Environmental Distribution, Phytotoxicity, and Bioremediation of Endocrine Disruptor Di-*n*-Butyl Phthalate

内分泌攪乱物質であるフタル酸ジブチルの環境分布、
植物毒性、およびバイオレメディエーションに関する研究

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A Dissertation Submitted to the Graduate School of Human Life Science, Osaka City University for the Degree of Doctor of Philosophy

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications:

Main papers:

1. **Liao, C. S.**, Nishikawa, Y., Shih, Y. T.., "Characterization of Di-*n*-Butyl Phthalate Phytoremediation by Garden lettuce (*Lactuca sativa* L. var. *longifolia*) through Kinetics and Proteome Analysis", *Sustainability*, vol. 11, pp. 1625, 2019.03.
2. **Liao, C. S.**, Yuan, S. Y., Hung, B. H., Chang, B. V., "Removal of organic toxic chemicals using the spent mushroom compost of *Ganoderma lucidum*" *J. Environ. Monitor*., vol. 14, pp. 1983-1988, 2012.07.
3. **Liao, C. S.,** "Biodegradation of di-*n*-butyl phthalate in a soil microcosm," *J. Environ. Sci. Health. B*, vol.45, pp.366-371, 2010.05.
4. **Liao, C. S.**, Chen, L. C., Chen, B. S., Lin, S. H., "Bioremediation of endocrine disruptor di-*n*-butyl phthalate ester by *Deinococcus radiodurans* and *Pseudomonas stutzeri*," *Chemosphere*, vol.78, pp.342-346, 2010.01.
5. **Liao, C. S.**, Yen, J. H., Wang, Y. S., "Growth inhibition in Chinese cabbage (*Brassica rapa* var. *chinensis*) growth exposed to di-*n*-butyl phthalate," *J. Hazard. Mater.*, vol.63, pp.625-631, 2009.04.

Reference papers:

1. Kaewlaoyoong, A., Vu, C. T., Lin, C., **Liao, C. S.**, Chen, J. R., "Occurrence of phthalate esters around the major plastic industrial area in southern Taiwan", *Environ. Earth Sci.,* vol. 77, pp. 457, 2018.06.
2. Chang, B. V., **Liao, C. S.**, Yuan, S. Y., "Anaerobic degradation of diethyl phthalate, di-*n*-butyl phthalate, and di-(2-ethylhexyl) phthalate from river sediment in Taiwan," *Chemosphere*, vol.58, pp.1601–1607, 2005.01.
3. Chang, B. V., **Liao, C. S.**, Yuan, S. Y., "Anaerobic degradation of di-*n*-butyl phthalate and di-(2-ethylhexyl) phthalate in sludge," *Bull. Environ. Contam. Toxicol.*, vol.75, pp.775-782, 2005.01.
4. Yuan, S. Y., Liu, C., **Liao, C. S.**, Chang, B. V., "Occurrence and microbial degradation of phthalate esters in Taiwan river sediments," *Chemosphere*, vol.49, pp.1295-1299, 2002.01.

ABBREVIATIONS

1. 2-DE, two-dimensional gel electrophoresis;
2. AaRS, aminoacyl-tRNA synthetase;
3. ABTS, 2,20-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid);
4. ACN, acetonitrile;
5. Acyl-ACP desaturase, acyl-[acyl-carrier-protein] desaturase;
6. AF, agricultural fields;
7. BG, back gardens;
8. BSA, bovine serum albumin;
9. BBP, benzylbutyl phthalate;
10. CMC, critical micelle concentration;
11. CTAC, cetyltrimethylammonium chloride;
12. DBP, di-*n*-butyl phthalate;
13. DEHP, di-2-ethylhexyl phthalate;
14. DEP, diethyl phthalate;
15. DPP, dipropyl phthalate;
16. DHP, dihexyl phthalate;
17. DPhP, diphenyl phthalate;
18. DCP, dicyclohexyl phthalate;
19. DMP, dimethyl phthalate;
20. D*n*OP, di-*n*-octyl phthalate;
21. DFR, dihydroflavonol-4-reductase;
22. DTT, dithiothreitol;
23. DI, deionized;
24. DCM, dichloromethane;
25. EDCs, endocrine-disrupting chemicals;
26. Fd-NiR, ferredoxin-nitrite reductase;
27. GC-MS, gas chromatography-mass selective detector;
28. GO, gene ontology;
29. GVP, greenhouse vegetable production;
30. IEF, isoelectric focusing;

31. IPG, immobilized pH gradient;
32. LC/MS/MS, liquid chromatography tandem mass spectrometry;
33. LMS, laccase mediator systems;
34. MDLs, method detection limit;
35. MALDI-TOF-MS, matrix assisted laser desorption ionization time of flight mass spectrometry;
36. MS, mass spectrometry;
37. ND, non-detected;
38. NEPZ, Nanzih Export Processing Zone;
39. PAEs, phthalate esters;
40. PMF, peptide mass fingerprinting;
41. POD, peroxidase;
42. PSII, photosystem II;
43. RPT3, root phototropism protein 3;
44. ROS, reactive oxygen species;
45. RS, roadside;
46. SMC, spent mushroom compost;
47. SVOC, semi-volatile organic compounds;
48. SOD, superoxide dismutase;
49. SPCC, proficiency calibration check;
50. TCA, trichloroacetic acid;
51. TEM, transmission electron microscopy;
52. TEMED, N,N,N',N'-tetramethylethylenediamine;
53. TFA, trifluoroacetic acid;

ABSTRACT

Nowadays, plastic products have been filled and disturbed in our living environment. After used, they would be discarded into the terrestrial or aquatic ecosystems and then break down to become to the different kinds of chemical pollutants. Therefore, the agricultural land which used the river water or groundwater for irrigation would be impacted. One of the most important chemical pollutants are plasticizers, phthalate esters (PAEs).

Di-*n*-butyl phthalate (DBP) is one of the most widely used PAEs and is mainly used as a plasticizer for plastics. Due to its high production and application figures, DBP is widely distributed in various environmental samples such as soil, air, wastewater, sewage sludge, river water, sediment, and groundwater. Then, it can be accumulated in seafood or agricultural products and represents a substantial risk to human health via the food chain. In recent years, several studies have shown that DBP has embryotoxicity and other effects on different organisms. Therefore, DBP had been classified as suspected endocrine-disrupting chemicals (EDCs). Since DBP is widely distributed in the environment, the purpose of this study is to assessing the related risk of DBP for environmental and agricultural protection.

In chapter 1, the result of PAEs environmental distribution shows that DBP and di-2-ethylhexyl phthalate (DEHP) were found to be higher than the other PAEs in the river water and sediment in Taiwan in 2000. The concentrations of DBP in the water and sediment samples were 1.0-13.5 μg L^{-1} (ppb) and 0.3-30.3 μg g^{-1} (ppm), respectively. On the other hand, the result of 2013 shows that DEHP and di-*n*-octyl phthalate (D*n*OP) accounted for the most of PAEs concentrations in soil (> 90%). DBP were almost found in each soil sample in Taiwan, the mean concentration was 0.01 mg kg^{-1}. In addition, major sources of PAEs in agricultural soil could be the use of chemical fertilizer and plastic products. These results show that the mean concentration of DBP decreased in Taiwan's environment from 2000 to 2013, but still could be found in each sampling sites in Taiwan.

In chapter 2, the results of DBP phytotoxicity shows that DBP caused the leaves of Chinese cabbage turned yellow and displayed etiolation. Meanwhile, DBP could be accumulated in different parts of Chinese cabbage. DBP even below 1 mg L^{-1} had a significant effect on the concentration of chlorophyll in Chinese cabbage and the biomass showed a severe decrease under treatment with more than 30 mg L^{-1} of DBP. In proteomics analysis, six protein spots derived

from the normal control and the DBP-treated Chinese cabbage plants showed reproducible differences in the expression in 2-DE. Three proteins appeared or increased while the other three proteins decreased or disappeared during growth in hydroponic culture with DBP added. Three spots of increasing protein were responsible for biosynthesis of fatty acids, signal transduction of phototropic response and nitrate assimilation in plant cells, respectively. The other three spots that disappear or show decrease in protein with DBP treatment were responsible for biosynthesis of flavonoids and floral color development in the cell, aminoacylation of tRNA in the cell, and synthesis of ATP in the cell to restrict growth and development in Chinese cabbage, respectively. In this study, DBP seems to be able to induce physiological reactions or metabolism disorder in the Chinese cabbage cell. We might say that DBP limits the growth and development of Chinese cabbage.

In chapter 3, the results of bioremediation show that aerobic biodegradation rates of PAEs were better than anaerobic biodegradations. The best biodegradation half live ($t_{1/2}$) of 5 ppm DBP in laboratory and soil are 0.65 and 2.23 days by *Deinococcus radiodurans*, respectively. Then, the removal efficiencies of 2 mg L^{-1} DBP by laccase which was extracted from the spent mushroom compost of *Ganoderma lucidum* was 100%, after 1 day of incubation. On the other hand, the best phytoremediation half live ($t_{1/2}$) of 5 ppm DBP is 2.69 days by Garden lettuce. DBP potentially causes osmotic and oxidative stress in Garden lettuce. In addition, since DBP had no significant effects on the morphology and physiological status of Garden lettuce, Garden lettuce can be recommended for use in the plant anti-DBP toxicity test, and also as the candidate plant for DBP phytoremediation.

Based on all the results of this study, we hope that these findings could provide useful information for applying the bioremediation of DBP and assessing the related risk of DBP in the environments. Meanwhile, we also believe that all we have done only the temporary solutions to the problem, the DBP and PAEs contaminated problems still depends on the wisdom of human beings to solve in the end in the future.

INTRODUCTION

Nowadays, our human beings using a lot of plastic products. When we used it, it would be wasted into our environments and then they could be distributed from the river into the soil, groundwater and ocean. In these processes, these plastic products would be break down and became to the different kinds of chemical pollutants. Therefore, the soil, lake, river and groundwater layer would be the sink of them. This result would directly impact the agricultural land which used the river water or groundwater for irrigation of everyday. Among these chemical pollutants, the most concerned group are EDCs.

EDCs are defined as chemicals which interfere with the normal function of hormones that control growth and reproduction in animals, plants, microorganisms and humans. Disruption of the endocrine system can occur in various ways. Some EDCs can mimic or partly mimic naturally occurring hormones in the body such as estrogens (the female sex hormone), androgens (the male sex hormone), and thyroid hormones. Some of them can fooling the body into over-responding to the stimulus, or responding at inappropriate times. Other EDCs can block the effects of a hormone from certain receptors by blocking the receptor site on a cell. Still others directly stimulate or inhibit the endocrine system and cause overproduction or underproduction of hormones.

PAEs which are suspected to be EDCs, have been extensively used as plasticizers in various fields such as food packaging, cosmetics, pesticides, building materials, chemical fertilizer and the pharmaceutical industry (Rudel et al., 2011; Abdel Daiem et al., 2012; Peng et al., 2013; Zhang et al., 2015). Figure 1 shows the mapping of three plasticization theories which are still used nowadays (Bocqué et al., 2016). In general, it is recognized that the low molecular weight of a plasticizer allows reducing secondary forces, such as hydrogen bonding and van der Waals forces, between the polymer chains by occupying intermolecular spaces. Thus, plasticizers change the three-dimensional molecular organization of polymers, decreasing the energy required for molecular motion. As plasticizers, PAEs are characterized by their low water solubility and high octanol/water partition coefficients, but they are not covalently bound to plastics (Staples et al., 1997). Due to their physicochemical properties and wide applications, PAEs are widely distributed in various environmental samples such as groundwater (Zhang et al., 2009), surface water (Kaewlaoyoong et al., 2018), sediment (Chen et al., 2018) and soil (Škrbić et al., 2018).

Meanwhile, some PAEs are also semi-volatile organic compounds (SVOC) and can be present as indoor pollutants and easily leach into air, car, air conditioner and household dust (Wolfe et al., 1980; Bui et al., 2016).

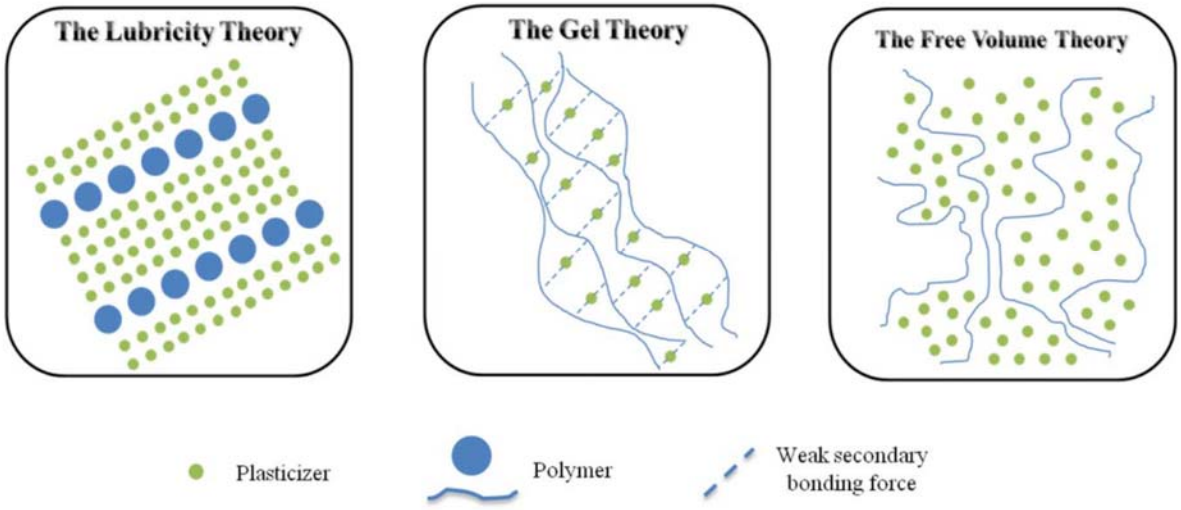

Figure 1. Mapping of three plasticization theories (Bocqué et al., 2016).

DBP is one of the most widely used PAEs and is mainly used as a plasticizer for plastics. The chemical structure of PAEs and DBP are shown in Figure 2. It is an oily liquid that is soluble in fat and slightly soluble in water. It is not very volatile, so it does not readily vaporize into the atmosphere. It has a water solubility of 10 mg L^{-1} at 20 °C and a half-life of 22 years in aqueous solution (Sugatt et al., 1984). In recent years, DBP has been reported to be found in surface water, sediments, drinking water (Ding et al., 2019) and even in human drugs (Kelley et al., 2011). According to the previous study, DBP was estimated to be 46.4% in drinking water and the other three PAEs dimethyl phthalate (DMP), diethyl phthalate (DEP), and DEHP were 4.5%, 6.8%, and 39.4%, respectively (Ji et al., 2014). In recent years, accumulation of DBP in aquatic environments and agricultural land has dramatically increased because of wastewater disposal and high agricultural products such as fertilizers, pesticides, and the use of agricultural plastic mulch bags (Gao and Wen, 2016).

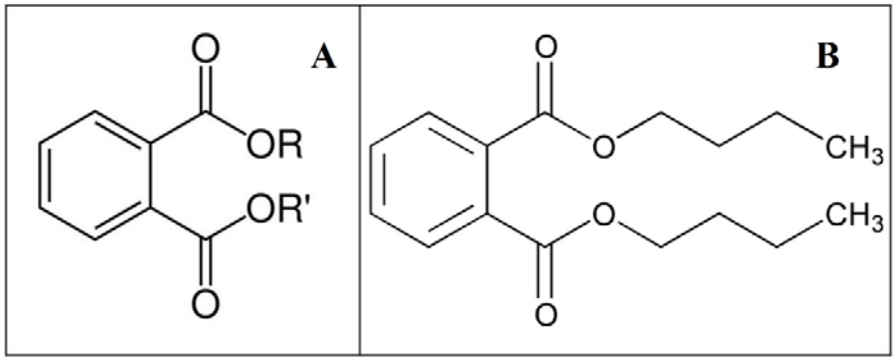

Figure 2. Chemical structure of (A) PAEs and (B) DBP.

While DBP is released into the environment, it can be taken up by crops and then can enter in the food supply chain system. It can also be released into the algae artificial culture system through the same route and might potentially impact the physiology of algae and contaminate its end-products. In terms of human toxicity, there is no evidence that DBP can cause human cancer, however, DBP is of particular concern because it is known to be a reproductive toxin (Rudel et al., 2003) that aggravates autoimmune thyroid disease (Duan et al., 2018). On the other hand, several studies related to the plant toxicity of DBP have been produced. First, the effect of DBP on carotene synthesis during seedling growth has been reported (Hemming et al., 1981; Herring et al., 1988). Subsequently, the relative sensitivity of DBP to cabbage (*Brassica oleracea*) and radish (*Raphanus sativus*) were also reported (Hardwick et al., 1984; Hannay et al., 1986). Some studies also reported DBP can reduce the capsaicin content in capsicum fruit (Capsicum annum) (Yin et al., 2003) and affect leaf color in six plant species (Dueck et al., 2003). A 2016 study indicated increased use of plastic film in greenhouse vegetable production (GVP) could result in PAEs contamination in vegetables. The total PAEs concentrations ranged from 0.14 to 2.13 mg kg^{-1} (mean 0.99 mg kg^{-1}) in soils and from 0.15 to 6.94 mg kg^{-1} (mean 1.49 mg kg^{-1}) in vegetables (Li et al., 2018). The latest study, carried out in 2018, indicated that the toxicity to lettuce of DBP was higher than that of DEHP in soil, and DBP treatment is associated with a decline in lettuce leaf size, as well as chlorophyll *a* and carotenoid content when compared to the control lettuce plants (Ma et al., 2018).

Several studies related to the algae biotoxicity of DBP have been reported. The effects of DBP on the growth rate and chlorophyll concentration was first reported (Chi et al., 2019). This

study revealed that the tolerance of the three microalgae species to DBP followed the order *Cylindrotheca closterium* > *Chaetoceros muelleri* > *Dunaliella salina*. Green alga (*D. salina*) was more sensitive to DBP while benthic diatom (*C. closterium*) exhibited strong adaptive ability to it. The other study investigated the photosynthetic performance of photosystem II (PSII), oxidative stress and the antioxidant reaction induced by reactive oxygen species (ROS) in *C. vulgaris* under the stress of cetyltrimethylammonium chloride (CTAC) (Zhang et al., 2019). However, only a few studies have reported the biotoxic effect of chemical pollutants on microalgae and without discussing the proteomics and GO analysis.

Proteomics analysis is one of the most popular methods to study plant responses to environmental stresses. Proteomics involves investigation of protein-protein interactions and the large-scale determination of gene and cellular function at the proteome level. Mass spectrometry (MS) such as liquid chromatography tandem mass spectrometry (LC/MS/MS), has increasingly become the method of choice for analysis of complex protein samples (Aebersold and Mann, 2003; Liang et al., 2013). The analyzed LC/MS/MS data is uploaded onto a software database such as FunRich (http://www.funrich.org) to obtain the cellular Gene Ontology (GO) annotations through computational comparison. GO is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species. There are three ontologies in GO, biological process, cellular component and molecular function. These three GO ontologies are disjoint, implying that there is no relation between the terms from the different ontologies. GO annotations consist of an association between a gene and a GO term, with supporting evidence in the form of a GO evidence code and either a published reference or description of the methodology used to create the annotation (Gene Ontology Consortium, 2016).

For phytotoxicity, proteomics has been used to study the expression of salt stress related proteins in several plants, which can provide a better indication of cellular activities under salt stress (Parker et al., 2006; Aghaei et al., 2008). However, only a few reports have focused on proteomics studies of DBP-treated plants. According to our previous study, the morphology, chlorophyll concentration and proteome changes on DBP-treated Bok choy were reported (Liao et al., 2006). In the proteomics analysis of Bok choy, stress proteins such as superoxide dismutase (SOD) and the peroxidase 21 precursor were identified. These two proteins were believed to increase in response to free radical exposure as a detoxification mechanism and they might also be used as important molecular markers for DBP tolerance. These reports illustrate that

proteomics analysis could help us to understand the responses of plants under DBP treatment.

Bioremediation is divided into two types: phytoremediation and biodegradation. Phytoremediation is defined as a kind of bioremediation technique using plants to remove or degrade organic and inorganic pollutants in soils, water or air environments (He et al., 2015). There are six types of phytoremediation, including phytosequestration, rhizodegradation, phytohydraulics, phytoextraction, phytovolatilization and phytodegradation. Several studies have reported the phytoremediation of PAEs (Cai et al., 2006; Cai et al., 2008), however, only a few studies have used the proteomics method to analyze plant responses in the phytoremediation of PAEs (Zhang et al., 2015).

Biodegradation is an effective strategy to remove organic pollutants from environments. The microbial degradation of the organic contaminants depends on the availability of molecular oxygen in the environment. Metabolic breakdown of DBP by microorganisms is considered to be one of the major routes of environmental degradation for this widespread pollutant (Wolfe et al., 1980). A number of studies have reported that the aerobic degradation of DBP is more effective than anaerobic conditions in soils, sediments and sludge (Yuan et al., 2002; Wang, 2004). In 2010, researchers used the strain *Rhodococcus jostii* RHA1 to degrade DBP and constructed the biodegradation model and processes, including transesterification, demethylation, and ester hydrolysis (Hara et al., 2010). Figure 3 shows a related study which has fully presented the microbial degradation of PAEs under aerobic and anaerobic conditions, including all intermediate products and required enzymes in 2015 (Benjamin et al., 2015). However, little is known about the survival status of DBP degrading bacteria under the original isolated environment such as river sediment and activated sludge.

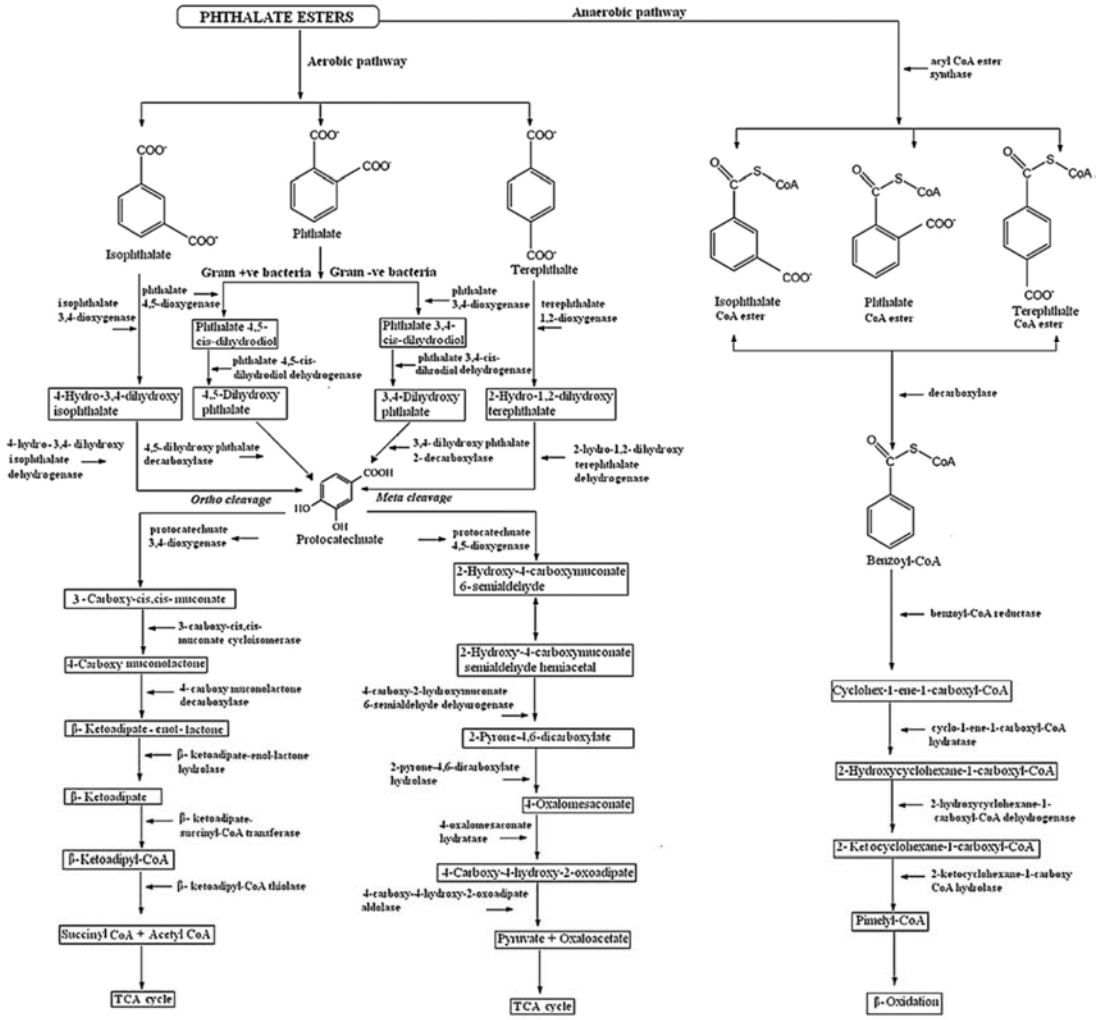

Figure 3. Microbial degradation of PAEs under aerobic and anaerobic conditions (Benjamin et al., 2015).

Therefore, based on the ecological remediation and agricultural protection, the objectives of this study are:

(1). To investigate the distribution of PAEs in terrestrial and aquatic environments of Taiwan.

(2). To evaluate the phytotoxicity of DBP on plants.

(3). To analyze the proteome changes of DBP treated plants.

(4). To construct the optimal culture conditions for DBP bioremediation by bacteria, fungi, and plant.

(5). To analyze the survival status of DBP degrading bacteria under the original isolated environment.

Figure 4. Flowchart of this study.

CHAPTER I:

Environmental distribution of phthalate esters in terrestrial and aquatic environments in Taiwan

1. INTRODUCTION

Located on the western edge of the Pacific Ocean, subtropical Taiwan is home to 129 rivers-21 classified as primary and 29 secondaries. As a newly industrialized country with only a recent history of environmental controls, severe damage has been done to the water quality of island. In sediment samples taken from points along the Tanshui River in the Taipei metropolitan area, Liu et al., (2000) identified 16 polycyclic aromatic hydrocarbons (PAHs), seven chlorobenzenes and two PAEs. PAEs, which are present in both urban and industrial wastes, show low persistence in water due to their solubility, adsorption on particulate matter, and biodegradation processes.

Soils and sediments are the major “sink” for PAEs (Tran et al., 2015). Plastic mulching films are considered the main anthropogenic source of PAEs contamination in agricultural areas due to the existence of great amount of PAEs in the films (Li et al., 2016). PAEs contamination can also come from pesticides, fertilizers, industrial discharge, sewage, etc. (Ma et al., 2013; Li et al., 2016). PAEs contamination of agricultural soil could result in PAE-contaminated agricultural products, therefore, raising concerns about subsequent health risks (Yang et al., 2015).

The incident that two Taiwanese chemical companies intentionally used PAEs as an emulsifier (or clouding agent) for their food processing has attracted wide public attention to health risks caused by PAEs exposure (Wu et al., 2012). Kaohsiung City, located in the South of Taiwan, is the most industrialized city in the island with a population of 2.77 million. Ever since having been founded in the seventeenth century, Kaohsiung City has rapidly grown into an important economic and industrial center of Asia. Along with that, the demand for food and agricultural products has risen intensively. The North of the city is a large agricultural production area, providing agricultural products for the city itself and other provinces of Taiwan. This area is also where industries have blossomed in the recent decades, leading to the arrival of many industrial plants (Lin et al., 2017; Vu et al., 2017). Therefore, there are increasing concerns about the potential contamination that those plants may cause to the agricultural production area and the potential associated health risks to humans.

The purpose of chapter 1 is to measure the distribution and concentration of PAEs in terrestrial and aquatic environments of Taiwan. We investigated the distribution of PAEs in 14 river surface water samples and 6 sediment samples of Taiwan in 2000. Then we investigated the distribution of PAEs in soil, fertilizers and plastics materials taken around the major industrial

area in Kaohsiung City, Taiwan in 2013. River surface water and sediment samples were collected from 14 main rivers of Taiwan. Soil samples were collected from agricultural fields (AF), household back gardens (BG) and along the roadside (RS) around the major industrial area in Kaohsiung City. Additionally, the non-cancer and carcinogenic risks of the exposure to PAEs in the soil for adults and children were estimated via dietary and non-dietary pathways. In addition, PAEs content in chemical and organic fertilizers, plastic mulching films and other types of plastics was measured and discussed concerning the potential sources of PAEs contamination. The results of this study will provide a baseline for PAEs contamination around the major industrial plants of Kaohsiung City, thus supporting a reliable reference for future environmental monitoring and protection plans.

2. MATERIALS AND METHODS

2.1 Chemicals

All 99.0% analytical standards PAEs, including diethyl phthalate (DEP), dipropyl phthalate (DPP), DBP, diphenyl phthalate (DPhP), benzylbutyl phthalate (BBP), dihexyl phthalate (DHP), dicyclohexyl phthalate (DCP), DEHP, dimethyl phthalate (DMP), and di-*n*-octyl phthalate (D*n*OP) were purchased from AccuStandard, Inc., New Haven, CT. All other chemicals were purchased from Sigma (St. Louis). Solvent was HPLC-graded.

2.2 Sampling

2.2.1 River Samples

The locations of the sampling sites are presented in Figure 5; samples were collected between January and August 2000. The 14 water samples were collected from the Zhonggang, Keya, Erren, Gaoping, Donggang, Danshui, Puzi, Bazhang, Jiangiun, Jishui, Zengwen, Agongdian, Beinan and Waishuang Rivers. The six sediment samples were collected from the Zhonggang, Keya, Erren, Gaoping, Donggang and Danshui Rivers. All sites are considered to be among the most heavily contaminated in Taiwan. Su rface (0-20 cm depth) water samples were collected in 10 L glass bottles and stored at 4 ℃. Sediment samples (top 10 cm layer) were collected with an Ekman grab sampler and stored at 4 ℃. There were five sample points for each site. All bottles or grabs of one site were manually mixed to visual homogeneous extent and then sampled for analysis. All experiments were performed in duplicate.

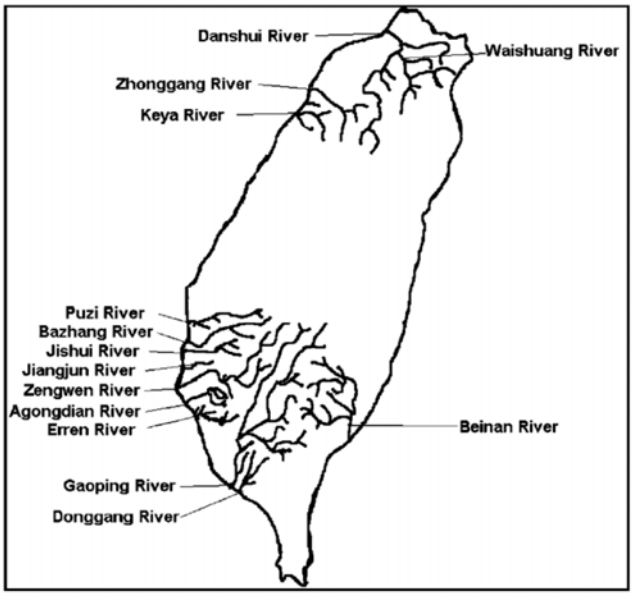

Figure 5. Taiwan river sampling sites.

2.2.2 Soil Samples

Kaohsiung City, located in the South of Taiwan, is one of the most rapidly growing industrial cities in Asia. Numbers of industrial plants with various large-scale industries have arrived in the northern area of the city, including Nanzih Export Processing Zone (NEPZ), Dashe Industrial Park and Renwu Industrial Park (Fig. 6). Renwu Industrial Park and Dashe Industrial Park specialize in plastic resin production, whereas NEPZ contains mostly metal processing industries. These are the major industrial plants of Kaohsiung City and have been reported to be associated with various types of contaminants (Lee et al., 2018; Lin et al., 2009, 2010). However, even though these industrial plants have resided in the northern agricultural and gardening area of the city for decades, there have been no studies examining the PAEs contamination in the soil and investigating potential PAEs contamination sources here. In this study, the sampling campaign covered a large area of three northern districts of Kaohsiung City, namely Renwu, Dashe and Nanzih districts. The vegetable planting area located within 2.5 km away from the abovementioned industrial plants were selected to be the sampling sites. The sampling sites included a large paddy/vegetable/flower field in the north-west of the city and numerous vegetable gardens located around the three industrial plants mentioned. To avoid the dilution and complication effects of precipitation, total 24 soil samples were collected in the agricultural fields

(AF), back gardens (BG) and roadsides (RS) sites around the three industrial plants within one week in the dry season (from February to June) of 2013. During this dry season, the ambient temperature fluctuated between 22 and 30 °C and precipitation was insignificant. Soil samples were collected on sunny days, between 9.00 am and 4.00 pm.

Before sampling, some piled litter and debris on the soil surface were removed. Five surface soil samples (located on the crossing diagonals: four in the corners and one in the crossing point, depth 0-10 cm below ground surface) were collected from 5 x 5 m^2 or other smaller plots with a pre-cleaned stainless-steel scoop and homogeneously mixed to form a composite sample. Approximately 50 g of soil sample was collected from each sampling point. In addition, 13 fertilizer and 27 plastic samples were collected to investigate the source of PAEs contamination in AF and BG sites. Similar to soil samples, five fertilizer samples were collected with the stainless-steel scoop and mixed together to create a composite sample. Meanwhile, plastic samples were rinsed with deionized (DI) water, cut into 0.5 × 0.5 cm pieces, and dried by a vacuum freezedrying machine (Eyela FDU-1200, Tokyo Rikakikai, Japan, -50 °C, 10 Pa, 24 h). At least, a duplicate sample (soil, fertilizer and plastic) was collected for each site. The samples were kept in aluminium bags at 6-8 °C by a temperature-controlled box during the transportation to the laboratory. Precautions were performed during the sampling and sample analysis to avoid PAEs contamination (Lin et al., 2009). Soil and fertilizer samples were homogeneously mixed and screened through a stainless-steel sieve (60 mesh). Then, all the samples were stored at 4 °C until sample extraction. Note that all samples were extracted within 14 days after the sampling.

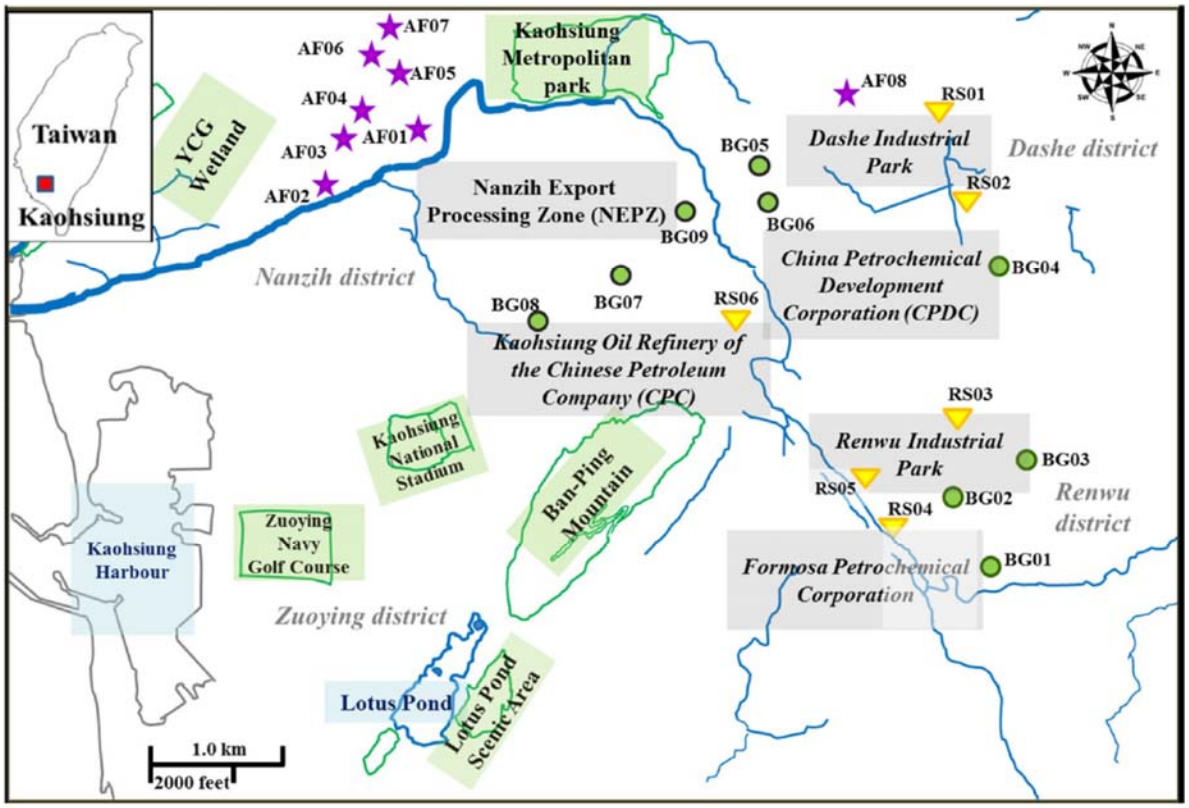

Figure 6. Sampling sites of agricultural fields (AF: purple star symbols), back gardens (BG: green circle symbols) and roadsides (RS: yellow trigonometry symbols) in Kaohsiung City, Southern Taiwan.

2.3 Analytical methodology

2.3.1 River Samples

With slight modifications, PAEs extraction and analysis were performed as described in USEPA SW-846 method 8270 (USEPA, 1995). Briefly, 10 g of dry sediment samples were extracted with 300 ml hexane for 18 h, and 200 ml of water samples were extracted with 100 ml hexane three times each. Extracts were reduced with a rotary evaporator to concentrates of less than 5 ml; remaining water was removed by the addition of anhydrous sodium sulfate. Co-extracted sulfur was removed by the addition of acid-washed copper powder. Extracts were then placed in Kuderna-Danish tubes and blown under a gentle stream of nitrogen to exactly 1 ml for GC/MS analysis. Analyses were performed with a Finnigan MAT Automass I gas chromatograph coupled with a mass selective detector and DB-5 capillary column (film thickness, 0.25 lm; inner diameter, 0.25 mm; length, 30 m) (J.W., USA). Injector and detector temperatures

were set at 250 and 320 ℃, respectively. Helium was used as carrier gas at a flow rate of 0.8 ml min^{-1}. Oven temperature was maintained at 50 ℃ for 4 min, increased at a rate of 6 ℃/min to 160 ℃ and held for 1 min, increased at a rate of 10 ℃ min^{-1} to 280 ℃ and held for 1 min, then increased at 10 ℃ min^{-1} to 300 ℃; total run time was approximately 60 min. Recovery percentages were 85.5%, 83.5%, 99.5%, 86.5%, 90.0%, 89.0%, 92.2% and 99.5% for DEP, DPP, DBP, DPhP, BBP, DHP, DCP and DEHP, respectively. Detection limits were 0.6, 0.8, 1.0, 0.6, 1.0, 0.8, 0.6 and 1.0 μg L^{-1}, also respectively.

2.3.2 Soil Samples

The procedure of sample extraction and analysis in this study followed that in our previous study (Lin et al., 2009). Basically, samples (10 g each for of soil and fertilizer and 0.5 g each for plastic) were added and shaken in a conical flask with 30 mL dichloromethane (DCM) for 15 min. The process was repeated three times. The supernatants were combined and concentrated to 0.5 mL in a Turbo Vap (Biotage, USA) using gentle nitrogen flow (5 psi). Known quantity of the internal standard was added to the final extracts (0.5 mL) prior to the injection into the GC/MS for analysis. A 6890-5973 gas chromatography-mass selective detector (GC-MS) system was employed to quantify PAEs in an HP-5MS (30 m × 0.25 mm × 0.5 μm) capillary column (injection volume 1 μL, helium flow rate 1 mL min^{-1}). Oven temperature was initially set at 45 °C for 1 min before rising to 310 °C at the rate of 10 °C min^{-1} and maintained there for 7 min. Selective ion monitoring (SIM) mode was performed with the specific retention time and characteristic ions in order to help increase the accuracy of an analysis. All calculation of concentrations and calibrations were performed according to the previously built standard calibration curves. Eight calibration levels (1.25, 5, 10, 20, 40, 60, 80 and 100 μg mL^{-1}) were considered. Relatives response factor of all compounds were above 0.05, whereas the percentage of deviation of each compound was lower than 20%. In order to guarantee good data quality, a decafluoro triphenyl phosphine (DFTPP) standard check was implemented to calibrate the mass selective detector before analysis. System proficiency calibration check (SPCC) was also performed once every 12 h of analysis. Blank and spike recovery tests were performed afterwards. Method detection limit (MDLs) were estimated from field blanks and seven samples of the same concentration. An individual MDL analyze was calculated from the product of the standard deviation by the appropriate one-sided 99% t statistic. The MDL values ranged from 0.11 mg kg^{-1}

dry weight for DEP to 0.13 mg kg^{-1} dry weight for DEHP. The blanks showed non-detected (ND) values. Standard mid-point and spiked-sample checks were performed every 10-sample batch. Standard mid-point and spiked-sample recoveries were from 94 to 108% and from 80 to 85%, respectively. Accuracies of the measures were 100 ± 10%.

2.4 Analytical methodology

SPSS for Windows, Version 22, was employed for statistical analysis in this study. Pearson's bilateral test was used to analyze the correlation between soil PAEs and basic soil properties. Student's t test was employed to examine the correlation between PAEs concentrations in two fertilizer groups and similarly in two plastic groups. Differences were regarded as significant with 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1 Occurrence of phthalate esters in Taiwan river water and sediments in 2000

Table 1 presents the PAEs concentration ranges and averages for the 14 water and six sediment samples. Respective concentrations of DEHP in water and sediment were ND-18.5 μg L^{-1} and 0.5-23.9 μg g^{-1}; of DBP, 1.0-13.5 μg L^{-1} and 0.3-30.3 μg g^{-1}; of DEP, ND-2.5 μg L^{-1} and 0.1-1.2 μg g^{-1}; and of DPP, ND-1.8 μg L^{-1} and ND-1.1 μg g^{-1}. Concentrations of DHP, BBP, DCP and DPhP were all below their detection limits. Categorized in terms of concentration levels in Taiwan rivers, DEHP and DBP were placed in the high category, DEP and DPP at the second category and the rest below the detection limit. This finding is supported by Vitali et al.,'s (1997) observation that DEHP and DBP are the most commonly produced PAEs. Furthermore, according to the results of a study conducted by the Environment Protection Agency Japan (EPAJ, 1993), 0.009-3.5 μg g^{-1} concentrations of DEHP were found in all 45 Japanese river sediment samples collected and tested. The DEHP concentrations we measured for the present study were much higher than those reported by the EPAJ for sediment samples taken from Japanese and Italian rivers. In addition, we found that concentrations of all eight PAEs were up to 1000 times higher in our sediment samples than in our surface water samples. This finding is supported by Al-Omran and Preston (1987) and Matsuda and Schnitzer (1971), who reported that PAEs are soluble by fulvic and humic acids, and are capable of being adsorbed onto particulate matter, with sediments serving as a final sink.

Table 1. Phthalate esters found in river surface waters and sediments in Taiwan.

PAEs	Water (μg/l)		Sediment (μg/g)	
	Range	Average	Range	Average
DEP	ND–2.5	0.5	0.1–1.1	0.2
DPP	ND–1.8	0.1	ND–1.1	0.1
DBP	1.0–13.5	4.9	0.3–30.3	6.3
DPhP	ND	ND	ND–1.2	0.1
BBP	ND	ND	ND–1.8	0.2
DHP	ND	ND	ND–1.9	0.2
DCP	ND	ND	ND–1.9	0.2
DEHP	ND–18.5	9.3	0.5–23.9	4.6

A comparison of total eight PAEs values in our river sediment samples revealed a high-to-low presence in the Zhonggang => Keya => Erren => Gaoping => Donggang => Danshui Rivers (Table 2). For the rest of this discussion we will focus on a comparison of PAEs concentration and biodegradation in the samples taken from the Zhonggang and Danshui Rivers, which had the highest and lowest concentrations, respectively. Both the rivers are heavily polluted. The Zhonggang River runs near the Hsinchu Science-Based Industrial Park, less than 100 km south of Taipei; many of the Park's factories discharge wastewater into the river. The Danshui skirts the western edge of Taipei City on its way to the Taiwan Strait; it has been subjected to continuous pollution for the last 40 years, with pollution sources including municipal sewage, industrial waste, and emission from pig farms. PAEs have been discharged into the Danshui River for much longer than they have into the Zhonggang River. One possible explanation for the lower levels of PAEs in Danshui River sediment may be the presence of PAEs-adapted anaerobic consortia, resulting in greater biodegradation activity based on microbial action.

Table 2. Total PAEs values in sediment samples.

River	PAEs (μg/g)
Zhonggang River	27.1 ± 3.8
Keya River	23.9 ± 2.5
Erren River	7.1 ± 0.8
Gaoping River	4.0 ± 0.5
Donggang River	3.2 ± 0.4
Danshui River	2.9 ± 0.3

3.2 Occurrence of PAEs around the major plastic industrial area in southern Taiwan in 2013

Figure 7 and Table 3 provide information about PAEs composition in soils taken from AF, BG and RS sites. For AF sites, the total of six PAEs concentrations ranged from 0.1 to 1.61 mg kg^{-1} (0.65 ± 0.47 mg kg^{-1}). Among the six PAEs, DEHP was the most popular PAEs compound in soil samples. The highest and second highest concentrations of DEHP were observed at the sites AF08 (1.53 mg kg^{-1}) and AF02 (0.95 mg kg^{-1}), respectively. In three other AF sites, DEHP was also the most popular PAEs compound, indicating the widespread contamination of DEHP in AF sites. A large area of the site AF08 was planted with lemon and gurva (Table 3). During the sampling, plastic bags used to cover the ripening fruit were observed on the ground and nearby grasses at this site. Those plastic bags were discarded after the harvesting of the fruit and not collected for further disposal and/or treatment. This environmentally unfriendly practice of the local farmers probably contributed to the very high PAEs concentrations in soil in site AF08. In addition, during the sampling, we observed that the sites AF01, AF02, AF05, AF06 and AF08 were receiving an additional amount of chemical fertilizer as these fields were near to the harvest. Chemical fertilizer was known to contain high concentrations of PAEs and other organic contaminants (Staples et al., 1997; Mo et al., 2008; Wang et al., 2013) and the effects of applying fertilizer on soil PAEs contamination will be discussed in "PAEs contamination in chemical and organic fertilizers". D*n*OP was the most popular PAEs at the sites AF05 (0.61 mg kg^{-1}) and AF06 (0.64 mg kg^{-1}), making it the second most popular PAEs compound in AF soils. DEHP and D*n*OP have been well documented to occur in plastic mulching film, and many chemicals and fertilizers used for farming practices (Mo et al., 2008; Wang et al., 2013; Li et al., 2016a). The differences in observed PAEs composition at different AF sites were mainly due to the use of different types of plastic mulching film, fertilizers and other plastic farming materials and

equipment.

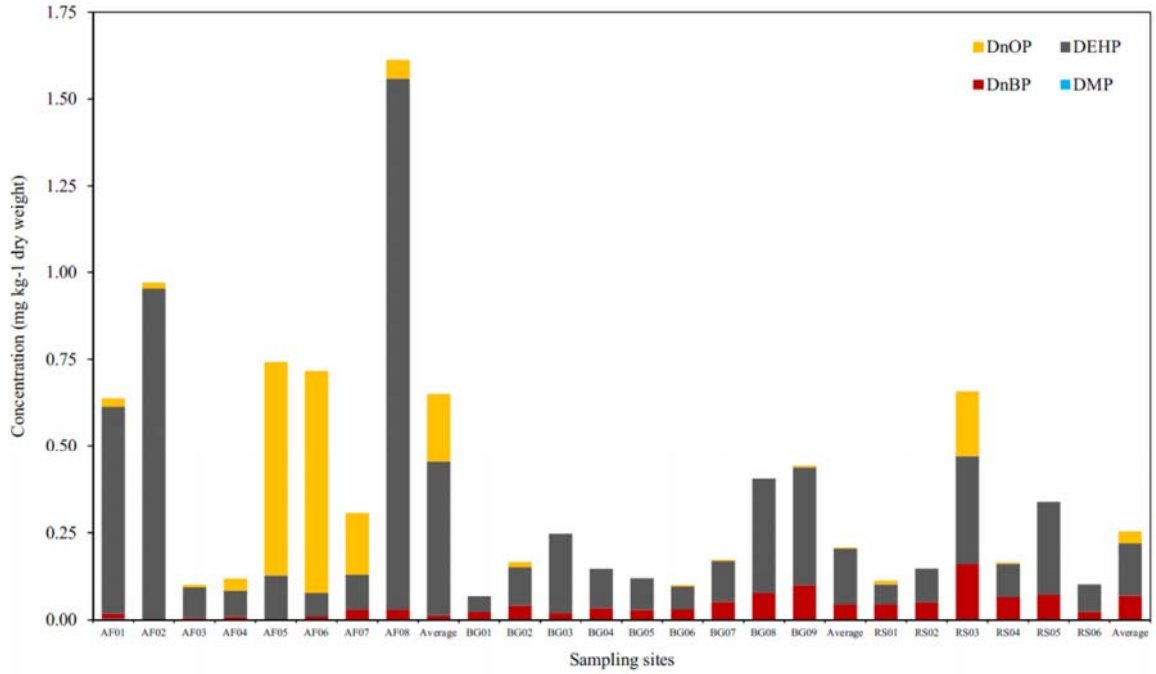

Figure 7. PAEs composition in soil samples taken from AF, BG and RS sites in Kaohsiung City, Taiwan. DEHP and D*n*OP accounted for the most of PAEs concentrations in soil (> 90%). DEP and BBP were not found, while DMP was only detected in negligible amount in the soil sample collected at site AF01.

Table 3. PAEs concentrations (mg kg^{-1}) taken from AF, BG and RS sites in Kaohsiung City, Taiwan; and allowable and cleanup levels issued by the New York State Department of Environmental Conservation.

PAEs	Agricultural fields (AF) (n = 8)						Back gardens (BG) (n = 10)						Roadside (RS) (n = 6)						Allowable levels	Cleanup levels
	Range	Mean ± SD	Median	S	K		Range	Mean ± SD	Median	S	K		Range	Mean ± SD	Median	S	K			
DEHP	0.07	1.53	0.44 ± 0.51	0.11	1.42	1.10	0.05	0.34	0.16 ± 0.10	0.11	0.93	-0.77	0.06	0.31	0.15 ± 0.10	0.10	0.96	-1.44	4.35	50
DBP	0.00	0.03	0.01 ± 0.01	0.01	0.78	-0.84	0.02	0.10	0.04 ± 0.03	0.03	1.29	0.76	0.02	0.16	0.07 ± 0.04	0.06	1.72	3.59	0.081	8.1
D*n*OP	0.00	0.64	0.20 ± 0.25	0.04	1.30	-0.25	0.00	0.01	0.003 ± 0.005	0.004	2.28	5.64	0.00	0.19	0.03 ± 0.07	0.002	2.43	5.93	1.2	50
BBP	ND	-	ND -	ND	-	-	ND	-	ND -	ND	-	-	ND	-	ND -	ND	-	-	1.125	50
DEP	ND	-	ND -	ND	-	-	ND	-	ND -	ND	-	-	ND	-	ND -	ND	-	-	0.071	7.1
DMP	0.00	0.003	0.001 -	0.001	2.83	8.00	ND	-	ND -	ND	-	-	ND	-	ND -	ND	-	-	0.02	2
Σ PAEs	0.10	1.61	0.65 ± 0.47	0.68	0.87	0.91	0.07	0.44	0.21 ± 0.13	0.17	1.08	-0.14	0.10	0.66	0.25 ± 0.20	0.16	1.74	2.73		

**** S.D.: Standard Deviation; ND: Not Detected; S: Skewness; K: Kurtosis***

PAEs contamination in AF sites of this study was compared to the findings of other similar studies around the world to briefly assess the PAEs contamination status in the agricultural soil of Kaohsiung City (Table 4). Most studies concerning PAEs contamination in agricultural soil were carried out in China, suggesting that this country has been incurred a heavy PAEs contamination (Li et al., 2016b). The comparison showed that PAEs contamination in Kaohsiung City was not as serious as that in several parts of China but was still more serious than that in some other areas of China and other countries. The most serious PAEs contamination in agricultural soil was found in the rapidly developed area of Shandong Peninsula (Li et al., 2016b), the intensive agricultural area of Heilongjiang (Xu et al., 2008) and the outskirts of Beijing (Ma et al., 2003) in China. High PAEs contamination in those areas could be due to their intensive farming and usage of plastic mulching films (Li et al., 2016b). It is noticeable that although our findings of DMP were three orders of magnitude lower than those of the highest found in agricultural soil in Shandong Peninsula (Li et al., 2016b), they were still one order of magnitude higher than the findings in the rural area fertilized by wastewater treatment sludge in Paris, France (Tran et al., 2015). DBP concentrations in our study were two to three orders of magnitude lower than those in the studies conducted in Beijing (Ma et al., 2003), Shandong Peninsula (Li et al., 2016b) and Heilongjiang (Xu et al., 2008). DEHP, though representing the highest concentrations among the six PAEs in Kaohsiung City's agricultural soil, showed much lower concentrations than those in the similar studies conducted in the agricultural and industrial areas of the Yellow River Delta (Yang et al., 2013), of the suburban areas of Nanjing (Wang et al., 2013), of Heilongjiang (Xu et al., 2008), of Beijing (Ma et al., 2003) in China. Yet, Kaohsiung City's DEHP content was approximately twice and three times higher than that of the suburban soils of Tianjin (Kong et al., 2012) and the wastewater-irrigated soils of Hebei (Zhang et al., 2015) in China, respectively. D*n*OP content in this study, however, was among the three highest D*n*OP findings. Kaohsiung City's D*n*OP content was only lower than that of Shandong Peninsula (Li et al., 2016b) and of Nanjing (Wang et al., 2013). Kaohsiung City's D*n*OP was in fact one and two orders of magnitude higher than that in the study conducted in 31 Chinese provinces (Niu et al., 2014) and the rapidly growing city of Guangzhou, China (Zeng et al., 2008), respectively.

Table 4. Comparison of PAEs concentrations (values are shown in mean ± standard deviation or mean) between different similar studies in different geographical areas around the world (mg kg^{-1}).

	DMP	DEP	D*n*BP	BBP	DEHP	D*n*OP	References
Agricultural soil							
Kaohsiung, Taiwan	0.0004 ± 0.001	ND	0.01 ± 0.01	ND	0.44 ± 0.51	**0.2 ± 0.25**	This study
Shandong Peninsula, China	**0.14 ± 0.2**	**0.34 ± 0.44**	**1.99 ± 2.55**	**0.49 ± 1.16**	0.29 ± 0.46	**0.36 ± 0.77**	Li et al. (2016b)
Paris, France	0.001	0.005	0.004	0.0004	0.12	0.004	Tran et al. (2015)
Hebei, China	0.02 ± 0.01	0.003 ± 0.003	0.05 ± 0.01	0.02 ± 0.03	0.14 ± 0.05	0.04 ± 0.02	Zhang et al. (2015)
31 provinces in China	0.01 ± 0.01	0.003 ± 0.002	0.07 ± 0.05	0.00004 ± 0.00004	0.82 ± 0.76	0.02 ± 0.03	Niu et al. (2014)
Nanjing, China	0.01 ± 0.01	0.01 ± 0.01	0.26 ± 0.38	0.01 ± 0.01	**2.45 ± 2.04**	**0.46 ± 0.45**	Wang et al. (2013a)
Yellow River Delta, China	0.03 ± 0.003	0	0.41 ± 0.05	0	**1.22 ± 0.11**	0.01 ± 0.001	Yang et al. (2013)
Five regions in Czech Republic			**1.1**		**1.31**		Daňková et al. (2012)
Tianjin, China	0.02 ± 0.02	0.03 ± 0.02	0.07 ± 0.04	0.07 ± 0.27	0.26 ± 0.4	0.07 ± 0.13	Kong et al. (2012)
Guangzhou, China	0.01	0.01	0.12	0.004	0.6	0.01	Zeng et al. (2008)
Heilongjiang, China			**15.46**		**4.61**		Xu et al. (2008)
Beijing, China			**2.96**		**2.7**		Ma et al. (2003)
Roskilde, Denmark			0.002	0.0001	0.03	0.004	Vikelsoe et al. (2002)
Chemical fertilizer							
Kaohsiung, Taiwan	ND	ND	0.01 ± 0.03	**0.01 ± 0.01**	0.22 ± 0.35	**0.1 ± 0.25**	This study
China	**0.02**	**0.0001**	**0.16**	0.002	0.07	0.0002	Mo et al. (2008)
Roskilde, Denmark			0.09 ± 0.002	**0.04 ± 0.01**	**1.1 ± 0.13**	0.04 ± 0.004	Vikelsoe et al. (2002)
Organic fertilizer							
Kaohsiung, Taiwan	ND	ND	0.03 ± 0.02	0.03 ± 0.02	0.05 ± 0.03	**0.04 ± 0.02**	This study
China	0.001	ND	0.0004	ND	0.0008	ND	Mo et al. (2008)
Roskilde, Denmark			0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.001 ± 0.001	Vikelsoe et al. (2002)
Plastic mulching films							
Kaohsiung, Taiwan	ND	ND	ND	**3.2 ± 1.8**	**67.06 ± 32.78**	**157.76 ± 96.23**	This study
Beijing, China	0.09	0.04	3.2	0.08	5.15	0.04	Li et al. (2016a)
Nanjing, China	3.95	**3.76**	**15**	**3.05**	**105**	75	Wang et al. (2016)
China	**1240 ± 111.6**	ND	ND			**270 ± 29.4**	Zhou et al. (2012)

In bold are values of 'high' concentrations for a specific PAE compound

ND not detected

Figure 8 shows PAEs concentrations in chemical and organic fertilizer samples. This sampling design was dedicated to measure differences between these two commonly used categories of fertilizer, which in turn should help to investigate the main source of PAEs contamination in soil. In this study, six samples of organic (compost) and seven samples of chemical fertilizer were collected in different AF and BG sites. The results showed that the content of BBP in organic fertilizer (0.03 ± 0.02 mg kg^{-1}) was higher than that in chemical fertilizer (0.005 ± 0.01 mg kg^{-1}) ($p < 0.05$). DEHP and D*n*OP concentrations in organic fertilizer

(0.05 ± 0.02 and 0.04 ± 0.02 mg kg^{-1}, respectively), on the other hand, were far lower than those in chemical fertilizer (0.22 ± 0.35 and 0.1 ± 0.25 mg kg^{-1}, respectively) ($p < 0.05$). The total PAEs concentrations in chemical fertilizer were higher than that in organic fertilizer ($p < 0.05$). Therefore, it is still reasonable to conclude that organic fertilizer was less PAE-contaminated than chemical fertilizer. Our finding was similar to that of Mo et al., (2008) when PAEs concentrations in the organic fertilizer were one to two orders of magnitude lower than those in the most commonly used commercial chemical fertilizer in China (Table 4). Likewise, in the study of Vikelsoe et al., (2002), PAEs content in chemical fertilizer (0.34 ± 0.64) was 2-40 times higher than that in organic fertilizer (0.15 ± 0.08). The production of chemical fertilizer could be plastic-involved in multiple sections, not to mention the packaging of this type of fertilizer (Vikelsoe et al., 2002). Organic fertilizer, however, is often produced naturally, making it less contaminated with hydrophobic contaminants (Mo et al., 2008). Therefore, future pollution management and human-wellbeing improvement plans should take into account the use of organic fertilizer in farming.

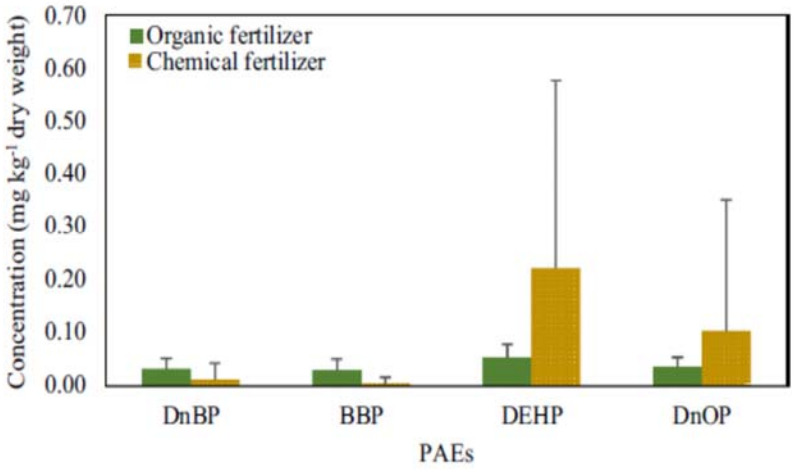

Figure 8. PAEs concentrations in organic and chemical fertilizer samples taken in AF and BG sites

Figure 9 shows PAEs concentrations in plastic samples of mulching films and other types of plastic materials used in agricultural and gardening activities taken from AF and BG sites. Initially, plastic mulching films were collected because they were reported to be the main source of PAEs contamination to agricultural soil (Li et al., 2016a). However, during the sampling, we noticed that there were other types of plastic materials that were frequently used in agricultural

and gardening activities, such as water hose, plastic bags used to cover the ripening fruit, plastic gloves, plastic boots and some other plastic gardening tools. These plastic materials occurred diffusely on the agricultural fields and the gardens. Although the amount of these plastic materials was not as high as that of mulching films, they could also add up to the PAEs contamination in soil given that they contain higher PAEs levels. The analytical results of 19 plastic mulching film samples and 8 samples of other types of plastic materials showed that PAEs concentrations in plastic mulching films were much lower than those of the other types of plastic materials, especially in the case DEHP (67.06 ± 32.78 vs 302 ± 208.58 mg kg^{-1}) and D*n*OP (157.76 ± 96.23 vs 862.28 ± 599.05 mg kg^{-1}) ($p < 0.05$). As for DBP (ND vs 7.73 ± 6.2 mg kg^{-1}) and BBP (3.2 ± 1.8 vs 13.1 ± 9.17 mg kg^{-1}), although the results were less different between the two plastic groups, high discrepancies between plastic mulching films and the other types of plastic materials occurred ($p < 0.05$). The finding of PAEs content in plastic mulching films in this study was comparable to that in the studies conducted in Nanjing (Wang et al., 2016) and across China (Zhou et al., 2012), but was far higher than that in the study conducted in Beijing (Li et al., 2016a) (Table 4). Besides, we could not make a similar comparison to PAEs content in the mulching films for our finding for the other types of plastic materials used in farming and gardening activities since they have not been studied in the literature. The results of this study suggest that in addition to the pollution control for PAEs levels in plastic mulching films, other plastics-related materials employed in agricultural and gardening activities should also be taken into consideration.

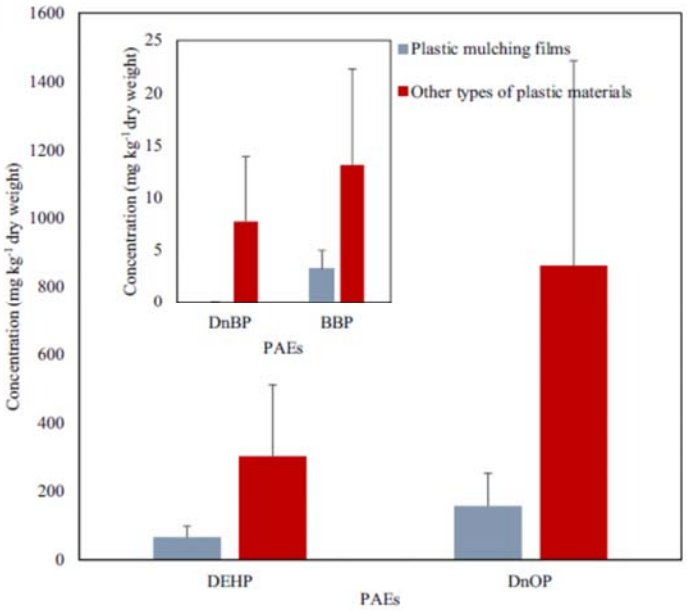

Figure 9. PAEs concentrations in plastic mulching films and other types of plastic materials used in farming and gardening activities taken in AF and BG sites.

4. BRIEF SUMMARY

The results of PAEs environmental distribution shows that DBP and DEHP were found to be higher than the other PAEs in the river water and sediment in Taiwan in 2000. DBP concentrations in the water and sediment samples were 1.0-13.5 μg L^{-1} (ppb) and 0.3-30.3 μg g^{-1} (ppm), respectively. On the other hand, DEHP and D*n*OP accounted for the most of PAEs concentrations in soil (> 90%) in 2013. DBP were almost found in each soil sample in Taiwan, the mean concentration was 0.01 mg kg^{-1}. In addition, major sources of PAEs in agricultural soil could be the use of chemical fertilizer and plastic products.

CHAPTER II:

Phytotoxicity of endocrine disruptor di-n-butyl phthalate on the proteome level of vegetables

1. INTRODUCTION

As we mentioned in the introduction section of this thesis, several related studies on the phytotoxicity of DBP have been published. These results indicated that DBP could cause different effects on different plants, such as growth inhibition, carotene synthesis disturbance, leaf crinkle, and chlorophyll concentration decreased. Although the results showed that DBP can be taken up and have adverse effects on plants, but only a few reports have focused on proteomics studies of DBP-treated plants.

In view of this, we used hydroponic methods to cultivate Bok choy (*Brassica rapa* subsp. *chinensis*) in our previous study with DBP addition to the water for cultivation (Liao et al., 2006). DBP was absorbed throughout the plant from root to leaf. In the proteomics analysis of Bok choy, stress proteins such as superoxide dismutase (SOD) and the peroxidase 21 precursor were identified. These two proteins were believed to increase in response to free radical exposure as a detoxification mechanism and they might also be used as important molecular markers for DBP tolerance. These reports illustrate that proteomics analysis could help us to understand the responses of plants under DBP treatment. The present research was designed to study Chinese cabbage (*Brassica rapa* var. *chinensis*), a common vegetable and commonly used in Chinese cuisine and have been cultivated for over 6000 years in East Asia, for understanding its phytotoxicity and proteomics level changes.

In order to investigate the potential effects of DBP on vegetables. The purpose of this study was to evaluate the phytotoxicity of DBP on common vegetable Chinese cabbage. In addition, proteome changes of Chinese cabbage exposed to DBP were also investigated. The ultimate goal of this chapter is to obtain more information on the phytotoxicity of plants exposed to DBP.

2. MATERIALS AND METHODS

2.1 Chemicals

The standard reagents, DBP (98.7% purity, CAS: 84-74-2) was purchased from Riedel-deHaën Co., Germany. Chlorophyll a standard (from spinach, CAS: 479618) was purchased from Sigma-Aldrich Chemical Co., MI, USA. The solvents used in this experiment including ethanol and acetone were purchased from E. Merck (Germany). All other chemicals were purchased from Sigma-Aldrich Chemical Co., MI, USA. All reagents used were reagent HPLC-grade. Individual DBP stock solutions were dissolved with acetone at 10000 mg L-1

before use. The glassware was thoroughly cleaned to reduce any background contamination of PAEs. All glassware was washed with deionized water and dried overnight in an oven at 80 °C. After cooling, the glassware was rinsed twice with acetone and air-dried for use.

2.2 Plant culture

Chinese cabbage (*Brassica rapa* var. *chinensis*) are the most popular leaf vegetables in Taiwan (Table 5). After germination and growth for eight days, four Chinese cabbage seedlings were planted in a 3-L pot containing 2.5 L hydroponic solution (Fig. 10). The seedlings were fixed by a styrofoam board. The hydroponic solution was modified from Hoagland's solution (Hoagland and Arnon, 1950), which consisted of (in g L^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (0.1), KNO_3 (0.08), $MgSO_4 \cdot 7H_2O$ (0.05), $NH_4H_2PO_4$ (0.02), Fe-EDTA (3), H_3BO_3 (3), Cu-EDTA (0.01), Zn-EDTA (0.03), Mn-EDTA (0.4), $NaMoO_4 \cdot 2H_2O$ (0.003). The pH of the hydroponic solution was adjusted to 6.5. Referring to the DBP data found in the aquatic environment of Taiwan (Yuan et al., 2002), DBP with the concentrations of 0, 10, 30, 50 and 100 mg L^{-1} was added to the solution. Seedlings were cultured under 25 ±1 ℃ during daytime for 16 h and under 20 ±1 ℃ at night for 8 h. All experiments were conducted in triplicate. The experiment was carried out in the phytotron of the College of Agriculture, National Taiwan University, Taiwan. Chinese cabbage were planted with a relative humidity variation from 70 % to 95 %. The appearance and morphology of plants, and leaf damage including chlorosis and necrosis were observed and recorded. After treatment in DBP amended hydroponic solution for 0, 7, 14, 21, 28, 35 and 42 days, the plant leaves were collected and the accumulations of DBP were analyzed, chlorophyll concentration were determined. The leaf tissue was observed with microscope at 42nd day and processed the proteomic analysis at 35th day.

Table 5. Name and biological classification of Chinese cabbage.

Family	Japanese name	English name	Scientific name
Brassicaceae	チンゲン菜	Chinese cabbage	*Brassica rapa* var. *chinensis*

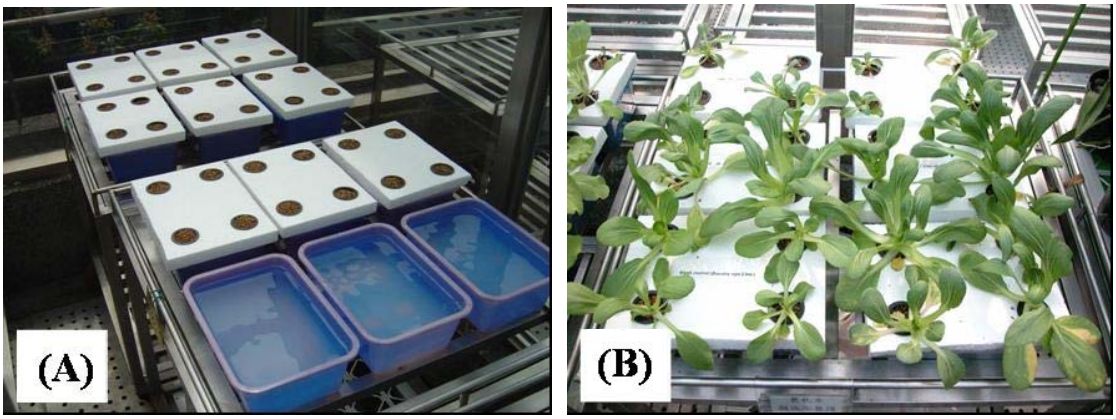

Figure 10. The hydroponic system. (A) before test and (B) Chinese cabbage, after 35 days cultivation.

2.3 Chlorophyll (a+b) concentration determination

The chlorophyll (a+b) concentration was measured by spectrophotometry using the modified method of Wintermans and De Mots (1965). One gram of fresh leaves was in 2 mL of sodium phosphate buffer (pH 6.8) with homogenized. Each 40 µL sample buffer was then extracted with 960 µL absolute ethanol for 30 min in the dark and centrifuged at 1,000×*g* for 10 min. Absorption of the extracts was measured with a spectrophotometer at 665 and 649 nm and chlorophyll concentration (µg mL^{-1} FW) was calculated using the relation: $6.1 \times A_{665\ nm} + 20.04 \times A_{649\ nm}$. All experiments were conducted in triplicate.

2.4 Residual DBP analysis

The amounts of DBP in leaf, stem and root samples and in the whole plant of Chinese cabbage were analyzed following the method by Chang et al., (2005) and Yin et al., (2003) with slight modifications. Plant leaves were rinsed three times with deionized water, then dried at 70 ℃, ground in a mortar and sieved to < 2 mm in size. Each 1.00 g sample in 2 mL *n*-hexane was added to the sample bottles and shaken with a rotating shaker at 160 rpm for 2 hrs. Residual DBP was extracted with *n*-hexane twice, the extracts were combined and analyzed on a Hewlett-Packard 6890 gas chromatograph coupled with a mass-selective detector and DB-5 capillary column (film thickness, 0.25 µm; inner diameter, 0.25 mm; length, 30 m). The injector temperature was set at 250 ℃. Helium was used as the carrier gas at a flow rate of 0.8 mL min^{-1}. Oven temperature was set initially at 50 ℃ for 4 min, then increased at a rate of 6 ℃ min^{-1} to 160

℃ and held for 1 min, increased at a rate of 10 ℃ min^{-1} to 280 ℃ and held for 1 min, and then increased at 10 ℃ min^{-1} to 300 ℃. The mass-selective detector was programmed to scan over a mass range of 50 to 400 m z^{-1} units at 4.1 scan s^{-1}. Recovery percentage of DBP was 96.5% and the method detection limit (MDL) was 80 μg L^{-1}. External calibration procedure was used to determine the identity and quantity of each analytic peak in the sample chromatogram.

2.5 Leaf tissue observation

In the transmission electron microscopy (TEM) study, the outer leaves of Chinese cabbage were collected and fixed in 5 % glutaraldehyde solution buffered in 0.22 mM NaCl, washed with 0.05 M sodium cacodylate containing 0.25 M sucrose (pH 7.5), postfixed with 2% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, then dehydrated through ethanol of 70, 80, 90, 95 and 100 %, respectively, and the samples were finally embedded in resin for fixing leaves by following the method of Spurr (1969). Ultrathin sections (90 nm) were obtained with a diamond knife on an LKB-NOVA microtome and collected on copper grids (200 mesh). Sections were then stained with 50 % uranyl acetate solution for 30 min in the dark at an ambient temperature of 25 ℃ (Gibbons and Grimstone, 1960) followed by staining with lead citrate for 20 min (Reynolds, 1963). Leaf tissues were observed with a JEM-100 CX II transmission electron microscope at 80 kV.

2.6 Proteomic analysis of Chinese cabbage

2.6.1 Protein extraction

Leaves were washed with deionized water and ground to a fine powder in liquid nitrogen, approximately 1.0 g of the ground tissue was added to 10 mL of Tris-HCl solution (consist of 50 mM Tris-HCl, 1 mM EDTA, 20 mM dithiothreitol (DTT), 0.01 % protease inhibitor, pH 8.0) for 30 min. A 0.2 mL of TCA/acetone solution (consisted of 10 % (w/v) trichloroacetic acid (TCA) and 0.1 % DTT in ice cold acetone) was added to a 1.5 mL plastic tube containing 0.8 mL of leaf sample solution. Followed by the growing at -20 ℃ for 1 h, the sample solution was centrifuged in a centrifuge (12,000 rpm) for 10 min and the supernatant was discarded. The pellet was washed as described above for additional two times. The pellet was vacuum-dried for 1 h, then suspended in 400 μL of sample buffer (6 M urea, 4 % CHAPS, 2 M Thiourea, 40 mM DTT), and the protein concentration was determined by 2-D Quant Kit (GE Healthcare, 80-6483-56). Figure

11 shows the standard curve of BSA (Bovine serum albumin) in this method.

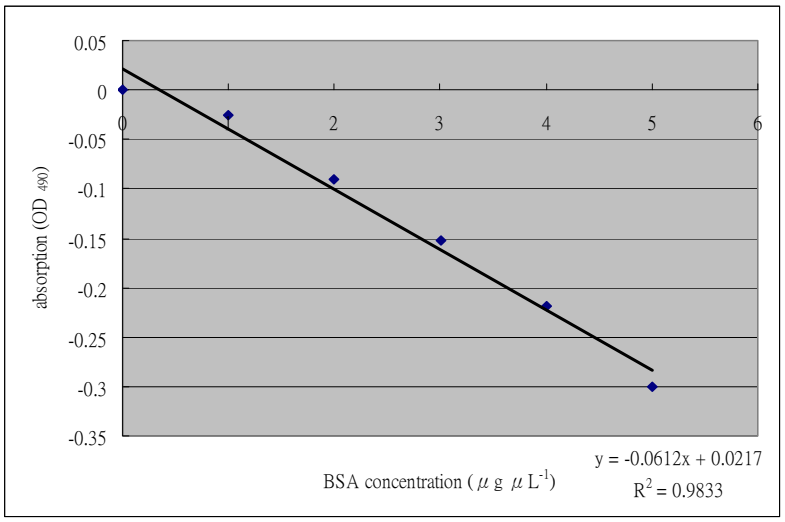

Figure 11. Standard curve of BSA (determined by 2-D Quant Kit).

2.6.2 Protein desalting

Experiments were performed from previous methods with modification. D-Tube Dialyzer Midi (Merck, 71506-3CN) and 2-D Clean-Up Kit (GE Healthcare, 80-6484-5) were used for desalting. The procedure by D-Tube was transferred the protein sample buffer 50 mL into the D-Tube Dialyzer Midi and dialyzed for 24 hours at 4 ℃. The 2-D Clean-Up Kit was prepared for 2-D electrophoresis that might otherwise produce poor 2-D spot-maps due to high conductivity, high levels of interfering substances, or low protein concentration. The procedure by 2-D Clean-Up Kit was transferred 1-100 µL protein sample (containing 1 µg to 1 mg of protein) into a 1.5mL of microcentrifuge tube and 300 µL of precipitant was added and mixed well by vortexing or inversion. The sample was then incubated on ice bath (4-5 ℃) for 15 min, 300 µL of co-precipitant was added, and mixed by vortexing. The mixture was centrifuged in a microcentrifuge set at maximum speed (at least 12,000×g) for 5 min, and the tubes were removed from the centrifuge, the supernatant was removed by decanting or careful pipetting twice. The supernatant was removed by a pipette tip until no visible liquid remaining in the tubes. Twenty-five µL of distilled or de-ionized water was pipetted on top of each pellet and vortex for 5-10 s. The mixture was added 1 ml of wash buffer (pre-chilled for at least 1 h at -20 ℃) and 5 µL wash additive and vortex until the pellet is fully dispersed. The sample was incubated at -20

℃ for more than 30 min, every 10 min vortexed for 20-30 s and then centrifuged in a microcentrifuge set as above. The supernatant was carefully removed and discard and a white pellet should be visible. The pellet was allowed to air dry briefly (for no more than 5 min) and resuspend each pellet in an appropriate volume of rehydration or IEF sample loading solution for first dimension IEF. The sample was vortexed for at least 30 s, incubated at room temperature and vortexed up and down to fully dissolve. The sample was centrifuged in a microcentrifuge set as above to remove insoluble material and to reduce foam. The supernatant may be loaded directly onto first dimension IEF or transferred to another tube and stored at -80 ℃ for later analysis.

2.6.3 Two-dimensional gel electrophoresis

The protein solution was loaded on IPG (immobilized pH gradient) gel strips (pH 3-10, 13 cm in length, GE Healthcare). The IPG strips were rehydrated overnight prior to use in a rehydration buffer solution (8 M urea, 0.5 % Triton X-100, 2 % IPG buffer, 65 mM DTT and 0.0002 % bromophenol blue). The first dimension, IEF (isoelectric focusing) was carried out using the IPG phor system (GE Healthcare) at 18 ℃. The IEF program of Chinese cabbage was shown in Table 6. After IEF, the IPG strips were put in the equilibration solution A (0.375 M Tris-HCl, 6 M urea, 2 % SDS, 20 % glycerol, 130 mM DTT, pH 8.8) and then in the equilibration solution B (6 M urea, 2% SDS, 0.375 M Tris-HCl, 20 % glycerol, 135 mM iodoacetamide, pH 8.8), separately, with gentle agitation for 15 min at room temperature. It was then attached with 0.5 % agarose to the top of a 12.5 % SDS-polyacrylamide gel (Table 7). The 2nd-dimension step was carried out at 45 mA *per* gel for 5 h with Hoefer SE 600 Ruby (GE Healthcare) until the bromophenol blue reached the bottom of the gel.

Staining was carried out by following the method of Hochstrasser et al., (1988) with slight modifications. The gels were fixed in 300 mL of 11.5 % TCA and 4.5 % sulfosalicylic acid at first followed by fixing in a mixture of 300 mL of 40 % ethanol and 10 % acetic acid. The gels were washed with water for 10 min, incubated in the sensitizer (a mixture of 0.5 M sodium acetate and 0.125 % glutaraldehyde, 250 mL) for 20 min and washed twice for 10 min with water. Then the gels were incubated in 300 mL of silver solution (24 mM $AgNO_3$, 9 mM NaOH, and 0.14 % NH_3) followed by washing in 500 mL of water for 1 min. The gels were developed in the mixture of citric acid (760 μM) and 0.0037 % formaldehyde (300 mL). The silver reaction was

stopped by adding 300 mL of solution containing 30 % ethanol and 7 % acetic acid.

Table 6. Program for IEF of Chinese cabbage after 12 hours of rehydration at 30V.

Steps	Chinese cabbage	
S1	500 V	500 Vhr
S2	1000 V	1000 Vhr
S3	8000 V	32000 Vhr

Table 7. Hardness percentage of SDS-polyacrylamide gel for 2DE.

Solutions	7.5 %	10 %	**12.5 %**	15 %
Monomer stock solution [a]	25 mL	33.3 mL	**41.7 mL**	50 mL
4X resolving gel buffer [b]	25 mL	25 mL	**25 mL**	25 mL
10% SDS	1 mL	1 mL	**1 mL**	1 mL
ddH_2O	48.5 mL	40.2 mL	**31.8 mL**	23.5 mL
TEMED	33 µL	33 µL	**33 µL**	33 µL
APS 10%	500 µL	500 µL	**500 µL**	500 µL
Total volume	100 mL	100 mL	**100 mL**	100 mL

[a] The formula of monomer stock solution (30%T, 2.6%C) are 15g of acrylamide and 0.4g of *N*,*N*-Methylenebisacrylamide solving in 50 mL of deionized water.

[b] The formula of 4X resolving gel buffer are 9.07g of Tris base solving in 50 mL of deionized water.

2.6.4 Digestion of in-gel protein

The in-gel protein was digested with following the method by Hellman et al (1995). Each spot of interest in the silver stained gel was sliced into 1 mm cubes and washed three times with 50% (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate buffer (pH 8.0) for 15 min at room temperature. In-gel protein digestion was preformed using porcine trypsin (Promega, USA). The gel pieces were soaked in 100 % ACN for 5 min, dried in a lyophilizer for 30 min and rehydrated in 25mM ammonium bicarbonate buffer (pH 8.0) containing 35 µL of 10 µg mL^{-1} trypsin until the gel pieces were fully immersed. After incubating for 20 h at 37 °C, the remaining

trypsin solution was transferred into a new microtube. The gel pieces were resuspended with 50 % ACN in 5.0 % trifluoroacetic acid (TFA) for 60 min then concentrated to dryness.

2.6.5 Mass spectrometry analyses of proteome

After in-gel protein digestion, lyophilized samples were premixed with 1:1 matrix solution (5 mg/ml CHCA in 50 % acetonitrile, 0.1 % v/v TFA and 2% w/v ammonium citrate) and spotted onto the 96-well format MALDI sample stage. Peptide mass fingerprinting (PMF) analysis was carried out with a dedicated MALDI-TOF-MS (matrix assisted laser desorption ionization time of flight mass spectrometry, Micromass, Manchester, UK). Samples were analyzed in the reflector mode at an accelerating voltage of 20 kV, 70 % grid voltage, 0 % guide wire voltage, 100 ns delay and a low mass gate of 500 Da. The PMF and individual MSMS ion data were saved as Mascot-searchable .txt file and .pkl files for independent searches against Swiss-Prot or NCBI database using the Mascot search engine (http://www.matrixscience.com/, Perkins et al., 1999). The procedures and conditions of our protein search were shown in Fig. 2.4. Additionally, references of each protein can be acquired in the ExPASy proteomics server (http://tw.expasy.org/) by using their accession number.

3. RESULTS AND DISCUSSION

3.1 Chinese cabbage (*Brassica rapa* var. *chinensis*)

3.1.1 Outer and inner injury of Chinese cabbage leaf

The morphological changes of Chinese cabbage between normal and DBP-treated are shown in Fig. 12, 13. The results shown that exposure of Chinese cabbage to 50 mg L^{-1} of DBP for 36 days (Fig. 12), the surface of the leaf became yellow partially. After exposure of Chinese cabbage to 50 mg L^{-1} of DBP for 42 days (Fig. 13), the etiolation appeared on the leaf followed by the whole leaf turning yellow. Examination of grana structure in chloroplast under the TEM revealed the organelle unchanged in the yellow colored leaf tissue (Fig. 14). The chloroplasts in the yellow leaves with remarkable difference in the grana structure were not found. These results show that DBP could induce the leaves of Chinese cabbage turning yellow but not remarkable change on the chloroplast and grana structure.

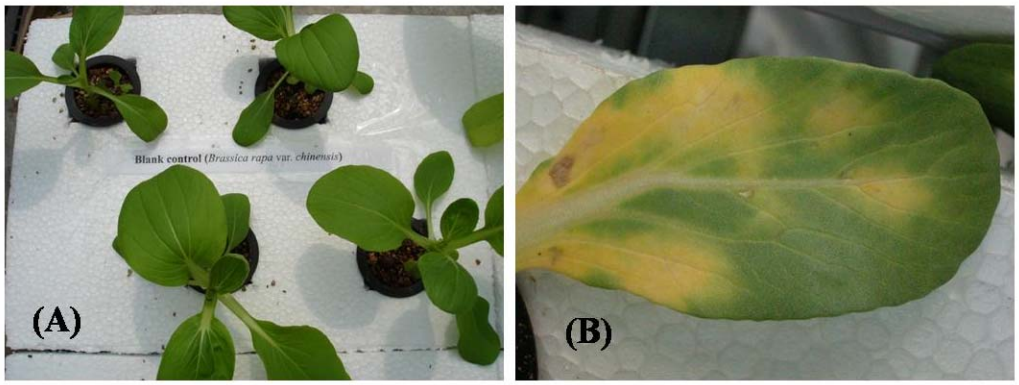

Figure 12. Effect of DBP-treatment for 36 days on the Chinese cabbage leaf. (A) control (normal) and (B) 50 mg L^{-1} of DBP treated.

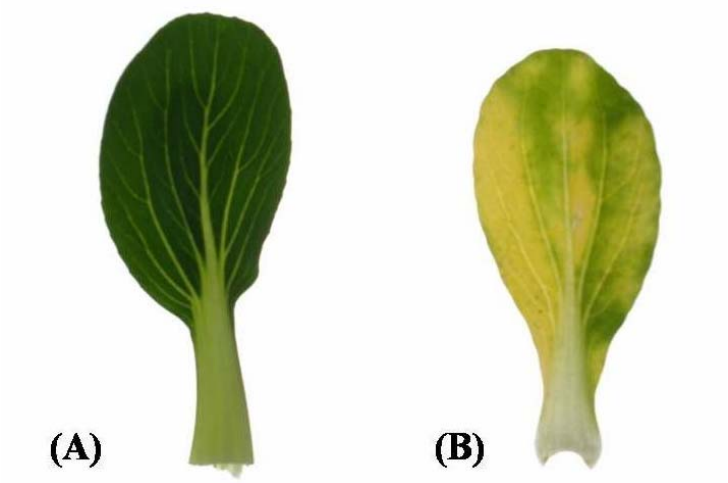

Figure 13. Effect of DBP-treatment for 42 days on the Chinese cabbage leaf. (A) control (normal) and (B) 50 mg L^{-1} of DBP treated.

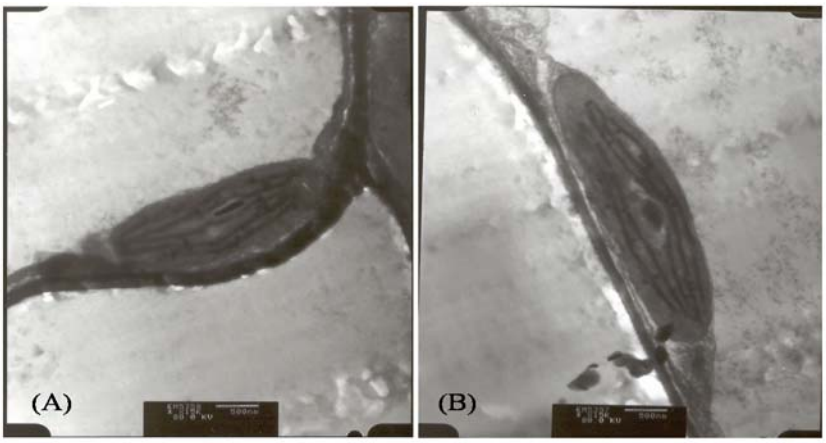

Figure 14. Transmission electron microscopic image of Chinese cabbage organelle. (A) normal green leaf (control) and (B) yellow leaf (50 mg L^{-1} DBP treated for 42 days).

3.1.2 Concentration of chlorophyll and accumulation of DBP in Chinese cabbage leaf

The DBP content and chlorophyll concentration in Chinese cabbage leaves were determined and compared. The result revealed that the growth of Chinese cabbage was significantly inhibited according to the amount of DBP treatment.

The concentration of DBP in the Chinese cabbage increased with increasing DBP added to the hydroponic solution (Fig. 15). With 1 mg L^{-1} of DBP, no significant difference was found when compared with control, but 10, 30, 50 and 100 mg L^{-1} all caused significant DBP accumulation ($p < 0.05$). Table 8 shows the DBP accumulation in different parts of Chinese cabbage by treatment with various amounts of DBP in hydroponic solution for 42 days. The result shown that DBP was taken up by the root and translocated to the whole plant. This result indicated that DBP could translocate from roots through the whole plant and finally accumulated in the leaf. The results from Fig. 16 and Table 8 shown that the accumulation of DBP in plant was increased with both the amount of DBP and growing period.

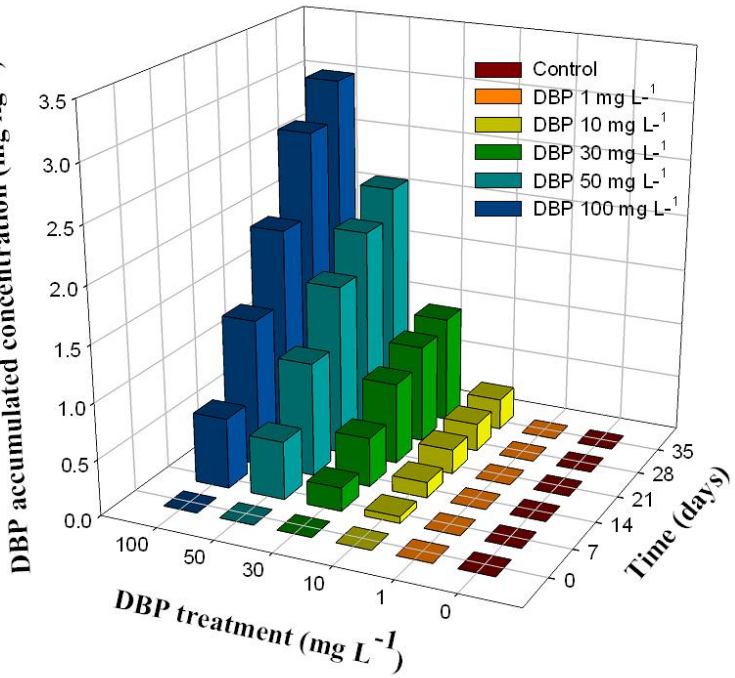

Figure 15. DBP accumulation in Chinese cabbage by treatment of various amounts of DBP for 35 days.

Table 8. DBP accumulation (μg g^{-1}) in different part of Chinese cabbage plant with various amounts of DBP-treated for 42 days.

	DBP treatment (mg L^{-1})					
	0	1	10	30	50	100
Root	n.d.[a]	n.d.[a]	0.14 ±0.06	0.26 ±0.12	0.38 ±0.18	0.64 ±0.22
Stem	n.d.[a]	n.d.[a]	0.16 ±0.06	0.22 ±0.14	0.54 ±0.26	0.95 ±0.46
Leaf	n.d.[a]	n.d.[a]	n.d.[a]	0.48 ±0.28	1.19 ±0.66	1.44 ±0.88

[a]n.d. = not detectable (below the detection limit of 0.1 μg g^{-1} in triplicate).

On the other hand, the chlorophyll concentration in leaf decreased upon increasing the amount of DBP and the growing period (Table 9). Change of chlorophyll concentration, DBP accumulation and biomass of Chinese cabbage upon treating with various levels of DBP for 42 days are given in Table 10. The results shown that the biomass of whole plant and chlorophyll in leaves was significantly decreased by increasing DBP treatment when compared with control. The biomass shown a severe decrease with more than 30 mg L^{-1} of DBP. The accumulation of DBP in whole plant increased with increasing the amount and exposure time of DBP (Tables 10). An average amount of 3.03 μg g^{-1} was found in whole plant upon treating with 100 mg L^{-1} of DBP for 42 days. Similar effects were found in the study on *Raphanus* and *Browallia* (Hemming et al., 1981), capsicum fruit (Yin et al., 2003) and *Brassica campestris* (Dueck et al., 2003). In these reports, DBP was delivered in the vapor phase or the closed glass-containers.

Table 9. Chlorophyll concentration (mg g^{-1} fresh wt) in Chinese cabbage leaf after treatment with DBP for different periods.

Days after treatment	DBP treatment (mg L^{-1})					
	0	1	10	30	50	100
0	0.2372 ±0.032	0.2319 ±0.028	0.2391 ±0.028	0.2334 ±0.025	0.2278 ±0.028	0.2312 ±0.025
7	0.2412 ±0.035	0.2347 ±0.034	0.2112 ±0.026	0.2012 ±0.027	0.1936 ±0.024	0.1827 ±0.028
14	0.2436 ±0.045	0.2366 ±0.042	0.1963 ±0.034	0.1868 ±0.032	0.1724 ±0.015	0.1726 ±0.025
21	0.2442 ±0.038	0.2420 ±0.042	0.1812 ±0.032	0.1747 ±0.038	0.1767 ±0.028	0.1564 ±0.032
28	0.2452 ±0.032	0.2428 ±0.044	0.1724 ±0.026	0.1652 ±0.022	0.1554 ±0.037	0.1582 ±0.046
35	0.2448 ±0.028	0.2436 ±0.042	0.1712 ±0.024	0.1592 ±0.026	0.1506 ±0.025	0.1493 ±0.034

Table 10. Biomass, chlorophyll concentration and DBP accumulation in Chinese cabbage by treatment with various amounts of DBP for 42 days.

	DBP treatment (mg L^{-1})					
	0	1	10	30	50	100
Biomass (g, dry wt)[a]	4.59 ±0.51	4.01 ±0.44	3.34 ±0.36	2.59 ±0.28	1.42 ±0.24	1.12 ±0.28
Chlorophyll concentration (mg g^{-1} fresh wt)	0.2693 ±0.048	0.2518 ±0.056	0.1906 ±0.032	0.1632 ±0.046	0.1607 ±0.038	0.1534 ±0.064
DBP accumulated in whole plant (μg g^{-1})	n.d.[b]	n.d.[b]	0.32 ±0.08	0.96 ±0.24	2.11 ±0.56	3.03 ±0.74

[a]Average of three plants.
[b]n.d. = not detectable (all three replicates were below the detection limit of 0.1 μg g^{-1} in triplicate).

3.1.3 Proteome differences of Chinese cabbage

Differences in the expression of proteins in the control and the 50 mg L^{-1} DBP treated Chinese cabbage leaf were studied after 35 days of growing. The proteins in whole leaf were extracted with TCA/acetone solution and separated by 2-DE followed by staining with silver as described above. The 2-DE photographs of the control and DBP-treated Chinese cabbage are shown in Fig. 16. There are also six protein spots that revealed significant alterations. Three proteins (spots 2, 4 and 5) were found or increased in amount and three proteins (spots 1, 3 and 6) were decreased or disappeared. The differentially expressed proteins were identified by MALDI-TOF MS and PMF analyses (listed in Table 11).

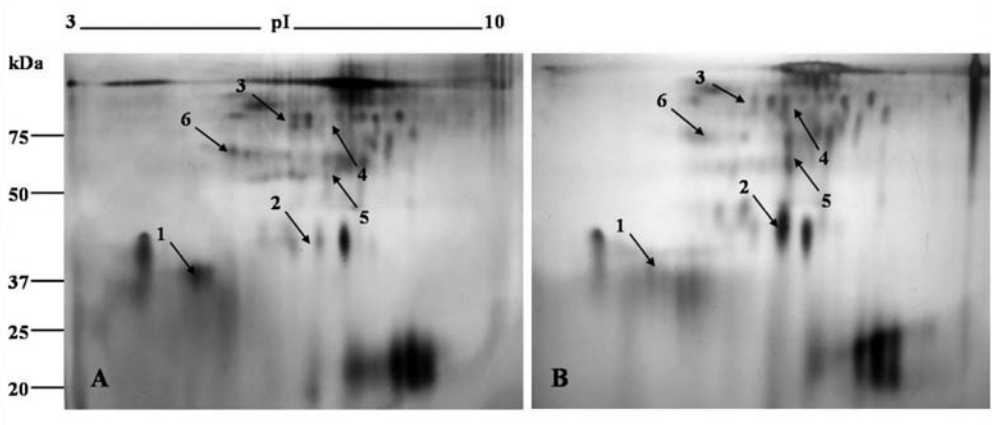

Figure 16. 2-DE photographs of Chinese cabbage leaf sample after growth for 35 days. (A) control and (B) 50 mg L^{-1} DBP treated. Each arrow shows the proteins that reproducible differences in expression. The name, estimated molecular weight and p*I* values of proteins list in Table 11.

Table 11. Proteomic characterization of polypeptide differences in expression between control and DBP treatment of Chinese cabbage.

Spots	Protein name	Accession no.	Estimated MW (kDa)	Estimated p*I*
1	Dihydroflavonol-4-reductase	P51102	42.7	5.43
2	Acyl-[acyl-carrier-protein] desaturase	P28645	45.6	5.94
3	Aminoacyl-tRNA synthetase	O23627	81.9	6.59
4	Root phototropism protein 3	Q9FMF5	81.7	7.85
5	Ferredoxin-nitrite reductase	P05314	66.4	6.51

6	ATP synthase subunit beta	Q9MTG8	53.7	5.68

Among the proteins, acyl-[acyl-carrier-protein] desaturase (acyl-ACP desaturase, spot 2), root phototropism protein 3 (RPT3, spot 4) and ferredoxin-nitrite reductase (Fd-NiR, spot 5) shown an increase with 50 mg L^{-1} DBP treated. Acyl-[acyl-carrier-protein] desaturase (acyl-ACP desaturase, spot 2) is involved in the fatty acid biosynthesis. Acyl-ACP desaturase was the primer of fatty acid biosynthesis and catalyzes the principal conversion of saturated fatty acids to unsaturated fatty acids in the synthesis of vegetable oils. In the reaction, acyl-ACP desaturase introduces the first double bond into C18 fatty acids in higher plants and, therefore, it is involved in controlling the extent of unsaturation of membrane lipids (Jaworski et al., 1993).

Root phototropism protein 3 (RPT3, spot 4) is a signal transducer of the phototropic response and photo-induced movements in plants (Sakai et al., 2000). Plant life is strongly dependent on the environment, and plants regulate their growth and development in response to many different environmental stimuli. One of these regulatory mechanisms is phototropism. Phototropism allows plants to change their growth direction in response to the location of the light source. So plants are able to sense and respond to changes in light quality, quantity, and direction through the action of a number of photoreceptors and associated signal-response systems which involved phototropism proteins.

Ferredoxin-nitrite reductase (Fd-NiR, spot 5) is essential for the nitrate assimilation in plants and catalyses the six-electron concerted reductions of nitrite to ammonia using ferrodoxin (Fd) as an electron donor. It contains siroheme and [4Fe-4S] clusters (Lancaster et al., 1979). Plant Fd-NiR shows low but significant sequence homology with Fd-sulphite reductase (SiR) and NADPH-sulphite reductase, and the regions forming the [4Fe-4S] cluster and the siroheme are well conserved. Plant SiR and NiR are each capable of catalysing the multielectron reduction rections of both sulphite and nitrite, but each enzyme has a Km for its preferred substrate about two orders of magnitude lower than that of the other substrate (Krueger et al., 1982).

Our results shown that acyl-ACP desaturase, RPT3 and Fd-NiR were increased in the protein pattern of leaf tissue on DBP-treated Chinese cabbage. These three proteins are responsible for the fatty acid biosynthesis, signal transduction of the phototropic response and nitrate assimilation in plant cells. According to this result, we might say that DBP seems to induce some physiological reactions increasing in the Chinese cabbage cell. We supposed that

these responses might be symbolizes of DBP-treated Chinese cabbage. However, the other three proteins (spots 1, 3 and 6) found in the control sample were significantly decreased in the 2-DE electrophotogram of DBP-treated Chinese cabbage. The protein identified as Dihydroflavonol-4-reductase (DFR, spot1) catalyzed the conversion of dihydroflavonols into flavan-3, 4-diols (flavanol). Flavanol is one of the flavonoid precursors which are the predominant secondary metabolites in higher plants. Flavanol and other flavonoids take charge of the color of flower. Therefore, we can say that DFR is an up-regulated enzyme during floral color development (Farzad et al., 2003).

Aminoacyl-tRNA synthetase (aaRS, spot 3) is an enzyme which was first discovered to ligate an amino acid to tRNA in 1958 (Hoagland et al., 1958). When tRNAs are charged with their cognate amino acids in the nucleus prior to export to the cytoplasm, the universal genetic code is determined. Although the same 20 amino acids are found in organisms in all taxonomic domains, tRNAs are more-highly differentiated and have undergone charges throughout evolution. These changes alter synthetase-tRNA contacts so that adaptations must occur (Shiba et al., 1997). AaRS also involved in a broad repertoire of functions that not only impact protein synthesis, but also extend to a number of other critical cellular activities (Martinis et al., 1999a). Specific aaRSs play roles in cellular fidelity, tRNA processing, RNA splicing, RNA trafficking, apoptosis and transcriptional and translational regulation. Recently, aaRS is indicated a transcription termination factor that may interact with the 3'-end of pre-mRNA to promote 3'-end formation. A EMBO workshop entitled "Structure and Function of Aminoacyl-tRNA Synthetases" highlighted the diversity of the aaRSs' role within the cell (Martinis et al., 1999).

ATP synthase subunit beta (spot 6) is one of the subunits of chloroplast ATP synthase. An ATP synthase is a general term for an enzyme that can synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate by utilizing some form of energy. In plants ATP synthase is present in chloroplasts (CF_0F_1-ATP synthase). The enzyme is integrated into thylakoid membrane; the CF_1-part sticks into stroma, where dark reactions of photosynthesis and ATP synthesis take place. The overall structure and the catalytic mechanism of the chloroplast ATP synthase are almost the same as those of the mitochondrial enzyme. The ATP synthase subunit beta contains the catalytic site for ATP synthesis during photooxidative phosphorylation in the chloroplast (Jiao et al., 2004). However, in chloroplasts the protonmotive force is generated not by respiratory electron transport chain, but by primary photosynthetic

proteins.

The results in this part revealed some damages or disorder of metabolism inside the Chinese cabbage cell that may partially be attributed to the change in the amounts of some proteins in the cell. Decrease of DFR indicated that DBP might affect the flavonoid biosynthesis and floral color development in the Chinese cabbage cell. This effect might the cause of Chinese cabbage leaf turning yellow. Decrease of aaRS indicated that DBP might affect the aminoacylation of tRNA and other transcriptional or translational regulation in the Chinese cabbage cell. This effect might cause some disorders of regular metabolism or development of Chinese cabbage. Decreased of ATP synthase subunit beta (spot 6) indicated that ATP synthesis in the Chinese cabbage cell might be affected by DBP and cause the restriction of growth and development in Chinese cabbage. Form our results of Chinese cabbage, we might say that DBP seems to induce some physiological reactions increasing and also cause the restriction of growth and development in Chinese cabbage.

4. BRIEF SUMMARY

Effects of DBP on the morphological and proteome-level changes in Chinese cabbage were noted. The results showed that the leaves of Chinese cabbage turned yellow and displayed etiolation with decrease in biomass and concentration of chlorophyll. Increase and accumulation of DBP in different parts of plant implied that DBP not only translocated from roots to the whole plant but also had a significant dose-response relationship on delivery from hydroponic culture. In the proteomic analysis, six protein spots derived from the normal control and the DBP-treated Chinese cabbage plants showed reproducible differences in the expression in 2-DE. Three proteins appeared or increased while the other three proteins decreased or disappeared during growth in hydroponic culture with DBP added. Three spots of increasing protein were responsible for biosynthesis of fatty acids, signal transduction of phototropic response and nitrate assimilation in plant cells, respectively. The other three spots that disappear or show decrease in protein with DBP treatment were responsible for biosynthesis of flavonoids and floral color development in the cell, aminoacylation of tRNA in the cell, and synthesis of ATP in the cell to restrict growth and development in Chinese cabbage, respectively. In conclusion, DBP seems to be able to induce physiological reactions or metabolism disorder in the Chinese cabbage cell. We might say that DBP limits the growth and development of Chinese cabbage; however, the outer injuries, proteomic patterns, pathogenic mechanisms are totally different from those found in Bok choy (*B. rapa* subsp. *chinensis*).

CHAPTER III:

Bioremediation of endocrine disruptor di-n-butyl phthalate by bacteria, fungi, and plant

1. INTRODUCTION

Microbial degradation is believed to be one of the major processes in the remediation of chemical pollutant contaminated soil. A number of studies have reported that the aerobic degradation of DBP is more effective than anaerobic degradation in soil (Yuan et al., 2002; Wang, 2004). Therefore, it is clear that the metabolic breakdown of DBP by micro-organisms is considered to be one of the major routes of environmental degradation for this widespread pollutant. However, little is known about the survival conditions of DBP-degrading bacteria in contaminated environments such as soil.

Although toxic chemicals may gradually be removed from the environment through sorption, chemical degradation, or photolysis, metabolic breakdown by microorganisms is considered to be one of the major routes of degradation for toxic chemicals (Liao et al., 2010). White rot fungi have been considered as a biological alternative for the removal of toxic chemicals due to their ability to produce non-specific oxidative enzymes involved in the degradation of lignin, these being mainly lignin peroxidase, manganese peroxidase, and laccase (Martínez et al., 2005). This is a non-specific oxidative metabolism and makes white rot fungi useful for a wide range of biotechnological applications, including but not limited to degrade recalcitrant compounds such as PAEs, polyaromatic hydrocarbons, aromatic dyes, and other pollutants (Wang et al., 2009).

Ganoderma lucidum is one of the most important and widespread white rot fungi in the world and is associated with the degradation of a wide variety of woods (D'souza et al., 1999). Most studies with *G. lucidum* are related to its medicinal and pharmacological properties (Ko et al., 2001). However, few studies have explored the use of *G. lucidum* and its enzymes in the degradation of xenobiotics (Murugesan et al., 2009). Among the major extracellular enzymes involved in lignin degradation, laccase appears to be the main enzyme produced by *G. lucidum* in the majority of culture conditions (Ting et al., 2011). This enzyme can catalyse the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, methoxyphenols, aromatic amines, and ascorbate, with concomitant four-electron reduction of oxygen to water. Because laccases have a tolerance to pollutants at high concentrations, they have attracted growing attention in many industrial and environmental fields. Enzyme activities and the removal rates of toxic chemicals are influenced by pH, temperature, substrate concentration, the presence of substrates (veratryl alcohol or cofactors such as Cu^{2+} and Mg^{2+}), and inhibitors (e.g. organic acids such as citric, oxalic, and tartaric acids) in the aqueous environment. The choice of the proper mediator substance also plays a key role by affecting applicability and effectiveness of the laccase mediator systems (LMS). The most commonly used are 2,20-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxy benzotriazole (HBT).

Spent mushroom compost (SMC) of G. lucidum, a waste product of the mushroom industry,

still contains many residual enzymes, such as proteases, cellulases, hemicellulases, lignin peroxidase, manganese peroxidase, and laccase. The utilization of SMC as a source material for ligninolytic enzymes to remove toxic chemicals is a win–win strategy as it not only converts the waste into a useful material, but also saves on the disposal costs of waste removal. In addition, the combination of ozonation and SMC-mediated aerobic biological treatment was also investigated in the removal of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil. In spite of the removal efficiency (35%) obtained by the SMC-mediated biological process as a stand-alone treatment, the combined process showed a benzo[a]pyrene concentration reduction higher than 75%; the best removal (82%) was observed after 10 min pre-ozonation treatment. Since little is known about the ability of laccase from SMC to remove organic toxic chemicals in the aqueous environment.

2. MATERIALS AND METHODS

2.1 Chemicals

Di-*n*-butyl phthalate (DBP) (98.7% purity, CAS: 84-74-2) was purchased from Riedel-deHaën Co, Germany. The solvents used in the experiment, including acetone and *n*-hexane (HPLC-grade) were purchased from E. Merck, Germany. All other chemicals were purchased from the Sigma Chemical Co, Saint Louis, MI, USA. A stock solution of DBP was dissolved in acetone at a concentration of 100 g L^{-1}. The glassware was thoroughly cleaned to reduce any background contamination of PAEs. All glassware was washed with deionized water and dried overnight in an oven at 80 °C. After cooling, the glassware was rinsed twice with acetone and air-dried for use.

2.2 Biodegradation by bacteria

2.2.1 Sampling and isolation of DBP-degrading bacteria

Activated sludge samples were collected from a wastewater treatment plant in the Chinese Petroleum Corp., Kaohsiung, Taiwan. All sludge samples were collected in 3L glass sterile bottles and stored at 4 ℃. The samples were mixed manually to visual homogeneous extent and then sampled for analysis. Bacteria in the activated sludge samples with the ability to degrade DBP were plated on the trypticase soy agar (BBL, USA) and R_2A agar (BBL, USA.). After 48 h of incubation at 30 ℃, a number of well separated individual colonies of different morphological types appeared and were further streaked onto fresh TSA plates which contained 5mg L^{-1} of DBP to purify the degrading bacteria. Subsequently, DBP-degrading bacteria were used for 16S rRNA identification. The primers which were used to amplify 16S rRNA gene were Pf: 50-AGAGTTTGATCCTGGCTCAG-30 and Pr: 50-ACGGCTACCTTGTTACGACT- 30 corresponding to 8–27 and 1495–1514 bases of coli 16S rRNA gene, respectively. The amplification

programs were carried out by PCR. The amplified products were subjected to gel electrophoresis in 1% agarose. The DNA sequencing services were carried out by Seeing Bioscience Corporation, Taiwan.

2.2.2 Experimental design of biodegradation

Pure DBP-degrading bacteria enrichment cultures were used with the Trypticase soy broth (BBL, U.S.A.). The microbial culture medium used in the experiments contained the following (all concentrations in mg l^{-1}): K_2HPO_4, 65.3; KH_2PO_4, 25.5; $Na_2HPO_4 \cdot 12H_2O$, 133.8; NH_4Cl, 5.1; $CaCl_2$, 82.5; $MgSO_4 \cdot 7H_2O$, 67.5; and $FeCl_3 \cdot 6H_2O$, 0.75. The pH of the medium was adjusted to 7 with potassium hydroxide before autoclaving at 121 °C for 25 min. Batch experiments were performed in 125 ml glass bottles containing 45 ml microbial culture medium and 5 ml pure DBP-degrading bacteria culture medium. Each experimental bottle was contained with 5 mg l^{-1} of DBP. The following factors were then modified in order to study their effects on DBP degradation in the medium: temperature (25, 30, 35, 40°C), initial pH levels (6.5, 7.0, 7.5, 8.0), yeast extract (50 mg l^{-1}), surfactants (Brij 35, Triton X-100, Tergitol) and 160 rpm shaking rate. The surfactants were all prepared at 1 critical micelle concentration (CMC). The bottles were incubated in an incubator at 30°C in dark under aerobic conditions. Aqueous samples were periodically collected in order to measure residual DBP concentrations.

2.2.3 Analysis of residual DBP

The residual DBP in the culture medium were analyzed following the method of Yuan et al., (2002) and Chang et al., (2005) with slight modifications. Briefly, 2 ml of culture medium was added to bottles containing 2 ml of *n*-hexane and shaken at 160 rpm for 2 h. Residual DBP was extracted three times. The extracts were combined and analyzed on a Perkin Elmer Clarus 400 gas chromatograph coupled with an electron capture detector and Elite-5ms capillary column (film thickness, 0.25 μm; inner diameter, 0.25 mm; length, 30 m). The injector temperature was set at 250 °C. Nitrogen was used as the carrier gas at a flow rate of 0.8 ml min^{-1} at a 10:1 split ratio. The initial column temperature was set at 150 °C for 1 min, increased at a rate of 6 °C min^{-1} to 220 °C, then increased by at a rate of 3 °C min^{-1} to 275 °C and held for 13 min. The detector temperature was set at 320 °C. The recovery percentage of DBP was 94.5% and the method detection limit (MDL) was 80 μg L^{-1}.

2.2.4 PCR-DGGE analysis of microbial community

Pure DBP-degrading bacteria isolated from the activated sludge were cultured with the optimal

condition of DBP degradation. After 48 h of incubation, 10 ml of culture medium were amended into 50 ml origin activated sludge for analyzing the survival condition of DBP-degrading bacteria under the original environment.

At each sampling date, DNA from the mixed cultures were obtained and analyzed. Extractions of the total microbial DNA from the activated sludge samples were performed using UltraClean Soil DNA kit (MO BIO laboratories, Inc). After extraction, the total microbial DNA was further purified to remove humic acids using electrophoresis of DNA extracts in a 1% agarose gel. After staining with ethidium bromide (EtBr), the total microbial DNA was excised from the gel and recovered with a QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). PCR reactions were carried out with a thermal cycler 9700 (Applied Biosystems, USA). The PCR primers 968f: 5'-AACGCGAAGAACCTTAC-3' and 1401r: 5'-CGGTGTGTACAAGACCC-3' were combined to amplify the segment of eubacterial 16S rDNA from the nucleotide 968 to nucleotide 1401, respectively (Nubel et al., 1996). For PCR amplification, 10 ng of sample DNA was added to a reaction mixture prepared with 2 μl 10×PCR buffer (Progema), 1.5 mM $MgCl_2$, 100 μM dNTPs (Roche Molecular Biochemical, Mannheim, Germany), 0.1 μM each primer, 1U *Taq* polymerase (Promega) and filter-sterilized milliQ water to a final volume of 20 μL. The PCR was carried out as follows: an initial DNA denaturation step at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, annealing for 1 min at 64 °C, extension for 1 min at 72 °C, and a final extension at 72 °C for 10 min. The PCR product was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

DGGE analysis was performed with a D-Code universal mutation detection system (Bio-Rad, Hercules, Calif.). The PCR products were separated on a double-gradient gel as described by Cremonesi et al., (1997). The gel consisted of an 8–10% polyacrylamide gradient with a 40–60% denaturing gradient (100% = 7 M urea with 40% formamide). Electrophoresis was performed at 75 V for 12 h in 1×TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 7.4) at a constant temperature of 60 °C. After the electrophoresis, polyacrylamide gel was stained with SYBR Green I nucleic acid gel stain, visualized on a UV transilluminator, and photographed with a digital camera.

2.2.5 Statistical analyses

The DBP degradation data collected for this study fit well with first-order kinetics: $S = S_0\exp(-k_1 t)$, $t_{1/2} = \ln 2k_1^{-1}$, where S_0 is the initial concentration, S is the substrate concentration, t is the time period and $k1$ is the degradation rate constant. The remaining percentage was calculated as the DBP residue concentration divided by the original DBP concentration, multiplied by 100. Each treatment was performed in triplicate. Statistical analysis was carried out using ANOVA.

2.3 Biodegradation by fungi

2.3.1 Microorganisms and SMC

G. lucidum BCRC36021 was obtained from the Bioresource Collection and Research Centre (BCRC), Hsinchu, Taiwan. SMC of *G. lucidum* was produced in a mushroom cultivation factory in Chiayi, Taiwan, after the harvest of edible crops, using wheat bran and sawdust-based fermented compost.

2.3.2 Enzyme extraction and dialysis

Experiments were performed using 1000 mL Erlenmeyer flasks containing 600 mL of sodium acetate–acetic acid buffer and 120 g of *G. lucidum* SMC. The following factors were adjusted to study their effects on the enzyme extraction: extraction time (3 h, 1 days, and 4 days), various buffers (phosphate buffer, pH 7.0; phosphate buffer, pH 5.8; sodium acetate buffer, pH 5.0; sodium acetate buffer, pH 4.5), solid: solution ratio (1 : 25, 1 : 10, and 1 : 5), and temperature (4, 20, and 30 °C). The samples were then centrifuged (10 000g x 10 min), and the supernatant was partially purified by precipitation with ammonium sulfate and dialysis. The enzyme was used as a crude laccase. The material was stored at -20 °C until use.

2.3.3 Enzyme stability and activity assays

After enzyme extraction and dialysis, the following factors were adjusted to study their effects on the enzyme stability: temperature (4, 20, and 30 °C), buffer (glycine buffer, pH 3.0; phosphate buffer, pH 7.0; phosphate buffer, pH 5.8; sodium acetate buffer, pH 5.0; and sodium acetate buffer, pH 4.5), and the addition of substrates (1% of triton X-100, 1% of TWEEN 80, 1 mM of EDTA, and 20 mM of b-mercaptoethanol). The laccase activity was measured by spectrophotometry at 405 nm. The reactive mixture contained 0.5 mL of enzyme supernatant, 0.25 mL of 100 mM glycine buffer (pH 3.0), and 0.25 mL of 4 mM ABTS. The enzyme reactive mixtures were incubated at 25 °C for 5 min before measurement. For the evaluation of the laccase activity, one activity unit was defined as the amount of enzyme necessary to oxidize 1 mmol of substrate per min. Each value presented in this paper represents the mean of three replicates.

2.3.4 Removal experiments

In this study, there are two different removal experiments to analyze the DBP or DEHP at concentrations of 2 mg L^{-1}. Experiment 1 was conducted by using a 125 mL serum bottle containing 45 mL of basal medium (BM), 5 mL of crude laccase, 2 mg L^{-1} of DBP, and the addition of $CuSO_4$ (1 mM), $MnSO_4$ (0.5 mM), tartaric acid (20 mM), ABTS (1 mM), or HBT (20 mg L^{-1}), respectively. Experiment 2 was performed with 45 mL of basal medium (BM), 5 mL of crude laccase, ABTS (1

mM), and DBP and DEHP (all at concentrations of 2 mg L^{-1}). The BM contained (per liter of dH2O) the following elements: corn steep liquor 10 g, yeast extract 2 g, KH_2PO_4 0.9 g, Na_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCl_2 \cdot 7H_2O$ 0.5 g, thiamine HCl 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.0005 g, $MnSO_4 \cdot 4H_2O$ 0.0007 g, $ZnSO_4 \cdot 7H_2O$ 0.043 g, and $CuSO_4 \cdot 5H_2O$ 0.0008. The pH of BM was 5.0 prior to sterilization. The experiments were incubated at 30 °C on a rotary shaker in the dark for 1 d. Before sampling, each flask was shaken vigorously to ensure mixing. The residues of toxic chemicals were determined periodically.

2.3.4 Analysis of toxic chemicals

PAEs were extracted twice using n-hexane, and then extracted again over 20 min at 30 °C with a Branson 5200 ultrasonic cleaner. The extracts were combined and analysed on an HP5890 series II gas chromatograph, coupled with a flame ionization detector and DB1701 capillary column (inner diameter, 0.53 mm; length, 30 m). The injector temperature was set at 150 °C. Nitrogen was used as the carrier gas at a flow rate of 1.0 mL min^{-1} at a 20 : 1 split ratio. The initial column temperature was set at 170 °C for 1 min, increased at a rate of 7 °C min^{-1} to 220 °C, then increased at a rate of 2.5 °C min_1 to 260 °C and held for 8 min. The detector temperature was set at 320 °C. The recovery percentages of DBP and DEHP were 95.5% and 97.5%, respectively.

2.4 Phytoremediation by plants

2.4.1 Cultivation of Plants

The eleven test plants used in this study were all typical plants from North-East Asia and Taiwan. The common and scientific names of them were Edible rape (*Brassica napus*), Chinese cabbage (*Brassica rapa* var. *chinensis*), Spinach (*Spinacia oleracea* L.), Chinese mustard (*Brassica rapa* L. *Chinensis Group*), Water cabbage (*Brassica rapa pekinensis*), Ceylon spinach (*Basella rubra.* L.), Garden lettuce (*Lactuca sativa* L. var. *longifolia*), Chinese celery (*Apium graveolens* L.), Edible amaranth (*Amaranthus tricolor* L.), Cauliflower (*Brassica oleracea* var. *botrytis*) and Chinese chive (*Allium tuberosum*), respectively. After germination and growth for fourteen days, all plant seedlings were each planted in a 3 L pot containing 2.5 L of hydroponic solution. The seedlings were fixed with a styrofoam board and every styrofoam board was used for four seedlings. The hydroponic solution was modified from Hoagland's solution [48], which consisted of (in g L^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (0.1), KNO_3 (0.08), $MgSO_4 \cdot 7H_2O$ (0.05), $NH_4H_2PO_4$ (0.02), Fe-EDTA (3), H_3BO_3 (3), Cu-EDTA (0.01), Zn-EDTA (0.03), Mn-EDTA (0.4) and $NaMoO_4 \cdot 2H_2O$ (0.003). The pH value of the hydroponic solution was adjusted to 6.5. The electrical conductivity (EC) value of the solution was 1.2 mS cm^{-1}. All experiments were carried out in a plant growth chamber and all test plants were cultivated with a controlled relative humidity variation from 70% to 80%, which was carried

out to simulate the environmental humidity of Taiwan.

2.4.2 Experimental Design

First, eleven test plants were used to evaluate the potential capability of DBP phytoremediation. Referring to the DBP data found in the aquatic environment of Taiwan (Yuan et al., 2002), DBP with a concentration of 5 mg L^{-1} was added to the hydroponic solution. Regarding the average temperature and daylight hours in Taiwan, the cultural temperature of daytime and nighttime were 25 ± 1 °C/23 ± 1 °C, the illumination times of light and dark were 13 h/11 h. The period of cultivation was 21 days and the sampling dates were 0, 7, 14 and 21 days, respectively.

After growing in the DBP-added hydroponic solution for 21 days, the biomass, leaf chlorophyll concentration, residual DBP in hydroponic solution and accumulated DBP in the whole plant were analyzed. The morphology of the plants was also observed and recorded during the period of phytoremediation. Meanwhile, the residual DBP in the hydroponic solution and the accumulated DBP in different parts of test plants were analyzed to represent the phytoremediation ability of test plants. In the end, the plant with the best DBP phytoremediation ability of the eleven test plants was selected for use in this study. The best phytoremediation ability means the plant has the highest rate of DBP removal.

Following the DBP phytoremediation capability evaluation, the selected plant was used to establish the optimal cultural conditions of phytoremediation. The following cultural conditions were modified to study their effects on DBP phytoremediation: Cultural temperature of daytime and night (25 °C /23 °C, 23 °C /21 °C, 21 °C /19 °C), light and dark illumination times (13 h/11 h, 12 h/12 h, 11 h/13 h), initial pH levels (6, 7, 8 or 9), DBP added concentration (1 mg L^{-1}, 3 mg L^{-1}, 5 mg L^{-1}) and surfactants (Brij 35, Brij 30, Triton X-100, Tergitol, Tween 80). The surfactants were all prepared at one critical micelle concentration (CMC). After growing for 21 days, the DBP phytoremediation efficiency was determined by measuring the residual DBP in the hydroponic solution. In the end, the phytoremediation efficiency with all optimal conditions was determined.

2.4.3 Plant Leaf Chlorophyll (*a* + *b*) Concentration Determination

Chlorophyll *a* and *b* were measured using the method adapted from our previous study (Liao et al., 2009). One gram of fresh plant leaf sample was immersed in a 2 mL sodium phosphate buffer (pH 6.8), then homogenized. After shaking with the buffer, 40 μL of sample solution was taken and extracted with 960 μL absolute ethanol in the dark. The extraction was carried out for 30 min and then centrifuged at 8000 *g* for 10 min. The absorption of the extracts was measured with a spectrophotometer at 665 and 649 nm and the chlorophyll concentration (mg L^{-1} FW) was calculated

using the relation: $6.1 \times A_{665\,nm} + 20.04 \times A_{649\,nm}$. All experiments were conducted in triplicate and identified using *t*-tests.

2.4.4 DBP Concentration Analyses

The residual DBP in the roots, stems and leaves of the plants was analyzed following the methods of our previous study (Liao et al., 2009). The plant samples were rinsed three times with deionized water, then dried at 70 °C, ground into a mortar and sieved to < 2 mm in size. Each 1.00 g sample in 2 mL *n*-hexane was added to the sample bottles and shaken with a rotating shaker at 160 rpm for 1 h. Residual DBP was extracted with *n*-hexane three times and then the extracts were combined to await analysis.

The analysis of residual DBP in the hydroponic solution followed the methods of our previous study (Liao et al., 2010) with slight modifications. Briefly, 2 mL of hydroponic solution was added to bottles containing 2 mL of *n*-hexane and shaken in a rotating shaker at 160 rpm for 10 min. The residual DBP was extracted three times with *n*-hexane twice, and then the extracts were combined to await analysis.

The DBP extracts and phthalic acid, which is the main metabolite of DBP, were analyzed on a Perkin Elmer Clarus 400 gas chromatograph, coupled with an electron capture detector and an Elite-5ms capillary column (film thickness of 0.25 μm, inner diameter of 0.25 mm, length of 30 m). The injector temperature was set to 250 °C. Nitrogen was used as the carrier gas at a flow rate of 0.8 mL min^{-1} and a 10:1 split ratio. The initial column temperature was set at 150 °C for 1 min, increasing by a rate of 8 °C min^{-1} to 220 °C, then increasing by a rate of 4 °C min^{-1} to 275 °C, where it was then held for 10 min. The detector temperature was set at 320 °C. The recovery percentage of DBP was 96.5% and the method detection limit (MDL) was 0.08 mg l^{-1}. The method of the previous study was that the initial column temperature was set at 160 °C for 1 min, increased by a rate of 10 °C min^{-1} to 280 °C, then held for 1 min, then increasing by a rate of 10 °C min^{-1} to 300 °C.

3. RESULTS AND DISCUSSION

3.1 Biodegradation by bacteria

3.1.1 Isolation and identification of the DBP-degrading bacteria

Twenty-three bacterial strains capable of utilizing DBP as the carbon source and energy were isolated from activated sludge. The incubation was done at 30 °C, pH 7.5 and initial concentration of DBP 5 mg l^{-1}. After 48 h of incubation, the two isolates showing the highest degradation rates were strains AS7 and AS16; the half-lives were 0.89 and 1.18 days, respectively (Table 12). Strain AS7

was coccus-shaped, gram positive, and formed orange colonies, and was identified with their 16S rRNA as *Deinococcus radiodurans*. Strain AS16 was rod-shaped, gram negative, and formed white colonies, and was identified with their 16S rRNA as *Pseudomonas stutzeri*.

Table 12. Comparison of the effects of various incubation factors on DBP degradation rate constants (k_1, day^{-1}) and half-lives ($t_{1/2}$, days) in the culture medium.

Treatment	*Pseudomonas stutzeri*			*Deinococcus radiodurans*		
	k_1 (day^{-1})	$t_{1/2}$ (days)	r^2	k_1 (day^{-1})	$t_{1/2}$ (days)	r^2
temperature						
25	0.35	1.98	0.87	0.45	1.53	0.86
30	0.58	1.18	0.94	0.77	0.89	0.91
35	0.54	1.27	0.92	0.22	3.14	0.81
40	0.24	2.87	0.76	0.05	12.63	0.91
pH						
6.5	0.53	1.32	0.92	0.34	2.01	0.87
7	0.41	1.69	0.85	0.27	2.59	0.86
7.5	0.58	1.18	0.94	0.77	0.89	0.91
8	0.45	1.56	0.85	0.37	1.85	0.94
surfactant						
Brij 35	0.43	1.58	0.88	0.51	1.37	0.97
Triton X-100	0.51	1.38	0.93	1.07	0.65	0.95
Tergitol	0.58	1.19	0.95	0.66	1.04	0.93
others						
shake	0.59	1.18	0.89	0.73	0.95	0.98
yeast extract	0.51	1.38	0.88	0.62	1.12	0.97

3.1.2 The effects of various incubation factors on DBP

The effects of various temperatures on the aerobic degradation of DBP in the culture medium are presented in Fig. 17 and Table 12. According to the result of Fig. 17, the optimal temperature for DBP degradation was 30 °C for both degrading strains. As shown in Table 12, the DBP degradation rate constant of *D. radiodurans* at 30 °C calculated by first-order kinetics was 0.77 day^{-1} and the half-life was 0.89 days. On the other hand, the DBP degradation rate constant of *P. stutzeri* at 30 °C calculated by the first-order kinetics was 0.58 day^{-1} and the half-life was 1.18 days. As can be seen, the DBP degradation rates of *D. radiodurans* at 30 °C were found to be slightly lower than the rates of *P. stutzeri*. When compared with the various temperatures, Fig. 17 shows that DBP degradation rates of *D. radiodurans* were higher than rates of *P. stutzeri* at 25 and 30 °C. However, DBP degradation rates of *D. radiodurans* were significantly decreased and lower than the rates of *P. stutzeri* at 35 and 40 °C. These results indicated that *D. radiodurans* have better degradation rates than *P. stutzeri* at 25 and 30 °C but not at 35 and 40 °C.

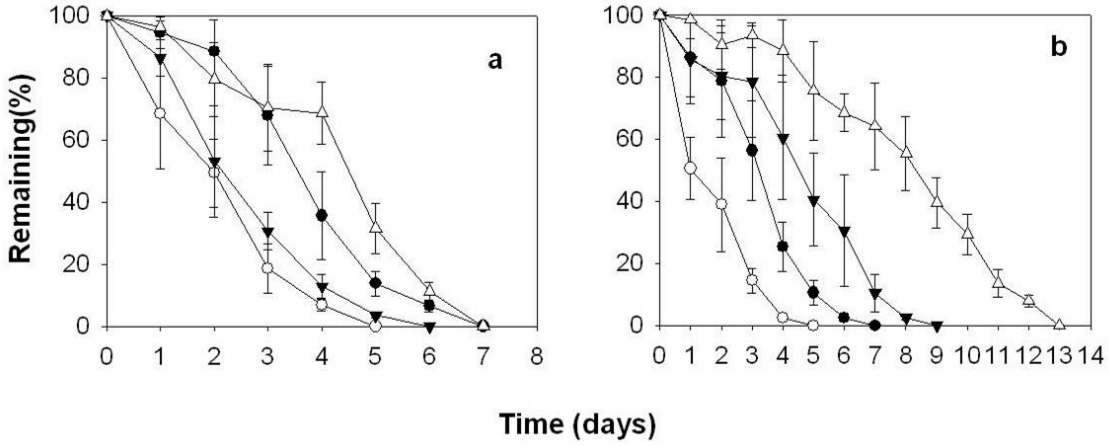

Figure 17. Biodegradation of DBP in a culture medium at various incubation temperatures. (a), *P. stutzeri* (b), *D. radiodurans*. Symbols: ●, 25°C; ○, 30°C; ▼, 35°C; △, 40°C.

The effects of various pH levels on the aerobic degradation of DBP in the culture medium are presented in Fig. 18 and Table 13. According to the result of Fig. 18, we found that the pH levels for DBP degradation were both 7.5 for these two degrading strains. When compared with the various pH levels, Fig. 18 shows that the DBP degradation rates of *D. radiodurans* were not significantly different from *P. stutzeri* at pH 6.5, 7 and 8. However, DBP degradation rates of *D. radiodurans* and *P. stutzeri* were significantly increased at pH 7.5. These results indicated that both *D. radiodurans* and *P. stutzeri* were preferred to culture in a weakly alkaline condition especially at pH 7.5.

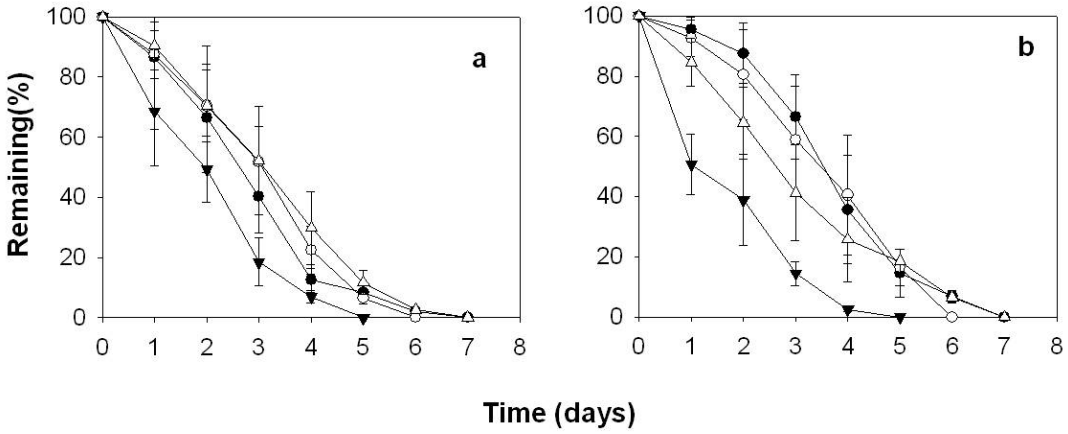

Figure 18. Biodegradation of DBP in the culture medium at various pH levels. (a), *P. stutzeri* (b), *D. radiodurans*. Symbols: ●, pH6.5; ○, pH7; ▼, pH7.5; △, pH8.

The effects of various surfactants on the aerobic degradation of DBP in the culture medium are presented in Fig. 19 and Table 13. As shown in Fig. 19, the addition of Brij 35 and Tergitol could not enhance the DBP degradation for these two degrading strains. However, the addition of Triton X-100

could enhance the DBP degradation for *D. radiodurans* when compared with the control. Triton X-100 is a non-ionic surfactant with a molecular weight of 624. The critical micelle concentration (CMC) of Triton X-100 is 0.23 mM. Under Triton X-100 treatment, DBP partitioned into the micellar phase of Triton X-100 was directly available to be acted on by a microorganism resulting in an enhanced degradation. As shown in Table 16, the DBP degradation rate constant of *D. radiodurans* under Triton X-100 treatment calculated by the first-order kinetics was 1.07 day-1 and the half-life was 0.65 days. As can be seen, the DBP degradation rate of *D. radiodurans* under Triton X-100 treatment could enhance the DBP degradation.

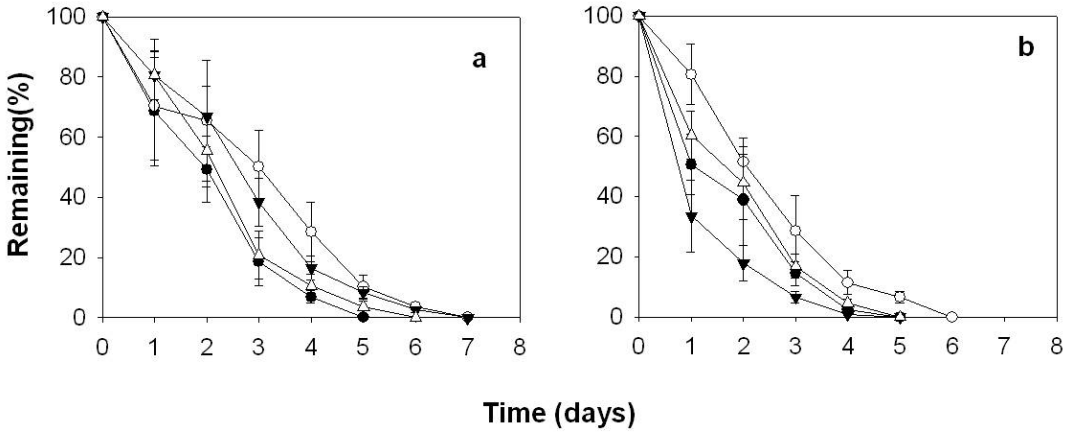

Figure 19. Biodegradation of DBP in the culture medium at various surfactants. (a), *P. stutzeri* (b), *D. radiodurans*. Symbols: ●, control; ○, Brij 35; ▼, Triton X-100; △, Tergitol.

Fig. 20 and Table 13 show the effects of yeast extract (50 mg l^{-1}) and shaking culture (160 rpm) on the aerobic degradation of DBP in a culture medium. According to the result of Fig. 20, we found that DBP degradation was not significantly enhanced by the addition of yeast extract and shaking culture when compared with the control.

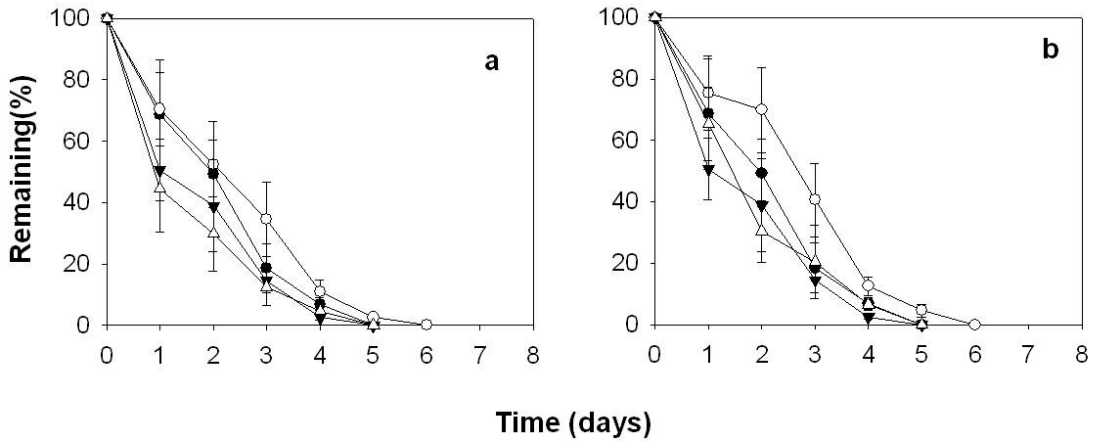

Figure 20. Biodegradation of DBP in the culture medium under treatment with shaking culture (160 rpm) and yeast extract (50 mg l^{-1}). (a), shaking culture (b), yeast extract. Symbols of fig. 4a: ●, *P. stutzeri* control; ○, *P. stutzeri* shaking culture; ▼, *D. radiodurans* control; △, *D. radiodurans* shaking culture; Symbols of fig. 4b: ●, *P. stutzeri* control; ○, *P. stutzeri* added yeast extract; ▼, *D. radiodurans* control; △, *D. radiodurans* added yeast extract.

3.1.3 PCR-DGGE analysis

PCR-DGGE analysis was used to measure the structure of microbial community in DBP-degrading cultures. *D. radiodurans* and *P. stutzeri* isolated from the activated sludge were cultured with the optimal conditions of DBP degradation. The optimal conditions of DBP degradation for these two strains are: 30 °C, pH 7.5 and static culture. After 48 h of incubation, 10 ml of culture medium was amended into 50 ml of original activated sludge which yielded 5 mg l^{-1} of DBP for analyzing the survival condition of DBP-degrading bacteria under the original environment. After 3, 5, and 7 days incubation, DNA from the mixed cultures were obtained and analyzed by PCR-DGGE. Fig. 26 shows the structures of microbial communities which mixed culture with pure DBP-degrading bacteria and original activated sludge. Lanes D and P were the DNA of *D. radiodurans* and *P. stutzeri*, respectively. Lanes D3, D5 and D7 were the DNA of *D. radiodurans* and original activated sludge mixed culture after 3, 5, and 7 days incubation, respectively. Lanes P3, P5 and P7 were the DNA of *P. stutzeri* and original activated sludge mixed culture after 3, 5, and 7 days of incubation, respectively. In this DGGE gel, we found that *D. radiodurans* could survive very well after 7 days of incubation with the original activated sludge (Fig. 21). Fig. 21 shows that band of *D. radiodurans* still appeared on lane D7 when compared with lanes D3 and D5. However, *P. stutzeri* amended into the original activated sludge could not survive well after 5 days of incubation. Fig. 21 shows that the band of *P. stutzeri* did not appear on lane P5 and P7 when compared with lanes P3. These results indicated that *P. stutzeri* might have ecological competition with the original microorganisms in the activated sludge.

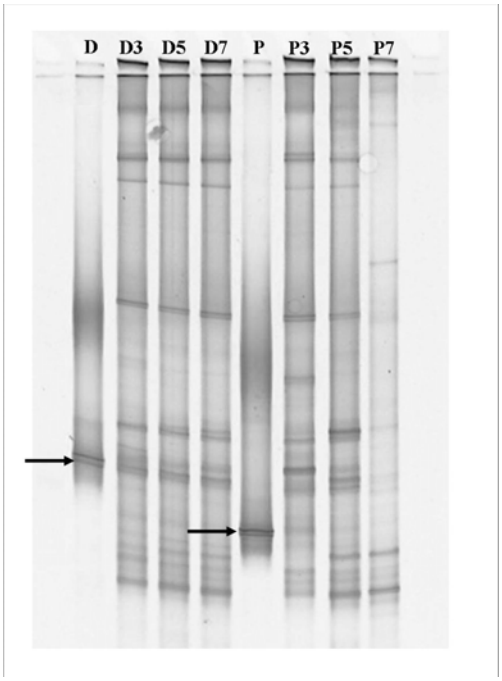

Figure 21. Analysis of the structure of microbial community in DBP-degrading bacteria and original activated sludge mixed cultures. Lanes D and P are the DNA of *D. radiodurans* and *P. stutzeri*, respectively. Lanes D3-7 are the DNA of *D. radiodurans* and original activated sludge mixed culture after 3, 5, and 7 days incubation, respectively. Lanes P3-7 are the DNA of *P. stutzeri* and original activated sludge mixed culture after 3, 5, and 7 days incubation, respectively.

3.2 Biodegradation by fungi

3.2.1 The effects of various extraction conditions on laccase activity

The effects of various extraction buffers on laccase activity are presented in Fig. 22. The effects of various extraction buffers in descending order of magnitude were sodium acetate buffer (pH 5.0) > sodium acetate buffer (pH 4.5) > phosphate buffer (pH 5.8) > phosphate buffer (pH 7.0). The removal efficiency over various extraction times, in descending order of magnitude, was 3 h > 1 d > 4 d. These results indicated both sodium acetate buffer (pH 5.0) and an extraction time of 3 h are the best running condition for measuring laccase activity. This is consistent with the findings of Lang *et al.,* (1998), who reported that the ligninolytic enzymes in the straw and soil layers of solid state cultures were extracted with sodium acetate buffer (pH 5.0) for 3 h. We thus set it as a rule to perform dialysis with sodium acetate-acetic acid (pH 5.0) for 3 h for enzyme extraction reported in this study.

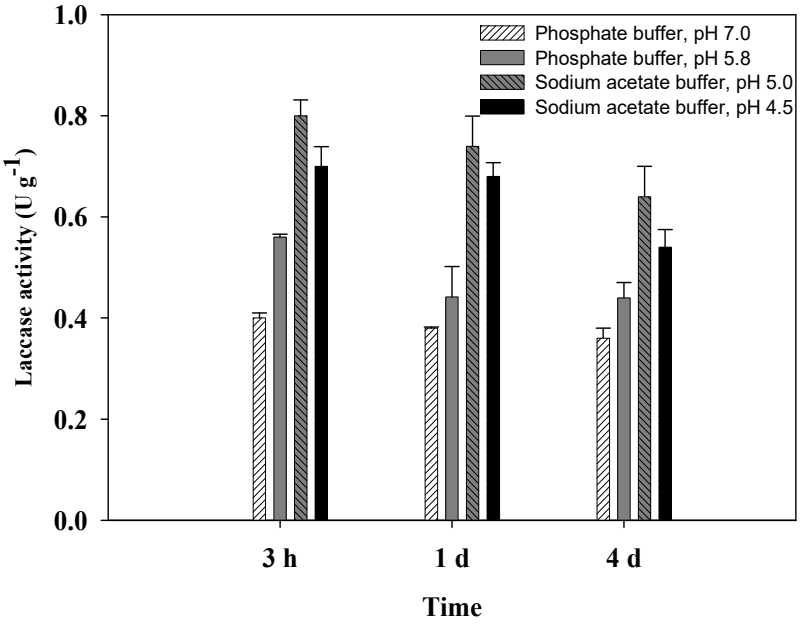

Figure 22. The effects of various extraction buffers on laccase activity.

The effects of various extraction buffers and solid/solution ratios on laccase activity are presented in Fig. 23. After 3 h of extraction at 4 °C, the effects of extraction buffer with various solid/solution ratios on laccase activity, in descending order of magnitude, were 1:5 > 1:10 > 1:25. These results indicated that a solid-to-solution ratio of 1:5 is the best extraction ratio for crude laccase activity. SMC of *G. lucidum* used wheat bran and sawdust as the substrate. Wheat bran and sawdust are abundant source for ferulic, coumaric acid and syringic acid. These phenolic acids are known to enhance the laccase production in white rot fungi. The higher the substrate present in the buffer, produced a higher laccase activity. Likewise, an extraction solid/solution ratio of 1:5 was set up as the optimal extraction ratio for the later experiment.

The effects of various temperatures on laccase activity are presented in Fig. 24. After extraction by various extraction buffers, the effects of various temperatures on laccase activity, in descending order of magnitude, were 4 °C > 20 °C > 30 °C. The results demonstrated that 4 °C is the best extraction temperature for laccase activity. Therefore, 4 °C was used as the optimal extraction temperature for the later experiment. However, when the phosphate buffer at pH 7.0 was used as the extraction buffer at 20 °C, the laccase activity was very close to that under the optimal conditions (Fig. 24). Therefore, this condition could be considered as an alternative extraction condition for crude laccase extraction.

When the enzyme extractions were performed from *G. lucidum* SMC, we detected the amount of total protein, total activity, and specific activity were 0.96 μg, 2.23 U, and 2.3 U mg^{-1}, respectively. However, after adding ammonium sulfate and dialysis performed, there is a significant change of

those three parameters for the enzymes extraction measured: total protein 1.2 µg, total activity 29.1 U, and specific activity 24.2 U mg^{-1}. This demonstrated that dialysis increase laccase activity 10.5 times than using extraction buffer alone while performing laccase extraction from SMC.

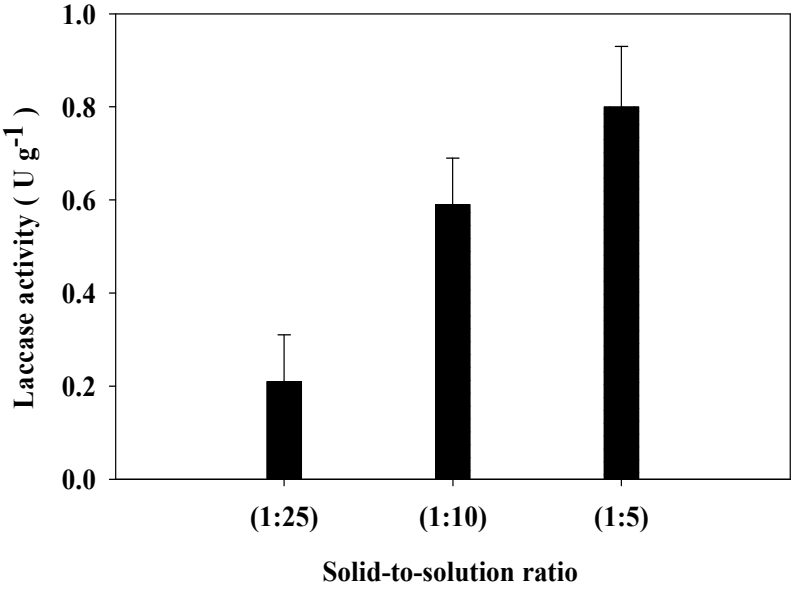

Figure 23. The effects of extraction buffer with various solid/solution ratios on laccase activity.

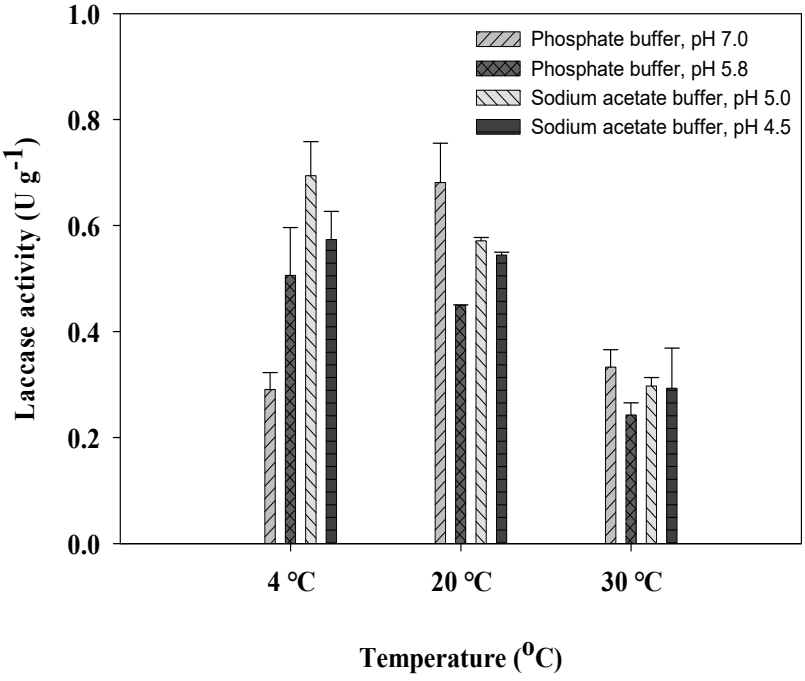

Figure 24. The effects of various temperatures on laccase activity.

3.2.2 The effects of various factors on laccase stability

After enzyme extraction and dialysis, the effects of temperature, buffer, and substrates on laccase stability were compared (Fig. 25). The effects of various stability buffers on laccase stability at 4 °C, in descending order of magnitude, are phosphate buffer, pH 5.8 > glycine buffer, pH 3.0 > sodium acetate buffer, pH 4.5 > sodium acetate buffer, pH 5.0 > phosphate buffer, pH 7.0. The laccase activity after treatment with these were 42.5, 35.8, 33.2, 28.7, and 25.4 U L^{-1}, respectively. Hence, the phosphate buffer, pH 5.8, was used as the stability buffer at 4 °C to create the optimal stability conditions in this study. Figure 25b shows that the addition of 1% triton X-100 could significantly increase the laccase stability after 8 d of incubation, but the addition of 20 mM β-mercaptoethanol would inhibit laccase stability. Other studies have indicated that some surfactants could increase the interaction between enzymes and hydrophobic molecules. Hence, the surfactant triton X-100 was used to create the optimal conditions for laccase stability in this study.

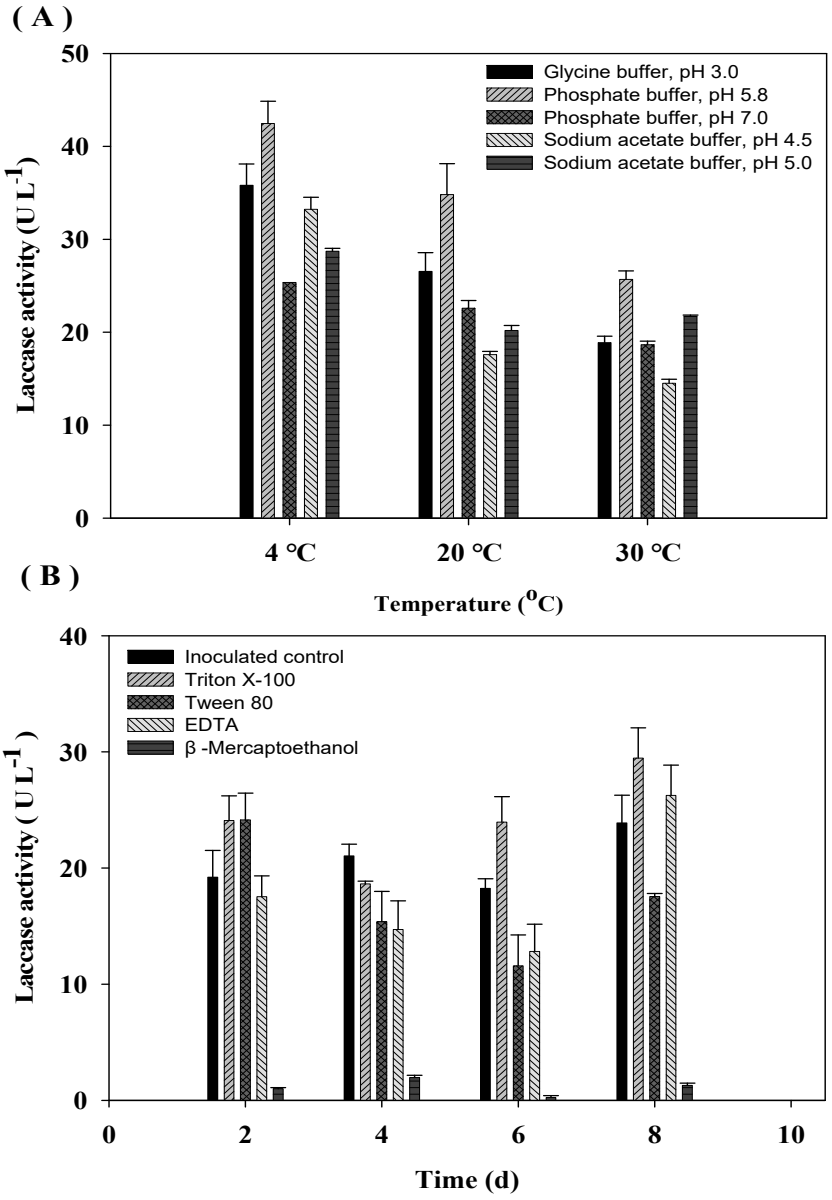

Figure 25. The effects of various factors on laccase stability.

3.2.3 The removal of toxic chemicals by the addition of crude laccase

The removal efficiencies of crude laccase for four toxic chemicals are shown in Table 13. The results indicated that the higher the concentration of toxic chemicals present, the slower the removal efficiencies observed. The removal efficiencies, in descending order of magnitude, were DBP > DEHP. Compounds with higher molecular weights and structural complexity are more resistant to biotransformation than those with lower molecular weights.

This result also shows that the four toxic chemicals, at a concentration of 2 mg L^{-1}, were almost completely removed by crude laccase obtained from *G. lucidum* SMC after 1 d of incubation. This result was also very similar to that of Lee *et al*, who reported that white rot fungi, *Daldinia*

concentrica, and *Trametes versicolor*, could obviously degrade DBP by the addition of laccase. When the 100 μM (28 mg L^{-1}) of DBP was added to the medium, the degradation ratios by *D. concentrica* and *T. versicolor* were 94 and 83%, respectively, after 1 days of incubation. In terms of converting waste into a useful material, the crude laccase obtained from *G. lucidum* SMC was shown to have a significant effect in removing various toxic chemicals when it was extracted under optimal conditions.

Table 13. Removal efficiency (%) of toxic chemicals by addition of crude laccase after 1 days of incubation.

	Concentration	Removal efficiency (%)
	DBP	DEHP
2 mg L^{-1}	100.0 ± 2.4 b	95.7 ± 6.4 c
20 mg L^{-1}	88.8 ± 3.4 B	74.8 ± 5.3 C

Data were analyzed by one-way ANOVA. The given values are mean ± SEM of three independent experiments. Different letters indicate significant differences ($P < 0.05$).

3.3 Phytoremediation by Plants

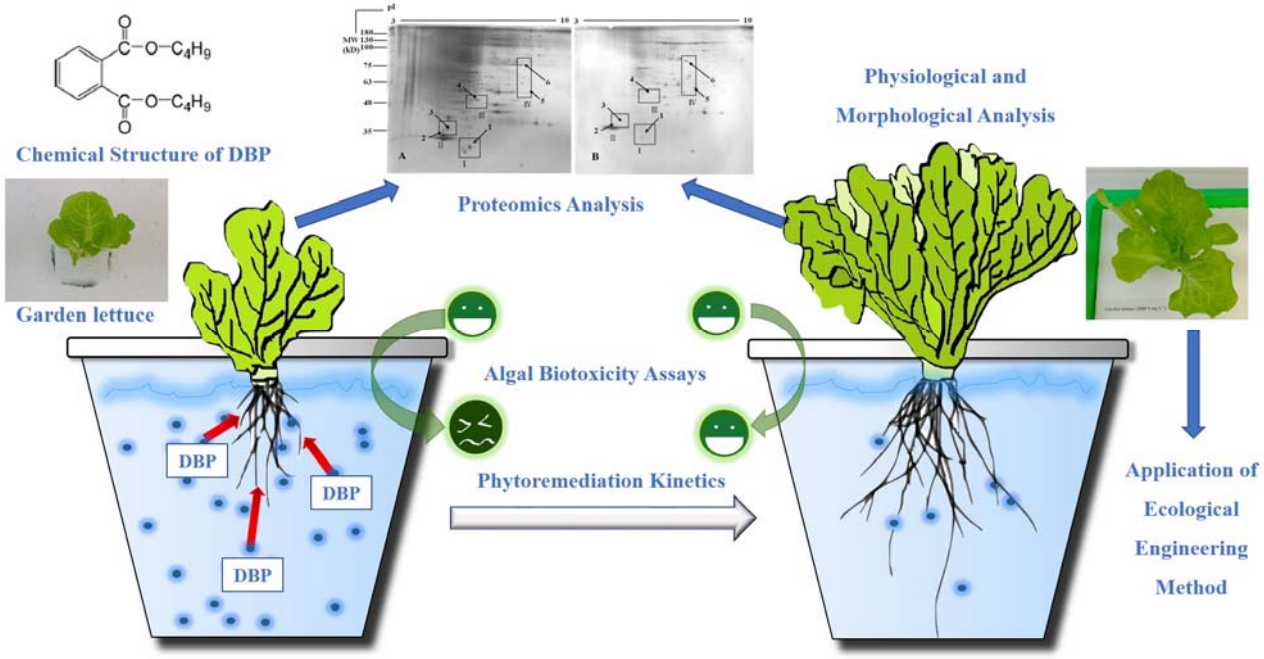

Figure 26. Graphical abstract of phytoremediation by Garden lettuce.

3.3.1 Evaluation of DBP Phytoremediation Capability in Eleven Test Plants

Eleven popular leaf vegetables in Taiwan were used to evaluate the potential of DBP phytoremediation capability in this study. Table 15 showed the results of biomass, chlorophyll

concentration and DBP residue in the hydroponic solution after 21 days of phytoremediation. Garden lettuce showed the best DBP phytoremediation capability of all the test plants (Table 14). Table 18 shows that the biomass and chlorophyll of the plant leaves had all normally increased after DBP phytoremediation. However, the results of residual DBP in the hydroponic solution and accumulated DBP in different parts of the eleven test plants (Table 15) showed Garden lettuce had significant DBP absorption capability (paired *t*-test; $p < 0.05$). Table 15 reveals that after DBP phytoremediation, the accumulated concentration of DBP in the roots, stems and leaves of Garden lettuce was 3.35 ± 0.42, 2.74 ± 0.38 and 4.35 ± 0.42 mg kg^{-1}, respectively. The other test plants all showed no significant DBP absorption capability following DBP phytoremediation. The morphology of Garden lettuce during the period of phytoremediation is shown in Fig. 27. The result displays the morphology of Garden lettuce, such as the leaf shape and color, revealing no significant changes during the exposure to DBP for 21 days.

Table 14. Biomass, chlorophyll concentration and DBP residue in hydroponic solution after DBP phytoremediation of eleven test plants.

Common Name	Biomass (g, dry wt) [a]		Chlorophyll Concentration (mg g^{-1}, fresh wt) [a]		Residual DBP in Hydroponic Solution (mg L^{-1}) [a]	
	0 day	21 days	0 day	21 days	0 day	21 days
Edible rape	2.64 ± 0.68	4.32 ± 0.52	0.227 ± 0.032	0.259 ± 0.048	5.02 ± 0.15	3.89 ± 0.18
Control [c]	2.83 ± 0.58	5.63 ± 0.82	0.264 ± 0.034	0.306 ± 0.066		
Chinese cabbage	2.82 ± 0.95	6.58 ± 1.52	0.132 ± 0.028	0.194 ± 0.042	5.02 ± 0.16	3.56 ± 0.13
Control [c]	2.74 ± 0.84	7.08 ± 1.38	0.144 ± 0.032	0.226 ± 0.064		
Spinach	2.35 ± 0.76	3.28 ± 0.85	0.253 ± 0.044	0.268 ± 0.048	4.98 ± 0.14	4.72 ± 0.15
Control [c]	2.39 ± 0.54	2.98 ± 0.78	0.244 ± 0.028	0.318 ± 0.054		
Chinese mustard	1.48 ± 0.36	4.63 ± 1.46	0.154 ± 0.035	0.204 ± 0.047	4.99 ± 0.16	4.79 ± 0.16
Control [c]	1.52 ± 0.26	5.79 ± 1.24	0.156 ± 0.028	0.417 ± 0.068		
Water cabbage	2.68 ± 0.28	7.18 ± 1.44	0.252 ± 0.046	0.309 ± 0.063	5.06 ± 0.21	2.24 ± 0.08
Control [c]	2.57 ± 0.23	6.92 ± 0.88	0.264 ± 0.058	0.364 ± 0.088		
Ceylon spinach	1.12 ± 0.25	3.76 ± 0.88	0.279 ± 0.055	0.319 ± 0.058	4.95 ± 0.12	3.52 ± 0.14
Control [c]	1.15 ± 0.22	4.21 ± 0.82	0.282 ± 0.048	0.355 ± 0.098		
Garden lettuce	1.45 ± 0.26	4.68 ± 0.52	0.165 ± 0.037	0.269 ± 0.042	4.99 ± 0.14	n.d. [b]
Control [c]	1.49 ± 0.31	4.72 ± 0.52	0.169 ± 0.037	0.272 ± 0.042		
Chinese celery	0.92 ± 0.32	4.06 ± 0.85	0.141 ± 0.032	0.191 ± 0.038	5.03 ± 0.18	4.72 ± 0.18
Control [c]	1.02 ± 0.29	4.54 ± 0.92	0.144 ± 0.038	0.255 ± 0.049		
Edible amaranth	1.54 ± 0.56	6.13 ± 1.22	0.153 ± 0.025	0.192 ± 0.035	5.05 ± 0.24	3.33 ± 0.16
Control [c]	1.47 ± 0.48	5.74 ± 0.89	0.149 ± 0.023	0.214 ± 0.042		
Cauliflower	3.14 ± 0.85	9.42 ± 1.98	0.082 ± 0.016	0.129 ± 0.023	5.02 ± 0.05	2.16 ± 0.05
Control [c]	3.06 ± 0.62	9.68 ± 1.42	0.083 ± 0.016	0.133 ± 0.031		
Chinese chive	0.65 ± 0.22	3.43 ± 0.34	0.203 ± 0.038	0.249 ± 0.046	4.99 ± 0.05	4.92 ± 0.06
Control [c]	0.66 ± 0.23	3.42 ± 0.29	0.205 ± 0.033	0.251 ± 0.044		
Blank [d]					5.04 ± 0.12	4.96 ± 0.08

[a] The given values are mean ± standard deviation (SD) of the three repeated tests. [b] n.d. = not detected (below the detection limit of 0.08 mg L^{-1} in triplicate). [c] Control: Without DBP treatment. [d] Blank: Without plant in hydroponic solution.

Table 15. DBP accumulated in different parts of eleven test plants after DBP phytoremediation for 21 days.

Common Name	DBP Accumulated Concentration (mg Kg^{-1}) [a]					
	Root		Stem		Leaf	
	0 day	21 days	0 day	21 days	0 day	21 days
Edible rape	n.d. [b]	0.05 ± 0.01	n.d. [b]	0.16 ± 0.04	n.d. [b]	0.68 ± 0.06
Chinese cabbage	n.d. [b]	0.10 ± 0.02	n.d. [b]	0.22 ± 0.06	n.d. [b]	0.74 ± 0.12
Spinach	n.d. [b]	0.03 ± 0.01	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]
Chinese mustard	n.d. [b]	0.06 ± 0.02	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]
Water cabbage	n.d. [b]	0.43 ± 0.12	n.d. [b]	0.88 ± 0.14	n.d. [b]	0.82 ± 0.18
Ceylon spinach	n.d. [b]	0.08 ± 0.02	n.d. [b]	0.26 ± 0.04	n.d. [b]	0.46 ± 0.12
Garden lettuce	n.d. [b]	3.35 ± 0.42	n.d. [b]	2.74 ± 0.38	n.d. [b]	4.35 ± 0.42
Chinese celery	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]
Edible amaranth	n.d. [b]	0.22 ± 0.04	n.d. [b]	0.12 ± 0.02	n.d. [b]	0.25 ± 0.06
Cauliflower	n.d. [b]	0.37 ± 0.02	n.d. [b]	0.45 ± 0.06	n.d. [b]	0.96 ± 0.08
Chinese chive	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]	n.d. [b]

[a] The given values are mean ± SD of three repeated tests. [b] n.d. = not detected (below the detection limit of 0.08 mg L^{-1} in triplicate).

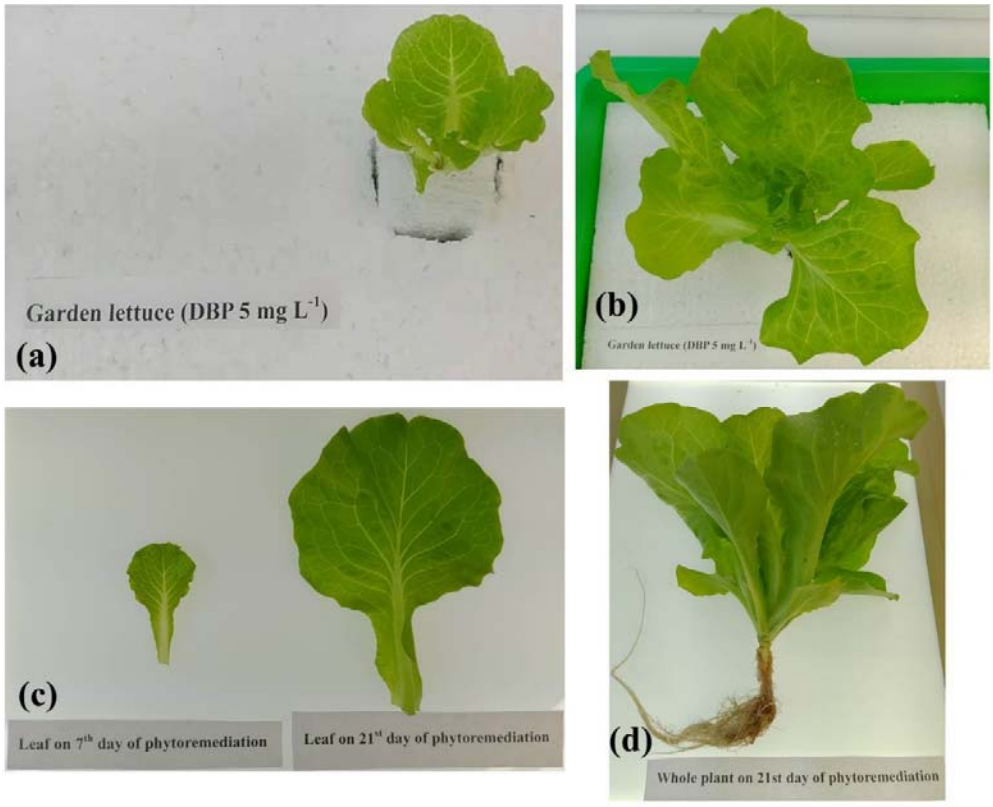

Figure 27. The morphology of Garden lettuce during the phytoremediation period. (a) Garden lettuce on 7th day of phytoremediation and (b) Garden lettuce on 21st day of phytoremediation and (c) single leaf on the 7th and 21st day of phytoremediation and (d) the whole plant on the 21st day of phytoremediation.

Previous studies indicated some plants had DBP absorption capability and accumulated distribution in the roots, stems, and leaves of plants (Li et al., 2016a; Liao et al., 2009; Liao et al., 2006). In this study, Garden lettuce could accumulate DBP in the root, stem, and leaf in 3.35 ± 0.42, 2.74 ± 0.38 and 4.35 ± 0.42 mg L^{-1} after 21 days, respectively. This capability was better than any of the other plants reported in previous studies, such as Bok choy (Liao et al., 2006) and Chinese cabbage (Liao et al., 2009).

3.3.2 Cultural Conditions of DBP Phytoremediation

The effects of various illumination times (light/dark) on the phytoremediation of DBP in the culture medium are presented in Table 16. According to the result, the optimal illumination times for DBP phytoremediation were 11 h light and 13 h dark. As shown in Table 16, the DBP

phytoremediation rate constants (k1, day^{-1}) of Garden lettuce at 11 h light and 13 h dark illumination times calculated by first-order kinetics were 2.283 and had a half-life ($t_{1/2}$, days) of 5.299 days. The reason this illumination time enhanced the efficiency of phytoremediation is because Garden lettuce is a vegetable which can be grown all year round, but the growth rate and quality in spring, autumn and winter is higher than in summer. Garden lettuce is a vegetable species which dislikes a long illumination time.

Table 16. Comparison of the effects of various cultural conditions on DBP phytoremediation rate constants (k1, day^{-1}) and half-lives ($t_{1/2}$, days).

Treatment	Garden lettuce (*Lactuca sativa* L. var. *longifolia.*)		
	k_1 (day^{-1})	$t_{1/2}$ (days)	r^2
Illumination times (light/dark)			
13h/11h	1.295	10.362	0.811
12h/12h	0.986	10.237	0.896
11h/13h	2.283	5.299	0.896
pH			
6	1.575	6.155	0.784
7	3.506	3.597	0.921
8	4.605	2.887	0.899
9	3.912	3.173	0.921
Temperature (daytime/night)			
25 °C /23 °C	3.616	3.265	0.942
23 °C /21 °C	4.609	2.991	0.820
21 °C /19 °C	2.477	4.834	0.926
DBP added concentration			
1 mg L^{-1}	4.741	2.504	0.866
3 mg L^{-1}	4.273	2.840	0.905
5 mg L^{-1}	3.783	3.193	0.931
Surfactants (1 CMC)			
Brij35	2.442	4.719	0.963
Brij30	1.677	7.706	0.894
Triton x-100	1.085	10.730	0.698
Tergitol	2.354	5.797	0.831
Tween 80	4.605	2.686	0.937

The effects of various pH levels on the phytoremediation of DBP in the culture medium are presented in Table 16. According to the results, the optimal pH level for DBP phytoremediation

was 8. As shown in Table 16, the DBP phytoremediation rate constants (k1, day^{-1}) of Garden lettuce at pH 8 were calculated by first-order kinetics and were 4.605, with a half-life ($t_{1/2}$, days) of 2.887 days. This result indicates that weakly alkaline conditions might improve the absorption capability of Garden lettuce and increased the efficiency of DBP phytoremediation.

The effects of various temperatures (daytime/night) on DBP phytoremediation in the culture medium are presented in Table 16. According to the results, the optimal cultural temperature for DBP phytoremediation was 23 °C during daytime and 21 °C at night. As shown in Table 16, the DBP phytoremediation rate constants (k1, day^{-1}) of Garden lettuce at a temperature 23 °C in daytime and 21 °C at night were calculated by first-order kinetics, providing a value of 4.609 and a half-life ($t_{1/2}$, days) of 2.991 days. This result indicates that 23°C during daytime and 21°C at night are the optimal physiological growth temperatures for Garden lettuce.

The effects of various DBP added concentrations on DBP phytoremediation in the culture medium are presented in Table 16. According to the results, the DBP added concentration for DBP phytoremediation was 1 mg L^{-1}. As shown in Table 16, the DBP phytoremediation rate constants (k1, day^{-1}) of Garden lettuce at a DBP added concentration of 1 mg L^{-1} were calculated by first-order kinetics, providing a value of 4.741 and a half-life ($t_{1/2}$, days) of 2.504 days. However, after considering the phytoremediation efficiency and the environmental pollution concentration, we decided on a DBP added concentration of 5 mg L^{-1} as the recommended optimal concentration of DBP to add. The DBP phytoremediation rate constant (k1, day^{-1}) at a DBP added concentration of 5 mg L^{-1} was 3.783, with a half-life ($t_{1/2}$, days) of 3.193 days.

The effects of various non-ionic surfactants on DBP phytoremediation in the culture medium are presented in Table 16. Our previous study found the 1 CMC of the surfactant could enhance the biodegradation efficiency of DBP, but 2 and 5 CMC would inhibit it. At 1 CMC of surfactant, DBP, which partitioned into the micellar phase of the surfactant, was directly available to be degraded on by a microorganism. However, at 2 or 5 CMC of surfactant, cellular toxicity would occur from the interaction of surfactant molecules with the cell membranes or membrane proteins. Therefore, we used 1 CMC as the added concentration of surfactants. According to the results, the optimal non-ionic surfactant condition for DBP phytoremediation was the addition of 1 CMC of Tween 80. The 1 CMC of Tween 80 was 0.012 mM (0.0016%, *w*/*v*). As shown in Table 16, the DBP phytoremediation rate constant (k1, day^{-1}) of Garden lettuce at 1 CMC of Tween 80 was 4.605, with a half-life ($t_{1/2}$, days) of 2.686 days. This result indicates that the addition of 1 CMC

of non-ionic surfactant Tween 80 is the optimal cultural condition for the DBP-based phytoremediation of Garden lettuce. In addition, phthalic acid, which is the main metabolite of DBP, was not be detected in the solution or plant samples after phytoremediation. Therefore, the type of Garden lettuce phytoremediation should be the phytoextraction, so that DBP can accumulate in the plant body.

3.3.3 Algal Biotoxicity of DBP

The biotoxicity effects of DBP before and after phytoremediation were examined using *C. vulgaris*. The median effective concentration (EC_{50}) of DBP for *C. vulgaris* was 4.9 mg L^{-1}. The latest study indicated the 96-hour median effective concentration values (96h-EC_{50}) of DBP on two typical freshwater algae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*) were 15.3 mg L^{-1} and 3.14 mg L^{-1}, respectively. Figure 28 shows the percentage of chlorophyll *a* of *C. vulgaris* compared with the blank after DBP phytoremediation. The blank test refers to biotoxicity analysis without DBP treatment. The result showed the chlorophyll *a* content of *C. vulgaris* increased after phytoremediation, suggesting after DBP was removed from the hydroponic solution, biotoxicity markedly decreased. Compared with the blank, the chlorophyll *a* content of *C. vulgaris* in the DBP solution after phytoremediation with optimal cultural conditions was 76.9%. These results indicate that DBP biotoxicity could be significantly reduced after phytoremediation with optimal cultural conditions.

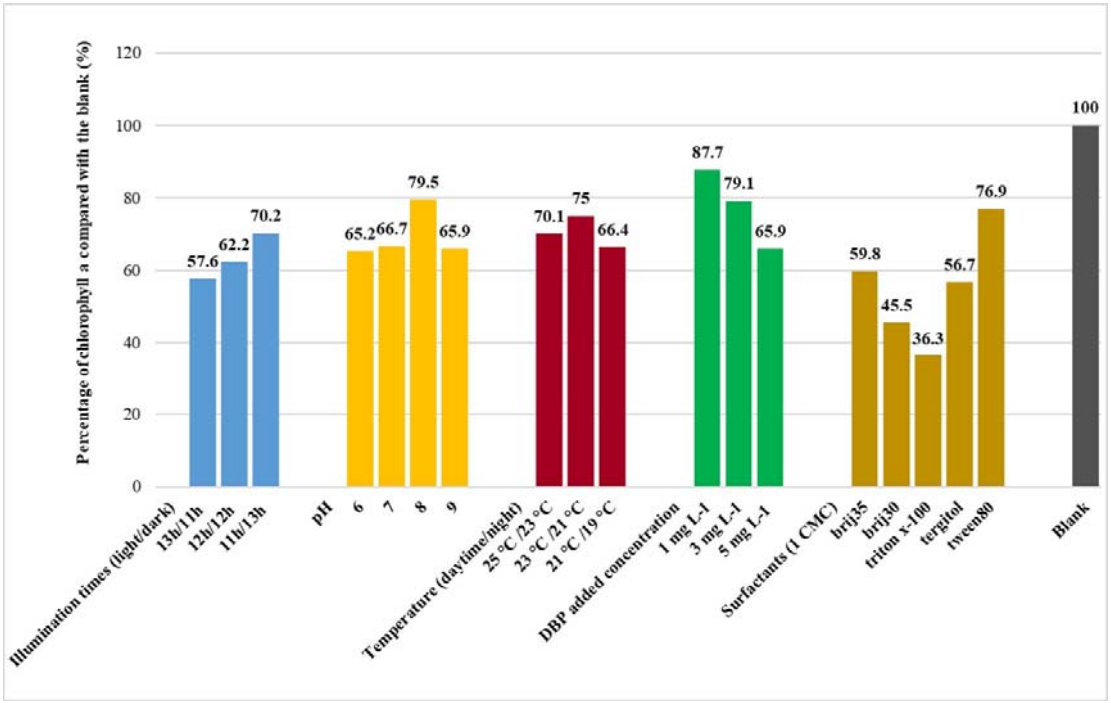

Figure 28. Percentage of chlorophyll *a* in *C. vulgaris* compared with the blank after DBP phytoremediation.

3.3.4 Proteomic Analysis

After separating with 2-DE, followed by staining with silver, 2-DE images of the Garden lettuce leaf proteomes were taken and are shown in Fig. 29. There are six protein spots, revealing significant alterations. Four proteins (spots 1, 2, 3 and 4) were identified: Photosystem II reaction center protein H (spot 1), putative Adenosine diphosphate (ADP)-ribosylation factor (spot 2) and chloroplastic 30S ribosomal protein S7 (spot 3). The protection of telomeres 1 protein (spot 2) decreased in amount or disappeared. The other two proteins (spots 5 and 6) identified as Nucleotide-binding site leucine-rich repeat (NBS-LRR) resistance-like protein RGC1F (spot 5) and DNA-directed RNA polymerase subunit beta (spot 6) was found or had increased. In the proteomic 2-DE analysis, the increase or decrease of each spot represented the strength or weakness of a protein activity in an organism. The differentially expressed proteins identified by MALDI-TOF MS and PMF analyses are listed in Table 17.

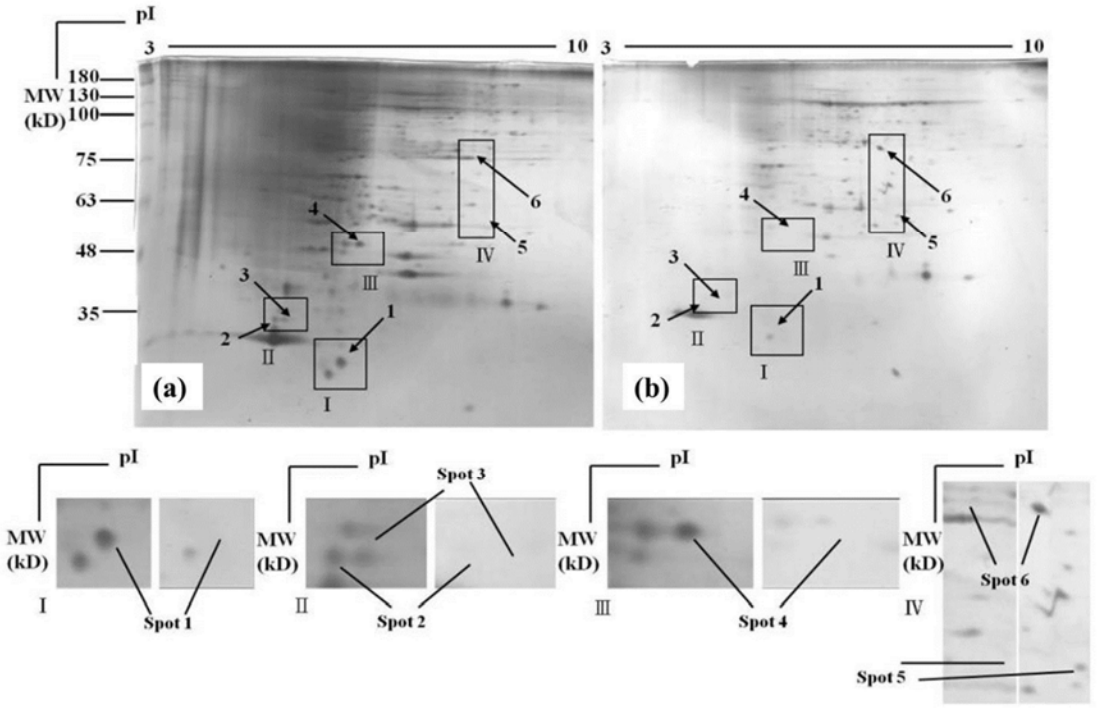

Figure 29. Two dimensional photographs of normal and DBP phytoremediated Garden lettuce. (**a**) Normal and (**b**) DBP phytoremediated Garden lettuce. Each arrow shows the proteins that are reproducible in expression. The name, accession number, estimated molecular weight and *pI* values of the proteins are listed in Table 20.

Table 17. Proteomic characterization of polypeptide differences in expression between normal and DBP treated Garden lettuce.

Spots	Protein Name	Accession no.	Estimated MW (kD)	Estimated p*I*	Amounts [a]
1	Photosystem II reaction center protein H	Q332U8	7.7	6.23	-
2	Putative ADP-ribosylation factor	A8QVJ0	15.6	4.89	-
3	Chloroplastic 30S ribosomal protein S7	Q332R9	17.2	5.35	-
4	Protection of telomeres 1 protein	B7T1J4	50.1	6.38	-
5	NBS-LRR resistance-like protein RGC1F	Q56P11	51.8	7.95	+
6	DNA-directed RNA polymerase subunit beta	Q91Fl1	80.4	7.51	+

[a] + = increase; - = decrease.

The reduced photosystem II reaction center protein H (spot 1) suggests the modulation of photosynthesis toward photosystem I (PSI). Since PSI is the terminal electron carrier in the chloroplast, this finding can be correlated to osmotic stress (Heazlewood et al., 2016). ADP ribosylation factor (spot 2) is one of the Guanosine triphosphate (GTP)-binding proteins. It is ubiquitous in eukaryotic cells, involved in catalyzing GTP/Guanosine diphosphate (GDP) exchange and acts as the regulator of vesicular traffic (Pasqualato et al., 2002). The chloroplastic 30S ribosomal protein (spot 3) and the protection of telomeres 1 protein (spot 4) are both proteins related to transcription and the protein metabolism (Heazlewood et al., 2016). Two proteins increased: NBS-LRR resistance-like protein RGC1F (spot 5), which occurs in response to osmotic and oxidative stress (Heazlewood et al., 2016), and DNA-directed RNA polymerase subunit beta (spot 6), which is the protein related to transcription and the protein metabolism (Heazlewood et al., 2016). According to these results, DBP potentially causes osmotic and oxidative stress in Garden lettuce. Compared with previous studies, DBP could accumulate in different parts of plants and cause free radical increases within the plants.

4. BRIEF SUMMARY

The results of DBP bioremediation shows that aerobic degradation of PAEs were better than anaerobic degradation. The best degradation half live ($t_{1/2}$) of 5 ppm DBP in lab. and soil are 0.65 and 2.23 days by *Deinococcus radiodurans*, respectively. Then, the removal efficiencies of 2 mg L^{-1} DBP by laccase which was extracted from the spent mushroom compost of *Ganoderma lucidum* was 100%, after 1 d of incubation. On the other hand, the best phytoremediation half live ($t_{1/2}$) of 5 ppm DBP is 2.69 days by Garden lettuce. DBP potentially causes osmotic and oxidative stress in Garden lettuce. In addition, since DBP had no significant effects on the morphology and physiological status of Garden lettuce, Garden lettuce can be recommended for use in the plant anti-DBP toxicity test, and also as the candidate plant for DBP phytoremediation.

CHAPTER 4
CONCLUSION AND FUTURE PLAN

In summary, in chapter 1, we investigated PAEs in 14 river surface water samples and 6 sediment samples of Taiwan in 2000. Then we investigated PAEs in soil, fertilizers and plastics materials taken around the major industrial area in Kaohsiung City, Taiwan in 2013. The result shown that concentrations of DBP was found to be higher than the other six PAEs and the concentration ranges of DBP in the water and sediment samples were 1.0-13.5 μg L^{-1} and 0.3-30.3 μg g^{-1}, respectively. The result also shown that concentrations of all eight PAEs were up to 1000 times higher in the sediment samples than in the surface water samples. This finding indicated that PAEs are soluble by fulvic and humic acids, and are capable of being adsorbed onto particulate matter, with sediments serving as a final sink. The result of 2013 shown that DEHP and di-*n*-octyl phthalate (D*n*OP) accounted for the most of PAEs concentrations in soil (> 90%). DBP were almost found in agricultural soil, chemical fertilizer, organic fertilizer, but not plastic mulching films samples in Taiwan, and the mean concentration was 0.01 mg kg^{-1}. Major sources of PAEs in agricultural soil could be the use of chemical fertilizer and plastic products.

In chapter 2, we evaluate the effects of DBP on vegetables. In 2009, etiolation occurred on leaves of Chinese cabbage plant treated with 50 mg l^{-1} of DBP for 42 days. DBP even below 1 mg l^{-1} had a significant effect on the concentration of chlorophyll in Chinese cabbage and the biomass shown a severe decrease under treatment with more than 30 mg l^{-1} of DBP. At a concentration below 1 mg l^{-1} of DBP, no significant difference in accumulation was found, but treatments with concentration exceeding 10, 30, 50 and 100 mg l^{-1} all resulted in significant accumulation of DBP. According to proteome level studies, three spots of increasing protein were responsible for biosynthesis of fatty acids, signal transduction of phototropic response and nitrate assimilation in plant cells, respectively.

In chapter 3, the results of DBP bioremediation shows that aerobic degradation of PAEs were better than anaerobic degradation. In 2010, we isolated two DBP degradable strains from activated sludge and these strains were identified with their 16S rRNA as *Deinococcus radiodurans* and *Pseudomonas stutzeri*. We constructed the optimal condition of DBP degradation by using different kinds of incubation factors such as temperature, initial pH, yeast extract and surfactants. The optimal conditions of DBP degradation for these two strains are: 30

°C, pH 7.5 and static culture. Besides, addition of 0.23 mM of Triton X-100 could enhance the DBP degradation for *D. radiodurans*. In the end, we amended these two strains into the origin activated sludge and analyzed the whole microbial community structure of mixed cultures by PCR-DGGE technique. The result shown that only *D. radiodurans* could survive in the activated sludge after 7 days of incubation. The other study of 2010 also shown that we added *D. radiodurans* into a soil microcosm and it could survive in the soil microcosm through 24 days of incubation. The best degradation half live ($t_{1/2}$) of 5 ppm DBP in lab. and soil are 0.65 and 2.23 days by *Deinococcus radiodurans*, respectively. In 2012, we found that the removal efficiencies of 2 mg L^{-1} DBP by laccase which was extracted from the spent mushroom compost of *Ganoderma lucidum* was 100%, after 1 day of incubation. In 2019, we used Garden lettuce (*Lactuca sativa* L. var. *longifolia*), which has a significant DBP absorption capability, as a test plant to measure phytoremediation kinetics and proteome changes after being exposed to DBP. The results show that DBP accumulated in different parts of the Garden lettuce but the physiological status and morphology shown no significant changes following DBP phytoremediation. The optimal condition for the DBP phytoremediation of Garden lettuce is one critical micelle concentration (CMC) of non-ionic surfactant Tween 80 and the half-life ($t_{1/2}$, days), which calculated by first-order kinetics, was 2.69 days for 5 mg L^{-1} of DBP. In addition, the results of biotoxicity shown that the median effective concentration (EC_{50}) of DBP for *C. vulgaris* is 4.9 mg L^{-1}. According to the information of proteomes, DBP potentially causes osmotic and oxidative stress in Garden lettuce. In addition, since DBP had no significant effects on the morphology and physiological status of Garden lettuce, Garden lettuce can be recommended for use in the plant anti-DBP toxicity test, and also as the candidate plant for DBP phytoremediation. Based on these works, we hope that these findings could provide useful information for applying the bioremediation of DBP and assessing the related risk of DBP in our environments. However, we also believe that all we have done only the temporary solutions to the problem, the DBP and PAEs contaminated problems still depends on the wisdom of human beings to solve in the future.

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