MARMARA UNIVERSITY

INSTITUTE FOR GRADUATE STUDIES

ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) IN A MOVING BED BIOFILM REACTOR (MBBR) FOR MAINSTREAM TREATMENT: A PILOT STUDY IN ISTANBUL ATAKOY SEWAGE TREATMENT PLANT (STP)

SUMEYYE CELIK

MASTER THESIS

Department of Environmental Engineering

THESIS SUPERVISOR

Bilge Alpaslan Kocamemi, PhD

Assoc.Prof

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Sümeyye ÇELİK, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "**Anaerobic Amonium Oxidation (Anammox) in a Moving Bed Biofilm Reactor (MBBR) For Mainstream Treatment: A Pilot Study in Istanbul Atakoy Sewage Treatment Plant**", on August 17, 2018 and has been found to be satisfactory by the jury members.

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ÖZET

HAREKETLİ YATAK BİYOFİLM REAKTÖRÜNDE (HYBR) ANAEROBİK AMONYUM OKSİDASYONU İLE ANA AKIM ATIKSULARININ ARITIMI: İSTANBUL ATAKÖY EVSEL ATIKSU ARITMA TESİSİ PİLOT ÇALIŞMASI

Atıksu arıtmında biyofilm sistemleri askıda büyüyen aktif çamur sistemlerine göre bir çok avantaja sahiptir. Özellikle Anammox gibi yavaş büyüme hızına sahip mikroorganizmalarla çalışan sistemlerde etkilidir. Bir çok uygulama şekli bulunan biyofilm sistemlerinden biri de tam karışımlı ve sürekli akış olarak işletilebilen hareketli yatak biyofilm rekatörleridir (HYBR). Anammox bakterilerinin keşfedilmesinden sonra değişen azot döngüsü ile atıksuların arıtımında yüksek hava ihtiyacı olan nitrifikasyon ve fazla çamur üreten, organik karbon ihtiyacı yüksek olan denitrifikasyon proseslerine kısmi nitrifikasyon ardından uygulanan Anammox prosesi (deammonifikasyon prosesi) ekonomik bir alternatif olmuştur.

HYBR Anammox sistemlerinin uzun başlangıç periyodlarını kısatmak için genel yaklaşım üzerlerinde önceden bakteri üretilmiş hazır medyaları kullanmaktır. Ancak, bakterili hazır medyalar lokal olarak her zaman mevcut olmamaktadır. Bu nedenle, demmonifikasyon HYBR sistemleri için Anammox aşılaması yapılmadan lokal aktif çamurun ana akım suyu ile beslenilerek optimum bir Anammox zenginleştirme stratejisinin geliştirilmesi için ilave araştırma yapılması gerekmektedir.

Bu çalışmada Anammox aşılaması yapılmadan lokal aktif çamur ve boş taşıyıcı malzeme kullanılarak Istanbul Atakoy AAT'ne kurulan pilot ölçekli HYBR sisteminde ana akım kısmi nitrifikasyon - Anammox prosesinin ilk işletmeye alma ve Anammox biyofilmi oluşturma aşamaları 374 günlük işletim sürecinde gözlemlenmiştir. Biyofilm oluşturma aşamasını etkileyen temel faktörler belirlenerek Anammox biyofilmi oluşması aşamasında gerçekleşen tüm prosesler detaylı olarak incelenmiştir.

Anahtar sözcükler: anaerobik amonyum oksidasyonu, azot giderimi, evsel atıksu, hareketli yatak biyofilm reaktör

ABSTRACT

ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) IN A MOVING BED BIOFILM REACTOR (MBBR) FOR SIDESTREAM TREATMENT: A PILOT STUDY IN ISTANBUL ATAKOY SEWAGE TREATMENT PLANT (STP)

Biofilm-based technologies have several advantages over floc-based systems and are very efficient for processes performed by slowly growing microorganisms, e.g. Anammox. One of these technologies is the moving bed biofilm reactors (MBBR) which are completely mixed and continuously operated systems. Currently, partial nitritation followed by Anammox process (deammonification) has gained great interest as a promising economic alternative for high amount of oxygen requiring nitrification and high organic carbon consuming and high amount of sludge producing denitrification processes.

The common approach to start a new deammonification MBBR system is to use pre-colonized media in order to shorten start-up period due to having long doubling time of Anammox species. However, pre-colonized media is not always available locally. Hence, further research is necessary to develop an optimum Anammox enrichment strategy for deammonification MBBR systems without Anammox pre-seeding and feeding local activated sludge seed with mainstream wastewater.

In this study, the start-up and Anammox biofilm development phases of mainstream partial nitritation-Anammox process was observed for 374 days in a pilot MBBR in Istanbul Atakoy STP without Anammox seeding using virgin carriers. The major factors affecting initial biofilm development on virgin carriers and all processes occurring during the development of Anammox biofilm were deeply investigated.

Keywords: anaerobic ammonium oxidation, mainstream, moving bed biofilm reactor, nitrogen removal

CLAIM FOR ORIGINALITY

The aim of this study is to investigate nitrogen removal from mainstream of STP using partial nitritation followed by Anammox process called as deammonification process. Nitrogen removal from mainstream using Anammox is a process still under investigation in all over the world. In Turkey, there is one two-stage granular deammonification pilot, which was constructed in Bursa Dogu STP in 2015 (Uflaz, 2018). The pilot plant, which has been constructed in Istanbul Atakoy STP in the scope of this thesis, is the first one-stage Moving Bed Biofilm Reactor (MBBR) type mainstream deammonification pilot plant in Turkey. The findings of the study will provide insight for the installation of full-scale mainstream Anammox applications in Turkey.

SYMBOLS

μ	: Specific growth rate of bacteria, g new cells/g cells.d
μ_m	: Maximum specific growth rate of bacteria, g new cells/g cells.d
K_n	: Half-velocity constant, g/m^3
K_d	: Endogenous decay coefficient, g VSS/g VSS.d
NH_4^+-N	: Ammonium ion
NH_3	: Ammonia
HCO_3^-	: Bicarbonate
N_2H_4	: Hydrazine
NH_2OH	: Hydroxyl-amine
NO_2^--N	: Nitrite
NO_3^--N	: Nitrate
g	: Gram
d	: Day
N	: Nitrogen
NO	: Nitric oxide
N_2	: Nitrogen gas
l	: Liter
m^2	: Meter square
CH_3OH	: Methanol
m^3	: Meter cube
mg	: Miligram
O_2	: Oxygen
PO_4^{3-}	: Phosphate
mm	: milimeter
N	: Nitrogen
^oC	: Degree celsius
pH	: Activity of hydrogen ion
rpm	: Revolutions per minute
t	: Time
T	: Temperature

ABBREVIATIONS

Accum	: Accumulation
AerAOB	: Ammonium oxidizing bacteria
AFM	: Atomic force microscopy
AMO	: Ammonia monooxygenase
AHO	: Ammonia oxidizing archaea
AnAOB	: Anaerobic ammonia-oxidizing bacteria
DO	: Dissolved oxygen
COD	: Chemical oxygen demand
CSTR	: Continuously stirred tank reactor
DNA	: Deoxyribonucleic acid
EDS	: Energy-dispersive X-ray spectroscopy
EPS	: Extracellular polymeric substances
EFF	: Effluent
FA	: Free ammonia
FTIR	: Fourier transform infrared
HRT	: Hydraulic retention time
HZO	: Hydrolase
HRT	: Hydraulic retention time
HZO	: Hydroxylamine oxidoreductase
HZO	: Hydroxylamine oxidoreductase
INF	: Influent
NOB	: Nitrite oxidizing bacteria
NOR	: Nitrite oxidoreductase
OHO	: Ordinary heterotrophic organism
OLR	: Organic loading rate
PCR	: Polymerase chain reaction
PP	: Polypropylene
PROD	: Produce
PTFE	: Polytetrafluoroethylene
PVC	: Polyvinyl chloride

rRNA	: Ribosomal ribonucleic acid
SEM	: Scanning electron microscopy
SRT	: Sludge retention time
STP	: Sludge treatment plant
TAN	: Total ammonium nitrogen
TGA	: Thermogravimetric analyses
TKN	: Total Kjeldahl nitrogen
TS	: Total solids
VSS	: Volatile suspended solids
STP	: Sewage treatment plant
XRD	: X-ray diffraction

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1. INTRODUCTION

1.1. Biological Nitrogen Removal from Wastewater

The municipal wastewater containing organic nitrogen and phosphorus has a serious environmental pollution potential. The removal of ammonium is of crucial point due to toxic effect on the aquatic organisms (Khin and Annachhatre, 2004). Ammonium can be treated from wastewaters by applying some processes. These methods can be physical, chemical or biological. Among them biological process is the most feasible and economical way to eliminate of nitrogen from the wastewater. These systems are built on the knowledge of microbial nitrogen cycling (Figure 1.1). The well-known nitrification and denitrification processes, are the most common biological methods nitrogen removal. Recently, novel nitrogen removal processes have developed in view of newly discovered biological pathways. These processes include mainly the ANaerobic AMMonium OXidation (Anammox) (van der Star et al., 2007).

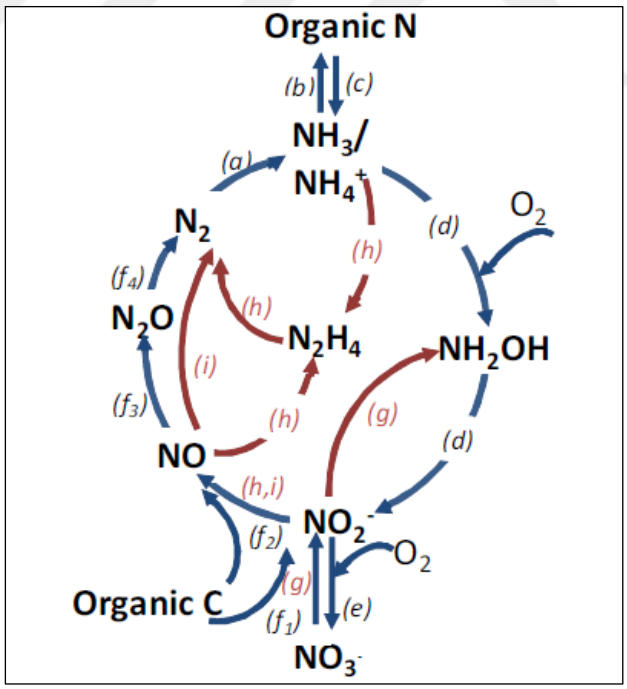


Figure 1.1 Major biological nitrogen removal processes in wastewater treatment (Langone, 2013)

1.1.1. Nitrification

The biological conversion of ammonium (NH_4^+) or ammonia (NH_3) to nitrate (NO_3^-) nitrogen is known as nitrification. Nitrification occurs in 2 steps. Each step involves different species of bacteria. In nitritation step ammonia-oxidizing bacteria AerAOB oxidize NH_4^+ to nitrite (NO_2^-). The second nitratation step NO_2^- oxidize to NO_3^- by nitrite oxidizing bacteria (NOB). These reactions are accompanied to each other and turned rapidly to the NO_3^- form; so that, NO_2^- concentration generally low (Khin and Annachhatre, 2004).

1.1.1.1. Stoichiometry of nitrification

Bacteria carrying out NH_3 oxidation to NO_3^- are autotrophs (chemolithotrophs) which use reduced nitrogen species for energy (electron donor) and fix CO_2 for cell compound synthesis. AerAOB use NH_3/NH_4^+ (oxidation state -3) as shown Equation 1.1.1 and NOB use NO_2^- (oxidation state +3) as electron donors as shown Equation 1.1.2. Product of AerAOB) metabolism is NO_2^- product of NOB metabolism is NO_3^-. Generally, but not always, oxygen is the electron acceptor as shown on the Equation 1.1.3.

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2 \qquad \Delta G° = -275 \text{ kJ mol}^{-1} \qquad (1.1.1)$$

$$NO_2^- + 5O_2 \rightarrow NO_3^- \qquad \Delta G° = -74 \text{ kJ mol}^{-1} \qquad (1.1.2)$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \qquad \Delta G° = -360 \text{ kJ mol}^{-1} \qquad (1.1.3)$$

In order for this reaction to go to competition, 4.57 mg of Oxygen (O_2) is required per mg NH_4^+ -N. Assimilative reactions occur during nitrification as shown Equation 1.1.4;

$$NH_4^+ + 4CO_2 + HCO_3^- + H_2O \rightarrow C_5H_7NO_2 + 5O_2 \quad (1.1.4)$$

In this reaction $C_5H_7NO_2$ represent the empirical formula of bacteria cell (Rittmann and McCarty, 2001).

Based on Equation 1.1.3 and Equation 1.1.4 the overall oxidation and assimilation reaction is;

$$22NH_4^+ + 37O_2 + 4CO_2 + HCO_3^- \rightarrow C_5H_7NO_2 + 21NO_3^- + 20H_2O + 42H^+ \quad (1.1.5)$$

Equation 1.1.5 indicates that alkalinity is consumed during the nitrification process. In common mass unit, the alkalinity consumption is 7.05 g as $CaCO_3$/g NH_4^+-N. From the equations, $\Delta G°$ of NH_4^+oxidation (Equation 1.1.2) is about 4 times greater than NO_2^- oxidation to NO_3^- (Equation 1.1.3).

1.1.1.2. Mechanisms of nitrification

Nitritation process, which is the oxidation of NH_4^+ /NH_3^+ to N_2 by AerAOB, can be divided into three sub-processes:

Step 1: NH_3^+ is oxidized to hydroxylamine (NH_2OH) by the enzyme ammonium monooxygenase (AMO) as shown Equation 1.1.6.

$$NH_3^+ + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1.1.6)$$

Step 2: NH_2OH is oxidized into NO_2^- by the catalysis hydroxylamine oxidoreductase (HAO) as seen Equation 1.1.7.

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (1.1.7)$$

Step 3: In this step oxygen and free hydrogen ions are turned into water (Equation 1.1.8)

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (1.1.8)$$

Equation 1.1.9 summarizes the overall energy reaction of nitritation process;

$$\Sigma: \quad NH_3 + 1.5O_2 \rightarrow NO_2^- + H^+ + H_2O \qquad (1.1.9)$$

Nitratation process, which is the oxidation of NO_2^- to NO_3^- by NOB can be divided two steps; NOB use the enzyme nitrite oxidoreductase (NOR) to conduct the process.

Step 1: NO_2^- is converted to NO_3^- with the catalysis of NOR enzyme as shown Equation 1.1.10.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \qquad (1.1.10)$$

Step 2: In this step oxygen, electrons and protons assemble into water as shown Equation 1.1.11.

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O \qquad (1.1.11)$$

Equation 1.1.12 summarized the overall energy reaction of nitritation process.

$$\Sigma: \quad NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \qquad (1.1.12)$$

1.1.1.3. Phylogeny

Microbial group of AerAOB can be (Figure 1.2) found in anywhere. If there are ammonia which they can consume: soils, freshwater, and marine habitats. AerAOB has slow growth rate in the population.

All isolated chemolithoautotrophic bacteria which are purified, NOB concern to four genera *Nitrobacter, Nitrococcus, Nitrospina* and *Nitrospira*. *Nitrobacter* species have been purified various environments.

NOBs are comprised of five genera (*Nitrobacter , Nitrospina , Nitrospira , Nitrospina* and *Nitrococcus*) which have been found in several lineages of Bacteria (Figure 1.3).

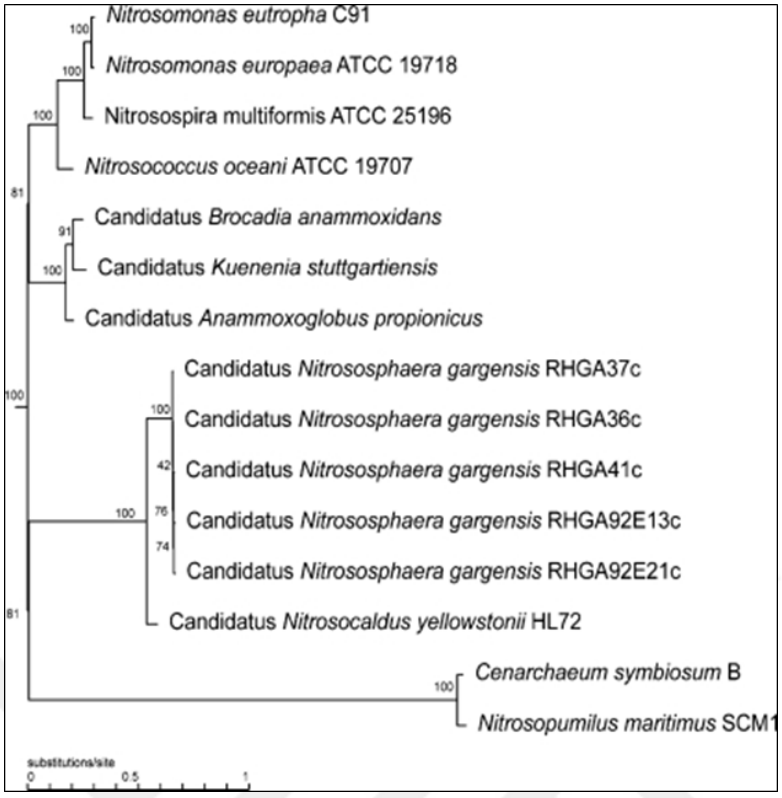


Figure 1.2 Phylogenetic tree of AerAOB

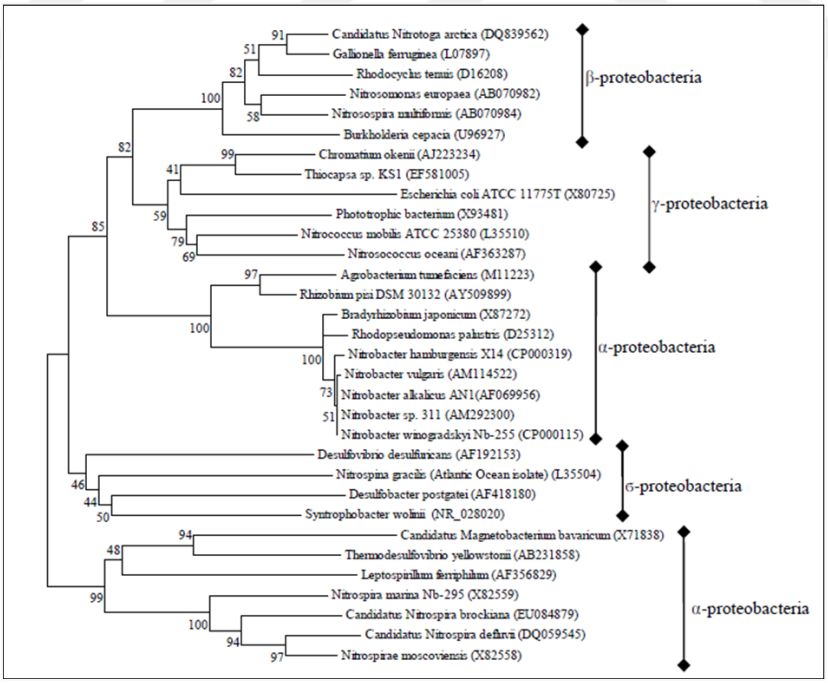


Figure 1.3 Phylogenetic tree of NOB (Daims et al., 2016).

1.1.1.4. Kinetics

The nitrification kinetic is generally mentioned in terms of the reactants as a double Michaelis-Menten function. The first step of nitrification – nitritation is influenced by NH_4^+ and O_2 concentration and the second step – nitratation is affected by NO_2^- and O_2 concentration (Tanaka et al., 1981). Growth kinetics for the NH_4^+ oxidation as given Equation 1.1.13 for nitrification process (Metcalf and Eddy, 2003).

$$\mu_n = \frac{\mu_{nm} N}{K_n + N} k_{dn} \quad (1.1.13)$$

μ_n = specific growth rate of the nitrifying bacteria, g new cells/g cells.d

μ_{nm} = maximum specific growth rate of the nitrifying bacteria, g new cells/g cells.d

N = nitrogen concentration, g/m^3

K_n = half- velocity constant, g/m^3

K_{dn} = endogenous decay coefficient of nitrifies, g VSS/g VSS.d

A great range of maximum specific growth rates have been noticed that directly affiliated to temperature. According to the studies μ_{nm} varies from 0.25 to 0.77 g VSS/g VSS at 20 °C (Metcalf and Eddy, 2003). The μ_{nm} values for the nitrifying bacteria are less according to the values for heterotrophic bacteria, thus requiring too long sludge retention time (SRT) for activated-sludge systems (Metcalf and Eddy, 2003).

Nitrification rates are affected by the level of dissolved oxygen (DO). To account the effects of DO, the expression for the specific growth rate Eqn. 1.1.13 is modified as follows (Eqn. 1.1.14):

$$\mu_n = \left(\frac{\mu_{nm} N}{K_n + N} k_{dn} \right) \left(\frac{DO}{K_O - DO} \right) - k_{do} \quad (1.1.14)$$

In this equation; DO = dissolved oxygen concentration, g/m^3

K_O = half –saturation coefficient for DO, g/m^3

There are several environmental factors (pH, DO, temperature, free ammonia concentration, toxic compounds) directly affecting the kinetics of both nitritation and nitratation steps of nitrification process.

1.1.2. Amonium oxidation by archea

The discovery of ammonia oxidizing archaea (AOA) has radically challenged nitrogen cycle. Until today as known, nitrification first step which oxidation of NH_3 to NO_2^- had catalyzed by AerAOB. AMO enzyme is responsible for the oxidation of NH_3 (Hatzenpichler, 2012). Until recently, Archaea known as obligate extremophiles. Könneke et al. (2005) was reported for the first time nitrification by the Archaea (2005).

1.1.3. Denitrification

The biological reduction of NO_3^- to N_2 (Equation 1.1.15) under limited O_2 conditions by facultative heterotrophic bacteria is called denitrification. NO_3^- is used to oxidize organic carbon instead of molecular O_2 into elemental N_2 and carbon dioxide (CO_2):

$$NO_3^- + organic\ carbon \rightarrow N_2 + CO_2 \quad (1.1.15)$$

Due to denitrification process is a respiratory denitrifying bacteria need an energy source. Denitrifying bacteria utilize complex organic substances such as organic carbon for bacteria metabolism and their demand for removal of one gram of N corresponds to 3-6 grams of COD. If the COD/N ratio of the wastewater becomes too low and additional carbon source like methanol must be added. For the denitrification; C source, low O_2 and sufficient NO_3^- must be present in the system (Martens, 2005).

1.1.3.1. Stoichiometry of denitrification

Biological denitrification pathways occurs by the indicated NO_3^- and NO_2^- reduction reactions. NO_3^- (an electron acceptor) is reduced to nitrogen gas following Equations 1.1.16-1.1.18.

$$0.25O_2 + H^+ + e^- \rightarrow 0.5H_2O \quad (1.1.16)$$

$$0.2NO_3^- + 1.2H^+ + e^- \rightarrow 1.2N_2 + 0.6H_2O \quad (1.1.17)$$

$$0.34NO_2^- + 1.36H^+ + e^- \rightarrow 0.17N_2 + 0.68H_2O \quad (1.1.18)$$

According to the oxidation-reduction half reactions, 0.25 mole of O_2 is equivalent to 0.2 mole NO_3^- for electron transfer. Hence, O_2 equivalent of NO_3^- is (0.25 X 32 g O_2)

divided by the NO_3^- equivalent (0.20 X 14 g N) equals to 2.86 g O_2/ g NO_3^--N (Metcalf and Eddy, 2003).

Dissimilation and synthesis reactions are showed (Equation 1.1.19, Equation 1.1.20).

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \quad (1.1.19)$$

$$NO_2^- + 0.53CH_3OH + 0.67H_2CO_3 \rightarrow 0.04C_5H_7O_2N + 0.48N_2 + 1.23H_2O + HCO_3^- \quad (1.1.20)$$

According to equations, methanol demand for the NO_3^- removal, considering the synthesis requirement. Is 2.46 mg CH_3OH per mg NO_3^--N and corresponding to 1.7 mg COD mg NO_3^--N (Metcalf and Eddy, 2003).

In denitrification process, when the NO_3^--N reduced, 3.57 g of alkalinity as $CaCO_3$ alkalinity is constituted.

1.1.3.2. Mechanism of denitrification

Denitrification is a respiratory process, in which NO_3^- or NO_2^- serve as an electron acceptors and generate ATP as an end product. During the transfer of electrons from the donor to the acceptor, the organism gains energy and bacteria can use this energy for the synthesis reactions (Matějů et al., 1992).

The enzymes associated with denitrification are synthetized under anaerobic conditions, although denitrification process can be under aerobic conditions. (Robertson and Kuenen, 1984). Reduction of NO_3^- to N_2 occurs in 4 steps with following order:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

All these steps are catalyzed by a specific enzyme. Nitrate reductases catalyze to this step. Today seven enzymes are known for the catalyzing the denitrification steps (Zumft 1997; Philippot 2002). At the first pathway of denitrification, NO_3^- decreases to NO_2^- by the help of reductases Nar or Nap. The second step reactions catalyzed by 1 of 2 nitrite reductases, NirK or NirS. NO is reduced to N_2O by the nitric oxide reductases cNor or

qNor. At the last N_2O is converted to N_2. This step is catalyzed by enzyme known as nitrous oxide reductase, Nos (Jones et al., 2008).

1.1.3.3. Phylogeny

Broad class of bacteria have been shown capable of denitrification, but similar microbial capability has not been found in fungi or algae. Bacteria capable of denitrification are both heterotrophic and autotrophic. The heterotrophic organisms include genera which is following: *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacerium, Corynebacterium, Pseudomonas, Vibrio, Rhizobium, Spirillum, Rhodoppseudumonas, Neisseria (Metcalf and Eddy, 2003). Pseudomonas* species are the most common and widely distributed of all the denitrifiers. Most of denitrifiers are facultative aerobic organisms with able to use O_2 and some can also carry out fermentation in the absence of NO_3^- or O_2. Other autotrophic bacteria can denitrify use hydrogen and reduced sulfur compounds as electron donors. Both groups of organisms can grow as an heterotrophic if there is an organic carbon source (Metcalf and Eddy, 2003).

1.1.3.4. Kinetics

Denitrification occurs in 4 steps. ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Usually it is described in 2 steps ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$) as the production of NO and N_2O do not contribute significantly to the total mass balance of nitrogen in the environmental system. Recently, N_2O modeling has received attention due to greenhouse gas effect. Most models only consider N_2O production by denitrifying microorganisms. A three step denitrification model including the reduction of NO_3^-, NO_2^- and NO has been formulated by von Schulthess (Schulthess and Gujer, 1996).

In the bio-kinetic equations describing the denitrification process, soluble substrate utilization rate is controlled by the soluble substrate concentration. NO_3^- concentration controls the substrate utilization kinetics only at very low NO_3^- concentration. NO_3^- serves as an e^- acceptor in the same way as O_2, thus NO_3^- utilization rate is comparatively with the rate of substrate utilization (Metcalf and Eddy, 2003).

Heterotrophic bacteria grow in both anoxic and aerobic tank with NO_3^- and O_2 consumption. The mixed liquor biomass concentration can count with the amount of

BOD removed. Bacteria that are strict aerobes can only use O_2 as an e^- acceptor. The substrate utilization rate r_{SU} modified by term to show a lower utilization rate as follow Equation 1.1.21.

$$r_{SU} = \frac{k\ X\ S_n}{K_s + S} \qquad (1.1.21)$$

n = fraction of denitrifying bacteria in biomass, g VSS/g VSS

k = maximum specific rate of substrate removal, g substrate/gVSS.d

X = concentration of microorganisms (mass/volume)

S = substrate concentration, g/m^3

Ks=half velocity constant, g/m^3

When NO_3^- is used as an e- acceptor instead of O_2, k may be lower than the rate of with O_2 as an e^- acceptor. In Eqn. 1.1.21. k value used is the same as that used for O_2 and any effect of lower kinetic rate is incorporated into the n term. k value found to be similar, whether NO_3^- or O_2 is the e^- acceptor (Metcalf and Eddy, 2003). N value has been found to between 0.20 and 0.80 for pre-anoxic denitrification process which operates domestic wastewater. The kinetics for methanol utilization are such as SRT required for denitrification suspended growth process are in the same range as SRTs for aerobic systems designed for BOD removal only, about 3 to 6 d (Metcalf and Eddy, 2003)

1.1.4. Anaerobic ammonium oxidation (Anammox)

Biological nitrogen removal can also be accomplished by heterotrophic- and autotrophic nitrifying bacteria under certain conditions and by unique bacteria associated with Anammox process. Autotrophic Anammox bacteria use NO_2^- as electron acceptor to oxidize NH_4^+ without DO and produce $N_2(g)$ as an end product (Broda, 1977). Anaerobic ammonium oxidizing (Anammox) bacteria were discovered in a denitrifying bioreactor in 1990. After that the researchers focused on Anammox process and Kuenen found that a great part of the nitrogen losses in the marine environment were due to the activity of Anammox bacteria (Kuenen, 2008). According to the Jetten's research these process is sustainable for the removed of nitrogen (Jetten et al., 2002). Optimum temperature for the growth of AnAOB is between 30 and 35 °C (Jetten et al.,

2002). According to the reports the range for the temperature value is between 20 and 43 °C for the AnAOB, with the optimum range is between 30 and 40 °C (WEF, 2015). Anammox bacteria could not be isolated and grown in pure cultures (Strous et al., 1999).

1.1.4.1. Stoichiometry of Anammox

The stoichiometry of Anammox process is shown through Equations 1.1.22-1.1.24 (Strous et al., 1998).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2.H_2O \qquad \Delta G° = -358 \text{ kJ mol}^{-1} \qquad (1.1.22)$$

$$0.26NO_2^- + 0.066CO_2^- + 0.66H_2O \rightarrow 0.26NO_2^- + 0.066HCO_3^- + 0.26NO_3^- + 0.66CH_2O_{0.5}N_{0.15} \qquad (1.1.23)$$

$$NH_4^+ + 01.32NO_2^- + 0.066CO_2^- + 0.13H^+ \rightarrow 1.2N_2 + 2.03H_2O + 0.26NO_3^- + 0.66CH_2O_{0.5}N_{0.15} \qquad (1.1.24)$$

The sole carbon source for biomass cell synthesis ($CH_2O_{0.5}N_{0.15}$) is bicarbonate (HCO_3^-) specifying these bacteria as chemolithoautotrophs. The oxidation of NO_2^- to NO_3^- produces the reducing equivalents for carbon fixation (WEF, 2015). Anammox reaction is related with the Gibbs free energy of bacteria, which are belong to the phylum of *Planctomycetes*. N_2(g) is the final product of the Anammox process. The nitrogen balance giives a ratio of NH_4^+ to NO_2^- conversion of 1:1.32 and a ratio of NO_2^- conversion to NO_3^- production of 1:0.26 (Equations. 1.1.23, 1.1.24). Nitrate production can be used as a measure of the growth of Anammox bacteria, at it is only involved in the anabolic reaction (Eq. 1.1.24).

Anammox bacteria are autotrophs hence there are used bicarbonate as the carbon source for the biomass generation. 1 mole of HCO_3^- is consumed to oxidize 15 mole of NH_4^+ and produce 0.23 mg of alkalinity as $CaCO_3$. The alkalinity consumption is lower than the requirements of nitritation process. However, during the Anammox reactions, hydrogen ions are consumed and pH can decrease of the system. Maximum specific growth rate of the Anammox bacteria around 0.054-0.2 d^{-1} (Strous et al., 1999).

1.1.4.2. Mechanism of Anammox

Anaerobic Ammonia-oxidizing bacteria (anAOB) use NH_4^+ as e^- donor and NO_2^- as e^- acceptor to produce N_2 gas in the complete absence of oxygen as shown in Figure 1.4 (Jetten et al., 2002).

The genome analysis showed that lack of a nitrite-hydroxylamine oxidoreductase and the presence of a nitrite-nitric oxide oxidoreductase, nirS, in the genome of *K. stuttgartiensis*. Specific biomarkers called as ladderane lipids surround the anammoxosome. Anammoxosome is a special compartment of the anammox cell and the anaerobic oxidation of ammonium to N_2 takes place in (Figure 1.4) (Kuypers et al., 2003). It contains large amount of a hydroxylamine oxidoreductase (HAO) like enzyme (Schalk et al. 2000). According to the Kartal and coauthors (Figure 1.4) this reaction involves three steps cyclic pathway (Kartal and Keltjens, 2016); (i) one e^- reduction of and NO_2^- to nitric oxide (NO) by nirS; (ii) the condensation of one molecule of NO with one molecule of the NH_4^+ with the concomitant input of three electrons generating hydrazine (N_2H_4); and (iii) the oxidation of N_2H_4, catalyzed by HZO, yielding four electrons and produce N_2.

1.1.4.3. Phylogeny

Anammox species belong to the order Brocadiales (Ali and Okabe, 2015). Figure 1.6 shows the phylogenetic tree of 16S rRNA gene *Planctomycetales.* Four "Candidatus" anammox genera have been enriched from activated sludge: "Kuenenia", "Brocadia" (Strous et al., 1999) "Anammoxoglobus"(Kartal et al., 2010, Kartal et al., 2007) and "Jettenia" (Quan et al., 2008). The fifth Anammox genus, "Candidatus Scalindua" (Kuypers et al., 2003) has often been detected in natural habitats, especially in marine sediments and oxygen minimum zones (OMZ) (Dalsgaard et al., 2005). According to the phylogenetic analysis, the Candidatus '*Scalindua sorokinii* from the deeper layer of the Black Sea exhibited 87.9% sequence similarity *to Kuenenia*, 87.6% to *Brocadia*.

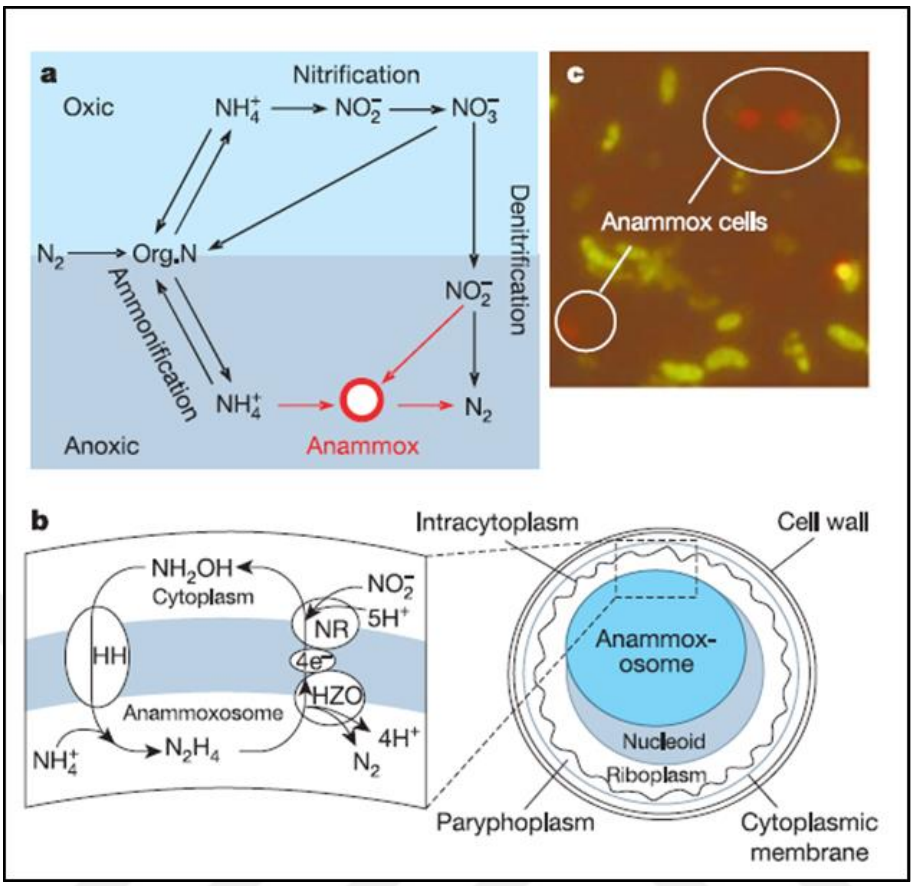


Figure 1.4 Morphology and physiology of Anammox bacteria (Jetten et al., 2002)

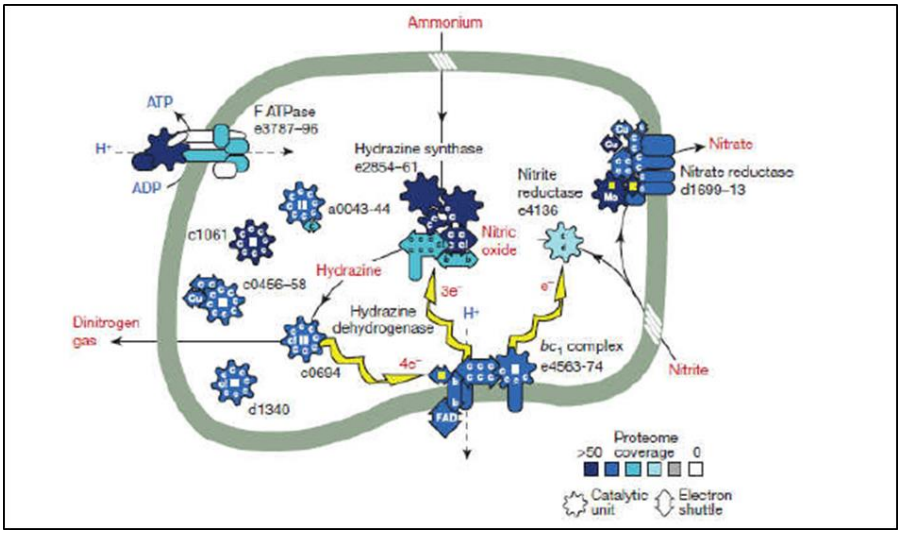


Figure 1.5 Biochemical pathway of *K. stuttgartiensis* (Kartal et al., 2010)

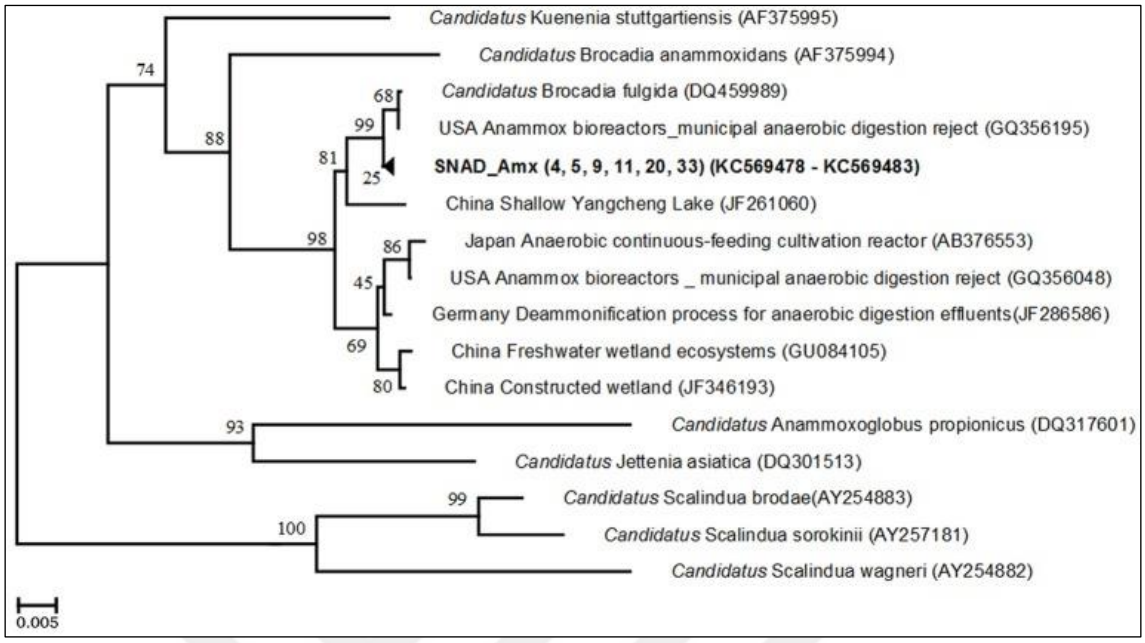


Figure 1.6 Phylogenetic tree of 16S rRNA gene *Planctomycetales* (Kuenen, 2008).

1.1.4.4. Kinetics

The specific Anammox growth rate (μ_{AN}) can be expressed by the Monod model that is used to describe the effect of limiting substrate on microbial growth (Equation.1.1.25) (Yao et al., 2015).

$$\mu_{AN} = \mu_{MAXAN} \frac{S_{NH_4}}{K_{ANSNH_4}+S_{NH_4}} x \frac{S_{NO_2}}{K_{ANSNO_2}+S_{NO_2}} \quad (1.1.25)$$

In this kinetic;

μ_{AN};	specific growth rate of anammox, g VSS/g VSS
μ_{ANMAX}	maximum specific growth rate, g VSS/g VSS
S_{NH4} and S_{NO2}:	NH_4^+-N and NO_2^--N concentrations in the bulk, mg/l
$K_{SNH4\ AN;}$	NH_4^+-N half saturation constants for anammox, mg/l
K_{SNO2AN};	NO_2^--N half saturation constants for anammox, mg/l

1.1.4.5. Challenges of anammox process

Application of Anammox process to mainstream has some challenges. Hence, application of deammonification process for the mainstream is still under investigation. First challenge is the high C/N ratio of wastewater (Lackner et al., 2014b). High C/N ratio makes heterotrophs (OHO) the dominant species of reactor and reduce population of slow growing microorganisms like AnAOB and AerAOB (Agrawal et al., 2018).

Another challenge is the low activity of AnAOB at low temperature (<15 °C) and fluctuations occurring with temperature variation of the wastewater (10–25 °C) (Vlaeminck et al., 2012) in comparison to the centrate (∼30 °C) treatment process (Kartal et al., 2010).

Retention of the Anammox bacteria is also problem for the partial nitritation followed by Anammox process. The main thing is to selectively retain AerAOB against NOB to sustain nitritation that is a prerequisite for Anammox. Anammox bacteria have been known as very slow-growing microorganisms, which need around 11 days for the doubling. For the keep in balance of amount of Anammox bacteria, long SRT is necessary Anammox. If there is organic matter heterotrophic denitrifiers (OHO) can easily out-compete with Anammox bacteria for nitrite and can become the dominant species in a very short time.

NO_2^--N is substrate of the Anammox process. However, high-concentrations of NO_2^--N has inhibitory effect on the Anammox bacteria. Anammox bacteria are strictly anaerobic and are inhibited by dissolved oxygen (Strous et al., 1999).

1.1.5. Anammox technologies

As discussed in Section 1.1.4, Anammox process stochiometrically requires a ratio of NH_4^+ to NO_2^- ratio of 1:1.32 to 1:1.5 in the influent. This necessity requires a prelimary partial nitritation step prior to Anammox applications. Combination of partial nitritation and Anammox process is called as deammonification process (WEF, 2015). Depending on the implementation of partial nitritation and Anammox processes, deammonification process can be classified either two-stage or one-stage process.

Depending on the sludge characteristics, two-stage and one-stage deammonification systems can be either suspended or attached growth system. The suspended growth systems can be further classified as granular or flocculants suspensions.

Early full scale approaches in sidestream deammonification used to two-stage configurations but due to more economic and easy operation of one-stage deammonification systems, nowadays one-stage deammonification applications have great attention.

1.1.5.1. One-stage configurations

In one-stage deammonification (Figure 1.7), both partial nitritation and Anammox processes occur in the same tank. There are various commercially available systems marketed for either sidestream or mainstream.

The type of Demon SBR® combines pH aeration/anaerobic control principle with sub-cycles with aeration followed unaerated phase to obtain the optimum conditions for the AerAOB and AnAOB (WEF, 2015).

DeAmmon® process is developed by Purac/Lackeby AB cooperate with the University of Hannover and Ruhr River Association (WEF, 2015). Reactors consist of three stages and intermittent aeration applies. The intermittent aeration strategy is provided to each zone to help AerAOB and AnAOB reactions.

Anita™ Mox process is an attached growth biofilm approach for the sidestream deammonification. The key parameter for controlling to the Anita™ Mox process is DO concentration to keep under balance the activities of AerAOB and AnAOB (WEF, 2015).

Early full-scale approaches in sidestream deammonification used two-stage configurations but due to more economic and easy operation of one-stage deammonification systems, nowadays one-stage deammonification applications have been received great attention.

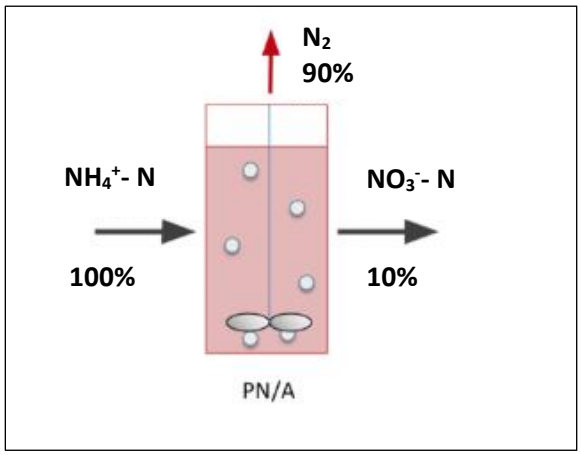


Figure 1.7 One-stage deammonification

One stage process performs partial nitritation followed by Anammox in the same reactor (Barwal and Chaudhary, 2014). One-stage configurations significantly reduce the costs of infrastructure and operation according to the two-stage process. Low costs are due to biofilm carriers with a high specific surface being applied to a single reactor (Zekker et al., 2012).

1.1.5.2. Two-stage configurations

In two-stage deammonification application (Figure 1.8), partial nitritation and Anammox process take place in 2 different reactors sequentially.

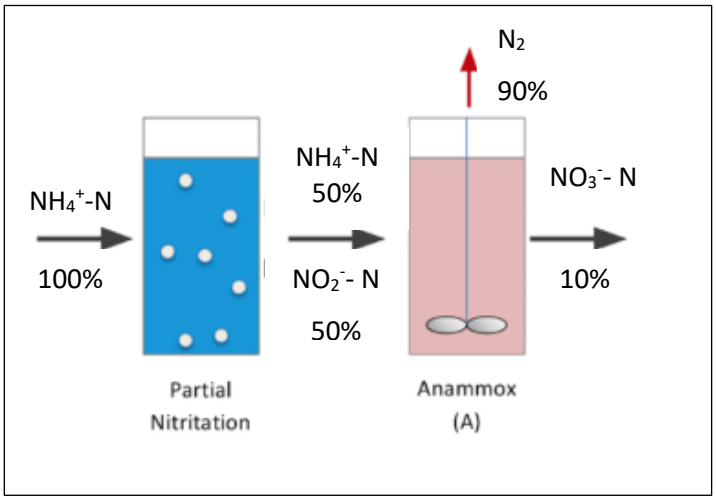


Figure 1.8 Two-stage deammonification

Two-stage deammonification systems;

SHARON Process (Figure 1.9) is the first two-stage process. SHARON process was developed by a group from Delft University and it is abbreviation for Single Reactor High activity Ammonia Removal over Nitrite (Hellinga et al., 1998). Sharon process exploits the advantages of the NOB out-selection mechanism of temperature and SRT control, coupled with close control of the DO in the system (WEF, 2015).

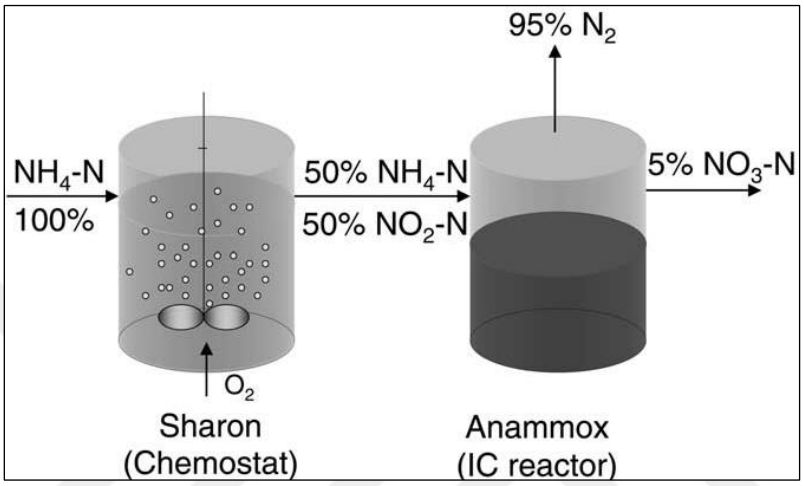


Figure 1.9 The SHARON process flow diagram (Jetten et al., 2002)

Today, there are 2 full-scale mainstream deammonification systems in the word; Strass WWTP, Austria and Changi WWPT, Singapore.

Strass Sequencing Batch Reactor Process (Strass SBR) developed in Strass facility in Australia for the nitrogen removal and it coupled with deammonification process. SBR operates with high SRT, coupled with primary sludge addition for carbon supplementation. In this process, nitrite shunt was achieved by switching the aeration on and off based on a tight pH dead band. Aeration started, pH would start to decrease until the low pH set point was reached aeration stopped. DO was depleted, followed by NO_2^- depleted for NH_4^+ oxidation in this anoxic period (WEF, 2015).

1.1.6. Advantages of Anammox process

Anammox is a novel and sustainable biotechnology due to lower energy consumption, high removal efficiencies and reduced greenhouse gas emission (Mao et al., 2017). Aeration energy consumption is less around 55-60% according to the conventional

nitrification/denitrification process. Anammox bacteria do not require organic carbon as being chemolitotrophic. Organic carbon demand in Anammox process is absent with respect to the conventional nitrification/denitrification processes Sludge production is very low due to slow growing characteristics of Anammox organisms (Lackner et al., 2014a).

1.2. Attached Growth (Biofilm) Processes

Attached-growth systems or fixed-film biofilm processes are biological processes in which the microorganism are attached to inert solid surface (Gavrilescu and Macoveanu, 2000).

1.2.1. Description of biofilm

In biofilm reactors, microorganisms are attached to a solid surface and hence do not require a settler. Although bacteria in suspension is washed out with flow, bacteria attached to a solid surface remains in the reactor. Small washout rates provide the development of biofilm. (Henze et al., 2008).

Biofilm has a hydrated biological structure attached to a surface owing to extracellular polymeric substances (EPS) which is producing by microorganism (Gavrilescu and Macoveanu, 2000). Their resistance to toxic shocks, retention of slow-growing microorganisms, flexible process, smaller area demand according to the suspended systems, lower hydraulic retention time and higher biomass retaining time make biofilms advantageous wastewater treatment facilities, with efficient removal of a variety of contaminants (Khaled Shahot et al., 2014). Due to these advantages, the application of biofilm systems increases the ability of controlling of the possible reaction process and population (Borkar et al., 2013).

In the Figure 1.10, different layers of biofilm are shown. These layers are bulk liquid, boundary layer, biofilm and biofilm support. Mass transport of substrates is based on molecular diffusion in the biofilm structure. Due to being a slow mechanism of mass transport according to the substrate removal, substrate gradients occur in the biofilm. These substrate gradients are resulted with mass transport limitation. This allows for the development of different ecological niches within the biofilm. Figure 1.11 is an example

for AerAOB and AnAOB development within the biofilm. The reactions take place in different layers of biofilm on the carrier. In the biofilm, DO does not diffuse freely through the biofilm structure, leading to the formation of an oxygen concentration gradient, which generates anaerobic conditions inner layer of biofilm and this structure allows the co-exist of different microbial groups. Aerobic bacteria are grow the outer layer and can use the DO and substrate directly. With the biological activity of bacteria, which are located at the outer layer of biofilm, some waste/ intermittent products occurs. The bacteria, which are located in deeper layer on biofilm, can use these products as a substrate for their biological activity.

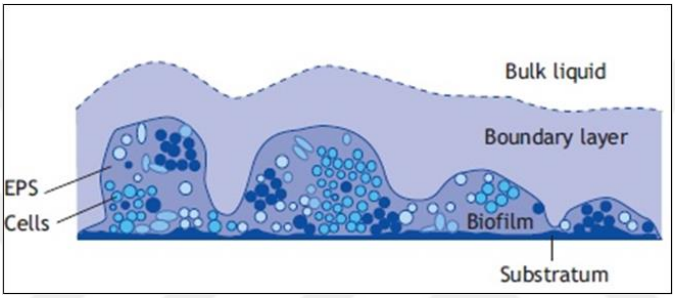


Figure 1.10 The schematic representation of the biofilm layers (Henze et al., 2008).

Diffusion of compounds in and out of the biofilm layers plays a key role in MBBR process as all the biofilm system. Because of the importance of diffusion, the thickness of the effective biofilm (the depth of the biofilm to which the substrates have penetrated) is significant. Depth of biofilm layer on the carrier is around 100 µm for the full substrate penetration and covered all the surface of the carrier (Rusten et al., 2006). Diffusion distance is increased in the biofilm mode of growth. (Figure 1.12). The biofilm and the substratum to which it is anchored impede flow near the biofilm, throttling convective transport. Inside cell clusters, the locally high cell densities and the presence of extracellular polymeric substances arrest the flow of water (Stewart, 2003).

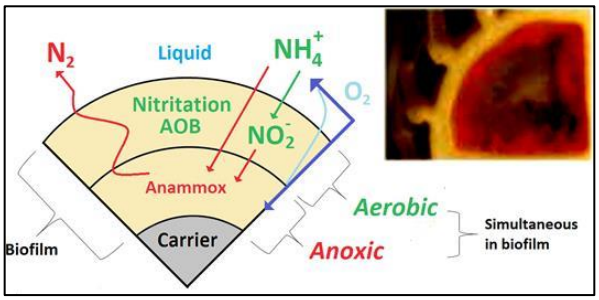


Figure 1.11 The schematic representation of AerAOB and AnAOB bacteria in biofilm (Capodaglio et al., 2016).

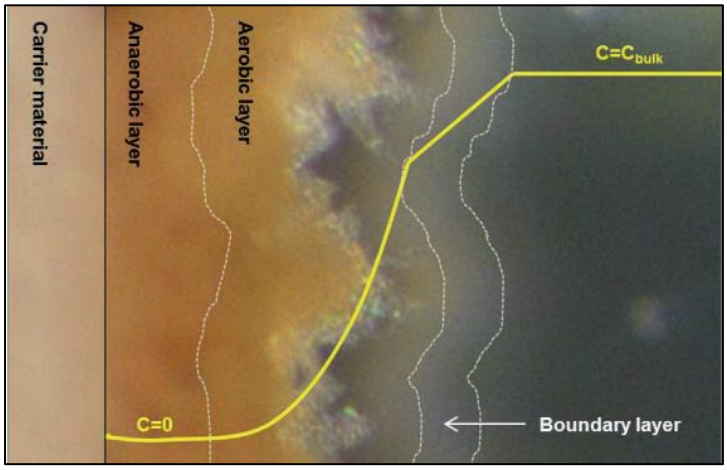


Figure 1.12 Transportation of substrate in biofilm layer (Piculell et al., 2016)

1.2.1.1. Attachment of biofilm

The development and formation of biofilm occurs in five stage as shown in Figure 1.13. Early level consists of bacterial attachment to medium. Bacterial proliferation then leads to colonization of the enclosing space and forms biofilm after dispersion (Cogan and Keener, 2004).

EPS is a sticky material produced by bacteria. This sticky material generates 90% of the biofilm and helps to the attachment of cells. Rock, gravel, slag, sand, redwood and plastic can be used as carrier materials. Attached growth process can be operated both aerobic and anaerobic processes (Metcalf and Eddy, 2003).

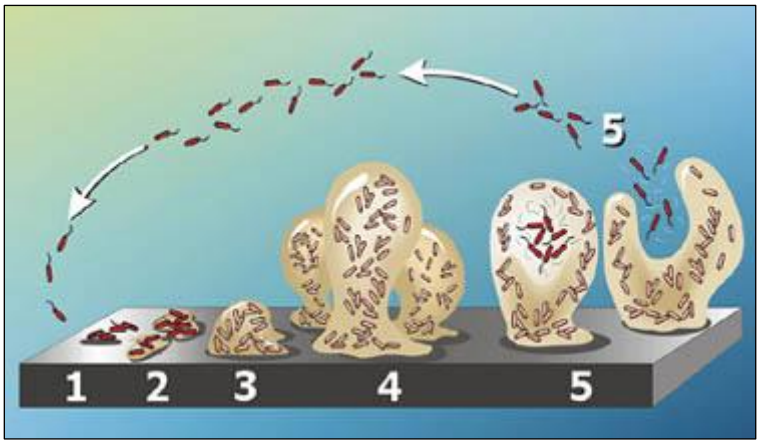


Figure 1.13 The stages of biofilm formation. (1) initial attachment, (2) irreversible attachment, (3) maturation 1, (4) maturation 2, (5) dispersion (Cogan and Keener, 2004).

1.2.1.2. Detachment of biofilm

Turbulence is necessary for the effective operation of biofilm process. High amount of turbulence detaches biomass from the surface of carriers. In addition, biofilm detaches from the outer surface of the carriers due to collision and corrosion. Due to this reason, the MBBR carriers have fins on the outside to prevent biofilm detach (Barwal and Chaudhary, 2014).

Biofilm attachment and detachment mechanisms are influenced by various processes, like adsorption and desorption of biomass, biofilm thickness (Christensen and Characklis, 1990). Interface between a carrier surface and bulk provides good conditions for attachment of cells. Other parameters which influence the attachment and detachment processes are pH, substrate, temperature and ionic strength (Barwal and Chaudhary, 2014).

Detachment of biofilm is generally based on four different processes, grazing (predators consumed bacteria from biofilm surface), sloughing (loss of large particles from biofilm), erosion (loss of small particles from the biofilm surface, mainly due to liquid shear stress), and abrasion (due to collisions of particles). The biofilm detachment rate depends on many variables such as velocity of flow, biofilm morphology, and carrier material characteristics.

1.2.2. Types of biofilm reactors

1.2.2.1. Non submerged attached growth systems

The trickling filter (TF) (Figure 1.14) is the most common aerobic attached growth process, which consist of a medium with high porosity. In this system, microorganisms responsible for treatment are attached to an inert packing material. Rock is the most common packing material for trickling filters (Metcalf and Eddy, 2003). Waste effluent is channeled across the top of the medium by a rotator. For the purification volume, plastic packing is used to replace the conventional trickling filters. The construction of filtration system is equipped with an under drain system to collect treated wastewater and removed organic material. Effluent collected is channeled to a settling chamber to allow the separation of solids from treated effluent (Eddy et al., 2003). Microorganisms

present in wastewater are retained on biofilm's surface during the influent comes the medium. This enables contact of attached microorganism to biodegrade organic substances from the influent (Boltz et al., 2009).

The rotating biological contactor (RBC) (Figure 1.14) is a fixed film biological secondary treatment methods (Metcalf & Eddy, 2003; Lee, 2000). The mechanism is similar with the trickling filter system. There was an increase use of RBC in the 1960s and 1970s but was soon decreased by various incurring problems such as the failure to meet expected performance, over substrate build ups, damage in shaft, lopsided disks due to instable biomass burden and proliferation of unwanted microorganisms. However, several of these issues have been overcome and the system is again widely applied (Ghawi and Kriš, 2009). The process consists of a set of disks normally made from plastic stacked beside each other on the same shaft is circulated across the flow of effluent. About 40% of the disks are submerged in the tank. The shaft circulates at the rate of 1 to 2 rpm and soon microbial growth and thickens on the moist surface area of the disks. The layer of microorganisms filters out organic constituents from influent to be assimilated in aerobic metabolism.

The rotation proceeds and leaves the wastewater towards the air, transferring oxygen to the layer of microbial build up. As the later re-submerges into the wastewater, excessive waste substances are removed as sloughing by transporting it with the flow of effluent. Mechanical failures are often experienced with the RBC's.

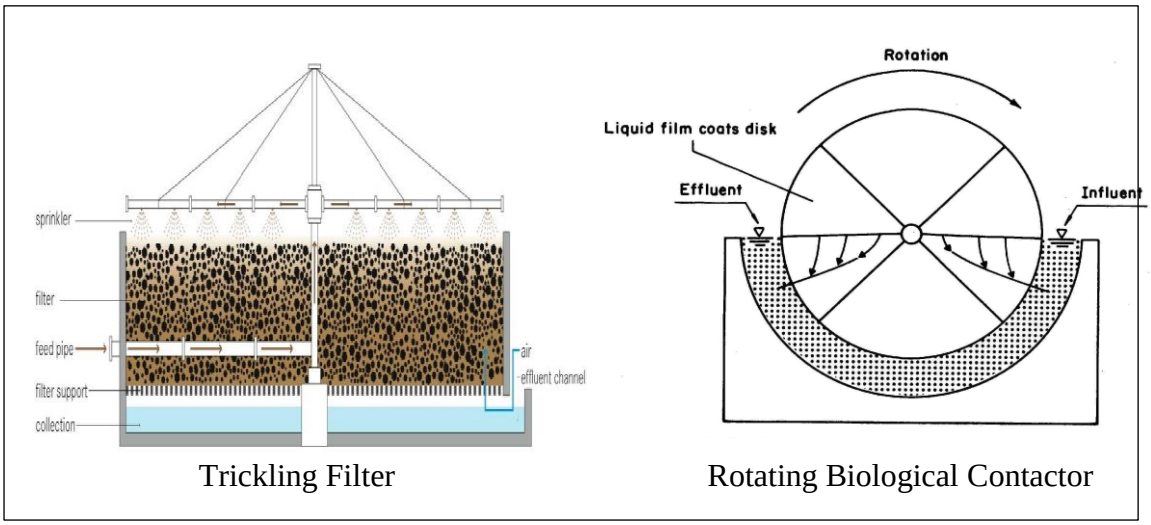


Figure 1.14 Schematic views of trickling filter and rotating biological contactor

1.2.2.2. Submerged fixed film systems

Fluidized Bed Biofilm Reactors (FBBR) (Figure 1.15) consist of a bed of a solid media which utilizes media for cell immobilization and retention. Aerobic and anaerobic FBBRs have received increasing attention recently (Burghate and Ingole, 2013) . This process provides the immobilization of biomass on the surface of particles, and this leads to obtain large surface area on which active biomass attached and this allows small bioreactor volumes use (Burghate and Ingole, 2013, Grady et al., 2011).

The Integrated Fixed Film Biofilm Processes (IFAS) process is a combination of the fixed-film and the suspended activated sludge processes (Figure 1.16). In IFAS, media can be fixed in the reactor or can be freely circulate**.** Thus, systems are used for the removal of organic carbon and beside this nitrogen and phosphorus. Interaction between suspended biomass and attached biomass has a great important in this process and still needs to investigate. In some cases, biomass from secondary clarifier is not recycled and reactor contains high amount of active attached biomass and this process called as Moving Bed Biofilm Reactor (MBBR) (Grady et al., 2011).

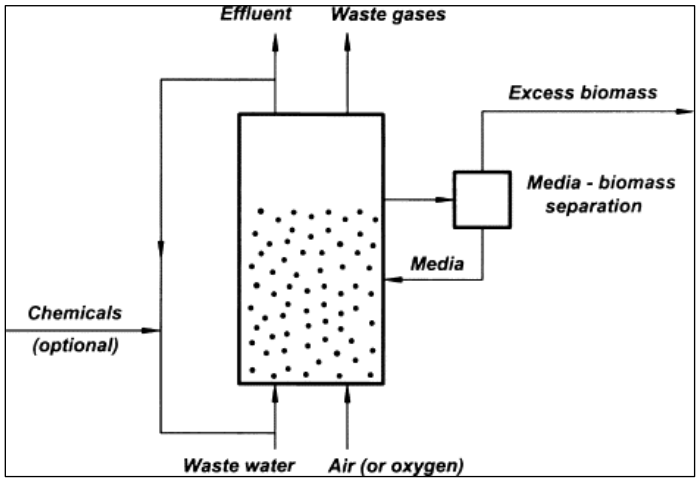


Figure 1.15 Fluidized Bed Biofilm Reactor (Sokół and Korpal, 2006)

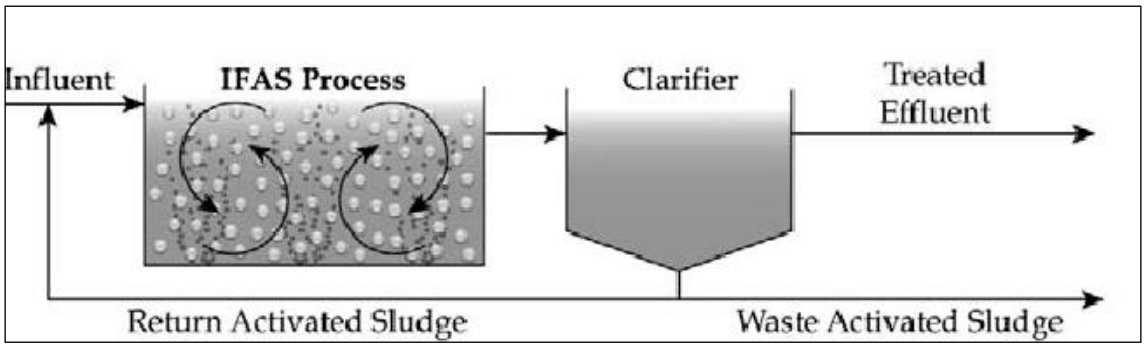


Figure 1.16 Integrated Fixed Film Biofilm Processes configuration

1.2.3. Moving Bed Biofilm Reactors (MBBR)

1.2.3.1. Description of process

Moving bed biofilm reactor (MBBR) has developed in Norway at the end of 1990's. This process consist of small cylindrical shaped polyethylene carrier elements which have specific density of 0.96 g/cm^3 (Metcalf and Eddy, 2003). Biomass attach and grow on these carriers. These carriers are circulating continuously by use of mixers and air agitation and carriers are maintained in the reactor by the use of sieve at the tank outlet. The specific surface area of carriers range between 200 to 1200 m^2/m^3 of bulk volume. Carrier filling ratio is an important parameter and it is between 25 to 50% of the tank volume. The MBBRs (Figure 1.17) can be operated both aerobic-anoxic and anaerobic processes (Ødegaard et al., 1998b).

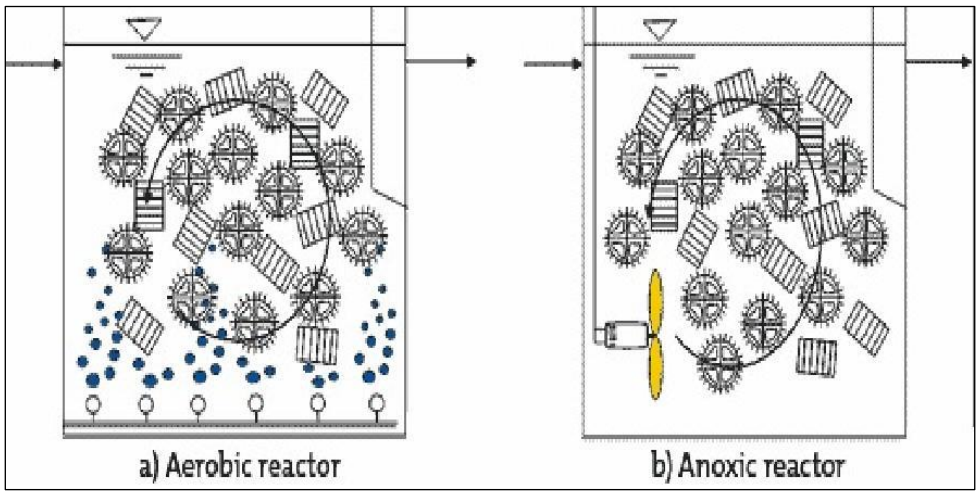


Figure 1.17 The principle of the moving bed biofilm reactor operation: a) for aerobic process, b) for anaerobic process (Odegaard, 1999).

1.2.3.2. Major operational factors

The major factors affecting the operation of MBBR are; biofilm carriers, mixing conditions, diffusion intensity and reactor filling ratio by carriers.

Biofilm carrier

The MBBR carrier was developed in Norway in the 80's (Ødegaard et al., 1994). MBBR carriers are designed to have a large protected surface area. Many types of MBBR carriers (Figure 1.18) have been developed and marketed within time. Now, there are several types of carriers available on the market. All these carriers have different surface area, shape and specific design. The available carrier area per packed volume can vary greatly, ranging from 200 to 1,200 m^2/m^3 for some of the most commonly used carrier types, where the carrier diameter ranging between 7 mm to 6.4 cm (Barwal and Chaudhary, 2014). Carriers can be made of various materials like porous ceramic, polyvinyl alcohol (PVA), plastic foam, high density polyethylene (HDPE), polypropylene (PP) or polyethylene (PE) with a typical density slightly less than water (density 0.95 g/cm^3) (Qiqi; et al., 2012). Some carrier types available in the market are shown in Figure 1.19.

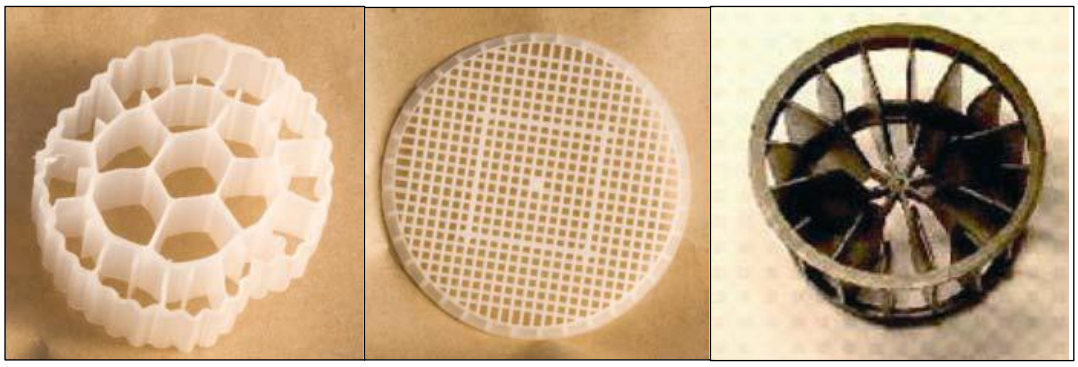


Figure 1.18 The different types of MBBR carriers (Henze et al., 2008).

Mixing conditions

Mixing and turbulence are key parameters in the MBBR systems. In the aerobic and anoxic processes, the biofilm carrier movement is accomplished by the air using course bubble diffusers. In anaerobic processes mechanical mixers are used normally horizontal shaft mounted banana mixers (Odegaard, 1999). According to the Kamstra et al. (2017), in a flow-through mixing creates more intensity due to added energy from the influent flow. Studies showed that movement of carriers do not change by the water velocity at above the 6 m/h air flows. The carrier movement by the air supply and course bubble aeration has been developed in aerobic and anoxic process (Odegaard, 1999). Mixing intensity is related with energy requirement and it depends on reactor dimensions and volumetric load, as well as on carrier design. The density of the carrier material, filling ratio will also affect the mixing requirements (Piculell, 2016).

Model	**Company**	**Material**	**Length (mm)**	**Diameter (mm)**	**Specific surface area (m^2/m^3)**	**Referances**
K1	AnoxKaldness™	HDPE	7	9	500	Aygun et al. (2008)
K2	AnoxKaldness™	HDPE	15	15	350	Das and Naga (2011)
K3	AnoxKaldness™	HDPE	12	25	500	Das and Naga (2011)
Natrix M2	AnoxKaldness™	HDPE	30	36	220	Das and Naga (2011)
Natrix C2	AnoxKaldness™	HDPE	50	64	200	Das and Naga (2011)
Biofilm Chip M	AnoxKaldness™	HDPE	2.2	48	1200	Das and Naga (2011)
Biofilm Chip p	AnoxKaldness™	PP	3	45	900	Kermani et al. (2008)
FLOCOR RMP	FLOCOR-Henderson Plastics LTD.	PP	10	15	260	FLOCOR
FLOCOR RS	FLOCOR-Henderson Plastics LTD.	PP	35	35	230	FLOCOR
Bio-media	Fxsino	PE	10	10	500	FXSİNO
Biosphere	Siemens	PE	9	13	800	BioSphere™
Biosphere N	Siemens	PE	12	12	650	BioSphere™
Spira 12	Siemens	PE	14	14	600	BioSphere™

Figure 1.19 Physical properties of different biofilm carrier elements

Diffusion intensity

Aerobic MBBR processes generally operate at higher DO concentrations because of diffusion limitations, in order to ensure sufficient substrate availability (Piculell et al., 2016). Aerobic MBBR processes have higher energy requirements to supply high

amount of DO. On the other hand, it can be used as an advantage; several different microbial niches can work co-existence in the same system and also microorganisms which live in the deeper layers of the biofilm will be less exposed to sudden toxic substances (Piculell et al., 2016).

Reactor filling ratio by carrriers

The performance of MBBR depends on the percent fill of carrier to allow the free carrier suspension, the carrier filling fraction (percentage of reactor volume occupied with carriers in empty tank) normally varies from 60 to 70 % (Barwal and Chaudhary, 2014).

1.2.3.3. Advantages and disadvantages of MBBR process

MBBR process has been proved to be economical, feasible option especially for the autotrophic nitrogen removal processes (because of lower volume reactors needed than in case of conventional activated sludge reactor (Chan et al., 2009). MBBR provide to utilize the whole tank volume for the biomass growth thus decreases the tank volume (Odegaard, 1999). MBBR provide more benefits according to the conventional activated sludge system such as high efficiency of oxygen transfer, low HRT and stability under shock organic loading rate (Chan et al., 2009). MBBRs do not need any sludge recycle like the other biofilm reactors. This is achieved by having the biomass grow on carriers that move freely in the reactor.

MBBR systems allow using the whole reactor volume as active, with no dead space or short-circuiting. It does not need sludge recirculation since the biomass grows on carriers and no clogging problem. MBBRs may operate for all aerobic, anoxic and anaerobic processes. It produce less sludge according to the activated sludge process (Ødegaard et al., 1994). Different types of biofilm carriers can be used and active biofilm surface area and can be increase up to 1200 m^2/m^3 reactor volume. One of the important advantages of the MBBR is the filling rate of carrier in the reactor The standard filling degree is 67 %, resulting in a total, according to the carrier type, this allows to work with high organic loading rates (Ødegaard et al., 1994).

The advantage over other biofilm processes is its flexibility. One can use almost any reactor shape. This process can be applied in existing plants for the retrofit. This process reduces the installation cost. System footprint can be reduced and this makes it more compact. Biofilm structure is more resistant to shock organic and hydraulic loading and abrupt changing value of pH, toxicity and temperature.

Beside these advantages, MBBR process has also some disadvantages. High-energy cost associated with aeration for the movement of carriers. However, with the correct design and choosing correct biological process oxygen demand can be reduced.

1.2.4 MBBR Anammox Applications

Within the last decade, several technologies MBBRs have been developed and successfully applied to the sidestream anaerobic treatment. More than 100 plants have been installed and still operating all over the world (Lackner et al., 2014b). With applying partial nitritation followed by Anammox process to mainstream treatment, energy requirements for the aeration could be reduced according to the conventional treatment process (Gao et al., 2014). Nowadays researchers are studying the application of partial nitritation and Anammox in mainstream wastewater, with using different processes and operation schemes (Piculell et al., 2016).

2. MATERIALS AND METHODS

2.1. Atakoy Sludge Treatment Plant

The municipal sewage treatment plant at Atakoy, Istanbul, Turkey, (Figure 2.1) has a treatment capacity of about 1.600.000 people equivalent (p.e) with a treatment capacity of 400.000 m³/ day. The treatment plant was designed as an advanced biological treatment process for the removal of carbon, nitrogen and phosphorus.



Figure 2.1 Atakoy STP

2.2. Description of Pilot Plant

The pilot plant (Figure 2.2) has been located in Atakoy sewage treatment plant (STP). The reactor with a diameter of 1.1 m and height of 1.2 m has a working volume of 0.76

m^3. The P&ID of the pilot reactor, which was operated as an SBR under continuous flow conditions, is shown in Figure 2.3. Sludge age in suspense was kept between 2 and 7 days. Hydraulic retention time (HRT) was 1 day.

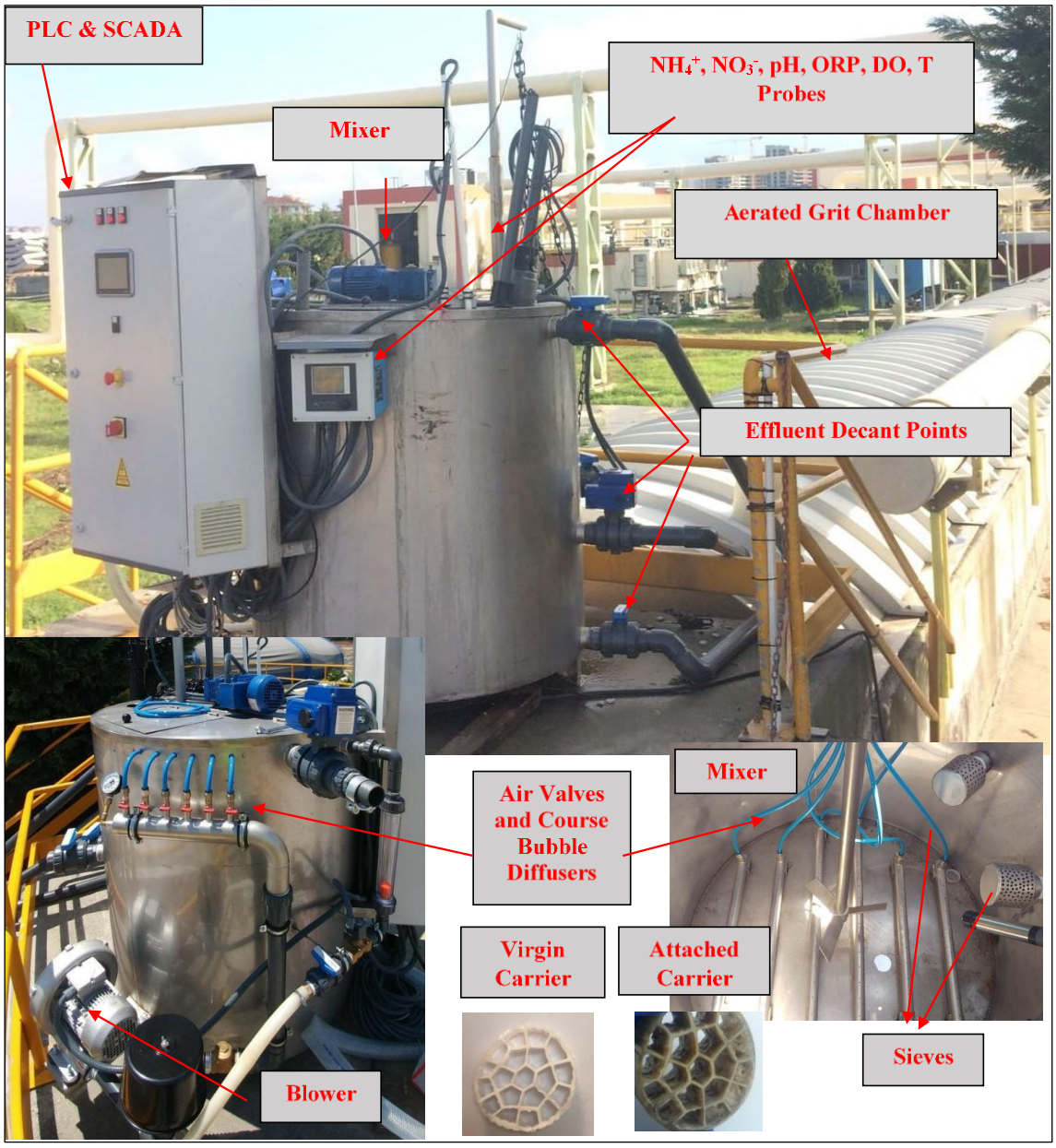


Figure 2.2 One-stage deammonification MBBR pilot system in Istanbul Atakoy STP

The reactor was equipped with a mechanical mixer, which has 45^0 pitched, 3 blade impellers having diameter of 55 cm (initially 36 cm) for the mixing both in aerobic and anaerobic cycles. The mixer worked with an electric motor which has a power of 2.2 kW (İmak). Oxygen supply during oxic periods was provided with a centrifugal blower

(Seko, 1.3 Kw) connected to tubular type course bubble diffusers. Feeding and decant levels were adjusted with hydrostatic type low and high water level sensors (Etrans–P Enelsan). The reactor feeding was accomplished with a submerged centrifuge pump that has 2.2 kW power. Decant was done with an electrical actuator valve controlled by PLC.

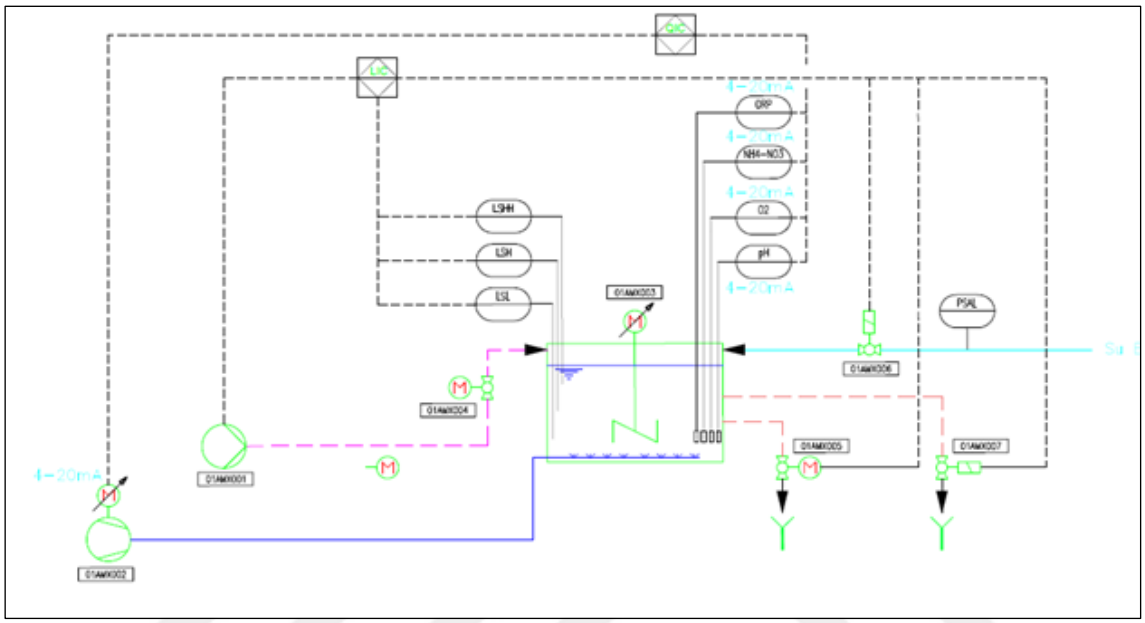


Figure 2.3 P&ID of the pilot reactor

The reactor was operated with a PLC & Scada system of which sample screenshot is shown in Figure 2.4. NH_4^+-N, NO_3^--N, Oxidation reduction potential (ORP), pH, temperature and DO values were continuously monitored via on-line probes (Endress and Hauser, WTW Varion plus). The DO level in the reactor was controlled with a PID system in order to maintain set DO level during oxic phase.

2.3 Start-up and Operation of the Pilot

Initial seeding of the pilot reactor was done with return activated sludge (RAS) from Atakoy STP. At the start-up operation, 1/3 of reactor volume was filled with the plastic carriers described in Table 2.1. The reactor was being continuously fed with aerated grit chamber effluent of Atakoy STP (usually one cycle/day) for about one year. The major influent wastewater characteristics of the Atakoy STP after screens are summarized in Table 2.2.

The pilot MBBR reactor was operated as SBR via PLC system shown in Figure 2.4. Each cycle of the reactor consisting fill, react, waste, settle, decant and idle phases is summarized schematically in Figure 2.5. In each cycle, fill, waste and decant phases durations were dependent on the signals coming from hydrostatic low/high level transmitter. The duration of react phase was adjusted in 2 different ways: (i) due to set NH_4^+-N level, (ii) time. In operation days of 10- 30, the react phase duration was adjusted depending on the set NH_4^+-N level. Decant phase started when the on-line measured NH_4^+-N level became less than or equal to set NH_4^+-N value on PLC. Later, throughout the study, react phase operated depending on set time value on PLC. Settle phase operated based on set time value on PLC.

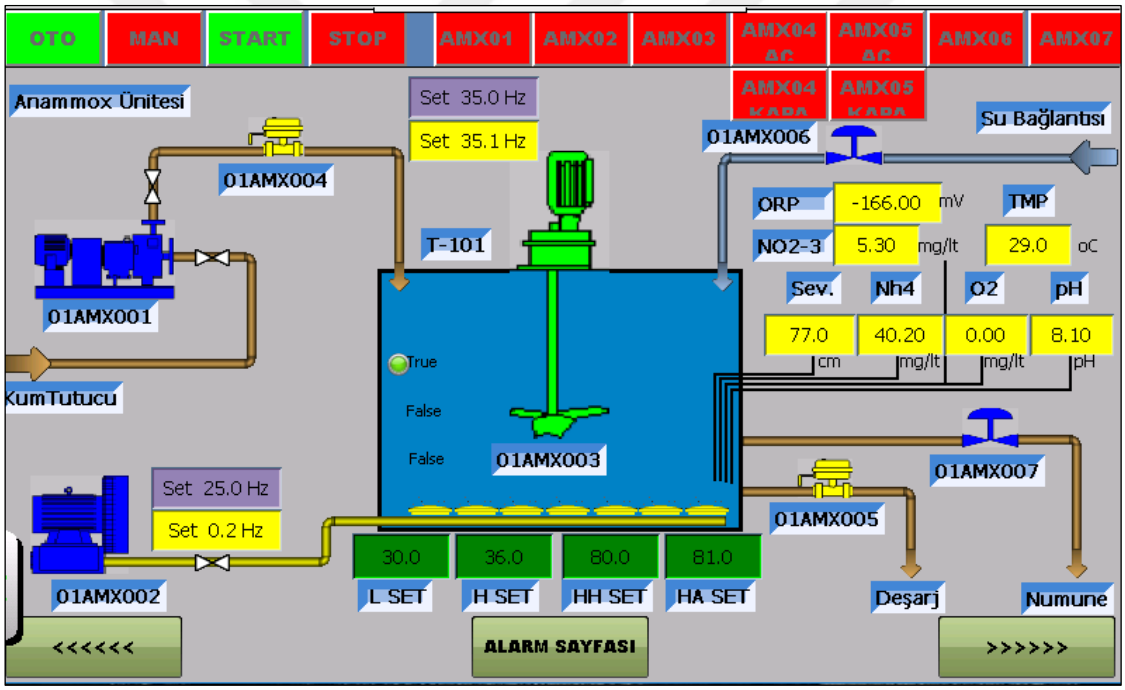


Figure 2.4 The PLC& SCADA panel sample screenshot

Table 2.1 Specifications of MBBR carriers

Parameter	Specification	Unit
Trademark	Aquaflex BIOAQUA	
Carrier Diameter	26	mm
Carrier Width	10	mm
Surface Area	650	m^2/m^3

Material	PP	
Density	0.95-0.97	g/m^3
Hole numbers	19	pcs

Table 2.2 Influent wastewater characteristics of the pilot

Parameter	**Average**	**Max.**	**Min**
$COD_{(Filtered)}$ mg/l	154.2±77.0	651	50
TN mg/l	59.1±16.8	101.9	22.8
NH_4^+-N mg/l	37.3±10.0	68	12.8
PO_4^{3-}-P mg/l	5.6±2.0	13	1.5
AKM mg/l	8205±5977	27870	480

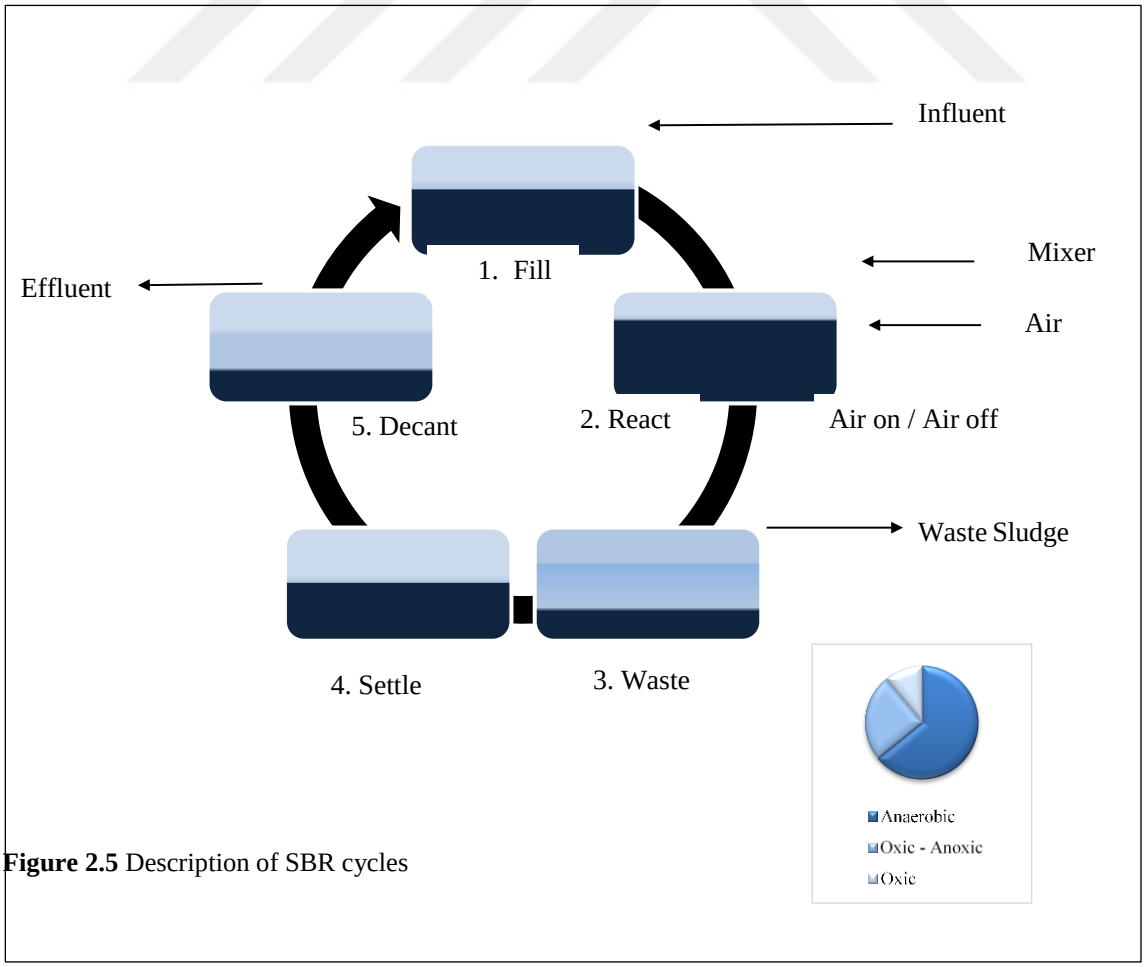


Figure 2.5 Description of SBR cycles

The operation of the pilot reactor as one-stage MBBR, i.e., partial nitritation and Anammox processes in the same tank, was accomplished by applying different operational strategies as described in Table 2.3. All operation strategies were based on control of DO concentration during oxic periods. The DO concentration was maintained around set DO value described in PLC by using PID control in SCADA system. In anaerobic periods, blower was always in off position.

During the operation of the pilot reactor for a time period of 374 days, daily influent and effluent samples were collected from the reactor and analyzed for NH_4^+-N, NO_2^--N, NO_3^--N, PO_4^--P, COD as described in Section 2.3.

The biofilm containing carriers were collected from the reactor regularly (usually once a week) for physical, microscopic and molecular characterization of the biofilm as described in Sections 2.4.

Visual inspection of biofilm attachment was also done regularly by taking photos of carriers every week.

Stock trace element solutions 1 and solution 2 were prepared by dissolving the following chemicals in 1L: $C_{10}H_{14}N_2Na_2O_8.2H_2O$ (15 g), $ZnSO_4.7H_2O$ (0.43 g), $COCl_2$ (0.12 g), $MnCl_2.4H_2O$ (0.99 g), $CuSO_4.5H_2O$ (0.25 g), $NaMoO_4.2H_2O$ (0.22 g), $NiCl_2.6H_2O$ (0.19 g), Na_2SeO_3 (0.1 g), H_3BO_3 (0.011 g) were added to the reactor. For the Solution 2 $C_{10}H_{14}N_2Na_2O_8.2H_2O$ (5 g) and $FeSO_4.7H_2O$ (5 g).

Table 2.3 Operational schemes of the pilot

Operation period (d)	**Continuous aeration controlled by set DO (PID control)**	**Anaerobic**	**Intermittent Aeration Air on (PID control) Air off**	**Anaerobic**
0-68	✓	X	X	X
68-164	X	X	✓	X
165-344	✓	X	✓	✓
345-374	✓	✓	X	X

2.4. Analytical measurements

The measurements were performed with samples filtered through 0.45 μm glass fiber filters unless stated as unfiltered (u) measurements. The spectrophotometric and ion selective electrode methods followed in this study are summarized in Table 2.4.

Table 2.4 Analytical measurement methods

Parameter	**Method**	**Description**
TN	TS EN 12260: 2005-(ASI-L)	Oxidation method
NH_4^+-N	SM 4500 NH_3 –D-	Nitrate electrode method
NO_3^--N	4500 NO_3-B	Selective electrode method
NO_2^--N	Method-4500 NO_2^--B Standard Methods (2005)	Colorimetric Method
COD	5220 C	Closed reflux titrimetric method
PO_4^{3-}-P	SM4500-P B SM4500-P C	Vanadomolybdophosphoric acid colorimetric

Ion chromatography measurements for NH_4^+-N, NO_2^--N, NO_3^--N and PO_4^{3-}-P were performed with Shimadzu SPD-20A UV-VIS Dual Injection Chromatography.

Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were in accordance with method 2540D in Standard Methods (1998).

2.5. Biofilm Characterization Tests

For the monitoring evaluations of biofilm, some tools were applied carrier surface.

2.5.1. Gravimetric measurements

Total biofilm solids on the carriers were measured by drying ten carrier samples collected from the reactor randomly. The collected carriers were initially dried at 105 °C for 2 h. The dried samples were weighed and the biomass removed by placing the carriers in EDTA solution (0.1 M). The carriers were then rinsed several times using pressurized deionized water, then re-dried 2 h at 105 °C, and cooled in a desiccator before weighing. The difference in initial and final weights was used to calculate the biomass on the carriers.

2.5.2. Atomic Force Microscopy (AFM)

AFM was applied to MBBR carriers by using AFM Workshop TT that has ACLA model SPM probe. The Cantilever made from Si, N-type and has L: 22 μm, W: 40 μm, T: 7.8 μm dimension. The carriers collected from the reactor were cut into small pieces and then either measured directly or stored at -20^{0}C before AFM test. No pretreatment was applied before AFM test**.**

2.5.3. X-ray Powder Diffraction (XRD)

XRD analyses were performed by Bruker D8 advanced X-Ray diffraction.

2.5.4. Fourier transform infrared (FT-IR)

Fourier transform infrared (FT-IR) analyses were performed with Jasco FT/IR equipped with total reflection Gladi and plate Diamond ATR crystal with using liquid nitrogen cooled mercury cadmium telluride (MCT) detector at 500 – 4000 cm-1 wavelength. Before FT-IR test, samples taken from the reactor were freeze-dried with lyophilizator (Lyovac GT32) over night.

2.5.5. Thermogravimetric analyses (TGA)

The thermogravimetric analyses (TGA) were conducted in accordance with European Standards: DIN 51718A/CEN/TS 1514–1/-2 using NETZSCH STA 409C/CD model TGA device. The test was applied directly to the biofilm scraped from the carriers (either directly taken from the reactor or stored at -20^{0}C).

2.5.6. Scanning electron microscopy (SEM)

SEM analysis was performed with fixed samples. For fixation, carriers with biofilm were initially cut into small pieces (1 cm x 1 cm) and then fixed with 2.5 % glutaraldehyde (GA) in a 0.1 M phosphate buffer and kept in the fridge at 4 °C overnight. Fixed samples were then dried sequentially with 30%, 50%, 70%, 90% and 100% ethanol. The SEM images were taken with Philips XL30.

2.5.7. Energy-dispersive X-ray spectroscopy (EDS)

Samples pre-treated as described in Section 2.5.6 were analyzed with Jeol JSM-5910LV device.

2.6. Molecular techniques

2.6.1. DNA extraction

Samples were taken from reactor regularly for DNA extraction. DNA was extracted with Fastprep DNA extraction kit (QBiogene, Carlsbad, CA). NanoDrop Lite spectrophotometer (Thermofisher, MA) was used for check the DNA quality.

2.6.2. QPCR analyses

Quantitative polymerase chain reaction (QPCR) analyses were applied by using SteponePlus (Applied Biosystems, USA) using evagreen Supermix (Biorad, USA). Copy number of total Bacteria and AerAOB, NOB (Nitrospira and Nitrobacter) and AnAOB were summarized in Table 2.5. QPCR were accomplished in 20 µL reaction mixtures, which was containing evagreen Supermix (10 µL), PCR water (7 µL), primers (0.3 µL each; 10 mmol/L), and template DNA (2 µL; 1–10 ng).

Table 2.5 Primer sets of PCR amplification of pilot plant

Primer	Sequence	Target	Annealing	References
341f	CCTACGGGAGGCAGCAG	bacterial 16S	60	Koike et al., 2007
534r	TTACCGCGGCTGCTGGCAC			
Pla46f	GGATTAGGCATGCAAGTC	anammox 16S	59	Schmid et al., 2003
Amx368R	CCTTTCGGGCATTGCGAA			
amoA-2r	CCCCTCKGSAAAGCCTTCTTC	AerAOB	55	Rotthauwe et al., 1997
amoA-1f	GGGGTTTCTACTGGTGGT			
P338f	ACTCCTACGGGAGGCAGCAG	NOB 16S rRNA genes of Nitrobacter	65	Regan et al., 2002
NIT3	CCTGTGCTCCATGCTCCG			
NSR1113f	CCTGCTTTCAGTTGCTACCG	NOB 16S rRNA genes of Nitrospira	60	Dionisi et al., 2002
NSR1264r	GTTTGCAGCGCTTTGTACCG			

3. RESULTS AND DISCUSSION

3.1. Operational Strategies of the Pilot

Through the operation of the mainstream one-stage deammonification MBBR pilot for a time period of 374 days, there were mainly 3 major challenges;

I. Initial attachment of bacteria on the surface of virgin carriers,

II. Managing competition between bacterial groups and start of Anammox species enrichment on virgin carriers,

III. Minimization of detachment of the bacteria from carriers and providing sustainable nitrogen removal

This study evaluated different operation strategies discussed in Sections 3.1.1- 3.1.3 in order to overcome these challenges.

3.1.1. Strategies for initial biofilm attachment

At the start of pilot operation (Phase I, 1-68 days), several operational factors (Table 2.6) were applied to shorten the period of initial biofilm attachment on virgin carriers.

One of the major factor affecting initial biofilm attachment on virgin carriers was the mixing conditions inside the reactor. Virgin carriers having slightly lighter density than water initially tended to float on the surface of water even though both the blower and the mixer were operating. In order to provide proper mixing conditions for carriers, the major changes applied for the reactor operation were as follows:

1. Change of mixer propeller,
2. Frequency adjustment of mixer,
3. Frequency adjustment of blower.

Impeller diameter of the 3-blade, 45° pitched mixer (Figure 2.2) was increased from 36 cm to 50 cm. Additionally, different mixing frequencies were applied in the range of 40 Hz to 50 Hz in order to provide proper turbulence (i.e., velocity gradient, G) in the reactor for both oxic and anoxic/anaeorobic phases. The centrifugal blower described in Section 2.2, was another equipment creating additional turbulence while introducing air

into the reactor during oxic phase. Different blower frequencies were tried between 10 Hz to 45 Hz to find optimum mixing conditions in the reactor. With these trials, the optimum conditions for proper mixing in the pilot reactor was identified as $D_{impeller}/D_{tank}$ ratio of 0.55, G_{mixer} of 50 sec^{-1}, G_{blower} of 542 sec^{-1}.

Start-up period of the pilot was in the summer season and there was a serious scum problem in Atakoy STP. It was observed that presence of scum in the reactor negatively affected initial biofilm attachment on carriers. Hence, in the first 68 days of operation antifoam agent (cationic water base polyelectrolyte) was sprayed regularly on to the water surface of the reactor three times in a week.

One of the another factor negatively affecting initial attachment was suspended solid content of the influent. Grit chamber effluent of Atakoy STP contains significant amount of suspended solids (373.1±95.72 mg/l, Section 2.3). Hence, the solids that enters into the reactor with the influent grazed the thin biofilm attached weakly on the virgin carriers and also behaved as a media for the attachment of the bacteria. To adjust the solids amount in the reactor different settlement periods were tried to wash-out these solids from the reactor. Initially, settle period was 120 min and decreased gradually to the level of 30 min. Gradual decrease of settlement period also helped to wash-out suspended-growth microbial species from the reactor and hence favored attached-growth microbial population on carriers.

The other actions done to accelerate initial attachment of biofilm on the carriers were regular trace elements addition and re-seeding of fresh activated sludge into the reactor. Trace elements solution described in Section 2.3 were added into the reactor three times in a week. Re-seeding of 100 L fresh return activated sludge of Atakoy STP was done two times in this period.

These strategies, especially mixer impeller size, frequencies of mixer and blower, helped to accelerate initial attachment and development of biofilm on the carriers. The initial biofilm attachment and biofilm development periods were observed simply with naked-eye, gravimetric measurements and thickness measurements (Section 3.3). The charactersitics of the biofilm were identified with instrumental physical characterization methods (Sections 3.4) and molecular analyses (Section 3.5).

Table 3.1 Operational Changes during initial biofilm attachment period

OPERATION TIME (d)	1 ↓	10 ↓	20 ↓	30 ↓	40 ↓	50 ↓	60 ↓	68 ↓
MIXER IMPELLER SIZE	36 cm			55 cm				
MIXER POWER	2.2 kW							
MIXER FREQUENCY	50 Hz		45 Hz	50 Hz		40 Hz	45 Hz	
BLOWER POWER	1.3 kW							
BLOWER FREQUENCY	45 Hz			30 Hz		10 Hz	30 Hz	
SCUM CONTROL WITH POLYELECTROLYTE ADDITION	SCUM			3 TIMES / WEEK				
SETTLEMENT PERIOD (min)	120		60	30				
RE-SEEDING (L)	100						100	

3.1.2. Strategies for one-stage deammonification process

The major strategy to start one-stage deammonification process (partial nitrititation and Anammox) is to wash-out NOB species from the system while providing spatial attachment of OHO, AerAOB and AnAOB species on the carriers. The major operational parameters that can be adjusted for this purpose are: (i) DO, (ii) pH, (iii) temperature, (iv) FA and (v) sludge age. In this study, an operational strategy based on mainly DO control and sludge age control was followed in order to wash-out NOB and enrich Anammox. The pilot reactor was operated under ambient temperature conditions without adjusting influent pH.

DO control strategy was described in detail in Section 2.3 Table 2.6. Depending on the changes in DO control scheme, the operational period of 374 days was divided into 4 different phases. The sample DO measurements belonging to each phase are shown in Figure 3.1 the T, pH and DO values given in Figure 3.2 are the average values on-line measured parameters in each cycle.

Phase I (0-68 days) – Only oxic phase : Initially, the aim was to provide attachment of OHO and AerAOB species on the carriers and wash-out NOB from the system under set DO level of 0.5-0.8 mg/l (0-30 days), 0.8-1 mg/l (31-48 days). The increase in set DO level was dependent on seasonal wastewater temperature increase. Later, between

operations days of 49-68, DO set level decreased to the range of 0.3-0.5 mg/l in order to develop anaerobic conditions in the inner part of thin biofilm already attached on carriers. The aim was to start AnAOB enrichment. In this phase, Proportional Integral Derivative (PID) control of DO was set by trying different (P), (I) and (D) values. The duration of reaction period in this phase varied significantly (between 50-750 minutes) since the react period ended up depending on set NH_4^+-N level.

Initially, sludge age was (0-30 days) around 5 days. Later (31-68 days), it was decreased to 3.5 days in order to wash-out NOB from the reactor.

Phase II (69-164 days) – Intermittent aeration (oxic→ anaerobic): The aim was to provide accumulation of NO_2^--N in oxic phase and in anaerobic phase to increase AnAOB population that would use accumulated NO_2^--N with NH_4^+-N . In the operation days of 69-87, for the reaction period of 1410 min, the oxic phase duration was 66% while anaerobic duration was 34% of one oxic-anaerobic cycle. There were around 15 oxic-anaerobic sub-cycles in a day. The set DO level was kept in the range of 0.8-1 mg/l. In the operation days of 88-164, for the total reaction period of 1410 min, oxic duration was gradually decreased with respect to anaerobic duration. 60% oxic- 40% anaerobic 2 sub-cycles in a day was decreased to 57% oxic – 43% anaerobic 1 sub-cycle in a day, 44% oxic- 46% anaerobic 1 sub-cycle in a day.

Throughout the entire phase, sludge age was kept around 3.5 days in order to further wash-out NOB from the reactor.

Phase III (165-344 days) – Oxic→ Anaerobic→ Intermittent aeration (Oxic→ Anaerobic) → Anaerobic: The aim was to provide constant level of rbCOD removal during initial oxic phase in order to prevent removal of accumulated nitrite in the intermittent aeration phase with denitritation process. The anaerobic cycle following to oxic phase was for contact of AnAOB species with both nitrite and ammonium. Intermittent aeration phase was for further nitrite accumulation in oxic and denitritation of possibly formed nitrate with sbCOD. In the last anaerobic phase, it was aimed to provide contact of AnAOB with ammonium and nitrite in the absence of significant amount of COD and hence without competition of AnAOB with OHO for nitrite. The total reaction period of 1440 min was operated as 16% oxic, 8% anaerobic, 26% intermittent aeration (50% oxic→ 50% anaerobic), 50% anaerobic until the operation

day of 230. Later, between operation days of 231-240, intermittent phase oxic – anaerobic durations were adjusted as 75% - 25% since the partial nitritation rate decreased significantly due to cold weather conditions. Since the cold weather conditions seriously halted nitrite accumulation, nitrite spike started in anaerobic phase in operation day of 206. After operation –day of 231, the total reaction period of 1440 min was operated as 8% oxic followed by 8% anaerobic period , 34% intermittent aeration (50% oxic $\rightarrow$ 50%anaerobic), 50% anaerobic. In this phase, DO set level in oxic periods was around 0.8- 1 mg/l.

Initially, sludge age was (169-250 days) around 4.5 days. Later, it was decreased to 2.7 between days of 251-280 days and then 1.9 days after operation day of 281 in order to wash-out NOB and excess OHO from the reactor.

Phase IV (345-374 days) – Oxic$\rightarrow$ Anaerobic: Since the nitrite accumulation could not successfully achieved in the previous phases, nitrite spike continued in this phase as well. Intermittent aeration was omitted since nitrite was spiked instead of accumulation through partial nitritation in the oxic phase. Oxic phase was only applied for the removal of rbCOD and hence to prevent OHO, AnAOB competition for spiked nitrite in the anaerobic phase. For the total reaction period of 1440 min, oxic duration was kept either 120 or 240 min depending on observed COD removal efficiency.

In this phase, it was aimed to discharge all suspended solids from the reactor by omitting settlement phase and discharging all suspended solids with decant. However, the measured SS values indicated that small amount of solids always reminded in the reactor and sludge age for suspended solids calculated around 1.8 days.

3.1.3. Strategies for minimization of detachment

The biofilm thickness on carriers can not be increased more $\approx$60 μm because of detachment of bacteria from the carriers due to turbulence inside the reactor and solids coming with the influent. In order to minimize detachment process, after initial biofilm was formed, blower and mixer frequencies were gradually decreased. Solids were wasted from the reactor as much as possible by omitting the settlement phase in the reactor.

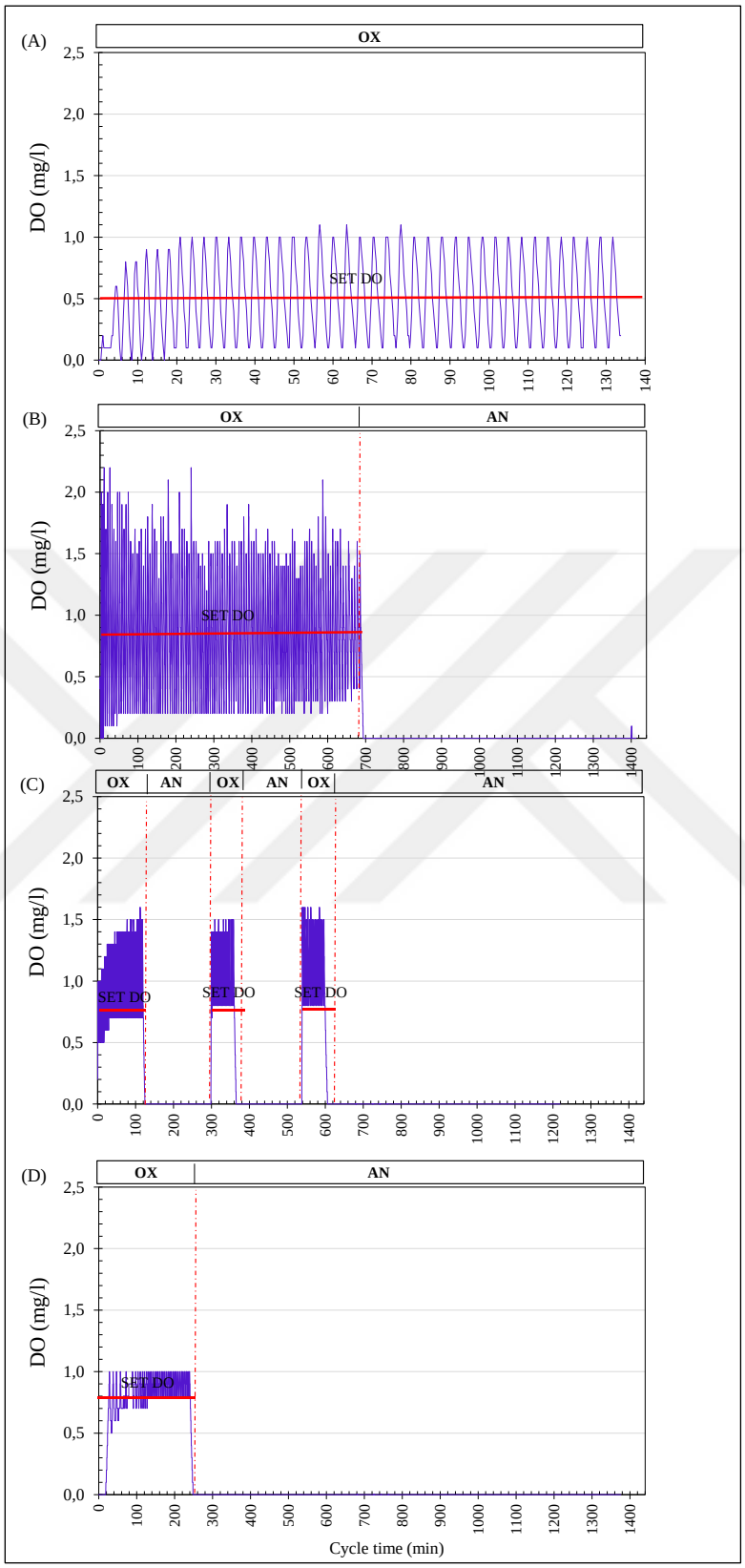


Figure 3.1 DO cycle samples A) Phase I, B) Phase II, C) Phase III, D) Phase IV Ox: Oxic, An: Anaerobic

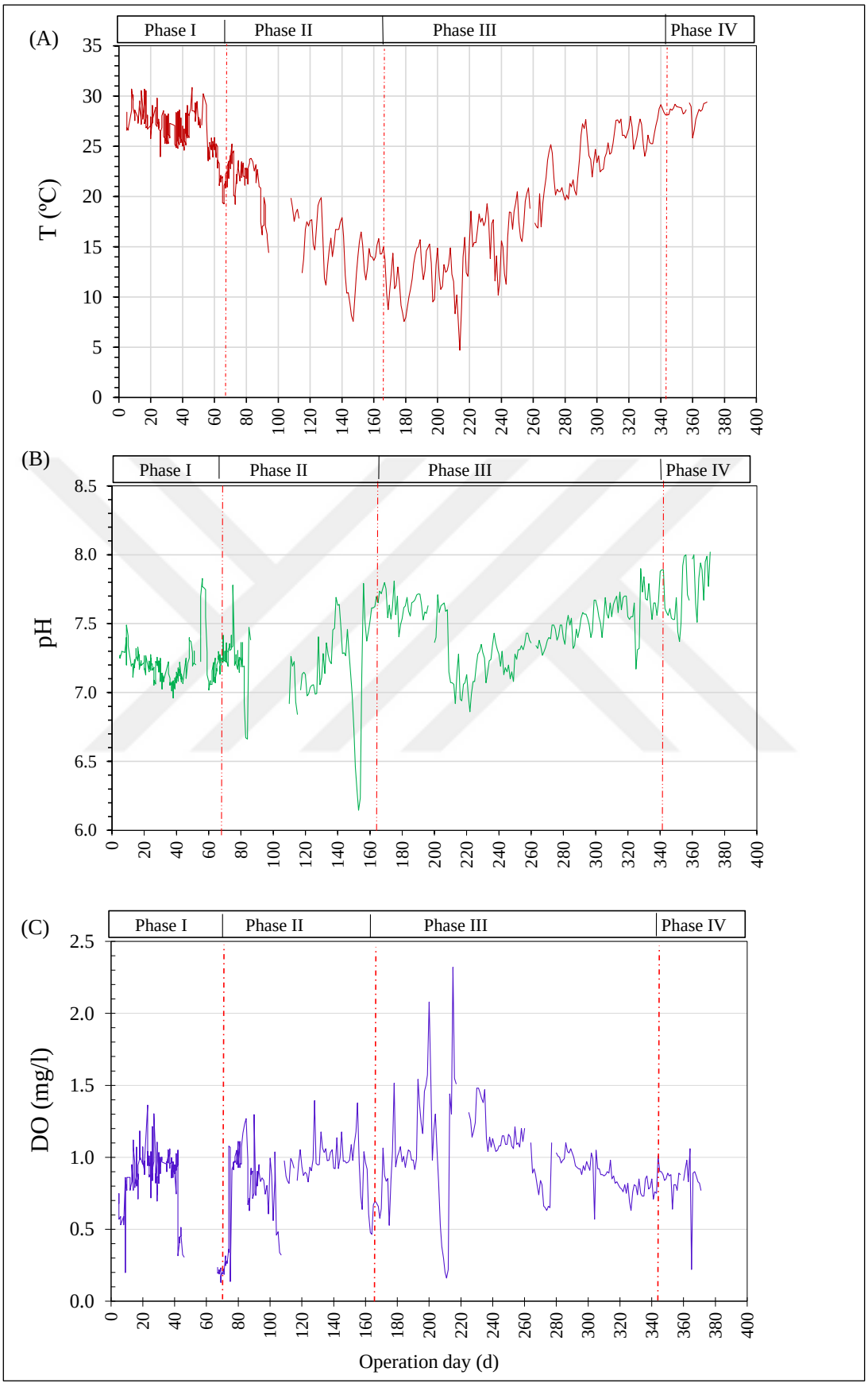


Figure 3.2 Time course major operational parameters A) temperature, B) pH, C) DO

3.2. Performance of Biological Nutrient Removal Processes in the Pilot

3.2.1. Organic carbon removal

Daily COD influent and effluent measurements were demonstrated in Figure 3.3. COD removal efficiencies were calculated from these data and shown in Figure 3.4. In all different operation schemes which were explained in Section 3.1.2, the reaction period was always started with oxic phase for the removal of rbCOD with oxygen and followed by anaerobic phase for the hinder of the denitrification process (via NO_3^--N or NO_2^--N route). As seen from Figure 3.4, total COD (COD_U) removal and soluble COD (COD_F) removal efficiencies were 40% ±25 - 45%±26 Phase I and 44% ±18 - 44 %± 20 for Phase II. In Phases 3 and 4, the removal efficiencies for total and soluble COD were 54%±18 - 52%±17 and 49%±17- 47%±20, respectively.

3.2.2. Nitrogen Removal

Figure 3.5 shows the influent and effluent values of TN, NH_4^+-N, NO_2^--N and NO_3^--N for 374 days operation period. Based on different operation schemes (Table 2.3, Section 3.1.2) tried through this study, the expected possible nitrogen removal processes are the followings.

(i) In oxic phase; full nitrification, partial nitritation and partial nitratation

(ii) In anoxic/anaerobic phases; denitrification, denitritation, denitratation and Anammox.

The data shown in Figure 3.5 were evaluated based on these expected processes. For this purpose, TN removal, NH_4^+-N removal, NO_2^--N removal/accumulation, NO_3^--N removal/ production efficiencies were calculated from the daily measurements as summarized in Figure 3.6.

At the start-up, Phase I, the dominant period was the oxic phase operating under low set DO values (0.3-0.5 mg/l) in the oxic phase. Nitrification process was observed as the dominant process expectedly under these operational conditions. But, NH_4^+-N removal efficiency was not 100% and it was around 59%±24 due to the low DO set points.

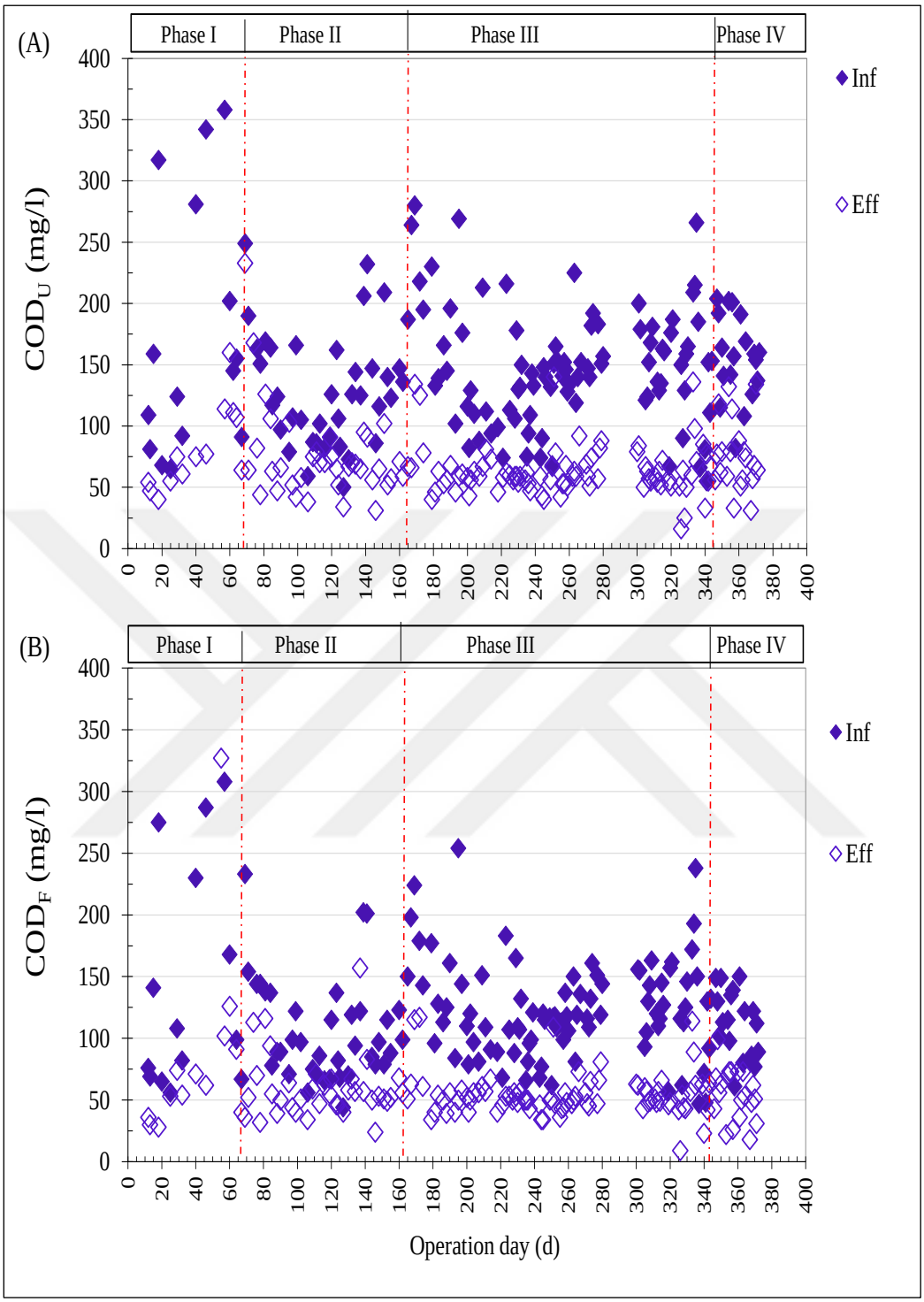


Figure 3.3 Daily COD influent and effluent measurements; A) Unfiltered COD (COD_U), B) Filtered COD (COD_F)

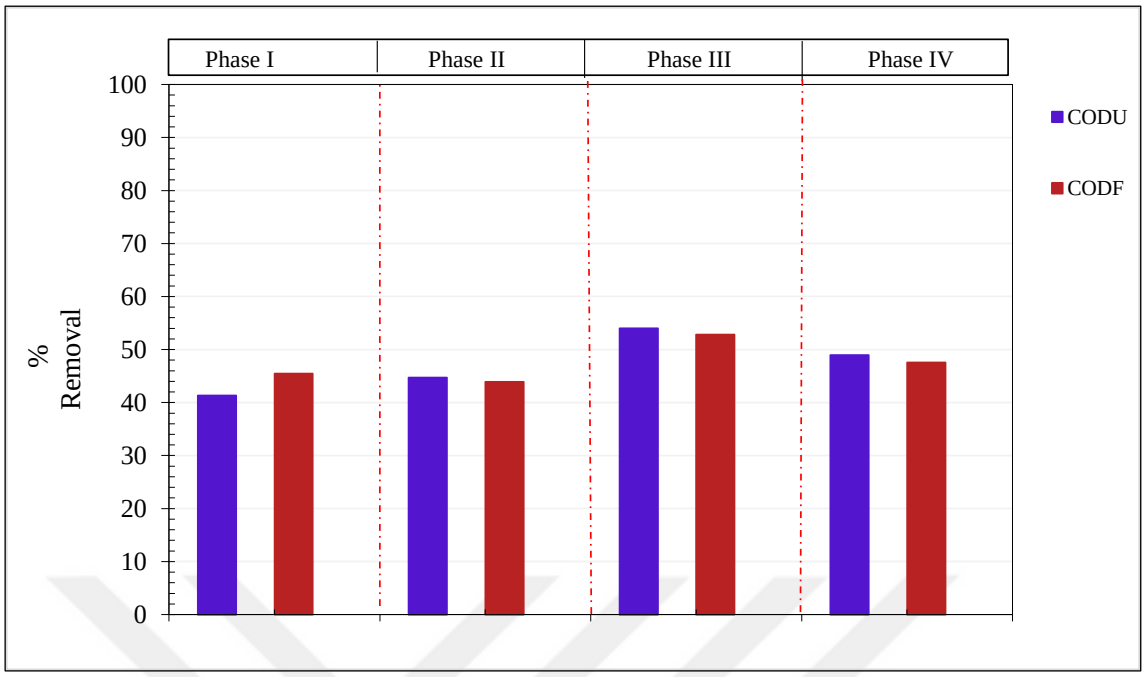


Figure 3.4 Time course COD removal efficiencies

Additionally, low DO levels allowed only partial conversion of ammonium to nitrate (partial nitratation) (11.8%±15 with high nitrate production) 52.8%±82 with nitritation process. TN removal rate observed around 36%±17might be due to assimilation of NH_4^+ by all groups of bacteria and denitrification process which might occur under low DO conditions. In consistent with this hypothesis, during almost half of the operation days of Phase I, 11%±16 NO_3^--N removal was observed instead of production. The increase in TN values observed rarely in few operational days of Phase I might be a result of increasing organic nitrogen and NH_4^+-N values because of cell lysis.

In Phase II, oxic period was followed by anaerobic period. NH_4^+-N removal efficiencies reached to the levels of 69%±27. In this phase, NO_2^--N mostly exhibited an accumulation trend. 59%±46 of influent ammonium accumulated as NO_2^--N. NO_2^--N removal of 25%±22 was observed rarely. NO_3^--N mostly exhibited a removal trend. But, the removed nitrate was only 4% ±6 of initial ammonium concentration. In few operational days, nitrate production was observed around 15%±10. These observations were a sign of decreasing denitrification activity in the system most probably closely linked to the significant amount of organic carbon removal in the oxic phase (Figure 3.2). Similar to the Phase I, TN removal efficiencies in Phase II were around 34%± 16.

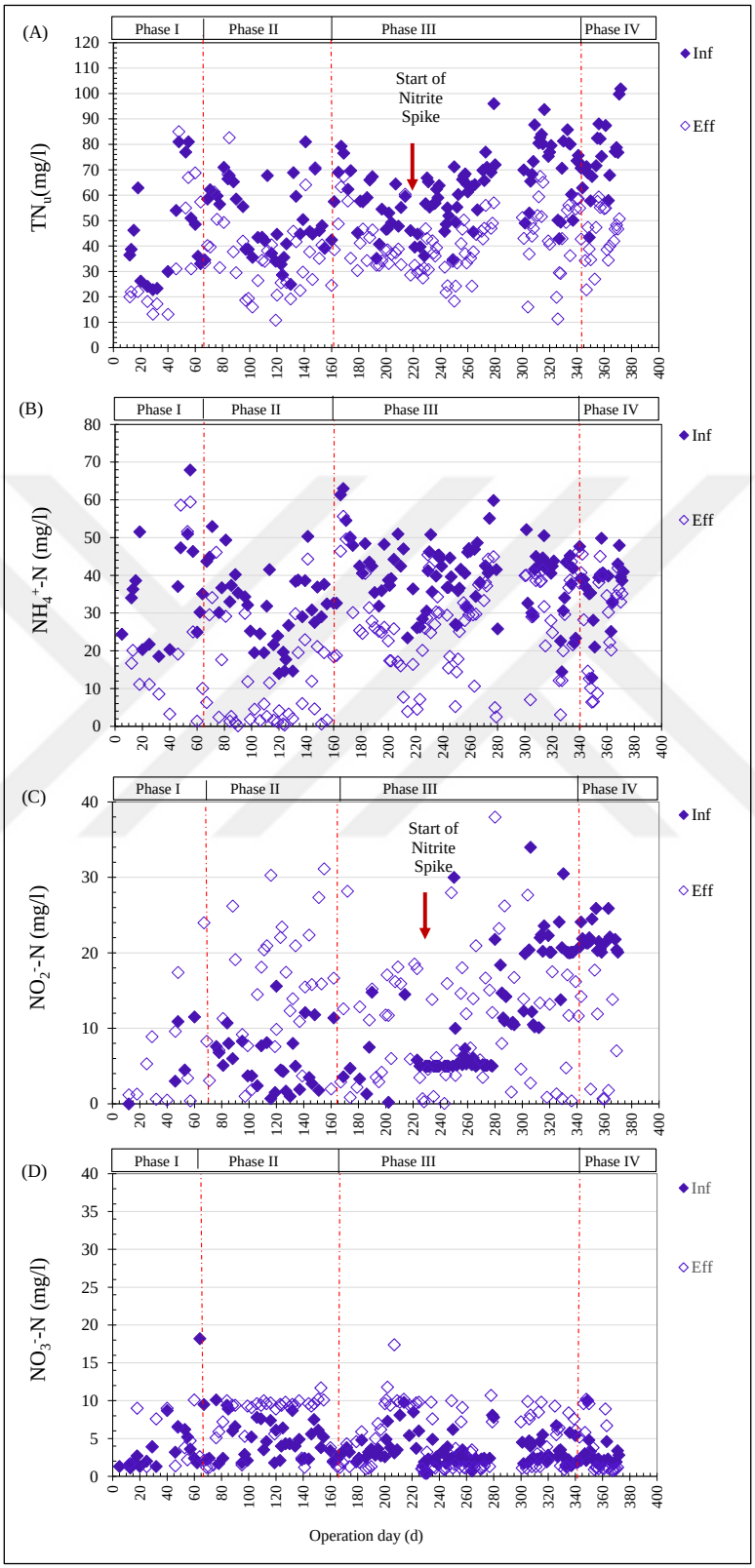


Figure 3.5 Time course influent and effluent parameters (A). TN, (B). NH_4^+-N, (C). NO_2^--N, (D). NO_3^--N

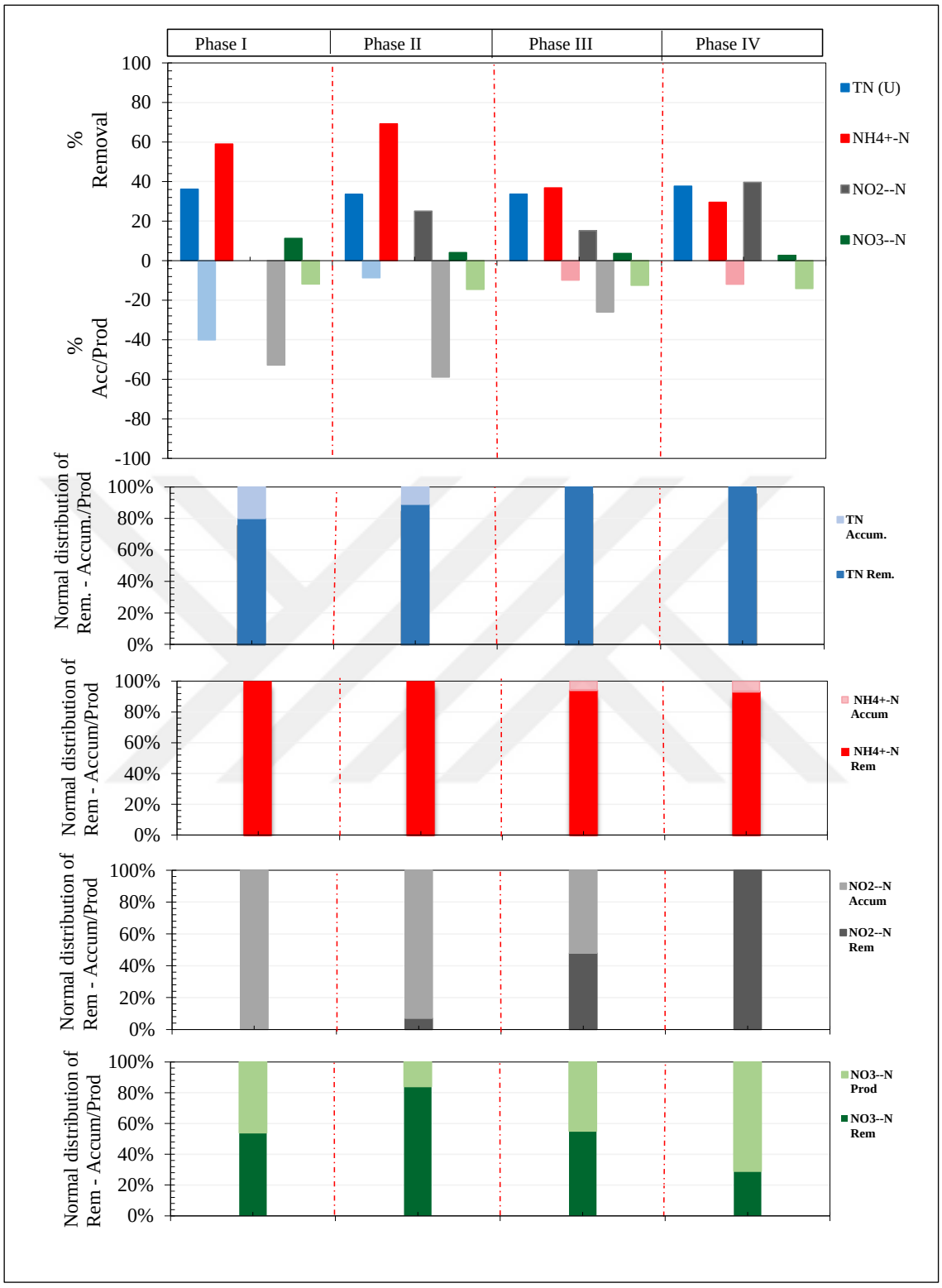


Figure 3.6 Time course nitrogen removal efficiencies during operation days

In spite of negligible denitrification activity in the system no changes observed in TN removal efficiency might be an indication of a newly starting Anammox activity.

In Phase III, with changing the seasonal conditions, wastewater temperature decreased dramatically (Figure 3.2). NO_2^--N accumulation efficiencies were decreased most probably due to increasing growth rate of NOB species in cold weather conditions with respect to AerAOB. Since nitrite accumulation was decreased more with time, at operation day of 250 NO_2^--N spiking to the reactor was started at the beginning of anaerobic phase. With decreasing temperature, NH_4^+-N removal rate dramatically decreased from 69% ±27 to 37%±24. NH_4^+-N accumulations observed in few operational days were most probably related with the release of organic nitrogen and ammonia during lysis of cells. In this phase, the number of operation days in which NO_2^--N accumulation or removal observed were almost equal in number. Similar trend was also observed for NO_3^--N productions and removals. Decreasing of NO_3^--N removal efficiency to the levels of 4%±3 is a clear indication of decreasing denitrification activity in the system in comparison to the previous phases. The sample hourly measurements of nitrogenous compounds together with COD in a batch cycle of this Phase are shown in Figure 3.7.

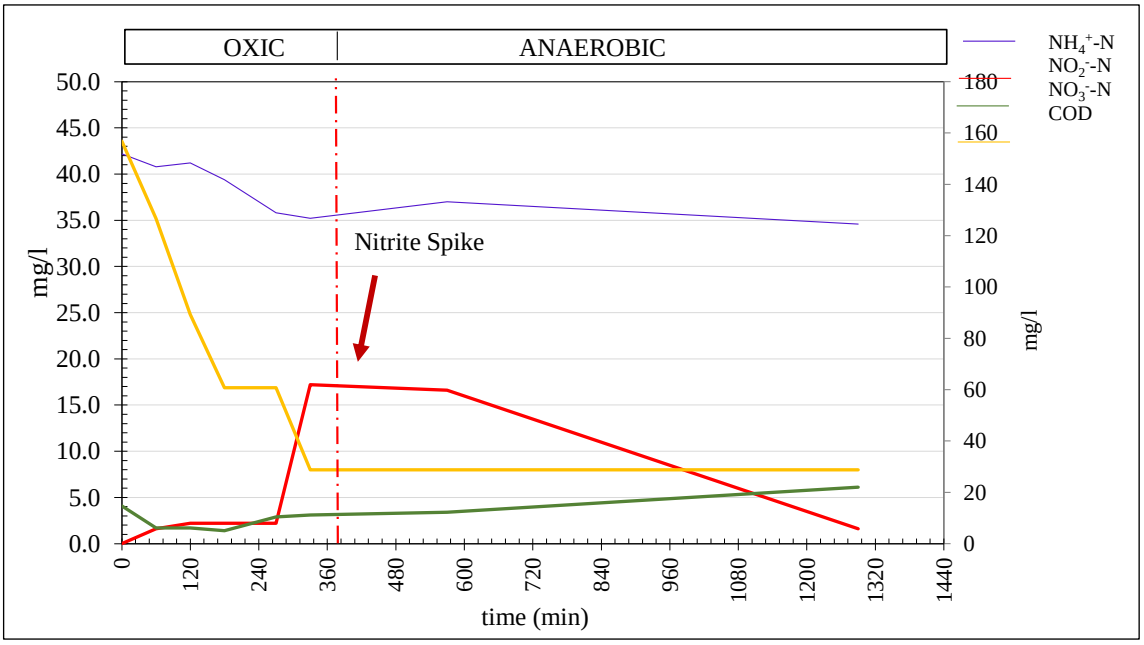


Figure 3.7 Sample batch cycle removal or accumulation efficiencies of NH_4^+-N, NO_2^--N, NO_3^--N and COD for Phase III

Figure 3.7 apparently shows nitrite and ammonium removal together with nitrate production trend in the anaerobic phase. This was a clear indication of the start of Anammox activity in the reactor.

In Phase IV, although all suspended bacteria were washed-out from the reactor and all reactions were accomplished by the attached biomass in the system NH_4^+-N removal efficiency was observed around 30%±27. NO_3^--N removal efficiency decreased to the levels of 14%±7 although NO_2^--N removal efficiency was around 30%±7. In the same period, COD removal efficiency was around 49%, which is even lower than the previous phase. Hence, nitrite and ammonium removals observed without significant change in NO_3^--N and COD removal efficiencies indicate prevalence of Anammox process in the system.

3.2.3. Phosphate removal

Influent and effluent PO_4^{3-}-P measurements are only available for Phase III and Phase IV and results were shown at Figure 3.8.

The data given in Figure 3.8 were evaluated in order to decide the degree of biological P uptake and release performance in the pilot reactor. As explained in Section 3.1, Phase III operation scheme was based on oxic followed by the intermittent aeration and anaerobic phases. In most of the operation days, 50%±21 PO_4^{3-}-P removal (uptake) was observed. However, in a few operational days released PO_4^{3-}-P cannot be up taken completely and PO_4^{3-}-P accumulation was observed around 28%±12. This might be related with the depletion of initial rbCOD in initial oxic phase prior to anaerobic phase and hence limitation of VFA storage in anaerobic phase and uptake during intermittent oxic periods. Another hypothesis can be secondary release of phosphorus during last anaerobic phase. In Phase IV, initial oxic followed by anaerobic operation scheme does not allow to uptake of released PO_4^{3-}-P in anaerobic zone. PO_4^{3-}-P uptake efficiency decreased almost 50% with respect to the Phase 3 and observed around 23%±16. Further research is necessary in order to clarify release and uptake mechanisms in anaerobic phase. In a few operational days, released PO_4^{3-}-P cannot be up taken completely and PO_4^{3-}-P accumulation was observed around 23%±16.

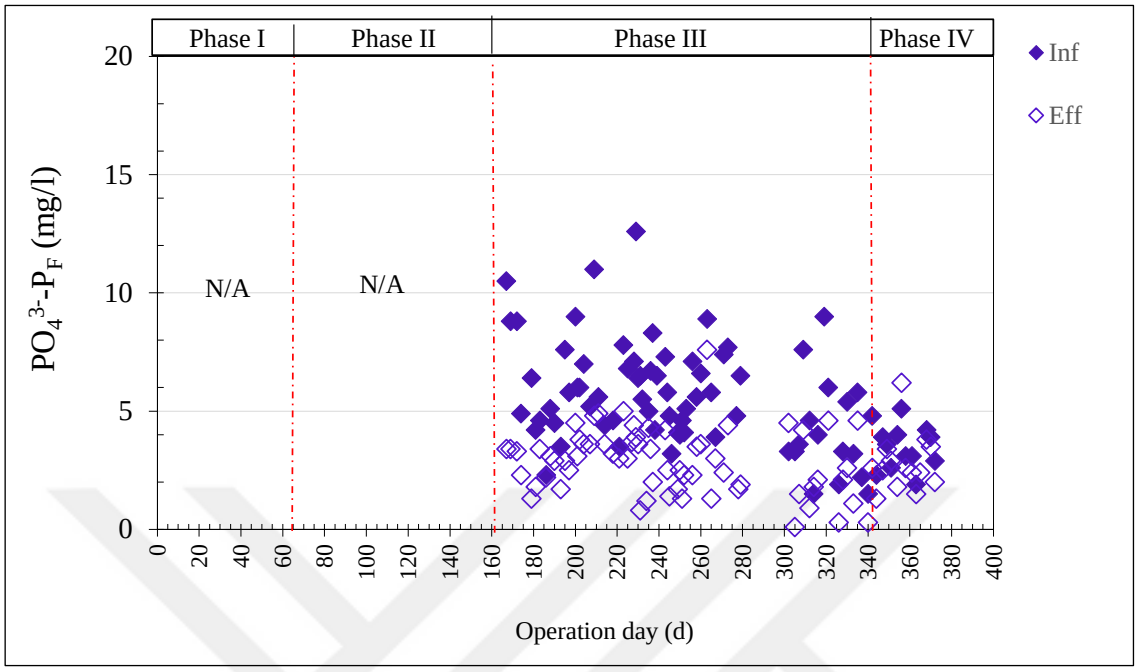


Figure 3.8 Time course influent and effluent values for filtered PO_4^{3-}-P, N/A: Not applicable

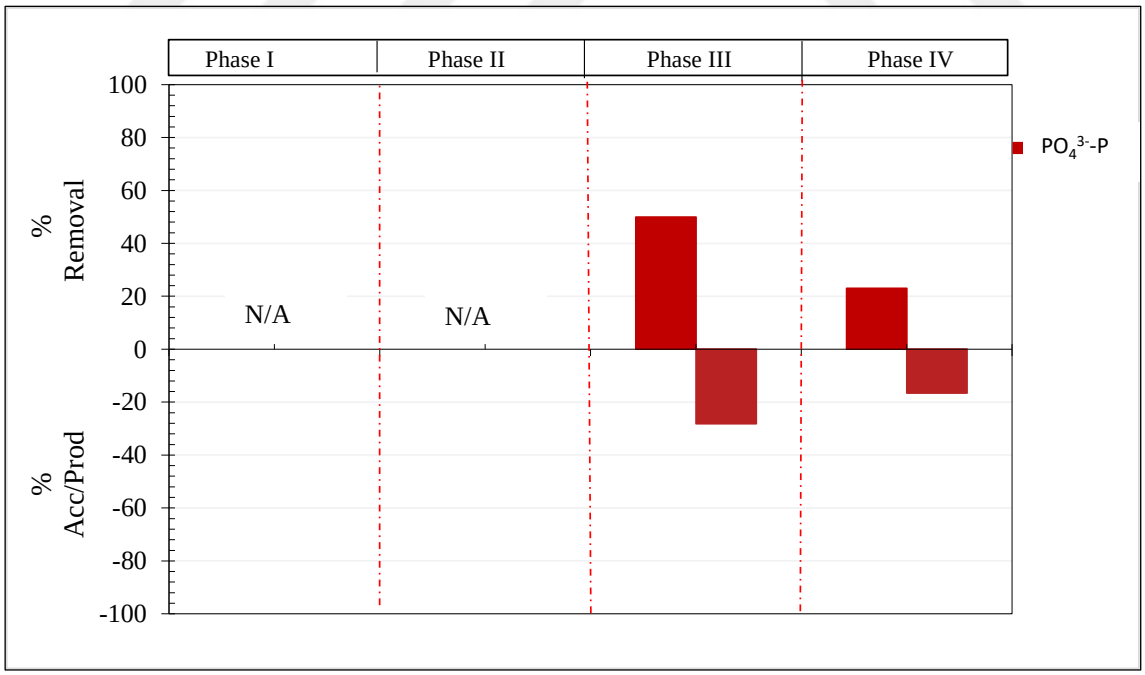


Figure 3.9 Time course phosphorus removal/ accumulation efficiencies, N/A: Not applicable

3.3. Biofilm Formation on Virgin Carriers

3.3.1. Visual inspection

The progress of initial biofilm attachment and development of biofilm was observed with naked eye as shown in photos in Figure 3.10. After 15 days of the start-up, solids were slightly apparent on carriers. Starting from the end of Phase I, biofilm attachment on carriers increased gradually. The biofilm reached to mature stage in Phase 3. In Phase 4 detachment of biofilm, especially in the outer layers of carriers were observed.

3.3.2. Gravimetric measurements

The biomass mount shown in Figure 3.11 represent the amount of biofilm attached on 10 randomly chosen carriers from the reactor by considering the total effective area of carriers reported by the carrier manufacturer. During Phase I and Phase II, biofilm amount on carriers mostly exhibited an increasing trend with fluctuations. Detachment of loosely attached biofilm was apparent in this period time to time. In Phase 3, biomass weight measurements almost reached a steady value indicating that irreversible attachment of the film was achieved with almost a constant film thickness.

3.4. Identification of the Characteristics of Biofilm Attaching on Carriers

3.4.1. Atomic force microscopy (AFM)

The surface roughness of the biofilm attaching on carriers was analyzed with AFM measurements as shown in Figure 3.12. Figure 3.11(a) shows AFM image of a virgin carrier which exhibited a smooth surface without any significant peaks and valleys. But, in AFM image belonging to Phase III, biofilm formation was apparent with peaks and valleys.

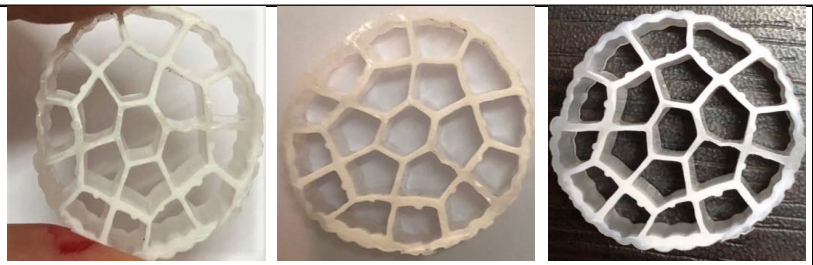
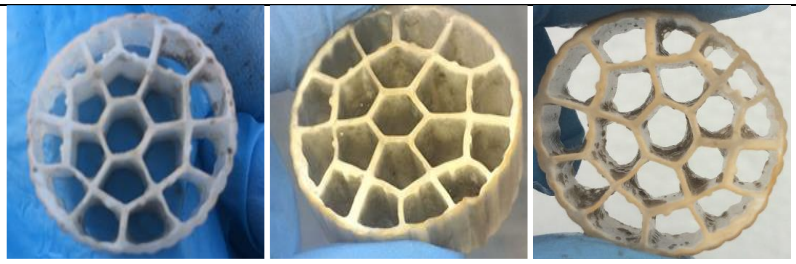
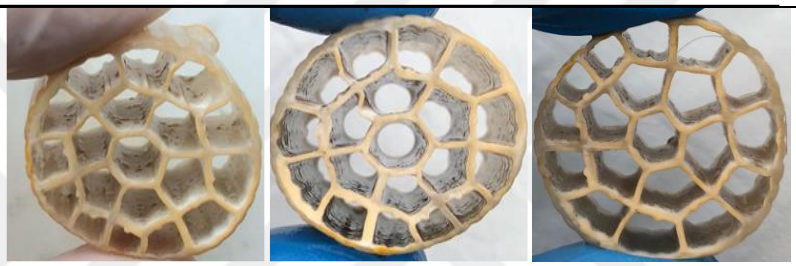
Virgin Carrier	
Phase I (1-68 days) *(15-46-64 days)*	
Phase II (69-168 days) *(74-146-162 days)*	
Phase III (169-344 days) *(186-218-320 days)*	
PHASE IV (345-374 days) (*347-355-372 days)*	

Figure 3.10 Time course visual inspection of carriers

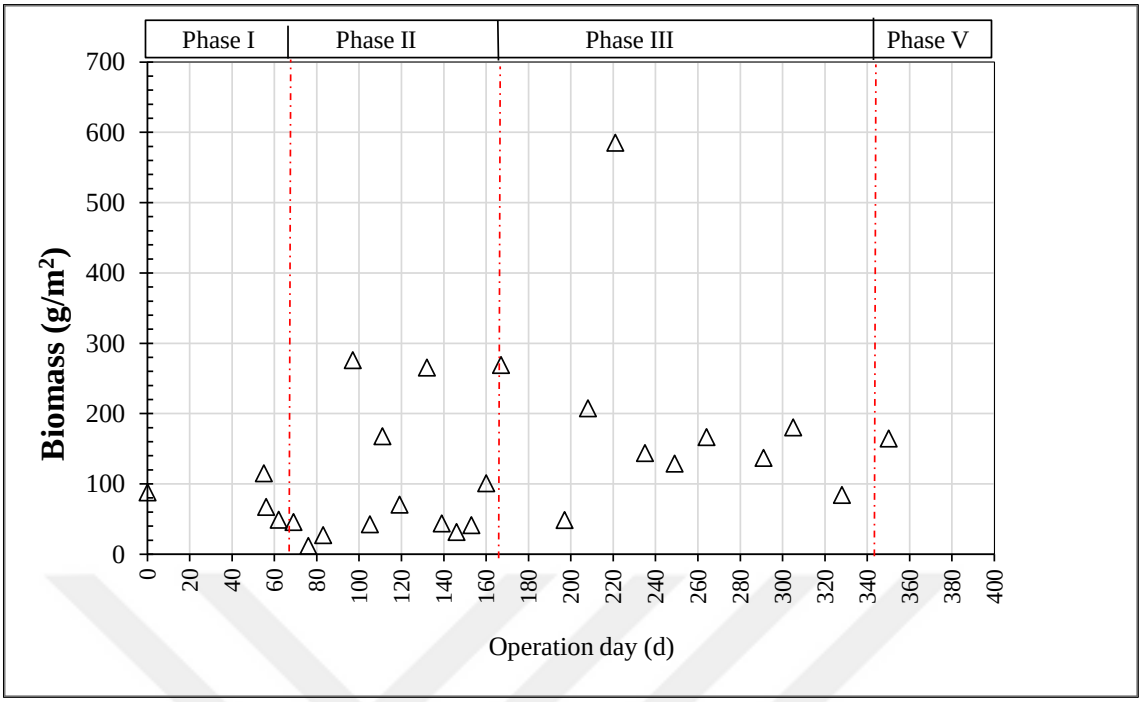


Figure 3.11 Time course carriers weight measurements

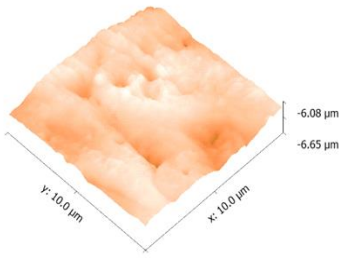
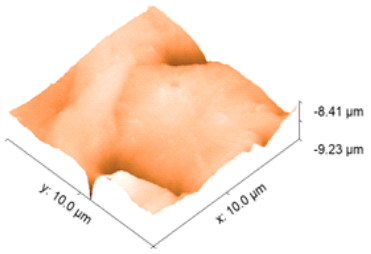
Virgin Carrier		
Phase III (165 - 344 days)		

Figure 3.12 Time course AFM images

3.4.2. X-ray Powder Diffraction (XRD)

XRD analysis was performed in order to analyze the crystalline structure in biofilm attached on carriers if any. However, XRD analysis of the biofilm samples given on carriers exhibited 100% amorphous structure by exhibiting the polypropylene characteristics of the carriers.

3.4.3. Fourier transform infrared (FT-IR)

Bacterial attachment on carriers analyzed with FTIR measurements are shown in spectrums in Figure 3.13. The peaks observed between 3000 cm^{-1} and 2500 cm^{-1} denote O-H, N-H, C-H groups found in soluble EPS. Other adsorption bands obtained at 1500-500 cm^{-1} result from bond vibrations of polysaccharides as well as DNA/RNA.

3.4.4. Thermogravimetric analyses (TGA)

TGA analysis of biofilm attached on carriers was shown in Figure 3.14. The mass loss from 20^0C-105^0C (≈12%) is strongly physically bound water. The mass loss between 105^0C-550^0C (≈80%) can be interpreted as loss of volatile solids due to pyrolysis of organics. The mass loss between 550^0C-800^0C (≈8%) represents loss of inorganic substances in the biofilm.

3.4.5. Scanning electron microscopy (SEM)

The morphology of bacteria in biofilm attached on carriers was monitored with SEM analyses. SEM images of the biofilm in Phase II and IV are shown in Figure 3.15 together with the surface image of a virgin carrier. The cocci shape bacteria were predominant species on these images. The clusteration of these cocci shape bacteria, especially in Phase IV indicates that they might be Anammox species. The biofilm thickness was measured roughly as 56 μm on SEM image belonging to Phase III.

3.4.6. Energy-dispersive X-ray spectroscopy (EDS)

EDS spectrums shown in Figure 3.16 showed that C and O are the dominant elements of the biofilm. Besides C and O, inorganic elements of Na, P, Cl, Al, Si and Ca were found in biofilm in trace quantities.

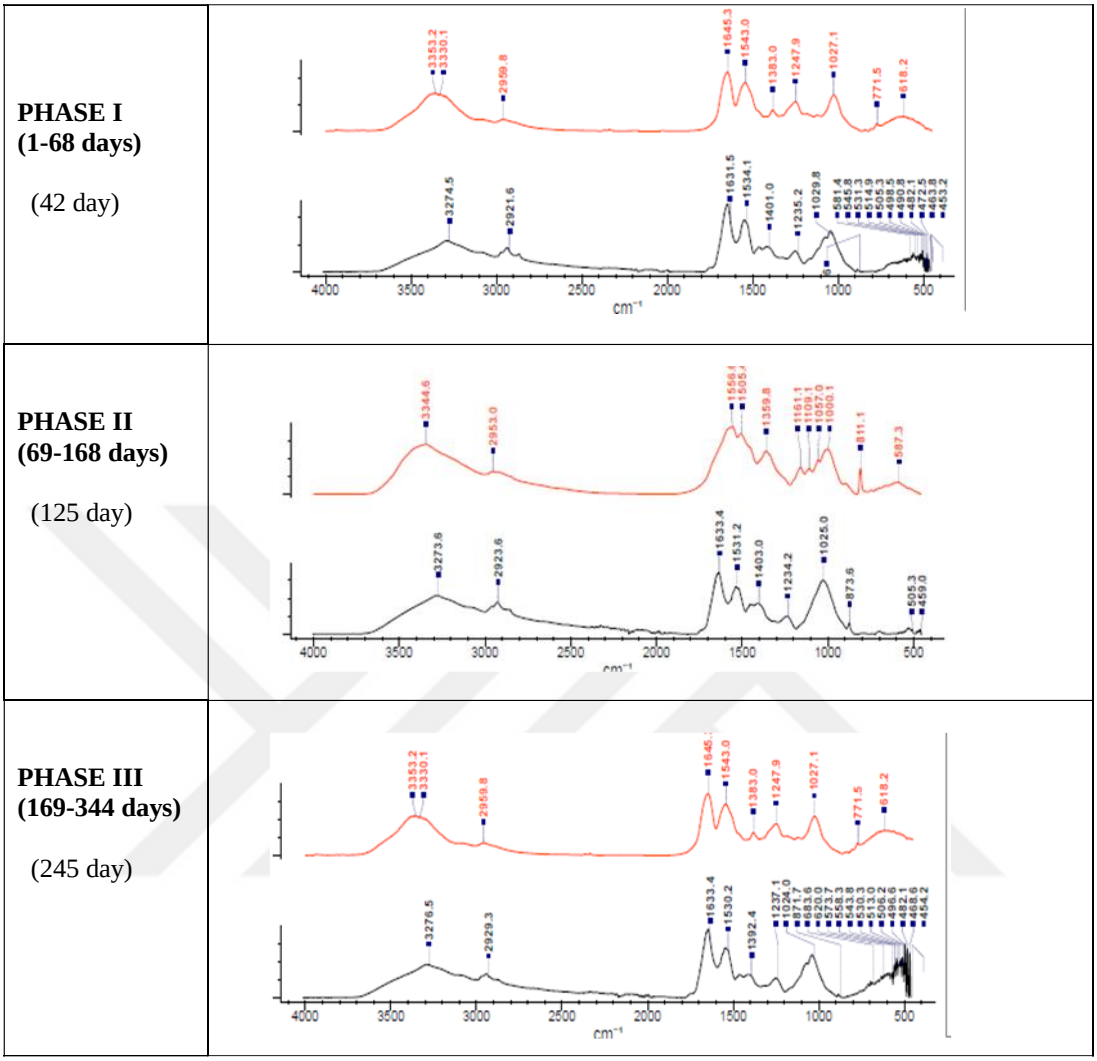


Figure 3.13 Time course FTIR analyses

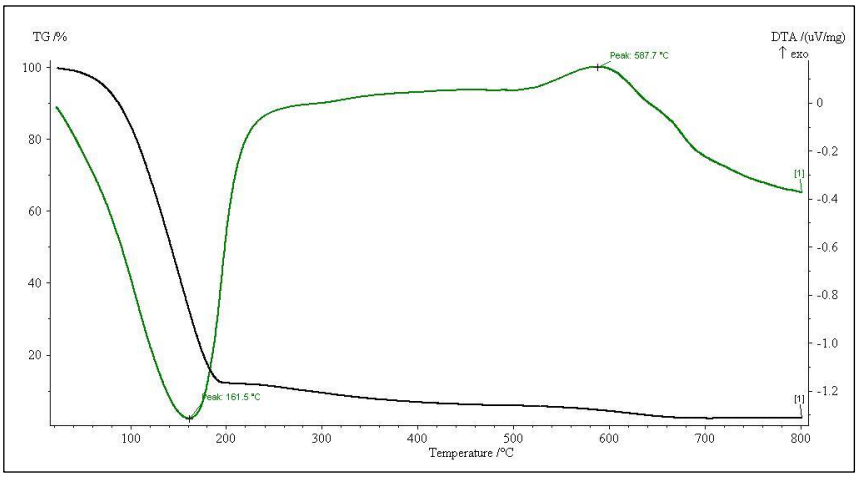


Figure 3.14 TG and DTG curves of biofilm on carriers in Phase II

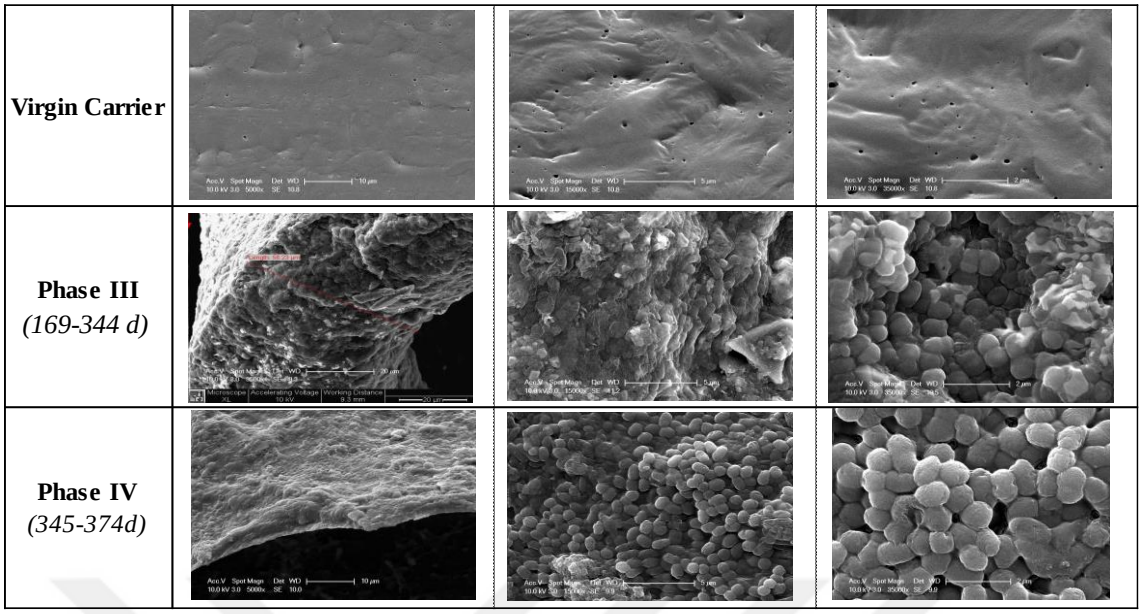


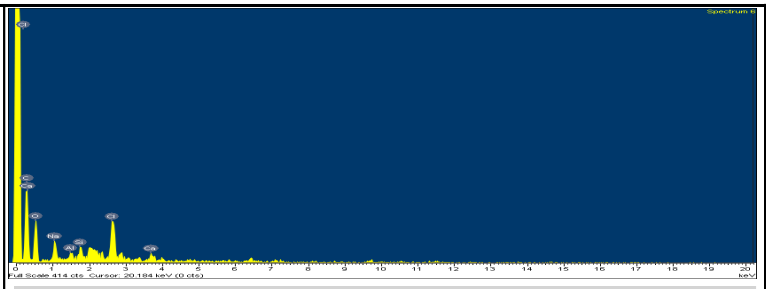
Figure 3.15 Time course SEM images

3.5 Microbial Community Analyses of Biofilm with Quantitative Polymerase Chain (qPCR)

qPCR results of Total Bacteria, ammonium oxidizers (AerAOB), nitrite oxidizers (NOB, Nitrospira, Nitrobacter) and Anammox (AnAOB) population in sludge samples taken from bulk liquid and biofilm scraped from carriers are shown in Figure 3.17.

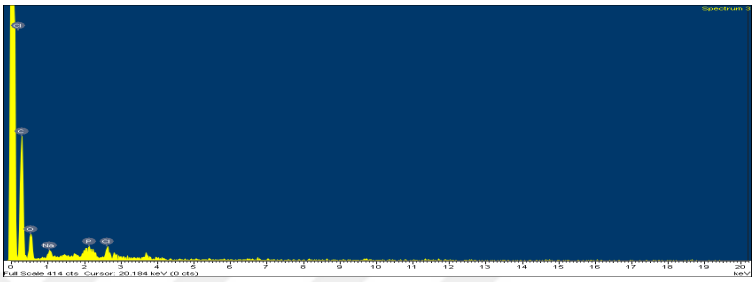
Total bacteria population which was around 10^8 copy number / ng until the mid stages of Phase III, increased to the levels of 10^9 copy number / ng at the end of Phase III. No change was observed in total bacteria population (10^9 copy number / ng) during Phase IV. AerAOB population, which was around 10^6 copy number / ng, decreased in Phase I and Phase II. After that, it was around 10^4 -10^5 copy number / ng. Initial NOB population (mainly Nitrospira species) of 10^3-10^4 copy number/ ng increased gradually till the mid stages of Phase II due to decreasing wastewater temperatures since low temperature favors the growth rate of NOB with respect to AerAOB. After that, NOB population decreased but not dropped below 10^4 copy number/ ng for Nitrospira species, 10^3 copy number/ ng for Nitrobacter species.

Phase I
(0-68 Days)



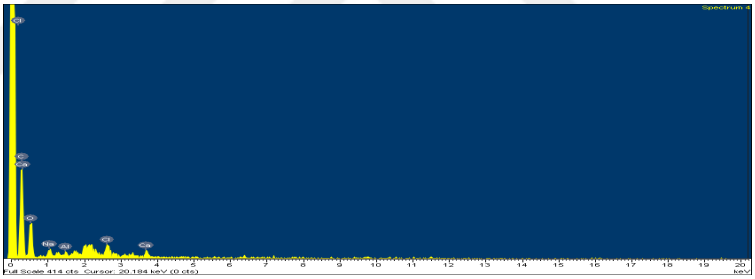
Element	C	O	Na	P	Cl	Al	Si	Ca
Weight %	67.55	29.02	1.41	0.42	1.26	-	-	-

Phase II
(69-164 Days)



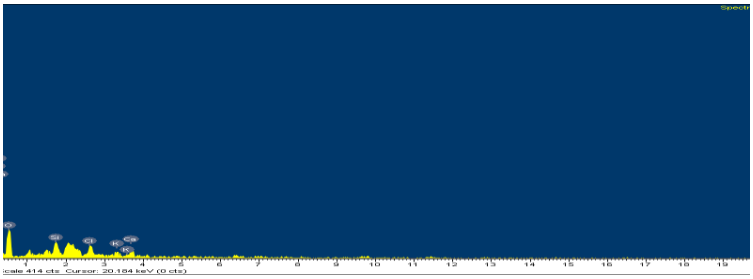
Element	C	O	Na	P	Cl	Al	Si	Ca
Weight %	55.27	33.87	2.99	-	-	0.67	1.17	0.91

Phase III
(165-344 Days)



Element	C	O	Na	P	Cl	Al	Si	Ca
Weight %	57.33	37.19	2.00	-	1.75	0.64	-	1.09

Phase IV
(345-374 Days)



Element	C	O	Na	P	Cl	Al	Si	Ca
Weight %	61.68	33.45	1.73	-	1.41	-	1.73	0.91

Figure 3.16 Time course EDS analyses of the biofilm attached to the carrier

Anammox population was initially around 10^4-10^5 copy number/ng DNA which was higher than the initially expected amount in activated sludge. This number did not increase significantly till the mid stages of Phase III. After that, it increased gradually and reached to the level of 10^6 - 10^7 copy number/ng and proved the enrichment of Anammox in the system.

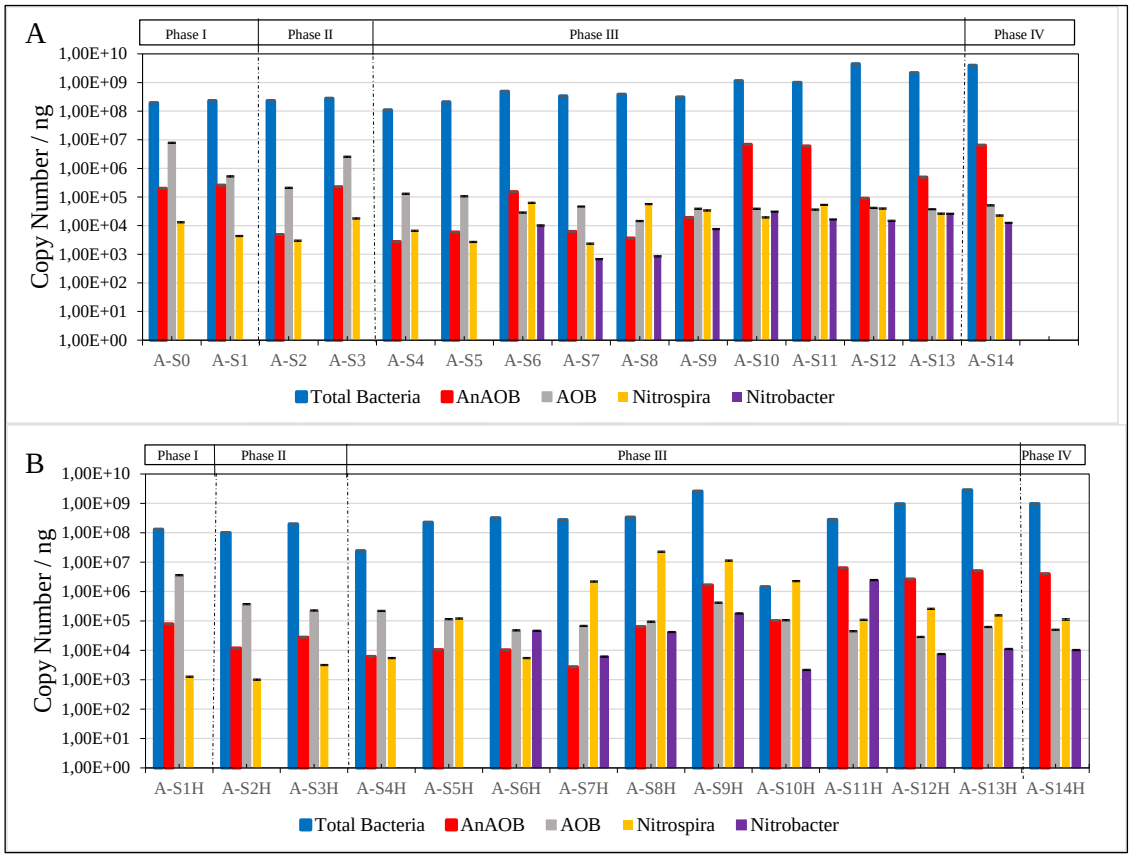


Figure 3.17 Time course QPCR results for (A) suspended biomass, (B) attached biomass.

4. CONCLUSION

In this study, the aim was firstly to provide the initial attachment of bacteria on surface of MBBR virgin carriers and then to initiate Anammox activity and enrich the Anammox species on carriers. Minimization detachment from the carriers and sustainability of nitrogen removal were also studied. For these purposes, a one-stage deammonification MBBR pilot reactor was started-up and operated in Atakoy STP with continuous feeding by Atakoy STP aerated grit chamber effluent for 374 days. The major conclusions obtained from this study are as follows:

1. The major factors affecting the initial attachment of biofilm on virgin carriers were determined as mixer impeller size ($D_{impeller}$) with respect to the tank diameter (D_{tank}), velocity gradient (G) created by the mixer and velocity gradient (G) created by blower. The optimum conditions were determined as $D_{impeller}/D_{tank}$ of 0.55 and G_{mixer} of 50 sec^{-1}. Operation of blower around G value of 542 sec^{-1} caused a detachment problem in the long term operation.
2. Scum control, wash-out of suspended solids with gradually decrease in settlement period and re-seeding with fresh activated sludge were determined as parameters positively affecting initial biofilm attachment period on virgin carriers.
3. DO based operational strategy was identified as a succesful operation strategy for the start-up of the mainstream one-stage deammonification process with activated sludge seed. However, dynamic changes in oxic-anaerobic operation schemes were required depending on process occuring in the system. NO_2^--N accumulation was identified as the limiting step. Nitrite spiking could be necessary to accelerate start-up period.
4. At the start-up of mainstream one-stage deammonification MBBR systems, an initial oxic phase is necessary for the purpose of rbCOD removal to hinder competition between OHO and Anammox species for the NO_2^--N in the aneorobic phase.
5. In the start-up of mainstream deammonification without using Anammox seed, partial nitritation and nitratation processes were simultaneously occured with denitritation and denitratation processes. The start-up of Anammox activity was found heavily dependent on NO_2^--N accumulation and exposure of microbial

population to NO_2^--N in the absence of rbCOD. Nitrite spiking could be necessary to accelerate start-up period especially under cold ambient conditons. First signs of the anammox activity was observed around operation day of 100. After operation days of 220-240, Anammox activity became clearly apparent.

6. Phosphate removal efficiency in a one-stage deammonification MBBR was found heavily dependent on operation scheme of the system, i.e., sequential arrangement of oxic and anaerobic phases. Oxic, short anaerobic, intermittent aeration, anaerobic operation scheme provided around 50% phosphate uptake.
7. AFM, FTIR, TGA, SEM and EDS were identified as instrumental methods that can be utilized for time course physico-chemical characterization of biofilm attachment on virgin carriers of MBBR systems.
8. The increase in Anammox population was proved with qPCR measurements. The initial population of Anammox species (10^4 copy number/ ng) increased to the level of almost 10^6 copy number/ ng indicating that enrichment of Anammox. qPCR results of NOB species (Nitrospira and Nitrobacter) indicated that a decrease in NOB population with the applied operation schemes. But, NOB population can not be washed-out from the system completely.

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