BIOTRANSFORMATION OF BENZALKONIUM CHLORIDES BY IMMOBILIZED CELLS OF *PSEUDOMONAS SP*. BIOMIG1

by

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…to ask why we science is to ask why the leaves fall,

it's in their nature…

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Mass use of quaternary ammonium compounds (QACs) caused these chemicals to become an environmental concern. Little or no elimination of QACs in wastewater treatment plants necessitates a removal policy for these chemicals within treatment system before discharge. In this study, an advanced treatment system after biological treatment is proposed for efficient removal of benzalkonium chlorides (BACs), the most common type of QACs in consumer products. Cultures of *Pseudomonas sp.* BIOMIG1^{BDMA} which can convert BACs into dimethylbenzylamine (BDMA) were immobilized into Ca-alginate beads. Beads were optimized with respect to $CaCl_2$ concentration and diameter for the best BAC biotransformation efficiency at a cell density of 10^7 CFU/mL. Optimal beads were 3 mm beads produced by using 0.15 M $CaCl_2$. Number of cells in these beads was found to be $4.6\pm1.4x10^6$ CFU/bead. BAC degradation kinetics of these beads were analyzed for C_{12}BDMA and C_{14}BDMA. The cell specific utilization rate constant was estimated to be 0.46 μM-BACs/hr.

Continuous flow packed bed reactors were prepared using alginate beads as packing material and operated at 4.7, 2.3, and 1.2 hrs empty bed contact times corresponding to 1.3 hrs, 0.8 hrs, and 0.3 hrs mean residence times. 85±5% removal efficiency was obtained with the setup of 0.3 hrs mean residence time at 20 μM BACs. The same flow rate gave 102±11% removal efficiency for 2 μM BAC concentration, and 95±5% removal efficiency for actual wastewater.

BENZALKONYUM KLORÜRÜN İMMOBİLİZE EDİLMİŞ *PSEUDOMONAS* CİNSİ BAKTERİ HÜCRELERİ TARAFINDAN BİYODÖNÜŞÜMÜ

Dördüncül amonyum bileşiklerinin (DAB) kitlesel kullanımı bu kimyasalların çevresel bir soruna dönüşmesine neden olmuştur. Atıksu arıtma tesislerinde DAB'lerin neredeyse hiç arıtılmaması, bu kimyasalların çevreye salınımından önce arıtım sistemi içerisinde giderimi için bir politika belirlenmesini gerektirmektedir. Bu çalışmada, DAB'ler arasında tüketici ürünlerinde en yaygın bulunan kimyasal olan benzalkonyum klorürlerin (BAK) etkili bir şekilde bertarafı için biyolojik arıtma sonrasına eklenebilecek bir ileri arıtma sistemi önerilmiştir. Daha önce keşfedilen ve BAK parçalama hızı yüksek bir *Pseudomonas* suşu olan BIOMIG1'in, BAK'ı benzildimetilamine (BDMA) dönüştüren alt suşu ($BIOMIG1^{BDMA}$) kültürde büyütülüp kalsiyum alginat bilyelerinin içerisine hapsedilmiştir. Bilyeler 10^7 CFU/mL hücre yoğunluğunda kalsiyum klorür ($CaCl_2$) konsantrasyonu ve bilye çapı açısından BAK biyodönüşüm verimliliği en yüksek olacak şekilde optimize edilmiştir. En uygun bilyeler 0.15 M $CaCl_2$ içerisinde üretilen 3 mm çapındaki bilyeler olarak belirlenmiştir. Bilyelerdeki hücre miktarı $4.6\pm1.4x10^6$ CFU/bilye olarak bulunmuştur. Bu bilyelerin BAK parçalama kinetiği, atıksularda en yaygın olarak bulunan BAK'lar, C_{12}BDMA ve C_{14}BDMA kullanılarak incelenmiştir. Hücreye özgü kullanım oranı sabitinin 0.46 µM-BAK/sa olduğu tahmin edilmiştir.

Alginat bilyeleri ile sürekli akışlı dolgu yataklı reaktörler hazırlanmıştır. Bu reaktörler, sırasıyla 1.3, 0.8 ve 0.3 saat ortalama bekletme süresine (OBS) karşılık gelen 4.7, 2.3 ve 1.2 saat boş yatak temas süresinde çalıştırılmıştır. 20 µM BAK giriş konsantrasyonunda 0.3 OBS'de çalıştırılan reaktörde %85±5 giderim verimi gözlenmiştir. Aynı koşullarda giderim verimi, 2 µM BAK giriş konsantrasyonunda %102±11, 20 µM BAK içeren atıksuda %95±5 olarak belirlenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units Used
[BAC]	BAC concentration	(μM)
[BDMA]	BDMA concentration	(μM)
AOP	Advanced Oxidation Processes	
APHA	American Public Health Association	
BA	Benzyl Amine	
BAC	Benzalkonium Chloride	
BAK	Benzalkonyum Klorür	
BDMA	Benzyl Dimethyl Amine	
BMA	Benzyl Methyl Amine	
BTMA	Benzyl Trimethyl Ammonium	
BTMA-Cl	Benzyl Trimethyl Ammonium Chloride	
C_{10}TMA	Decyl Trimethyl Ammonium	
C_{12}BDMA	Dodecyl Benzyl Dimethyl Ammonium	
C_{12}BDMA-Cl	Dodecyl Benzyl Dimethyl Ammonium Chloride	
C_{12}TMA	Dodecyl Trimethyl Ammonium	
C_{12}TMA-Cl	Dodecyl Trimethyl Ammonium Chloride	
C_{14}BDMA	Tetradecyl Benzyl Dimethyl Ammonium	
C_{14}BDMA-Cl	Tetradecyl Benzyl Dimethyl Ammonium Chloride	
C_{14}TMA	Tetradecyl Trimethyl Ammonium	
C_{16}BDMA	Hexadecyl Benzyl Dimethyl Ammonium	
C_{16}BDMA-Cl	Hexadecyl Benzyl Dimethyl Ammonium Chloride	
C_{16}TMA	Hexadecyl Trimethyl Ammonium	
C_{16}TMA-Cl	Hexadecyl Trimethyl Ammonium Chloride	
C_{18}BDMA	Octadecyl Benzyl Dimethyl Ammonium	
CFU	Colony Forming Unit	(CFU/mL)
COD	Chemical Oxygen Demand	(mg/L)
DAB	Dördüncül Amonyum Bileşikleri	
DNA	Deoxyribo Nucleic Acid	

HPLC	High Performance Liquid Chromatography	
HRT	Hydraulic Retention Time	(hrs)
K_{BAC}	Half-saturation constant	(µM)
k_d	Decay constant	(1/hr)
k'	Cell specific rate of BAC utilization	(µM/hr)
LB	Luria Bertani	
MIC	Minimum Inhibitory Concentration	
MOPS	3-N-morpholino Propane Sulfonic Acid	
OBS	Ortalama Bekletme Süresi	(sa)
QAC	Quaternary Ammonium Compounds	
R	Alkyl or aryl functional group	
TMA	Trimethyl Amine	
TOC	Total Organic Carbon	(mg/L)
US EPA	U.S. Environmental Protection Agency	
UV	Ultraviolet light	
X	Cell density	(cells/L)
Y	Yield coefficient	(cells/µmoles BAC)

1. INTRODUCTION

Biocides have been used for hygene purposes in living and working environments, for over a century. While the simplest biocides such as alcohols or silver that are widely used nowadays have a history going back to 1880s, most of the organic biocides originated in mid 20th century (McDonnell and Pretzer, 2001). Since the disinfection became an essential practice in modern life, demand for biocides increased. However, extensive utilization of biocides became a concern recently due to their environmental impact and role in the antibiotic resistance. As a result, both developing alternative approaches to limit biocide use and treatment of biocide bearing waste streams are now trending research topics (Gerecke et al., 2002; Hegstad et al., 2010; Lejars et al., 2012; Patel et al.; 2014 Scott and Jones, 2000).

Biocides are active ingredients of many consumers products. As these products are utilized by purchasers for relevant purposes, biocides mainly end up in wastewater unless they are directly released into the environment. Fate of antimicrobials in the wastewater treatment system is a major concern since they may directly affect the performance of biological treatment since they are inhibitory (Flores et al., 2015; Hajaya et al., 2011; Tezel et al., 2006; Tezel et al., 2007). Additionally and maybe more importantly, biocides escaping from the treatment systems results in exposure of microorganisms to those chemicals leading to development and dissemination of biocide resistance in the environment. Given the fact that some of biocide resistance mechanisms also affective against antibiotics, biocide contamination in the environment is a growing concern on the human health (Martinez, 2008).

Quaternary ammonium compounds (QACs), biocides that attracted attention in 2000s, almost a century after their start of use at 1916 (McDonnell and Pretzer, 2001) are widely used as active ingredient in disinfectants as well as as additives in consumer products. The QAC consumption worldwide was reported to be 500,000 tones and expected to escalate (Hauthal, 2004). Area of QAC applications is varying from water

treatment, industrial applications as surfactants, emulsifiers, fabric softeners, disinfectants, pesticides, corrosion inhibitors, to even eyedrops as a preservative ingredient (Hegstad et al., 2010; Tezel, 2009, Marple et al., 2014).

75% of QACs utilized enters into wastewater treatment systems and the remaining is directly discharged into environment (Tezel, 2009). In treatment systems QACs bear toxicity to microorganisms, i.e methanogens (Garcia et al., 2000). QACs may have other implications beside toxicity. QACs being used widely in clinical purposes, for instance, may emerge bacterial resistance as in the case of the study performed by Buffet-Battaillon et al. (2011), where higher minimum inhibitory concentration of QACs on clinical *E. coli* was correlated with antibiotic resistance. Modifications in the membrane composition, expression of stress response and repair systems, or increased expression of efflux pump genes excluding these agents from the cell are among mechanisms that facilitate QAC resistance (Hegstad et al., 2010). QACs which get out from the wastewater streams untreated are a concern of ecotoxicity and antimicrobial resistance. Treatment of QACs in sewage systems before spreading into environment is required to prevent toxicity and emerging bacterial resistance to QACs. Although studies about biotransformation and biodegradation patterns of QACs were present in the literature (Van Ginkel et al., 1992; Larson, 1983; Masuda et al., 1976), Kreuzinger et al. (2007) reported QACs as prior substances requiring further investigation and assets.

Benzalkonium chlorides (BAC) are among the most commonly used QACs in disinfection. US EPA (2006) considers BAC (or ADBAC) as the model compound for quaternary ammonium compounds. BAC consists of two methyl groups, a benzyl group, and a long chain alkyl group varying in the size ranged in 8-18 Carbon bound to Nitrogen which is bound to chloride. BAC is highly toxic to fish and aquatic invertebrates on an acute basis (US EPA, 2006). No concern of direct risk to human by dermal exposure is signified by the same authors. Hence BACs entering into receiving environments does not pose primary health risk to human. The impact may occur, however, on an environmental basis. Untreated BACs may select resistant bacteria, or may alter aquatic community of diverse kinds where this became an issue of society and human health as well as that of

environment. In order to alleviate the environmental and human health impacts of BACs, BACs have to be treated in wastewater treatment systems.

Advanced oxidation processes (AOPs) are a joint of chemical treatment processes for removal of organics via oxidation with the help of hydroxyl radicals that occur in such processes. A study showed that AOPs could increase BAC bioavailability (Adams & Kuzhikannil, 2000). AOPs can be utilized for reducing the toxicity of BACs, or complete degradation (Hidaka et al., 1992; Loveira et al., 2012). The treatment could be as short as 3 hrs, whereas the toxic byproducts remain in the system. AOPs coupled with biodegradation, however could be more effective as in the study conducted by Adams & Kuzhikannil (2000), where TOC values in a bioassay performed by the authors decreased by 90% after application of UV/H_2O_2 coupled with biodegradation.

Biodegradation processes are considered rather clean since by-products occured in chemical operations do not appear. Additionally, the outcomes are also biodegradable (Straathof et al., 2002). Biotransformation of QACs was first identified when a monoalkonium QAC in a media was transformed into trimethylamine (Dean-Raymond and Alexander, 1977). There have been many studies in the literature about biodegradation of non-benzyl QACs but studies on BAC degradation is limited (Zhang et al., 2015). Complete mineralization of BACs via biodegradation was reported lately by Tezel et al. (2012). They selectively grew a community on BACs and reported that 98% of the community gene pool was consisted of *Pseudomonas* species. Patrauchan and Oriel (2003) reported that 40% of BACs were eliminated by *Aeromonas hydrophila* K, leading to an accumulation of biotransformation products and inhibition of further degaradation. Given the fact that not all bacteria can utilize BACs, BAC degrading bacteria is valuable and can be utilized in single-culture systems for the treatment of BAC-bearing secondary effluent or industrial wastewater. Therefore conservation of BAC-degrading bacteria in a continuous flow systems is crucial.

Immobilization of cells or enzymes produces static biocatalysts. Immobilization avoids valuable cells and enzymes to escape from flow-thru catalytic systems. Common immobilization methods are; adsorption or covalent binding onto a surface, encapsulation, entrapment into a polymer, and cross-linking (Bickerstaff, 1997). An economical and simple method to immobilize cells is entrapment into calcium alginate (Ca-alginate) beads. Beads containing microorganisms can be used in continuous flow systems for treatment. As an example, (Bettmann & Rehm, 1984) a *Psuedomonas sp.* immobilized in alginate and polyacrylamide-hydrazide degraded 2 g/L phenol within two days whereas free cells were not able to grow on those phenol concentrations.

This study aims to develop an advanced treatment technology utilizing immobilized cells of a novel bacteria degrading a common micropollutant, BACs. Our main objective is to immobilize cells of a BAC-degrading bacterial strain, *Pseudomonas sp.* BIOMIG1 into Ca-alginate beads and to test BAC biotreatment in a continuous flow packed-bed reactor.

2. LITERATURE SURVEY

2.1. QACs in General

Quaternary ammonium compounds (QACs) are the group of compounds having four functional groups which are either alkyl, aryl or methyl groups, bound to a nitrogen atom ($N^+R_1R_2R_3R_4$) (Figure 2.1). The functional groups are used to classify the QACs: it is monoalkonium when one of the fuctional groups is alkyl and rest is methyl (Figure 2.2.A); dialkonium having two long chains and 2 methyl groups (Figure 2.2.B), or benzalkonium having a benzyl group, alkyl group and two methyl groups (Figure 2.2.C). The extensive use of QACs in various domestic, industrial, or agricultural applications made this compound enter the high production volume chemicals list of US EPA (2006b).

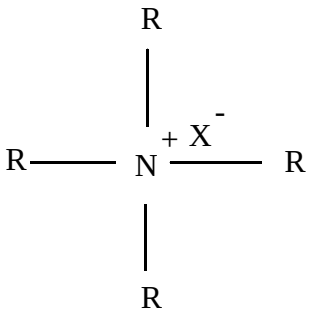


Figure 2.1: Schematic representation of QACs where R can be an alkyl or benzyl group, and X^- is a halide of Cl^-, Br^-, or NO_3^-

Since QACs have four functional groups bound to a nitrogen atom, they are positively charged in aquous solutions. This feature of these chemicals is the reason that they are also called cationic surfactants (Tezel and Pavlostathis, 2011). Chloride salt of benzalkonium (BAC) is the most abundant type of QACs used in disinfection purposes. The most common BACs are benzyldimethyldodecylammonium chloride (C_{12}BDMA-Cl, MW: 340.0 g/mole, Figure 2.3.A), benzyldimethyltetradecylammonium chloride (C_{14}BDMA-Cl, MW: 368.0 g/mole, Figure 2.3.B) found in the wastewater systems.

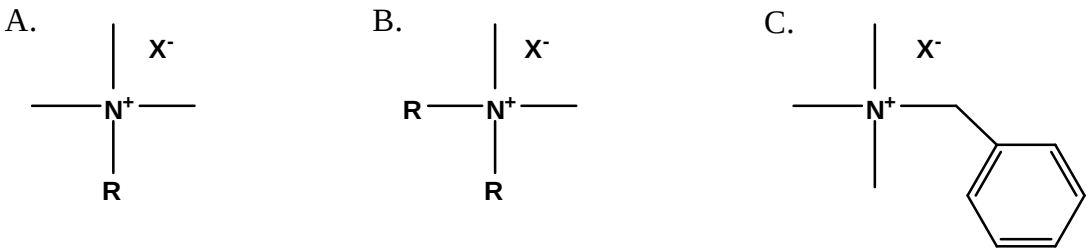
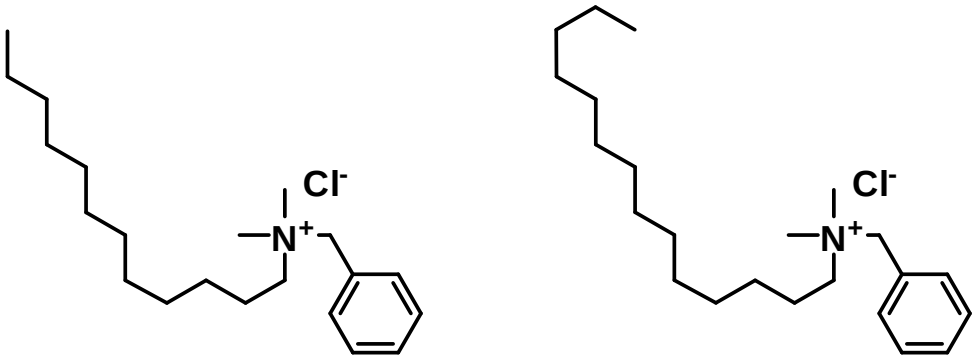


Figure 2.2: The general chemical structure of main types of QACs, namely, monoalkonium (A), dialkonium (B), and benzalkonium halides (C)

Having hydrophobic alkyl chain and hydrophilic central ammonia, BACs are excellent surfactants for dissolution of various organics in water. Their solubity in water is high, melting point is low and they are not volatile. Chemicals with this feature are also known as ionic liquids. These chemicals are preferred because of their ability to dissolve cellulose, their convenience as a transfer and storage media for solar-thermal systems, their ionic properties to be used as batteries instead of water solution, and their ability to separate gaseous chemicals because of selective bonding (Armand et al., 2009; Barghi et al., 2010; Emedian, 2015; Wu et al., 2001).



A B

Figure 2.3: C_{12}BDMA-Cl (A), and C_{14}BDMA-Cl (B)

2.2. Occurrence of QACs in the Environment

QACs are the active ingredients of many disinfectant formulations and used as additives in personal care products and detergents. They have been utilized extensively since 1930 at the time when first antibiotic, sulfonamides, was introduced into the market. They are classified as “high production volume” chemicals (Tezel and Pavlostathis, 2009). Disinfectant and antiseptic formulations containing QACs as active ingredient are utilized in homes, human and animal healthcare facilities, agriculture and industry. These formulations are applied on surfaces at concetrations typically between 400 and 1000 ppm to kill about 99.9% of bacteria, fungi and viruses. Domestic, hospital and industrial use of QACs results in QACs-bearing waste/wastewater (Tezel and Pavlostathis, 2009). Because typical wastewater treatment plants are designed to remove major, easily degradable organics, most trace contaminants, including QACs, pass through wastewater treatment plants and are released into the environment.

QACs along with the other surface active agents took attention recently and their occurrence in the environmental compartments has been reviewed (Clara et al., 2007; Olkowska et al., 2014). Monoalkonium, dialkonium, and benzalkonium compounds differ in concentration in different environmental compartments like surface waters, wastewaters, or sewage sludge. Determination of the surfactants in different environmental compartments is difficult due to the facts that: a) the composition of such matrices are highly complex, b) individual chemicals are in rather low concentrations, c) chemical structures of the surfactants varies, and d) amphiphilic structure may cause loss in aquous form (Olkowska et al., 2011). High-performance liquid chromatography (HPLC) can be coupled with different kinds of detectors like fluorescence, ultra-violet, mass spectrometry, and derivatives of these to determine the surfactants in a matrix (Olkowska et al., 2012).

Martinez-Carballo et al. (2007a) recently investigated the levels of QACs in wastewaters and surface waters. They found that BACs, particularly C12BDMA, were abundant in wastewater. The maximum concenctration of C12BDMA measured was QACs

170 μg/L as C_{12}BDMA, whereas monoalkonium and dialkonium QACs were detected at 9.9 μg/L and 40 μg/L, respectively. Merino et al. (2013a) reported that concentration of BACs ranged between 0.14 and 49 μg/L in influent samples taken from two WWTPs in Spain. The concentrations in wastewater influents, effluents, and surface waters give an insight on the measure to what extent QACs leave wastewater treatment plants and reach to surface waters.

In addition, hospital and laundary effluents are the major source of QACs, especially BACs and dialkonium QACs in the wastewater. For instance, C_{12}BDMA levels in hospital and laundry effluents which subsequently combine into the sewage were 2.8, and 2.1 mg/L, respectively (Martinez-Carballo et al., 2007a). In another study, Kümmerer et al. (1997) reported BACs concentration of European hospitals to be between 0.05 and 6 mg/L. BAC concentrations in effluents of an industrial park in Taiwan were reported to be in a range between 0.5 and 100 μg/L where the highest concentration belonged to octodecyl BAC (C_{18}BDMA) (Ding and Liao, 2001). Hospitals and laundries as well as industrial parks are indeed important sources for occurrence of QACs in the wastewaters and main type of QACs which are received from these indirect sources are BACs.

In the effluents of wastewater treatment plants, QAC concentrations are generally below 1 μg/L. In surface waters, concentrations detected are at ng/L levels. The most common QACs detected in surface waters were C_{12}BDMA, C_{14}BDMA, and homologous dialkonium QACs having 10 carbon long alkyl chain length. The concentration of C_{12}BDMA, which was reported as 1.9 μg/L, is the highest among others (Martinez-Carballo et al., 2007a). Ding and Liao (2001) reported that total BACs concentration in selected rivers in Taiwan varied from 2.5 to 65 μg/L. When the occurance of QACs in the sediments was investigated, the values escalate up to 3.6 mg/kg dry sample levels and the highest concentrations belonged to BACs as C_{12}BDMA and C_{14}BDMA (Martinez-Carballo et al., 2007b). Ferrer and Furlong reported BAC concentrations in surface waters downstream to a wastewater treatment plant (WWTP) between 1.2 and 36.6 μg/L (2001), and between 21 and 260 μg/kg in sediments in riversides downstream to a WWTP (2002). Concentration of QACs in sewage sludge was reported to be between 22-103 mg/kg dry

weight as the maximum value observed was that of a big city WWTP where industrial wastewater discharge was high (Martinez-Carballo et al., 2007b). Merino et al. (2003b) reported that concentrations of monoalkonium and dialkonium QACs, and BACs in sludge samples collected from two WWTPs in Spain were between 0.1 and 34 mg/kg. Another study showed that BAC concentrations in municipal sewage sludge in China ranged from 0.94 to 191 mg/kg with C_{12}BDMA having the highest concentration among BACs (Ruan et al., 2014).

The QACs can be found in wastewater, WWTP effluents, effluents of mainly hospitals and laundries, and in the sewage and digested sludge. They occur also in receiving surface water bodies, and sediments underneath. Almost in all of these engineered or natural environmental compartments, BACs are the major type, or among the major types of QACs present. A summary of QACs distribution in different compartments of either engineered or natural systems is given in Figure 2.4.

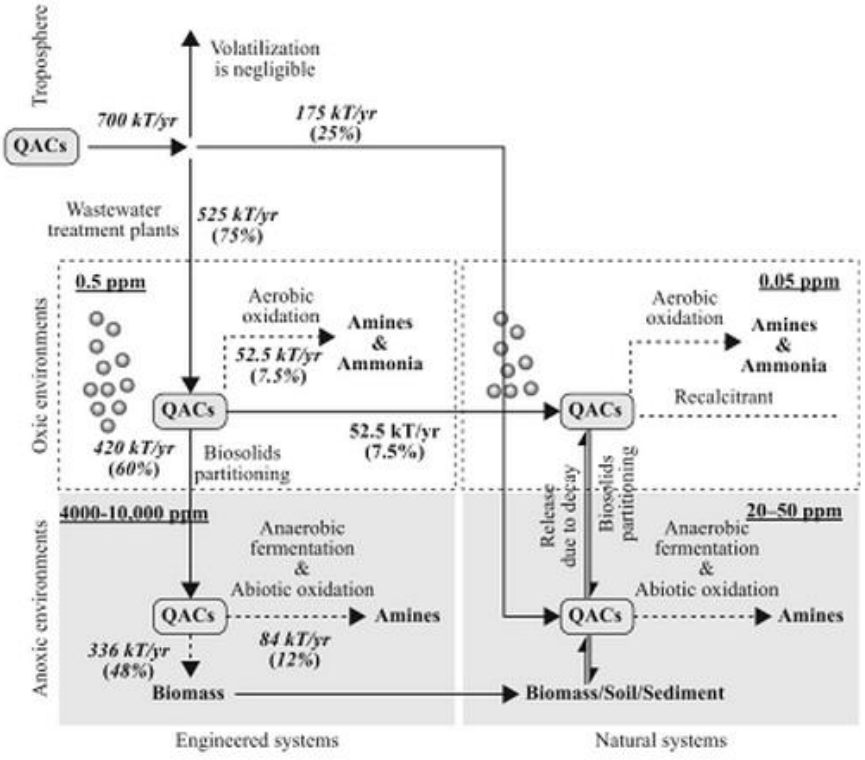


Figure 2.4: Transport of QACs in different environmental systems according to global QAC consumption (Tezel and Pavlostathis, 2011)

2.3. Fate and Effect of QACs

Unique physical/chemical properties of QACs promotes surfactancy, detergency, and biocidal activity which make them useful for various industrial, agricultural, or domestic applications. These properties have also made QACs an environmental concern as the effect of QACs in different environmental systems are directly associated with those properties.

Fate of QACs in the environment depends mainly on sorption and biodegradation (Zhang et al., 2015). QACs have a high affinity of adsorption onto surfaces of different kinds such as sludge, clay, sediments, humic substances and cell wall of microorganisms (van Wijk et al., 2009). Adsorption affinity depends on the type and structure of QACs, type of the surface that they adsorb, and various environmental parameters like pH and temperature (Ying, 2006). Adsorption affinity is mainly related to alkyl chain length, where increasing length increases adsorption onto sludge (Clara et al., 2007). Ismail et al. (2010) conducted a study on adsorption of monoalkonium QACs with chain lengths having 12 or 16 carbon (C_{12}TMA-Cl and C_{16}TMA-Cl), and BACs (C_{12}BDMA and C_{16}BDMA). They found that benzyl group increases affinity to solid surface for shorter alkyl chains. Ren et al. (2011) reported that adsorption increased as the particle size of the adsorbent and temperature decreased.

QACs present in the sludge may inhibit methanogenesis. Flores et al. (2015) reported that BAC concentrations led to a gradual decrease in methane production between 0.02 and 100 mg/L. At 0.02 mg/L there was no apparent effect whereas at 100 mg/L methanogenesis was almost completely inhibited. Tezel et al. (2006) found that dialkonium and benzalkonium QACs had short- and long-term effects on the mixed methanogenic culture at and above 25 mg/L concentration. According to their findings, QAC inhibition was more significant on methanogenesis than acidogenesis. Anaerobic treatment instead of aerobic biological treatment for high organic load wastewaters is a cost effective and beneficial option. However, presence of QACs in these treatment systems may decrease

the treatment efficiency as well as the methane yield. Tezel et al. (2007) in their study on poultry processing wastewater treatment system reported that at concentrations 50 mg/L or above QACs adversely affected the COD removal.

Harmful effects of QACs on nitrification have been investigated recently (Hajaya and Pavlostathis, 2012; Kreuzinger et al., 2007). Hajaya and Pavlostathis (2012) reported that BACs affected nitrogen removal in non-acclimated reactors by inhibiting nitrification. Nitrification was recovered after QACs were eliminated in mixed heterotrophic system. Kreuzinger et al. (2007) reported that nitrification could be affected from QACs at concentrations as low as 2 mg/L. Yang et al. (2015) demonstrated that the nitrification would not recover after elimination of BACs from the system when the temperature decreased to 10°C. In soil, parameters affecting the inhibition of nitrification are the nature of QACs as well as the carbon content of soils (Sarkar et al., 2010).

QACs present in the environmental compartments have further effects besides the inhibition in engineered systems. Toxicity of QACs is a major concern. Ivanković and Hrenović (2010) reported that toxicity of the surfactants is highly specific for different types and classes of the surfactants and for the organisms tested. QACs are toxic to aquatic vertebrates and invertebrates, algae, and protozoa (Zhang et al., 2015). The structure of QACs determines antimicrobial activity. Biocidal activity occurs by penetration of alkyl chain of QACs into microorganism's membrane and alteration of phospholipid bilayer (Wessels and Ingmer, 2015). Membrane is disrupted and intracellular components leak out (Ioannou et al., 2007; Pérez et al., 2009; Sutterlin et al., 2008).

On eukaryotic level, QACs toxicity has been investigated in the literature by several authors lately. It was demonstrated via *in vitro* studies that QAC concentration range found in wastewater and nasal drops is sufficient to bear genotoxic effects such as induction of DNA migration in the liver cells at 1 mg/L BAC. (Ferk et al., 2007). Deutschle et al. (2006) reported that BAC concentrations in the range 200-500 mg/L might cause DNA damage on epithelial cells surviving at those concentrations. Among studied

mulfunctions are mitochondrial dysfunction in mammalian epithelial cells, decrease in fertility in mice, and lung effects to mice due to inhalation of QAC aerosols (Inacio et al., 2013; Larsen et al., 2012; Melin et al., 2014). van Wijk et al. (2009) reported that algae are more sensitive to QACs than fish or crustaceans. Similar findings have been demonstrated in other studies (Jing et al., 2012; Liang et al., 2013; Zhu et al., 2010).

QACs toxicity in accordance with other toxic substances have also been investigated in the literature. Toxicity to microorganisms can be caused by QACs themselves; hence, there may be interactions between QACs and other surfactants present in wastewater systems and they may effect toxicity. In their study about toxic chemical uptake by the cells of green algae, Yu et al. (2013) reported synergistic effect between C_{16}TMA-Cl and fluoranthene at fluoranthene levels up to 50 μg/L, whereas they resembled antagonistic effect at concentrations between 50 and 200 μg/L to *Chlorella vulgaris*. Flores et al. (2015) reported that BACs almost completely inhibited anaerobic digestion at 100 mg/L but the authors found no synergistic effect between BACs and certain commercial biocides such as Proxel LV (PRX), Triton X-100 (TRX), and DOWFAX 63N10 (DWF). Sutterlin et al. (2008) reported that BAC toxicity on *P. putida* and *V. fischeri* decreased when sulfonic acids, a kind of anionic surfactants were present. Alkyl chain lengths and mixture ratios of QACs with anionic surfactants are important parameters in toxicity of surfactants against bacteria (Wong et al., 2012).

Games et al. (1982) showed that biodegradation and adsorption were two competing removal mechanisms of QACs in sewage treatment systems. QACs are readily biodegradable under aerobic conditions, but the portion adsorbed to sludge and transported to anaerobic engineered and natural systems is significant and leads to accumulation of QACs in the environment since QACs are not biodegradable under anaerobic conditions (Zhang, 2015). In addition to toxicity, the QACs present in the environment select resistant bacteria which may also be resistant to other biocidal agents (Ishikawa et al., 2002; Mc Cay et al., 2010).

2.4. Resistance Mechanisms

QACs are toxic to various organisms and can be used for disinfection against bacteria for over 99% efficiency at proper levels (Tezel and Pavlostathis, 2011). The QAC levels in sewage, effluent of WWTP, sewage sludge and surface water are notably lower than concentrations of domestic, industrial, and agricultural applications (Tezel and Pavlostathis, 2015). Although there is no clear evidence for development of resistance in the literature so far that is debated by Gerba (2015; and many QAC producer companies), the exposure to QACs causes propagation of integrons, mobile genetic elements that may carry genetic material within and between genomes (Tezel and Pavlostathis, 2015). The presence of those integrons, and additionally, multidrug-resistant gene cassettes shared between clinical and soil samples analyzed by Forsberg et al. (2012) reveals a relation between pathogens and soil bacteria which are exposed to QACs.

Susceptibility to QACs can be tested via minimum inhibitory concentration (MIC), biocidal tests, or standardized challenge tests (Buffet-Bataillon et al., 2012). In MIC test the microorganism is grown on a nutrient agar containing serial dilutions of the antimicrobial or antibiotic. The minimum concentration that bacteria cannot grow is the MIC. It is an easy and quick test where several strains or various antimicrobial agents may be tested in a short time. Biocidal tests include application of antimicrobial on a target organism for a time and then observing the viable cells. Standardized challenge tests are used to determine preservation capacity of the antimicrobial additives in consumer products (Nicoletti et al., 1993; Poole, 2002; Russel, 2003).

QACs mainly attack cells by disrupting the integrity of the cell membrane. Classical thought was that the antimicrobial agent selected the bacterial strains that were already resistant to such agent, and the remaining strains died. Above MIC levels this assumption applies (Tezel and Pavlostathis, 2015). Under MIC levels, however, the strains that are originally susceptible to the antimicrobial may have individuals that survive via a non-hereditary (not transferred to offspring) phenotype called persistence (Blázquez et al.,

2012). Persistent bacteria arise from the stress on cellular processes leading to SOS response which further prompts error-prone DNA replication with mutations (Ceragioli et al., 2010). Bacteria may respond to sub-MIC antimicrobials via modifying the cell membrane, porin (cellular channel protein) structure or density, via increasing the efflux pump expression, and by retrieval of mobile genetical elements expressing efflux pumps against QACs (Tezel and Pavlostathis, 2011).

Bacterial response against antibiotics occurs through similar mechanisms. Above MIC only the resistants survive. Nonetheless, lower levels of antibiotics does not kill bacteria, and a wide range of mutated bacteria survive (Andersson and Hughes, 2014). The mechanisms are similar to QAC tolerance mechanisms. Sub-MIC level of QACs promotes oxidative stress in bacteria. Nakata et al. (2011) reported that *E. coli* produced intracellular superoxides and hydrogenperoxide after exposed to C_{16}BDMA-Br (bromide salt of that benzalkonium). Gene transfer via mobile genetic elements like poly-antimicrobial resistance gene cassettes or transposons escalates upon oxidative stress. Gullberg et al. (2011) reported that application of antibiotics at concentrations considerably lower than MIC of subjected bacteria might choose brand new mutant lines which might have even been present in a very low initial fraction.

Resistance mechanisms to QACs include biodegradation, reduction of number of porins, mobile genetic elements, enhanced biofilm formation, and overexpression of efflux pumps (Tezel and Pavlostathis, 2015; Buffet-Bataillon et al., 2011). Bore et al. (2007) reported that *E. coli* altered the levels of outer membrane proteins such as porins and drug transporters after repeated exposure to low level BAC. Karatzas et al. (2008) found reduced levels of porins in a *Salmonella* strain which was exposed to low level QACs. Machado et al. (2013) in their study of alteration of proteomics of *Pseudomonas aeruginosa* after continuous exposure to increasing doses of BAC reported that porin underexpression was concentration specific. Langsgrud et al. (2003) reported that exposure to QACs led to slime formation causing adaptation in *Pseudomonas aeruginosa*. When the slime was eliminated by addition of chemicals partial loss of the acquired resistance occurred. Pagedar et al. (2012) showed strong relation between biofilm formation and

adaptation to BAC on their study with dairy-originated *E. coli* isolates. Bjorland et al. (2003) discovered a QAC resistance gene on a plasmid in *Staphylococcus* strains where strains having the plasmid have high MIC against BAC.

Microorganisms that are exposed to QACs may response in a single or multiple mechanisms, or inherit a bundle of genes which result in resistance to other antimicrobials (Tabata et al., 2003). If the genetical material, or stress response of the surviving cells is responsible for an efflux pump effective against multiple antimicrobials the process is called cross-resistance (Buffet-Bataillon et al., 2011). Grkovic et al. (2002) stated that microorganisms might respond to QACs by overexpression of drug transporter genes which might be effective against a bunch of other antimicrobials and also against antibiotics. Integrons and multidrug resistance families play a crucial role in antimicrobial resistance (Braga et al., 2010; Cambray et al., 2010; He et al., 2011).

Oh et al. (2013) reported that the MIC value for BAC of a community from river sediments in USA increased from 125 mg/L to 250 mg/L in 43 days upon exposure to BACs for 3 years. Guo et al. (2012) reported that mutants of *Salmonella typhimurium* which exposed to BAC and survived, had increased MIC against various antibiotics. Müller et al. (2013) identified a transposon, Tn6188, related to tolerance to BAC in a bacterial strain isolated from a food chain. Mester et al. (2015) reported that adaptation to BAC at a short time could develop adaptation to other ionic liquids in food chain bacteria. Tandukar et al. (2013) found that an aerobic microbial community exposed to BACs for a long-term became less susceptible to BACs as well as a number of commercially produced antibiotics. Authors also noted that *Pseudomonas* predominated the community after exposure. Katharios-Lanwermeyer et al. (2012) suggested that conjugation of gene cassettes from non-pathogenic bacteria to pathogenic caused pathogen bacteria resistant to BAC. They also proposed a cross resistance mechanism that bacteria adapted to BACs might also gain adaptation to cadmium.

Antimicrobial agents select for resistance of bacteria through cellular mechanisms. By some alteration in the metabolism after exposure to antimicrobials, cells prompt an SOS mechanism which ultimately leads to emergence of mutations. By means of these processes and via mobile genetic elements, the bacteria may gain resistance to antimicrobials. Misuse in environments like hospitals and food chain where QACs are extensively used may bring out multi-resistant bacteria more common in such environments, leading to sanitary issues. The release of QACs from sewage to the environment may also cause emergence of such adaptive bacteria, which may pose a serious threat to human health. QACs, hence, should be eliminated in WWTP before before wastewater is discharged into the environment.

2.5. Advanced Oxidation of QACs

QACs that are not treated in WWTPs reach to surface waters, sludge due to adsorption, aquatic sediments and soil due to land application of digested sludge. Presence of QACs in the environment is not desired as discussed above, therefore treatment technologies that transform QACs to not toxic and non-antimicrobial chemicals are desired. Since QACs are not-easily-biodegradable chemicals, treatment of QACs by advanced oxidation has been studied. Advanced oxidation which is a chemical treatment process used extensively for removal of organic pollutants from water and wastewater by oxidation through reactions with hydroxyl radicals (•OH) generated by strong oxidants such as ozone, hydrogen peroxide (H_2O_2) and/or UV (Calvosa et al., 1991; Gilbert, 1987; Hu and Yu, 1994; Kitis et al., 1999). AOPs are generally used prior to biological treatment in order to enhance the biodegradation of recalcitrant chemicals or following the biological treatment to clean up the toxic metabolites generated during the biotransformation of degradable chemicals (Alvares et al., 2001).

AOPs are more beneficial than the classical chemical oxidation with chlorine or permanganate that generates residuals which are toxic to bacteria and may affect the biodegradation afterwards, when the chemical oxidation is used as a preliminary step

before biodegradation. AOPs have no such by-products. The higher oxidation potential of AOPs over chemical oxidants is another benefit (Ikehata and El-Din, 2004).

When ozone is used in the advanced oxidation process, different parameters such as pH or functional groups determine how ozone acts as an oxidant (Alvares et al., 2001; Ikehata and El-Din, 2004). Molecular ozone reactions, or ozonolyses, predominate at lower pH. Electrophilic attack of ozone to organic compounds will end up with carboxylic acids. C=C double bonds, hydroxyl, methyl, or methoxy side chains, negatively charged atoms are among the features of compounds susceptible to ozonolysis (Langlais et al, 1991). At pH values over 8 the combination of ozone with hydroxyl radical leads to occurrence of free radicals like hydroperoxyl, superoxide, hydrogen trioxide, and hydrogen tetraoxide in a series of reactions (Wang et al., 2003).

Corless et al. (1989) studied the impact of ozonation on monoalkyl and dialkyl ammonium compounds having saturated and unsaturated alkyl chains. Authors observed quaternary ammonium carboxylic acids, aliphatic aldehyde, and aliphatic carboxylic acids occurred after treating 500 µg/L QACs at an ozone concetration of 5 mg/L for 30 min at 10°C and at pH between 4.5-5. Adams and Kuzhikannil (2000) reported significant change in bioavailability of BACs after 30 min treatment with UV/H_2O_2. They also found no change in biodegradability of dialkonium compounds with 18 carbon alkyl chain length after 120 minutes of oxidation. In their study with C_{12}BDMA, Hidaka et al. (1992) showed that aromatic ring of the BACs was more prone to photocatalytic titanium dioxide (TiO_2) oxidation than the alkyl chain. Loveira et al. (2012) pointed that photocatalytical TiO_2 oxidation was an efficient method for reducing the BAC toxicity in wastewater as a pretreatment process before biological treatment. There are also AOPs of photocatalytic Titania with UV for complete degradation or with solar light present in the literature (Hidaka et al., 1989, 1990).

QACs, especially BACs, as recalcitrant pollutants in wastewater, should be eliminated in WWTP before discharge. Advanced oxidation is valid for only partial

treatment because of its cost ineffectiveness and high energy demand. The arguments in the literature also support that biodegradation is the cost effective and sustainable alternative for removal of QACs/BACs from wastewater.

2.6. Biodegradation of QACs

Biodegradation of QACs has recently been reviewed in the literature (Brycki et al., 2014; Tezel and Pavlostathis, 2015). Among the genera of *Pseudomonas, Xanthomonas, Aeromonas, Bacillus* and *Thalassospira* there are certain species or strains that metabolize QACs of different types (Dean-Raymond, 1977; Patrauchan and Oriel, 2003; Tandukar et al., 2013). The biodegradation requires oxygen, and under methanogenic conditions there is no evidence for biodegradation of usual QACs of types monoalkonium, dialkonium or benzalkonium (Garcia et al., 2001; Tezel et al.; 2006). QACs having alkyls replaced by esters, so called esterquats, however, can be degraded under methanogenic conditions (Garcia et al., 2000; Watson et al., 2012). Biodegradation of esterquats or dimeric QACs will not be explained further as they are out of the scope of this study.

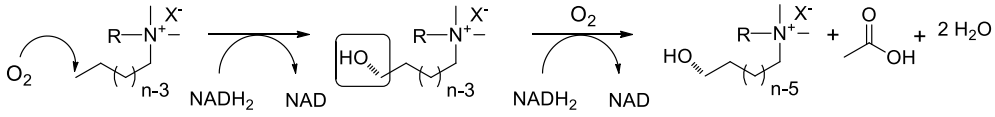
Liffourrena et al. (2008) isolated a *Pseudomonas putida* strain which can achieve degradation of monoalkonium QACs having alkyl group with 14 carbon chain length (C_{14}TMA). At 50 mg/L C_{14}TMA, the growth was observed where there was no other carbon or nitrogen source. Trimethylamine (TMA) was formed as a result of monooxygenase activity. Dean-Raymond and Alexander (1977) reported partial degradation of monoalkonium having alkyl group with 10 carbon chain (C_{10}TMA) and alkyl group with 14 carbon chain length (C_{14}TMA), where the latter compound was degraded only in lower concentrations, by the co-culture of a *Pseudomonas* and a *Xanthomonas* strain. Patrauchan and Oriel (2003) reported an *Aeromonas* strain degrading BAC by utilizing it as a sole carbon and nitrogen source. Nishihara et al. (2000) showed a *Pseudomonas fluorescens* strain degrading dialkonium QAC as well as assimilating monoalkonium salts and BAC. Bassey and Grigson (2011) isolated *Bacillus niabensis* and *Thalassospira sp.* which could degrade C_{16}BDMA at concentrations 2-4 g/L from marine

sediments. Bacteria reported to degrade QACs are capable of it only when the concentrations are low, and degradability as well as degradation rate decreases with increasing alkyl chain length because of toxicity.

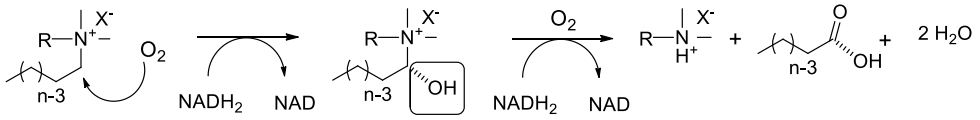
When the degradation is accomplished via co-culture, one of the important parameter is how the sequential biotransformation processes occur. If biotransformation product of a strain is the substrate of the other, sufficient amount of products must be metabolized by the former strain in a time interval. The latter strain must utilize the products of former strain, otherwise latter strain might be removed from the system. The solid retention time (SRT) should be very carefully determined (Van Ginkel, 1996). Toxicity is also a problem, because when the bacterial strain or community do not degrade QACs it is unclear whether the QAC is not biodegradable by that strain, or it is too toxic that the growth of the strain is inhibited before biodegradation (Reynolds et al., 1987). Biodegradability is also dependent on non-methyl alkyl groups (Van Ginkel and Kolvenbach, 1991). As the number of alkyl groups bound to central nitrogen increases, biodegradability decreases. It is reported in the literature that QACs having benzyl group are less biodegradable than the monoalkoniums having the same alkyl chain length (Garcia et al., 2001, Tezel et al., 2012).

There are three pathways of degradation of QACs suggested in the literature so far (Figure 2.5). The first pathway starts with ω-hydroxylation of alkyl group. Upon oxidation of the C farthest to the central nitrogen on alkyl group, one or more β-oxidation processes follows decreasing the chain length by two carbons at a time. Dean-Raymond and Alexander (1977) first predicted the pathway when they observed 9-carboxynonyl-trimethylammonium and 7-carboxyheptyltrimethylammonium in the media as C_{10}TMA was degraded. Another pathway was proposed by Van Ginkel et al. (1992) as the authors isolated a *Pseudomonas* strain from activated sludge where the strain could only degrade monoalkonium compounds among QACs. The predicted pathway is as follows: C_{16}TMA undergoes α-hydroxylation where the C nearest to central N is oxidized and cleaved (N-dealkylation). Trimethylammonia and an alcohol form. Alcohol is converted to carboxylic acid by alkanol dehydrogenase enzyme. Carboxylic acid is then mineralized via several β-

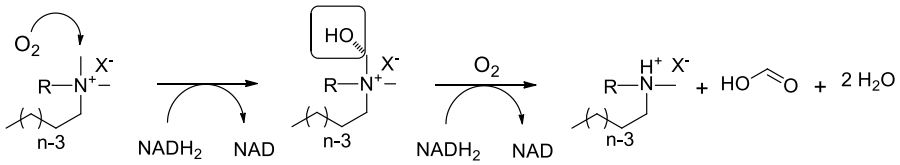
oxidation chains. N-dealkylation process is reported to take place in also BAC biodegradation resulting in benzyldimethylamine (BDMA). The process is followed by further demethylations resulting in benzylmethylamine (BMA) and benzylamine (BA), which further cleaved to benzaldehyde and ammonia by deamination (Patrauchan and Oriel, 2003). Tezel et al. (2012) isolated a microbial community mainly consisted of *Pseudomonas spp.* where degradation pathway continued with debenzylation from BDMA and then demethylation. Authors suggested that there was different bacterial strains that might involve in degradation of BDMA because there was a retardation in the process and partial accumulation of BDMA. The last pathway incorporates hydroxylation of a methyl group bound to central N. Takenaka et al. (2007) proposed this pathway when they observed formic acid and dodecyldimethylamine along with the usual residuals of first pathway as a result of biodegradation of dodecyltrimethyl ammonium (C_{12}TMA).



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 2.5: Aerobic biotransformation pathways of QACs (Tezel and Pavlostathis, 2011)

QACs are biodegradable by certain bacterial strains. There are QAC-degrading bacteria which can be found in wastewater or in activated sludge as explained above. Recently, a BAC degrading bacterial strain, *Pseudomonas sp.* BIOMIG1 was isolated from

sewage samples analyzed in our laboratory (Ertekin et al., 2015). Among QAC degraders reported in the literature so far only *Pseudomonas sp.* BIOMIG1 have the ability to degrade non-alkyl containing amines (Tezel and Pavlostathis, 2015). Therefore, this strain is a preferable option to be used in eliminating QACs from sewage systems.

2.7. Immobilized Cell Technology for Treatment of QACs

Immobilization of cells for achieving solid catalysts is a promising technique for biocatalysis. As the technology is developing and new alternatives are arising, the design and application of immobilization become more feasible. Immobilized organisms are useful in a way that: a) they can be kept still when there is a flow regime in the reactor; b) they will be protected from chemical exposure for some extent. Due to these benefits of immobilization, cells can be used in various biotransformation applications, otherwise may not be applicable. Immobilization is attracting more and more attention in the research arena (Liu et al., 2009; Wang et al., 2008; Zhang et al., 2008).

When selecting the immobilization material, physical and chemical properties as well as economical attributes should be considered. Surface area, compressibility, porosity and empty space for growth in the pores are among physical properties of the material (Bickerstaff, 1997). The yield may decrease, for example, if the pores are too small to allow biomass growth, or if the solid is not durable against physical pressure in a flow. The solid material has also to be inert against cells, has to have some hydrophilicity to interact with water by diffusion, and has to be cheap and easily producible.

Immobilization has basically five types of interaction between cells/enzymes and solid material: 1) Adsorption, as cells/enzymes are interacted with a solid surface by weak chemical interactions (Messing, 1976), 2) covalent binding, where the interaction between cells/enzymes and solid surface is chemical rather than physical (Porath and Axén, 1975), 3) entrapment, as the cells or enzymes are not bound to the immobilization material but

they are entrapped in the mesh structure inside the solid which can allow water pass through (Tan et al., 2014), 4) encapsulation, where a semi-permeable membrane, through which substrate in water may pass but cells/enzymes cannot, is enclosing the cells/enzymes (Nicodemus and Bryant, 2008), and 5) cross-linking of cells without need of a solid support, as in flocculation for wastewater treatment.

Calcium alginate (Ca-alginate) entrapment is one of the most commonly used immobilization methods among those because of its economical, practical, and physicochemical properties (de-Bashan and Bashan, 2010). By adding dropwise somewhat water soluble Na-alginate into calcium chloride ($CaCl_2$) via a needle from a height, insoluble Ca-alginate beads are formed. Among important parameters in formation of the gel beads are $CaCl_2$ and alginate concentrations which determine the porosity and toughness of the beads, the diameter of the needle which determines the bead size, curing time which is the contact time of $CaCl_2$ and alginate concentrations, and presence of a coating around the beads (Talekar and Chavare, 2012; Won et al., 2005). Increased curing time enhances rigidity of the beads whereas coating is useful for prevention of leakage.

For biodegradation of QACs Ca-alginate beads were reinforced few times in the literature. Bergero and Luchessi (2013) immobilized a *Pseudomonas putida* strain in Ca-alginate beads for investigation of the effects of concentration of sodium alginate, pH, temperature, agitation rate and initial concentration of C_{14}TMA on this compounds biodegradation. Authors suggest optimum conditions for C_{14}TMA biodegradation as 4% Na-alginate, 0.3 M $CaCl_2$, $2x10^8$ CFU/mL cell concentration. They also found optimum pH, temperature, and shake velocity to be 7.4, 30°C, and 100 rpm, respectively. The same authors investigated BAC removal with immobilized and free cells using C_{14}BDMA, C_{16}BDMA, and a mixture of them as substrate (Bergero and Luchessi, 2015). Beads with cells removed 90% of the BACs. They also found 80% removal in QAC bearing industrial wastewater after 48 hours of contact time.

3. HYPOTHESES AND OBJECTIVES

In wastewater, increasing concentration of QACs due to wide variety of use leads to spreading of these compounds in the environment, which causes environmental toxicity as well as an increase in the QAC-resistant bacteria. An advanced treatment unit is necessary to reduce the QAC levels in wastewater effluent to acceptable levels. This necessity is based on three assumptions: 1) The antibiotic resistance is spreading around in the environment (Martinez, 2008), 2) pollution due to disinfectants is crucial in the spread of resistance mechanisms (Tandukar et al., 2013), 3) the spread of bacterial resistance to antibiotics may be prevented if the disposal of the disinfectant pollution is before discharge into receiving environments (Oh et al., 2013). In accordance with these assumptions the main objective of this study is to achieve biotransformation of BAC in the laboratory by immobilized cells of a BAC-degrading bacterial strain, *Pseudomonas sp.* BIOMIG1, which was recently isolated from activated sludge in our lab. The hypothesis below is going to be tested in this study.

Hypothesis

Immobilized cells of a BAC-degrading microorganism may be used for treatment of BAC-bearing wastewater.

Objective: To develop a packed bed reactor composed of immobilized cells for the treatment of BAC-bearing wastewater.

Approach: BAC-transforming cells will be immobilized into Ca-alginate and cell beads will be added both into LB (Luria Bertani) broth containing BAC and into BAC-bearing municipal wastewater, decrease in BAC concentration will be monitored. A packed-bed reactor will be made from cell beads and used for treatment of BAC-bearing municipal wastewater.

4. MATERIALS AND METHODS

4.1. Materials and Instruments

A BAC mixture (Lonza Inc., Switzerland) of 40% (w/w) dodecyl benzyl dimethyl 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), and 10% (w/w) hexadecyl benzyl dimethyl ammonium chloride (C_{16}BDMA-Cl, $C_{25}H_{46}NCl$, 396.1 g/mole) was used in batch experiments, and for preparation of LB-BAC medium. C_{12}BDMA-Cl and C_{14}BDMA-Cl (Tokyo Chemical Industry Co., Japan) in the pure form was used for continuous flow packed bed reactor experiments.

All the chemicals used throughout the experiments are purchased from Sigma Aldrich Chemicals Company (Germany). In high performance liquid chromatography (HPLC, Agilent 1260, U.S.), Luna® 5 µm C18 (2) 100 Å (Phenomenex, USA) column was used to measure BAC and BDMA concentration at 210 nm. Every sample was centrifuged at 10000 rpm for 10 min in microcentrifuge tubes and 0.6 mL of supernatant was taken for HPLC analysis. For wastewater characterization, inductively coupled plasma optical emission spectrometry (ICP-OES), atomic absorbtion spectroscopy (AAS), and ion chromatography (IC) were used to determine heavy metals, potassium (K^+), and anions, respectively.

4.2. Solutions and Media Preparation

Mineral salt medium (modified M9) was 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, 0.1 M $MgSO_4.7H_2O$, 0.01 M $CaCl_2$ and 0.1% (v/v) trace metal solution (0.5g/L $ZnCl_2$, 0.30g/L $MnCl_2.4H_2O$, 3.0g/L H_3BO_3, 2.0g/L $CoCl_2.6H_2O$, 0.10g/L $CuCl_2.2H_2O$, 0.20g/L $NiSO_4.6H_2O$ and 0.30g/L $Na_2MoO_4.2H_2O$).

After adding these except for $MgSO_4$ and $CaCl_2$, it was autoclaved at 121°C for 15 min, sterile 0.1 M $MgSO_4.7H_2O$ (6.16 g in 250 mL DI water) and sterile 0.01 M $CaCl_2.2H_2O$ (0.37 g in 250 mL DI water) were added as 10 mL into 1 L under aseptic onditions. LB-BAC broth was prepared adding BAC mixture into LB after sterilization of LB at 121°C for 15 min at a final concentration of 50 ppm. LB-BAC agar was prepared the same way, with an addition of 1.5 % (w/v) agar in the solution. Chromagar Pseudomonas (PS agar; CHROMagar Microbiology, France) was prepared adding 8.30 g of dry PS agar into 250 mL deionized (DI) water and boiling on a magnetic stirrer. 10 mM MOPS (3-n-morpholinopropanesulfonic acid) was prepared adding 2.1 g MOPS into 1 L DI water. The pH was then adjusted to 7.2 with 1 M NaOH (4 g NaOH in 100 mL DI water) and solution was autoclaved at 121°C for 15 min. MOPS buffered modified M9 was prepared excluding K_2HPO_4 and KH_2PO_4 in the recipe above and adding MOPS to have 10 mM MOPS instead. For 3% (w/v) sterile Na-alginate preparation, 6 g Na-alginate was poured slowly into a bottle containing 200 mL DI water on a stirrer. After about 45 minutes the solution was taken into autoclave at 121°C for 15 min. 0.15 M and 0.5 M $CaCl_2$ were prepared by addition of 22.1 g and 73.5 g $CaCl_2$ into DI water, respectively. Sterilization of $CaCl_2$ solutions were done by autoclaving them at 121°C for 15 min. In preparation of BAC containing media, either 10000 ppm (1 g BAC in 100 mL sterile DI water) or 1000 ppm BAC (1/10 diluted from 10000 ppm with sterile DI water) was utilized in solutions. For C_{12}BDMA-Cl and C_{14}BDMA-Cl 10 mM stock was prepared adding 0.34 g C_{12}BDMA-Cl and 0.38 g C_{14}BDMA-Cl in 100 mL sterile DI water. Sterile 0.85% (w/v) saline solution was prepared by adding 2.13 g NaCl in 250 mL DI water and autoclave. Eluent for HPLC contains 60:40 acetonitrile: 50 mM phosphate buffer (v/v, pH: 3.5). For packed-bed reactor setting, as an internal standard 20 μM BTMA was prepared by adding 0.371 g BTMA-Cl into 100 mL DI water and autoclaved at 121°C for 15 min. 60 mM BDMA stock was prepared by adding 9.2 mL BDMA into sterile DI water under aseptic conditions.

Wastewater used in this study was obtained from ISKI Paşaköy Advanced Wastewater Treatment Plant, Istanbul, Turkey. Sample was taken from the effluent of the secondary clarifier. Before use, the WW samples were filtered from Whatmann paper (11 μm pore size), autoclaved at 121°C for 30 min, filtered again with sterile 0.45 μm filter

under aseptic conditions. Characterization was accomplished after these treatments according to standard methods (APHA, 2012).

4.3. Culture Preparation

A pure culture of *Pseudomonas sp.* BIOMIG1 was previously isolated in our lab (Ertekin et al., 2015). We used a special strain of BIOMIG1 (BIOMIG1^{BDMA}), which could biotransform BACs to BDMA. Culture of BIOMIG1 used in all experiments was prepared as follows: 1 mL stock previously stored at -20°C in a 1.5 mL microcentrifuge tube was taken out and thawed. It was added into LB broth containing 50 mg/L BAC to a final volume of 5 mL in a 50 mL falcon tube. The culture was grown overnight on an orbital shaker at 130 rpm. Serial dilutions of 10^{-6}, 10^{-7} and 10^{-8} with sterile saline solution were prepared in microcentrifuge tubes and spread onto Chromagar Pseudomonas plates the next day. After an overnight growth on Chromagar Pseudomonas at 28°C the single colonies were patched onto LB-BAC agar via sterile wooden applicator. After overnight growth on LB-BAC agar at 28°C, half of the patch was mixed into 1 mL LB-BAC broth, and the other half into 2 mL modified M9 containing 50 mg/L BAC. The next day the overnight grown culture was added 180 µL glycerol and left in -20°C. The M9 media was analyzed in HPLC after 5 days of incubation at 130 rpm to confirm the BAC degradation activity of the culture used. Figure 4.1 depicts the schematic representation of these processes.

The culture used in the preparation of the alginate-beads was grown as follows: Four patches from 4 separate colonies on PS agar that had been previously confirmed as BAC-degraders were dipped into 4 separate Erlenmeyers containing 200 mL sterile LB-BAC broth. After overnight growth at 130 rpm the next day the media were transferred into aseptic centrifuge tubes and centrifuged at 10000 rpm for 10 min. The supernatants were discarded, pellets were resuspended in saline and centrifuged in the same conditions for washing, and supernatants were discarded. Pellets were resuspended in sterile saline

solution as 4 mL for each, and all the suspensions were collected into a sterile falcon tube. The tube containing dense culture was stored at 4°C for at most one day before use.

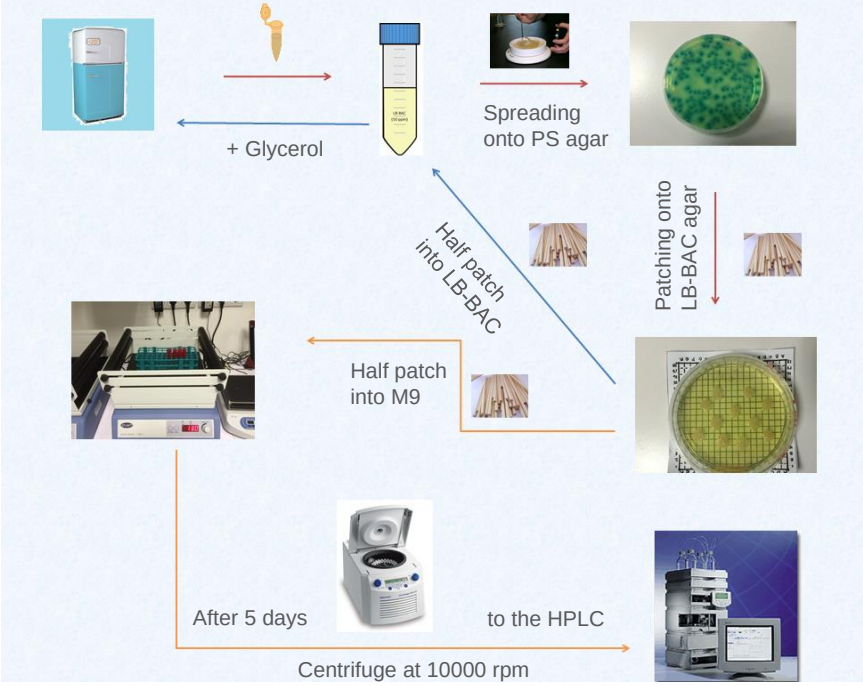


Figure 4.1: Culture stock preparation

4.4. Ca-Alginate Bead Preparation

Beads were formed via dripping 3% Na-alginate solution into $CaCl_2$ solution from a 20 cm height via peristaltic pump operated at 2.5 mL/min flowrate. After 20 min the pump was stopped and the beads were separated from $CaCl_2$ solution via a Buchner funnel. Beads on the funnel were washed with sterile 10 mM MOPS, and added into 10 mM MOPS solution with a sterile spoon. The beads in the solution were kept at +4°C. For the preparation of beads that contain cells, dense culture was added into Na-alginate under aseptic conditions. All the procedure was done under a laminar flow hood.

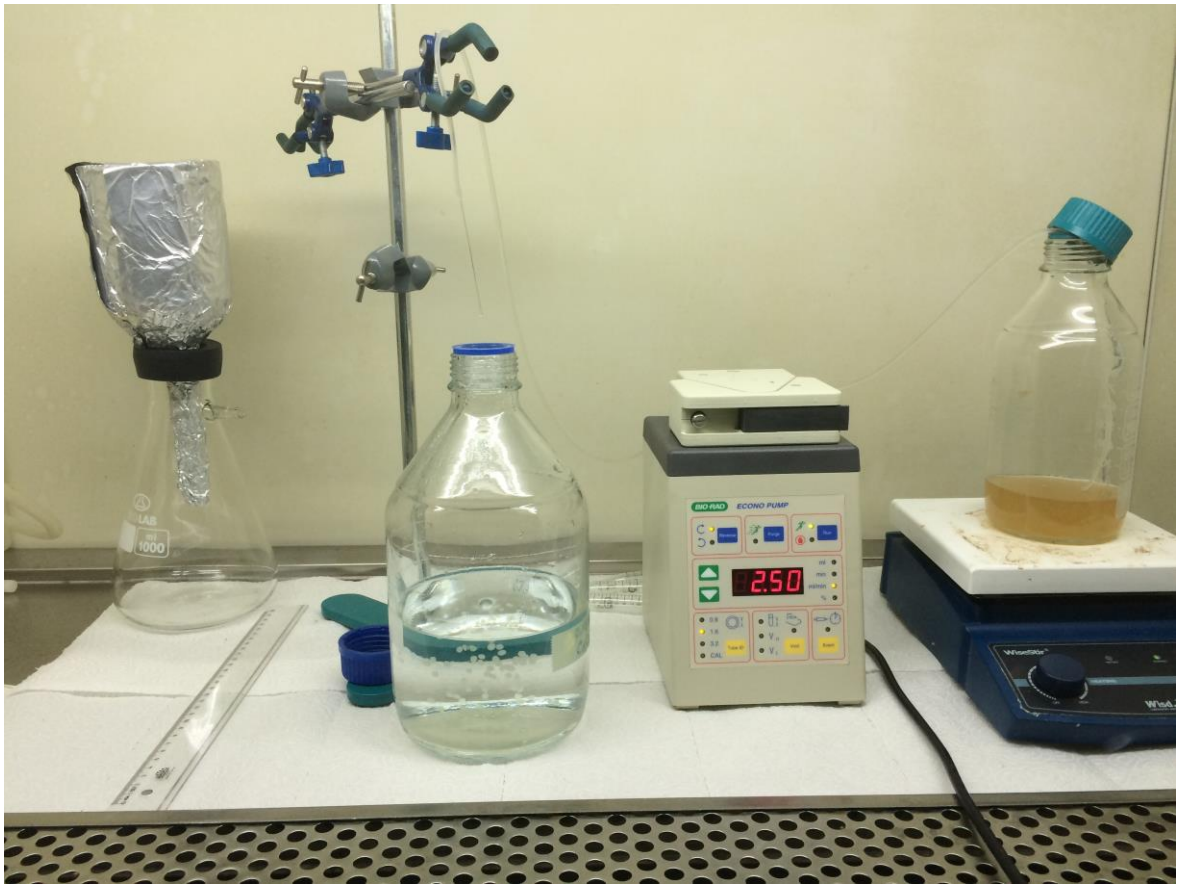


Figure 4.2: Ca-alginate bead preparation setup

4.5. Cell Concentration Measurements

Cell concentration in the effluent of the packed-bed reactor columns loaded with cell beads and empty beads, and in the reservoir bottles were analyzed in the course of the reactor operation. Samples were diluted with saline solution and spreaded over either PS agar, LB or LB-BAC agars. Colonies formed were counted and cell concentration was reported as colony forming units per mL (CFU/mL). All cell counts were done at least with three replicates.

Viable cell concetration in the alginate beads was determined as follows: A bead was transferred into 1.5 mL microcentrifuge tube and washed twice with saline solution.

After washing, the bead was dissolved in M9 buffer under strong agitation over a vortexer. The content was then appropriately diluted and spreaded over PS, LB-BAC agars.

4.6. Testing the BAC Biotransformation Activity of Beads

Four types of beads with different sizes and strength were synthesized. First two types of beads had 2 mm diameter, synthesized by dripping 3% alginate solution in to either 0.15 M or 0.5 M $CaCl_2$ through a 23 gauge needle. Second set of beads had 3 mm diameter and synthesized by dripping 3% alginate solution in to either 0.15 M or 0.5 M $CaCl_2$ through a Pasteur pipette. The cell concentration in the beads in which BIOMIG1^{BDMA} was entrapped was around 5 million CFU/3-mm bead, and 3 million CFU/2-mm bead. About 592±23 3-mm beads or 1239±35 2-mm beads were transferred into Erlenmeyer flasks containing 80 mL 100 times diluted M9 medium at 50 mg/L initial BAC concentration. Number of cells in each flask was around $3x10^9$ CFU. Flask containing beads with same type but without cells were prepared as negative controls. The flasks containing cell beads as well as empty beads as negative control were incubated at room temperature agitated on orbital shaker at 130 rpm for 5 days. Each day BAC concentrations were monitored using HPLC method described above. Same set of experiments were performed in MOPS buffered M9 as well.

4.7. BAC Biotransformation Kinetics of Beads

The BAC biotransformation kinetics of beads was investigated in 250-mL Erlenmeyer flasks using 3 mm beads synthesized in 0.15 M $CaCl_2$. Four parallels and a control were set using in MOPS buffered M9 containing 10 μM C_{12}BDMA and 10 μM C_{14}BDMA as described in the previous section. Samples taken every hour were measured using the HPLC method described above. The BAC biotransformation kinetics were simulated according to Michaelis-Menten growth model (Rittmann and McCarty, 2001). The model employs BAC concentration ($[BAC]$, μM), maximum specific rate of BAC

utilization (k, μmoles/cell·hr), cell density (X, cells/L) half saturation constant (K_{BAC}, μM) in the equation below:

$$-\frac{d[BAC]}{dt} = \frac{k[BAC]X}{K_{BAC} + [BAC]} \quad (1)$$

Bacterial growth has a rate directly related to BAC concentration when it is the only C source:

$$\frac{dX}{dt} = Y\ \frac{k[BAC]X}{K_{BAC} + [BAC]} - \ k_d X \quad (2)$$

Where Y (cells/μmoles BAC) is the yield coefficient and k_d (1/hr) is decay constant. If we assume that the bacterial growth is 0 at such low concentrations of substrate we can simplify the equation further as follows:

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

Where k' (μM/hr) is cell specific rate of BAC utilization. Since there is two substrates and one product in the reaction we can further simplify the equation as follows:

$$\frac{d[BDMA]}{dt} = \frac{k'[BAC]}{K_{BAC} + [BAC]} \quad (4)$$

The simulation was done in Berkeley-Madonna software using Runge-Kutta 4th order integration method.

4.8. Packed-Bed Reactor Setup

Two 32-mL glass chromatography columns were used as reactors. Each column was sterilized via autoclaving at 121°C for 15 min. Sterile glass beads occupying 4 mL of the effective column volume were transferred into each column as the supporting media. Then columns were filled with alginate beads. One column received beads with BIOMIG1 cells (a total of $3x10^9$ CFU) whereas the other was packed with beads containing no microorganism. Each column was attached to a Bunsen holder. A bottle containg aqueous medium with BACs was connected to the columns through a peristaltic pump via sterile Teflon tubings penetrated through bottle's cap. Exit of the column packed with BIOMIG1 beads was connected to a fraction collector to get samples at desired time intervals. On the other hand, the effluent of the other reactor was collected in a beaker. The reactors were set as up-flow. Flowrate in each reactor were adjusted using the peristaltic pump. Fraction collector was adjusted to collect 2 mL composite samples at 0.1 mL/min and 0.2 mL/min flow rates, and 4 mL composite samples at 0.4 mL/min and 0.8 mL/min flow rates. For 0.1 and 0.2 mL/min flow rates fractions were collected as 55 drops per fraction, and for 0.4 mL/min and 0.8 mL/min flow rates as 10 min and 5 min fractions, respectively. The packed-bed reactor setting is presented in Figure 4.3.

Mean residence time of the reactors at 0.1 mL/min, 0.2 mL/min, and 0.4 mL/min flow rates was determined as follows: Reactor packed with beads containing no microorganisms was fed with 10 mM MOPS containing 20 μM BTMA for 10 hydraulic retention times (HRTs). A solution containing 60 mM BDMA was introduced into the reactor for 1 min, 30 sec, and 15 sec when the reactor was operated at 0.1 mL/min, 0.2 mL/min, and 0.4 mL/min flow rates, respectively. Samples were collected at the exit of the reactor for 24 hrs and analyzed by HPLC.

Continuous flow reactor experiments were performed in three tiers. In the first tier, effect of HRT on removal of 10 μM C_{12}BDMA and C_{14}BDMA from MOPS buffered M9 medium was investigated. In the course of this tier, reactors were fed with aforementioned

medium at 0.1, 0.2, 0.4 and 0.8 mL/min flowrates. BAC removal performance of the reactor was determined not only using the difference between the influent and effluent BAC concetrations, but also the amount of BDMA produced. Maximum flowrate, thus the minimum HRT, at which over 90% BAC removal efficiency was achieved, was determined. In the second tier, the reactors were operated at the HRT/flowrate identified at the first tier but at 1 μM C_{12}BDMA and C_{14}BDMA concentration. In the third tier, the reactors were operated at the HRT/flowrate identified at the first tier and fed with secondary effluent taken from Pasakoy WWTP and fortified with 10 μM C_{12}BDMA and C_{14}BDMA.

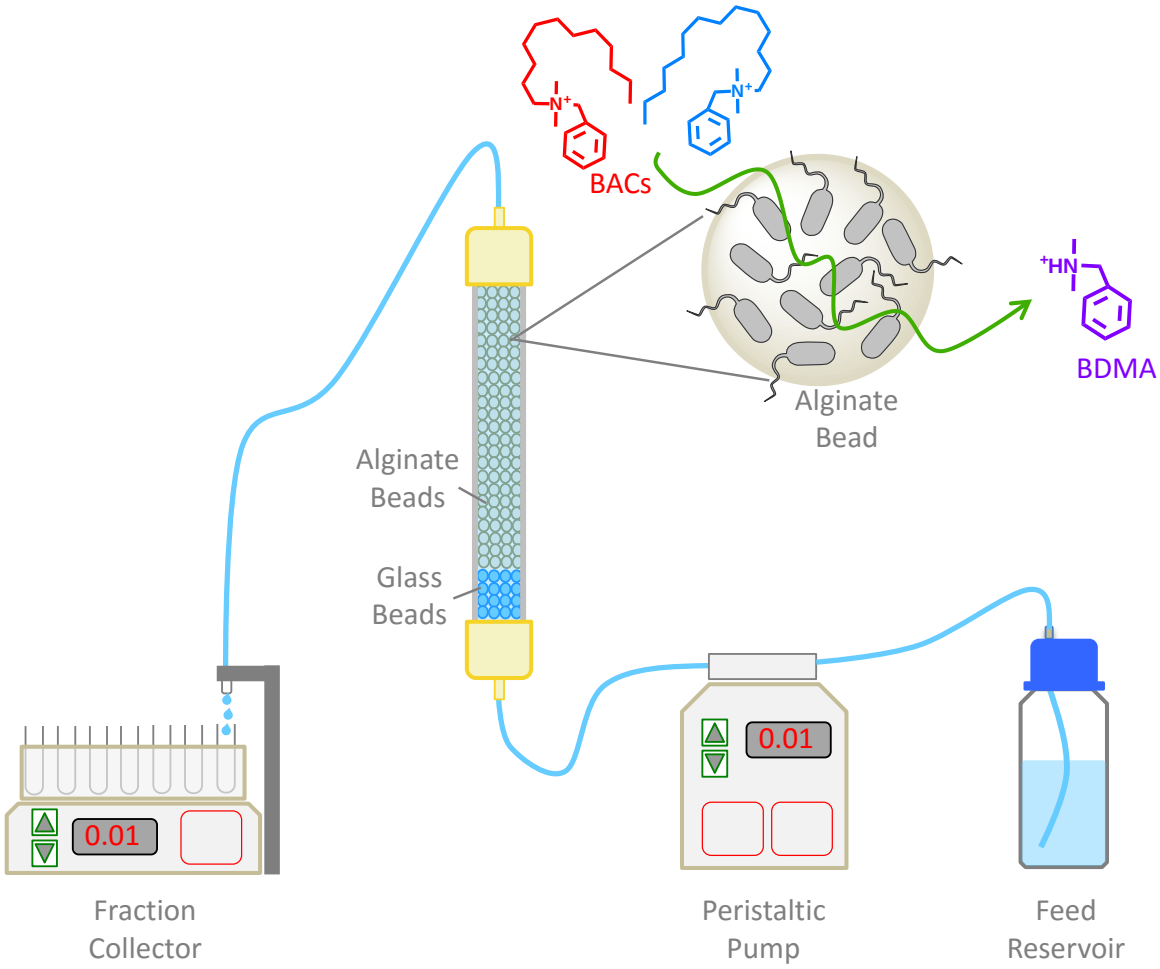


Figure 4.3: Schematic representation (upper) and actual photo (below) of packed-bed reactor setup

5. RESULTS AND DISCUSSION

5.1. Optimization of Alginate Entrapment and BAC Biotransformation Activity of Beads

Four type of beads were synthesized by dripping 3% alginate solution onto either 0.15 M or 0.5 M $CaCl_2$ solution via either 23 gauge needle or a Pasteur pipette. The beads generated using a Pasteur pipette were about 3 mm in size, whereas the beads formed via 23 gauge needle were 2 mm (Figure 5.1).

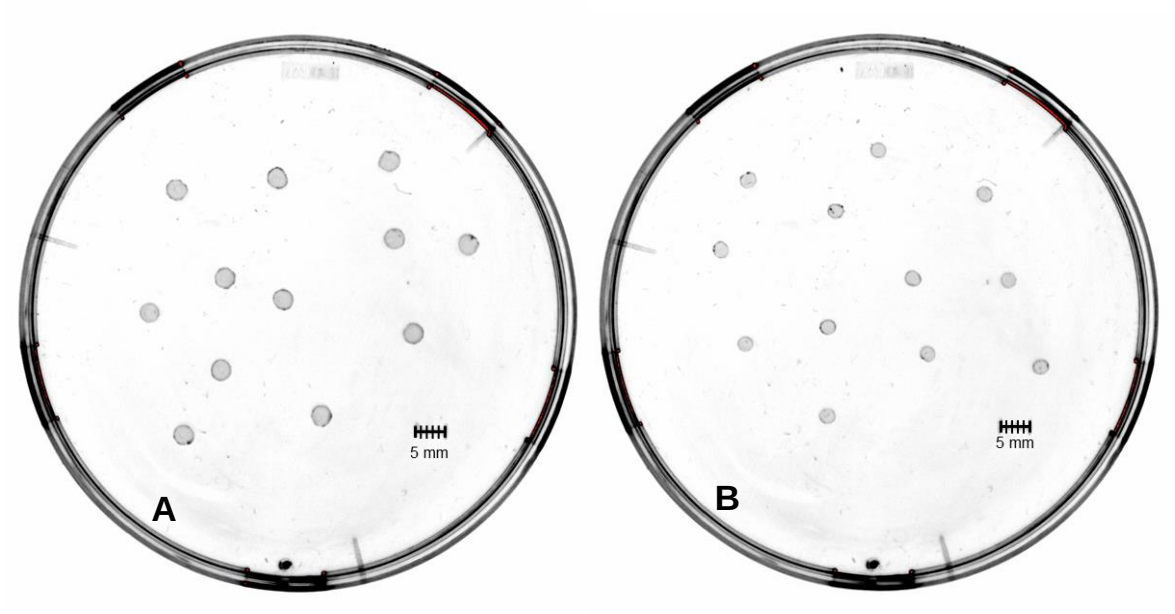


Figure 5.1: Ca-alginate beads generated via (A) Pasteur pipette, and (B) 23 gauge needle

An experiment was performed to test the BAC biotransformation activity of 4 types of beads synthesized in either 100 times diluted M9 medium or MOPS buffered M9 medium. About 20 g of beads transferred in to 250-mL Erlenmeyer flasks containing 80 mL medium at 140 μM BACs. The cell concentration in each flask was around $3x10^9$ CFU (Table 5.1).

Table 5.1: Bead characterization for batch BAC biotransformation assay

Bead type[a]	# of cells/bead ($x10^6$)	Weight of beads added to each reactor (g)	Initial cell concentration of the reactors ($x10^9$)
A1	4.6±1.4	17±2	2.7±0.8
A2	4.7±1.5	17±2	2.8±0.6
B1	2.4±0.9	20±1	3.0±1.1
B2	3.8±1.3	20±1	4.7±1.6

[a]Beads were formed dripping Na-alginate with cells into (1) 0.15 M or (2) 0.5 M $CaCl_2$ via (A) Pasteur pipette, or (B) 23 gauge needle.

BACs were converted to equimolar amount of BDMA within four days in all flasks prepared using 100 times diluted M9 medium. However BDMA formation was faster in the flasks containing beads prepared in 0.15 M $CaCl_2$ solution (Figure 5.2 A1 and B1). When effect of bead size on BAC biotransformation was delineated, we observed that biotransformation was relatively faster in the flasks prepared with 3-mm beads (Figure 5.2 A1). In the rest of the flasks we observed that BDMA formation was ceased within the second and third days and finally reached to the expected concentration at the end of the fourth day (Figure 5.2 A2, B1 and B2). On the contrary, BDMA formation was progressive in the flask containing 3-mm beads prepared in 0.15 M $CaCl_2$ solution.

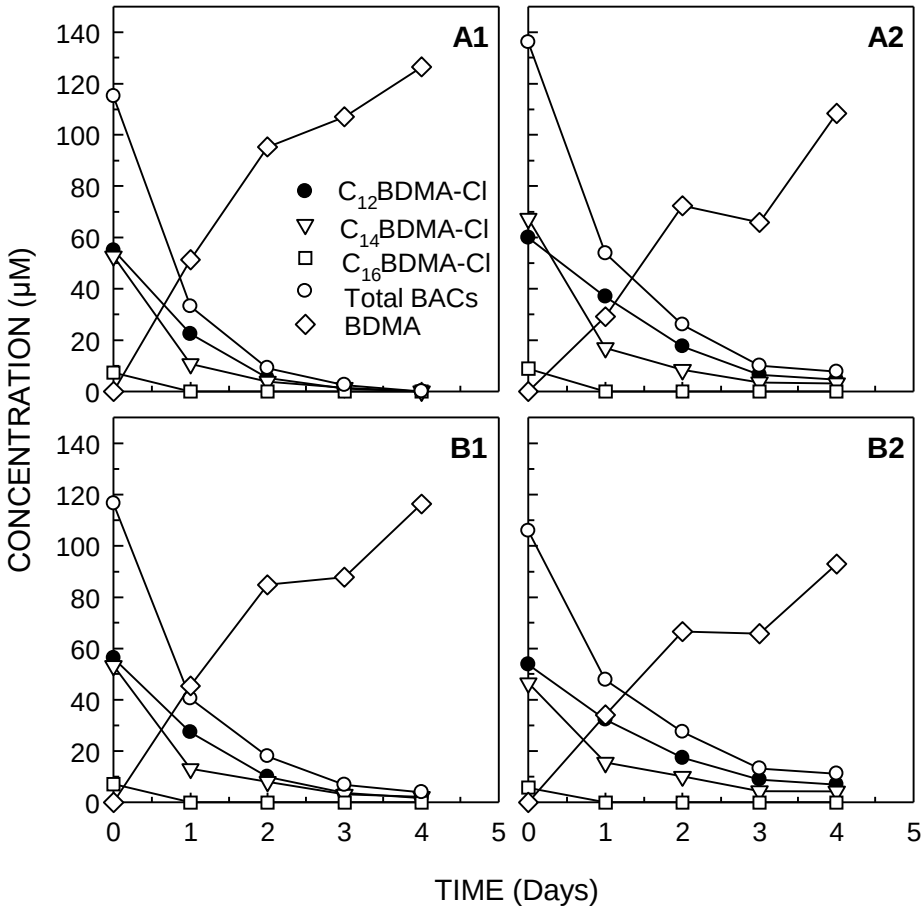


Figure 5.2: Profiles of BAC utilization and BDMA formation by (A) 3-mm and (B) 2-mm BIOMIG1^{BDMA} beads prepared with (1) 0.15 M and (2) 0.5 M $CaCl_2$ in 100 times diluted M9 medium

BAC biotransformation to BDMA was faster in MOPS buffered M9 medium for all type of beads and lasted 3 days (Figure 5.3). When BAC biotransformation rates of beads were compared, a similar trend observed in the flasks prepared using 100 times diluted M9 was reported. However, delay of BDMA formation in the flask containing 2-mm beads prepared in 0.15 M $CaCl_2$ solution was not observed in the MOPS buffered M9 medium (Figure 5.3 B1). Similarly, BAC biotransformation to BDMA was more progressive and efficient in the flask containing 3-mm beads prepared in 0.15 M $CaCl_2$ (Figure 5.3 A1).

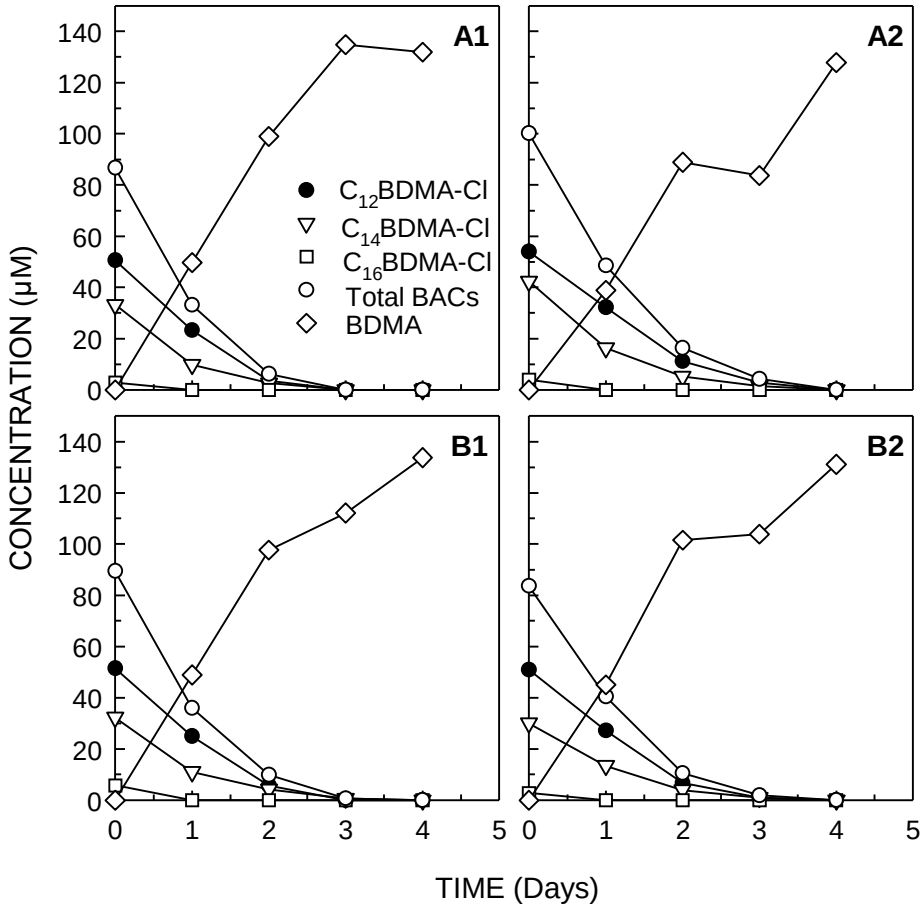


Figure 5.3: Profiles of BAC utilization and BDMA formation by (A) 3-mm and (B) 2-mm BIOMIG1^{BDMA} beads prepared with (1) 0.15 M and (2) 0.5 M $CaCl_2$ in MOPS buffered M9 medium

In control flasks where beads containing no microorganisms were used, the concentration of C_{14}BDMA-Cl and C_{16}BDMA-Cl slightly decreased whereas the concentration of C_{12}BDMA-Cl did not change (Figure 5.4 and 5.5). On the contrary, BDMA was not detected in any of the flasks. We attributed the disappearance of relatively hydrophobic BACs to their adsorption on the alginate beads. Other studies also showed that BAC with longer alkyl chain length have strong tendency to sorb onto organic surfaces (Garcia et al., 2006; Clara et al., 2007; Ismail et al., 2010). In addition, we suggested that the delays in the BDMA formation mentioned above was due to the effect of adsorption; that is bioavailability of BACs decreased as they sorbed onto the alginate.

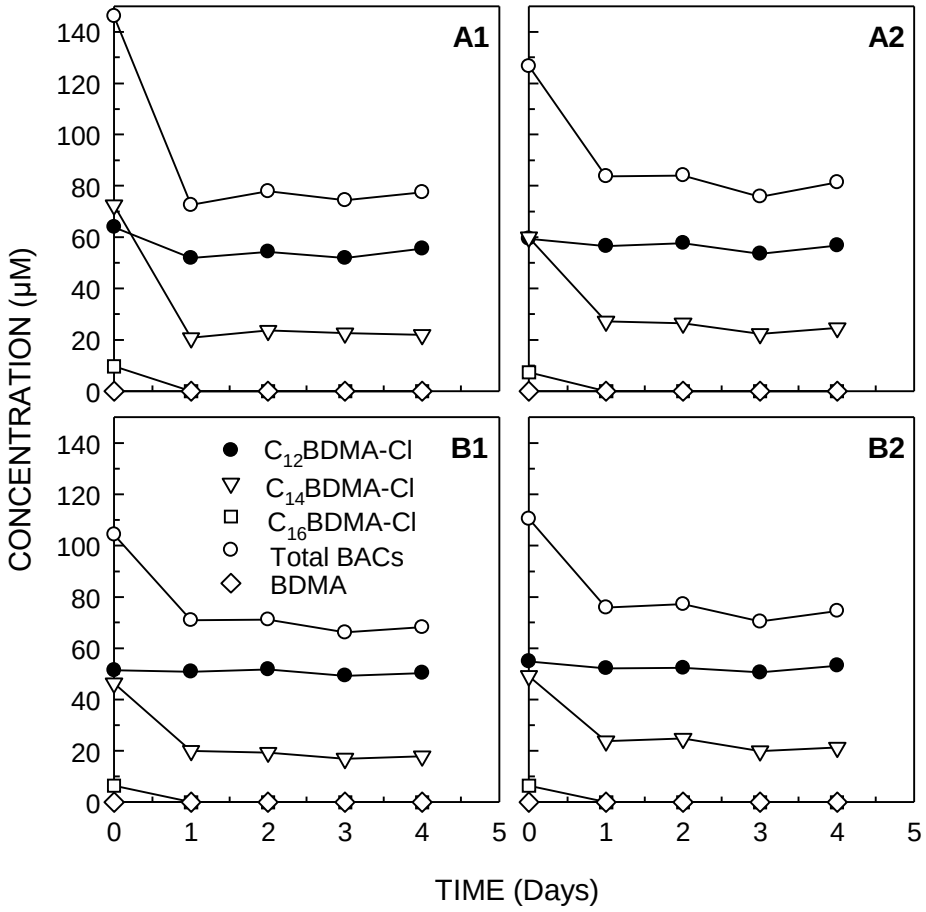


Figure 5.4: Profile of BACs in the flasks containing (A) 3-mm and (B) 2-mm alginate beads without microorganisms with (1) 0.15 M and (2) 0.5 M $CaCl_2$ in 100 times diluted M9 medium

Based on our observations, we proposed a sequence of processes involved in the biotransformation of BACs by the alginate beads as follows (Figure 5.6): (1) BACs enter into alginate beads via diffusion; (2) some portion of the BACs adsorp onto alginate beads. The portion adsorbed depends on the hydrophobicity of BACs; (3) BACs that do not adsorp are transformed into BDMA by $BIOMIG1^{BDMA}$ entrapped in alginate beads; (4) BACs that are adsorbed desorp and are transformed into BDMA by $BIOMIG1^{BDMA}$.

In both media, we reported that the most progressive and efficient BAC biotransformation was achieved with 3-mm beads prepared in 0.15 M $CaCl_2$. Based on the mechanisms decribed above, we suggest that although diffusion resistance is higher in 3-

mm beads compared to 2-mm beads due to lower surface area-to-volume ratio (Talekar and Chavare, 2012), adsorption is less due to the same reason. In addition to that, more cells are entrapped into 3-mm beads than 2-mm beads. Limited adsorption and high cell density in 3-mm beads facilitated efficient BAC biotransformation.

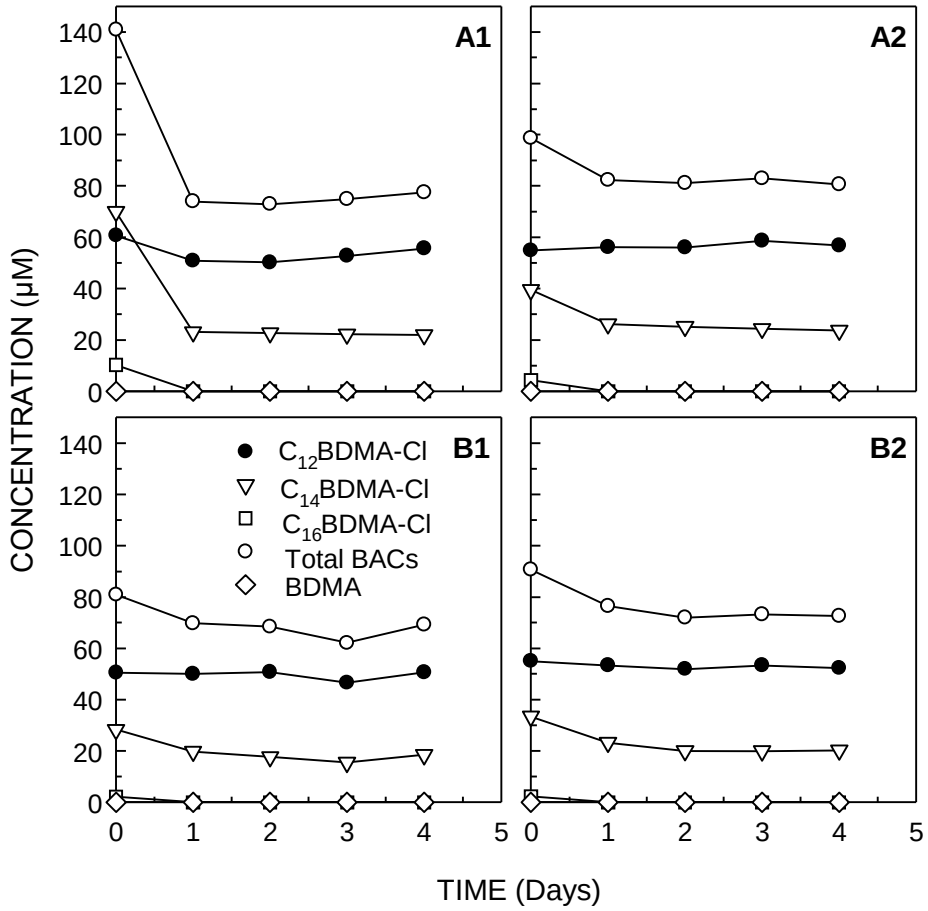


Figure 5.5: Profile of BACs in the flasks containing (A) 3-mm and (B) 2-mm alginate beads without microorganism with (1) 0.15 M and (2) 0.5 M $CaCl_2$ in MOPS buffered M9 medium

The effect of $CaCl_2$ concentration on formation of Ca-alginate beads is extensively discussed in the literature. In their study on α-amylase entrapment in Ca-alginate beads, Talekar and Chavare (2012) discovered that above 1 M $CaCl_2$ the immobilization yield significantly decreased. Won et al. (2005) confirmed these findings in $CaCl_2$ concentration range 0.05-0.3 M, although reduction in the immobilization yield was small. We also

observed higher efficiency of entrapment done in lower concentration of $CaCl_2$ (0.15 M) in different media and at different bead sizes.

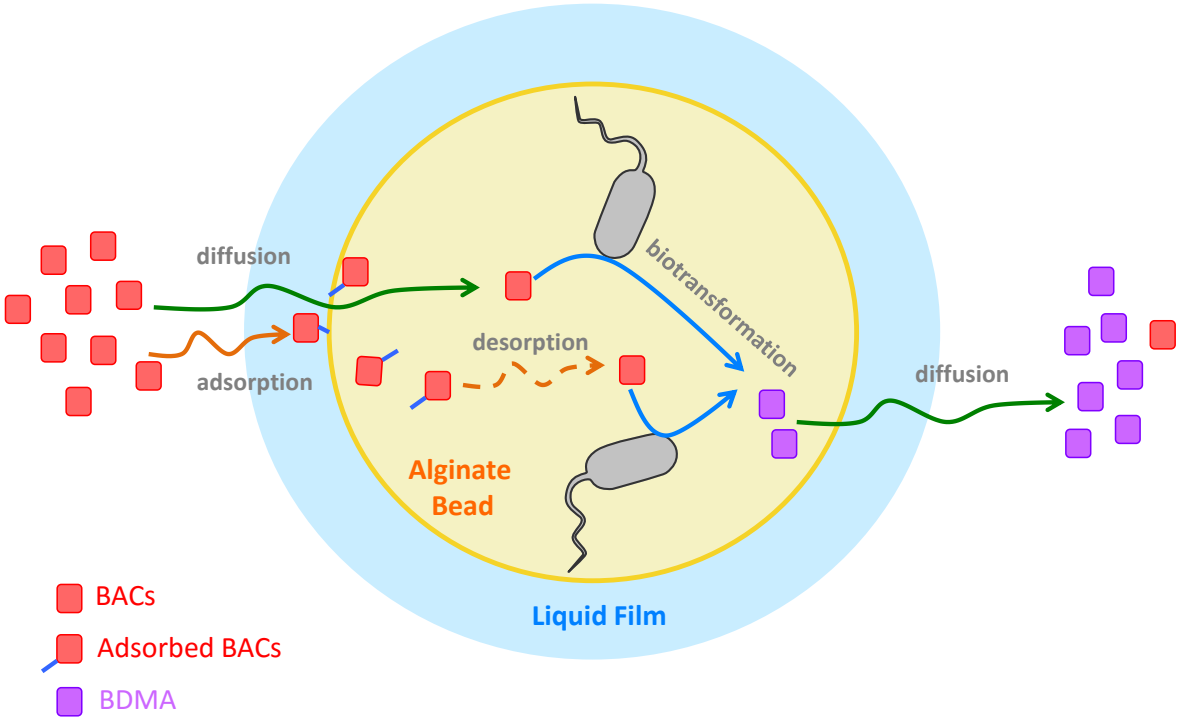


Figure 5.6: Proposed mechanisms governing the biotransformation of BACs to BDMA

Cell-entrapped beads in MOPS buffered media showed higher removal of BACs than the beads in 100 times diluted M9. These might have been partially due to reduced buffering capacity of 100 times diluted M9. Bickerstaff (1997) reported that phosphate buffers might cause deformation in the Ca-alginate beads. 100 times diluted M9 had phosphate salts for buffering. These salts might have altered the lattice structure of the beads.

In conclusion, 3-mm beads prepared in 0.15 M $CaCl_2$ and assayed in MOPS buffered M9 were found to be optimal and kinetic experiments were conducted at the same conditions.

BAC biotransformation kinetic experiment was conducted in the same conditions with the bead optimization assay, except the BAC concentration in the flasks was, 10 µM C_{12}BDMA and 10 µM C_{14}BDMA. All of the BACs was utilized within 50 hrs resulting in accumulation of equimolar amount of BDMA in the medium (Figure 5.7). Given the fact that not only biotransformation but also adsorption was a BAC removal mechanism, the rate of BAC biotransformation was estimated based on BDMA formation (Eqn-4). K_{BAC} value in the equation was derived from Yılmaz et al. (2014) by taking the average of the values for the individual compounds weighing by molar fraction (in this case molar fractions were equal). As a result, K_{BAC} was taken as 0.54 µM. The cell specific rate of BAC utilization (k') was calculated as 0.46 µM/hr. R^2 of the estimation was 97.8% which was consistent with the value reported by Yilmaz et al. (2014).

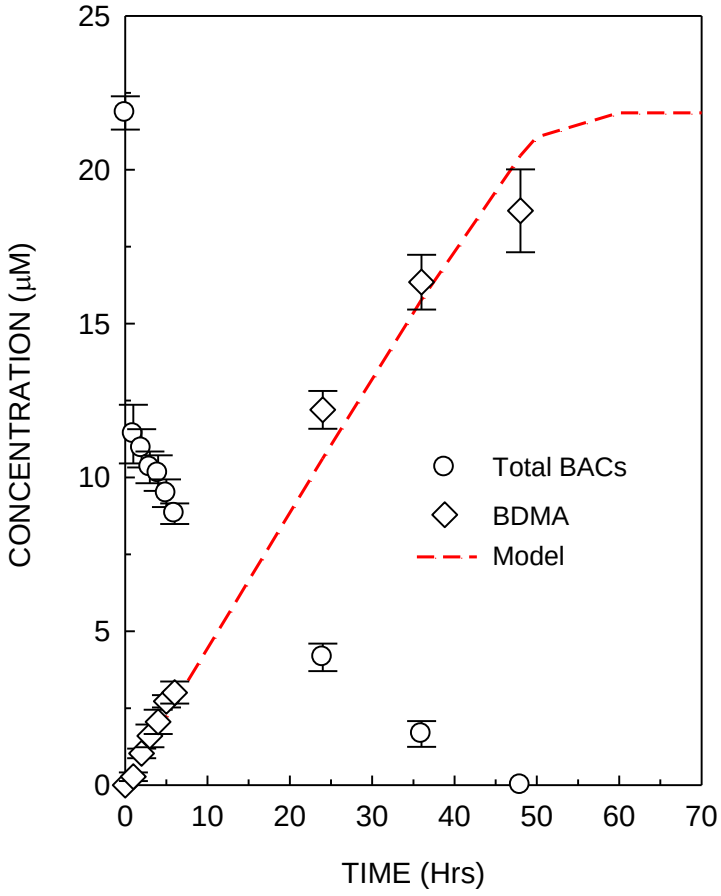


Figure 5.7: Profile of BAC utilization and BDMA formation 3-mm beads prepared in 0.15 M $CaCl_2$ at 10 µM C_{12}BDMA-Cl and C_{14}BDMA-Cl (R^2 = 0.98)

5.2. Effect of HRT on BAC Removal in the Packed-Bed Reactor

A column reactor was prepared by packing 28 mL empty volume with 3-mm alginate beads prepared in 0.15 M $CaCl_2$ solution and containing $BIOMIG1^{BDMA}$. The reactor was fed with MOPS buffered M9 medium prepared at 10 μM C_{12}BDMA-Cl and 10 μM C_{14}BDMA-Cl. The feed was introduced into the reactor through a peristaltic pump at a flowrate 0.1, 0.2, 0.4 or 0.8 mL/min for almost a month. The corresponding operational HRTs were 4.67, 2.33, 1.17 and 0.58 hrs.

In order to determine the mean residence time (MRT) in the reactor, a slug dose of BDMA was injected into the reactor while operating at 0.1, 0.2 or 0.4 mL/min flow rates. Samples were collected at the exit of the reactor via fraction collector and the BDMA was quantified using HPLC. Peak BDMA concentration was detected after 1.3 hrs, 0.83 hrs, and 0.33 hrs of BDMA dosing at 0.1 mL/min, 0.2 mL/min and 0.4 mL/min flowrates, respectively (Figure 5.8). Those values corresponded to MRTs at each flowrate. BDMA was not eluted completely from the reactor after 24, 12 and 6 hrs suggesting that ideal plug flow could not be sustained and diffusion was effective in the reactor (Figure 5.8).

BAC and BDMA concetration in the influent and the effluents of the test and control reactors were monitored. BAC concentration in the influent before the feed bottle was connected to the setup and after it was disconnected from the setup displayed no significant difference. Samples taken from the feed bottle during the operation period was spreaded on PS or LB agar to check if there was any microbial contamination in the influent. Bacterial growth was not observed suggesting that reactors was fed aseptically during the operation period. Microorganisms were not detected even at the exit of the control reactor suggesting that our set was contamination free in the course of operation. Therefore we attributed $BIOMIG1^{BDMA}$ in the test reactor was the only microorganism responsible for any BAC removal and BDMA formation in the test reactor.

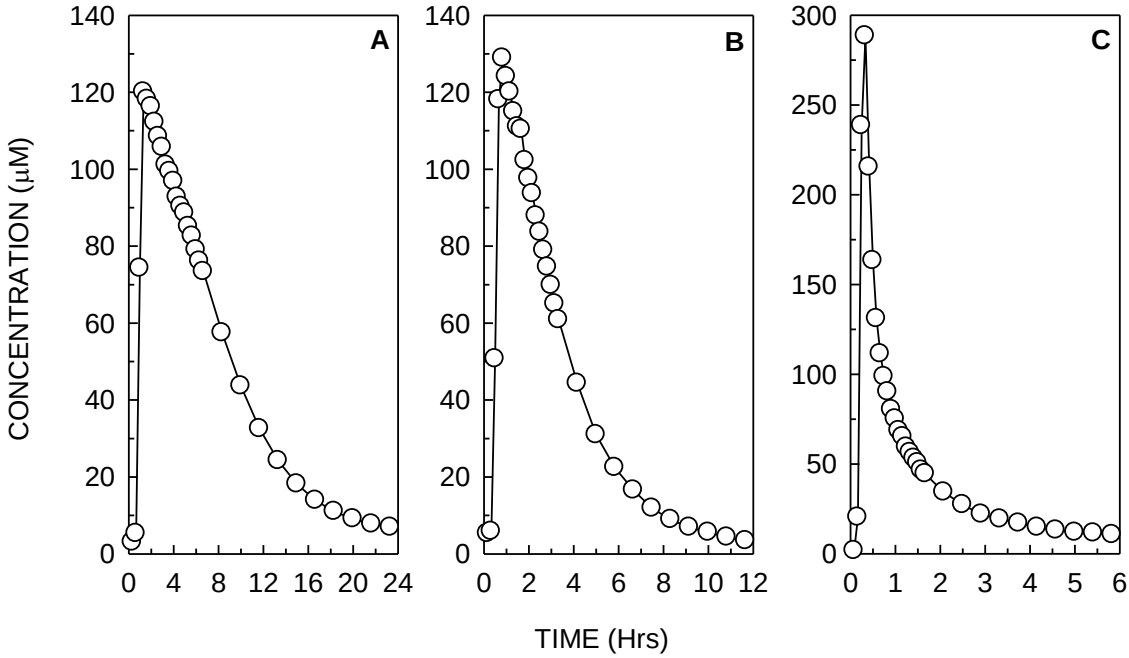


Figure 5.8: MRT determination for flow regimes (A) 0.1 mL/min, (B) 0.2 mL/min, and (C) 0.4 mL/min

Influent mean BAC concentration was 19.0±0.7 μM and did not change in the course of the reactor operation, suggesting that reactors were fed with a stable feed composition throughout the operation period and there was no abiotic or biotic transformation process affecting the feed composition (Figure 5.9).

Total BAC concentration at the effluent of the control reactor was lower than that of the influent for initial three days (Figure 5.10B). When individual BACs were taken into the account, we observed that C_{12}BDMA-Cl concentration was equal to its concentration in the influent, whereas the concentration of C_{14}BDMA-Cl was significantly lower than the concentration in the effluent for the three days (Figure 5.10B). Depending on our previous observations obtained during batch experiments, lower C_{14}BDMA-Cl concentration observed in the first three days of operation was attributed to the sorption of this BAC to the alginate beads. After three days, C_{14}BDMA-Cl concentration gradually reached to about 10 μM and stayed constant in the course of the operation period (Figure 5.10B).

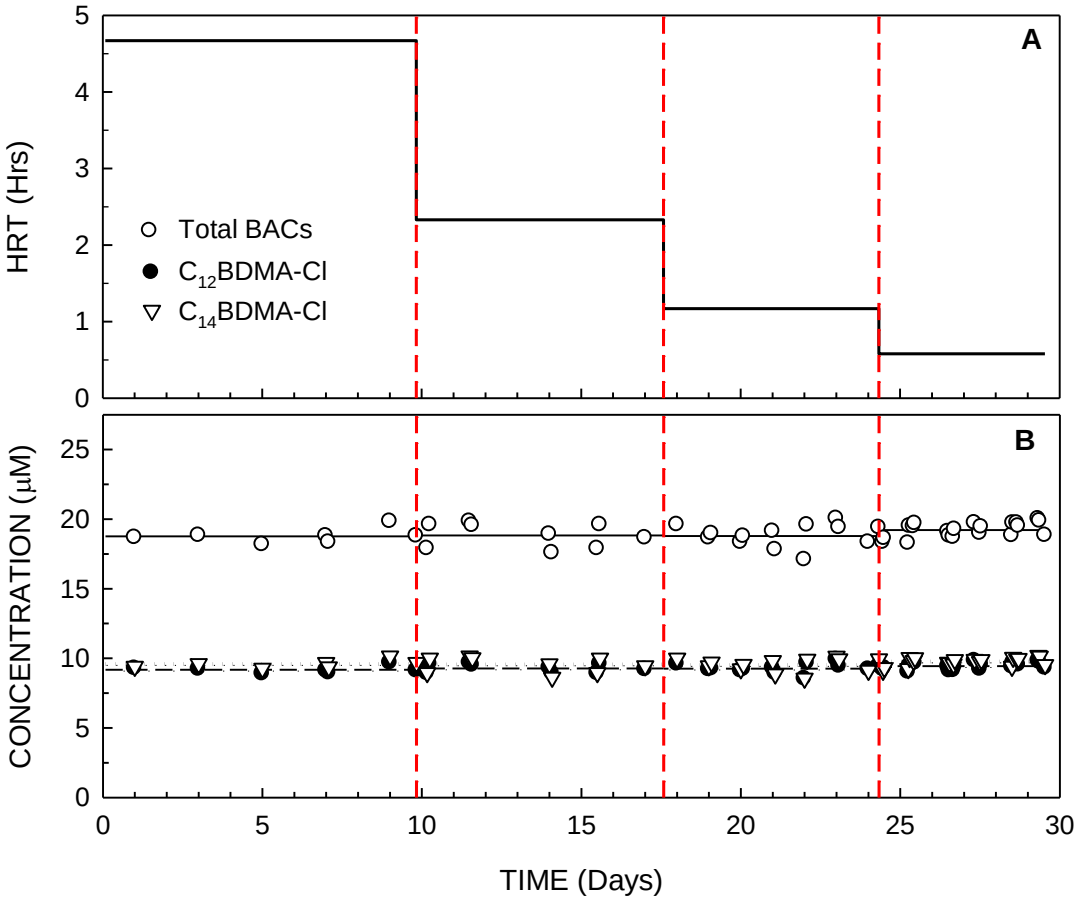


Figure 5.9: (A) HRTs applied and (B) influent BAC concentration during the course of reactor operation

When the test reactor containing BIOMIG1^{BDMA} entrapped in Ca-alginate beads was operated at 4.67 hrs HRT, BACs at the effluent of the reactor gradually decreased and could not be detected after 3 days (Figure 5.11B). At the same time, BDMA concentration gradually increased and reached to a concentration equal to total BAC concentration fed into the reactor, suggesting that all BACs were transformed into BDMA by BIOMIG1^{BDMA} beads (Figure 5.11C).

After about 10 days of operation at 4.67 hrs HRT, the flowrate of the feed was elevated to 0.2 mL/min which maintained an HRT of 2.33 hrs in the reactor. The reactor was operated about a week at this HRT. During the operation period, the total BAC

concentration in the effluent was 0.3±0.2 µM (Figure 5.11B) whereas the BDMA concentration was 17.2±0.6 µM (Figure 5.11C).

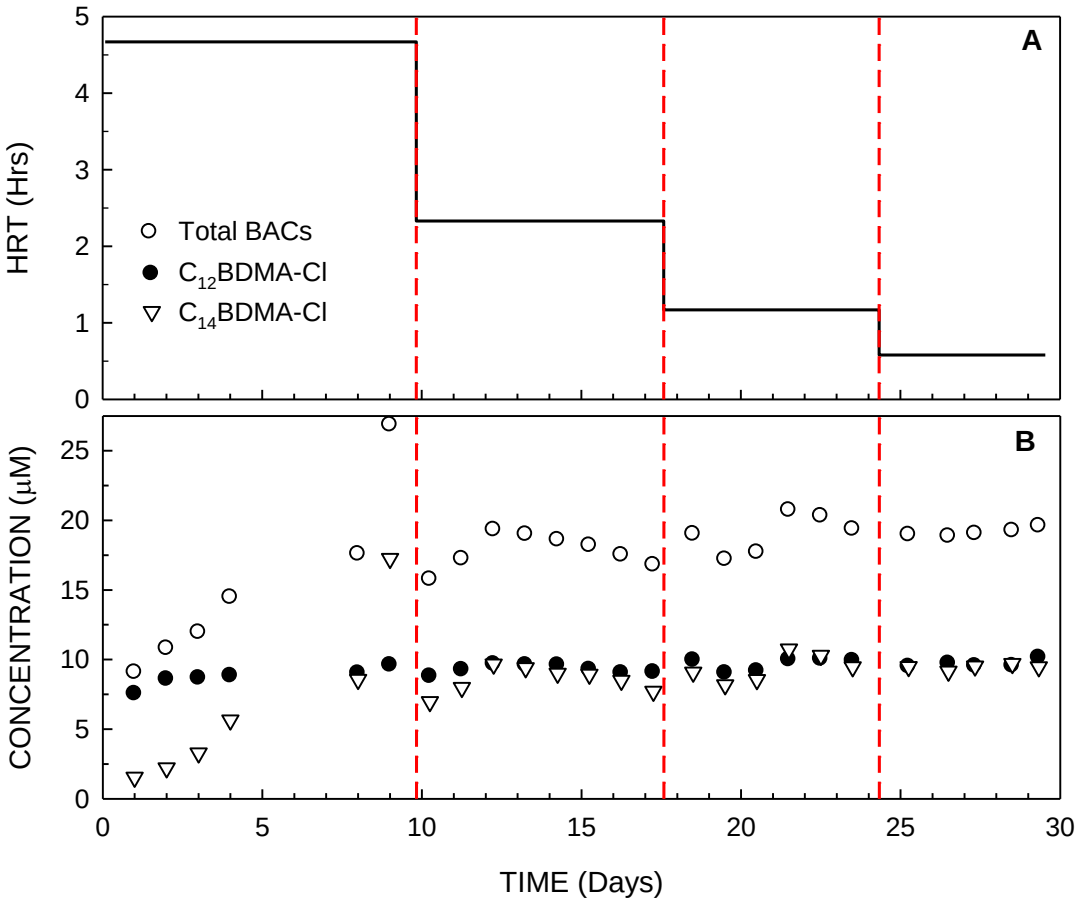


Figure 5.10: (A) HRTs applied and (B) profile of BAC concentration in the effluent of the control reactor during the course of operation

Flowrate of the feed was further increased to 0.4 mL/min. The HRT of the reactor was 1.17 hrs at this flowrate. The reactor was operated for 7 days at this HRT. Total BAC concentration in the effluent of the reactor increased to 1.5±0.5 µM (Figure 5.11B) whereas BDMA concentration decreased to 16.0±0.6 µM (Figure 5.11C).

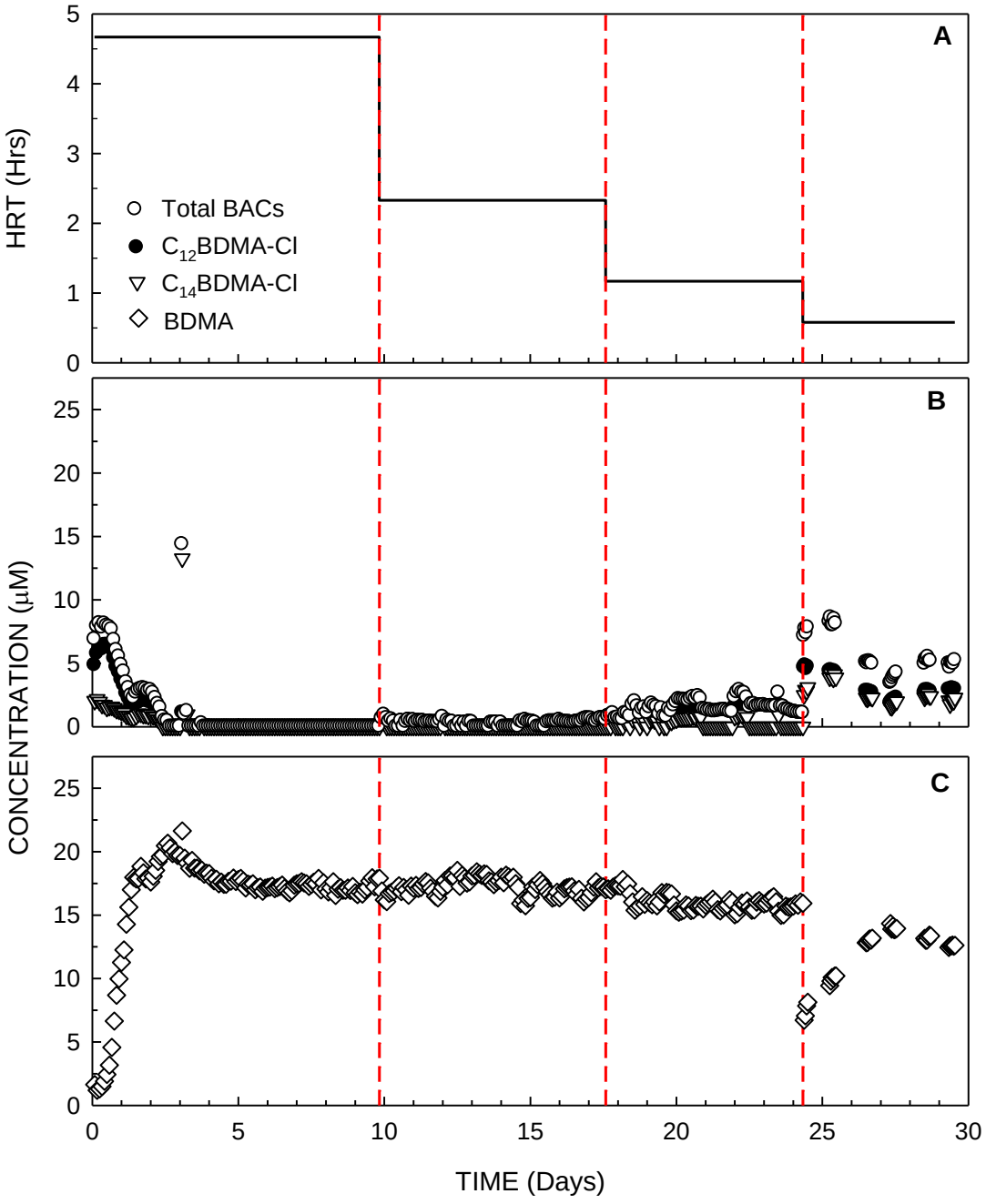


Figure 5.11: (A) HRTs applied and profile of (B) BAC and (C) BDMA concentration in the effluent of the test reactor during the course of operation

At the last stage of the operation, flowrate of the feed was increased to 0.8 mL/min corresponding to an HRT of 0.58 hrs. At this HRT, the mean total BAC concentration in the effluent increased significantly and reached to 5.7±1.6 μM. As a result, BDMA concentration decreased to 11.9±2.1 μM. In addition, the BIOMIG1^{BDMA} cells in the

effluent of test reactor at different HRTs were quantified. Effluent cell concentrations were 8.3±1.0 x10^5 CFU/mL, 2.2±0.2 x10^5 CFU/mL, and 2.0±1.5 x10^5 CFU/mL in the effluents of settings with 2.33 hrs, 1.17 hrs, and 0.58 hrs HRT, respectively. This result suggests that cells grown in the beads escaped from the reactor.

The efficiency of BAC removal in the test reactor at different HRT values is given in Figure 5.12. 90-100% interval is highlighted in green, 85-90% interval is highlighted in pink. The efficiency was calculated by dividing the BDMA concentration of individual effluent fraction to the mean value of influent total BAC concentration. When the stability in the efficiency was established, average of the efficiencies in that stable time interval was considered as removal efficiency at such HRT. Observed mean efficiencies at 4.67 hrs HRT and 2.33 hrs HRT were over 90% (0.93±0.02 and 0.91±0.03, respectively). In the flow regime with 1.17 hrs HRT 85±3% mean efficiency was observed. Minimum HRT at which above 85% BAC removal efficiency achieved was identified as 1.17 hrs. Both the control and test reactor was operated at this HRT in the rest of the experiments.

5.3. BAC Removal in the Packed-Bed Reactor at a Low BAC Concentration

Both control and test reactor was operated at 1.17 hrs HRT with a feed containing 2 μM total BAC (1 μM C_{12}BDMA-Cl + 1 μM C_{14}BDMA-Cl) concentration for 7 days. The BAC concentration in the influent was stable and equal to 1.9±0.2 μM (Figure 5.13A). The BAC concentration in the effluent of the control was similar to the influent BAC concentration suggesting that biotic and abiotic transformation processes were not present in the absence of BIOMIG1^{BDMA} (Figure 5.13B). BACs were not detected in the effluent of the test reactor (Figure 5.13C). On the other hand, BDMA at a concentration equal to the total BAC concentration fed into the reactor was reported in the effluent of the reactor (Figure 5.14D). The average BAC removal efficiency of the test reactor after 2nd day was 102±11% (Figure 5.14E). About 4.1±0.6 x10^4 CFU/mL cells were detected in the effluent of the test reactor. Significantly lower amount of cell concentration at the effluent of the test reactor operated at 2 μM total BAC concentration compared to the reactor operated at

20 µM was attributed to the slower growth rate at low substrate concetrations which can be explained by microbial kinetics (Eqns, 1 and 2).

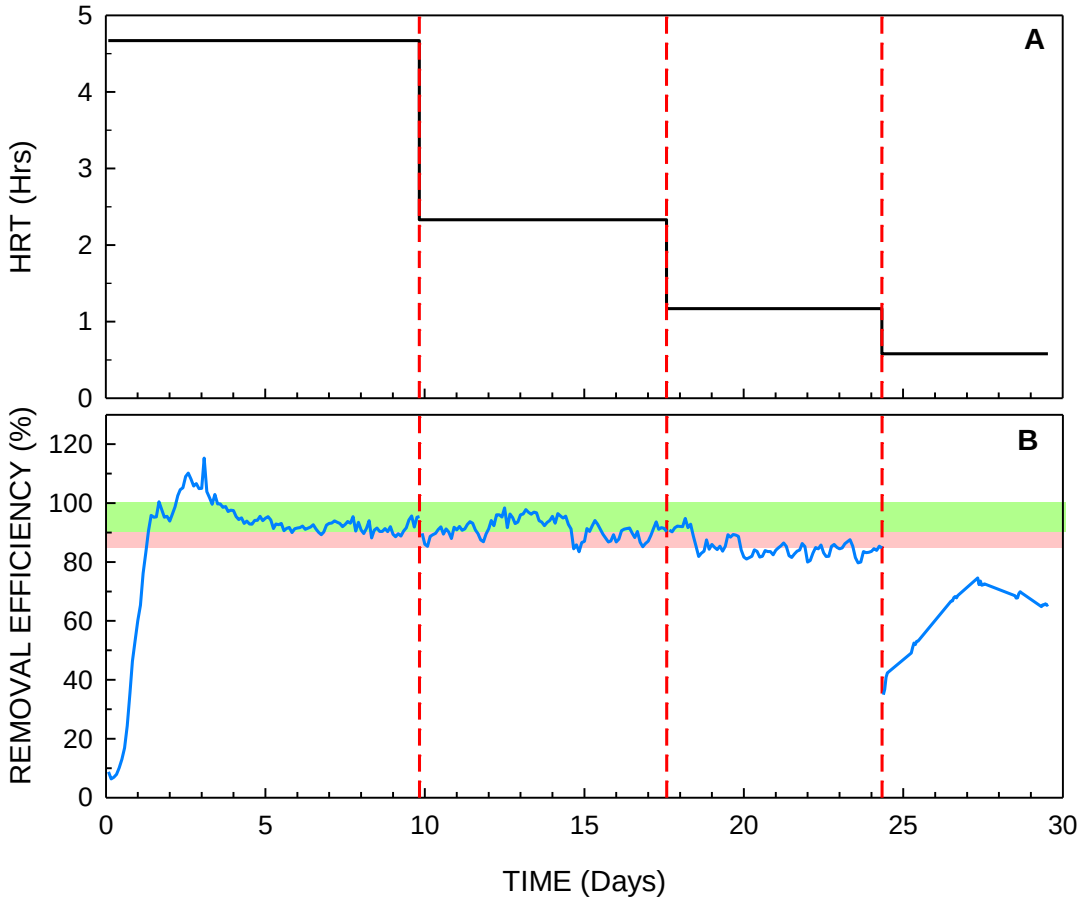


Figure 5.12: (A) HRTs applied and (B) BAC removal in the test reactor during the course of operation

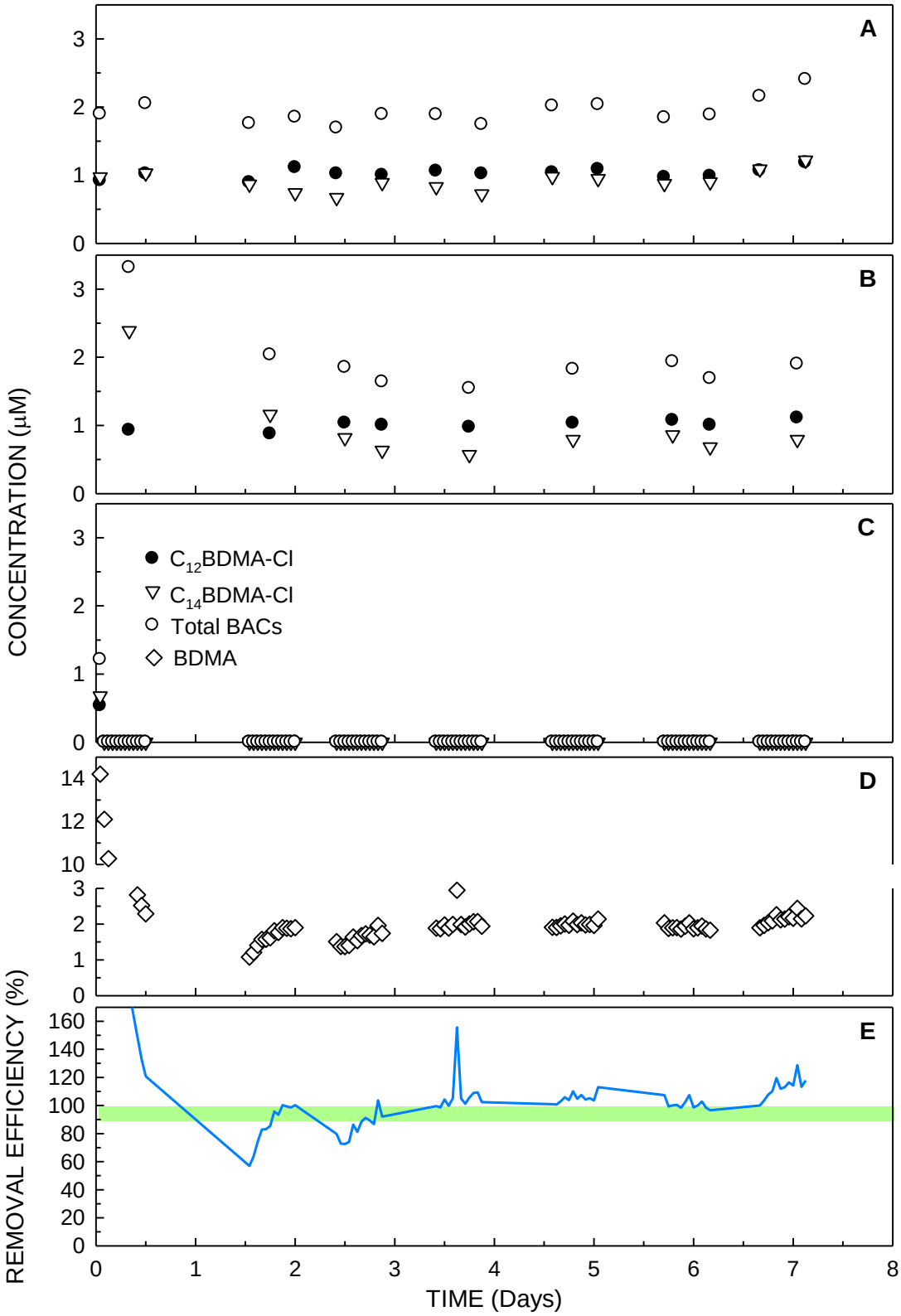


Figure 5.13: Profile of BAC concentration in the (A) influent, (B) effluent of the control reactor, (C) effluent of the test reactor, (D) BDMA in the effluent of the test reactor, and (D) BAC removal efficiency at 1.17 hrs HRT and 2 µM BAC concentration

5.4. BAC Removal from Secondary Effluent in the Packed-Bed Reactor

Control and test reactors were operated at 1.17 hrs HRT and fed with wastewater taken from the effluent of the secondary clarifier of Pasakoy WWTP. Wastewater was fortified with BACs to maintain 10 μM C_{12}BDMA-Cl and 10 μM C_{14}BDMA-Cl. The reactors were operated for 7 days. After preparative operations defined in Materials and Methods (section 4.2) were done, wastewater had a pH of 8.63±0.02, and total organic carbon (TOC) of 14.1±1.5 mgC/L. Concentrations of Ni^{2+}, Si^{2+}, and Al^{3+} were 40±0.6 μg/L, 42±2 mg/L, and 25±2 μg/L, respectively. Concentrations of Mo, Zn, Pb, Co, Cd, Fe, Mn, Cr, and Cu were below the detection limit. Concentrations of K^{+}, F^{-}, NO_3^{-}, PO_4^{3-}, Cl-, and SO_4^{2-} were 21±0.4 mg/L, 0.28±0.09 mg/L, 9.2±0.14 mg/L, 2.7±0.04 mg/L, 104±1.5 mg/L, and 100±2.4 mg/L, respectively.

Average BAC concentration in the influent was measured as 18.1±1 μM (Figure 5.14A). In the first 2 days of operation, there was a course of adaptation of BIOMIG1^{BDMA} to incoming wastewater. After those days, total BAC concentration was below 1 μM and BDMA concentration was 17.2±0.9 μM as an average throughout the study. Average of the efficiencies after day 2 was found to be 95±5%. Effluent of the test reactor contained 1.2±1.1 x10^5 CFU/mL cells.

Beads in the test reactor were also analyzed for cell concentration; it was 1.4±1.1 x10^6 CFU/mL. This value is lower than the initial cell concentration of the beads. This might be due to operating conditions. If the flow rate is too high against cell growth, cell release might occur.

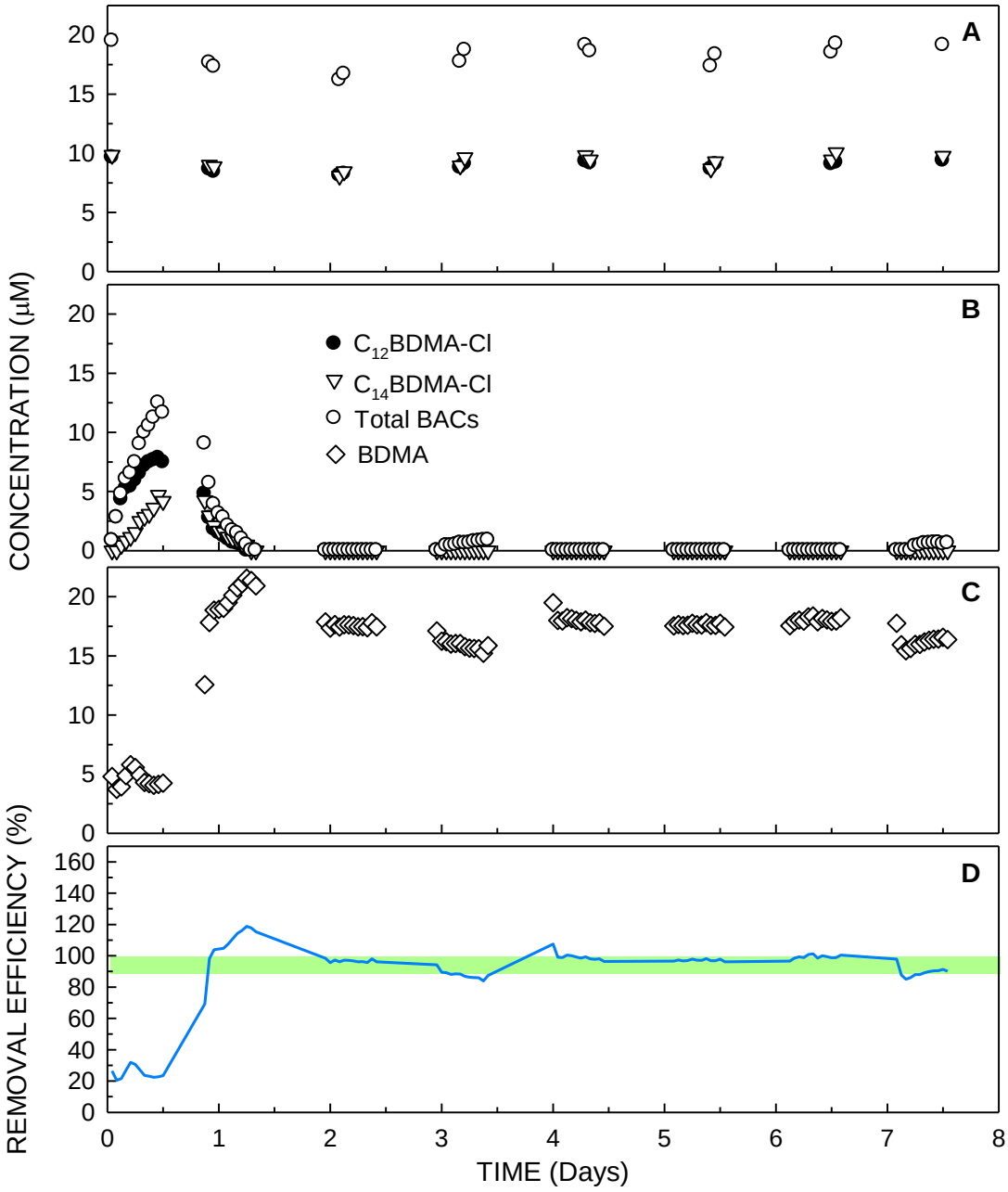


Figure 5.14: Profile of BACs in the (A) influent and (B) effluent of the test reactor, and (C) BDMA in the effluent of the test reactor. (D) BAC removal efficiency in the reactor operated at 1.17 hrs HRT with secondary effluent as the feed

Biodegradation of BACs is studied in the literature at community level and at species level (Oh et al., 2014, Tandukar et al., 2013). Tandukar et al. (2013) reported that *Pseudomonas* spp. predominated the community after prolonged exposure to BACs. Oh et al. (2014) reported partial degradation of BACs by *Pseudomonas nitroreducens*. BIOMIG1 is the only species reported in the literature so far, which can degrade BACs completely (Tezel and Pavlostathis, 2015). This study showed that this strain could be utilized for a specialized wastewater treatment technology.

With these experiments it was demonstrated that BAC removal was possible from continuous flow systems using Ca-alginate gel beads for entrapment of a novel BAC degrader, *Pseudomonas sp.* BIOMIG1^{BDMA}, a mutant strain that transforms BACs into BDMA. With the results obtained in this study it is suggested that BAC biodegradation in the effluents of biological wastewater treatment plants is feasible with immobilized cells for observed >90% removal of C_{12}BDMA and C_{14}BDMA. Metagenomics and proteomics studies on BAC resistance and degradation is present in the literature (Machado et al., 2013; Oh et al., 2013; Oh et al., 2014). It is proposed that the immobilization of identified enzymes responsible for BAC degradation for investigating enzyme activity in continuous flow systems as a future research.

6. CONCLUSIONS

In the course of this research, *Pseudomonas sp.* BIOMIG1^{BDMA} which can transform BACs into BDMA was immobilized into Ca-alginate gel beads. Biotransformation assays showed that optimum beads were the ones which were produced with a diameter of 3 mm in 0.15M $CaCl_2$. A considerable decrease in aquous C_{14}BDMA concentrations in negative controls having empty beads pointed out adsorption of that compound onto bead surface. Kinetic experiments showed lower activity of immobilized cells than that of the free cells. The cell specific BAC utilization rate was found to be 0.46 μM/hr. Lower rate might be explained by diffusion resistance against substrate movement into the beads. Adsorption of C_{14}BDMA also lowered the utilization rate. The continuous flow packed bed reactor experiments showed optimal mean residence time as 0.3 hrs where the efficiency was 85±5%. This was further confirmed by low level BAC concentration experiments (2 μM) and the ones with wastewater. These two experiments yielded removal efficiencies of 102±11% and 95±5%, respectively.

In conclusion, immobilized BIOMIG1^{BDMA} in Ca-alginate for treatment of a continuous flow system showed that this method is efficient for removal of BACs from wastewater streams.

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