İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EXPERIMENTAL ASSESSMENT OF ANAMMOX PROCESS RESPONSE TO DIFFERENT CARBON COMPOUNDS

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ANAMMOX PROSESİNİN FARKLI KARBON KAYNAKLARINA TEPKİSİNİN DENEYSEL YÖNTEMLERLE BELİRLENMESİ

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EXPERIMENTAL ASSESSMENT OF ANAMMOX PROCESS RESPONSE TO DIFFERENT CARBON COMPOUNDS

SUMMARY

Anammox is the oxidation of ammonium to dinitrogen gas anaerobically with nitrite as the electron acceptor. The microorganism that carries out this process has been identified as a novel autotrophic member of the order Planctomycetales, one of the major distinct divisions of the bacterial domain. It is an almost completely unexplored part of the biological nitrogen cycle and the process offers new opportunities. Application of this process in nitrogen removal from wastewater could replace traditional nitrification-denitrification, reducing treatment cost.

Oxidation of ammonium anaerobically with nitrite yields in mainly nitrogen gas and small amount of nitrate. In anammox process, an electron donor is required for CO_2 fixation and nitrite acts also as electron donor. Thus, part of the nitrite is converted to nitrate. Although produced nitrate is very small portion of the totally removed nitrogen, especially for treatment of wastewaters containing high ammonium loads, produced nitrate would reach to significant concentrations in the effluent which is not desired for the water quality and needs to be removed in the wastewater treatment plants.

In this study, the influence of the representative organic compounds on the activity of anammox and nitrate reduction mechanism in the anammox process was investigated. For the assessment of the response of the anammox process in the presence of an organic carbon compound, batch and continuous flow reactor experiments were planned. In the first part of the experimental studies, stimulatory and inhibitory effects of different carbon compounds on the anammox process were examined. For this purpose parallel batch experiments were conducted with 8 different carbon compounds such as acetate, propionate, methanol, starch, glucose, ethanol, formate and amino acids. Glucose, ethanol and methanol were used to represent readily biodegradable substrate, starch was used to represent particular substrate and propionate, formate, acetate and amino acids were used to represent volatile fatty acids as readily biodegradable substrate mainly formed in sludge digestion tanks. Different concentrations of each compound were studied. Results indicated that methanol strongly inhibited the anammox activity where acetate and propionate were found to have stimulatory effect on the anammox process. All the other compounds were inhibited some degree of the anammox activity.

For the continuous flow reactor experiments methanol, acetate and propionate were selected. Three different continuous flow reactors inoculated with 74% enriched

anammox biomass and one was fed with methanol, one with acetate and the other with propionate. Methanol supplement was stopped due to immediate inhibition of anammox reaction. Flushing of the culture with sole mineral medium did not restore the anammox activity. Acetate feeding was supplied to the reactor at constant concentration. Propionate feeding was supplied to the reactor with gradually increasing concentrations. To examine the response of the anammox population to organic carbon supply was monitored for carbon and nitrogen compounds and also for population dynamics with Fluorescent in situ Hybridization analysis. During acetate feeding for 21 days it was observed that acetate was completely consumed in the reactor. Whereas, it was not detected any group of organism which would be an indication of denitrifiers. It was obtained from the mass balances of the acetate feeding reactor that less nitrate was produced compared to stoichiometrically expected amount. It would be an indication of nitrate reduction in presence of acetate. Slight change in anammox population was detected with Fluorescent in situ hybridization analysis which would indicate new strain(s) of anammox bacteria.

Especially in the beginning period of propionate supply, although propionate was completely consumed in the reactor it was not detected any change in the nitrate concentration. During this period, anaerobic ammonium oxidation activity in the reactor did not significantly change. As soon as the propionate load was increased, significant decrease in nitrate concentration in the effluent was observed.

FISH analyses were also showed that there was not any group of organism which would be an indication of denitrifiers. FISH analyses were also showed that anammox population was significantly changed under propionate feeding conditions which might be the indication of new anammox strains. Significant decrease in nitrate concentration was only observed under reasonably high load of propionate to the reactor. Propionate feeding reactor was examined also searching for denitrification activity with batch experiments. Results obtained from propionate feeding reactor directed the research on propionate consumption by anammox bacteria. For the investigation of this theory pulse-addition experiments with propionate in an anammox reactor were performed. For this purpose 74% enriched anammox bacteria which has never met with an external organic carbon compound, were used. Since biomass was 74% enriched, not a pure culture of anammox bacteria, to prove the theory of propionate consumption a pure culture of anammox cells has to be obtained. Since anammox bacteria have an extremely low growth rate classical microbiological isolation techniques are not practicable. A pure culture of anammox cells was obtained using density gradient centrifugation. Purified anammox cells were analyzed for degree of purification with Fluorescent in situ Hybridization. A micro batch experiment was conducted with purified anammox cells in presence of propionate in order to observe propionate consumption.

The mechanism of nitrate reduction coupled to propionate oxidation by anammox bacteria was investigated in the batch experiments using both labeled ($^{15}NO_3^-$) and unlabeled N-compounds. Both batch and continuous cultivation experiments showed that, nitrate was the electron acceptor coupled to propionate. Propionate oxidation only occurred in the presence of nitrate and when nitrate was present as the only electron acceptor, a transient accumulation of nitrite was observed. This indicated that nitrite was a free intermediate of nitrate reduction and that the reduction of nitrite was the rate-limiting step.

Considering the experimental circumstances associated with this study, a model was proposed to explain possible reaction mechanism of nitrate reduction in anammox process. According to proposed model, part of the nitrate is converted to nitrite and the rest is converted to dinitrogen gas. Nitrite formation was observed due to nitrate reduction and formed nitrite was simultaneously converted to dinitrogen gas with ammonium as expected anammox activity. This study demonstrates that anammox bacteria which are known as chemolithoautotroph can reduce nitrate using propionate as the electron donor.

ANAMMOX PROSESİNİN FARKLI KARBON KAYNAKLARINA TEPKİSİNİN DENEYSEL YÖNTEMLERLE BELİRLENMESİ

ÖZET

Anammox prosesi, amonyağın anaerobik koşullar altında azot gazına indirgendiği bir prosestir. Bu proseste amonyak elektron vericisi, nitrit elektron alıcısı olarak kullanılır. Anaerobik amonyum oksidasyonu, çok kısa bir zaman öncesine kadar biyolojik azot döngüsünün keşfedilmemiş bir bölümünü oluşturmakta idi. Bu prosesi oluşturan mikroorganizma topluluğu Planktomaysit türünün ototrofik grubunun üyesi olarak tanımlanmaktadır.

Nitritin elektron alıcısı olarak kullanıldığı anaerobik amonyum oksidasyonunda ana ürün azot gazı olmakla birlikte bir miktar da nitrat oluşmaktadır. Nitrat, biyosentez reaksiyonunda CO_2 fiksasyonu için gerekli olan elektron alıcısı olarak da kullanılmasıyla oluşmaktadır. Oluşan nitrat, anammox reaksiyonu uyarınca giderilen toplam azotun çok küçük bir parçasını oluşturmakla birlikte özellikle yüksek giriş amonyak konsantrasyonlarında önemli miktarlarda nitrat oluşumuna sebep olabilmektedir. Çıkış kalitesinde istenen standartların sağlanabilmesi için oluşan bu nitratın giderilmesi gerekmektedir.

Bu çalışma çerçevesinde, karbon kaynaklarının anammox prosesi üzerindeki etkileri ve anammox prosesinde oluşan nitratın giderim mekanizmasının belirlenmesi amaçlanmıştır. Anammox reaktörüne organik karbon bileşenlerinin beslenmesi ve anammox popülasyonunun bu koşullara tepkisinin belirlenmesi hedeflenmiştir. Çalışmanın ilk bölümünde farklı karbon kaynaklarının anammox aktivitesi üzerindeki etkilerinin belirlenmesi için 8 farklı karbon kaynağından oluşan kesikli deneyler gerçekleştirilmiştir. Kesikli deneylerde asetat, propiyonat, metanol, nişasta, glükoz, etanol, format ve aminoasitler farklı konsantrasyonlarda kullanılmıştır. Deneylerde; glükoz, etanol ve metanol kolay ayrışan substratı, nişasta partiküler substratı, format, asetat, propiyonat ve amino asitler genellikle anaerobik çamur çürütmenin asidifikasyon fazında oluşan fermantasyon ürünlerini temsil etmek üzere kullanılmıştır. Kesikli deneylerden elde edilen sonuçlar, metanolun anammox aktivitesini tamamen durdurduğunu, asetat ve propiyonatın anammox aktivitesini teşvik ettiğini, diğer bileşenlerin anammox aktivitesini farklı derecelerde inhibe ettiğini göstermiştir.

Sürekli sistem reaktör deneyleri için metanol, asetat ve propiyonat seçilmiştir. Bunun için üç ayrı reaktör %74 derecesinde zenginleştirilmiş aynı anammox kültürü ile aşılanmıştır. Rektörlerin biri metanol, diğeri asetat beslemesi için, diğeri de propiyonat beslemesi için kullanılmıştır. Metanol beslemesi, anammox aktivitesini derhal ve geri dönüşümsüz şekilde inhibe ettiği görülmesi üzerine kısa sürede durdurulmuştur. Sistemin bir hafta süre ile mineral ortam ile yıkanması ile, anammox aktivitesinin tekrar geri kazanımını sağlanamamıştır. Asetat beslemesi sabit asetat

yükünde çalıştırılırken, propiyonat beslemesi giderek artan yüklerde gerçekleştirilmiştir. Karbon kaynağı beslemesi koşulları altında anammox prosesinin tepkisini belirlemek için reaktörler karbon ve azot bileşenleri yanında popülasyon dinamiği açısından da izlenmiştir.

Asetat ile beslenen anammox reaktöründen elde edilen sonuçlara göre reaktöre beslenen asetatın tüketilmesine rağmen nitrat konsantrasyonunda bir düşüş görülmemiştir. Ayrıca FISH analizleri ile elde edilen bulgularda denitrifikasyon bakterilerini temsil edebilecek bir organizma grubuna rastlanmamıştır. Fakat, 21 günlük süre için yapılan kütle dengesinden elde edilen sonuçlar, proseste oluşan nitratın anammox stokiyometrisine göre beklenenden daha az olduğunu göstermiştir. Bu sonuç, asetatın varlığında nitratın indirgenebileceğinin göstergesi olarak kabul edilebilir. FISH analizleri ayrıca asetatın varlığında anammox popülasyonunda küçük bir değişikliğin olduğunu da göstermiştir.

Özellikle propiyonat ilavesinin başladığı ilk periyotta reaktöre beslenen propiyonatın tümünün tüketilmesine rağmen nitrat konsantrasyonunda bir değişiklik görülmemiş, nitrat konsantrasyonu uzun süre sabit kalmıştır. Bu süre zarfında ayrıca anammox stokiyometrisinin önemli ölçüde değişmediği görülmüştür. Ancak sisteme sağlanan propiyonat yükünün artırılmasıyla nitrat konsantrasyonunda önemli bir düşüş görülmüştür ki bu da sistemde yeterli miktarda propiyonatın varlığında nitratın tüketildiğini göstermiştir.

Yapılan FISH analizlerinde uzun süreli propiyonat beslemesine rağmen denitrifiyer topluluğunu temsil edecek bir organizma grubu belirlenmemiştir. FISH analizleri reaktördeki anammox topluluğunun baskınlığının sürdüğünü fakat önemli ölçüde değiştiğini, yeni anammox türlerinin geliştiğini göstermiştir.

Propiyonat ile beslenen anammox reaktöründen elde edilen bulgular, propiyonat tüketiminden anammox bakterisinin sorumlu olabileceğini göstermiştir. Bu sebeple çalışma kemolithoototrof olarak bilinen anammox bakterisinin propiyonat tüketimi konusuna yönlendirilmiştir. Bu amaçla, önce %74 oranında zenginleştirilmiş anammox biyo-reaktöründe propiyonat tüketimini izlemek için anlık propiyonat ilavesi ile deneyler yapılmıştır. Bu deneylerde propiyonat konsantrasyonun lineer olarak düştüğü görülmüştür. Anammox biyo-reaktörünün saf kültürden oluşmadığı sebebiyle anammox hücreleri yoğunluk gradyanı santrifüj yöntemi ile saf kültürü elde edilerek bir mikro ölçekli kesikli deneyle propiyonat tüketimi izlenerek %74 zenginleştirilmiş kültürde elde edilen sonucun ispatı sağlanmıştır. Elde edilen kültürün saflık derecesi FISH analizi ile değerlendirilmiştir ve yaklaşık %99.5 derecesinde saflığa ulaşıldığı görülmüştür.

Propiyonatın varlığında nitratın anammox bakterisi tarafından giderim mekanizması farklı azot bileşenleri ile yapılan kesikli deneylerde ve $^{15}NO_3^-$ izotopu işaretli kesikli deneyleriyle açıklanmıştır. Bu kesikli deneylerle ve daha önce yapılan sürekli akımlı reaktör deneylerinde nitratın propiyonat oksidasyonunda elektron alıcısı olduğu bulunmuştur. Propiyonat oksidasyonu sadece nitratın varlığında gerçekleşmiş ve nitrit ara birikimi görülmüştür. Bu şekilde nitritin nitrat gideriminde serbest ara ürün olduğunu ve bu şekilde hız kısıtlayıcı adım olduğunu göstermektedir.

Tüm bu bulgular ışığında anammox bakterisinin propiyonatı kullanarak nitratı indirgeme reaksiyonunun mekanizması bir model ile açıklanmıştır. Bu modele göre

nitratın bir kısmı nitrite dönüşürken, bir kısmı doğrudan azot gazına indirgenmektedir. Bu modele göre oluşan nitrit, anammox prosesinde tekrar döngüye girerek anaerobik amonyum oksidasyonu reaksiyonunu gerçekleştirmektedir. Bu bulgu, elektron alıcısı olarak nitriti kullanarak amonyağı anaerobik ototrofik koşullar altında oksitleyen anammox bakterisinin propiyonatı elektron vericisi olarak kullanarak nitratı indirgeyebildiğini ortaya koymaktadır.

1 INTRODUCTION

1.1 Significance of the study

It is widely acknowledged that nitrogen in wastewater has become one of the major pollutants to our water resources. Nitrate, nitrite and ammonium are important pollutants in municipal and industrial wastewaters.

Conventional methods for the biological removal of these compounds involve two discrete steps namely nitrification and denitrification. First off all, nitrification is an energy demanded process for aeration and due to low growth rate of nitrifiers, large nitrification volumes are required. Secondly, denitrification requires organic carbon to be efficient. If the carbon content in the wastewater is not sufficient, an extra carbon source has to be supplied which contributes to an increase of overall treatment costs.

In wastewater treatment plants with anaerobic sludge digestion, 15-20% of the inlet nitrogen load is recycled with the return liquors from sludge dewatering. Separate treatment of this digester supernatant would significantly reduce the nitrogen load of the main stream and improve nitrogen elimination. Chemical elimination of ammonium with magnesium –ammonium –phosphate (MAP) precipitation or with air stripping is feasible but much more expensive than classical nitrification and denitrification of an organic carbon source

Anaerobic ammonium oxidation process (Anammox) is a new low-cost alternative to conventional denitrification systems. If the anammox process is combined with a preceding nitrification step, only part of the ammonium needs to be nitrified to nitrite while the anammox process combines the remaining ammonium with this nitrite to yield dinitrogen gas and small amount of nitrate. This will reduce oxygen demand in the nitrification reactor and lead to a second reduction in costs. The biomass yield of the anammox process is very low; consequently little sludge is produced. The low

sludge production is the third factor that contributes to the substantially lower operation costs compared to conventional denitrification systems.

Separate treatment of anaerobic sludge digestion effluents using Anammox process is a new opportunity for wastewater treatment plants. The effluents from sludge digesters contain ammonium in high concentrations. Although these ammonium-rich streams were reported to have relatively low BOD_5, they may still contain residual biodegradable carbon compounds due to low efficiency of the digester or a breakdown in the system. Since Anammox bacteria are known as obligate chemolithoautotrophs, the effect of residual carbon in the effluent needs to be investigated to determine the inhibitory or stimulatory effects on the anammox process. Additionally, in the anammox process, because reducing power is required for CO_2 fixation in biosynthesis, part of the nitrite is converted to nitrate. Although relatively small amount of nitrate is produced, especially wastewaters containing high ammonium, such as sludge digestion effluents, produced nitrate would be reached to significant concentrations in the effluent which has to be removed.

This study focuses on the assessment of the influence of the representative organic compounds on the activity of anammox and the investigation of the nitrate reduction mechanism in presence of an organic compound in the anammox process.

1.2 Scope and Outline

In the first part of the experimental studies batch experiments were performed for the assessment of the inhibitory or stimulatory effects of different organic compounds on the anammox processes. The experimental assessment was obtained by performing parallel batch tests with methanol, acetate, propionate, starch, ethanol, glucose, formate and amino acids. Glucose, ethanol and methanol were used to represent readily biodegradable substrate, starch was used to represent particular substrate and propionate, formate, acetate and amino acids were used to represent volatile fatty acids as readily biodegradable substrate mainly formed in sludge digestion tanks. Stimulatory and inhibitory effects of these carbon compounds were evaluated and the chemostat experiments were performed with methanol, acetate and propionate. During the operation of the chemostat, together with nitrogen compounds and mass

balances, changes in the bacterial populations were also monitored with the Fluorescence *in situ* hybridization analysis.

In view of the consideration of the results of the first part of the study, propionate consumption in the anammox reactor was examined. For the investigation of propionate consumption by anammox bacteria, pure culture of anammox cells were obtained and micro batch experiments were performed.

The third period of the experimental studies involves the investigation of the possible nitrogenous compound coupled to propionate. The batch experiments were conducted with different configurations of nitrogen compounds in presence of propionate. ^{15}N-labelling experiments were performed to explore the possible reaction mechanism of nitrate conversion coupled with propionate consumption by anammox bacteria.

2 FUNDAMENTAL BASIS OF ANAEROBIC AMMONIUM OXIDATION AND DENITRIFICATION

2.1 Stoichiometry and kinetics of the anammox process

Anaerobic ammonium oxidation is the oxidation of ammonium to dinitrogen gas with nitrite as the electron acceptor. The process is mediated by autotrophic bacteria that use CO_2 as the only carbon source.

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2\,H_2O \qquad (2.1.)$$

The process was first described by Broda (1977) as a microbial metabolism and the existence of chemolithoautotrophic bacteria capable of anaerobic ammonium oxidation was predicted. Until its discovery, bacteria capable of oxidizing ammonia anaerobically had never been found and were known as 'lithotrophs missing from nature (Strous et al., 1999).

Later, the anammox process was discovered in a denitrifying pilot plant for the treatment of wastewater from the Gist brocades yeast factory in Delft (Mulder et al., 1995). In this system, ammonium disappearance was observed under anoxic conditions. The discovery was important for two reasons: Firstly, the anammox process is very attractive to wastewater treatment, secondly, it had been known that ammonia could not be oxidised under anoxic conditions.

Initially, nitrate was believed to be the electron acceptor. The continuous experiments with the systematic, stepwise addition of various supplements to the wastewater showed that nitrite was the main electron acceptor used in the anammox process. (Van de Graaf et al., 1995).

Only after the role of nitrite was recognized, enrichment and study of the responsible microorganism became possible. A first successful step in the partial enrichment of the organisms catalysing anaerobic ammonium oxidation was the use of fluidized bed cultures fed with mineral media containing ammonium and nitrite only (Van de Graaf et al., 1996, 1997).

Van De Graaf et al. (1996) operated a fluidized bed reactor inoculated with a large amount of sand covered with denitrifying and anammox biofilms originating from the 27 l installation settled by Mulder et al. (1995). This anammox fluidized bed culture was maintained using an autotrophic mineral medium with ammonium, nitrite and carbonate. A conversion of 3.1 kg NH_4^+-N/m^3.day was achieved with a feed of 30 mM NH_4^+ while the original sludge had an anaerobic ammonium removal rate of 0.4 kg N/m^3.day which was obtained by Mulder et al. (1995). The total nitrogen removal of the anammox reactor, including the conversion of nitrite, was 4.8 kg N/m^3.day.

The complete anammox stoichiometry was first estimated by van de Van de Graaf et al. (1996) conducting mass balances over a fluidized bed reactor (FBR). The nitrogen balance obtained from the FBR run depicted a ratio that 1:1.31:0.22. This estimation has been modified slightly with the introduction of the sequencing batch reactor (SBR) (Strous, et al., 1998).

Results obtained from the 15 l SBR showed that the biomass specific activity in the reactor was constant during the operation of 90 days of exponential growth at 20±6 μmol NH_4^+/g protein.min. Mass balances over the SBR were compiled and with this mass balances, the conversions of nitrogen and carbon compounds were calculated. The overall nitrogen balance obtained from the SBR run showed a ratio of 1:1.32:0.26 for the conversion of ammonium and nitrite to the production of nitrate. Biomass yield were determined as 0.066 ± 0.01 C mol/mol NH_4^+. The maximum growth rate was calculated as 0.0027 h^{-1} which equals to a doubling time of 11 days. The stoichiometry of the anammox process (at nitrite limitation, in the presence of 5 mM surplus ammonium and nitrate) was calculated as:

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \longrightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

Nitrate presumably produced from nitrite to generate reducing equivalents for CO_2 fixation and therefore is a measure for biomass growth (Van de Graaf et al., 1996; Strous, et al., 1999). The maximum specific ammonium consumption (q_{max}; in the presence of excess nitrite) was obtained as 45±5 μmol NH_4^+/g protein.min. The maximum specific ammonium consumption (q_{max} = 45 μmol NH_4^+/g protein.min) and the maximum growth rate (μ_{max} = 0.0027 h^{-1}; doubling time 11 days) were much higher than reported previously by Van de Graaf et al. (1996) (q_{max} = 26 μmol NH_4^+/g protein.min, μ_{max} = 0.001 h^{-1}). Strous et al. (1998) concluded that this result could be partly because of the higher degree of enrichment was obtained in the SBR than that of the FBR.

2.2 Effects of environmental conditions on the anammox process

2.2.1 Influence of temperature and pH on the anammox process

The maximum specific substrate (nitrite, ammonium) conversion rate of anammox process was measured at different temperature and pH values. The linear decrease of the substrates in each single experiment showed that the substrate conversion rate was constant at each temperature and pH value. The specific substrate conversion rates were presented in Figure 2.1 (Strous, 2000).

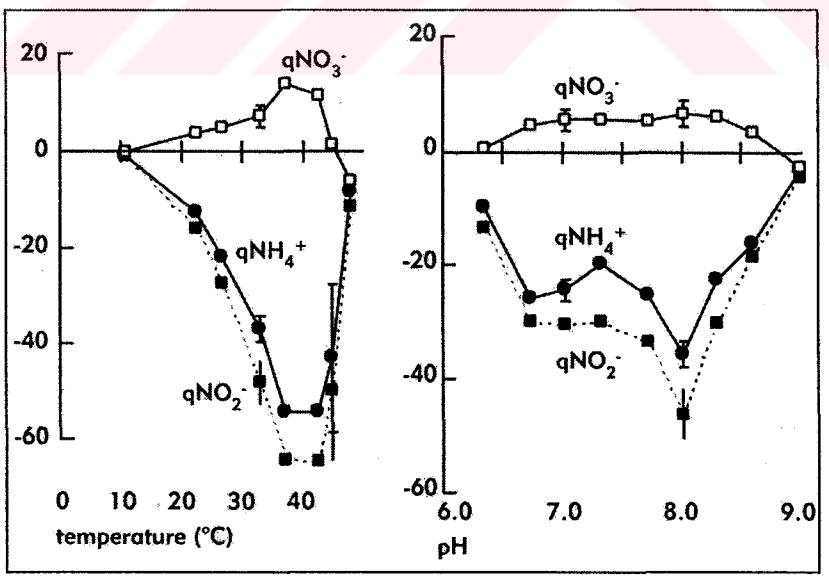


Figure 2.1 Influence of temperature and pH on the anammox process (Strous, 2000).

The figure shows that the optimum temperature of the anammox biomass was 40 ± 3^0C. The physiological pH range of the anammox biomass was 6.7 to 8.3 with an optimum at pH 8.

2.2.2 Substrate affinity and substrate inhibition

The affinity for the substrates ammonium and nitrite was very high and affinity constants for ammonium and nitrite were less than 5 μM respectively (Jetten et al., 1999). Strous et al., (1999) concluded that the anammox process was not inhibited by ammonium and by the product nitrate. The anammox process was inhibited by nitrite in presence of more than 0.1 g NO_2^- - N/l.

2.2.3 Influence of oxygen on anaerobic ammonium oxidation

The influence of oxygen on the anammox process was investigated in both batch and continuos systems (Jetten et al., 1999). Initial batch experiments showed that oxygen completely inhibited the anammox activity when it was deliberately introduced into the enrichment cultures (Van de Graaf et al., 1996; Jetten et al., 1997).

In a flow up study, an intermittently oxic (2h) and anoxic (2h) reactor system was used to study the reversibility of oxygen inhibition for 20 days (Strous et al., 1997). From these studies, it became clear that ammonium was not oxidized in the oxic periods, but that the anammox activity in the anoxic periods remained constant throughout the experiment, indicating that the inhibitory effect of oxygen was indeed reversible (Jetten et al., 1999).

The sensitivity of the enriched anammox culture to oxygen was further investigated under micoaerobic conditions (Strous, et al., 1997). Four batch experiments were conducted at different oxygen concentrations; 2%, 1%, 0.5 % and 0% air saturation. No ammonium was oxidized in presence of 2%, 1%, 0.5% air. Only at 0% air saturation, ammonium and nitrite were consumed.

Figure 2.2 shows the influence of oxygen on anammox activity. These results indicated that the anammox activity in these enrichment cultures is only possible under strict anoxic conditions (Jetten et al., 1999).

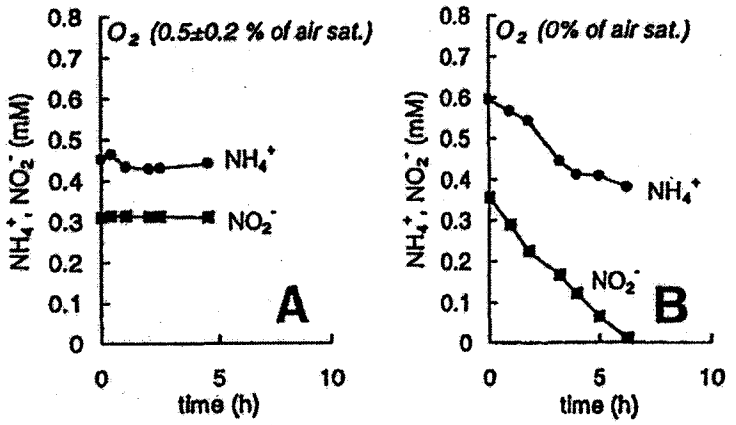


Figure 2.2 Influence of oxygen on anammox activity (Jetten et al., 1999).

2.3 Possible reaction mechanisms for anammox

The possible metabolic pathway for anaerobic ammonium oxidation was investigated using ^{15}N labelling experiments. These experiments showed that the electron acceptor nitrite reduced to hydroxylamine and that hydroxylamine reacts with the electron donor ammonium, leading to the ultimate production of dinitrogen gas (Jetten et al., 1999). Although the main product of the reaction was dinitrogen gas, approximately 20% of the nitrite supplied was recovered as nitrate. The production of nitrate from nitrite was verified with ^{15}N–NMR analysis (van de Graaf et al., 1997). Only when labelled nitrite was supplied to the cultures, formation of $^{15}NO_3^-$ was observed. The function of this nitrate formation is presumably the generation of reducing equivalents necessary for the reduction of CO_2.

In batch experiments with excess hydroxylamine and ammonium, a transient accumulation of hydrazine was observed indicating that hydrazine is the intermediate of this final step (Van de Graaf et al., 1997). The occurrence of hydrazine as an intermediate in microbial nitrogen metabolism is rare (Schalk et al., 1998). The oxidation of hydrazine to dinitrogen gas is also mediated by the HAO (hydroxylamine oxidoreductase) enzyme of *N. europaea*. High HAO activity in cell extracts of B. anammoxidans indicated that a similar enzyme might be operative in the anammox mechanism (Schalk et al., 2000).

From these results, they postulated that the oxidation of hydrazine to dinitrogen gas generates the electrons for the initial reduction of nitrite to hydroxylamine. Possible mechanism of anaerobic ammonium oxidation given by Jetten et al. (2001) is illustrated in Figure 2.3.

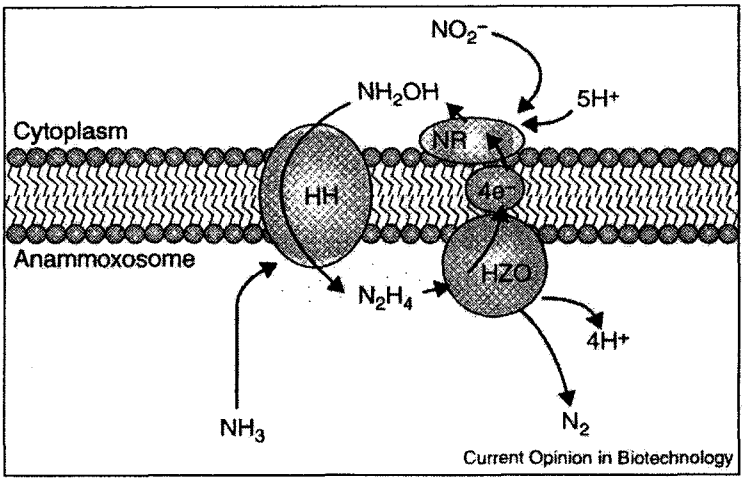


Figure 2.3 Possible mechanism of anaerobic ammonium oxidation (Jetten et al., 2001).

2.4 Microbiology of anaerobic ammonium oxidation

Experiments on enrichment culture obtained from a fluidized bed reactor showed that the dominant micro-organism was gram-negative, with an unusual and irregular morphology. After 177 days of enrichment, 64 % of all cells counted were of the described dominant type and it was observed that this enrichment culture had an extremely low growth rate around 0.001 h^{-1}. It was also observed during the enrichment of anaerobic ammonium oxidizers on synthetic medium, the colour of the biomass changed from brownish to red. Spectra taken from whole cells showed a clear increase in typical haem spectra, in a particular of the cyctochorome *c* (540-554nm; α-region) (Van de Graaf et al., 1996).

Van de Graaf et al (1996) reported that the level of ether lipids increased during the enrichment for anammox microorganisms. The presence of ether lipids seems to be confined to the most ancient microorganism (e.g. Archaea or the deepest phylogenetic branches with in the bacteria), thus suggesting that the ether linkage in the lipids could have appeared during the evolution of life before the ester types.

The findings reported by Van de Graaf et al (1996) also showed that the observed increase in anammox capacity of the enrichment is directly related to an increase in morphological conspicuous microorganism, an increase in ether lipids and an increase in cytochromes. They concluded that these properties might all be due to one and the same organism could be responsible for the anammox reaction.

However, the cultivation using a fluidised bed reactor was not satisfactory, because the operation of laboratory scale fluidised beds was difficult and the biomass retention in this reactor some times was not sufficient to maintain the anammox culture (Strous et al., 1998). Since currently available microbiological techniques are not designed very well to deal with very slowly growing microorganisms such as the anammox culture, a sequencing batch reactor (SBR) was applied and optimised for the quantitative study for anammox (Strous et al., 1998). The SBR was a powerful experimental set up in which the biomass was retained very efficiently (>90%). The use of a medium for strict autotrophic growth in the SBR system gave rise to persisting stable and strongly selective conditions (Jetten et al, 2002). Furthermore a homogenous distribution of substrates, products and biomass aggregates over the reactor was achieved and the reactor has been in operation reliably for more than 2 years under substrate-limiting conditions. In this system due to the reliable operation and the efficient biomass retention, large amounts of enriched anammox biomass could be produced and the degree of enrichment in the SBR aggregates was achieved 74 % (Strous, et al., 1998).

This value is 15 % higher than that 64 % which was reported by Van de Graaf et al. (1996) in a fluidized bed reactor. It is still not clear that why the degree of enrichment of the anammox population stabilised at 74 %, and what the other 26 % of the population contributes the process (Strous et al., 1998). Strous et al. also stressed that, although the biomass used in their experiments was not a pure culture (74 % enriched culture), they never detected any biological activity in the enrichment culture except for anammox.

Attempts to isolate this microorganism from the enrichment cultures using classical microbiological techniques were unsuccessful mainly because of the extremely slow growth of the bacteria. Dominant bacterium from the anammox enrichment culture was purified physically using percoll density gradient centrifugation (Strous et al.,

1999). The resulting cell suspensions almost completely consisted of cells of the target bacterium and were shown to have high anammox activity and fix CO_2 while oxidizing ammonium anaerobically. Strous also reported that both purified and unpurified cell suspensions were only active at high cell densities. Strous et al., (1999) concluded that, cells might need to twin over hydrazine in the periplasm. Since it cannot be avoided that free hydrazine diffuses away from the cells, when the cells are far apart, they might loose too much hydrazine to their environment and become inactive. In a dense suspension, no hydrazine would be lost because each cell benefits from its neighbour's losses. Thus, hydrazine would function as the auto-inducer substance in these cells. The purified cells were even more dependent on the hydrazine pulse than has been described for the enrichment culture. Due to cyclic nature of the anammox mechanism, the anammox cells need to invest reducing power (obtained from internal storage material) to start their catabolism by reducing NH_2OH from nitrite. Since hydrazine is an established anammox metabolite (Van de Graaf et al., 1997), the cells must have a hydrazine pool that is continuously turned over.

The purity of the obtained cell suspension was assessed using phase contrast microscopy, fluorescence in situ hybridization, staining with STYO-9 and electron microscopy. The obtained purity was between 99.5 and 99.8 % (less than one contaminating bacterium per 200 target bacteria). Extensive screening by electron microscopy showed that among the few remaining contaminants at least 7 morphologically different bacteria were present. The dominant morphotypical cells were coccoid shaped. Because the responsible bacterium was purified but not isolated, contaminating organisms might contribute to the observed activity. However, from kinetic and stoichiometric perspectives it is extremely unlikely that the contaminating organisms (1 in 200) contribute significantly to anammox catabolism or anabolism. However it is quite possible that other organisms are required for growth of the anammox organism. Possible symbionts might, for example, supply growth factors and scavenge by–products of anammox anabolism. These purified cell suspensions were shown to have high anammox activity and fix CO_2 while oxidizing ammonium anaerobically. Using fluorescence in situ hybridization with specific gene probes (targeted at the numerically dominant 16S rDNA sequence obtained from the purified cells), target organism was identified as a

new, deeply branching planctomycete. Based on this finding, the anaerobic ammonium oxidizing planctomycete-like bacterium was named '*Candidatus Brocadia Anammoxidans*' (Strous et al., 1999).

Since planctomycetes have been known as aerobic organoheterotrophs (Fuerest, 1995), the identification of anammox as a planctomycete was surprising although relatively little is known about their physiology in general (Strous et al., 1999). Study of planctomycetes has been focused on cell wall composition, phylogeny and ultrastructure, because in these aspects the planctomycetes are unique among the prokaryotes (Fuerest, 1995).

The order *Planctomycetales* is one of the approximately 40 currently defined distinct divisions that form the domain bacteria (Hugenholtz et al., 1998). The cell walls of planctomycetes do not contain peptidoglycan, like in gram positive or negative bacteria. Instead, these bacteria have proteinaceous cell walls like Archaea. Molecular ecology has indicated that the planctomycetes are one of the bacterial divisions represented in almost every ecosystem investigated (Hugenholtz et al., 1998; Neef et al., 1998). Planctomycetes were isolated from sea and fresh water, oligotrophic and polluted habitats, from alkaline (pH 11.6) and acid (pH 4.2) environments and were found to be meso and thermophilic environments (up to 55°C) (Schlesner, 1994). Very recently, it is reported that the bacteria, anaerobically oxidize ammonium with nitrite to N_2, was found in the world's largest anoxic basin, the Black Sea. Phylogenetic analysis of 16 S rRNA gene sequences showed that these bacteria are related to members of the order *Planctomycetales* performing the anammox process (Kuypers et al., 2003).

The oligonucleotid probes were used to survey the presence of planctomycete–like anammox bacteria in wastewater treatment systems (Schmid et al., 2000; Van Dongen et al., 2001). The observed probe binding patterns indicated that in addition to *B. anammoxidans*, other planctomycete-like bacteria occurred in these treatment systems. Table 2.1 presents the results of survey of various nitrogen removal systems for the presence of planctomycete-like anammox bacteria (Jetten, 2002). Schmid et al. (2000) investigated the diversity of planctomycete-like bacteria in those wastewater systems and showed new anammox bacteria which one of them named *Candidatus Kuennenia Stuttgartiensis*. FISH with specific probes for *K.*

Stuttgartiensis demonstrated that these anammox bacteria dominated the microbial biofilm communities of the investigated plants.

It is reported very recently that, the bacteria anaerobically oxidize ammonium with nitrite to N_2, was found in the world largest anoxic basin, The Black Sea. Phylogenetic analysis of 16S rRNA gene sequences showed that these bacteria are related to members of the order Planctomycetales performing the anammox (anaerobic ammonium oxidation) process (Kuypers, et. al. 2003).

Table 2.1 Survey of various nitrogen removal systems for the presence of planctomycete-like anammox bacteria (Jetten 2002).

System	Source of ammonia	Mode of detections	Reference
Rotating Biological Contractor (Kollikon, CH)	Leachate	Anammox activity, FISH	Egli et al. 2001
Rotating Biological Contractor (Mechernich, BRD)	Leachate	Anammox activity, FISH	Helmer et al. 2001, Schmid&Wagner
Trickling filter (Stuttgart, BRD)	Wastewater	N-loss, Anammox activity, FISH	Schmid et al. 2000
Biofilm reactor (Sydney, Australia)	Coke oven water	Anammox activity, FISH	Jetten, 2001
Freshwater wetland (Uganda)	Water	Nitrogen loss, FISH	Strous&van Kuijck, pers. comm.
Fluidized bed reactor	Mineral medium	Anammox activity	Van de Graaf et al. 1996
Sequencing batch reactor	Mineral medium	Anammox activity, FISH	Strous et al. 1998
Sequencing batch reactor	Wastewater	Anammox activity, FISH	Van Dongen et al. 2001

2.5 Anammox for nitrogen removal

Recent studies showed that lithotrophic ammonia oxidizers can also denitrify under both oxygen-limited and anoxic conditions (Schmidt and Bock, 1997). It was also reported that these group of bacteria can oxidize ammonia with N_2O_4 (dimeric form of NO_2) as oxidant under both oxic and anoxic conditions (Schmidt et. al. 2002a). The two groups of lithoautotrophic ammonia oxidizers, Anammox and *Nitrosomonas* (Nitrobacteraceae) are supposed to be natural partners in ecosystems with limited oxygen supply. Under these conditions aerobic ammonia oxidizers are able to oxidize ammonia to nitrite which will be consumed by anammox bacteria together with ammonia. In this system that was named CANON (Completely Autotrophic Nitrogen Removal Over Nitrite) the main product is N_2 and small amounts of nitrate are detectable (Sliekers, et. al., 2002). Very recently it was reported that these two groups of aerobic and anaerobic ammonia oxidizers can cooperate for ammonia conversion when the NO_2-dependent ammonia oxidation of Nitrosomonas supplies Brocadia anammoxidans with nitrite as oxidant (Schmidt et. al. 2002b). The introduction of partial nitrification led to the implementation of the Anammox process for treatment of high-strength wastewaters and to substantial savings of energy and resources.

2.5.1 Sharon-Anammox process

The SHARON (**S**ingle reactor system for **H**igh **A**mmonium **R**emoval **O**ver **N**itrite) process takes place in an intermittently aerated, completely stirred continuous flow reactor without sludge retention (Stowa, 2001). In the Sharon process, ammonium is converted to nitrite under aerobic conditions by ammonium-oxidizing bacteria (nitritification). The following equation describes this process:

$$NH_4^+ + 1.5\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+$$

When nitrite oxidizing bacteria are present in the reactor as well as ammonium-oxidizing organisms, the following reaction takes place under aerobic conditions where nitrite is oxidized to nitrate (nitratification):

$$NO_2^- + 0.5\ O_2 \longrightarrow NO_3^-$$

Due to a short retention time of approximately 1 day and high temperature (35°C), the nitrite oxidizers are washed out (Stowa, 2001) and only nitrite is formed in the Sharon reactor. Both nitrite and nitrate can be removed under anoxic conditions in the SHARON reactor by heterotrophic organisms with addition of an external electron donor such as methanol.

Denitrification of nitrite: $6NO_2^- + 3CH_3OH + 6H^+ \longrightarrow 3N_2 + 3CO_2 + 6H_2O$

Denitrification of nitrate: $6NO_3^- + 5CH_3OH + 6H^+ \longrightarrow 3N_2 + 5CO_2 + 13H_2O$

Thus for denitrification of nitrite 40% less methanol is needed than for denitrification of nitrate. This means that the nitrite route for N-removal needs 25% less of oxygen and 40% less of methanol than the nitrate route.

Instead of such a nitrifying/denitrifying Sharon reactor, a combined Sharon-Anammox process was developed by Van Dongen et al. (2001). When the Sharon process is coupled to the Anammox process, only 50% of the ammonium needs to be converted to nitrite. This implies that no extra addition of base is necessary, since most of the wastewater resulting from sludge digestion will contain enough alkalinity (in the form of bicarbonate) to compensate for the acid production (Jetten et al., 2001).

The microbial principles of the two processes have been elucidated (Hellinga et al., 1998; Jetten et al., 1997; Jetten et al., 1998) and biomass formation excluded reaction equations of the Sharon and Anammox process are presented in Table 2.2 (Jetten et al., 2001).

Because the classical nitrification-denitrification processes are needed large amount of energy, air supply, external carbon source addition and large reactor systems, partial oxidation of ammonium to nitrite and conversion of nitrite with ammonium to nitrogen gas in such a combined Sharon - Anammox system would leads to be overcome these disadvantages (Jetten et al., 2001).

Table 2.2 Reaction equations of the Sharon and Anammox processes (biomass formation not included (Jetten et al., 2001).

Sharon	$2\ NH_4^+ + 2HCO_3^- + 1{,}5\ O_2 \rightarrow NH_4^+ + NO_2^- + 2CO_2 + 3\ H_2O$
Anammox	$1\ NH_4^+ + 1.32\ NO_2^- \rightarrow N_2 + 2H_2O$
Combined process	$2\ NH_4^+ + 2HCO_3^- + 1{,}5\ O_2 \rightarrow N_2 + 2CO_2 + 5\ H_2O$

2.5.2 CANON process

The CANON process (**C**ompletely **A**utotrophic **N**itrogen **R**emoval **O**ver **N**itrite) has been patented by Dijkman and Strous (1999). Further studies on such a system in one single reactor were performed by Sliekers et al. (2002). The process relies on the interaction of two groups of autotrophic bacteria under oxygen-limiting conditions that perform two sequential reactions, simultaneously. Under oxygen limitations, ammonium is oxidized to nitrite by aerobic ammonium oxidizers, and produced nitrite can be used by planctomycete-like Anammox bacteria.

Experiments with *Brocadia anammoxidans* enrichment have showed that anammox bacteria are reversibly inhibited by low concentrations (0.5% air saturation) of oxygen (Strous et al., 1997). Nitrifiers were known to be present in low number in the 64% enriched anammox biomass (Van de Graaf et al., 1996). Since they would be able to coexist under oxygen-limiting conditions, nitrifiers oxidize ammonium to nitrite and keep the oxygen concentration low, while anammox converts the toxic nitrite and the remaining ammonium to dinitrogen gas. Such a system was established by gradually supplying more and more air into an anammox SBR reactor (Strous et al., 1998). In this reactor, *N. eutropha* or related bacteria consumed the oxygen effectively so that the actual oxygen concentration remained below the detection limit of 2 μM. Meanwhile, nitrite concentrations never exceed 1 mM, indicating that B. anammoxidans was active as well.

FISH analysis also confirmed that presence of oxic nitrifiers using probes specific for anammox and nitrifiers meanwhile aerobic nitrite oxidizers such as *Nitrobacter winogradskii* or *Nitrospira moscoviensis*, were never detected consistent with the absence of nitrite oxidizing activity in oxic batch tests (Jetten et al, 2001).

With this symbiosis of aerobic and anaerobic ammonium oxidizing bacteria, ammonium removal was established in a single oxygen-limiting step, by performing two sequential equations simultaneously (Sliekers et al., 2002):

$$1NH_3 + 1.5\ O_2 \longrightarrow 1NO_2^- + H_2O + H^+$$

$$1NH_3 + 1.32\ NO_2^- + H^+ \longrightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2H_2O + H^+$$

$$1NH_3 + + 0.855\ O_2 \longrightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$$

Jetten et al (2002) stressed that important for such an implementation of the technology are; effective on line monitoring of nitrogen compounds, very efficient biomass retention, a good balance between aerobic and anaerobic ammonium oxidation and long term stability of the process.

2.6 The Microbiology of Denitrification

Denitrification, or dissimilatory nitrate reduction, is the biological reduction of nitrate (and nitrite) to gaseous products, namely N_2, NO and N_2O, by anaerobically respiring chemoheterotrophs (Robertson and Kuenen, 1991). The process requires an anoxic or reducing environment, an organic or inorganic electron donor. In this system the nitrate, nitrite and other nitrogen oxides are used as electron acceptors instead of oxygen. Nitrate is converted to nitrogen gas (N_2) via the intermediates NO_2, NO and N_2O. However, not all of the denitrifiers possess all of the enzymes required to complete this conversion and the enzymes' efficiencies are sensitive to environmental changes. Additionally, some strains may themselves use the intermediates in this cascade as substrates, so these then become and products for some strains and substrates for others, depending upon the microbe and the prevailing conditions (Robertson and Kuenen, 1991).

A diverse group of Gram-negative and Gram-positive heterotrophic and autotrophic bacteria are capable of denitrifying and most will use oxygen if available, in presence of nitrate as an electron acceptor (Robertson and Kuenen, 1991). They are therefore facultative anaerobes. The autotrophic denitrifiers will use either bicarbonate or CO_2 as their carbon source, while the heterotrophic denitrifiers depend upon an organic

carbon source, and their overall growth during denitrification will therefore depend upon both the nature and concentration of the carbon source.

Denitrification is considered an anoxic process, but under certain conditions, it appears it can occur in the presence of oxygen. In suspended growth systems like activated sludge, the possibility exists of both aerobic layers and anoxic sublayers situated next to each other, allowing both nitrification and denitrification. *Nitrosomonas* and *Nitrobacter* are also capable of growth in the presence of limited dissolved oxygen concentrations, and nitrate and nitrite can be used under these conditions by *Nitrobacter* and *Nitrosomonas* respectively as electron acceptors, instead of oxygen. In *Nitrosomonas*, N_2, NO and N_2O are produced, while in *Nitrobacter* the final products are mainly ammonia, N_2O and NO_2 (Seviour and Blackall, 1998).

Robertson and Kuenen (1991) reported an attracting increasing interest in biological nitrogen removal technology namely **S**imultaneous **N**itrification and **D**enitrification (SND). SND or aerobic denitrification is considered as a physical event where both nitrification and denitrification occur in very close proximity. SND may take place as a result of an existing oxygen gradient, where nitrifiers will metabolize in the areas of high dissolved oxygen concentrations, and denitrifiers in the areas of low dissolved oxygen concentrations, or anoxic regions. These situations are common in biofilms in rotating contactors and trickling filters (Bock, et al., 1988). Conversely, Robertson and Kuenen (1991, 1995) reported that the heterotrophic nitrifier *T. pantotropha* can denitrify under aerobic conditions by using both nitrogen oxides and oxygen as terminal electron acceptors.

A wide taxonomic range of bacteria can denitrify, and all are aerobes which have an alternative method for carrying out electron transport phosphorylation by reducing nitrogen oxides if O_2 becomes limiting. The selection of denitrifiers does not require strictly anaerobic conditions, and organisms like *Paracoccus denitrificans, Alcaligenes faecalis, Pseudomonas aeruginosa* and even *Zoogloea ramigera* will all denitrify under conditions of O_2 saturation of 98% to 25%, but this process occurs at a slower rate than does anoxic denitrification (Robertson and Kuenen 1992). Examples of denitrifiers, and the reactions they carry out, are given in Table 2.3 (Seviour and Blackall, 1998).

2.7 Effects of carbon sources on denitrification

In most single sludge biological nutrient removal processes (BNR), denitrification is largely accomplished in a primary anoxic reactor receiving three feed streams. One is influent wastewater, which may have depleted degradable organic matter if the denitrification basin follows an anaerobic fermentation process for phosphorus release. The other two are re-circulated nitrified wastewater from the aeration basin and recycled activated sludge solids, both of which contain little soluble organic substrate for the denitrifying organisms.

Dissimilatory denitrification to nitrogen gas is a series of nitrate reduction steps with several intermediates:

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

In this context, nitrite appears to be the major intermediate product in denitrification. The accumulation of nitrite during denitrification is particularly troublesome for BNR processes because any nitrite leaving the primary denitrification reactor will be re-nitrified during subsequent aeration. Thus, nitrite accumulation during denitrification represents a futile process whereby that nitrite becomes the electron donor for nitrification increasing the oxygen required for nitrogen removal. Additionally, nitrite accumulation is often not considered in designing of BNR systems thus, may lead to serious errors, as it is likely to yield higher rates than the real NUR (Nitrogen Utilization Rate) values in denitrification (Sözen and Orhon, 1999).

Individual reductase enzymes catalyze each step in bacterial denitrification. Although some bacteria can carry out the complete reduction of nitrate to nitrogen gas, others contain enzymes for only part of the reduction pathway and do not reduce nitrate completely to nitrogen gas (Tiedje, 1988). Partial denitrification produces one or more oxidized nitrogen species; NO_2^-, NO, or N_2O as an end product under certain environmental conditions, one of which may be limitation of carbon and energy substrate required by heterotrophic denitrifying bacteria. Nitrite accumulation has been reported in growth of both *Paracoccus denitrificans* and *Pseudomonas fluorescens* in pure cultures (Thomsen et al. 1994; Almeida et al. 1995).

Table 2.3 Examples of denitrifying bacteria and their taxonomic affiliations (Seviour and Blackall, 1998).

Taxon	Habitat	Comments
Archaea		
Several extreme halophiles including *Halobacterium mediteranei*	solar saltern	Produces N_2 from NO_3^- or NO_2^-
Proteobacteria		
Subclass-α		
Phototrophic bacteria like *Rhodobacter spheroids*	sewage	Not all strains denitrifying
Budding bacteria like *Hyphomicrobium* spp.	water	Uses C-1 compounds as energy sources
Helical bacteria like some *Azospirillum* spp.	soil	Not all strains denitrifying
Gram-nagative aerobic rods like *Agrobacterium tumefaciens, Rhizobium* spp.	soil	Some produce N_2O which is then used as an electron acceptor by others
Gram-negative cocci and coccibacilli like *Gluconobacter* spp. and *Paracoccus denitrificans*	sewage and soil	N_2 and N_2O produced
Subclass-β		
Gram-negative aerobic and facultatively anaerobic bacteria including *Pseudomonas* spp.	soil and water	
Gram-negative cocci like *Neisseria* spp	animals	Some reduce NO_2 to gas
Subclass-γ		
Gram-negative aerobic bacteria like	commonly	NO_3^- and NO_2^- reduced but
Moraxella spp.	animals	not always to gas
Pseudomunas spp	Range of habitats	Metabolically diverse
Zoogloea ramigera	sewage	NO_3^- reduced to N_2
Gram-negative chemoautotrphs	fresh water	Sulphide-dependent
Like *Beggiatoa alba*		reduction of NO_3^- to N_2
Flavobacterium group	sewage	Not understood
like *Empedobacter* sp.		
Gram-positive bacteria		
Endospore-forming rods like many *Bacillus* spp., including some halophiles	Range of habitats including sewage	Metabolically diverse
Non-spore-forming bacteria like *Tsukamurella paurometabola*	humans	NO_2^- utilized by some isolates

Almeida et al. (1995) proposed a kinetic model based on competitive inhibition of nitrite reduction by nitrate. In this model, nitrite accumulation was the result of competition between nitrate and nitrite reductases for the electron generated by oxidation of common electron donors.

Van Rijn et al. (1996) observed that when acetate is the carbon-energy source, nitrite reduction is slowed significantly in the presence of nitrate in denitrifying cultures of *Pseudomonas stutzeri*, leading to nitrite accumulation. Furthermore, acetate electrons preferentially oxidized nitrate reductase and associated cytochromes rather than nitrite reductase if nitrate and nitrite both were present.

van Rijn et al. (1996) also reported that more nitrite accumulated when acetate was used as the carbon and energy substrate during denitrification by *Pseudomonas stutzeri* than when more complex electron donors such as butyrate were added. In these experiments, both volatile fatty acid compounds were added in a molar volatile fatty acid: nitrogen ratio of 1.28:1. However, for the more carbon-rich substrate, butyrate, the equivalent carbon to nitrogen mass (C/N) ratio was 4.4:1 compared with 2.2:1 for acetate. Moreover, butyrate is a better electron donor, supplying 20 moles of electrons per mole, compared with 8 moles of electrons per mole of acetate. It might be concluded that nitrite accumulated because electrons from acetate were limited and reduced nitrate reductase first (Oh and Silverstein, 1999).

However, van Rijn et al. (1996) reported that butyrate was actually consumed more slowly than acetate, and therefore electron flow was limited in spite of the electron-rich butyrate substrate. They concluded that in the presence of both nitrate and nitrite, acetate electrons flowed preferentially to nitrate reductase, whereas electrons from butyrate oxidation reduced nitrite reductase more quickly than nitrate reductase.

Blaszczyk (1993) reported that significant accumulation of nitrite when simple carbon sources such as methanol, acetate, or ethanol alone were used for growth of *Paracoccus denitrificans*. The C/N ratio for acetate, ethanol and methanol substrates was approximately 2.6:1. However, when the simple substrate was supplemented with a small amount of yeast extract and vitamins, nitrate was completely reduced to nitrogen gas without accumulation of nitrite.

Mycielski et al. (1985) reported that when starch was used as a carbon source, the number of denitrifying bacteria clearly depended on the ratio of starch concentration to nitrate concentration in the medium. Therefore, starch at certain concentrations resulted in the selection of fermenting bacteria capable of reducing nitrate to only nitrite.

The levels of accumulated nitrites have also been found to depend on the initial concentration of nitrate in the medium. At high concentrations; 3-4 g of NO_3^--N/l, the amount of accumulated nitrite was almost 1 g NO_2^--N/l (Blaszczyk et al., 1985).

The type of electron donor may also influence the selection of particular populations of denitrifying bacteria in a mixed culture. Wilderer at al. (1987) hypothesized that two kinds of dissimilatory denitrifying bacteria existed in activated sludge cultures: (1) Nitrate respiring bacteria that only reduce nitrate to nitrite; and (2) 'true' denitrifying bacteria that reduce nitrate and nitrite simultaneously to either N_2O or N_2. They reported that nitrite accumulation increased over time when glucose was fermented before denitrification and suggested that nitrate respiring bacteria were selected by this growth conditions instead of true denitrifiers. On the other hand, while activated sludge cultures receiving only acetate during the anaerobic-aerobic-anoxic sequence showed transient nitrite accumulation, all the nitrite was consumed by the end of reaction. They also suggested that, true denitrifying bacteria were favoured when acetate was the substrate throughout the multiple-phase reaction period, whereas fermentation of glucose seemed to be associated with a decrease in nitrite reducing bacteria, although no bacterial population measurements were reported.

It has been shown that the accumulation of nitrite is also strongly affected by the species composition within the denitrifying community itself. Comparative studies on the denitrification of nitrate by three strains of denitrifying bacteria isolated from wastewater treatment plants (*Paracoccus denitrificans, Pseudomonas stutzeri, Pseudomonas aeroginosa*) revealed the existence of three patterns of nitrate denitrification (Blaszczyk, 1992). *Paracoccus denitrificans* did not accumulate nitrite, *Pseudomonas aeroginosa* denitrified nitrate with transient accumulation of nitrite, *Pseudomonas stutzeri* first completely transformed nitrate to nitrite and then nitrite was reduced to nitrogen gas.

Martienssen and Schöps (1999) conducted experiments to examine the influence of substrate and growth conditions on the composition of a denitrifying biocommunity. They proposed a model population to distinguish three different groups of nitrate reducing bacteria with respect their capability to reduce nitrate and nitrite. A first group (group A) represented facultative anaerobic bacteria which reduce nitrate only

to nitrite. Group B contained bacteria capable of reducing nitrate and nitrite without any nitrite accumulation. The nitrite reduction rates of these bacteria were always higher than the corresponding nitrate reduction rates. Group C contained bacteria capable of reducing nitrate as well as nitrite. But nitrate reduction was associated with a transient accumulation of different amounts of nitrite. Batch and continuous culture experiments showed that the carbon supply determines the efficiency of denitrification rather than individual enzyme activities. Facultative anaerobes reducing nitrate only to nitrite play an important role in this process.

Experiments with acetate as carbon an energy source in an SBR (Oh and Silverstein, 1999) showed that, when the C/N ratio during completely anoxic denitrification was decreased from 2:1 to 1:1, significant nitrite accumulation was observed with an average total accumulated nitrite value of 33 mg NO_2^--N/l that was reduced only to 14.3 mg NO_2^--N/l by the end of 10.5 h SBR cycle. In addition, under substrate limitation (C/N=1:1), the specific rate of nitrate reduction was significantly increased from 0.023 mgNO_3^--N/mg MLVSS.h. to 0.034 mgNO_3^--N/mg MLVSS.h, although not all of the nitrate was consumed by the end of the reaction. Meanwhile the acetate consumption rate did not change, supporting the conclusion that in substrate-limited conditions electrons are distributed unequally to favour nitrate reductase resulting in nitrite accumulation.

Gommers et al. (1988) reported that when propionate was used as electron donor for denitrification nitrate was mainly reduced only to nitrite. Laboratory experiments with activated sludge grown on acetic acid showed denitrification rates of 4.5 mg NO_3^--N/g SS.h, 1.7 mg NO_3^--N/g SS.h, and 0.7 mg NO_3^--N/g SS.h when respectively acetate, propionate and butyrate was used as electron donor (Ødegaard et al., 1990).

Continuous culture experiments for denitrification with activated sludge using a mixture of VFA's such as acetate, propionate, butyrate and valerate were conducted by Fass et al., (1994). Results obtained from a chemostat denitrifying reactor showed that all four organic acids, as well as nitrate, were immediately consumed. Transient accumulation of nitrite was observed. They concluded that the rate of carbon consumption, nitrate conversion and nitrite production were depended on the type of VFA. Batch experiments with biomass taken from a chemostat denitrifying reactor, addition of VFA's individually showed that propionate was more slowly used by the

biomass when it was the sole source of carbon than was acetate, butyrate, valerate and mixture of VFA. The finding that biodegradation rates of acetate, butyrate and valerate were significantly the same and they also reduced nitrate 30 mg NO_3^--N/g SS. h, produced nitrite 10 mg NO_2^--N/g SS. h, and reduced nitrite 18 mg NO_2^--N/g SS. h at the same rate.

3 THEORETICAL APPROACH

Anaerobic ammonium oxidation (anammox) is the oxidation of ammonium to dinitrogen gas with nitrite as the electron acceptor. In this process nitrite also serves as an electron donor for CO_2 fixation in biosynthesis and part of the nitrite is converted to nitrate. According to overall reaction equation:

$$1\ NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow$$

$$1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03 H_2O \qquad (3.1)$$

In this process, part of the nitrite is converted to nitrate in biosynthesis since reducing power is required for CO_2 fixation (van de Graaf et al., 1996). Although relatively small amount of nitrate (0.26 mol nitrate per mol of ammonium oxidized) is produced in the anammox process, especially in the wastewaters containing high ammonium loads, such as sludge digester effluents, this amount would reach to significant concentrations, which has to be removed. Even though these ammonium-rich streams were reported to have relatively low BOD_5, they may still contain biodegradable carbon compounds due to low efficiency of the digester or a breakdown in the system. Since the potential effects of the organic compounds on the anammox process are fairly unknown, the assessment of the influence of the organic compounds on the anammox process is of great importance.

For the assessment of nitrate removal and the effect of organic compounds, supply of different compounds was proposed in batch and continuous anammox cultures. The most important factors affecting nitrate reduction were reported as the type and the amount of the carbon source (Tiedje, 1988; Thomsen et al., 1994; Almeida et al., 1995; Rijn et al., 1996; Oh and Silverstein, 1999; Blaszczyk, 1993; Wilderer et al., 1987). Since insufficient data is available for the understanding of the influence of organic compounds on the anammox process, there is great need for detailed experimental studies in for the compilation of a reliable data base.

Theoretically, if an organic compound is supplied in to an anammox reactor, this would result in the following expectations:

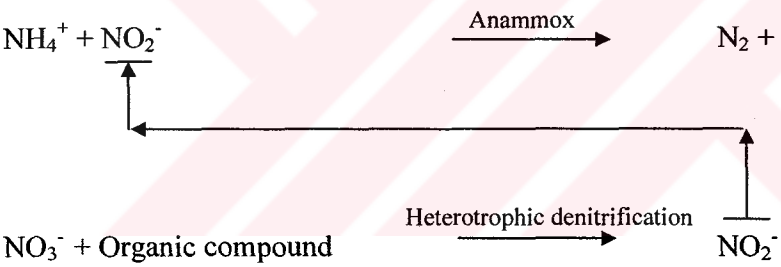
1. Heterotrophs build up in the reactor and a part of the heterotrophs could denitrify nitrate, using the organic compound as the electron donor.

$$NH_4^+ + NO_2^- \xrightarrow{\text{Anammox}} N_2 + NO_3^-$$

$$NO_3^- + \text{Organic compound} \xrightarrow{\text{Heterotrophic denitrification}} N_2$$

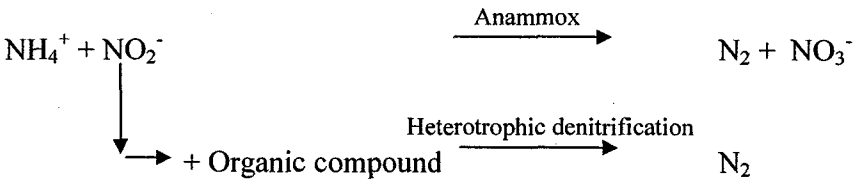
Since the highest degree of the anammox culture enrichment is approximately 70-80%, under the laboratory conditions, the presumably very limited amounts of heterotrophs might survive in an anammox reactor using lysed cells as the carbon source. Thus, nitrate produced during the anammox activity could be reduced to dinitrogen gas in presence of an organic compound by heterotrophic bacteria.

2. Denitrifiers reduce nitrate only to nitrite, thus extra nitrite would be available for the anammox activity;



If denitrifier population would be able to reduce nitrate only to nitrite, in this way this would supply extra nitrite for anammox bacteria results in less nitrite supplement in the feeding.

3. Denitrifiers use nitrite instead of nitrate, thus nitrite will be limited and denitrifiers and anammox would compete for nitrite and decrease in anammox activity resulting in increase in ammonium concentration.



4. Presence of an organic compound may strongly influence the anammox metabolism that may be resulted in a significant change in the anaerobic ammonium oxidation activity.

Under these circumstances, batch and continuous culture experiments were proposed to investigate the response of anammox bacteria to different organic compounds and to explore the mechanism of nitrate reduction in the anammox enrichment culture in the presence of organic compounds.

4 MATERIALS AND METHODS

4.1 Experimental set-up

The biomass used for reactor inoculations and batch experiments was supplied from a 15 l Anammox-Completely stirred tank reactor in which 74% of the biomass consisted of planctomycete-like Anammox (anaerobic ammonium oxidizing) bacteria. This reactor was fed with a mineral medium containing 60 mM NH_4^+ (840 mg NH_4^+-N/l) and 60 mM NO_2^- (840 mg NO_2^--N/l). The composition of the mineral medium is presented in Table 4.1. The medium in the reactor was kept constant at a value of 10 l. Anaerobiosis was maintained by flushing with N_2/CO_2 gas mixture. The CO_2 present in the gas was sufficient to buffer the solution and to keep the pH between 7.0 and 8.0. Reactor was operated in a continuous flow mode with a hydraulic retention time of 5 days and stirred at 100±10 rpm. Almost complete biomass retention was achieved in the reactor and the temperature was kept at 30±1°C. Reactor was operated under substrate (NO_2^-) limitation.

Table 4.1 The composition of the mineral medium

Components	Concentration(g/l)
$KHCO_3$	1.25
KH_2PO_4	0.025
$CaCl_2.2H_2O$	0.3
$MgSO_4.7H_2O$	0.2
$FeSO_4$	0.00625
EDTA	0.00625
1M HCL	1.25 ml/l
trace elements solution:	1.25 ml/l
EDTA	15
$ZnSO_4.7H_2O$	0.43
$CoCl_2.6H_2O$	0.24
$MnCl_2.4H_2O$	0.99
$CuSO_4.5H_2O$	0.25
$NaMoO_4.2H_2O$	0.22
$NiCl_2.6H_2O$	0.19
$NaSeO_4.10H_2O$	0.21
H_3BO_4	0.014
$NaWO_4.2H_2O$	0.050

4.2 Batch experiments with different organic compounds

Batch experiments were carried out in 50 ml serum bottles and each bottle contained 40 ml anammox biomass suspension taken from the biomass source reactor. Serum bottles were sealed with butyl rubber stoppers and aluminum caps. Bottles were placed on a magnetic stirrer with a magnet to maintain all biomass granules in suspension and flushed for 15 minutes with a gas mixture of Argon/CO_2 to remove oxygen. Nitrite was added to all bottles in the same amounts (specified in the results section) in the form of $NaNO_2$. There was no need to add ammonium, since the medium already contained ammonium in excessive amounts. For each carbon source different concentrations of the compound were applied (specified in the results section). Argon/CO_2 gas flushing was continued during the experiments to maintain anaerobiosis. All sets were studied in duplicate. Samples were taken from all sets in the same intervals and stored at –20°C until measurement. Experiments were also monitored with nitrite test strips. Before measurement, samples were centrifuged at 13.000 rpm for 5 min and supernatant was analyzed for ammonium, nitrite, and nitrate.

4.3 Operation and continuous flow experiments with the CSTR

Continuous flow experiments were conducted for methanol, acetate and propionate feedings. A 15 l reactor was inoculated with 2 l biomass from the anammox source reactor for continuous cultivation with methanol. The final volume of the mixed liquor in the reactor was 8 l and kept constant during operation. Reactor was fed with mineral medium containing increasing amounts of ammonium and nitrite (3 to 12 mM). Reactor was operated in a continuous flow mode with a hydraulic retention time of 4 days. The same 15 l reactor was used for acetate feeding. The biomass in the reactor was removed after the methanol feeding was stopped, and reactor was re-inoculated with 2 l biomass. The final volume of the mixed liquor in the reactor was 5 l and kept constant during the operation. Increasing amount of ammonium and nitrite (1 to 6 mM) in mineral medium was supplied to the system. The reactor was operated in a continuous flow mode with a hydraulic retention time of 3 days.

Continuous cultivation of anammox with propionate was conducted in an 8 l fermentor and 2 l anammox biomass was used for inoculation. Final volume of the mixed liquor in the reactor was 5 l and kept constant with a level controller. Reactor was controlled with a computer and monitored for the parameters; pH, temperature and dissolved oxygen and stirred at 100±10 rpm. The influent containing 30 mM nitrite and ammonium in mineral medium was supplied to the reactor. Reactor was operated in a continuous flow mode with a hydraulic retention time of 3.5 days.

Continuous methanol, acetate and propionate feedings were supplied to the reactors from separate bottles with a pump at the rates of 260, 84 and 180 ml/day, respectively. Temperature was kept constant at 30±1°C and stirred at 100±10 rpm in all three reactor systems. Methanol and acetate supplemented reactors were flushed continuously with N_2/CO_2 gas mixture, while propionate supplemented reactor was flushed with Argon/CO_2 gas mixture in order to maintain anaerobiosis. Samples from the influent and effluent of the reactors were taken once or twice a day and analyzed for ammonium, nitrite, nitrate, propionate and acetate. Biomass samples were also taken for certain intervals for monitoring bacterial populations with FISH analysis.

Experimental set-up of the reactor system is illustrated in Figure 4.1.

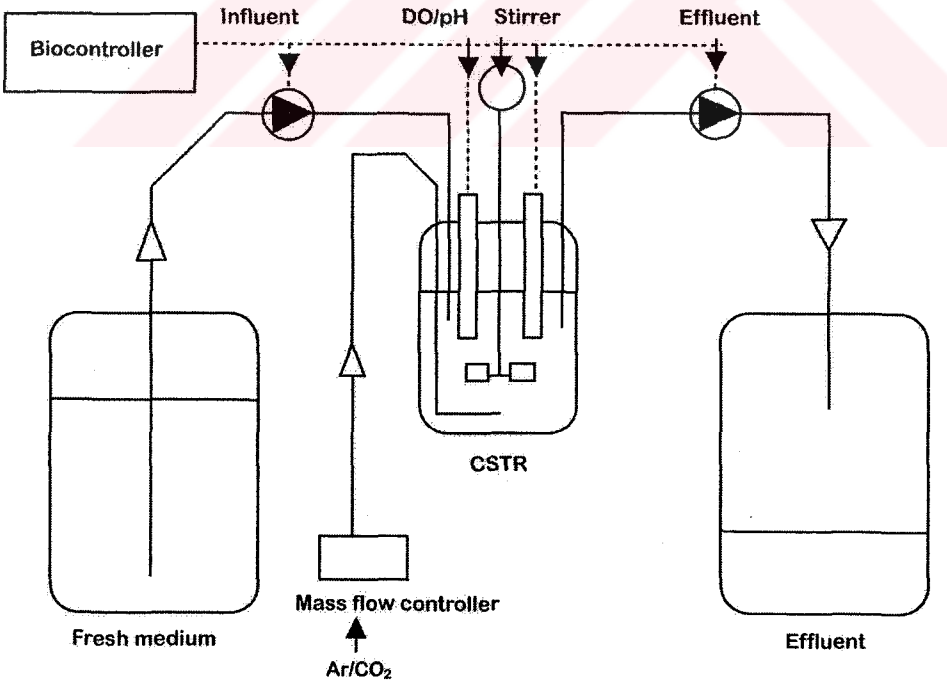


Figure 4.1 Experimental set-up of the reactor system.

4.4 Monitoring of bacterial populations

During operation of the reactors, feeding with acetate and propionate, together with nitrogen compounds and mass balances, changes in the bacterial populations were also monitored. Fluorescence in situ hybridization (FISH) analysis was applied to investigate changes in the composition of the biomass in the reactor with addition of the organic carbon compound. The bacterial population in the propionate feeding reactor was monitored also by fluorescence in situ hybridization (FISH) and immunofluorescence analysis according to Schmid et al. 2003. The antibodies against periplasmic nitrate reductase napA of Escherichia coli were a generous gift of S.B. Mohan and J.A. Cole (Birmingham UK). The Cy3 labelled secondary antibody was a sheep-anti-rabit for napA.

4.4.1 Fluorescence in situ Hybridization (FISH) protocol

Fluorescence in situ Hybridization protocol was adapted from Neef et al. (1998).

Fixation of cells for in situ hybridization: Cells were washed in 10 mM phosphate buffer (pH 7.2, containing 130 mM NaCl) and re-suspended in the same buffer. One volume of the cell suspension was mixed with 3 volumes of the same buffer (freshly prepared and containing 4% paraformaldehyde) and kept on ice for 1-3 hours. The fixative was then removed by centrifugation and the cells were re-suspended in 0.5 ml phosphate buffer. Finally, 0.5 ml of absolute ice-cold methanol was added and the cells stored at –20°C until used.

Immobilization of fixed cells on microscope slides: 5-10 µl aliquots of cell suspension were loaded in each gelatine-coated well of a teflon-printed slide (Nutacon, the Netherlands). After loading, the slide was dried for 10 minutes at 46°C. The cells were then dehydrated by successive passage through 50, 80, and 100% ethanol (3 minutes each) and air dried.

Hybridization of immobilized cells: To each well on the teflon-printed slide, 10 µl of freshly prepared hybridization buffer (pH 8, 20 mM Tris/HCl, 180 mM NaCl, formamide as specified in Table 5.10), and 1 µl probe working solution containing 5ng of each of the respective dye-labeled probes were added.

The slides were incubated at 46°C for 90 minutes in 50 ml Falcon tubes, containing a tissue paper moisturized with 2 ml of hybridization buffer. After hybridization, the slides were washed rapidly with 20 mM Tris/HCl (pH 8, 48°C and containing 5 mM EDTA and NaCl concentrations depending on the applied formamide concentration in the buffer. The slides were rapidly immersed in the same buffer and incubated at 48°C. After 20 minutes the slides were rinsed with cold Milli Q water, air dried rapidly and stored at -20°C until studied under the microscope.

Epifluorescence microscopy and documentation: The slides were embedded in vectashield (Vector laboratories, USA) and analyzed with a Zeiss Axioplan 2 imaging fluorescent microscope. Photomicrographs were taken using LeicaQFluoro imaging software (Leica, The Hague).

4.5 Pulse addition experiments with propionate

Pulse addition experiments were carried out in the 15 l, 74% enriched source anammox reactor to observe propionate consumption. Reactor was feeding with mineral medium containing 60 mM ammonium and nitrite and maintained same feeding during the experiment. Biomass in the source reactor has never saw before an external organic carbon compound. Pulse addition of propionate was conducted in two separate experiments. In the first experiment, feeding was continued during the whole course of the experiment in order to supply required electron acceptor (nitrite) and electron donor (ammonium). A 50 ml propionate solution was added to the reactor to obtain a final concentration of approximately 1mM. In the second experiment, influent and effluent were switched off and reactor was converted to batch mode. Experiment was run for 24 hours. Samples from the effluent were taken routinely and analyzed for ammonium, nitrite and nitrate and acetate. Operation conditions of the reactor were mentioned previously.

4.6 Purification of dominant anammox cells and micro batch experiments for propionate consumption

4.6.1 Cell purification

74% enriched anammox bacteria in granules were used as a source of biomass. Granules were homogenized prior to the aggregate disruption; by passing them through a thin needle three times. 50 ml concentrated anammox granules were disrupted in 5 ml portions by mild sonication (30 s, 150 W, tip diameter 9.5 mm) in 10 ml serum bottles. After sonication, the partially disrupted aggregates were centrifuged (5 min., 10.000 x g). The single cells were recovered in a dense, dark-red pellet at the bottom of the tube after removal of the remaining aggregates (light red, less dense pellet on top of the dense pellet). The cells were re-suspended in 4.3 ml Hepes/bicarbonate buffer (75/5 mM, pH 7.8). 4.3 ml of the cell suspension was mixed with 5.7 ml Percoll (Pharmacia) and centrifuged (4°C, 15 min., 10.000 x g). The target cells were recovered as a red band at the bottom of the gradient. The cells were washed in Hepes/bicarbonate buffer (75/5 mM, pH 7.8) to remove the Percoll and stored on ice until use for subsequent experiments.

4.6.2 Determination of the purity of the cell suspensions

In order to determine and confirm degree of purification, fluorescence *in situ* hybridization (FISH) analysis was applied to a part of the cell suspension after purification process. The applied oligonucleotide probes are specified in the results section.

4.6.3 Micro batch experiments with purified anammox cells

The purified cells were washed in Hepes/bicarbonate buffer (75/5 mM, pH 7.8) containing 2 mM NO_3^- and 1 mM propionate. The cells were concentrated in 200 µl of the same buffer. This mixture was transferred to a 0.6 ml eppendorf cup and the cup was placed in a 10 ml glass container with a butyl rubber stopper. Container was purged with a gas mixture of Argon/CO_2 to remove oxygen. At the end of the experiment, the cells were separated from the liquid fraction by centrifugation and the supernatant was analyzed for ammonium, nitrite, nitrate and propionate.

4.7 Batch experiments with anammox bacteria using different nitrogenous compounds and propionate

A set of batch experiments were performed to determine nitrogenous compound that acts as electron acceptor coupled with propionate. Biomass taken from a 15 l, 74% enriched anammox source reactor was used as the source of biomass. Prior to the batch test, biomass was washed with mineral medium, until all N-compounds were removed. Experiments were carried out in 10 ml serum bottles. Serum bottles were sealed with butyl rubber stoppers and aluminum caps. Total mixed liquor volume of the bottles was 5 ml. Bottles were placed on a magnetic stirrer with a magnet to maintain all biomass in suspension. Bottles were flushed for 15 minutes with Argon/CO_2 gas mixture to remove oxygen and then components were added in the required amounts. Compounds and their concentrations are specified in the results section. Argon/CO_2 gas flushing was continued during experiment to maintain anaerobiosis. All sets were studied in duplicate. Experiment was carried out for 24 hrs. Samples were taken from all sets in the same intervals and stored at –20°C until measurement. Before measurement, samples were centrifuged at 13.000 rpm for 5 min and supernatant was analyzed for ammonium, nitrite, nitrate and propionate.

4.8 ^{15}N-labeling studies

Experiments with $^{15}NO_2^-$, and $^{15}NO_3^-$ were performed to follow the conversion of nitrogen compounds in the presence or absence of propionate. Granules from a 15 l, 74% enriched source anammox reactor were used as the source of biomass. Prior to the batch test, granules were washed with mineral medium, until all N-compounds were removed. The experiments were conducted in 10 ml serum bottles containing 5 ml concentrated cell suspension and sealed with butyl rubber stoppers. After gassing for 15 min with the N_2/CO_2 (80/20 %) gas mixture, the experiments were started by adding the ^{15}N-substrates from an anoxic stock solution with a syringe. Compounds and their concentrations are specified in the results section. $^{15}NaNO_3$ and $^{15}NaNO_2$ isotopes were used for the labeled compounds. During the experiments the cell suspension were stirred. All sets were studied in duplicate. Samples were taken in the same intervals and centrifuged at 13.000 rpm for 5 minutes and supernatant was removed and stored -20 °C prior to analysis for ammonium, nitrite, nitrate and propionate.

4.9 Analytical procedures

Nitrate was measured colorimetrically at 420 nm after reaction (20 min.) of 50 µl sample (containing 0.1-5 mM nitrate) with 10 µl saturated sulfamic acid solution (to remove nitrite), 0.2 ml 5% salicylic acid in 98% sulfuric acid and 2 ml cold (4°C) 4M NaOH. Nitrite was measured colorimetrically at 540 nm after reaction (30 min.) of 10 µl sample (containing 0-4mM nitrite) with 1 ml 1% sulfanilic acid in 1M HCL and 1 ml 0.1% N-naphtylethylenediamine in a 4 ml disposable cuvette (Griess-Romijn, 1996). Ammonium was measured colorimetrically at 420 nm after reaction (20 min) of 40 µl sample (containing 0-10 mM NH_4^+) with 760 µl 0.54% ortophtaldialdehyde in 10 ml ethanol, 50 µl mercaptoethanol diluted in 7.3 P-buffer. Samples were stored in the freezer (-20°C) and centrifuged just before measurement. Acetate and propionate were measured on a HP 5890 GC (230°C; innowax capillary column) equipped with a FID detector. The ^{15}N-analysis was performed by gas chromatography followed N_2 detection on an isotope ratio mass spectrometry (Thermo Finnigan Delta plus). The isotope ratios of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ were measured according to Risgaard-Petersen et al. (1995).

5 EXPERIMENTAL RESULTS

Experimental studies for the investigation of the influence of different carbon sources on the anammox process were conducted in three fundamental bases. In the first part of the study, batch experiments were carried out with different carbon compounds, such as acetate, propionate, methanol, ethanol, glucose, starch, formate and amino acids. In the second part of the study, methanol, acetate and propionate were supplied separately and continuously to the anammox reactors for the assessment of long term effects of the compounds. All through the continuous cultivation of anammox supplemented with the organic compound the bacterial population was monitored by Fluorescence In Situ Hybridization (FISH) analysis to investigate the changes in the composition of the culture. For the investigation of propionate consumption propionate pulse addition experiments were carried out in the highly enriched anammox culture. Anammox cells were purified and micro batch experiments were conducted so as to prove propionate consumption by anammox bacteria. Finally, the nitrogenous compound coupled to propionate oxidation was investigated in batch experiments with different nitrogenous compounds in the presence of propionate and ^{15}N-labeling experiments were carried out to explore possible reaction mechanism.

5.1 Experimental studies for the assessment of the influence of organic carbon compounds on anammox process

Batch experiments were carried out with eight different organic carbon compounds, such as methanol, acetate, propionate, ethanol, glucose, formate, starch and amino acids. For each set of experiment, different concentrations of the compound were applied and each was run in 4 parallel sets. Glucose, ethanol and methanol were used to represent readily biodegradable substrate, starch was used to represent particular substrate and propionate, formate, acetate and amino acids were used to represent volatile fatty acids as readily biodegradable substrate mainly formed in sludge digesttion tanks. Experimental set-up is specified in Table 5.1. The first letter of the

carbon compound used as the identification of experimental set; M: methanol, A: acetate, P: propionate, S: starch, E: ethanol, G: glucose, F: formate, AA: aminoacids. All sets were studied in parallel and in duplicate. In all experiments, sets with a subscript of 11 and its duplicate 12 were run under the absence of a carbon source as a control set.

Table 5.1 Experimental set-up of the batch experiments with different carbon compounds.

Compound	Set no.	Concentration mmol/l	Compound	Set no.	Concentration mmol/l
Methanol	M_{11}	0	Ethanol	E_{11}	0
	M_{12}	0		E_{12}	0
	M_{21}	0,5		E_{21}	0,5
	M_{22}	0,5		E_{22}	0,5
	M_{31}	1		E_{31}	1
	M_{32}	1		E_{32}	1
	M_{41}	2		E_{41}	2
	M_{42}	2		E_{42}	2
Acetate	A_{11}	0	Glucose	G_{11}	0
	A_{12}	0		G_{12}	0
	A_{21}	1		G_{21}	1
	A_{22}	1		G_{22}	1
	A_{31}	2		G_{31}	2
	A_{32}	2		G_{32}	2
	A_{41}	3		G_{41}	3
	A_{42}	3		G_{42}	3
Propionate	P_{11}	0	Formate	F_{11}	0
	P_{12}	0		F_{12}	0
	P_{21}	0,5		F_{21}	0,5
	P_{22}	0,5		F_{22}	0,5
	P_{31}	1		F_{31}	1
	P_{32}	1		F_{32}	1
	P_{41}	3		F_{41}	2
	P_{42}	3		F_{42}	2
Starch	S_{11}	0	Amino acids	AA_{11}	0
	S_{12}	0		AA_{12}	0
	S_{21}	0,25		AA_{21}	0,5
	S_{22}	0,25		AA_{22}	0,5
	S_{31}	0,5		AA_{31}	1
	S_{32}	0,5		AA_{32}	1
	S_{41}	1		AA_{41}	2
	S_{42}	1		AA_{42}	2

*1 mmol methanol/l = 32 mg/l
* 1mmol/l acetate = 60 mg/l
* 1mmol/l propionate = 74.1 mg/l
* 1mmol/l starch = 342.3 mg/l
* 1mmol/l ethanol = 46.1 mg/l
* 1mmol/l glucose = 180 mg/l
* 1mmol/l formate = 50 mg/l
* 1mmol/l L-alanine = 89.1 mg/l

5.1.1 Batch experiments with methanol

Batch experiments with methanol were run in four parallel sets for 280 minutes. Initial conditions of the experiment are presented in Table 5.2. Ammonia, nitrite and

nitrate profiles are shown in Figures 5.1a to 5.1d. In sets M_{11} and its duplicate M_{12}, anammox without methanol as a control, decrease in nitrite and ammonia concentrations and increase in nitrate concentration were observed as a result of anammox activity. Meanwhile in sets M_{21}, M_{31}, and M_{41} and their duplicates M_{22} and M_{32}, there were no change in nitrite, ammonia and nitrate concentrations during experiment. As can be seen from the figures, duplicates are well fit to each other.

Table 5.2 Initial conditions of the batch experiments with methanol.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Methanol (mmol/l)
M_{12}	1.44	1.79	7.47	0
M_{11}	1.36	1.73	7.88	0
M_{22}	1.44	1.77	7.08	0.5
M_{21}	1.57	1.76	7.09	0.5
M_{32}	1.53	1.77	6.19	1
M_{31}	1.43	2.04	6.19	1
M_{41}	1.39	1.54	7.18	2

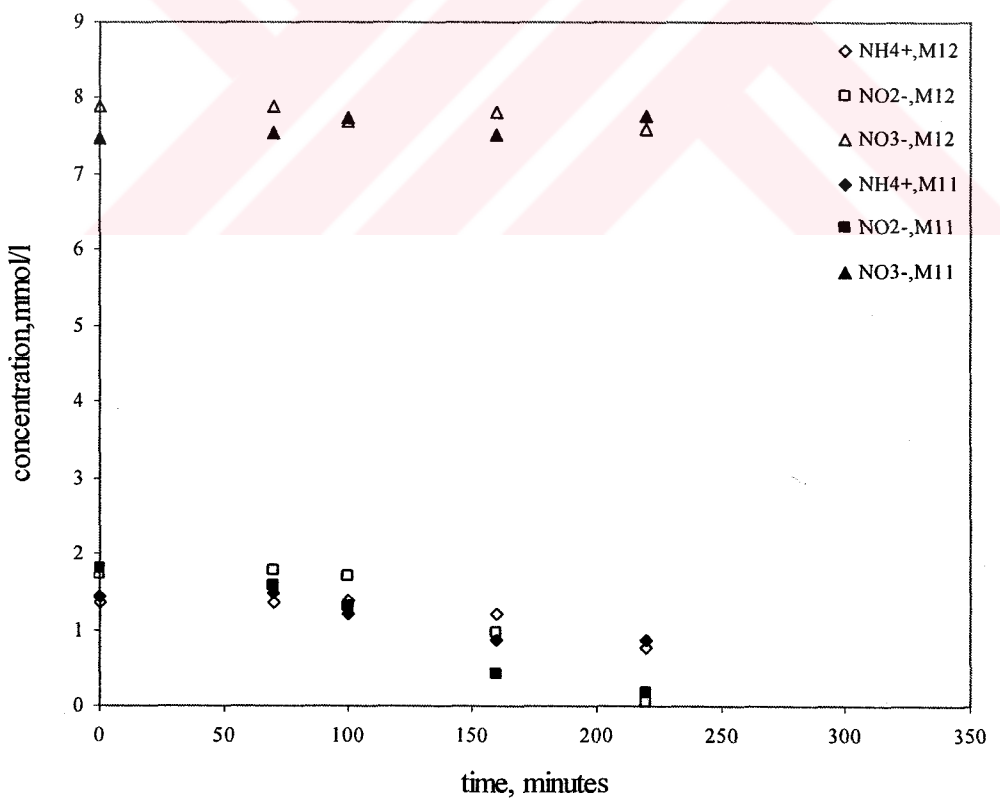


Figure 5.1a Results of the experimental sets 11 and 12 of methanol batch experiments.

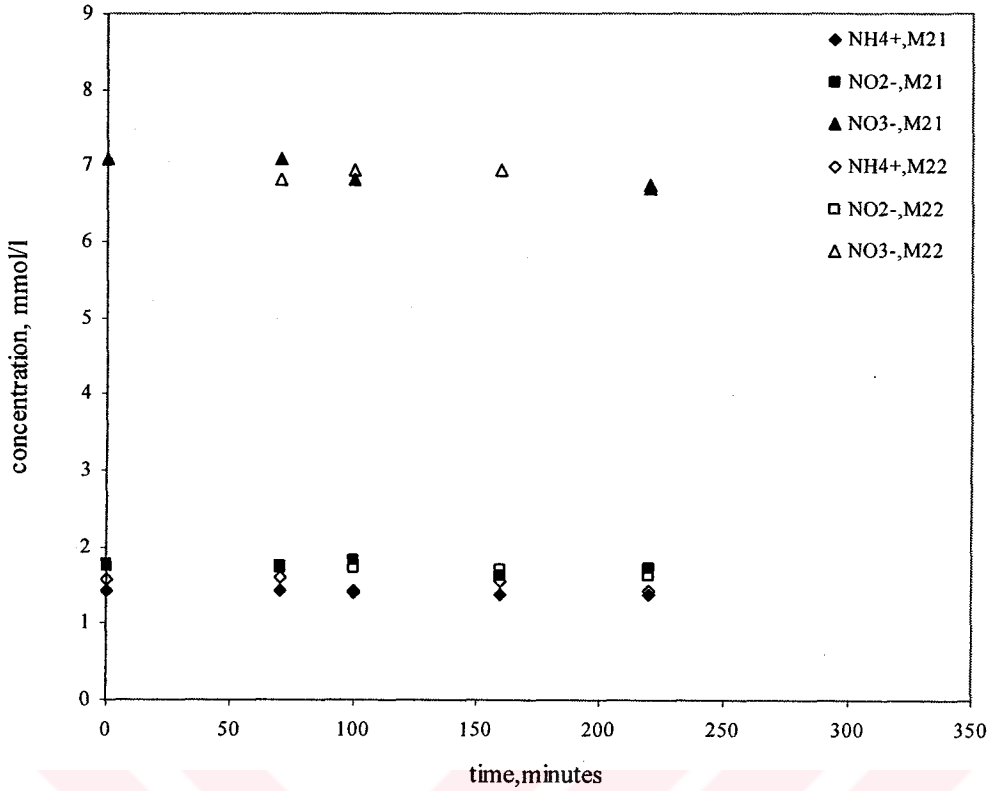


Figure 5.1b Results of the experimental sets 21 and 22 of methanol batch experiments.

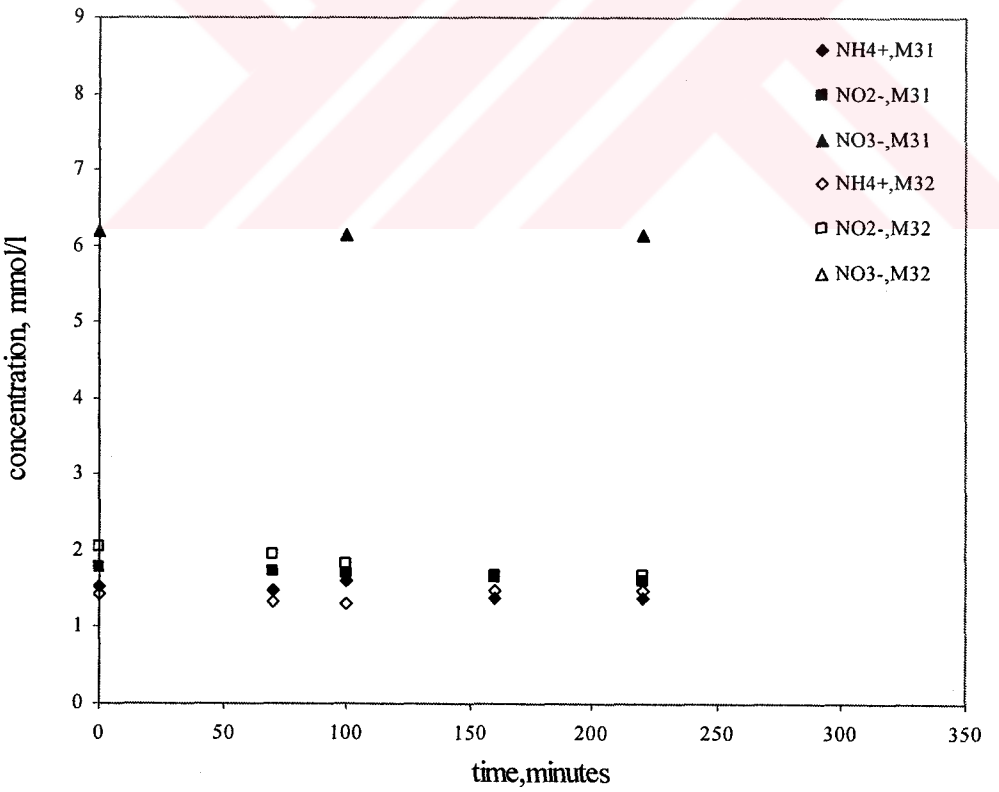


Figure 5.1c Results of the experimental sets 31 and 32 of methanol batch experiments.

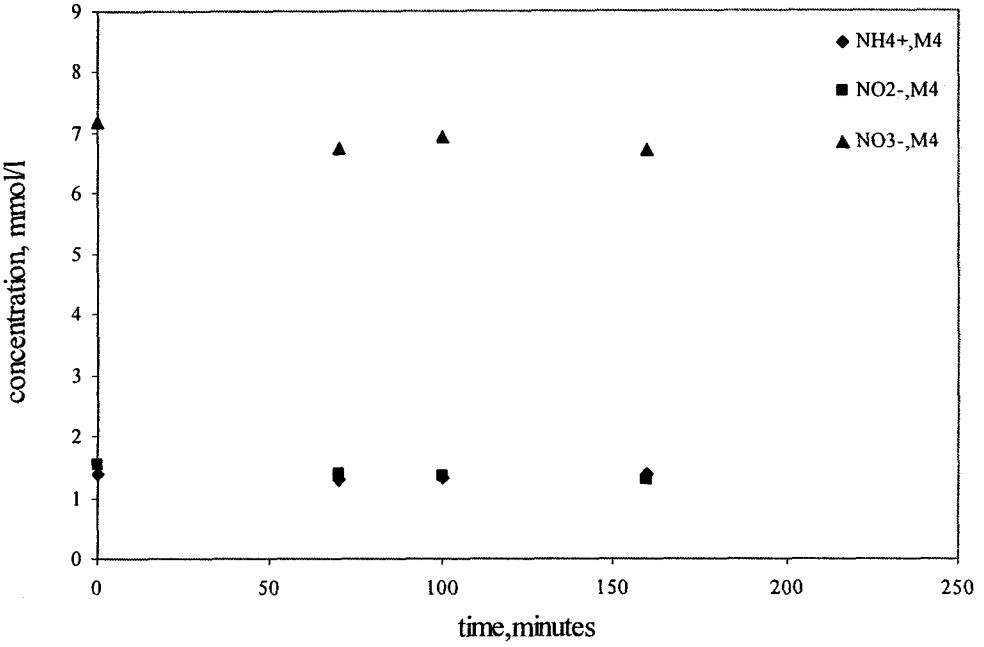


Figure 5.1d Results of the experimental sets 41 and 42 of methanol batch experiments.

5.1.2 Batch experiments with acetate

Batch experiments with acetate were conducted for 330 minutes. Table 5.3 shows the initial conditions of the experiments. Results obtained from the experiments are illustrated in Figures 5.2a to 5.2d. As soon as oxygen was removed (app. in 90 min.), nitrite conversion was started and completely converted in 180 minutes in all sets. Nitrite and ammonia were converted faster in presence of 1 and 2 mmol acetate/l compared to control set. Meanwhile, nitrate productions were not different. Results obtained from the duplicates are compatible.

Table 5.3 Initial conditions of the batch experiments with acetate.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Acetate (mmol/l)
A_{11}	4.39	1.73	6.16	0
A_{12}	4.31	1.69	6.06	0
A_{12}	4.34	1.80	5.87	1
A_{22}	4.30	1.76	6.37	1
A_{31}	5.04	1.72	5.92	2
A_{32}	5.00	1.71	6.19	2
A_{41}	4.56	1.63	6.51	3
A_{42}	4.17	1.66	6.30	3

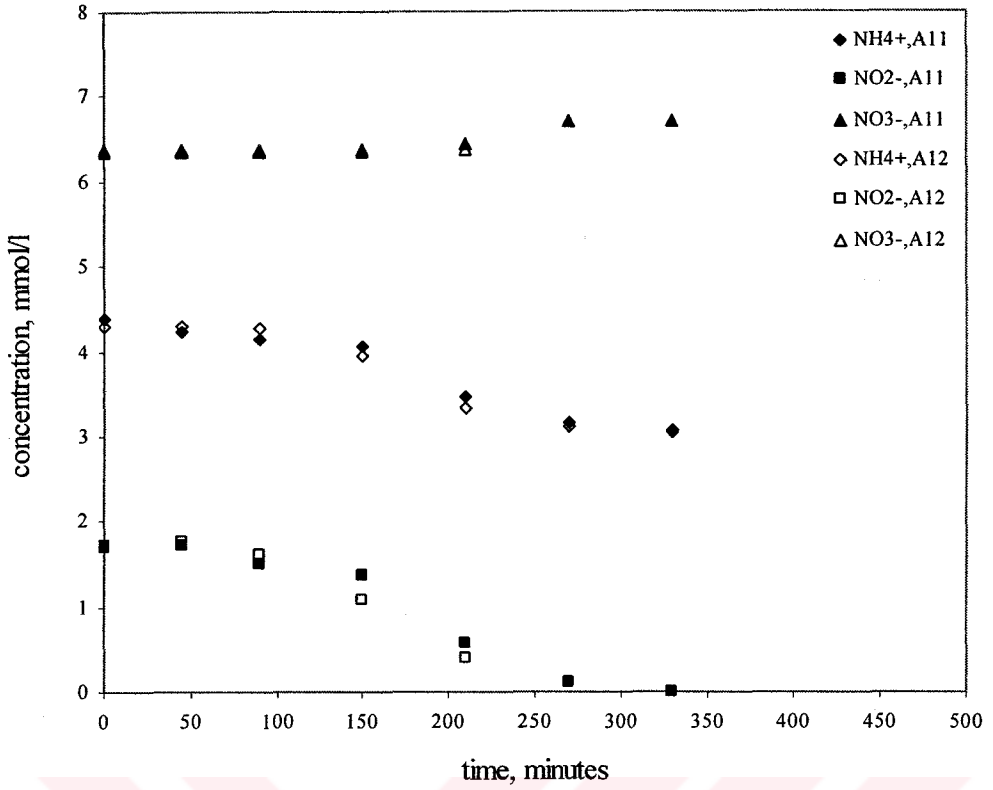


Figure 5.2a Results of the experimental sets 11 and 12 of acetate batch experiments.

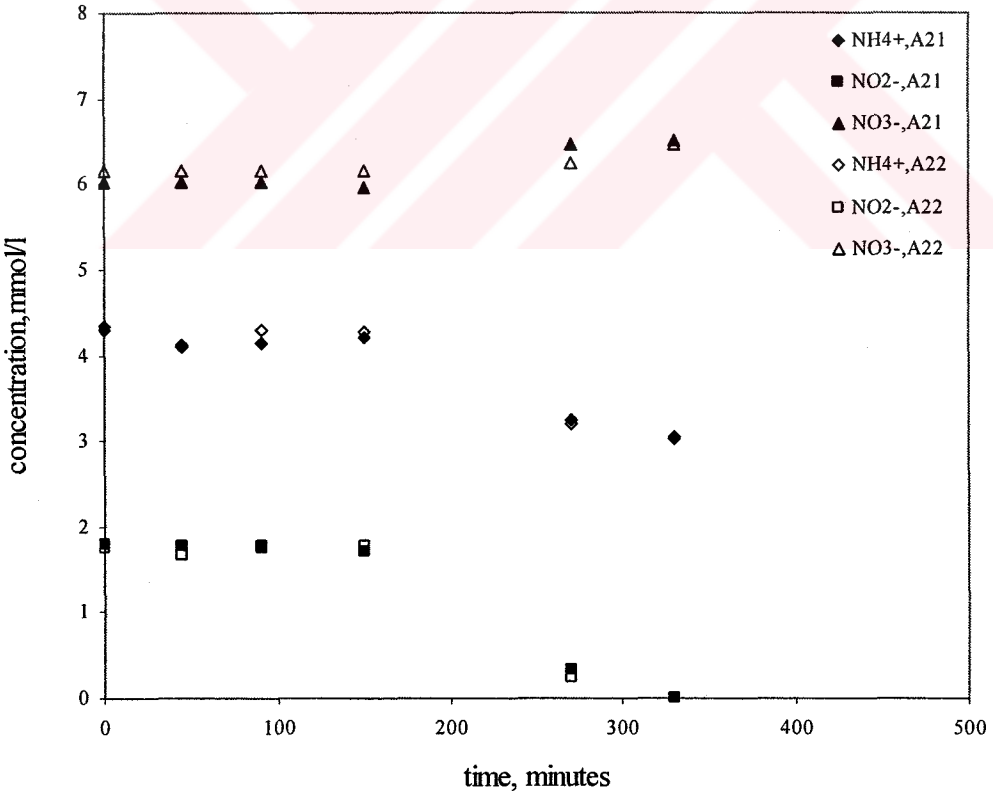


Figure 5.2b Results of the experimental sets 21 and 22 of acetate batch experiments.

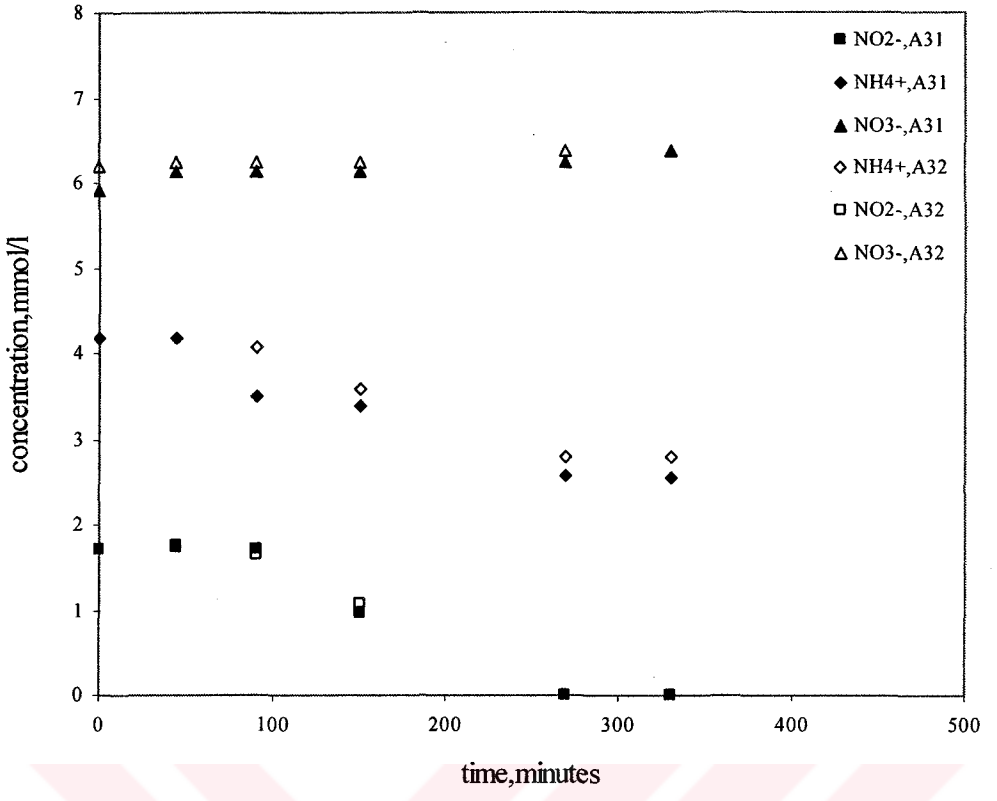


Figure 5.2c Results of the experimental sets 31 and 32 of acetate batch experiments.

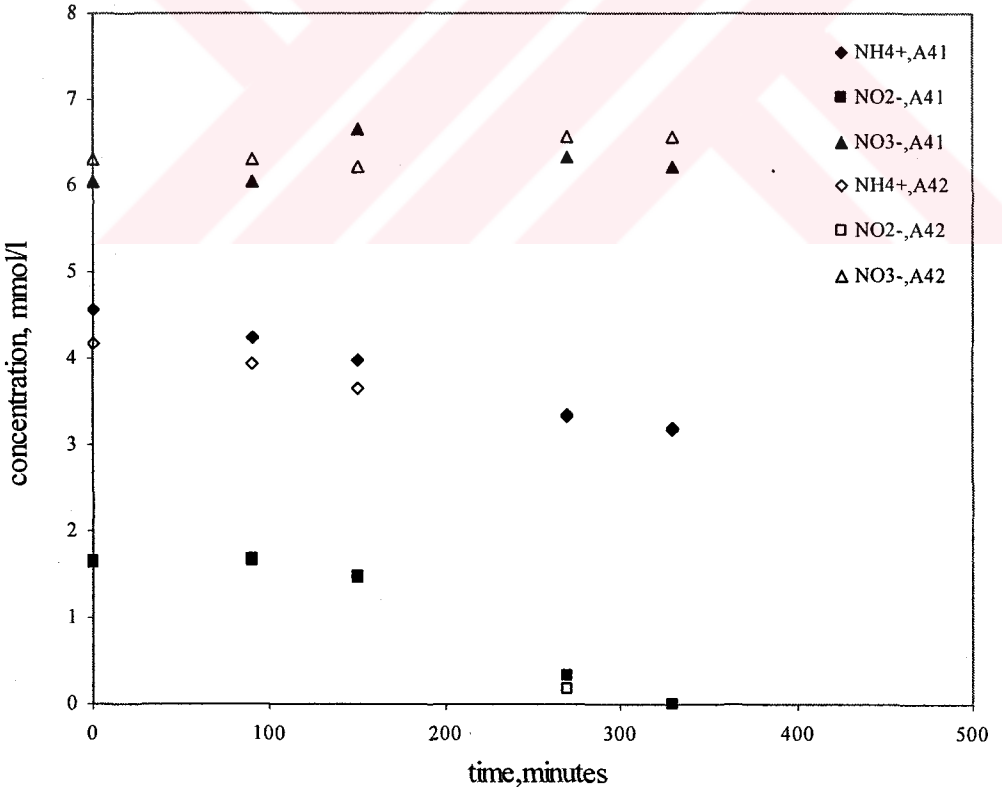


Figure 5.2d Results of the experimental sets 41 and 42 of acetate batch experiments.

5.1.3 Batch experiments with propionate

Batch experiments with propionate were carried out for 300 minutes. Initial conditions of the experiments are presented in Table 5.4. Ammonia, nitrite and nitrate profiles are shown in Figures 5.3a to 5.3d. In control set, nitrite converted completely in 210 minutes. Meanwhile in presence of 0.5 and 1 mmol/l propionate, same amount of nitrite was converted in 155 minutes. Less ammonia conversion was observed in control set compared to other sets in the same period. Compatible results were obtained from the duplicates.

Table 5.4 Initial conditions of the batch experiments in presence of propionate.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Propionate (mmol/l)
P_{11}	5.05	1.78	6.99	0
P_{12}	5.17	1.76	7.36	0
P_{21}	5.00	1.71	6.77	0,5
P_{22}	5.15	1.63	6.80	0,5
P_{31}	4.88	1.70	6.48	1
P_{32}	4.98	1.66	6.80	1
P_{41}	4.90	1.72	7.32	3
P_{42}	4.76	1.66	-	3

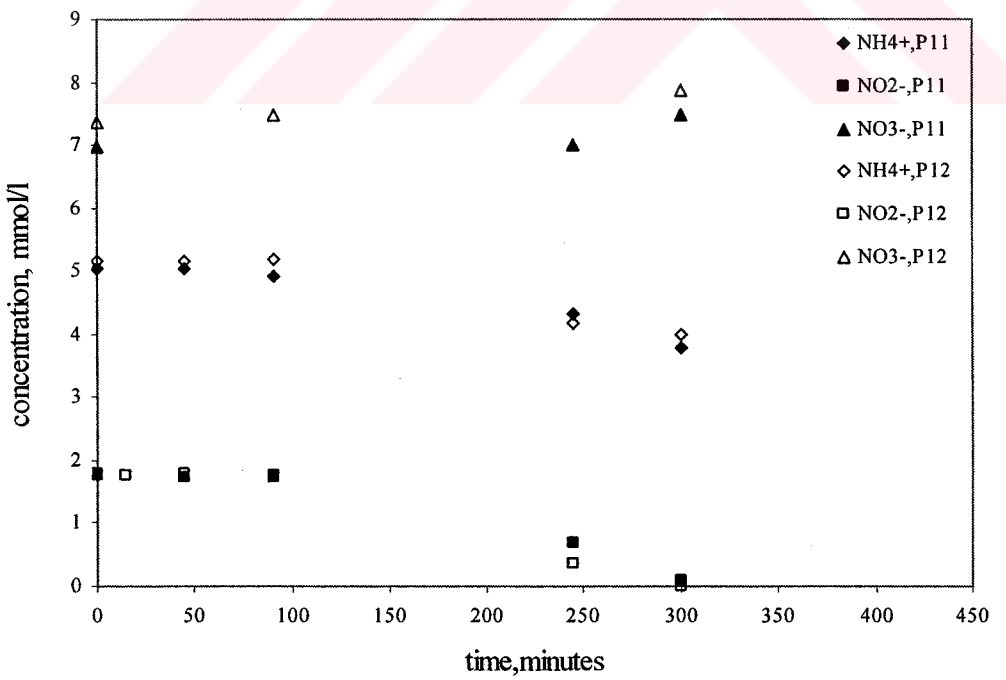


Figure 5.3a Results of the experimental sets 11 and 12 of propionate batch experiments.

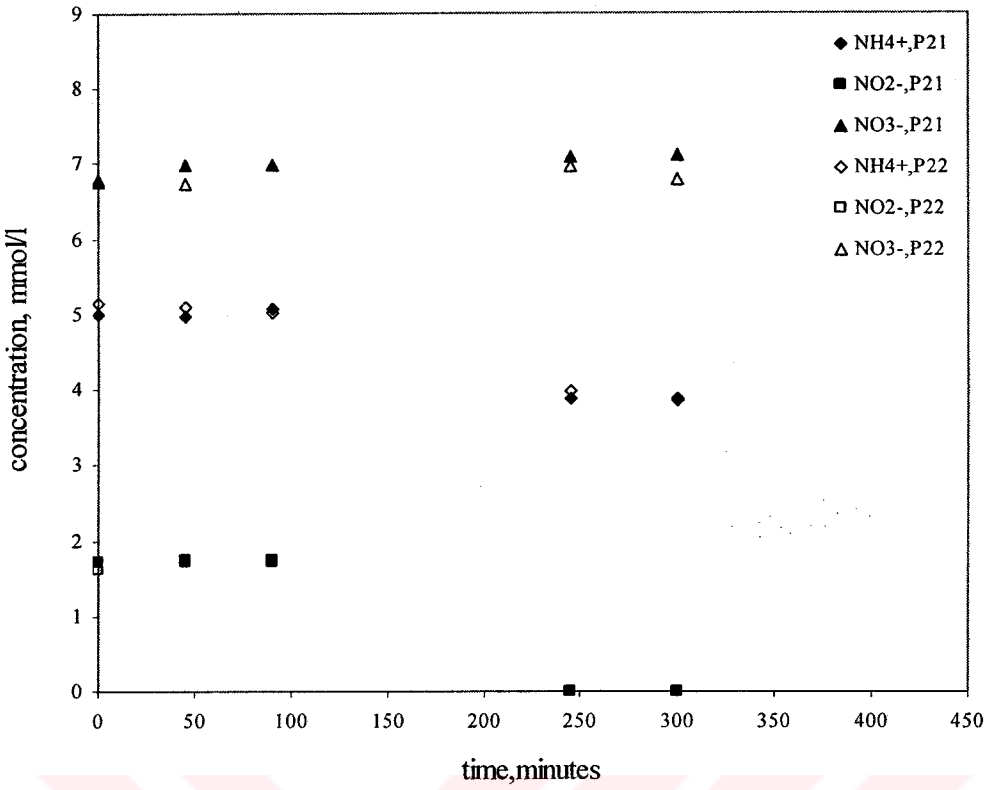


Figure 5.3b Results of the experimental sets 21 and 22 of propionate batch experiments.

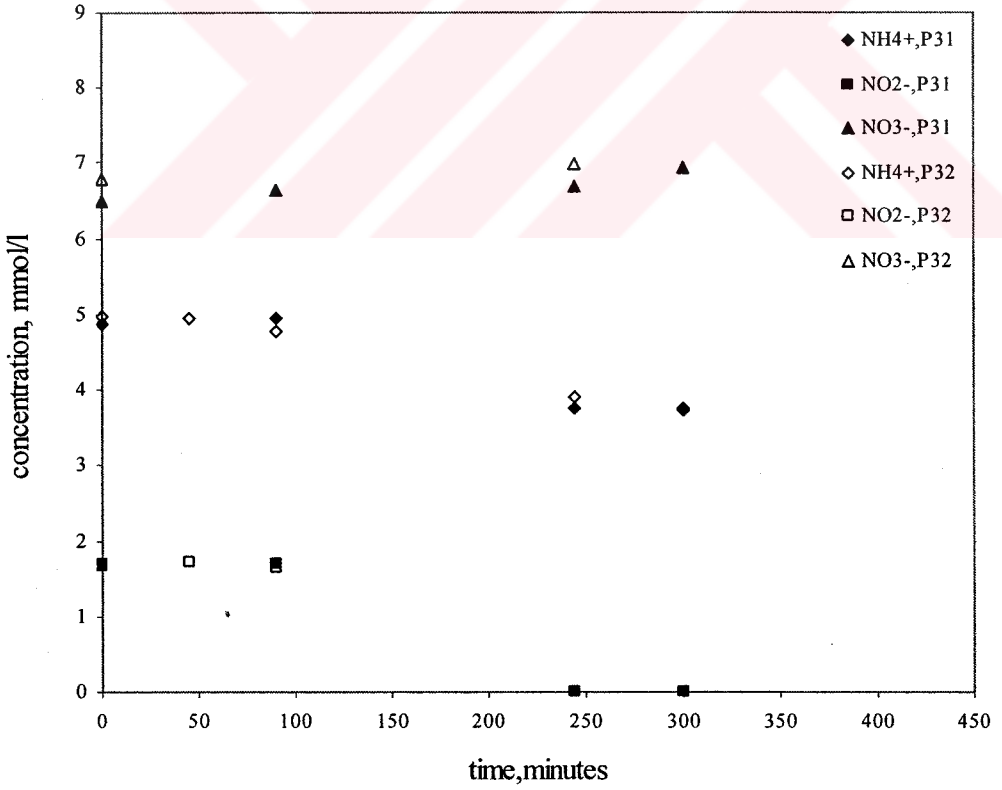


Figure 5.3c Results of the experimental sets 31 and 32 of propionate batch experiments.

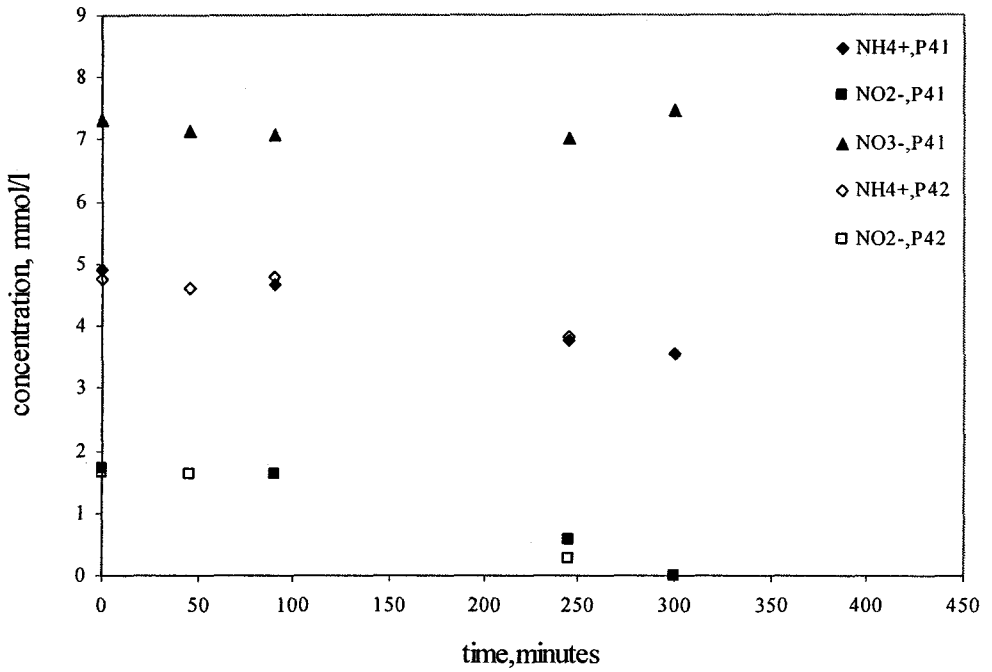


Figure 5.3d Results of the experimental sets 41 and 42 of propionate batch experiments.

5.1.4 Batch experiments with starch

Batch experiments performed with starch were run for 250 min. Initial conditions of the experiments are presented in Table 5.5. Nitrite, nitrate and ammonia profiles are shown in Figures 5.4a to 5.4d. In control set, anammox activity was started as soon as oxygen was completely removed and nitrite converted completely in 100 min. Where in presence of 0.25, 0.5 and 1 mmol starch/l, observed nitrite conversions were lower. Ammonia conversions and nitrate productions were also lower in the presence of starch. Same results were obtained from duplicates.

Table 5.5 Initial conditions of the batch experiments in presence of starch.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Starch (mmol/l)
S_{11}	3.83	1.75	6.84	0
S_{12}	3.71	1.75	6.91	0
S_{21}	3.52	1.58	6.05	0,25
S_{22}	3.73	1.65	6.76	0,25
S_{31}	3.64	1.66	5.88	0,5
S_{32}	3.64	1.72	6.24	0,5
S_{41}	3.61	1.63	5.87	1
S_{42}	3.64	1630	6.04	1

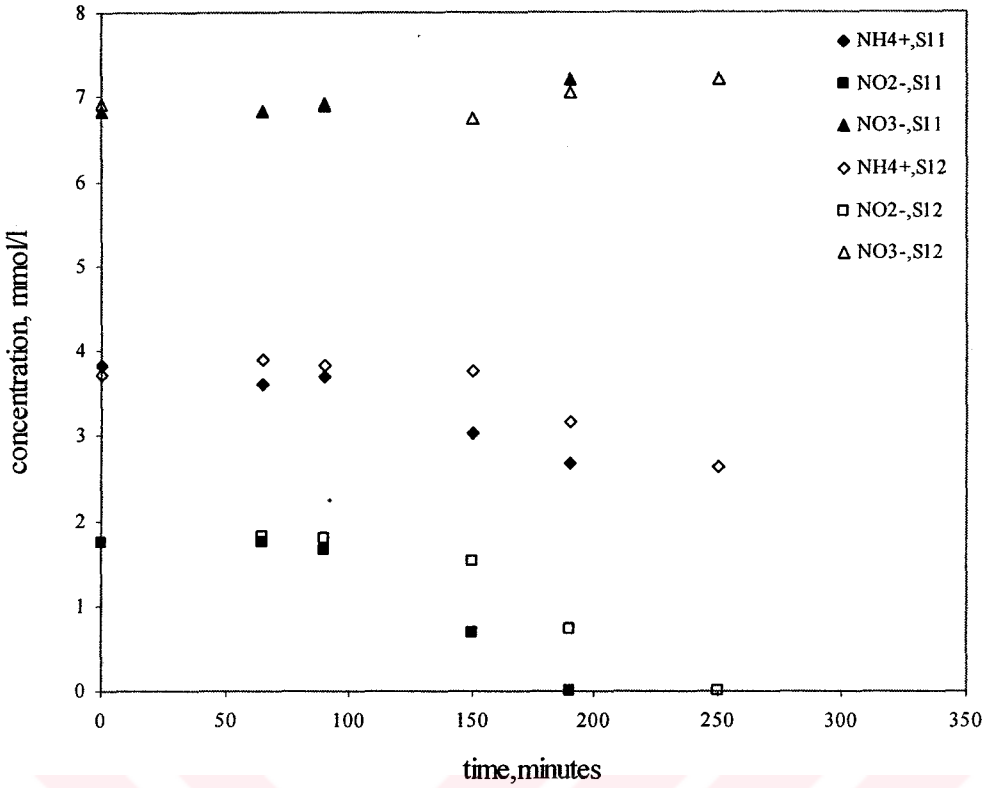


Figure 5.4a Results of the experimental sets 11 and 12 of starch batch experiments.

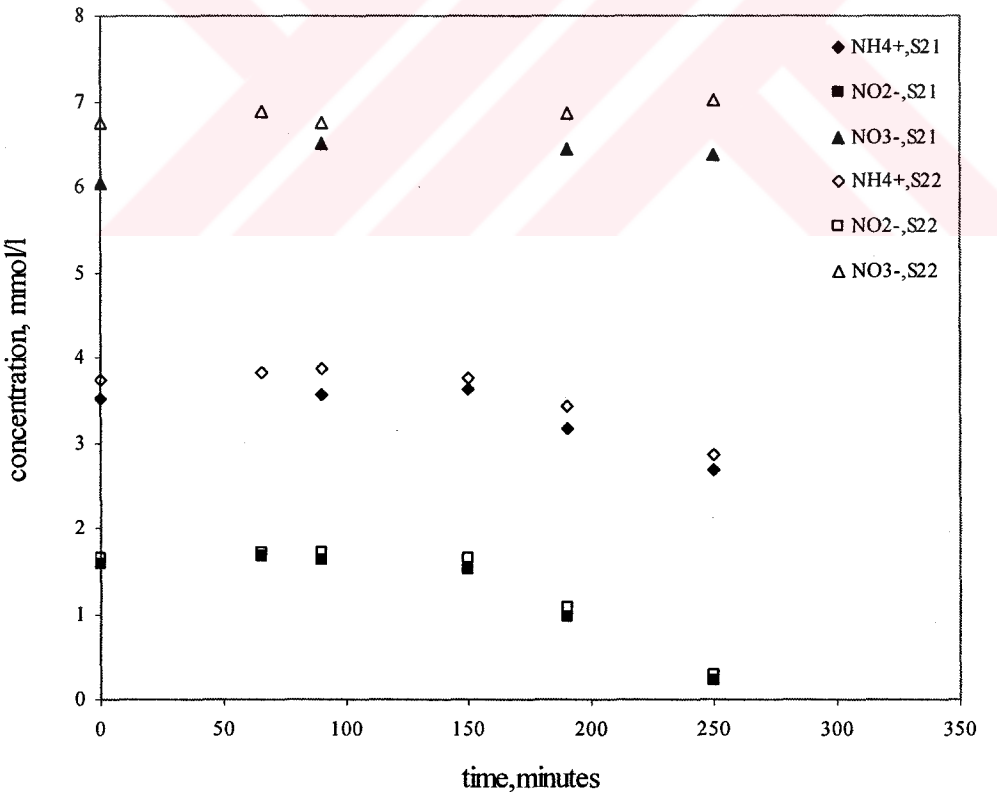


Figure 5.4b Results of the experimental sets 21 and 22 of starch batch experiments.

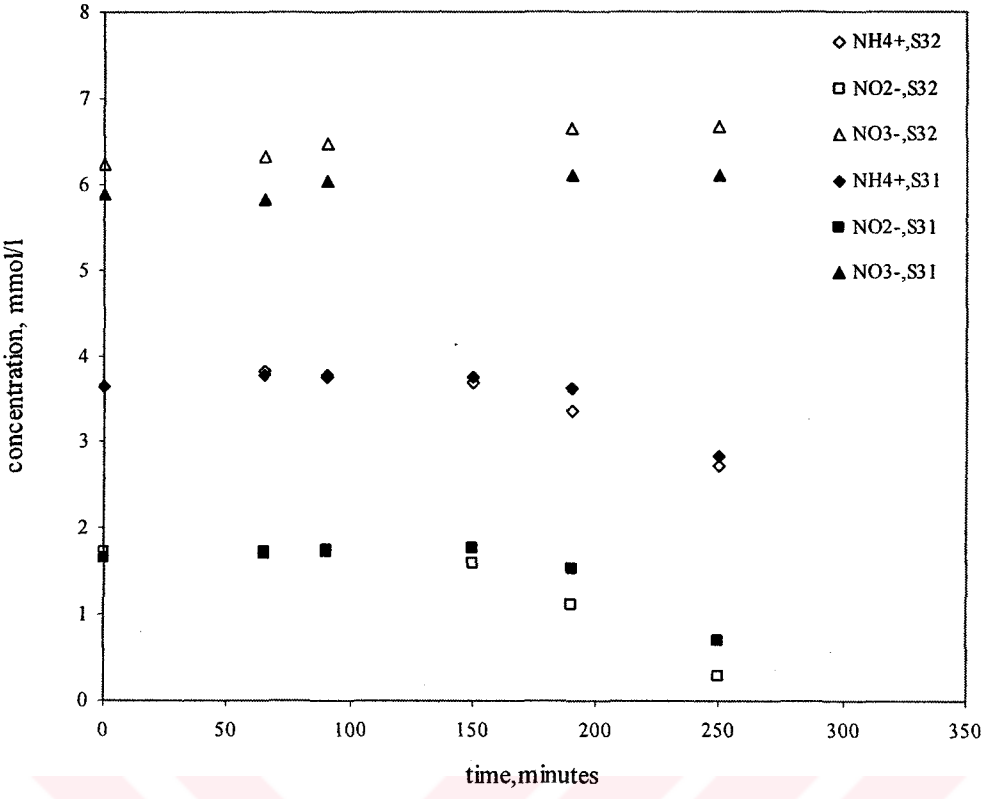


Figure 5.4c Results of the experimental sets 31 and 32 of starch batch experiments.

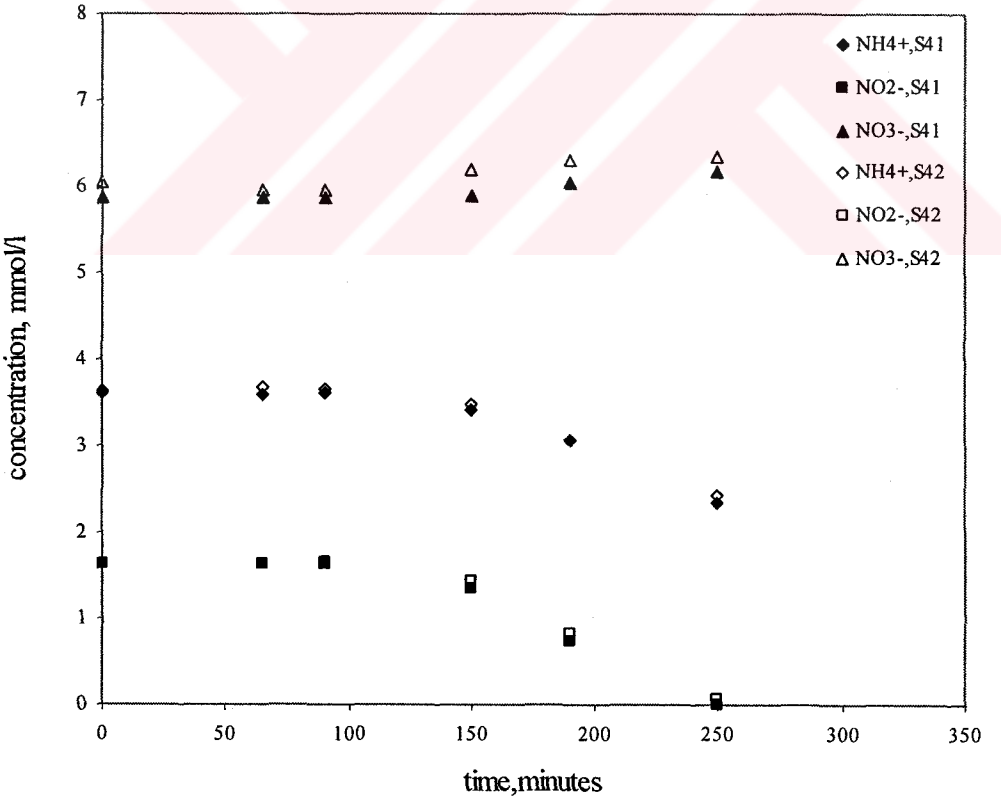


Figure 5.4d Results of the experimental sets 41 and 42 of starch batch experiment.

5.1.5 Batch experiments with ethanol

Batch experiments with ethanol were conducted for 240 min. Table 5.6 shows the initial conditions of the experiments. Results obtained from the experiments are illustrated in Figures 5.5a to 5.5d. In control set, nitrite completely converted in 180 minutes. Results showed that, especially at higher concentrations of ethanol nitrite and ammonia conversions were lower in the same period. Interestingly, in presence of 0.5 mmol ethanol/l both nitrite and ammonia conversions were higher than that of control set. Compatible results were obtained from duplicates.

Table 5.6 Initial conditions of the batch experiments with ethanol.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Ethanol (mmol/l)
E_{11}	8.48	1.84	8.09	0
E_{12}	7.96	1.83	7.84	0
E_{21}	8.6	1.82	8.00	0,25
E_{22}	-	-	-	0,25
E_{31}	8.10	1.83	8.12	0,5
E_{32}	7.71	1.89	7.68	0,5
E_{41}	8.56	1.96	7.76	1
E_{42}	9.02	1.77	6.56	1

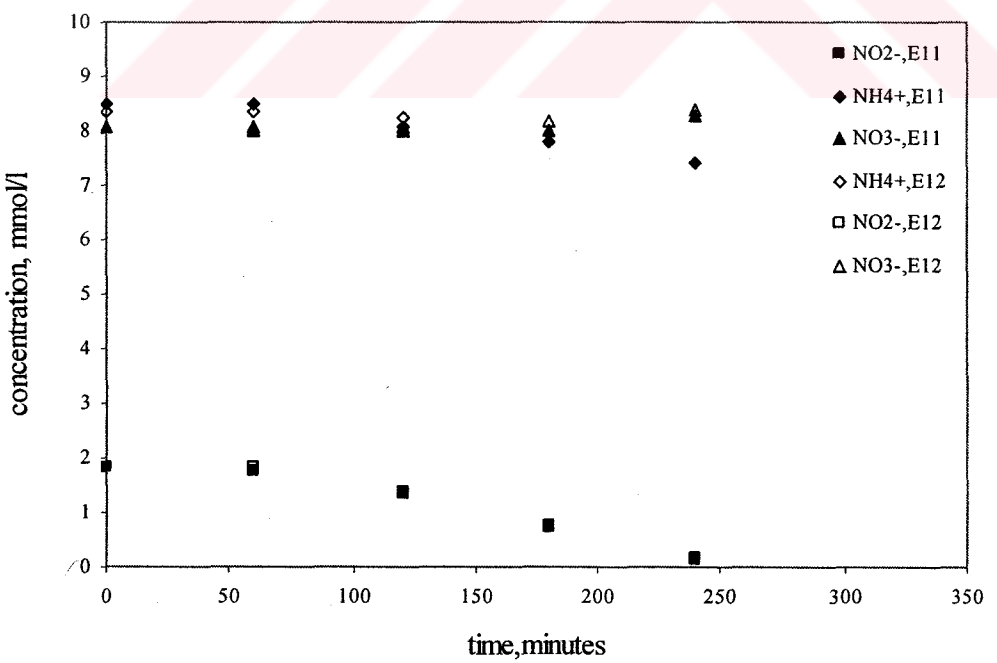


Figure 5.5a Results of the experimental sets 11 and 12 of ethanol batch experiments.

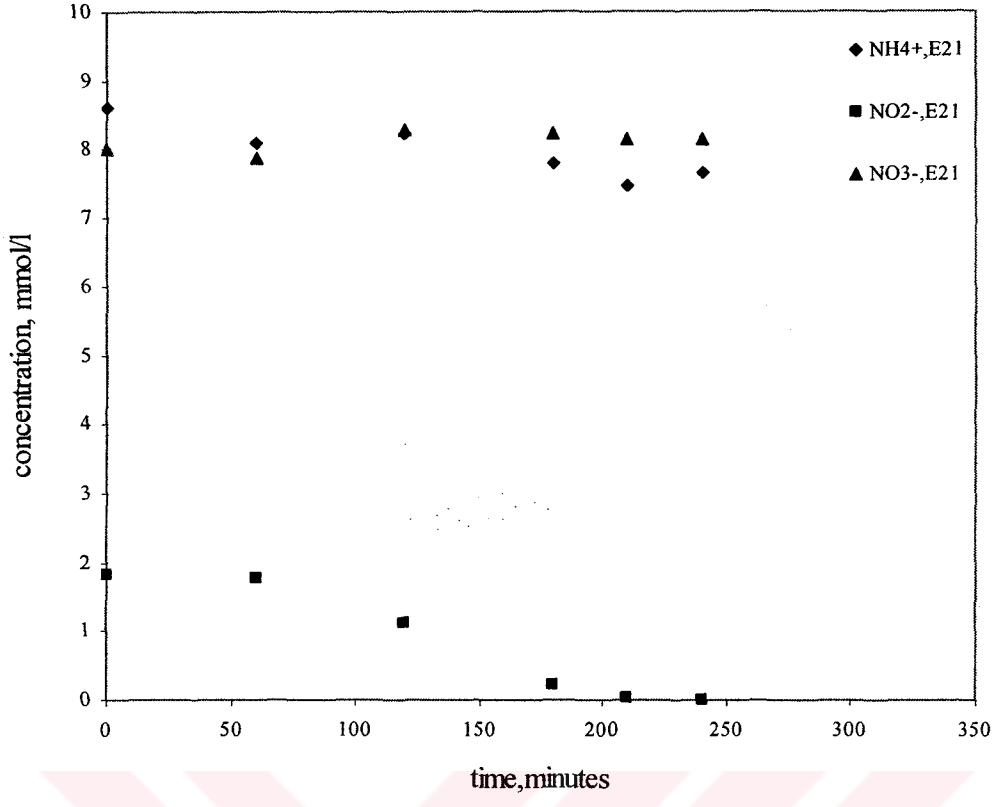


Figure 5.5b Results of the experimental sets 21 and 22 of ethanol batch experiments.

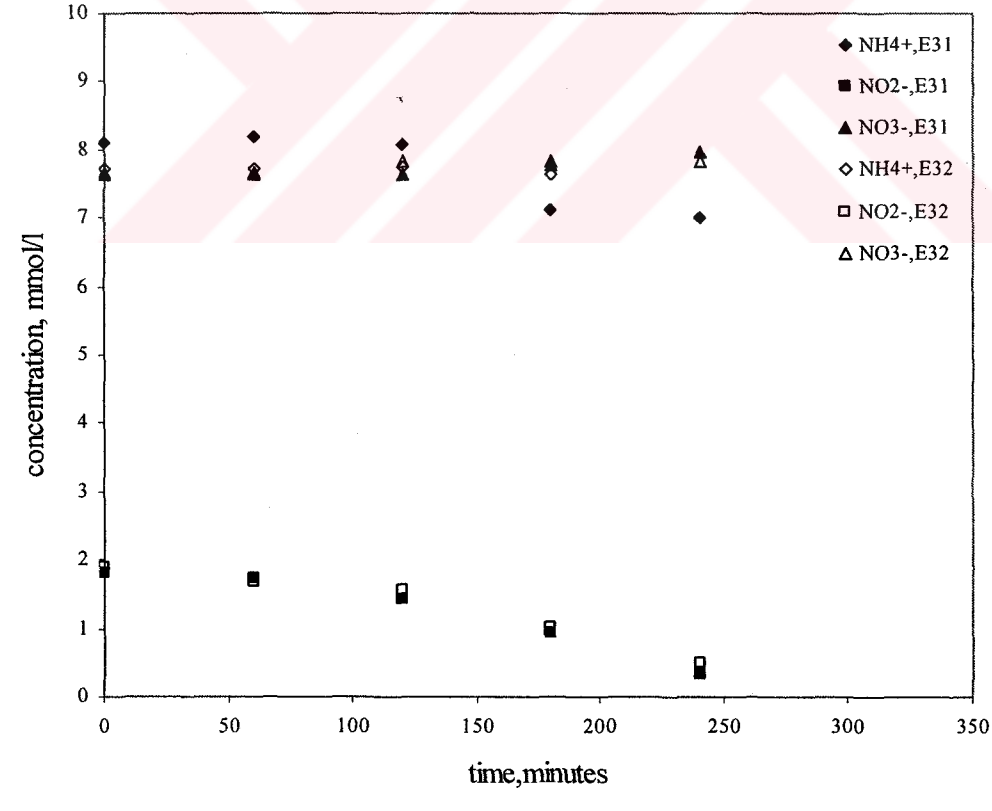


Figure 5.5c Results of the experimental sets 31 and 32 of ethanol batch experiments.

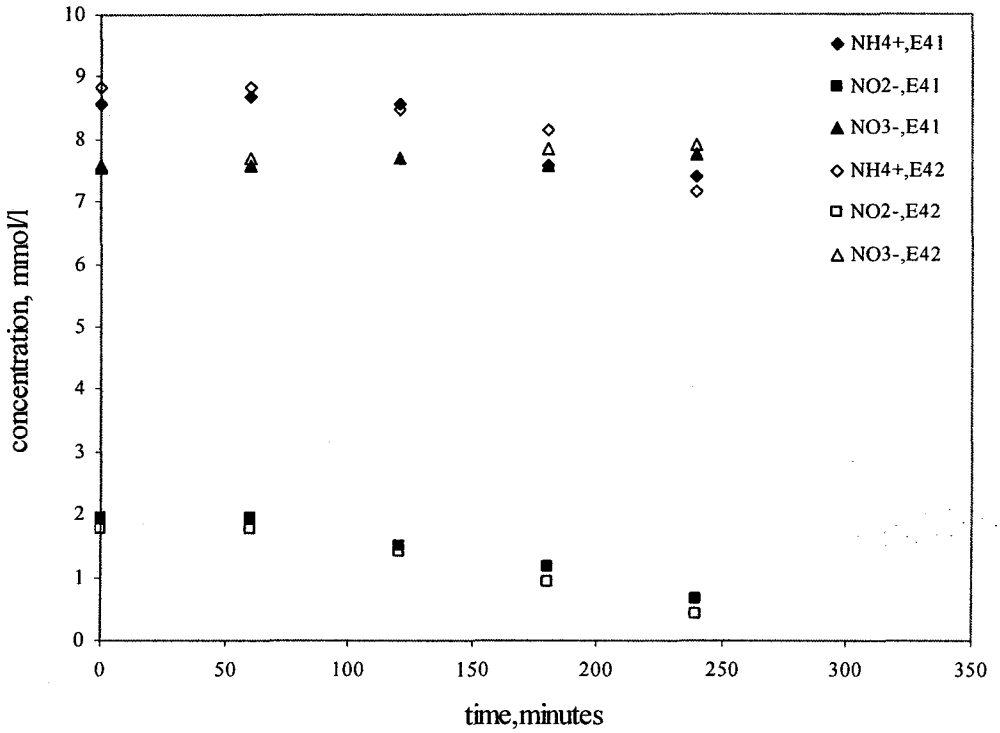


Figure 5.5d Results of the experimental sets 41 and 42 of ethanol batch experiments.

5.1.6 Batch experiments with glucose

Experiments were carried out for 240 min. Initial conditions of the experiments are presented in Table 5.7. Ammonia, nitrite and nitrate profiles are shown in Figures 5.6a to 5.6d. In control sets G_{11} and G_{12}, nitrite was completely converted in 180 min. In presence of 2 and 3 mmol/l glucose, observed nitrite conversion was lower. It can also be clearly seen from the duplicates, reliable results were obtained.

Table 5.7 Initial conditions of the batch experiments with glucose.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Glucose (mmol/l)
G_{11}	3.03	1.54	6.25	0
G_{12}	3.23	1.64	6.35	0
G_{21}	3.13	1.68	6.56	1
G_{22}	3.93	1.75	6.29	1
G_{31}	2.99	1.77	6.65	2
G_{32}	3.12	1.74	7.12	2
G_{41}	3.02	1.71	5.76	3
G_{42}	2.99	1.74	6.26	3

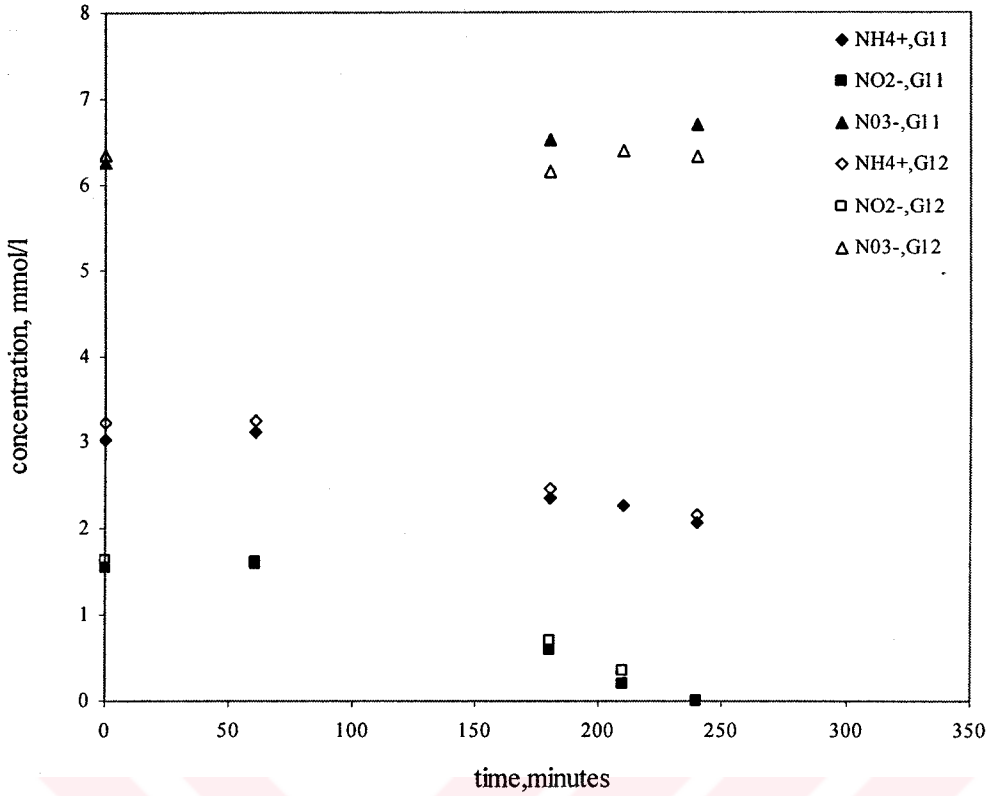


Figure 5.6a Results of the experimental sets 11 and 12 of glucose batch experiments.

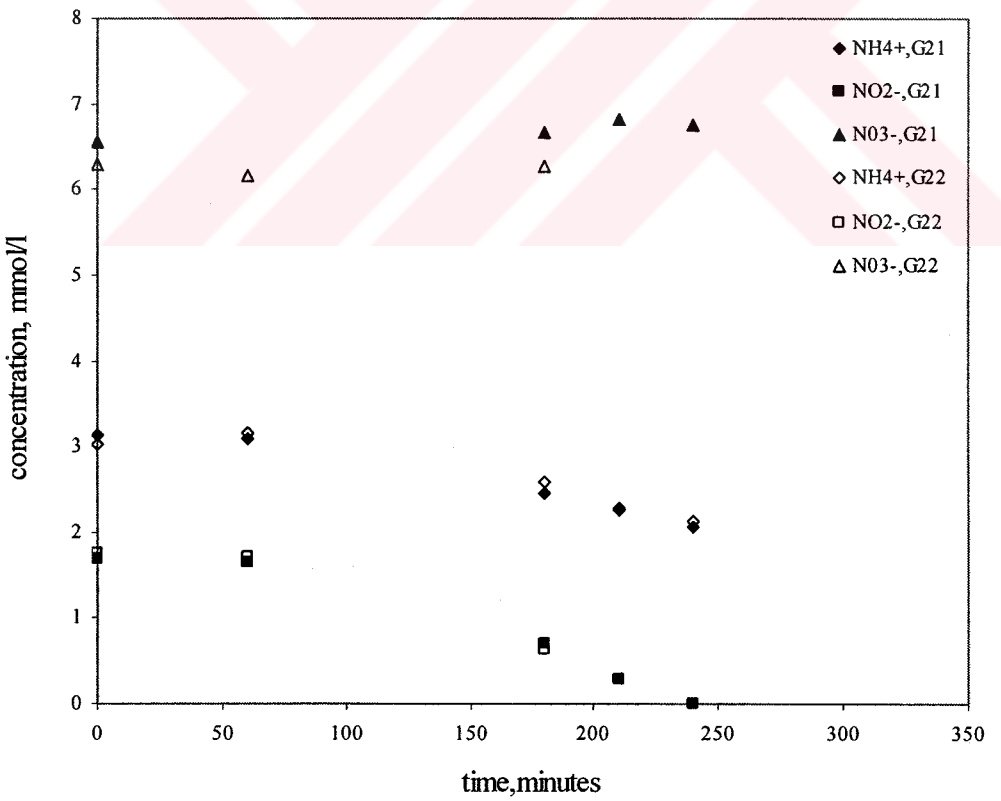


Figure 5.6b Results of the experimental sets 21 and 22 of glucose batch experiments.

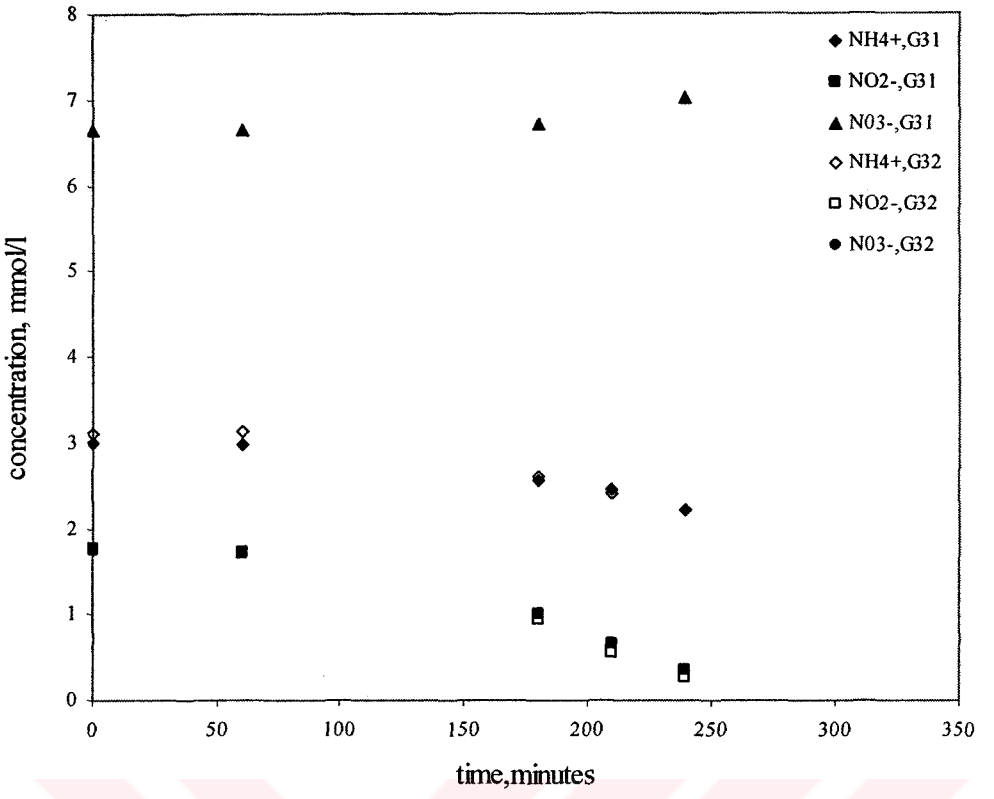


Figure 5.6c Results of the experimental sets 31 and 32 of glucose batch experiments.

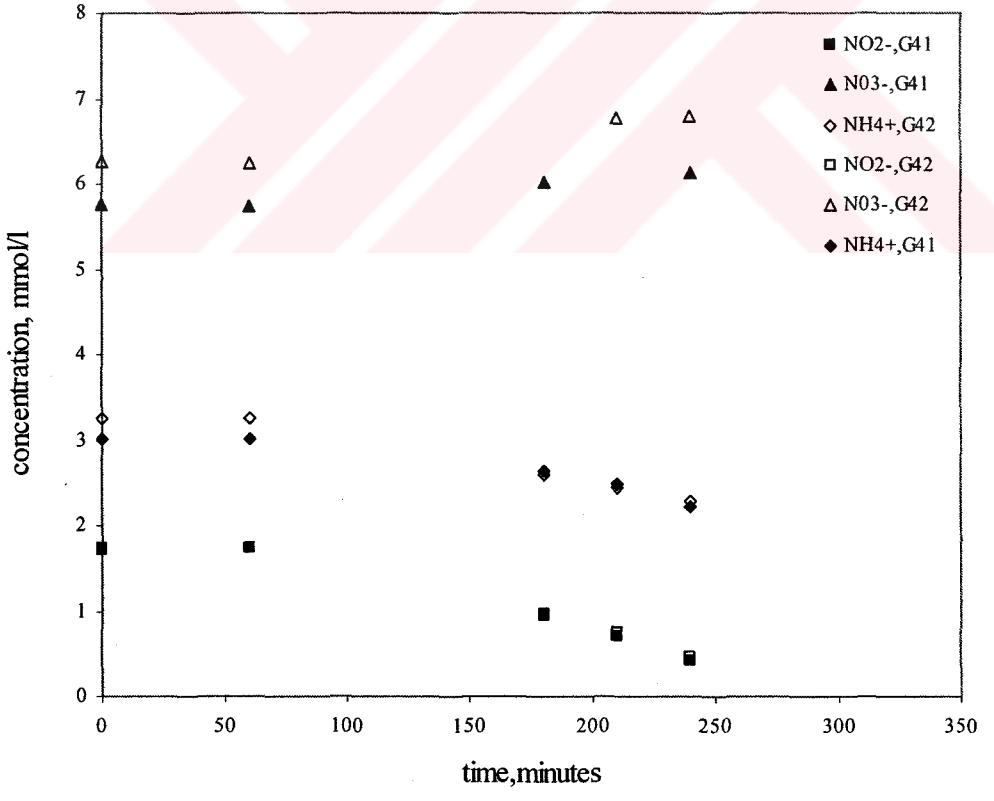


Figure 5.6d Results of the experimental sets 41 and 42 of glucose batch experiments.

5.1.7 Batch experiments with formate

Batch experiments with formate were run for 165 min. Initial conditions of the experiment are presented in Table 5.8. Results obtained from the experiments are illustrated in Figures 5.7a to 5.7d. Results showed that, in control set between time 60 and 120 min, 1.34 mmol/l nitrite was converted. Where in the other sets, observed nitrite conversions were less than 1 mmol/l. Similarly, ammonia conversions were also less than that of control set in the same period.

Table 5.8 Initial conditions of the batch experiments with formate.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Formate (mmol/l)
F_{11}	5.40	1.82	9.55	0
F_{12}	5.26	1.70	9.78	0
F_{21}	5.30	1.74	9.46	0,5
F_{22}	5.16	1.72	9.37	0,5
F_{31}	4.98	1.75	9.34	1
F_{32}	4.75	1.71	9.04	1
F_{41}	4.82	1.66	7.96	2
F_{42}	4.80	1.73	8.68	2

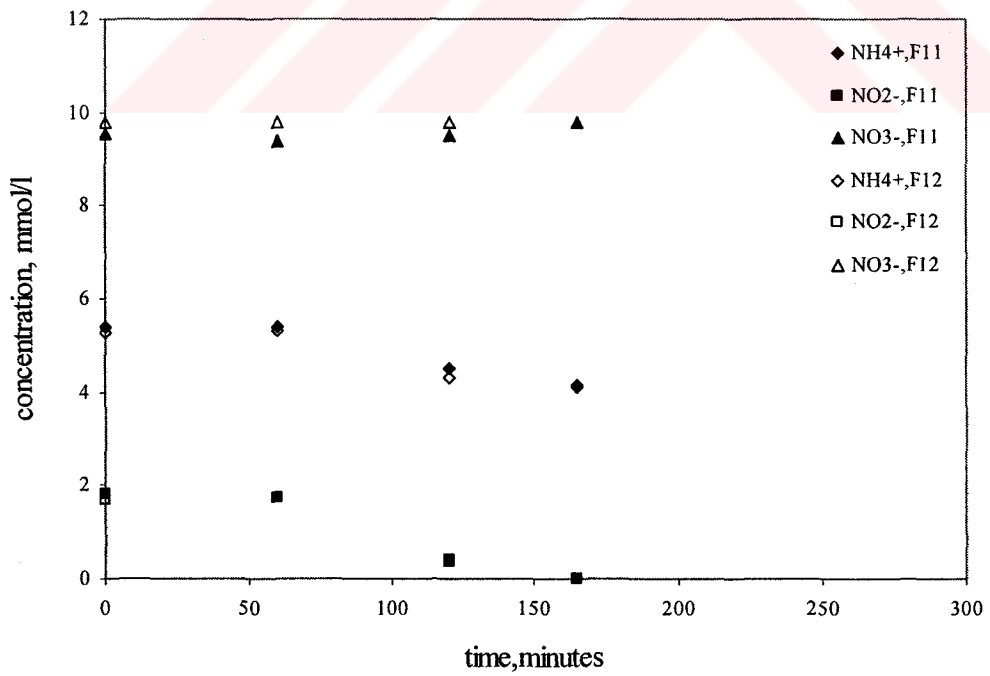


Figure 5.7a Results of the experimental sets 11 and 12 of formate batch experiments.

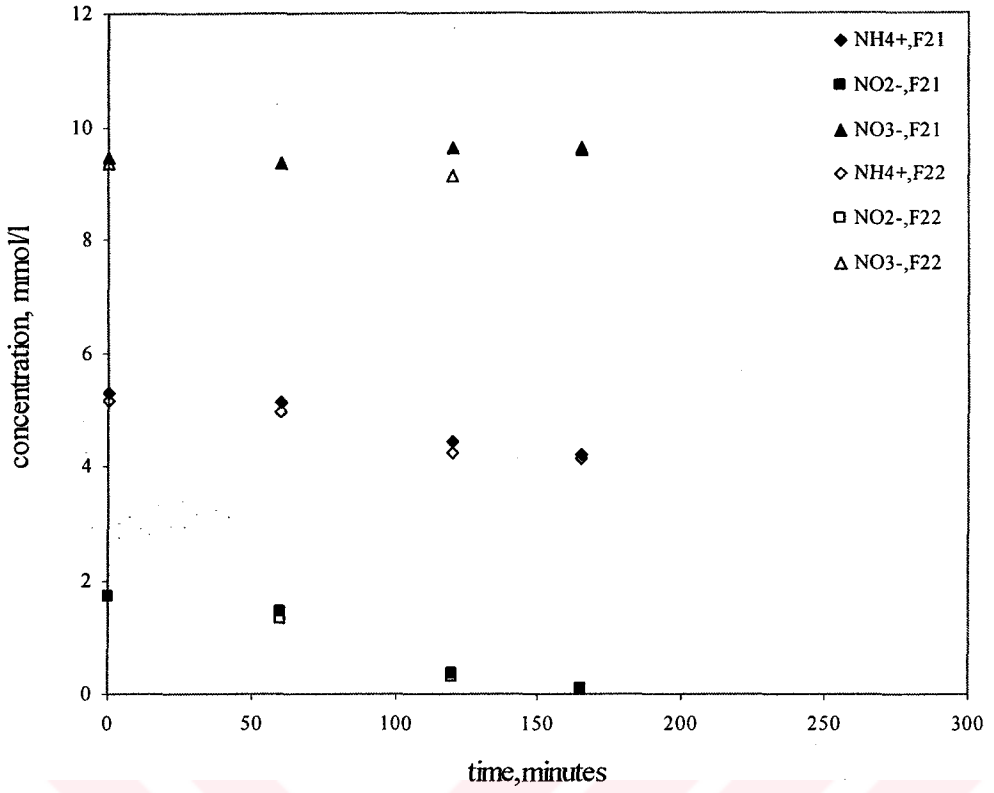


Figure 5.7b Results of the experimental sets 21 and 22 of formate batch experiments.

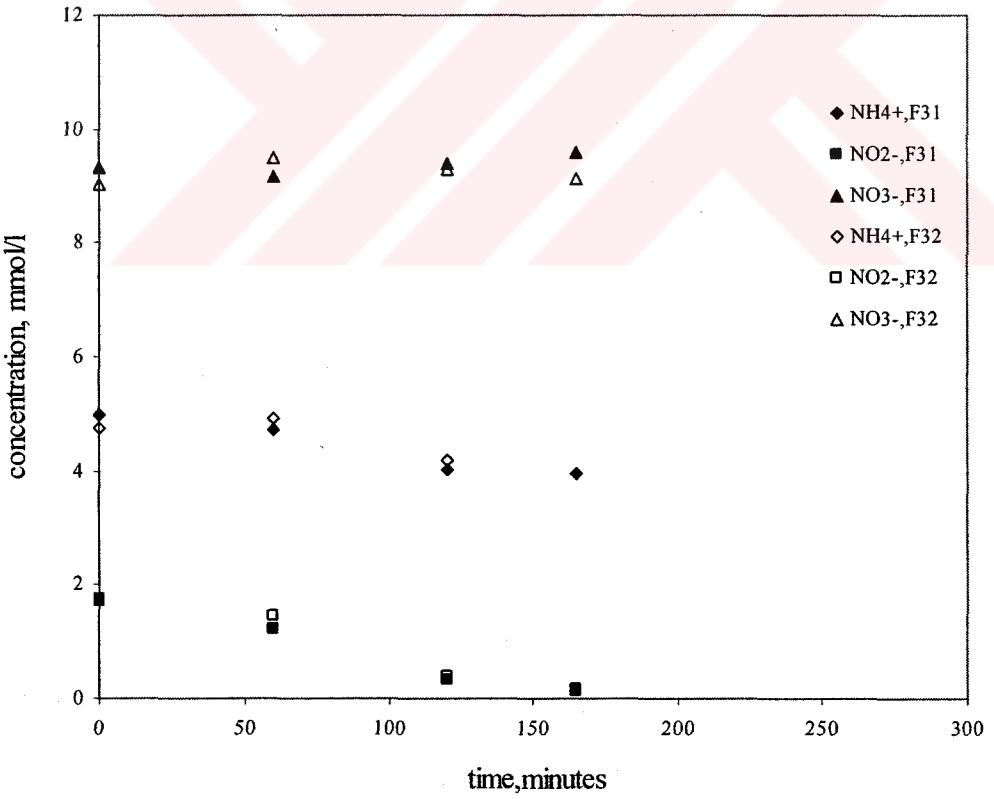


Figure 5.7c Results of the experimental sets 31 and 32 of formate batch experiments.

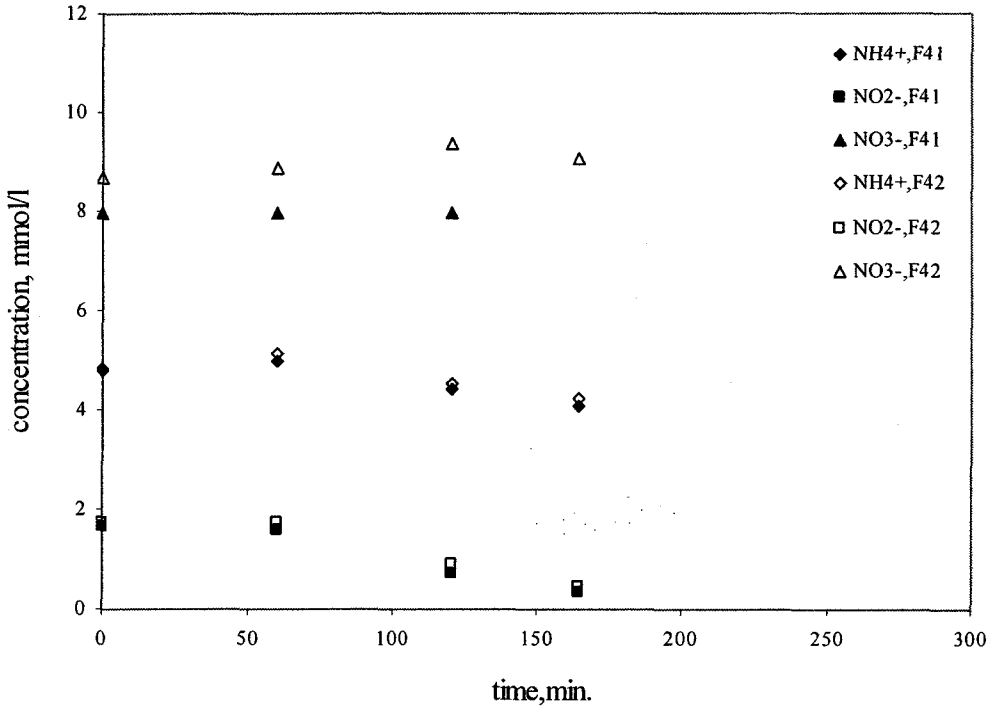


Figure 5.7d Results of the experimental sets 41 and 42 of formate batch experiments.

5.1.8 Batch experiments with amino acids

L-alanine was used for the experiments and run for 210 min. Table 5.9 presents the initial conditions of the experiments. Ammonia, nitrite and nitrate profiles are illustrated in Figures 5.8a to 5.8d. In control set, nitrite was converted completely in 115 min. Meanwhile, observed nitrite and ammonia conversions were lower in presence of 0.5, 1 and 2 mmol/l alanine concentrations.

Table 5.9 Initial conditions of the batch experiments with amino acids.

Set no.	NH_4^+ (mmol/l)	NO_2^- (mmol/l)	NO_3^- (mmol/l)	Amino acids (mmol/l)
AA_{11}	4.04	1.65	10.28	0
AA_{12}	3.96	1.84	10.26	0
AA_{21}	4.32	1.87	10.35	0.5
AA_{22}	4.01	1.92	10.54	0.5
AA_{31}	3.99	1.76	9.96	1
AA_{32}	4.12	1.85	10.06	1
AA_{41}	3.96	1.76	10.49	2
AA_{42}	3.88	1.94	10.14	2

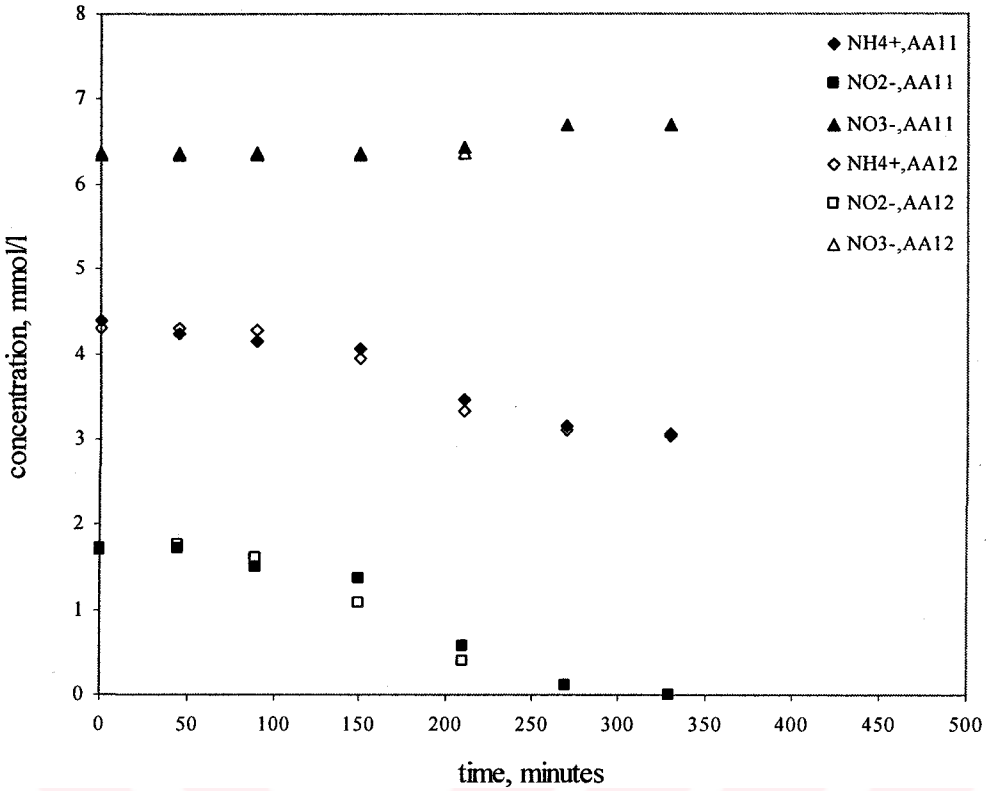


Figure 5.8a Results of the experimental sets 11 and 12 of aminoacids batch experiments.

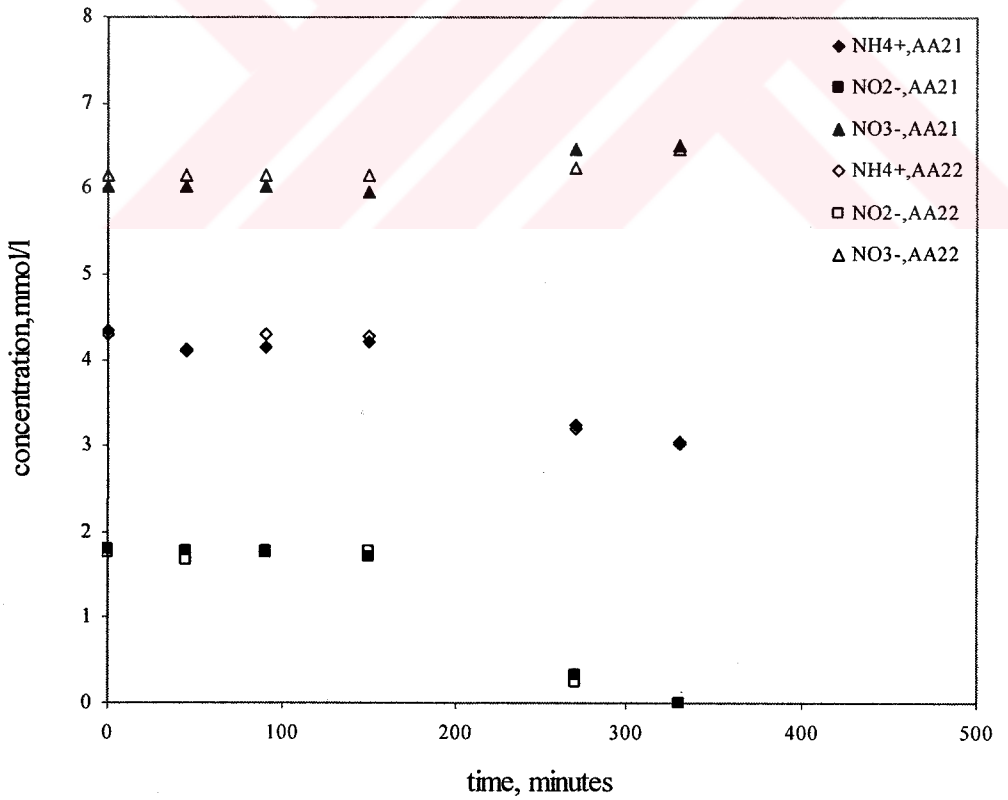


Figure 5.8b Results of the experimental sets 21 and 22 of aminoacids batch experiments.

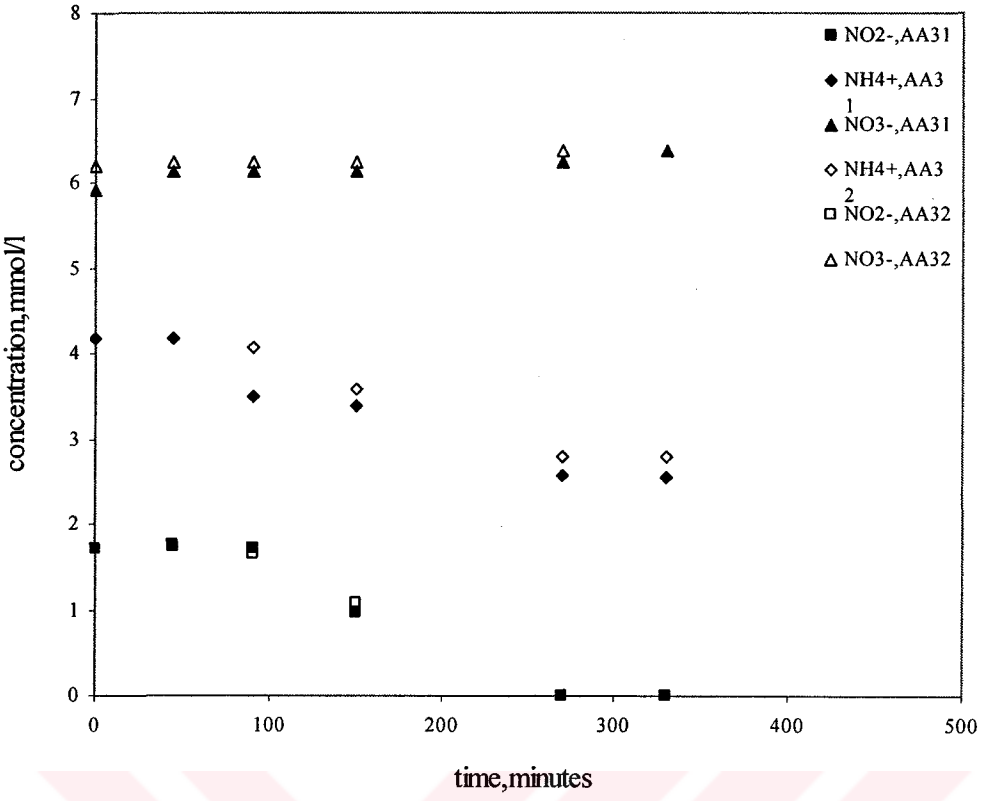


Figure 5.8c Results of the experimental sets 31 and 32 of aminoacids batch experiments.

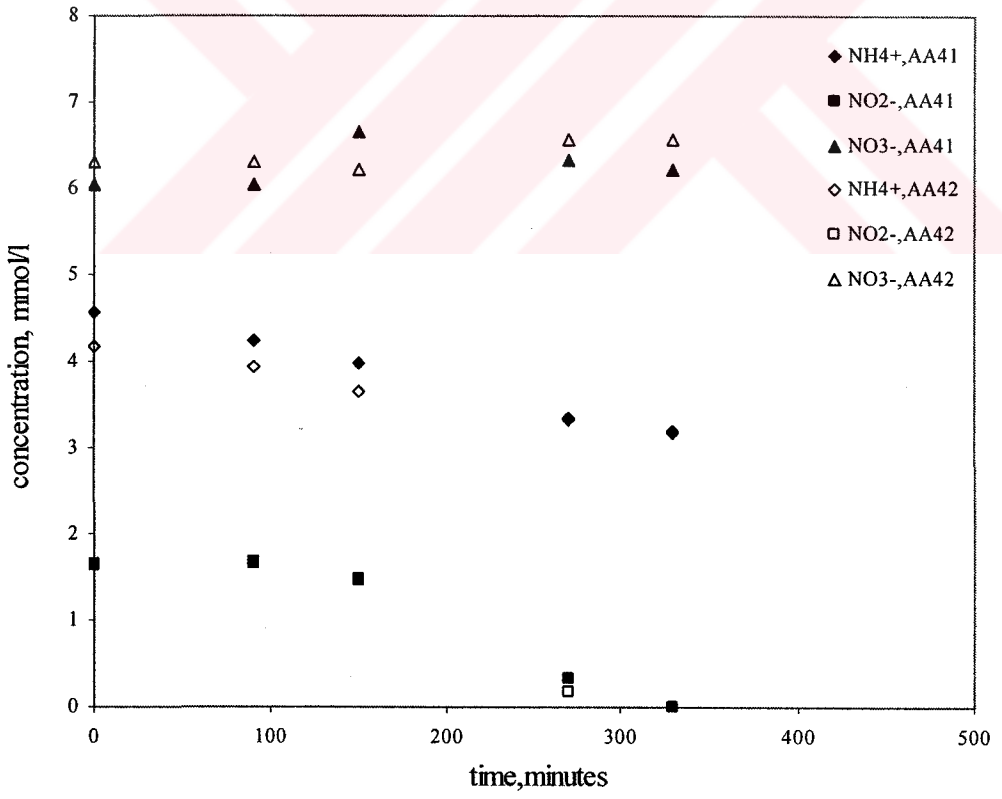


Figure 5.8d Results of the experimental sets 41 and 42 of aminoacids batch experiments.

5.2 Continuous cultivation experiments in the anammox reactor with organic compounds

Results obtained from the batch experiments showed that, anammox activity was stopped in the presence of methanol, where in the presence of acetate or propionate the activity of anammox measured higher compared to control set. In that respect, for the evaluation of the influence of the organic carbon feeding and examine the metabolic activities in the anammox enrichment culture, continuous cultivation experiments were performed. For this purpose 3 different reactors were studied.

5.2.1 Continuous cultivation experiment with methanol

The first experiment was carried out with methanol in a reactor with a volume of 15 l. Reactor was inoculated with 2 l anammox granules taken from the source anammox reactor which consists of 74% enriched anammox bacteria. The operating conditions of the reactor were described in Chapter 4. Methanol feeding was started continuously at 10^{th} day of the run. For this purpose, 2.4 mmol/l methanol solution was prepared and fed to the reactor with a rate of 260 ml/day, supplying 0.63 mmol propionate/day. Reactor was run with a mineral medium containing 12 mmol/l nitrite and 12 mmol/l ammonia and 2.4 mmol methanol/l for 72 hours. As shown in Figure 5.9, within the first 24 hours of methanol feeding, a dramatic increase in nitrite and ammonia concentrations was observed in the effluent. Increase in nitrite and ammonia concentrations are continued in the remaining period of the run. At the end of the 72 hours, both influent and methanol feeding were stopped and biomass in the reactor was started to wash with sole mineral medium to remove excess nitrite and ammonia from the reactor. Results obtained from the experiment are shown in Figure 5.9.

5.2.2 Continuous cultivation experiment with acetate

The same reactor with a 15 l volume was used for acetate feeding. After methanol feeding experiment was finished, the biomass in the reactor was removed and reactor was re-inoculated with 2 l biomass from the source anammox reactor. The reactor was run with an influent mineral medium containing 6 mmol/l of ammonium and 5 mmol/l of nitrite

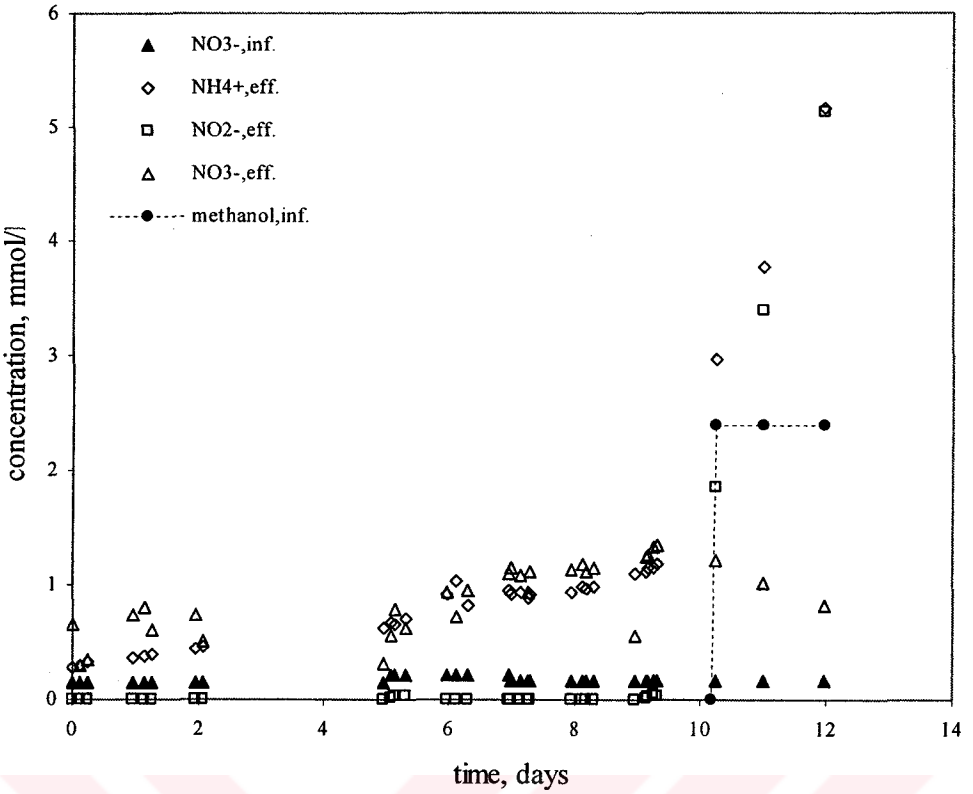


Figure 5.9 Results of continuous flow reactor experiment with methanol.

with a rate of 1.5 l/day. Acetate feeding was started from a stock solution of 10 mmol acetate/l and supplied continuously to the reactor with a rate of 84 ml/day, corresponding to 0.84 mmol acetate/day. Acetate feeding continued to the end of the run at 21st day and during the whole run, reactor was followed for nitrite, ammonia, nitrate and acetate. For the duration of the run, nitrate build-up was observed in the influent bottle, possibly because of nitrification activity. Increase in nitrate concentration in the influent resulted in an increase of nitrate concentration in the effluent. This nitrate interference made difficult to evaluate the results. For this reason, mass balances for N-compounds were done over the reactor. Results obtained from this experiment are shown in Figure 5.10.

5.2.3 Continuous cultivation experiment with propionate

For the continuous cultivation experiment with propionate, an 8 l reactor was used. Reactor was controlled with a computer and monitored for the parameters pH, temperature and dissolved oxygen and stirred at 100±10 rpm. Reactor was started to run with an influent mineral medium containing 29 mmol/l and 27 mmol/l ammonium.

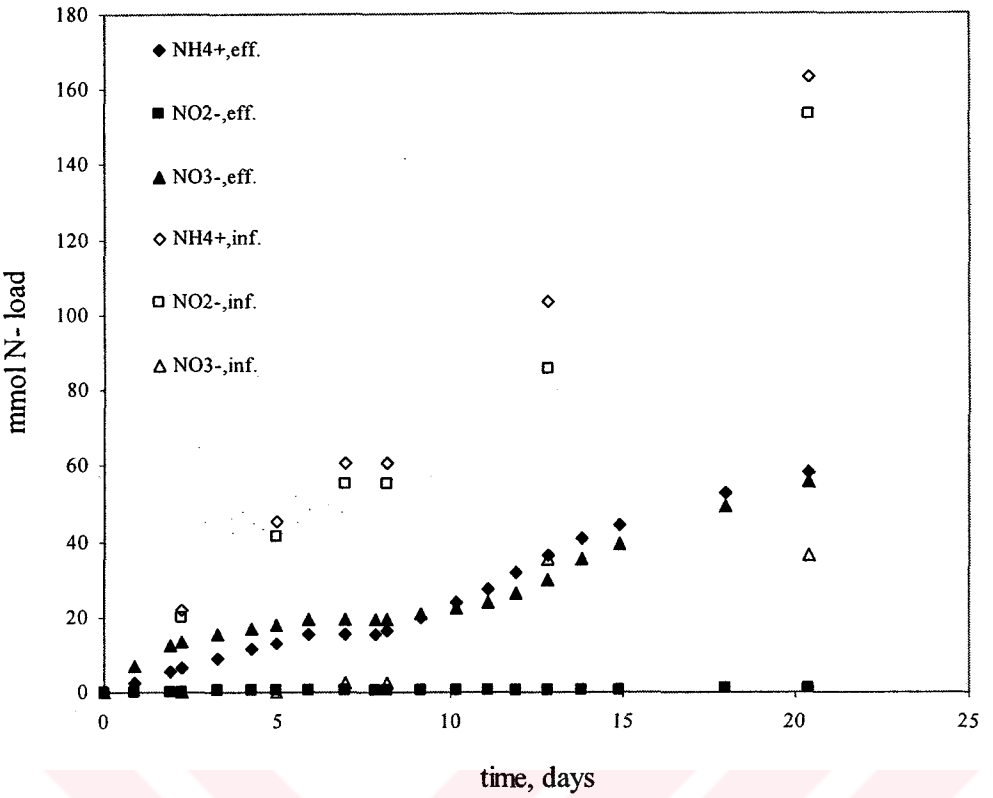


Figure 5.10 Results of continuous cultivation experiment with acetate.

After 15 days, 1 mmol propionate/l stock solution was started to feed with a rate of 180 ml/day supplying 0.18 mmol propionate/day and continued for 3 days. Right after, propionate feeding was doubled to 0.36 mmol propionate/day from a 2 mmol propionate/l stock solution and continued for 23 days. At the end of 23 days propionate feeding was doubled again to 0.72 mmol propionate/day from a 4 mmol propionate/l stock solution. Meanwhile, nitrite, nitrate and ammonia concentrations in the effluent were stable.

Propionate feeding was increased from 0.72 to 1.44 mmol/day and 2.88 mmol/day within 15 days with doubling the concentration of the stock solutions. Propionate concentration in the effluent was measured during feeding period and it was observed that propionate was consumed completely in the reactor. When 2.88 mmol/day propionate feeding was started from the stock solution of 16 mmol/l, decrease in nitrate and increase in ammonia concentrations was observed in the effluent.

In order to investigate whether the nitrate decrease resulted from the high load off propionate, the propionate supply was decreased to 0.72 mmol/day again. It was observed that nitrate concentration was started to increase slowly again. For this reason, 20 mmol/l propionate stock solution was started to feed to the reactor supplying 3.6 mmol propionate/day. At this time, more evident decrease in nitrate concentration was observed. During this period, increase in ammonia concentration was continued. After 6 days, propionate feeding was reduced again to 0.72 mmol/day for 14 days and increase in nitrate concentration and decrease in ammonia concentration was observed. Results obtained from the experiment are illustrated in Figure 5.11.

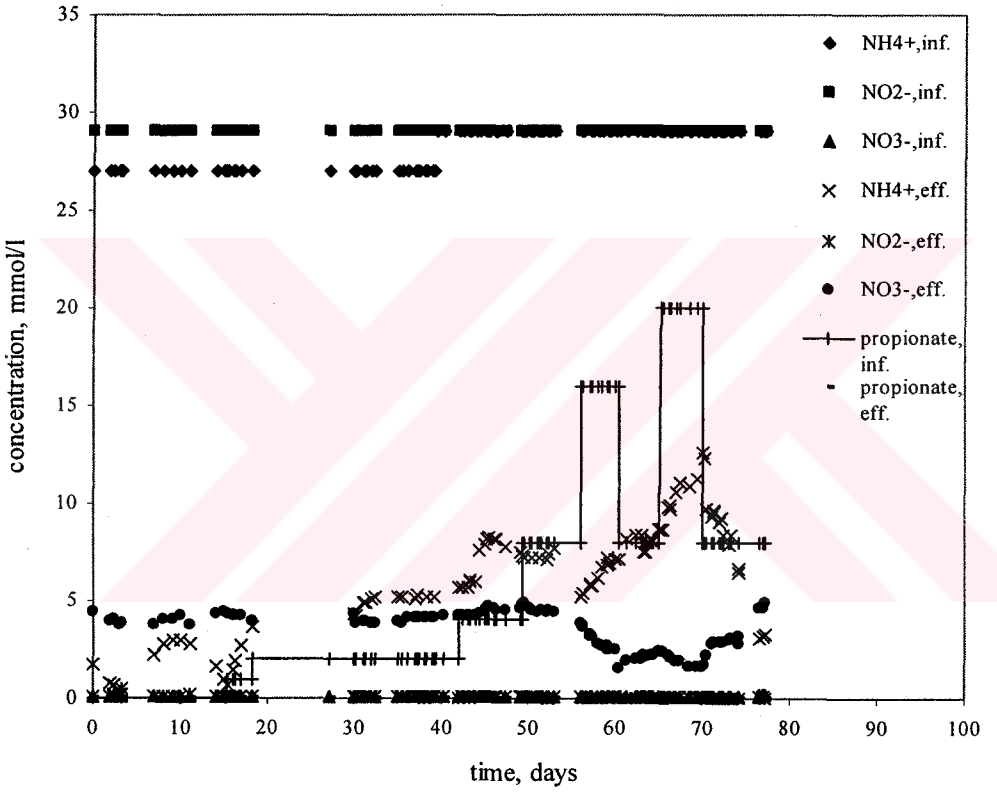


Figure 5.11 Results of continuous flow experiment with propionate.

5.3 Population shift due to acetate and propionate feeding

During operation of the reactors feeding with acetate and propionate, together with nitrogen compounds and mass balances, changes in the bacterial populations were also monitored. Fluorescence in situ hybridization (FISH) analysis was applied to investigate the changes in the composition of the culture in the reactor with addition

of the organic compound. For this purpose, biomass samples were taken before the onset of acetate and propionate feeding. Further, biomass samples were taken for certain intervals to present each acetate/propionate feeding period.

Bacterial composition related with the inoculum of the acetate feeding reactor was analyzed and hybridization signals were obtained by using probes; DH_2-432 (cy3-labeled), EUB338 (fluos-labeled), and PLA46 (cy5-labelled). FISH micrographs of the analysis are presented in Figure 5.12a. Changes in the bacterial population due to continuous acetate supply, which comprise a period of 25 days, were examined with probes PLA46 (cy3-labeled) and AMX820 (fluos-labeled). Obtained hybridization signals are presented in Figure 5.12b.

Bio-community of the inoculum of the propionate feeding reactor was determined by applying the probes DH_2-432 (cy3-labeled), EUB338 (fluos-labeled), and AMX820 (cy5-labeled) and micrographs are shown in Figure 5.13a. Changes in the bacterial population after 10 days of propionate addition was examined with probes DH_2-432 (cy3-labeled), PLA46 (fluos-labeled) and AMX820 (cy5-labeled). Hybridization signals are presented in Figure 5.13b.

FISH pictures illustrated in Figure 5.13c presents the hybridization signals obtained by probes BET42a (cy3-labeled) and AMX820 (fuos-labeled) and demostrates build-up of β-proteobacteria which might indicate denitrifiers in the anammox reactor after 34 days of the onset of propionate feeding (1.44 mmol/day propionate feeding period).

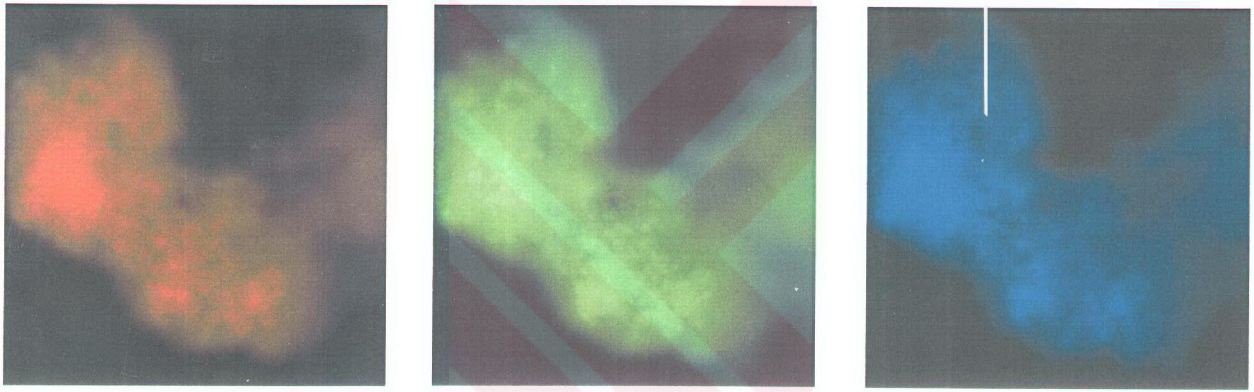
Probes targeting α and γ- Proteobacteria, and several probes specific for certain denitrifiers (*Zoogloea ramigera, Spherotilens natans, Paracoccus denitrificans, Alcaligenes faecalis and Azospirillum)* were applied but no hybridization signals were detected (pictures not shown).

Mc Ewan et al (1984) demonstrated that nitrate reducing organisms have periplasmic nitrate reductase enzyme (NAP). Immunofluorescence was applied to seek out the organism group, namely true denitrifiers which has NAP gene. For the determination of possible denitrifiers versus anammox, combination of immunofluorescence and FISH analysis was applied (Schmid et al. 2003). Immunofluorescence with antibodies raised against the periplasmic nitrate reductase NapA showed that only

when the propionate in the influent was raised to 3.6 mmol propionate/day, NapA positive cells were detectable. In Figures 13d and 13e, Immunofluorescence + FISH analysis are presented reflecting the 75th day of the run. Figures showed that very small amount of denitrifying bacteria were detected although such a long period of propionate feeding. Probes used for FISH analysis are specified in Table 5.10.

Table 5.10 Probes used for in situ hybridization and the formamide concentration used during hybridization.

Probe name	**Probe sequence**	**Target organisms**	**Formamide %**
EUB338 [S-D-Bact-0338-a-A-18]	5 '-GCT GCC TCC CGT AGG AGT-3 '	Most eubacteria	20
PLA46 [S-P-Planc-0046-a-A-18]	5 '-GAC TTG CAT GCC TAA TCC-3 '	Planctomycetes	20
Amx820 [S-G-Amx-0820-a-A-22]	5 '-AAA ACC CCT CTA CTT AGT GCC C-3 '	Most Anammox	20
DH2-432	5 '-CCT AAC TCC CGA CAG CGG-3'	Dokhaven-2	20
ALFA968 [S-P-Alph-968-a-A-18]	5 '-CGT AAG GTT CGT CGC GTT-3'	α-Proteobacteria	20
BET42a [L-P-Beta-1027-a-A-17]	5 '-GCC TTC CCA CTT CGT TT-3'	β-Proteobacteria	35
GAM42a [L-P-Gamm-1027-a-A-17]	5 '-GCC TTC CCA CAT CGT TT-3'	γ-Proteobacteria	35



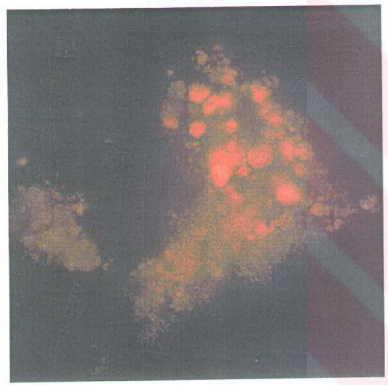
(1) (2) (3)

Figure 5.12a FISH micrographs (views of the same microscopic field) of the biomass in the anammox reactor before acetate feeding period.

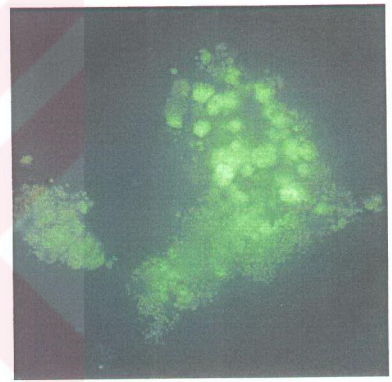
(1): Red: Hybridization with cy-3 labeled probe DH_2-432 which represents dominant type of the anammox bacteria in the reactor.

(2): Green: Hybridization with fluos labeled probe EUB-338, targeting most eubacteria.

(3): Blue: Hybridization with cy-5 labeled probe Pla46, targeting all planctomycetes.



(1)



(2)

Figure 5.12b FISH micrographs (views of the same microscopic field) of the flocs in the anammox reactor under acetate feeding.

(1): Red: Hybridization with cy-3 labeled probe Pla46.

(2): Green: Hybridization with fluos labeled probe Amx-820 presenting most of the identified anammox cells.

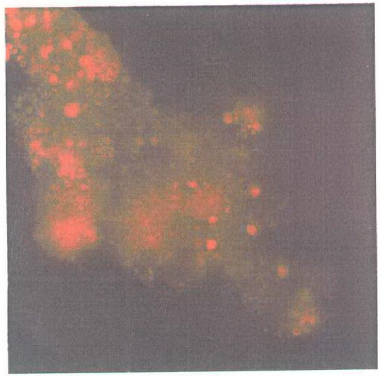
(1) (2) (3)

Figure 5.13a FISH micrographs (views of the same microscopic field) of the biomass in the anammox reactor before propionate feeding period.

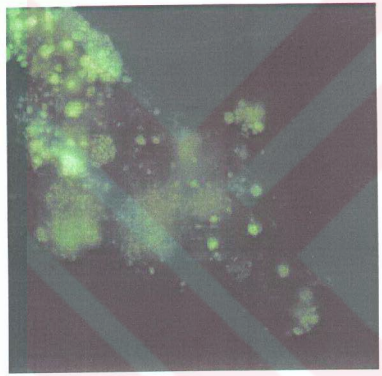
(1): Red: Hybridization with cy-3 labeled probe DH_2-432.

(2): Green: Hybridization with fluos labeled probe EUB-338.

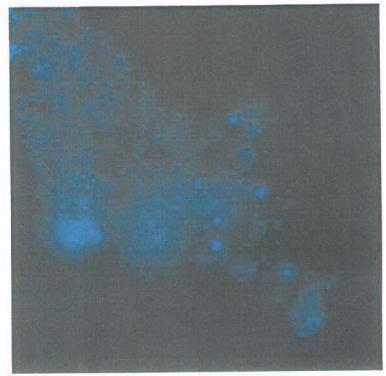
(3): Blue: Hybridization with cy-5 labeled probe Amx-820.



(1)



(2)



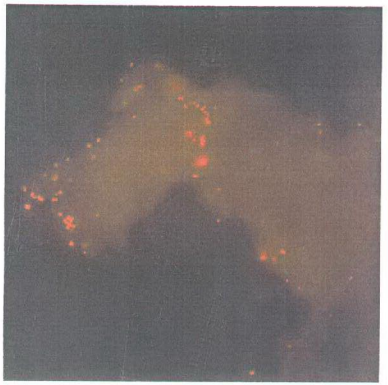
(3)

Figure 5.13b FISH micrographs (views of the same microscopic field) of the biomass in the anammox reactor under 2 mmol/l propionate feeding conditions.

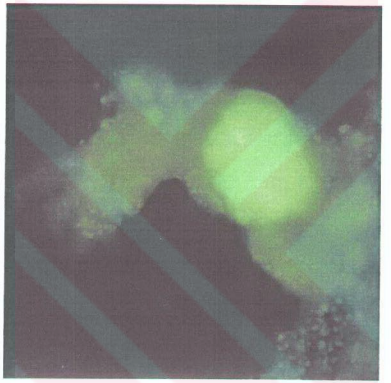
(1): Red: Hybridization with cy-3 labeled probe DH_2-432.

(2): Green: Hybridization with fluos labeled probe Pla46.

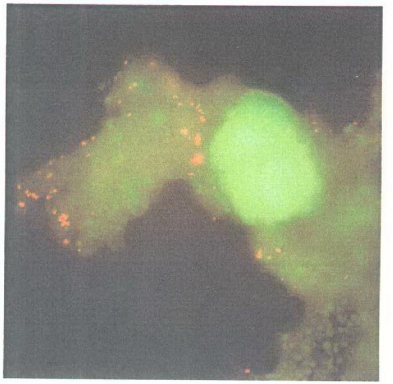
(3): Blue: Hybridization with cy-5 labeled probe Amx-820.



(1)



(2)



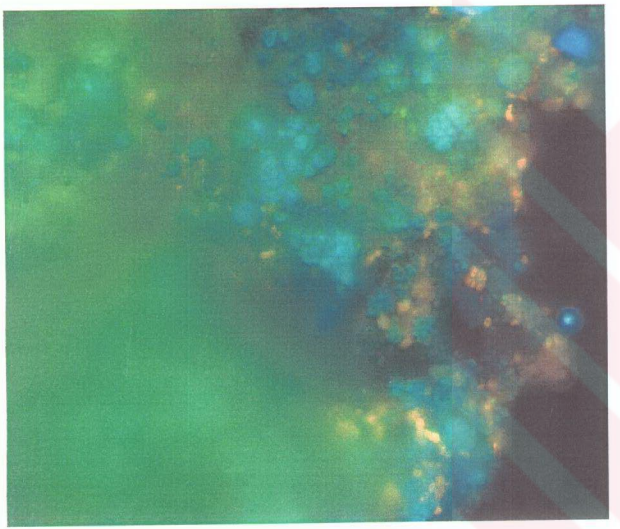
(3)

Figure 5.13c FISH micrographs (views of the same microscopic field) of the biomass in the anammox reactor under 8 mmol/l propionate feeding conditions.

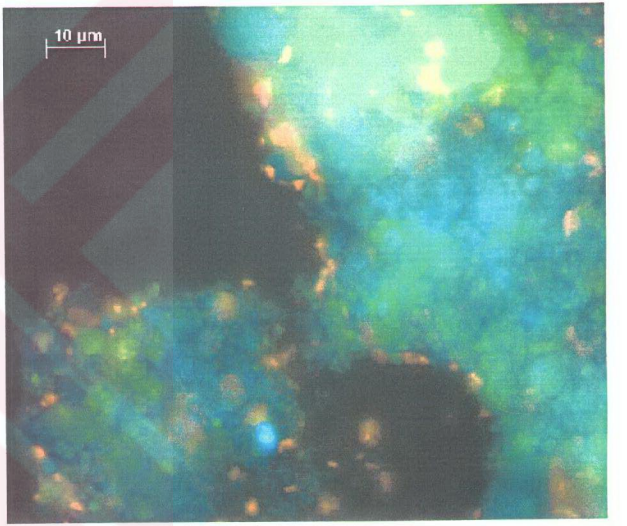
(1): Red: Hybridization with cy-3 labeled probe Bet42a.

(2): Green: Hybridization with fluos labeled probe Amx820.

(3): Same microscopic section showing hybridization with probes Bet42a+Amx820 together.



(d)



(e)

Figure 5.13d FISH + immunofluorescence micrograph reflecting the bio-community in the anammox reactor 75th days of propionate feeding run. Red: Immunofluorescence, signals of NAP gene; Turquoise: Pla46+EUB; Green: EUB

Figure 5.13e FISH + immunofluorescence micrograph reflecting the bio-community in the anammox reactor 75th days of propionate feeding run. Red: Immunofluorescence, indicates NAP gene; Turquoise: Pla46+Amx820; Green: Pla46

5.4 Investigation of propionate consumption in the anammox reactor

Since anammox is known as chemolithoautotrophic bacteria, another group of bacteria could be responsible for propionate consumption. Theoretically, addition of an organic compound to an anammox reactor, would lead to build up of heterotrophs and some of them could denitrify nitrate, using the organic compound as an electron donor. In the frame of these considerations, batch and continuous flow experiments were carried out to investigate propionate consumption in the anammox reactor.

5.4.1 Search for denitrifier activity

In order to distinguish anammox and possible denitrifier activity in the propionate supplemented anammox reactor, batch experiments were performed at first. The objective of the experiments was to inhibit anammox activity and give a chance for denitrifiers which would possibly grow in the anammox reactor under propionate feeding conditions.

Results obtained from the former batch experiments with organic compounds showed that anammox was completely inactive in presence of methanol. On the other hand, methanol is a well known electron donor for denitrifiers. So that, methanol was used as an inhibitor for the anammox activity in the experiments. For this purpose, 40 ml biomass was taken from the anammox reactor feeding with propionate, reflecting the 58^{th} day of the run. Two parallel sets of experiments were performed and studied in duplicate. Experiments were carried out as described for batch experiments with different carbon compounds in Chapter 4. Experimental set-up is presented in Table 5.11. Same amounts of $NaNO_2$ solution were added to the sets.

One of the sets was run as a control without methanol presented as D_1. Propionate was also added to the sets to supply extra carbon source for denitrifier activity. Ammonia, nitrite and nitrate profiles of the experiments are illustrated in Figure 5.14a and 5.14b. It can be clearly seen that nitrite and ammonia were decreased by the time in control set without methanol, where in culture exposed to methanol, any change was observed neither in nitrite nor in ammonia concentrations. This can be explained by the fact that there was no denitrifier activity in the reactor. That shows the dominancy of the anammox activity in the propionate feeding reactor.

Table 5.11 Initial conditions of the experiment search for denitrifier activity.

Set no.	NH_4^+ mmol/l	NO_2^- mmol/l	NO_3^- mmol/l	Methanol mmol/l	Propionate mmol/l
D_{11}	9.00	1.84	2.55	-	2.00
D_{12}	9.22	1.85	2.50	-	2.00
D_{21}	8.80	1.72	2.48	0.50	2.00
D_{22}	9.14	1.75	2.29	0.50	2.00

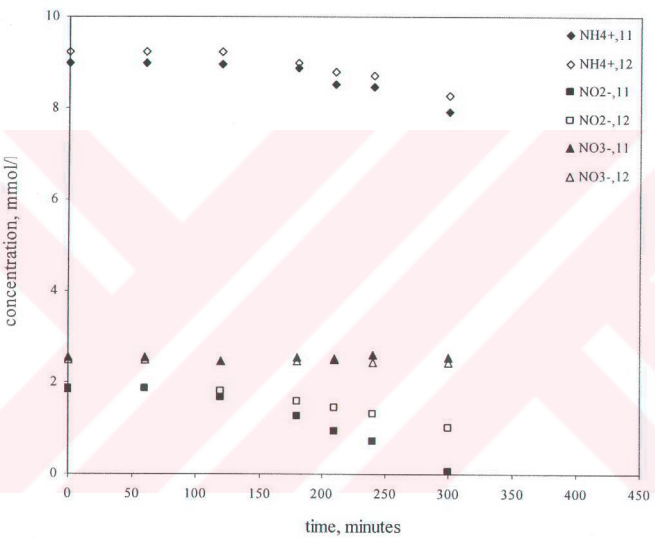


Figure 5.14a Results of the batch sets D_{11} and D_{12} (without methanol).

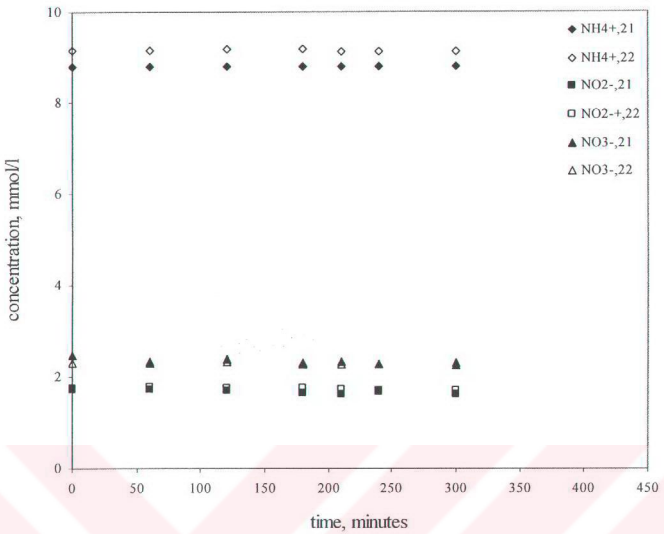


Figure 5.14b Results of the batch sets D_{21} and D_{22} (with methanol).

5.4.2 Pulse addition experiments with propionate in the anammox reactor

Results of the batch experiments for the investigation of denitrifier activity in the propionate supplemented reactor supported the idea of propionate consumption by anammox bacteria. In order to clarify the doubt about propionate consumption by anammox bacteria, pulse-addition experiments were performed in the 74% enriched anammox source reactor. Biomass in the source reactor has never met before an organic compound.

Experiments were carried out in two parts. In the first experiment, influent containing 60 mmol/l ammonium and nitrite was continued during the experiment in order to supply electron donor (ammonia) and electron acceptor (nitrite). A 50 ml propionate solution was added to the reactor to obtain a final concentration of approximately 1mmol propionate/l. Experiment was run for 24 hours. Throughout the experiment, nitrite, nitrate, ammonia and propionate concentrations were measured. Results are illustrated in Figure 5.15a.

The second experiment was carried out in an alternating on and off period of influent in order to observe the system in the absence of electron acceptor (nitrite). Experiment was performed same as described for the previous one. Results obtained from the experiment are presented in Figure 5.15b.

Throughout the first pulse propionate addition experiment propionate consumption was observed as 1 mmol/l. Meanwhile in the second experiment, propionate consumption was measured as 1.4 mmol/l. As can be clearly seen from the Figures 5.15a and 5.15b, propionate was consumed without a lag phase in both experiments. This is an imperative indication of the dominancy of the anammox activity for propionate consumption.

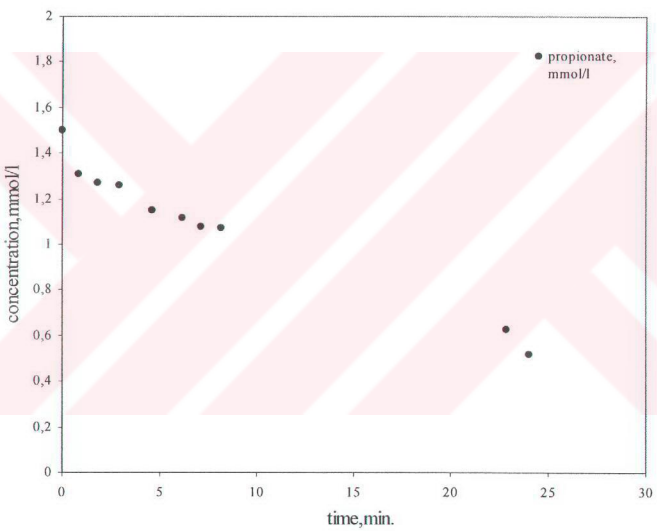


Figure 5.15a Propionate pulse addition experiment1.

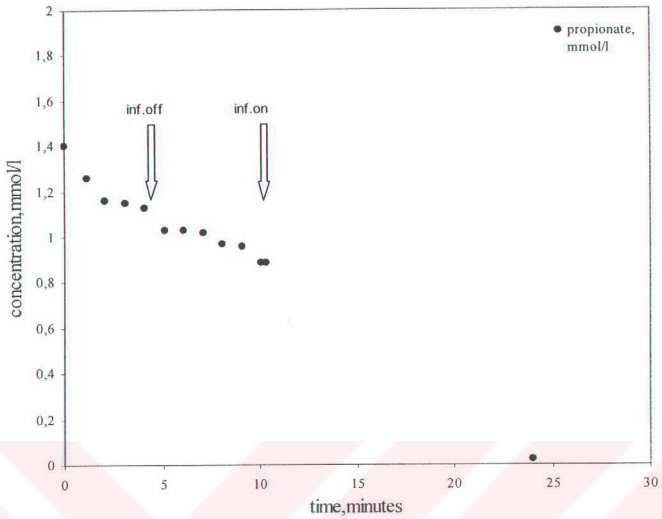


Figure 5.15b Propionate pulse addition experiment2.

5.4.3 Proof of propionate consumption by anammox bacteria

Results of the propionate pulse addition experiments demonstrated that propionate was consumed also in 74% enriched anammox population. These results were strengthened the theory of propionate consumption by anammox bacteria which is known as a chemolithoautotroph.

However, these observations were still not enough to prove this claim, since anammox source reactor was consisted of 74% enriched, not pure, culture. In this context, to prove this theory, a pure culture of anammox cells has to be obtained from the anammox enriched culture and observed the same results with pure culture. For this purpose, dominant anammox cells were purified from the enrichment culture and a micro batch experiment was performed.

5.4.3.1 Purification of dominant anammox cells

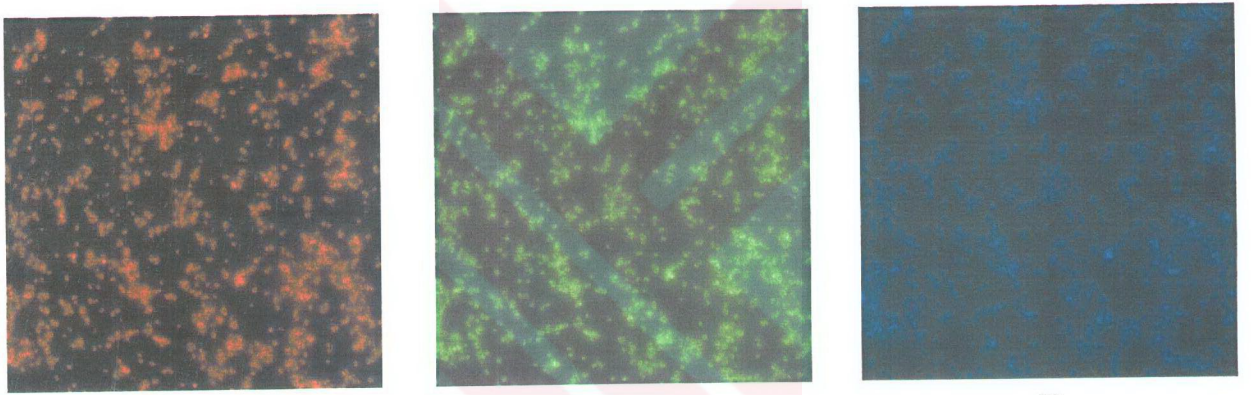
Since anammox is an extremely slowly growing bacteria, attempts to isolate this microorganism from the enrichment culture using classical microbiological techniques (dilution series, obtaining colonies from single cells on a range of media) were unsuccessful but it was accomplished physically using density gradient centrifugation (Strous, M., 2000). In order to obtain a pure culture of dominant anammox cells from the enrichment culture, density gradient centrifugation method was modified which was described by Strous (2000) as described in Chapter 4.

After density gradient centrifugation, mainly two bands containing single cells were obtained in the centrifuge tube. The target cells were recovered as a red band in the bottom part of the gradient. No other bands containing any other cell types were recovered.

The determination and confirmation of the degree of purification were obtained by the application of fluorescence *in situ* hybridization (FISH) analysis to a part of the cell suspension after purification process. Applied oligonucleotide probes are specified in Table 5.12. Pictures obtained from FISH analysis are presented in Figure 5.16. FISH pictures showed that obtained purity was between 99% and 99.5.

Table 5.12 Probes used for in situ hybridization and the formamide concentration used during hybridization.

Probe name	Probe sequence	Target organisms	Formamide %
EUB338 [S-D-Bact-0338-a-A-18]	5 '-GCT GCC TCC CGT AGG AGT-3 '	Most eubacteria	20
PLA46 [S-P-Planc-0046-a-A-18]	5 '-GAC TTG CAT GCC TAA TCC-3 '	All Planctomycetes	20
Amx820 [S-G-Amx-0820-a-A-22]	5 '-AAA ACC CCT CTA CTT AGT GCC C-3 '	Most anammox	20



(1) (2) (3)

Figure 5.16 FISH micrographs (views of the same microscopic field) of the purified anammox cells.

(1): Red: Hybridization with cy-3 labeled probe EUB338

(2): Green: Hybridization with fluos labeled probe PLA46.

(3): Blue: Hybridization with cy-5 labeled probe Amx820.

5.4.3.2 Micro batch experiment with pure culture of anammox cells for propionate consumption

The purified cell suspensions were concentrated to a final concentration of more than 4 g protein/l and incubated in 200µl (1 mmol/l propionate and 2.4 mmol/l nitrate in Hepes/bicarbonate buffer) activity tests in 0.6 ml eppendorf tube. Experiment was run for 150 minutes. Results obtained from the experiment are illustrated in Figure 5.17. Figure shows that the cells consumed propionate at a rate of 92 µl/h. Meanwhile slight increase in nitrite and ammonia concentrations and remarkable decrease in nitrate concentration were perceived.

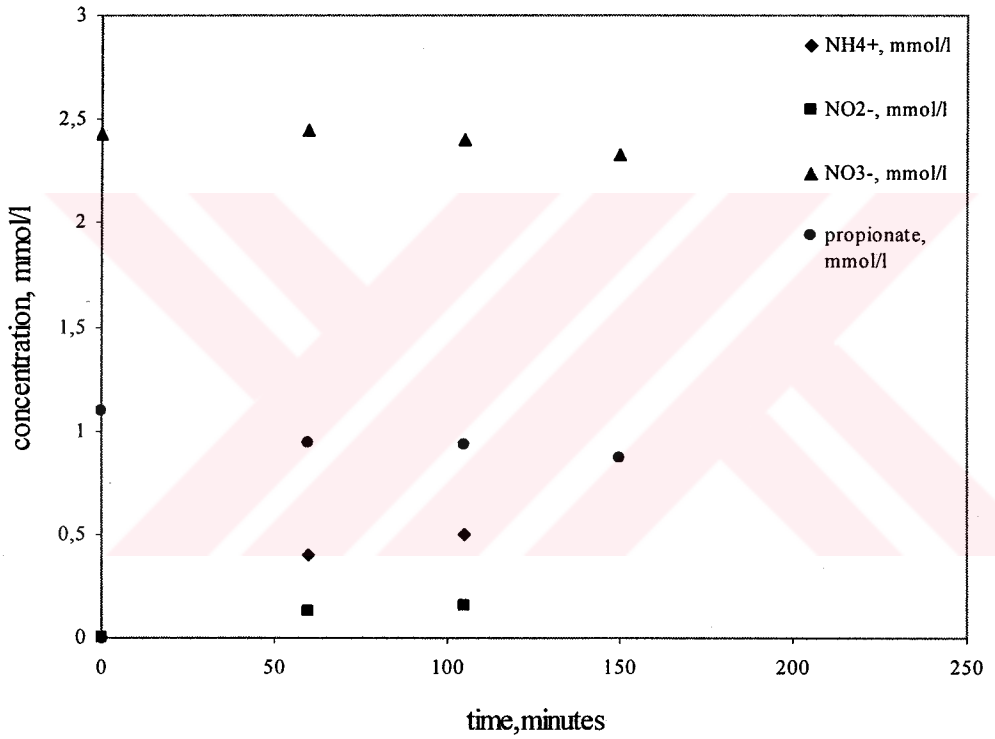


Figure 5.17 Results of micro batch experiment with pure culture of anammox.

5.5 Investigation of nitrogenous compound coupled to propionate oxidation and possible reaction mechanism

In order to investigate nitrogen compound(s) coupled to propionate oxidation, batch experiments were performed with several configurations of nitrogen compounds. Further, isotope labeling experiments were performed to explore reaction mechanism using ^{15}N-labelled nitrate in presence of propionate.

5.5.1 Batch experiments with different nitrogenous compounds and propionate

Biomass were taken from the source anammox reactor and washed with mineral medium until all N compounds were removed and distributed to 10 ml serum bottles in the same volume of biomass to obtain final volume of 5 ml as described in Chapter 4. 6 different experimental sets were performed and initial conditions of the sets are specified in Table 5.13. Experiments were run for 24 hours. All batch sets were studied in duplo (except set 1) and duplicates are depicted as small letters.

Table 5.13 Experimental set-up of the batch experiments with configurations of N-compounds.

Experimental set no.	Compounds (mmol/l)			
	NH_4^+	NO_2^-	NO_3^-	Propionate
1	-	-	-	0.7
2a	5.74	-	-	2.30
2b	5.42	-	-	1.70
3a	-	5.25	-	2.35
3b	-	5.75	-	2.45
4a	-	-	5.50	2.60
4b	-	-	6.41	2.80
5a	3.98	-	5.20	2.00
5b	4.79	-	6.15	1.90
6a	3.82	4.69	7.23	1.65
6b	5.21	4.36	7.08	1.70

In experimental set 1, only propionate was added to observe if propionate would be used by anammox in absence of an electron acceptor. Figure 5.18a to 5.18d illustrates the results obtained from experimental set 1. Figure 5.15a shows propionate profile and it can be clearly seen that propionate concentration remained constant during 24 hours of the experiment. In Figure 5.18b ammonia profile is presented. Increase in ammonia concentration was observed especially in the last 6 hours of the run. Meanwhile, nitrite and nitrate concentrations remained constant around 0 mmol/l during the whole course of the experiment as illustrated in Figures 5.18c and 5.18d respectively.

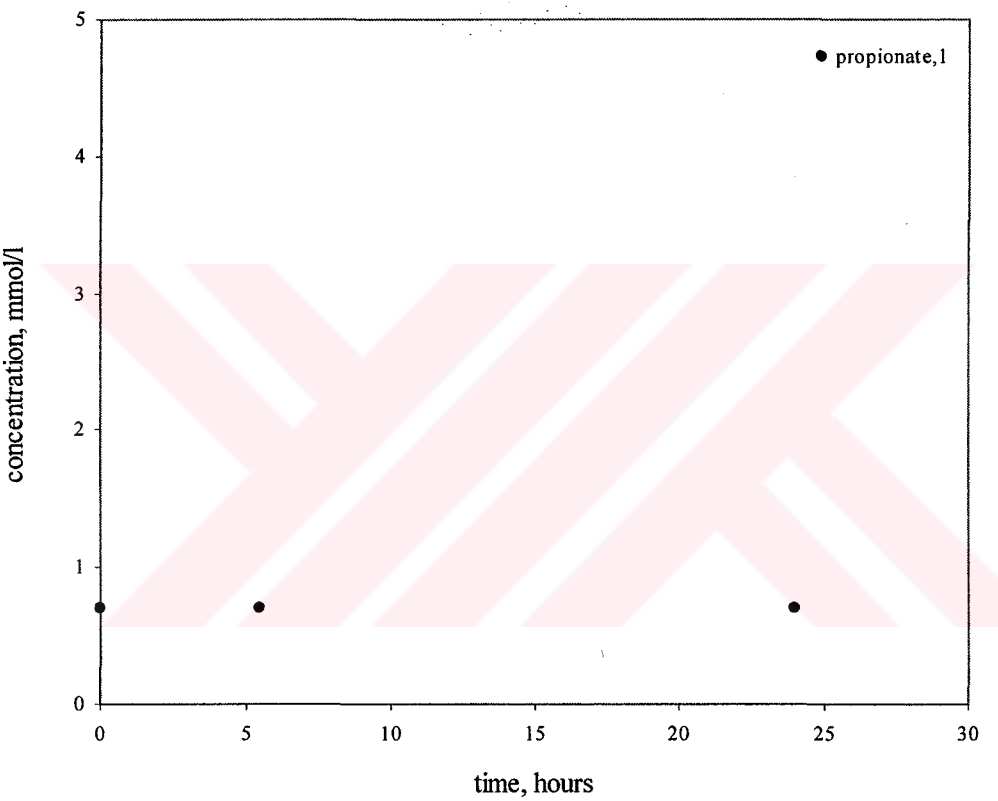


Figure 5.18a Propionate profile of the experimental set 1

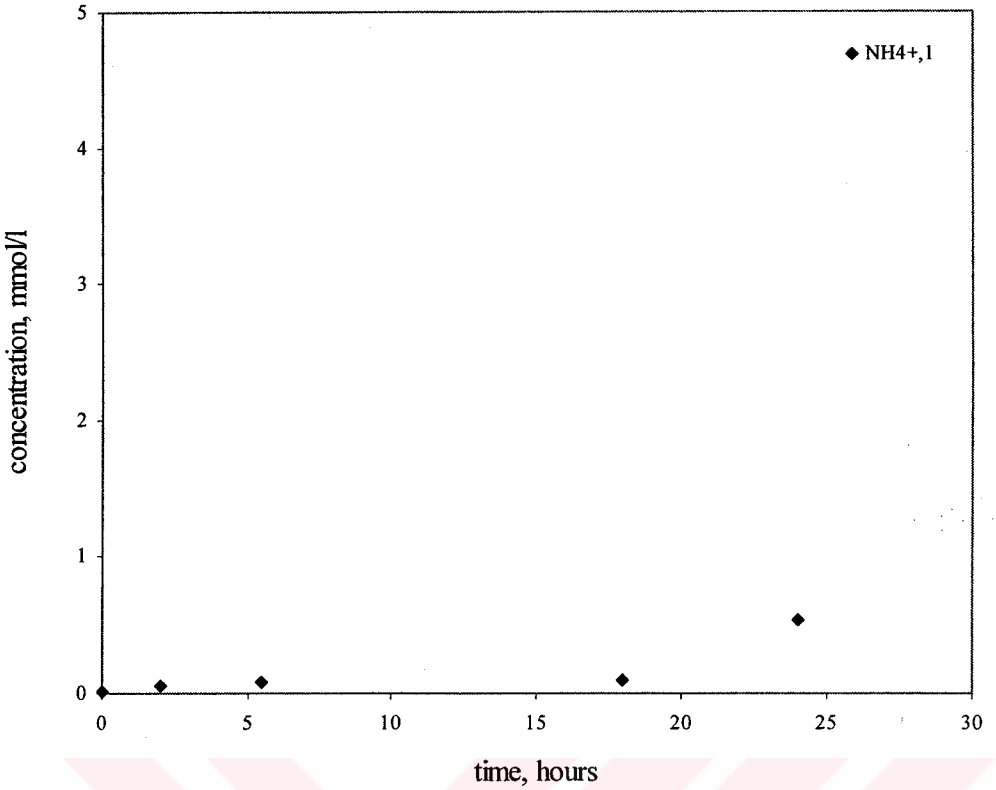


Figure 5.18b Ammonia profile of the experimental set 1.

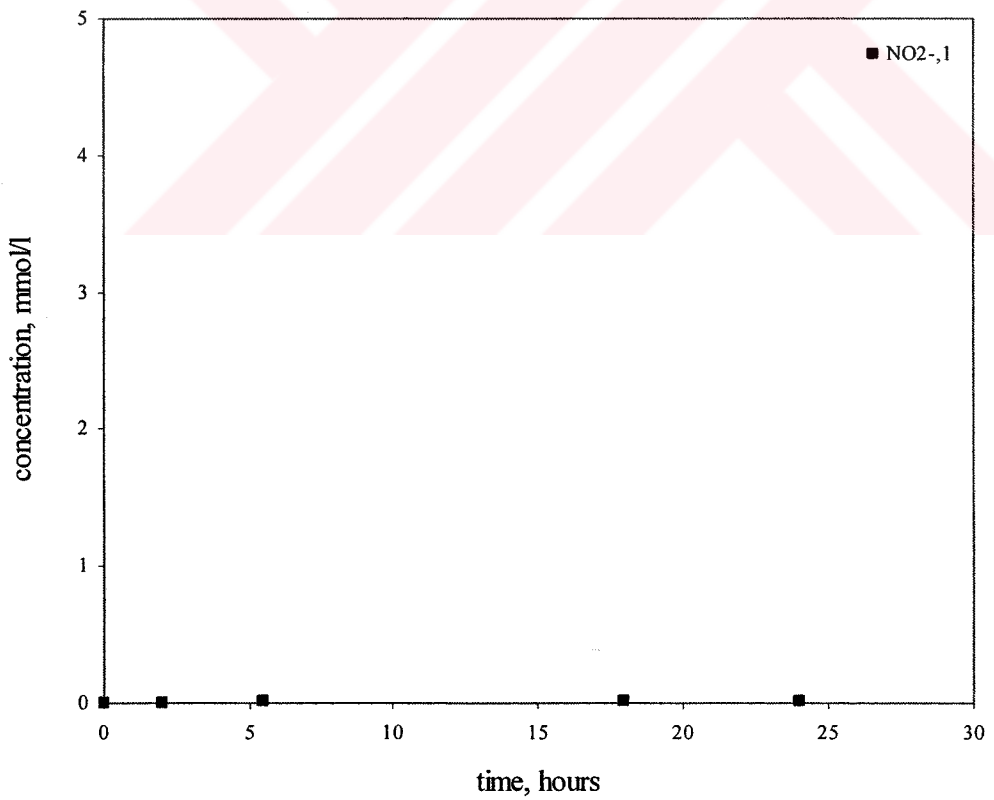


Figure 5.18c Nitrite profile of the experimental set 1.

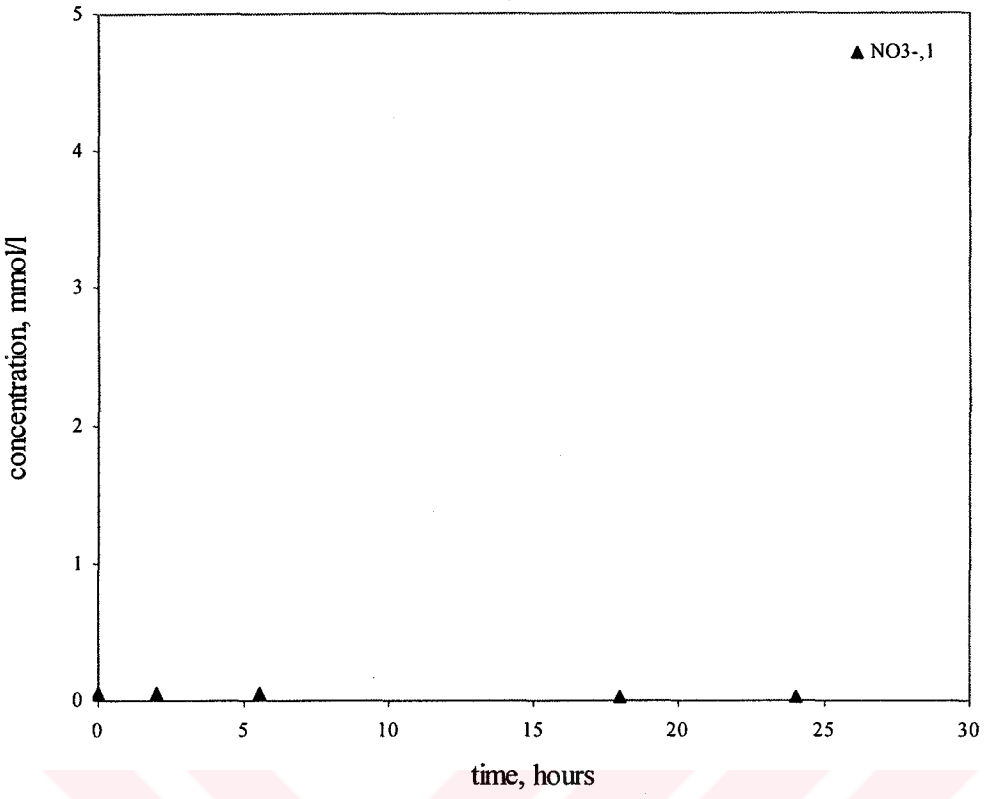


Figure 5.18d Nitrate profile of the experimental set 1.

Experimental set 2 was performed for the assessment of the behavior of the anammox activity in presence of ammonia and propionate. Results are presented for duplicates 2a and 2b in Figures 5.19a to 5.19d. Propionate profile is illustrated in Figure 5.19a and it can be clearly seen that there were no change in propionate concentration in both sets during the 24 hour experiment. Figure 5.19b shows ammonia profiles for sets 2a and 2b. In both sets, ammonia concentrations remained stable during the first 18 hours of the experiment; further significant increase was noted in both sets in the last 6 hours of the run. Meanwhile nitrite and nitrate concentrations stayed stable at around 0 mmol/l as illustrated in Figures 5.19c and 5.19d.

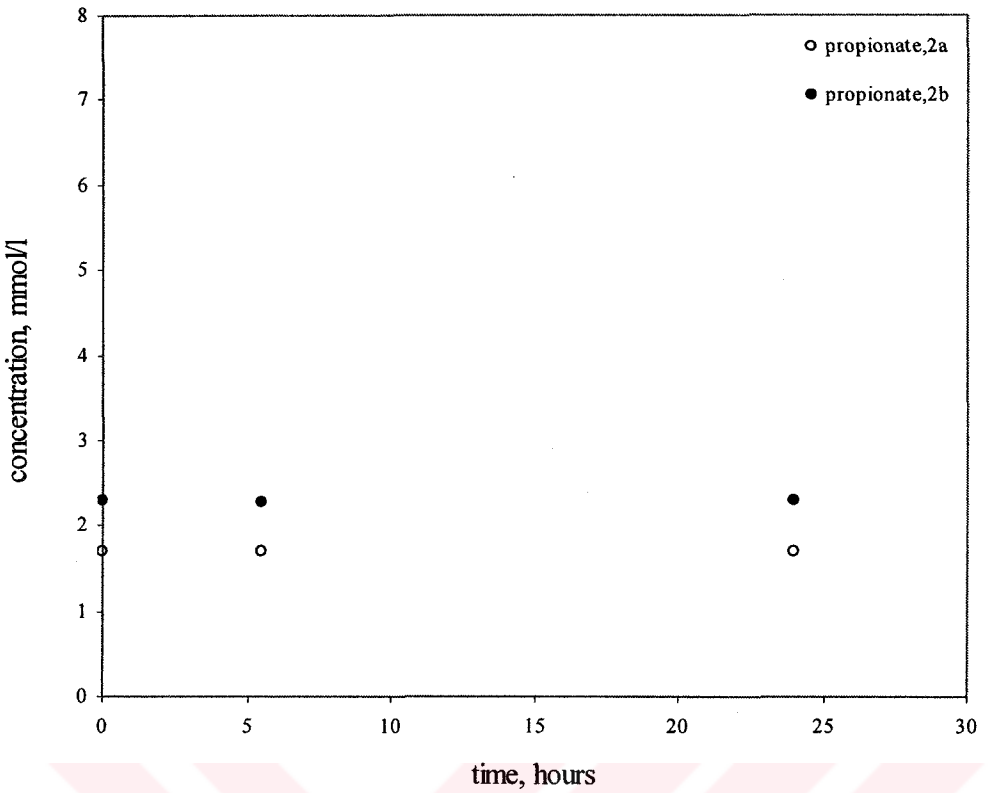


Figure 5.19a Propionate profile of the experimental set 2.

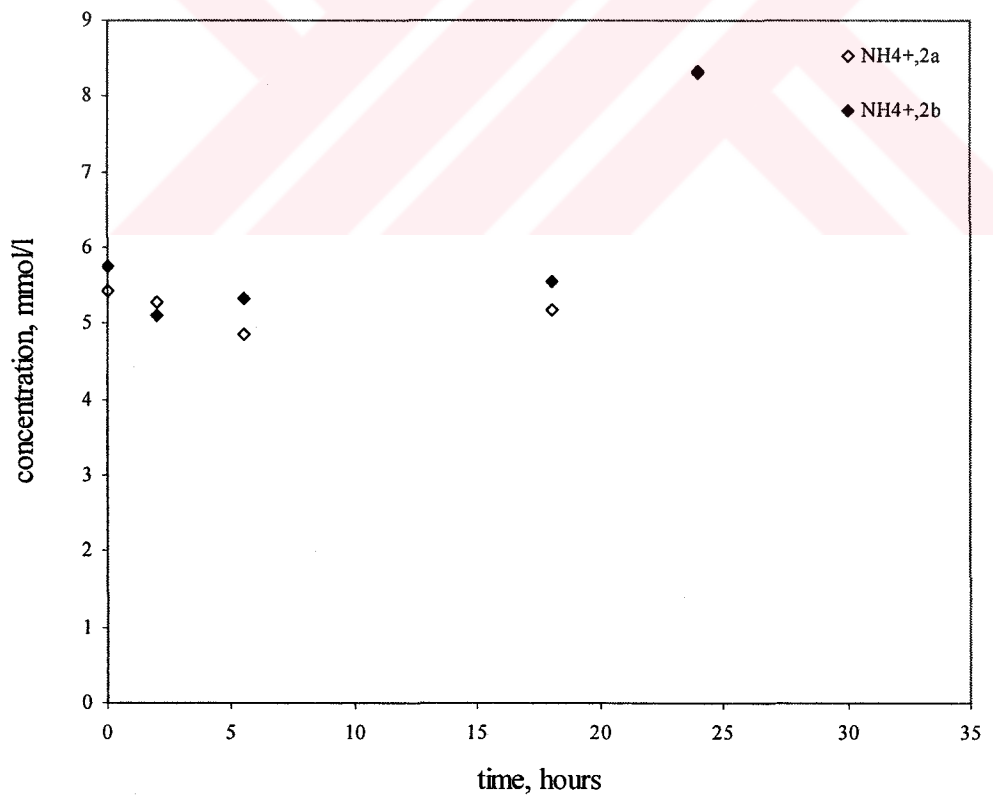


Figure 5.19b Ammonia profile of the experimental set 2.

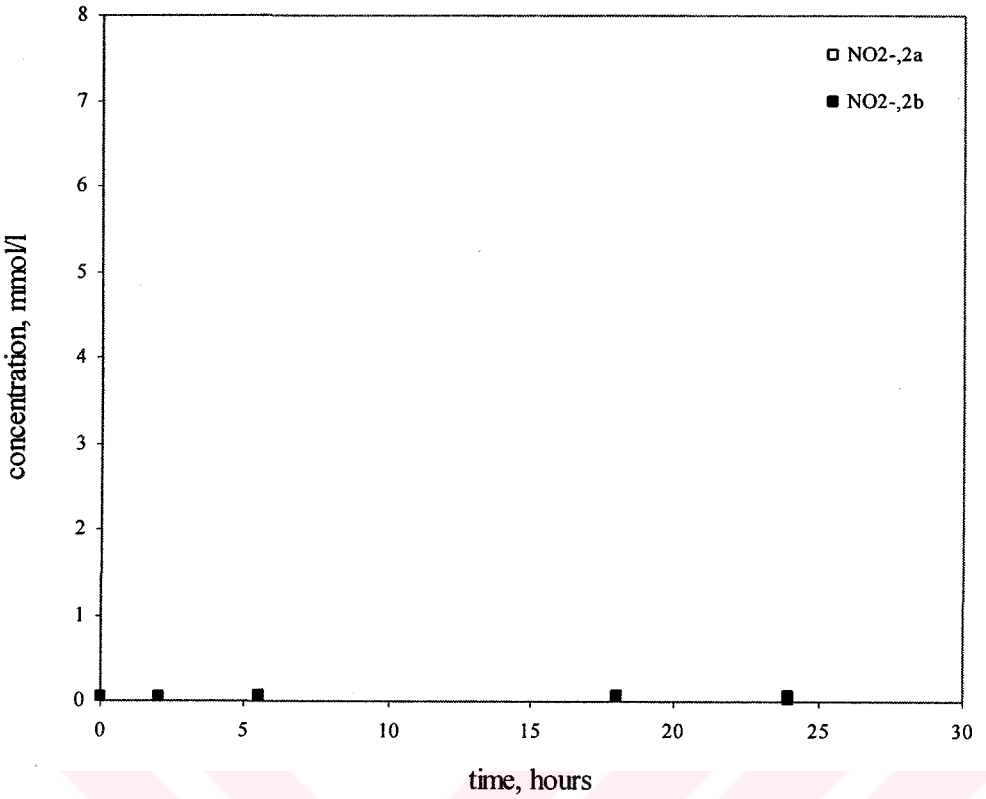


Figure 5.19c Nitrite profile of the experimental set 2.

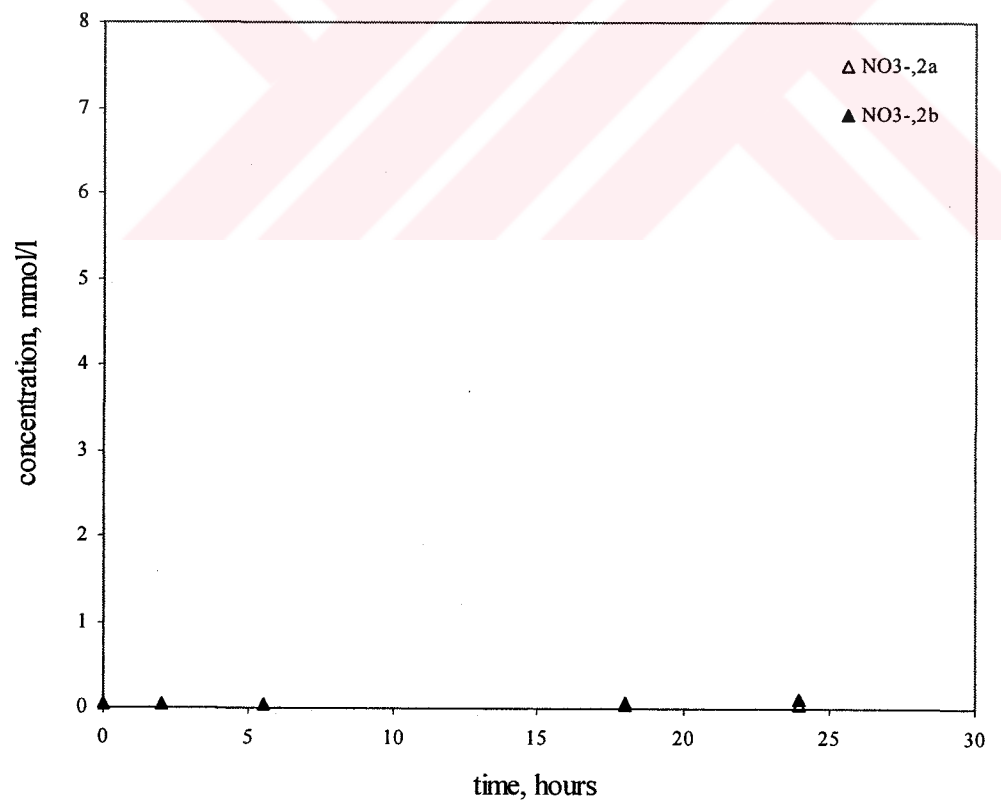


Figure 5.19d Nitrate profile of the experimental set 2.

Experimental set 3 was run in presence of nitrite and propionate. It was expected to determine if nitrite would be coupled to propionate oxidation as electron acceptor. Figure 5.20a shows propionate profile for sets 3a and 3b. No change was noted in propionate concentration during the whole course of the experiment. In Figure 5.20b ammonia profiles are presented. Slight increase in ammonia concentration was perceived especially in the first 18 hours of the experiment. Ammonia concentration was further decreased to 0 mmol/l at the end of the experiment. Nitrite profile is illustrated in Figure 5.20c for sets 3a and 3b respectively. It can be clearly seen that nitrite concentration was decreased significantly during the whole course of the experiment. In the first five hours of the run, 0.57 and 0.20 mmol/l nitrite conversion was detected in set 3a and 3b respectively. In the following 13 hours, nitrite conversions were recorded as 0.51 and 1.54 mmol/l in sets 3a and 3b, respectively. Nitrite conversions were persisted in the last 6 hours of the experiment as 1.09 and 1.41 mmol/l in sets 3a and 3b, respectively. Nitrate concentrations were stable around 0 mmol/l during the whole course of the experiment as illustrated in Figure 5.20d.

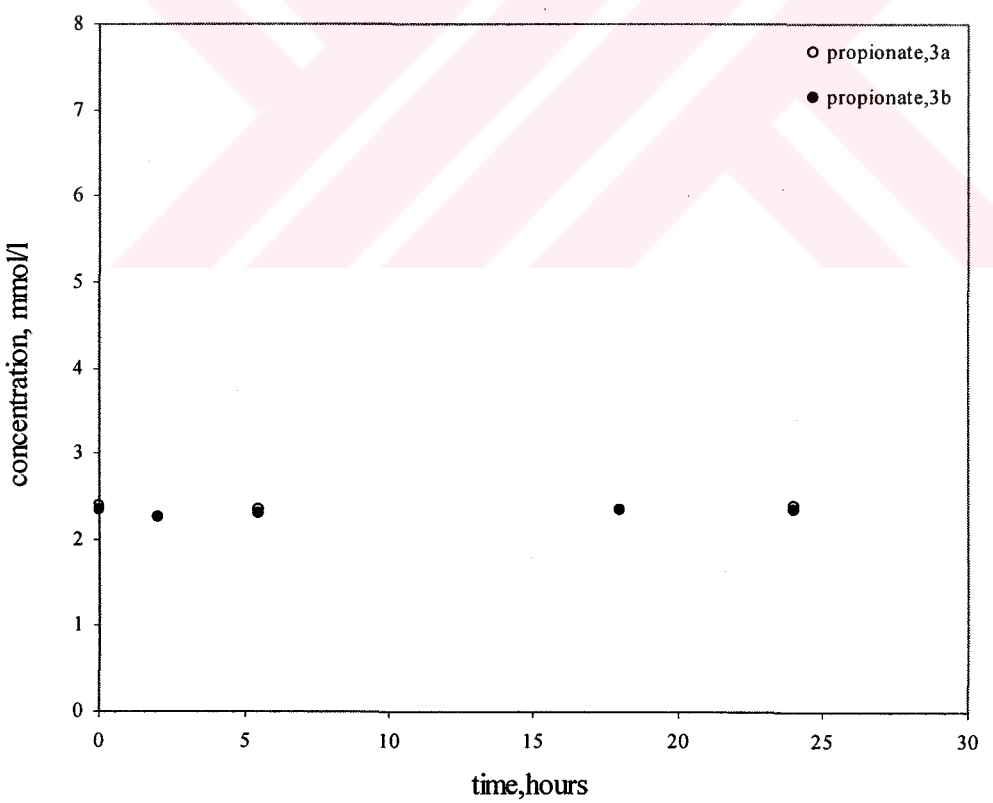


Figure 5.20a Propionate profiles of the experimental sets 3a and 3b.

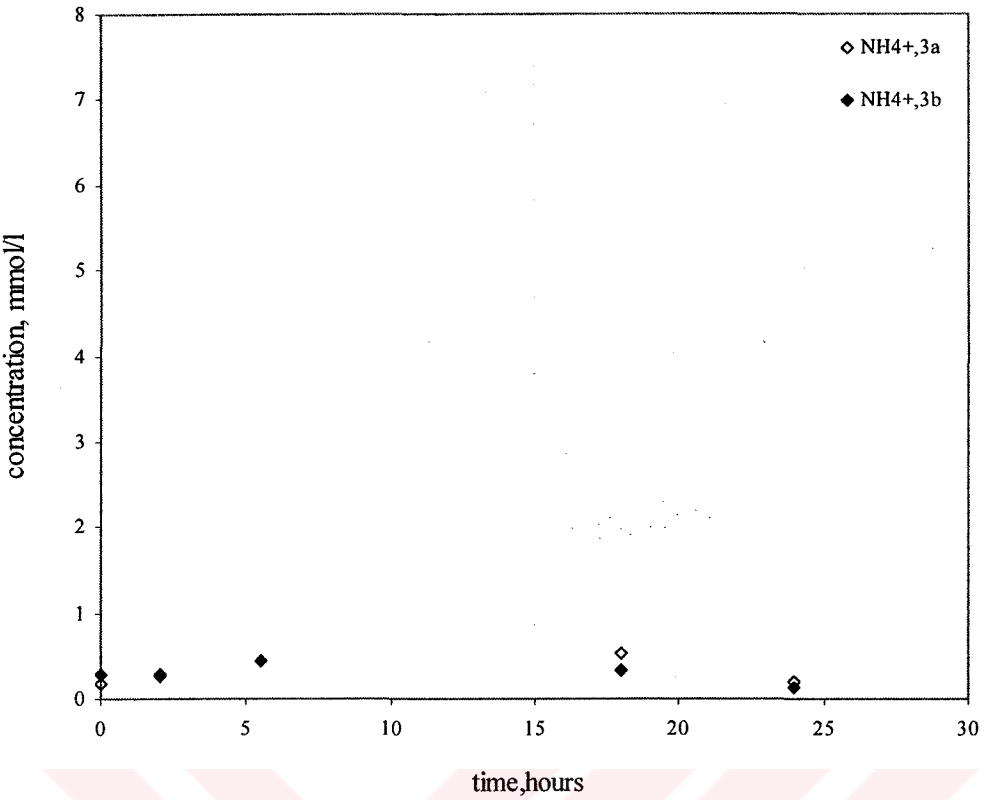


Figure 5.20b Ammonia profiles of the experimental sets 3a and 3b.

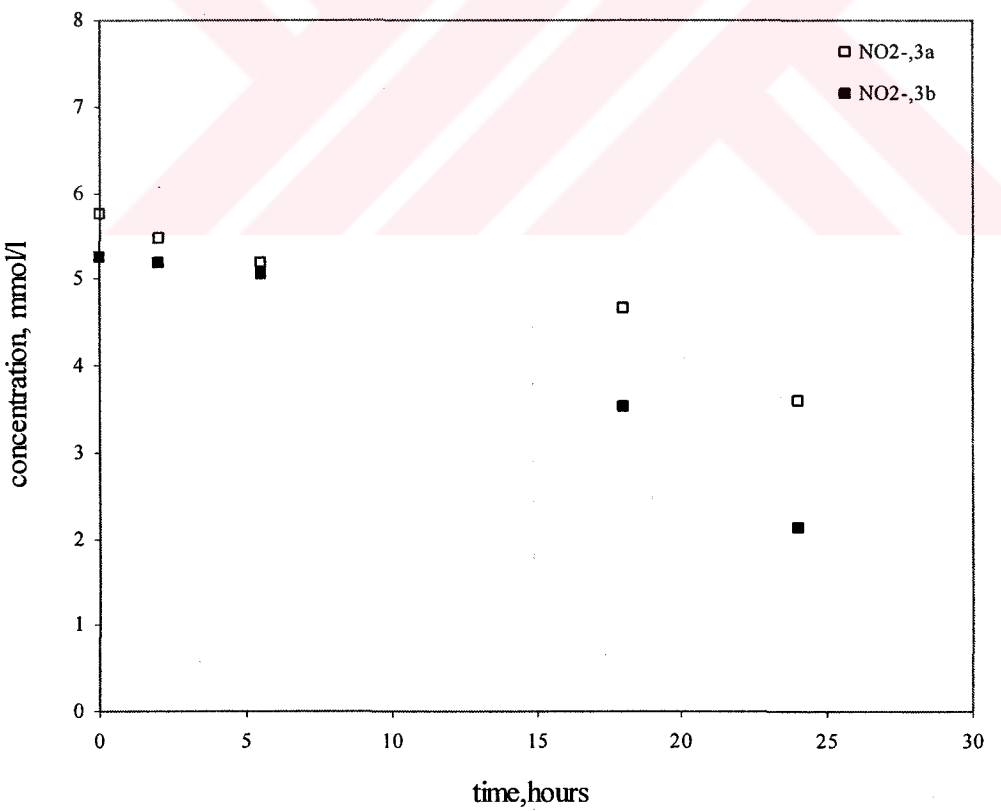


Figure 5.20c Nitrite profiles of the experimental sets 3a and 3b.

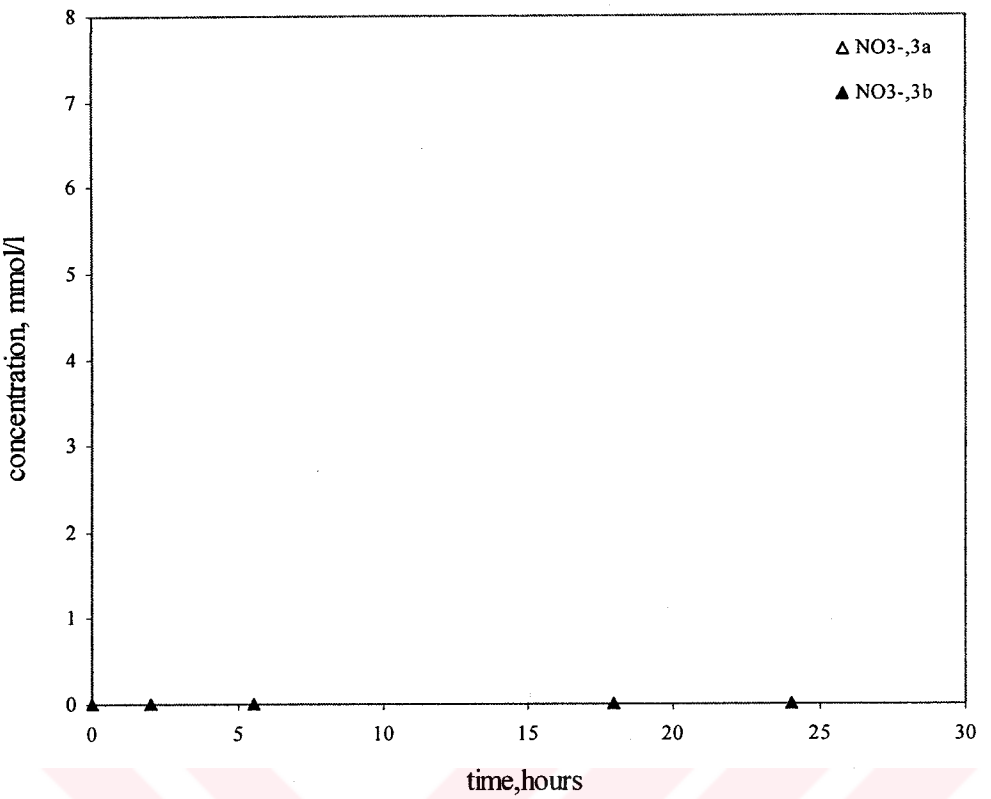


Figure 5.20d Nitrate profiles of the experimental sets 3a and 3b.

Experimental set 4 was performed in the presence of nitrate and propionate. Propionate profiles of the run are shown in Figure 5.21a related with sets 4a and 4b. In the first 5.5 hours of the experiment, slight decrease in propionate concentration was recorded in both sets. During this period, propionate concentration decreased from 2.60 to 2.45 and from 2.80 to 2.60 in sets 4a and 4b respectively. However, in the remaining 19 hours of the experiment, more evident decrease was observed and propionate was completely consumed. Ammonium was ever detected throughout the experiment as shown in Figure 5.21b. Figure 5.21c shows nitrite profiles related with set 4a and 4b. Although no nitrite was added at the beginning of the run, a significant increase was observed in between 5.5-18 hours in set 4b. Nitrite concentration increased from 0.08 to 1.63 mmol/l and further decreased to 0 mmol/l in period of the last 6 hours. Meanwhile in set 4a, minor increase from 0.05 to 0.11 mmol/l was obtained and converted in the remaining 6 hours. In Figure 5.21d, nitrate profiles are illustrated. In the first 5.5 hours of the experiment, there was a slight decrease in nitrate concentration in set 4b, whereas in set 4a there was no clear change. A meaningful decrease was perceived in the remaining 18.5 hours of the run and nitrate converted completely in both sets.

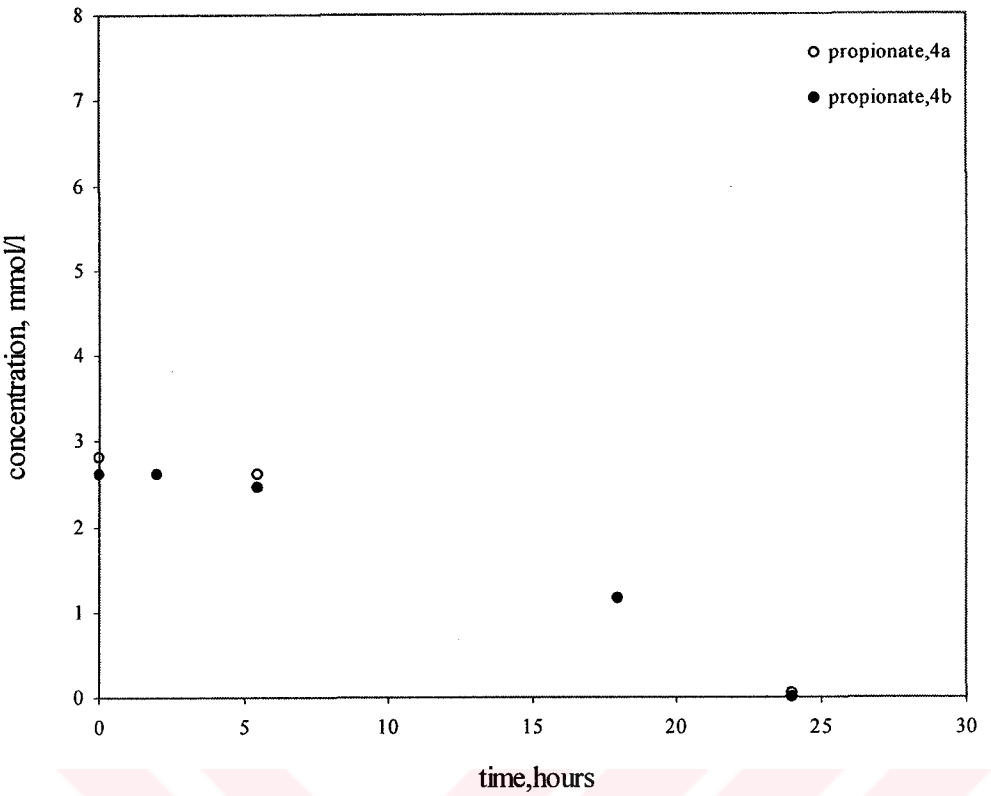


Figure 5.21a Propionate profiles of the experimental sets 4a and 4b.

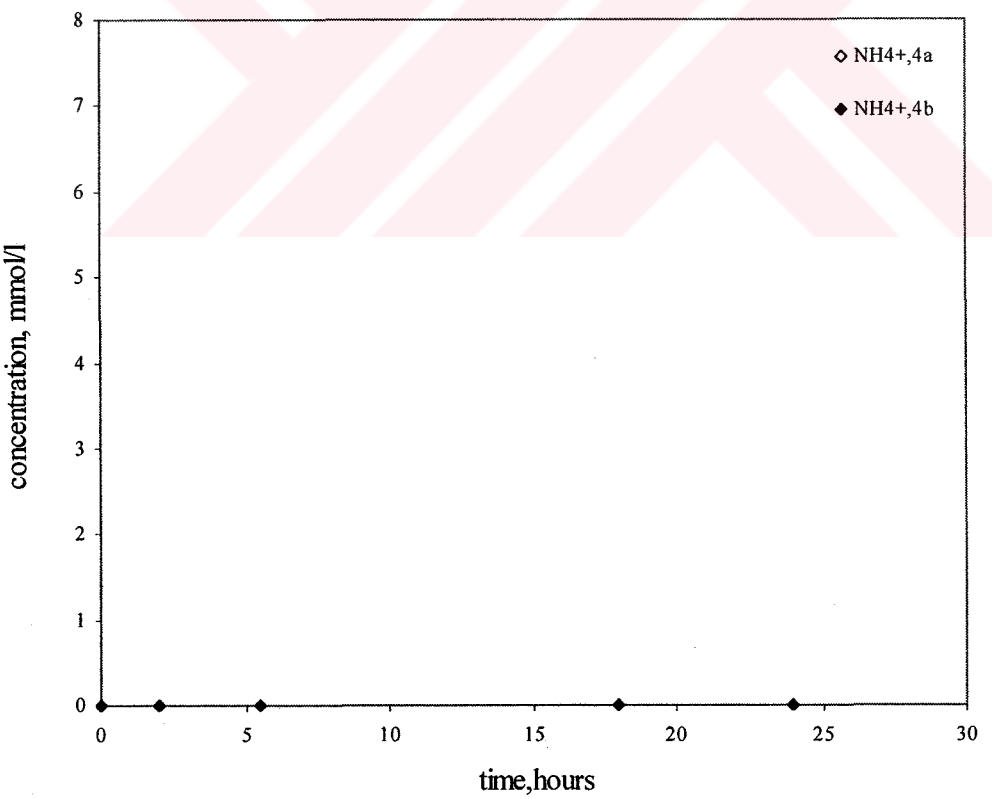


Figure 5.21b Ammonia profiles of the experimental sets 4a and 4b.

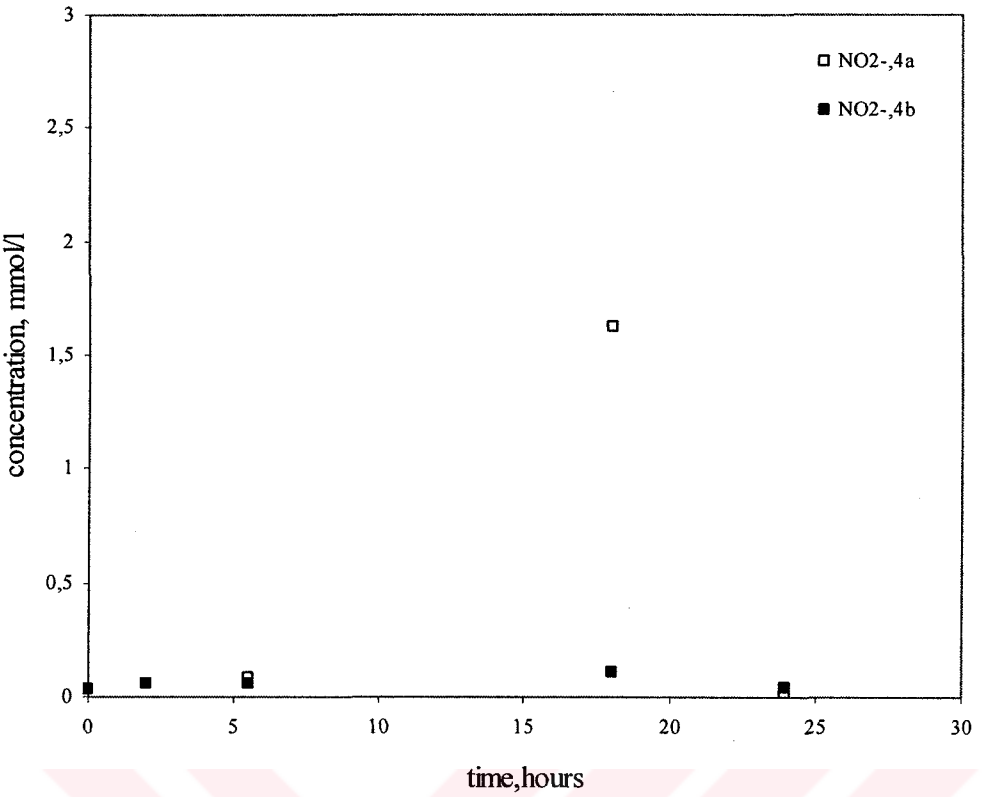


Figure 5.21c Nitrite profiles of the experimental sets 4a and 4b.

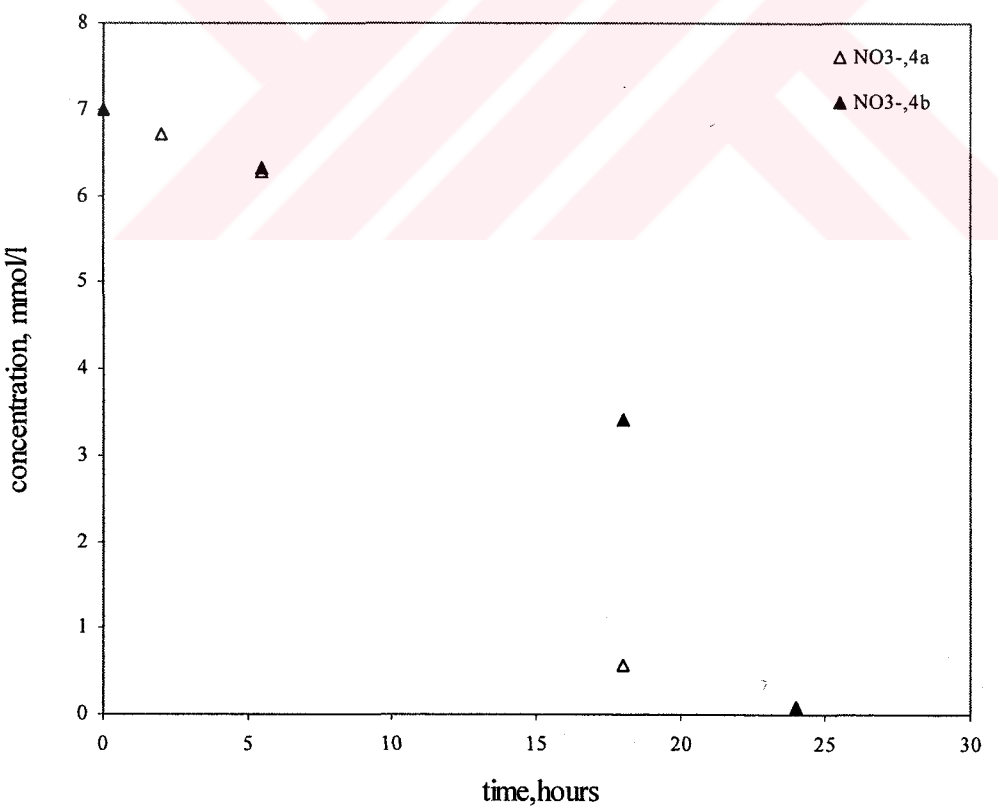


Figure 5.21d Nitrate profiles of the experimental sets 4a and 4b.

However, nitrate conversion trend was dissimilar in set 4a and 4b. In set 4b, nitrate was converted completely in 13 hours, where in set 4a observed conversion was 2.19 mmol/l.

Experimental set 5 was run in presence of ammonia, nitrate and propionate. In Figure 5.22a propionate profile is presented. In the first 5 hours of the experiment, propionate concentration decreased from 2.00 to 1.80 mmol/l and 1.90 to 1.75 mmol/l in set 5a and 5b respectively. Propionate consumption continued in the period between 5.5-18.5 hours and decreased from 1.80 to 1.30 mmol/l in set 5a and from 1.75 to 1.25 mmol/l in set 5b. During the last 6 hours of the experiment, much more propionate consumption was recorded and concentration decreased from 1.30 to 0.60 mmol/l and 1.25 to 0.40 mmol/l in set 5a and 5b respectively. Figure 5.22b illustrates ammonia profiles related with sets 5a and 5b. As can be clearly seen from the figure that ammonia concentration remained constant in the first 5 hours of the experiment in set 5a, where in set 5b, decreased from 4.79 to 4.39 mmol/l. Although nitrite is absent in the medium, ammonia conversions were observed between 5.5-18.5 hours of the experiment in both sets.

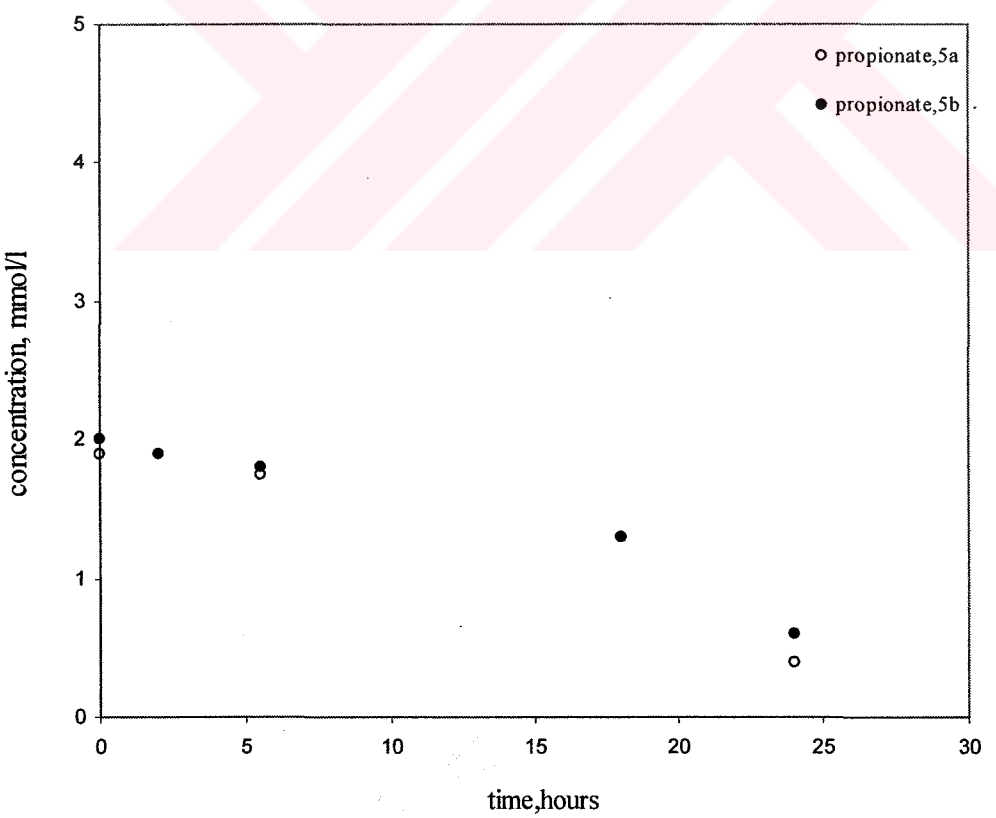


Figure 5.22a Propionate profiles of the experimental sets 5a and 5b.

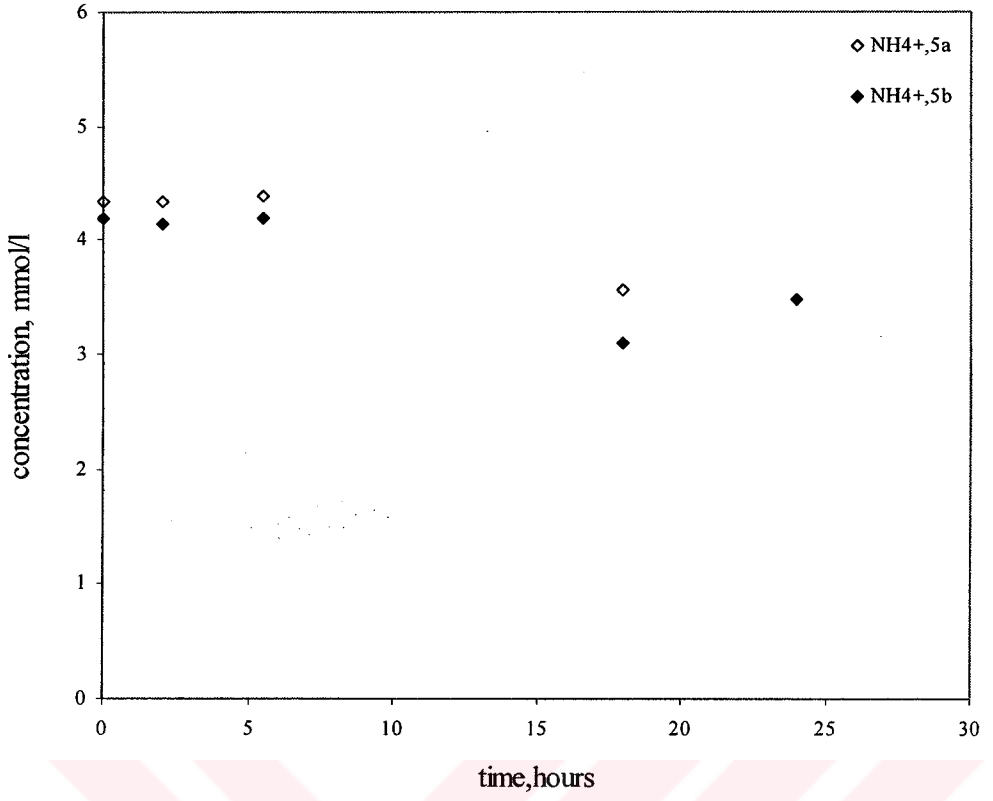


Figure 5.22b Ammonia profiles of the experimental sets 5a and 5b.

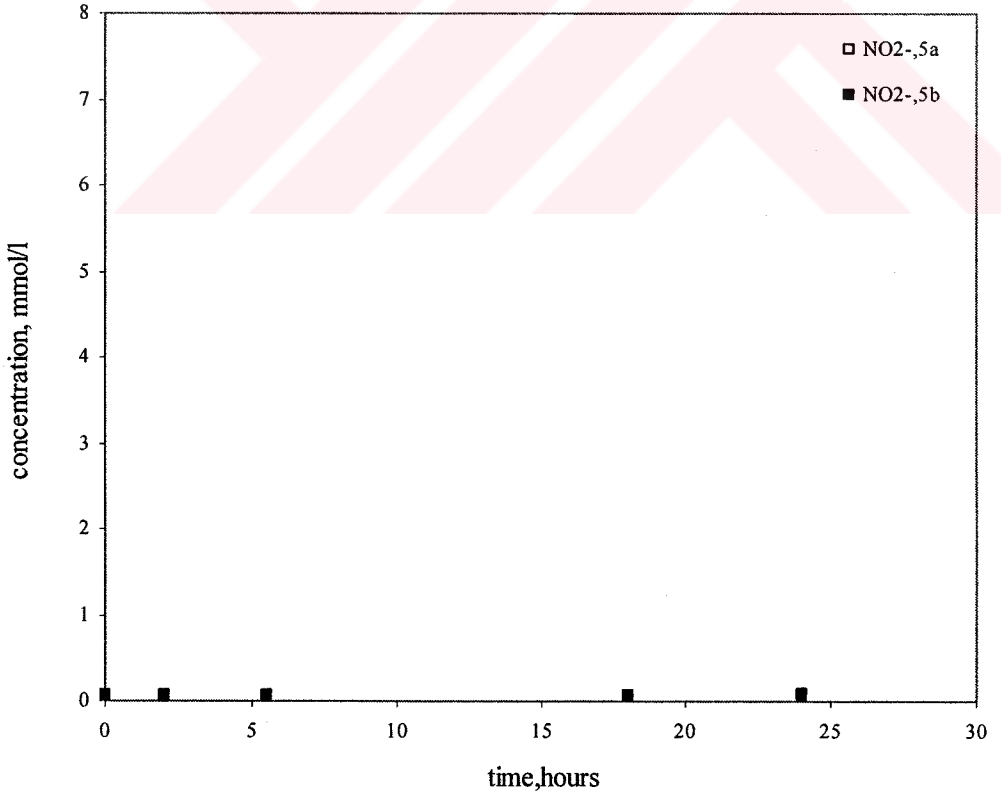


Figure 5.22c Nitrite profiles of the experimental sets 5a and 5b.

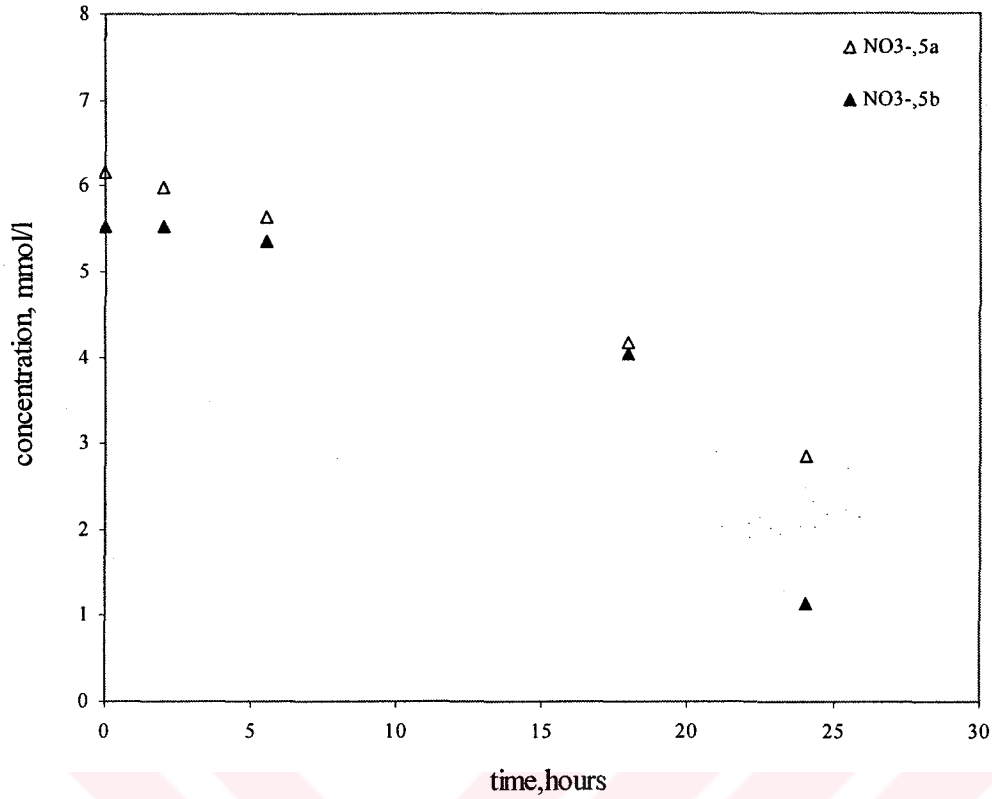


Figure 5.22d Nitrate profiles of the experimental sets 5a and 5b.

During this period, conversion of ammonium was measured as 1.08 and 0.83 mmol/l in set 5a and 5b, respectively. Increase in ammonia concentrations from 3.10 to 3.48 mmol/l in set 5a and from 3.56 to 4.66 mmol/l in set 5b was noted at the end of the run, possibly due to cell lysis. Nitrate profiles are illustrated in Figure 5.22c. Similar nitrate conversion rates were observed for set 5a and 5b in the first 5.5 hours. Nitrate concentration decreased from 6.00 to 5.35 mmol/l and from 6.15 to 5.63 mmol/l in sets 5a and 5b respectively. Nitrate conversion was continued at the same rate in the following 13 hours and decreased from 5.35 to 4.03 mmol/l in set 5a and from 5.63 to 4.17 mmol/l in set 5b. During the last 5.5 hours of the run nitrate concentration was decreased from 4.03 to 1.15 mmol7l and 4.17 to 2.85 mmol/l in set 5a and 5b, respectively. Nitrite concentration remained around 0 mmol/l for both sets during the whole course of the experiment, as illustrated in Figure 5.22d.

Experimental set 6 was run in presence of ammonia, nitrite, nitrate and propionate. Small amount of propionate was consumed in the first 5.5 hours of the run in both sets 6a and 6b as shown in Figure 5.23a. Further, more evident propionate consumption was noticed in the remaining 18.5 hours of the experiment.

Figure 5.23b shows ammonia profiles for both sets. Since both ammonia and nitrite were present in the medium, anaerobic ammonium oxidation activity was observed in the first 5.5 hours. Ammonia concentration decreased from 3.82 to 2.16 mmol/l in set 6a and from 5.21 to 2.92 mmol/l in set 6b. Although nitrite was converted completely in the first 5.5 hours, ammonia concentration was continued to decrease for further 13 hours of the run, in both sets. Interestingly, ammonia conversion is continued till the end of the run in set 6a, where in set 6b increase in ammonia concentration was detected possibly due to cell lysis.

Figure 5.23c shows nitrite profiles related with sets 6a and 6b. As can be clearly seen that nitrite was fully converted in the first 5.5 hours of the experiment by the anammox activity. No change in nitrite concentration was noticed in the remaining period of the experiment.

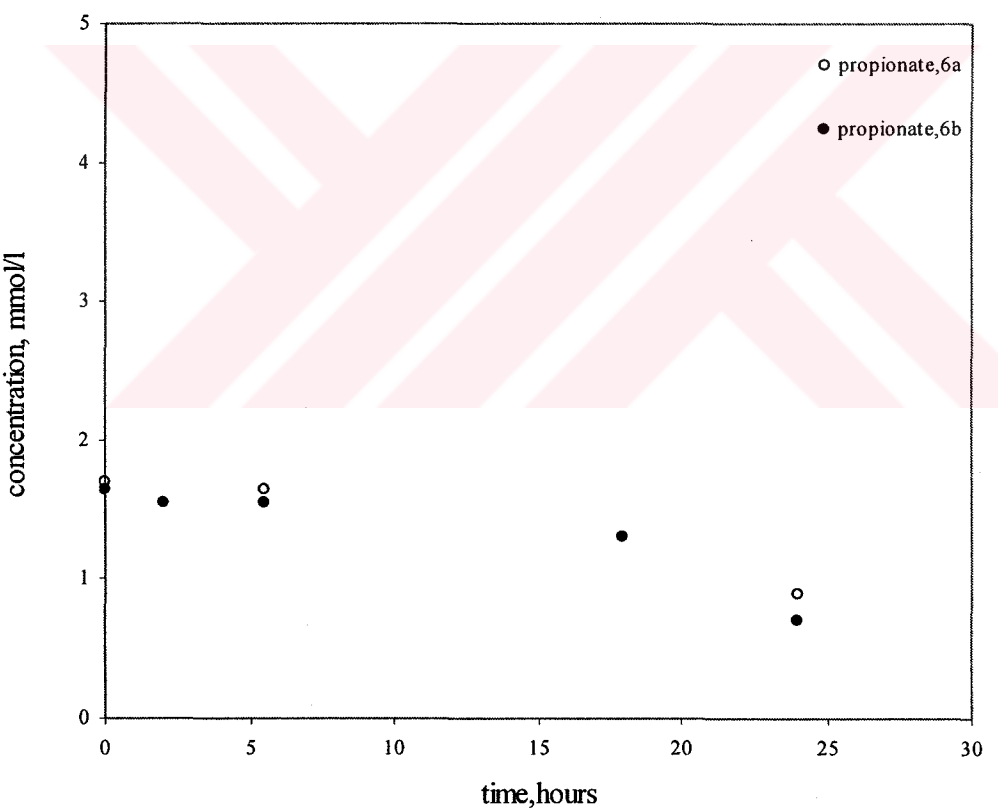


Figure 5.23a Propionate profiles of the experimental sets 6a and 6b.

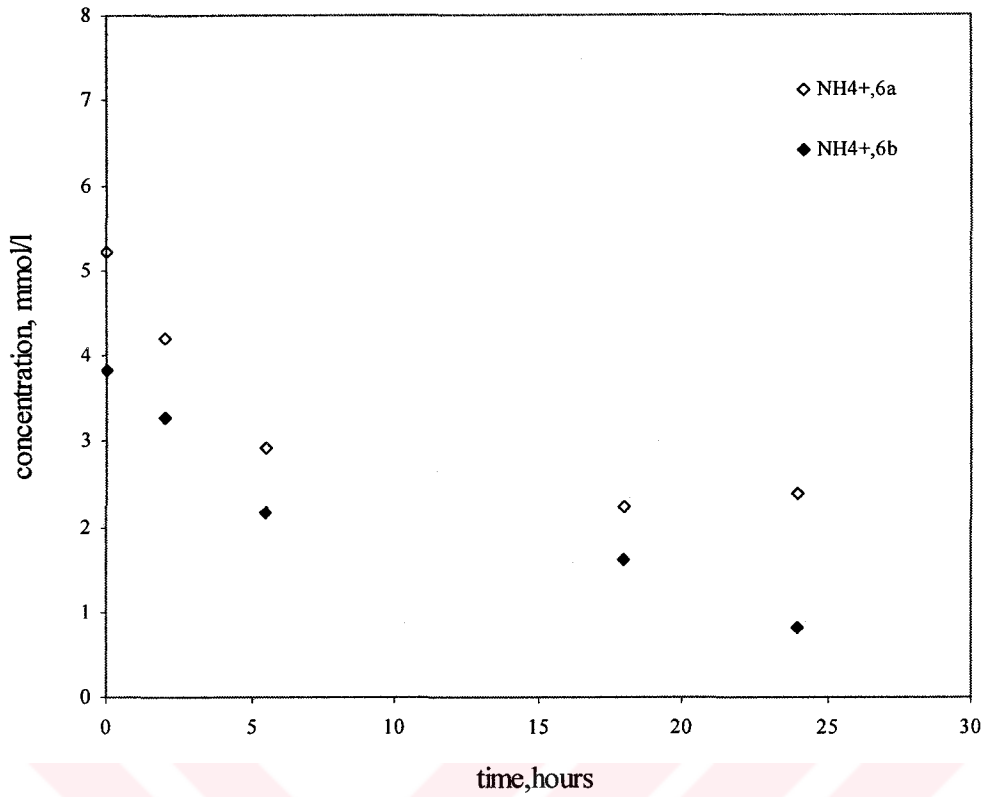


Figure 5.23b Ammonia profiles of the experimental sets 6a and 6b.

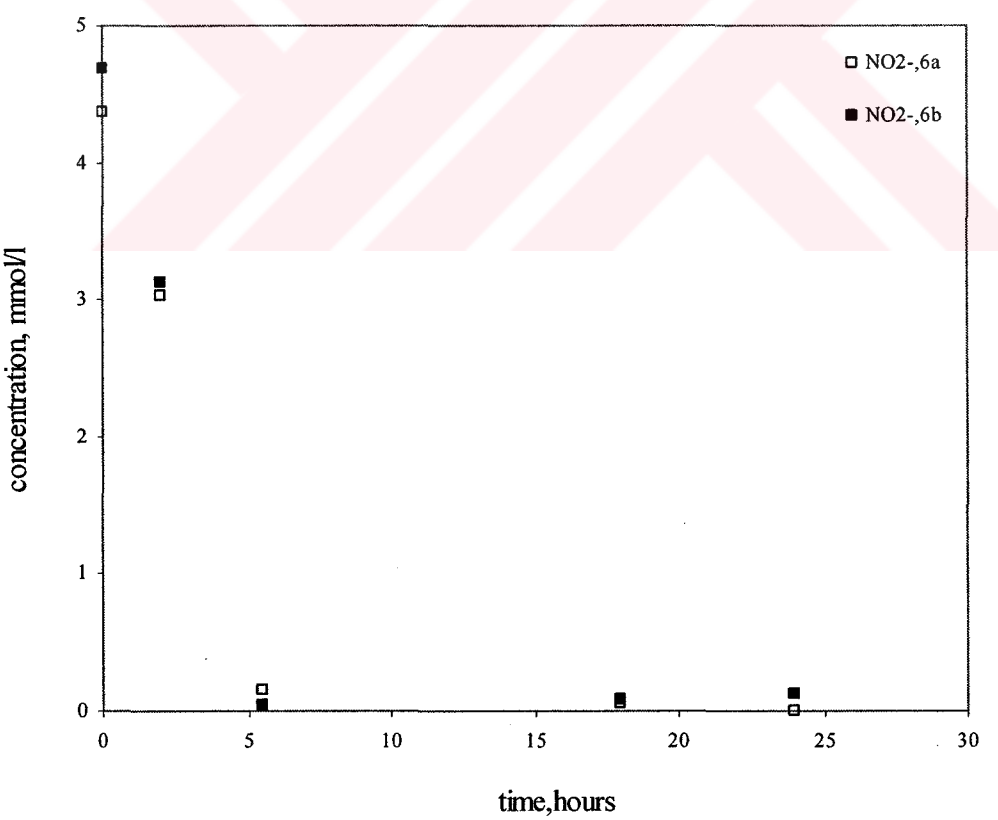


Figure 5.23c Nitrite profiles of the experimental sets 6a and 6b.

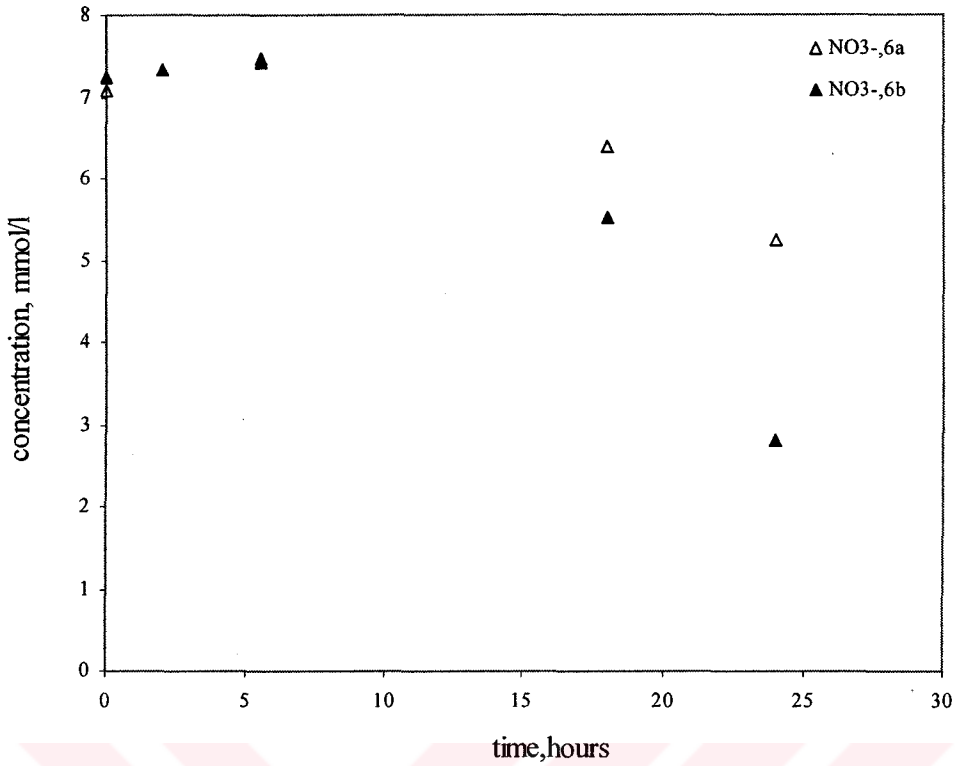


Figure 5.23d Nitrate profiles of the experimental sets 6a and 6b.

Nitrate profiles are presented in Figure 5.23d. A slight increase in nitrate concentration was measured due to anammox activity in the first 5.5 hours of the run. After nitrite was converted completely, an imperative decrease in nitrate concentration was detected. Nitrate concentration was decreased from 7.43 to 5.52 mmol/l in experimental set 6a and from 7.46 to 6.39 mmol/l in set 6b between 5.5 - 18.5 hours. Nitrate conversion was continued in the last 5.5 hours of the run and decreased from 5.52 to 2.80 mmol/l and from 6.39 to 5.25 mmol/l in set 6a and 6b respectively.

5.5.2 $^{15}NO_3^-$ isotope labeling experiments

Batch experiments where $^{15}NO_3^-$ were performed so as to inspect the possible reaction mechanism of the propionate oxidation by anammox bacteria. Experiments were conducted as described in Chapter 4. Table 5.14 depicts the set-up of the experiments. ^{15}N- labeled compounds, except $^{15}NH_4^+$ ($^{15}NO_3^-$, $^{15}NO_2^-$, $^{14,15}N_2$ and $^{15,15}N_2$) were measured with a sensitive mass spectrometer together with gas chromatography. Samples were measured also by colorimetric method.

Table 5.14 Experimental set-up of the $^{15}NO_3^-$-labeling experiments.

Experimental sets	Components				
	$^{14}NH_4^+$ mmol/l	$^{14}NO_2^-$ mmol/l	$^{14}NO_3^-$ mmol/l	$^{15}NO_3^-$ mmol/l	propionate mmol/l
1a	-	-	-	1.9	0.74
1b	-	-	-	1.94	0.75
2a	-	1.63	-	1.83	0.80
2b	-	1.46	-	1.85	0.75
3a	2.14	2.81	-	0.96	0.80
3b	2.10	2.73	-	1.14	0.82

Experimental set 1 was performed in presence of $^{15}NO_3^-$ and propionate. Results are presented in Figure 5.24a to 5.24f. As can be seen from the Figure 5.24a, all $^{15}NO_3^-$ was converted in 20 hours in both sets. No $^{15}NO_2^-$ formation was detected in set 1a, while in set 1b 90 μmol $^{15}NO_2^-$/l was noticed as shown in Figure 5.24b. Produced $^{14,15}N_2$ and $^{15,15}N_2$ gases are presented in Figures 5.24c and 5.24d.

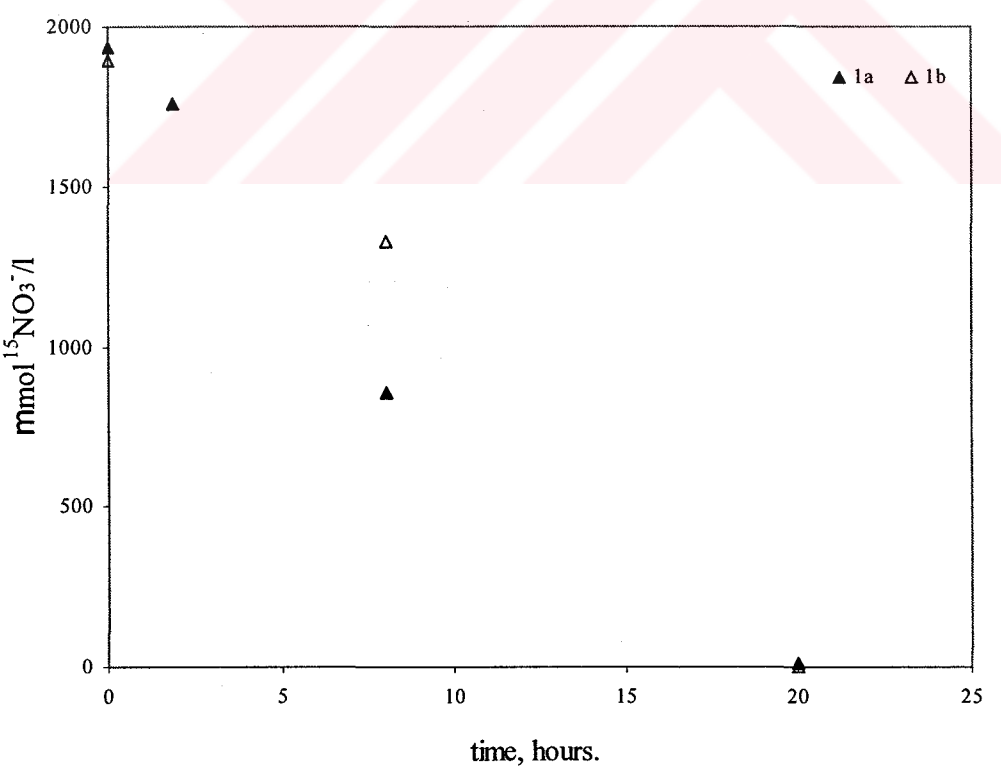


Figure 5.24a $^{15}NO_3^-$ profiles of the experimental sets 1a and 1b.

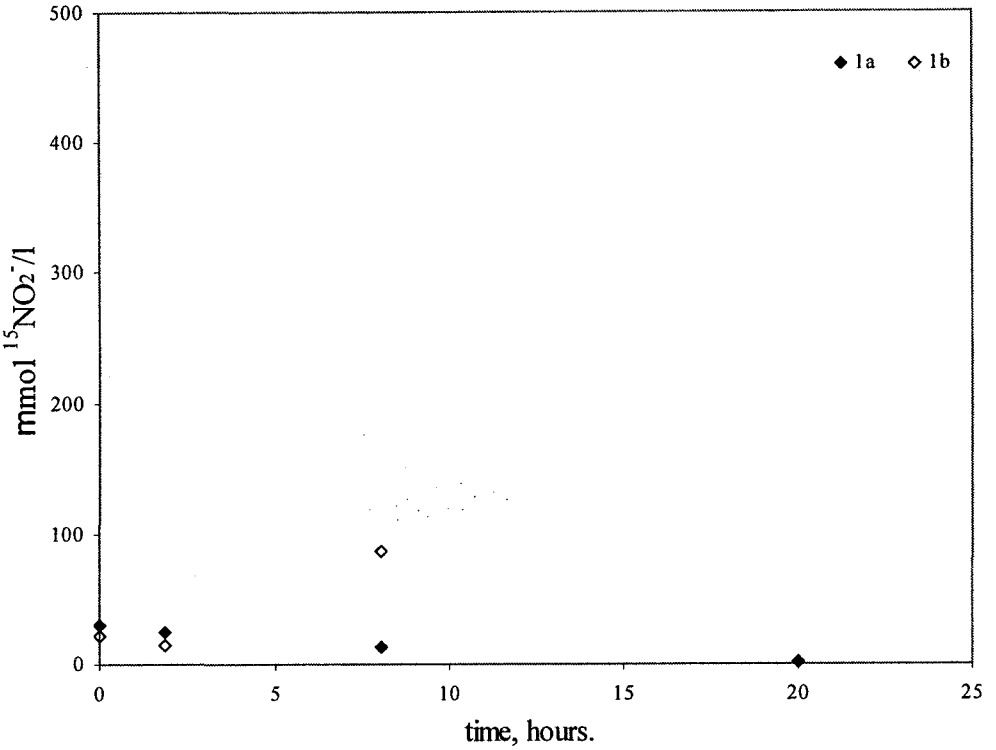


Figure 5.24b $^{15}NO_2^-$ profiles of the experimental sets 1a and 1b.

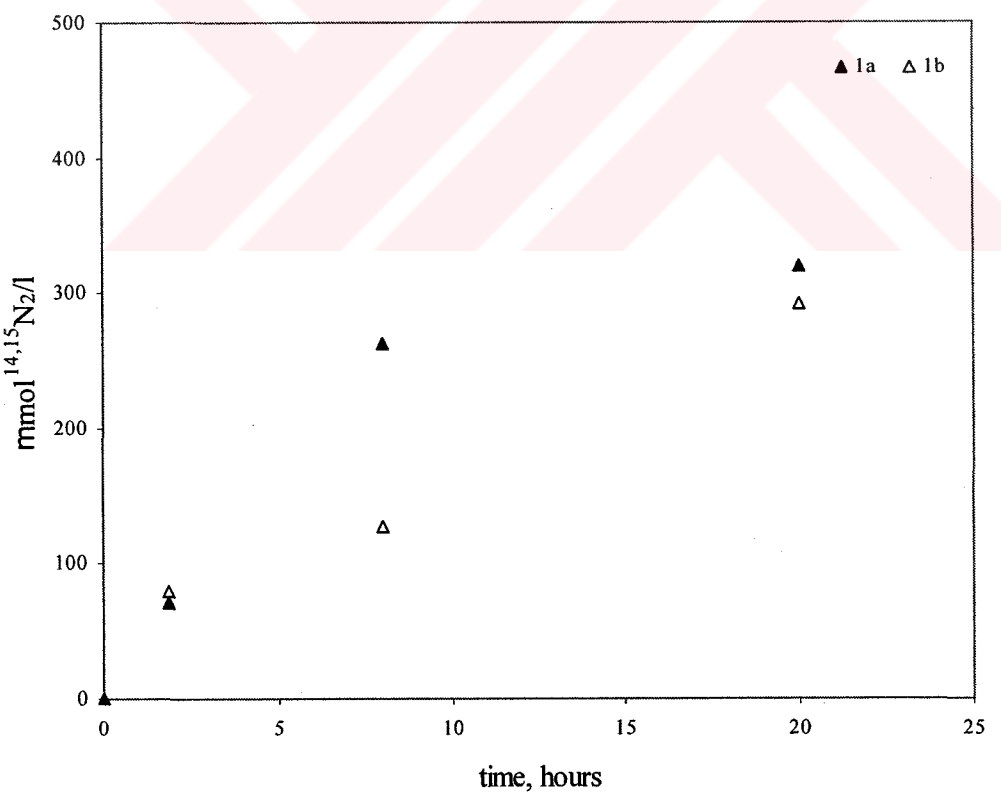


Figure 5.24c $^{14,15}N_2$ profiles of the experimental sets 1a and 1b.

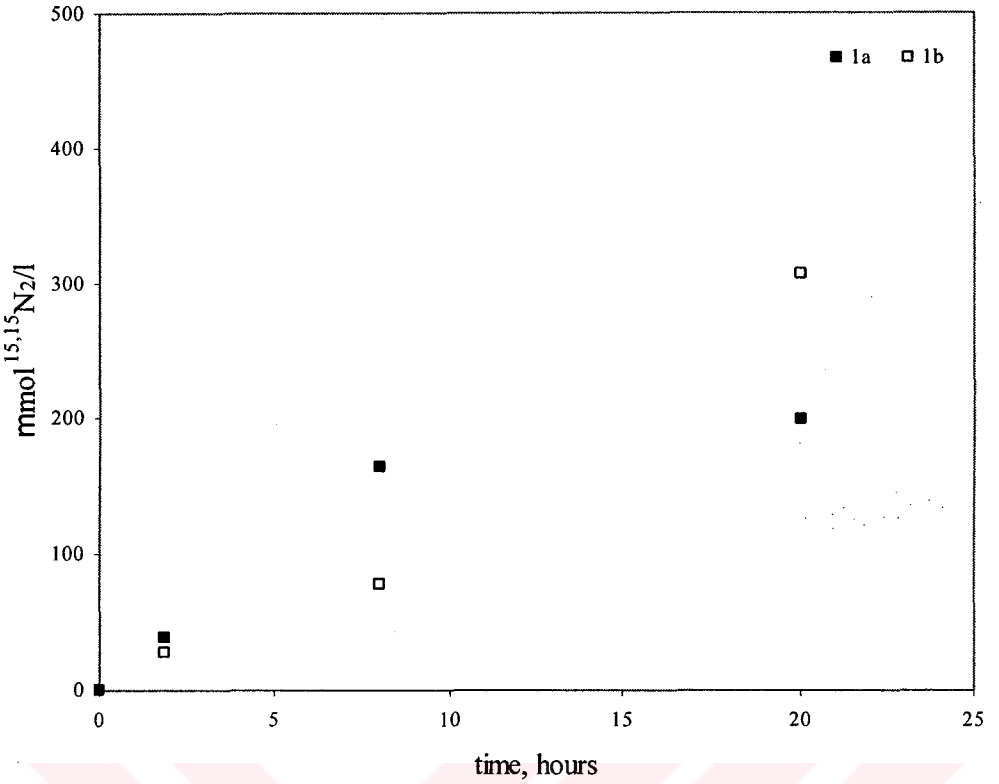


Figure 5.24d $^{15,15}N_2$ profiles of the experimental sets 1a and 1b.

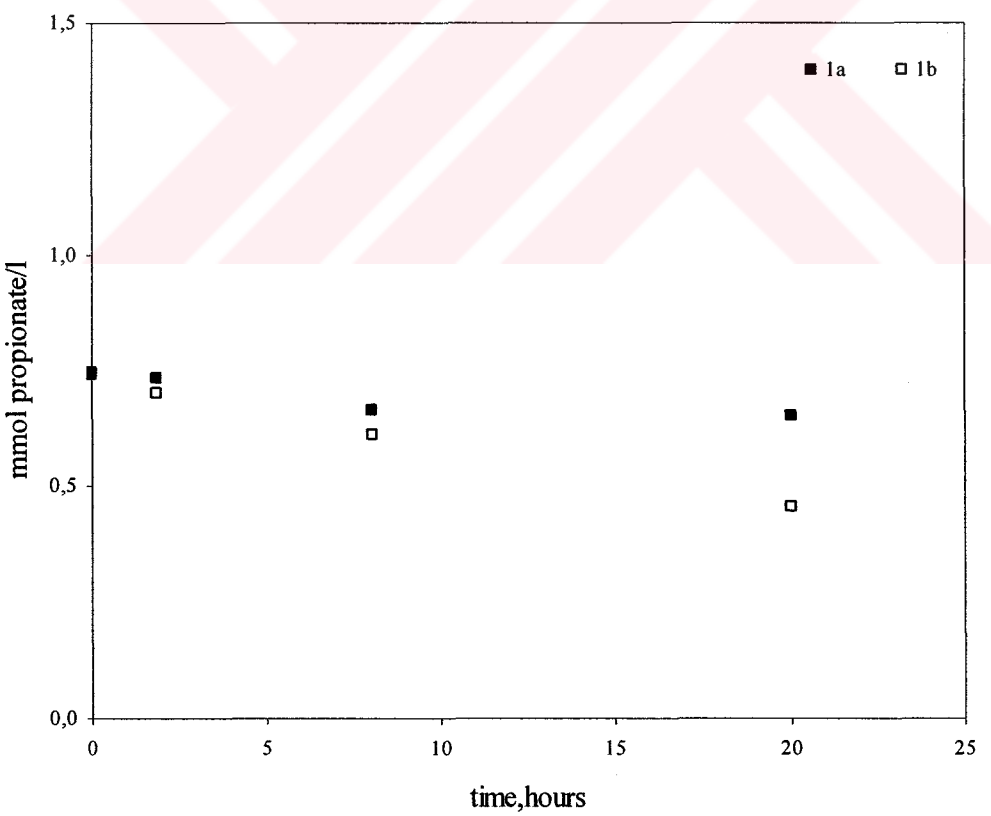


Figure 5.24e Propionate profiles of the experimental sets 1a and 1b.

Measured $^{14,15}N_2$ concentrations were identical in both sets as approximately 300 μmol $^{14,15}N_2$/l while produced $^{15,15}N_2$ was measured as 300 μmol/l and 200 μmol/l for set 1a and 1b, correspondingly. In that order, 110 μmol/l and 300 μmol/l propionate consumptions were perceived for the duration of the experiment. Propionate profiles are illustrated in Figure 5.24e. Samples were measured also by colorimetric method to detect the formation of the unlabeled N-compounds. In this experimental set, unlabeled ammonium formation was identified possibly due to cell lysis. As shown in Figure 5.24f, considerable amount of ammonia formation was noticed in both duplicates at the end of the experiment. Nitrite accumulation was measured as 200 μmol $^{14}NO_2^-$/l for set 1b which is not compatible with $^{15}NO_2^-$ measurement. While in set 1a, nitrite formation was ever detected. Nitrite profiles of the replicates are presented in Figure 5.25g.

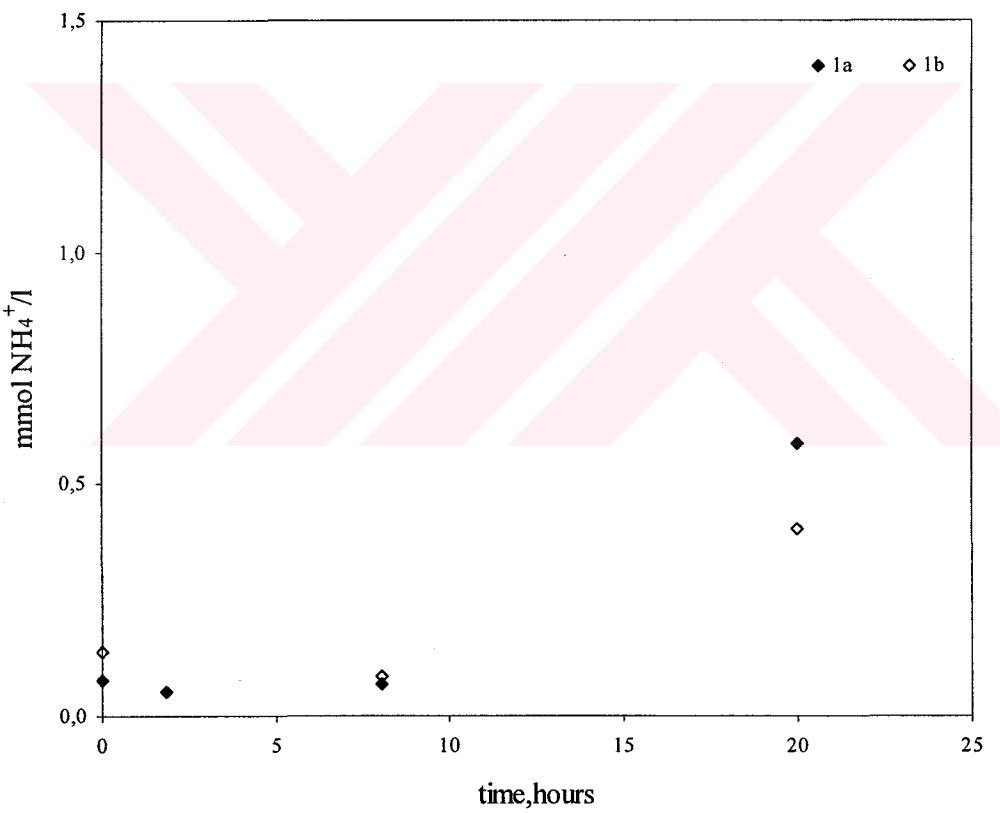


Figure 5.24f Ammonia profiles of the experimental sets 1a and 1b.

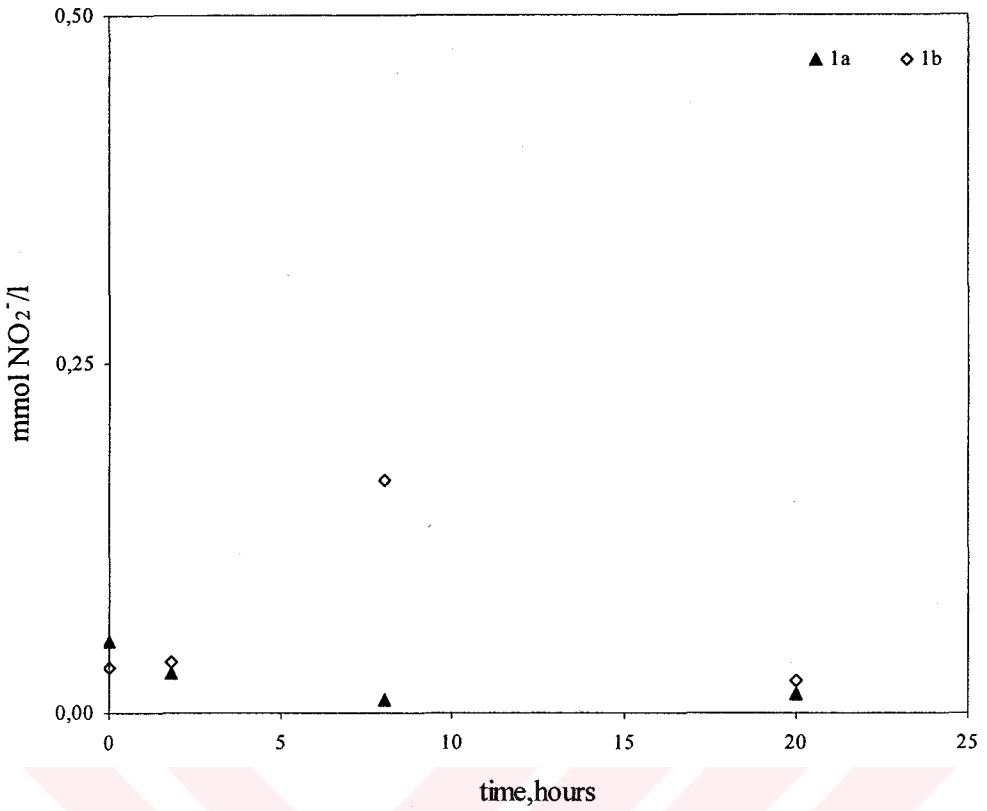


Figure 5.24g $^{14}NO_2^-$ profiles of the experimental sets 1a and 1b.

Experimental set 2 was conducted in presence of $^{15}NO_3^-$, $^{14}NO_2^-$ and propionate. Complete reduction of $^{15}NO_3^-$ was obtained in 20 hours in both repetitions repetitions, as depicted in Figure 5.25a. In the same period, transitional $^{15}NO_2^-$ production was observed as 422 μmol/l and 342 μmol/l, correspondingly in set 2a and 2b and illustrated in Figure 5.25b. As can be clearly seen from the Figure 5.25c, measured $^{14,15}N_2$ concentrations were compatible for set 2a and 2b as 615 μmol/l and 545 μmol/l, respectively. Likewise, produced $^{15,15}N_2$ were identical for both replicas around 200 μmol/l as presented in Figure 5.25d.

Propionate consumption profiles for the experimental sets 2a and 2b are sketched in Figure 5.25e. A linear consumption of around 200 μmol propionate/l was detected in both replicas. No $^{14}NH_4^+$ formation was observed for the duration of the experiment as shown in Figure 5.25f. Meanwhile, the existing $^{14}NO_2^-$ in the medium was also fully utilized. Nitrite conversions as 1.63 and 1.46 mmol/l were measured for the whole period of the run in both sets. Figure 5.25g demonstrates the nitrite profiles associated with sets 2a and 2b.

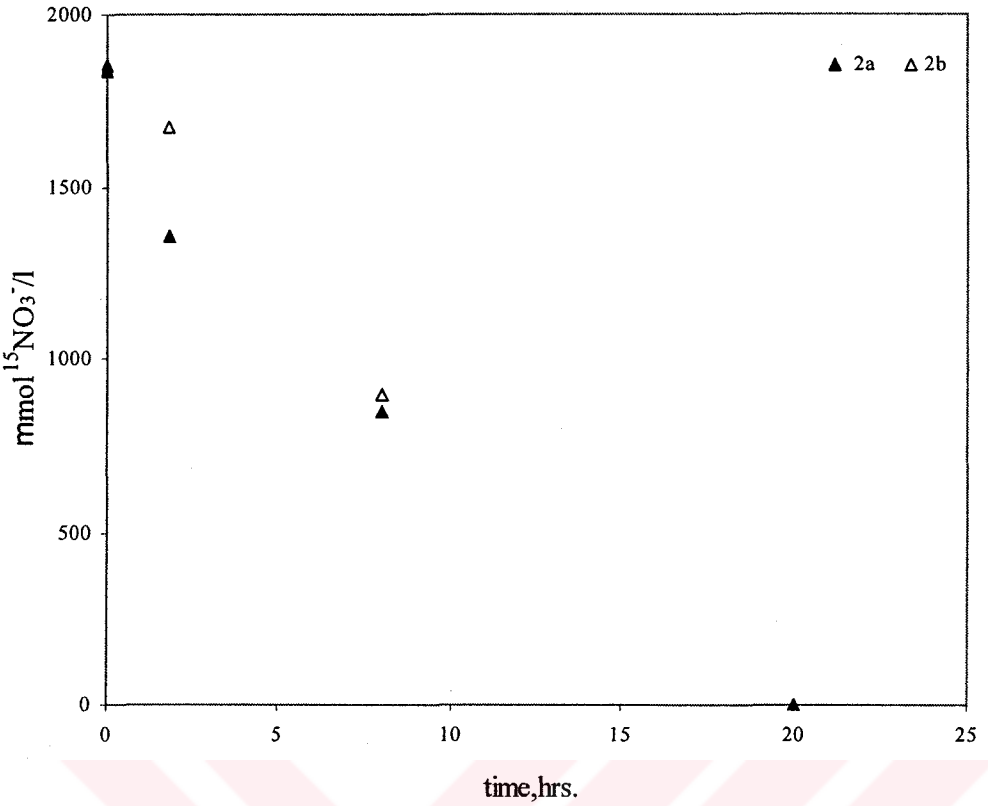


Figure 5.25a $^{15}NO_3^-$ profiles of the experimental sets 2a and 2b.

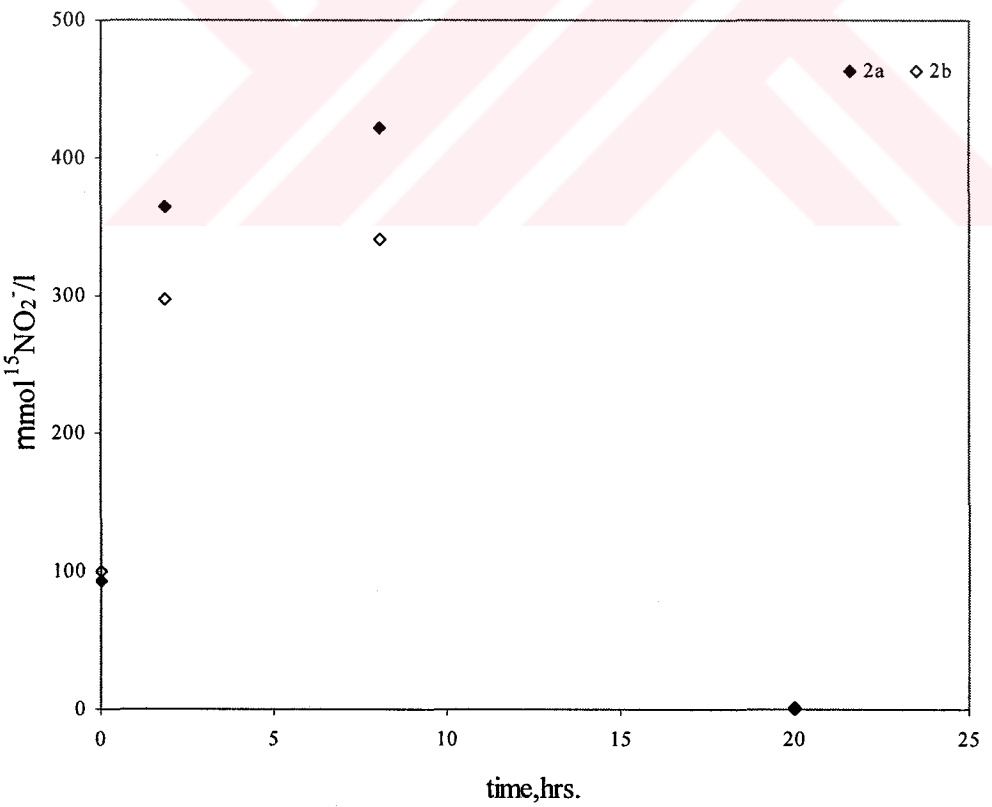


Figure 5.25b $^{15}NO_2^-$ profiles of the experimental sets 2a and 2b.

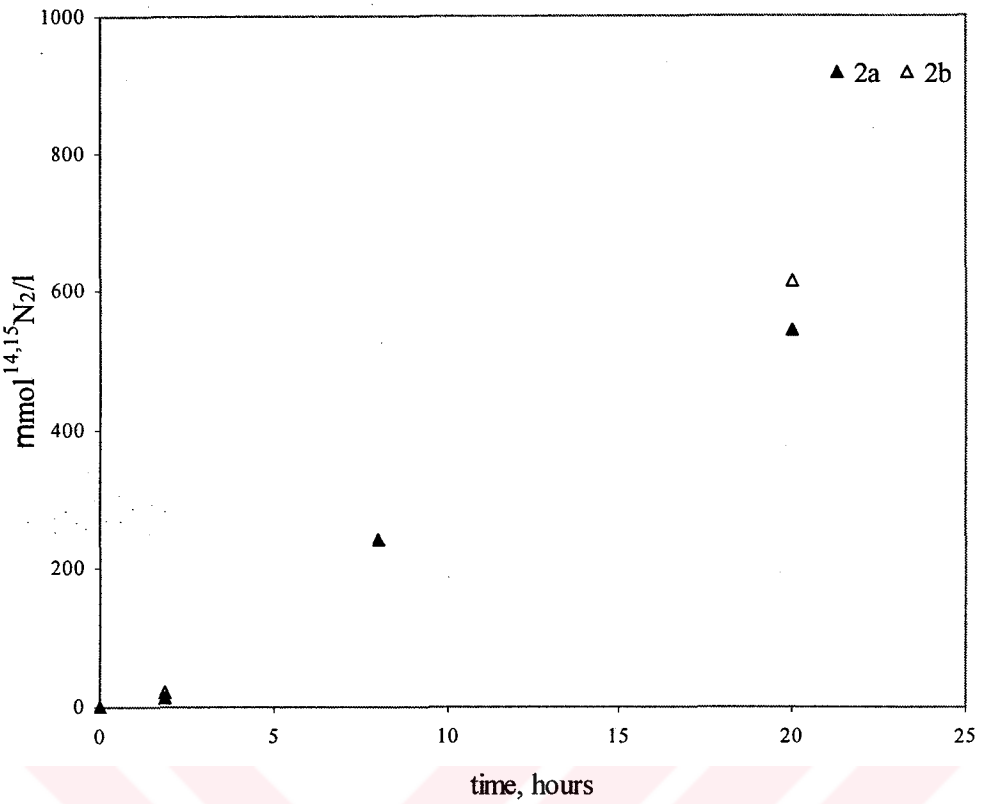


Figure 5.25c $^{14,15}N_2$ profiles of the experimental sets 2a and 2b.

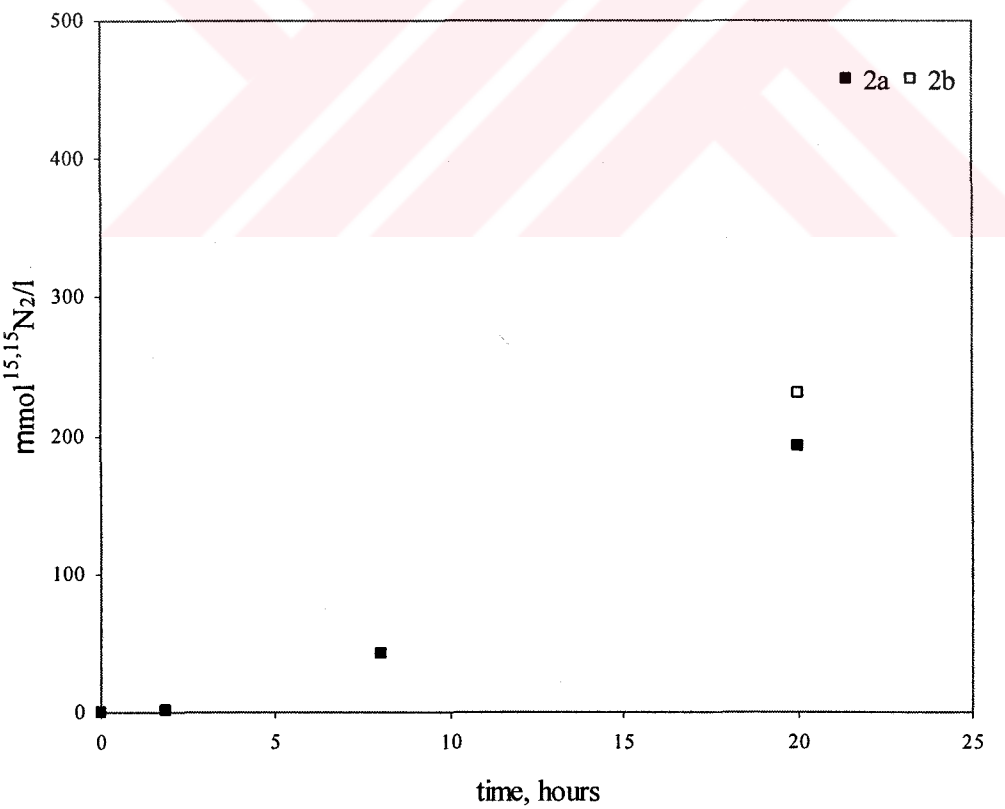


Figure 5.25d $^{15,15}N_2$ profiles of the experimental sets 2a and 2b.

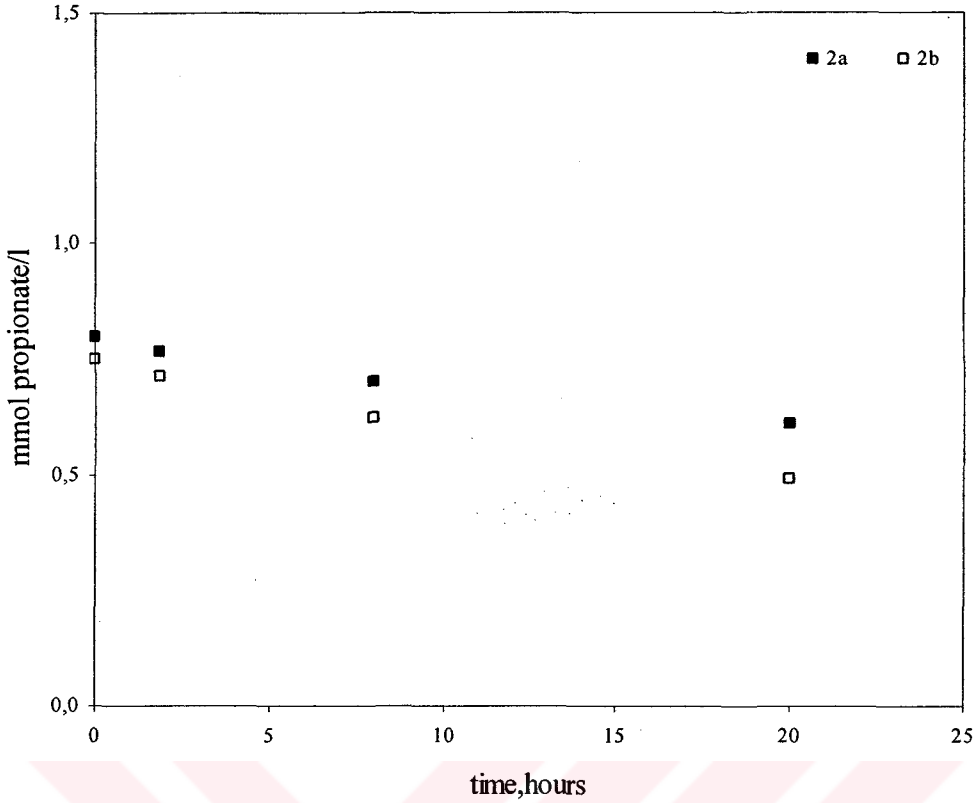


Figure 5.25e Propionate profiles of the experimental sets 2a and 2b.

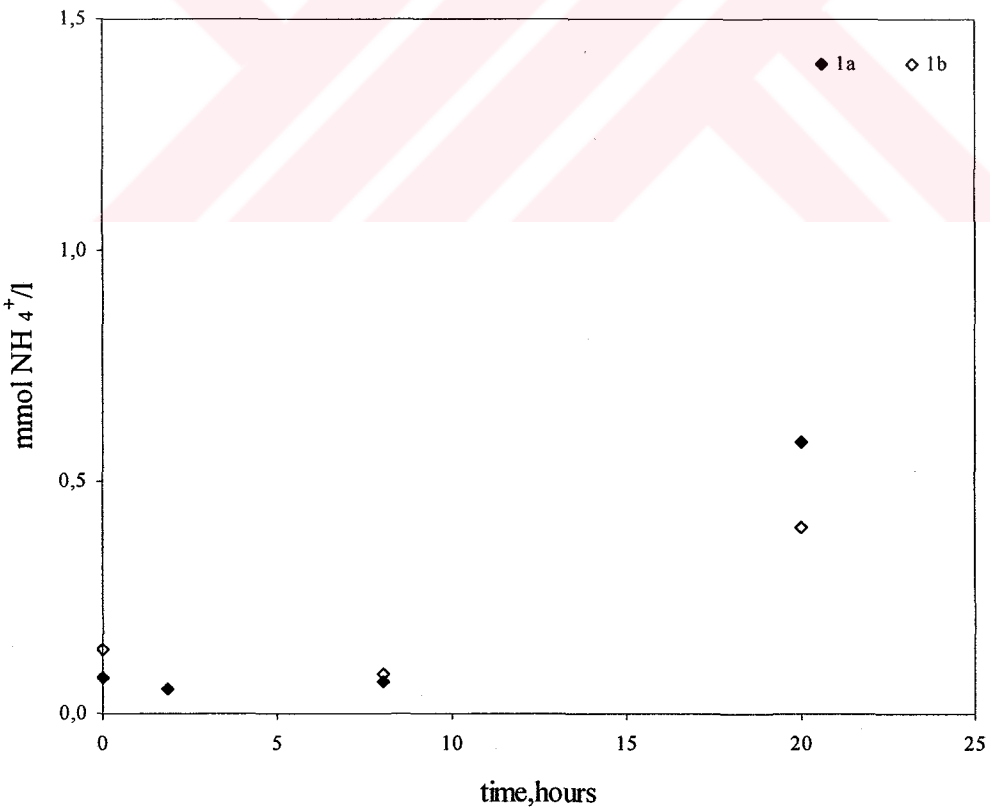


Figure 5.25f $^{14}NH_4^+$ profiles of the experimental sets 2a and 2b.

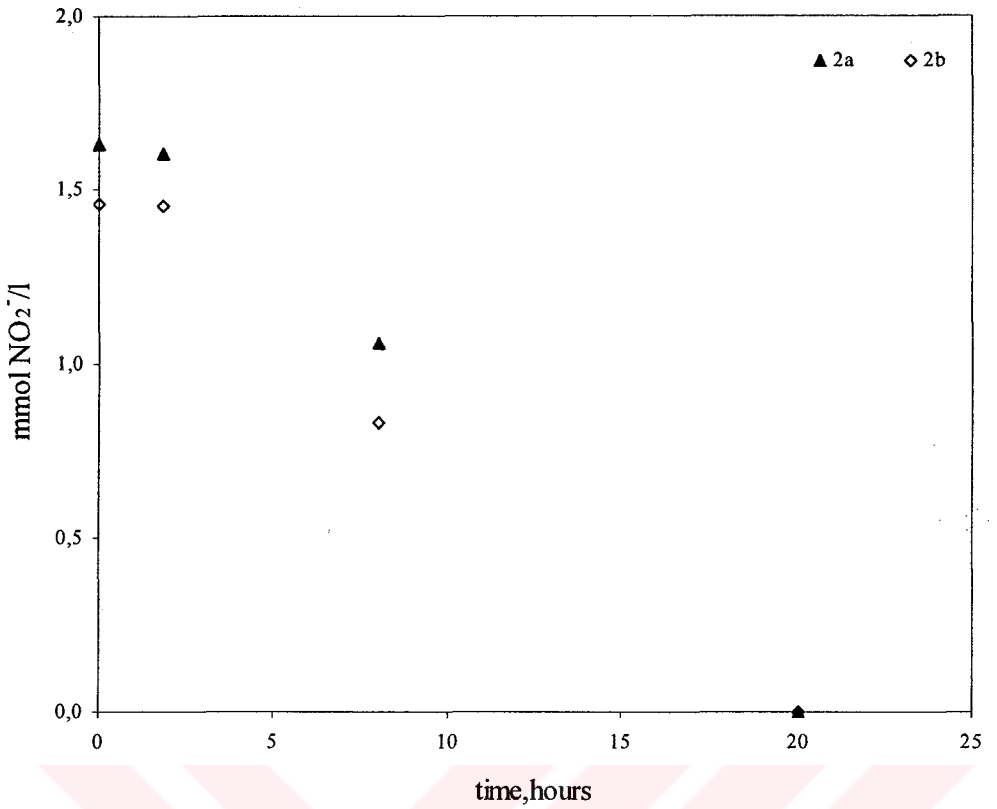


Figure 5.25g $^{14}NO_2^-$ profiles of the experimental sets 2a and 2b.

Experimental set 3 was run in presence of $^{15}NO_3^-$, $^{14}NO_2^-$, $^{14}NH_4^+$ and propionate to examine the consequence of the anaerobic ammonium oxidation activity in presence of propionate. As soon as nitrite was completely converted, experiment was stopped at the 3rd hour of the run so as to prevent the system become more complicated with the components that would be formed by further reactions in the system.

$^{15}NO_3^-$ profiles are presented in Figure 5.26a. Figure shows that $^{15}NO_3^-$ concentration decreased from 962 to 525 and 1135 to 664 μmol/l within 1.85 hours in sets 3a and 3b, respectively. Transient $^{15}NO_2^-$ accumulation was detected as 220 and 100 μmol/l in replicas as illustrated in Figure 5.26b. $^{14,15}N_2$ production was measured as 418 and 332 μmol/l correspondingly for sets 3a and 3b as presented in Figure 5.26c. Meanwhile $^{15,15}N_2$ production was ever detected as depicted in Figure 5.26d.

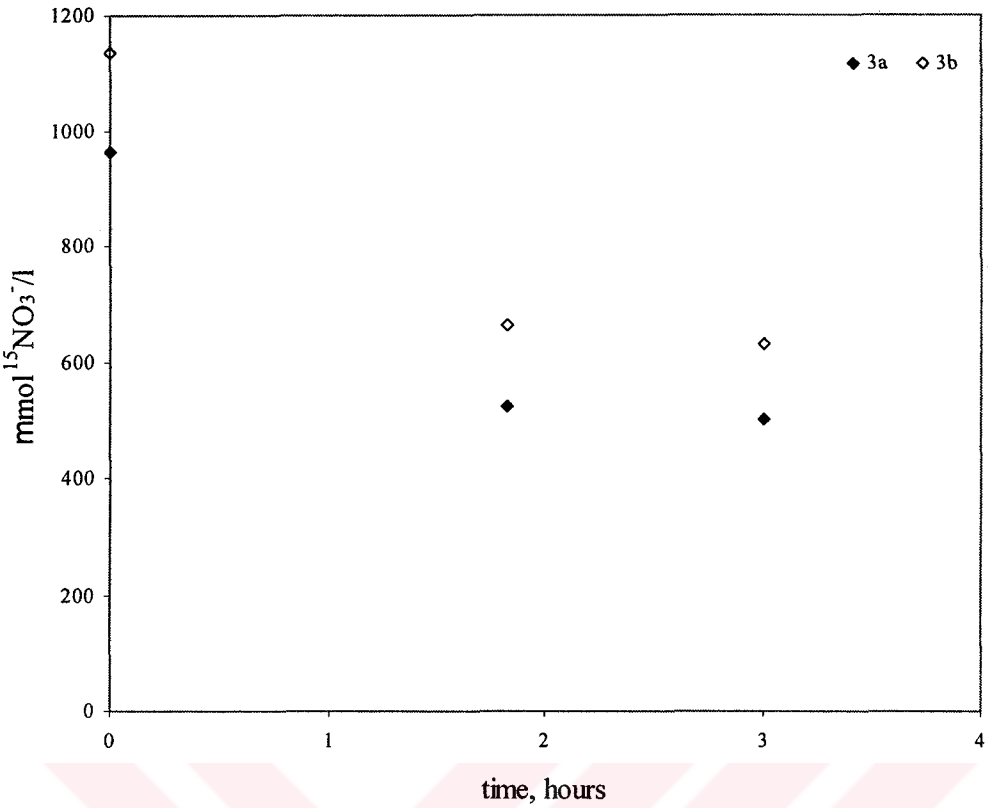


Figure 5.26a $^{15}NO_3^-$ profiles of the experimental sets 3a and 3b.

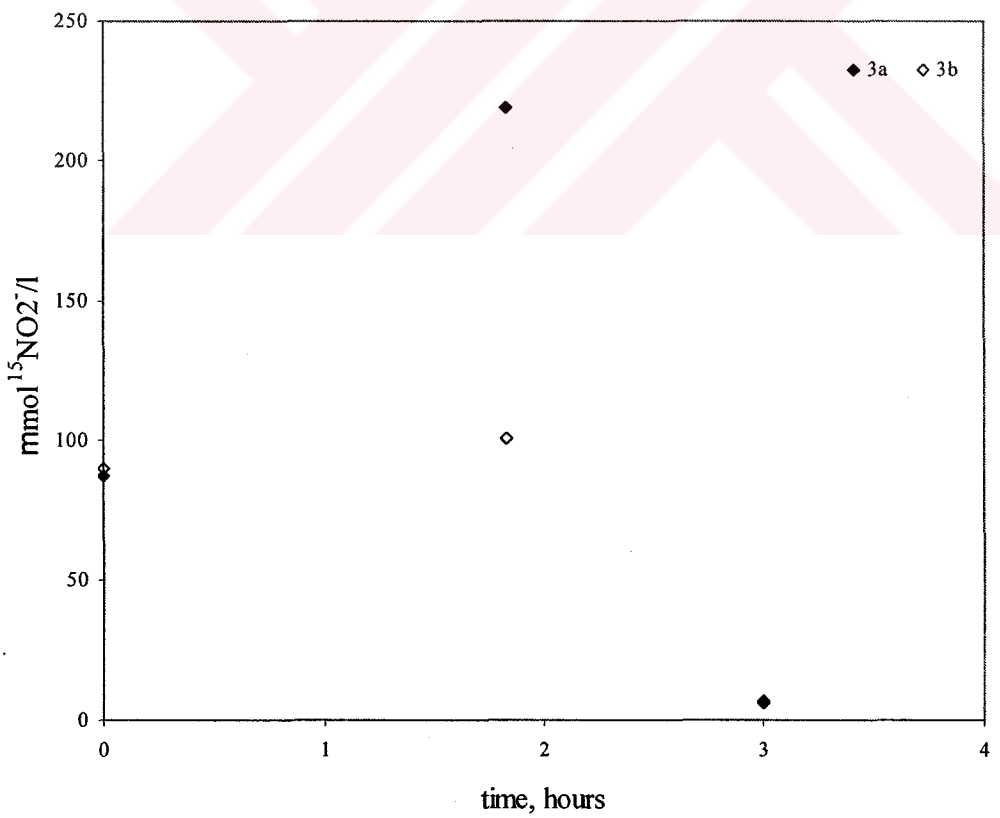


Figure 5.26b $^{15}NO_2^-$ profiles of the experimental sets 3a and 3b.

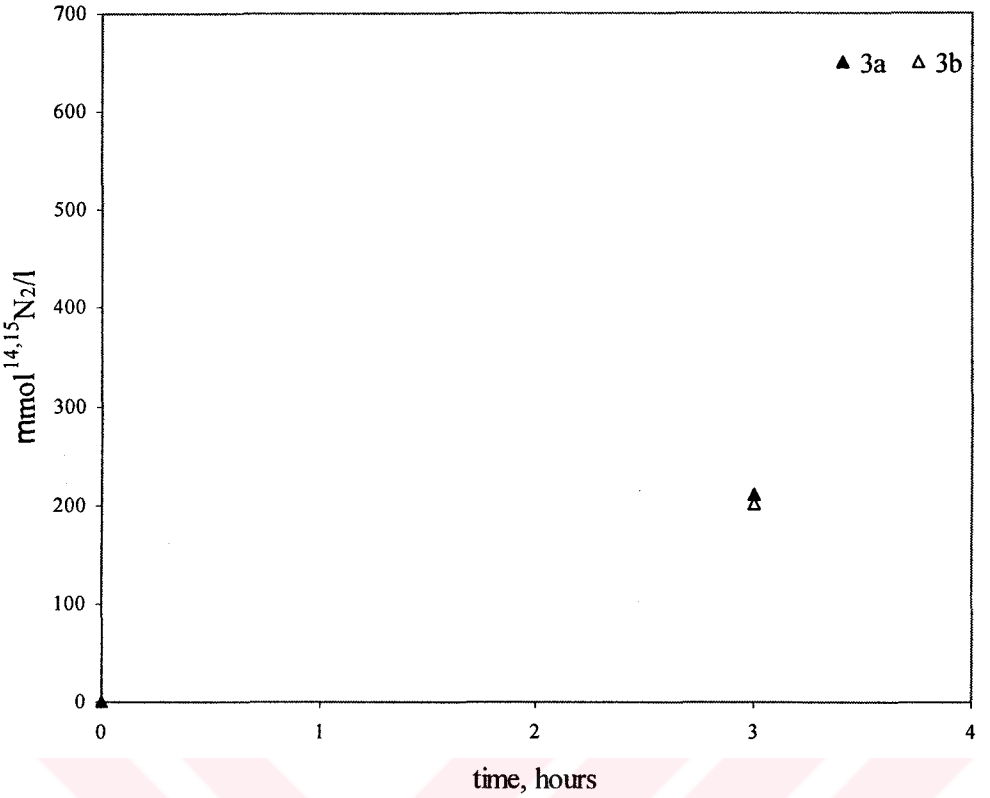


Figure 5.26c $^{14,15}N_2$ profiles of the experimental sets 3a and 3b.

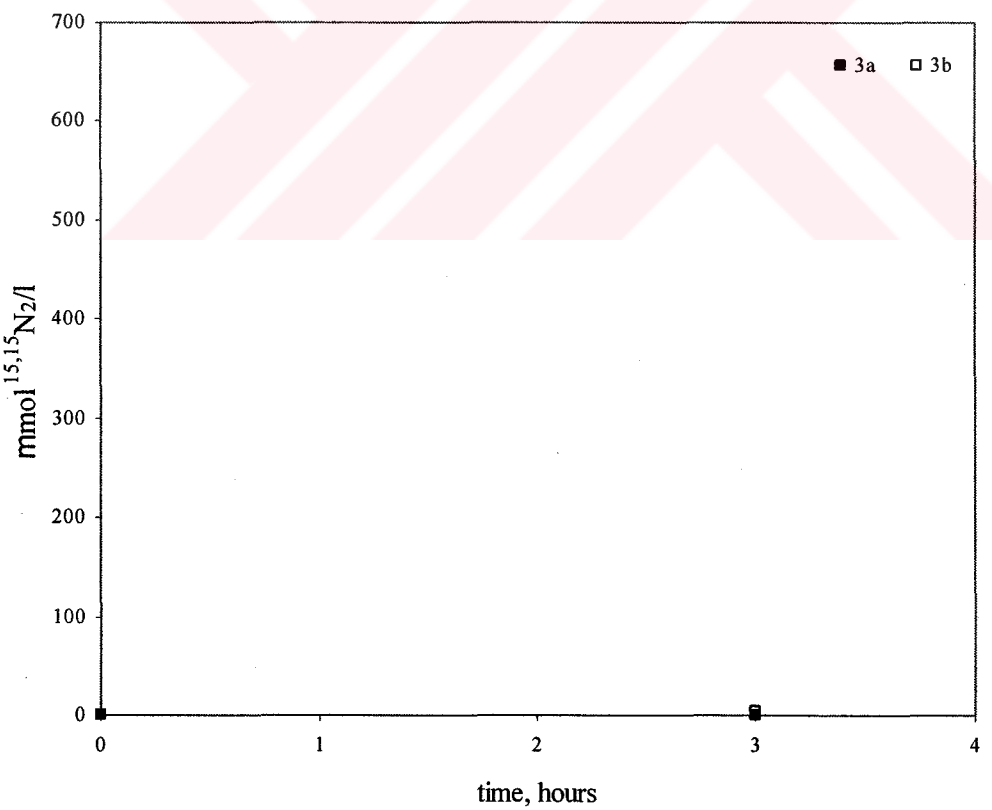


Figure 5.26d $^{15,15}N_2$ profiles of the experimental sets 3a and 3b.

For the duration of the 3 hour experiment, propionate consumptions were detected as 120 μmol/l from both replicates as presented in Figure 5.26e. In the same period, 1.5 mmol/l ammonium conversions were observed in both sets as illustrated in Figure 5.26f. As a result of anammox activity, 2.8 and 2.7 mmol/l nitrite was converted in sets 3a and 3b, respectively. $^{14}NO_2^-$ profiles of the replicates are presented in Figure 5.26g. Meanwhile, slight increase in nitrate concentration was observed from 0.78 to1.18 mmol/l and 1.02 to 1.17 mmol/l in set 3a and 3b, respectively. $^{14}NO_3^-$ profiles of the duplos are presented in Figure 5.26h.

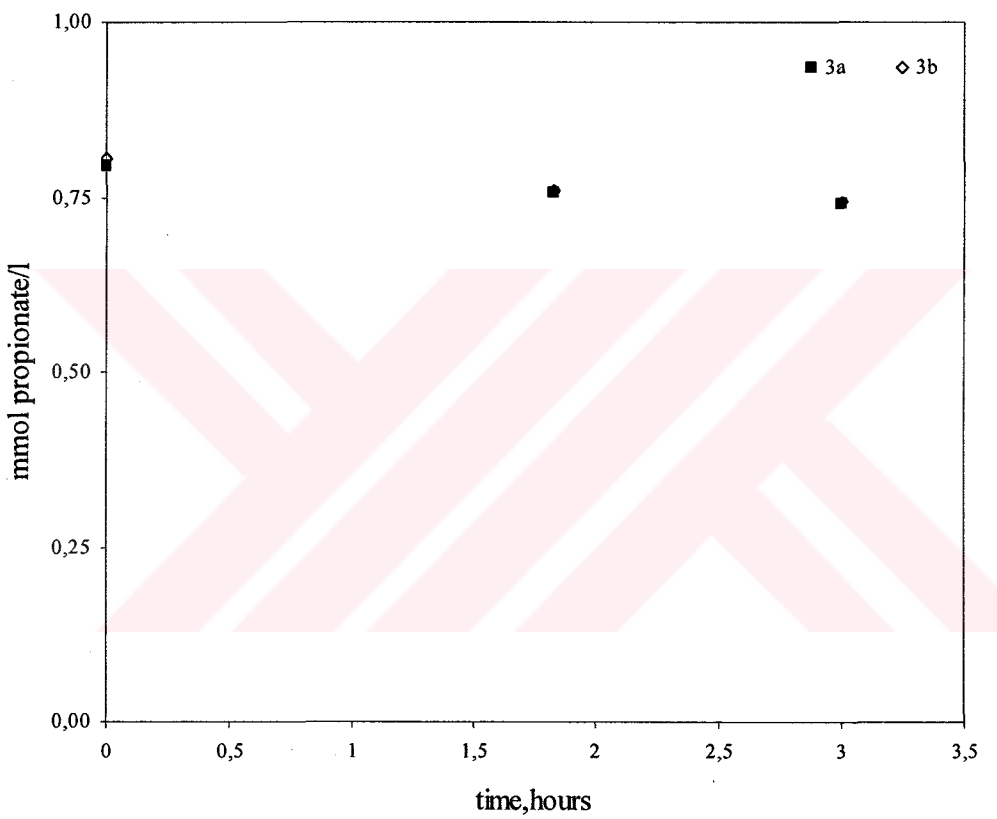


Figure 5.26e Propionate profiles of the experimental sets 3a and 3b.

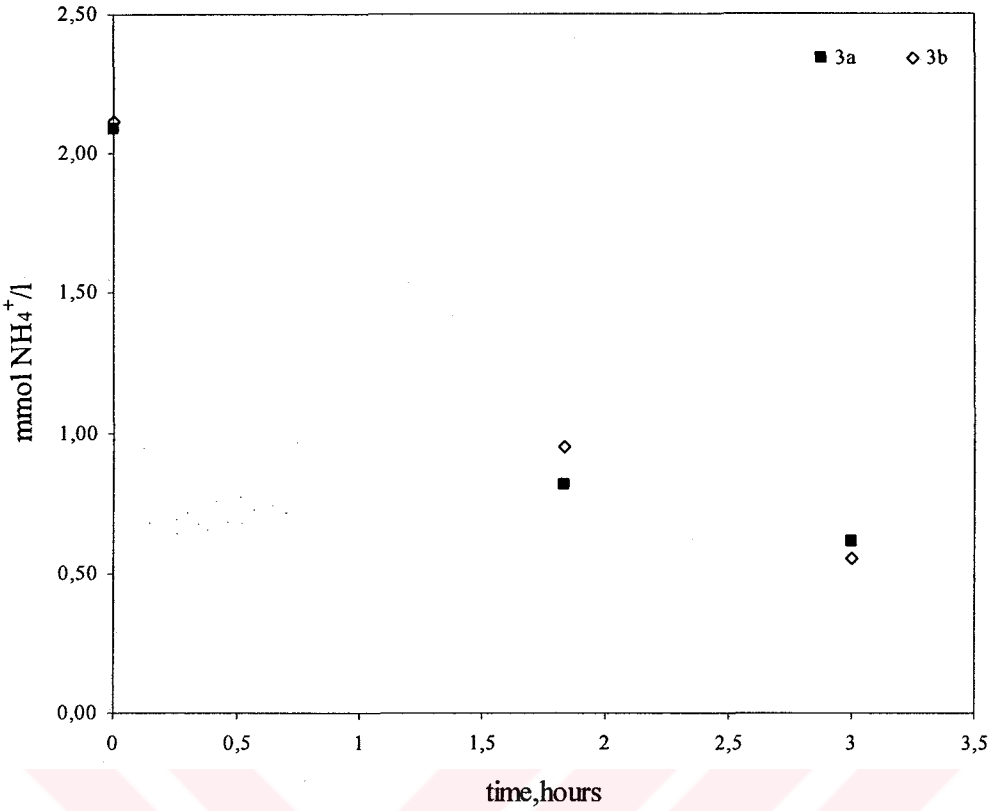


Figure 5.26f $^{14}NH_4^+$ profiles of the experimental sets 3a and 3b.

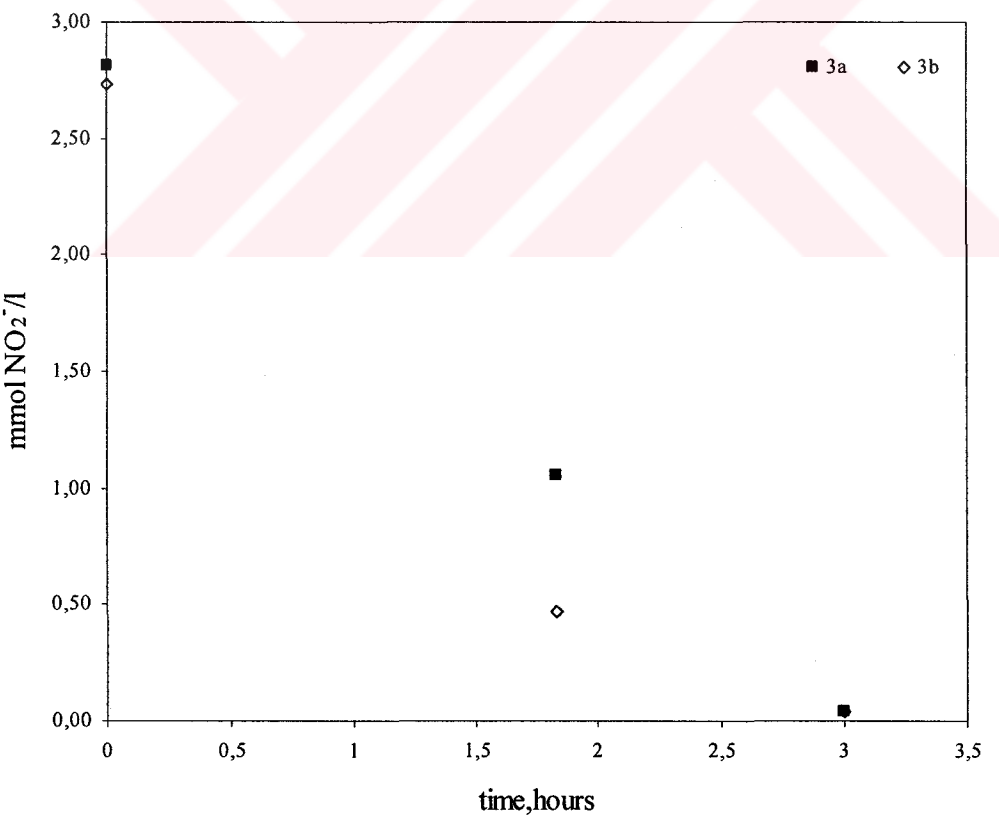


Figure 5.26g $^{14}NO_2^-$ profiles of the experimental sets 3a and 3b.

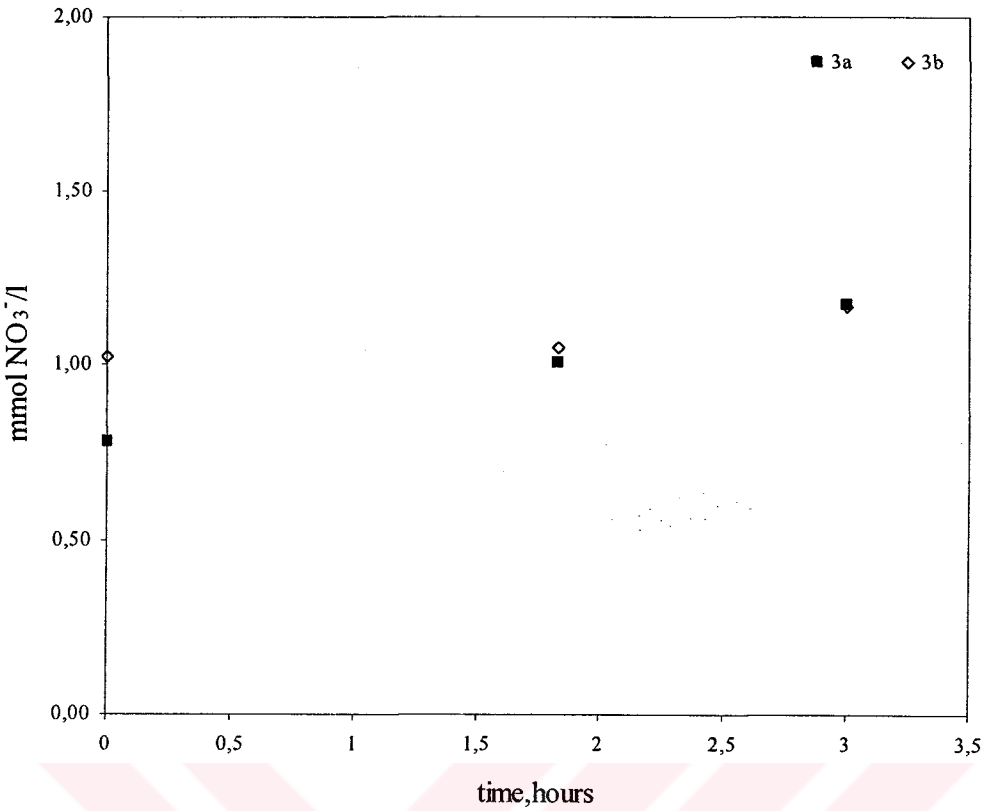


Figure 5.26h $^{14}NO_3^-$ profiles of the experimental sets 3a and 3b.

6 EVALUATION OF THE RESULTS

6.1 Effects of organic compounds on the anammox process

Batch experiments with different carbon compounds showed that anammox is inhibited by methanol, ethanol, formate, amino acids, glucose and starch. Conversely, acetate and propionate were found to have stimulating effect on the anammox activity. The influence of different carbon sources on the activity of anammox are presented in Table 6.1 based on the maximum nitrite conversion rates as activity. In this table, decrease or increase in activity is presented as a percentage compared to their own control set which is presented as 100%.

Table 6.1 Effects of organic compounds on the anammox activity.

Compound	Concentration mmol/l	Effect on anammox activity, %
Control set	0	100
Methanol	0-2	0
Acetate	0-3	116
Propionate	0-3	124
Ethanol	0-2	70
Glucose	0-3	90
Formate	0-2	72
Aminoacids	0-2	82
Starch	0-1	70

However, in batch experiments carried out with various carbon compounds, the precise influence of added components on the growth of anaerobic ammonium oxidizers was not measured. These experiments only provided insight into the inhibitory or stimulatory potential of certain components. Results obtained from the batch experiments with acetate are in a good agreement with findings reported by Van de Graaf et al. (1996) that are presented in Table 6.2.However, results that

observed for glucose and propionate are inconsistent with each other. This inconsistency is possibly due to the degree of enrichment of the biomass used in the experiments. Since 64% enriched anammox culture was used by Van de Graaf et al. (1996), presence of heterotrophic activity might negatively affected the anammox activity.

Batch experiments indicated that methanol has a strong inhibitory effect on the anammox activity even in very low concentrations. In control experiment without methanol, nitrite and ammonia concentrations were decreased by the time. Meanwhile, in sets 2, 3 and 4 nitrite and ammonium concentrations were stable indicating that anammox process was inhibited by methanol. Comparative ammonia, nitrite and nitrate profiles for different concentrations of methanol are presented in Figure 6.2a to 6.2c. It can be clearly seen from the figures that anammox was thoroughly inactive even in presence of 0.5 mmol methanol/l.

Table 6.2 Effects of addition of various carbon compounds on the anammox activity in batch experiments reported by Van de Graaf et al. (1996).

Compound	Concentration	Effect on Anammox activity
Acetate	1 or 5 mM	Increase, nitrite formation
Propionate	1 mM	No effect
Glucose	1 mM	Increase, nitrite formation

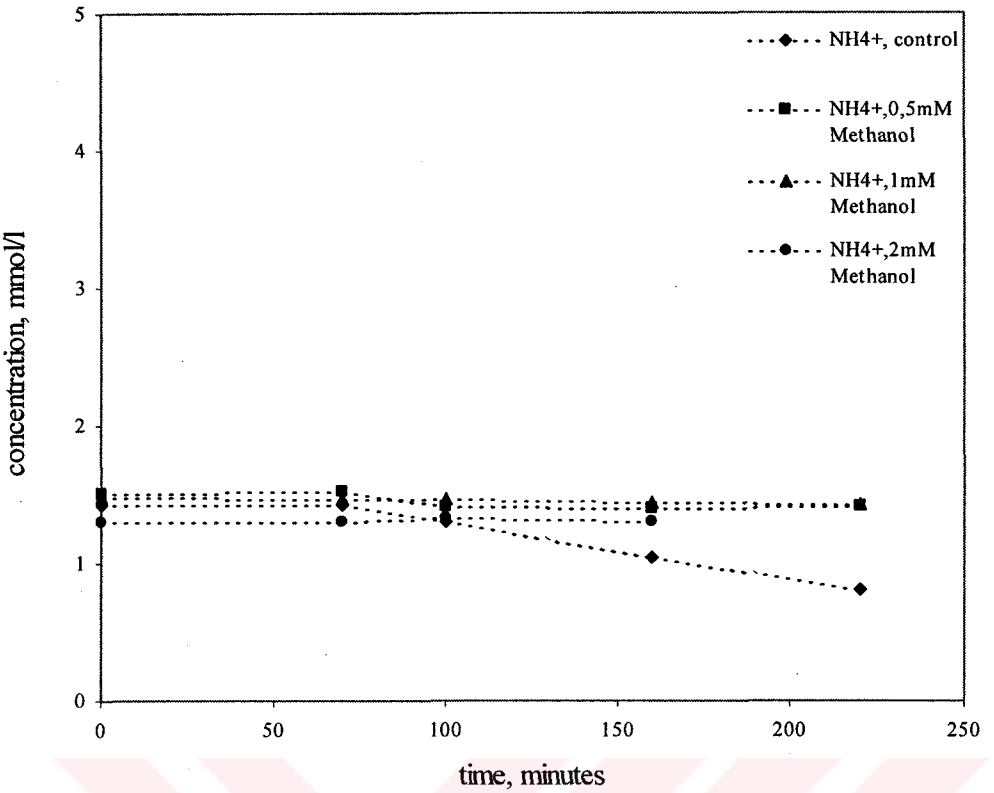


Figure 6.1a Ammonia profiles for 0-0.5-1-2 mmol methanol/l concentrations.

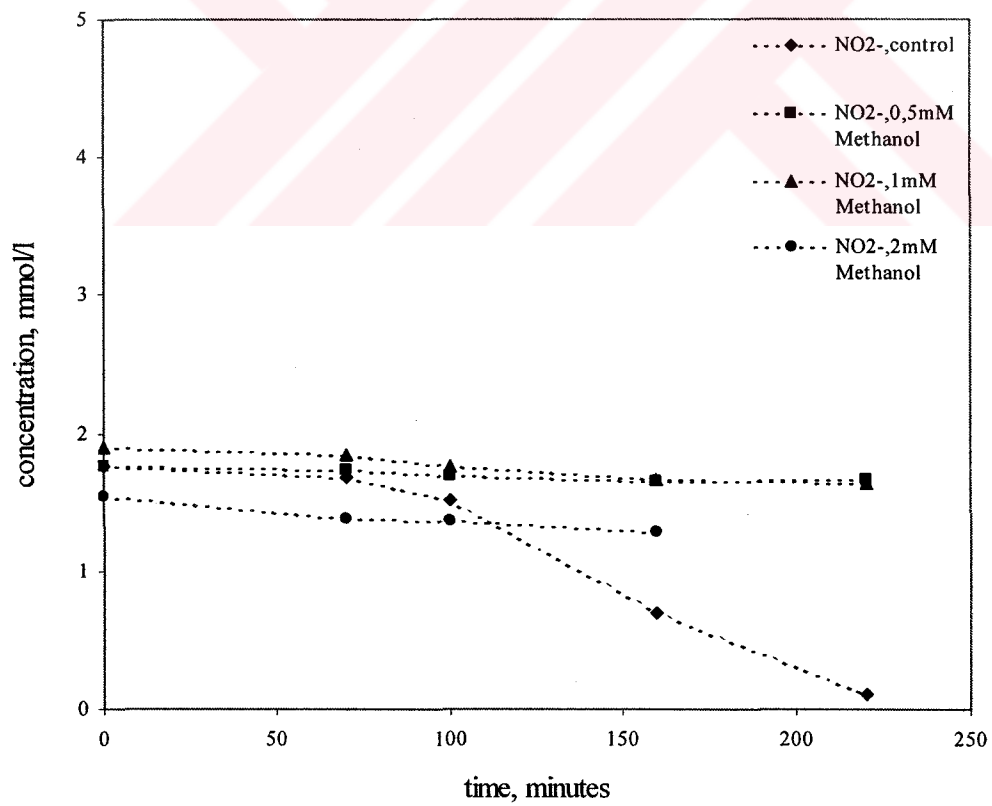


Figure 6.1b Nitrite profiles for 0-0.5-1-2 mmol methanol/l concentrations.

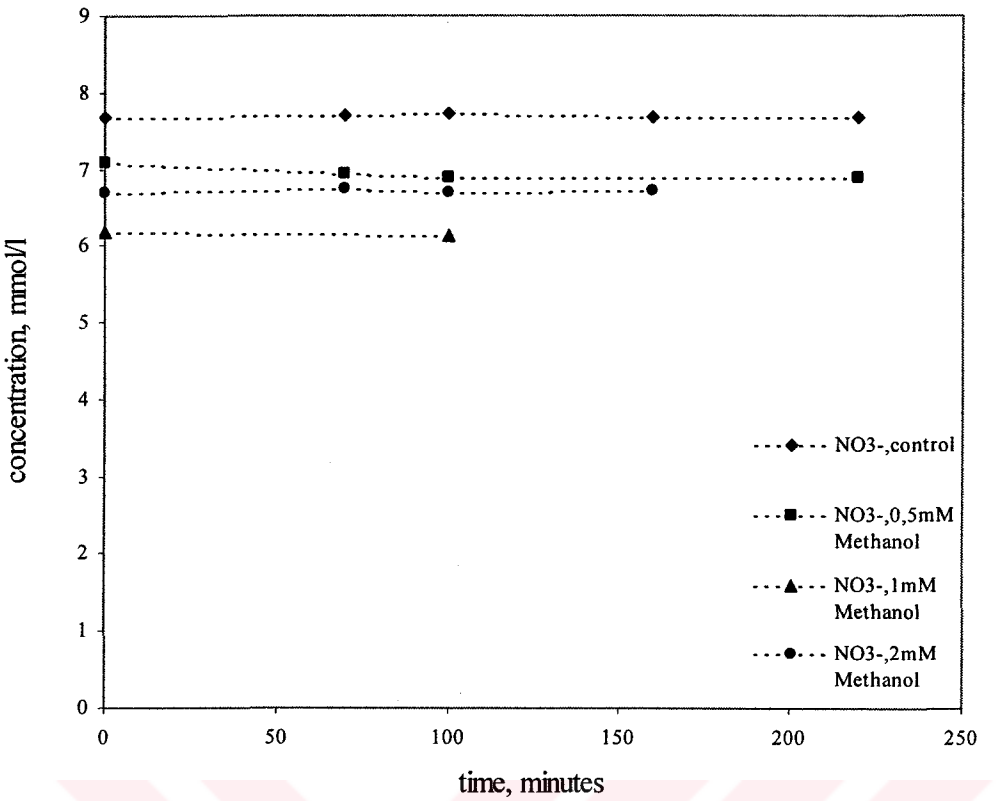


Figure 6.1c Nitrate profiles for 0-0.5-1-2 mmol methanol/l concentrations.

Figures 6.2a to 6.2c shows ammonia, nitrite and nitrate profiles in presence of different acetate concentrations. Results indicated that anammox activity was not decreased in presence of acetate, whereas slight increase in activity was observed. The highest activity was obtained at 1 mmol/l acetate concentration. Stimulating effect of acetate on anammox activity was calculated as 16%.

Batch experiments with propionate showed that propionate had a stimulating effect on the activity of anammox. As can be seen from the Figure 6.3a and 6.3b, ammonia and nitrite conversions were faster in the presence of 0.5, 1 and 3 mmol propionate/l than that of in the control experiment. Stimulating effect of propionate on the activity of anammox was calculated as 24%. Figure 6.3c shows nitrate profiles for various concentrations of propionate.

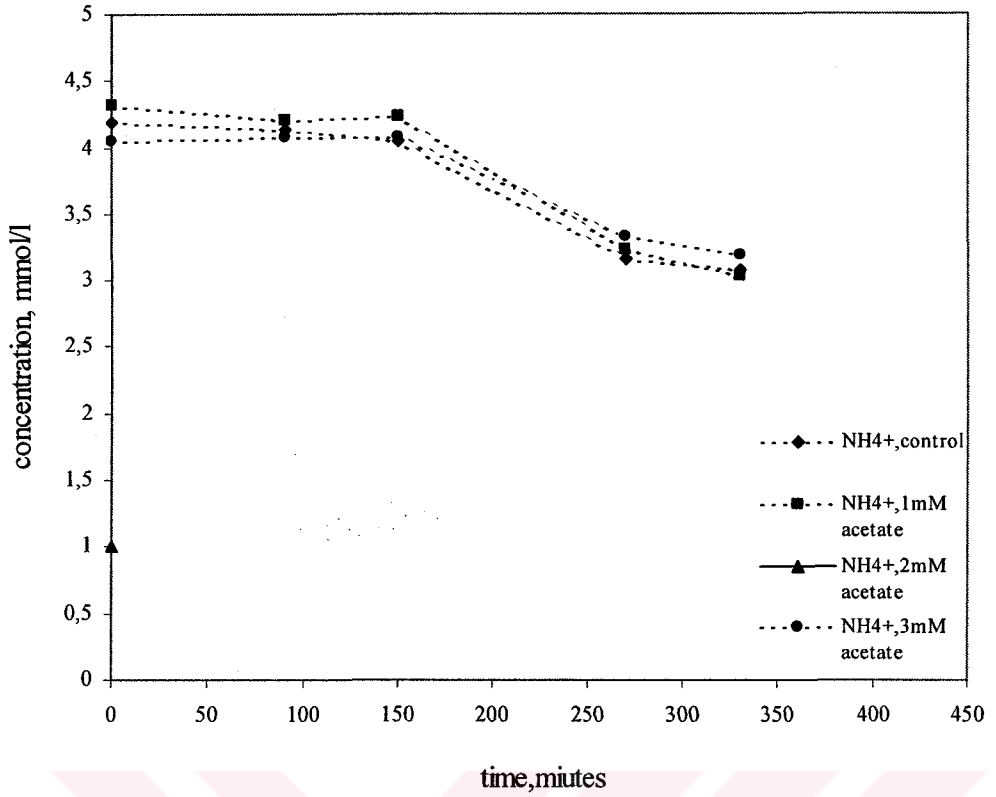


Figure 6.2a Ammonia profiles for 0-1-2-3 mmol acetate/l concentrations.

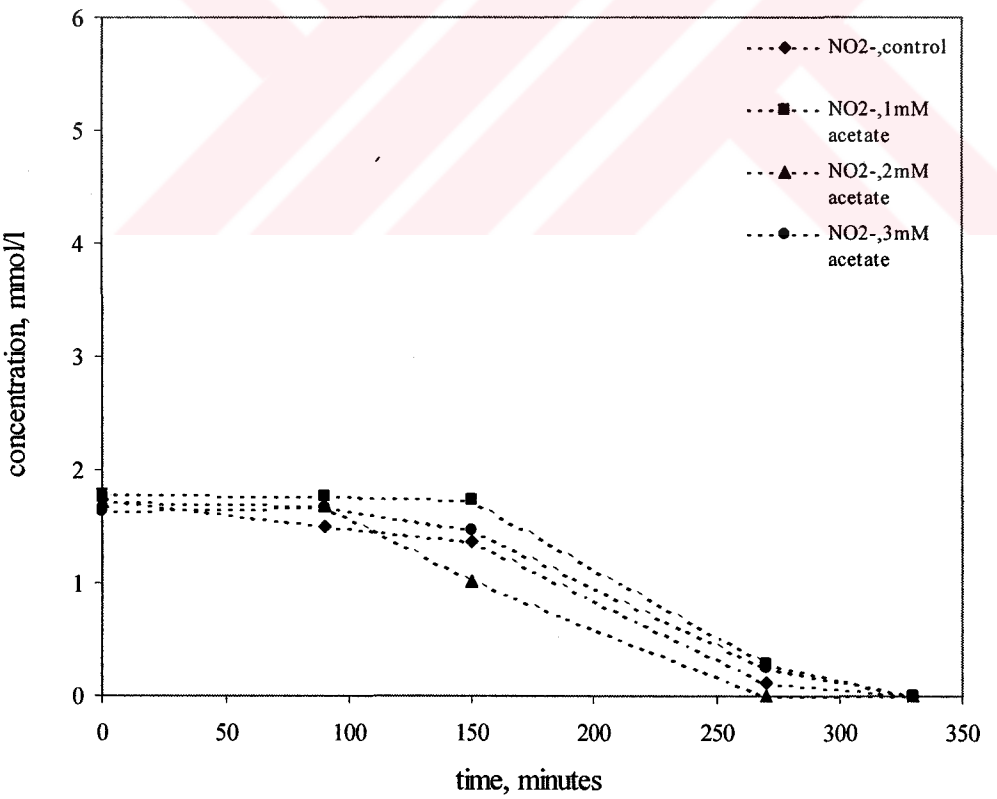


Figure 6.2b Nitrite profiles for 0-1-2-3 mmol acetate/l concentrations.

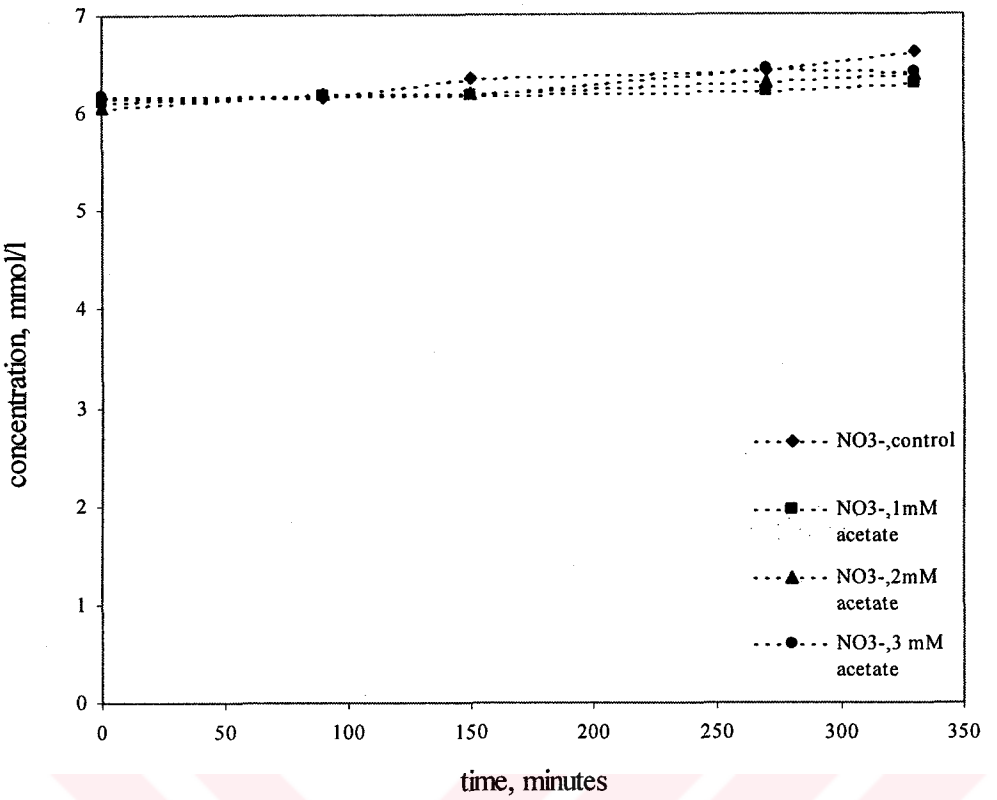


Figure 6.2c Nitrate profiles for 0-1-2-3 mmol acetate/l concentrations.

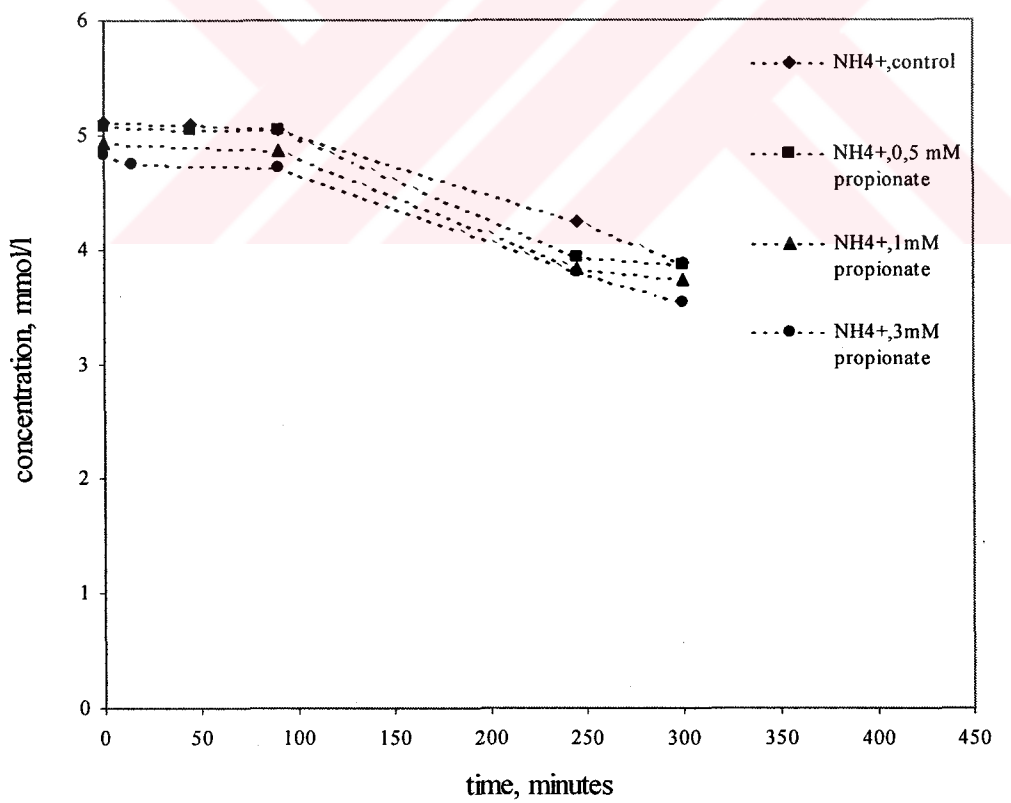


Figure 6.3a Ammonia profiles for 0-0.5-1-3 mmol propionate/l concentrations.

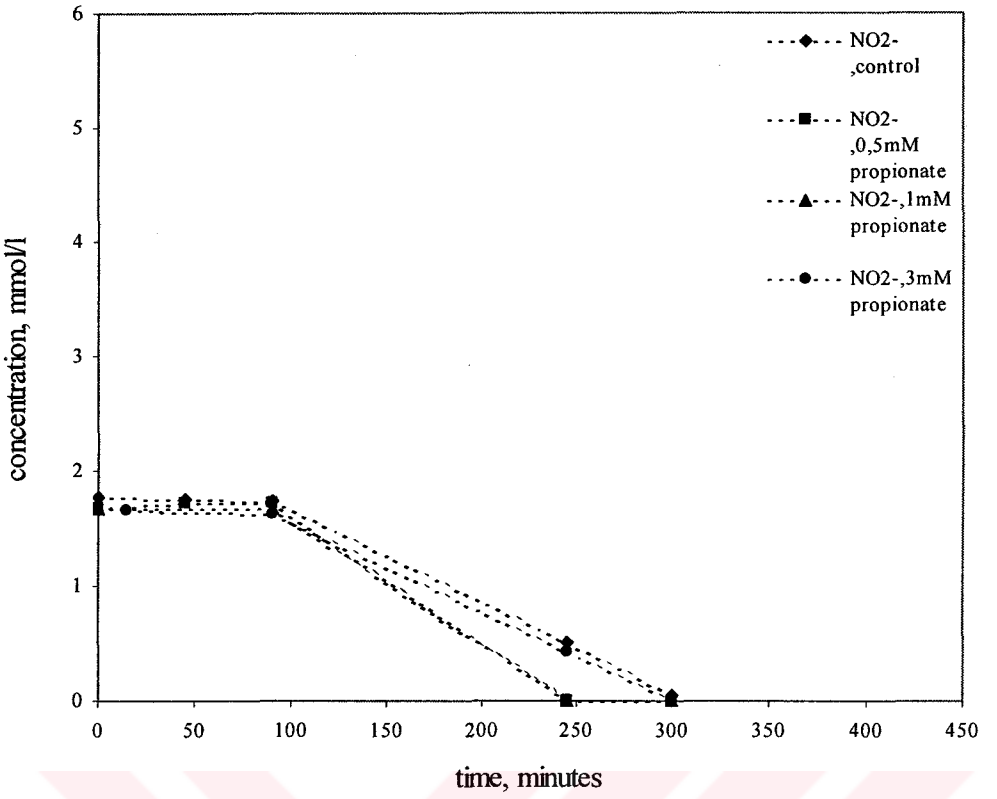


Figure 6.3b Nitrite profiles for 0-0.5-1-3 mmol propionate/l concentrations.

Results obtained from batch experiments with starch showed that anammox activity was lower in the presence of 0.25, 0.5 and 1 mmol/l starch concentrations compared to control set without starch. This observation indicates inhibitory effect of starch on the anammox activity. Figures 6.4a to 6.4c shows comparative ammonia, nitrite and nitrate profiles in presence of different concentrations of starch. Inhibitory effect of starch on the activity of anammox was calculated as 30%.

Batch experiments performed with ethanol indicated that ethanol had an inhibitory effect on the anammox activity especially at 1 and 2 mmol/l ethanol concentrations. Figures 6.5a to 6.5c illustrates ammonia, nitrite and nitrate profiles in the presence of different concentrations of ethanol. The most interesting point in the Figure 6.5b, nitrite reduction was faster in ethanol concentration of 0.5 mmol/l compared to control set. Whereas in presence of 1 and 2 mmol ethanol/l, inhibitory effect was calculated as 30%.

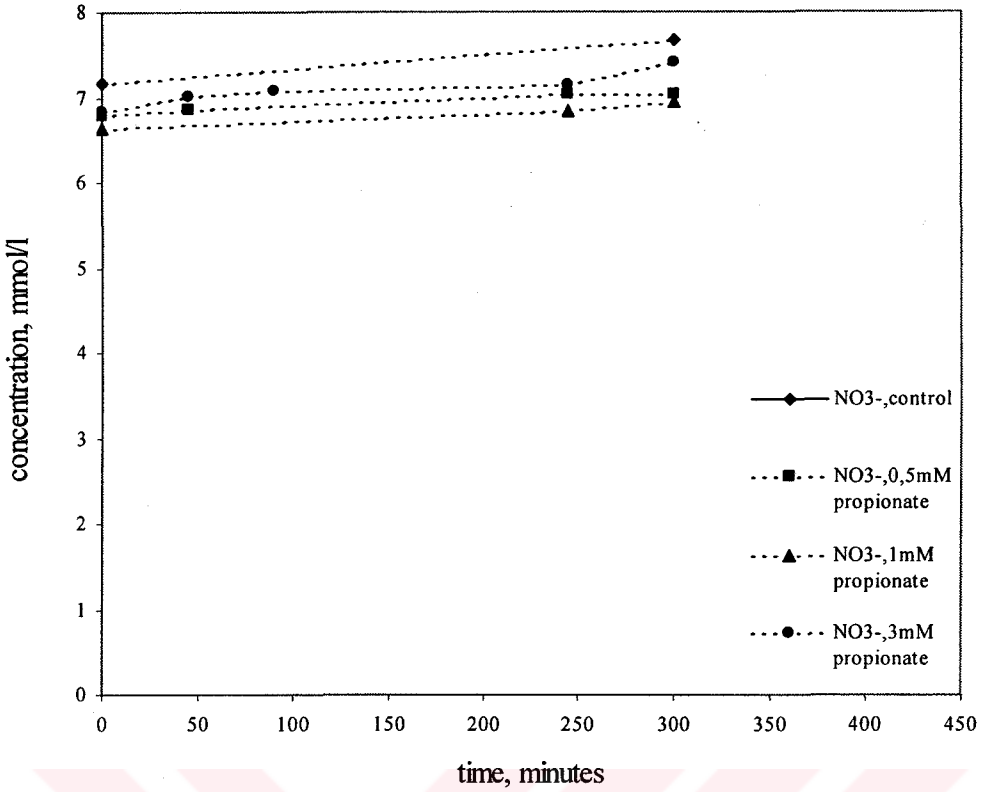


Figure 6.3c Nitrate profiles for 0-0.5-1-3 mmol propionate/l concentrations

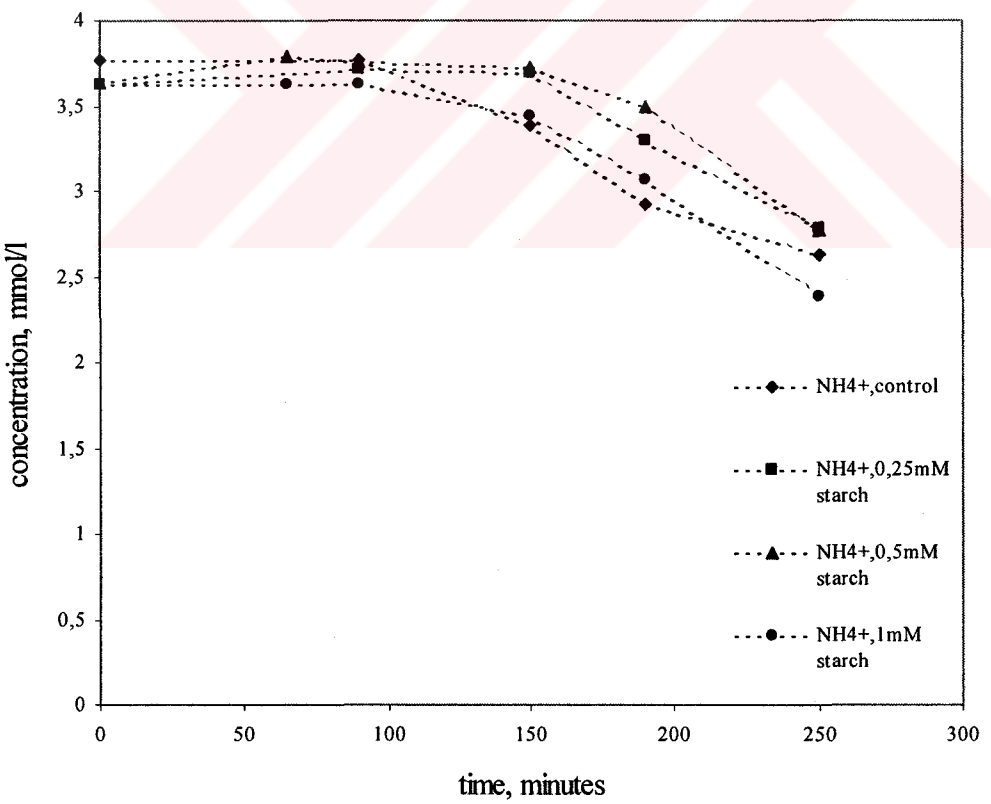


Figure 6.4a Ammonia profiles for 0-0.25-0.5-1 mmol starch/l concentrations.

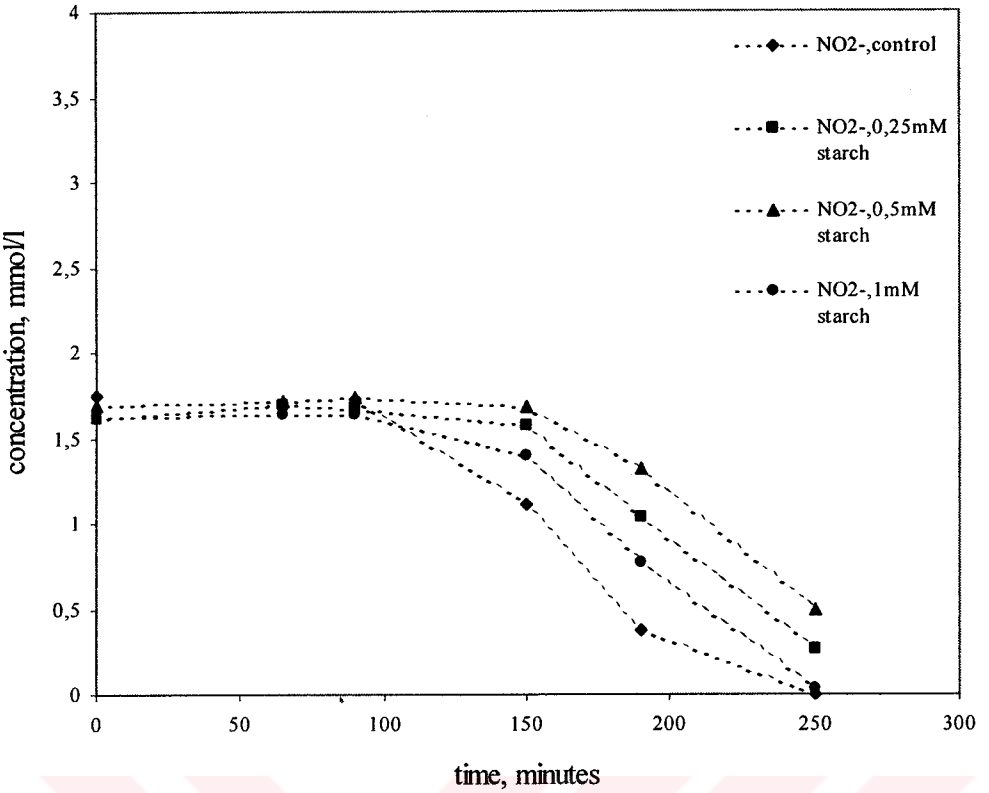


Figure 6.4b Nitrite profiles for 0-0.25-0.5-1 mmol starch /l concentrations.

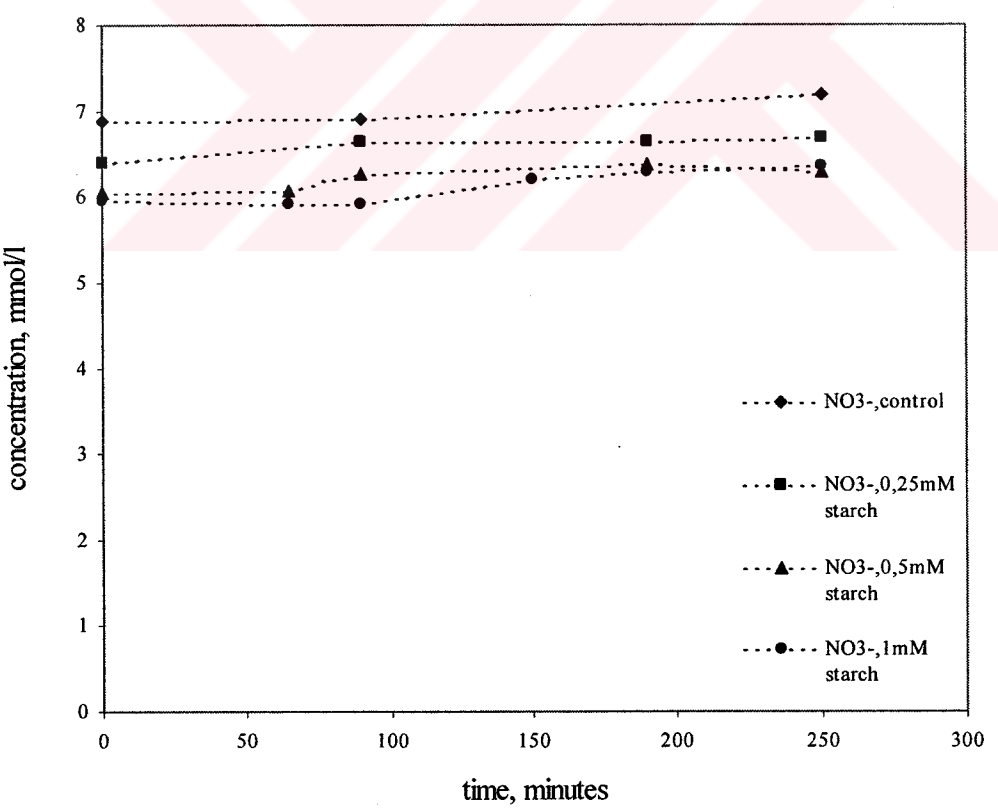


Figure 6.4c Nitrate profiles for 0-0.25-0.5-1 mmol starch /l concentrations.

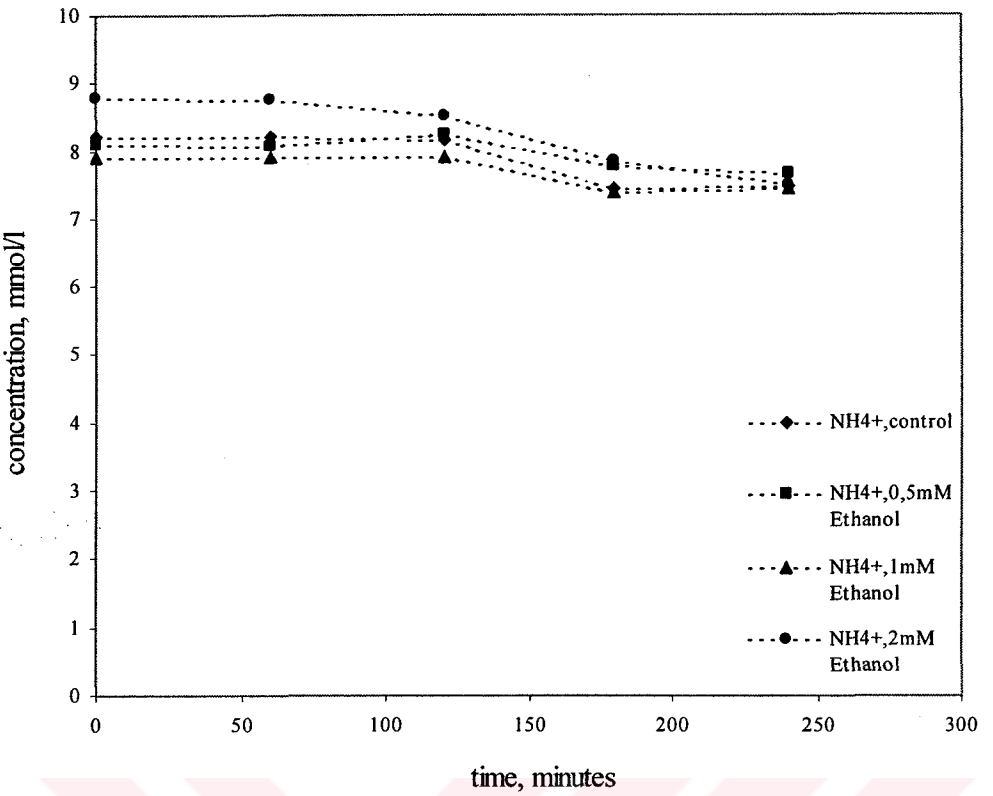


Figure 6.5a Ammonia profiles for 0-0.5-1-2 mmol ethanol/l concentrations.

Figures 6.6a through 6.6c depict ammonia, nitrite and nitrate profiles related with various concentrations of glucose. In presence 1 mmol/l glucose, ammonia and nitrite conversion rates were close to that of control set. As can be seen from the Figures 6.6a and 6.6b, at 2 and 3 mmol/l glucose concentrations, anammox activity was decreased. Inhibitory effect of glucose on the anammox activity was calculated as 10%.

Results obtained from the batch experiments with formate showed that the activity of anammox decreased in presence of formate indicating that inhibitory effect of formate. The comparative ammonia, nitrite and nitrate profiles related with different formate concentrations are illustrated in Figures 6.7a to 6.7c. As can be seen from the figures, observed anammox activity was lower in presence of 0.5, 1 and 2 mmol/l formate compared to that of control set. Inhibitory effect of formate on anammox activity was calculated as 28%.

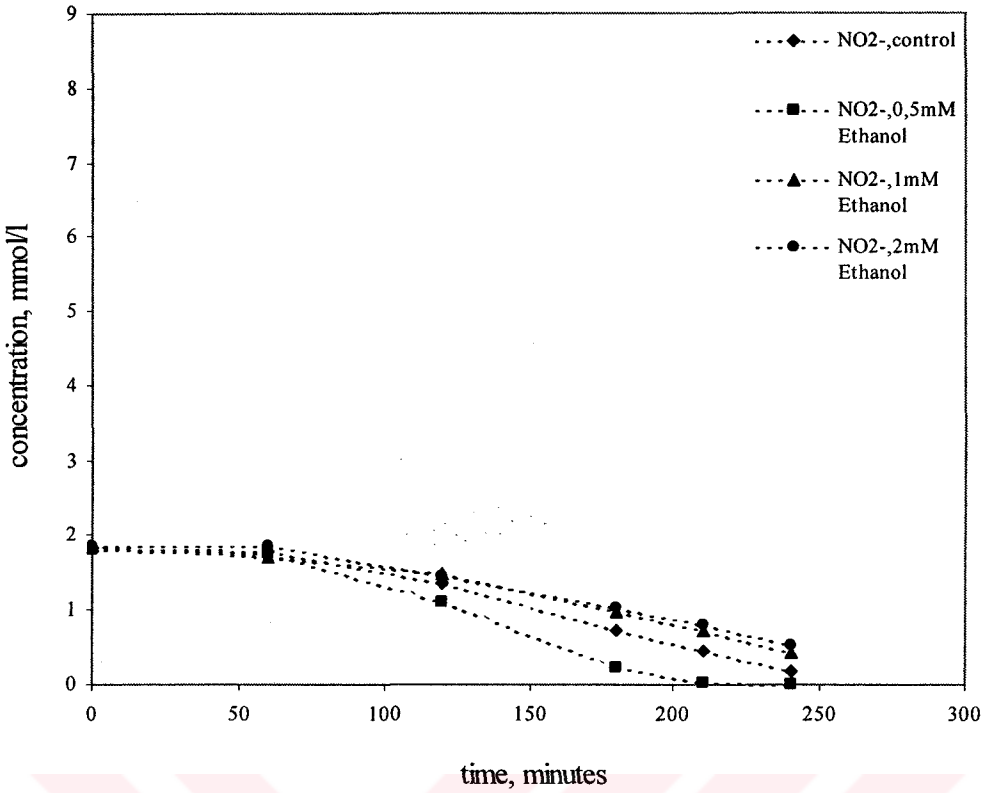


Figure 6.5b Nitrite profiles for 0-0.5-1-2 mmol ethanol/l concentrations.

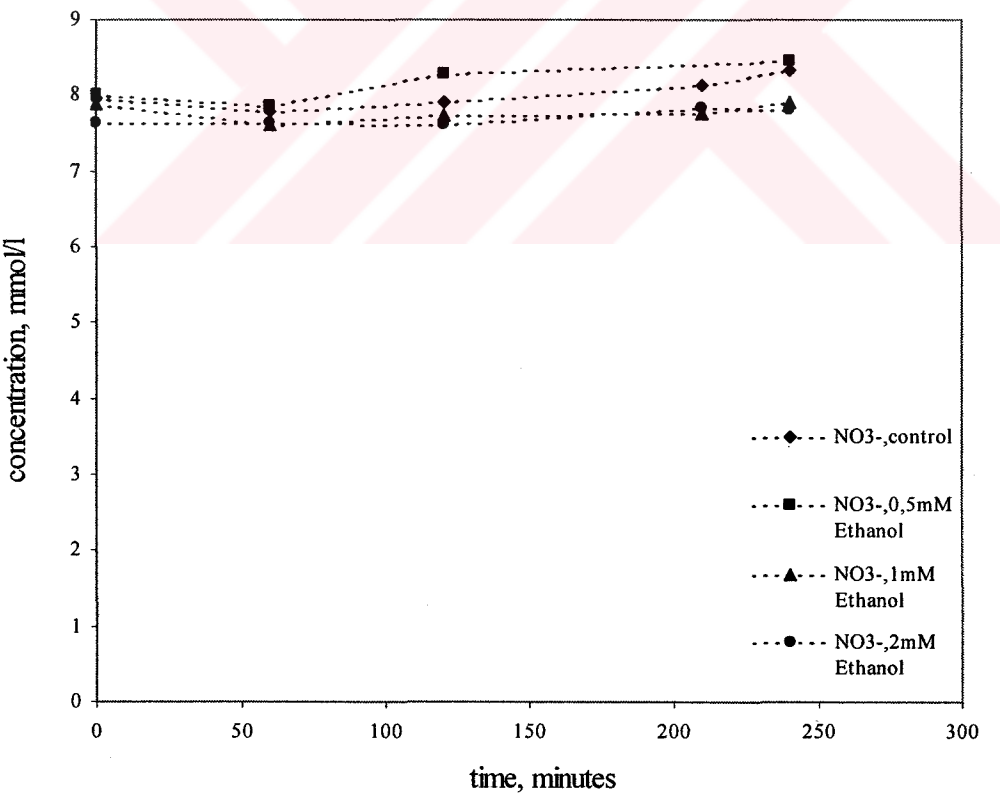


Figure 6.5c Nitrate profiles for 0-0.5-1-2 mmol ethanol/l concentrations.

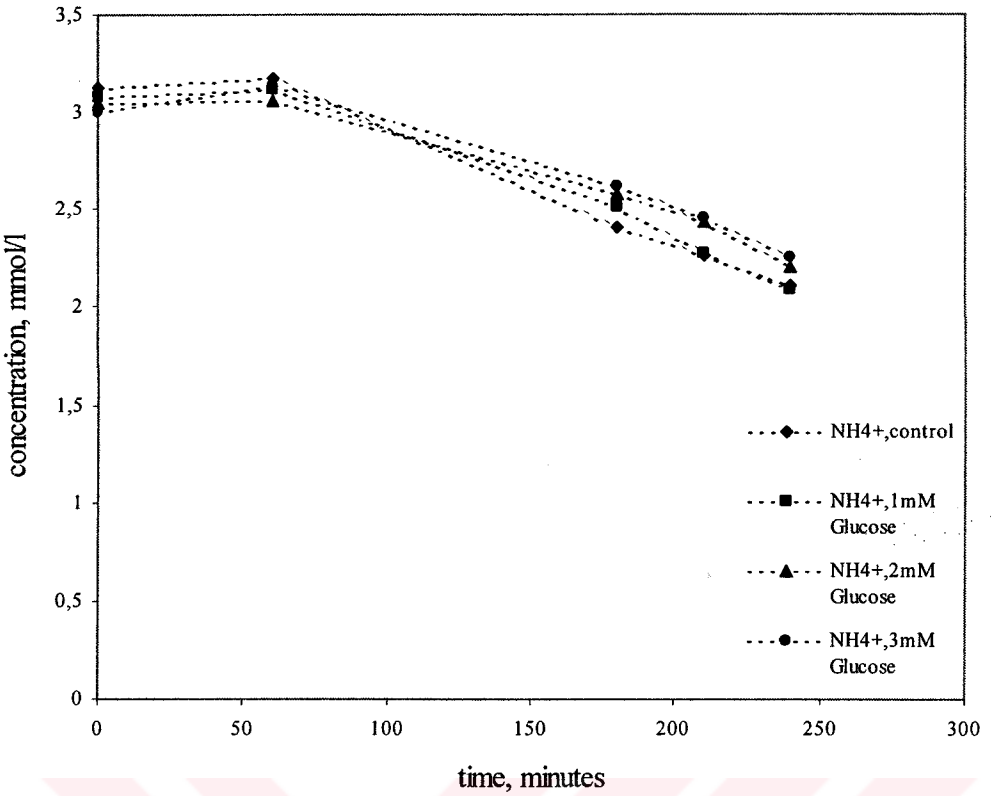


Figure 6.6a Ammonia profiles for 0-1-2-3 mmol glucose/l concentrations.

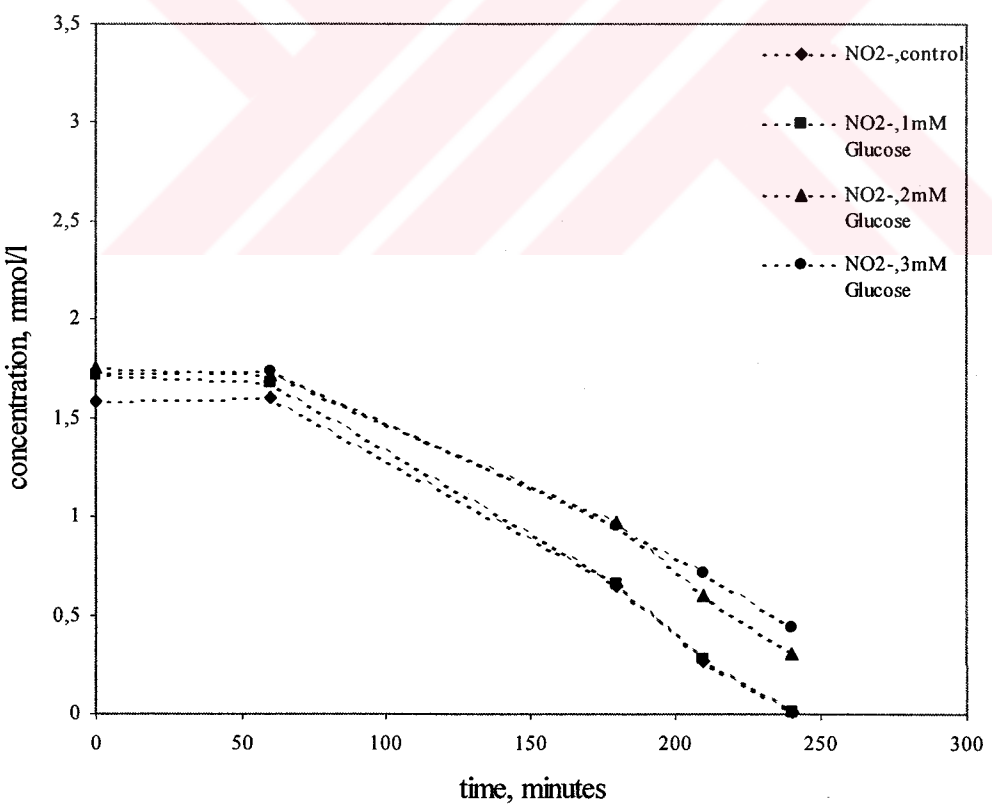


Figure 6.6b Nitrite profiles for 0-1-2-3 mmol glucose/l concentrations.

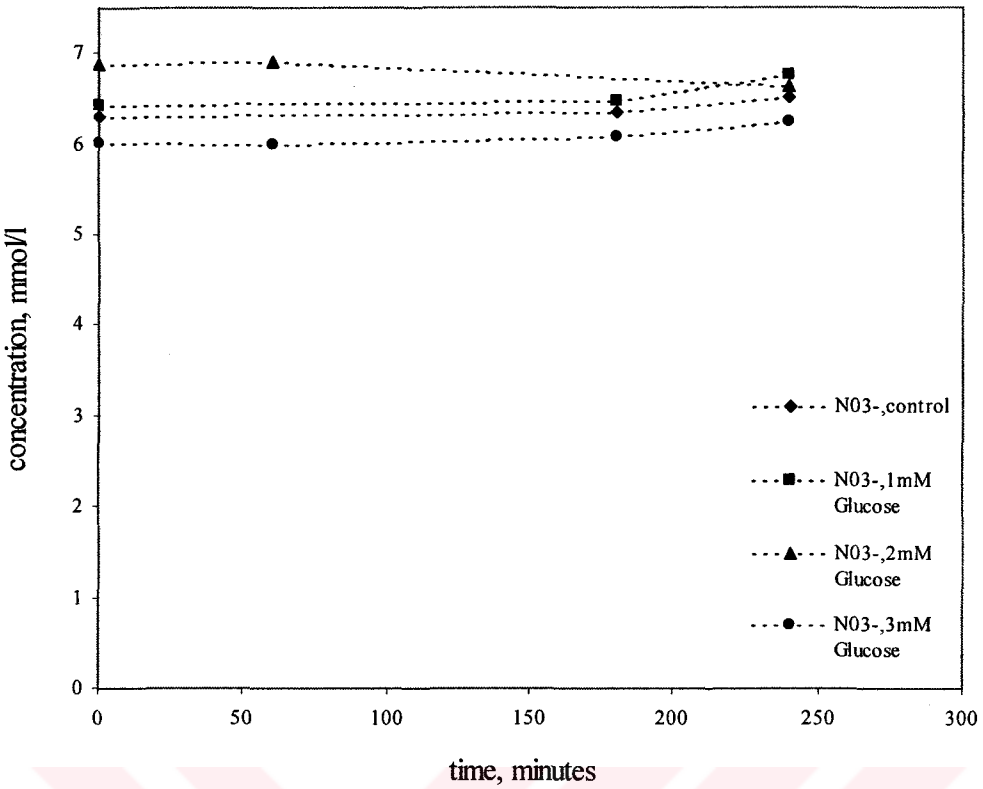


Figure 6.6c Nitrate profiles for 0-1-2-3 mmol glucose/l concentrations.

Figures 6.8a through 6.8c depict ammonia, nitrite and nitrate profiles related with batch experiments with L-alanine. Figures indicated that anammox activity was lower in presence of 0.5, 1 and 2 mmol /l L-alanine, than that observed in the control set. Inhibitory effect of L-alanine on the activity of anammox was calculated as18%.

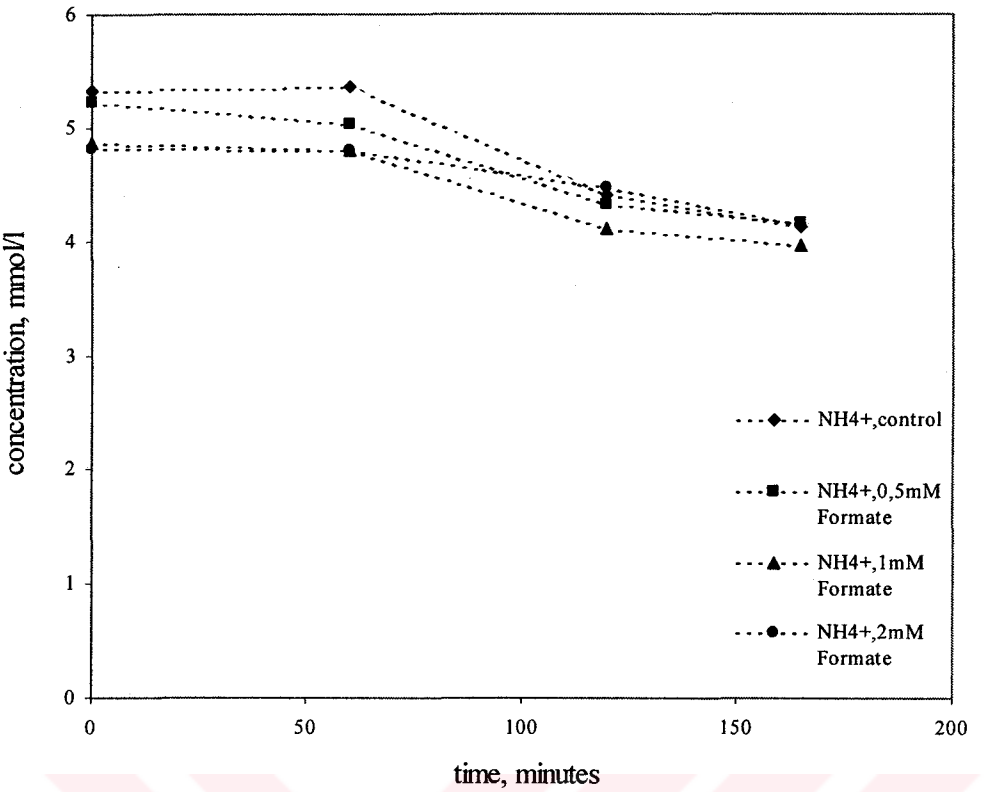


Figure 6.7a Ammonia profiles for 0-0.5-1-2 mmol formate/l concentrations.

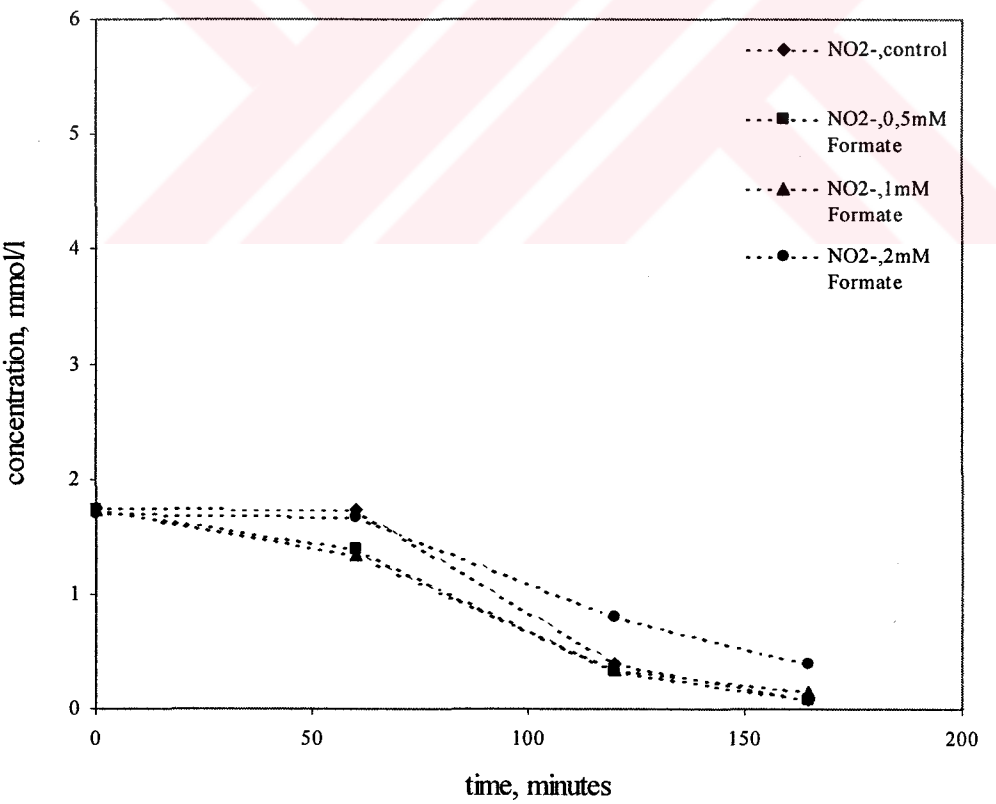


Figure 6.7b Nitrite profiles for 0-0.5-1-2 mmol formate/l concentrations.

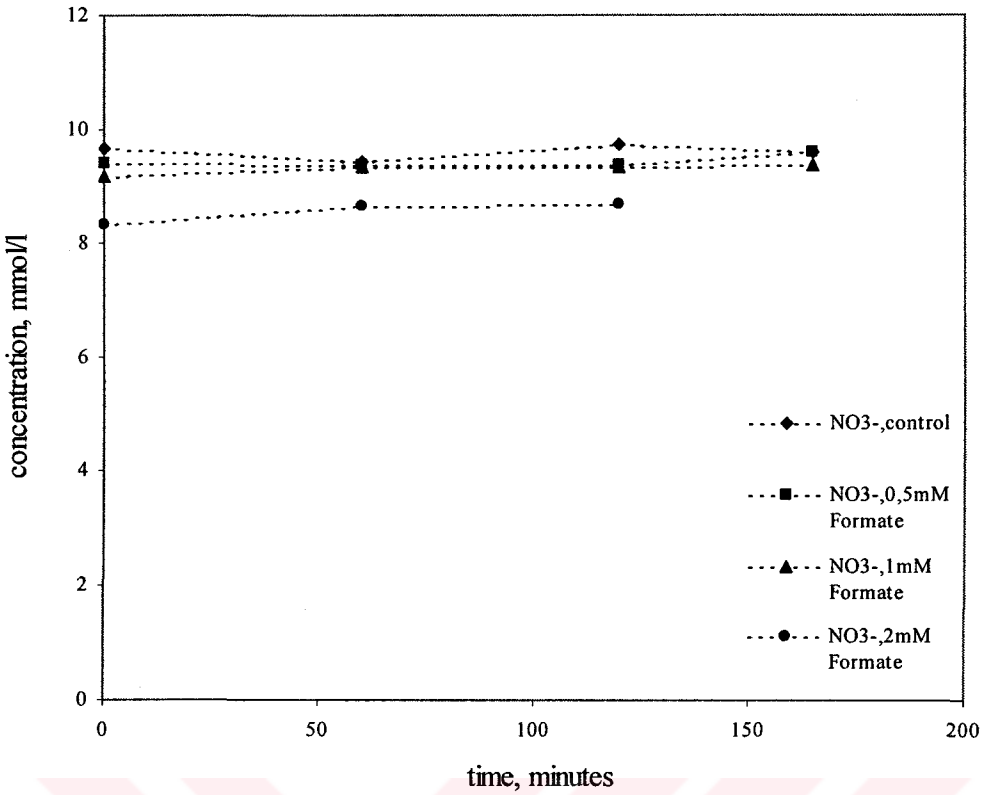


Figure 6.7c Nitrate profiles for 0-0.5-1-2 mmol formate/l concentrations.

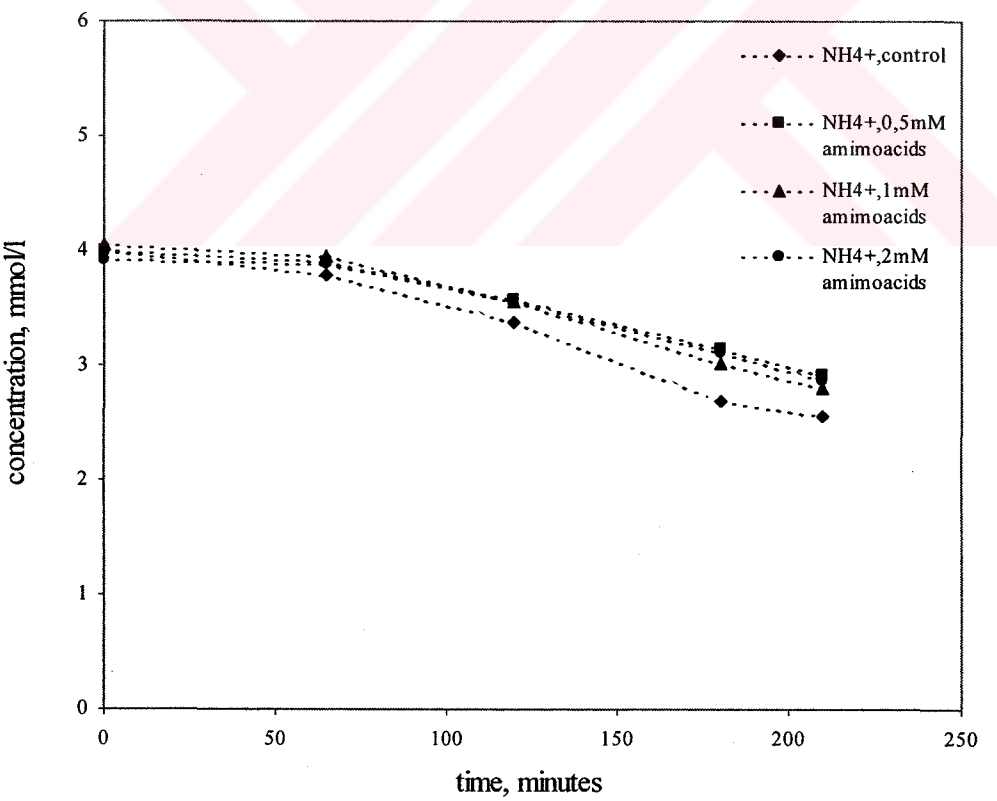


Figure 6.8a Ammonia profiles for 0-1-2-3 mmol amino acids/l concentrations.

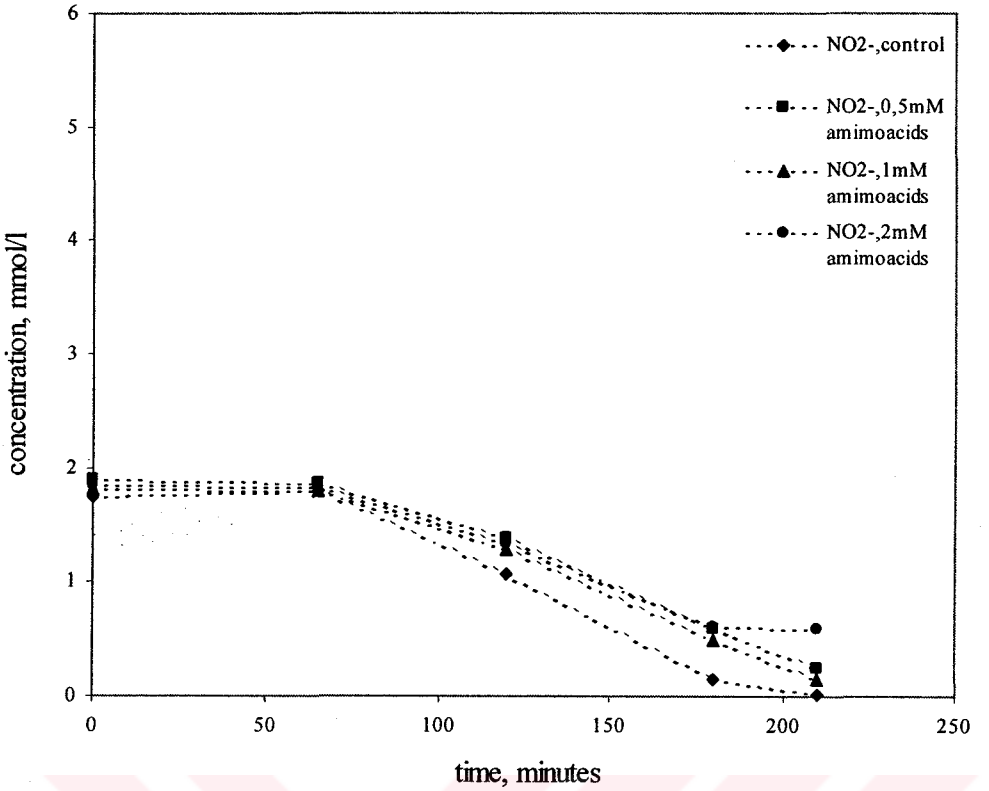


Figure 6.8b Nitrite profiles for 0-1-2-3 amino acids /l concentrations.

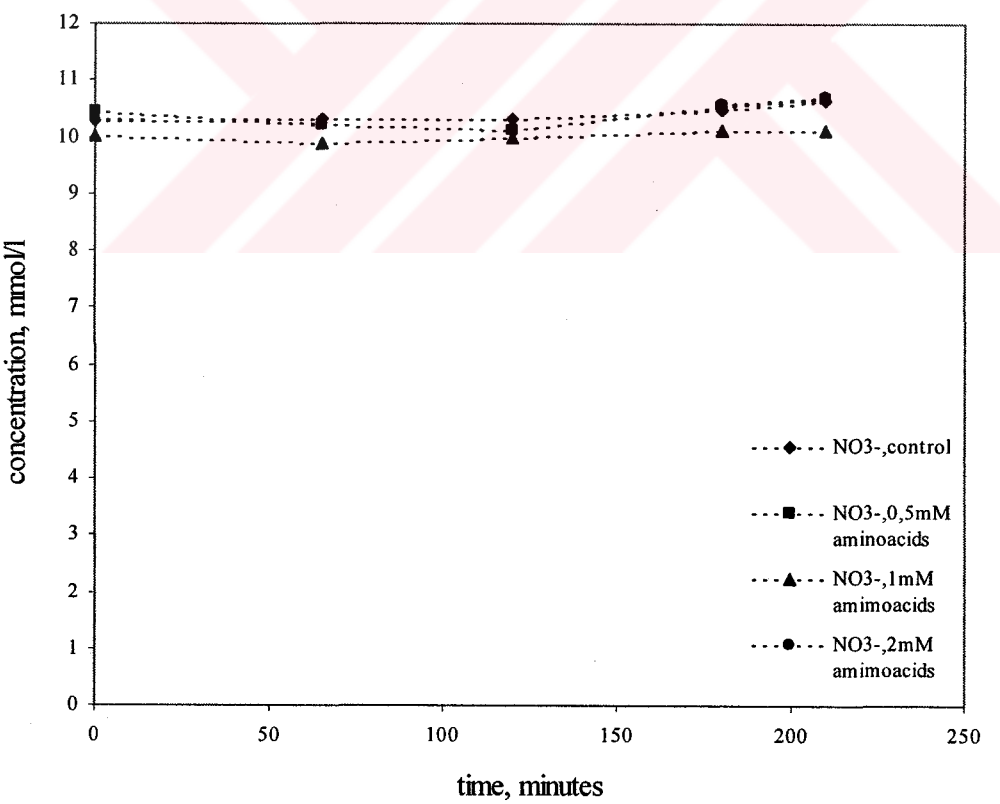


Figure 6.8c Nitrate profiles for 0-1-2-3 amino acids /l concentrations.

6.2 Continuous cultivation of anammox in a bio-reactor

6.2.1 Continuous methanol feeding

Results obtained from the continuous run of the anammox reactor under methanol feeding conditions demonstrated that anammox activity was completely lost in presence of methanol. Flushing a period of 1 week of the culture with sole mineral medium without methanol did not restore the anammox activity. Both batch and continuous cultivation experiments revealed that methanol has a strong inhibitory effect on the anammox process.

6.2.2 Continuous acetate feeding

For the duration of the run, nitrate build-up was observed in the influent possibly due to growth of nitrifiers in the feeding bottle and brought about an increase in the nitrate concentration in the effluent. Although it made difficult to evaluate the results, mass balances were done over the period of 21 days of the run and results were illustrated in Figure 4.10, previously. Figure 6.9 shows theoretically expected total nitrate and measured nitrate load in the effluent. As can be seen from the figure, especially 10 days after the onset of the run, observed nitrate in the effluent was lower than that of stoichiometrically expected values possibly due to denitrification activity of anammox bacteria.

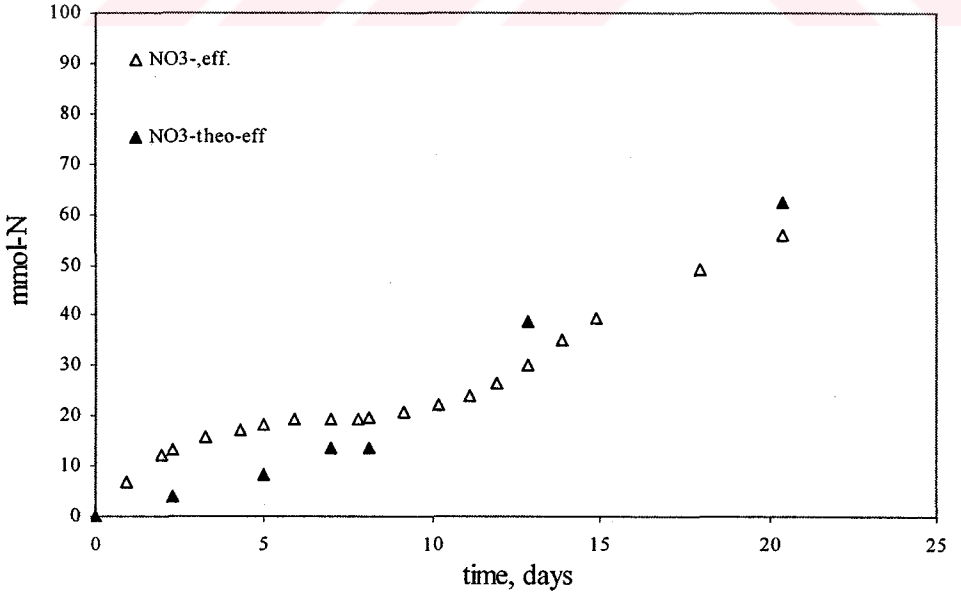


Figure 6.9 Observed and theoretically expected nitrate loads in the effluent.

6.2.3 Continuous propionate feeding

Results obtained from the reactor run are presented in Table 6.3. Results indicated that, until the period of 2.88 mmol/day propionate feeding, ammonia conversion rate was compatible with stochiometrical values. However, observed nitrate production rate was lower than stochiometrically expected values. The lowest nitrate concentration in the effluent was observed in the period of 3.6 mmol propionate/day feeding. Meanwhile, nitrite was completely converted in the reactor but observed ammonia conversion was decreased especially in the period of 3.6 mmol/day propionate feeding. When propionate feeding was decreased again to 1.44 mmol/day ammonia conversion was increased again that could be seen as decrease in ammonia concentration. Increase in ammonia concentration during period of increased propionate supply could be concluded as decrease in the activity of anaerobic ammonium oxidation in presence of propionate. Decrease in nitrate concentration could be explained by either a result of decreased anammox activity that would be resulted in less nitrate production or denitrification activity of anammox in presence of propionate as an alternative electron donor. Figure 6.10 shows observed and theoretical nitrate profiles in the propionate feeding reactor.

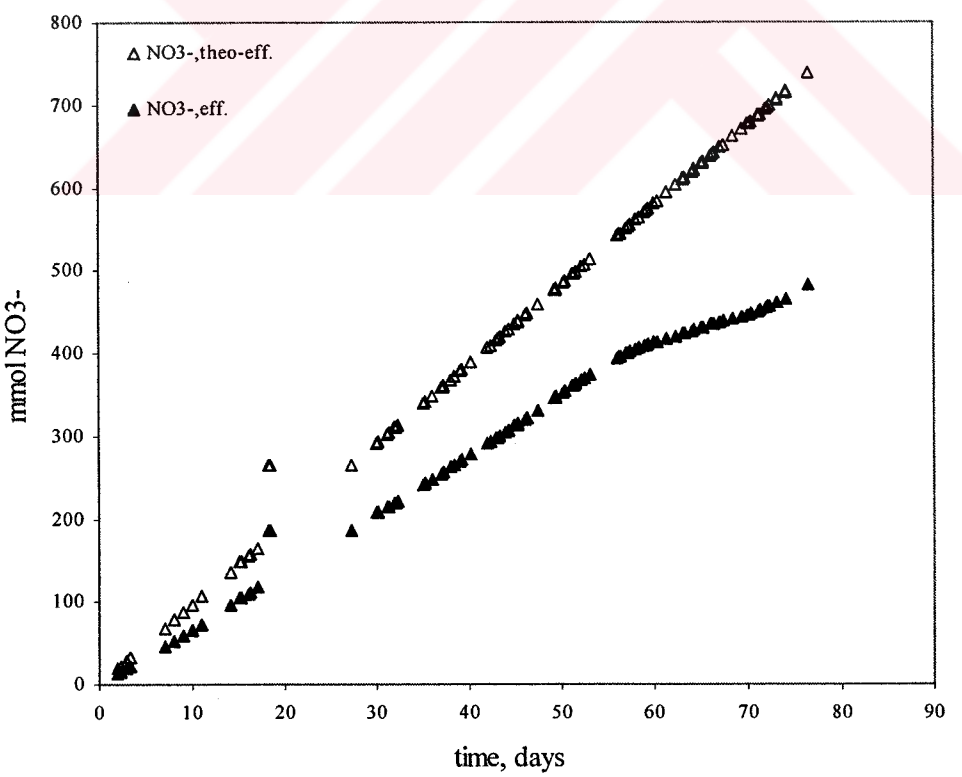


Figure 6.10 Observed and theoretically expected nitrate profiles in the effluent.

Table 6.3 Mass balances for propionate feeding reactor for the parameters ammonia, nitrite and nitrate.

Time [days]	Propionate feeding [mmol/d]	Propionate out [mM]	Ammonium in [mmol/d]	Nitrite in [mmol/d]	Nitrate in [mmol/d]	Ammonium out [mmol/d]	Nitrite out [mmol/d]	Nitrate out [mmol/d]	Ammonium out expected [mmol/d]*	Diff.** [mmol/d]	Nitrate out expected [mmol/d]*	Diff.*** [mmol/d]
27-42	0.36	0.001	45.9	49.3	0.13	8.11	0.08	7.65	8.55	-0.44	9.69	-2.04
42-50	0.72	0.001	49.3	49.3	0.15	12.12	0.1	7.65	11.95	0.17	9.69	-2.04
50-56	1.44	0.001	49.3	49.3	0.13	11.4	0.12	7.63	11.95	-0.55	9.69	-2.06
56-61	2.88	0.002	49.3	49.3	0.13	11.2	0.07	4.85	11.95	-0.75	9.69	-4.85
61-65	1.44	0.002	49.3	49.3	0.13	13.5	0.07	3.66	11.95	1.55	9.69	-6.04
65-70	3.6	0.004	49.3	49.3	0.15	17.5	0.12	3.4	11.95	5.55	9.69	-6.29
70-76	1.44	0.001	49.3	49.3	0.15	14.56	0.07	4.85	11.95	2.61	9.69	-4.85

*Expected ammonium conversion and nitrate production rate based on the stoichiometry of anaerobic ammonium oxidation process.

**Difference between observed ammonium conversion rate and stoichiometrically expected rate.

***Difference between observed nitrate production rate and stoichiometrically expected rate.

6.3 Population shift due to acetate and propionate feeding

A slight change in the anammox population was detected after the onset of the acetate feeding as can be seen from the Figure 6.11a. In the picture, the yellow area shows Pla46 and Amx820 binding and a minor area (red) was detectable which was bind with Pla46 probe but not with Amx820 probe. Whereas strong signals with Pla46 probe were obtained from the whole clusture which indicates dominancy of the planctomycetes. Since Amx820 probe targets all known Anammox bacteria, this minor area could be an indication of new species of anammox due to acetate feeding.

Fluorescence in situ hybridization analysis showed that the bio-community composition was significantly changed under propionate feeding conditions. Figure 6.11b reveals the change in anammox population. Purple denotes the hybridizations with probes PLA46, DH_2-432, and Amx820. Whereas the yellow part of the cluster shows the cells that were not bind with Amx820 probe. This strongly indicates the new species of anammox due to propionate feeding.

FISH analysis were also performed using probes targeting α, β and γ- Proteobacteria to search denitrifiers in both acetate and propionate feeding reactors. Any bacterial community belong to these subclasses of Proteobacteria was ever detected in the acetate feeding reactor. While in the propionate feeding reactor, 34 days after the onset of the propionate feeding, few β- Proteobacteria were detected that might indicated denitrifiers. However, these β-proteobacteria might also indicated nitrifiers which were known to be present in low numbers in the 64% and 74% enriched anammox culture (van De Graaf, et. al, 1995; Strous, 2002). Although it is known that a significant portion of denitrifiers belong to β subclass of the Proteobacteria, this detection could not be an absolute indication of denitrifiers. Moreover, a clear denitrifying activity was not observed although propionate was completely consumed in the reactor. Since it is known that a wide taxonomic range of bacteria can denitrify, it is difficult to identify denitrifiers using currently designed 16S rDNA specific oligonucleotide probes. A new molecular technique; combination of immunofluorescence and FISH analysis was applied. The method applied to the biomass sample reflecting the 84^{th} day of the propionate feeding period. Results showed that very small amount of denitrifying bacteria were detectable although such a long period of propionate feeding.

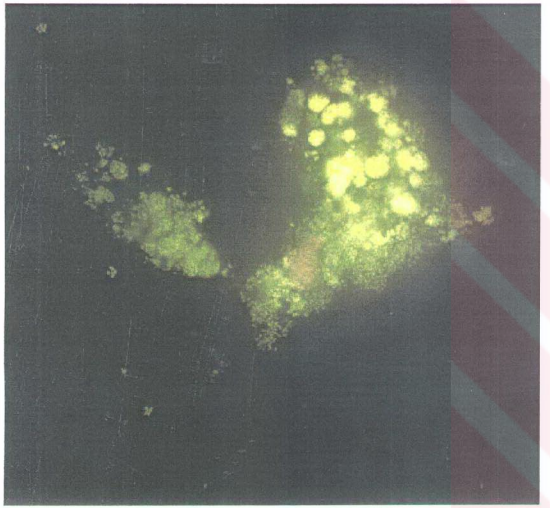


Figure 6.11a Change in population after acetate feeding

Yellow: PLA46+Amx820

Red: PLA46

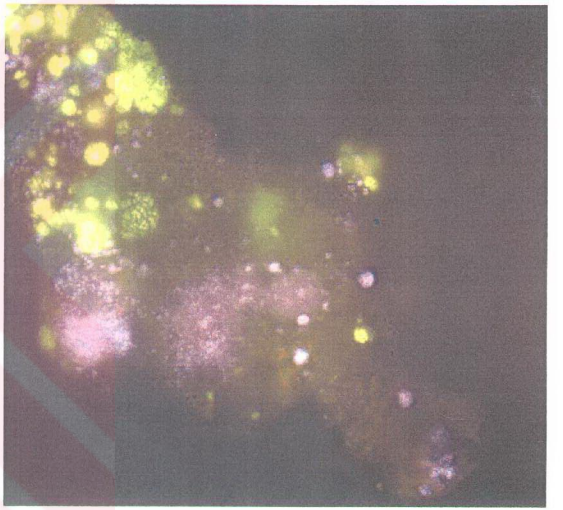


Figure 6.11b Change in population after propionate feeding

Yellow: DH_2-432 + PLA46

Purple: DH_2-432 + PLA46 + Amx820

6.4 Evaluations of the results for the investigation of propionate consumption by anammox bacteria

So far anammox is known as obligate chemolithoautotroph, another group of bacteria could be responsible for propionate consumption. In theory, addition of an organic compound to an anammox reactor would lead to build up of heterotrophs and some of them could denitrify nitrate, using the organic compound as the electron donor. However, FISH results obtained from the continuous cultivation experiments with propionate showed that, there were not detectable amount of organism group able to denitrify. In addition, nitrate concentration stayed stable especially until 2.88 mmol propionate/day whereas, propionate was consumed completely in the reactor. Here we can claim about mainly three alternatives:

1. Limited amount of denitrifiers were build-up in the propionate reactor and there is a possibility that they use nitrite instead of nitrate, thus nitrate concentration stayed stable,

2. Denitrifiers were active but their nitrate reduction rate was slower than nitrate production rate of anammox, thus nitrate conversion can not be detected,

3. Anammox bacteria could be responsible for propionate consumption.

Results obtained from the batch experiments performed for searching the denitrifier activity showed that nitrate or nitrite conversion was detected in presence of methanol and propionate. Where, in the absence of methanol, ammonia and nitrite concentrations were decreased as the confirmation of the anammox activity. This indicates that there was no denitrifier activity in the propionate feeding reactor. That shows the dominancy of the anammox activity in the propionate feeding reactor in this time period. Since results obtained from the batch sets D_{11} and D_{12} are quite consistent with each other (similarly D_{21} and D_{22}) results belong to experimental sets D_{11} and D_{21} were used for comparison as illustrated in Figure 6.12.

Pulse addition experiments with propionate in a 74% enriched anammox reactor showed that propionate was consumed without a lag phase. These results indicated that presently active and dominant group of organism should be responsible for

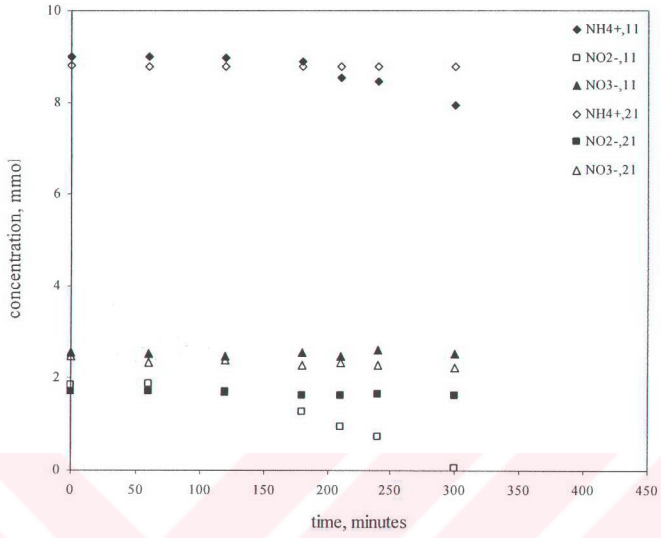
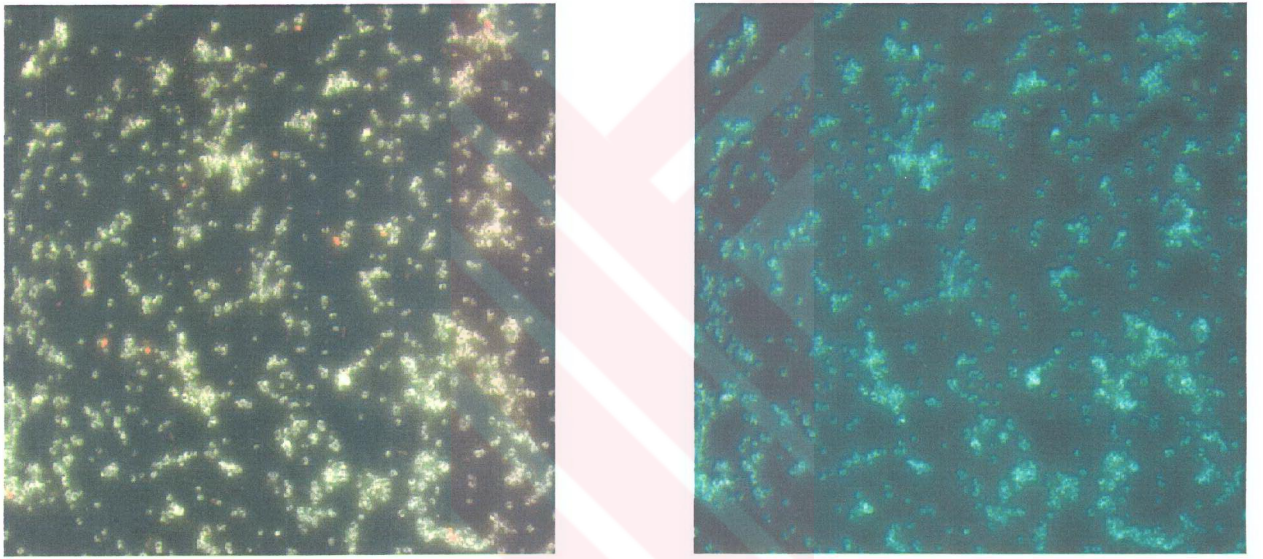


Figure 6.12 Comparative ammonia, nitrite and nitrate profiles of the sets D_{11} and D_{21}.

propionate consumption. Considering the circumstances of the experiments have been done until this point, anammox bacteria could be responsible for propionate consumption.

Although the theory of propionate consumption by anammox bacteria was strengthened by these results, observations were still not enough to prove this claim since anammox source reactor was consisted of 74% enriched culture. In this context, a pure culture of anammox cells was obtained from the anammox enrichment culture using density gradient centrifugation. Micro batch experiment was conducted with purified anammox cells and propionate consumption by anammox was proved. The purity of the obtained cell suspension was assessed using Fluorescence in situ hybridization analysis. In Figure 6.13 FISH pictures are presented and result showed that obtained purity was approximately 99.5%. All cells were bound with EUB338 probe. As can be seen from the picture (1) almost all single cells in the culture were bound with PLA 46 and Amx820 probe, only very few signals were detected which are bound with EUB338 but not with PLA 46 or Amx820 probe as shown in picture (2).



(1) (2)

Figure 6.13 FISH pictures of purified anammox cells, views of the same microscobic field.
Yellow: EUB338 + PLA46 + Amx820
Red: EUB
Turquoise:PLA46+Amx820

6.5 Evaluations of the experimental results of the investigation of the nitrogen compound coupled to propionate oxidation and possible reaction mechanism

6.5.1 Survey of the nitrogenous compound

In experimental set 1, only propionate was added to the anammox enrichment culture to observe the end of propionate in the absence of an electron acceptor. Figure 6.14 illustrates ammonia, nitrite, nitrate and propionate profiles of the experimental set 1.

In order to explain the results of the batch sets, the better way would be divide the graph in 3 periods. Periods 1, 2 and 3 represent hours between 0-5.5 and 5.5-18 and 18-24 respectively. Table 6.4 presents the changes in ammonia, nitrite, nitrate and propionate concentrations. It can be clearly seen that propionate concentration was stayed stable. Figure also indicates that ammonia concentration increased especially in the last 6 hours of the run possibly due to cell lysis.

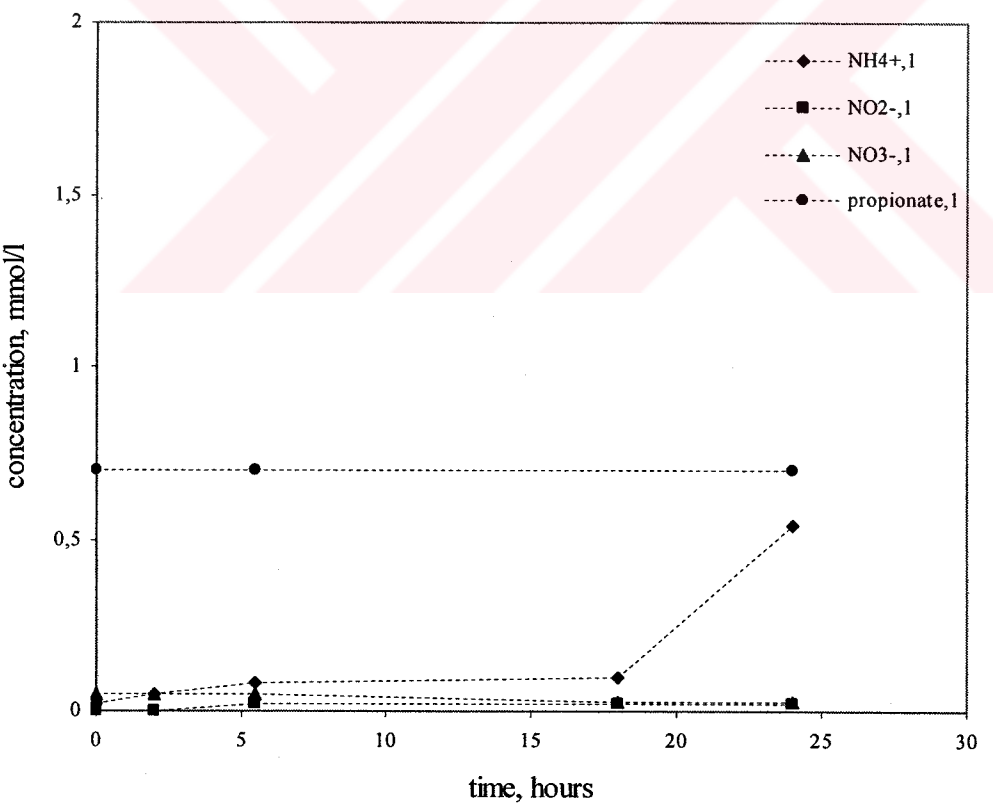


Figure 6.14 Ammonia, nitrite, nitrate and propionate profiles of the experimental set1.

Table 6.4 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental set1.

Set no	Period	NH_4^+ µmol/l	NO_2^- µmol/l	NO_3^- µmol/l	Propionate µmol/l
set 1	1	-	-	-	-
set 1	2	-	-	-	-
set 1	3	+460	-	-	-
	Total	**+460**	-	-	-

Set 2 was consisted of ammonia and propionate. Results obtained from the experiment were previously presented in chapter 4 Figure 4.19a to 4.19d. Figures 6.15a and 6.15b illustrates comparative ammonia, nitrite, nitrate and propionate profiles related with sets 2a and 2b. Changes in ammonia, nitrite, nitrate and propionate concentrations are presented in Table 6.5. It can be clearly seen from the table, ammonia concentration was not changed during 18 hours, however significant increase in ammonia concentration was observed hours between 18 to24, possibly due to cell lysis. Propionate was not consumed during the whole course of the experiment.

Table 6.5 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental sets 2a and 2b.

Set no	Period	NH_4^+ µmol/l	NO_2^- µmol/l	NO_3^- µmol/l	Propionate µmol/l
2a	1	-	-	-	-
2b		-	-	-	-
2a	2	+320	-	-	-
2b		+210	-	-	-
2a	3	+2640	-	-	-
2b		+2760	-	-	-
2a	**Total**	**+2960**	-	-	-
2b		**+2970**	-	-	-

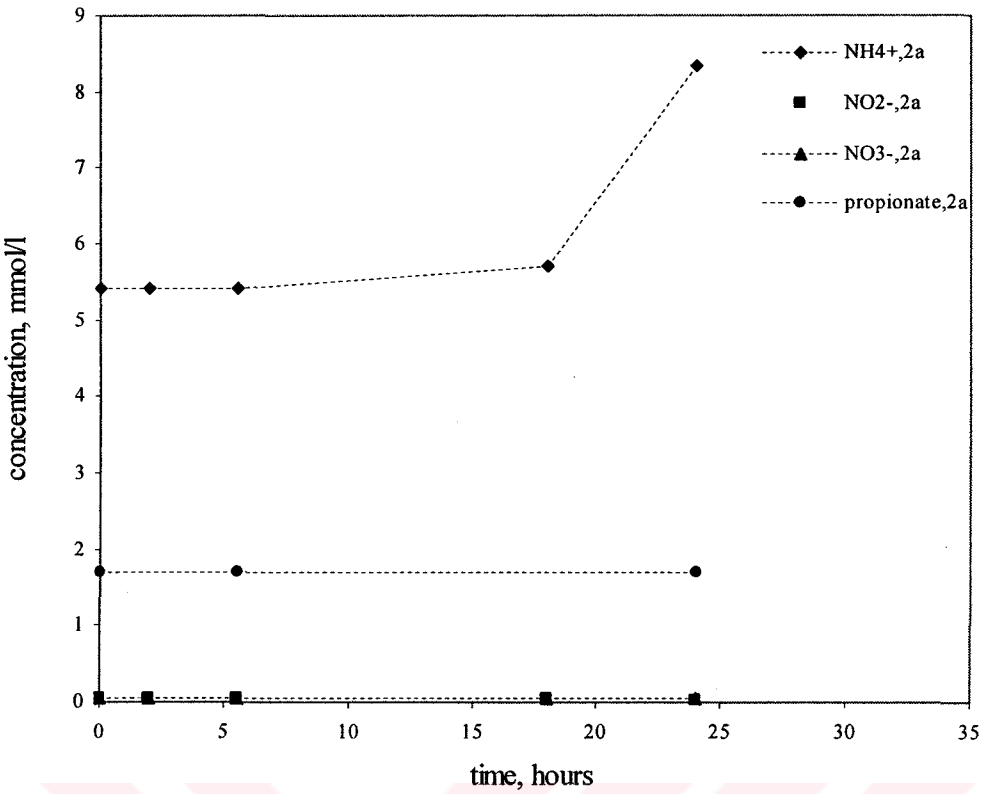


Figure 6.15a Ammonia, nitrite, nitrate and propionate profiles of the experimental set 2a

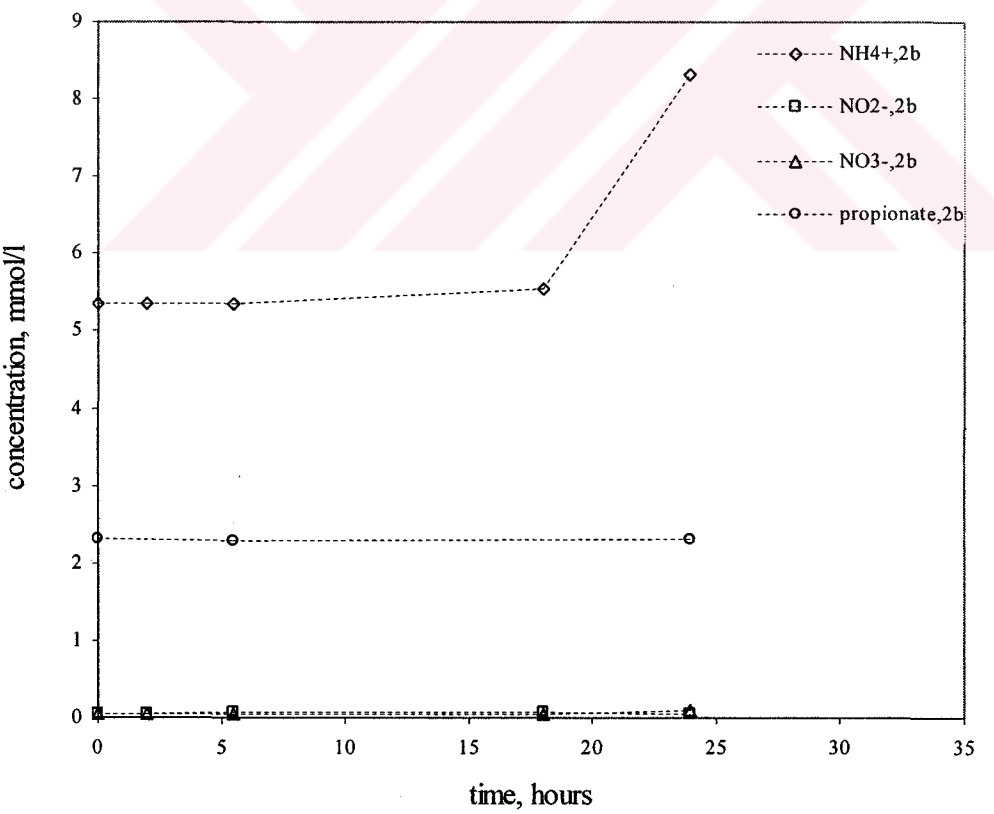


Figure 6.15b Ammonia, nitrite, nitrate and propionate profiles of the experimental set2b

Experiment 3 was conducted in presence of nitrite and propionate. Table 6.6 presents observed changes in the concentrations ammonia, nitrite, nitrate and propionate. As can be seen from the table, in the whole course of the run 3135 and 2170 μM nitrite conversion was observed in set 3a and set 3b, respectively. Meanwhile propionate was not consumed. Since medium was not contained ammonia initially, the end of nitrite is unclear. Ammonia that released due to cell lysis or produced from the reduction of nitrite (Equation 6.1) may conduct the anammox metabolism. Comparative ammonia, nitrite, nitrate and propionate profiles are illustrated in Figure 6.16a and 6.16b related with experimental sets 3a and 3b.

$$C_3H_6O_2 + 2.34\,NO_2 + 2.34H^+ \longrightarrow 3CO_2 + 2.34NH_3 + 0.67H_2O \qquad (6.1)$$

Table 6.6 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental sets 3a and 3b.

Set no	Period	NH_4^+	NO_2^-	NO_3^-	Propionate
		μmol/l	μmol/l	μmol/l	μmol/l
3a	1	+260	-570	-	-
3b		+150	-205	-	-
3a	2	+80	-510	-	-
3b		-110	-1535	-	-
3a	3	-320	-1090	-	-
3b		-180	-1395	-	-
3a	**Total**	-	**-2170**	-	-
3b		-	**-3135**	-	-

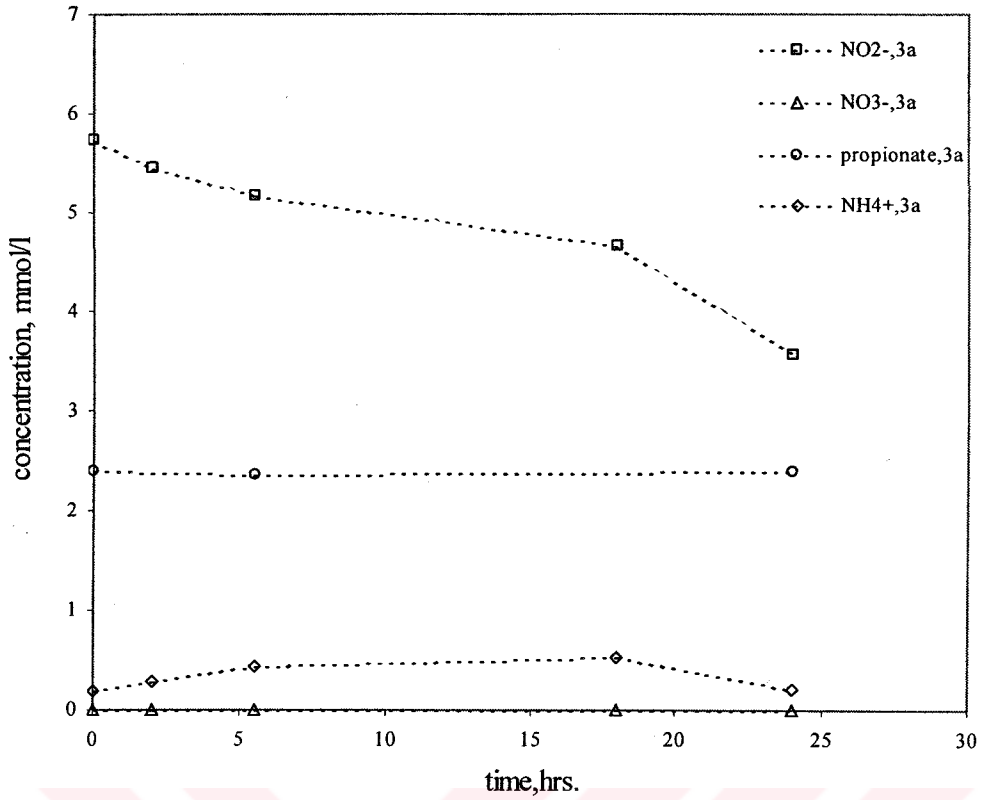


Figure 6.16a Ammonia, nitrite, nitrate and propionate profiles of the experimental set3a.

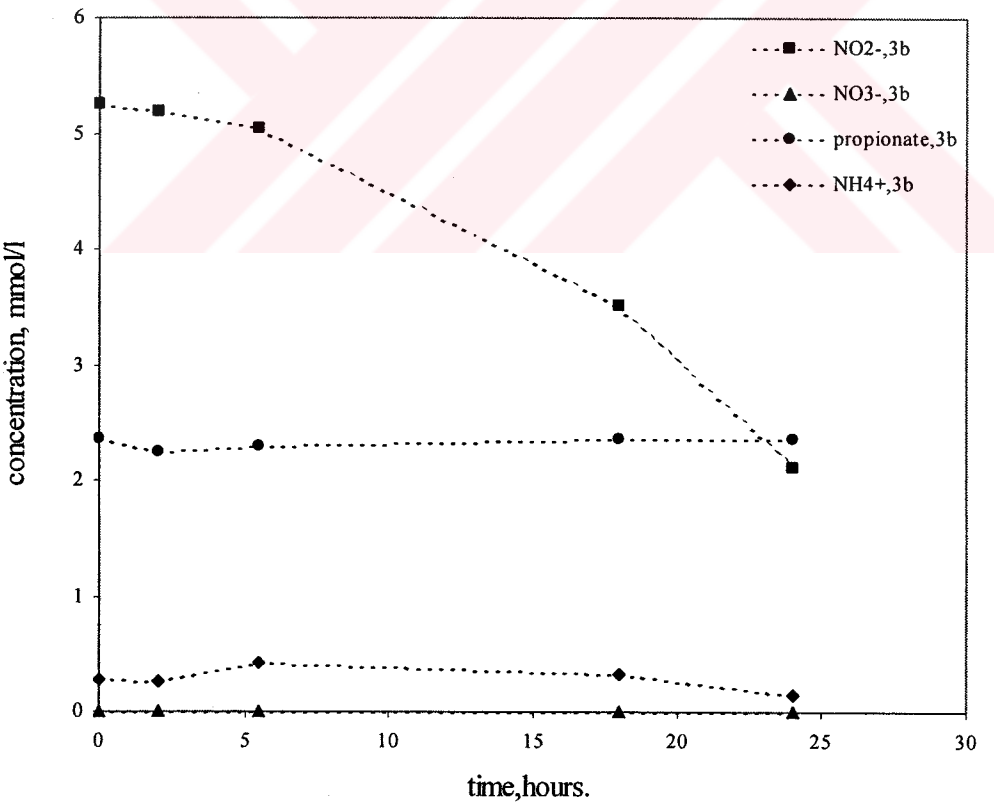


Figure 6.16b Ammonia, nitrite, nitrate and propionate profiles of the experimental set3b.

In set 4 components were nitrate and propionate. Ammonia, nitrite, nitrate and propionate conversions and productions are presented in Table 6.7. Ammonia, nitrite, nitrate and propionate profiles related with set 4a and 4b are illustrated in Figure 6.20a and 6.20b. In period 1, observed nitrate and propionate consumptions were resulted in a nitrate to propionate ratio of 2.18 and 2.5 in sets 4a and 4b, respectively. In period 2, in sets 4a and 4b nitrate and propionate conversions were obtained with a nitrate/propionate ratio of 2.23 and 2.26, respectively. These values are close to the ratio of 2.8 which is obtained from energy reaction of denitrification of NO_3^- to N_2 with propionate, which is presented as Equation 6.2.

$$C_3H_6O_2 + 2.8\ NO_3^- + 2.8\ H^+ \longrightarrow 3CO_2 + 1.4N_2 + 4.4H_2O \quad (6.2)$$

$$C_3H_6O_2 + 7\ NO_3^- \longrightarrow 3CO_2 + 7NO_2^- + 3H_2O \quad (6.3)$$

Interestingly, nitrite production was observed during period 2; 1625 μM in set 4a, 100 μM in set 4b and converted completely in period 3. Nitrate and propionate conversions continued during period 3 in set 4b whereas it was almost completed in set 4b in period 2. This observation may also explain differences between set 4a and 4b for nitrite production during period 2 as a delay. To explain nitrate reduction with propionate and temporary nitrite accumulation a model was developed which is presented in Figure 6.17. According to proposed model, denitrifying activity of anammox (AD) takes place using nitrate as electron acceptor and propionate as electron donor. Part of the nitrate converted to nitrite as an intermediate and simultaneously converted to dinitrogen gas with ammonia as anammox (A) activity, while the rest is converted to dinitrogen gas with denitrification activity of anammox.

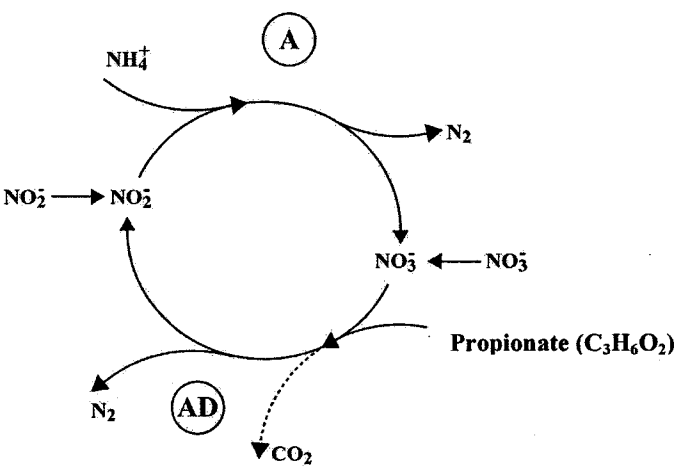


Figure 6.17 Proposed model for nitrate reduction with propionate by anammox.

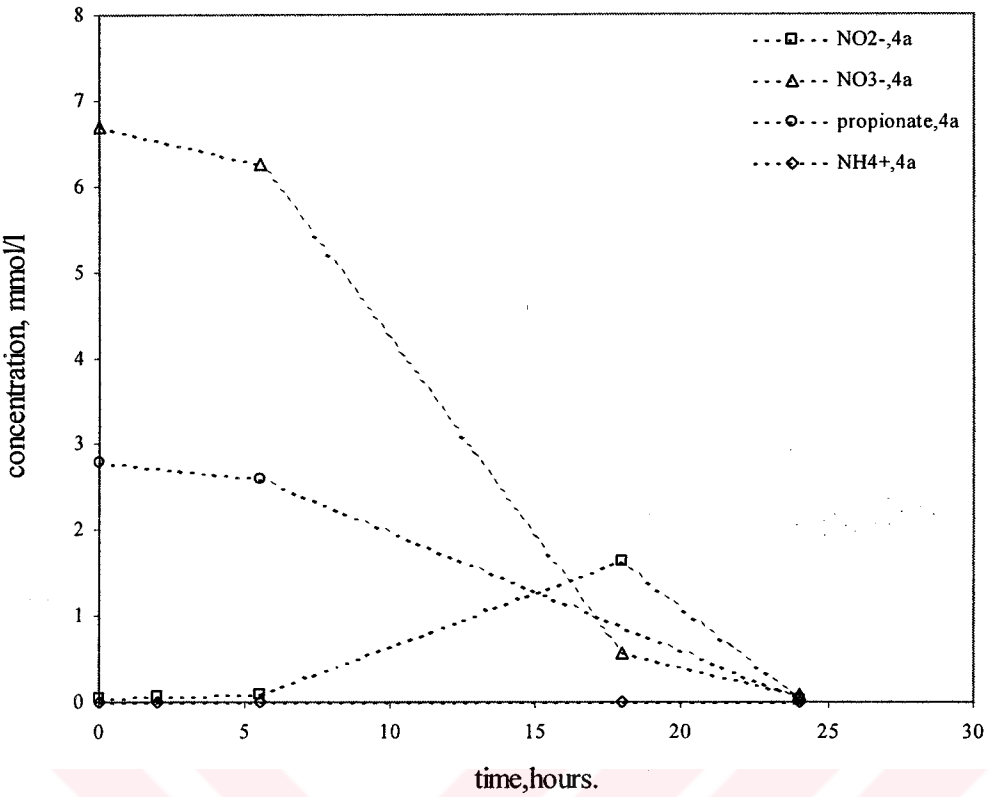


Figure 6.18a Ammonia, nitrite, nitrate and propionate profiles of the experimental set4a.

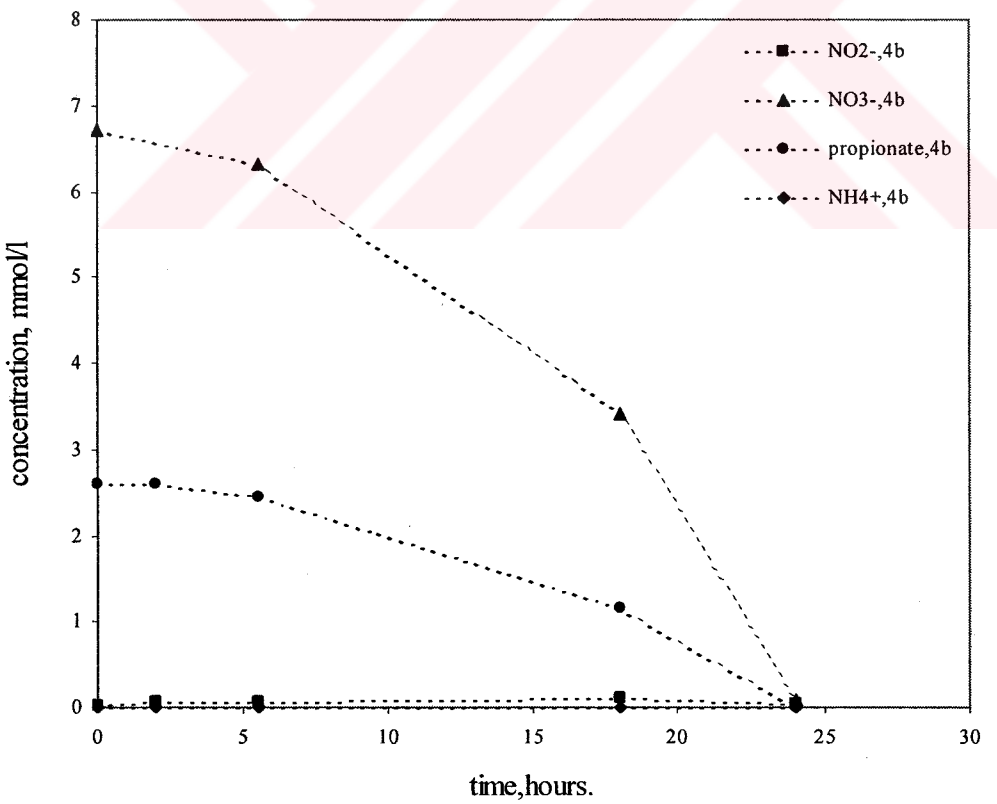


Figure 6.18b Ammonia, nitrite, nitrate and propionate profiles of the experimental set4b.

Table 6.7 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental sets 4a and 4b.

Set no	Period	NH_4^+ µmol/l	NO_2^- µmol/l	NO_3^- µmol/l	Propionate µmol/l	NO_3^-/propionate
4a	1	-	-	-435	-200	2.18
4b		-	-	-375	-150	2.5
4a	2	-	+1625	-5705	-2600	2.23
4b		-	+100	-2900	-1300	2.26
4a	3	-	-1600	-475	-60	-
4b		-	-100	-3420	-1150	2.97
4a	**Total**	-	-	**-6615**	**-2860**	**2.3**
4b				**-6695**	**-2600**	**2.58**

Set 5 was consisted of ammonia, nitrate and propionate and observed conversions are presented in Table 6.8. Ammonia, nitrite, nitrate and propionate profiles are illustrated in Figures 6.20a and 6.20b. Although results obtained from 5a and 5b were not in agreement in terms of NO_3^-/propionate ratio in especially period 1 and 2, the ratios of total nitrate conversion and propionate consumption were quite consistent with each other, 2.5 and 2.74 in set 5a and 5b, respectively. These ratios are compatible with stoichiometrically obtained ratio of 2.8. Interestingly, ammonia conversion was observed in period 2. This can be explained by the proposed model as additional ammonia conversion with nitrite which was partially formed by nitrate conversion as denitrification activity of anammox.

Table 6.8 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental sets 5a and 5b.

Set no	Period	NH_4^+ µmol/l	NO_2^- µmol/l	NO_3^- µmol/l	Propionate µmol/l	NO_3^-/ propionate
5a	1	-	-	-520	-150	3.5
5b		-	-	-180	-200	0.9
5a	2	-830	-	-1460	-450	3.2
5b		-1080	-	-1324	-500	2.65
5a	3	+380	-	-1325	-720	1.84
5b		-	-	-2880	-900	3.2
5a	**Total**	-	-	**-3305**	**-1320**	**2.5**
5b				**-4384**	**-1600**	**2.74**

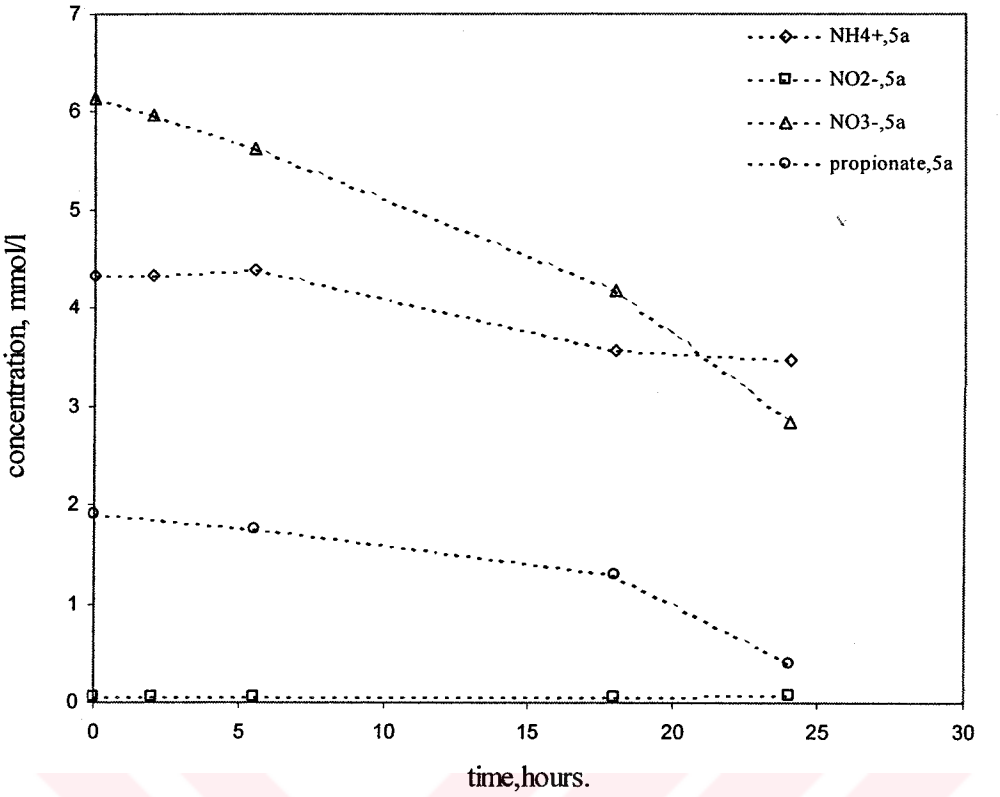


Figure 6.19a Ammonia, nitrite, nitrate and propionate profiles of the experimental set5a.

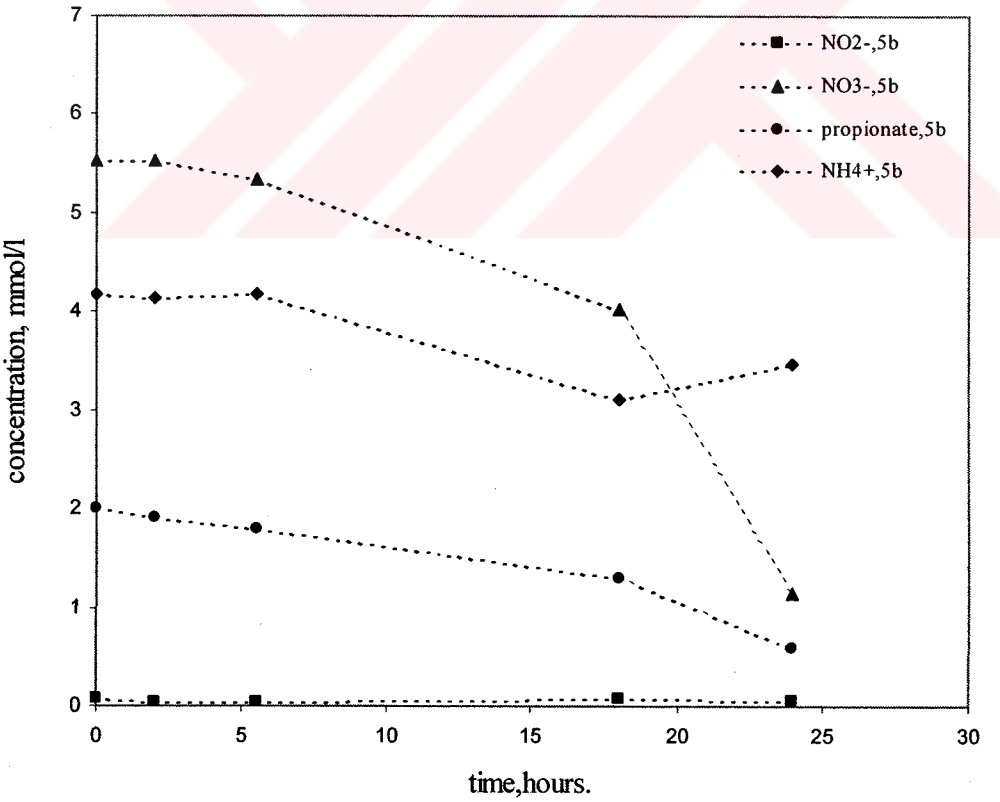


Figure 6.19b Ammonia, nitrite, nitrate and propionate profiles of the experimental set5b.

Set 6 was consisted of ammonia, nitrite, nitrate and propionate. Results obtained from sets 6a and 6b are illustrated in Figure 6.20a and 6.20b. Ammonia, nitrite, nitrate and propionate conversions and productions are presented in Table 6.9. Table 6.10 depicts comparison of measured conversions and stoichiometrically expected values.

Table 6.9 Changes in ammonia, nitrite, nitrate and propionate concentrations in experimental sets 5a and 5b.

Set no	Period	$^{*}NH_4^+$ µmol/l	$^{*}NO_2^-$ µmol/l	$^{*}NO_3^-$ µmol/l	Propionate µmol/l
6a	1	-2290	-4215	+385	-100
6b		-1660	-4645	+195	-50
6a	2	-690	-	-1075	n.a
6b		-550	-	-1905	-250
6a	3	+160	-	-1135	n.a
6b		-800	-	-2725	-600
	Total	**-2980**	**-4215**	**-2210**	**-800**
		-3010	**-4645**	**-4630**	**-950**

* Measured conversion values
n.a: not available

Table 6.10 Comparison of measured conversions and stoichiometrically expected values related with sets 6a and 6b.

Set no	Period	$^{*}NO_3$/ propionate	$^{**}NH_4^+$ µmol/l	$^{**}NO_3^-$ µmol/l	Difference NH_4^+,µmol/l	Difference NO_3^-,µmol/l
6a	1	-	-3200	+830	-1860	-720
6b		-	-3520	+915	-910	-635
6a	2	n.a.	-	-	-690	-1075
6b		7.62	-	-	-550	-1905
6a	3	n.a.	-	-	+160	-1135
6b		4.54	-	-	-800	-2725
6a	**Total**	**2.76**				
6b		**4.87**				

** Calculated conversions according to anammox stochiometry
* Measured conversion values
n.a: not available

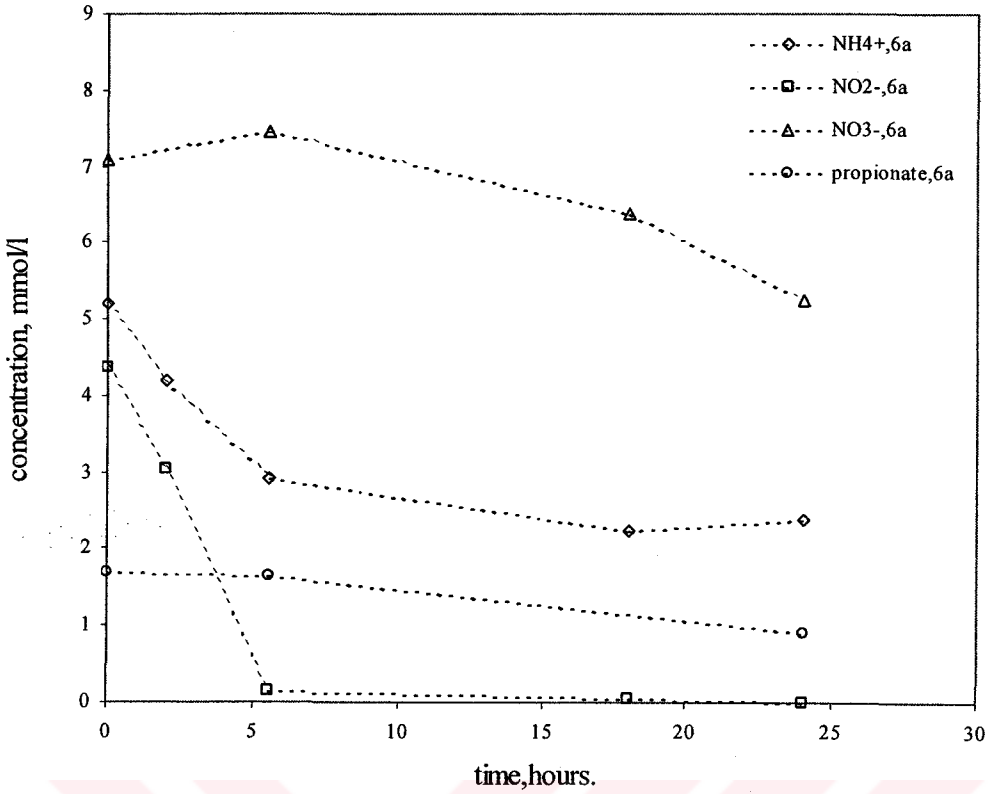


Figure 6.20a Ammonia, nitrite, nitrate and propionate profiles of the experimental set6a.

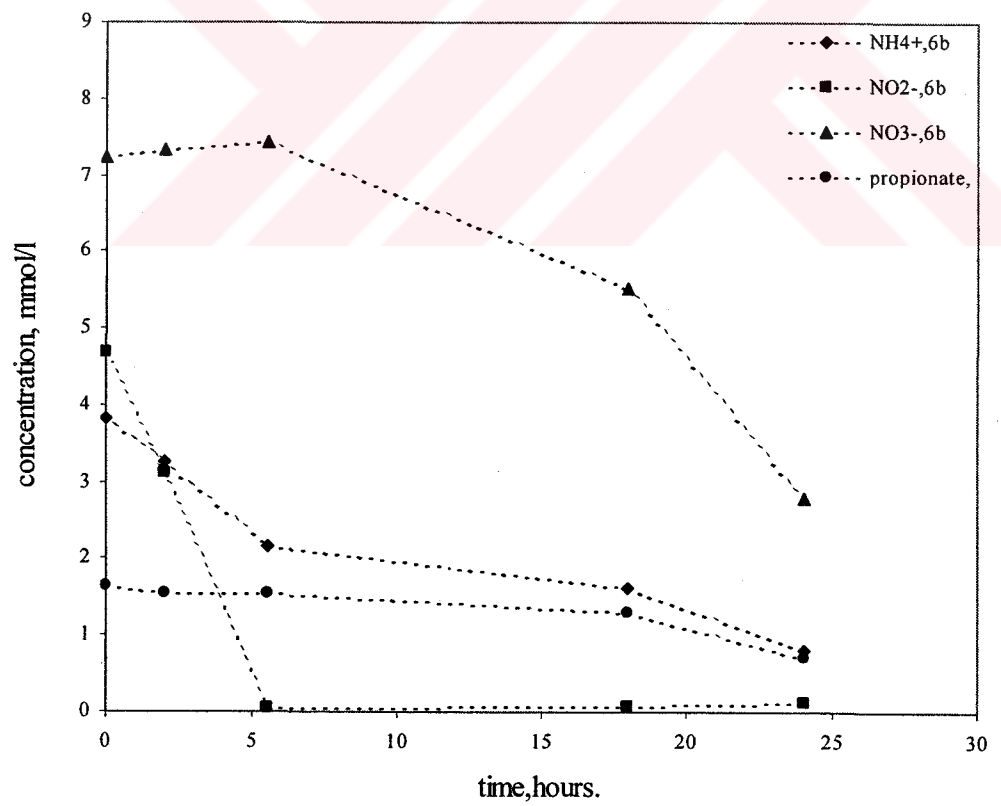


Figure 6.20b Ammonia, nitrite, nitrate and propionate profiles of the experimental set6b.

In both experimental sets 6a and 6b, it was observed that anammox activity (A) was the main activity in period 1. Meanwhile, propionate consumption was observed as 100 μM and 50 μM which indicates the propionate consumption during anammox activity. It can be clearly seen from the Table 6.9 that less ammonia conversion and less nitrate production were obtained compared to anammox stoichiometry. Less nitrate production can be explained by reduction of nitrate with propionate. In period 2, nitrate conversion was observed with a nitrate to propionate ratio of 7.62 in set 6 a. This ratio is quite high compared to 2.8 which was presented in Equation 6.2 previously. It is interesting that obtained ratio of 7.62 is in good agreement with the nitrate to propionate ratio of 7 obtained from the energy reaction of denitrification of nitrate to nitrite, as presented in Equation 6.3. Although nitrite formation was not detected during the whole course of the experiment, this result may also as the indication of partial conversion of nitrate to nitrite in presence of propionate as electron donor.

Since nitrite was completely converted in period 1, anammox activity was not expected anymore in period 2. Whereas, decrease in ammonia concentrations was observed in both sets 6a and 6b. Results obtained from the experimental set 1 and set 2 showed that during 18 hours of the run there were no ammonia conversion in presence of only ammonia or ammonia + propionate in the medium. So that, ammonia is not coupled to propionate and anammox converts ammonia only in presence of nitrite as electron acceptor. Thus, observed ammonia conversion in experimental set 6a in period 2 was possibly due to additional anammox activity with nitrite which is formed from reduction of nitrate with propionate.

6.5.2 Evaluation of the ^{15}N-labeling experiments

The reaction mechanism and end-product of nitrate reduction coupled to propionate oxidation was investigated by batch experiments with anammox enrichment cultures supplying ^{15}N labeled nitrate and nitrite. ^{15}N labeling pattern of N_2 was monitored by measuring $^{15}NO_3^-$, $^{15}NO_2^-$, $^{14,15}N_2$, and $^{15,15}N_2$.

When ^{15}N- nitrate incubated with only propionate, as stated in experimental Set 1, $^{14,14}N_2$ and $^{15,15}N_2$ were both detected. Thus, the experiment clearly showed that N_2 was the end-product of nitrate reduction. Approximately same amount of $^{14,14}N_2$ and

$^{15,15}N_2$ productions as about 300 μmol/l were measured in duplicates of Set 1. Conversions of N-compounds and propionate are illustrated in Figure 6.21a and 6.21b. Although observed nitrite accumulation was insignificant relatively to that of observed in the former experiments, results of the experimental set 1 strongly indicated that nitrite was a free intermediate, as shown in Figure 6.21b.

In the experiments where ^{15}N-nitrate was incubated with nitrite and propionate, as presented in Set 2, both ^{15}N-nitrate and nitrite were converted to yield higher level of $^{14,15}N_2$, as around compared to experimental set 1. Figure 6.21c and 6.21d shows the profiles of the N-compounds and propionate, related to the duplicates 2a and 2b. Since excess nitrite was present in the medium, transient accumulation of labeled nitrite was pretty clear. Thus, it could be concluded as nitrite reduction is the rate limiting step.

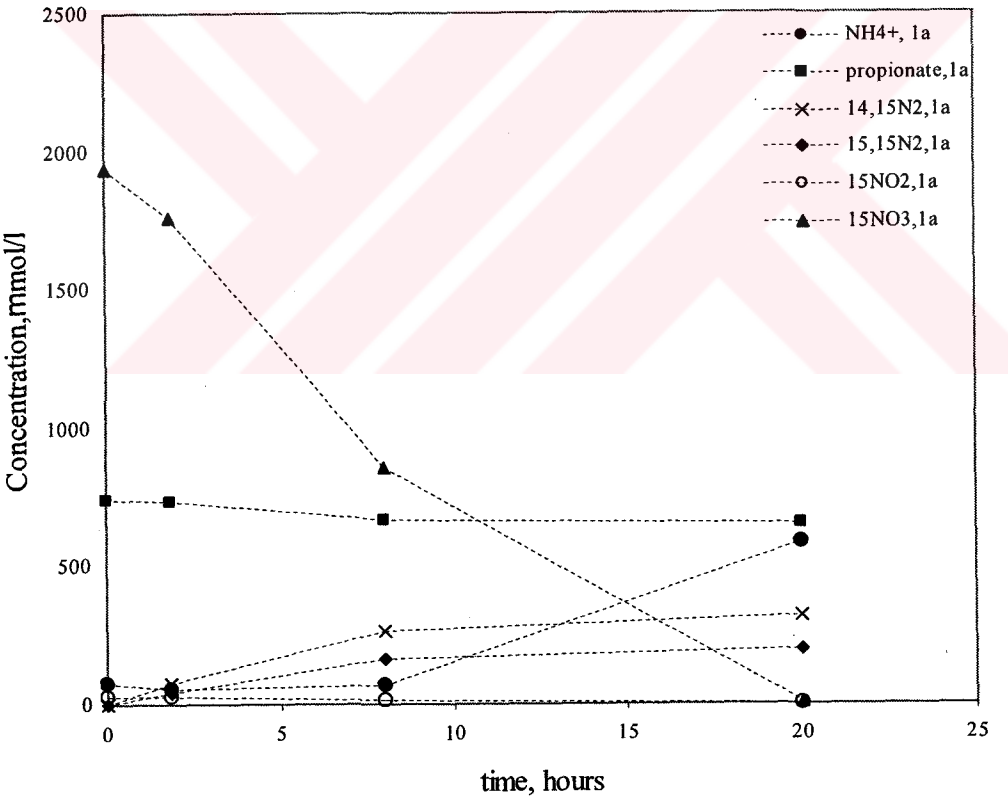


Figure 6.21a Conversions in the experimental set1a.

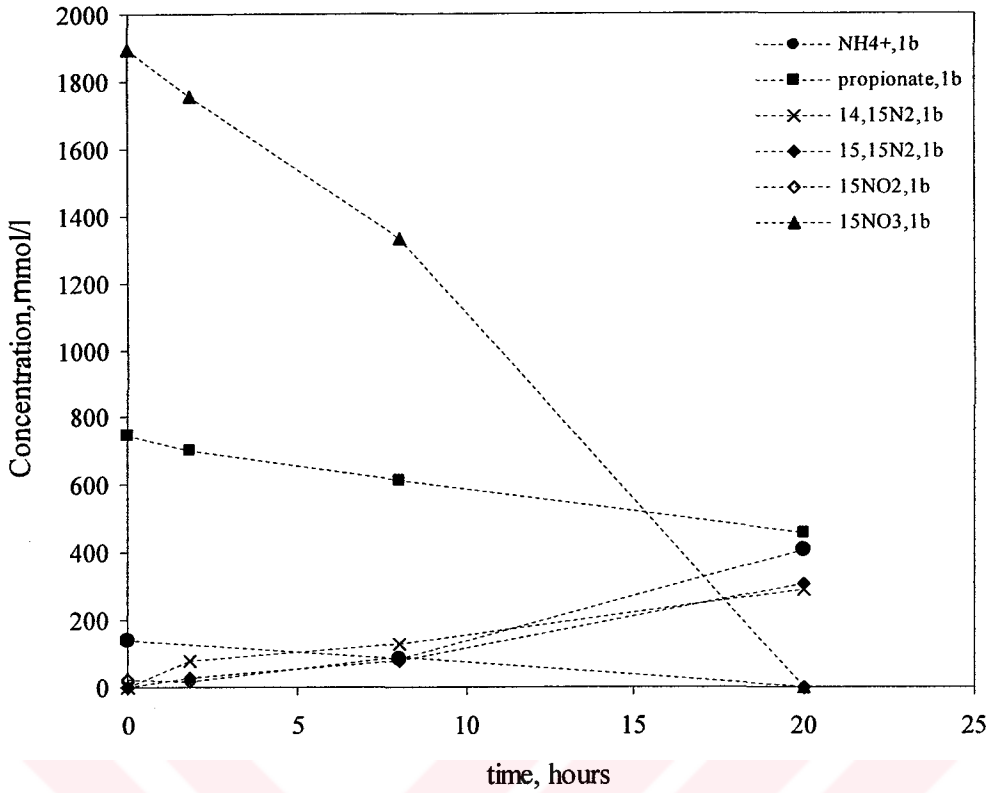


Figure 6.21b Conversions in the experimental set1b.

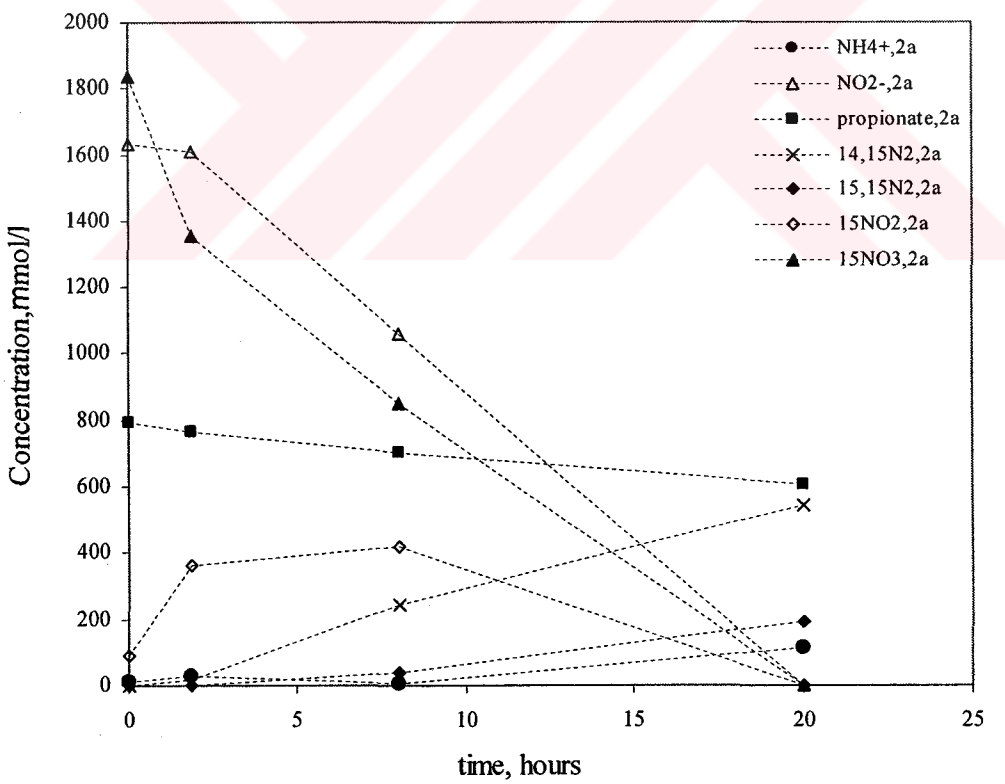


Figure 6.21c Conversions in the experimental set 2a.

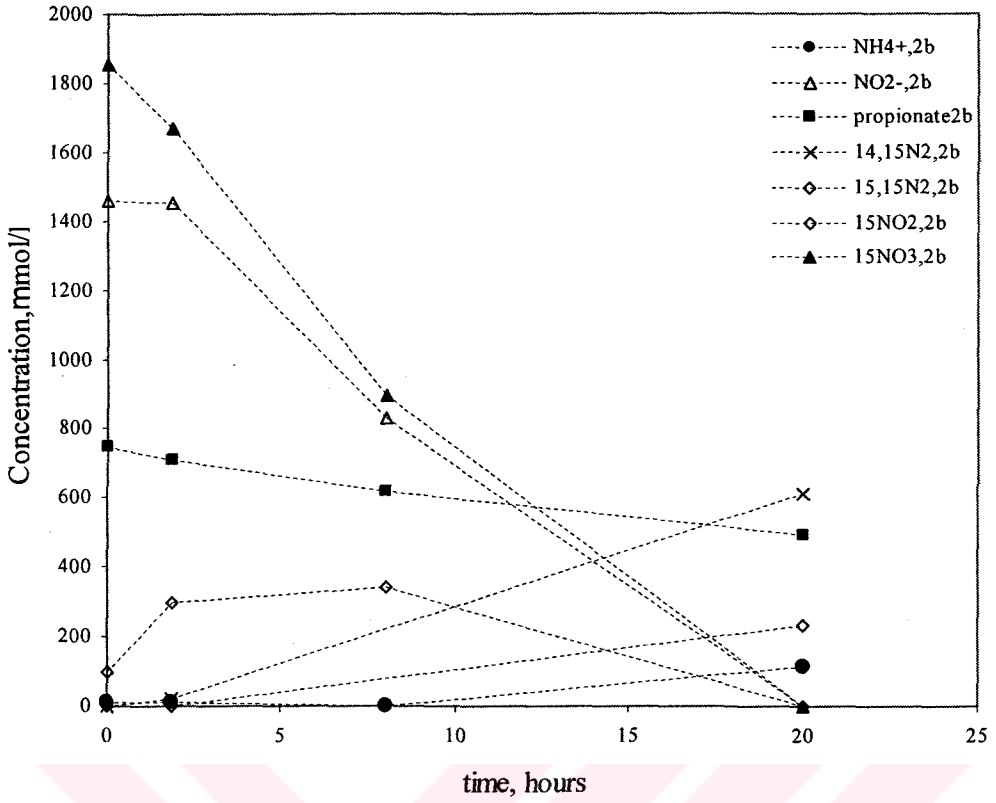


Figure 6.21d Results of the experimental set 2b.

Experimental Set 3 was performed with the aim of the examination of the behavior of the anammox bacteria in presence of ^{15}N-nitrate, nitrite, ammonium and propionate. Rapid conversion of ammonium and nitrite, and production of nitrate proved that, anaerobic ammonium oxidation was the dominant reaction in the system. However, detectable amount of propionate consumption and decrease in ^{15}N-nitrate concentration demonstrated that nitrate reduction could be run in parallel to anaerobic ammonium oxidation. Transient $^{15}NO_2^-$ formation was also noticed which explains $^{14,15}N_2$ formation. This indicated that the $^{15}NO_2^-$ produced from the reduction of $^{15}NO_3^-$ was subsequently combined with $^{14}NH_4^+$ to form $^{14,15}N_2$. Conversion profiles of N-compounds and propionate are illustrated in Figure 6.21e and 6.21f. Hardly any $^{15,15}N_2$ was formed that shows $^{15}NO_3^-$ reduction did not proceed to the whole pattern to produce $^{15,15}N_2$.

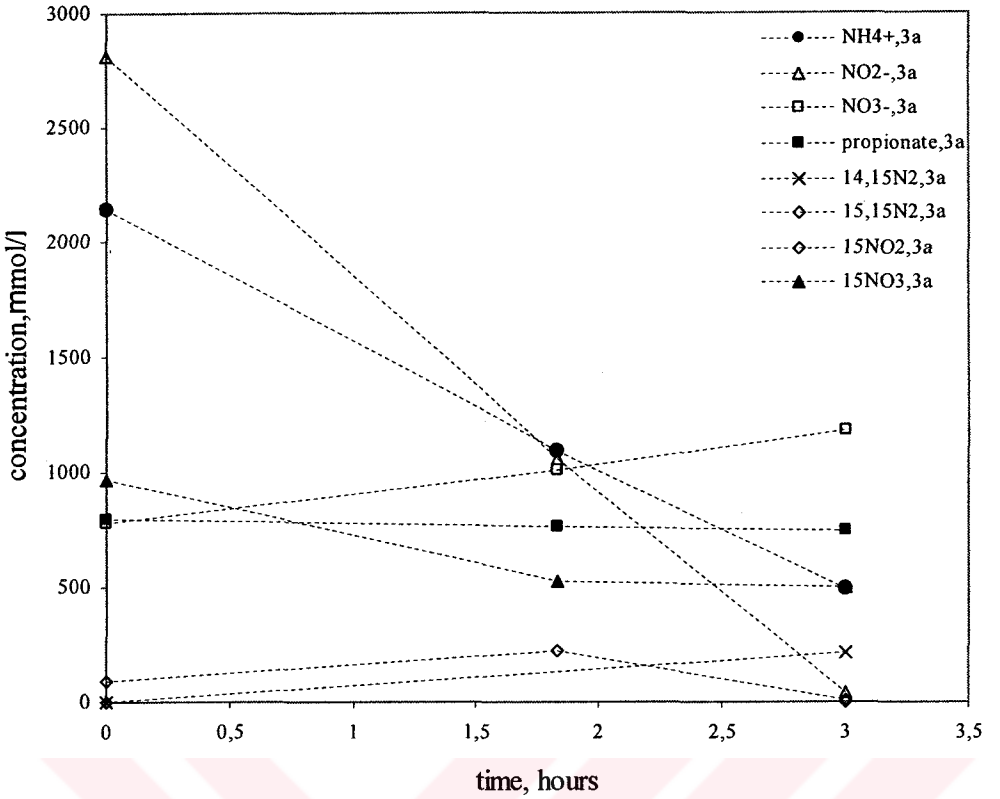


Figure 6.21e Results of the experimental set 3a.

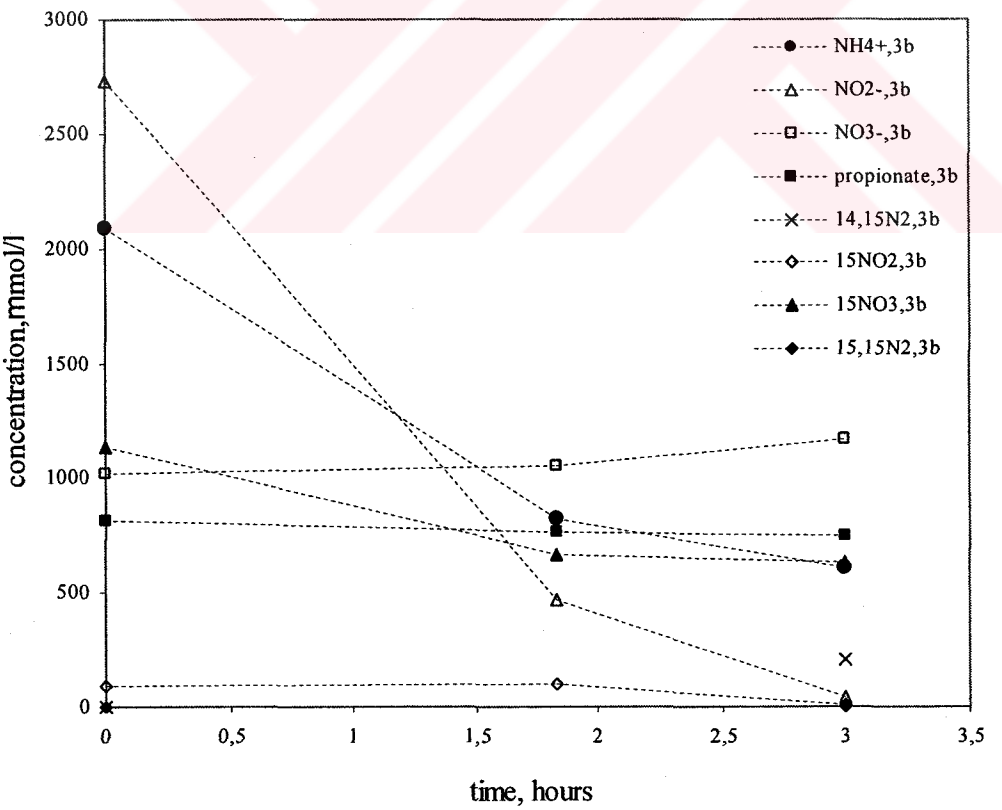


Figure 6.21f Results of the experimental set 3b.

7 DISCUSSION AND CONCLUSIONS

This study proves that organic compounds significantly influence the anammox process (inhibit or stimulate) and some of organic compounds can be converted by anammox bacteria.

The influence of different organic compounds such as acetate, propionate, methanol, ethanol, glucose, starch, formate and amino acids on the activity of anammox was investigated in batch experiments. Anammox was inhibited by some degree of ethanol (> 1mM; 30%), formate (> 1 mM; 28%), amino acids (>0.5 mM; 18%), glucose (> 2 mM; 10%), and starch (>0.25 mM; 30%). The batch experiments indicated that methanol had a strong inhibitory effect on anammox bacteria.

The anammox activity was completely lost in the presence of very low methanol concentrations. Methanol inhibition was experienced also in the continuous culture of anammox enrichment by continuous supply of methanol which resulted in immediate and irreversible inhibition of anammox, supporting the results of the batch experiments. Flushing a period of 1 week of the culture with medium without methanol did not restore the anammox activity. Consequently, the revelation of anammox to methanol has to be prevented.

Interestingly, among the applied 8 different carbon compounds, acetate and propionate was found to have stimulatory effect on the activity of anammox. Therefore, continuous cultivation experiments with acetate and propionate were set up in a chemostat for the assessment of long term effects on the anammox process, thus it would make possible to examine metabolic activities, mass balances, conversion rates and the population dynamics.

Results showed that acetate was completely consumed but did not changed the stoichiometry of ammonium and nitrite conversion in the reactor. Interestingly, production of nitrate was found fewer compared to stoichiometrically expected values.

FISH analysis indicated that the degree of enrichment of the anammox culture was not changed and any bacterial community indicating denitrifiers could not be detected. The influence of acetate on anammox bacteria and acetate oxidation mechanism should be clarified the in further studies.

Throughout the propionate supplement to the reactor, propionate was ever detected in the effluent. So far anammox bacteria are known as obligate chemoautolithotroph, initially it was considered to detect denitrifier activity which would be involved in propionate consumption. Theoretically, addition of an organic compound to an anammox reactor, would lead to build up of heterotrophs and some of them could denitrify nitrate, using the organic compound as the electron donor. Since the experiments were carried out with enrichment cultures (70-80% anammox bacteria), it was not clear who was responsible for the propionate consumption. Methanol was used to inhibit anammox activity to find out whether heterotrophic denitrifiers were involved in propionate consumption in the batch experiments. No nitrite or ammonium conversions were detected denoting the dominancy of the anammox activity in the reactor. This indicated that the anammox bacteria themselves might be responsible for the propionate consumption.

Pulse-addition of propionate to the anammox reactor that never saw propionate before, in two separate experiments, led to an immediate conversion of propionate without a lag phase. This is also an imperative indication of the dominancy of the anammox activity for propionate consumption.

Results also confirmed by monitoring population shifts of the enrichment culture during the continuous cultivation. FISH analysis showed that anammox bacteria dominated the community throughout the experiment. FISH analysis with probes targeting alpha, and gamma proteobacteria showed that any bacterial community belongs to these groups of organism was ever detected in the reactor. Very few beta proteobacteria were detectable in the propionate feeding reactor after 8 mM propionate feeding was started. In addition, several probes specific for certain denitrifiers (*Zoogloea ramigera, Spherotiles natans, Paracoccus denitrificans, Alcaligenes faecalis and Azospirillum*) did not give detectable hybridization signals. Furthermore immunofluorescence with antibodies against the periplasmic nitrate reductase NapA, showed that NapA positive cells were only detectable after the

propionate in the influent was raised to 20 mM. FISH analyses were also showed that anammox population was significantly changed under propionate feeding conditions that might be an indication of the new strain/strains of anammox bacteria.

Anaerobic ammonium oxidation activity in the reactor did not significantly change with the relatively low propionate supply and observed ammonium conversion rate was compatible with the stoichiometry of anammox. However, nitrate production rate was considerably lower at around 2 mmol nitrate/day than stoichiometrically expected values. This indicates produced nitrate was reduced in the presence of propionate. The lowest nitrate concentration in the effluent was observed in the 20 mM propionate feeding period. This indicated that the net nitrate production was changed into a net nitrate consumption.

Finally, propionate consumption by anammox bacteria was proved undoubtedly, by purifying the anammox cells from the enrichment culture with percoll density gradient centrifugation. It appeared that a 99.5% pure cell suspension of anammox bacteria consumed propionate with nitrate as the electron acceptor, sufficient to explain propionate conversion in the continuous enrichment culture experiment.

The mechanism of nitrate reduction with propionate oxidation by anammox bacteria was investigated in the batch experiments using both labeled ($^{15}NO_3^-$) and unlabeled N-compounds. Both continuous cultivation and batch experiments showed that, nitrate was the electron acceptor coupled to propionate. Experiments also showed that ammonia was not consumed in presence of propionate. Propionate oxidation only occurred in the presence of nitrate. Ammonium was not required for propionate oxidation, indicating that anaerobic ammonium oxidation and propionate oxidation were two independent processes. In the presence of propionate, ammonium oxidation was observed only if nitrate and/or nitrite were also available in the medium. When nitrate was present as the only electron acceptor, a transient accumulation of nitrite was observed. This indicated that nitrite was a free intermediate of nitrate reduction and that the reduction of nitrite was the rate-limiting step.

The consumption ratio of nitrate/propionate was about 2.7 in good agreement with the theoretically expected values for complete reduction of nitrate to dinitrogen gas. In some experiments where ammonium and/or nitrite were also present in the

medium, the ratio of nitrate/propionate was found around 7 which is identical with the expected ratio of the reduction of nitrate to nitrite with propionate. This can be concluded as produced nitrite was combined with ammonium to form dinitrogen gas as a result of anaerobic ammonium oxidation reaction.

^{15}N-labeling experiments proved that N_2 was the end-product of nitrate reduction. In the experiments where ^{15}N-nitrate was incubated with nitrite and propionate and with only propionate $^{14,14}N_2$ and $^{15,15}N_2$ were both detected. Transient accumulation of nitrite was also confirmed by these experiments. However, it is still unclear whether nitrite is reduced to N_2 via NO and N_2O in denitrification, or via dissimilatory reduction to ammonium followed by anaerobic ammonium oxidation.

In the light of experimental results summarized and evaluated in the preceding sections the concluding remarks of this study may be expressed as follows:

- Methanol inhibited the anammox process, completely and irreversibly. The revelation of anammox to methanol has to be prevented.
- Acetate was consumed in the anammox enrichment culture and found to have a stimulating effect on anammox activity.
- Anammox bacteria are able to reduce nitrate coupling to propionate oxidation.

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