EVALUATION OF FACTORS AFFECTING THE BIOTRANSFORMATION OF BENZALKONIUM CHLORIDES BY *PSEUDOMONAS* SPP.

by

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EVALUATION OF FACTORS AFFECTING THE BIOTRANSFORMATION OF BENZALKONIUM CHLORIDES BY *PSEUDOMONAS* SPP.

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dedicated to my family

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ABSTRACT

EVALUATION OF FACTORS AFFECTING THE BIOTRANSFORMATION OF BENZALKONIUM CHLORIDES BY *PSEUDOMONAS* SPP.

Quaternary ammonium compounds (QACs) are cationic surfactants that are active ingredients of many commercial and industrial disinfectants. Therefore they are major micropollutants found in industrial effluents, sewage, activated sludge, treated wastewater and receiving waters. Biodegradation is considered as the ultimate process to alleviate the impacts of QACs on the environment.

The main objective of this study is to determine the optimum conditions for biotransformation of benzalkonium chlorides (BACs), an extensively used QACs, in biological systems. Strain BIOMIG1, a new *Pseudomonas* species that belongs to none of the previously described *Pseudomonas* groups, was identified as the key microorganism degrading BACs in activated sludge. BIOMIG1 is an oxidase and catalase positive γ-*Proteobacterium* that has an optimum growth temperature at 28 ^{o}C. BIOMIG1 can degrade BACs up to 2.8 mM (1000 mg/L) with a maximum rate of 4 μM/hr (1.4 mg/L-hr). A set of kinetic assays testing the effect of various bio-physico-chemical factors on BAC degradation by BIOMIG1 was performed. At each condition, kinetic parameters such as maximum growth rate and half-saturation coefficient of BAC degradation by BIOMIG1 were calculated by curve fitting to the experimental data using Michaelis-Menten growth model. Results of kinetic assays and modeling can be summarized as follows: (1) BAC biotransformation experienced a lag-period below 10^{8} CFU/mL cell density and the lag period increased as cell density decreases; (2) Biodegradation rate of BAC with 14 carbon chain length was the highest followed by BACs with 12 and 16 carbon chain length; (3) Optimum BAC degradation temperature was determined as 42 ^{o}C. On the other hand, BAC degradation resulted in accumulation of benzyldimethylamine at 45 ^{o}C or ceased at 50 ^{o}C. Moreover, threshold oxygen concentration for BAC biotransformation was measured as 0.83 mg O_2/L.

ÖZET

BENZALKONYUM KLORÜRÜN *PSEUDOMONAS* CİNSİ BAKTERİLER TARAFINDAN BİYODÖNÜŞÜMÜNÜ ETKİLEYEN FAKTÖRLERİN DEĞERLENDİRİLMESİ

Dördüncül amonyum bileşikleri (DAB'lar) evsel ve endüstriyel bir çok dezenfektan formülasyonunda kullanılan katyonik yüzey aktif kimyasallardır. Bu nedenle evsel ve endüstriyel atıksuda en sık karşılaşılan mikrokirleticilerdendir. Biyodegredasyon DABların çevre üzerindeki olumsuz etkilerini azaltan en önemli mekanizmalardan biridir.

Bu çalışmanın temel amacı en sık kullanılan DABlardan biri olan benzalkonyum klorürlerin (BAK) biyolojik sistemlerde biyotransformasyonunun gerçekleştiği optimum koşulları belirlemektir. Daha önce hiçbir *Pseudomonas* grubuna ait olmayan yeni bir cinse ait BIOMIG1 suşu aktif çamurda BAK biyodegredasyonunu gerçekleştiren anahtar mikroorganizma olarak belirlendi. Bu suş optimum büyüme sıcaklığı 28 ^{o}C olan oksidaz ve katalaz pozitif bir γ-*Proteobacterium* olarak tanımlandı. BIOMIG1 BAK'ı 2.8 mM (1000 mg/L) konsantrasyona kadar maksimum 4 μM/saat (1.4 mg/L-saat) hızda parçalayabilmektedir. Çeşitli biyo-fiziko-kimyasal koşulların BIOMIG1'in BAK degredasyonu üzerindeki etkileri bir set deney ile araştırılmıştır. Herbir koşulda, maksimum büyüme hızı ve yarı doygunluk sabiti gibi kinetik parametreleri deneysel verilerin Michaelis-Menten modeline uydurularak hesaplanmıştır. Kinetik deneyleri ve modelleme sonuçları şöyle özetlenebilir: (1) BAK biyotransformasyonu 10^8 CFU/mL nin altındaki hücre yoğunluklarında belirli bir gecikmeden sonra başlamaktadır, bu gecikme süresi hücre yoğunluğu azaldıkça artmaktadır; (2) 14 karbonlu alkil grubuna sahip BAKın biyodegradasyon hızı en yüksek olup onu sırasıyla 12 ve 16 karbonlu alkil grubuna sahip BAKlar takip etmektedir; (3) Optimum BAK degredasyon sıcaklığı 42 ^{o}C'dir. Buna karşın 45 ^{o}C'de BAK mineralize olamayıp, ortamda benzildimetilamin birikimi gözlenmiş, 50 ^{o}C'de ise BAK biyotransformasyonu tamamen durmuştur. Bunlara ek olarak BAK biyotransformasyonunun gerçekleşmesi için gereli olan eşik çözünmüş oksijen konsantrasyonu 0.83 mg O_2/L olarak tepit edilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units Used
QAC	Quaternary Ammonium Compounds	
BAC	Benzalkonium Chloride	
R	Functional group	
HPV	High Production Volume	
U.S. EPA	U.S. Environmental Protection Agency	
OECD	Organization for Economic Co-operation and Development	
Cl	Chloride	
Br	Bromide	
N	Nitrogen	
LAS	Linear Alkyl Benzene Sulfonate	
BSA	Benzene Sulfonic Acid	
NSA	Naphthalene Sulfonic Acid	
SDS	Sodium Dodecyl Sulfonate	
C_{12}BDMA	Dodecyl benzyl dimethyl ammonium	
C_{14}BDMA	Tetradecyl benzyl dimethyl ammonium	
C_{12}BDMA-Cl	Dodecyl benzyl dimethyl ammonium chloride	
C_{14}BDMA-Cl	Tetradecyl benzyl dimethyl ammonium chloride	
C_{16}BDMA-Cl	Hexadecyl benzyl dimethyl ammonium chloride	
K_I	Half-saturation competitive inhibition constant	(mg/L)
HPB	Hexadecyl Pridinium Bromide	
HTMAB	Hexadecyl Trimethyl Ammonium Chloride	
DNA	Deoxyribo Nucleic Acid	
ABC	ATP (Adenosine TriPhosphate) binding cassette	
ATP	Adenosine Tri Phosphate	
ICE	Integrative Conjugative Elements	
SMR	Small Multidrug Resistance	
NADH	Nicotinamide Adenine Dinucleotide	
C_{12}TMA-Br	Dodecyl Trimethyl Ammonium Bromide	

C_{14}TMA-Br	Tetradecyl Trimethyl Ammonium Bromide	
C_{16}TMA-Br	Hexadecyl Trimethyl Ammonium Bromide	
BDMA	Benzyl Dimethyl Amine	
BMA	Benzyl Methyl Amine	
BA	Benzyl Amine	
FAD	Flavin Adenine Dinucleotide	
WHO	World Health Organization	
TCI Chemicals	Tokyo Chemical Industry Co., Ltd., Tokyo, Japan	
LB	Luria Bertani	
MIC	Minimum Inhibitory Concentration	
N	Number of cells after certain time	
No	Beginning cell number	
μ	Specific growth rate	(hr)
td	Time required for cells to double itself	(1/hr)
OD	Optical density	(nm)
[BAC]	BAC concentration	(μM)
K_{BAC}	Half-saturation constant	(μM)
X	Cell density	(cells/L)
Y	Yield coefficient	(cells/ μmoles BAC)
k_d	Decay constant	(1/hr)
k'	Cell specific rate of BAC utilization	(μM/hr)
DMSO	Dimethyl sulfoxide	
HPLC	High Pressure Liquid Chromatography	
CLSI	Clinical and Laboratory Standards Institute	
IC_{50}	Inhibitor concentration that causes 50% inhibition	(mg/L)
IC_{90}	Inhibitor concentration that causes 50% inhibition	(mg/L)
IC_{99}	Inhibitor concentration that causes 50% inhibition	(mg/L)
EC_{50}	Inhibitor concentration that causes 50% inhibition	(mg/L)
DTMAC	Dodecyl Trimethyl Ammonium Chloride	
D	Dilution factor	
k_{enzyme}	Enzyme synthesis rate	(1/hr)
β	Biodegradation proportionality factor	(μM/s.K)
T	Absolute temperature	(K)

Ea	Activation energy	(kcal/mol)
R	Gas constant	(1.987×10^{-3} kcal/mol·K)
ΔS	Entropy change of enzyme deactivation	(kcal/mol.K)
ΔH	Enthalpy change of enzyme deactivation	(kcal/mol)

1. INTRODUCTION

We all scare of "microbes" that make us sick. Therefore we use disinfectants to get rid of them and preserve our living environment free of disease causing microbes. However, our hygiene obsession has created a bigger problem; we taught microbes how to tackle with disinfectants. The talent that they have developed due to our unintended use of disinfectants is "antimicrobial resistance". Many antimicrobial resistance mechanisms are also used against antibiotics which makes the problem a significant threat to human health (Scenihr, 2009; Hegstad et al., 2010; Tezel and Pavlostathis, 2012a). Recently, it is discovered that microbes can consume disinfectants and most of the antibiotics as their carbon and energy source (Dantas et al., 2008). Are we in trouble? Theoretically yes, but let's be optimistic.

After use, active ingredients of disinfectants, i.e. biocides, are blended into wastewater. Wastewater containing organics and many other trace contaminants is treated and discharged in to a receiving body. Since many wastewater treatment plants are only designed to remove major easily degradable organics, most of the trace contaminants, including biocides, pass through wastewater treatment plants and are released into the environment. Biocides are very abundant in the environment (Martinez-Carballo et al., 2007a; Hughes et al., 2012). As a result, it is believed that the main cause of development of antimicrobial resistance is exposure of microorganisms to antimicrobial agents (biocides) at low concentrations in the environment (Martinez, 2008). Antimicrobial resistance is the major health threat that the human society is facing today (WHO, 2000).

Benzalkonium chlorides (BAC), which are quaternary ammonium biocides (QACs), include a mixture of alkyl benzyl dimethyl ammonium chlorides with C_8 to C_{18} alkyl groups. BACs are the active ingredient of many pharmaceutical formulations, cosmetics, commercial disinfectants, industrial sanitizers and food preservatives (Tezel and Pavlostathis, 2009). BAC is the most frequently found QAC group worldwide in domestic wastewater at concentrations ranging between 20 and 300 μg/L. (Martinez-Carballo et al., 2007a; Clara et al., 2007). On the other hand, wastewaters of hospitals and poultry

processing facilities generally contain QACs/BACs up to 10 mg/L (Kummerer et al., 1997; Martinez-Carballo et al., 2007b).

Although BACs are toxic to microorganisms at typical concentrations found in the wastewater (<10 mg/L, domestic and industrial), continuous exposure to BACs during the treatment of BAC-bearing wastewater selects and enriches specific group of microorganisms that are both resistant to BACs and capable of degrading QACs. The microbial diversity in a benzalkonium chloride (BAC) enrichment microbial community which utilizes BACs as carbon and energy source, was determined by 16S rDNA phylogenetic analysis of the clone library derived from the DNA extracted from the community (Tezel et al., 2012a). A clone library was constructed representing a total of 47 *Bacteria* 16S rDNA clones. Analysis of the 47 rDNA *Bacteria* clones indicated a fair phylogenetic diversity in the clone library composed of nine unique bacterial species. All of the sequenced clones belonged to the *Proteobacteria*. Of these, 98% were related to *Pseudomonas* genus of *γ-Proteobacteria*, including the most numerically dominant phylotype, BAC54, which is most similar to *Pseudomonas nitroreducens* (99% similarity). Although the full extent of *Bacteria* diversity in the 16S rDNA clone library was not revealed, numerous dominant RFLP groups were obtained. The vast majority of the sequenced clones (98% of the clone library) belonged to *Pseudomonas* genus of *γ-Proteobacteria* which suggests that *Pseudomonas* spp. was responsible for the degradation of BAC in the BAC enrichment community (Tezel et al., 2012a). In addition, biotransformation pathway of n-tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl) by this community was delineated. Biotransformation of C_{14}BDMA-Cl commenced with cleavage of the C_{alkyl}–N bond and formation of benzyldimethylamine (BDMA). Mineralization of BDMA achieved through debenzylation to dimethylamine and benzoic acid followed by the catabolism of these product to ammonium and carbon dioxide.

Biotransformation of BACs by pure culture of *Aeromonas hydrophila* sp. was studied (Patrauchan and Oriel, 2003). *Aeromonas hydrophila* sp. converted BACs to BDMA following the same mechanism described above. However, accumulation of BDMA in the culture media inhibited complete transformation of BACs. It was also shown

that when *Aeromonas hydrophila* was incubated with BDMA as sole carbon source, it transformed BDMA to benzyl methyl amine then to benzyl amine following two demethylation steps other than debenzylation opposed to the pathway proposed by Tezel et al. (2012a).

Toxicity to organisms, and contribution to the development and dissemination of antimicrobial resistance, which is one of the most important human health threat declared by World Health Organization (WHO), are the major implications of QACs. These implications can be controlled by alleviating QACs in wastewater treatment plants before they are discharged into the environment. Biological treatment is the ultimate process to remove QACs from the wastewater. Since the biological treatment units of wastewater treatment plants are not designed for the treatment of QACs, QAC removal from 0 to 90% has been reported in the literature. Such a broad removal efficiency reported shows that several factors are playing, individually or simultaneously, a role in the biological treatment of QACs (Boethling, 1994).

These factors may include presence of BAC degraders and their abundance in activated sludge, BAC concentration and structure, oxygen concentration, temperature, adsorption to organic/inorganic surfaces, and concentration and type of readily biodegradable organics. Although several studies focusing on the biotransformation of BACs are available in the literature, none of them investigated the biological, chemical and physical factors given above on the biotransformation systematically. Therefore, the aim of this project is to identify the key microbial species/community responsible for the biotransformation of BACs and evaluate the effect of some of the aforementioned chemical and physical factors on biotransformation of BACs by that species/community in a representative biological treatment system.

The results obtained in this study would be used to develop post-treatment technologies or process modifications to achieve complete removal of BACs/QACs in the wastewater treatment plants. The outcomes of this research would help to develop strategies to control BAC related toxicity and antimicrobial resistance and therefore the advancement of human and environmental health.

2. LITERATURE SURVEY

QACs are organic compounds that contain four functional groups attached covalently to a central nitrogen atom (R_4N^+) (Fig 2.1). The functional groups (R) include at least one long-chain alkyl group and the rest are either methyl, benzyl, or ester groups. QACs are among the high production volume chemicals (HPVs, i.e., chemicals manufactured or imported in amounts equal to or greater than one million pounds per year) found on the lists of both the U.S. Environmental Protection Agency (U.S. EPA, 2006) and the Organization for Economic Co-operation and Development (OECD).

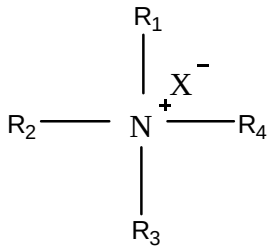
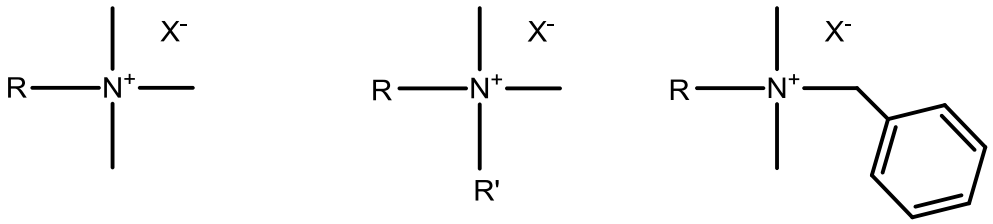


Figure 2.1. General molecular structure of a quaternary ammonium compound (R represents a functional group, X^- represents a halide such as Cl^-, Br^-).

QACs are large molecules having molecular weights typically between 300 and 400 g/mole and are composed of two different moieties: hydrophobic alkyl groups and a hydrophilic, positively charged central N atom, which retains its cationic character at all pH values. The two moieties not only affect the QACs' physical and chemical properties but also may have a decisive role in the fate and effects of these compounds in the environment (Boethling, 1994; Garcia et al., 1997).

QACs can be classified in three major groups depending on the type of functional groups (R's): monoalkonium, dialkonium and benzalkonium halides (Fig 2.2).



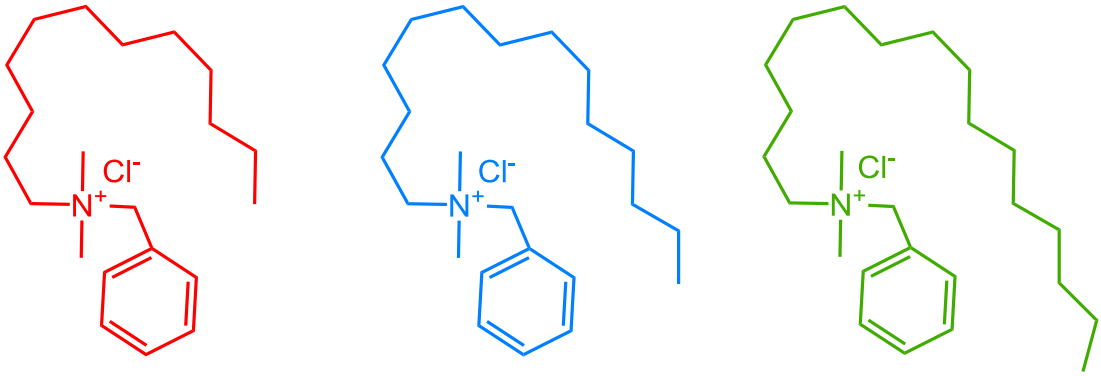
Monoalkonium halides
C_nTMA-X

Dialkonium halides
$DC_{n\text{-}n}$DMA-X

Benzalkonium halides
C_nBDMA-X

Figure 2.2. Representative QAC groups, their general structure and abbreviations used in this study (X^- is a halide counter-ion).

BACs contain a quaternary N bound to two methyl groups, a benzyl group, and an alkyl group that varies in length (typically, C_{12}, C_{14}, and C_{16}) (Fig 2.3).



(A) **(B)** **(C)**

Figure 2.3. Typical Benzalkonium Chlorides; (A) n-dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, MW: 340.0 g/mole, ThOD: 944 g/mole), (B) n-tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, MW: 368.0 g/mole, ThOD: 1040 g/mole),.(C) n-hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, MW: 396.0 g/mole, ThOD: 1136 g/mole).

2.1. Applications of QACs

QACs have unique physical/chemical properties such as; surface-active properties, self-assembly characteristics, detergency, and antimicrobial properties. This important characteristics gain QACs a wide and extensive variety of use such as fabric softeners, surfactants, emulsifiers, disinfectants, pesticides, corrosion inhibitors, and personal care products (Garcia et al., 1999; Steichen, 2001; Patrauchan and Oriel, 2003). The 2004 worldwide annual consumption of QACs was 500,000 tons and expected to reach or exceed 700,000 tons (Hauthal, 2004).

QACs are mostly used as fabric softeners that comprises %80 of total consumption. QACs are cationic surfactants, by the help of their positive charge they can adsorb onto negatively charged surfaces, which makes them favourable fabric softener agents (Garcia et al., 1999). Dialkonium and diesteralkonium halides are most commonly used fabric softener agents in today's market. QACs also take part in laundry detergents. Monoalkonium QACs with an alkyl chain length of 12–18 carbons are utilized in laundry detergents which also supply fabric softening properties (Zachvieja, 2001).

QACs have biocidal properties and maintain its features in a wide range of pH. They are used in domestic, industrial, agricultural, and medical applications as antimicrobial agents. Even at low concentrations they are effective on against a variety of bacteria, fungi, and viruses. Typical application concentrations are between 400 and 500 ppm when they are used as disinfectants. Monoalkonium (C_{16}), dialkonium ($C_{8–10}$), and benzalkonium ($C_{12–16}$) chlorides are most extensively used QACs as bioactive agents (Tiedink, 2001). The same types of QACs are also used in agricultural applications to sanitize animal houses. QACs are also used in agricultural applications as active ingredients or adjuvants, to provide better penetration of pesticides and enhance solubility of pesticides (Pesticide Action Network). Sanitizer formulations commonly contain benzalkonium chloride (BAC) homologs of different alkyl chain lengths, mainly C_{12}, C_{14}, and C_{16} (Sutterlin et al., 2008a).

QACs are used in cosmetic products because of their ability to reduce interfacial tension by sorbing onto a surface. As an example BAC is used in as such products; eye drops, artificial tears, decongestion nose drops, facial moisturizers, treatment formulations, facial cleansers, acne treatment formulations, sun protection creams and lotions, baby lotions, moisturizers, pain relief, and hand sanitizers (Gilbert and Moore, 2005). Skin care products and hair conditioners contain alkyl QACs in their formulas such as; mono-, di-, and trialkonium salts and polymeric QACs (e.g., polyquaternium 7 and 11) (Tang, 2001). QACs also used in mouthwashes to prevent dental biofilm formation (Hegstad et al., 2010).

Benzalkonium chlorides (BACs) are the most extensively used QAC group, employed in domestic and industrial applications such as pharmaceutics, cosmetics, disinfectants, sanitizers and food preservatives (Tezel and Pavlostathis, 2009). As an outcome of high excercising, BACs are the most common QACs found in engineered and natural systems (Clara et al., 2007; Kreuzinger et al., 2007; Kummerer et al., 1997; Martinez-Carballo et al., 2007a; Martinez-Carballo et al., 2007b).

Taken into consideration all this practices of QACs mentioned above, it is clear that QACs are important, widespread chemicals due to their usage in high quantities in domestic, agricultural, industrial, and clinical applications. This comes with the conclusion of humans and microorganisms being in constant contact with them. Since BACs are the most extensively used QAC group, in this study BACs constitute the primary focus.

2.2. Distribution of QACs in the Environment

QACs are chemicals used in multiple fields, and as a consequence they are released into the environment after their utilization. Approximately 25 % is released into the environment directly while the rest reaches to wastewater treatment plants. Occurance of QACs in different environments such as effluents of laundries and hospitals (Kummerer et al., 1997; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a), industrial wastewater (Kreuzinger et al., 2007; Martinez-Carballo et al., 2007b), sewage (Kreuzinger et al., 2007;

Martinez-Carballo et al., 2007a), sewage sludge (Martinez-Carballo et al., 2007b; Sutterlin et al., 2007), treated wastewater (Clara et al., 2007; Kreuzinger et al., 2007), surface waters (Ding and Liao, 2001; Kreuzinger et al., 2007; Martinez-Carballo et al., 2007a) and aquatic sediments (Kreuzinger et al., 2007; Martinez-Carballo et al., 2007b; Li and Brownawell, 2010) has been reported. According to the studies mentioned above, typical concentration of QACs in domestic wastewater is around 0.5 mg/L, in treated effluent wastewater is around 0.05 mg/L, in sewage sludge is around 5000 mg/kg dry weight and in surface water is around 0.04 mg/L.

QAC concentration in some treatment plants in Switzerland, which had different inputs from metallurgical processes and the textile industry, ranged between 0.04 to 0.45 mg/L (Michelsen, 1978). In a study performed in Austria, QAC concetrations in influents and effluents of five municipal wastewater treatment plants ranged from 25 to 300 μg/L and from 0.3 to 3.6 μg/L, respectively (Martinez-Carballo et al., 2007a). BAC is the most common QAC group found in wastewater followed by dialkonium and monoalkonium salts. BACs were detected at concentrations ranging between 20 and 300 μg/L in municipal wastewaters (Martinez-Carballo et al., 2007a; Clara et. al., 2007). Frequent occurance of BACs in wastewater was attributed to their use as the main active ingredients of disinfectants and sanitizers coupled with their persistence in natural and engineered systems.

QACs are hydrophobic and positively charged chemicals and they sorb onto negatively charged organic surfaces such as sludge and sediments (Garcia et al., 1997; Ferrer and Furlong, 2002; Sun et al., 2003) therefore they are transferred to anaerobic digesters (Boethling, 1994; Tezel et al., 2006; Ismail et al., 2010). QAC concentrations may reach up to 50 mg/L in anaerobic digesters of sewage treatment plants (Ecetoc, 1993; Garcia et al., 1999).

QACs were detected at higher concentrations in the wastewater of some industrial facilities compared to municipal wastewater. Kummerer et al. (1997) found QAC concentrations between 0.05 and 6.03 mg/L in effluent samples from European hospitals and Martinez-Carballo et al. (2007a) found QACs around 3 mg/L in laundry effluents and

5 mg/L in hospital effluents. The most common QAC detected in those studies was C_{12}BDMA-Cl.

QACs are detected in receiving waters in the low microgram per liter range. In Main River, Germany, QAC concentration ranging from 5 to 20 μg/L was reported (Huber et al., 1979). Dialkonium chlorides were detected in surface water samples collected in Germany at a concentration between 6 and 12 μg/L (OECD, 1994). Monoalkonium chlorides were measured in rivers in the United States at a range of 5 and 30 μg/L (Wee and Kennedy, 1982; Wee, 1984). Dialkonium chloride concentrations were 2, 24, 17, and 33 μg/L for four different rivers in the United States (Lewis and Wee, 1983). Monoalkonium, dialkonium, and benzalkonium halides were detected in 22 surface water samples at nanogram per liter levels. C_{12}BDMA and C_{14}BDMA were the most frequently detected QACs among others (Martinez-Carballo et al., 2007a). Dialkonium and benzalkonium chlorides (BACs) were found at 26 and 1.5 mg/kg, respectively, in river sediments, higher than other traditional organic pollutants (e.g. polycyclic aromatic hydrocarbons) (Li and Brownawell, 2010).

Studies on the occurance of QACs in the environment show that QACs are widely distributed and can be found in a broad range of different environments such as some industrial effluents, sewage sludge, activated sludge, treated wastewater and receiving waters. BACs are the most common QAC group detected in those environments.

2.3. Toxicity of QACs

75% of QACs consumed annualy reaches to wastewater treatment plants. Biological units of wastewater treatment plants have variable units which require physiologically different microoorganisms. Sensitivity of physiologically different microorganisms to QACs may be different as well; such as, QACs are particularly toxic to nitrifiers compared to heterotrophic bacteria.

Toxicity of BACs in activated sludge has been studied by some researchers. The EC_{50} value for C_{12}BDMA-Cl in activated sludge ranged between 10 and 40 mg/L (Reynolds et al., 1987). BACs were found to be inhibitory to a mixed, enriched nitrifying culture at 10–15 mg/L (Yang, 2007). In a different study, initially, complete inhibition of nitrification was observed and nitrogen removal efficiency decreased in a biological nutrient removal system (BNR) at 5 mg/L BAC (Hajaya and Pavlostathis, 2012). Inhibition of denitrification by BAC was observed at BAC concentrations around or higher than 50mg/L (Tezel et al., 2008; Tezel and Pavlostathis, 2009). Further QACs are toxic to methanogenesis at 25 mg/L or higher (Tezel et al., 2006). These results indicated that BACs cause inhibition in biological systems at and above 5 mg/L which is higher than the concetrations expected in wastewater. In addition, nitrifiers are the most sensitive physiological group in biological systems to BACs (Sutterlin et al., 2008b).

Several factors affect the inbition of biological systems by BACs. Several studies have shown that BAC adsorption to biomass substantially decreases the BAC inhibition in biological systems (Zhang et al., 2011; Hajaya and Pavlostathis, 2012). Zhang et al. (2011) studied the inhibitory effect of BACs (C_{12}–C_{16}) at a concentration range from 5 to 20 mg/L and at different biomass concentrations in an activated sludge system. The mode of action of BACs was determined to be inhibition of respiratory enzymes and the half-saturation competitive inhibition constant (K_I), which is equivalent to EC_{50} of benzalkonium chlorides, was found in between 0.12 and 3.60 mg/L for the activated sludge tested depending on the biomass concetration. At high biomass concentration, BACs were less inhibitory suggesting that adsorption to biomass decreases the bioavailability of BACs so do the the inhibitory effects.

Moreover, BACs can form ionic pairs in the presence of anionic compounds such as linear alkyl benzene sulfonates (LAS), benzene sulfonic acid (BSA), naphthalene sulfonic acid (NSA) and sodium dodecylsulfonate (SDS); the polarity changes and this phenomenon affects the distribution of BACs in the environment. Normally easily biodegradable surfactants LAS and SDS becomes less biodegradable when they form an ionic pair with QACs (Gerike et al., 1978; Kohler et al., 1997; Kummerer et al., 2002). The reason for that is, ionic pairs become less available for the bacteria because of bigger size

and lower polarity of the compounds formed. Further, ionic pairs reduce enormously and even eliminate the toxicity of BACs to microorganisms. Pitter (1961) claimed that QACs are not toxic in the presence of anionic surfactants at relative concetrations.

Acclimation plays an important role in QAC toxicity, unacclimated sludge was inhibited by 50% at 10 mg/L tetradecyl dimethyl benzyl ammonium chloride (C_{14}BDMA-Cl), however acclimated sludge was inhibited by 50% at 20 mg/L C_{14}BDMA-Cl. Larson and Schaeffer (1982), reported IC_{50} value of alkyl trimethyl ammonium chloride for unacclimated sludge as 28 mg/L using glucose uptake inhibition method. Pitter and Horska (1968), studied inhibitory effect of hexadecyl pridinium bromide (HPB) and hexadecyl trimethyl ammonium bromide (HTMAB) in unacclimated sludge fed with glucose or peptone and reported IC_{50} values as 9 mg/L for HPB and 19 mg/L for HTMAB. Reynolds et al. (1987), studied HTMAB and C_{12}BDMA-Cl toxicity with a respirometric assay with activated sludge and found EC_{50} values range between 10 and 40 mg/L. Boethling (1984), reported EC_{50} value of BAC for acclimated and unacclimated activated sludge range between 20 and 50 mg/L.

25% of all used QACs are released into the environment. It was shown that monoalkonium and dialkonium QACs affected the heterotrophic bacterial activity of aquatic microbial communities in a lake ecosystem at even 1 mg/L (Ventullo and Larson 1986). Also it was reported that all QACs were acutely toxic to aquatic invertebrates and fish by affecting the reproduction and larval growth and development (Lewis, 1991; Boethling, 1994; Utsunomiya et al., 1997). The EC_{50} of BACs to fish was studied and found between 0.5 and 5.0 ppm, and the EC_{50} to daphnids were from 0.1 to 1.0 ppm (Kummerer et al., 1997). Algae represent a group of organisms which is very sensitive to QACs.

Tezel (2009) studied the acute toxicity of nine QAC homologs belonging to the monoalkonium, dialkonium, or benzalkonium chloride group and with different alkyl chain lengths on a luminescent marine organism, *Vibrio fischeri* using Microtox assays, and showed that toxicity of QACs increases as the exposure time increases, meaning that a certain exposure time is necessary for QACs to fully reach the target site(s) and/or for cells

to react to the QACs. And also the lowest EC_{50}, which indicates the highest toxicity, in each QAC group was for QACs with the shortest alkyl chain within a homologous group. The EC_{50} values obtained in this study were consistent with previously reported values obtained using the same toxicity assay (Leal et al., 1994; Garcia et al., 2001; Nalecz-Jawecki et al., 2003; Sutterlin et al., 2008b).

Sutterlin et al. (2008b), elucidated the effect of BACs on *Pseudomonas putida*, an important gram-negative soil bacterium. The EC_{50} of benzalkonium chlorides was 6 mg/L, and the toxicity was not significantly affected by the presence of linear alkyl sulfonate, an anionic surfactant and common detergent ingredient. On the contrary; Russell and Hugo (1992), showed that anionic surfactants forms ionic pairs with QACs leading to an increase in hydrophobicity thus decrease the inhibitory effects of QACs.

2.4. Sub-inhibitory concentration and QAC resistance

The QAC concentration in a disinfectant application is around 1000 mg/L, which is significantly higher than the MIC of many pathogens. On the other hand, environmental concentrations of QACs are well below the MIC values. Sewage, biological wastewater treatment units, surface waters and sediments are environments where QACs are present at sub-inhibitory concentrations. When the high microbial diversity in these environments is coupled to sub-inhibitory QAC concentrations, such environments become selective, resulting in the emergence and dissemination of QAC resistance among different bacterial genera, which may also include clinically important pathogens.

The mode of action of QACs above MIC in bacteria is the disruption of cell membrane's physical and ionic stability (Wessels and Ingmer, 2013). For example, benzalkonium chlorides (BACs) bind to the cell membrane of *Pseudomonas fluorescens* by ionic and hydrophobic interactions, bringing about changes of membrane properties and function, followed by cellular disruption, loss of membrane integrity, ultimately resulting in leakage of essential intracellar constituents (Ferreira et al., 2011; Morente et al., 2013). Above MIC, microorganisms with durable cell membrane are selected and proliferate. On

the other hand, the mode of action of QACs at sub-MICs is complicated and always includes multiple processes such as loss of membrane osmoregulation, inhibition of respiratory enzymes, the dissipation of proton motive force and oxidative stress, which triggers SOS response, inducing error-prone DNA replication leading to mutations and gene transfers (Blazquez et al., 2012; Ceragioli et al., 2010). Adaptation to QACs at sub-MICs is achieved by modification of the outer membrane, cell membrane, density and structure of porins, regulatory hyperexpression of efflux pumps, and acquisition of QAC-specific efflux pumps through mobile recombinational elements, such as plasmids and integrons through oxidative stress or (followed by) stress-induced mutagenesis (Tezel and Pavlostathis, 2012a).

2.5. Pathways and mechanisms of QAC resistance at sub-inhibitory concentrations

Major pathways in the evolution of resistance at sub-inhibitory antibiotic concentrations have been recently reviewed (Andersson and Hughes, 2012; Andersson and Hughes, 2014). Microorganisms follow similar pathways while adapting to QACs. Exposure to QACs at sub-MIC creates (oxidative) stress. For instance, exposure of E. coli to cetyltrimethylammonium bromide resulted in intracellular production of superoxide and hydrogen peroxide (Nakata et al., 2011). Bacteria compensate for oxidative stress by SOS-response and induction of stress-response sigma factors rpoS, promoting cell survival by DNA repair, while nucleotide polymorphism may occur, resulting in mutations. Oxidative stress responses also boost gene transfer and recombination events via prophages, transposons, integrons and integrative-conjugative elements (ICE). As a result, resistant sub-populations evolve and dominate in a microbial community (Andersson and Hughes, 2012). Major mechanisms of adaptation to QACs include modification of cell membrane structure and composition, enhanced biofilm formation, acquisition of efflux genes, overexpression of efflux pump systems, and biodegradation. Generally, multiple mechanisms co-develop during adaptation of bacteria to QACs (Moen et al., 2012).

Exposure to QACs at sub-MICs enhances biofilm formation (Pagedar et al., 2012). QAC resistant strains of bacteria form biofilms faster and these species are less susceptible to QACs than planktonic species (Nakamura et al., 2013). Presence of multiple species in

biofilms increases QAC resistance (Giaouris et al., 2013; van der Veen and Abee, 2011). Expression of certain genes enhanced biofilm formation by QAC resistant *Listeria monocytogenes* (van der Veen and Abee, 2010a; van der Veen and Abee, 2010b).

Several mutations result in selection of microorganisms with reduced cell permeability. Resistant cells have modified cell membrane fatty acids, phospholipids, and outer membrane lipopolysaccharides (Fox et al., 2011), resulting in a more anionic and hydrophobic cell surface, thus restricting easy passage of the QACs through the cell surface. Other cell modifications in response to exposure to QACs are density reduction and composition change of the porins (Machado et al., 2013; Tabata et al., 2003), as well as change of the outer membrane protein composition (Mavri and Mozina, 2013).

Efflux-mediated QAC resistance has gained significant interest because it has a genetic origin, confers co-resistance to antibiotics and is transferable among microbial species through horizontal gene transfer. Multidrug efflux pumps mediate the transfer of biocides from the inside to the outside of the cell through an energy or proton-dependent mechanism. QAC resistance via efflux pumps follows two mechanisms. First, QAC resistance is induced by overexpression of efflux pumps upon exposure to QAC or as a result of QAC-induced stress. Such stress either triggers a regulatory system that controls the expression of an efflux determinant or causes a mutation resulting in the overexpression of the efflux determinant or increase in its extrusion efficiency (Grkovic et al., 2002). These efflux pumps are generally chromosomally encoded and act against a wide array of antimicrobial agents (Guo et al., 2014; Buffet-Bataillon et al., 2012; Holdsworth and Law, 2013; Mc Cay et al., 2010; Morita et al., 2014). Overexpression of these efflux pumps results in a two to eight fold increased tolerance of the adapted microorganism to QACs and other substrates of these pumps.

The second mechanism is through acquisition of genes for specialized QAC efflux pumps, which belong to the SMR family. Among them, EmrE, smr and SugE are multidrug efflux pumps (He et al., 2011), whereas QacE, QacΔE, QacF, QacG, QacH, QacI, QacJ and QacZ are QAC-specific efflux determinants (Braga et al., 2011). The genes of these efflux proteins are mainly found in mobile genetic elements such as transposons,

ICEs, plasmids and integrons. As a result, they can be horizontally transferred between microorganisms of the same or different genera. Such genes are abundant in the environment.

Acquisition of QAC efflux genes by microorganisms involves integration of genes to integrons and plasmids or recombination of efflux gene containing ICE and transposons into the recipient genome. Oxidative stress response plays a crucial role in both mechanisms. Integrons, which are promoterless mobile recombinational elements, play a significant role in the acquisition and mobilization of QAC resistance genes (Crambray et al., 2010). Integrons are found in many environmental bacterial species, particularly in those exposed to QACs and/or antibiotic residues (Gaze et al., 2011; Gaze et al., 2005). QAC contamination is also responsible for the stabilization of integrons and their gene cassettes (Gillings et al., 2009). A direct link between SOS response and the expression of integron integrases was demonstrated. SOS regulation enhances cassette swapping and capture under stressful conditions, such as during QAC exposure (Cambray et al., 2011).

Plasmids also play a significant role in harboring and disseminating genes related to resistance to QACs and other biocides. Plasmids of the incompatibility (Inc) group IncP-1, also called IncP, as extrachromosomal genetic elements can be transferred and replicated virtually in all Gram-negative bacteria. IncP-1group plasmids are broadly distributed in environments with sub-inhibitory QAC concentrations, such as soils and wastewater treatment plants. IncP plasmids commonly harbor QAC resistance genes along with many other resistance genes (Popowska and Krawczyk-Balska, 2013; Dutta et al., 2013; Elhanafi et al., 2010; Marti and Balcazar, 2012). In addition, plasmid-associated QAC resistance genes were transferred between non-pathogenic and pathogenic bacteria exposed to QACs, a process that also leads to the co-selection of resistance to other contaminants (Katharios-Lanwermeyer et al., 2012).

Using the SOS-response, ICEs are transferred from one bacterial chromosome to another. ICEs containing multiple QAC resistance genes were identified in bacterial strains of *Vibrio scophthalmi, V. splendidus, V. alginolyticus*, *Shewanella haliotis*, and *Enterovibrio nigricans* isolated from fish grown in marine aquaculture systems

(Rodriguez-Blanco et al., 2012). Exposure to biocides below inhibitory concentrations also facilitates the conjugation of transposons (Seier-Petersen et al., 2014). Novel transposons carrying QAC resistance genes have been identified in both Gram-negative and positive microorganisms (Muller et al., 2013; Ciric et al., 2011).

2.6. QAC Biodegradation: Ecology, Mechanism and Genetics

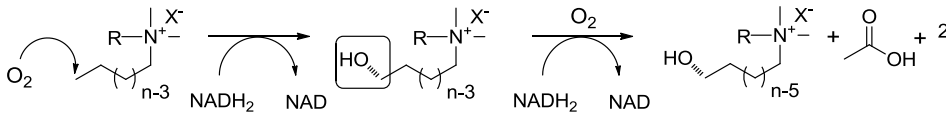
Wastewater, wastewater treatment plants, wastewater sludge, sludge applied soil, surface waters, and aquatic sediments are environments where QAC resistance evolves and proliferates. QAC resistant microorganisms may be transferred from outdoor environments to indoors such as homes and healthcare facilities. QAC resistance genes, especially efflux genes, are frequently found in bacterial isolates obtained from human/animal healthcare facilities. These genes either confer resistance to multiple biocides and antibiotics (co-resistance), or mobile genetic elements that they are attached to carry resistance genes to other biocides and antibiotics. Therefore, QAC resistant microorganisms, if they are pathogenic, may pose a serious threat to human health. In addition, QAC resistant microorganisms may decrease the efficacy of QAC disinfectants by creating microenvironments habitable by QAC susceptible microorganisms, as for example is the case of biofilms. Moreover, QAC degrading microorganisms create QAC gradients in which susceptible microorganisms survive or even develop QAC resistance and proliferate.

Aerobic biodegradation of QACs has been attributed mainly to bacterial species in the genera of. *Xanthomonas*, *Aeromonas*, and *Pseudomonas* (Tezel and Pavlostathis, 2012a). More recently, several bacteria have been identified that are capable of QAC degradation, such as *Pseudomonas putida* A ATCC 12633 (Liffourrena and Lucchesi, 2014), *Pseudomonas nitroreducens* B and DB (Oh et al., 2014), *Pseudomonas* sp. and *Stenotrophomonas* sp. B27 (unpublished). In addition, non-culture-based techniques such as cloning, metagenomics and pyrosequencing have been used to elucidate the composition of QAC degrading, enriched microbial communities. A microbial community originating from a river sediment, utilizing BACs as the sole carbon and energy source had a dramatically decreased phylogenetic diversity as compared to the control (without BAC exposure), and was dominated by *Pseudomonas* species (> 50% of the total) (Oh et al.,

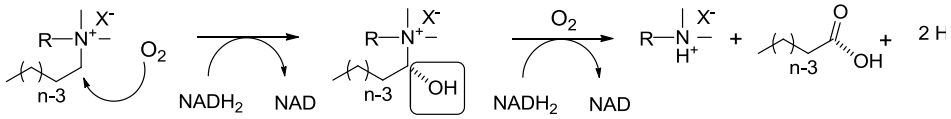
2013; Tezel et al., 2012b; Tandukar et al., 2013). Metagenomic analysis revealed that community adaptation to BACs occurred primarily via selective enrichment of BAC-degrading *Pseudomonas* species, particularly *P. nitroreducens*, and secondarily via amino acid substitutions and horizontal transfer of selected genes, including a gene encoding a PAS/PAC sensor protein and ring-hydroxylating dioxygenase genes (Oh et al., 2014; Oh et al., 2013). In addition, multi-drug efflux pump genes such as sugE, PmpM, mexAB-oprM and mexEF-oprN were enriched in the BAC-degrading community (Tandukar et al., 2013).

Based on the phylogenetic tree prepared using the 16S rDNA sequences of QAC degrading isolates and predominant species in QAC degrading microbial communities, *Pseudomonas* spp., particularly the *P. putida* and *P. aeruginosa* group, are key species in QAC degradation. In addition *Stenetrophomonas* spp. (γ-proteobacteria), and *Achromobacter* spp. (α-proteobacteria) are frequently identified in communities degrading QACs, indicating that they play a role in the biodegradation of QACs (Oh et al., 2013).

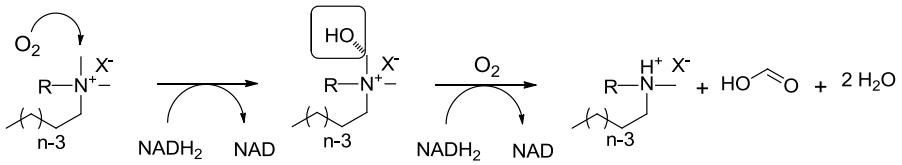
Three aerobic QAC biotransformation pathways, which differ in the location of the initial reaction, have been observed (Figure 2.4.): a) hydroxylation of the terminal C of the alkyl chain (ω-hydroxylation), followed by multiple β-oxidation cycles, progressing toward the hydrophilic moiety; b) hydroxylation of the C adjacent to the central N (α-hydroxylation), followed by central fission, resulting in the separation of the hydrophobic from the hydrophilic moiety; and c) hydroxylation of the methyl-C attached to the central N, followed by fission of the methyl group (Tezel et al., 2012a). In spite of the fact that QACs have a relatively high adsorption capacity resulting in their transfer to anoxic/anaerobic environments, such as anaerobic digesters and aquatic sediments, limited information exists relatively to the fate of QACs under such conditions. The transformation of BACs under anoxic, nitrate reducing conditions was recently reported to be initiated by means of an abiotic nitrite nucleophilic substitution reaction (modified Hofmann reaction) producing alkyl dimethyl amines (tertiary amines) (Tezel et al., 2012a; Tezel and Pavlostathis, 2009). Under anaerobic conditions, there is no evidence of mineralization of QACs that contain alkyl or benzyl groups (Tezel et al., 2012a).



a. ω-hydroxylation of terminal C of alkyl group



b. α-hydroxylation of C adjacent to N of alkyl group



c. α-hydroxylation of C of methyl group

Figure 2.4. Aerobic QAC biotransformation pathways

In spite of the fact that the energetic burden of the above-discussed QAC biotransformation pathways (initial reaction) is the same, pathway-b starting with the cleavage of the C_{alkyl}-N bond is the most predominant. Although, pathway-a was the first identified QAC biotransformation pathway, it could not be demonstrated in later studies for similar QACs. Moreover, the product of pathway-b, a tertiary amine, is less toxic than the products of pathway-a and -c (Tezel et al., 2012b). The combination of these two observations suggests that pathway-b is naturally selected as a mechanism for QAC biotransformation to cope with the toxic effects of QACs and to eliminate the detrimental consequences of the other biotransformation pathways. As a result, QAC biotransformation may have evolved as a QAC-resistance mechanism.

As an example, the biotransformation of n-tetra decyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl), under aerobic conditions by an enriched microbial community growing on benzalkonium chlorides (BACs) was investigated (Tezel et al. 2012b). Biotransformation of C_{14}BDMA-Cl commenced with cleavage of the C_{alkyl}–N bond and formation of benzyldimethylamine (BDMA). Tezel showed that; in contrast to the findings of a previous study; while BDMA was being degraded, no other benzyl-containing amines (i.e., BMA and BA) were detected in the present study. This observation suggests that

either BMA and BA are not the products of BDMA transformation or they are degraded faster than BDMA and thus they were not detected during the incubation. Batch kinetics of BDMA, BMA, and BA biotransformation in the BAC degrading community were evaluated by using BDMA, BMA, and BA as the carbon and energy source in three separate assays. The BDMA biotransformation rate was 4 times higher than the biotransformation rate of C_{14}BDMA-Cl, which explains why BDMA was not detected during the course of C_{14}BDMA-Cl degradation. On the other hand, the rate of BDMA biotransformation was twice as high as that of BMA. The results of the simulations suggest that at least BMA should have been detected at a concentration equal to about half the initial BDMA concentration. On the basis of the foregoing analysis, sequential demethylation of BDMA to BA was not observed in the BAC enriched community. Thus, BDMA is thought to be transformed to dimethylamine and benzoic acid via debenzylation, and dimethylamine is then mineralized resulting in the formation of ammonium and carbon dioxide (Figure 2.5.).

None of the isolates described above, except *Pseudomonas* sp. BIOMIG1, was able to grow on the non-alkyl containing amines, such as trimethyl amine and dimethyl amine, after dealkylation. Activation of the C-H bond in the QAC alkyl group was thought to commence with NADH-dependent hydroxylation by a monooxygenase in the presence of oxygen (Tezel et al., 2012a). Recently, two enzymes responsible for QAC dealkylation have been identified. Tetradecyl trimethyl ammonium bromide monooxygenase (TTABMO) identified in *Pseudomonas putida* ATCC 12633 is a typical flavoprotein that utilizes NADPH and FAD as cofactor (Liffourrena and Lucchesi, 2014). On the other hand, the enzyme responsible for dealkylating BACs by *Pseudomonas nitroreducens* was identified as a FAD-using amine oxidase (AOx-BAC) (Oh et al., 2014). AOx-BAC is phylogeneticaly more closely related to *Pseudomonas putida* amine oxidase and *Pseudomonas* sp. pseudooxynicotine amine oxidase than TTABMO, which suggests that there may be multiple enzymes responsible for the initial steps in QAC degradation.

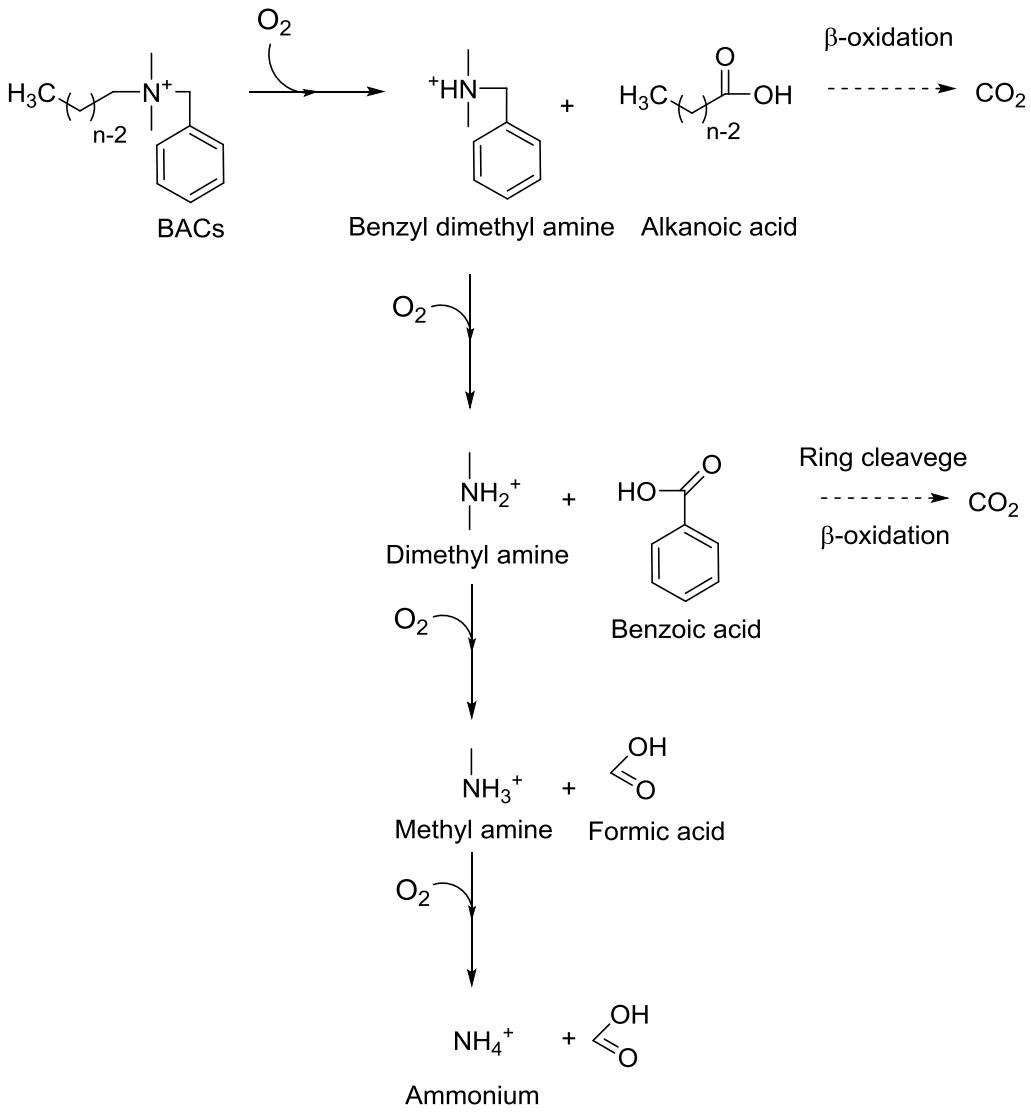


Figure 2.5. Proposed BAC biotransformation pathway by enrichment *Pseudomonas* spp. community (n: carbon number in the alkyl chain).

QAC degraders play an important role in mitigating QAC contamination in the environment. However, proliferation of QAC degraders and their transfer in indoor environments are not desired because it would decrease their antimicrobial efficacy, resulting in public health problems. Efforts should be devoted to develop technologies based on immobilized QAC degraders or their enzymes for the treatment of QAC bearing wastewater. To this end, QAC degraders have to be physiologically and metabolically well characterized and factors affecting QAC biodegradation identified. In case QAC detoxifying enzymes are used, strategies to enhance enzyme functionality by optimizing

cofactor requirements using directed enzyme evolution approaches will need to be evaluated.

2.7. Effect of Biophysicochemical Factors on Biodegradation

Biotransformation is a biochemical process of converting an (in)organic compound to another with a different structure. As all biochemical process, biotransformation is affected by many biotic and abiotic factors since enzymes are involved.

The most significant abiotic factors affecting microbial biotransformation are temperature, pH, salinity and toxins. Each microbial strain has its own minimum and maximum tolerance levels in the sense on abiotic factors. The conditions favoring the maximum growth rate are defined as “optimum conditions”. Temperature of the environment plays an important role. In general, biodegradation rate decreases at low temperatures. However, biodegradation is also adversely affected at high temperatures mainly due to denaturation of the enzymes essential for biotransformation and growth. pH around neutral is optimum for many biotransformation processes; acidic and basic pH inhibit biotransformation by disturbing ionic properties of enzymes especially involed in electron transfer (Alexander, 1999).

Heterotrophic bacteria are in need of nutrients, trace elements and an electron acceptor for biodegradation. While aerobes use oxygen as an electron acceptor, nitrate, sulfate, carbon dioxide, ferric ion or organic compounds might serve as electron acceptor for some specific bacteria. Macro and micronutrients essential for bacteria may include nitrogen, phosphorus, sulfur, amino acids, B vitamins, fat-soluble vitamins or other organic molecules which are mainly used as co factors (Alexander, 1999).

Calcium, iron and magnesium abundance in environment alters the availability of phosphorus because they precipitate phosphorus and since pH affects abundance of these cations, pH indirectly affects phosphorus abundance in the environment. Also addition of organic materials or individual chemicals can stimulate biodegradation. Electron acceptor

supply is an important factor, and mostly in many environments it is not sufficient, most common electron acceptor for aerobic bacteria used is oxygen (Alexander, 1999).

Presence of readily biodegradable substrates may affect the biodegradation of chemicals which are difficult to degrade. In the case of multiple substrates both is possible; either one substrate enhances the rate of biodegradation of other substrate or it may slow down the process. Additional carbon source may enhance growth of biomass thus promote biodegradation or if the target substrate is degraded cometabolically then it would facilitate its biodegradation. Sequential biodegradation of substrates is also observed, frequently results in diauxie, second substrate is inhibited during the utilization of first substrate and there is a lag phase in between utilization of two substrates (Alexander, 1999).

Biodegradation of some chemicals may require synergism of microorganisms. Synergism is essential for chemicals, biodegradation of which occurs in multiple steps and at least two of these steps can be achieved by two different microbial species. Generally, by-product of one step causes feedback inhibition which ceases the biotransformation of the parent chemical unless one of the microorganisms is not present in the environment. In synergetic relation, one microorganism can supply growth factors to the other microorganism and/or one species can grow on the intermediate of other species so that complete degradation of the target chemical can be achieved (Alexander, 1999).

Many physical and chemical factors may affect the kinetics, extent and pathway of biotransformation of QACs in the environment, but limited information was present on few of them such as QAC structure, the presence of readily biodegradable substrates and anionic organic compounds, and adsorption. Under aerobic conditions, the biodegradability of QACs generally decreases with the number of alkyl groups as R_4N^+< R_3MeN^+< $R_2Me_2N^+$< RMe_3N^+< Me_4N^+. Moreover, substitution of a methyl with a benzyl group can decrease biodegradability further (Ying, 2006). A comparison of the degradation rates of benzalkonium chlorides and monoalkonium bromides under aerobic conditions was undertaken. The rate of degradation of C_{12}BDMA-Cl, C_{14}BDMA-Cl and C_{16}BDMA-Cl, and C_{12}TMA-Br, C_{14}TMA-Br and C_{16}TMA-Br was inversely related to the length of the alkyl group (C_n) and the presence of a benzyl group decreased the rate as well. Indeed,

C_{16}BDMA-Cl was the most recalcitrant of the tested compounds, with an extent of only 30% degradation after 10 days. Likewise, it was reported that the aerobic degradation of QACs was dependent on the length of the alkyl group; however, the number of alkyl groups had a more pronounced effect on biodegradability. For example, dialkonium QACs were degraded five times slower than monoalkonium QACs (van Ginkel and Kolvenbach 1991).

Zhang et al. (2011) investigated the inhibitory effect and biodegradation of BAC in a microbial community derived from activated sludge. They showed that the presence of glucose, a readily biodegradable substrate, delayed the utilization of BAC. Degradation of BAC could only begin after a major fraction of glucose was depleted. At high concentrations, BAC resulted in the inhibition of glucose utilization, which in turn resulted in an extended delay in BAC biodegradation. As a result, QAC degradation did not begin as long as a readily biodegradable substrate was available. In a similar study investigating the factors affecting the biotransformation of a monoalkonium chloride, C_{12}TMA-Br, by *Pseudomonas fluorescens* F7 revealed that glucose halted the biodegradation of the QAC, whereas the presence of anionic organic compounds, such as fatty acids and sodium dodecyl sulfate, enhanced its biodegradation (Nishiyama and Nishihara, 2002).

QACs rapidly and strongly sorb onto a wide variety of materials of environmental relevance such as biomass, sediment, clay, and minerals. Zhang et al. (2011), showed that biomass-associated BAC was degraded twenty times slower than the BAC in the liquid-phase (bioavailable fraction). Sorption to sediment, clay and inorganic minerals was observed to retard and/or even prevent the biotransformation of QACs (Boethling, 1984). On the other hand, how adsoption to different organic/inorganic media affect the biotransformation pathway has not been elucidated.

In addition, oxygen concentration is another factor that affects the extent of biotransformation of QACs. Under anaerobic conditions, there is no evidence of mineralization of QACs that contain alkyl or benzyl groups (Battersby and Wilson 1989; Federle and Schwab 1992; Garcia et al. 1999 and 2000), most likely because of the highly

reduced nature of these substituent groups. On the other hand, oxygen threshold concentration for the biotransformation of QACs is not known.

3. HYPOTHESES AND OBJECTIVES

The overall objective of this research is to identify biological, chemical and physical factors and systematically investigate their role on the biotransformation of BACs by *Pseudomonas* spp. which is the key microorganism in the biotransformation of BACs in natural and engineered systems.

The following hypotheses were tested during the course of the research:

Hypothesis 1: BAC degrading microorganisms may be present in activated sludge

Approach: An activated sludge sample was taken from a municipal wastewater treatment plant. An enrichment community feeding with BACs was generated. BAC degrading species were isolated from the BAC enrichment activated sludge microbial community and identified based on 16S rDNA sequence.

Objective: Isolation of BAC degraders in an activated sludge microbial community.

Hypothesis 2: BAC biotransformation rate may follow Michaelis-Menten kinetics thus it is affected by BAC concentration

Approach: BAC biotransformation by a *Pseudomonas* spp. isolate was followed in culture flasks spiked with different BAC concentrations during a course of incubation.

Objective: Eluciting BAC biotransformation rate at a range of BAC concentration.

Hypothesis 3: Different BAC homologues may have different biotransformation rate and extent.

Approach: Biotransformation kinetics of three BAC structures i.e. dodecyl benzyl dimethyl ammonium (C_{12}BDMA-Cl), tetradecyl benzyl dimethyl ammonium (C_{14}BDMA-Cl) and hexadecyl benzyl dimethylammonium (C_{16}BDMA-Cl) chlorides by a BAC degrading *Pseudomonas* spp. isolate were determined.

Objective: Clarifying molecular structure effect on biotransformation kinetics of BACs.

Hypothesis 4: BAC degrader abundance may affect BAC biotransformation kinetics.

Approach: BAC biotransformation was followed at different *Pseudomonas* spp. isolate concentrations.

Objective: Identifying the critical BAC degrader population size for efficient BAC biotransformation

Hypothesis 5: Temperature may affect BAC biotransformation rate.

Approach: BAC biotransformation by the *Pseudomonas* spp. isolate was followed at different incubation temperatures

Objective: Determining optimum temperature for BAC biotransformaiton.

4. MATERIALS AND METHODS

4.1. Chemicals

BAC mixture used in this study is obtained from Lonza Inc. (Switzerland). Benzalkonium chloride types and percentages in this mixture are as well; (Abbreviation, molecular formula and weight): 40% (w/w) dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl, $C_{21}H_{38}NCl$, 340 g/mole), 50% (w/w) tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl, $C_{23}H_{42}NCl$, 368 g/mole), 10% (w/w) hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, 396.1 g/mole).

Every individual BAC mentioned above was obtained in high purity from TCI Chemicals (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Mineral salts and organic solvents used in experiments and instrumental analysis are purchased from Merck and Sigma Aldrich Chemicals Company.

4.2. Development of BAC Degrading Activated Sludge Microbial Community

An activated sludge sample was provided from biological the treatment unit of Paşaköy Municipal Wastewater Treatment Plant, İstanbul. A 20 mL of the sample was transferred aseptically into a 500-mL glass bottle with a screw-cap containing 180 mL of mineral salt medium (modified M9 medium) composed of 7.4 g/L K_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.00 g/L NH_4Cl, 0.25 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2$ and 1 mL/L trace metal solution (0.5 g/L $ZnCl_2$, 0.30 g/L $MnCl_2.4H_2O$, 3.0 g/L H_3BO_3, 2.0 g/L $CoCl_2.6H_2O$, 0.10 g/L $CuCl_2.2H_2O$, 0.20 g/L $NiSO_4.6H_2O$ and 0.30 g/L $Na_2MoO_4.2H_2O$). BAC solution composed of 40% (% w/w) C_{12}BDMA-Cl, 50% (% w/w) C_{14}BDMA-Cl and 10% (% w/w) C_{16}BDMA-Cl amended aseptically into this 200 mL sterile media as carbon and energy source, 50 mg/L (ca 140 μM) total BAC concentration provided in the bottle. BAC concentration in the bottle monitored on a daily basis using HPLC method described below. As soon as all BAC was consumed in the bottle, 20 mL sample from this bottle was

transferred aseptically into 180 mL sterile M9 media, total BAC concentration in the bottle set to 50 mg/L by spiking 1 mL from 10,000 mg/L BAC solution. This serial dilution procedure was repeated for 10 times to obtain a specialized BAC-degrading activated sludge microbial community. After about 50 days, we started to operate the BAC-degrading community in fed-batch mode by replacing 100 mL mixed liquor with fresh M9 media and amending 50 mg/L BAC three times a week. Resulting hydraulic retention time applied to the community is 4.7 days.

4.3. Isolation and Preliminary Identification of BAC-degraders in the Enrichment Community

A 100 μL of appropriately diluted sample from the BAC-degrading microbial community was spread on a CHROM®Agar Orientation plate. After 48 hrs incubation at room temperature, about twenty colonies grown on the agar plate picked and streaked on Luria-Bertani (LB) plates. When adequate growth was observed at room temperature on LB plates, a single colony of each isolate was picked and purified by streaking on LB plate. After purification was complete, one of the colonies was transferred from each twenty agar into 2 mL sterile M9 medium which contains 50 mg/L BAC and incubated at room temperature and BAC concentration in each tube was measured at 5^{th} and 11^{th} days. The isolates that consumed BAC were assigned as BAC degraders. Among BAC degrading isolates, ones that grow on CHROMAgar® *Pseudomonas* as “blue” flocks were assigned as *Pseudomonas* spp. and used in the following experiments.

4.4. Susceptibility Testing for *Pseudomonas* spp. Isolates

The susceptibility of each isolate to BAC was determined using macro-dilution assay as described by Clinical and Laboratory Standards Institute (CLSI 2006). Isolates were grown overnight in Mueller-Hinton broth then diluted in Mueller-Hinton broth to a turbidity comparable to that of a 0.5 McFarland turbidity standard (c.a. 0.5×10^8 CFU/mL). This suspension was further diluted 1:100 with deionized water. A 1 mL of the diluted culture sample was transferred to culture tubes containing 1 mL broth and a range

of BAC concentrations from 2 to 1000 mg/L (BAC is diluted by factor 2) (Figure 4.1.). The tubes incubated at room temperature (22 ^{o}C) for 24 hours and the growth measured with a UV/Vis spectrometer at 600 nm wavelength. Tubes containing nutrient broth having the same BAC concentrations but without culture were used as blanks. The susceptibility of each isolate to BAC was reported in terms of inhibitory concentration 50% (IC50), 90% (IC90) and 99% (IC99) which was assumed as minimum inhibitory concentration (MIC).

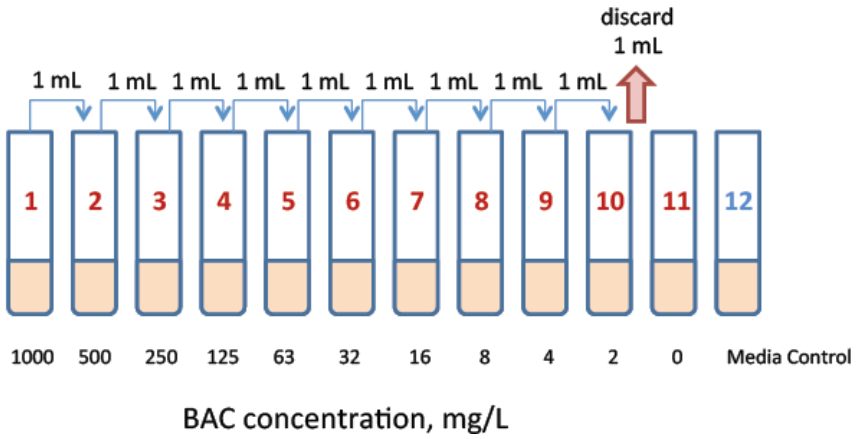


Figure 4.1. Preparation of susceptibility testing culture tubes using serial dilution.

4.5. Identification of BAC Degrader Based on 16S rDNA

A BAC-degrading *Pseudomonas* spp. having a moderate MIC was selected as a representative BAC degrader in an activated sludge community and used through the rest of the experiments. Prior to DNA extraction, 200 µL of isolate culture from the freeze stocks was transferred to 5 mL LB broth medium, and grown overnight to get sufficient cell yield. Genomic DNA of harvested cells was extracted using BioBasic BS 434 DNA extraction kit following the manufacturer's instructions. Extracted genomic DNA was quantified using Implen® P360 nanophotometer. Its quality was also checked visually on 0.7% Agarose gel after electrophoresis. 16S rDNA of the isolate was amplified by PCR using TaKaRa Premix TaqTM Kit (TaKaRa Bio, Shiga, Japan) with the following primers: 27F forward primer (27F: 5'-AGAGTTTGATCMTGGCTCAG-3') (0.4 µM), and 1492R reverse primer (1492R: 5'-TACGGYTACCTTGTTACGACTT-3') (0.4 µM). PCR conditions included 35 cycles at 94 ^{o}C (30 sec), 55 ^{o}C (30 sec) and 72 ^{o}C (1min), with a

final extension at 72 ^{o}C for 7 min. Amplified 16S rDNA was electroporated on 1% agarose gel (SeaKem LE Agarose, Lonza Inc., Basel, Switzerland), stained with Green DNA Dye (Bio Basic Inc., Onterio, Canada), UV-illumunated (GelDOC2000, BioRad, CA) and purified using EZ-10 PCR Purificaion Kit (Bio Basic Inc., Onterio, Canada). Purified 16S rDNA sample of the isolate was sequenced by MacroGen Inc. Europe (Amsterdam, Netherlands). Forward and reverse sequences were trimmed and assembled using Geneious Software (Biomatters Ltd., Auckland, New Zealand) to yield about 1350 bp length and above 95% quality 16S rDNA sequence. The sequence was then queried against the NCBI database (NCBI, 2012) using MEGABLAST algorithm and closest neighbor sequence to quired sequence was determined. Phylogenetic relationship between our isolate and other *Pseudomonas* species and BAC degraders in the literature was performed using Geneious Software following appropriate alignment and tree building algorithms.

4.6. LB Growth Assay

Specific growth rate and optimum temperature for the isolate were determined as follows: 0.1 mL overnight grown culture sample was well agitated and transferred into 99.9 mL sterile LB broth in Erlenmeyer flasks. The flasks were incubated at 5, 10, 15, 22, 30, 35, 40, 45 and 50 ^{o}C. At appropriate time intervals, 1 mL sample was taken and optical density was measured with a UV/Vis spectrometer at 600 nm wavelength. 99.9 mL sterile LB broth without bacteria was used as control. Growth rate and doubling time at each temperature was are calculated using the following equations (Eq. 1 and 2);

$$\frac{dOD}{dt} = \mu OD \qquad (1)$$

$$\mu = \frac{0.693}{t_d} \qquad (2)$$

4.7. Kinetic Assays

A set of biotransformation assays were performed at different biophysicochemical conditions to determine optimum conditions for BAC biotransformation by the *Pseudomonas* spp. isolate. All assays were performed using the following procedure: All assays were performed in 250-mL Erlenmeyer flasks. 98 mL of M9 medium was transferred to each flask along with a 1.5 mL of overnight grown isolate culture to give an initial turbidity equivalent to 1.0 McFarland (c.a. 1 x 10^8 CFU/mL). A 0.5 mL 10,000 mg/L BAC solution was added in to each flask to sustain 50 mg/L (~138 μM) initial BAC concentration in the flasks. The content in each flask was agitated on an orbital shaker at 130 rpm at room temperature till all BACs were utilized. As soon as BACs were consumed, the flasks were respiked with BAC(s) at desired concetration. A sample was taken in every 30 minutes and BACs in the samples were quantified using an HPLC method described below. Flasks prepared exactly the same way described above but containing no microorganisms were used as controls and monitored in the course of incubation. All flasks were prepared in triplicate.

BAC biotransformation was simulated using Michaelis-Menten growth model (Eq. 3) (Rittmann and McCarty, 2001).

$$\frac{d[BAC]}{dt} = \frac{k[BAC]X}{K_{BAC} + [BAC]} \quad (3)$$

$$\frac{dX}{dt} = Y\,\frac{k[BAC]X}{K_{BAC} + [BAC]} - \; k_d X \quad (4)$$

Where, *[BAC]* is BAC concentration (μM); *k* is maximum specific rate of BAC utilization (μmoles/cell·hr); K_{BAC} is half-saturation constant (μM); *X* is cell density (cells/L), *Y* is yield coefficient (cells/ μmoles BAC) and k_d is decay constant (1/hr). Given that the initial concentration of BACs used in all experiments is very low and the course of

experiments are less than the doubling time of the isolate used as well as the same cell density was used in all experiments, Eqns 3 and 4 were simplified to Eqn. 5.

$$\frac{d[BAC]}{dt} = \frac{k'[BAC]}{K_{BAC} + [BAC]} \quad 5$$

Where k' is cell specific rate of BAC utilization (μM/hr). Eqn 5 was fitted to experimental data to estimate k' and K_{BAC} values of BAC biotransformation by *Pseudomonas* isolate using Berkeley-Madonna software following error minimization employing Runge-Kutta 4 integration method.

All the k' values reported in this thesis can be converted to k (μmoles/cell·hr, μmoles/mgVSS·hr, gCOD/gVSS.d) by multiplying k with 10^{-10}, 0.286 and 7.4, respectively.

4.8. Measuring Oxygen Threshold Concentration

The threshold O_2 concentration for BAC transformation by the isolate was determined in a closed 120-mL serum bottle. The bottle was sterilized and filled with 100 mL M9 medium containing the isolate at 1 x 10^8 CFU/mL. The bottle was closed with a rubber stopper which was penetrated with a 10-mm needle used for sampling (Figure 4.2.). Bottle was incubated for 4 days at room temperature in order to confirm that all residual organics in the media had been depleted. At the fourth day, the bottle was spiked with 140 μM C_{14}BDMA-Cl. Both C_{14}BDMA-Cl and O_2 were monitored.



Figure 4.2. Closed bottle set-up used for determining the threshold O_2 concentration for BAC degradation.

4.9. BAC and By-product Analysis Methods

Benzalkonium chloride homologues as well as benzyl dimethyl amine (BDMA), benzyl methyl amine (BMA) and benzyl amine (BA), which are possible BAC transformation products, were measured using a high performance liquid chromatography (HPLC) unit equipped with a Phenomenex Luna SCX column (250 x 4.6 mm, 5µ) (Phenomenex, Inc., Torrance, CA) followed by a Polaris C_{18}A column (50 × 4.6 mm, 3.2 µ) (Agilent Technologies, Palo Alto, CA, USA). A Phenomenex SCX SecurityGuard cartridge (4 × 3.0 mm) is used as a precolumn. A 60:40 (v/v) mixture of acetonitrile and 50 mM phosphate buffer (pH 2.5) was used as the mobile phase at a flow rate of 1.0 ml/min and the columns were maintained at 35ºC. Detection was achieved with UV-Vis diode array detector at a wavelength of 210 nm.

An example HPLC chromatogram of 0.5 µg/mL analytes in DI, mobile phase and modified M9 culture medium is given in Figure 4.3.. The retention time of all analytes were almost the same in deionized water and mobile phase matrices, in fact analyte peaks comes earlier in M9 medium matrix. Since many of the samples that were analyzed using this method were in M9 medium, it was preferred to mix the actual samples with the equal

amount of mobile phase before analysis. This step during sample preparation not only eliminated retention time inconsistencies but also enhanced the peak quality. Culture samples in which BACs were measured also centrifuged at 10,000 xg before analysis. BAC recovery from the samples were above 98%.

The calibration curves of the analytes are linear (r2 >0.99) within 2 µg/mL and 100 µg/mL (~7 µM-300 µM for BACs and 20 µM-900 µM for amines). Expected quantitation detection limit for all analytes is 1 µM (Figure 4.4.).

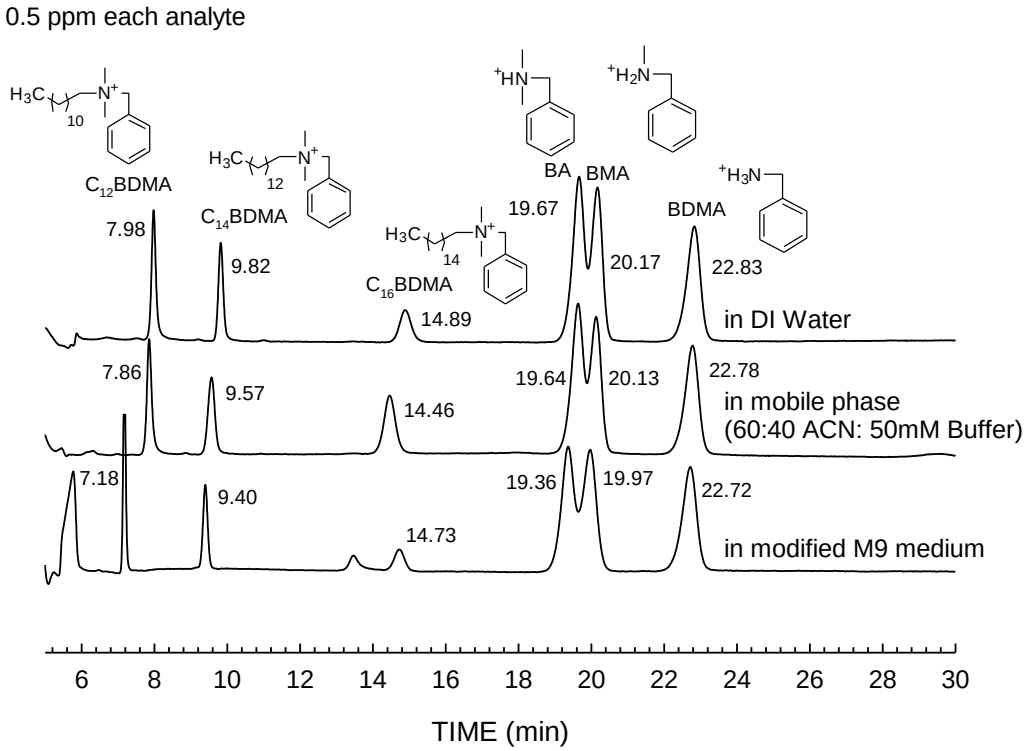


Figure 4.3. Sample HPLC chromatogram of dodecylbenzyl dimethyl ammonium chloride (C_{12}BDMA-Cl), tetradecylbenzyl dimethyl ammonium chloride (C_{14}BDMA-Cl), hexadecylbenzyl dimethyl ammonium chloride (C_{16}BDMA-Cl), benzyl amine (BA), benzyl methyl amine (BMA), benzyl dimethyl amine (BDMA) at 0.5 µg/L in DI water, mobile phase and modified M9 medium.

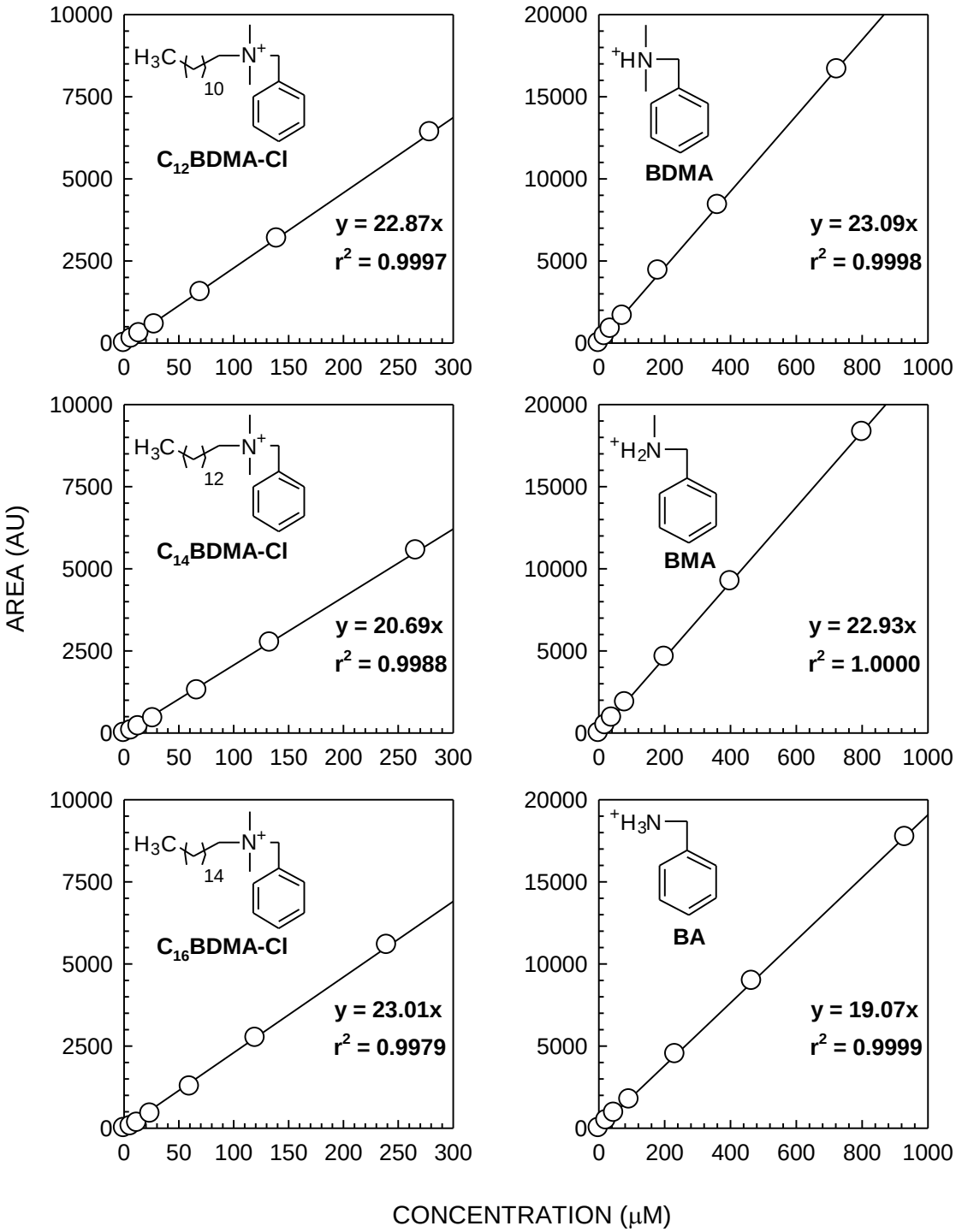


Figure 4.4. Molar calibration curves of C_{12}BDMA-Cl, C_{14}BDMA-Cl, C_{16}BDMA-Cl, BA, BMA, BDMA.

5. RESULTS AND DISCUSSION

5.1. Activity of BAC Degrading Community

BACs were consumed completely without any delay and accumulation intermediates during culture development and fed-batch operation through 1.5 years. pH was around 7.0 and did not change during this course (Figure 5.1). Given the fact that BAC concetration did not change in the control reactor set up without inoculumn from activated sludge, the dissapearance of BACs in the microbial community bottle was attributed to biodegradation.

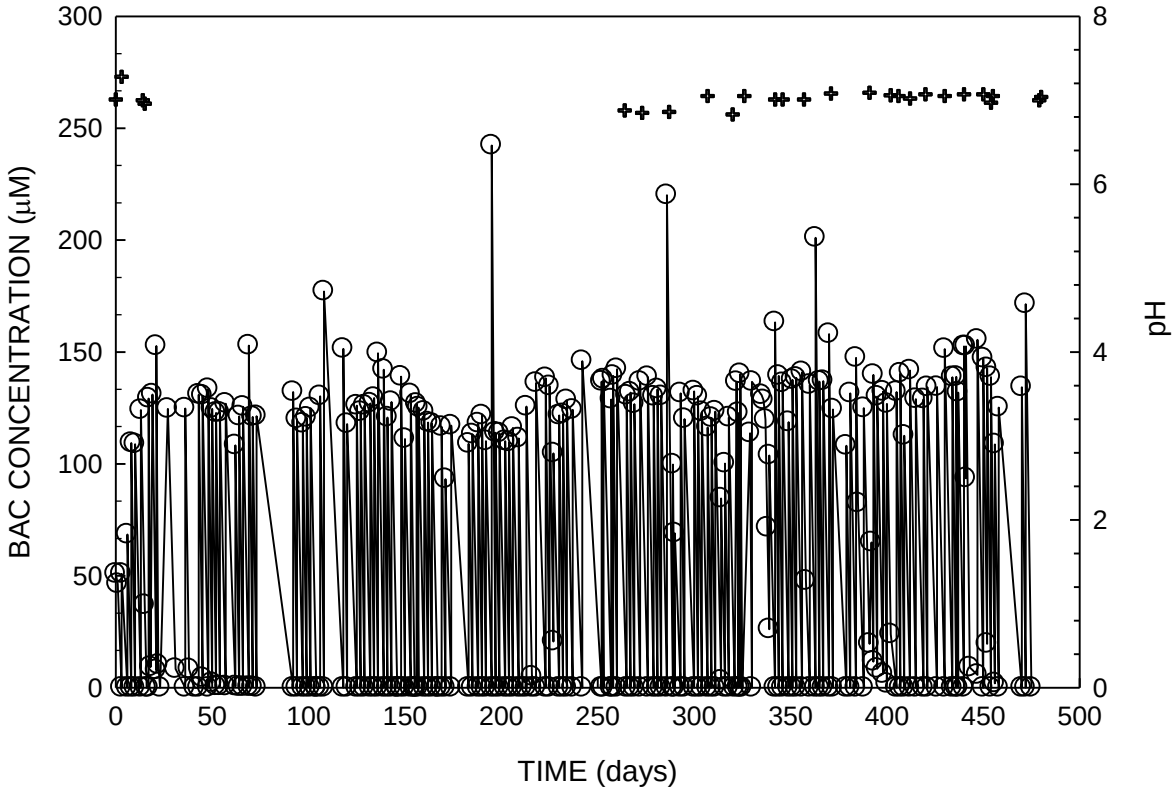


Figure 5.1.BAC utilization profile and pH in activated sludge microbial community during start-up and fed-batch operation period.

The biodiversity in the community at each generation was determined robustly by plating a sample on a chromogenic agar (CHROMAgar Orientation). This method assumes that colonies growing with different colors on this agar are bacteria belong to different genera, i.e. different phenotypes. Although this assumption is not always true, we used this method to follow the change in the activated sludge community structure during the development of BAC-degrading community. In addition, instead of attributing different colors to different phenotypes, we use the term "chromotype" to designate bacteria producing same color on the CHROMAgar Orientation (OR). In the initial activated sludge community, there were 8 chromotypes. As the community got exposed to BACs as the only carbon and energy source after multiple generations, the number of chromotypes decreased to 3 (Figure 5.2). We suggest that a specialized microbial community composed of 3 chromotypes was developed after 10 transfers/generations and each chromotype has a role on the biotransformation of BACs in this community

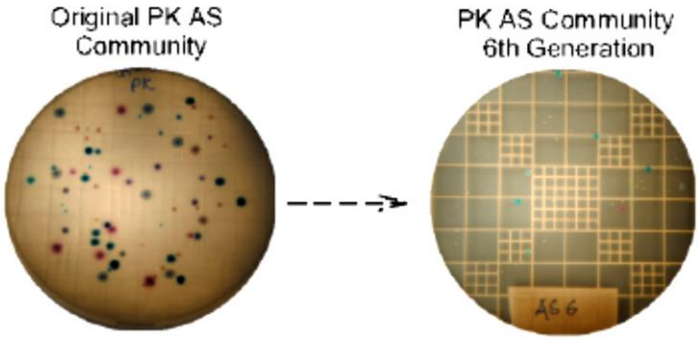


Figure 5.2. Biodiversity change in activated sludge community.

Yang (2007) developed an aerobic culture from a contaminated sediment sample, fed with dextrin, peptone and yeast extract semi-continously twice a week, from which four more communities developed (20 mg/L BAC and dextrin/peptone fed community, 50 mg/L BAC and dextrin/peptone fed community, 20 mg/L BAC fed community and 50 mg/L BAC fed community). They observed that biomass concentration susbstantially decresed in the communities fed only with BACs. In each only-BAC-fed community, BACs were almost completely consumed without an accumulation of any intermediate.

A metagenomic study was performed on the dextrin/peptone, BAC and dextrin/peptone and only BAC fed communities developed by Yang (2007) where microbial community adaptation to BAC was investigated (Oh et al., 2013). Comparing dextrin/peptone-fed community against BAC-fed community, it was revealed that there was a dramatic decrease in phylogenetic diversity in BAC-fed community. BAC-fed community was dominated by *Pseudomonas* species, particularly *P. nitroreducens.* Tezel et al. (2012b) revealed that the aforementioned BAC-fed also contained *Pseudomonas sp. WAI-21, Pseudomonas sp. Lin 2-2, and Pseudomonas sp. YG-1, P. putida* besides *P. nitroreducens* which altogether were responsible for BAC degradation in the community.

5.2. Characterization of BAC Degraders

A 100 μL of appropriately diluted sample from the BAC-degrading microbial was spread on a CHROMAgar Orientation plate. After 48 hrs incubation at room temperature, 3 green chromotypes, 1 purple chromotype and 16 beige chromotypes, which were predominant in the BAC-degrading community, were picked and streaked on LB plates. After 48 hrs of incubation at room temperature, a single colony of each isolate is transferred into a sterile glass test tube containing 2 mL M9 medium at 50 mg/L initial BAC concentration. BAC concentration in each tube was measured at 5th and 11th days of incubation. Isolates AS 12, 13, 17 and 20 degraded BACs completely (Table 5.1). BAC concentration in the tubes inoculated with the other isolates did not change significantly which implies that those isolates were not capable of BAC biotransformation.

BAC-degrading isolates AS 12, 13, 17 and 20 were patched on CHROMAgar *Pseudomonas*, they grew blue, which indicates that these isolates belong to *Pseudomonas* genus (Figure 5.3.). The exact identity of these isolates was confirmed using a16S rDNA based molecular biology method.

Table 5.1. BACs biotransformation extent of the isolates after 5 and 11 days of incubation.

Isolate	Biotransformation Extent (%)		Color on OR
	5 Days	11 Days	
AS 1	19	31	Green
AS 2	13	20	Beige
AS 3	12	20	Beige
AS 4	12	18	Beige
AS 5	16	20	Beige
AS 6	10	20	Beige
AS 7	17	18	Green
AS 8	10	15	Purple
AS 9	17	20	Beige
AS 10	16	39	Green
AS 11	10	23	Beige
AS 12	100	100	Beige
AS 13	100	100	Beige
AS 14	13	19	Beige
AS 15	12	18	Beige
AS 16	15	23	Beige
AS 17	100	100	Beige
AS 18	12	25	Beige
AS 19	13	52	Beige
AS 20	100	100	Beige

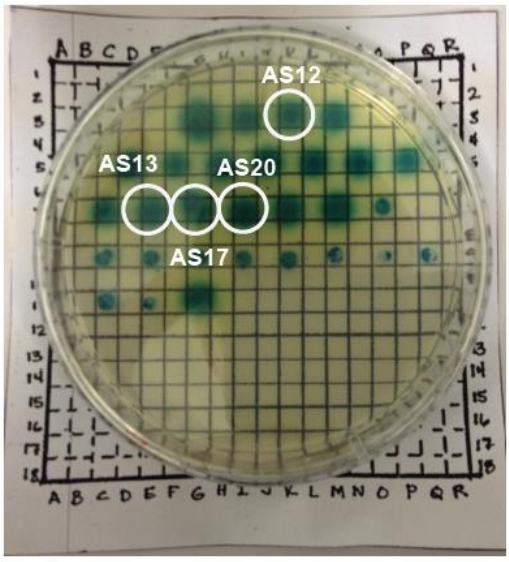


Figure 5.3. Isolates on CHROMAgar *Pseudomonas.*

The susceptibility of each isolate to BACs was tested using macro-dilution assay as described by Clinical and Laboratory Standards Institute (CLSI 2006). The growth of each isolate was inhibited in the tubes having BAC concentration at and above 32 mg/L (Figure 5.4.). The susceptibility of each isolate to BAC was reported in terms of inhibitory concentration 50% (IC_{50}), 90% (IC_{90}) and 99% (IC_{99}) which was assumed as minimum inhibitory concentration (MIC). The BAC MIC for the isolates ranged from 350 to 580 mg/L. AS 12 was the least and AS 13 was the most susceptible isolates tested (Table 5.2).

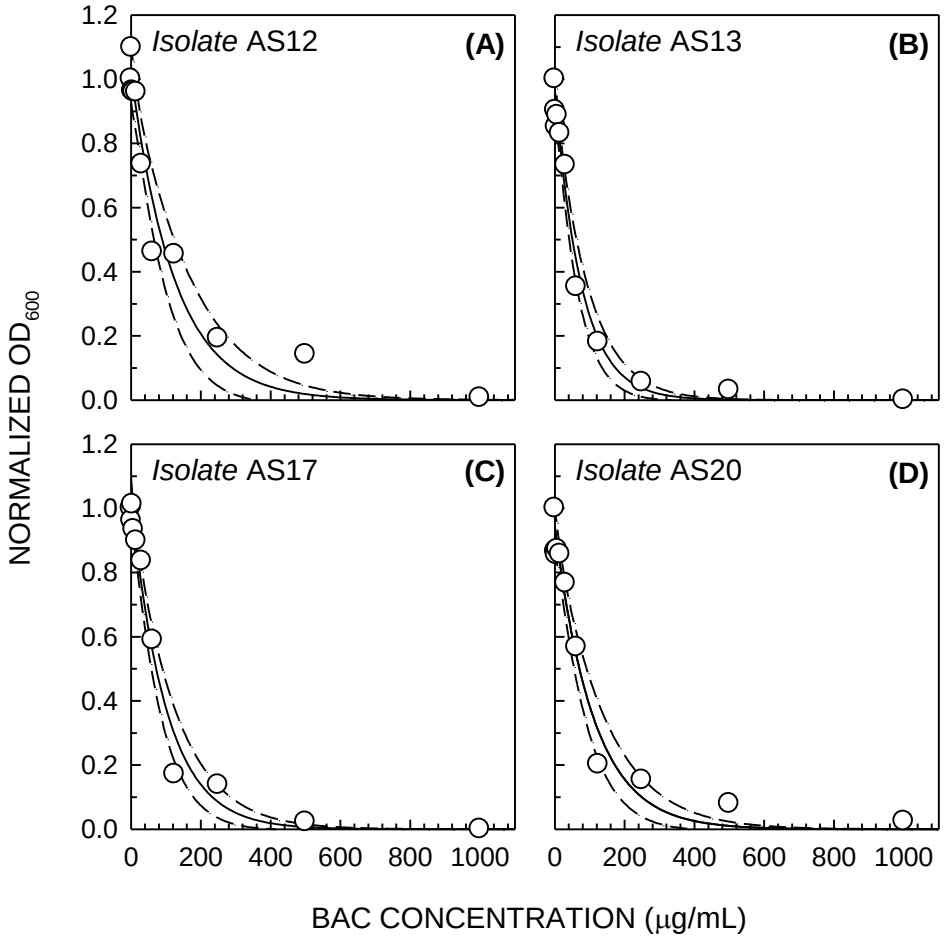


Figure 5.4. Normalized growth of isolate (A) AS 12, (B) AS 13, (C) AS 17 and (D) AS 20 at different BAC concentrations between 0 and 1000 mg/L (solid lines are the regression lines whereas the dashed lines represent 95% confidence intervals of the regressions).

McDonnell and Russell (1999) found the MIC of BACs for *Pseudomonas aeruginosa* as 700 mg/L whereas Cords (1983) tested MIC of BACs on *Pseudomonas aeruginosa* and *Escherichia coli*, found 200 and 300 mg/L respectively. BAC MIC for the aforementioned *Pseudomonas* dominated only-BAC fed community was 460 mg/L (Tandukar et al., 2012). Thus, MIC values reported in this study were comparable with the literature values. On the contrary, Sutterlin et al. (2008a) estimated EC_{50} of BAC for *Pseudomonas putida* as 6 mg/L which was almost one order of magnitude lower than IC_{50} values reported in our study.

Table 5.2. BAC susceptibility values for the BAC-degrading isolates.

Bacteria	IC_{50} µg/mL	IC_{90} µg/mL	IC_{99} µg/mL	R^2
Isolate AS 12	90 [65-125][a]	288 [193-382]	576 [314-718]	0.96
Isolate AS 13	52 [43-65]	172 [133-210]	348 [245-408]	0.99
Isolate AS 17	73 [60-90]	232 [176-284]	464 [318-546]	0.98
Isolate AS 20	69 [56-90]	249 [180-314]	502 [322-606]	0.98

[a]The values in the brackets are 95 % confidence intervals of the IC values

5.3. Selection of Working Strain and Identification Based on 16S rDNA

AS20, a BAC degrading isolate with a moderate BAC tolerance was selected as the working strain and used in all experiments. At the first step, 16S rDNA sequence of AS20 blasted against the sequences in the NCBI GenBank database. The closest sequence to AS20 16S rDNA belonged to *Pseudomonas sp.* CMR12a, a species which was isolated from the root of red cocoyam plant (99% similarity) (Perneel et al., 2007). AS20 16S rDNA sequence was then aligned with the sequences of other BAC/QAC degraders (Tezel et al., 2012b, Oh et al., 2014), *P. putida* ATCC 1233 and *P sp* 7-6, and other select well-characterized *Pseudomonas* species from each *Pseudomonas* group using MUltiple Sequence Comparison by Log- Expectation (MUSCLE). A similarity matrix was created to

compare the sequences. A neighbor joining phylogenetic tree was prepared from a distance matrix created by a maximum likelihood method (Figure 5.5.). Based on phylogenetic analysis, AS20 is significantly different than other BAC/QAC degraders and forms a distinct clade close to *P. putida* group. Further phylogenetic analysis performed based on 16S rDNA sequences using 68 well-characterized *Pseudomonas* species as well as strains within *Pseudomonas putida* group of bacteria suggests that AS20 is a new *Pseudomonas* species has not been characterized and grouped before. Therefore, AS20 is a novel BAC degrading *Pseudomonas* species. AS20 was called as BIOMIG1 here after.

5.4. Growth Kinetics of BIOMIG1

Growth of BIOMIG1 in LB was monitored at temperatures between 5 and 50 ^{o}C (Figure 5.6.A and F). Growth was not observed at 5 and above 35 ^{o}C. Growth was observed after 40 hrs at 10 ^{o}C (Figure 5.6.B). Specific growth rate and the corresponding doubling time at 10 ^{o}C were 0.18 1/hr and 3.85 hr, respectively (Table 5.3). At 15 ^{o}C, growth started after 15 hrs which was earlier than the growth at 10 ^{o}C (Figure 5.6.C). Moreover, doubling time of BIOMIG1 was 0.84 hr shorter at 15 ^{o}C than at 10 ^{o}C. BIOMIG1 grew in LB broth with a rate of 0.62 1/hr which corresponds to a doubling time of 1.1 hr (Figure 5.6.D). Doubling time of BIOMIG1 is comparable to that of *Pseudomonas aeruginosa* PA01, *putida* KT2440 and *syringae tomato* DC3000 which is equal to 0.5, 1.1 ve 1.47 hr (Bionumbers database). Fastest growth was observed at 30 ^{o}C. Lag phase was only 4 hours and exponential growth last for 6 hours. Growth was completed in 10 hours and growth rate was estimated as 1.07 1/hr, almost twice as fast as growth at 22 ^{o}C (Figure 5.6.E).

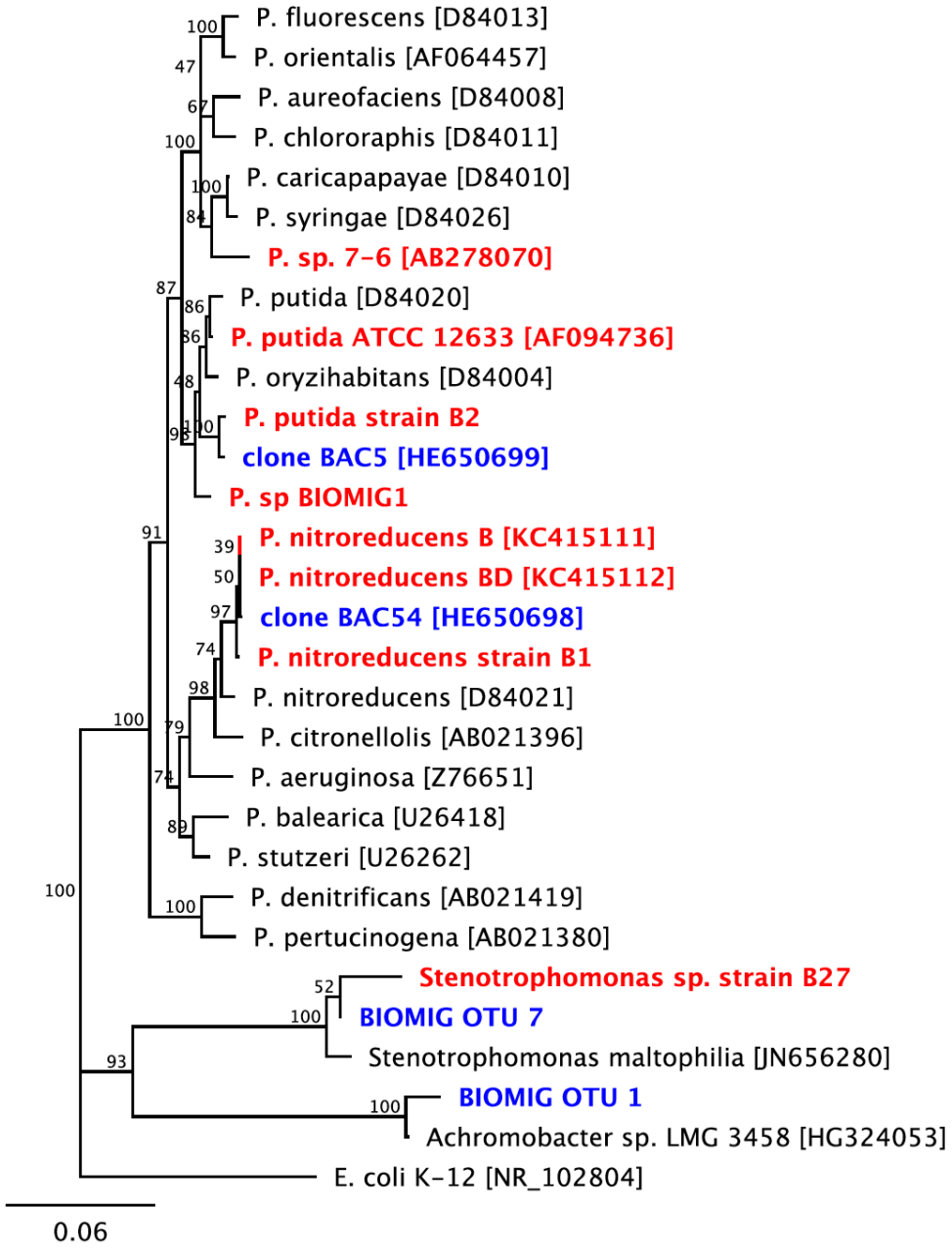


Figure 5.5. Phylogenetic tree of relationships of 16S rDNA sequence of BIOMIG1, determined by maximum likelihood followed by neighbor joining tree building method and Hasegawa-Kishino-Yano genetic distance model, relative to other BAC/QAC degraders and select *Pseudomonas* spp. Bootstrap values represents 100 replicates. The scale bar represents 0.06 substitution per nucleotide position. *E. coli* was used as the outgroup. (Branches in red are QAC-degrading isolates, whereas branches in blue are nucleotide sequences of species present in QAC-degrading communities identified in community clone libraries or by pyrosequencing).

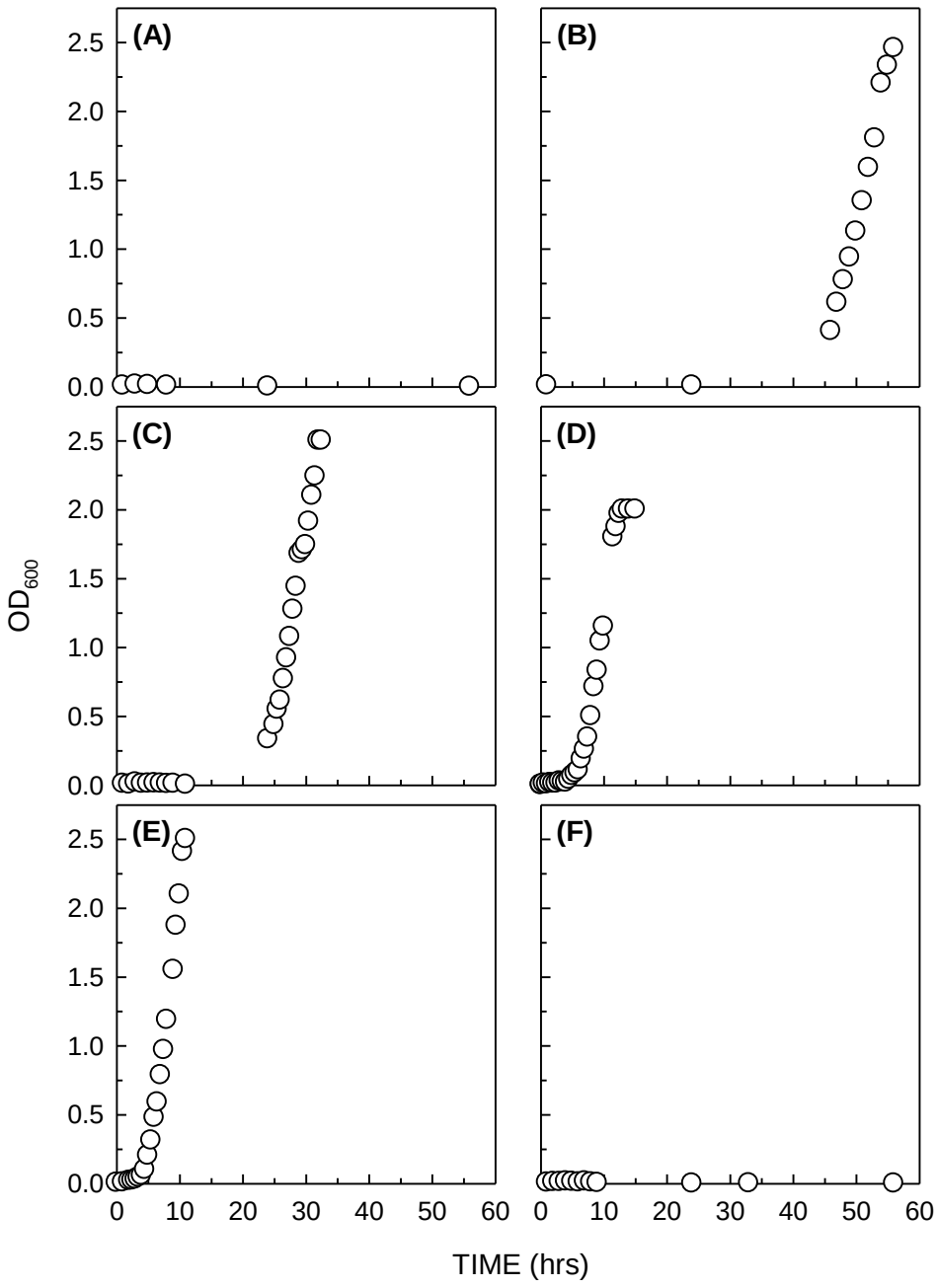


Figure 5.6. Growth profile of BIOMIG1in LB broth at (A) 5 ^{o}C, (B) 10 ^{o}C, (C) 15 ^{o}C, (D) 22 ^{o}C, (E) 30 ^{o}C and (F) 35 ^{o}C.

Table 5.3. Calculated specific growth rate and doubling time in LB broth at 10, 15, 22, 30 and 35 ^{o}C.

Parameter	**Temperature**					
	5 ^{o}C	10 ^{o}C	15 ^{o}C	22 ^{o}C	30 ^{o}C	35 ^{o}C
μ (1/hr)	0	0.18	0.23	0.62	1.07	0
t_d (hr)	0	3.85	3.01	1.11	0.64	0

Relationship between growth rate and temperature obeyed a modified Arrhenius model (Eqn. 6). As a result, optimum growth temperature of BIOMIG1 in LB broth was determined as 28 ^{o}C. Optimum growth temperature of *Pseudomonas* sp. CMR12a which is phlogenetically closest strain to BIOMIG1 was also reported as 28 ^{o}C (Perneel et al., 2007).

$$\mu = \frac{\beta T \, exp\left[\frac{-E_a}{RT}\right]}{1 + \exp\left[\frac{\Delta S}{R}\right] exp\left[\frac{-\Delta H}{RT}\right]} \qquad 6$$

Where β is biodegradation proportionality factor (μM/hr·K), T is absolute temperature (K), E_a is activation energy (kcal/mol), R is ideal gas constant (1.987 x 10^{-3} kcal/mol·K), ΔS is entropy change of enzyme deactivation (kcal/mol·K) and ΔH is enthalpy change of enzyme deactivation (kcal/mol).

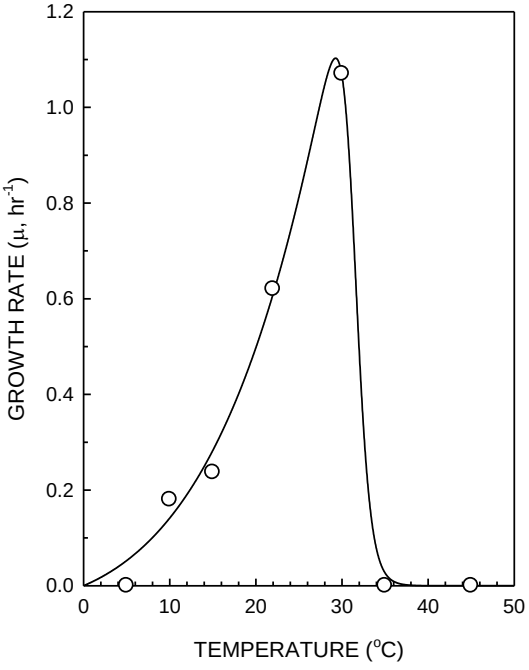


Figure 5.7. Relationship between temperature and growth rate.

Table 5.4. shows minimum, optimum and maximum growth temperatures of different bacterial species. Minimum growth temperature according to test results are above 5 and below 10 ^{o}C, but Arrhenius model predicts minimum temperature that growth is observed as 0 ^{o}C (Figure 5.7.). When compared to bacteria in Table 5.4., most of the species including *Pseudomonas*, grow even at 0 ^{o}C. Optiumum growth temperatures are mostly in good agreement with BIOMIG1 which 28 ^{o}C (Figure 5.7.) except *Aeromonas*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* and *Vibrio marinus* which are commensal pathogens.

Table 5.4. Minimum, optimum and maximum growth temperatures of various bacteria (Ratkowsky et al., 1983).

	T min (^{o}C)	**T opt (^{o}C)**	**T max (^{o}C)**
Acinetobacter sp. 4.41	-2	30	38
Aeromonas sp. 4.29	4	36	47
Flavobactetrium (Cytophaga) sp. 6.32	-3	28	37
Moraxella sp. 4.16	-1	30	41
Pseudomonas sp. 4.54	-1	31	37
Pseudomonas sp. 6.4	-6	26	31
Pseudomonas sp. 16L16	-6	29	37
Pseudomonas sp. 2.3	-7	27	37
Pseudomonas sp. 5.16	-4	29	40
Pseudomonas fluorescens	4	39	47
Pseudomonas aeruginosa	-1	39	47
Vibrio marinus ATCC 15382	4	24	30

5.5. BAC biotransformation by BIOMIG1

A BAC biotransformation kinetics assay delineating the effect of initial BAC concentration was performed following the same procedure described above by re-spiking the flasks at an initial total BAC concentration of 5 mg/L, 10 mg/L and 25 mg/L. Corresponding molar concentrations of C_{12}BDMA-Cl and C_{14}BDMA-Cl in the flasks were approximately equal to 6, 12 and 30 µM.

C_{12}BDMA-Cl was consumed within 5, 6 and 9 hours in the flasks amended at 6, 12 and 30 µM initial concentration, respectively (Fig. 5.8.A). On the other hand biotransformation of C_{14}BDMA-Cl was faster and it was utilized within 3, 5 and 8 hrs at the same initial concentrations, respectively (Fig. 5.8.B). BAC biotransformation kinetics was successfully simulated using Michaelis-Menten model (R^2 of fits >0.98). Estimated K_{BAC} values for C_{12} and C_{14}BDMA-Cl biotransformation were 0.02±0.01 µM and 1.13±0.33 µM, respectively. Likewise, Zhang et al. (2011) reported K_{BAC} for C_{14}BDMA-Cl as 1.7 µM for a microbial community generated from activated sludge which mainly fed with C_{14}BDMA-Cl. Estimated k' values for C_{12}BDMA-Cl at 6, 12 and 30 µM initial concentration were 1.34, 1.77 and 3.34 µM/hr, respectively. k' values for C_{14}BDMA-Cl were estimated as 2.58, 3.00 ve 4.86 µM/hr at 6, 12 and 30 µM initial concentration, respectively. Specific C_{14}BDMA-Cl utilization rate was reported as 6.9 µM/hr for an aerobic microbial community composed mainly of *Pseudomonas* at 130 µM. These results suggest that BIOMIG1 has higher affinity to C_{12}BDMA-Cl but degrades faster than C_{12}BDMA-Cl and degradation rate depends on BAC concentration.

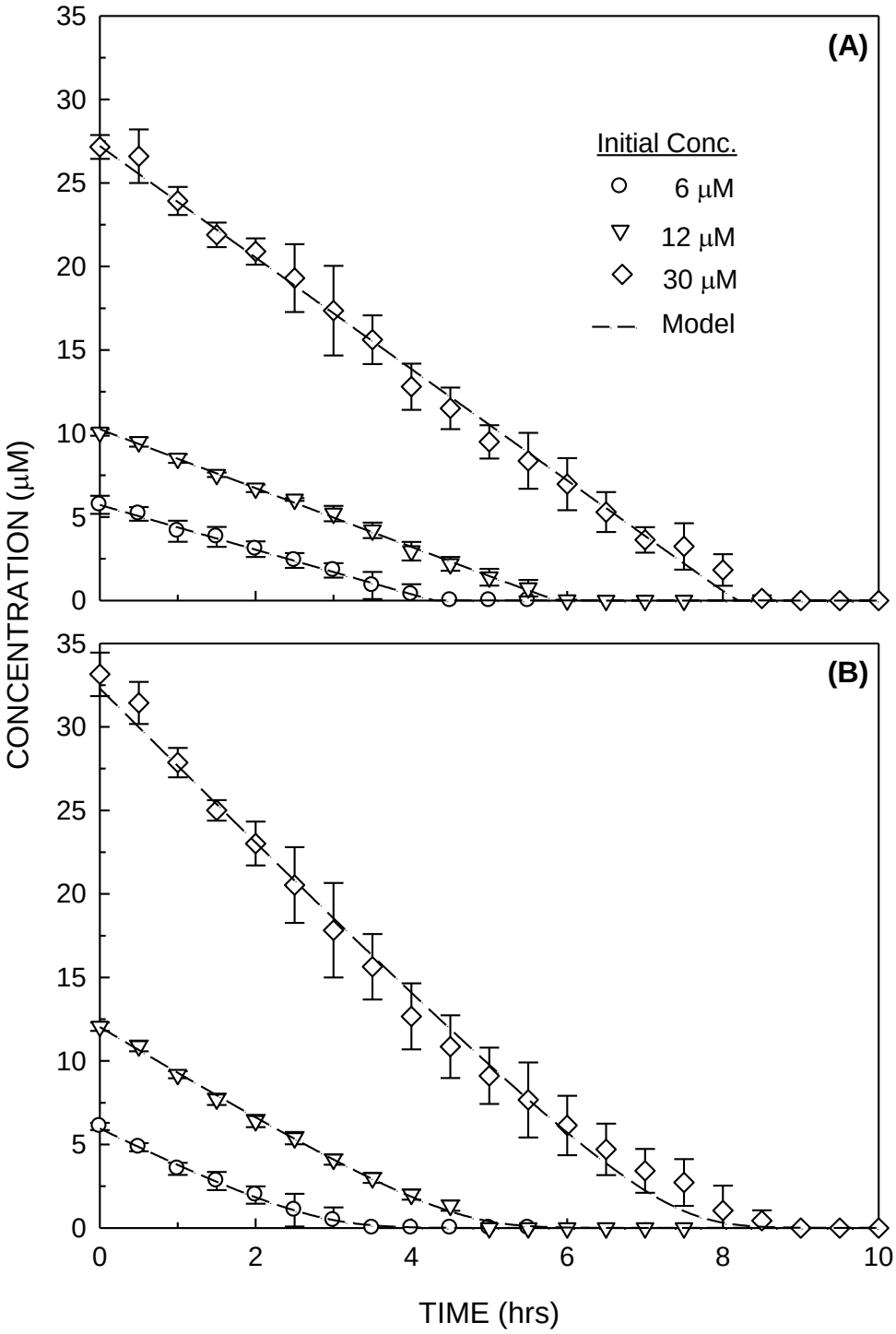


Figure 5.8. Profiles of (A) C_{12}BDMA-Cl and (B) C_{14}BDMA-Cl utilization by BIOMIG1 at 6, 12 and 30 μM concentration at 22 ^{o}C (error bars represent one standard deviation of the mean, n=3).

Table 5.5. Specific BAC growth rates at different BAC concentrations.

	C_{12}BDMA-Cl			**C_{14}BDMA-Cl**		
	6 µM	**12 µM**	**30 µM**	**6 µM**	**12 µM**	**30 µM**
k', µM/hr	1.34	1.77	3.34	2.58	3.00	4.86
k, µmoles/cell•hr	$1.34x10^{-10}$	$1.77\ x10^{-10}$	$3.34\ x10^{-10}$	$2.58x10^{-10}$	$3.00\ x10^{-10}$	$4.86\ x10^{-10}$
k, µmoles/mgVSS•hr	0.38	0.51	0.96	0.73	0.85	1.38
k, gCOD/gVSS•d	9.92	13.10	24.71	19.09	22.20	35.96

In addition, biotransformation of BACs at 1000 and 10,000 mg/L initial concentration was tested. BACs were completely degraded at 1000 mg/L within 20 days (Figure 5.9.). This result suggests that BIOMIG1 can degrade BACs at a concentration higher than its MIC. Although the BIOMIG1 degrades both BACs at a rate equal to 4.0 µM/hr at an initial total BAC concentration of 1000 mg/L, transient accumulation of BDMA was observed at that concentration which was not observed at low concentrations (Figure 5.9.). As proposed by others, dealkylation of BAC resulting in formation of BDMA is the first step in the BAC degradation (Tezel et al., 2012b). Formation of BDMA by BIOMIG1 at high BACs concentration suggested that BIOMIG1 also follows the same biotransformation pathway (Figure 2.4) *Pseudomonas nitroreducens* can convert BAC to BDMA and cannot degrade BDMA (Oh et al., 2014). On the contrary, a microbial community composed of multiple *Pseudomonas* species can utilize both BACs and BDMA. Therefore, BAC and BDMA biotransformation may be facilitated by two different enzymes. Depending on our current knowledge, BIOMIG1 is the first microorganism that can utilize both BAC and BDMA simultaneously. Accumulation of BDMA at high BAC concentration, on the other hand, was attributed to the inhibition of the enzyme responsible for BDMA biotransformation by BACs.

On the other hand, biotransformation of BACs at 10,000 mg/L initial concentration was not observed probably due to extreme cell lysis and denaturation of proteins at that high concentration (Figure 5.10.).

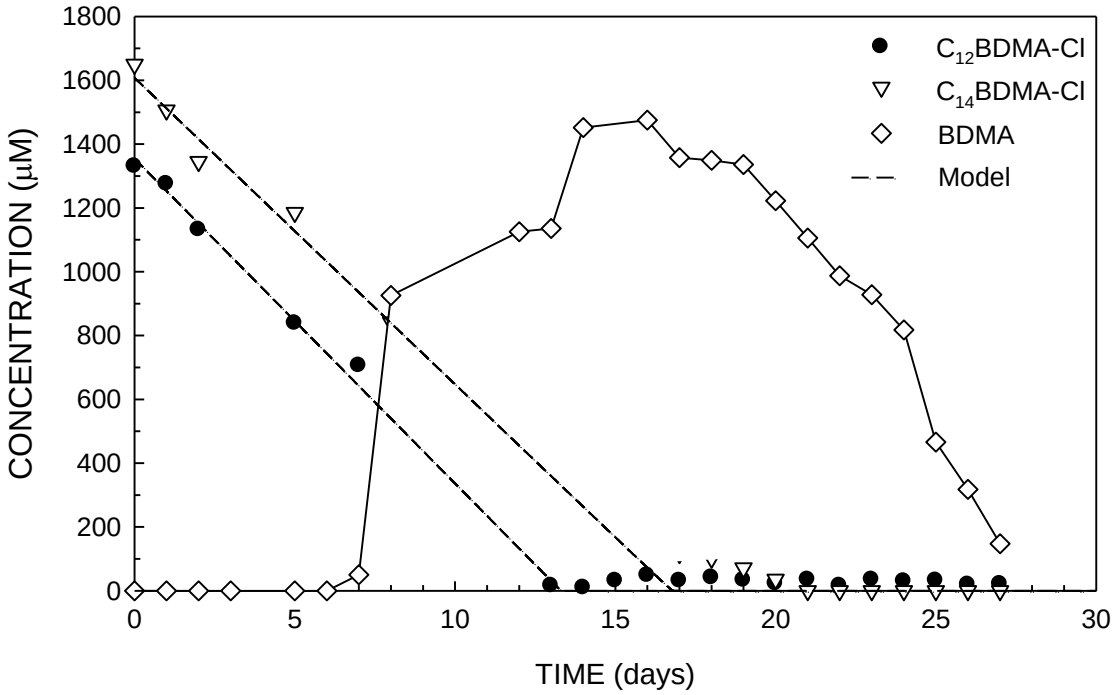


Figure 5.9. Profiles of C_{12}BDMA-Cl and C_{14}BDMA-Cl utilization and BDMA production and consumption by BIOMIG1 at 1000 mg/L initial total BAC concentration.

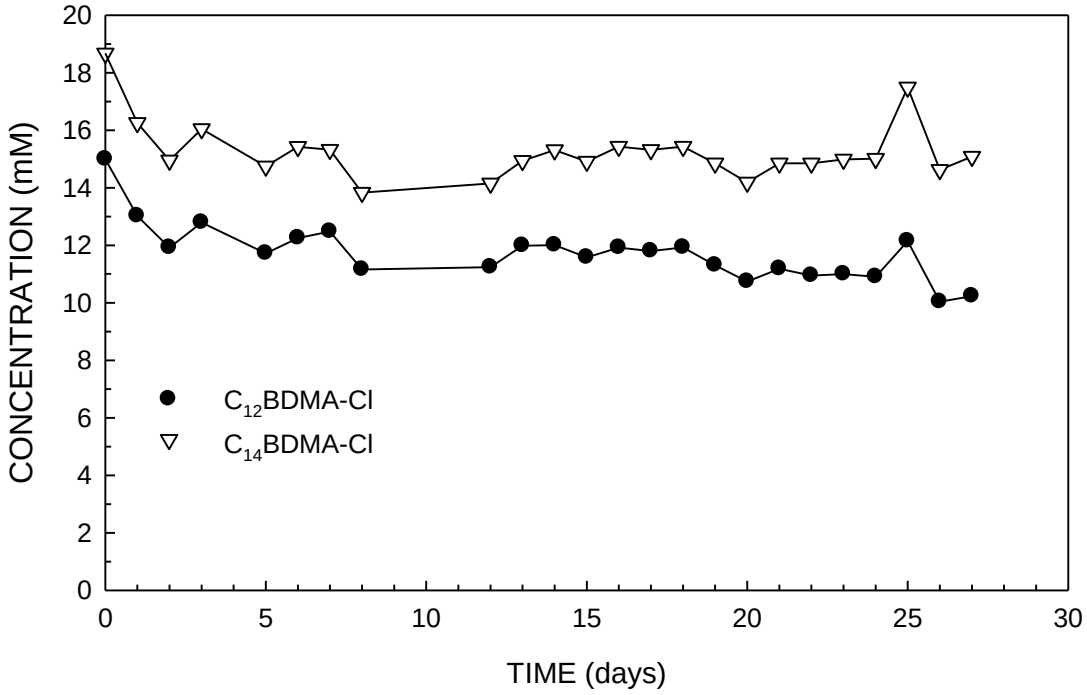


Figure 5.10. Profiles of C_{12}BDMA-Cl and C_{14}BDMA-Cl utilization by BIOMIG1 at 10,000 mg/L initial total BAC concentration.

5.6. Biotransformation of BAC Homologues

Biotransformation kinetics of individual BACs, C_{12}, C_{14} and C_{16}BDMA-Cl were determined following the same kinetic assay procedure described above. In this case each BAC was spiked individually into flasks at an initial concentration of 12 μM or 25 μM. C_{12} and C_{14}BDMA were completely utilized within 4 hrs by BIOMIG1 at 12 μM (Figure 5.11.A and B). On the contrary, 20% of the initially amended C_{16}BDMA-Cl was still present in the flasks after 10 hrs of incubation and expected to be depleted in 15 hrs (Figure 5.11.C). Estimated specific BAC utilization rates for C_{12}, C_{14} and C_{16}BDMA-Cl were 3.09, 3.17 ve 1.12 μM/hr. Although biotransformation rates of C_{12} and C_{14}BDMA-Cl were comparable when amended individually, biotransformation of C_{16}BDMA-Cl was three times slower than the others.

Biotransformation of C_{12} and C_{14}BDMA were completed in 6 hrs at 25 μM (Figure 5.12.A and B). On the other hand, utilization of C_{16}BDMA-Cl lasted 15 hrs (Figure 5.12.C). At 25 μM, specific utilization rate of C_{12} and C_{14}BDMA were 4.5 μM/hr whereas it was 2.0 μM/hr for C_{16}BDMA-Cl. At both concentrations, BAC with the longest alkyl chain length which is the host hydrophobic, C_{16}BDMA-Cl, was degraded the slowest. This suggests that adsorption on to cells may slow down its biotransformation. Zhang et al. (2011) showed that utilization rate of biomass-associated BACs were orders of magnitudes slower than free BACs. In another study, Garcia et al. (1999) reported that biodegradation rate of C_{16}TMA-Br was less than C_{14}TMA-Br followed by C_{12}TMA-Br. Those results agree well with current findings of this study.

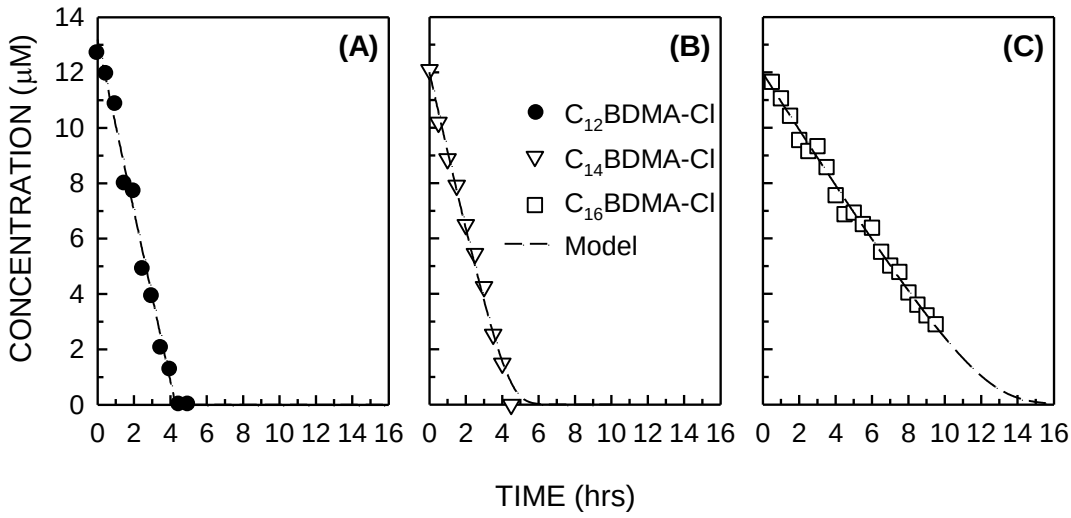


Figure 5.11. Profiles of (A) C_{12}BDMA-Cl, (B) C_{14}BDMA-Cl and (C) C_{16}BDMA-Cl utilization by BIOMIG1 at 12 μM initial concentration (error bars represent one standard deviation of the mean, n=3).

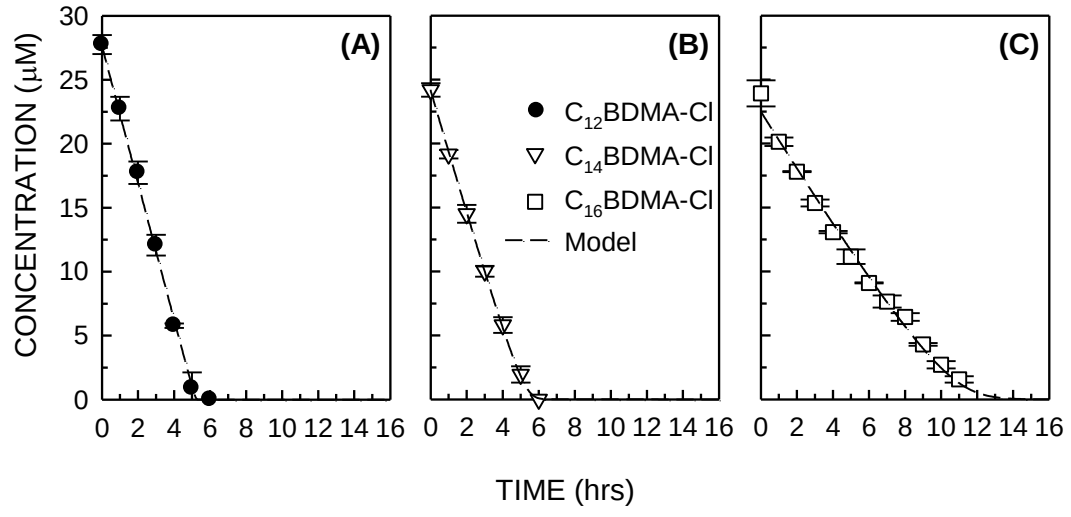


Figure 5.12. Profiles of (A) C_{12}BDMA-Cl, (B) C_{14}BDMA-Cl and (C) C_{16}BDMA-Cl utilization by BIOMIG1 at 25 μM initial concentration (error bars represent one standard deviation of the mean, n=3).

5.7. BAC biotransformation at Different Biomass Concentrations

Biotransformation of BACs by BIOMIG1 at different cell densities ranging from 10^8 to 10^5 CFU/mL was evaluated at 25 mg/L initial total BAC concentration. Biodegradation of C_{12}BDMA-Cl lasted 8 hrs at 10^8 CFU/mL (Figure 5.13.A). At every cell density below 10^8 CFU/mL, the biodegradation span increased gradualy from 15 hrs to 50 hrs. On the other hand BAC utilization rate decreased from 3.34 μM/hr to 1.00 μM/hr at 10^7 CFU/mL and stayed constant below this cell density. A similar trend was observed for C_{14}BDMA-Cl biodegradation at different cell densities (Figure 5.13.A). C_{14}BDMA-Cl was completely utilized in 8 hrs at 10^8 CFU/mL cell density. Biodegradation took longer times in the flask prepared at lower cell densities. For instance, complete removal of C_{14}BDMA-Cl from the flasks at 10^5 CFU/mL cell density achieved in 70 hrs. In addition, BAC utilization rate decreased from 4.86 μM/hr to 1.55 μM/hr at 10^7 CFU/mL and stayed constant below this cell density.

As a result, the major effect of lower cell densities on BAC biotransformation was the delay in the initiation of the biotransformation. At lower cell densities, not only BAC biotransformation delayed but also rate decreased. Optimum cell density for efficient BAC biotransformation is about 10^8 CFU/mL, below this concentration not only biotransformation experiences long lag periods but also rate of biotransformation decreases.

Yang (2007) reported that BAC degradation was possible even in low biomass concentration, in a mixed liquor developed from activated sludge which only fed with BAC as energy and carbon source. Ruiz Cruz (1979, 1981) made a research about biodegradation factors of QAC biodegradation in river water at an initial 5 mg/L QAC concentration. Effect of biomass was simulated with serial dilution of river water. Dilution of river water caused increase in the lag period and decrease in the biodegradation rate of DTMAC and DDMBAC. Paris et al. (1981) reported similar results between biomass and biodegradation rates of organic compounds.

Effect of cell density on biotransformation was simulated using Equation 5 which was modified by integrating a time dependet dilution factor, *D*. *D* in Equation 7 as a multiplier to Equation 5 and yielded Equation 8.

$$\frac{dD}{dt} = k_{enzyme} D \qquad 7$$

$$\frac{d[BAC]}{dt} = \frac{k'[BAC]}{K_{BAC} + [BAC]} D \qquad 8$$

Where *D* is dilution factor (unitless, ≤ 1) and k_{enzyme} is the enzyme synthesis rate (1/hr). Given the fact that BACs at the tested concentration do not promote growth, we assumed a cell may synthesize the BAC degrading enzymes in multiple copies to initiate BAC degradation. Therefore, instead of using a biomass growth equation, we used enzyme synthesis rate. Equation 8 successfully simulated the lag periods and following biodegradation events in the cultures at different cell densities. Average k_{enzyme} was estimated as 0.62±0.47 1/hr (n = 10) which is comparable to the specific growth rate (μ) of the BIOMIG1.

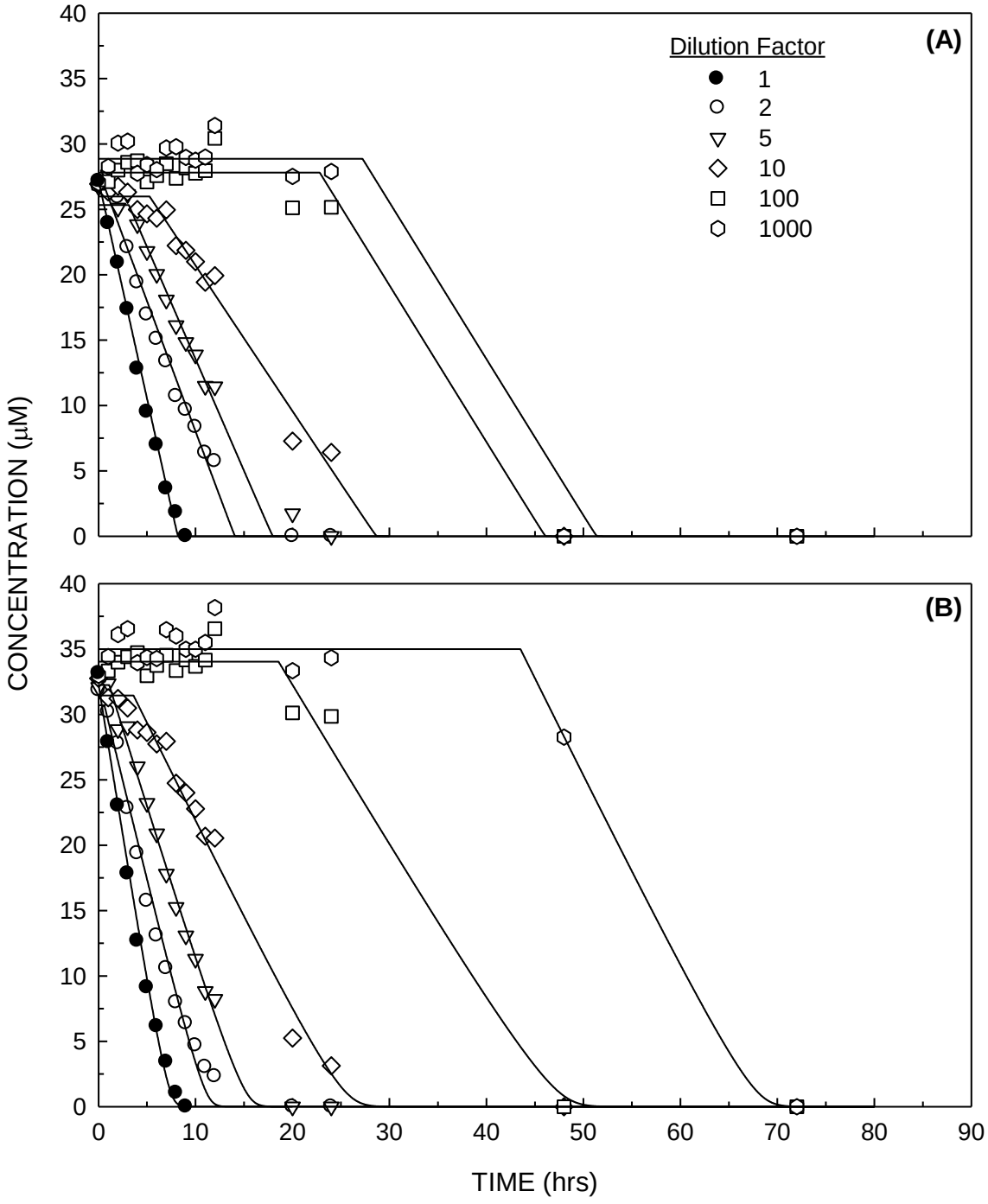


Figure 5.13. Profiles of (A) C_{12}BDMA-Cl and (B) C_{14}BDMA-Cl utilization by BIOMIG1 at 30 μM initial concentration and cell densities between 10^8 and 10^5 CFU/mL.

5.8. Determination of Optimum BAC Biotransformation Temperature

BAC biotransformation was evaluated at temperatures 5, 15, 22, 35, 45 and 50 ^{o}C. Biotransformation of both C_{12} and C_{14}BDMA-Cl was slow and lasted 140 and 100 hrs at 5 ^{o}C (Figures 5.14.A and 5.15.A). Duration of C_{12}BDMA-Cl biotransformation decreased to 24, 6 and 3 hrs at 15, 22 and 35 ^{o}C, respectively (Figure 5.14.B, C and D). Similarly, C_{14}BDMA-Cl biotransformation completed in 15, 5 and 2 hrs at 15, 22 and 35 ^{o}C, respectively (Figure 5.15.B, C and D). The rate of BAC biotransformation was almost zero for both C_{12} and C_{14}BDMA-Cl at 5 ^{o}C and reached to maximum of 4.41 and 6.07 μM/hr at 35 ^{o}C, respectively.

Moreover, both BACs were degraded rapidly but not completely at 45 ^{o}C (Figures 5.14.E and 5.15.E). Only 70% of the BACs were degraded at 50 ^{o}C (Figures 5.14.F and 5.15.F). Interestingly, all BACs were converted to BDMA and BDMA was not utilized by BIOMIG1 at 45^{o}C (Figure 5.16.A). This evidence suggests that optimum temperature for BAC biotransformation to BDMA and BDMA biotransformation to DMA are different. As a result, complete BAC biotransformation involves multiple enzymes that have different characteristics.

Dependence of BAC biotransformation to temperature obeyed a modified Arrhenius equation (Equation 6) (Figure 5.17.). The estimated parameters of relationship for for C_{12}BDMA-Cl; $\beta = 5.06 \times 10^{7}$ μM/d·K; E_a= 12.20 kcal/mol; ΔS = 0.189 kcal/mol·K; ΔH = 59.39 kcal/mol, and for C_{14}BDMA-Cl; $\beta = 6.99 \times 10^{8}$ μM/d·K; Ea= 11.93 kcal/mol; ΔS = 0.179 kcal/mol·K; ΔH = 55.93 kcal/mol. The same parameter's being close each other for different BACs suggests that both BACs are transformed by the same enzyme. The optimum temperature for BAC biotransformation was estimated as 42 ^{o}C. On the contrary, it was observed that BIOMIG1 is not able to grow above 35 ^{o}C (Figure 5.6). This evidence suggests that enzyme responsible for BAC to BDMA conversion originally may not belong to BIOMIG1 but acquired from a different microorganism having an optimum growth temperature of 42 ^{o}C.

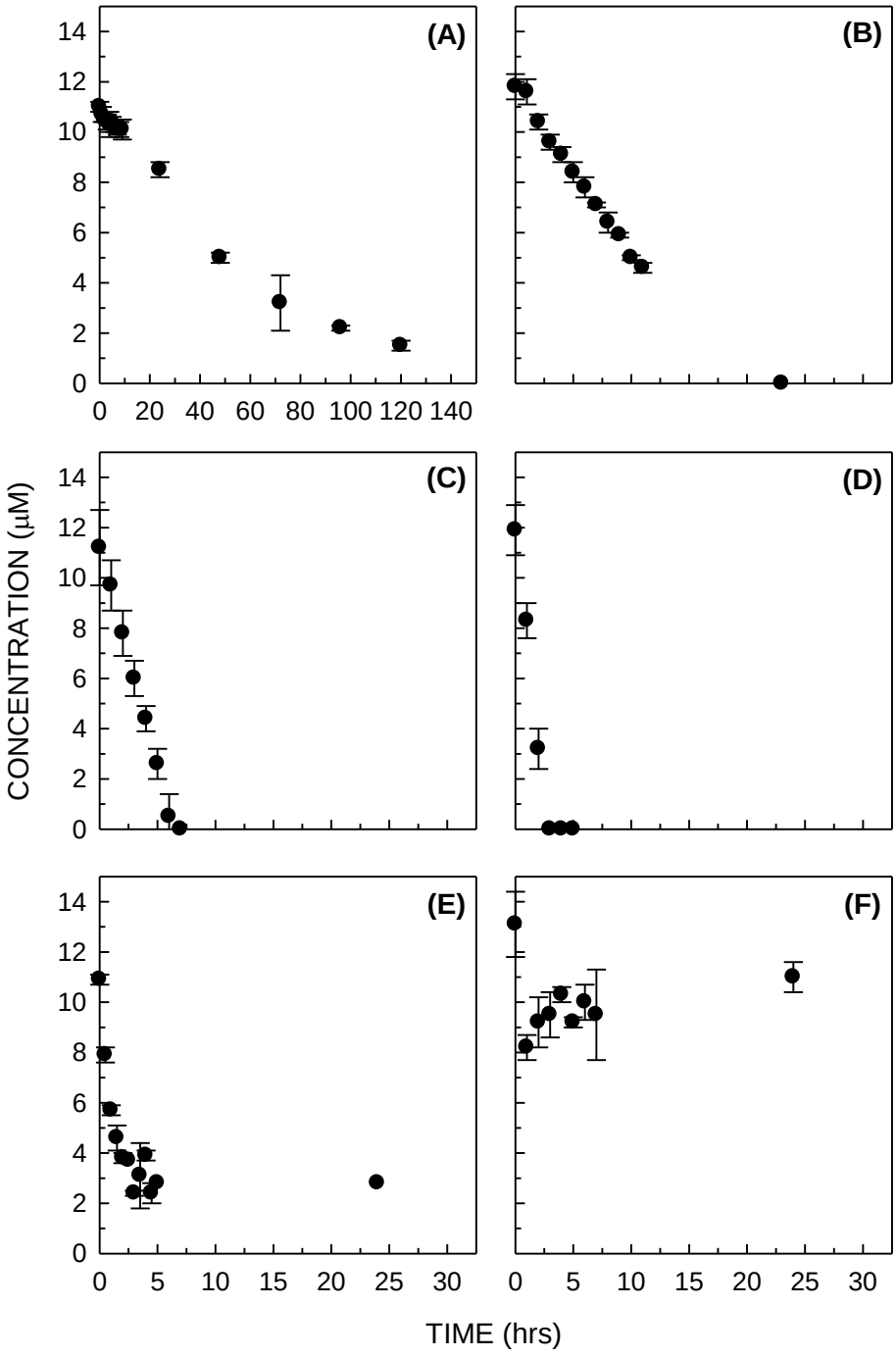


Figure 5.14. Biodegradation profile of C_{12}BDMA-Cl by BIOMIG1 at (A) 5 ^{o}C, (B)15 ^{o}C , (C) 22 ^{o}C, (D) 35 ^{o}C, (E) 45 ^{o}C and (F) 50 ^{o}C at 10 μM (error bars represent one standard deviation of the mean, n=3).

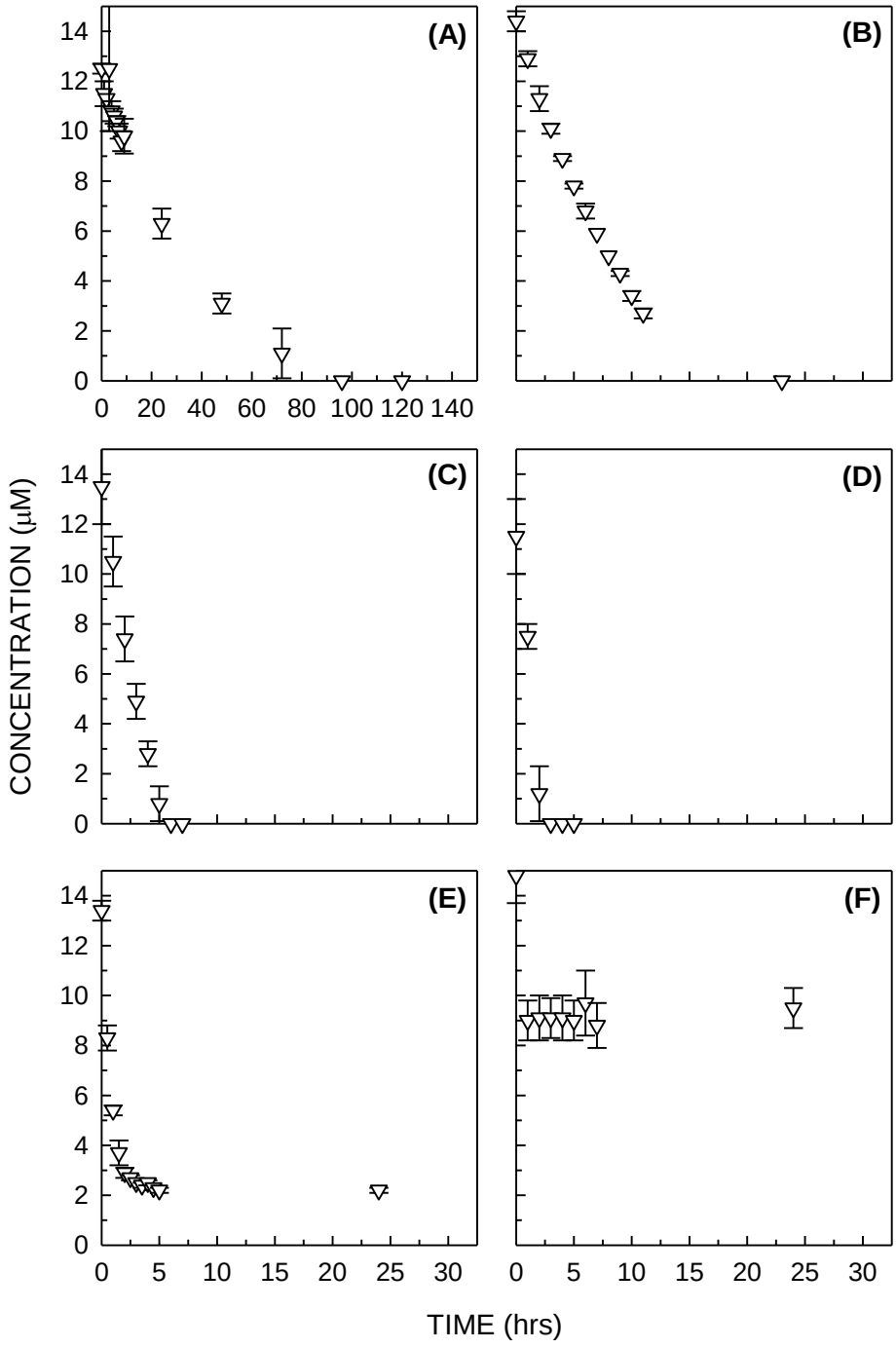


Figure 5.15. Biodegradation profile of C_{12}BDMA-Cl by BIOMIG1 at (A) 5 ^{o}C, (B) 15 ^{o}C, (C) 22 ^{o}C, (D) 35 ^{o}C, (E) 45 ^{o}C and (F) 50 ^{o}C at 10 μM (error bars represent one standard deviation of the mean, n=3).

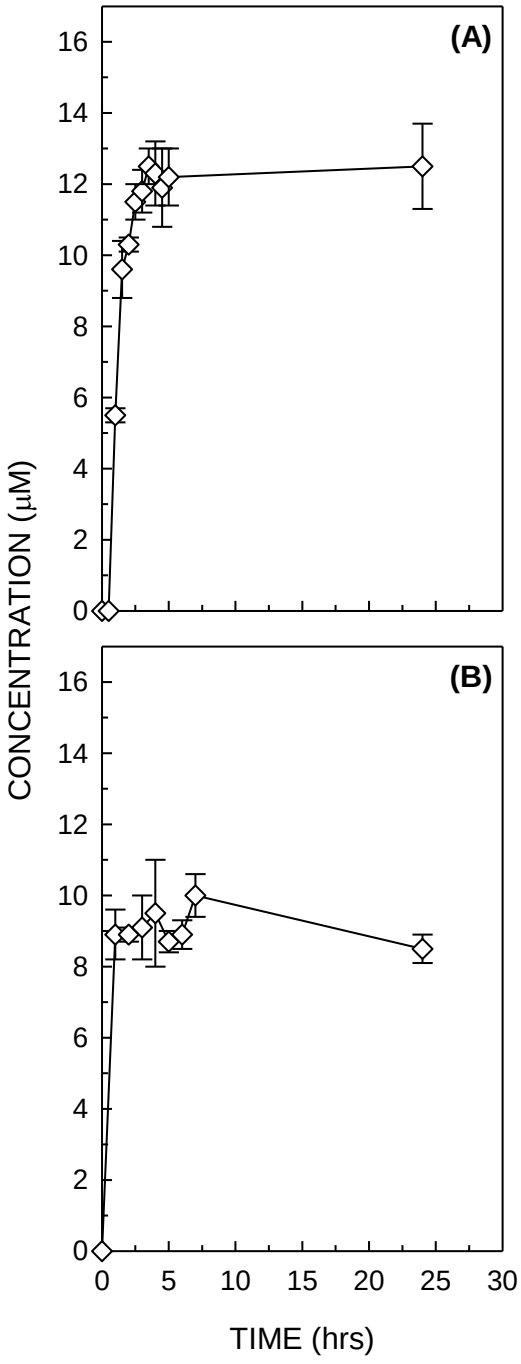


Figure 5.16. Formation of BDMA from BACs by BIOMIG1 at (A) 45 ^{o}C and (B) 50 ^{o}C (error bars represent one standard deviation of the mean, n=3).

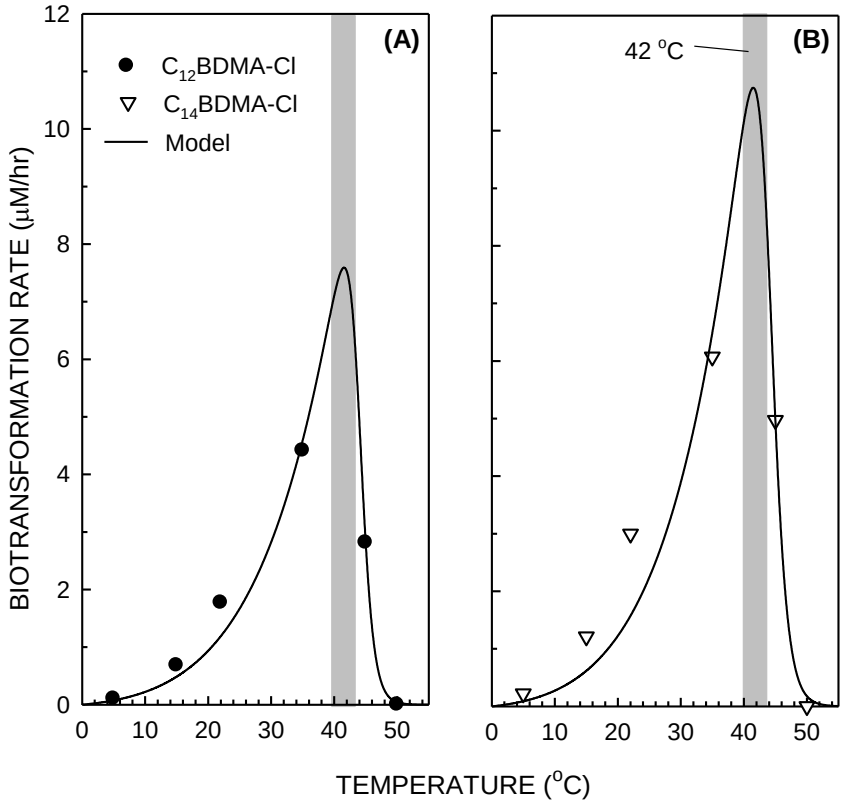


Figure 5.17. Estimated rates for (A) C_{12}BDMA-Cl and (B) C_{14}BDMA-Cl biotransformation by BIOMIG1 at 12 μM initial concentration between 5 and 50 °C.

5.9. Oxygen Threshold Concetration for BAC Biotransformation

A closed serum bottle containing BIOMIG1 was monitored for 18 days. At the fourth day, the bottle was spiked with 140 μM C_{14}BDMA-Cl. As soon as BAC was amended, both C_{14}BDMA-Cl and O_2 were consumed. The headspace O_2 partial pressure dropped to 0.05 atm after 3 days whereas C_{14}BDMA-Cl concentration dropped to 80 μM in one day and stayed constant following 2 days. At the 7th day, the bottle was re-spiked with UV-sterilized pure O_2 to maintain 0.2 atm partial pressure. As soon as the O_2 introduced, C_{14}BDMA-Cl concentration started to decrease. Biotransformation of BAC stopped at 20 μM when O_2 partial pressure reached to 0.02 atm (0.832 mg O_2/L in aqueous phase). After day 12, C_{14}BDMA-Cl concentration in the bottle was elevated to 130 μM and O_2 was injected to the headspace to maintain 0.15 atm partial pressure. C_{14}BDMA-Cl concentration decreased by c.a. 30 μM as O_2 concentration dropped to 0.02 atm. Bottle was amended with C_{14}BDMA-Cl and O_2 in 5 subsequent days where the same trend was

observed. During these days, BDMA started to accumulate in the bottle and its concentration reached to approximately 50 μM. The O_2 concentration at which BAC degradation stopped and BDMA accumulated was determined as 0.02 atm (0.83 mg O_2/L in the aqueous phase). This concentration is O_2 threshold concentration for BAC degradation below which BAC degradation does not occur.

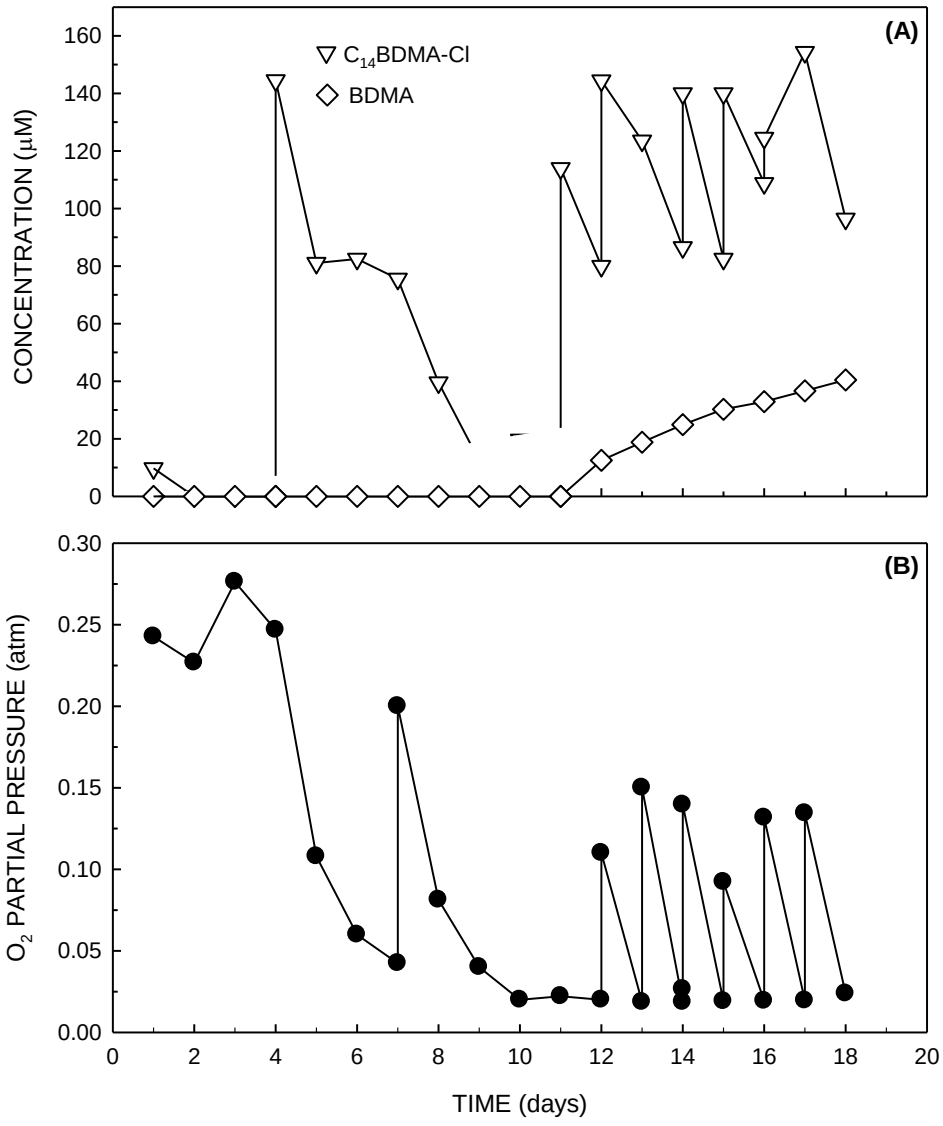


Figure 5.18. Profiles of (A) C_{14}BDMA-Cl utilization and BDMA formation and utilization as well as (B) headspace O_2 concentration in the closed-bottle inoculated with BIOMIG1 and received 140 μM C_{14}BDMA-Cl and 0.21 atm O_2 at the beginning of the incubation.

6. CONCLUSIONS

In the course of this research, a microorganism which can mineralize BACs was isolated from activated sludge. *Pseudomonas sp.* BIOMIG1 is a novel bacteria phlogenetically distinct from other *Pseudomonas* species based on its 16S rDNA. BIOMIG1 can degrade all BAC homologues tested including C_{12}, C_{14} and C_{16}BDMA-Cl. It can degrade these BACs up to 1000 mg/L. Biodegradation rate of C_{14}BDMA-Cl was higher than C_{12}BDMA-Cl followed by C_{16}BDMA-Cl which is the most hydrophobic BAC tested. BAC biodegradation follows Michaelis-Menten kinetics with a maximum specific BAC utilization rate of approximately 4 μM/hr. On the other hand utilization rate substantially decreases as initial BAC concentration decreases. BAC biotransformation is also affected by cell density; below a critical density of 10^8 CFU/mL, BAC degredation delays and rate decreases. Optimum BAC degredation temperature is 42 ^oC although the BIOMIG1 cannot survive at this temperature. BIOMIG1 cannot degrade BACs below 0.83 mg/L dissolved O_2 concentration.

In conclusion, BIOMIG1 is a novel bacterium which has potential applications on controlling QAC pollution in the environment. This study identified optimum conditions of BAC degradation by BIOMIG1 which may facilitate development of treatment biotechnologies using this novel strain.

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