/A	247.4±39.76	N/A
	30	250.66±110.29	N/A	320.92±65.40	N/A
Natural attenuation	0	267.41±70.61	-	260.93±48.7	-
	2	96±23.53	41.29±8.8	318.95±63.21	N/A
	4	38.28±17.82	75.53±6.66	199.29±29.99	8.55±11.49
	6	11.05±6.92	86.72±2.59	200.49±7.94	9.01±23.16
	10	0	78.71	122.31±68.7	35.52±26.33
	20	0	100	155.71±28.28	40.32±10.84

Treatment	Day	DBP Residual (mg kg^{-1})	%Deg DBP (%)	DEHP Residual (mg kg^{-1})	%Deg DEHP (%)
	30	1.76±3.94	99.34±1.47	142.23±32.1	45.49±12.3
Bioaugmentation	0	189.49±28.89		184.93±101.2	
	2	58.03±26.06	46.56±13.76	238.41±88.04	0
	4	8.62±8.23	85.3±4.35	148.9±49.12	27.86±18.8
	6	1.86±4.15	89.88±2.19	166.71±54.35	21.95±20.83
	10	0	78.71	137.06±50.18	42.36±19.23
	20	0	100	121.06±44.11	53.6±16.91
	30	2.64±5.91	98.6±3.12	131.64±60.61	49.55±23.23
Exogenous consortia	0	250.25±41.68		260.28±32.40	
	2	172.09±9.59	11.77±4.92	261.21±47.60	0
	4	155.25±7.38	10.25±3.78	164.53±14.0	21.87±5.46
	6	159.16±15.6	9.26±7.99	179.31±18.6	17.12±7.13
	10	91.19±34.84	31.95±17.86	155.45±11.48	22.82±4.4
	20	17.03±8.14	91.27±4.17	166.66±28.61	36.12±10.96
	30	1.72±3.83	99.12±1.97	188.97±85.45	41.49±11.8

Table 22 Enumeration of total heterotrophic bacteria and PAE-degrading bacteria in agricultural soil microcosm study

Treatment	Day	Total heterotrophic bacteria (log MPN/ g soil)	PAE-degrading bacteria (log MPN/g soil)
Natural attenuation	0	7.70 ±3.85	-
	2	8.60 ±4.77	-
	4	8.28 ±4.45	5.88 ±2.34
	6	8.56 ±4.73	4.81 ±2.15
	10	8.48 ±4.68	4.87 ±2.34
	20	8.93 ±4.00	4.87 ±2.34
	30	8.59 ±4.76	4.34 ±1.90
Bioaugmentation	0	8.63 ±4.83	-

Treatment	Day	Total heterotrophic bacteria (log MPN/ g soil)	PAE-degrading bacteria (log MPN/g soil)
	2	8.70 ±3.85	-
	4	8.39 ±4.58	7.73 ±3.30
	6	9.37 ±4.53	5.89 ±3.40
	10	8.98 ±4.08	5.68 ±3.11
	20	9.13 ±4.28	5.68 ±3.11
	30	8.63 ±4.77	5.62 ±3.04
Exogenous consortia	0	8.44 ±4.34	-
	2	9.06 ±4.36	-
	4	8.56 ±4.78	7.34 ±3.60
	6	9.15 ±4.23	9.09 ±3.68
	10	9.13 ±4.30	8.16 ±3.73
	20	8.63 ±4.77	8.16 ±3.73
	30	8.30 ±4.48	8.16 ±3.78

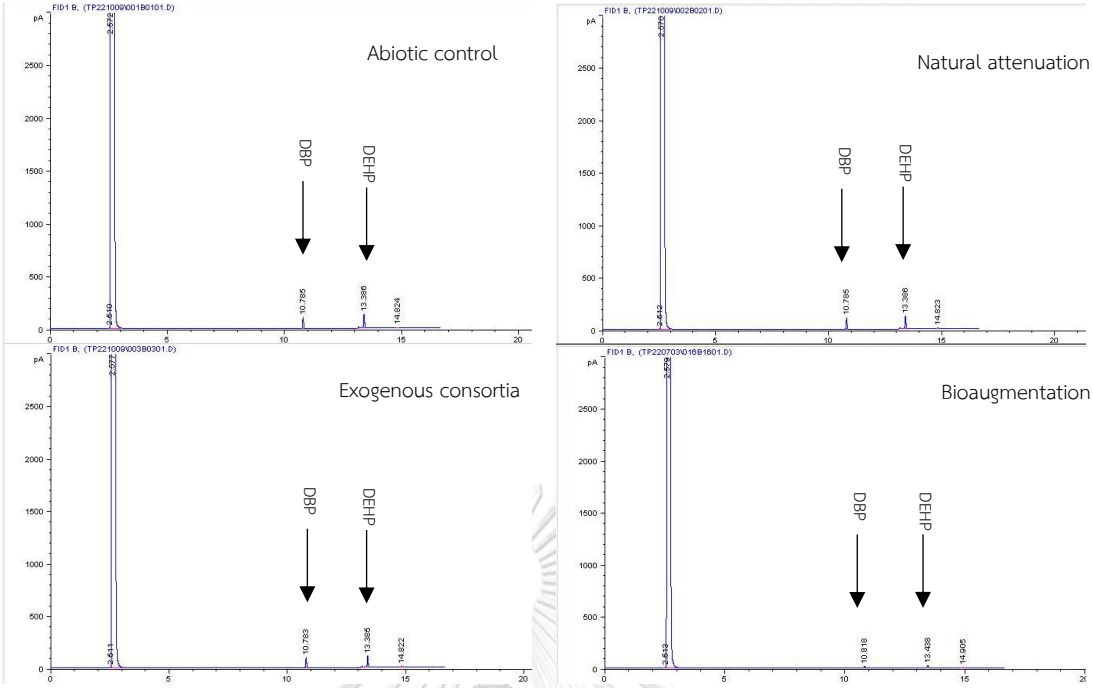

Figure 38 Chromatogram of soil microcosm sample Day 2

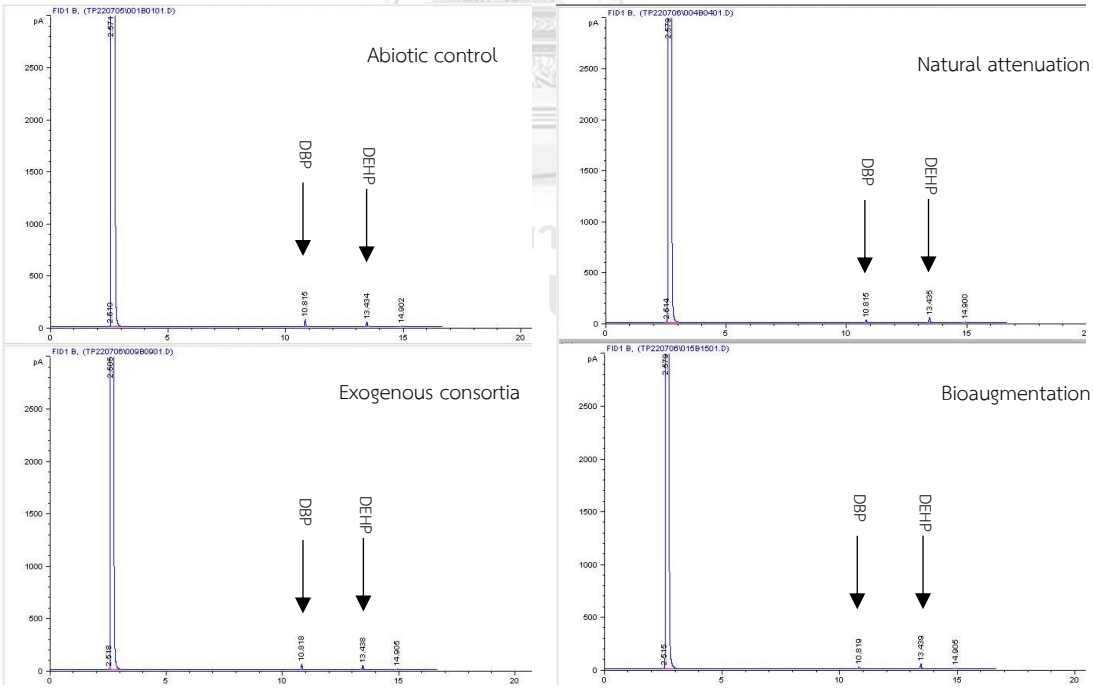

Figure 39 Chromatogram of soil microcosm sample Day 4

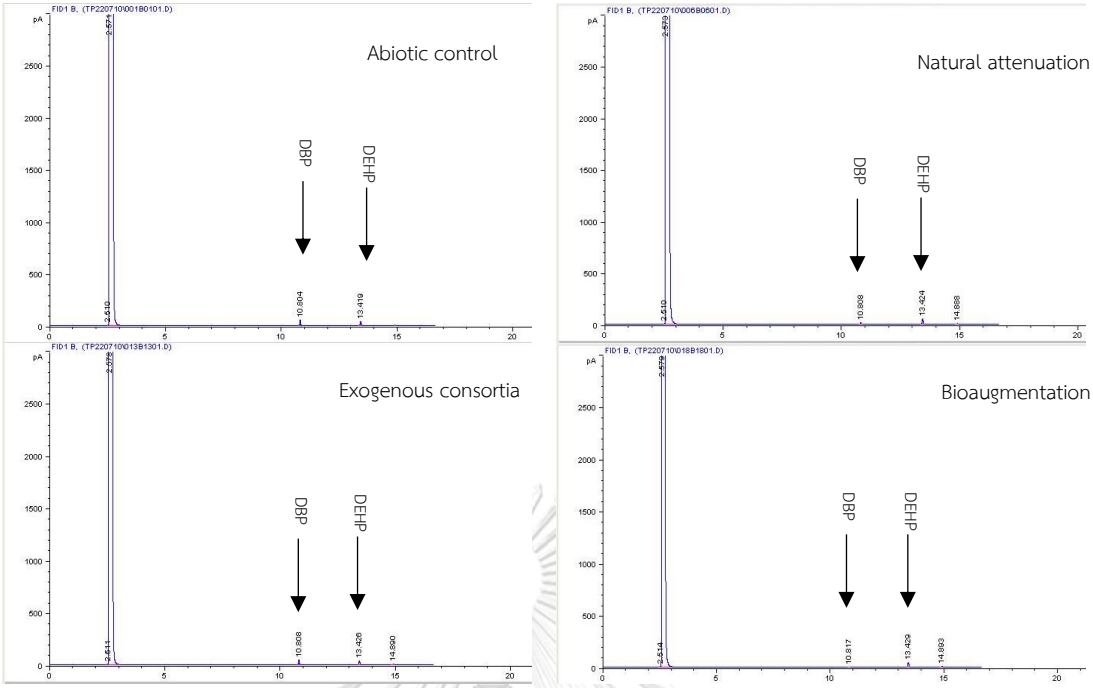

Figure 40 Chromatogram of soil microcosm sample Day 6

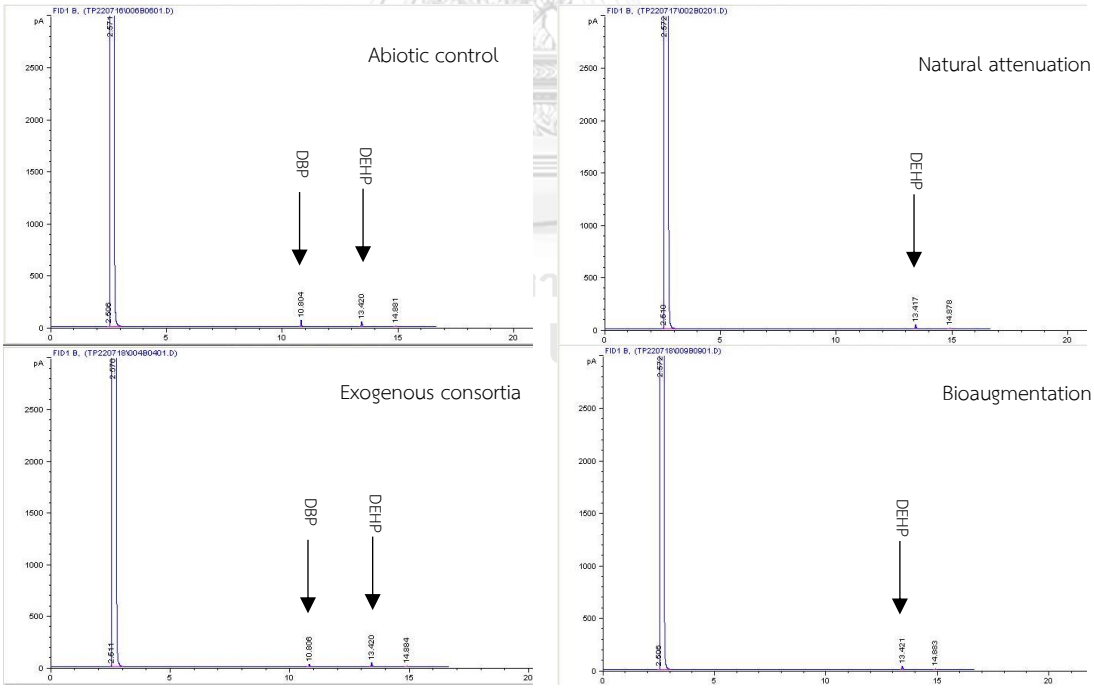

Figure 41 Chromatogram of soil microcosm sample Day 10

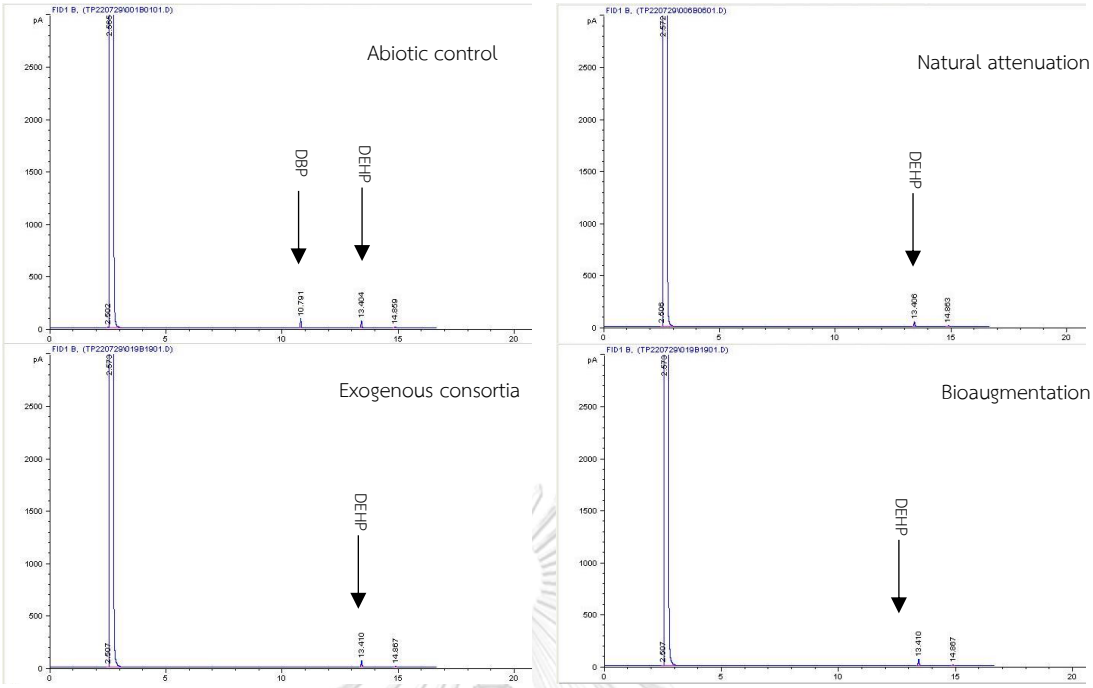

Figure 42 Chromatogram of soil microcosm sample Day 20

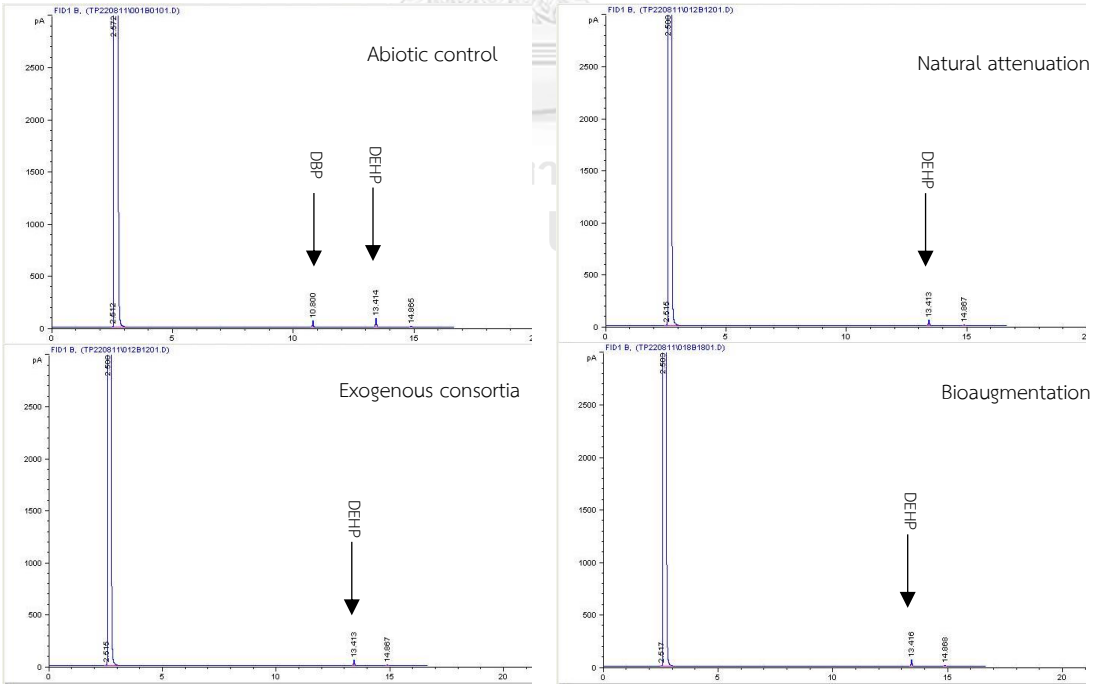

Figure 43 Chromatogram of soil microcosm sample Day 30

Table 23 Agricultural soil physicochemical properties

pH[1]	pH value	5.64
	pH level	Slightly acid
$CaCO_3$ requirement[2]	kg $CaCO_3$/ Rai	403
Soil particles (%)[3]	Sand	65
	Silt	22
	Clay	13
Soil texture		Sand loam
Organic matter[4]	g/kg	27
	level	Moderate
Phosphorus[5]	mg/kg	248
	level	Very high
Potassium[6]	mg/kg	96
	level	High
Calcium[7]	mg/kg	517
	level	Moderate
Magnesium[7]	mg/kg	76
	level	Moderate
EC 1:5	dS/m	0.07
OC	%	1.56
Total N	g/kg	0.91
Total P	g/kg	0.29
Total K	g/kg	0.40
AWCA	%	8.0

Analytical techniques: [1]Thomas (1996) [2]Woodruff (1948) [3]Gee and Bauder (1979) [4]Walkley and Black (1934) [5]Bray and Kurtz (1945) [6]Helmke and Sparks (1996) [7]Suarez (1996)

Table 24 Phytotoxicity test on mung bean seeds for soil post-microcosm study

Treatment	Dilution	Fresh weight (g)	Shoot length (cm)	Root length (cm)	Germination index
Water control	-	0.329 ± 0.07	3.390 ± 1.10	5.450 ± 1.58	-
Abiotic control	2.5 g l^{-1}	0.330 ± 0.06	3.333 ± 1.80	5.211 ± 1.08	0.983
	10X	0.295 ± 0.03	4.200 ± 1.60	4.990 ± 1.17	1.239
	100X	0.292 ± 0.06	3.589 ± 1.74	3.778 ± 1.16	1.377
Natural attenuation	2.5 g l^{-1}	0.265 ± 0.06	2.838 ± 2.03	3.563 ± 1.33	0.837
	10X	0.269 ± 0.08	2.840 ± 2.25	3.640 ± 1.58	0.838
	100X	0.242 ± 0.05	1.089 ± 1.09	2.500 ± 0.57	0.321
Bioaugmentation	2.5 g l^{-1}	0.268 ± 0.08	2.667 ± 1.15	4.767 ± 2.02	0.787
	10X	0.270 ± 0.05	2.938 ± 1.58	2.963 ± 1.17	0.867
	100X	0.231 ± 0.02	1.986 ± 1.07	2.486 ± 1.00	0.586
Exogenous consortia	2.5 g l^{-1}	0.303 ± 0.04	2.544 ± 1.63	4.867 ± 1.49	0.844
	10X	0.329 ± 0.07	5.063 ± 1.61	6.125 ± 1.61	1.707
	100X	0.335 ± 0.07	3.250 ± 1.38	5.090 ± 1.72	1.065

Table 25 Fertilizer addition soil microcosm study result

Treatment	**Day**	**DBP Residual (mg kg^{-1})**	**%Deg DBP (%)**	**DEHP Residual (mg kg^{-1})**	**%Deg DEHP (%)**
Abiotic control	0	165.87 ± 11.7		165.41 ± 15	
	1	167.28 ± 8	N/A	127.48± 12.4	22.93 ±7.5
	3	103.14 ± 11.3	37.82 ± 6.8	106.96± 4.86	35.34 ±2.9
	7	169. 52 ± 31.5	N/A	163.39± 21.7	1.22± 1.3
	15	178.85 ± 13.2	N/A	172.06±12.8	N/A
	25	229.33 ± 11.9	N/A	206.81± 21.5	N/A
Natural attenuation	0	156.3		165.41	
	1	57.81 ± 6.58	63.03 ± 4.21	106.68± 11.4	12.57± 6.86
	3	6.59 ± 4.92	57.96 ± 3.14	103.57± 16.8	2.04± 10.1
	7	1.83 ± 1.75	98.83 ± 1.12	95.38± 10.1	41.11± 6.09
	15	0	100	96.82± 45.78	41.46± 11.9
	25	0	100	66.73± 10.16	59.66± 6.14
Bioaugmentation	0	156.3		165.41	
	1	9.52 ± 1.45	93.91 ± 0.93	99.76± 7.49	16.75± 4.52
	3	0	62.18	90.01± 15.3	10.24± 9.26
	7	0	100	56.38± 7.46	64.68± 4.51
	15	0	100	87.72± 17.9	46.97± 10.8
	25	0	100	56.56± 12.6	65.81± 7.61
Exogenous consortia + Fertilizer	0	156.3		165.41	
	1	21.96 ± 1.51	85.95 ± 0.96	117.31± 15.6	6.15± 9.44
	3	0.11 ± 0.19	62.11 ± 0.12	165.6± 24.9	0

Treatment	Day	DBP Residual (mg kg^{-1})	%Deg DBP (%)	DEHP Residual (mg kg^{-1})	%Deg DEHP (%)
	7	0	100	82.9± 7.96	49.91 ±4.82
	15	0	100	103.8± 21.1	37.23± 12.8
	25	0	100	69.31± 10.3	58.1± 6.21
Natural attenuation + Fertilizer	0	156.3		165.41	
	1	47.86 ± 10.45	69.38 ± 6.66	85.9± 2.37	25.12± 1.43
	3	2.36 ± 0.16	60.67 ± 0.1	100.8± 12.7	3.72± 7.67
	7	1.06 ± 1.84	99.32 ± 1.17	105.8± 9.16	34.81± 5.5
	15	0	100	63.2± 17.18	61.79± 10.4
	25	0	100	66.57± 7.44	59.76± 4.5
Bioaugmentation + Fertilizer	0	156.3		165.41	
	1	20.49 ± 2.74	86.89 ± 1.76	93.65± 11.9	20.45± 7.2
	3	0.205 ± 0.197	62.05 ± 0.13	82.2± 7.55	14.97± 4.56
	7	0	100	104.07± 4.54	35.86± 2.74
	15	0	100	51.4± 10.76	68.92± 6.51
	25	0	100	39.91± 3.59	75.87± 2.17

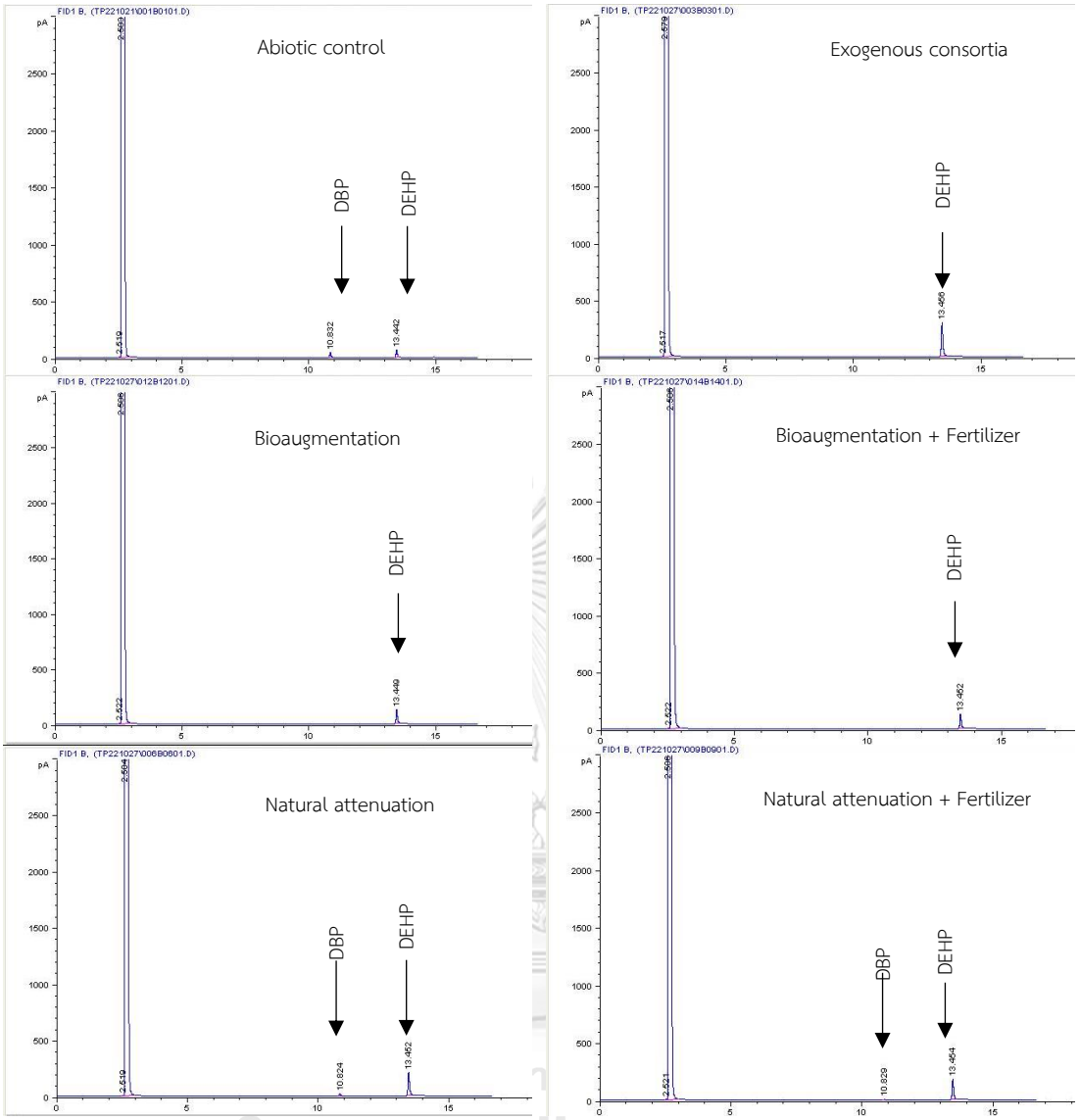

Figure 44 Chromatogram fertilizer-added soil microcosm sample Day 3

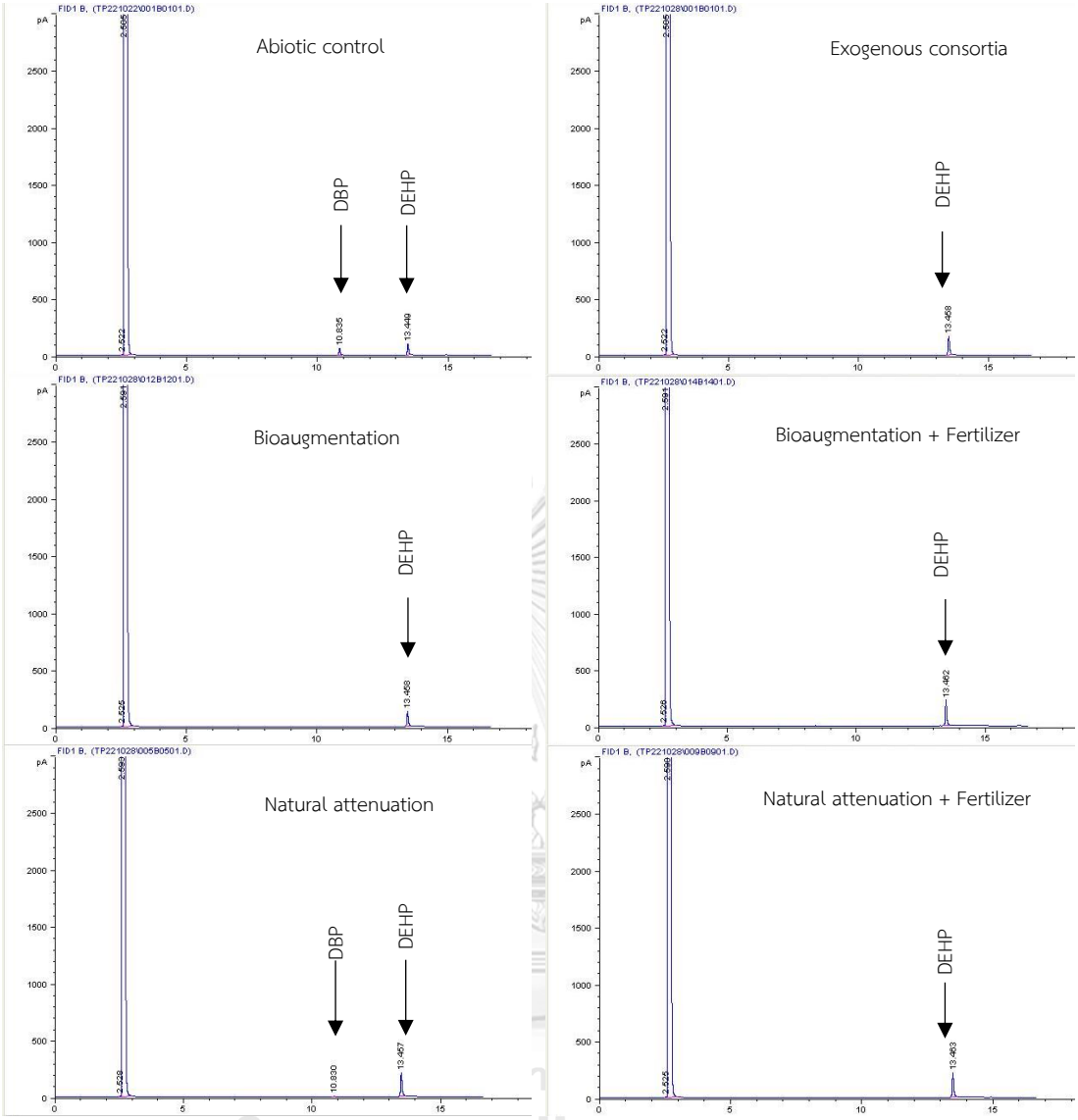

Figure 45 Chromatogram fertilizer-added soil microcosm sample Day 7

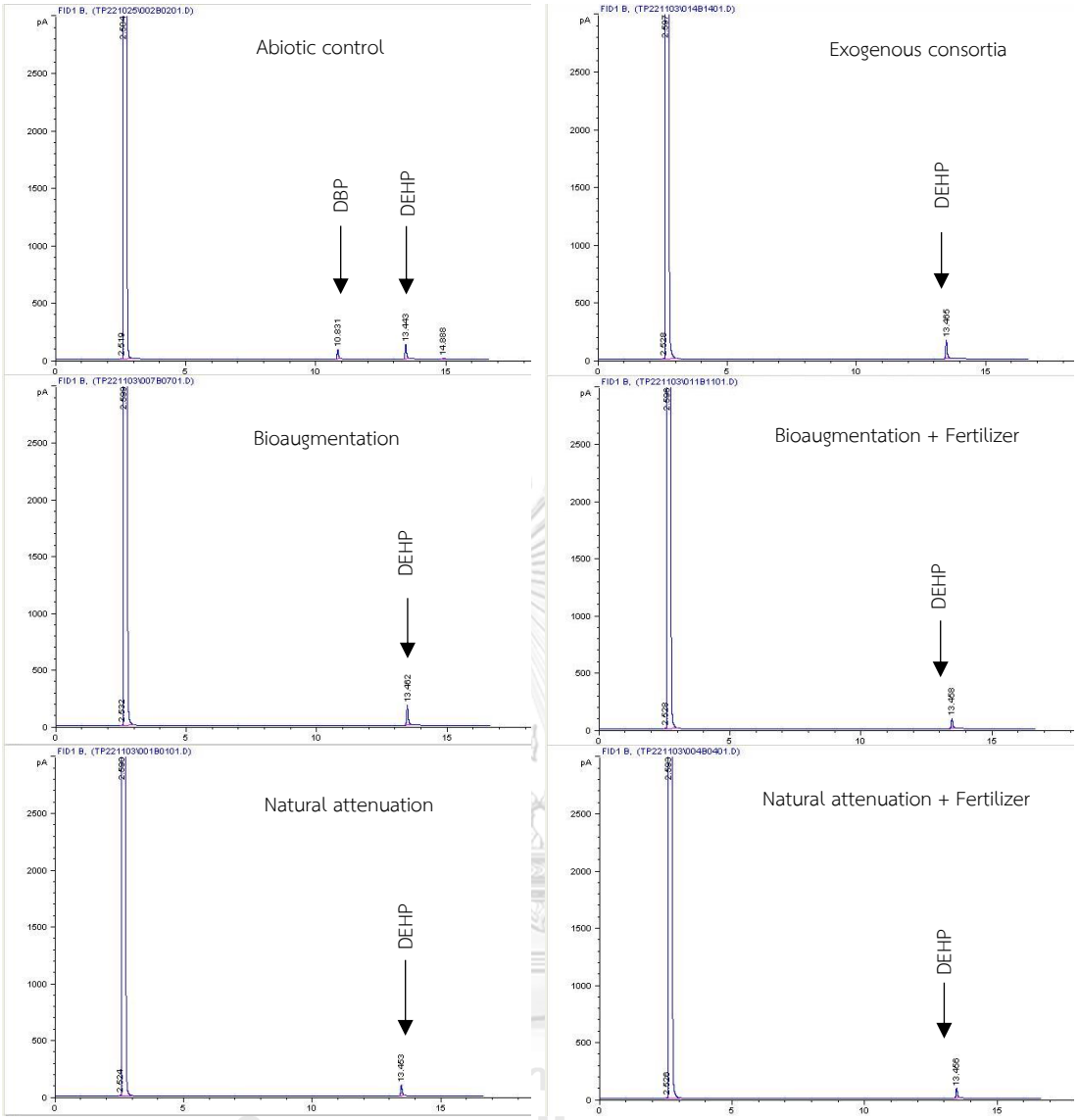

Figure 46 Chromatogram fertilizer-added soil microcosm sample Day 15

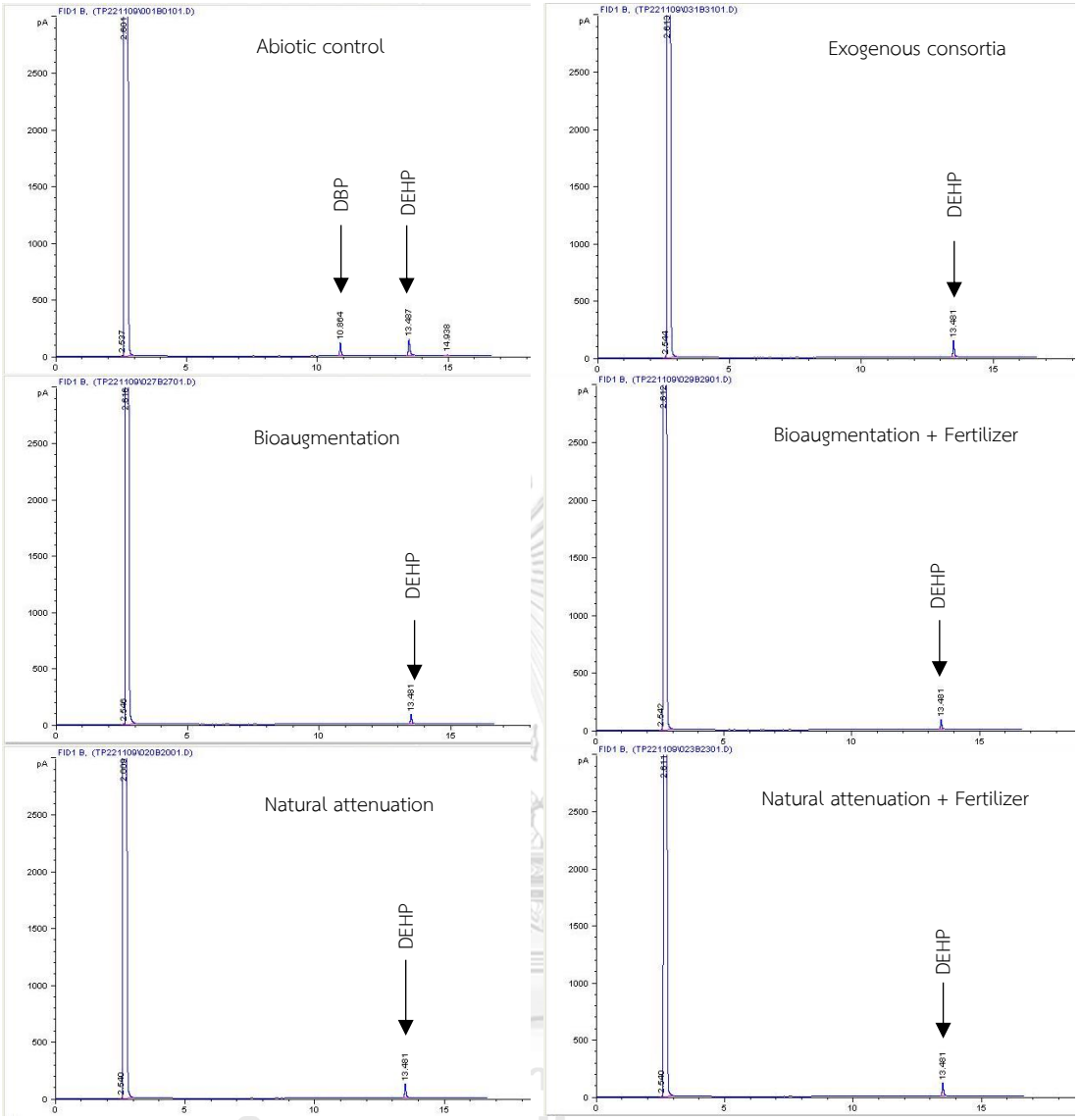

Figure 47 Chromatogram fertilizer-added soil microcosm sample Day 25

Table 26 Enumeration of total heterotrophic bacteria and PAE-degrading bacteria in fertilizer addition soil microcosm study

Treatment	**Day**	**Total heterotrophic bacteria (log MPN/ g soil)**	**PAE-degrading bacteria (log MPN/g soil)**
Natural attenuation	0	6.026 ± 0.11	
	3	5.753 ± 0.38	3.870 ± 0.42
	7	6.999 ± 0.11	3.786 ± 0.61
	15	5.928 ± 0.23	5.229 ± 1.23
	25	6.376 ± 0.09	5.170 ± 1.02
Natural attenuation + Fertilizer	0	6.24 ± 0.15	
	3	6.015 ± 0.33	6.515 ± 0.47
	7	6.795 ± 0.23	5.330 ± 1.24
	15	6.227 ± 0.32	5.515 ± 0.47
	25	6.956 ± 0.59	6.119 ± 0.34
Bioaugmentation	0	7.999 ± 0.11	
	3	7.387 ± 0.36	5.727 ± 2.89
	7	7.735 ± 1.08	4.786 ± 1.29
	15	6.403 ± 0.31	5.927 ± 0.51
	25	6.403 ± 0.31	5.343 ± 1.87
Bioaugmentation + Fertilizer	0	7.710 ± 0.54	
	3	7.394 ± 0.58	6.131 ± 2.05
	7	7.609 ± 0.37	3.997 ± 0.50
	15	6.682 ± 0.37	5.535 ± 0.50
	25	6.682 ± 0.37	6.766 ± 0.74
Exogenous consortia + Fertilizer	0	8.62 ± 0.76	
	3	7.736 ± 0.3	7.262 ± 0.51
	7	7.753 ± 0.64	5.656 ± 1.04
	15	7.086 ± 0.22	5.727 ± 1.15
	25	6.622 ± 0.39	5.036 ± 0.9

Table 27 Phytotoxicity test on Chinese convolvulus seeds for fertilizer addition post-microcosm study

Treatment	Dilution	Total fresh weight (g)	Shoot length (cm)	Root length (cm)	Germination index
Water control	-	0.48	4.6 ± 0.57	4.60 ± 0.2	1
Abiotic control	2.5 g l^{-1}	0.81	3.57 ± 1	3.1 ± 0.36	2.16
	10X	1.24	2.2 ± 0.45	3.4 ± 1.64	2.64
	100X	0.79	2.37 ± 2.03	2.5 ± 1.32	0.7
Natural attenuation	2.5 g l^{-1}	0.48	2.25 ± 0.35	1.75 ± 0.3	0.54
	10X	1.49	2.88 ± 0.8	2.08 ± 1.6	1.93
	100X	0.32	3.5	2.50	0.23
Natural attenuation + Fertilizer	2.5 g l^{-1}	0.5	2.15 ± 0.21	1.30 ± 1.7	1.21
	10X	0.76	2.57 ± 1.29	0.90 ± 1.4	0.63
	100X	0.98	3.73 ± 0.46	2.33 ± 1.15	0.65
Bioaugmentation	2.5 g l^{-1}	1.07	3.75 ± 1.71	1.75 ± 0.96	3.26
	10X	0.45	2.5	1.05 ± 1.34	0.49
	100X	1.05	5 ± 1.47	2.75 ± 0.29	1.71
Bioaugmentation + Fertilizer	2.5 g l^{-1}	1.12	2.95 ± 1.38	3.65 ± 1.06	2.26
	10X	1.75	3.62 ± 1.53	2.95 ± 0.39	2.06
	100X	1.04	3.68 ± 0.54	2.75 ± 0.29	2.56
Exogenous consortia	2.5 g l^{-1}	1.15	3.13 ± 0.85	2.38 ± 0.75	1.1

Treatment	Dilution	Total fresh weight (g)	Shoot length (cm)	Root length (cm)	Germination index
	10X	1.92	3.17 ± 1.5	2.92 ± 0.66	2.04
	100X	0.48	2.96 ± 0.98	2.09 ± 1.1	1.70

Table 28 Phytotoxicity test on corn seeds for fertilizer addition post-microcosm study

Treatment	Dilution	Total fresh weight (g)	Shoot length (cm)	Root length (cm)	Germination index
Water control	-	0.77	1.75 ± 0.63	3.1 ± 0.56	1
Abiotic control	2.5 g l^{-1}	0	0	0	0
	10X	0.46	4.5	5	1.613
	100X	0.40	3	4.5	1.452
Natural attenuation	2.5 g l^{-1}	0.89	2.35 ± 0.21	3.75 ± 1.77	1.210
	10X	0.32	0.5	4	0.645
	100X	0.70	1.55 ± 0.78	2.05 ± 2.77	1.323
Natural attenuation + Fertilizer	2.5 g l^{-1}	0.74	1.65 ± 0.21	3.5 ± 2.12	2.258
	10X	0.47	1	3.5	0.565
	100X	0.51	3	3	0.484
Bioaugmentation	2.5 g l^{-1}	0.77	1.5 ± 0.71	2.5	1.613
	10X	0.44	2.5	5	0.806
	100X	0.45	4.5	5	0.806
Biaougmentation + Fertilizer	2.5 g l^{-1}	0.79	1.25 ± 0.35	2.65 ± 3.32	1.710
	10X	0.94	2.75 ± 0.63	4.85 ± 1.91	3.129
	100X	0.78	3 ± 2.8	4.95 ± 2.76	3.194
Exogenous consortia	2.5 g l^{-1}	0.90	2.5 ± 0.71	3.75 ± 1.06	2.419
	10X	0.42	2.25 ± 1.06	3	1.935
	100X	0.90	2.75 ± 1.77	4.1 ± 0.14	2.645

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