Evaluation of gasoline-denatured ethanol as a carbon source for wastewater denitrification

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ABSTRACT

Methanol (MeOH) is a common external carbon source for wastewater denitrification, because of its low cost and low sludge yield. Ethanol (EtOH), on the other hand, is more expensive, but yields higher denitrification rates. This study introduces gasoline-denatured ethanol (dEtOH), which is now being produced in large quantities for the production of E10 gasoline, as an alternative carbon source. The gasoline added, as the denaturant, is known as “straight-run” gasoline; a lower grade material that contains mostly aliphatic compounds, but lacks the components that normally boost the octane rating, such as benzene, toluene, ethylbenzene and xylenes (BTEX). Herein are presented the results of using dEtOH, EtOH (95.5% ethanol-4.5% water) and MeOH for denitrification in lab-scale, sequencing batch reactors (SBRs). We also focused on the quantification of BTEX present in dEtOH solution and the inhibition potential of these compounds on both nitrification and denitrification. BTEX content in the dEtOH solution had low and consistent concentration. Ethylbenzene and o-xylene were not detected in the reactor. The removal rates of benzene, toluene and m-xylene were 3.1±1.4, 3.4±1.9 and 0.6±0.4 μg/L·h, respectively. BTEX were not detected in the effluent and did not inhibit nitrification and denitrification. The denaturant did not affect biomass production or the settling properties of the sludge. The yield (COD/NOx-N) and denitrification rates of dEtOH

were similar to those of EtOH and higher than those of MeOH. The cost of dEtOH ($0.91//lb NO_3^--N removed) is slightly higher than that of methanol ($0.74/lb NO_3^--N removed). Using dEtOH as an external carbon source is, therefore, very promising and utilities will have to decide if it is worth paying a little extra to take advantage of dEtOH's benefits.

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Attribution

The author was responsible for setting up, maintaining, and operating the reactors. Data collection and analysis were also under her responsibility.

Dr. Boardman and Dr. Bott, co-authors of the two manuscripts, offered valuable information about the design of experiments and guided the author throughout the experimental period. They reviewed her work, and contributed to its amelioration through their well-aimed comments on data interpretation.

List of abbreviations

ATF	Bureau of Alcohol, Tobacco, Firearms, and Explosives
ATP	Adenosine Triphosphate
BTEX	Benzene-Toluene-Ethylbenzene-Xylenes
C/N	Carbon to Nitrogen ratio
CNS	Central Nervous System
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
dEtOH	Ethanol denatured with straight-run gasoline
DO	Dissolved Oxygen
EPA	Environmental Protection Agency
EtOH	Ethanol (95.5% ethanol and 4.5% water)
F/M	Food to Microorganisms ratio
GC/MS	Gas Chromatography/Mass Spectrometry
HRT	Hydraulic Retention Time
MCL	Maximum Contaminant Level
M:D	Monovalent to Divalent ratio
MeOH	Methanol
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
SBR	Sequencing Batch Reactor
sCOD	Soluble Chemical Oxygen Demand
SRT	Solids Retention Time
SVI	Sludge Volumetric Index
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WWTP	Waste Water Treatment Plant

Greek Letters

α	significance level
β_0	intercept

β_1	coefficient of regressor for type 1
β_2	coefficient differences from β_1 for type 2
β_3	coefficient differences from β_1 for type 3
γ_{ij}	random effect
ε_{ijk}	random error

1 Introduction

1.1 Objective

Currently, nitrogen removal from wastewater is ranked among the major environmental problems. Combination of nitrification and denitrification processes are recommended and applied to deal with this issue. Nitrification has already been extensively studied and therefore optimized. Denitrification, on the other hand, is enhanced by the addition of external carbon sources and still offers great potential for improvement because there are a great variety of compounds that satisfy this need. This paper is in line with this endeavor and describes the results of a study aimed at the evaluation of an alternative carbon source in terms of its viability, practical and financial, to be used for wastewater denitrification improvement.

During the review of the literature that preceded this research, the majority of studies were focused on methanol and ethanol. However, comparisons made regarding the performance of denitrification with these two carbon sources resulted in ambiguous conclusions. Some of the studies indicated that ethanol improves denitrification rates over methanol (Christensson et al., 1994; Hallin and Pell, 1998; Trela, 1998), whereas others indicated that methanol yields higher rates (Henze, 1991). Therefore, for the specific study ethanol and methanol were selected as two of the three carbon sources tested. The third one, ethanol denatured with gasoline, is a newly introduced carbon source with great potential.

Hence, the objectives of this research were:

- to compare denitrification rates for organisms grown using methanol, ethanol and gasoline-denatured ethanol as external carbon sources;
- to preliminarily compare the cost of the substrates under study;
- to quantify certain aromatic hydrocarbons, i.e. benzene, toluene, ethylbenzene, and xylenes, known as BTEX, present in the ethanol-gasoline solution;
- to study the inhibition potential of the gasoline-denatured ethanol on both nitrification and denitrification and

- to determine the effluent BTEX concentrations.

The comparison was made based on the determination of:

- The amount of biomass produced in each reactor.
- The profiles of soluble chemical oxygen demand (sCOD), ammonium-nitrogen (NH_4^+-N), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N) and BTEX.
- The amount of ethanol, methanol, and denatured ethanol (expressed as mg COD) consumed per mg NOx-N removed.
- The amount of ethanol, methanol, and denatured ethanol consumed per NO_3^--N removed.
- The cost of external carbon source added per lb NO_3^--N.

1.2 Thesis organization

The thesis is divided into six major chapters. Chapter 2 provides an overview of the biological nitrogen removal, a synopsis of the fundamentals of nitrification and denitrification and the description of the three carbon sources under study. The operation of sequencing batch reactors is also discussed. In addition, information about BTEX compounds' degradation and their effect on the above-mentioned biological processes is given. In Chapter 3 follows a description of the experimental setup, its operation and maintenance. Information about sampling procedures and analyses are also provided. The chapter concludes with statistical methods used for the interpretation of data. Next, gasoline-denatured ethanol as an alternative carbon source for denitrification in terms of the BTEX effect on nitrification and denitrification is evaluated in Chapter 4. Chapter 5 contains additional information about the composition of denatured ethanol and BTEX degradation. Two additional chapters are included in the thesis consisting of a summary of the main conclusions and suggestions for future actions.

1.3 References

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2 Literature Review

2.1 Biological Nitrogen Removal

The presence of high nitrogen concentrations in the final effluent of an activated sludge process can adversely affect the quality of the receiving water bodies. The major pollution concerns related to high concentrations of nitrogen are:

1. Eutrophication. Nitrogen plays an important role, being a necessary nutrient for bacteria and algae growth. Excessive algal and bacteria growth, though, can result in hypoxic conditions, which can adversely affect aquatic life forms.
2. Hypoxia. Ammonia is responsible for dissolved oxygen depletion in water bodies due to nitrification.
3. Toxicity. Free ammonia is also toxic to fish and other aquatic organisms.
4. Methemoglobinemia. Infants may experience the “blue baby syndrome”, if they are exposed to high nitrate concentrations. Nitrate is reduced to nitrite inside the baby’s digestive tract, which can oxidize the iron in hemoglobin, preventing oxygen transfer in the body. In addition, nitrate reduction to nitrite might contribute to the formation of nitrosoamines, which are known carcinogens. Therefore, it is very important to achieve low nitrite and nitrate concentrations in the effluent of wastewater treatment plants (WWTP), before discharging it to surface and ground waters, the sources of potable water (Matějů et al., 1992; Metcalf and Eddy, 2003).

The main sources of nitrogen are wastewater effluents, storm water and agricultural runoff, ground water, and air emissions. Wastewater effluent contributes the most to nutrient loading (nitrogen and phosphorus), but it is the only controllable parameter to reduce their high concentrations. For this reason, regulatory agencies and organizations established laws and rules to protect surface and underground waters. Among the most affected regions around the world is Chesapeake Bay, the largest estuary in the U.S.A. The most important step toward the protection of the bay was the decision to decrease total nitrogen and phosphorus effluent concentration from WWTP to 3.0 mg/L and 0.3 mg/L, respectively (HRSD, 2011).

In the present study, we focused on nitrogen, so no further information will be given about phosphorus and its effect on the environment. Nitrogen removal can be accomplished by physico-chemical and/or biological treatment. Biological treatment, though, is highly preferred nowadays, because of its low cost. It can be accomplished by two processes: nitrification and denitrification. The former involves the oxidation of ammonia-nitrogen to nitrite/nitrate, whereas in the latter, nitrite/nitrate is reduced to nitrogen gas (N_2). More details about these processes are provided in the following sections.

2.2 Nitrification

Nitrification is a very important process in the nitrogen cycle, because it produces nitrate ions, the primary substrate for denitrification. Ammonia is oxidized in two stages, first to nitrite and then to nitrate in the presence of oxygen:

$$2NH_4^+ + 3O_2 \xrightarrow{AOB} 2NO_2^- + 4H^+ + 2H_2O + 58\text{-}84 \text{ kcal/mole} \quad (1)$$

$$2NO_2^- + O_2 \xrightarrow{NOB} 2NO_3^- + 15.4\text{-}20.9 \text{ kcal/mole} \quad (2)$$

Total reaction

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (3)$$

Reactions (1) and (2) are performed by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively. Both types of bacteria are autotrophs (consume carbon dioxide, via the Calvin cycle, as the main carbon source) and chemolithotrophs (use ammonia as the sole energy source). The amount of energy produced during nitrification is small, which is the main reason why nitrifiers constitute a small fraction of microorganisms present in the activated sludge (WEF, 2007). Nitrifiers usually comprise 1-2% (w/w) of activated sludge grown under conventional conditions and increase when the proportion of ammonium-nitrogen in the wastewater increases. In addition, they have a low growth yield, which results in the production of a small amount of biomass in the activated sludge process (Painter, 1986).

Okabe et al. (2011) reported that *Nitrosomonas* was the dominant AOB in several activated sludge systems. They attributed this to the fact that they have a higher growth rate than other nitrifying bacteria. Ammonia-oxidizing archaea have also been detected (Park et al., 2006). On the other hand, there are several studies indicating that

Nitrobacter and *Nitrospira* groups are the dominant NOB. AOB and NOB benefit from each other, since AOB produce nitrite and NOB consume nitrite. The importance of this interaction is evidenced by the fact that nitrite is toxic to AOB (Okabe et al., 2011). Regarding their yield coefficients, AOB produces more biomass than NOB, because the free energy released during the reaction (1) is greater than that during reaction (2).

Nitrifying bacteria are sensitive to several environmental and non-environmental factors, like pH, dissolved oxygen (DO), temperature, solids retention time (SRT) and toxic compounds. Different ranges of optimum pH are available in the literature. For example, the Water Environment Federation (2007) suggests 8.0-8.5, Metcalf and Eddy (2003) 7.5-8.0, and Focht and Chang (1975) 7.2-8.9. pH values near 5.8 and 6.0 decrease nitrification rates to 10 or 20% of the rate at pH 7.0. Lower values may inhibit the process, whereas higher ones favor the formation of free ammonia (Focht and Chang, 1975; Metcalf and Eddy, 2003). On the other hand, high pH values makes it easier for CO_2 (a necessary component for the growth of AOB and NOB) to dissolve into water, and become readily available (Okabe et al., 2011). In conclusion, neutral and slightly basic pH are recommended. To keep pH in the desired range, buffer solutions are added to activated sludge systems to ensure that there is enough alkalinity in the reactor.

In addition, according to Grady et al. (1999) the minimum DO concentration for nitrification is 2 mg/L. Nitrification is being inhibited when the available DO is less than this critical value. However, maintaining DO at levels higher than 2 mg/L inside the flocs during aerobic conditions is a very challenging task due to the difficulties imposed by the geometry of the flocs, the different respiration rates, and the varying diffusion coefficients.

Temperature is another crucial factor in the growth of AOB and NOB. Under mesophilic conditions (20-45°C), nitrifiers function best. The solids retention time (SRT) is directly associated with temperature. Complete nitrification occurs in high SRTs and low temperatures or low SRTs and high temperatures (Painter, 1986). More specifically, an SRT of 10 to 20 days is required at 10°C and 4 to 7 days at 20°C for complete nitrification (Metcalf and Eddy, 2003). Lower SRTs will result in nitrite accumulation and high food to microorganism (F/M) ratios. Other factors inhibiting nitrification are related

to the high concentrations of free ammonia and free nitrous oxide acid. Finally, ammonia oxidation is also affected by toxic materials, like benzene (Painter, 1986; Metcalf and Eddy, 2003).

2.3 Denitrification

Early in the 19th century, Gayon and Dupetit realized that nitrate and nitrite could biologically be converted to nitrogen gas (Focht and Chang, 1975). Since then extended research has established denitrification as an integral part of biological nitrogen removal. Denitrification is a microbial respiratory process, where nitrate is reduced to nitrogen gas. It is a reduction-oxidation reaction, where electrons are transferred from the electron donor (carbon source) to the electron acceptor (nitrate). In this way, the organism gains energy, which can then be used for the synthesis of a new cell mass and the maintenance of the existing cell mass.

There are two types of denitrification: assimilatory and dissimilatory. During assimilatory denitrification, nitrate is reduced to ammonia, which is used for cell synthesis. Dissimilatory denitrification involves the reduction of nitrate. It takes place under anoxic conditions, if nitrate is present according to the following reactions (Metcalf and Eddy, 2003; WEF, 2007):

$$NO_3^- + \text{organic matter} + H_2CO_3 + \text{denitrifiers} \rightarrow \text{new microbes} + N_2 + H_2O + HCO_3^- \quad (4)$$

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (5)$$

Different enzymes are involved in each of the above steps. Membrane-bound nitrate reductase enzymes catalyze the conversion of nitrate to nitrite, whereas nitrite is reduced to nitrous oxide by nitric oxide and nitrite reductases. Finally, nitrous oxide reductase enzymes catalyze the reduction of nitrous oxide to nitrogen gas. During this step adenosine triphosphate (ATP), which transfers energy in the cell, is produced (Matějů et al., 1992). Nitrogen gas (N_2) is released to the environment causing no adverse effects, because it is an inert gas and makes up most of the nitrogen found in the atmosphere and in natural waters.

A great variety of bacteria can undergo denitrification. Among them are *Alcaligenes*, *Paracoccus*, *Pseudomonas*, *Achromobacter, Aeromonas, Bacillus*, and *Rhodobacter* (Zumft, 1991; Matějů et al., 1992). Denitrifiers are heterotrophic, autotrophic and

photolithotrophic prokaryotes. Heterotrophs do not consume much energy for synthesis. This explains their faster growth and increased mass compared to those of autotrophs. Most heterotrophs are facultative aerobic bacteria, so it is important to maintain low DO values during denitrification to avoid using oxygen instead of nitrate. Microorganisms prefer oxygen to nitrate or nitrite as a terminal electron acceptor, because more energy is available from its reduction (Grady et al., 1999).

The availability and the type of the electron donor significantly affect denitrification. Banchuen (2002) mentions that simple organic compounds with small molecular weights are more easily degraded by denitrifying bacteria. The existence of more complex compounds may lead to nitrite accumulation, which hinders the rate of denitrification (Albanez et al., 2009). Moreover, high organic loading may cause the production of ammonia. Ammonia induces all the denitrification reductase enzymes. As a result, intermediate products of denitrification are accumulated.

Denitrification occurs in the absence of oxygen. If DO is present in the mixed liquor, denitrification rates are reduced, because DO blocks the activity of the nitrate reduction enzymes. Most researchers set 0.2 mg/L as the maximum allowed DO concentration (Banchuen, 2002).

According to Focht and Chang (1975) denitrification increases at pH 4 and decreases at 9.5. Between 7 and 8 denitrification rates are faster. On the other hand, Metcalf and Eddy (2003) noted that denitrification is independent of pH. Nevertheless, alkalinity is added in the activated sludge systems to maintain a pH in the range desired. Maintaining 50 mg/L of alkalinity (as mg/L $CaCO_3$) in the effluent prevents inhibiting drops of pH (WEF, 2002). Denitrification rate increases with temperature, with the optimal range being 25-30°C, although it has been reported that the maximum denitrification rate can be achieved at 40°C (Payne, 1981; van Haandel and van der Lubbe, 2007). Another significant factor that might affect denitrification is the presence of toxic compounds, for example benzene, toluene, ethylbenzene and xylenes (BTEX). More information about BTEX effects on both nitrification and denitrification are provided in section 2.5.

2.3.1 Carbon sources for denitrification

Nitrate reduction is only possible in the presence of energy and carbon. Carbon may be supplied by endogenous respiration, stored-induced carbon and wastewater itself. Endogenous respiration gives low denitrification rates, whereas wastewater may cause the system to be carbon-limited, if it is consumed in the processes involved before denitrification. Thus, for post-denitrification processes the addition of an external carbon source is necessary (Abufayed and Schroeder, 1986). Carbon addition is beneficial, because it increases the fraction of readily biodegradable compounds and the carbon to nitrogen ratio (C/N). Consequently, in the former case the required hydraulic retention time (HRT) is reduced and the volume of the anoxic basins is minimized. High C/N ratios are related to enhanced denitrification rates (Kang et al., 1992; Trela, 1998; Albanez et al., 2009).

External carbon sources are actually electron donors that give up electrons during cellular respiration. The electron exchanged is accompanied with the release of energy. The main characteristics that make an electron donor preferable are summarized below (Ginige et al., 2009; Swinarski et al., 2009):

1. Enhancement of denitrification by increasing the specific denitrification rates (SDNR).
2. Yield, measured as C/N.
3. Chemical oxygen demand (COD) of the compound.
4. Low price, e.g. \$/lb NO_3^--N denitrified.
5. Steady performance regardless of the temperature (winter-summer).
6. Availability at frequent intervals and required quantities.
7. Good sludge characteristics (production and settling properties).
8. Degree of carbon utilization that results to low effluent BOD.
9. Handling and storage - Safe to handle.
10. Easily biodegradable.
11. Free of nitrogen and phosphorus.
12. Free of non-biodegradable/toxic compounds.
13. Consistent composition.
14. Rapid bacteria acclimation.

Examples of electron donors that have been studied so far include methanol, ethanol, acetate, glycerol, primary sludge, acetic acid, and hydrolyzed starch. For the purposes of this study, methanol and two types of ethanol were used. Basic information about their performance as electron donors is given below.

2.3.1.1 MeOH

Methanol has been widely tested and used for the last twenty years as an electron donor for denitrification both in lab- and full-scale experiments (Nyberg et al., 1992; Onnis-Hayden, 2008). It is oxidized according to the following reaction:

$$NO_3^- + 1.08CH_3OH + H^+ \rightarrow 0.065C_5H_7NO_2 + 0.467N_2 + 0.76CO_2 + 2.44H_2O \quad (6)$$

which takes into consideration the amount of methanol required for deoxygenation and cell synthesis (Matějů et al., 1992).

The amount of methanol required for denitrification is given by the following equation based on experimental data, assuming no biomass growth (McCarty et al., 1969):

$$C_{MeOH} = 2.47NO_3^-\text{-}N + 1.53NO_2^-\text{-}N + 0.87DO_0 \quad (7)$$

where,

C_{MeOH} = required methanol concentration, mg/L as methanol

NO_3^--N = nitrate-nitrogen concentration, mg/L

NO_2^--N = nitrite-nitrogen concentration, mg/L

DO_0 = initial DO concentration, mg/L.

Methanol satisfies many of the criteria for an ideal carbon source as mentioned above. More specifically, it is cheaper than other suggested carbon sources; it is commercially available and widely used offering a wide range of performance data that can be employed for system's optimization. Another advantage that results from its wide use is the fact that it is thoroughly regulated. In addition, it is readily biodegradable and has low sludge production rate (low sludge yield). Moreover, the carbon oxidation is almost complete, resulting in low effluent BOD concentrations. According to McCarty et al. (1969) it is not considered a toxic chemical to fish life, given that fish can tolerate concentrations over 100 mg/L. Finally, it does not contain any phosphorus or nitrogen that could aggravate the influent nutrient load (Hallin et al., 2006; Mokhayeri et al., 2008b; Onnis-Hayden and Gu, 2008; Swinarski et al., 2009).

On the other hand, methanol has some important disadvantages. First of all, specific denitrification rates (SDNRs) are lower than that of other electron donors. Methanol is a single-carbon compound, not easily entering the tricarboxylic acid (TCA) cycle, unless it is reduced to form higher carbon intermediates (3-C and 4-C) (Mokhayeri et al., 2008b). In addition, it requires longer acclimation and recovery periods. It has been demonstrated that when methanol is used as an external carbon source a new population of methylotrophic bacteria with a slower growth rate is established (Hallin et al., 1996; Hallin et al., 2006; Swinarski et al., 2009). These kinds of bacteria are facultative organisms, which belong to the *Paracoccus* and *Hyphomicrobium* genera (Claus and Kutzner, 1985; Onnis-Hayden and Gu, 2008). In addition, according to Hallin and Pell (1998) methylotrophs inhibit the growth of other denitrifying bacteria present in the activated sludge. Therefore, substitution of methanol with other carbon sources may result to loss of denitrification. Moreover, it is produced from the methane present in natural gas and inevitably, its price is linked to that of fossil fuels. Therefore, an increase on fuel price corresponds to an increase in methanol price. According to the National Fire Protection Association (NFPA), methanol is classified as flammable. Hence, specific measures should be taken when transporting or storing. Furthermore, it is classified as poisonous and toxic according to European Union legislation, because it affects the central nervous system (CNS). McCarty et al. (1969) reported that 100-250 mL is considered toxic to humans. Finally, it was demonstrated by Mokhayeri et al. (2008a) and Dold et al. (2008) that during winter (at cold temperatures), methylotrophs' growth rate is low; therefore, methanol addition does not have the desired effect on nitrogen removal.

2.3.1.2 EtOH

Researchers have considered other carbon sources to substitute for methanol. These include ethanol, acetate, glycerol, primary sludge, acetic acid, and industrial and agricultural byproducts (Abufayed and Schroeder, 1986; Lee and Welander, 1996; Onnis-Hayden, 2008; Ginige et al., 2009; Swinarski et al., 2009; Adav et al., 2010). If ethanol is used as a carbon source, the stoichiometric relationship is described by the following reaction (Matějů et al., 1992):

$NO_3^-+0.613C_2H_5OH \rightarrow 0.102C_5H_7NO_2+0.449N_2+0.714CO_2+0.98H_2O+0.286OH^-$ (8)

According to Matějů et al. (1992) the amount of ethanol required for denitrification, without considering biomass growth can be calculated by:

$$C_{EtOH}=2.00NO_3^-\text{-}N+1.28NO_2^-\text{-}N \quad (9)$$

where,

C_{EtOH} = required ethanol concentration, mg/L as ethanol

NO_3^--N = nitrate-nitrogen concentration, mg/L

NO_2^--N = nitrite-nitrogen concentration, mg/L

Ethanol satisfies many of the criteria described above that characterize the ideal carbon source. To begin with, SDNRs achieved are two to three times higher than methanol's (Trela, 1998; Ginige et al., 2009), whereas Mokhayeri et al. (2008b) reported that the SDNR for methylotrophs was lower than for biomass grown on ethanol even at 13°C. Microbes quickly adapt to ethanol within a few hours (Trela, 1998). Moreover, denitrifiers grown on ethanol are able to use other carbon sources, such as acetate, propionate, and butyrate without losing their capacity to denitrify (Hallin and Pell, 1998). Thus, facilities can interchange carbon sources without affecting the performance of the plant. Moreover, ethanol is commercially available. It can be found in many industrial wastes; e.g., in pharmaceutical industries. Furthermore, it is readily biodegradable by many microorganisms, because it is a low-carbon compound, which easily enters the TCA cycle as acetyl-SCoA, as does acetate (Henze, 1994). Microbes are quickly adapted to ethanol when added as a carbon source, because they are already acclimated to the 5-10% acetate in wastewater. Besides, ethanol production is increasing, leading ethanol to be more commercial and less expensive.

Flammability, toxicity, and strict regulation issues are the main disadvantages hindering the more widespread use of ethanol as a carbon source. Ethanol is heavily taxed and difficult to obtain without complicated permits from the bureau of alcohol, tobacco, firearms, and explosives (ATF). Like methanol, it is ranked as a flammable chemical. The oral lethal dose (LD_{50}) in rats is 5,828 mg/kg. Finally, it is still more expensive than methanol.

2.3.1.3 dEtOH

The Hampton Roads Sanitation District (HRSD) is currently interested in the use of ethanol denatured with “straight-run” gasoline as an alternative carbon source for denitrification. In general, ethanol is denatured at the ethanol plant with additives, like acetone and gasoline, to prevent people from consuming it. The gasoline added, in our case, is known as virgin, straight-run, or natural gasoline, and it is blended with ethanol at 2-15%. This mixture is then added to gasoline to make it a cleaner fuel and protect the engine from knocking.

More specifically, “straight-run” gasoline is a lower grade material that contains mostly aliphatic compounds. It lacks the components that normally boost the octane rating, such as benzene, toluene, ethylbenzene and xylenes. In addition, the non-cyclic hydrocarbons contained have the potential to be good carbon sources. More information about its composition will be provided in Chapter 5. The denaturant does not alter the chemical composition of ethanol; therefore, if denatured ethanol is used as a carbon source, the stoichiometric relationship is also described by equation (8).

Because this substance is a new entry in the field of wastewater, little is known about its composition and its capacity to improve SDNRs. Given the fact that it is mostly ethanol, it is reasonable to assume that it shares the same advantages and disadvantages with common ethanol. There are also other issues to consider; in the market, its price is lower than ethanol’s. It is locally available, in large quantities for blending with gasoline to make E10[1]. Because it is denatured, it is declared unfit to drink and is not controlled by ATF.

Possible disadvantages evaluated herein are as follows. First, it is slightly more expensive than methanol. Second, the content of the denaturant (“straight-run” gasoline) and the concentration of hydrocarbons, like benzene, toluene, ethylbenzene, and xylenes contained in it, might vary. Third, the degree of carbon oxidation is unknown. It is possible that a high effluent COD, an indication that gasoline constituents cannot be biologically degraded, will be observed, and this excess will be discharged in the receiving water bodies. Dold (1989) stated that this problem might be tackled with the addition of hydrogen peroxide (H_2O_2) prior to activated sludge treatment, which will

[1] Fuel blend that contains 10% ethanol and 90% gasoline.

improve the adsorption of the compounds under investigation. Fourth, high concentrations of hydrocarbons in the influent might affect sludge properties (Dold, 1989). Oil could potentially accumulate on the flocs, forming a hydrophobic layer; thereby affecting the settling characteristics. In addition, the effect of the denaturant on WWTP is undetermined. Accumulation of gasoline constituents, like the BTEX compounds, in WWTP may affect the performance of the biological process. Lastly, it is possible that the sludge will contain high amounts of gasoline constituents, which require special techniques for sludge treatment and disposal.

2.4 Sequencing Batch Reactor (SBR)

The main characteristics that promote the use of SBRs in WWTP are briefly described. First of all, they operate like ideal plug flow reactors with respect to kinetic response. Secondly, their performance can be controlled; environmental conditions, like mixing, dissolved oxygen (DO) and cycle length, as well as the reaction time and maintenance of sludge solids, are easily regulated. In addition, the fact that nitrification and denitrification occur simultaneously in the same reactor eliminates the need of separate clarifiers and reduces the cost related to oxygen transfer. Moreover, activated sludge is completely mixed resulting to a well-homogenized liquor. The need for an additional equalization tank and peak loadings control is eliminated. A neutral pH and less demand for alkalinity can also be accomplished in the reactor. It is well known that alkalinity is consumed during nitrification, but produced during denitrification. Having the two processes occurring in the same reactor keeps the system balanced. Finally, SBRs are relatively cheap to build and operate (Silverstein and Schroeder, 1983; Abufayed and Schroeder, 1986; Albanez et al., 2009).

The modified Ludzack-Ettinger (MLE) process is commonly used for nitrogen removal. This pre-denitrification system is comprised of two compartments. The first one operates under anoxic conditions to denitrify, while the second one is under aerobic conditions (nitrification occurs). An internal recycle stream enriches the anoxic basin with nitrate produced in the aerobic basin. Thus, nitrate is provided for the heterotrophs to grow and denitrify. In this way, the need to add extra carbon source is eliminated and operating costs are decreased compared to other configurations. In SBRs, though, the recycled sludge is the sludge left in the reactors at the end of the cycle, which contains

nitrate (Grady et al., 1999). The MLE process can achieve effluent nitrate-nitrogen concentrations between 4 to 8 mg/L (Grady et al., 1999; Metcalf and Eddy, 2003).

2.5 Benzene-Toluene-Ethylbenzene-Xylenes (BTEX)

Among the numerous compounds present in gasoline, monoaromatic compounds, such as benzene, toluene, ethylbenzene, and xylenes (BTEX), are of particular interest, because of their potential toxicity. They have been detected at groundwater in high concentrations, ranging from 9 to 14 mg/L of benzene, 23 to 81 mg/L of toluene, and 13 to 171 mg/L of xylene isomers (Peña-Calva et al., 2004). The US EPA classifies BTEX compounds among the top 20 hazardous substances and priority pollutants (EPA, 2009). They are water soluble and highly volatile. Table 2.1 summarizes some of their physical properties.

Table 2.1-Properties of BTEX compounds at 20°C (Hartley and Ohanian, 1989; Weelink et al., 2010).

Compound	Solubility, mg/L	Density, kg/L	Vapor Pressure, kPa	K_H[2], atm·m^3/mol
Benzene	1740-1860	0.878	10.13	$5.5 \cdot 10^{-3}$
Toluene	500-627	0.867	2.93	$6.6 \cdot 10^{-3}$
Ethylbenzene	131-208	0.867	0.93	$8.4 \cdot 10^{-3}$
o-xylene	167-213	0.880	0.67	$5.3 \cdot 10^{-3}$
m-xylene	134-196	0.864	0.80	$6.9 \cdot 10^{-3}$
p-xylene	198	0.860	0.87	$7.0 \cdot 10^{-3}$

[2] At 25°C.

BTEX compounds contaminate soil and groundwater, as they leak from underground petroleum storage tanks. Hence, BTEX removal has been extensively studied by soil scientists for many years. Among the remediation techniques efficiently applied was the in-situ microbial degradation under both aerobic and anaerobic conditions (Karlson and Frankenberger, 1989; Molnaa and Grubbs, 1989; Block et al., 1990).

The effect of BTEX compounds on nitrification is very important for wastewater engineers, especially, those dealing with industrial wastewater. Under aerobic

conditions, oxygen not only serves as an electron acceptor, but also triggers microbial enzymatic activity. Thus, carbon mineralization is induced. More specifically, AOB and NOB use BTEX as carbon and energy sources, in the following order: *o*-xylene>*m*-xylene>toluene>benzene (Weelink et al., 2010). However, the presence of high BTEX concentrations can hinder the ability of bacteria to degrade them (Richardson, 1985).

Three factors play an important role to BTEX's inhibition of nitrification: the presence of functional groups in the compounds, their hydrophobic properties, and their concentrations. Toluene and *m*-xylene, for example, have methyl groups in their molecules, and tend to react with other compounds. Benzene, on the other hand, is more stable, because it lacks functional groups and is less reactive. In addition, polycyclic compounds are hydrophobic and accumulate in the cell membrane inhibiting the respiratory function of enzymes. High concentrations of BTEX have proven to be harmful to nitrifiers. Zepeda et al. (2006) noticed that the maximum amount of BTEX that nitrifiers could tolerate was 5 mg of carbon per liter. Microbes did not recover after they were exposed to higher concentrations (20-50 mg carbon per liter) of toluene and *m*-xylene and only partially recovered when exposed to 20-50 mg C/L of benzene. Exposure of cells to benzene decreased the activity of both AOB and NOB (Sikkema et al., 1992; Denich et al., 2003; Zepeda et al., 2006).

Gersberg et al. (1989) suggested denitrification instead of the addition of oxygen as a feasible way to remove BTEX from aquifers, due to the high amount of oxygen required and therefore the high cost associated with this practice. BTEX compounds are rapidly degraded under aerobic conditions, but they are more recalcitrant under anaerobic conditions. Anaerobic degradation was first demonstrated by Vogel and Grbìc-Galìc (1986). In general, BTEX decomposition under anaerobic conditions can be achieved in the presence of a variety of electron acceptors, such as nitrate, sulfate, ferric iron, manganese, carbon dioxide, or perchlorate. Zeyer et al. (1986) and Peña-Calva et al. (2004) concluded that toluene and *m*-xylene are biologically converted to CO_2 under denitrifying conditions. On the other hand, benzene is not easily degraded (Peña-Calva et al., 2004; Weelink et al., 2010).

Toluene and *m*-xylene though can be inhibitory to denitrification, when present at high concentrations. In such cases, nitrite might accumulate, because the nitrite oxide

reductase and nitrose reductase enzymes are affected (Weelink et al., 2010). Table 2.2 provides the effect each of the BTEX compounds has on human health, as well as the maximum contaminant levels (MCLs).

Table 2.2-Effects of BTEX on human and their MCL (Hartley and Ohanian, 1989; EPA, 2009).

Compound	Effect	MCL
Benzene	CNS[3], cancer	5 µg/L
Toluene	CNS, blood	1 mg/L
Ethylbenzene	CNS, liver, kidney	0.7 mg/L
Xylenes	CNS, liver, body weight	10 mg/L

[3] Central Nervous System

2.5.1 Benzene

Benzene, its structural formula is given in Figure 2.1, is a colorless liquid with a sweet odor. It evaporates into the air very quickly and dissolves slightly in water. It is also highly flammable. Benzene can pass through the soil into underground water, where it degrades slowly.

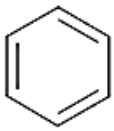


Figure 2.1-Structural formula of benzene.

It is classified as a carcinogen according to EPA and the International Agency for Research on Cancer (IARC, 2011). Maximum permissible level of benzene in drinking water is 5 ppb (EPA, 2009). The Occupational Safety and Health Administration (OSHA) has set limits of 1 ppm of workplace air for 8-hour shifts and 40-hour work weeks (ATSDR, 2011).

Benzene can be used as an electron donor either aerobically or anaerobically, according to the following reactions:

$$C_6H_6+3.04O_2+0.32H_2O+0.89NH_4^+ \rightarrow 1.54HCO_3^-+0.89C_5H_7O_2N+2.43H^+ \quad (10)$$

$$C_6H_6+2.52NO_3^-+0.87NH_4^+ \rightarrow 1.65HCO_3^-+1.26N_2+0.87C_5H_7O_2N+0.87H_2O \quad (11)$$

Bacteria cannot always recover after previous exposure to benzene, as it was mentioned before. They lose their ability to break it down and require long acclimation periods before they resume their activity.

Texier et al. (2003) demonstrated that benzene is converted to phenol and finally to acetate under aerobic conditions. Benzene's biodegradation pathway under anaerobic conditions has yet to be described in full detail. Theoretically, there are four possible initial steps that incorporate its transformation to carbon dioxide; hydroxylation, carboxylation, methylation and reduction of the benzene ring and ring cleavage. *Dechloromonas* and *Azoarcus* *β*-proteobacteria are known to degrade benzene (Chakraborty and Coates, 2004; Weelink et al., 2010).

2.5.2 Toluene

Toluene is a mono-substituted benzene derivative, in which one hydrogen is replaced by a methyl-group (Figure 2.2). It is a clear, colorless liquid with a distinctive smell. Usually it does not accumulate in the environment. It is not classified as carcinogen, but can enhance carcinogenesis by other compounds (Weelink et al., 2010). EPA's (2009) limit is 1 mg/L in drinking water, whereas OSHA has set a limit of 2,000 ppm of workplace air (ATSDR, 2011).

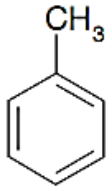


Figure 2.2-Structural formula of toluene.

Under aerobic conditions, microbial metabolism of toluene commonly occurs via the hydroxylation of the methyl group leading to benzyl alcohol and then to butyrate. Low concentrations of propionate, acetate, and benzoate can also be detected (Schwarzenbach et al., 1988; Peña-Calva et al., 2004; Zepeda et al., 2006).

Under anaerobic conditions, it affects the cytoplasmic membrane of the bacteria (Peña-Calva et al., 2004). In contrast with benzene, toluene can be initiated by three enzymatic mechanisms; reduction of the aromatic ring, methylation, or hydroxylation.

Thauera, *Azoarcus, Dechloromonas,* and *Georgfuchsia toluolica* bacteria are responsible for the anaerobic degradation of toluene. All species are facultative bacteria and members of *β*-proteobacteria. There are also four *α*-proteobacteria that can metabolize toluene (Chakraborty and Coates, 2004; Weelink et al., 2010).

2.5.3 Ethylbenzene

Ethylbenzene, its structural formula is given in Figure 2.3, is a colorless, flammable liquid that smells like gasoline. It transfers easily into the air from water and soil. It takes about 3 days to be broken down in air and can move through soil into groundwater. In aquatic environments, it reacts with other natural substances. The final product in all cases is carbon dioxide.

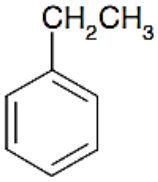


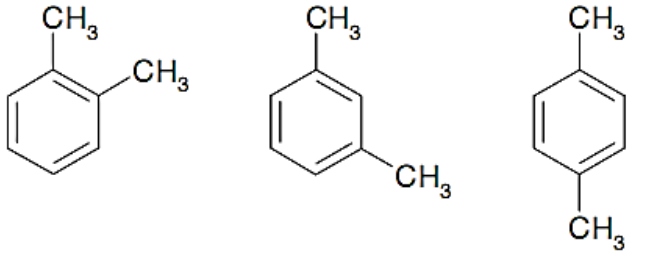
Figure 2.3-Structural formula of ethylbenzene.

Ethylbenzene is classified as a possible human carcinogen by IARC (2011). EPA (2009) established the safe exposure threshold for children to 30 ppm for 1 day or 3 ppm for 10 days. Lifetime exposure equal to 0.7 ppm does not cause any effects either. OSHA has limited workers' exposure to an average of 100 ppm of workplace air for an 8-hour workday, 40-hour workweek (ATSDR, 2011).

Ethylbenzene is metabolized under anoxic conditions by the *Azoarcus* and *Thauera* species (Chakraborty and Coates, 2004; Weelink et al., 2010). Its degradation is initiated by ethylbenzene dehydrogenase and in general, its degradation pathway is different from that of toluene, despite the fact that ethylbenzene and toluene have similar structure.

2.5.4 Xylenes

There are three isomers of xylene (Figure 2.4): *meta*-xylene (*m*-xylene), *ortho*-xylene (*o*-xylene), and *para*-xylene (*p*-xylene). It is a colorless, sweet-smelling, flammable liquid. It evaporates quickly from soil and surface water. In the air, it is broken down by sunlight.



o-xylene *m*-xylene *p*-xylene

Figure 2.4-Structural formula of *ortho*-xylene, *meta*-xylene and *para*-xylene.

EPA (2009) and IARC (2011) do not have sufficient evidence to classify it as carcinogen. Environmental Protection Agency has set a limit of 10 ppm in drinking water, whereas OSHA 100 ppm of workplace air for 8-hour shifts and 40-hour workweeks.

Zepeda et al. (2006) found that *m*-xylene is oxidized to acetate and butyrate under nitrifying conditions. Peña-Calva et al. (2004) reported that acetate and propionate were detected as intermediate products under anoxic conditions. Also, *m*-xylene can anaerobically be oxidized in the presence of nitrate, but not nitrite (Schwarzenbach et al., 1988).

Although there is general agreement regarding the anoxic mineralization of *m*-xylene to carbon dioxide (Zeyer et al., 1986; Chakraborty and Coates, 2004; Peña-Calva et al., 2004; Weelink et al., 2010), the literature is not consistent regarding *o*- and *p*- isomers. Some researchers state that *p*-xylene cannot be fully converted to carbon dioxide under anoxic conditions, while others propose that *o*-xylene can (Chakraborty and Coates, 2004). Weelink et al. (2010) in their review presented studies, according to which the *p*-isomer could also be anoxically degraded, but this was not the case with *o*-xylene. *O*-xylene, though, can be degraded under sulfate-reducing conditions. In increasing order of ease of degradability, xylenes are ranked as:

m-xylene > *p*-xylene > *o*-xylene

The degradation pathway of *m*-xylene is similar to toluene's. *Azoarcus* and *Thauera* *β*-proteobacteria are responsible for *p*- and *m*-xylenes biodegradation (Chakraborty and Coates, 2004; Weelink et al., 2010).

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3 Materials and Methods

3.1 SBR setup, operation, and maintenance

To accomplish the objectives of this research, three 4-L glass beakers were operated as sequencing batch reactors (SBRs). The reactors mimicked the Modified Ludzack-Ettinger process (MLE), the configuration of which is shown in Figure 3.1.

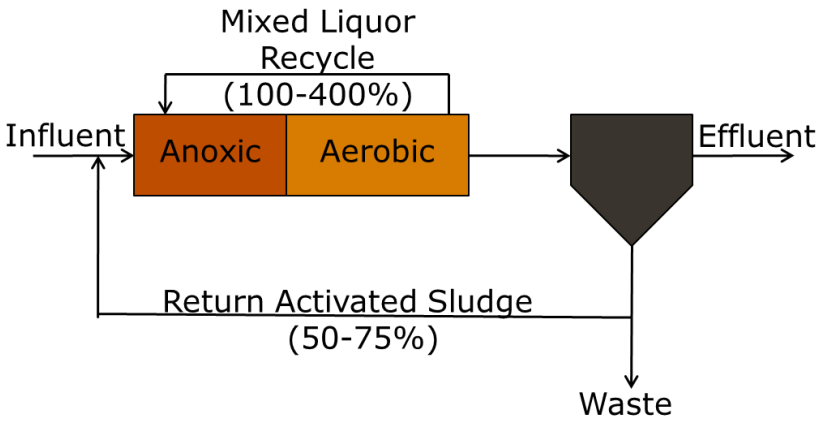
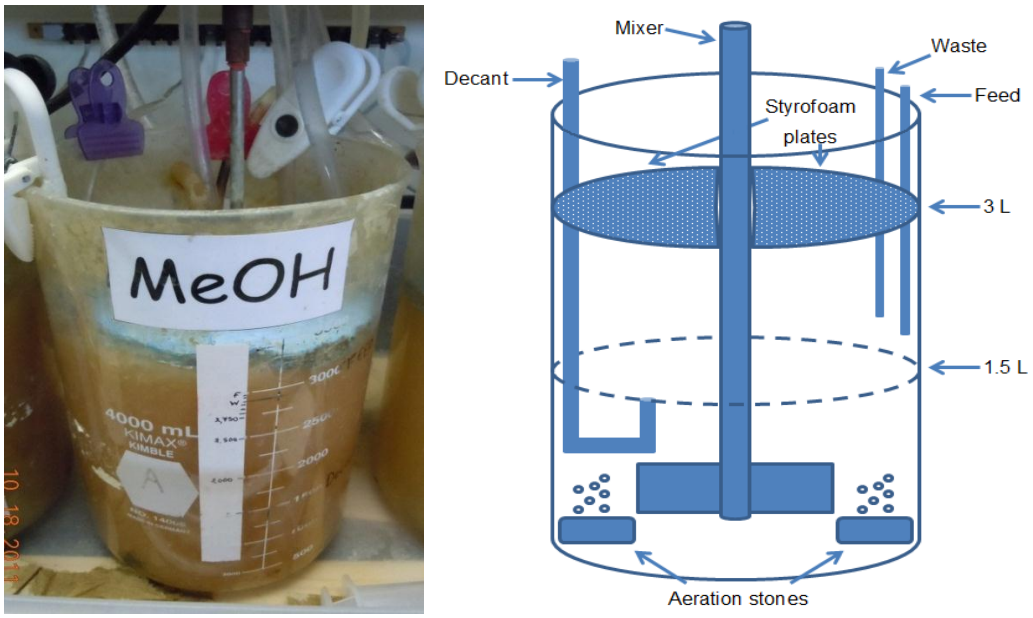


Figure 3.1-Modified Ludzack-Ettinger (MLE) process for nitrogen removal.

Figure 3.2 shows a photo of an SBR used in this study (a) and a schematic of the reactor (b).



(a) (b)

Figure 3.2-Photo (a) and schematic (b) of a reactor.

Initially, reactors were uncovered. Atmospheric oxygen intrusion into the mixed liquor inhibited denitrification. Thus, reactors' surfaces were covered eventually with floating styrofoam. The floating plates were an efficient countermeasure as DO values dropped from 0.2 to 0.04 mg/L.

In Figure 3.3 and Figure 3.4 the actual experimental setup and a more detailed schematic, respectively, are given. Four peristaltic pumps (Cole-Parmer, Vernon Hills, IL) were used; two of them to feed alcohols and wastewater, one for decanting and one for wasting sludge. A fish pump (Aquatic Ecosystems, Inc., Apopka, FL) supplied air to the reactors through two diffuser stones in each reactor for providing process oxygen requirements and to maintain complete mixing of the biomass. Mechanical stirring at 66 rpm was also provided by means of stainless steel 2.54cm x 7.62cm paddles and DAYTON Gearmotors (Grainger, Roanoke, VA). Three magnetic stirrers were inserted in the alcohol containers to ensure homogeneity of the solutions. Mixing was kept at a low speed to avoid loss due to volatilization.

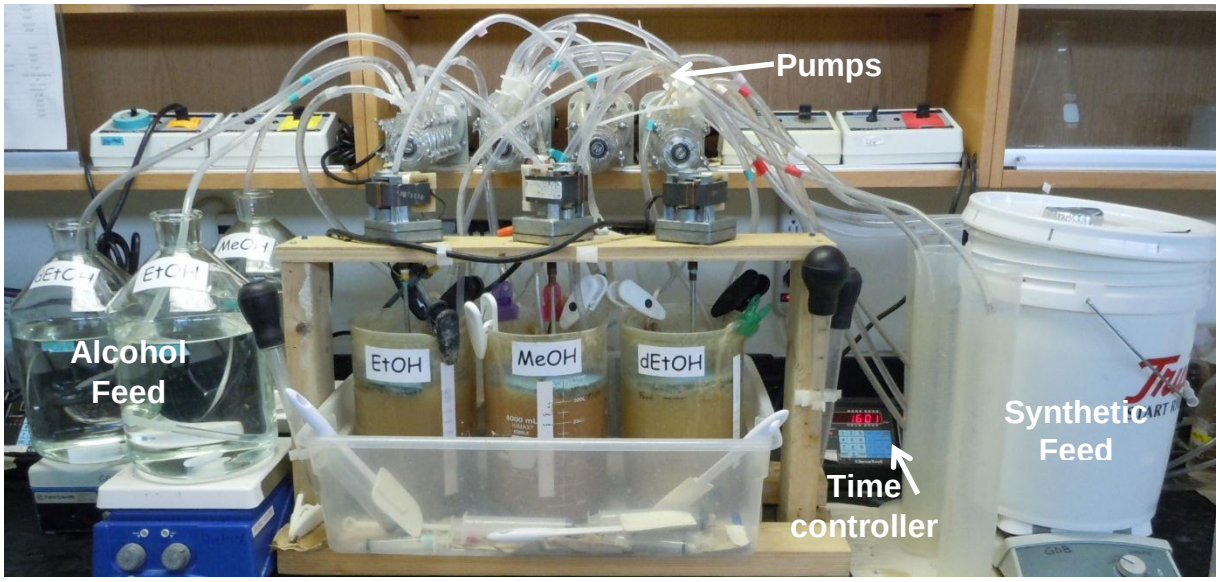


Figure 3.3-Experimental Setup.

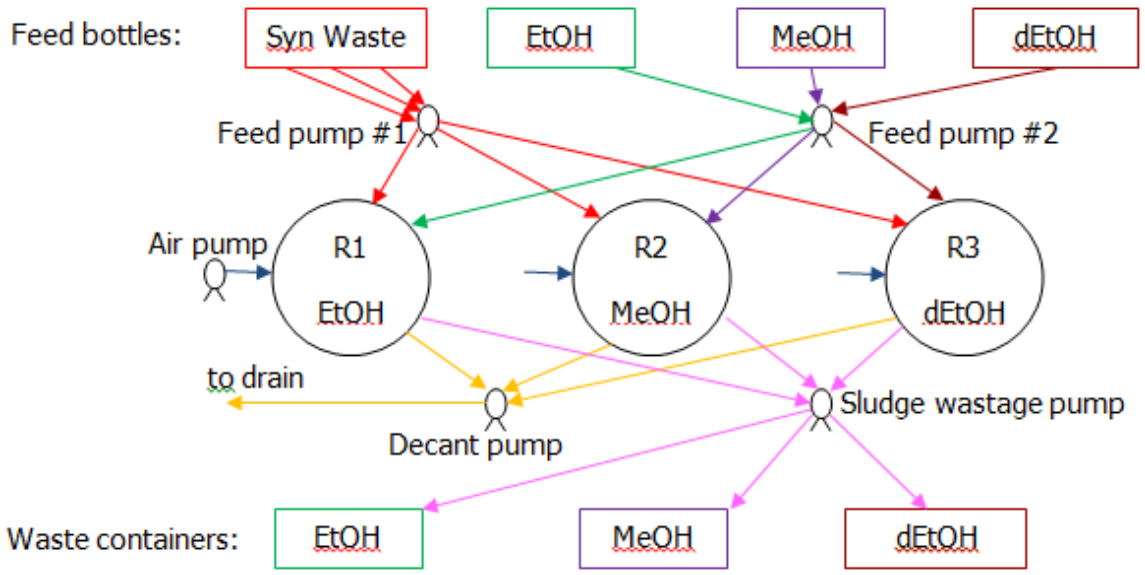


Figure 3.4-System schematic.

The system was fully automated with two time controllers (ChronTrol Corporation, San Diego, CA). Their 24-hr program is shown in Table 3.1.

Table 3.1-Automated Phase Cycles[4].

		CYCLE #	ON	OFF
CONTROL BOX 1	**Alcohol feed pump** Run Time per cycle = 5 min Volume transferred per cycle = 0.75 L	1	07:30	07:35
		2	13:30	13:35
		3	19:30	19:35
		4	01:30	01:35
	Synthetic wastewater feed pump Run Time per cycle = 5 min Volume transferred per cycle = 0.75 L	1	07:30	07:35
		2	13:30	13:35
		3	19:30	19:35
		4	01:30	01:35
	Stirrers Run Time per cycle = 305 min	1	07:30	12:35
		2	13:30	18:35
		3	19:30	00:35
		4	01:30	06:35
	Feed Stirrers Run Time per cycle = 1 min	1	07:29	07:30
		2	13.29	13.30
		3	19.29	13.30
		4	01:29	01:30
CONTROL BOX 2	**Decant pump** Run Time per cycle = 9 min Volume transferred per cycle = 1.45 L	1	13:20	13:29
		2	19:20	19:29
		3	01:20	01:29
		4	07:20	07:29
	Wastage pump Run Time per cycle = 2 min Volume transferred per cycle = 50 ml	1	12:20	12:22
		2	18:20	18:22
		3	00:20	00:22
		4	06:20	06:22
	Air pump Run time per cycle = 210 min	1	09:05	12:35
		2	15:05	18:35
		3	21:05	00:35
		4	03:05	06:35

[4] Table format adopted from Maharajh (2010).

Figure 3.5 illustrates the sequence schedule of a cycle. Nitrification and denitrification were achieved by turning on and off the air supply. All reactors were operated with four 6-hr cycles. Each cycle included a 5-min feed period, a 90-min

anoxic period, a 210-min aerobic period, and 55 min for settle, decant and idle periods. For decanting, a J-tube was placed at mid-depth to avoid disturbing the settled sludge. The treated effluent flowed into the drain. By aerating the system after denitrification, gases produced by denitrification were purged and thus, no rising sludge occurred (Leung and Tam, 1994). The maximum and minimum volumes of the reactors were 3.0 and 1.5 L, respectively, resulting in 1.5 L being decanted per cycle. This maintained the HRT at 12 hr.

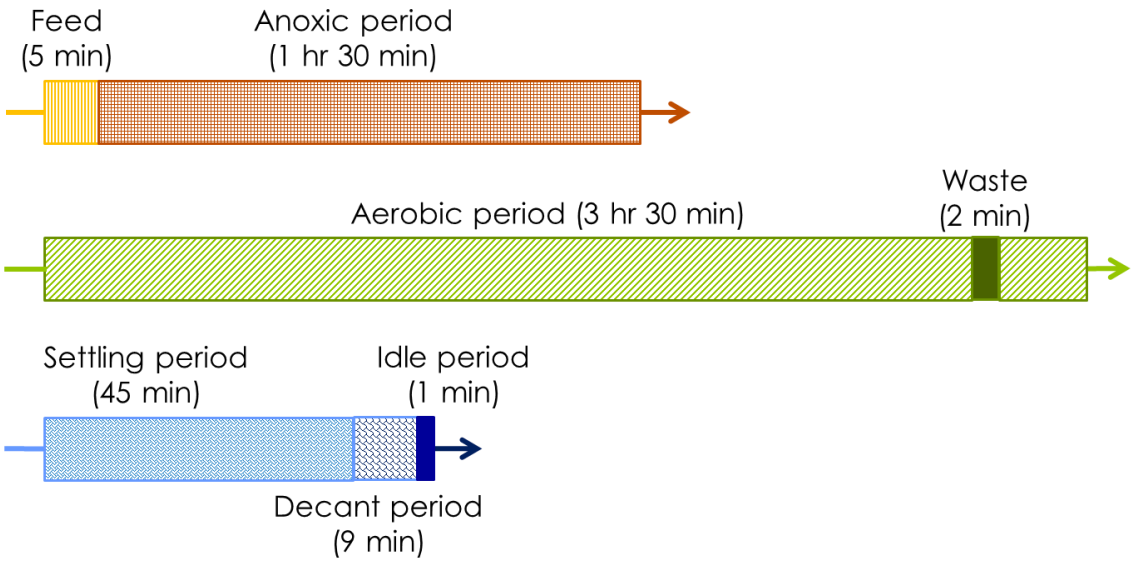


Figure 3.5-Sequence schedule.

All reactors were operated at an ambient room temperature between 21 and 23°C. The pH was maintained in the desired ranges for both nitrification (7.5-8.0) and denitrification (7.0-8.0).

The solids retention time (SRT) was maintained at about 12 d by wasting and returning the proper amount of mixed liquor. Wasting occurred at the end of the aerobic phase. Effluent total suspended solids, volume removed for sample analysis and variations of the waste pump flows were taken into consideration for SRT correction. The formula used for SRT correction is given in the following equation (WEF, 2007):

$$SRT = \frac{V \cdot X}{(Q_w + Q_s) \cdot X + (Q - Q_w) \cdot X_e} \quad (12)$$

where,

SRT = solids retention time, days

V = reactor's volume, L

X = mixed liquor suspended solids, mg/L

Q_w = flow rate of liquid containing microorganisms to be wasted, L/day

Q_s = flow rate of liquid containing microorganisms to be sampled, L/day

Q = wastewater flow rate into the reactor, L/day

X_e = effluent suspended solids, mg/L

Sludge was returned every two or three days. During sampling, wastage pumps were turned off to compensate for the volume of samples removed. The additional volume was returned to each reactor from the respective waste container.

Regarding the system's maintenance, this included dismantling of the pumps and rinsing of the tubing with bleach once per week, scraping the reactor walls with a spatula several times per day, and rinsing the feed containers with bleach, whenever feed was prepared (every day for alcohol containers, every two days for synthetic wastewater container).

3.2 Reactor Feed

The reactors were all seeded with 1.5 L of returned activated sludge (RAS) from the Blacksburg/VPI Municipal Wastewater Treatment Plant. The reactors were seeded twice, due to the washout of nitrifiers. Synthetic domestic wastewater was then used to feed the reactors at a rate of 150 mL/min. The synthetic feed was comprised of bactopeptone (Spectrum Chemical Mfg. Corp, New Brunswick, NJ), an enzymatic digest of animal protein, and salts, whose concentrations are given in Table 3.2.

Table 3.2-Cation influent concentration.

Cation	Concentration (meq/L or mg/L)	Compound used	Desired influent concentration (mg/L)
Ca^{2+}	1.8 meq/L	$CaCl_2.2H_2O$	132
	0.7 meq/L	CaO	20
Mg^{2+}	2 meq/L	$MgSO_4.7H_2O$	246
K^+	0.75 meq/L	KH_2PO_4	102
	0.75 meq/L	K_2HPO_4	130
Na^+	5 meq/L	$NaHCO_3$	420
	0.5 meq/L	Na_2CO_3	134
Fe^{3+}	6 mg/L	$FeCl_3$	17
Al^{3+}	3 mg/L	$Al_2(SO_4)_3.18H_2O$	37

Cation concentrations in the feed were taken from Maharajh (2010) and adjusted accordingly for the particular experiment. The monovalent to divalent (M:D) ratio was maintained at approximately 3:2. This ratio was within the acceptable range suggested by Higgins and Novak (1997) to positively affect floc formation and settling properties.

Inorganic nitrogen of about 230 mg/l was added in the form of NH_4Cl. Neither NO_3^--N nor NO_2^--N were detected in the feed solution. All compounds were diluted in tap water. No additional micronutrients were provided, because it was assumed that the necessary quantity for the bacteria to grow could be found in the tap water. With the addition of buffer solutions, the pH was maintained at 8. The synthetic wastewater was prepared every two days.

Bactopeptone only contributed 25% of the influent sCOD. The remaining 75% was provided by different carbon sources: ethanol (mixture of 95.5% ethanol and 4.5% water-EtOH) bought by Ricca Chemical Company, Arlington, TX, laboratory grade methanol (MeOH) (Fisher Scientific, Pittsburg, PA) and denatured ethanol (dEtOH) (Allied Terminals, Norfolk, VA). Denatured ethanol was a product of straight-run gasoline addition to ethanol. More information about its composition will be given in the Results and Discussion section of Chapter 5. The total influent soluble chemical oxygen

demand (COD) concentration for the EtOH, MeOH and dEtOH reactors was 113±49 mg/L, 162±58 mg/L and 95±35 mg/L, respectively. Alcohol feeds were prepared every day.

3.3 Collection of samples

Sampling from one SBR cycle was performed every other day initially, and then every three days during the monitoring period of the study. Profile sampling was conducted every two days. Samples were taken every 3 minutes during the first 20 minutes of the anoxic period. A more detailed description of the profile sampling intervals is given in Table 3.3. Actually, samples were taken before COD or NO_3^--N were depleted (COD and NO_3^--N concentration: 10-20 mg/L and 1-2 mg/L, respectively). Due to the lower nitrification rates, samples were collected every 15 minutes after aeration began to ensure that DO concentration was higher than 2 mg/L. Samples were collected using a 60-ml plastic syringe, filtered immediately using 25 mm syringe filters and analyzed within a 6-hr period. They were kept refrigerated at 4°C until analyzed. Samples (35-mL aliquots) were taken from each reactor during the anoxic and aerobic periods. An additional 10 mL was withdrawn from the dEtOH reactor, to be used for BTEX analysis. Samples (0.5 L) from each reactor's treated effluent was retained for the duplicate analysis of TSS and VSS.

Table 3.3-Profile sampling schedule.

	sCOD	NH_4-N	NO_3-N	NO_2-N	BTEX	TSS	VSS	pH	DO	T
Feed (5 min)										
7:30	X	X	-	-	X	-	-		-	-
Anoxic (90 min)										
7:35	X	X	X	X	X	-	-	-	-	-
7:38	X	X	X	X	X	-	-	-	-	-
7:41	X	X	X	X	X	-	-	-	-	-
7:44	X	X	X	X	X	-	-	X	X	X
7:47	X	X	X	X	X	-	-	-	-	-
8:15	X	X	X	X	X	-	-	-	-	-
-	X	X	X	X	X	-	-	-	-	-
8:55	X	X	X	X	X	-	-	X	X	X
Aerobic (210 min)										
9:05	X	X	X	X	X	-	-	-	-	-
9:20	X	X	X	X	X	-	-	X	X	X
9:35	X	X	X	X	X	-	-	-	-	-
9:50	X	X	X	X	X	-	-	-	-	-
-	X	X	X	X	X	-	-	-	-	-
10:20	X	X	X	X	X	-	-	-	-	-
-	X	X	X	X	X	-	-	-	-	-
11:50	X	X	X	X	X	X	X	X	X	X
Settling (45 min)										
13:05	X	X	X	X	X	-	-	-	-	-
Decanting (9 min)	-	-	-	-	-	X	X	-	-	-
Idle (1 min)	-	-	-	-	-	-	-	-	-	-

3.4 Steady state determination

Steady state was visually determined by observing the concentration of effluent ammonia. From Figure 3.6 it can be concluded that steady state was reached between 110 and 125 days. Usually the system is stabilized in a period equal to three times the SRT. Therefore, the expected day for steady state determination would approximately be 36 days. The same was applied here, but it is not obvious from Figure 3.6, because

the particular figure includes the two times that the reactors had to be reseeded due to nitrifiers wash out.

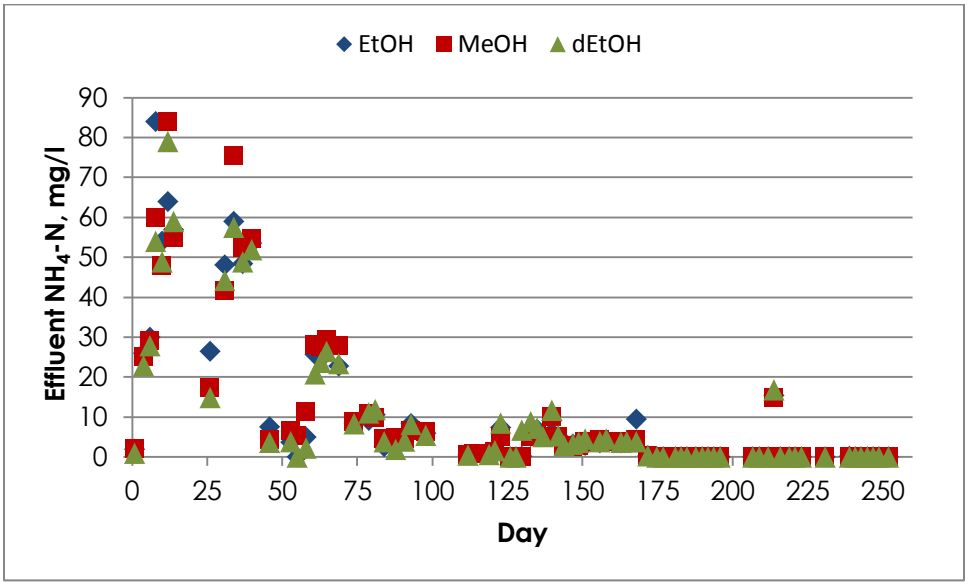


Figure 3.6-Steady state determination.

3.5 Analytical Methods

Routine analyses included sCOD (mg/L), ammonium (mg/L as N), nitrate (mg/L as N), nitrite (mg/L as N), MLSS (mg/L), MLVSS (mg/L), TSS (mg/L), and VSS (mg/L). Gas chromatography/mass spectrometry was employed for the determination of the denatured ethanol's composition, whereas gas chromatography with a photoionization detector was used for the quantification of BTEX concentration in the dEtOH reactor. Samples taken from the feed bottles of EtOH and MeOH were also analyzed for BTEX, but none of the BTEX compounds was detected. The sludge volumetric index (SVI) was also measured at regular intervals throughout the reactors' operation, according to Methods 2710C and D set forth by Standard Methods (APHA, 2005). Alkalinity was determined by the procedure described by Method 2320B (APHA, 2005). For monitoring purposes pH, DO and T were measured every two or three days using handheld probes. More specifically, pH was measured using an Oakton pH 110 Series meter with an Accumet electrode four times during the 6-hr cycle; within the first 10 min of the anoxic and aerobic periods, and 10 min before the end of each period. Temperature and DO were measured using a model 85 YSI meter and probe. Total solids in the mixed liquor and effluent were determined according to Method 2540D, whereas volatile

suspended solids according to Method 2540E (APHA, 2005). In the following paragraphs, the methods applied for the determination of COD, NH_4^+-N, NO_3^--N, NO_2^--N are described. All methods are colorimetric and incorporate the use of a HACH DR2800 spectrophotometer (Hach Company, Loveland, Colorado).

3.5.1 Chemical Oxygen Demand (COD)

Chemical oxygen demand was analyzed using Hach Test N' Tube COD reagent sets (Hach Method 8000). Method 8000 is USEPA approved for wastewater analyses and corresponds to Standard Method 5220B (APHA, 2005).

3.5.2 Ammonia-nitrogen (NH_3-N)

Ammonia-nitrogen was measured using Hach Test N' Tube Reagent sets. Two methods were employed; Method 10023 for low concentrations (0.02-2.50 mg/L) and Method 10031 for high concentrations (0.4-50.0 mg/L). Both techniques use the salicylate method for the determination of ammonia-nitrogen. The method is based on ammonia compounds combining with chlorine to form monochloramine. Monochloramine reacts with salicylate to form 5-aminosalicylate. The 5-aminosalicylate is oxidized in the presence of a sodium nitroprusside catalyst to form a blue colored compound. The blue color is masked by the yellow color from the excess reagent present to give a green-colored solution.

3.5.3 Nitrite-nitrogen (NO_2^--N)

Due to the great variability in the range of nitrite detected in the mixed liquor and the effluent, three methods were applied for their determination. First, Test N' Tube NitriVer3 Nitrite Reagent sets (Hach Method 8507-USEPA approved) were used for the detection of low levels of nitrite-nitrogen (0.002-0.300 mg/L NO_2^--N). The procedure follows the diazotization method where nitrite in the sample reacts with sulfanilic acid to form an intermediate diazonium salt. This couples with chromotropic acid to produce a pink colored complex directly proportional to the amount of nitrite present.

For higher concentrations of nitrite-nitrogen methods TNT839 (0.015-0.600 mg/L NO_2^--N) and TNT840 (0.6-6.0 mg/L NO_2^--N) were used. Methods TNT839 and TNT840 are equivalent to EPA Method 353.2. In these methods, nitrite in the sample reacts with

a primary aromatic amine in acidic solution to form a diazonium salt. This couples with an aromatic compound to form a colored complex that is directly proportional to the amount of nitrite present.

3.5.4 Nitrate-nitrogen (NO_3^--N)

Nitrate-nitrogen was analyzed by means of a DIONEX DX-120 ion chromatograph (IC) with a AS-9HC column (DIONEX Corp., Sunnyvale, CA), according to method 4110B (APHA, 2005). The sample is injected into a stream of eluent (9.0 mM Na_2CO_3) and passes through a series of ion exchangers. Nitrate is separated based on its relative affinity for a low-capacity, strongly basic anion exchange resin. Then, nitrate is directed through a suppressor, which provides continuous suppression of eluent conductivity and enhances analyte response. In the suppressor, the separated anions are converted to their highly conductive acid forms, while the conductivity of the eluent is decreased. Anions are measured by conductivity and are quantified based on the peak areas. External anions standards were used to check the accuracy of the results.

3.5.5 Gas Chromatography/Mass Spectrometry (GC/MS)

The composition of denatured ethanol was analyzed with a FOCUS gas chromatograph (GC) equipped with a DSQII mass spectrometer (MS), and an AS-3000 autosampler (Thermo Scientific, West Palm Beach, FL). Gas chromatography offers excellent separation, whereas a mass spectrometer can detect a wide variety of compounds and give qualitative information about the type of molecules present in the solution.

The procedure is as follows: the sample (1 µL) is vaporized and carried by helium through a column coated with a stationary phase where separation takes place. As the separated sample component molecules elute from the column into the MS, they are bombarded with energy. This causes them to lose an electron and form ions with a positive charge. Fragmentation of the molecules occurs during the process. The masses of each of the ionized fragments are recorded and give the unique fingerprint of a molecule (mass spectra) that is used for its identification.

3.5.6 Benzene-Toluene-Ethylbenzene-Xylenes (BTEX)

Standard Method 6200C, a purge and trap capillary column GC method was used for the quantification of BTEX compounds. For the particular analyses, a purge and trap concentrator (Tekmar-Dohrmann, Cincinnati, OH) and a gas chromatograph (Tremetrics, Austin, TX) equipped with a photoionization detector were used. BTEX analytical external standards were made using methanol as solvent.

Helium is bubbled through the water sample (5 mL) at ambient temperature for 11 minutes. The volatile organic compounds are transferred from aqueous to gaseous phase. The vapor passes through a trap, which contains absorbent materials. Volatile compounds are retained on the trap. After purging is complete, the trap is heated to the desorb temperature (250°C) and then held at this temperature for two minutes to thermally desorb the analytes into the carrier gas (helium). The trap is back-flashed with helium and the volatile compounds are carried to the GC by a heated transfer line (100°C). The organic compounds travel through the GC column at different rates and are separated depending on differences in partition coefficients between the mobile (helium) and stationary (capillary column) phases by the photoionization detector (PID).

3.5.7 Statistical analysis

For the statistical analysis of data, the statistical software packages, R (Version 2.14.0) and JMP 9.0.0 were employed. The significance level, α, was globally set equal to 0.05. An overview of the statistical techniques used in this study follows.

The validity of conclusions drawn from any statistical analysis depends on the validity of the underlying assumptions. The basic assumptions for the statistical methods applied to this study were with respect to population independency, normality and equal variance. Initially, data were examined to identify possible violations from these prerequisites. Independence of data across cycles and treatments (carbon sources) was established from the way sampling was performed. Normality was checked by the Shapiro-Wilk normality test, with the null hypothesis being that the population means were normally distributed. Equal variance and Bartlett tests were performed to check the homogeneity of variances between two and three populations, respectively. Depending on the results of the analyses mentioned before, the appropriate statistical tests were applied. More specifically, to detect the significance of

the difference between the means of two normally distributed sampling populations with equal variances, t-test was selected. ANOVA compared the means between three sampling populations, with normal distribution and equal variances. The non-parametric Kruskal-Wallis rank sum test was used instead of ANOVA, in cases where the assumption of normally distributed data was not met. ANOVA and Kruskal-Wallis test provided generic information on whether the tested means were different or not. To further identify exactly which means were significantly different from each other, Tukey's HSD (Honestly Significant Difference) test was used. Finally, for the cases that the assumption of equal variance did not hold, the one-way analysis of means test was performed.

Although every observation was considered independent from each other, across cycles and treatments, those within one cycle for a specific treatment were correlated. Thus, a linear mixed model was applied. The general equation that describes the model is given below:

$$y_{ijk} = \beta_0 + \gamma_{ij} + \beta_1 \cdot x_{ijk} + \beta_2\, x_{ijk} \cdot I(i,2) + \beta_3 \cdot x_{ijk} \cdot I(I,3) + \varepsilon_{ijk} \quad (13)$$

where,

y_{ijk} = response variable, (i = 1, 2, 3, the 3 treatment types; j = 1, …, 6, the 6 cycle replicates for each treatment type; k = 1, 2, …, the index of observations from a particular cycle)

x_{ijk} = regressor

β_0 = intercept

β_1 = coefficient of regressor for type 1

β_2 = coefficient differences from β_1 for type 2

β_3 = coefficient differences from β_1 for type 3

γ_{ij} = random effect (adjusts the intercept for each treatment*cycle combination and accounts for the correlation among observations within each combination)

ε_{ijk} = random error

I(i, α') = indicator function ($\begin{cases} 1, if\ i = \alpha' \\ 0, if\ i \neq a' \end{cases}$

or more specifically,

$$y_{1jk} = \beta_0 + \gamma_{1j} + \beta_1 \cdot x_{1jk} + \varepsilon_{1jk} \quad (14)$$

$$y_{2jk} = \beta_0 + \gamma_{2j} + (\beta_1+\beta_2)\cdot x_{2jk} + \varepsilon_{2jk} \quad (15)$$

$$y_{3jk} = \beta_0 + \gamma_{3j} + (\beta_1+\beta_3)\cdot x_{3jk} + \varepsilon_{3jk} \quad (16)$$

Statistical tests determined the intercept and coefficient differences.

3.6 References

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4 Evaluation of gasoline-denatured ethanol as an alternative carbon source for wastewater denitrification

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ABSTRACT: In this study, the performance of three carbon sources, methanol, ethanol and gasoline-denatured ethanol, was compared and evaluated on the basis of treatment efficiency and cost per amount of denitrification. To accomplish the research objectives, three 4-L glass beakers were operated as sequencing batch reactors (SBRs) at the same SRT of 12.0±0.9 days. Methanol and ethanol (95.5% ethanol, 4.5% water) were used in control reactors. Denitrification rates with denatured ethanol were similar to those of ethanol (14.5±0.6 and 14.9±2.4 mg NO_3^-N/L·h, respectively), and higher than those of methanol (8.9±0.9 mg NO_3^-N/L·h). The denaturant affected neither biomass production, nor nitrogen removal processes. In addition, no BTEX was detected in the effluent. The cost of dEtOH ($0.91//lb NO_3^--N removed) is slightly higher than that of methanol ($0.74/lb NO_3^--N removed). Using dEtOH as an external carbon source is, therefore, very promising and utilities will have to decide if it is worth paying a little extra to take advantage of dEtOH's benefits.

KEYWORDS: Denitrification, alternative carbon source, ethanol, methanol, SBRs

4.1 Introduction

High nitrogen concentrations within the final effluent of an activated sludge process can adversely affect the quality of receiving water bodies. The major pollution concerns are eutrophication, hypoxia caused by ammonia nitrification, ammonia toxicity to fish and other aquatic organisms and methemoglobinemia, which is related to the presence of nitrate in potable water (Metcalf and Eddy, 2003). Among the most affected regions worldwide is the Chesapeake Bay, the largest estuary in the U.S.A. In order to protect the Bay, regulatory agencies and organizations have worked to establish lower total nitrogen and phosphorus effluent standards for wastewater treatment plants (WWTP) (3.0 mg/L and 0.3 mg/L, respectively) (HRSD, 2011).

This study focuses on nitrogen removal, which can be accomplished by the combination of two biological processes: nitrification and denitrification. The former includes the oxidation of ammonia-nitrogen to nitrate, whereas the latter the reduction of nitrate to nitrogen gas (N_2). Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) carry out nitrification. These bacteria are sensitive to several environmental and non-environmental factors, such as pH, dissolved oxygen (DO), temperature, solids retention time (SRT) and toxic compounds (Painter, 1986; Metcalf and Eddy, 2003).

For complete nitrogen removal, nitrification is always accompanied by denitrification. Denitrification is a microbial respiratory process, where nitrate is reduced to nitrogen gas, which is inert and causes no harm to the environment. It takes place through a sequence of steps and involves different enzymes for its catalysis (Metcalf and Eddy, 2003). An electron donor (carbon source) and an electron acceptor (nitrate) are necessary for denitrification to occur. Energy produced by the electron transfer is used for cell synthesis and growth. The required denitrifiers are heterotrophic, autotrophic and photolithotrophic prokaryotes (Zumft, 1991; Matějů et al., 1992). Denitrification is inhibited by the presence of DO and low pH values (Focht and Chang, 1975). Toxic compounds, such as benzene, toluene, ethylbenzene and xylenes (BTEX) might also adversely affect denitrification.

In post-denitrification processes, an external carbon source is supplied to ensure that the system is not carbon-limited (Abufayed and Schroeder, 1986). The addition of

external carbon is beneficial, because it increases the fraction of readily biodegradable compounds and the carbon to nitrogen ratio (C/N). Consequently, lower hydraulic retention time (HRT) and minimum anoxic volumes are required. High C/N ratios have been reported to enhance denitrification rates (Kang et al., 1992; Trela, 1998; Albanez et al., 2009).

Examples of electron donors studied so far include methanol, ethanol, acetate, glycerol, primary sludge, acetic acid, and hydrolyzed starch (Abufayed and Schroeder, 1986; Lee and Welander, 1996; Onnis-Hayden, 2008; Ginige et al., 2009; Swinarski et al., 2009; Adav et al., 2010). For the purposes of this study, methanol, ethanol, and gasoline-denatured ethanol were compared.

Methanol has been tested and used extensively during the last twenty years as an electron donor for denitrification in both lab- and full-scale experiments, due to its numerous advantages (Nyberg et al., 1992; Onnis-Hayden and Gu, 2008). More specifically, it is cheaper than other suggested carbon sources, commercially available and widely used, offering a wide range of performance data that can be employed for system optimization. Another advantage that results from its widespread use is the fact that it is thoroughly regulated. In addition, it is readily biodegradable and has low sludge yield. Moreover, the carbon oxidation is almost complete resulting in low effluent COD concentrations. It is not considered a toxic chemical to fish life, given that fish can tolerate concentrations over 100 mg/L. Finally, it does not contain any phosphorus or nitrogen that could aggravate the influent nutrient load (McCarty et al., 1969; Hallin et al., 2006; Mokhayeri et al., 2008b; Onnis-Hayden and Gu, 2008; Swinarski et al., 2009).

On the other hand, methanol has some important disadvantages. First of all, its specific denitrification rates (SDNRs) are lower than those of other electron donors. Methanol is a single-carbon compound, not easily entering the tricarboxylic acid (TCA) cycle, unless it is reduced to form higher carbon intermediates (3-C and 4-C) (Mokhayeri et al., 2008a). In addition, it requires longer acclimation and recovery periods. It has been demonstrated that when methanol is used as an external carbon source a new population of methylotrophic bacteria with a slower growth rate is established (Hallin et al., 1996; Hallin et al., 2006; Swinarski et al., 2009). These kinds of bacteria are facultative organisms, which belong to the *Paracoccus* and

Hyphomicrobium genera (Claus and Kutzner, 1985; Onnis-Hayden and Gu, 2008). In addition, according to Hallin and Pell (1998) methylotrophs inhibit the growth of other denitrifying bacteria present in activated sludge. Therefore, substitution of methanol with other carbon sources may result in loss of denitrification. Moreover, it is produced from the methane present in natural gas and inevitably, its price is linked to that of fossil fuels. According to the National Fire Protection Association (NFPA) methanol is classified as flammable. Hence, specific measures should be taken while transported or stored. Furthermore, it is classified as poisonous and toxic according to European Union legislation, because it affects the central nervous system. McCarty et al. (1969) reported that 100-250 mL is considered toxic to humans. Finally, it was demonstrated by Mokhayeri et al. (2008a) and Dold et al. (2008) that during winter (at cold temperatures), methylotrophs' growth rate is low; therefore, methanol addition does not have the desired effect on nitrogen removal.

Trying to overcome methanol's disadvantages, researchers have considered the use of ethanol, which appears to be a better carbon source. SDNRs are two to three times higher compared to methanol's (Trela, 1998; Ginige et al., 2009). Microbes quickly adapt to ethanol within a few hours (Trela, 1998) or one sludge age, according to Hallin et al. (1996). Moreover, denitrifiers grown with ethanol are able to use other carbon sources, such as acetate, propionate, butyrate and methanol without losing their capacity to denitrify (Hallin and Pell, 1998). Thus, utilities can interchange carbon sources without affecting the performance of the plant. Moreover, ethanol is commercially available. It can be found in many industrial wastes; for example, in pharmaceutical industries. It is readily biodegradable by many microorganisms, because it is a low-carbon compound, which easily enters the TCA cycle (Henze, 1994). The demand for ethanol is increasing, leading manufacturing companies to increase their productivity and reduce their selling prices to make ethanol more competitive.

Flammability, toxicity, and strict regulation issues are the main disadvantages hindering the more widespread use of ethanol as a carbon source. Ethanol is heavily taxed and it is difficult to procure without complicated permits from the bureau of alcohol, tobacco, firearms, and explosives (ATF). Like methanol, it is ranked as a flammable chemical by NFPA.

The Hampton Roads Sanitation District (HRSD) is currently interested in the use of ethanol denatured with “straight-run” gasoline as an alternative carbon source for denitrification. In general, ethanol is denatured at the ethanol plant with additives, like acetone and gasoline, to prevent people from consuming it. The gasoline added, in our case, is known as virgin, straight-run, or natural gasoline, and it is blended with ethanol at 2-15%. This mixture is then added to gasoline to make it a cleaner fuel and protect the engine from knocking.

More specifically, “straight-run” gasoline is a lower grade material that contains mostly aliphatic compounds. It lacks the components that normally boost the octane rating, such as benzene, toluene, ethylbenzene and xylenes. In addition, the non-cyclic hydrocarbons contained have the potential to be good carbon sources.

Because this substance is a new entry in the field of wastewater, little is known about its composition and its capacity to improve SDNRs. Possible disadvantages evaluated herein are as follows. First, it is slightly more expensive than methanol. Second, the content of the denaturant (“straight-run” gasoline) and associated hydrocarbons, like BTEX, vary from batch to batch. Third, the degree to which it is biodegraded is unknown. It is possible that a high effluent COD will be observed; an indication that gasoline constituents cannot be biologically degraded. Dold (1989) stated that this problem could be addressed with the addition of hydrogen peroxide (H_2O_2) prior to activated sludge treatment, so that the adsorption of the compounds under investigation is enhanced. Fourth, high concentrations of hydrocarbons in the influent may affect sludge properties (Dold, 1989). Gasoline could possibly accumulate on the flocs forming a hydrophobic layer and thereby affecting settling characteristics. In addition, the effect of the denaturant on WWTP is undetermined. Accumulation of gasoline constituents, like the BTEX compounds, in a WWTP may have an undesirable impact on the performance of the biological process. Lastly, it is possible that the sludge will contain high amounts of gasoline constituents, which require special techniques for sludge treatment and disposal.

In summary, the issue of alternative carbon sources for nitrogen removal is ranked among the highest priorities in the area of nutrient removal. This study is in line with this

need. Herein are presented the results of the comparison of three carbon sources on the denitrification capability of three lab-scale sequencing batch reactors (SBRs).

The overall objectives of this research were: 1) to compare nitrification and denitrification rates for organisms grown using different external carbon sources; 2) to compare the cost of the substrates under study, and 3) to determine if the BTEX compounds inhibit nitrification and denitrification.

4.2 Materials and Methods

4.2.1 Experimental setup

The experiment was conducted in three 4-L SBRs, which were used to mimic the Modified Ludzack-Ettinger process (MLE). The maximum and minimum volumes of the reactors were 3.0 and 1.5 L, respectively, resulting to 1.5 L being decanted per cycle. This corresponded to a 12-hr hydraulic retention time (HRT). The solids retention time (SRT) was maintained at 12±0.9 days by wasting and returning the proper amount of mixed liquor. Effluent total suspended solids (TSS), liquid removed for sample analysis and variations of the waste pump flows were taken into consideration for SRT correction. Figure 4.1 shows an image of the physical setup.

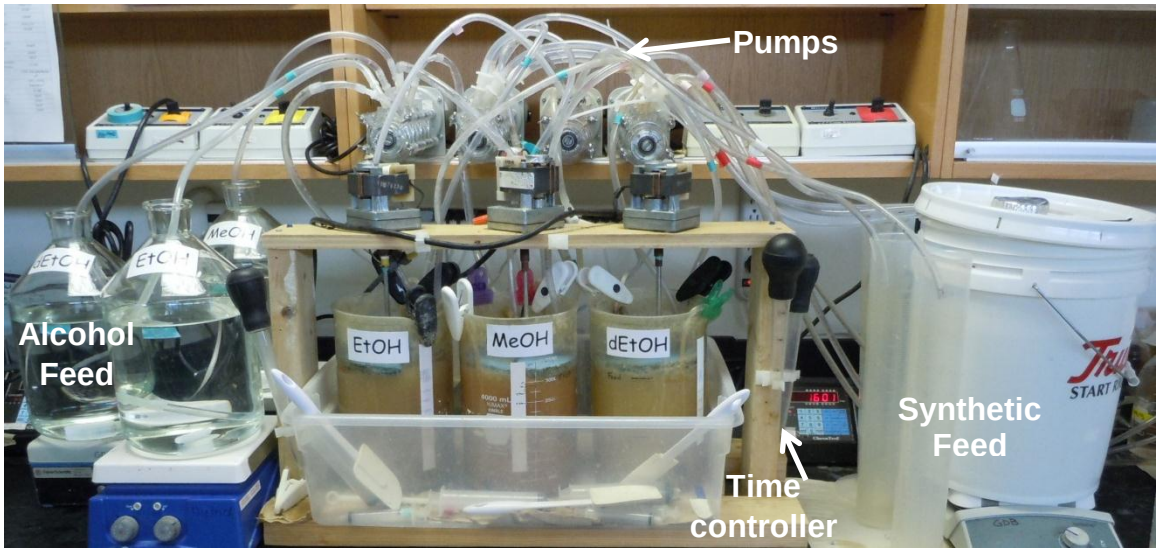
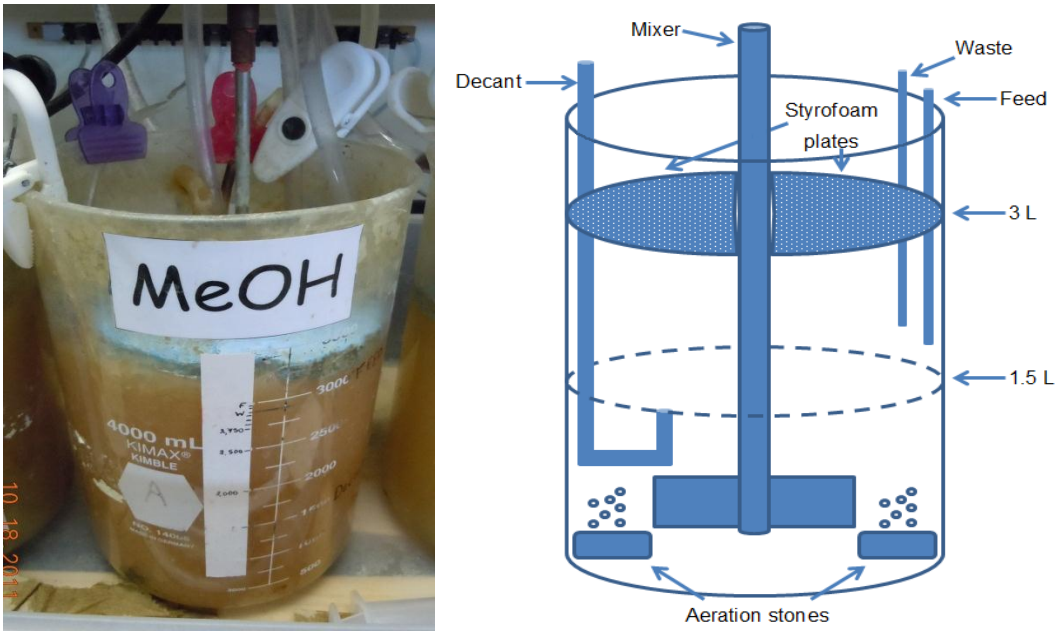


Figure 4.1-Experimental setup.

The system consisted of three 4-L cylindrical glass beakers, which served as bioreactors, three 5-L glass bottles for alcohol feeds, one 19-L plastic bucket for

synthetic wastewater feed, and three 1-L graduated cylinders for the collection of wasted solids. The system was also equipped with four peristaltic pumps (Cole-Parmer, Vernon Hills, IL) for wastewater inflow, solids wastage, and decanting. Reactors were mixed using stainless steel 2.54cm x 7.62cm paddles rotated at 66 rpm by Dayton Gearmotors (Grainger, Roanoke, VA). Three magnetic stirrers were inserted in the alcohol containers to ensure homogeneity of the solution. Mixing was kept at low speed to avoid losses due to volatilization. The system was controlled by two programmable timing units (ChronTrol Corporation, San Diego, CA). A fish pump provided air through aeration stones. The reactors were covered with Styrofoam plates to minimize oxygen intrusion during mixing (Figure 4.2).



(a) (b)

Figure 4.2 - Actual image (a) and schematic (b) of a reactor.

All reactors were operated at room temperature for approximately five months under state conditions. The pH was maintained in the desired ranges to facilitate nitrification (7.5-8.0) and denitrification (7.0-8.0) processes.

4.2.2 Sequence schedule

Both nitrification and denitrification were achieved by turning on and off the air supply. All reactors were operated with four 6-hr cycles. Each cycle consisted of 5 minutes for feeding, 90 minutes of anoxic period (for denitrification), 210 minutes of

aerobic period (for nitrification), and 55 min for settling, decanting and idling. Mixed liquor withdrawal lasted 2 min and occurred at the end of the aerobic phase. Effluent was withdrawn using a J-tube to avoid disturbing the settled sludge and was discharged to the drain. Mixing was turned on at the beginning of the feed period and stopped when aeration ended. By aerating the system after denitrification, gases produced by denitrification were purged and thus, no rising sludge occurred (Leung and Tam, 1994).

4.2.3 Reactor Feed

The reactors were all inoculated with 1.5 L of returned activated sludge (RAS) from the Blacksburg/VPI Municipal Wastewater Treatment Plant. Synthetic wastewater was fed into the reactors at a rate of 150 mL/min containing (in tap water): 188.7 mg/L bactopeptone (Spectrum Chemical Mfg. Corp, New Brunswick, NJ), 132.2 mg/L $CaCl_2.2H_2O$, 8.4 mg/L CaO, 246.3 mg/L $MgSO_4.7H_2O$, 102.0 mg/L KH_2PO_4, 130.6 mg/L KH_2PO_4, 17.4 mg/L $FeCl_3$, 37.0 mg/L $Al_2(SO_4)_3.18H_2O$, 419.8 mg/L $NaHCO_3$, and 133.9 mg/L Na_2CO_3. Inorganic nitrogen of about 230 mg/l was added in the form of NH_4Cl. Neither NO_3^--N nor NO_2^--N were detected in the influent. No additional micronutrients were used, assuming they were present in tap water. Wastewater was prepared every two days.

Bactopeptone provided 25% of the influent soluble chemical oxygen demand (sCOD). The remaining amount was supplemented by the addition of the electron donors: 95.5% ethanol mixed with water (EtOH) (Ricca Chemical Company, Arlington, TX), laboratory grade methanol (MeOH) (Fisher Scientific, Pittsburg, PA) and denatured ethanol (dEtOH) (Allied Terminals, Norfolk, VA). Electron donors were pumped directly into their respective reactors at a rate of 150 mL/min. The total influent sCOD concentration for the EtOH, MeOH and dEtOH reactors was 113±49 mg/L, 162±58 mg/L and 95±35 mg/L, respectively. Alcohol feeds were prepared every day.

4.2.4 Collection and analysis of samples

Sampling from one SBR cycle was performed every other day initially, and then every three days during the monitoring period of the system. Profile sampling was conducted every two days for two weeks, resulting in six sample sessions. During profiling, samples were taken every 3 minutes during the first 20 minutes of the anoxic

period to attain sufficient data for analysis. Because nitrification occurs at a slower rate, samples were collected every 15 minutes after aeration began to ensure that the dissolved oxygen (DO) concentration was sufficient for nitrification to occur. Samples were collected using a 60-ml plastic syringe, filtered immediately using 25 mm syringe filters (0.45 µm filter pore size) and kept refrigerated at 4°C until analyzed. Samples (35-mL) were taken from each reactor during the anoxic and aerobic periods. One half liter (0.5 L) of each reactor's treated effluent was retained for the duplicate analysis of TSS and VSS.

4.2.5 Analytical Methods

All analytical procedures were performed according to Standard methods (APHA, 2005). The parameters measured included nitrate (mg/L as N), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), total suspended solids (TSS), volatile suspended solids (VSS), and the sludge volumetric index (SVI). Nitrate was determined with a DIONEX DX-120 ion chromatograph (IC) with a AS-9HC column (DIONEX Corp., Sunnyvale, CA). The eluent was 9.0 mM sodium carbonate (Na_2CO_3) and the sample volume 5 mL.

The other measured parameters, such as sCOD, ammonium-nitrogen (NH_4^+-N) and nitrite-nitrogen (NO_2^--N) were determined colorimetrically using a HACH DR2800 spectrophotometer (Hach Company, Loveland, CO). sCOD was analyzed using Hach Test N' Tube COD Reagent sets (Method 8000). For the determination of ammonia-nitrogen, two methods were applied: Hach Method 10023 for low concentrations (0.02-2.50 mg N/L) and Hach Method 10031 for higher concentrations (0.4-50.0 mg N/L). Due to the high range of nitrite detected in the mixed liquor, three methods were employed for its analysis. Hach Method 8507 was employed for low levels of nitrite-nitrogen (0.002-0.300 mg/L NO_2-N), while, for higher concentrations the applied methods were TNT839 (0.015-0.600 mg/L NO_2-N) and TNT840 (0.6-6.0 mg/L NO_2-N).

For monitoring purposes, pH, DO and temperature were measured every two or three days using handheld probes. The pH was measured using a pH/mV meter (Fisher Scientific, Pittsburgh, PA) four times during the 6-hr cycle; within the first 10 min and 10

min before the end of the anoxic and aerobic periods, respectively. Temperature and DO concentration were recorded using a YSI DO probe (YSI, Inc., Yellow Springs, OH).

4.2.6 Statistical methods

For the statistical analysis of data, the statistical software packages, R (Version 2.14.0) and JMP 9.0.0 were employed. The significance level, α, was globally set equal to 0.05. An overview of the statistical techniques used in this study follows.

The validity of conclusions drawn from any statistical analysis depends on the validity of the underlying assumptions. The basic assumptions for the statistical methods applied to this study were with respect to population independency, normality and equal variance. Initially, data were examined to identify possible violations from these prerequisites. Independence of data across cycles and treatments (carbon sources) was established from the way sampling was performed. Normality was checked by the Shapiro-Wilk normality test, with the null hypothesis being that the population means were normally distributed. Equal variance and Bartlett tests were performed to check the homogeneity of variances between two and three populations, respectively. Depending on the results of the analyses mentioned before, the appropriate statistical tests were applied. More specifically, to detect the significance of the difference between the means of two normally distributed sampling populations with equal variances, t-test was selected. ANOVA compared the means between three sampling populations, with normal distribution and equal variances. The non-parametric Kruskal-Wallis rank sum test was used instead of ANOVA, in cases where the assumption of normally distributed data was not met. ANOVA and Kruskal-Wallis test provided generic information on whether the tested means were different or not. To further identify exactly which means were significantly different from each other, Tukey's HSD (Honestly Significant Difference) test was used. Finally, for the cases that the assumption of equal variance did not hold, the one-way analysis of means test was performed.

Although every observation was considered independent from each other, across replicates and treatments, those within one replicate for a specific treatment were correlated. Thus, a linear mixed model was applied. The general equation that describes the model is given below:

$$y_{ijk} = \beta_0 + \gamma_{ij} + \beta_1 \cdot x_{ijk} + \beta_2\, x_{ijk} \cdot I(i,2) + \beta_3 \cdot x_{ijk} \cdot I(I,3) + \varepsilon_{ijk} \quad (17)$$

where,

y_{ijk} = response variable, (i = 1, 2, 3, the 3 treatment types; j = 1, …, 6, the 6 cycle replicates for each treatment type; k = 1, 2, …, the index of observations from a particular cycle)

x_{ijk} = regressor

β_0 = intercept

β_1 = coefficient of regressor for type 1

β_2 = coefficient differences from β_1 for type 2

β_3 = coefficient differences from β_1 for type 3

γ_{ij} = random effect (adjusts the intercept for each treatment*cycle combination and accounts for the correlation among observations within each combination)

ε_{ijk} = random error

$I(i, \alpha')$ = indicator function $\begin{cases} 1, if\ i = \alpha' \\ 0, if\ i \neq \alpha' \end{cases}$

or more specifically,

$$y_{1jk} = \beta_0 + \gamma_{1j} + \beta_1 \cdot x_{1jk} + \varepsilon_{1jk} \quad (18)$$

$$y_{2jk} = \beta_0 + \gamma_{2j} + (\beta_1 + \beta_2) \cdot x_{2jk} + \varepsilon_{2jk} \quad (19)$$

$$y_{3jk} = \beta_0 + \gamma_{3j} + (\beta_1 + \beta_3) \cdot x_{3jk} + \varepsilon_{3jk} \quad (20)$$

Statistical tests determined the intercept and coefficient differences.

4.3 Results and Discussion

The adopted methodology to test the research objectives is summarized in the following steps:

- Comparing the amount of biomass produced in each reactor.
- Plotting changes of sCOD, NH_4^+-N, NO_3^--N, and NO_2^--N against time.
- Calculating the amount of ethanol, methanol, and denatured ethanol (expressed as mg COD) consumed per mg NOx-N removed.
- Comparing the cost of external carbon sources per lb NO_3^--N removed.

4.3.1 Biomass production

The average MLVSS/MLSS ratio was approximately 0.7 for all reactors throughout the experimental period. As expected, the amount of biomass present in the two ethanol reactors was similar and higher than that in the methanol reactor (1453±248 mg/L, 1458±255 mg/L and 1213±185 mg/L for EtOH, dEtOH and MeOH reactors, respectively). This was also statistically verified. Initially, an ANOVA test indicated that the means of MLVSS in the reactors were statistically different ($p=3.05x10^{-7}$). Tukey's HSD test for multiple comparisons of means, was selected to determine the relationship between the mean values of MLVSS. Results indicated that MLVSS mean values of EtOH and dEtOH were statistically different from that of MeOH ($p_{EtOH-MeOH} = 6.2x10^{-6}$, $p_{dEtOH-MeOH} = 3.7x10^{-6}$, $p_{dEtOH-EtOH} = 0.99$). Thus, it can be inferred that the presence of BTEX compounds in dEtOH did not affect biomass production. Figure 4.3 presents the results of Tukey's HSD test.

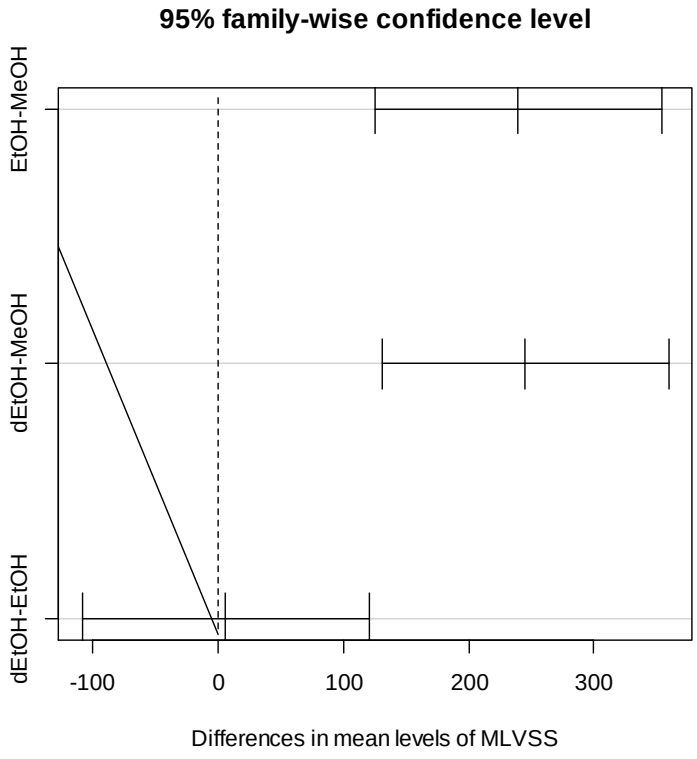


Figure 4.3-Correlation of EtOH, MeOH and dEtOH mean MLVSS values.

The sludge volumetric indices (SVI) were 58±7.8, 57±3.5 and 56±6.1 mL/mg for EtOH, MeOH and dEtOH reactors, respectively, indicating that sludge settling properties were not affected by BTEX.

4.3.2 Effect on denitrification

A representative time series of NO_3^--N concentration during a 6-hr cycle of each of the reactors is shown in Figure 4.4. Denitrification was not affected by the BTEX compounds; on the contrary, it occurred rapidly within the first minutes of the anoxic period (0-12 min). For this reason, data collected during this period were used to test some of the objectives. All profile data refer to steady state conditions.

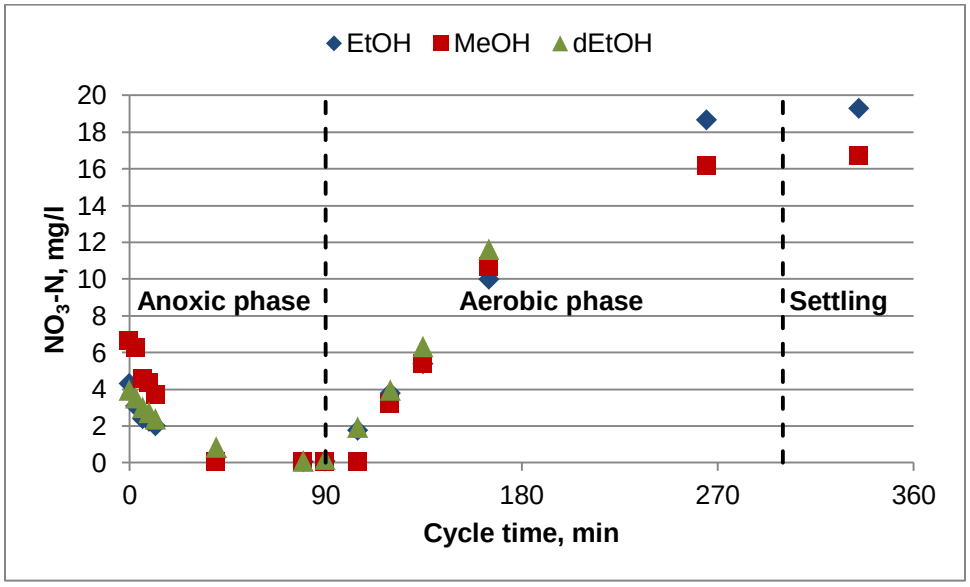


Figure 4.4-Representative NO_3^--N profile.

Figure 4.5 depicts the change of sCOD with cycle time in each reactor. BTEX compounds were not detected in the effluent of the dEtOH reactor.

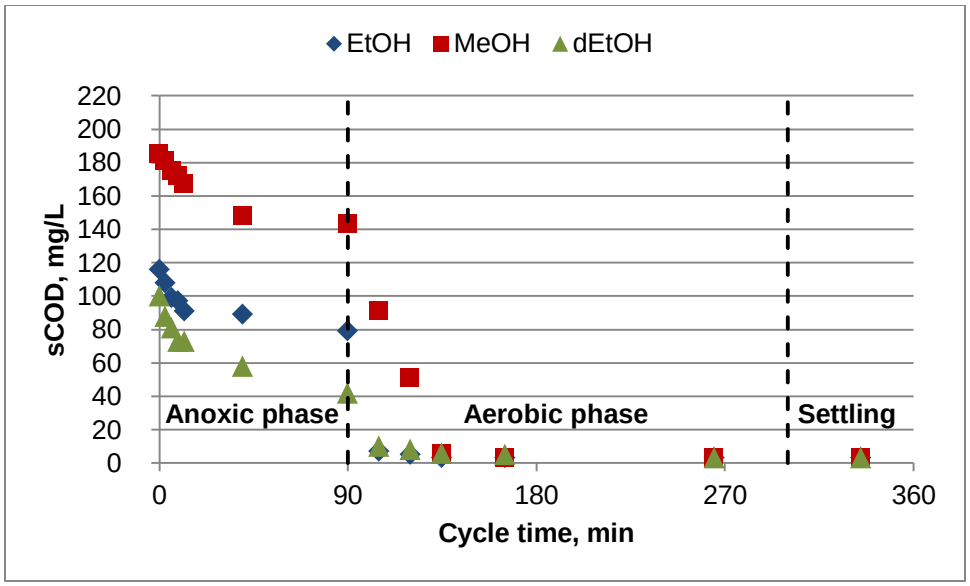


Figure 4.5-Typical sCOD profile.

A linear regression model was fit to the data. More specifically, a mixed model was applied to compare the carbon removal rates during the anoxic period. Figure 4.6 presents the data acquired within the first 12 min of the cycle and the best-fit model plotted against time. The results indicated that the carbon removal rates of EtOH and dEtOH were not significantly different ($p = 0.17$), but both were different from MeOH ($p = 0.0009$). Removal rates (slopes) calculated by the model were 2.31 mg/L·min, 2.00 mg/L·min and 1.55 mg/L·min for dEtOH, EtOH and MeOH, respectively.

Carbon removal efficiency was higher than 90% for all reactors. The change of sCOD during the anoxic phase indicates that the system was not carbon limited in any of the reactors.

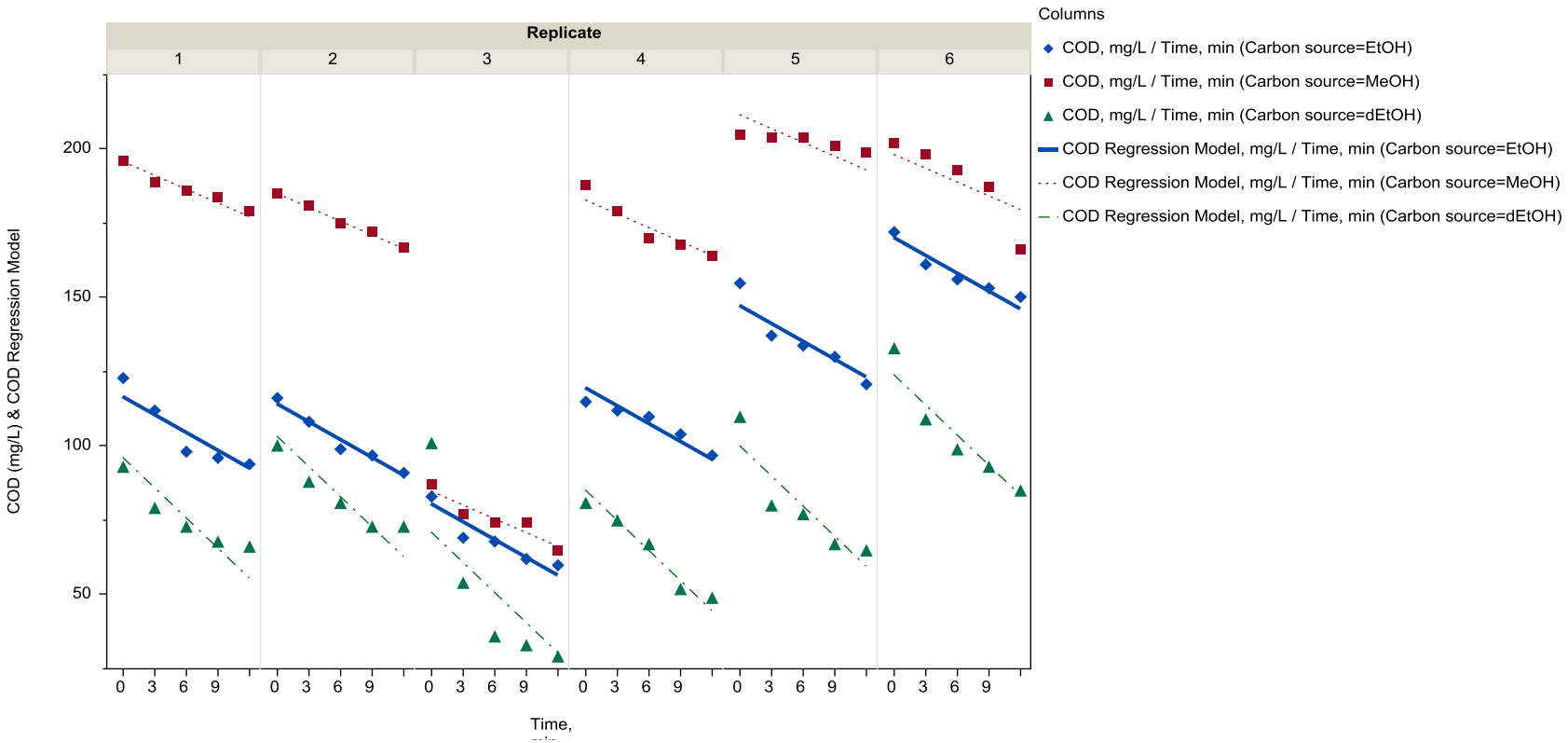


Figure 4.6-COD (mg/L) and COD Regression Model vs. Time (min) by Replicate and Carbon source.

A similar model was employed to quantify the relationship between COD and the oxidized forms of nitrogen (NOx-N) by carbon source and replicate. Although the analysis indicated that EtOH and dEtOH were statistically different from MeOH ($p = 0.0312$), their means were the same ($p = 0.7385$). The amount of COD consumed per NOx-N denitrified was determined by the slopes of lines connecting the regressed data (Figure 4.7). These values were 8.66 mg COD/mg NOx-N, 8.33 mg COD/mg NOx-N and 6.72 mg COD/mg NOx-N for dEtOH, EtOH and MeOH, respectively.

Christensson et al. (1994) studied the denitrification capability of methanol and ethanol in two continuous stirred tank reactors (CSTRs) at 25°C. They reported COD/NO_3^--N ratios equal to 5.81 mg COD/mg NO_3^--N for ethanol and 4.16 mg COD/mg NO_3^--N for methanol, which are comparable to the theoretical values of 4.8 mg methanol as COD required per mg NO_3^--N reduced and of 5.5 mg ethanol as COD required per mg NO_3^--N reduced. Obviously, the values reported here deviate from the above mentioned values. This might be attributed to the high DO concentration in the beginning of the anoxic period. As a result, a lot of carbon was being consumed within this period to remove it. Another possible interpretation for the identified differences might be due to the fact that in this study NOx-N and not NO_3^--N was used.

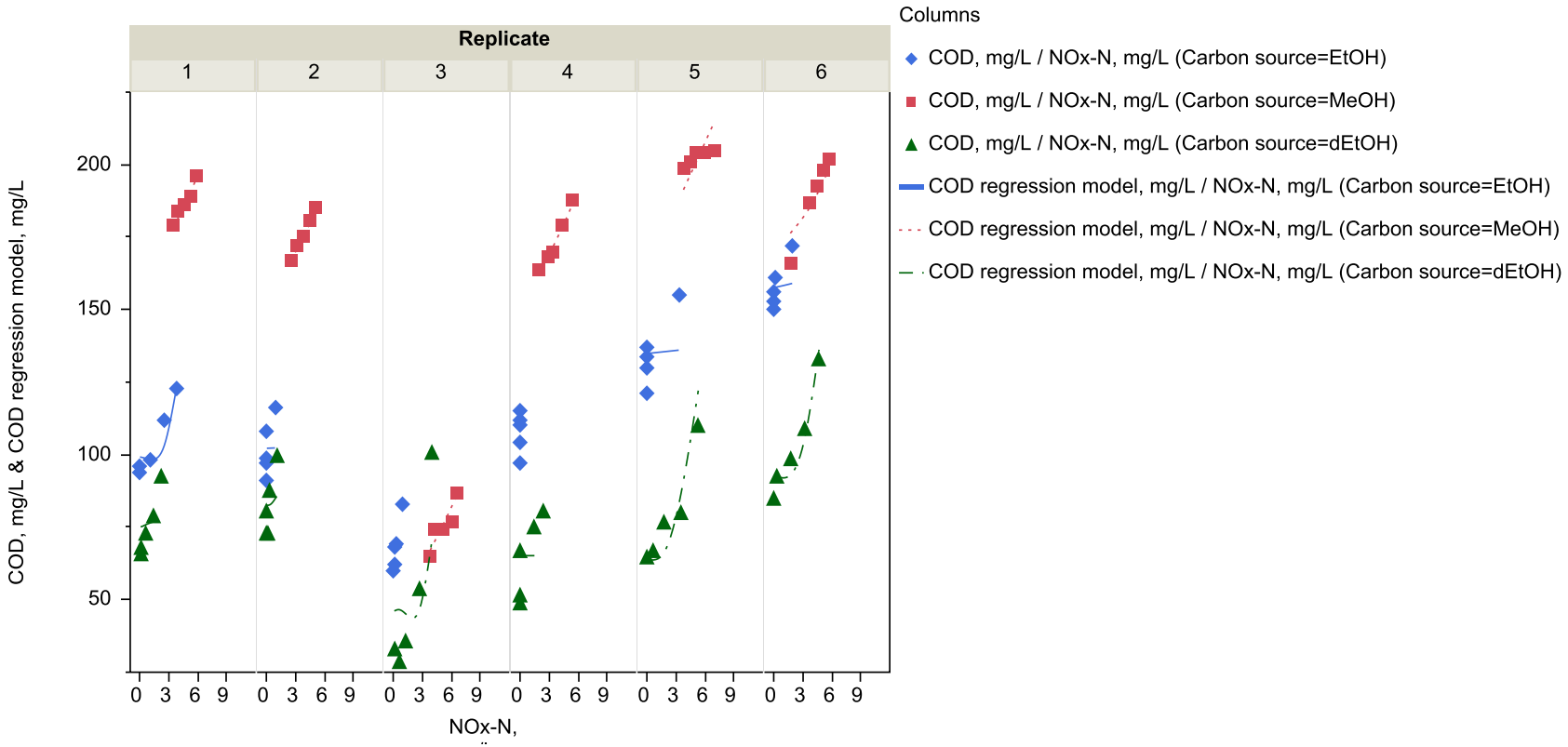


Figure 4.7-COD (mg/L) and COD regression model vs. NOx-N (mg/L) by Replicate & Carbon source within 12 min in the anoxic phase.

NO_3-N and COD were plotted with time, as shown in Figure 4.8. As mentioned before, in neither case was the system carbon limited. In addition, NO_3-N concentration was very low at the end of the anoxic period. Consequently, the system was efficiently denitrified.

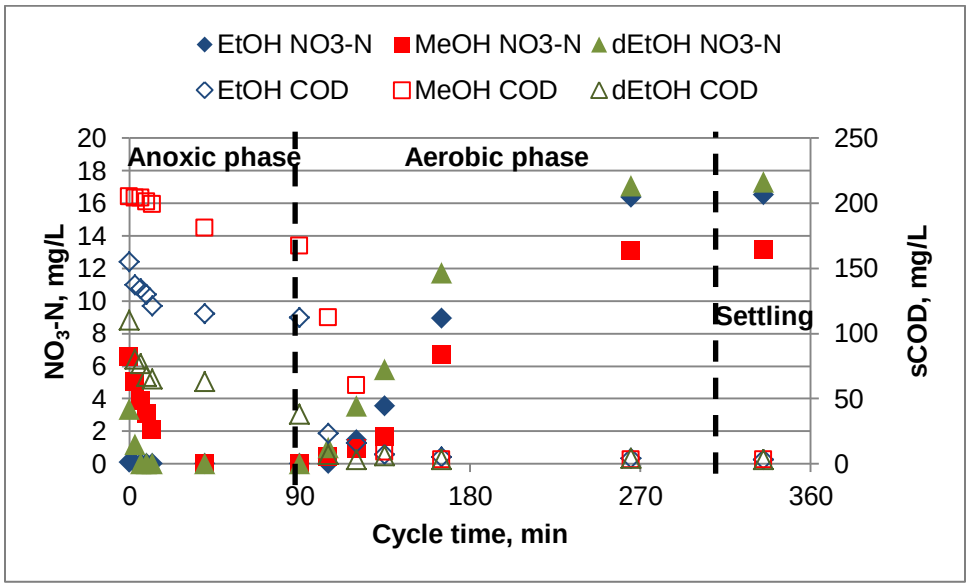


Figure 4.8-Representative change of NO_3-N and COD with time.

Denitrification rates (DNR), presented in Table 4.1 were calculated based on the residual concentrations of NO_3^--N (1-2 mg/L) and sCOD (10-20 mg/L) from data taken during the feed period, because during this period the rates were higher. P-values from Tukey's HSD test showed that the DNRs of dEtOH were similar to those of EtOH, while both EtOH and dEtOH achieved significantly higher denitrification rates than MeOH ($p_{EtOH\text{-}MeOH}$ = 0.009, $p_{dEtOH\text{-}MeOH}$ = 0.007 and $p_{dEtOH\text{-}EtOH}$ = 0.940). On the other hand, the Kruskal-Wallis Rank Sum test indicated that SDNRs were not statistically different (p = 0.528); which might be attributed to the higher concentration of biomass present in the ethanol reactors. Nevertheless, dEtOH's SDNR was approximately 1.2 times higher than MeOH's.

Table 4.1-Denitrification (DNR) and specific denitrification rates (SDNR).

	DNR, mg NO_3^-N/L·h	SDNR, mg NO_3^-N/g MLVSS·d		
	This study	This study	Literature	Reference
MeOH	8.9±0.9	165±49	221 (13°C) 101 (20-22.8°C) 19 (12.4-15.7°C) 32 (30°C)	Mokhayeri et al. (2008b) Swinarski et al. (2009) Louzeiro et al. (2002) Santos et al. (2004)
EtOH	14.5±0.6	197±28	730 (13°C) 234 (13°C) 230 (20-22°C)	Mokhayeri et al. (2008b) Fillos et al. (2007) Peng et al. (2007)
dEtOH	14.9±2.4	201±50	-	-

From the contents of Table 4.1 it is inferred that the calculated SDNRs for MeOH can be compared with those reported by Mokhayeri et al. (2008b) and Swinarski et al. (2009), but they differ from other reported values. This might be attributed to the different way rates were calculated. For instance, Mokhayeri et al. (2008b) and Swinarski et al. (2009), as well as the authors of this study, took into account data corresponding to the faster linear part of the NO_3^--N curves. On the other hand, Louzeiro et al. (2002) and Santos et al. (2004) applied first- and zero-order kinetics. Calculated SDNRs for EtOH were in the same range with values found in the literature.

4.3.3 Effect on nitrification

Aromatic compounds caused a significant decrease in SNR values at 5-20 mg C/L, according to Zepeda et al. (2006). In this study, though, nitrification was not inhibited as

shown in Figure 4.9. The behavior of the reactor fed with dEtOH was similar to that of the control reactors.

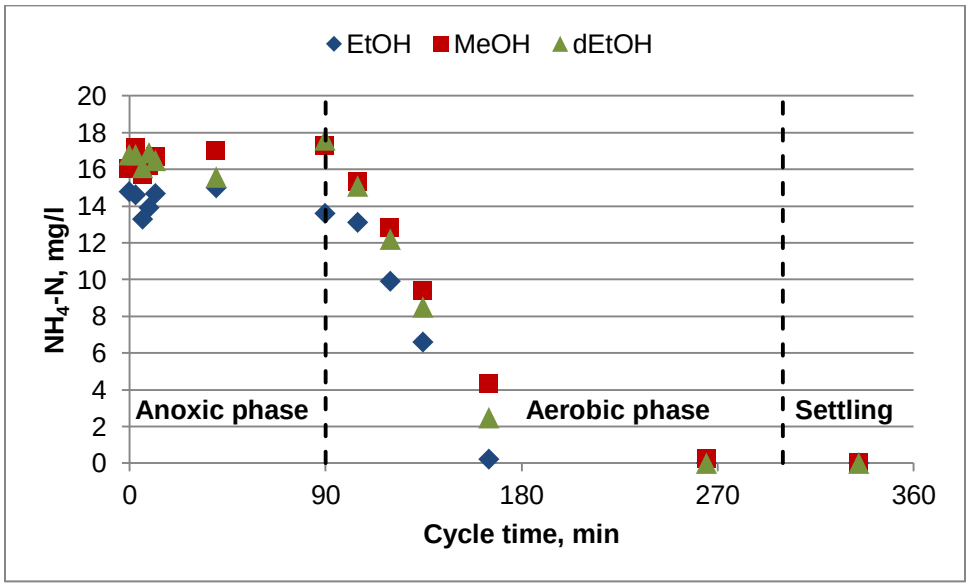


Figure 4.9-Ammonium-nitrogen profile.

SNR presented in Table 4.2 did not have equal variances. Therefore, the data were log-transformed to determine which of the carbon sources were different. Statistical comparison of the transformed data showed that the means of the rates for EtOH and dEtOH were the same ($p_{EtOH\text{-}dEtOH}$ = 0.76); on the contrary, MEOH rate means were statistically different from both ethanol reactors ($p_{MeOH\text{-}EtOH}$ = 0.003 and $p_{MeOH\text{-}dEtOH}$ = 0.014). Nitrification rates, on the other hand, were statistically the same. It can be inferred that the differences in MLVSS is responsible for this dissimilarity between SNR and SDNR.

Table 4.2-Nitrification rates (NR) and specific nitrification rates (SNR).

Carbon source	NR, mg NH_4^+N oxidized/L·h	SNR, mg NH_4^+N oxidized/g MLSS·d	MLVSS, mg/L
EtOH	5.1±0.7	12.2±1.2	1453±248
dEtOH	5.5±0.4	12.9±1.5	1458±255
MeOH	5.6±0.5	16.9±3.7	1213±185

Aerobic nitrate-nitrogen data were fit to a linear model, as shown in Figure 4.10. P-values obtained by the model were 0.32 and 0.81 for MeOH-dEtOH and MeOH-EtOH

reactors, respectively, indicating that their slopes were the same (0.087 mg/L·min for MeOH, 0.09 mg/L·min for EtOH and 0.088 mg/L·min for dEtOH). Therefore, the presence of BTEX in the dEtOH reactor did not inhibit nitrification, verifying the tentative conclusion reached by plain observation of the profile data of ammonium-nitrogen.

To further establish the potential of dEtOH as an alternative carbon source, a cost comparison was considered. Table 4.3 compares the three carbon sources in terms of their anoxic yield and cost per amount of nitrate denitrified. The lower yield and lower theoretical COD for methanol is counterbalanced by the higher yield and higher theoretical COD for ethanol.

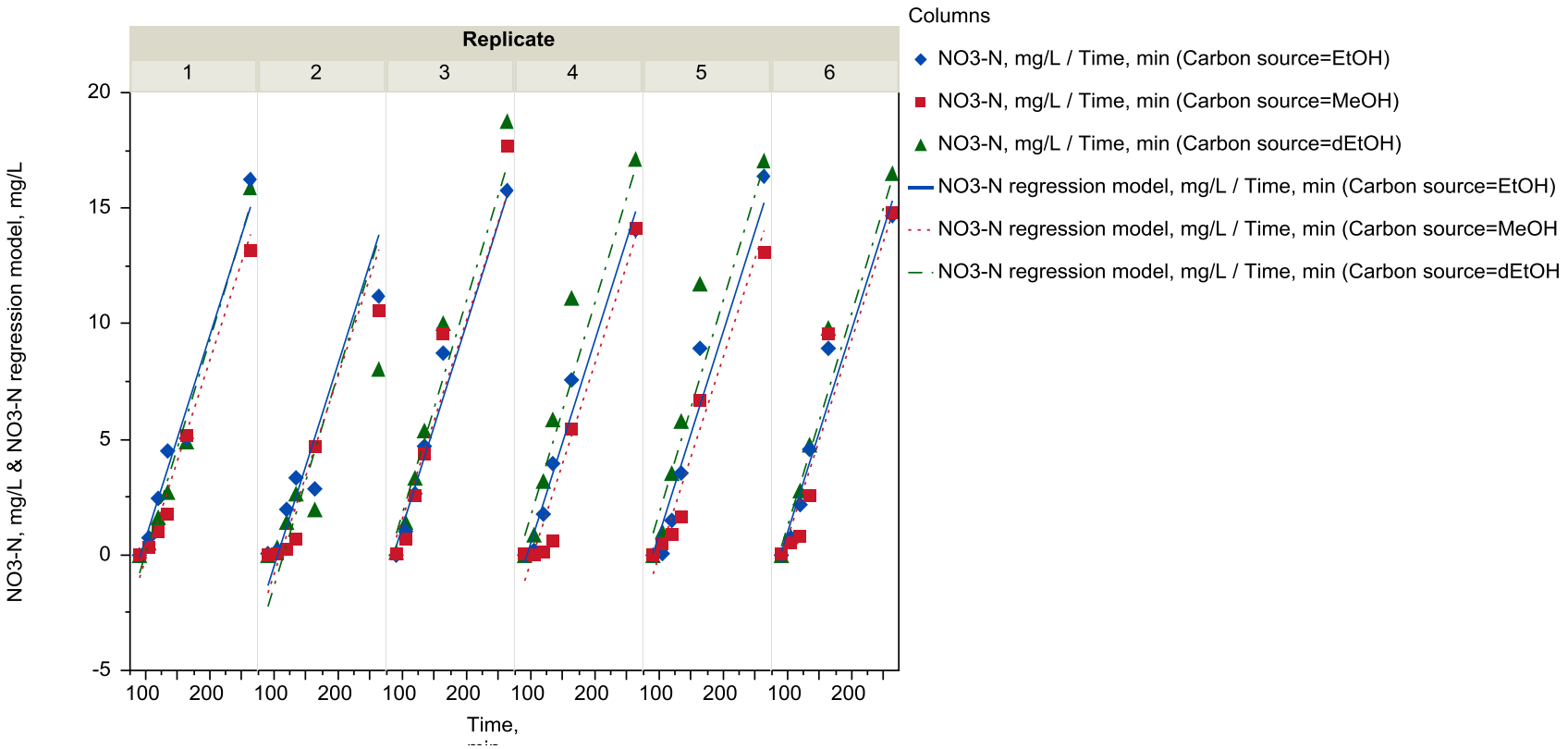


Figure 4.10-NO_3^--N (mg/L) and NO_3^--N regression model vs. Time (min) by Replicate & Carbon source during the aerobic phase.

Table 4.3-Comparison of the three tested carbon sources.

Electron donor	Anoxic yield, g COD/g COD	Cost[5], $/gal	Theoretical COD, g COD/g chemical	COD:NO_3^--N, g COD/g NO_3^--N	lb COD req/lb NO_3^--N denitrified	Cost, $/lb NO_3^--N
MeOH	0.4	1.54	1.50	4.8	3.20	0.74
EtOH	0.5	3.85	2.09	5.5	2.64	1.56
dEtOH	0.5	2.25	2.09	5.5[6]	2.64	0.91

[5]Dr. Bott provided the costs (September, 2011)

[6]Assumed to be equal with ethanol's.

4.4 Conclusions

The performance of three carbon sources, methanol, ethanol and gasoline-denatured ethanol, was compared and evaluated on the basis of treatment efficiency and cost per amount of denitrification. The specific denitrification rates of denatured ethanol were similar to those of ethanol, as expected, and higher than those of methanol. The denaturant affected neither the biomass production, nor the nitrogen removal processes. In addition, the effluent sCOD concentrations were low, indicating that no BTEX accumulation occurred. The cost of denatured ethanol calculated as $/lb NO_3^--N is slightly higher than that of methanol. The results are promising for the use of denatured ethanol provided that paying the additional cost of purchasing dEtOH is preferable to covering the cost of building larger storage tanks and implementing more safety features for methanol.

4.5 Acknowledgements

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5 Evaluation of gasoline-denatured ethanol as an alternative carbon source for denitrification: Effects of benzene, toluene, ethylbenzene and xylenes (BTEX) on nitrification and denitrification

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ABSTRACT: The overall objective of this project was to evaluate the potential of using gasoline-denatured ethanol (dEtOH) as an alternative carbon source for denitrification. In this paper, we focused on the quantification of benzene, toluene, ethylbenzene, and xylenes (BTEX) in the dEtOH solution, the inhibition potential of BTEX on both nitrification and denitrification, and the compliance of their effluent concentrations with drinking water standards. The experiment was conducted in three, 4-L sequencing batch reactors (SBRs) operated at 6-hr cycles. Methanol and pure ethanol were used in control reactors. The amount of BTEX contained in the dEtOH solution was low; 57.5-95.6 mg/L benzene, 29.3-52.6 mg/L toluene, 2.0-41 mg/L ethylbenzene, 4.5-9.6 mg/L *m,p*-xylenes, and 2.9-7.1 mg/L *o*-xylenes. Only benzene, toluene and *m,p*-xylenes were detected at the beginning of the each cycle in low concentrations. Neither nitrification nor denitrification was inhibited, and BTEX were completely removed from the reactor. Nitrification and denitrification rates in the two ethanol reactors were similar and higher than those in the methanol reactor. Therefore, dEtOH appears to be a good carbon source for wastewater denitrification.

KEYWORDS: Denitrification, methanol, ethanol, denatured ethanol, BTEX, SBRs

5.1 Introduction

Early in the 19th century Gayon and Dupetit realized that nitrate and nitrite could biologically be converted to nitrogen gas (Focht and Chang, 1975). Since then extensive research has established denitrification as an integral part of biological nitrogen removal. Denitrification is actually a reduction-oxidation reaction, where electrons are transferred from the electron donor (carbon source) to the electron acceptor (nitrate). In this way, the organism gains energy, required for the synthesis of new biomass and the maintenance of the existing biomass.

The availability and the type of the electron donor significantly affect denitrification. Banchuen (2002) reported that simple organic compounds with small molecular weights are more easily degraded by denitrifying bacteria. The existence of more complex compounds may lead to nitrite accumulation, which hinders the rate of denitrification. Another desirable characteristic of an electron donor is its ability to enhance denitrification by increasing the specific denitrification rates (SDNR) and yield, measured as carbon to nitrogen (C/N) ratio. Its performance should be steady regardless of the temperature (winter-summer). In addition, a high chemical oxygen demand (COD) mass equivalent of the carbon source is recommended. Microbes should be able to effectively use it, leaving a low COD concentration in the treated effluent. Moreover, a good electron donor should not affect sludge production and settling properties. Furthermore, utilities should have quick access to the source at frequent intervals and required quantities, to minimize transportation costs. Safety issues related to handling and storage are equally important. The presence of non-biodegradable/toxic compounds, like benzene, toluene, ethylbenzene, and xylenes (BTEX) might inhibit denitrification. Therefore, the electron donor should have little or no non-biodegradable and/or toxic compounds. An electron donor with consistent composition, to which bacteria can quickly acclimate, is preferable. Last but not least, a low price (e.g. \$/lb NO_3^--N denitrified) plays an important role in the selection of an electron donor (Ginige et al., 2009; Swinarski et al., 2009).

A number of electron donors have been studied. Among them are methanol, ethanol, acetate, glycerol, primary sludge, acetic acid, and industrial and agricultural byproducts (Abufayed and Schroeder, 1986; Lee and Welander, 1996; Onnis-Hayden,

2008; Ginige et al., 2009; Swinarski et al., 2009; Adav et al., 2010). However, comparisons made regarding denitrification, especially with methanol and ethanol resulted in ambiguous conclusions. Some of the studies indicated that ethanol improves denitrification rates over methanol (Christensson et al., 1994; Hallin and Pell, 1998; Trela, 1998), whereas others indicated that methanol yields higher rates (Henze, 1991). Therefore, in this study ethanol and methanol were selected as two of the three carbon sources considered and were used as control feeds. The third, ethanol denatured with gasoline, is a newly introduced carbon source with great potential.

Methanol satisfies many of the criteria mentioned above. It is cheaper than other suggested carbon sources, commercially available, and has yielded a wide range of performance data that can be employed for system optimization. Another related advantage is the fact that it is thoroughly regulated. In addition, it is readily biodegradable and has a low sludge production rate (low sludge yield). Moreover, the carbon oxidation is almost complete resulting in low effluent BOD concentrations. According to McCarty et al. (1969), it is not considered toxic to fish life, given that fish can tolerate concentrations over 100 mg/L. Finally, it does not contain any phosphorus or nitrogen that could aggravate the influent nutrient load (Hallin et al., 2006; Mokhayeri et al., 2008a; Onnis-Hayden, 2008; Swinarski et al., 2009).

On the other hand, methanol has some important disadvantages. First, specific denitrification rates (SDNRs) are lower than those of other electron donors. Methanol is a single-carbon compound, and does not easily enter the tricarboxylic acid (TCA) cycle, unless it is reduced to form higher carbon intermediates (3-C and 4-C) (Mokhayeri et al., 2008b). It requires longer acclimation and recovery periods. It has been demonstrated that when methanol is used as an external carbon source a new population of methylotrophic bacteria with a slower growth rate is established (Hallin et al., 1996; Hallin et al., 2006; Swinarski et al., 2009). These kinds of bacteria are facultative organisms, which belong to the *Paracoccus* and *Hyphomicrobium* genera (Claus and Kutzner, 1985; Onnis-Hayden and Gu, 2008). In addition, according to Hallin and Pell (1998), methylotrophs inhibit the growth of other denitrifying bacteria present in the activated sludge. Therefore, substitution of methanol with other carbon sources may result in a loss of denitrification. Methanol is produced from the methane present in

natural gas, so its price is linked to that of fossil fuels. According to the National Fire Protection Association, methanol is classified as flammable. Hence, specific safety measures are necessary during transportation and storage. It is classified as poisonous and toxic according to European Union legislation, because it affects the central nervous system. McCarty et al. (1969) reported that 100-250 mL is considered toxic to humans. Finally, it was shown by Mokhayeri et al. (2008b) that during winter months, methanol addition does not enhance nitrogen removal.

Ethanol also demonstrates the ability to be used as an external carbon source. To begin with, SDNRs achieved are two to three times higher than that of methanol (Trela, 1998; Ginige et al., 2009). Microbes quickly adapt to ethanol. Trela (1998) reported adaptation within a few hours. Denitrifiers grown with ethanol are able to use other carbon sources, such as acetate, propionate, butyrate and methanol without losing their capacity to denitrify (Hallin and Pell, 1998). Thus, utilities can interchange carbon sources without affecting the performance of the plant. Ethanol is commercially available and can be found in many industrial wastes; e.g., in pharmaceutical industries. It is readily biodegradable, because it is a low-carbon compound, which easily enters the TCA cycle as acetyl-SCoA (Henze et al., 1994), and more readily available than methanol.

Flammability, toxicity, and strict regulation issues are the main disadvantages that inhibit the commercial use of ethanol. Ethanol is heavily taxed and difficult to procure due to the complicated permits from the bureau of alcohol, tobacco, firearms, and explosives (ATF). Like methanol, it is ranked as flammable chemical. The oral lethal dose (LD_{50}) in rats is 5,828 mg/kg. Finally, it produces more biomass than methanol.

The Hampton Roads Sanitation District (HRSD) is currently interested in using ethanol denatured with “straight-run” gasoline (dEtOH) as an alternative carbon source for denitrification. In general, ethanol is denatured at the ethanol plant with additives, like acetone and gasoline, to prevent people from consuming it. The gasoline added, in our case, is known as virgin, straight-run, or natural gasoline, and it is blended with ethanol at 2-15%. This mixture is then added to gasoline to make it a cleaner fuel and protect the engine from knocking.

More specifically, “straight-run” gasoline is a lower grade material that contains mostly aliphatic compounds. It lacks the components that normally boost the octane rating, such as benzene, toluene, ethylbenzene and xylenes. In addition, the non-cyclic hydrocarbons contained have the potential to be good carbon sources. More information about its composition will be provided below.

Because this substance is a new entry in the field of wastewater, little is known about its composition and its capacity to improve SDNRs. Given that it is mostly ethanol, it is reasonable to assume that it shares the same advantages and disadvantages with common ethanol. There are also other issues to consider; in the market, its price is lower than ethanol’s. It is locally available, in large quantities for blending with gasoline to make E10 (fuel blend that contains 10% ethanol and 90% gasoline). Because it is denatured, it is declared unfit to drink and is not controlled by ATF.

Possible disadvantages evaluated herein are as follows. First, it is more expensive than methanol. Second, the content of the denaturant (“straight-run” gasoline) and the concentration of hydrocarbons, like benzene, toluene, ethylbenzene, and xylenes contained in it, might vary. Third, the degree of carbon oxidation is unknown. It is possible that a high effluent COD, an indication that gasoline constituents cannot be biologically degraded, will be observed. The chemicals passing through biological treatment would be discharged to receiving waters, possibly affecting aquatic life. Dold (1989) stated that this problem could be addressed with the addition of hydrogen peroxide (H_2O_2) prior to activated sludge treatment, which will improve the adsorption of the compounds under investigation. Fourth, high concentrations of hydrocarbons in the influent might affect sludge properties (Dold, 1989). Oil could potentially accumulate on the flocs, forming a hydrophobic layer; thereby affecting the settling characteristics. In addition, the effect of the denaturant on WWTP is undetermined. Accumulation of gasoline constituents, like the BTEX compounds, in the WWTP may affect the performance of the biological process. Lastly, it is possible that the sludge will contain high amounts of gasoline constituents, which require special techniques for sludge treatment and disposal. BTEX compounds are of particular interest, because of their

potential toxicity. The US EPA (2009) classifies them among the top 20 hazardous substances and priority pollutants.

The effect of BTEX compounds on nitrification is very important for wastewater engineers, especially those dealing with industrial wastewater. Under aerobic conditions, oxygen not only serves as an electron acceptor, but also triggers microbial enzymatic activity. Thus, carbon mineralization is induced (Weelink, 2010). However, the presence of high BTEX concentrations can hinder the ability of bacteria to degrade them (Richardson, 1985).

Three factors play an important role in BTEX's inhibition of nitrification: the presence of functional groups in the compounds, their hydrophobic properties, and their concentrations. Toluene and *m*-xylene, for example, have methyl groups, and tend to react with other compounds. Benzene, on the other hand, is more stable, because it lacks functional groups and is less reactive. In addition, polycyclic compounds are hydrophobic. They accumulate in the cell membrane and inhibit the respiratory function of enzymes. Moreover, high concentrations of BTEX have proven to be harmful to nitrifiers. Zepeda et al. (2006) noticed that the maximum amount of BTEX that nitrifiers could bear was 5 mg of carbon per liter. Microbes did not recover after they were exposed to higher concentrations (20-50 mg carbon per liter) of toluene and *m*-xylene and only partially recovered when exposed to 20-50 mg C/L of benzene. Exposure of cells to benzene decreased the activity of both AOB and NOB (Sikkema et al., 1992; Denich et al., 2003; Zepeda et al., 2006).

Gersberg et al. (1989) suggested using denitrification instead of nitrification as a feasible way to remove BTEX from aquifers, due to the high amount of oxygen required and cost associated with this practice. BTEX compounds are rapidly degraded under aerobic conditions, but they are more recalcitrant under anaerobic conditions. Anaerobic degradation was first demonstrated by Vogel and Grbìc-Galìc (1986). In general, BTEX decomposition under anaerobic conditions can be achieved in the presence of a variety of electron acceptors, such as nitrate, sulfate, ferric iron, manganese, carbon dioxide, or perchlorate. Zeyer et al. (1986) and Peña-Calva et al. (2004) concluded that toluene and *m*-xylene are biologically converted to CO_2 under denitrifying conditions. *M*-xylene, in particular, cannot be oxidized in the presence of nitrite (Schwarzenbach et al., 1988).

On the other hand, benzene is not easily degraded (Peña-Calva et al., 2004; Weelink et al., 2010).

Although there is general agreement regarding the anoxic mineralization of *m*-xylene to carbon dioxide (Zeyer et al., 1986; Chakraborty and Coates, 2004; Peña-Calva et al., 2004; Weelink et al., 2010), the literature is not consistent regarding *o*- and *p*- isomers. Some researchers state that *p*-xylene cannot be fully converted to carbon dioxide under anoxic conditions (Chakraborty and Coates, 2004), while others propose that *o*-xylene can (Kuhn et al., 1985; Zeyer et al., 1986). Weelink et al. (2010) presented studies where the *p*-isomer could also be anoxically degraded, but this was not the case with *o*-xylene. However, *o*-xylene, was degraded under sulfate-reducing conditions. In decreasing order of degradability, xylenes are ranked as: *m*-xylene > *p*-xylene > *o*-xylene.

Toluene and *m*-xylene can be inhibitory to denitrification, when present at high concentrations. In such cases, nitrite might accumulate, because the nitrite oxide reductase and nitrose reductase enzymes are affected (Weelink et al., 2010).

The overall goal of this project was to evaluate the practicability of using gasoline-denatured ethanol (dEtOH), as compared to 95.5% ethanol mixed with water (EtOH) and methanol (MeOH) for wastewater denitrification. In this paper, we focused on the quantification of BTEX compounds, present in dEtOH, the inhibition potential of dEtOH on both nitrification and denitrification, and the compliance of BTEX concentrations in the treated effluent with drinking water standards.

5.2 Materials and Methods

5.2.1 Experimental setup

To accomplish the objectives of this research, three, lab-scale, anoxic-aerobic SBRs, with working volumes of 3L, were seeded with sludge from VPI Wastewater Treatment Plant (Blacksburg, VA). The reactors were operated at a cycle time of 6 hours. Each cycle consisted of influent feeding, anoxic reaction, aerobic reaction, waste discharge, sludge settling, effluent decanting, and idling. Synthetic wastewater and methanol, ethanol, or denatured ethanol were pumped into each reactor in the first 5 minutes (min) of the anoxic period in a 50:50 ratio. They mimicked the Modified

Ludzack-Ettinger process (MLE), with the recycled sludge of MLE being the sludge left in the SBRs at the end of the cycle. All reactors were operated at an ambient room temperature varying between 21°C and 23°C. The pH was maintained in the desired range for both nitrification (7.5-8.0) and denitrification (7.0-8.0).

The hydraulic retention time (HRT) was maintained at 12 hours, by decanting half of the reactor's volume, whereas the solids retention time (SRT) was kept at 12±0.9 days by wasting and returning the proper amount of mixed liquor. Effluent total suspended solids, volume removed for sample analysis, and variations of the waste pump flows were taken into consideration for SRT correction. Sludge was returned every 2 or 3 days per week. The system was equipped with four peristaltic pumps (Cole-Parmer, Vernon Hills, IL) for wastewater inflow, solids wastage, and decanting. The reactors were mixed using stainless steel 2.54cm x 7.62cm paddles rotated at 66 rpm by Dayton Gearmotors (Grainger, Roanoke, VA). Three magnetic stirrers were inserted in the alcohol containers to ensure homogeneity of the solution. Mixing was kept at low speed to avoid losses due to volatilization. The operation of the various components of the system was controlled electronically by two programmable timers (ChronTrol Corporation, San Diego, CA). Figure 5.1 shows a picture of the setup.

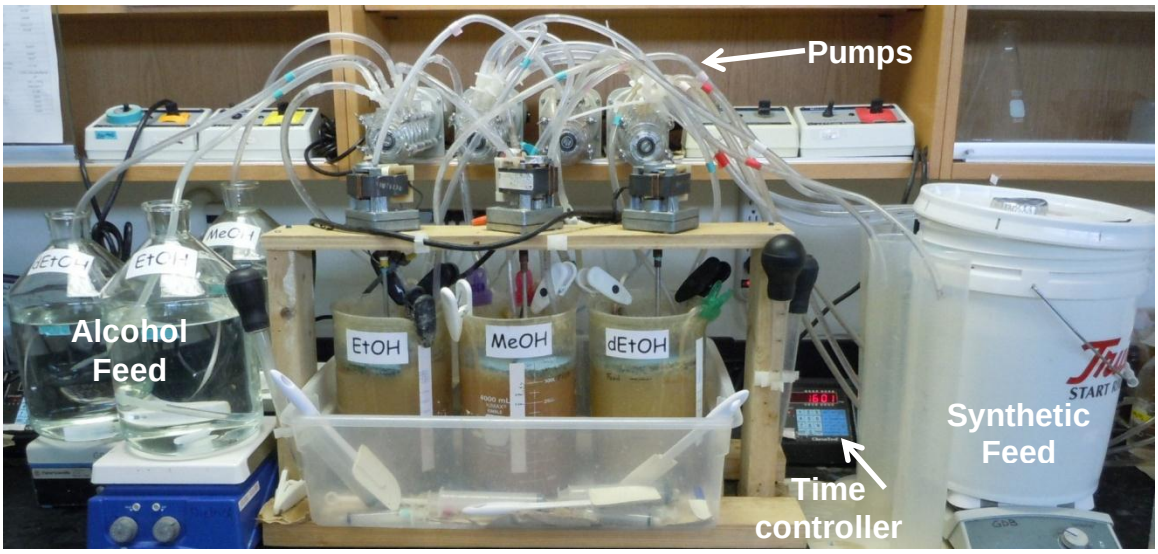

Figure 5.1-Experimental setup.

5.2.2 Sequence schedule

Both nitrification and denitrification were achieved by turning on and off the air supply. The SBRs were operated using a 6-hr cycle, including fill (5 minutes), react (5 hours) with a 90-min anoxic period and a 210-min aerobic period, settle (45 minutes), draw (9 minutes), and idle (1 min) phases. Mixed liquor was withdrawn for two min and occurred at the end of the aerobic phase. Effluent was withdrawn using a J-tube to avoid stirring the settled sludge and was wasted to the drain. Figure 5.2 describes the sequence schedule.

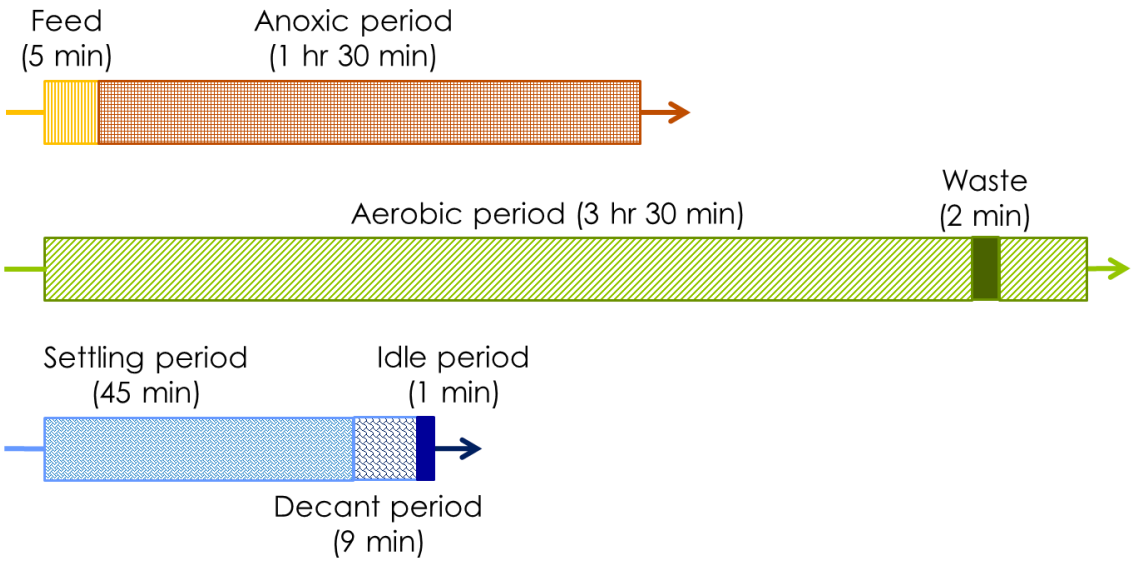

Figure 5.2-Sequence schedule.

Mixing was initiated at the beginning of the feed period and stopped when aeration ended. By aerating the system after denitrification, gases produced by denitrification were purged and thus, no rising sludge occurred (Leung and Tam, 1994).

5.2.3 Reactor Feed

Returned activated sludge from the Blacksburg/VPI Municipal Wastewater Treatment was used to seed the reactors. Synthetic wastewater (SW) was then used to feed the reactors at a rate of 150 mL/min. SW was prepared every two days in accordance with

Table 5.1. The soluble chemical oxygen demand (sCOD) and ammonium-nitrogen concentrations contained in the SW feed were maintained approximately at 175 mg/l and 58 mg/L, respectively.

Table 5.1-Synthetic wastewater composition.

Constituent	Concentration, mg/L
Bactopeptone[7]	188.7
NH_4Cl	230
$CaCl_2.2H_2O$	132
CaO	8.4
$MgSO_4.7H_2O$	246
KH_2PO_4	102
K_2HPO_4	131
$NaHCO_3$	420
Na_2CO_3	134
$FeCl_3$	17.4
$Al_2(SO_4)_3.18H_2O$	37.0

[7]Enzymatic digest of protein (Spectrum Chemical Mfg. Corp, New Brunswick, NJ).

Neither NO_3^--N nor NO_2^--N were detected in the influent. All constituents were diluted in tap water. No additional micronutrients were used, because it was assumed that the necessary quantity for the bacteria to grow could be found in the tap water. With the addition of buffer solutions ($NaHCO_3$, Na_2CO_3 and CaO) the pH was maintained at 8.

Bactopeptone only contributed 25% of the influent sCOD. The remaining 75% was provided by the different carbon sources: 95.5% ethanol (Ricca Chemical Company, Arlington, TX), laboratory grade methanol (Fisher Scientific, Pittsburg, PA) or denatured ethanol (Allied Terminals, Norfolk, VA). Soluble COD in stock solutions of EtOH, MeOH, and dEtOH were 770±172 mg/L, 844±188 mg/L and 612±94 mg/l, respectively.

5.2.4 Collection and analysis of samples

Samples were collected during the anoxic, aerobic, and settling periods. Profiling sampling was conducted every two days for two weeks. To achieve a better understanding of the rapid rate of denitrification, samples were collected every 3

minutes during the first 20 minutes of the anoxic period. Since nitrification is a slower reaction, samples were taken every 15 minutes after aeration began to ensure that dissolves oxygen (DO) concentration was higher than 2 mg/L.

A 60-ml plastic syringe was used for the collection of samples, which were filtered immediately using 0.45 μm filters. Sample (35-mL) aliquots were taken from each reactor during the anoxic, aerobic, and settling periods. An additional volume of 10 mL was withdrawn from the dEtOH reactor, to be used for BTEX analysis[2]. For the duplicate analysis of total suspended solids (TSS) and volatile suspended solids (VSS), 500 mL were retained from each reactor's effluent.

5.2.5 Analytical Methods

Soluble chemical oxygen demand (sCOD), ammonia, and nitrite, were determined colorimetrically using Hach reagents and a DR2800 spectrophotometer (Hach Company, Loveland, CO). More specifically, method 8000 was used to measure sCOD and methods 10023 and 10031 to quantify ammonia-nitrogen. Due to the great variability in the range of nitrite-nitrogen detected in the mixed liquor and the effluent, three methods were applied for its determination; 8507, TNT839 and TNT840.

Nitrate-nitrogen, TSS in the mixed liquor and in the effluent, and the sludge volumetric index (SVI) were determined according to Standard Methods (APHA, 2005).

The composition of the concentrated solution of denatured ethanol was analyzed with a FOCUS gas chromatograph (GC) equipped with a DSQII mass spectrometer (MS), and an AS-3000 autosampler (Thermo Scientific, West Palm Beach, FL). The volume of the sample was 1 μL.

A purge and trap capillary column gas chromatographic method, Standard Method 6200C, was applied for the quantification of BTEX in aqueous samples. A purge and trap concentrator (Tekmar, Cincinnati, OH) and a gas chromatograph with photoionization controller (Treemetrics, Austin, TX) were used. 5 mL unfiltered samples were analyzed. The analytes were desorbed into the carrier gas (helium) at 250°C in the concentrator and the volatile compounds were carried at 100°C to the GC.

[2] It should be pointed out that samples taken from the feed bottles of EtOH and MeOH were also analyzed for BTEX, but the compounds were undetectable.

For monitoring purposes pH, DO and temperature were measured every two or three days, four times within a cycle, using handheld probes. The pH was measured using an Oakton pH/mV meter (Fisher Scientific, Pittsburgh, PA), while temperature and DO concentration were recorded using a YSI DO probe (YSI, Inc., Yellow Springs, OH).

5.2.6 Statistical analysis

Computer software packages, R (Version 2.14.0) and JMP 9.0.0 were used for statistical analysis. The significance level, α, was set equal to 0.05. More information are given below.

5.3 Results and Discussion

5.3.1 Composition analysis of dEtOH

Severe air pollution problems are caused by the emission of carbon monoxide. A countermeasure used to deal with this issue is the addition of oxygenated compounds in gasoline, because additional oxygen completely oxidizes carbon monoxide to carbon dioxide. An example of an oxygenated additive is gasoline-denatured ethanol.

For the needs of this experiment, two batches of denatured ethanol were analyzed. The first batch of dEtOH contained 2.19%(v/v) of the denaturant and the second batch 2.31%(v/v). The results taken from the GC/MS and GC/PID analyses for the BTEX content of dEtOH are presented in Table 5.2. In the last column, the amount of BTEX in a feed with a sCOD of 100 mg/L highlights the difference of magnitude between the concentration of BTEX in dEtOH (mg/L) and in the reactor (μg/L).

Table 5.2-BTEX concentrations present in dEtOH batches (mg/L) and in the reactor (µg/L).

Component	GC/PID[8]		GC/MS[8]		µg/L added for a feed of 100 mg/L sCOD
	1st batch	2nd batch	1st batch	2nd batch	
Benzene	70.9	80.0	84.5	105.3	8.2±1.77
Toluene	35.4	44.1	112.7	143.3	7.9±1.51
Ethylbenzene	2.5	3.0	3.7	4.6	-
m,p-xylene	5.8	7.5	15.5	16.1	1.3±0.31
o-xylene	3.7	4.7	13.7	14.4	-

[8]Mean values

From Table 5.2 it is concluded that the two batches had a similar composition of BTEX compounds, which is encouraging for the subsequent use of denatured ethanol as a carbon source. Three aliquots from each batch were analyzed. The statistical significance of the results taken from the GC/PID analysis was established employing two-sample t-tests for benzene (pb = 0.4309), toluene (pt = 0.1892), m,p-xylenes (pm,p-x = 0.2652) and o-xylenes (po = 0.5179). For ethylbenzene, the Wilcoxon Rank Sum Test indicated that the population means were the same (p = 1). Prior to running tests, all necessary assumptions were verified. More specifically, the Shapiro-Wilk normality and F-tests were used to check if data were normally distributed and if they had equal variances.

Data acquired from the GC/MS analysis were also compared and showed that benzene (p_b = 0.08), toluene (p_t = 0.06), ethylbenzene (p_e = 0.32), *m,p*-xylenes ($p_{m,p-x}$ = 0.76), and o-xylenes (p_o = 0.76) content in the two batches was statistically the same.

The results of the two techniques were statistically compared by using a one-way ANOVA test. The test showed that the mean values were statistically different (p-value = 0.02). A possible interpretation is that the concentrated sample needed to be diluted in water before GC/PID could be used. In addition, xylene isomers are not soluble in water and have a lower density. Thus, during the GC/PID analysis, they might have risen to the surface of the sample and not been completely purged. This could also explain why the concentrations detected with GC/PID were lower than those detected with GC/MS.

Composition analysis with GC/MS verified the assumption that dEtOH was rich in alkanes (Table 5.3). Library search results also showed that among other organic compounds butane, heptane, and octane, were detected in both batches.

Table 5.3-Alkanes present in dEtOH concentrated solutions (mg/L).

Component	1st batch	2nd batch
Pentane	1,105	1,077
Hexane	1,131	981
Octane	47.9	45.1

5.3.2 BTEX degradation

After steady state was reached, profiling data were collected. BTEX concentrations were plotted against cycle time. One representative plot is given below.

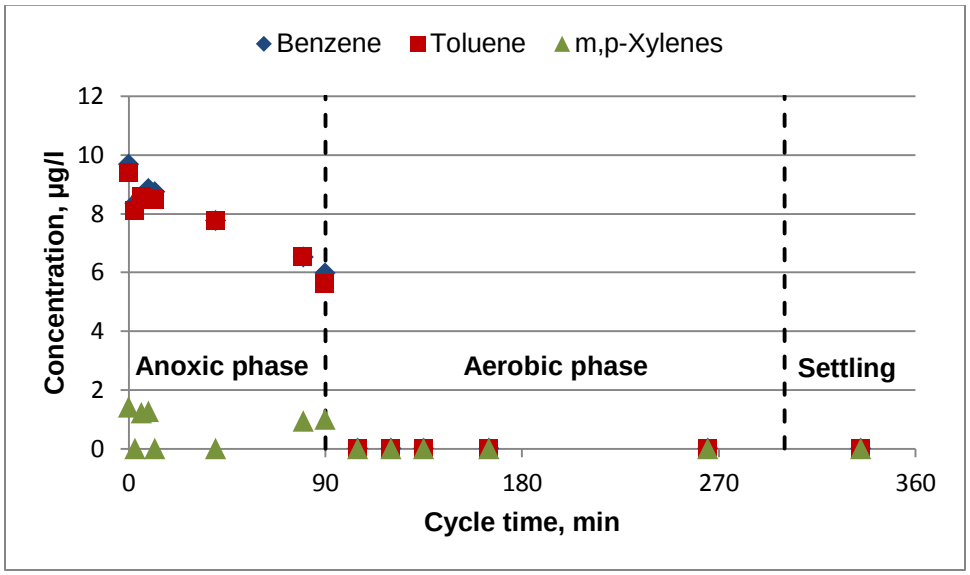

Figure 5.3-Representative BTEX profile for dEtOH reactor.

Benzene, toluene and *m,p*-xylenes were detected in the beginning of the cycle and during the anoxic phase, but not during the aerobic and settling phases. This could be attributed to either biodegradation or volatilization.

Although Peña-Calva et al. (2004) reported that benzene is recalcitrant to biological treatment, Wu et al. (2009) found that benzene degradation is enhanced by the presence of low concentrations of ethanol under denitrifying conditions. Ethanol in high concentrations is known to inhibit benzene's degradation under aerobic conditions,

because microorganisms prefer it over benzene. According to Hunt et al. (1997), high concentrations of ethanol damage the cell membranes and other cellular structures of microbes. The fact that benzene in dEtOH was diluted in ethanol might have contributed to its complete removal during a 6-hr cycle. On the contrary, toluene was completely removed under anoxic conditions (Peña-Calva et al., 2004). They tested the effect of different concentrations of toluene (15, 25, 40, 55, 70, 85, and 100 mg/L of toluene-C) on a sludge with the VSS concentration being equal to 4,000 mg/L.

Ethylbenzene and *o*-xylene were not detected in the reactor. The calculated removal rates of benzene, toluene and m-xylene were 3.1±1.4, 3.4±1.9 and 0.6±0.4 µg/L·h, respectively. Their average consumption efficiency was 58±26.9%, 70±28.4% and 80±31.3%, respectively, during the anoxic cycle. Karlson and Frankenberger (1989) reported 95% and 100% removal of benzene and toluene from groundwater samples with removal rates equal to 29.1 µg/L·h and 12.7 µg/L·h, respectively, when additional nitrate was provided to the bacteria, for toluene and benzene. This difference can be attributed to the significantly lower initial concentration of the compounds in our experiment. Gersberg et al. (1989) studied BTX anoxic biodegradation in groundwater samples in the presence of 13.3-13.7 mg/L benzene, 33.7-39.5 mg/L toluene, and 15.4-23.2 mg/L xylenes. They calculated the removal efficiencies of benzene, toluene, and xylenes equal to 80%, 95%, and 47%, respectively. They observed that *m,p*-xylenes were more slowly degraded than benzene and toluene.

According to Dold (1989), who reviewed the biological treatment of refinery wastewater, one problem associated with biologically treating refinery waste is the high effluent COD. However, in our case, the effluent COD was below detection, and BTEX concentration was within the acceptable limits for potable water (0.005, 1.0, 0.7 and 10.0 mg/L for benzene, toluene, ethylbenzene and total xylenes, respectively) (US EPA, 2009). The fate of each of the carbon sources, expressed as COD, is illustrated in Figure 5.4.

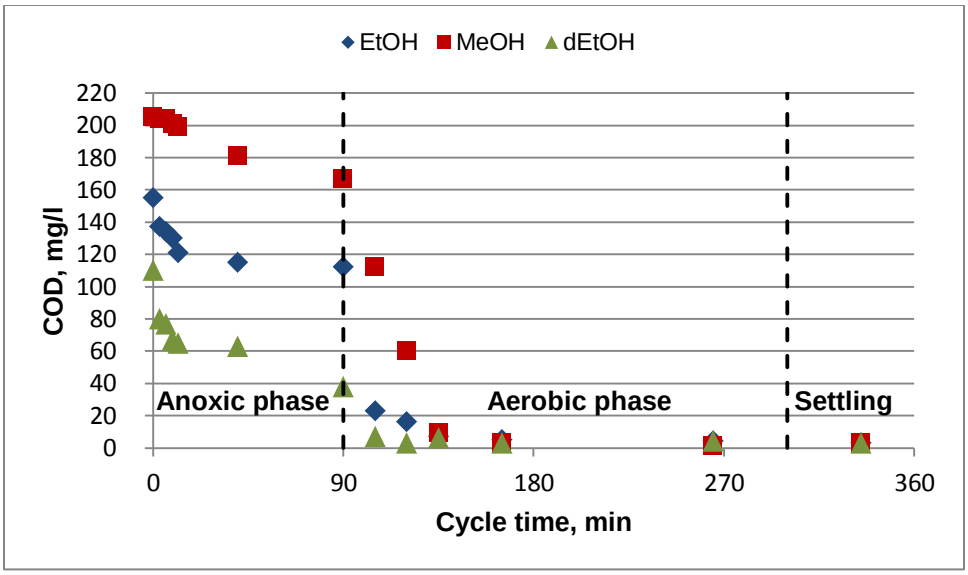

Figure 5.4-Representative COD profile.

Aromatic compounds caused a significant decrease in the values for specific nitrification rates at 5-20 mg C/L, according to Zepeda et al. (2006). No inhibition of nitrification and denitrification was observed, according to Figure 5.5 and Table 5.4. The behavior of the reactor fed with dEtOH was similar to that of the reactor fed with EtOH.

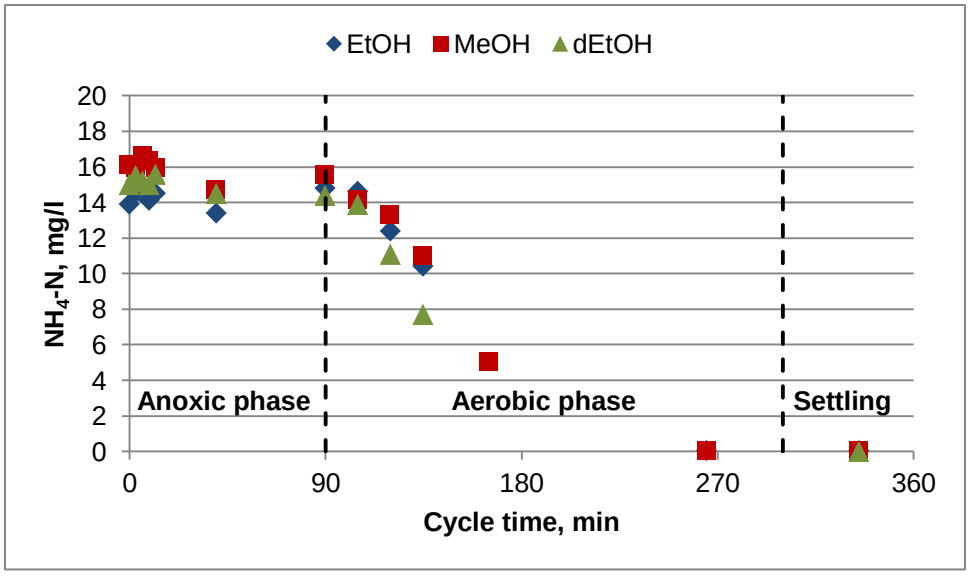

Figure 5.5-Representative ammonia-nitrogen profile.

Table 5.4-Nitrification rates (NR) and specific nitrification rates (SNR).

Carbon source	NR, mg NH_4^+N oxidized/L·h	SNR, mg NH_4^+N oxidized/g MLSS·d	MLVSS, mg/L
EtOH	5.1±0.7	12.2±1.2	1453±248
dEtOH	5.5±0.4	12.9±1.5	1458±255
MeOH	5.6±0.5	16.9±3.7	1213±185

In Figure 5.6 the horizontal straight lines correspond to the average values of MLSS after steady state was reached and during the profiling period. No increase of VSS was observed in the dEtOH reactor due to the existence of the BTEX compounds as compared to the EtOH reactor. MLSS concentrations for methanol, ethanol, and denatured ethanol feeds were 1955±315 mg/L, 2224±361 mg/L and 2258±398 mg/L, respectively. Peña-Calva et al. (2004) reported a similar observation and attributed this fact to the consumption of BTX as an energy source and not as carbon source for cell synthesis. The addition of dEtOH had no apparent effect on the overall settling properties of the biomass. The sludge volumetric indices (SVI) were 58±7.8, 57±3.5 and 56±6.1 mL/mg for EtOH, MeOH and dEtOH reactors, respectively.

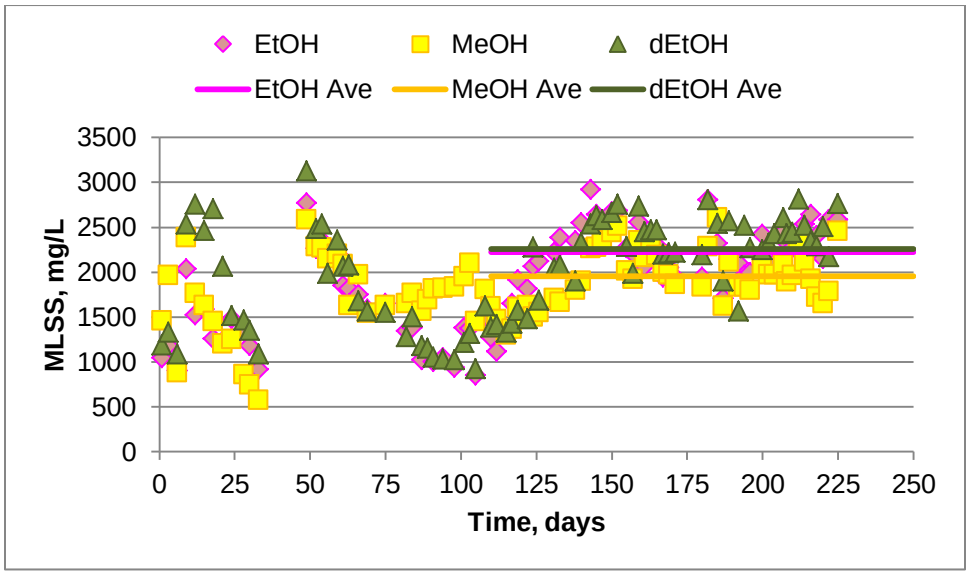

Figure 5.6-MLSS concentration throughout the experimental period.

Other processes, such as volatilization and sorption can remove BTEX compounds from the reactors, so the fate of the compounds is hard to track.

5.4 Conclusions

Ethanol denatured with gasoline (dEtOH), a new entry for wastewater denitrification, was analyzed and tested as a carbon source. Benzene, toluene, ethylbenzene, and xylenes (BTEX), present in the dEtOH solution were quantified and their inhibition potential on both nitrification and denitrification was studied. The basic conclusions are summarized as follows:

- Nitrification and denitrification rates of denatured ethanol were similar to those of ethanol and higher than those of methanol.
- Denaturant concentration in the two denatured ethanol solutions tested was about the same.
- The gasoline additive and BTEX compounds in dEtOH did not affect nitrification or denitrification.
- Biomass production was not influenced by the denaturant.
- BTEX effluent concentrations were below the detection limit (1 ppb).
- BTEX effluent concentrations were well below drinking water standards.

5.5 Acknowledgements

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6 Conclusions

The performance of three carbon sources, methanol, ethanol and gasoline-denatured ethanol, was compared and evaluated on the basis of treatment efficiency and cost per amount of denitrification. The specific denitrification rates of denatured ethanol were similar to those of ethanol, as expected, and higher than those of methanol. The added gasoline affected neither the biomass production, nor the nitrogen removal processes. In addition, the effluent sCOD concentrations were below the detection limit. The concentration of the denaturant in the two denatured ethanol batches tested was about the same. Finally, the cost of denatured ethanol calculated as \$/lb NO_3^--N is slightly higher than that of methanol. The results are promising for the use of denatured ethanol provided that paying the additional cost of purchasing dEtOH is preferable to covering the cost of building larger storage tanks and implementing more safety features for methanol.

7 Future work

Further research on the following issues could further enhance the theoretical and practical background of using denatured ethanol:

- To ensure that the composition of dEtOH regarding its BTEX composition is consistent, more batches should be analyzed.
- It could be tested in long-term operation at larger scale, to determine its potential in real-life situations.
- It could also be evaluated by using continuous flow reactors.
- Increasing the strength of wastewater to monitor the effect of elevated BTEX concentration on nitrification/denitrification, could be beneficial.
- The efficiency of denatured ethanol at cold temperatures could be tested.
- The possibility of using substrates interchangeably could be assessed.
- Air quality problems caused by BTEX emission are worth evaluating.
- Identification of specific bacteria related to anoxic BTEX degradation might worth studying.
- Batch tests to determine μ_{max} and K_s.

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