T.C.
HARRAN UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

M.Sc. THESIS

INVESTIGATION OF PROKARYOTIC COMMUNITY STRUCTURE OF ANAMMOX REACTORS DURING START-UP

Ozan Kadir BEKMEZCİ

DEPARTMENT OF ENVIRONMENTAL ENGINEERING

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Doç. Dr. Sinan UYANIK danışmanlığında, Ozan Kadir BEKMEZCİ'nin hazırladığı "Investigation of Prokaryotic Community Structure of Anammox Reactors During Startup (Anammox Reaktörlerinin Başlangıç Aşmasındaki Prokaryotik Komünite Yapısının İncelenmesi)" konulu bu çalışma 08.08.2011 tarihinde aşağıdaki jüri tarafından Çevre Mühendisliği Anabilim Dalında Yüksek Lisans tezi olarak kabul edilmiştir.

Danışman : Doç. Dr. Sinan UYANIK

Üye : Yrd. Doç. Dr. Sevgi DEMİREL

Üye : Doç. Dr. Osman GÜLNAZ

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Bu Çalışma TÜBİTAK (Proje No: 108Y271) ve HÜBAK (Proje No: 1051) tarafından desteklenmiştir.

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ABSTRACT

M.Sc. Thesis

INVESTIGATION OF PROKARYOTIC COMMUNITY STRUCTURE OF ANAMMOX REACTORS DURING START-UP

Ozan Kadir BEKMEZCİ

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Graduate School of Natural and Applied Sciences
Department of Environmental Engineering

Supervisor: Assoc. Prof. Dr. Sinan UYANIK
Year: 2011, Page: 53

In order to study reactor performance and microbial community structure during anammox start-up, a sequencing batch reactor was set up. The reactor was seeded with 1.75 L denitrification sludge and 5 mL enriched anammox culture. It was operated under different conditions for 192 days for anammox enrichment, which was followed by a period of irregular feeding and maintenance.

Studies on the reactor's performance showed that simultaneous increase of NO_2^- and NH_4^+ concentrations by 25% in the feed was tolerated immediately when steady state had been achieved or in less than 20 days if the reactor had recently reached to its pick performance, that short term reactor shutdown (up to 18 days) was well tolerated after steady state had been established, that the long term irregularity in feeding frequency did not affect performance of the reactor.

Based on the DGGE images, it was assumed that increasing NO_2^- and NH_4^+ concentrations accompanied by increasing nitrogen loading rate altered prokaryotic community structure, that short term (up to 18 days) system shutdown did not affect the prokaryotic community structure, and that long term irregular feeding (>70 days) altered the prokaryotic community structure.

KEYWORDS: Anammox, enrichment, prokaryotic community structure, DGGE

ÖZ

Yüksek Lisans Tezi

ANAMMOX REAKTÖRLERİNİN BAŞLANGIÇ AŞMASINDAKİ PROKARYOTİK KOMÜNİTE YAPISININ İNCELENMESİ

Ozan Kadir BEKMEZCİ

Harran Üniversitesi
Fen Bilimleri Enstitüsü
Çevre Mühendisliği Anabilim Dalı

Danışman: Doç. Dr. Sinan UYANIK
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Anammox başlangıç evresinde reaktör performansı ve mikrobik komünite yapısının incelenmesi için, bir sıralı kesikli reaktör kurulmuştur. Reaktöre 1.75 L denitrifikasyon çamuru konularak 5 mL zenginleştirilmiş anammox kültürü aşılanmıştır. Anammox zenginleştirmesi için 192 gün boyunca farklı koşullarda işletimini düzensiz beslemeli ve işletimli bir dönem takip etmiştir.

Reaktör performansı üzerine çalışmalar göstermiştir ki; reaktör kararlı duruma ulaşmışsa veya en yüksek performansına ulaştıktan sonraki 20 gün içerisinde ise, besindeki NO_2^- ve NH_4^+'nın eş zamanlı olarak %25 arttırılması, anında tolere edilmiştir; reaktörün kısa bir süre için durdurulması (18 güne kadar), kararlı durum oluştuktan sonra kolayca tolere edilmiştir; besleme sıklığındaki uzun süreli düzensizlikler reaktör performansını etkilememiştir.

DGGE görüntülerinden anlaşılmıştır ki; artan azot yüküyle NO_2^- ve NH_4^+ konsantrasyonları, prokaryotik komünite yapısını değiştirmiştir; sistemin kısa süreyle durdurulması (18 güne kadar), prokaryotik komünite yapısını etkilememiştir; ve uzun süreli düzensiz besleme (>70 gün), prokaryotik komünite yapısını değiştirmiştir.

ANAHTAR KELİMELER: Anammox, zenginleştirme, prokaryotik komünite yapısı, DGGE.

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Ozan K. Bekmezci, 2011.

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ABBREVIATIONS

16S rRNA gene	DNA fragment coding 16S ribosomal RNA of prokaryotic small subunit
16S rDNA	DNA fragment coding 16S ribosomal RNA of prokaryotic small subunit
18S rRNA gene	DNA fragment coding 18S ribosomal RNA of eukaryotic small subunit
18S rDNA	DNA fragment coding 18S ribosomal RNA of eukaryotic small subunit
341F	A forward primer for 16S rRNA gene
907R	A reverse primer for 16S rRNA gene
Anammox	Anaerobic(anoxic) ammonium oxidation
APS	Ammonium persulphate
Ar/CO_2	Gas mixture of 95% of argon and 5% of CO_2
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DGG1	Denaturation Gradient Gel 1: 6% acrylamide gel with gradient from 40% to 70%
DGG2	Denaturation Gradient Gel 2: 6% acrylamide gel with gradient from 35% to 60%
DGGE	Denaturation Gradient Gel Electrophoresis
DO	Dissolved Oxygen
FISH	Florescent In Situ Hybridization
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
SBR	Sequencing Batch Reactor
TAE	Buffer solution containing tris base, acetic acid, and EDTA
TEMED	Tetramethylethylenediamine
U1F	A forward primer for both 16S rRNA gene and 18S rRNA gene
U1R	A reverse primer for both 16S rRNA gene and 18S rRNA gene
UV	Ultraviolet
VSS	Volatile(combustible) suspended solid

1. INTRODUCTION

Inevitably, the development of industrial society causes more and more wastes. Since the load of our wastes has long been over the capacity of natural compensation, the environmental awareness is increasing, day by day. Wastewaters have been receiving their share in this awareness. This trend not only resulted in the first version of wastewater treatment plants, but also leads the increasing importance and demand of tertiary treatment.

In traditional treatment plants with only up to secondary treatment, when nitrogen in the wastewater is significantly higher than what the microorganisms of secondary treatment require, the discharge is made without enough treatment, which causes serious environmental problems in receiving water bodies.

A major result of the problem is eutrophication. Excessive nitrogen and phosphorus concentrations are the sole chemical reasons for eutrophication in water bodies. Water clarity and food web structure is changed in eutrophic ecosystems. That can results in decrease in water quality, biodiversity, goods, and services (Beklioglu et al., 2003). Hence, protection of water resources becoming increasingly important; and therefore, nitrogen discharge limits are very tight for wastewaters which can cause eutrophication.

Biological nitrification-denitrification process is the most common, traditional, and even *de facto* standard method for nitrogen removal. However, due to operational costs of this process, **an**aerobic **amm**onium **ox**idation (anammox) processes have been developed. In contrast to traditional nitrification-denitrification process, anammox processes require less oxygen and no organic carbon loading, produce much less sludge, and do not emit greenhouse gasses.

On the other hand, since anammox bacteria grow very slowly (minimum duplication time is 10-12 days), the startup periods are very long like 6-18 months (Van der Star et al., 2007). This common handicap of all anammox based process

needs to be further studied in order to develop faster and easier startup periods as well as to forecast possible problems in startup together with solutions.

Anammox reaction, which is the key component of all anammox processes, is sustained by some eubacteria. Those bacteria, which may cover only 16% of bacterial community of anammox sludge, produce N_2 gas by oxidizing NH_4^+ with NO_2^{-2} as electron acceptor (Li et al., 2009). Reaction 1.1 gives the consensus on the stoichiometry of anammox reaction.

$$1NH_4^+ + 1.32NO_2^- + 0{,}066HCO_3^- + 0.13H^+ \rightarrow$$
$$1.02N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.26NO_3^- + 2.03H_2O \qquad \text{(Reaction 1.1)}$$

During the startup periods of anammox reactors, anammox bacteria (and accompanying community) are being enriched. Therefore, "enrichment" and "start-up" terms refers to same efforts and can be used interchangeably in this thesis. The difference between the two terms lies within the concept.

At Environmental Engineering Department, Harran University, Turkey, a sequencing batch reactor (bioreactor) was set up in order to enrich anammox bacteria, i.e. to obtain anammox sludge. The sludge will be used in further studies.

After achieving initial anammox activity, operational conditions were changed in order to evaluate reactor's response. Meanwhile, sludge samples were taken at 9 temporal points in order to evaluate the community structure, and were stored at -20 °C.

At the end of the enrichment, chromosomal DNAs were extracted from each sludge sample. Each extract was amplified by PCR with prokaryotic primers (16S rRNA gene) and with universal primers (rRNA small subunit gene). Those PCR products were run at DGGE for microbial community assessment.

This study aimed to find information which can help to predict microbial changes during startup of full-scale anammox reactors by determining changes in bacterial community structure as well as the reactor's performance during anammox enrichment at laboratory scale. By determining response of microbial community together with the reactor's chemical response to known changes in operational factors during enrichment, it may be possible to predict the process of microbial change during startup periods of full-scale anammox reactors with similar configurations. Therefore, it may be possible to plan more efficient startup periods.

2. LITERATURE REVIEW

2.1 Nitrogen Removal and Anammox

Recently, quality of both fresh and marine waters is rapidly deteriorated with increasing population size and industrialization. That pollution is caused by discharge of wastewaters without enough or any treatment. Nitrogen in wastewaters is one of the major pollutants, which can be prevented by proper treatment. High concentrations of nitrogen are present in agricultural, industrial, and domestic wastewaters. Runoff waters of treatment sludge, wastewaters due to cleaning animal wastes, and wastewaters of molasses industry also contain high concentrations of nitrogen (Lau et al., 2006, Kalyuzhnyi et al., 2006, Hansen et al., 1999). When those wastewaters are discharged to receiving environments without enough treatment, unwanted conditions like eutrophication may occur in water bodies. Therefore, nitrogen removal methods have been developed.

There exist several physicochemical nitrogen removal methods like ion exchange and chemical precipitation. Nevertheless, high operational cost and unreliability of those processes limited their applications. The most economic and successful nitrogen treatment method is the application of biological nitrogen cycle in reactors of treatment plants. Conventionally, the most common biological nitrogen removal method is achieved by successive uses of nitrification and denitrification.

Nitrification is achieved in two steps. Different bacterial genera are responsible for each step, namely *Nitrosomonas* and *Nitrobakter*. In the first step, ammonium is oxidized to nitrite by *Nitrosomonas*:

$$NH_4^+ + 3/2\ O_2 \rightarrow 2H^+ + H_2O + NO_2^- \qquad \text{(Reaction 2.1)}$$

In the second step of nitrification, nitrite is oxidized to nitrate by *Nitrobacter*:

$$NO_2^- + ½\ O_2 \rightarrow NO_3^- \qquad \text{(Reaction 2.2)}$$

In denitrification, nitrate and nitrite ions produced at nitrification are reduced to nitrogen gas by heterotrophic denitrification bacteria under anoxic conditions (Khin and Annachhatre, 2004):

$$2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 2OH^- + 4H_2O \quad \text{(Reaction 2.3)}$$

$$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2OH^- + 2H_2O \quad \text{(Reaction 2.4)}$$

Due to relatively high operational costs of this process, new biological nitrogen removal processes have been developed including anammox, SHARON, and CANON processes which minimize oxygen consumption and reduce or remove organic carbon requirement. Details of those processes are reviewed by Khin and Annachhatre (2004). Those new processes based on **an**aerobic **amm**onium **ox**idation (anammox) reaction (also named anoxic ammonium oxidation).

$$NH_4^+ + NO_2^- \rightarrow N_2 + H_20 \quad \text{(Reaction 2.5)}$$

In 1977, Broda suggested the existence of a missing litotrophy which could gain energy from anammox reaction based on thermodynamic calculations (Broda, 1977). After two decades, Mulder et al. (1995) observed oxidation of ammonium under anaerobic conditions in a fluidized bad denitrification reactor for treatment of metanogenic reactor effluent. They suggested that Reaction 2.6 had occurred under anaerobic conditions. In this reaction, nitrate is electron acceptor while ammonium is the electron donor. They gave the name "anammox" to the process and patented it.

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \quad \text{(Reaction 2.6)}$$

Later, van de Graaf et al. (1995) suggested that the process occurred according to Reaction 2.5 in which nitrite is electron acceptor while ammonium is the electron donor.

Nonetheless, after long discussions, a consensus has been established on stoichiometry given by Reaction 2.7, in which anaerobic ammonium oxidation uses nitrite as electron acceptor (Strous et al., 1998)

$$1NH_4^+ + 1.32NO_2^- + 0{,}066HCO_3^- + 0.13H^+ \rightarrow$$
$$1.02N_2 + 0.066CH_{1.8}O_{0.5}N_{0.2} + 0.26N0_3^- + 2.03H_20 \quad \text{(Reaction 2.7)}$$

2.2 Microbial Community Structure of Anammox Reactors

With developing molecular methods, presentation of microbial community structure data became common. Most common method, FISH (Florescent In Situ Hybridization), determines presence and physical location of specified target taxon (Egli et al., 2003; Schmid et al., 2005; Tal et al., 2006). This method does not allow determination of new taxa and does not give any information about non-targeted taxa.

If a researcher aims to determine unknown taxa in the community or to see whole bacterial scale, conventional method is clone libraries (Penton et al., 2006; Amano et al., 2007; Woebken et al, 2007). In this method, after a specific DNA fragment is multiplied with PCR by using a primer couple, specific to a group or universal, the PCR products are transferred to a clone library. In the clone library, a finite number of colonies were chosen as sample. Then, sequence of PCR product on the plasmid of each sample clone is determined. Therefore, the researcher tries to deduce information about whole community with limited number of samples (maximum number of sample microorganisms is equal to the number of sampled colonies).

On the other hand, in DGGE (Denaturation Gradient Gel Electrophoresis), it is possible to have a different band on the gel for each different strain in a sludge sample. Theoretically, even one base difference can be seen as a different band (Guldberg and Güttler, 1993; Nollau et al., 1997). The weakness of this system is that long PCR products (>1000bp) may not be run properly on the gels (Sheffield et al., 1989). Therefore, magnification to smaller taxons may not be possible. Nevertheless, it may not be consider as a handicap because determination of functional taxonomic unit is sufficient in environmental engineering point of view.

In DGGE, in order to multiply target DNA fragment, a PCR procedure is carried out by using primers, one or both of which has extra G and C nucleotides

(typically 40 bases) at 5' end, called GC tail. The resulting PCR products have GC rich ends, called GC clamp (Sheffield et al., 1989). The GC clamp end of a PCR product resists melting in comparison to other regions of the PCR product. Such PCR products are run by electrophoresis on a gel which has an increasing denaturation field (denaturation gradient gel) prepared by gradual mixing of two different concentrations of denaturation chemicals. GC and AT bonds within a PCR product starts to dissolve (melting) while it runs on the gel. In the forward regions of the gel, the melting is higher. Therefore, PCR products with same length but different sequence, i.e. belongs to different taxa, forms different three dimensional structure since each has different GC/AT ration and order. AT rich PCR products and AT rich segment in any PCR product tents to melt easily in comparison to GC rich ones. Due to GC clamp, PCR product does not melt completely even at the highest denaturant concentration.

In such gels, PCR products belonging to different taxa run at different speeds and forms different bands. Afterwards, DNA sample can be isolated from each band, and then it can be sequenced in order to reach taxon information by BLAST search.

3. MATERIAL and METHODS

3.1 Reactor Design

A BIOFLO 110 Modular Benchtop Fermentor (New Brunswick Scientific) with 7.5 L vessel was used as Sequencing Batch Reactor (Figure 3.1). BIOFLO 110 includes a set of monitoring and controlling devices for pH, temperature, and mixing (rotor, impeller). The reactor's maximum working volume was 5.5 L.

In order to keep reactor's temperature constant at 35 °C, the SBR was equipped with a heating blanket and an internal cold water pipe controlled by its automatic thermostat system.

3.2 Reactor Operation

3.2.1 The inoculum

The bioreactor was seeded with 1.75 L of the sludge taken from denitrification unit of Pasaköy Advanced Wastewater Treatment Plant, Istanbul, Turkey. The sludge was sieved in order to remove rough particles before seeding. The volatile suspended solid of the sieved sludge was 12000 mg/L. In order to ensure presence of significant amount of anammox bacteria, 5 mL of enriched anammox culture was inoculated to the reactor. The enriched anammox culture was given by Dr. Bilge ALPASLAN KOCAMEMİ, Environmental Engineering Department of Marmara University, Istanbul, Turkey.

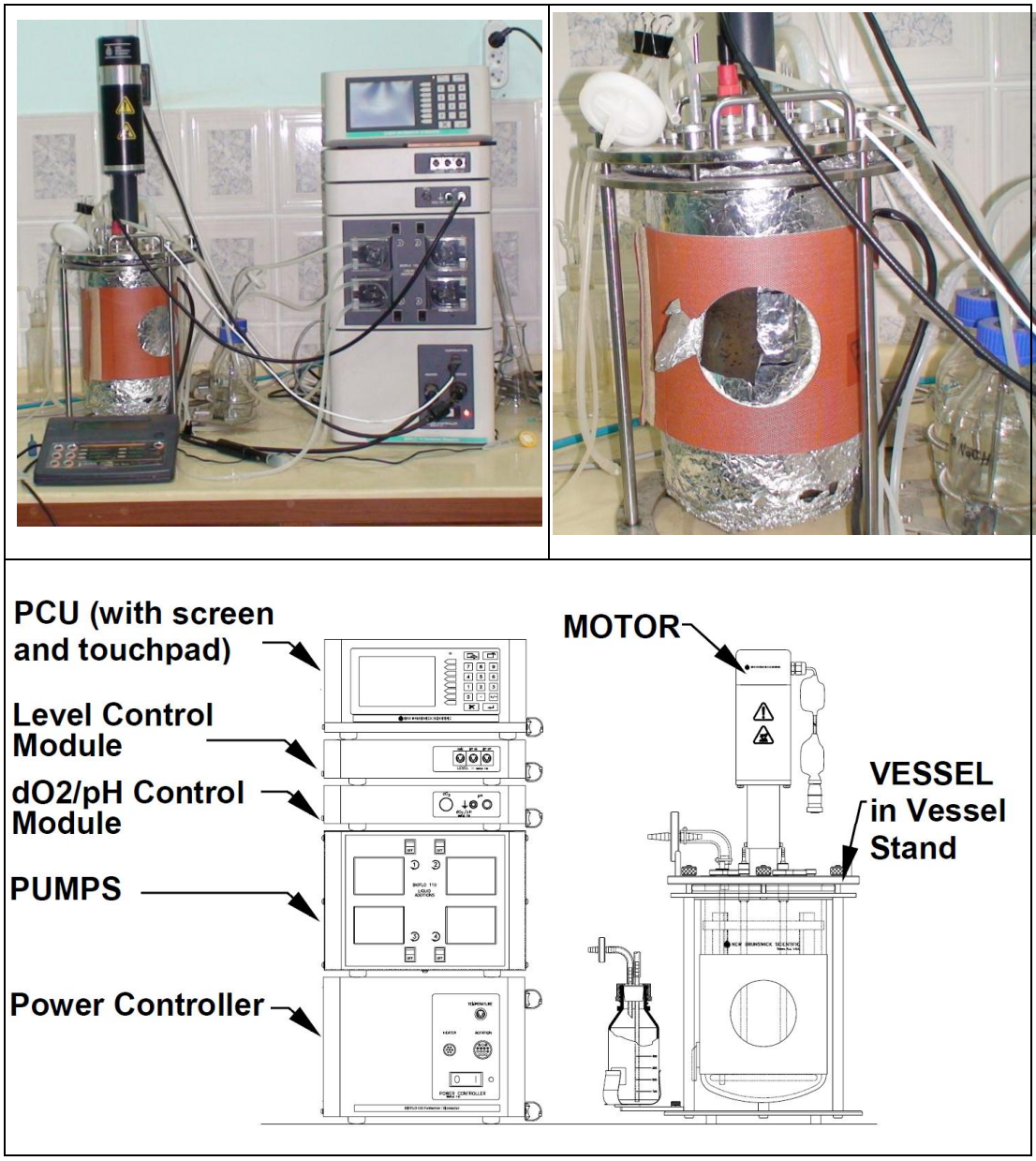

Figure 3.1. The bioreactor used for the experiment

3.2.2 The feed

The reactors were fed with a synthetic autotrophic medium adapted from the medium previously described by Van de Graaf et al. (1996). The feed composition has been given in Table 3.1. Nitrogen loading rates was changed during trials as described in the following section.

Table 3.1. Composition of the feed

Macro Elements		Trace Elements	
Compound	**Concentration (mg/L)**	**Compound**	**Concentration (mg/L)**
$NaNO_2$	246.4 – 462	EDTA	15000
$(NH_4)_2 SO_4$	188.8 – 354	$ZnSO_4.7H_2O$	430
KNO_3	0 – 81.5	$MnCl_2.4H_2O$	990
$KHCO_3$	1250	$CuSO_4.5H_2O$	250
KH_2PO_4	25	$NaMoO_4.2H_2O$	220
$MgSO_4.7H_2O$	200	$NiCl_2.6\ H_2O$	190
$FeSO_4$	6.25	H_3BO_4	14
EDTA	6.25	$NaSeO_4$	210
$CaCl_2.2H_2O$	300	$NaWO_4.2H_2O$	50
Trace Elements	1.25 (mL/L)	$CoCl_2.6\ H_2O$	240

3.2.3 Operational conditions

The operational conditions were based on the principles described by Uyanik et al. (2011) in order to ensure anammox enrichment.

In this study, several different operational condition sets were applied in order to test different conditions and due to operational necessities which can be interpreted to understand the reactor's reaction to changing operational conditions. Therefore, the experiment was divided into 8 periods. Details of each period were given at Table 3.2.

One of the main operational changes was that the control unit of the BIOFLO 110 Modular Benchtop Fermentor was installed in Day 126 (start of Period V). Therefore, operational factors described below were controlled differently during first four periods and last four periods.

Table 3.2. Operational conditions at 8 periods.

	I	II	III	IV	V	VI	VII	VIII
Starting and ending days	1-11	11-95	95-109	109-126	126-139	139-174	174-192	192-374
Time length (days)	11	84	14	17	13	35	18	182
Mixing	Manual 3/day	Manual 3/day	Manual 3/day	Manual 3/day	Auto Continues	Auto Continues	Auto Continues	Auto Continues
Feeding frequency	1/day	1/day	1/day	1/day	1/day	3/week	3/week	2-3/week
Feeding volume	1.75 L	1.75 L	1.75 L	1.75 L	1.75 L	3.5 L	3.5 L	3.5 L
pH regulation	Manual	Manual	Manual	Manual	Auto	Auto	Auto	Auto
Nitrogen loading rate [1] (mg-N/week)	1103	1017	919	1152	1152	1187	1481	Not specific
Light [2]	D+C [2]	D+C [2]	D+C [2]	D+C [2]	C [2]	C [2]	C [2]	C [2]
Control unit	Absent	Absent	Absent	Absent	Present	Present	Present	Present
Feed content								
Total nitrogen [1] (mg/L)	90	83	75	94	94	113	141	141
NO_2^--N / NH_4^+-N	1.25	1.50	1.25	1.25	1.25	1.25	1.25	1.25
NO_2^--N (mg/L)	50	50	42	52	52	63	78	78
NH_4^+-N (mg/L)	40	33	33	42	42	50	63	63
NO_3^--N (mg/L)	11	11	0	0	0	0	0	0

1) Total Nitrogen and nitrogen loading rate regards only NO_2^- and NH_4^+ (excluding NO_3^-) in the feed, which will be diluted in the reactor.
2) D: The reactor was stored in a dark room; C: The reactor was covered with aluminium foil.

3.2.3.1 Light and temperature control

The reactor was covered with aluminum foil in order to prevent light penetration during entire experiment.

During first four periods, the reactor was kept in a temperature controlled dark room. The room's temperature was regulated at 35±2 °C.

During last four periods, the reactor's temperature was regulated at 35±1 °C by the control unit via cooling pipes and heat blanket supplied with the reactor. The new room was subjected to light.

3.2.3.2 Feeding

The feeding was performed by the same principles during whole study, but the details were changed according to other operational conditions. The general principles were:

1) Deoxygenation of the feed by Ar/CO_2 gas,
2) Settling of the sludge in previously mixed reactor content,
3) Removal of the supernatant by leaving 1.75 L of the reactor content without oxygen entry to the reactor,
4) Filling the deoxygenated feed to the reactor while continuing the deoxygenation of the feeding,
5) Deoxygenation of the reactor content after filling.

During the first four periods, following feeding procedure was performed:

1) Mix the reactor content by Ar/CO_2 gas flow for 5-10 minutes for homogenization of the liquid phase,
2) Let it settle down for 30 minutes,
3) Pump out the supernatant until the reactor content is reduced to 1.75 L,
4) Supply Ar/CO_2 mixture to the reactor's gas bearing portion in order to prevent entry of oxygen into the reactor during pump out,
5) Deoxygenate the feed by Ar/CO_2 mixture until the feed's DO level drops below 0.02 mg DO/L,
6) Control the pH of the feed and adjust to pH7.5 by adding 0.25 M NaOH if needed, during deoxygenation of the feed (there is no need for pH adjustment downwards),
7) Pump in 1.75 L of the deoxygenated feed while continuing the deoxygenation of the feeding,
8) Mix and deoxygenate the reactor content by Ar/CO_2 gas flow for 30 minutes,
9) Seal the reactors non-air-tight parts before stopping Ar/CO_2 gas flow.

During the last four periods, following feeding procedure was performed:

1) Let the reactor content settle down by stopping the impeller of the fermentor for 1 hour,
2) Pump out the supernatant until the reactor content is reduced to 1.75 L,
3) Supply Ar/CO_2 mixture to the reactor's gas bearing portion in order to prevent entry of oxygen into the reactor during pump out,
4) Deoxygenate the feed by Ar/CO_2 mixture until the feed's DO level drops below 0.02 mg DO/L,
5) Pump in 1.75 or 3.5 L of the deoxygenated feed while continuing the deoxygenation of the feeding,
6) Start the impeller of the fermentor,
7) Deoxygenate the reactor content by Ar/CO_2 gas flow for 30-45 minutes,
8) Seal the reactors non-air-tight parts before stopping Ar/CO_2 gas flow,
9) Check the pH of the reactor content from the monitor of the control unit and manually initiate the pH adjustment if needed.

3.2.3.3 Oxygen control

Dissolved O_2 was controlled in the feed as described in "3.2.3.5 Feeding" and in the effluents by a bench top DO meter, HANNA INSTRUMENTS HI 2400.

3.2.3.4 pH control

During first four periods, the feed's pH was measured by a portable pH meter (Mettler Toledo SG8-ELK - SEVENGO PRO pH/IYON) and the action regarding pH control was described under "3.2.3.1 Feeding".

During last four periods, owing to operation of the control unit, pH was monitored and regulated with the built-in pH regulation system of the bioreactor. It

was kept around pH7.5. The system was supplied with 0.25 M HCl and 0.25 M NaOH solutions.

3.2.3.5 Mixing

During first four periods, the mixing was performed manually by 10 minutes of Ar/CO_2 gas flow two times per day in addition to the mixing during daily feeding.

During first four periods, the reactor was continuously mixed by the built-in impeller.

3.3 Analytic Methods

3.3.1 NH_4^+ measurement

Ammonium was analyzed with test kits (Merck, Spectroquant® Test Kits 1.00683.0001) by a spectrophotometer (Shimadzu UV-1601) according to Standard Methods (APHA 1995).

3.3.2 NO_3^- and NO_2^- measurement

Nitrate and nitrite were measured with anion exchange chromatography by anionic column SHIM-PACK IC-A3 with SHIMADZU HIC-20A SUPER.

3.4 Evaluation of Microbial Community

In order to evaluate microbial community, sludge samples were taken, their DNA was extracted, 16S rRNA genes were amplified by PCR, and the PCR products were run under DGGE as explained below.

3.4.1 Sludge sampling

9 sludge samples were taken from the reactor at Day 35, 63, 138, 179, 273, 280, 294, 332, and 374. First 8 samples were 12 mL and stored at -20 °C. Sample preparation was performed at the day of final sample; therefore, it did not stored under refrigeration before sample preparation.

3.4.2 Sample preparation and VSS equalization

The frozen samples were left to defreeze for about 30 minutes. All samples were centrifuged at 10000 rpm for 20 minutes by Hettich Centrifuge ROTOFIX 32. The supernatants were removed. The pellets were transferred to 1.5 mL micro-centrifuge tubes, otherwise known as Eppendorf tubes. The tubes were filled with sterile ultra-pure water up to 1 mL of total volume. Then, the samples were re-suspended. 200 µL of each re-suspended sample was taken for VSS measurement. Remainder samples were stored at -20 °C.

VSS samples were dropt on previously dried incombustible filter papers. The filters were transferred to clean crucibles and left to dry at 104±1 °C for 24 ours in an own, Electro-Mag M5040 P Hot Air Sterilizer. Dried filters in crucibles were left to cool down in a desiccator for 30 minutes. Then, the filter papers were weighted (W_1 = filter paper + total suspended solid). The filter papers were again transferred to the crucibles and combusted at 550 °C for 40 minutes in a furnace, Thermolyne 48000 Furnace F48020-80. After the combustion, the filters in crucibles were left to cool down in a desiccator for 30 minutes. Then, the filter papers were weighted again (W_2 = filter paper + non-volatile suspended solid). VSS values were calculated according to following formula:

$$VSS = (W_1 - W_2) / V \qquad \text{Equation 3.1}$$

where VSS is volatile(combustible) suspended solid in mg/mL, W_1 is the weight after drying in mg, W_2 is the weight after combustion in mg, and V is the volume of the re-suspended sludge sample as 0.2 mL.

After obtaining VSS values, samples were defrosted and the other samples were diluted to the VSS level of the sample with lowest VSS level. Therefore, the VSS levels of all samples were equalized.

3.4.3 DNA extraction

The DNA extraction was performed by using MO-BIO 12888 PowerSoil DNA Isolation Kit. DNA of 200µL of each sample with equalized VSS was extracted

according to manufacturer's instructions expect the sample size and that transferred supernatant were kept at the same volume for every sample.

3.4.4 Polymerase chain reaction (PCR)

The extracted DNA templates were amplified by two primer pairs (Table 3.3).

Each PCR solution contained 25 µL PCR master mix (Fermentas PCR Master Mix (2X) #K0171), 5 µL of 5µM forward primer, 5 µL of 5 µM reverse primer, 15 µL DNA template. PCR was performed with 1 cycle of 95 °C for 5 minutes, 31 cycles of 94 °C for 30 seconds, 50 °C for 1 minute, and 72 °C for 2 minutes with final extension of 72 °C for 10 minutes.

Table 3.3. Primers for PCR

Target	Primer	Sequence(5' – 3')	Reference
Prokaryotic 16S rRNA gene	GC-341F	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G**CC TAC GGG AGG CAG CAG**	Muyzer et al. (1993)
	907R	CCG TCA ATT CMT TTR AGT TT	Lane et al. (1985)
Universal Small subunit rRNA gene	GC-U1F	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G**CT YAA AKR AAT TGR CGG RRR SSC**	Rivas et al. (2003)
	U1R	**CGG GCG GTG TGT RCA ARR SSC**	

3.4.5 Agarose gel electrophoresis

An agarose gel electrophoresis was performed in order to verify successful reaction. The PCR products were run on 1% agarose gel under 80 V for 15 minutes with TBE buffer. Then, the gel was stained by bleaching in 10 µg/L ethidium bromide solution for 35 minutes before UV imagery.

3.4.6 Denaturation gradient gel electrophoresis (DGGE)

The PCR products were run on 6% acrylamide gel (DGG1) with 40% to 70% denaturation gradient and on 6% acrylamide gel (DGG2) with 35% to 60% denaturation gradient by using BIO-RAD DCode System for DGGE.

Low grade gel solutions, high grade gel solutions, and stacking gel solutions were prepared according to Table 3.4 and Table 3.5.

14.4 µL of 0.2g/L APS solution (10% ammonium persulfate) and 14.4 µL of TEMED were added to each of low grade and high grade denaturation gel solution immediately before casting gradient gels according to manufacturer's interactions. The denaturation gradient was increasing downwards. 1-2 mL of 2-propanol (i.e. iso-propanol) was carefully added on top of the gradient gel in order to cut the contact between air and gradient gel. Otherwise, the upper portion of the gel will not be polymerized.

Table 3.4. Chemicals for 6% acrylamide gel (DGG1) with 40% to 70% denaturation gradient

	Gradient Gel Solutions (6% Acrylime-BIS)		Stacking Gel Solution (4% Acrylime-BIS)
	Low Grade (40%)	High Grade (70%)	
40% Acrylamide-BIS	2.4 mL	2.4 mL	1.0 mL
50x TAE buffer	0.32 mL	0.32 mL	0.2 mL
Urea	2. 688 g	4.704 g	-
Formamide	2.56 mL	4.48 mL	-
PCR grade water	upto 16 mL	upto 16 mL	upto 10 mL

Table 3.5. Chemicals for 6% acrylamide gel (DGG2) with 35% to 60% denaturation gradient

	Gradient Gel Solutions (6% Acrylime-BIS)		Stacking Gel Solution (4% Acrylime-BIS)
	Low Grade (35%)	High Grade (60%)	
40% Acrylamide-BIS	2.4 mL	2.4 mL	1.0 mL
50x TAE buffer	0.32 mL	0.32 mL	0.2 mL
Urea	2.352 g	4.032 g	-
Formamide	2.24 mL	3.84 mL	-
PCR grade water	up to 16 mL	up to 16 mL	up to 10 mL

One hour later, the gradient gel was completely polymerized. The 2-propanol was removed and upper portion of the casting sandwich was rinsed with ultra-pure water. The rinsed region was dried with filter paper.

50 µL of 0.2g/L APS solution and 5 µL TEMED was added in to stacking gel solution. The sufficient stacking gel solution was poured on the gradient gel by leaving enough empty volume in order not to allow overflow during comb placement. Because of quick polymerization of the gel, immediately, a 20-well-comb was placed into the stacking gel solution. It should be noted that the comb should not enter or touch to gradient gel in order for smooth entry of the PCR products into the gradient gel. The stacking gel was totally polymerized in less than 20 minutes.

Before loading, the system had filled with 1x TAE buffer and heated to 60°C, which was also the operational condition. 20 µL of each PCR product mixed 4 µL of a three-phase loading dye. 20 µL of each mixture was loaded to the gel. For 10 minutes, 150 V was applied, which was sufficient for the entry to gradient gel (Figure 3.1). Then, the system was run at 85 V (yielding ~48 mA) for 16 hours.

After the electrophoresis, the gel was stained by bleaching in 10 µg/L ethidium bromide solution for 60 minutes, before UV imagery. It should be noted

that ethidium bromide is mutagenic and all contact with even scarcely contaminated substances should be avoided. Any ethidium bromide contaminated material should be either disposed according to local or national regulations or washed completely with water.

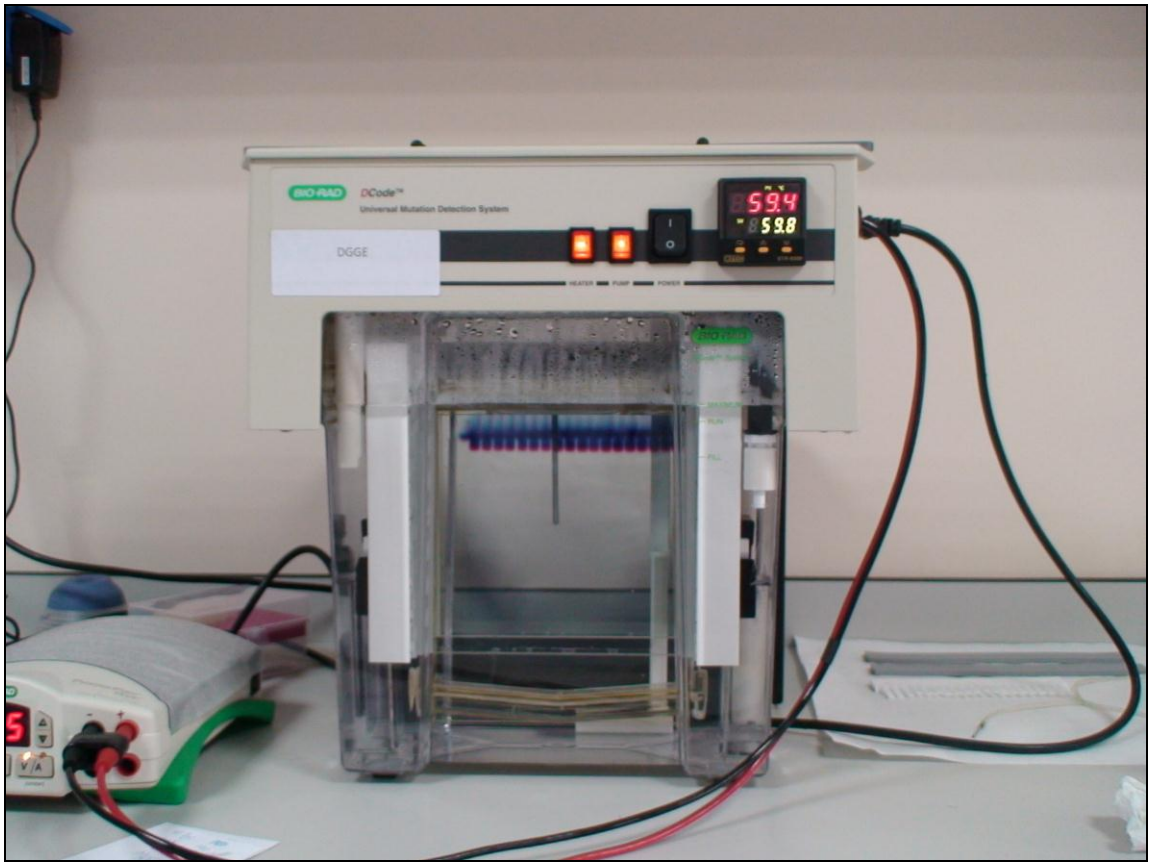

Figure 3.2. Loading dye entering to the gradient gel

4. RESULTS and DISCUSSION

4.1 Fate of Nitrogen

Influent and effluent NO_2^-, NH_4^+, NO_3^- concentrations and nitrogen removal rates have been given at Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4, respectively, for the first seven periods.

NO_2^- consumption and NO_3^- production in mg-N were calculated for each sampling day and divided to NH_4^+ consumption in mg-N. Those rates for the first seven periods have been given at Table 4.1 in order to evaluate the reactors performance for anammox stoichiometry.

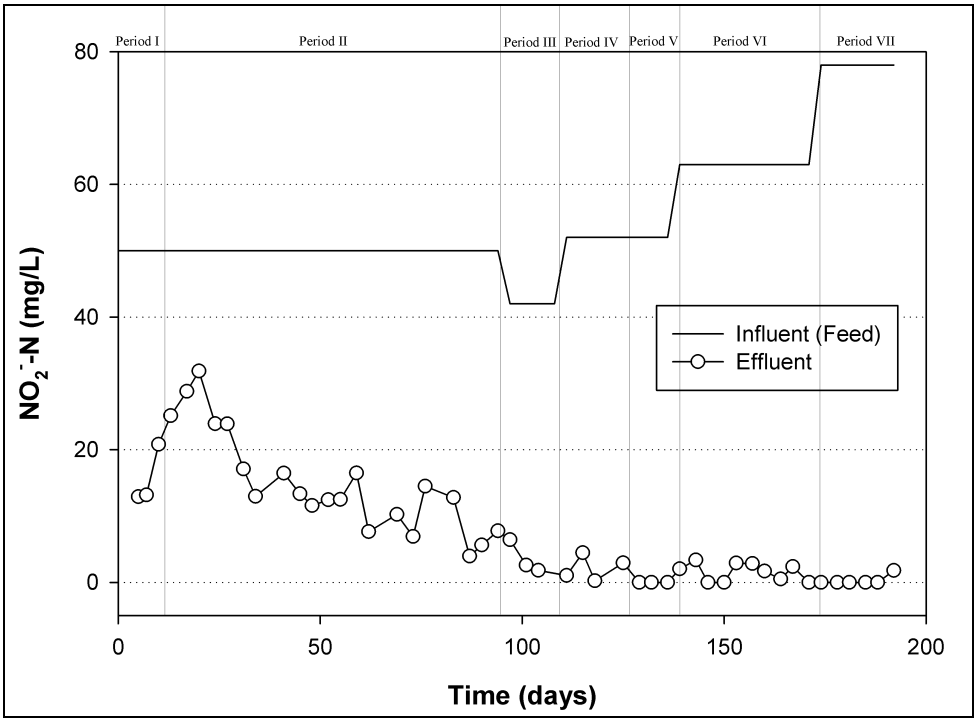

Figure 4.1. NO_2^--N levels during first 7 periods

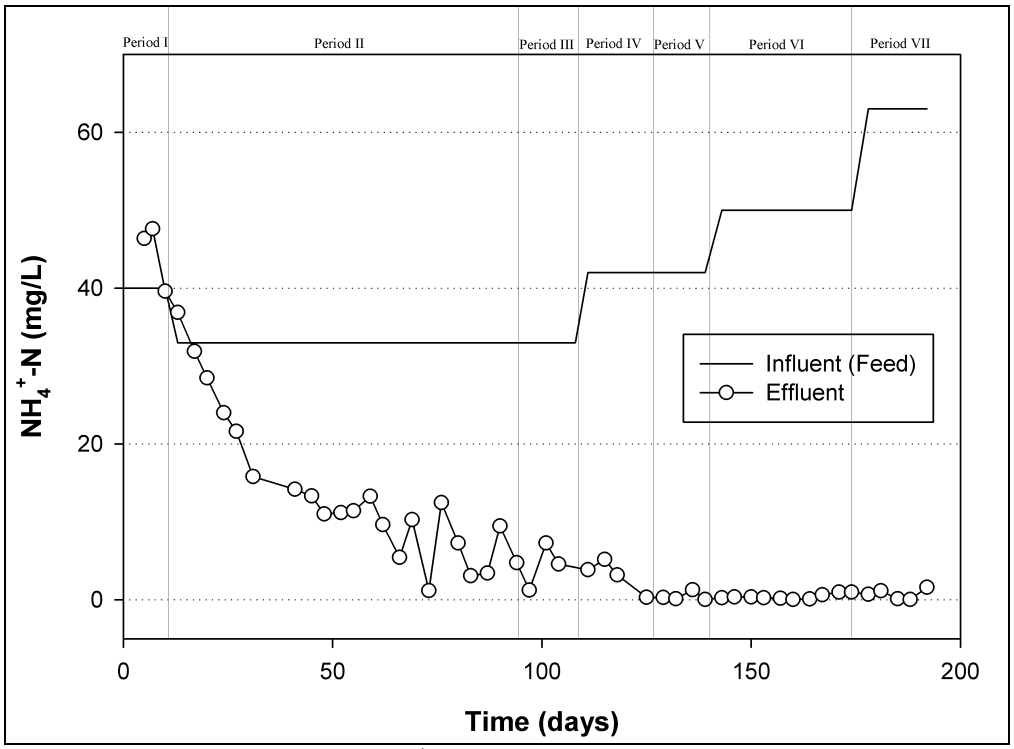

Figure 4.2. NH_4^+-N levels during first 7 periods

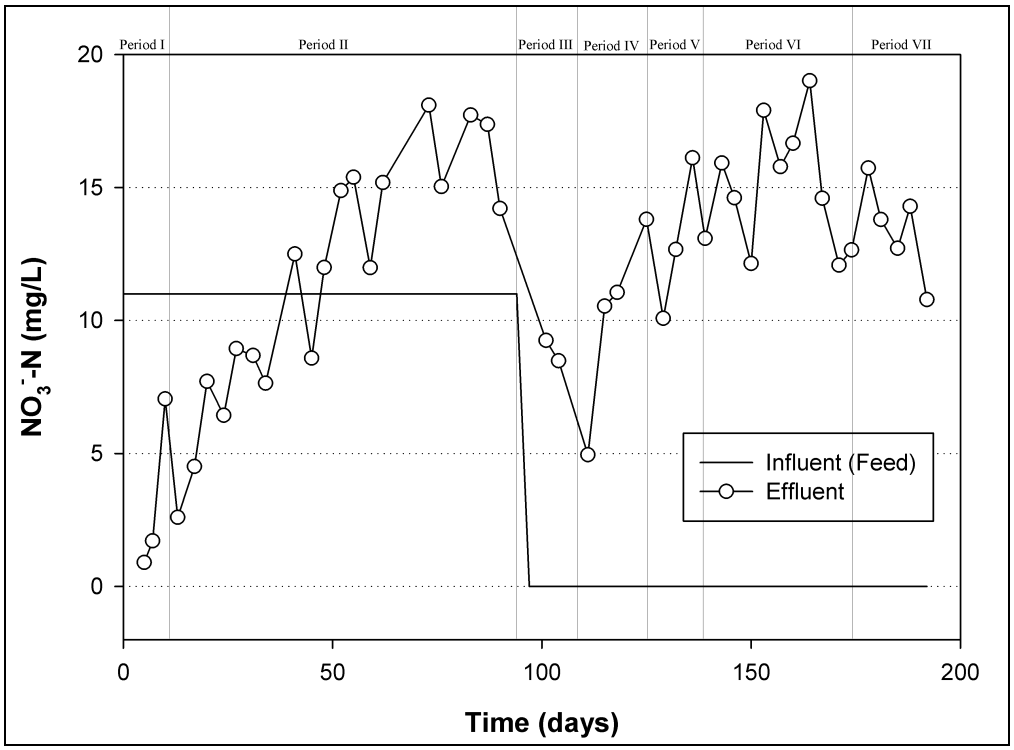

Figure 4.3. NO_3^--N levels during first 7 periods

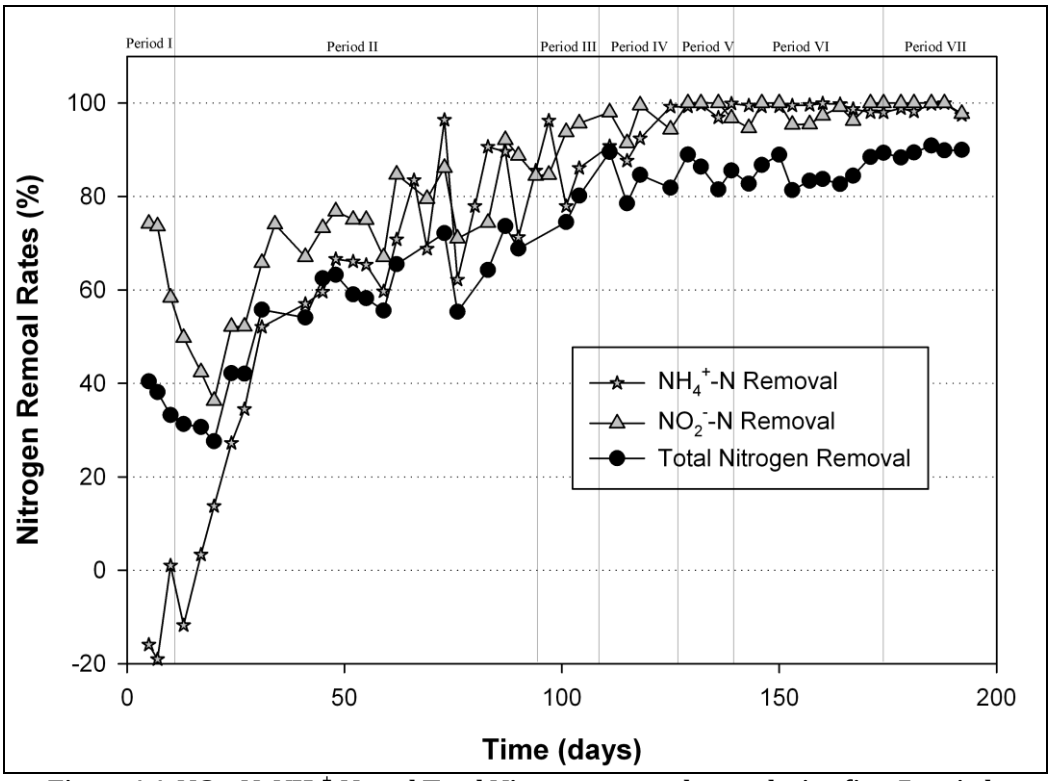

Figure 4.4. NO_2^--N, NH_4^+-N, and Total Nitrogen removal rates during first 7 periods

Table 4.1. The reactor's stoichiometric ratios regarding anammox reaction during first 7 periods

Period	Day	NO_2^--N removal / NH_4^+-N removal ratio	NO_3^--N production / NH_4^+-N removal ratio
I	10	72.98	
II	17	19.28	
	20	4.02	
	24	2.90	
	27	2.30	
	31	1.91	
	41	1.78	0.08
	45	1.86	
	48	1.75	0.05
	52	1.72	0.18
	55	1.74	0.20
	59	1.70	0.05
	62	1.81	0.18
	69	1.75	
	73	1.35	0.22
	76	1.73	0.20
	83	1.24	0.22
	87	1.56	0.22
	90	1.89	0.14
	94	1.50	
III	97	1.37	
	101	1.53	0.36
	104	1.41	0.30
IV	111	1.34	0.29
	115	1.29	0.29
	118	1.33	0.28
	125	1.18	0.33
V	129	1.25	0.24
	132	1.24	0.30
	136	1.28	0.40
	139	1.19	0.31
VI	143	1.20	0.32
	146	1.27	0.29
	150	1.27	0.24
	153	1.21	0.36
	157	1.21	0.32
	160	1.23	0.33
	164	1.25	0.38
	167	1.23	0.30
	171	1.29	0.25
	174	1.29	0.26
VII	178	1.25	0.25
	181	1.26	0.22
	185	1.24	0.20
	188	1.24	0.23
	192	1.24	0.18
Anammox Reaction		1.32	0.26

4.1.1 Period I: Starting with ammonium production

This period was characterized by;

1) Nitrite production instead of removal (Figure 4.1),

2) Ammonium production instead of removal (Figure 4.2),

3) Nitrate consumption instead of production (Figure 4.3).

The feeding medium was prepared to favor anammox bacteria. Most of the other microorganisms present in the seeding medium (denitrification sludge) are prone to death and decay due to changing medium. Such decay definitely increases BOD as organic matter. The significant presence of organic nutrients is likely to diminish enrichment of anammox bacteria which are autotrophic. In order to consume that organic matter, nitrate was included in the enrichment medium although nitrate was not required for anammox reaction. This was expected to sustain denitrification for removal of organic matters. Figure 4.3 shows that nitrate was consumed as expected.

Ammonium production was also expected due to decaying microorganisms and detritus. In order to overcome excess ammonium and to favor anammox reaction, NH_4^+-N/NO_2^--N ratio was increased to 1.50 from 1.25 at Day 11 as suggested by Uyanik et al. (2011).

4.1.2 Period II: Achieving anammox reaction

This was the main enrichment period with above mentioned principles. The main goal of Period II was to observe anammox reaction as the main reaction at the reactor.

Towards the end of Period II, observation of nitrite and ammonium removal rates above 80% were the first indications of anammox reaction (Figure 4.4). Even before this, nitrate consumption shifted to production. Therefore, it can be safely assumed that sufficient anammox activity was achieved at the reactor. On the other

hand, stoichiometric values at Table 4.1 indicates that there are other minor but significant reactions in the reactor.

4.1.3 Period III: Discontinuation of nitrate addition and high nitrite ratio

In Period II, production of nitrate removed the need for nitrate addition. Therefore, the addition of nitrate to the feeding medium discontinued at the beginning of Period III, at Day 95.

Due to presence of anammox reaction and lack of apparent ammonium production, high NO_2^--N/NH_4^+-N ratio was not required anymore. Therefore, NO_2^--N/NH_4^+-N ratio was reduced to 1.25 from 1.50. This ratio is 1.32 in anammox reaction (Reaction 1.1). By keeping it below reaction value, nitrite becomes limiting factor. Consequently, nitrite is expected to be depleted before ammonium, which is preferred due to higher toxicity of nitrite.

Due to above mentioned rational and because excess nitrogen levels cause irreversible inhibition of anammox bacteria (Strous et al., 1999), nitrite concentration decrease was preferred instead of ammonium increase in the feed for sustaining 1.25 NO_2^--N/NH_4^+-N ratio.

As a result, ammonium removal efficiency decreased from 96% to 86% between Day 97 and 104. On the contrary, nitrite removal efficiency increased from 84% to 96% during same period. This can be comprehended as successful application of decreasing NO_2^--N/NH_4^+-N ratio in the feed (Figure 4.4).

"NO_2^--N consumption to NH_4^+-N consumption ratio" did not change significantly within Period III, but it was closer to anammox reaction stoichiometry than the ones at the end of Period II. On the other hand, "NO_3^--N production to NH_4^+-N consumption ratio" increased above anammox reaction stoichiometry.

4.1.4 Period IV: Increasing nitrite and ammonium concentrations by 25%

After achieving clear anammox reaction with sufficient nitrite and ammonium removal (Figure 4.1, 4.2, 4.4), their concentrations in the feed were increased by 25% at Day 109, which started Period IV.

The reactor well tolerated the increased nitrogen concentration. Stoichiometric ratios further approached to stoichiometric ratios of anammox reaction (Table 4.1).

4.1.5 Period V: Continuous mixing

As described at "3.2.3 Operational Conditions", the reactor was mixed manually during first four periods. By the end of Period IV, a new control unit for the fermentor was received. Its operation was started at Day 126 which initiated Period V without any change in the feed and feeding regime.

The new control unit also sustained continuous mixing by its impeller instead of previous daily manual gas blowing, which can be considered as the main change in the reactor's operation.

With the new control unit, the reactor was moved to a room with light and unregulated temperature form a temperature controlled dark room. The reactor had been and was covered with aluminum foil for light prevention as a precaution against oxygen inhibition (Strous et al., 1997; Griffin et al., 2007; Uyanik et al., 2011). The new control unit allowed the use of cooling pipes and heat blanket supplied with the reactor. The temperature of the reactor was monitored from and was regulated by the control unit until the end of the study. No significant temperature fluctuations were observed within the reactor during remaining periods, except System Halt I and System Halt II which will be discussed later.

These changes did not adversely effect ammonium and nitrogen removal. Both of the removal rates went above 99% (Figure 4.1, 4.2, 4.4). This high removal should be the cause of decreasing "NO_2^--N consumption to NH_4^+-N consumption ratio" to near NO_2^--N/NH_4^+-H ratio in the feed (1.25) which is below 1.32, the stoichiometric ratio of anammox reaction (Table 4.1).

Initially "NO_3^--N production to NH_4^+-N consumption ratio" decreased to 0.24 which is fairly close to 0.26 of anammox stoichiometry (Figure 4.3, Table 4.1). Combined with complete removal of nitrate and ammonium, this could be an evidence for complete dominance of anammox reaction in the reactor. But, later, it was further increased above anammox stoichiometry (Figure 4.3, Table 4.1). A

possible cause might be phototrophic activity. Since the reactor moved to a light bearing room, some light might have penetrated in to the reactor, which can have caused phototrophic activity resulting in limited oxygen production. That oxygen may have been used for limited denitrification of ammonium to nitrate.

This logic can also explain almost complete removal of ammonium regardless of anammox stoichiometry. According to anammox stoichiometry (reaction 1.1), "NO_2^--N consumption to NH_4^+-N consumption ratio" should be 1.32, but our feed has 1.25 NO_2^--N/NH_4^+-N ratio. Therefore, if all of the nitrite in our feed is consumed by anammox reaction and there exists no other reaction the reactor, 5.3% of the ammonium should remain in reactor. But the ammonium removal rate was above 99% in most of the time. The portion which was not removed by anammox reaction may have been removed by limited denitrification to nitrate.

For example, at Day 132 when nitrite removal rate was 100%, if we subtract the NH_4^--N quantity from produced NO_3^--N quantity and recalculate stoichiometric rates, "NO_3^--N production to NH_4^+-N consumption ratio" becomes 0.26 instead of 0.30, and 0.26 is a perfect mach for anammox stoichiometry.

By the end of Period V; the main goal of the start-up study was achieved, which was anammox reaction as highly dominant reaction in the reactor. However, the enrichment can continue with decreasing growth rate due to nitrogen loading rate not sufficiently increasing with growing anammox population.

4.1.6 Period VI: Feeding three times per week

As the main objective of the start-up and operational functionality of the fermentor with its control unit had been achieved, the feeding frequency was lowered to three times per week in order to ease labor force, at Day 139 which became the start of Period VI. In order to sustain continuity of anammox enrichment, feeding volume was increased to practical maximum of 3.5 L for each feeding, and also nitrite and ammonium concentrations were increased by 20%. Therefore, weekly nitrogen loading was slightly increased to 1187 mg-N from 1152 mg-N. By this way, anammox population was intended to be in active growth phase but considering it as

lag phase would be pretentious due to limited increase in the nitrogen load which can have limited the growth of anammox bacteria.

In Period VI, ammonium removal rate remained fairly stable above 99% until Day 167 (Figure 4.4). Meanwhile nitrite removal rate fluctuated above 94% as well as fluctuation of nitrate production (Figure 4.3 and 4.4). If the explanation based on limited phototrophic oxygen production, as stated in section "4.1.5 Period V", is assumed to be valid, then the fluctuation may have been caused by changes either in the presence of light at the room or in the quality of aluminum foil cover of the reactor.

At the end of Period VI, ammonium removal rate decreased to around 98% while nitrite removal rate increased to 100% (Figure 4.4). Therefore, "NO_2^--N consumption to NH_4^+-N consumption ratio" (1.29) approached closely to anammox stoichiometry (1.32) (Table 4.1). Meanwhile, nitrate production decreased and "NO_3^--N production to NH_4^+-N consumption ratio" became 0.26 as a perfect match for anammox stoichiometry (Figure 4.3, Table 4.1). Those can be interpreted as increasing tendency towards anammox reaction.

4.1.7 Period VII: Increasing nitrite and ammonium concentrations by 25%

After achieving 100% nitrite removal rate at Period VI, nitrite and ammonium concentrations were increased by 25% at Day 174. Therefore, weekly nitrogen loading was increased to 1481 mg-N from 1187 mg-N (Table 3.2).

Even at the beginning, the new nitrogen load was well tolerated. Nitrite removal rate remained 100% during most of Period VII (Figure 4.4). Nevertheless, both "NO_2^--N consumption to NH_4^+-N consumption ratio" and "NO_3^--N production to NH_4^+-N consumption ratio" slightly decreased (Table 4.1), indicating deviations from anammox reaction.

By Day 192, the goal of start-up or enrichment was considered to be achieved without any reservation.

4.1.8 Period VIII: Unsteady operation

After successful completion of the start-up or enrichment by Period VII, the reactor was continued to be feed 3 times a week with the same feed as in Period VII in order to keep the anammox bacteria alive. But, two discontinuations of feeding happed during Period VIII, as discussed below (System Halt I and System Halt II). Nevertheless, the feeding was not totally regular after System Halt II, as it would have been in non-experimental enrichments in idle stage or suspension. During this time, the feeding was performed 2 or 3 times a week as per conditions. Limited numbers of samples were taken during Period VIII, in order to evaluate such irregular feeding regimens.

Influent and effluent NO_2^-, NH_4^+, NO_3^- concentrations and nitrogen removal rates has been given at Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8, respectively, for Period VIII.

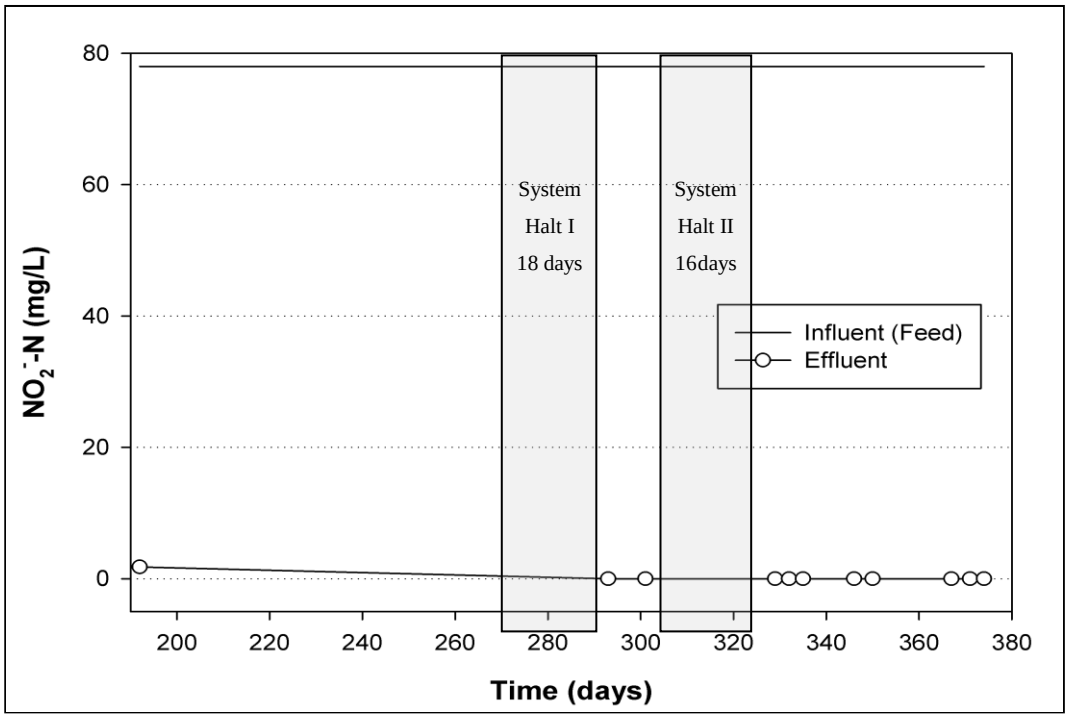

Figure 4.5. NO_2^--N levels during Period VIII

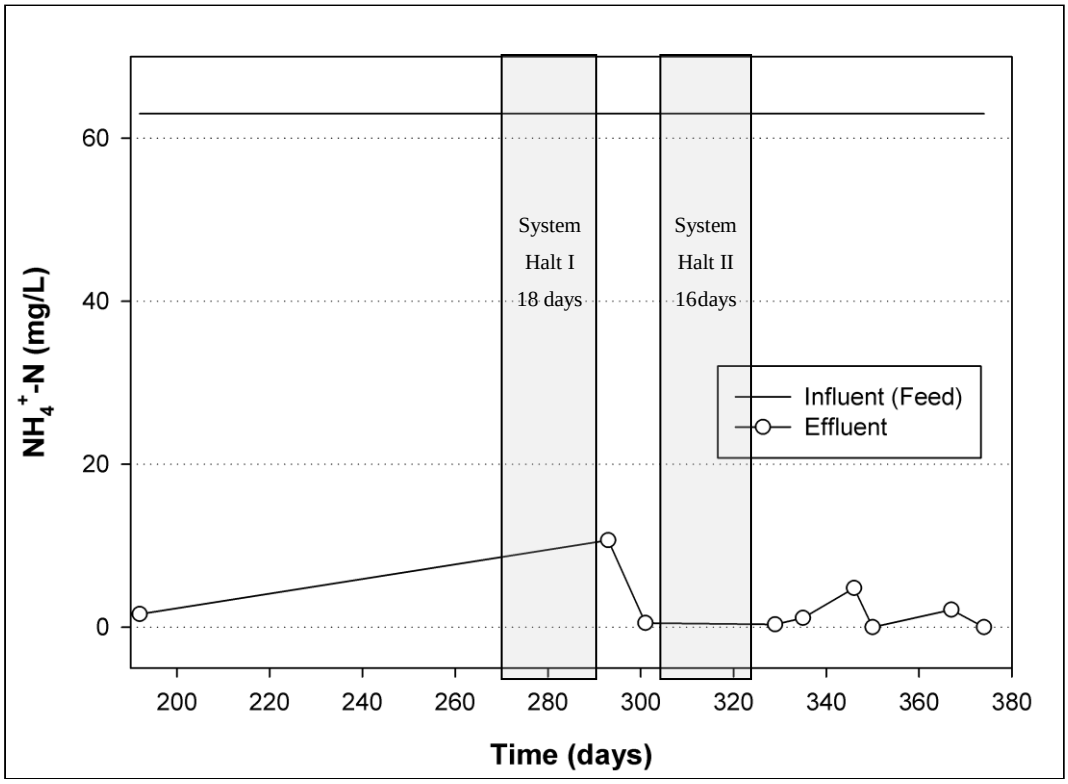

Figure 4.6. NH_4^+-N levels during Period VIII

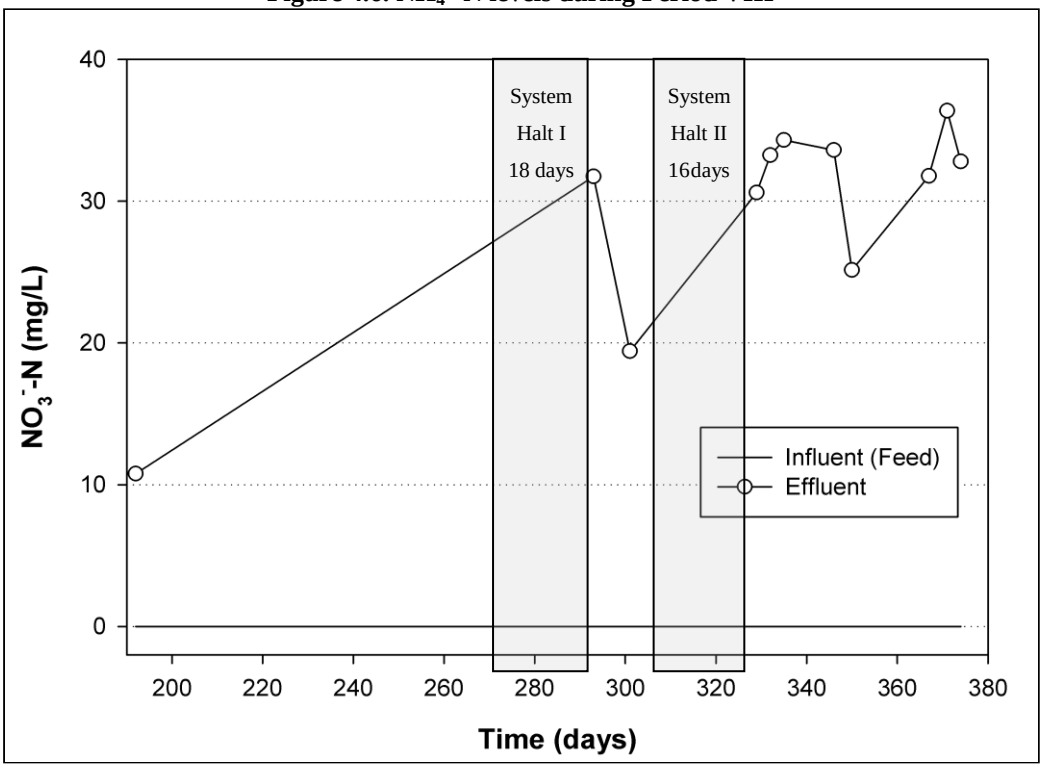

Figure 4.7. NO_3^--N levels during Period VIII

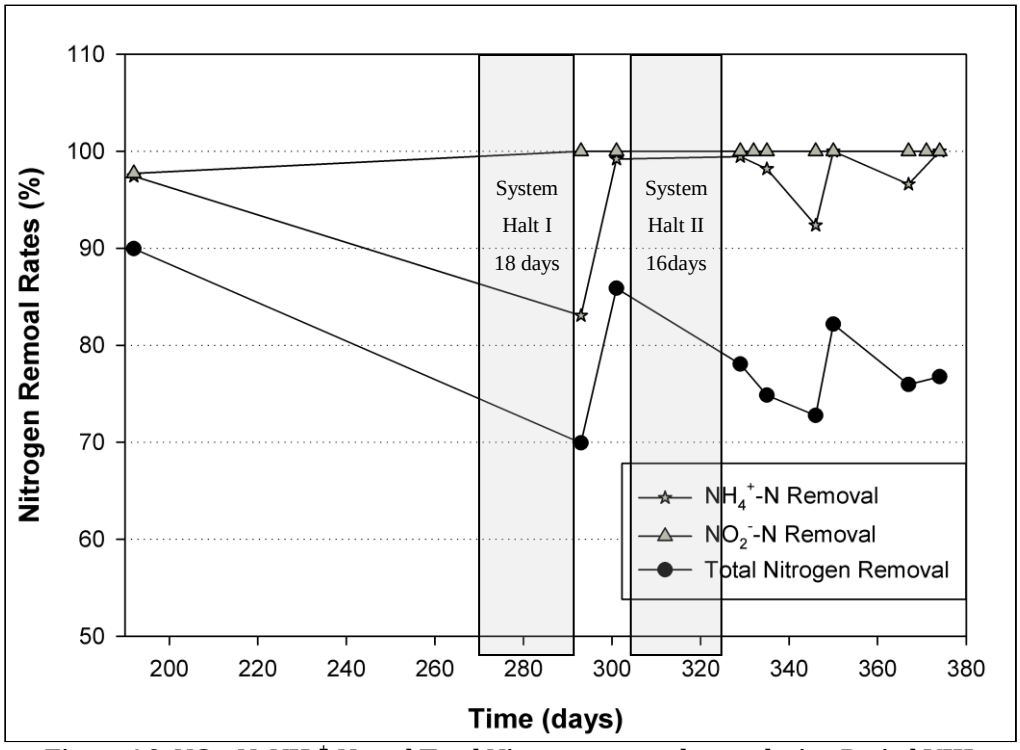

Figure 4.8. NO_2^--N, NH_4^+-N, and Total Nitrogen removal rates during Period VIII

NO_2^- consumption and NO_3^- production in mg-N were calculated for each sampling day and divided to NH_4^+ consumption in mg-N. Those rates for Period VIII have been given at Table 4.1 in order to evaluate the reactors performance for anammox stoichiometry.

Although the ammonium levels were severely fluctuating and nitrate levels were both fluctuating and having an increasing trend, nitrite removal rate was sustained at 100% until the end of Period VIII (Figure 4.6, 4.7, and 4.8).

Table 4.2. The reactor's stoichiometric ratios regarding anammox reaction during Period VIII

Day	NO_2^--N removal / NH_4^+-H removal ratio	NO_3^--N production / NH_4^+-H removal ratio
192	1.24	0.18
272-290	System Halt I	
293	1.49	0.61
301	1.25	0.31
306-322	System Halt II	
329	1.25	0.49
335	1.26	0.55
346	1.34	0.58
350	1.24	0.40
367	1.28	0.52
374	1.24	0.52
Anammox Reaction	1.32	0.26

4.1.8.1 System Halt I

Between Day 272 and 290, the system was halted for 18 days due to problems in Ar/CO_2 gas supply which was required for deoxygenation during feeding. In order to minimize microbial activity and associated damage, whole system was shutdown, including mixing and temperature control.

Three days after the restart, at Day 293, nitrite removal was 100% but ammonium and nitrate levels were elevated (Figure 4.6, 4.7, and 4.8). Accordingly, corresponding stoichiometric ratios were significantly altered (Table 4.2). A possible cause may have been the decay of microorganisms during the halt. Even if so, the decay should have been limited because the elevations were limited and four days later, at Day 301, the elevated nitrate level dropped, ammonium removal rate reached back to 99%, and stoichiometric ratios got close to stoichiometry of anammox reaction as well as zero nitrite presence in the effluent (Figure 4.5, 4.7, 4.8 and Table 4.2). In other words, the system was recovered within 11 days.

4.1.8.2 System Halt II

Between Day 306 and 322, the system was halted again for 16 days due to the very same reason. The same precautions were taken as in System Halt I.

The nitrite and ammonium removal was not effected from System Halt II (Figure 4.5, 4.6, and 4.8). On the other hand, nitrate production increased and consequently Total Nitrogen removal decreased (Figure 4.7 and 4.8). Therefore, stoichiometric ratio of nitrate was shifted (Table 4.2).

4.1.8.3 Irregular feeding

After System Halt II, the reactor was feed irregularly as described above.

During this period, the nitrite removal remained 100%. This can be comprehended as the success of the feed's 1.25 NO_2^--N/NH_4^--N aiming complete removal of nitrite.

Ammonium removal decreased time to time (Figure 4.5 and 4.8). Minimum levels were within the capacity of anammox reaction without giving necessity to explain nitrite removal by any other means. Those lower ammonium removals coincided with higher nitrate production (Figure 4.6 and 4.7), which yielded higher "NO_3^--N production to NH_4^+-H removal ratio" (Table 4.2).

4.2 Evaluation of Microbial Community

Community structure of the reactor was tried to be evaluated by running PCR products of sludge samples on DGGE.

4.2.1 PCR

Two different primer couples were used for PCR.

The first primer couple (341F and 907R) was targeting 16S rRNA gene in all prokaryots (Lane et al., 1985; Muyzer et al., 1993). This primer couple has been used commonly for determination or evaluation of microbial communities in DGGE studies including anammox based ones (Nakagawa et al., 2006; Innerebnera et al.,

2007; Sahinkaya et al., 2007; Kataoka et al., 2008; Liu et al., 2008; Yan et al., 2008; Sànchez-Melsió et al., 2009; Xiao et al., 2009).

The second primer couple (U1F and U1R) is targeting a reserved region present in both 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes (Rivas et al., 2003). Therefore, this primer couple promises determination of all organisms in the sludge.

U1F corresponds to *E. coli* positions 909–932 and U1F corresponds to *E. coli* positions 1383–1404 (Rivas et al., 2003), yielding PCR products of around 450 to 500 bp. This length might have been fairly close to the length of PCR products by the first primer couple (around 566 bp), in order to let both type of PCR products to be run at the same denaturation gradient gel.

In all DGGE studies, to the author's best knowledge, a GC clamp, which is 40 bp length guanine and cytosine rich oligomer, was added to the 5' end of the forward primer. In the absence of GC clamb, separation of the two strands of PCR products has higher chance during DGGE run, which shall devastate DGGE operation (Muyzer et al., 1993). The GC clamp creates a strongly bonding long site for the PCR product which does not split apart even at higher denaturant concentrations (Sheffield et al., 1989). Therefore, a GC clamp was included in 5' end of the both forward primers.

As can be seen on agarose gel electrophoresis image (Figure 4.9), polymerization of all DNA samples by PCR with both primer sets was achieved. In order to address each PCR product, a simple coding has been issued as in Table 4.3.

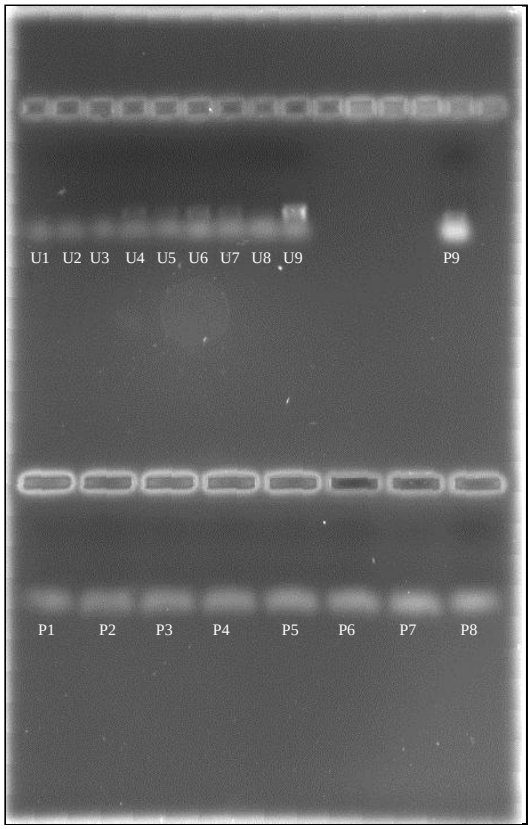

Figure 4.9. Image of agarose gel electrophoresis of all PCR products

Table 4.3. Coding for PCR products and sludge samples

Sludge Sampling Day	Period	Sludge Sample	PCR Products	
			341F and 907R (Prokaryotic)	U1F and U1R (Universal)
35	II	S1	P1	U1
63	II	S2	P2	U2
138	V	S3	P3	U3
179	VII	S4	P4	U4
273	VIII (Beginning of System Halt I)	S5	P5	U5
280	VIII (Middle of System Halt I)	S6	P6	U6
294	VIII (After System Halt I)	S7	P7	U7
332	VIII (After System Halt II)	S8	P8	U8
402	VIII (After long irregular feeding period)	S9	P9	U9

4.2.2 DGGE

First three deductions from the gel images are that band occurrence decreases with increasing sample storing time, that denaturant concentrations of DGG2 were two low for PCR products of the bacterial primers and vice versa for PCR products of the universal primers and that universal primers yielded weak or practically no bands as seen in Figure 4.10 and 4.11. Those results will be discussed before discussion of DGGE bands for evaluation microbial community.

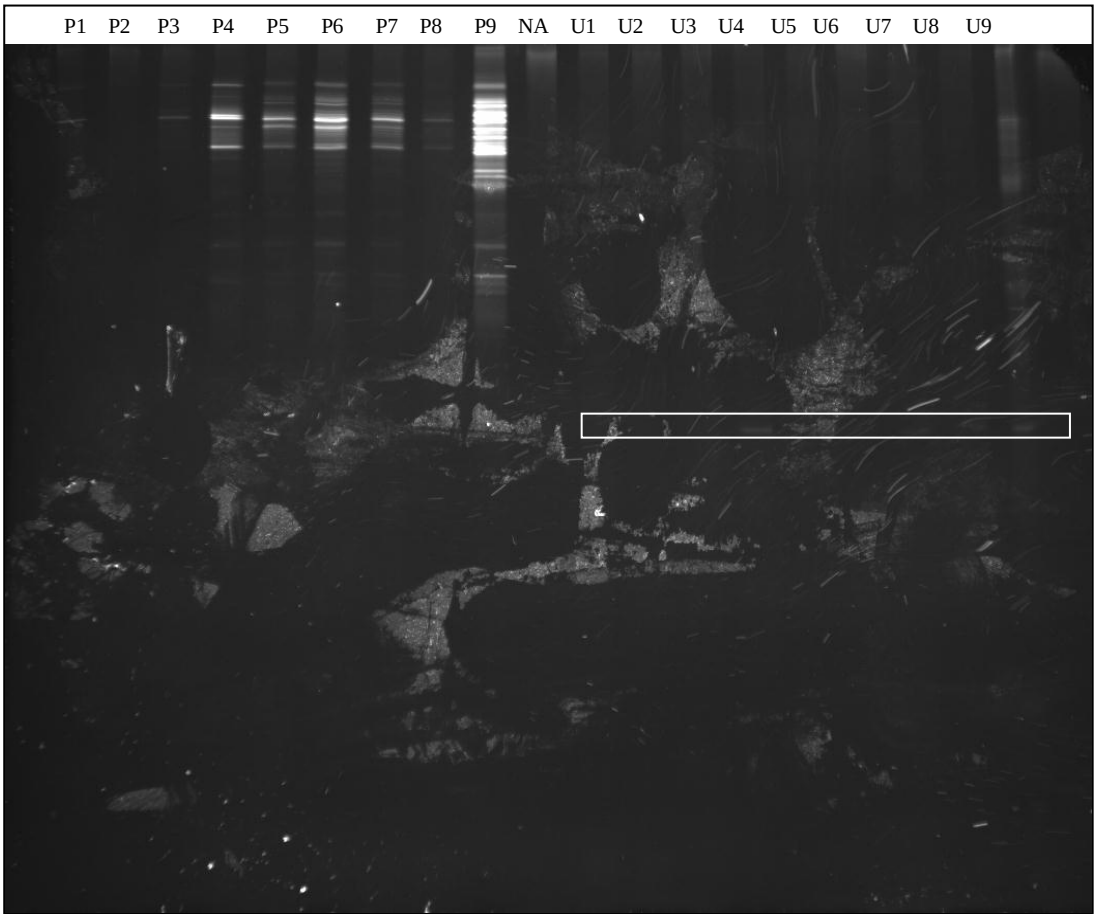

Figure 4.10. Image of denaturation gradient gel (DGG1, 40% to 70%)

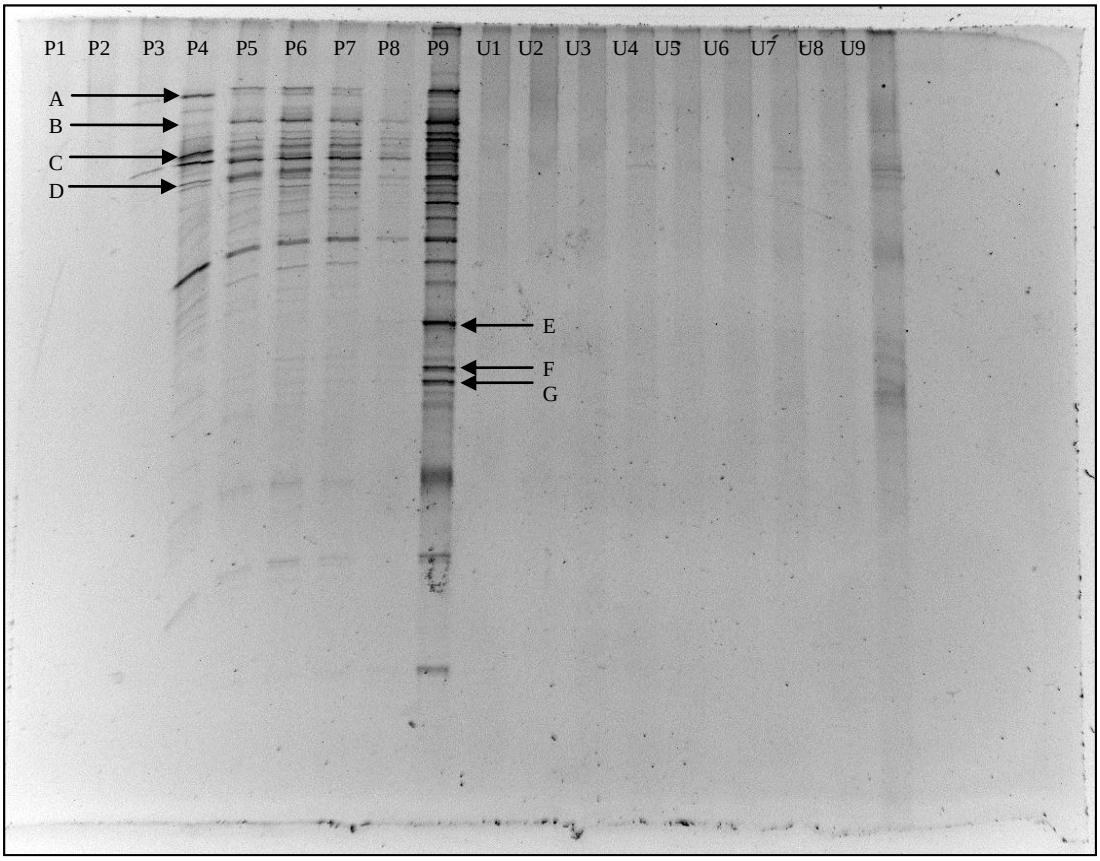

Figure 4.11. Image of denaturation gradient gel (DGG2, 35% to 60%)

4.2.2.1 Decreasing band occurrence with increasing sample age

It is known that extended period of storage and freezing samples before DNA extraction lowers the quality and quantity of the extracted DNA (Vu et al., 1999; Gessoni et al., 2004).

Corinaldesi et al. (2005) demonstrated that frozen coastal and deep-sea sediment samples lacked amplifiable 16 rDNA in extracellular material. Luna et al. (2006) comprehended this finding as absence of bacterial cellular DNA release during the freezing and de-freezing process. On the other hand repetitive freezing and de-freezing is commonly used for cell lyses including DNA extraction procedures as mentioned by Luna et al. (2006). Therefore, bacterial DNAs should be released to extracellular environment together with other cytoplasmic materials including nucleases of both prokaryotic and eukaryotic origin. In such extracellular environments, released intracellular DNases of an organism are likely to cleave released DNAs of other organisms in addition to extracellular DNase activity. For

example, DNases of organisms with methylated DNA may chop DNAs of non-methylated DNA and vice versa. This can be suggested as an explanation of the findings of Corinaldesi et al. (2005). In that case, repetitive freezing and de-freezing is expected to enhance degeneration total DNA in a sample due to increased cell lyses.

The last sludge sample was taken at the arrival of DNA extraction kit. Therefore, all samples but S9 had to be stored as sludge before DNA extraction.

As described under "3.4.2 Sample preparation and VSS equalization", all sludge samples but S9 was de-frozen for VSS measurement at the sampling day of S9. Then, all sludge samples were frozen and de-frozen before DNA extraction which was performed one day after the first de-frosting. To sum up, S9 was de-frosted once but the other samples were de-frosted twice. Therefore, the high intensity and high number of visible bands for S9 can be explained by number of frosting and de-frosting.

On the other hand frosting and de-frosting is not likely to explain absence or near absence of visible bands in P1, P2, and P3. Absence of significant microbial community may not be valid also, because source sludge used for inoculation was biochemically active and even at the beginning of the study there was biochemical activity (Table 4.1, 4.2, 4.3, and 4.4). A possible explanation, other than an unknown electric cut between third and fourth sampling, might be relatively longer refrigeration periods of those three sludge samples.

4.2.2.2 Poor universal primer bands

PCR products of the universal primers yielded very weak bands or no bands at all. It was expected to observe at least the same number of band with the PCR products of the other primer couple because both primer sets target all prokaryotic taxons and the universal primers targets eukaryotic taxons too, and it is assumed that DGGE can separate PCR products of each taxon as different band on a line.

As explained in "3.4.4. Polymerase chain reaction (PCR)", same amounts of primers were used in PCR of both primer sets for same aliquot of DNA templates.

But, Table 3.1 shows that each primer has different number of alternative nucleotide points. 341F has no alternative nucleotide point. 907R has 2 points with two alternative nucleotides, which yields 4 different oligonucleotide sequences, each of which yields one fourth of the primer molecules in its stock solution. Similarly, U1F has 9 points with two alternative nucleotides, which yields 512 different oligonucleotide sequences. Finally, U1R has 5 points with two alternative nucleotides, which yields 32 different oligonucleotide sequences.

One single taxon would have only one DNA sequence for complementary sites of the primers. Therefore, for PCR amplification of that taxon's target zone, the PCR solution will contain 512 times diluted U1F concentration and decreased U1F amount. While dilution hinders the performance of the reaction, such a decreased amount is enough to consume primers of 9 PCR cycles. Such PCR solutions would be prone to contain single strand PCR products which can go faster than any double stranded PCR product of the same PCR reaction in DGGE. This can also explain the double band formation in agarose gel electrophoresis (Figure 4.9) as well as weak bands or no bands at all in DGG1 and DGG2 (Figure 4.10 and Figure 4.11).

Increasing the concentration of primers having points with alternative nucleotides by factors equal to number of different oligonucleotide sequences may solve this problem.

4.2.2.3 Choosing denaturation gradient range for PCR products

Bands of PCR products of the bacterial primers stayed within the first half of DGG1. Therefore, several bands were unrepeatable or even on top of each other. In the lower gradient range of DGG2, they reached near the forward edge (Figure 4.10 and 4.11). Therefore, it can be safely stated that 6% acrylamide gel with 35% to 70% denaturation gradient is suitable for electrophoresis of PCR products of GC-341F and 907R primers with electrophoretic conditions of 60 °C, 85 V, and 16 hours in 1x TAE buffer.

On the other hand, neither of the gels worked for PCR products of the universal primer set. In DGG1, leading bands marked by a rectangular perimeter on Figure 4.10 run haft of the gel. On the other hand, those leading bands did not appear in

DGG2, meaning that they run out of the gel. When proximity of the leading bands of the universal primers to the leading bands of the bacterial primers in DGG1 is considered, chemistry of DGG2 might be sufficient for running PCR products of the universal primers if one of the following changes is applied:

1) Decreasing voltage to 75 V or 80 V,

2) Decreasing run time to 14 or 15 hours,

3) Increasing the length of the gradient gel.

4.2.2.4 Evaluation of DGGE bands for microbial community

Due to above mentioned problems, comparison of same bands on different lines based on only their luminescence is not likely to be meaningful. On the other hand, band composition in one line can be compared with other band compositions. This may allow us to deduce information about presence of an effect of changing operational conditions on prokaryotic community by comparing P4 to P9 at Figure 4.10 and Figure 4.11. The bands significant in this discussion were labeled on only Figure 4.11. Although the same conclusions are possible by Figure 4.10, its bands were not labeled because of the low resolution of the bands and high band overlaps.

The main difference between P4 to P5 sampling is that nitrogen concentration in the feed and nitrogen loading rate were increased (Table 3.2). This change resulted in decreasing abundance of Band A and C, increasing abundance of band B and D, and appearance of a new band above Band A (Figure 4.11). Therefore, it can be deduced that increasing NO_2^- and NH_4^- concentrations and increasing nitrogen load had significantly affected prokaryotic community structure. This change is expected to be on the favor of anammox bacteria, which complies with that "NO_2^--N consumption to NH_4^--N consumption ratio" was not changed significantly but "NO_3^--N production to NH_4^--N consumption ratio" decreased almost to anammox stoichiometry (Table 4.1).

From P5 to P8, there is no significant change in band composition (Figure 4.11). Such unchanging community structure can explain quick recovery of the

system following elevated ammonium and nitrate levels immediately after System Halt I. If the assumption of decaying organisms as an explanation elevated levels of ammonium and nitrate is true, then the decaying organisms were likely to be eukaryotic (see 4.1.8.1. System Halt I). The reactor was not affected from System Halt I, which also complies with stability of the prokaryotic community (see 4.1.8.2. System Halt II).

P9 has appearance of Band E and significant increase in the abundance of Band F and G (Figure 4.11). Therefore, irregular feeding seems to have a significant effect on community structure.

5. CONCLUSION

This study has shown the following regarding anammox enrichment and its prokaryotic community structure:

1) Successful anammox enrichment also in sequencing batch reactors can be achieved by following the guide line for anammox enrichment described by Uyanik et al. (2011).

2) Simultaneous increase of NO_2^- and NH_4^+ concentrations by 25% can be tolerated immediately if steady state has been achieved or in less than 20 days if the reactor has recently reached to pick performance, but it can alter prokaryotic community.

3) Short term reactor shutdown (up to 18 days) can be well tolerated after steady state has been established, but it may not affect the prokaryotic community structure.

4) Long term irregularity in feeding frequency may not affect performance of the reactor but it can alter prokaryotic community structure.

5) It can be safely stated that 6% acrylamide gel with 35% to 70% denaturation gradient is suitable for electrophoresis of PCR products by GC-341F and 907R primers for anammox sludge sample with electrophoretic conditions of 60 °C, 85 V, and 16 hours in 1x TAE buffer.

During DGGE studies, old sludge samples and PCR of U1F and U1R primer couple did not resulted in comprehendible results by means of project goals. Therefore, development of prokaryotic community in early stages of the study and all of the eukaryotic microbial community went beyond comprehension. In order to obtain meaningful data in terms of interpreting microbial community structure, following precautions can be suggested for future studies:

1) DNA of sludge samples should be extracted immediately after sampling and DNA samples should be stored instead of sludge samples.

2) Increasing the primer concentrations in PCR reaction solutions according to number of single sites with two alternative nucleotides may be useful for obtaining successful PCR products.

3) DGGE of PCR products by U1F and U2R primer couple will likely to be successful if voltage is decreased to 75 V or 80 V, if run time is decreased to 14 or 15 hours, or if a different DGGE system is used with longer gel length.

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RESUME

The author was born in 1978 at İzmir. After finishing primary and high education in Adana, he moved to Ankara for undergraduate study and received his B.Sc. degree on Biology from Middle East Technical University, in 2002. His first serious research experience was the internship at the Netherlands Institute of Ecology.

After graduation and completion of military service, he worked at regulatory affairs department of a human vaccine company as translator, as translation responsible and as regulatory responsible, in a clinical research company as a clinical trial assistant, even as international trade responsible in a pharmaceutical company. Although they were economically satisfactory, all those experiences helped him to realize that he was born to be an academician.

He moved to Urfa for M.Sc. study at Environmental Engineering Department of Harran University, in 2008.

He started to work at Environmental Engineering Department of Bitlis Eren University, in July 2011.

SUMMARY

In order to study reactor performance and microbial community structure during anammox startup, a sequencing batch reactor was set up. The reactor was seeded with 1.75 L denitrification sludge and 5 mL enriched anammox culture. It was operated under different conditions for 192 days for anammox enrichment. The enrichment was divided into 7 periods according to changing operational conditions. That was followed by an 8^{th} period with irregular feeding and maintenance.

NO_2^-, NO_3^-, NH_4^+ concentrations of the effluent was measured in order to evaluate the reactor's performance. Sludge samples were collected and their DNA was isolated for DGGE, in order to evaluate microbial community structure.

Studies on the reactor's performance showed that simultaneous increase of NO_2^- and NH_4^+ concentrations by 25% in the feed was tolerated immediately when steady state had been achieved or in less than 20 days if the reactor had recently reached to its pick performance, that short term reactor shutdown (up to 18 days) was well tolerated after steady state had been established, that the long term irregularity in feeding frequency did not affect performance of the reactor.

Results of DGGE studies did not allow a detailed analysis of the microbial community structure. First, older samples did not yielded DGGE bands at all. Extraction of DNA form sludge samples immediately after sampling and refrigerated storage of DNA samples instead of sludge samples will likely to solve this problem in further studies. Second, PCR products of U1F and U1R primer couple did not yield comprehendible DGGE bands. Increasing the primer concentrations in PCR reaction solutions according to number of single sites with two alternative nucleotides may be useful for obtaining successful PCR products by U1F and U1R. Third, neither of the DGGE gels were optimum for running PCR products of U1F and U1R primer couple. DGGE of PCR products by U1F and U2R primer couple will likely to be successful if voltage is decreased to 75 V or 80 V, if run time is decreased to 14 or 15 hours, or if a different DGGE system is used with longer gel length.

Based on the DGGE images, it was assumed that increasing NO_2^- and NH_4^+ concentrations accompanied by increasing nitrogen loading rate altered prokaryotic community structure, that short term (up to 18 days) system shutdown did not affect the prokaryotic community structure, and that long term irregular feeding (>70 days) altered the prokaryotic community structure.

ÖZET

Anammox başlangıç evresinde reaktör performansı ve mikrobik komünite yapısının incelenmesi için, bir sıralı kesikli reaktör kurulmuştur. Reaktöre 1.75 L denitrifikasyon çamuru konularak 5 mL zenginleştirilmiş anammox kültürü aşılanmıştır. Anammox zenginleştirmesi için 192 gün boyunca farklı koşunlarda işletilmiştir. Zenginleştirme, değişen işletim koşullarına göre 7 döneme ayrılmıştır. Zenginleştirmeyi, düzensiz beslemeli ve işletimli sekizinci bir dönem takip etmiştir.

Reaktörün performansını değerlendirmek için çıkış suyunda NO_2^-, NO_3^-, NH_4^+ konsantrasyonları ölçülmüştür. Mikrobik komünite yapısını değerlendirmek için çamur örnekleri alınmış ve DDGE çalışması için DNA'ları izole edilmiştir.

Reaktör performansı üzerine çalışmalar göstermiştir ki; reaktör kararlı duruma ulaşmışsa veya en yüksek performansına ulaştıktan sonraki 20 gün içerisinde ise, besindeki NO_2^- ve NH_4^+'nın eş zamanlı olarak %25 arttırılması, anında tolere edilmiştir; reaktörün kısa bir süre için durdurulması (18 güne kadar), kararlı durum oluştuktan sonra kolayca tolere edilmiştir; besleme sıklığındaki uzun süreli düzensizlikler reaktör performansını etkilememiştir.

DGGE çalışmasının sonuçları, detaylı bir mikrobik komünite yapısı analizine imkan tanımamıştır. İlk olarak, yaşlı örneklerde, neredeyse hiç DGGE bandı gözlenmemiştir. İleriki çalışmalarda, örneklemeden hemen sonra çamur örneklerinden DNA ekstrakte edilmesi ve çamur örnekleri yerine ekstrakte DNA örneklerinin buzlukta saklanmasının bu sorunu çözmesi olasıdır. İkinci olarak, U1F ve U1R primer çiftinin PCR ürünleri, değerlendirilebilir DGGE bantları oluşturmamıştır. İki alternatif nükleotidli tek noktaların sayısına göre PCR tepkime çözeltisinde primer konsantrasyonlarının arttırılması, U1F ve U1R primer çiftinin PCR ürünlerinin başarı ile elde edilmesinde faydalı olabilir. Üçüncü olarak, DGGE jellerinin her ikisi de U1F ve U1R primer çiftinin PCR ürünlerinin yürütülmesinde optimum olmamıştır. Voltajın 75-80 V'ye düşürülmesi, yürütme süresinin 14-15 saate düşürülmesi veya daha uzun jel uzunluğuna sahip bir başka DGGE sistemi

kullanılması durumunda U1F ve U2F primer çiftinin PCR ürünlerinin DGGE çalışması başarılı olabilir.

DGGE görüntülerinden anlaşılmıştır ki; artan azot yüküyle NO_2^- ve NH_4^+ konsantrasyonları, prokaryotik komünite yapısını değiştirmiştir; sistemin kısa süreyle durdurulması (18 güne kadar), prokaryotik komünite yapısını etkilememiştir; ve uzun süreli düzensiz besleme (>70 gün), prokaryotik komünite yapısını değiştirmiştir.