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Effect of the C-source and COD/N on Nitrite and Partial Nitrate Denitrification

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Abstract

Nitrogen in wastewater, along with phosphorus, needs to be removed to control eutrophication that affects the aquatic life when it occurs in water bodies. Recent developments in total nitrogen removal technology involve partial nitrification-denitrification and partial denitrification-anammox (N-PDN-anammox) processes, which reduce the energy and the external carbon source requirement. The objectives of this study were to investigate dynamic specific nitrite denitrification rates (SDNRs) and kinetic parameters in a process that makes use of municipal wastewater alone (objective-1) and MWW with methanol as a complementary carbon source (Objective-2). Another objective of the study was to investigate and compare complete nitrite versus partial nitrate denitrification at a lower COD/N ratio and identify process control strategies and microbial composition for a successful partial denitrification (PDN) process.

To achieve the first objective, a sequencing batch reactor (SBR) continuously fed with primary effluent and nitrite solution were operated at a hydraulic and solids retention time of 8.4 hrs and 26-30 days, respectively. The SDNRs from the SBR, using MWW as a carbon source, were compared with those determined in four 1 L-batch reactors using acetate as a carbon source. The SDNR was directly related to COD/N until a maximum SDNR (mgNO2-N/mgVSS/d) of 0.07 for MWW and 0.4 for acetate occurred at COD/N ratios of 6 and 13, respectively; beyond this COD/N ratio, SDNR decreased. The relationships of SDNR with COD/N and F/M ratios were developed. This is the first nitrite denitrification study using MWW as a carbon source to determine the SDNRs and kinetic parameters and model a direct relation between SDNR and F/M ratio. Subsequently, the nitrite denitrification process was assessed using methanol as a complementary carbon source for the first time. An SBR fed with primary effluent diluted 50% with distilled water by volume and nitrite solution was operated at an HRT of 8.4 hrs (30 L/d) and SRT of 16-17 days for 110 days. The SDNR from the SBR was compared with those determined in 23 batch reactors employing municipal wastewater (MWW), methanol, nitrite, nitrate and a combination of nitrite and nitrate in various ratios. The maximum SDNR was 0.52 mgN/mgVSS/d corresponding to COD/N ratios of 5.9 in the SBR, much higher than with MWW. However, the maximum nitrite SDNR was lower than the maximum observed batch nitrate 1.06 mgNO_3-N/mgVSS-d SDNR using the nitrite acclimatized biomass from the SBR. The nitrite denitrifier yields were also determined for all cases. Lower SDNR values were determined at lower nitrite concentrations below the half-saturation coefficient (KNO2). The higher SDNR rates due to methanol addition could be translated into 37% and 86% of the capital and operating cost savings for post and pre-anoxic bioreactor, respectively; hence the addition of methanol as a complementary carbon source for nitrite denitrification can be considered as a mainstream process even for conditions carbon is not limiting in the MWW.

Lastly, until today, understanding the fundamentals of partial denitrification (PDN) affecting nitrite accumulation over nitrate denitrification was not well understood. This includes characterizing partial denitrification versus complete denitrification with respect to kinetic parameters, microbiological composition and the overall denitrification performance. A study is conducted to investigate and compare the dynamic specific denitrification rates (SDNRs) from nitrate and nitrite at various COD/N ratios in two SBRs systems that use acetate. Both SBRs were 12 L with a flow of 18 L/d. SBR1 (47 d-SRT) and SBR2 (31 d-SRT) were operated on for 126 days, with HRT of 16 hrs, and fed with nitrite and nitrate, respectively. The maximum SDNRNO2 and SDNRNO3-NO2 during the cyclic tests were 0.69 and 2.67 mgN/mgVSS-d in SBR1 and SBR2, respectively. The optimum operating condition for PDN was found to be at minimum COD/N of 2.7-3 with 79% nitrite accumulation. Higher COD/N of 6.7 and 9.8 also accumulate nitrite, but nitrite accumulated was denitrified. Kinetic parameters were also determined for nitrite in offline batch experiments (half-saturation coefficient KNO2 and the maximum specific growth rate) of 0.77 d-1. The taxonomic analysis showed Zoogloea genus (42% RA) in SBR1 was more responsible for nitrite denitrification, while Thauera genus in SBR2 with RA of 10% was more responsible for PDN. We relate the difference in microbial structure to the NiR reductase and NaR reductase distribution among microbial structure, indicating NiR reductase enzymes are higher in Zoogolea genera than NaR reductase. In contrast, NaR is higher than NiR in Thauera. This study considered to be the first study to compare the microbial community structure for denitrification from nitrite and nitrate, which proves that denitrification from nitrate is much higher than from nitrite and nitrite accumulation will occur at high COD/N; however, this study clarified that more than 80% nitrite accumulation at high COD/N of 10.

Keywords: denitrification, partial denitrification, nitrite, nitrate, COD/N ratio, municipal wastewater, external carbon source, specific denitrification rate, microbial analysis.

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Co-Authorship Statement

This Ph.D. thesis contains material that is published or “under review” for publication in peer reviewed journals, as listed below.

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Title of the Paper: Effect of COD/N ratio on denitrification from nitrite
Authors: Ahmed Badia, Mingu Kim, George Nakhla, Madhumita B. Ray
Status: Published
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Chapter 5
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Table of Contents

LIST OF TABLES

List of figures

List of Acronyms

ANAMMOX: Anaerobic ammonium oxidation

AOB Ammonia-oxidizing bacteria

BNR: Biological nitrogen removal

CANON: Completely autotrophic nitrogen removal over nitrite

CSTR: Continuous stirred-tank reactor

FDN: Full denitrification (complete denitrification)

MABR: Membrane aerated biofilm reactor

MBR: membrane biofilm reactor

MBBR: Moving bed biofilm reactor

MWW: Municipal wastewater

NOB: Nitrite-oxidizing bacteria

NaR: Nitrate reductase enzyme

NiR: Nitrite reductase enzyme

N-PDN-anammox: nitrification-partial denitrification-anammox

OLAND: Oxygen limited nitrification denitrification

PNA: Partial nitrification-anammox

PNA-DN: Nitrite shunt (partial nitrification-denitrification)

SBR: Sequencing batch reactor

SHARON: Partial nitrification in continuous chemostat reactor

SND: simultaneous nitrification and denitrification

TKN: Total Kjeldahl Nitrogen

Nomenclature

Symbol	**Description**	**Units**
b/K_d	decay coefficient	d^{-1}
BOD	Biochemical oxygen demand	mgO2/L
COD	Chemical oxygen demand	mgO2/L
DO	Dissolved oxygen	mgO2/L
FA	Free ammonia	mgN/L
FNA	Free nitrous acid	mgN/L
F/M	Food to microorganisms	mgCOD/mgVSS-d
HRT	Hydraulic retention time	Hr
K_{NO2}	Monod half-saturation concentration for nitrite	mgN/L
$Ko^{/}$	DO-inhibition concentration	mgO2/L
MLSS	Mixed liquor suspended solids	mgSS/L
MLVSS	Mixed liquor volatile suspended solids	mgVSS/L
ORP	Oxidation reduction potential	mV
SCOD	Soluble COD	mgCOD/L
$SDNR_{NO2}$	Specific denitrification rate from nitrite	mgN/mgVSS-d
$SDNR_{NO3}$	Specific denitrification rate from nitrate	mgN/mgVSS-d
SNAR	Specific nitrite accumulation rate	mgN/mgVSS-d
SN	Soluble nitrogen	mgN/L
SNe	Effluent soluble nitrogen	mgN/L
SNi	Influent soluble nitrogen	mgN/L
SP	Soluble phosphorus	mgP/L
SRT	Solids retention time	D
SSUR	Specific substrate utilization rate	mgCOD/mgVSS-d
TCOD	Total COD	mgCOD/L
TN	Total nitrogen	mgN/L
TP	Total phosphorous	mgP/L
TSS	Total suspended solids	mgTSS/L
VSS	Volatile suspended solids	mgVSS/L
Y_{HD}	Biomass yield coefficient based on COD removed	mgVSS/mgCOD
Y_N	Biomass yield coefficient based on nitrite/nitrate removed	mgVSS/mgN
μ_{max}	Biomass specific growth rate	d^{-1}

Chapter 1

Introduction

1.1 Rational

The forms of Nitrogen in water bodies, in general, are organic nitrogen, ammonia, nitrate and nitrite, which have a deleterious effect on human health and in aquatic life, causing eutrophication. Moreover, intermediate nitrogen oxides have very harmful effects. Nitrite is very toxic to microorganisms compared with nitrate, nitric oxide (NO) has a toxic effect on bacteria, and nitrous oxide (N_2O) is one of the major greenhouse gases that cause ozone depletion, which is increasing globally at an alarming rate of 0.31% per year (Pan et al., 2013), and has radiative effect 300 times stronger than carbon dioxide. Therefore, the removal of nitrogen compounds from wastewater is essential.

The traditional biological nutrient removal (BNR) processes involve high dissolved oxygen (high energy), high quantities of external carbon source, larger footprint of the treatment facilities and huge sludge quantities that requires sludge treatment facilities. Recently, emerging BNR technologies, such as partial nitrification-anammox (PNA), nitrite shunt (partial nitrification-denitrification, PNA-DN), and nitrification-partial denitrification-anammox (N-PDN-anammox) are of researchers' interest because of their economic and sustainable values.Of these nitrite shunt and N-PDN-anammox have shown promise for mainstream municipal wastewater treatment; both processes require an understanding of denitrification from nitrite.

Extensive research has been conducted on denitrification from nitrate to determine the optimum process parameters (Beccari et al., 1983; Frison et al., 2013); however, not much work has been conducted on denitrification from nitrite as such, it has become an emerging topic. A review of the literature showed the electron distribution among the four types of nitrogen oxide (NO_3^-/NO_2^-/NO/N_2O) with methanol as electron donor, but the effect of internal carbon source (municipal wastewater) on the denitrification rates was not considered, which will provide better expectation in the design of biological nitrogen removal in wastewater treatment plants (Adav et al., 2010). Thus, the optimum COD/N for external and internal carbon source that provides the maximum

SDNR when nitrite is used as the electron acceptor must be determined (Ginige et al., 2009 and Akunna et al., 1992). Often due to low carbon to nitrogen ratio in internal carbon source (municipal wastewater), the denitrification process in full-scale wastewater treatment plants employs external carbon sources to minimize total nitrogen effluent. Previous research studied the effect of a mixture of carbon sources on nitrate denitrification. As per the literature review, the effect of the mixture of real wastewater and external carbon source on denitrification from nitrite has not yet been reported. Accordingly, an optimum ratio of external and internal carbon (municipal wastewater) sources, kinetics and potential competitive effects of the two types of carbon sources are unknown.

Partial denitrification is the latest advance in the second generation total nitroen (TN) removal processes. The process is used to partially denitrify nitrate and maximize nitrite accumulation that will be used as an electron acceptor in a downstream anammox process. In this process, the PDN, more specifically, the nitrite accumulation is the limiting step. A review of the literature showed different strategies to enhance nitrite accumulation during denitrification from nitrate (Du et al., 2016 and Ma et al., 2017). However, the fundamentals behind nitrite accumulation over nitrate denitrification were not well understood, and conflicting reports were observed concerning the impact of COD/N ratio, carbon source type, pH level and/or nitrate/nitrite concentration on the accumulation rate. As a result, robust engineering process control strategies were not identified. Therefore research comparing complete and partial processes would be required to understand the process kinetics and microbial diversity and ultimately contributing to a robust PDN process.

1.2 Thesis objectives

Denitrification from nitrite is a new trend, with still many challenges to overcome to achieve nitrite shunt and partial denitrification. Nitrite ion is very toxic to microorganisms, which requires careful control of the kinetic and operation parameters, and microorganisms require appropriate time to acclimatize to the electron donor (carbon source). Extensive research has been conducted on full denitrification from nitrate (FDN) to determine the optimum parameters; however, not much work has been conducted on denitrification from nitrite as the starting electron acceptor. Furthermore, partial denitrification from nitrate to nitrite (PDN) is a new trend. The following are the specific thesis objectives:

1. Investigating the effect of COD/N ratio on specific nitrite denitrification rate when the carbon source is municipal wastewater and studying the kinetic parameters as well as modelling a relationship between carbon source consumption rate to nitrite removal rate.
2. Exploring the long-term effect of methanol usage as a complementary carbon source on the specific denitrification rate and nitrite denitrification kinetics parameters for municipal wastewater and their economic value.
3. Comparing the long-term specific denitrification rate (SDNR) for nitrite and nitrate at lower COD/N ratio with acetate as a carbon source and characterizing kinetic parameters and microbial structure during complete versus partial denitrification of nitrite.

1.3 Thesis organization

The thesis is organized in article-integrated format, and thesis-chapters are presented below:

Chapter 1 includes a background of denitrification from nitrate, nitrite and partial denitrification. Research objectives are also included.

Chapter 2 is a literature review that includes the fundamental background of denitrification from nitrate and nitrite. The chapter also presents the operational and inhibition parameter, which affect the denitrification process.

Chapter 3 presents a research article named “Effect of COD/N ratio on denitrification from nitrite” A sequencing batch reactor (SBR) operated and continuously fed with primary effluent and nitrite. Influent MWW characteristics varied significantly during the study, i.e. 200-810 mgCOD/L and 6-80 mgN/L. The SDNR from the SBR were compared with those determined in four batch reactors using acetate. The SDNR was directly related to COD/N, relationships of SDNR with COD/N and F/M ratios were developed, and the kinetic parameters including biomass yield coefficients were determined.

Chapter 4 presents a research article named “Denitrification of nitrite using methanol as a complementary carbon source.” An SBR fed with primary effluent and methanol as complementary carbon source, and nitrite solution was operated at an HRT of 8.4 hrs and SRT of 16-17 days for 110 days. The SDNR from the SBR was compared with those determined in 23 batch reactors employing MWW, methanol, nitrite, and nitrate. A study of cost analysis for using methanol as a complementary carbon source with nitrite was also conducted.

Chapter 5 presents a research article named “Effect of low COD/N ratio on denitrification from nitrite and nitrate: the case for complete versus partial nitrite denitrification.” Two SBRs were operated for 126 days, SBR1 fed with nitrite and acetate and SBR2 fed with nitrate and acetate. Impact of process operating conditions such as COD/N ratio and DO level, nitrate and nitrite SDNRs and microbial yields were characterized from the longe term study. The specific denitrification rate from nitrite ($SDNR_{NO2}$), nitrate ($SDNR_{NO3}$) and nitrite accumulation rate (NAR) were studied at various COD/N ratios based on cyclic tests and the optimum operation condition for PDN was identified. The taxonomic analysis was also conducted to compare microbial structure in a nitrite versus partial nitrate denitrifying reactor.

Chapter 6 includes summaries conclusions and future research recommendations.

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Chapter 2

Literature Review

2.1 Introduction

Discharging nitrogen and phosphorus compounds in rivers or lakes can cause eutrophication, which clogs water mains, decomposes and causes dissolved oxygen depletion, and kills aquatic life. In addition, nitrogen compounds can have deleterious effects on human health. The primary health hazard from drinking water containing nitrate/nitrite-nitrogen is when nitrite exists in the human digestive system, or nitrate is reduced to nitrite during digestion. Generally, nitrite oxidizes iron, which is the main component of hemoglobin of the red blood cells, forming a condition called methemoglobin. This creates a condition known as " blue baby syndrome," in which blood cannot carry sufficient oxygen to the individual body cells causing the veins and skin to appear blue. Due to this health hazard, EPA (2009) stipulated that the maximum level of nitrate and nitrite should not exceed 10 mg/L and 1 mg/L, respectively in any wastewater discharged, and TP of 1 mg /L (EPA, 2009),. On the other hand, Constantine(2008) reported chronic and acute toxicity limits of ammonia as 0.02 and 0.1 mgNH_4^+/L, respectively. Therefore, the removal of nitrogen compounds from wastewater is essential.

Nitrogen in raw wastewater is mostly in the form of ammonia and organic nitrogen. In wastewater treatment plants majority of the nitrogen is removed by biological treatment and some by physical treatment. Typical nitrogen removing wastewater treatment plants employ first-generation nitrification and denitrification processes. X. Yang et al., 2012 reported that in biological denitrification, oxidized nitrogen compounds (nitrate and nitrite) are utilized as electron acceptors by a wide range of heterotrophic bacteria for their metabolic activity, and release nitrogen gas, which is harmless to the human health. This process is accompanied by the removal of organic carbon, which acts as an electron donor and carbon source. As denitrification occurs under anoxic conditions, the process is affected by various parameters such as dissolved oxygen (DO), type and availability of organic carbon, COD-to-nitrogen ratio, temperature, and pH. Most advances in nitrogen removal processes include the use of anaerobic ammonium oxidation (ANAMMOX) bacteria and forming either a combined partial nitritation-anammox (PNA) or partial denitrification-anammox (PDN-anammox) process for total nitrogen removal (Schmidt et al., 2003).

2.1.1 First-generation total nitrogen removal processes

The first generation nitrogen removal processes involve the conventional nitrification-denitrification pathway. There are many types of process configurations that are currently used for total nitrogen removal based on the nitrification-denitrification pathway. The most common include the original Ludzac-Ettinger, the Modified Ludzac-Ettinger (MLE) and Bardenpho configurations (Eddy et al., 2014). The MLE configuration consisted of a pre-anoxic denitrification reactor followed by an aerobic reactor. The process involves nitrification in the aerobic zone and recycling the nitrate that is produced in the aerobic zone to the anoxic zone where denitrification occurs. The MLE differs from the original Ludzac-Ettinger configuration, where the denitrification (anoxic) reactor is located post the aerobic reactor, often referred to as post-denitrification. In most post-denitrification configurations, the external carbon source is added to the anoxic zone to compensate for the carbon source that is already utilized (oxidized) in the aerobic zone. The most effective TN removal configuration is the Bardenpho that combines pre anoxic, aeration, post anoxic, and secondary aerobic zones with internal recirculation from the first oxic zone to the first anoxic zone, while denitrification in the second anoxic zone mainly relies on the carbon source released during endogenous respiration.

Carousel ditch is yet another process where simultaneous nitrification-denitrification (SND) occurs within the bio-floc at low DO (Eddy et al., 2014), with floc size ranges 80-100 µm. Figure 2-1 shows that autotrophic bacteria (*ammonia-oxidizing and nitrite-oxidizing bacteria*) grow on the activated sludge surface, while denitrifiers grow inside the bio-floc. This process is possible due to the dissolved oxygen gradient from outside (high DO, aerobic zone) to the inside (low DO) of the floc (anoxic zone).

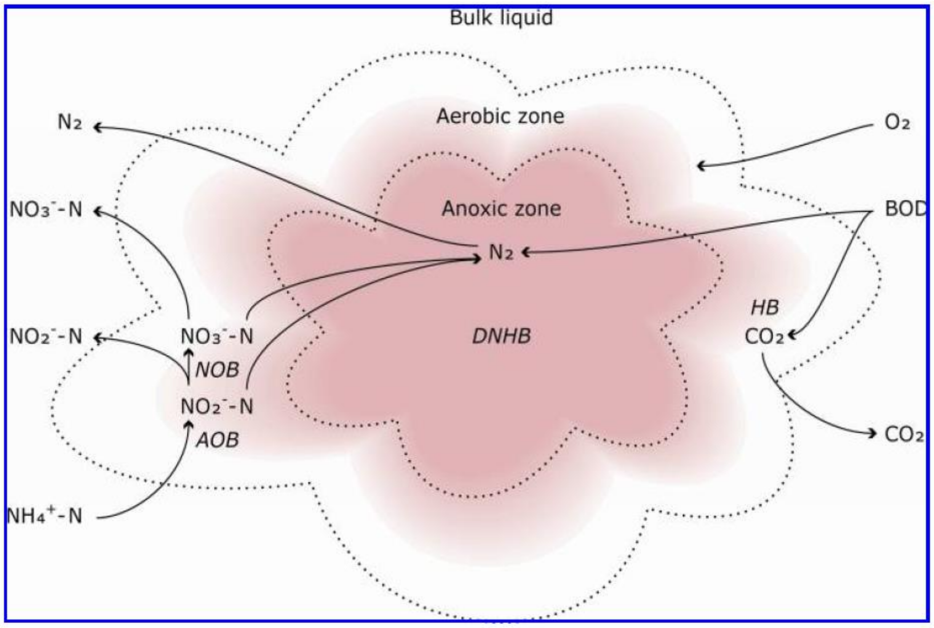

Figure 2. 1 Schematic diagram of simultaneous nitrification-denitrification (SND), **(S.-P. Sun et al., 2009)**

In conventional nitrification-denitrification processes, ammonia is nitrified to NO_2-N, and then it is oxidized to NO_3-N by autotrophic microorganisms (Figure 2-1). After that, the NO_3-N (electron acceptor) gets biologically reduced (denitrified) to nitrogen gas in the absence of dissolved oxygen by denitrifying heterotrophic bacteria (DNHB). Thus the NO_2-N and NO_3-N along with organic carbon (electron donor) removed in the anoxic environment (Capodaglio et al., 2016). The following is the denitrification stoichiometric bioreaction (equation 2-1) using acetate as a carbon source and ammonia as a nitrogen source (mole based):

$$1.0\ \mathrm{NO3}^- + 1.0\ \mathrm{H}^+ + 1.45\ \mathrm{CH3COO}^- + 0.33\ \mathrm{NH}_4^+ = \mathrm{N}_2 + 0.33\ \mathrm{C}_5\mathrm{H}_7\mathrm{NO}_2 + 1.12\ \mathrm{HCO}_3^- + 1.62\ \mathrm{H}_2\mathrm{O} + 0.13\mathrm{CO}_2 \qquad (2\text{-}1)$$

According to the above equation 2-1, the amount of acetate required is 6.5 g COD per g NO_3-N, the biomass produced is 0.4 g VSS per g of COD (2.67 g VSS/g NO_3-N), and the alkalinity produced is 3.57 g $CaCO_3$ per g of NO_3-N removed. A schematic diagram of the nitrogen cycle as it occurs in activated sludge treatment is shown in Figure 2-2. The figure includes nitrogen assimilation and dissimilation pathways. Several parameters should be controlled to achieve full nitrification (NH_4 to NO_3^-) or partial nitrification (NH_4 to NO_2^-). For full nitrification, a minimum bioreactor operating DO of 0.5-1.0 mg/L, pH of 7.8-8.2 and minimum alkalinity of 7.14 mg alkalinity as $CaCO_3$ / mg N oxidized is required. Similarly, for denitrification (NO_2^- to N_2), operating parameters including lower DO (< 0.5 mg/L) and internal and/or external carbon sources should be maintained.

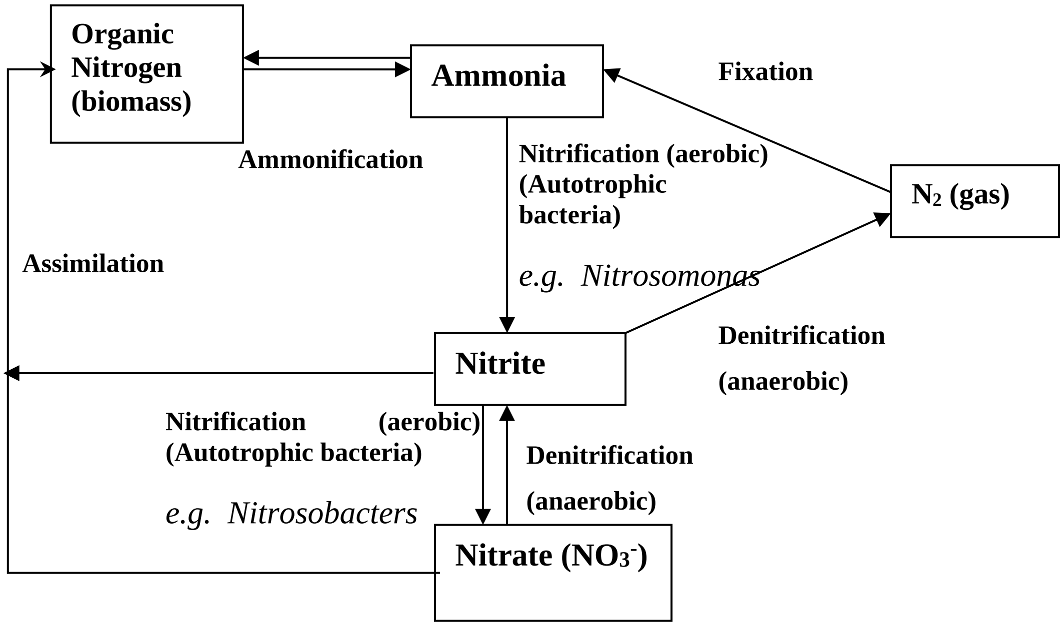

Figure 2. 2 Schematic of the nitrogen cycle in wastewater treatment plants

2.1.2 Second generation total nitrogen (TN) removal processes

The current research trend in TN removal is towards second-generation processes, which are configured based on partial nitrification or partial denitrification pathway. Processes under this category include the nitrite shunt, the partial nitrification-anammox, and the partial denitrification-anammox processes. Compared to the first-generation processes, the second generation processes are considered sustainable requiring limited aeration input, limited or no organic carbon requirement leaving the carbon for recovery, and minimal waste sludge generation. However, the full-scale application of these processes for mainstream wastewater has not yet been implemented.

In the nitrite shunt process, ammonia is first nitrified to nitrite according to the stoichiometric equation (Equation 2-4, Table 2-1) and then denitrified according to equation 2-4 (Table 2-1). The fundamental advantages of nitrite shunt over the traditional nitrification/denitrification include 25% lower oxygen demand and 40% less external carbon source (estimated based on Equation 2-

4, Table 2-1). The only drawback of denitrification from nitrite so far is nitrite toxicity to the microorganisms and the limited biodiversity of the microbial community (Simon & Klotz, 2013; Sun et al., 2009).

The PNA-anammox process started to take researchers' interests 20 years ago; the process saves 62.5% of oxygen, 90% of external carbon source, and substantial reduction in the sludge production. Approximately 50% of the ammonia is first oxidized to nitrite (which acts as an electron acceptor), which reacts biologically with the remaining 50% of ammonia to produce nitrogen gas (Equation 2-4, Table 2-1), by anammox bacteria according to the anammox stoichiometric equation shown below (equation 2-2):

$$1.0\ \mathrm{NH_4^+} + 1.32\mathrm{NO_2^-} + 0.066\ \mathrm{HCO_3^-} + 0.13\mathrm{H^+} = 1.02\mathrm{N_2} + 0.26\mathrm{NO_3^-} + 0.33\ \mathrm{CH_2O_{0.5}N_{0.15}} + 2.03\ \mathrm{H_2O} \tag{2-2}$$

It can be observed from the above equation that each mole of ammonia oxidized, one mole of nitrite reduced, and 0.26 mole of nitrate produced. The anammox bacteria are autotrophs and utilize carbon dioxide or alkalinity for their cell growth. Certain conditions are needed to partially nitrify to nitrite (Equation 2-4, Table 2-1); these conditions include pH, temperature, dissolved oxygen, sludge age, free ammonia concentration, and nitrous acid concentration. Park et al., 2010 has combined these parameters in one contour-graph at a specific temperature in order to accumulate nitrite by allowing the ammonia-oxidizing bacteria (AOB) to grow and the nitrite-oxidizing bacteria (NOB) to washout. The partial nitrification-anammox process has been successfully implemented in full-scale to treat ammonia-rich streams (the sidestream wastewater). Current full-scale installations use various reactor configurations, including granular sludge sequential batch reactor (single sludge), Sharon-anammox (two sludge), suspended granular sludge anammox (single sludge) and moving bed bioreactor (single sludge). To date, the PNA process is not implemented for mainstream wastewater treatment. Demonstrating a robust PNA process has been a challenge due to the inherent mainstream wastewater treatment conditions, which favour nitrite-oxidizing bacteria domination over anammox bacteria for nitrite. Due to this limitation, current research has been shifted to the partial denitrification anammox (PDNA) process. In partial denitrification, ammonia is allowed to be nitrified fully to nitrate (equations 2-4 and 1-5, Table 2-1), then partially denitrified to nitrite at a condition that maximizes nitrite accumulation. The process saves 45% oxygen (aeration energy) and 79% of the external carbon source. The main advantage of PDN-anammox over the PNA-anammox is that it does not need as much control to wash out NOBs. The following stoichiometric equation (equation 2-3) shows the partial denitrification reaction from nitrate (Du, 2019):

$$1.32\ NO_3^- + 0.55\ CH_3COO^- + 0.088\ NH_4^+ = 1.32\ NO_2^- + 0.088\ C_5H_7NO_2 + 0.66\ HCO_3^- + 0.198\ H^+ + 0.264\ H_2O \qquad (2\text{-}3)$$

Table 2.1 Stoichiometric Nitrification/Denitrification **(Paredes et al., 2007)**)

No.	**Process**	**Biological conversion**
2-4	Nitritification	$NH_4^+ + 1.5\ O_2 + 2\ HCO_3^- = NO_2^- + 2CO_2 + 3\ H_2O$
2-5	Nitratation	$NO_2^- + 0.5\ O_2 = NO_3^-$
2-4+2-5	Nitrification	$NH_4^+ + 2\ O_2 + 2\ HCO_3^- = NO_3^- + 2\ CO_2 + 3\ H_2O$
2-6	Denitratation	$2\ NO_3^- + C = 2NO_2^- + CO_2$
2-7	Denitrification via Nitrite (Denitritification)	$4\ NO_2^- + 3\ C + 2\ H_2O + CO_2 = 2\ N_2 + 4\ HCO_3^-$
2-6+2-7	Denitrification	$4\ NO_3^- + 5\ C + 2\ H_2O = 2\ N_2 + 4\ HCO_3^- + CO_2$
2-8	Partial nitrification (50% conversion)	$NH_4^+ + 0.75\ O_2 + HCO_3^- = 0.5NO_2^- + 0.5\ NH_4^+ + CO_2 + 1.5\ H_2O$
2-8a	Anammox (without cell synthesis)	$NH_4^+ + NO_2^- = N_2 + 2\ H_2O$
2-8b	Anammox (with cell synthesis)	$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- = 1.02\ N_2 + 0.26\ NO_3^- + 0.66\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$
2-4 + 2-5+ 2-6+2-7	Traditional Nitrification Denitrification	$4\ NH_4^+ + 8\ O_2 + 5\ C + 4\ HCO_3^- = 2\ N_2 + 9\ CO_2 + 10\ H_2O$
2-4+2-8a	CANON	$NH_3 + 0.895\ O_2 = 0.12\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$
2-9	OLAND	$NH_4^+ + 0.75\ O_2 = 0.5\ N_2 + H^+ + 1.5\ H_2O$

2.1.3 Denitrification process fundamentals

The complete heterotrophic denitrification process consists of sequential reductive reactions from nitrate NO_3^- to nitrite NO_2^-, nitric oxide (NO), nitrous oxide (N_2O), and finally to nitrogen gas (N_2). The process involves four different enzymes responsible for denitrification reduction: nitrate reductase (Nar), nitrite reductase (NiR), NO reductase (NoR), and N_2O reductase (NoS) (Pan, Ni,

& Yuan, 2013). These enzymatic reduction reactions are shown in the following enzymatic reaction sequence (H. Sun et al., 2009):

$$NO_3^{-}\text{-}N \xrightarrow{NaR} NO_2^{-}\text{-}N \xrightarrow{NiR} NO\text{-}N \xrightarrow{NoR} N_2O\text{-}N \xrightarrow{NoS} N_2$$

NO_2^-, NO, and N_2O are obligate intermediates of heterotrophic denitrification. Under typical denitrifying conditions found in a biological wastewater treatment process, NO and N_2O reductases have higher maximum nitrogen turnover than NO_3^- and NO_2^- reductases (Law et al., 2012) observed that the maximum N_2O reduction rate is almost four times faster than the NO_3^- and NO_2^- reduction rate.It is generally considered that the nitrogen reduction rate decreases as the oxidation state increases. As such nitrite reduction rate is faster than nitrate, the nitric oxide reduction rate is faster than nitrite, and nitrous oxide reduction rate is faster than nitric oxide, implying that during denitrification to nitrogen gas, no accumulation of the intermediates occurs. Pan et al., 2012 found that the maximum specific denitrification rates (SDNR) for NO_3^-, NO_2^-, N_2O are 45 (pH=7.0), 86 (pH=7.5), 340 (pH=8.0) mg N/g VSS-hr, respectively.

Some general properties of the biologically relevant nitrogen compounds, which reflect toxicity levels to microorganisms and their chemical properties, are listed in Table 2-2. Nitrogen-based catabolism, including properties of respiration as well as detoxification of harmful nitrogen transformers and enzymes ("reactive nitrogen species"), were explained by Simon & Klotz, 2013. There are detrimental effects of these by-products to the environment. For example, nitric oxide (NO) has a toxic effect on bacteria, and nitrous oxide (N_2O) is one of the major greenhouse gases that cause ozone depletion. N_2O is increasing globally at an alarming rate of 0.31% per year (Pan, Ni, & Yuan, 2013) and has a radiative effect of 300 times stronger than carbon dioxide.

Many factors such as external and internal carbon amount and type (COD/N), electron acceptor type ($NO_3^-/NO_2^-/NO/N_2O$), pH, alkalinity, temperature, and DO concentration affect denitrification. Carbon limitation has been widely reported as a condition leading to incomplete denitrification (Ginige et al., 2009). In wastewater treatment plants, carbon originates mostly from the influent wastewater. The availability of carbon sources compared with nitrate/nitrite is often expressed as the COD (chemical oxygen demand) or BOD (biochemical oxygen demand) to N ratio (COD/N, BOD/N) (Eddy et al., 2014). The COD/N ratio should be ≥ four to achieve a high level of nitrogen removal from nitrate (estimated from Equations 2-6 and 2-7, Table 2-1) and ≥

three from nitrite (Equation 2-7, Table 2-1). However, there is often an inadequate quantity of carbon in the influent wastewater for full denitrification in the treatment plants.

Table 2.2 Properties of nitrogen compounds **(Simon & Klotz, 2013)**

Nitrogen compound	**Oxidation state**	**Toxicity to microbial cells**	**Other properties**
Nitrate (NO_3^-)	+5	Non-toxic in physiological concentrations	The anion of strongly oxidizing and toxic nitric acid (HNO_3; pKa=−1.4).
Nitrogen dioxide (NO_2)	+4	Toxic orange gas. Arises from the oxidation of nitric oxide by oxygen	In equilibrium with the colorless gas dinitrogen tetroxide (N_2O_4).
Nitrite (NO_2^-)	+3	Toxic; binds to cellular iron atoms, for example, in hemoglobin.	Forms nitrosonium in acidic conditions according to $HNO_2+H^+ \rightarrow NO^+ + H_2O$. The anion of unstable nitrous acid (HNO_2; pKa=3.4).
Nitric oxide (NO)	+2	Highly reactive toxic radical. Binds to heme iron atoms and Fe/S centers and forms dinitrosyl iron complexes [$Fe(NO)_2$]. Causes nitrosation of thiol groups to form S-nitrosothiols (-S-N=O).	Generation of nitrogen dioxide (NO_2) or peroxynitrite ($ONOO^-$) in the presence of oxygen and superoxide radicals, respectively. Redox-related reactive species are the nitroxyl (NO^-) and nitrosonium (NO^+) ions.
Nitrous oxide (N_2O)	+1	Chemically inert and non-toxic in physiological concentrations	--
Dinitrogen (N_2)	0	Chemically inert and non-toxic.	--
Hydroxylamine (NH_2OH)	−1	Toxic by binding to heme groups.	--
Hydrazine (N_2H_4)	−2	Highly toxic.	--
Ammonium(NH_4^+)	−3	Non-toxic in physiological concentrations.	Cation of ammonia (NH_3), which is a toxic uncoupling agent (pKa=9.25).

Previous studies have monitored greenhouse gas emissions such as NO or N_2O in denitrifying bioreactors that are operated under lower organic carbon to nitrogen ratios. Pan, Ni, & Yuan, 2013 reported this ratio as 1.5 or less, as this ratio was not sufficient for denitrification. A previous study by (Pan, Ni, & Yuan, 2013), who also mentioned that 20% of N_2O was accumulated during denitrification with methanol as a carbon source and 40% accumulated during the endogenous denitrification phase (pH 6.0), while no accumulation of N_2O with methanol and 30% accumulation in endogenous phase at pH 6.5. Hanaki et al. (1992) observed that 10% of N_2O emissions at the same COD/N (1.5) when acetate was used as a carbon source, 20 to 30% of the nitrogen load was emitted as N_2O at COD/N ratios below 3.5, accompanied by nitrite accumulation. Kishida et al., 2004 observed that the N_2O emission rate at a BOD_5/N ratio (five-day BOD to N ratio) of 2.6 was 270 times higher than that at a BOD_5/N ratio of 4.5 in swine wastewater. Alternatively, denitrification from nitrite with an external carbon source in the pre- and post-anoxic bioreactors do not seem to produce these intermediates, which needs further investigation.

2.2 Microbiology and kinetics in denitrification

2.2.1 Heterotrophic-denitrifiers and enzymatic reactions

Microbial community and structure and the associated specialized enzymes play an essential role in the biological treatment. The most common denitrifiers are heterotrophic bacteria. H. Sun et al., 2009 summarized the characteristics of denitrifying species (Table 2-3). There are specialized microbes that degrade specific organic carbon sources such as *methanotrophs* that consume methane as electron donor and nitrate or nitrite as electron acceptor (He et al., 2015), and *methylotrophs* that consume methanol (Cherchi et al., 2008), *Saccharibacteria* for glucose (Yan et al., 2019). Moreover, some genera are more specialized for electron acceptor type, such as *zoogloea* for nitrite (Chai et al., 2019).

Table 2.3 Summary of characteristics of denitrifiers for nitrate and nitrite reduction **(H. Sun et al., 2009)**

Species	**Denitrification characteristic of bacteria**	**Reduction rates comparison**
P flavobacterium sp	Nitrate reduction, nitrite reduction, no nitrite accumulation	$r_{Red,\ NO_3\text{-}N} < r_{Red,\ NO_2\text{-}N}$
P fluorescens	Nitrate reduction, nitrite reduction, nitrite accumulation	$r_{Red,\ NO_3\text{-}N} > r_{Red,\ NO_2\text{-}N}$
Alcaligene	Nitrate reduction, nitrite accumulation, nitrite as the only final product	--
Acinetobcter Pseudomonas	Nitrate reduction, nitrite reduction, no nitrite accumulation, for coexisting nitrate and nitrite preferential reduction of nitrite	$r_{Red,\ NO_3\text{-}N} < r_{Red,\ NO_2\text{-}N}$
Alcaligenes	Nitrate reduction, nitrite reduction, no nitrite accumulation with glucose	$r_{Red,\ NO_3\text{-}N} > r_{Red,\ NO_2\text{-}N}$

Ruiz et al., 2006 have concluded in their research using the up-flow sludge blanket reactor (USB) technology that nitrite accumulation can occur during nitrification at dissolved oxygen (DO) concentration of 1 mg /L. They also examined the acclimatization period of the denitrifying microorganisms. In this case, denitrifiers that were previously adapted to a lower nitrite concentration can be acclimatized to a high nitrite feed as long as the nitrite concentration remains low inside the reactor. Pishgar et al., 2019 studied the dynamicity of microbial structure for nitrite and nitrate denitrification in two up-flow anaerobic/oxic/anoxic/oxic granular SBRs (18 L each, 4-hr-cycle, 6.7-HRT). The authors divided denitrifiers into four groups (Table 2-4): (i) complete aerobic denitrifiers: *Dokdonella*, *Flavobacterium,* and *Accumulibacter*; (ii) complete anoxic denitrifiers: *Acinetobacter*, *Pseudomonas*, *Arcobacter, and Comamonas*; (iii) incomplete nitrite denitrifier: *Diaphorobacter* (aerobic/anoxic) and (iv) incomplete nitrate denitrifiers: *Thauera* (aerobic/anoxic) and *Zoogloea* (strictly-aerobic). A more detailed literature regarding the microbial community is presented in Chapter 5.

Table 2.4 Classification of putative complete and incomplete denitrifiers from the ecophysiological perspective **(Pishgar et al., 2019)**.

Complete denitrifier		**Incomplete denitrifier**			
Aerobic	Anoxic	Aerobic via NO_2^-	Aerobic via NO_3^-	Anoxic via NO_2^-	Anoxic via NO_3^-
Dokdonella	*Acinetobacter*	*Diaphorobacter*	*Zoogloea*	*Diaphorobacter*	*Thauera*
Flavobacterium	*Pseudomonas*	*Acinetobacter*	*Thauera*	*Ca. Microthrix*	*Dechloromonas*
Ca. Accumulibacter	*Arcobacter*	*Rhodobacter*	*Haliangium*	*Acholeplasma*	*Flavobacterium*
Ca. Microthrix	*Comamonas*		*Hydrogenophaga*		*Cloacibacterium*
Simplicispira	*Paludibacter*				*Erysipelothrix*
Dechloromonas	*Simplicispira*				*Fusibacter*
Rhodoferax					

2.2.2 Denitrifier kinetics: SDNR and Half saturation coefficient

Specific denitrification rate (SDNR, mgN/mgVSS-d) is the removal of nitrite or nitrate as N concentration normalized to the time (mgN/L.d) and divided by the biomass concentration as (mgVSS/L). Knowing the SDNR allows sizing the denitrification bioreactor; however, other factors affect SDNR. Half saturation concentration (Ks) is the concentration at which specific growth rate and SDNR is 50% of the maximum specific growth rate ($\mu_{max,}$ 1/d). The maximum SDNR can be determined at the lab by conducting kinetic batch experiments in the absence of oxygen using excess carbon source concentration to avoid interference between K_N and K_{COD} (Equations 2-10 through 2-12 and Table 2-5), and a pH between 7-8 (low pH may induce nitrous acid inhibition).

Table 2.5 Half-saturation coefficients and stoichiometric coefficients depending on the limiting substrate. **(Shaw, 2015)**)

Limiting component (S)	**Half saturation coefficient (K)**	**Stoichiometric coefficient (Stcoeff.)**	**Process**
S_{NO3}	K_{NO3}	$(1 – Y_{Anox})/(2.86\ Y_{Anox})$	Denitrification, nitrate-limited
S_{NO2}	K_{NO2}	$(1 – Y_{Anox})/(1.72\ Y_{Anox})$	Denitrification, nitrite-limited
S_S	K_S	$1/Y_{Anox}$	Denitrification, carbon-limited
S_{O2}	K_{O2}	$(1 – Y_{Aer})\ /Y_{Aer}$	Oxygen uptake, oxygen-limited

Dissolved oxygen with small concentration (0.1- 0.2 mg/l) can inhibit the enzymatic reduction of nitrate and nitrite in the denitrification process, as shown below (Eddy et al., 2014):

$$\mathrm{SDNR}_{\mathrm{NO3}} = \left(\frac{1-1.42\mathrm{Y_h}}{2.86}\right)\left[\frac{\mu_{\mathrm{hmax}}*\mathrm{S_s}}{\mathrm{Y_h}\ (\mathrm{K_s}+\mathrm{S_s})}\right]\left(\frac{\mathrm{S_{NO3}}}{\mathrm{K_{NO3}}+\mathrm{S_{NO3}}}\right)\left(\frac{\mathrm{K'_o}}{\mathrm{K'_o}+\mathrm{S_o}}\right)(\eta)\left(\frac{\mathrm{X_h}}{\mathrm{X_{vss}}}\right) \tag{2-10}$$

$$\mathrm{SDNR}_{\mathrm{NO2}} = \left(\frac{1-1.42\mathrm{Y_h}}{1.72}\right)\left[\frac{\mu_{\mathrm{hmax}}*\mathrm{S_s}}{\mathrm{Y_h}\ (\mathrm{K_s}+\mathrm{S_s})}\right]\left(\frac{\mathrm{S_{NO2}}}{\mathrm{K_{NO2}}+\mathrm{S_{NO2}}}\right)\left(\frac{\mathrm{K'_o}}{\mathrm{K'_o}+\mathrm{S_o}}\right)(\eta)\left(\frac{\mathrm{X_h}}{\mathrm{X_{vss}}}\right) \tag{2-11}$$

$$\mathrm{SDNR}_{\mathrm{NO3-NO2}} = \left(\frac{1-1.42\mathrm{Y_h}}{1.14}\right)\left[\frac{\mu_{\mathrm{hmax}}*\mathrm{S_s}}{\mathrm{Y_h}\ (\mathrm{K_s}+\mathrm{S_s})}\right]\left(\frac{\mathrm{S_{NO2}}}{\mathrm{K_{NO2}}+\mathrm{S_{NO2}}}\right)\left(\frac{\mathrm{K'_o}}{\mathrm{K'_o}+\mathrm{S_o}}\right)(\eta)\left(\frac{\mathrm{X_h}}{\mathrm{X_{vss}}}\right) \tag{2-12}$$

Where SDNR is specific denitrification rate from nitrate or nitrite (mg N/mgVSS-hr), $\boldsymbol{\mu}_{hmax}$ is the maximum specific growth rate of heterotrophic-denitrifiers (1/hr), Y_h is the yield coefficient for heterotrophs for specific carbon source (for methanol Y_h= 0.18 to 0.2 mg VSS cells produced /COD removed), Ks is substrate utilization half-saturation coefficient (mg/L), Ss is the carbon source (methanol, ethanol, acetate, and glycerol) concentration in mg/L, S_{NO3} is the concentration of nitrate (mg/l), K_{NO3} is the half-saturation concentration of nitrate in mg/L, K_o' is the inhibiting DO concentration (ranges between 0.1 to 0.2 mg/L for nitrate), η is the fraction of biomass able to degrade the external carbon with nitrate reduction, X_h is the biomass concentration in the mixed liquor suspended solids (mg/L), and X_{vss} is the concentration of volatile mixed liquor suspended solids (mg/L).

A similar equation can be applied to determine the SDNR from nitrite with the modification of a stoichiometric coefficient of 2.86 by 1.72, while the coefficient is 1.14 for partial denitrification. Moreover, the DO inhibition coefficient K_o' is only reported for denitrification from nitrate. To the best of our knowledge, no values were reported when denitrification occurred from nitrite, also under the partial denitrification condition. Furthermore, not many studies were conducted to determine kinetic parameters such as $\boldsymbol{\mu}_{hmax}$, K_{NO2}, and Θ (Arrhenius coefficient) for denitrification from nitrite with an external and internal carbon source. Dosta et al., 2007 reported K_{NO2} and K_{NO3} concentrations (half-saturation constant for nitrite and nitrate) of 0.9 and 1.5 mgN/L, respectively. Kornaros et al., 1996 reported these values as 0.28 mg NO_2^--N/L and 0.77 mg NO_3^--N/L with L-glutamic acid as carbon source. Therefore, nitrite has higher denitrification rates when both nitrite and nitrate have a similar concentration (Pan et al., 2012).

Eddy et al., 2014 reported simplified equations to calculate SDNR for denitrification from nitrate with methanol and ethanol as an external carbon source with different Arrhenius coefficients. These rates can be amended for denitrification from nitrite.

For methanol: $\text{SDNR} = 0.0738\ (1.11)^{T-20}$ (2-13)

For ethanol: $\text{SDNR} = 0.161\ (1.13)^{T-20}$ (2-14)

However, these equations cannot be used in the design of pre-anoxic zones where external carbon source is added as a complementary carbon source, in addition to the influent wastewater main carbon source. In case of the pre-anoxic denitrification zone, Eddy et al., 2014 recommended the use of the following equations to calculate SDNR:

$$\text{SDNR}_{20} = (F/M)\ (F_b/0.3) + 0.029 \tag{2-15}$$

$$F_b = \left[\frac{\frac{Y_h}{1+B_h(SRT)}}{\frac{Yh}{1+B_h(SRT)}+Y_i}\right] \tag{2-16}$$

Where: (i) SDNR (specific denitrification rate at 20^0C in g NO_3^--N/g MLVSS-d), (ii) F/M is g COD applied/g MLSS-d in the anoxic zone, (iii) Y_h is the yield of heterotrophic biomass (0.67 g VSS/g COD removed-domestic sewage), (iv) B_h is endogenous decay rate for heterotrophs g VSS/g VSS-d, (v) F_b is the active biomass fraction, and (vi) Y_i is the inverse VSS fraction of non-biodegradable g nbVSS/g COD (0.1-0.3 for secondary treatment with primary treatment, and 0.3-0.5 without primary treatment).

2.2.3 Denitrifier kinetics: Denitrifiers yield

The yield coefficient Y_{HD} is defined as the amount of biomass produced divided by the COD removed ($mgVSS_{produced}/COD_{removed}$). The $VSS_{produced}$ can also be presented as COD of the biomass produced multiplying by 1.42 (1.42 mgCOD/mgVSS), it can also be calculated in relation to N removed (mg $VSS_{produced}$/mg $N_{removed}$) (Eddy et al., 2014). The relation between Y, SDNR, and μ_{max} is $Y_N = \mu_{max}/SDNR$. The relation between Y_N and Y_{HD} can be calculated as per the following equation:

$$Y_N = \frac{1-1.42Y_{HD}}{ST\ Y_{HD}} \tag{2-17}$$

Many factors affect the yield coefficient, such as carbon source type and an electron acceptor (oxygen, nitrate, nitrite), as shown in Table 2-6. Frison et al., 2013 estimated the total consumption of COD per gram of nitrite converted to nitrogen gas as 1.72, which is obtained from half-reaction (Eddy et al., 2014) and equivalent to 2.86 for nitrate, 1.72 for nitrite and 1.14 for partial denitrification (denitrification from nitrate to nitrite); hence, the heterotrophic sludge yield can be calculated as follows:

$$Y_{HD(NO3-N2)} = 1 - \frac{2.86\ NO2-N_{reduced}}{COD_{consumed}} \tag{2-18}$$

$$Y_{HD(NO2-N2)} = 1 - \frac{1.72\ NO2-N_{reduced}}{COD_{consumed}} \tag{2-19}$$

$$Y_{HD(NO3-NO2)} = 1 - \frac{1.14\ NO2-N_{reduced}}{COD_{consumed}} \tag{2-20}$$

Where Y_{HD} is the yield coefficient for anoxic heterotrophs (g COD cells produced/g COD removed), Frison et al., 2013 also estimated the Y_{HD} for acetic acid and glycerol 2.1 and 2.3, respectively.

Table 2.6 Effect of the investigated external carbon sources on COD consumption and sludge yields. **(Frison et al., 2013)**)

Parameter	**Acetic acid (80%)**	**Glycerol**	**OFMSW liquid drainage**	**OFMSW fermentation liquid**	**CM&MS fermentation liquid**
Specific COD consumption **(KgCOD kgNO_2-N $^{-1}$)**	2.1	2.3	2.7	3.1	3.0
Sludge yields in anoxic phase (Y_{HD}) (KgVSS kgCOD^{-1})	0.18	0.24	0.36	0.45	0.31

2.3 Denitrification from nitrite

During a nitrite shunt process, ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB); nitrite is then consumed by heterotrophic denitrifiers in an anoxic environment as an electron acceptor with an organic carbon source (electron donor) such as methanol, ethanol, acetate, glycerol and glucose. The process requires the competitive advantage of denitrifying bacteria over nitrite-oxidizing bacteria (NOB). The advantages of the process include: (i) 25% process air reduction, (ii) 40% external carbon saving, (iii) reduced biological sludge production, which intern reduces sludge treatment equipment sizes (Figure 2-3), (Bilyk et al., 2011)) and (iv) an overall reduction of aerobic and anoxic bioreactor size and the footprint. However, the process has challenges during the first partial ammonia nitrification to nitrite step and also during the subsequent denitrification from nitrite step, the latter associated with nitrite toxicity to the microorganisms (Ruiz et al., 2006). Some researches indicated that denitrification from nitrite requires certain acclimatization periods under specific operational conditions (Chung & Bae, 2002). The nitrite denitrification process is carried out anoxically by a bacteria that induce nitrite reductase enzyme (NiR), mostly in the cytoplasm rather than in periplasm, which limits the induction of nitrate reductase enzyme (NaR) (Mellor et al., 1992). Detailed literature in denitrification from nitrite can be found in Chapters 3 and 4, including a summary of the literature in Table 3-1.

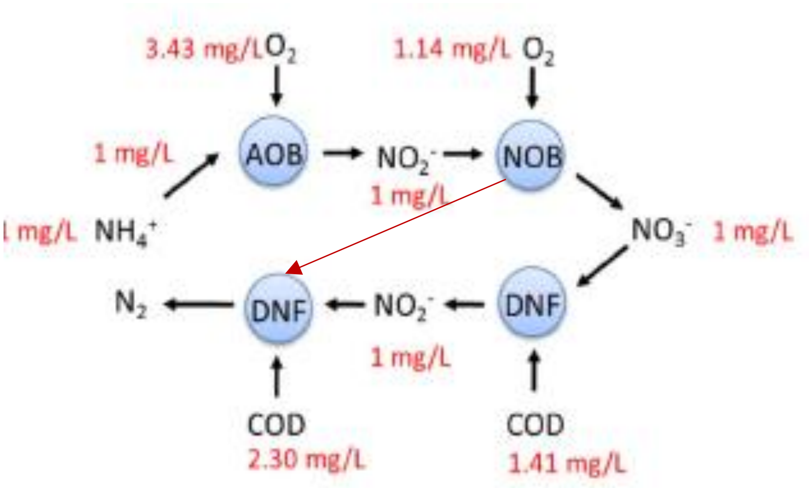

Figure 2.3 Nitrification/Denitrification through nitrite pathway **(Ma et al., 2016)**

2.3.1 Effect of pH and use of ORP on nitrite-denitrification process

The pH and ORP play a vital role during denitrification from nitrite. In this process, alkalinity (3.57 mg$CaCO_3$) is produced during denitrification from nitrite resulting in a pH increase (Eddy et al., 2014). Pan et al., 2012 conducted denitrification from nitrite and nitrate study in a sequencing batch reactor (SBR) (6 hr-cycle) and found that substantial N_2O accumulation occurs at relatively lower pH levels of 6.0 to 6.5. The maximum specific nitrate and nitrite reduction rates vary with pH, and the highest specific denitrification rates of 1.08 and 2.06 mg N/mg VSS-d occurred at 7.2 to 7.8 pH, respectively. However, they did not study this effect with various types of external carbon sources and COD/N ratios and different pH values.

S. Ge et al., 2012 examined denitrification from nitrate and the denitrification from the produced nitrite on the nitrate denitrification-pathway with various carbon sources (glucose, acetate, methanol). The authors relied on the use of pH and ORP control strategies. Their study confirmed that the alkalinity produced during denitrification is mainly from nitrite denitrification (not from nitrate). Hence the pH increase is due to OH^- produced during denitrification from the nitrite denitrification process, as shown in the following denitrification equations:

$$3NO_3^- + CH_3OH \rightarrow 3NO_2^- + 2H_2O + CO_2 \qquad (2\text{-}21)$$

$$2NO_2^- + CH_3OH \rightarrow N_2 + H_2O + CO_2 + 2OH^- \qquad (2\text{-}22)$$

Figure 2-4 variation of ORP, pH and nitrogen concentration with methanol as a carbon source (S. Ge et al., 2012). The study showed a gradual pH increase during the initial reaction stage. The CO_2 acidity produced by nitrate reduction is counteracted by alkalinity production followed by hydroxide alkalinity generation resulting in a relatively sharp pH increase to the peak pH of 8.5 point-B (Figure 2.4). The increase in pH was due to the denitrification of nitrite (Equation 2-22).

Overall, pH could be chosen as a control parameter during denitrification (e.g. SBR process) to indicate the end of the nitrate or nitrite reduction and the starting point for denitrification from nitrite as well as to start the aeration-nitrification. Moreover, the ORP for both denitrification phases has two major inflection points, A and B, corresponding to pH values (Figure 2.4). They also proposed that carbon source addition and denitrification could be controlled by monitoring these inflection points in the pH and ORP curves without additional chemical analyses.

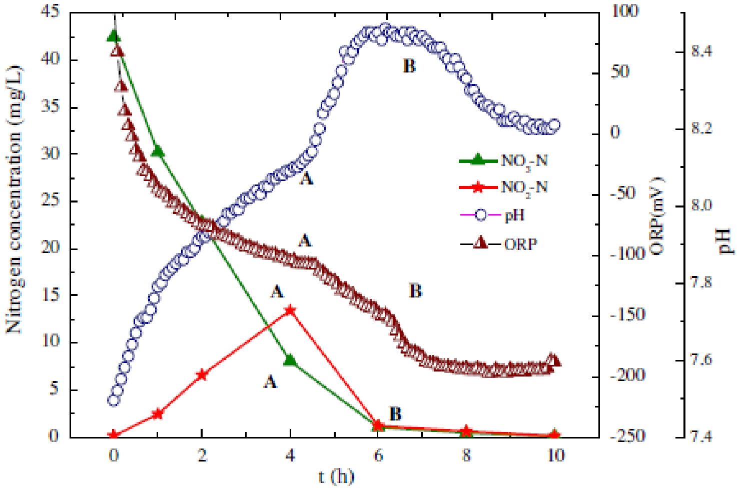

Figure 2.4 Variation of ORP, pH and nitrogen concentration with methanol as a carbon source. (Ge et al., 2012)

During denitrification, electrons are transferred from the organic carbon source (electron donor) to the electron acceptor (nitrite) until it reaches the equilibrium. During this process, the oxidation-reduction potential (ORP) can measure electrons transferred in m.volt during the process. However, because this process is carried out by the bacteria and the surrounding environment, the equilibrium conditions will never be reached (Cheng et al., 2012). In the aerobic process, oxygen is the electron acceptor; the ORP reads +ve. In contrast, in the anaerobic or anoxic process, the ORP reads -ve, further ORP in the biological process will never read zero because the biological reaction is always spontaneous. H. Sun et al., 2009 calculated ORP based on equation 2-23, where E^0 is the standard oxidation-reduction potential (V), n is the numbers of transferred electrons, R is the molar gas constant, 8.314 J/(mol·K), T is the temperature(K); F is Faraday constant, 96487 C/mol, [Red] concentration of the reduced species, [Ox] is the concentration of the oxidized species.

$$E = E^o - \frac{2.303\ RT}{nF} \log \frac{(Red)}{(OX)} \qquad (1\text{-}23)$$

The reduction of NO_3^- and NO_2^- and the number of electrons involved in the reactions are shown below:

$$NO_3^- + 2H^+ + 2e \xrightarrow{NaR} NO_2^- + H_2O \quad (-82.9\ KJ/mol) \qquad (2\text{-}24)$$

$$NO_2^- + 4H^+ + 3e \xrightarrow{NiR} \frac{1}{2} N_2^- + 2H_2O \quad (-277\ KJ/mol) \qquad (2\text{-}25)$$

These equations confirm that the rate of denitrification from nitrite to nitrogen gas is more spontaneous (-277 KJ/mol) than denitrification from nitrate to nitrite (-82.9KJ/mol).

The bioreactor pH also impacts the generation of free nitrous acid (FNA) concentration in a denitrifier reactor. FNA is one of the most inhibitory parameters in the denitrification reaction, and its generation is based on the following equation (equation 2-26) illustrated by Anthonisen et al., 1976, who reported a minimum concentration of 0.2 mgFNA/L for complete denitrification inhibition. Y. Zhou et al., 2011 suggested at the FNA concentration range of 0.01 to 0.025, 40% inhibition may occur during denitrification from nitrate, mainly when nitrite ion exists (i.e. competition between two-electron acceptors). Zhou also suggested that at the same levels of FNA, a similar level of inhibition might occur when denitrifying from nitrite. On another note, FNA inhibition has not been reported for domestic wastewater due to the lower nitrite concentration (20-30mg/L) and the neutral pH (7.0).

$$FNA\ mg/l = \frac{47}{14} \frac{NO_2{-}N\ mg/l}{K_a x\ 10^{pH}} \qquad (1\text{-}26)$$

Where K_a is the ionization constant of the nitrous acid, K_a varies with temperature as $K_a = e^{-2300/(273+T)}$ and T is the temperature in ^0C.

2.3.2 Denitrification kinetics (yield, half-saturation, growth rate)

Denitrification kinetics are model input parameters, which describe specific denitrification rates (SDNR) according to the Monod model, which allows the design of biological treatment facilities. The well defined kinetic parameters are half-saturation concentration (K_{NO3} and K_{NO2}, mg N/L), maximum specific growth rate (μ_{max} , 1/d) and the yield coefficient (mg VSS/mg COD or mg VSS/mg N) (Kornaros et al., 1996). Karanasios et al. (2010) summarizes these parameters with hydrogen as electron donor, as it is shown in Table 2-7, these kinetic parameters varied widely and depends on the operation conditions such as temperature, initial nitrite or nitrate concentrations, carbon source type and concentrations, and the biomass type and diversity which has the highest effect on the SDNR. It is also important to note that the literature values are highly variable owing to the difficulty of reproducibility of any kinetic test due to the change of microbial diversity and measurement accuracy. There is minimal literature on yield data of denitrification from nitrite, especially with MWW as a carbon source. However, the available literature is for complete denitrification from nitrate with external carbon sources such as acetate: Yalda Mokhayeri et al. (2008), Cherchi et al. (2009) and Zhang et al., (2016) reported 0.42, 0.35 amd 0.38 mg VSS/mg COD, respectively. Peng et al., 2007 estimated the yield of 0.4, 0.42, and 0.65 mgVSS/mgCOD for methanol, ethanol, and acetate, respectively. Guven et al., 2018 also reported a yield coefficient of 0.45 mgVSS/mgCOD for a mixture of carbon sources (acetate, propionate, ethanol, glucose).

Similarily limited data is available on half-saturation coefficient and μ_{max} for nitrite denitrification. For example, Dosta et al., 2007 reported half-saturation coefficients for K_{NO2} and K_{NO3} 0.9 mg NO_2-N/L and 1.4 mg NO_3-N/L for acetate. Kornaros et al., 1996 found K_{NO2} of 0.28 mg NO_2-N/L and K_{NO3} of 0.77 mgNO_3-N/L for L-glutamic acid. On the other hand, Her and Huang ,1995a reported K_{NO2} of 10.9 mg/L and K_{NO3} of 14.3 mg/L with methanol. The value of μ_{max} for nitrate and acetate was found to be 0.5 d^{-1} by Yalda Mokhayeri et al., 2008, 1.3 d^{-1} by Dold et al. 2008, and 1.25 d^{-1} by Nichols et al., 2007..

Table 2.7 Values of saturation constants, maximum specific growth rates, growth yield coefficients for nitrite and nitrate denitrification with hydrogen **(Karanasios et al., 2010)**

K_{NO3}, mgN/L	K_{NO2}, mgN/L	μ_{max} (NO_3)- 1/d* 10^{-3}	μ_{max} (NO_2), 1/d *10^{-3}	Y_{NO3}, mgVSS/mgN	Y_{NO2}, mgVSS/mgN
28.63	4.79	1.164	13.2	0.4207	0.082
0.5-8.82	0.778-28.45	2.1-3.72	0.455-0.868	0.719-1.077	0.0047-1.467
37.8	42.98	0.5088	0.20	2.055	1.497
2.09	1.55				
0.18	0.16				
317.9					
0.0001		0.0552		0.345	
9.1	39.1	0.276	22.0	0.132	0.00806
8.3	38.4	3.648	20.0	0.128	0.000106

Additional detailed literature on yield can be found in chapter 3 (section 3.3.3.3), chapter 4 (sections 4.4.3.2, 4.4.4.1 and 4.4.4.2) and chapter 5 (section 5.3.5.1). A further detailed discussion of K_{NO2} and μ_{max} can be also be found in chapter 3 (section 3.3.3.4), chapter 4 (sections 4.3 and 4.4.3.3) and chapter 5 (section 5.3.5.3). In summary, these kinetic parameters are an essential tool for biological denitrification design, and currently, there are not available literature for nitrite. The literature review showed that kinetic information is available for nitrate; however, even for nitrate kinetics, data is limited for various carbon sources and partial denitrification. Limitation of denitrification kinetics data was identified as a research gap and will be addressed in the present study.

2.3.3 Effect of carbon source type and COD/N Ratio on SDNR

In addition to an internal carbon source (municipal wastewater-MWW), a range of external carbon sources can be used in denitrification such as methanol, acetate, ethanol, glycerol, glucose and sugar. Other external carbon sources from organic waste or food waste are also used, including MicroC (Cherchi et al., 2008) and organic fraction of municipal solid water (Frison et al., 2013). Carbon sources can have various effects on SDNR, microbial biodiversity, yield, Ks (carbon source), and maximum specific growth rate. Henze et al., 1994 classified the carbon source according to their biodegradability; (i) directly degradable (acetate and acetic acid) which directly metabolized has the highest SDNR, (ii) easily degradable (higher VFA, lower alcohols, lower amino acids, simple carbohydrates) and (iii) the second highest, and slowly degradable which hydrolyzed before becoming readily biodegradable which has the lowest SDNR rate..

The biodegradability of municipal wastewater (MWW) varies from readily to slowly biodegradable organic carbon such as volatile fatty acids (VFA), lipids, carbohydrates and proteins. Municipal wastewater COD comprises of various organic carbon source fractions such as readily biodegradable (39% of total COD) and slowly biodegradable (23%) organics (Makowska & Spychała, 2014). Compared to other external caron sources, MWW is much less biodegradable (Henze et al., 1994). Acetate can enter the tricarboxylic acid cycle (TCA) cycle directly. In contrast, methanol is used by methylotrophs and converted to glycine through the serine-glyoxylate pathway, which could induce 3-4 c intermediates until converted to acetate (Cherchi et al., 2008). When glucose is used as a carbon source, it passed through many steps until it enters the TCA cycle. Accordingly, the carbon source utilization rates are different, which in turn affects the SDNR and the kinetic parameters. Methanol is the most used external carbon source for denitrification in the USA (Theis & Hicks, 2012). The extra capital safety system cost for methanol was estimated to be in the range of 25% to 31% compared to other non-flammable external carbon sources (Cherchi et al., 2008). Although methanol is most commonly used, due to increasing price and shortage of supply, there is a trend to look for cheaper, safer, and effective alternatives such as MicroC^{TM} (Cherchi et al., 2008).

Adav et al., 2010 conducted tests to denitrify a high concentration of nitrite (200 mg/l) with different types of external carbon sources (acetate, ethanol, and methanol). The highest SDNR was obtained for acetate concentration (1500 mg/l): 2.07 mg N/g VSS-hr at a $COD_{acetate}/NO_2^{-}$N ratio of 8, whereas the highest SDNR for ethanol and methanol at a concentration of 700 mg/L (i.e. $COD_{methanol}$/ NO_2^{-}—N ratio of 5.25, and $COD_{ethanol}/NO_2^{-}$—N of 7.3) were 1.2 and 1.61 mg N/g VSS.hr, respectively. The NiR enzyme activities were highest at the highest denitrification rates for the three carbon sources (i.e., 44.2, 39.3, and 34 mM $NO2^{-}$-N/g VSS-hr for acetate, ethanol, and methanol, respectively). However, when the ratios increased more than the ratios mentioned above for the three types of electron donors, SDNR, and enzymatic activities decreased. (Adav et al., 2010 mentioned that their study was the first for denitrification from high nitrite concentration with three external carbon sources. Analyzing the data given by Adav et al., 2010, we may conclude that for high nitrite concentration, at COD/N ratios equal to 5 (maximum economic ratio used for domestic wastewater with external carbon source), SDNR of the three-carbon source are equal (1.2 mg N/g VSS-hr, Figure 2-5). Moreover, the shape of the three curves in the same figure indicates substrate inhibition because SDNR reaches the maximum and then dropped to a lower level at higher COD/N. The same trend also applies to the relationship between the SDNR and F/M ratio. We may also conclude from the same study (Figure 2-6) that enzymatic activity increased as COD/N ratio increases up to 11.4 mgN/mgVSS-d at COD/N ratio equal 5 (for methanol), and decreased gradually, and levelling off at enzymatic activity of 3 mgN/mgVSS-d at COD/N ratio of 10. However, enzyme activity for ethanol and acetate was much higher than methanol at COD/N ratio of more than 10 (Figure 2-6), which indicates the importance of finding out the optimum COD/N at which SDNR is maximum.

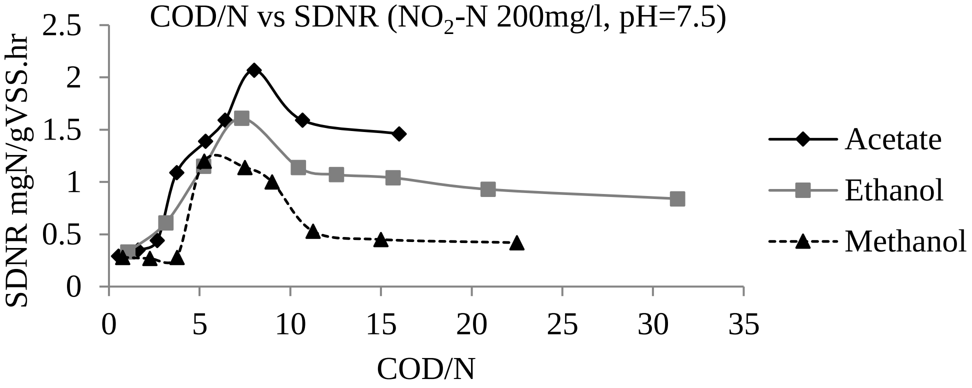

Figure 2.5 COD/N vs SDNR (data adopted from **Adav et al., 2010**)

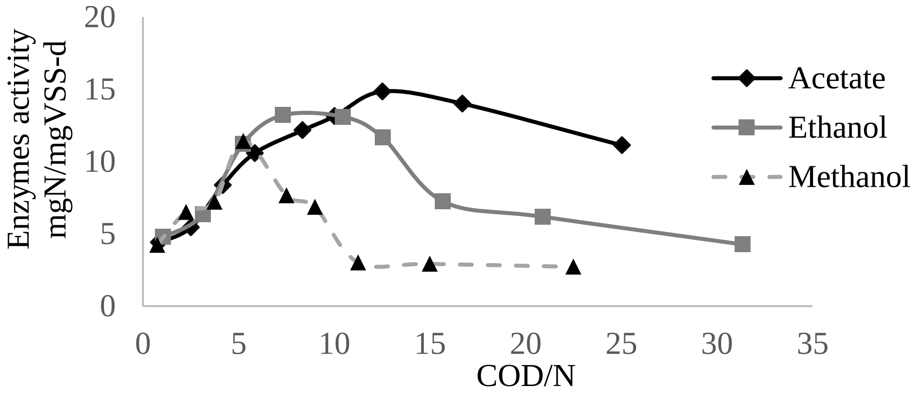

Figure 2.6 COD/N vs Enzyme activity (data adopted from **Adav et al., 2010**)

There are limited studies on denitrification from nitrite. However, several studies that compared the impact of carbon source type and COD/N ratio on nitrate denitrification are discussed below.

Yalda Mokhayeri et al., 2008 have conducted a study on the effect of changing the carbon source on the specific denitrification rates from nitrate. In the study mentioned above, they used three main batches to acclimatize the sludge on three carbon sources: methanol, ethanol, and acetate. Then, they alternated the type of carbon source for the batches with sludge that was acclimatized with another carbon source and measured the specific denitrification rate for different cases. Table 2-8 shows a comparison of SDNR rates for the different substrates added to each of the grown biomass for 24 hours. All experiments were conducted at 13 ^{0}C in batch reactors to evaluate SDNR for different external carbon sources. As it is known that denitrification with methanol is more sensitive to cold temperature than other carbon sources, therefore, the total sludge age for methanol reactor was 26 days, and 14 days for ethanol and acetate. The authors selected a longer sludge age for methanol reactor in order to maintain MLSS in the methanol bioreactor and to prevent sludge washout at low temperatures. Therefore, the anoxic operation parameters condition were: SRT for methanol, acetate, and ethanol were 12, 7, and 7 respectively, DO<0.4 mg/L, pH range 7.4-7.63, the temperature 13^{0}C, and COD/N (600/100 mg/L) ratio was 6. As it is clearly seen from the Table 2-8 that the highest denitrification rates (from nitrate) were 31.7 and 30.4 mg NO_3^--N/g VSS-hr, respectively, when acetate and ethanol were the carbon sources, while methanol exhibited the lowest rate (9.2 mg NO_3^--N/g VSS-hr). However, acetate acclimatized sludge may take a long time to acclimatize to methanol as a carbon source (denitrification rate after 24 hrs is only 0.8 hr^{-1}).

Bernat et al., 2008 also reported that acetate could produce the highest denitrification rate, followed by methanol and glucose. Bernat et al., 2008 observed that COD/N requirements in practice should be in the range of 5–10 g COD/ g NO_3^-- N and a minimum ratio of 3.5–4 g COD /g NO_3^-- N is necessary, and when the influent COD/N ratio is lower than 3.4 g COD /g NO_3^--N, extra COD should be added to remove residual nitrate. On the other hand, when the influent ratio is higher, a short aerobic phase should be introduced after the anoxic phase to remove any additional carbon source remaining and to liberate N_2 gas.

Table 2.8 Comparison of *ex-situ* SDNR rates for the different substrates added to each of the grown biomass during 24 hours **(Yalda Mokhayeri et al., 2008)**

Substrate to addition	Biomass acclimatized to	SDNR (mg NO_3^--N/g-VSS-h		Percent increase in rates, (%)
		Instantaneous	After 24 hr period	
Methanol	Methanol	9.2	9.2	-
Ethanol		7.2	9.7	34.7
Acetate		4.3	7.3	69.8
Methanol	Ethanol	3.2	5.4	68.8
Ethanol		30.4	30.4	-
Acetate		18.9	25.6	35.4
Methanol	Acetate	0.8	0.8	0.0
Ethanol		4.7	5.5	17.0
Acetate		31.7	31.7	-

Ginige et al., 2009 conducted denitrification experiments using 3 SBR batches, and 4-hr-cycles/batch at average SRT of 15 days, where batch one was operated as primary anoxic zone, batch two was operated with primary wastewater feed followed by secondary anoxic phase, and batch three was used as control (no methanol). Methanol was added to SBR 1 (primary as complementary carbon source) and 2 (secondary anoxic), acclimatization period (3-6 weeks) (Ginige et al., 2009). The COD/NO_3^-- N ratios for stages 1, 3, and 4 were 62.5/33.1=1.9, 51.5/36.1= 1.4, and 40.1/33= 1.2, respectively. The authors concluded that methanol addition enhances the growth of specific methanol-denitrifiers, improving denitrification rates, as the capability of sludge to use methanol and ethanol for denitrification is improved, and unlike acetate sludge, settlebility also improved. On the other hand, methanol reduced the capabilities of denitrifiers that utilize normal wastewater COD (denitrification rate is reduced when the carbon source is wastewater); however, a little amount of methanol (low COD/N) could lead to nitrite accumulation. They mentioned that the best location for external carbon is in the post anoxic zone, and in small amounts as the remaining amount of nitrite/nitrate to be denitrified is small. However, methanol may be added to the primary anoxic zone as a complementary external carbon to adjust COD/N ratio, when a small amount is needed as sludge may require acclimatization every time, which is not feasible.

Timmermans & Van Haute, 1983 studied specific denitrification rates of nitrite produced during nitrate denitrification (Table 2-9). When the nitrite concentration was 9.2 mg/L, and nitrate concentration was 55 mg/l, the SDNR of nitrate (0.038 mg N/mg VSS-hr) was half that of nitrite (0.077 mg N/mg VSS-hr). Timmermans & Van Haute, 1983 also concluded that when nitrite exists in higher concentrations than nitrate, nitrite inhibits nitrate denitrification. However, if nitrate is the only electron acceptor, normal SDNR from nitrate is obtained. Moreover, the optimum ratio of methanol to nitrate for complete denitrification (mg CH_3OH/mg NO_3^--N) was 2.52, whereas the ratio of methanol to nitrite is 1.8 (Table 2-9).

Table 2.9 Reaction rates of *Hyphomicrobium spp*. at different concentrations of nitrate and nitrite in batch reactors at a temperature of 25°C **(Timmermans & Van Haute, 1983)**)

Initial concentration, NO_2 -N, mgN/L	**Initial concentration,NO_3 -N, mgN/L**	**RD** **gNO_2-N/gMLVSS-h**	**RD** **gNO_3-N/g MLVSS-h**	**RM** **gCH_3OH/gMLVSS-h**	**pH**
92	0	0.060	0	0.11	
71.7	0	0.074	0	0.13	
98.5	1.5	0.069	0	0.11	
97	3.8	0.065	0	0.11	9.4
49.6	9.2	0.074	0.015	0.14	
47	6.0	0.069	0.012	0.13	
9.2	55	0.077	0.038	0.12	
92.8	25.7	0.077	0.006	-	8.3
80.4	28.4	0.079	0.005	0.13	

Frison et al., 2013 compared denitrification rates for nitrite and nitrate with synthetic digested sludge supernatant as a carbon source (Table 2-10), which illustrates various rates for various carbon sources. For example, the organic fraction of municipal solids waste (OFMSW), the fermentation or the drainage liquid had a close range of SDNR (0.51 and 0.65 mgNO_2–N/mgVSS-day), and their performance in denitrification is 50% of CM&MS (1.16 mgNO_2-N/mgVSS-d). Glycerol showed very low performance (0.11 mgNO_2-N/mgVSS-d) compared with acetate (0.603 mgNO_3-N/mgVSS-d) and acetic acid (1.14 mgNO_2-N/mgVSS-d). Table 2-10 also shows that the highest SDNRs occurred with nitrite using as acetic acid and CM&MS (cattle manure and maize silage) as a carbon source.

Table 2.10 Nitrate and nitrite rates with various organic carbon sources **(Frison et al., 2013)**

External carbon source	Nitrate/nitrite denitrification rate
Acetate	0.603 kg NO_3-N/kgVSS^{-1} day^{-1}
Propionate	0.362 kg NO_3-N/kgVSS^{-1} day^{-1}
Effluent VFA	0.054 g NO_3-N/gVSS^{-1} day^{-1}
Effluent VFA	0.28 g NO_3-N/gVSS^{-1} day^{-1}
Acetic acid	1.14 kg NO_2-N/kgVSS^{-1} day^{-1}
Glycerol	0.11 kg NO_2-N/kgVSS^{-1} day^{-1}
OFMSW drainage liquid	0.51 kg NO_2-N/kgVSS^{-1} day^{-1}
OFMSW fermentation liquid	0.65 kg NO_2-N/kgVSS^{-1} day^{-1}
CM&MS fermentation liquid	1.16 kg NO_2 -N kgVSS^{-1} day^{-1}

Frison et al., 2013 conducted a cost analysis to compare different carbon sources for denitrification (Table 2-11). Where OFMSW represents the organic fraction of the municipal solid waste, and CM&MS represents cattle manure and maize silage (fermentation liquid). Based on methanol prices in 2004, Frison et al. (2013) concluded a 22% decrease in overall specific cost when using OFMSW fermentation liquid compared to methanol (3.24-3.64 Euros/kg N removed for methanol, and 2.85 Euros/kg N removed for OFMSW fermentation liquid) for denitrification.

Denitrification rate is primarily governed by COD/N ratio (Bernat et al., 2008). Studies on nitrate denitrification presented that optimal COD/N ratios were reported (> 4.0) (Eddy et al., 2014). In contrast, the effect of carbon source type and COD/N ratio on nitrite denitrification was not available in the literature. The possible effect of COD/N ratio includes inhibition of a high dosage of carbon to denitrification. In this study, the effect of COD/N ratio on nitrite SDNR and the mechanism behind will be studied using municipal wastewater, methanol and acetate as a carbon source by giving emphasis on nitrite concentration that can be found in wastewater.

Table 2. 11 Cost analyses related to the investigated carbon sources **(Frison et al., 2013)**

Parameter	Acetic acid (80%)	Glycerol	OFMSW drainage liquid	OFMSW fermentation liquid	CM&MS fermentation liquid
Unit cost of raw material (Euro ton-1)	620-700	500-550	-	75	62.5
Raw material needed (kgraw/kgN-1 red)	2.69	1.98	-	13.1	9.7
Equivelent loss biogas production (Nm3 kgN -1 red)	-	-	-	-	1.75
Estimated cost per kg of COD added (Euro kgCOD-1)	0.79-0.90	0.42-0.46	-	≤0.1	0.51
Estimated cost per kg of nitrogen removed (Euro kgN- 1red)	1.67-1.88	0.99-1.09	-	≤0.1	0.74

2.4 Partial denitrification (PDN)

Partial denitrification is a second-generation TN removal process. It is the second and limiting step of the nitrification-partial denitrification-anammox (PDNA) process for TN removal. In nitrification-PDNA, ammonia is allowed to be nitrified fully to nitrate, then partially denitrified to nitrite at a condition that maximizes nitrite accumulation. The process (Figure 2-7) saves 45% oxygen (aeration energy) and 79% of the external carbon source (Ma et al., 2016). The nitrification-PDNA process is currently considered to be the most promising second-generation process for mainstream wastewater treatment. The process also can be very successful for nitrogen-rich industrial wastewater treatment such as chemical fertilizer wastewater, explosives wastewater and other production wastewater (Yang Zhang et al., 2019). A summary of previous detailed studies can be found in Chapter 5, Table 5-1. However, some general information is highlighted here.

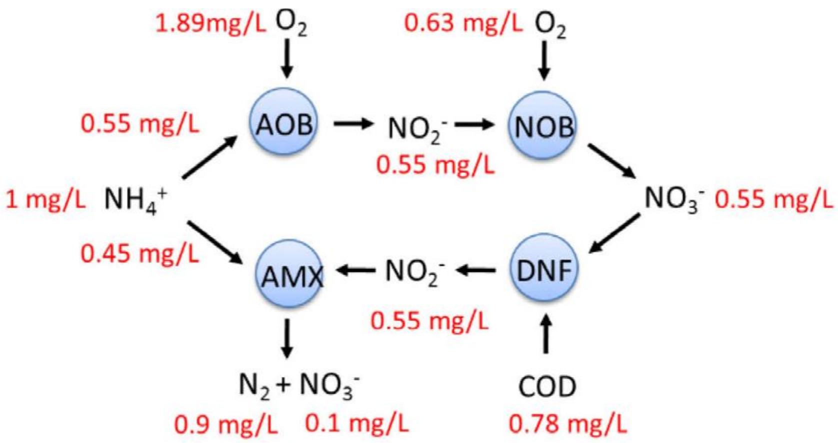

Figure 2. 7 Partial denitrification-anammox **(Ma et al., 2016)**

In addition to S. Ge et al., 2012 studied the effect of COD/N ratio in a batch reactor for different types of electron donors (methanol, acetate, and glucose), on the denitrification rates from nitrate, with ratios of 1, 6, 10, 15, 20, and 25 (VSS was 1250 mg/L). They also studied specific nitrite accumulation rates (SNAR) during transformation as well as the denitrification rates from nitrite and concluded that the initial COD/NO_3^--N affected the level of (i) nitrate denitrification rate (initial, phase 1), (ii) nitrite denitrification (during nitrate denitrification), (iii) nitrate denitrification (phase 2) and (iv) nitrite denitrification (phase 2) and (v) nitrite accumulation during denitrification from nitrate. The highest SDNRs were 0.64 (glucose), 0.35 (acetate), 0.029 (methanol) and 0.04 mgN/mgVSS-d, respectively. Moreover, the highest accumulation occurred with glucose (22.32 mgN/L) at SNAR of 0.34 mgN/mgVSS-d. Ge et al. attributed nitrite accumulation to the competition between electron acceptors enzymes (Nir and Nar reductase). They also recommended the use of pH and ORP to control the denitrification process (real-time control). S. Ge et al., 2012 found two high accumulation points occurred with glucose of 22.32 and 20.01 mgN/L at COD/N of 15 and 10, respectively (table 2-12). Whereas the maximum nitrite accumulated with methanol was 19.34 mgN/L at COD/N of 25. Acetate was found to cause the lowest-highest accumulation among the three carbon sources with nitrite accumulation of 17 mgN/L at COD/N of 25. It can be concluded from Table 2-13 the highest nitrite accumulation occurred at the highest SNAR of 0.34 mgN/mgVSS-d.

Table 2.12 Effects of carbon sources on the nitrite accumulation in the first phase **(Ge et al., 2012)**.

COD/NO3-N	Carbon source	Accumulation time, hr	Nitrite acumulation, mgN/L	SNAR-NO2, mgN/mgVSS-d	SDNR-NO3, mgN/mgVSS-d
1	Acetate	2	1.86±0.94	0.013±0.005	0.052±0.01
	Methanol	1	3.17±0.45	0.05±0.014	0.073±0.004
	Glucose	1	2.1±0.05	0.025±0.002	0.096±0.008
6	Acetate	2	8.44±0.81	0.062±0.011	0.28±0.03
	Methanol	4	10.82±1.52	0.043±0.002	0.13±0.04
	Glucose	2	14.51±1.05	0.11±0.02	0.24±0.07
10	Acetate	2	12.03±0.47	0.095±0.004	0.32±0.03
	Methanol	4	12.03±2.41	0.062±0.004	0.14±0.03
	Glucose	1	20.01±1.44	0.31±0.05	0.55±0.12
15	Acetate	1	15.68±3.04	0.24±0.03	0.55±0.05
	Methanol	4	14.68±2.16	0.057±0.016	0.16±0.07
	Glucose	1	22.32±1.85	0.34±0.03	0.64±0.2
25	Acetate	1	17.19±2.08	0.22±0.01	0.6±0.09
	Methanol	2	19.35±1.26	0.25±0.05	0.4±0.04
	Glucose	4	10.43±0.23	0.14±0.03	0.04±0.04

Shi et al., 2019 studied the effect of pH (adjusted to 9) on achieving partial denitrification (PDN) in a 6 L anoxic-sequencing batch biofilm reactor (SBBR, sponge carrier media,), the cycles were varied started with 6 hr-cycle (116 days) and then reduced to 2hr-cycle. However, the authors use 3 reaction times (5 hrs and 3hrs-period 1, 1 hr-period 2, no settling), the nitrate feed and diluted MWW-COD (205 mgCOD/L before dilution) feed were 30mgN/L and 145 mgCOD/L, respectively, and the COD/N ranged between 4-6 and DO≤0.1 mg/L. The results showed a high nitrite accumulation of nitrite 23.5 and 25.2 at H of 7 and 9, respectively, implying that there is no effectct of COD/N ratio and pH on PDN. However, in batch tests with acetate carbon source Shi found a higher accumulation at pH 9 than pH 7 with nitrite accumulation percentage and concentrations of (90.3%, 22.5 mgN/L) and (91.8%, 22), respectively. In the same study, Shi found that the maximum nitrite accumulated reached after 60 minutes in day after day 126 while before that day the was reached after 180 minutes, implying long term operation improve the microbial structure to carry out nitrite accumulation in a shorter period. However, he concluded possible optimum COD/N ratio 6.2, which is higher than previously believed ratio of 3 (Cui et al., 2017). Shi finally found that *thauera* has the highest relative abundance (21.9%), followed by *Flavobacterium* (9.6%) and *Thiobacillus* (6.3%).

Yang Zhang et al., 2019. also investigated partial denitrification (COD of 100mg/L, acetate) at COD/N of 3, 5 and 7 with cultured *Acinetobacter Johnsonii* , the maximum nitrite accumulation were 80.9%, 94.2 and 87.4%, respectively, and occurred after 13 hours from the experiment which indicating the optimum COD/N of 4-5 is the optimum. However, Zhang et al. illustrated that NaR is less competitive than NiR when COD/N is low (less than 3), and when COD/N increases the gene level of *napA*, which is located in the periplasm, increases and the denitrification from nitrate become much higher than nitrite. This imply that the nitrite and nitrate reductase enzymes availability as well as the COD/N ratio plays an importat role in nitrite and nitrate denitrification rates, and partial denitrification. He also found that there is no major pH effect on the PDN in the rang of 6-9.

Xiujie et al., 2019 studied partial denitrification with glucose in 3 SBRs (6 hrs cycle, 2cycle/d), the nitrite accumulated in SBR1 (anoxic) was 8.29 mgN/L , 9.94 mgN/L in SBR2 (aerobic-anoxic), 11.63 mgN/L in SBR3 (low DO) and at COD/N of 3 (30 mgNO3-N/L), and the denitrification rates from nitrate and nitrite were 17.24 mgN/L-hr and 3.38 mgN/L-hr in SBR1, 10.16 mgN/L-hr and 1.72 mgN/L-hr in SBR2 and 7.98 mgN/L-hr and 0.8 mgN/L-hr in SBR3, respectively, for the first 2.5 hr of the reaction and then reduced to 0.52 mgN/L-hr and 0.9 mgN/L-hr (2.5-8 hrs) for nitrate and nitrite, respectively. These results imply that nitrate reductase is more affected by carbon source deficiency than nitrite, whereas when carbon source available, the nitrate reductase activity is much higher than nitrite activity. Further explanation by Xiujie that the biomass in SBR1 has higher denitrification ability than SBR2 and SBR3 and nitrite reductase is more sensitive to DO than nitrate reductase, which causes nitrite accumulation. He also detected various phyla, and they are arranged from the highest to the lowest relative abundance *Proteobacteria* (44%) in SBR1, *Bacteroidetes* (19%) in SBR3, *Acidobacteria*(12.9%), *Planctomycetes, Candidatus saccharibacteria, Chlorofexi*). In the genus level *Saccharibacteria*, which is related to glucose, with a relative abundance of 5.35% in SBR1, 45.44% in SBR2, and 34.96% in SBR3. The relative abundance in SBR2 and SBR3 is related to the favourability of *Saccharibacteria* to DO. Other genera that was found and arranged according to relative abundance *are Aridibacter, Pseudomonas*, *Thauera, Gemmobacter* and *Citrobacter*, implying that the relative abundance of the genera are related to the carbon source (glucose).

In a study by Du et al., 2017 in SBR followed UASB, the $SDNR_{NO3-NO2}$ and $SDNR_{NO2}$ with acetate were 0.47 and 0.42 mgN/mgVSS-d, respectively, whereas $SDNR_{NO3-NO2}$ and $SDNR_{NO2}$ with ethanol were 0.29 and 0.2 mgN/mgVSS-d. However, Le et al., 2019a and Le et al., 2019b were not able to accumulate nitrite with methanol, illustrating two elucidation for PDN which are 1- acetate and glycerol can enter directly to TCA cycle and stored in the cell in the form of poly-3-hydroxybutyrate and then utilized as an electron donor during denitrification. A large pool of electrons is released within the cell during denitrification. While NaR has a higher electron capacity (2 e^-/mole-nitrate) than NiR (e^-/mole-nitrite), NaR could react with more electrons, which creates a much higher reduction rate than NiR reaction rate. The other explanation 2- when acetate or glycerol are used, NaR reacts with the electrons in the cytochrome b, which is located in the upstream region of the cytoplasm. At the same time, NiR accepts electrons from cytochrome c located in the downstream region. Acetate and glycerol donate electrons in the upstream region (cytoplasm b) which allow nitrite to accumulate, whereas methanol donate electrons in the down stream region which nitrite is not accumulated with methanol.

Moreover, Le et al., 2019a and Le et al., 2019b in the partial denitrification study (PDN-anammox) accumulated more than 80% nitrite at COD/N ratio of 2-3, and nitrite started to be denitrified when nitrate concentration was below 3 mgN/L. However, they did not consider denitrification from nitrite (partial nitrification/denitrification) to measure specific denitrification rates from nitrite in relation to COD/N ratios. To do so, an acclimatization step is required for denitrifies (denitrifying-bacteria and heterotrophs) with nitrite as an electron acceptor. Nitrite with a higher toxicity effect on the microbes than nitrate requires longer acclimatization time. Table 2-12 shows denitrification rates from accumulated nitrite with different carbon sources. It is clear that in the first phase of SDNR, acetate exhibited the highest denitrification rate from nitrate (0.35 d^{-1}), while the denitrification rate from nitrite was highest with methanol in the second phase (0.041 d^{-1}).

In conclusion, several early-stage trials to investigate partial denitrification (PDN) (Table5-1, chapter 5) are in the research process; the study in chapter 5 shows PDN-trials through the control of COD/N, final nitrate concentration, high pH, and high DO parameters. However, until now, these parameters do not show they govern the PDN process since various researchers selected different COD/N with a wide gap. Controls such as increasing the pH will not make any difference since pH typically increases with alkalinity during denitrification; however, increasing DO will be very critical since nitrite may convert again to nitrate. The control through the effluent nitrate concentration was not proven because the effluent nitrate concentration varies with the carbon source and is also directly related to K_{NO3}. Most of the recent researches combine PDN-anammox in one reactor, which did not analyze nitrite accumulation but anammox activity. Finally, none of the previous research studied the partial denitrification mechanism and compared nitrite and nitrate denitrifying biomass structure (type and biodiversity) and critically analyze the partial denitrification kinetics.

2.5 Research knowledge gaps

Nitrite shunt and partial denitrification processes are new trends in the TN removal process from municipal wastewater. Nitrite ion is very toxic to microorganisms, which requires careful control of the kinetic and operation parameters, and microorganisms require a long time to acclimatize to the electron donor. Extensive research has been conducted on denitrification from nitrate to determine the optimum parameters; however, not much work has been conducted on denitrification from nitrite as the starting electron acceptor. The following are some specific knowledge gaps that were found in the existing literature, which will be addressed in the present work:

1. Review of the literature showed the electron distribution among the four types of nitrogen oxide (NO_3^-/NO_2^-/NO/N_2O) with methanol as electron donor, but the effect of internal carbon source (municipal wastewater) on the denitrification rates was not considered, which will provide better expectation in the design of biological nitrogen removal in wastewater treatment plants (Eddy et al., 2014). Thus, the optimum COD/N for external and internal carbon source that provides the maximum SDNR when nitrite is used as the electron acceptor must be determined. This also will include the determination of K_N (for nitrite), K_S (for carbon source), μmax for heterotrophic denitrifiers, and the denitrification inhibition effect. Moreover, DO inhibition concentration (K_O') has been reported to be 0.1 to 0.2 mg/L for denitrification from nitrate. However, K_O' value has not been reported in case of denitrification from nitrite.
2. In relation to the carbon source, and due to low carbon to nitrogen ratio in real wastewater (municipal wastewater-MWW), denitrification process in full-scale wastewater treatment plants employ external carbon sources to minimize total nitrogen effluent. Peng et al., 2007

studied the effect of a mixture of carbon sources (starch-wastewater, mixed with methanol, ethanol and acetate in three bio-reactors) on nitrate denitrification. As per the literature review, the effect of the mixture of real wastewater and external carbon source on denitrification from nitrite has not yet been reported. Accordingly, an optimum ratio of external and internal carbon (real wastewater) sources, kinetics and potential competitive effects of the two types of carbon sources are unknown. However, this is vital information for the design and operation of the nitrite denitrification system.

3. Partial denitrification is the latest advance in the second generation TN removal processes. The process is used to partially denitrify nitrate and nitrite and maximize nitrite accumulation that will be used as an electron acceptor in a downstream anammox process. In this process, the PDN, more specifically, the nitrite accumulation is the limiting step. A review of the literature showed different strategies to enhance nitrite accumulation during denitrification from nitrate. However, several conflicting reports were observed with regard to the impact of COD/N ratio, carbon source type, pH level and/or nitrate/nitrite concentration on the accumulation rate. However, the fundamentals behind nitrite accumulation over nitrate denitrification were not well understood. As a result, robust engineering process control strategies were not identified. Therefore research comparing nitrite and partial denitrification processes would be required to understand the process kinetics and ultimately device a robust control strategy that enables sustainable PDN processes.
4. Microbial biodiversity and structure comparison for nitrite and nitrate denitrification on various taxonomic levels (phylum, class, genus and species) level had never been studied before. Such a comparison will allow us to compare between nitrate and nitrite denitrification rates and kinetics also, the possibility of PDN study in nitrate denitrification bioreactor.
5. The effect of introducing both nitrate and nitrite in different ratios as electron acceptors with internal carbon sources was never studied. Such a scenario occurs in many treatment plants during nitrification when changes in DO, pH, and alkalinity enhance or impede NOB or AOB growth.

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Chapter 3

Effect of COD/N Ratio on Denitrification from Nitrite

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The objective of this study was to investigate dynamic specific denitrification rates (SDNR) from nitrite at various chemical oxygen demand (COD) / nitrogen (N) ratios using municipal wastewater (MWW). A sequencing batch reactor (SBR) continuously fed with primary effluent and nitrite solution were operated at hydraulic retention time of 8.4 hrs and solids retention time of 26-30 days for 3 months. Influent MWW characteristics varied significantly during the study i.e. 200-810 mgCOD/L and 6-80 mgN/L. The SDNR from the SBR were compared with those determined in four batch reactors using acetate. The SDNR was directly related to COD/N until a maximum SDNR (mgNO$_2$-N/mgVSS/d) of 0.07 for MWW and 0.4 for acetate occurred at COD/N ratios of 6 and 13, respectively; beyond this COD/N ratio, SDNR decreased. The biomass yield coefficients (mgVSS/mgCOD) were 0.33 for MWW and 0.51 for acetate. The relationships of SDNR with COD/N and F/M ratios were developed.

3.1 Introduction

Biological nitrogen removal processes typically used in municipal wastewater (MWW) treatment operations involve nitrification of ammonia to nitrate via nitrite followed by denitrification of nitrite and nitrate to nitrogen gas. Denitrification processes are often carbon-limited, requiring supplementation of carbon either by fermentation of biosolids or use of external carbon sources such as methanol, glycerol, acetate etc. In order to reduce energy and carbon demand, shortcut nitrogen removal through nitrite is gaining popularity (Capodaglio et al., 2016). Particularly, shortcut denitrification processes can also be effectively employed with short-cut nitrification processes, which partially nitrify ammonia nitrogen to nitrites through suppressing the activity of nitrite oxidizing bacteria (Liu et al., 2017). The emerging second generation technologies, such as Sharon (stable high rate ammonia removal over nitrite), Anammox (anaerobic ammonium oxidation), and Canon (completely autotrophic nitrogen removal) employ short-cut nitrification through enhancement of ammonia oxidizing bacteria (AOB), and washout of nitrite oxidizing bacteria (NOB), by controlling temperature, DO, and pH (Paredes et al., 2007; Li et al.,2008; and Zhang et al.,2008). Thus, the integration of denitrification or denitrification specifically in these processes is timely to ensure environmental sustainability in light of the benefits compared to denitrification from nitrates which include up to 40 % reduction in carbon, 25% reduction aeration energy, and minimal nitrogen oxides concentration in the final effluent.

Previous studies addressed that shortcut denitrification is influenced by the effect of COD type and COD/N ratio similar to denitrification from nitrate. Various denitrification studies related to COD/N ratios are presented in Table 3.1. Different studies used various types of COD sources such as methanol, acetic acid, glycerin, glucose, and employed different concentrations of COD (≤ 3000 mg/L), nitrogen (≤2500 mgNO$_2$-N/L and ≤2500 mgNO$_3$-N/L), COD/N ratios (1-106) to optimize denitrification performance. Some studies highlighted the effect of COD/N ratio on the overall denitrification rates. For example, according to a study by Ge et al. (2012), who investigated a wide range of COD/N ratio with three different carbon sources, i.e. acetate, methanol, and glucose for denitrification from nitrate, the SDNRs increased from 0.05 to 0.6 mgNO$_3$-N/mgVSS/d for acetate, and from 0.07 to 0.4 mgNO$_3$-N/mgVSS/d for methanol as the COD/N ratio increased from 1 to 25. However, the authors also observed that SDNR with glucose decreased from 0.64 to 0.04 mgNO$_3$-N/mgVSS/d with the increase in COD/N ratio from 15 to 25, indicating that the variation of SDNR and optimum COD/N for maximum SDNR depends on the carbon type. Similarly, a recent study by Katarzyna et al. (2015) who operated an SBR process fed with glycerin for nitrite-denitrification at different COD/N ratios of 2-4 (nitrite concentration of 100 mg/L) also reported a SDNR of 0.23 to 0.45 mg NO$_2$-N/mgVSS/d, indicating better denitrification with higher COD/N ratio.

Table 3. 1 Comparison of denitrification studies from nitrate and nitrite with different c-sources

C-source	**NO_2-N and (NO_3-N) mg/L**	**SCOD, mg/L**	**COD/NO_3-N**	**COD/NO_2-N**	**SDNR mgNO_2-N/mgVSS/d (mgNO_3-N/mgVSS/d)**	**Inhibition**	**Reference**
methanol, acetic acid, benzoic acid, glucose	0-50 (0-50)	500-833	(0-40)	0-40	96-100% removal for all sources	Methanol (COD/N of 27), benzoic acid (COD/N of 40), C-source inhibition	Her et al. (1995a)
glycerin	100	200-400	NA	one cycle/d (2, 3, 4). two cycles (2.5, 3, 3.5)	one cycle 0.26, 0.24, 0.34, Two cycles 0.23, 0.28, 0.45	NA	Katarzyna et al. (2015)
acetate, methanol, glucose	(40)	40, 240, 400, 600, 800, 900	1, 6, 10, 15, 20, 25	NA	COD/$N_{acetate}$ of 25 (0.6), COD/$N_{methanol}$l of 25 (0.4), COD/$N_{glucose}$ of 15 (0.64)	SDNR reduced to 0.04mgNO3-N/mgVSS-d at COD/$N_{glucose}$ of 25 due to substrate inhibition	Ge et al. (2012)
sweet-production waste(CS1), soft drinks waste(CS2), dairy waste(CS3)	(700)	C-source was increased or reduced based on studied COD/N (155, 850, 370)	CS1 (5, 5.5, 6, 6.5), CS2 (4.8, 5.5, 6, 6.5), CS3 (4.3, 4.5, 4.6, 4.7)	NA	CS1 (0.73, 0.96, 0.92, 1.0), CS2 (0.76, 1.15, 1.12, 1.15), CS3 (0.87, 0.92, 1.06, 0.95)	NA	Fernández-Nava et al. (2010)
methanol	7-18 (50-100)	NA	5.9, 4.1	No COD data	0.524, 0.585 (0.208, 0.321)	Nitrate denitrification reduced when COD of methanol below 40 mg/L. Nitrite-denitrification inhibition due to nitrite	Beccari et al. (1983)

						concentration under nonlimited C-source	
methanol, ethanol, acetate	SBR (30), Ex-situ batches (100)	SBR (100), Ex-situ batches (600)	SBR (3.3), Ex-situ batches (6)		(0.221, 0.73, 0.76)	ethanol and methanol can be replaced each other for the same type of biomass without major change in SDNR	Mokhayeri et al. (2008)
phenol	40-50 (2.5), 59-70 (4), 76-98 (6.5)	2400	NA	48, 34, 24, (ΔCOD/ΔN= 6 for phase1)	0.034, 0.02, 0.0 for phase1, 2, and 3, respectively	Nitrite concentration inhibition at 70 mg NO_2-N/L when phenol is c-source	Queiroz et al. (2011)
acetate (mixed with WW-denitrified effluent	(20-30)	40-120	2.18-6.02		ranged (0.0816-0.222)	NA	Hyden et al. (2007)
acetic acid, glycerol, organic fraction of the municipal solid waste (OFMSW) fermentation liquid, OFMSW drainage liquid, cattle manure and maize silage (CM&MS) fermentation liquid	120(<1)	500-3000	NA	3.1-3.5 (ΔCOD/ΔN were 2.1, 2.3, 3.1, 2.7, and 3)	0.05-1.14, 0.02-0.11, 0.19-0.65, 0.35-0.51, 0.34-1.16	NA	Frison et al. (2013)
glucose, methanol, ethanol, bacto-peptone	50, 300 (50)	400	10 (ΔCOD/ΔNO3-N was in range of 5.3-6.3)	10 (ΔCOD/ΔNO2-N was in range of 3.8-4.3)	0.76-01.59 (0.29-0.37), cells were acclimatized on glucose	when nitrite and nitrate present together, initial NO_3-N/NO_2-N >0.5 cause inhibition. Nitrite concentration of 200mg/L decreased SDNR	Chung et al. (2002)

						to 0.09 mgNO_2-N/mgVSS-d.	
Municipal wastewater+ Ethanol	25	140 + 60	NA	5.6	0.119	NA	Yang et al. (2007)
glucose	50-2500 (50-2500)	5318	Varied (2.13-106), 8.86 is the optimum	Varied (2.13-106), 6.65 is the optimum	0.024 (0.023) mg NOx-N/gMLSS/d	FNA inhibition	Akunna et al. (1992)
acetate	54 (20)	NA	NA	NA	108 (28) mg NOx-N/L/d	NA	Al-Samawi and Shamkhi (2014)
MicroCTM, methanol, acetate	(20-40)	0-300	6.5, 4.8, 5.7		(0.15, 0.15, 0.333)	NA	Cherchi et al. (2009)
glucose, glycerol, acetic acid, lactic acid, methanol	200 (200)	3000	15	15	0.14, 0.24, 0.57, 0.57, 0.133 (0.065, 0.18, 0.67, 0.67, 0.06)	NA	Akunna et al. (1993)

2

In order to better understand the effect of COD/N ratio on dentirification, $COD_{consumed}/N_{removed}$ ($\Delta COD/\Delta N$) needs to be considered. Chung et al. (2002) who studied denitrification from nitrite with glucose, methanol, ethanol, and peptone (COD/N ratio 10 with NO_2-N of 100 mg/L and COD of 400 mg/L) mentioned that the ratio of $\Delta COD/\Delta NO_2$-N varied from 3.8 to 4.3 with SDNR ranging from 0.76 to 1.59 mgNO_2-N/mgVSS/d. Similarly, a comprehensive study by Frison et al., (2013), who tested the effect of five different carbon types on SDNR and $\Delta COD/\Delta N$ ratio, found that SDNR with acetic acid, glycerol, organic fraction of the municipal solid waste (OFMSW, fermentation liquid), OFMSW drainage liquid, and cattle manure and maize silage (CMMS) were 1.14, 0.11, 0.65, 0.51, 1.16 mgNO_2-N/mgVSS/d at $\Delta COD/\Delta N$ ratios of 2.1, 2.3, 3.1, 2.7, 3.0, respectively, indicating that $\Delta COD/\Delta N$ was carbon type specific without direct relationship between SDNR and $\Delta COD/\Delta N$.

Scrutiny of the previous studies (Table 3.1) indicated that while the effect of COD/N on denitrification from nitrate has been extensively studied, the research on the effect of COD/N on denitrification from nitrite is limited (Chung et al., 2002; Frison et al., 2013; Beccari et al., 1983; Adav et al., 2010). In addition, most nitrite denitrification studies used synthetic wastewater and solid waste by-products. As far as our knowledge goes, studies on denitrification of nitrites with real wastewater are not reported. Furthermore, most of the previous studies were conducted in narrow ranges of COD/N ratios with optimum values of 2-5. However, since wastewater influent characteristics vary widely, the information on the effect of COD/N on denitrification in a wide range with real wastewater is lacking. Furthermore, the relationship between initial COD/N ratio and $\Delta COD/\Delta N$ ratio is not readily available as earlier studies merely reported these values (Beccari et al., 1983; Chung et al., 2002; Katarzyna et al., 2015; Frison et al., 2013).

Hence, the objective of this research work was to explore the denitrification of nitrite using municipal wastewater at different initial COD/N and corresponding $\Delta COD/\Delta N$ ratios. The nitrite-denitrification performance was monitored using an SBR fed with municipal wastewater to determine the effects of a wide range of COD/N ratios on nitrogen removal. Since the COD/N in MWW varies seasonally and temporally in a wide range; this study elucidates the dynamic denitrification rates at various conditions. In addition, the effect of COD/N ratio on denitrification rate using nitrite was determined using pure acetate as the carbon source.

3.2 Materials and Methods

3.2.1 Bioreactor setup and operation

A bioreactor (shown in Figure 3.1) with a working volume of 10.5 L, equipped with a mechanical stirrer, dissolved oxygen sensor, ORP and pH meters, connected to a control unit, was used in the experiments. The system included air diffuser and DO control units for operation at different dissolved oxygen levels to control nitritation and nitratation; however, for the purpose of this study, the system was anoxically operated without aeration. The volumes of the influent and effluent tanks were 60 L and 50 L, respectively, while a 3.8 L tank was used for feeding additional nitrite (100 mL/min for 2 min) to control the initial nitrite concentration in the reactor.

The SBR cycle length was 240 min (6 cycles per day) with 20 min MWW and 1min nitrite feed, 110 min react phase under completely-mixed anoxic conditions, 90 min settling time, and 20 min decanting. The fill was around 48% i.e. in each cycle, 5L were decanted and replaced with 5L of feed, corresponding to a daily feed flow of 30L. The solids retention time (SRT) was maintained between 26 -30 days by wasting 0.35-0.41 L/d of the mixed liquor during the anoxic react phase. Mixing in the SBR was only used during the reaction period. The bioreactor was operated at room temperature. . pH and ORP were monitored to ensure anoxic conditions during the study and were in the ranges of 8-9 and -250 to -350 mV, respectively. Although the influent MWW had a pH in the range of 7.0-7.2, alkalinity production due to denitrification increased the SBR operating pH.

The influent wastewater, which was primary effluent of the Greenway Wastewater Treatment plant (London, ON, Canada), was collected every 7-10 days. A synthetic nitrite stock solution was prepared using sodium nitrite with concentrations of 140-1900 mg NO_2-N/L, corresponding to 2.75-36 mg NO_2-N/L in the SBR. Prior to the beginning of the experiments, the biomass underwent a three-week acclimatization period (4 cycles per day for two weeks, 20 L/d), introducing 5 L of activated sludge per day for 3 times in the first week. Due to phosphorus release during the first 2 weeks of the start-up, the operation cycle was reduced from 6 hr to 4 hr.

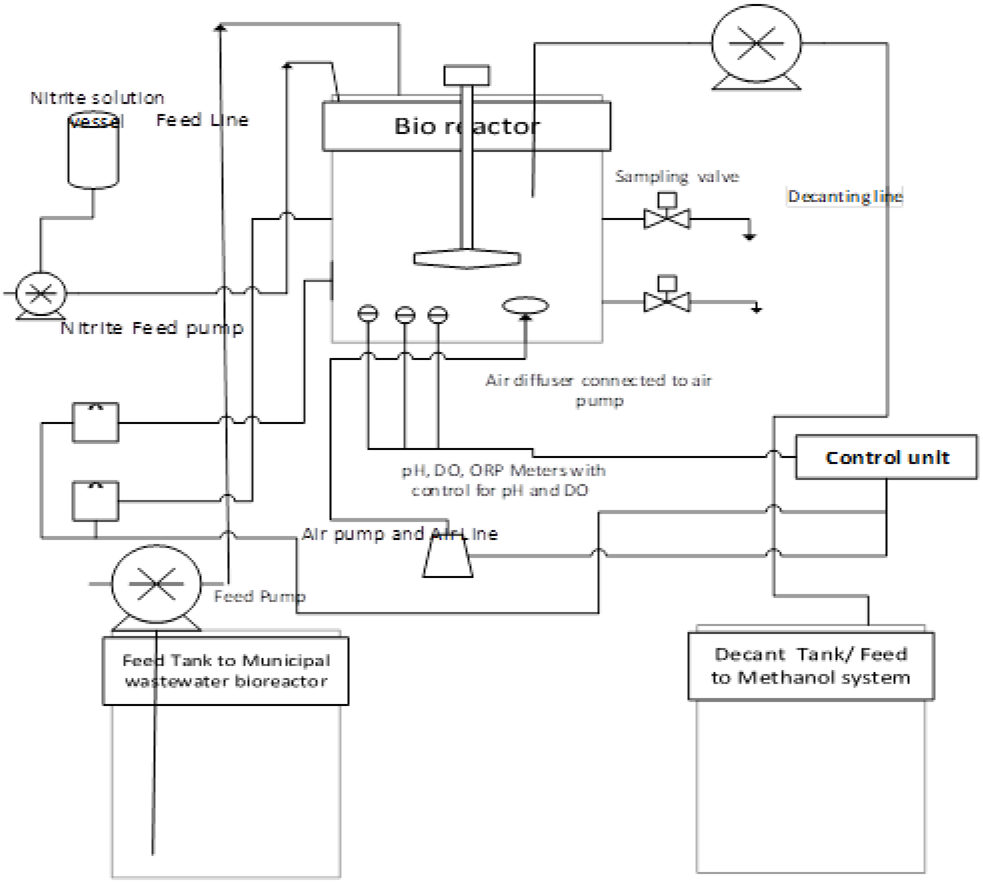


Figure 3. 1 Bioreactor configuration

3.2.2 Batch tests

In order to examine the effect of different COD/N ratios on the denitrification rate from nitrite using acetate, the offline batch tests (B1, B2, B3, B4) were conducted using acetate in four 1 L flasks for a period of 4 hr with the MWW-acclimatized biomass collected from the SBR and concentrated by centrifugation for 10 min at 3000 rpm. One liter of distilled water was added to each flask, containing $NaHCO_3$ (120 mg/L) (alkalinity source), KH_2PO_4, $MgSO_4{\cdot}7H_2O$ (100 mg/L), $CaCl_2$ (100 mg/L), 1 ml/L of trace elements solution (composition in g/L for all as: EDTA 15, $ZnSO_4$ 0.43, $CoCl_2$ 0.24, $MnCl_2$ 0.63, $CuSO_4$ 0.25, Na_2MoO_4 0.22, $NiCl_2$ 0.19, Na_2SeO_4 0.21, H_3BO_3 0.01 and $NaWO_4$ 0.05). Acetate and sodium nitrite were added to the solution to obtain various COD/N ratios such as 6.6, 13.5, 19.6, and 28.4. The concentrations of various water quality parameters for the batch tests are shown in Table 3.2.

Table 3. 2 Concentration of synthetic wastewater using acetate and sodium nitrite and biomass used in the batch tests

Conditions	**B1**	**B2**	**B3**	**B4**
COD (mg/L)	102	186	312	362
NO_2-N (mg/L)	15.3	13.8	15.9	12.6
MLSS (mg/L)	3120	3060	2960	2960
MLVSS (mg/L)	1820	1740	1740	1680
pH-initial	7.4	7.45	7.44	7.42
pH-final	8.42	8.65	8.74	8.75

3.2.3 Analytical methods

The SBR influent and effluent, as well samples from the kinetic tests conducted in the SBR, were analyzed weekly. A total of 17 samples from influent and effluent of SBR, 80 samples (10 kinetic tests x 8 sample per test) from the SBR, and 32 samples for acetate test (4 kinetic tests x 8 samples per test) were analyzed to calculate SDNR and $\Delta COD_{consumed}/\Delta N_{removed}$. The collected samples were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), total COD (TCOD), soluble COD (SCOD), ammonia, nitrite (NO_2^-), total nitrogen (TN), soluble nitrogen (SN), total phosphorus (TP), and soluble phosphorus (SP). Sterile 0.45 µm membrane filter papers (VWR International, Canada) were used for filtration of the samples, and 1.2 µm filters were used for TSS and VSS analyses in accordance with Standard Methods (APHA et al., 2005). HACH methods were used to measure total phosphorous (Method 10127), ammonia (Method 10031), total nitrogen (Method 10072), nitrite (Method 8153), and COD (Method 8000).

3.3 Results and Discussion

3.3.1 Reactor pH and ORP

As a measure of the denitrification conditions, ORP and pH of the reactor were monitored, and representative data are shown in Figure 3.2. With the addition of nitrite to the SBR, the ORP rapidly increased, with the magnitude of increase depend on the concentration of nitrite. For example, ORP increased from -350 to -250 mV, corresponding to a nitrite concentration of 9.0 mg/L (Figure 3.2). The increase in ORP was due to the increase in the concentration of oxidants (i.e. nitrite), however, the ORP dropped with time with the reduction of nitrite, and dropped further upon addition of MWW.

The pH sharply increased from 8.70 to 9.1 during denitrification in each cycle (Figure 3.2), and decreased to 8.7 again when denitrification ended, and municipal wastewater was fed to the bioreactor at a pH of 7.0-7.2. This can be related to the following stoichiometric equation (Ge et al. 2012):

$$2NO_2^- + CH_3OH \rightarrow N_2 + H_2O + CO_2 + 2OH^-$$

Kim et al. (2004) reported that the optimum denitrification rates from nitrite were not affected by pH up to 11.0, and thus there was no inhibition due to pH during this study.

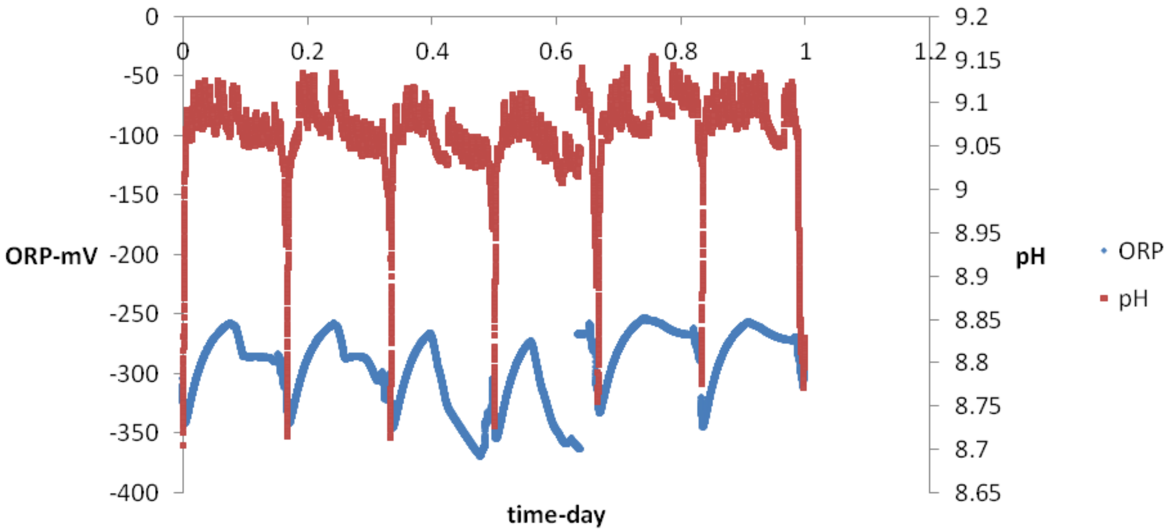


Figure 3. 2 ORP and pH (NO_2-N of 9mg/L and SDNR of 0.04 mg NO_2-N/mg VSS-d)

3.3.2 Influent and effluent characteristics

The summary of influent and effluent water quality parameters is presented in Table 3.3, which shows that TCOD varied from 200 to 800 mg/L (average 526±332 mg/L), and SCOD varied from 83 to 350 mg/L (average 176±55 mg/L). The average primary effluent BOD_5 during the study was 232mg/L or 44% of the average measured TCOD. The NO_2-N fed to the SBR varied between 2.75 and 40 mg/L, with an average of 30 mg/L. With highly variable influent characteristics, a steady-state condition was not achieved in the SBR, as can be seen in Figures 3.3A-3.3D. Since the 4-hr-cycle in closed SBR system was anoxic and DO was near zero, no nitrates were detected in the effluent.

Table 3. 3 Influent (primary effluent from Greenway Wastewater Treatment Centre) and effluent characteristics (average ± standard deviation of 15-17 samples)

Parameter	Influent (mg/L)	Effluent (mg/L)
TCOD	526±332	158±30
SCOD	176±55	80±11
NO_2-N	30±14	10±10
TN	81±24.4	49±23
SN	65±22.6	41±19.1
TP	8±4.9	2.9±1.06
SP	2.3±1.4	1.2±1.03
NH_3-N	30±5.7	22±7.8
TSS	254±198	63±12
VSS	236±251	47±8
	Bioreactor	
TCOD/NO_2-N	20±23	
SCOD/ NO_2-N	9±8	
MLSS	7000±1617	
MLVSS	4500±831	

As indicated by MLVSS variations during this study in Figure 3.3A, complete acclimatization was considered after 20 days as the biomass levels showed an increasing trend with consistent COD and nitrite removal ratio, reflected by SDNR/SSUR (specific substrate utilization rate) in the denitrifying SBR (Figures 3.3C and 3.3D). TN_i concentrations estimated based on the combined concentrations of TN in the influent wastewater and the nitrite added in the SBR according to Eqs. 3-1, 3-2, and 3-3 varied between 6 and 36 mg/L (Figure 3.2B).

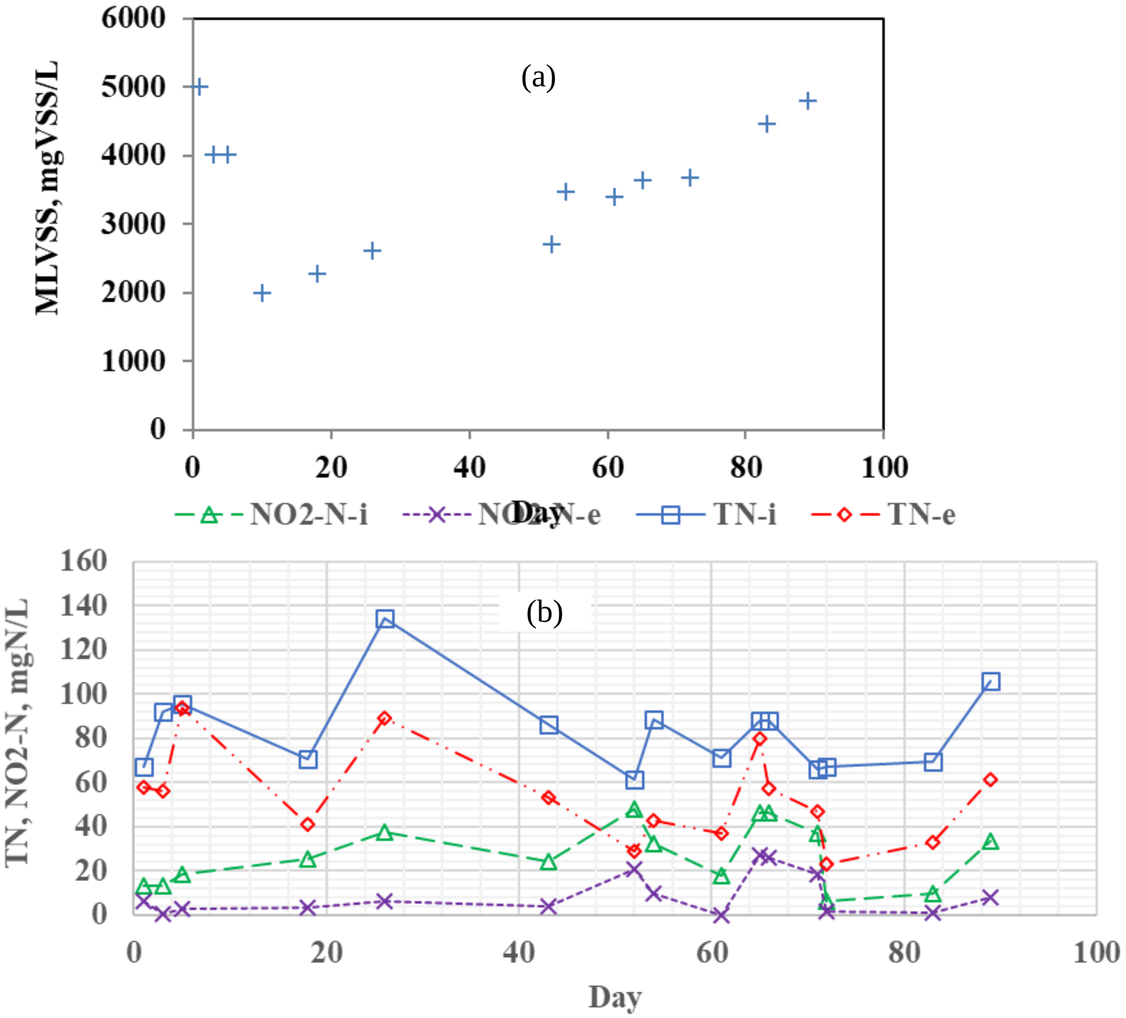
$$NO_2\text{-}N_i = \frac{NO2-N\ mg/L(measured\ at\ time\ 0\ in\ the\ bioreactor\ )*10.5\ L-\ 5.5L*NO2-Ne\ mg/L}{5L} \tag{3-1}$$

$$TN_i\ mg/L = TN(measured\ in\ feed\ tank)\ +\ NO_2 - N_i \tag{3-2}$$

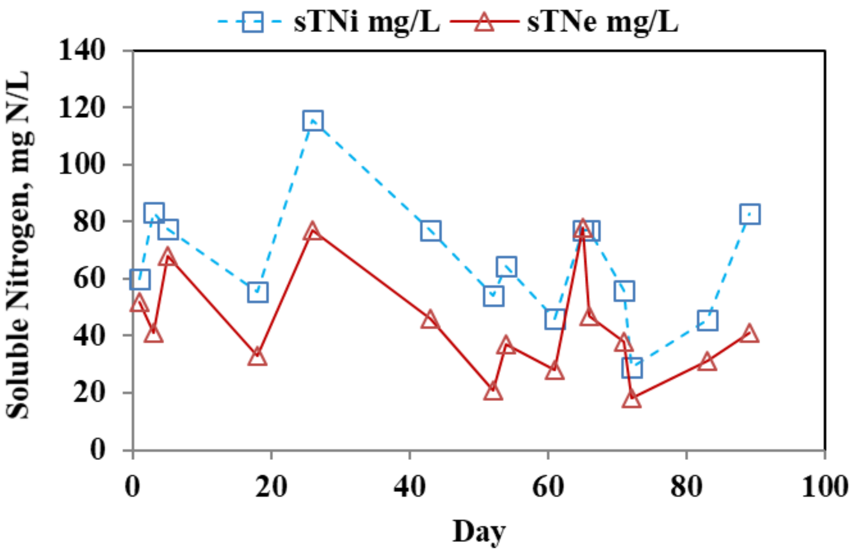
$$SN_i\ \ mg/L = SN(measured\ in\ feed\ tank)\ + NO_2 - N_i \tag{3-3}$$

Similarly, the average SN_i (influent) concentration was 65 mg/L comprised of NH_3-N, NO_2-N, and soluble organic nitrogen concentrations of 30 mg/L, 30 mg/L, and 5 mg/L, respectively (Figure 3.2C). Conversely, the average SN_e (effluent) was 41 mg/L including 22 mg/L NH_3-N, 10 mg/L NO_2-N, and 9 mg/L of soluble organic nitrogen. An average of 12 mg/L TKN (TKN_i of 51 mg/L less TKN_e of 39 mg/L) including 8 mg/L of NH_3-N was removed, and 20 mg/L nitrite was decreased as well due to anabolic metabolism (assimilation in biomass) and catabolic metabolism (dissimilation to N_2), respectively. The observed difference between influent and effluent soluble organic nitrogen of 4 mg/L is attributed to the hydrolysis of the effluent biomass in the effluent tank, which had a retention time of ≤ 1.7 day. The average removal efficiencies of COD and nitrite varied between 32%-88% (average 64% for TCOD and 56% for SCOD) and 50%-91% (average 72%), respectively.

Figure 3.4A presents the relationship between the nitrite removal efficiency and the influent TCOD/NO_2-N ratios, indicating that there is an optimum COD/NO_2-N ratio beyond which nitrite removal efficiency decreased. Further explanation of the effect of COD/NO_2-N ratio is shown in the kinetic section below. Previous research indicated that complete denitrification using a synthetic wastewater required a stable BOD/N ratio of 3 to 3.2 during the process (Rocher et al., 2015). However, in this study it was very difficult to maintain a stable COD/N ratio due to the high variability of the influent COD. Therefore, influent nitrite concentration varied depending on the influent COD. Although the COD/N ratio varied widely at 9±8 with a relative standard deviation (RSD) of 89%, the average nitrite removal efficiency in the SBR was 72%. Furthermore, the ΔCOD/ΔN ratios, calculated from the ratio of the specific substrate (SCOD) uptake rate (SSUR) and SDNR (SSUR/SDNR), showed a relatively lower variation at 3.3±1.8 with a RSD of 55%. Total nitrogen in the effluent (Figure 3.3B) was high due to the remaining nitrite and ammonia nitrogen. Although influent ammonia concentration was 30 mg/L, accounting for 50% of SN_i (Figure 3.3C), only a small portion of TKN was removed (11 mg/L) including 8.0 mg/L of ammonia by biomass synthesis and the remaining ammonia was included in the effluent soluble nitrogen (SN_e) of 41 mg/L.


(a)
MLVSS, mgVSS/L
0
1000
2000
3000
4000
5000
6000
0
20
40
60
80
100
Day
NO2-N-i
NO2-N-e
TN-i
TN-e
(b)
TN, NO2-N, mgN/L
0
20
40
60
80
100
120
140
160
0
20
40
60
80
100
Day

(c)



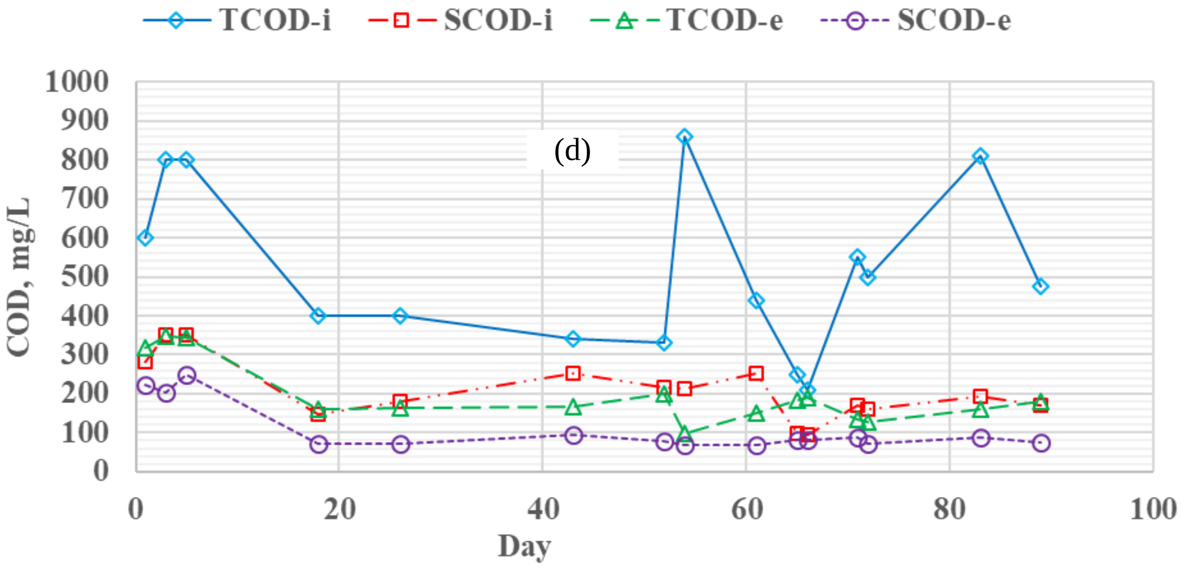
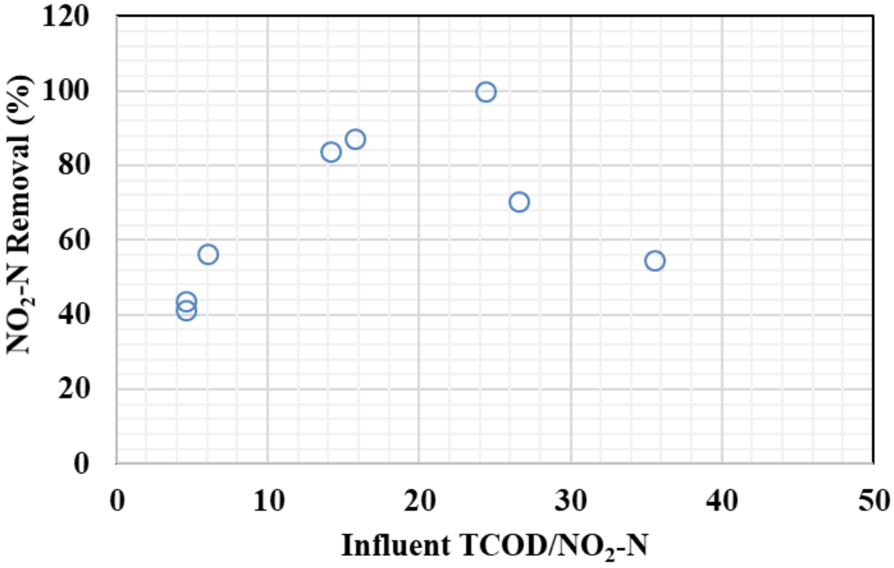


Figure 3. 3 Variation of solids, nitrogen, COD concentration in the SBR with time (A) MLVSS (B) influent (TNi, NO_2-Ni) and effluent (TNe, NO_2-Ne) (C) influent and effluent (STNe) (D) influent (CODi) and effluent (CODe)

A



B

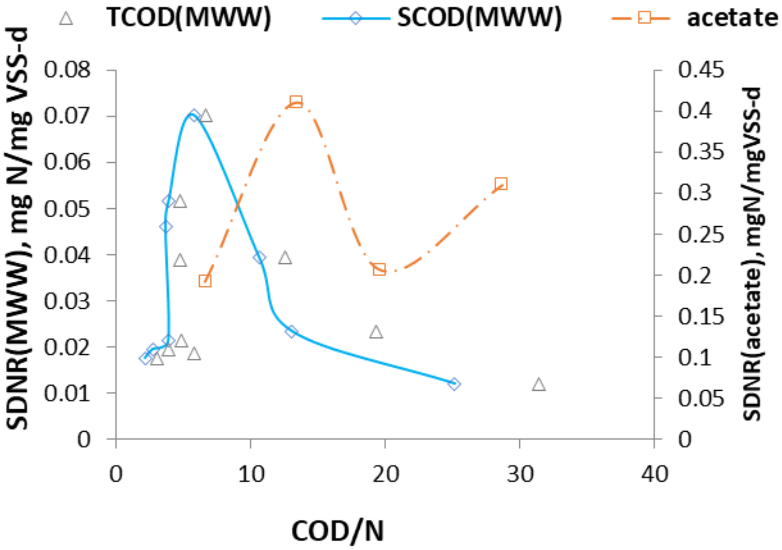


Figure 3. 4 (A) Relationship between influent COD/NO_2-N and nitrite removal efficiency (B) SDNR vs COD/N (initial COD and NO_2-N) in the bioreactor for municipal wastewater-MWW (SCOD range of 75-130 mg/L and NO_2-N of 2.75-36 mg/L), MWW (TCOD range of 85-145 mg/L and NO_2-N of 2.75-36 mg/L), and acetate (SCOD range of 102-362 mg/L and NO_2-N of 12.6-15.9 mg/L) as carbon source

3.3.3 Kinetic Studies at different COD/N ratios

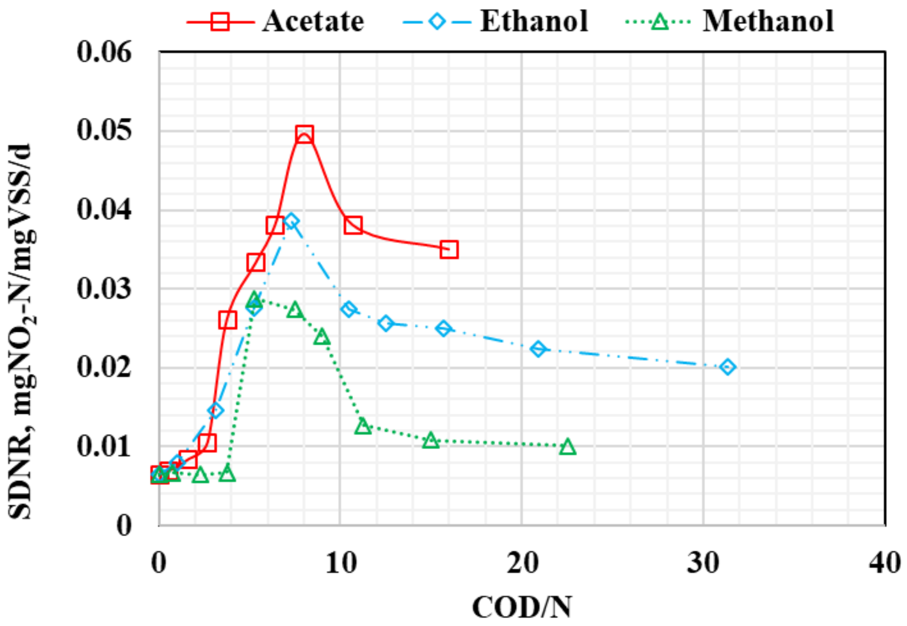
3.3.3.1 FNA and COD Inhibition in relation to COD/N

The denitrification kinetic studies were performed at different total and soluble COD to nitrite-N (COD/NO_2-N) ratios in the SBR with real wastewater and in batch reactors with different concentrations of acetate to determine the specific denitrification rate (SDNR) for both carbon sources. The COD/N ratios represent the initial TCOD, SCOD, and NO_2-N in the tests. The kinetic data followed zero-order kinetics and the SDNRs were calculated by dividing the slope of the line of nitrite concentration removed during the SBR anoxic cycle normalized to the time-day (mgNO_2-N/L/d) by the MLVSS concentration. Similarly, the SSURs were calculated by dividing the slope of the line of COD concentration removed during the SBR anoxic cycle normalized to the time-day (mgCOD/L/d) by the MLVSS concentration. During the SBR kinetic tests, the initial SCOD/N ratios ranged from 2-25. It must be asserted that the SSUR was calculated based on SCOD removed, taking into consideration the COD solubilized from the hydrolysis of particulate organics using a hydrolysis coefficient of 1.8 d^{-1} (Kappeler and Gujer, 1992; Jeppsson,1996; Drolka et al., 2001) of the average influent VSS. The adjusted TCOD/N was estimated by adding a COD of 1.42 gCOD/gVSS to the measured SCOD during each 4-hr-cycle, yielding the TCOD/N ratio of 3-35. In the range of COD/N ratios, SDNR varied between 0.01-0.07 mgN/mgVSS/d, (Figure 3.4B). A similar maximum SDNR of 0.07 mgN/mgVSS/d was reported by Raboni et al. (2014) for wastewater, but using nitrate in the pre-anoxic reactor.

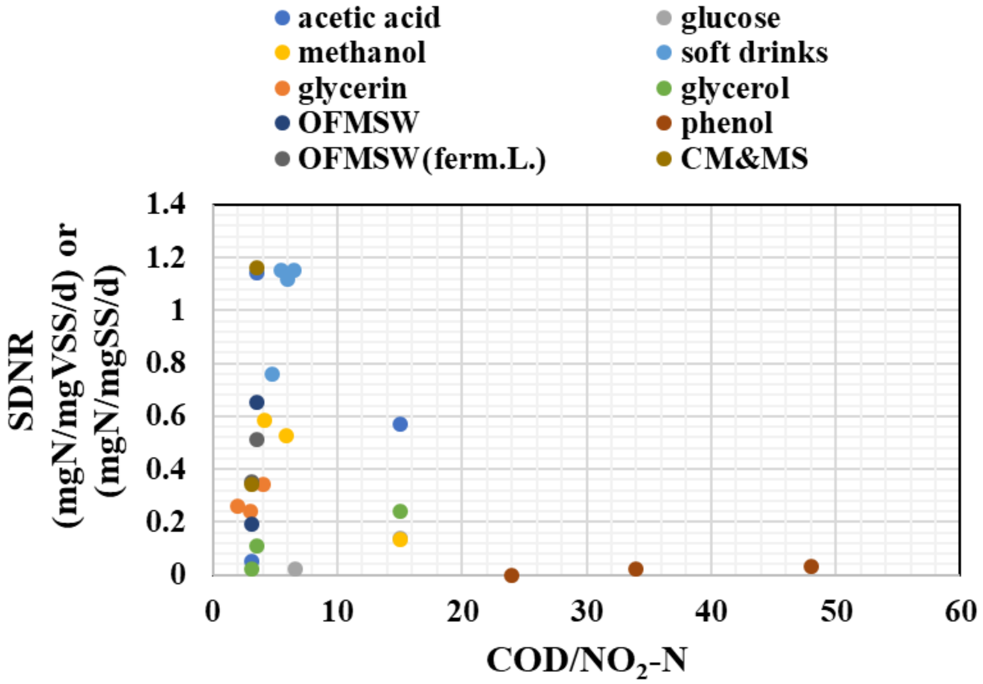
The SDNR with MWW linearly increased with the increase in SCOD/N ratio in the 3-6.6 range. There was an optimum SCOD/N ratio beyond which SDNR decreased, with similar trend and values (Figure 3.4B). With acetate as a carbon source, much greater SDNRs were obtained compared to those of municipal wastewater. The highest SDNR (0.4 mgN/mgVSS/d) with acetate was observed at COD/N ratio of 13.5 beyond which SDNR decreased (Figure 3.4B). The higher SDNR achieved with acetate relative to wastewater was due to higher and faster biodegradability of acetate (100% biodegradable), as compared with the MWW organics which are typically a complex mix of carbohydrates, proteins, lipids, and VFA. Nevertheless, both carbon sources (wastewater and acetate) showed the similar trend with respect to the effect of COD/N ratio on SDNR. It should be noted that at SCOD/NO_2-N ratios $<$ 6.6 the NO_2-N concentrations ranged from 17 to 36 mg/L. On the other hand, at SCOD/NO_2-N ratios $>$ 6.6 the NO_2-N concentrations ranged from 2.75 to 9 mg/L. As shown later, the K_{NO2} derived from the kinetic studies was 4.07 mgNO_2-N/L and therefore the observed decline in SDNR with MWW can be explained by the low ambient NO_2-N concentrations relative to K_{NO2}, as per the Monod model. As shown in Figure 3.4B, SDNR with acetate peaked at 0.41 mgNO_2-N/mgVSS-d, at COD/N ratio of 13.5 and NO_2-N concentration of 13.8 mg/L (Table 3.2). The observed reduction in SDNR occurred at high acetate concentrations (B3 and B4 – Table 3.2) of $>$ 300 mgCOD/L, and may be attributed to the lack of biomass acclimatization to high acetate concentrations.

Earlier researchers had shown that SDNR (NO_x-N) increased with an increase in COD/N; however, these tests were conducted at low COD/N ratios (2-5) with external carbon sources (Oh and Silverstein, 1999; Fernández-Nava et al., 2010; Beccari et al., 1983; Chung et al., 2002; Katarzyna et al., 2015). The negative effect of high COD/N ratio on SDNR was reported in very few studies (Adav et al., 2010; Akuna et al., 1993; Ge et al., 2012). Additionally, denitrification studies with nitrite were relatively fewer than those conducted using nitrate. Adav et al. (2010) conducted SDNR tests using three different carbon sources (acetate, ethanol, methanol) at different COD/N ratios ranging <1 to 35 with carbon source concentrations of 100-3000 mg/L, and NO_2 concentration of 200 mg N/L. The data of Adav et al. (2010) were analyzed, and replotted in Figure 3.5A to demonstrate the effect of COD/N ratio on SDNR. The optimum COD/N ratio for nitrite-denitrification rate varied with the type of carbon source, i.e. 8 for acetate, 7.5 for ethanol, and 5 for methanol with respective SDNR of 0.048, 0.041, and 0.03 mgN/mgVSS/d. Since the tests were conducted at high substrate concentrations (NO_2^- and COD), the decrease in SDNR at high COD/N ratios could be attributed to organic substrate inhibition, as suggested by Ge et al. (2012), who reported a decrease in SDNR with glucose from 0.64 mg NO_3-N/mgVSS/d at COD/N of 15, and COD of 600 mg/L, to 0.04 mgNO_3-N/mgVSS/d at COD/N of 25, and a COD of 1000 mg/L.

A


Acetate
Ethanol
Methanol
SDNR, mgNO$_2$-N/mgVSS/d
0.06
0.05
0.04
0.03
0.02
0.01
0
0
10
20
30
40
COD/N

B


acetic acid
methanol
glycerin
OFMSW
OFMSW(ferm.L.)
glucose
soft drinks
glycerol
phenol
CM&MS
SDNR
(mgN/mgVSS/d) or
(mgN/mgSS/d)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
COD/NO$_2$-N

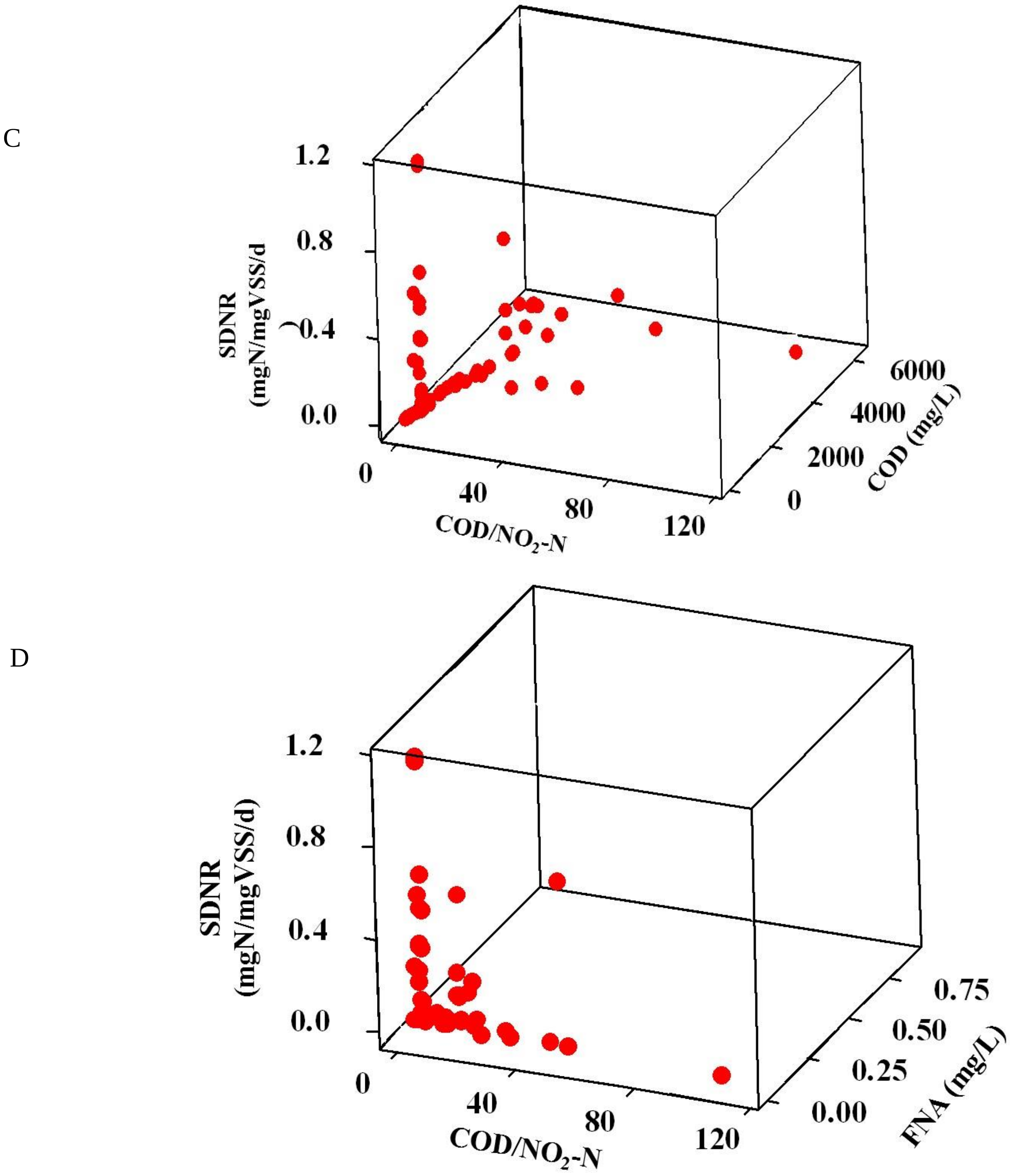


Figure 3. 5 (A) COD/N vs SDNR (NO_2-N 200 mg/l, pH=7.5, FNA of 0.04 mg/L) (Adopted and modified from Adav et al., 2012) (B) Collective graph showing the relation between SDNR mgNO_2-N/mgVSS-d and COD/NO_2-N ratio (initial COD and NO_2-N) (C) Effect of COD/NO_2-N and COD on SDNR collected from Table 3.1 (D) Effect of COD/NO_2-N and FNA on SDNR collected from Table 3.1

Akunna et al. (1992), who investigated the denitrification potential in a digester using glucose as a carbon source at a COD/N ratio of 2.3-106 (COD concentration = 5318 mg/L and nitrite concentration = 50-2500 mg/L), and found the optimum COD/N ratio to be 8.86 for nitrate removal (removal efficiency of 64 %), and 7.0 for nitrite removal efficiency (removal efficiency of 52 %) based on the influent and effluent nitrate and nitrite concentrations. The authors concluded that denitrification rates declined at high nitrite concentrations due to inhibition by free nitrous acid (FNA), which ranged between 0.017 and 0.88 mg/L.

A graph (Figure 3.5B) was drawn to further investigate the effect of COD/N ratio on the SDNR (data were taken from Table 3.1). Figure 3.5B shows that SDNR increased as COD/N ratio increases until a certain point beyond which SDNR decreased. Further examination of the effect of COD and FNA on SDNR (Figures 3.5C and 3.5D) showed that the SDNR widely varied in the low range of COD/N ratio (<6), indicating the effect of different substrate type. However, average SDNR at COD (<2g/L) and COD (>2 g/L) were 0.19 and 0.08 mgN/mgVSS/d, respectively, indicating inhibition. FNA ranged from <0.01 to 0.88 mg/L, mostly less than 0.05 mg/L, and 8.2% of 61 data points >0.1 mg/L. SDNR at FNA of <0.05 mg/L were spread over the range of <0.01 to 1.16 mgN/mgVSS/d, with an average 0.16 mgN/mgVSS/d, much higher than the average 0.02 mgN/mgVSS/d at FNA concentrations of 0.1-0.88 mg/L, indicating the minimal effect of FNA below 0.05 mg/L and the significant negative effect of high FNA on SDNR.

Hence, the data from these previous studies indicated that the negative effect of high COD/N ratios on SDNR was possibly due to substrate and FNA inhibition. However, in this study, the SCOD concentrations in the SBR and batch reactors were approximately 100 mg/L and 360 mg/L for municipal wastewater and acetate, respectively, minimizing the effect of substrate inhibition. Similarly, FNA concentration in the reactor was estimated at 0.0026 mg/L (NO_2-N concentration of 35 mg/L at pH of 8.2, pK_A 5.6×10^{-4}), indicating the insignificant effect of FNA.

The relationship between TCOD/N (including the hydrolyzed particulate COD) and ΔTCOD/ΔN is presented in Figure 3.6A. ΔTCOD/ΔN were in range of 3 - 5 at the same TCOD/N ratio for 50% of the measured points which confirms the stoichiometric ratio of ($\Delta TCOD/\Delta N = 1.71/(1-1.42Y)$ since the yield is constant for the same carbon source. However, ΔTCOD/ΔN increased as

TCOD/N increased for TCOD/N ratio of 8-15, before stabilizing at ΔTCOD/ΔN of 14 in the TCOD/N range of 20-35. Since the ratio of ΔTCOD/ΔN is in essence of the ratio of SSUR/SDNR, the high ΔTCOD/ΔN are due to high apparent SSUR as a result of COD consumption for maintenance. Further scrutiny of the 10 data points reveals that 8 points have TCOD/N ratio of 3-12, corresponding to ΔTCOD/ΔN range of 3.0 to 7.0 which lies within the normal removal ratios (ΔTCOD/ΔN). Nevertheless, the other 2 points, corresponding to ΔTCOD/ΔN of 11and 13.3, with respect to TCOD/N of 20 and 32, occurred at nitrite removals of 1.8 and 3.9 mg/L, respectively. Thus, ΔTCOD removed in the SBR were 24 and 43 mg/L for the initial nitrite concentrations of 2.7 and 4.8 mg/L, respectively, or 4.5% and 8.1% of the influent COD and hence can be due merely to experimental errors.

3.3.3.2 Impact of F/M ratio on SDNR

Furthermore, the data shown in Figure 3.5A can be plotted based on the Lineweaver-Burk model (Lai et al., 2014) (Figure 3.5B). Since ΔSCOD/ΔN is proportional to SSUR/SDNR ratio, the following model was developed for wastewater (Eq. 3-4, R^2=0.85):

$$SDNR = 1.66\, SSUR \left(\frac{N}{SCOD}\right)_i \tag{3-4}$$

Where SDNR is specific denitrification rate (mgNO_2-N/mgVSS/d), SSUR specific substrate utilization rate (mgSCOD/mgVSS/d), i denotes the initial feed concentrations (mg/L) of SCOD and NO_2-N. The Lineweaver-Burk model fitted the experimental data for wastewater quite well. For acetate, the experiments were conducted mostly in the high COD/N ratios, which coincided very well with the data collected for wastewater as can be seen in Figure 3.6B; indicating that a similar model can be used for acetate in that range.

Metcalf and Eddy Inc. et al. (2014) presented an empirical relation between $SDNR_{NO3}$ and F/M ratio (mgBOD_5/mg$VSS_{\text{active biomass}}$/d) as shown below (Eq. 3-5):

$$SDNR = 0.029 + 0.03\, F/M \tag{3-5}$$

Eq 3-5 was further modified considering that the DO concentration in the pre-anoxic reactor due to the internal recirculation ratio (IR) and return activated sludge (RAS) reversely affect SDNR. Raboni et al. (2014) mentioned that nitrogen removal occurs due to anabolic assimilation (cell synthesis from nitrate) and catabolic dissimilation (denitrification to N_2). The assimilative part for denitrifiers is 0.05ΔBOD_5×Q/(X×V_{anox}) or (0.05 F/M). Considering the optimum ΔBOD_5/ΔNO_3-

N as 4 (Raboni et al., 2014) and substituting it in the above expression, we obtain $0.05 \times 4 NO_3$-$N \times Q/XV_{anox}$, the assimilative N = 0.2 $SDNR_{dissimilative}$, which results in a NO_x overall removal rate of 1.2 $SDNR_{dissimilative}$. The other assimilative contribution was due to aerobic heterotrophs cells (in both RAS and IR) that still consume nitrogen for cell synthesis even with low DO according to the following Eq. 3-6:

$$SDNR = 1.2\, SDNR_{\text{dissimilative}} \frac{K_o^{/}}{K_o^{/} + DO} + 0.05\, \frac{F}{M}\, \eta_{BOD} \frac{DO}{0.2 + DO} \tag{3-6}$$

Where $K_o^- = 0.18$ mg/L, $SDNR_{dissimilative}$ = 0.07 $mgNO_3$-N/mgVSS/d, $\eta_{BOD} = 0.9 - 0.95$, DO =0.2-0.3 mg/L in the pre-anoxic zone (Raboni et al., 2014), the equation was further reduced based on the above assumption to Eq. 3-7:

$$SDNR = 0.026 + 0.036\, F/M \tag{3-7}$$

In this study, assimilation of nitrogen in the biomass was predominantly due to the used ammonia from the wastewater which, affected anoxic removal of ammonia from wastewater, and the nitrite removal was directly related to $SDNR_{dissimilative}$ as the DO was near zero, and the biomass was strictly denitrifiers (no nitrogen assimilation by aerobic microorganisms). With the above conditions and applying similar principles to those mentioned by Raboni et al. (2014), an empirical relation between $SDNR_{dissimilative}$ ($mgNO_2$-N/mgVSS/d) and F/M (mgSCOD/mgVSS/d) was derived (Eq. 3-8) and the results are presented in Figure 3.7A:

$$SDNR_{dissimilative} = 0.32\, \frac{F}{M} \tag{3-8}$$

The difference between these two models (Eqs. 3-7 and 3-8) was due to the following: (i) Raboni et al. (2014) used BOD/N ratio in contrast to SCOD/N ratio used in this work, (ii) only one BOD/N ratio was used compared to the wide range of COD/N ratio used in this work, and (iii) Raboni et al. (2014) considered denitrification from NO_3 as compared to NO_2 in this work.

3.3.3.3 Observed yields in the SBR and batch tests

The observed yield (Y_H), was estimated according to a study by Rahman et al. (2016). The carbon to nitrogen ratio (COD/N) was calculated by plotting COD concentration versus nitrite concentration during the anoxic cycle of the SBR and the slope is the ratio of removal of nitrite to COD nitrogen (or the ratio of SDNR/SSUR). The value of the yield depends on the operational conditions including substrate type. In this study, the Y_{HD} values are estimated based on 95% confidence limit for both municipal wastewater and acetate.

The observed yield (Y_{HD} – mgVSS/mgCOD) is then calculated based on the following Eq. 3-9:

$$Y_{HD} = (1 - \frac{1.71\ SDNR}{SSUR})/1.42 \qquad (3\text{-}9)$$

Where SDNR is specific denitrification rate and SSUR is specific substrate utilization rate mg COD/mg VSS/d. The ratio of SDNR/SSUR was plotted for wastewater and acetate as shown in Figure 3.7B, and the observed yield (Y_{HD}) was 0.33 and 0.51 mgVSS/mgTCOD for municipal wastewater (MWW) and acetate, respectively. Based on the 0.33 mgVSS/mgTCOD for municipal wastewater, the removed TCOD of 368 mg/L (Table 3.3), and the estimated nitrogen content of VSS (0.1 mgN/mgVSS), the TKN consumed for cell growth was estimated as 13 mgN/L, very close to the measured average of 12 mg/L with Y_{HD} of 0.32 (Table 3.3).

The biomass yield based on nitrite consumption (Y_N, mgVSS/mgNO_2-N) was calculated according to the stoichiometric Eq. 3-10 (Metcalf and Eddy Inc. et al., 2014):

$$Y_N = (\ \frac{1.71\, Y_H}{1 - 1.42\ Y_H}) \qquad (3\text{-}10)$$

Substituting in the above equation Y_N (mgVSS/mgNO_2-N) is obtained as 1.062 for MWW (TCOD), and 3.16 for acetate. Kornaros et al. (1996) reported Y_N of 1.41 with nitrite and 2.43 mgVSS/mgNO_2-N for nitrate with glutamate as carbon source.

The yield was also calculated from half reactions with ammonia as the nitrogen source for biomass and nitrite as the electron acceptor as shown in the following overall stoichiometric reaction:

$$\begin{aligned} 0.02C_{10}H_{19}O_3N + 0.19NO_2{}^- + 0.19H^+ + 0.094\ CO_2 + 0.094HCO_3{}^- + 0.0015NH_4{}^+ \\ = 0.0215C_5H_7O_2N + 0.095N_2 + 0.2135H_2O \end{aligned}$$

From the above equation Y_H is 0.3 mgVSS/mgCOD, close to measured yield value of 0.33mgVSS/mgCOD (10% difference).

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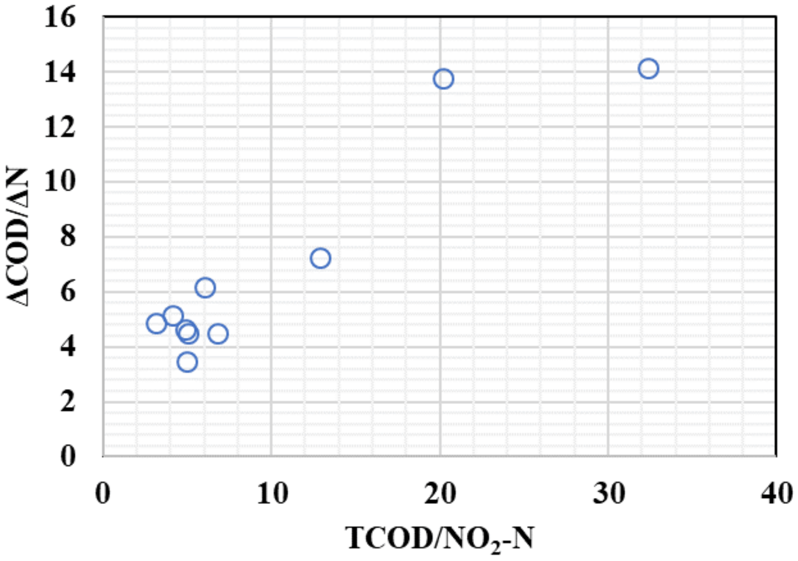

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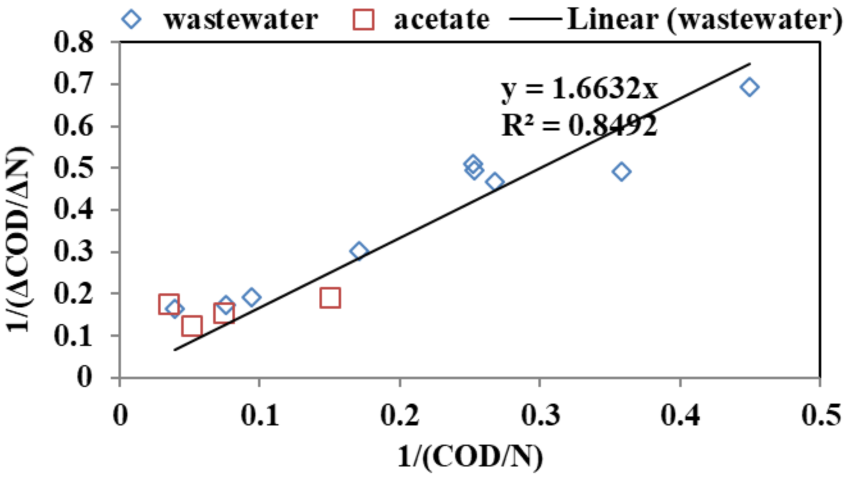

Figure 3. 6 (A) Relationship between TCOD/N (initial COD and NO_2-N) and ΔTCOD/ΔN for municipal wastewater (MWW) (B) Relationship between the inverse of the TCOD/N and ΔTCOD/ΔN ratios

3.3.3.4 Monod Kinetics

In order to determine other kinetic parameters such as $\boldsymbol{\mu}_{hmax}$ and K_{NO2}, the Monod equation (Eq. 3-11) (Metcalf and Eddy Inc. et al., 2014) was used.

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\mu_{hmax}*S_s}{Y_h\,(K_s+S_s)}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right)\left(\frac{K'_o}{K'_o+S_o}\right)(\eta)\left(\frac{X_h}{X_{vss}}\right) \qquad (3\text{-}11)$$

Where K_{NO2} is half saturation concentration (mg/L), K_S is half saturation concentration (mg/L) $\boldsymbol{\mu}_{hmax}$ is biomass specific growth rate (d^{-1}), K'_o is DO denitrification-inhibition concentration (mg/L), and S_s, S_{NO2}, S_o are the concentrations of COD (mg/L), NO_2-N (mg/L), and DO (mg/L), respectively. Eq. 3-11 can be simplified to Eq. 3-12 since DO is close to zero in this work and the denitrifying consortia are strictly anoxic ($\eta = 1$). Additionally, substrate concentration is significantly higher than the reported Ks of 4 mg/L for wastewater and 1 mg/L for acetate (Kujawa and Klapwijk, 1999).

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\mu_{hmax}}{Y_h}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right) = \frac{\mu_{hmax}}{Y_N}\,\frac{S_{NO2}}{K_{NO2}+S_{NO2}} \qquad (3\text{-}12)$$

However, $\boldsymbol{\mu}_{hmax}$ can be directly related to Y_N (1.26 mgVSS/mgNO_2-N for MWW and 3.16 mgVSS/mgNO_2-N for acetate) and K_{Dmax} (maximum SDNR) of 0.07 mgNO_2-N/mgVSS/d for MWW and 0.4 mgNO_2-N/mgVSS/d for acetate, according to the relation of $K_{Dmax}=\mu_{max}/Y_N$, hence, $\boldsymbol{\mu}_{hmax}$ was obtained as 0.075 d^{-1} for municipal wastewater and 1.26 d^{-1} for acetate.

Using Eq. 3-12 with the replacement Y_H by Y_N, the Hanes plot was drawn (Eq. 3-13, and Figure 3.7C).

$$\frac{NO2-N}{SDNR} = \frac{NO2-N}{K_{Dmax}} + \frac{K_N}{K_{Dmax}} \qquad (3\text{-}13)$$

Where K_{Dmax} is substituted by $\boldsymbol{\mu}_{hmax}/Y_N$, K_N was determined as 4.07 mg/L (for MWW). This explains why complete removal of NO_2-N was not possible in the SBR with initial NO_2-N concentration of 2.75 and 4.8 mg/L due to the switching function of NO_2-N/(K_N+NO_2-N). The obtained $\boldsymbol{\mu}_{hmax}$ is in accordance with the reported values (1 - 4 d^{-1}) for external carbon sources (deBarbadillo et al., 2008; Kornaros et al., 1996; Yang et al., 2007; Koike and Hattori, 1975). The reported half-saturation coefficients (K_{NO2} and K_{NO3}) are 0.9 mgNO_2-N/L and 1.4 mgNO_3-N/L for acetate (Dosta et al., 2006) and 0.28 mg NO_2-N/L and 0.77 mgNO_3-N/L for L-glutamic acid

(Kornaros et al., 1996). However, Her and Huang (1995b) reported K_{NO2} of 10.9 mg/L and K_{NO3} of 14.3 mg/L with methanol which are higher than the values reported in this work.

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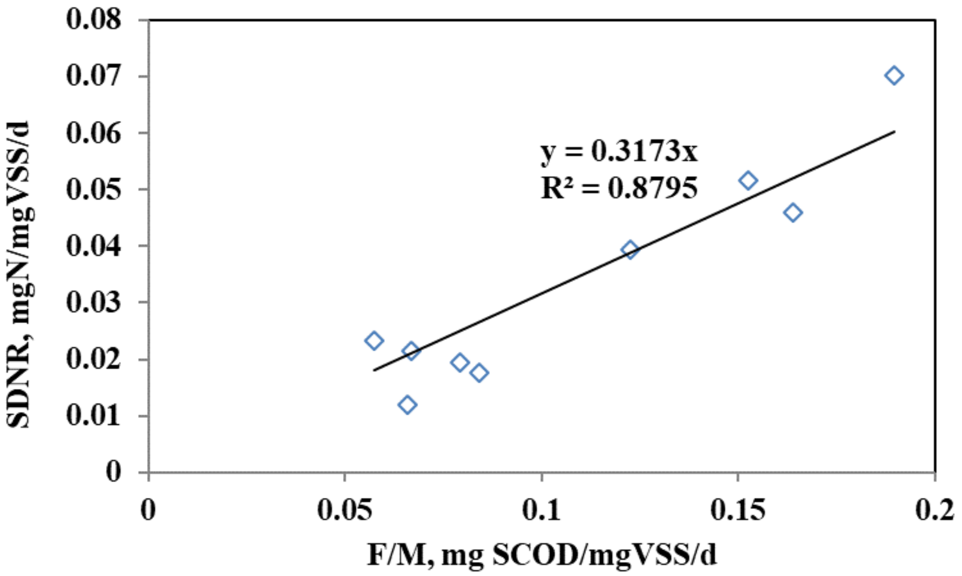

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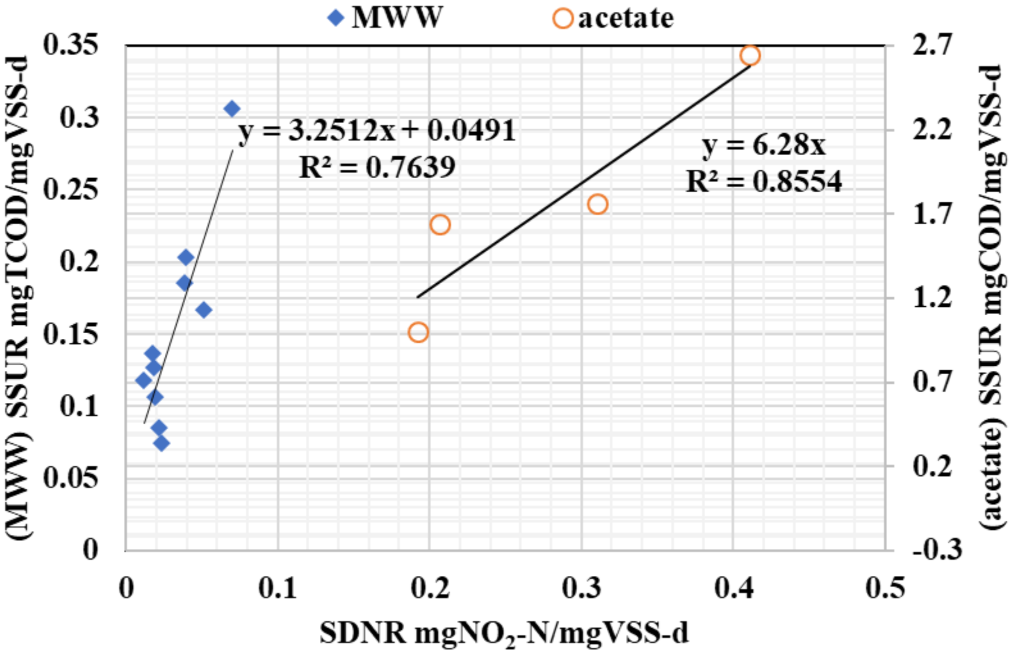

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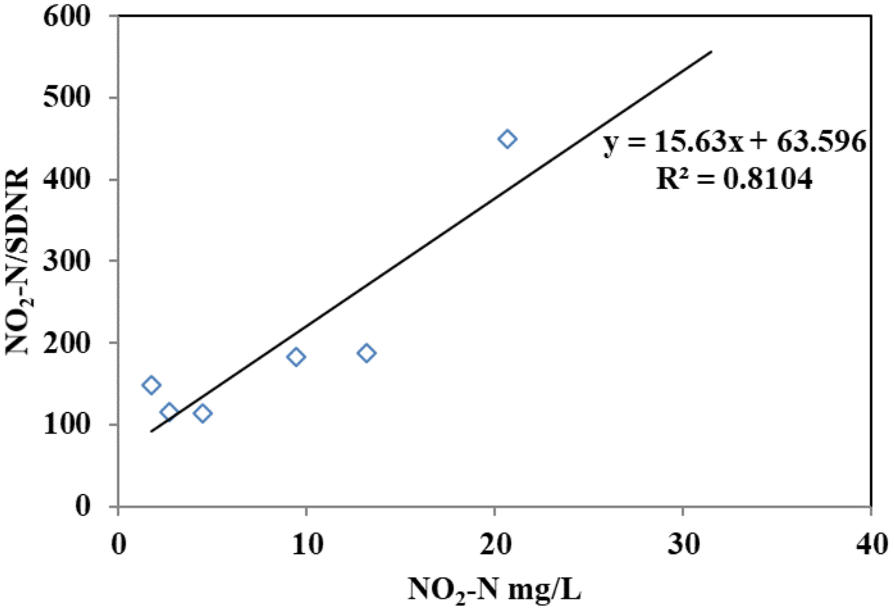

Figure 3. 7 (A) Relationship between SDNR and F/M ratio (B) Y_H from SSUR vs SDNR for municipal wastewater (MWW) and acetate (C) Hanes-plot to determine K_{NO2} and $\boldsymbol{\mu_{hmax}}$

3.4 Conclusions

A comprehensive study on denitrification from nitrite with real municipal wastewater and acetate as carbon sources was conducted at varying initial COD/N ratios. The specific denitrification rate (SDNR) was directly related to COD/N ratio, and a maximum SDNR of 0.07 (for wastewater) and 0.4 mgNO$_2$-N/mgVSS/d for acetate occurred at COD/N ratios of 6 and 13 respectively. At higher COD/N ratios, SDNR decreased. The yield coefficients were found to be 0.33 mgVSS/mgTCOD for municipal wastewater and 0.51 mgVSS/mgCOD for acetate. Predictive models were developed to determine SDNR as a function of initial COD/N ratio and F/M ratio.

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Chapter 4

Nitrite Denitrification using Biomass Acclimatized with Methanol as Complementary carbon source: Long-term Performance and Kinetics study

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The objective of this study was to investigate the dynamic specific denitrification rates (SDNRs) from nitrite at various COD/N ratios in a system that used methanol as a complementary carbon source. An SBR fed with municipal wastewater (MWW), methanol and nitrite solution was operated at an HRT of 8.4 hrs and SRT of 16-17 days for 110 days. The SDNR from the SBR employing methanol as a complementary carbon source was compared with those determined in 23 batch reactors employing MWW, methanol, nitrite, and nitrate. The batch tests were conducted using the biomass acclimatized with nitrite and methanol as a complementary carbon source. The maximum SDNR was 0.52 mgN/mgVSS/d, corresponding to COD/N ratios of 5.9 in the SBR, which was about eight times higher than the literature reported value for systems fed with MWW. However, the maximum nitrite SDNR was lower than the observed batch nitrate 1.06 mgNO_3^--N/mgVSS-d SDNR using the nitrite acclimatized biomass. In the batch test, a lower nitrite SDNR (0.38 mgNO_2^--N/mgVSS/d) was achieved using methanol as a sole carbon source. The nitrite denitrifier yields were 0.39 and 0.34 mgVSS/mgCOD using methanol as a complementary and sole carbon source, respectively. The half-saturation coefficient of nitrite (K_{NO2}) during post-denitrification with methanol was found to be 9.2 mg/L, which confirmed the lower SDNR values at lower nitrite concentrations below the K_{NO2}. The higher SDNR rates in methanol usage as a complementary carbon source versus sole carbon source could be translated into 37% capital and operating cost savings.

4.1 Introduction

Conventional nitrogen removal from domestic wastewater is a process that involves nitrification of ammonia and soluble biodegradable organic nitrogen to nitrate and nitrite. Then, these oxidized forms of nitrogen can be reduced to nitrogen gas by facultative heterotrophs when a carbon source is available. Most of the nitrogen removal treatment technologies in wastewater treatment plants employ nitrification and denitrification processes. This process is accompanied by the removal of organic carbon, which acts as an electron donor. As denitrification occurs under anoxic conditions, the process is affected by various parameters such as dissolved oxygen (DO), type and availability of organic carbon, COD-to-nitrogen ratio, temperature, and pH.(Almeida et al., 1995; Badia et al., 2019; Her & Huang, 1995b; Peng et al., 2007; Zhao et al., 1999)

Further, new processes are being developed for biological nitrogen removals (BNR) such as anaerobic ammonium oxidation (ANAMMOX) and partial nitrification-denitrification (Campos et al., 2019; Schmidt et al., 2003; Zhang et al., 2019). Notably, ANAMMOX technology, which removes ammonia nitrogen with nitrite under anaerobic condition, received substantial attention as it significantly reduces aeration cost and external carbon use. In order to achieve a successful ANAMMOX process, partial nitrification technology, by converting part of the ammonia to nitrite, was also extensively studied (Castro-Barros et al., 2017; Fudala-Ksiazek et al., 2014; Liu et al., 2010; Yang et al., 2007; Zeng et al., 2014). However, conventional biological nutrient removal (BNR) technologies are still widely used due to system stability and the scale-up issue of emerging technologies (Daigger et al., 2019; Du et al., 2019; Fofana et al., 2019; Gao et al., 2010; Onnis-Hayden et al., 2007; Wu et al., 2007). One viable option for saving energy without significant modification of the system is the combination of partial nitrification and nitrite denitrification, the nitrite shunt process. Compared to denitrification from nitrates, nitrite shunt processes saves up to 40% reduction in carbon, 25% reduction aeration energy, and lower capital cost.

Nitrite denitrification naturally occurs in ammonia-rich wastewater (ammonia ≥100 mgN/L) treatment processes such as side-stream wastewater treatment processes (Ruiz et al., 2006). However, cases for nitrite accumulation and further direct denitrification from nitrite is an emerging concept in mainstream municipal wastewater treatment observed either during nitrite shunt or partial denitrification processes. In the nitrite shunt processes, oxygen is limiting that creates out-competition of the nitrite-oxidizing bacteria by the denitrifying bacteria for nitrite (Gu et al., 2018; Mehrabi et al., 2020). However, most of these studies, the nitrite accumulation and nitrite denitrification, were not decoupled; hence the much-needed nitrite denitrification kinetics information was not available. Another application that reported nitrite accumulation was during denitrification from nitrate. Some of the cases where nitrite accumulation or nitrite denitrification has been discussed were in mainstream partial denitrification processes. In side-stream wastewater treatment, nitrite appears as an end product during nitrate denitrification. Mohan et al. (2016) studied denitrification of high strength wastewater with acetate and observed significant nitrite accumulation of 400, 1000, 2000, 2700 mgNO_2^--N/L corresponding to 677, 1354, 2031, 2708 mgNO_3^--N/L feed nitrate concentration, respectively. Partial denitrification from nitrate has also been reported in municipal wastewater treatment under limited carbon availability. Ge et al.(2012) observed nitrite accumulation of 22 mgNO_2^--N/L with glucose during denitrification from nitrate (40 mgNO_3^--N/L) at COD/N of 15 and a maximum accumulation of 17 and 19 mgNO_2^--N/L with acetate and methanol, respectively, at COD/N of 25. Similarly, a study by Li et al. (2008) showed more than 90% nitrite accumulation (90 mgNO_2^--N/L) during denitrification from nitrate using pyridine at 2, 4, 9, and 13 COD/N ratios. A high nitrate SDNR of 1.8 mgNO_3^--N/mgMLSS-d was obtained at all COD/N ratios. In the same process, the nitrite SDNR was 1.15 mgNO_2^--N/mgMLSS-d, slightly lower than the nitrate SDNR for all COD/N ratios. However, the authors reported significant nitrite accumulation as the carbon source was exhausted before complete denitrification from nitrite took place. One option to achieve complete denitrification is by adding methanol as a complementary carbon source.

Methanol has been used as a supplementary carbon source for denitrification from nitrate in Europe since 1990 to reduce total nitrogen to 2.2 mg/L, and in China, since 2003 to meet the environmental regulations for total nitrogen less than 5 mg/L (Theis & Hicks, 2012). The selection of methanol over other carbon sources is mainly related to the methanol cost as well as the environmental life cycle analysis (LCA). LCA is used to assess environmental impact parameters: ozone depletion, global warming, acidification, eutrophication, smog formation, ecotoxicity, particulate respiratory effects, human carcinogenic effects, and human non-carcinogenic effects. Methanol has the lowest impact on the LCA parameters compared to ethanol and acetic acid. For instance, the impact of methanol on CO_2 emission was 1.4 kgCO_2 produced per kgNO_3 removed, lower than ethanol (2.07 kgCO_2/kgNO_3), and acetic acid (2.71 kgCO_2/kg NO_3).(Theis & Hicks, 2012) The acetate was found to have the highest impact on the ozone depletion; the ethanol impact was reported as 0.146 kg CFC-11eq, which is lower than the methanol (0.165 kg CFC -11 eq) and acetate (0.339 kg CFC -11 eq) impact.

Ginige et al. (2009) studied the denitrification from nitrate with methanol as a complementary carbon source in an SBR with different COD/N ratios, i.e., methanol-COD to NO_3-N (MCOD/N) of 0, 0.5, 1.0, 3.3, and TCOD/N of 1.2, 1.3, 2, 4.5. The study showed that the maximum SDNR was 0.36 mgN/mgVSS-d after sludge acclimatization at COD/N of 4.5. The above and other previous denitrification studies addressed the optimization of carbon type and COD/N ratio for complete denitrification from nitrate. To the best of the authors' knowledge, none of the previous research studied the effect of methanol as a complementary carbon source for denitrification of municipal wastewater from nitrite. Such information is essential to optimize denitrification from nitrite. The objectives of this study were to (i) identify the long-term effect of methanol usage as a complementary carbon source on SDNR and nitrite denitrification kinetics parameters, (ii) characterize the short-term performance of biomass grown on complementary methanol addition when fed with either internal carbon source (MWW), methanol or glucose carbon sources and in the presence of nitrite/nitrate as an electron acceptor. The latter will address the biomass's short-term performance under shock conditions where, for example, nitrate, as oppose to nitrite, is the electron acceptor or when fed with other carbon sources for the short term.

4.2 Materials and Methods

4.2.1 Bench scale setup and operation

A bioreactor (shown in Figure 4.1) with a working volume of 10.5 L, equipped with a mechanical stirrer (60 rpm), dissolved oxygen sensor, ORP and pH meters, connected to a control unit, was used in the experiments to ensure anoxic conditions. The system included air diffuser and DO control units for operation at different dissolved oxygen levels; however, for this study, the system was anoxically operated without aeration. The volumes of the influent and effluent tanks were 60 and 50 L, respectively, while a 3.8-L tank was used for supplying nitrite (100 mL/min for 2 min) to control the initial nitrite concentration in the reactor.

The influent wastewater was collected from the Greenway Pollution Control Plant (PCP) (London, ON, Canada) every 7–10 days and stored at 4°C before using. Previous analysis of Greenway's wastewater showed high strength primary effluent with high COD up to 1000 mg/L, (Badia et al., 2019); therefore, the reactors were fed with diluted MWW (1:1) representing the typical North American wastewater strength, nitrite, and methanol. High purity methanol (99.9%) was added to meet the desired MCOD/TCOD ratio, and the nitrite was added to maintain various COD/N ratios and to characterize the effect of COD/N on the SDNR. A synthetic nitrite stock solution was prepared using potassium nitrite with concentrations of 5000–17500 mg NO_2^--N/L, corresponding to 20–71 mg NO_2^--N/L in the SBR. A 99.9% purity methanol Optima^{TM} Fisher Chemical (Fisher Scientific) was used as a complementary carbon source.

The SBR was operated for 110 days in which the system reached a pseudo-steady-state (acclimatized biomass) after 73 days or four turnovers of the mean solids retention time (SRT). The SRT was maintained between 14-16 days, which was estimated based on the bioreactor-VSS divided by final effluent-VSS in the decant tank. During the pseudo-steady state period, biomass concentrations varied within 17%; however, despite the four turnovers and less variability of the biomass, a true steady-state was not achieved due to the high variability of the influent wastewater. The SBR cycle length was 240 min (6 cycles per day) with 20-min diluted MWW feeding, 1-min nitrite feeding, 1 min pure methanol feed within the desired MCOD/N range, 110-min react phase under complete mix anoxic conditions, 90-min settling, and 20-min decanting time. The fill was around 48%; in each cycle, 5 L was decanted and replaced with 5 L of feed, corresponding to a daily feed flow of 30 L (8.4 hr of hydraulic retention time, 10.5 L of effective bioreactor volume). Mixing in the SBR was only used during the reaction period. The bioreactor was operated at room temperature.

4.2.2 Sampling and Analysis

Influent, bioreactor and effluent samples were collected one to two times a week and analyzed for total suspended solids (TSS), volatile suspended solids (VSS), total COD (TCOD), soluble COD (SCOD), ammonia, nitrite (NO_2^-), total nitrogen (TN), soluble nitrogen (SN), total phosphorus (TP), and soluble phosphorus (SP). Samples were filtered using 0.45 µm membrane filter papers (VWR International, Canada) for soluble component analysis, and 1.2 µm filters were used for

TSS and VSS analyses in accordance with Standard Methods.(Ahmad & Reynolds, 1999; Beutler et al., 2014; Xu, n.d.) HACH methods were used to measure total phosphorous (Method 10127), ammonia (Method 10031), total nitrogen (Method 10072), nitrite (Method 8153), and COD (Method 8000). The pH and ORP in the reactor were monitored using Atlas Scientific Sensors.

The bioreactor feed concentration was determined by analyzing the SBR samples at the beginning of the SBR reaction time (time zero) and the end of the SBR cycle as per equations 4-1 to 4-6. The influent nitrite concentration (NO_2^--N_i) was the nitrite concentration fed to the system; it was calculated by subtracting effluent nitrite mass from the nitrite mass at time zero and dividing by the feed volume per cycle (Eq. 4-1). The total feed nitrogen (TN*i*) concentration was estimated based on the combined concentration of TN in the influent wastewater and the nitrite added in the SBR (Eq. 4-2). The feed soluble nitrogen concentration (SN_i) was calculated similar to TN*i*, the concentration was estimated based on the combined soluble nitrogen (SN) in the influent wastewater and the nitrite dosed to the SBR (NO_2^--Ni) (Eq. 4-3)

$$NO_2^-\text{-}N_i = \frac{NO_2\_No * V1 - NO_2\_Ne*(V1-V2)}{V2} \tag{4-1}$$

$$TN_i = TN(measured\ in\ feed\ tank) + NO_2^-\text{-}N_i \tag{4-2}$$

$$SN_i = SN(measured\ in\ feed\ tank) + NO_2^-\text{-}Ni \tag{4-3}$$

Where NO_2^--$N0$ and NO_2^--N_e represent the initial and final SBR nitrite concentration at the beginning (time zero) and end of the SBR cycle, respectively. V1 represents the SBR volume, and V2 represents the exchange volume or feed volume per cycle (5 L, 48% of SBR volume).

Similarly, the feed COD concentration, including the methanol COD (MCODi), the total MWW+methanol feed COD concentration (TCODi), and the soluble MWW COD ($SCOD$)+methanol feed COD concentration (SCODi) was calculated according to Equations 4-4, 4-5, and 4-6. The added methanol COD concentration (MCODi) was determined by subtracting effluent SCOD mass ($SCODe$) and MWW $SCOD$ mass from SCOD mass at time zero in the reactor ($SCODo$) and dividing by the feed volume per cycle (Eq. 4-4).

$$MCOD_i = \frac{SCODo * V1 - [SCODe*(V1-V2) + SCOD\ (measured\ in\ feed\ tank)*V2]}{V2} \tag{4-4}$$

$$TCOD_i = TCOD(measured\ in\ feed\ tank) + MCODi \tag{4-5}$$

$$SCOD_i = SCOD(measured\ in\ feed\ tank) + MCODi \tag{4-6}$$

Where $SCODo$ and $SCODe$ represent the initial and final SBR soluble COD concentration at the beginning (time zero) and end of the SBR cycle.

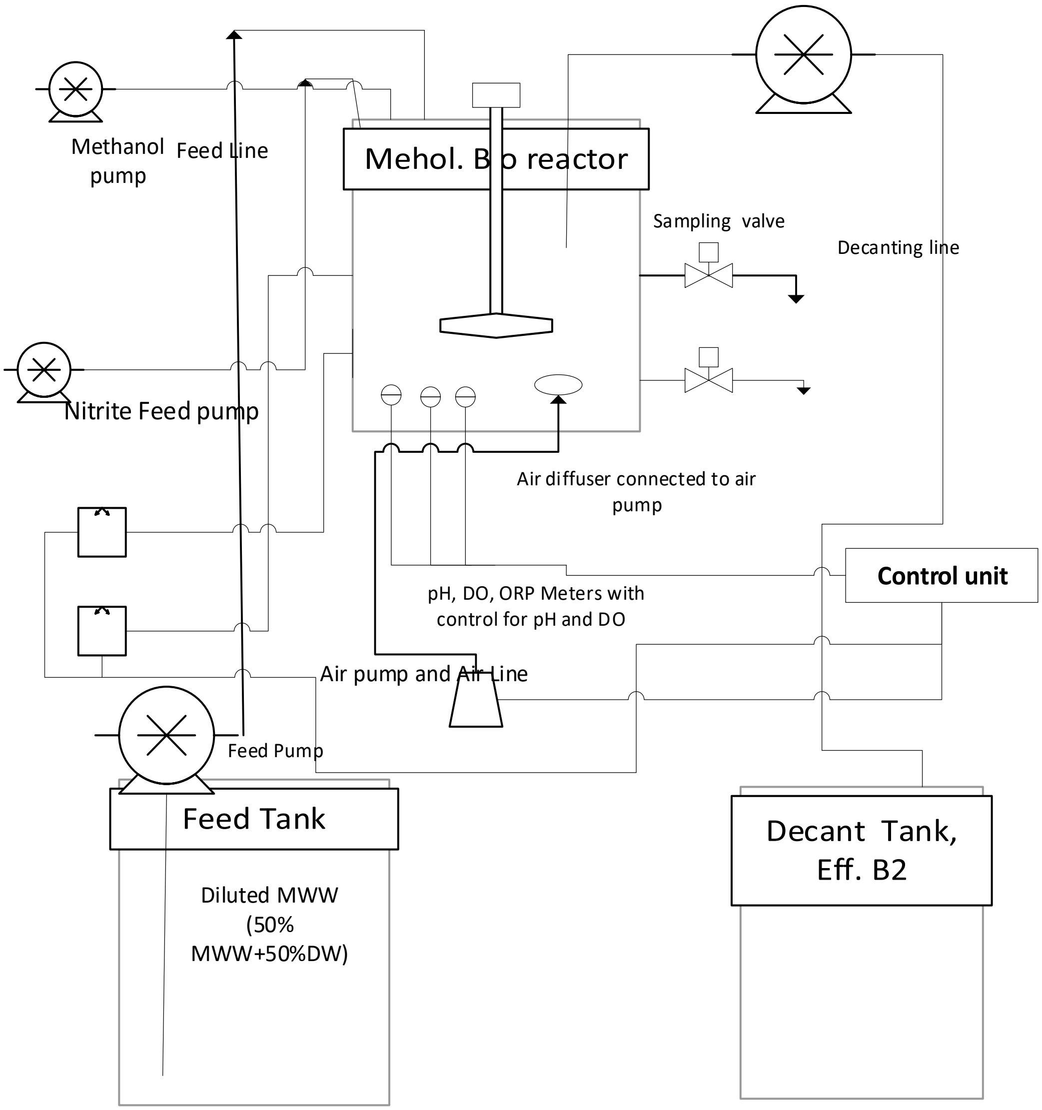


Figure 4. 1 Bioreactor set up

4.2.3 Batch tests

A total of 23 offline batch tests and 11 cyclic tests in the SBR were conducted. The cyclic tests were conducted in the SBRs to determine the maximum SDNR and kinetic parameters using carbon source similar to the long-term study (MWW+ Methanol) at different COD:N ratios, each lasting four hours. The offline batch tests were implemented to characterize the short-term performance of biomass grown on complementary methanol addition when fed with either internal carbon source (MWW), methanol or glucose carbon sources and in the presence of nitrite/nitrate as an electron acceptor (Table 4.1). The 23 offline batch experiments were conducted at the end of the long term SBR study, using a concentrated (centrifuged for 10 min at 1,800 *g*) nitrite-methanol+MWW acclimatized biomass from the SBR. The batch experiments were conducted in a 1-L flask, at room temperature and under complete mix (180 rpm) and anoxic condition. The batch tests were conducted in 7 phases; each phase consisted of 4 to 6 parallel experiments that were carried out at the same time. The batch tests were completed over two weeks, including a one-day layover between the runs. One liter of distilled water was added to each flask for external carbon source experiments, containing $NaHCO_3$ (120 mg/L; alkalinity source), KH_2PO_4, $MgSO_4 \cdot 7H_2O$ (100 mg/L), $CaCl_2$ (100 mg/L), 1 mL/L of trace elements solution (composition in g/L for all as: EDTA 15, $ZnSO_4$ 0.43, $CoCl_2$ 0.24, $MnCl_2$ 0.63, $CuSO_4$ 0.25, Na_2MoO_4 0.22, $NiCl_2$ 0.19, Na_2SeO_4 0.21, H_3BO_3 0.01, and $NaWO_4$ 0.05). For tests with the MWW, the design COD/N ratio was maintained by varying NO_3^--N and NO_2^--N concentrations. For external carbon source based batch tests, the methanol and glucose varied in concentrations while maintaining a constant nitrite and nitrate concentration. The dissolved oxygen was removed by flushing the flasks for 5 minutes with N_2 gas in all the batch experiments. Each test was conducted for 4-18 hours, depending on the time it takes to complete the removal of the substrate (Table 4.1). Samples were collected from each batch (cyclic and offline) at time zero, and then every 15 minutes for the first 2 hours; after that, samples were collected every 1 to 2 hrs for COD, nitrite, nitrate, and DO analysis. Alkalinity, ammonia, MLSS, and MLVSS were measured at the start and the end of each batch test. The results were then used to determine the SDNR and the corresponding specific substrate utilization rate (SSUR) at different COD/N ratios for the various test conditions.

Table 4. 1 Batch test summary

Phases	Electron acceptor	Carbon source	COD (mg/L)	NO_2^--N or NO_3^--N (mg/L)	COD/N ratios	Number of different C/N cases
1	NO_2^--N	MWW	144-326	15.6-85	3.6-9.3	6
2	NO_3^--N	MWW	174-195	46-122	1.6-4.2	4
3	NO_2^--N	Methanol	148-1346	54-57	2.6-24.5	5
4	NO_3^--N	Methanol	133-1207	55-58	2.3-21.6	5
5	NO_2^--N	Glucose	145-1430	56-61	2.6-23.4	6
6	NO_3^--N	Glucose	135-1340	55-60	2.5-22.3	5
7	NO_3+NO_2	MWW	192-197	60 (20-40 each)*	3 (5-11)**	3

*The sum of nitrite and nitrate concentration was maintained at 60mg/L, the nitrite and nitrate concentration used varied from 20-60 mg/L

** A COD/NO_X-N ratio of 3, COD/NO_3-N of 5 and COD/NO_2-N of 11 was maintained

4.2.4 Kinetics calculations

The heterotrophic denitrifier yield (Y_{HD} – mgVSS/mgCOD) in the SBR and batch tests was calculated as per Eq (4-7), (Badia et al., 2019; Rahman et al., 2016).

$$Y_{HD} = (1 - \frac{1.71\ SDNR}{SSUR})/1.42 \qquad (4\text{-}7)$$

The theoretical yield was also calculated from half-reactions with ammonia as the nitrogen source for biomass, nitrite as the electron acceptor, and methanol as electron donor, as shown in the following overall stoichiometric reaction

$$0.167CH_3OH + 0.167NO_2{}^- + 0.17H^+ + 0.025HCO_3{}^- + 0.025NH_4{}^+$$
$$= 0.025C_5H_7O_2N + 0.083N_2 + 0.39H_2O + 0.067CO_2$$

The denitrifier yield (Y_N, mgVSS/mgNO_2^--N) were also calculated based on nitrite removed as per Eq. 4-8

$$Y_N = (\ \frac{1.71\ Y_H}{1 - 1.42\ Y_H}) \qquad (4\text{-}8)$$

The Monod equation (Eq. 4-9) was used to determine the maximum specific growth rate ($\boldsymbol{\mu}_{hmax}$) and half-saturation coefficient (K_{NO2}) (Eddy et al., 2014).

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\boldsymbol{\mu}_{hmax}*S_S}{Y_h\ (K_S+S_S)}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right)\left(\frac{K_O'}{K_O'+S_O}\right)(\eta) \qquad (4\text{-}9)$$

Eq. 9 can be simplified to Eq. 4-10 because DO is close to zero in the study, and the heterotrophic biomass (denitrifiers) is completely anoxic ($\eta = 1, DO \simeq 0.0$). Additionally, unlimited COD-substrate (methanol) concentration significantly higher than the reported Ks of 3.8-15 mg/L for methanol (Kornaros et al., 1996; Mokhayeri et al., 2008; Torres Ortiz, 2013) was used in the batch test.

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\boldsymbol{\mu}_{hmax}}{Y_h}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right) = \frac{\boldsymbol{\mu}_{hmax}}{Y_N}\frac{S_{NO2}}{K_{NO2}+S_{NO2}} \tag{4-10}$$

Where K_{NO2} is the nitrite half-saturation concentration (mg/L), K_S is the half-saturation concentration for the carbon source-methanol (mg/L), $\boldsymbol{\mu}_{hmax}$ is biomass specific growth rate (d^{-1}), K_O' is DO denitrification-inhibition concentration (mg/L), and S_s, S_{NO2}, S_o are the concentrations of COD (mg/L), NO_2^--N (mg/L), and DO (mg/L), respectively.

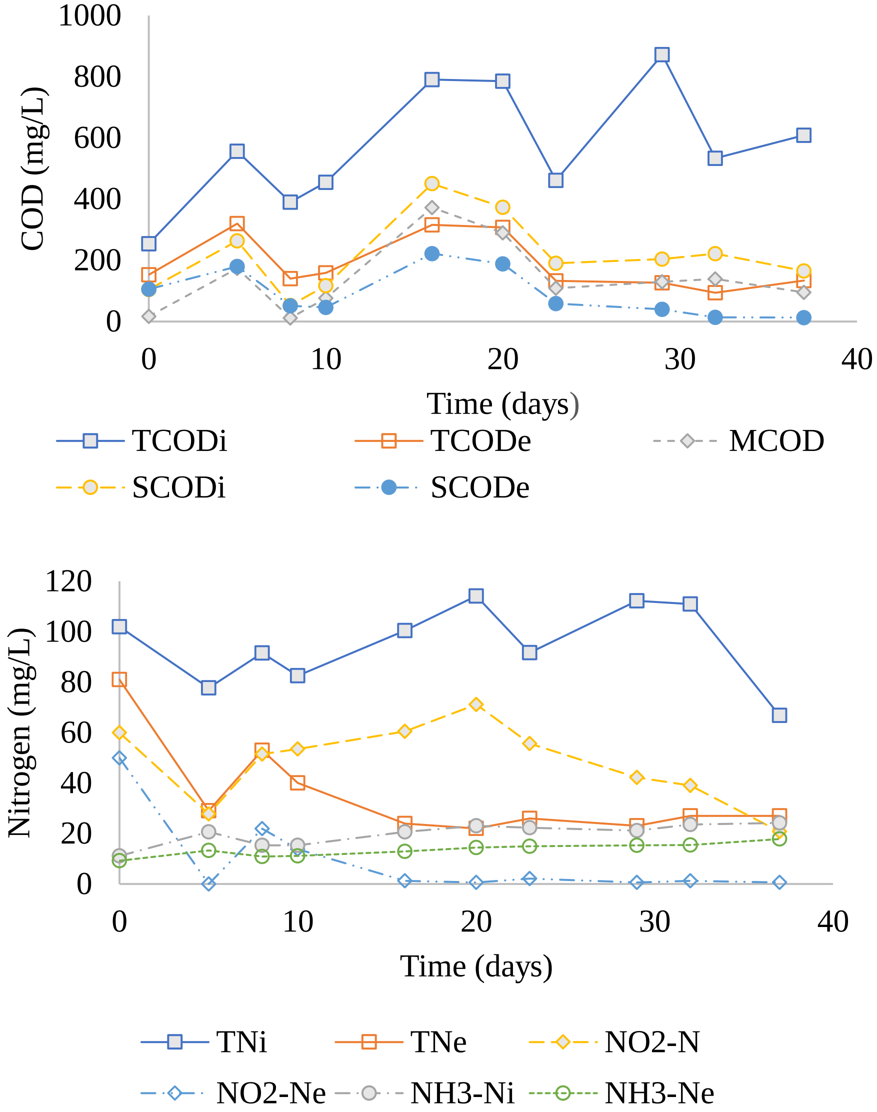
4.3 Results and Discussion

4.3.1 SBR denitrification performance overview

The pH and ORP in the reactor were monitored to ensure anoxic conditions during the study; the values remained between 8–9 and −250 to −350 mV, respectively. Although the influent diluted MWW had a pH in the range of 7.0–7.2, the reactor pH increased due to alkalinity production by the denitrification process. According to (Eddy et al., 2014), the stoichiometric alkalinity removed during nitrification is about 7.14 $mgCaCO_3/mgNH_3$-N-removed and 50% of this amount is recovered during denitrification. In this study, alkalinity produced per NO_2^--N removed was 3.27, close to the theoretical value. This relationship between alkalinity and nitrogen removal can be found in the supporting document (Figure 4.1SD).

The SBR COD and nitrogen profile during the pseudo-steady-state are presented in Figures 4.2 (a) and (b). The summary of influent and effluent quality parameters data can be found in the supporting document (Table 4.1SD). The TCODi (MWW+ Methanol) varied from 237 to 724 mg/L (average 571 ± 332 mg/L) with an average of 430±134 and 141±112 mg/L for municipal wastewater COD and methanol COD, respectively. Similarly, SCODi varied from 54 to 450 mg/L (average 214 ± 120 mg/L), including methanol added to the system. The average primary effluent BOD_5 during the study was 232 mg/L or 54% of the average measured wastewater TCOD before dilution and methanol addition. The average TCOD and sCOD removed were 68% and 57%, respectively, and the corresponding nitrite removal ranged from 67-100% with an average 96% removal. It can be observed that the carbon source removed was not only due to methanol removal but was also due to an internal carbon source (MWW). This can be observed by comparing the average difference between inf-MCOD and eff-SCOD of 42 mg/L, and 122 mg/L between inf-SCOD and eff-COD.

The TNi varied between 66 and 111 mgN/L (with an average of 95 mgN/L), including the 20 to 71 mg/L NO_2^--N added to the SBR. The average influent soluble nitrogen (SN_i) concentration was 72 mg/L with NH_3-N of 19.2 mg/L, NO_2^--Ni of 48 mg/L, and soluble organic nitrogen concentrations of 4.8 mg/L. Conversely, the average effluent soluble nitrogen (SN_e) was 23 mg/L, including 11.5 mg/L NH_3-N, 9.2 mg/L NO_2^--N, and 2.3 mg/L soluble organic nitrogen. An average 13.5 mg/L of TKN (TKN_i of 32.5 mg/ L less TKN_e of 19 mg/L) including, 7.7 mg/L of NH_3-N and 39 mg/L of nitrite, was removed due to anabolic metabolism (assimilation in biomass) and catabolic metabolism (dissimilation to N_2), respectively. The average MLSS and MLVSS were 4439 and 3392, respectively (Table 4.1SD). During the pseudo-steady-state period, the MLVSS was 4500mg/L for seven days and stabilized at 3392 for the rest of the operating period.



(a)

(b)

Figure 4.2. Influent and Effluent (a) COD and (b) nitrogen compounds profile (during the last 40 days of operation, 25°C)

4.3.2 Effect of complementary methanol COD to N ratio on nitrite removal efficiency

Figures 4.3 and 4.4 present the relationship between the nitrite removal efficiency and the influent TCOD/N and MCOD/TCOD ratios in the SBR, respectively. The COD/N and MCOD/COD ratios represent the initial TCODi (254-871 mgCOD/L), MCODi (17-372 mgCOD/L), and NO_2^--Ni (20-60 mgN/L) in the tests. When the TCOD/N ratio approaches 8.3, the nitrite denitrification efficiency increased to 98%, even with low methanol-mixture content of 20% (Figure 4.3). Further addition of methanol to make up 50% methanol-MWW mixture content showed no effect on the nitrite denitrification. However, COD/N lower than 7.5 reduced the denitrification efficiency to less than 70%. It can also be observed that the increase of COD/NO_2^--N ratio using methanol as a complementary carbon source improve the denitrification efficiency due to the synergy between MWW and MCOD in denitrification. As discussed in the introduction, there is no previous work conducted on the evaluation of the impact of COD/N ratio on denitrification from nitrite under combined MWW and methanol carbon source. However, the results from the current study are compared with the previous denitrification with external carbon studies. For example, Akunna et al., 1992 found during their denitrification study with glucose (5318 mgCOD/L), the optimum nitrite and nitrate (50-2500 mgN/L) removal efficiency of 54 and 70% were at COD/N ratios of 7.0 and 8.86, respectively. Previous research indicated that complete denitrification using synthetic wastewater required (methanol, ethanol, glycerol, acetate, starch, glucose, saccharose, propionate, lactase) a stable BOD/N ratio of 3 to 3.2 (COD/N of 6-6.5) during the process of denitrification of nitrite.(Rocher et al., 2015) Therefore, it is recommended when optimizing the usage of methanol as a complementary carbon source to consider the optimum COD/N ratio to maximize nitrite removal efficiency, which is considered in the following discussion.

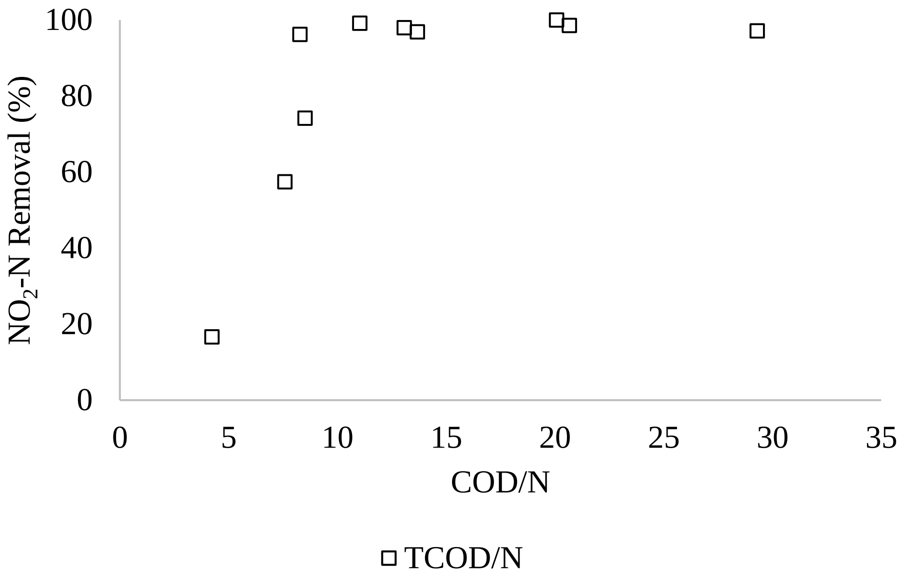


Figure 4. 2 NO_2-N removal in relation to the COD/N ratio in the SBR

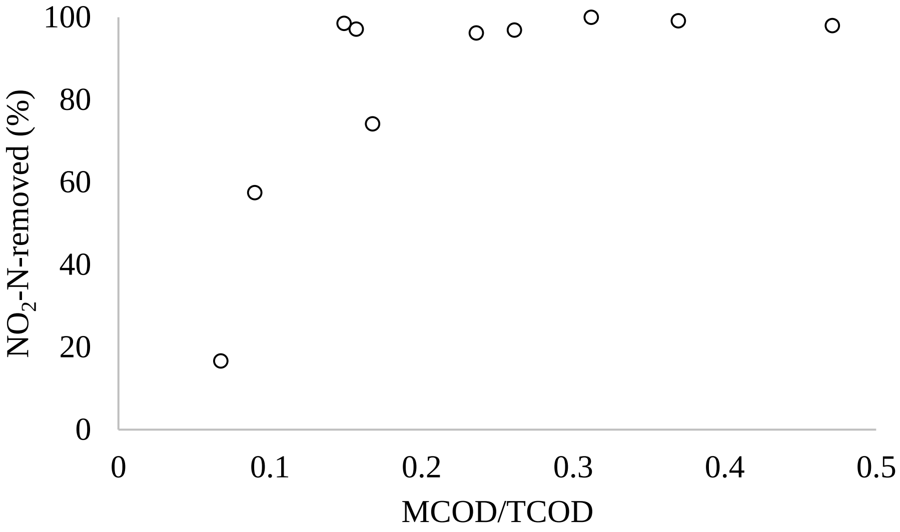


Figure 4. 3 Effect of MCOD/TCOD on nitrite removal

4.3.3 Impact of complementary methanol on nitrite denitrification kinetics

At the end of the continuous SBR study, 11 cyclic tests in the SBR were conducted to identify the impact of complementary methanol on nitrite denitrification kinetics parameters, including the denitrifier yield, maximum SDNR, and half-saturation coefficient. During this period, the biomass was considered to be fully acclimatized for nitrite. This was indicated through the stable MLVSS data shown in Figure 4.2 SD and also reflected by the %NO_2^--N removal, which indicates the stabilization of the denitrifying SBR and the %N removal at various COD/N ratios (Figure 4.3) for methanol and the TCOD (Figure 4.4).

4.3.3.1 Maximum Specific Denitrification Rate (SDNR)

With the variation of internal COD concentrations of MWW during our experiments, we also varied the ratio of MCOD/TCOD in the cyclic test in order to examine the optimum ratio for the maximum SDNR. The COD/N ratios represent the initial TCOD, SCOD, NO_3^--N, and NO_2^--N in the tests. The SDNRs were calculated by dividing the slope of the line of nitrite concentration removed over time (mgNO_2^--N/L/day) during the SBR anoxic cycle by the MLVSS concentration. Similarly, the specific substrate (sCOD) uptake rates (SSURs) were calculated by dividing the slope of the line of COD concentration removed over time (mgCOD/L/day) during the SBR anoxic cycle by the MLVSS concentration.

During the SBR cyclic tests, the initial SCOD/N ratios ranged from 1.8 to 9.5, and TCOD/N of 2-12.5 after considering particulate COD. It must be asserted that the SSUR was calculated based on SCOD removed, taking into consideration of the MCOD and the COD hydrolysed from particulate organics (average influent VSS) using a hydrolysis coefficient of 1.8 day^{-1} (Badia et al., 2019; Drolka et al., 2001; Kappeler & Gujer, 1992). The adjusted TCOD/N was estimated by adding a COD of 1.42 gCOD/gVSS to the measured SCOD during each 4-hr cycle, yielding the TCOD/N ratio of 2–12.5. In the range of COD/N ratios, SDNR varied between 0.01 and 0.5 mgN/mgVSS/day (Figure 4.5). According to Fig 4.5, the optimum SDNR occurred at COD/N ratio of 5.9. The SDNR with MWW and methanol linearly increased with the increase in TCOD/N ratio in the 2.0–5.9 range. There was an optimum TCOD/N ratio beyond which SDNR decreased. The main reason for the decrease of SDNR is related to the high K_{NO2} of 9.2 mgN/L derived from the kinetic studies (section 4.3.3.3), and therefore, the observed decline in SDNR with MWW can be explained by the low ambient NO_2^--N concentrations relative to K_{NO2}, as per the Monod model. The maximum SDNR of 0.52 mgN/mgVSS/day higher than the 0.03-0.3 mgN/mgVSS-d reported in the previous denitrification from nitrite studies using external carbon sources (Adav et al., 2010; Akunna et al., 1993; Badia et al., 2019; Chung & Bae, 2002; Ge et al., 2012), and comparable to the value reported by (Beccari et al., 1983). The maximum SDNR was achieved at MCOD/TCOD of 0.64, meaning with 64% of the organic carbon coming from methanol. The relatively higher SDNR in this study showed synergy between methanol as a supplementary carbon source and the MWW. In summary, the results showed that with an increase of the MCOD/TCOD to 50-60%, a 7.4 fold improvement of SDNR could be achieved compared to the SDNR in using MWW alone as a carbon source (SDNR=0.07 mgN/mgVSS-d, Badia et al.(2019), which can make huge savings in the anoxic bioreactor volume, mixing equipment and power.

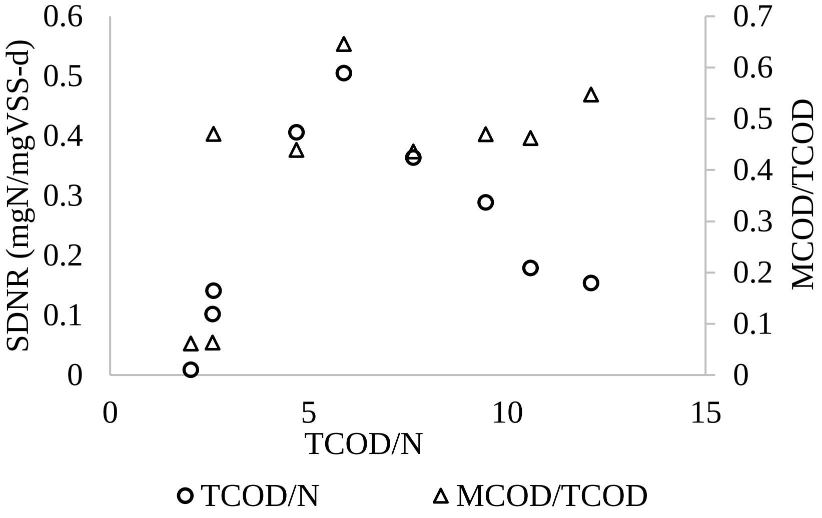


Figure 4. 4 Effect of COD/N and MCOD/TCOD ratio on the SDNR

4.3.3.2 Heterotrophic denitrifier yield (Y_{HD})

The observed heterotrophic denitrifier yield (Y_{HD}) in the SBR was estimated after determining the slope of the SDNR/SSUR (Data in supporting document, Figure 4.3SD) and substituting the value in Eq. 4-7. The Y_{HD} was found to be 0.39 mgVSS/mgTCOD for methanol + MWW. Further, based on the estimated Y_{HD} of 0.39 mgVSS/mgTCOD for the mixture of methanol and municipal wastewater, the removed TCOD of 383 mg/L (from Table 4.1SD), and the estimated nitrogen content of VSS (0.1 mgN/mgVSS), the TKN consumed for cell growth was estimated as 16 mgN/L. On another note, the $Y_{HD,}$ according to the 13.6 mg/L average ammonia removed (TKN used for biomass = (Influent NH_3 – Effluent NH_3)/0.6) in the SBR resulted in Y_{HD} =0.35, close to the estimated value that is comparable to the yield of MWW (Badia et al., 2019). The Y_{HD} is lower than the 0.46 obtained for methanol when used in post denitrification (data not shown), which indicates that using methanol as a complementary carbon source improves not only the SDNR but also lowers the sludge produced compared with methanol usage in the post denitrification. The current nitrite denitrifier yield value was compared with the nitrate denitrifier yield using various carbon sources. Peng et al. (2007) estimated a yield 0.4, 0.42, and 0.65 mgVSS/mgCOD for methanol, ethanol, and acetate, respectively, which is close to the yield estimated in this study. Other yield studies by Guven et al. (2018) reported a yield coefficient of 0.45 mgVSS/mgCOD for a mixture of acetate, propionate, ethanol, and glucose, and Sobieszuk & Szewczyk (2006) reported 0.44 mgVSS/mgCOD for methanol. Both were studies for the denitrification from nitrate, which are in good agreement with the yield estimated in the current study. On another note, Bernat et al. (2015) reported a daily variation on yield (ranging from 0.27 to 0.44), which depended on the daily operation conditions and COD/N ratio. Using Eq (4-8), the denitrifier yield (Y_N, mgVSS/mgNO_2^--N) was estimated as 2.27 and 1.48 mgVSS/mgN for methanol only (post denitrification) and methanol with MWW (pre-denitrification), respectively. The estimated values were higher than the estimated Y_N of 1.062 mgVSS/mgNO_2^--N using MWW only (Badia et al., 2019), lower for values that made use of acetate 3.16 mgVSS/mgNO_2^--N as a carbon source and comparable to Kornaros et al. (1996) that stated a yield Y_N of 1.41 mgVSS/mgNO_2^--N in a kinetic study using glutamate. The literature showed that the acetate yield is higher than the methanol or MWW yield; in this study, the combination of methanol and MWW provide yield close to the MWW only yield (0.33 mgVSS/mgCOD, (Badia et al., 2019)).

4.3.3.3 Half saturation concentration and biomass specific growth rate

The Monod equation (Eq. 4-9) was used to determine other kinetic parameters such as the maximum specific growth rate ($\boldsymbol{\mu}_{hmax}$) and half-saturation coefficient (K_{NO2}) (Eddy et al., 2014) The analysis was based on 4-hr-10 cyclic tests in a post-denitrifying-SBR with methanol as a sole carbon source and nitrite as an electron acceptor. The $\boldsymbol{\mu}_{hmax}$ can be directly related to Y_N (2.27 mgVSS/mgNO_2^--N) and post denitrification $SDNR_{max}$ (maximum SDNR) of 0.62 mgNO_2^--N/mgVSS/d, according to the relation of $SDNR_{max}=\mu_{max}/Y_N$. Hence, $\boldsymbol{\mu}_{hmax}$ was obtained as 1.4 d^{-1} for methanol and nitrite in the post-denitrification SBR. The value is higher than the previously reported value from denitrification with nitrate, i.e., 0.5 d^{-1} (Mokhayeri et al., 2008), however close to the estimated 1.3 d^{-1} (Dold et al., 2008) and 1.25 d^{-1} (Nichols et al., 2007). The obtained $\boldsymbol{\mu}_{hmax}$ is in accordance with the reported values (0.5 - 4 d^{-1}) for different external carbon sources (deBarbadillo et al., 2008; Dold et al., 2008; Kornaros et al., 1996; Stensel et al., 1973; Yang et al., 2007). Using Eq. 4-8 with the replacement Y_H by Y_N, the Lineweaver-Burk plot was developed (Eq.4-11, and Figure 4.6) to determine K_{NO2}.

$$\frac{1}{SDNR} = \frac{Y_N}{\mu_{hmax}} K_N \left(\frac{1}{NO2-N}\right) + \frac{Y_N}{\mu_{hmax}} \tag{4-11}$$

Substituting $SDNR_{max}$ with $\boldsymbol{\mu}_{hmax}/Y_N$, K_N was determined as 9.2 mg/L. This explains why complete removal of NO_2^--N was not possible in the SBR during the experimental period, and this was due to the switching function of NO_2^--N/(K_N+NO_2^--N), effluent NO_2^--N is 9.2 mg/L (Table 4.1SD). In order to decrease effluent nitrite quality, the SBR system needs to be optimized by increasing reaction time, SRT, and biomass concentrations. The reported half-saturation coefficients (K_{NO2} and K_{NO3}) are 0.9 mgNO_2^--N/L and 1.4 mgNO_3^--N/L for acetate (Dosta et al., 2007) and 0.28 mg NO_2^--N/L and 0.77 mgNO_3^--N/L for L-glutamic acid(Kornaros et al., 1996). However, Her & Huang (1995a) reported K_{NO2} of 10.9 mg/L and K_{NO3} of 14.3 mg/L with methanol, which is comparable to the values reported in this work. Badia et al. (2019) determined the K_{NO2} value of 4.07 mgN/L for MWW. The high variability in K_{NO2} is mainly due to the testing conditions, including carbon source type and concentrations and COD/N range. The reported K_{NO2} for acetate and glutamic acid were much lower than the ones determined for methanol and MWW. It is important to note that the K_{NO2} reported for acetate and glutamic study were during denitrification from nitrate as oppose to nitrite as being the sole electron acceptor (this study). Thus, the much lower K_{NO2} values for acetate and glutamic acid could be a function of the carbon source and, to some extent, due to the nitrate denitrifier biomass diversity.

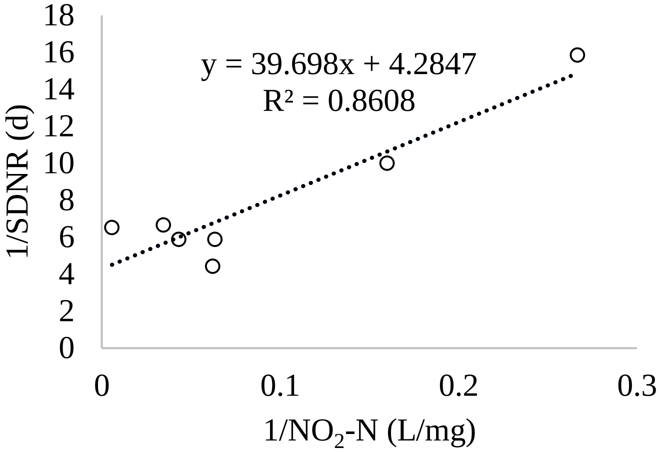


Figure 4. 5 Lineweaver-Burk plot for kinetic parameters determinations

4.3.4 Batch tests

The batch tests were conducted to characterize the performance of biomass acclimatized to nitrite as an electron acceptor and methanol as a complementary carbon source upon short-term exposure to a different carbon source or an electron acceptor. The carbon sources studied include MWW only, methanol, and glucose, whereas the alternative electron acceptor was nitrate. Any of these conditions could happen in a practical setting, and it would be of benefit to understand how the nitrite acclimatized biomass performs under such circumstances. There was no nitrite removal with glucose after 8-24 hours of sampling; therefore, results are not discussed further in this paper.

4.3.4.1 Short-term Nitrite or Nitrate Denitrification using MWW as a sole carbon source

Six batch tests were conducted at room temperature to investigate the acclimatized biomass denitrification potential from nitrite and the actual impact of carbon source change on SDNR from the combination of methanol and MWW to MWW as a sole carbon source (Phase 1, Table 4.1). The tests were conducted by employing different nitrite concentrations (15.6, 22.8, 37, 58, 71 and 85 mgN/L) to achieve COD/N ratios of 3.6, 4.3, 5.3, 7.7, 6.3, and 9.3 at MWW-TCOD concentrations 144, 144, 286, 307, 326 and 305 mg/L, respectively. The maximum SDNR showed 0.47 mgN/mgVSS-d at COD/N of 5.3 (Table 4.3 and Supporting document Figures 4.4SD a and b). It is of importance to mention that B1 and B2 experiments were conducted one week before the other (B3, B4, B5and B6) batch tests. The estimated yield coefficient and kinetic parameters were Y_H of 0.52 mgVSS/mgCOD, μ_{max} of 1.23 d^{-1}, and K_{NO2} of 6.48 mgN/L. The denitrifier yield is within the range obtained in the SBR, which can be related to the biomass type developed within the SBR. The SDNR and the yield were as high as the values measured in the SBR despite the fact that the batch reactor was primarily fed with MWW. The half-saturation coefficient (K_{NO2}) was higher than the one observed for MWW of 4.07 mg/L (Badia et al., 2019) and lower than the one obtained using methanol (9.3 mg/L). It is also clear that for the MWW-nitrite range, the optimum COD/N ratio for the highest SDNR was 5.3 (Table 4.3), which was close to the COD/N in the SBR (5.88). The ∆COD/∆N (SSUR/SDNR) showed an inverse relation to COD/N, while COD/N decreased from 9.3 to 3.6, ∆COD/∆N increased from 5.5 to 7.6. A similar trend was observed from the long term performance with methanol+MWW as carbon source; however, while the trend is consistent, the magnitude of ∆COD/∆N seems to be more carbon source-specific. The above results showed that the MWW as a sole carbon source behaved similarly to the SBR study that used MWW+methanol and better compared to the earlier studies that used MWW as a sole carbon source. The result implies that (i) the kinetics is highly dependent on the nature of the biomass: the biomass acclimatized with methanol+MWW behaved differently compared with the MWW acclimatized biomass, and (ii) a short term switch from the MWW+Methanol to MWW as a sole carbon source, will not affect the denitrification performance.

Similarly, four batch tests were conducted with MWW and nitrate (Phase 2, Tables 4.1 and 4.3). The tests were conducted under varying COD/N ratios of 1.6, 2, 3, and 4.2 by employing various nitrate concentrations (46, 64, 86 and, 122 mgN/L) and TCOD concentrations of 195, 193, 174 and 195 mg/L (Figure 4.4SD). All SDNR values were very close at the employed COD/N ratios of 1.6-4.2. The maximum nitrate SDNR (0.15 mgN/mgVSS-d) was lower than the corresponding nitrite SDNR (0.47 mgN/mgVSS-d); however, we attribute the decrease of SDNR could be due to the nature of the biomass and the low COD/N ratio. It is plausible that the optimum SDNR was not wholly achieved due to the lower COD/N data range (1.6-4.2) used in this test. (Akunna et al., 1992) obtained the optimum SDNR with glucose at COD/N of 8.86 and 7 for nitrate and nitrite, respectively. The yield coefficient, according to the 4 data points, was found to be 0.3 ±0.05 mgVSS/mgCOD, which agrees with (Muller et al., 2003). The yield of 0.3 was lower than the nitrite yield; this is per the stoichiometry.

During the tests with nitrite and nitrate, dissimilatory nitrate or nitrite reduction to ammonium (DNRA) was observed, and ammonia concentration increased at the end of each test (5-11 mgNH_4-N/L). According to van den Berg et al. (2015) and Kraft et al. (2014), the leading cause that affects the dissimilatory nitrite/nitrate reduction-denitrification to ammonia is the nirF enzyme. Baideme et al. (2019) who studied the condition of the ammonia restoration during denitrification reported a relative abundance of nirF with an accumulation of ammonia when sludge age (SRT) exceeded 12 days. In this study, alkalinity changes were related to the accumulation of ammonia, and alkalinity recovery per ammonia increase was 7.14 mg$CaCO_3$ per mgN, higher than the theoretical recovery of 3.57 mg$CaCO_3$/mgN denitrified from denitrification. The DNRA ranged between 5-11 mgNH_3-N, corresponding to 35-78 mg$CaCO_3$ alkalinity restored.

Table 4. 2 Batch tests: MWW with Nitrite and Nitrate

Parameter	**Nitrite and MWW**						**Nitrate and MWW**			
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B1**	**B2**	**B3**	**B4**
TCOD mg/L	144	144	286	307	326	305	195	193	174	195
CODf mg/L	72	63	132	154	162	213	43	47	38	70
NO2/NO3 mgN/L	15.6	22.8	37	58	71	85	46	64	86	122
NO2f/NO3f mgN/L	0.9	0.9	13	20	68	70	2	22	50	50
MLSS mg/L	8500	6600	1030	1060	1010	1050	820	750	910	860
MVSS mg/L	4080	3700	790	820	810	820	650	620	740	720
SDNR mgN/mgVSS-d	0.08	0.18	0.46	0.47	0.25	0.31	0.14	0.14	0.11	0.15
COD/N	9.3	6.3	7.7	5.3	4.6	3.6	4.2	3.0	2.0	1.6
ΔCOD/ΔN	5.5	2.8	6.3	6.2	8.1	7.6	4.4	5.2	4.5	5.6
Intial ALK. mgCaCO3	280	280	430	430	430	430	418	418	418	418
Final ALK. mgCaCO3	345	346	581	576	576	577	640	620	630	620

4.3.4.2 Short-term Nitrite and Nitrate denitrification using Methanol as a sole carbon source

Additional batch tests were conducted to the ones discussed above in order to discern the short-term impact of carbon source change, from the combination of methanol and MWW to methanol as a sole carbon source, on the SDNR (Phases 3 and 4, Tables 4.1 and 4.4). The maximum SDNR in the main SBR of 0.52 mgN/mgVSS-d occurred at optimum COD/NO_2^--N of 5.88 based on biomass acclimatized for nitrite fed with a mixture of methanol and MWW. Using the acclimatized biomass, the nitrite denitrification with methanol as a sole carbon source showed a relatively lower SDNR ranging between 0.27 to 0.38 mgN/mgVSS-d SDNR (Table 4.4) but a comparable yield coefficient of Y_H=0.34 mgVSS/mgCOD. The half-saturation coefficient was not obtainable because the nitrite concentration used was higher than the $K_{NO2,}$ which was chosen based on the requirement of the SDNR data. The above result confirmed the observed synergy between methanol+MWW, which resulted in higher SDNR compared to the methanol addition as a sole carbon source. The main reason for the reduction in SDNR could be attributed to the biodiversity of biomass that is acclimatized to both carbon sources together in the SBR, and when exposed to a methanol carbon source (methanol) in the short term test led to the μ_{max} reduction while Y_H remained the same. It can also be observed, the ΔCOD/ΔN (SSUR/SDNR) has an inverse relation to COD/N, while COD/N increased from 2.6 to 18.1, ΔCOD/ΔN decreased from 4 to 2.1 (Table 4.3). The highest SDNR of 0.38 mgN/mgVSS-d occurred at COD/N of 2.6 with a relative consumption of ΔCOD/ΔN (SSUR/SDNR) of 4.0 higher than the consumption in the SBR (2.8), which can be attributed to the lower SDNR while the COD consumption (SSUR) is almost similar. On the other hand, it can be observed that the relation between COD/N and SDNR is more carbon source-specific; in our case, the SBR-COD/N and the COD/N-batch are different.

Similarly, five batch tests were conducted with methanol and nitrate (Phase 4, Table 4.3) as a carbon source and electron acceptor, respectively. The highest SDNR among the five nitrate batches were 1.06 mgN/mgVSS-d (Table 4.3 and in supporting document Figure 4.5SD) occurred at the typical optimum COD/NO_3^--N of 10 and the minimum $\Delta COD_{consumed}/\Delta N_{reduced}$ of 1.5. The nitrate SDNR was much higher than the one reported in section 4.3.4.1 (0.15 mgN/mgVSS-d), confirming that the lower SDNR has associated with the COD/N ratio as opposed to the nature of the biomass. It indicates that biomass acclimatized with nitrite can also denitrify nitrate with high performance; however, the maximum SDNR was achieved at a higher COD/N (10) in the case of nitrate vs 5.88 in the case of nitrite. The high COD/N can be attributed to the higher COD required for denitrification from nitrate due to extra 2-electrons transferred during the bioreaction. Though the COD/N ratio has a wide range (2.6-24.5), the removal rate ΔCOD/ΔN was high 4.0 and 4.9 at low COD/N (2.6) and high COD/N (24.5), which can be attributed to the higher nitrate-SDNR and SSUR. The yield coefficient parameter was estimated as Y_H=0.28 mgVSS/mgCOD. The yield was also calculated according to the half-reactions (with nitrate as the nitrogen source for biomass and nitrate as the electron acceptor):

$$0.167CH_3OH + 0.1NO_3{}^- + 0.1H^+ = 0.0218C_5H_7O_2N + 0.039N_2 + 0.307H_2O + 0.058CO_2$$

From the above equation, Y_H is 0.28 mgVSS/mgCOD, which matched exactly with the estimated experimental yield value. The lower nitrate yield was in agreement with the yield reported in section 4.3.4.2 and also comparable to the stoichiometric yield. The alkalinity produced during methanol/nitrate experiments matches the ratio of 3.57 mgCaCO3/$N_{denitrified.}$

Further, the alkalinity produced during methanol experiments did not show ammonia release, indicating that DNRA is not only affected by the sludge age but also the carbon source type. Various studies reported DNRA phenomena during denitrification at different carbon sources, i.e., organic soil (Friedl et al., 2018) and (Nizzoli et al., 2010), glycerol (Baideme et al., 2019), and glucose (Lu et al., 2013).

Table 4. 3 Batch tests: Methanol and Nitrite

Parameter	Nitrite and Methanol					Nitrate and Methanol				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
TCOD mg/L	148	459	723	996	1346	133	356	603	912	1207
CODf mg/L	83	415	651	905	1234	45	281	557	751	1109
NO2/NO3 mgN/L	57	55.2	54	55	55	57.1	57	58	55	56
NO2f/NO3f mgN/L	31.2	33.6	31	31	32	33	36	34	34	31.4
MLSS mg/L	820	750	910	860	880	750	720	850	800	830
MVSS mg/L	650	620	740	720	740	660	640	750	700	740
SDNR mgN/mgVSS-d	0.38	0.32	0.28	0.30	0.27	0.34	0.37	1.06	0.36	0.38
COD/N	2.6	8.3	13.4	18.1	24.5	2.3	6.3	10.4	14.0	21.6
ΔCOD/ΔN	4.0	3.4	3.3	2.1	4.9	4.2	4.7	1.5	7.4	4.0
Intial ALK. mgCaCO3	66	64	64	64	64	64	62	64	64	64
Final ALK. mgCaCO3	126	124	112	124	124	84	88	124	96	94

4.3.4.3 Short-term Combined Nitrite and Nitrate Denitrification using MWW as a sole carbon source

Three batch tests were also conducted to examine SDNR from a mixture of nitrate and nitrite with MWW with three different NO_2:NO_3 ratios of 1:2, 1:1, and 2:1, with nitrite concentrations of 20, 40 and 60 mgN/L (NO_2^--N+NO_3^--N= 60 mgN/L), maintaining a COD/NOx ratio of 3 (Table 4.4). In the three batches, nitrite was reduced sharply while the nitrate removal was minimal. It can also be seen that the highest nitrite SDNR was 50% lower than the one observed when nitrite was used as the only electron acceptor, indicating that nitrite and nitrate existence partially inhibited the denitrification rate with decreasing both nitrite and nitrate SDNRs.

Figure 4.7 shows the denitrification rates of nitrite and nitrate at the NO_3/NO_2 ratio of 2 (B1). The results indicate that when high nitrate and nitrite was present, nitrate denitrification was limited until nitrite concentration reduces to a lower level (below 6 mg/L). Compared to observations in sections 4.3.4.1 and 4.3.4.2, a lower nitrate-SDNR was obtained (0.037mgN/mgVSS-d, Figures 4.7 and 4.6SD, Table 4.4). Mehrabi et al., 2020 reported similar, lower nitrate denitrification performance under the presence of nitrate and nitrite in a nitrite acclimatized biomass. The lack of nitrate denitrification could be due to the biomass that was acclimatized to nitrite resulting in a dominance of specific types of nitrite denitrifier species; however, this needs further investigation. Another reason for nitrate denitrification inhibition suggested by (Tian, 2004) is that nitrite is very toxic to the denitrifying microorganisms, and due to this toxicity, the nitrite concentration inside the cells is much lower than the bulk liquid nitrite concentration. Due to this condition, microorganisms use nitrite as an electron acceptor instead of nitrate to reduce the bulk-liquid external toxicity stress from nitrite. However, recent partial denitrification results from nitrate and a rather accumulation of nitrite over nitrate do not support the latter claim (Badia & Dagnew, 2020).

Table 4. 4 Batch tests: MWW and Nitrite/Nitrate

Parameter	Batches		
	B1	B2	B3
TCOD, mg/L	197	192	193
COD_f, mg/L	53	57	67
NO_2-N, mg/L	20	30	40
NO_2-N_f, mg/L	0	0	0.3
NO_3-N, mg/L	40	30	20
NO_3-N_f, mg/L	32.6	27.5	11.5
VSS, mg/L	950	920	950
SDNR, mgNO_2-N/mgVSS-d	0.21	0.21	0.26
SDNR, mgNO_3-N/mgVSS-d	0.037	0.006	0.014
SDNR, mgNO_x-N/mgVSS-d	0.21	0.260	0.234
COD/NO_2-N	11	7	5
COD/NO_3-N	5	6	8
COD/NO_x-N	3	3	3
ΔCOD/ΔNO_2-N	9.7	8.4	6.2
ΔCOD/ΔNO_3-N	54	275	112
ΔCOD/ΔNO_x-N	9.7	6.7	6.9
Initial ALK, mg$CaCO_3$/L	373.5	373.5	373.5
Final Alk, mg$CaCO_3$/L	437	418	470

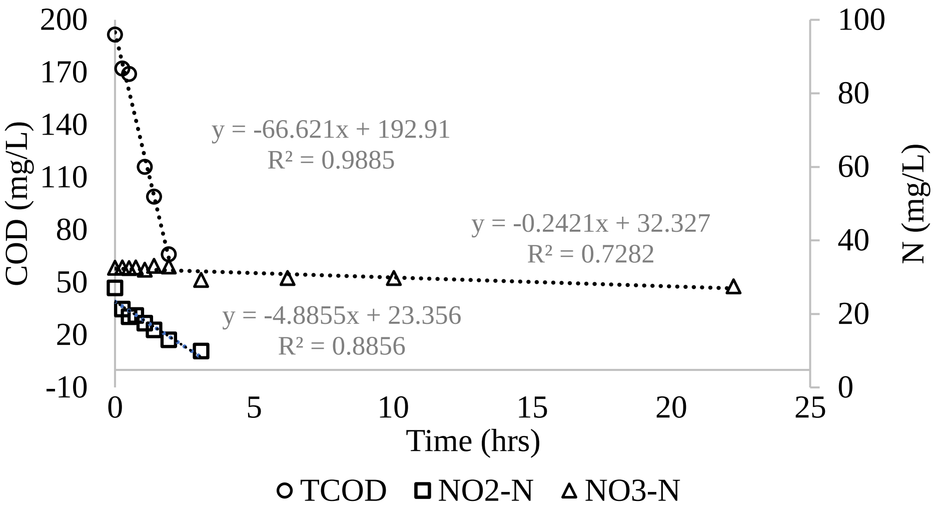


Figure 4. 6 Denitrification rates of a mixture of nitrite and nitrate with MWW(B2)

4.3.5 Making a case for complementary carbon source addition: capital and operating cost analysis

Methanol, as a complementary carbon source, can be employed in low concentration in the pre-denitrification and with a high concentration in the post-denitrification. The methanol addition in the pre-denitrification is used when TCOD/N is lower than the pre anoxic zone denitrification capacity and the addition of methanol, i.e., the MCOD/TCOD must be adjusted to minimize the operation cost. Similarly, using methanol in the post-denitrification will result in higher capital cost for an extra anoxic tank (concrete and mixing equipment) in addition to the operational cost of methanol when MCOD/TCOD of 1. Based on the results in this study, the COD/N ratios can be categorized into three levels: low level of 0-3, a medium of 4-7, and a high level ≥ 8 (Table 4.5). The highest SDNR 0.4-0.52 mgN/mgVSS-d would occur when MCOD/TCOD in the range of 0.4-0.6 (medium range of TCOD/N), i.e., where methanol was used as a complementary carbon source in the pre-denitrification anoxic tank (Figure 8 and Table 4.5). Moreover, high SDNR of 0.32 mgN/mgVSS-d with methanol alone would be considered when methanol is used as the sole carbon source in the post denitrification.

According to the cost of methanol addition of 2.5 CAD/gallon (Eleanor Key, 2018), the cost comparison between using the methanol in the pre and post nitrite denitrification based on the methanol added to achieve the respective maximum SDNR's of 0.52 and 0.38 mgN/mgVSS-d was similar. However, the higher SDNR in the pre nitrite denitrification is translated to 37% volume and mixing power reduction (supporting document, Table 4.2SD), which can be translated to a capital cost reduction associated with civil infrastructure and operating cost reduction associated with mixing power. Furthermore, when we compare the cost of methanol as an external complementary carbon source in the pre-anoxic reactor (SDNR of 0.52 mgNO2--N/mgVSS-d) to the internal carbon source of MWW (SDNR of 0.07 mgNO2--N/mgVSS-d), the anoxic volume will be reduced by 86% of reactor volume. However, the methanol supply shall be added to the operating cost, but its continuous addition may not be required, as demonstrated in the batch reactors that showed higher SDNRs even after switching the carbon source from MWW + methanol to just MWW.

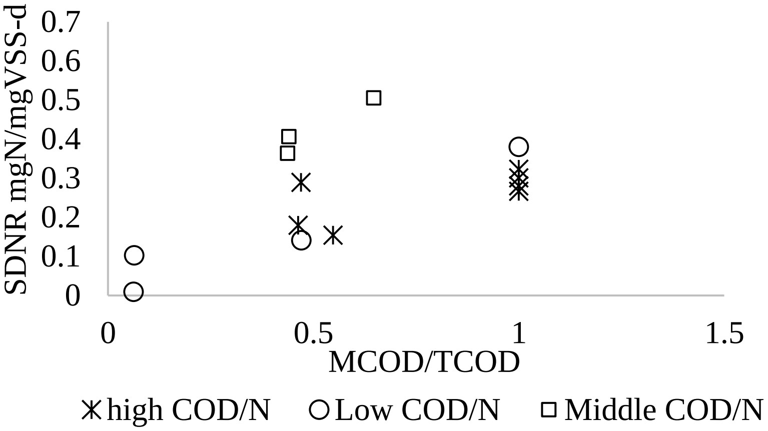


Figure 4. 7 Effect of MCOD/N on the SDNR

Table 4. 5 Cost analysis for denitrification with methanol as a complementary carbon source

Parameter	**Low COD/N**	**Mid COD/N**	**High COD/N**
COD/N-range	0-3.0	4.0-7.0	≥8.0
SDNR, mgN/mgVSS-d	0.01-0.38*	0.36-0.5**	0.15-0.38***

*Higher at MCOD/TCOD of 1.0
**Higher at MCOD/TCOD of 0.5
***Higher at MCOD/TCOD of 0.5 and COD/N of 8-9

4.4 Conclusion

Due to the low carbon to nitrogen ratio in MWW, the denitrification process in full-scale wastewater treatment plants employs external carbon sources to minimize total nitrogen in the effluent. This comprehensive study demofnstrated that compared to MWW as a sole carbon source, denitrifier's kinetics (the half-saturation nitrite denitrification coefficient, denitrifier yield, and maximum specific growth rate) and its activity (SDNR) was higher with MWW+methanol, which enhances denitrification performance. Batch studies also confirmed that a short term switch from MWW+methanol to MWW as a sole carbon source will not affect the denitrification performance, creating the concept of intermittent methanol addition. Comparatively, upon using methanol as a sole carbon source, a lower SDNR (0.38 mgNO_2^--N/mgVSS/d) was achieved, which implies that when methanol is supplemented with the MWW, a synergic effect occurs which enhances the SDNR. The addition of methanol as a complementary carbon source for nitrite denitrification can be considered as a mainstream process even for conditions carbon is not limiting in the MWW.

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Chapter 5

Effect of low COD/N ratio on denitrification from nitrite and nitrate: the case for complete versus partial nitrate denitrification

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The objective of this study was to investigate and compare the dynamic specific denitrification rates (SDNRs) from nitrate and nitrite at various COD/N ratios in two systems that use acetate as a carbon source. SBR1 (47 d-SRT) and SBR2(31 d-SRT) were operated for 126 days, with HRT of 16 hrs, and fed with nitrite and nitrate, respectively. The maximum $SDNR_{NO2}$ and $SDNR_{NO3}$ during the cyclic tests were 0.69 and 2.67 mgN/mgVSS-d in SBR1 and SBR2, respectively. However, the maximum $SDNR_{NO2}$1 occurred during nitrate denitrification was 1.27 mgN/mgVSS-d in SBR2, which is double the rate in SBR1 due to nitrite accumulation stress and the difference in microbial structure and diversity in SBR2. The optimum operating condition for PDN was found to be at minimum COD/N of 2.7-3, with 79% nitrite accumulation. Higher COD/N of 6.7 and 9.8 also accumulate nitrite, but nitrite accumulated was denitrified with a maximum $SDNR_{NO2}$2 of 1.04 mg N/mgVSS-d at CODN of 9.8. Kinetic parameters were also determined for nitrite in offline batch experiments, and the half-saturation coefficient K_{NO2} was found to be 3.17 mgN/L and the maximum specific growth rate of 0.77 d^{-1}. The taxonomic analysis showed *Zoogloea* genus (42% RA) in SBR1 was more responsible for nitrite denitrification, while *Thauera* genus in SBR2 with RA of 10% was more responsible for PDN. We relate the difference in microbial structure to the NiR reductase and NaR reductase distribution among microbial structure, indicating NiR reductase enzymes is higher in *Zoogolea* genera than NaR reductase , whereas NaR is higher than NiR in *Thauera*.

5.1 Introduction

It has been known that nitrogen compounds in the form of ammonia, nitrite, and nitrate are the primary source of severe environmental contamination problems. Ammonia and nitrite are reduced forms of nitrogen; hence if directly discharged to freshwater bodies, these compounds will be oxidized to nitrate consuming the oxygen in the aquatic environment. Further, the presence of any of the nitrogen compounds stimulates the growth of aquatic plants and algae. When algae grow excessively, water force main and intakes can be clogged; algae can also block the sunlight and decomposes anaerobically and causes the depletion of oxygen, which in turn kill the aquatic life (*Scientific Investigations Report*, 2017). Subsequent decomposition of aquatic life in freshwater bodies (rivers, lakes, estuaries) increase the deleterious effect on aquatic life and human health. The presence of nitrate and nitrite in drinking water has been reported to cause methemoglobinemia in the infants, a blue baby syndrome. Therefore, conventional (first-generation) biological nitrification-denitrification (BNR) processes are applied to overcome such problems. However, employing these first-generation processes require (i) high power input for biological process air supply, (ii) external carbon source addition for denitrification (for wastewater with a lower carbon to nitrogen content), (iii) larger footprint, and (iv) more extensive sludge treatment facilities to handle large quantities of waste activated sludge produced during the process. Maktabifard et al., 2018 reported that aeration consumes 50% of the wastewater treatment facility's power.

Recently there is an increased interest in second-generation total nitrogen removal processes. One of these processes is called nitrite shunt that involves partial oxidation of ammonia to nitrite and direct denitrification from nitrite. The process saves 25% of oxygen demand and reduces bioreactor size due to shorter pathways from nitrite to nitrogen gas (3 electrons) during denitrification, and 5 electrons are transferred to nitrate during full denitrification . Over the last ten years, integrating partial nitritation with the anammox process has been the most researched second-generation TN removal process. The anammox process allows ammonia removal as an electron donor with nitrite as an electron acceptor under an anoxic condition and in the absence of an organic carbon source (Chen et al., 2016) and (Zeng et al., 2014), producing nitrogen gas and 11% nitrate as end-product. The process saves 60% of oxygen if we nitrify 50% of influent ammonia and 90% of external carbon source as electron donor, much less sludge production due to the low yield of anammox bacteria (0.114 mgCOD/mgN) (Al-Omari et al., 2015; De Cocker et al., 2018; Guo et al., 2018). Despite the substantial research on the topic, a robust partial nitrification-anammox process configuration for mainstream wastewater treatment are yet to be obtained (Antwi et al., 2019; C.-H. Ge et al., 2019; Jin et al., 2019; Tomaszewski et al., 2017; Trojanowicz et al., 2016; Wang et al., 2018; Y. Yang et al., 2018). However, the process has been proven for sidestream (ammonia-rich) wastewater treatment. Currently, the bottleneck for adopting the PNA process is the difficulty in achieving partial nitrification to nitrite due to difficulty to washout or suppress the nitrite-oxidizing bacteria (NOB), which requires (i) process operation at high temperature, (ii) short sludge age, (iii) lower organic matter content (COD/N<0.5), and (iv) lower DO concentration (Sui et al., 2020; Wang et al., 2018; J. Yang et al., 2016; D. Zhang et al., 2019). Therefore, there is a need to employ an alternative nitrite generation pathway that can be integrated with an anammox process for mainstream domestic wastewater treatment. Most recently, the nitrification-partial denitrification-anammox (nitrification-PDN-anammox) process had started to receive the attention of researchers; the process is attractive mainly for facilities adding external carbon source for denitrification. Compared to the first generation BNR processes, the nitrification-PDN-anammox process saves 45% of oxygen and 79% of the external carbon source addition (X. Xu et al., 2020).

The nitrification-PDN-anammox process takes advantage of previous observations that reported nitrite accumulation during denitrification from nitrate using pure cultures (Betlach & Tiedje, 1981; S. Ge et al., 2012; X. Yang et al., 2012). For example, Almeida et al., 1995 observed a 0.33 and 28% nitrite accumulation during nitrate denitrification by *Pseudomonas Fluorescence*, at 600 and 900 mg/L nitrate concentrations and COD/N ratios of 4.9 and 1.8, respectively. S. Ge et al., 2012, X. Yang et al., 2012, and Cao et al., 2013 also observed nitrite accumulation during nitrate denitrification with various carbon sources and varying COD/N ratios. S. Ge et al., 2012 observed a maximum of 55% nitrite accumulation with glucose at COD/N of 15 and a 42% and 47% accumulation with acetate and methanol, respectively, at COD/N of 25 during denitrification from nitrate (40 mgNO$_3$-N/L). Rocher et al., 2015 also observed nitrite accumulation during denitrification; the accumulation was 20-30% for most of the carbohydrates and organic acids, 34% saccharose, 28% propanoate, 24% glucose, 24% acetate, 23% starch, 12% lactose, while alcohols accumulated less than 10%, methanol 5%, ethanol 11% and glycerol 9%. Akunna et al., 1993 performed batch tests on nitrate (140 to 200 mgNO$_3$-N/L) reductions with anaerobic sludge using various carbon sources at COD/N ratio of 13-15; while no accumulation of nitrite occurs with methanol, 14%, 70% and 86% nitrite accumulated with acetic acid, glycerol and lactic acid, respectively. Overall the above studies showed the impact of carbon source type and COD:N ratio on nitrite accumulation. However, in all the studies, the observed nitrite accumulation was relatively lower.

Mohan et al., 2016 examined the denitrification of high strength nitrate-wastewater (700-2710 mgNO_3-N/L) at various COD/N ratios (1.5, 2, 3). The study showed 50%, 70%, 90% and 100% nitrite accumulation at nitrate concentrations of 677, 1354, 2031 and 2710 mg/L, respectively. These results indicate that there is a prorated increase in nitrite accumulation with the increase of initial nitrate concentration, whereas no direct relation between COD/N and nitrite accumulation. It was also found the optimum SDNR of 19.85 mgNO_3-N/mgVSS-d and 8.76 mgNO_2-N/mgVSS-d ($SDNR2_{NO2}$-nitrite denitrification after complete nitrate exhaustion) at COD/N of 1.5, the higher the nitrate concentration, the slower the denitrification from nitrite.

Several authors studied the impact of pH, oxygen level and free nitrous acid inhibition on PDN. (Qian et al., 2019) studied the effect of pH of 5, 7, 9, along with COD/N of 3 with acetate (90 mgCOD/L and 30mgNO_3-N/L) on PDN in 3-SBRs. The authors obtained 57% nitrite accumulation at a pH of 9 higher than at pH of 7 (38%) and 5 (21%). The higher nitrite accumulation at pH of 9 was due to a difference between nitrate denitrification rate and nitrite denitrification (21.2 >9.5 mg/L-hr), while lower difference at pH of 5 (17.3 >13.4 mg/L-hr) and pH of 7 (17.2 >11 mg/L-hr). J. Zhao et al., 2019 conducted batch tests at various nitrite and nitrate concentrations of 5-35 mgNO_3-N/L, 7.5-17.5 mgNO_2-N/L, and various NO_2/NO_3 ratios, it was found that when initial nitrite concentration was 12.5 and 17.5 with NO_2/NO_3 ratios of 1, nitrite accumulated to 21 and 31, respectively, suggesting that nitrite concentration of 15mgN/L causes FNA inhibition which slows nitrite-SDNR and leads to nitrite accumulation. Though FNA inhibition slows both nitrite and nitrate SDNR, nitrite-SDNR was more affected. In addition to the above, the dissolved oxygen (DO) can inhibit nitrate and nitrite denitrification (Eddy et al., 2014) and can lead to nitrite accumulation. Oh & Silverstein, 1999a examined the effect of DO by aerating the SBR for the first 80 min (COD/N of 5.4), and the air was then shut off for the remaining reaction period (660 minutes). In order to compare the effect of the dissolved oxygen, during the second step (660 minutes), one SBR was operated at DO of 0.0 mg/L, while a second SBR was set at DO of 0.8

mg/L. Results showed both systems accumulate nitrite but at different COD/N ratios. In the absence of DO, the $SDNR_{NO3}$ increased from 0.55 to 0.82 mgN/mgVSS-d with a decrease in COD/N from 5.4 – 8.1 to 2.7, whereas in the SBR of 0.8 mg DO/L, accumulation only observed after oxygen was exhausted entirely and high COD/N of 5.4-8.1, the SDNR was found to be 0.8 $mgNO_2$-N/mgVSS-d.

More recent researches started to emphasize employing the anammox process through PDN pathway for mainstream wastewater treatment. The literature on domestic wastewater concentrations is summarized in Table 5.1. Campolong et al., 2019 combined deammonification and partial denitrification in 2hr-RT- $0.34m^3$ - tertiary MBBR and was able to obtain nitrite accumulation of 91.0 ± 9.0% with glycerol, 89.0 ± 8.3%, with acetate and 74.0 ± 8.4% with methanol. The optimum COD/N ratio was found to be 2.7 for acetate and glycerol but was 3.8 for methanol. Campolongo also conducted cost analysis and found that methanol is the cheapest ($1.23/kgTIN), followed by glycerol ($1.31/kgTIN), and acetate was the most expensive ($3.28/kgTIN). Another study on partial denitrification-anammox in SBR (2L-8hr-cycle-SBR), NH_4-N of 25 mg/L and NO_3-N of 30mg/L (1200mgMLVSS/L, DEAMOX sludge), 97% N removal at optimum COD/N of 3 (Cao et al., 2016). Ji et al., 2020 accumulated 90% of nitrite in a partial denitrification/anammox study employing a 5L UASB reactor, feeding domestic wastewater collected from the septic tank (224.6mgCOD/L, 67.7 $mgNH_4$-N/L), with the addition of synthetic nitrate solution of 70-180 mgN/L (COD/N of 3.3). They also employed batch experiments at COD/N of 3.0. Similar studies indicated that denitrification from nitrite starts when nitrate is wholly exhausted or reached as low as 3 mg/L (Le, Peng et al., 2019a) and (Le et al., 2019b). Most of these studies demonstrated higher nitrite accumulation rates. However, the studies did not address why nitrite accumulates during nitrate denitrification.

Table 5. 1 Summary of partial denitrification studies

System	Carbon source(mgCOD/L)	NO_3-N (mgN/L)	COD/N	PDN (%)	max-SDNR (mgNO_3-N/mgVSS-d)	Notes	Reference
SBR (5 L)	acetate+domestic wastewater (186+136mg/L)	80	1.7	≥80	4.56	COD/N is for acetate only	(Cao et al., 2017)
Continuous flow (360 L)	acetate (64 mg/L)	29 mgTIN/L	1.2 (2.2 COD added/TIN removed)	≥80	NA	Nitrification followed by PDN-anammox process	(Le et al., 2019a)
3x20L continuous systems (48-1L- batches)	acetate, glycerol, methanol, ethanol (22-58)	44	0, 0.25, 0.5, 0.75, 1 and 2	≥90 acetate and glycerol	0.25 for glycerol 0.54 for acetate	Nitrification followed by PDN-anammox process	(Le et al., 2019b)
CSTR	acetate (1590, 2950)	900, 600	1.8, 4.9	28, 0.33	12 mgN /mgCELL-d	Pure culture (denitrification)	(Almeida et al., 1995)
SBR (5 L, 2-cycles/d+0.5L batches), pH of 5.5 and 6.5	acetate(90 for SBR), batches (64, 256)	SBR (30), batches (20, 40, 80)	SBR (3), batches (3.2)	80	1.5-2.4	SNAR (0.228-1.8 mgNO2-N/mgVSS-d), FNA of 0.2 mg/L stops denitrification during low pH(5.5)	(Du et al., 2016)
Static culture (flasks)	citrate, acetate, glucose (1050-2400)	350	3-6.7	less than 10%	citrate (0.11), acetate (0.12), glucose (0.08)	Increasing DO increases PDN	(X. Yang et al., 2012)
3 up flow SBRs (1 L each), pH of 5, 7, 9	acetate (90)	30	3	83% (pH of 9)	21.2 mgN/L-hr (pH of 9)	Increasing pH increase nitrite accumulation.	(Qian et al., 2019)
1.6 L UASB (HRT of 1.1 hr)	acetate (60-100)	30	2-3.4	89	2.7 for nitrate and 2.46 for nitrite (NH_4/NO_3=1)	Nitrification-PDN-anammox (DEAMOX), one sludge system	(Du et al., 2019)
6L UASB (SBR, 2cycles/d)	acetate , ethanol (150)	50	3	95 (acetate) 89 (ethanol)	acetate (0.47 mgNO_3-N and 0.42 mgNO_2-N/mgvss-d), ethanol (0.29 mgNO_3-N and 0.2 mgNO_2-N/mgvss-d)	Nitrification-PDN-anammox (DEAMOX), one sludge system	(Du et al., 2017)
10L-SBR followed by 3.2L-UASB	acetate (187) combined with MWW	20-40	2.6-2.8 (based on acetate)	85.6	acetate (2.5 mgNO_3-N/mgVSS-d, 2.0 mgNO_2-N/mgvss-d)	Nitrification - PDN-anammox (DEAMOX), one sludge system	(Cao et al., 2019)
1L-UASB (0.6-1.2l HRT)	60	20 41	1.51 2.5-3	<80% >80%?	NRR of 0.8 kgN/m^3-d	Nitrification-PDN-anammox (DEAMOX), one sludge	(X. Xu et al., 2020)
1.2 L DEAMOX reactor (A/O biofilm process) and batch tests	acetate (156)	60	2.6	90 (42% in batches)	1.4-1.97 (0.175-0.211 mgNO_2-N/mgVSS-d)	Nitrification-PDN-anammox (DEAMOX), one sludge	(Ma et al., 2017)
5L-SBR (2 cycles/d) batch tests (0.5 L)	acetate (90), batch-1 (75,100, 200, 400) batch-2 (16, 32, 64, 80, 160)	30 batches (20, 40, 80, 150)	3 batches (0.8, 1.6, 2.4, 3.2, 4, 8)	90	1.4 (0.175 mgNO_2-N/mgVSS-d)	Partial denitrification, higher nitrate initial concentration the higher the SDNR	(Du et al., 2016)

Overall the review of the literature showed the potential of nitrite accumulation through the PDN pathway. In most of the earlier studies (1990 to 2015), the accumulated nitrite concentration was relatively lower (< 50%). The intent of most of the earlier research was basically to study kinetics for denitrification from nitrate and to identify factors causing incomplete denitrification, hence use that information to minimize nitrite accumulation during nitrate denitrification. However, recent studies (2016-2020) mostly implement a combined PDN-anammox process and demonstrated higher nitrite accumulation. However, nitrite accumulation was not directly observed instead, quantified indirectly based on ammonia consumption and nitrate reduction. The recent studies indicated contrasting information behind nitrite accumulation, making the control strategies less robust, requiring additional information on PDN processes. One way to clearly understand the fundamentals is through a parallel comparison of nitrite versus partial nitrate denitrification process, to the best of the authors' knowledge, none of the previous research compared kinetic parameters and microbiological composition under this condition. This study provides an assessment of denitrification from nitrite and nitrate for the mainstream wastewater with lower COD:N ratio and targets to (i) characterize and compare the long-term performance of nitrite versus nitrate and partial denitrification process under varying operating conditions such as COD:N and DO level (ii) compare the COD/N plays on nitrite denitrification and PDN process fundamentals including nitrate and nitrite SDNR, SNAR and half-saturation coefficient (iii) classify and compare the biomass community structure grown in partial denitrification and complete nitrite denitrification reactor and compare the microbial composition with the process performance.

5.2 Materials and Methods

5.2.1 Bench scale setup and operation

Two anoxic sequencing batch bioreactors referred herein as SBR1 and SBR2 were setup for nitrite and nitrate denitrification study, respectively (Figure 5-1). Each reactor had a 12 L working volume, equipped with custom made mechanical mixer (ARROW JR4000, 200 rpm) connected to a controller to ensure complete mix condition under anoxic environment. The mixer was programmed to be turned on during the feed and react phase of the SBR. Each reactor was also equipped with influent and effluent pumps (Masterflex, ColePaR, model no: 07528-10) and acetate pump (Fisher brand, Mini pump, S/N:192057692). The two systems were also purged with nitrogen gas (304 CF, 2400 PSIG, 99.998% purity) during the 10 minutes influent feed cycle and during the first 5 minutes of the react cycle for a total 15 minutes/cycle, to maintain an anoxic condition that was demonstrated through daily dissolved oxygen (DO) measurement inside the bioreactors. In addition to the reactors, the setup had influent and effluent tanks (each 40 L with 36 L effective volume). The influent and effluent tanks were refilled and emptied every two days, respectively. The influent tank was filled with synthetic wastewater composed of 120 mg/L $NaHCO_3$, 100 mg/L KH_2PO_4, 100 mg/L $MgSO_4 \cdot 7H_2O$, 100 mg/L $CaCl_2$, and 1 ml/L of trace elements solution. The stock trace element solution were composed of 15, 0.43, 0.24, 0.63, 0.25, 0.22, 0.19, 0.21, 0.01 and 0.05 g/L $ZnSO_4$, $CoCl_2$, $MnCl_2$, $CuSO_4$, Na_2MoO_4, $NiCl_2$, Na_2SeO_4, H_3BO_3 and $NaWO_4$, respectively (Badia et al., 2019). Sodium nitrite (127 mg/L, 97% purity) and sodium nitrate (156.5 mg/L, 97% purity) were added to the SBR1 and SBR2 synthetic wastewater solution, respectively, to obtain a concentration of 25 mg/L. At the beginning of the feed sequence, in addition to the synthetic wastewater, a 10 mL of sodium acetate solution was also directly pumped to each bioreactor for one minute to achieve 50 mgCOD/L in each bioreactor. The sodium acetate solution was prepared by dissolving 40 g of CH_3COONa (97% powder purity) in one litre of DI water. Accordingly, the COD/N ratio for both bioreactors was maintained at about 2, which is typically considered to be a low COD/N ratio (Le et al., 2019b).

The SBRs were operated for 126 days at solids retention time (SRT) of 41 and 31 days for nitrite and nitrate (SBR1 and SBR2), respectively. The bioreactor was operated at room temperature (25 C^0). The SBRs (SBR1 and SBR2) cycle length was 480 min (3 cycles per day) with 10-min feed, 1-min sodium acetate feed, 430-min react phase under complete mix anoxic conditions, 30-min settling, and 10-min decanting time. The fill was around 50% of the SBR volume; in each cycle, 6 L was decanted and replaced with 6 L of feed, corresponding to a daily feed flow of 18 L. At the end of the long term continuous study at COD/N ratio of 2, the COD/N in SBR1 and SBR2 was changed sequentially to study the effect of the COD/N on complete and partial nitrite denitrification., Table 5.1 shows the COD concentration employed during the sequential COD:N study, while nitrite and nitrate were not changed, the COD/N ratio were varied by varying the COD concentration. These ratios varied between 3-25 for nitrite and 1-10 for nitrate to examine partial denitrification during nitrate denitrification.

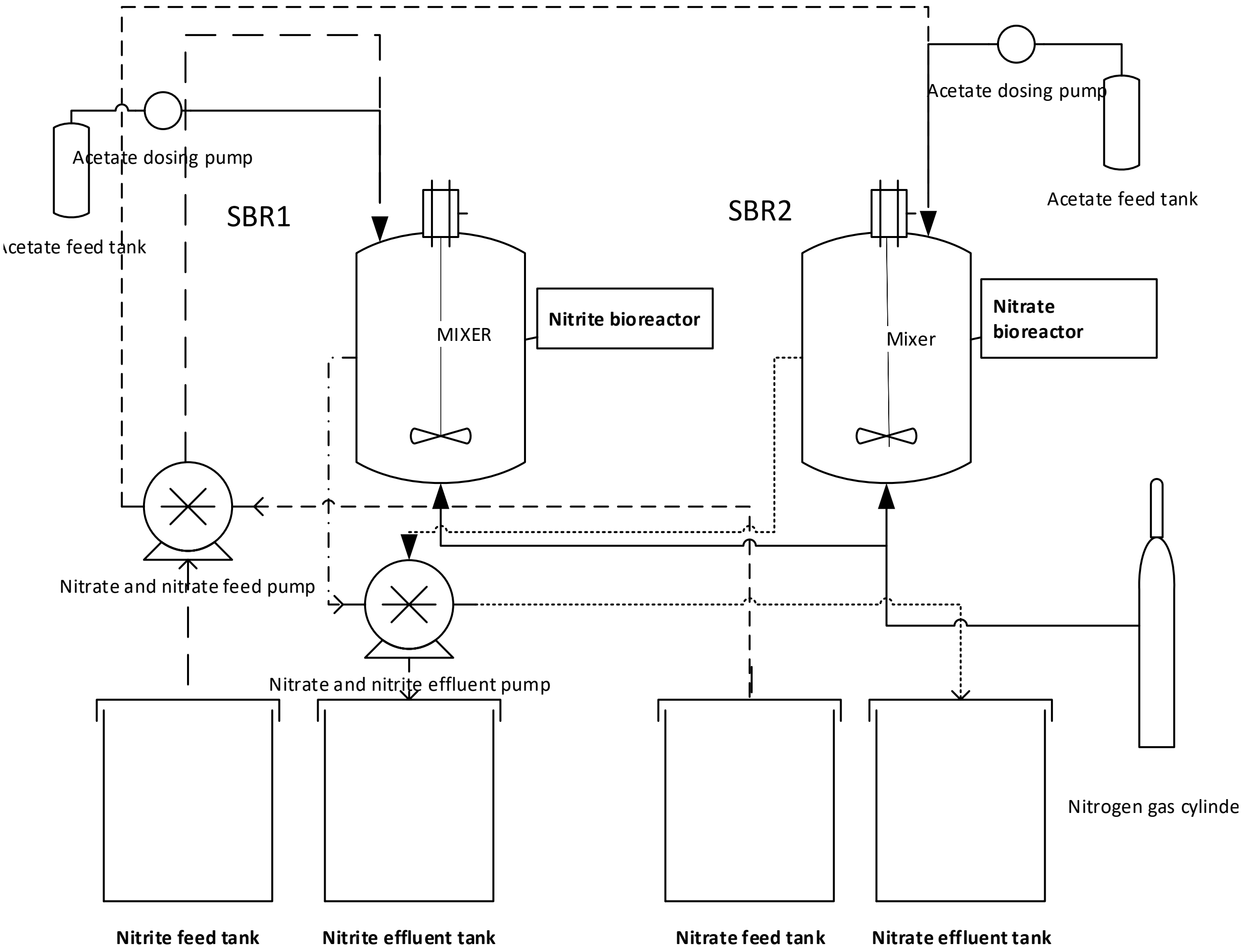

Figure 5.1 SBR1 and SBR2 set up

Table 5.2 Cyclic tests in SBR1 and SBR2

SBR1				SBR2			
NO_2-N, mg/L	COD, mg/L	COD/N	VSS, mg/L	NO_3-N, mg/L	COD, mg/L	COD/N	VSS, mg/L
11	29	3	1465	17	18	1	945
12	50	4	4745	18	32	2	435
10	111	12	1805	16	43	3	650
11	112	10	1700	18	122	7	650
12	300	25	1845	16	158	10	960

5.2.2 Batch tests

The specific denitrification rate (SDNR) is a critical kinetics parameter used for the sizing of a denitrification reactor. The half-saturation concentration (K_N) has a direct effect on the denitrification rate; according to the Monod kinetics, the SDNR is inversely related to the $K_{N,}$ as shown by the switching function $N/(K_N+N)$. At higher $K_{N,}$ the SDNR is significantly lower; however, a lower K_N does not affect the SDNR, and the switching function will be approximately equal to 1. In order to obtain the half-saturation concentration of nitrite (K_N), the SDNR from nitrite with acetate was obtained by varying the nitrite concentration using a series of offline batch kinetic tests, and the K_N was calculated as per equation 5-6 (section 5.3.5.3). The tests were conducted at relatively higher COD concentrations (unlimited-carbon source) and with low nitrite concentration (limited-nitrite concentration), respectively. The batch kinetic studies were conducted using five-1-L flasks referred herein as B1, B2, B3, B4, and B5. Each test was conducted for four hours using the acetate-nitrite-acclimatized biomass collected from the SBR1 after concentrating by centrifugation (10 min at 3000 rpm) and subsequently resuspending to the desired 1000 mg/L MLSS concentration (Table 5-3).

Table 5.3 Batch test summary

Parameter	B1	B2	B3	B4	B5
COD, mg/L	101	98.5	114.7	104.5	98
NO_2-N, mg/L	3.6	7.5	11.7	17.1	21.9
TSS, mg/L	947.5	747.5	810	845	1205
VSS, mg/L	342.5	270	300	297.5	422.5

5.2.3 Sampling and Analysis

Influent and effluent samples of the SBRs were collected two to three times a week and analyzed for total suspended solids (TSS), volatile suspended solids (VSS), total COD (TCOD), soluble COD (SCOD), ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), total phosphorus (TP), and soluble phosphorus (SP). Sterile 0.45 µm membrane filter papers (VWR International, Canada) were used for filtration of the samples for SCOD, NH_3, NO_2^- and NO_3^- analysis and 1.2 µm filters were used for TSS and VSS analyses in accordance with the Standard Methods (APHA et al., 2005). HACH methods were used to measure total phosphorous (Method 10127), ammonia (Method 10031), total nitrogen (Method 10072), nitrite (Method 8153), nitrate (Method 10020) and COD (Method

8000). The DO, pH, and ORP in the reactor were monitored using Atlas Scientific Sensors. For the cyclic and batch study, grab samples were collected every 15 minutes during the length of each test. The high-frequency sampling enabled us to capture the process dynamics and understand the nitrite and nitrate denitrification process fundamentals.

The SBRs were fed with acetate from a one-litre separate tank; therefore, the residual bioreactor COD and fed COD concentration were analyzed by taking and analyzing samples from the SBR at time zero of the react phase (after feeding acetate). The initial bioreactor concentration was determined by taking into account the concentrations before and after feeding (equation 5-1). The influent COD was further estimated by taking the bioreactor COD concentration at time zero of the react phase, the COD concentration before feeding (effluent from the previous cycle), and the exchange volume into consideration (equation 5-1). In this equation, the COD equivalent of nitrite was subtracted to maintain COD primarily related to organic carbon.

$$COD_i = \frac{COD_0 * SBR\ volume\ -\ COD_e * V_e}{V_e} - COD_{NO2} \tag{5-1}$$

Where COD_i = the influent COD (calculated)

COD_e = the effluent COD (measured)

COD_0 = the COD in the SBR at time zero of the react phase (measured)

COD_{NO2}= the amount of COD measured in the COD test corresponding to nitrite oxidation (calculated based on the COD equivalent of nitrite, mgCOD/L ≃1.1 mgNO2-N/L)

V_e = Exchange volume per cycle (6 L, 50% of SBRs volume)

5.2.4 Microbial analysis

The microbial analysis was conducted to discern the denitrifying microbial population composition and relative abundance within the nitrite versus nitrate denitrifier bioreactors. Duplicate sludge samples were collected from the SBRs (after3 months of operation) and submitted to a molecular research DNA LAB (Shallowater, TX, USA). The samples were analyzed using the MiSeq sequencing method under bTEFAP. The 16S rRNA gene V4 variable-region PCR primers 515/806 were used in a 30–35 PCR with the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 minute after final elongation at 72 °C for 5 minutes. The PCR end-product was examined in 2% of agarose gel after amplification in order to assure successful amplification. Several samples in equal proportions were pooled together (e.g., 50 samples) depending on their molecular weight and DNA concentrations. Calibrated Ampure XP beads were then used to purify the pooled samples, and the Illumina DNA library was prepared by using the purified PCR product. Sequencing was performed at MR DNA (www.mrdnalab.com), Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using the MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). To sum-up, sequences were joined, depleted of barcodes, then sequences <150bp removed, sequences with vague base calls removed. Sequences were denoised, OTUs generated, and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at a 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu). Finally, the classification of the microbial communities was made based on the % relative abundance (RA%); microbes with RA greater than 1% were considered for the classification.

5.3 Results and Discussion

5.3.1 SBRs denitrification performance overview

The SBRs were operated for 126 days in which the biomass showed acclimatization after two weeks. The average influent and effluent quality parameters are summarized in Table 5.4 and shown in Figures 5-2 a to d. The average values were calculated using the last 40 days of data that showed stable performance. The average effluent TSS and VSS from nitrite SBR1 were 15.2±3.8 and 6.5±2.07 mg/L with a range of 10-23 and 4-8 mg/L, respectively, while the average effluent TSS and VSS from nitrate SBR2 were 22.8±3.7 and 13±2 mg/L with a range of 19-30 and 10-15 mg/L, respectively. The effluent TSS (VSS) concentration differences were mainly due to the difference of the type of microorganisms grown in SBR1 and SBR2 (as discussed in section 3.4) that resulted in different settling characteristics as indicated by the SVI for SBR1 (19 mL/g) vs. SBR2 (96 mL/g). Correspondingly, the average MLSS (MLVSS) in SBR1 was 5495 (2392) mg/L with an average SRT of 47 days; however, the concentration was lower in SBR2 with MLSS (MLVSS) concentration of 2338 (1228) mg/L and 31 days SRT (Table 5.4). The decline in SRT and observed decrease in MLSS concentration in SBR2 was due to poor settling and biomass loss associated with the type of microbial structure grown in each bioreactor (section 5.3.4). A decrease in the MLVSS/MLSS ratio from 0.6 to 0.43 was observed in SBR1 and from 0.6 to 0.52 in SBR2. The main reason for the MLVSS/MLSS decrease is the accumulation of fixed non-volatile matter such as calcium carbonate that was supplied with synthetic wastewater, which quickly settled at the bottom of SBRs. The accumulation was relatively higher in SBR1 because of the longer SRT.

The pH and ORP for influent effluent in both SBRs were monitored to ensure anoxic conditions during the study. The influent pH for both SBRs remained constant, ranging from 6.9-7.2 with an average value of 7.1 throughout the study. However, the effluent pH increased mainly due to the alkalinity production during the denitrification from nitrite (Badia et al., 2019; Cao et al., 2017; Du et al., 2016; Peng et al., 2006). SBR1 was setup to denitrify nitrite as an electron acceptor in which 3 electrons were transferred from nitrite to nitrogen gas during the bioreaction; thus, the average bioreactor pH was 8.45. However, SBR2 was partially denitrifying nitrate in which only 2 electrons were transferred during the bioreaction; therefore, SBR2's effluent pH was 7.5 (Table 5-4). The increase of pH that occurred during the PDN-cycle (SBR2) was mainly due to the dissociation of sodium acetate (carbon source) (Cao et al., 2017). The difference between the oxidation-reduction potential (ORP) for influent and effluent, Table 5-4, showed higher -ve △ORP difference for SBR1 (-126 mv) than SBR2 (-105 mv). The increase of △ORP in SBR2 was mainly due to the portion of nitrite that was fully denitrified (26% of N removal).

The average influent acetate COD varied from 43.3 to 82.9 mg/L (average 59±21 mg/L) for SBR1 and from 33.3 to 55.5 mg/L (average 47±9.7) for SBR2 (Figures 5-2a and c), respectively. Most COD was consumed in SBR1 for the nitrite denitrification (95% N removal efficiency) with average effluent of 3.7 mgCOD/L (94% COD removal efficiency), while the average effluent of SBR2 (6.8 mg/L) was higher than SBR2 with 85% removal efficiency. The NO_2–N fed to the SBR1 varied between 23.7 and 27.3 mg/L with an average of 25±1.1 mg/L (Figure 5-2b, Table 5-4), and the influent NO_3–N fed to the SBR2 varied between 25 and 28.6 mg/L with an average of 28±1 mg/L (Figure 5-2 d, Table 5-4). The nitrite effluent showed variability until day 80 when the final effluent reached to less than 1 mgN/L (Figure 5-2 b), which matches with stable effluent COD (soluble COD) for the same period in SBR1. The △COD/△N was found to be 2.5, which was calculated from Table 5-4 with consideration of a 2 mgN/L consumed for biomass synthesis. In comparison, a △COD/△N calculated from the yield equation 5-4 (Section 5.3.5) (△COD/△N

=1.72/(1-1.42Y)) was equal to 3, which implies that a portion of denitrification was due to endogenous respiration COD. In SBR2, NO_3-N was not completely removed during the first 80 days of operation (Figure 5-2 d), and early on, NO_2-N accumulation was not observed except for some days (12 mgN/L-day 35, 5-9mgN/L-between days 55-75). However, during the last 40 days of the SBR2 operation, stable effluent NO_2-N, and NO_3-N concentration with 26% N removal efficiency was observed. The average effluent NO_2-N accumulated at the end of the cycle for the last 40 days of operation exceeded 11±2 mg/L (Figure 5-2 d, Table 5-4), which indicates that PDN can be maintained with COD/N and DO control. Additional discussion on COD/N and DO control is presented in sections 5.3.2 and 5.3.3.

Table 5.4 Average influent and effluent characteristics for SBR1 and SBR2[*]

Parameter	SBR1(Nitrite), mean ± SD[*]		SBR2(Nitrate), mean ± SD[*]	
	Influent	Effluent	Influent	Effluent
NO_2-N, mgN/L	25±1.1	0.69±7.4		11±2.9
NO_3-N, mgN/L		0.55±0.5	28±1	5.0±3
TCOD		9.5±4.35		31±5
COD, mg/L[**]	59±21	3.7±4.38	47±9.7	6.8±5
ALK, mgCaCO3/L	109.9±3.7	223.1±3.63	104.2.5	207±9
pH	7.1±0.3	8.45±0.15	7.1±0.2	7.5±0.1
DO, mg/L	7.18±1.4	0.31±0.07	6.4±1.9	0.34±0.2
ΔORP	(-126.2)±63		(-105.6)±55	
TSS, mg/L		15.2±3.8		22.8±3.7
VSS, mg/L		6.5±2.07		13±2
$\%N_{removal}$	95.2±3.18		26±9.3	
SRT, d	47±3		31±3.5	
MLSS, mg/L	5495±1725		2338±463	
MLVSS, mg/L	2392±1443.547		1228±233	

[*] Average data during the last 40 days of operation

[**] COD = COD_i, calculated as per equation 5-1.

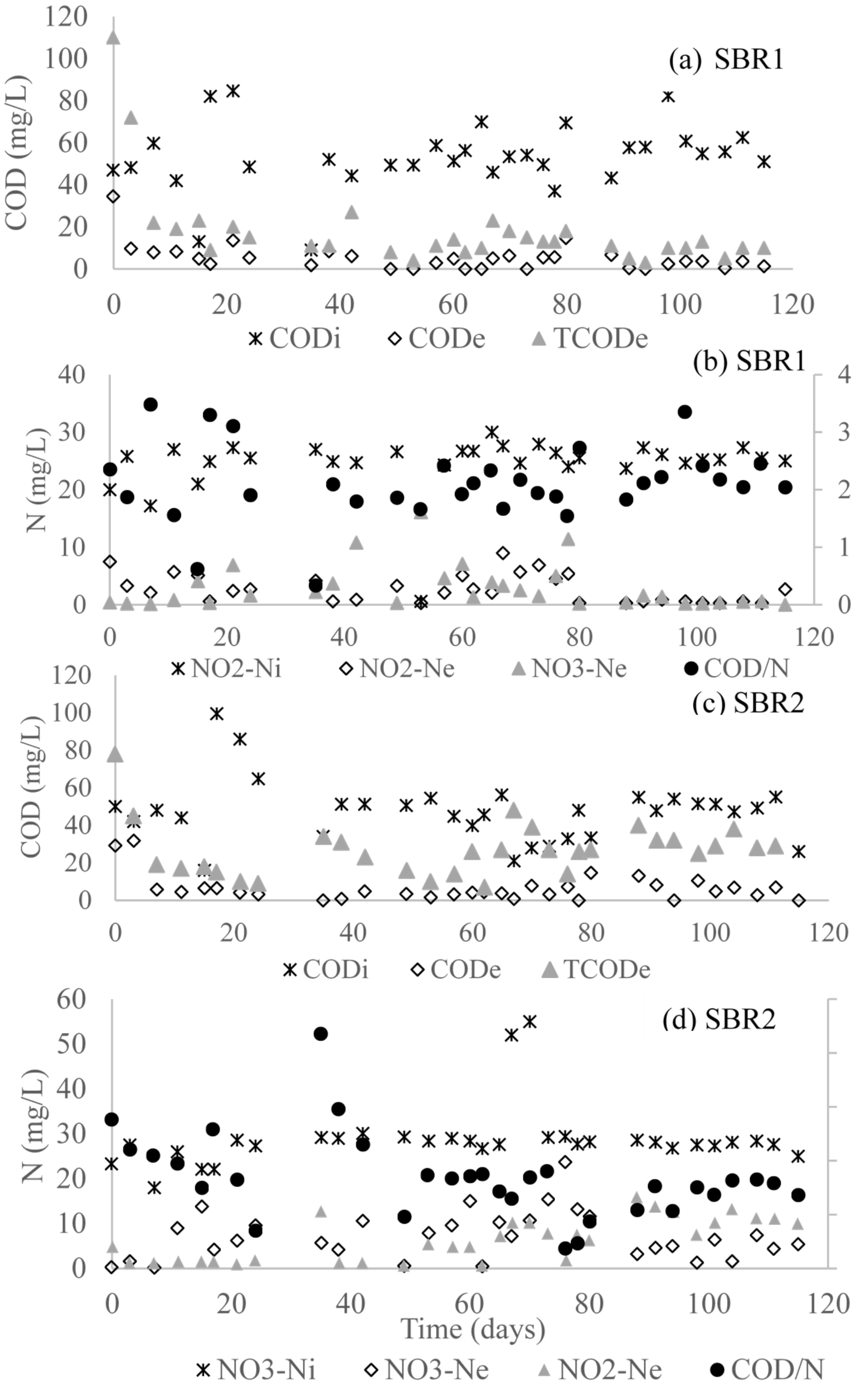

Figure 5.2 Influent and effluent characteristics for SBR1 and SBR2: (a) influent soluble CODi and effluent soluble CODe, (b) influent NO$_2$-Ni, effluent NO$_2$-Ne, and effluent NO$_3$-Ne, (c) influent soluble CODi and effluent soluble CODe, (d) influent NO$_3$-Ni, effluent NO$_2$-Ne, and effluent NO$_3$-Ne

5.3.2 COD/N and DO impact on the long term nitrite and nitrate denitrification

Both SBRs were operated at the COD/N ratio of 2 (Figure 5-2 a to d), with nitrite and nitrate concentration of 25 mgN/L in the influent for 126 days. A high nitrite removal efficiency was obtained (more than 80%) in the first week of operation. However, due to high DO intrusion from the influent tank (1 mg/L in the bioreactor and 6 mg/L in the influent tank) or at times due to low COD/N ratio during some instances (between days 11 to 70), the nitrite removal efficiency was not stable. During most of the operation period, the SBRs DO was maintained less than 0.5 mg/L by purging it with nitrogen. A similar reduction in denitrification was also reflected in SBR2 since both SBRs were purged from one nitrogen-cylinder (Figure 5-3a). During increased DO levels, the specific denitrification rate (SDNR) decreases according to the DO inhibition-switching function ($K_o^{/}/DO+ K_o^{/}$), (where $K_o^{/}$ is the inhibition concentration (ranges from 0.1 to 0.2 mgDO/L Oh (Oh & Silverstein, 1999b)), and denitrification from nitrate/nitrite would be negatively affected if it is higher than 0.5 mgDO/L, (Eddy et al., 2014). Lower COD/N also reduced nitrite removal efficiency due to the lack of carbon source, as 3 electrons are donated from the carbon source (sodium acetate) to nitrite (electron acceptor) to produce nitrogen gas. Theoretically, each mgNO_2-N requires 1.72 mgCOD to be converted to nitrogen gas divided by 0.4 for the yield consideration (1-Y), which increases the COD/N needed to more than 4; therefore, one of the reasons for not having full nitrite denitrification could be the lower COD/N of 2 (Figure 5-3 a). However, Figure 5-3a also clearly showed that with a slight COD/N increase to 2.5, the nitrite removal efficiency was more than 95% with an average final effluent NO_2-N concentration of 0.69 mg/L (80 10 120 days). A COD/N ratio of 2.5 is still lower than the theoretical 4, and it is believed that the higher removal could also be due to consistent DO that was below 0.5 mg/L and due to endogenous respiration COD.

Similarly, in denitrification from nitrate, there were disturbances in the DO, and the bioreactor DO reach as high as 1 mgDO/L between days 10 to 70, which inhibited denitrification from nitrate and nitrite and reduced the SDNR. Comparatively, the COD/N was relatively stable, with an average of 1.8 except some days with disturbance showing COD/N less than 1. During the initial 80 days, the NOx-N removal ranged from 15 to 92%, and the lower removals were associated with higher DOs (1 mg/L) and the higher removals with lower DOs (0.3 mg/L). During the last 40 days of operation (DO<0.5 mg/L, COD/N of 1.8), the nitrate concentration was reduced from 28 mgN/L to 5 mgN/L (93% NO_3-N removal). However, most of the nitrate was accumulated as nitrite (up to 68%), and approximately 25% nitrite is denitrified. At this point, it was not clear why the nitrite was not accumulated during the first 80 days of operation. It is suspected that, during the first 80 days, it was possible that nitrate was partially denitrified; however, due to the high DO level in the bioreactor, the nitrite might be further oxidized by nitrite-oxidizing bacteria (NOB), forming nitrate as an end product. Oxidation of nitrite to nitrate was also observed coincident with higher DOs (0.5 to 1 mg/L) during the first 80 days of SBR1 operation (Figure 5-2 a and 5-3 a). Considering the last 40 days of SBR2 data (Figure 5-3 b), it can be observed that a 1.8 COD/N did not allow for complete denitrification from nitrate because of the deficiency of the number of electrons. Five electrons are needed and donated from acetate to the electron acceptor nitrate, and theoretically, each mgNO$_3$-N requires 2.86 mgCOD to be converted to nitrogen as divided by 0.4 for the yield consideration (1-Y), which increase the ratio to 7. Understanding the COD/N ratio impact on a long term base is difficult due to the additional COD from endogenous decay. Thus a cyclic test was performed inside the SBRs to understand the impact of acetate COD/N ratio on the corresponding nitrite accumulation rates, nitrate denitrification rates, and nitrite denitrification rates, and the results are discussed below.

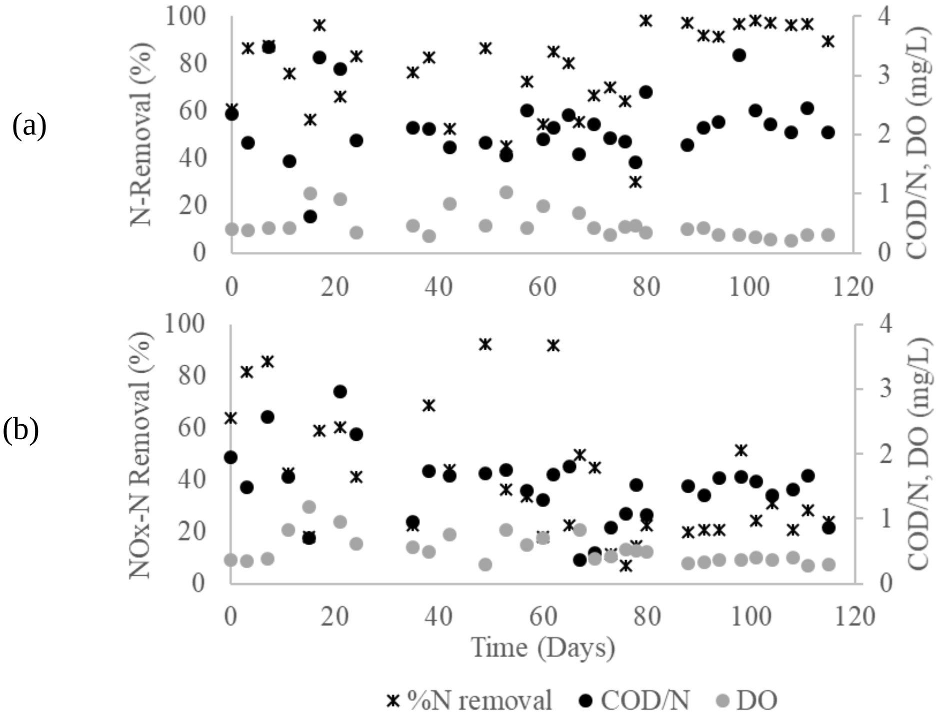

Figure 5.3 Effect of the COD/N and DO on nitrogen removal (a) SBR1-nitrite (b) SBR2-nitrate

5.3.3 Effect of COD/N on partial denitrification

Various COD/N ratios were conducted in SBR 1 and SBR2 to study the effect of COD/N ratio on the denitrification nitrate and identify the optimum COD/N to maximize nitrite accumulation. Partial denitrification of nitrate was reported to depend on several factors (Ji et al., 2018; Peng et al., 2006; Pishgar et al., 2019). A study by Akunna et al., 1993 illustrated that the pathway during denitrification from nitrate with volatile fatty acids such as acetate is nitrogen gas, while the pathway with glucose is dissimilatory nitrate reduction to ammonium. The reason for this is that acetate enters the tricarboxylic acid cycle (TCA) directly, whereas glucose will first Glycolysis before entering the TCA cycle. As a result, PDN may not be observed on some carbon sources. However, the occurrence of PDN during processes that made use of volatile fatty acids as carbon sources were not conclusive. Some researchers obtained PDN at a lower COD/N ratio (Ji et al., 2018), higher COD/N ratio (Shi et al., 2019), at a higher pH (Peng et al., 2006; Qian et al., 2019; Shi et al., 2019), carbon source (Le et al., 2019b), and type of seeding sludge (Cao et al., 2013; Chen et al., 2016). So the intent here is to understand the PDN process better and generate fundamental knowledge to aid in developing control strategies.

Figures 5-4 a to e shows cyclic test results in SBR2 at COD/N ratios of 1.1, 1.8, 2.7, 6.7 and 9.8, respectively, each test lasted 7.5 hours and samples were collected every 15 minutes for the first 4 hours. The data were also used to calculate (i) the specific denitrification rate from nitrate ($SDNR_{NO3}$), (ii) specific nitrite accumulation rate (SNAR), (iii) the first specific denitrification rate from nitrite ($SDNR_{NO2}$-1), (iv) the second specific denitrification rate from nitrite ($SDNR_{NO2}$-2) and (v) the nitrate to nitrite transformation rate (NTR%). The $SDNR_{NO3}$ ($SDNR_{NO2}$) was calculated by dividing the slope of the nitrate (nitrite) concentration removed over time (mg N/L/day) by the MLVSS concentration. The SNARs followed zero-order kinetics and were calculated by dividing the slope of the line of nitrite concentration accumulated during the SBR2 anoxic cycles normalized to the time-day (mgNO_2–N/L/day) and divided by the MLVSS concentration. The nitrate to nitrite transformation ratio (NTR%) was calculated according to the following formula (Shi et al., 2019):

$$\mathrm{NTR\%} = \frac{(\mathrm{NO_2{-}N})_{\mathrm{accmulated}} - (\mathrm{NO_2{-}N})_0}{(\mathrm{NO_3{-}N})_0 - (\mathrm{NO_3{-}N})_{\mathrm{max.acc}}} * 100 \qquad (2)$$

Where $NO_2\text{-}N_{accumulated}$ represents the maximum nitrite accumulation, $NO_2\text{-}N_0$ and $NO_3\text{-}N_0$ represent nitrite and nitrate concentration at time zero and $NO_3\text{-}N_{mac.\ acc}$ represents nitrate concentration at nitrite maximum accumulation.

At very low COD/N of 1.1 there was a slight nitrite accumulation, nitrite increased from 3.6 mgN/L to 5.7 mgN/L with NTR (nitrite to nitrate transformation ratio) of 17.1% (Figure not shown here). The kinetic rates $SDNR_{NO3}$, SNAR and $SDNR_{NO2}$-1 were meagre 0.078, 0.068 and 0.01 mgN/mgVSS-d (Table 5-5), respectively, and $SDNR_{NO2}$-2 was zero because there was not enough carbon for further denitrification from nitrate and nitrite. At slightly higher COD/N of 1.8, nitrite accumulated to 11.7 mgN/L with NTR of 68% while nitrate reduced from 18.1 mgN/L to 9.3 mg/N/L after 90 minutes from the cycle start time (Figure 5-4 a). Beyond this point of time, both nitrate and nitrite were further reduced to 9.0 mgN/L and 10.2 mgN/L, respectively. These minor reductions can be attributed to endogenous respiration since COD was very low 4.2 mg/L at the maximum nitrite accumulation, which can also be observed from the NO_X-N profile at 90 minutes (Figure 5-4a). Relatively higher kinetic rates were also observed; the $SDNR_{NO3}$, SNAR and $SDNR_{NO2}$-1 were 2.119, 0.884 and 1.27 mgN/mgVSS-d, respectively (Table 5-5).

At COD/N of 2.7, the amount of nitrite accumulated increased from 5.4 mg/L to 16.2 mgN/L with NTR of 79.4% while nitrate reduced from 15.7 mgN/L to 2.1 mgN/L at 240 minutes from cycle start time. After 240 minutes, while the nitrate concentration remained unchanged at 2.1 mgN/L, the nitrite was reduced to 14.7 mgN/L, which can be attributed to the utilization of carbon source from endogenous respiration. This can be confirmed by the remaining COD of 3.6 mg/L at the maximum nitrite accumulation and can also be observed from NO_X-N rate at 150 minutes (Figure 5-4 b). While the $SDNR_{NO3}$ remained comparable to the 1.8 COD:N test (2.127mgN/mgVSS-d), the SNAR increased to 1.62 mgN/mg VSS-d while the $SDNR_{NO2}$-1 decreased to 0.465 mgN/mgVSS-d (Table 5-5).

At high COD/N of 6.7, while nitrite increased from 4.5 to 14 mgN/L (maximum accumulation) with NTR of 81.4%, nitrate reduced from 18.1 mgN/L to 6.3 mgN/L, and this accumulation occurred within 15 minutes from the cycle start time (Figure 5-4 c). After 60 minutes from cycle start time, nitrate and nitrite were further reduced to 1.3 and 6 mgN/L, respectively, and COD was consumed entirely. Beyond this point time (60 minutes), the nitrite reduced to 2.9 mg/L; however, the carbon source was supplied through endogenous respiration (Figure 5-4 c). This result is significant, showing that with an increase in COD/N ratio, the maximum accumulation happens very fast, and we may have higher NTR but wasn't captured because samples were collected every 15 minutes. The $SDNR_{NO3}$ slightly increased to 2.61 mgN/mgVSS-d, SNAR increased 2.127 mgN/mg VSS-d and $SDNR_{NO2}$-1 almost remained unchanged 0.483 mgN/mgVSS-d, due to the availability of carbon source and a 0.83mgN/mgVSS-d $SDNR_{NO2}$-2 was observed(Table 5.5). At the highest COD/N of 9.8, nitrite accumulated from 4.8 to 15.3 mgN/L with NTR of 73%, nitrate was then reduced from 16.1 mgN/L to 1.7 mgN/L, same as above (COD/N of 6.7) this accumulation also occurred within the first 15 minutes from the cycle start. It is suspected that the actual NTR would be much higher if the sampling period were shorter than 15 minutes. Nitrate and nitrite are then reduced to 0.7 and 1.5 mgN/L after 45 minutes from cycle start (COD of 44 mg/L), respectively (Figure 5-4 d). The $SDNR_{NO3}$ slightly decreased to 2.16 mgN/mgVSS-d , and SNAR decreased to 1.575 mgN/mg VSS-d, and $SDNR_{NO2}$-1 almost remained unchanged 0.585 mgN/mgVSS-d, and this is due to the availability of carbon source $SDNR_{NO2}$-2 increased to 1.04 mgN/mgVSS-d (Table 5.5).

Previous research by Le et al., 2019 stated that maximum nitrite accumulation occurred at nitrate concentration of 3 mgN/L. Fundamentally, the minimum nitrate concentration in the final effluent is directly affected by K_{NO3}, which was reported as 6.9 mg/L by Cao et al., 2017. Thus in the case of high initial nitrate concentration, maximum nitrite accumulation can happen at a high remaining nitrate concentration because the switching function will be 1 ($NO_3 - N/(K_{NO3} + NO_3 - N) = 1$). The cyclic studies (Figures 5-4 a to d), clearly uncover that nitrite accumulation is not affected by higher levels of COD/N ratio; actually, accumulation occurs when COD/N $\geq$ 2.7. Therefore, in order to accumulate more than 80% nitrite, the threshold COD/N ratio has to be higher than 2.7. Moreover, SNAR (2.127 mgN/mgVSS-d) needs to be maximized to accumulate nitrite to higher than 80%, and $SDNR_{NO2}$-1 to be minimized to a lower value (0.46 mgN/mgVSS-d), which is usually reduced due to the competitive inhibition between nitrite and nitrate. Moreover, a lower COD/N ratio of 2.5 to 3 can be used to control further denitrification from nitrite. However, the analysis showed higher NTR at COD/N of 6.7, but nitrite was further reduced due to the availability of the carbon source, which can be controlled by either reducing the retention time and/or inhibiting the nitrite reductase (NiR) enzyme. All the cyclic studies in SBR2 showed that despite the COD:N ratio, nitrate denitrification in SBR2 occurs at a much higher rate compared to nitrite denitrification. This goes against the denitrification thermodynamics. The ΔG for nitrate denitrification (-93.23 Kj/e$^-$) is lower than the ΔG for nitrite denitrification (-71.67 Kj/e$^-$) (Eddy et al., 2014), which indicates that under carbon limiting condition, the nitrite denitrification is favoured over nitrate denitrification thermodynamically. However, this study clearly showed that this is not the case, which is further clarified by comparing the kinetics and microbiological makeup of the nitrite (SBR1) versus nitrate (SBR2) reactors. Moreover, the analysis illustrates that △COD/△NO_3-N of 4.3 and 4.1 at COD/NO_3-N of 6.7 and 9.8, respectively. This indicates that COD/NO_3-N could be further optimized in the range of 4-4.5.

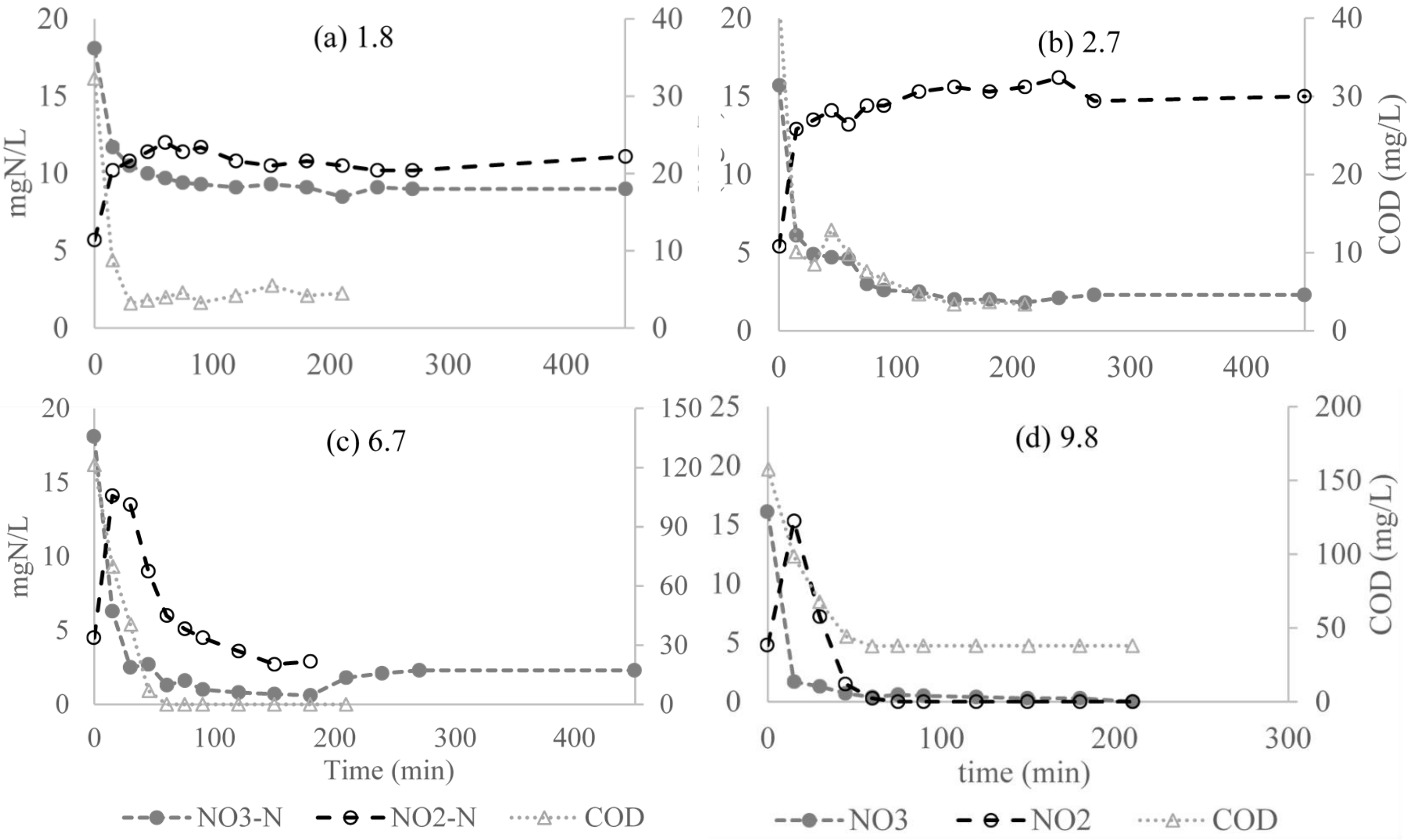

Figure 5.4 Cyclic test in SBR2: (a) COD/N of 1.8, (b) COD/N of 2.7, (c) COD/N of 6.7 and (d) COD/N of 9.8

Table 5.5 Effect of COD/N on PDN in SBR2

NO_3-Ni[1]	NO3-N[2]	NO2-N[2]	CODi[1]	COD[2]	SDNR[3]	SNAR[4]	SDNR-1[4]	SDNR-2[4]	COD/N	VSS[1]
17	12.6	5.4	18.4	28.0	0.078	0.068	0.01	0	1.1	945
18.1	9.3	11.7	32.3	4.2	2.119	0.844	1.27	0	1.8	435
15.7	2.1	16.2	42.6	3.4	2.127	1.662	0.465	0	2.7	650
18.1	6.3	14.1	121.5	84	2.610	2.127	0.483	0.83	6.7	650
16.1	1.7	15.3	157.5	98.6	2.160	1.575	0.585	1.04	9.8	960

[1] mg/L
[2] N at max-accumulation, mg/L
[3] mgNO_3-N/mgVSS-d
[4] mgNO_2-N/mgVSS-d

5.3.4 Bacterial identification

In order to distinguish between the nitrite and nitrate denitrifiers community diversity and a structure, the taxonomic analysis was conducted. Previous studies have been conducted to investigate the community structure for complete and partial denitrification of nitrate (Andalib et al., 2018a; Du et al., 2016; Miao et al., 2017; Qian et al., 2019; Y. Yang et al., 2018). However, these studies did not delineate if there is any microbial composition difference between a process that was under complete nitrite denitrification vs partial denitrification.

5.3.4.1 Phylum level classification

The relative taxonomic microbial communities were studied up to the phylum level for both SBR1 and SBR2 (Supporting document: Figure SD-5-1 a and b). The most identified predominant phylum was *Proteobacteria* phylum, which was found to be 79% in SBR1 (nitrite) and 69.3% in SBR2 (nitrate). The *Proteobacteria* phylum is typically found in municipal wastewater treatment facilities and responsible for denitrification (Du et al., 2017). Du et al., 2017, who studied partial denitrification from nitrate, reported a comparable 65% RA of the *Proteobacteria* phylum. The decrease in *Proteobacteria* phylum RA in SBR2 could be related to the lower available carbon source for nitrate (5 electrons needed) than nitrite (3 electrons needed). The second-highest relative abundance phylum in SBR1 was *Acidobacteria* with RA of 7.1%, the *Acidobacteria* was identified as the third dominant phylum in SBR2 (2.4%). Most bacteria in this phylum grow at a lower pH, and under aerobic conditions however, some are facultative heterotopic denitrifiers (Eichorst et al., 2018). The *Bacteroidetes* was the second for SBR2 (19%) and the third for SBR1 (6%), and also classified as facultative heterotrophic denitrifiers and found in an anoxic denitrifying environment (Xia et al., 2019).

The next taxonomic classification is the class level (supporting document, Figure-SD 5.2a (SBR1) and Figure-SD 5.2b (SBR2)), *Betaproteobacteria* class showed to dominate the denitrification process with the highest relative abundance of 67.5 and 50.4% for SBR1 and SBR2, respectively. It was interesting to observe the presence of *Nitrospira* with a relative abundance of 1.8% and 0.31% in SBR1 and SBR2, respectively which indicate the existence of nitrite-oxidizing bacteria (NOB), which can be related to the intrusion of oxygen and an increase in DO level to 1 mg/L while nitrogen cylinder ran out of gas. The presence of *Nitorspira* confirmed the decline in nitrite and nitrate removal efficiency, and the lack of nitrite accumulation during the first 80 days of operation was associated with NOx-N oxidation. An overall comparison of the microbial composition in SBR1 and SBR2 showed that there was no substantial difference in composition and relative abundance differences on a phylum and class level.

5.3.4.2 Microbial genus and species

In general, most denitrifying bacteria are heterotrophic because they require organic carbon source, which is needed for cell growth and nitrate reduction, (X. Xu et al., 2020). During denitrification from nitrite, a nitrite may accumulate due to nitrite reductase (Nir) inhibition, lag in synthesis, and nitrate reductase (NaR) inhibition because nitrite is on the nitrate denitrification pathway, (Andalib et al., 2018a). (Andalib et al., 2018a) divided denitrifiers into four groups; (i) Group A: reduces NO_3 to NO_2, which lack Nir, (ii) Group B: reduces nitrate to further nitrogen oxides in the denitrification pathway (NO_2, NO, N_2O), (iii) Group C: reduces nitrate and nitrite, but with a higher nitrite denitrification rate (no nitrite accumulation), and (iv) Group D: reduce nitrate and nitrite but with higher nitrate denitrification rate (nitrite accumulation may be observed).

There were 17 genera identified in SBR1 and 21 in SBR2 with a relative abundance of 1% and above. The relative abundance distribution on the genus level is shown in Figure 5-5 a., and Table 5-6 shows the classification of the genera level, relative abundance (RA%), class, and phylum levels along with the importance of each genus found in SBR1 and SBR2. The dominant genus in the SBR1(nitrite) was *Zoogloea* with a relative abundance of 42.2%, followed by *Acidobacterium* (6.6%), *Thauera* (4.5%), and *Dokdonella* (2.9%). However, the genera relative abundance distribution in SBR2 were utterly different, the *Thauera* comes first (10%) followed by *Zoogloea*

(7.2%), *Sterolibacterium* (5.9), *Denitratisoma* (5.45%), *Niastella* (4.87%), *Dokdonella* (4.24%), *Fluviicola* (3.84%), *Dechloromonas* (3.47%), *Terrimonas* (3.32%), *Methyloversatilis* (2.61%) and *Lewinell*a (2.05%). It can also be observed that the nitrate reactor had a wide range of genera (11) at 2 to 10% RA, whereas in the case of the nitrite reactor only 4 genera were identified with higher RA. *Zoogloea* is well known in wastewater treatment for its higher EPS concentration and floc formation and often associated with sludge having SVI as low as 19 mL/g (An et al., 2016). The higher relative abundance of *Zoogloea* in SBR1 (nitrite denitrifying bioreactor) could indicate that this genus may be associated with group C denitrifiers where microbe consisted of higher NiR enzyme over NaR enzyme. In this study, the nitrite reactor had also shown good settling characteristics (section 5.3.1), confirming the microbiological and the process data. In comparison, the higher *Thauera* with a relative abundance of (9.96%) in SBR2 could be attributed to group D, which has higher NaR enzymes than NiR enzymes. This is consistent with the observation by Qian et al., 2019. The authors found a relatively higher relative abundance of *Thauera at a pH of 9* (17.04%), but the abundance dropped to 2.21% at a pH 7. The authors also showed a higher NaR enzyme of 8089 hits than NiR enzyme of 2950 hits associated with *Thauera* genera. The higher NaR enzyme associated with *Thauera* and *Thauera* abundance in SBR2 may explain the preferential nitrate denitrification but nitrite accumulation in the SBR2 reactor. Z. Xu et al., 2018 also found RA of *Thauera* 38% at higher COD/N of 6, showing a RA(%) increase with higher carbon source availability. This is in agreement with a previous study by W. Zhou et al., 2017 that detected *Thauera* in the three bioreactors using thiosulfate (0.38%), sulfide (0.12%), elemental sulfur (most abundant 2.24%) and ethanol (0.17%) as an electron donor in a study of autotrophic denitrifiers. McIlroy et al., 2016 reported a high acetate uptake rate with nitrite by *Dechloromonas* genus, which part of their species belongs to the denitrifying phosphorus accumulating organism (DPAO) group. Also, *Dokdonella* was studied and considered as complete nitrite and nitrate denitrifiers in both the oxic and anoxic environment (Du et al., 2017) and (Pishgar et al., 2019). It is crystal clear that the microbial community is more diverse in SBR2, where *Thauera* has a highest relative abundance of 10% followed by *Zoogloea* (7.2%), while SBR1 has *Zoogloea* with a very high relative abundance of 42.2% and contains a relatively smaller fraction of *Thauera* (4.5%).

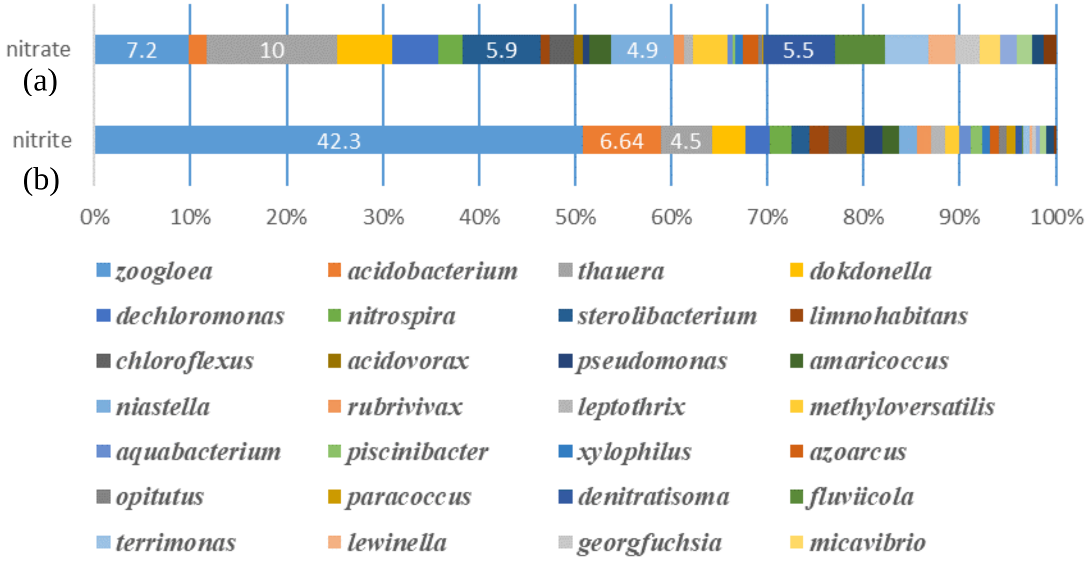

Figure 5. 5 Microbial classification on genus lever (a) SBR1 and (b) SBR2

Table 5. 6 Characteristics of each genus detected with high RA%

Genus	SBR-1 RA%	SBR-2 RA%	Class/ Phylum	Important notes	Reference
Zoogloea	*42.3*	*7.2*	*Betaproteobacteria/ Proteobacteria*	Capable of denitrifying from nitrate and nitrite but more specifically from nitrite anoxically	(Chai et al., 2019), (McIlroy et al., 2016)
Acidobacterium	*6.64*	*1.4*	*Acidobacteria/ Acidobacteria*	Facultative heterotrophs. G-ve. Reduces nitrate and nitrite with acetate anoxically.	(Pankratov et al., 2012)
Thauera	*4.6*	*10*	*Betaproteobacteria Proteobacteria*	Facultative heterotrophs. G-ve. Denitrify nitrate to nitrite aerobically and anoxically.	(Pishgar et al., 2019), (X. Xu et al., 2020)
Dokdonella	*2.9*	*4.25*	*Gammaproteobacteria Proteobacteria*	G-ve. Denitrify nitrite and nitrate to nitrogen gas aerobically. Grew specifically with acetate	(Y. Li et al., 2013), (Pishgar et al., 2019)
Dechloromonas	*2.1*	*3.5*	*Betaproteobacteria Proteobacteria*	G-ve. Denitrify nitrate to nitrite anoxically. Grew specifically with acetate. Contains autotrophic denitrifiers species. Some strains can produce N_2O. Some species are DPAOs	(X. Xu et al., 2020), (W. Zhou et al., 2017), (McIlroy et al., 2016)
Sterolibacterium	*1.60*	*5.94*	*Betaproteobacteria Proteobacteria*	G-ve. Denitrifirs and appears in denitrifying communities. Recovered from contaminated groundwater (56), lake sediment, cattle manure, and the gut of medicinal leeches, but their roles in these ecosystems are largely unknown.	(Wei et al., 2018)
Denitratisoma	*0.52*	*5.5*	*Betaproteobacteria Proteobacteria*	Similar to *thauera*, denitrify nitrate to nitrite aerobically and anoxically. Grew specifically with acetate. Contains autotrophic denitrifiers species.	(Xia et al., 2019)
Niastella	*1.47*	*4.88*	*Flavobacteria Bacteroidetes*	Found in most MWW treatment facilities	(Qian et al., 2019)

5.3.4.3 Microbial species

Similar to the genus, the species level shows a functional classification; hence it was expected to observe a difference in the species-level classification. There were 16 species identified in SBR1 and 19 in SBR2, with a relative abundance of 1% and above (Figure 5.8). The most common species in SBR1 (nitrite) were *Zoogloea Resiniphila* with a relative abundance of 24.5%, followed by *Zoogloea Ramigera* with a relative abundance of 16.7%. Both of the two species belong to *Zoogloea* genus and the class of *Betaproteobacteria* under the phylum of *Proteobacteria*. However, the relative abundance of these species in nitrate SBR2 was 0.99 and 6% for *Zoogloea resiniphila* and *Zoogloea Ramigera*, respectively, which indicates that *Zoogloea Resiniphila* is more responsible for the denitrification from nitrite (group C- more NiR). In contrast, *Zoogloea Ramigera* can denitrify from both electron acceptors (group B, balanced NiR, and NaR). Furthermore, the third species in SBR1 is *Acidobacterium spp*. (6.59%) followed by *Dokdonella spp*. (2.84%), and *Thauera spp*. (2.55%). On the other hand, the most relative abundance in the SBR2 (nitrate) was *Thauera spp*. (6.5%) which has a higher NaR compared to NiR enzymes. This variation in the enzymes is confirmed by Ma et al., 2017, who observed a relative abundance of 1.23% of *Thauera spp*., in association with lower Nir enzymes (588 hits) than Nar enzymes (5892 hits) and confirmed by X. Xu et al., 2020, who observed a 5.7% *Thauera Phenylacetica* in UASB. In this current study, the SBR2 reactor that is dominated by the *Thauera spp.* showed a lower NO_2-N removal rate than NO_3-N removal, which, together with a lower COD/N ratio, leads to partial denitrification (nitrite accumulation). Therefore, the nitrite accumulated by *Thauera* is due to the availability of nitrate above K_{NO3}; however, NO_3-N below K_{NO3}, nitrite-denitrifiers starts to reduce nitrite by inducing nirS, mRNA increases, and the accumulated nitrite is then reduced. The second species in the SBR2 is *Zoogloea Ramigera* (6%), which is classified in group D as above, *Sterolibacterium spp*. (5.8%) and *Denitratisoma spp*. (4.8%) all of which are classified under class *Betaproteobacteria* and phylum of *Proteobacteria*. While heterotrophic denitrifiers are inhibited by high oxygen concentration, *Zoogolea Ramigera* can significantly denitrify nitrate at as high as 8 mgO_2/L, and the rate could be higher when ammonia exists with high concentrations (Pishgar et al., 2019); thus $SDNR_{NO2}$ could be augmented in the presence of high ammonium concentration (Pishgar et al., 2019). This indicates that *Zoogolea*, which belongs to the denitrifying glycogen accumulating organisms (DGAO) group, uses ammonia for cell growth (assimilation), while denitrify nitrate aerobically (nitrate dissimilation) (Pishgar et al., 2019).

In conclusion, the bacterial community in SBR1 and SBR2 were acclimatized to denitrify from nitrite and nitrate, respectively. The study showed a clear microbial community difference between the two reactors that yield a significant difference between the two denitrification processes. In this study, the change in the microbial community resulted in two complete denitrification patterns, high nitrite denitrification in SBR1, whereas higher nitrate denitrification but lower nitrite denitrification in SBR2 due to the diversity of the microbial communities. Moreover, *Zoogloea* and *Thauera* genera played a vital role in denitrification from nitrite and nitrate, respectively. For complete and partial nitrite denitrification, it would be favourable to analyze NiR and NaR enzymes in SBR1 and SBR2, respectively.

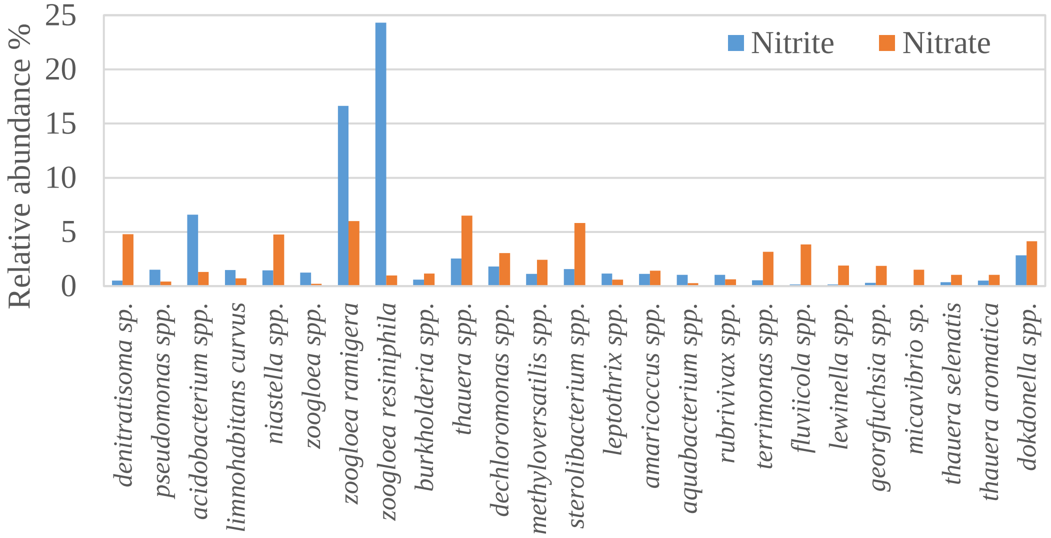

Figure 5. 6 Taxonomic microbial analysis on the species level in SBR1 and SBR2

5.3.5 Nitrate versus nitrite denitrification Kinetics

The kinetics parameters, including denitrifier yield, SDNR, and half-saturation coefficient, were compared from a complete nitrite denitrifying (SBR1) and a complete nitrate and partially nitrite denitrification (SBR2) system. This information will further explain the differences between the two systems. Also, this knowledge will contribute to model these systems accurately. Currently, limited kinetic data is available for such systems; therefore, the conventional modelling practice assumes similar kinetic values for NOx-N denitrification.

5.3.5.1 Heterotrophic denitrifier yield (Y_{HD})

The heterotrophic denitrifier yield (Y_{HD}) is defined as the biomass produced per the COD removed or nitrite (nitrate) - nitrogen removed. Determining the rate of biomass produced in the system allows us to determine the wasted biomass, and the parameter is used for process modelling to size the denitrifying bioreactor, estimate effluent quality, and also used to determine other kinetic parameters. The heterotrophic denitrifier yield (Y_{HD} – mgVSS/mgCOD) was calculated from the long term SBR 1 and SBR 2 data as per the equations below (Badia et al., 2019), (Bill et al., 2009) and Equation 9 by Y. Mokhayeri et al., 2009:

$$Y_{\mathrm{HD\ (NO2)}} = (1 - \frac{1.72\ \triangle \mathrm{NO_2{-}N}}{\triangle \mathrm{COD}})/1.42 \tag{5-2}$$

$$Y_{\mathrm{HD\ (NO3-NO2)}} = (1 - \frac{1.14 \triangle \mathrm{NO_2{-}N}}{\triangle \mathrm{COD}})/1.42 \tag{5-3}$$

$$Y_{\mathrm{HD\ (NO3-N2)}} = (1 - \frac{2.86 \triangle \mathrm{NO_3{-}N}}{\triangle \mathrm{COD}})/1.42 \tag{5-4}$$

Equations 2-3 can be rewritten for SBR2 as:

$$Y_{\mathrm{HD\ (NO3-N2)}} = \frac{(1-(1.14\ \mathrm{NO_2{-}N\ produced} + 1.71\ \mathrm{NO_2{-}N\ removed}) + 2.86\ \mathrm{NO_3{-}N\ removed})/\ \Delta COD)}{1.42} \tag{5-5}$$

Where $\triangle NO_2$-N represent the nitrite removed in the SBR1 (equation 5-2), 1.14$\triangle NO_3$-N represent nitrate removed to nitrite in SBR2 (equation 5-3), $\triangle$COD represent the COD removed in the respective SBR1 or SBR2 bioreactors (equations 5-3 to 5-5), the NO_2-N produced represents the SNAR, the NO_2-N removed represents $SDNR1_{NO2}$ + $SDNR2_{NO2}$, and the NO_3-N removed represents the $SDNR_{NO3}$. The kinetic data followed zero-order kinetics, and the SDNRs ($\triangle NO_2$-N and $\triangle NO_3$-N) were calculated by dividing the removed nitrite in the SBR1 and the removed nitrate in SBR2 normalized to the cycle time and MLVSS concentration. The substrate utilization rate, SSURs ($\triangle$COD) were calculated by dividing the removed COD concentration during the SBR anoxic cycle normalized to the cycle time-day (mgCOD/L/day) and the MLVSS concentration.

The heterotrophic denitrifier yield (Y_{HD}) in the SBR was estimated after determining the substrate utilized in both SBRs or calculating the slope of the SDNR/SSUR ($\triangle$N/$\triangle$COD) for each data point multiplied by the stoichiometric coefficient, 1.72 for SBR1 and SBR2, and substituting the values in Equation 3 for SBR1 and Equation 5-4 for SBR2. The Y_{HD} (SBR1-nitrite) was found to be 0.3±0.1 mgVSS/mgTCOD (Figure SD 5-3 a), and 0.34±0.13 mgVSS/mgTCOD for SBR2 (PDN) (Figure SD 5-3 b). Cherchi et al., 2009, Yongmei Zhang et al., 2016 and Yalda Mokhayeri et al., 2008 reported a yield of 0.35, 0.38 and 0.42 mgVSS/mgCOD, respectively during complete nitrate denitrification studies with acetate as carbon source. Peng et al., 2007 estimated yield of 0.4, 0.42, and 0.65 mgVSS/mgCOD for methanol, ethanol, and acetate, respectively. Other yield studies by (Guven et al., 2018), who reported a yield coefficient of 0.45 mgVSS/mgCOD for a mixture of acetate, propionate, ethanol, and glucose. Bernat et al., 2015 reported a daily variation on yield (0.27-0.44), which depends on the daily operation conditions and COD/N ratio. All literature yield data are based on full nitrate denitrification; however, the yield estimated in the current study (during PDN) is in good agreement with literature. According to the following PDN stoichiometric equation (half-reaction stoichiometry below), the yield would be 0.13 mgVSS/mgCOD, which occurred due to PDN only. However, the PDN calculated yield in this study was due to a combination of full denitrification $SDNR_{NO3}$, $SDNR_{NO3-NO2}$, SNAR (specific nitrite accumulation rate), and SDNR1(first specific denitrification rate from nitrite).

$$\begin{gathered}0.125CH_3COO + 0.38NO_3{}^{-} + 0.009H^{+} \\ = 0.009C_5H_7O_2N + 0.38NO_2{}^{-} + 0.1H_2O + 0.125HCO_3{}^{-} + 0.086CO_2\end{gathered}$$

There is limited literature on the nitrite yield with acetate. (Badia et al., 2019) (Chapter 3) reported a yield of 0.51 mgVSS/mgCOD during offline testing of nitrite and acetate, which was much higher than the current yield data. The higher yield can be attributed to the use of unacclimatized sludge to acetate; rather, the sludge was acclimatized to MWW in the long-term process. Although there was not much difference yield between the complete denitrification from nitrate in the above literature, the sludge produced in the denitrification from nitrite and for PDN is lower due to less COD consumed (3 electron transferred for nitrite and 2 electron transferred for PDN).

5.3.5.2 Effect of COD/N on nitrate and nitrite SDNR during SBR cyclic tests

Various COD/N ratios were conducted in SBR1 and SBR2 to study the effect of COD/N ratio on the denitrification from nitrite versus nitrate, find the optimum SDNR for both nitrite and nitrate, and compare the nitrite versus nitrate denitrification rate.

Figure 5-7a and Table 5.7 show the effect of COD/N in SBR1 on denitrification from nitrite (SDNR) during cyclic tests. The SDNR linearly increased with the increase of COD/N in the range of 2.6-10.1 (COD range 29-300 mg/L) to a maximum value of 0.69 mgN/mgVSS-d at COD/N of 10.1 beyond which SDNR declined and dropped to as low as 0.31 mgN/mgVSS-d at COD/N of

25. These findings agreed with (Adav et al., 2010; Akunna et al., 1992; Badia et al., 2019, 2020; S. Ge et al., 2012). As shown later, the K_{NO2} (half-saturation coefficient of nitrite) obtained from the kinetic studies was 3.17 $mgNO_2$–N/L, and hence, the observed decline in SDNR at higher COD/N is associated with the lower bioreactor NO_2–N concentration relative to K_{NO2}. Furthermore, Table 5-7 shows that the nitrite and COD concentrations are much lower than the substrate inhibition limits for nitrite (0.2 mgFNA/L) and COD (acetate) (Badia et al., 2019) and (Adav et al., 2010) which indicate that there will be optimum COD/N at which maximum SDNR will occur, and this optimum COD/N ratio mainly depends on the carbon type, for example, the ratio was 6 for MWW (Badia et al., 2019). In a study by (Badia et al., 2019), the maximum nitrite SDNR (carbon source: acetate) was lower (0.41 $mgNO_2$-N/mgVSS-d, at the optimum COD/N ratio of 13) which could be due to the nature of the biomass that was acclimatized to MWW as oppose to acetate. This implies that along with K_N and K_S, a low COD/N ratio (low carbon source) is needed to maximize SDNR, compared to nitrate, and this ratio has to be analyzed to find the maximum SDNR whenever carbon source changes. Also, the analysis should be preferably conducted using acclimatized biomass to the carbon source and electron acceptors before the batch tests.

Similar cyclic tests were conducted in the SBR2, and kinetic data including (i) nitrate specific denitrification rate ($SDNR_{NO3}$), (ii) nitrite denitrification before nitrate exhaustion ($SDNR1_{NO2}$) and (iii) nitrite denitrification after nitrate exhaustion ($SDNR2_{NO2}$) was generated (Section 5.3.3). Most of this data has been discussed in section 5.3.3 with the focus of nitrite accumulation. In this section, the discussion is on the SDNR. Figure 5-7b shows the $SDNR_{NO3}$, $SDNR1_{NO2,}$ and $SDNR2_{NO2}$ trends with various COD/N ratio. At a low COD/N ratio of 1.1, very low $SDNR_{NO3}$ was observed (0.078 $mgNO_3$-N/mgVSS-d), and $SDNR1_{NO2}$ ($mgNO_2$-N/mgVSS-d) was also very low, which can be attributed to the low COD/N ratio as well as the competition between nitrate and nitrite denitrifies. When the COD/N ratio increased to 1.8 the $SDNR_{NO3}$ and $SDNR_{NO2}$-1 increased to 2.129 $mgNO_3$-N/mgVSS-d and 1.27 $mgNO_2$-N/mgVSS-d, respectively, due to more carbon source available for the two-electron acceptors. This also indicated that NiR reductase is not inhibited, but the accumulation was basically due to the difference between nitrate and nitrite SDNRs, which is in agreement with (Andalib et al., 2018a). Furthermore, $SDNR_{NO3}$ stabilized, and the curve approximately flattened with COD/N ratio increase; comparatively, the nitrite SDNR (SBR1) dropped by half with an increase in COD/N ratio from 10 to 25. This was not observed with $SDNR_{NO3}$; however, it is essential to note that the maximum COD/N ratio was 10; hence the $SDNR_{NO3}$ (SBR2) could behave similarly to SDNR (SBR1). Akunna et al., 1993 observed a reduction in nitrate removal rate beyond COD/N of 20, which indicates that if we carry out the cyclic test at higher COD/N (20), we may observe a decline in $SDNR_{NO3}$.

$SDNR_{NO2}$-1 also increased from 0.01 mgNO_2-N/mgVSS-d at COD/N of 1.1 to 1.29 mgNO_2-N/mgVSS-d at COD/N of 1.8, and then decreased with the increase of COD/N to 1.8 to 0.465 mgNO_2-N/mgVSS-d. Any further increase in the COD/N did not impact the $SDNR_{NO2}$-1 of approximately 0.5 mgNO_2-N/mgVSS-d (Figure 5-7b), which proves the direct relation between $SDNR_{NO2}$-1 and COD/N ratio and agrees with (Badia et al., 2019, 2020). The second specific denitrification rate ($SDNR_{NO2}$-2) is directly related to the COD/N ratio and final NO_3-N concentration (Figure 5-7b); there was no denitrification rate (zero) from nitrite ($SDNR_{NO2}$-2) at COD/N of 1.1, 1.8 and 2.7, which can be attributed to the deficiency of carbon source. However, the $SDNR2_{NO2}$ increased at COD/N of 6.7 and 9.8 to 0.83 and 1.04 mgNO2-N/mgVSS-d, respectively, higher than the nitrite SDNR in SBR1. This can be attributed to the higher denitrifiers diversity in SBR2, and since nitrite is generated in the pathway of the denitrification from nitrate, the SBR2-biomass acclimatized to nitrite. The $SDNR_{NO2}$-2 in SBR2 of 1.04 mgNO2-N/mgVSS-d was much higher than $SDNR_{NO2}$ in SBR1 of 0.69 mgNO2-N/mgVSS-d, which can also be attributed to the higher microbial diversity in SBR2.

It can also be concluded that denitrification from nitrate is more than double of denitrification from nitrite, which agrees with H. Sun et al., 2009, who found $SDNR_{NO2\_1}$ is always less nitrate-$SDNR_{NO3}$, and this is proved according to our experiments in SBR2. Ma et al., 2017 found during partial denitrification study nitrate $SDNR_{NO3}$ range between 1.43-1.98 mgN/mgVSS-d while nitrite $SDNR_{NO2}$-1 ranged 0.175-0.211 mgN/mgVSS-d, and nitrite $SDNR_{NO2}$-2 ranged 0.54-0.77 mgN/mgVSS-d. This indicates that $SDNR_{NO3}$ is much higher than $SDNR_{NO2,}$ which agrees with our findings. These are the maximum $SDNR_{NO2}$-1 during the cyclic test was 0.69 mgNO_2-N/mgVSS-d in SBR1, and the maximum $SDNR_{NO2}$-2 in the SBR2 was 1.27 mgNO_2-N/mgVSS-d. High $SDNR_{NO2}$-1 compared with the maximum $SDNR_{NO2}$ in SBR1 (0.69mgN/mgVSS-d) can be attributed to (i) the accumulated nitrite causes high stress on the NiR reductase-enzymes which increase the denitrification rate to reduce the nitrite toxicity effect (Andalib et al., 2018b), (ii) the difference between the microbial structure in both SBRs (iii) K_{COD} (COD-half saturation concentration) for acetate is as high as 34 mgCOD/L while there is no effect of K_{COD} at the start of the test (unlimited carbon source) on $SDNR_{NO2}$-1, the $SDNR_{NO2}$-2 is reduced according to switching function due to limited carbon source ($K_{COD}/(K_{COD}+COD)$). Though high COD/N of 6.7 and 9.8 (Figures 5-4 c and d), nitrite still accumulates and appeared for a short period, and this was due to the higher $SDNR_{NO3}$ than $SDNR_{NO2}$-1 which agrees with S. Ge et al., 2012.

To sum up, $SDNR_{NO3}$ is always higher than $SDNR_{NO2}$ for specific carbon sources such as acetate, which causes PDN. While denitrification from nitrite saves 25% of oxygen and 40% of external carbon source, PDN can be a much better option since higher $SDNR_{NO3}$ can be obtained, 45% and 80% of external carbon source can be saved.

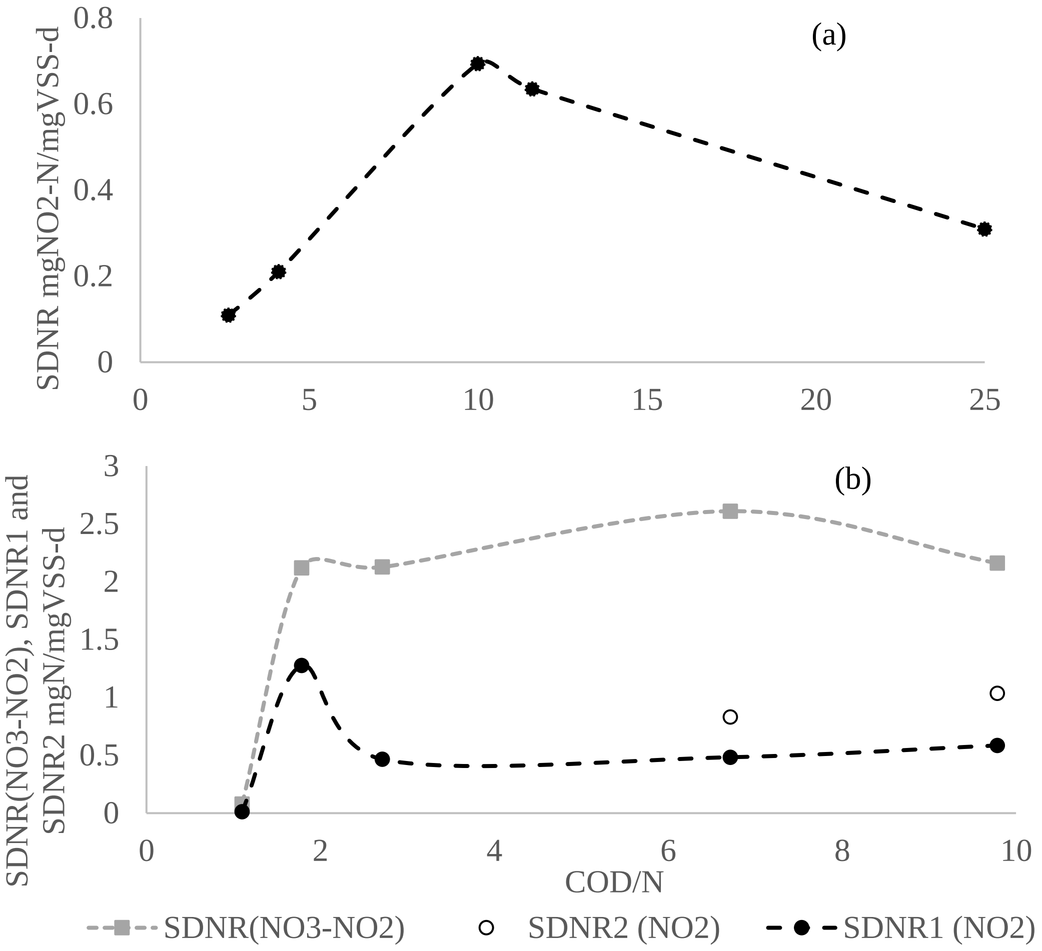

Figure 5. 7 Relationship between COD/N and SDNR: (a) SBR1 and (b) SBR2

Table 5. 7 Effect COD/N on SDNR IN SBR1

SBR1 (nitrite)						
NO2-Ni, mg/L	**NO2-Nf, mg/L**	**CODi, mg/L**	**CODf, mg/L**	**SDNR, mgN/mgVSS-d**	**COD/N**	**VSS, mg/L**
11.2	2.7	29	3	0.11	2.6	1465
12	0	50	2	0.21	4.2	4745
9.6	0	111	46	0.64	11.6	1805
11.1	0.3	112	45	0.69	10.1	1700
12	0	300	241	0.31	25.0	1845

5.3.5.3 Nitrite half-saturation concentration and biomass specific growth rate

Half saturation concentration for nitrite and nitrate is an important parameter because a high half-saturation concentration will have a negative impact on the SDNR (SDNR is reduced). Therefore, K_N is a useful tool to estimate the SDNR during the design of a wastewater treatment facility. Half saturation concentration for nitrate with acetate was previously studied by Cao et al., 2017 and was found to be 6.9 mgN/L; therefore, it wasn't studied in this work. The Monod equation (Equation 5-6) was used to determine other kinetic parameters such as $\boldsymbol{\mu}_{hmax}$ and K_{NO2} (Eddy et al., 2014). This analysis was conducted in 5 batches with biomass withdrawn from SBR1 using sodium acetate as a sole carbon source and nitrite as an electron acceptor. The analysis was based on the specific denitrification and COD specific consumption rates in each batch.

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\mu_{hmax} * S_S}{Y_h\,(K_S+S_S)}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right)\left(\frac{K_O'}{K_O'+S_O}\right)(\eta) \qquad (6)$$

Where K_{NO2} is the half-saturation concentration (mg/L), K_S is the half-saturation concentration for the carbon source-acetate (mgCOD/L), $\boldsymbol{\mu}_{hmax}$ is biomass specific growth rate (d^{-1}), K_O' is DO denitrification-inhibition concentration (mg/L), and S_s, S_{NO2}, S_o are the concentrations of COD (mg/L), NO_2-N (mg/L), and DO (mg/L), respectively. Equation 5-6 can be simplified to Equation 5-7 because DO is close to zero in each batch, and the heterotrophic biomass (denitrifiers) is completely anoxic ($\eta = 1, DO \simeq 0.0$). Additionally, the unlimited COD-substrate concentration for acetate was used (100 mgCOD/L), is significantly higher than the reported Ks of 15-34 mg/L for acetate (Stensel et al., 1973; Torres Ortiz, 2013), and was reported as 38.1 mg/L by (Cherchi et al., 2009). Therefore, equation 6 can be reduced to equation 7.

$$SDNR = \left(\frac{1-1.42Y_h}{1.71}\right)\left[\frac{\mu_{hmax} * S_S}{Y_h\,(K_S+S_S)}\right]\left(\frac{S_{NO2}}{K_{NO2}+S_{NO2}}\right) = \frac{\mu_{hmax}}{Y_N}\;\frac{S_{NO2}}{K_{NO2}+S_{NO2}} \qquad (5\text{-}7)$$

However, $\boldsymbol{\mu}_{hmax}$ can be directly related to Y_N (1.12 mgVSS/mgNO_2-N), which can be calculated from equation 5-8 (Badia et al., 2019) and SDNR (maximum SDNR) of 0.69 mgNO_2-N/mgVSS/d, according to the relation of $SDNR_{max}$=μ_{max}/Y_N, hence, $\boldsymbol{\mu}_{hmax}$ was obtained as 0.77 d^{-1} for acetate and nitrite , which is higher than the previously reported values of 0.121 d^{-1} for nitrite and o.434 d^{-1} for nitrate with acetate (Cherchi et al., 2009), and reported 1.26 d^{-1} but with MWW-biomass (Badia et al., 2019). values which were estimated from denitrification with nitrate, i.e., 0.5 d^{-1} (Yalda Mokhayeri et al., 2008), it is also close to the estimated 1.3 d^{-1} (Dold et al., 2008), and 1.25 d^{-1} (Nichols et al., 2007). The obtained $\boldsymbol{\mu}_{hmax}$ is in accordance with the reported values (0.5 - 4 d^{-1}) for different external carbon sources (deBarbadillo et al., 2008; Dold et al., 2008; Kornaros et al., 1996; Stensel et al., 1973; Q. Yang et al., 2007).

$$Y_N = \left(\frac{1.71\, Y_H}{1 - 1.42\; Y_H} \right) \qquad (5\text{-}8)$$

Equation 5-7 is rearranged to get equation 5-9 with the replacement Y_H by Y_N, the Lineweaver-Burk plot was drawn (Equation 5-9, and Figure 5-8).

$$\frac{1}{SDNR} = \frac{Y_N}{\mu_{hmax}}\, K_N \left(\frac{1}{NO2-N}\right) + \frac{Y_N}{\mu_{hmax}} \qquad (5\text{-}9)$$

Where $SDNR_{max}$ is substituted by $\boldsymbol{\mu}_{hmax}/Y_N$, K_N was determined as 3.17 mg/L. This explains why complete removal of NO_2-N was not possible in the SBR1 during the experimental period, and this was due to the switching function of NO_2-N/(K_N+NO_2-N), effluent NO_2-N is 3.5 mg/L for the hole period (126 days) (Table 5-2, shows 40 days average). The reported half-saturation coefficients (K_{NO2} and K_{NO3}) are 0.9 mgNO_2-N/L and 1.4 mgNO_3-N/L for acetate (Dosta et al., 2007), (Almeida, Júlio, et al., 1995) found K_{NO2} and K_{NO3} less than 0.3mgN/L, and 0.28 mg NO_2-N/L and 0.77 mgNO_3-N/L for L-glutamic acid (Kornaros et al., 1996). Moreover, (Badia et al., 2019) determined the K_{NO2} value of 4.2 mgN/L for MWW and 9.2mgN/L for methanol (Badia et al., 2020), (Her & Huang, 1995a) reported K_{NO2} of 10.9 mg/L and K_{NO3} of 14.3 mg/L with methanol. This indicates that K_{NO2} and K_{NO3} are mainly affected by other factors such as initial electron donor and electron acceptor concentrations, electron donor type, temperature, low pH, biomass structure, and COD/N ratio. The high half-saturation concentration implies that the SDNR will be highly reduced (50%) when nitrite concentration is equal to the K_N, and the final nitrite effluent needs a longer time to reach to zero.

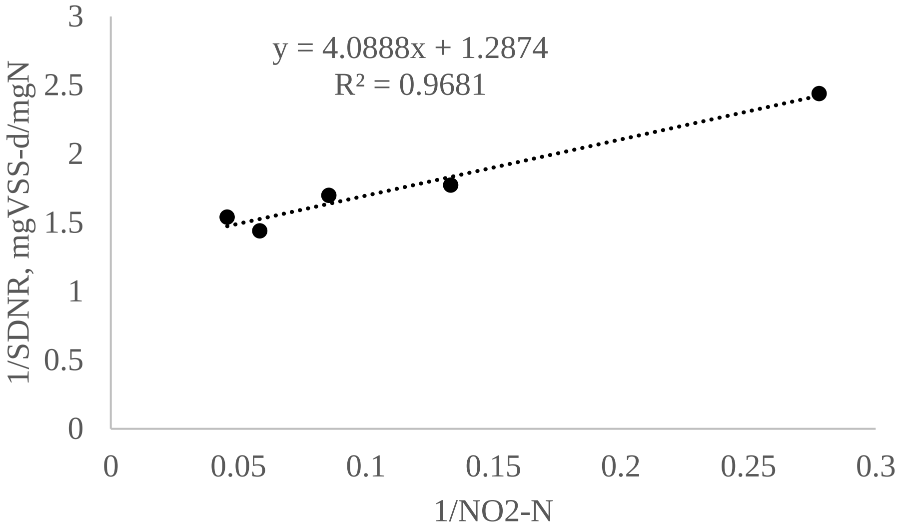

Figure 5. 8 Lineweaver-Burk plot for kinetic parameters determinations

5.4 Conclusion

The partial denitrification process is used to partially denitrify nitrate and maximize nitrite accumulation that will be used potentially as an electron acceptor in a downstream anammox process. In this process, the PDN, more specifically, the nitrite accumulation is the limiting step. Hence the fundamentals behind nitrite denitrification versus nitrite accumulation over nitrate denitrification need to be well understood. The third comprehensive study characterized nitrite versus nitrate denitrification under lower COD/N ratios using acetate as a carbon source. The study identified that:

- nitrate SDNR ($SDNR_{NO3}$, 2.67 mgN/mgVSS-d) was much higher than nitrite SDNR ($SDNR_{NO2,}$ 0.69 mgN/mgVSS-d). Under carbon limiting condition, thermodynamically, nitrite denitrification is favoured over nitrate denitrification. Interestingly the current study shows this does not apply, which explained the nitrite accumulation observed in partial denitrification processes.

- During nitrate denitrification, nitrite accumulation of 79% to 100% was observed at COD/N 2.6 to 9.8, contradictory to previous research that suggested that nitrite accumulated only at a lower COD/N ratio. A COD/N of 2.7 was found to be the minimum COD/NO_3-N ratio to accumulate more than 79% of nitrite; however, at higher COD/N ratios, a strategy to maintain the accumulated nitrite is required.
- In the partial denitrification process, two nitrite SDNRs were identified, one before nitrate exhaustion ($SDNR_{NO2}$-1) and the second one after exhaustion ($SDNR_{NO2}$-2). It was found that the $SDNR_{NO2}$-2 appeared only after nitrate concentrations dipped below the nitrate half-saturation coefficient (K_{NO3}). Moreover, the maximum $SDNR_{NO2}$-1 (1.27mgN/mgVSS-d) occurred at $SDNR_{NO2}$-2 of 0 and was much higher than all nitrite SDNRs observed in this study, which could be attributed to enhanced rate by the nitrite denitrifiers to overcome the nitrite accumulation stress during PDN.
- The taxonomic analysis clearly showed that there is no phylum and class level difference in denitrifiers grown in nitrate and nitrite reactors. However, genus and species level differences were observed with respect to composition and diversity. The nitrate reactor consisted of a more diverse community, the *Zoogloea* (RA of 42%) and *Thauera* genus (RA-10%), further causing the imbalance of NiR and NaR reductaseenzymes, hence different reaction rates. The microbiology data supported the observed difference in the SDNRs, the myth behind partial denitrification and potentially the lack of agreement with thermodynamics principles.

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Chapter 6

Conclusion and recommendations

6.1 Conclusion

Denitrification from nitrite is a new trend, with still many challenges to overcome to achieve nitrite shunt and partial denitrification. Firstly, a comprehensive study on denitrification from nitrite with real municipal wastewater as carbon sources was conducted at varying initial COD/N ratios. The specific denitrification rate (SDNR) was directly related to the COD/N ratio, and a maximum SDNR of 0.07 (for wastewater) and 0.4 mgNO_2-N/mgVSS/d for acetate occurred at COD/N ratios of 6 and 13, respectively. At higher COD/N ratios, SDNR decreased. The yield coefficients were found to be 0.33 mgVSS/mgTCOD for municipal wastewater and 0.51 mgVSS/mgCOD for acetate. Predictive models were developed to determine SDNR as a function of initial COD/N ratio and F/M ratio.

In relation to the carbon source, and due to the low carbon to nitrogen ratio in real wastewater (municipal wastewater-MWW), the denitrification process in full-scale wastewater treatment plants employs external carbon sources to minimize total nitrogen effluent. A comprehensive study on denitrification from nitrite with a mixture of real municipal wastewater and methanol as carbon sources was conducted at varying initial COD/N ratios. The study showed kinetic parameters including (i) the half-saturation nitrite denitrification coefficient (9.5 mg NO_2-N/L), (ii) denitrifier yield (0.39 mgVSS/mgTCOD) and (iii) maximum specific growth rate (1.4 d^{-1}) were higher than the ones obtained during the denitrification process that uses municipal wastewater as a sole carbon source. The specific denitrification rate (SDNR) was directly related to COD/N ratio, and a maximum SDNR of 0.5 (for MWW+methanol) and 0.47 mgNO_2-N/mgVSS/d (MWW) was found at COD/N of 5.9, much higher than the SDNR by using municipal wastewater as a sole carbon source. Comparatively, upon using methanol as a sole carbon source, a lower SDNR (0.38 mgNO_2-N/mgVSS/d) was achieved, which implies that when methanol is supplemented with the MWW, a synergic effect occurs which enhances the SDNR. The higher SDNR rates could be translated into 28% and 30% capital and operating cost savings, respectively; hence the addition of methanol as a complementary carbon source for nitrite denitrification can be considered as a mainstream process even for conditions carbon is not limiting in the MWW.

The partial denitrification process is used to partially denitrify nitrate and maximize nitrite accumulation that will be used potentially as an electron acceptor in a downstream anammox process. In this process, the PDN, more specifically, the nitrite accumulation is the limiting step. Hence the fundamentals behind nitrite denitrification versus nitrite accumulation over nitrate denitrification need to be well understood. The third comprehensive study characterized nitrite versus nitrate denitrification under lower COD/N ratios using acetate as a carbon source. The study identified that:

- nitrate SDNR ($SDNR_{NO3}$, 2.67 mgN/mgVSS-d) was much higher than nitrite SDNR ($SDNR_{NO2,}$ 0.69 mgN/mgVSS-d). Under carbon limiting condition, thermodynamically, nitrite denitrification is favoured over nitrate denitrification. Interestingly the current study showed that this does not apply, which explained the nitrite accumulation observed in partial denitrification processes.
- During nitrate denitrification, nitrite accumulation of 79% to 100% was observed at COD/N 2.6 to 9.8, contradictory to previous research that suggested that nitrite accumulated only at a lower COD/N ratio. A COD/N of 2.7 was found to be the minimum COD/NO_3-N ratio to accumulate more than 79% of nitrite; however, at higher COD/N ratios, a strategy to maintain the accumulated nitrite is required.
- In the partial denitrification process, two nitrite SDNRs were identified, one before nitrate exhaustion ($SDNR_{NO2}$-1) and the second one after nitrate exhaustion ($SDNR_{NO2}$-2). It was found that the $SDNR_{NO2}$-2 appeared only after nitrate concentrations dipped below the nitrate half-saturation coefficient (K_{NO3}). Moreover, the maximum $SDNR_{NO2}$-1 (1.27mgN/mgVSS-d) occurred at $SDNR_{NO2}$-2 of 0 and was much higher than all nitrite SDNRs observed in this study, which could be attributed to enhanced rate by the nitrite denitrifiers to overcome the nitrite accumulation stress during PDN.
- The taxonomic analysis clearly showed that there is no phylum and class level difference in denitrifiers grown in nitrate and nitrite reactors. However, genus and species level differences were observed with respect to composition and diversity. The nitrate reactor consisted of a more diverse community, the *zoogloea* (RA of 42%) and *thauera* genus (RA-10%), further causing the imbalance of NiR and NaR reductaseenzymes, hence different reaction rates. The microbiology data supported the observed difference in the SDNRs, the myth behind partial denitrification and potentially the lack of agreement with thermodynamics principles.

6.2 Recommendations

According to the above research conclusions, the following are recommended future work in the research area:

1- Study the effect of various carbon source on the biomass acclimatized to nitrite and MWW, and model the DO effect on denitrification from nitrite (DO inhibition concentration)
2- Study the effect of various supplementary carbon source other than methanol in denitrification from nitrite, and the effect of this carbon source in denitrification from nitrite
3- PDN study to control accumulated nitrite at high COD/N
4- Study PDN with various MWW and various carbon sources
5- Study the microbial shift in the acclimatized biomass (i) during long term study for the nitrite and nitrate bioreactors with various carbon sources, (ii)when nitrite is replaced by nitrate, and nitrate is replaced by nitrite (in reverse).
6- Study PDN-anammox in two bio reactors

Appendices

Appendix for chapter 4 (Supporting Document)

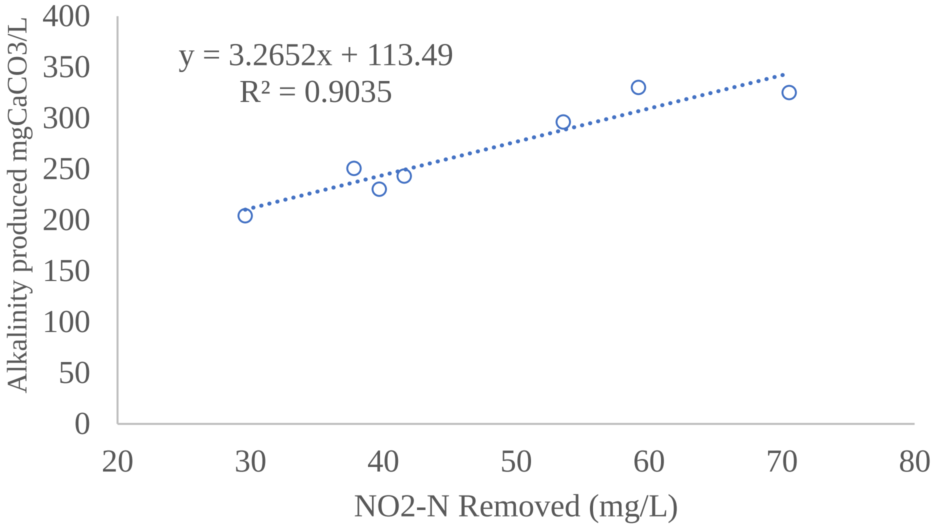


Figure SD 4. 1 Relation between the alkalinity produced and nitrogen removed in the SBR (influent and effluent data, room temp. 25^0)

Table SD 4. 1 Pseudo steady-state influent and effluent characteristics (10 samples)

Parameter	Influent (mg/L)	Effluent (mg/L)
	Average±SD	**Average**±SD
TCOD	571±171	188±88
SCOD	214±120	92±77
Methanol	141±112	
NO_2-N	48±16	9.2±16
Total N	95±17	27±18
Soluble N	72±16	23±18
Total P	6.9±3.2	1.8±0.9
Soluble P	1.3±0.5	0.4±0.38
NH_3-N	21.2±3	11.5±2.6
Alkalinity ($CaCO_3$/L)	228±41	487±86
TSS	340±80	96±24
VSS	250±76	62±14
Bioreactor parameters		
MLSS	4439±190	
MLVSS	3392±125	
SRT	17.4	

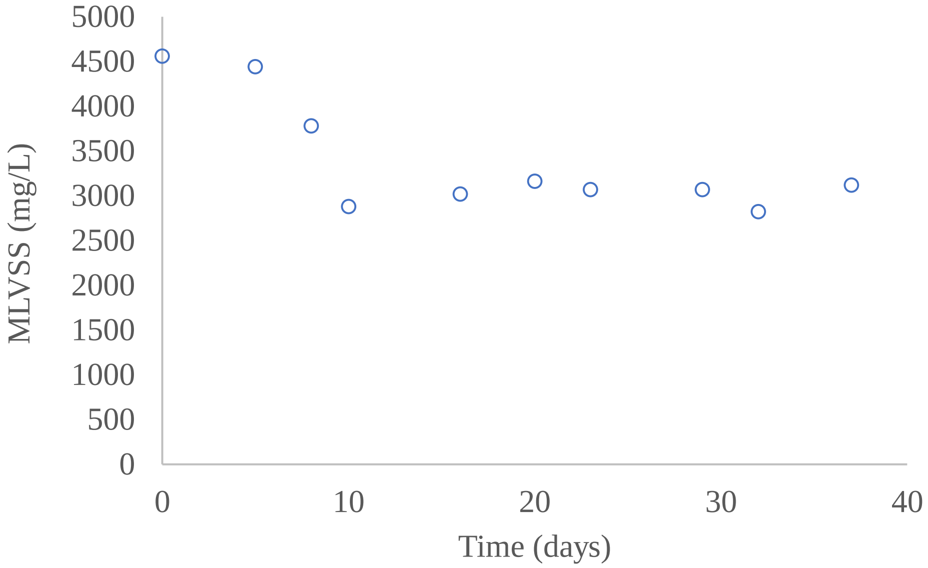


Figure SD 4. 2 MLSS concentration in the SBR during steady state operation (room temp. 25^0)

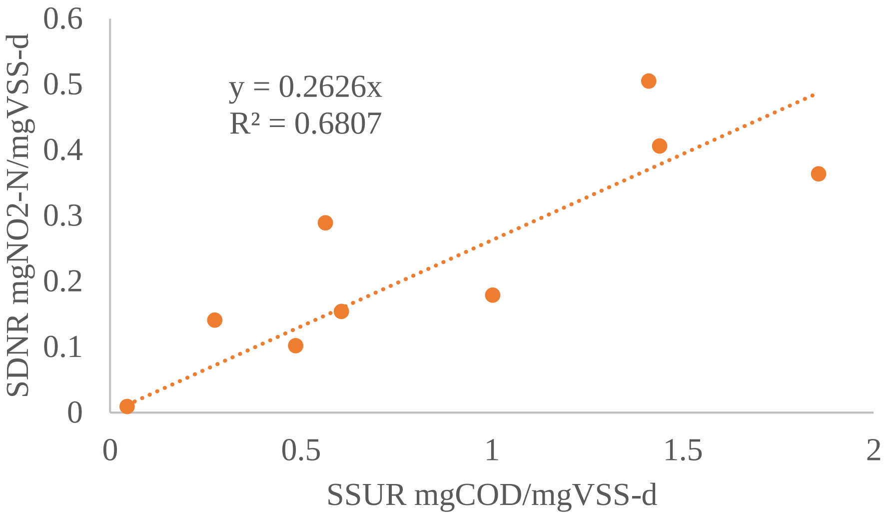
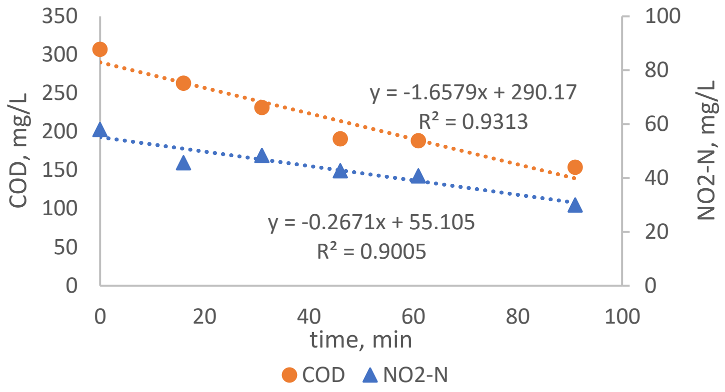
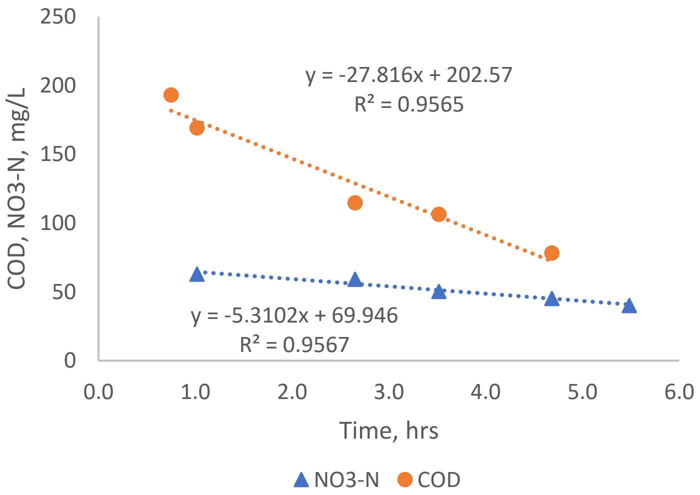


Figure SD 4. 3 Relation between SDNR and SSUR to estimate the yield coefficient-Post denitrification (kinetic study in the post-denitrification SBR, room temp. 25^0)



(a)



(b)

Figure SD 4. 4 Denitrification rates for (a) nitrite and (b) nitrate with MWW as a sole carbon source (batch kinetics, room temp. 25^0)

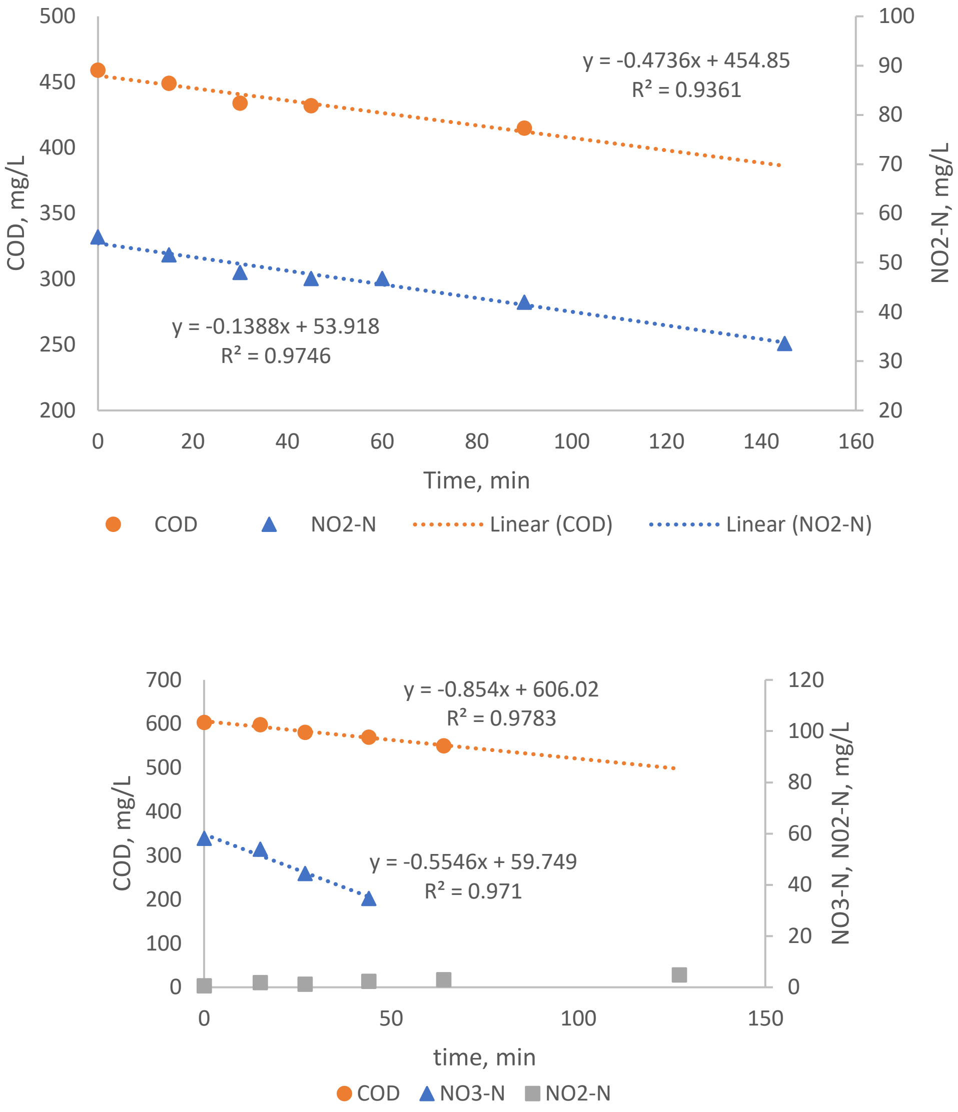


Figure SD 4. 5 Denitrification rates for (a) nitrite and (b) nitrate with methanol as a sole carbon source (batch kinetics, room temp. 25^0)

Table SD4. 2 Pre and post denitrification cost calculation

Pre-denitrification:

Step-1 Assume

- The bioreactor volume ($V1=1000\ m^3$)
- MLVSS=3000 mg/L

Step-2 Calculate the nitrite mass removed per day under pre-denitrification scenario

- Consider maximum nitrite SDNR of 0.52 mgN/mgVSS-d (based on this study, carbon source MWW+methanol)
- NO_2-N = 0.52 mgNO_2-N/mgVSS-d x 3000 mgVSS/L x 1000 m^3 = 1560 gNO_2-N/d

Step -3 Estimate the equivalent post denitrification tank volume

- Consider maximum nitrite SDNR of 0.38 mgNO_2-N/mgVSS-d (based on this study, Carbon source – methanol only)
- V2 = 1560 gNO_2-N/(0.38 mgNO_2-N/mgVSS-d X 3 gVSS)= 1368.42 m^3

Step -4 Calculate percent saving

% Savings (equipment, power and volume) = 100 x (1368.42 -1000)/1000 = 36.8%

Appendix for chapter 5 (Supporting Document)

Figure SD 5.1 a

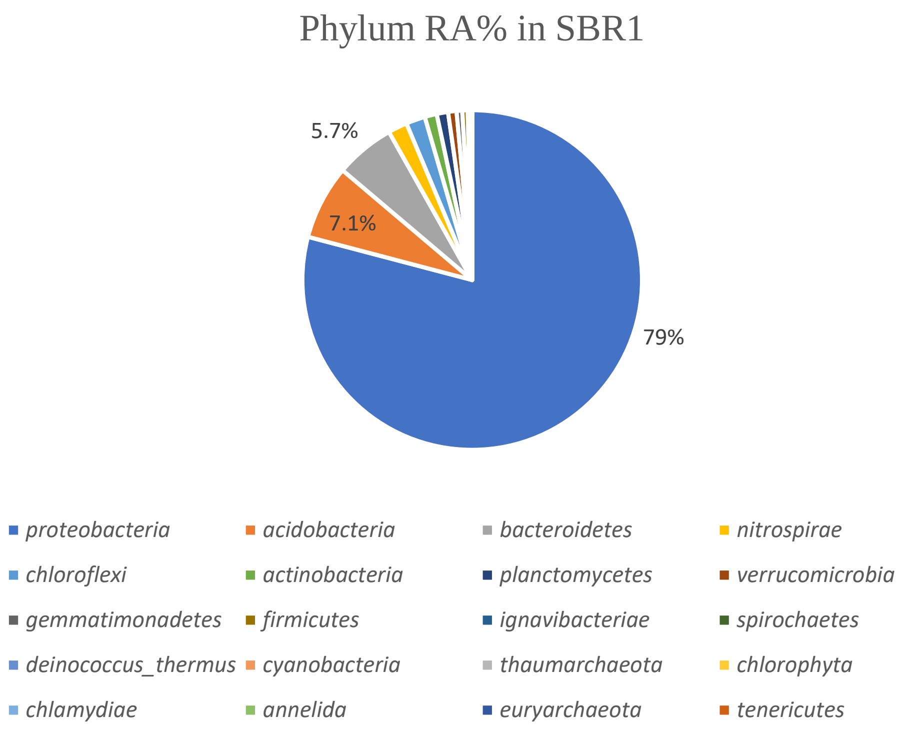


Figure SD 5.1 b

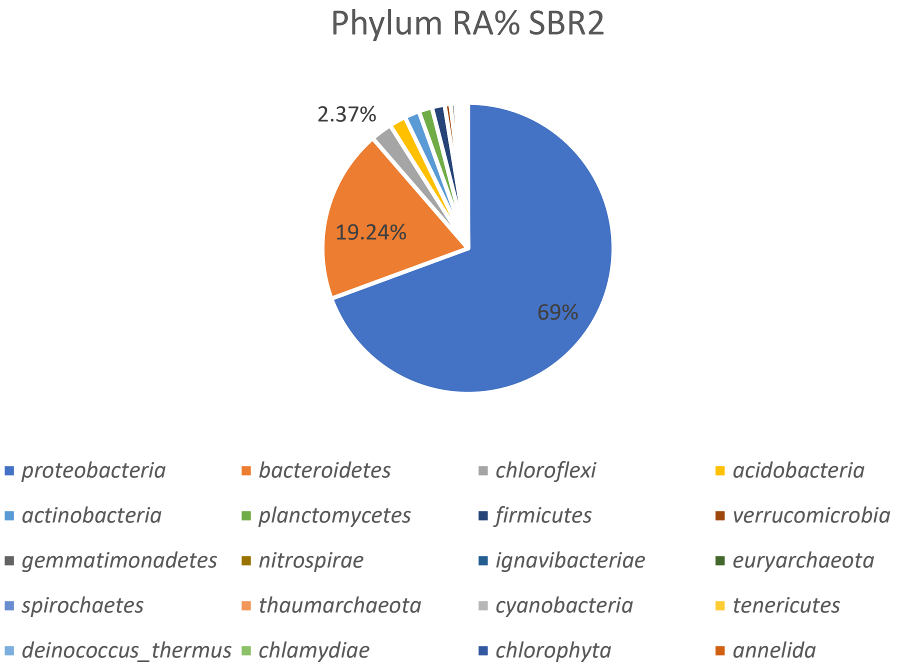


Figure SD 5. 1 Microbial classification on phylum lever (a) SBR1, (b) SBR2

Figure SD 5.2 a

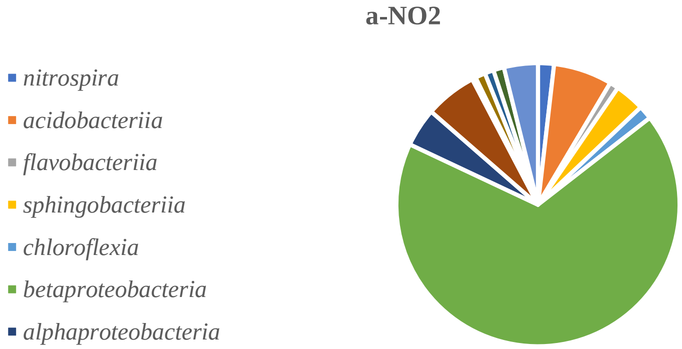


Figure SD 5.2 b

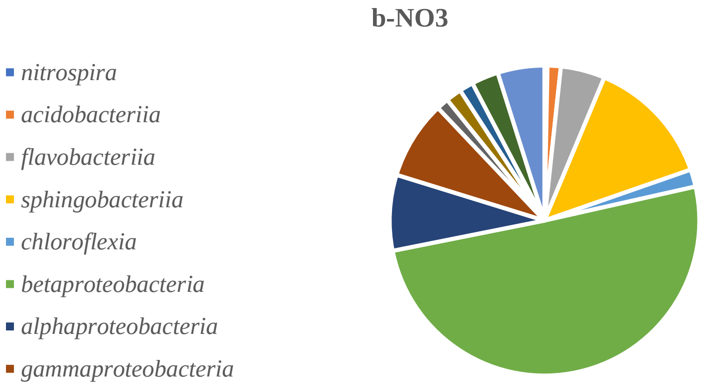


Figure SD 5. 2 Microbial classification on class lever (a) SBR1, (b) SBR2

Figure SD 5.3a (SBR1)

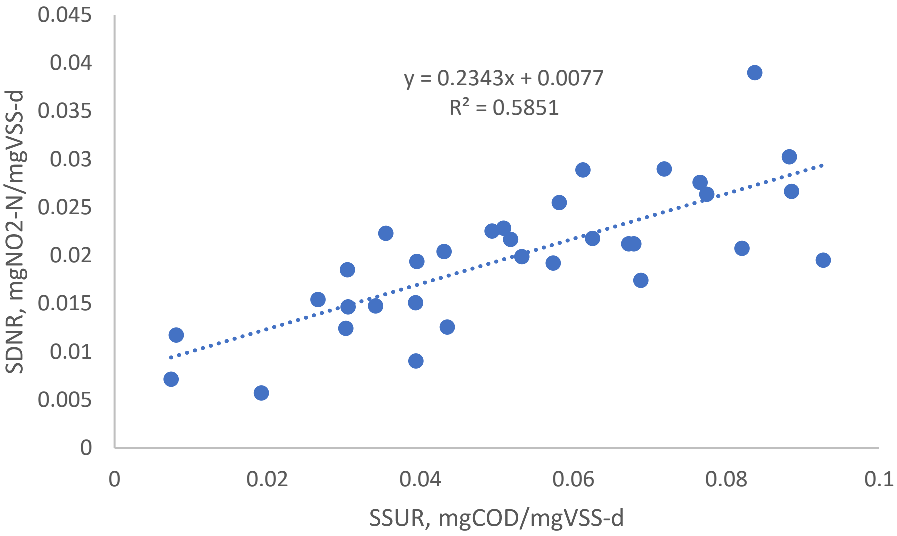


Figure SD 5-3b

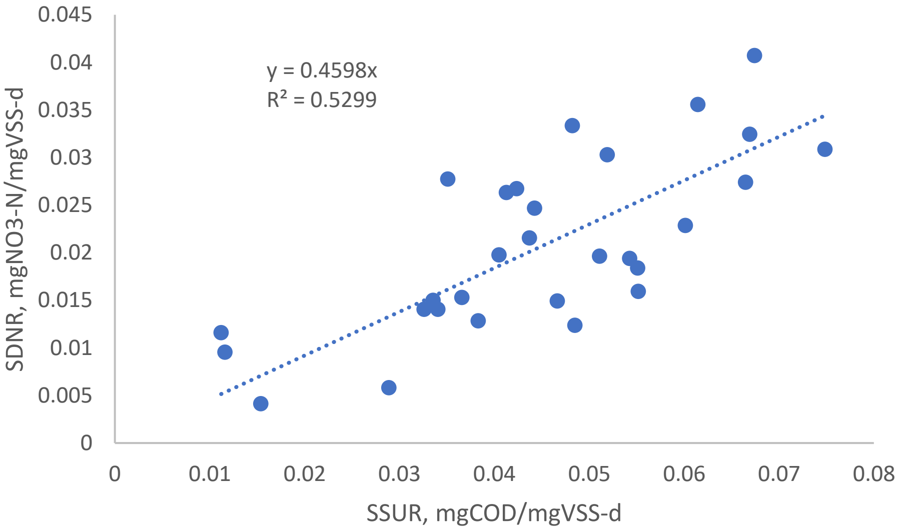


Figure SD 5. 3 Yield determination in according to equations 2-5:(a) SBR1, (b) SBR2

Curriculum Vitae

PROFESSIONAL PROFILE

Professional engineer (PEng) and project manager with significant experience in the design, installation, and execution of water and wastewater including process and hydraulic design, Equipment sizing and selection, preparing proposals for sales, Equipment manufacturing, design of storm drainage management systems, water structures, and water resource management.

Experienced in project tendering, procurement, negotiation, Operation and Maintenance (O&M), and subsurface contamination bioremediation.

SUMMARY OF KEY EXPERIENCE

- Design and construction of water and wastewater treatment plants, sewerage networks, water supply works and all water and wastewater related works
- Expertise in Environmental Engineering, water and wastewater process and hydraulic, mechanical equipment sizing and selection, preparing proposals for sales, manufacturing, design of irrigation systems, storm drainage management, water resource management, water structure, plumbing, and desalination plants such as R.O. (Reverse osmoses desalination) plants and softeners
- Strengths in management and coordination, and project follow-up with customers, government authorities and staff engineers
- Successful in managing and leading engineering teams including design engineers and project engineers for water and wastewater treatment plants (process, hydraulics, contracting, tendering, proposals, equipment selection, installation, customer service and maintenance, cost control, workshop and fabrication, procurement and negotiation with both clients and suppliers)
- Work flow management: development, improvement and implementation of process-oriented management systems

PROFESSIONAL EXPERIENCE (Projects are shown in the appendix)

Project Manager *July 2018-present*
JACOBS (CH2M)
Project manager for 200,000 m3/d wastewater treatment plant (ICEAS process)

Internship
The City of Toronto *Aug.2017-Dec.2017*
Studying the performance of Highland Creek wastewater treatment plant and optimizing the final effluent quality (professional in using BioWin).

Teaching and Research Assistant *2014-Jun 2018*
University of Western Ontario
Doing research for BNR system and teaching Municipal engineering design, Environmental engineering, water quality management, and wastewater treatment. I am evaluating denitrification from nitrite with Municipal wastewater treatment and synthetic wastewater as carbon sources.

Wastewater Design Engineer
Environmental Engineering services, Waterloo, Ontario, Canada *2014 - 2015*

I was responsible for reviewing the submitted, Engineering drawings and technical reports associated with the new sub-division applications, Environmental Compliance Approval applications, site plans and the Infrastructure capital projects, to confirm the compliance with the City design specification & standards; as well to ensure the compliance with the Official plan, Zoning By-Law and other applicable By-Laws and Acts; In addition to Performing the calculations and analysis to ensure the safety and sufficiency of the designed elements of the Sanitary system, Storm water system and retaining walls

Engineering Manager
Al Arrab (ACC) General Contracting, Water Section, Riyadh, Saudi Arabia. *2010 - 2013*
Engineering Manager responsible for major projects including Water and Wastewater treatment plants including pumping station projects with a value of $ 500,000,000 million USD. Performing and reviewing hydraulic and process design for different process-systems for water and wastewater treatment plants including infrastructure works. Managed ongoing projects, including procurement of electromechanical equipment, supervision of the project execution and acted as team leader for project and engineering staff. Monitoring the site work execution including cost to prevent project going over budget. Identifying, analyzing and developing responses to commercial risks, including providing advice on contractual issues and claims, and ensuring implementation of health and safety regulations.

Engineering Manager *2008 - 2010*
A&F ARASHED WATER AND ENVIRONMENT, Riyadh, Saudi Arabia
Handled all operational activities, planned and developed business strategies and policies; engineering and project management, procurement, customer services, execution of works at site, electrical works, sales and marketing, cost control and workshop activities.

Operations Director *2007 - 2008*
WASHNAH Water Equipment, Jeddah, Saudi Arabia
Managed company’s operational activities comprising engineering and project management, procurement, customer services, execution of works at site, electrical works, cost control and workshop activities

Projects & Engineering Department Manager *2001 - 2007*
Saudi Berkefeld Co. Ltd.(WETICO), Riyadh, Saudi Arabia
Managed environmental and project engineering staff (scope: large scale water and wastewater treatment plants) from contract review to project hand over, handled process and hydraulic design, equipment selection, and technical submittals. Provided technical support to projects under execution, installation of equipment, and negotiations with clients and equipment suppliers

Senior Environmental Engineer *1999 - 2001*
Reza Investment Co. Ltd., Environmental Engineering Division, Jeddah, Saudi Arabia
Process and hydraulic design of water and wastewater treatment plants. Procurement and installation of equipment. Preparation of proposals for clients for new projects, commissioning, operation and maintenance of water and wastewater treatment plants.

Project Manager *1995 - 1999*
Saudconsult, Saudi Consulting Services, Riyadh, Saudi Arabia
Design storm drainage and management systems, sewers networks, pumping stations, water and wastewater treatment plants, irrigation systems and supervise infrastructure

Senior Sanitary Engineer *1993 - 1995*
Riyadh Water and Sewerage Authority, Riyadh, Saudi Arabia
Design of wastewater treatment plants, design of sewerage network systems, design of water supply networks, design of pumping stations, preparing tender documents for projects

Sanitary Engineer *1990 - 1993*
General Organization Greater Cairo Sanitary Drainage Utility, (Supervision Sector), Cairo, Egypt
Supervision of construction of pumping stations, sewerage networks systems and wastewater treatment plants

Sanitary Engineer *1988-1990*
General Organization Greater Cairo Sanitary Drainage Utility GOSD, Design Sector, Cairo, Egypt
Design of wastewater treatment plants, sewerage networks systems and pumping stations
(structure, hydraulic and process design, and survey and leveling of pipe line routes).

EDUCATION AND PROFESSIONAL DEVELOPMENT

P. Eng. Licensure 2015
Professional Engineers of Ontario
Ph.D. Civil and Environmental Engineering University of Western Ontario Water/Wastewater Engineering *Expected 2020*
MEng- Master of Civil and Environmental Engineering University of Western Ontario Environmental/ Water Engineering *2014*
M.ESc. Civil Engineering Sanitary (Environmental) Engineering *1992*
Faculty of Engineering, Cairo University Cairo, Egypt
Evaluated by World Education Services Canada as equivalent to Canadian Master's degree.
Postgrad. Diploma in Wastewater and Irrigation Loughborough University of Technology, WEDC England *1992*
B.Sc. Civil Engineering Specialization in water and wastewater engineering *1987*

Faculty of Engineering, Cairo University Cairo, Egypt
Evaluated by World Education Services Canada as equivalent to Bachelor's degree (four years).
Publications:
Badia, A.; Kim, M.; Nakhla, G.; Ray, M.; 2018. "Effect of COD/N ratio on the denitrification from Nitrite". Wat Environ Res.
Eini, E.; Badia, A.; Susan Atlin, S.; Anthony Pigaidoulis; A. 2018. **"**A Window into the Highland Creek WWTP: Review of liquid stream performance". Water Environmental Association of Ontario
Badia, A.; Kim, M.; Dagnew, M. (2020). "Denitrification of nitrite using methanol as a complementary carbon source". Under review
Badia, A., Pan, Y., Dagnew, M. (2020). "Effect of low COD/N ratio on denitrification from nitrite and nitrate: the case for complete versus partial nitrite denitrification". Under preparation.
Memberships
PEO, ASCE, WEF, WEAO
References
Are available upon request
Appendix
Projects (ACC): Design, execution, procurement, commissioning and handover of the following:
Aug.2010-Jul.2013

- “Al-Kharj road STP phase-3 with average flow capacity of 200,000 m3/day and 1.5 peak factor”. “Sludge dewatering project centrifuge decanters 8000 m3/day and 3% solids concentration”.
- “Jizan Water Treatment Plant- RO system with a capacity of 20,000 m3/day”
- “Jizan housing-1 STP 3000 m3/day and Jizan housing -2 STP 4000 m3/day (SBR system)”.
- “Alrugi, 2000 m3/day MBR-BNR system”.
- “Shenan STP(rural) 6000 m3/day (carousel ditch –BNR system)”.

Projects A&F ARASHED: Design, execution, procurement, commissioning and handover of the following *Jul.2008-Jul.2010*

- “Qassim 15 RO Brackish water plants ranging from 500m3/day to 2000m3/day”
- “ Nestlah 1000 M3/day (brackish water R.O)”
- “King Faisl Hospital MBR with average flow of 8000 m3/day and peak flow of 16000 m3/day”.
- “Hail STP 75,000 m3/day (MBR system)”. Retrofit and upgrade of Hail Aerated lagoons of 6000 m3/d to 75,000 m3/d MBR (membrane bioreactor) technology. The scope of work was design and built. The process (bioreactor) design was based on a suspended-growth activated sludge. The effluent was extracted was via Toray flat plate membranes by suction pumps. The configuration consists of headworks (coarse screens, grit and grease removal units, 3.0 mm drum screens, equalization tank), membrane bioreactor (MBR) for biological nutrient reduction (BNR), followed by gas chlorination for disinfection. The effluent was disposed to Wadi, sludge centrifugal decanter was used to reach to 25% solids concentration.

Projects (**WASHNAH**): Design, execution, procurement, commissioning and handover of the following: *Jul.2007-Jun 2008*

- “Albatha 7200 m3/day Sea water R.O plant”**.**
- “Majmaa STP phase two with average flow of 5000 m3/day and peak flow of 10000 m3/day (carousel ditch –BNR system)”.

Projects(**Wetico**): *Jan. 2000- Jul. 2007*

Municipal Water and Waste water treatment plants

(design, execution, procurement, commissioning and handover), Oxidation Ditches-BNR, MBR, MBBR, SBR

- “Riyadh STP complete design proposal and final design with a capacity of 200,000 m^3/day average flow (300,000 peak flow) using oxidation ditch system”.
- “Majmaa & Shaqra STPs complete design proposal and final design with a capacity of 3,500 m^3/day each plant average flow (5,250 m3/day peak flow) using SBR system”.
- “Participation of Khobar, Dammam & Qattif tertiary treatment design proposa”l.
- “Arar, Najran, Rafha, and Hail STP design proposal complete design with a capacity of 12,000 m^3/day plant average flow (19,200 peak flow) using aerated lagoons systems”.
- “Onayza STP with a capacity of 25000m3/day average flow (37,500 m3/day peak flow)”.
- “Alghat and Zulfi STPs(rural) with a capacity of 3500m3/day average flow(5,250 peak flow)”.
- “Wadi Dawasir STP with a capacity of 25,500m3/day average flow (48,480 peak flow)”.
- “Domat Al Jandal STP(rural) with a capacity of 5,500m3/day average flow (11,000 peak flow)”.
- “Khamis Mushatt STP design proposal with a capacity of 40,000m3/day average flow (80,000 peak flow) using MLE-BNR systems”.
- “Rabigh and Khorsania MBR 4000 m3/ day , Aramco standard”
- “Najran STP 60,000 m3/day (MBR system)”. Retrofit and upgrade of Hail Aerated lagoons of 6000 m3/d to 60,000 m3/d MBR (membrane bioreactor) technology. The scope of work was design and built. The process (bioreactor) design was based on a suspended-growth activated sludge. The effluent was extracted was via Toray flat plate membranes by suction

pumps. The configuration consists of headworks (coarse screens, grit and grease removal units, 3.0 mm drum screens, equalization tank), membrane bioreactor (MBR) for biological nutrient reduction (BNR), followed by gas chlorination for disinfection. The effluent was disposed to Wadi, sludge centrifugal decanter was used to reach to 25% solids concentration before it's sold to farmers.

Industrial water and waste water treatment plants (**Wetico**)
(design, execution, procurement, commissioning and handover)

- Jun2003-Jul.2005"Jeddah Industrial city waste water treatment plant (BOT project); Oxidation ditch system with average capacity of 25000 m3/day and 4000 m3/day followed by MBR and RO for a capacity of 8000m3/day".
- "Obeikan, Saudi Paper Mills, and Waraq paper mill WWTP- 6000 m3/day".
- "Hadco and AKOLID slaughter houses WWTP – Average flow 1,800, Peak flow 2,400 m3/day".
- "Aujan Soft drinks WWTP – 1,000 m3/day".
- "Almarai MBR dairy, 1000 m3/day ".
- "Many others of small and package plants of industrial & domestic water and wastewater".

Projects (**Reza**):

- Design and review of Jizan sewage treatment plant with a capacity of 20,000 m³/day using Carousel® system,
- Design proposal of Yanbu sewage treatment plant with a capacity of 60,000 m³/day using single activated sludge system
- "Design and preparation of a proposal for Makkah Al-Mukkarmah (The Holy City) sewage treatment plant with a capacity of 250,000 m³/day using AIWPS® (Advanced Integrated Wastewater Pond System)".
- "Design and commissioning of Sludge handling and disposal for Riyadh STP (the system comprising of four thickeners and six belt presses)".
- "Design and Commissioning of Khamis Mushatt sewage treatment plant Carousel® system with a capacity of 22,500 m³/day)".

Projects (**Saudconsult**):

- *Jun 1997-Jul.1998"Design of Sultan City tertiary treatment plant (rural) to produce non-potable water and design water transmission line to provide irrigation water to Abha Airport and some suburbs around it".*
- "Resident Engineer for Khamis Mushatt wastewater treatment plant (22,500 m3/day capacity)".
- "Project Manager for storm management system and flood control for Buraidah City (construction of sewerage network – pipes diameter ranging from 200mm to1600mm and storm emergency lagoons)".
- "Design of Makkah oxidation ponds (90,000 m^3/day)".
- "Design and construction supervision for Khamis Mushatt STP, Abha STP and Bisha STP with capacities of 7500m3/day, 20,000m3/day (A2O Process), and 7500 m3/day respectively".
- "Follow up and design of Al-Ghat (rural) blending tanks; mixing of desalinated water with well water according to SASO Potable Water Regulations".
- "Design of Buraidah sewage treatment plant with a capacity of 150,000 m³/day".