DISSERTATION

BIODEGRADATION OF BISPHENOL A BY ALGAL-BACTERIAL SYSTEMS

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BIODEGRADATION OF BISPHENOL A BY ALGAL-BACTERIAL SYSTEMS

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We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Engineering.

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ABSTRACT

Emerging pollutants in water, such as endocrine-disrupting compounds have generated significant interest because of their adverse effect on wildlife. Bisphenol A (BPA) is an endocrine disruptor that is extensively used in industry as a monomer for polycarbonate production. Because of its high demand and production, it is increasing concern that BPA-contaminated wastewater may intrude into our water environments. The source of BPA was believed to be discharged from industrial wastewater or landfill leachate. In many developing countries, BPA-contaminated wastewater i.e. landfill leachate is discharged to water environment with only primary or without treatment because of high cost of treatment. In this context, low-cost biological treatment is required. Recently, an algal-bacterial system has been receiving attention due to its promising treatment and low operational cost. However, the feasibility of an algal-bacterial system for degrading a moderately toxic and oestrogenic compound such as BPA has not been studied. The major aim of this thesis was to investigate the feasible use of an algal-bacterial system for the degradation of BPA. In order to investigate the feasibility of using an algal-bacterial system to degrade BPA, three major experiments were conducted. First, BPA degradation by a bacterial consortium. Second, BPA degradation by an algal-bacterial system in a batch experiment and third, BPA degradation by an algal-bacterial system in a semi-continuous experiment.

The bacterial consortium biodegraded BPA 10, 20, and 50 mg L^{-1} to below the detection limit under mechanical aeration. Similarly, the algal-bacterial systems also degraded BPA to below

the detection limit in all BPA concentrations under photosynthetic oxygen in the batch experiments. These results suggest that photosynthetic oxygen provided by the algae support the degradation of BPA. Furthermore, the semi-continuous experiment of an algal-bacterial system biodegraded BPA up to 50 mg L^{-1} d^{-1}, indicating the feasibility use of algal-bacterial system for the degradation of BPA.

The partial 16S rDNA sequencing results show that Proteobacteria dominated (90-93%) the inoculum of the batch experiment and the semi-continuous experiment. Besides, 7 phylotypes were detected in the inoculum of the both batch experiment and the semi-continuous experiment. Amongst the 7 phylotypes, 6 of them were known bacteria that degraded various monocyclic and polycyclic aromatic hydrocarbon (MAH and PAH). Therefore, these bacteria might also degrade BPA and some of the BPA biodegradation intermediates that showed a similar chemical structure to the MAHs and PAHs. Moreover, the treated effluent in the batch experiment showed a relatively low amount (<5%) of oestrogenic BPA biodegradation intermediates. The treated effluent is then recommended to be discharged to the environment with the dilution of river or rainwater. The semi-continuous experiment of an algal-bacterial system has shown a mutualistic relationship between the algae and the BPA-degrading bacteria for the degradation of BPA.

This is the first report showing the feasibility of an algal-bacterial system to degrade a moderately toxic and oestrogenic activity compound. Furthermore, the present study demonstrated the potential of an algal-bacterial system to treat 50 mg L^{-1} of BPA which concentration is as high as industrial effluent in a semi-continuous BPA loading. Based on the overall results, the algal-bacterial system may serve as a low-cost alternative treatment for degradation of BPA in developing countries.

Chapter I

General Introduction

1.1. Background

Bisphenol A (BPA) is a synthetic compound that is widely used in the production of plastics. It is also a ubiquitous pollutant that is receiving attention due to its chemical toxicity and oestrogenic activity (Alexander et al., 1988; Krishnan et al., 1993). The International Union of Pure and Applied Chemistry (IUPAC) described that the effect of acute toxicity should occur in short time i.e. 24 hours; whereas, a chronic toxicity effect usually takes a longer time i.e. from 7 days or more to occur (McNaught and Wilkinson, 1997). BPA has both acute and chronic toxicity effects on algae depending on the species and concentration of exposure (Zhang et al., 2014). The acute and chronic effects of BPA on the survival and growth of invertebrates, fish, bird, and mammals ranged from μg L^{-1} to mg L^{-1} (Flint et al., 2012). Moreover, BPA is known to have oestrogenic effect that can interfere with the proper function of the endocrine system of several organisms (Krishnan et al., 1993). Even algae that are non-target organisms without an endocrine system are affected by EDCs on photosystem II energy fluxes (Perron and Juneau, 2011).

Although accumulated evidence shows that BPA exhibits toxic effects on organisms, the regulations of BPA are still scarcely implemented in the world. Several developed countries, except the government of Japan, have considered regulating BPA or established some guidelines for it (Table 1.1). For example, an effective concentration (EC50) and lethal concentration (LC50) of BPA to

aquatic biota ranging from 1.0 to 10 mg L^{-1} were set by Canada (Environment Canada, 2008). BPA is classified as “moderately toxic” to aquatic organisms by the European Commission (Commission of the European Communities, 1996). The United States Environmental Protection Agency (US EPA), has classified BPA as “toxic” to aquatic organisms (Alexander et al., 1988). However, in Japan, an ecological risk evaluation for BPA concluded, “The current exposure levels of BPA will not pose unacceptable risks to the local populations of aquatic life, particularly fish” (AIST, 2007). No BPA guidelines or regulations have been established for developing countries.

The systematic name of BPA is 2,2-bis(4-hydroxydiphenyl) or 4,4’-Isopropylidenebisphenol with a molecular weight of 168.2 g mol^{-1}. BPA is moderately soluble in water with a solubility of 300 mg L^{-1} at room temperature. The acid dissociation constant (pKa) value, a consequence of the thermodynamics of the dissociation reaction, of BPA, ranged from 9.9 to 11.3. BPA is produced from phenol and acetone through acid catalyzed condensation reaction and is then crystallized to form high purity BPA (Fig. 1.1; Shimizu et al., 2008). Because of its purity, BPA is an important monomer that is easily bound to other molecules to form a polymer (Fiege et al., 2000). For example, combining BPA with phosgene produces polycarbonate, and combining BPA with epichlorohydrin produces epoxy resin. Polycarbonate is crystal-clear transparent, heat resistant, and low-cost material, which is suitable to manufacture plastic-based products, such as automobile parts, sports equipment, plastic containers, and toys. Epoxy resin is used as canned food coating, water pipe lining, and thermal papermaking.

BPA ranks as one of the highest production volume chemicals in the world, with the global

production capacity of 2.2 $\times 10^6$ tonnes annually in 2003 (Burridge, 2003). Because of the high demand for polycarbonates and epoxy resin, the global production capacity of BPA reached 4.7 $\times 10^6$ tonnes annually in 2007 (Jiao et al., 2008). This figure is expected to rise to about 9.6 $\times 10^6$ tonnes by 2020. Since BPA is a high production volume chemical, it should be screen for its toxicity as internationally agreed (USEPA, 2004).

Due to the extensive use of BPA in commercial products, it raises the concern if it ends up leaking or discharging from manufacturing to aquatic environments. BPA could be released into aquatic environments through several pathways. For instance, an intentional or unintentional discharged from manufacturing and a release from wastewater treatment plants (WWTP) (Meesters and Schroder, 2002). The release of BPA from landfill leachate is another important route of BPA exposure to the environment (Wintgens et al., 2003). The concentrations of BPA in landfill leachate are one to five orders higher than in WWTP, which ranged from 1.4- 17,200 μg L^{-1} (Crain et al., 2007). In a landfill, BPA can leach out from the incomplete polymerized products via hydrolysis of BPA from plastics, especially during the initial stage of landfilling (Asakura et al., 2004). Moreover, when the landfill is associated with much garbage, the leachate that contains BPA is not biodegradable in an anaerobic condition (Ying and Kookana, 2005). BPA will, therefore, accumulate in landfill leachate and if the leachate is not treated properly and leaks into the environment, it would cause direct BPA pollution.

Developing countries are vulnerable to landfill leachate because they do not have proper facilities for leachate treatment. Landfill leachate that is contaminated with BPA is directly discharged

into the water environment or after only primary treatment because they cannot afford the high treatment cost (Pugh et al., 1999). In reality, conventional leachate treatment methods are costly owing to the initial outlay of constructing the treatment plant, energy necessities and regular use of additional chemicals. In this context, low-cost aerobic biological treatment for BPA-containing wastewater, especially in landfill leachate, is required.

There are several methods to remove BPA from wastewater including Advanced Oxidation Process (AOP) and biological treatments (BT). AOPs include Fenton and sono-Fenton; photodegradation; ultrafiltration, pre-ozonation and ozone disinfection; TiO_2 based photocatalytic suspended system, photo-Fenton and ultrasonification (Gonze et al., 1999; Chiang et al., 2004; Kaneco et al., 2004; Katsumata et al., 2004; Nomiyama et al., 2007). In AOPs, BPA is degraded by hydroxyl radicals that are generated from various technologies i.e. ozone, UV light, chemicals, etc. However, the advanced technology consumed high energy to generate hydroxyl radicals. Although AOPs are effective for eliminating BPA but have required high energy and the high cost of treatment, which hinders their use in removing BPA, especially for high-volume wastewater treatment. Moreover, AOPs are known to degrade BPA to some intermediates that exhibit oestrogenic activity and are more toxic than the mother compound of BPA (Olmez-Hanci et al., 2013; Plahuta et al., 2014). The toxic and oestrogenic intermediates may lead to a secondary pollution. BT is considered as one of the most economical and promising methods to biodegrade BPA (Staples et al., 1998; Fürhacker et al., 2004). They include membrane bioreactors and continuous activated sludge reactors.

1.2. BPA biological treatment and BPA-degrading capacity

In biological treatment, bacteria have the primary role of degrading BPA because they use it as a carbon sources for their growth (Lobos et al., 1992). BPA-degrading bacteria have been isolated from various environments from soils, activated sludge, river, and seawater (Table 1.2). Lobos et al. (1992) and Spivack et al. (1994) were the first groups of researchers that studied the biodegradation of BPA by a bacterium. They described the degradation pathways of BPA by an unidentified single-strain bacterium *Sphingomonas* sp. MV1. (Fig. 1.2). BPA was biodegraded to its biodegradation intermediates before being transformed into bacterial cells and carbon dioxide (CO_2). However, not only BPA but also its biodegradation intermediates exhibit oestrogenic activity. The BPA biodegradation intermediates include 4,4-dihydroxy-alpha-methylstilbene, 2,2-bis(4-hydroxyphenyl) propanoic acid (IV) and *p*-hydroxyacetophenone (*p*-HAP) exhibit oestrogenic activity (Nishihara et al., 2000; Yoshihara et al., 2001; Ike et al., 2002; Suzuki et al., 2004). Therefore, monitoring the BPA biodegradation intermediates is essential to evaluate the treatment efficiency.

Although single-strain bacteria have been found to be able to degrade BPA, employing single-strain of bacteria in the wastewater treatment process is not practical. Because it is hard to maintain a pure culture, amplifying the pure strain to augment a practical wastewater process became problematic (Quan et al., 2004). Moreover, a single-strain bacterium *Sphingomonas* sp. MV1 degraded BPA to *p*-HAP that inhibited its growth (Lobos et al., 1992). On the other hand, a mixed bacteria is more capable in degrading organic pollutants than a single-strain of bacteria (Sorkhoh et al., 1995). Mixed bacteria may comprise of various enzymes and a series of sequential reactions that

enable the degradation of the targeted compound and its biodegradation intermediates (Holben et al., 1998). Although, mixed bacteria may be beneficial to be used for pollutant degradation, the degradation capability of a microbial community is influenced by the existence of the important populations in the community. The dominant and functionally important populations in a mixed bacterial community often changed following the environmental conditions (Lee et al., 2008). However, bacterial acclimatization to the targeted compound can result in a significant population composition in the microbial communities for the removal of quinoline (Liu et al., 2006). In the present study, the BPA acclimatized bacterial community is defined as bacterial consortium. Therefore, investigation of the microbial community structure of the bacterial consortium is critical to identify the microorganisms that are functionally responsible for BPA biodegradation. Since the structure of a microbial community can be encoded by a phylogenetic tree (Tanaseichuk et al., 2014), the study of the phylogenetic tree is used to elucidate the functional members in the community.

It is known that BPA is biodegraded in an aerobic condition (Dorn et al., 1987). Under aerobic respiration, bacteria use oxygen (O_2) to oxidize carbon in BPA to form CO_2 and produce bacterial cells. In this process, O_2 acts as an electron donor for the biodegradation of BPA. In a similar manner, mechanical aeration is applied in conventional activated sludge treatment for biodegradation purposes. In fact, the aeration system consumes approximately 50 to 65% of the net power demand of a typical activated sludge wastewater treatment plant (USEPA, 1999). If the cost of aeration in a biological treatment could be reduced, a relatively low-cost treatment system for BPA degradation could be realized.

1.3. BPA biodegradation by photosynthetic algal-bacterial system

An algal-bacterial system represents one type of low-cost biological treatment systems (Oswald et al., 1951). Because, in this system, algae supply the O_2 necessary for the heterotrophic bacteria to degrade organic pollutants and produce the CO_2, which in turn are needed by the algae for photosynthesis (Fig. 1.3). An algal-bacterial systems were first studied for the removal of nutrients in wastewater treatment ponds by Oswald et al. (1951). These systems were then extended to treat agriculture wastewaters and domestic wastewaters because of the potential cost-effective photosynthetic O_2. Moreover, algae supported the aerobic degradation of organic compounds i.e. phenanthrene, acetonitrile, phenol, and salicylate by the bacteria through providing the photosynthetic O_2 (Guieysse et al., 2002; Borde et al., 2003; Muñoz et al., 2003a, 2003b, 2004; Safonova et al., 2004; Muñoz et al., 2005a, 2005b). The application of the algal-bacterial system to a moderately toxic compounds such as BPA remains to be elucidated.

BPA is toxic to both marine and freshwater microalgae (Li et al., 2009; Zhang et al., 2014). However, algae could still removed it either through oxidization (Hirooka et al., 2005), glycosylation (Nakajima et al., 2007) or bioaccumulation (Li et al., 2009). Hirooka et al. (2005) reported that *Chlorella fusca* biodegraded BPA through oxidative degradation that were similar to those found in higher plants. Glycosylation is a detoxification reaction in the plant kingdom (Pridham, 1964). This detoxification reaction has also been observed in freshwater green algae when exposed to BPA. In this process, algae hydroxylated BPA to glycosides that are non-toxic or less toxic forms (Nakajima et al., 2007). On the other hand, if bioaccumulation occurs, the increase of BPA toxicity in the algae

cell can damage the cytoarchitecture and cause disruption of physiological and biological processes (Li et al., 2009). As bioaccumulation may not only adversely affect the algae, it could also create another concern of post-treatment biomass management if it is to be used for removing BPA. Since, the mechanism of BPA removal by algae can differ depending on the species, algal BPA degradation should be determined in different species of algae. Even though some compounds may be toxic to algae, algal-bacterial systems have been shown to be able to remove organic pollutants such as acetonitrile, salicylate, phenol, phenanthrene, and black oil (Table. 1.3). In the case of BPA, it has been shown that BPA is toxic to algae alone but the response of algae to BPA in algal-bacterial systems has not been reported.

Chlorella is a genus of unicellular green algae belonging to the phylum Chlorophyta. This genus has been well studied in the field of wastewater treatment for removing organic pollutants and nutrients. For example, *C. sorokiniana* is very efficient in removing ammonium from wastewater treatment (de-Bashan et al., 2008). This alga is known to have high tolerance in an extreme condition, which would be unfavorable for other algal species (de-Bashan et al., 2008). *C. sorokiniana* has also been shown to be tolerant of high concentrations of salicylate and O_2 production (Muñoz et al., 2003). While *C. vulgaris* has a high tolerance of xenobiotics and has often been used to remove nonylphenol and tributyltin (Gao et al., 2011; Jin et al., 2011). *C. vulgaris* has also been reported to be able to remove BPA through bioaccumulation and biodegradation (Ji et al., 2014), but its biodegradation ability together with bacteria has yet to be elucidated. However, these two species are very different in size (*C. sorokiniana* < 8 µm and *C. vulgaris* < 5 µm). Larger algal cell size has been shown to

contribute to a higher O_2 production rate (Park and Lee, 2000). Since an algal-bacterial system depends critically on the photosynthetic oxygenation by algae, the algal cell size may affect the O_2 production and thereby affect the BPA biodegradation. Therefore, *C. sorokiniana* and *C. vulgaris* were chosen to represent a big and small size of *Chlorella*, respectively for the algal-bacterial systems.

Light plays a vital role in algal photosynthesis, but heterotrophic bacterial growth does not need light. Visible light has been previously reported to affect negatively bacterial growth (Aas et al., 1996; Sommaruga et al., 1997). Thus, an algal-bacterial system that required light prompted the question whether light affects bacterial growth in an algal-bacterial system during BPA degradation. Because many of recently designed bioreactors such as algal-bacterial pond systems and photo-bioreactors involve light, this study attempts to fill the research gap of algal-bacterial research.

Although some batch experimental studies on the biodegradation of organic pollutants by algal-bacterial systems have been applied in the field, a feasibility study of using an algal-bacterial system for the degradation of BPA is required. However, the information on semi-continuous loading of organic compounds is limited. There is only one report in the literature showing a semi-continuous degradation of salicylate by *C. sorokiniana* and *Ralstonia basilensis* (Guieysse et al., 2002). They concluded that salicylate degradation was always limited by the O_2 generated by algae. However, whether the limitation could be overcome by using a bacteria consortium with algae is to be elucidated. Therefore, a feasibility of using an algal-bacterial system was attempted and the potential application of the algal-bacterial system for BPA biodegradation was discussed.

1.4. Aims of the thesis

The ultimate aim of this thesis was to investigate the feasibility of an algal-bacterial system to biodegrade BPA. The specific objectives of this study are:

1. To investigate the BPA degradation capability of a bacterial consortium
2. To examine whether light affects BPA biodegradation by the bacterial consortium
3. To investigate the BPA degradation capability of an algal-bacterial system
4. To inquire into the feasibility of an algal-bacterial system for biodegrading BPA in a semi-continuous BPA loading

1.5. Outline of the thesis

This thesis is comprised of four chapters include the background of the dissertation and the treatment of BPA. Moreover, the aim of the study is also addressed. In the second chapter, BPA biodegradation by a bacterial consortium is presented. The third chapter discusses the biodegradation of BPA by algal-bacterial systems. The tolerance of algae both in an algal monoculture and in an algal-bacterial system is also presented in Chapter III. A study of the feasibility of algal-bacterial systems to biodegrade a semi-continuous loading of BPA is addressed. Since BPA is an oestrogenic compound, the biodegradation intermediates of BPA were also monitored to confirm the effluent quality. The results and discussion of the biodegradation intermediates of both the bacterial and algal-bacterial systems are presented in Chapter II and Chapter III, respectively. The functionally important

bacteria of the bacterial consortium for BPA degradation was also investigated. The microbial community study of both the bacterial and the algal-bacterial systems is presented in Chapter II and Chapter III, respectively. Finally, a general discussion of the study, application of an algal-bacterial system, and future study is also proposed in Chapter IV.

Table 1.1. Standard, limits and guidelines for BPA in different countries.

Country/ continent	**BPA Standard, limit and guideline**	**References**
Canada	EC_{50}[1] and LC_{50}[2] of BPA to aquatic biota ranged from 1.0 to 10 mg L^{-1}	Environment Canada (2008)
Europe	BPA is moderately toxic to aquatic organisms	Commission of the European Communities (1996)
US	BPA is toxic to aquatic organisms	Alexander et al. (1988)
Japan	"The current exposure levels of BPA will not pose unacceptable risks to the local populations of aquatic life, particularly fish."	AIST[3] (2007)

[1]Half maximal effective concentration

[2]Half maximal lethal concentration

[3]National Institute of Advanced Industrial Science and Technology

Table 1.2. BPA-degrading bacteria and source of the environment.

Bacterial strains	**Environment**	**References**
Pseudomonas putida strain KA5		Kang and Kondo (2002)
Pseudomonas sp. strain KA4		Kang and Kondo (2002)
Sphingomonas sp. strain BP-7		Sakai et al. (2007)
Sphingomonas sp. strain BP-7	River	Yamanaka et al. (2008)
Sphingomonas sp. strain MV1		Lobos et al. (1992)
Sphingomonas sp. strain MV1		Spivack et al. (1994)
Pseudomonas paucimobilis strain FJ-4		Ike et al. (1995)
Sphingomonas sp. strain TTNP3		Tanghe et al. (1999)
Sphingomonas sp. strain TTNP3	Bed reactor/	Kolvenbach et al. (2007)
Cupriavidus basilensis strain JF1	Activated sludge	Fischer et al. (2010)
Achromobacter xylosoxidans strain B-16	Composting Leachate	Zhang et al. (2007)
Sphingomonas sp. strain AO1		Sasaki et al. (2005)
Sphingomonas sp. strain AO1		Oshiman et al. (2007)
Pseudomonas sp. strain LBC1		Telke et al. (2009)
Bacillus sp. strain GZB		Li et al. (2012)
Unidentified strain WH1	Sediment/soil	Ronen and Abeliovich (2000)

Table 1.3. Organic pollutants degraded by the algal-bacterial systems.

Organic compound	**Algae**	**Bacteria**	**Removal rate (mg L^{-1} d^{-1})**	**Reference**
Acetonitrile	*Chlorella sorokiniana*	Bacterial consortium	432	Muñoz et al. (2005b)
Salicylate	*C. sorokiniana*	*Ralstonia basilensis*	7.4	Borde et al. (2003)
Phenol		*Acinetobacter haemolyticus*	3.5	
Phenanthrene		*Pseudomonas migulae* *Sphingomonas yanoikuyae*	0.08	
Black oil	*Chlorella* spp. *Scenedesmus* sp. *Stichococcus* sp. *Phormidium* sp.	*Rhodococcus* sp. *Kibdelosporangium aridum*	5.5	Safonova et al. (2004)
Phenanthrene	Cyanobacteria	*Pseudomonas* sp.	0.00001	Abed (2010)

Phenanthrene	*C. sorokiniana*	*Pseudomonas migulae*	576	Muñoz et al. (2003a)
Phenol	*C. vulgaris*	*Alcaligenes* sp.	90	Essam et al. (2006)

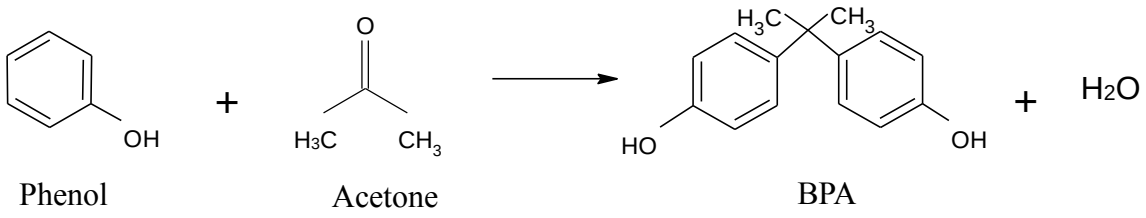


Fig. 1.1. The chemical formation of BPA.

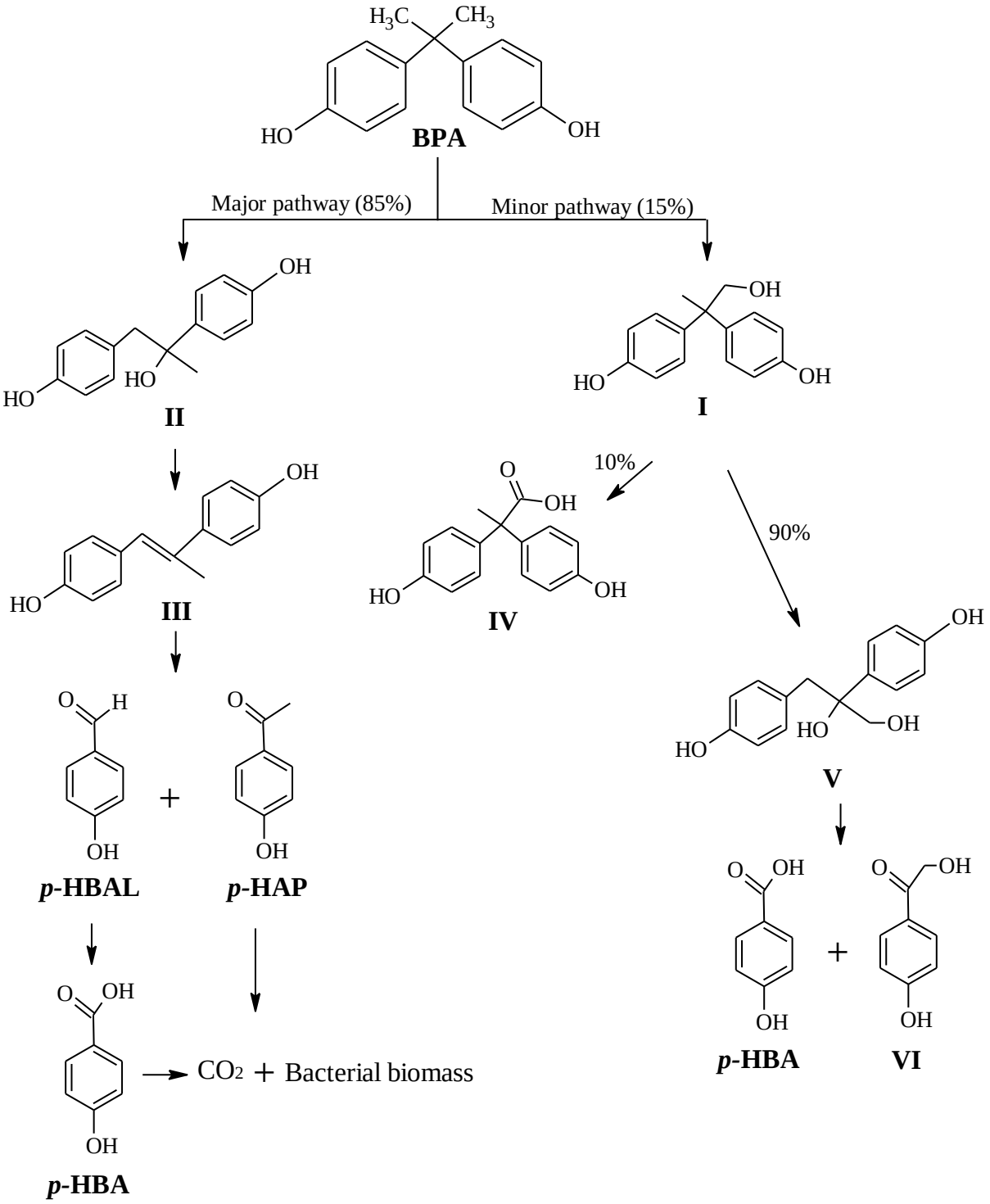

H3C
CH3
HO
OH
BPA
Major pathway (85%)
Minor pathway (15%)
HO
HO
OH
II
HO
OH
OH
I
10%
90%
HO
OH
III
O
OH
HO
OH
IV
O
H
OH
p-HBAL
+
O
OH
p-HAP
OH
HO
OH
V
O
OH
OH
p-HBA
+
O
OH
OH
VI
O
OH
OH
p-HBA
CO2 + Bacterial biomass

Fig. 1.2. Metabolic pathway for BPA biodegradation by *Sphingomonas* strain MV1 (Lobos et al., 1992; Spivack et al., 1994).

(I) 2,2-bis(4-hydroxyphenyl)-1-propanol,

(II) 1,2-bis(4-hydroxyphenyl)-2-propanol,

(III) 4,4-dihydroxy-alpha-methylstilbene,

(IV) 2,2-bis(4-hydroxyphenyl) propanoic acid,

(V) 2,3-bis(4-hydroxyphenyl)-1,2-propanediol,

(VI) *p*-hydroxybenzolmethanol,

(*p*-HBAL) *p*-hydroxybenzaldehyde,

(*p*-HAP) *p*-hydroxyacetophenone,

(*p*-HBA) *p*-hydroxybenzoic acid

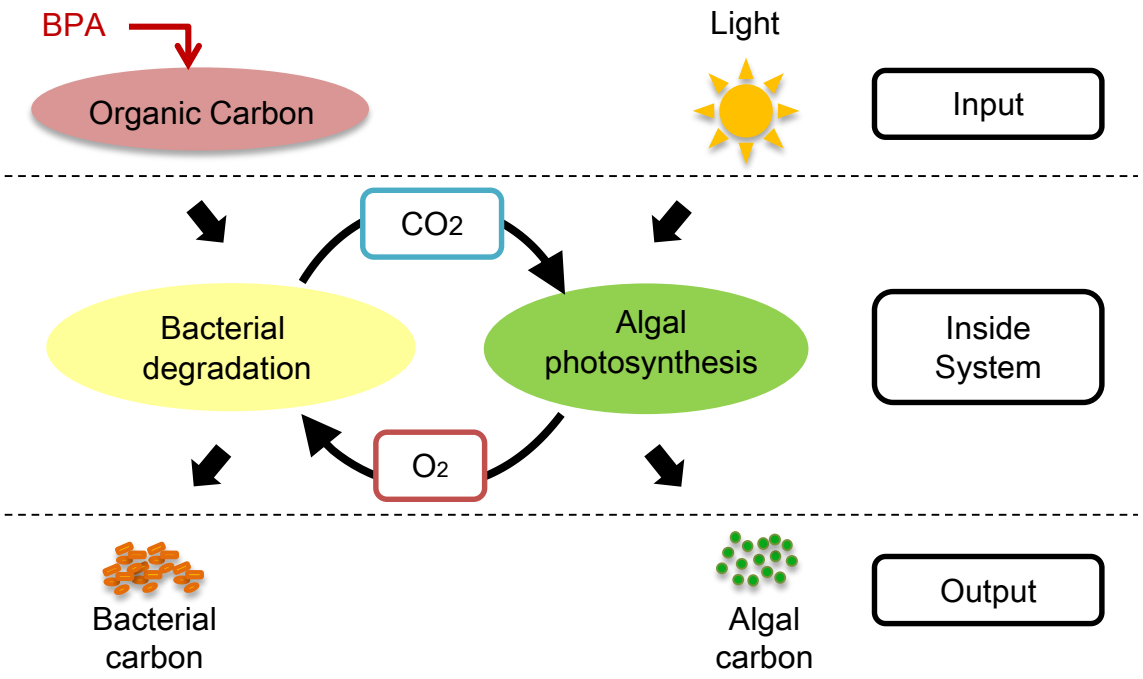


Fig. 1.3. General schematic diagram of an algal-bacterial system.

Chapter II

Bacterial BPA Degradation in Light and Dark Conditions

2.1. Introduction

BPA biodegradation studies have focused mainly on single-strain bacteria, but rarely on bacterial consortia (Zhao et al., 2008; Roh et al., 2009). Single-strain BPA degraders were individually isolated from diverse environments. They include *Sphingomonas* sp. MV1 (Lobos et al., 1992), *Sphingomonas* sp. strain AO1 (Sasaki et al., 2005), *Achromobacter xylosoxidans* strain B-16 (Zhang et al., 2007), and *Bacillus* sp. strain GZB (Li et al., 2012). However, single-strain bacteria would have to compete with the autochthonous population in the wastewater treatment process, in which they may be implanted. Because of a weak flocculation capability of single-strain bacteria, it may also be difficult to produce enough biomass to augment a practical wastewater process (Quan et al., 2004).

Since bacterial consortia contained in WWTP are composed of a broad range of bacterial species, BPA biodegradation by bacterial consortia should be evaluated to understand the degradation capability. Bacterial consortia have an excellent resistance to substrate inhibition (Marrot et al., 2006) and are more efficient in the utilization of phenol and potentially toxic biodegradation intermediates (Hanson et al., 1999). A mixed bacterial community has shown a promising BPA removal in activated sludge (Zhao et al., 2008; Roh et al., 2009). Ferro Orozco et al. (2013) also reported that mixed bacteria from activated sludge were able to deal with higher BPA concentrations because of the presence of high microbial diversity with a broad physiological capability. For BPA biodegradation

studies, mixed bacteria in the form of activated sludge have been used (Zhao et al., 2008; Roh et al., 2009; Ferro Orozco et al., 2013). Since BPA is a hydrophobic compound, its removal in activated sludge could be affected by sludge absorption and bacterial degradation. Some studies show that in conventional activated sludge treatment, a significant amount of BPA is adsorbed on sludge (Mohapatra et al., 2011; Nguyen et al., 2013). In this study, BPA biodegradation capability of a bacterial consortium but not sludge was examined.

Previous BPA degradation studies were mainly conducted in the dark probably because bacteria do not need light for their growth, and thus the effect of light on BPA degradation by bacteria has been neglected. The fate of BPA in river water has been conducted in the dark but not in the light. The researchers concluded that BPA is degradable in non-sterilised river water (Kang and Kondo, 2002; Suzuki et al., 2004). However, some studies showed that visible light has an inhibitory effect on heterotrophic bacteria production (Aas et al., 1996; Sommaruga et al., 1997). Thus, whether light affects bacterial growth on BPA was investigated.

Nevertheless, under the light condition, those substances that are photodegradable may serve as extra carbon sources for bacterial growth and may cause an overestimation of the bacterial growth. Mineral salts medium (MSM) is a common culture medium for bacteria, algae, and algal-bacterial cultures (Weissenfels et al., 1992; Guieysse et al., 2002; Muñoz et al., 2005a), which is composed of mineral chemicals required for microbial growth. To keep the mineral chemicals in solution so that microbial can easily absorb it, artificial metal chelator, EDTA (ethylenediaminetetraacetic acid) is commonly added. Among the metal complexes formed in the medium, Fe-EDTA is photodegraded to

biodegradable metabolites (Kari and Giger, 1995) that may serve as carbon sources for bacterial growth. Since light may have various effects on bacterial growth, the effect of light on BPA biodegradation by bacterial consortium was investigated in this study.

2.2. Materials and Methods

2.2.1. Culture medium and pre-culture

All experiments were performed using sterile mineral salt medium (MSM) consisting of the following components (in g L^{-1}): KNO_3, 1.25; $MgSO_4 \cdot 7H_2O$, 1.00; $CaCl_2$, 0.0835; H_3BO_3, 0.1142; $FeSO_4 \cdot 7H_2O$, 0.0498; $ZnSO_4 \cdot 7H_2O$, 0.0882; $MnCl_2 \cdot 4H_2O$, 0.0144; MoO_3, 0.0071; $CuSO_4 \cdot 5H_2O$, 0.0157; $Co(NO_3)_2 \cdot 6H_2O$, 0.0049; EDTA, 0.5; and KH_2PO_4, 1.25 (Sorokin and Krauss, 1958).

BPA ($C_{15}H_{16}O_2$) was obtained from Wako, Japan. It was dissolved in alkaline; a stock solution (1 mg mL^{-1}) of BPA was prepared with KOH solution. The medium was set to pH 7 with KOH and was autoclaved at 121 ℃ for 20 min.

The activated sludge was obtained from the Hachioji Kitano Wastewater Treatment Plant in Tokyo, Japan and was filtered through a 4.7 cm diameter GF/F filter (Whatman) to filter out the suspended solids. Approximately 20 mL of the filtrate was inoculated into a 500 mL conical flask with 250 mL of MSM containing 50 mg L^{-1} BPA. The culture was then incubated at 25 ± 1 ℃ in an incubator in the dark under continuous aeration. A substrate of 50 mg L^{-1} BPA was fed to the cultures once a week for three months. The bacterial consortium was then collected and maintained at a temperature of 4 ℃ before bacterial BPA degradation assay.

2.2.2. Bacterial BPA degradation assays in the light and dark conditions

The highest BPA concentration in landfill leachate was 17 mg L^{-1} (Yamamoto and Yasuhara, 1999). Thus, in the degradation assays, BPA was used at concentrations 2-3 folds the highest reported value to assess the level of bacterial tolerance. Degradation assays were performed in serum bottles containing 100 mL of MSM and 0, 10, 20, and 50 mg L^{-1} of BPA. The initial bacterial biomass was adjusted at OD_{600nm} = 0.007 ± 0.001. Bacterial growth in the liquid medium was measured by monitoring the OD_{600nm} using a UV-VIS spectrophotometer (Jasco, V-530, Japan).

The bottles were closed with rubber septa and mechanically aerated through glass tubes with a flow rate of 0.2 L min^{-1}. Milli-Q water was added daily to compensate for evaporation. Samples (1.5 mL) were withdrawn daily to measure the microbial growth, BPA concentration, BPA metabolites, and EDTA concentration. BPA degradation experiments were performed in triplicate and cultures were incubated at 25 ± 1 ℃ in an incubator in the dark. To investigate the effect of light on the degradation of BPA, an experiment was conducted with a 12-h light/12-h dark cycle. The light intensity was 300 μmol photons m^{-2} s^{-1} (Biospherical Instrument Inc., QSPL2101).

2.2.3. Analytical methods

2.2.3.1. Analysis of BPA and EDTA by HPLC

BPA concentration was determined by HPLC (600E system, Waters). Prior to analysis, samples (1 mL) were centrifuged in a high-speed refrigerated microcentrifuge (Model 3700, Kubota) at 10,000 × g for 10 min. Subsequently, 2.5 μL of supernatant were injected into the HPLC system

equipped with a UV-VIS detector (SSC-5410, Senshu Scientific) that was connected to a column (Brownlee SPP C18, 4.6 mm × 75 mm × 2.7 μm, PerkinElmer). BPA was eluted with a mobile phase composed of acetonitrile, Milli-Q water, and acetic acid (37/63/0.1, v/v/v) at a flow rate of 0.4 mL min^{-1}. The oven temperature was set to 30 ℃. The detection wavelength was 222 nm (Inoue et al., 2003), and the detection limit of BPA was approximately 0.5 mg L^{-1}.

For the EDTA concentration analysis, a solution of 0.001 M $FeCl_3$ in 0.01 M HCl was used to convert the EDTA into a Fe(III)-EDTA complex (Loyaux-Lawniczak et al., 1999). The $FeCl_3$ was diluted 1:1 with the EDTA sample plus a small amount of acetic acid and left overnight in the dark to form the Fe(III)-EDTA complex prior to the HPLC analysis. A previous study (Loyaux-Lawniczak et al., 1999) reported that in the pH range of 3.5 to 5, the Fe(III)-EDTA complex is 100% deprotonated. Therefore, the eluent for EDTA was set at pH 4 in this study by using 0.03 M acetate buffer. Acetate buffer was selected as there was no absorption within the Fe(EDTA) detection wavelength. The mobile phase was composed of 30 mM of tetra-butylammoniumbromide, Milli-Q water and 2% of methanol. The solution was filtered through a GF/F (Whatman) prior to analysis. Fe(III)-EDTA complex was detected at 256 nm, and the chromatographic conditions were identical to those described above for BPA (Loyaux-Lawniczak et al., 1999).

2.2.3.2. Analysis of BPA and EDTA degradation metabolites by GC-MS

In order to elucidate the BPA degradation pathways by the bacterial consortium, biodegradation intermediates produced from BPA at a concentration of 20 mg L^{-1} were identified by

GC-MS. The pH of the samples were adjusted to 2 with 6 M HCl and extracted with diethyl ether (Spivack et al., 1994). The extracts were dried *in vacuo*. The dried extracts were derivatized by adding 50 μL *N, O*-bis(trimethylsilyl)trifluoroacetamide (Wako, Japan) and incubating at 80 ℃ for 30 min. GC-MS analyses were performed by injecting the derivatized samples into a HP 6890N GC coupled to an HP MSD 5973 (Agilent Technologies) and fitted with a DB-5MS capillary column (30 m ×0.25 mm ø, 0.25-μm film thickness, Agilent Technologies). High-grade helium was used as the carrier gas with a flow rate of 1.0 mL min^{-1}. The oven temperature was programmed as follows: initial temperature of 60 ℃ for 2 min; ramp 8 ℃ min^{-1} to 120 ℃; 6 ℃ min^{-1} to 310 ℃; isothermal at 310 ℃ for 20 min. The samples were analysed with the splitless injection.

BPA metabolites, such as *p*-HAP, *p*-hydroxybenzaldehyde (*p*-HBAL), and *p*-hydroxybenzoic acid (*p*-HBA), were identified using respective standards (Wako, Japan). Hydroxy-bisphenol A (OH-BPA) and *p*-hydroquinone (HQ) were identified based on the mass spectra reported in previous studies (Kolvenbach et al., 2007; Nomiyama et al., 2007). 2,2-*bis*(4-hydroxyphenyl)-1-propanol (I), 1,2-bis(4-hydroxyphenyl)-2-propanol (II), 2,2-bis(4-hydroxyphenyl) propanoic acid (IV), and *p*-hydroxyphenacyl alcohol (VI) were identified based on the interpretations of their fragmentation patterns (*m/z*) and by comparison with previous studies (Lobos et al., 1992; Spivack et al., 1994). The time course of the BPA degradation intermediates was expressed as relative percentage peak area (%) to the peak area of the initial BPA concentration.

For EDTA metabolite, ketopiperazinediacetate (KPDA) was identified based on the interpretation of its fragmentation pattern.

2.2.4. BPA degradation rate per cell

In order to investigate whether at t=48, light delays bacterial growth, BPA degradation rate per cell was calculated.

BPA degradation rate per cell used in this study was defined as

$$\text{BPA degradation rate per cell} = \frac{C_i - C_{t=48}}{48 \times \text{Cell number}_{t=48}}, \quad (2.1)$$

where C_i is the initial BPA concentration (mg L-1), $C_{t=48}$ is the residual BPA concentration at t_{48}, and $\text{Cell number}_{t=48}$ is the cell number at t_{48}. The BPA degradation rate per cell was measured in mg L^{-1} h^{-1} $cell^{-1}$.

2.2.5. BPA degradation kinetics calculation

The delay of biodegradation is the time, before the linear (k) of the plot ln [C] versus time can be drawn. BPA degradation was calculated based on the first-order reaction kinetics. The first-order degradation kinetics are expressed as follow:

$$\ln [C] = -kt + \ln[C]_o \quad (2.2)$$

where $[C]_o$ is the initial BPA concentration, k is the first-order degradation rate constant, and t is time. The degradation half-life can be expressed as follows:

$$t_{1/2} = \ln 2/k \quad (2.3)$$

2.2.6. Cell yield calculation

Carbon conversion efficiency is the efficiency of bacterial growth on an organic compound.

In order to further clarify the biodegradation of BPA by the bacterial consortium in the light and dark conditions, the carbon conversion efficiency of the bacteria was calculated. A volume of 30 mL of culture was centrifuged at 10,000 × g for 10 min. The supernatant was discarded, and the pellet was filtered through a pre-weighed 0.20 μm hydrophilic PTFE membrane filter (Advantec, Japan). The filter were then dried at 105 ℃ for 24 h (Valentine et al., 1996). The biomass dry weight was determined using an ultra-microbalance (Mettler Toledo, XP6U Ultra Micro Comparator, USA). The linear regression between cell dry weight (mg mL^{-1}) and OD_{600nm} was obtained according to the following expression: cell dry weight (mg mL^{-1}) = 1.682 OD_{600nm} + 0.0016 (n = 4, r = 0.997, p < 0.01). The cell yield was calculated based on the amount of produced cell dry weight obtained per unit of BPA consumed (g-cell g-BPA^{-1}).

Assuming that all BPA was converted to CO_2, soluble organic carbon, and cell biomass, a carbon mass balance analysis was performed based on the bacterial cell yield. The activated sludge biomass is represented by the empirical chemical formula of $C_5H_7NO_2$ (Porges et al., 1956). The bacterial cell yield in the dark was calculated based on the final day of the experiment as below,

BPA, $C_{15}H_{16}O_2$, molecular weight = 228.29 g mol^{-1} (2.4)

Bacterial biomass, $C_5H_7NO_2$, molecular weight = 113.0 g mol^{-1} (2.5)

Cell dry weight (μg mL^{-1}) = Cell yield (g g^{-1}) × Substrate (mg L^{-1}) (2.6)

Therefore, the bacterial cell yield is equal to the slope of the cell dry weight gained from BPA, in which the slope is 0.973 g g^{-1}.

Cell dry weight per mol of BPA = 228.29 × 0.973 = 222.1 g (2.7)

Mol of biomass per mol of BPA = 222.1 g ÷ 113.0 g mol^{-1} (2.8)

= 1.965 mol bacterial biomass

1.965 mol of bacterial biomass x (5.00 mol C/ mol bacterial biomass) x (12.00 g/ mol C) = 118 g-C

(2.9)

1.00 mol substrate x (15.00 mol C/ mol substrate) x (12.00 g/ mol C) = 180 g-C

(2.10)

2.2.7. Microbial community analysis

2.2.7.1. Community DNA extraction and purification

The community DNA of day 0 sample of the batch experiment sample was extracted. For the community DNA extraction, sample (1 mL) without replicate was used. A 200 µL of TE buffer and 4 µl of 10 % Triton X-100 were added to the sample. The mixture was shaken vigorously before being heated at 75 ℃ for 5 min. The extracted community DNA was then purified by using illustra™ GFX™ PCR DNA and Gel Band Purification Kits (GE Healthcare Life Sciences, USA), following the manufacturer's instructions.

2.2.7.2. PCR amplification and construction of 16S rRNA gene clone libraries

The bacterial 16S rRNA gene (16S rDNA) was amplified using the PCR primer set B27F (5'-AGAGTTTGATCCTGGCTCAG) and U1492RM (5'-GGYTACCTTGTTACGACTT) (Kurosawa et al., 1998). A total of 25 µL mixtures was prepared with a composition of 12.5 µL of

Emerald Amp (a pre-mixed PCR reagent; TaKaRa Bio), 0.5 μM of each primer, 2 μL of community DNA, and 8 μL of sterilized mili-Q water. Amplification was performed by a thermal cycler PCR using the following temperature program: initial denaturation at 94 ℃ for 3 min, 30 cycles of 94 ℃ for 30 sec, 60 ℃ for 30 sec, 72 ℃ for 1 min 40 sec, and a final extension step at 72 ℃ for 5 min. To check the PCR products, electrophoresis on 1% agarose gel was used. The PCR products were purified using the same kit mentioned above. Electrophoresis on 1% agarose gel again was used to check the purified PCR products. They were then ligated into pT7 Blue T-vector (50 ng/μL; Novagen, Germany). The ligation reaction was performed by mixing 1 μL of T-vector, 6 μL of ligation high, and 5 μL of purified DNA and then incubating the mixture at 16 ℃ for 2 hours. The produced recombinant plasmids were transformed into competent *Escherichia coli* DH5α (TaKaRa Bio), and then plated on LB plates containing 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal (TaKaRa Bio), and 0.5 mM IPTG (TaKaRa Bio). Detection occurred through Blue/white selection (the successfully ligated recombinants make white colonies), in which 100 of white colonies were picked randomly. The colonies were subcultured in 100 μL of LB medium containing 100 μg mL^{-1} ampicillin in a 96-well plate at 37 ℃ overnight. Sample 1 μL of culture was used as the template DNA for amplification of the inserted 16S rDNA by PCR. The PCR amplification were performed using Premix Ex *Taq* (TaKaRa Bio) with primers T7P-F (5'-TAATACGACTCACTATAGGG) and T7U-R (5'-GTT TTCCCAGTCACGA CGT) under following temperature program: initial denaturation at 94 ℃ for 3 min, 35 cycles of denaturation at 94 ℃ for 30 sec, 51 ℃ for 30 sec, 72 ℃ for 2 min, and 72 ℃ for 5 min.

2.2.7.3. Sequence and phylogenetic analysis

The sequencing was done by Eurofins Genomics, a DNA sequencing services provider. The obtained sequences were then aligned using CLUSTALW program of the Molecular Evolutionary Genetics Analysis (MEGA 6). The sequences were grouped into representative clones using a 98% similarity threshold (Shaw et al., 2008) by GENETYX ver. 11. The sequences of each group were compared to their closest relatives in NCBI database using BLASTN (Altschul et al., 1990). About 600 bp of the each 16S rDNA clone was sequenced. Phylogenetic relationships among the representative clones, closest sequences obtained by using BLASTN, and the previously reported BPA-degrading bacterial 16S rDNA sequences were analyzed using (MEGA) version 6.0 (Tamura et al., 2011). CLUSTAL W was first used to align the sequence, and then the phylogenetic trees were constructed using the Neighbor-joining trees of Kimura-2 model. Samplings bootstrap of 1000 were used to confirm the stability of branching pattern of the tree (Thompson et al., 1994). To determine whether the representative clones in the sample are similar to each other, homologous coverage calculation was used. The equation was

$$C = 1 - (n/N) \tag{2.11}$$

where n is the number of total number of clones in the sample, and N is the total number of phylotypes (Good, 1953; Singleton et al., 2001).

2.3. Results and Discussion

2.3.1. Photolysis of EDTA

Although, EDTA concentration for cells grown in the dark were unchanged in all BPA concentrations (Fig. 2.3.1a), the EDTA concentration in the bacterial cultures decreased gradually with time for cells grown in the presence of light (Fig. 2.3.1b). These findings indicate that EDTA may have been photolysed. The EDTA photolysis products are CO_2, formaldehyde, ethylenediaminetriacetic acid (ED3A), iminodiacetic acid (IDA), N-aminoethyleneglycine, and glycine (Lockhart and Blakeley, 1975; Nowack and Baumann, 1998). The carboxylic acid (COOH) of EDTA (4-COOH) and its metabolic compounds, such as ED3A (3-COOH), *N,N'*-ethylenediglycine (2-COOH) and IDA (2-COOH) are highly polarities. Therefore, determination of these compounds by GC-MS would have been impractical. However, KPDA (2-COOH), the intra-molecular crystallized compound of ED3A, was detected in the medium under the light conditions only (Fig. 2.3.2). EDTA was photolysed to ED3A that was easily biodegradable, and then spontaneously transformed to KPDA (Yuan and VanBriesen, 2006). The detection of KPDA in the medium indicates that ED3A is present in the medium under the light condition. The EDTA metabolites may have contributed as additional carbon sources for bacterial growth. However, the contribution of EDTA metabolites should be estimated quantitatively.

2.3.2. Bacterial degradation of BPA and growth in the light and dark conditions

The results showed that BPA was removed both in the light and dark conditions regardless

of the initial BPA concentrations (Fig. 2.3.3). The degradation kinetics showed that the degradation rate constants ranged from 0.032 to 0.055 h^{-1} with BPA concentrations of 10 to 50 mg L^{-1} in the light condition and from 0.053 to 0.069 h^{-1} in the dark condition. The degradation half-life ranged from 21.9 to 12.6 h in the light condition and from 13.1 to 10.2 h in the dark condition (Table. 2.3.1). However, when Zhang et al. (2007) used a BPA-acclimatized single-strain of *A. xylosoxidans* B-16 to degrade BPA, a degradation rate constant of 0.0004-0.0021 h^{-1} was observed. The significantly lower degradation rate of the single-strain bacteria than the bacterial consortium used in the present study suggests that using bacterial consortium may induce better BPA degradation.

A 24-h delay of BPA degradation was observed in the dark for all BPA concentrations (Table 2.3.1). The delay may be attributed to the lag phase of bacterial growth. While in the light condition, the 48-h delay suggests that light may have delayed the degradation process at the early stage of growth (Table 2.3.1). Moreover, the degradation half-life under light conditions, which was calculated after excluding the delay period due to the lag phase, was longer than in the dark for all BPA concentrations (Table 2.3.1). This result suggests that light caused a delay in BPA degradation not only during the early stages of degradation, but also the entire degradation process. Nevertheless, BPA was removed to the concentration below the detection limit by the end of the experiment under both the light and dark conditions (Fig. 2.3.3).

Bacteria grew in the dark to reach a stationary phase at the dry weight of 29.6, 49.6, 52.6, and 81.2 μg mL^{-1} for initial BPA concentrations of 0, 10, 20, and 50 mg L^{-1}, respectively (Fig. 2.3.4). Bacterial growth was concomitant with a decrease in BPA concentration (Fig. 2.3.3) suggesting that

BPA served as a carbon source for growth. The bacteria cultured in the light condition achieved maximal dry weight of 71.7, 93.0, 100.8, and 121.6 μg mL^{-1} for initial BPA concentrations of 0, 10, 20, and 50 mg L^{-1}, respectively (Fig. 2.3.4). The BPA degradation rate per bacterial cell ranged from 1×10^{-8} to 1×10^{-10} mg L^{-1} h^{-1} $cell^{-1}$ in all conditions at t=48 hours. Although light caused a delay in BPA degradation at t=48 hours, the BPA degradation rate per bacterial cell was not significantly different between the light and dark conditions. This result indicates that BPA degradation capacity of each single cell is not affected by the light. Furthermore, a significant correlation of BPA degradation rate and cell density was found (r= 0.882) (Fig. 2.3.5). The results show that a higher degradation rate was found to be related to a higher cell density in the dark. On the other hand, a lower BPA degradation rate was found to be related to lower cell density in the light. This finding suggests that light limits the cell growth and is thereby affecting the BPA degradation rate. Nevertheless, bacteria under light conditions continued to grow even though BPA was removed to below the detection limit (Fig. 2.3.4b). The continued growth of bacteria in the light conditions was believed to be related to the ongoing decrease of the EDTA concentration (Fig. 2.3.1b). A further investigation of the source of bacterial growth in the light conditions was carried out and shown in section 2.3.3.

2.3.3. Cell yield

A significant correlation between the biomass dry weight and the initial concentration of BPA was found in both light and dark incubations (Fig. 2.3.6). The y-intercepts, which are higher

than 0 mg DW μg mL $^{-1}$ for both the light and dark incubations (Fig. 2.3.6), suggest other sources of carbon were available in the MSM medium regardless of light condition. The presence of chemicals introduced during the pre-culture may explain the value of 27.4 μg mL^{-1} for the y-intercept of the bacterial culture grown in the dark condition. These unidentified chemicals may also explain in part, the intercept of 75.3 μg mL^{-1} for cells grown in light conditions. The bacterial dry weight that was incubated in the light condition may have been higher due to the photolysis of EDTA. The average yield was 0.96 and 0.97 g-cell g-BPA^{-1} in the light and dark conditions, respectively. The similar cell yields for both light- and dark-grown cultures shows consistent bacterial yield, even with the occurrence of EDTA photolysis in the culture medium. Therefore, the effect of EDTA on BPA degradation under light could be minor.

The carbon balance analysis for BPA biodegradation showed that the percentage of substrate carbon found in the biomass was 65.5% (118 ÷ 180 x 100%). Whereas, the percentage of carbon released as CO_2 and dissolved organic carbon was 34.5% (100% - 65.5%). *Sphingomonas* sp. MV1 mineralized 60% of the carbon in BPA to CO_2 and approximately 20% to dissolved organic carbon. The remaining 20% of the carbon in BPA were utilized to maintain the biomass (Lobos et al., 1992). In the present study, the bacterial biomass gaining from BPA was 45.5% higher than the previous study. This finding suggests that the bacterial consortium degraded BPA efficiently and converted most of the carbon from BPA to maintain the cell yield. Previous study showed that pure cultures grown on methyl tert-butyl ether (MTBE) were reported to have low cell yields. The reason was a lack of necessary downstream metabolic pathways to degrade MTBE efficiently (Steffan et al., 1997;

Hanson et al., 1999). The high cell yield coefficient (0.95–0.97 g g^{-1}) of the bacterial consortium in this study was likely because of the existence of various bacteria. Therefore, through this study we understand that bacterial consortium are more efficient than single-strain bacteria in converting the carbon of BPA to bacterial biomass. This finding will be helpful for applying bacterial consortium in the degradation of BPA.

2.3.4. Microbial community

A total of 60 clones were grouped into 17 phylotypes (Fig. 2.3.7). The homologous coverage showed 0.71 in the microbial community. This result indicates that the representative clones in the sample are similar to each other. Moreover, the microbial community analysis showed that bacteria in the batch experiment can be categorized into four phyla and Proteobacteria was the dominant phylum that accounted for 90% of the clones. Alphaproteobacteria was the dominant class that accounted for 54% of the clones followed by Gammaproteobacteria with 35% of the clones and the least observed phylotype was Beta-proteobacteria that accounted for only 11% of the total clone of Proteobacteria. The bacterial community are shown in Fig. 2.3.8. The 16S rDNA sequence of the 17 phylotypes were compared with the NCBI database (BLASTN), and the phylogenetic tree is shown in Fig. 2.3.8. Clone B04 was the dominant phylotype that accounted for 41.7% of the total clones (Fig. 2.3.8), which was 99% similarity to *Sphingobium jiangsuense* strain BA3 (Zhang et al., 2012). This strain has been shown as a 3-phenoxybenzoic acid degrader. Besides, Clone B34 was the second dominant phylotype that accounted for 16.7% of the total clones (Fig. 2.3.8), with sequence identities

of 99% to *Pseudomonas putida* that was known as BPA-degrading bacterium (Kang and Kondo, 2002). Therefore, the detection of *Sphingobium jiangsuense* and *Pseudomonas putida* in the sample with 57.4% of the population of the total clones suggests the potential to remove BPA in the system.

2.3.5. BPA degradation pathways and intermediates

BPA degradation pathways and degradation intermediates were examined because of their potential oestrogenic activity (Suzuki et al., 2004). BPA biodegradation intermediates that either exhibit oestrogenic activity or not are summarized in Table 2.3.2. BPA metabolites, *p*-HAP and compound IV, have low oestrogenic activity compared to BPA (Ike et al., 2002; Suzuki et al., 2004). Moreover, Yoshihara et al. (2001) reported that Compound III exhibited 100 times higher oestrogenic activity than BPA. Therefore, the biochemical properties of BPA metabolites and their accumulation must be considered in BPA remediation.

In this study, a total of ten BPA degradation intermediates were detected from the biodegradation of BPA under the light condition, and nine BPA degradation intermediates were detected in the dark (Table 2.3.3). The pathways that are presumed to be responsible for the production of these metabolites by the bacterial consortium are presented in Fig. 2.3.9. In the first proposed pathway (Pathway I), BPA was oxidised to compound II, and then it was further degraded to generate *p*-HBAL and *p*-HAP through compound III (not detected). Compound III is a transient metabolite that is dehydrated from compound II (Spivack et al., 1994) and is oxidized to *p*-HBAL and *p*-HAP rapidly, which explains its absence in the culture medium. *p*-HBAL was metabolized to

p-HBA and then to CO_2 and bacterial biomass. The time course of BPA metabolites for cells grown in the presence or absence of light is shown in Fig. 2.3.10.

In the proposed pathway I (Fig. 2.3.10a, b), the first compounds to be detected were *p*-HBAL and *p*-HBA in the light (Fig. 2.3.10a). Whereas in the dark, the first compounds were *p*-HBAL and *p*-HAP at 24-h (Fig. 2.3.10b). After 24-h, most of the metabolites decreased both in the light and dark conditions, except for *p*-HBAL, which fluctuated in the dark and was still detected at the end of the experiment. *p*-HBAL is neither toxic nor oestrogenic (Ike et al., 2002) and is safe to be discharged. *p*-HAP is known to be a common BPA degradation metabolite (Zhang et al., 2013) with slight oestrogenic activity and may serve as a bacterial growth substrate (Lobos et al., 1992). However, to further degrade this compound, bacteria that possess the catechol pathway are required (Lobos et al., 1992). In this study, *p*-HAP increased with time and then decreased to a lower level both in the light and dark conditions. I, therefore, speculate that the bacterial consortium that were used in this study may contain bacteria possessing a catechol pathway.

In the second proposed pathway (Pathway II), BPA was probably oxidised to compound I which was further oxidized to IV and V. Although compound V was not detected, it was presumed to be oxidized to *p*-HBA and VI that were both detected. Several bacteria were shown to degrade BPA following pathways I and II that are proposed. These bacteria were mainly isolated from WWTP sludge, including *Sphingomonas* sp. strain MV1 and *S. paucimobilis* strain FJ-4 (Lobos et al., 1992; Spivack et al., 1994; Ike et al., 1995). Compound VI and *p*-HBA first accumulated in the presence of light (Fig. 2.3.10c). Whereas compound I accumulated in the first 24 h and then gradually decreased

with time in the dark (Fig. 2.3.10d). The decline of compound I followed by a large increase of IV and VI indicates that compound I is metabolized to IV and VI. All metabolites belonging to this pathway decreased to a low level at the end of the experiment.

However, for cultures grown in the presence of light, compound I was detected at 72 h (Fig. 2.3.10c), which was delayed by 48 h compared with the culture in the dark (Fig. 2.3.10d). The delay of detecting compound I may suggest that light inhibited the degradation of BPA through pathway II. Pathway II has been reported to be a minor pathway (20%) in BPA-degrading bacteria compared to pathway I (80%) and has a tendency to release several dead-end products (Lobos et al., 1992; Ike et al., 1995). For example, compound I (Lobos et al., 1992; Ike et al., 1995), V and VI (Ike et al., 1995) accumulated in the culture because they were not able to support cell growth. However, this was not observed in this study, both in the light and dark conditions.

In the third proposed pathway (Pathway III), BPA is metabolized to HQ and 4-(2-propanol)-phenol; HQ was detected, but 4-(2-propanol)-phenol was not detected. It is expected that 4-(2-propanol)-phenol will degrade to *p*-HAP. Although 4-(2-propanol)-phenol was not detected in this study, this compound is expected to be present during BPA degradation because of the presence of HQ. This is consistent with the pathway in *Cupriavidus basilensis* JF1 and *Bacillus* sp. strain GZB, whereby BPA is degraded to HQ and 4-(2-propanol)-phenol (Fischer et al., 2010; Li et al., 2012). However, HQ has also been reported to be a photocatalytic product of *p*-isopropenylphenol (Kaneco et al., 2004), as shown in the fourth proposed pathway (Pathway IV).

BPA reacted with the OH radical to generate OH-BPA (Nomiyama et al., 2007). The OH-

BPA further reacted with OH radicals to produce HQ. Because HQ was detected both in the light and dark conditions, HQ may not only be a photodegradation product but also a biodegradation intermediate. Nevertheless, HQ was detected in relatively small amounts (Fig. 2.3.10e, f), both in the light and dark conditions. Thus, this appears to be a minor pathway for BPA degradation. Conversely, OH-BPA was detected only in the light; therefore, OH-BPA might be a photodegradation product. This compound was first detected at 24-h but then decreased gradually with time to a relatively low level at the end of the experiment (Fig. 2.3.10g). The results could reflect phenomena in the natural system in which OH-BPA would be produced from BPA in the presence of light in rivers. OH-BPA exhibited low oestrogenic activity in an oestrogen receptor assay (Nomiyama et al., 2007). Therefore, besides the natural system mentioned above, a BPA biodegradation process with lighting requires extra care and attention because of the production of OH-BPA.

It is notable that many biodegradation studies mainly focused on the degradation of the targeted mother compounds and neglected the biodegradation intermediates that may be harmful or oestrogenic. The negligence of biodegradation intermediates may cause another undesirable pollution through the discharge of the treatment effluent. This study provided information on the degradation of BPA as well as the biodegradation intermediates.

Table 2.3.1. Biodegradation kinetic equations, degradation half-life, and delay of BPA degradation in the light and the dark.

Initial	Light				Dark			
BPA (mg L^{-1})	Equation	r	Half-life (h)	Delay (h)	Equation	r	Half-life (h)	Delay (h)
10	lnC = - 0.0317t + 1.212	0.998	21.9	48	lnC = - 0.0529t + 1.811	0.984	13.1	24
20	lnC = - 0.0402t + 1.325	0.983	17.2	48	lnC = - 0.0639t + 1.522	0.992	10.8	24
50	lnC = - 0.0549t + 2.735	0.993	12.6	48	lnC = - 0.0680t + 1.151	0.988	10.2	24

Table 2.3.2. Oestrogenic activity of BPA biodegradation intermediates.

Chemical compounds	Oestrogenic activity	Assay	References
BPA	5 OM< potent E2	Yeast two-hybrid assay	Nishihara et al. (2000)
I	No oestrogenic activity	Yeast two-hybrid assay	Ike et al. (2002)
III	3 OM< potent E2	ERE-luciferase reporter assay in MCF-7 cells	Yoshihara et al. (2001)
IV	Slight oestrogenic activity	MCF-7 cell	Suzuki et al. (2004)
VI	No oestrogenic activity	Yeast two-hybrid assay	Ike et al. (2002)
p-HBAL	No oestrogenic activity	Yeast two-hybrid assay	Ike et al. (2002)
p-HAP	Slight oestrogenic activity; 7 OM< potent E2	Yeast two-hybrid assay	Ike et al. (2002)
p-HBA	No oestrogenic activity	Yeast two-hybrid assay	Ike et al. (2002)
HQ	8 OM< potent E2	Yeast two-hybrid assay	Nishihara et al. (2000)
OH-BPA	Slight oestrogenic activity	Medaka estrogen receptor α	Nomiyama et al. (2007)

OM: orders of magnitude

Table 2.3.3. Mass spectra of BPA and its degradation intermediates.

Chemical compounds	Retention time (min)	Characteristic Fragment Peaks (*m/z*)
BPA	26.78	372: (M^+); 357: (M^+-CH_3); 341; 207: (M^+-C_6H_4O-TMS); 191; 117; 73
I	29.23	460: (M^+); 445: (M^+-CH_3); 371; 357; 191; 73
II	29.96	460: (M^+); 445: (M^+-CH_3); 343; 254; 73
IV	29.77	474: (M^+); 459: (M^+-CH_3); 371; 357; 191; 73
VI	20.01	310: (M^+); 295: (M^+-CH_3); 251; 221; 103; 73
p-HBAL	12.57	194: (M^+); 179: (M^+-CH_3); 161; 151; 73
p-HAP	14.37	208: (M^+); 193: (M^+-CH_3); 151; 135; 73
p-HBA	16.01	282: (M^+); 267: (M^+-CH_3); 223: (M^+-$COOCH_3$); 197; 73
HQ	12.89	254: (M^+); 239 (M^+-CH_3); 223; 133; 112; 73
OH-BPA*	28.44	460: (M^+); 445: (M^+-CH_3); 357; 207: (M^+-C_6H_4O-TMS); 191; 73

* Represents BPA intermediate detected only in the light.

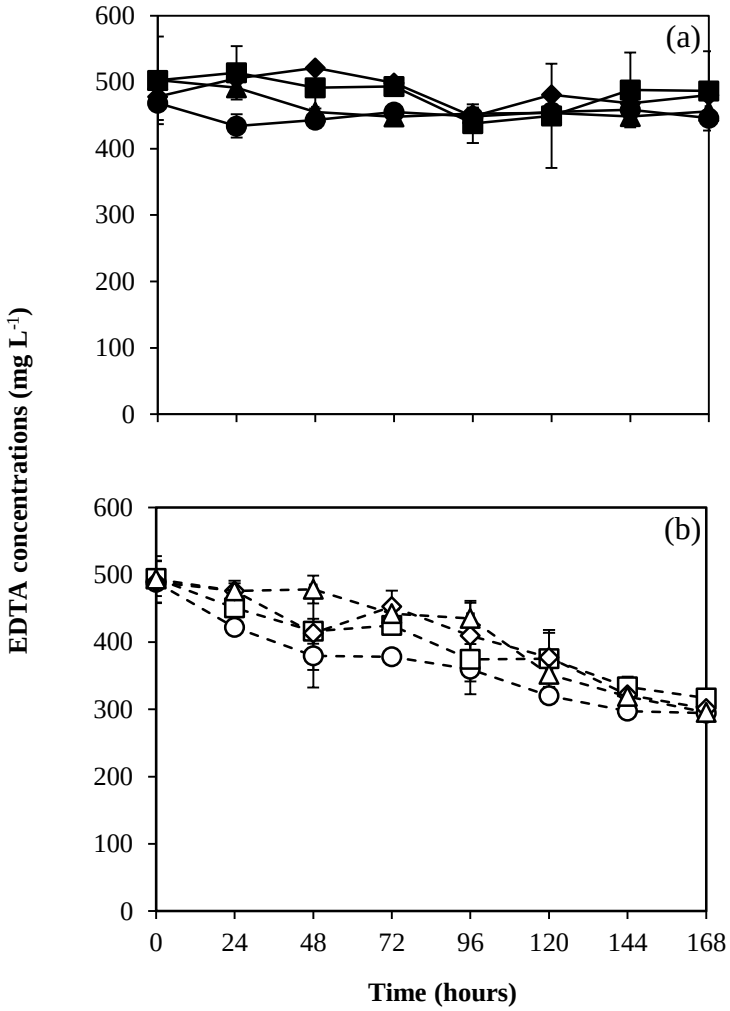


Fig. 2.3.1. Time course variations of EDTA concentrations at various initial BPA concentrations (mg L^{-1}): (◆) 0; (■) 10; (▲) 20; (●) 50 in the dark (a) (◇) 0; (□) 10; (△) 20; (○) 50 in the light (b).

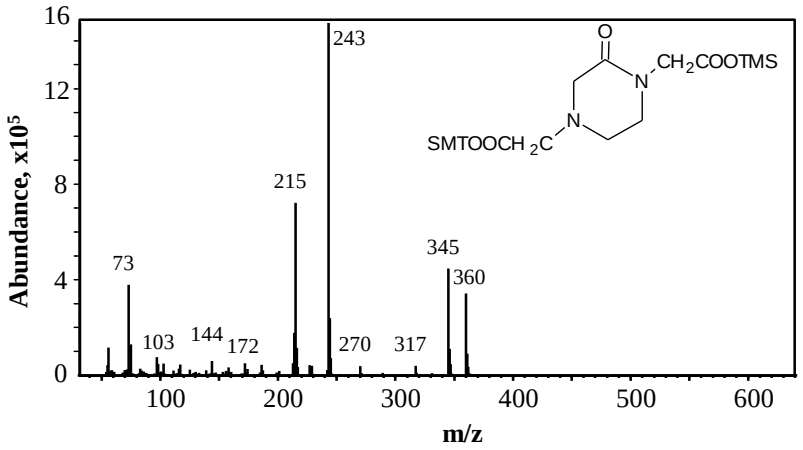


Fig. 2.3.2. Mass spectrometrum of TMS-derivatized of ketopiperazinediacetate (KPDA). The compound was detected at a retention time of 25.83 min.

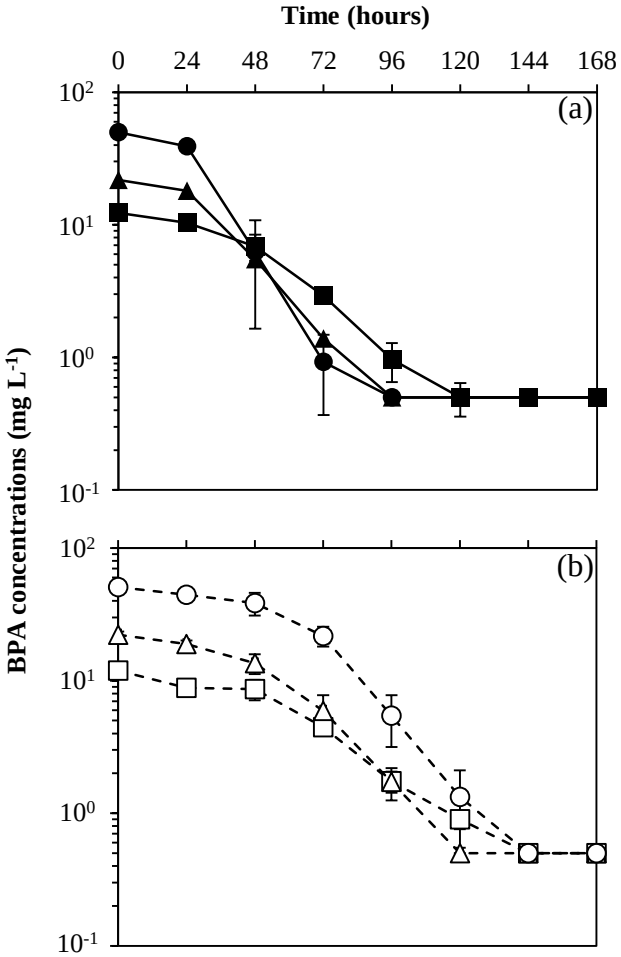


Fig. 2.3.3. Time course of BPA concentrations (mg L^{-1}): (■) 10; (▲) 20; (●) 50 in the dark (a); (□) 10; (△) 20; (○) 50 in the light (b).

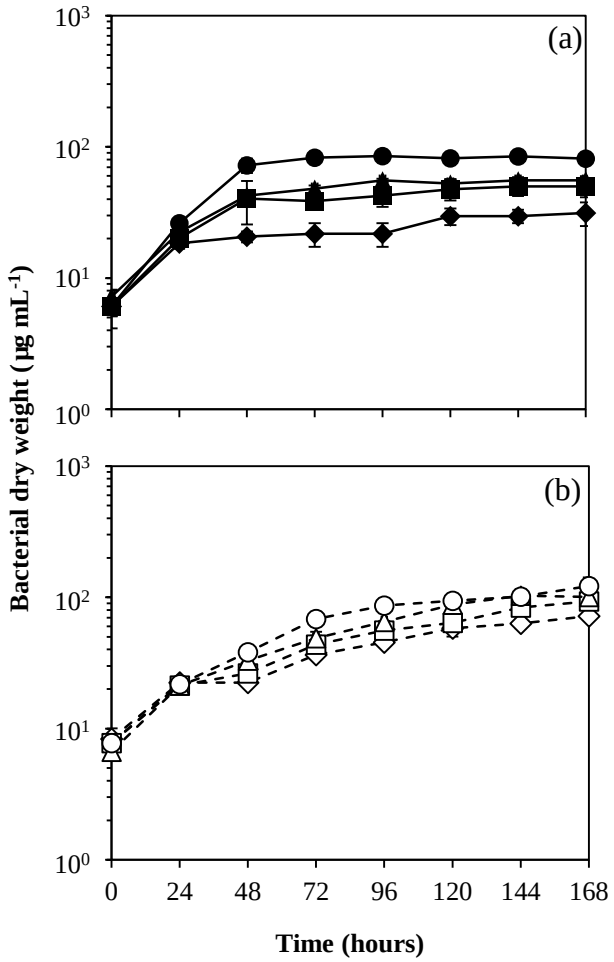


Fig. 2.3.4. Time course of bacterial dry weight in BPA concentrations (mg L^{-1}): (◆) 0; (■) 10; (▲) 20; (●) 50 in the dark (a) (◇) 0; (□) 10; (△) 20; (○) 50 in the light (b).

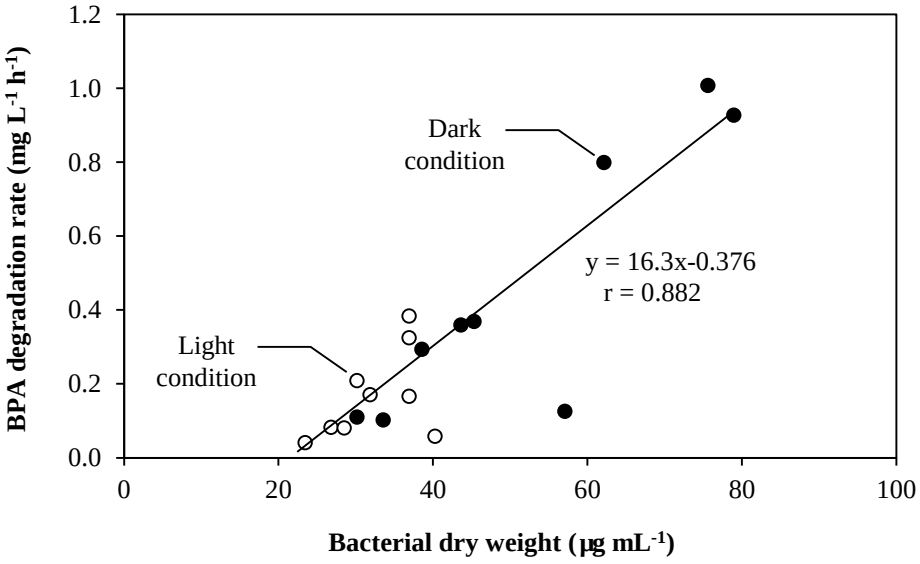


Fig. 2.3.5. Correlation between BPA degradation rate and bacterial dry weight at the 48-hours in the dark (closed symbols) and in the light (open symbols) conditions.

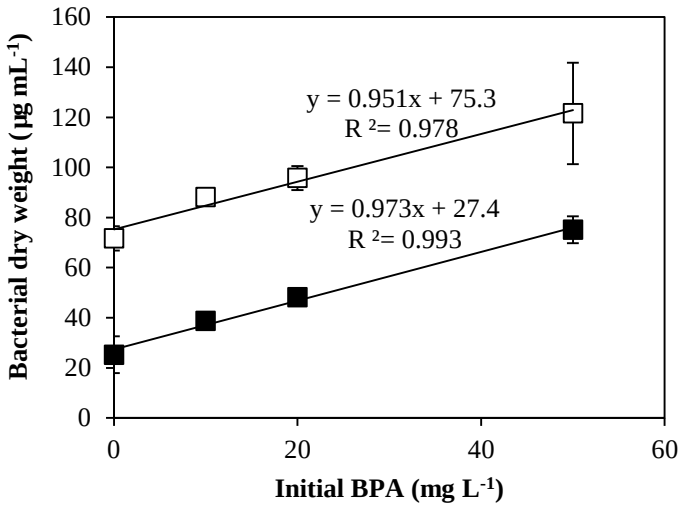


Fig. 2.3.6. Bacterial biomass produced from BPA in the dark (closed symbols) and in the light (open symbols) conditions.

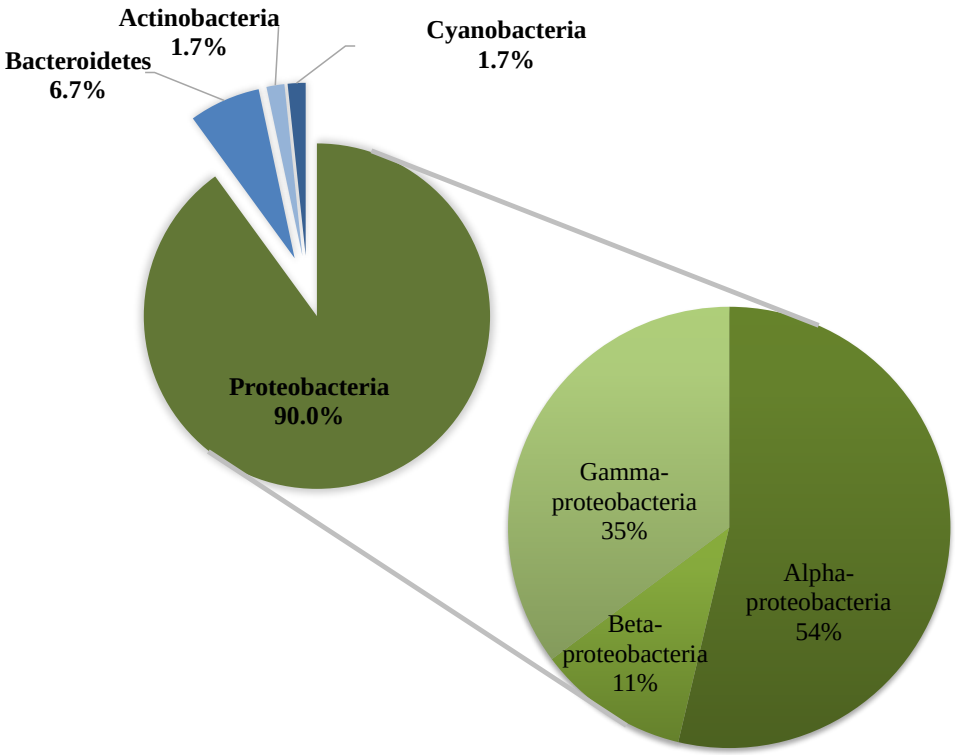


Fig. 2.3.7. Microbial community structure of clone libraries in the batch experiment. The larger circle represents the phylotypes composition at the phylum level, whereas the smaller circle represents the phylotypes composition of Proteobacteria class.

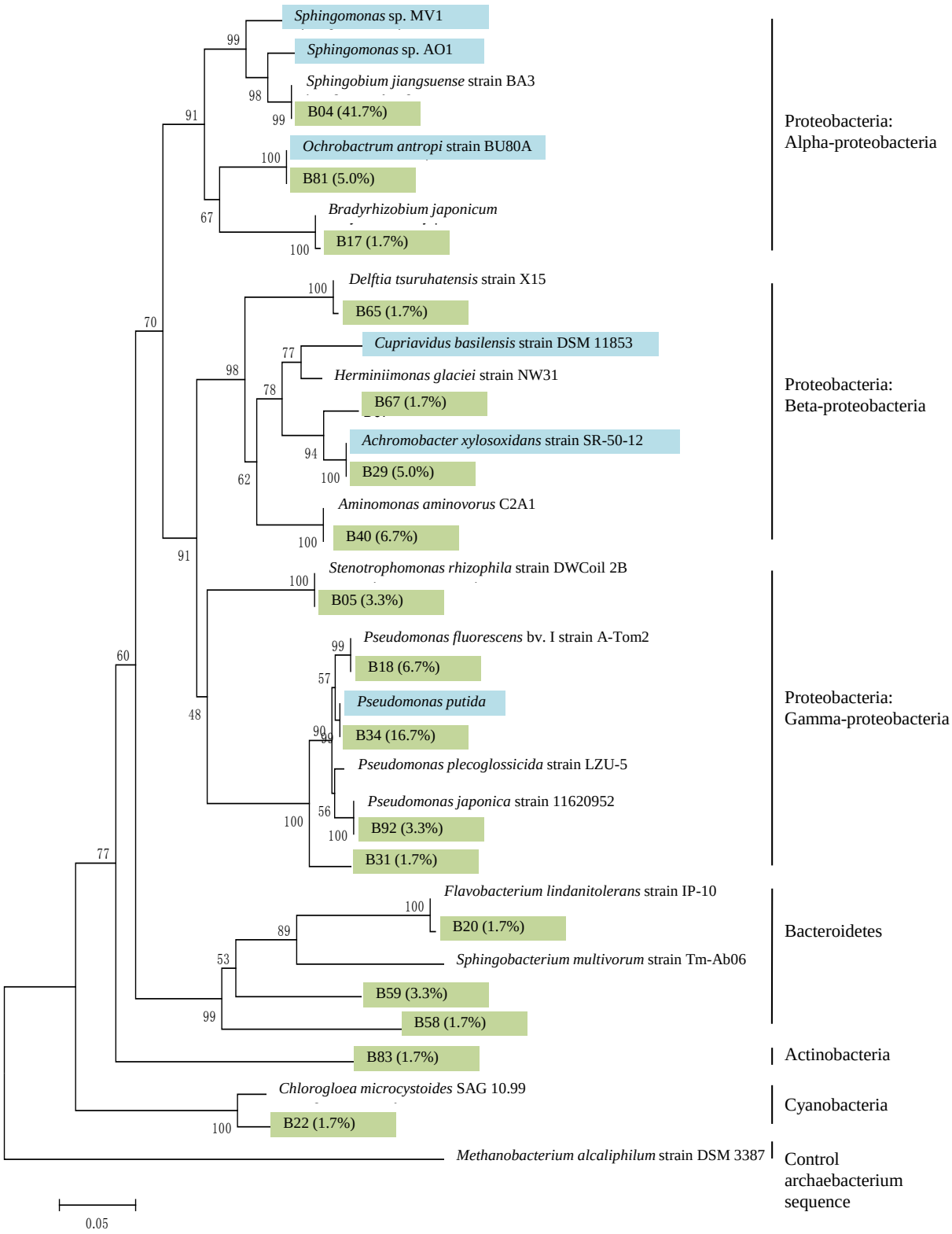

Sphingomonas sp. MV1
Sphingomonas sp. AO1
Sphingobium jiangsuense strain BA3
B04 (41.7%)
Ochrobactrum antropi strain BU80A
B81 (5.0%)
Bradyrhizobium japonicum
B17 (1.7%)
Proteobacteria: Alpha-proteobacteria
Delftia tsuruhatensis strain X15
B65 (1.7%)
Cupriavidus basilensis strain DSM 11853
Herminiimonas glaciei strain NW31
B67 (1.7%)
Achromobacter xylosoxidans strain SR-50-12
B29 (5.0%)
Aminomonas aminovorus C2A1
B40 (6.7%)
Proteobacteria: Beta-proteobacteria
Stenotrophomonas rhizophila strain DWCoil 2B
B05 (3.3%)
Pseudomonas fluorescens bv. I strain A-Tom2
B18 (6.7%)
Pseudomonas putida
B34 (16.7%)
Pseudomonas plecoglossicida strain LZU-5
Pseudomonas japonica strain 11620952
B92 (3.3%)
B31 (1.7%)
Proteobacteria: Gamma-proteobacteria
Flavobacterium lindanitolerans strain IP-10
B20 (1.7%)
Sphingobacterium multivorum strain Tm-Ab06
B59 (3.3%)
B58 (1.7%)
Bacteroidetes
B83 (1.7%)
Actinobacteria
Chlorogloea microcystoides SAG 10.99
B22 (1.7%)
Cyanobacteria
Methanobacterium alcaliphilum strain DSM 3387
Control archaebacterium sequence
0.05

Fig. 2.3.8. Phylogenetic tree based on a comparison of the partial 16rRNA gene sequence of BPA-degrading bacterial consortium in the batch experiment (1,000 bootstraps for confidence level). The parentheses are the percentage of the phylotypes in the total clones. The green bars represent phylotypes in the batch experiment; the blue bars represent previously reported BPA-degrading bacterial strains.

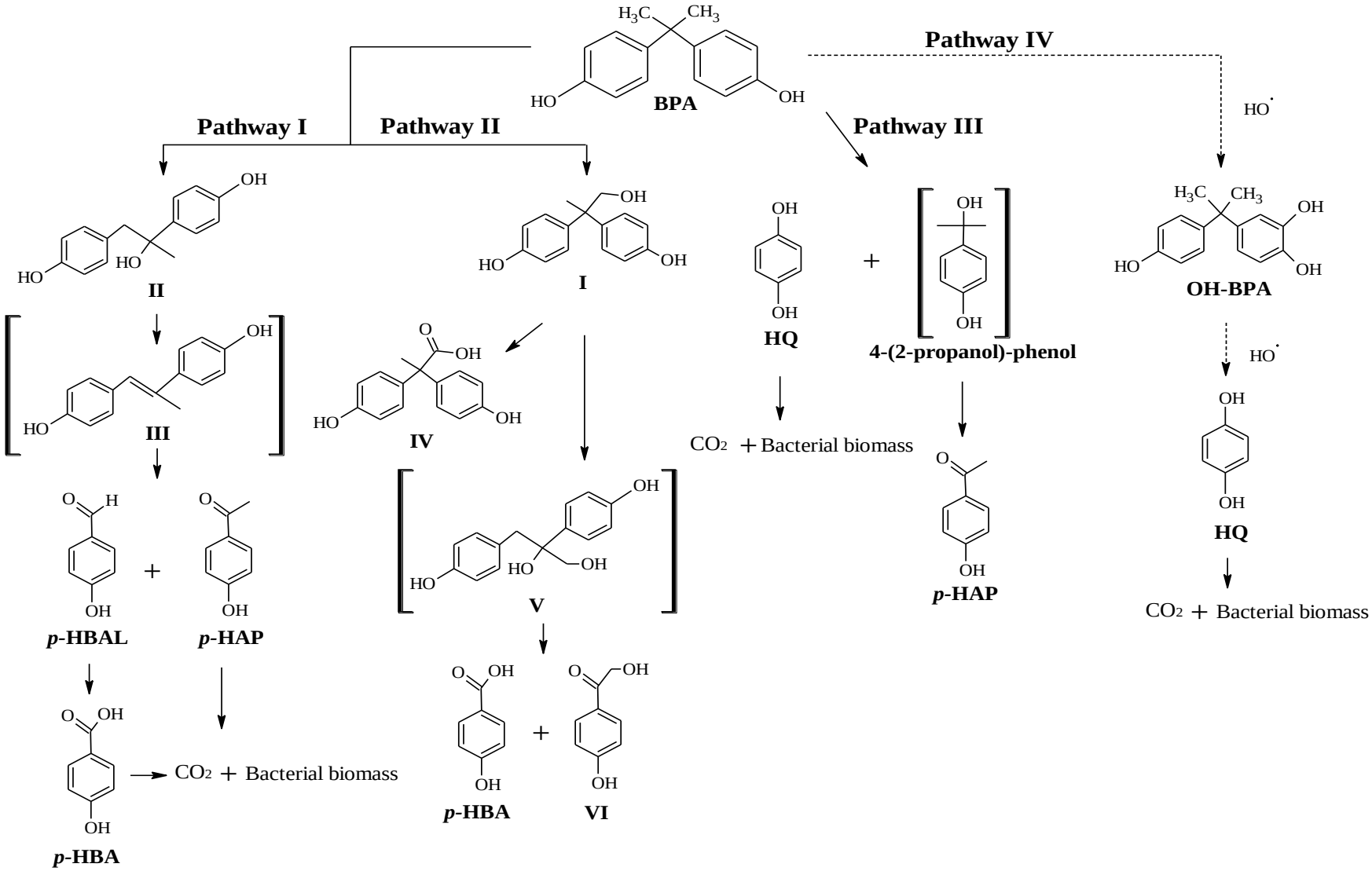

BPA
Pathway I
Pathway II
Pathway III
Pathway IV
II
I
III
IV
V
HQ
4-(2-propanol)-phenol
OH-BPA
p-HBAL
p-HAP
p-HBA
VI
CO2 + Bacterial biomass
CO2 + Bacterial biomass
CO2 + Bacterial biomass
HO·
HO·

Fig. 2.3.9. Proposed pathways used by the bacterial consortium to metabolize bisphenol A. The intermediates in parentheses were not detected but assumed to be produced. Solid arrows represent bacterial degradation pathways and dashed arrows represent the photodegradation pathway. 2,2-bis(4-hydroxyphenyl)-1-propanol (I), 1,2-bis(4-hydroxyphenyl)-2-propanol (II), 4,4-dihydroxy-alpha-methylstilbene (III), 2,2-bis(4-hydroxyphenyl) propanoic acid (IV), 2,3-bis(4-hydroxyphenyl)-1,2-propanediol (V), *p*-hydroxybenzolmethanol (VI), *p*-hydroxybenzaldehyde (*p*-HBAL), *p*-hydroxyacetophenone (*p*-HAP), *p*-hydroxybenzoic acid (*p*-HBA), *p*-hydroquinone (HQ), and hydroxy-BPA (OH-BPA).

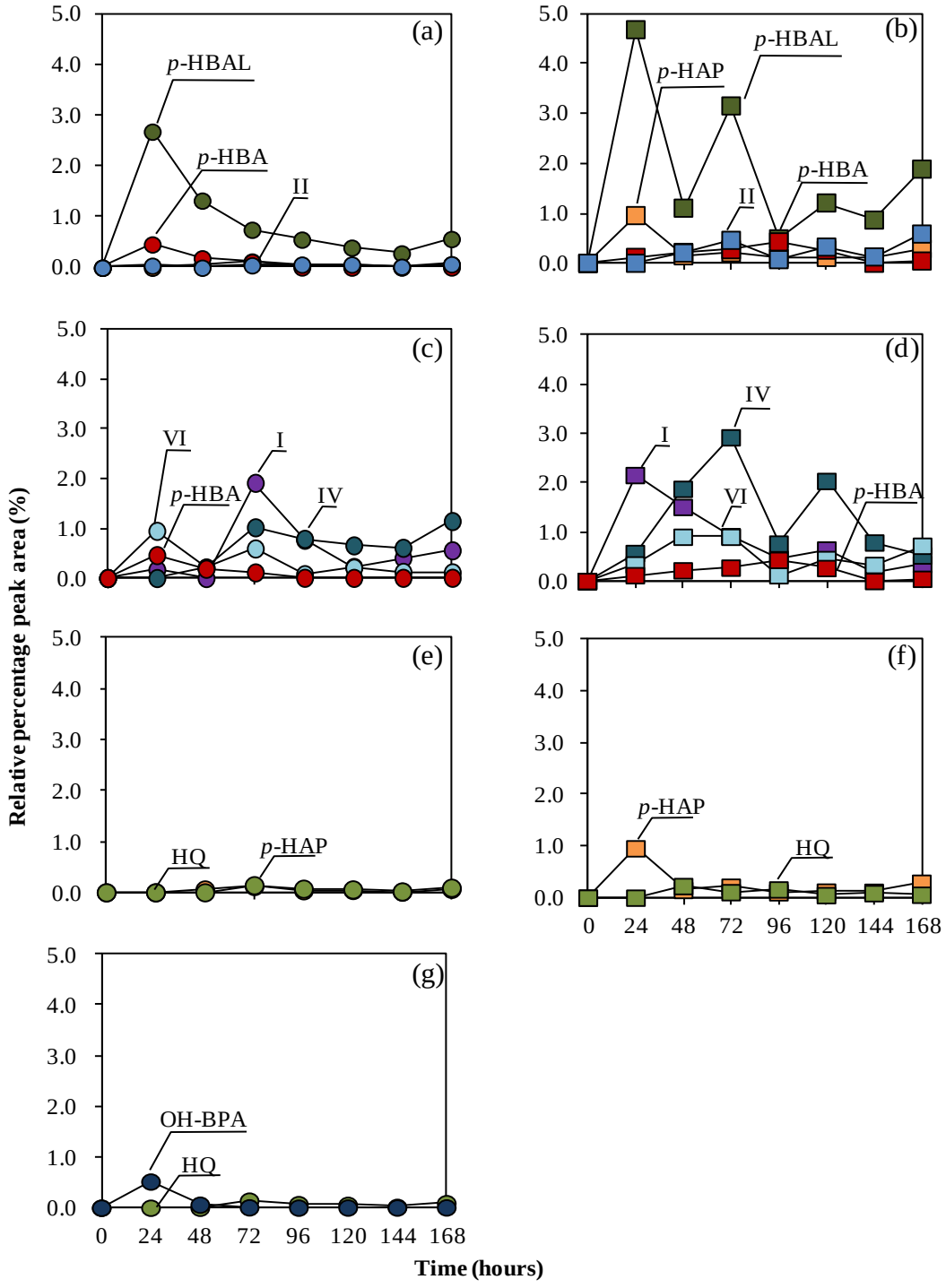


Fig. 2.3.10. Time course of BPA degradation intermediates for 20 mg L^{-1} BPA following Pathway I (a, b), Pathway II (c, d), Pathway III (e, f), and Pathway IV (g). Circles represent metabolites in the light condition, and squares represent metabolites in the dark.

Chapter III

Biodegradation of Bisphenol A by an Algal-Bacterial System

3.1. Introduction

The advances of bioremediation, which used mixed bacteria to biodegrade BPA, showed its promising removal of BPA (Zhao et al., 2008; Roh et al., 2009). In Chapter II, BPA-acclimatized bacterial consortium from activated sludge could biodegrade BPA to below the detection limit and transformed 65.5% of the carbon of BPA to bacterial biomass under mechanical aeration. However, the aerobic treatment is an expensive treatment, because of the energy required for aeration. For this reason, a low-cost aeration system is necessary to reduce the operation cost. Photosynthetic oxygenation of microalgae can provide an alternative source of oxygen with relatively low-energy through photosynthesis requiring light and nutrients.

An algal-bacterial system represents one type of low-cost water treatment. This treatment was originally invented by Oswald et al. (1988) almost 30 years ago for treating domestic wastewater. Although this is not a new technology, it has recently been revived due to the promotion of low-carbon society throughout the globe. An algal-bacterial system is designed to utilize the mutualistic relationship between algae and bacteria to treat organic pollutants. For instance, algae supply the oxygen necessary for the heterotrophic bacteria to degrade organic pollutants. The bacteria biodegrade organic compounds and subsequently release the carbon dioxide for algal photosynthesis (Oswald et al., 1988). In this context, the energy for external aeration required for the conventional

aerobic system could greatly be reduced thereby reducing carbon dioxide emission.

Recently, the application of algal-bacterial systems has been expanded to treat industrial wastewater and the removal of nutrients from livestock effluent (Muñoz and Guieysse, 2006; Posadas et al., 2014). The growth in popularity of the algal-bacterial system has led to a better understanding of the synergistic relationships between algae and bacteria for treating organic wastewater. Furthermore, the increase of bacteria can promote algal growth through generating CO_2 and stabilizing the pH (Gonzalez and Bashan, 2000; de-Bashan et al., 2002). Despite the significant development of research into nutrient removal (Karya et al., 2013), there is a lack of knowledge regarding the application of an algal-bacterial system to a moderately toxic and oestrogenic pollutant biodegradation such as BPA. Since microalgae are sensitive to toxic pollutants, the inhibition of algae by the pollutant can potentially result in the partial failure of an algal system (Muñoz et al., 2006). However, an algal-bacterial system is more tolerant of hazardous pollutants in comparison to algae alone (Borde et al., 2003). The role of bacteria in combination with selected algae has not been elucidated in the biodegradation of BPA. Since, BPA, as well as its biodegradation intermediates, show oestrogenic activity, BPA biodegradation intermediates were also monitored.

C. sorokiniana and *C. vulgaris* was chosen to represent big and small algae, respectively for the degradation of BPA in algal and algal-bacterial systems. Moreover, these two species have also been shown different in developing mucilage (Luo et al., 2008). Therefore, the degradation of BPA in the algal-bacterial systems of two species was also compared. Since BPA is toxic to algae (Li et al., 2009; Zhang et al., 2014), the tolerance of algae towards BPA in both the algal and the algal-

bacterial systems was also examined. In the algal-bacterial system, the O_2 and CO_2 mass balance was calculated to understand the mutualistic relationship between the algae and bacteria for CO_2 and O_2 demand, respectively. BPA concentrations from plastic plants have been recorded up to 47 mg L^{-1} (Staples et al., 1998). A further investigation of the feasibility of using an algal-bacterial system to biodegrade BPA from the industrial effluent is required. Therefore, a feasibility study of an algal-bacterial system in semi-continuous BPA loading was conducted. It is believed that the degradation capability of the bacterial consortium is related to the bacterial species present (Cavalca et al., 2000). The present study investigated the dominant and the functionally important members of the bacterial consortium of the inoculum of a semi-continuous algal-bacterial system.

3.2. Materials and Methods

3.2.1. Microorganisms and culture conditions

The BPA-degrading bacterial consortium used in this study was enriched from the activated sludge of Kitano Wastewater Treatment Plan Hachioji, Tokyo. The activated sludge bacteria were acclimatised with 50 mg L^{-1} BPA in MSM at 25 ± 1 ℃ in an incubator in the dark under continuous aeration. The bacteria were fed with BPA 50 mg L^{-1} once a week for three months. The bacterial consortium was then collected and maintained at a temperature of 4 ℃ for use in further experiments.

C. sorokiniana (NIES- 2168) and *C. vulgaris* (NIES- 2170) were obtained from the National Institute of Environmental Studies (NIES) of Japan. The algae were pre-cultivated in mineral salts medium (MSM) described by Sorokin and Krauss (1958) for seven days before starting the experiments.

All experiments were performed in an incubator at 25 ± 1 ℃ under a 12-h light/12-h dark cycle with a light intensity of 300 μmol photons m^{-2} s^{-1}. The light intensity was measured by a quantum scalar photosynthetic active radiation irradiance sensor QSPL- 2101 (Biospherical Instrument, San Diego, USA). All of the experiments were performed in triplicate.

The microorganisms were cultivated in MSM as composed of (g l^{-1}): KNO_3, 1.25; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2$, 0.0835; H_3BO_3, 0.1142; $FeSO_4 \cdot 7H_2O$, 0.0498; $ZnSO_4 \cdot 7H_2O$, 0.0882; $MnCl_2 \cdot 4H_2O$, 0.0144; MoO_3, 0.0071; $CuSO_4 \cdot 5H_2O$, 0.0157; $Co(NO_3)_2 \cdot 6H_2O$, 0.0049; EDTA, 0.5; and KH_2PO_4, 1.25. KOH was used to adjusted the pH to 7. All medium was sterilized by autoclave at 121 ℃ for 20 minutes before use. BPA ($C_{15}H_{16}O_2$) was purchased (Wako, Japan). Although BPA solubility in water is around 100-300 mg L^{-1}, it can be dissolved in high pH. Therefore, BPA stock solution of 1 mg mL^{-1} was prepared by first dissolving the weighed BPA into MSM with a tablet of KOH. The concentration of 1 mg mL^{-1} was then adjusted by adding MSM. At this stage, the pH was not adjusted. The pH was only adjusted immediately before inoculation.

3.2.2. Algal BPA inhibition and degradation assay

The algal BPA inhibition was studied by using sterilized serum bottles containing 100 mL of MSM. The algae were cultivated with 10, 20, and 50 mg L^{-1} BPA, which were prepared from the 1 mg mL^{-1} stock solution. The control experiment was performed under the same culture conditions without BPA. Both of *C. sorokiniana* and *C. vulgaris* were inoculated into the bottle with an initial chlorophyll a (Chl *a*) 0.5 mg L^{-1}. To create an enclosed system for algal growth, the headspace of the

bottle was filled with N_2/CO_2 (70/30 v/v) and then sealed with a rubber septum and an aluminium cap. Samples (1.5 mL) were withdrawn daily using a sterilised syringe to monitor the algal density and BPA concentration.

The abiotic loss of 10, 20, and 50 mg L^{-1} BPA in MSM without microbial addition was also determined during the experimental period in a 12-h light/12-h dark cycle and a 24-h dark. All of the samples were stored at 4 ℃ in the dark prior to measurement, and the samples were measured within 36 h of sampling.

To investigate the algal BPA adsorption and bioaccumulation, a separate set of control experiments was conducted using four different algal cell concentrations (10^4, 10^5, 10^6, and 10^7 cells mL^{-1}). The algal cells were incubated in serum bottles with the same culture condition as the algal inhibition assay, but the headspace was the air. In the algal BPA adsorption control, only BPA 10 mg L^{-1} was injected. For the surface adsorption, 45 mL algal culture at Day 7 after inoculation was harvested by centrifugation. The pellet was then washed with 10% methanol. BPA in the 10% methanol washed solution is considered as the algal surface adsorption. The washed cell pellet was then extracted with dichloromethane–methanol (1:2 v/v) solution to obtain the bioaccumulation of BPA (Correa-Reyes et al., 2007). Both surface adsorption and bioaccumulation solution were then quantified by HPLC.

3.2.3. Algal-bacterial BPA degradation assay in batch experiment

The algal-bacterial BPA degradation assays (batch experiment) were performed in serum

bottles containing 100 mL of MSM. The bottles were filled with MSM and BPA at a final concentration of 0, 10, 20, or 50 mg L^{-1}. The algal and bacterial cells were then inoculated into the bottle at the same initial Chl *a* concentration as in the algal BPA inhibition assay. To obtain the desired algal and bacterial cell density, the inoculate stock cultures of both algae and bacteria were counted before the inoculation. The initial bacterial cell density was 2×10^5 cells mL^{-1}. In order to examine the mutualistic effect of algae and bacteria, gas exchange with the atmosphere was precluded. Thus, the headspace was filled with air, and the bottle was then sealed with rubber septa. The air consisted of 0.033% CO_2, at which concentration on algal growth can be omitted. Samples (2.5 mL) were withdrawn daily to measure the microbial growth, pH and BPA concentration.

3.2.4. Algal-bacterial BPA degradation assay in semi-continuous experiment

In a review by Staples et al. (1998), the highest BPA concentration of 46.9 mg L^{-1} was found in the decant pond of an oil production plant. In landfill leachate, BPA concentrations of 17.2 mg L^{-1} have been reported (Yamamoto et al., 2001). Therefore, in this study BPA was used at concentrations corresponding to the highest reported value to assess the level of algal tolerance in semi-continuous BPA loading, which was 50 mg L^{-1}. The algal-bacterial BPA degradation assay (semi-continuous experiment) was performed in a round flask containing 1 L of MSM. The algae (approximately 10^4 cells mL^{-1}) and bacteria (approximately 10^6 cells mL^{-1}) were then inoculated into the flask. In order to determine the mutualistic relationship of algae and bacteria, gas exchange was precluded using silicon stopper and the headspace was filled with air. The algae and bacteria were cultured at 25 ±

1 ℃ under a 12-h light/12-h dark cycle with a light intensity of 300 μmol photons m^{-2} s^{-1}. Samples (2 mL) were withdrawn daily to measure the Chl *a*, bacterial dry weight and BPA concentration. When the BPA concentration was below the detection limit, BPA in the flask was adjusted to the initial BPA concentration of 50 mg L^{-1}. In the semi-continuous algal-bacterial BPA degradation experiment, *C. sorokiniana* + Bacteria decreased 50 mg L^{-1} of BPA to below the detection limit after 6 days of inoculation. The subsequent BPA removal was rapidly achieved in a day of the new adjustment, but the degradation rate could not maintained. The following BPA concentration was determined within two days of the new adjustment and continued for two adjustments. After day 11, a new BPA was added to maintain the BPA concentration at 50 mg L^{-1} daily four days. In the semi-continuous algal-bacterial BPA degradation experiment, *C. vulgaris* + Bacteria decreased BPA to below the detection limit after 4 days of inoculation. The subsequent BPA was adjusted on day 4 and day 6 every other day. On the second day of both adjustments, BPA concentrations decreased to below the detection limit. On day 8, BPA was adjusted daily to maintain the BPA concentration at 50 mg L^{-1}.

Before adjusting the BPA concentration, a sample (50 mL) was withdrawn for further analysis and then the same amount of fresh MSM was replenished. To adjust the BPA concentration, 50 mg of BPA was dissolved in MSM using a tablet of KOH. The dissolved BPA was then injected into the round flask containing algae and bacteria. The pH was maintained at 7 with KOH or HCL after adjusting the BPA. A sample (1 mL) was withdrawn to check the adjusted BPA concentration. The dissolved oxygen (DO) concentration in the medium was monitored every 12 hours by a dissolved oxygen meter (Mettler Toledo, Switzerland) immediately after the light was switched off

and on.

3.2.5. Analytical methods

For the BPA analyses, samples (1 mL) were centrifuged in a high-speed refrigerated microcentrifuge (Model 3700, Kubota) at 10,000 × g for 10 min. The supernatant of 2.5 µl was then injected into the HPLC system (600E, Waters) equipped with a UV-VIS detector (SSC-5410, Senshu Scientific) set at 222 nm. The samples were isocratically eluted using a mobile phase composed of acetonitrile, Milli-Q water, and acetic acid (37/63/0.1, v/v/v) at a flow rate of 0.4 mL min^{-1}. The detection limit and quantification limit (LOQ) of BPA was approximately 0.5 mg L^{-1} and 1.8 mg L^{-1}, respectively.

BPA biodegradation intermediates produced by the algal system and the algal-bacterial system during the degradation of BPA at the concentration of 20 mg L^{-1} were analysed by GC-MS. Because BPA 20 mg L^{-1} did not significantly affect the growth of both *C. sorokiniana* and *C. vulgaris*, this concentration was chosen to monitor the biodegradation intermediates. The samples were derivatized with *N, O*-bis(trimethylsilyl) trifluoroacetamide (Wako, Japan). The GC-MS analysis was performed with an HP 6890N coupled to an HP MSD 5973 (Agilent Technologies). A DB-5MS capillary column (30 m × 0.25 mm ø, 0.25-µm film thickness, Agilent Technologies) was used with high-grade helium as the carrier gas with a flow rate of 1.0 mL min^{-1}. The BPA degradation intermediates were speculated according to the interpretations of the fragment peaks (*m/z*), and then compared with previous studies (Lobos et al., 1992; Spivack et al., 1994; Kolvenbach et al., 2007;

Nomiyama et al., 2007). The time course of the BPA degradation intermediates by the algal-bacterial system was expressed as relative percentage peak area to the peak area of the initial BPA concentration.

3.2.6. BPA degradation kinetics calculation

BPA degradation was calculated based on the first-order reaction kinetics. The first-order degradation kinetics are expressed as Eq. 2.2.

3.2.7. Bacterial and algal growth

The bacterial cell density was determined by the cells counting. For bacterial cell counting, the bacterial samples were stained with SYBR Gold, and the cell counting was conducted under a fluorescent microscope with 1000× magnification (Shibata et al., 2006).

The algal growth was evaluated by measuring the concentration of Chl *a*. Chl *a* of algae was extracted by *N*, *N*-dimethyl-formamide using the extraction method described by Suzuki and Ishimaru, (1990). The Chl *a* in the extracts were then measured using a fluorometer (Model 10AU-Fluorometer, Turner) with Welschmeyer's (1994) method.

3.2.8. Algal specific growth rate

To further examine the effect of BPA on the algal growth, specific growth rate (SGR) of the algal system and the algal-bacterial system for both algae species were calculated from the equation

below,

specific growth rate = μ

$$\mu = \frac{\ln (N_2 / N_1)}{(t_2 - t_1)}, \quad (3.1)$$

Where, N_1 and N_2 are Chl *a* concentration at time1 (t_1) and time2 (t_2), respectively (Guillard, 1973).

3.2.9. Hypothetical carbon mass balance

In order to understand the mutualistic relationship between algae and bacteria, a mass balance of O_2 and CO_2 was calculated. However, this is just a hypothetical calculation with an assumption that BPA has been completely removed into either bacterial biomass or CO_2, and the calculation omitted the BPA biodegradation intermediates that were detected in this study. The carbon balance was based on the measured values and stoichiometry of bacterial BPA mineralization reaction

$$C_{15}H_{16}O_2 + 8O_2 + 2NH_3 \rightarrow 2C_5H_7O_2N + 5CO_2 + 4H_2O \quad (3.2)$$

from Chapter II. The BPA oxidation was calculated based on equation

$$C_{15}H_{16}O_2 + 18O_2 \rightarrow 15CO_2 + 8H_2O \quad (3.3)$$

Algal photosynthesis stoichiometry

$$4CO_2 + \text{nutrients} + H_2O + h\nu \rightarrow 4CH_{1.7}O_{0.4}N_{0.15}P_{0.0094} + 3.5O_2 \quad (3.4)$$

proposed by Hill and Bendall (1960). Based on the biomass composition of $C_5H_7O_2N$ reported by Porges et al. (1956) for bacteria, and that of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$ reported by Oswald et al. (1988) for algae, the C-biomass of the microbial content was calculated. The calculation was based upon the initial algal biomass and bacterial biomass, which was 8.6 x 10^{-4} mol (0.02 mg mL^{-1} / 23.2 g mol^{-1})

and 5.8 x 10^{-5} mol (6.6 μg mL^{-1}/ 113 g mol^{-1}) for algae and bacteria, respectively. The ratio between algae and bacteria was 15: 1. The algal photosynthesis stoichiometry became

$$5CO_2 + 1.25\text{nutrients} + 1.25H_2O + h\nu \rightarrow 5CH_{1.7}O_{0.4}N_{0.15}P_{0.0094} + 4.38O_2 \quad (3.5)$$

The stoichiometry of BPA biodegradation by an algal-bacterial system can be expressed as

$$C_{15}H_{16}O_2 + 3.6O_2 + 2NH_3 + 1.3 \text{ nutrients} + h\nu \rightarrow 2C_5H_7O_2N + 5CH_{1.7}O_{0.4}N_{0.15}P_{0.0094} + 2.8H_2O. \quad (3.6)$$

EDTA, $C_{10}H_{16}O_8N_2$, molecular weight = 292.24 g mol^{-1} (3.7)

Bacterial biomass, $C_5H_7O_2N$, molecular weight = 113.0 g mol^{-1} (3.8)

Cell dry weight (μg mL^{-1}) = Cell yield (g g^{-1}) × Substrate (mg L^{-1}) (3.9)

Therefore, the bacterial cell yield is equal to the slope of the cell dry weight gained from EDTA, is 0.25 g g^{-1}.

Cell dry weight per mol of EDTA = 292.24 × 0.25 = 73.1 g (3.10)

Mol of biomass per mol of EDTA= 73.1 g ÷ 113.0 g mol^{-1}

= 0.646 mol bacterial biomass (3.11)

Based on the equation above, EDTA mineralization reaction can be expressed as $C_{10}H_{16}N_2O_8 + 6.2O_2 + NH_3 \rightarrow 0.6C_5H_7O_2N + 7CO_2 + 6.2H_2O + 2.4N_{\text{alkylated intermediates}}$ (3.12)

3.2.10. Mass balance in the semi-continuous algal-bacterial system

In order to clarify the data of the bacterial dry weight in the semi-continuous experiment, the theoretical bacterial dry weight was calculated and the actual bacterial dry weight was measured.

The theoretical biomass was calculated based on the injected carbon sources concentration from the cell yield using Eq 2.6 and Eq. 3.9 for BPA and EDTA, respectively. Whereas, the bacterial dry weight was calculated from the equation below.

$$\text{Bacterial dry weight } (mg\ mL^{-1}) =$$

$$\text{Total dry weight } (mg\ mL^{-1}) - \text{Algal dry weight}(mg\ ml^{-1}) \quad (3.13)$$

For the total dry weight, a volume of 5 mL of *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria was filtered through and pre-weighed GF/F filter and dried at 60 ℃ for 24 h. The total dry weight was determined using an ultra-microbalance. Since the theoretical bacterial dry weight of *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria at Day 15 and Day 13, respectively were similar, the actual bacterial dry weight of the days were used to compared with the theoretical bacterial dry weight.

The algal dry weight was converted from the linear regression between dry weight (mg mL^{-1}) and Chl *a* (mg L^{-1}). The dry weight of *C. sorokiniana* was expressed as dry weight (mg mL^{-1}) = 0.005 Chl *a* + 0.003 (n = 4, r = 0.9997, $p < 0.001$). While, the dry weight of *C. vulgaris* was expressed as dry weight (mg mL^{-1}) = 0.031 Chl *a* + 0.058 (n = 4, r = 0.9998, $p < 0.001$).

Biodegradation percentage (P_b) of BPA by algae was calculated according to the following equation (Li et al., 2009):

$$P_b\ (\%) = (A_t - A_r - A_a - A_d - A_c) \times 100/A_t, \quad (3.14)$$

where A_t is the initial amount of BPA added to the medium, A_r is the residual amount of BPA in the medium, A_a is the amount of BPA abiotic loss, A_d is the amount of BPA adsorbed on the algal

cell surface, and A_c is the amount BPA accumulated in the algal cells.

3.2.11. BPA concentration in the algal-bacterial biomass of the semi-continuous experiment

A volume of 20 mL *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria at Day 15 and Day 13, respectively was harvested by centrifugation. The pellet was then washed with mili-Q water. The washed cell pellet was then extracted with dichloromethane–methanol (1:2 v/v) solution with 25 min sonication. The solution was then quantified by HPLC.

3.2.12. Algal-bacterial flocculation

In order to take the picture of the algal-bacterial flocculation, the sample was stained with SYBR Gold same as section 3.2.7 and the microscope was attached with a Canon EOS Kiss X7i C-mount camera.

3.2.13. Statistical analysis

The statistical analyses of the experimental data were conducted using Student's *t* assay and one-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey-Kramer or Dunnett's tests. Confidence levels of 95% and above was used to determine the significant differences.

3.2.14. Microbial community analysis

The same microbial community analysis as Chapter II was conducted.

3.3. Results and Discussion

3.3.1. BPA degradation by algal and algal-bacterial systems

The BPA concentrations in the medium of the abiotic control (without algae and bacteria) in the dark did not decrease throughout the experiment. While the BPA concentrations in the light conditions decreased gradually with the time in all BPA conditions (Fig. 3.3.1). The abiotic reduction in the light conditions at the end of the experiment was 11.3, 13.2, and 18.8% for the initial BPA concentrations of 10, 20, and 50 mg L^{-1}, respectively. These decreases indicate that BPA could be degraded mainly by photo-degradation in the presence of light. This result agreed closely with a previous study that photolysis of BPA in f/2 medium under the light intensity of 80 μmol photons m^{-2} s^{-1} was an average of 19.7% for BPA 0.01 to 9.00 mg L^{-1} (Li et al., 2009). Therefore, BPA photolysis occurs irrespective of light intensity and medium.

In the *C. sorokiniana* BPA adsorption study, 0.15, 0.32, 0.36, and 0.60% of the injected BPA was adsorbed on the surface of algae for the initial cell density of 10^4, 10^5, 10^6, and 10^7 cells mL^{-1}, respectively. On the other hand, the algal BPA bioaccumulation accounted for under detection (UD), UD, 0.05, and 0.11% of the initial cell density of 10^4, 10^5, 10^6, and 10^7 cells mL^{-1}, respectively. The results indicate that the total BPA adsorbed and accumulated in *C. sorokiniana* cells was less than 1%. Similarly, in *C. vulgaris*, both algal BPA surface adsorption and accumulation were UD in all initial cell densities. These results suggest that BPA removal by algal cell surface adsorption and bioaccumulation for both *C. sorokiniana* and *C. vulgaris* was less than 1%.

The BPA concentrations in the algal system and the algal-bacterial system of *C. sorokiniana*

and *C. vulgaris* are shown in Fig. 3.3.2a, b, c, d. The BPA concentrations in both the algal system and the algal-bacterial system were reduced. This reduction in BPA concentrations in both the algal and algal-bacterial systems might be partly caused by photo-degradation. In *C. sorokiniana*, the remaining BPA accounted for 50.2, 56.1, and 60.5% of the initial BPA concentrations of 10, 20 and, 50 mg L^{-1}, respectively at the end of experiments. While, the remaining BPA in *C. vulgaris* accounted for 65.9, 64.0, and 65.6% of the initial BPA concentrations of 10, 20 and, 50 mg L^{-1}, respectively at the end of experiments. The findings suggest that both *C. sorokiniana* and *C. vulgaris* alone are not able to remove BPA to below the detection limit. By adding bacteria into the algal system, BPA was removed to below the detection limit in both *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria for all BPA concentrations by the end of the experiments (Fig. 3.3.2c, d). Since algae alone could only partially degrade BPA, the further reduction of BPA concentrations in the algal-bacterial system was the result of bacterial activity. This result suggests that the complementary activity of bacteria and algae may have enhanced BPA degradation in an algal-bacterial system. Moreover, *C. sorokiniana* + Bacteria showed the first order degradation rate constant (Eq. 2.2) of 0.05, 0.06, 0.06 h^{-1} for BPA 50, 20, 10 mg L^{-1}, respectively. While *C. vulgaris* + Bacteria showed a BPA biodegradation rate constant of 0.03, 0.05, 0.06 h^{-1} for BPA 50, 20, 10 mg L^{-1}, respectively. *C. sorokiniana* + Bacteria showed significantly higher BPA degradation rate constant than *C. vulgaris* + Bacteria in BPA 10 and 20 mg L^{-1}. Although the BPA biodegradation rate constants of *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria were no significant difference at BPA 50 mg L^{-1}, the delay of BPA biodegradation in *C. vulgaris* + Bacteria was one day longer than *C. sorokiniana* + Bacteria in all BPA concentrations (Fig. 3.3.2c, d). The

delay of BPA degradation in *C. vulgaris* + Bacteria could be due to the smaller size of algae that reduce the O_2 production rate (Park and Lee, 2000) thereby affecting the BPA degradation. A bacterial cell growth in the algal-bacterial system was observed in all BPA concentrations (Fig. 3.3.2e, f). The initial bacterial cell density (2×10^5 cells mL^{-1}) increased gradually with time to the range of $2 \times 10^7 - 4 \times 10^7$ cells mL^{-1} in *C. sorokiniana* + Bacteria (Fig. 3.3.2e) and $1 \times 10^7 - 2 \times 10^7$ cells mL^{-1} in *C. vulgaris* + Bacteria (Fig. 3.3.2f) of all BPA concentrations. The decrease in BPA concentration was accompanied by an increased in bacterial cell density, indicating that bacteria might use BPA as a carbon source for their growth. These findings agreed with the discussion in Chapter II that bacterial consortium used BPA as growth source. However, in Chapter II, the bacterial consortium was cultured under mechanical aeration. Therefore, the results suggest that photosynthetic oxygenation of the algal-bacterial systems able to support the degradation of BPA. These results agreed with previous studies that photosynthetic oxygen of the algal-bacterial system supports the degradation of organic pollutants by bacteria even in the absence of mechanical aeration (Guieysse et al., 2002; Borde et al., 2003; Muñoz et al., 2003b, 2004).

3.3.2. Algal growth in algal and algal-bacterial systems

The growth response of *C. sorokiniana* and *C. vulgaris* after exposure to four BPA concentrations at the initial Chl *a* concentration of 0.5 mg L^{-1} are shown in Fig. 3.3.3a, b. The Chl *a* concentrations in the *C. sorokiniana* at the end of the experiment reached 2.2, 1.9, 1.2, and 0.24 mg L^{-1} for BPA 0, 10, 20, and 50 mg L^{-1}, respectively. On the other hand, The Chl *a* concentrations in the

C. vulgaris at the end of experiment reached 2.5, 2.2, 2.2, and 1.6 mg L^{-1} for BPA 0, 10, 20 and 50 mg L^{-1}, respectively. The results of algal BPA inhibition showed that the Chl *a* concentration of *C. sorokiniana* was significantly lower than the control at BPA concentrations of 20 and 50 mg L^{-1} (Fig. 3.3.3a). For *C. vulgaris*, the concentration of Chl *a* was significantly lower than that of the control when the BPA concentration reached 50 mg L^{-1} (Fig. 3.3.3b). In a comparison of *C. sorokiniana* and *C. vulgaris* at BPA 50 mg L^{-1}, the Chl *a* concentration of *C. sorokiniana* did not increase; whereas the Chl *a* concentration of *C. vulgaris* showed an increase by the end of the experiment. Thus, the results suggest that *C. sorokiniana* was more sensitive to BPA than *C. vulgaris*. Similar reduction effects were also found in previous studies i.e. *C. fusca* growth was limited by BPA concentrations > 9.12 mg L^{-1} (Hirooka et al., 2005) and *C. pyrenoidosa* was limited by BPA concentrations $\geq$ 25 mg L^{-1} (Zhang et al., 2014). According to Zhang et al., 2014, the inhibitory effect was probably due to the interference of BPA in the synthesis of protochlorophyll or protein and its subsequent conversion to chlorophyll.

When the bacteria were combined with *C. sorokiniana* in the algal-bacterial system, the effect of BPA concentration on the algal growth in the algal-bacterial system is presented in Fig. 3.3.3c. The Chl *a* concentration of the algal-bacterial system at the end of the experiment reached 1.3, 1.8, 1.9, and 2.2 mg L^{-1} for BPA 0, 10, 20 and 50 mg L^{-1}, respectively. Even at BPA concentrations of 20 and 50 mg L^{-1}, which limited the growth of algae in *C. sorokiniana* monoculture, the algae grew significantly better in the algal-bacterial system (Fig. 3.3.3c). Similarly in *C. vulgaris* + Bacteria, the Chl *a* concentration reached 1.3, 1.5, 1.6 and 2.1 mg L^{-1} for BPA 0, 10, 20 and 50 mg L^{-1}, respectively

at the end of the experiment (Fig. 3.3.3d). Even with a BPA concentration of 50 mg L^{-1}, algae grew significantly higher than the control at the end of the experiment. These results indicate that the growth of the bacteria in an algal-bacterial system counteracts the reduced growth of algae in the presence of BPA. The attenuation of the effect was probably due to the rapid reduction of BPA by bacteria (Fig.3.3.2c,d). All of the BPA concentrations in the algal-bacterial system decreased rapidly and achieved the residual BPA concentrations of < 5 mg L^{-1} by day 3 and day 5 for *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria, respectively.

The SGR of the *C. sorokiniana* in the algal system reduced from 0.8 to -1.1 d^{-1} with an increase of BPA concentrations from 0 to 50 mg L^{-1} (Fig. 3.3.4a). Similarly in the algal system of *C. vulgaris*, the SGR of *C. vulgaris* reduced from 1.0 to 0.4 d^{-1} with an increase of BPA concentrations from 0 to 50 mg L^{-1} (Fig. 3.3.4b). This finding indicates that BPA limits the algal growth. In contrast, a significant positive correlation ($SGR= 0.0136\ BPA_{(t=0)} + 0.3941$, $r = 0.9840$; $p < 0.001$) between BPA concentrations and the SGR of *C. sorokiniana* in *C. sorokiniana* + Bacteria, and ($SGR= 0.0126\ BPA_{(t=0)} + 0.3061$, $r = 0.9996$; $p < 0.001$) between BPA concentrations and the SGR of *C. vulgaris* in *C. vulgaris* + Bacteria were observed. These findings suggest that the bacteria probably stimulated the growth of algae in the algal-bacterial system through the release of CO_2 from BPA biodegradation. Specifically, because the experiment was conducted in an enclosed system, CO_2 required for algal growth might be delivered by the bacterial biodegradation of BPA. These results confirmed the mutualistic relationship between algae and bacteria through their gas exchange.

Interestingly, at BPA 0 mg L^{-1}, the algal SGR of the algal systems were higher than that of

the algal-bacterial systems in both algal species. This can be explained by the 30% initial CO_2 in the headspace of the algal system that promoted a higher SGR. On the other hand, in an algal-bacterial system, there was no BPA as carbon sources for bacteria to grow, and, therefore, low CO_2 rate would be found, thereby causing a relatively low SGR.

3.3.3. BPA biodegradation intermediates

In the algal system, three peaks corresponding to OH-BPA, HQ and BPA were detected (Table 3.3.1). OH-BPA was detected in both the algal and the algal-bacterial systems as well as the abiotic control exposed to light, but not in the abiotic control incubated in the dark. These observations indicate that OH-BPA was a photo-degradation intermediate rather than a degradation intermediate of BPA. Although Hirooka et al. (2005) reported that *C. fusca* transformed BPA to OH-BPA, a contrasting opinion was reported by Nakajima et al. (2007) that green algae did not have BPA-oxidizing enzymes. In this study, BPA was partially degraded by the monoculture of *C. sorokiniana* and *C. vulgaris*, but degradation products could not be detected. However, Nakajima et al. (2007) reported that green algae mineralized BPA to glycosides that are non-or less toxic forms.

In both species of the algal-bacterial system, ten degradation intermediates were detected (Table 3.3.1) that were the same as those described in Chapter II using the same bacterial consortium. Furthermore, these BPA biodegradation intermediates have been reported by several researchers using single-strain bacteria such as *Sphingomonas* sp. MV 1 and TTP3 (Lobos et al., 1992; Kolvenbach et al., 2007), but were slightly different from those who used single-strain bacteria, such as *Cupriavidus*

basilensis strain JF1 and *Bacillus* sp. Strain GZB (Fischer et al., 2010; Li et al., 2012). This finding indicates that different BPA biodegradation intermediates may be obtained from different bacteria.

Not only BPA but also its biodegradation intermediates exhibits oestrogenic activity (Nishihara et al., 2000; Yoshihara et al., 2001; Ike et al., 2002; Suzuki et al., 2004; Nomiyama et al., 2007). The time course for the oestrogenic and non-oestrogenic biodegradation intermediates are given in Fig. 3.3.5. BPA biodegradation intermediates that exhibit oestrogenic activity are *p*-HAP, compound IV, HQ, and OH-BPA (Fig. 3.3.5a,c). For *C. sorokiniana* + Bacteria, the maximum relative abundance of the detected biodegradation intermediates was relatively low (less than 1.5%). *p*-HAP, a common biodegradation intermediates of BPA (Zhang et al., 2013), was among the oestrogenic intermediates that increased initially during the biodegradation of BPA. The compound then decreased to a lower level at the end of the experiment. Compound IV, OH-BPA, and HQ were maintained at a lower level from the beginning to the end of the experiment. However, in *C. vulgaris* + Bacteria the oestrogenic biodegradation intermediates remained at below 0.5% throughout the experimental period.

The concentration of non-oestrogenic activity biodegradation intermediates increased during the biodegradation of BPA and decreased to low level at the end of experiment except *p*-HBAL and compound I in *C. sorokiniana* + Bacteria and *C. vulgaris* + Bacteria, respectively (Fig. 3.3.5b,d). *p*-HAP, *p*-HBA and *p*-HBAL are known intermediates in the biodegradation of BPA (Zhang et al., 2013). These compounds are also known as lignin phenols, which are hardly photodegradable (Benner and Kaiser, 2011). Thus, the decrease in the levels of *p*-HAP (Fig. 3.3.5a,c) and *p*-HBA (Fig.

3.3.5b,d) indicates that these compounds were biodegraded in the algal-bacterial system. By contrast, *p*-HBAL accumulates in *C. sorokiniana* + Bacteria at the end of the experiment and thus appears to be a biodegradation dead-end product. Although *p*-HBAL accumulated in the *C. sorokiniana* + Bacteria, it is not toxic and has no oestrogenic activity (Ike et al., 2002). However, compound I seems to be resistant to degradation by both the algal-bacterial systems before day 3. This observation suggests that compound I is not the preferred carbon source for the microbial system because the degradation of compound I only occurs once the level of BPA was depleted (Fig. 3.3.2c,d). Indeed, compound I is reported to be a recalcitrant compound that cannot support bacteria cell growth (Lobos et al., 1992). Biodegradation intermediate compound II and compound VI were detected in a relatively small concentration throughout the entire BPA degradation period. The results demonstrate that the algal-bacterial systems degraded BPA and produced low concentrations of biodegradation intermediates. Since a relatively low concentration of oestrogenic activity biodegradation intermediates was detected, the effluent was proposed to be discharged to the environment with a dilution of rain or river water.

3.3.4. Hypothetical carbon mass balance

From the Eq 3.2 and Eq. 3.3, BPA mineralization required additional O_2 per mol C for complete biodegradation. However, according to Eq. 3.4, a mol of CO_2 could produce $3.5O_2/ 4CO_2$= 0.875 mol of O_2 by the algal photosynthesis. Furthermore, the stoichiometry of an algal-bacterial system Eq. 3.6 showed that to biodegrade one mol of BPA, additional 3.6 mol of O_2 are required. This

finding, therefore, suggests that there is an imbalance between O_2 and CO_2 in the algal-bacterial system for complete BPA biodegradation. These results agreed with previous studies that additional O_2 was required in the algal-bacterial system for complete biodegradation of more recalcitrant compound such as methane and methanol than glucose (Bahr et al., 2011). For this reason, some researchers suggest an external O_2 or CO_2 supply in an algal-bacterial system for recalcitrant pollutant removal (Bordel et al., 2009; Bahr et al., 2011).

However, in this study, BPA was removed to below the detection limit in the algal-bacterial system irrespective of the initial BPA concentration (i.e. up to at least 50 mg L^{-1}). These findings indicate that an imbalance between O_2 and CO_2 was not occurring in the present experimental setup. The photolysis of EDTA can explain this phenomenon. The concentration of EDTA decreased gradually with time (Fig. 3.3.6). The removal of EDTA accounted for 114.3 mg L^{-1} at the end of the experiment. From the Eq. 3.12, the biodegradation of EDTA could release 2.7 mmol of CO_2 (114.3 / 292.24 × 7). The CO_2 then promoted the photosynthesis of algae (Eq. 3.5) and released 2.4 mmol of O_2. Based on the Eq. 3.6, an additional 0.79 mmol of O_2 is needed to biodegrade 50 mg L^{-1} BPA. Therefore, the 2.4 mmol of O_2 released from EDTA biodegradation could compensate for the O_2 shortage. These findings suggest that instead of supplying additional O_2 or CO_2 to the system, other organic pollutants such as EDTA could act as an additional carbon source for bacterial growth and this would compensate for the shortage of CO_2. Nevertheless, since BPA biodegradation intermediates were detected in this study, the hypothetical carbon mass balance was imprecise. To further confirm the calculation, C-labelled BPA should be used.

As photosynthesis involved CO_2 assimilation by algae that released of H^+ and CO_3^-, it acquires an increase in pH. In the study, pH increased gradually with time in the batch experiment (Fig. 3.3.7). The result indicates that algal photosynthesis progressed well. The pH is one of the important parameters that reflect and affect the growth of algae (Mayo et al., 1997; Muñoz and Guieysse, 2006). Thus, in order not to allow any changes in the pH, pH 7 was adopted for the semi-continuous study.

3.3.5. BPA biodegradation by a semi-continuous algal-bacterial system

In the semi-continuous experiment, the lag growth phase (4 days) of *C. sorokiniana* in Chl *a* was observed (Fig. 3.3.8). The lag phase in *C. sorokiniana* was probably due to algal BPA inhibition. This can be explained from the result of the batch experiment of *C. sorokiniana* + Bacteria, as 50 mg L^{-1} BPA slightly delayed *C. sorokiniana* growth by day 2 (Fig. 3.3.3c). The Chl *a* concentration of *C. sorokiniana* increased in a log phase when BPA concentration decreased to 27 mg L^{-1} (Fig. 3.3.2c). However, the Chl *a* concentration of the semi-continuous *C. sorokiniana* + Bacteria (Fig. 3.3.8) increased in a log phase from day 4 to day 6. Even though, a semi-continuous BPA loading (Fig. 3.3.9) was performed in the *C. sorokiniana* + Bacteria, the Chl *a* concentration of *C. sorokiniana* did not decrease (Fig. 3.3.8). This finding indicates that *C. sorokiniana* BPA inhibition might be recovered through BPA acclimatization. On the other hand, the Chl *a* of *C. vulgaris* showed a relatively short lag phase in its growth (1 day) (Fig. 3.3.8). The Chl *a* concentration increased after the inoculation and reached the maximum growth of 3.2 mg L^{-1} at day 9. These results demonstrate that even with

the semi-continuous loading (Fig. 3.3.9) of BPA 50 mg L^{-1}, algal growth was not reduced (Fig. 3.3.8). This finding suggests that the bacterial consortium protect *C. vulgaris* from a reduction in growth in the algal-bacterial systems.

In the semi-continuous algal-bacterial BPA degradation experiment, *C. sorokiniana* + Bacteria, the DO concentrations after the light and after the dark phases before day 6 were less than 1 mg L^{-1} (Fig. 3.3.10a). The duration of the low DO concentrations coincided with the lag phase of *C. sorokiniana* + Bacteria (Fig 3.3.8). The low DO in the medium also affected the BPA degradation because DO < 1 mg L^{-1} may reduce the rate of bacterial growth. The results suggest that algae and bacteria are in a mutually limiting relationship when treating 50 mg L^{-1} of BPA. These results agreed with a previous study that bacteria growth might be limited by oxygen when poor photosynthesis occurs (Guieysse et al., 2002). However, as the Chl *a* of *C. sorokiniana* + Bacteria entered a log phase (Fig. 3.3.8), the DO after day 6 was high after light phase ranging from 3.3-15.2 mg L^{-1} (Fig. 3.3.10a). A low DO was observed after dark phase ranging from 0-5.3 mg L^{-1}.

On the other hand, in the semi-continuous algal-bacterial BPA degradation experiment of *C. vulgaris* + Bacteria, the DO concentration never reached to the under the detection limit in both light and dark phases during the period of lag and log phase (Fig. 3.3.10b). The DO concentrations ranged from 8.3 to 16.7 mg L^{-1} after the light phase and from 0.6 to 7.5 mg L^{-1} after the dark phase. Despite the fact that the bacterial BPA degradation consumed O_2 during light phase, the DO concentration after the light phase remained at a higher level. The fluctuation of DO after the light and dark phase during BPA degradation indicates that the algae sustained DO in the medium. Therefore, this finding

suggests that algae can act as O_2 supplier to replace the mechanical aeration for bacterial BPA biodegradation. However, whether the O_2 that supply by the algae are sufficient for the degradation of BPA, a mass balance was calculated.

3.3.6. Mass balance in the semi-continuous algal-bacterial system

The bacterial dry weight increased from the initial bacterial dry weight of 1.5 μg mL^{-1} to 390.9 μg mL^{-1} in *C. vulgaris* + Bacteria by Day 13 (Table 3.3.2). Based on the injected BPA and the EDTA concentration in the semi-continuous experiments, the theoretical bacterial dry weight of the bacterial dry weight was 441.1 μg mL^{-1} for *C. vulgaris* + Bacteria. The result showed the bacterial dry weight was (11.4%) lower than the theoretical bacterial dry weight. This result agreed with the stoichiometry Eq. 3.9 that additional O_2 is required by the bacteria to degrade the injected BPA. However, BPA in *C. vulgaris* + Bacteria was degraded to below the detection limit in several BPA injections. Moreover, the BPA concentration in the algal-bacterial biomass was detected at 0.05 μg L^{-1}. These findings suggest that BPA was probably transformed by algae. Indeed, in the algal system, the BPA biodegradation percentage (Eq.14) was about 23.1%. Hence, the removed BPA that was not transformed into bacterial cell (11.4%) was speculated to be removed by *C. vulgaris*.

On the other hand, the bacterial dry weight increased from the initial bacterial dry weight of 0.39 μg mL^{-1} to 496.1 μg mL^{-1} in *C. sorokiniana* + Bacteria by Day 15 (Table 3.3.2). Based on the injected BPA and the EDTA concentration in the semi-continuous experiments, the theoretical value of the bacterial dry weight was 441.7 μg mL^{-1} for *C. sorokiniana* + Bacteria. The bacterial dry weight

of *C. sorokiniana* + Bacteria was (12.3%) higher than the theoretical value, which was probably caused by the algal mucilage. Although *C. sorokiniana* is one of the closest relatives of *C. vulgaris*, this strain develops mucilage (Luo et al., 2008). A previous study showed that *C. sorokiniana* released mucilage (Imase et al., 2008) that consists of saccharides and proteins (Watanabe et al., 2006) for strengthening the algal-bacterial flocculation. In the present study, *C. sorokiniana* + Bacteria flocculation was also observed (Fig. 3.3.11). Therefore, the total dry weight after subtracting algal dry weight could be the combination of bacterial and mucilage dry weight. Hence, the bacterial dry weight in *C. sorokiniana* + Bacteria could be overestimated. Nevertheless, the BPA concentration in the floc of *C. sorokiniana* + Bacteria was below the detection limit. This result agreed with the algal BPA adsorption study. In fact, BPA biodegradation percentage (Eq.14) by *C. sorokiniana* cell was about 20.1% in the algal system. Thus, the degradation of BPA in *C. sorokiniana* + Bacteria could be a synergistic action of algae and bacteria. Although, in the batch experiment, the algal-bacterial stoichiometry showed that an imbalance of O_2 and CO_2 could be compensated by the addition of EDTA. However, in the semi-continuous experiment, only a small amount of EDTA was injected during the new BPA injections, BPA was also removed to below the detection limit for several BPA injections. The biodegradation of BPA was probably due to a synergistic effect of algae and bacteria.

3.3.7. Microbial community

The homologous coverage showed 0.72 in the microbial community of the semi-continuous experiment. This result indicates that the representative clones in the sample are similar to each other.

A total of 15 phylotypes were detected out of 51 clones in the semi-continuous experiment culture. Based on the phylogenetic reconstruction of the 16S rDNA gene sequences, the bacteria belong to two Phyla, which accounted for 7% and 93% of the Bacteroidetes and Proteobacteria, respectively (Fig. 3.3.12). Amongst the Proteobacteria, Betaproteobacteria and gammaproteobacteria were the dominant classes and the representative phylotypes are SC08 (29.4%) and SC01 (29.4%) (Fig. 3.3.13). The clone SC08 was closely affiliated to *Herminiimonas glaciei* strain DSM 11853 that is known to be a toluene-degrading bacterium (Kim et al., 2014). Whereas, clone SC01 was closely affiliated to *Pseudomonas fluorescens* that were reported to be able to degrade hydrocarbon (Vasudevan et al., 2007) and phenol via meta-cleavage pathway (Mahiudddin et al., 2012). Since both BPA and its biodegradation possess of benzene ring, these bacteria may degrade them through meta-cleavage of the benzene ring. Furthermore, amongst the detected phylotypes, clone SC22 (2%) and SC41 (4%) shared 98% similarity with BPA-degrading bacteria such as *Ochrobactrum antropi* strain BU80A (Zu et al., 2014) and *Pseudomonas putida* (Kang and Kondo, 2002), respectively. Although, the BPA-degrading bacteria was not the dominant strain in the bacterial consortium but it may play an important role in functionally degrading BPA. Therefore, the presence of BPA-degrading bacteria in the consortium may explain the BPA degradation capability in the present study.

Table 3.3.1. BPA degradation intermediates in the algal (A) and the algal-bacterial systems (AB).

Abbreviation	Chemical compounds	Systems	Retention time (min)	Oestrogenic activity
BPA	Bisphenol A	A, AB	26.78	Yes
p-HAP	*p*-hydroxyacetophenone	AB	14.37	Yes
HQ	*p*-hydroquinone	A, AB	12.89	Yes
OH-BPA	hydroxy-BPA	A, AB	28.44	Yes
IV	2,2-bis(4-hydroxyphenyl) propanoic acid	AB	29.77	Yes
I	2,2-bis(4-hydroxyphenyl)-1-propanol	AB	29.23	No
II	1,2-bis(4-hydroxyphenyl)-2-propanol	AB	29.96	No
VI	*p*-hydroxybenzoylmethanol	AB	20.01	No
p-HBA	*p*-hydroxybenzoic acid	AB	16.01	No
p-HBAL	*p*-hydroxybenzaldehyde	AB	12.57	No

Table 3.3.2. Microbial dry weight in the semi-continuous experiments.

	Dry weight ($\mu g\ mL^{-1}$)			
	Total	Algae	Bacteria	Theoretical bacteria
C. vulgaris + Bacteria (Day 13)	487.8	96.9	390.9	441.1
C. sorokiniana + Bacteria (Day 15)	562.0	65.9	496.1	441.7

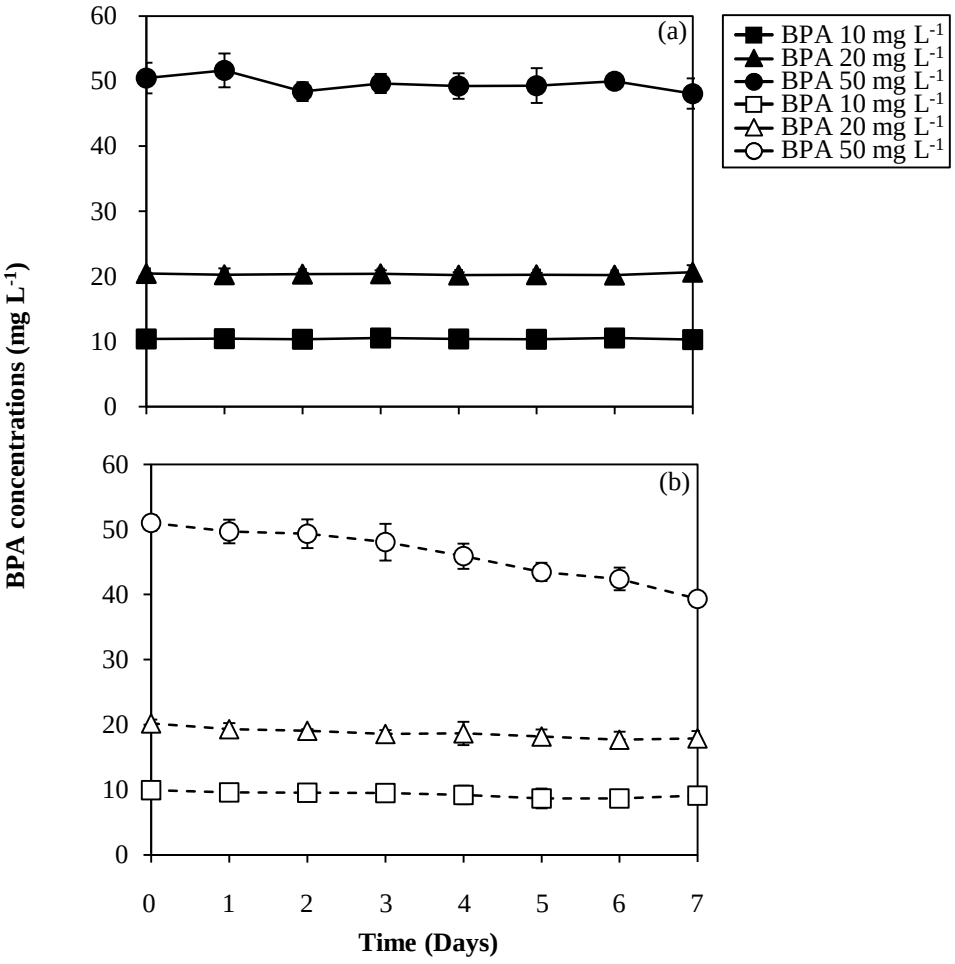


Fig. 3.3.1. Time course of BPA concentrations in abiotic control under the dark (a) and under the light conditions (b) in the batch experiment. The square is 10 mg L^{-1}, the triangle is 20 mg L^{-1}, and the circle is 50 mg L^{-1}.

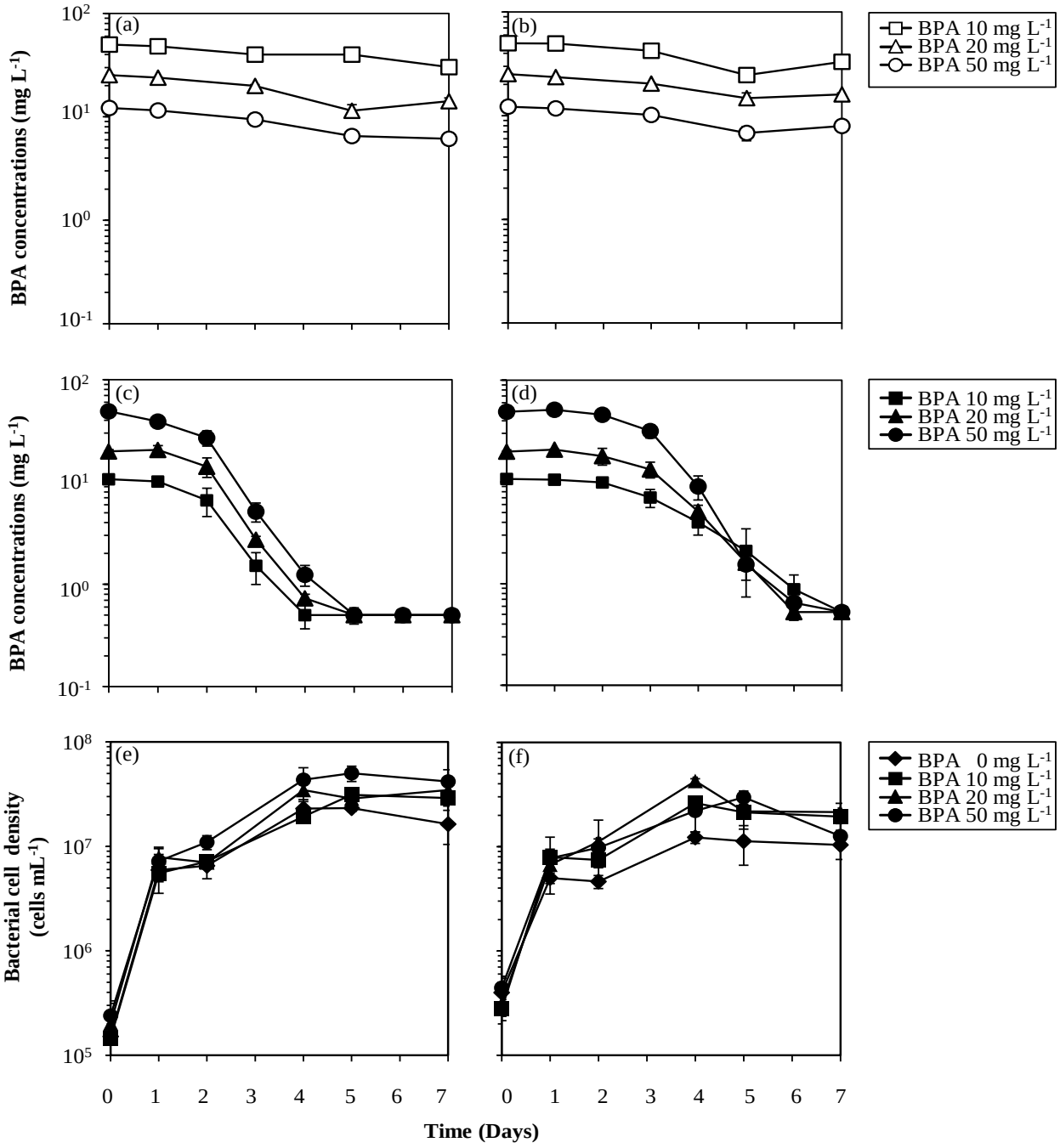


Fig. 3.3.2. Time course of BPA concentrations in *C. sorokiniana* (a), *C. vulgaris* (b), *C. sorokiniana* + Bacteria (c), and *C. vulgaris* + Bacteria (d); and the bacterial cell density in *C. sorokiniana* + Bacteria (e) and *C. vulgaris* + Bacteria (f) in batch experiment. Open symbols represent the algal system, and closed symbols represent the algal-bacterial system for different initial BPA concentrations. Diamond is BPA 0 mg L^{-1}, square is 10 mg L^{-1}, triangle is 20 mg L^{-1}, and circle is 50 mg L^{-1}.

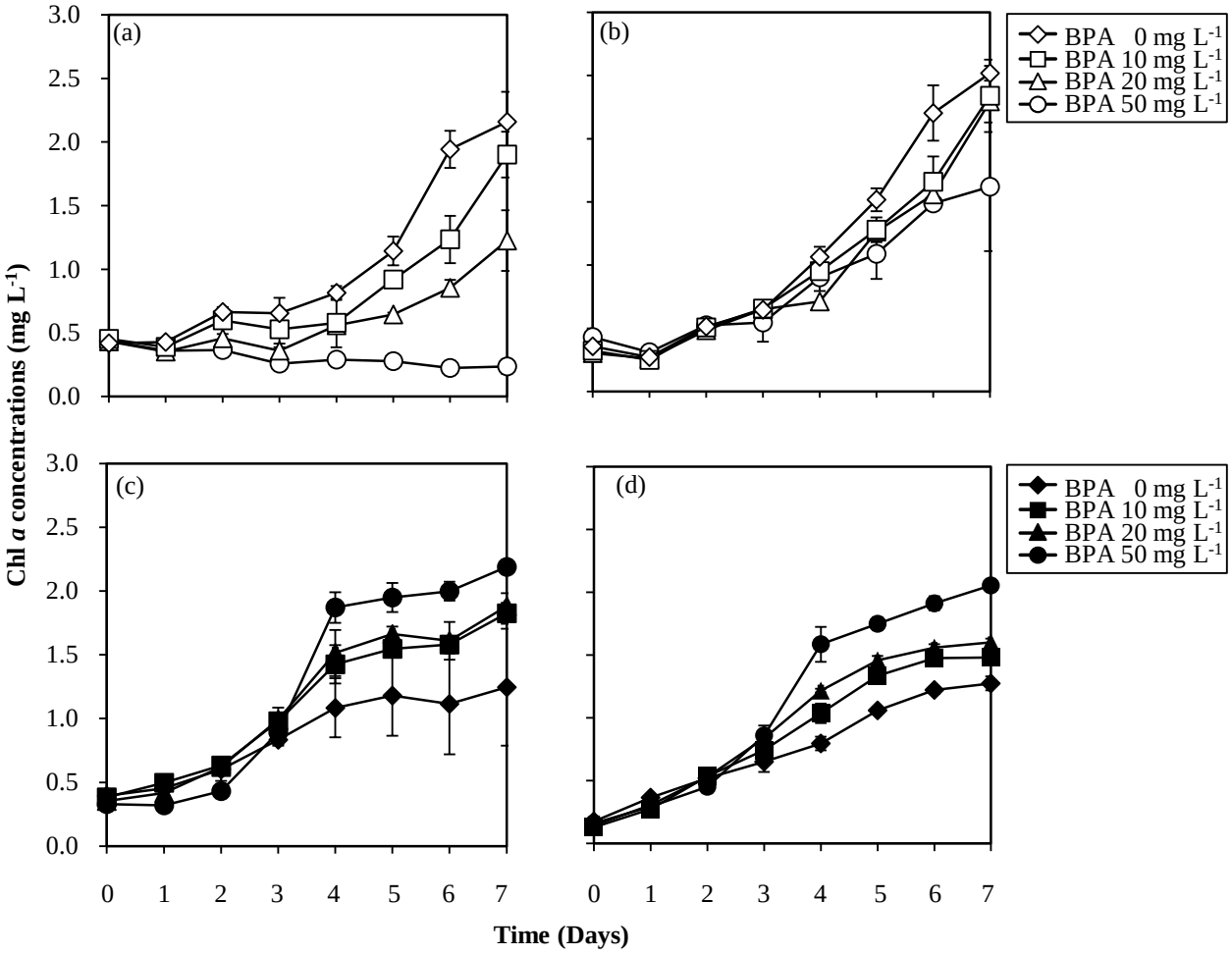


Fig. 3.3.3. Effect of BPA concentrations on the amount of Chl *a* synthesis by *C. sorokiniana* (a), *C. vulgaris* (b), *C. sorokiniana* + Bacteria (c) and *C. vulgaris* + Bacteria (d). Diamond is BPA 0 mg L^{-1}, square is 10 mg L^{-1}, triangle is 20 mg L^{-1}, and circle is 50 mg L^{-1}. The open symbols represent the algal systems, and the closed symbols represent the algal-bacterial systems.

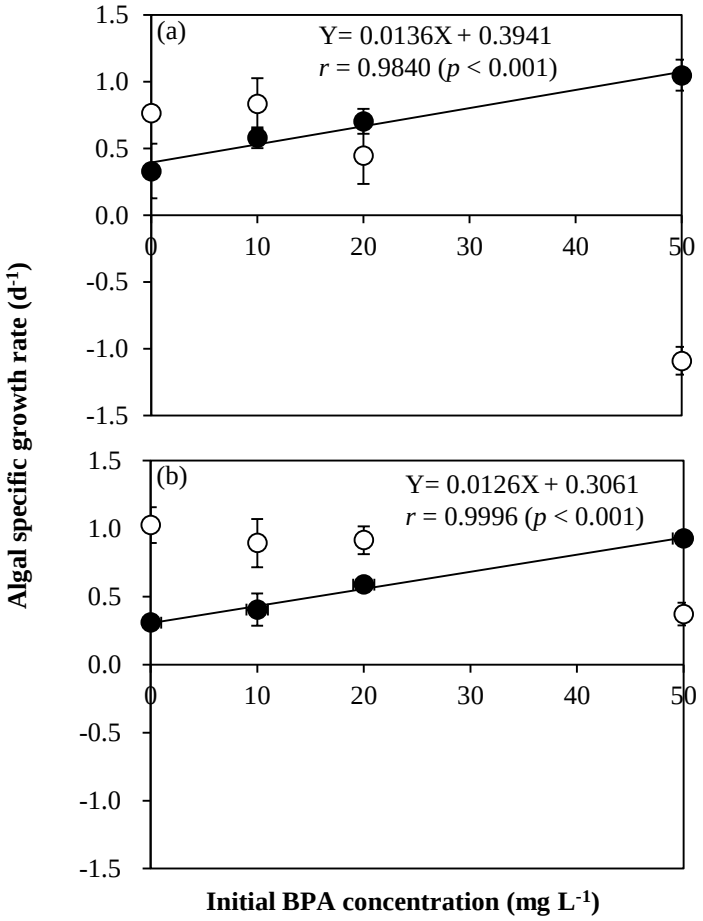


Fig. 3.3.4. Effect of BPA concentrations on the specific growth rate of *C. sorokiniana* and *C. sorokiniana* + Bacteria (a) and *C. vulgaris* and *C. vulgaris* + Bacteria (b) in the batch experiment. The open symbols represent the algal systems, and the closed symbols represent the algal-bacterial systems.

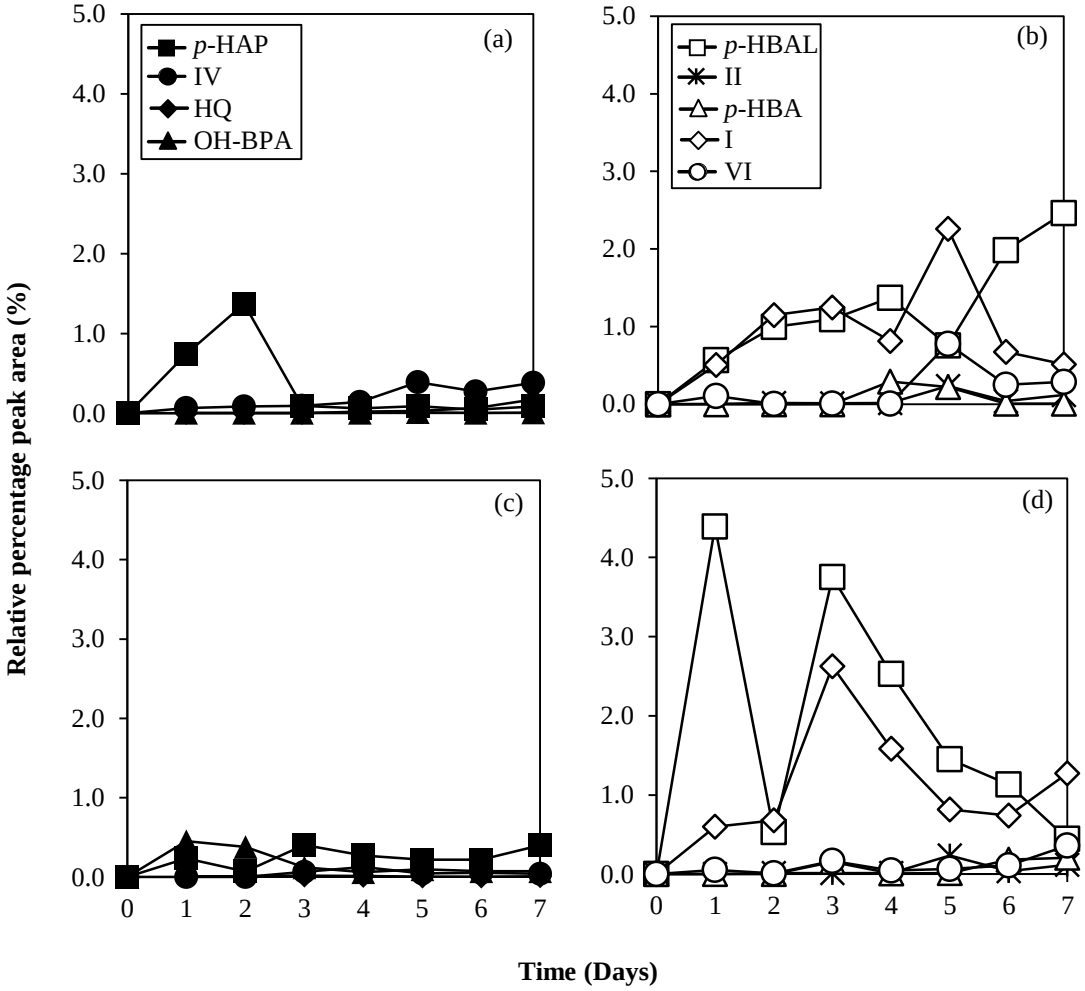


Fig. 3.3.5. Time course of major BPA biodegradation intermediates generated from 20 mg L^{-1} BPA by *C. sorokiniana* + Bacteria (a,b) and by *C. vulgaris* + Bacteria (c,d) in the batch experiment. Closed symbols represent oestrogenic biodegradation intermediates, and open symbols represent non-oestrogenic activity biodegradation intermediates. Abbreviations for the biodegradation intermediates are shown in Table 3.3.1.

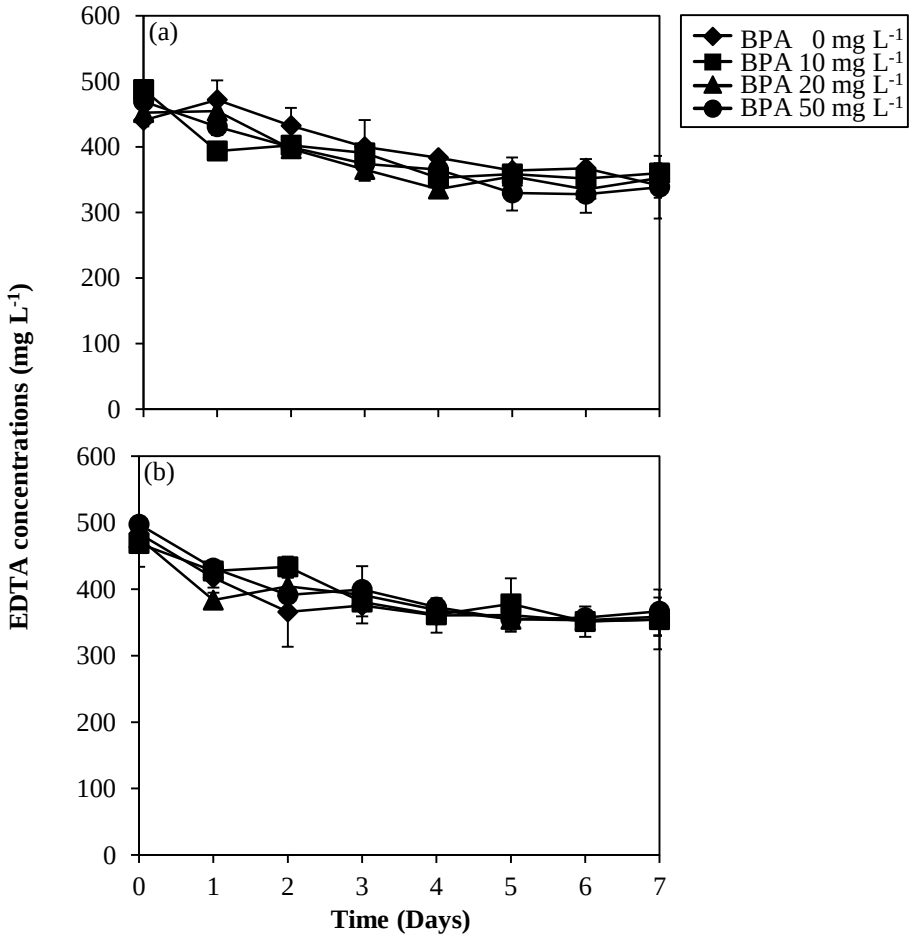


Fig. 3.3.6. EDTA concentrations in *C. sorokiniana* + Bacteria (a) and *C. vulgaris* + Bacteria (b) in the batch experiment.

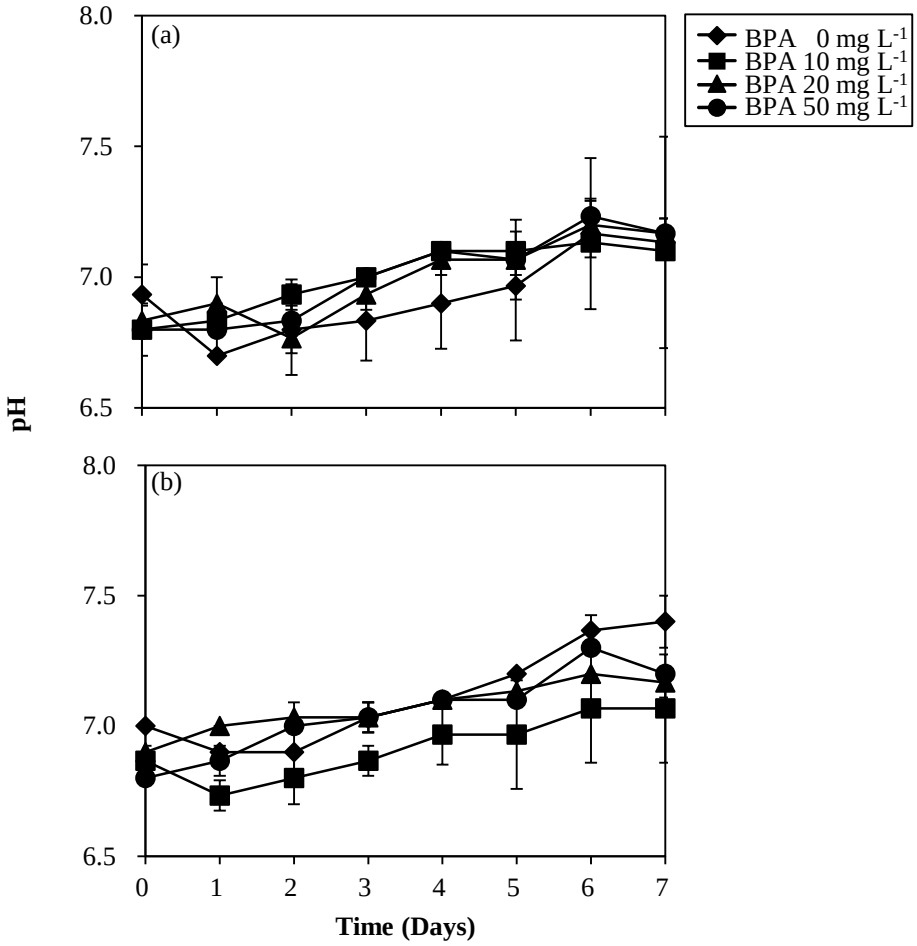


Fig. 3.3.7. Change of pH in *C. sorokiniana* + Bacteria (a) and *C. vulgaris* + Bacteria (b) in the batch experiment.

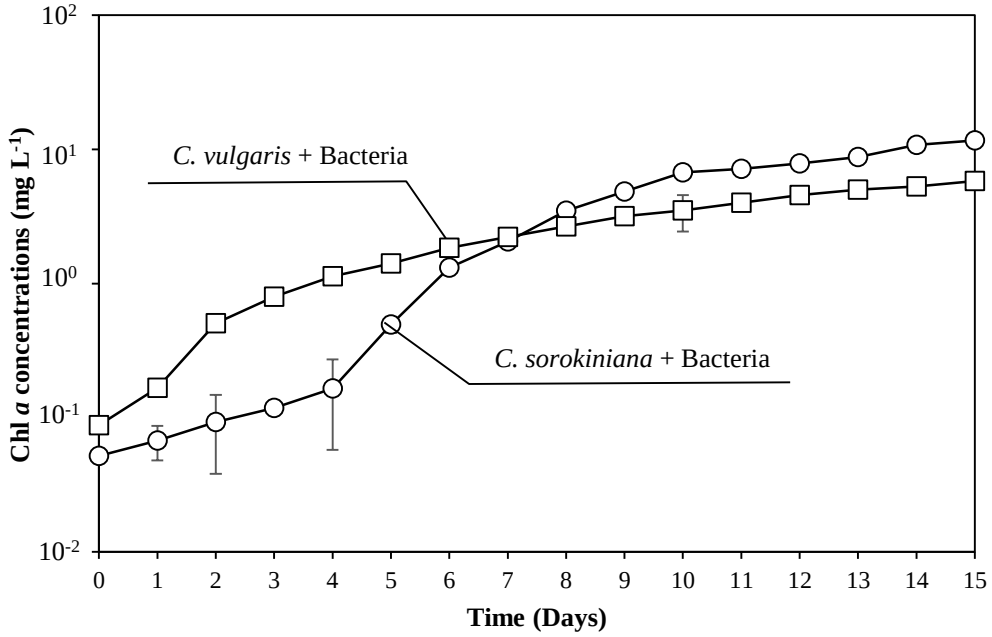


Fig. 3.3.8. Change of Chl *a* concentrations in the semi-continuous algal-bacterial systems. Circle represents *C. sorokiniana* + Bacteria and square represents *C. vulgaris* + Bacteria.

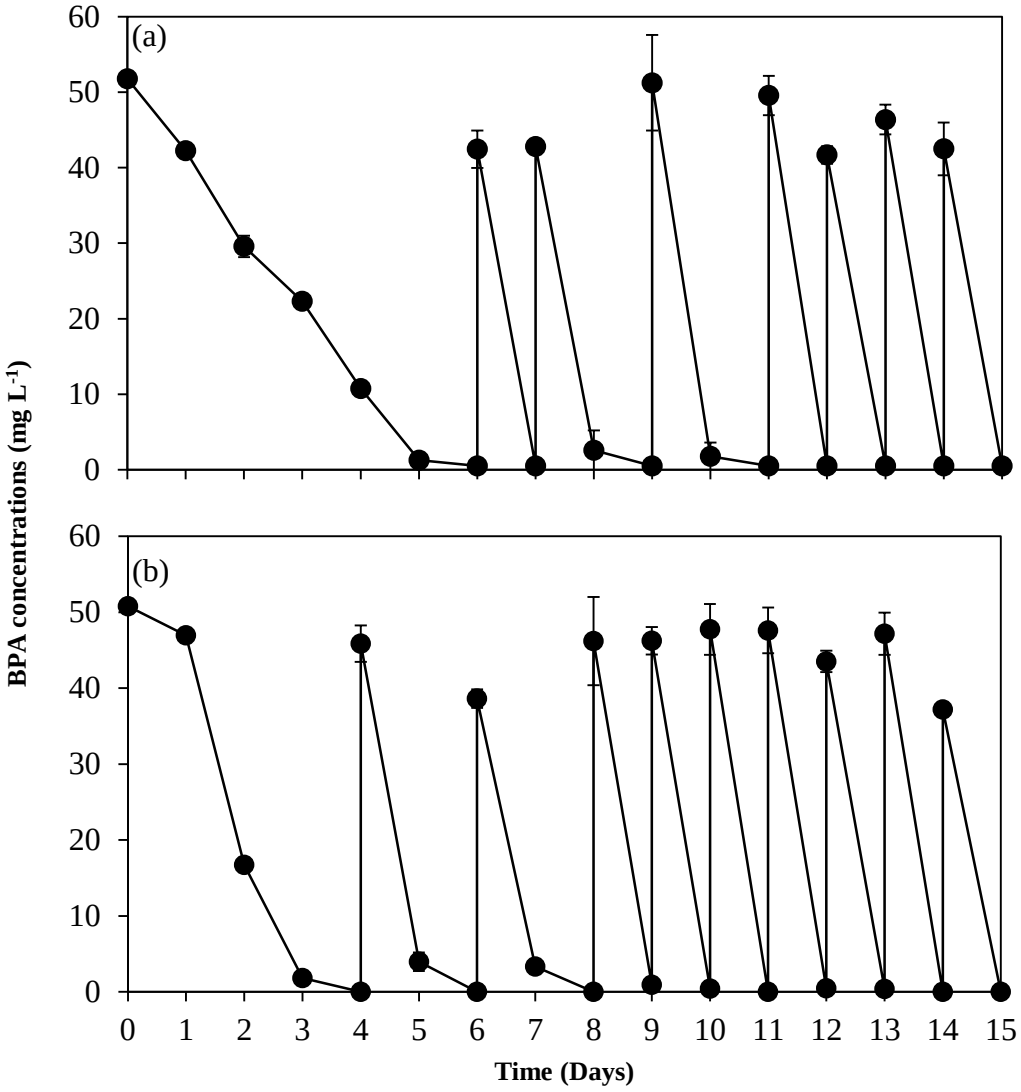


Fig. 3.3.9. Changes of BPA concentrations in semi-continuous *C. sorokiniana* + Bacteria (a) and semi-continuous *C. vulgaris* + Bacteria (b).

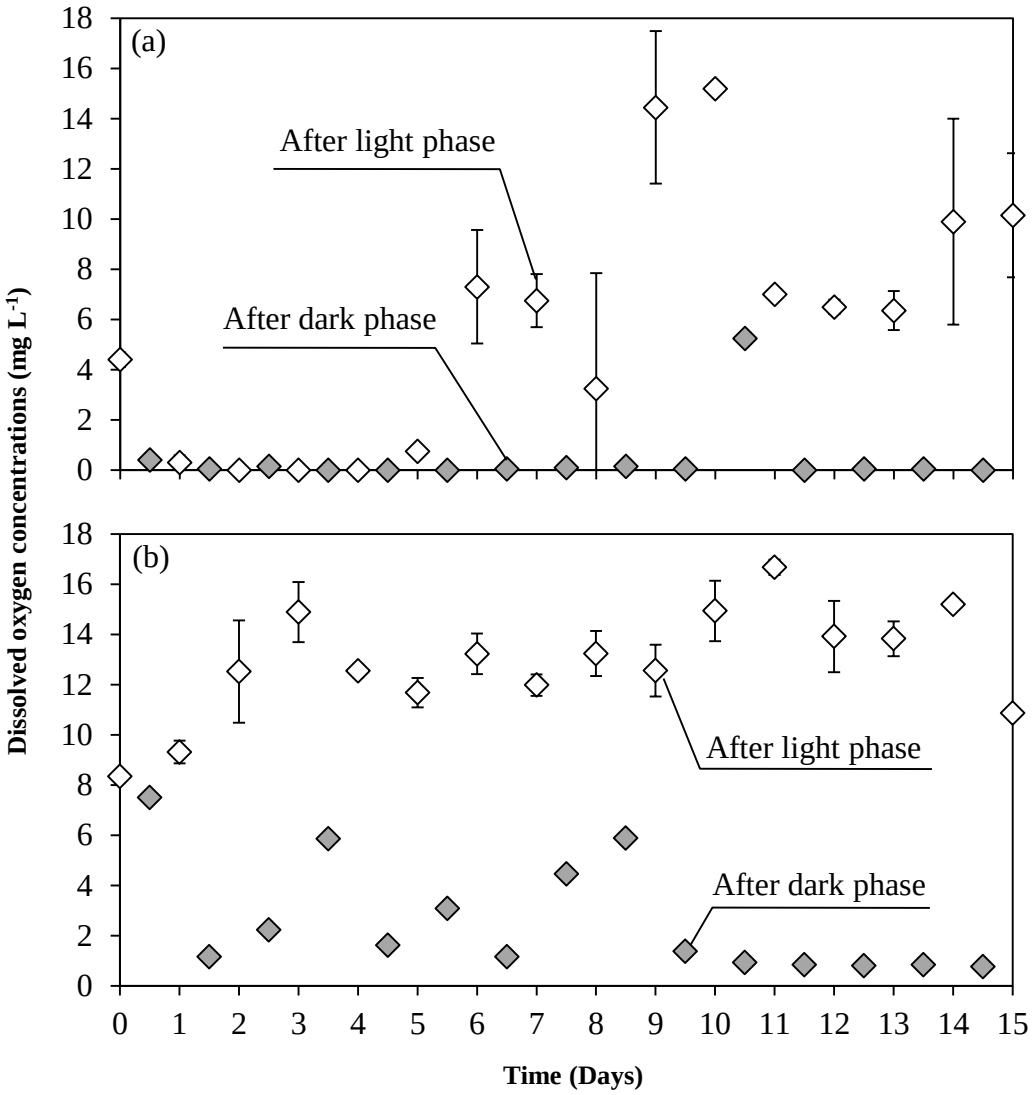


Fig. 3.3.10. Changes of dissolved oxygen (DO) concentrations during BPA degradation in semi-continuous *C. sorokiniana* + Bacteria (a) and *C. vulgaris*+ Bacteria (b). Open symbols represent DO after light phase while closed grey symbols represent DO after dark phase.

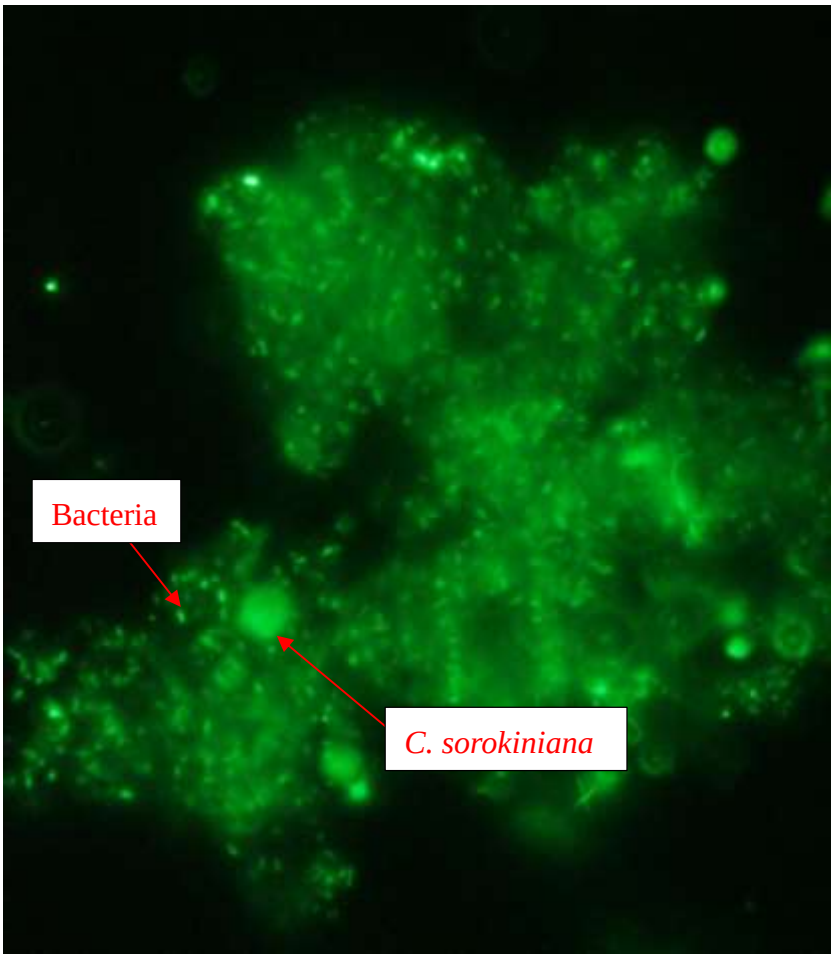


Fig. 3.3.11. Flocculation of algae and bacteria in *C. sorokiniana* + Bacteria.

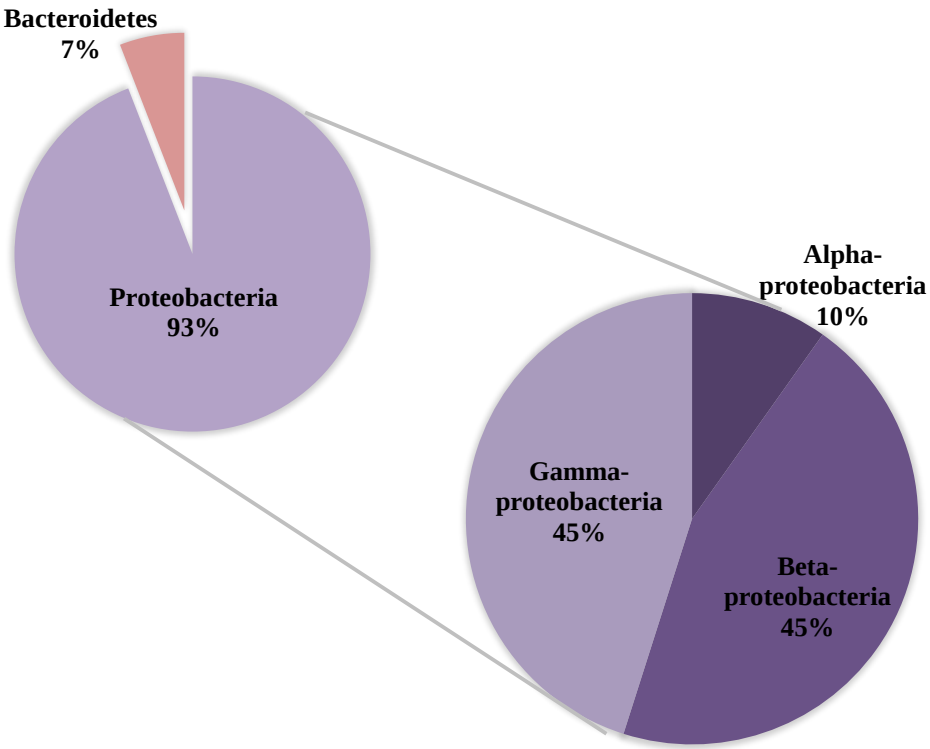


Fig. 3.3.12. Microbial community structure of clone libraries in the semi-continuous experiment. The smaller circle represents the phylotypes composition at the phylum level, whereas the larger circle represents the phylotypes composition of Proteobacteria class.

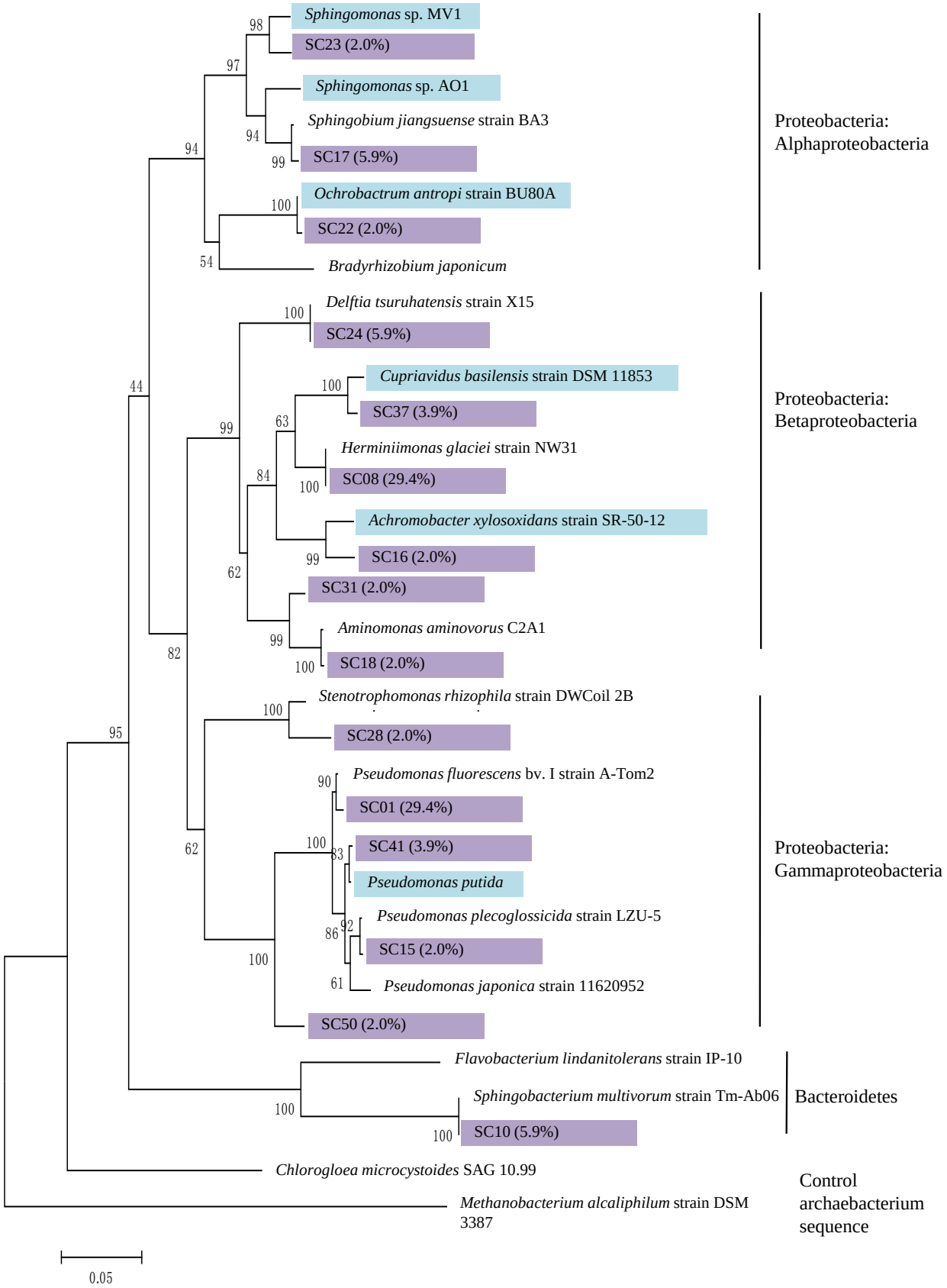

Sphingomonas sp. MV1
SC23 (2.0%)
Sphingomonas sp. AO1
Sphingobium jiangsuense strain BA3
SC17 (5.9%)
Ochrobactrum antropi strain BU80A
SC22 (2.0%)
Bradyrhizobium japonicum
Proteobacteria: Alphaproteobacteria
Delftia tsuruhatensis strain X15
SC24 (5.9%)
Cupriavidus basilensis strain DSM 11853
SC37 (3.9%)
Herminiimonas glaciei strain NW31
SC08 (29.4%)
Achromobacter xylosoxidans strain SR-50-12
SC16 (2.0%)
SC31 (2.0%)
Aminomonas aminovorus C2A1
SC18 (2.0%)
Proteobacteria: Betaproteobacteria
Stenotrophomonas rhizophila strain DWCoil 2B
SC28 (2.0%)
Pseudomonas fluorescens bv. I strain A-Tom2
SC01 (29.4%)
SC41 (3.9%)
Pseudomonas putida
Pseudomonas plecoglossicida strain LZU-5
SC15 (2.0%)
Pseudomonas japonica strain 11620952
SC50 (2.0%)
Proteobacteria: Gammaproteobacteria
Flavobacterium lindanitolerans strain IP-10
Sphingobacterium multivorum strain Tm-Ab06
SC10 (5.9%)
Bacteroidetes
Chlorogloea microcystoides SAG 10.99
Methanobacterium alcaliphilum strain DSM 3387
Control archaebacterium sequence
0.05

Fig. 3.3.13. Phylogenetic tree based on a comparison of the partial 16rRNA gene sequence of BPA-degrading bacterial consortium in the semi-continuous experiment (1,000 bootstraps for confidence level). The parentheses are the percentage of the phylotypes in the total clones. The purple bars represent the phylotypes in the semi-continuous experiment the blue bars represent previously reported BPA-degrading bacterial strains.

Chapter IV

General Discussion

4.1. BPA-degrading capacity of a bacterial consortium

The present study investigated microbial community in the inoculum of the bacterial consortium (Chapter II) and the semi-continuous algal-bacterial system (Chapter III). A phylogenetic tree that comprised of the clones from the two samples was constructed (Fig. 4.1). A total of 7 phylotypes was detected in both of the samples, including *Pseudomonas putida* (B34; SC41), *Ochrobactrum anthropi* BU80A (B81; SC22), *Sphingobium jiangsuense* BA3 (B04; SC17), *Delftia tsuruhatensis* strain X15 (B65; SC24), *Pseudomonas fluorescens* bv I strain A-Tom 2 (B18; SC01), *Aminomonas omnivorous* C2A1 (B40; SC18), an uncultured bacterium (B67; SC16; Table 4.1). Hence, these phylotypes may be functionally important bacteria for the degradation of BPA. Of these bacteria, BPA- and its biodegradation intermediates, *p*-HAP-degrading bacteria, i.e. *Pseudomonas putida* and *Pseudomonas fluorescens* were also detected (Table 4.2). Thus, it explained the degradation of BPA and *p*-HAP in the present study. Besides, the 7 phylotypes, with the exception of the uncultured bacterium, are also known to degrade various monocyclic (MAH) such as phenol, 4-chlorophenol, aniline, and microcystin (Liu et al., 2002; Tamburro et al., 2004; Hu et al., 2009; Mahiudddin et al., 2012), and polycyclic aromatic hydrocarbon (PAH) including naphthalene, phenanthrene, tetrabomobisphenol A, and 3-phenolxybenzoic acid (Lloyd-Jones et al., 1997; Zhang et al., 2012; Zu et al., 2014; Table 4.2). Therefore, these bacteria might also degrade some of the BPA

biodegradation intermediates (Fig. 2.3.9), that showed a similar chemical structure to the MAHs and PAHs.

Mixed bacteria are more effective than pure cultures for biodegradation due to broad enzymatic capacities (Sorkhoh et al., 1995). In the present study, several enzymes were believed to have been involved in the degradation of BPA. For instance, *Pseudomonas putida* and *Ochrobactrum* sp., were reported to degrade aromatic compounds through Glutathione S-tranferases (Tamburro et al., 2004). *Sphingobium* sp. and *Delftia tsuruhatensis* degraded aromatic compounds through meta-cleavage pathway enzymes (Zhang et al., 2008; Roy et al., 2012). While, the degradation of *p*-HAP by *Pseudomonas fluorescens* involved Baeyer-Villger monooxygenases (Kamerbeek et al., 2001). Although, the enzymes involved in *Aminomonas aminovorus* for aromatic compounds degradation were unstated, the degradation was predicted to be oxygen-dependent (Hu et al., 2009). Therefore, the BPA-degrading capability of the bacterial consortium used in Chapter II and Chapter III could be explained by the robustness of enzymes. Moreover, 3 of the 7 phylotypes were coincided with the genera (*Pseudomonas, Ochrobactrum, Aminomonas*) that were found by Viñas et al. (2005) in PAH-degrading microbial consortium. These genera were also well known for oil degradation (Van Gestel et al., 2003; Viñas et al., 2005). It is therefore suggested that the bacterial consortium in the present study has a potential for the degradation of both MAHs and PAHs.

4.2. BPA biodegradation by photosynthetic algal-bacterial system

An algal-bacterial system for the degradation of BPA has been investigated. This system may

serve as a low-cost alternative treatment for developing countries which cannot afford the expensive wastewater treatment process such as AOPs. Although BPA is a moderately toxic compound (Commission of the European Communities, 1996), algae alone reduced a small portion of BPA. This finding agreed with Hirooka et al. (1995), the first study of BPA biodegradation by algae. They reported that *C. fusca* biodegraded BPA to OH-BPA in light conditions but did not show the result in the abiotic control. However, in the present study, OH-BPA was detected in both the algal culture and the abiotic control in the light, but not in the abiotic cultured in the dark. The difference between the abiotic cultured in the light and dark conditions suggests that OH-BPA was not a biodegradation intermediate of algae, but rather was a BPA photodegradation intermediate. Nakajima et al. (2007) also reported that green algae may not have oxidative enzymes to degrade BPA. Instead, green algae hydroxylated BPA to glycosides as a detoxification function. Glycosides were not detected in the present study, possibly because long-chained glycosylation products have easily been separated during GC-MS detection.

When bacterial consortium was inoculated into the algal system to form an algal-bacterial system, BPA was removed to below the detection limit. By using algae alone, the BPA concentration could not be degraded to below the detection limit, suggesting that further reduction of BPA in the algal-bacterial system was due to the bacteria. Although bacteria alone biodegraded BPA to below the detection limit, they were cultured under mechanical aeration. Therefore, the depletion of BPA in the algal-bacterial system suggests that photosynthetic oxygenation in the algal-bacterial system supports the degradation of BPA. This suggestion is consistent with previous studies that an algal-

bacterial system supports the degradation of organic pollutants by bacteria in the absence of mechanical aeration (Borde et al., 2003; Muñoz et al., 2003a, 2004). Although the *C. vulgaris* showed a longer delay of BPA degradation than *C. sorokiniana* owing to the smaller cell size that reduce the O_2 production rate (Park and Lee, 2000), *C. vulgaris* is still recommended for the degradation of BPA in the actual wastewater application because it has a higher tolerance of BPA. *C. sorokiniana* was inhibited by 50 mg L^{-1} of BPA at the start-up stage of the semi-continuous experiment, which resulted in a continuous low DO in the system. The algal inhibition may cause a failure of the system with *C. sorokiniana* if BPA was continuously loaded into the system.

In the present study, BPA was removed to below the detection limit in both the bacterial (Chapter II) and the algal-bacterial systems (Chapter III). However, the BPA degradation rate constants under the light conditions (the bacterial and the algal-bacterial systems) were significantly lower than in the dark conditions (bacterial system) with BPA 20 mg L^{-1} and 50 mg L^{-1} (Table 4.3). In Chapter II, light limited bacterial growth and, therefore it was suggested, delayed the BPA degradation. This finding agreed with a review that the bactericidal effect was related to visible light (Lubart et al., 2011). They summarised that endogenous photosensitizers in bacteria could generate reactive oxygen species (ROS) under visible light and in the presence of oxygen, which kill bacteria. Blue light, especially at 415 nm has a higher affinity for a bactericidal effect than red light. Light intensity was also found to be positively correlated with the amount of the generated ROS and thus leading to bacteriolysis (Kotelevets et al., 1988). Although light limited bacterial growth was observed, the BPA degradation rate in the algal-bacterial system was significantly higher than that of

the bacterial system cultured in the light condition at BPA 50 mg L^{-1} (Table 4.3). The enhancement of BPA biodegradation in the algal-bacterial systems was probably due to a complementary activity of bacteria and algae.

Because algal photosynthesis requires light, the current photoreactors are designed with an optimum light intensity for algal photosynthesis (Carvalho et al., 2011). However, the increase in light intensity may bring about a negative effect on bacteria (Kotelevets et al., 1988) and possibly to algae through photo-inhibition (Carvalho et al., 2011). Therefore, when algal cell densities are relatively low during the start-up stage of wastewater treatment, a lower light intensity should be used to reduce the inhibitive effect of light on the microbials. However, to enhance the photosynthetic oxygen production, the light intensity should be increased. Therefore, further investigation should be focused on the optimum light intensity for an algal-bacterial system.

4.3. Application of an algal-bacterial system

Although satisfactory BPA removal could be observed by the algal-bacterial system in the laboratory scale, further field application is still needed. Future research should investigate the real wastewater application to evaluate and optimize the algal-bacterial system. BPA has been detected in landfill leachate all over the world at the concentrations ranging from 0.07-25,000 μg L^{-1} (Table 4.4). Thus, BPA in landfill leachate should be targeted to treat. However, not all leachates are suitable for biological treatment. Landfill leachate has a lifecycle of five phases. Young leachates in Phase I are in an aerobic phase, followed by an anaerobic acidic phase in Phase II. Phase III is an intermediate

methanogenic phase and Phase IV is a stabilized methanogenic phase. Phase V is the final phase of leachates, which is an aerobic phase (Tchobanoglous et al., 1993). Moreover, the pH in Phase II is relatively low, but then the pH increases to 7.5–9.2 in Phase III (Kjeldsen et al., 2002). Thus, Phase III leachates are suitable for biological treatment (Chiemchaisri et al., 2009). Whereas, old leachate is low in biodegradability that provides only low biodegradable carbon sources for bacterial growth (Kjeldsen et al., 2002; Renou et al., 2008). Therefore, BPA in the leachate of Phase III should be treated before it is chemically changed to old leachate.

However, Phase III leachate may not be suitable to be directly treated by an algal-bacterial system. Leachate generally contains some heavy metals that could inhibit algal growth (Koukal et al., 2003). Anaerobic digestion is a low-cost biological treatment, which is designed for the treatment of high COD wastewater. Moreover, anaerobic digestion has shown to be effective in removing heavy metal through adsorption (Haytoglu et al., 2001). Therefore, it is proposed that an anaerobic digestion treats Phase III landfill leachate that contains BPA as a secondary treatment after the primary treatment of sedimentation (Fig. 4.2). However, BPA is not biodegradable under anaerobic conditions (Ying and Kookana, 2005). The effluent from the anaerobic digestion (ADE) that contains BPA is transferred to an open-type of algal-bacterial system, for example, a high rate algal pond (HRAP). HRAP is proposed as a tertiary treatment to treat ADE because it is a cost effective aerobically biological treatment, which driven by natural solar energy. Since, ADE usually contains a relatively high nutrient (Table 4.5) and COD concentrations ranging from 500-5000 mg L^{-1} (Renou et al., 2008), the nutrient and the trace metals from the ADE could be used as growth substrates of the algae in

HRAP (Wahal, 2010); while, BPA and other organic compounds could be used as growth substrates for bacteria in HRAP.

In Chapter III, both of the batch experiments and the semi-continuous experiment demonstrated that the algal-bacterial system successfully biodegraded BPA up to 50 mg L^{-1}. Moreover, the treated effluent in the batch experiment showed a relatively low amount (<5%) of oestrogenic BPA biodegradation intermediates. The treated effluent is then recommended to be discharged to the environment with the dilution of river or rainwater. In comparison to the untreated and direct discharge of leachate that is contaminated with BPA in developing countries, the effluent of an algal-bacterial system may greatly improve the water quality of the environment. It is not known whether HRAP could perform the same way as an enclosed system due to the mixture of the existing microbials in the leachate. Therefore, further study should pay attention to the effect of the autochthonous population in the leachate on the degradation of BPA. If the bacterial consortium in the present study is not compromised in its ability to degrade BPA, HRAP treatment might give a similar result.

Nevertheless, based on results of the present study, BPA degradation may be optimised in HRAP by performing the BPA loading in the dark phase to reduce algal BPA inhibition. This is because O_2 is supplied through algal photosynthesis in the light phase and carried forward to the dark phase. The bacterial BPA degradation could be achieved in the dark phase by consuming the O_2 supplied by the algae and, therefore, the decrease in the toxicity of the BPA concentration would be low enough for algal activity.

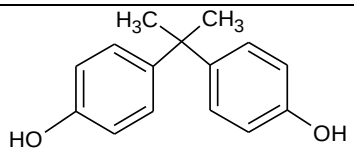
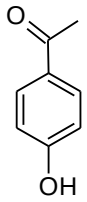
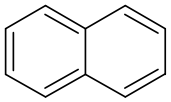
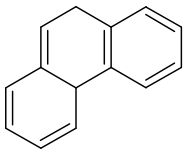
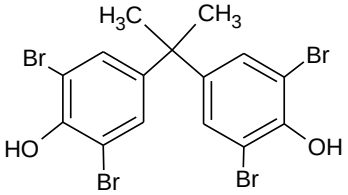
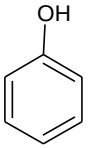
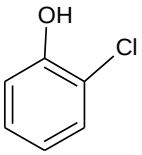
4.4. Future study

The current study indicated that BPA up to an initial concentration 50 mg L^{-1} was removed by an algal-bacterial system in the 1-L flask. However, in order to generate a deeper understanding of the practical application of an algal-bacterial system: (1) determine BPA biodegradation using isotopic labelling to confirm the calculation of mass balance analysis in Chapter II, (2) determine which combinations of bacteria are responsible for most efficient BPA degradation and stable operation for at least 15 days as it was shown in the semi-continuous algal-bacterial system, and (3) investigate the feasibility of using HRAP for the degradation of BPA from landfill leachate. Because, the chemical BPA used in present study was a pure chemical and it might be different from landfill leachate owing to the mixture of various chemicals.

Table 4.1. Closest strains of the clones present the inoculum of both the batch and the semi-continuous experiments.

Strain	**Clone number**
Pseudomonas putida	B34; SC41
Ochrobactrum antropi	B81; SC22
Sphingobium jiangsuense	B04; SC17
Delftia tsuruhatensis	B65; SC24
Pseudomonas fluorescens	B18; SC01
Aminomonas aminovorus	B40; SC18
Uncultured bacterium	B67; SC16

Table 4.2. MAHs and PAHs degradation by bacterial strains.

Strain	Compound	Reference
	 BPA	Kang and Kondo (2002)
	 *p*-HAP	Tanner and Hopper (2000)
	 Naphthalene	Lloyd-Jones et al. (1997)
	 Phenanthrene	Lloyd-Jones et al. (1997)
Pseudomonas putida	 Tetrabromobisphenol A	Zu et al. (2014)
	 Phenol	Tamburro et al. (2004)
	 4-chlorolphenol	Tamburro et al. (2004)
Ochrobactrum sp.		

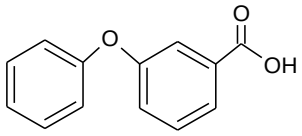
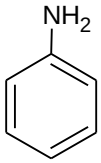
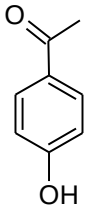
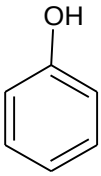
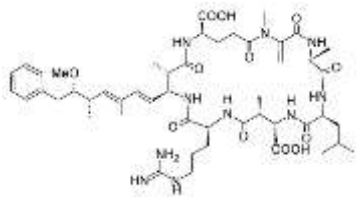
		
Sphingobium jiangsuense	3-phenoxybenzoic acid	Zhang et al. (2012)
		
Delftia tsuruhatensis	Aniline	Liu et al. (2002)
		
	p-HAP	Moonen et al. (2008)
		
Pseudomonas fluorescens	Phenol	Mahiudddin et al. (2012)
		
Aminomonas aminovorus Uncultured bacterium	Microcystin	Hu et al. (2009)

Table 4.3. BPA biodegradation rate constants (h^{-1}) in the bacterial systems cultured in the dark and light condition and the algal-bacterial systems.

BPA concentration	Bacteria in the dark condition	Bacteria in the light condition	*C. sorokiniana* + Bacteria	*C. vulgaris* + Bacteria
10 mg L^{-1}	0.03^{a}	0.03^{a}	0.05^{b}	0.03^{a}
20 mg L^{-1}	0.05^{c}	0.04^{d}	0.06^{c}	0.04^{d}
50 mg L^{-1}	0.08^{e}	0.05^{f}	0.06^{g}	0.06^{g}

a-f Subscripts of the different alphabet indicate significant difference between the bacterial system and the algal-bacterial system in the light and dark conditions

Table 4.4. BPA concentrations found in landfill leachates of various countries.

Country	**BPA concentration (μg L^{-1})**	**Reference**
Japan	0.149 – 12.3	Yasuhara et al. (1997)
Japan	1.3 – 17,200	Yamamoto et al. (2001)
Japan	1 – 8,000	Urase et al. (2003)
Japan	0.009 – 3,600	Kurata et al. (2008)
Japan	0.07 – 288	Asakura et al. (2004)
Japan	4,000 – 6,000	Teuten et al. (2009)
Germany	3,610	Coors et al. (2003)
Germany	4,200 – 25,000	Schwarzbauer et al. (2002)
Sweden	36	Svenson et al. (2011)
Sweden	0.01– 107	Kalmykova et al. (2013)
Italy	33.4	Baderna et al. (2011)
China	0.09 – 200	Li et al. (2006)
Laos	0.18 – 20	Teuten et al. (2009)
Cambodia	0.7 – 60	Teuten et al. (2009)
Vietnam	2.5 – 30	Teuten et al. (2009)
Thailand	20 – 200	Teuten et al. (2009)
Thailand	<100	Boonyaroj et al. (2012)

Philippines	8 – 1,000	Teuten et al. (2009)
Philippines	9,000	Urase et al. (2003)
Malaysia	60 – 4,300	Teuten et al. (2009)
Brazil	26.8	Viecelli et al. (2014)
US	1.7	Federal Register (2011)

Table 4.5. Nutrient concentrations in anaerobic digestion effluent.

Anaerobic digestion effluent	TN	NH_4^+N	TP	Reference
Livestock waste	1240	1200	140	Park et al. (2010)
Commercial scale anaerobic digestion	2667	2266	381	Cai et al. (2013)
Dried *Spirulina* sp.	430	361	86	Borges et al. (2012)
Sewage sludge	943	630	42	Song et al. (2004)

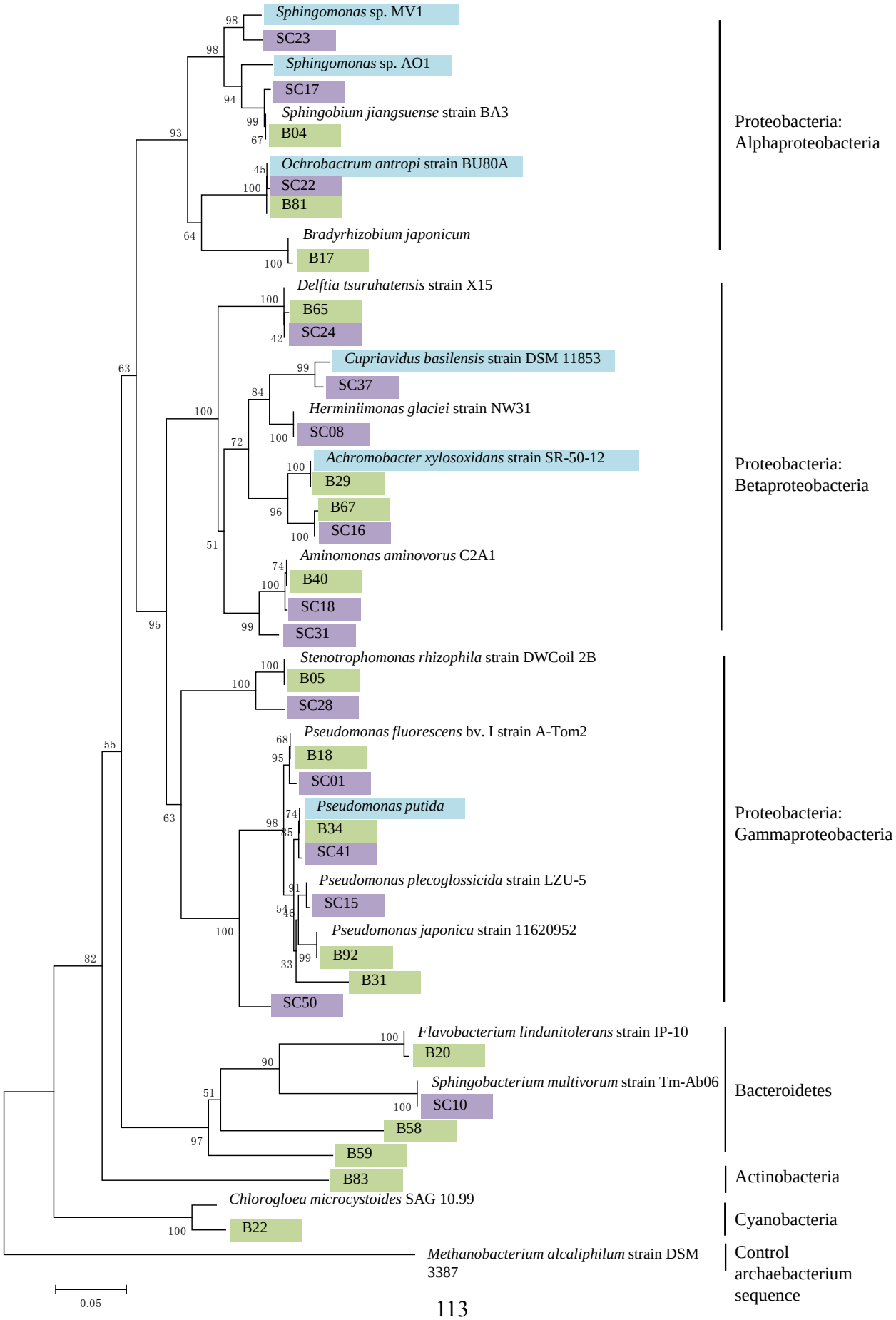

Sphingomonas sp. MV1
SC23
Sphingomonas sp. AO1
SC17
Sphingobium jiangsuense strain BA3
B04
Ochrobactrum antropi strain BU80A
SC22
B81
Bradyrhizobium japonicum
B17
Delftia tsuruhatensis strain X15
B65
SC24
Cupriavidus basilensis strain DSM 11853
SC37
Herminiimonas glaciei strain NW31
SC08
Achromobacter xylosoxidans strain SR-50-12
B29
B67
SC16
Aminomonas aminovorus C2A1
B40
SC18
SC31
Stenotrophomonas rhizophila strain DWCoil 2B
B05
SC28
Pseudomonas fluorescens bv. I strain A-Tom2
B18
SC01
Pseudomonas putida
B34
SC41
Pseudomonas plecoglossicida strain LZU-5
SC15
Pseudomonas japonica strain 11620952
B92
B31
SC50
Flavobacterium lindanitolerans strain IP-10
B20
Sphingobacterium multivorum strain Tm-Ab06
SC10
B58
B59
B83
Chlorogloea microcystoides SAG 10.99
B22
Methanobacterium alcaliphilum strain DSM 3387
0.05
Proteobacteria: Alphaproteobacteria
Proteobacteria: Betaproteobacteria
Proteobacteria: Gammaproteobacteria
Bacteroidetes
Actinobacteria
Cyanobacteria
Control archaebacterium sequence

Fig. 4.1. Phylogenetic tree based on the comparison of the partial 16rRNA gene sequence of BPA-degrading bacterial consortium in both the batch and the semi-continuous experiment (1,000 bootstraps for confidence level). The green bars represent the phylotypes in the batch experiment (Chapter II); the purple bars represent the phylotypes in the semi-continuous experiment (Chapter III); the blue bars represent previously reported BPA-degrading bacterial strains.

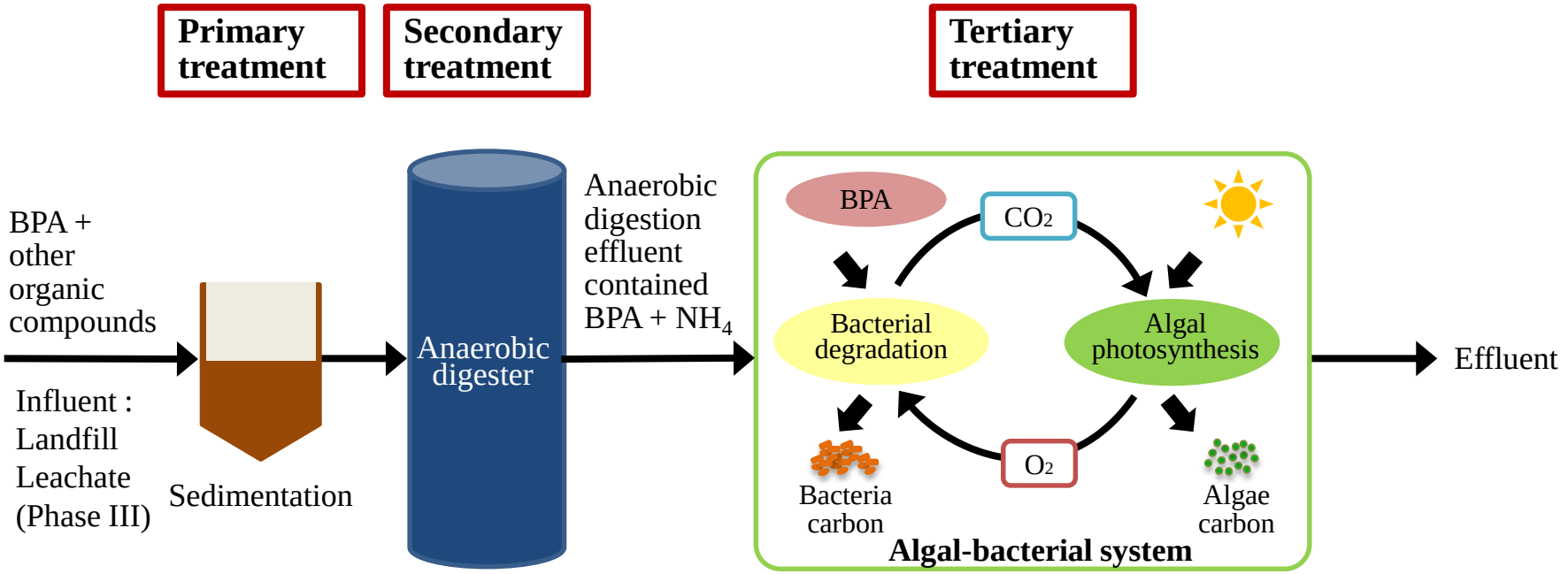


Fig. 4.2. A proposed system for BPA treatment involves an anaerobic digestion and an algal-bacterial system.

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