BIODEGRADATION OF TRICLOSAN BY AEROBIC MICROORGANISMS

A Dissertation

by

DO GYUN LEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Kung-hui Chu
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ABSTRACT

Biodegradation of Triclosan by Aerobic Microorganisms. (August 2012)

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Triclosan, a synthetic antimicrobial agent, is an emerging environmental contaminant. Due to incomplete removal of triclosan by wastewater treatment plants, treated wastewater is one major source of environmental triclosan. Biodegradation of triclosan has been observed in activated sludge and the environment, suggesting that it is possible to develop a cost-effective biotreatment strategy for triclosan removal from wastewater. However, current knowledge on triclosan biodegradation is scarce and limited. To bridge this knowledge gap, this dissertation characterized cultivable triclosan-degrading microorganisms, identified uncultivable triclosan-utilizing bacteria, and elucidated triclosan biodegradation pathways. Furthermore, two treatment strategies were examined to enhance triclosan biodegradation in nitrifying activated sludge (NAS).

A wastewater bacterial isolate, *Sphingopyxis* strain KCY1 (hereafter referred as strain KCY1), can completely degrade triclosan with a stoichiometric release of chloride. This strain can retain its degradation ability toward triclosan when after grown in complex nutrient medium containing triclosan as low as 5 μg/L. Based on five identified metabolites, a *meta*-cleavage pathway was proposed for triclosan

biodegradation by strain KCY1. By using [$^{13}C_{12}$]-triclosan stable isotope probing, eleven uncultured triclosan-utilizing bacteria in a triclosan-degrading microbial consortium were identified. These clones are distributed among α-, β-, or γ-Proteobacteria, suggesting that triclosan-utilizing bacteria are phylogenetically diverse. None of these clone sequences were similar to known triclosan degraders.

Growth substrates affected the triclosan degradation potential of four selected oxygenase-expressing bacteria. Biphenyl-grown *Burkholderia xenovorans* LB400 and propane-grown *Rhodococcus ruber* ENV425 cannot degrade triclosan. On the other hand, propane- and 2-propanol-grown *Mycobacterium vaccae* JOB5 can degrade triclosan completely. Due to product toxicity, finite transformation capacities for triclosan were observed for *Rhodococcus jostii RHA1* grown on biphenyl, propane, and LB medium with dicyclopropylketone (alkane monooxygenase inducer). Four chlorinated metabolites were detected during triclosan degradation by biphenyl-grown RHA1 and a *meta*-cleavage pathway was proposed.

Complete triclosan (5 mg/L) degradation was observed within 96 hrs in NAS receiving ammonia amendment (0 to 75 mg/L of NH4-N). The fastest triclosan degradation was observed in the NAS exhibiting the highest amount of ammonia. When ammonia oxidation was active in NAS, the amendment of strain KCY1 did not further enhance triclosan removal. Overall, the results suggested that triclosan biodegradation can be enhanced by increasing the activity of ammonia oxidation in NAS.

Dedicated to

my beloved parents, my wife, my sister, and my brother

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1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

A public health concern about triclosan has recently emerged due to its widespread occurrence in surface waters, wastewater, sediment, soils, and even in urine, human blood, and breast milk (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Dayan, 2007; Kinney et al., 2008; Kolpin et al., 2002; Morrall et al., 2004; Nakada et al., 2010; Queckenberg et al., 2010; Sandborgh-Englund et al., 2006; Singer et al., 2002). Triclosan is a synthetic broad-spectrum antimicrobial agent. Due to its effectiveness, triclosan has been incorporated into numerous personal care, household, and industrial products since 1965 (Schweizer, 2001; Yazdankhah et al., 2006). Global production of triclosan was estimated to exceed 1500 tons per year, of which more than 43% of the production was used in the US and Europe (Halden and Paull, 2005; Singer et al., 2002). Once triclosan is released into the environment, triclosan can be potentially transformed into more toxic compounds, like chlorodioxins, chlorophenol, chloroform, *p*-hydroquinone, and 2,4-dichlorophenol (Latch et al., 2003) when interacted with strong oxidants or under UV light. Furthermore, trace levels of triclosan could promote the development of cross-resistance to antibiotics (Braoudaki and Hilton, 2004), and cause adverse health effects within the ecosystem (Tatarazako et al., 2004).

This dissertation follows the style of Water Research.

Triclosan is also a weak androgenic and estrogenic compound (Foran et al., 2000; Gee et al., 2008; Ishibashi et al., 2004b).

Through commercial or residential washing of equipment outdoors with triclosan-containing products, triclosan may be transported into the stormwater drain systems; untreated triclosan is released directly into creeks, rivers, and ultimately to the Bay. Moreover, wastewater is a major source of triclosan to the environment to incomplete removal of triclosan by conventional wastewater treatment processes. Researchers have been actively seeking effective treatment strategies for triclosan and this task is challenging. Many advanced physical/chemical processes like UV irradiation, ozonation, sorption, advanced oxidation processes (Behera et al., 2010; Bokare et al., 2010; Jiang et al., 2009; Liyanapatirana et al., 2010; Rafqah et al., 2006; Sanchez-Prado et al., 2006b; Singer et al., 2002; Suarez et al., 2007; Tixier et al., 2002; Zhang and Huang, 2003) have shown effective for the removal of triclosan. However, these techniques are costly and can potentially generate more toxic by-products. On the other hand, rapid biodegradation of triclosan by many fungi, bacterial consortia, activated sludge and pure strains has been reported (Hay et al., 2001; Hundt et al., 2000; Kim et al., 2011; Meade et al., 2001; Roh et al., 2009a; Stasinakis et al., 2010), suggesting that the biological removal of triclosan could be an inexpensive and effective alternative for triclosan removal.

Knowledge of the microbiology of triclosan biodegradation in wastewater is essential for developing an effective biological treatment approach for triclosan. Several studies have shown triclosan biodegradation by nitrifying activated sludge, wastewater

bacteria, and *Nitrosomonas europaea* (Hay et al., 2001; Kim et al., 2011; Roh et al., 2009a; Stasinakis et al., 2010). However, these studies only revealed a small fraction of microorganisms that are capable of degrading triclosan in wastewater, and did not investigate the degradative enzymes, or degradation pathways for triclosan. Recently, in Chu's laboratory, a triclosan-degrading bacterium, named strain KCY1, was isolated from activated sludge. The strain KCY1 showed rapid cometabolic degradation of triclosan at a high concentration (5 mg/L). In this study, I used strain KCY1 as a model strain to quantify its degradation ability toward triclosan and investigated the pathway used for triclosan degradation. Meanwhile, I also attempted to use both culture-dependent and culture-independent methods to investigate and identify triclosan-degrading cultures in wastewater.

Ammonia-oxidizing bacteria (AOB) are an important group of microorganisms responsible for nitrification, nutrient removal process in wastewater treatment. The oxidation of ammonia to nitrite is catalyzed by ammonia monooxygenase (AMO) expressed by AOB. A study reported that *amoA* gene encodes the membrane-associated active-site polypeptide of the AMO from *Nitrosomonas europaea*, a representative AOB (McTavish et al., 1993). AMO has a wide substrate specificity and is known to degrade a wide range of environmental pollutants (Chang et al., 2002; Hyman et al., 1988; Keener and Arp, 1994; Rasche et al., 1991). Roh et al. (Roh et al., 2009a) reported that both nitrifying activated sludge and AOB can degrade triclosan, suggesting that AMO might be responsible for triclosan biodegradation. Many other bacterial oxygenases, including monooxygenase and dioxygenase, are also known to oxidize a wide variety of

xenobiotic compounds (Mahendra and Alvarez-Cohen, 2006; Robrock et al., 2011; Sharp et al., 2007; Sharp et al., 2005; Sinha et al., 2009; Spain and Nishino, 1987). It is possible that different oxygenases are capable of degrading triclosan. Thus, in this study, I investigated the degradation ability toward triclosan by a selected set of oxygenase-expressing cultures. A new degradation pathway for triclosan by one of the oxygenase-expressing cultures surveyed was also elucidated.

Bioaugmentation of activated sludge with specialized bacteria could be a powerful tool to enhance the removal of undesired contaminants (McLaughlin et al., 2006; Van Limbergen et al., 1998). Since bioaugmentation of activated sludge with the addition of specialized strains does not always work due to less predictable and uncontrollable problems (Goldstein et al., 1985; Van Limbergen et al., 1998), only a few successful bioaugmentation studies using activated sludge with special microorganims have been reported (Boon et al., 2000; Hajji et al., 2000; McClure et al., 1991). In this study, I examined whether the bioaugmentation of nitrifying activated sludge with strain KCY1 could enhance the removal efficiency of triclosan in wastewater. To investigate the effects of inoculation with strain KCY1 on triclosan degradation in nitrifying activated sludge, changes in microbial populations of triclosan-degrading bacteria (strain KCY1 and AOB), and *amoA* gene in nitrifying activated sludge were investigated using real-time PCR assays.

1.2. Goal, objectives, and hypotheses

The ***overall goal*** of this research is to gain a better understanding of microorganisms capable of degrading triclosan and the enhanced biological removal of triclosan in the environment. To accomplish this goal, experiments were performed to achieve the following four specific objectives:

Objective 1: Identify cultivable and uncultivable triclosan-degrading bacteria in wastewater.

Hypothesis: Wastewater contains many different triclosan-degrading bacteria, both cultivable and uncultivable.

Task 1a: Isolate, identify and quantify cultivable triclosan-degrading bacteria from wastewater

Task 1b: Apply molecular methods to identify uncultivable triclosan-degrading bacteria

Objective 2: Characterize a newly isolated triclosan-degrading culture, strain KCY1.

Hypothesis: Strain KCY1 can degrade triclosan effectively in wastewater

Task 2a: Determine triclosan degradation kinetics

Task 2b: Investigate its ability to degrade or utilize common wastewater organics

Task 2c: Examine factors influencing triclosan degradation

Task 2d: Determine androgenicity/estrogenicity of triclosan degradation products

Task 2e: Identify triclosan degradation metabolites and determine degradation pathways

Objective 3: Demonstrate triclosan biodegradation by different oxygenase-expressing cultures.

Hypothesis: Bacteria expressing mono- and di-oxygenases can cometabolize triclosan.

Task 3a: Examine triclosan biodegradation by model propane-monooxygenase-expressing bacteria

Task 3b: Examine triclosan biodegradation by model biphenyl-dioxygenase-expressing bacteria

Task 3c: Identify triclosan degradation metabolites and determine degradation pathways

Objective 4: Investigate the effects of ammonia amendment and bioaugmentation with strain KCY1 on triclosan biodegradation in nitrifying activated sludge

Hypothesis: Ammonia amendment and bioaugmentation with strain KCY1 in nitrifying activated sludge can enhance triclosan biodegradation.

Task 4a: Examine triclosan biodegradation by nitrifying activated sludge amended with the different concentrations of ammonia

Task 4b: Examine triclosan biodegradation by nitrifying activated sludge augmented with strain KCY1

Task 4d: Examine triclosan biodegradation by nitrifying activated sludge amended with ammonia and strain KCY1

Task 4e: Investigate the temporal changes in the abundance of strain KCY1, AOB and *amoA* gene in nitrifying activated sludge after the amendment with ammonia and/or strain KCY1

1.3. Dissertation overview

This dissertation is organized into seven sections. The research hypothesis and specific objectives of this study are outlined in **Section 1**. The literature review for this research is described in **Section 2**. In **Section 3**, the isolation and characterization of a wastewater triclosan-degrading bacterium, *Sphingopyxis* strain KCY1 is addressed. The research described in Section 3 has been published in (Lee et al., 2012). The identification of triclosan-degrading wastewater microorganisms using [^{13}C]-stable isotope probing is present in **Section 4**. Most of research described in this section has been submitted to *Applied Microbiology and Biotechnology* for publication. The ability of oxygenase enzymes in catalyzing the biodegradation of triclosan is described in **Section 5**. In **Section 6**, the effects of ammonia amendment and bioaugmentation with strain KCY1 on triclosan biodegradation in nitrifying activated sludge, and the abundance of strain KCY1, ammonia-oxidizing bacteria, and *amoA* gene were investigated. Manuscripts containing portions of the materials presented in Section 5 and 6 are in preparation for submission. Finally, **Section 7** summarizes key conclusions of this research and recommendations for future study.

2. LITERATURE REVIEW

2.1. Chemical property and regulation

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; CAS 3380-34-5) is a nonionic, lipophilic, and chlorinated phenolic compound that has been used as a synthetic broad-spectrum antimicrobial agent, since the Ciba Specialty Chemicals (Basel, Switzerland) first produced triclosan in 1965 (Schweizer, 2001; Yazdankhah et al., 2006). Due to its effective antimicrobial ability, triclosan has been widely incorporated into numerous personal care and household products, and industrial products such as deodorants, soaps, skin cream, toothpastes, laundry detergents, socks, sport footwear, toys, fabrics, computer keyboards, countertops, and many different plastic kitchenware all over America, Europe and Asia. It has been calculated that triclosan has been globally produced over 1500 tons per year, especially, more than 300 tons and more than 350 tons of triclosan are yearly used in the US and in Europe, respectively (Halden and Paull, 2005; Singer et al., 2002). Triclosan is referred to as a halogenated biphenyl ether due to functional groups of ether and phenol. Alternative names for triclosan include 2,4,4'-trichloro-2'-hydroxydiphenyl ether, 5-chloro-2-(2,4-dichlorophenoxy)phenol or trichloro-2'-hydroxydiphenyl ether. In addition, triclosan has commercial names such as Irgasan DP-300, Lexol 300, Ster-Zac, Cloxifenolum, Microban in plastics and clothing, and Biofresh in acrylic fibers (Adolfsson-Erici et al., 2002). The chemical formula for triclosan is $C_{12}H_7Cl_3O_2$ and its molecular weight is 289.55 g/mol. Triclosan is white to off-white crystalline powder with a hardly detectable

phenolic odor. Triclosan is thermally stable; it has a melting point between 56-60 °C and a boiling point between 280-290 °C (Kroon and van Ginkel, 2001). Triclosan is relatively non-volatile and non-soluble in water (solubility: 10 mg/L at 20 °C; log K_{ow} = 4.76), but readily soluble in a wide range of organic solvents (Bhargava and Leonard, 1996; Kroon and van Ginkel, 2001). The chemical structure of triclosan (Figure 2.1) is similar to several emerging contaminants such as polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), dioxine, bispenol A, diethylstilbestrol (synthetic nonsteroidal estrogen), and thyroxine (thyroid hormone) (Allmyr et al., 2008; Crofton et al., 2007; Veldhoen et al., 2006). Triclosan has been banned in Canada and Japan. In the European Union, triclosan is categorized as a dangerous irritant to the environment and aquatic life (Winter, 1994). The use of triclosan in food contact plastics was banned in September 2009 in Germany. It has been advised to consumers not to use antibacterial products in Finland and Denmark (Fuerhacker and Haile, 2011). In the U.S., the non-pesticide use of triclosan is regulated by the U.S. Food and Drug Administration (FDA) and the pesticide use of triclosan is regulated by the Environmental Protection Agency (EPA). There is no established regulation for triclosan in surface waters yet.

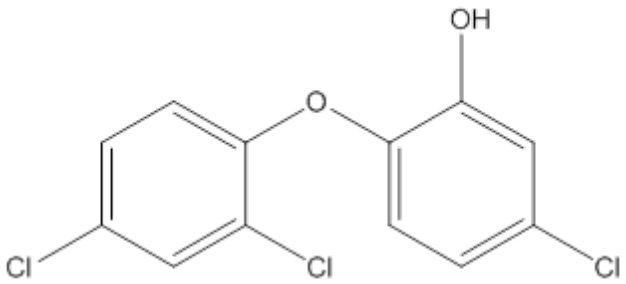

Figure 2.1. Chemical structure of triclosan

2.2. Toxicology

2.2.1. Toxicity of triclosan

Triclosan has a low level of toxicity to humans and other mammals (Bhargava and Leonard, 1996; Rodricks et al., 2010). Triclosan showed a low acute toxicity in animal studies that the dermal LD_{50} (median lethal dose) is 5000 mg/kg for rats, and the oral LD_{50} are 4000 mg/kg for mice, 4500-5000 mg/kg for rats, and over 5000 mg/kg for dogs (Kroon and van Ginkel, 2001). One study in male mice also reported that triclosan showed a low acute toxicity (LD_{50} > 1000 mg/kg) (Kanetoshi et al., 1992). However, several studies reported that triclosan is acutely and chronically toxic to several different types of aquatic life such as fish and algae in the range of 34.2 – 200 μg/L (Capdevielle M and Inauen J, 2008; Dussault et al., 2008; Ishibashi et al., 2004a; Orvos et al., 2002). (Ricart et al., 2010) reported that toxicity of triclosan to biofilm algae and bacteria caused an increase of bacterial mortality with a No Effect Concentration (NEC) of 0.21 μg/L and triclosan was more toxic to bacteria than algae.

Also, as shown in two fish studies, the acute toxicity of triclosan depended on the different life stages of species, especially, showing more toxic effects on juveniles (Nassef et al., 2009; Oliveira et al., 2009).

2.2.2. Triclosan as a class of endocrine disrupting compounds (EDCs)

PCBs and PBDEs are known thyroid disrupting compounds and several studies reported that triclosan is a thyroid disruptors in animals due to the structural similarity of triclosan to thyroid hormones such as thyroxine (Allmyr et al., 2008; Crofton et al., 2007; Norris DO, 2006; Veldhoen et al., 2006). Damage caused by disrupting thyroid systems may affect normal growth and development of humans, especially, the development of Children's brain. Using frogs that have a primary thyroid hormone-signaling mechanism which is almost identical to human's, two studies showed that low levels of triclosan interfered with thyroid-mediated developmental processes of tadpoles into frogs (Fort et al., 2011; Fort et al., 2010; Veldhoen et al., 2006). Further, triclosan may disrupt other critical hormone systems in mammals due to the structural similarity to anthropogenic estrogens (bisphenol A and diethylstilbestrol), and the anti-estrogen (2,3,7,8 tetrachloro-*p*-dioxin) (Jacobs et al., 2005). An *in vitro* study showed that triclosan exerted androgenic and estrogenic activities in human breast cancer cells (Gee et al., 2008). Two i*n vivo*-fish studies suggested that triclosan may act as anti-estrogen (Matsumura et al., 2005) or weak androgen (Foran et al., 2000). Several male rat studies demonstrated that triclosan lowered serum levels of testosterone and several steroidogenic enzyme activities (Kumar et al., 2009; Zorrilla et al., 2009). One study

also reported that a triclosan metabolite may have a weak estrogenic activity (Ishibashi et al., 2004a).

2.2.3. Development of antibiotic cross-resistance

Several studies raised a concern that triclosan may promote the development of cross-resistance to antibiotics in microorganism and lead to the emergence of bacteria resistant to antibiotics in the environment. While antimicrobial agents are generally not intended to destroy particular cellular constituents in bacteria, antibiotics attack specific cellular target to inhibit the growth of cell and the synthesis of cell wall or cell contents (Jones et al., 2000). Because triclosan kills bacteria in a similar way as antibiotics by inhibiting the active sites of the specific bacterial fatty acid biosynthetic enzyme, *enoyl-acyl* carrier protein reductase, which is necessary to build and reproduce cell membranes (McMurry et al., 1998), bacteria that become resistant to triclosan may have the potential of antibiotic resistance. A laboratory study demonstrated that cross-resistance between triclosan and ciprofloxacin (oral antibiotic) was found in *Pseudomonas aeruginosa* (Russell, 2003). Actually, there is no evidence that triclosan causes a mutation in bacteria. However, since triclosan kills normal bacteria that are not resistant to triclosan in the environment, mutated bacteria that are resistant to triclosan are more likely to survive and reproduce in the triclosan contaminated environment. Laboratory studies found that a number of different strains of mutant bacteria that are resistant to triclosan also showed the resistance to a number of clinical-use antibiotics (Levy, 2001).

2.3. Source, fate and transport in the environment

Disposal and usage of triclosan-laden products resulted in triclosan-containing wastewater. Triclosan is released into the environment via effluent discharge to surface water or via biosolid applications into soils (Morrall et al., 2004; Singer et al., 2002).

The environmental triclosan can be transformed into more toxic products through photodegradation and/or biodegradation. Under anaerobic conditions, methylation of triclosan into methyl-triclosan in surface waters and wastewater has been reported (Lindström A, 2002). Due to the lipophilic property of methyl-triclosan (K_{ow} = 5.2), it may bioacummulate in wildlife and human. A fish study found the concentrated methyl-triclosan in fatty tissue (Balmer et al., 2004) and another study reported that methyl-triclosan has acute toxicities in the marine bacterium *Vibrio fischeri* at low levels of triclosan (Farre et al., 2008). A great concern about the rapid transformation of triclosan into 2,8-dichlorodibenzo-*p*-dioxin (2,8-DCDD) during the photo-degradation in natural water and wastewater has been raised (Buth et al., 2009; Latch et al., 2003; Latch et al., 2005; Lores et al., 2005; Mezcua et al., 2004; Sanchez-Prado et al., 2006a) because dioxin is highly carcinogenic and can cause such severe health problems as affecting reproductive abilities and weakening the immune system (Mason G, 1986). Triclosan can be transformed into chlorophenols (2,4-dichlorophenol and 2,4,6-trichlorophenol), which are listed as priority pollutant by EPA, and are potentially toxic and stable in the environment (Canosa et al., 2005), in the presence of chlorine (Canosa et al., 2005; Fiss et al., 2007; Rule et al., 2005) or chloramines (Greyshock and Vikesland, 2006). Recent

studies have shown that triclosan in tap/surface water or in common triclosan-containing hygienic products reacts with residual chlorine to form potentially carcinogenic chloroform (Fiss et al., 2007; Greyshock and Vikesland, 2006; Rule et al., 2005).

Triclosan has been detected in the aquatic environment including surface waters, wastewater, activated sludge, lake, marine, and river sediments (Aguera et al., 2003; Chalew and Halden, 2009; Kolpin et al., 2002; McAvoy et al., 2002; Miller et al., 2008; Morales et al., 2005; Morrall et al., 2004; Nakada et al., 2010; Singer et al., 2002), in agricultural soils, and even in soybean plants (Wu et al., 2010). Because triclosan is primarily a water-borne contaminant, it is detected ubiquitously in aquatic environments at some of the highest concentrations among 95 organic wastewater pollutants (Kolpin et al., 2002). About 96% of household and consumer products containing triclosan eventually flows down the drain (Reiss et al., 2002) and small amounts of triclosan is still discharged with treated-water effluent into receiving water systems (Morrall et al., 2004; Nakada et al., 2010; Singer et al., 2002). Through commercial or residential washing of equipment outdoors with triclosan-containing products, runoff containing triclosan goes into stormwater drain systems without treatment, and flows directly into creeks, rivers and eventually to the bay or oceans.

Consumer product wastes containing triclosan, and sludges from wastewater treatment plants are mostly sent to landfill for disposal, and triclosan can be released into the environment through landfill leachate (NICNAS, 2009). According to a recent USGS study, approximately 58 % of 139 U.S. streams were contaminated with triclosan, with concentrations ranging from 0.14 μg/L to 2.3 μg/L (Kolpin et al., 2002). Triclosan

concentrations in activated sludge have been detected in the range of 500 to 15600 μg/kg of dry weight (McAvoy et al., 2002; Morales et al., 2005). Triclosan concentrations in wastewater influent range from 1.86 to 26.8 μg/L, and triclosan in wastewater effluents are detected at the concentrations between 0.027 and 2.7 μg/L (Chalew and Halden, 2009; McAvoy et al., 2002; Morrall et al., 2004; Nakada et al., 2010; Singer et al., 2002). Also triclosan was found in lake, marine, and river sediments at the concentrations in the range from 37 to 53 ng/g, from 0.27 to 130.7 ng/g, and from 4.4 to 35.7 ng/g, respectively (Aguera et al., 2003; Miller et al., 2008; Singer et al., 2002).

Due to the application of biosolids to an agricultural field and irrigation of treated wastewater contaminated with triclosan, triclosan has been observed in agricultural areas (Cha and Cupples, 2009; Lozano et al., 2010). Of the triclosan entering wastewater treatment plants (WWTPs), 15% was adsorbed onto biosolids, 79% was removed by biodegradation, and the remaining 6% was discharged continuously into the receiving surface water (Morrall et al., 2004; Singer et al., 2002). Triclosan in biosolids have been detected at the concentrations between 90 and 30,000 μg/kg (Chu and Metcalfe, 2007; Kinney et al., 2008; Lee and Peart, 2002; Morales et al., 2005; Ying and Kookana, 2007). Triclosan was also detected at concentrations ranging from 69 to 833 ng/g in agricultural soil amended with biosolids (Kinney et al., 2008). Further, the source of triclosan in vegetation contributed from the biosolid application to an agricultural field and the irrigation of treated wastewater containing triclosan (Cha and Cupples, 2009; Lozano et al., 2010). One field study reported that triclosan in soybean plants was detected at concentrations between 36 and 80 ng/g of dry weight from irrigation treatment samples,

and between 13 and 136 ng/g of dry weight from biosolids application treatment samples, respectively (Wu et al., 2010).

Furthermore, several studies reported that triclosan was detected in 75% of 2517 human urine samples at concentrations of 2.4–3790 μg/L (Calafat et al., 2008) and in the 61% of 90 urine samples from age 6-8 year-old girls (Wolff et al., 2007). Triclosan was also detected in human blood (Allmyr et al., 2006; Allmyr et al., 2008; Dirtu et al., 2008; Sandborgh-Englund et al., 2006). For example, triclosan was detected in the range between 4.1-19 ng/g in blood serum samples (Allmyr et al., 2008). A recent study showed that triclosan in the range of 100-2100 μg/kg of lipid was detected in the 96.8% of 62 samples of breast milk (Dayan, 2007) and concentrations of triclosan in breast milk ranged from 0.018 to 0.95 ng/g (Allmyr et al., 2006). Moreover, triclosan was found in indoor dusts (~ 1.1 μg/g) (Canosa et al., 2007), and foods (0.02-0.15 ng/g) such as dairy products, meat, fish and egg (Adolfsson-Erici and Allmyr, 2007).

2.4. Treatment technologies for triclosan removal

2.4.1. Physical/Chemical treatment technologies

A granular activated carbon (GAC) treatment system was effective at decreasing 2032 ng/L of influent triclosan concentration to 102 ng/L of triclosan in effluent (Hernandez-Leal et al., 2011). Powdered activated carbon achieved a 95% reduction in triclosan during drinking water treatment (Westerhoff et al., 2005). Different sorbents like kaolinite and montmorillonite are effective for triclosan removal (Behera et al., 2010). Advanced membrane technologies such as nanofiltration/reverse osmosis

membranes are effective for triclosan removal, but more expensive than other treatment options (Nghiem and Coleman, 2008). 60% of initial triclosan in domestic wastewater was removed by ultrasonication (Sanchez-Prado et al., 2008). However, conventional sand filtration showed no removal of triclosan (Lundstrom et al., 2010).

Triclosan can be effectively removed by chemical oxidation processes such as UV irradiation, ozonation, UV/hydrogen peroxide-advanced oxidation process, and chlorination. One study reported that 93% of initial triclosan was removed by UV irradiation in wastewater (Sanchez-Prado et al., 2006b). Ozonation can rapidly oxidize triclosan during wastewater treatment (Suarez et al., 2007). UV/hydrogen peroxide-advanced oxidation process showed high removal efficiency of triclosan (Yu et al., 2006). Triclosan was degraded during chlorination at neutral pH (7.3-8.3) (Canosa et al., 2005). UV light in combination with TiO_2 achieved a 75-82% reduction in triclosan, whereas 30% of triclosan was removed by TiO_2 as a sole oxidant (Zoh et al., 2009). Triclosan was almost completely removed within 60 min by Fe(II)/UV (Zoh et al., 2010). Oxidants such as manganese oxides (δ-MnO_2 and MnOOH), titanium dioxide, permanganate (Mn(VII)), and Fe(III)-saturated montmorillonite have shown to oxidize triclosan effectively (Jiang et al., 2009; Liyanapatirana et al., 2010; Rafqah et al., 2006; Sanchez-Prado et al., 2008; Suarez et al., 2007; Zhang and Huang, 2003).

2.4.2. Biological treatment technologies

Several studies reported the removal of triclosan in wastewater treatment plants (WWTPs) by biological treatment process (McAvoy et al., 2002; Singer et al., 2002; Thompson et al., 2005; Yu and Chu, 2009). The removal efficiency of triclosan is dependent on the operations and configurations of WWTPs. Thompson et al. reported that overall removal of triclosan ranged between 95-98% (activated sludge), 86-97% (trickling filter), and 58-96% (rotating biological contactors) (Thompson et al., 2005). McAvoy et al. found that triclosan was removed with rates of 96 %, 71 %, and 32%, for activated sludge plants, trickling filter plants, and primary treatment plants, respectively (McAvoy et al., 2002). In general, activated sludge treatment enables the greatest removal of triclosan. One study estimated that 79% and 15% of triclosan was removed by biodegradation and adsorption onto biosolids, respectively, and then the remaining 6% of triclosan was discharged into the receiving water (Singer et al., 2002).

Several studies demonstrated the aerobic biodegradation of triclosan by wastewater enrichment consortium (Hay et al., 2001; Stasinakis et al., 2010), nitrifying activated sludge (Roh et al., 2009a), wastewater microorganisms (*Sphingomonas* sp. Rd1 and *Sphingomonas* sp. PH-07) (Hay et al., 2001; Kim et al., 2011), soil microorganisms (Meade et al., 2001), and two white rot fungi (*Trametes versicolor* and *Pycnoporus cinnavarinus*) (Hundt et al., 2000). 0.15-0.18 μg/mL of triclosan was degraded cometabolically by all these known strains and cultures. The two soil bacteria, *Pseudomonas putida* TriRY and *Alcaligenes* x*ylosoxidans* subsp. *denitrificans* TR1 utilized triclosan as a carbon source and degraded it within 4 days and 10 days (Meade et

al., 2001). A recent study suggested that a diphenyl-ether degrading bacterium, *Sphingomonas* sp. PH-07 may use 2,3-dioxygease enzyme to cometabolically degrade 25% of 10 mg/L triclosan in 8 days and thus proposed a possible degradation pathway for triclosan (Kim et al., 2011). Nitrifying activated sludge and an ammonia-oxidizing bacterium (AOB), *Nitrosomonas europaea,* were found to degrade triclosan cometabolically (Roh et al., 2009a). 45% of 1 mg/L triclosan was degraded by *Nitrosomonas europaea* in 24 hours. This study demonstrated the involvement of ammonia monooxygenase in *Nitrosomonas europaea* in triclosan biodegradation.

Triclosan in aerobic condition shows relatively rapid degradation; however, triclosan is slowly degraded and persistent under anaerobic condition. Anaerobic degradation of triclosan was not observed in 70 days (Ying et al., 2007). Similarly, a study reported remaining of 91% of 14C-triclosan after incubation under anaerobic conditions for 147 days (Lundstrom et al., 2010). A recent study did not observe anaerobic triclosan biodegradation, but 75% of triclosan removal by aerobic activated sludge after 150-hr incubation (Chen et al., 2011).

2.5. Culture-independent methods

2.5.1. Real-time PCR assay

Information about the abundance of triclosan-degrading bacteria in current wastewater treatment plants is important to assess the triclosan biodegradation potential in wastewater. Real-time PCR assay is an easy, fast, and highly specific culture-independent method that has been used to quantify target gene (in our case the 16S

rRNA gene). The method relies on the detection of real-time fluorescence that was emitted due to amplicon products at the end of each PCR cycle (Gibson et al., 1996; Higuchi et al., 1992). Increase of PCR products with each cycle of amplification corresponds to the increase in fluorescence signal, resulting from fluorescence dye binding to the double-stranded DNA (i.e. SYBR Green) or sequence specific probes (i.e. *Taq*Man Probes) (Chen et al., 1997; Morrison et al., 1998). The initial amount of target gene is correlated to the first significant increase in fluorescence signals. If a sample has a higher starting copy number of target gene, a significant increase in fluorescence (i.e. above background) will be detected earlier. Then the copy number of the target gene can be quantified by comparing between significant increase in fluorescence in target gene and in standard curves constructed a wide of known copy numbers in templates.

In this study, a real-time PCR assay by designing primers/probes specific to a target triclosan-degrading culture, strain KCY1 was developed (Task 1a). The data of the copy number of the target gene was used to determine the abundance of triclosan-degrading bacteria.

2.5.2. Quantitative assay for linking microbial community function and structure (Q-FAST) assay

To indentify and quantify active microorganisms responsible for triclosan biodegradation in a given microbial community, a culture-independent and quantitative assay called Q-FAST will be used in this study. As shown in Figure 2.2, Q-FAST, short for linking microbial community function and structure, can simultaneously indentify

and quantify active microorganisms responsible for utilizing specific compounds in a particular microbial community (Yu and Chu, 2005). Q-FAST is a combination of stable isotope probing (SIP) technique and a real-time terminal restriction fragment polymorphism (real-time-T-RFLP) assay. SIP is a method to investigate the metabolic activity of microorganisms responsible for the biodegradation of contaminants by assimilating isotopically labeled compounds into the nucleic acids of microbial communities (Murrell et al., 2000). SIP using DNA is applied primarily with ^{13}C or ^{15}N-labeled compounds (Dumont and Murrell, 2005; Friedrich, 2006; Meselson and Stahl, 1958; Radajewski et al., 2002). Real-time-T-RFLP, a combination of real-time PCR and T-RFLP, is a quantitative fingerprinting method to simultaneously determine the composition of microbial community and the abundance of specific microbial groups (Yu and Chu, 2005). T-RFLP is a molecular method to generate distinct profiles of a microbial community by digesting fluorescently end-labeled PCR products (in our case the 16S rRNA gene) and detecting a series of terminal restriction fragment (T-RF) profiles on an automated sequence analyzer (Kitts, 2001; Liu et al., 1997). The results of Q-FAST for Task 1b (as described below) was beneficial to identify bacteria capable of utilizing triclosan as a sole carbon source, and simultaneously to analyze microbial community structure.

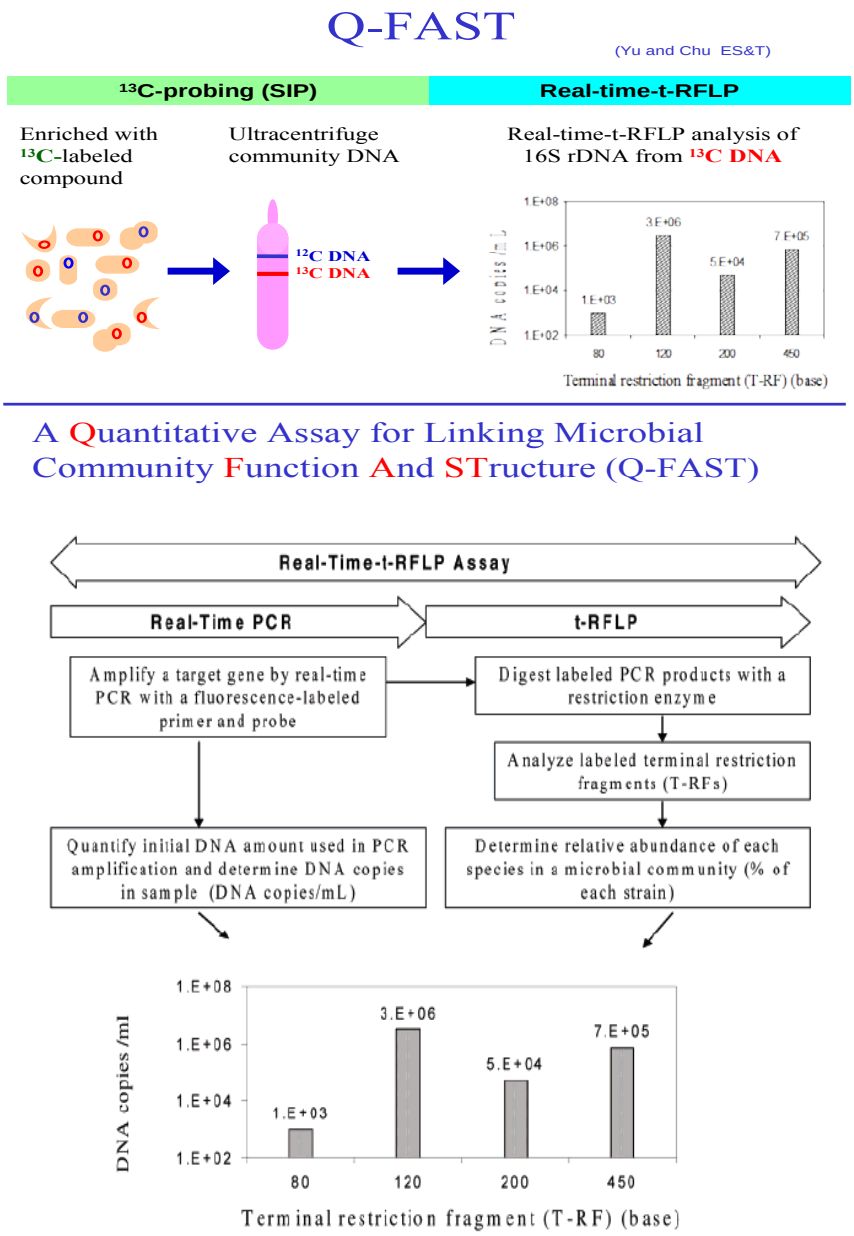

Figure 2.2. Illustration of quantitative functional assay Q-FAST for linking microbial community function and structure. Cited from (Yu and Chu, 2005).

2.6. *Saccharomyces cerevisiae* Bioluminescent Yeast Estrogen and Androgen Assays (BLYES and BLYAS)

Due to the androgenicity and estrogenicity of triclosan, it is important to assess androgenic and estrogenic potential of triclosan biodegradation metabolites/end products in samples. Two yeast-based bioluminescent assays, *S. cerevisiae* BLYES and *S. cerevisiae* BLYAS, can quickly detect the quantifiable bioluminescence signal in response to either an estrogenic or androgenic compound (Arnold, 1996) (Figure 2.3). BLYES has two plasmids, pUTK404 containing *P. luminescens* aldehyde synthesis genes (*luxCDE*) and the *Vibrio harveyi* flavin oxidoreductase gene (*frp*), and pUTK407 containing *P. luminescens luciferase* genes (*luxAB*), respectively. The human estrogen receptor (*hER*) gene is located on the chromosome. When BLYES is exposed to an estrogenic compound, this chemical binds to the human estrogen receptor. This complex binds to the EREs on pUTK407, and then transcription of the *luxAB* genes begins. This genetic expression is incorporated with the *luxCDE* genes and produces a measurable bioluminescence signal (Sanseverino et al., 2005). Similarly, BLYAS have the human androgen receptor (*hAR*) gene in the chromosome, instead of *hER* gene in BLYES (Eldridge et al., 2007). The bioluminescence assay was used in Task 2d to assess androgenic and estrogenic potential of triclosan biodegradation samples.

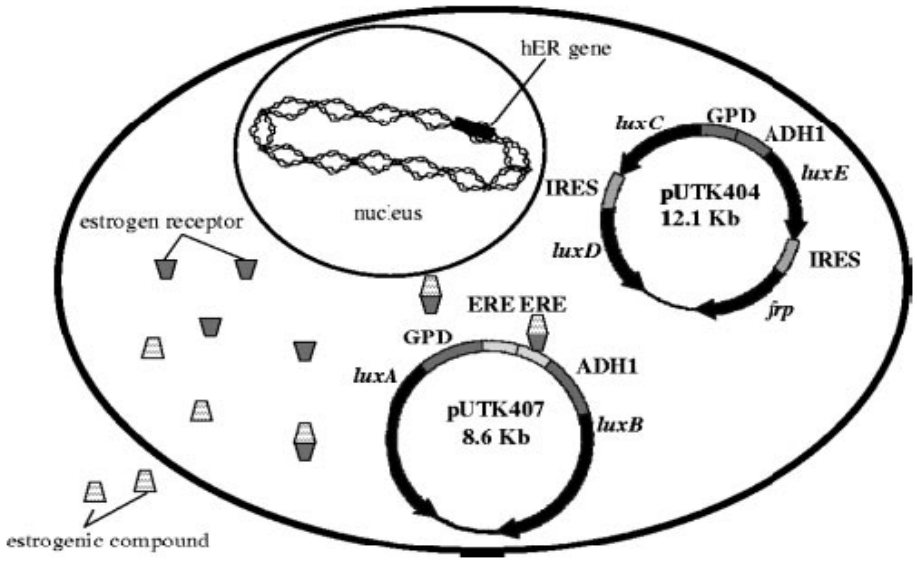

Figure 2.3. Schematic representation of *S. cerevisiae* BLYES. Cited from (Sanseverino et al., 2005).

3. BIODEGRADATION OF TRICLOSAN BY A WASTEWATER MICROORGANISM, SPHINGOPYXIS STRAIN KCY1*

3.1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a common synthetic antimicrobial agent that has been incorporated into more than 700 different industrial and personal care products. These products, including deodorants, soaps, toothpastes, and various plastic products, contain 0.1 - 0.3% triclosan (Sabaliunas et al., 2003; Schweizer, 2001; Singer et al., 2002). Not surprisingly, triclosan, ranging from 0.14 to 2.3 μg/L, was detected in 58% of 139 U.S. streams (Kolpin et al., 2002). The widespread of triclosan in the environment has raised a concern, because the trace level of triclosan might promote the development of antimicrobial-resistant microorganims (Braoudaki and Hilton, 2004) and cause adverse effects on the ecosystem (Tatarazako et al., 2004). When exposed to UV, triclosan can potentially be transformed into more toxic chemicals like chlorodioxins (Latch et al., 2003; Rule et al., 2005). Triclosan has shown weak androgenic activity in aquatic species (Foran et al., 2000) and both estrogenic and androgenic responses in human breast cancer cells (Gee et al., 2008), suggesting triclosan itself is an endocrine-disrupting compound.

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*Reprinted from Water Research, 46 (13), Lee, D.G., Zhao, F., Rezenom, Y.H., Russell, D.H., Chu, K.H. Biodegradation of triclosan by a wastewater microorganism, 4266-4234.

Biodegradation of triclosan in the environment and wastewater has recently become an interesting research topic (Hay et al., 2001; Kim et al., 2011; Meade et al., 2001; Roh et al., 2009a; Sabaliunas et al., 2003; Schweizer, 2001; Singer et al., 2002). A previous study reported that approximately 79% of triclosan was removed by biological wastewater treatment processes (Singer et al., 2002), suggesting that (i) biodegradation can be an important removal mechanism in wastewater and (ii) triclosan-degrading bacteria are present in the activated sludge. These two aspects were supported by two later studies. For example, biodegradation of triclosan was observed for two wastewater microorganisms, *Sphingomonas* sp. Rd1 (Hay et al., 2001) and *Nitrosomonas europaea* (Roh et al., 2009a), and by nitrifying activated sludge (Roh et al., 2009a). Still, knowledge about wastewater microorganisms capable of degrading triclosan is limited. Recently, a known diphenyl ether degrader, *Sphingomonas* sp. PH-07, showed an ability to partially degrade triclosan and produce three metabolites (hydroxylated triclosan, 4-chlorphenol, and 2,4-dichlorophenol) (Kim et al., 2011). To date, no complete dechlorination of triclosan has been observed and triclosan degradation kinetics and pathways still remain unclear.

In this study, the isolation and characterization of a wastewater triclosan-degrading bacterium, *Sphingopyxis* strain KCY1 was reported. This isolate showed complete dechlorination of triclosan based on stoichiometric release of chloride. We also determined triclosan degradation kinetics, a proposed possible degradation pathway for triclosan, and assessed the potential significance of this isolate to triclosan biodegradation in wastewater.

3.2. Material and methods

3.2.1. Chemicals

Triclosan (TCS) (97% pure) was purchased from Aldrich Chemical Inc. (Milwaukee, WI). Dimethylformanide (DMF) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Biotechnology Inc. (Rockford, IL). Diphenyl ether (DE), 2,4,4'-Tribromodiphenyl ether (tri-BDE) and 2,2',4,4'-tetrabromodiphenyl ether (tetra-BDE) were purchased from Accustandard (New Haven, CT) (Figure 3.1). 3-Fluorocatechol was purchased from Alfar Aesar (Ward Hill, MA). Fast DNA kit was purchased from Q-Biogene (Carlsbad, CA). Stock solution of 1 g/L triclosan was prepared in acetone.

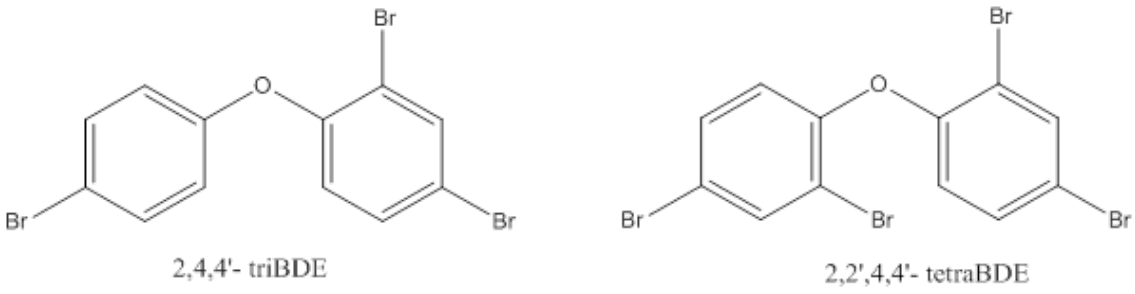


Figure 3.1. Chemical structure of tri-BDE and tetra-BDE

3.2.2. Isolation and identification of triclosan-degrading bacteria

A triclosan-degrading consortium, originally inoculated with activated sludge, was used as a source for the isolation of triclosan-degrading bacteria. Briefly, a loopful of the consortium was streaked onto nitrate mineral salts (NMS)-triclosan (5 mg/L) agar plates that were incubated at 30 oC (Chu and Alvarez-Cohen, 1996). Due to slow and poor growth of colonies on the NMS-triclosan agar plates, colonies with different morphology were picked and re-streaked onto 1/10-strength R2A-triclosan agar plates (containing 5 mg/L of triclosan, 10% of R2A broth, and 10.8 g/L BBL selected agar). Only one of three isolates, named strain KCY1, was later confirmed with an ability to degrade triclosan in liquid medium. The 16S rRNA gene of strain KCY1 was sequenced as previously described (Yu et al., 2007). The sequence was aligned with other known triclosan-degrading bacteria using Clustal X2. A phylogenetic tree was constructed using TreeView software. The GenBank accession number for 16S rRNA sequence of strain KCY1 was DQ983313.2.

Detailed descriptions of enrichment, isolation, and confirmation of triclosan degradation ability of this isolate are described as follows. Five mL of the activated sludge was added into a 250 mL flask containing 95 mL of nitrate mineral salt (NMS) medium (Chu and Alvarez-Cohen, 1996) and 5 mg/L of triclosan. The flask was incubated on a rotary shaker (150 rpm) at 30 ^{o}C. After 20 days of initial incubation, 10 mL of the cell suspension was transferred to a new flask containing the same growth medium. Every 10 days, the enrichment culture was transferred to a new flask and this procedure was repeated four times. The specific degradation rate of triclosan of the

enrichment culture increased from 0.03 to 0.17 nmol/min/mg of protein. One loopful of the consortium was streaked onto noble agar plates containing NMS medium and 5 mg/L of triclosan. The plates were incubated at 30 ^{o}C. Since the colonies grew slowly on the NMS-triclosan noble agar plates, these colonies were picked and re-streaked onto 1/10-strength R2A agar plates containing triclosan (5 mg/L triclosan, 10% of R2A broth, and 10.8 g/L BBL selected agar). After numerous streaking, three colonies with different morphology were selected to examine their ability to degrade triclosan in NMS medium containing 5 mg/L of triclosan (triclosan was used as a sole carbon source). The concentrations of biomass were determined as optical density at absorbance 600 nm using UV-visible spectrophotometer (HP G1130A) and as protein content using a BCA protein assay kit, respectively.

Cell morphology was examined by transmission electron microscopy (TEM) performed with JEOL 1200EX equipped with the SIA-15C camera (JEOL, Tokyo Japan), using the exponentially growing cells. Strain KCY1 was prepared with the gram-stain, and then observed by TEM.

3.2.3. Determination of degradation ability toward triclosan and compounds structurally similar to triclosan

The isolate was initially grown in 20% R2A medium with 5 mg/L triclosan for three days. The cell suspension was harvested by centrifugation and the pellet was washed with 50 mM phosphate-buffered saline and then resuspended in fresh NMS medium for experimental use. The degradation tests were conducted in 250 mL flasks

containing the resting cell suspension and 5 mg/L of triclosan. The flasks were incubated on a rotary shaker at 150 rpm and at 30 ^{o}C, and liquid samples were collected over time for triclosan measurements. A subset of collected liquid samples was used for BLYES and BLYAS assays. Liquid samples collected from degradation experiments were also used to measure concentrations of chloride.

A parallel set of experiments was conducted to determine whether the isolate could degrade compounds that are structurally similar to triclosan. Three compounds, tri-BDE (1 mg/L), tetra-BDE (1 mg/L), and DE (1 g/L) (Kim et al., 2007; Robrock et al., 2009), were selected. Particularly, PDBEs, used as flame retardants, are toxic and known as endocrine disruptors (Meerts et al., 2000). The degradation experiments were conducted similarly as described above, except using resting cell suspension (OD_{600} = 0.6-0.8) and each of these three compounds.

3.2.4. Resistance to other antimicrobial agents

Experiments were performed to determine whether strain KCY1 was resistant to three common antimicrobial agents: kanamycin, trimethoprim and ampicillin. Strain KCY1 was streaked onto three 20% R2A agar plates containing 50 mg/L of kanamycin, 10 mg/L of trimethoprim, and 10 mg/L of ampicillin, respectively (Boehme et al., 2004). Also strain KCY1 was streaked onto 10% R2A agar plates containing 5 mg/L of triclosan as a positive control of the cell growth. The plates were then incubated at 30 ^{o}C.

3.2.5. Determination of Monod kinetic parameters for triclosan degradation

Monod degradation kinetic model, $\boldsymbol{q = \frac{q_m \cdot S}{K_s + S}}$, was used to describe triclosan degradation by strain KCY1. The kinetic experiments were conducted in a series of 40 mL EPA glass vials containing resting cells of strain KCY1 (OD_{600} = 0.4) and triclosan (ranging from 0.3 to 5 mg/L) in NMS medium. The vials were incubated on a rotary shaker at 150 rpm and at 30 ^{o}C for 3 hrs, and then used for triclosan and protein measurements. The incubation duration (3 hr) was determined in the laboratory where initial degradation rates remained linear. Experimental data obtained from kinetic tests were plotted as specific triclosan degradation rates (q, mass of substrate/mass of cell protein/time) against triclosan concentrations (S, mass/volume). The maximum specific triclosan degradation rate (q_m, mass of triclosan/mass of cell protein/time) and the half-velocity constant (K_s, mass of triclosan/volume) were determined by curve fitting using Sigmaplot 8.0 (SPSS Inc.) as previously described (Roh and Chu, 2010). All kinetic experiments were conducted in duplicate.

3.2.6. Determination of degradation/utilization ability toward other organics

Experiments were performed to determine whether the isolate could grow on three common macro-organics in wastewater: glucose (300 mg/L), sodium acetate (175 mg/L), and sodium succinate (300 mg/L) (Roh and Chu, 2010). These compounds were selected for the experiments because glucose is a common carbohydrate, and sodium succinate and sodium acetate are components present inside the tricarboxylic acid cycle (TCA cycle). Cell growth expressed as optical density (OD600), protein contents, and

volatile suspended solids (VSS) was monitored over time. Doubling times were determined from the exponential growth phase curves. Autoclave-killed cells were used as negative controls.

The ability of strain KCY1 to grow on catechol was determined using agar plates containing 1.5% noble agar, catechol, and NMS medium. The plates were prepared as follows. After agar was solidified, the strain was streaked on the plates and crystal of catechol was placed on one side of the plate so that a concentration gradient of catechol was developed. The plates were incubated at 30 ^{o}C. No growth was observed on the plates, suggesting that the strain was unable to grow on catechol.

3.2.7. Effect of complex nutrients on triclosan degradation

Cells grown in a complex nutrient medium without prior exposure to triclosan were tested for their ability to retain its biodegradation of triclosan. Experiments were conducted as follows. Strain KCY1 was grown in 100% R2A (fully nutrient-rich) medium without or with (5 or 500 μg/L) triclosan and transferred to its respective growth medium every two days. After four consecutive transfers, the cells were harvested as described above for degradation tests. The degradation experiments were conducted in glass vials containing 5 mg/L triclosan and the resting cells in NMS medium.

3.2.8. Bioluminescent androgenic/estrogenic screening assays

To evaluate androgenic and estrogenic potential of triclosan degradation metabolites and end products, the bioluminescent androgenic and estrogenic screening (BLYES and BLYAS) assays were performed as described previously (Eldridge et al., 2007; Sanseverino et al., 2005). The *S. cerevisiae* BLYES and BLYAS were obtained from the University of Tennessee, Knoxville, Tennessee. After 6 hours of incubation, the bioluminescence was measured using a BioTek Synergy 4 Microplate Reader (BioTek, Winooski, VA). Testosterone (from 0.7 μM to 0.3 nM) and 17β-estradiol (E2) (from 0.1 μM to 5.4 nM) were used as reference compounds for androgenic and estrogenic responses, respectively. The 50% effective concentration (EC_{50}) of the reference compounds and samples were determined as described by Sanseverino et al. (Sanseverino et al., 2009).

3.2.9. Determination of enzymes responsible for triclosan degradation

The isolate was screened for the presence of catechol 2,3-dioxygenase and/or catechol 1,2-dioxygenase using a spectrophotometric method as described previously (Klecka and Gibson, 1981; Nakai et al., 1988). Cells of strain KCY1 grown on triclosan were harvested by centrifugation, washed twice with 50 mM phosphate buffer, and resuspended in the same buffer (Min et al., 2009). The cells were lyzed by ultrasonication (Min et al., 2009; Toyama et al., 2010) and the cell lysates were centrifuged at 15,000 × g at 4 ^{o}C for 30 min. The supernatant was used for enzyme assay and protein concentration measurement. Activity assays for catechol 2,3-

dioxygenase and catechol 1,2-dioxygenase were conducted at 25 ^{o}C as describe previously (Klecka and Gibson, 1981; Nakai et al., 1988). The reaction mixture contained 1.9 mL of 20 mg/L of catechol and 0.1 mL of cell extract in a final volume of 2 mL. Formation of 2-hydroxymuconic semialdehyde was determined at 375 nm and *cis*-*cis*-muconic acid was determined at 260 nm with a spectrophotometer (HP G1130A, USA). Protein content was measured using a BCA protein assay kit. Enzyme activity was expressed as a micromole of substrate per minute per mg of protein (Toyama et al., 2010).

In addition, triclosan degradation via *meta*-cleavage pathway was tested by adding 3-fluorocatechol (50 mg/L) or in the absence of it. 3-Fluorocatechol is an inhibitor of catechol 2,3-dioxygenase that catalyzes *meta*-cleavage reactions (Bartels et al., 1984; Toyama et al., 2010). Lack of triclosan degradation in the presence of 3-fluorocatechol would, therefore, suggest that a *meta*-cleavage reaction is essential for triclosan degradation.

3.2.10. Chemical analysis

Chloride concentrations were measured using a DX-80 Ion Chromatography (IC) system (Dionex, Sunnyvale, CA) equipped with an IonPac AS14A-5µm analytical column (3 × 150 mm). An eluent solution containing 0.16 M Na_2CO_3 and 0.02 M $NaHCO_3$ was used. The detection limit for chloride ion was 0.05 mg/L.

Triclosan, tri-BDE, tetra-BDE, and DE concentrations and degradation metabolites were determined using a GC (Agilent 6890) /MS (Agilent 5973) equipped

with DB-5 column. In addition, to detect possible degradation metabolites, LC/MS analysis was performed using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) interfaced with quadruple ion trap mass spectrometer (LCQ-DECA; ThermoFinnigan) APCI-MS.

Liquid samples were collected and acidified to reach pH 2-3 by adding concentrated sulfuric acid. The acidified liquid samples were extracted with ethyl acetate (1:1 v/v) at 350 rpm for 60 min, before adding anhydrous sodium sulfate. Then ethyl acetate layer (i.e. the upper-layer) was transferred to a new glass vial, purged with nitrogen gas to dryness, and then reconstituted in 450 μL of acetone. For GC/MS analysis, the reconstituted samples were derivatized with BSTFA (50 μL) in acetone. Triclosan concentrations were determined using a GC/MS in selective ion monitoring (SIM) mode as described by Roh et al. (Roh et al., 2009a). In addition, different GC oven temperatures (Masai et al., 1997) in full scan mode was used to detect triclosan biodegradation metabolites.

The reconstituted samples were used directly for LC/MS analysis. An Aquasil C18 column (2.1 × 150 mm, 3 μm; Thermo Hypersil-Keyston, Bellafonte, PA) was used for separation with water (A) and methanol (B) both containing 0.1% formic acid as mobile phases. The elution gradient was as follows: 5% B for 2 minutes increased to 100% B in 25 minutes and held for 5 minutes. An APCI probe in negative ion mode was used for ionization. The MS operating conditions were optimized as follows: sheath gas and auxiliary gas flow rate, 50 and 10 arbitrary units, respectively; APCI vaporizer temperature, 450°C; corona current, 5 μA; and transfer capillary temperature, 150°C.

Liquid samples of tri- and tetra-BDE, and DE were prepared as described previously. (Kim et al., 2007; Robrock et al., 2009). The GC oven temperature program for BDEs concentrations was the same as previously described (Li et al., 2009). Standard concentration curves for BDEs ranging from 0.1 to 3 mg/L were used.

3.3. Results and discussion

3.3.1. Identification of a triclosan-degrading microorganism, strain KCY1

Among three presumptive triclosan-degrading colonies, one isolate (yellow-mucoid), designated strain KCY1, showed the ability to degrade triclosan in NMS medium. Strain KCY1 is a short, rod-shaped (0.5 μm × 1.7 μm) Gram-negative bacterium with a flagellum (Figure 3.2).

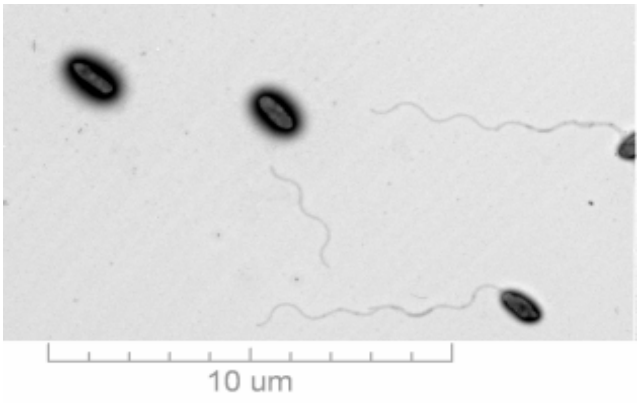
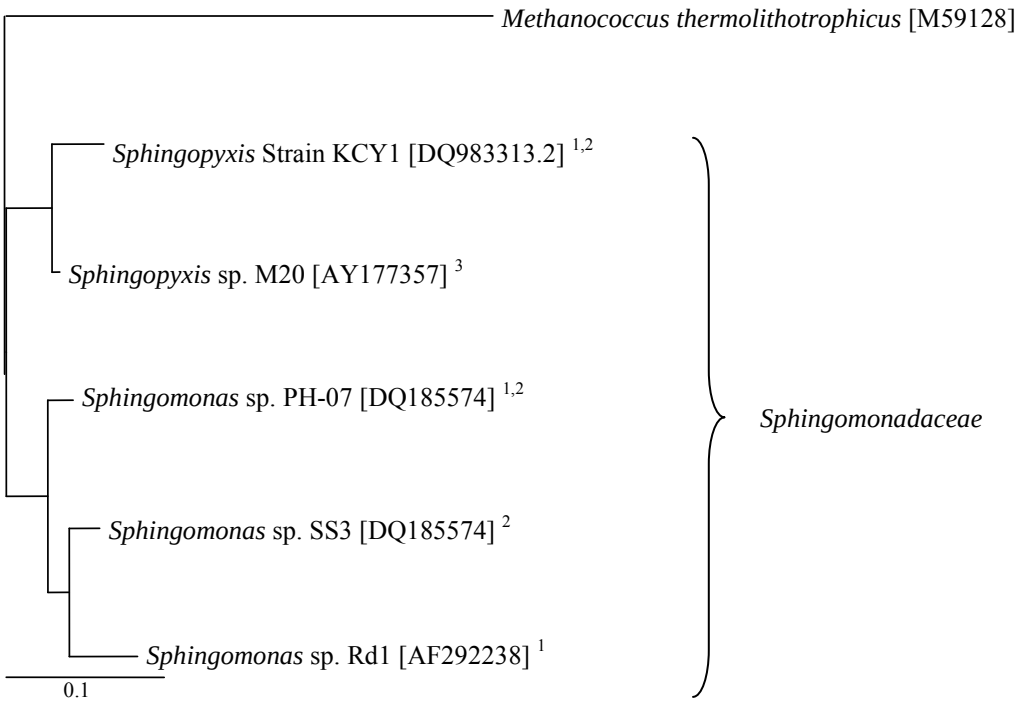


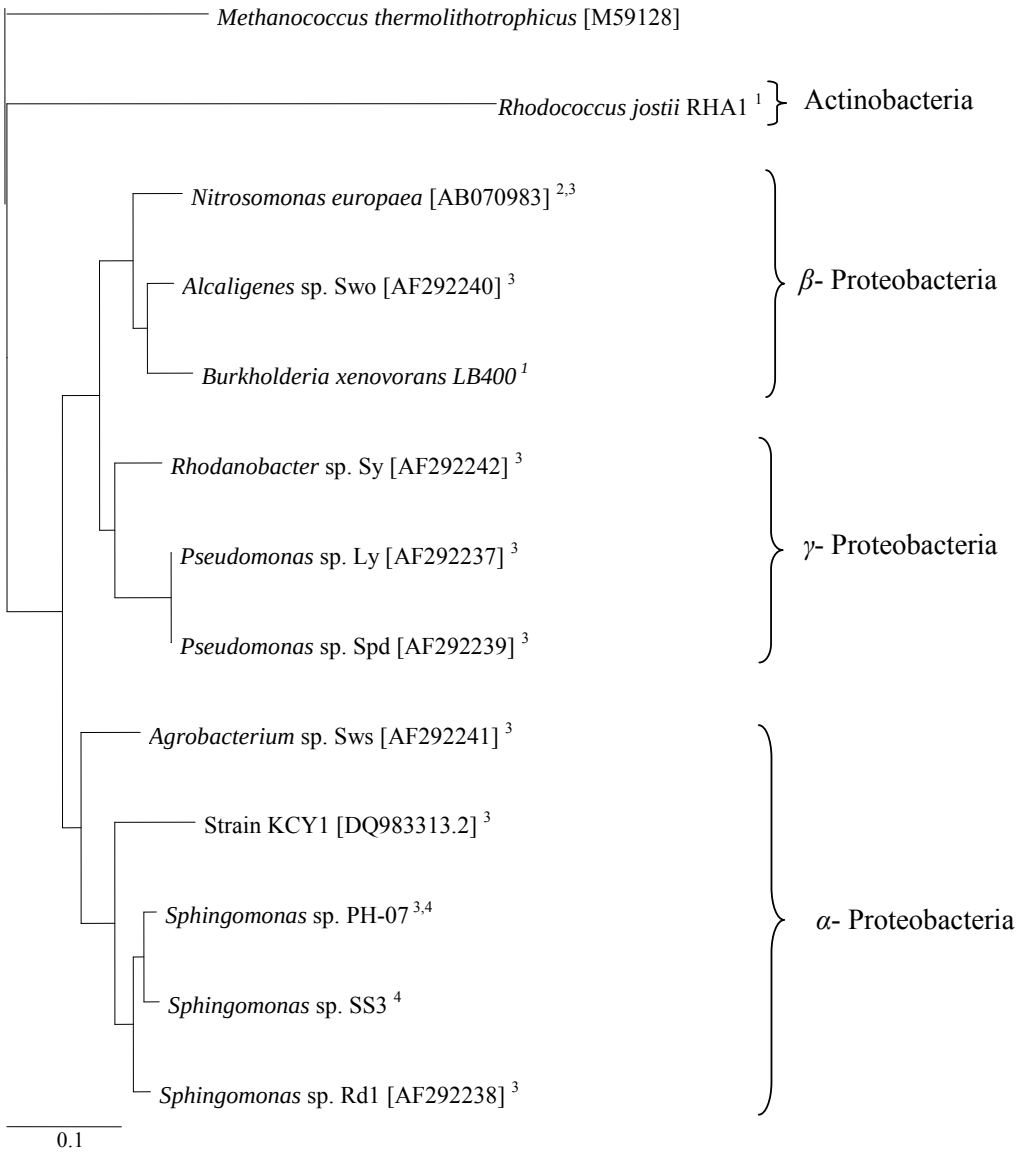
Figure 3.2. Transmission electron micrograph of *Sphingopyxis* strain KCY1. The strain is rod-shaped (with a diameter of 1.5 μm and a length of 2.0 μm) and has a flagellum. Bar: 10 μm.

Based on its 16S rRNA gene sequence, strain KCY1 is a member of the genus *Sphingopyxis*. As shown in Figure 3.3, strain KCY1 had 98 % similarity to *Sphingopyxis* sp. M20, a known phenanthrene-degrading bacterium (Bodour et al., 2003). Strain KCY1 also has 93% similarity to two DE degraders (*Sphingomonas* sp. PH-07 and *Sphingomonas* sp. SS3) and one known triclosan degrader (*Sphingomonas* sp. Rd1) (Hay et al., 2001; Kim et al., 2007; Schmidt et al., 1992). In addition, strain KCY1 has 80% homology to two well studied biphenyl degraders, *Burkholderia xenovorans* LB400 and *Rhodococcus* sp. RHA1, indicating no substantial relationship between strain KCY1 and these PCB-degrading strains (Figure 3.4). It is not surprising to observe the high homology of strain KCY1 to other known pollutant degraders, since many members in the genera *Sphingopyxis*, and the sphingomonads were known for their ability to degrade a wide range of xenobiotic-pollutants (Stolz, 2009; Tani et al., 2011).



[1] Triclosan-degrading bacteria; [2] Diphenyl-ether degrading bacteria; [3] Phenanthrene-degrading bacteria.

Figure 3.3. A phylogenetic tree showing relative relationship between strain KCY1 and related species (family: *Sphingomonadaceae*). The phylogenetic tree was constructed using the neighbor-joining method with bootstrapping and rooted by referring to *Methanococcus thermolithotrophicus*. Bootstrap support values from 1000 replicates are indicated at branch nodes. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

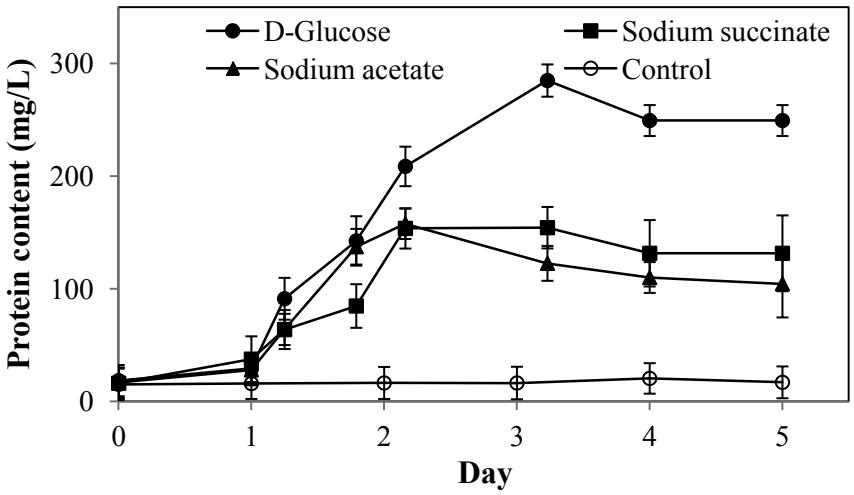


[1] PCBs-degrading bacteria; [2] Ammonia oxidizing bacteria; [3] Triclosan-degrading bacteria; [4] Diphenyl-ether degrading bacteria.

Figure 3.4. A phylogenetic tree showing relative relationship between strain KCY1 and known triclosan degraders. The phylogenetic tree was constructed using the neighbor-joining method with bootstrapping and rooted by referring to *Methanococcus thermolithotrophicus*. Bootstrap support values from 1000 replicates are indicated at branch nodes. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

3.3.2. Characteristics of strain KCY1

Since strain KCY1 can grow rapidly on R2A agar, it is expected that the strain can also grow on glucose, sodium succinate, and sodium acetate (Figure 3.5). Higher growth yield was observed when sodium acetate was supplied as a carbon source. The observed average yields (Y) ranged from 0.10 to 0.22 mg-protein/mg-BOD_L. Doubling times ranging between 5 and 9 hrs were observed. The ability of strain KCY1 to grow in these organics suggests that the strain can grow in the presence of common organics in wastewater. Although strain KCY1 can degrade triclosan, no biomass/protein increase was observed for more than 30 days when the strain was supplied with triclosan as a sole carbon source in NMS medium (data not shown). This result suggested that strain KCY1 was unable to grow with triclosan. Furthermore, strain KCY1 was found to be kanamycin and trimethoprim resistant, but ampicillin sensitive (data not shown).



Parameters	Glucose	Sodium succinate	Sodium acetate	Typical value
Y (g-VSS/g-BOD_L)[a, b]	0.11	0.10	0.22	0.42-0.49
Doubling Time (hr)	5	9	6	-

[a] Y = yield coefficient. [b] Based on theoretical oxygen demand: 1 mg-glucose/L = 1.1 mg-BOD_L/L; 1 mg-sodium succinate/L = 0.6 mg-BOD_L/L; 1 mg-sodium acetate/L = 0.5 mg-BOD_L/L. 1g-protein = 1.5 g-VSS (measured in laboratory).

* The yields of aerobic heterotrophs range from 0.42 gVSS/gBOD_L with other electron donors to 0.49 gVSS/gBOD_L with carbohydrate BOD (Rittmann and McCarty, 2001).

Figure 3.5. Growth curves and kinetics of strain KCY1 with three different organics: glucose, sodium acetate, and sodium succinate. Growth yields and doubling times during exponential growth were calculated.

3.3.3. Degradation of triclosan by strain KCY1

As shown in Figure 3.6, strain KCY1 degraded approximately 90% of ithe nitial triclosan (5 mg/L) in 24 hrs and reached about 100% removal on day 2. The initial specific degradation rate for triclosan ranged from 0.099 to 0.103 mg-triclosan/mg-protein/day. Strain KCY1 was capable of completely degrading triclosan at a faster degradation rate than those of known triclosan degraders (Hay et al., 2001; Kim et al., 2011; Meade et al., 2001; Roh et al., 2009a). As previous studies did not contain sufficient data for the calculation of specific degradation rates, only qualitative comparison can be made. For example, 4 to 9 days was needed for two soil triclosan-degrading bacteria, *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp *denitrificans* to degrade 0.15-0.18 mg/L of triclosan (Meade et al., 2001). Incomplete triclosan degradation was reported by *Sphingomonas* sp. Rd1 and triclosan-degrading consortium (35% of triclosan (500 mg/L) in 14 days) (Hay et al., 2001), and by *Nitrosomonas europaea* (50% of triclosan (1 mg/L) in 1 day) (Roh et al., 2009a). A recent study showed that *Sphingomonas* sp. PH-07 can degrade 25% of triclosan (10 mg/L) in 8 days (Kim et al., 2011).

Since triclosan is a chlorinated compound, the amount of chloride release from triclosan biodegradation could be used to assess the degree of dechlorination. Assuming that 5 mg/L triclosan is completely dechlorinated, a theoretical chloride concentration of 1.84 mg/L is expected (3 mole of chloride is theoretically released per mole of triclosan added). At the end of the degradation experiment (i.e. on day 3), 107% of the theoretical amount of chloride release (1.96 mg/L) was observed, suggesting that triclosan was fully

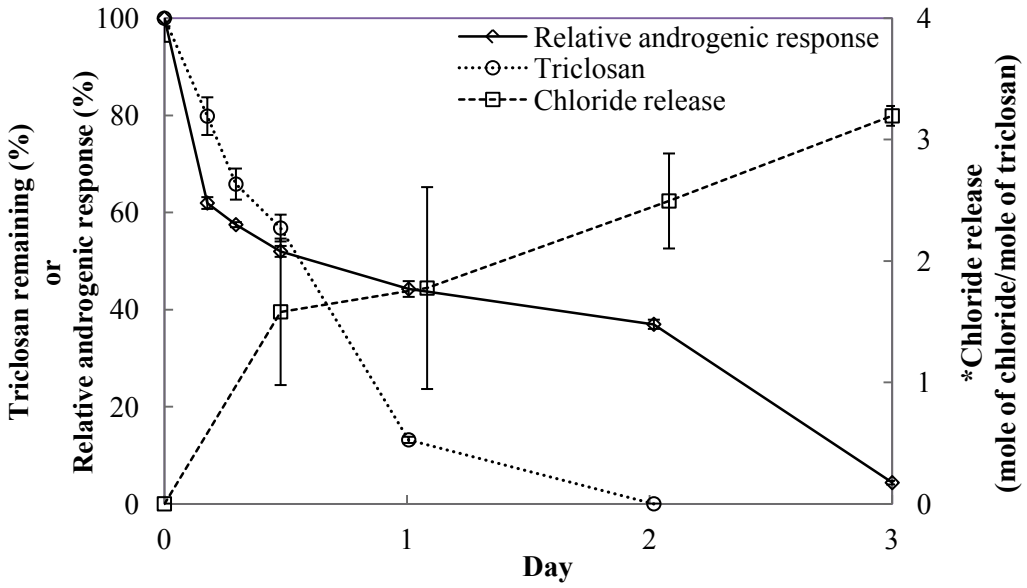
dechlorinated by strain KCY1. To our knowledge, this is the first study reporting complete dechlorination of triclosan by a rapid triclosan-degrading wastewater microorganism.

3.3.4. Androgenic and estrogenic potential of triclosan degradation metabolites and end products

BYLES and BLYAS assays were used to evaluate estrogenic and androgenic potential of triclosan degradation metabolites and end products. Triclosan itself triggered weak androgenic activity in the BLYAS assay, but not estrogenic activity in the BLYES assay in this study. The different results observed by the BLYES assay and by the human breast cancer cells (Gee et al., 2008) might be due to the different responses and sensitivities between the yeast cells and human breast cancer cells. As shown in Figure 3.6, the androgenic potential of the liquid samples decreased at three different paces: a very rapid decrease in the first 6 hours, followed by a much slower decrease rate between hour 6 and day 2, and then declined at a moderate rate between day 2 and day 3. This decline pattern did not correspond well to the trend of triclosan degradation – approximately 95% of triclosan was degraded on day 1 and triclosan concentration was below detection limit on day 2. Some unknown intermediates with higher or less androgenic potential might have been also produced during triclosan transformation. At the end of triclosan degradation, these known metabolites and unknown intermediates converted to end-products/ metabolites with non- and/or less

androgenic potential. The above result suggested that triclosan was converted to metabolites with non- and/or less androgenic potential.

Since triclosan is a chlorinated organic compound, the decrease of androgenic potential over time might correlate to the extent of dechlorination during the triclosan degradation. The decline of androgenic potential could be explained by the decrease in initial triclosan concentration and the transformation into less-chlorinated metabolites that were detected in this study before day 1 (see identification of metabolites below). Between day 1 and day 2, the reduction rate of androgenic activity became slower than the triclosan degradation rate, suggesting that (i) other androgenic metabolites might be produced during this period or (ii) androgenic metabolites might be transformed at a slower rate than the triclosan degradation rate. Interestingly, the androgenic response (~40% of original response) was observed even though triclosan was no longer detected after day 2. This indicates a slow transformation of triclosan metabolites with androgenicity, like 2,4-dichlorophenol (detected). Previous studies have reported that 2, 4-dichloropehnol exhibited *in-vivo* androgenic activity in zebrafish embryos (Sawle et al., 2010) and in human prostate cancer cells (Kim et al., 2005). The statement is supported by 84% of the stoichiometric chloride release on day 2. On day 3, no androgenic potential of the sample was detected and greater than 100% recovery of chlorides was obtained, suggesting dechlorination and breakdown of the chlorine-containing metabolites into none-chlorinated end products with no androgenic activity.



*3 mole of chloride is theoretically released per mole of triclosan added.

Figure 3.6. Biodegradation of triclosan by the resting cells of strain KCY1. Reduction of androgenic potential in samples was observed during triclosan degradation. The relative androgenic responses of the samples over time were determined by dividing the initial response measured at time zero. Triclosan remaining (open circles); relative androgenic responses (open diamonds); chloride release (open squares).

3.3.5. Factors affecting triclosan degradation

As wastewater contains a wide range of complex organics that are readily available for microbial growth, it is important to know whether strain KCY1 would retain its ability to degrade triclosan after it has grown on complex nutrients. To determine the effects of nutrients on triclosan biodegradation by strain KCY1, the strain was initially grown in nutrient-rich medium (100% R2A) without triclosan for 8 days (4 consecutive transfers every 2-day). After growing on nutrient rich medium without triclosan, strain KCY1 lost its degradation ability toward triclosan (Table 3.1). However, when triclosan was present in the growth medium (either 5 or 500 μg/L), the strain was able to completely degrade 5 mg/L of triclosan within in four days (data not shown). As shown in Table 3.1, a much slower initial specific triclosan degradation rate (mass of triclosan/biomass/time) was observed for cells grown with 100% R2A and 5 μg/L of triclosan than the cells grown with 100% R2A and 500 μg/L of triclosan. However, after exposure to 5 mg/L of triclosan for 1 day, similar specific triclosan degradation rates (between day 1 and day 2) were observed for both cells with prior exposure to triclosan. In fact, these degradation rates observed between day 1 and day 2 were similar to the rates shown by the cells pre-grown in 100% R2A medium with 5 mg/L triclosan. These results suggested that it is necessary for strain KCY1 to have prior exposure to triclosan, regardless of the concentrations, in order to maintain its ability to degrade triclosan. Moreover, low strength rich-nutrient medium (20% vs. 100% of R2A medium) could enhance the triclosan degradation rate of strain KCY1. As ambient triclosan concentrations in wastewater range from 0.61 to 5.1 μg/L (Thompson et al., 2005; Yu

and Chu, 2009) and wastewater is full of readily available organics, strain KCY1 demonstrates the potential to grow effectively in wastewater while degrading triclosan at a reasonable rate. The phenomenon of losing triclosan degradation ability after growing on rich-nutrient medium without triclosan suggested that the gene involved in triclosan biodegradation might be in a plasmid (Singh et al., 2004; Vega et al., 1988). It is also possible that the strain KCY1 requires a longer period of time to recover its triclosan degradation ability after pregrown in nutrient rich medium. This aspect was not explored in this study. Further studies are needed to isolate the plasmid and identify the catabolic genes that are responsible for triclosan biodegradation.

3.3.6. Degradation kinetic parameters for triclosan

The results of triclosan degradation tests were used to develop the relationship between the specific triclosan degradation rate (q) and triclosan concentrations (Figure 3.7). The Monod kinetic parameters were calculated by fitting nonlinear regression to the experimental data (Sigmaplot, SPSS Inc, version 16.0). Initial degradation rates were calculated by dividing the amount of triclosan degraded within 3 hours over the initial biomass. No significant change in biomass was observed at the end of the 3-hour kinetic experiments. The maximum specific triclosan degradation rate (q_m) and the half velocity constant (K_s, mass of substrate/volume) were 0.13 mg-triclosan/mg-protein/day (or 1.7 mg-BOD_L/mg-protein/day) and 2.8 mg-triclosan/L (or 3.7 mg-BOD_L/L), respectively. The K_m value is much higher than ambient concentrations of triclosan in wastewater (ranging from 0.61 to 5.1 μg/L) (Thompson et al., 2005; Yu and Chu, 2009),

suggesting that triclosan degradation by strain KCY1 would follow the first order degradation kinetics in wastewater.

Table 3.1. Effects of growth medium on specific triclosan degradation rates by strain KCY1.

Treatments	Specific Degradation Rate (mg-triclosan/mg-protein/day)	
	Day 0-1	**Day 1-2**
A. 100% R2A *	no degradation	no degradation
B. 100% R2A + 5 µg/L triclosan*	0.009 ± 0.004	0.060 ± 0.015
C. 100% R2A + 500 µg/L triclosan*	0.022 ± 0.023	0.057± 0.022
D. 100% R2A + 5 mg/L triclosan‡	0.059 ± 0.008	
E. 20% R2A + 5 mg/L triclosan‡	0.101	

* Cells (A,B, and C) were subcultured four times (every 2 days) into the respective growth medium with different concentrations of triclosan ranging from 0, 5, or 500 µg/L before used for triclosan (5 mg/L) degradation experiments in NMS medium.

‡ Cells (D and E) were not subcultured before used for triclosan (5 mg/L) degradation experiments in NMS medium.

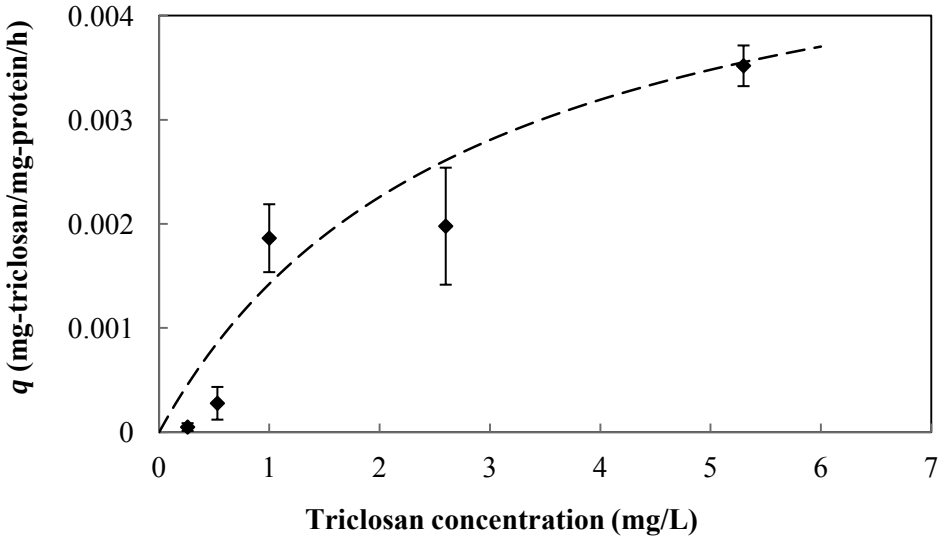


Figure 3.7. Degradation kinetic of triclosan by strain KCY1. Nonlinear regression was used to determine Monod degradation kinetic parameters. Solid diamonds are experimental data. The dash line is showing a fitted Monod kinetic model.

3.3.7. Degradation ability toward compounds which are structurally similar to triclosan

Since two BDEs (tri- and tetra- BDEs) and DE are structurally similar to triclosan and strain KCY1 can degrade triclosan, I hypothesized that strain KCY1 could degrade these compounds as well. Strain KCY1 was able to degrade approximately 78% of DE (1 g/L) within 5 days, but unable to use DE as a sole carbon source (data not shown). Although earlier it was reported that C-Br and C-Cl bonds are at least equally viable for enzymatic reaction (Dos Santos et al., 1999), strain KCY1 was unable to degrade tri-BDE and tetra-BDE. The inability of strain KCY1 to degrade these two BDEs could be due to a combination of various factors, including the difference in electron-withdrawing effects that would result from the difference between the halogen

species (Cl vs Br) and the absence of hydroxyl group in both BDEs that could contribute to selectivity of the enzymes. The reason why strain KCY1 can degrade DE but not tri- and tetra- BDEs was unclear in this study.

3.3.8. Enzymes involved in triclosan biodegradation

Another set of experiments was conducted to examine whether 3-flurocatechol, a *meta*-cleavage inhibitor, would affect triclosan biodegradation. As shown in Figure 3.8, the degradation of triclosan ceased after the addition of 3-flurocatechol at 23-hr, suggesting that strain KCY1 might use a *meta*-cleavage pathway to degrade triclosan. In addition, only the activity of catechol 2,3-dioxygenase, not catechol 1,2-dioxygenase, was detected in the cell extract of strain KCY1 grown on triclosan (specific enzyme activity = 337 nmol/min/mg-protein). The overall result strongly suggests that a *meta*-cleavage pathway was involved in triclosan degradation. More studies are needed to further elucidate oxygenase enzymes that are responsible for triclosan degradation.

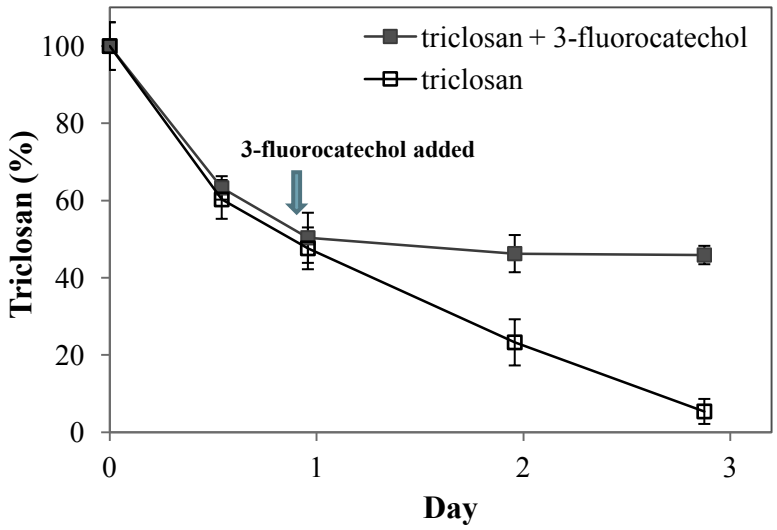


Figure 3.8. Effects of 3-fluorocatechol on triclosan degradation by strain KCY1. Cells with the addition of 3-fluorocatechol at 23hrs (closed squares); cells without the addition of 3-fluorocatechol (open diamonds).

3.3.9. Degradation metabolites and possible degradation pathway for triclosan

During the triclosan biodegradation, five metabolites were identified (Figure 3.9). These metabolites are monohydroxy-triclosan, 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal, 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione, and 2,4-dichlorophenol (Figure 3.10 and Figure 3.11). 2,4-Dichlorophenol was confirmed via the use of authentic standard. The standards for the other four metabolites are not commercially available and they were tentatively indentified based on mass spectra and the fragmentation patterns. The two identified metabolites (monohydroxy-triclosan and 2,4-dicholophenol) were among the seven

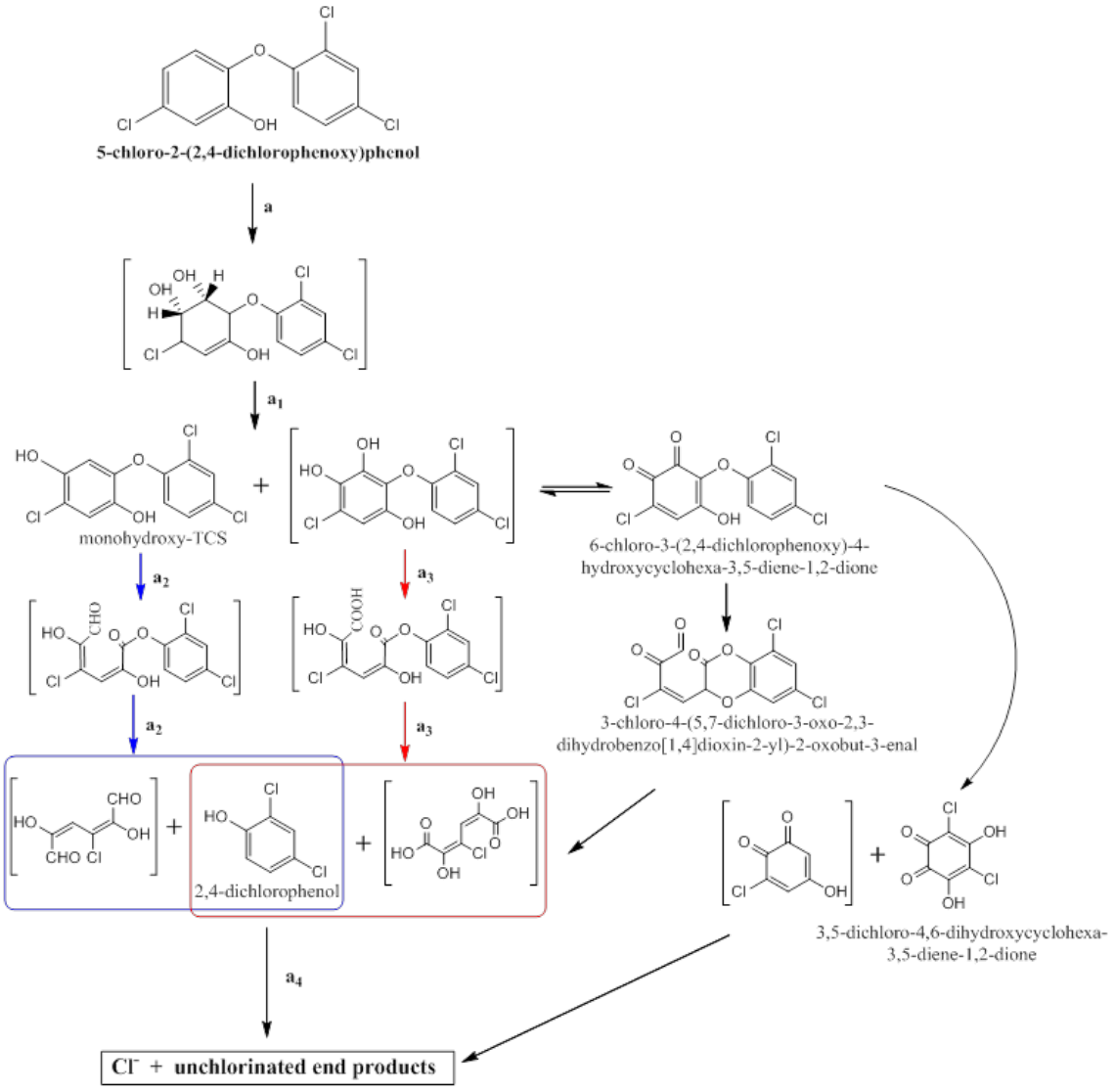


Figure 3.9. A possible biodegradation pathway for triclosan by strain KCY1.

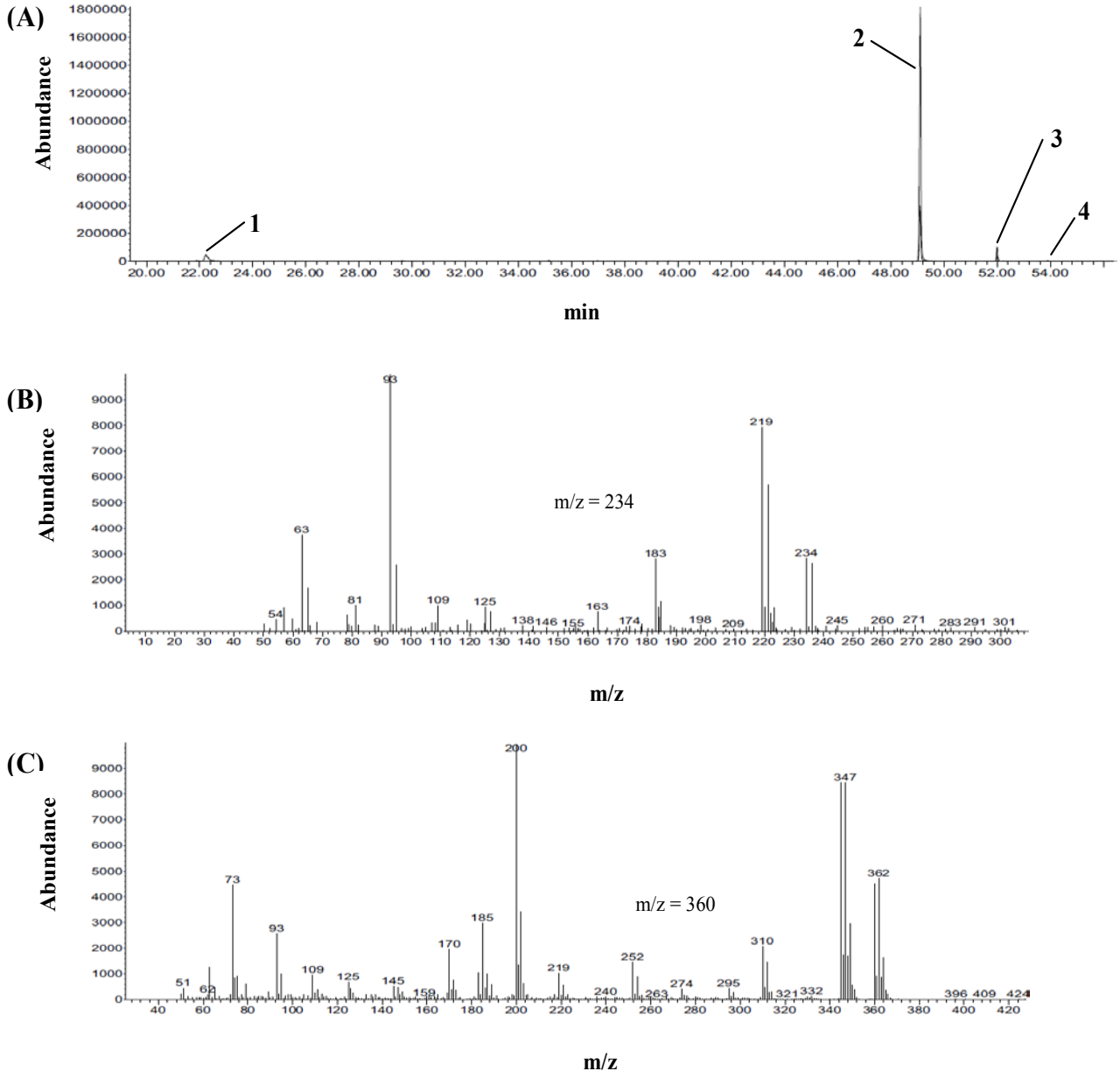


Figure 3.10. GC-MS chromatogram of triclosan degradation metabolites by strain KCY1. (A) GC-MS chromatogram of triclosan degradation metabolites as the TMS derivatives; GC mass spectrum of TMS-derivatized, (B) 2,4-dichlorophenol (peak 1), (C) triclosan (peak 2), (D) 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione (peak 3) and (E) monohydroxy-triclosan (peak 4).

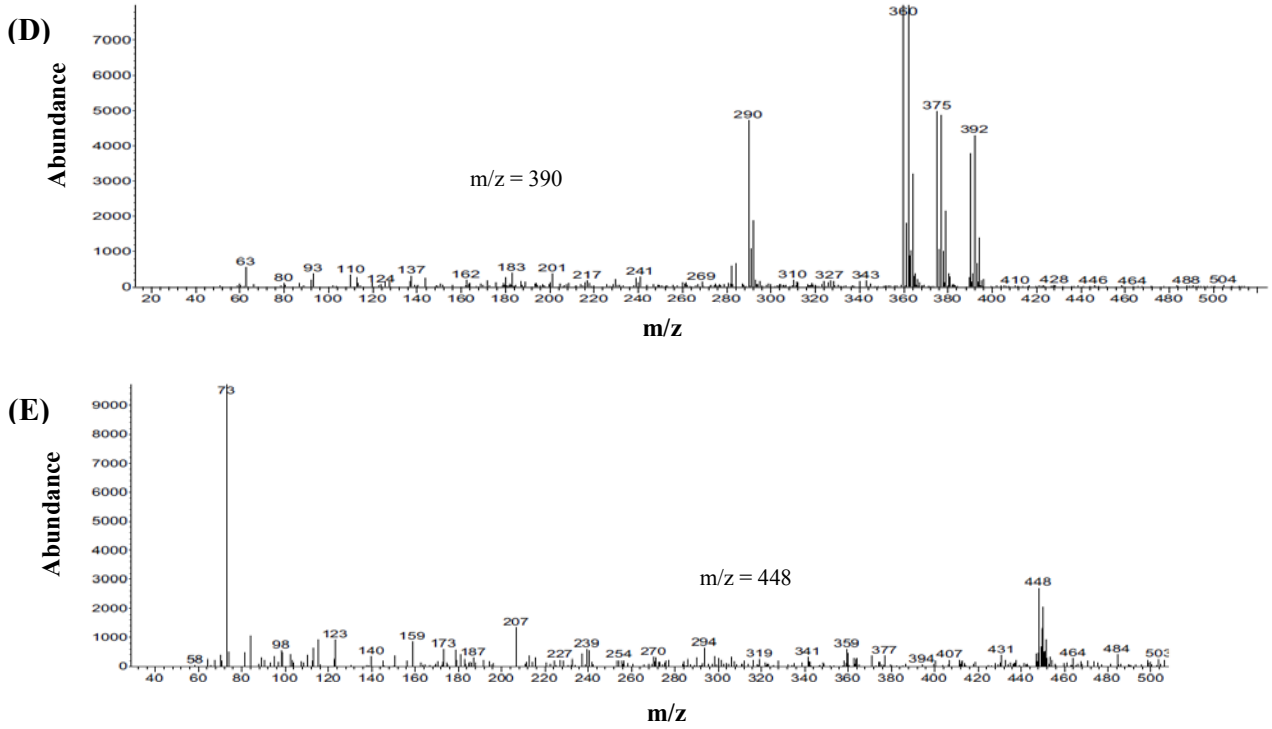


Figure 3.10. Continued.

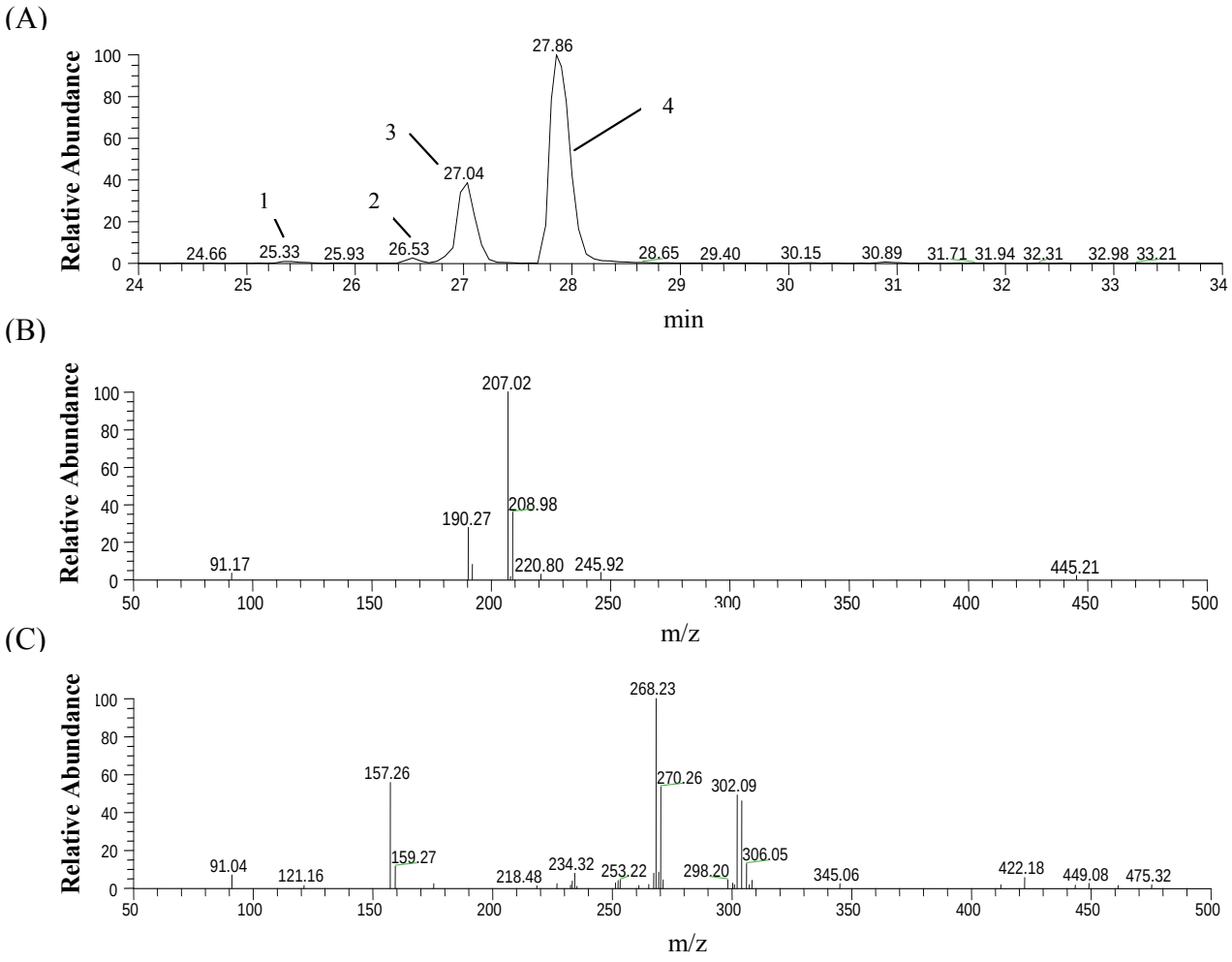


Figure 3.11. APCI-LC/MS of triclosan degradation metabolites by strain KCY1. (A) APCI-LC/MS of triclosan degradation metabolites; APCI mass spectrum at retention time (B) 25.3 min (peak 1), 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione; (C) 26.5 min (peak 2), monohydroxy-triclosan; (D) 27.0 min (peak 3), 3-chloro-4-(5,7-dichloro-3-oxo-2,3-dihydrobenzo[1,4]dioxin-2-yl)-2-oxobut-3-enal; (E) 27.9 (peak 4), triclosan.

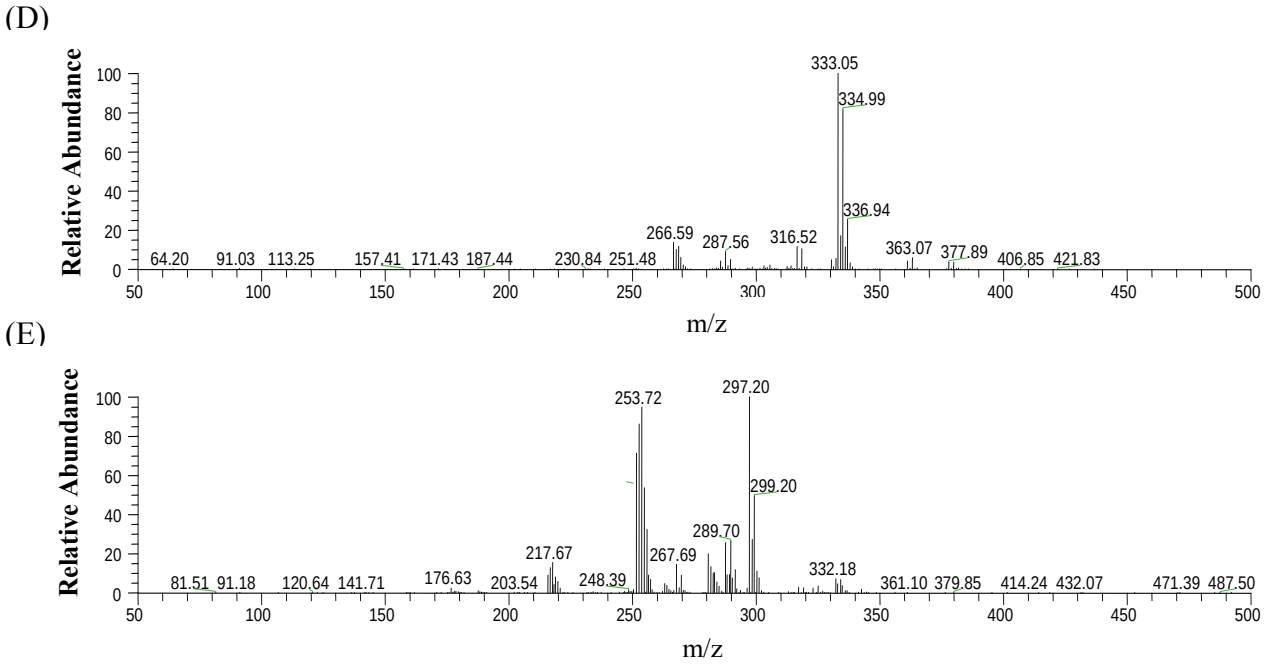


Figure 3.11. Continued.

triclosan metabolites previously observed during triclosan degradation by a diphenyl ether degrader, *Sphingomonas* sp. PH-07 (Kim et al., 2011). The other three identified metabolites have not been reported before. All five metabolites were observed in the first 8-hr degradation time. However, past this time (8 hrs), only 2,4-dichlorophenol was observed in 24-hr and 32-hr samples, indicating continuous degradation of these metabolites. At the end of triclosan degradation experiments, a stoichiometric release of chloride ions was observed, suggesting complete dechlorination of triclosan by strain KCY1.

The proposed biodegradation pathway for triclosan by strain KCY1 is presented in Figure 3.9. Based on the results of inhibition tests and enzyme activity assays, triclosan degradation is likely to follow a *meta*-cleavage pathway. Given that strain KCY1 was able to degrade DE and that DE has been known to be degraded via a *meta*-cleavage pathway (Kim et al., 2007; Pfeifer et al., 1989), I proposed that an initial attack of a regioselective dioxygenase at the 2,3-position of triclosan resulted in the formation of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione, and monohydroxy-triclosan (detected) and dihydroxy-triclosan (not detected) (reactions a and a_1 in Figure 3.9). (Pfeifer et al., 1989) had reported that a further dioxygenation with simultaneous ether-bond cleavage occurred during diphenyl ether degradation. Thus, it is possible that the monohydroxy- and dihydroxy-triclosan were further attacked by 2,3-dioxygenase and then subjected to an ether cleavage to produce 2,4-dichlorophenol (shown as reaction a_2 or a_3 or a_4 in Figure 3.9). Two other ring-fission intermediates might be formed; however, none of them were detected in this study. The detection of 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione also suggested another degradation route involving the occurrence of an ether-bond cleavage of 6-chloro-3-(2,4-dichlorophenoxy)-4-hydroxycyclohexa-3,5-diene-1,2-dione (shown as reaction a_5 in Figure 3.9). Interestingly, strain KCY1 can grow on phenol, but not on catechol, suggesting that the utilization of phenol by strain KCY1 may not follow a catechol pathway, but a hydroquinone pathway (Bae et al., 1996). The complete recovery of chloride ions suggests that 2,4-dichlorophenol and 3,5-dichloro-4,6-dihydroxycyclohexa-3,5-diene-1,2-dione were completely dechlorinated (the reaction a_6

in Figure 3.9). The chlorinated metabolites might explain the delayed decrease of androgenic activity even after triclosan concentration decreased to zero on day 2 (Figure 3.6).

4. PROBING TRICLOSAN-UTILIZING BACTERIA IN A TRICLOSAN-DEGRADING MICROBIAL CONSORTIUM ENRICHED FROM ACTIVATED SLUDGE

4.1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a widely used, broad spectrum bactericide. Triclosan blocks enzyme enoyl-acyl carrier protein reductase to prevent bacterial lipid biosynthesis (McMurry et al., 1998). Due to its effectiveness and thermal stability, triclosan is incorporated into numerous consumer products, including soaps, toothpastes, disinfectants, cosmetics, and detergents (Dann and Hontela, 2011). After the products are used or disposed, triclosan is released from these triclosan-containing products and consequently enters wastewater. It is estimated that triclosan-containing products contributes to 96% of triclosan in wastewater (Ciba Specialty Chemical, 1998). The triclosan in wastewater is biodegraded by activated sludge or adsorbed onto the biosolids. Through effluent discharge into surface water or biosolid applications into fields, triclosan is continuously released into the environment (Morrall et al., 2004; Singer et al., 2002).

Triclosan and its metabolites are not only detected in rivers, lakes, soils, and sediments, but also in human urine, blood, and breast milk samples (Allmyr et al., 2006; Calafat et al., 2008; Kolpin et al., 2002; Singer et al., 2002). The prevalence of triclosan poses a potential threat to ecosystems and human health. For example, low levels of environmental triclosan could lead to the higher occurrences antimicrobial-resistant

microorganisms (Braoudaki and Hilton, 2004; Yazdankhah et al., 2006). Environmental triclosan is also a precursor of chloroform and dioxins (Latch et al., 2003; Rule et al., 2005). Furthermore, triclosan is an endocrine-disrupting compound, as indicated by *in-vitro* cancer breast cell bioassays (Gee et al., 2008).

Biodegradation of triclosan observed in activated sludge systems indicated that biodegradation can potentially be an important removal mechanism for triclosan in wastewater (Singer et al., 2002). Several wastewater microorganisms, including *Sphingomonas* sp. Rd1 (Hay et al., 2001), *Nitrosomonas europaea* (Roh et al., 2009a), *Sphingomonas* sp. PH-07 (Kim et al., 2011), and *Sphingopyxis* strain KCY1 (Lee et al., 2012), are known to degrade triclosan cometabolically. Although, two soil bacteria are known to use triclosan as a sole carbon source (Meade et al., 2001), it remains unknown if any microorganism in activated sludge is capable of using triclosan as a carbon source. However, all attempts to obtain a pure culture of a triclosan-utilizing bacterium from activated sludge have been unsuccessful to date.

The objective of this study was to use culture-independent molecular methods to identify the presence of active triclosan-utilizing bacteria in a triclosan-degrading microbial consortium. The consortium was originally enriched from activated sludge. A stable-isotope probing (SIP)-based method, called Q-FAST (Yu and Chu, 2005), along with universally ^{13}C-labeled triclosan was applied to the consortium. The ^{13}C-labeled triclosan was used to track the flow of the heavier carbon (^{13}C) from the compound into the genetic biomarker (DNA) of the active triclosan-utilizing microorganisms in the triclosan-degrading microbial consortium. Meanwhile, the structure of active triclosan-

utilizing microbial community was characterized using real-time-t-RFLP assay (Yu and Chu, 2005). Identification of these uncultured triclosan-utilizing bacteria in the degrading consortium is important as it can provide insights to the range of microorganisms in activated sludge that can potentially degrade and utilizing triclosan. Additionally, our results have potentially to be used as biomarkers for assessing triclosan degradation potential in different built and natural systems.

4.2. Material and methods

4.2.1. Chemicals

Unlabeled triclosan (97% pure) and [$^{13}C_{12}$]-labeled triclosan (universally labled, isotopic purity 99% or greater) were purchased from Aldrich Chemicals (Milwaukee, WI) and Wellington Laboratories (Ontario, Canada), respectively. Unlabeled glucose and ^{13}C-labeled glucose were purchased from Isotec Inc. (Miamisburg,OH). Cesium chloride (purity 99.99%) was purchased from Fisher Scientific (Fair Lawn, NJ). Ethidium bromide (EtBr) was obtained from Promega Corporation (Madison, WI). GeneScan 500 ROX Size Standard was purchased from Applied Biosystems (Warrington, UK). *Taq* PCR Master Mix was purchased from QIAGEN Inc. (Valencia, CA). The stock solution of unlabeled and ^{13}C-labled triclosan was prepared in acetone (1g/L of acetone).

4.2.2. Triclosan-degrading microbial consortium and identification of triclosan-utilizing bacteria

The triclosan-degrading consortium was established in a 500-mL flask containing activated sludge (the final concentration of mixed liquor volatile suspended solids (MLVSS) of 500 mg/L), 200 mL of nitrate mineral salts (NMS) medium (Chu and Alvarez-Cohen, 1996), and 5 mg/L of unlabeled triclosan. The activated sludge was collected from a local wastewater treatment plant (WWTP) in College Station, TX. The flask was incubated on a rotary shaker at 150 rpm at 30 ^{o}C. After 5 days of incubation, triclosan was completely degraded and the cell suspension (40 mL) was then transferred to a new flask containing NMS medium (80 mL) and unlabeled triclosan (5 mg/L). The amended triclosan was degraded rapidly within 3 days and the transfer was repeated every 3 days for 5 times. After the fifth transfer, the triclosan-degrading consortium was used as an inoculum for Q-FAST experiment to identify active triclosan-utilizing bacteria.

To identify active triclosan-utilizing bacteria in the degrading consortium, the Q-FAST experiment was conducted as follows. Prior to the experiment, acetone-free ^{13}C-triclosan solution was prepared by adding a known amount of stock solution of ^{13}C-triclosan to an autoclaved 300-mL flask. After acetone in the flask was completely evaporated to the dryness, NMS medium was added into the flask to dissolve the ^{13}C-triclosan. The flask was shaken at 150 rpm for one day to ensure that the ^{13}C-triclosan was completely dissolved into the NMS medium (the duration of one day was determined previously in the laboratory). The Q-FAST experiment was then initiated in

a new 300-mL flask by adding 1 mL of the triclosan-degrading consortium into 85 mL of the ^{13}C-labeled triclosan-containing NMS medium (the final concentration of ^{13}C-labeled triclosan is 5 mg/L). The inoculated flask was then incubated on a rotary shaker at 150 rpm at 30 ^{o}C. A parallel experiment using unlabeled triclosan was performed as a live control to monitor the progress of triclosan degradation. When the unlabeled triclosan in the live control was completely degraded, liquid sample was collected from the flask amended with ^{13}C-labeled triclosan. The cells in the liquid sample was harvested and used for genomic DNA extraction. The genomic DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedical LLC, Solon, OH), following the manufacturer's instructions. The extracted DNA was further used for cloning and real-time-T-RFLP analysis. Additionally, the genomic DNAs were extracted from original activated sludge sample and triclosan-degrading consortium for real-time-T-RFLP analysis.

4.2.3. Ultracentrifugation, restriction digestion, PCR cloning, sequencing, and phylogenetic analysis

The extracted DNA was further separated into ^{12}C- (lighter) and ^{13}C- (heavier) DNA fractions. The ^{13}C-DNA/^{12}C-DNA separation and purification were performed as described previously (Yu and Chu, 2005). The positive control for the ^{12}C- and ^{13}C-DNA bands was prepared from *Escherichia coli* grown on unlabeled glucose and ^{13}C-glucose, respectively (Roh et al., 2009b; Yu and Chu, 2005). Although no visible ^{13}C-DNA band was observed in the ultracentrifugation tube, approximately 200-300 μL of

^{13}C-DNA was carefully withdrawn from the expected location of that in the positive control tube using a disposable syringe with a sterile 21-gauge hypodermic needle. The extracted ^{13}C-DNA was then used as a template for 16S rRNA gene sequencing to identify the triclosan-utilizing bacteria, and for real-time-T-RFLP analysis as described by Yu et al. (2005).

The 16S rRNA genes in ^{13}C-DNA fraction were amplified with bacterial universal primers as previously described (Yu et al., 2005), except that 35 of PCR cycles were used. The PCR product was cloned into the vector TOP10 (TOPO TA cloning kit) (Invitrogen, Carlsbad, CA). .Clones with inserts were firstly verified by PCR with M13 primers. Eighty clones with inserts were digested with *Msp*I and *Hha*I, and then clones showing unique RFLP patterns were selected. Plasmids of the selected clones were extracted and the insertions were sequenced at the Gene Technology laboratory, Texas A&M University, College Station, TX (Yu et al., 2005). 16S rRNA sequences were assembled and checked for chimera as previously described (Yu et al., 2005). Eleven clones were identified by comparing related sequences of 16S rRNA gene sequences in the GenBank using BLAST. The closest relatives identified from searches were aligned using the bootstrap neighbor-joining method in the CLUSTAL X2 software. A phylogenetic tree was constructed using TreeView software. The 16S rRNA sequences of clones have been deposited in the GenBank database under accession numbers JQ965772-JQ965782.

4.2.4. Real-time-T-RFLP analysis of triclosan-degrading microbial community

The extracted ^{13}C-DNA was used as a template to characterize the active triclosan-degrading microbial community structure using real-time-T-RFLP as described previously (Yu et al., 2005). Additionally, genomic DNAs extracted from original activated sludge and triclosan-degrading consortium were used for real-time-T-RFLP analysis. The PCR products were digested with *Msp*I to generate a profile of terminal-restriction fragments (T-RFs). The lengths of T-RFs were determined automatically using ABI 3130xl Genetic Analyzer (Applied Biosystems Instruments, CA). The microbial community profile is analyzed and expressed as the 16S rRNA gene copies per unit volume for each ribotype (i.e., T-RF). The diversity of microbial community was determined using four indices: richness (S), Shannon-Weaver diversity index (H), evenness (E), and reciprocal of Simpson's index (1/D). These four indices were determined as described previously (Saikaly et al., 2005; Shannon and Weaver, 1963; Simpson, 1949). The *in silico* analysis of 16 rRNA gene sequences (11 clones) was also performed to correlate to the lengths of T-RF obtained from the ^{13}C-DNA fraction.

4.2.5. Monitoring of triclosan degradation by GC/MS analysis

Triclosan concentrations were determined using gas chromatograph/ mass spectrometer (GC/MS) (Lee et al., 2012). Briefly, the liquid samples were extracted with ethyl acetate (1:1 v/v) at 350 rpm for 60 min, followed by adding anhydrous sodium sulfate for dehydration. Then ethyl acetate layer (i.e. the upper-layer) was transferred to a new glass vial, evaporated to dryness, and then reconstituted in 450 μL

of acetone. The reconstituted samples were derivatized with BSTFA (50 μL) in acetone, before used for GC/MS analysis with SIM mode.

4.3. Results and discussion

4.3.1. Identification of active triclosan-utilizing microorganisms in the triclosan-degrading consortium

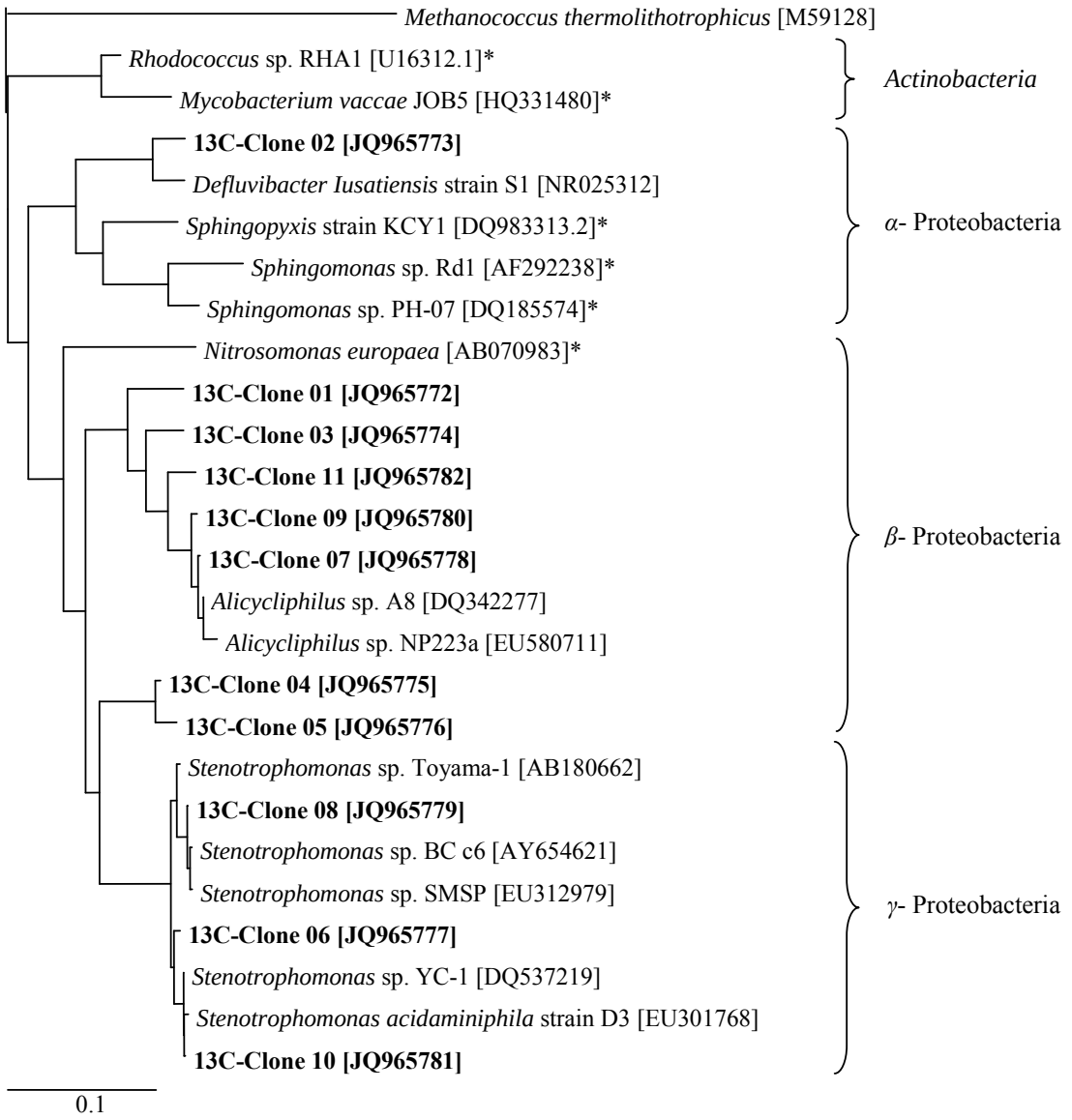
Eleven clones were obtained from the ^{13}C-DNA fraction, suggesting the presence of triclosan-utilizing microorganisms in the consortium. By comparing the 16S rRNA sequences in GenBank, these 11 clones were phylogenetically diverse and affiliated to three different genera: one clone (Clone #2) is related to the genus *Defluvibacter* of *α*-Proteobacteria, seven clones (Clone #1, 3, 4, 5, 7, 9, and 11) to the genus *Alicycliphilus* of *β*-Proteobacteria, and three clones (Clone #6, 8, and 10) to the genus *Stenotrophomonas* of *γ*-Proteobacteria (see Figure 4.1). However, none of the eleven clones are similar to known triclosan-degraders/utilizers.

Bacteria in these three genera are ubiquitous in various natural and engineered environments including WWTPs. Many of those in WWTPs are capable of degrading surfactants, nonyphenols, and recalcitrant chlorinated aromatic compounds (Boonchan et al., 1998; Di Gioia et al., 2004; Fritsche et al., 1999; Liu et al., 2007; Soares et al., 2003; Weelink et al., 2007). The Clone #2 sequence (in *α*-Proteobacteria) had 97% similarity to *Defluvibacter iusatiensis* strain S1, which is known to degrade chlorophenol (Fritsche et al., 1999). Among the seven clones in *β*-Proteobacteria, Clone #7 sequence showed 98% similarity to a known benzene-degrader, *Alicycliphilus* sp. A8 (DQ342277), and

Clone #9 sequence showed 99% similarity to a known nonylphenol ethoxylates-degrader, *Alicycliphilus* sp. NP223a (EU580711) (Weelink et al., 2007). The Clone #6, #8, and #10 sequences had 97-99% similarity to three *Stenotrophomonas* sp. (AB180662, EU312979, and DQ537219), which are capable of degrading dibenzoflan (AB180662), fenitrothion (EU312979), and chlorpyrifos (DQ537219) (Yang et al., 2006). Most of all, these eleven clone sequences are different from known triclosan-degrading bacteria (i.e., less than 93% similarity), suggesting that the wastewater triclosan-utilizing bacteria are phylogenetically diverse.

Cross-feeding is one of limitations for SIP-based applications (Friedrich, 2006; Leigh et al., 2007). In addition to labeling the target degraders (primary), non-target microorganisms might be indirectly labeled through growth on metabolic intermediates released by the primary degraders, or on the labeled biomass of the primary degraders (Leigh et al., 2007; Manefield et al., 2002). A long incubation period during SIP experiments is most likely to contribute to the cross-feeding effect (Gallagher et al., 2005; Leigh et al., 2007). One possible approach to minimize the effect of cross-feeding is to use a RNA-SIP method, because RNA is produced at a higher rate that can be labeled 6.5 times faster than DNA (Manefield et al., 2007) and the RNA production is independent of cellular replication (Whiteley et al., 2002). However, application of RNA-based SIP is also subjected to its own technical limitations, such as broad and fuzzy bands after ultracentrifugation and short-lived RNA in samples (Hirsch et al., 2010; Whiteley et al., 2002).

In this study, the enrichment culture has been transferred to fresh growth medium with unlabeled triclosan every 3 days for 5 times before its use for the SIP experiment (i.e. the triclosan-degrading consortium was used). Through the successive enrichment, it was expected to increase the population of active triclosan-utilizing bacteria in the 5th enrichment culture, making a short incubation time possible during the SIP experiment. In our experiment, an incubation time of 6 days with ^{13}C-triclosan was used. This incubation time was relatively shorter than the incubation times previously reported (> 21 days) (Gallagher et al., 2005; Lin et al., 2004; Radajewski et al., 2002). As mentioned earlier, some heterotrophic wastewater microorganisms are known to degrade triclosan cometabolically (Hay et al., 2001; Kim et al., 2011; Lee et al., 2012; Roh et al., 2009a). Cometabolic biodegradation of triclosan might potentially result in labeling bacteria that are not capable of utilizing triclosan as a carbon source (i.e., non-target bacteria). Our experimental results were unable to exclude the possibility of labeling those non-target bacteria. However, the likelihood of unintended cross-feeding in our experiment is minimal due to the antimicrobial nature of triclosan and the use of the 5th triclosan-enrichment culture.



* Known triclosan-degrading bacteria. It was found that *Rhodococcus* sp. RHA1 and *Mycobacterium vaccae* JOB5 were capable of degrading triclosan (described in Section 5)

Figure 4.1. A phylogenetic tree showing the relative relationship between 16 clones derived from ^{13}C-DNA fraction and known pollutant degraders including triclosan-degrading bacteria. The phylogenetic tree was constructed using the neighbor-joining method with bootstrapping and rooted by referring to *Methanococcus thermolithotrophicus.* Bootstrap support values from 1000 replicates are indicated at branch nodes. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

4.3.2. Active triclosan-degrading microbial community

The active triclosan-degrading microbial community from ^{13}C-DNA fraction was characterized using real-time-T-RFLP analysis. As shown in Figure 4.2, five different ribotypes (T-RF = 99, 104, 106, 108, and 116 bp) were observed in the active triclosan-degrading microbial community. Using *in silico* analysis with *Msp*I, the predicted T-RFs of the eleven clone sequences matched with four T-RFs (99, 104, 106, and 108 bp). Clones #3, #4, #7, and #9 related to the *Alicycliphilus* showed the predicted T-RF of 99 bp. The predicted T-RFs for Clones #1 and #5 related to the *Alicycliphilus*, and for Clone #2 related to the *Defluvibacter* were 104 bp and 105 bp, respectively. Clones #6, 8, 10, and 11 related to the *Stenotrophomonas and Alicycliphilus* had the predicted T-RF of 108 bp. However, given that the measured T-RFs were typically 0-3 bases shorter than the predicted T-RFs (Yu et al., 2005), it is possible that the clones with a predicted T-RF of 108 bp may also contribute to the measured T-RF of 106 bp. Two T-RFs (99- and 108- bp) contributed to approximately 87% of total 16S rRNA gene copies derived from the ^{13}C-DNA fraction, suggesting that dominant *Alicycliphilus*- and *Defluvibacter*-related phylotypes (Clone #2, 3, 4, 7, and 9) may play important role in utilizing triclosan as a carbon source in the triclosan-degrading consortium established from activated sludge.

Clones corresponding to the measured T-RF of 116 bp were not obtained. The lack of clones corresponding to T-RF of 116 bp after screening based on the unique RFLP patterns might be explained by the bias in PCR-based sequencing and cloning. It is known that bacteria in low abundance are difficult to be picked up for cloning and

sequencing (Brose et al., 2003; Wintzingerode et al., 1997). Yet, the low abundance microbial population cannot be undervalued because they may still perform an important function in a mixed culture. For example, ammonia-oxidizing bacteria are present at low concentrations in nitrifying activated sludge and natural environments; but they are responsible for the first step of nitrification (Morales et al., 2009; Rappe and Giovannoni, 2003). With the same reasoning, it is possible that the low abundance strains in the triclosan-enrichment culture might play an important role in triclosan utilization. Application of SIP to label slow-growing bacteria in a mixed consortium is also challenging due to the requirement of long incubation time, which in term might lead to cross-feeding effects (Radajewski et al., 2002). Application of a high-throughput DNA-sequencing method, such as 454 pyrosequencing, might be able to detect the presence of slow-growing and low abundance microorganisms in a given mixed culture (Roesch et al., 2007; Sogin et al., 2006). Future studies are also needed to link the function and the low abundant microorganisms in a mixed culture.

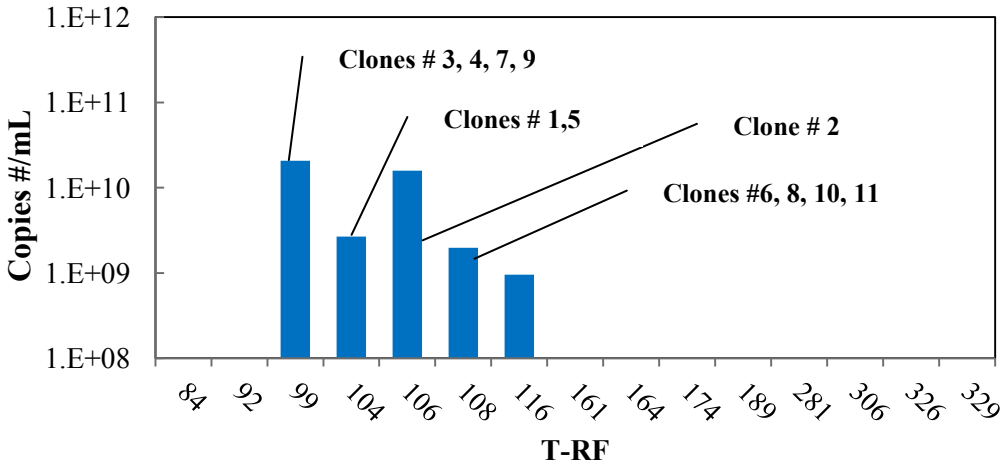


Figure 4.2. Active triclosan-degrading microbial community profile derived from ^{13}C-DNA fraction. Five different ribotypes were observed and the predicted T-RFs of 16 clones corresponded to the measured T-RFs with the copies of 16S rRNA/mL.

4.3.3. Changes in microbial community structure due to triclosan additions

The Change in triclosan-enriched microbial community structure was observed during the consecutive transfers. As shown in Figure 4.3, fourteen different T-RFs, ranging from 1.3×10^{10} to 3.5×10^{11} 16S rRNA gene copies/mL, were detected in the original activated sludge sample. The number of ribotypes (i.e. T-RFs) decreased from 14 in the activated sludge to 9 in the triclosan-degrading consortium. In addition, the biomass of the triclosan-degrading consortium decreased 10-fold from its initial inoculation biomass. The nine ribotypes (T-RFs = 84, 99, 104, 106, 108, 116, 306, 326, and 329 bp) were persisted in the profile during consecutive transfers, suggesting that these ribotypes were either resistant to a high triclosan concentration (5 mg/L) and/or involved in degrading/utilizing triclosan. While microorganisms corresponding to the

nine T-RFs were stably maintained in the microbial community, the bacterial species represented by the 306 bp T-RF appeared during enrichment with triclosan, suggesting that this ribotype might be actively associated with triclosan degradation. However, we cannot exclude the possibility that bacterial species corresponding to the 306 bp T-RF may obtain carbon from dead biomass of wastewater microorganisms.

By comparing to the microbial community profile of the activated sludge (as the inoculum), six ribotypes (T-RFs = 92, 161, 164, 174, 189, and 281 bp) disappeared during the enrichment with triclosan, suggesting that bacterial species corresponding to these six T-RFs were susceptible to triclosan, an antimicrobial agent. Due to analytical detection limits and potential analytical error in low concentrations a higher triclosan concentration than ambient concentrations in wastewater (0.61 to 5.1 μg/L) (Thompson et al., 2005; Yu and Chu, 2009) was used in this study. Hence, we cannot rule out that we may reduce the chance to identify other microorganisms associated with triclosan degradation, but less resistant to a higher triclosan concentration in wastewater.

In silico analysis using the 16S rRNA gene sequences of known wastewater microorganisms in the GenBank was performed in an attempt to link any known wastewater microorganisms to the disappeared, newly emerged, or remaining ribotypes in the triclosan-degrading consortium (Figure 4.3 and Table 4.1). None of known wastewater microorganisms showed a predicted T-RF of 92, 161, 164, 174, 189, or 306 bp (i.e., 6 of 7 ribotypes that disappeared from the triclosan-degrading consortium). *Clostridium sticklandii* (predicted T-RF of 281 bp), which was previously observed in

nitrifying-denitrifying activated sludge (Juretschko et al., 2002), might contribute to 281-bp T-RF and be susceptible to triclosan.

As mentioned earlier, there were 9 remaining ribotypes (T-RFs = 84, 99, 104, 106, 108, 116, 306, 326, and 329) in the triclosan-degrading consortium. Table 4.1 shows that a number of known wastewater microorganisms, including foam-causing bacteria (*Actinobacteria*), filamentous bacteria (*β*-Proteobacteria and *Firmicutes*), nitrifying-denitrifying bacteria (*α*- and *β*-Proteobacteria), and phosphorus-accumulating bacteria (*β*-Proteobacteria and *Actinobacteria*), could contribute to the measured T-RFs of 84, 99, 104, 106, or 108 bp (Yu et al., 2005). Several known triclosan-degrading bacteria could also contribute to the T-RFs of 104 and 106 bp. For example, *Sphingomonas* sp. PH-07, *Sphingomonas* sp. Rd1, and *Sphingopyxis* Strain KCY1 have a predicted T-RF of 106 bp, and *Nitrosomonas europaea* has a predicted T-RF of 107 bp. Due to the complexity of microbial community and the redundancy of microbial function in activated sludge, our real-time-T-RFLP results can only reveal the change of microbial community structure in response to successive triclosan additions. More studies are needed to determine which specific functional groups of wastewater microorganisms are affected by triclosan addition.

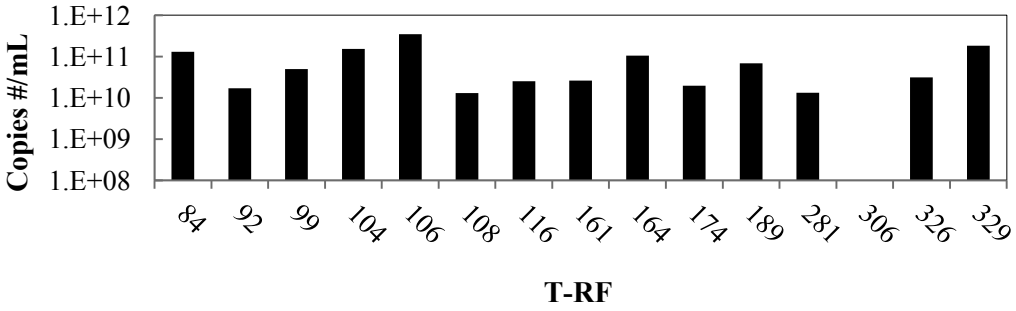
The diversity (richness and evenness) of microbial community structures of original activated sludge and the triclosan-degrading consortium was also examined using four indices (species richness (*S*), the Shannon-Weaver diversity index (*H*), species evenness (*E*), and the reciprocal of Simpson's index (*1/D*)) (Saikaly et al., 2005; Shannon and Weaver, 1963; Simpson, 1949). In response to triclosan addition, a

decrease trend was observed in *S*, *H*, and *E*, but not in *1/D* (Figure 4.3), indicating that the microbial community structure became less diverse, mainly due to fewer species.

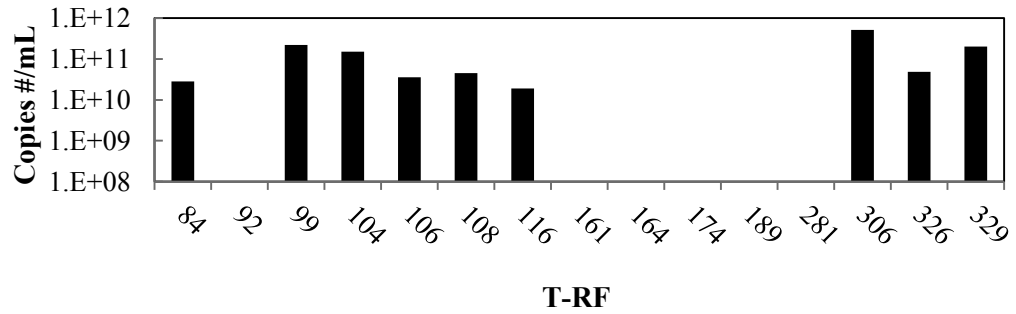
As expected, the decrease in the microbial community diversity is possibly due to the toxicity of triclosan on the wastewater microorganisms. Despite the microbial functional redundancy in a mixed culture like activated sludge, the decrease in diversity in microbial community could ultimately affect the performance of biological wastewater treatment processes. A previous study showed that inhibitory effects on peptone biodegradation sewage sludge at 2 mg/L of triclosan (Offhaus et al., 1978). When activated sludge microorganisms were exposed to triclosan concentrations ranging between 3 and 10 mg/L, D-^{14}C-glucose utilization was inhibited (Orvos et al., 2002). Neumegen et al. (2005) reported that an effective concentration (EC_{50}) of 1.82 mg-triclosan/L for activated sludge microorganisms that are responsible for BOD removal. At 0.75 mg/L of triclosan, little adverse effects on BOD removal and nitrification process were observed (Federle et al., 2002). A decrease trend of mean EC_{50} values (from 38.2 to 9.97 mg/L of triclosan) was observed in activated sludge when solid retention times increased from 5 to 15 days, suggesting that nitrifying bacteria are more sensitive than heterotrophs and potential deterioration performance of nitrification was suggested (Stasinakis et al., 2008). Addition of 0.5 mg/L of triclosan into a non-acclimated activated sludge could significantly inhibit nitrification process (Stasinakis et al., 2007). Liu (2008) reported that decrease in the growth rate of bacteria in activated sludge was a function of triclosan concentration. However, there are no studies examining the long-term toxic effects of triclosan at environmental relevant levels on the

diversity and function of microbial communities in activated sludge, surface water, soils and sediments.

(A)



(B)



Sample	$S^{(a)}$	$H^{(b)}$	$E^{(C)}$	$1/D^{(d)}$
Activated sludge	14	4.126	1.084	3.887
triclosan-degrading consortium	9	2.450	0.779	4.177

(a) *S*: Richness; (b) *H*: Shannon-Weaver Index; (c) *E*: Evenness; (d) *1/D*: Reciprocal of Simpson's Index

Figure 4.3. Comparison of real-time-T-RFLP profiles and the diversity indices between (A) activated sludge and (B) triclosan-degrading consortium.

Table 4.1. Comparison of observed T-RFs to the T-RFs of known wastewater microorganisms

Wastewater microorganims[1,2]		**Predicted T-RF size (bp)[3]**
foam-causing bacteria	*Gordona amarae*	84
filamentous bacteria	Eikelboom Type 1863 *Leptothrix cholodnii* *Leptothrix mobilis* *Leptothrix discophora* *Sphaerotilus natans* *Lactosphaera pasteurii*	84, 99, 104, 106
nitrifying-denitrifying bacteria	*Comamonas denitrificans* *Pseudomonas stutzeri* *Nitrosomonas europaea* *Hyphomicrobium zavarzinii* *Nitrobacter winogradskyi* *Nitrosomonas marina* *Nitrosomonas eutropha*	104, 106
phosphorus-accumulating bacteria	*Rhodocyclus* sp. strain R6 *Tetrasphaera australienesis* *Tetrasphaera elongata*	104, 106, 108
triclosan-degrading bacteria	*Sphingomonas* sp. PH-07 *Sphingomonas* sp. Rd1 *Sphingopyxis* strain KCY1 *Nitrosomonas europaea*	104, 106

[1] Common wastewater microorganims cited from previous studies (Yan et al., 2007; Yu et al., 2005).

[2] Known triclosan degrading bacteria cited from previous studies (Hay et al., 2001; Kim et al., 2011; Lee et al., 2012; Roh et al., 2009a).

[3] The predicted T-RFs (*in silico*) digested with *Msp*I restriction enzyme with the assumption that T-RFs measured by the real-time-T-RFLP are typically 0-3 bases shorter than the predicted T-RFs (Yu et al., 2005).

5. EFFECTS OF GROWTH SUBSTRATE ON TRICLOSAN BIODEGRADATION POTENTIAL OF OXYGENASE-EXPRESSING BACTERIA

5.1. Introduction

Triclosan is one of concerned pharmaceutical and personal care products that are present in treated wastewater. In the past several decades, triclosan has been used as an antimicrobial agent in soaps, deodorants, toothpastes, and various plastic products (Dann and Hontela, 2011; Latch et al., 2003). Triclosan has been detected in surface water, wastewater, and soil, and even in human breast milk, blood, and urine samples (Dann and Hontela, 2011). Detection of environmental triclosan has raised a great concern, since triclosan is a weak endocrine disrupting compound (Foran et al., 2000) and can be transformed into more toxic chlorinated compounds in the environment (Latch et al., 2003). Previous studies also suggest that trace triclosan could promote the development of cross-resistance to antibiotics among bacteria (Schweizer, 2001). While triclosan is not regulated in the United States, it is currently restricted or banned in many countries, including Canada, Japan, and Denmark (Dann and Hontela, 2011; Winter, 1994).

Except two soil triclosan-utilizing bacteria (Meade et al., 2001), several aerobic bacteria are known to degrade triclosan by oxygenases via cometabolic reactions (Hay et al., 2001; Kim et al., 2011; Lee et al., 2012; Roh et al., 2009a). Roh et al. (2009a) reported that ammonia-oxidizing bacteria (AOB) can degrade triclosan and suggested that ammonia monooxygenase (AMO) is responsible for the degradation. Triclosan was

degraded by *Sphingomonas* sp. Rd1 grown on complex medium (Hay et al., 2001). A diphenyl ether-grown *Sphingomonas* sp. PH-07, presumably expressing diphenyl ether dioxygenase, is also known to degrade triclosan (Kim et al., 2011). Recently, Lee et al. (2012) reported biodegradation of triclosan by a wastewater isolate, *Sphingopyxis* strain KCY1 when grown with 20% R2A medium containing triclosan. Strain KCY1 can also degrade biphenyl cometabolically. Both AMO (Chang et al., 2002; Hyman et al., 1988; Keener and Arp, 1994; Rasche et al., 1991; Sayavedra-Soto et al., 2010) and biphenyl dioxygenases (Fritsche and Hofrichter, 2005; Furukawa et al., 2004; Haritash and Kaushik, 2009; Robrock et al., 2011) are broad-substrate monooxygenases and known for their ability to cometabolize a wide range of compounds, including aliphatic and/or aromatic compounds. Accordingly, it is possible that other oxygenase-expressing bacteria can degrade triclosan via cometabolic reactions.

Product toxicity is an unfavorable phenomenon during cometabolic degradation. The degradation metabolites of a cometabolic substrate can inactivate degradative microorganisms/enzymes, resulting in a finite transformation capacity for the cometabolic substrate, T_c (defined as mass of cometabolic substrate degraded over mass of biomass inactivated) (Chu and Alvarez-Cohen, 1998). When triclosan is a cometabolic substrate, the degradation of the degradative microorganisms might be potentially limited by triclosan product toxicity.

This study examined the biodegradation potential of triclosan by several model oxygenase-expressing bacteria grown on different substrates. As triclosan is polychlorinated hydroxydiphenyl ether, four oxygenase-expressing bacteria capable of

degrading ethers and/or biphenyl, were selected for this study. The selected bacteria are *Mycobacterium vaccae* JOB5 (Perry, 1968), *Rhodococcus ruber* ENV425 (Steffan et al., 1997), *Rhodococcus jostii* RHA1(Seto et al., 1995), and *Burkholderia xenovorans* LB400 (Seeger et al., 1997) (hereafter, called JOB5, ENV425, RHA1, and LB400, respectively). When grown on propane, JOB5 and ENV425 express propane monooxygenases (PMO) to degrade different ethers, including methyl *tert*-butyl ether (MTBE) (Steffan et al., 1997; Vainberg et al., 2006; Wackett et al., 1989). Biphenyl-grown RHA1 and LB400 are known for their ability to express biphenyl dioxygenase for the biodegradation of polychlorinated biphenyls (PCBs) (Arnett et al., 2000; Masai et al., 1997) and polybrominated diphenyl ethers (PBDEs) (Robrock et al., 2009).

Interestingly, all these four strains can grow on a wide range of carbon sources and express different oxygenases in responding to their growth substrates. For example, RHA1 can grow on biphenyl and propane to express biphenyl dioxygenase and PMO, respectively (Masai et al., 1995; McLeod et al., 2006). JOB5 can express PMO when grown on propane (Vanderberg and Perry, 1994), or express butane monooxygenase when grown on butane (Hamamura et al., 1999), or express alkane monooxygenase (AlkMO) when grown on alkanes (Ferreira et al. 2007). RHA1 also contains AlkMO gene clusters (McLeod et al., 2006). Thus, in this study, we investigated the effects of different growth substrates (propane, 2-propanol, biphenyl, and complex medium like Luria-Bertani (LB) medium) on triclosan degradation potential of these four model strains. The biodegradation potential of each strain grown under different growth conditions was evaluated in terms of its initial degradation rate, degree of dechlorination,

and transformation capacity (T_c) if product toxicity occurs. Viability of cells before and after exposure to triclosan was determined by counting the colonies on a plate or by quantifying the 16S rRNA gene copies of the viable cells. In addition, triclosan degradation metabolites and pathway by these strains were examined.

5.2. Material and methods

5.2.1. Chemicals

Triclosan (97%) and biphenyl (99%) were purchased from Aldrich Chemical Inc. (Milwaukee, WI). Propane gas (> 99.9%) and sodium formate were purchased from MP Biomedicals Inc. (Solon, Ohio). Tetrazotized *o*-dianisidine was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). 2-Propanol (> 99%), dicyclopropylketone (DCPK) (95%), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). 3-Fluorocatechol and naphthalene (99.6%) was purchased from Alfar Aesar (Ward Hill, MA). Propidium monoazide (PMA) was purchased from Biotium Inc. (Hayward, CA). Bicinchoninic acid (BCA) protein assay reagent kit and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Stock solution of triclosan (1 g/L) was prepared in acetone.

5.2.2. Strains

JOB5 and ENV425 were kindly provided by Dr. Robert Steffan, Shaw Environmental Inc. (Lawrenceville, NJ). LB400 was kindly provided by Dr. Rebecca

Parales, University of California, Davis, CA. RHA1 was kindly provided by Dr. Bill Mohn, University of British Columbia, Canada.

5.2.3. Culture conditions

Four different growth substrates (propane, 2-propanol, biphenyl, and Luria-Bertani (LB) medium) were used in this study (Table 5.1). To induce the expression of PMO, JOB5, ENV425, and RHA1 were pregrown in nitrate mineral salts (NMS) medium (Chu and Alvarez-Cohen, 1996) with propane (40%, vol/vol) in glass bottles (280 mL) sealed with Teflon mininert-valved screw caps. JOB5 and RHA1 were also pregrown on NMS medium containing 2-propanol (10 mM). 2-Propanol was used as a growth substrate to presumably avoid the induction of propane monooxygenase (Kulikova and Bezborodov, 2001). To induce the expression of biphenyl dioxygenases, LB400 and RHA1 were pregrown in NMS medium with 10 mM of biphenyl. LB medium was used as a model rich complex medium to avoid expression of PMO or biphenyl dioxygenase in the four strains. As degradative ability of strain KCY1 was induced when triclosan (5 mg/L) was present in 20% LB medium, all strains were also grown in LB medium containing triclosan (5 mg/L) to determine whether triclosan addition can trigger the induction of enzymes for triclosan degradation. Additionally, all strains were pregrown in LB medium and 1 mM of dicyclopropylketone (DCPK), a gratuitous inducer of *alk*B genes for alkane monooxygenase activity (Smith and Hyman, 2004).

All cultures were incubated aerobically on a rotary shaker at 160 rpm at 30 ^{o}C. The culture medium occupies no more than 20% of the total flask/glass bottle volume to avoid oxygen limitations during the course of experiments. Cell biomass was quantified as protein content using a BCA protein assay kit and/or determined as optical density (OD_{600}) at 600 nm using a UV-visible spectrophotometer (HP G1130A). The activity of non-specific monooxygenase enzyme in the pregrown cells was confirmed using colorimetric naphthalene oxidation assay as described previously (Chu & Alvarez-Cohen, 1998). Briefly, 1 mL of cell suspension (OD_{600} ~ 0.2) was incubated at 150 rpm and 35 ^{o}C for 60 min with 1 mL of 243 μM naphthalene and 1 mL of 20 mM sodium formate. Then, 100 μL of 0.2% (wt/vol, freshly made) tetrazotized *o*-dianisidine was added to the reaction mixture. The purple color appearance (absorbance at 530 nm) in the assay indicated the positive monooxygenase activity. A mixture containing cell suspension and sodium formate (without naphthalene) was used as a negative control. Duplicate samples were used.

5.2.4. Triclosan degradation tests with resting bacterial strains

Degradation ability toward triclosan of the resting cells pregrown on different growth substrates was determined as follows. The pregrown cells were harvested in the late exponential growth phase (OD_{600} ~ 1.0) by centrifugation at 10,000g for 5 min, and then resuspended in fresh NMS medium for degradation tests. The experiments were conducted similarly as described by (Lee et al., 2012). Briefly, the degradation tests were initiated in 40 mL glass vials containing 5 mg/L of triclosan and 10 mL of the

resting cell suspensions, and then the vials were incubated on a rotary shaker at 160 rpm at 30 ^{o}C. Liquid samples were taken over time for triclosan and chloride measurements as described previously (Lee et al., 2012).

Parallel sets of experiments amended with acetylene (an inhibitor of non-specific monooxygenases) were used to assess whether non-specific monooxygenases are responsible for triclosan degradation. After 6 hrs of the initiation of the degradation tests, acetylene (5%) was amended into the headspace of the vials. After 10 min, the residual acetylene gas was purged from the vials. Cessation of triclosan degradation after acetylene treatment would indicate the involvement of non-specific monooxygenases in triclosan degradation. Killed controls were used. Duplicates were used in all experiments.

5.2.5. Tests for determination of toxicity due to triclosan and/or its transformation products

Experiments were conducted to examine whether triclosan itself and/or its degradation metabolites would exert toxicity on the degradative cells. The toxicity experiments were conducted similarly as described in degradation tests. Viability of cells before and after triclosan was determined by plate colony counts and by quantifying the 16S rRNA gene copies of the viable cells. The 16S rRNA gene copies of the viable cells were determined using real-time PCR with the pretreatment of propidium monoazide (PMA), a DNA intercalating dye (Nocker et al., 2007). PMA can readily penetrate into cells with compromised membrane (presumably dead) to form covalent

bonds with the DNA of the dead cells, which cannot be PCR amplified (Bae and Wuertz, 2009; Kobayashi et al., 2010). Thus, the pretreatment of samples with PMA will allow for discriminating between viable and dead cells. Transformation capacity (T_c) (Chu and Alvarez-Cohen, 1998) for triclosan was calculated with a minor modification, i.e., by dividing the amount of triclosan degraded by the 16S rRNA gene copy number of cells inactivated.

5.2.6. Detection of triclosan degradation metabolites transformed by resting cells

Experiments were conducted to identify triclosan metabolites. Only cells shown triclosan degradation ability were used in this experiment. Briefly, the experiment was initiated in 40 mL glass vials containing 5 mg/L of triclosan and 10 mL of the resting cell suspensions. The vials were incubated on a rotary shaker at 160 rpm at 30 ^{o}C (Lee et al., 2012).

5.2.7. Chemical analysis

Triclosan concentrations in the liquid samples were analyzed as described previously (Lee et al., 2012). Briefly, Liquid samples were collected over time and extracted with ethyl acetate (1:1 v/v) at 350 rpm for 60 min. The extracts were derivatized BSTFA which replace active hydrogens with a –Si $(CH_3)_3$ (trimethylsilyl) group, before use for analyses. Triclosan concentrations and triclosan biodegradation metabolites were determined using a GC (Agilent 6890 system II)/MS (Agilent 5973 MS system) equipped with DB-5 column in selective ion monitoring (SIM) mode and full

scan mode as described previously (Lee et al., 2012). The detection limit for triclosan in SIM mode was 0.05 mg/L.

Because triclosan is a chlorinated compound, the amount of chloride release from triclosan biodegradation could be used to assess the degree of dechlorination. The chloride concentrations were measured using a DX-80 ion chromatography (IC) (Dionex, Sunnyvale, CA) equipped with an IonPac AS14A-5μm analytical column (3 × 150 mm). An eluent solution containing 0.16 M Na_2CO_3 and 0.02 M $NaHCO_3$ was used. The detection limit for chloride was 0.05 mg/L.

5.2.8. Quantification of viable cells

Viable cells were determined by colony plate counts or by quantifying the gene copies of the viable cells. Cell suspensions before and after triclosan exposure were serially diluted and used for conventional colony counts on R2A plates. A subset of the cell suspensions were pretreated with PMA before real-time PCR analysis for 16S rRNA gene copies.

A 20 mM of PMA was prepared in 20% (v/v) DMSO as a stock solution. A known amount of the PMA stock solution (1.3 μl) was added to a cell suspension (500-μl) in a 2-mL transparent microcentrfuge tube to reach a final concentration of 50 μM. PMA cross-linking procedure was carried out according to the manufacturer's instruction (Biotium Inc., Hayward, CA).

After the PMA cross-linking reaction, the genomic DNA was extracted from each 500-μl aliquot using a FastDNA kit as described previously (Yu et al., 2005). The

extracted DNA was used as a template for real-time PCR amplification for 16S rRNA gene copies using a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Each 25 μL PCR mixture contained 600 nM forward (1055F) and reverse primers (1392R) and 12.5 μL SYBR Green I mix (Qiagen, Valencia, CA). The thermal cycling protocol as follows: 95 °C for 15 min; 45 cycles of 95 °C for 30s, 50 °C for 1 min, and 72 °C for 2 min. Standard curves for the bacterial 16S rRNA gene concentration were constructed as described previously (Yu et al., 2005).

5.3. Results and discussion

5.3.1. Degradation of triclosan by resting cells grown on propane or 2-propanol

The triclosan degradation potential of the cells grown on propane was examined. Propane-grown RHA1 and JOB5 were able to degrade triclosan (5 mg/L); however, propane-grown ENV425 was unable to degrade triclosan (Figure 5.1). Propane-grown RHA1 degraded only 35% of the added triclosan, with an initial degradation rate of 0.018 ± 0.002 mg-triclosan/mg-protein/day and no release of chloride (Table 5.1). Propane-grown JOB5 showed a better degradation ability toward triclosan than that of propane-grown RHA1. While JOB5 degraded approximately 95% of triclosan with an initial degradation rate of 0.021 ± 0.003 mg-triclosan/mg-protein/day, small amount of chloride was detected (0.27 mg/L) (Table 5.1).

Table 5.1. Triclosan degradation ability of bacterial strains grown on different substrates.

Growth substrate	Bacterial strains	Triclosan degradation [Cl^- released (mg/L)]	Reference
Propane	Strain JOB5	yes [0.27]	This study
	Strain RHA1	yes [ND]	
	Strain ENV425	no	
2-Propanol	Strain JOB5	yes [0.12]	This study
	Strain RHA1	no	
Biphenyl	Strain RHA1	yes [0.26]	This study
	Strain LB400	no	This study & (Kim et al., 2011)
Diphenyl ether	*Sphingomonas* sp. PH-07	yes [NA]	(Kim et al., 2011)
LB	Strain JOB5	no	This study
	Strain RHA1	no	
	Strain ENV425	no	
	Strain LB400	no	
LB + triclosan	Strain JOB5	no	This study
	Strain RHA1	no	
	Strain ENV425	no	
	Strain LB400	no	
LB + DCPK	Strain JOB5	no	This study
	Strain RHA1	yes [0.13]	
	Strain ENV425	no	
	Strain LB400	no	
[*] **R2A + triclosan**	Strain KCY1	yes [1.96]	(Lee et al., 2012)

[ǂ] 3 mole of chloride is theoretically released per mole of triclosan.

[*] R2A medium (20 %); triclosan concentration: 5 mg/L.

ND= not detected; NA= not available.

Assuming 1 mol of triclosan degraded will release 3 mol of chloride, this chloride concentration (0.27 mg/L) is 83-fold lower than the theoretical value (1.74 mg/L). Unlike 100% dechlorination of triclosan exhibited by strain KCY1 (Lee et al., 2012), these results suggest that triclosan was partially dechlorination by these two propane-grown JOB5 and RHA1.

All propane-grown cells showed positive results with the naphthalene assay, indicating non-specific PMO enzyme was active. The activity of PMO in propane-grown RHA1 and JOB5 was inhibited by exposing to acetylene. RHA1 is known to express PMO when grown on propane (Sharp et al., 2007). As triclosan was degraded by propane-grown RHA1 but not by acetylene-treated, propane-grown RHA1 (Figure 5.1A), our results suggest that PMO was responsible for the oxidation of triclosan. Interestingly, triclosan degradation was observed in acetylene-treated, propane-grown JOB5 (Figure 5.1B), suggesting that other unidentified enzymes, in addition to PMO, could degrade triclosan. To our surprise, propane-grown ENV425 was unable to degrade triclosan. The inability of ENV425 to degrade triclosan was unclear. One possible explanation is that the PMO expressed by strain ENV425 might have a narrower substrate specificity range than those expressed by strains RHA1 and JOB5.

No triclosan degradation was observed for RHA1 grown on 2-propanol (Figure 5.2), further supporting that PMO was responsible for triclosan degradation (Figure 5.1A). On the other hand, 2-propanol-grown JOB5 degraded triclosan at an initial degradation rate of 0.022 ± 0.001 mg-triclosan/mg-protein/day. This degradation rate was comparable to that observed in propane-grown JOB5. However, the amount of chloride released (0.12 mg/L) by 2-propanol-grown JOB5 was two-fold less than that by propane-grown JOB5 (0.27 mg/L) (Table 5.1).

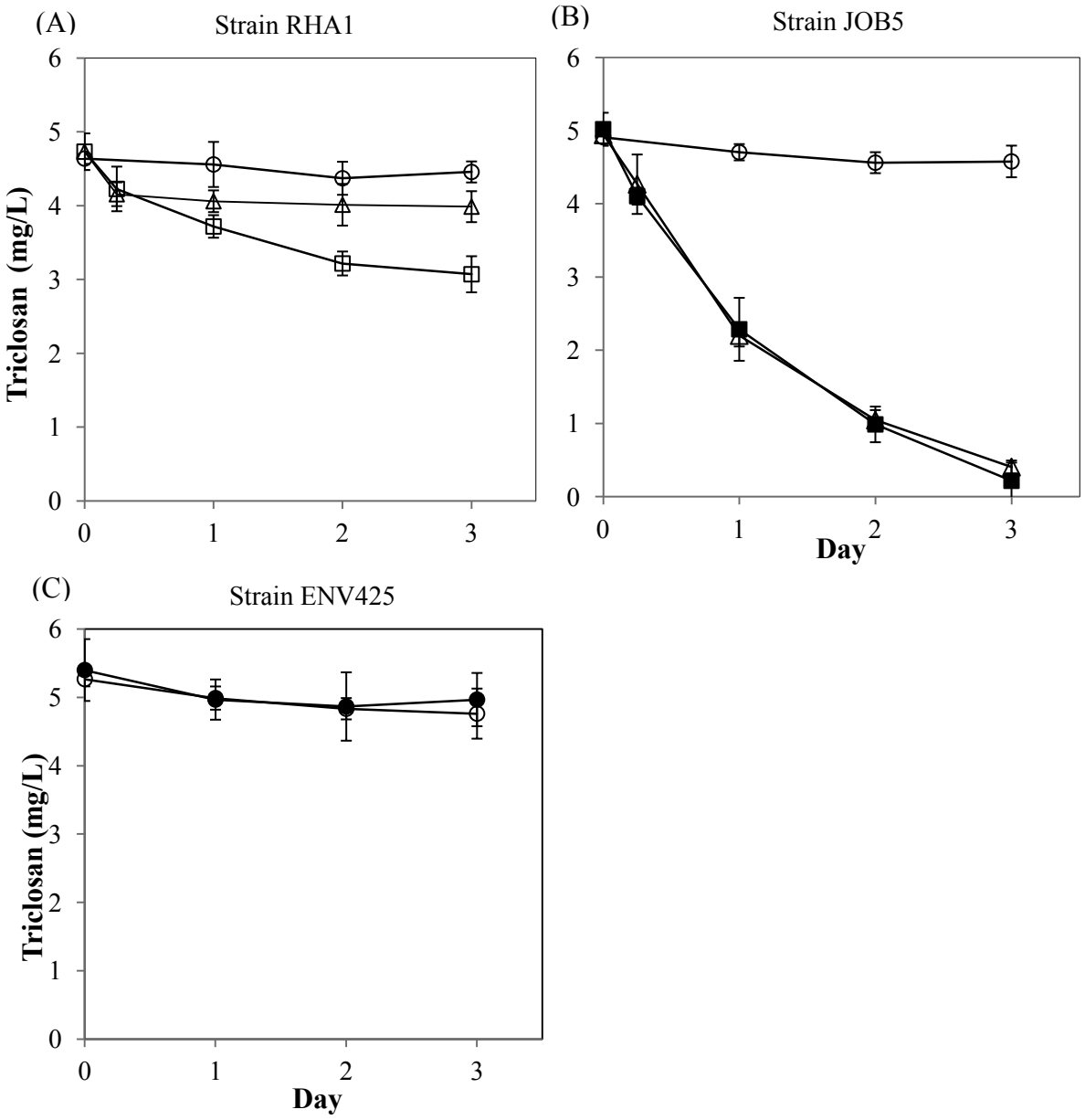


Figure 5.1. Triclosan degradation by propane-grown resting cells: (A) RHA1, (B) JOB5 and (C) ENV425. Symbols: □, strain RHA1; ■, strain JOB5; ●, strain ENV425; ○, killed control; Δ, cells treated with acetylene gas. The error bars represent the range of duplicates.

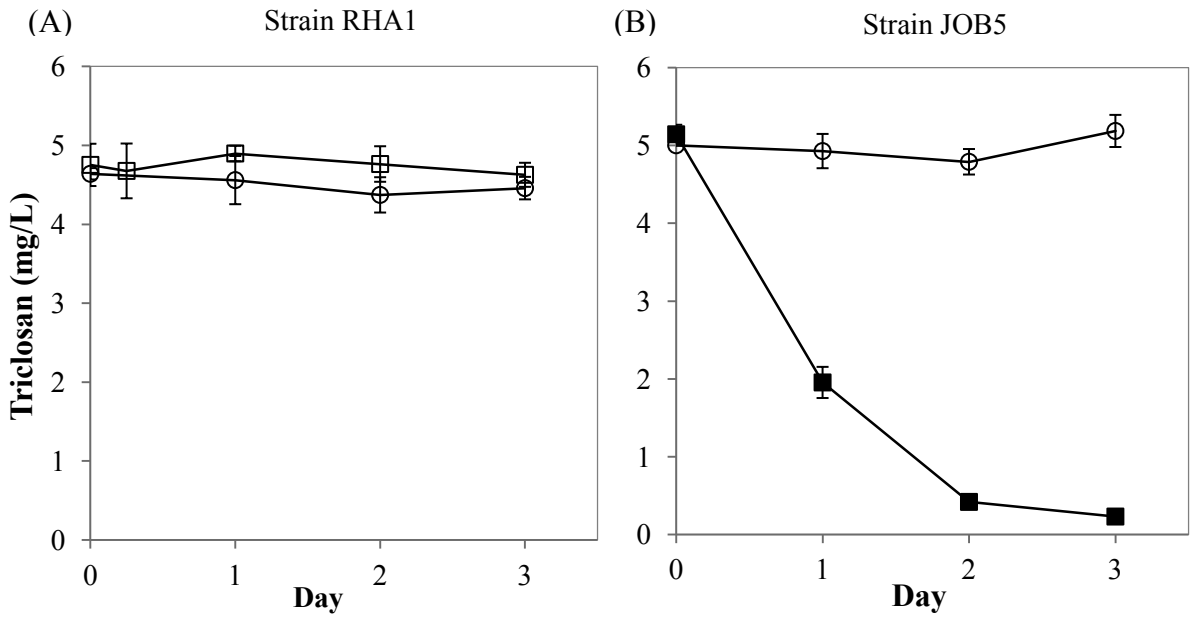


Figure 5.2. Triclosan degradation by 2-propanol grown resting cells: (A) RHA1 and (B) JOB5. Symbols: □, strain RHA1; ■, strain JOB5; ○, killed control. The error bars represent the range of duplicates.

Although it is unclear if 2-propanol can also induce PMO or other enzymes in JOB5, our results suggest that triclosan can be degraded by PMO and/or other unknown enzymes in JOB5. Future studies are needed to identify the unknown degradative enzymes in JOB5.

5.3.2. Degradation of triclosan by resting cells grown on biphenyl

As triclosan is structurally similar to polychloro-biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), it was hypothesized that bacteria capable of degrading PCBs and PBDEs, such as biphenyl-gown RHA1 and LB400, might be able to degrade triclosan. As expected, biphenyl-grown RHA1 degraded triclosan (64% of

triclosan added) at an initial degradation rate of 0.030 ± 0.008 mg-triclosan/mg-protein/day (Figure 5.3) and a release of chloride (0.26 mg/L) (Table 5.1). Based on initial triclosan degradation rate, amount of triclosan degraded and chloride released, biphenyl-grown RHA1 showed a better degradation ability toward triclosan than that of propane-grown RHA1. Biphenyl-grown RHA1 can effectively degrade 2,4,4'- tri-CB, 2,2',4-tri-CB, and 2,2',4,4'-tetra-CB (Seto et al., 1995) and fully debrominate tetra-BDE 47 (2,2',4,4') (Robrock et al., 2009). However, in this study, the extents of triclosan dechlorination by both propane- and biphenyl-grown RHA1 were small. The small dechlorination extents for triclosan might be due to the difference in substrate regiospecificity of the biphenyl dioxygenase expressed by RHA1. Although triclosan is structurally similar to PBDEs and PCBs, the hydroxyl-group in triclosan may affect its polarity, reactivity, and dielectric characteristics (Arnett et al., 2000; Dos Santos et al., 1999; Norris and Cortese, 1927), leading to a change in the regiospecificity of the biphenyl dioxygenase.

Triclosan degradation was not observed for biphenyl-grown LB400. The result was similar to the observation that biphenyl-ether-grown LB400 did not degrade triclosan (Kim et al., 2011). The inability of LB400 to degrade triclosan might be due to the direct triclosan toxicity (see the results of toxicity below).

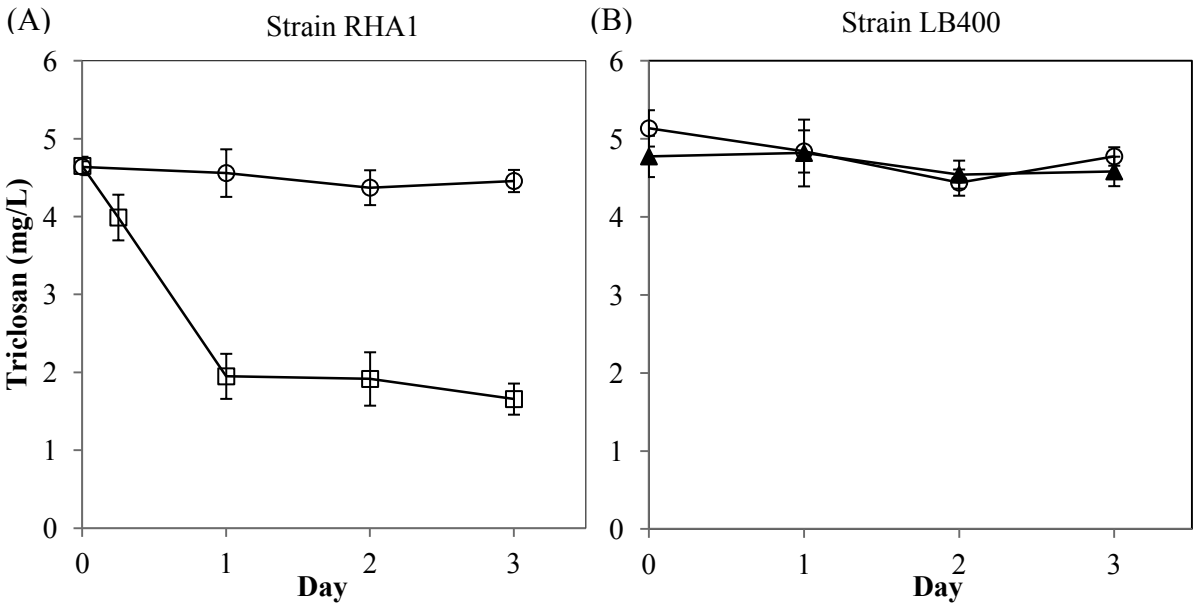


Figure 5.3. Triclosan degradation by biphenyl-grown resting cells: (A) RHA1 and (B) LB400. Symbols: □, strain RHA1; ▲, strain LB400; ○, killed control. The error bars represent the range of duplicates.

5.3.3. Degradation of triclosan by resting cells grown on LB medium

All strains (JOB5, ENV425, LB400 and RHA1) can grow in LB medium. This complex medium was used to promote the growth of cells without the induction of a non-specific oxygenase enzyme. As expected, no triclosan degradation was observed for all LB-grown strains (Figure 5.4). Similarly none of LB + triclosan (5 mg/L)-grown cells showed an ability to degrade triclosan.

Since both JOB5 and RHA1 contain alkane monooxygenase (AlkMO) gene clusters (Lopes Ferreira et al., 2007; McLeod et al., 2006), experiments were conducted to determine if triclosan can be degraded by AlkMO expressed by these two strains. DCPK was amended in LB medium to presumably induce the expression of AlkMO. No triclosan degradation was observed in the experiments using LB+DCPK-grown- LB400, ENV425 and JOB5. However, LB+DCPK-grown RHA1 degraded triclosan (63%) at an initial degradation rate of 0.101 ± 0.003 mg-triclosan/mg-protein/day (Figure 5.4) with a release of the small amount of chloride (0.13 mg/L) (Table 5.1). The observations suggest that AlkMO was involved in triclosan degradation by LB+DCPK-grown RHA1. Previous studies have shown that RHA1 has both PMO and AlkMO gene clusters (McLeod et al., 2006) and AlkMO in RHA1 is co-upregulated by propane (Sharp et al., 2007). Thus, it is possible that AlkMO was also involved in triclosan oxidation by propane-grown RHA1. Overall, our current data suggest that both AlkMO and PMO expressed by RHA1 can degrade triclosan.

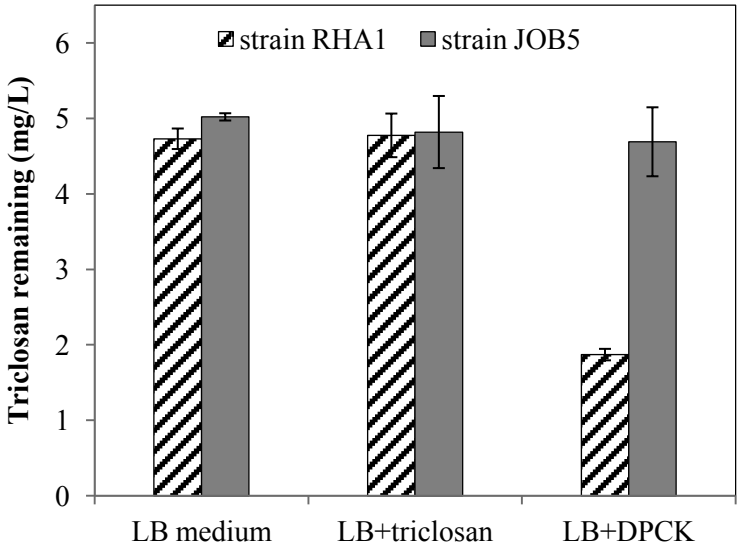


Figure 5.4. Effects of growth conditions on triclosan degradation by RHA1 and JOB5: LB medium; LB with triclosan; and LB with DPCK. The error bars represent the range of duplicates.

5.3.4. Toxicity of triclosan, product toxicity, and transformation capacity

As triclosan is an antimicrobial agent and was cometabolically degraded by the model strains, additional experiments were conducted to determine if their degradation potential was affected by the direct toxicity of triclosan and/or triclosan transformation metabolites. A significant decrease in cell viability (by counting colony formation) were observed between before and after exposed cells of LB400 to triclosan (Figure 5.5A), indicating that triclosan has direct toxic effect on LB400. The triclosan toxicity could explain the inability of biphenyl-grown LB400 to degrade triclosan (Figure 5.3B).

In contrast, no significant difference in the cell viability was observed for triclosan-exposed ENV425 (Figure 5.5A), suggesting that ENV425 is resistant to triclosan. The inability to degrade triclosan by propane-grown ENV425 (Figure 5.1C) is

unknown. Although the PMO of ENV425 was 91% homologous to the PMO of RHA1 (Hatzinger and Hawari, 2008), it might be due to the difference in the enzyme regiospecificity for triclosan.

Cessation of triclosan degradation by RHA1 (propane-, biphenyl-, or LB+DCPK-grown RHA1) was observed in Figure 5.1A, 3A, and 4. The phenomenon could be caused by the lack to reducing energy (i.e., NADH) or product toxicity during triclosan degradation. To examine the effects of reducing energy on triclosan degradation, a parallel set of degradation tests was conducted using 20 mM sodium formate as external reducing energy source and resting cells of LB+DCPK- or propane-grow RHA1. The addition of formate did not enhance triclosan degradation (data not shown), suggesting that product toxicity possibly caused the cessation of triclosan degradation by RHA1.

The effects of product toxicity on triclosan degradation by RHA1 were observed. The number of viable RHA1 decreased significantly after triclosan degradation, suggesting the occurrence of product toxicity from triclosan degradation on RHA1 (Figure 5.5B). The product toxicity caused the cessation of triclosan degradation and resulted in a finite transformation capacity (T_c) for triclosan, defined as amount of triclosan degraded/number of cell inactivated. The estimated T_c values were $5.63 \pm 0.38 \times 10^{-3}$ ng-triclosan/16S rRNA gene copies for biphenyl-grown RHA1, $0.62 \pm 0.02 \times 10^{-3}$ ng-triclosan/16S rRNA gene copies for LB+DCPK-grown RHA1, and $0.20 \pm 0.03 \times 10^{-3}$ ng-triclosan/16S rRNA gene copies for propane-grown RHA1 (Figure 5.5C).

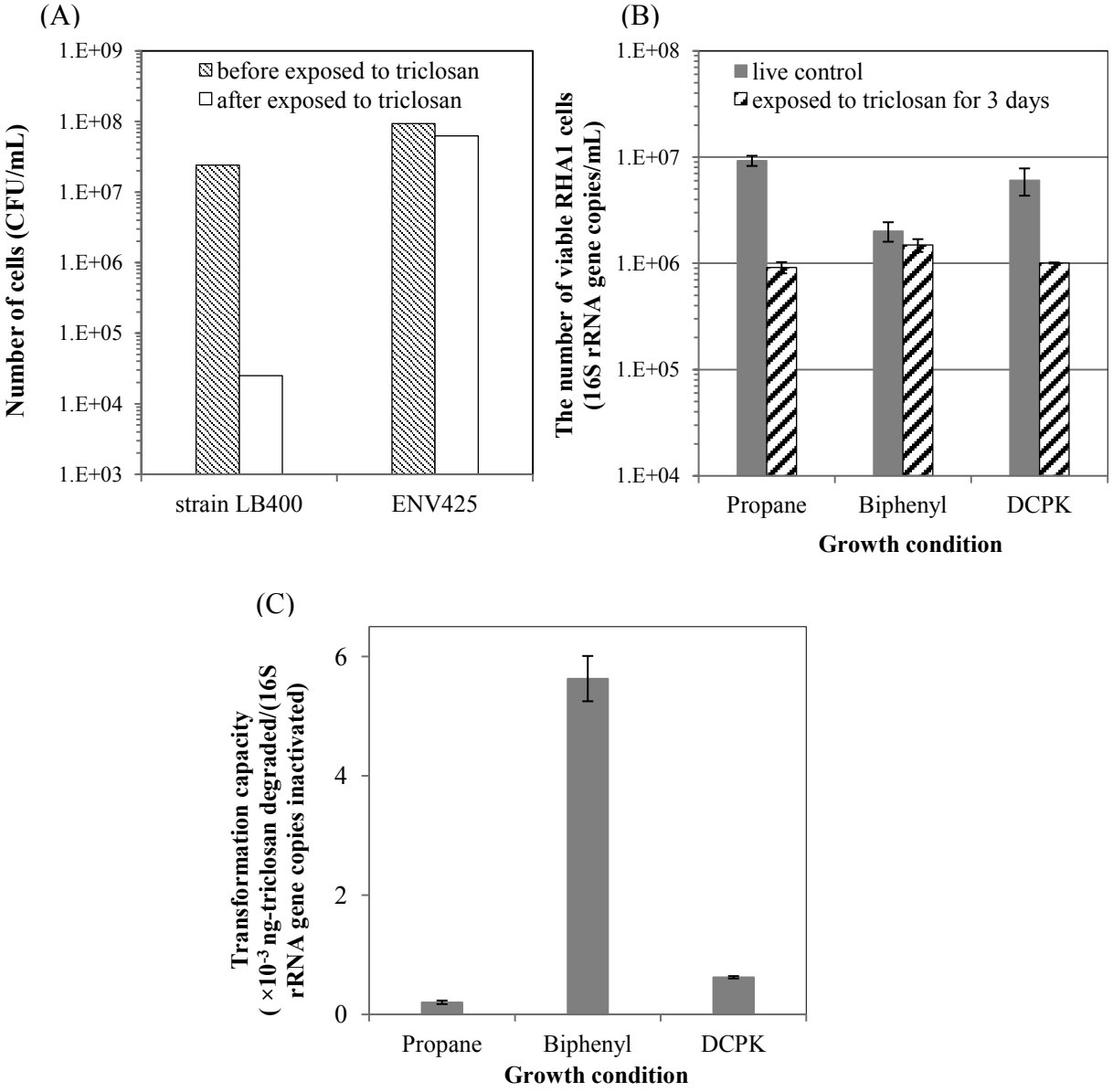


Figure 5.5. (A) The number of LB400 and ENV425 cells before/after exposed to triclosan, (B) the number of 16S rRNA gene copies of RHA1 with different growth conditions, and (C) transformation capacity of RHA1 under different growth conditions. The error bars represent the range of duplicates.

RHA1 is known to contain more than 200 di- or monooxygenase enzymes and the expression of these enzymes is growth substrate dependent (McLeod et al., 2006). In this study, RHA1 was experienced with different extents of triclosan product toxicity which was growth substrate dependent. The propane- and LB+DCPK-grown RHA1 (expressing PMO and AlkMO, respectively) were more susceptible to triclosan degradation product toxicity than the biphenyl-grown RHA1 (expressing biphenyl-dioxygenase). A general trend—degradation product toxicity was more significant to RHA1 cells when monooxygenases were involved in triclosan degradation—was observed.

5.3.5. Triclosan transformation metabolites and degradation pathway

Experiments using degradative strains were conducted to identify degradation metabolites during triclosan degradation. Despite repeated efforts, no triclosan metabolites were detected during triclosan degradation by propane-grown JOB5, propane-grown RHA1 and LB+DCPK-grown RHA1. When using biphenyl-grown RHA1 for triclosan degradation, four metabolites (2,4-dichlorophenol, 2-chlorohydroquinone, monohydroxy-triclosan, and dihydroxy-triclosan) were detected. Monohydroxy-triclosan and 2,4-dichlorophenol were detected in the 6-hr and 12-hr samples. 2-Chlorohydroquinone was detected. Monohydroxy-triclosan and 2,4-dichlorophenol were detected in 6-hr and 12-hr samples. 2-Chlorohydroquinone was detected in 12-hr and 24-hr samples. Detectable chloride was observed in samples collected after 24 hr (data not shown). Dihydroxy-triclosan was detected in the 6-hr, 12-

hr and 24-hr samples. Monohydroxy-triclosan, dihydroxy-triclosan, and 2,4-dichlorophenol were among the seven triclosan metabolites reported previously (Kim et al., 2011). Chlorohydroquinone was a new triclosan metabolite that has not been previously reported.

Since biphenyl-grown RHA1 is known to degrade PCBs via a *meta*-cleavage (Masai et al., 1997) and triclosan degradation was observed in this study, new experiments were performed to determine whether triclosan was degraded via similar pathway. The experiments were conducted using biphenyl-grown RHA1 with and without 3-flurocatechol (50 mg/L), a *meta*-cleavage inhibitor (Toyama et al., 2010). Triclosan degradation was observed in absence, but not in the presence of 3-flurocatechol (data not shown), the results suggested that a *meta*-cleavage pathway was involved in triclosan degradation by biphenyl-grown RHA1.

Based on the inhibition results and metabolites identified in this study, a biodegradation pathway for triclosan by biphenyl-grown RHA1 was proposed in Figure 5.6. The proposed pathway is similar to the degradation pathway for PCBs (Seeger et al., 1997). Given that biphenyl and ethylbenzene 2,3-dioxygenase genes in RHA1 were co-regulated by biphenyl (Robrock et al., 2011), we proposed that a biphenyl dioxygenase (*Bph*A) and ethylbenzene dioxygenase (*Etb*A) were involved in the the initial attack at the 2,3-position of triclosan to form dihydroxy-triclosan. Previous study showed that 2,3-dihydroxydiphenyl ether 1,2-dioxygenases (2,3-DHBD) (*Bph*C and *Etb*C) have broad substrate specificities and co-expressed in biphenyl-grown RHA1 (Robrock et al., 2011). Thus, a *meta*-ring-fission of dihydroxy-triclosan was possible, i.e., a further

dioxygenation at 1,2-position of dihydroxy-triclosan by 2,3-DHBD. This ring-fission could yield 2,4-dichlorophenol (detected) and 3-chloro-2,5-dihydroxy-6-oxohexa-2,4-dienoic acid (not detected). This interpretation is supported by a study reported that 2,3-DHBD catalyzed a variety of PCB congeners into *meta*-cleavage products (Hauschild et al., 1996).

Then, 2,4-dichlorophenol might be further transformed to chlorohydroquinone (detected) with a release of one chloride. The low chloride recovery at the end of degradation (0.26 mg/L, corresponding to only 0.7 mol of Cl^- produced/mole of triclosan degraded) suggests that 2,4-dichlorophenol was not completely transformed into chlorohydroquinone. Furthermore, since methylsilylation was used as a derivatization method, it is difficult to detect all metabolites and many other chlorinated metabolites might have not detected in this study.

Detection of large proportion of chlorinated metabolites in triclosan degradation samples supported the observations of product toxicity exerted on RHA1 cells (Figure 5.5). Previous study showed that RHA1 experienced the product toxicity during cometabolic degradation of PCBs (Parnell et al., 2006). Makinen et al. (1993) also reported that the toxicity of chlorophenols to microorganisms caused the failure of removing chlorophenols in activated sludge systems. Although it is unclear that dihydroxy-triclosan is toxic to degradative microorganisms, several studies have demonstrated the toxic effect of hydroxylated intermediates of PCBs on PCB-degrading microorganisms (Camara et al., 2004). Additionally, other unknown metabolites might exert toxicity to the degradative microorganisms.

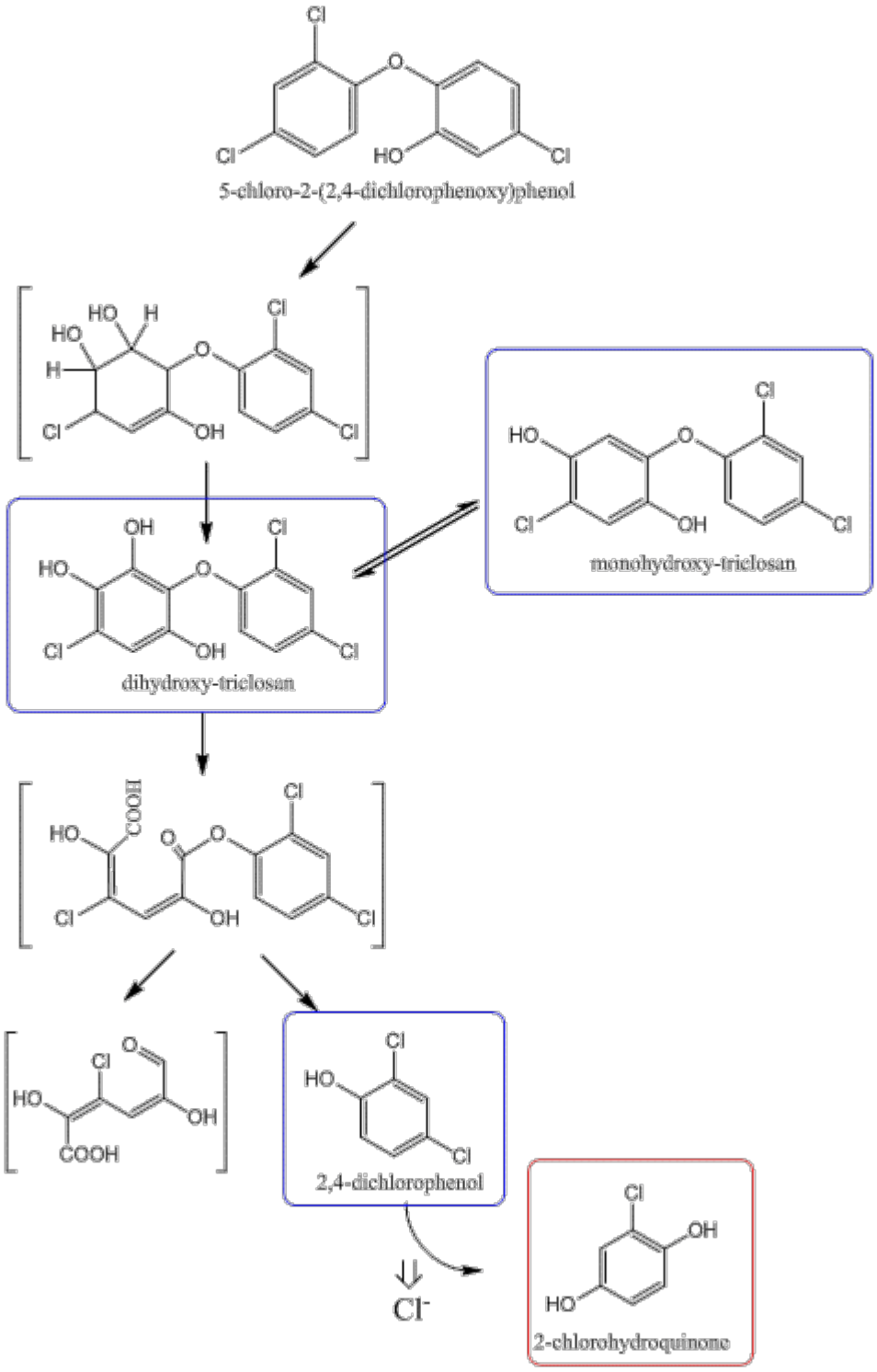


Figure 5.6. Possible biodegradation pathway for triclosan by biphenyl-grown RHA1

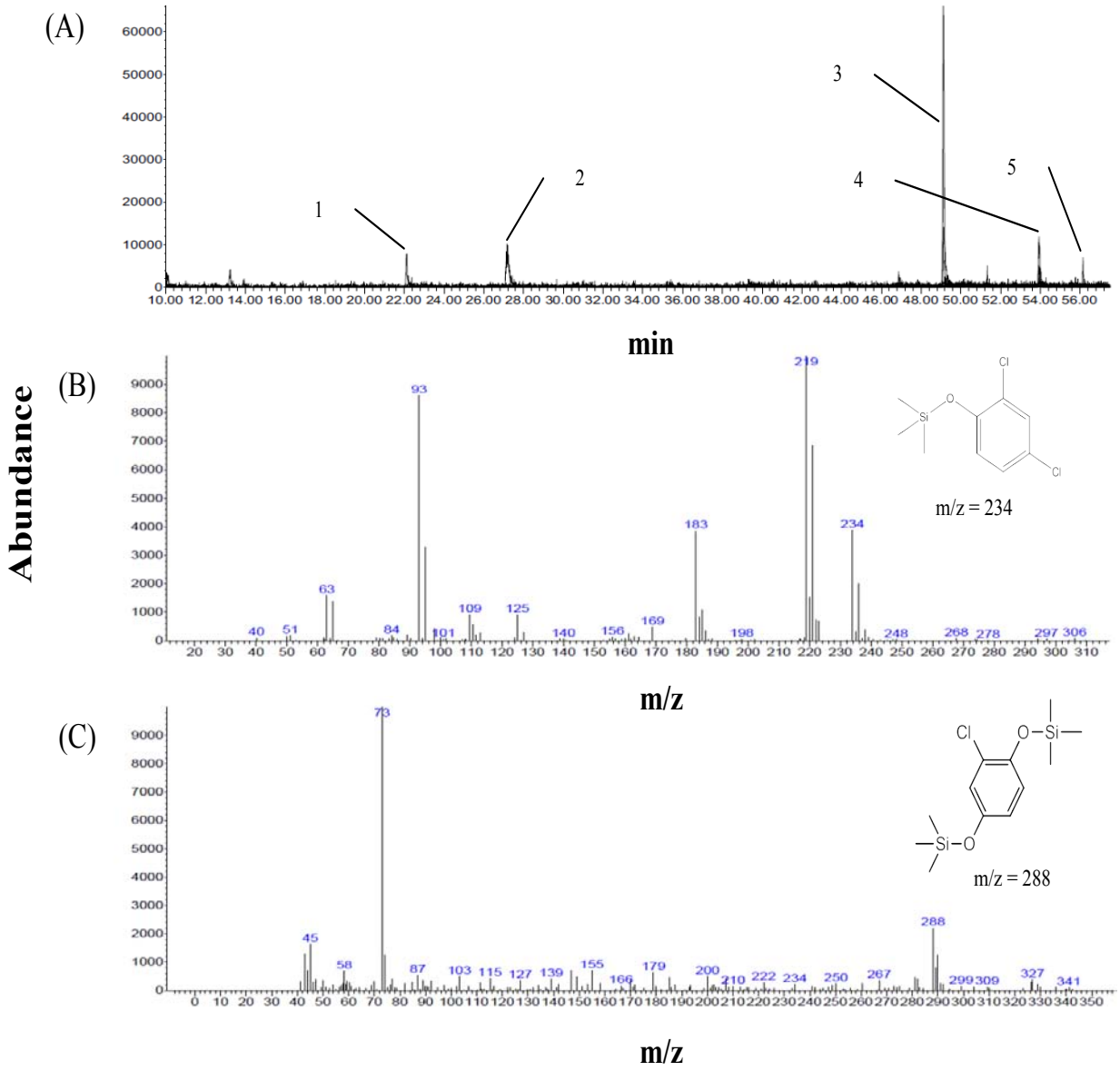


Figure 5.7. GC-MS chromatogram of triclosan degradation metabolites by biphenyl-grown RHA1. (A) GC-MS chromatogram of triclosan degradation metabolites as the TMS derivatives; (B) GC mass spectrum of TMS-deivatized 2,4-dichlorophenol (peak 1); (C) GC mass spectrum of TMS-deivatized chlorohydroquinone (peak 2); (D) GC mass spectrum of TMS-deivatized triclosan (peak 3). (E) GC mass spectrum of TMS-derivatized monohydroxy-triclosan (peak 4); (F) GC mass spectrum of TMS-derivatized dihydroxy-triclosan (peak 5).

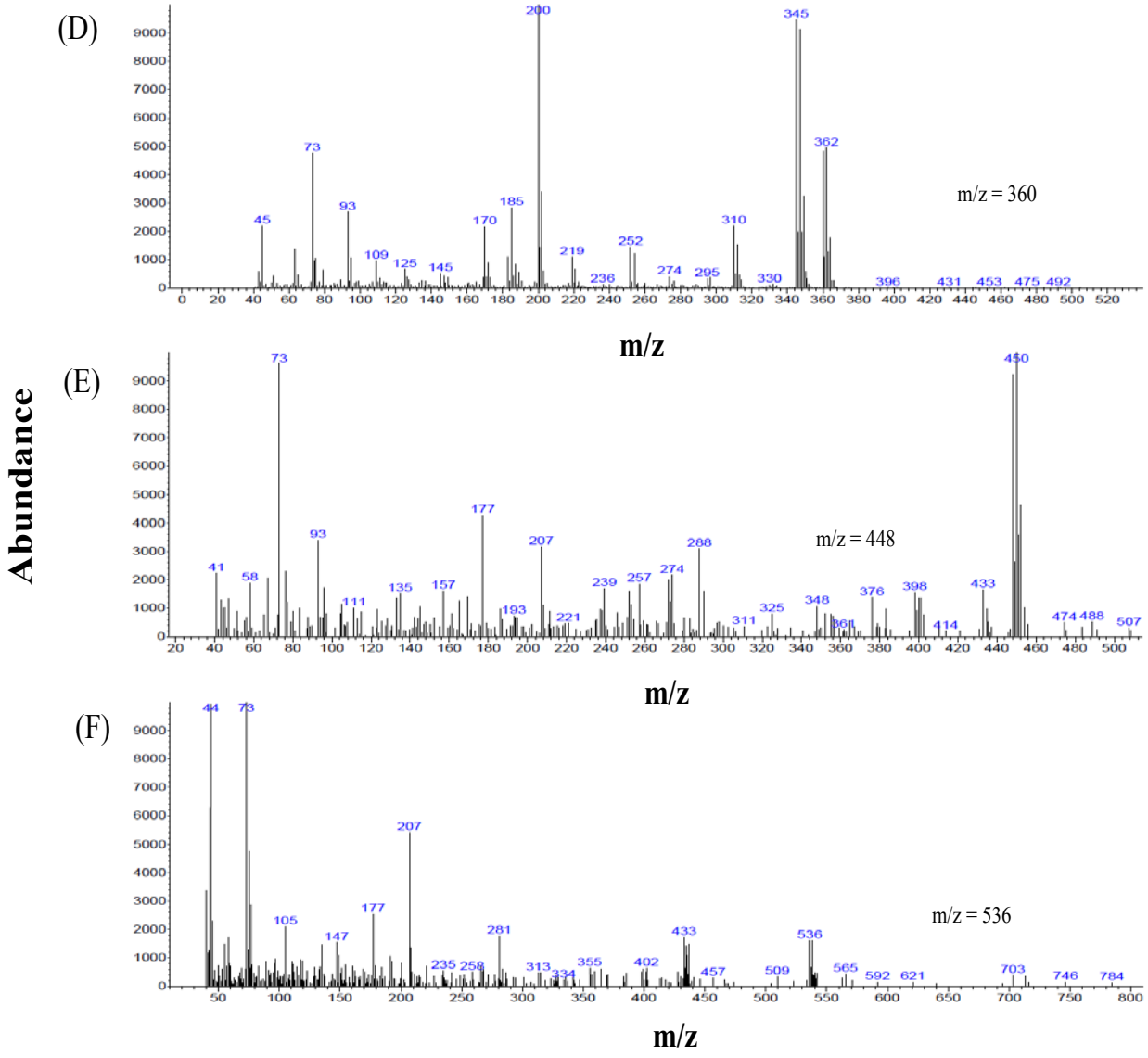


Figure 5.7. Continued.

6. EFFECTS OF AMMONIA AMENDMENT AND BIOAUGMENTATION ON TRICLOSAN BIODEGRADATION IN NITRIFYING ACTIVATED SLUDGE

6.1. Introduction

Numerous household and personal care products contain triclosan, a broad-spectrum antimicrobial agent (Bhargava and Leonard, 1996; Sabaliunas et al., 2003; Schweizer, 2001). Triclosan is also an endocrine-disrupting compound (Gee et al., 2008) and has been frequently detected in the environment. The occurrences of environmental triclosan have raised a great public health concern because environmental triclosan may pose a potential public health risk by promoting antibiotic-resistant microorganisms (Braoudaki and Hilton, 2004). Moreover, upon exposure to sunlight or UV light, the environmental triclosan can be transformed into more toxic compounds like chlorinated dioxins (Latch et al., 2003; Latch et al., 2005).

Wastewater is one major source of environmental triclosan. Approximately 72-94% of triclosan in wastewater was removed by the activated sludge systems in wastewater treatment plants (WWTPs) (Morrall et al., 2004; Roh et al., 2009a; Singer et al., 2002), leaving a considerable amount of triclosan in treated wastewater which is then discharged into receiving waters. As a result, triclosan was commonly detected in surface water, marine, soil, sediments, and even in human (Aguera et al., 2003; Allmyr et al., 2006; Calafat et al., 2008; Singer et al., 2002). Thus, improving triclosan removal during wastewater treatment processes is a key to minimize the occurrence of triclosan in the environment.

Although triclosan is an antimicrobial agent, a few wastewater microorganisms, including ammonia-oxidizing bacteria (AOB) (Roh et al., 2009a) and *Sphingopyxis* strain KCY1 (hereafter referred as strain KCY1) (Lee et al., 2012), are known to degrade triclosan. AOB is an important group of autotrophic bacteria in nitrifying activated sludge (NAS); AOB can express ammonia monooxygenase (AMO) enzymes to oxidize ammonia to nitrite, which is the first and important step of nitrification process. The same enzyme (i.e., AMO) is also responsible for the degradation of triclosan (Roh et al., 2009a). Potentially, triclosan removal can be enhanced by increasing the population of AOB or the activity of AMO in NAS.

Strain KCY1 is a heterotrophic wastewater bacterium and has exhibited favorable triclosan degradative capabilities, including (i) complete dechlorination of triclosan; (ii) reduction of androgenicity of triclosan following degradation; and (iii) retaining its triclosan degradation ability when grown in complex nutrient medium with ambient concentrations of triclosan (Lee et al., 2012). The unique degradation characteristics of strain KCY1 offer a possible treatment approach - bioaugmentation - for removing triclosan in NAS. Bioaugmentation could be an effective treatment strategy to enhance removal of specific compounds in natural/engineered systems when effective degradative microorganisms are absence (McLaughlin et al., 2006; Van Limbergen et al., 1998). The strain KCY1 appears to be an ideal candidate as a bioaugmenting agent for enhanced triclosan removal in NAS.

In this study, I hypothesized that increasing ammonia oxidation activity and/or bioaugmenting with strain KCY1 can enhance removal of triclosan in NAS. A series of

laboratory-scale bioreactors were used to investigate whether triclosan removal can be enhanced by amending ammonia and/or strain KCY1. The effects of these amendments on the changes in strain KCY1, AOB, and *amo*A gene (catabolic gene responsible for ammonia oxidation) in the bioreactors were also examined. The population of strain KCY1 and AOB, and the quantity of *amo*A gene were molecularly quantified using real-time PCR assays. The obtained results were used to establish any possible correlations to assess triclosan biodegradation potential in three different NAS surveyed in this study.

6.2. Material and methods

6.2.1. Chemicals

Triclosan (TCS) (97% pure) was purchased from Aldrich Chemical Inc. (Milwaukee, WI). Stock solution of 10 g/L triclosan was prepared in acetone. N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce Biotechnology Inc. (Rockford, IL). FastDNA SPIN kit and FastDNA SPIN kit for soil was purchased from MP Biomedicals (Solon, OH). Primers and probes for real-time-PCR assays were purchased from Sigma–Aldrich (St. Louis, MO). Quanti-Tect probe PCR supermix and SYBR Green I were purchased from Qiagen (Valencia,CA).

6.2.2. Bacterial strains and culture conditions

Strain KCY1, a triclosan-degrading bacterium isolated from activated sludge (Lee et al., 2012), was initially grown in a flask containing 20% R2A medium and 2 mg/L of triclosan. The flask was incubated aerobically on a rotary shaker at 160 rpm at

30 ^{o}C for 2 days**.** The cells in the late exponential phase (OD_{600} ~ 0.8-1.0) were harvested by centrifugation at 10,000×g for 5 min for experimental use.

6.2.3. Activated sludge samples

Nitrifying activated sludge (NAS) samples were collected in May 2009 from three different WWTPs in College Station (WWTP#1), Austin (WWTP #2), and Houston (WWTP #3) in Texas. These three WWTPs use different process configurations and operating conditions for BOD removal and nitrification (Table 6.1). WWTP #1 and WWTP #2 use single-stage activated sludge system. The WWTP#1 treats 9.5 million gallons per day (MGD) and is operated at a solid retention time (SRT) of 7–8 days to achieve 97-98% BOD removal and 95% ammonia removal. The WWTP#2 processes 75MGD at a SRT of 10-12 days to achieve 97-99% BOD removal and 99% ammonia removal. The WWTP #3 uses two-stage activated sludge system to treat 200 MGD. By operating at a SRT of 2-20 days, the WWTP#3 can remove 91-99% of BOD and 84-99% of ammonia.

The genomic DNAs of activated sludge samples from these three WWTPs were used as template to quantify total microbial population (Yu et al., 2005), strain KCY1, AOB, and *amo*A gene. The activated sludge collected from WWTP #1 was also used for bioreactor setup. The ammonia amendment (Nov. 2011) and bioaugmentation experiments (May 2012) as described below.

Table 6.1. Prevalence of *Sphingopyxis* strain KCY1, AOB, and *amoA* gene in selected WWTPs.

WWTPs Operating Parameters	**WWTP #1**			**WWTP #2**	**WWTP #3**	
					1st Stage	2nd Stage
Treatment capacity (MGD*)	9.5			75	200	
SRT (d)	7-8			10-12	2	20
MLVSS (g/L)	2.15			1.52	1.35	1.10
BOD_5 removal (%)	95			97-99	91-99	
Ammonia removal (%) (influent concentration)	97-98 (19-22 mg/L)			99 (24-30 mg/L)	84-99 (16-20 mg/L)	
	May 2009	Nov. 2011	May 2012			
Total bacteria population (# copies/mL)	1.7×10^9	8.2×10^9	4.9×10^9	2.1×10^9	3.0×10^9	1.4×10^9
Strain KCY1 (# copies /mL)	1.2×10^7	2.2×10^5	1.4×10^6	2.4×10^6	4.5×10^6	6.4×10^5
ǂ AOB (# copies /mL)	1.5×10^5	5.4×10^5	4.2×10^5	1.6×10^6	1.9×10^5	7.4×10^5
ǂ *amoA* gene (# copies /mL)	1.7×10^4	3.6×10^4	7.1×10^4	4.6×10^4	2.1×10^4	1.5×10^4

* Million gallon per day. Activated sludge samples from three WWTPs were collected in May 2009.
ǂ Data about AOB and *amo*A were cited from Roh et al (Roh and Chu, 2010).

6.2.4. Bioreactor setup and experimental approach

A total number of twenty batch reactors were constructed using 1-L glass flasks. The twenty reactors were divided into three sets, eight reactors in Set A, eight reactors in Set B, and four reactors in Set C. The Set A was used for ammonia amendment, the Set B was used for bioaugmentation with strain KCY1, and the Set C was used for both ammonia amendment and bioaugmentation with strain KCY1. Activated sludge collected from WWTP#1 in November 2011 was used for bioreactors in Set A and in Set C. The sludge collected in May 2012 was used for bioreactors in Set B. The collected

activated sludge was aerated for 1 day to reduce its dissolved organic matter content (Batt et al., 2006) before used for bioreactor setup. Each bioreactor contains 200 mL of the aerated mixed liquor volatile suspended solids (MLVSS, 2870 mg/L) and wrapped with aluminum foil to avoid development of phototrophic reactions in the reactors. The average ammonia concentrations in the MLVSS was 23 mg NH_4-N/L for November 2011 samples and 20 NH_4-N/L for May 2012 samples, respectively. The initial concentration of triclosan was 2 mg/L in all bioreactors.

To determine the effects of ammonia amendment on triclosan degradation, the reactors in Set A were amended with different concentrations of ammonia (0, 5, 25, and 75 mg of NH_4-N/L). These reactors were referred as #A0, A5, A25, and A75 (duplicate), respectively. The range of ammonia added in the reactor was determined based on the typical ammonia concentrations ranging from 12 to 45 mg NH_4-N/L, in domestic wastewater (Tchobanoglous et al., 2003). Ammonia sulfate ($(NH_4)_2SO_4$) was used to prepare an aqueous stock of ammonia.

To determine the effects of bioaugmentation with strain KCY1 on triclosan degradation in NAS, the reactors in Set B (#B0, B0*, B0-KCY1, and B0-KCY1*) were amended with strain KCY1. Two different types of live controls were used; they were live controls without allylthiourea (ATU) (#B0) and live controls with ATU (#B0*). ATU (10 mg/L) was added to inhibit the activity of ammonia-oxidizing bacteria (Rasche et al., 1991). The reactors #B0-KCY1 (duplicate) in Set B were spiked with the cell suspension of strain KCY1 (prepared as described above) to result in an initial strain KCY1 concentration of 8.7×10^8 16S rRNA gene copies/mL. The other two reactors B0-

KCY1* were amended with strain KCY1 and ATU to assess the degradative role of strain KCY1 in triclosan degradation in the absence of ammonia oxidation activity.

Bioreactors in Set C (A25-KCY1 and A25-KCY1*) were amended with ammonia (25 mg NH_4-N/L) and strain KCY1 with and without ATU. This set of experiments was conducted to determine the effects of bioaugmentation when ammonia oxidation is active in NAS. As the fastest triclosan degradation was observed in bioreactors when 25 mg/L of NH_4-N was amended, the reactors in Set C were thus amended with 25 mg/L of NH_4-N and strain KCY1.

Liquid in the bioreactor was completely mixed at 300 rpm using a magnetic stirrer. Experiments were conducted at room temperature. The first samples were collected within 6 min after initiating the experiments. Liquid samples were collected from each bioreactor at 0, 24, 54, and 96 hours. The collected samples were used for genomic DNA extractions, and triclosan, ammonia and nitrate measurements.

6.2.5. Development of a real-time PCR assay for strain KCY1

A real-time PCR assay targeting the 16S rRNA gene of strain KCY1 was developed. A set of forward and reverse primers, and a *Taqman* probe (Table 6.2) were designed by aligning the 16S rRNA gene sequences of fifteen cultured bacteria

Table 6.2. Primers, probes, and PCR protocols of real-time PCR assays used in this study.

Assay Target	Primer Name & Sequence (5'-3')	Reference
KCY1 16S rRNA gene	Forward Primer (KCY1-F): 5'- ATCCTGATCGCGGATTAGAGAGATCTT -3' T_m= 57.6 °C, G+C= 44.4% Reverse Primer (KCY1-R): 5'- TGAGACAACTTTTGGAGATTAGCTACCC -3' T_m= 58.0 °C, G+C= 42.9% Probe (KCY1-Taq): 5'-[6-FAM]-TGGCAACTACAGTGGGCAGCAACCTCGC-[TAMRA-Q]-3' T_m= 68.0 °C, G+C= 60.7%	This study
PCR Protocol	95 °C for 15 min; 40 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 20 s, and final at 4°C	
Total bacterial 16rRNA gene	Forward Primer (1055F): 5'-ATGGCTGTCGTCAGCT-3' T_m= 57.7 °C Reverse Primer (1392R): 5'-ACGGGCGGTGTGTAC-3' T_m=58.9 °C Probe (16S*Taq*1115f): 5'-[6-FAM]-CAACGAGCGCAACCC-[6-TAMRA]-3' T_m=62.9 °C	(Harms et al., 2003)
PCR Protocol	95 °C for 15min; 45 cycles of 95 °C for 30s, 50 °C for 1min, and 72 °C for 2min	

T_m: melting temperature, G+C: G and C content
[6-FAM] = 6-carboxyfluorescein, [TAMRA] = 6-carboxytetramethylrhodamine

(AB15049, AB033950, EU188914, DQ983313, AB280000, EU448286, EU143356, EF494193, EF534729, EU44081, AY177357, DQ137852, EU816422, EU240203, and DQ205298) with greater than 98% similarity to KCY1 16S rRNA gene sequence (DQ983313.2). The primers and probe were examined for their uniqueness to the target

16S rRNA gene sequence region of strain KCY1 against bacterial 16S rRNA gene sequences deposited in the GenBank using the Basic Local Alignment Search Tool (BLAST). The designed primers and probe had at least three total mismatches when comparing with more than bacterial 16S rRNA gene sequences in the GenBank (accessed on 02/05/2009).

6.2.6. DNA extraction and real-time PCR assays

The genomic DNA of activated sludge samples and strain KCY1 were extracted using a FastDNA kit as described previously (Yu et al., 2005). The extracted DNA concentrations were determined using a Hoefer DQ 300 Fluorometer (Hoefer Inc., San Francisco, CA). The genomic DNA of strain KCY1 was used for the development of the real-time PCR assay for strain KCY1. The bacterial population, strain KCY1, AOB, and *amoA* gene in the activated sludge samples were determined using real-time PCR with the genomic DNAs of the activated sludge samples as templates (Roh and Chu, 2011). The PCR amplification reactions were performed using a Bio-Rad iQ5Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Information for primers, probes and thermal cycle protocol were listed in Table 6.2. The standard curve for the bacterial 16S rRNA gene, AOB, and *amoA* gene concentration was constructed as described previously (Yu et al., 2005). Similarly, the standard curve for the strain KCY1 gene concentration (ranging from 1.0×10^{1} to 1.0×10^{7} copies of the strain KCY1 partial 16S rRNA gene) was constructed using a plasmid # 931 containing the partial 16S rRNA gene of strain KCY1.

6.2.7. Chemical analysis

Triclosan concentrations were monitored by gas chromatograph/mass spectrometer (GC/MS) equipped with a DB-5 Column in selective ion monitoring (SIM) mode as described previously (Lee et al., 2012). Liquid samples were filtrated with Whatman 0.2 μm membrane filters (Piscataway, NJ) before use for ammonia and nitrate analysis. Ammonia concentrations were measured by the phenate method (Eaton et al., 1995). The detection limit for ammonia is 0.02 mg/L as NH_4-N. Nitrate concentrations were measured using a DX-80 Ion Chromatography (IC) system (Dionex, Sunnyvale, CA) equipped with an IonPac AS14A-5μm analytical column (3 × 150 mm). The detection limit for nitrate is 0.05 mg/L as NO_3-N.

6.3. Results and Discussion

6.3.1. Effect of ammonia amendment on triclosan removal in NAS

Bioreactors in Set A (#A0, #A5, #A25, and #A75) were conducted to investigate the effects of NH_4-N amendment on triclosan degradation by NAS. The average concentrations of NH_4-N and NO_3-N in MLVSS used for the bioreactor setup were 23 mg/L and 10 mg/L, respectively.

Active nitrification was observed in all bioreactors in Set A. Regardless the amount of ammonia amended in the bioreactors, ammonia concentrations decreased over time, corresponding to the production of nitrate (Figure 6.1). The highest ammonia oxidation and nitrate production were observed in the reactor #A25; 27.7 mg/L of NH_4-N was removed and 20 mg/L of NO_3-N was produced over 96 hr. To our surprise, less

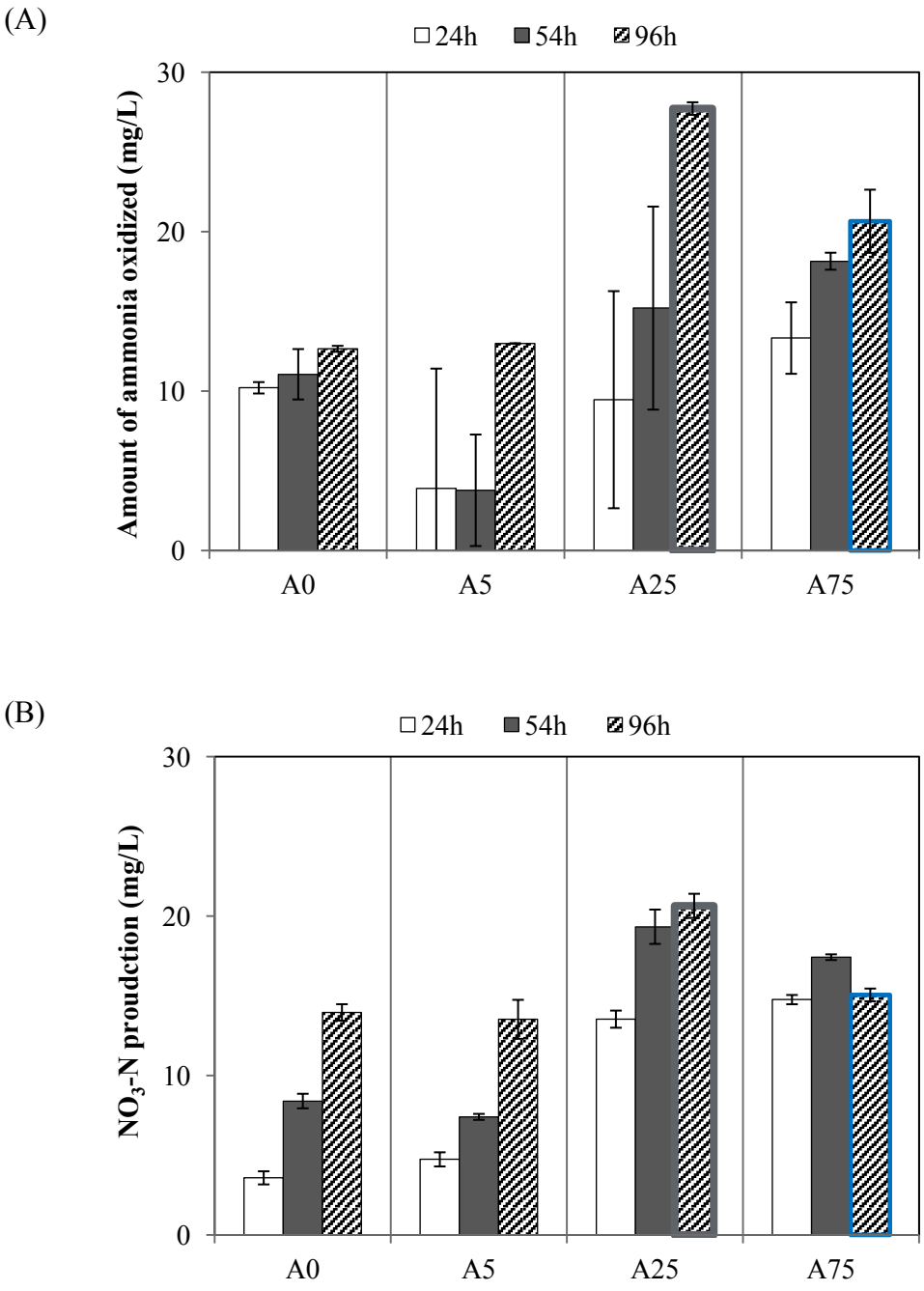
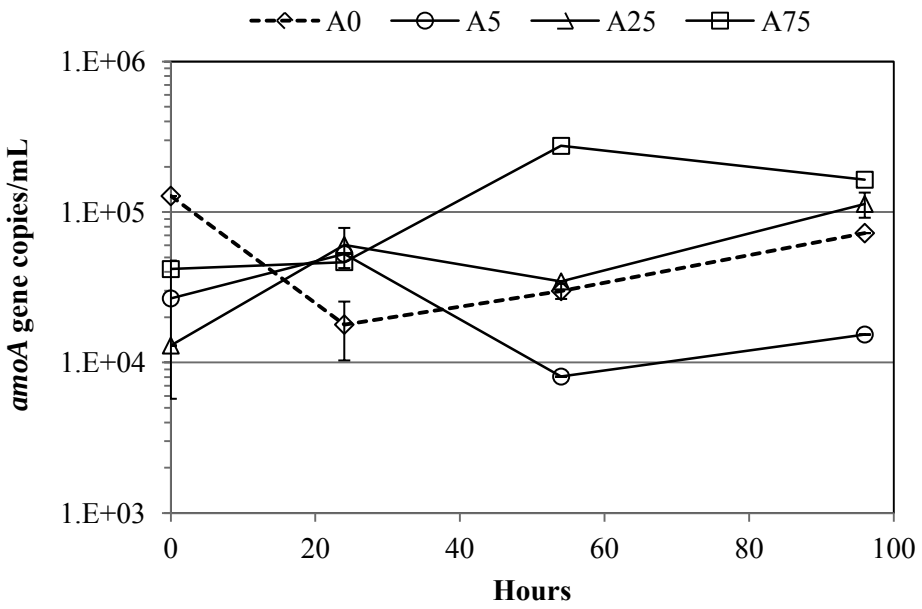


Figure 6.1. (A) Amount of ammonia oxidized and (B) production of NO_3-N over 96 hours in reactors amended with different concentrations of ammonia.

ammonia removal and nitrate production were observed in the reactor #A75 (receiving the highest ammonia concentrations) than those in reactor #A25 (Figure 6.1). The lower ammonia removal in the reactor #A75 might be due to the toxicity effects of the high initial ammonia concentrations on ammonia-oxidizing bacteria (Claros et al., 2010). Free ammonia concentrations ranging from 22 to 150 mg/L can inhibit the activities of both *Nitrobacter* and *Nitrosomonas* (Anthonisen et al., 1976; Bae et al., 2001; Turk and Mavinic, 1989).

The temporal changes in the abundances of AOB and *amoA* gene (encodes the active site of the ammonia monooxygenase enzyme for ammonia oxidation in AOB) in each reactor was monitored using real-time PCR assays (Figure 6.2). The NAS (collected in Nov. 2011) contained averagely 1.6×10^6 16S rRNA gene copies of AOB/mL and 4.6×10^4 *amoA* gene copies/mL. After 96 hrs of incubation, the number of *amoA* gene copies increased 10-fold in reactor #A25 and 32-fold in reactor #A75. As expected, no significant change in the number of AOB 16S rRNA gene copies was observed for reactors #A0-A75, since AOB are slower growers. However, the increase of amoA gene copies was unable to correlate the amounts or the rates of ammonia oxidation observed in reactors #A0-A75.

(A)



(B)

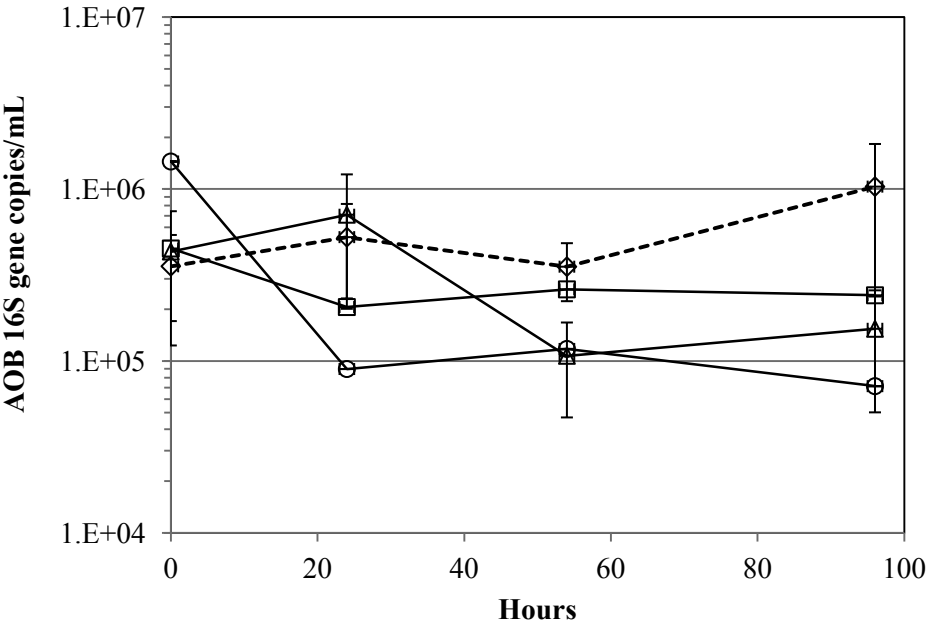


Figure 6.2. Changes in concentrations of (A) *amoA* gene copies and (B) AOB 16S rRNA gene copies over 96 hrs in reactors amended with different concentrations of NH_4-N. Symbols: A0 (◊), A5 (○), A25 (Δ) and A75 (□).

Over 96 hrs of incubation, complete degradation of triclosan was observed in the reactors without and with ammonia amendment (#A0-A75) (Figure 6.3). However, a complete removal of triclosan was observed earlier (i.e., within 54 hours) in reactor #A25. The initial triclosan degradation rates of reactor #A25 (0.144 mg/L-triclosan/hour) were 2-fold faster than those in other ammonia-amended bioreactors (degradation rate of 0.071-0.095 mg/L-triclosan/hour) (Figure 6.3). In reactors #A25, the fastest triclosan degradation rate corresponded to the largest amount of ammonia oxidized (Figure 6.1), supporting the hypothesis that triclosan removal can be enhanced by increasing ammonia oxidation activity in NAS.

To correlate ammonia oxidation activity with triclosan removal, the amount of ammonia oxidized was plotted against % of triclosan removal (Figure 6.4A). While the data are scattered in Figure 6.4A, complete removal of triclosan was observed when 6 to 22 mg/L of NH_4-N was oxidized. Thus, it is suggested that more than 22 mg/L of ammonia oxidation is possibly required to ensure 100% removal of triclosan (2 mg/L) in NAS. These results implicates that triclosan can be completely removed within 96 hrs in NAS if ammonia is present in its typical concentration range (12 to 45 mg/L) in wastewater. As typical hydraulic retention time (HRT) for NAS is 24-48 hr and ambient triclosan concentrations range from 0.61 to 5.1 μg/L, more studies are needed to examine the effects of HRT on removing ambient triclosan in NAS. Nevertheless, maintaining the conditions favorable for nitrification processes in WWTPs can expect the better performance of triclosan degradation in NAS.

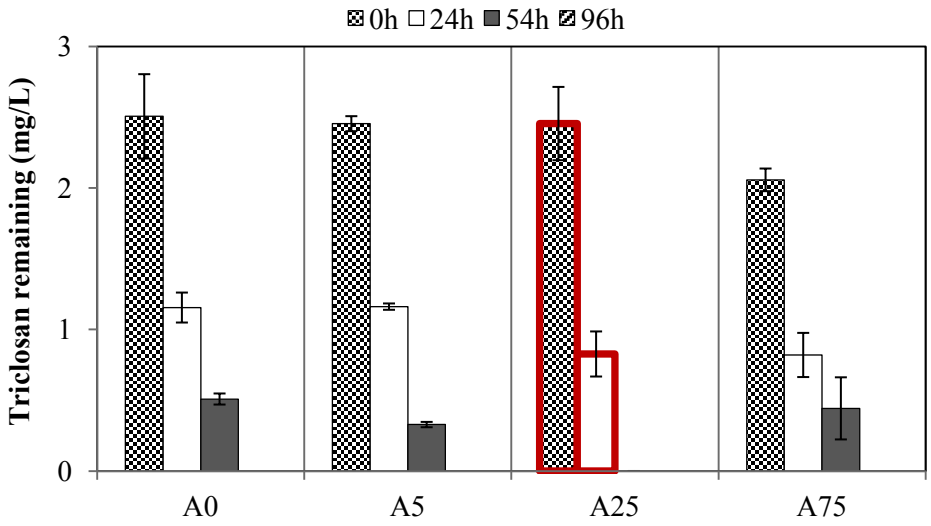
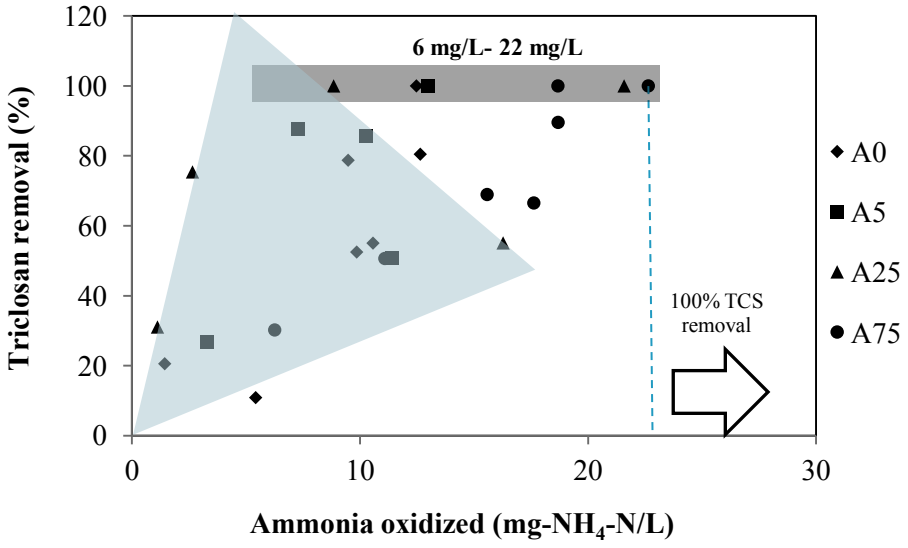


Figure 6.3. Biodegradation of triclosan (2 mg/L) in NAS amended with different concentrations of ammonia-N.

(A)



(B)

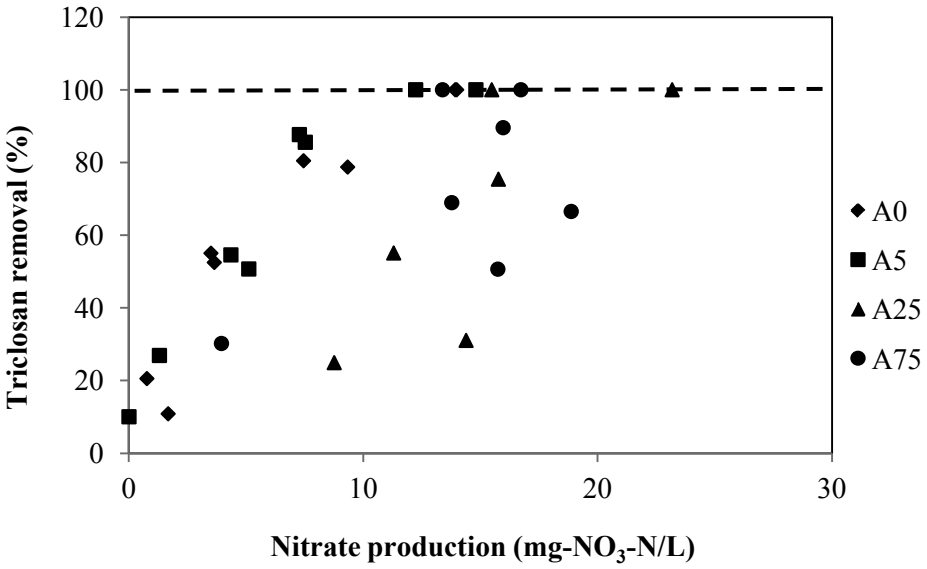


Figure 6.4. Relationship (A) between the amount of ammonia oxidized (mg-NH_4-N/L) and triclosan removal (%) and (B) between the amount of nitrate production (mg-NO_3-N/L) and triclosan removal (%).

6.3.2. Effects of bioaugmentation with strain KCY1 on triclosan removal in NAS

Another set of reactors (#B0, B0*, B0-KCY1, and B0-KCY1*) was conducted to investigate if triclosan degradation can be enhanced in NAS by increasing the population of strain KCY1. All the reactors were amended with strain KCY1 with an average initial concentration of 8.7 $\times 10^8$ 16S rRNA gene copies/mL. The reactors (#B0 and #B0-KCY1) showed a decrease in ammonia concentrations and an increase in nitrate production over time, indicating that nitrification was active in these reactors (Figure 6.5). In the presence of ATU (an AOB inhibitor), the smaller changes in ammonia and nitrate concentrations in the reactors #B0* and #B0-KCY1* were observed, indicating that the activity of AOB was inhibited but not completely in these reactors (Figure 6.5).

After hour 24, a significant decrease in strain KCY1 was observed in the reactor #B0-KCY* while the number of strain KCY1 16S rRNA gene copies was 5-fold lower than that in the reactor #B0-KCY1. The results suggested that ATU also affected the activity of strain KCY1 (Figure 6.6). The NAS (collected in May 2012) contained average concentrations of 4.0 $\times 10^5$ 16S rRNA gene copies of AOB/mL and 7.1×10^4 *amoA* gene copies/mL. After 96 hrs of incubation, the number of *amoA* gene copies in the reactors #B0 and B0-KCY1 increased, but not in the reactors #B0* and B0-KCY1*. No significant change in the number of AOB 16S rRNA gene copies was observed for the reactors in #B0, B0*, B0-KCY1, and B0-KCY1* (data not shown).

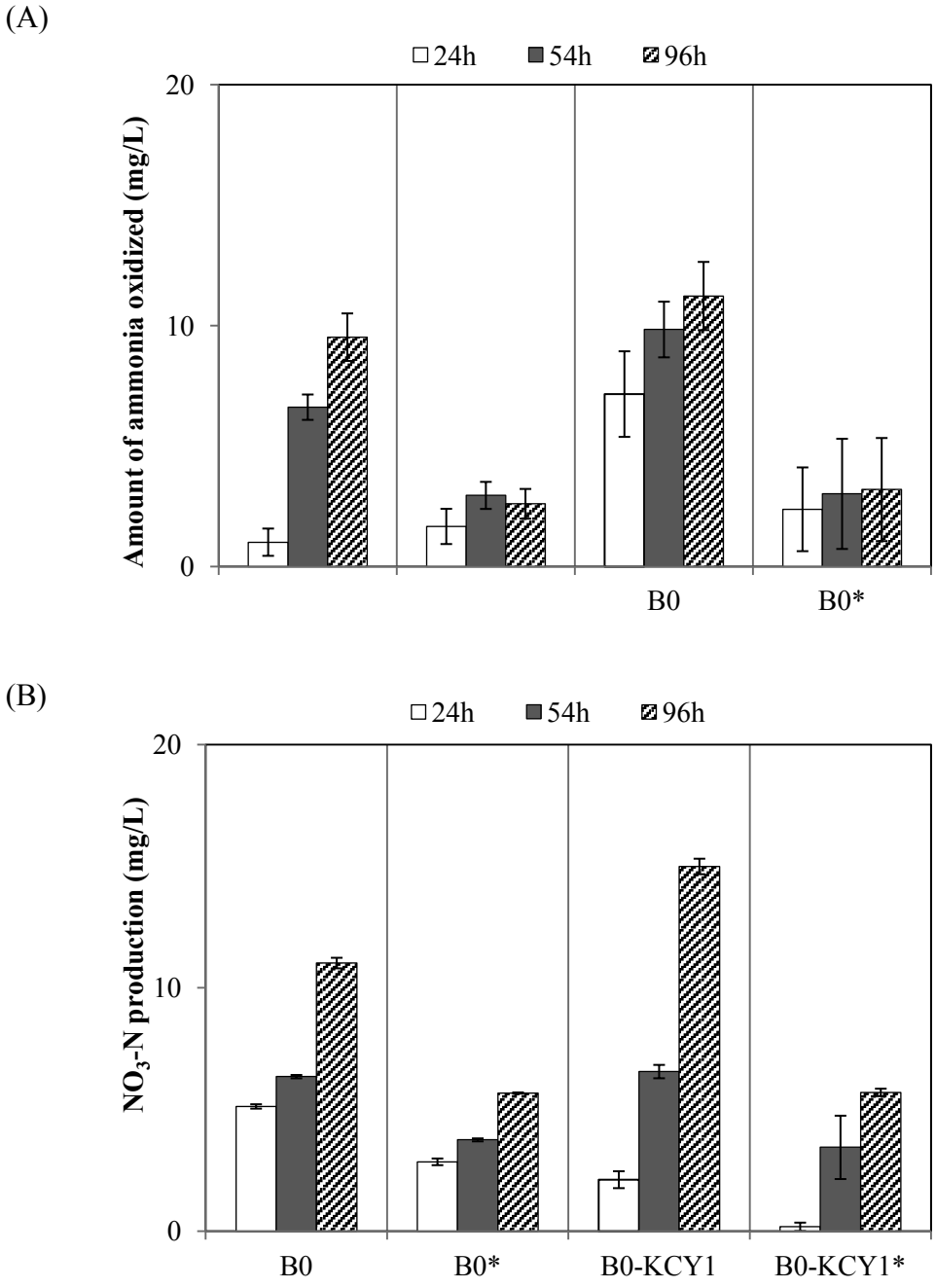


Figure 6.5. (A) Amount of ammonia oxidized and (B) production of NO_3-N over 96 hours in reactors bioaugmented with strain KCY1.

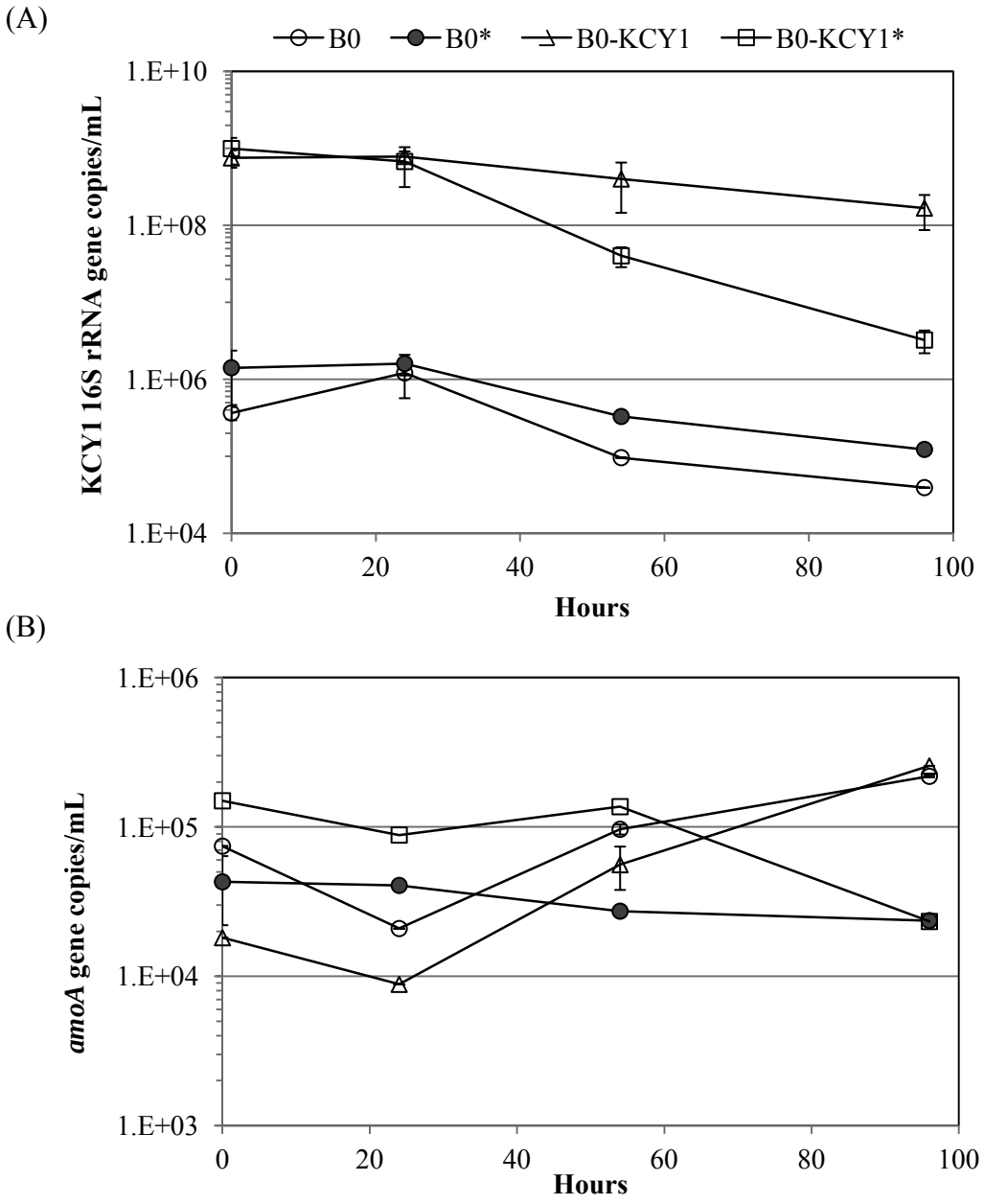


Figure 6.6. Abundance of (A) strain KCY1 16S rRNA gene and (B) *amoA* gene in NAS bioaugmented with strain KCY1 during 96-hour triclosan degradation. Symbols: B0 (○), B0* (●), B0-KCY1 (Δ), B25-KCY1* (□).

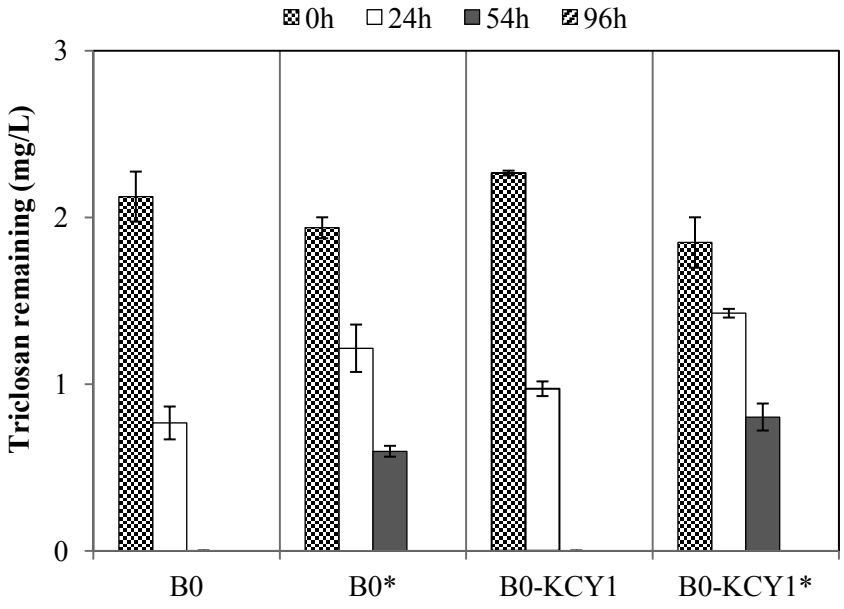


Figure 6.7. Biodegradation of triclosan (2 mg/L) by NAS bioaugmented with strain KCY1.

In the reactors #B0 and B0-KCY1, 2 mg/L of triclosan was completely degraded in 54 hrs. This similar degradation pattern for triclosan observed in B0 and B0-KCY1 reactors suggested that bioaugmentation with strain KCY1 did not enhance triclosan degradation. Complete removal of triclosan was also observed in the reactors #B0* and B0-KCY1* after 54 hrs of incubation. The degradation was slower than that observed in the reactors B0 and B0-KCY1. However, since the activity of AOB and strain KCY1 were both inhibited by ATU, the complete removal of triclosan after hr 54 might be explained by other unknown triclosan-degraders in the bioreactors #B0* and B0-KCY1*. This result also consist to previous observation that other non-AOB was responsible for triclosan biodegradation in NAS (Roh et al., 2009a).

The effects of bioaugmentation with strain KCY1 were not significant in enhancing triclosan degradation. One possible explanation might be due to the differences in degradation half-velocity parameters among strain KCY1, AOB, and unknown triclosan-degraders in NAS. Another possible reason for the ineffectiveness of the bioaugmentation might be due to that strain KCY1 may prefer other readily degradable compounds than triclosan in the bioreactors.

While experimental data of Set B were unable to differentiate the weight of different roles of AOB, strain KCY1 and unknown triclosan degraders during triclosan degradation. By plotting triclosan removed (mg/L) verses concentrations of strain KCY1 (Figure 6.8), one can expect approximately 50% of triclosan removal (i.e. around 1 mg/L of triclosan) when AOB activity was inhibited.

6.3.3. Effects of ammonia and strain KCY1 amendments on triclosan removal in NAS

To determine whether both ammonia amendment and bioaugmentation with strain KCY1 can further enhance triclosan degradation in NAS, two reactors were amended with 25 mg/L of NH_4-N (based on results of Set A showing the fastest triclosan degradation when 25 mg/L of NH_4-N was added) and strain KCY1 (an initial strain KCY1 concentration of 2.0×10^9 16S rRNA gene copies/mL). These reactors were referred as A25-KCY1. The other reactors #A25-KCY1*(duplicate) were prepared similarly but with addition of 10 mg/L of allylthiourea (ATU).

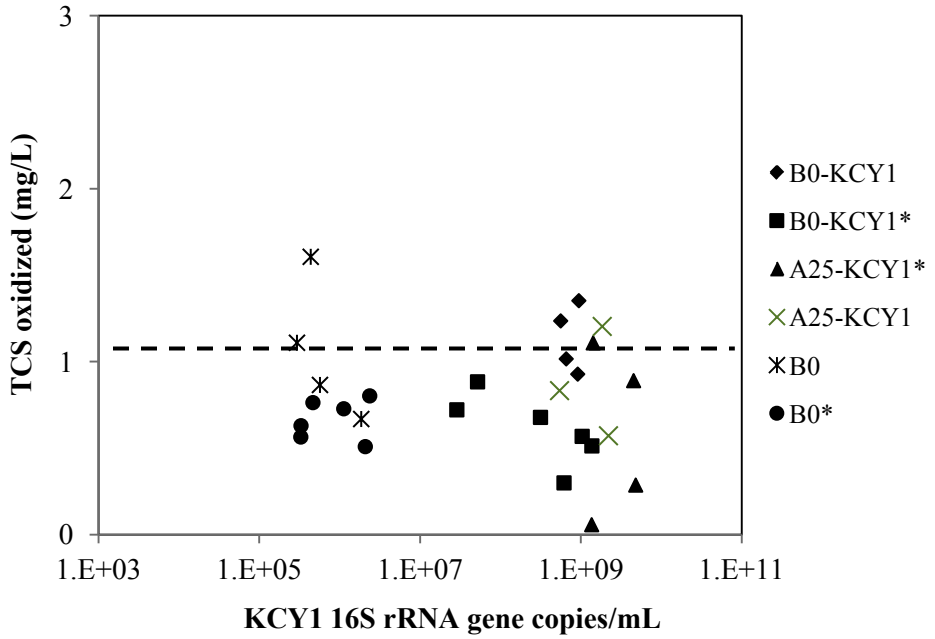


Figure 6.8. Relationship between the number of strain KCY1 (16S rRNA gene copies/mL) and the amount of triclosan oxidized (mg/L).

The reactor #A25-KCY1 showed a decrease trend in ammonia concentration, indicating that AOB were active (Figure 6.9). In the presence of ATU, small amounts of ammonia was oxidized and nitrate was produced, indicating that the activity of AOB was inhibited in the reactor #A25-KCY1* (Figure 6.9).

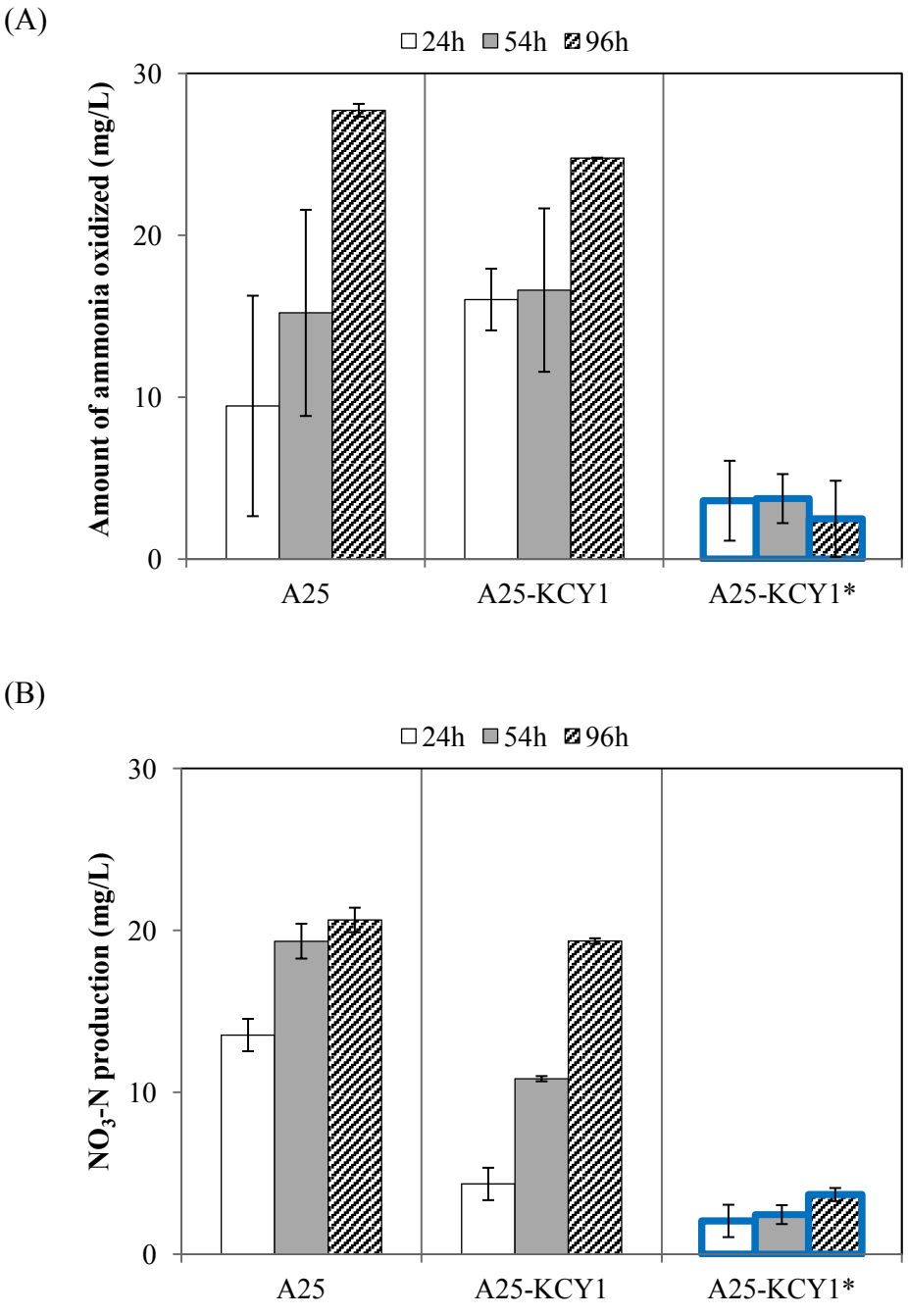
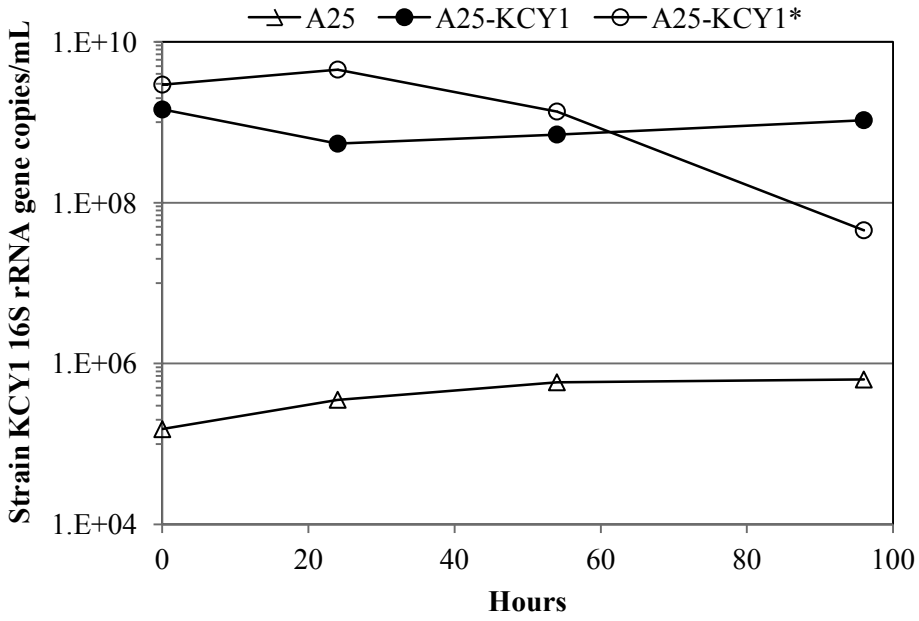


Figure 6.9. (A) Amount of ammonia oxidized and (B) production of NO_3-N over 96 hrs in reactors amended with both 25 mg/L of ammonia-N and strain KCY1.

No significant changes in 16S rRNA genes numbers of strain KCY1, ranging between 5.7×10^8 and 1.8×10^9 16S rRNA gene copies/mL, were observed in the reactor #A25-KCY1 during 96-hr incubation period (Figure 6.10). Similarly, in the presence of ATU, a significant decrease in strain KCY1 was observed after hour 24 in the reactor #A25-KCY1*, similar to that observed in the reactor #B0-KCY1* (Figure 6.6).

Triclosan was completely removed within 54 hours in the reactor #A25-KCY1 (Figure 6.10). This trend was similar to those observed in the reactor #A25, suggesting that triclosan degradation was not further enhanced by the bioaugmentation with strain KCY1 when ammonia oxidation was highly active. Unlike complete triclosan degradation observed in the reactor #B0-KCY1*, triclosan was not degraded completely during 96-hr incubation in the reactor #A25-KCY1*. The reasons for the incomplete degradation were unclear. One possible explanation might be the presence of different unknown triclosan-degraders in activated sludge samples collected on different dates, in November 2011 and in May 2012.

(A)



(B)

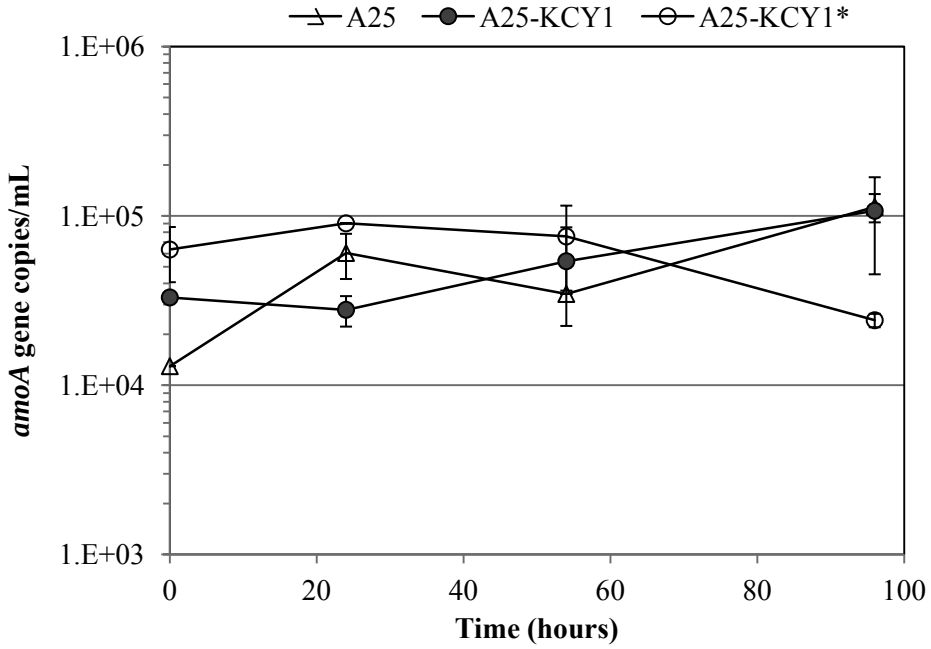


Figure 6.10. Abundance of strain KCY1 16S rRNA gene and *amoA* gene in NAS amend with 25 mg/L of ammonia-N and strain KCY1 during 96-hour triclosan degradation. Symbols: A25(Δ), A25-KCY1(●), and A25-KCY1*(○).

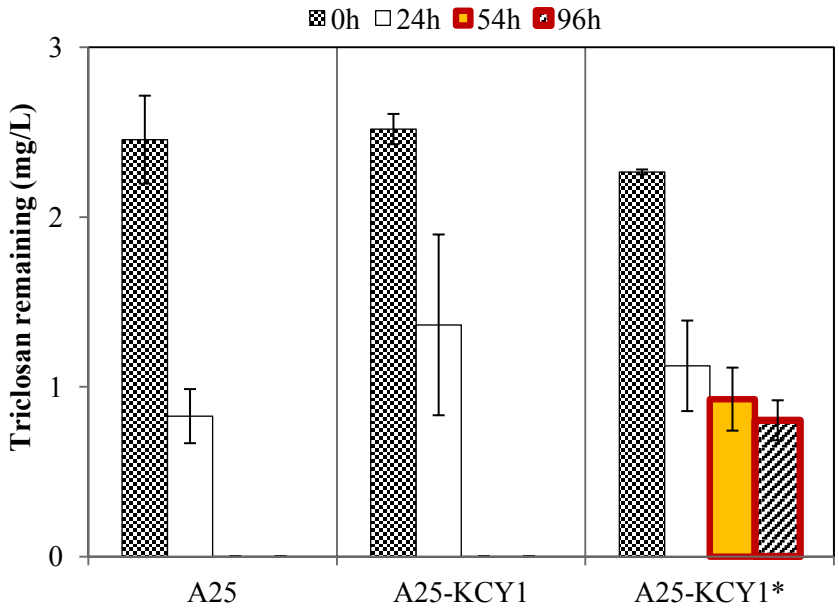


Figure 6.11. Biodegradation of triclosan (2 mg/L) in NAS amend with 25 mg/L of ammonia-N and strain KCY1.

6.3.4. Prevalence of triclosan-degrading bacteria in NAS

Real-time PCR assay for strain KCY1 was developed and validated before used for quantifying strain KCY1 in activated sludge samples collected from three WWTPs. The developed real-time PCR assay was validated using the activated sludge spiked with known amounts of strain KCY1. The C_T values were inversely proportional to DNA template between 0.5 ng and 250 ng of strain KCY1 DNA ($R^2 = 0.997$), and between 0.5 ng and 200 ng of the spiked samples ($R^2 = 0.994$) (Figure 6.12). The linear relationships between threshold cycle values and the logarithm of the DNA template concentrations suggest that the range of DNA concentrations can be precisely quantified using the developed real-time PCR assay.

Populations of strain KCY1 and AOB in three activated sludge samples were comparable, despite the fact that these three WWTPs have different configurations and operating conditions (Table 6.1). Strain KCY1 was observed in all of these three WWTPs with the similar populations, indicating that strain KCY1 is an indigenous wastewater microorganisms. However, the population of strain KCY1in the activated sludge collected from WWTP #1 in November 2011 (used for biodegradation experiments) was lower than that in activated sludge sample collected in May 2009 and May 2012, suggesting that the number of strain KCY1 may be varied seasonally.

These activated sludge samples contained similar number of *amoA* gene (1.5-2.1 $\times 10^4$ gene copies/mL) and AOB genes (1.5-7.4 $\times 10^5$ gene copies/mL) to the activated sludge tested for triclosan degradation in lab-scale bioreactors (Table 6.1). As the bioreactor results suggest that AOB play an important role in triclosan degradation, theses three activated sludge could have the potential for triclosan removal. In addition, based on the results of bioreactors, triclosan can be completely removed within 96 hrs if ammonia is present within its typical concentration range (12 to 45 mg/L) in wastewater. As the reactor #A25 containing initially 33 mg/L of NH_4-N (highest nitrification occurred) showed the fastest triclosan degradation, the best performance in triclosan removal would be possibly observed in WWTP #2, receiving 24-30 mg/L of NH_4-N. If ammonia concentrations in NAS which nitrification is highly active are determined, initial concentrations of ammonia could be used as an indicator to evaluate the potential of triclosan removal in NAS within a given HRT in a selected WWTP. To confirm this, further studies are required by conducting triclosan degradation tests using NAS

collected from several WWTPs. Establishing the conditions favorable for the nitrification process could be one of the strategies to enhance triclosan degradation in NAS.

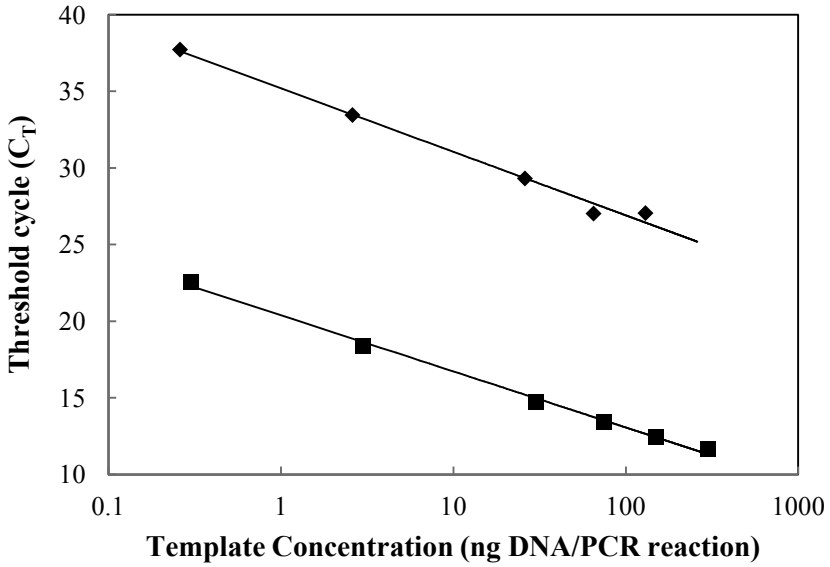


Figure 6.12. Validation of the developed real-time PCR assay for strain KCY1. Genomic DNA extracted from strain KCY1 and community DNA extracted from spiked samples (i.e. adding known amounts of strain KCY1 into activated sludge) was used. Solid diamonds (regression equation: y= -1.8ln(x) +35.2, R^2 = 0.994) represent spiked samples. Solid squares (regression equation: y= -1.6ln(x) +20.4, R^2 = 0.997) represent strain KCY1.

7. SUMMARY, CONCLUSIONS, AND FUTURE RESEARCH

7.1. Research summary and conclusions

Triclosan is a common synthetic antimicrobial agent used in numerous industrial and personal care products. Detection of triclosan in water, sediments, and even in human milk and urine has raised a public health concern because of the formation of toxic metabolites and the potential of promoting antibiotic resistance in microorganisms. While biodegradation of triclosan has been suggested in wastewater treatment plants (WWTPs), little is known about the microbiology of triclosan in wastewater and essential knowledge for developing strategies of the enhanced removal of triclosan in wastewater.

In this study, a wastewater isolate, *Sphingopyxis* strain KCY1, showed an ability to completely degrade triclosan with a stoichiometric release of chloride. This strain can grow with glucose, sodium succinate, and sodium acetate but not triclosan (data not shown) or catechol. Also, its triclosan degradation ability can be retained when the growth medium contained trace amount of triclosan, as low as 5 μg/L. Given that ambient triclosan concentrations in wastewater typically range from 0.61 to 5.1 μg/L and wastewater is full of readily available organics, strain KCY1 may play an important role in triclosan biodegradation in wastewater. Five different transformation metabolites were detected during triclosan biodegradation. Our results also suggested that 2,3 dioxygenases and *meta*-cleavage are important for triclosan degradation. Future studies are needed to investigate the abundance of strain KCY1 in different built and natural

environments, the significance of its role during triclosan degradation in wastewater, and the possibility of bioaugmentation with this strain for enhanced triclosan biodegradation in engineered systems.

This was the first study to molecularly identify eleven phylogenetically diverse microorganisms capable of utilizing triclosan as a carbon source sing culture-independent method, Q-FAST assay. These 11 clones were related to the phylum of Proteobacteria: seven in *β*-Proteobacteria (mainly *Alicycliphilus* genus-related), three in *γ*-Proteobacteria (mainly *Stenotrophomonas* genus-related), and one in *α*-Proteobacteria (mainly *Defluvibacter* genus-related). These genera *Alicycliphilus, Stenotrophomonas,* and *Defluvibacter* have not been known yet to degrade triclosan, although they were reported to degrade recalcitrant chlorinated aromatic compounds, suggesting that these wastewater microorganisms which are capable of degrading chlorinated compounds could be also involved in triclosan utilization. These clones also were corresponded to four measured T-RFs (sizes of 99, 104, 106, and 108 bp) observed in the triclosan-utilizing microbial community profile, but none of clones corresponded to 116 bp of T-RF. Two T-RFs (99 and 108 bps) contributed to approximately 87% of total 16S rRNA gene copies derived from the ^{13}C-DNA fraction, suggesting that Clones #2, #3, #4, #7, and #9 are dominant phylotypes in the triclosan-degrading consortium. In the response to successive triclosan additions, the biomass and diversity of microbial community in the triclosan-degrading consortium decreased significantly, possibly due to the toxicity of triclosan on the wastewater microorganisms.

Triclosan degradation potential of strains examined in this study was determined

with respect to their sensitivity to triclosan and product toxicity and effects of their growth substrates. LB400 was unable to degrade triclosan due to its sensitivity to triclosan toxicity. Propane-grown EVN425 was resistant to triclosan but didn't degrade triclosan. Propane- and 2-propanol-grown JOB5 degraded triclosan completely. Unlike JOB5, the triclosan degradation ability of RHA1 was growth substrate dependent. Due to product toxicity, RHA1 showed different transformation capacities for triclosan, with an order from high to low: biphenyl-grown > LB+DCPK-grown > propane-grown RHA1. Biphenyl-grown RHA1 degraded triclosan via *meta*-cleavage pathway. Four chlorinated metabolites were detected during triclosan degradation. Overall, the results of this study provide a new understanding of triclosan degradation by different oxygenase-expressing cultures. The metabolites and degradation pathway could be used to predict and assess the fate and transport of triclosan in the environment.

We also reported in this study that triclosan can be completely removed in 96 hrs whether ammonia is present within its typical concentration range (12 to 45 mg/L) in nitrifying activated sludge collected from the WWTP. Particularly, triclosan was degraded within 54 hrs by NAS containing 33 mg/L of NH_4-N when the most active nitrification in activated sludge occurred, suggesting that the conditions favorable for nitrification processes are related to the performance of triclosan degradation in NAS. No exact correlation between AOB 16S rRNA genes (or amoA genes) and triclosan degradation rates was observed on the basis of the results of real-time PCR assay. However, After 54 hrs, the increase trend in the number of *amoA* gene was observed in the batch reactors amended with different ammonia concentrations, supporting that

ammonia oxidation was active. Bioaugmentation NAS with strain KCY1 in NAS was not able to enhance the degradation of triclosan. The degradation rate in the bioaugmented batch reactor with ATU was similar to that in the augmented reactor without ATU, suggesting that non-ammonia oxidizing wastewater microorganisms also play an important role in triclosan biodegradation in NAS. Strain KCY1 were observed in three surveyed WWTPS operated with different configurations and operating conditions, indicating that strain KCY1 is an indigenous wastewater bacterium. In addition, similar population of *amoA* gene and AOB were also observed in these WWTPs. This study provides beneficial information about environmental microorganims capable of degrading triclosan and their responsible enzymes to enhance the biological removal of triclosan ubiquitously presented in the environments including wastewater.

7.2. Future research

(1) Sequencing of the complete genome of strain KCY1 characterized in this study will provide insights into factors regulating the unique enzymatic system which is involved in the cometabolic degradation of triclosan. The putative triclosan dioxygenase in this strain has not yet been characterized, and subsequent enzymes in triclosan degradation pathway have not been identified. The availability of the genome sequence will significantly accelerate the analysis of the genes and enzymes involved in triclosan degradation.

(2) Strain KCY1 also demonstrated the ability to biodegrade diphenyl ether which is similar to triclosan. Thus, it is possible that this strain could be degrade other xenobiotics or endocrine disrupting compounds that have the similar chemical structure to triclosan, and exhibit the metabolism or cometabolism of a wide variety of these toxic pollutants. Further investigation about which chemicals can be degraded by strain KCY1 will be useful to better understand this strain in the aspect of its degradation ability.

(3) This study has shown the role of monooxygenase enzymes expressed in several selected bacteria in degrading triclosan. Genetic knockouts can be useful approach to confirming that the omission of particular enzymes eliminates the ability for triclosan degradation. In future study, it would be interesting to clone candidate enzymes such as propane and alkane monooxygenase into tractable host strains.

(4) Triclosan biodegradation by pure cultures used in this study are not representative of the true environment such as contaminated hazard waste sites or receiving-waters/wastewater, due to the lack of interactions with other species in indigenous microbial community, oxygen, nitrogen sources, cell densities, inducing substrates and nutrient conditions, pH and temperature. Microcosm studies of triclosan biodegradation in true environment must be studied to provide comprehensive information of degradation kinetics at ambient concentrations by indigenous microorganisms present in natural conditions.

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