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Simultaneous Elimination of Carbon and Nitrogen Compounds of Petrochemical Effluents by Nitrification and Denitrification

Anne-Claire Texier¹, Alejandro Zepeda²,
Jorge Gómez¹ and Flor Cuervo-López^{1*}

¹Universidad Autónoma Metropolitana-Iztapalapa

²Universidad Autónoma de Yucatán
Mexico

1. Introduction

Human activities have resulted in the increase of nitrogen and carbon content in wastewater and groundwater affecting the environment (Bremmen, 2002). The average water consumption in Mexico is close to 0.25 m³/d, resulting in municipal and industrial wastewater generation between 168 and 232 m³/s, respectively. Only 12 and 20% of wastewater has received some treatment (Monroy et al., 2000). The increase of nitrogen compounds such as nitrate, nitrite and ammonium in superficial and groundwater has caused several environmental effects such as eutrophication, toxicity to aquatic organisms, loss of biodiversity (Galloway, 1998; Mateju et al., 1992; Schimel et al., 1996); and human health damages as methahemoglobinemia (Mateju et al., 1992; Morgan-Sagastumen et al., 1994); formation of nitrosoamines which are potentially carcinogenic compounds (Cerhan et al., 2001, as cited in González-Blanco et al., 2011) and gastric cancer (Knobeloch et al., 2000; Ward et al., 2005). In the north of Gulf of Mexico, an important hypoxic zone has been detected where oxygen concentration is lower than 2 mg/l due to high nitrate discharges and eutrophication (Alexander et al., 2000; McIsaac et al., 2002). Nitrate concentrations between 7 and 156 mg/l (Antón & Díaz, 2000) and higher than 80 mg/l (Muñoz et al., 2004; Pacheco et al., 2001) have been determined in aquifers of middle and south of Mexico, respectively. These nitrate concentrations are higher than the maximum levels established by Secretary of Environmental and Natural Resources (SEMARNAT), NOM-003-ECOL-1997 (15 and 40 mg total nitrogen/l) (Diario Oficial de la Federación, 1998) and the United States Environmental Protection Agency (USEPA, 2007) (10 mg N-NO₃⁻/l, 1 mg N-NO₂⁻/l and 10 mg N-NH₄⁺/l). Therefore, it is clear the need of applying effective wastewater treatments for reducing nitrogen contamination.

One of the most important sources of carbon pollution is the petrochemical industry. In 2009, more than 85 million of crude oil barrels were produced in the world in order to satisfy the energy and derivatives demand for industrial uses. At present, Russia is the first crude oil producer followed by Saudi Arabia and USA. Both of them produce almost 30% of

the total crude oil. In 2010, Mexico was the seventh petroleum crude oil producer (close to 2.5 million barrel/d) accounting to 0.05 million barrel (eq)/d of derivatives. Monoaromatic hydrocarbons such as benzene, toluene and xylenes (so-called BTX) are also produced from crude oil (Kirkeleit et al., 2006) by catalytic reforming (dehydrogenating process of the aliphatic compounds) and by alkylation and transalkylation processes (Perego & Ingallina, 2004).

A crescent BTX production has been observed all over the world. For instance, in 2000, the USA toluene production was close to 6.5 million of tons. In 2002, xylenes production was around 7 million of tons while in 2005, the USA benzene production was around 7 million of tons with a higher production of toluene and xylenes. BTX are widely used for producing paints, rubber, adhesives, varnishes, agrochemicals, polymers among many other products. In fact, almost 30% of gasoline content corresponds to BTX (Hartley & Englande, 1992; Sander et al., 2010). The average daily gasoline sales in Mexico in 2001 were close to 547,400 barrels (PEMEX Petrochemical, 2001). Accidental spills, evaporation of industrial sources, leakage from storage tanks in gas stations, industrial discharges and car combustion have resulted in environmental pollution with BTX. The great mobility abilities and toxicity of the BTX compounds are of major concern for environment and human health (Coates et al., 2002). These aromatic compounds have a high vapor pressure and are relatively water-soluble. Some of the physical and chemical properties of BTX are illustrated in Table 1. In this sense, BTX presence in soil, air, surface and groundwater has been increasingly reported (Jain et al., 2011; Martínez et al. 2009; Peña-Calva et al., 2004b; Steen-Cristenesen & Elton, 1996).

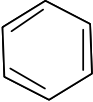
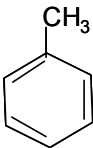
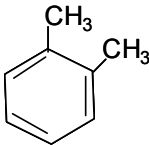
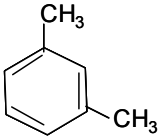
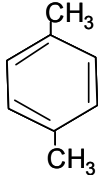
Compound	Benzene	Toluene	Xylenes		
Chemical structure					
			<i>o</i> -xylene	<i>m</i> -xylene	<i>p</i> -xylene
Molecular weight (g/mol)	78	92	106		
Density (g/ml)	0.87	0.87	0.87 ± 0.02		
Water solubility (g/l) at 25°C	1.87	0.47	0.17 ± 0.032		
Vapor pressure (mm Hg)	95	28	5.83 ± 0.76		
Partition coefficient octanol/water (log K _{ow})	2.13	2.69	3.04 ± 0.23		
Henry's law constant at 25°C (kPa m ³ /mol)	0.56	0.67	0.657 ± 0.094		
Polarity	Non polar	Non polar	Non polar		

Table 1. Some physical-chemical properties of benzene, toluene and xylenes (average values for *orto*, *meta*, and *para* isomers).

Concentrations (mg/l) ranging from 9-14, 23-81 and 13-171 of benzene, toluene and isomers of xylene have been respectively reported in groundwater (Gersberg et al., 1989). Likewise, considering the non polar characteristic of these compounds, there is an important bioaccumulation of BTX in the lipid fraction of cell membrane. Benzene is the most dangerous of the BTX compounds as it is a known human carcinogen (Huff, 2007). Breathing low levels of benzene can cause drowsiness, dizziness, rapid heart rate, confusion, and unconsciousness whereas higher levels of this compound can result in death (Ashly et al., 1994). It has been reported that benzene may induce damages to the bone marrow and cause anemia (Fishbein, 1985; Vereb et al., 2011), depression in the immune system and leukemia (Huff, 2007). The narcotic and neurotoxic properties of toluene are the major health hazards in humans due to its tendency to accumulate in adipose tissue. Toluene is highly lipophilic affecting the central nervous system (Fornazzari et al., 2003) where it seems to inhibit neuronal transmission by causing changes in protein conformation and hence in the membranal transport (Rosenberg et al, 1988). Acute intoxication from inhalation is characterized by euphoria, hallucinations, dizziness, confusion, headache, ataxia, stupor, and coma. Exposition over long periods of time may produce neuropsychosis, cerebral degeneration with ataxia, peripheral neuropathies, cognitive ability problems, ototoxicity and deafness (Waniusiow et al., 2008). However, there are no epidemiological evidences whether toluene induces cancer in persons exposed to the solvent (Fishbein, 1984; Weelink, et al., 2010). Xylenes seem not to be as toxic as benzene and toluene since their values of LD50 range from 200 to 4000 mg/kg for animals (Fishbein, 1985). According to Mexican legislation (NOM-127-SSA1-1994), (Diario Oficial de la Federación, 1994), the maximum levels established in potable water for BTX are (mg/l): benzene 0.01; toluene 0.3 and isomers of xylene 0.5. In the United States the maximum levels are 0.0005, 1 and 10 mg/l for benzene, toluene and mixed xylenes, respectively (USEPA, 2006, as cited in Farhadian et al., 2008). All of these BTX characteristics clearly illustrate why these compounds represent an environmental and health challenge.

There are different alternatives for nitrogen and carbon compounds removal from contaminated water, such as physical-chemical and biological processes. Gravity separation, volatilization, adsorption, dialysis, inverse osmosis, ultrasonic radiation or chemical reactions as phentom are some of the most used physical-chemical processes (Jacobs & Testa, 2003; Thoma et al., 2006). Nevertheless, most of these processes are not cost-effective, are poorly efficient when highly contaminated streams are treated and generally give undesirable residues (Farhadian et al., 2008). On the other hand, biological treatment processes are economically feasible, result in highly nitrogen and carbon consumption efficiencies and conversion to innocuous products such as N₂, CO₂ and water, respectively. Nitrogen compounds can be biologically removed from water by the coupling of nitrification and denitrification processes (Cuervo-López et al., 2009).

BTX are chemically reduced compounds, thus, their biologic oxidation is thermodynamically favored (Table 7). Many biological attempts have been conducted in order to remove these volatile organic compounds from the environment. Aerobic treatments have been widely proposed for BTX elimination (Duetz et al., 1994; Haigler et al., 1992; Rozkov et al., 1998; Yerushalmi et al., 1999). Removal of BTX mixtures appeared to be favored at aerobic conditions (Deeb & Alvarez-Cohen, 1999; Prenafeta-Boldú et al., 2002); however, negative effects such as inhibition or catabolic repression have also been reported at these conditions

(Chang et al., 1993; Duetz et al., 1997). Nevertheless, aerobic conditions might lead to important BTX stripping to atmosphere due to their volatilization characteristics. In fact, depending on aeration system, losses of BTX have reached up to 30% of their content (Zytner et al., 1994, as cited in Farhadian et al., 2008). In spite of complete removal of these compounds has been achieved (Deeb & Alvarez-Cohen, 2000), high conversion into biomass has also been reported (Chang et al., 1993; Reardon et al., 2000; Reardon et al., 2002). Biomass generation, stripping into atmosphere, besides their relatively low water-solubility seemed to be the most important disadvantages for aerobic treatment utilization. Therefore, anaerobic biological treatments appeared to be an adequate alternative for BTX removal.

Attempts for BTX removal using different electron acceptors such as nitrate (Burland & Edwards, 1999; Major et al., 1988; Martinez et al., 2007; Peña-Calva et al., 2004b; Weelink et al., 2010), sulfate (Craig et al., 1996; Feris et al., 2008) and CO₂ (Corseuil et al., 1998; Lovley, 2000; Morgan et al., 1993; Weelink 2010) have been conducted. There is scarce information describing interaction of BTX mixtures under anaerobic conditions. Benzene appeared to be recalcitrant in most of the cases (Weelink 2010); however, some investigations reported benzene removal under nitrate reducing conditions (Burland & Edwards, 1999). Rapid toluene consumption has been reported under sulfate (Beller & Spormann, 1997), hem (Kane et al., 2002) and methanogenic (Washer & Edwards, 2007) conditions. Most of these studies are focused on the disappearance of the aromatic compounds and the electron acceptors; however, no information about products formation is mentioned as no mass balances are indicated. Emphasize on the dissimilative catabolism has not been made, and further research on this topic is needed. Likewise, scarce consumption rate values are included; however, most of the values reported suggest that the processes are slow due to the microbial growth is slow and scarce. Information about these parameters would be useful for practical applications.

This chapter pretends to succinctly present the current knowledge of biological removal of ammonium, nitrate, and BTX compounds by nitrification and denitrification and describe some environmental factors that affect these microbial processes. Likewise, the goal of this work is to give practical information in order to obtain processes mainly mineralizing. The work is divided into two parts. In the first part, the simultaneous elimination of ammonium and BTX compounds by nitrification is described. In the second part, the simultaneous consumption of nitrate and BTX compounds by denitrification is presented. A general description of microbiology and physiology of nitrification and denitrification is made. Inhibitory effects of BTX compounds on the nitrifying and denitrifying processes are also described. In the case of toluene consumption by denitrification, studies carried out in batch and continuous cultures are also included. The importance of performing a complete evaluation of the biological processes through response variables such as efficiencies, yields, consumption and production rates, mass balances for the development of environmentally acceptable wastewater treatment processes is emphasized.

2. Nitrification

Nitrification has been extensively investigated as a very useful process in the first step of nitrogen removal in biological wastewater treatment. The oxidation of ammonia and nitrite by nitrifying processes generates nitrate for denitrifying processes where nitrate is converted to molecular nitrogen.

2.1 Definition of respiratory process

Nitrification is the biological oxidation of ammonium (NH₄⁺) to nitrate (NO₃⁻) via nitrite (NO₂⁻). It is an aerobic respiratory process where nitrifying bacteria use ammonium and nitrite as energy sources, carbon dioxide as a carbon source, and molecular oxygen as the final electron acceptor (Prosser, 1989). The nitrifying process is composed of two consecutive steps: 1) the oxidation of ammonium to nitrite that is mainly carried out by ammonium-oxidizing bacteria (AOB) and 2) the oxidation of nitrite to nitrate that is catalyzed by nitrite-oxidizing bacteria (NOB) (Table 2). As indicated by the free energy change (ΔG°') values, the oxidation of hydroxylamine (NH₂OH) to nitrite is the main step where the AOB obtain energy. The ΔG°' is lower for nitrite oxidation than for ammonia oxidation and the consequence is a lower growth yield for NOB than for AOB. Due to the low energy availability for cellular biosynthesis in the respiratory process, growth of nitrifying bacteria is slow and scarce, even in optimal conditions. Growth yields have been found to be 0.08 g cells/g NH₄⁺-N for AOB and 0.05 g cells/g NO₂⁻-N for NOB, respectively (Wiesmann, 1994). Doubling times have been reported to vary between 7 and 24 h for ammonium-oxidizing species and 10 and 140 h for *Nitrobacter* species (Bock et al., 1991).

Reactions	Equations	ΔG°' (kJ/reaction)
Ammonium oxidation	NH ₄ ⁺ + 0.5O ₂ → NH ₂ OH + H ⁺	- 8
	NH ₂ OH + O ₂ → NO ₂ ⁻ + H ⁺ + H ₂ O	- 267
Global reaction	NH ₄ ⁺ + 1.5O ₂ → NO ₂ ⁻ + 2H ⁺ + H ₂ O	- 275
Nitrite oxidation	NO ₂ ⁻ + 0.5O ₂ → NO ₃ ⁻	- 74

Table 2. Reactions and ΔG°' values of ammonium and nitrite oxidizing processes in nitrification.

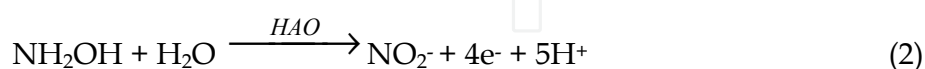
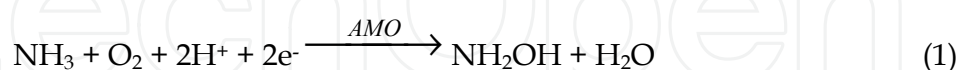
2.2 Microbiological aspects

Nitrification is carried out by two groups of Gram negative chemolithoautotrophic bacteria that belong to the *Nitrobacteraceae* family: the ammonium- and nitrite-oxidizing bacteria. There are no known autotrophic bacteria that can catalyze the production of nitrate from ammonium (Kowalchuk & Stephen, 2001). The AOB are species of the following genera: *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, *Nitrosococcus*, and *Nitrosovibrio*, being *Nitrosomonas* and *Nitrosomonas europaea* the genus and species better studied, respectively. Ammonia can also be transformed by a number of heterotrophic fungi and bacteria but it is generally accepted that chemolithotrophs are the primary nitrifiers in many systems (Kowalchuk & Stephen, 2001). While AOB are considered critical in nitrification, recently it has been reported that two major microbial groups are now believed to be involved in ammonium oxidation: AOB and ammonium-oxidizing archaea (You et al., 2009). *Candidatus "Nitrosopumilus maritimus"* and *Candidatus "Cenarchaeum symbiosum"* have been shown to be ammonia-oxidizing archaea containing the genes for all three subunits (*amoA*, *amoB*, and *amoC*) of ammonia monooxygenase, the enzyme responsible for ammonia oxidation (Hallam et al., 2006; Könneke et al., 2005, as cited in You et al., 2009). The genera of NOB are: *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira*. Most of the physiological and biochemical investigations have been carried out with members of the genus *Nitrobacter*. Some strains of

Nitrobacter have been shown to be able to grow heterotrophically but their growth is very slow (Bock et al., 1991).

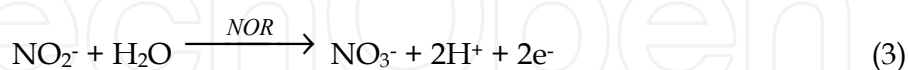
2.3 Biochemistry aspects

In the ammonium-oxidizing process, the first reaction is catalyzed by an ammonia monooxygenase (AMO) (Eq. 1) and the second one by a hydroxylamine oxidoreductase (HAO) (Eq. 2).



In the first reaction of ammonia conversion into hydroxylamine (NH_2OH), one of the oxygen atoms from O_2 is transferred to NH_3 , producing hydroxylamine, while the other is involved in H_2O formation (Hollecher et al., 1981). It is generally accepted that ammonia (NH_3) rather than ammonium (NH_4^+) is the real substrate for the enzyme AMO. AMO is located in the cytoplasmic membrane. The broad specificity of the AMO has shown to permit the co-oxidation of numerous organic compounds, including recalcitrant aliphatic, aromatic, and halogenated molecules (Juliette et al., 1993; Keener & Arp, 1993, 1994; McCarty, 1999). The second reaction of the ammonia oxidation, the NH_2OH conversion into NO_2^- , is catalyzed by the complex enzyme system HAO. HAO is located in the periplasmic space (Whittaker et al., 2000). Two of the electrons produced in the second reaction are used to compensate the electron input of the first reaction, whereas the other two are passed via an electron transport chain to the terminal oxidase, thereby generating a proton motive force (Kowalchuk & Stephen, 2001). This proton motive force is used as the energy source for ATP production.

The second step in nitrification is the oxidation of nitrite to nitrate and is catalyzed by the enzyme nitrite oxidoreductase (NOR) (Eq. 3). In this reaction, the oxygen atom is derived from water. NOR is located in the cytoplasmic membrane and is composed of cytochromes *a* and *c*, a quinone and a deshydrogenase dependent on NADH (Aleem & Sewel, 1981; Spieck et al., 1998).



2.4 Environmental factors affecting nitrification

Nitrification is affected by environmental factors such as temperature, pH, substrates concentrations (O_2 , NH_3 , and NO_2^-), and organic matter (Bernet & Spérandio, 2009). Such parameters can cause an effect on both the anabolic (biosynthetic process) and catabolic (respiratory process) processes.

Growth conditions are optimal at 25-30°C and pH 7.5-8.0 for AOB and at 28-30°C and pH 7.6-7.8 for NOB (Bock et al., 1991). In nitrifying processes, ranges of 25-30°C and pH 7.5-8.0 are generally used to obtain a successful nitrification. However, nitrification has been shown to occur in acidic conditions at pH = 5 (De Boer & Kowalchuk, 2001). pH has also an indirect

effect on nitrification by acting on the equilibrium ammonium/ammonia ($\text{NH}_4^+/\text{NH}_3$, $\text{pK}_a = 9.25$ at 25°C) (Hoffman, 2004). At alkaline pH values, the availability of NH_3 is not limited and this would be favorable for nitrification considering that NH_3 is the substrate for AMO (Suzuki et al., 1974). However, it is also known that high concentrations of free ammonia inhibit nitrification (Anthonisen et al., 1976; Vadivelu et al., 2006b). In aerobic nitrifying reactors operated under constant aeration and agitation, NH_3 can be lost from the system by volatilization. On the other hand, the ammonia oxidation to nitrate produces protons and leads to an acidification of the environment. This decrease in the pH value can inhibit nitrification by diminishing the availability of NH_3 . pH also establishes the concentrations of nitrite (NO_2^-) and nitrous acid (HNO_2) with a pK_a value of 3.34 at 25°C (Whiten et al., 2008). It has been observed that high concentrations of nitrite can inhibit the nitrification process and that could be mainly related to the pH values through the formation of HNO_2 . Nitrous acid is known to inhibit nitrification, thus, low pH values must be avoided in nitrifying processes (Anthonisen et al., 1976; Vadivelu et al., 2006a, 2006b). Recently, Silva et al. (2011) reported that the nitrite-oxidizing process was more sensitive to the presence of nitrite than the ammonium-oxidizing process, suggesting that nitrite accumulation in nitrification systems should be controlled to avoid a higher accumulation of nitrite and a decrease in nitrate yield. In consequence, pH value is generally controlled in nitrifying systems and maintained between 7.5 and 8.0 to avoid operational problems.

In the nitrifying respiratory process, oxygen is used as the final electron acceptor. It is generally assumed that oxygen concentrations higher than 2 mg/l are adequate for a successful nitrification (Gerardi, 2002). Nitrification is very sensitive to low dissolved oxygen concentrations. AOB and NOB present different affinities for oxygen (K_{O_2}) and NOB are more sensitive to O_2 limitation than AOB (Laanbroek & Gerards, 1993). Therefore, low dissolved oxygen concentration, around 0.2 to 0.5 mg/l, is a possible condition for limiting nitrite-oxidizing activity in nitrification systems (Bernet & Spérandio, 2009). Partial nitrification is used to obtain nitrite instead of nitrate as end product. Partial nitrification is involved in several processes (OLAND, CANON, SHARON, ANAMMOX, denitrification via nitrite) where the shortcut of nitrate could mean considerable savings in demand for oxygen and carbon source (Ahn et al., 2011; Moore et al., 2011; Peng & Zhu, 2006).

The high sensitivity of nitrifying bacteria to the toxic or inhibitory effects of organic compounds is well-documented, and it is known that the stability of nitrifying systems in wastewater treatment can be altered by the presence of organic matter (Schweighofer et al., 1996). Most of the studies on effects of organic compounds on nitrification have used axenic cultures or consortia such as activated sludge as inoculums. It has been shown that the effects mainly depend on the type and chemical structure of the organic pollutant as well as its concentration and hydrophobicity but also the type of culture (axenic or consortium) and the origin of the sludge (Gómez et al., 2000; Zepeda et al., 2006).

2.5 Ammonium and BTX removal

2.5.1 Inhibitory effects of organic matter and BTX on nitrification process

There are numerous studies in the literature on the inhibitory effects of organic compounds on nitrification. Some works with axenic cultures of *Nitrosomonas* sp. or *Nitrobacter* sp. have been focused on the growth inhibition of bacteria (Jensen, 1950; Steinmüller & Bock, 1976),

others on the activity of the AMO (McCarty, 1999). There is also information on the effects of different organic substances on the nitrifying respiratory process of microbial consortia (Gómez et al., 2000, Silva et al., 2009; Zepeda et al., 2003, 2006). Various hypotheses have been proposed to explain the negative effects of organic matter on nitrification. In axenic cultures of *Nitrosomonas europaea*, it has been observed that the AMO had a broad substrate range for catalytic oxidation and the inhibitory effects of many organic substances would be due to competition for the active site (Chang et al., 2002; Hyman et al., 1985; Juliette et al., 1993; Keener & Arp, 1993). The enzyme AMO contains copper and iron, and metal chelators such as allylthiourea are inhibitory (Bremmer & Bundy, 1974; Hauck, 1980; Hooper & Terry, 1973, as cited in Bock et al., 1991; Hyman et al., 1990; Zahn et al., 1996). In the literature, it was also mentioned that nitrifying bacteria are susceptible to organic compounds because of a limitation of reducing power (Iizumi et al., 1998; Shiemke et al., 2004), the formation of covalent binding with the enzyme AMO (Hyman & Wood, 1985), or the hydrophobic character of the organic molecules and their effects on biological membranes (Sikkema et al., 1994, as cited by Zepeda et al., 2006; Takahashi et al., 1997). In microbial consortia, the competition between heterotrophs and autotrophs for ammonia and oxygen is another hypothesis commonly mentioned for explaining the nitrification inhibition by organic matter (Hanaki et al., 1990).

Some aromatic compounds are known to inhibit nitrification (Amor et al., 2005; Brandt et al., 2001; Texier & Gómez, 2002). Little attention has been paid to the inhibitory influence of benzene, toluene, and xylenes (BTX) on nitrifying microorganisms and further work is required for understanding how BTX compounds can affect the performance of nitrifying treatment systems. Inhibition of nitrification by toxic chemicals, such as BTX compounds, focuses mainly on the modes of action of inhibitors on the enzyme AMO by using axenic cultures of *N. europaea* (Keener & Arp, 1994; McCarty, 1999). Main results are consequently in relation to the ammonium-oxidizing process and very little information is available on the effects of BTX compounds on the nitrite-oxidizing process. Keener & Arp (1994) in their study on the inhibition of NH_4^+ oxidation by *N. europaea* from various aromatic compounds reported that benzene concentrations of 14.0 and 23.4 mg/l inhibited the NO_2^- production by 60% and 80%, respectively. Dyreborg & Arvin (1995) observed that the level of benzene concentration provoking 100% inhibition of the ammonium oxidation was 10.7 mg/l. In the studies of Zepeda et al. (2003, 2006), the effect of different initial concentrations of benzene, toluene, and *m*-xylene on a nitrifying consortium produced in steady-state nitrification was evaluated in batch reactors. The values for the ammonium consumption efficiency, nitrate yield and nitrification specific rates were determined. These values are needed for characterizing the physiological behavior of the nitrifying sludge in the presence of BTX compounds. Zepeda et al. (2006) observed that at 5 mg C/l of each aromatic compound, there was no significant effect on nitrification efficiency and the ammonium removal efficiency was of $95 \pm 7\%$ after 16 h. Benzene and *m*-xylene at 10 mg C/l decreased ammonium consumption efficiency by 57% and 26%, respectively, whereas toluene did not affect the efficiency of the ammonium oxidation process. In all cases, the consumed $\text{NH}_4^+\text{-N}$ was totally oxidized to $\text{NO}_3^-\text{-N}$ after 16 h. The nitrifying yield was close to 1.0 and nitrite concentration was negligible. This indicated that the process was mainly dissimilative and biomass formation was limited. These results suggested that wastewaters containing up to 5 mg C/l of benzene, toluene, or *m*-xylene would not affect the efficiency of ammonium

conversion to nitrate in a treatment system. However, BTX (5-20 mg C/l) induced a significant decrease in the values for specific rates of NH_4^+ consumption (76-99%) and NO_3^- production (45-98%), affecting mainly the ammonium-oxidizing pathway (Table 3). These results indicated that, in the presence of BTX compounds, the nitrification process was inhibited but the nitrifying metabolic pathway was only altered at the specific rate level as nitrate was still the main end product. At 10 mg C/l of BTX compounds, the inhibition order on nitrate production was: benzene > *m*-xylene > toluene while at 20 mg C/l, the sequence changed to *m*-xylene > toluene > benzene. The same authors also reported that at 5 mg C/l of BTX compounds, there was no toxic effect on the sludge whereas bacteria did not totally recover their nitrifying activity from 10 to 50 mg C/l. Results indicated that mechanisms involved in the inhibition of nitrification processes by BTX compounds depend on various factors, such as the initial concentration, the chemical structure, and the hydrophobic character of the pollutants. Zepeda et al. (2003, 2006) also observed that the inhibitory effects of BTX compounds seemed to be related to their persistence in the nitrifying cultures. At low concentrations, the chemical structure of the BTX compounds appeared to be the predominant factor whereas at higher concentrations, their hydrophobicity played an important role. The presence of different functional groups as well as the nature of the substituent can influence the metabolism and toxicity of aromatic compounds (O'Connor & Young, 1996). The absence of functional groups may confer to benzene higher stability and persistence to biotransformation while the presence of methyl groups may facilitate the mechanisms of biotransformation for toluene and *m*-xylene. Moreover, previous studies reported that many cyclic hydrocarbons are toxic and inhibitory to microorganisms because of their hydrophobic character and their devastating effects on biological membranes (Radniecki et al., 2011; Sikkema et al., 1995; Tsitko et al., 1999).

	Initial concn (mg C/l)	Specific rates (g N/g microbial protein-N.h)	
		NH_4^+ -N consumption	NO_3^- -N production
Control	0	1.389 ± 0.079	0.577 ± 0.030
Benzene	6.5	0.266 ± 0.008 (– 81%) ^a	0.306 ± 0.024 (– 47%)
	10	0.226 ± 0.009 (– 84%)	0.141 ± 0.010 (– 76%)
	20	0.155 ± 0.004 (– 89%)	0.090 ± 0.003 (– 84%)
Toluene	5	0.317 ± 0.012 (– 77%)	0.310 ± 0.007 (– 46%)
	10	0.225 ± 0.006 (– 84%)	0.221 ± 0.004 (– 62%)
	20	0.054 ± 0.004 (– 96%)	0.047 ± 0.007 (– 92%)
<i>m</i> -xylene	5	0.328 ± 0.002 (– 76%)	0.320 ± 0.002 (– 45%)
	10	0.219 ± 0.009 (– 84%)	0.201 ± 0.020 (– 65%)
	20	0.010 ± 0.002 (– 99%)	0.009 ± 0.001 (– 98%)

^aPercentages of decrease for nitrification specific rates were calculated by using the values obtained in the control culture as references.

Table 3. Nitrification specific rates in the nitrifying cultures in the absence and presence of BTX compounds (from Zepeda et al., 2006).

As it can be seen in Table 4, in nitrifying cultures, binary and ternary mixtures of benzene, toluene, and *m*-xylene provoked also a significant decrease in the values of specific rates for

NH₄⁺ consumption (from 72 to 90%) and NO₃⁻ production (from 39 to 79%) (Zepeda et al., 2007). The inhibitory effect of BTX compounds on the nitrifying process seemed to be higher when they were present in mixtures than individually. Results showed that benzene, toluene, and *m*-xylene individually or in mixtures significantly inhibited the nitrifying activity of the sludge by decreasing the nitrification specific rates. However, it was found that the nitrifying sludge can tolerate up to 5 mg C/l of BTX in single solutions or 2.5 mg C/l in mixed solutions, maintaining high the NH₄⁺-N oxidation efficiency and the nitrifying yield.

	NH ₄ ⁺ -N consumption		Specific rates	
	g N/g protein-N.h	Decrease (%) ^a	NO ₃ ⁻ -N production g N/g protein-N.h	Decrease (%) ^a
Control	1.389 ± 0.079	-	0.577 ± 0.030	-
Single solutions				
Benzene	0.266 ± 0.008	81	0.306 ± 0.024	47
Toluene	0.317 ± 0.012	77	0.310 ± 0.007	46
<i>m</i> -Xylene	0.328 ± 0.002	76	0.320 ± 0.002	45
Mixed solutions ^b				
BT	0.389 ± 0.002	72	0.352 ± 0.006	39
BX	0.194 ± 0.003	86	0.202 ± 0.006	65
TX	0.208 ± 0.007	85	0.173 ± 0.002	70
BTX	0.139 ± 0.012	90	0.121 ± 0.012	79

Table 4. Specific rates of the nitrification cultures in the absence (control) and presence of benzene, toluene, and *m*-xylene at 5.0 ± 0.5 mg C/l for single solutions and 2.5 ± 0.2 mg C/l for each compound of the mixtures (from Zepeda et al., 2007). ^aPercentages of decrease for nitrification specific rates were calculated by using the values obtained in the control culture without BTX compounds as references. ^bBT, Benzene-Toluene; BX, Benzene-*m*-Xylene; TX, Toluene-*m*-Xylene; BTX, Benzene-Toluene-*m*-Xylene.

As observed by Zepeda et al. (2006, 2007) in the case of BTX compounds, in spite of the inhibitory effects of organic compounds on nitrification, it was demonstrated that in some cases and under controlled experimental conditions, nitrification processes could successfully proceed (Texier and Gómez, 2007). Moreover, microbial consortia under nitrifying conditions have been shown to be able to oxidize simultaneously ammonia and organic compounds, suggesting that nitrifying consortium coupled to a denitrification system may have promising applications for complete removal of nitrogen and organic compounds from wastewaters. This topic is detailed in the following section.

2.5.2 Simultaneous elimination of ammonium, organic matter and BTX removal

Previous studies have shown that the ammonia-oxidizing bacterium *N. europaea* was able to oxidize a broad range of hydrocarbons, including non-aromatic and aromatic compounds, and it is believed that the AMO is participating. The role of the AMO was demonstrated in experiments undertaken with selective inhibitors of the enzyme such as allylthiourea. Table 5 presents some examples of organic substances oxidized by *N. europaea* cultures. In the majority of these studies, kinetic data are missing for ammonium and nitrite oxidation

processes as for organic compounds, thus, more investigation is required to understand better the involved mechanisms.

Several investigations have shown that *N. europaea* oxidizes recalcitrant aromatic compounds, and it is also believed that these oxidations are mediated by the AMO (Chang et al., 2002; Hyman et al., 1985; Keener & Arp, 1994; Vannelli & Hooper, 1995). However, in these studies, there was an accumulation of more oxidized aromatic products in the culture medium. For example, in the case of benzene, ring cleavage did not occur and phenol was accumulated in cultures of *N. europaea*. In contrast, Zepeda et al. (2003, 2006) observed that when a nitrifying consortium was used, benzene, toluene, and *m*-xylene were converted to volatile fatty acids. These authors proposed that the aromatic compounds oxidation with ring fission could be the result of the coexistence and participation of both, lithoautotrophic nitrifying bacteria and heterotrophic microorganisms present in the consortium. It can be considered that the high diversity of microorganisms, enzymatic material and possible metabolic pathways which characterizes microbial consortia could contribute to obtain these results. However, further work is required in this direction to elucidate the underlying processes involved in the transformation of BTX by nitrifying consortia.

Organic compound	Main product	Reference
Methane	Methanol	Hyman & Wood, 1983
Ethylene	Ethylene oxide	Hyman & Wood, 1984
Methanol	Formaldehyde	Voysey & Wood, 1987
Carbon monoxide	Carbon dioxide	Tsang & Suzuki, 1982
Methyl Fluoride and Dimethyl Ether	Formaldehyde and methanol-formaldehyde	Hyman et al., 1994
Alcane (up to C ₈)	Alcohol	Hyman et al., 1988
Alkene (up to C ₅)	Epoxide and alcohol	Hyman et al., 1988
<i>Trans</i> -2-butene	2-butene-1-ol	Vannelli et al., 1990
Methyl sulphur	Methyl sulfoxide	Juliette et al., 1993
Iodoethane	Acetaldehyde	Rasche et al., 1990a
Fluoromethane	Formaldehyde	Rasche et al., 1990a
Bromoethane	Acetaldehyde	Rasche et al., 1990b
Cloromethane	Formaldehyde	Rasche et al., 1990b
Benzene	Phenol	Keener & Arp, 1994
Toluene	Benzyl alcohol and benzaldehyde	Keener & Arp, 1994
Ethylbenzene	Phenethyl alcohol	Keener & Arp, 1994
<i>p</i> -Xylene	4-Methylbenzyl alcohol	Keener & Arp, 1994
Naphtalene	2-Naphtol	Chang et al., 2002
Styrene	Phenylglyoxal	Keener & Arp, 1994
Clorobenzene	4-Clorophenol	Keener & Arp, 1994
Aniline	Nitrobenzene	Keener & Arp, 1994
Nitrobenzene	3-Nitrophenol	Keener & Arp, 1994
Acetophenone	2-Hidroxyacetophenone	Keener & Arp, 1994
Benzyl alcohol	Benzaldehyde	Keener & Arp, 1994
Phenol	Hydroquinone	Hyman et al., 1985
Nitrapirine (aerobic)	6-Acid chloro picolinico	Vannelli & Hooper, 1992
Nitrapirine (anaerobic)	2-Chloro-6-dicloromethyl piridine	Vannelli & Hooper, 1993

Table 5. Oxidation of organic compounds in *Nitrosomonas europaea* cultures.

In their study, Zepeda et al. (2006) reported that the nitrifying sludge was able to completely consume benzene (6.5 mg C/l), toluene (5 mg C/l), and *m*-xylene (5 mg C/l) over 21 h. Specific rates of BTX consumption are presented in Table 6. These results indicate that for initial concentrations below 6.5 mg C/l, toluene and *m*-xylene disappeared faster from the cultures than benzene, which appeared to be most persistent. This is in accordance with the fact that benzene at low concentrations was the highest inhibitory compound on nitrification. Individually as in BTX mixtures, the nitrifying consortium presented the following sequence of biotransformation: *m*-xylene > toluene > benzene. However, in mixtures, significant differences in the values for specific rates of BTX compounds removal were found. The work of Zepeda et al. (2007) emphasizes the importance for considering the possible component interactions in the biotransformation of mixed BTX compounds in nitrification systems.

Up to a concentration of 10 mg C/l, benzene was first oxidized to phenol, which was later totally oxidized to acetate. At a concentration of 5 mg C/l, toluene was first oxidized to benzyl alcohol, which was later oxidized to butyrate while *m*-xylene was oxidized to acetate and butyrate (Zepeda et al., 2006). As it has been previously shown that the AMO oxidized benzene to phenol, then it is likely that benzyl alcohol be an intermediate of toluene oxidation by the AMO (Hyman et al., 1985; Keener and Arp, 1994; Zepeda et al., 2003). It is presumed that *N. europaea* may initiate oxidation of BTX to provide intermediates, such as phenol and benzyl alcohol, which could later be transformed to VFA by heterotrophic microorganisms from the nitrifying consortium. In nitrifying cultures added with benzene, toluene, and *m*-xylene compounds in individual (5 mg C/l) and mixed solutions (2.5 mg C/l for each one), Zepeda et al. (2007) observed that after 24 h, ammonium consumption efficiency and conversion of consumed ammonium into nitrate were close to 100%. These results confirmed that a nitrifying consortium produced in steady state was able to convert benzene, toluene, and *m*-xylene to VFA and simultaneously oxidize ammonium to nitrate through a dissimilative process. This type of nitrifying consortium coupled with a denitrification system may have promising applications for complete removal of both nitrogen and BTX compounds from wastewater streams into N₂ and CO₂.

In the last decades, several nitrifying consortia have been reported to oxidize simultaneously ammonium and various recalcitrant aromatic compounds, such as phenolic compounds (phenol, 2-chlorophenol, *p*-cresol, *p*-hydroxybenzaldehyde) (Amor et al., 2005; Martínez-Hernández et al., 2011; Silva et al., 2009, 2011; Vázquez et al., 2006; Yamaghisi et al., 2001) and BTX compounds (Zepeda et al., 2003, 2006, 2007). In some cases, nitrate and carbon dioxide were the major products from the process. As a consequence, nitrifying processes have been recently proposed as novel alternative technologies for the simultaneous removal of ammonium and aromatic pollutants from industrial wastewaters of chemical complexity such as effluents generated by the petrochemical industry (Beristain-Cardoso et al., 2011, Silva et al., 2011). Nitrification as the initial step in the removal of ammonia from wastewaters might be also used to oxidize simultaneously ammonia and aromatic compounds, allowing their mineralization or the production of intermediates that can be totally oxidized by denitrification (Beristain-Cardoso et al., 2009). However, additional studies in both batch and continuous bioreactors are needed to obtain more information about physiological, kinetic, ecological and engineering aspects in order to control the sludge metabolic capacity in nitrification processes.

	Specific rates (g C/g microbial protein-N.h)
Individual solutions	
Benzene	0.034 ± 0.003
Toluene	0.045 ± 0.003
m-Xylene	0.055 ± 0.001
Binary mixtures ^b	
Benzene	0.005 ± 0.001
Toluene	0.055 ± 0.005
Benzene	0.051 ± 0.005
m-Xylene	0.044 ± 0.005
Toluene	0.012 ± 0.002
m-Xylene	0.053 ± 0.005
Ternary mixtures ^b	
Benzene	0.017 ± 0.002
Toluene	0.039 ± 0.004
m-Xylene	0.050 ± 0.005

^aThe initial concentration of BTX compounds in individual solutions was 5.0 ± 0.5 mg C/l.

^bThe initial concentration for each BTX compound of the mixtures was 2.5 ± 0.2 mg C/l.

Table 6. Specific rates of biotransformation for benzene, toluene, and *m*-xylene in individual, binary, and ternary solutions in nitrifying cultures (from Zepeda et al., 2007).

3. Denitrification

Denitrification is a biological process that microorganisms use for obtaining energy from the reduction of nitrate to dinitrogen gas (Knowles, 1982; Mateju et al., 1992). Denitrification requires the concomitant oxidation of an electron donor in order to reduce nitrate to N₂ gas. Diverse compounds can be used for this purpose. Organic sources are used in organotrophic denitrification whereas inorganic compounds are used for lithotrophic denitrification. Different organic compounds have been used in organotrophic denitrification such as methanol, glycerol, benzoic acid, acetate, glucose, lactate (Akunna et al., 1993; Cuervo-López et al., 1999; Martínez et al., 2009). Recalcitrant compounds such as *p*-xylene, benzene, toluene and *p*-cresol have been successfully removed by organotrophic denitrification (Cervantes et al., 2009; Dou et al., 2008; Hänner et al., 1995; Martínez et al., 2007; Peña-Calva et al., 2004b). Therefore, denitrification seems a good option for treating wastewater and underground water contaminated with BTX as simultaneous removal of nitrogen and carbon compounds can be obtained. Nevertheless, it is essential to remark that for biological wastewater treatment, the denitrification process must be dissimilative and dissipative to prevent biomass generation.

The type of electron source will determine the performance of the denitrifying process in terms of change in Gibbs free energy (ΔG°) and consumption rate (Cuervo-López, et al., 2009). Several respiratory process, including denitrification, with different electron sources and their respective ΔG° values are illustrated in Table 7. In all cases, the denitrification process is exergonic and appears to be thermodynamically favored when compared with anaerobic processes such as methanogenesis or sulfate reduction.

Electron donor	Equation Denitrification	ΔG° (kJ/reaction)
Acetic acid	$\text{CH}_3\text{COOH} + 1.6\text{NO}_3^- \rightarrow 2\text{CO}_2 + 0.8\text{N}_2 + 1.6\text{OH}^- + 1.2\text{H}_2\text{O}$	-843
Ethanol	$\text{C}_2\text{H}_6\text{O} + 2.4\text{NO}_3^- + 0.4\text{H}^+ \rightarrow 1.2\text{N}_2 + 2\text{HCO}_3^- + 2.2\text{H}_2\text{O}$	-1230
Phenol	$\text{C}_6\text{H}_6\text{O} + 5.6\text{NO}_3^- + 0.2\text{H}_2\text{O} \rightarrow 2.8\text{N}_2 + 6\text{HCO}_3^- + 0.4\text{H}^+$	-2818
<i>p</i> -Cresol	$\text{C}_7\text{H}_8\text{O} + 6.8\text{NO}_3^- \rightarrow 3.4\text{N}_2 + 7\text{HCO}_3^- + 0.2\text{H}^+ + 0.4\text{H}_2\text{O}$	-3422
Benzene	$\text{C}_6\text{H}_6 + 6\text{NO}_3^- \rightarrow 3\text{N}_2 + 6\text{HCO}_3^-$	-2977
Toluene	$\text{C}_7\text{H}_8 + 7.2\text{NO}_3^- + 0.2\text{H}^+ \rightarrow 3.6\text{N}_2 + 7\text{HCO}_3^- + 0.6\text{H}_2\text{O}$	-3524
Xylene	$\text{C}_8\text{H}_{10} + 8.4\text{NO}_3^- + 0.4\text{H}^+ \rightarrow 4.2\text{N}_2 + 8\text{HCO}_3^- + 1.2\text{H}_2\text{O}$	-4136
Aerobic respiration		
Toluene	$\text{C}_7\text{H}_8 + 9\text{O}_2 \rightarrow 7\text{CO}_2 + 4\text{H}_2\text{O}$	-3831
Benzene	$\text{C}_6\text{H}_6 + 7.5\text{O}_2 + 3\text{H}_2\text{O} \rightarrow 6\text{HCO}_3^- + 6\text{H}^+$	-3173
Methanogenesis		
Toluene	$\text{C}_7\text{H}_8 + 7.5\text{H}_2\text{O} \rightarrow 4.5\text{CH}_4 + 2.5\text{HCO}_3^- + 2.5\text{H}^+$	-131
Benzene	$\text{C}_6\text{H}_6 + 6.75\text{H}_2\text{O} \rightarrow 3.75\text{CH}_4 + 2.25\text{HCO}_3^- + 2.25\text{H}^+$	-124
Sulfate reduction		
Toluene	$\text{C}_7\text{H}_8 + 4.5\text{SO}_4^{2-} + 3\text{H}_2\text{O} \rightarrow 7\text{HCO}_3^- + 2.5\text{H}^+ + 4.5\text{HS}^-$	-205
Benzene	$\text{C}_6\text{H}_6 + 3.75\text{SO}_4^{2-} + 3\text{H}_2\text{O} \rightarrow 6\text{HCO}_3^- + 0.325\text{H}^+ + 3.75\text{HS}^-$	-186
Hem reduction		
Toluene	$\text{C}_7\text{H}_8 + 94\text{Fe(OH)}_3 \rightarrow 7\text{FeCO}_3 + 29\text{Fe}_3\text{O}_4 + 145\text{H}_2\text{O}$	-3398
Benzene	$\text{C}_6\text{H}_6 + 30\text{Fe}^{3+} + 3\text{H}_2\text{O} \rightarrow 6\text{HCO}_3^- + 30\text{Fe}^{2+} + 36\text{H}^+$	-3040

Table 7. ΔG° values of denitrification and several respiratory processes in presence of different electron sources.

3.1 Microbiological aspects

Different taxonomic groups are involved in denitrification including Gram negative and positive bacteria. Their remarkable characteristic is their facultative respiration. Some of them are phototrophic, whereas many others can use organic sources or sulfur compounds. Some of them are illustrated in Table 8.

Denitrifiers able to consume BTX have been isolated from different places such as forest soils, sediments of aquifers, beaches, and contaminated soils with hydrocarbons. Toluene and xylene consumers as *Thauera aromatica* and *Azoarcus evansii* (Anders et al., 1995; Song et al., 1998, as cited in Peña-Calva, 2007), and BTX consumers as *Pseudomonas putida* and *Pseudomonas fluorescences* have been isolated from these sites (Shim et al., 2005). Axenic and enriched cultures of *Rhodococcus pyridinovorans* have been reported to be able to consume *m*-xylene. Enrichment cultures of *Betaproteobacteria* growing with *p*-xylene and nitrate have been reported (Rotaru et al., 2010 as cited in Sander et al., 2010), whereas the elimination of the three xylene isomers can be carried out by microbial consortia (Jung & Park, 2004). Some evidences on benzene consumption have been reported for *Dechloromonas* (Coates et al., 2001), likewise, there are evidences indicating that this strain is able to use BTX mixtures for microbial growth (Chakraborty et al., 2005).

3.2 Biochemistry aspects

The denitrification process could be described as a modular organization in which every biochemical reaction is catalyzed by specific reductase enzymes (Cuervo-López et al., 2009). Four enzymatic reactions take place in the cell as follows: (i) nitrate is reduced to nitrite by nitrate reductase (*Nar*); (ii) a subsequent reduction of nitrite to nitric oxide is carried out by nitrite reductase (*Nir*); (iii) afterwards, nitric oxide is reduced to nitrous oxide by the enzyme nitric oxide reductase (*Nor*); (iv) finally, nitrous oxide is reduced to N₂ by the enzyme nitrous oxide reductase (*Nos*) (Lalucat et al., 2006) (Table 9). These reactions take place when environmental conditions become anaerobic (Berks et al., 1995; Hochstein & Tomlinson, 1988). The enzymatic reactions, which are thermodynamically favored, are carried out in the cell membrane and periplasmic space. Small half saturation constant values (*Km*) have been reported for different nitrogen substrates for some denitrifying bacteria, indicating that denitrifying enzymes have a high affinity for their substrate. However, several factors have to be considered, as the presence of small quantities of molybdenum, cooper and hem to ensure the successful enzymatic activity, as they are known cofactors for denitrifying enzymes.

Dominio	Type	Genera	Species	References (as cited in González-Blanco et al., 2011)
Archaea	Organotrophic	<i>Haloarcula</i> <i>Halobacterium</i>	<i>marismortui</i> <i>denitrificans</i>	Ichiki et al., 2001 Tomlinson et al., 1986
Bacteria (Gram +)	Organotrophic	<i>Bacillus</i>		Zumft, 1997
Bacteria (Gram -)	Litotrophic	<i>Beggiatoa</i>		Kamp et al., 2006
		<i>Thiobacillus</i> <i>Paracoccus</i> <i>Pseudomonas</i> <i>Ralstonia</i>	<i>denitrificans</i> <i>denitrificans</i> <i>eutropha</i>	Beller et al., 2006 Ludwig et al., 1993 Schwartz et al., 2003
	Phototrophic	<i>Rhodobacter</i>		Zumft, 1997
	Organotrophic	<i>Alcaligenes</i>	<i>faecalis</i>	van Niel et al., 1992
		<i>Pseudomonas</i> <i>Pseudovibrio</i> <i>Paracoccus</i> <i>Bacillus</i> <i>Aquaspirillum</i> <i>Tahuera</i> <i>Halomonas</i>	<i>denitrificans</i> <i>denitrificans</i> <i>infernus</i> <i>aromatic</i> <i>nitroreducens</i>	Zumft, 1997 Shieh et al., 2004 Baumann et al., 2006 Boone et al., 1995 Thomsen et al., 2007 Beristain et al., 2009 González-Domenech et al., 2008
		<i>Hyphomicrobium</i>	<i>denitrificans</i>	Yamaguchi et al., 2003
		<i>Flavobacterium</i>	<i>denitrificans</i> sp. nov	Horn et al., 2005

Table 8. Some microbial genera carrying out the denitrifying process (modified from González-Blanco et al., 2011).

Enzyme	Reaction	Km	ΔG°' KJ/reaction	Localization
Nitrate reductase (Nar)	$\text{NO}_3^- + \text{UQH}_2 \rightarrow \text{NO}_2^- + \text{UQ} + \text{H}_2\text{O}$	3.8 mM* <i>Pseudomonas stutzeri</i>	- 274. 38	Cell membrane and periplasmic space
Nitrite reductase (Nir)	$\text{NO}_2^- + \text{Cu}^{1+} + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O} + \text{Cu}^{2+}$ $\text{NO}_2^- + \text{c}^{2+} + 2\text{H}^+ \rightarrow \text{NO} + \text{H}_2\text{O} + \text{c}^3$	230 μM+ <i>Alcaligenes xylosoxidans</i> 53 μM++ <i>Pseudomonas aeruginosa</i>	- 76.2	Periplasmic space
Nitric oxide reductase (Nor)	$2\text{NO} + 2\text{c}^{2+} + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} + 2 \text{c}^{3+}$	2.4 nM** <i>Pseudomonas stutzeri</i>	- 306.3	Cell membrane
Nitrous oxide reductase (Nos)	$\text{N}_2\text{O} + 2 \text{c}^{2+} + 2\text{H}^+ \rightarrow \text{N}_2 + \text{H}_2\text{O} + 2\text{c}^{3+}$	60 μM*** <i>Pseudomonas stutzeri</i>	- 339.5	Periplasmic space

Table 9. Some characteristics of the denitrifying enzymes and its location in the cell. UQH₂: reduced ubiquinone, UQ: ubiquinone, c²⁺: reduced cytochrome, c³⁺: oxidized cytochrome. (*Blümle et al., 1991; +Dood et al., 1997; ++Greenwood et al., 1978; **Remde & Conrad, 1991; ***Zumft et al., 1992, as cited in Zumft, 1997).

3.3 Environmental factors affecting denitrification

Low oxygen tension and the presence of nitrogen oxides are required as an inductor of denitrification (Cuervo-López et al., 2009). Denitrifying activity is inhibited in reversible manner under aerobic conditions. Oxygen concentrations ranging from 0.09 to 2.15 mg/l have resulted in a decrease of nitrate consumption and denitrifying rate or accumulation of intermediaries as nitrous oxide (Bonin et al., 1989; Oh & Silverstein, 1999; Wu & Knowles, 1994). Thus, in order to maintain high consumption efficiency of the reducing source and high denitrifying yield values (Y_{N₂}, conversion of nitrate to N₂) in denitrifying systems, oxygen concentrations should be maintained lower than 2 mg/l.

Carbon/nitrogen (C/N) ratio is an important factor which determines the denitrifying performance. This variable is related with the accumulation of denitrification intermediaries and could determine the dissimilative reduction of nitrate to ammonium (DNRA) (Cuervo-López, 2009). Several works focused on BTX removal have resulted in nitrite accumulation due to the low C/N ratio established (Burland & Edwards 1999; Dou et al. 2008; Evans et al., 1992). This behavior is in contrast with that reported by Peña-Calva et al. (2004a, 2004b) and Martínez et al. (2007, 2009), where BTX were converted to CO₂ whereas nitrate was reduced to N₂ at C/N ratios close to stoichiometric values. Thus, these results evidenced that complete elimination of both, carbon and nitrogen compounds will outcome with no accumulation of undesirable intermediaries if C/N ratios are close to stoichiometric values. According to Peña-Calva et al. (2004b), the type of electron source has also an important effect in the accumulation of nitrogen intermediaries, as NO₂⁻ and N₂O were detected when

m-xylene was used whereas no intermediaries were observed when toluene was assayed. Another important parameter which affects denitrification, particularly its thermodynamics, is the pH value, as the rate of denitrifying enzymes could be affected. In this sense, batch studies with *Paracoccus denitrificans* indicate important differences in the rate of nitrite and nitrous oxide consumption and production (Thomsen et al., 1994). Another important effect of pH might be observed on the transport mechanisms of substrates as membrane properties could be affected. As a result, changes in consumption efficiency and consumption rate will be observed. Denitrification could be carried out in a temperature range between 5 and 35°C (Lalucat et al., 2006). However, temperature effects can be observed in rates and consumption efficiency of substrates and possibly in the products formation yield. Similarly to pH, effects of low and high temperatures are mainly related with the physicochemical changes in the cell membrane structures, either for lipids and proteins. It has been reported that toluene denitrification was performed with influents at 5°C in a continuous UASB reactor (Martínez et al., 2007). Therefore, for obtaining a constant and acceptable denitrifying rate it is recommendable to conduct biological wastewater treatment at C/N stoichiometric values, neutral pH and temperature values between 20 and 35°C.

Finally, kinetics considerations have to be taken into account for a successful denitrifying process (Cuervo-López et al., 2009). According to these authors, the metabolism of denitrifying cultures can be described by the following equation:

$$q_s = \frac{\mu}{Y_{x/S_{cx}}} + \frac{q_p}{Y_{p/S_{cp}}} \quad (4)$$

Where q_s is the specific consumption rate of substrate, a constant of first order which its value is dependent on the environmental culture conditions; μ is the specific growth rate; q_p is the specific product formation rate; $Y_{x/S_{cx}}$ and $Y_{p/S_{cp}}$ are substrate conversion or yield for biomass and products respect to the consumed substrate, respectively. The equation summarizes the metabolism of many cell cultures; where $\mu/Y_{x/S_{cx}}$ expresses the biosynthetic process and $q_p/Y_{p/S_{cp}}$ the respiratory process. In denitrification, it is desirable to obtain dissimilative processes where μ and $Y_{x/S_{cx}}$ will be very small for obtaining negligible biomass production but high substrate conversions to molecular nitrogen and bicarbonate. This situation is highly recommended for practical purposes, as operational costs by sludge disposition could be minimized. In this regard, Peña-Calva et al. (2004a, 2004b), Hernández et al. (2008) and Martínez et al. (2007, 2009) have shown that denitrification process with BTX could be clearly dissimilative.

3.4 Nitrate and BTX removal

First evidences indicating toluene consumption by denitrification and its conversion to CO₂ are those obtained by Zeyer et al. (1986). Similar results have been reported by several authors (Alvarez & Vogel, 1995; Chakraborty et al., 2005; Elmén et al., 1997; Kobayashi et al., 2000). However, in these works nitrate consumption and N₂ production have not been mentioned, thus, the occurrence of denitrification process has not been verified. Evans et al. (1991, 1992) have reported toluene consumption and production of CO₂, biomass and intermediaries as benzilsuccinate with a concomitant accumulation of nitrite. Toluene

elimination close to 80% was achieved by Schocher et al. (1991). The authors determined that 50% of the aromatic compound was oxidized to CO_2 whereas they assumed that the other fraction was assimilated as biomass. High nitrate concentrations corresponding to low C/N ratio values (close to 0.2) have been used in most of these works, resulting in nitrite accumulation. The three isomers of xylene have been eliminated under denitrifying conditions, where *m*-xylene was firstly consumed followed by *p*-xylene and finally by *o*-xylene (Haner et al., 1995; Kuhn et al., 1988). It has been reported a removal of *m*-xylene close to 55% but there is no mention about the fate of the aromatic compound or nitrate (Elmén et al., 1997; Evans et al., 1991a). In most of these works enrichment cultures have been extensively used as inocula. Peña-Calva et al. (2004b) evidenced that toluene and *m*-xylene concentrations ranging from 15 to 100 mg of toluene-C/l and 15 to 70 mg of *m*-xylene-C/l were used as electron sources for a denitrifying dissimilative respiratory process when sludge without previous acclimation to BTX was used as inoculum. However, the physiologic stability of the sludge was important. Likewise, these authors conducted their assays at C/N ratio close to stoichiometric values (C/N = 1). At these conditions, nitrate consumed was completely converted into N_2 , while toluene and *m*-xylene were completely converted into HCO_3^- within 4 to 7 days of culture. Under these conditions, the efficiency of the denitrification pathway was not influenced by toluene or *m*-xylene; and the denitrifying yield values were close to 1. However, the authors reported that benzene was recalcitrant. Benzene elimination by denitrification has been reported by using sediments (Major et al., 1988), enriched cultures (Burland & Edwards, 1999) and axenic cultures of *Dechloromonas* RCB (Coates et al., 2001; Kasai et al., 2006, as cited in Weelink et al., 2010). These authors have determined that benzene was oxidized to CO_2 and assimilated into biomass. Dou et al. (2008) have assessed BTX concentrations ranging from 1.3 to 15 mg C/l for each aromatic compound with an enriched mixed bacterial consortium. Similarly to other works, an excess of nitrate (C/N = 0.05) was established for achieving consumption of the aromatic compounds by denitrification. BTX and nitrate were consumed within a period of 20 and 40 days of culture, however, significant nitrite accumulation was observed concomitant to N_2 production. Therefore, benzene is still considered as the most recalcitrant of the BTX under denitrifying conditions (Alvarez & Vogel, 1995; Cunningham et al., 2001; Da Silva & Alvarez, 2002; Peña-Calva et al., 2004b; Phelps & Young, 1999; Weelink et al., 2010).

BTX are usually present in contaminated aquifers as a mixture of them. There are some reports under denitrifying conditions where BTX interactions were evaluated (Alvarez & Vogel, 1995; Ma & Love, 2001). These studies showed that only toluene and xylene were consumed but no benzene. In spite of toluene and xylene were mineralized, reduction of nitrate to molecular nitrogen was not evidenced. The denitrification of different concentrations (50, 70 and 90 mg C/l) of a mixture of BTX using a stabilized sludge without previous contact to BTX was studied by Peña-Calva et al. (2004a). Firstly, toluene was completely consumed and oxidized to HCO_3^- whereas nitrate was reduced to N_2 . After this, partial *m*-xylene consumption (close to 55%) and partial reduction of nitrate to N_2 was observed as NO_2^- and N_2O were accumulated. Benzene was not consumed in any of the cultures. Nevertheless, oxidation of an equal mixture of benzene and toluene coupled to nitrate reduction has been reported in a pure culture of *Dechloromonas* (Chakraborty et al., 2005) and enriched mixed bacteria culture (Dou et al., 2008). However, most studies with consortia have shown that benzene seems to be recalcitrant to biodegradation under

denitrifying conditions in both cases: in mixtures and as a sole electron donor. In this sense, it has been suggested that benzene is not consumed due to the lack of oxidizing enzymes (Gülensoy & Alvarez, 1999) and/or to the high structural stability of benzene (Cadwell & Sufita, 2000). Likewise, toxic effects on bacteria cell membranes have been proposed (Sikkema et al., 1994). Limitation in cell transport system for benzene incorporation into the cell has also been proposed (Peña-Calva, 2007). More investigation is required to understand better the involved mechanisms.

Mixtures of BTX are often present in different types of water along with other carbon sources which might influence the rate and extent of its elimination (Lovanh & Alvarez, 2004). Several studies about the elimination of mixtures of readily consumable carbon compounds with BTX have been made. Numerous substrate interactions among BTX and other carbon compounds have been mentioned that affect the elimination of the aromatic hydrocarbons. Under aerobic conditions, high BTX consumption was obtained with mixtures of these compounds (Deeb & Alvarez-Cohen, 1999; Prenafeta-Boldú et al., 2002). However, negative effects such as competitive inhibition with toluene in presence of phenol (Lovanh & Álvarez, 2004) or ethanol (Da Silva et al. 2005) and catabolic repression in presence of succinate or acetate (Lovanh & Álvarez, 2004) have been reported. The effect of alternate carbon sources on BTX consumption associated to microbial growth has been evaluated at denitrifying conditions. Su & Kafkewitz (1994) demonstrated that a nitrate-reducing strain of *Pseudomonas maltophilia* was capable of utilizing toluene and succinate simultaneously. Both substrates were consumed for microbial growth. Data about the effect of organic compounds on oxidation and consumption rate of BTX are seldom found in the literature. In this regard, Gusmao et al. (2006) evaluated toluene, benzene, ethylbenzene and xylenes consumption separately (between 14 and 33 mg/l of each BTEX) or in mixtures (5 mg/l of each hydrocarbon) in the presence of ethanol (566 mg/l) resulting in high toluene removal efficiencies. However, no information about the fate of ethanol or BTEX consumed was made. Evidences of microbial consortia capable of accomplish denitrification with different ratios of acetate-C/toluene-C concentrations at C/N ratio of 1.4 have been reported by Martínez et al. (2007). These authors achieved elimination efficiencies of toluene, acetate and nitrate close to 100%, as well as yields formation of HCO_3^- and N_2 close to 1, indicating complete mineralization of both organic compounds. Likewise, denitrifying process was clearly dissimilative, as no biomass formation was detected.

All these results evidenced the feasibility of denitrification for BTX removal. Nevertheless, in spite of N_2 production was achieved in some of the works; significant nitrite accumulation was also obtained due to the high nitrate concentrations used in the assays. This problem could be overtaken by establishing C/N ratios close to stoichiometric values considering that it was not required an excess of nitrate in order to achieve effective BTX consumption. Moreover, the control and definition of C/N ratio seemed to be an option for negligible biomass production and obtaining dissimilative process during ground and wastewater treatment. Likewise, the use of sludge with no previously contact to BTX but physiologically stable, that is, in denitrifying steady state, could be an economical and less time-costly option when compared to the use of enrichment cultures. This type of denitrifying consortium may have promising applications for complete removal of both nitrogen and BTX compounds from wastewater streams into N_2 and CO_2 .

3.5 Kinetic data for BTX consumption

Some kinetic data for BTX consumption obtained in batch denitrifying cultures are illustrated in Table 10, whereas data obtained for mixtures of BTX are mentioned in Table 11. It is difficult to make comparisons among these data considering that operational and environmental conditions, as well as biomass concentration used are different. However, it is possible to see that specific consumption rate values are generally low. Elmen et al. (1997) reported in axenic cultures of *Azoarcus tolulyticus* that specific toluene consumption rate decreased in 25% at toluene concentrations of 102 mg/l, whereas Peña-Calva et al. (2004b) using a denitrifying sludge without BTX acclimation determined a decrease of 21% in specific toluene consumption rate (q_T) at 100 mg toluene-C/l. According to Peña-Calva et al. (2004b), the maximum value of specific *m*-xylene consumption rate (q_X) was reached at 70 mg *m*-xylene-C/l, whereas a significant inhibition of the denitrifying pathway was observed at higher *m*-xylene concentrations, as they observed accumulation of NO₂⁻ and N₂O. It is important to remark that at the same experimental conditions, the q_X reported by these authors was 6 times lower than toluene consumption rate. Similar results have been obtained by Dou et al. (2008) as they reported that q_T values were higher than q_X values. Likewise, they observed an inhibitory effect of xylenes on biodegradation rate of BTX. These results suggested that wastewaters containing less than 100 mg/l of toluene or *m*-xylene would not affect the removal rate in a treatment system. Nevertheless, generalizations about this situation must be avoided as previous physiological state of sludge, biomass concentration and the rest of culture conditions might affect kinetic data and performance of the respiratory process.

Initial concentration of single BTX in batch assays	Inoculum	BTX removal rate	Reference
70 mg toluene/l	Denitrifying consortium	0.025 mg toluene-C /mg VSS-d	Peña-Calva et al., 2004b
50 mg toluene/l	Enriched mixed culture	1.67 mg/l/d	Dou et al., 2008
55 mg toluene/l	<i>Azoarcus tolulyticus</i>	0.89 mg toluene/mg biomass-d	Elmen et al., 1997
20 mg toluene/l	Denitrifying consortium	0.007 mg toluene-C /mg VSS-d	Martínez et al., 2009
50 mg benzene/l	Enriched mixed culture	1.46 mg benzene/l/d	Dou et al., 2008
70 mg <i>m</i> -xylene/l	Denitrifying consortium	0.0037 mg xylene-C /mg VSS-d	Peña-Calva et al., 2004b
50 mg <i>m</i> -xylene/l	Enriched mixed culture	1.49 mg <i>m</i> -xylene/l/d	Dou et al., 2008

Table 10. Some kinetic data obtained for BTX consumption at different denitrifying culture conditions.

Several assays with mixtures of BTX have indicated substrate interactions among these compounds. Differences in consumption rate values have been observed when mixtures of BTX are assayed (Table 11) and compared with the values obtained in assays with the single

BTX compound (Table 10). In this sense, enhancement in xylene and toluene consumption rate between two to three times was determined in mixtures of toluene, benzene and xylene (Peña-Calva et al., 2004b), suggesting a positive interaction in hydrocarbons consumption when they are in mixture (Table 11). In contrast Dou et al. (2008) reported a diminishing in BTX consumption rate when mixtures of BTX concentrations higher than 5 mg/l were assayed and compared with those obtained in single BTX tests (Table 10). On the other hand, reports on mixtures of easy consumable substrate such as acetate and toluene conducted with microbial consortia with no previous contact to BTX, have indicated that at the acetate/toluene ratio of 65/20 the specific consumption rate of toluene value was twice compared to that obtained at 20 mg toluene-C/l alone (Martínez et al., 2009) (Table 11). Therefore, whereas the acetate concentration is clearly higher than toluene the consumption rate for toluene was increased. Authors have suggested that the specific consumption rate of toluene was biochemically enhanced by the presence of acetate. Noteworthy, at these conditions the denitrifying process was clearly dissimilative, as no biomass formation was detected. All these results indicate that substrate interaction among BTX and organic compounds mixtures are complicated as beneficial and deleterious effects on consumption rate values have been observed. More investigation is required on this topic. The wastewater treatment should be focused on obtaining dissimilative process with high consumption rates values. The use of some BTX mixtures or addition of easy consumable substrates might be helpful.

Initial BTX mixture concentration in batch assays (mg/l)	Corresponding single compound concentration in the BTX mixture in batch assays	BTX removal rate	Reference
65 acetate/20 toluene	20 mg toluene/l	0.014 mg toluene-C /mg SSV-d	Martínez et al., 2009
90	39 mg toluene/l	0.021 mg toluene-C/mg SSV-d	Peña-Calva et al., 2004a
300	50 mg toluene/l	1.29 mg toluene/l	Dou et al., 2008
90	41 mg <i>m</i> - xylene /l	0.0032 mg <i>m</i> -xylene-C/mg SSV-d	Peña-Calva et al., 2004a
300	50 mg <i>m</i> - xylene /l	0.92 mg <i>m</i> -xylene /l	Dou et al., 2008

Table 11. Kinetic data of BTX consumption at different concentrations of BTX mixtures at different denitrifying culture conditions.

3.6 Examples of simultaneous elimination of BTX and nitrate in reactors

Different configurations of reactors have been proposed for conducting biological BTX remediation (Farhadian et al., 2008). Some studies on BTX removal by denitrifying bioreactors have been carried out in recent years using different configurations such as horizontal anaerobic immobilized bioreactors (HAIB) (Gusmao et al., 2006, 2007), up flow anaerobic sludge blanket (UASB) reactors (Martínez et al., 2007, 2009) and sequencing batch reactors (SBR) (Hernández et al., 2008).

A horizontal-flow anaerobic immobilized biomass reactor (HAIB) inoculated with a denitrifying consortium was used for treating both a mixture solution of approximately 5.0 mg/l of each BTEX and the hydrocarbons separately dissolved in a solution containing ethanol (Gusmao et al., 2006). Organic matter removal efficiencies were of 95% with benzene and toluene amendments and about 76% with ethylbenzene, *m*-xylene and the BTEX-mix amendments. Ethanol was removed with an average efficiency of 86% whereas a concomitant nitrate removal of 97% was determined. Another HAIB reactor inoculated with a denitrifying pure culture resulted in nitrate and BTEX removal close to 97% (Gusmao et al., 2007). These systems showed to be an alternative for treating wastewater contaminated with nitrate, BTX and ethanol, however, no data about N_2 formation and BTEX lost due to volatilization or adsorption in the polyurethane used as packing material in the reactors was mentioned. Martínez et al. (2007), reported a successful denitrification of different toluene-C loading rates (25, 50, 75, 100 and 125) mixed with acetate (accounting for a total carbon loading rate of 250 mg C/l/d) in a continuously fed UASB reactor inoculated with denitrifying sludge physiologically stabilized. At these operational conditions, the C/N ratio was adjusted to 1.4. Authors indicated that even at 125 mg toluene-C/l/d the biological process was not affected as carbon consumption efficiency values were close to 100%, whereas Y_{HCO_3} and Y_{N_2} values were higher than 0.71 and 0.88, respectively. This work evidenced that acetate and toluene can be effectively consumed by denitrification at C/N ratios close to stoichiometric values in continuous culture. These results suggest that the simple UASB denitrifying reactor system have promising applications for complete conversion of nitrate, toluene and acetate into N_2 and CO_2 with a minimal sludge production. Likewise, Martínez et al. (2009) reported in a continuous UASB reactor that no sludge acclimation was required for removing toluene from synthetic wastewater. They evidenced that the addition of acetate was useful for improving the specific removal rate of toluene. In order to discriminate whether the acetate effect was on the microbial community or on their metabolic denitrifying activity, these authors conducted molecular biology and ecological studies. Results showed no close effect of acetate on the microbial community composition suggesting that acetate could act as a biochemical enhancer. Thus, practical applications could be derived as no enrichment or previously adapted inocula have to be used in order to achieve successful BTX elimination by denitrifying respiratory process. Finally, using a sequencing batch reactor (SBR), Hernández et al. (2008) determined that at 70 mg toluene-C/l, toluene and nitrate consumption efficiencies were 84 and 100%, respectively whereas Y_{N_2} , Y_{HCO_3} values were close to 1, thus, the process was clearly dissimilative. Authors observed that specific toluene (q_T) and nitrate (q_N) consumption rate values were increased in twofold after 17 cycles of operation. They also evaluated the settleability properties of the denitrifying sludge by means of sludge volume index (SVI). Under these operational conditions, toluene (r_T) and nitrate (r_N) volumetric consumption rates were 46 mg toluene $L^{-1}d^{-1}$ and 50 mg nitrate $L^{-1}d^{-1}$, respectively, whereas the SVI values remained close to 40 ml/g. Considering that operational troubles due to bad sludge settleability properties are often observed at SVI values higher than 200 ml/g, the results obtained by these authors suggest that SBR could be useful for effectively treating wastewaters contaminated with toluene. However, additional studies in bioreactors are needed to obtain more information about physiological, kinetic, ecological and engineering aspects in order to control the sludge metabolic capacity in denitrification processes for treating BTX removal.

4. Conclusion

As described in this chapter, the progress made over the last decades in the understanding of the metabolic capabilities of nitrifying and denitrifying microorganisms for the biotransformation of carbon and nitrogen compounds has allowed the simultaneous elimination of BTX compounds, ammonium, and nitrate from wastewaters by nitrification and denitrification processes. The coupling of nitrifying and denitrifying processes could constitute a technological alternative for the biological treatment of petrochemical effluents. However, more investigation is required on various aspects such as physiology, engineering, and ecology information among others, to control better nitrifying and denitrifying bioreactors for the simultaneous consumption of BTX and nitrogen compounds from industrial effluents of chemical complexity.

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Universidad Autónoma Metropolitana, Consejo Divisional, DCBS. México.

6. References

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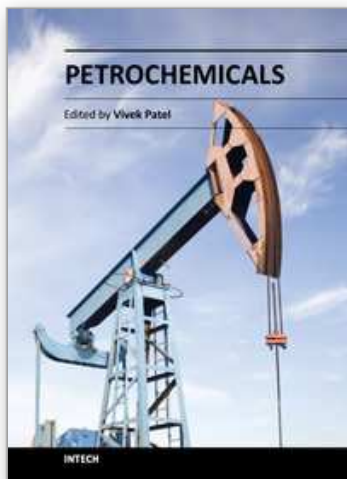
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Petrochemicals

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The petrochemical industry is an important constituent in our pursuit of economic growth, employment generation and basic needs. It is a huge field that encompasses many commercial chemicals and polymers. This book is designed to help the reader, particularly students and researchers of petroleum science and engineering, understand the mechanics and techniques. The selection of topics addressed and the examples, tables and graphs used to illustrate them are governed, to a large extent, by the fact that this book is aimed primarily at the petroleum science and engineering technologist. This book is must-read material for students, engineers, and researchers working in the petrochemical and petroleum area. It gives a valuable and cost-effective insight into the relevant mechanisms and chemical reactions. The book aims to be concise, self-explanatory and informative.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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