BIODEGRADATION OF TRICLOSAN BY A TRICLOSAN-DEGRADING

ISOLATE AND AN AMMONIA-OXIDIZING BACTERIUM

A Thesis

by

FUMAN ZHAO

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

MASTER OF SCIENCE

May 2006

Major Subject: Civil Engineering

BIODEGRADATION OF TRICLOSAN BY A TRICLOSAN-DEGRADING

ISOLATE AND AN AMMONIA-OXIDIZING BACTERIUM

A Thesis

by

FUMAN ZHAO

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

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Kung-Hui Chu
Committee Members,	Timothy Kramer
	Hongbin Zhan
Head of Department,	David V. Rosowsky

May 2006

Major Subject: Civil Engineering

ABSTRACT

Biodegradation of Triclosan by a Triclosan-degrading

Isolate and an Ammonia-oxidizing Bacterium. (May 2006)

Fuman Zhao, B.S., University of Tianjin, China

Chair of Advisory Committee: Dr. Kung-Hui Chu

Triclosan is incorporated in a wide array of medical and consumer products as an antimicrobial agent or preservative. Disposal of these products transport triclosan into wastewater and later into soils and surface waters. Due to incomplete removal of triclosan in wastewater treatment plants, contamination of triclosan in the environment has raised several concerns, including: (i) an aid to the development of cross-resistance to antibiotics, (ii) the toxicity to ecological health, (iii) the formation of chlorodioxins from triclosan and its metabolites. By using ^{14}C-labeled triclosan, ^{14}CO$_2$ was observed in activated sludge samples, suggesting that triclosan was biodegraded. However, little is known about the microorganisms responsible for triclosan biodegradation in activated sludge.

The goal of this study is to better understand biodegradation of triclosan in activated sludge. Two specific objectives are: (i) isolating and characterizing triclosan-degrading bacteria from activated sludge, (ii) characterizing the cometabolic degradation of triclosan through an ammonia-oxidizing bacterium *Nitrosomonas europaea*.

A triclosan-degrading strain, KCY1, was successfully isolated from the activated sludge. The strain KCY1 completely degraded triclosan in three days when OD_{600} was 0.4. Based on 16S rRNA analysis, the strain KCY1 has 97% similarity with *Phingomonas* or *Phingopyxis*. Negative results of oxygenase activity assays suggested that other enzymes rather than oxygenases might be responsible for the triclosan biodegradation. Experiments using *N. europaea* showed that triclosan could be cometabolized. In the presence of inhibitor for ammonia monooxygenase (AMO), *N. europaea* was unable to degrade triclosan, suggesting that AMO might be responsible for triclosan degradation. Triclosan appeared to competitively inhibit ammonia oxidation by *N. europaea*. Results of this study showed that triclosan might be effectively biodegraded by triclosan-degrading cultures, strain KCY1 and *N. europaea*.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Triclosan, an antimicrobial agent and preservative, has been widely used for over 30 years since its first introduction into the health care industry in a surgical scrub in 1972 (Glaser, 2004). Triclosan can block lipid biosynthesis by inhibiting the enzyme enoyl acyl carrier protein reductase and may induce bacterial resistance development (McMurry et al., 1998; Levy et al., 1999). Currently, Triclosan has been incorporated in a broad array of personal care products (e.g. hand disinfecting soaps, medical skin creams, dental products, deodorants, toothpastes), consumer products (e.g. fabrics, plastic kitchenware, sport footwear), and cleaners or disinfectants in hospitals or households. It is estimated that between 1992 and 1999 over 700 consumer products with antibacterial properties, the vast majority of them containing triclosan, entered the consumer market (Schweizer, 2001). In recent years, approximately 1,500 tons of triclosan are presumably produced annually, and approximately 350 tons of those are applied in Europe (Singer et al., 2002).

The widespread use of triclosan resulted in the discharge of this compound to wastewater treatment plants (WWTPs) and then into soils and surface waters due to incomplete removal of triclosan in WWTPs. Triclosan and its derivatives have been identified in various countries, including the USA (Lopez-Avila and Hites, 1980;

_____

This thesis follows the style of Chemosphere.

McAvoy et al., 2002), Sweden (Paxeus, 1996) and Switzerland (Lindstrom et al., 2002). The triclosan concentrations in WWTP influent range from 0.062-21.39 μg/L (Lindstrom et al., 2002; McAvoy et al., 2002; Kanda et al., 2003; Bester, 2003; Sabaliunas et al., 2003). The removal of triclosan by WWTPs varies from 0% to 100%, depending on the types of secondary and tertiary treatments utilized (McAvoy et al., 2002; Kanda et al., 2003; Bester, 2003; Singer et al., 2002; Federle et al., 2002). High triclosan removal (> 90%) was observed by activated sludge processes (McAvoy et al., 2002; Bester, 2003; Federle et al., 2002). While, the removal of triclosan by attached growth processes was much lower and less consistent (Sabaliunas et al., 2003). Triclosan has a high octanol-water partition coefficient, $logK_{ow}$ 4.8 (Lopez-Avila and Hites, 1980), leading to approximately 30% of influent triclosan absorbed onto biosolids (Bester, 2003; Singer et al., 2002; Federle et al., 2002). Triclosan concentrations in WWTP effluent range from 0.042 to 22.1 μg/L (Sabaliunas et al., 2003; Singer et al., 2002; Aguera, 2003). A comprehensive study performed by the United State Geological Survey detected triclosan in 57.6% of 139 U.S. streams at a medium concentration of 0.14 μg/L and a maximum concentration of 2.3 μg/L (Kolpin et al., 2002).

The broad contamination of triclosan in the environment has raised several concerns, including an aid to the development of cross-resistance to antibiotics (Braoudaki and Hilton, 2004), the toxicity to ecological health (Tatarazako et al., 2004), and the formation of chlorodioxins from triclosan and its metabolites (Latch, et al., 2003). A recent study also suggested that triclosan is potentially weakly androgenic (Foran et al., 2000).

The fate of triclosan in WWTPs has been reported: 79% biodegradation, 15% absorption to biosolids, and 6% release into the receiving surface water (Singer H et al., 2002). By using ^{14}C-labeled triclosan, Hay et al. reported that triclosan was biodegraded in batch reactors containing activated sludge (Hay et al., 2001). However, little is known about the microorganisms that are responsible for degrading triclosan in activated sludge.

The research efforts of this study include isolation, identification and characterization of triclosan-degrading cultures in activated sludge. The study goal is to better understand biodegradation of triclosan in activated sludge. The specific objectives are to isolate and characterize triclosan-degrading bacteria from activated sludge, and to characterize the cometabolic degradation of triclosan through an ammonia-oxidizing bacterium *Nitrosomonas europaea*. The following chapters consist of: Chapter II, background; Chapter III, materials and methods; Chapter IV, triclosan-degrading consortium and an isolate KCY1; Chapter V, biodegradation of triclosan by *Nitrosomonas europaea*; Chapter VI, conclusion and future studies.

CHAPTER II

BACKGROUND

Physical and Chemical Properties

Triclosan is a synthetic chlorinated aromatic compound with functional groups of ethers and phenols (Fig. 2.1) (General Chemistry Online, 1999). Its chemical formula is $C_{12}H_7Cl_3O_2$ and the molecular weight is 289.5 g/mol, the CAS number is [3380-34-5] and the EINECS number is [222-182-2]. Triclosan is a white to off-white powder at room temperature and standard pressure with a melting point of about 55-57 ºC and a weak aromatic/phenolic odor. It is slightly soluble in water (17 mg/L) while very soluble in fat, ethanol, diethyl ether and stronger basic solutions such as 1 M sodium hydroxide like other phenols (Chemical Land21).
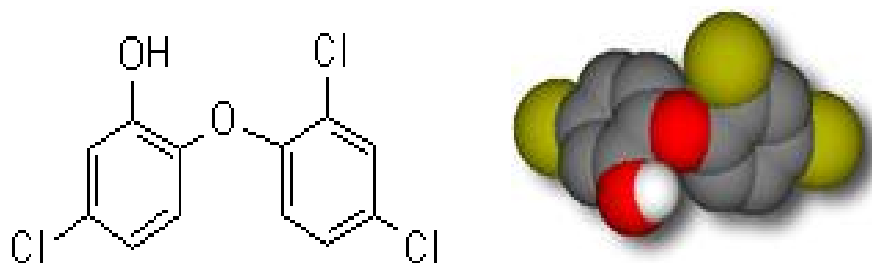
Fig. 2.1. Triclosan chemical structure and molecular model.

Triclosan has several chemical names: 2,4,4'-Trichloro-2'-hydroxydiphenyl ether, Trichloro-2'-hydroxy-diphenylether; 5-Chloro-2-(2,4-dichlorophenoxy)phenol;

2,4,4'-Trichloro-2-hydroxydi-phenyl ether; 5-Chloro-2-(2,4-dichlorophenoxy) phenol (Chemical Land21). Although the name "triclosan" is popularly used, some synonyms also prevail such as Irgasan DP 300, Irgasan CH3536, Microban, Lexol, Ster-zac, Cloxifenolum, Biofresh etc (FDA, 2001).

Under ordinary conditions triclosan is stable, but at relatively high temperature, for example in autoclaving at 121 ºC, triclosan will partially decompose. In the aquatic environment, triclosan will undergo a series of transformation reactions to produce, in some cases, more toxic and/or bioaccumulative compounds (Mezcua, et al., 2004; Lindstrom et al., 2002). One of the fastest and most favorable is the oxidation of triclosan by free chlorine (Canosa er al., 2005), which is already present in tap water, or is induced into waste water sewers by domestic disinfecting products. Moreover, thiclosan is a dioxin precursor and can be converted to 2, 8-dichlorodibenzo-p-dioxin by an intramolecular photochemical substitution reaction in aqueous solutions buffered at PH 8 or above (Latch et al., 2000).

Activity Mechanism and Effectiveness

Early research suggested that the bacterial inner cytoplasmic membrane was the major target for triclosan action (Russell, 2003). It was later demonstrated that triclosan blocks fatty acid synthesis, which is necessary for building cell membranes and for reproduction, by interacting with the NADP binding site of the enoyl-acyl carrier protein (ACP) reductase, FabI in *Escherichia coli* (McMurry et al., 1998) and InhA in *mycobacterium tuberculosis* (Parikh et al., 2000). However, *Streptococcus pneunomiae*, which is also sensitive to triclosan, does not contain a FabI homolog. Instead, *S.*

pneunomiae contains FabK, an isofunctional FAD-dependent enoyl-ACP reductase which is not sensitive to triclosan. This indicates that triclosan must have some other targets in *S. pneunomiae* (Heath and Rock, 2000). It is found that triclosan can induce potassium ion leakage, indicative of membrane damage (Suller and Russell, 1999). Membrane-destabilizing effects have also been demonstrated (Villalain et al., 2001).

Under proper settings and conditions, triclosan has been proved to be an effective antimicrobial agent in everyday usage, such as in hospitals, in order to prevent hospital-acquired infections. Experiments demonstrate that triclosan is a very potent inhibitor, and only a small amount is needed to perform a powerful antibiotic action (Levy et al., 1999). However, current reports suggest that its effectiveness is compromised due to inappropriate utilization. A study suggested that triclosan acted on a specific bacterial target rather than as a non-specific biocide, which would facilitate the acquisition of bacterial resistance (McMurry et al., 1998). Consequently, bacterial pathogens can survive the threat of antibiotics and biocides, they may also thrive (Russell, 2002). Also cross-resistance between triclosan and antibiotics occurs in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157 (Braoudaki and Hilton, 2004). In a healthy household, no current data demonstrates any extra health benefits from having antibacterial-containing cleansers (Levy, 2001). According to the centers for Disease Control and Prevention, antibacterial soaps are not necessary in everyday use, and washing hands with ordinary soap and warm water is an effective way to ward off infections (Lurie, 2004).

Toxicity

Although triclosan is a chlorophenol, a class of chemicals which are suspected of causing cancer in humans, it has been treated as relatively non-toxic to humans and other mammals for a long time (Bhargava and Leonard, 1996). However, in current years, some reports have suggested that triclosan is registered as a pesticide and EPA gives it high scores both as a human risk and an environmental risk (Jackson, 2004). Triclosan may cause photoallergic contact dermatitis (PACD), which occurs when a part of skin exposed to triclosan is also exposed to sunlight. PACD can cause an eczematous rash, usually on the face, neck, the back of the hands, and on the sun-exposed area (Durbize et al., 2003). The dermal LD_{50} (the lethal dose that kill 50 percent of a population of a test animals) for rats is 5000 mg/kg. The oral LD_{50} for rats is 4500-5000 mg/kg, for mice it is 4000 mg/kg, and for dogs it is over 5000 mg/kg (Triclosan. Material Safety Data Sheet, CNCCC). Data comparing triclosan's EC_{50} (effective concentration of a given compound that reduces the specified effect to half of the original response) with phenol and copper indicate that triclosan is the most toxic compound, while phenol is the least toxic one shown in Table 2.1 (Neumegen et al., 2004). Additionally, triclosan is lipophilic, and can bioaccumulate in fatty tissues. Concerns over interfering with the body's thyroid hormone metabolism led to a study which found that triclosan had a marked hypothermic effect, lowering the body temperature, and, overall, causing a "nonspecific depressant effect on the central nervous system" of mice (Miller et al., 1983). Although the chemical structure of triclosan closely resembles certain estrogens, a study on a Japanese species of fish did not

demonstrate estrogenic effects. However, triclosan was found weakly androgenic, causing changes in fin length and sex ratios (Foran et al., 2000).

Table 2.1
EC_{50} values for triclosan, phenol, and copper.

Toxicant	EC_{50} Culculated (mg/L)	EC_{50} Literature value (mg/L)	Organism/Test used	Reference
Triclosan	1.82±0.1	0.4	Daphnia, EC_{50}	Jones et al., (2002)
		0.2	Algae, EC_{50}	Jones et al., (2002)
		0.34	Daphnia magna, EC_{50}, 48h	Orvos et al., (2002)
		0.001	Algae (Scenedesums), EC_{50},96h	Orvos et al., (2002)
		0.26	Fathead minnow, LC_{50}, 96h	Orvos et al., (2002)
		0.37	Bluegill, LC_{50}, 96h	Orvos et al., (2002)
Phenol	270±0.26	482	Shkl assay, Bioluminescent bacterium EC_{50}	Ren and Frymier (2002)
		1.8 mmol/L	2,4-dimethyl phenol, IC_{50}	Hall et al., (1996)
		0.19 mmol/L	2,5-dichlorophenol, IC_{50}	Hall et al., (1996)
		107.4	Biotox, EC_{50}, min	Kahru et al., (1996)
		17.3	Microtox, EC_{50}, 5 min	Kahru et al., (1996)
		28	Microtex	Arretxe et al., (1997)
$CuSO_4 \cdot 5H_2O$	18.3±0.37	449.42	Providencia rettgeri, MIC, Oxoid broth	Hassen et al., (1998)
		399.49	Pseudomonas aeruginosa, MIC, Oxoid broth	Hassen et al., (1998)
		49.94	Citrobacter freundii, MIC, Oxoid broth	Hassen et al., (1998)
		0.1-2.5	Harlequin fish, LC_{50}, 96h	Merck (2001)
		0.1-2.5	Rainbow trout, LC_{50}, 96h	Merck (2001)
		0.24	Daphnia, EC_{50}, 48h	Merck (2001)
		0.1-2.5	Goldfish, LC_{50}, 96h	Merck (2001)
		0.39	Pond snails, LC_{50}, 96h	OMRI (2001)

Recent attention has been focused on triclosan's link to dioxin. According to EPA's draft Dioxin Reassessment, triclosan is listed as "could be" and "suspected to be" contaminated with dioxins (U.S. EPA, 1994). Due to the chemical structure similarity (as a polychlorinated phenoxy phenol), it has been suggested that dioxin would be a synthesis impurity during triclosan manufacture (Menoutis and Parisi, 2001). Recent studies reported that a certain part of triclosan can change to dioxins in processes of manufacturing (Menoutis and Parisi, 2001), incineration (Kanetoshi et al., 1988), and

exposure to sunlight or ultraviolet light (Latch et al., 2000). Dioxin can be highly carcinogenic and can cause health problems as severe as weakening of the immune system, decreasing fertility, altering sex hormones, bringing about miscarriage, birth defects, and cancers (US Dept of Health and Human Services, 1998). Furthermore, triclosan can experience oxidation by free chlorine and the products are health-concerning compounds like chloroform, tetraclosan, pentaclosan, 2,4-DCP and 2,4,6-TCP (Rule et al., 2005).

Environmental Effects

Over 95% of the uses of triclosan are in consumer products that are disposed of in residential drains (Reiss et al., 2002). Since wastewater treatment plants can not completely remove triclosan from the water, the discharged triclosan will distribute broadly to different environments such as streams, lakes, rivers, surface waters and sediments (Danish EPA, 2003). Research has found triclosan is high stable for long periods of time in the environment. It has given rise to the absorption and bioaccumulation in aquatic organisms and even in human breast (Adolfsson-Erici et al., 2002). A study in bodies of water in Switzerland also found low levels of methyl triclosan, the breakdown by-product of triclosan. Methyl triclosan, which is formed by a process called biological methylation, is actually more lipophilic and more bioaccumulative than triclosan (Lindstrom et al., 2002).

Triclosan can have detrimental impacts on aquatic ecosystems. In a marine algae growth experiment with different triclosan strengths, it has been found that triclosan can stunt the growth of algae, and the higher the concentration of triclosan, the

slower the growth of algae (Wofberg, 2004). Because algae are the first-step producers in aquatic ecosystems, high levels of triclosan discharged into the environment may cause possible balance destruction of aquatic ecosystems (Tatarazako et al., 2004; Orvos et al., 2002). The risks are especially high immediately downstream from wastewater treatment plants (Reiss et al., 2002).

Because of its lipophilic nature and resistance to degradation, triclosan in waterways has produced some toxic effects to aquatic organisms. It has been found that triclosan is highly toxic to Japanese medaka fish in their early life stages and may be a weak endocrine disruptor (Ishibashi et al., 2004). An experiment showed that increasing amount of triclosan not only stunts artemia growth, but inhibits the artemia completely (Bich and Vu, 2004). Also a group of studies to measure the acute toxicity of triclosan to a microtox bacterium (*Vibrio fischeri*), a crustacean (*Ceriodaphnia dubia*), a microalga (*Selenastrum capricornutum*) and fish (*Danio rerio* and *Oryzias latipes*) were conducted by Japanese researchers. The results indicated that the microtox bacterium, crustacean and fish had similar sensitivities towards triclosan toxicity (i.e., IC_{25} from 0.07 to 0.29 mg/L trclosan, while the microalga was about 30-80-fold (IC_{25} 0.0034 mg/L triclosan) more sensitive to triclosan toxicity than the bacterium and fish (Tatarazako et al., 2004).

Regulation and History

In the USA, the EPA and FDA share responsibility for regulating antimicrobial products. In general, EPA regulates all of the pesticidal uses of triclosan and the FDA regulates all food and drug uses of triclosan. Although triclosan has possessed some risks to human health and environment, EPA and FDA have done little to warm

consumers of the possible health and environmental effects of triclosan (Glaser, 2004). In Europe, there is a different story to treat this chemical. In 2000, the Danish EPA, National Board of Health, National Central Laboratory and the Danish Consumer Information Center issued a joint statement advising consumers against the routine use of antibacterial household and personal hygiene products, stating that their use is unnecessary for domestic use and potentially harmful to the environment as they "are extremely persistent and highly toxic in the marine environment" (Environment News Daily, 2000). Six Finnish public authorities also issued a statement urging consumers to not use certain antibacterial chemicals, stating they are unnecessary and that their growing use increases the risk of spreading antibiotic resistance in microbial populations. The joint statement also stated that even Finnish hospitals should not use triclosan for routine cleaning operations. In households, we see more disadvantages than advantages (Environment News Daily, 2001). The following year, German environment minister Jurgen Trittin called on consumers to not use cleaning agents containing anti-bacterial agents and on industries to stop marketing and advertising the antibacterial qualities of their products, calling their use in households, "superfluous and risky," He also demanded that industries stop suggesting to consumers that they are "surrounded by enemy germs which they had to fight aggressively" (Environment News Daily, 2001).

Degradation

Due to wide use of triclosan in our routine life, this has caused its ubiquitous distribution in the environment. Simultaneously, triclosan degradation takes place at different sites such as in WWTPs, surface waters, sedimentation solids and drinking

water. The degradation activities occur through different ways like physical absorption, chemical reactions, and biodegradation.

Research about triclosan's occurrence and fate has found that around 15% of triclosan is absorbed by sludge in WWTPs and 6% of triclosan was released into natural receiving water (Singer et al., 2002). The relatively high octonal-water partition coefficient ($logK_{ow}$ 4.8) of triclosan can lead to its easy absorption to sediments in streams, lakes or rivers when the treated effluent wastewater disperses in the environment. At the same time, triclosan's strength will reduce by dilution in the large water reservoirs.
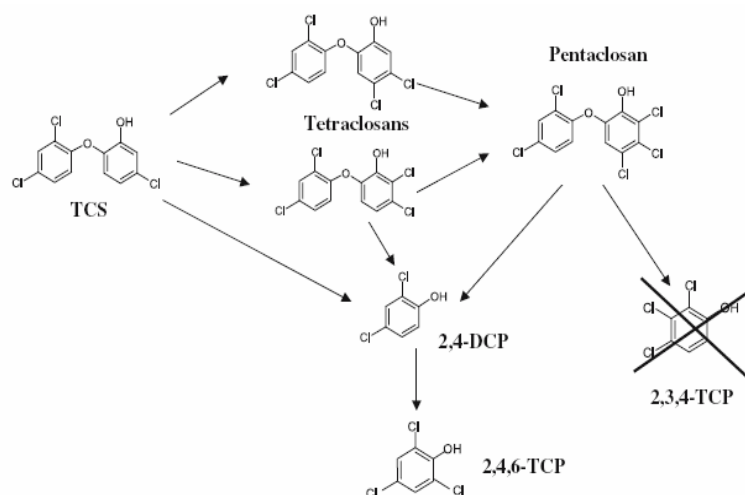
Fig. 2.2. Proposed triclosan degradation pathways in the presence of free chlorine.

Triclosan can experience many chemical reactions to lower its concentration. Under laboratory conditions, triclosan was found to be easily decomposed when it is autoclaved. Some studies have shown that triclosan can be photodegraded. When we

shined sunlight or ultraviolet light on the surface water containing triclosan, up to 12% of dissolved triclosan will be converted to dioxin (Singer et al., 2002). During drinking water treatment, triclosan will react with free chlorine to the form two tetra- and a penta-chlorinated hydroxylated diphenyl ether, 2,4-dichlorophenol, as well as significant amounts of 2,4,6- tricloro-phenol while 2,3,4- triclorophenol was not detected shown in Fig. 2.2 (Canosa et al., 2005). Triclosan can be rapidly oxidized by manganese oxides δ-MnO_2 and $MnOOH$ (Zhang and Huang, 2003). It was suggested that this oxidation process might be significant under the conditions where biological transformation might not occur.

Compared to chemical and physical degradations, biodegradation of triclosan is the most effective and comprehensive method to remove it from natural environments. Experimental data have been reported about triclosan average removal efficiency of 79% in WWTPs under aerobic and anaerobic conditions (Singer et al., 2002). Triclosan degraders have been studied in laboratory conditions. It was found that a bacterial consortium, which was enriched from activated sludge in a WWTP, could degrade 35% of total ^{14}C-labeled triclosan with an initial concentration 500 mg/L in 13 days, and an isolate *Sphingomonas sp.* RD1 from the consortium was detected to partially degraded triclosan in a complex medium through cometobolism (Hay et al, 2001). In addition, some researchers have investigated that two soil bacteria, *Pseudomonas putida* and *Alcaligenes xylosoxidans,* had high levels of triclosan resistance and were able to use triclosan as a sole carbon source to clear particulate triclosan from agar plates (Meade et al., 2001). Also, it was found that two fungi, *Trametes versicolor* and *Pycnoporus*

cinnavarinus, could degrade triclosan. Actually, *T. versicolor* metabolized triclosan and produced three metabolites, 2-O-(2,4,4′-trichlorodiphenyl ether)-β-D-xylopyranoside trichlorodiphenyl ether)-β-D- xylopyranoside, 2-O-(2,4,4′-trichlorodiphenyl ether)-β-D-glucopyranoside, and 2,4-di- chlorophenol. *P. cinnabarinus* converted triclosan to 2,4,4′-tricloro-2′-methoxydiphenyl ether and the glucoside conjugate known from *T. versicolor*. The conjugates showed a distinctly lower cytotoxic and microbicidal activity than triclosan did (Hundt et al., 2000).

Ammonia-oxidizing Bacteria and Ammonia Monooxygenase

Ammonia-oxidizing bacteria, a group of chemolithoautotrophic microorganisms, play an important role in the global cycling of nitrogen by converting ammonia to nitrite (Prosser, 1989; Sprent, 1987). They comprise two monophyletic lineages within the class proteobateria based on 16S rRNA gene sequences: the genus *Nitrosococcus* is in the γ subgroup and the genera *Nitrosomonas* and *Nitrosospira* in the β subgroup (Head et al., 1993; Teske et al., 1994). It is thought that the genus *Nitrosococcus* is restricted to marine habitats (Ward and Carlucci, 1985), and the *Nitrosomonas* and *Nitrosospira* of proteobacterial ammonia oxidizers appear to occur in a broad range of environments (Belser, 1979). This group of microorganisms has been refractory to conventional techniques for isolation of pure cultures. As a result, most of studies of the physiology and ecology of ammonia oxidation have focused on a single strain of *Nitrosomonas europaea*, which was originally isolated from soil and can be grown most conveniently under laboratory conditions (Prosser, 1989).

As an obligately lithoautotrophic soil nitrifying bacterium, *Nitrosomonas europaea* obtains all of its energy for growth from the oxidation of ammonia to nitrite and satisfies all of its carbon requirements by fixing carbon dioxide (Wood et al., 1986). It lives in several environments such as soil, sewage, freshwater, the walls of buildings and on the surface of monuments especially in polluted areas where air contains high levels of nitrogen compounds. This microbe prefers an optimum PH of 6.0-9.0, fairly neutral conditions, and a temperature range of 20-30 °C. Most are motile with flagella located in the Polar Regions although some species are non-motile (Regents of University of California, 2005). *N. europeae* is capable of oxidizing various hydrocarbon compounds such as methane, methanol, phenol, and benzene, as well as halogenated hydrocarbons such as TCE (Hyman et al., 1988; Rasche, et al., 1991).

The oxidation of ammonia to nitrite is a two-step reaction catalyzed by two enzymes (Fig. 2.3). The initial oxidation of ammonia yields hydroxylamine (NH_2OH) by the membrane-bound enzyme ammonia monooxygenase (AMO). Subsequently, hydroxylamine is oxidized to nitrite catalyzed by a multi-heme-containing periplasmic hydroxylamine oxidoreductase (HAO) (Wood et al., 1986). AMO produced by *Nitrisomonas europaea* can oxidize various hydrocarbon and aromatic compounds. These compounds were co-metabolic substrates for AMO. Unlike AMO, the substrate range of HAO is restricted to small molecules such as hydroxylamine, hydrazine and N-methylhydroxylamine (Hyman and Wood, 1983).

Generation of NH_2OH from NH_3:

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \longrightarrow NH_2OH + H_2O + energy$$
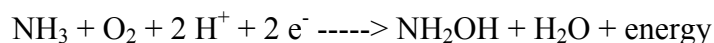
Production of NO_2^- from NH_2OH:

$$NH_2OH + H_2O \text{ -----> } NO_2^- + 5\ H^+ + 4\ e^- + energy$$
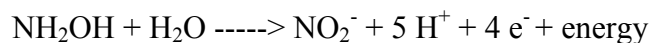
In recent years, the ever-extended substrate range of AMO is reported. The ability of *Nitrosomonas europaea* to degrade a variety of organic contaminants in nitrifying soils and waters makes itself an attractive organism for bioremediation (Chang et al., 2002).
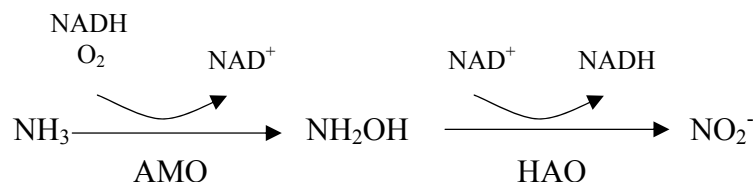
Fig. 2.3. Ammonia oxidation by *N. europaea* by enzymes AMO and HAO.

CHAPTER III

MATERIALS AND METHODS

Chemicals

Triclosan (purity 97%) was obtained from Aldrich (Aldrich Chemical Company, Inc., Milwaukee, WI). Stock solution of triclosan was prepared in acetone. Allylthiourea, an inhibitor for ammonia monooxynase enzymes, was purchased from Sigma-Aldrich, Inc. (Louis, MO). Sodium formate was purchased from MP Biomedicals, Inc. (Solon, Ohio). Ethyl ether was used to extract triclosan from liquid samples. Dimethylformanide (DMF) was purchased from J.I. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) and N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) from Pierce (Pierce Biotechnology Inc., Rockford, IL, USA). All other reagents used were commercial products of high grade available.

Bacteriological Media

A chemically defined nitrate mineral salts (NMS) medium was used for the triclosan-degrading culture enrichment (Chu and Alvarez-Cohen, 1996). The NMS medium contains 11.76 mM $NaNO_3$, 6.1 mM Na_2HPO_4, 0.98 mM K_2SO_4, 0.15 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM H_2SO_4, 3.9 mM KH_2PO_4, 0.07 mM $CaSO_4 \cdot 2H_2O$, 0.08 mM $FeSO_4 \cdot 7H_2O$, 0.001 mM KI, 0.002 mM $ZnSO_4 \cdot 7H_2O$, 0.002 mM $MnSO_4$, 0.002 mM H_3BO_4, and 0.004 mM $CoMoO_4 \cdot 7H_2O$. The consortium members were streaked on MNS-triclosan agar plates (5 mg/L triclosan and 1.5% Noble agar dissolved in NMS medium). The R2A-triclosan agar plates (5 mg/L triclosan, 1.52 g/L or 10% of R2A agar,

and 10.8 g/L BBL selected agar) were used for subcultivation. NMS agar plates without triclosan (only 1.5% Noble agar dissolved in NMS medium) were employed too. Pure colonies were grown in 10% Bacto Tryptic Soy Broth (TSB) containing 5 mg/L triclosan and 3 mg/L TSB powder).

The *N. europaea* growth medium was utilized for the ammonia-oxidizing bacterium growth. It contains 3.3 g $(NH_4)_2SO_4$, 0.41 g KH_2PO_4, 0.75 ml of 1 M $MgSO_4$, 0.2 ml of 1 M $CaCl_2$, 0.33 ml of 30 mM $FeSO_4$/50 mM EDTA, 0.01 ml of 50 mM $CuSO_4$, 5.444 g KH_2PO_4, 0.48 g NaH_2PO_4, 0.04% Na_2CO_3 in 1 liter of volume. The final PH of the medium was adjust to 8 .0 by 10 N of NaOH.

Growth of *Nitrosomonas europaea*

N. europaea was a gift from Dr. Michael Hyman, Department of Microbiology, North Carolina State University. The cells were grown in 1000 ml glass bottles. The bottles were incubated in the dark at 150 rpm, at 30 °C for 3 days before harvesting for triclosan degradation studies. The cells were pelleted by centrifugation (at 10000×g rpm and 4 °C for 30 min), and then washed once with phosphate buffer solution (50 mM NaH_2PO_4 [PH was adjusted to 7.8 through 1 N NaOH], 2 mM $MgSO_4•7H_2O$). The washed cell pellets were resuspended in the *N. europaea* growth medium and the protein concentration was measured. All cell suspensions were stored at 4 °C in the dark before used.

Triclosan-degrading Consortium

Activated sludge collected from a local wastewater treatment plant in Knoxville, Tennessee, was used as an inoculum for establishing triclosan-degrading consortium.

After this initial enrichment, the cell suspension was transferred to a new 250 ml autoclaved flask containing NMS medium and 5 mg/L triclosan (40 ml/80 ml ratio of cell suspension to NMS medium). Because triclosan stock solution was prepared in acetone which is a possible carbon source for bacterial growth, the acetone was removed as described below. Triclosan stock solution was added to an empty autoclaved flask to let acetone evaporate entirely before NMS medium was added into the flask. Before transferring the cell suspension to this flask, at least 2 days were needed for triclosan dissolving in NMS medium completely. The consortium was incubated at 150 rpm at 30°C for 10 days. Three more times cell transfers were conducted by following the same procedures and conditions described above. The triclosan concentration was monitored at the 0th day, 2nd day, 4th day, 7th day, and 10th day during each enrichment cycle. Killed controls were used.

Isolation

After the last cycle of enrichment, the triclosan-degrading consortium was streaked on the NMS-triclosan agar plates for isolating triclosan-degrading bacteria. The streaked plates were incubated at 30 °C under aerobic conditions. After 10 days of incubation, single colonies were picked and streaked on R2A-triclosan agar plates. Repeated streakings on NMS-triclosan and on R2A-triclosan were conducted four times until colonial morphology (e.g. color, texture, light transmission, form, margin and elevation) appeared homogeneous. MNS agar plates (no triclosan) were also used to check the purity of presumptive triclosan-degrading colonies. Isolation schematic presentation is shown in Fig. 3.1.

```
                          ┌─────────────┐
                          │     NMS     │
                          │  Agar Plate │
                          └─────────────┘
                                 ↑
┌─────────────┐      ┌─────────────┐      ┌─────────────┐
│NMS-Triclosan│ ───→ │R2A-Triclosan│ ───→ │R2A-Triclosan│
│  Agar Plate │      │  Agar Plate │      │  Agar Plate │
└─────────────┘      └─────────────┘      └─────────────┘
                                                 │
                                                 ↓
                     ┌─────────────┐      ┌─────────────┐
                     │R2A-Triclosan│ ←─── │R2A-Triclosan│
                     │  Agar Plate │      │  Agar Plate │
                     └─────────────┘      └─────────────┘
```
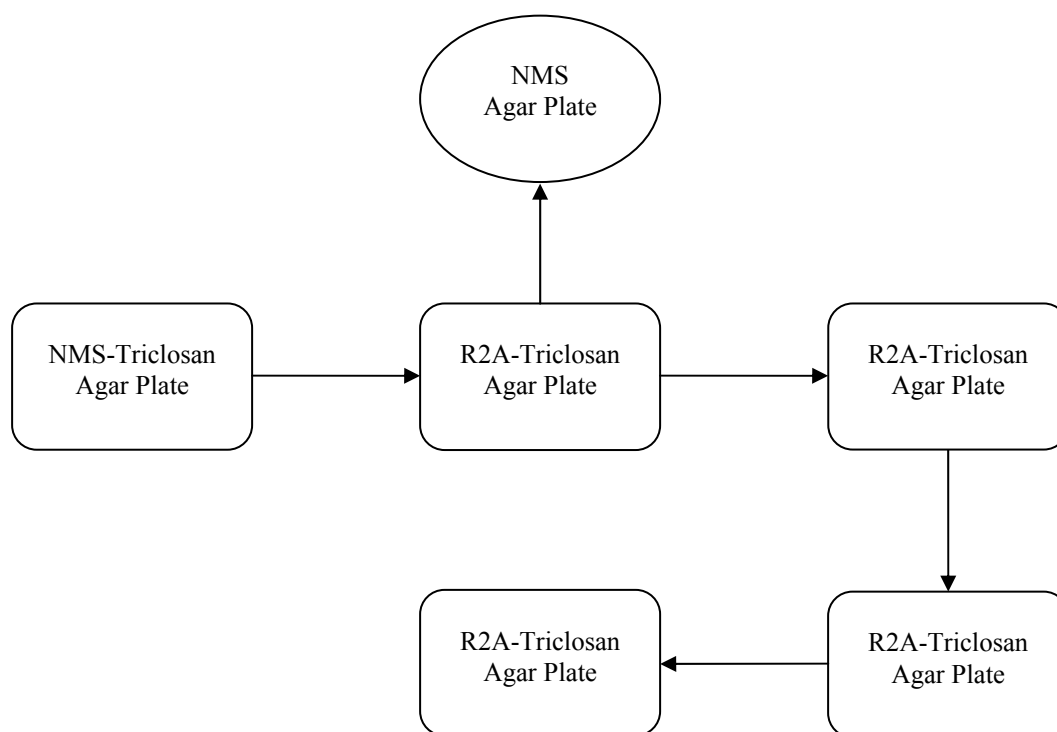
Fig. 3.1. Schematic procedures for isolating triclosan-degrading cultures.

The presumptive triclosan-degrading colonies were further examined for their ability to degrade triclosan in MNS medium containing 5 mg/L triclosan. Triclosan concentrations over time were measured by GC-MS analysis at the day 0, day 1, day 3, day 5 and day 7. The experiments were conducted in batch mode at 30 °C at 150 rpm under aerobic conditions. The colonies were pregrown in 10% TSB media for two days. The cell suspension was centrifuged at 10000×g rpm and 4 °C for 10 minutes. The supernatant was decanted and the combined cell pellets were "washed" two times in Phosphate-Buffered Saline (PSB) solution (including 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na$_2$HPO$_4$, 0.24 g/L KH$_2$PO$_4$, and the final PH was adjusted to 7.4 with HCl). After that, an exact volume of cell suspension was added to NMS medium to examine triclosan

degradation through the isolate. Optical density (600 nm) 0.4 was used as initial cell concentrations. Killed controls were used to estimate abiotic loss of triclosan. Chloride concentrations before and after experiments were measured by using a DX-80 Ion Chromatography (IC) (Dionex Corporation, Sunnyvale, CA). The DX-80 was equipped with an IonPac AS14A-5μm Analytical Column (3 x 150 mm) for anion separation. An eluent solution of 0.16 M Na_2CO_3 and 0.02 $MNaHCO_3$ was used. The Regeneration solution is 70 mN H_2SO_4. Flow rates were 1 ml/min and a sample size of 1.0 mL volume was for IC analysis. The production of chloride production was determined by substracting chloride concentrations measured before and after experiments.

DNA Extraction and PCR Amplification

The triclosan-degradation isolate was pregrown in 10% TSB medium containing with 5 mg/L triclosan for two days before use for DNA extraction. The genomic DNA of the strain was extracted by following the manufacture's instructions of FastDNA kit (Q-Biogene Bio 101, Carlsbad, CA). For soil samples, FastDNA SPIN kit was used with a minor modification: the silica binding matrix-DNA complex was washed twice with 80% (V/V) ethanol after the recommended salt-ethanol wash step (Dionisi et al., 2002).

The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the extracted DNA as a template. The PCR reactions were carried out by using a machine of PCR Sprint Thermal Cycler (Thermo Electron Corporation, Milford, MA). The total volume of PCR reaction was 25 μl, with 12.5 μl Taq PCR Master Mix (QIAGEN Inc. Valencia, CA), 1 μl of 40 mM forward primers 8F (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1 μl of 40 mM reverse primers 1407R (5'-ACGGGCGGTGTGTACA-3') (Braker et al., 2001), 9.5 μl HPLC water and 1 μl of extracted DNA templates. The PCR thermal cycle program was 95 °C for 10 min, followed by 30 cycles of 95 °C for 45 sec, 57 °C for 1 min, and 72 °C for 2 min. A final elongation step of 72 °C for 10 min was included. PCR products were separated through agarose (1.2% w/v) gel electrophoresis in Tris-acetate-EDTA (TAE) buffer. DNA ladder, 1 Kb plus DNA Ladder (Invitrogen, carlsbad, CA.), was used to estimate the size of the amplified DNA.

After the PCR products were separated from genomic DNA on agarose gel, the violet-visible DNA bands of expected size (about 1400 bp) were excised from the agarose gel with a clean, sharp scalpel. The PCR products in the excised gel were purified by using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA.).

16S rRNA Sequence

The 16S rRNA sequences were determined by using an Applied Biosystem 300 DNA sequencer located at the DNA Technologies Laboratory, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX. The primer set, 16S 8F and 16S 1047R, was used for PCR amplification. The obtained raw sequence data from both strands were assembled into full-length sequences by using the BLAST 2 Sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The 16S rRNA sequences of triclosan-degrading strain was compared against with the reference sequences in GenBank by using the BLAST, the related sequences were identified and the closest relatives were selected for sequence alignment. Phylogenetic relationships were

completed with CLUSTAL X software, and the bootstrap support values from 1000 replication are indicated at branch nodes. A phylogenetic tree was plotted by using a software Tree View.

Tests of Triclosan Degradation by an Isolate

Indole oxidation analysis: Dioxygenase activity in cells was measured in term of the oxidation of indole into cis-indole-2,3- dihydrodiol, indoxyl, and finally indigo. The assays were performed as described by Jenkins and Dalton (Jenkins and Dalton, 1985). The isolate was pregrown in 10% TSB medium for two days before harvested through centrifuge at 10000xg rpm and 4 °C for 30 min. After washing with 0.05 M KH_2PO_4 buffer solution, the pellets were resuspended with cell solution (containing 50 ml DI water, 10 ml pure glycerol, 10 ml filtered alcohol, 10 ml of 0.05 M KH_2PO_4, 10 ml of 100 mM DTT and 10 ml of 0.05 M NADH). French pressure was used to break the cells. The experimental samples were prepared with a volume of 3 ml including 0.15 ml of 0.5 KH_2PO_4 buffer solution, 3 µl of 0.1 M $FeSO_4 \cdot 7H_20$, 0.02 ml of 0.05 M NADH, 0.3 ml of 2 mM indole and 2.53 ml cell suspension. Dioxygenase activity was determined by measure the absorbance at A_{400} after 90 minutes of incubation. The absorbance was detected by a Pierce spectrophotometer. A blank containing all ingredients except indole was used as background absorbance.

Naphthalene oxidation assays: The activity of monooxygenase was measured by naphthalene oxidation assay as described by Chu and Alvarez-Cohen (Chu and Alvarez-Cohen, 1998). The assay is based on that monooxygenase can oxidize naphthalene to 1- or 2-naphthol which reacts with tetrazotized o-dianisidine to form a purple naphthol-

diazo complex. The quality of naphthol-diazo complex is measured through absorbance at 530nm. Isolate was pregrown with 10% TSB medium to optical density (600 nm) 0.2. Glass vials were amended with 1 ml cell suspension, 1 ml of saturated naphthalene stock solution and 1 ml of 20 mM sodium formate. The amended vials were incubated at 30 °C, at 160 rpm for 1 hour. After adding 100 μl of the freshly made 0.2% (w/v) tetrazotized o-dianisidine, the absorbance (530 nm) was measured promptly in two minutes by using a Pierce spectrophotometer. Blank controls containing only cells and formate (no naphthalene) were employed. Differences in absorbance between blanks and samples were determined after incubation for 24 hours.

Tests of Triclosan Degradation by *N. europaea*

Experiments were conducted to examine triclosan degradation by *N. europaea* at two different triclosan concentrations (0.5 mg/L and 2.0 mg/L). The flasks contained 300 ml of acetone-free growth medium that was prepared as follows: First, adding a known amount of triclosan-acetone stock solution into empty flasks; After acetone completely evaporated, adding the *N. europaea* growth medium and waiting for at least 2 days to allow triclosan to completely dissolve in the growth medium; and finally adding cell suspension prepared previously. Additional sets of flasks were amended with 10 mg/L allylthiourea as an AMO inhibitor (Rasche et al, 1991), and 20 mM sodium formate as an oxygenase reducing power. Positive controls (without triclosan) were used to assess ammonia oxidation activities of *N. europaea*. Initial protein concentrations were measured by using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Concentrations of triclosan, ammonia, and nitrite were monitored over time and

measured by GC-MC, IC, and ammonia-selective electrode probe, respectively. Samples were filtered with Millex-GP filter unit (0.22 μm pore size) before ammonia and nitrite analyses.

Analytical Methods

Concentrations of triclosan were determined by GC-MS analysis. Triclosan in liquid samples was extracted with ethyl ether overnight, and the extracts were derivitized with 50 ml DMF and 450 ml BSTFA. The derivitized samples were injected into a Hewlet Packard 5890 Series II Gas Chromatograph and detected by a Hewlett Packard 5972 Mass Spectrometer (Lab Extreme Inc., Kent city, MI). The GC was equipped with a HP-5MS capillary column (30 m x 0.25 mm i.d., df: 0.25 um) purchased from Agilent (Wilmington, DE, USA). Helium (purity 99.999%) was used as carrier gas at a constant flow of 1.2 ml/min. Automatic injections were performed in splitless flow (split ratio equals 1.00:1) 1.2 ml/min. The each injected volume was 1 μl. The GC oven temperature was operated as follows: starting at 80 °C, increased with a rate of 30 °C/min to 280 °C and held for 3 minutes, finally to 300 °C and held for 3 minutes. The GC-MS interface temperature was 290 °C. Mass spectra were obtained in selected ion monitoring (SIM) mode (600 eV) at the two mass to charge values 200 and 360 for triclosan. The monitoring peak areas were converted to triclosan concentrations by comparing them with the standard curves constructed from standard solutions.

CHAPTER IV

TRICLOSAN-DEGRADING CONSORTIUM AND AN ISOLATE KCY1

Triclosan-degrading Consortium

After depletion of added triclosan, a known amount of cell suspension was transferred to a new glass bottle containing NMS medium and 5 mg/L of triclosan. Four successive transfers were conducted (Fig. 4.1). The ratio of cell suspension to NMS medium for each transfer was 40 mL/80 mL (v/v). Despite triclosan was degraded in transfer bottles, no significant changes in turbidity were observed.
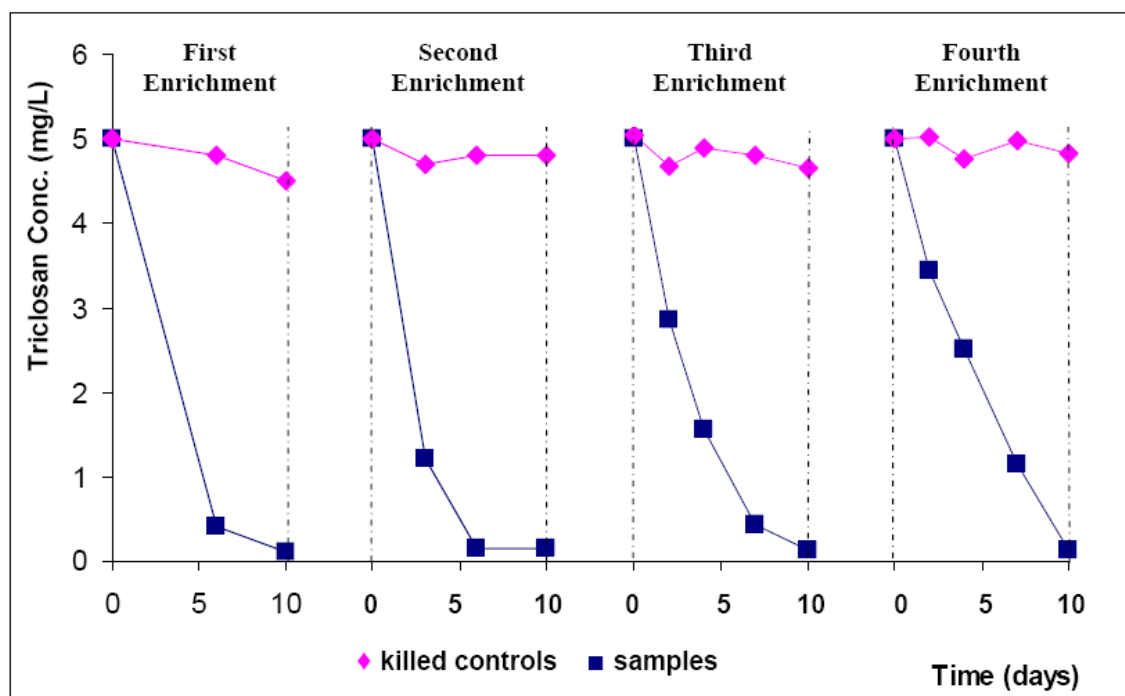

Fig. 4.1. Degradation of triclosan in four successive culture transfers.

An additional set of the fourth enrichment culture was used to estimate initial degradation rate of triclosan (Fig. 4.2).
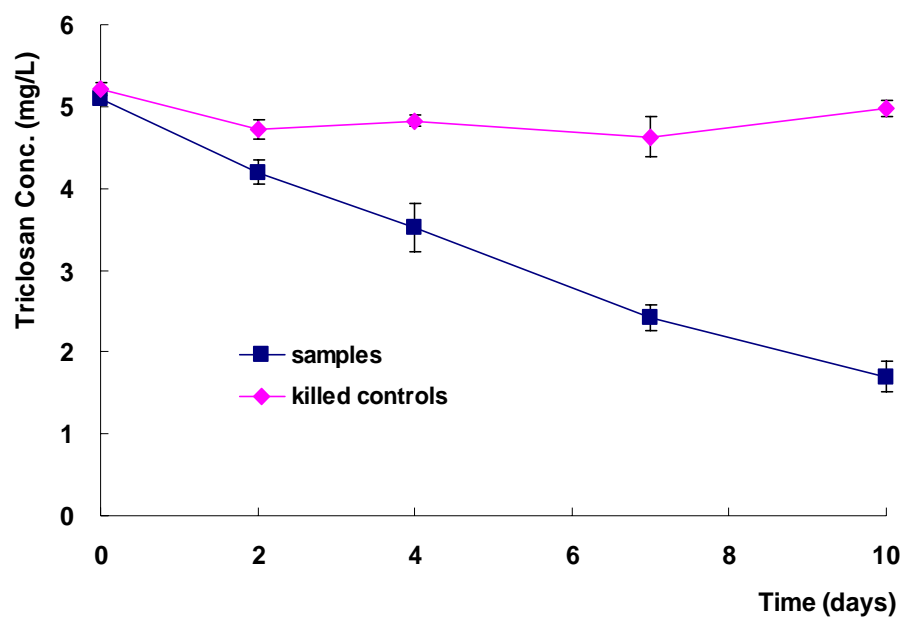
Fig. 4.2. Biodegradation of triclosan by the fourth enrichment culture. The volume ratio of cell suspension to NMS medium is 10 ml to 90 ml. Spiked triclosan concentration was 5 mg/L, and in aerobic condition. The bars indicate ranges of duplicate samples.

The initial specific degradation rate of triclosan was 0.17 nmol/min/mg of protein, based on 11.2 mg protein/L measured in the fourth enrichment culture. Since protein contents were not measured in the first three enrichment cultures, theoretical calculations were conducted. The calculations were made by using 0.2 mg protein/L and and known dilution factors employed during culture transfers. The protein content was estimated by assuming 5 mg/L triclosan was used as a sole carbon source (70% of substrate metabolized for biomass growth; 13% of protein content in biomass). The

calculated degradation rates for the first, second, and third enrichment cultures were 0.029 nmol/min/mg of protein, 0.047 nmol/min/mg of protein, and 0.076 nmol/min/mg of protein, respectively. Therefore, triclosan-degrading consortium degrading capacity increased through the enrichment processes.

Isolation of Triclosan-degrading Strain KCY1

After the fourth enrichment, cell suspension was used for isolation. Isolation of triclosan-degrading cultures was conducted by streaking on NMS-triclosan agar plates, after one time streaking, morphologically distinct colonies were selected and substreaked on R2A-triclosan agar plates four times to check for the purity of strains. Three presumptive triclosan-degrading colonies grew much faster on R2A-triclosan agar plates than on NMS-triclosan agar plates. There three presumptive cultures showed distinguishable colonies on R2A-triclosan agar plates: one is white and shiny, one is opaque and whitish, and the third is yellow and mucoid (Fig. 4.3).
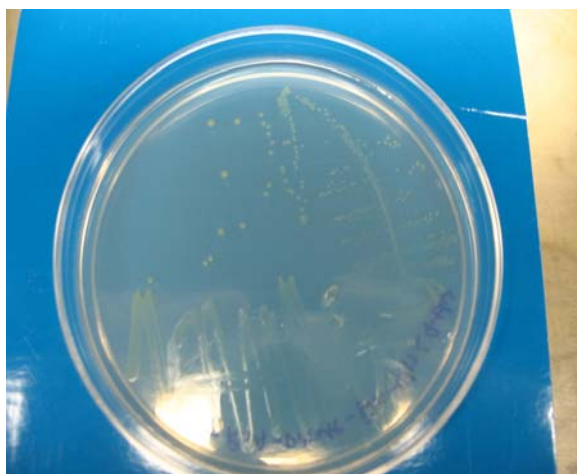

Fig. 4.3. Isolated yellow and mucoid colony on R2A-triclosan agar plates. The plates were incubated under aerobic conditions at 30 °C.

When streaking these three single colonies back onto NMS plates (no triclosan), the white-and-shiny colony and the opaque-and-whitish colony were able to grow on the plates. No growth of the yellow-and-mucoid colony was observed. This result demonstrated that the white-and-shiny colony, the opaque-and-whitish colony could use other carbon sources except triclosan or other than triclosan to satisfy their carbon and energy requirements for their growths.

Experiments using resting cells were conducted to examine colonies' ability to degrade triclosan. Each isolate was pregrown with 10% TSB medium for 2 days and harvested for experimental use. The cells were spun down and resuspended with NMS medium to 0.4 of $OD_{600.}$ Experiments were conducted in 250 ml glass vials containing 100 ml cell suspensions and 5 mg/L of triclosan. The vials were incubated at 30 °C and 150 rpm. Concentrations of triclosan were measured over time. After 7 days of incubation, no degradation of triclosan was observed in vials containing the white-and-shiny colony and the opaque-and-whitish colony. Triclosan degradation was only observed in vials containing the yellow-and-mucoid colony. About 80% of triclosan added was degraded within 1 day. After 3 days, all the spiked triclosan concentration almost approached zero (Fig. 4.4). Thus, only one strain of triclosan-degrading bacteria was successfully isolated, and named as strain KCY1.

Because triclosan is a chlorinated organic compound, the chloride ion loss from the parent compound can be used to evaluate if triclosan was fully mineralized by the strain KCY1. If 5 mg/L triclosan is completely mineralized, it will release 1.84 mg/L of

chloride ion. The measured chloride ion concentration was 1.88 mg/L, suggesting that triclosan was completely mineralized by the strain KCY1 (Table 4.1).
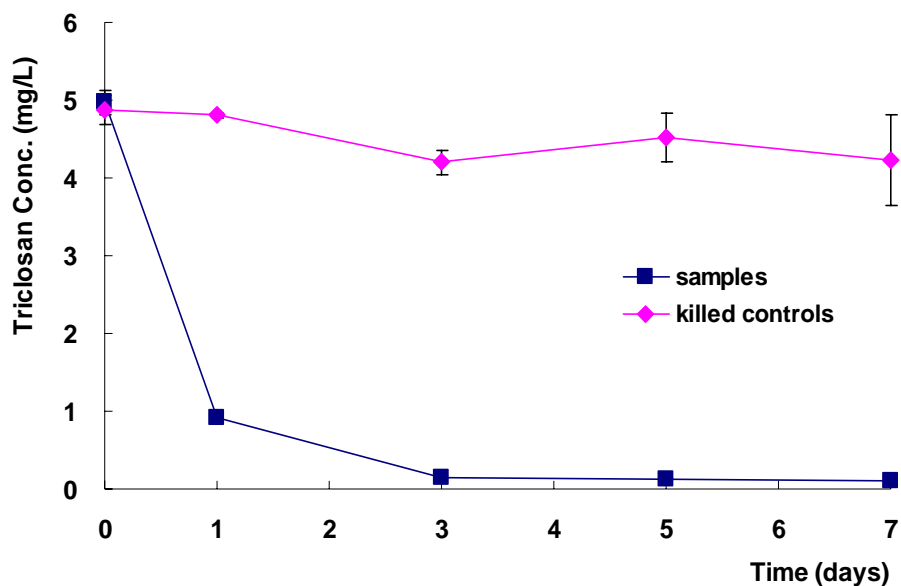
Fig. 4.4. Biodegradation of triclosan by using resting cells of strain KCY1.
The bars indicate ranges of duplicate samples

Table 4.1
Recovery of chloride following triclosan degradation by strain KCY1.
The initial triclosan concentration was 5 mg/L.

	Initial Cl⁻ concentration (mg/L)	18.87
Measurements	Final Cl⁻ concentration (mg/L)	20.75
	Produced Cl⁻ concentration (mg/L)	1.88
Theoretical prediction*	% of Cl⁻ in triclosan	36.8%
	Cl⁻ production (mg/L)	1.84

* 3 moles of chloride ion released per 1 moles of triclosan mineralized.

Phylogenetic Analysis of Strain KCY1

After PCR amplification of 16S rRNA of strain KCY1, the PCR products were separated from genomic DNA in agarose gel electrophoresis. As shown in Fig. 4.5, the 16S rRNA fragment size was consistent with the expected size (~1400 bp) determined by FluorChem 5500 (Alpha Innotech, San Leandro, CA), suggesting that 16S rRNA was successfully amplified and separated.

According to 16S rRNA sequence analysis, isolate KCY1 belongs to a member of the genus *Sphigomonas* within α-proteobacteria or the genus of *Sphigopyxis* within α-proteobacteria (Table 4.2). The phylogenetic tree (Fig. 4.6) shows the relationship between strain KCY1 and the most closely related bacteria that was deposited in GeneBank database. Strain KCY1 shows 97% similarity to two well-studied organic contaminant degraders, phenanthrene-degrading bacterium and *Sphingomonas sp*. DB-1. The known triclosan-degrading isolate *Sphingomonas sp*. Rd1 (Hay et al., 2001) has 91% similarity to strain KCY1. Interestingly, five estrogen-degrading bacteria KC11, KC9, KC10, KC14 and KC8 (isolated in our lab) were found to have high similarities (96%, 95%, 95%, 92%, and 92% respectively) to strain KCY1.
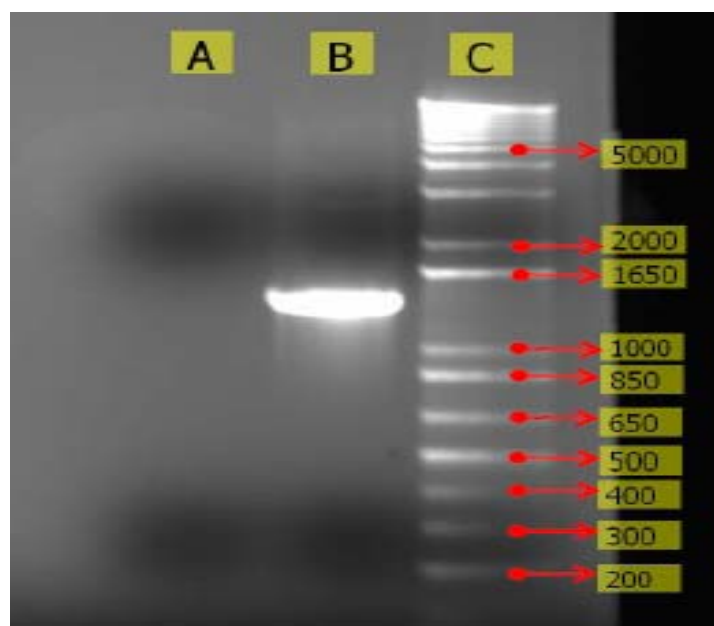
Fig. 4.5, Agarose gel electrophoresis of PCR-amplified product DNA. Lane A shows the negative control without PCR product template; Lane B shows the sample with PCR product template; Lane C shows 1 Kb plus DNA ladder.

Table 4.2
Bacteria with a high homology in the 16S rRNA sequence of strain KCY1.

Name of bacteria	Genus	Homology
Kartchner Caverns bacterium HI-I1	unclassified	97%
Phenanthrene-degrading bacterium	unclassified	97%
Sphingopyxis sp. Geo24	*sphingopyxis*	97%
Sphingomonas sp. strain:MBIC3365	*sphingomonas*	97%
Sphingopyxis sp. C-1	*Sphingopyxis*	96%
Sphingopyxis sp. DG892	*Sphingopyxis*	96%
Sphingopyxis alaskensis	*Sphingopyxis*	96%
Sphingopyxis alaskensis	*Sphingopyxis*	96%
Sphingomonas alaskensis	*Sphingomonas*	96%
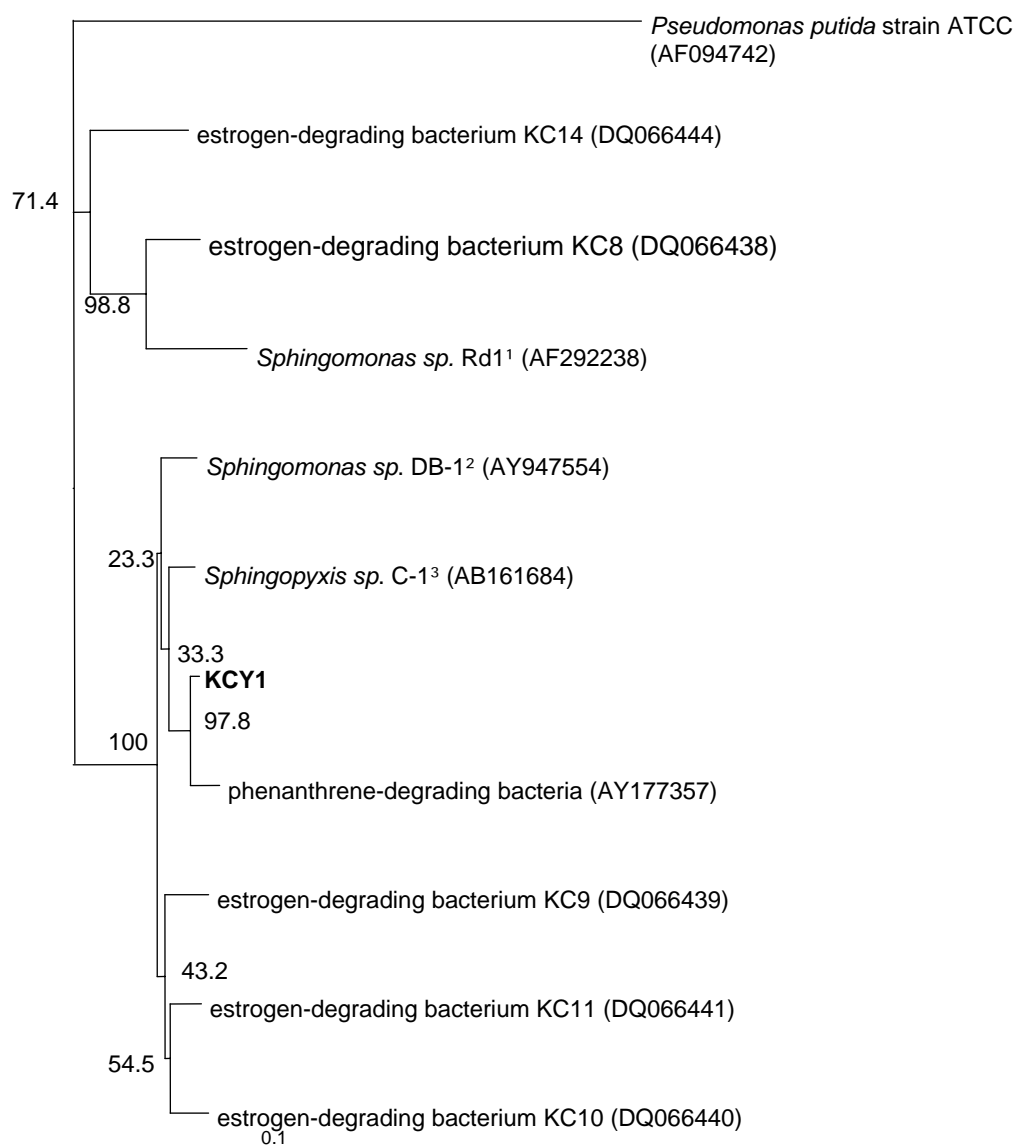
Fig. 4.6. Phylogenetic relationships between strain KCY1 and other known bacteria. [1]previously isolated triclosan-degrading strain; [2] DDT-degrading bacteria; [3] alkaliphilic microcystin-degrading bacteria. Bootstrap support values from 1000 replicates are indicated at branch nodes.

KCY1's Ability to Use Triclosan as a Sole Carbon Source

Additional experiment was conducted to determine whether strain KCY1 can grow on triclosan. When strain KCY1 was grown in NMS medium with 5 mg/L triclosan (not experiencing enrichment through 10% TSB medium), a very long lag phase appeared before triclosan degradation occurred (Fig. 4.7). After 33 days of incubation, about 80% of spiked triclosan was degraded. However, no significant increase in optical density or measurable protein content was observed. Despite that triclosan was the only carbon source available for the pure culture KCY1, this result was insufficient to determine whether KCY1 can use triclosan as a sole carbon and energy source.
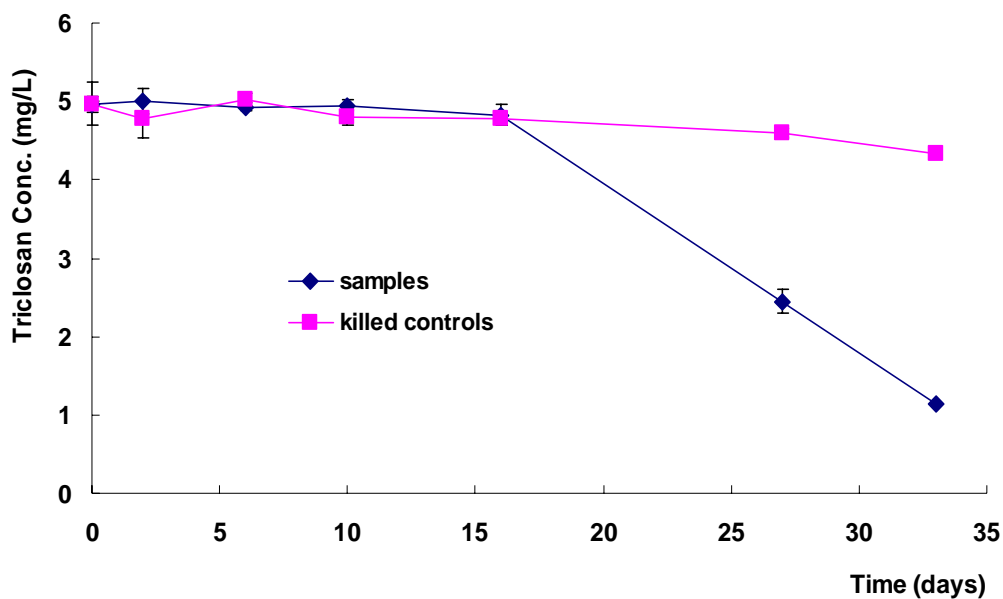
Fig. 4.7. Degradation of triclosan by strain KCY1 growing in NMS-triclosan medium. Triclosan with concentration 5 mg/L was contained as a sole carbon source.The bars indicate ranges of duplicate samples

Oxygenase Activity Assays

Indole oxidation assay and naphthalene oxidation assay were employed to measure non-specific di- and mono- oxygenases activities in strain KCY1. Positive color change indicates the presence of enzyme activity. No color and absorbance changes were observed in indole oxidation assay (Table. 4.3) and in naphthalene oxidation assay (Table. 4.4).

Table 4.3
Indole oxidation assay: Absorbance (A_{400}) changes in 90 min

Time (min)	Absorbance-1	Absorbance-2
2	-0.0338	-0.0312
5	-0.0327	-0.0322
10	-0.0324	-0.0363
15	-0.0318	-0.0330
20	-0.0330	-0.0369
25	-0.0298	-0.0330
30	-0.0370	-0.0310
35	-0.0355	-0.0366
45	-0.0358	-0.0366
60	-0.0313	-0.0290
90	-0.0366	-0.0340

Table 4.4
Naphthalene oxidation assay: Absorbance (A_{530}) changes in 24 hours

Time (hour)	Absorbance-1	Absorbance-2
1	-0.0168	-0.0155
24	-0.0210	-0.0193

The negative results of di- and mono-oxygenase activity assays suggested that non-specific oxygenases were not present in strain KCY1.

CHAPTER V

BIODEGRADATION OF TRICLOSAN BY *NITROSOMONAS EUROPAEA*

Introduction

Organic compounds can be biotransformed by microorganisms through two different mechanisms: growth-linked (using these compounds as growth substrates) and non-growth-linked (using these compounds as cometabolic substrates). In the growth-linked processes, biodegradation occurs when the organism consumes the organic compound as a primary substrate to satisfy its energy and organic carbon needs. On the other hand, non-growth-linked processes are a fortuitous transformation of an organic compound by enzymes or other biomolecules that are produced by microorganisms for other purposes. The microorganisms obtain no obvious or direct benefit through cometabolic degradation. In many case, cometabolic metabolites are harmful to microorganisms (Ward et al., 1997).

Extended studies show that microorganisms with non-specific monooxygenase or dioxygenase have the potential to initiate the cometabolic transformation (Ward et al., 1997). Ammonia monooxygenase (AMO) is a type of non-specific monooxygenase enzyme. Previous researches revealed that the physiological role of AMO is to metabolize growth-supporting substrate ammonia and simultaneously cometabolize a large range of non-growth-supporting substrates including hydrocarbon organic compounds, halogenated aliphatic organic compounds, and halogenated aromatic organic compounds (Vannelli et al, 1990; Keener and Arp, 1994). Triclosan belongs to a

chlorinated aromatic organic compound, and it is possible to detect its cometabolic transformation by *Nitrosomonas europaea* under proper conditions.

This study will address several questions concerning with the activity of AMO. The first question is to determine whether triclosan can be cometabolically transformed by an ammonia-oxidizing bacterium, *N. europaea*. The second question is to determine whether AMO plays an important role for the transformation, if degradation of triclosan by *N. europaea* observed. If AMO is found to involve in triclosan degradation, questions such as competitive inhibition and limitation of reducing will be addressed.

Results and Discussion

Triclosan degradation by *N. europaea*

Ammonia-oxidizing bacterium *N. europaea* was capable of degrading triclosan at concentrations of 2 mg/L and 0.5 mg/L (Fig. 5.1 and Fig. 5.2). These experiments were conducted with the same initial cell concentration (13 mg of protein /L) and ammonia concentration (700 mg-N/L). No triclosan degradation was observed in killed controls or in the presence of allylthiourea, an AMO inhibitor (Fig. 5.3).
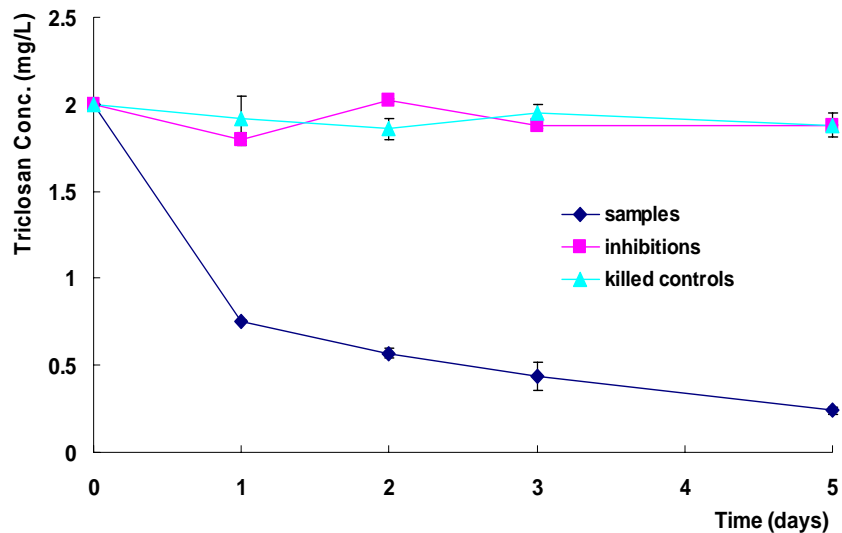
Fig. 5.1. Triclosan degradation by *N. europaea* with 2 mg/L triclosan. Initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic conditions at 30 °C. The bars indicate ranges of duplicate samples

.
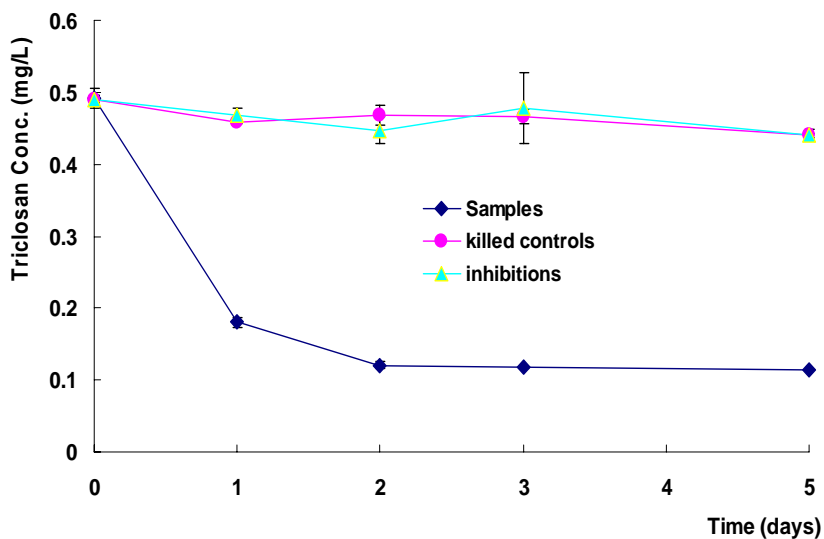
Fig. 5.2. Triclosan degradation by *N. europaea* with 0.5 mg/L triclosan. Initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic condition at 30 °C. The bars indicate ranges of duplicate samples

Similarly, neither production of nitrite (Fig. 5.4), nor depletion of ammonia (Fig. 5.5) were observed, indicating that allylthiourea completely inhibited the activity of AMO enzyme.

Fig. 5.3. Triclosan degradation by *N. europaea* in the presence of AMO inhibitor. Initial triclosan concentration 2.0 mg/L and 0.5 mg/L, initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic condition at 30 °C.
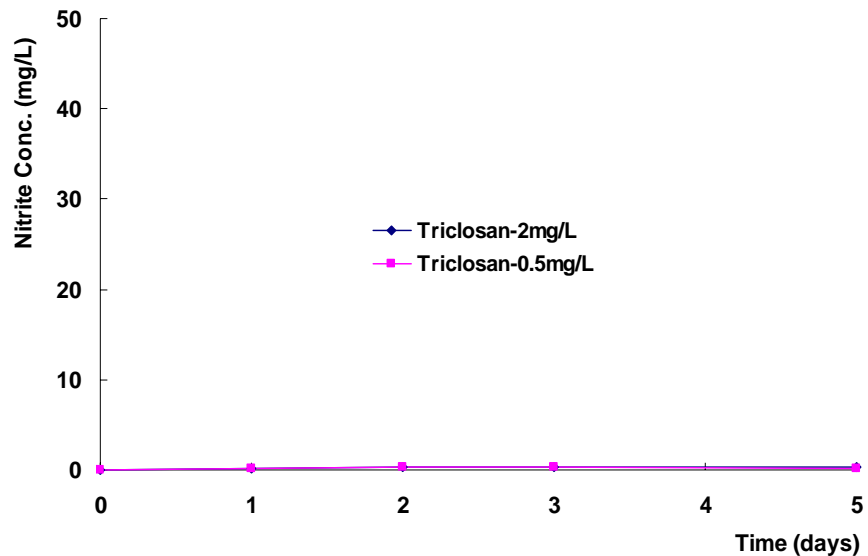
Fig. 5.4. Nitrite production by *N. europaea* in the presence of AMO inhibitor. Initial triclosan concentration 2.0 mg/L and 0.5 mg/L, initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic condition at 30 °C.
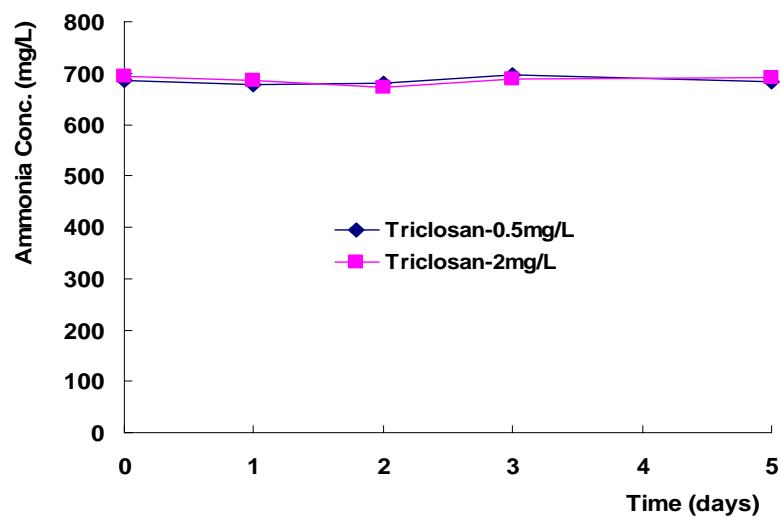
Fig. 5.5. Ammonia oxidation by *N. europaea* in the presence of AMO inhibitor. Initial triclosan concentration 2.0 mg/L and 0.5 mg/L, initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic condition at 30 °C.

Effects of NADH on triclosan degradation

Experiments were conducted to examine the effects of NADH on triclosan degradation by *N. europaea*. Sodium formate was provided as an external NADH source. Interestingly, no effects of NADH on triclosan degradation were observed (Fig. 5.6). Furthermore, the production of nitrite was not affected by the addition of formate (Fig. 5.7).
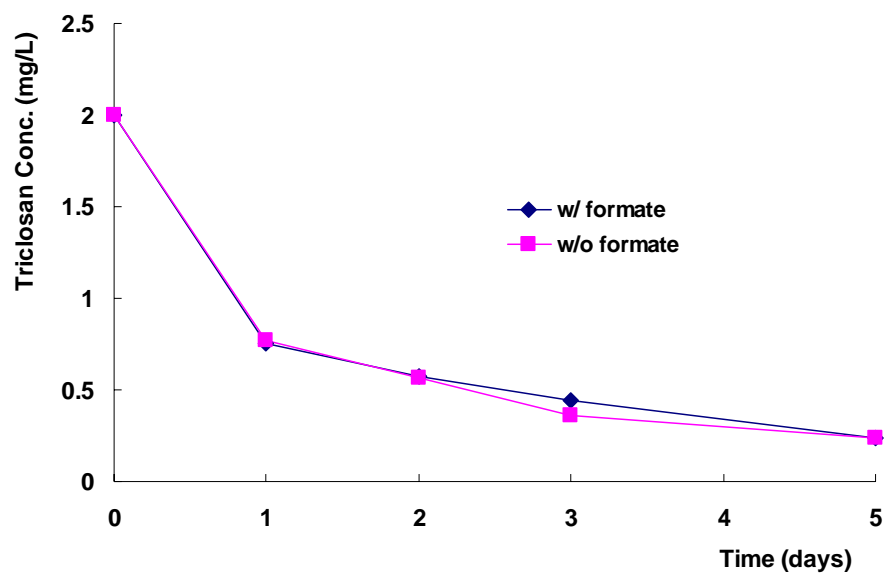
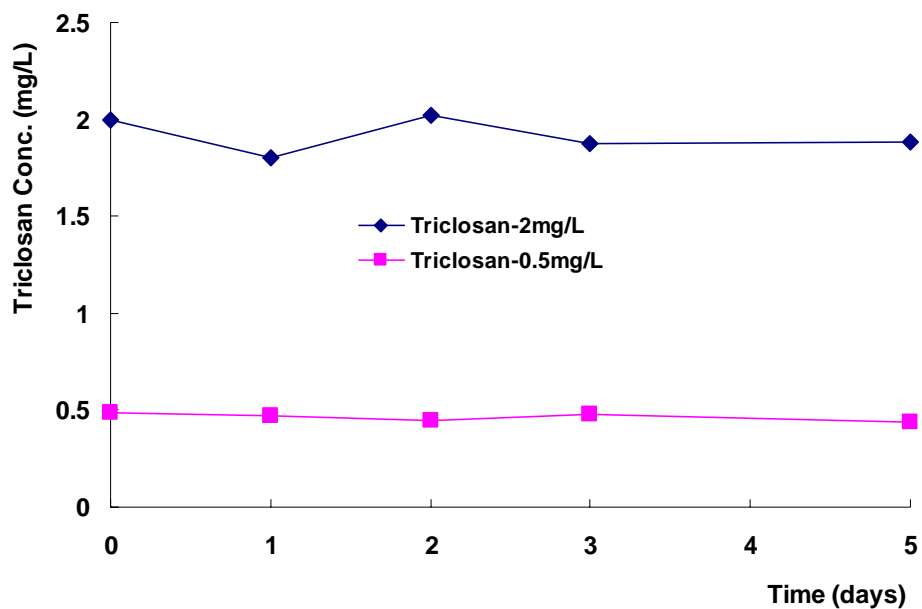
Fig. 5.6. Triclosan degradation by *N. europaea* in the presence of formate. Initial triclosan concentration 2.0 mg/L, initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic conditions at 30 °C.
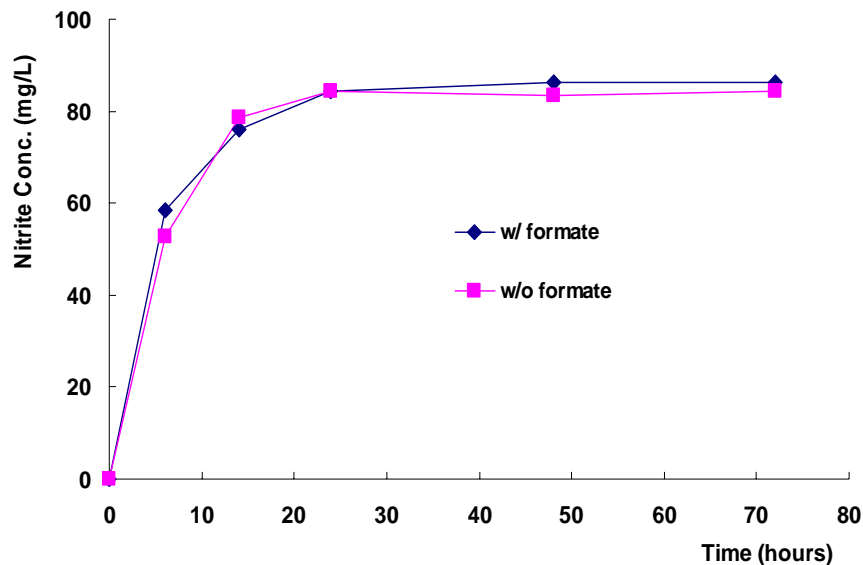
Fig. 5.7. Nitrite production over time by *N. europaea* in the presence of formate. Initial triclosan concentration 2.0 mg/L, initial cell protein concentration was 13 mg/L, and initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic conditions at 30 °C.

Competitive inhibition between ammonia and triclosan

Previous studies with *N. europaea* have demonstrated that alternative substrates for AMO could often exert an inhibitory effect on ammonia oxidation rates (Juliette et al., 1993; Keener and Arp, 1993). This inhibitory effect occurred because of competitive interactions between ammonia and the alternative substrate for AMO. The effects of this competition could be most easily detected by examining accumulation rates of nitrite. In this experiment, ammonia oxidation and triclosan degradation revealed an inhibitory competition between each other (Fig. 5.8).
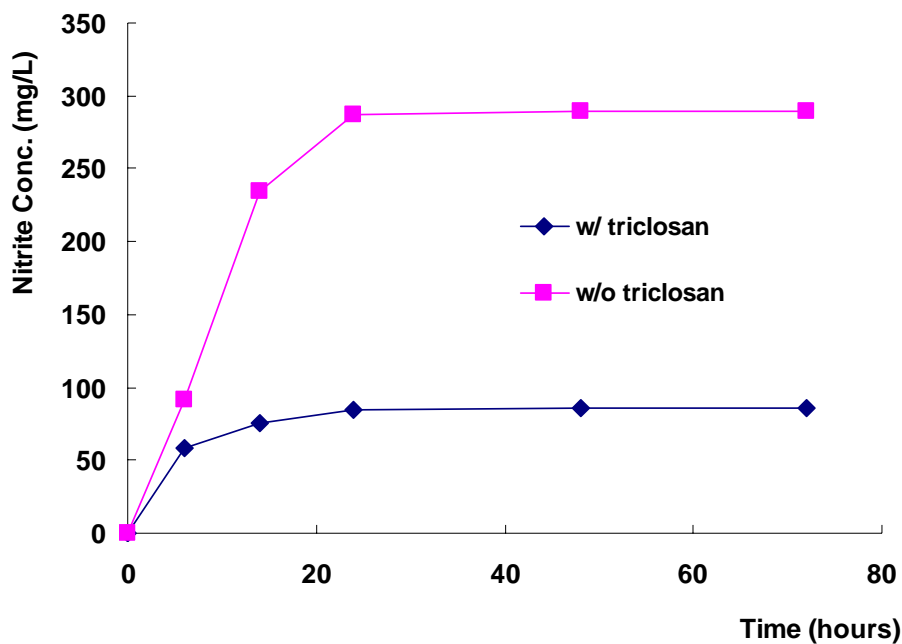
Fig. 5.8. Nitrite production by *N. europaea* in the presence or absence of triclosan. Initial triclosan concentration was 2.0 mg/L. Initial cell concentration was 13 mg protein /L. Initial ammonia concentration was 700 mg-N/L. Experiment was conducted under aerobic conditions at 30 °C.

Discussion

Previous studies have reported wide substrate ranges of AMO enzyme produced by *N. europaea* (Hyman et al. 1988; Rasche et al., 1991). However, rarely the chlorinated aromatic compounds have been shown to be degraded by AMO (Keener and Arp, 1994). This experiment showed that triclosan could be degraded completely through AMO oxidizing-activity, suggesting that AMO might be capable of cometabolizing complex chlorinated aromatic compounds.

Triclosan is a widely used antimicrobial agent. In this study, *N. europaea* exhibited a capacity to degrade triclosan as high as 2 mg/L, suggesting that *N. europaea*

is also resistant to antibacterial agents, like triclosan. More studies are needed to explore the ability of *N. europaea* to degrade other antimicrobial agents.

During triclosan transformation, the nitrite production rates decreased after several hours and eventually approached near zero at the end of the 2nd day (Fig. 5.8). Some possible reasons might explain the observations. First, products of triclosan degradation might inhibit AMO activity. This explanation was not valid, because the positive controls (no triclosan) showed the same trends. Secondly, there was no available ammonia to fuel ammonia oxidation. This reason might not be true either, because on the 2nd day, about 90% ammonia remained in the culture medium. The most possible explanation was the inhibition of AMO activity by high nitrite concentrations (Stein and Arp, 1998). This is supported by the fact that in *N. europaea* are grown with ammonia concentration of 280 mg/L which was used in a prior research (Shi et al., 2004). In this study, ammonia concentration of 700mg/L was used.

The results of this study indicated that no effects of formate on the oxidation of triclosan and ammonia. The possible explanations follow: (1), the generated NADH from ammonia oxidation could sufficiently satisfy the requirements of triclosan and ammonia oxidation since ammonia oxidation process is accompanied with energy generation; (2), sodium formate is not an effective external reducing energy source for ammonia-oxidizing bacteria. Both explanations will require further studies for validation.

CHAPTER VI

CONCLUSION AND FUTURE STUDIES

In this study, two separate tasks were conducted: (1) Isolation and characterization of triclosan-degrading bacteria; (2) Triclosan degradation by ammonia-oxidizing bacterium *N. europaea*. The following paragraphs summarize the findings and conclusions of this study.

Chapter IV descripbed the isolation and identification of triclosan-degraing isolate KCY1. Activated sludge was used to enrich tirclosan-degrading consortium that could completely degrade 5 mg/L triclosan in 10 days. Through successive streakings on NMS-triclosan (5 mg/L) and R2A-triclosan (5 mg/L) agar plates, three distinguishable isolates were obtained, in which only one isolate, strain KCY1, positively confirmed as a triclosan-degrading bacterium. According to 16S rRNA and phylogenetic analyses, strain KCY1 belongs to genus of *Sphingomonas* (97% similarity) or *Sphingopyxis* (97% similarity). However, results of this study were insufficient to determine whether strain KCY1 can use triclosan as a sole carbon and energy source. Further more no non-specific oxygenase activity was detected in strain KCY1. More studies are needed to elucidate enzymes responsible for triclosan degradation by KCY1.

Chapter V focused on triclosan oxidation by an ammonia-oxidizing bacterium *Nitrosomonas europaea.* Experimental data indicated that *N. europaea* could degrade a relatively high strength of triclosan (2mg/L). AMO,,a non-specific monooxygenase enzyme produced *N. europaea,* appeared to play a key role in the oxidation of ammonia

and the degradation of triclosan. When the AMO inhibitor allylthiourea was amended, the oxidations of triclosan and ammonia were stunted completely. Generally, oxygenation needs reducing power as an investment in form of NADH (or NADPH) to initiate the reaction. However, in this experiment, the addition of formate as an external NADH source could not stimulate ammonia oxidation and triclosan degradation. Furthermore, ammonia and triclosan were competitively inhibited each other by competing for AMO enzymes. Overall, our experimental results indicated that triclosan was degraded by *N. europaea* via cometabolic processes, which is different from KCY1's activity to degrade triclosan.

The significance of this study includes (1) the strain KCY1 was successfully isolated, which is the first triclosan-degrading bacterium isolated from activated sludge in waste water treatment plants. With a better understanding of triclosan-degrading cultures, one can predict the fate of triclosan in engineered and natural environments. Potnetially, a better strategy can be developed for controlling triclosan released into the environment, (2) ammonia-oxidizing bacterium, *Nitrosomonas europaea* was first found to have the ability to degrade triclosan through cometabolism. *N. europaea* is an important nitrification bacterium in waste water treatment plant. By using its degradation capacity of triclosan, it is possible to optimize the designing parameters for enhanced triclosan removal in wastewater treatment plants. Future research can focus on using *N. europaea* to degrade concerned emerging contaminants in wastewater.

Future studies should be conducted to further characterize isolate KCY1 as well as the degradation capacity of triclosan by N. *europaea*.   It is possible that KCY1 can

completely mineralize triclosan. Detailed studies are needed to determine whether the strain KCY1 can use triclosan as a sole carbon and energy source. Since triclosan are present in low concentrations in wastewater and the environment, it is important to investigate whether *N. europaea* can cometabolically degrade triclosan at ambient concentrations. Furthermore, the prevalence of KCY1 and *N. europaea* in different wastewater treatments should be examined. Fundamental knowledge, such as growth and degradation kinetics, degradation pathway, can be obtained by using these two bacteria as model strains to study triclosan degradation.

REFERENCES

Adolfsson-Erici, M., M. Pettersson, J. Parkkonen, and J. Sturve. 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 46, 1485-1489.

Aguera, A., Fernandez-Alba, A.R., Piedra, L., Mezcua, M., Gomez, M.J., 2003. Evaluation of triclosan and biphenylol in marine sediments and urban wastewaters by pressurized liquid extraction and solid phase extraction followed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry. Anal. Chim. Acta. 480, 193-205.

Belser, L.W., 1979. Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. 33, 309-333.

Bester, K., 2003. Triclosan in a sewage treatment process-balances and monitoring data. Water Res. 37, 3891-3896.

Bhargava, H.N., Leonard, P.A., 1996. Triclosan: applications and safety. Am. J. Infect. Control. 24, 209-218.

Bich, C., Vu, D., 2004. The effect of triclosan on artemia. California State Science Fair: 2004 Project summary. Project number: S1421. Available from: http://www.usc.edu/CSSF/History/2004/Projects/S1421.pdf

Braker, G., Ayala-del-Rio, H.L., Devol, A.H., Fesefeldt, A., Tiedje, J.M., 2001. Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length

polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. Appl. Environ. Microbiol. 67, 880-887.

Braoudaki, M., Hilton, A.C., 2004. Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157. FEMS Microbiol. Lett. 235**,** 305-309.

Canosa, P., Morales, S., Rodriguez, I., Rubi, E., Cela, R., Gomez, M., 2005. Aquatic degradation of triclosan and formation of toxic chlorophenols in presence of low concentration of free chlorine. Anal. Bioanal. Chem. Vol., No (11/2/2005): pp. 1-8.

Chang, S.W., Hyman, M.R., Williamson, K.J., 2002. Cooxidation of naphthalene and other polycyclic aromatic hydrocarbons by the nitrifying bacterium, *Nitrosomonas europaea.* Biodegradation 13, 373-381.

Chemical Land21. Life Science Products: Triclosan. Available from:

http://www.chemicalland21.com/arokorhi/lifescience/phar/TRICLOSAN.htm

Chu, K.H., Alvarez-Cohen, L., 1996. Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources. Water Environ. Res. 68**,** 76-82.

Chu, K.H., Alvarez-Cohen, L., 1998. Effect of nitrogen source on growth and trichloroethylene degradation by methane-oxidizing bacteria. Appl. Environ. Microbiol. 64, 3451-3457.

Danish EPA, 2003. Fate and Effects of Triclosan. Environmental Project No. 861. Available from:

http://www.mst.dk/udgiv/publications/2003/87-7972-984-3/html/kap05_eng.htm

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas* oligotropha-like ammonia-oxidizing bacteria and *Nitrospira sp.* from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol. 68, 245-253.

Durbize, E., Vigan, M., Puzenat E., 2003. Spectrum of cross-photosensitization in 18 consecutive patients with contact photoallergy to ketoprofen: associated photoallergies to non-benzophenone-containing microbe. Contact Dermatitis. 48, 144-149.

Environment News Daily (ENDS), 2000. Denmark discourages household antibacterials. London, UK. October 26.

Environment News Daily (ENDS), 2001. Finnish warning on anti-bacterial chemicals. London, UK. February 16.

Environment News Daily (ENDS), 2001. German appeal to limit anti-bacterial use. London, UK. March 22.

FDA. 2001 Glossary of pesticide Chemicals. Available from:

http://vm.cfsan.fda.gov/~acrobat/pestglos.pdf

Federle, T.W., Kaiser, S.K., Nuck, B.A., 2002. Fate and effects of triclosan in the activated sludge. Environ. Toxicol. Chem. 21, 1330-1337.

Foran, C.M., Bennett, E.R., and Benson, W.H., 2000. Developmental evaluation of a potential non-steroidal estrogen: triclosan. Mar. Environ. Res. 50**,** 153-156.

General Chemistry Online, 1999. What is the triclocarbon and triclosan? Available from:

http://antoine.frostburg.edu/chem/senese/101/consumer/faq/triclosan.shtml

Glaser, A., 2004. The ubiquitous triclsan. Pesticide and You. Vol. 24, 12-17.

Hay, A.G., Dees, P.M., Sayler, G. S., 2001. Growth of a bacterial consortium on triclosan. FEMS Microbiol. Ecol. 36**,** 105-112.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., Saunders, J.R., 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. 139, 1147-1153.

Heath, R.J., Rock, C.O., 2000. A triclosan-resistant bacterial enzyme. Nature. 406, 145-146.

Hundt, K., Martin, D., Hammer, E., Jonas, U., Kindermann, M.K., Schauer, F., 2000. Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*. Appl. Environ. Microbiol. 9, 4157-4160.

Hyman, M.R., Murton, I.R., Arp, D.J., 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes and alkynes. Appl. Environ. Microbiol. 54, 3187-3190.

Hyman M.R., Wood, P.M., 1983. Methane oxidation by *Nitrosomonas europaea*. Biochem. J. 212, 31-37.

Ishibashi, H., Matsumura, N., Hirano, M., Matsuoka, M., 2004. Effects of triclosan on the early life stages and reproduction of medake *oryzias latipes* and induction of hepatic vitellogenin. Aquat. Toxicol. 67, 167-179.

Jackson, T., 2004. Pesticide found in everyday products. North Texas Daily. Available from:

http://www.ntdaily.com/vnews/display.v?TARGET=printable&article_id=41b01c50 b0067

Jenkins, R.O., Dalton, H., 1985. The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiol. Lett. 30, 227-231.

Juliette, L.Y., Hyman, M.R., Arp, D.J., 1993. Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfate compounds: Thioethers are oxidized to sulfoxides by ammonia oxygenase. Appl. Environ. Microbiol. 59 (11), 3718-3727.

Kanda, R., Griffin, P., James, H.A., Fothergill, J., 2003. Pharmaceutical and personal care products in sewage treatment work. J. Environ. Monit. 5, 823-830.

Kanetoshi, A., Ogawa, H., Katsura, E., Haneshima, H., Miura, T., 1988. Formation of polychlorinated dibenzo-p-dioxins upon combustion of commercial textile products containing 2,4,4′-tricloro-2′-hydroxydiphenyl ether (irgasan® DP300). J. Chromatogr. 422, 289-299.

Keener, W.K., Arp, D.J., 1993. Kinetic studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. Appl. Environ. Microbiol. 59 (8), 2501-2510.

Keener, W.K, Arp, D.J., 1994. Transformations of aromatic compound by *Nitrosomonas europaea*. Appl. Environ. Microbiol. 60, 1914-1920.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. stream, 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202-1211.

Latch, D.E., Packer, J.L., Arnold, W.A., and Mcneill, K., 2003. Photochemical conversion of triclosan to 2,8-dichlorodibenzo-p-dioxin in aqueous solution. J. Photochem. Photobiol., A : Chemistry. 158, 63-66.

Levy, C.W., Roujeinikovai, A., Sedelnikova, S., Baker P.J., Stuitje, A.R., Slabas, A.R., Rice, D., Rafferty, J.B., 1999. Molecular basis of triclosan activity. Nature. 398, 393-384.

Levy, S.B., 2001. Antibacterial household product: cause for concern. Emerg. Infect. Dis. 7, 512-515.

Lindstrom, A., Buerge I.J., Poiger T., Bergqvist P.A., Muller M.D., Buser H.R., 2002. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. Environ. Sci. Technol. 36, 2322-2329.

Lopez-Avila, V., Hites, R.A., 1980. Organic compounds in an industrial wastewater: Their transport into sediments. Environ. Sci. Technol. 14, 1382-1390.

Lurie, Z., 2004. Engaging in germ warfare. Journal Gazette. Avail from: http://www.fortwayne.com/mld/journalgazette/929628.htm

McAvoy, D.C., Schatowitz, B., Jacob,M., Hauk, A., Eckhoff, W.S., 2002. Measurement of triclosan in wastewater treatment systems. Environ. Toxicol. Chem. 21, 1323-1329.

McMurry, L.M., Oethinger, M., Levy, S.B., 1998. Triclosan targets lipid synthesis. Nature. 394, 531-532.

Meade, M.J., Waddell, R.L., Callahan, T.M., 2001. Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans subsp*. denitrificans inactivate triclosan in liquid and solid substrates. FEMS Microbiol. Lett. 204, 45-48.

Menoutis, J., Parisi, A.I., 2001. Triclosan and its impurities. Triclosan Review Series, Quantex Laboratories, Inc. Available from:

http://www.quantexlabs.com/About%20Triclosan/ABOUT%20TRICLOSAN.HTM

Mezcua, M., Gomez, I.J., Ferrer, I., Aguera A., Hernando, M.D., Fernandez-Alba, A.R., 2004. Evidence of 2,7/2,8 – dibenzodichloro-p-dioxin as a photodegradation procuct of triclosan in water and wastewater samples. Anal. Chim. Acta. 524, 241-247.

Miller, T.L., Lorusso, D.J., Walsh, M.L., Deinzer, M.L., 1983. The acute toxicity of penta-, hexa-, and heptachlorohydroxydiphenyl ethers in mice. J. Toxicol. Environ. Health. 12, 245-253.

Neumegen, R.A., Fernandez-Alba, A.R., Christi, Y., 2004. Toxicities of triclosan, phenol and copper sulfate in activated sludge. Available from:

http://www.massey.ac.nz/~ychisti/EnvTox.pdf#search='triclosan toxicity'

Orvos, D.R., Versteeg, D.J., Inauen, J., Capdevielle, M., Rothenstein, A., Cunningham, V., 2002. Aquatic toxicity of triclosan. Environ. Toxicol. Chem. 21(7), 1338-1349.

Parikh, S., Xiao, G., Tonge, P.J., 2000. Inhibition of InhA, the enoyl reductase from *Mycobacterium tuberculosis*, by triclosan and isoniazid. Biochemistry. 39, 7645-7650.

Paxeus, N., 1996. Organic pollutants in the effluents of large wastewater treatment plants in Sweden. Water Res. 30, 1115-1122.

Prosser, S.J., 1989. Autotrophic nitrification in bacteria. Adv. Microbiol. Physiol. 30, 125-181.

Rasche, M.E., Hyman, M.R., Arp, D.J., 1991. Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. 57, 2986-2994.

Regents of University of California, 2005. *Nitrosomonas europaea*. JGI. Available from: http://genome.jgi-psf.org/finished_microbes/niteu/niteu.home.html

Reiss, R., Machay, N., Habig, C., Griffin, J., 2002. An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. Environ. Toxicol. Chem. 21, 2483-2492.

Rule, K.L., Ebbett, V.R., Vikesland, P.J., 2005. Formation of chloroform and chlorinated organics by free-chlorinate-mediated oxidation of triclosan. Environ. Sci. Technol. 39, 3176-3185.

Russell, A.D., 2003. Similarities and differences in the responses of microorganisms to biocides. J. Antimicrob. Chemother. 52, 750-763.

Russell, A.D., 2002. Introduction of biocides into clinical practice and the impact on antibiotic resistant bacteria. J. Appl. Microbiol. Symp. Suppl. 92, 121-125.

Sabaliunas, D., Webb, S.F., Hauk, A., Jacob, M., Eckhoff, W.S., 2003. Environmental fate of triclosan in the River Aire basin, UK. Water Res. 37, 3145-3154.

Schweizer, H.P. 2001. Triclosan: A widely used biocide and its link to antibiotics. FEMS Microbial. Lett. 202, 1-7.

Shi, J., fujisawa,, S., Nakai, S., Hosomi, M., 2004. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. Water Res. 38, 2323-2330.

Singer, H., Muller, S., Tixier, C., Pillonel, L., 2002. Triclosan: Occurrence and fate of a widely used biocide in the aquatic environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments. Environ. Sci. Toxicol. 36, 4998-5004.

Sprent, J.I., 1987. The Ecology of the Nitrogen Cycle. Cambridge University Press, Cambridge, UK.

Stein, L.Y., Arp, D.J., 1998. Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. Appl. Environ. Microbiol. 64, 4098-4102.

Suller, M.T., Russell, A.D., 1999. Triclosan and antibiotic resistance in staphylococcus aureus. J. Antimicrob. Chemother. 46, 11-18.

Tatarazako, N., Ishibashi, H., Teshima, K., Kishi, K., and Arizono, K., 2004. Effects of triclosan on various aquatic organisms. Environ. Sci. 11**,** 133-140.

Teske, A., Elm, E., Regan, J.M., Toze, S., Rittmann, B.E., Stahl, D.A., 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. 176, 6623-6630.

Vannelli, T., Logan, M., Arciero, D.M., Hooper, A.B., 1990. Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea.* Appl. Environ. Microbiol. 56, 1169-1171.

U.S. Dept of Health and Human Services, 1998. Toxicological profile for chlorinated dibenzo-p-dioxins. Public Health Service, Agency for Toxic Substances and Disease Registry. Atlanta, GA.

U.S. EPA, 1994. Estimating exposure to dioxin-like compounds, Vol. II: Properties, sources, occurrence and background exposures. Office of Research and Development. Review draft. Washington DC, June. pp 3-54.

Villalain, J., Mateo, C. R., Aranda, F. J., Shapiro, S., Micol, V., 2001. Membranotropic effects of the antibacterial agent triclosan. Arch. Biochem. Biophys. 390, 128–136.

Ward, B.B., Carlucci, A.F., 1985. Marine ammonia- and nitrite-oxidizing bacteria: serological diversity determined by immunofluorescence in culture and in the environment. Appl. Environ. Microbiol. 50, 194-201.

Ward, C.H., Cherry, J.A., Scalf, M.R., 1997. Subsurface restoration. Ann Arbor Press, Inc. Chelsea, Michigan, pp. 373-395.

Wolfberg, A.L., 2004. Triclosan's effect on a marine alga. California State Science Fair: 2004 Project summary. Project number: J0929. Available from:

http://www.usc.edu/CSSF/History/2004/Projects/J0929.pdf

Wood, P.M., 1986. Nitrification as a bacterial energy source. In. J.I. Prosser (ed.), Nitrification. Special Publ. Soc. Gen. Microbiol. Vol. 20, pp. 39-62. IRL Press, Oxford, UK.

Zhang, H., Huang, C.H., 2003. Oxidative transformation of triclosan and chloroprene by manganese oxides. Environ. Sci. Technol. 37, 2421-2430.

APPENDIX

16S rDNA Gene Sequences and Accession Numbers

KCY1

```
   1 ATGCCTAANCATGCAAGTCGAACGAGNNCTTCGGATCTAGTGGCGCACGGGTGCGTAACG
  61 CGTGGGAATCTGCCCTTGGGTGCGGAATAACTTTCCGAAAGGAATGCTAATACCGCATAA
 121 TGTCGTAAGACCAAAGATTTATCGCCCAAGGATGAGCCCGCGTAAGATTAGCTAGTTGGT
 181 GGGGTAAAAGCCTACCAAGGCGACGATCTTTAGCTGGTCTGAGAGGATGATCAGCCACAC
 241 TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
 301 GGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTC
 361 TTTTACCCGGGATGATAATGACAGTACCGGGAGAATAAGCTCCGGCTAACTCCGTGCCAG
 421 CAGCCGCGGTAATACGGAGGGAGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCGCG
 481 TAGGCGGTTTTTTAAGTCAGAGGTGAAAGCCCGGGGCTCAACCCCGGAATTGCCTTTGAA
 541 ACTGGAAAACTTGAATCTTGNAGAGGTCAGTGGAATTCCGAGTGTAGAGGTGAAATTCGT
 601 AGATATTCGGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGAGG
 661 TGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
 721 GATAACNANNTGTCCGGGTTCATAGAACTTGGGGTGGCGCAGCTAACGCNTTAAGTTATC
 781 CGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGNGNGCCTGCACAAG
 841 CGGTGGAGCATGTGGTTTAATTNGAAGCANNGCGCAGAACCTTACCAGCGTTTGACATCC
 901 TGATCGCGGATTAGAGAGATCTTTTCCTTCAGTTCGGCTGGATCAGTGACAGGTGCTGCA
 961 TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
1021 CATCCCTAGTTGCCATCATTAAGTTGGGCACTCTAAGGAAACTGCCGGTGATAAGCCGGA
1081 GGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGCGCTGGGCTACACACGTGCTA
1141 CAATGGCAACTACAGTGGGCAGCAACCTCGCGAGGGGTAGCTAATCTCCAAAAGTTGNCT
1201 CAGTTCGGATTGTTCTCTGCAACTCGAGAGCANGAAGGCGGAATCGCTAGTNNNCGCGNN
1261 NAGCNNGCNNNNNNNNNNNNNNNNNNNNNT
```

Sphingomonas sp. Rd1 [AF292238]

```
1 AAGGGGTGCTAATACCGTATGATGTCGTAAGACCAAAGAT TTTTCCCCCA GGGATGAGCC
```

61 GGCGTAGGAT TAGNTAGTTG GTGGGGTAAA GGCTCACCAA GGGGACGATC CTTAGTTGTT

121 CTGAGAGGAT GATCAGCCAC ACTGGGACTG AGACACGGCC CAGATTCTTA CGGGAGGCAG

181 CAGTGGGGAA TATTGGACAA TGGGCGAAAG CCTGATCCAG CAATGCCGCG TGAGTGATGA

241 AGGCCTTAGG GTTGTAAAGT CTTTTAACCC GGGAAGATAA TGACTGTACC GGGAGAATAA

301 GCCCCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA GGGGGCTAGC GTTGTTCGGA

361 ATTACTGGGC GTAAAGCGCA CGTAGGCGGC TTTGTAAGTT AGAGGTGAAA GCCCGGGGCT

421 CAACCCCGGA ATTGCCTTTA AGACTGCATC GCTTGAACGT CGGAGAGGTG AGTGGAATTC

481 CGAGTGTAGA GGTGAAATTC GTAGATATTC GGAAGAACAC CAGTGGCGAA GGCGGCTCAC

541 TGGACGACTG TTGACGCTGA GGTGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG

601 GTAGTCCACG CCGTAAACGA TGATAACTAG CTGTCCGGGC ACTTGGTGCT GGGGTGGCGC

661 AGCTAACGCA TTAAGTTATC CGCCTGGGGA GTACGGCCGC AAGGTTAAAA CTCAAAGAAA

721TTGACGGGGG CCTGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGCAGAAC

781 CTTACCAACG TTTGACATCC CTAGTGTGGA TCGTGGAGAC ACTTTCCTTC AGTTCGGCTG

841 GCTAGGTGAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT

901 CCCGCAACGA GCGCAACCCT CGCCTTTAGT TACCATCATT TAGTTGGGTA CTCTAAAGGA

961 ACCGCCGGTG ATAAGCCGGA GGAAGGTGGG GATGACGTCA AGTCCTCATG GCCCTTACGC

1021GTTGGGCTAC ACACGTGCTA CAATGGCAAC TACAGTGGGC AGCGACCCCG CGAGGGCGAG

1081 CTAATCTCCA AAAGTTGTCT CAGTTCGGAT TGTTCTCTGC AACTCGAGAG CATGAAGGCG

1141 GAATCGCTAG TAATCGCGGA TCAGCATGCC GCGGTGAATA CGTTCCCAGG CCTTGTACAC

1201 ACCGCCCGTC ACACCATGGG AGTTGGATTC ACCCGAAGGC GCTGCGCTAA CCGCAAGGAG

1261 GCAGGCGACC ACGGTGGGTT TAGCGACTGG GGTGA

Phenanthrene-degrading bacterium M20 [AY177357]

1 GCCTAACACA TGCAAGTCGA ACGAGACCTT CGGGTCTAGT GGCGCACGGG TGCGTAACGC

61 GTGGGAATCT GCCCTTGGGT GCGGAATAAC TTTCCGAAAG GAATGCTAAT ACCGCATAAT

121 GTCGTAAGAC CAAAGATTTA TCGCCCAAGG ATGAGCCCGC GTAAGATTAG CTAGTTGGTG

181 GGGTAAAAGC CTACCAAGGC GACGATCTTT AGCTGGTCTG AGAGGATGAT CAGCCACACT

241 GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT GGACAATGG

301 GCGAAAGCCT GATCCAGCAA TGCCGCGTGA GTGATGAAGG CCCTAGGGTT GTAAAGCTCT

361 TTTACCCGGG ATGATAATGA CAGTACCGGG AGAATAAGCT CCGGCTAACT TCGTGCCAGC

421 AGCCGCGGTA ATACGAGGGG AGCTAGCGTT GTTCGGAATT ACTGGGCGTA AAGCGCGCGT

481 AGGCGGCTTT TTAAGTCAGA GGTGAAAGCC CGGGGCTCAA CCCCGGAATA GCCTTTGAAA

541 CTGGAAAGCT AGAATCTTGG AGAGGTCAGT GGAATTCCGA GTGTAGAGGT GAAATTCGTA

601 GATATTCGGA AGAACACCAG TGGCGAAGGC GACTGACTGG ACAAGTATTG ACGCTGAGGT

661 GCGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACGCCG TAAACGATGA

721 TAACTAGCTG TCCGGGTTCA TAGAACTTGG GTGGCGCAGC TAACGCATTA AGTTATCCGC

781 CTGGGGAGTA CGGTCGCAAG ATTAAAACTC AAAGGAATTG ACGGGGGCCT GCACAAGCGG

841 TGGAGCATGT GGTTTAATTC GAAGCAACGC GCAGAACCTT ACCAGCGTTT GACATCCTGA

901 TCGCGGATTA GAGAGATCTT TTCCTTCAGT TCGGCTGGAT CAGTGACAGG TGCTGCATGG

961 CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG CAACCCTCAT

1021 CCCTAGTTGC CATCATTCAG TTGGGCACTC TAAGGAAACT GCCGGTGATA AGCCGGAGGA

1081 AGGTGGGGAT GACGTCAAGT CCTCATGGCC CTTACGCGCT GGGCTACACA CGTGCTACAA

1141 TGGCAACTAC AGTGGGCAGC AACCGTGCGA GCGGTAGCTA ATCTCCAAAA GTTGTCTCAG

1201 TTCGGATTGT TCTCTGCAAC TCGAGAGCAT GAAGGCGGAA TCGCTAGTAA TCGCGGATCA

1261 GCATGCCGCG GTGAATACGT TCCCAGGCCT TGTACACACC GCCCGTCACA CCATGGGAGT

1321 TGGTTTCACC CGAAGGCAGT GCTCTAACCC GCAAGGG

Estrogen-degrading bacterium KC11 [DQ066441]

1 CCACTGGCGG CTGCCTACAC ATGCAAGTCG AACGAAGTCT TCGGACTTAG TGGCGCACGG

61 GTGCGTAACG CGTGGGAATC TGCCCTTGGG TACGGAATAA CTCAGAGAAA TTTGTGCTAA

121 TACCGTATAA TGACTTCGGT CCAAAGATTT ATCGCCCAAG GATGAGCCCG CGTAAGATTA

181 GCTAGTTGGT GGGGTAAAGG CCCACCAAGG CGACGATCTT TAGCTGGTCT GAGAGGATGA

241 TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA

301 TTGGACAATG GGCGAAAGCC TGATCCAGCA ATGCCGCGTG AGTGATGAAG GCCCTAGGGT

361 TGTAAAGCTC TTTTACCCGG GATGATAATG ACAGTACCGG GAGAATAAGC TCCGGCTAAC

421 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GAGCTAGCGT TGTTCGGAAT TACTGGGCGT

481 AAAGCGCGCG TAGGCGGTTT TTCAAGTCAG AGGTGAAAGC CCGGGGCTCA ACCCCGGAAT

541 TGCCTTTGAA ACTGGAAGAC TTGAATCTTG AGAGGTCAG TGGAATTCCG AGTGTAGAGG

601 TGAAATTCGT AGATATTCGG AAGAACACCA GTGGCGAAGG CGACTGACTG ACAAGTATT

661 GACGCTGAGG TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC

721 GTAAACGATG ATAACTAGCT GTCCGGGTTC ATAGAACTTG GGTGGCGCAG CTAACGCATT

781 AAGTTATCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT GACGGGGGCC

841 TGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGCAGAACCT TACCAGCGTT

901 TGACATCCTG ATCGCGGATT AGAGAGATCT TTTCCTTCAG TTCGGCTGGA TCAGTGACAG

961 GTGCTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC

1021 GCAACCCTCA TCCCTAGTTG CCATCATTCA GTTGGGCACT CTAAGGAAAC TGCCGGTGAT

1081 AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTACGCGC TGGGCTACAC

1141 ACGTGCTACA ATGGCGGTGA CAGTGGGCAG CAACCCTGCG AGGGGTAGCT AATCTCCAAA

1201 AGCCGTCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGGCGGA ATCGCTAGTA

1261 ATCGCGGATC AGCATGCCGC GGAATNCG

Estrogen-degrading bacterium KC9 [DQ066439]

1 CCACTGGCGG CTGCCTACCA TGCAAGTCGA ACGAGATCTT CGGATCTAGT GGCGCACGGG

61 TGCGTAACGC GTGGGAATCT GCCCTTGGGT ACGGAATAAC TCAGAGAAAT TTGTGCTAAT

121 ACCGTATAAT GTCTTCGGAC CAAAGATTTA TCGCCCAAGG ATGAGCCCGC GTAGGATTAG

181 CTAGTTGGTG AGGTAAAAGC TCACCAAGGC GACGATCCTT AGCTGGTCTG AGAGGATGAT

241 CAGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG TGGGGAATAT

301 TGGACAATGG GCGAAAGCCT GATCCAGCAA TGCCGCGTGA GTGATGAAGG CCCTAGGGTT

361 GTAAAGCTCT TTTACCCGGG ATGATAATGA CAGTACCGGG AGAATAAGCT CCGGCTAACT

421 TCGTGCCAGC AGCCGCGGTA ATACGAGGGG AGCTAGCGTT GTTCGGAATT ACTGGGCGTA

481 AAGCGCGCGT AGGCGGTTTT TTAAGTCAGA GGTGAAAGCC CGGGGCTCAA CCCCGGAATT

541 GCCTTTGAAA CTGGAAAACT AGAATCTTGG AGAGGTCAGT GGAATTCCGA GTGTAGAGGT

601 GAAATTCGTA GATATTCGGA AGAACACCAG TGGCGAAGGC GACTGACTGG ACAAGTATTG

661 ACGCTGAGGT GCGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACGCCG

721 TAAACGATGA TAACTAGCTG TCCGGGTTCA TAGAACTTGG GTGGCGCAGC TAACGCATTA

781 AGTTATCCGC CTGGGGAGTA CGGTCGCAAG ATTAAAACTC AAAGGAATTG ACGGGGGCCT

841 GCACAAGCGG TGGAGCATGT GGTTTAATTC GAAGCAACGC GCAGAACCTT ACCAGCGTTT

901 GACATCCTGA TCGCGGTTAC CAGAGATGGT TTCCTTCAGT TCGGCTGGAT CAGTGACAGG

961 TGCTGCATGG CTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG

1021 CAACCCTCAT CCCTAGTTGC CATCATTAAG TTGGGCACTC TAAGGAAACT GCCGGTGATA

1081 AGCCGGAGGA AGGTGGGGAT GACGTCAAGT CCTCATGGCC CTTACGCGCT GGGCTACACA

1141 CGTGCTACAA TGGCGGTGAC AGTGGGCAGC AACCTCGCGA GAGGTAGCTA ATCTCCAAAA

1201 GCCGTCTCAG TTCGGATTGT TCTCTGCAAC TGCGAGAGCA TGAAGGCGGA ATCGCTAGTA

1261 ATCGCGGATC AGCATGCCGC GGA

Estrogen-degrading bacterium KC10 [DQ066440]

1 CCACTGGCGG CTGCCTACAC ATGCAAGTCG AACGAAGTCT TCGGACTTAG TGGCGCACGG

61 GTGCGTAACG CGTGGGAATC TGCCCTTGGG TACGGAATAA CTCAGAGAAA TTTGTGCTAA

121 TACCGTATAA TGTCTTCGGA CCAAAGATTT ATCGCCCAAG GATGAGCCCG CGTAAGATTA

181 GCTAGTTGGT GAGGTAAGAG CTCACCAAGG CGACGATCTT TAGCTGGTCT GAGAGGATGA

241 TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA

301 TTGGACAATG GGCGAAAGCC TGATCCAGCA ATGCCGCGTG AGTGATGAAG GCCTTAGGGT

361 TGTAAAGCTC TTTTACCCGG GATGATAATG ACAGTACCGG GAGAATAAGC TCCGGCTAAC

421 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GAGCTAGCGT TGTTCGGAAT TACTGGGCGT

481 AAAGCGCGCG TAGGCGGTTT TTTAAGTCAG AGGTGAAAGC CCAGTGCTCA ACACTGGAAC

541 TGCCTTTGAA ACTGGAAAAC TTGAATCTTG GAGAGGTCAG TGGAATTCCG AGTGTAGAGG

601 TGAAATTCGT AGATATTCGG AAGAACACCA GTGGCGAAGG CGACTGACTG GACAAGTATT

661 GACGCTGAGG TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC

721 GTAAACGATG ATAACTAGCT GTCCGGGCTC ATAGAGCTTG GGTGGCGCAG CTAACGCATT

781 AAGTTATCCG CCTGGGGAGT ACGGTCGCAA GATTAAAACT CAAAGGAATT GACGGGGGCC

841 TGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGCAGAACCT TACCAGCGTT

901 TGACATCCTG ATCGCGGATT AGAGAGATCT TTTCCTTCAG TTCGGCTGGA TCAGTGACAG

961 GTGCTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC

1021 GCAACCCTCA TCCCTAGTTG CCATCATTCA GTTGGGCACT CTAAGGAAAC TGCCGGTGAT

1081 AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTACGCGC TGGGCTACAC

1141 ACGTGCTACA ATGGCGGTGA CAGTGGGCAG CAACCGGGCG ACCGGTAGCT AATCTCCAAA

1201 AGCCGTCTCA GTTCGGATTG TTCTCTGCAA CTGCGAGAGC ATGAAGGCGG AATCGCTAGT

1261 AATCGCGGAT CAGCATGCCG CGGAATTCCN GG

Estrogen-degrading bacterium KC14 [DQ066444]

1 AGAGTTTGAT CATGGCTCAG AACGAACGCT GGCGGCATGC CTAATACATG CAAGTCGAAC

61 GAGATCTTCG GATCTAGTGG CGCACGGGTG CGTAACGCGT GGGAATCTGC CCTTGGGTTC

121 GGAATAACTT CTGGAAACGG AAGCTAATAC CGGATGATGA CGTAAGTCCA AAGATTTATC

181 GCCCAAGGAT GAGCCCGCGT AGGATTAGCT AGTTGGTGGG GTAAAGGCCC ACCAAGGCGA

241 CGATCCTTAG CTGGTCTGAG AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC

301 TCCTACGGGA GGCAGCAGTA GGGAATATTG GACAATGGGC GAAAGCCTGA TCCAGCAATG

361 CCGCGTGAGT GATGAAGGCC TTAGGGTTGT AAAGCTCTTT ACCCGGGAT GATAATGACA

421 GTACCGGGAG AATAAGCTCC GGCTAACTCC GTGCCAGCAG CCGCGGTAAT ACGGAGGGAG

481 CTAGCGTTGT TCGGAATTAC TGGGCGTAAA GCGCACGTAG GCGGCTATTC AAGTCAGAGG

541 TGAAAGCCCG GGGCTCAACC CCGGAACTGC CTTTGAAACT AGATAGCTTG AATCCAGGAG

601 AGGTGAGTGG AATTCCGAGT GTAGAGGTGA AATTCGTAGA TATTCGGAAG AACACCAGTG

661 GCGAAGGCGG CTCACTGGAC TGGTATTGAC GCTGAGGTGC GAAAGCGTGG GGAGCAAACA

721 GGATTAGATA CCCTGGTAGT CCACGCCGTA AACGATGATA ACTAGCTGTC AGGGCACATG

781 GTGTTTTGGT GGCGCAGCTA ACGCATTAAG TTATCCGCCT GGGGAGTACG GTCGCAAGAT

841 TAAAACTCAA AGGAATTGAC GGGGGCCTGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA

901 AGCAACGCGC AGAACCTTAC CAACGTTTGA CATCCCTATC GCGGATCGTG GAGACACTTT

961 CCTTCAGTTC GGCTGGATAG GTGACAGGTG CTGCATGGCT GTCGTCAGCT CGTGTCGTGA

1021 GATGTTGGGT TAAGTCCCGC AACGAGCGCA ACCCTCGCCT TTAGTTGCCA GCATTTAGTT

1081GGGTACTCTA AAGGAACCGC CGGTGATAAG CCGGAGGAAG GTGGGGATGA CGTCAAGTTC

1141 TCATGGCCCT TACGCGTTGG GCTACACACG TGCTACAATG GCGACTACAG TGGGCAGCCA

1201 CCTCGCGAGA GGGAGCTAAT CTCCAAAAGT CGTCTCAGTT CGGATCGTTC TCTGCAACTC

1261 GAGAGCGTGA AGGCGGAATC GCTAGTAATC GCGGATCAGC ATGCCGCGGT GAATACGTTC

1321 CCAGGCCTTG TACACACCGC CCGT

Estrogen degrading bacterium KC8 [DQ066438]

1 CCACTGGCGG CTGCCTACAC ATGCAAGTCG AACGAAGGCT TCGGCCTTAG TGGCGCACGG

61 GTGCGTAACG CGTGGGAATC TGCCCTTAGG TACGGAATAA CAGTGAGAAA TTACTGCTAA

121 TACCGTATGA TGTCGCAAGA CCAAAGATTT ATCGCCTAAG GATGAGCCCG CGTAGGATTA

181 GCTAGTTGGT GAGGTAAAAG CTCACCAAGG CGACGATCCT TAGCTGGTCT GAGAGGATGA

241 TCAGCCACAC TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA

301 TTGGACAATG GGCGAAAGCC TGATCCAGCA ATGCCGCGTG AGTGATGAAG GCCTTAGGGT

361 TGTAAAGCTC TTTTACCCGG GAAGATAATG ACTGTACCGG GAGAATAAGC CCCGGCTAAC

421 TCCGTGCCAG CAGCCGCGGT AATACGGAGG GGGCTAGCGT TGTTCGGAAT TACTGGGCGT

481 AAAGCGTACG TAGGCGGCTT TGTAAGTTAG AGGTGAAAGC CCGGGGCTCA ACCCCGGAAT

541 TGCCTTTAAG ACTGCATCGC TTGAACGTCG GAGAGGTGAG TGGAATTCCG AGTGTAGAGG

601 TGAAATTCGT AGATATTCGG AAGAACACCA GTGGCGAAGG CGGCTCACTG GACGACTGTT

661 GACGCTGAGG TACGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC

721 GTAAACGATG ATAACTAGCT GTCCGGGTAC TTGGTACTTG GGTGGCGCAG CTAACGCATT

781 AAGTTATCCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT CAAAGAAATT GACGGGGGCC

841 TGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGCAGAACCT TACCAACGTT

901 TGACATCCCT ATCGCGGTTA CCAGAGATGG TTTCCTTCAG TTCGGCTGGA TAGGTGACAG

961 GTGCTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC

1021 GCAACCCTCG CCTTTAGTTG CCATCATTTA GTTGGGCACT CTAAAGGAAC CGCCGGTGAT

1081 AAGCCGGAGG AAGGTGGGGA TGACGTCAAG TCCTCATGGC CCTTACGCGT TGGGCTACAC

1141 ACGTGCTACA ATGGCAACTA CAGTGGGCAG CAAGCCGGCG ACGGTGAGCT AATCTCCAAA

1201 AGTTGTCTCA GTTCGGATTG TTCTCTGCAA CTCGAGAGCA TGAAGGCGGA ATCGCTAGTA

1261 ATCGCGGATC AGCATGCCGC GGA

Sphingopyxis sp. C-1 [AB161684]

1 TGGAGAGTTT GATCCTGGCT CAGAACGAAC GCTGGCGGCA TGCCTAACAC ATGCAAGTCG

61 AACGAAGTCT TCGGACTTAG TGGCGCACGG GTGCGTAACG CGTGGGAATC TGCCCTTGGG

121 TACGGAATAA CTCAGAGAAA TTTGTGCTAA TACCGTATAA TGTCTTCGGA CCAAAGATTT

181 ATCGCCCAAG GATGAGCCCG CGTAAGATTA GCTAGTTGGT GGGGTAAAAG CCTACCAAGG

241 CGACGATCTT TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGGACTGAG ACACGGCCCA

301 GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGAAAGCC TGATCCAGCA

361 ATGCCGCGTG AGTGATGAAG GCCTTAGGGT TGTAAAGCTC TTTTACCCGG GATGATAATG

421 ACAGTACCGG GAGAATAAGC TCCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG

481 GAGCTAGCGT TGTTCGGAAT TACTGGGCGT AAAGCGCGCG TAGGCGGTTT TTTAAGTCAG

541 AGGTGAAAGC CCAGTGCTCA ACACTGGAAC TGCCTTTGAA ACTGGAAAAC TTGAATCTTG

601 GAGAGGTCAG TGGAATTCCG AGTGTAGAGG TGAAATTCGT AGATATTCGG AAGAACACCA

661 GTGGCGAAGG CGACTGACTG GACAAGTATT GACGCTGAGG TGCGAAAGCGTGGGGAGCAA

721 ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG ATAACTAGCT GTCCGGGTTC

781 ATAGAACTTG GGTGGCGCAG CTAACGCATT AAGTTATCCG CCTGGGGAGT ACGGTCGCAA

841 GATTAAAACT CAAAGGAATT GACGGGGGCC TGCACAAGCG GTGGAGCATG TGGTTTAATT

901 CGAAGCAACG CGCAGAACCT TACCAGCGTT TGACATCCTG ATCGCGGATT AGAGAGATCT

961 TTTCCTTCAG TTCGGCTGGA TCAGTGACAG GTGCTGCATG GCTGTCGTCA GCTCGTGTCG

1021 TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTCA TCCCTAGTTG CCATCATTAA

1081GTTGGGCACT CTAAGGAAAC TGCCGGTGAT AAGCCGGAGG AAGGTGGGGA TGACGTCAAG

1141 TCCTCATGGC CCTTACGCGC TGGGCTACAC ACGTGCTACA ATGGCAACTA CAGTGGGCAG

1201 CAACCTCGCG AGGGGTAGCT AATCTCCAAA AGTTGTCTCA GTTCGGATTG TTCTCTGCAA

1261 CTCGAGAGCA TGAAGGCGGA ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG

1321 TTCCCAGGCC TTGTACACAC CGCCCGTCAC ACCATGGGAG TTGGTTTCAC CCGAAGGCAG

1381 TGCTCTAACC CGCAAGGGAG GAAGCTGACC ACGGTGGGAT CAGCGACTGG GTGAAGTCG

1441 TAACAAGGTA GCCGTAGGGG AACCTGCGGC TGGATCACCT CCTT

Sphingomonas sp. DB-1 [AY947554]

1 ATTAGAGTTT GATCCTGGCT CAGAACGAAC GCTGGCGGCA TGCCTAACAC ATGCAAGTCG

61 AACGAAGTCT TCGGACTTAG TGGCGCACGG GTGCGTAACG CGTGGGAATC TGCCCTTGGG

121 TACGGAATAA CTCAGAGAAA TTTGTGCTAA TACCGTATAA TGTCTTCGGA CCAAAGATTT

181 ATCGCCCAAG GATGAGCCCG CGTAAGATTA GCTAGTTGGT GAGGTAAAAG CTCACCAAGG

241 CGACGATCTT TAGCTGGTCT GAGAGGATGA TCAGCCACAC TGGGACTAAG ACACGGCCCA

301 GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGGACAATG GGCGAAAGCC TGATCCAGCA

361 ATGCCGCGTG AGTGATGAAG GCCTTAGGGT TGTAAAGCTC TTTTACCCGG GATGATAATG

421 ACAGTACCGG GAGAATAAGC TCCGGCTAAC TCCGTGCCAG CAGCCACGGT AATACGGAGG

481 GAGCTAGCGT TGTTCGGAAT TACTGGGCGT AAAGCGCGCG TAGGCGGTTT TTTAAGTCAG

541 AGGTGAAAGC CCGGGGCTCA ACCCCGGAAT TGCCTTTGAA ACTGGAAAAC TTGAATCTTG

601 GAGAGGTCAG TGGAATTCCG AGTGTAGAGG TGAAATTCGT AGATATTCGG AAGAACACCC

661 AGTGGCGAAG GCGACTGACT GGACAAGTAT TGACGCTGAG GTGCGAAAGC TGGGGAGCA

721 AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GATAACTAGC TGTCCGGGTT

781 CATAGAACTT GGGTGGCGCA GCTAACGCAT TAAGTTATCC GCCTGGGGAG TACGGTCGCA

841 AGATTAAAAC TCAAAGGAAT TGACGGGGGC CTGCACAAGC GGTGGAGCAT GTGGTTTAAT

901 TCGAAGCAAC GCGCAGAACC TTACCAGCGT TTGACATCCT GATCGCGGAT AGTGGAGACA

961 CTTTCCTTCA GTTCGGCTGG ATCAGTGACA GGTGCTGCAT GGCTGTCGTC AGCTCGTGTC

1021 GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTC ATCCCTAGTT GCCATCATTC

1081 AGTTGGGCAC TCTAAGGAAA CTGCCGGTGA TAAGCCGGAG GAAGGTGGGG ATGACGTCAA

1141 GTCCTCATGG CCCTTACGCG CTGGGCTACA CACGTGCTAC AATGGCAACT ACAGTGGGCA

1201 GCAACCTCGC GAGGGGTAGC TAATCTCCAA AAGTTGTCTC AGTTCGGATT GTTCTCTGCA

1261 ACTCGAGAGC ATGAAGGCGG AATCGCTAGT AATCGCGGAT CAGCATGCCG CGGTGAATAC

1321 GTTCCCAGGC CTTGTACACA CCGCCCGTCA CACCATGGGA GTTGGTTTCA CCCGAAGGCA

1381 GTGCTCTAAC CCGCAAGGGA GGAAGCTGAC CACGGTGGGA TCAGCGACTG GGTGAAGTC

1441 GTAACAAGGT AAATCT

Pseudomonas putida strain ATCC 17522 [AF094742]

1 ATCATGGCTC AGATTGAACG CTGGCGGCAG GCCTAACACA TGCAAGTCGA GCGGATGAAG

61 AGAGCTTGCT CTCTGATTCA GCGGCGGACG GGTGAGTAAT GCCTAGGAAT CTGCCTGGTA

121 GTGGGGGACA ACGTCTCGAA AGGGACGCTA ATACCGCATA CGTCCTACGG GAGAAAGCAG

181 GGGACCTTCG GGCCTTGCGC TATCAGATGA GCCTAGGTCG GATTAGCTAG TTGGTGAGGT

241 AATGGCTCAC CAAGGCGACG ATCCGTAACT GGTCTGAGAG GATGATCAGT CACACTGGAA

301 CTGAGACACG GTCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGGA CAATGGGCGA

361 AAGCCTGATC CAGCCATGCC GCGTGTGTGA AGAAGGTCTT CGGATTGTAA AGCACTTTAA

421 GTTGGGAGGA AGGGCAGTAR ATTAATACTC TGCTGTTTTG ACGTTACCGA CAGAATAAGC

481 ACCGGCTAAC TCTGTGCCAG CAGCCGCGGT AATACAGAGG GTGCAAGCGT TAATCGGAAT

541 TACTGGGCGT AAAGCGCGCG TAGGTGGTTT GTTAAGTTGG ATGTGAAAGC CCCGGGCTCA

601 ACCTGGGAAC TGCATTCAAA ACTGACAAGC TAGAGTATGG TAGAGGGTGG TGGAATTTCC

661 TGTGTAGCGG TGAAATGCGT AGATATAGGA AGGAACACCA GTGGCGAAGG CGACCACCTG

721 GACTGATACT GACACTGAGG TGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT

781 AGTCCACGCC GTAAACGATG TCAACTAGCC GTTGGGAGCC TTGAGCTCTT AGTGGCGCAG

841 CTAACGCATT AAGTTGACCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT CAAATGAATT

901 GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG CGAAGAACCT

961 TACCAGGCCT TGACATCCAA TGAACTTTCC AGAGATGGAT TGGTGCCTTC GGGAACATTG

1021 AGACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGTA

1081 ACGAGCGCAA CCCTTGTCCT TAGTTACCAG CACGTAATGG TGGGCACTCT AAGGAGACTG

1141 CCGGTGACAA ACCGGAGGAA GGTGGGGATG ACGTCAAGTC ATCATGGCCC TTACGGCCTG

1201 GGCTACACAC GTGCTACAAT GGTCGGTACA GAGGGTTGCC AAGCCGCGAG GTGGAGCTAA

1261 TCCCAYAAAA CCGATCGTAG TCCGGATCGC AGTCTGCAAC TCGACTGCGT GAAGTCGGAA

1321 TCGCTAGTAA TCGCGAATCA GAATGTCGCG GTGAATACGT TCCCGGGCCT TGTACACACC

1381 GCCCGTCACA CCATGGGAGT GGGTTGCACC AGAAGTAGCT AGTCTAACCT TCGGGAGGAC

1441 GGTTACCACG GTGTGATTCA TGACTGGGGT GAAGTCGTAA CAAGGTAG

VITA

Name: Fuman Zhao

Address: Environmental Engineering Division
Zachry Department of Civil Engineering
Texas A&M University
College Station, TX 77843-3136

E-mail Address: zhaofuman@neo.tamu.edu

Education: B.S., Chemical Engineering, 1998
University of Tianjin, China

M.S. Environmental Engineering, 2006
Texas A&M University