FATE AND EFFECT OF ALKYL BENZYL DIMETHYL AMMONIUM CHLORIDE IN MIXED AEROBIC AND NITRIFYING CULTURES

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Dedicated to

My family

for their eternal love and support

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SUMMARY

Quaternary ammonium compounds (QACs) are widely used in commercial and consumer applications as disinfectants, fabric softeners, hair conditioners, and emulsifying agents. The massive production and utilization of QACs has led to their extensive discharge into the environment, raising concerns globally. Several studies have reported on potential risks and detrimental effects of QACs on the natural environment and public wastewater treatment plants. Biological treatment has been found to be an effective way to remove QACs and especially aerobic treatment processes can provide rapid biodegradation via a consortium of bacteria. Although extensive research has been conducted on the fate and effect of QACs, relatively little is known about their effect on aerobic biological treatment processes, especially on nitrification.

Research was conducted on the fate and effect of alkyl benzyl dimethyl ammonium chloride (AB), a QAC widely used as disinfectant, in mixed aerobic and nitrifying cultures. The results of this study demonstrated that up to 50 mg/L AB was efficiently degraded in a mixed aerobic culture fed with dextrin and peptone, although trace residual AB levels were observed. Nitrification of the produced ammonia was complete at an AB concentration of 20 mg/L after an acclimation period, but was almost completely inhibited at 50 mg/L. Mixed aerobic cultures maintained only with AB as external nitrogen and carbon source achieved a high degree of AB degradation at both 20 and 50 mg/L.

Ammonia oxidation by a nitrifying culture, enriched with ammonium chloride and sodium bicarbonate, was inhibited with increasing AB concentration and completely

ceased at 15 mg/L AB. Degradation or utilization of AB was not observed for all tested AB concentrations between 2 to 20 mg/L. Based on these experimental results, and assuming non-competitive inhibition, a relatively low value of the AB inhibition coefficient was obtained, which indicates a relatively high susceptibility of the ammonia oxidizers to AB. The results of this study have significant implications for both engineered and natural systems relative to the fate and effect of QACs.

CHAPTER 1

INTRODUCTION

Quaternary ammonium compounds (QACs) play an important role in many industrial fields due to their versatile physico-chemical properties. Such widespread uses as disinfectants, fabric softening agents, foam depressants, and antistatic agents lead to massive discharge into the environment with its associated concerns (Wee and Kennedy, 1982). Potential risks have been reported in many previous studies that repeated exposure to QACs can induce microbial resistance against antibiotics in many pathogenic microorganisms (McDonnell and Russell, 1999). In addition, discharge of QACs can disturb the purifying activities of natural aquatic systems or public wastewater treatment plants because of their toxicity to microbial life (Laopaiboon et al., 2002; Tubbing and Admiraal, 1991).

The possibility to effectively eliminate QACs-related pollution has been investigated in many ways. As one of the most economical means, biological treatment of QACs is found to be an effective way to remove QACs and especially aerobic treatment processes can provide rapid biodegradation via a consortium of bacteria (Scott and Jones, 2000). There are also different types of treatment approaches using advanced oxidation processes. For example, UV radiation resulted in 20 to 30 % of COD removal for a series of alkyl benzyl dimethyl ammonium chloride and the biodegradation rate of QACs could be greatly enhanced when combined with an advanced oxidation process (Adams and Kuzhikannil, 1999; Ikehata and El-Din, 2004).

Although extensive research has been conducted on the fate and effect of QACs, relatively little is known about their effect on aerobic biological treatment processes, especially on nitrification. Limited information found in the literature shows that QACs are highly toxic on the nitrification process, but their detrimental effect could be mitigated as a result of the biodegradation by heterotrophs (Munao et al., 1990). Therefore, an in-depth study of the effect of QACs on aerobic mixed biological treatment systems, their biodegradability, and the extent of inhibition on the nitrification process is needed.

Alkyl benzyl dimethyl ammonium chloride (AB), one of the commonly used QACs in many industrial fields, was selected for this study. Two aerobic, mixed cultures -- a heterotrophic and a nitrifying (autotrophic) culture -- were developed and used to assess the biodegradation of AB and its long-term effect on aerobic biological treatment processes.

CHAPTER 2

BACKGROUND

2.1. General Aspects of QACs

2.1.1. Production and Uses

The term *surfactant* refers to substances that interfacially congregate when added to a solvent. This distinguished trait is contrary to that of other solutes which generally activate solute-solvent interaction. The origin of surfactants' interesting properties comes from the existence of two distinct parts of the molecule, a hydrophilic and a hydrophobic moiety. Since the surfactant molecules are large enough (typical molecular weight of between 300 and 400), two zones are able to act independently. When the hydrophilic region contains a positively charged group, the molecule is referred to as a cationic surfactant. The center of cationic surfactants consists of one or more sulfur, phosphorus, arsenic, or nitrogen atoms. However due to the significantly lower cost to produce nitrogenous cationic surfactants, other types are rarely found in the market. Quaternary ammonium compounds, abbreviated as QACs or quats, are the most prevalent forms of cationic surfactants used today. Cationic surfactants have been important since their bactericidal properties were recognized in the 1930s. Production of cationic surfactants accounts for only 8% in the surfactant market (estimation of cationic surfactants produced worldwidely in 1986 was more than 250,000 metric tons), but they hold a substantial position in a variety of applications.

Reviews of cationic surfactants and their numerous applications have been presented before (Linfield, 1970; Richmond, 1990; Rubingh and Holland, 1991). QACs

can be used for many purposes such as cosmetics, pharmaceuticals, and sanitizers. The wide range of QACs applications is attributed to their adsorption onto a variety of solid surfaces. The majority of minerals and organic substances are hydrophilic and polar in general. Silica contents of minerals possessing hydroxyl group and carboxylate groups of organic substances result in highly negative charges on their surfaces where cationic surfactants can bind strongly. The usage of QACs can be described as follows (%): fabric softners, 66; coated clays, 16; biocides, 8; oil-field chemicals, 6; asphalt additives, 2; and textiles, 1. Therefore, the use of cationic surfactants as fabric softeners significantly dominates over all other applications. Fabric softeners function to make clothes and fabrics soft and pleasant while in use, to dry easily, to reduce electrical charge, and to maintain the fragrance for a long time. Although QACs have many applications in addition to their biocidal properties, the large amount of QACs released from laundries to sewage systems appears not to cause serious problems in the purification and nitrification of wastewater treatment plants (Gerike et al., 1978). This might be explained by the fact that QACs can lose their toxicity upon interaction with anionic surfactants and adsorption on particulate matter. Cationic surfactants have a crucial role in metal separation during mining in that the adsorption of a surfactant on the polar surfaces of minerals is essential in order to make mineral particles hydrophobic and hence afloat in the solution.

Organophilic clays are produced by displacing inorganic cations of clay particles by large organic cations, using exclusively QACs with more than one long alkyl chain and most commonly dimethyl (dihydrogenated-tallow) ammonium or dimethyl (hydrogenated-tallow) benzyl ammonium. Since this type of clay has great properties in

lubrication and prevention of pigment settling, organophilic clays containing QACs are widely added in lubricants, paints, printing inks, oil-drilling mud, etc.

Another use of cationic surfactants is to enhance the binding ability between asphalt/bitumen and crushed rock. The hydrophilic surface of crushed rock results in less affinity to apolar asphalt/bitumen matrix. The bonding power, however, can be considerably improved by the addition of cationic surfactants whose polar moieties are adsorbed onto the mineral surface whereas their hydrophobic chains freely spread over bitumen.

QACs have a broad spectrum of microbiological activities over wide pH ranges. As a result, they are used in many fields including industry, agriculture, hospitals, and housekeeping. They are very effective agents against bacteria, fungi, and viruses although there are few signs of posing threats to mankind and other higher forms of life.

2.1.2. Properties

QACs are produced by the nucleophilic substitution reaction of tertiary amines by an alkyl halide, benzyl chloride, or similar material (Fredell, 1994). The general feature of QACs as shown in Figure 2.1, is that the R_1 - R_4 groups are covalently bonded alkyl and/or benzyl groups and the X represents the anion which is a halide, usually a chloride. The central nitrogen atom generates a positive charge which is the functional region of the molecule. The structure and chain length of the R groups can vary. QACs are basically odorless and have no malignant effect on synthetic materials including plastics, rubber, ceramic, and stainless steel. QACs have a number of versatile properties such as surface-activation, detergency, and bactericidal ability, all of which bring QACs into

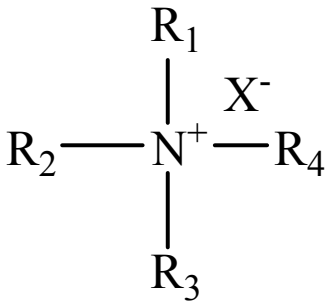


Figure 2.1. General structure of quaternary ammonium compounds (R_1 – R_4 represent alkyl and/or benzyl moieties).

wide uses and popularity throughout the world. Structural features and industrial usage of several QACs are represented in Table 2.1. The largest application of QACs is now for sanitation and disinfection. The first report regarding the antibacterial properties of QACs was announced in 1915 and then in 1935 another milestone demonstrating the antibacterial activity of long chain QACs took place. According to previous studies, the bactericidal efficiency of QACs is dependent on the length of the alkyl chain. Experiments conducted with homologues of alkyl benzyl dimethyl ammonium chloride demonstrated that the compounds containing alkyl chains with 12 to 16 carbon atoms had the greatest bactericidal activity (Fredell, 1994; Petrocci, 1983). Consequently the majority of QACs consist mainly of C_{12} to C_{16} alkyl chains, C_{14} is the most common. Table 2.2 shows the minimum inhibitory concentrations for ten QACs. From these data, it can be concluded that QACs are generally more effective against Gram positive bacteria (i.e., *Bacillus subtilis* and *Staphylococcus aureus*) rather than Gram negative bacteria (i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aspergillus niger*).

The antibacterial property of QACs is closely linked with the chemical properties of cationic surfactants. QACs are positively charged compounds that are naturally attracted to negatively charged substances such as bacterial proteins essential for the structure and enzymatic activities of the cell. QACs are known to exert their antibacterial activity by disorganizing and denaturing these proteins. The mechanisms of the biocidal action of QACs are schematically depicted in Figure 2.2 (Gilbert and Moore, 2005).

QACs start their inhibitory action with binding to the cell surface. Resulting changes at the outer layer, cytoplasmic membrane, or within the cytoplasm are related to the antibacterial effect of QACs. For instance, benzalkonium chloride, one of the most

Table 2.1. Structural features of QACs and their industrial usage

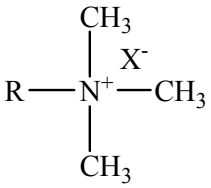
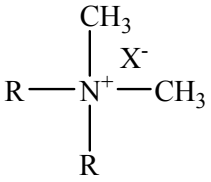
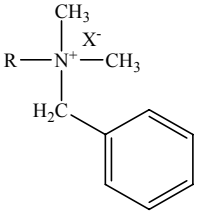
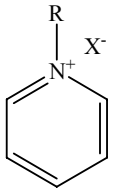
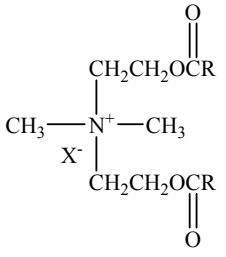
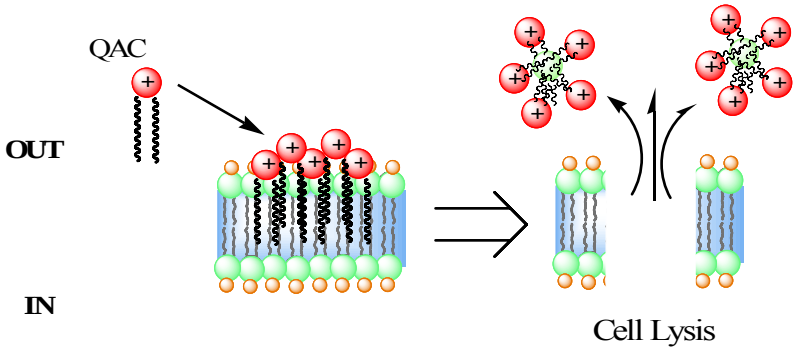
QAC Group	Molecular Structure	Applications
Alkyl trimethyl ammonium		Organoclays, phase transfer catalyst, oilfield applications
Dialkyl dimethyl ammonium		Biocides, wood preservatives, oilfield applications
Alkyl benzyl dimethyl ammonium		Biocides, cosmetics, wood preservation, phase-transfer catalyst, organoclays
Alkyl pyridinium		Phase transfer catalyst, pesticides
Diethylester dimethyl ammonium (Esterquat)		Active ingredient in fabric softeners

Table 2.2. Minimum inhibitory concentration (ppm)[a] of QACs for several bacteria and fungi (Cords, 1983)

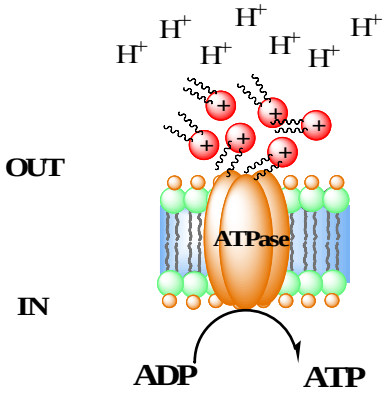
Compound	Organism				
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Aspergillus niger*
One R group is fatty acid					
Benzalkonium chloride	200	300	3	4	60
Dodecyltrimethylammonium chloride	500	500	5	5	500
Tetradecyltrimethylammonium chloride	150	100	1.5	5	50
Hexadecyltrimethylammonium chloride	5000	5000	5	5	5000
Dodecylbenzyldimethylammonium chloride	750	750	2	2	75
Two R groups are fatty acids					
Dioctyldimethylammonium chloride	40	75	20	20	225
Didecyldimethylammonium chloride	225	750	> 0.7	7	75
Ditetradecyldimethylammonium chloride	2250	> 2250	225	750	2250
Three R groups are fatty acids					
Tri(octyldecyl)methylammonium chloride	500	500	3	5	150
Tridodecylmethylammonium chloride	> 5000	> 5000	500	1500	1500

[a]Values in ppm are for bacteriostatic or fungistatic activity, not cidal

(A)



(B)



(C)

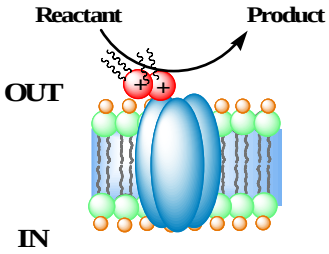


Figure 2.2. Mechanisms of biocidal action of QACs: physical disruption of the membrane (A), dissipation of the proton motive force (B), inhibition of membrane-associated enzyme activity (C).

widely used QACs, was observed to alter the hydrophobicity of *Pseudomonas aeruginosa* (El-Falaha et al., 1985), which may promote damage of the cell wall and outer membrane of bacteria. However, the extent of the biocidal effect varies depending on the species of bacteria. Gram-negative bacteria are in general less sensitive to biocides than Gram-positive bacteria due to the existence of their outer membrane. The cytoplasmic membrane, surrounding the cytoplasm of a cell and comprised of a phospholipid bilayer with embedded proteins, is commonly regarded as the main target site of biocides. The cytoplasmic membrane can be disrupted leading to the leakage of intracellular components, which is thought as a means of disruption of the cell permeability barrier (Lambert and Hammond, 1973). Previous studies proved that QACs induced leakage of intracellular components and subsequently damaged the cell membrane (Figure 2.2A) (Davies et al., 1968). In addition, QACs primarily affect the membrane phospholipids leading to cell lysis (Gilby and Few, 1960; Takasaki et al., 1994).

The proton motive force (PMF), which is initiated by a proton gradient across the cytoplasmic membrane, is involved in all forms of respiratory and photosynthetic processes, such as active transport, oxidative phosphorylation, and ATP synthesis. QACs and some organic acids were found to inhibit ATP synthesis by neutralizing the PMF and denaturing responsible proteins (Figure 2.2B). For instance, Denyer and Hugo (1977) observed that cetyl trimethyl ammonium bromide (CTAB) discharged the membrane potential and led to PMF dissipation.

Many enzymatic proteins, anchored in the cytoplasmic membrane, are involved in the electron transport chain. Certain types of biocides inhibit these kinds of proteins so that

many vital processes, for example glycolysis, fatty acid and phospholipid synthesis, and solute uptake, are significantly blocked (Figure 2.2C). Other biocidal agents react with the thiol group in proteins which is necessary for many enzymatic activities. These reactions also lead to cell inhibition or cell inactivation (Maillard, 2002).

The thermal stability of QACs varies depending on the associated counterions. For example, alkyl trimethyl ammonium chlorides are stable up to 130°C, above which they start breaking down, but their homologues substituted with bromide and iodide become unstable at a lower temperature (Laughlin, 1991). More important is the fact that the anion affects the phase equilibrium between the surfactants and water due to hydration of the entire QAC compound within the liquid and liquid crystal phase. In dilute solutions, moreover, the anion has a remarkable impact on the micelle formation (Zana, 1991). Other physico-chemical properties, such as viscosity and solubility are influenced by the anion as well. Chloride and methosulfate are representative anions commonly used (Cross, 1994).

2.1.3. Toxicity

The high toxicity of cationic surfactants against microorganisms, in contrast to the relatively low toxic effect on humankind and mammals, has justified the use of these compounds as disinfecting and sanitizing agents. Nevertheless, it is evident that QACs can create toxic effects to humans and animals by all ways of exposure including inhalation, ingestion, dermal absorption and irrigation of body cavities. Condensed solutions of QACs can cause burns to skin and mucosal membrane, whereas diluted solutions result in benign irritation. QACs are known to create systemic toxicity and allergic reactions as well.

Among the numerous QACs, benzalkonium chloride (BAC), also called alkyl benzyl dimethyl ammonium chloride, is the most comprehensively investigated compound.

Exposure to QACs can happen even in an ordinary life via several routes as described below. QACs have been used in cosmetics, ophthalmic medications, contact lens solutions, hand washes, antimicrobial soaps, skin wound cleanser, and preoperative skin preparation solutions (Drobeck, 1994). Such uses thus result in frequent contact with body surfaces and cavities, increasing the likelihood of absorption, inhalation, and unexpected ingestion. Oral ingestion is the most common way for QACs to enter. Used widely in inhalers and nasal sprays, as wetting agents and preservatives, QACs can be easily inhaled. In addition, dermal contact by accidental spillage of QACs on skin or clothes is usual since they are present in a number of domestic chemicals like shampoos, detergents, cleaning agents, or disinfectants, although adsorption can rarely occur unless there is wounded skin. In addition, eye contact, irrigation of body cavities, or parenteral exposure could be other means of entry. It has been estimated that 100 to 400 mg/kg (oral) or 5 to 15 mg/kg (parenteral) dose of QACs could be fatal to human adults (Ellenhorn et al., 1997), and 1 to 3 g was regarded as a fatal dose (Arena, 1964). The LD_{50} of benzalkonium chloride varies, depending on the animal species and the route of exposure (Wade and Weller, 1994), as follows (in mg/kg): guinea pig, oral 200; mouse, intraperitoneal 10; mouse, intravenous 10; mouse, oral 175; mouse, subcutaneous 62; rat, intraperitoneal 14.5; rat, intravenous 13.9; rat, oral 240; rat, subcutaneous 400; rat, skin 1,560.

Acute poisoning by QACs may bring about diverse symptoms depending on the route(s) of exposure. Acute (single-dose) toxicity of the QACs, at lethal level, is usually

defined by peripheral paralysis and central nervous system stimulant-like effect, whereas chronic (multiple-dose) toxicity is characterized by body weight loss, reduced food consumption, dehydration, and increased mortality. Ingestion of benzalkonium chloride in higher concentrations results in caustic burns on the lips, tongue, mouth, throat, or stomach, all of which can come along with hypersalivation, vomiting, diarrhea, and confusion. Much lethal symptoms, such as hypotension, shock, respiratory paralysis, and coma, may also happen (Mathieu-Nolf et al., 1985). In case of inhalation, bronchoconstriction by BAC-containing medications was reported (Graf et al., 1995; Hallen and Graf, 1995), and nasal stiffness as well as nasal mucosa swelling are other potent side effects. Dermal burnings caused by concentrated cetrimide and caustic actions with benzalkonium chloride are the effect of skin exposure (Mercer, 1983; Nicola et al., 1997). Eye contact with QACs is likely to generate mild discomfort (0.1% solution) to fatal corneal damage (10% solution) depending on species and concentration. There have been some reports proving the occurrence of ocular inflammation in human eyes (Reynolds, 1996; Swan, 1944).

When it comes to chronic poisoning, in contrast, QACs have relatively not been the topics of interest. Since usual kitchen detergents contain QACs, low amounts of them can be easily ingested. Experimental measurements as well as estimations expect that an average oral intake of surfactants would reach about 100 mg/man-year but this value does not produce a toxic effect (Gloxhuber, 1974). Analogous to acute poisoning, it is reported that those who were exposed to QACs, especially to benzalkonium chloride, showed occupational asthma or irritable dermatitis.

Poisonings due to diluted solutions can be of little concern and easily dealt with, but those in high concentrations can be sometimes lethally dangerous if urgent and proper treatments are not given. Death due to heavy exposure is associated with cardiorespiratory collapse, bronchoconstriction, or acute pulmonary oedema (Ellenhorn et al., 1997). Patients can die within 1 to 3 hours after ingestion.

2.1.4. Biodegradation

Biodegradation is the major means of removing cationic surfactants in wastewater treatment plants and the environment. In spite of frequent releases of cationic surfactants into wastewater treatment plants, little damage has been reported, which is attributed to the fact that not only are the influent concentrations low but also cationic surfactants tend to be strongly adsorbed on particulate matter, to form charge-neutralized complexes with anions, and to lead to microbial acclimation by extended exposure to microorganisms. Nonetheless, accidental discharge of these compounds can lead to temporal disturbance of treatment systems, especially for nitrification, which is known to be sensitive to inhibition (Boethling, 1984). The removal rate of cationic surfactants in wastewater treatment plants reaches 90%. As these compounds are immediately adsorbed, most degradation processes take place on suspended solids. The susceptibility of cationic surfactants to biodegradation was first reported in the 1950s. Gerike (1982) confirmed this previous study, using activated sludge and cetyl trimethyl ammonium bromide (CTAB), dodecyl benzyl dimethyl ammonium chloride, and didecyl dimethyl ammonium chloride. Adsorption accounted for 8 to 29% removal, and the previously reported removal efficiency (more than 90%) provided a good

evidence of biodegradation as reported in previous studies (Fenger et al., 1973; Janicke and Hilge, 1979).

A general agreement was made in terms of what type of cationic surfactants was readily degradable, or which one was most obstinate (Dean-Raymond and Alexander, 1977; Larson and Vashon, 1983; Masuda et al., 1978). Mono alkyl quaternaries are exceedingly favorable to biodegradation, and alkyl pyridinium classes are least degraded. Alkyl benzyl dimethyl and dialkyl dimethyl ammonium compounds are placed in the midst of them.

Biodegradation appears to lie under the effect of various factors besides chemical structure. Studies done by Ruiz Cruz (1979; 1981) assessed the influence of several parameters on the biodegradation of cationic agents in river water. The variables adopted were biomass concentration, acclimation, temperature, aeration, and nutrients. It was evident in these studies that biomass concentration as well as temperature was positively correlated with biodegradation of QACs, and that the presence of nutrients and pre-acclimation enhanced the microbes' degrading ability. The role of aeration was also regarded as beneficial, although to a lower degree. Ventullo and Larson (1986) investigated the effect of pre-exposure to alkyl trimethyl ammonium chloride at a lake ecosystem in southern Ohio.

Studies on the biodegradation pathways have been conducted for various QACs so far. The utilization of tetramethyl ammonium chloride by *Pseudomonas* sp. was described by Hampton and Zatman (1973). According to their work, the oxidation of this compound is initiated by splitting the C-N bond, therefore producing methanal and trimethyl amine. The intermediate trimethyl amine is oxidized to trimethyl amine N-oxide (Large et al.,

1972) or transformed to methanal and dimethyl amine (Colby and Zatman, 1973; Meiberg and Harder, 1978). The trimethyl amine N-oxide is further converted to dimethyl amine (Large, 1971; Myers and Zatman, 1971), followed by the generation of methanal and methyl amine (Colby and Zatman, 1973). Methyl amine is then converted to methanal and ammonium. Based on an investigation of the utilization of alkyl trimethyl ammonium bromides by microorganisms derived from sewage and soil, Dean-Raymond and Alexander (1977) demonstrated that the first step of degradation occurs with hydroxylation of the terminal carbon of the alkyl group and the resulting carboxylic acid undergoes β-oxidation. Another study dealing with hexadecyl trimethyl ammonium chloride suggested a central fission of C_{alkyl}-N bond as the first step of degradation (van Ginkel et al., 1992). Not only hexadecyl trimethyl ammonium chloride, but also hexadecanal and hexadecanoate produced as metabolites, were all utilized by *Pseudomonas* sp. In contrast, this strain did not grow on trimethyl amine, suggesting that other species of microorganisms must get involved for the complete degradation of this compound (Figure 2.3). The degradation mechanism of alkyl benzyl dimethyl ammonium chloride, the target compound for this study, was proposed in recent years (Patrauchan and Oriel, 2003). Dodecyl and tetradecyl benzyl dimethyl ammonium chloride were completely metabolized by *Aeromonas hydrophilia* sp. K. The suggested pathway is shown in Figure 2.4. It is believed that the central C_{alkyl}-N bond fission is first carried out by an oxygen dependent dehydrogenase as suggested for alkyl trimethyl ammonium compounds (van Ginkel et al., 1992) and then intermediates such as benzyl amines and benzoic acid, are formed consecutively by N-demethylation reactions while *Aeromonas hydrophilia* sp. K is utilizing all these

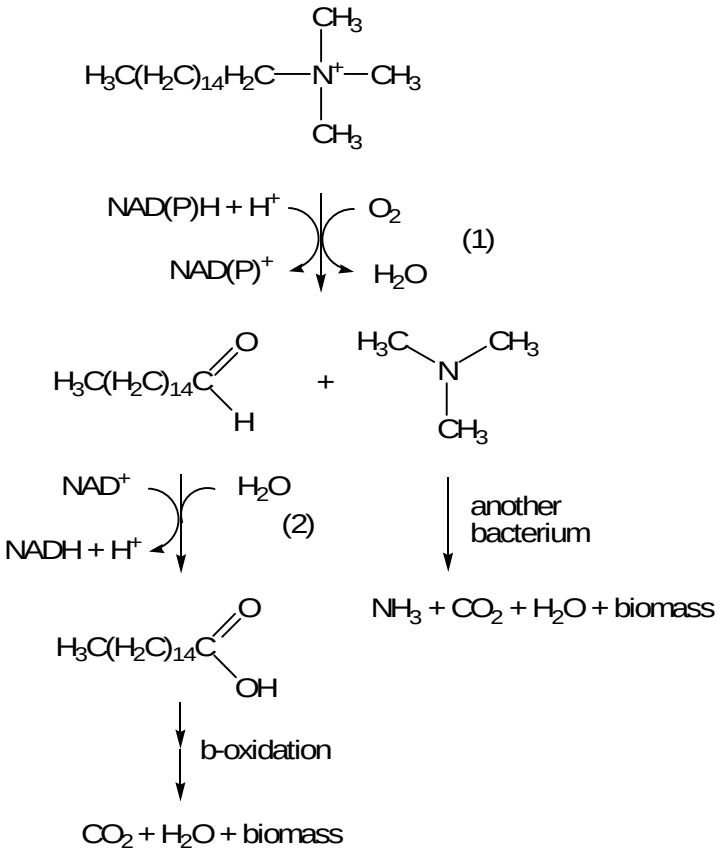


Figure 2.3. Proposed degradation pathway of hexadecyl trimethyl ammonium chloride by *Pseudomonas* sp. monooxygenase (1), and alkanal dehydrogenase (2) (van Ginkel et al., 1992).

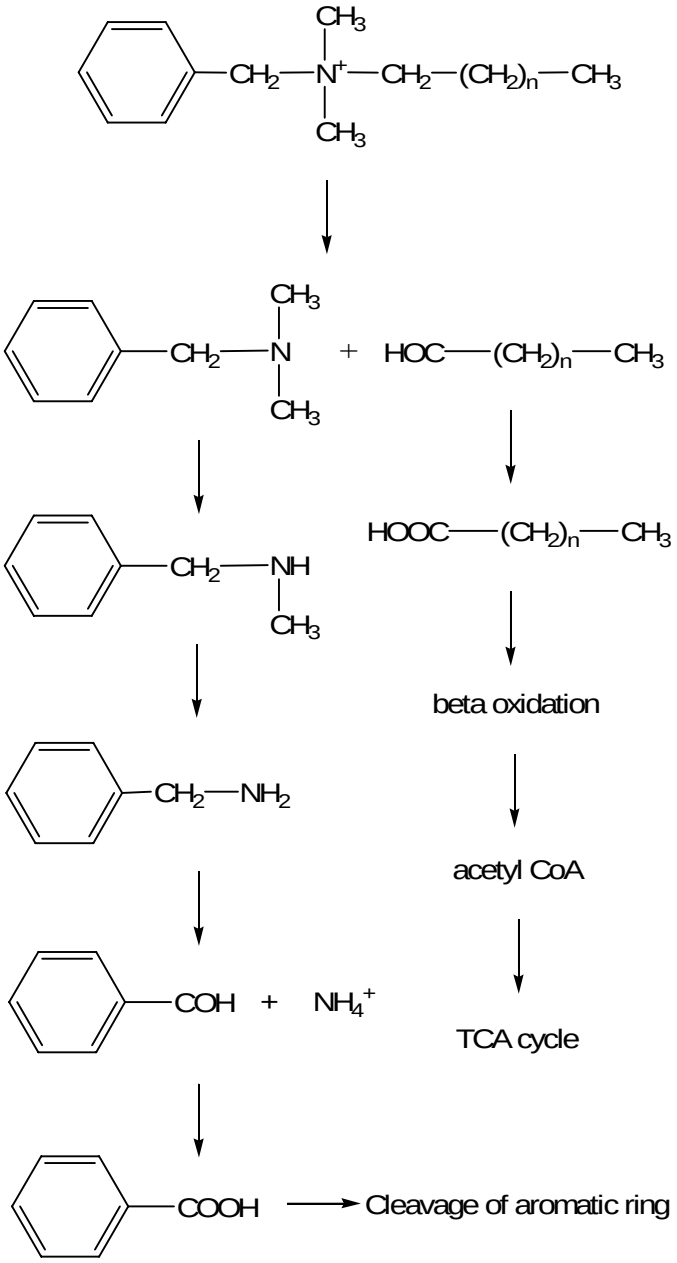


Figure 2.4. Proposed degradation pathway of alkyl benzyl dimethyl ammonium chloride by *Aeromonas hydrophilia* sp. K (Patrauchan and Oriel, 2003).

intermediates as carbon and nitrogen source. Also the fact that this strain cannot utilize alkyl dimethyl amines firmly demonstrates that C_{benzyl}-N bond fission is indeed hard to occur. In contrast to the previous findings, however, a recent study proposed the degradation of dodecyl trimethyl ammonium chloride by *Pseudomonas* sp. strain 7-6, isolated from a wastewater treatment plant, via dual pathways, which besides an initial attack on the C_{alkyl}-N bond also initiates degradation via cleavage of a C_{methyl}-N bond, hence producing dodecyl dimethyl amine as an intermediate (Takenaka et al., 2007). Overall, general degradation pathways of cationic surfactants shown in Figure 2.5 indicate that biodegradation is mostly commenced with cleavage of the C_{alkyl}-N bond irrespective of the type of QACs and the degradation of the produced alkanals proceeds via β-oxidation for complete mineralization (van Ginkel, 2004).

Because of the surfactants' amphiphilic (i.e., both hydrophilic and hydrophobic), character and the limited enzymatic ability of individual microorganisms, only a few known surfactants, alkane sulphonates, alkyl sulphates, and alkyl amines, are completely degraded by a single microorganism (van Ginkel, 1996). For this reason, consortia of microorganisms are highly efficient, as well as necessary for the complete degradation of surfactants (Scott and Jones, 2000; van Ginkel, 1996). Such consortia of microorganisms are classified as either commensal (one benefits, whereas the other is not affected) or synergistic. An example of commensalism is found in the case of hexadecyl trimethyl ammonium chloride degradation (van Ginkel et al., 1992). A *Pseudomonas* sp. attacks the C_{alkyl}-N bond of the compound and releases trimethyl amine on which methylotrophs can grow together. As mentioned above, *Aeromonas hydrophilia* sp. K was not capable of

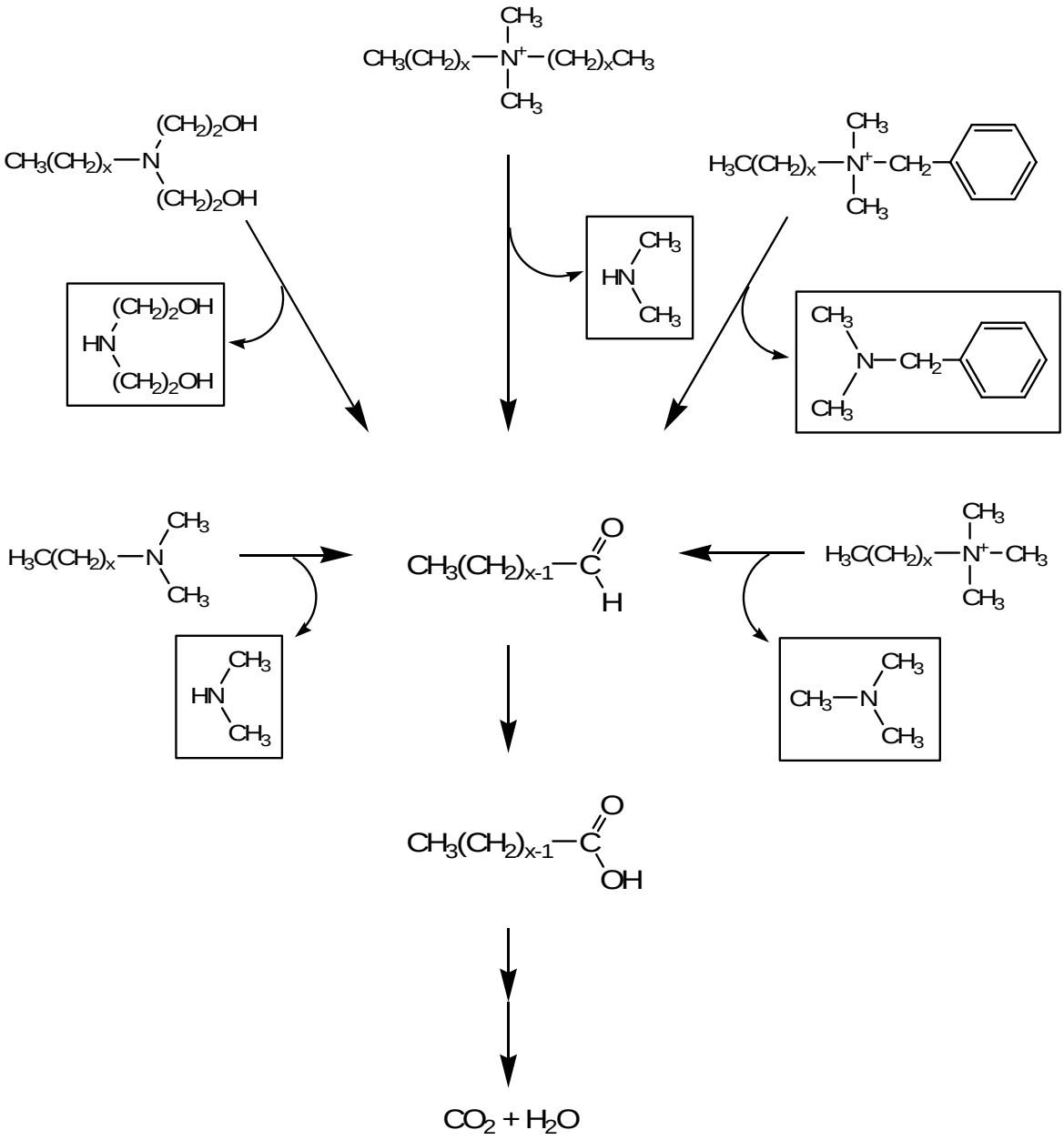


Figure 2.5. General degradation pathway of cationic surfactants. The alkanals formed through the oxidation of the α-carbon of the alkyl chain are further metabolized via β-oxidation (van Ginkel, 2004).

utilizing alkyl dimethyl amine produced as a result of the aerobic degradation of alkyl benzyl dimethyl chloride. The complete degradation of this compound requires three microorganisms that metabolize the alkyl chain, the aromatic moiety, and dimethyl amine (van Ginkel, 2004). On the other hand, the degradation of decyl trimethyl ammonium bromide assessed by Dean-Raymond and Alexander (1977) exhibited synergistic relation. Decyl trimethyl ammonium chloride was extensively utilized by two microorganisms, *Pseudomonas* sp. and *Xanthomanas* sp. Interestingly, any one of these strains did not grow when inoculated separately, but together growth and metabolism took place without the addition of growth factors. Such results suggest that the products of partial degradation by one strain were necessary for the survival and growth of the other one.

2.2. Problem Identification

Despite the considerable number of studies covering various kinds of cationic surfactants, especially for quaternary ammonium compounds (QACs), relatively limited information is available with respect to the effect of QACs on nitrification. Nitrification is a crucial step in wastewater treatment systems since such process is the starting point of biological nitrogen removal. However, nitrifiers are more sensitive than other heterotrophic microorganisms and thus susceptible to inhibition by toxic chemicals (Boethling, 1984). Pitter (1962) found that 3 mg/L of both hexadecyl pyridinium bromide (HPB) and hexadecyl trimethyl ammonium bromide (HTMAB) strongly inhibited nitrification and at 6 mg/L nitrification completely failed. In another study, the inhibition of nitrification was observed at 16 mg/L of dioctadecyl dimethyl ammonium chloride (DODMAC) in the

presence of 20 mg/L linear alkyl benzene sulphonate (LAS) (Gerike et al., 1978). Although these previous studies can provide general insights on the effect of QACs on aerobic biological treatment systems, detailed information about the fate and effect of alkyl benzyl dimethyl ammonium chloride (benzalkonium chloride), popularly used as a disinfectant agent currently, on a mixed aerobic heterotrophic culture and its nitrification process is still scarce. To this end, this study was initiated with the goal of understanding the fate, biodegradability and toxicity of alkyl benzyl dimethyl ammonium chloride in mixed cultures representative of aerobic treatment processes.

2.3. Research Objectives

Based on the above-presented literature review, information about the inhibitory effect on mixed aerobic heterotrophic cultures and their nitrification process by alkyl benzyl dimethyl ammonium chloride has been relatively insufficient. Furthermore, the biodegradation pathway of this compound was unveiled relatively recently as compared to that of other QACs. Thus, further study on the biodegradability of this compound under various culture conditions is warranted. Therefore the main goal of this study was to assess the fate and effect of alkyl benzyl dimethyl ammonium chloride on mixed aerobic cultures with respect to both heterotrophic and autotrophic (i.e., nitrifying) metabolism.

The specific objectives of this study were to:

1. Assess the fate and inhibitory effect of alkyl benzyl dimethyl ammonium chloride on both heterotrophic and nitrifying mixed cultures.

2. Investigate the effect of microbial acclimation on the biodegradation of alkyl benzyl dimethyl ammonium chloride and its use as the sole source of carbon and nitrogen.
3. Determine the toxicity of alkyl benzyl dimethyl ammonium chloride and its biotransformation products.

CHAPTER 3

MATERIALS AND ANALYTICAL METHODS

3.1. pH

All pH measurements were performed using the potentiometric method with a Model 370 digital pH meter (Orion Research, Inc., Boston, MA) and a gel-filled combination pH electrode (VWR International, West Chester, PA). The meter was calibrated weekly with pH 4.0, 7.0, and 10.0 standard buffer solutions (Fisher Scientific, Pittsburgh, PA). The electrode was rinsed with deionized (DI) water before sample readings and stored in an electrode storage solution (Fisher Scientific, Pittsburgh, PA) of pH 4 when not in use. Although the sensitivity of the meter display was 0.01 units, the limit of accuracy was taken to be only 0.1 pH units (Eaton et al., 2005).

3.2. Dissolved Organic Carbon (DOC)

DOC measurements were performed using a Shimadzu TOC-5050A Total Organic Carbon Analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a non-dispersive infrared detector for the analysis of total, organic (by difference), and inorganic carbon of liquid samples. Autosampler tubes were washed with weak bleach solution, rinsed with deionized (DI) water and baked at 300°C for 30 minutes before use to ensure the absence of any residual carbon. In order to measure DOC, liquid samples were filtered through 0.2 μm membrane filters (Fisher Scientific, Pittsburgh, PA) and acidified (pH < 2.0) using a 0.2 N HCl solution. Then, 4 mL of acidified samples (3.6 mL 0.2 N HCl

solution + 0.4 mL sample) were transferred to autosampler tubes. Triplicate measurements were performed for each sample. The injection volume was 25 μL. Carbon analysis was based on catalytic combustion of the sample at 680°C. A calibration curve was prepared using standard solutions of potassium hydrogen phthalate (KHP).

3.3. Chemical Oxygen Demand (COD)

COD of the samples was measured using the closed reflux, colorimetric method as described in *Standard Methods* (Eaton et al., 2005). An aliquot of 3 mL digestion solution was transferred to HACH COD digestion vials (HACH Company, Loveland, CO) and then 2 mL of pre-filtered sample was added to the vial. After shaking, the vials were heated at 150°C for 120 minutes and then cooled down to room temperature. Absorbance was measured at 620 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. All samples were prepared at least in duplicate and a calibration curve was also prepared using standard solutions of potassium hydrogen phthalate (KHP)

3.4. Ammonia

The ammonia distillation method as per *Standard Methods* (Eaton et al., 2005) was used to determine the liquid phase ammonia concentration in the cultures. Culture samples were centrifuged at 12,000 rpm for 15 minutes and filtered through a 0.2 μm membrane filter (Fisher Scientific, Pittsburgh, PA). Then, the samples were added to an ammonia

distillation apparatus (Labconco Corp., Kansas City, MO). The pH of all samples was kept at 9.5 by addition of 6 N NaOH and borate buffer was added to increase hydrolysis of organic nitrogen compounds (Eaton et al., 2005). Ammonia vapors from the boiling samples were condensed and captured through the immersed outlet of the distillation apparatus in indicating boric acid solution. Ammonia captured in the solution was quantified titrimetrically with 0.2 N H_2SO_4.

3.5. Gas Composition

The gas composition was determined by an Agilent Technologies Model 6890N GC unit (Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Oxygen (O_2) was separated with a 15 m HP-Molesieve fused silica, 0.53 mm i.d. column (Agilent Technologies, Inc.); carbon dioxide (CO_2) was separated with a 25 m CP-PoraPLOT Q fused silica, 0.53 mm i.d. column (Varian, Inc., Palo Alto, CA). Both columns were operated with helium as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150°C, and the detector temperature was set at 150°C. All gas analyses were performed by injecting a 100 μL gas sample. Calibration curves were prepared using the above-described procedure and pure gas standards.

3.6. Total and Volatile Suspended Solids

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to procedures described in *Standard Methods* (Eaton et al., 2005). All filters were

washed with deionized (DI) water and ignited at 550°C for 20 minutes in a Fisher Isotemp Model 550-126 muffle furnace (Fisher Scientific, Pittsburgh, PA) before use. The filters were then cooled in a desiccator and weighed using an Ohaus AP250D analytical balance (Ohaus Corp., Pine Brook, NJ). Culture samples of known volume (typically 10-20 mL) were filtered through 47 mm diameter Whatman GF/C glass fiber filters (1.2 μm nominal pore size; Whatman, Springfield Mill, England). The filters were then rinsed with equal volumes of deionized (DI) water to remove dissolved organic carbon, residual inorganic carbon, and salt. The filters containing the samples were dried at 105°C for at least 1.5 hours in a Fisher Isotemp Model 750G oven (Fisher Scientific, Pittsburgh, PA). After cooling in a desiccator, the dry weight was recorded and the filters containing the dry samples were transferred to a Fisher Isotemp Model 550-126 muffle furnace and ignited at 550°C for 20 minutes. After ignition, the samples were cooled down in a desiccator. The residual solid weight was measured, and then the TSS and VSS concentrations were calculated.

3.7. Nitrite and Nitrate

Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Corp., Sunnyvale, CA) equipped with a conductivity detector, a Dionex IonPac AG14A (4×50 mm) precolumn, and a Dionex IonPac AS14A (4×250 mm) analytical column. The unit was operated in autosuppression mode with 1 mM $NaHCO_3$/8 mM Na_2CO_3 eluent with a flow rate of 1 mL/min. The injection volume was 1 mL. Calibration curves were prepared using standards prepared by

dissolving reagent grade sodium salts of each analyte in deionized (DI) water. All standards and samples were filtered through 0.22 μm membrane syringe filters (National Scientific Company, Rockwood, TN) prior to injection.

3.8. Dissolved Oxygen (DO)

The DO concentration of the cultures used in this study was measured using the polarographic method (Eaton et al., 2005) with a YSI Model 58 oxygen meter in conjunction with a YSI 5750 oxygen probe (Yellow Springs Instrument, Yellow Springs, OH). The instrument was calibrated to water-saturated air (at a given temperature) before each use and the probe electrolytic solution and membrane were changed periodically.

3.9. QACs

Barquat MB-80™ was used in this study. Barquat MB-80™ (Lonza, Inc., Fair Lawn, NJ) is comprised of three alkyl benzyl dimethyl ammonium chloride compounds and ethanol as follows (molecular formula, % w/w): dodecyl dimethyl ammonium chloride ($C_{21}H_{38}NCl$, 40%), tetradecyl dimethyl ammonium chloride ($C_{23}H_{42}NCl$, 50%), hexadecyl dimethyl ammonium chloride ($C_{25}H_{46}NCl$, 10%), and ethanol (C_2H_5OH, 10%). A stock solution (10,000 mg/L) was prepared based on the active ingredient purity and concentration. Quantification of QACs was accomplished using the modified disulfine blue (DSB) method (Tezel et al., 2006). The basis of this method, schematically shown in Figure 3.1, is the reaction of QACs with an intensively colored anionic dye resulting in the formation of an anionic dye-QAC ion pair which is extracted with a solvent and then the

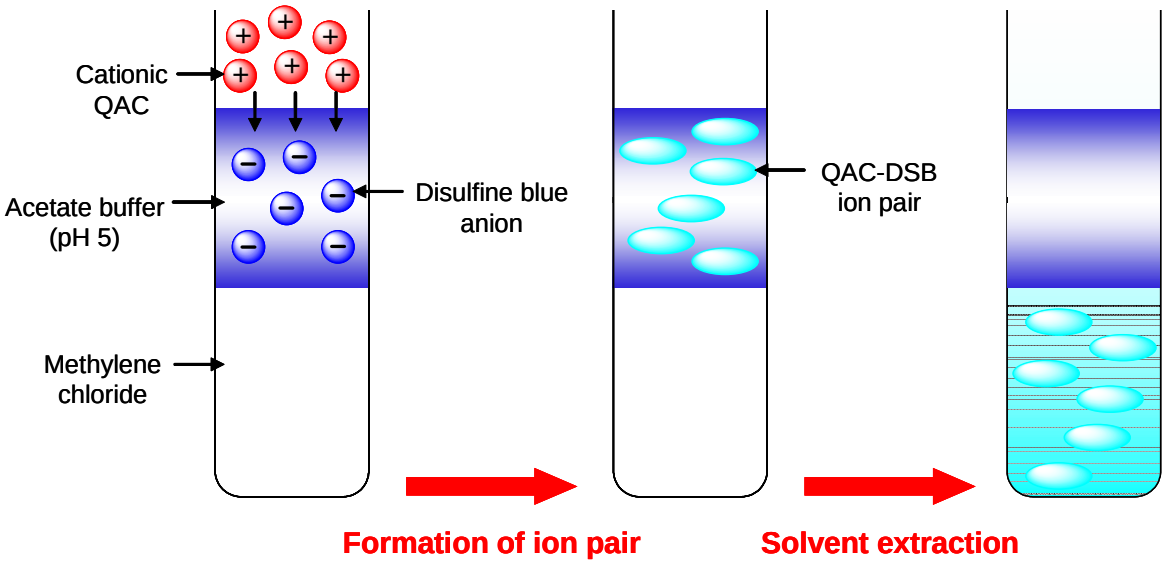


Figure 3.1. Schematic of the disulfine blue (DSB) method.

color intensity in the solvent phase measured spectrophotometrically (HMSO, 1981; Waters and Kupfer, 1976). Analyses were carried out in 25 mL test tubes by adding 5 mL of acetate buffer, 2 mL of disulfine blue dye solution, 10 mL methylene chloride, and 2 mL of the sample. The acetate buffer was made by adding 115 g anhydrous sodium acetate and 35 mL glacial acetic acid in 1 L deionized (DI) water. The dye solution was prepared by dissolving 0.16 g of Patent Blue VF (Acros Organics, Morris Plains, NJ) in 2 mL ethanol and diluting it to 250 mL with deionized (DI) water. After 24 hours of tumbling at 6.9 rpm, the bottom solvent layer was transferred into a 2 mL clear glass vial and the color intensity was measured with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). The QAC concentration was quantified based on sample absorbance at the characteristic maximum wavelength of 628 nm and a previously prepared calibration curve at a concentration range 0 to 30 mg/L (Figure 3.2). The minimum detection limit was estimated as 0.2 mg/L.

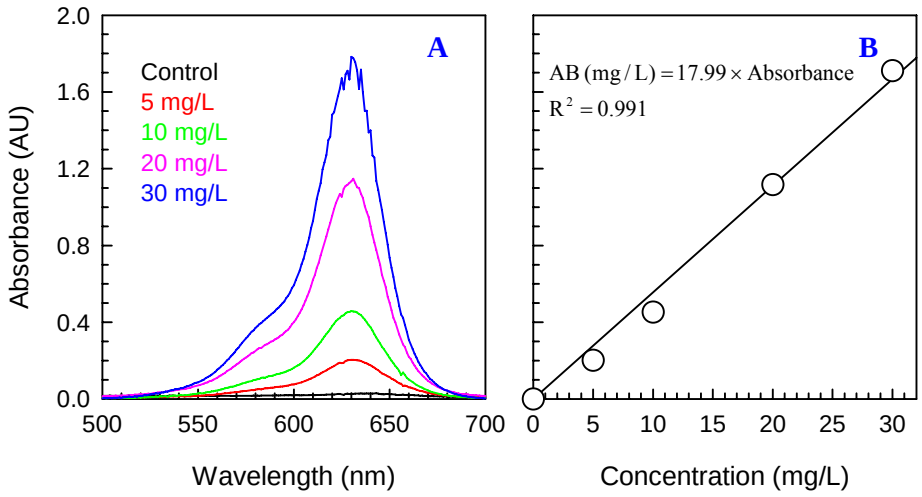


Figure 3.2. UV spectra (A) and calibration curve (B) of alkyl benzyl dimethyl ammonium chloride based on absorbance at 628 nm.

CHAPTER 4
MIXED AEROBIC CULTURES

4.1. Introduction

Quaternary ammonium compounds (QACs) are massively produced throughout the world and used extensively in both industrial and household applications as fabric softners, antibacterial agents, paint additives, and cosmetics (Games et al., 1982). As a result, worldwide consumption of QACs amounted to 0.5 million tons in 2003 (Hauthal, 2004). Such a widespread use of QACs causes them to be released and accumulate in aquatic environments as well as wastewater treatment plants. However, their biocidal property can impact natural purification processes mainly driven by microorganisms. Previous research has pointed out that QACs may not be a threat to microorganisms under normal circumstances in wastewater treatment systems (Boethling, 1984) and, at nontoxic levels, they can be removed at about 90% (Boethling, 1994). However, only limited research has been performed to investigate the effect of QACs on aerobic biological treatment processes, especially on nitrification. It was revealed in a literature that QACs inhibit nitrification and they are more toxic than anionic surfactants (Munao et al., 1990).

The objective of this study was to assess the biodegradation and toxicity of alkyl benzyl dimethyl ammonium chloride, one of commonly used QACs as a sanitizer, on an aerobic heterotrophic and nitrifying mixed culture.

4.2. Materials and Methods

4.2.1. Alkyl Benzyl Dimethyl Ammonium Chloride (AB)

A stock solution of 10,000 mg/L AB stock solution was diluted to 200 mg/L and was used to measure both soluble COD and DOC according to the corresponding methods described in Chapter 3. Samples were run in triplicate and the obtained experimental values were expressed as mean ± error. For reference, theoretical values were calculated based on a stoichiometric equation and composition ratio.

Physico-chemical properties of AB were estimated with a software package named Estimation Programs Interface (EPI) Suite™, Version 3.12. EPI Suite™ is an interface program developed by the US Environmental Protection Agency's Office of Pollution Prevention & Toxics and Syracuse Research Corporation (SRC). With this software using structure information of a compound, several properties, such as melting and boiling point, vapor pressure, octanol-water partition coefficient (K_{ow}), water solubility, and biodegradability, were estimated to enlighten the inherent characteristics and environmental behavior of AB.

4.2.2. Control Mixed Aerobic Culture

A suspended growth, mixed aerobic culture was developed from a contaminated sediment sample obtained from Bayou d'Inde, a tributary of Calcasieu River near Lake Charles, LA. The location and details on the sediment sampling and analysis have been reported elsewhere (Gess and Pavlostathis, 1997; Prytula and Pavlostathis, 1996). The 2 L culture was semi-continuously fed twice a week, at intervals of 3 and 4 days, with peptone

(Sigma-Aldrich, Inc., St. Louis, MO), dextrin (Tate & Lyle PLC, London, UK), and yeast extract (Becton, Dickinson and Company, Sparks, MD) dissolved in deionized (DI) water. The initial loading of peptone, dextrin, and yeast extract was 250, 250, and 50 mg/L-day, respectively. Before each feeding, 500 mL of culture mixed liquor was removed and replaced with fresh culture media. The composition of the culture media and the trace metal solution is shown in Table 4.1 and Table 4.2, respectively. The culture solids retention time (SRT) and hydraulic retention time (HRT) was 14 days. Pre-humidified air passed through a water tap was supplied through a fine pore diffuser in order to maintain DO at or above 6 mg/L. The culture was maintained at room temperature (22 to 23^{o}C), stirred continuously with a Teflon-coated stirring bar over a magnetic stir plate, and its pH maintained at 7.6 ± 0.5 (mean ± standard deviation) with sodium bicarbonate (0 to 1 g/L-media depending on culture condition). The following analyses were periodically performed according to the procedures outlined in *Standard Methods* (Eaton et al., 2005) (see Chapter 3): pH, total and volatile suspended solids (TSS, VSS), soluble COD, DOC, ammonia, nitrite, and nitrate.

4.2.3. AB-amended Cultures

Starting with the control culture, four AB-amended cultures were developed and were divided into two groups, depending on whether they were fed with organic carbon sources, other than AB. One group was fed with dextrin, peptone, and yeast extract as well as AB, whereas the other group was only fed with AB. For each group, one culture was amended with an initial AB concentration of 20 mg/L, whereas the second culture was

amended with an initial AB concentration of 50 mg/L. A summary description of the AB-amended cultures is given in Table 4.3.

The dextrin/peptone fed AB20 culture (denoted as AB20+D/P) was prepared first by using the waste from the control culture as inoculum. The duration of each feeding cycle of AB20+D/P varied in the first 10 days in order for the culture to acclimate to AB. After 10 days of operation, all culture conditions, such as feeding cycles, HRT/SRT, and feed composition, were exactly the same as in the control culture, except that 20 mg/L of AB was added at the beginning of each feeding cycle. The dextrin/peptone fed AB50 culture (denoted as AB50+D/P) was developed using the waste from the AB20+D/P which had been in operation for 10 days. After 21 days of acclimation, all culture conditions were exactly the same as in the control and AB20+D/P cultures, except the initial AB concentration, which was equal to 50 mg/L.

Two additional cultures maintained without dextrin/peptone addition (denoted as AB20-D/P and AB50-D/P), were developed from their counterpart cultures enriched with dextrin/peptone and AB (AB20+D/P and AB50+D/P, respectively). The two dextrin/peptone-free cultures were initially maintained with minimal wastage, except for regular samplings and analyses, in order to preserve most biomass and the only externally added growth and energy source was AB. However, after 53 and 39 days of operation of the AB20-D/P and AB50-D/P, respectively, both cultures were subjected to wasting twice a week, hence imposing an HRT/SRT value of 14 days.

For all AB-amended cultures, at the end of each feeding cycle (3 or 4 days), the appropriate volume of mixed liquor was removed and replaced by an equal volume of

Table 4.1. Composition of the stock culture media

Component	Concentration[a]
1. K_2HPO_4	600 mg/L
2. KH_2PO_4	335 mg/L
3. $CaCl_2 \cdot 2H_2O$	67.5 mg/L
4. $MgCl_2 \cdot 6H_2O$	135 mg/L
5. $MgSO_4 \cdot 7H_2O$	267.5 mg/L
6. $FeCl_2 \cdot 4H_2O$	67.5 mg/L
7. Tracer metal stock solution[b]	0.67 mL/L

[a] Based on the total feed volume (i.e., nutrients plus media) per feeding
[b] Composition given in Table 4.2

Table 4.2. Composition of the trace metal stock solution[a]

Component	Concentration (mg/L)
1. $ZnCl_2$	25
2. $MnCl_2 \cdot 4H_2O$	15
3. H_3BO_3	150
4. $CoCl_2 \cdot 6H_2O$	100
5. $CuCl_2 \cdot 2H_2O$	5
6. $NiSO_4 \cdot 6H_2O$	10
7. $NaMoO_4 \cdot 2H_2O$	15

[a] Adopted from Wolin et al. (1963)

Table 4.3. Description of AB-amended cultures

Parameter	AB-Amended Cultures			
	AB20+D/P	AB50+D/P	AB20-D/P	AB50-D/P
Main carbon source(s)	Dextrin/Peptone		AB	
Total volume (L)	1.6	1.6	1.6	1.6
AB conc. (mg/L)	20	50	20	50
HRT/SRT (days)	14[a]	14[b]	Phase 1 & 2[c]	Phase 1 & 2[c]

[a] After 10 days of stable operation
[b] After 21 days of stable operation
[c] Phase 1: minimal biomass waste; Phase 2: 14 days of HRT/SRT

culture media and an aliquot of AB solution. The following analyses were periodically performed: pH, TSS, VSS, soluble COD, DOC, ammonia, nitrite, and nitrate.

4.2.4. Toxicity Assessments

4.2.4.1. Oxygen Consumption

The inhibitory effect of QACs on the oxygen utilization of the mixed aerobic control culture was assessed at different QAC concentrations. Various concentrations (i.e., 0, 10, 20, 30, 50, 75, 100 mg/L) of QAC solution were prepared by diluting 1000 mg/L QAC stock solutions with culture media. Along with AB, three other QAC species were tested as well: didecyl dimethyl ammonium chloride (denoted as Didecyl), dioctyl dimethyl ammonium chloride (denoted as Dioctyl), and octyl decyl dimethyl ammonium chloride (denoted as Octyl decyl). An aliquot of 200 mL control culture was transferred to an Erlenmeyer flask where the DO was monitored until zero. Then, aliquots of 5 mL oxygen-free culture were transferred into 14.5 mL glass vials, and 5 mL prepared QAC solution was added into each vial. The vials were then sealed with rubber stoppers and aluminum seals and 5 mL fresh air was injected to the headspace of each sealed vial. The vials were then placed on a tumbler (6.9 rpm) and incubated at room temperature (22 to 23^{o}C). The oxygen concentration in the headspace of each vial was measured every six hours up to 24 hours (i.e., 0, 6, 12, 18, and 24 h), while the carbon dioxide concentration was measured only at the end of the assay (i.e., 24 h). Due to the limitation of wasted culture volume, an assay for each QAC was conducted at a different time. Prior to the initiation of each assay,

however, the oxygen uptake rate (OUR) of the culture sample was measured to obtain an OUR in a close range for all tested QACs.

4.2.4.2. OUR/SOUR

The effect of AB and its products on oxygen uptake rate (OUR) of the control culture was investigated. To assess the effect of the products of AB, enough volume of each culture (control, AB20+D/P, AB50+D/P) was taken at the last day of a feeding cycle in order to assure that all degradable soluble COD and residual QAC were removed. Culture aliquots were stored under sufficient aeration until the next step. Aliquots of AB20+D/P and AB50+D/P cultures were centrifuged for 10 minutes at 12,000 rpm to obtain supernatants which were then filtered with 0.2 μm membrane syringe filters. An aliquot of 30 mL of the control culture was mixed with fresh media and the filtered supernatant of both AB20+D/P and AB50+D/P cultures, in a ratio of 1:1, respectively. After mixing well, each sample was aerated for about 5 minutes to achieve oxygen saturation and then transferred to a 50 mL tapered-neck Erlenmeyer flask which was baked before at 350^{o}C to remove all organic carbon. The DO probe was inserted and the DO concentration over time was recorded.

The influence of AB on OUR was assessed in a similar manner. AB solutions of 20, 50, and 100 mg/L were prepared by mixing fresh media and a stock AB solution. The control culture was combined with the same volume of respective AB solutions, hence resulting in half of original concentration. The well-mixed sample was aerated and

transferred to a 50 mL tapered-neck Erlenmeyer flask to measure DO. The same experimental procedures were repeated with AB+20 and AB+50 cultures.

VSS of the cultures and pH of all samples were recorded before measuring DO. Based on DO changes over time, linearized OUR values were estimated and specific OUR values were calculated based on OUR and VSS values.

4.2.4.3. Microtox® Test

The acute toxicity of AB and its aerobic degradation product(s) in the AB-amended and dextrin/peptone fed cultures was assessed using the standard Microtox® test. The Microtox® system consists of the Azur Environmental M500 Analyzer with Microtox® Omni Software (Strategic Diagnostics Inc., Newark, DE). The acute toxicity is measured by detecting the change in luminescence of *Vibrio fischeri*, a marine bacterium that naturally gives off luminescent light. All samples were adjusted to 2% NaCl before analysis in order to have the proper osmotic pressure for the bacteria and different sample dilutions were tested in 15 minutes. The luminescence given off by the different dilutions of the sample was compared to that of the control, which consisted of 2% NaCl in DI water (pH 6.5). The effective concentration of an analyte (i.e., AB in this case) that causes the bacteria to emit light at 50% of their maximum response is the EC_{50} concentration. The 95% confidence range and R^2 values were calculated by linear regression of the data (x-axis: log of the concentration; y-axis: log of the fractional change in fluorescence after the sample is added and incubated for 15 minutes taking into account the change of fluorescence in the control). The linear regression line is extrapolated to calculate the EC_{50} value when the sample

concentration is not high enough to depress the florescence by 50%. Five samples were tested in this study: culture media, supernatant from the control, AB20+D/P and AB50+D/P cultures, and a 20 mg/L AB solution in culture media. All samples were filtered through 0.2 μm membrane filters (Fisherbrand, Pittsburgh, PA, USA). Samples from AB-amended cultures were taken at the last day of the feeding cycle in order for all AB to have been removed and transformed to byproduct(s) at the time of the analysis.

4.3. Results and Discussions

4.3.1. Alkyl Benzyl Dimethyl Ammonium Chloride (AB)

The general formula of AB is represented as $C_{n+9}H_{2n+14}NCl$, where n refers to the number of carbon on the alkyl chain. With this formula, the stoichiometric oxidation equation for AB can be expressed as

$$C_{n+9}H_{2n+14}NCl + \frac{3n+23}{2}O_2 \rightarrow NH_3 + (n+9)CO_2 + (n+5)H_2O + HCl \quad (4\text{-}1)$$

Barquat MB-80™, a commercial sanitizer that was used for this study, mainly consists of three different AB homologues with C_{12}, C_{14}, and C_{16} alkyl groups, respectively. A detailed description of Barquat MB-80™ is given in Table 4.4. Based on the composition ratio and theoretical values in terms of organic carbon and oxygen demand for each component, the estimated values of organic carbon and oxygen demand were 0.651 g C/g mixture and 2.44 g O_2/g mixture, respectively. The experimental values of DOC and COD were 0.693 ± 0.003 g/g mixture and 2.52 ± 0.11 g/g mixture, which agree well with the calculated values.

Table 4.4. Characteristics of the AB mixture (Barquat MB-80™)

	Active ingredients			Inert ingredients	
Components	C_{12}-AB	C_{14}-AB	C_{16}-AB	Ethanol	Water
Ratio (%, w/w)	32	40	8	10	10
Molecular formula	$C_{21}H_{38}NCl$	$C_{23}H_{42}NCl$	$C_{25}H_{46}NCl$	C_2H_5OH	H_2O
MW (g/mol)	339.99	368.04	396.09	46.07	18.02
ThOC (g/g)	0.742	0.751	0.758	0.521	-
ThOD (g/g)	2.72	2.83	2.87	2.08	-

Summarized physico-chemical values of three AB compounds are shown in Table 4.5, estimated using the EPI Suite™ software. This estimation program employs a chemical notation system called SMILES (Simplified Molecular Input Line Entry System) to depict the molecular structure of a target compound and SMILES covers all information about atoms, bonds, and branches of a compound. Because of its amphiphilic character having both an apolar and a polar functional group, octanol is a versatile solvent that embraces any kind of solutes (Schwarzenbach et al., 2003) and in turn octanol-water coefficient K_{ow} plays a good role to predict partitioning of a compound between organic and aqueous phase. The K_{ow} values of AB compounds listed in Table 4.5 are quite large and tend to become larger by an order of magnitude as the length of alkyl chain gets longer, meaning that the alkyl chain of an AB compound increasingly affects its hydrophobicity. Nevertheless, predicted or experimental K_{ow} values are confined to limited practicality in that cationic surfactants inherently accumulate at the interface between two phases. For this reason, the accurate measurement of the K_{ow} for a surfactant is difficult and, even if the accuracy of measurement can be assured enough, K_{ow} is not a proper indicator of hydrophobicity for predicting environmental behavior because cationic surfactants are likely to form complexes immediately in contact with negatively charged constituents in soil, sludge, and sewage in the environment (Boethling, 1984). Estimation of boiling/melting points as well as vapor pressure is computed with a methodology based on group contributions of a molecule (Stein and Brown, 1994). Not to mention their large molecular sizes and complexity of AB compounds, high melting/boiling points and substantially low vapor pressures are indicative of their non-volatility. As for water solubility, it decreases

Table 4.5. Summary of physico-chemical properties of AB homologues[a]

	C_{12}-AB	C_{14}-AB	C_{16}-AB
Octanol-water partition coeff. (K_{ow})	8.51E+02	8.13E+03	7.76E+04
Boiling/Melting point (ºC)	537.63 / 230.18	560.84 / 241.02	584.04 / 251.86
Vapor pressure (mm Hg)	1.88E-11	3.53E-12	9.28E-11
Water solubility (mg/L at 25 ºC)	22.47	2.203	0.215
Soil adsorption coeff. ($K_{oc,}$ mL/g solid)	8.424E+05	2.865E+06	9.747E+06

[a]Estimated using the EPI Stuie™

approximately by an order of magnitude as the alkyl chain length increases and these predictions are consistent with increasing hydrophobicity of a longer alkyl chain AB compound. However, it should be noted that these estimations are poorly comparable with those, ranging from 0.85 to 75% w/v for C_{12} to C_{16}-AB, in a previously published work (Linfield, 1970). An experimental result conducted with the homologues of dialkyl dimethyl ammonium chloride and alkyl trimethyl ammonium chloride demonstrates that water solubility decreased by about an order of magnitude as their alkyl chain(s) became longer (Kunieda and Shinoda, 1978). K_{oc}, referred to as soil adsorption coefficient, can be defined as

$$K_{oc} = \frac{C_{oc}}{C_w} \tag{4-2}$$

where C_{oc} is the amount of chemical adsorbed per unit mass of organic carbon in the soil or sediment and C_w is the concentration of chemical in the solution at equilibrium (Schwarzenbach et al., 2003). K_{oc} provides information of the extent to which a chemical partitions between solid and aqueous phases in soil, or between sediment and water in aquatic environments. The results in Table 4.5 indicate that soil adsorption of AB is also closely associated with alkyl chain length, namely hydrophobicity. The tendency that adsorption of AB largely increases as the alkyl chain increases is consistent with some studies (Garcia et al., 2004; Kwolek et al., 2003) suggesting that Van der Waals hydrophobic interaction is a major driving force for the adsorption of long-chain AB homologues.

4.3.2. Control Culture Performance

4.3.2.1. Culture Monitoring

The control culture was maintained in excess of 230 days. Throughout this period, its pH was 7.6 ± 0.5, and was adjusted with sodium bicarbonate providing alkalinity consumed by nitrification.

The TSS and VSS concentration, measured over the last 140 days was equal to 3310 ± 190 mg/L and 2640 ± 170 mg/L, respectively. As seen in Figure 4.1, both TSS and VSS were stably maintained over the measurement period. Every week, 1 L of culture volume was wasted, thus resulting in 14 days of SRT. As a result, it is expected that approximately 2640 mg microbial mass should be reproduced weekly to maintain the reported VSS concentration and this value accounts for about 190 mg VSS produced/L-day. Combining with the widely-used empirical formula for bacterial cells (Rittmann and McCarty, 2001), that is $C_5H_7O_2N$, 23 mg/L-day of nitrogen is required to synthesize the above-stated biomass. The nitrogen content of the peptone and yeast extract used in this study is reported as 8.0% and 10.9% (w/w), respectively. Based on these values and taking into account the feeding protocol of the control culture, 25.5 mg N/L-day should be released in each feeding cycle, assuming that all organic nitrogen is bioavailable and therefore degraded. Upon a simple assumption that there is no decay of microorganisms, 2.5 mg/L-day of nitrogen which is the difference subtracting 23 from 25.5 mg/L-day is expected to remain in the culture.

The soluble chemical oxygen demand (sCOD) and the dissolved organic carbon (DOC) were measured in samples taken at the last day of each feeding cycle and results are

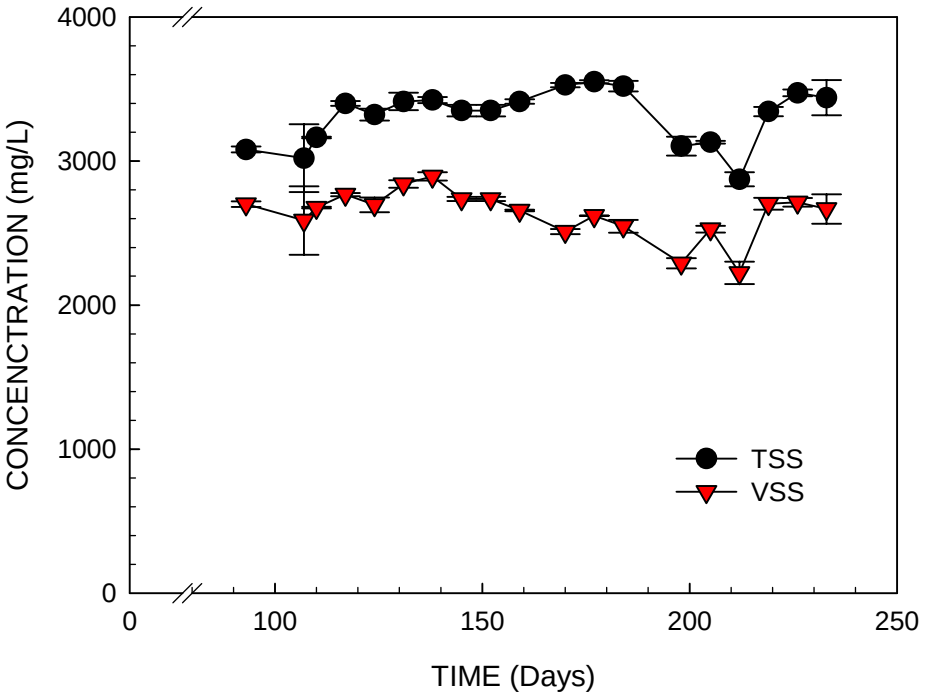


Figure 4.1. Suspended solids in the control culture over the test period.

shown in Figure 4.2. The soluble COD values varied more than the DOC values. Over the last 100 days of operation, the mean ± standard deviation of the soluble COD and DOC values was 205 ± 43 mg/L and 43 ± 6 mg/L, respectively. Because, as it is shown below, all degradable organic material was removed within the first 24 hours of each feeding cycle, the soluble COD and DOC values shown in Figure 4.2 are attributed to dissolved non-biodegradable, or inert organic materials.

The ammonia, nitrite, and nitrate concentrations, measured at the end of each feeding cycle and at the beginning of the subsequent feeding cycle, over time are shown in Figure 4.3. Ammonia, produced as a result of peptone degradation, was always low (less than 13 mg N/L). The highest nitrite concentration (40 mg N/L) was detected at the initial feeding cycles, but with increased time, nitrite was not detected most of the time and when detected was below 12 mg N/L. The culture nitrate concentration increased constantly during the first 15 days of operation and ranged between 110 and 185 mg N/L for the next 65 days of operation. During the last 100 days, the nitrate concentration ranged between 65 and 135 mg N/L. The frequent swings in the nitrate concentration are due to culture wastage and feeding every 3 or 4 days. Based on the above observations, it is concluded that the enriched mixed culture degraded the organic feed (dextrin and peptone), and converted the resulting ammonia to nitrate without any significant accumulation of either ammonia or nitrite. The high nitrification capacity of the control culture is attributed to the fact that its SRT value of 14 days was significantly higher than the typical design SRT values of 4 to 7 days of nitrifying activated sludge systems operated at 20ºC (Metcalf & Eddy Inc., 2003).

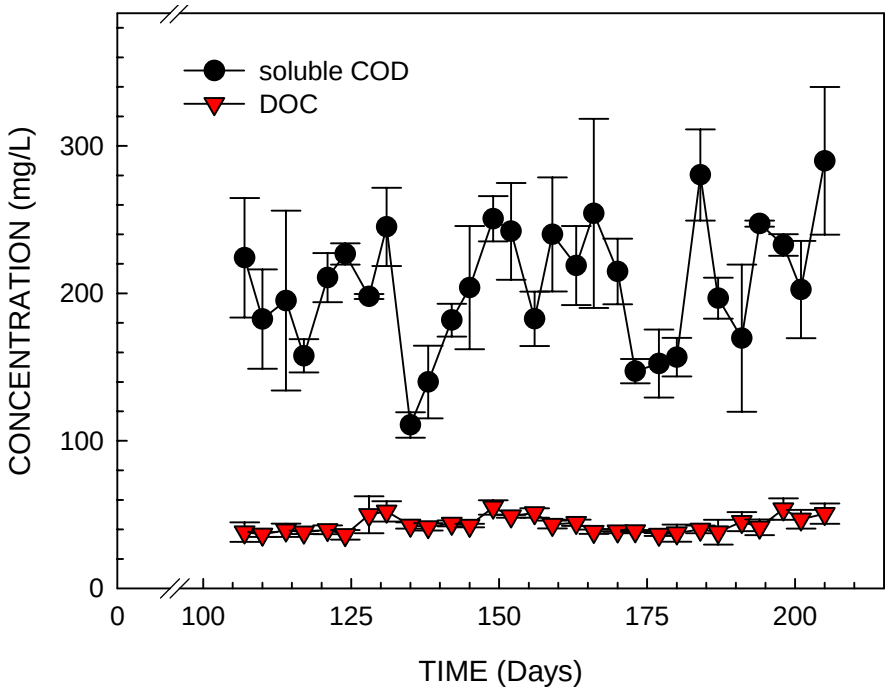


Figure 4.2. Soluble COD and DOC in the control culture over the test period.

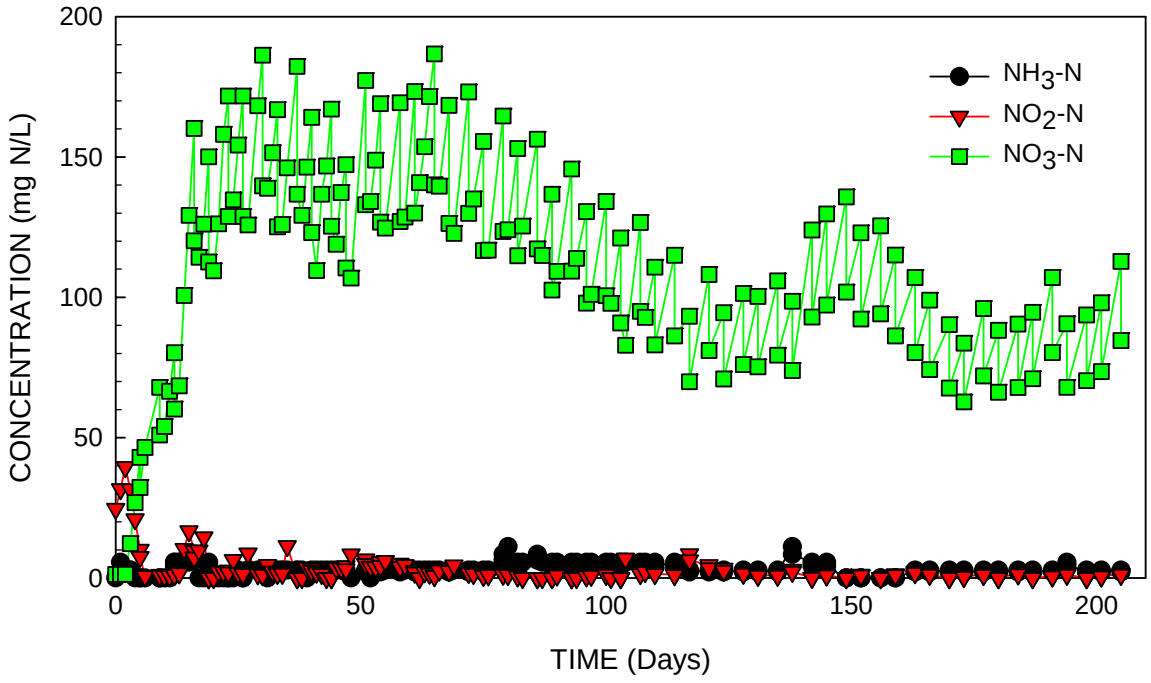


Figure 4.3. Profiles of the three nitrogen species in the control culture over the test period.

Lower SRT results in a washout of nitrifying bacteria due to their relatively low growth rate (Campos et al., 1998).

4.3.2.2. Feeding cycle characterization

A detailed characterization of a typical, 4-day feeding cycle (between 61 to 65 days) was conducted to better understand the dynamics of feed degradation, ammonia production and nitrification. Just before the initiation of this feeding cycle, 500 mL of culture mixed liquor was replaced with an equal volume of culture media containing 1g/L sodium bicarbonate to provide alkalinity. The following parameters were monitored over the 4-day feeding cycle: pH, DOC, soluble COD, ammonia, nitrite, and nitrate. The results of pH, soluble COD, DOC and three nitrogen species variation during the feeding cycle are shown in Figure 4.4. Within 24 hours, the pH increased from an initial value of 7.2 to 7.8 as added sodium bicarbonate dissolved in the culture and then gradually decreased to 7.4 by the end of the cycle, a result of nitrification of ammonia released by the degradation of the feed organic nitrogen (Figure 4.4A). The pattern of soluble COD and DOC decrease over the 4-day cycle is shown in Figure 4.4B. All degradable organic feed was removed within the first 24 hours and a certain fraction, considered to be non-biodegradable remained. Therefore, the heterotrophic population of the mixed culture underwent endogenous respiration for the remaining three days of the cycle. The pattern of the three nitrogen species is shown in Figure 4.4C. Ammonia was released, but its concentration was kept below 8 mg N/L as a result of oxidation to nitrate, which increased from an initial value of 130 mg N/L to 187 mg N/L. Very low concentrations (below 6 mg N/L) of nitrite were

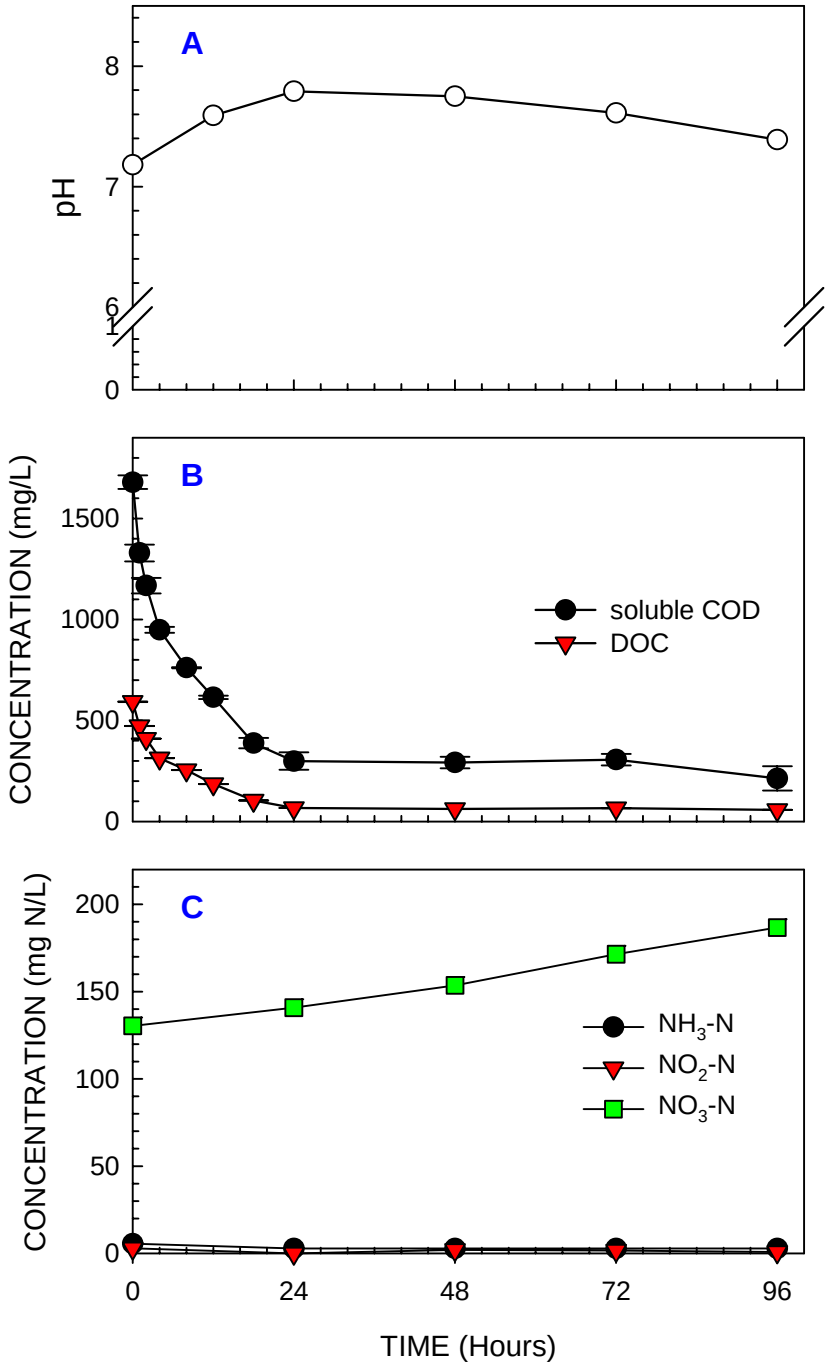


Figure 4.4. pH (A), soluble COD and DOC (B), and three nitrogen species (C) variation in the control culture during one feeding cycle.

observed. Combination of the results shown in Figure 4.4 supports the successful activity of nitrifiers in the culture. It should be also deduced that heterotrophs in the culture recovered the loss of biomass caused by wastage within a short time and underwent endogenous respiration after 24 hours, while nitrifiers were active throughout the entire feeding cycle.

4.3.3. AB-amended Cultures

4.3.3.1. AB20+D/P

Initially developed from the control culture, a culture designated as AB20+D/P, was amended AB resulting in an initial AB concentration of 20 mg/L and was fed similarly to the control culture. After an initial operation of 10 days in which ordinary wastage and feed were suspended until AB was removed, the culture was fed every 3 or 4 days and maintained with a 14-day SRT. At the beginning of each feeding cycle, sodium bicarbonate (up to 0.4 g/L-media) was added. The mean pH of the culture was 7.5 ± 0.6 throughout the entire test period.

Figure 4.5A shows the pattern of multiple AB additions at an initial concentration of 20 mg/L. In all cases, AB was completely removed by the second day of the feeding cycle without any sign of inhibition. The soluble COD and DOC concentrations within a feeding cycle were monitored and shown in Figure 4.6. Like in the control culture, all degradable feed was utilized within 24 hours. In comparison with Figure 4.4B, soluble COD of 1800 mg/L and DOC of 700 mg/L at the beginning were higher than those of the control culture because of the contribution of added AB, but residual values after 24 hours were similar.

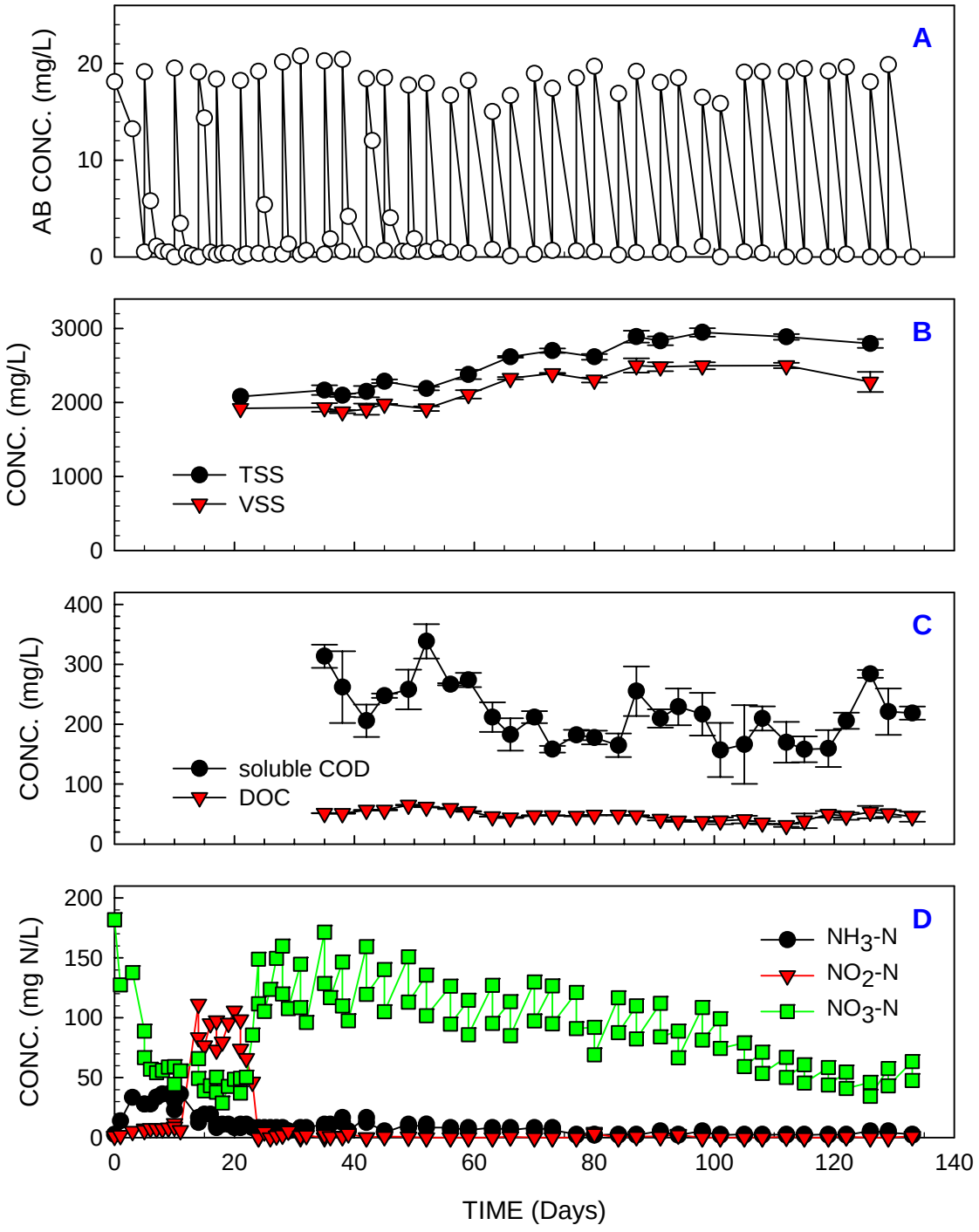


Figure 4.5. AB (A), TSS and VSS (B), soluble COD and DOC (C), and three nitrogen species (D) in the AB20+D/P culture over the test period.

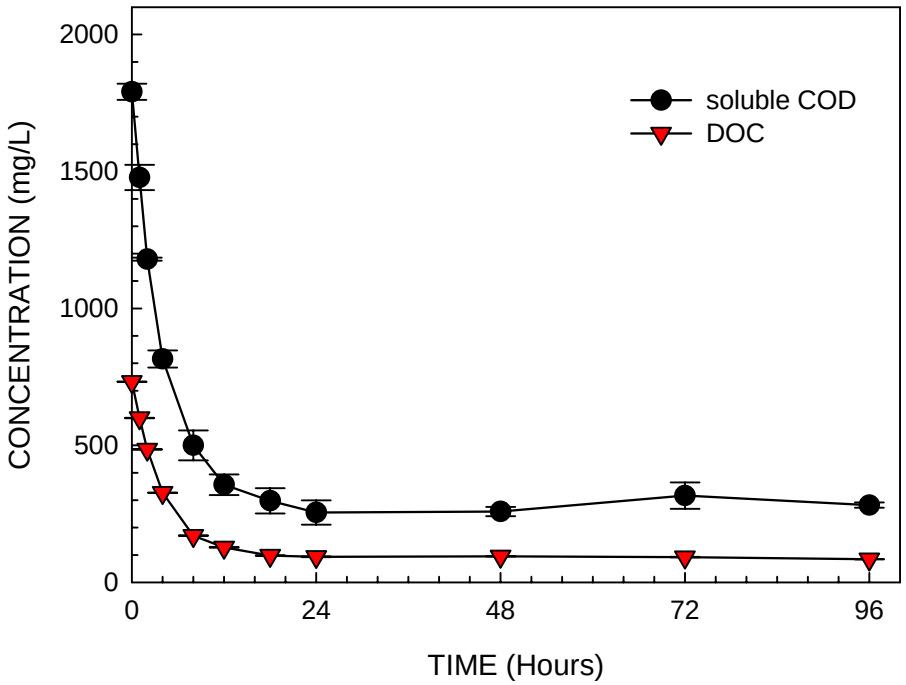


Figure 4.6. Soluble COD and DOC variation in the AB20+D/P culture during one feeding cycle.

These results show that 20 mg/L AB did not have any inhibitory effect on the culture and the degradation of AB took place simultaneously with the degradation of the feed.

The culture VSS concentration dropped to about 1900 mg/L between 25 and 50 days of operation, but in the latter 50 days of operation, the VSS concentration increased to about 2500 mg/L, a value similar to that of the control culture (Figure 4.5B). The culture soluble COD concentration shown in Figure 4.5C, measured at the end of the feeding cycles, for the initial test period (i.e., up to 50 days) ranged between 205 and 338 mg/L, but towards the end of the test (around 125 days), the mean soluble COD concentration was about 200 mg/L. The culture DOC concentration, measured at the end of the feeding cycles, over the entire test period ranged between 31 and 65 mg/L, respectively. The initial decrease in VSS and increase in soluble COD suggest that significant cell lysis was caused by AB (McDonnell and Russell, 1999). However, with prolonged culture operation, selection of more resistant species took place. A previous study dealing with dodecyl trimethyl ammonium chloride (DTMAC) also reported the selection of more tolerant bacterial species by chronic exposure and recovery of heterotrophic activity (Ventullo and Larson, 1986).

The concentrations of all nitrogen species measured in the AB20+D/P culture over the duration of this test are shown in Figure 4.5D. Initially, the ammonia concentration increased and remained around 33 mg N/L for about 11 days, and then decreased and remained below 17 mg N/L for the entire test period. The nitrite concentration was about 8 mg N/L for 11 days, suddenly increased to as high as 111 mg N/L for a period of 12 days, and then decreased to and remained at non-detectable levels for the rest of the test. The

nitrate concentration decreased initially and remained between 29 and 65 mg N/L for about 23 days of operation, and then increased again following a pattern similar to that of the control culture as discussed above. Towards the end of this test, the mean nitrate concentration was about 46 mg N/L. These results indicate that initially, while ammonification was not inhibited, both nitrification steps (i.e., ammonia to nitrite and nitrite to nitrate) were inhibited. However, with prolonged culture operation, complete nitrification was achieved, more likely as a result of acclimation and/or selection of nitrifying bacterial species more tolerant to AB. In a previous study (Pitter, 1962), the QACs hexadecyl pyridinium bromide (HPB) and hexadecyl trimethyl ammonium chloride (HTMAB) strongly inhibited nitrification at 3 mg/L and completely blocked the process at 6 mg/L. However, these results were conducted for a relatively short period (8 hours), which probably precluded any culture acclimation to these QACs. Another study conducted with an acclimated sewage sludge showed that nitrification was not impacted at 16 mg/L of AB (C_{12} alkyl chain) (Gerike et al., 1978).

4.3.3.2. AB50+D/P

This culture, developed with mixed liquor from the AB20+D/P culture which had been maintained for 10 days, was amended with AB resulting in an initial AB concentration of 50 mg/L at each feeding cycle. After a period of minimal culture wastage for 21 days, the culture was maintained with an SRT value of 14 days. Although sodium bicarbonate was not added to this culture, its pH was 8.3 ± 0.4 as a result of almost complete inhibition of the nitrification process as discussed below.

Figure 4.7A shows the pattern of multiple AB additions at an initial concentration of 50 mg/L. With the exception of the second and fourth feeding cycle, AB was degraded without any delay. However, after the fifth feeding cycle, the disulfine blue method used for the quantification of AB yielded a low level of absorbance equivalent to about 3 to 7 mg/L AB. Based on HPLC analysis it was shown that these residual values were indeed C_{12} and C_{14} AB homologues. The culture biomass concentration varied between 810 and 1430 mg TSS/L and between 610 and 1070 mg VSS/L (Figure 4.7B). These values are significantly lower than those of the control and AB20+D/P cultures. The mean soluble COD and DOC values, shown in Figure 4.7C, were 420 ± 60 mg/L and 100 ± 20 mg/L, respectively, which again are much higher than the values observed in the control and the AB20+D/P cultures. These results show that AB at an initial concentration of 50 mg/L resulted in a high extent of cell lysis.

Figure 4.7D shows the concentration of all nitrogen species measured in the AB50+D/P culture over the test period. After about 5 days, the ammonia concentration was first decreased and then gradually increased and remained between 63 and 123 mg N/L after 40 days of operation. For the first 30 days of incubation, nitrite was the predominant ammonia oxidation product reaching a value as high as 100 mg N/L, and then decreased to non detectable levels after 38 days of operation. Nitrate decreased initially and its concentration was below 9 mg N/L for the entire test period. In spite the fact that AB50+D/P started with mixed liquor from the AB20+D/P culture, which achieved complete nitrification of the ammonia released due to ammonification of the organic feed,

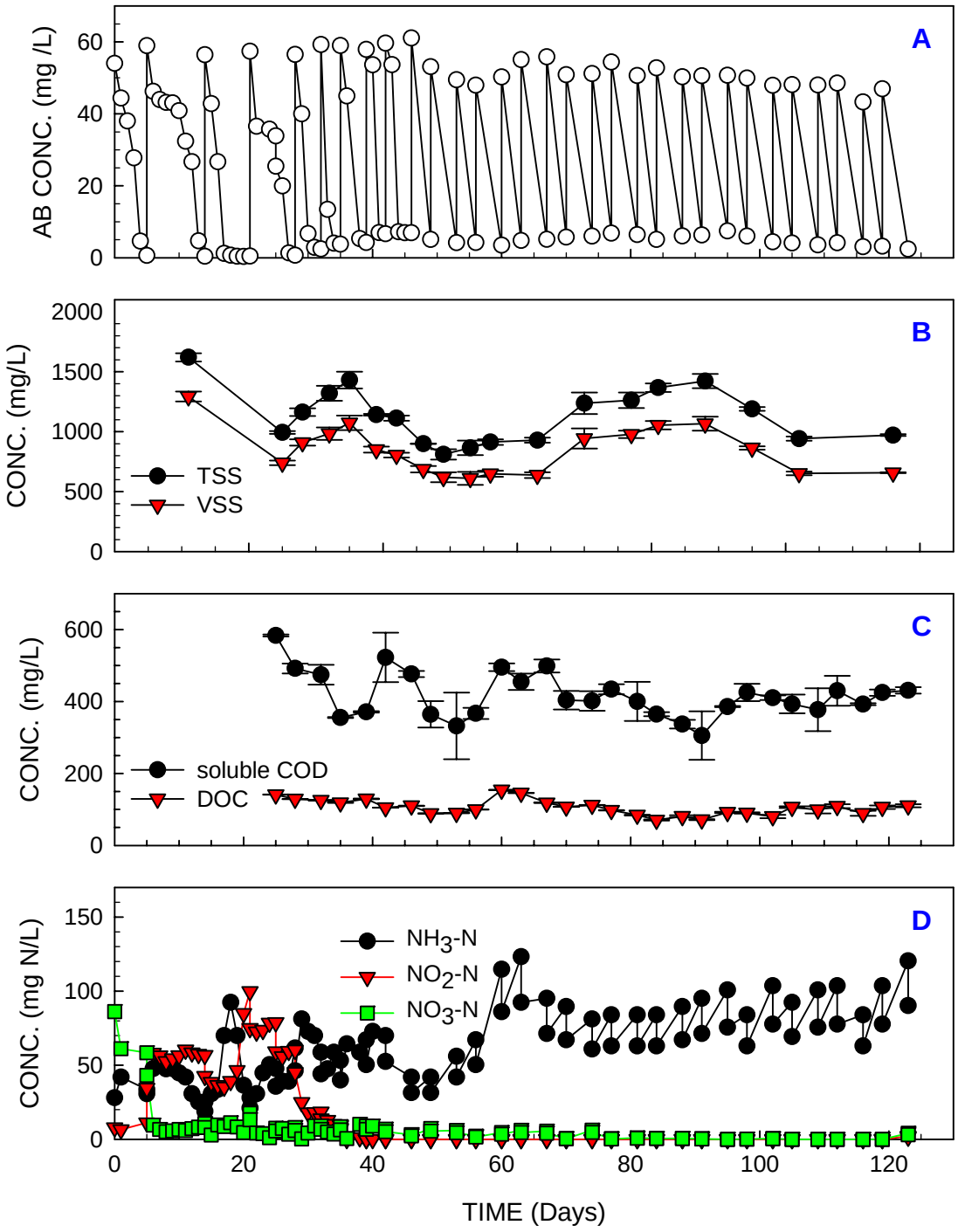


Figure 4.7. AB (A), TSS and VSS (B), soluble COD and DOC (C), and three nitrogen species (D) in the AB50+D/P over the test period.

AB at an initial concentration of 50 mg/L resulted in severe inhibition of the nitrification process.

4.3.3.3. AB20-D/P

This culture, which was developed using AB20+D/P culture mixed liquor after the culture had been in operation for 38 days, was maintained similarly to the AB20+D/P culture, except that it was fed with culture media and AB was the only externally added carbon source. The mean pH of the culture was 6.8 ± 0.3 throughout the entire test period and was periodically adjusted with the addition of sodium bicarbonate (up to 40 mg for each feed).

Figure 4.8A shows the pattern of multiple AB additions at an initial concentration of 20 mg/L. As was the case with culture AB20+D/P, AB was completely removed by the second day of the feeding cycle. The TSS and VSS concentration of the culture decreased over time as a result of biomass decay because of the low external carbon source (i.e., AB), as well as cell lysis (Figure 4.8B). A further decrease in the culture solids concentration at 53 days of operation is due to the fact that the culture was wasted frequently to maintain an SRT of 14 days. Under these conditions, the culture VSS concentration reached a final value of about 50 mg/L. In spite of this low biomass concentration, this culture was able to degrade AB as well as to achieve nitrification as discussed below. The culture soluble COD and DOC, measured at the end of the feeding cycles, declined over time but to a lower degree as compared to the decline of the culture VSS, indicating that a significant fraction of the cell lysis products was either slowly degraded or non-degradable (Figure 4.8C).

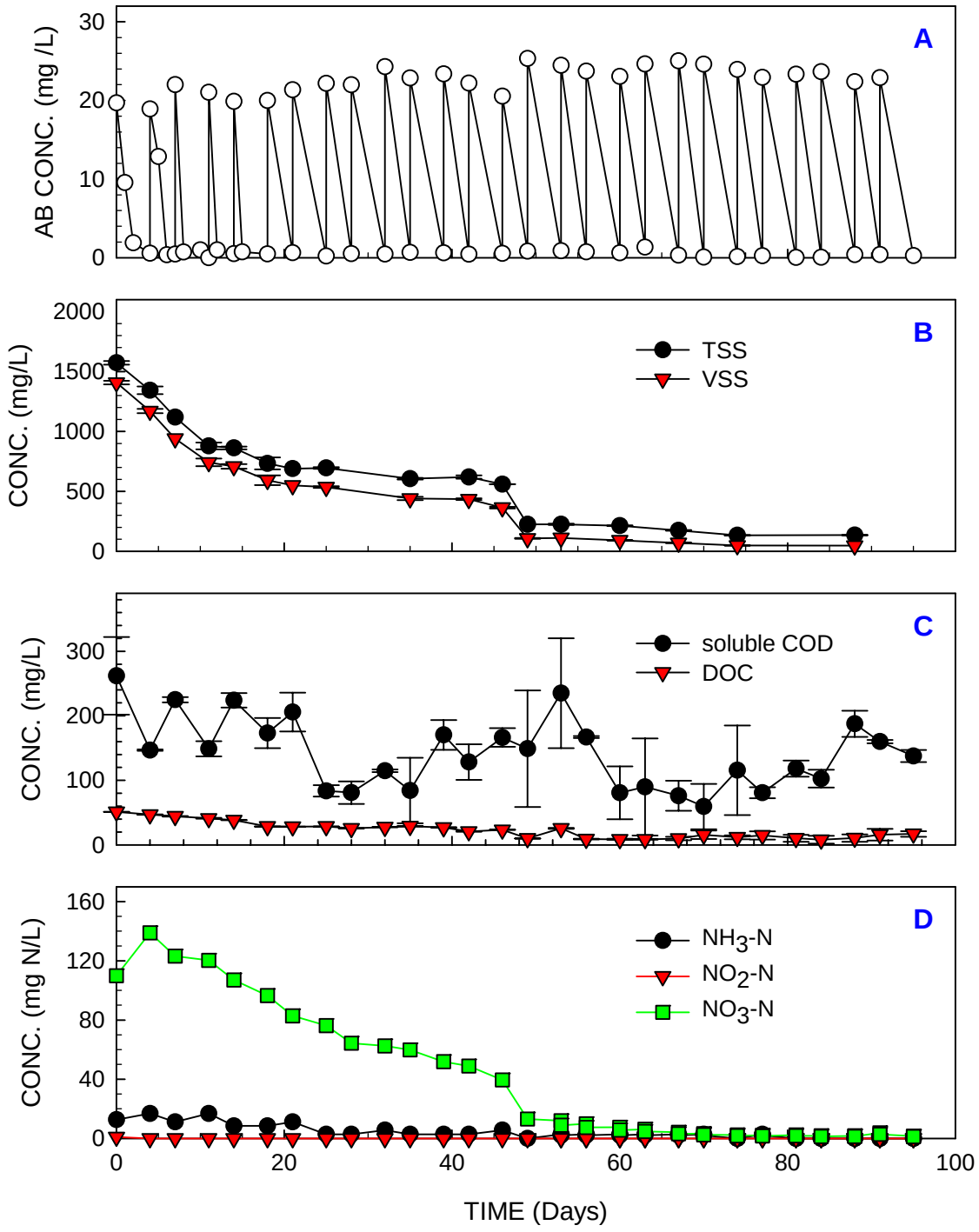


Figure 4.8. AB (A), TSS and VSS (B), soluble COD and DOC (C), and three nitrogen species (D) in the AB20-D/P over the test period.

The ammonia concentration was about 10 mg N/L for the first 20 days and gradually declined to non-detectable levels (Figure 4.8D). The nitrate concentration decreased constantly as a result of culture volume loss due to regular samplings and replacement with fresh media, and reached a steady state value of about 2 mg N/L. Nitrite was not detected throughout the test period. Note that the only external nitrogen source for this culture was AB. For the level of AB fed, the expected nitrogen released upon AB degradation is about 1 mg N/L. Therefore, during the last 20 to 30 days, this culture must have been nitrogen-limited. In spite of such conditions, this culture was consistently able to degrade AB at an initial concentration of 20 mg/L over a long period.

4.3.3.4. AB50-D/P

This culture, which was developed using AB50+D/P culture mixed liquor after the culture had been in operation for 42 days, was maintained similarly to the AB50+D/P culture, except that it was fed with culture media and AB was the only externally added carbon source. After 39 days of operation, the culture was maintained at an SRT of 14 days. The mean pH was 6.9 ± 0.1 adjusted with sodium bicarbonate (up to 80 mg for each feed).

The pattern of AB additions at an initial concentration of 50 mg/L and its removal are shown in Figure 4.9A. With the exception of two feeding periods (day 55 to 67 and 105 to 116) where the AB removal was slow, 50 mg/L of AB was removed within the feeding cycle. As was the case with the AB50+D/P culture, a residual AB concentration between 3 and 12 mg/L was observed. The slow period of AB removal, which resulted in relatively high AB concentrations for a significant time, were accompanied by excessive cell lysis

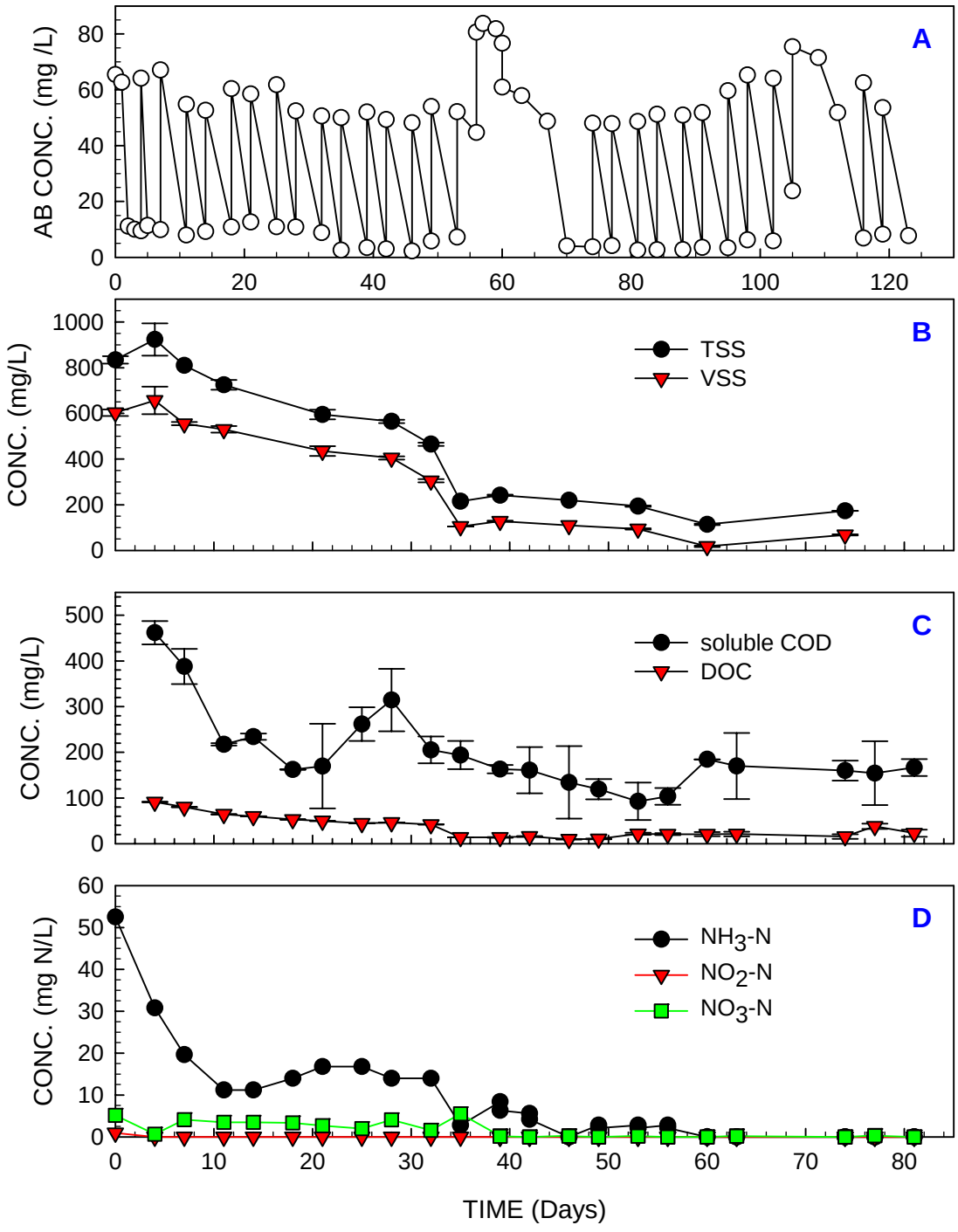


Figure 4.9. AB (A), TSS and VSS (B), soluble COD and DOC (C), and three nitrogen species (D) in the AB50-D/P over the test period.

demonstrated by both a sudden decrease of the culture biomass concentration and an increase of the soluble COD concentration (Figure 4.9B and Figure 4.9C). However, it is remarkable that even at a very low biomass concentration, the culture was able to recover and resume fast AB degradation.

The concentration of the three nitrogen species in the AB50-D/P culture over time are shown in Figure 4.9D. Nitrite was not detected and the concentration of both ammonia and nitrate decreased over time and were not detected after 60 days of operation, indicating that all nitrogen made available by the degradation of AB was used for cell synthesis.

4.3.4. Toxicity Assessments

4.3.4.1. Oxygen Consumption

The first work of this assay was to estimate OURs of the control culture which was used for each QAC assay. OUR values in Figure 4.10 were observed to be in a close range so the profiles of oxygen consumption for all tested QACs could be comparable. As shown in Figure 4.11A, in the case of AB, the rate of oxygen consumption was low until 12 hours as the AB concentration increased and then the rates were similar for all AB concentrations. In the case of didecyl (Figure 4.11B), the difference in oxygen consumption was evident until 18 hours, but the amount of headspace oxygen measured at 24 hours was the same in all tested concentrations. In contrast to the results obtained with AB and didecyl, the addition of dioctyl decreased the oxygen consumption of the culture significantly at 75 mg/L and above (Figure 4.11C). For octyl decyl, Figure 4.11D shows that the difference in oxygen consumption between the various concentrations were clearer than for other tested

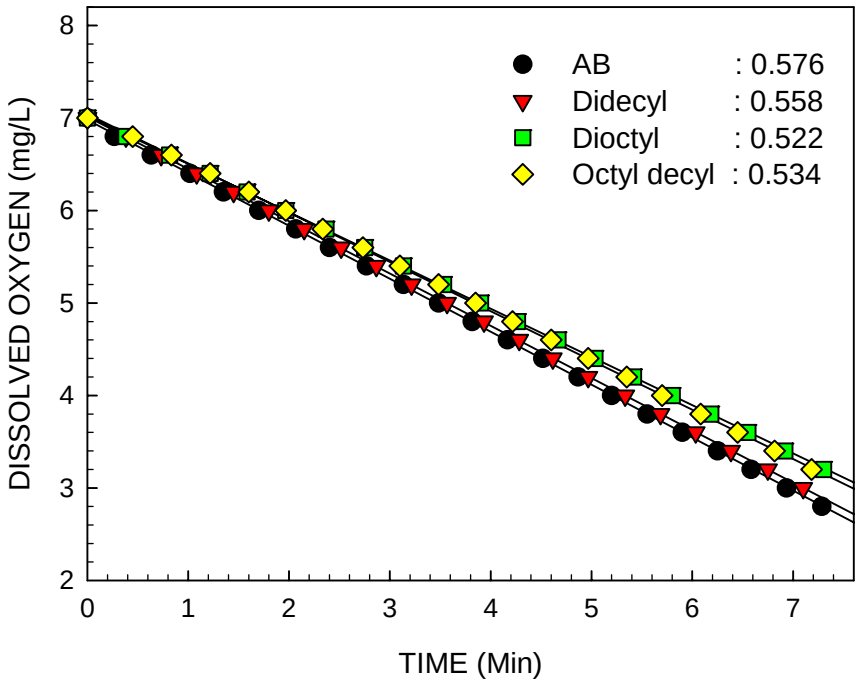


Figure 4.10. DO profile of the control culture samples used to test the oxygen consumption rate with different QACs (numbers represent OUR values in mg/L-min for each test).

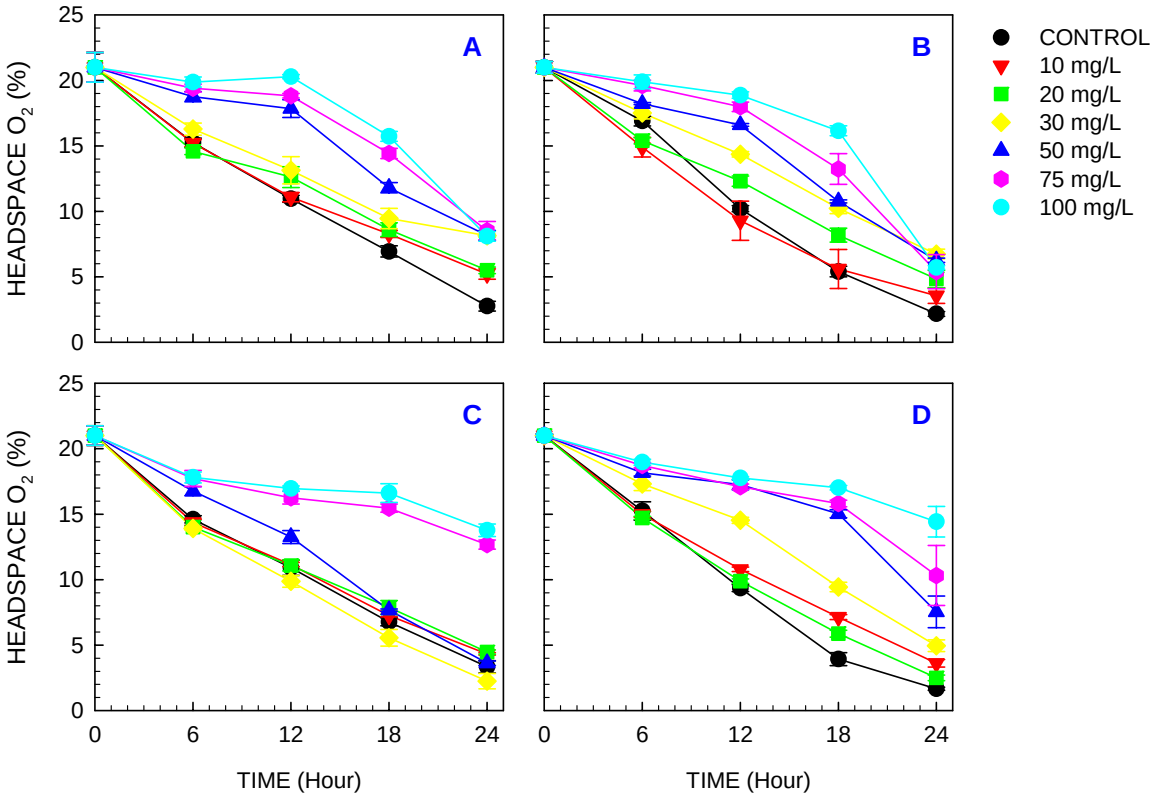


Figure 4.11. Oxygen consumption in the headspace of aerobic mixed culture samples amended with AB (A), Didecyl (B), Dioctyl (C), and Octyl decyl (D).

QACs over time. At 24 hours, the ratio of oxygen in headspace changed from 21 to 15% at 100 mg/L whereas it decreased to 2% at 0 mg/L (i.e., control). For all tested QACs, the rates of oxygen consumption were distinctly affected with increasing concentrations and the headspace oxygen decreased very slowly at higher QAC concentrations, especially during the early test period. After between 12 to 18 hours, depending on a species, however, the consumption rates tended to increase even at high QAC concentrations.

The headspace CO_2 generated after 24 hours of incubation, depicted in Figure 4.12, is consistent with the results from the measurements of oxygen consumption. The CO_2 production decreased gradually with increasing octyl decyl concentration although for other QAC species the amount of CO_2 formed did not differ notably over a certain level of QAC concentration.

At the end of the test, the QACs in the samples were measured and the results are shown in Figure 4.13. Recovery did not reach 100% for all samples, which may be the result of partial incorporation and/or biodegradation of QACs. As shown above, the mixed culture utilized up to 50 mg/L AB despite the occurrence of significant cell lysis. Therefore, partial degradation of QACs at higher concentrations before significant cell lysis took place during the relatively short incubation period is possible.

4.3.4.2. OUR/SOUR

The impact of AB and its product(s) on the oxygen uptake rate of both control and AB-amended cultures was assessed. The specific oxygen uptake rate (SOUR) is a useful indicator to estimate the microbial activity of a culture in that it considers not only the rate

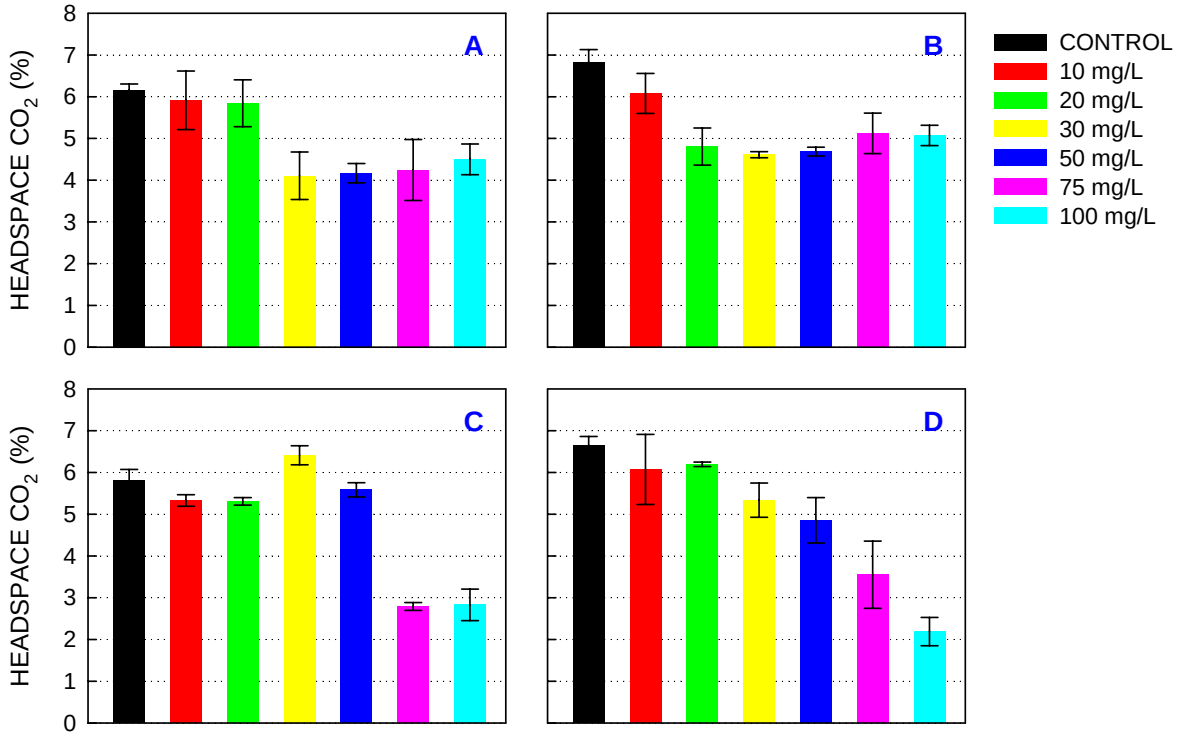


Figure 4.12. CO_2 formation in the headspace of the aerobic mixed culture amended with AB (A), Didecyl (B), Dioctyl (C), and Octyl decyl (D) after 24 hours of incubation.

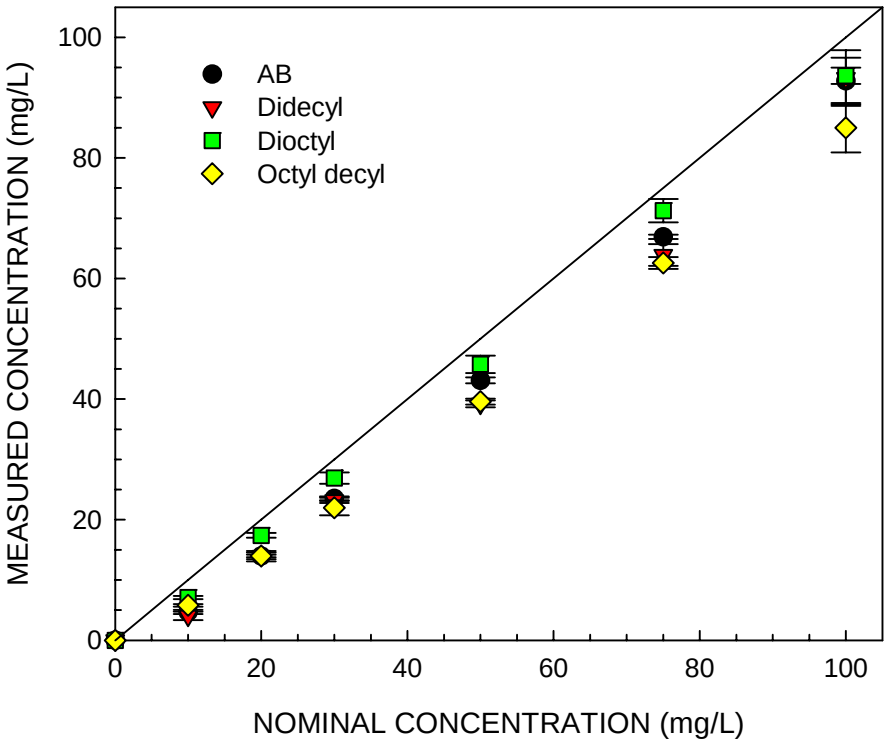


Figure 4.13. Recovery of the tested QACs after 24 hours of incubation under aerobic conditions.

of oxygen consumption but also the effect of microbial density. The measured OUR/SOUR values are listed in Table 4.6. When mixed with AB or supernatant of AB-amended cultures, OUR/SOUR of all tested cultures was found to be higher than only with culture media. This observation suggests the utilization of AB and its product(s). Filtered supernatant of AB-amended cultures might contain AB product(s) such as amines and benzoic acid, residual AB itself, or soluble lysis products all of which could be used as organic carbon sources. Moreover, the prepared AB solution also contained a small amount of other ingredients like ethanol (Table 4.4) and this can also facilitate microbial respiration. In the case of the control culture, the higher organic content in the AB50+D/P supernatant led to a higher SOUR than when the AB20+D/P supernatant was used. In addition, the SOUR of the control culture increased as the AB concentration increased up to 25 mg/L, but at 50 mg/L the SOUR value noticeably decreased. These results indicate that the microbial activity in this culture was significantly impacted at 50 mg/L in the short incubation time. Interestingly, the SOUR of the AB20+D/P culture increased up to 50 mg/L but remained about the same at 100 mg/L. In the case of the AB50+D/P culture, the SOUR increased steadily with increasing AB concentration, even at 100 mg/L. These results demonstrate the effect of the long-term acclimation of the AB-amended cultures, which led to selecting AB-resistant species.

4.3.4.3. Microtox® Test

Using the Microtox® test, the acute toxicity of five samples was assessed: culture media, 20 mg/L AB in culture media, and supernatant from the control, AB20+D/P, and

Table 4.6. OUR, SOUR, pH, and VSS values of control culture samples amended with different test solutions[a]

CULTURE		MEDIA	AB20+D/P PRODUCT(S)	AB50+D/P PRODUCT(S)	10 mg/L AB	20 mg/L AB	25 mg/L AB	50 mg/L AB	100 mg/L AB
CONTROL	OUR	5.3	7.6	11.3	11.7	N/A	13.9	6.1	N/A
	SOUR	4.0	5.7	8.4	8.8		10.4	4.6	
	pH	7.0	7.0	8.5	7.1		7.0	7.0	
	VSS	1340	1340	1340	1340		1340	1340	
AB20+D/P	OUR	3.1	N/A	N/A	N/A	7.3	N/A	10.1	11.2
	SOUR	3.2				7.4		10.2	9.7
	pH	6.7				6.7		6.7	6.9
	VSS	990				990		990	1160
AB50+D/P	OUR	4.3	N/A	N/A	N/A	8.6	N/A	14.9	12.5
	SOUR	8.1				15.9		27.5	40.3
	pH	7.7				7.7		7.7	7.8
	VSS	540				540		540	310

[a]Units: OUR, mg DO/L-h; SOUR, mg DO/g VSS-h; VSS, mg/L

AB50+D/P cultures obtained at 121, 49, and 39 days of operation, respectively. The results shown in Figure 4.14 point out that culture media and supernatant from both the control and the AB20+D/P cultures had no acute toxicity (Figure 4.14A to C). On the other hand, the effect of the supernatant of the AB50+D/P increased with increasing supernatant volumetric fraction, but did not exceed a 17% effect even at a supernatant fraction as high as 45 % (Figure 4.14D). This result is consistent with the previous finding that a trace amount of residual AB existed in the AB50+D/P culture. In contrast, 20 mg/L AB in culture media exhibited a very high toxicity (Figure 4.14E). The 5-min EC_{50} was 0.23 mg/L (95% confidence range: 0.16 to 0.30; $R^2 = 0.91$) and the 15-min EC_{50} was 0.15 mg/L (95% confidence range: 0.09 to 0.25; $R^2 = 0.83$). Based on the above results, it is concluded that although AB has a high acute toxicity, the aerobic mixed culture was able to degrade AB to products which had almost insignificant toxic effects. Previously reported acute Microtox® 15-min EC_{50} values ranged between 0.07 and 0.16 mg/L (Nalecz-Jawecki et al., 2003) for AB compounds and was equal to 0.20 mg/L for C_{12}-AB (Kaiser and Palabrica, 1991). The EC_{50} values measured in the present study agree well with those previously reported.

4.4. Summary

Efficient removal and degradation of a mixture of AB homologues was achieved with a mixed, aerobic culture at an AB concentration up to 50 mg/L when dextrin and peptone were the main carbon and energy source of this culture. Degradation of the added protein and ammonification were not impacted by AB even at an initial concentration of 50 mg/L. Nitrification of the produced ammonia was complete at an AB level of 20 mg/L, but

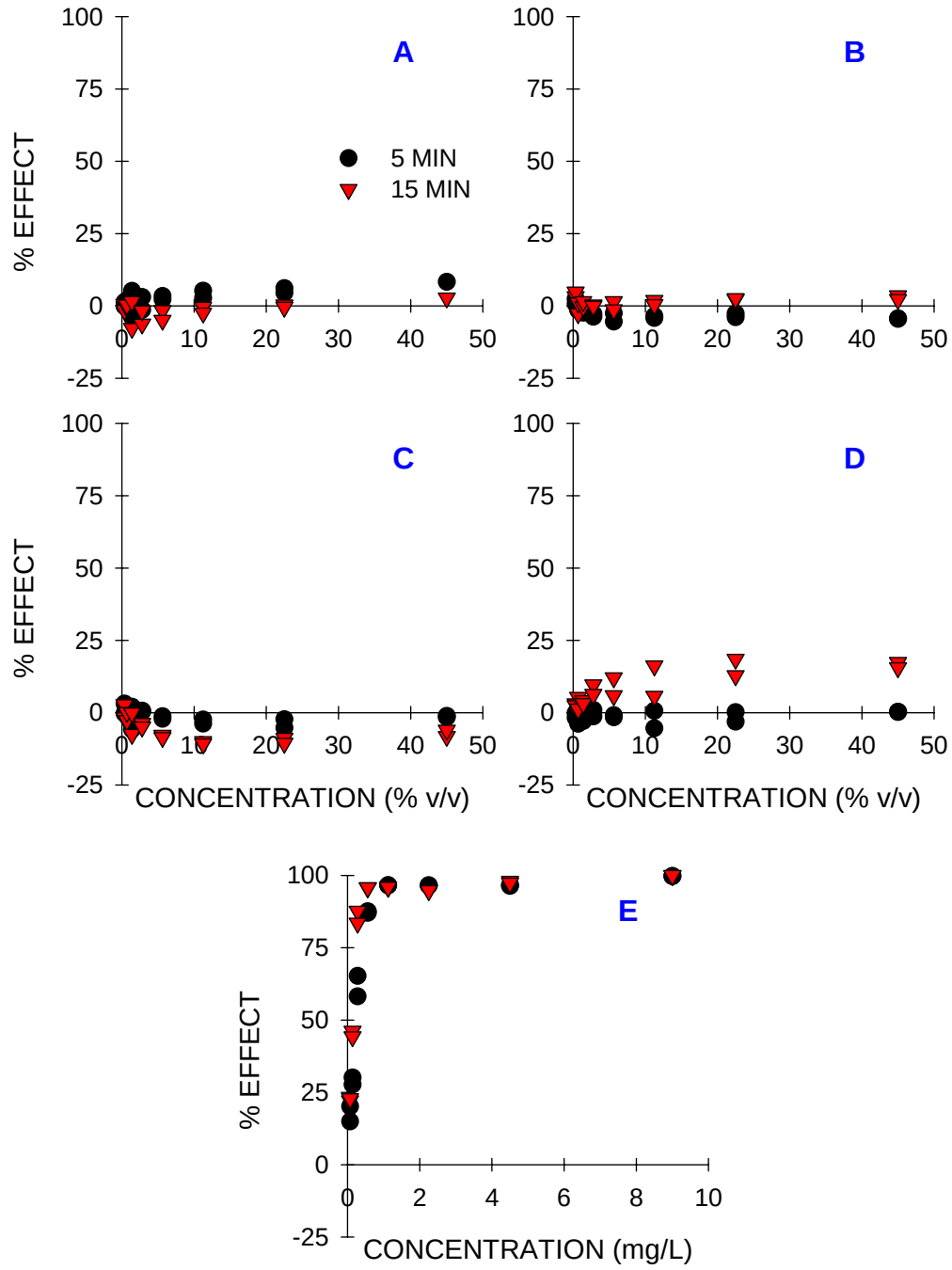


Figure 4.14. Results of the Microtox® test conducted with culture media (A), supernatant of the control (B), the AB20+D/P (C), and the AB50+D/P (D) cultures, and 20 mg/L AB solution in culture media (E).

at 50 mg/L, almost complete inhibition of nitrification was observed. Mixed cultures which were maintained with only AB as the externally supplied carbon and nitrogen source achieved a high degree of AB degradation at both 20 and 50 mg AB/L. The mixed aerobic cultures used in this study were able to degrade AB and eliminate its acute Microtox® toxicity.

CHAPTER 5

NITRIFYING CULTURE

5.1. Introduction

Nitrification plays an important role in wastewater treatment for the purpose of nitrogen removal and numerous efforts have been devoted to understand the mechanism of autotrophic ammonia oxidation. Oxidation of ammonia is achieved by a group of obligate chemolithoautotrophic bacteria which use the obtained energy for the fixation of CO_2 through the Calvin cycle (Bedard and Knowles, 1989; McCarty, 1999). Two different types of bacteria are responsible for nitrification, which is accomplished in two steps: conversion of ammonia to nitrite, by so-called ammonia oxidizers, and conversion of nitrite to nitrate, by nitrite oxidizers. Detailed investigation on the classification, distribution, and physiology of nitrifying microorganisms can be found in a number of previous studies (Bothe et al., 2000; Koops and Pommerening-Roser, 2001). In spite of its important implications, nitrification is a very sensitive process easily affected by external factors such as pH, temperature, microbial density, and inhibiting compounds (Anthonisen et al., 1976). A number of previous studies were performed with inhibitory compounds to elucidate their effect on autotrophic nitrifying bacteria (Dincer and Kargi, 2001; Kelly et al., 2004; Lee et al., 2000).

In contrast to the work presented in Chapter 4, which involved heterotroph-dominant cultures, the objectives of the work presented in this chapter were to enrich a

nitrifying culture and to assess the biodegradability and the toxic effect of AB on the nitrifying activity of the culture.

5.2. Materials and Methods

5.2.1. Control Nitrifying Culture

An aerobic mixed nitrifying culture was developed using as inoculum mixed liquor from the activated sludge reactor of the RM Clayton wastewater treatment plant, in Atlanta, GA. Initially, the culture was amended with ammonium chloride for about 70 days in order to selectively grow nitrifying bacteria. During this initial period, 1.5 L culture was semi-continuously fed twice a week, following the same intervals as the cultures described in Chapter 4. At each feeding, 100 mg N/L NH_4Cl and 1200 mg/L $NaHCO_3$ were added. The sodium bicarbonate was added to not only provide alkalinity, but also to be used as the carbon source. For the purpose of enriching slowly-growing nitrifying bacteria, before each feeding time, the culture was settled for more than 30 minutes and then 1250 mL of culture supernatant was wasted and replaced with fresh culture media. The stock culture media and trace metal solution were as those used with the mixed heterotrophic culture (see Table 4.1 and Table 4.2, respectively). A change in the feeding/wasting procedure by which 400 mL of culture was wasted before each feeding (twice a week) without culture settling, resulted in poor nitrification, more likely caused by accumulated chloride due to NH_4Cl addition. This modified procedure lasted for 25 days. The culture was then maintained as follows: twice a week, the culture was allowed to settle for more than 30 minutes, 1350 mL of culture supernatant was wasted and replaced with fresh media. The culture setup is

illustrated in Figure 5.1. The culture was maintained in a doubleside arm flask (Wheaton Science, Millville, NJ) equipped with a glass and Teflon®-coated paddle blade impeller and a Teflon®-coated magnetic stirrer. A HD-pH/P pH controller (Etatron, Italy) was also used to maintain stable pH via a pH probe (Orion Research Inc., Boston, MA) and a pump connected to a 0.5 N $NaHCO_3$ solution stored in a graduated cylinder. The set pH was 7.5 and the amount of $NaHCO_3$ solution consumed was recorded. Pre-humidified air was supplied through a fine pore diffuser in order to provide a sufficient DO level. The culture was maintained at room temperature (22 to 23^{o}C). pH, TSS, VSS, ammonia, nitrite, and nitrate were periodically measured following the procedures outlined in *Standard Methods* (Eaton et al., 2005) (see Chapter 3).

5.2.2. Oxygen Uptake Rate

At the 85th day of operation of the mixed nitrifying culture, aliquots of this culture, which had oxidized all ammonia and undergone starvation, were removed and aerated in order to achieve oxygen saturation. Then, aliquots of the aerated culture were transferred to a 50 mL tapered-neck Erlenmeyer flask and changes in DO over time were recorded using a DO meter (Yellow Springs Instrument, Yellow Springs, OH) equipped with a membrane probe (Yellow Springs Instrument, Yellow Springs, OH). The oxygen uptake rate (OUR) was calculated as the slope of the DO vs. time graph. The specific oxygen uptake rate (SOUR) was then calculated by dividing the OUR values by the culture VSS concentration. Several OUR runs were performed with the addition of either NH_4Cl or glucose as explained in the Results and Discussion section.

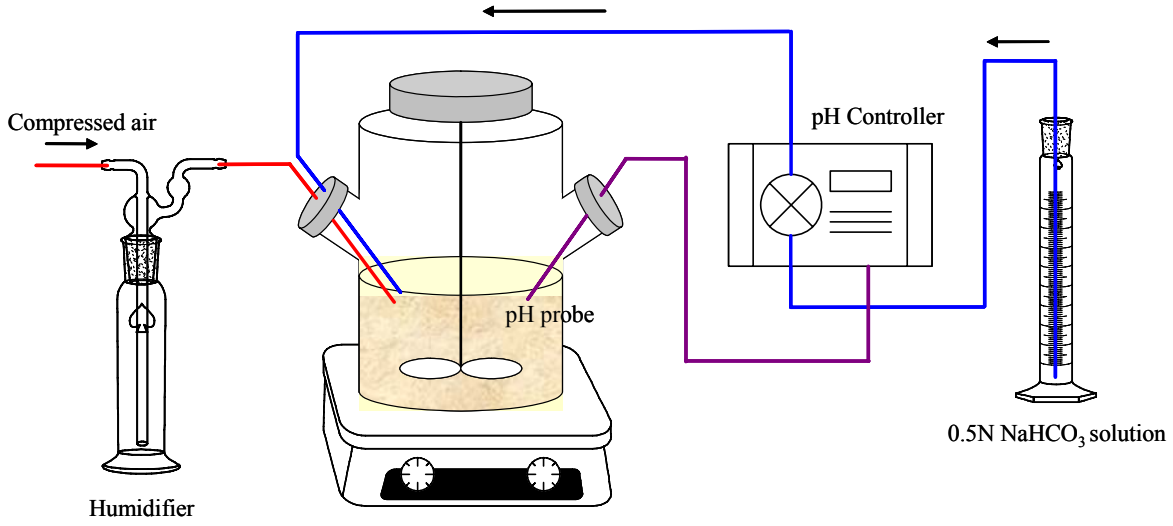


Figure 5.1. Schematic of control nitrifying culture setup.

5.2.3. Toxicity Assays

In order to assess the toxic effect of AB on nitrification, the following assay was performed. An aliquot of 200 mL of the mixed nitrifying culture was removed on the 68th day of operation immediately after the replacement of the culture supernatant and the addition of 100 mg N/L NH_4Cl. This culture sample was then transferred to an Erlenmeyer flask where it was continuously aerated and mixed. Then, an aliquot of an AB stock solution was added to the culture resulting in an initial AB concentration of 20 mg/L. Four more culture samples with initial AB concentrations of 2, 5, 10, and 15 mg/L were prepared in the same manner after three days. For all five culture samples, the concentration of nitrogen species and AB were measured at intervals of 24 hours for 4 days and at each sampling time, the pH was adjusted around 7 with a concentrated $NaHCO_3$ solution.

5.3. Results and Discussions

5.3.1. Culture Monitoring

The nitrifying activity of the mixed culture after more than 60 days of enrichment was monitored for 89 days. The culture pH was controlled using a controller set at pH 7.5, but its pH was 7.6 ± 0.7 (mean ± standard deviation). Stoichiometrically, 100 mg N/L requires alkalinity of 14.3 meq/L, which corresponds to a concentration of 1200 mg/L $NaHCO_3$ for complete nitrification to nitrate. Experimental values obtained by measuring the consumed amount of 0.5 N $NaHCO_3$ agreed well with the theoretical value.

With less frequent replacement of the culture volume, which led to a relatively long hydraulic retention time (more than 60 days) at the early test period, a sign of nitrification

inhibition was witnessed. Figure 5.2 shows a gradual increase in nitrite over time until about 20 days. One of the plausible reasons is the increasing chloride concentration which has been known to be an inhibitor of nitrification (Cui et al., 2006; Faller and Ilic, 1974). A previous study reported that chloride mainly inhibits *Nitrobacter*, which oxidizes nitrite, and a complete inhibition of nitrification was observed at 6 g Cl^-/L (Schenk and Hegemann, 1995). The mechanism of inhibition by chloride is known to be related to the changes in osmotic pressure in a cell (Darrah et al., 1987; Darrah et al., 1985; Darrah et al., 1986). The concentration of chloride in the culture was predicted by simulating the culture removal and feed addition volumes per feeding cycle over 60 days. The results of this simulation are shown in Figure 5.3. According to this, the chloride concentration was initially about 0.4 g/L at steady state. However, after changing the feeding pattern the chloride concentration should have doubled in about 11 days of operation and, finally in about 30 days, the concentration should have reached 1.1 g/L at steady state. Resuming the previous culture wasting scheme, i.e., leaving 250 mL culture after settling, nitrite oxidation was enhanced and complete conversion of ammonia to nitrate was observed after about the 55^{th} day and lasted till the end of this test (Figure 5.2).

Characterization of a feeding cycle was conducted at the 57^{th} day and results are shown in Figure 5.4. The culture VSS concentration at the time the feeding cycle characterization was conducted was 250 ± 10 (mean ± standard deviation). The data shown in Figure 5.4 is a typical nitrification pattern. Ammonia tended to disappear completely within 48 hours while nitrite increased until 24 hours and then decreased to none at 72 hours. Along with the decrease in both ammonia and nitrite, nitrate increased almost

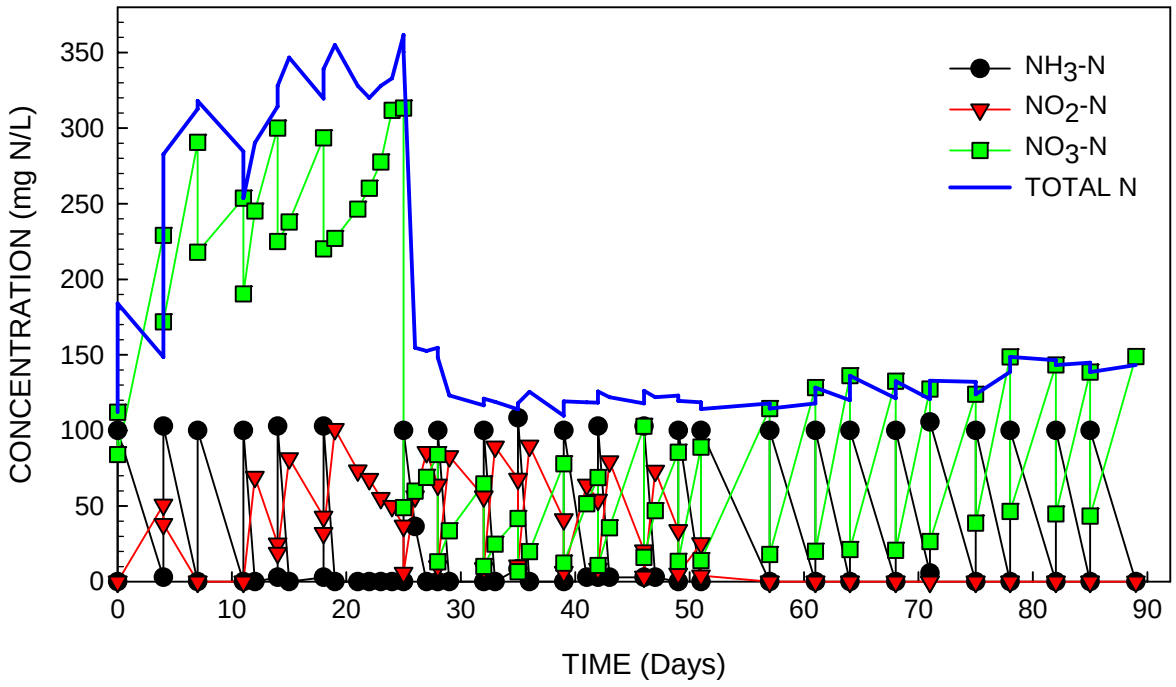


Figure 5.2. Profiles of the three nitrogen species in the nitrifying culture over the test period.

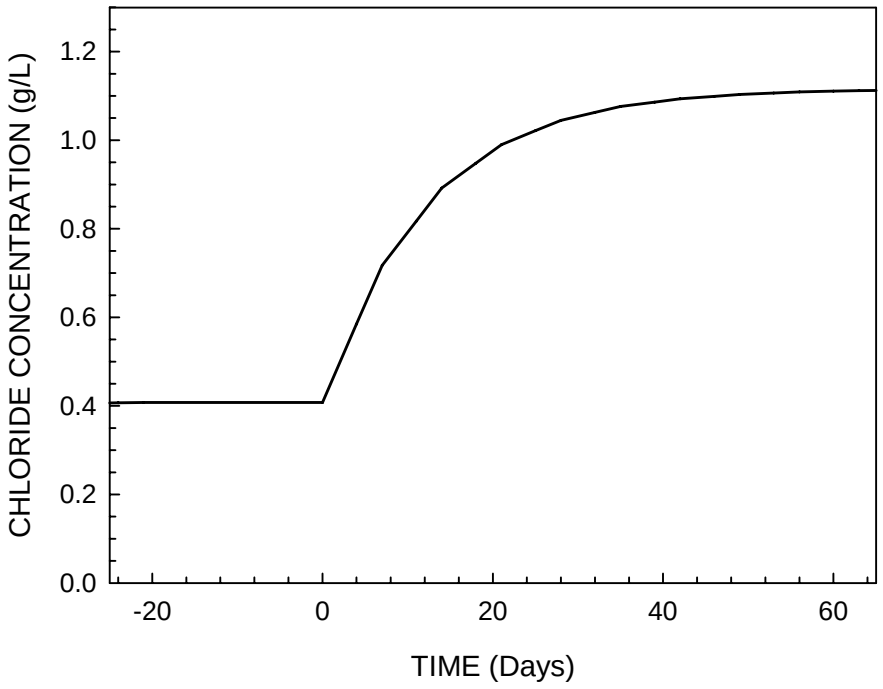


Figure 5.3. Simulation of chloride concentration in the nitrifying culture (Time zero signifies the change in feeding pattern).

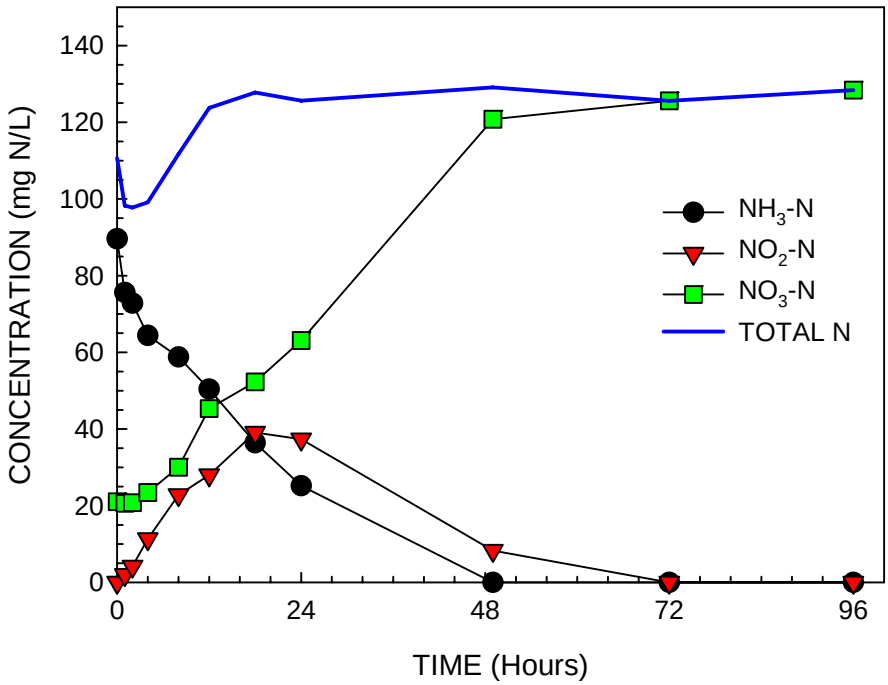


Figure 5.4. Nitrogen species in the nitrifying culture during one feeding cycle.

linearly from about 20 to 120 mg N/L within 48 hours. The amount of nitrogen generated as nitrate compares well with the ammonia-N fed. The fact that the sum of the three nitrogen species in the culture was constant after about 20 hours demonstrates complete nitrification as well as low nitrogen utilization for cell synthesis due to the slow growth rate of nitrifiers. The lower total nitrogen concentration at the early stage may be due to ammonia conversion to hydroxylamine, the first intermediate of ammonia monooxygenase, which was not monitored in this study.

5.3.2. OUR/SOUR

Measuring oxygen uptake rate (OUR), or specific oxygen uptake rate (SOUR), after the addition of a substrate could be an indirect way to assess the activities of relevant microorganisms in the culture. Based upon oxygen solubility for which external factors such as ambient temperature and altitude were taken into account, an equivalent amount of substrate, ammonium chloride and glucose in this case, was added respectively to the prepared culture samples and then changes in OUR/SOUR were compared. Initial concentrations of ammonium chloride and glucose added were 6.7 mg/L and 7.5 mg/L, respectively. The VSS concentration of the culture was 290 ± 30 mg/L and all measurements were conducted at the 84^{th} day, the last day of a feeding cycle.

Measured OUR and SOUR values are shown in Table 5.1. Under starvation conditions, the oxygen uptake rate by the culture was very slow and the DO decreased very little within 25 minutes (Figure 5.5A). When ammonium chloride was added, the OUR was practically constant and more than 10 times faster than that of the control until about 25

Table 5.1. OUR and SOUR values of the nitrifying culture amended with different substrates

	Control starved	Ammonium chloride amendment[a]	Glucose amendment[b]
OUR (mg DO/L-h)	0.901	11.3	1.08
SOUR (mg DO/g VSS-h)	3.11	39.1	3.72
R^2	0.998	1.00	0.996

[a]Regression considering DO values up to 14 minutes
[b]Regression considering DO values up to 93 minutes

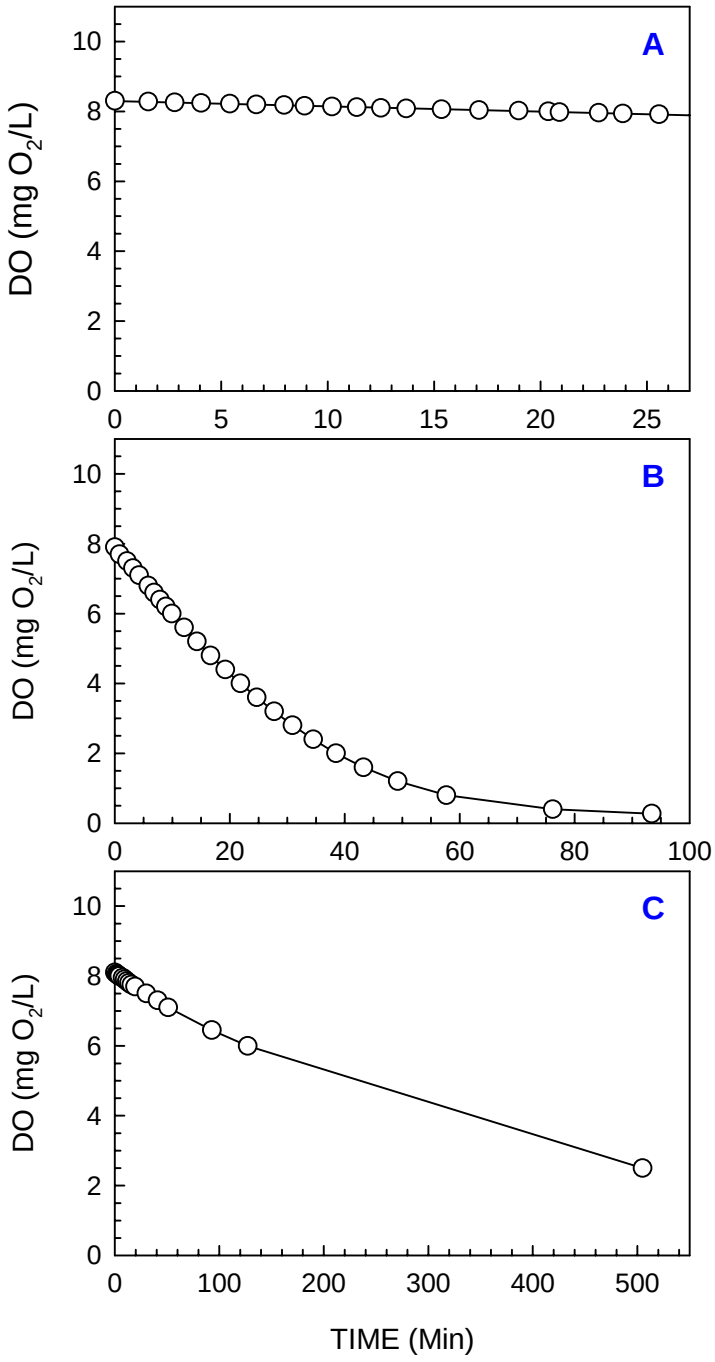


Figure 5.5. DO profiles of the starved control nitrifying culture (A), culture amended with 6.7 mg/L ammonium chloride (B), and culture amended with 7.5 mg/L glucose (C).

minutes when the availability of substrate and oxygen was limited. When glucose was added, the OUR exerted by the heterotrophs in the mixed culture increased by about 20% in comparison with the control, but the rate was still very low and DO was not depleted for about 9 hours (Figure 5.5C). Therefore, the fraction of heterotophs in the mixed nitrifying culture is considered to be negligible.

5.3.3. Toxicity Assessment

The inhibitory effect of AB on the nitrifying culture was assessed at a range for AB concentrations from 2 to 20 mg/L and data are shown in Figure 5.6. The nitrification pattern at both 2 and 5 mg/L AB was similar to that of the control (i.e., in the absence of AB) shown in Figure 5.4 in that most of ammonia was oxidized within 48 hours and nitrite accumulation did not occur. These findings suggest that at these AB concentrations, nitrification was not impacted, although the rate of ammonia to nitrite oxidation was slower. It is generally accepted that ammonia oxidizers are less affected than the nitrite oxidizers by toxicants (Carvallo et al., 2002; Selivanovskaya et al., 2004). The inhibitory effect of AB was apparent at 10 mg/L at which ammonia was not fully utilized within the test period. Nitrification was completely inhibited at 15 mg AB/L and above. Combined with the above mentioned observation of no accumulation of nitrite in the AB-amended culture series, and in contrast to previous studies on the effect of various toxicants on nitrification, it is clear that in our case the ammonia oxidizing bacteria were more sensitive to AB than the nitrite oxidizers. Despite the observed variation in the AB concentration over time (Figure 5.7), these data indicate that AB was not degraded even at a low

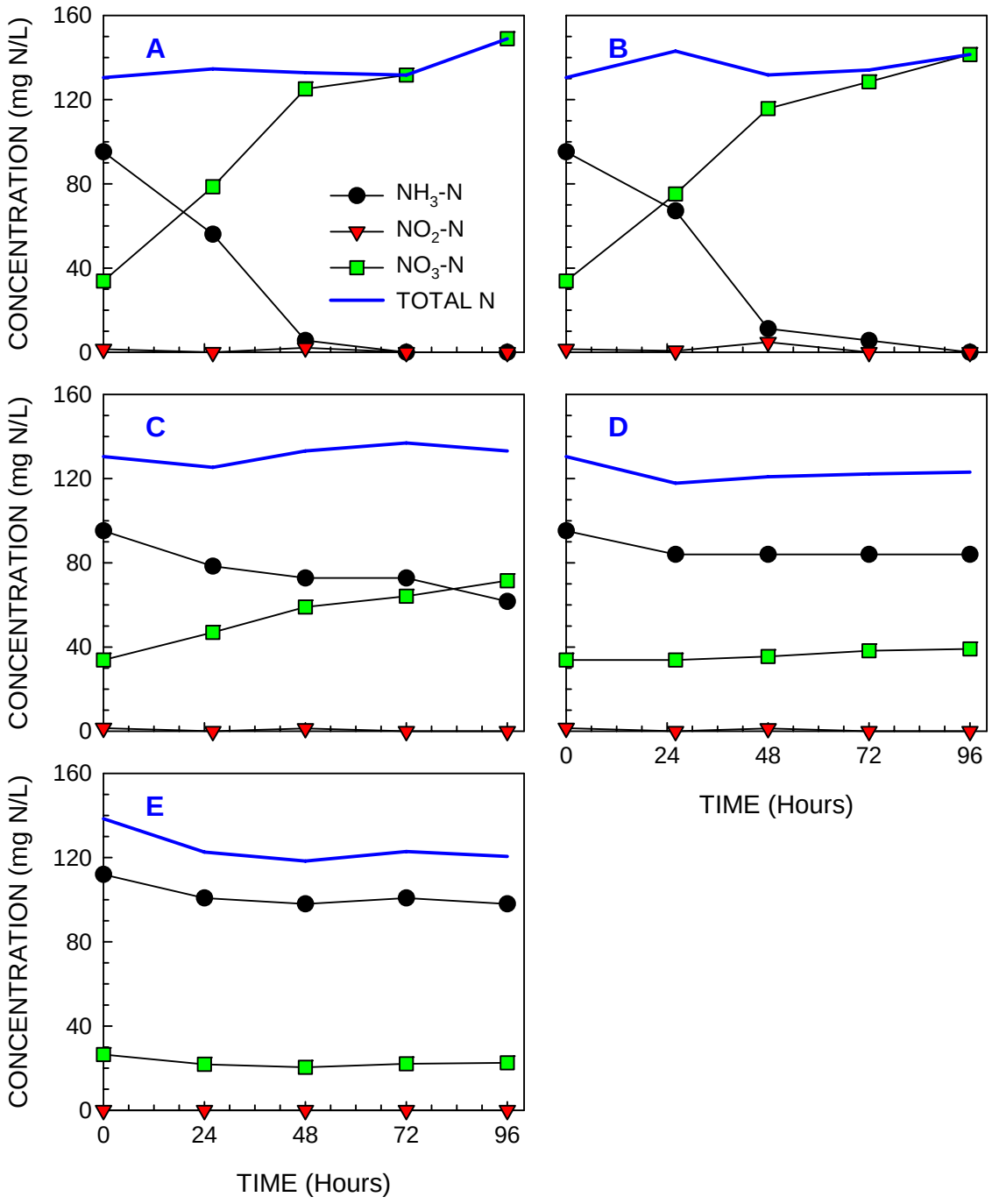


Figure 5.6. Effect of AB concentration on the oxidation of 100 mg N/L NH_4Cl at AB concentrations of 2 mg/L (A), 5 mg/L (B), 10 mg/L (C), 15 mg/L (D), and 20 mg/L (E).

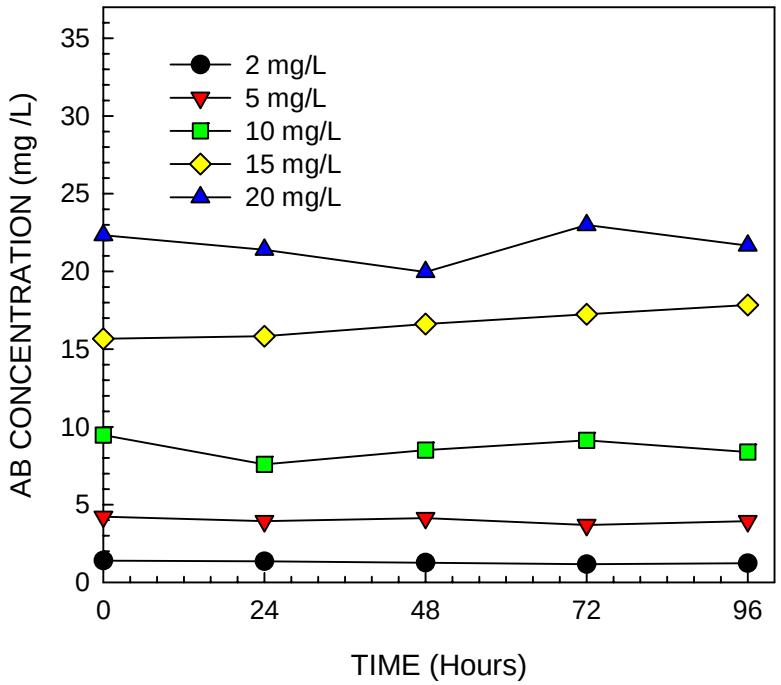


Figure 5.7. Fate of the different concentrations of AB in the nitrifying culture.

concentration over the test period. Based on the results of the OUR/SOUR assay, the number of heterotrophs in the mixed nitrifying culture is very small, which further explains the lack of AB degradation in the nitrifying culture. The process of nitrification involves a group of membrane-bound enzymes, such as ammonia monooxygenase (AMO) and nitrite oxidoreductase (Kelly et al., 2004; McCarty, 1999). Thus, the sorptive affinity of AB for the cell membrane, documented in a number of previous studies, significantly affects the enzymatic activities related to nitrification.

The final step was to establish the inhibition coefficient K_i using the data presented in Figures 5.4 and 5.6. Assuming a non-competitive inhibition, the relation between the rate of reaction and the concentration of the inhibitor can be expressed with a modified Michaelis-Menten equation as,

$$v = \frac{V_{max} \cdot S \cdot K_i}{(K_m + S)(K_i + I)} \tag{5.1}$$

where

v = reaction rate (mg NH_3-N/L-h)

V_{max} = maximum rate of reaction (mg NH_3-N/L-h)

S = substrate concentration (mg NH_3-N/L)

I = inhibitor concentration (mg AB/L)

K_m = Michaelis-Menten coefficient (mg NH_3-N/L)

K_i = inhibition coefficient (mg AB/L)

The inhibition coefficient K_i refers to the inhibitor concentration where the reaction rate reaches half of its maximum value. In the case of high substrate (i.e., ammonium chloride) concentration, equation 5.1 can be simplified as,

$$v = \frac{V_{max} \cdot K_i}{K_i + I} \tag{5.2}$$

The determination of K_i could be done graphically using the linearized version of equation 5.2 as follows:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{I}{V_{max} \cdot K_i} \tag{5.3}$$

The reaction rate "v" was calculated with the data from four samples, from 0 (i.e., the control) to 10 mg/L AB-amended cultures since the reaction rates of 15 and 20 mg/L AB amended cultures were apparently zero. In determining the "v" value in each sample, regressions were performed with the experimental data measured up to 48 hours (24 hours in the case of the control) in order to exclude the effect of substrate limitation on the results. A plot of the data according to equation 5.3 is shown in Figure 5.8. The estimated V_{max} and K_i values were 3.8 ± 2.3 mg NH_3-N/L-h and 1.5 ± 0.9 mg AB/L (mean ± standard error; R^2 = 0.985), respectively. The Michaelis-Menten coefficient (i.e., K_m) for nitrification in activated sludge is typically between 0.5 and 1.0 mg NH_3-N/L (Metcalf & Eddy Inc., 2003). When compared with K_i values of various nitrification inhibitors shown in Table 5.2, the K_i value of AB suggests a high susceptibility of nitrifying bacteria (mainly ammonia oxidizers) to AB. For reference, inhibition coefficients of surfactants, including QACs, on denitrifiers were assessed previously by Seifert and Domka (2005) and ranged from 0.2 to 102 mg/L.

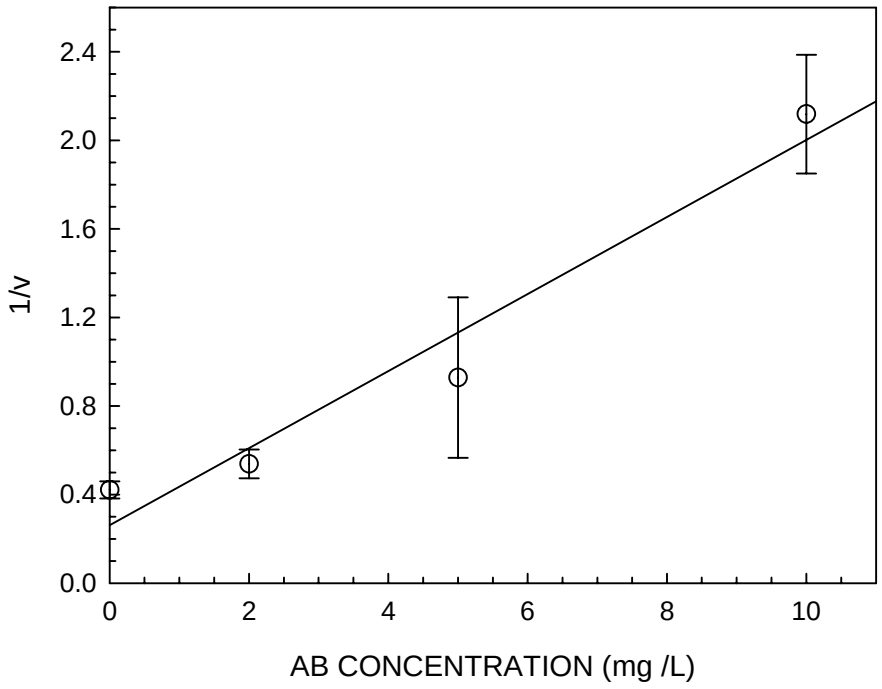


Figure 5.8. Nitrification rate versus AB concentration used for the determination of the inhibition coefficient K_i.

Table 5.2. Nitrification inhibitors and their inhibition coefficient values

Inhibitor	Inhibition coefficient (K_i, mg/L)
2-Propanone[a]	804
Aniline[b]	3
Arsenic[c]	292
Chromium[c]	50
Fluoride[c]	1218
Formaldehyde[a]	61
Methanol[a]	116

[a]Oslislo and Lewandowski, 1985
[b]Gheewala et al., 2004
[c]Beg et al., 1982

5.4. Summary

A nitrifying culture was enriched by feeding ammonium chloride and sodium bicarbonate. Despite an inhibition caused by accumulated chloride at an early stage, successful nitrification was observed in the control culture where ammonia at an initial concentration of 100 mg N/L was completely oxidized within 48 hours and completely converted to nitrate. The results from measuring OUR/SOUR of culture samples fed with different substrates indicated that the proportion of heterotrophs in the culture was very low. A test to investigate the effect of AB at different concentrations between 2 to 20 mg/L on nitrification was conducted and showed that ammonia oxidation was inhibited with increasing AB concentration and ceased at 15 mg/L AB. For all tested AB concentrations, AB was not utilized or degraded in the nitrifying culture within 4 days of the test period. Based upon the above results, the AB inhibition coefficient was estimated as 1.5 ± 0.9 mg AB/L, indicating significant susceptibility of the nitrifying culture, especially the ammonia oxidizers, to AB.

CHAPTER 6

CONCLUSIONS

One of the most commercially used quaternary ammonium compounds (QACs), alkyl benzyl dimethyl ammonium chloride (AB) was chosen for this study. The biodegradation of AB and its effect on nitrification were investigated. Reliable removal and degradation of a mixture of AB homologues up to 50 mg/L were observed with a mixed, aerobic culture over a prolonged incubation and repetitive feedings. However, nitrification in this culture was affected to some extent depending on the AB concentration added and the duration of the operation. A short-term experiment with a nitrifying culture fed with ammonium chloride and inorganic carbon source showed a significant inhibition of AB on the nitrifying activity of the culture.

Based on the results of the present study, the following specific conclusions can be drawn:

1. A mixed, aerobic culture fed with dextrin and peptone completely removed an initial concentration of 20 mg/L AB without any sign of delay throughout the entire period and degradation of the added protein and ammonification were not impacted by the AB.
2. Nitrification was initially affected at 20 mg/L AB but, with prolonged culture operation, complete nitrification was achieved as a result of acclimation and/or selection of tolerant nitrifying bacterial species.

3. An initial concentration of 50 mg/L was degraded without any delay after the fourth feeding cycle, but low levels of C_{12} and C_{14} were present at a concentration range from 3 to 7 mg/L. Complete degradation of organic nutrients and ammonification took place.
4. Nitrification was severely inhibited at 50 mg/L AB and did not recover over time. Both nitrite and nitrate were less than 10 mg N/L for the entire test period.
5. The cultures fed with AB as a sole organic carbon and nitrogen source utilized AB up to 50 mg/L despite their low biomass concentration.
6. The relatively lower VSS and higher soluble COD and DOC values of the AB-fed cultures than in the AB-free, control culture, indicate the occurrence of cell lysis by AB. The degree of cell lysis was significant with increasing AB concentration.
7. The nitrifying culture fed with ammonium chloride and sodium bicarbonate over a prolonged incubation period was susceptible to AB. Nitrification became noticeably inhibited with increasing AB concentrations and ceased at a concentration of 15 mg/L AB.

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