MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES

SHORT AND LONG TERM EFFECTS OF HEAVY METAL EXPOSURE ON ANAMMOX PROCESS

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Ph.D. THESIS

Department of Environmental Engineering

Thesis Supervisor

Assoc. Prof. Kozet BAKIRCI

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Dedicated to my daughters; Miray and Tanem

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ÖZET

ANAMMOX SİSTEMİ ÜZERİNDE UZUN VE KISA VADEDE AĞIR METAL İNHİBİSYONUN ETKİLERİ

Son yirmi beş yılda, özellikle azot konsantrasyonu yüksek atık suların arıtımında gelecek vaat eden Anaerobik Amonyak Oksidasyon(Anammox) prosesi ile ilgili araştırmalar hız kazanmaya başladı. Yüksek azot konsantrasyonu içeren atık sular biyolojik arıtma sistemlerinde inhibisyona sebep olan ağır metalleri önemli oranda içerebilmektedir. Ancak, literatürde ağır metallerin kısa ve uzun vadedeki etkileri ile ilgili çalışmalar sınırlı sayıda yapılmıştır.

Bu çalışma kapsamında kadmiyum, bakır, nikel ve çinkoya kısa süreli etkileşimi ve uzun vadede kadmiyum ve bakır maruziyetinin Anammox sistemi üzerindeki etkileri incelenmiştir. Bu güne kadar, ağır metallerin Anammox üzerindeki inhibe edici etkilerinin incelendiği çalışmalar yalnızca toplam ağır metal konstrasyonu baz alınarak yapılmıştır. Ancak, bu yaklaşımla metallerin toksik etkilerini öngörmede doğru olmayan bir metodolojiye sebep olabilir. Bu nedenle, bizim çalışmamızda, belirli inorganik / organik türlerin varlığında, ağır metallerin hücre içi, yüzeye-bağlı, çözünebilir, serbest-iyon ve zayıf (kararsız) kompleksleri dâhil olmak üzere, biyoyararlanılabilir metal bileşenleri de dikkate alınmıştır.

Metallerin kısa vadedeki etkileri belirlemek amacı ile giderek artan konsantrasyonlarda Cu,Cd,Ni, ve Zn kullanılarak kesikli deneyler gerçekleştirildi. Sonuçlarımız gösterdi ki toplam uygulanan metal konsantrasyonları ve çözünür metal konsantrasyonun göre inhibisyon etkisi sıralaması Cu>Cd>Ni>Zn şeklinde gerçekleşmiştir. Bu sıralama hücre-içi konsantrasyonlara bakıldığı zaman Ni>Cd>Cu>Zn olarak bulunmuştur. Uygulanan metaller arasında en düşük toksik etkiye sahip olan metal çinko olarak bulunmuştur. Sonuçlarımıza göre inhibisyon etkisi ile çözünür, hücreiçi, hücre ile ilişkili fraksiyonlarda pozitif bir ilişki test edilen tüm metallerde gözlemlenmiştir. Serbest iyon ve Anammox inhibisyonu arasında bakır hariç iyi bir korelasyon görülmüştür. Bakır için hesaplanan serbest iyon konsantrasyonları çok düşük olduğundan serbest Cu iyonu konsantrasyonunun Anammox sistemi üzerinde önemli bir inhibitör etkisi olmadığı varsayılmıştır. Çinko ve kadmiyuma bağlı inhibisyonlar çözünebilir, hücre-içi ve serbest iyon fraksiyonları ile açıklanabilse de yüzeye bağlı metal konsantrasyonları ile iyi bir

korelasyon bulunamamıştır. Modifiye edilmiş rekabetçi olmayan inhibisyon ve 4-noktalı lojistik modeller, test edilen inhibisyon senaryolarının tamamında en iyi uyan inhibisyon modelleri olmuştur.

Uzun vadede ağır metal maruziyetinin Anammox üzerindeki etkilerini araştırmak için artan konsantrasyonlarda Cu(II) ve Cd(II) kullanılarak azot giderimine etkisi incelenmiştir. Literatürde çoğu çalışmada kadmiyumun kısa süreli inhibitör etkilerine odaklanılmıştır. Şimdiye kadar yapılan çalışmalarda Anammox sisteminin kadmiyuma uzun süreli maruz kalma etkisi incelenmemiştir. Çalışmamızda, uzun vadede kadmiyum maruziyeti için azot giderim oranı baz alınarak yarışmasız inhibisyon modeline göre IC50 değeri 6.75 mg L^{-1} olarak hesaplanmıştır.

Önceki çalışmalarda Cu'nun kısa dönemli etkileri daha çok incelenmesine rağmen, birçok çalışmada uzun vadeli etkiler de söz konusudur. Bizim çalışmamızda bakır için IC50 değeri azot giderim oranı baz alınarak 6.77 mg L^{-1} olarak hesaplandı. Sonuçlar uzun süreli maruziyette bakır için IC50 seviyesinin kısa süreli maruziyete göre daha yüksek olduğunu göstermiştir. Bunun muhtemel sebebi uzun süreli maruz kalmada aklimasyon ve bakteriyel adaptasyonu olabileceği düşünülmektedir.

Ayrıca, Anammox popülasyonunun ağır metal maruziyetine tepkisinin gözlemlenmesi için numunelerdeki bakteri türleri moleküler teknikler kullanılarak belirlenmiştir. Uzun vadede inhibisyon deneylerine başlamadan önce metagenomik analizler sonucunda *Candidatus Kuenenia* toplam bakteri popülasyonu içerisinde en baskın tür olarak gözlemlendi. Gerçek zamanlı PCR sonuçlarına göre, Anammox bakterilerinin nispi bolluğu, uygulanan metallere kendiliğinden adaptasyona bağlı olarak ağır metale maruz bırakılma süresince bazı dalgalanmalar gösterdiği gözlemlenmiştir. Ancak uzun süreli metal etkileşimden sonra, Anammox bakterilerinin nispi bolluğu, azalma eğilimi göstermiştir.

Anahtar Kelimeler: Anammox, inhibisyon, ağır metal, yeni nesil dizi analizi

ABSTRACT

SHORT AND LONG TERM EFFECTS OF HEAVY METAL EXPOSURE ON ANAMMOX PROCESS

For the past twenty-five years, there has been a rapid rise in the field of research about Anaerobic Ammonium Oxidation (Anammox) process which has a crucial potential to treat high nitrogen load wastewater streams. High-strength wastewaters may encompass a significant amount of heavy metal which lead to inhibition in biological treatment systems. However, little attention has been paid to inhibitory effects of heavy metals in short-term and little is known about the long-term effect of heavy metals.

This study provides insight into the short-term effects of cadmium, copper, nickel, and zinc and also long-term effects of cadmium and copper exposure on Anammox system. Up to now, the inhibitory effect of heavy metals on Anammox merely considered based on total applied heavy metal concentration. However, this approach may result in inaccurate methodology to predict the toxic effect of metals. Therefore, in our study also possible bioavailable fractions of metals were taken into account including the intracellular, surface-bound, soluble, free-ion, and weak (labile) complexes of heavy metals, in the presence of certain inorganic/organic species.

In order to determine the short-term effect of Cu, Cd, Ni, and Zn on Anammox, batch tests were carried out by using variable concentration of heavy metal. Our results revealed that the inhibitory order of metals was Cu>Cd>Ni>Zn in terms of applied concentration and soluble fraction. This order can be given for intracellular concentrations as Ni>Cd>Cu>Zn and for cell-associated fraction Ni>Cu>Cd>Zn. The lowest toxic effect was found for Zn among the tested heavy metals and their fractions. Our results showed that there is a positive correlation between the intracellular, cell-associated, and soluble fractions of tested heavy metals and Anammox inhibition. Anammox inhibition and free-ion concentration was correlated well for applied heavy metals except Cu. Since the free ion concentration of Cu was calculated too low, it was assumed that free Cu ion concentration has no significant inhibitory effect over Anammox system. Inhibition by Zn and Cd could be explained by soluble, intracellular and free ion fractions but not by surface-bound fractions. The modified non-competitive inhibition and 4-points logistic models were the main inhibition models that best described the inhibition response for

the full suite of tested inhibition scenarios.

The long-term impact of Cu (II) and Cd (II) in elevated concentration on nitrogen removal rate has been investigated. In literature, most studies have only tended to focus on inhibitory effect of Cd on Anammox system for short-term exposure. The prolonged exposure effect of Cd has not been studied up to now. The half maximal inhibitory level for long-term exposure of Cd was calculated as 6.75 mg L^{-1} with the modified non-competitive inhibition model based on nitrogen removal efficiency. Our results showed that 50 % activity loss observed at lower concentration of Cd comparing with the short-term exposure.

Although short-term effects of Cu were mostly examined in the previous studies long-term effects were also concerned in several studies. The results indicated IC50 value for Cu was calculated as 6.77 mg L^{-1} based on nitrogen removal efficiency. Results indicated that the IC50 level for copper in long-term exposure was higher than in short-term exposure.

Moreover, to observe the response of Anammox community to heavy metal exposure bacterial species in samples was determined using molecular techniques. In the studied SBR, *Candidatus Kuenenia* was found to be the dominant genera using metagenomics analysis with next generation sequencing. According to real-time PCR results, relative abundances of Anammox bacteria shows some fluctuations during the exposure depending on self-adaptation to copper. After long exposure period relative abundances of Anammox bacteria showed a tendency to decrease.

Keywords: Anammox, inhibition, heavy metal, next generation sequencing

CLAIM FOR ORIGINALITY

Evaluation of inhibition of Anammox based on fractionation of heavy metals was done for the first time in such a degree of detail. Additionally, metagenomics analysis with next-generation sequencing was applied to examine abundance and diversity of bacterial communities in the biological samples used for inhibition studies.

Anammox process is a novel nitrogen removal method generally applied to wastewaters with high nitrogen levels. However, wastewaters which are suitable to apply Anammox come together with high heavy metal concentrations. Up to now, inhibition of Anammox bacteria by heavy metals were evaluated on the basis of total applied heavy metal concentration. The primary factor for inhibition, however, is not the total metal concentration but the distribution of metal among the solid and bulk phases. The extent of inhibition of nitrogen removal rate due to Cu, Cd, Ni and Zn was evaluated on the basis of soluble, intracellular, surface-bound, cell-associated and free heavy metal concentrations. These fractions can be regarded as fractions which are bioavailable to Anammox bacteria. By this way, fractions of heavy metals which do not play a key role on inhibition could be taken out of consideration. This is the major novelty of this study.

Furthermore, the long-term exposure effect of Cd on Anammox process was investigated for the first time in literature.

Additionally, total bacterial community in samples was determined using next generation sequencing technology. The metabolic response to an inhibitory chemical is mostly related with microbial characteristics. This means that response to heavy metal exposure for each species may be variable. Therefore, it is important to report inhibitory levels based on active species accomplishing the degradation. This is another novelty of this work, bacterial community exposed to Cu and Cd are identified for the first time in such a degree of detail. The discrepancy between the reported IC50 values could be surmounted to a significant extent when IC50 values are given as genera-specific and also based on bioavailable fractions.

SYMBOLS

%	: Percent
$(NH_4)_2SO_4$	: Ammonium sulfate
µm	: Micrometer
Ca	: Calcium
$CaCO_3$	: Calcium carbonate
CH_4	: Methane
cm	: centimeter
Co	: Cobalt
CO_2	: Carbon dioxide
Cr	: Chrome
Cu	: Copper
Fe	: Iron
g	: Gram
h	: Hour
H_2S	: Hydrogen sulfide
H_2SO_4	: Sulfuric acid
K	: Potassium
kg	: Kilogram
L	: Liter
M	: Molar
µM	: Micro molar
m^2	: Meter square
m^3	: Meter cube
mg	: Miligram
PO_4^{3-}	: Posphate
Mg	: Magnesium
mm	: milimeter
Mo	: Molybdenum
N	: Nitrogen

Na	: Sodium
NaOH	: Sodium hydroxide
NH_3	: Ammonia
NH_4HCO_3	: Ammonum bicarbonate
NH_4	: Ammonium
Ni	: Nickel
^{o}	: Degree
^oC	: Degree celcius
pH	: Activity of hydrogen ion
pKa	: Ionization constant
rpm	: Revolutions per minute
t	: Time
T	: Temperature
K_d	: Distribution coefficient
Zn	: Zinc

ABBREVIATIONS

ANAMMOX : Anaerobic Ammonium Oxidation

SHARON : Single Reactor High Activity Ammonia Removal Over Nitrite

CANON : Completely autotrophic nitrogen removal over nitrite

DEMOX :Single-stage nitrogen removal using Anammox and partial nitritation

OLAND : Oxygen Limited Autotrophic Nitrification Denitrification

AOB : Ammonia-Oxidizing Bacteria

NOB : Nitrite-Oxidizing Bacteria

AAS : Atomic Absorbtion Spectrometry

AOB : Ammonia Oxidizing Bacteria

CANON : Completely Autotrophic Nitrogen Removal over Nitrite

COD : Chemical Oxygen Demand

DAPI : 4,6-diamidino-2-phenylindole

DEAMOX : Combined Denitrification and Anammox

DO : Dissolved Oxygen

EDTA : Ethylenedinitrilotetraacetic Acid

EPS : Extracellular Polymeric Substances

FA : Free Ammonia

FISH : Fluorescence In-Situ Hybridization

EEC : European Union Countries

HAO : Hydroxylamine Oxidoreductase enzyme

HCO_3^- : Bicarbonate ion

Heme : Hemochrome / Cytochrome

HPLC : High Performance Liquid Chromatography

HZS : Hydrazine Synthase enzyme

IC50 : Half Maximal Inhibitory Concentration

MLVSS : Mixed Liquor Volatile Suspended Solids

mM : Milimolar

Nir : Nitrite Reductase enzyme

NOB : Nitrite Oxidizing Bacteria

NRR : Nitrogen Removal Rate

SBR : Sequencing Batch Reactor

SHARON : Single Reactor High Activity Ammonia Removal Over Nitrite

TSS : Total Suspended Solids

UV-VIS : Ultra Violet - Visible

VSS : Volatile Suspended Solids

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1. INTRODUCTION

1.1. Nitrogen Pollution

Industrial and domestic wastewater discharges are the most widespread environmental problem throughout the world. Mainly, besides organic pollutants, these wastewater sources contain nitrogen, phosphorus, detergents, pesticides, and heavy metals. The most common and major problem among these contaminants is nitrogen and phosphorus (nutrients) (Larsdotter, 2006). Excessive concentration of nitrogen compounds which are found in wastewater may cause undesirable adverse effects on public health and the environment. Ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, and organic nitrogen are of primary concern in wastewater treatment (Hurse and Connor, 1999). Uncontrolled amount of nitrogen and phosphorus discharge into the water sources may lead to eutrophication problem (Tchobanoglous et al., 2003). In parallel with eutrophication, enormous plant growth and decay are promoted with a consequent increase in the growth of toxic cyanobacteria and algae (Akpor, 2011). Correspondingly it leads to attenuation of water quality, devastation to aquatic life and severe damages to the ecosystem (Carpenter, 2005). Toxins produced by cyanobacteria (cyanotoxins) may cause liver damage, skin irritation, and gastroenteritis (Akpor, 2011). Also, the high concentration of nitrate in drinking water may cause fatal illness for children methemoglobinemia ("blue baby syndrome"). Nitrates are converted to nitrite in digestive systems of infants and nitrite react with hemoglobin and convert it to methemoglobin, a form of hemoglobin which cannot transport oxygen in the blood (Knobeloch et al., 2000). Hence, there is an emerging effort for developing new technologies to remove nitrogen from wastewater and by this way to avert the public and environmental problems (Zhu et al., 2008).

Physicochemical and biological approaches can be used to eliminate the nitrogen compounds from wastewater. Some of the physicochemical technologies are; ammonia stripping and distillation, ion exchange, activated carbon usage for removal of chloramine, advanced oxidation processes and breakpoint chlorination (Andrea G. Capodaglio, Massimo Raboni, 2014). However, biological processes are known to be more convenient and cost-effective, hence more beneficial than the physicochemical processes in nitrogen removal (EPA, 2013).

1.2. Effluent Discharge Standards for Nitrogen

Environmental quality standards restrict the certain pollutants to protect the human health, environment, and resources. In order to reduce the adverse impacts of nitrogen polluted effluents to water sources, stringent legislations are being implemented by the countries.

In drinking water, the U.S EPA has set the 10 ppm nitrate-nitrogen concentration, with a maximum nitrite-nitrogen concentration of 1 ppm [Royer et al, 2008]. Even though nitrate concentrations in well water generally higher than 50 ppm, nitrite concentrations less than a few ppm and nitrate concentration is not higher than 10 ppm in drinking-water derived from surface water in many countries (Gorchev and Ozolins, 2011).

For the discharge from urban wastewater treatments to the sensitive areas maximum allowable limit concentration of total nitrogen was set as 10 ppm (for the p.e more than 10^5) for European Union countries, by the Council Directive on Urban Wastewater Treatment (91/271/EEC). According to the directive for sensitive area discharges, secondary treatment is required for whole wastewater that enters a collection system, while primary treatment may be adequate for less sensitive area discharges.

The Environmental Protection Act 2002 regulates the discharge of polluted effluents. Maximum allowable limits were set for ammoniacal nitrogen as 1 ppm, nitrate nitrogen as 10 ppm and Total Kjeldahl Nitrogen as 25 ppm.

1.3. Conventional Nitrogen Removal from Wastewater

Biological nitrogen removal from wastewater is achieved by microbial transformation of total nitrogen (Figure 1.1). There is an emerging concern to develop new technologies to get rid of nitrogen compounds (Zhu et al., 2008). Still, the most common approach is conventional nitrogen removal systems. Up to now, improvements and modifications in biological nitrogen removal processes have been achieved, such as pre-denitrification, post denitrification, Bardenpho, oxidation ditch, step feeding processes, University of Cape Town (UCT), and Virginia Initiative Plant (VIP) (Dapena-Mora et al., 2004; Tchobanoglous et al., 2003).

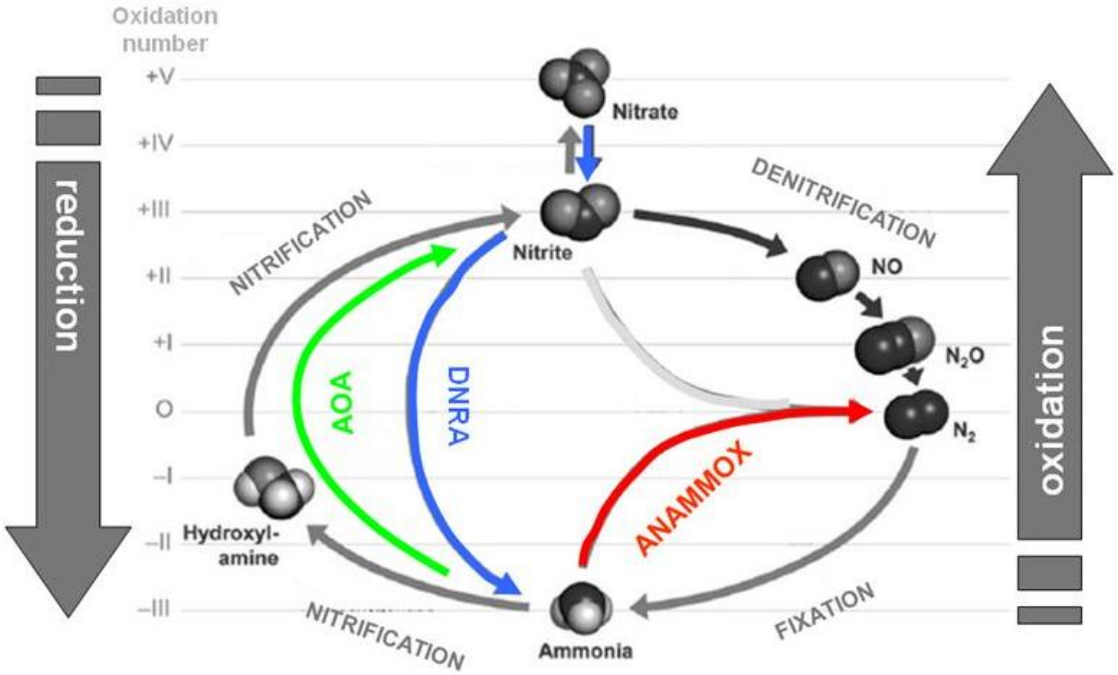


Figure 1. 1 Biological Nitrogen Cycle. Adapted from Colasanti (2011)

Conventional nitrogen removal systems consist of two distinct steps; nitrification (aerobic) and denitrification (anoxic) processes. Nitrification and denitrification processes will be discussed in the following section.

1.3.1. Nitrification

Nitrification is a two-step microbial process to remove nitrogenous compounds by consecutive oxidation of ammonia (NH_4-N) to nitrite (NO_2-N) and nitrate (NO_3-N). Two groups of autotrophic bacteria are responsible for nitrification. These bacteria are strict aerobes, autotrophs, and chemolithotrophs (Rittmann and McCarty, 2001). In the first stage (ammonia oxidation), NH_4^+ is oxidized to NO_2^- by ammonia-oxidizing bacteria (AOB). In the second stage of nitrification (nitrite oxidation), NO_2^- is oxidized to NO_3^- by nitrite-oxidizing bacteria (NOB). The first step of nitrification can be divided into two sub-processes.

1. Ammonia oxidation to the intermediate product hydroxylamine with the ammonia monooxygenase(AMO) enzyme that catalyzes this reaction (Equation 1.1)

$$2\mathrm{NH_4} + 0.5\mathrm{O_2} + 2e^- \rightarrow NH_2\mathrm{OH} + \mathrm{H^+} \qquad (1.1)$$

2. Hydroxylamine oxidizes to form nitrite and this reaction is initiated by hydroxylamine oxidoreductase(HAO) enzyme (Equation 1.2) (Bock and Wagner, 2006).

$$NH_2OH + H_2O \rightarrow 2NO_2^- + 5H + 4e^- \qquad (1.2)$$

In the second step of nitrification, nitrite oxidoreductase enzyme catalyzes nitrite oxidation reaction (Equation 1.3-1.4)

$$NO_2 + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \qquad (1.3)$$

$$2H^+ + 2e^- + 0.5O_2 \rightarrow H_2O \qquad (1.4)$$

For these oxidation steps, *Nitrosomonas* and *Nitrobacter* are the most noted bacteria genera in wastewater treatment (Tchobanoglous et al., 2003). Besides, *Nitrosococcus*, *Nitrosospira, Nitrosovibrio,* and *Nitrosolobus* are the other genera that can also perform in nitritation step. *Nitrospina, Nitrococcus,* and *Nitrospira* are the other actors of the nitratation (Meincke et al., 1989; Watson et al., 1981).

The overall reaction can be shown in Equation 1.5 and to carry out the reaction required amount of alkalinity given in Equation 1.5.

$$NH_4^+ + 2O_2 + 2HCO_3 \rightarrow NO_3^- + 2CO_2 + 3H_2O \qquad (1.5)$$

Based on the stoichiometry, neglecting the cell synthesis, 4.57 g O_2/NH_4-N is required to oxidize ammonium nitrogen to nitrate nitrogen and 7.14 g alkalinity is consumed during these reactions (Tchobanoglous et al., 2003). The required amount of oxygen and consumed amount of alkalinity will be decreased when the bacterial cell synthesis is counted because some amount of ammonia will be consumed due to bacterial cell synthesis.

The overall reaction for synthetic-oxidation can be represented as follows:

$$NH_4^+ + 1.83O_2 + 1.98\ HCO_3 \rightarrow 0.98NO_3^- + 0.021C_5H_7O_2N + 1.041H_2O + 1.88H_2CO_3 \qquad (1.6)$$

Biomass yield was reported as 0.15 g VSS /g NH_4-N for AOB and 0.02g VSS/ g NO2-N

oxidized for NOB in the literature (Haug and Mccarty, 1972). According to these yields, for ammonia oxidation to nitrate, the actual oxygen requirement is 4.33 g O_2/g NH_4-N. Also, the consumed amount of alkalinity can be calculated as 7.07 mg as $CaCO_3$/g NH_4-N oxidized Equation 1.6 (US and EPA, 1975).

The efficiency of nitrification process is depending on several environmental factors including pH, alkalinity, DO concentration, substrate concentration, temperature etc. Nitrification rates decrease rapidly at pH values below 6.8 and optimal nitrification rates occur in the range 7.5 to 8.0 (Tchobanoglous et al., 2003). Hence, it is important to provide sufficient alkalinity to assure adequate nitrification rates. Moreover, long solid retention times and a low food to microorganism ratio are required for nitrification processes. Wastewater temperature affects the rate of the process, the optimum temperature for nitrification at between 30 and 35 ^oC (Tchobanoglous et al., 2003). Nitrification rates decrease at higher or lower temperatures. Nitrifiers are also influenced negatively by most of the heavy metals and toxic compounds. Similarly, free ammonia and nitrous acid adversely affect the nitrification process (Anthonisen et al., 1976; Zhou et al., 2011).

1.3.2. Denitrification

Denitrification is a microbial reduction of nitrate to gaseous nitrogen species including nitric oxide, nitrous oxide, and nitrogen gas mostly by facultative heterotrophic bacteria. In contrast to nitrification, a wide variety of bacteria and also eukaryotes and archaea have been pointed as capable of denitrification (Zumft, 1997). Most of this microbial community are facultative aerobic organisms that use NO_3^- or NO_2^- rather than O_2 as an electron acceptor. Ettwig et al. (2010) found that as an electron donor methane usage is possible during the denitrification process. It has been signified that microorganisms capable of heterotrophic nitrification can also denitrify under anoxic and aerobic conditions, called as aerobic denitrification (Robertson et al., 1995).

Same enzymes are involved in each reduction step of denitrification regardless of the electron donor used, include nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) (Loosdrecht et al., 2016).

The overall pathway is;

$$NO_3^- \xrightarrow[NAR]{} 2NO_2^- \xrightarrow[NIR]{} 2NO \xrightarrow[NOR]{} N_2O \xrightarrow[NOS]{} N_2 \qquad (1.7)$$

Denitrification process is carried out under anoxic conditions when the dissolved oxygen concentration is lower than 0.5 mgL^{-1} and denitrification occur efficiently when the oxygen is absent. Nitrate or nitrite replace oxygen with microbial respiration (Tchobanoglous et al., 2003). Microorganisms capable of denitrification are generally among the Gram-negative *Proteobacteria* principally *Pseudomonas* and *Alcaligenes* and to a lesser extent Gram-positive bacteria like *Basillus* (Robertson and Grofmann, 2007) The bacteria capable of accomplishing denitrification use organic and inorganic compounds as an electron donor. Generally, methanol and organics in wastewater are used as an electron donor during the denitrification process. For wastewater with lower C/N ratios to provide enough carbon for reduction of nitrate and nitrite exogenous carbon source has been needed in many biological nutrient removal processes. For different electron donors oxidation reactions are shown in Equation 1.8-1.11;

Wastewater ; $C_{10}H_{19}O_3N + 10NO_3^- \rightarrow 5N_2 + 10\,CO_2 + 3H_2O + NH_3 + 10OH^-$ (1.8)

Methanol : $5CH_3OH + 6NO_3^- \rightarrow 3N_2 + 5\,CO_2 + 7H_2O + 6OH^-$ (1.9)

Acetate : $5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10\,CO_2 + 6\,O + 8OH^-$ (1.10)

Ethanol : $5CH_3CH2OH + 12NO_3^- \rightarrow 6N_2 + 10\,CO_2 + 9H_2O + 12OH^-$ (1.11)

During denitrification process, bicarbonate alkalinity is produced and carbonic acid concentrations are reduced. In the above reactions, for one mole of one mole of NO_3 reduction, one mole of hydroxide alkalinity is produced. This corresponds to 3.57 mg alkalinity as $CaCO_3$/mg NO_3-N reduced to nitrogen gas (USEPA, 2010). Therefore, as opposed to effects of nitrification, generally, pH is elevated in denitrification reactions. Commonly denitrifiers have less sensibility to pH variations than the nitrifying organisms. Studies show that there is a decrease in denitrification rates and also an accumulation of intermediate compounds is possible for pH value below 6.0 and above pH 8.0 (US and EPA, 1975). Also, high dissolved oxygen concentration or low concentration of electron donors may lead to the accumulation of intermediates (Rittmann, 2007).

Based on the combined nitrification-denitrification treatment process, different configurations and technologies have been developed to remove the nitrogen compounds (Figure 1.2).

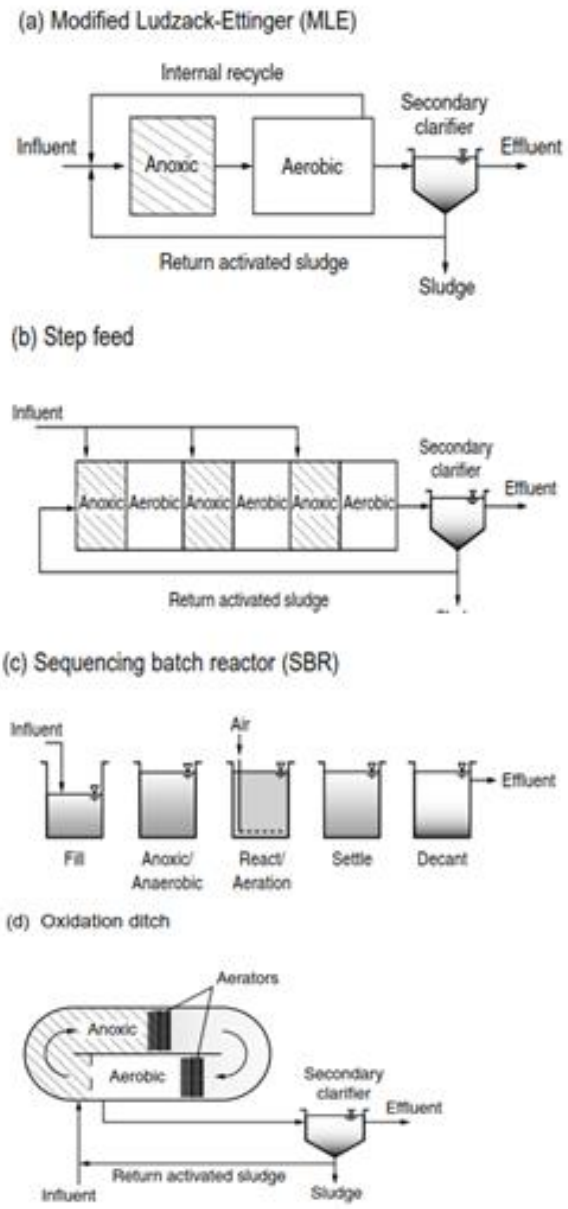


Figure 1. 2 Different configurations of Conventional Nitrification/Denitrification Process Adapted from (Tchobanoglous et al., 2003).

These processes are designed for both carbon and nitrogen removal. Nitrification takes place in the aeration tanks and nitrate produced in this step is eliminated in the anoxic zones. However, in conventional treatment processes, a large oxygen consumption and

carbon source requirement for denitrification renders high operational costs. In the presence of oxygen, generally, methane has been used as an electron donor in denitrification process that is relatively causing lower cost (Lee et al., 2001; Werner and Kayser, 1991). Although it is possible to use methane as an electron donor in denitrification, it is moderately slow process (Werner and Kayser, 1991).

Wastewater containing high nitrogen concentration needs a considerable amount of carbon source and high oxygen requisite for the removal of nitrogen in conventional treatment systems (Van Dongen et al., 2001) resulting in high-cost requirements. Therefore, generally biological nitrification/denitrification processes were not been implemented for high-strength wastewater because of these limitations. In order to cope with such problems, there is an emerging concern to develop new low-cost and effective technologies to get rid of nitrogen compounds from wastewaters. Although, total nitrogen removal can be achieved by conventional nitrogen removal processes, alternative biological nutrient removal processes have been developed that are novel and cost-effective, including OLAND (Oxygen Limited Autotrophic Nitrification Denitrification), ANAMMOX (Anaerobic Ammonium Oxidation) and CANON (Completely Autotrophic Nitrogen Removal Over Nitrite).

Anammox is a biological process that able to oxidation of ammonium to nitrogen gas by using nitrite as an electron acceptor by the planctomycete-like bacteria (Jetten et al., 2005) and has a crucial potential to treat high nitrogen loaded wastewater streams.

More detailed information about Anammox process will be given in the following section.

1.4. Anaerobic Ammonium Oxidation (Anammox)

Anammox was firstly discovered in the Netherlands in which ammonia was removed under anoxic conditions in a laboratory-scale denitrifying fluidized bed reactor (Mulder et al., 1995) and named as Anammox in the mid-1990s. Later its first discovery researches revolved around the mechanism of microorganisms responsible for the Anammox process.

The Anammox process is a chemolithoautotrophic biotransformation process carried out by the Planctomycete group of bacteria and these bacteria oxidize the ammonium nitrogen to nitrogen gas by using nitrite as the electron acceptor (Jetten et al., 2002; Kartal et al.,

2006; Villaverde, 2004). The process is autotrophic and bicarbonate/CO_2 are the carbon sources for anabolic reaction to biomass production .The overall catabolic reaction for the Anammox process was shown in Equation 1.12 and Equation 1.13 (Ahn, 2006).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (1.12)$$

$$CO_2 + 2NO_2^- + H_2O \rightarrow CH_2O + 2NO_3^- \qquad (1.13)$$

The determined stoichiometry for the combination of anabolism and catabolism reactions overall reaction for Anammox metabolism is shown in Equation 1.14 (Lotti et al., 2014).

$$11NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 2.002H_2O + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} \qquad (1.14)$$

Dinitrogen gas is the key product of the Anammox process, but also about 16% of the nitrogen feed is converted to nitrate. Based on the experiments the ratio between consumption of ammonium to nitrite and production to nitrate was found as 1:1.146:0.161 (Lotti et al., 2014). A number of intermediates occur during the catabolism of Anammox bacteria like other biochemical reactions. Nitric oxide and hydrazine are the main intermediates of the Anammox process. The reduction of nitrite to nitric oxide (NO) is carried out by a nitrite oxidoreductase (NirS) and later hydrazine (N_2H_4) is formed by a hydrazine synthase enzyme (HZS) via ammonia and nitric oxide. Subsequently, hydrazine is oxidized to dinitrogen gas by hydrazine dehydrogenase (HDH) enzyme (Kartal et al., 2013).

Anammox bacteria have slow growth rates and the doubling time has been reported in a range between 7 to 20 days hinge on species (Oshiki et al., 2011; Strous et al., 1998; Van Der Star et al., 2008b). However, Lotti et al. (2015) reported the doubling time very low approximately 3 days for Anammox bacteria. Anammox process has a crucial potential to treat high nitrogen loaded wastewater streams without organic carbon depending on its high affinity for ammonia and nitrite (Ks<0.1mgN L^{-1}) relatively high maximum specific nitrogen consumption rate about 0.8 kg N (kg dry weight)$^{-1}$ day^{-1} (Kartal et al., 2004; U. Van Dongen et al., 2001). Table 1.1 shows some operational parameters for different Anammox species.

Table 1. 1 Operational parameters for different Anammox species

Parameters/Species	*Brocadia Anammoxidans '*	*Kuenenia stuttgartiensis*	*Brocadia sinica*	*Jettenia caeni*
pH range	6.7-8.3	6.5-9	7-8.8	6.5-8.5
Temperature range , ^{o}C	20-43	25-37	25-46	20-42,5
Doubling Time ,d	10.70	8-11	7	
Growth Rate, h^{-1}	0.0027	0.0026-0.0035	0.0041	0.002
K_s for NH_4,µM	<5	n.d	28 ± 4	17.1 ± 4.3
K_s for NO_2,µM	<5	0,2-3	34 ± 21	35.6 ± 0.92
References	(Strous et al., 1999, 1998)	(Dapena-Mora et al., 2007; Egli et al., 2001; Van Der Star et al., 2008a)	(Oshiki et al., 2011)	(Ali et al., 2015)

Anammox process has several major advantages comparing with conventional nitrification-denitrification processes. Comparison between conventional nitrogen removal systems and Anammox is shown in Table 1.2.

Anammox is a promising alternative to conventional treatment methods for high nitrogen loaded wastewater streams. The process is found to be economically attractive due to its low sludge production, low aeration requirement (Ma et al., 2016; van der Star et al., 2007) and since Anammox is autotrophic bacteria, there is no external organic carbon requirement. Additionally, the process is environment- friendly due to lower greenhouse emissions (Kartal et al., 2010). Anammox process is a good alternative to treat wastewater streams such as landfill leachate, reject effluents, and several industrial wastewaters however, the complexity of effluent composition and inhibitory factors, make it difficult to operate the Anammox process (Jin et al., 2012).

Table 1. 2 Comparison of Biological Nitrogen Removal System

Treatment Process	**Oxygen Demand (kg O_2/kg NH_4-N)**	**Alkalinity Consumption (kg CaCO3/kgNH_4-N)**	**COD demand (gCOD/gN)**	**Sludge production (kgVSS/kgN)**	**Biomass Yield (kg DS/kgNH4-N)**	**N removal Efficiency**
Conventional N/DN	4.57(4.18)	7.07	4	1	0,8	95%
Nitritation/DN	3.43 (3.16)	7.07	2.4	-	0,5	-
PN / Anammox	1.71-2.06	3.57	0	0,1	0,1	83-90%
References	(Rittmann and McCarty, 2001; Tchobanoglous et al., 2003; Van Dongen et al., 2001)	(Rittmann and McCarty, 2001; Tchobanoglous et al., 2003)	(Van Hulle et al., 2010)	(Mulder, 2003)	(Ma et al,2016)	(Jetten et al, 1997;Fux et al, 2002)

*N: Nitrification, DN Denitrification, PN : Partial Nitritation

Partial nitritation is a prerequisite for the Anammox system to accomplish efficient nitrogen removal providing appropriate nitrite/ammonium nitrogen ratio (Cho et al., 2011). It is hard to provide stable nitrite effluents for long periods of time especially for wastewaters with high organic load such as leachate (Akgul et al., 2013). The inappropriate ratio of nitrite/ammonium may cause decreasing in nitrogen removal rates (Akgul et al., 2013). Therefore partial nitritation can be identified as rate limiting step for the Anammox process. Ammonia could directly be converted to dinitrogen gas by combining Anammox with a preceding partial nitrification step. In partial nitrification step, only half of the aeration capacity that used in nitrification is necessary.

1.4.1. Microbiology of the Anammox Process

Based on the phylogenetic analysis, the Anammox bacteria form a deep-branching in the *Planctomycetes* phylum as shown in Figure 1.3 (Op den Camp et al., 2007).

Up to now, there have been classified six "Canditatus" genera of Anammox bacteria; Candidatus *Brocadia*, Candidatus *Scalindua*, Candidatus *Kuenenia*, Candidatus *Anammoxoglobus*, Candidatus *Jettenia* and, Candidatus *Anammoxomicrobium* (Ding et al., 2013; Ibrahim et al., 2016; Kartal et al., 2007b) and 14 species of Anammox have been discovered as yet. Since the Anammox culture has not been isolated in pure culture, the status of *Candidatus* was given.

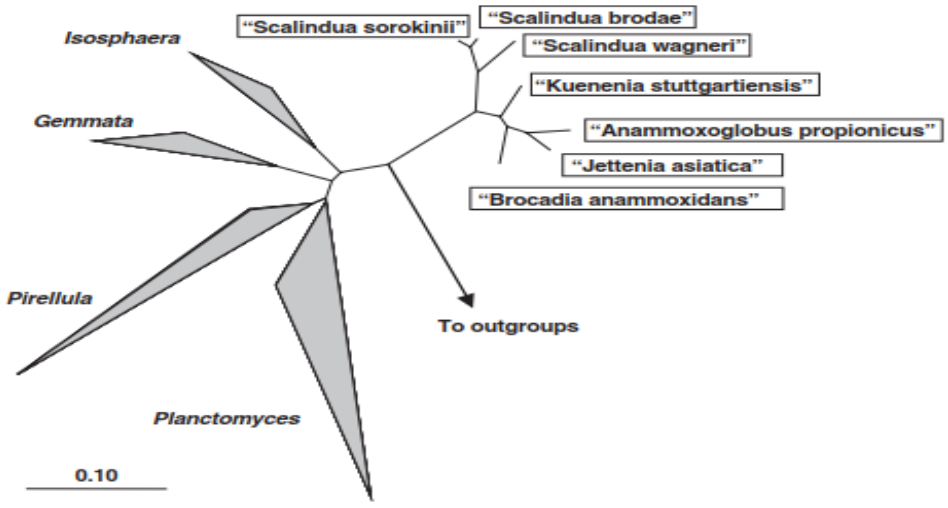


Figure 1. 3 Phylogenetic relationship of Anammox 16S rRNA gene sequences (Op den Camp et al., 2007)

Identified Anammox organisms are associated with monophyletic clade called Brocadiales and deeply branching inside the phylum Planctomycetes (Schmid et al., 2005; Strous et al., 1999). Many of these Anammox genera were identified in wastewater treatment plants includes; Kuenenia, Brocadia, Jettenia, Anammoxoglobus, and Anammoxomicrobium (Strous et al., 2006, 1999). Only the genus Scalindua has generally been detected in marine environments (Dalsgaard et al., 2005; Kuypers et al., 2003; Schmid et al., 2003) and also some fresh water source and wastewater treatment plant. Table1.3 shows the classification of Anammox bacteria (Ibrahim et al., 2016).

Anammox bacteria has a coccoid cell with a diameter of 0.8-110 μm. Differentiation of cytoplasm is unique in Anammox bacteria. The cytoplasm of Anammox consisting of three separate compartments is divided by three individual bilayer membranes. These compartments are the paryphoplasm, riboplasm, and Anammoxosome from outside to inside.

Table 1.3 Classification of Anammox bacteria discovered up-to-date. Adopted from Ibrahim et al. (2016).

Source	Genus	Species	Reference
Wastewater	*Brocadia*	*CandidatusBrocadia Anammoxidans*	Strous et al., 1999
Wastewater		*Candidatus Brocadia fulgida*	Kartal et al., 2004
Wastewater		*Candidatus Brocadia sinica*	Oshiki et al., 2011
Wastewater, Freshwater	*Kuenenia*	*Candidatus Kuenenia stuttgartiensis*	Penton et al., 2006
Marine wastewater Black sea	*Scalindua*	*CandidatusScalindua brodae*	Schmid et al., 2003
Wastewater		*Candidatus Scalindua wagneri*	Schmid et al., 2003
Sea water		*Candidatus Scalindua sorokinii*	Kuypers et al., 2003
Marine Arabian sea		*Candidatus Scalindua arabica*	Woebken et al., 2008
Marine sediments		*CandidatusScalindua marina*	Brandsma et al., 2011
Marine		*CandidatusScalindua profunda*	Vossenberg et al., 2012
Synthetic wastewater	*Anammoxoglobus*	*CandidatusAnammoxoglobus propinicus*	Kartal et al., 2007
Wastewater	*Jettenia*	*CandidatusJettenia asiatica*	Quan et al., 2008
Wastewater		*CandidatusJettenia caeni*	Ali et al., 2015
Wastewater sludge	*Anammoxomicrobium*	*Candidatus Anammoxomicrobium moscowii*	Khramenkov et al., 2013

The Anammox cell layout is illustrated in Figure 1.4.

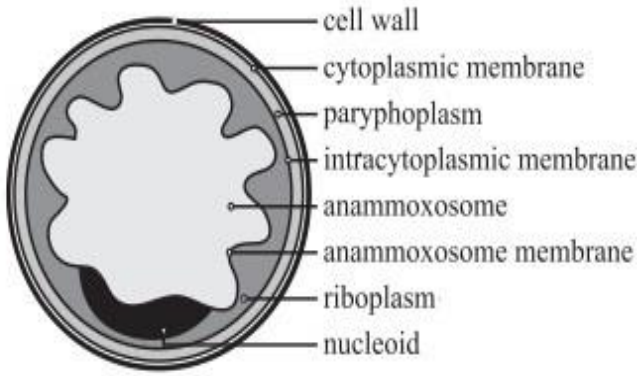


Figure 1. 4 Schematic views of cellular compartmentalization in Anammox bacteria. Adapted from (van Niftrik, et al., 2008)

The outermost cellular compartment is paryphoplasm bound by the cytoplasmic membrane. The riboplasm is the middle compartment that is encircled by an intracytoplasmic membrane and includes ribosomes and nucleoid (van Niftrik and Jetten, 2012). The anammoxsome is the inner ribosome-free part that is surrounded by the Anammoxosome membrane and has a tubule-like structure (Van Niftrik et al., 2004). The Anammoxosome compartment constitutes a bigger part of the cell volume and devoted to energy metabolism by creating a proton motive force for ATP synthesis and mitochondria in eukaryotic cells are functionally equivalent as an energy-producing organelle (van Niftrik and Jetten, 2012). Anammoxosome is the unique part of the cell structure of Anammox bacteria, it contains ladderane lipids that includes linearly concatenated cyclobutane moieties (Rattray et al., 2008; Sinninghe Damsté et al., 2002). This unique organelle is the location where the hydrazine biosynthesis and all catabolic processes of Anammox process (Figure 1.4) (Almeida et al., 2015; Van Niftrik et al., 2010). Using electron microscopic measurements, Fuerst and Sagulenko (2013) revealed that peptidoglycan was not observed in the cell wall of the Anammox like Planctomycetes. Therefore, according to its structural cell wall type, Anammox bacteria can be classified as neither Gram-positive nor Gram-negative bacteria. However, van Teeseling et al. (2015) found that Anammox bacterium *Kuenenia stuttgartiensis* contains peptidoglycan in their cell wall and they redefine the Anammox bacteria as Gram-negative based on its thickness, location, and composition.

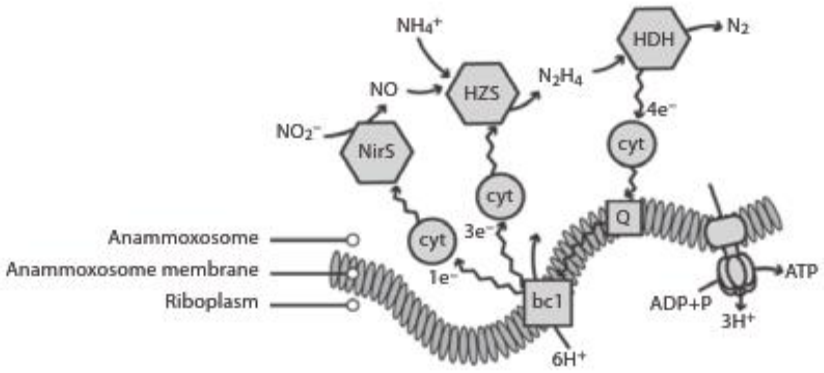


Figure 1. 5 Schematic overview of Anammox catabolism. Nitric oxide (NO) and hydrazine (N_2H_4) are the intermediates. Coenzyme Q, Q; Nitrite reductase, NirS; cytochrome, cyt ; cytochrome bc1 complex, bc1. Adapted from Van Teeseling et al., 2013.

Based on biochemical studies, reduction of nitrite to nitric oxide by NiRs with a byproduct nitrate, subsequently hydrazine production by combination of ammonium and nitric oxide and finally nitrogen gas formation due to four electron oxidation of hydrazine steps is carried out in the anammoxosome compartment (Figure1.4) (Strous et al., 2006; Van Teeseling et al., 2013) .

1.4.2. Applications and Reactor Configurations of the Anammox process

Anammox process is applicable especially for ammonium-rich wastewaters such as landfill leachates (Akgul et al., 2013; Egli et al., 2001), anaerobic digester reject water (Dapena-Mora et al., 2006; Furukawa et al., 2009), wastewater from semiconductor factories (Tokutomi et al., 2011), and sludge digester liquors (Fux et al., 2004).

As mentioned before, the molar ratio of NH_4^+: NO_2^- should be approximately 1:1.15 (Lotti et al., 2014) for the proper application of Anammox process. Since the major nitrogen species in the wastewater is mostly NH_4-N, it needs to be converted to NO_2-N by ammonia oxidizing bacteria by partial nitritation (PN) process. Successful operation of the PN/Anammox resulted in growing numbers of full-scale Anammox installations in the field (Lackner et al., 2014) for the high-strength ammonia wastewater with low C:N ratios. Partial nitritation and Anammox processes can be applied both in single reactors (one–stage) and in separate units (two-stage).

Different configurations for one stage and two-stage applications exist, including; Combined SHARON / Anammox process, CANON process, Single-stage nitrogen

removal using Anammox and partial nitritation (SNAP or DEMOX), denitrifying ammonium oxidation (DEAMOX), Deammonification system (DEMON). In the CANON process 63% oxygen consumption and 100% carbon consumption saving is provided in comparison with the traditional processes (Third et al., 2005). Although the Anammox process is economically favorable compared to conventional treatment methods, this process has been restricted by environmental and operational conditions due to vulnerable structure and slow growth rate of Anammox microorganisms (Jin et al., 2012; van der Star et al., 2007). Due to its slow growth rate, enrichment of Anammox bacteria has been challenge long since. Consequently, various reactor configurations have been studied to sustain efficient biomass retention for Anammox bacteria. The reactor configuration for the Anammox system preponderantly is chosen as sequencing batch reactor (SBR) and fluidized-bed reactors (Op den Camp et al., 2006).

Both attached growth and suspended growth types bioreactors were designed and successfully operated in the literature. Moving bed biofilm reactor (MBBR) (Gut et al., 2006), upflow biofilters (Jin et al., 2008), fluidized bed reactors (FBR) (Van De Graaf et al., 1996) and rotating biological contactor (RBC) (Lv et al., 2011) configurations were used for attached growth systems with different type of supporting materials such as; polypropylene, polyurethane, or membrane surfaces (Abma et al., 2007)

The sequencing batch reactors (SBR) (Arrojo et al., 2006; Du et al., 2014; He et al., 2007; Strous et al., 1998), membrane bioreactor (MBR) (Van Der Star et al., 2008a; Xue et al., 2009), up-flow anaerobic sludge blanket (UASB) (Ma et al., 2017; Tang et al., 2011) and anaerobic membrane bioreactors (anMBR) (Suneethi and Joseph, 2011) were used for suspended growth types reactors.

There are several operational key parameters that affect the activity of Anammox bacteria, such as temperature, dissolved oxygen concentration, and pH. The optimum temperature for Anammox systems has been reported between 30-40^{o}C by several authors (Strous et al., 1999). However, there are some other studies where Anammox could be successfully operatedat low temperatures (Cema et al., 2007; Vázquez-Padín et al., 2009).

Table 1. 4 Examples of Anammox configurations for different type of wastewater

Reactor Configuration	Wastewater	Scale	Reference
SHARON-Anammox	Digester Supernatant	Lab-scale	Gali et al ,2007
SHARON-Anammox	Sludge Liquor	Lab-scale	van Dongen et al, 2001
SHARON-Anammox	Digester Supernatant	Full Scale	van de Star et al.,2007
Partial Nitrification-Anammox	Piggery wastewater	Lab-scale	Ahn et al, 2004
Partial Nitrification-Anammox	Piggery wastewater	Lab-scale	Hwang et al., 2005
Anammox	Monosodium glutamate wastewater	Lab-scale	Chen et al.,2007
MBR-SHARON-Anammox	Landfill leachate	Lab-scale	Akgul et al., 2012
Partial Nitrification-Anammox	Landfill leachate	Lab-scale	Ganigue et al., 2007
Partial Nitrification-Anammox-Soil Infiltration	Landfill leachate	Lab-scale	Liang and Liu,2008
Partial Nitrification-Anammox	Semiconductor factory wastewater	Full Scale	Tokutomi et al., 2011

In the literature, it has been reported that the activity of Anammox bacteria is inhibited in the presence of 0.2-1.0 mg L^{-1} of dissolved oxygen concentration (Strous et al., 1997). Therefore, in order to prevent the inhibition of oxygen in the Anammox process, it is important to monitor dissolved oxygen concentration in the reactor. Besides that, pH is another key parameter for the process. While low pH values increase the free nitrous acid (FNA) concentration and high pH values might cause the rise in the free ammonia (FA) concentration, Anammox bacteria are adversely affected from increasing concentrations of FA and FNA (Fernández et al., 2012). For this reason, it is important to control the FA and FNA concentration in the process. Optimum pH range for the Anammox biomass was reported between 7.7 to 8.3 in the study of Strous et al. (1997).

1.4.3. Inhibition of the Anammox Process

As mentioned before, Anammox is both environmentally and economically good alternative for especially nitrogen-concentrated wastewater with low C: N ratio. Although this process is applicable for ammonium-rich wastewaters, the complexity of influent composition and inhibitory factors in wastewater make it difficult to operate Anammox process (Jin et al., 2012). A wide range of inhibitory compounds usually come together with ammonium-rich wastewaters. Therefore, the cultivation and application of Anammox process have been restricted by environmental and operational conditions. Also, the low growth rate of biomass (Jetten et al., 1998) and vulnerable structure adversely affect the process efficiency. Consequently, researchers have deepened their studies on the inhibition effects of various organic and inorganic substances on Anammox process.

1.4.3.1. Substrate inhibition

Ammonium and nitrite are the substrates of the Anammox process and at high concentration, these substrates can inhibit the biomass activity.

Although Anammox process seems as an advantageous alternative to treat the high-strength ammonium wastewater, high ammonia concentration may cause an inhibitory effect on the efficiency of the process, especially at high pH values. The increase of ammonium concentration at high pH values resulted in high free ammonia concentrations. In the Anammox system, ammonia inhibition is related to unionized free ammonia (FA) concentration rather than total ammonia concentration (Aktan et al., 2012). Since free ammonia can easily penetrate through the cell membrane, it has a more inhibitory effect than ammonium (Gallert and Winter, 1997). Strous et al. (1999) concluded that free ammonia concentrations up to 1 g N L^{-1} did not lead to any inhibition in the Anammox process. There are some studies that report free ammonia inhibition at higher levels. Waki et al. (2007) reported that free ammonia concentration between 13-90 mg L^{-1} influenced negatively the Anammox activity. In another study (Fernández et al., 2012) short-term and long-term in SBR influences of FA were studied and IC_{50} levels were found 38 mg $L^{-1}$for short-term studies. For the long-term tests, removal efficiency decrease to zero when the concentrations of FA reached to 35-40 mg L^{-1} in the same study. When the ammonium concentration reached up to 770 mg L^{-1}, 50 % activity was observed by

Dapena-Mora et al. (2007). In one of our previous studies, we showed that there was no negative effect on Anammox process up to 150 mg L^{-1} free ammonia concentration (Aktan et al., 2012). The variances among the literature on free ammonia inhibition values may ascribable to differences in reactor configuration, operational conditions and Anammox species (Cho et al., 2010).

The inhibition effects of nitrite, which is the other main substrate of the process, due to its accumulation or free nitrous acid (FNA) formation have been widely studied in the literature. However, there is no agreement on the suppression threshold values of nitrite. Although some researchers reported that NO_2^- causes inhibition on Anammox activity (Fux et al., 2004), some other researchers (Egli et al., 2001; Fernández et al., 2012) claim that inhibition does not only depend on total NO_2^- concentration also it is related with an unionized form of nitrite (FNA). Since accumulation of nitrite may cause a decline in the catabolic activity of Anammox biomass, it is essential to prevent accumulation of nitrite for effective biomass retention in Anammox systems. Strous et al. (1999) observed complete activity loss when the NO_2^- concentration reaches 100 mg N L^{-1}, while Egli et al. reported complete inhibition at 180 mg N L^{-1} of NO_2^- concentration. Dapena and Mora (2007) observed 50 % activity loss in the batch system when the concentration of nitrite was reached to 350 mg N L^{-1}. Fernández et al. (2012) reported that IC_{50} levels for FNA as 11 µg L^{-1} HNO_2 in the study of and also activity did not change when the concentration lower than the 0.5 µg L^{-1} HNO_2 .

The inhibition threshold concentration ranging between 5 and 400 mg N L^{-1} in the literature, the differences can be attributed to different operational parameters, reactor types, and biomass species.

1.4.3.2. Organic Matter inhibition

Anammox bacteria are obligate chemoautotrophic microorganisms which use CO_2 as the main carbon source (Kimura et al., 2011).The existence of high organic matter/carbon affects the Anammox process negatively. The inhibition mechanism for the nontoxic organic matter transpires in two different types. One of the inhibition mechanism is called as “out –competition”. In the presence of organic matter, a competition takes place between heterotrophic denitrifies and the Anammox culture, heterotrophs suppress the growth of Anammox bacteria by promoting denitrification process and consequently

nitrogen removal rate decreases (Chamchoi et al., 2008; Guven et al., 2005; Lackner et al., 2008). The second inhibition mechanism is called as "metabolic pathway conversion inhibition" (Jin et al., 2012). In this inhibition mechanism, Anammox bacteria use organic matter as a substrate rather than nitrite or ammonium (Guven et al., 2005). Even though the dominant species are Anammox bacteria, reduction in the nitrogen removal efficiency is observed.

Additionally, toxic organic compounds may also inhibit the Anammox bacteria generally irreversibly such as; antibiotics, alcohol, and aldehydes. Inhibitory effect of methanol has been studied in literature and various level of inhibition concentrations have been reported in different Anammox species. Jensen et al. (2007) state that methanol cause almost complete inhibition over Anammox in marine sediment at a concentration range of 98-126 mg L^{-1}. Isaka et al. (2008) reported 71% reduction in Anammox activity at 160 mg L^{-1} methanol concentrations and the main species was mentioned as "*Candidatus Kuenenia stuttgartiensis*" in the study. Although there are different inhibitory threshold values for methanol reported in the literature, Tang et al (2010) achieved successful enrichment of Anammox biomass from methanogenesis sludge that includes high methanol and acetate. Isaka et al. (2008) stated that methanol was converted to formaldehyde form during the Anammox process and terminate the enzyme and protein activity and irreversible inhibition of Anammox ensue from aldehyde production.

Furthermore, since antibiotics have a broad range of usage in many fields, inhibitory impacts of antibiotics have been widely discussed in the literature. Some studies about the influences of the antibiotics were shown in Table 1.5.

Table 1. 5 Influences of antibiotics on Anammox process

Antibiotics	Concentrations (mg L^{-1})	Inhibitory effect	Reference
Chloramphenicol	200[a]	68% ±10	van de Graaf et al., 1995
	420[a]	IC50	Fernández et al., 2009
	20[b]	25%	Fernández et al., 2009
Ampicillin	400	71%±3	van de Graaf et al., 1995
Penicillin	100	36%±10	van de Graaf et al., 1995
	100	No inhibitory effect	Jetten et al. ,1999
Tetracycline hydrochloride	94	IC50	Fernández et al., 2009
	10[b]	60%	Fernández et al., 2009
Tetracycline	1100	IC50	Lotti et.al, 2012
Oxytetracycline	5 ± 3.5[b]	Completely inhibit	Noophan et al., 2012
	517.5	IC50	Yang et al., 2013
	155-1731[c]	SAA reduce 1.4%	Zhang et al., 2014

[a] short-term , [b] long-term , [c] shock loading

1.4.3.3. Other inhibitory compounds of wastewater

Salinity

Ammonium-rich wastewater, especially industrial wastewater generally contains a larger amount of salt and thus, have a high osmotic pressure (Vredenbregt et al., 1997). Under high osmotic pressure commonly microorganisms tend to die or become plasmolyzed and dormant (Madigan et al., 2003). The negative influences of salinity in anaerobic treatment processes have been widely investigated (Jin et al., 2012). Since Anammox have also been found in marine environments, acclimation and enrichment of this bacteria were implemented at high salt concentrations in some studies (Kartal et al., 2006). Fernandez et al (2008) claimed that salinity in the range of 3-15 mg L^{-1} had a positive effect on Anammox retention and formation of granule. However, most researchers showed that high salt concentrations decreased the Anammox activity (Dapena-Mora et al., 2007;

Isaka et al., 2007; Jetten et al., 1998) and different types of salts had a variable effect on the process. Dapena-Mora et al. (2007) studied different types of salts without acclimatization and stated the IC_{50} values for NaCl, Na_2SO_4, and KCl were 13.46 g L^1, 11.36 g L^{-1}, and 14.9 g L^{-1}, respectively. They found that NaCl had no inhibitory effect if the concentration was less than 8.78 g L^{-1}. Fernández et al. (2008) reported that retention of Anammox biomass improved and specific Anammox activity gradually increased when 10 g L^{-1}NaCl was given to non-adapted Anammox biomass. In another study, conjugate influences of NaCl (90%) and KCl (10%) were examined for adapted and non-adapted biomass (Kartal et al., 2007a) and the salt concentration increased in a stepwise manner from 5 to 90 g L^{-1}. They observed that Anammox biomass were effectively acclimated at 30 g L^{-1} salt concentration and activity lost have been reported at 90 g L^{-1} of salt concentration.

Sulfide and phosphate

The inhibitory effect of other common constituents in wastewater such as sulfide and phosphate have been investigated in the literature. In the presence of SO_4^{2-} in the Anammox process bacteria may use SO_4^{2-} as an electron acceptor rather than NO_2^- (Yang et al., 2009). Under anaerobic conditions, SO_4^{2-} is reduced to H_2S and the inhibitory effect is generally expressed as H_2S. Since H_2S is highly soluble in lipids and is uncharged, it can penetrate into the cell membrane easier than SO_4^{2-} therefore, it is considered more toxic to the microbial community (Wang, 2002). The adverse effects of sulfide over Anammox process was associated with sulfide interaction with heme centers of cytochrome oxidase that decreases the heme iron in cytochrome c (Pietri et al., 2011). Dapena-Mora et al. (2007) observed total activity loss in batch tests at a concentration of 0.65 mM H_2S. Similar results have been reported by Carvajal-Arroyo et al. (2013), they found IC_{50} levels for H_2S as 0.03 mM. However, van de Graff et al. (1996) indicated that 2mM sulfide provides promoting effect on Anammox activity.

On the other hand, a common constituent of wastewater, phosphate, shows lower toxicity than sulfate effect over Anammox biomass. While van de Graff et al. (1996) reported complete loss of activity Anammox when reached the 5 mM phosphate concentration, Dapena-Mora et al. (2007) found the IC_{50} value for phosphate is 20 mM. Conversely, in another study, any inhibitory effect was not observed up to 20 mM (Egli et al., 2001).

The discrepancies between inhibitory thresholds concentrations of phosphate might be attributed to the presence of different species of Anammox which show different tolerance to phosphate concentrations.

1.4.3.4. Heavy Metal Inhibition

Anammox process has been identified as a promising novel technology for especially high-strength ammonium wastewater with low organic content (van der Star et al., 2007) wastewaters such as landfill leachates (Akgul et al., 2013; Egli et al., 2001), anaerobic digester reject water (Dapena-Mora et al., 2006; Furukawa et al., 2009), wastewater from semiconductor factories (Tokutomi et al., 2011) and sludge liquors (Fux et al., 2004). Ammonium-rich wastewater streams like landfill leachates may contain high concentration of heavy metals including; cadmium, chromium, copper , lead, nickel, and zinc (Kjeldsen et al., 2002). Some metals are critical for cell metabolism as constituents of co-enzyme and enzymes (Nies, 1999). For example, copper is a crucial component of nitrite reductase enzyme, molybdenum is an essential trace element for nitrate reductase enzyme, iron contributes to nitrite reductase, nitrate reductase, and dehydrogenation coenzymes synthesis (Fermoso et al., 2009) and nickel is an important constituent of dehydrogenation coenzymes (Hira et al., 2012). Although some of the heavy metals are fundamental for microbial cell production, they are also common inhibitors for biological processes in high concentrations. High concentration of these metals can inhibit the activity of Anammox by deactivating the enzymes (Fermoso et al., 2009).

In the literature, there are numerous studies about the toxicity of heavy metals on microorganisms in biological nitrogen removal systems (Çeçen et al., 2010a; Hu et al., 2003; Semerci and Çeçen, 2007). However, far too little attention has been paid to inhibitory effects of heavy metals (Li et al., 2015; Daverey, Chen, et al., 2014; Yang et al., 2013; Zhang et al., 2016; Zhang et al., 2015). The relevant studies result about the heavy metal inhibition on Anammox process is shown in Table 1.6.

Table 1. 6 Literature about heavy metal inhibition on Anammox system

Metal	Operation Mode	Applied Concentration mg L^{-1}	Inhibitory effect	Reference
Cu	Batch	1.9	IC50	Lotti et al., 2012
	Batch	12.9	IC50	Yang et al., 2013
	Batch	4.2	IC50	G. Li et al., 2015
	Long-term	5	Strongly inhibited	Yang et al., 2013
	Long-term	5	10% activity loss	Kimura and Isaka, 2014
	Batch	4.2	IC50	Daverey et al., 2014
	Batch	32.5	IC50	Z. Z. Zhang et al., 2015
	Batch	19.3	IC50	Val del Río et al., 2017
	Long-term	1	57.3 % decrease NRR	Ma et al., 2018
Ni	Long-term	5	10% activity loss	Kimura and Isaka, 2014
	Batch	69.2	IC50	Val del Río et al., 2017
	Batch	48.6	IC50	G. Li et al., 2015
Zn	Batch	59.1	IC50	Val del Río et al., 2017
	Batch	3.9	IC50	Lotti et al., 2012
	Batch	6.9	IC50	Daverey et al., 2014
	Batch	7.6	IC50	G. Li et al., 2015
	Batch	25	IC50	Q. Q. Zhang et al., 2015b
	Long-term	20	Temporary inhibiton	Daverey et al., 2014
	Long-term	10	10% activity loss	Kimura and Isaka, 2014
Cr	Batch	9.84	IC50	Yu et al., 2016
	Batch	26,9	IC50	Val del Río et al., 2017
As	Batch	60	29,67% inhibition	Yu et al,2016
Cd	Batch	174.6	IC50	Val del Río et al., 2017
	Batch	11.16	IC50	Bi et al., 2014
	Batch	11.2	IC50	Li et al., 2015
	Batch	7	IC50	Yu et al., 2016
Ag	Batch	11.52	IC50	Bi et al., 2014
Hg	Batch	60.35	IC50	Bi et al., 2014
	Batch	2.33	IC50	Yu et al., 2016
Mo	Long-Term	0.2	72% activity loss	Kimura and Isaka, 2014
Pb	Batch	45.6	IC50	Val del Río et al., 2017
	Batch	40	7.19 % decrease NRR	Bi et al., 2014
	Batch	4.3	20% activity loss	G. Li et al., 2015
	Batch	10.4	IC50	Yu et al., 2016

Although the individual inhibitory effect of some heavy metals have been investigated, the discrepancy between the results shows that there is no general agreement on inhibitory concentrations. The variance between the suppression threshold levels might be related to the operational conditions, reactor types and different Anammox species.

1.5. Toxicity of Heavy Metal to Microorganisms

Metals which have a density higher than 5 g/cm^3 are defined as heavy metal. Most of the heavy metals have a high specific gravity (greater than 4.0), atomic weight and number. Most heavy metals are essential for microbial production in biological systems. It is projected that approximately one-third of the structurally defined proteins are metal content and about half of the all known proteins are metalloproteins (Degtyarenko, 2000). Essential heavy metals which are necessary for the biological and physiological functions were reported as copper (Cu), iron (Fe), chromium (Cr), cobalt (Co), magnesium (Mg), manganese (Mn), nickel (Ni), selenium (Se), molybdenum (Mo) and zinc (Zn) (WHO, 1996). Although some of the heavy metals are crucial for cell synthesis, heavy metal ions may lead toxic effects at high concentrations due to their toxic complexes in the cell (Nies, 1999). The physical and chemical properties of the accessible donor ligands and metals are affected adversely the cell growth process in intracellular biomolecules when these metals reach the toxic concentrations (Lemire et al., 2013). Heavy metal ions must penetrate inside the cell, to have an inhibitory effect. For this reason, the intracellular heavy metal concentrations must be carefully controlled due to their potential to form toxic complexes (Nies, 1999). For many microorganisms, uptake of metal ions occurs in different ways such as, transmembrane transport, extracellular sorption, and intracellular accumulation (Hu et al., 2003). In the metal transport process, active metabolism can be considered as less important since both living and dead cells are capable of metal uptake (Nies, 1999). As a result of a number of active sites capable of binding metal ions of bacterial cell surfaces and extracellular polymers, sorption plays an important role in metal transport.

There are two major uptake systems for heavy metal ions; one includes nonspecific binding of the metal to cell surfaces or extracellular matrices, etc., while the second type of uptake involves metabolism dependent intracellular uptake. Intracellular metal uptake

generally is driven by chemiosmotic gradient across the cytoplasmic membrane of bacteria. Firstly, metal diffusion takes place through the outer membrane and subsequently metal transport of metal across the cell membrane (Hu et al., 2003; Nies, 1999). Nonessential toxic metals could starve cells by competitively hindering the transport of the essential metals (Lemire et al., 2013). Intracellular accumulation could occur after uptake when the metals present in high concentration and since nonessential metals have the similar structure they can enter the cell through the essential metal uptake system (Nies, 1999). Afterwards, metals can damage the structure of the proteins and may inhibit the function of various physiological cations. Additionally, redox-active metals, such as copper, may damage the membrane function via endorsing the stress through redox cycling activity and catalyzing the production of hydroxyl radicals, thus they are considered more toxic (Howlett and Avery, 1997) .

The fate of heavy metal is affected by microbial bioremediation. Heavy metals are not removed during bacterial metabolism or co- metabolism processes. Metals are degraded and converted to less toxic form by bioremediation which depends on the metabolic potential of the microorganism through redox processes. Mainly four mechanisms have been identified in the bioremediation of heavy metal that is shown schematically in Figure 1.6.

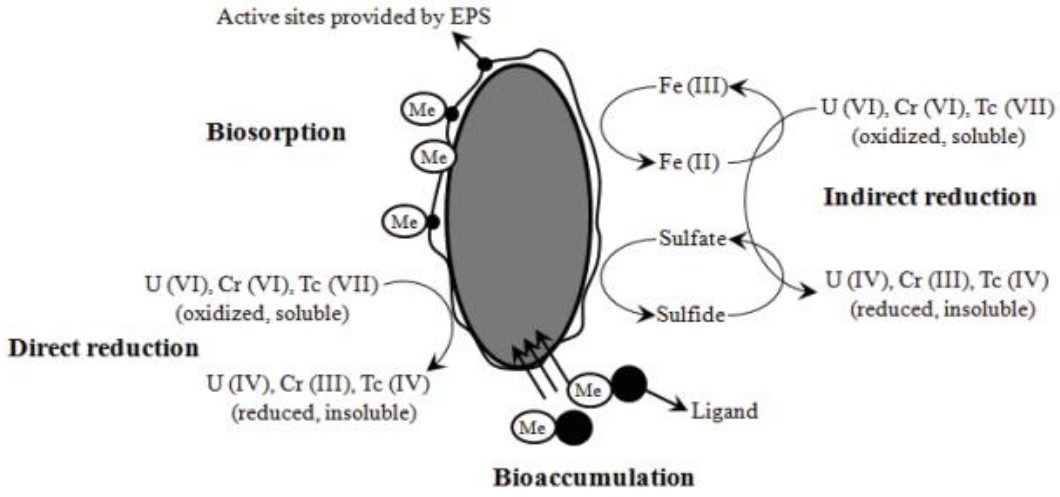


Figure 1. 6 Possible way of microorganisms-metal interaction (Adapted from Wang et al, 2010)

1.5.1. Heavy Metal Speciation

Metals can exist as many different chemical forms in any environment. There are several factors that specify the proportion and availability of these chemical species such as, pH, reduction potential, ionic strength and temperature (Lemire et al., 2013). Complexes occur when metal ions or atom is associated with ligands. Ligands, which are the species that bind to metal ions, can consist of wide variety of atoms, molecules or ions. If the ligands are joined to the atom in more than one place, they are called as multidentate ligands or chelating agent. Common examples of chelates are ethylenediaminetetraacetic acid (EDTA), ethylenediamine (EN), acetylacetone (ACAC), nitrilotriacetic acid (NTA) (Xu et al., 2010). Especially EDTA and NTA are the most widely found synthetic ligands in industrial and domestic wastewaters (Alder et al., 1990).

About one-third of the elements in the periodic table can react with organic ligands. Organic compounds, their by-products and also metal ions in solutions, soils or sediments arise from natural processes or industrial activities. Metal-binding ligands, mixed ligand complexes, adsorption complexes are formed by biological, photochemical and redox reactions in the environment (Fernando, 1995). Studies show that these metal complexes have been transported in soil, air and natural water sources and disrupt the environment. Since bioavailability and toxicity of these complexes is related to their stability, it is important to identify metal speciation rather than its total concentration (Allen and Hansen, 1996; Fernando, 1995).

The stability of complex ions is related with the ligands and nature of central atoms. The term of “stability constant” can be found from the dissociation reaction of complex in solution. The higher stability constant, the more stable the complex. Complexations results the decrease in the affinity of metals to precipitate out of solution or adsorb to the surface of solids associated with the solution. The occurrence of stronger complexes may cause the reduction in free ion activity (Benjamin, 2002)

1.6. Inhibition Mechanisms

Heavy metal species disrupt the enzyme activity by tightly binding to a functional group at the active site of an enzyme and damage the membrane function or DNA. Heavy metals may inhibit excess amount of enzymes at high concentration (Sharma, 2012).

Enzyme inhibitors which are classified as reversible or irreversible can reduce or completely inhibit catalytic activity of enzymes. There are two types of inhibitors specific and non-specific inhibitors. The specific inhibitors affect a specific component of a single enzyme while non-specific inhibitors may inhibit multiple enzyme targets. In irreversible inhibition target enzyme dissociate very slowly since inhibitors form covalent bonds with enzyme (Berg et al., 2002)

Reversible inhibition is subdivided into four categories related to their binding behavior;

- Competitive inhibition
- Non-competitive inhibition
- Uncompetitive inhibition
- Mixed inhibition

The description of these inhibition categories is done below (Cornish-Bowden, 2013; Segel, 1993) ;

Competitive inhibition; at the active side of the enzyme both inhibitor and substrate have an affinity to bind. Thus, the substrate and inhibitors compete for binding to active side of the enzyme.

Figure 1. 7 illustrates the competitive inhibition mechanism.

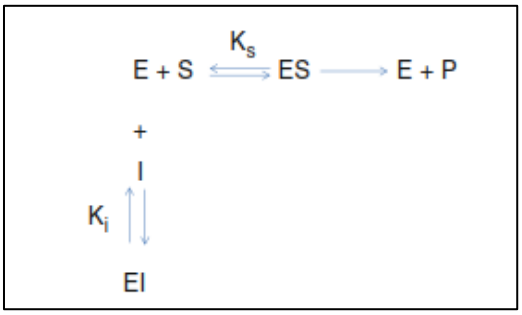


Figure 1. 7 Competitive inhibition mechanism (Adapted from Ring et al., 2014)

To overcome the inhibition effect substrate concentration should be increased sufficiently to displace the inhibitor. V_{max} (the maximum rate of enzyme hydrolysis) of the reaction does not change.

Non-competitive inhibition; the inhibitor shows same binding affinity for enzyme and also enzyme-substrate complex. Non-competitive inhibitor has no effect on the binding

of substrate since it reacts with the enzyme but generally not the active side of the enzyme. Thus, in the non-competitive inhibition V_{max} of the reaction is decreased. Figure 1. 8 illustrates the non-competitive inhibition mechanism.

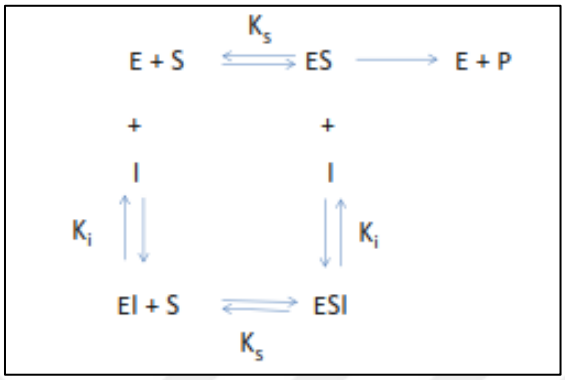


Figure 1. 8 Non-competitive inhibition mechanism (Adapted from Ring et al., 2014)

Uncompetitive inhibition; Unlike competitive inhibition, inhibitors has no affinity to bind the free enzyme, only binds to substrate-enzyme complex. The high concentration of substrate promotes the uncompetitive inhibition effect. Furthermore, uncompetitive inhibitors cause the decrease of V_{max} of the reaction.

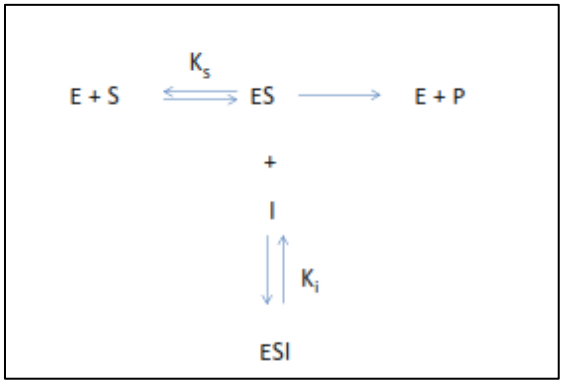


Figure 1. 9 Uncompetitive inhibition mechanism (Adapted from Ring et al., 2014)

Mixed inhibition; in this type inhibition both substrate-enzyme complex and enzyme is affected adversely like non-competitive inhibition. Although inhibition mechanisms look similar, in this type of inhibition inhibitors shows different affinities to bind free enzyme or substrate-enzyme complex. Inhibitors may bind to the active side and different site of a free enzyme. **Figure** 1. 10 illustrates the non-competitive inhibition mechanism.

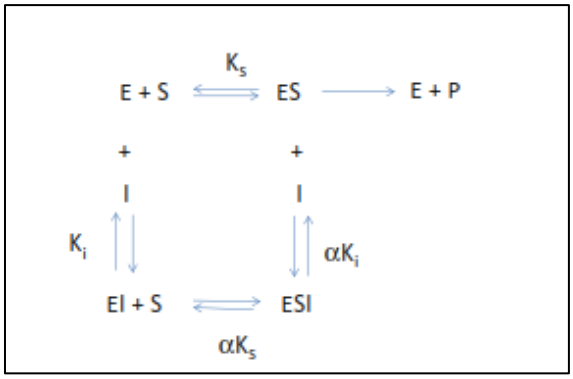


Figure 1. 10 Mixed inhibition mechanism (Adapted from Ring et al., 2014)

1.7. The scope of the Thesis Study

The nitrogen-rich wastewaters generally contain high concentration of heavy metals. For instance, landfill leachate, depending on the waste composition, waste age, and, landfill technology, contain substantial quantities of heavy metals, including copper, zinc, lead, cadmium, and nickel (Baun and Christensen, 2004) that leach from the waste. Similarly, livestock wastewaters are associated with high concentrations of nitrogen as well as heavy metals such as copper and zinc (Lotti et al., 2012). Such heavy metals do exist in such wastewaters because their feed is supplemented as a dietary supply with them and the excess amount is excreted (L'Herroux et al., 1997). Likewise, high concentrations of metal-bound nitrogen species are present in metal refinery wastewater (Milia et al., 2015) because nitrogenous compounds are used during production (Manipura et al., 2007).

In fact, the effect of various metals, including Cu, Zn, Co, Mn, and Ni is dose-dependent: when supplied in sufficient quantities they are necessary for major or trace elements for biochemical reactions. However when their concentration exceeds beyond the nutritional level, they form unspecific complex compounds within the cell and become inhibitory (Nies, 1999). The range of concentrations between beneficial and toxic effects may be narrow; therefore, it is important to understand the concentrations which cause inhibition of Anammox activity.

Recently, there has been some effort to determine the individual effects in the presence of specific heavy metals, such as Cd, Ag, Hg, Pb, Cu, Zn and Ni on Anammoxactivity (Bi et al., 2014; Daverey et al., 2014; Guo et al., 2015; Kimura and Isaka, 2014; G. Li et al., 2015; Liu et al., 2013; Lotti et al., 2012; Yang et al., 2017, 2013, Q. Q. Zhang et al., 2015b, 2015a, Zhang et al., 2016a, 2016b). However, the experimental data are rather controversial, and there is no general agreement about inhibitory concentrations. The discrepancy between the results may be explained by a number of operational factors which play a role on all biochemical reactions such as; mean cell residence time, pH, reactor type and configuration and microorganism concentration (Wang et al., 1999). The variability should also be examined in terms of Anammox species that take a role in the conversion. There still remains, a paucity of documented data on heavy metal inhibition that provide sufficient information regarding the Anammox species that are exposed to these chemicals. Such data have a major influence on the inhibition levels, since the response of each Anammox species to heavy metal may be different in a given biological system because the metabolic response to an inhibitory chemical is mostly related with microbial characteristics (Li et al., 2016).

Therefore, the present study aims to analyze the effects of Cu, Zn, Ni and Cd on the Anammox system. The experiments were performed in batch and continuous-flow reactors that were enriched with Anammox biomass, to evaluate the inhibitory effect of heavy metal in all aspects.

In order to determine the short-term effect of these metals batch tests were conducted for 24 h. Since the inhibition effect of Cu and Cd metals were found to be stronger than the others in short-term batch experiments, continuous-flow reactors were also tested to determine the prolonged effect of Cu and Cd.

So far, inhibition of Anammox by heavy metal were examined by some researchers, however; the effect of heavy metal speciation on inhibition is generally overlooked in the literature. Therefore, the heavy metal speciation and related inhibition effects were examined and the effective concentration that causes 50% inhibition was calculated for each metal by using linear and non-linear regression models. Metal speciation was evaluated theoretically by using Visual MINTEQ (Version 3.1).

Additionally, microbiological analyses were done via real-time PCR method and next-generation sequencing techniques to examine the possible shift of the bacterial community and for a better understanding of the inhibition behavior of Anammox bacteria facing heavy metals.

The methodology of this study is summarized in Table 1.7

2. MATERIALS AND METHOD

Table 2. 1 Methodology of the study

PERIOD 1	**PERIOD 2**	**PERIOD 3**	**PERIOD 4**
Enrichment of Anammox Biomass *Set-up and start-up of the SBR to provide effective biomass retention of Anammox culture for long-term experiments * Set-up and start-up Up-flow reactor to provide effective biomass retention of Anammox culture for long-term experiments * For short-term experiments, the start-up of the SBR reactor to maintain the enriched culture of Anammox	**Short-Term Experiments** * Design and start-up of batch experiment setup □ Determination of the individual effects of Cu, Ni, Zn and Cd during short-term exposure **Metal Measurements** * Development of digestion methods * Development of heavy metal measurement methods **Metal Speciation Calculations** * Visual Minteq applications	**Long-Term Experiments** * Start-up of Cu experiments in up-flow columns after achieving 90 % removal efficiency in column * Start-up of Cd experiments in SBR reactor after achieving 90 % removal efficiency in reactor * Determination of the effect of Cu and Cd in long-term exposure * Data evaluation	**Molecular Analysis** * Analysis of the microbial community during the study by using ; □ Real-time PCR □ Next-generation sequencing

This study consists of four parts to comply with the above-mentioned objectives.

Firstly, to investigate the individual short-term inhibitory effects of several heavy metals (Cu, Zn, Ni, and Cd), variable concentrations of heavy metals were contacted with Anammox biomass and threshold inhibitory were determined in a batch reactor.

In the second part, to observe the impacts of Cd during long-term exposure, lab-scale sequencing batch reactor was operated for approximately 330 days.

Additionally, the lab-scale continuous up-flow reactor was operated for 240 days to determine the impacts of long-term exposure of Cu on the Anammox system.

Furthermore, microbial consortia exposed to heavy metal was identified using real-time PCR and metagenomics analysis.

Figure 2.1 illustrate the reactor configurations during the study.

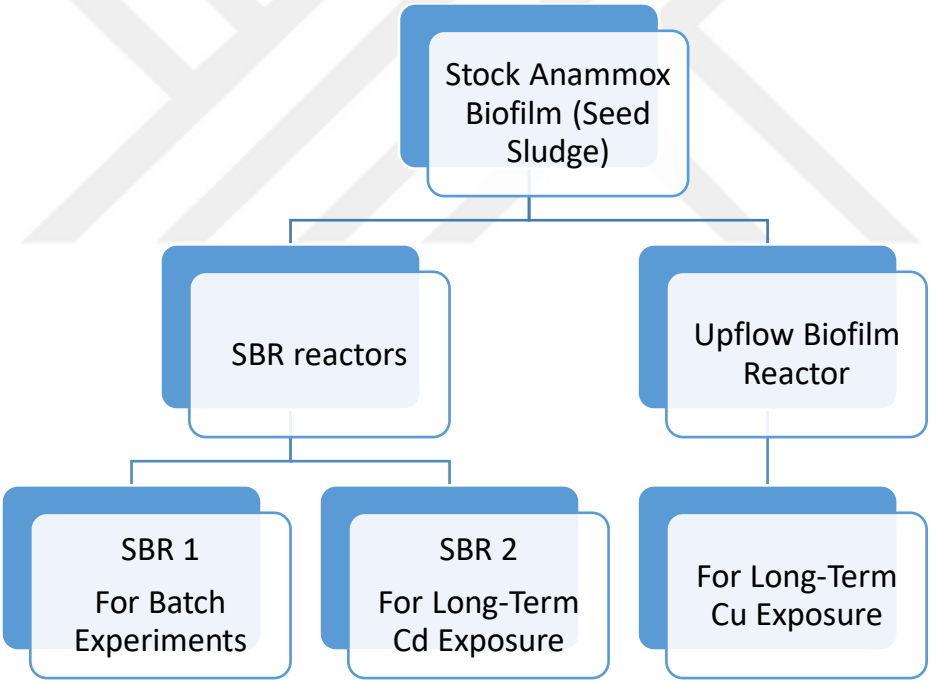


Figure 2. 1 Reactor Configurations

2.1. Inoculum and Synthetic wastewater

Sequencing batch reactors and up-flow continuous reactor were inoculated with enriched Anammox sludge which was obtained from an ongoing lab-scale up-flow column reactor which is in operation for over six years.

Approximately 300 mL of Anammox seed sludge with an approximately 1000 mg L^{-1} MLVSS concentration were inoculated to both reactors.

As a feed solution, a synthetic wastewater was prepared similarly as reported by Egli et al. (2001). The composition of the synthetic medium was shown in Table 2.2.

Synthetic feed solution also contains mainly 1:1.1 ratio ammonium to nitrite in the form of $(NH_4)_2SO_4$ and $NaNO_2$, as 100 mg L^{-1} NH_4-N and 110 mg L^{-1} NO_2-N for SBR reactor and batch test analysis, for up-flow column reactor 300 mg L^{-1} NH_4-N and 330 mg L^{-1} NO_2-N. Besides, 50 mg L^{-1} nitrate was added to feed solution to prevent decay of Anammox bacteria and to provide the anoxic conditions.

Table 2. 2 The composition of synthetic wastewater

Constituents	Stock Concentration	Reactor Concentrations
$(NH_4)_2.SO_4$	100 g L^{-1} as NH_4-N	100-300 mg L^{-1} as NH_4-N
$NaNO_2$	100 g L^{-1} as NO_2-N	110-330 mg L^{-1} as NO_2-N
KNO_3	25 g L^{-1} as NO_3-N	50 mg L^{-1} as NO_3-N
$NaHCO_3$		1.049 g L^{-1}
K_2HPO_4	174.2 g L^{-1}	174.2 mg L^{-1}
$CaCl_2.2H_2O$	73.5 g L^{-1}	73.5 mg L^{-1}
$MgCl_2.7H_2O$	102 g L^{-1}	102 mg L^{-1}
Mineral Solution 1		1 mL L^{-1}
$Na_2EDTA.2H_2O$	10 g L^{-1}	
$ZnSO_4.7H_2O$	0.43 g L^{-1}	
$CoCl_2.6H_2O$	0.24 g L^{-1}	
$MnCl_2.4H_2O$	0.99 g L^{-1}	
CuSO4.5H2O	0.25 g L^{-1}	
$NiCl_2.6H_2O$	0.19 g L^{-1}	
H_3BO_4	0.014 g L^{-1}	
Mineral Solution 2		1 mL L^{-1}
$Na_2EDTA.2H_2O$	10 g L^{-1}	
$FeSO_4$	5 g L^{-1}	

2.2. Experimental Setup

2.2.1. Short-Term Effect Experiments (Cadmium (Cd), Copper (Cu), Nickel (Ni) and, Zinc (Zn))

To observe the influences of heavy metals short-term exposure batch tests were carried out in side armed glass reactors with 100 ml total volume and 50 ml liquid phase volume. Batch system configuration is shown in Figure 2.1. Side-armed reactors are specifically designed and produced for these batch experiments. Side-arms are used to take samples in each run.

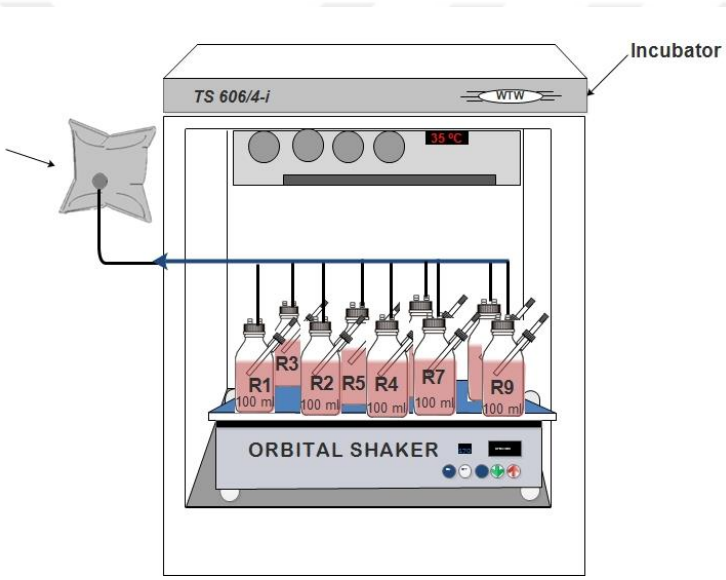


Figure 2. 2 Batch system configuration

In order to provide biomass for batch experiments, Anammox bacteria were enriched in a sequencing batch reactor (SBR) operated at a sludge retention time of 50 days (Figure 2.3). After steady-state condition is reached and 90 % nitrogen removal efficiencies were achieved in the SBR, mixed liquor was taken from the reactor for batch inhibition studies. The reactors were inoculated with Anammox biomass approximately as 900± 100 mg L^{-1} MLVSS concentration. To provide anoxic conditions liquid phase and headspace was purged with dinitrogen gas. The batch reactor system is kept in an incubator (VTW Model No: TS606-G/2-i), this way operating temperature was kept at 35±0.5 °C.

Short-term inhibition responses were tested using different concentrations of metals (Cd, Ni, Zn, and Cu) on Anammox biomass. The concentration ranges for heavy metals were shown in Table 2.3.

To monitor the ammonia nitrogen and nitrite nitrogen removal rates samples were taken every three hours and analyzed during the 24-h time period. Tests were performed at least in triplicate for each heavy metal concentration in 100 ml side armed bottles placed on a gyratory shaker (125 rpm). In each run, nine bottles were operated in parallel where three of them were served as control rector. In order to maintain oxygen-free conditions during the course of batch experiments and especially during sampling a gas bag filled with nitrogen gas was used. Gas bag provides a positive pressure on the headspace of bottles. Nitrogen gas is distributed to the system via the manifold and is connected to each bottle through a normal open-side of a three-way solenoid valve. After the mixed liquor settled for two minutes, the sample was drawn off from the sampling valve via a syringe.

Table 2. 3 Applied concentrations of heavy metals during batch experiments

Constituents	Concentration Range mg L^{-1}
Cd	0-5-7.5-10-15-17.5-20-30
Ni	0-5-6-8-10
Zn	0-7.5-10-15-20
Cu	0-1-2.5-5-10

Nitrification inhibition estimations were done based on total nitrogen (ammonium + nitrite nitrogen) removal rate. NRR was calculated based on the fast initial rate and was calculated by using Equation 2.1.

$$NRR\left(\frac{\frac{mgN}{gVSS}}{h}\right)=\frac{\left(TN_{(\mathrm{inf})}-TN_{(eff)}\right)}{t\times VSS} \quad (2.1)$$

where $TN_{(inf)}$ and $TN_{(eff)}$ were initial and final total nitrogen concentrations. Percent inhibition values were calculated according to Equation 2.2.

$$\%\ inhibition=\left(\frac{NRR_{\mathrm{blank}}-NRR_{\mathrm{Heavymetal}}}{NRR_{blank}}\right)\mathrm{X}\ 100 \quad (2.2)$$

The values obtained from heavy metal exposure experiments were compared to those collected from control samples (without heavy metal) to calculate percent inhibition

response. The percent inhibition of Anammox activity in the presence of heavy metals was calculated based on the rate in the absence or presence of the inhibitor (Eqn (2.2).

2.2.2. Long-Term Experiments

To investigate the long-term effects of cadmium (Cd) and copper (Cu), two different reactor configurations were used during the study.

2.2.2.1. Sequencing Batch Reactor Configuration

In order to determine the long-term effects of cadmium (Cd), a sequencing batch reactor (SBR) was designed which had an active volume of 2 L and had a solid retention time of 40 days. Figure 2.3 shows the schematic figure of the SBR systems.

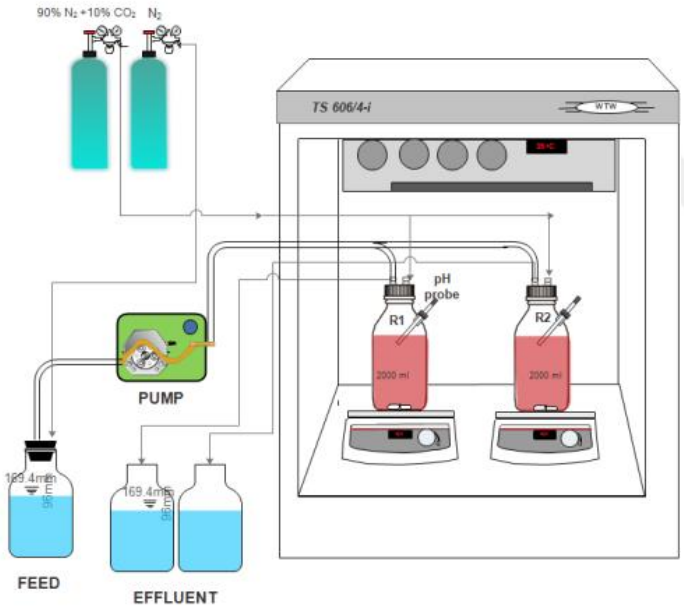


Figure 2. 3 Schematic figure of the SBR reactors (R1: Enriched culture that was used for batch experiments, R2: Reactor that was used for investigating the Cd effect)

The SBR was operated on four cycles in 24 h and each cycle consisted of the following phases; 25 min fill, 22.6 h react, 30 min settlement and 25 min draw/idle. In each SBR cycle, 1L of treated wastewater was exchanged with a new batch of synthetic wastewater. The hydraulic retention time (HRT) of the system was kept as 2 days. The reactor was fed with synthetic wastewater composed of essential macro and micronutrients according to Egli et. al. (2001) with 100 mg L^{-1} NH_4-N and 110 mg L^{-1} NO_2-N. N_2-CO_2 (90%-10%) gas mixture supplied to reactor enabled to maintain an oxygen-free environment for

Anammox enrichment.



Figure 2. 4 Experimental setups for the SBR reactors

To examine the long-term effect of cadmium, heavy metal concentration was increased gradually starting from 0.2 mg L^{-1}.

2.2.2.2. Up-flow Continuous Reactor Configuration

To observe the response of Anammox system to prolonged copper exposure, a plexiglass continuous flow up-flow reactor was designed which has an active volume of 2.31 L with diameter 7 cm and height of 60 cm. The schematic figure of the system is shown in Figure 2.5.

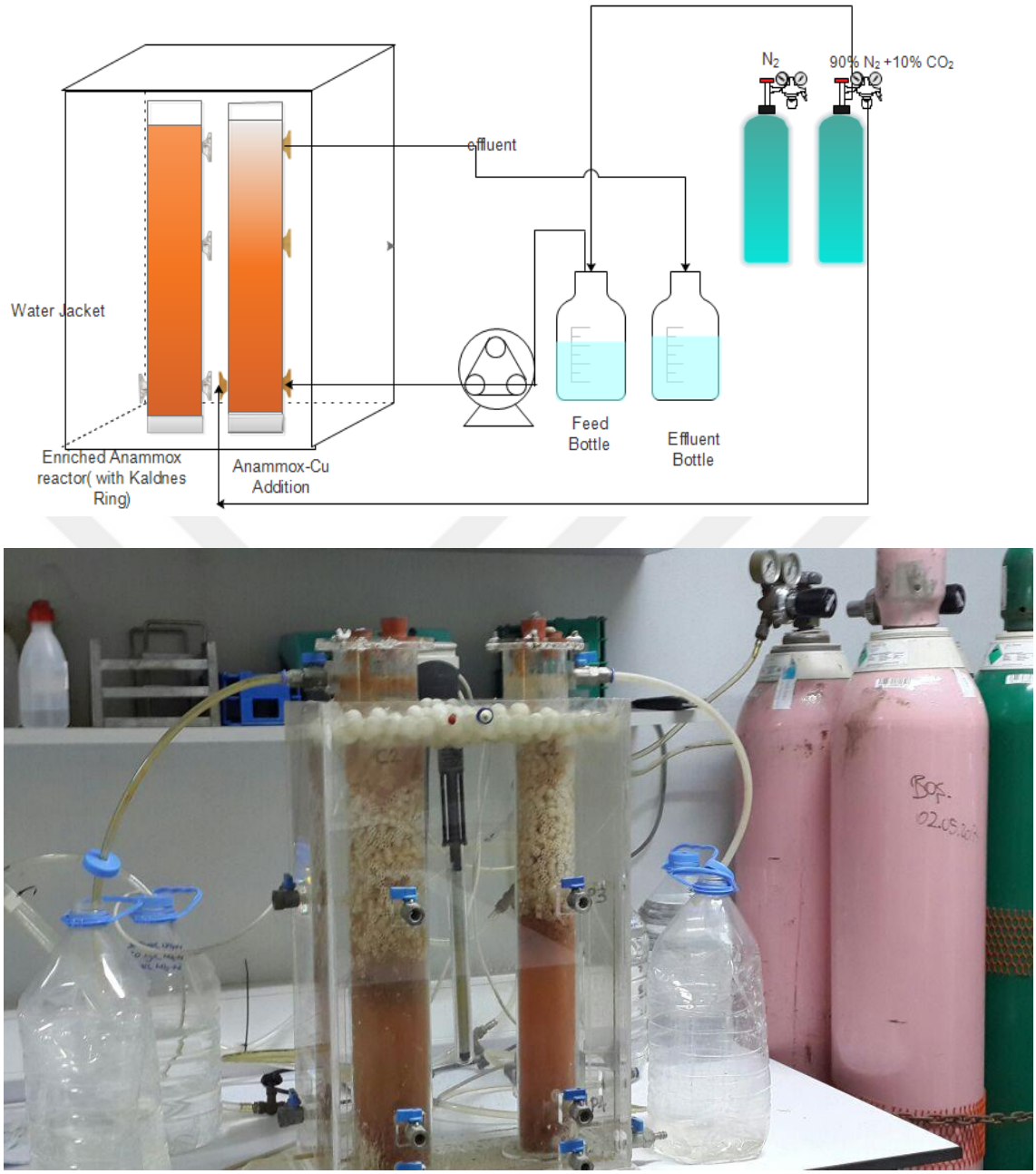


Figure 2. 5 The experimental setup of up-flow continuous Anammox reactors

The average flow rate of the system was 1800mL d^{-1} and hydraulic retention time was 1.28 d. The reactor was half full filled up with Type K1 Kaldnes rings packing material to provide abundant biomass retention. The carrier materials were provided by AnoxKaldnes Company and made of polyethylene (PEHD) material with nominal length and diameter 7.2 mm and 9.1 mm, respectively. The reactor was operated under 35±1°C to provide constant temperature water bath was used around the reactor. To enable the anoxic conditions inside the reactor 90% N_2 + 10% CO_2 gas combination was supplied

from the bottom of the column. Carbon dioxide gas is used to serve as the source of inorganic carbon along with the bicarbonate in the feed.

In order to test the long-term effect of copper metal, concentration was increased gradually starting from 0.2 mg L^{-1}.

2.3. Analytical Methods

Nitrite and nitrate concentrations were analyzed by using high-pressure liquid chromatography (HPLC) (Shimadzu Prominence LC-20AD Shimadzu Corp, Kyoto, Japan) which was equipped with SPD-20A UV-VIS detector and reverse phase C-18 column (Knauer Vertex Plus 125x4 Eurosil Bioselect 300-5) was employed for separation. The length of the column 125 mm and the inner diameter is 4 mm with 5 µm pore size.

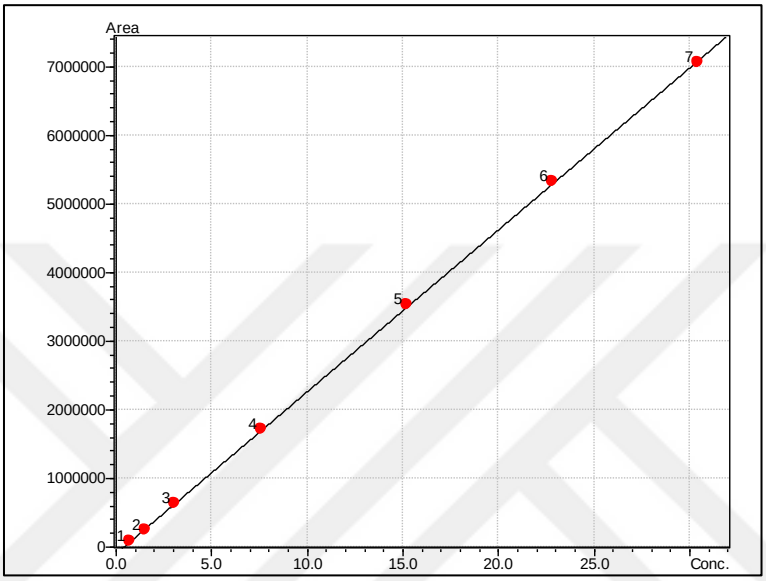
0.01 M octylamine solution was used as a mobile phase solution. pH of the solution was adjusted to around 4.0-4.5 using acetic acid. Pumping rate of the mobile phase was arranged as 1 mL min^{-1}. The following conditions were set for the measurements; UV wavelength of 210 nm, column temperature of 35 ^{o}C and injection volume of 10 µL. Samples were filtered through a 0.45 µm PVDF syringe filter before introduced to HPLC.



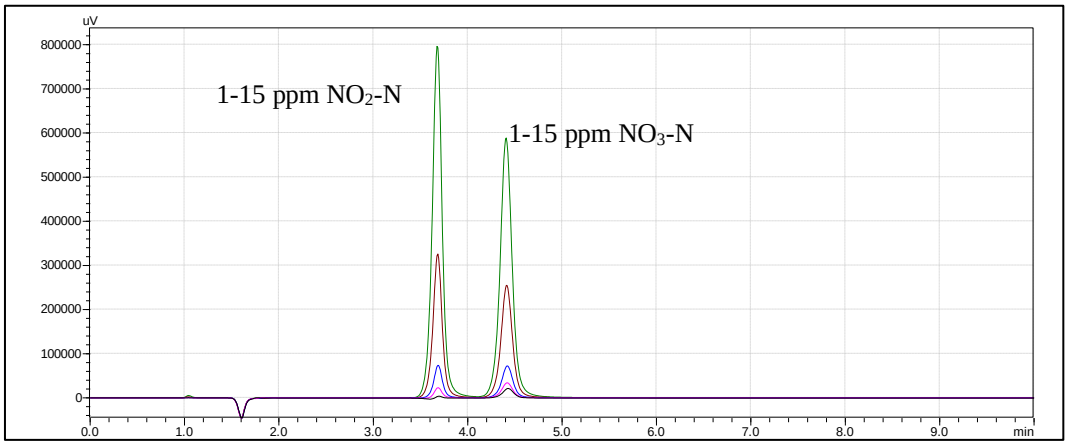
Figure 2. 6 Shimadzu High-Pressure Liquid Chromatography with Prominence UFLC system.

The ammonium concentrations were measured by Nesslerization method using HACH DR2500 spectrophotometer (Hach Method No: 8038). This procedure is a modification of the method from Standard methods 4500-NH_3 B&C.

Volatile suspended solids (VSS) and suspended solids (SS) measurements were done using gravimetric methods according to Standard methods 2450 D.



(a)



(b)

Figure 2. 7 An example from an (a) HPLC Calibration curve and (b) chromatogram

2.4. Evaluation of IC_{50} values

The half maximal inhibitory concentration (IC_{50}), which is the concentration of a toxic or inhibitory compound that results in 50% decrease in biological activity, is estimated using

several inhibition models for each metal. In order to calculate IC_{50} value, both linear and nonlinear regression models were applied using GraphPad Prism (version 7.03) software. IC_{50} values were calculated based on total nitrogen (ammonium nitrogen +nitrite nitrogen) removal rate.

- Non-competitive inhibition model

$$I\% = 100 \times \left(1 - \frac{1}{1+\frac{HM}{a}}\right) \quad (2.3)$$

- Modified non-competitive inhibition model

$$I\% = 100 \times \left(1 - \frac{1}{1+\left(\frac{HM}{a}\right)^{b}}\right) \quad (2.4)$$

- Linear model

$$I\% = m \times HM + n \quad (2.5)$$

- 4-point logistic model

$$\mathrm{I\%} = 100 \times \left(1 - \frac{\mathrm{c-d}}{1+\left(\frac{\mathrm{HM}}{\mathrm{a}}\right)^{\mathrm{b}}} + \mathrm{d}\right) \quad (2.6)$$

where I% is the inhibition response, HM is the heavy metal concentration, a is the concentration causing 50% inhibition in the nitrogen removal rate, b is a fitting parameter, c and d are the maximum and minimum inhibition responses, respectively.

2.5. Heavy Metal Measurements

Heavy metal concentrations were measured by using Perkin Elmer AAnalyst 400 Flame Atomic Absorption Spectrometry (AAS). Samples were filtered through 0.45 µm PVDF syringe filter and acidified with concentrated nitric acid before introduced to AAS. Dilution of the samples was prepared by using 3% nitric acid solution. In order to construct the calibration curve of each heavy metal, several dilutions of standard stock solutions were used (1000 mg L^{-1}). At least five-point calibration curves were established. Measurement of each heavy metal concentration was performed at least triplicate to minimize the errors. Wavelengths used for each heavy metal measurement were shown in Table 2.4.

Table 2. 4 Slit width wavelengths of the lamps

Metal	Wavelength (nm)	Slit Width (mm)	Characteristic Concentration (mg L^{-1})	Sensitivity Check (mg L^{-1})
Zn	213.9	2.7/1.80	0.006	0.30
Cd	228.8	2.7/1.35	0.010	0.50
Ni	232.0	1.8/1.35	0.060	3.00
Cu	324.8	2.7/0.80	0.025	1.30

2.5.1. Metal Partitioning Experiments

The heavy metal measurements consist of three form; total metal, soluble metal and intracellular metal. Soluble and intracellular metal measurements were done after 24 h exposure time in batch tests. A simple mass balance of copper concentration can be signified as follows;

$$HM_{Total} = HM_s + HM_{sb} + HM_{int} + HM_{ppt}$$

where "s" is soluble, "sb" is the surface-bound, "int" is the intracellular heavy metal and "ppt" is the precipitate. Measurement of total, soluble and intracellular copper concentrations were done mainly as described by Wang et al. (2016).

The biomass bounded fraction of heavy metals was considered as the sum of the intracellular and surface-bound fractions and was called as a cell-associated heavy metal.

2.5.2. Sample Digestion and Preparation

50 mL mixed liquor was taken from batch reactors and test bottles directly and 10 mL mixed liquor was used to measure the total heavy metal concentration and digested according to Standard Acid Digestion Methods 3030E (APHA, 2005). 2 ml concentrated HNO_3 and 10 mL sample were put into the polypropylene falcon tubes. Samples were digested at a temperature of 105^oC for 4 hours and sample loss was measured during digestion.

40 ml mixed liquor was taken and filtered through 0.70 µm glass fiber filter (Whatman GF/C 47mm) and the filtrate was digested and measured as soluble part of heavy metals.

In order to determine the intracellular heavy metal concentration modified EDTA washing procedure (Hu et al., 2003) was applied to the retentate on the filter. For the preparation of washing solution 1mM EDTA and 0.1M NaCl were mixed and to prevent the osmotic shock pH is kept at 7.0. Remaining microbial pellets were re-suspended in washing solution to remove surface-bound heavy metal for 30 min at 150 rpm. Subsequently, centrifugation of samples was done twice at 1600 g for 5 min. The supernatant part was removed between each centrifugation and resuspension of pellets was done by using a washing solution. Finally, to measure the intracellular metal concentration nitric acid digestion was applied to the final pellet.

In the absence of bacteria, filtered and unfiltered solution concentrations also measured the difference between measurements was used to find the precipitated amount of heavy metal in the solution. To calculate the surface-bound heavy metal concentration the formula given above was used for HM_{sb}. Flow diagram of the metal analysis is shown in Figure 2.7.

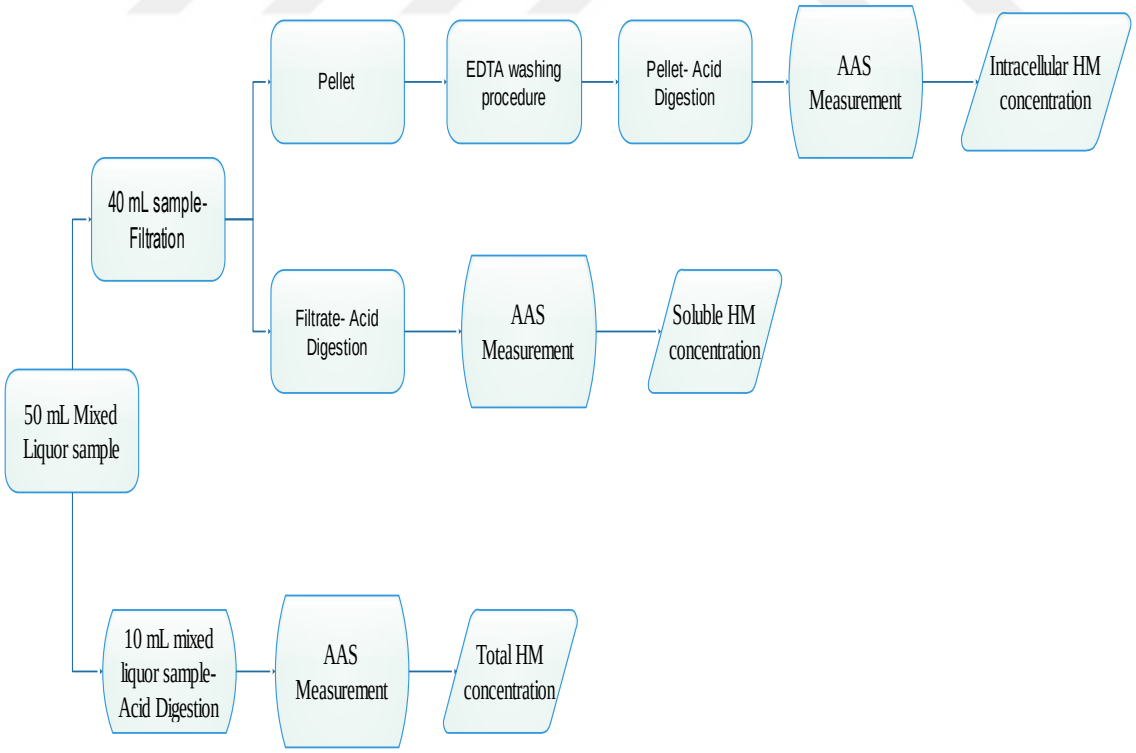


Figure 2. 8 Flow diagram of the heavy metal measurement procedure

2.6. Calculations of Chemical Speciation

The expression of speciation indicates both chemical form and oxidation state of the element in which that element exists in the environment. According to the International Union of Pure and Applied Chemistry (IUPAC) "speciation" refers to the distribution of an element among the chemical species defined in the system (Templeton et al., 2000). The chemical speciation should be taken into account instead of measuring only the total concentration to estimate the heavy metal effect on aquatic organisms. To correlate the chemical speciation with biological effect of metal, the bioavailability of the metals has been explained by some models such as the free ion activity model (FIAM) and the biotic ligand model (BLM).

The computer speciation models that have a huge default database are wide to calculate the distribution of inorganic aqueous species (Baun and Christensen, 2004). Speciation calculations for each metal (Cd, Ni, Zn, and Cu) were simulated by using a chemical equilibrium software Visual Minteq (Version 3.1). All free metal concentrations and inorganic metal complexes were predicted by using this software. Computation was accomplished using heavy metal concentrations, temperature, pH and all other chemical species as input data. All speciation calculations were carried out under 35^{o}C and at a pH value of 8.0.

The distribution coefficient (K_d) was incorporated to the software to estimate sorption to biomass (i.e. cell associated heavy metal, HM_{int} + HM_{sb}).

Isotherm models (Freundlich, Langmuir, and Linear) were applied to calculate K_d for each metal using data pairs on cell-associated heavy metal concentration (mg metal/kg MLVSS) and soluble metal concentration (HM_s).

2.7. Molecular Analyses

2.7.1. Sample Collection and DNA extraction

Samples were stored at -20 ^{o}C before extraction. The bead-beating based FastPrep-24 instrument was used at a speed setting of 4 m/s for 20 sec for the nucleic acid extraction of samples. Then, the supernatant portion was separated by using 14000 rpm

centrifugation for 10 min. Nucleic acid extraction was performed following the procedure in our previous work (Aktan et al., 2012) using Bio 101 FastDNA® SPIN Kit (Q-BIOgene). Extracted DNA samples were stored at -20 ºC in the freezer.

2.7.2. Nanophotometer Measurements

DNA yield can be measured directly by absorbance method under ultraviolet light. Although this method is not highly sensitive, is still the preferred method when working with a small amount of sample. This method is based on the measurement of absorbance at 260nm (A_{260}) where the lights most intensely absorb by DNA, to calculate the concentration of DNA according to Lambert Beer's Law. Although DNA has a great absorbance at 260 nm UV light, RNA also has a strong absorbance at the same wavelength and aromatic amino acids absorb at 280nm. This means that these contaminants may cause interferences in the measurement of DNA concentration. Therefore, to evaluate the purity of DNA samples A_{260}/A_{280} ratio is used and pure nucleic acids samples would have an A_{260}/A_{280} ratio 2.0 and ratio around 1.80 is expected if there is no protein contamination at DNA samples.

After nucleic acid extraction, the obtained amount was quantified and checked for purity by NanoDrop™ 2000c (Thermo Scientific, USA) at a 260 nm wavelength as µg/mL.

2.7.3. Real-time Polymerase Chain Reaction

A variable region of DNA or RNA is amplified over and over again within a short period of time by PCR method and exact results are obtained. However, there are some troubleshoots during the execution of the samples in an agarose gel and monitoring under UV using ethidium bromide. Real-time PCR is a technique for simultaneously monitoring and quantifying the results of DNA amplification from a monitor using fluorescent dyes. Contamination that may occur during DNA amplification or the ability to stop the program immediately rather than waiting for a long time at any point is a great advantage over conventional PCR in terms of avoiding time loss.

The quantity and purity of the extracted DNA samples were predicted by Quant-iT™ PicoGreen dsDNA Reagent Kit (Molecular Probes) following the manufacturer's protocol. Real-time PCR reactions were performed using LightCycler Instrument (Roche, Mannheim, Germany) with Fast- Start DNA Master SYBR Green I kit (Roche). To

provide suitable PCR amplicons as templates for subsequent PCRs DNA samples were diluted. An external DNA standard curve was formed using serial dilutions of a 3605 pg/µl and 567 bp template (Figure 2.7).

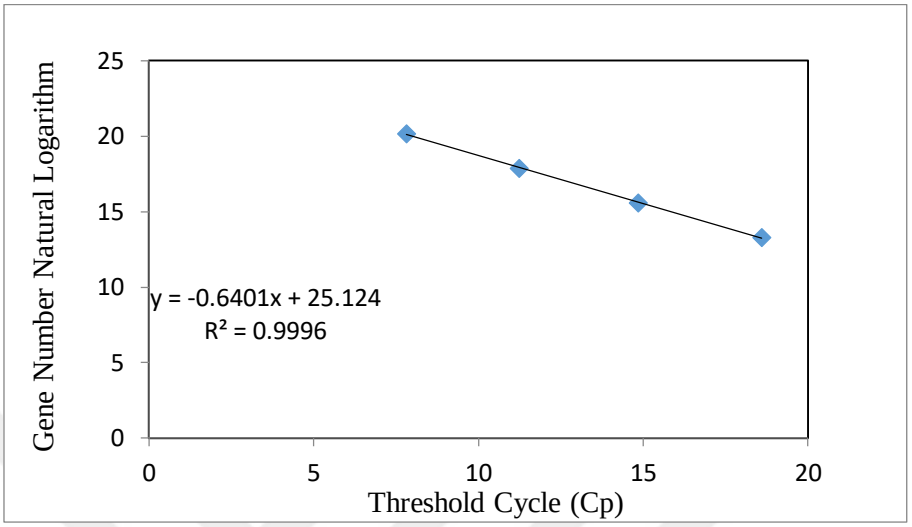


Figure 2. 9 Standard Curve for real-time PCR

By using the generated graph gene copy number for bacteria was calculated according to Equation 2.6.

$$\text{Gene copy number} = e^{(25.12-(Cp*0.64))} \quad (2.6)$$

The applied equation was revised for the Anammox measurements due to the differences between amplicon sizes of 16S and AMX primer sets. Equation 2.7 was used to calculate the gene copy number of Anammox bacteria and total gene copy number ratio to the relative quantification of Anammox bacteria in the system was examined.

$$\text{Gene copy number} = \left(\frac{567}{621}\right) * e^{(25.12-(Cp*0.64))} \quad (2.7)$$

16S DNAs fragments were amplified with 341F-907R and AMX368R-AMX820R primers shown in Table2.5.

Table 2. 5 Primer sets used in PCR

Primer Name	Primer Sequence (5'-3')	Reference
341F	CCT ACG GGA GGC AGC AG	Teske et al., 1997
907R	CCG TCA ATT CCT TTR AGT TT	
AMX368F	TTC GCA ATG CCC GAA AGG	Schmid et al., 2003
AMX820R	AAA ACC CCT CTA CTT AGT GCC C	

The PCR amplification reaction was carried out in a Progene thermocycler (Techne, Cambridge, UK) and the following program was used: pre-denaturation (95°C, 10 min), 40 amplification cycle each consist denaturation (95°C, 10 sec), annealing (57°C, 10 sec), elongation (72°C, 10 sec) and post-elongation (78°C, 0 sec), melting curve (65°C, 15 sec), cooling (40°C, 30 sec). The reactions were subsequently cooled to 4°C.

2.7.4. Metagenomic Analysis using Next-Generation Sequencing

DNA library construction and sequencing were conducted by Bioeksen Company (Istanbul, Turkey). Primer pair sequences that aim V3 and V4 region and create a single amplicon of approximately 460 bp were used to create amplicon libraries. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences as in Klindwoth et al. (Klindworth et al., 2013). DNA samples are amplified by PCR (Biorad CFX Connect) using forward primer (5'TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and reverse primer (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC-3'). Following amplification, PCR products are checked by agarose gel electrophoresis to verify size (approximately 550 bp) and later on were purified using Biospeedy PCR Product Purification Kit (Bioeksen, Turkey).

A second stage PCR was applied to purified PCR products to attach dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. The additional purification procedure was applied to clean up the final library before quantification. Final library was size verified (approximately 630 bp) again using Bioanalyzer DNA 1000 chip. In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Sequencing data were processed by Mothur version 1.36.1 for taxonomic classification and RDP database for reference.

3. RESULTS and DISCUSSION

3.1. Start-up and Enrichment Process

The up-flow reactor and SBR reactors were inoculated with enriched Anammox sludge which was obtained from ongoing lab-scale up-flow column reactor. Each reactor was continuously fed with synthetic wastewater and prior to the heavy metal exposure, the continuous up-flow system was operated approximately one year and SBR reactors were operated for approximately six months. Afterward, 94% nitrogen removal efficiency was achieved, exposure tests were started.

3.2. Short-Term Exposure to Heavy Metals

To investigate the individual short-term impacts of Cu, Cd, Ni, and Zn on Anammox biomass, batch tests were carried out using variable heavy metal concentrations. Former studies have clearly demonstrated that the inhibition level in the bioreactor is dependent on the heavy metal concentration. Likewise, the increment of the heavy metal concentration caused a decrease in the Anammox activity in terms of the nitrogen removal rate in our study. Batch short-term assays were analyzed for ammonium and nitrite nitrogen at regular intervals over the course of the 24-hour period. However, the rate was calculated based on the fast initial rate. Nitrogen removal rates decreased gradually in the presence of increasing concentrations of heavy metals. Firstly, the relationship between heavy metal concentration and inhibition level was interpreted regarding total applied heavy metal concentration under stable conditions for short-term exposure. However, considering the only total applied metal concentration to evaluate the inhibitory behavior and undervalue the metal speciation may result in incorrect approximation. Expressing inhibitory concentrations in terms of total heavy metal is of little use because not all of the fractions of heavy metals are bioavailable. Thus, the assessment of heavy metal toxicity should be evaluated based on bioavailable fractions that are influenced by biological/chemical speciation. It is considered that heavy metals that added to bioreactors are formed into four forms.

1. Soluble form (in mixed liquor); HM_s
2. Intracellular heavy metal (the portion that enters the biomass cell) ; HM_{int}
3. Surface-bound heavy metal (extracellular sorption); HM_{sb}

4. Precipitate (insoluble part); HM_{ppt}

Intracellular and surface-bound fractions together can be called as cell-associated heavy metal. The soluble moiety may react and form strong or labile complexes with certain compounds or may remain solely as a free ion. It is believed that heavy metal toxicity is directly related with the free metal ion activity in the solution (Hu et al., 2002), metals may be uptaken in this form via the bacterial transport system (Nies, 1999).

3.2.1. Short-Term Impact of Cd

To evaluate the inhibitory effect of cadmium on the Anammox system, batch tests were performed by using variable Cd concentrations in the range of 5-30 mg L^{-1}. In each batch test, along with heavy metal, control samples (without heavy metal addition) were also used. Control experiments showed that NH_4-N and NO_2-N were depleted within 9 hours. As seen in Figure 3.1, with increasing concentrations of Cd ammonia and nitrite removal ratio progressively decreased.

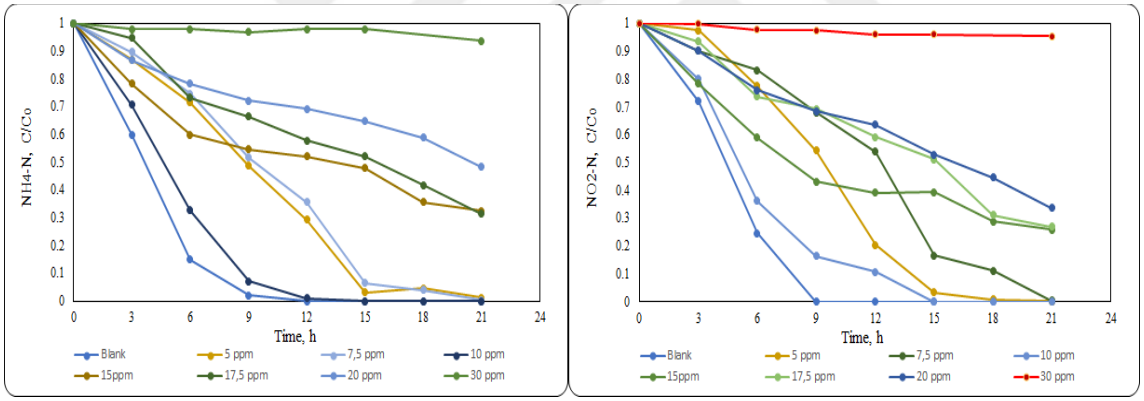


Figure 3. 1 The effect of Cd on removal efficiency of (a) ammonium-nitrogen and (b) nitrite-nitrogen

The increase in Cd concentration intensified the inhibitory effect, therefore, nitrogen removal rates trended down in response to the increase in Cd addition (Figure 3.2). For instance, NRR values for the control samples were calculated as 22.5 mg N g^{-1} VSS h^{-1} on average while at a dose of 10 mg L^{-1} Cd this value decreased to 13.6 mg N g^{-1} VSS h^{-1} and after 15 mg L^{-1} Cd addition, a sharp decrease of NRR was observed and finally approached to zero (Figure 3.2).

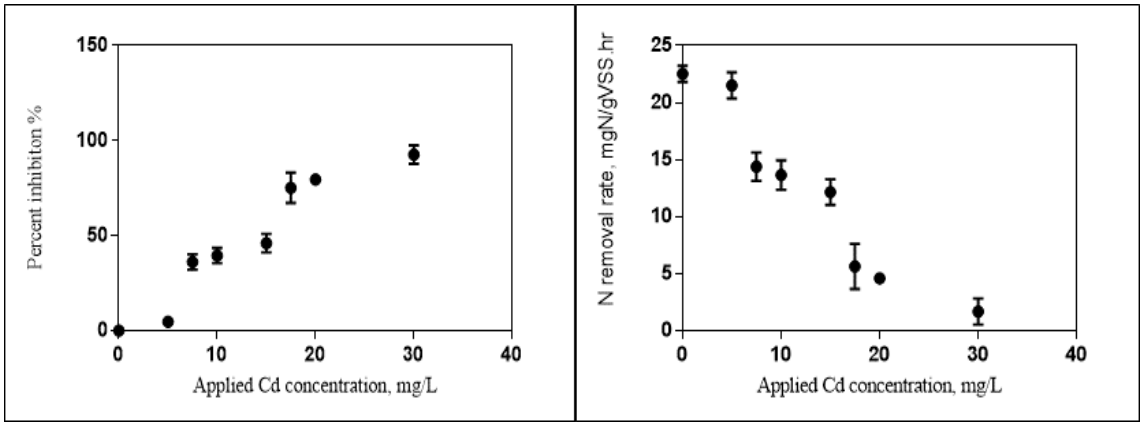


Figure 3. 2 Nitrogen removal rate response to increasing Cd concentration

Up to date, there are several studies that examine the inhibitory effect of heavy metal on the basis of total applied concentration. Despite the fact that several inhibition studies were done to understand the effect of heavy metal speciation over nitrification inhibition (Çeçen et al., 2010a, 2010b, Hu et al., 2003, 2002; Semerci and Çeçen, 2007), no research has been found that focus on the inhibitory effect of metal speciation for the Anammox process. It is important to predict or determine the chemical speciation of metals since bioavailability and mobility of metals related to chemical forms of metals (Sekhar et al., 2002). Metal partitioning experiments were applied to determine fractionation of Cd between solid and liquid phases. Partitioning of Cd between solid and liquid phases are shown in Table 3.1.

Precipitated part of the heavy metal was calculated based on the difference between heavy metal concentrations of filtered and unfiltered Anammox feed solutions in the lack of biomass. As a result, a precipitate fraction of Cd was found to be approximately 6 % of the total applied amount for the applied Cd concentration range. It is considered that the possible Cd precipitation was due to complexes of bicarbonate or carbonate species with Cd which have a relatively high complex stability (Çeçen et al., 2010b). As shown in Figure 3.3 (a), a linear relationship was observed between the total applied Cd concentration and soluble Cd amount (with R^2=0.97). Even though the soluble concentration of Cd increased as the applied Cd concentration increased, the percentage of soluble Cd in total applied concentration trended down and meanwhile, the percentage of intracellular portion increased.

Table 3. 1 Partitioning of Cd between liquid and solid phases

Applied Cd concentration*	Cd conc. in mixed liquor	Soluble Cd conc.	Precipitated Cd conc.	Intracellular Cd conc.	Surface-bound Cd conc.
5	4.23	4.08	0.13	0.10	0.00
7.5	6.53	5.67	0.39	0.19	0.29
10	9.89	5.68	0.59	1.33	2.28
15	13.50	7.02	0.86	3.26	2.35
17.5	18.10	7.65	1.15	7.92	1.37
20	25.34	9.53	1.97	8.81	5.03
30	34.86	10.77	1.74	14.47	7.88

* All concentrations in this table are given in mg L^{-1}

A linear partitioning between the intracellular and the total applied heavy metal concentration was inferred (Cd_{intr}=0.37 x Cd_{total} with R^2=0.89) (Figure 3.3 (c)).

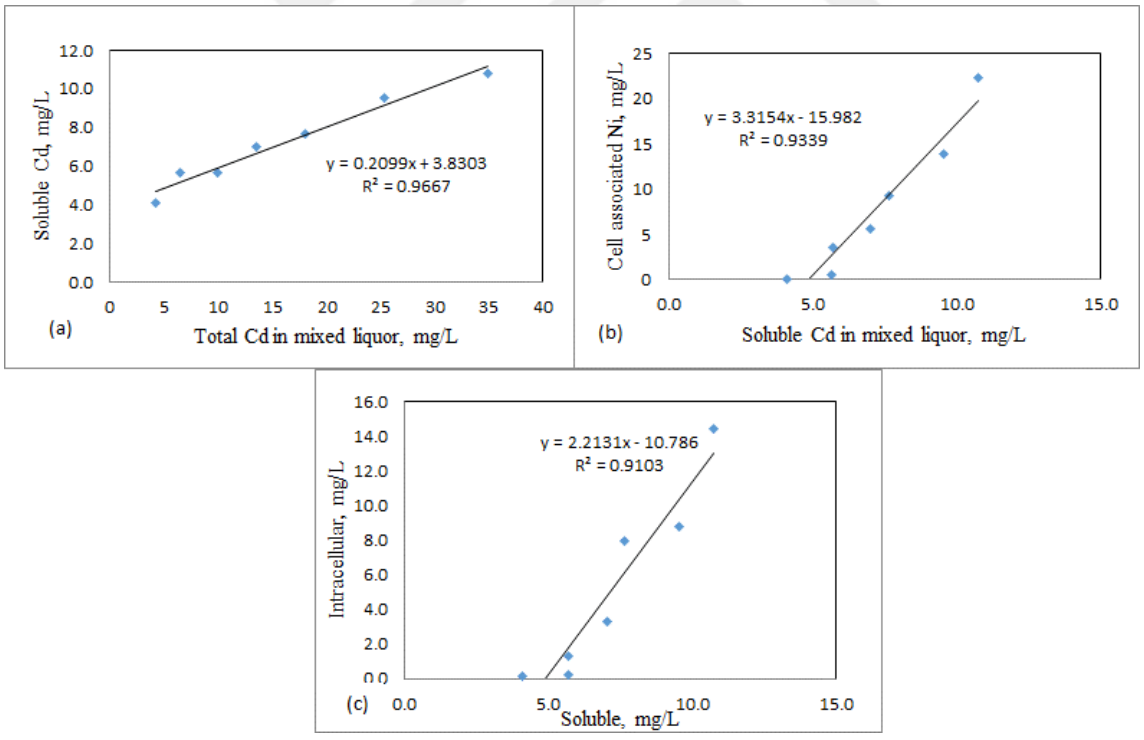


Figure 3. 3 Cd fractions relationships between the solid and bulk phases (a) Total applied Cd vs Soluble Cd, (b) Total Cd vs intracellular Cd, (c) Soluble Cd vs Cell-associated Cd concentration

The cell-associated fraction of Cd was taken as the sum of the intracellular and surface-bound fractions and measurements indicated that a significant percentage of mixed liquor Cd was composed of the cell-associated part.

Table 3.2 and Figure 3.4 show the 50 % inhibition evaluations based on applied, soluble, intracellular, surface-bound, cell-associated and free Cd ion concentrations. Initially, the inhibitory effect of Cd calculated with regard to the total applied concentration of Cd, however, total applied metal as the inhibitor alone is not a proper approach. This approach may lead to inaccurate estimation for inhibitory manners of Cd over Anammox process. Since the high stable complexes of Cd with EDTA or carbonate do not allow it to dissociate into a free ion and reduce the possibility of Cd entering other reactions, the bioavailability of these complexes decrease. Therefore, it is considered that the inhibition effect of these complexes does not occur to a significant degree. On the other hand, labile complexes have the tendency to dissociate into a free ion and hence, they can be regarded to be of significance to toxicity.

Although, there are some studies that worked on inhibition of heavy metal on sewage system in a comparative way via taking into account the metal complexes, the studies that investigate the inhibitory effect of heavy metal on Anammox process only total applied amount taking into consideration until now and only our study that is about the speciation of nickel and zinc , its inhibition effect on Anammox is available in the literature (Kalkan Aktan et al., 2018).

In order to calculate the IC_{50} values, both linear and non-linear regression models were applied and most of the time, a better fit was obtained using the modified non-competitive inhibition model.

In the case of the total applied amount of Cd considered as an inhibitor, IC_{50} was found as 12.14 mg L^{-1} and an R^2 value of 0.93 with a modified non-competitive inhibition model (Table 3.2). Among the inhibition models, the 4-point logistic model was provided more consistent curve for the evaluation of the inhibition due to soluble Cd and IC_{50} value 6.50 mg L^{-1} with an R^2 value of 0.95.

Table 3. 2 IC_{50} values for Cd-based on applied, soluble, intracellular, cell-associated, surface bound and free ion concentrations

	Applied concentration	Soluble	Intracellular	Cell-associated	Surface-bound	Free ion
IC_{50} (mg L^{-1})	12.14	6.50	1.25	2.94	1.07	4.07
Confidence Range	10.99 to 13.34	6.32 to 6.84	0.57 to 2.30	1.68 to 4.67	0.57 to 1.76	2.45 to 58.71
R^2	0.93	0.95	0.89	0.89	0.81	0.94
Inhibition model best fit	Modified non-competitive	4-point logistic	Modified non-competitive	Modified non-competitive	Modified non-competitive	4-point logistic

Intracellular accumulation of heavy metals is an important part of the toxicity. Nonessential metals have the similar structure they can enter the cell through the essential metal uptake system (Nies, 1999). Cadmium enters the cell via the manganese uptake system in a type of bacteria (Tynecka and Malm, 1995). Cd^{2+} competes with Mn^{2+} for transport in gram-positive bacteria such as *Lactobacillus plantarum, Bacillus subtilis* (Archibald and Duong, 1984). Cd has a higher affinity than Mn and they are a competitive inhibitor for each other. It is reported that *L.plantarum* has highly active Mn uptake system and also takes up Cd. Therefore, accumulation of metals may occur and destroy the structure of the proteins and may inhibit the function of various process within cells and results in toxic effects (Hu et al., 2003; Nies, 1999).

The IC_{50} value for the intracellular Cd inhibition was evaluated as 1.25 mg L^{-1} and non-modified was provided the best fit curve among the inhibition models with an R^2=0.89 (Table 3.2 and Figure 3.4).

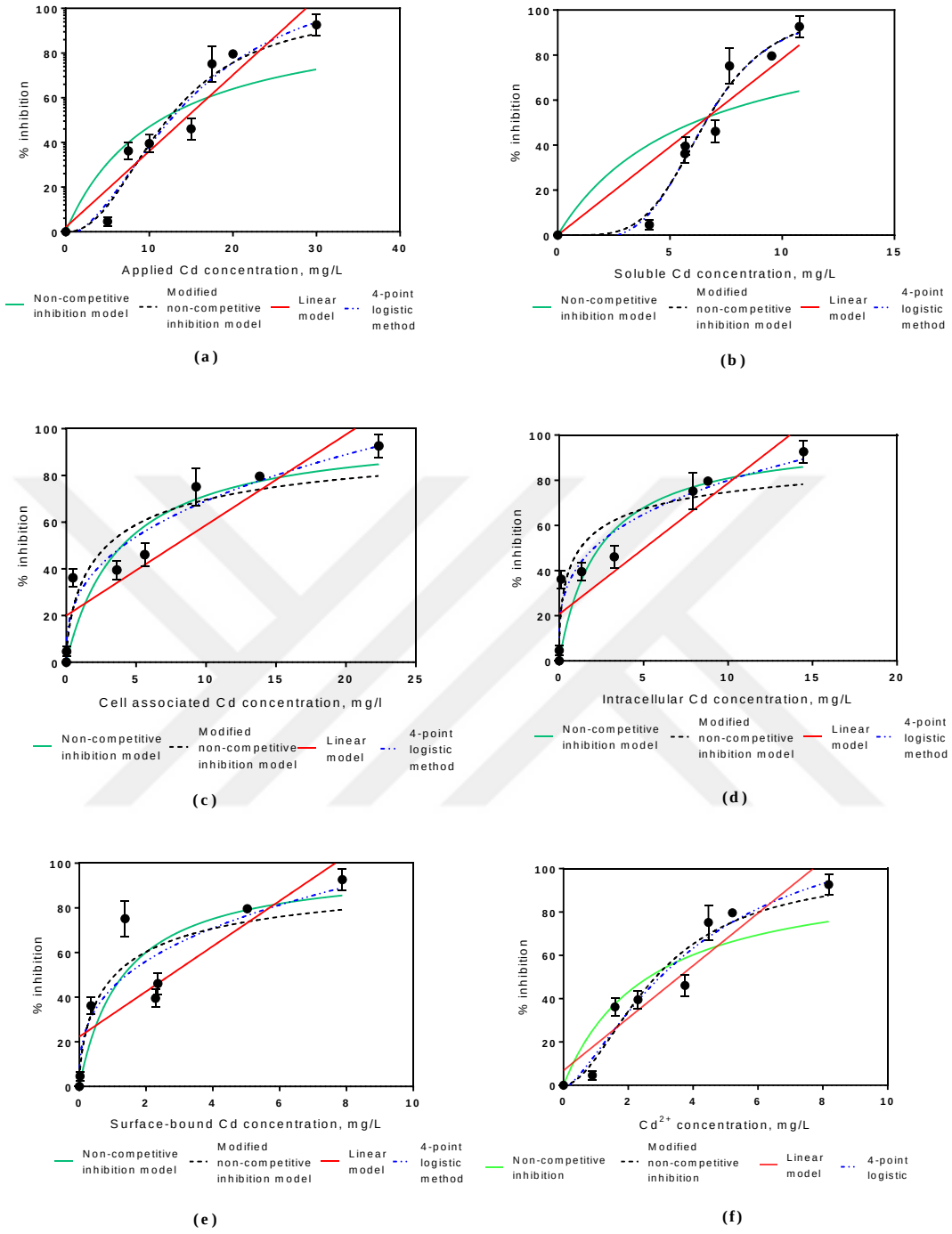


Figure 3. 4 Inhibition response of (a) Applied Cd concentration, (b) Soluble Cd concentration, (c) Cell-associated Cd concentration, (d) Intracellular Cd concentration, (e) Surface-bound Cd concentration, (f) Cd^{2+} concentration with the application of various inhibition models.

Pertinent literature concerning the inhibitory effect of heavy metal over microorganisms showed that surface-bound metals also play a crucial role during metal uptake since it is the first step in biosorption involves binding of metal ions to bacterial cell surfaces before the heavy metal enter the cell membrane and cytoplasm (Cox et al., 1999; Texier et al., 2000). Metals may also precipitate or bind on bacterial cell surfaces through interactions including proteins or cell-associated polysaccharides (Mullen et al., 1989). Heavy metals which are bound to the cell surface as a result of ion-exchange, complexation, and physical adsorption mechanisms are then internalized via uptake mechanism(s) of the cell (Abbas et al., 2014). For the cell-associated and a surface-bound fraction of Cd, IC_{50} values were calculated as 1.07 and 2.94, respectively. A better fit was obtained with the modified non-competitive inhibition model for both fractions.

In order to predict the chemical compounds of Cd in the solution and distribution speciation, the chemical equilibrium program Visual MINTEQ (version 3.1) was used. The resulting labile, stable complexes, cell-associated fraction, various anions and molecules were shown in Table 3.3. Furthermore, the inhibitory effect of heavy metals is directly related to the free ion concentration (Hu et al., 2002). The toxicity and bioavailability of cationic metals mostly governed by free ion activity model (FIAM) that explains the vital role of activity of the free metal ion to determine the uptake or toxicity between aquatic organisms and metals (Brown and Markich, 2000).

Free heavy metal concentrations, as well as the other inorganic metal complexes, are predicted using a chemical equilibrium software application (Visual MINTEQ version 3.1). Equilibrium concentrations of Cd species at soluble phase (all species were assumed as soluble) were calculated by the chemical equilibrium simulation program (Table 3.3). Heavy metal concentrations, temperature, pH and all other chemical species were used as input data. Distribution coefficient (K_d) was incorporated into the software to estimate sorption to biomass (i.e. cell associated heavy metal, HM_{int} + HM_{sb}). The theoretical speciation calculations were previously examined in the absence of biomass (initial speciation) as a control measurement.

In order to calculate K_d for each metal isotherm models (Freundlich, Linear and Langmuir models) are applied (Appendix C, Table C.1) by using data pairs on cell-associated heavy metal concentration (mg metal/kg MLVSS) and soluble metal

concentration. A linear model was found to best describe the sorption of heavy metal to biomass with a K_d =2885 L/kg VSS (R^2=0.94) (Figure 3.5).

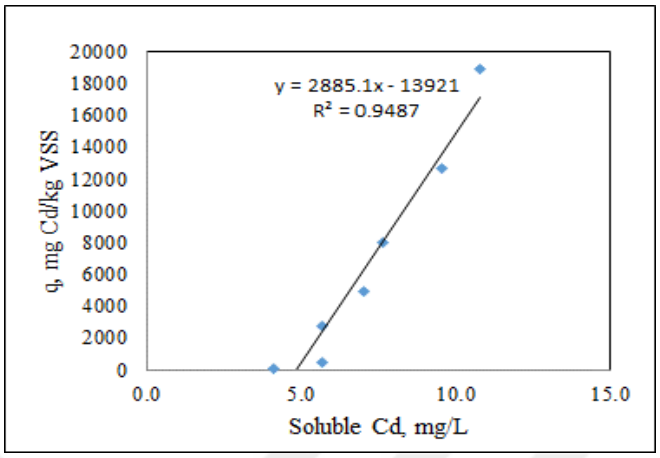


Figure 3. 5 Linear adsorption isotherm according to soluble Cd and cell-associated Cd concentration

On Table 3.3, initial speciation exemplifies the distribution of species before biomass addition, i.e. before any biochemical reaction commences in the medium. According to chemical speciation modeling, since ammonium and nitrite concentrations are high at the beginning of the reaction, Cd forms labile complexes with ammonia and nitrite, which constitutes around 4% and 8% of the total applied Cd concentration, respectively. However, these complexes are not formed in a significant amount after equilibrium concentrations are reached.

According to these calculations, the foremost part of the Cd for both initial and final speciation in the form of free Cd^{+2} ion and concentration is approximately 23% of the total applied amount in the final speciation. In order to evaluate IC_{50} values due to free Cd^{+2} ion concentration, these results were incorporated into the regression models and 4-point logistic model fits best with an R^2 value of 0.94. The IC_{50} value was calculated as 4.07 mg L^{-1}.

Cd formed stable complexes with phosphate, carbonate, chloride, sulfate, and EDTA. Among the complexes formed quite stable complexes with inorganic ligands carbonate, phosphate and organic ligand EDTA for instance; $CdHPO_4$ (with high stability constant, log β: 23.23), $CdHEDTA^-$ (log β: 21.43), $CdEDTA^{-2}$ (log β: 18.10), $CdCO_3$ (log β: 4.37) and $CdHCO_3^+$ (log β: 11.83). Around 25% of the total Cd concentration is formed as soluble Cd-phosphate complex ($CdHPO_4$) with a high stability constant 16.08 (Appendix

B, Table B.1)

The labile complexes of Cd for the final speciation like hydroxides, chloride, and nitrite is 4% of the total applied Cd metal concentration.

Table 3. 3 Theoretical speciations of Cd determined by chemical speciation program

Speciation of Cd	Total applied Cd concentration, mg/L						
	5 ppm	7.5 ppm	10 ppm	15 ppm	17.5 ppm	20 ppm	30 ppm
Initial Speciation							
Cd^{+2}	0.80	1.44	2.10	3.42	4.09	4.76	7.46
$CdOH^{+}$	0.01	0.01	0.02	0.03	0.04	0.04	0.07
$CdCl^{+}$	0.13	0.24	0.35	0.58	0.69	0.80	1.26
$CdCl_2$ (aq)	0.00	0.00	0.00	0.01	0.01	0.01	0.01
$CdSO_4$ (aq)	0.16	0.29	0.43	0.69	0.83	0.96	1.51
$Cd(SO_4)_2^{-2}$	0.01	0.01	0.02	0.03	0.03	0.04	0.06
$Cd(NH_3)_2^{+2}$	0.01	0.01	0.02	0.03	0.04	0.04	0.06
$CdNH_3^{+2}$	0.14	0.26	0.37	0.61	0.73	0.85	1.33
$Cd(NO_2)^2$ (aq)	0.05	0.09	0.13	0.21	0.25	0.29	0.45
$CdNO_2^{+}$	0.25	0.45	0.66	1.08	1.29	1.50	2.35
$CdNO_3^{+}$	0.00	0.01	0.01	0.02	0.02	0.02	0.03
$CdHPO_4$ (aq)	0.83	1.49	2.16	3.48	4.13	4.78	7.31
$CdHCO_3^{+}$	0.16	0.28	0.41	0.67	0.80	0.93	1.46
$CdCO_3$ (aq)	0.45	0.82	1.19	1.94	2.31	2.69	4.21
$Cd(CO_3)_2^{-2}$	0.03	0.06	0.08	0.14	0.16	0.19	0.30
$CdEDTA^{-2}$	1.98	2.02	2.05	2.08	2.09	2.10	2.12
Final Speciation							
Cd^{+2}	0.83	1.51	2.21	3.62	4.33	5.04	7.94
$CdOH^{+}$	0.01	0.01	0.02	0.03	0.04	0.05	0.08
CdCl+	0.15	0.27	0.40	0.65	0.77	0.90	1.42
$CdCl_2$ (aq)	0.00	0.00	0.00	0.01	0.01	0.01	0.02
$CdSO_4$ (aq)	0.19	0.35	0.52	0.85	1.01	1.18	1.85
$Cd(SO_4)_2^{-2}$	0.01	0.01	0.02	0.03	0.04	0.05	0.07
$CdNH_3^{+2}$	0.00	0.00	0.00	0.01	0.01	0.01	0.01
$CdNO_2^{+}$	0.00	0.00	0.00	0.00	0.00	0.00	0.01
$CdNO_3^{+}$	0.00	0.01	0.01	0.02	0.02	0.02	0.04
$CdHPO_4$(aq)	0.95	1.72	2.50	4.03	4.79	5.54	8.49
$CdHCO_3^{+}$	0.18	0.33	0.48	0.79	0.94	1.10	1.73
$CdCO_3$ (aq)	0.53	0.97	1.42	2.32	2.77	3.23	5.08
$Cd(CO_3)_2^{-2}$	0.04	0.07	0.10	0.17	0.20	0.23	0.36
$CdEDTA^{-2}$	1.98	2.03	2.06	2.09	2.10	2.11	2.13
Cell-associated	0.13	0.20	0.26	0.39	0.46	0.52	0.79

3.2.2. Short-Term Impact of Cu

Batch tests were done within a concentration range of 1–10 mg L^{-1} Cu to determine the negative effects of copper on Anammox system. Short-term exposure to increasing concentrations of copper resulted in a decrease in Anammox activity expressed in terms of the nitrogen removal rate (Figure 3.6)

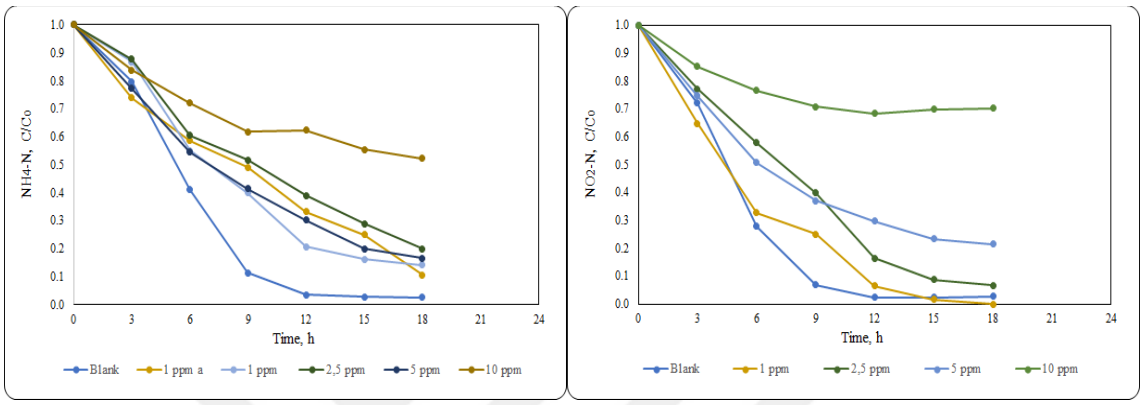


Figure 3. 6 The effect of Cu on removal efficiency of (a) ammonium-nitrogen and (b) nitrite-nitrogen

As seen in Figure 3.7 NRR values trended down with the increasing of the applied Cd concentration. The removal rate was calculated as 37.1 mg N g^{-1} VSS h^{-1} on average for the control samples. After 10 ppm Cu addition, NRR reduced to 11.6 mg N g^{-1} VSS h^{-1}.

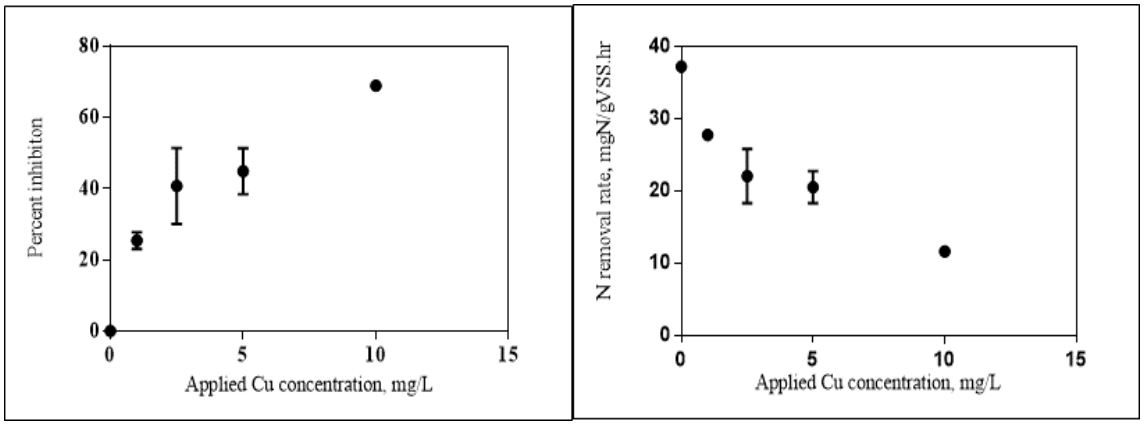


Figure 3.7 Nitrogen removal rate in response to increasing Cu concentration

The fractionation of the Cu was obtained via metal partitioning experiments. Table 3.4 shows the partitioning of Cu metal between solid and bulk phases. Among the applied heavy metals, Cu showed the highest precipitate fraction at higher concentrations. It is considered that the precipitate mainly consisted of the complexes of copper ion with SO_4^{2},

NO_3^-, HCO_3, OH^- and CO_3^{2-} when the solubility product is surpassed (Cuppett et al., 2006).

Table 3. 4 Partitioning of Cu between liquid and solid phases

Applied Concentration	Cu conc. in mixed liquor	Soluble Cu conc.	Precipitated Cu conc.	Intracellular Cu conc.	Surface-bound Cu conc.
1	1.33	1.28	0.05	0.25	0.00
2.5	2.68	1.94	0.74	0.33	0.17
5	6.99	3.51	3.48	1.04	0.50
10	11.55	5.44	6.11	1.37	1.20

* All concentrations in this table are given in mg L^{-1}

The results of metal partitioning showed that intracellular uptake of Cu metal was between 25% on average of the applied amount. The surface-bound fraction of Cu was found the lowest among the surface-bound fraction of applied heavy metals tested. This is presumably related to the internalization mechanism of copper. Even though Cu may interact with the active sites of enzyme and cellular nucleic acids, the main mechanism of Cu microbial toxicity is related with the cytoplasmic membrane destruction (Avery et al., 1996; Hu et al., 2003). Toxic concentration of Cu may lead damage of membrane integrity and cause loss of cell vitality. Our results showed that surface-bound Cu concentration was low meanwhile intracellular concentration was high, this may also support the idea of disruption of cell membrane at toxic concentration of copper.

According to partitioning experiment results, linear relationships between soluble Cu and total applied, cell-associated, and intracellular Cu concentrations was inferred with R^2=0.99, R^2=0.99 and R^2=0.92, respectively (Figure 3.8). Our results shows that there was a positive correlation between Anammox inhibition and intracellular Cu concentration (r=0.89) according to Pearson correlation analyses. Results indicate that the major part of the Cu tends to precipitate immediately (especially for concentrations higher than 5 mg L^{-1}) while rest was taken as surface bound or intracellular. As it mentioned before, the precipitated amount of Cu mostly related with the stable complexes of copper ion. The detailed information about this complexes will be given in the theoretical speciation calculations.

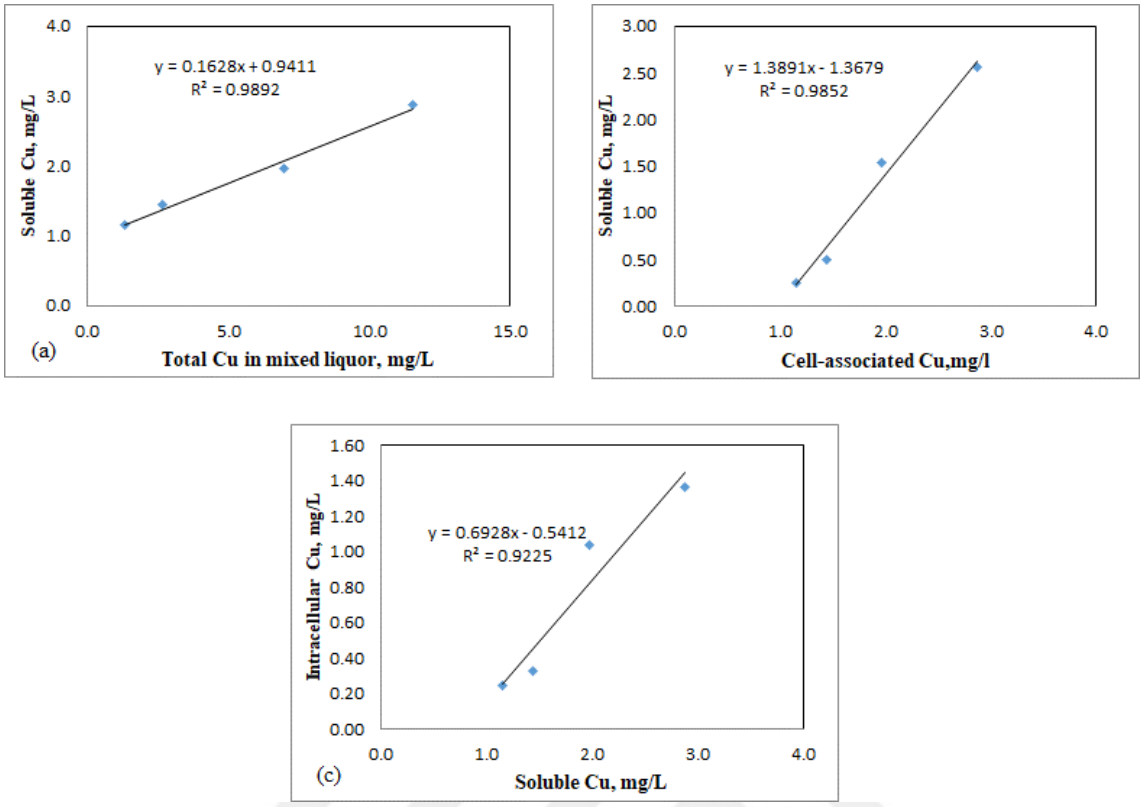


Figure 3. 8 Cu fractions between the solid and bulk phases (a) Total applied Cu vs Soluble Cu, (b) Soluble Cu vs intracellular Cu, (c) Soluble Cu vs Cell-associated Cu

To calculate K_d for Cu metal, several isotherm models (Freundlich, Linear and Langmuir models) are applied by using data pairs on cell-associated heavy metal concentration (mg metal/kg MLVSS) and soluble metal concentration. The sorption of Cu to biomass was best represented by a linear model with a K_d =1153 L/kg VSS (R^2=0.99) (Figure 3.9)

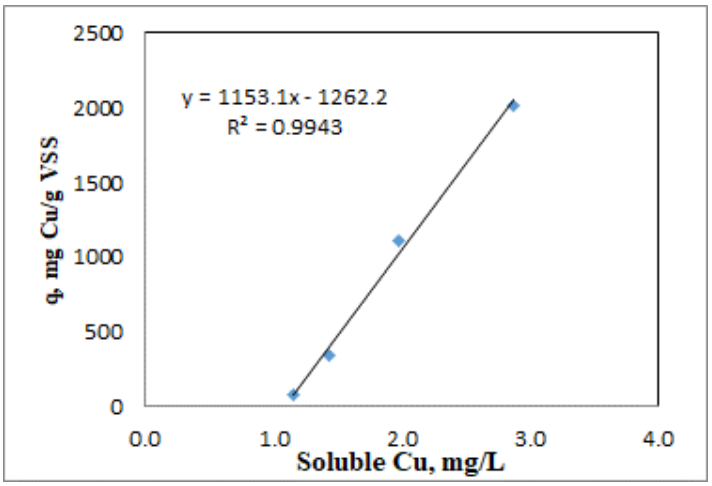


Figure 3. 9 Linear adsorption isotherm according to soluble Cu and cell-associated Cu concentration

The degree of nitrogen removal rate inhibition due to Cu was evaluated on the basis of the soluble, intracellular, surface-bound, cell-associated concentrations and free ion. (Table 3.5). Several inhibition models were tested for the determination of the IC50 values. Figure 3.10 demonstrates the inhibition model graphs for each fraction of Cu.

Table 3. 5 IC_{50}values for Cu-based on applied, soluble, intracellular, cell-associated, surface bound and free ion concentrations

	Applied concentration	Soluble	Intracellular	Cell-associated	Surface-bound	Free ion
IC50 mg L-1	4.57	2.43	1.16	1.38	0.152	0.009
Confidence Range	3.61-5.95	2.10-2.86	0.851-1.68	0.951-2.15	0.057-0.525	0.004-0.025
R^2	0.93	0.93	0.92	0.91	0.87	0.88
Inhibition model best fit	Modified non-competitive	Modified non-competitive	Modified non-competitive	Modified non-competitive	Modified non-competitive	Modified non-competitive

As shown in Table 3.5, the modified non-competitive inhibition model was found to best describe the inhibition response of all Cu fractions. The IC50 value was calculated as 4.57 mg L^{-1} (R^2= 0.97 according to the 95% confidence interval) based on total applied Cu concentration. The IC50 values were calculated as 2.43 mg L^{-1} (R^2= 0.93) and 1.16mg L^{-1} (R^2= 0.92) for the case of soluble and intracellular Cu considered as an inhibitor, respectively.

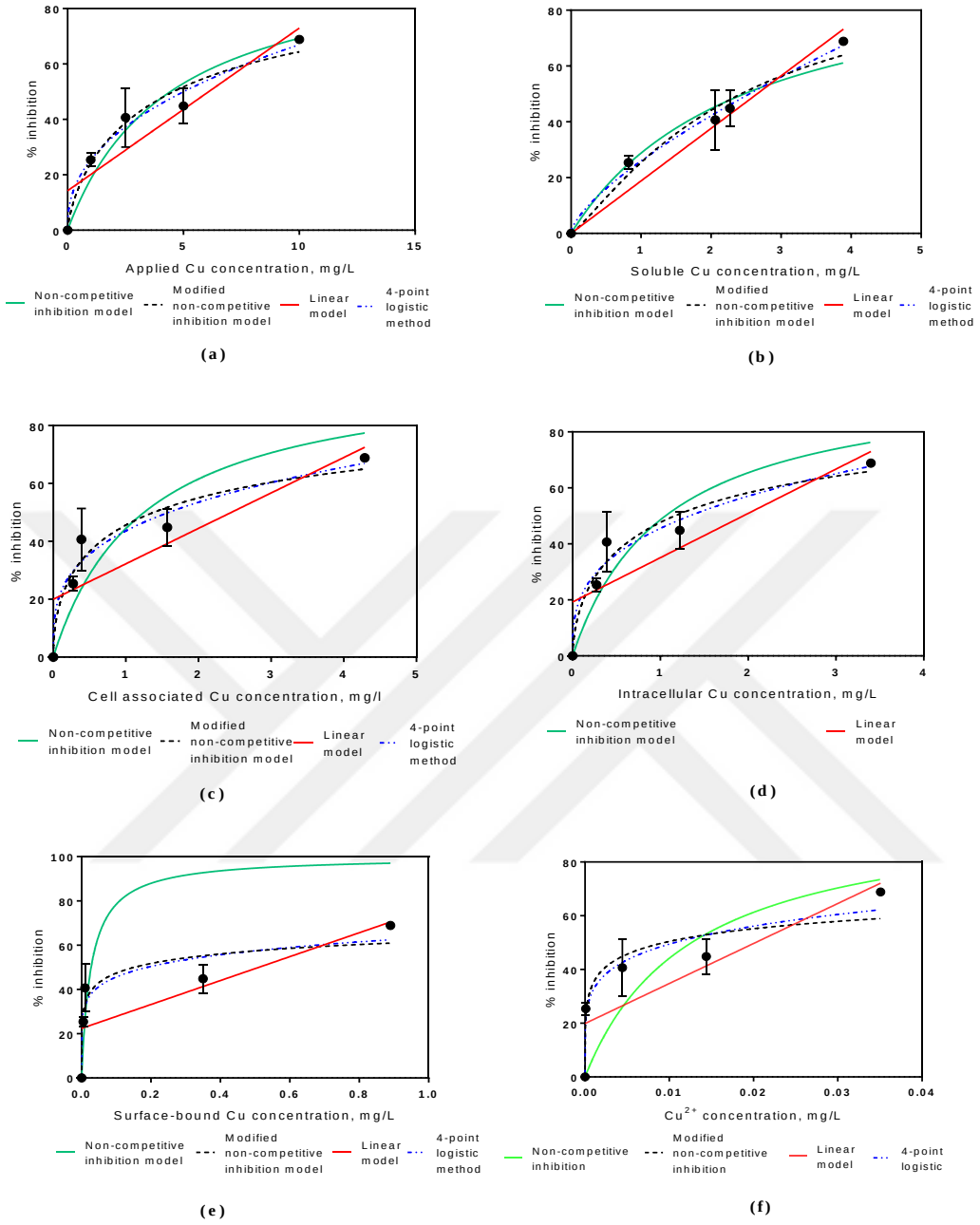


Figure 3. 10 Inhibition response of (a) Applied Cu concentration, (b) Soluble Cu concentration, (c) Cell-associated Cu concentration, (d) Intracellular Cu concentration, (e) Surface-bound Cu concentration, (f) Cu^{2+} concentration with the application of various inhibition models.

To evaluate the chemical speciation of copper ions in the solution pH, temperature conditions, all inorganic, and organic compounds were integrated to the software application. The resulting labile, stable complexes, cell-associated fraction, various anions and molecules were shown in Table 3.6. The chemical speciation was performed

based on the assumption that all fractions are in soluble form and precipitation did not form. The initial speciation calculations were performed again in the absence of biomass and calculated K_d value (according to metal partitioning experiments) was incorporated to the software to estimate sorption to biomass for the final speciation considering biomass. According to speciation predictions, a very small part of the total metal concentration consists of the free ion. The correlation between free ion and toxicity of heavy metals has been revealed in many studies (Braam and Klapwijk, 1981; Çeçen et al., 2010b; Hu et al., 2004), however; free ion activity model may not be proper for our study to relate the effect of the Cu exposure with inhibition. Similarly, Çeçen et al. (2010a) also reported that low concentration of Cu based on free and labile form may result in inhibition in nitrification bacteria, free Cu ion concentration was not found to be effective. The labile complexes of Cu with ammonia formation was insignificant in the case of both initial and final speciation. According to chemical speciation calculations, the notable part of the Cu for both initial and final speciation in the form of $CuCO_3$ that is highly stable complex and approximately 54% of the total applied amount for the final speciation. In addition, Cu formed stable complexes with hydroxides, sulfate, phosphate, carbonate, and EDTA. CuH_2EDTA ($\log\beta = 26.2$), $CuHEDTA^-$($\log\beta = 24.0$), $CuEDTA^{2-}$ ($\log\beta = 20.5$), $CuHPO_4$ ($\log\beta = 16.5$), $CuHCO_3^+$ ($\log\beta = 12.13$), $Cu(CO_3)_2^{2-}$ ($\log\beta = 10.1$), and $CuCO_3$ ($\log\beta = 6.77$) were the most stable complexes.

Furthermore, the fraction of the labile complexes of Cu was too low. It is usually thought that labile and free forms of heavy metals are responsible for the toxic effect. However, a free and labile form of Cu was much lower than the other applied heavy metals. If the free ion concentration was considered as the inhibitor IC50 value would be 0.009 mg L^{-1} (0.0001mmol L^{-1}) that is the lowest value among the other metals.

The cell-associated fraction was calculated via using adsorption constant that was found by linear adsorption isotherm with using the experimental data. One way analysis of variance indicated that differences of cell-associated fractions between experimental data and software data are statistically non-significant at the 0.05 significance level ($p=0.49$). Since the concentration of free ion and labile complexes are too low, a positive correlation can be found with cell-associated part and percent inhibition of Anammox.

In numerous studies, it was revealed that the role of extracellular polymeric substances (EPS) in heavy metal biosorption is critical (Comte et al., 2008; Liu et al., 2001). Zhang and his co-workers (2015) found a positive relationship between cell-associated Cu and EPS-bound Cu. In another study, Comte et al. (2008) indicated that the heavy metal sorption capacity of EPS of Cu is higher than Cd at pH 7 and 8 in activated sludge systems. The high affinity between EPS and soluble Cu may cause the formation of strong complexes with EPS. Therefore, the toxic effect of Cu over Anammox system might be related with the cell-associated portion.

The toxic effect of Cu on the Anammox process has been shown in relevant literature up to date. Lotti et al (2012) reported IC50 as 1.9 mg L^{-1}, on the other hand Zhang et al reported that the short-term inhibition IC50 level as 32.5 mg L^{-1} and Van del Rio and his co-workers (2017) found for the same level of 50% inhibition concentration was 19.3 mg L^{-1}. However, the effect of the inhibition was evaluated only taking into account the total applied heavy metal concentrations and speciation was not considered in these studies. Only, Li et al. (2015) studied the inhibitory effect of heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+}, MoO_4^{-2}, and Cd^{2+}) based on both total concentration and soluble concentration. They found the IC 50 value as 5.8 mg L^{-1} and 4.2 mg L^{-1} for the total concentration and soluble concentration, respectively. Up to now reports related to heavy metal inhibition on Anammox process used either applied or soluble concentrations. However, the speciation of heavy metals should be identified briefly to evaluate the inhibitory effects, since the bioavailability and mobility of metals related to chemical forms of metals. Inhibitory concentrations need to be determined based on bioavailable fractions. Since the total applied concentration of heavy metal give only a rough idea about threshold levels that microorganisms are exposed, the inconsistency between the results in the literature may be explained by occurrence of possible precipitations and strong complexes of copper. Furthermore, the variability of the results may also be explained by the presence of different Anammox species.

Table 3. 6 Theoretical speciations of Cu determined by chemical speciation program

Speciation of Cu after equilibrium, mg L^{-1}	Total applied Cu concentration, mg L^{-1}			
	1	2,5	5	10
Initial Speciation				
Cu^{+2}	0.005	0.013	0.025	0.045
$CuOH^{+}$	0.014	0.036	0.073	0.130
$Cu(OH)_2$ (aq)	0.004	0.011	0.022	0.039
$Cu2(OH)_2^{+2}$	0.000	0.001	0.004	0.012
$CuSO_4$ (aq)	0.001	0.002	0.005	0.009
$Cu(NH3)_4^{+2}$	0.000	0.001	0.002	0.003
$Cu(NH3)_3^{+2}$	0.007	0.018	0.036	0.064
$Cu(NH3)_2^{+2}$	0.025	0.062	0.125	0.223
$CuNH_3^{+2}$	0.024	0.060	0.120	0.214
$CuNO_2^{+}$	0.002	0.005	0.010	0.018
$Cu(NO_2)_2$ (aq)	0.000	0.000	0.001	0.001
$CuHPO_4$ (aq)	0.014	0.034	0.069	0.124
$CuCO_3$ (aq)	0.704	1.760	3.520	6.239
$CuHCO_3^{+}$	0.000	0.000	0.000	0.000
$Cu(CO_3)_2^{-2}$	0.185	0.460	0.914	1.610
$CuEDTA^{-2}$	0.011	0.028	0.054	1.221
Final Speciation				
Cu^{+2}	0.0001	0.004	0.014	0.034
$CuOH^{+}$	0.000	0.013	0.042	0.103
$Cu(OH)_2$ (aq)	0.000	0.004	0.013	0.032
$Cu2(OH)_2^{+2}$	0.000	0.000	0.001	0.007
$CuSO_4$ (aq)	0.000	0.001	0.003	0.008
$CuNH_3^{+2}$	0.000	0.000	0.000	0.000
$CuHPO_4$ (aq)	0.000	0.013	0.043	0.104
$CuCO_3$ (aq)	0.017	0.691	2.246	5.399
$CuHCO_3^{+}$	0.000	0.002	0.006	0.015
$Cu(CO_3)_2^{-2}$	0.004	0.183	0.591	1.406
$CuEDTA^{-2}$	0.812	1.173	1.207	1.219
Cell-associated	0.165	0.413	0.827	1.652

3.2.3. Short-Term Impact of Ni

In order to evaluate the IC_{50} levels and NRR value for short-term responses to Ni, batch tests were performed by using increasing level of Ni in the range of 5-10 mg L^{-1}

concentrations. The effect of metal concentration on ammonia and nitrite nitrogen removal were shown in Figure 3.11.

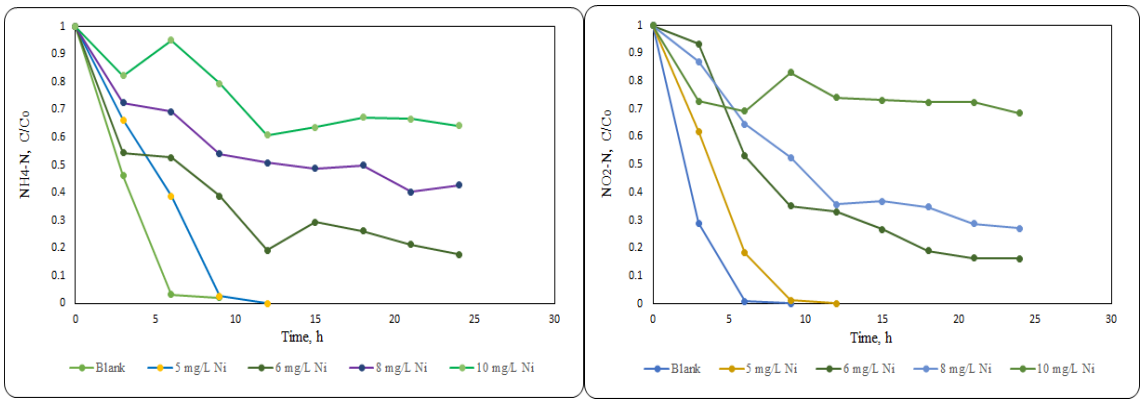


Figure 3. 11 The effect of Ni on the removal efficiency of (a) ammonium-nitrogen and (b) nitrite-nitrogen

NRR values were determined by using ammonia and nitrite nitrogen removal values. The nitrogen removal rates gradually decreased as the Ni concentration increased during experiments (Figure 3.12).

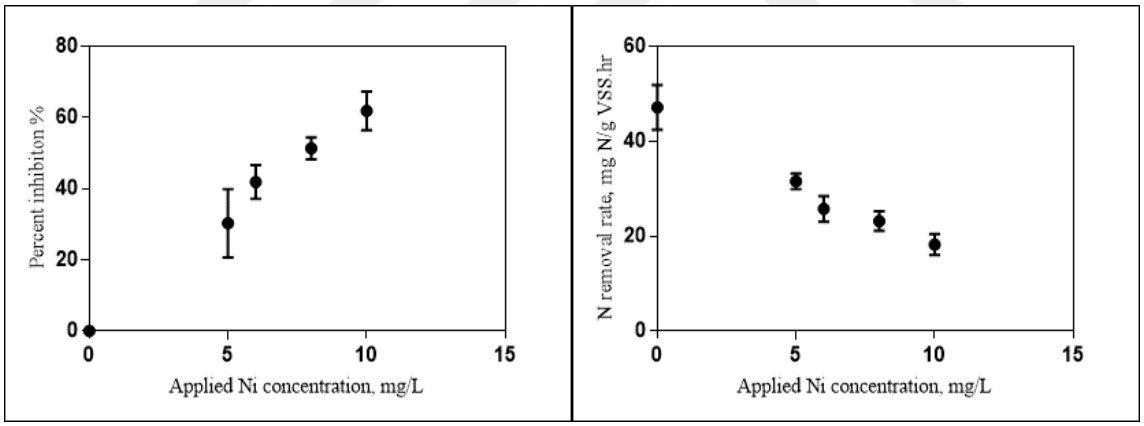


Figure 3. 12 Nitrogen removal rate in the presence of variable concentrations of Ni

In order to determine the Ni fractions that partition between the solid and bulk phases metal partitioning experiments was performed. Table 3.7 shows the portioning of Ni between liquid and solid phases. Results have shown that around 10-15 % of metal precipitation was formed within the system. The insoluble species can be possibly due to the precipitation of stable complexes like $NiCO_3$ (aq) and $NiHCO_3^+$ (Çeçen et al., 2010a).

Table 3. 7 Partitioning of Ni between liquid and solid phases

Applied Ni concentration	Ni conc. in mixed liquor *	Soluble Ni conc.	Precipitated Ni conc.	Intracellular Ni conc.	Surface-bound Ni conc.
5	4.96	4.00	0.49	0.16	0.31
6	5.93	4.52	0.81	0.19	0.42
8	7.66	5.84	0.94	0.22	0.66
10	11.45	7.90	1.77	0.42	1.37

* All concentrations in this table are given in mg L^{-1}

As a result of these experiments, a linear relationship was observed between the soluble portion and the total applied heavy metal concentrations (Ni_s=0.73 x Ni_{total} with R^2=0.95) (Figure 3.13a). The relationship between soluble Ni and intracellular Ni was also investigated and approximately 4.5% of the soluble Ni was incorporated as intracellular Ni (Figure 3.13b).Sum of the intracellular and surface-bound fractions was taken as a cell-associated fraction of Ni. Results indicated that 18% of soluble Ni tends to adsorb onto instead of adsorbing into biomass (Figure 3.13 c).

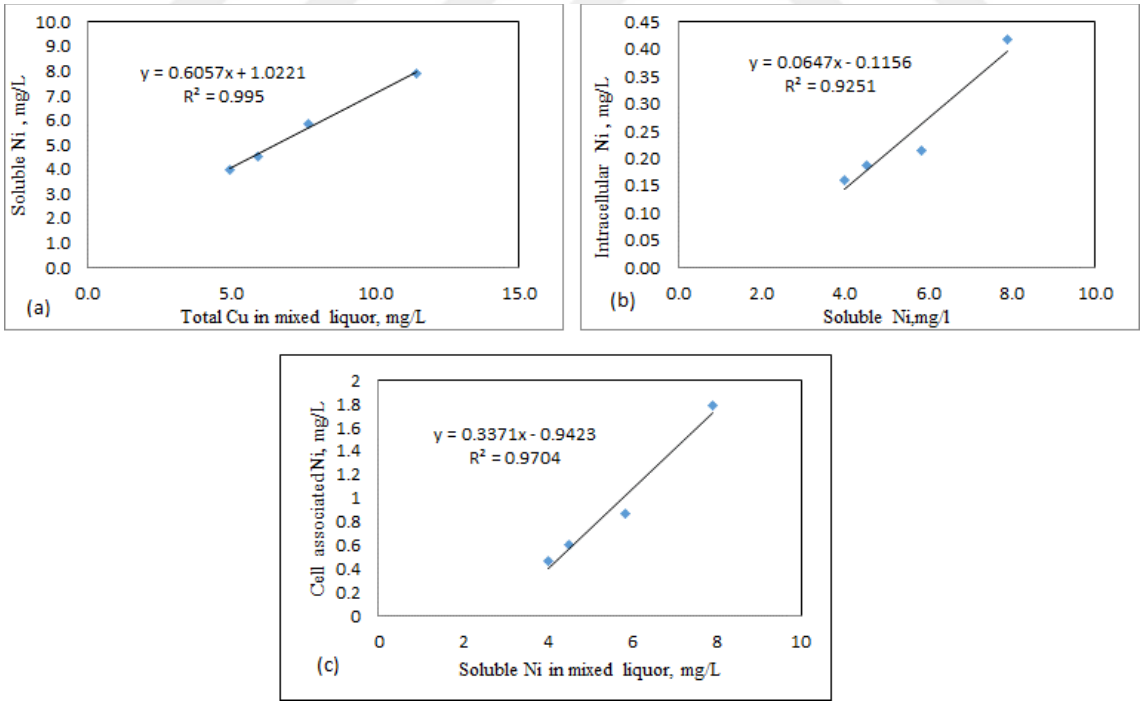


Figure 3. 13 Ni fractions relationships between the solid and bulk phases (a) Total applied Ni vs Soluble Ni, (b) Soluble Ni vs intracellular Ni, (c) Soluble Ni vs Cell-associated Ni

In order to calculate K_d for each metal isotherm models (Freundlich, Linear and Langmuir models) are applied (Appendix C, Table C.1) by using data pairs on cell-associated heavy metal concentration (mg metal/kg MLVSS) and soluble metal concentration. A linear model was found to best describe the sorption of heavy metal to biomass with a K_d=747.8 L/kg VSS (R^2=0.96) (Figure 3.14)

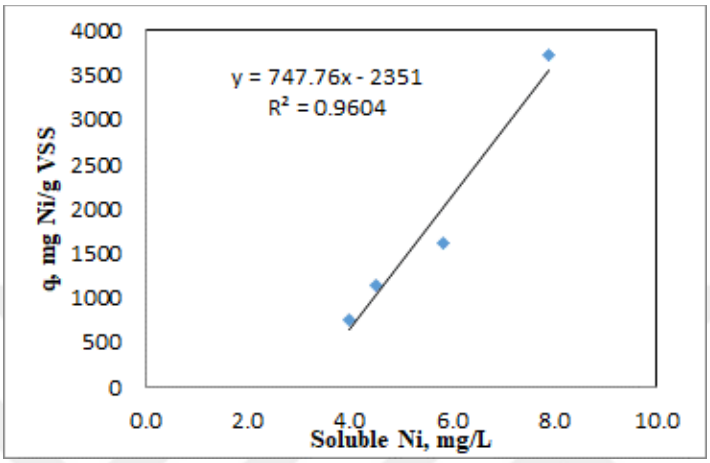


Figure 3. 14 Linear adsorption isotherm according to soluble Ni and cell-associated Ni concentration

To determine the inhibition degree of Ni on Anammox biomass soluble, intracellular, surface-bounded and cell-associated fractions of Ni was evaluated on the basis of the nitrogen removal rate. Figure 3.15 shows inhibition response of different fractions of Ni concentrations. The IC_{50} values were calculated based on applied, soluble, intracellular, cell-associated, surface bound and free ion concentrations. Table 3.8 summarizes the IC50 values for variable Ni concentrations.

It is important to consider the soluble part of the heavy metal to examine the inhibition degree since the precipitate fraction of metal is not included. Both linear and non-linear regression was applied to calculate inhibition percentages due to soluble Ni concentrations. For all models, a strong correlation was found between the soluble Ni concentration and inhibition percentage. Among the inhibition models, linear inhibition model was provided the best fit for the calculation of inhibition due to soluble Ni with an IC_{50} value of 5.99 mg L^{-1} and an R^2 value of 0.97.

Table 3. 8 IC_{50} values for Ni-based on applied, soluble, intracellular, cell-associated, surface bound and free ion concentrations

	Applied conc.	Soluble	Intracellular	Cell-associated	Surface-bound	Free ion
IC_{50} (mg L^{-1})	7.78	5.99	0.25	0.93	0.68	1.77
Confidence Range	5.77-7.09	5.27-10.65	0.21-0.32	0.75-1.20	0.53-0.92	1.47-1.98
R^2	0.97	0.97	0.9	0.92	0.92	0.93
Best fit inhibition model	Linear	Linear	Modified non-competitive	Modified non-competitive	Modified non-competitive	Modified non-competitive

For the case of intracellular Ni was the inhibitor non-competitive inhibition model fit more consistent curve than the other regression models with an IC_{50} value of 0.25 mgL^{-1} with an R^2 value of 0.90 (Table 3.8).

As seen in Table 3.8 and Figure 3.15, modified non-competitive inhibition model was provided the best fit for the calculation of IC value for both the fractions of surface-bound Ni and cell-associated Ni and IC_{50} values were calculated as 0.68 mg L^{-1} and 0.93 mgL^{-1} (with R^2=0.92), respectively.

Results indicated that the cell-associated fractions of total applied Ni and the surface-bound fraction was in conformity with the inhibition percentage. As it was shown in the Ni speciation calculations for free heavy metal concentrations and other inorganic metal complexes were predicted by using Visual MINTEQ (version 3.1) as explained in section 2.6. The results demonstrated that both labile and stable complexes with numerous anions and molecules such as, EDTA, phosphate, ammonia, sulfate, carbonate, and bicarbonate were formed (Table 3. 9) The theoretical speciation calculations were previously checked in the absence of biomass (initial speciation) as a control measurement.

For the initial speciation Ni forms, complexes with ammonia and nitrite around 6% and

2% of the total applied Ni concentration, respectively. However, these complexes are not formed after biomass addition and equilibrium concentrations are reached.

The labile complexes of Ni for the final speciation exist in the system that formed with hydroxide, ammonia, and chloride is the 5 % of the total heavy metal concentration. Due to low pH values, hydroxylated complexes are a negligibly small fraction in speciation. Similarly, since most of the ammonium nitrogen was eliminated by Anammox reaction during the reaction period the complexes of ammonia have a very small fraction.

Free Ni ion concentrations were calculated by using Visual MINTEQ and approximately 22% of the total applied concentration was constituted as free Ni ion concentration. IC_{50} values also calculated for the situation of free Ni as an inhibitor and model outputs showed very close agreement with free Ni ion concentrations. IC_{50} concentration was determined as 1.77 mg L^{-1} using linear inhibition model with an R^2 value of 0.95. Equilibrium concentrations of Ni species at soluble phase (all species were assumed as soluble) were calculated by the chemical equilibrium simulation program (Table 3.9). However, as previously discussed, some part of the stable complexes such as $NiCO_3$ (aq) and $NiHCO_3^+$ seem to precipitate during the test. The cell-associated fraction of Ni was calculated by implicated in adsorption constant that was found by linear adsorption isotherms with using the experimental data. Two-way analysis of variance indicated that differences between findings are statistically non-significant at the 0.05 significance level (p=0.55).

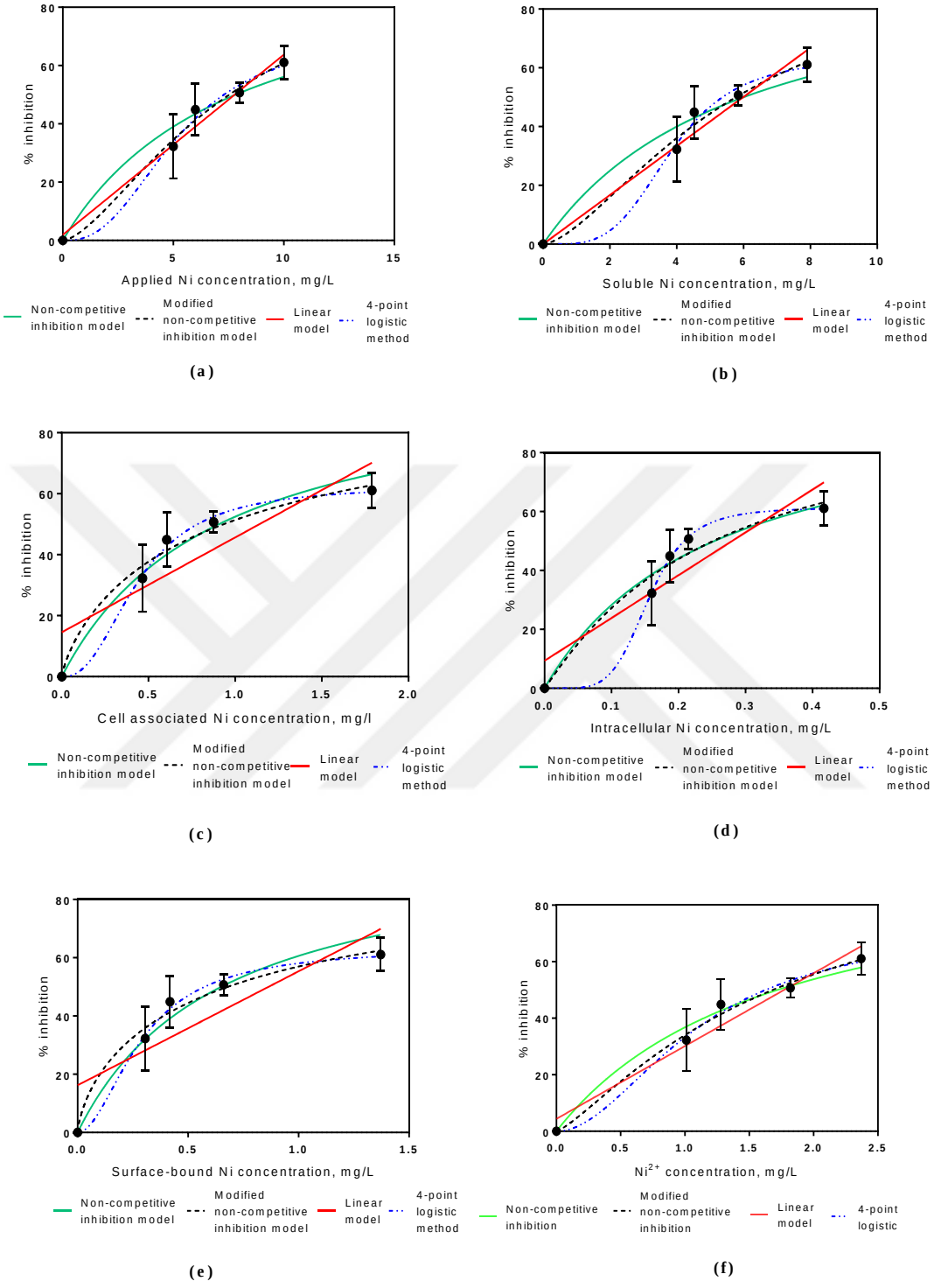


Figure 3. 15 . Inhibition response of the (a) the applied Ni concentration, (b) soluble Ni concentration, (c) cell-associated Ni concentration, (d) intracellular Ni concentration, (e) surface-bound Ni concentration, and (f) Ni^{2+} concentration for various inhibition models

Table 3. 9 Theoretical speciations of Ni determined by chemical speciation program

Speciation of Ni	Total applied Ni concentration, mg L^{-1}			
	5	6	8	10
Initial Speciation				
Ni^{2+}	1.14	1.43	2.03	2.62
$NiOH^{+}$	0.02	0.02	0.03	0.04
$Ni(OH)_2$ (aq)	0.00	0.00	0.00	0.00
$NiCl^{+}$	0.00	0.00	0.00	0.00
$NiSO_4$ (aq)	0.19	0.24	0.34	0.44
$NiNH_3^{+2}$	0.29	0.36	0.51	0.66
$Ni(NH_3)_2^{+2}$	0.02	0.02	0.04	0.05
$NiNO_2^{+}$	0.11	0.14	0.19	0.25
$NiNO_3^{+}$	0.01	0.01	0.01	0.01
$NiHPO_4$ (aq)	0.21	0.27	0.38	0.48
$NiCO_3$ (aq)	1.02	1.28	1.81	2.33
$NiHCO_3^{+}$	0.86	1.09	1.53	1.98
$NiEDTA^{2-}$	1.13	1.14	1.14	1.14
Final Speciation				
Ni^{2+}	1.01	1.28	1.82	2.37
$NiOH^{+}$	0.01	0.02	0.03	0.03
$Ni(OH)_2$ (aq)	0.00	0.00	0.00	0.00
$NiCl^{+}$	0.00	0.00	0.00	0.00
$NiSO_4$ (aq)	0.19	0.25	0.35	0.46
$NiNH_3^{+2}$	0.00	0.00	0.00	0.00
$NiNO_3^{+}$	0.00	0.01	0.01	0.01
$NiHPO_4$ (aq)	0.21	0.26	0.37	0.48
$NiCO_3$ (aq)	1.02	1.29	1.84	2.39
$NiHCO_3^{+}$	0.85	1.08	1.54	1.99
$NiEDTA^{2-}$	1.13	1.13	1.14	1.14
Cell associated	0.56	0.67	0.90	1.12

3.2.4. Short-Term Impact of Zn

Four different Zn concentration were tested (12, 16, 24 and 32 mg L^{-1}) to determine the level of inhibition and the effect of metal concentration on ammonia and nitrite nitrogen removal were shown in Figure3.13 and NRR values that are calculated according to this removal rates were shown in Figure 3. 16.

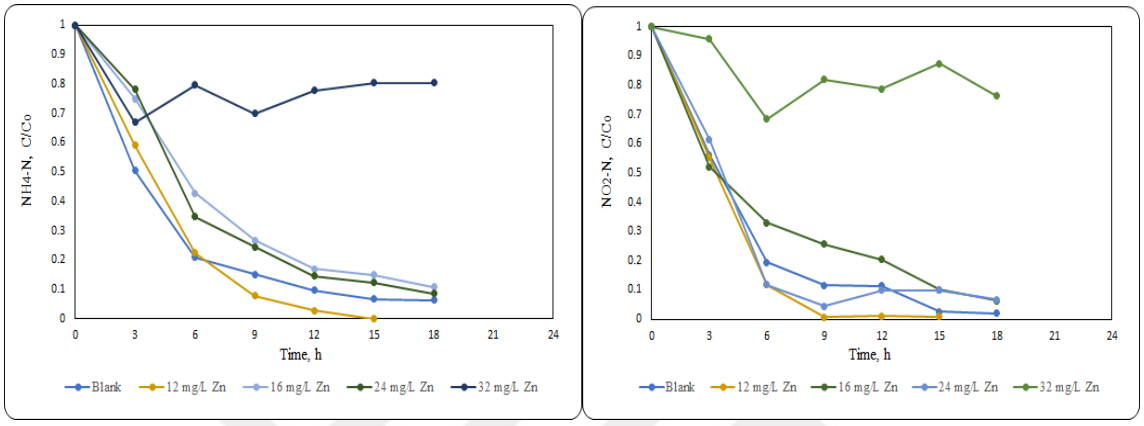


Figure 3. 16 The effect of Zn on the removal efficiency of (a) ammonium-nitrogen and (b) nitrite-nitrogen

Results show that NRR values gradually decreased as the Zn concentration increased as in the others (Figure 3.17)

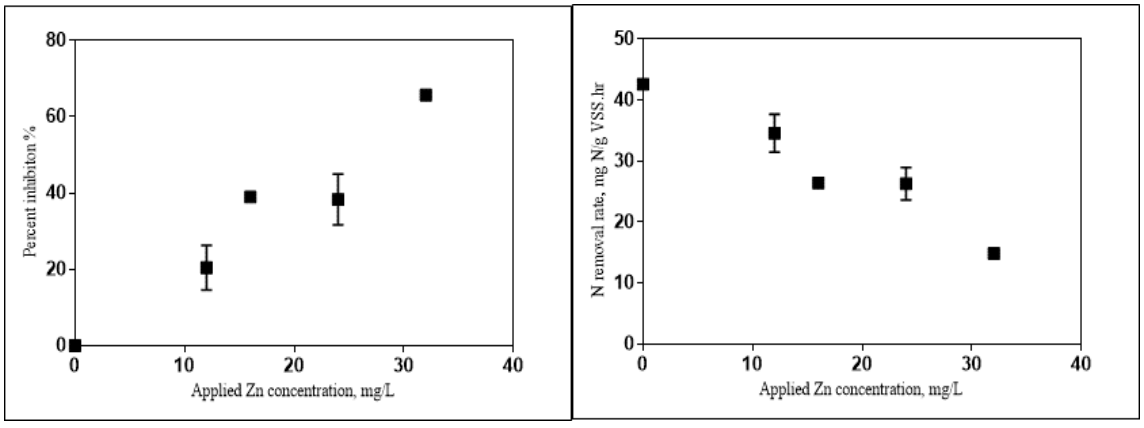


Figure 3. 17 Nitrogen removal rate in the presence of variable concentrations of Zn

Zn fractions were identified by metal partitioning experiments. Table 3.9 shows the partitioning of Zn metal between solid and bulk phases. According to metal partitioning experiments, a maximum precipitate fraction of Zn was determined as 9% it is a much lower value when we compared to the precipitate part of the Ni (Table3.10).

Table 3. 10 Partitioning of Zn between liquid and solid phases

Applied Zn Concentration*	Zn conc. in mixed liquor	Soluble Zn conc.	Precipitated Zn conc.	Intracellular Zn conc.	Surface-bound Zn conc.
12	11.16	4.15	6.05	1.00	0.00
16	16.29	5.02	7.80	1.07	2.39
24	22.13	6.94	10.39	1.26	3.54
32	33.54	8.36	10.59	1.57	13.02

* All concentrations in this table are given in mg L^{-1}

The precipitate part of the Zn can be possible due to the stable complexes with carbonate and bicarbonate. These experiments showed that intracellular uptake of Zn metal is around 32-54 % of the total applied amount and cell-associated part is between 54-70% of total applied heavy metal. The results indicated that the soluble portion of the Zn is around %30 that is the lowest fraction when we compared the soluble fraction of applied heavy metals. Similar the earlier reports about biosorption of heavy metals by activated sludge (Bakkaloglu et al., 1998; Hammaini et al., 2007), greater sorption capacity for Zn metal with respect to Ni was observed according to metal partitioning experiments (Table 3.7 and Table 3.10).

In order to evaluate the effects of Zn on Anammox process inhibition percentages calculations were correlated to soluble, surface-bound, intracellular, cell-associated and free ion concentration of Zn and IC_{50} values were determined for each one. To determine the IC 50 values for each fractionation of Zn several linear and non-linear inhibition models were applied. Table 3. 11 shows the IC_{50} values for variable Zn fractionations. Figure 3.18 illustrates the correlation between the inhibition responses with different fractionations of Zn for variable regression models.

Table 3. 11 IC_{50} values for Zn-based on applied, soluble, intracellular, cell-associated, surface bound and free ion concentrations

	Applied concentration	Soluble	Intracellular	Cell-associated	Surface-bound	Free ion
IC_{50} (mgL^{-1})	23.59	6.76	11.93	15.1	4.82	2.71
Confidence Range	21.94-24.60	6.37-7.23	6.07-infinity	14.12-16.20	2.43-10.03	2.49-2.98
R^2	0.97	0.96	0.92	0.98	0.79	0.97
Inhibition model best fit	Modified non-competitive	Modified non-competitive	4-point logistic model	Modified non-competitive	Modified non-competitive	Modified non-competitive

According to the total applied concentration of Zn, non-competitive inhibition model fit created a more reliable curve than the others with an IC_{50} value of 23.59 mg L^{-1} (R^2 value of 0.90). Besides that, a strong correlation was found between the soluble Zn and inhibition percentages and modified non-competitive inhibition model was provided the best fit curve to calculate the IC_{50} value that was 6.76 mg L^{-1} with an R^2=0.96.

As seen in Table 3.11, the 4- point logistic model gave the best result for inhibition due to intracellular Ni with an IC_{50} value of 11.3 mg L^{-1}(R^2 value of 0.92). For the cell-associated portion of Zn, IC 50 value was calculated as 15.1 mg L^{-1}(R^2 value of 0.98) by using modified non-competitive inhibition model. All inhibition models were tested for each fraction of Zn and a strong correlation was obtained between the inhibition response and almost all fractionations of Zn, except surface bound fraction. This result shows that surface-bound fraction of Zn may hardly predict the inhibitory effect of Anammox activity.

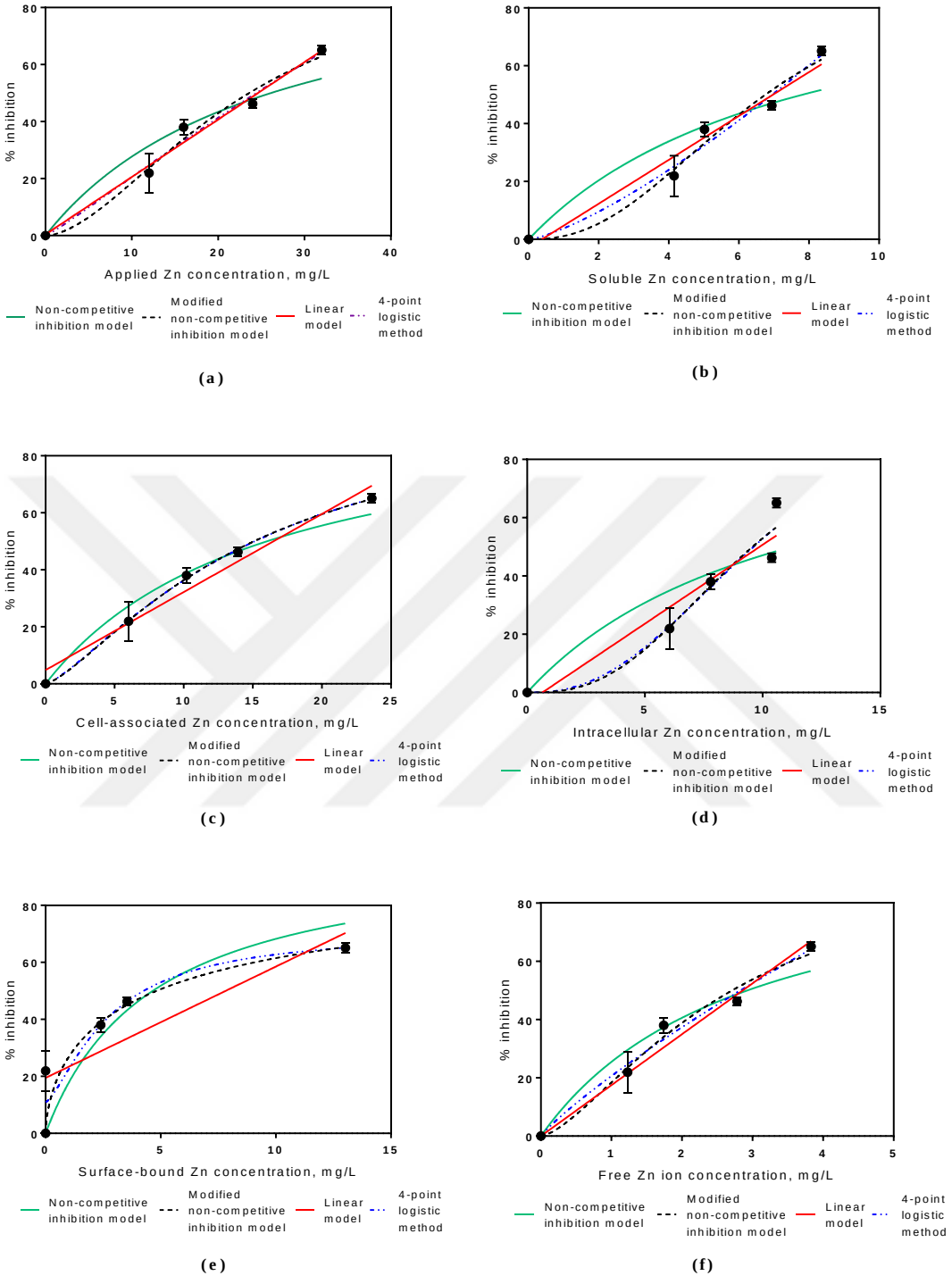


Figure 3. 18 Inhibition response of the (a) applied Zn concentration, (b) soluble Zn concentration, (c) cell-associated Zn concentration, (d) intracellular Zn concentration, (e) surface-bound Zn concentration, and (f) Zn^{2+} concentration for various inhibition models.

The partition coefficient was determined by using linear isotherm models shown in Figure

3.19. The adsorption constant was found 7101 L/kg VSS (R^2=0.93) and then incorporated in the Visual MINTEQ (Version 3.1) to simulate the theoretical speciation under equilibrium conditions.

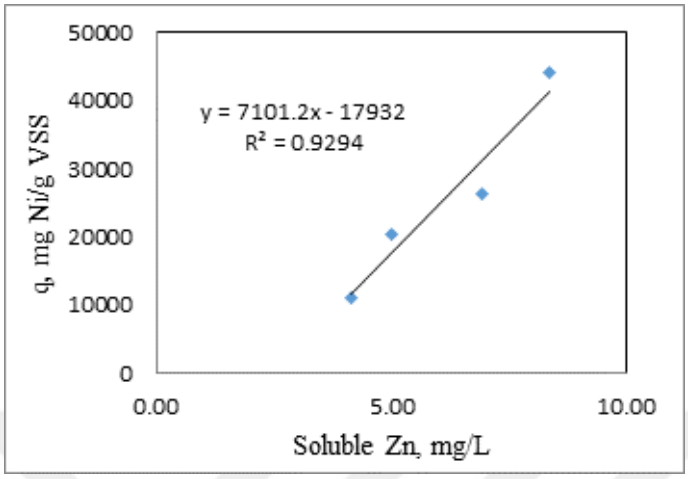


Figure 3. 19 Linear adsorption isotherm according to soluble Zn and cell-associated Zn concentration

Table 3.12 shows the initial speciation for control theoretical speciation of Zn where all species were assumed as soluble with the exception of the cell-associated part. All inorganic ions and complexes determined by using the partition coefficient that was calculated in linear adsorption isotherm. The labile complexes of Zn for the final speciation like hydroxides, chloride, and nitrate is 1% of the total applied Zn metal concentration. Zn formed stable complexes with hydroxides, chloride, sulfate, phosphate, carbonate, and EDTA. Zn-$EDTA^{-2}$ (with a high stability constant, $\log\beta$, of 18.00), $ZnHPO_4$ ($\log\beta = 15.69$), $ZnHCO_3^+$ ($\log\beta = 11.83$), $Zn(CO_3)_2^{2-}$ ($\log\beta = 7.30$), and $ZnCO_3$ ($\log\beta = 5.30$) were the most stable complexes. The insoluble part of the Zn probably is related with these stable complexes and these complexes constitute an average of 32% of the total concentration hence stable part has an insignificant inhibitory effect over Anammox system. According to both theoretical speciation and actual fractionation experiments the soluble part of Zn most probably free Zn^{+2} and labile species may cause the inhibition. Since labile complexes may dissociate into free ion form, the concentration of labile complexes has an important inhibitory effect. The labile complexes accounted for approximately 4.6 % and the free ion 11 % of the total Zn concentration. To investigate the IC_{50} value due to the free Zn ion concentration four different inhibition model was studied and 4-point logistic method gave the most reliable value with the R^2 value of 0.94. As seen in Table 3.11 and Figure 3.18, the half-maximal inhibition value for free Zn ion

was calculated as 2.71 mg L^{-1}.

Table 3. 12 Theoretical speciations of Zn determined by chemical speciation program

	Total applied Zn concentration, mg L^{-1}			
Speciation of Zn	12	16	24	32
Initial Speciation				
Zn^{2+}	2.91	4.01	6.22	8.47
$ZnNH_3^{+2}$	0.00	0.00	0.00	0.01
$ZnOH^+$	0.35	0.48	0.75	1.03
$Zn(OH)_{2\ (aq)}$	0.20	0.27	0.42	0.57
$ZnCl^+$	0.02	0.02	0.04	0.05
$ZnSO_{4\ (aq)}$	0.62	0.85	1.31	1.79
$Zn(SO_4)_2^{2-}$	0.02	0.02	0.03	0.05
$ZnNO_3^+$	0.01	0.02	0.03	0.04
$ZnHPO_{4\ (aq)}$	1.35	1.84	2.81	3.76
$ZnCO_{3\ (aq)}$	4.53	6.20	9.52	12.82
$ZnHCO_3^+$	0.63	0.86	1.32	1.77
$Zn(CO_3)_2^{2-}$	0.15	0.21	0.32	0.42
$ZnEDTA^{2-}$	1.23	1.23	1.24	1.25
Final Speciation				
Zn^{2+}	1.23	1.74	2.78	3.83
$ZnOH^+$	0.15	0.21	0.34	0.46
$Zn(OH)_{2\ (aq)}$	0.08	0.12	0.19	0.26
$ZnCl^+$	0.01	0.01	0.02	0.02
$ZnSO_{4\ (aq)}$	0.26	0.37	0.59	0.81
$Zn(SO_4)_2^{2-}$	0.01	0.01	0.02	0.02
$ZnNO_3^+$	0.01	0.01	0.01	0.02
$ZnHPO_{4\ (aq)}$	0.57	0.81	1.29	1.76
$ZnCO_{3\ (aq)}$	1.92	2.72	4.32	5.93
$ZnHCO_3^+$	0.27	0.38	0.6	0.82
$Zn(CO_3)_2^{2-}$	0.07	0.09	0.15	0.2
$ZnEDTA^{2-}$	1.2	1.21	1.22	1.23
Cell associated	6.25	8.33	12.5	16.66

3.3. Comparison of inhibitory effects of metals

Our results show that among the applied heavy metals copper has the most severe effect on Anammox biomass for short-term exposure. To compare the toxic influence of the different heavy metals IC50 values are stated according to molar concentration (Table 3.13)

Table 3. 13 Comparison of the IC50 values for the short-term exposure

Species	Applied concentration	Soluble	Intracellular	Cell-associated	Surface-bound	Free ion
Ni	0.133	0.102	0.004	0.016	0.012	0.023
Zn	0.361	0.103	0.182	0.231	0.074	0.041
Cd	0.108	0.058	0.011	0.026	0.010	0.036
Cu	0.072	0.038	0.018	0.022	0.002	0.0001

* All concentration

As it is seen in Table 3.13 the inhibitory order of metals was found as Cu>Cd>Ni>Zn in terms of applied concentration. The inhibitory order of tested metals was shown in Table 3.14 for all the parameters compared based on IC50 values. Among the tested metals and their partitioning, the lowest toxic effect was observed for Zn. Although Cu formed almost the same type of complexes with Zn shows the highest toxic effect on the basis of total concentration, soluble, and surface-bound fractions.

Table 3. 14 Comparison of the inhibitory order of Cd, Cu, Ni, and Zn

Parameters	Toxic Effect Comparison
Applied concentration	Cu>Cd>Ni>Zn
Soluble	Cu>Cd>Ni>Zn
Intracellular	Ni>Cd>Cu>Zn
Cell-associated	Ni>Cu>Cd>Zn
Surface-bound	Cu>Cd>Ni>Zn
Free ion	Cu>Ni>Cd>Zn

The chemical speciation estimations show that almost the same type of complexes formed in the Anammox medium for each tested metal.

According to results, inhibition by Cu associated well with the intracellular, cell-associated, and soluble fractions but not a free-ion fraction. Ni inhibition could be correlated well with soluble, intracellular, surface-bound and free ion fractions. Inhibition by Zn and Cd could be explained by soluble, intracellular and free ion fractions but not by surface-bound fractions.

The modified non-competitive inhibition and 4-points logistic models were the primary inhibition models that best described the inhibition response for the full suite of tested inhibition scenarios. To date, even though the individual toxic effect of Cu, Cd, Zn, and Ni over Anammox system has been examined for short-term exposure, there has been disagreement concerning the inhibitory concentration of the metals. For instance, the reported IC50 values for Cd varied between 7.00 mg L^{-1} (Yu et al., 2016) to 174.6 mg L^{-1}(Val del Río et al., 2017) while the range for Zn is between 3.90 mg L^{-1}(Lotti et al., 2012) to 59.1 mg L^{-1}(Val del Río et al., 2017). Similarly, there are perplexing results for Cu and Ni, the IC 50 values for Cu can be ranged from 1.9 mg L^{-1}(Lotti et al., 2012) to 32.5 mg L^{-1}(Q. Q. Zhang et al., 2015a).

To date, Anammox inhibition studies in literature take into account only the total or soluble concentration of heavy metals and underestimate the significance of the speciation. However, it has become essential to measure the speciation of metals in the presence of inorganic and organic ligands in order to an improved understanding of their toxic effects.

The inhibitory concentrations should be calculated based on the bioavailable fraction of metals because the total applied concentration consists of fractions such as stable complexes and precipitates that have probably no significant inhibitory effect on microorganisms (Kalkan Aktan et al., 2018). For example, it is possible to see either no inhibition in a reactor that uses a ligand like EDTA (which makes very stable complexes and therefore decreases the bioavailability of heavy metal to biomass) or observes inhibition in the other reactor that contains no such ligand, in two identical bioreactors with the same applied heavy metal dosages (Di Capua et al., 2017). There are a few studies in the literature that the effect of fractionation on biomass inhibition was considered on nitrification systems (Çeçen et al., 2010b, 2010a, Hu et al., 2003, 2002; Semerci and Çeçen, 2007). It is hard to compare with the other Anammox studies in the

literature since it is not yet known the effect of heavy metal speciation over the Anammox process in these studies.

3.4. Long-Term Exposure to Heavy Metals (for Cu and Cd)

In literature, there has been little attention has been paid to inhibitory effects of heavy metals (Daverey et al., 2014; Guangbin Li et al., 2015; Yang et al., 2013; Z. Z. Zhang et al., 2015; Zhang et al., 2016a) and little is known about the long-term effect of heavy metals on Anammox activity. Most of these studies are related to the short-term exposure effect of heavy metals. Although in literature, prolonged exposure effect of heavy metals has been worked in activated sludge systems and nitrification processes (Hu et al., 2004), there are limited studies on the response of Anammox bacteria for long-term exposure of heavy metals (Daverey et al., 2014; Kimura and Isaka, 2014). It is recognized that relying on only short-term batch experiment may lead to an inaccurate approach to predict the long-term toxic effect of heavy metals (Hu et al., 2004). Furthermore, examination of long-term exposure of heavy metals is the best way for a comprehensive understanding of adaptation, acclimation, shifts, and changes in the bacterial community.

Since, the order of inhibitory effect was found Cu> Cd>Ni>Zn for both soluble and total applied concentrations during the short- term exposure batch tests, Cu and Cd were selected for continuous-flow reactors to examine the prolonged exposure effect on Anammox system.

3.4.1. Long-Term Effects of Cd on Anammox Activity

After inoculation from ongoing lab-scale up-flow column reactor, SBR reactor was operated for 150 days to provide enriched Anammox culture prior to prolonged Cd exposure. Inhibition experiments were carried out after steady state conditions are achieved, approximately 100 days after reactor inoculated nitrogen removal rates reached more than 90%. NRR values were calculated as 4.18 mg N g^{-1} VSS h^{-1} at the beginning of the enrichment process. After steady-state conditions provided, NRR values were calculated as 14.0 mg N g^{-1} VSS h^{-1} on average. Figure 3.20 illustrates the influent and effluent values of ammonia and nitrite and NRR values during the enrichment phase.

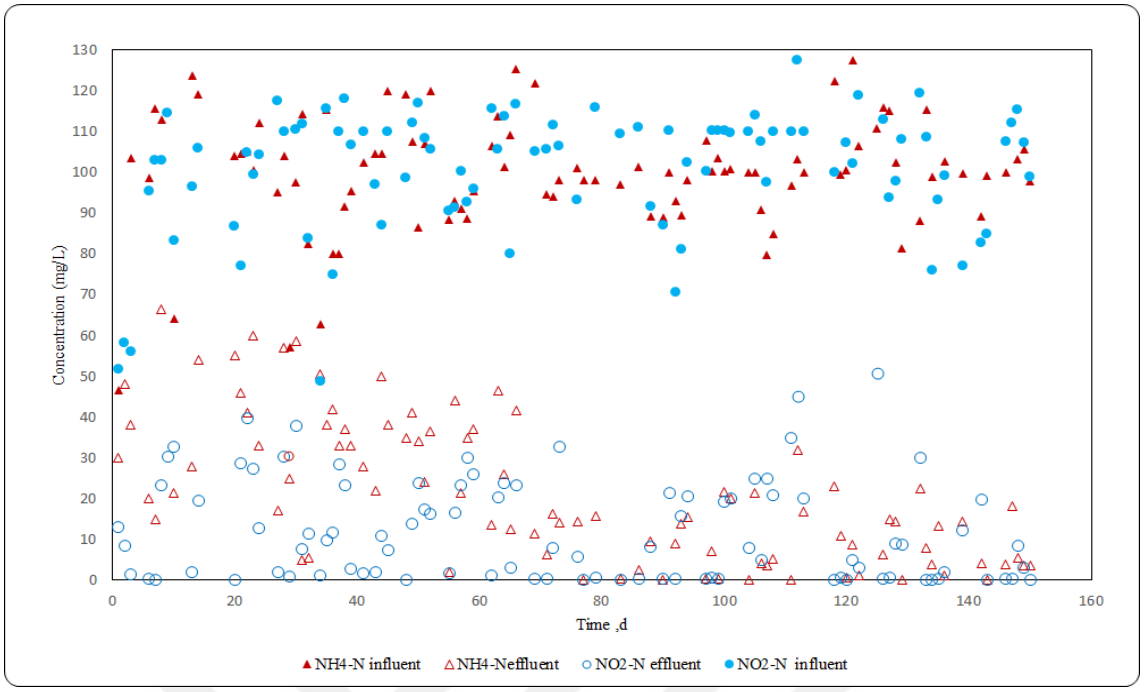


Figure 3. 20 Influent and effluent concentrations of ammonia nitrogen and nitrite nitrogen during the enrichment phase

After the enrichment phase, to observe the prolonged Cd exposure effect on Anammox activity, the system was operated for 340 days under gradually increased Cd concentration. Heavy metal exposures started with a concentration of 0.2 mg L^{-1} and were applied for 50 days. 0.4 mg L^{-1} Cd concentration was provided for another 50 days. Afterward, Cd concentration was increased in a stepwise manner, every 15 days until 9 mg L^{-1}. No inhibition was observed until 5 mg L^{-1} Cd was started to be given to the system (day 267) and the average nitrogen removal efficiency than was found as 91%.

There was no significant change between the means of each Cd concentration based on nitrogen removal efficiencies. An analysis variance (ANOVA) was used to test the significance of results at the 0.05 significance level. The results indicate that no significant difference exists between each datasets (p values ranging between 0.06 and 0.99). The decline in nitrogen removal rate started after 5.5 mg L^{-1} concentration applied.

The removal efficiency decreased to 82% on average at 5-6 mg L^{-1} and around 70% between 6.5-7 mg L^{-1}.Subsequently, the nitrogen removal rate reduced suddenly to 25% on average after 8 mg L^{-1} Cd was given to the system. Figure 3.21 shows the influent and effluent ammonia and nitrite concentration and Cd concentrations during the operation.

The nitrogen removal rate between 0.2 to 5 mg L^{-1} was calculated as 14.5± 0.17 mg N g^{-1} VSS h^{-1} (according to 95% confidence interval) and after that point removal rates started to decrease within the reactor. Figure 3.23 (a) shows the nitrogen removal rates under a stepwise increase of Cd concentrations.

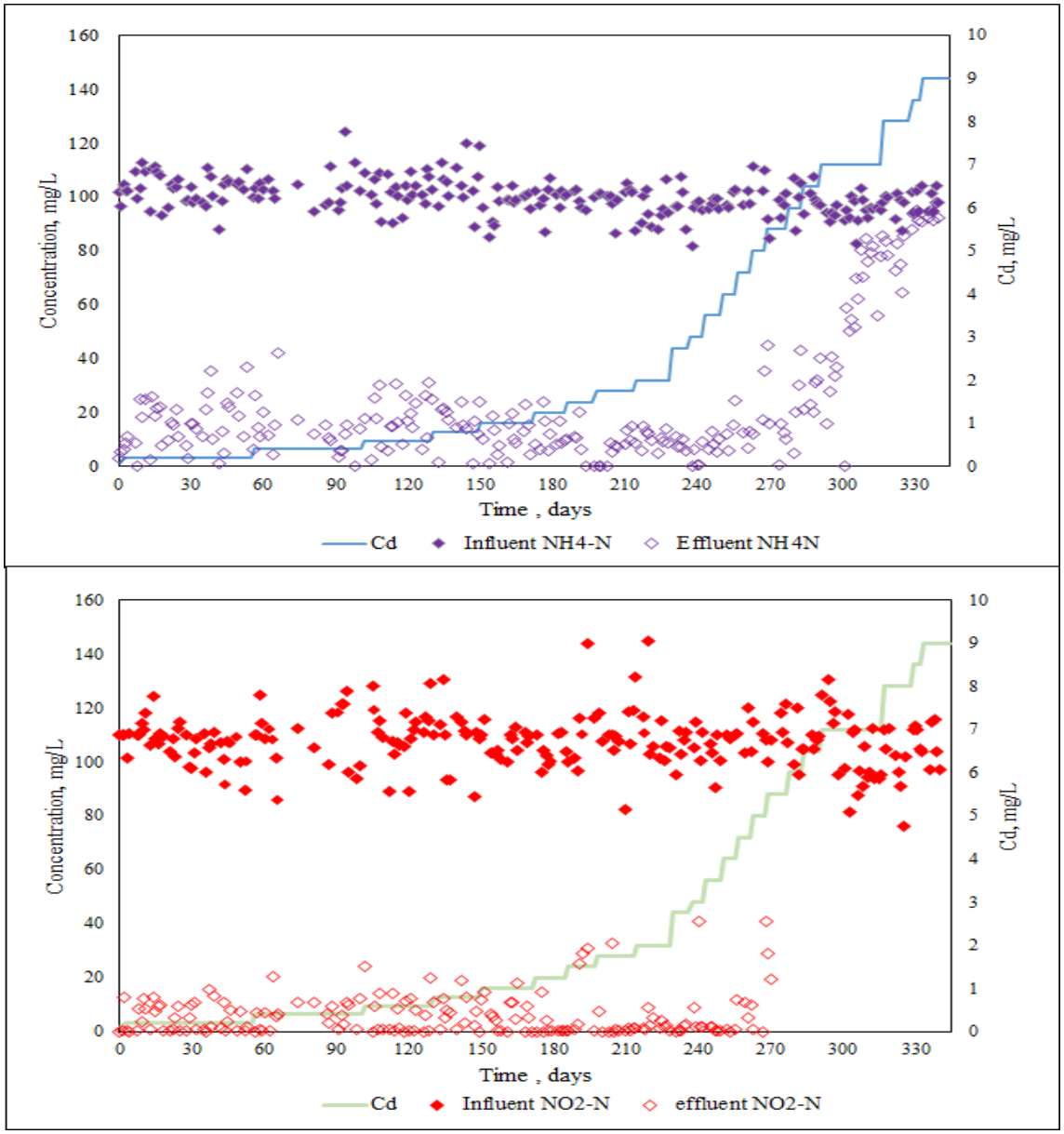


Figure 3. 21 Influent and effluent ammonia (a) and nitrite (b) nitrogen profiles during Cd exposure

Figure 3.21 shows the influent, effluent and intracellular Cd concentrations during the Cd exposure. In order to measure intracellular cadmium concentrations, mixed liquor

samples were taken monthly. Intracellular metal uptake was not detected up to a concentration of 5.5 mg L^{-1} (Figure 3.22(b)), i.e. influent and effluent Cd values were not significantly different from each other (determined at the 0.05 significance level using one-way ANOVA and p= 0.38). After that point, intracellular Cd concentration started to increase and intracellular accumulation occurs after 8 mg L^{-1} Cd applied where approximately 86% inhibition observed within the reactor.

This result is therefore in consistent with our findings in speciation experiments where a soluble and intracellular fraction of 5 mg L^{-1} Cd was found to be 96.5% and 2.4% of the applied concentration. In speciation experiments, our results also show that intracellular fraction of Cd started to increase after 10 mg L^{-1} Cd applied. The intracellular and soluble fractions were found as 13% and 57%, respectively.

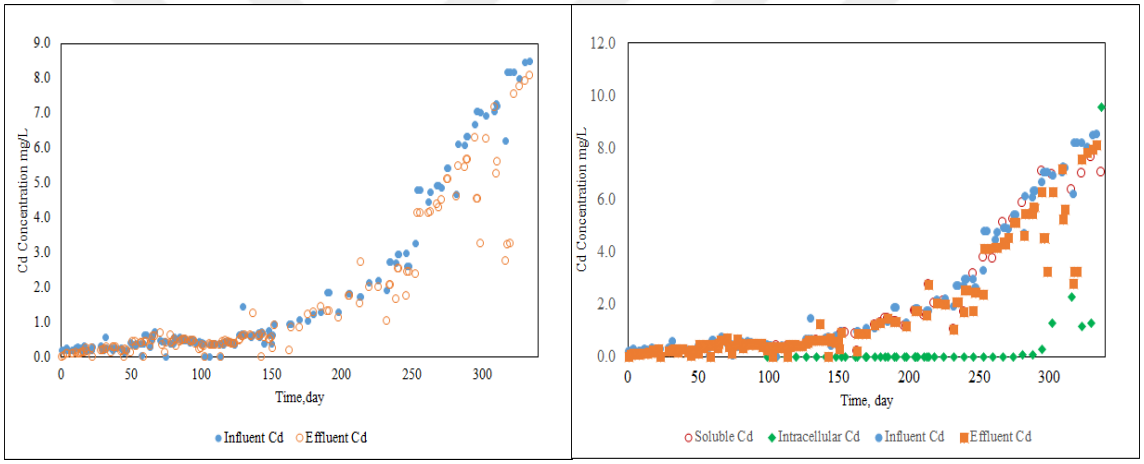


Figure 3. 22 (a) Influent and Effluent Cd concentrations during the exposure (b) Influent-Soluble-Intracellular Cd Concentrations

To identify the IC50 levels for the long-term exposure of Cd the experimental data were fitted with the modified non-competitive inhibition model and calculated as 6.75 mg L^{-1} (R^2: 0.81 according to 95% confidence interval) based on removal efficiency of nitrogen. Figure 3.23 (b) shows the applied Cd concentration versus the percent inhibition values according to modified non-competitive inhibition model. These results indicate long-term exposure of Cd had more detrimental effects than short-term exposure (IC50=12.14 mg L^{-1}) since 50 % of activity loss has been observed at a lower concentration. The reason of that may be related with the uptake system of Cd. In short-term exposure tests, the equilibrium between Cd and surface of the cell could not be established, since metal

uptake proceeded very slowly (Hu et. al, 2004). This might be the significant reason of observe the higher inhibitory effect during the prolonged exposure of Cd. These results can be also related to the relative abundance of the Anammox species during the exposure. According to qPCR results, the relative quantity of the Anammox bacteria among the all bacterial populations decreased by around 37% after 5.5 mg L^{-1} Cd applied (Table 3.16). Previous studies show that the impact Cd for short-term expose was detrimental. To date, even the long-term effects of some metals, such as Cu (Kimura and Isaka, 2014; Yang et al., 2013), Zn (Zhang et al., 2018), Mo and Ni (Kimura and Isaka, 2014) was studied by some researchers, the prolonged exposure of Cd has not been investigated in Anammox system so far. However, there are some studies that worked on the prolonged effect of Cd on nitrification systems (H. B. Chen et al., 2014; Semerci and Çeçen, 2007). Chen et al. (2014) studied the short and long-term effect of Cd(II) on biological nitrogen and phosphorous removal system in wastewater and they reported that there was no significant inhibitory effect up to 10 mg L^{-1} after short-term exposure. However, they found that for the long-term exposure of 10 mg L^{-1} Cd lead to 97% inhibition in terms of nitrogen removal efficiencies. Similar to our study, long-term exposure of Cd (II) lead to a decrease in the abundance of the nitrite oxidizing bacteria.

Çeçen et al. (2010b) performed short-term inhibition tests for nitrification system and they reported the 50% inhibition concentrations of cadmium as 10.65 and 19.90 mg L^{-1} on the basis of O_2 consumption and CO_2 production, respectively. However, they also reported the IC50 value for the long-term exposure of Cd between 2-2.5 mg L^{-1} on the basis of ammonium utilization rate.

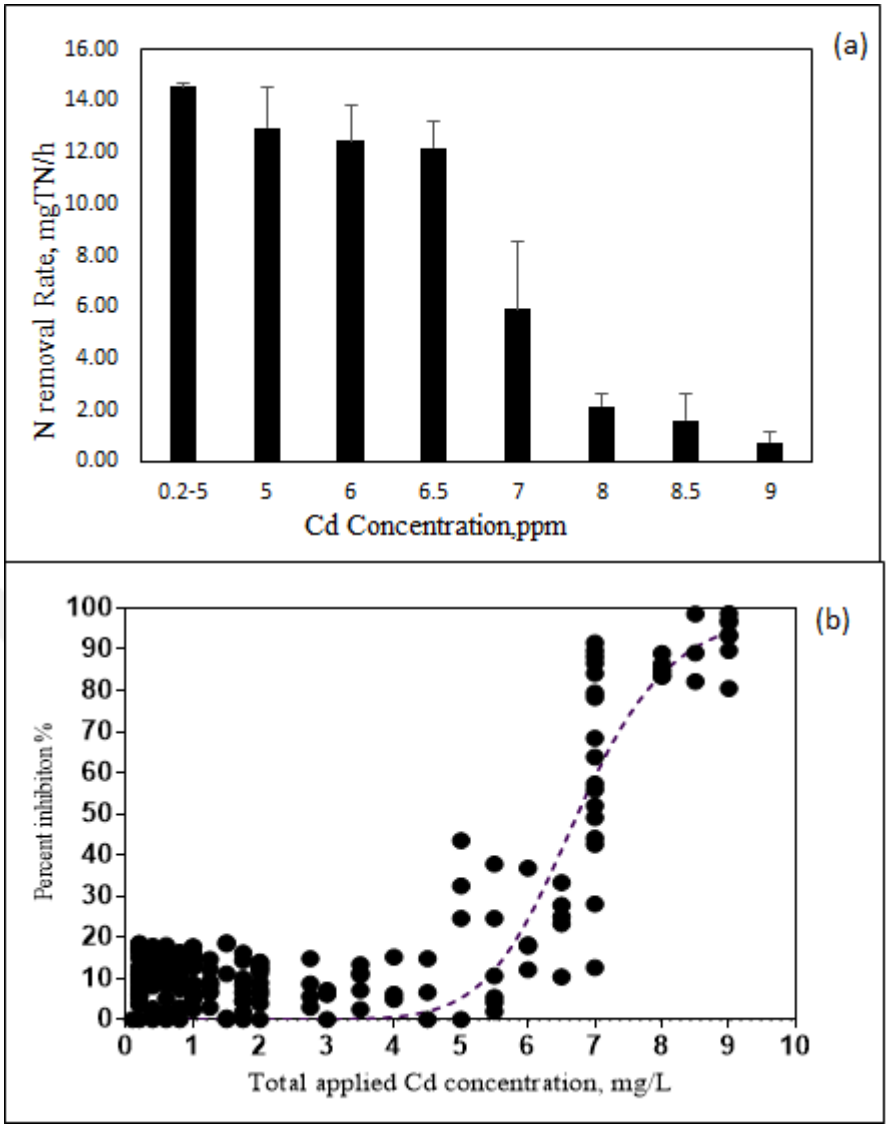


Figure 3. 23 (a) Nitrogen removal rates during long-term exposure (b) Relative Percent inhibition vs Cd concentration

Therefore, it can be concluded that in the long-term exposure of Anammox bacteria and nitrifiers shows the same trend, they are both more sensitive for the long-term exposure of this metal. Since Cd is a nonessential metal for both nitrifiers and Anammox bacteria it can be considered that Cd (II) may cause a more toxic effect in the case of prolonged exposure.

3.4.2. Long-Term Effects of Cu on Anammox Activity

Prior to the heavy metal exposure, continuous flow system was operated approximately one year and enrichment of the reactor was achieved with 94% nitrogen removal efficiency (Figure 3.24).

After steady state conditions were achieved, copper addition to the system was started with a concentration of 0.2 mg L^{-1}. In order to test the long-term inhibitory effect of Cu on the reactor performance, the system was operated for 240 days under gradually increased Cu loadings.

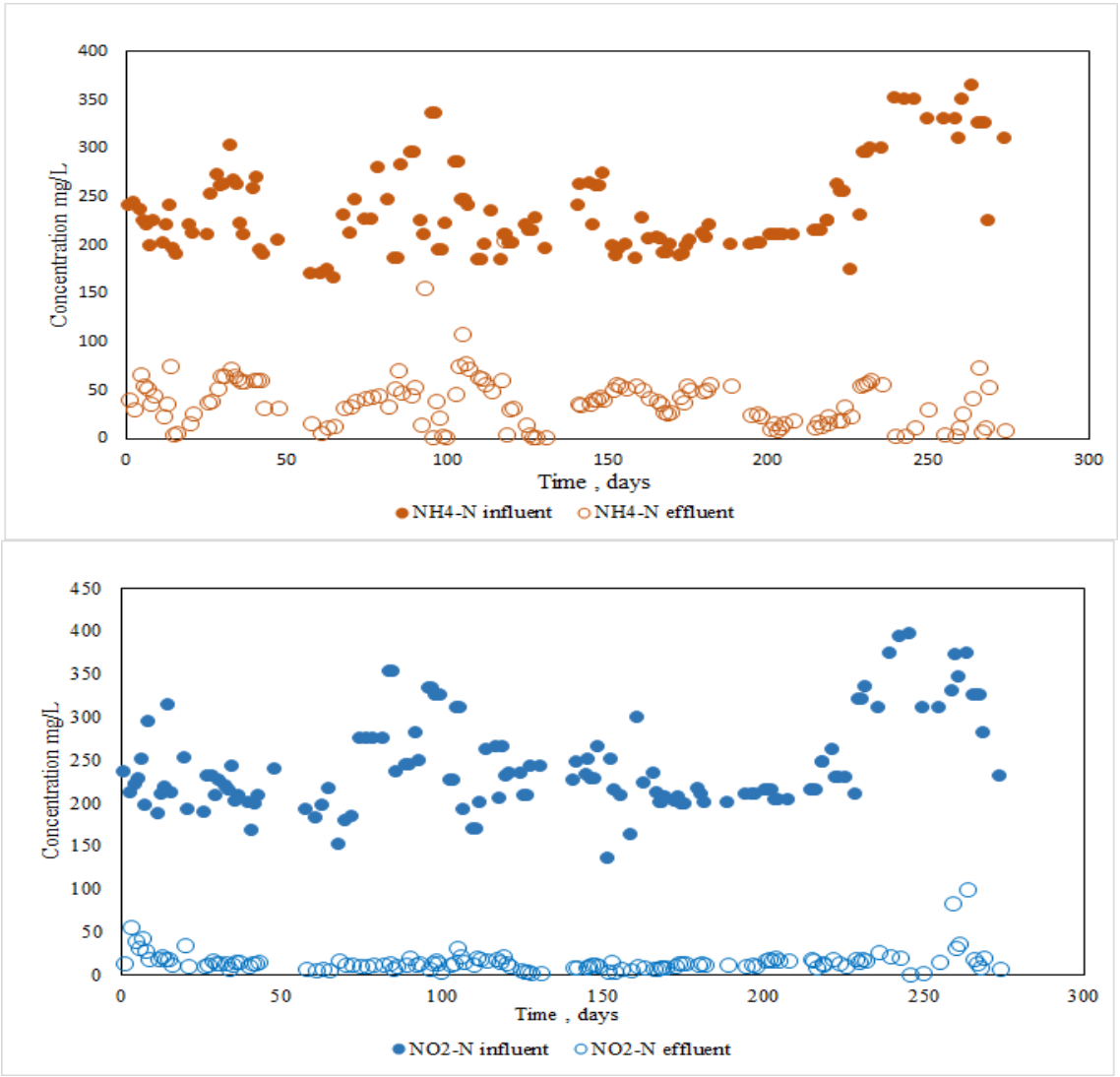


Figure 3. 24 Influent and effluent ammonia and nitrite nitrogen profiles of the up-flow reactor during an enrichment period

Figure 3.25 shows the influent and effluent ammonia and nitrite concentration and Cu concentrations during the operation. Heavy metal concentration was increased stepwise from 0.2 mg L^{-1} to 8 mg L^{-1}.

For 30 days, 0.2 mg L^{-1} Cu was given to the system and then the concentration of copper was increased step by step at every 15 days till 8 mg L^{-1}. No inhibition was observed until

6 mg L^{-1} Cu was started to be given to the system (day 180) and the average removal rate of nitrogen was found to be 97% at 6 ppm Cu addition.

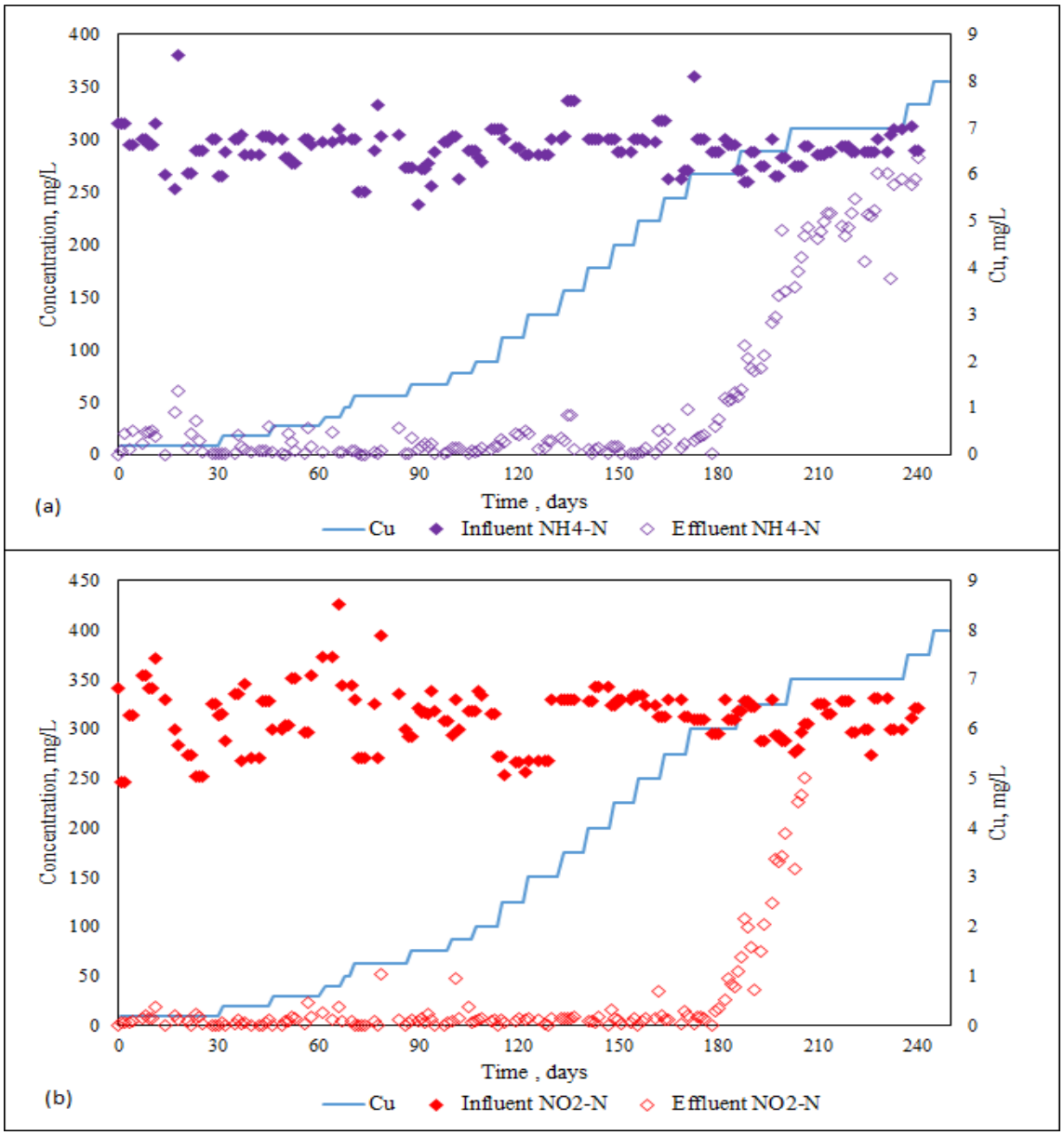


Figure 3. 25 Influent and effluent ammonia (a) and nitrite nitrogen (b) profiles during Cu exposure

There was no significant change between the means of each Cu concentration based on nitrogen removal efficiencies. An analysis variance (ANOVA) was used to test the significance of results at the 0.05 significance level. The results indicate that no significant difference exists between each datasets (p values ranging between 0.13 and 0.99). The decline in nitrogen removal rate was sharp after concentration of 6.5 mg L^{-1} applied. Therefore, exposure to a Cu concentration up to 6 mg/L resulted in higher nitrogen

removal efficiency.

The enhancement effect of Cu addition at low concentrations has been reported by many researchers (H. Chen et al., 2014; Zhang et al., 2016a). In literature, the presence of some heavy metals at low concentrations play a crucial role to stimulate metabolism of microorganisms. Cu is one of the essential micronutrients for many enzymes of microorganisms. Chen and his co-workers (2014) reported that Cu concentration should be kept below 4 mg L^{-1} to provide good reactor capacity and the optimum dosage to improve the specific Anammox activity indicated as 1.18 mg L^{-1}. In our study, we observed the enhancement of the nitrogen removal rate until Cu concentration reached up to 6 ppm. The NRR value was calculated as 19.1± 0.60 mg N g^{-1} VSS h^{-1} (according to the 95% confidence interval) up to 6 mg L^{-1}. As it was shown in Figure 3.26 (a) the nitrogen removal efficiency started to decrease after180 days at 6 mg L^{-1} Cu addition. The nitrogen removal rates decreased to 2.2 mg N L^{-1} h^{-1} when Cu concentration reached up to 8 ppm.

In order to examine the response of Anammox to long-term copper exposure, non-competitive, modified non-competitive,linear and 4-point logistic inhibition models were tested. The results indicated that inhibition response was more successfully represented by a modified non-competitive inhibition model and IC50 value was calculated as 6.77 mg L^{-1} (R^2: 0.95 according to 95% confidence interval) based on removal efficiency of nitrogen (Figure 3.26 (a)).

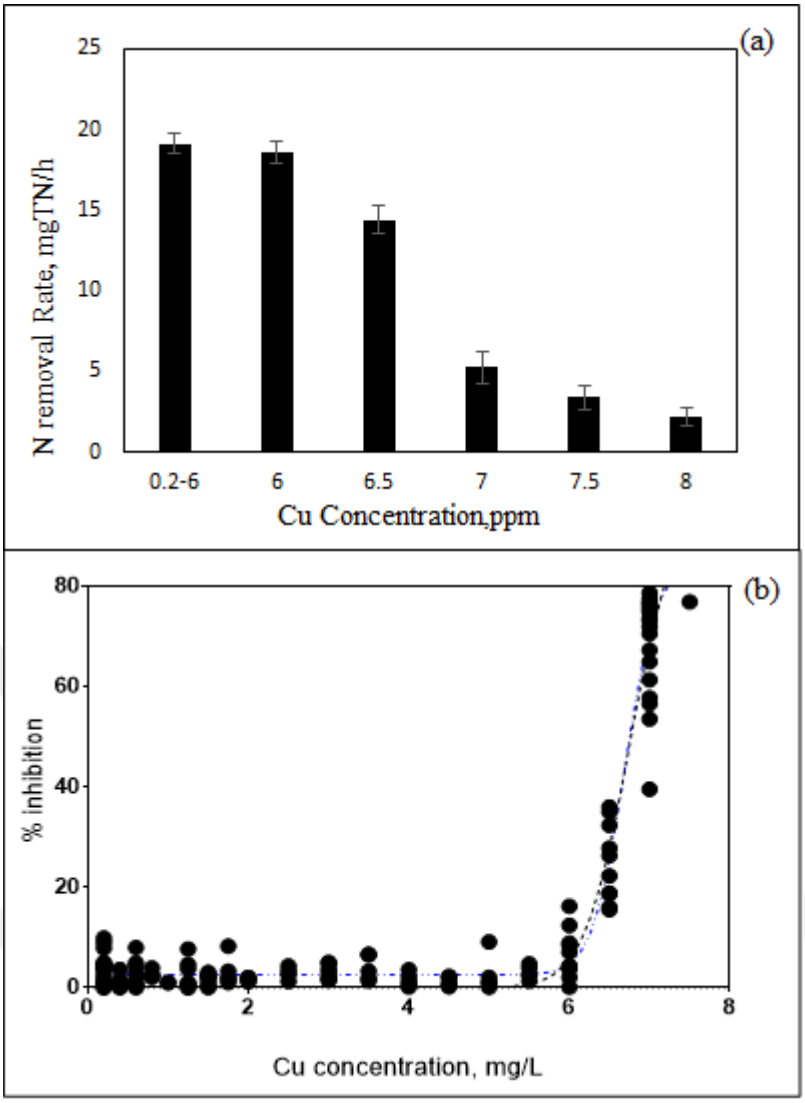


Figure 3. 26 (a) Nitrogen removal rates during long-term exposure (b) Relative Percent inhibition vs Cu concentration

To date, the inhibitory effect of copper on Anammox activity has been studied in some publications. Although short-term effects were mostly examined, long-term effects were also concerned in several studies. In a recent study conducted by Ma et al (2018), long-term exposure effect of Cu(II) was studied with a constant elevated concentration and they reported that NRR decreased at 57.3 %, 71.0% and, 100 %, respectively in 1, 5 and, 10 mg L^{-1} Cu (II) addition. Yang and his coworkers (2013) studied both short and long-term inhibition effect of Cu and stated that the IC50 value as 12. 9 mg L^{-1} for the short-term inhibition, while they investigated the long-term inhibition in up-flow anaerobic sludge blanket reactor and found 94% activity loss when 5 mg L^{-1} Cu added to the system. Zhang et al (2015a) also reported a 65% reduction in NRR when Cu concentration was

increased from 5 to 8 mg L^{-1}. For both studies, they observed a higher tolerance to Cu in short-term exposures rather than long-term. Furthermore, Kimura and Isaka (2014) studied the inhibitory effects of heavy metals (Cu, Ni, Zn, Mo, and Co) via continuous feeding test and reported 22% and 65% decrease in Anammox activity when the Cu concentration was increased to 5 mg L^{-1} and 7.5 mg L^{-1}, respectively.

Our findings do not support previous research in this area. In contrast to earlier findings, we have obtained higher median inhibition levels in long-term exposure. Besides our results indicated that the inhibitory effect of copper was lower for long-term exposure when comparing with the short-term effect. The possible reason for that starting from very low concentrations of Cu in our study may have provided suitable conditions for adaptation of the microorganisms. The reason for the discrepancy between short-term and long-term inhibition most probably is related to the acclimation and bacterial adaptation. There are numerous studies that have been discussed the potential for microbial adaptation to elevated metal concentration (Bååth, 1989; Rusk et al., 2004). The adaptation of the microbial communities to the elevated metal concentration may result in genotypic shifts or alteration in community structure (Silver and Walderhaug, 1992). The variability between the results should also be related with the microbial abundances and community diversity. Therefore, the species that take a role in the conversions was also investigated by molecular analysis.

3.5. The Response of Microbial Community to Heavy Metal Exposure (Molecular Analysis)

In reactors exposed to metals (Cu and Cd) for long-term, samples were regularly taken at every dose change to quantify Anammox species. After samples were taken, DNA extraction was performed and extracted DNA were analyzed quantitatively using real-time PCR method. To determine contamination in the extracted nucleic acid measurements were performed at 260 nm and 280 nm wavelengths. In DNA samples without protein contamination, the A260 / A280 ratio is close to 1.8 (Glasel, 1995). Protein contamination was not observed in most of the samples (Table 3.15).

Table 3. 15 The extracted DNA concentrations

Sample	Dosage (mg L^{-1})	DNA (ng/µl)	A260/ A280
Cd Long-Term Exposure			
1th day	0.00	35.5	1.67
8th day	0.20	29.0	1.65
15th day	0.20	35.5	1.63
46th day	0.20	29.5	1.69
74th day	0.40	23.5	1.74
102th day	0.40	25.5	1.70
128th day	0.60	21.5	1.72
151th day	0.80	21.0	1.75
164th day	1.00	23.5	1.81
178th day	1.20	27.0	1.80
221th day	1.50	25.0	1.79
206th day	1.75	26.5	1.71
221th day	2.00	23.0	1.73
235th day	2.75	20.0	1.74
247th day	3.75	24.5	1.82
294th day	4.50	25.0	1.72
276th day	5.50	22.0	1.76
290th day	6.50	21.5	1.72
304th day	8.00	36.0	1.71
337th day	9.00	38.0	1.79
Cu Long-Term Exposure			
2nd day	0.10	21.5	1.79
31th day	0.40	33.5	1.76
58th day	0.80	26.5	1.71
73th day	1.25	22.5	1.61
109th day	2.00	29.0	1.66
135th day	3.50	28.0	1.70
165th day	5.50	29.5	1.74
195th day	6.50	41.5	1.69
235th day	8.00	44.0	1.63

Quantification of the Anammox species by real-time PCR enabled us to confirm that Anammox was the dominant process in our study.

In literature, generally quantification comparison has been carried out by using gene copy numbers and total MLVSS values but the analysis that based on16S rRNA is the most appropriate way to give information about the total cellular activity. In order to evaluate the percentage of target species in total microorganisms, 16S rRNA gene specific for all bacterial species was calculated and 16S standard curve was created to compare the target

gene's rates. To determine the Anammox species percentage in reactors bacterial ratio was detected via AMX/16S rRNA gene copy ratios with real-time PCR techniques. Table 3.16 shows the variation of the Anammox bacteria percentage in the bacterial community depending on heavy metal concentration.

For the reactor that is exposed to Cd metal for the time period, initial Anammox percentages were calculated as 25 % among all microorganisms. However, Anammox ratio started to decrease for a while when the applied Cd concentration was increased step by step. After the given Cd concentration reached 0.8 mg L^{-1} (at day 150), increase in the Anammox bacteria percentage was observed. This increase reached up to 46% when the Cd concentration reached to 2.75 mg L^{-1}. The continuation of this increase of Anammox abundance may be related to that cadmium does not adversely affect the process until 2.75 mg L^{-1} after a long enrichment period (150 days).After that, nitrogen removal efficiency started to decrease after 5 mg L^{-1} Cd was applied to the system and correspondingly the Anammox ratio started to decrease in the reactor. According to the regression analysis, the relationship between the nitrogen removal rate and Anammox percentage is statistically significant at the 0.05 significance level (p=0.026). Pearson correlation analyses indicated that Anammox quantity positively correlated with the nitrogen removal rate (r=0. 522) during the cadmium exposure. In addition, the half maximal inhibitory concentration was calculated as 6.75 mg L^{-1} and the Anammox quantity was decreased to 11 % when 6.5 mg L^{-1} Cd-exposed to the reactor approximately half of the initial quantity.

Table 3. 16 The gene copy number of 16s rRNA and Anammox and their relative quantification (Cd Long-term Exposure)

Applied Heavy Metal Concentration	Target gene Copy number		AMX/16S rRNA ratio
	16s rRNA	Anammox (AMX)	
0.00	2.37E+05	5.82E+04	25%
0.20	9.34E+04	1.89E+04	20%
0.20	2.19E+05	4.55E+04	21%
0.40	6.64E+04	1.13E+04	17%
0.40	4.05E+05	6.21E+04	15%
0.60	6.48E+04	8.00E+03	12%
0.80	2.43E+05	6.35E+04	26%
1.00	6.50E+04	2.06E+04	32%
1.25	6.99E+04	2.17E+04	31%
1.50	1.36E+05	5.69E+04	42%
1.75	1.29E+05	4.73E+04	37%
2.00	1.25E+05	5.48E+04	44%
2.75	2.35E+05	1.08E+05	46%
4.50	1.32E+05	3.18E+04	24%
5.50	6.27E+04	1.07E+04	17%
6.50	5.94E+04	6.69E+03	11%
8.00	9.37E+04	9.68E+03	10%
9.00	3.05E+05	2.25E+04	7%

Metagenomic sequencing analyses were applied to obtain detailed information about the microbial community in the reactors by next-generation sequencing (NGS). For this purpose, two different samples were taken from the beginning of the reactor and day 245th. Figure 3. 27 shows the initial bacterial diversity results in both of the reactors that exposed Cd and Cu for long-term, according to NGS analysis. The analysis results indicated the most dominant phylum was the *Ignavibacteriae*, which accounted for 30.8% of the bacterial population. The second most abundant phyla were Anammox-associated *Planctomycetes* phylum with an abundance of 28%. The third and fourth most-abundant

phyla were *Proteobacteria* and *Chloroflexi* with abundances of 24.7 and 13.55 %, respectively. They were followed by *Actinobacteria* (0.38%), *Bacteroidetes* (0.22%) with each observed in a relatively low abundance. In line with our results, Mardanov et al. (2017) found the main phyla *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Chloroflexi*, *Ignavibacteriae*, and *Acidobacteria* in a Nitritation/ Anammox bioreactor.



Figure 3. 27 The bacterial community structure of the reactors obtained by NGS analysis of 16S rRNA fragments (start-up)

There have been classified six different genera of Anammox bacteria; *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Scalindua*, *Candidatus Jettenia*, *Candidatus Anammoxoglobus* and, Candidatus *Anammoxomicrobium* from previous studies (Ding et al., 2013; Ibrahim et al., 2016; Kartal et al., 2007b). In our study, the majority of the Anammox bacteria was related to *Candidatus Kuenenia* (89.3%) at the genus level of the total Anammox sequences. In accordance with the previous studies (Gonzalez-Gil et al., 2015; Zheng et al., 2016), one genus of Anammox bacteria was found to be dominant under the applied growth conditions.

The analysis indicates that the relative abundance of *Candidatus Kuenenia* is 25% of the all bacterial population which is also consistent with the qPCR results. According to NGS analyses, a substantial numbers of the bacterial population comprises heterotrophic bacteria, such as *Ignavibacteria* (30.8%) and *Anaerolineae* (7.18%). *Ignavibacteria* has been reported in Anammox reactors frequently (Bhattacharjee et al., 2017; Gonzalez-Gil et al., 2015; Mardanov et al., 2017; Speth et al., 2016). It has been reported that *Ignavibacterium album* is facultative anaerobe and possesses CO_2-fixing enzyme, therefore, have an ability to grow mixotrophically at worst case use inorganic carbon as a carbon source and utilize nitrogen for energy generation (Liu et al., 2012). The high fraction of *Ignavibacteria* in an autotrophic medium here may indicate that it has functions other than organic carbon oxidation. However, further studies are needed to test this hypothesis.

Nitrifying ammonia-oxidizing bacteria and nitrite-oxidizing bacteria were almost completely suppressed, as the relative abundances of *Nitrosomonas* (0.036%) *and* Nitrobacter (0.010%) were very low at the startup of the reactors.

Figure 3.28 shows the bacterial community structure on day 245 (3.5 mg L^{-1} Cd was started to applied) of the reactor. As it was mentioned before in qPCR results after 2.75 mg L^{-1} Cd exposure the relative quantity of Anammox started to decrease to 24% from 46 % in agreement with these result the relative abundances of *Candidatus Kuenenia* was found 26% of all the bacterial community. Therefore, it can be concluded that the relative percentage of *Candidatus Kuenenia* declines suddenly between 2.75 and 3.5 mg L^{-1} of Cd exposure. Moreover, Candidatus *Brocadia* was observed which accounted for 0.7% of the all bacterial population. Here the majority of the Anammox bacteria was related to *Candidatus Kuenenia* (89%) at the genus level, with *Candidatus Brocadia* comprising only 3% of the total Anammox sequences. Van Der Star et al. (2008a) hypothesized "Kuenenia" as a K (high substrate affinity) strategist and ''Brocadia'' as a r (high growth rate) strategist since *Candidatus Kuenenia* is known to have a lower nitrite apparent half-saturation constant (K_m) compared to *Candidatus Brocadia*, such that the dominance of *Candidatus Kuenenia* in some Anammox systems is explained by its high affinity for nitrite.

In another study (Zhang et al., 2018) also observed that, while the relative abundances of

Candidatus Kuenenia decreased, *Candidatus Brocadia* tended to increase under the elevated Zn exposure. They also observed an increase in the relative abundance of *Candidatus Kuenenia* up to 5 mg L^{-1}, afterward higher concentrations lead to decrease the relative quantity. *Candidatus Kuenenia* and *Candidatus Brocadia* have been identified as the dominant genera in Anammox bioreactors in previous studies via 16SrRNA gene analysis, such as fluorescence in situ hybridization (FISH) and/or PCR (Hu et al., 2013), and metagenome analysis (Guo et al., 2016).

As it was shown in Figure 3.28, the fraction of *Chloroflexi* in the system significantly increases to a level of 22.4% from 13.5%. Kindaichi et al. (2012) reported that *Chloroflexi* species degraded the organic compounds that resultant from dead biomass of the Anammox bacteria. The presence of a relatively high fraction of this phylum confirms biomass degradation in the studied reactor.

Furthermore, a new genus *Parcubacteria* observed at that concentration with a relative abundance of 7% of the bacterial population. These bacteria have been identified in an anoxic environment in previous studies(Nelson and Stegen, 2015). Some of the *Parcubacteria* is regarded as capable of degrading complex carbon sources and capable of fermenting simple sugars to organic acids (Nelson and Stegen, 2015). Some of the *Parcubacteria* members has been stated as potential denitrifiers, since the nitrite reductase enzyme (*nirK*), which is responsible for the reduction of nitrite in denitrification process, was found in some these genomes (Danczak et al., 2017). Therefore, the presence of these genomes may indicate that the Anammox inhibition was started via increasing levels of cadmium concentrations while denitrification started to takes place.

The degradation of bio-refractory compounds can be accomplished by fermentative bacteria and bacteria in an autotrophic environment. Therefore, the presence of these bacteria can be shown as an evidence of biomass decay in the SBR reactor after long-term exposure within Cd. A 50-d solids retention time in the current study would be long enough to observe such biomass decay. The hydrolysis of larger biomass residues into smaller organic materials, such as alcohols and short-chain volatile fatty acids, may cause heterotrophic denitrifiers to proliferate within the system. Microorganisms capable of denitrification are reported generally from the *Proteobacteria*, *Bacterioidetes*, *Chloroflexi*, *Firmicutes*, and *Chlorobi* phyla (Lu et al., 2014). Since the nitrate generated

by Anammox bacteria can be used by the denitrifying bacteria, the groups of heterotrophic bacteria and Anammox could result in the enhanced removal of both nitrogen and carbon.

However, since the denitrification process is thermodynamically more favorable than the Anammox reaction (Sabumon, 2007), attention should be given to the C/N ratio in the influent against the out-competition of Anammox.

Although, the relative abundances were very low *Nitrosospira* (0.03%) and *Nitrospira* (0.01%) has been detected after long-term exposure within Cd and a small increase was observed in the relative abundances of *Nitrosomonas* (0.23%) and *Nitrobacter* (0.03%).

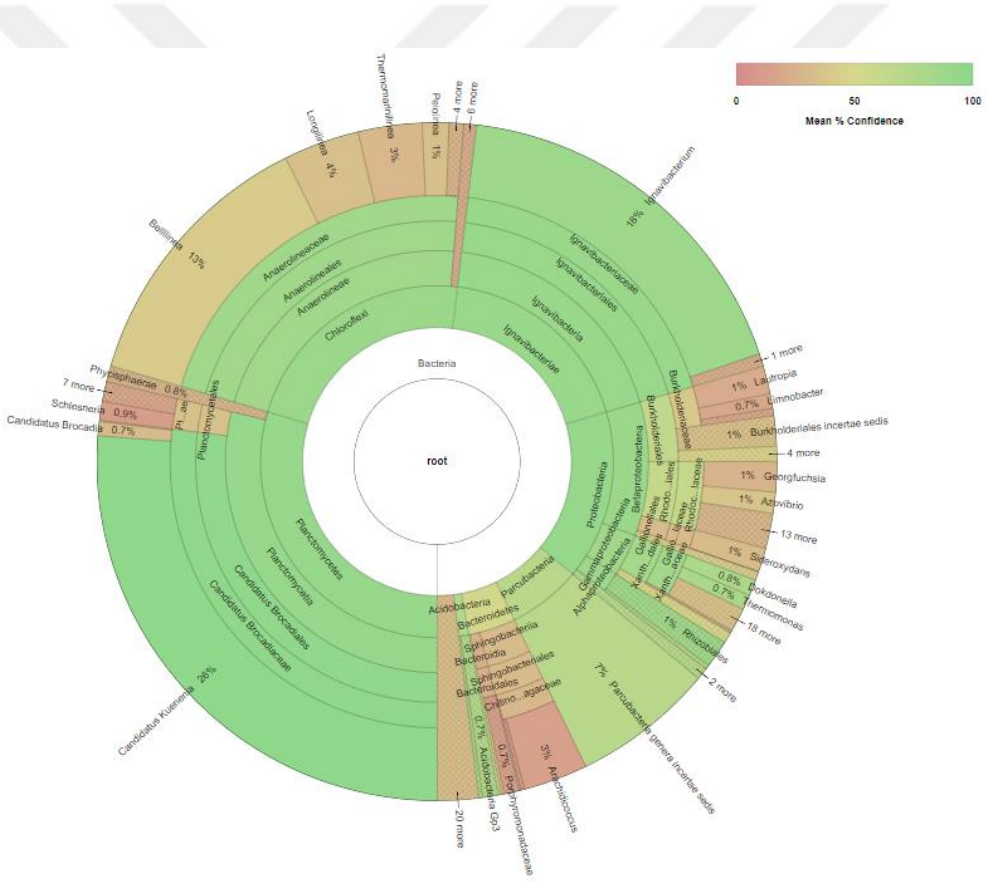


Figure 3. 28 The bacterial community structure of the Cd exposure reactor obtained by NGS analysis of 16S rRNA fragments (Day 245, 3.5 mg L^{-1} Cd)

Table 3. 17 shows the real-time PCR results for the prolonged Cu exposed reactor. Similar results were also obtained for the reactor that is exposed to Cu metal. The initial

Anammox percentages were calculated as 31 % and after a short period of decline, the quantity of Anammox started to increase similar to the other reactor. However, this reactor showed a more stable profile based on Anammox quantity. A plausible explanation for this observation can be related with the reactor type. Attached growth system was used investigate the long-term exposure effect of copper in this study. According to relative quantitation, Anammox percentage in the reactor sharply decrease after an exposure of 6.5 mg L^{-1} Cu. These results also are in compliance with the nitrogen removal rates, NRR values for the reactor also started to decline after 6 mg L^{-1} of Cu addition and the sudden decrease was observed between 6 and 8 mg L^{-1} of Cu. In line with the sharp decrease of NRR values Anammox percentage in the reactor suddenly decreased to 15 % according to the qPCR analysis (Table 3.17). Pearson correlation analyses indicated that Anammox quantity positively correlated with the nitrogen removal rate (r=0.539) during the copper exposure. Similarly, Ma and his coworkers (2018) also indicated the decrease in the relative abundance of Anammox species during the long-term exposure to elevated Cu(II).

Table 3. 17 The gene copy number of 16s rRNA and Anammox and their relative quantification (Cu Long-term Exposure)

Applied Heavy Metal Concentration	Target gene Copy number		AMX/16S rRNA ratio
	16s rRNA	Anammox (AMX)	
0.10	6.17E+04	1.92E+04	31%
0.40	1.75E+05	3.88E+04	22%
0.80	2.96E+05	6.60E+04	22%
1.25	3.02E+05	8.06E+04	27%
2.00	5.24E+04	1.74E+04	33%
3.50	2.26E+05	8.01E+04	36%
5.50	1.11E+05	4.54E+04	41%
6.50	6.00E+04	1.98E+04	33%
8.00	1.68E+05	2.54E+04	15%

Figure 3. 29 represent the bacterial diversity on day 87 of the reactor the given concentration of Cu was 1.25 mg L^{-1}. As it was explained before, the initial bacterial diversity of both reactors (Cu and Cd-exposed reactors) was the same (Figure 3.27).



Figure 3. 29 The bacterial community structure of the Cu exposure reactor obtained by NGS analysis of 16S rRNA fragments (Day 87, 1.25 mg L^{-1} Cu)

As mentioned before, the enhancement effect of Cu in nitrogen removal rate was observed up to 6 mg L^{-1} Cu exposure. In accordance with this enhancement, after elevated Cu exposure within 1.25 mg L^{-1}, NGS results also indicated the increase in the *Planctomycetes* phylum which accounted for 31% of the bacterial population. Besides, the abundances of *Candidatus Kuenenia* also increased to a level of 27% from 25% and was found to be the dominant genus. This results are also consistent with the qPCR results; the relative quantity of Anammox genus was found as 27% among all bacterial population after 1.25 mg L^{-1} Cd exposure.

In contrast with our study, Zheng et al. (2016) employed quantitative PCR and cloning/sequencing analysis to show that *Candidatus Brocadia* preferred to grow in the biofilm, while *Candidatus Kuenenia* became the key player in the suspended sludge.

At the startup of the process, *Ignavibacteriae* was the dominant phylum with relative abundances 30.8%, up to 23% a descent was observed for the relative quantification of this phylum.

Ma and his coworkers (2018) also examined the microbial diversity in the Anammox reactor after long-term exposure within elevated Cu(II). In contradiction with our results, they reported significant suppression on Anammox species at lower concentrations and observed a decreasing trend in the abundance of *Candidatus Kuenenia* in response to increasing copper concentrations. Although the dominant genus was found *Candidatus Kuenenia* in their study among anaerobic ammonium oxidizing bacteria, they found the relative abundance of the *Candidatus Kuenenia* much lower than our study and the most dominant phyla as *Proteobacteria*. Therefore, the differences between our results may also be related to the abundance of Anammox bacteria.

Besides, metagenomics analysis was done also for the reactor that was used to examine the short-term exposure of heavy metals. As a result of NGS analysis, the most abundant phylum in the reactor *Planctomycetes* phylum with an abundance of 25%. The order of the other four dominant phyla; *Chloroflexi* (20%), *Ignavibacteriae* (19,8%), *Proteobacteria* (16,5%) and, *Parcubacteria* (9 %).

The analysis indicates that the relative abundance of *Candidatus Kuenenia* is 20% of the all bacterial population which is the most dominant species among the *Planctomycetes* phylum.

4. CONCLUSION

The primary aim of this thesis is to examine the inhibitory effect of some heavy metals on Anammox biomass by evaluating the fractions that may be bioavailable to Anammox bacteria. The study highlights that the inhibitory effect of metals should not be examined directly without paying attention to speciation properties of each metal. Expressing inhibitory concentrations in terms of total heavy metal is of little use because not all of the fractions of heavy metals are bioavailable. Starting from this point, inhibitory levels are determined based on speciation.

In order to determine the short-term effect of Cu, Cd, Ni, and Zn batch tests were carried out using an increasing level of heavy metal concentrations. Results showed that the IC50 values for total applied, soluble, intracellular, cell-associated, surface-bound, and free-ion Cd concentrations are 12.14, 6.50, 1.25, 2.94, 1.07, and 4.07 mg L^{-1}, respectively. The IC 50 values of Cu are 4.57, 2.43, 1.16, 1.38, 1.16, and 0.152 for total applied, soluble, intracellular, cell-associated, and surface-bound Cu concentrations. Since the free ion concentration of Cu was calculated too low, it was assumed that free Cu ion concentration had no significant inhibitory effect over Anammox system. The inhibitory effect of Ni with IC50 values for total applied, soluble, intracellular, cell-associated, surface-bound, and free-ion Ni concentrations are 7.78, 5.99, 0.250, 0.930, 0.680, and 1.36 mg L^{-1}, respectively. The IC50 values for total applied, soluble, intracellular, cell-associated, surface-bound, and free-ion Zn concentrations are 23.6, 6.76, 11.9, 15.1, 4.82, and 2.71 mg L^{-1}, respectively.

Our findings showed that the inhibitory order of metals was Cu>Cd>Ni>Zn in terms of applied concentration and soluble fraction. This order can be given for intracellular concentrations as Ni>Cd>Cu>Zn and for cell-associated fraction Ni>Cu>Cd>Zn.

The discrepancy between the reported IC50 values could be surmounted to a significant extent when IC50 values are given as genera-specific and also based on bioavailable fractions.

The long-term impact of Cu and Cd in elevated concentration on nitrogen removal rate has been investigated. In literature, most studies have only tended to focus on the inhibitory effect of Cd on the Anammox system for short-term exposure. The prolonged

exposure effect of Cd has not been studied up to now. The half maximal inhibitory level for long-term exposure of Cd was calculated as 6.75 mg L^{-1} (R^2: 0.81 according to 95% confidence interval) with the modified non-competitive inhibition model based on removal efficiency of nitrogen. The results demonstrated that 50% inhibition was observed at a lower concentration of Cd at prolonged exposure. One possible explanation for this findings might be related with the relative abundance of the Anammox species during the exposure. Besides, it can be considered that since cadmium not an essential metal for Anammox bacteria it may cause more detrimental effect in the case of prolonged exposure. The reason of that may be related with the uptake system of Cd. Since uptake rate of Cd is too slow, short-term responses may lead to wrong outcomes. This might be the significant reason to observe a higher inhibitory effect during the prolonged exposure of Cd.

Although the short-term effects of Cu were mostly examined in the previous studies long-term effects were also concerned in several studies. The results indicated an IC50 value for Cu was calculated as 6.77 mg L^{-1} (R^2: 0.95 according to the 95% confidence interval) which was higher than the value that found in short-term exposure (4.57 mg L^{-1}). The reason for the discrepancy between short-term and long-term inhibition most probably is related to the acclimation and bacterial adaptation.

Moreover, IC50 values obtained by short terms experiments can also be considered as genera-specific because response or resistance to inhibition may vary depending on the dominant population. Before starting the inhibition experiments, the relative abundance of *Candidatus Kuenenia* constitutes 25 % of the entire bacterial population according to metagenomics analysis which is also consistent with the qPCR results. A substantial number of the bacterial population comprises heterotrophic bacteria, such as Ignavibacteria (30.8%) and Anaerolineae (7.18%) was also found according to NGS analyses. According to real-time PCR results, relative abundances of Anammox bacteria shows some fluctuations during the exposure depending on self-adaptation to copper. After a long exposure period, the relative abundance of Anammox bacteria showed a tendency to decrease.

qPCR results illustrated that the relative quantity of Anammox started to decrease to 24% from 46 % in agreement with these result the relative abundances of *Candidatus Kuenenia*

was found 26% of all the bacterial community after 2.75 mg L^{-1} Cd exposure. Additionally, Candidatus *Brocadia* was observed that had not been observed before which accounted for 7% of the all bacterial population at that dosage. Thus, it can be concluded that there might be sudden decrease at the relative percentage of *Candidatus Kuenenia* between 2.75 and 3.5 mg L^{-1} of Cd exposure.

The following recommendations are made for future studies based on our findings;

1. In the present study, long-term exposure effect of heavy metals could only be carried out with cadmium and copper, IC50 values determined for nickel and zinc for only short-term exposure. Further studies are needed to understand inhibition levels during long-term exposure.

2. It is possible to meet with a shock loading problems in the wastewaters which are suitable to apply Anammox process. In the scenario of the shock loading of heavy metals in full-scale treatment plant, it is very critical to take necessary precautions to recover from inhibition. Therefore recovery of Anammox performance after inhibition by heavy metals should be investigated in detailed.

3. In the current study the inhibitory effect of Cd, Cu, Zn and Ni were examined individually. In real case, however, the wastewater may contain two or more of these substances together. Therefore, in order to develop a control strategies the avoidance of the inhibition in full-scale Anammox process, combined effect of these heavy metal should be investigated in detail.

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6. APPENDIXES

6.1. APPENDIX A

Table A. 1 Comparison of inhibition models

		Applied heavy metal			Soluble			Intracellular		
Metal	Model	IC50	Confidence Range	R^2	IC50	Confidence Range	R^2	IC50	Confidence Range	R^2
Cu	Noncomp.	4.43	3.57-5.52	0.91	2.41	1.99-2.91	0.91	0.12	0.10-0.15	0.91
	Modified non.comp	4.57	3.61-5.95	0.93	2.35	2.02-2.77	0.92	0.12	0.11-0.13	0.93
	Linear	6.09	1.34-10.40	0.5	2.51	17.0-22.8	-	0.13	255-466	0.97
	4-PL	-	very wide	0.94	-	very wide	0.94	-	very wide	0.94
Cd	Noncomp.	11.28	8.49-14.92	0.78	6.02	4.20-8.65	0.61	2.37	1.59-3.39	0.84
	Modified non.comp	12.14	10.99-13.34	0.93	6.578	6.32-6.84	0.95	1.26	0.57-2.30	0.88
	Linear	14.08	2.32-4.50	0.91	3.80	6.08-13.07	0.88	5.05	3.27-38.08	0.83
	4-PL	14.72	10.35-50.92	0.93	6.50	5.89-8.01	0.94	-	very wide	0.94
Ni	Noncomp.	7.82	6.55-9.35	0.90	6	5.00-7.21	0.90	0.25	0.21-0.30	0.90
	Modified non.comp	7.53	6.75-8.52	0.93	5.77	5.16-6.66	0.92	0.25	0.21-0.32	0.90
	Linear	7.78	5.77-7.09	0.97	5.99	5.27-10.65	0.97	0.28	0.16-1.27	0.85
	4-PL	5.23	3.69-∞	0.92	3.85	2.93-∞	0.92	0.16	0.12-0.32	0.93
Zn	Noncomp.	26.1	21.8-31.4	0.90	7.81	6.24-9.86	0.84	11.24		0.8
	Modified non.comp	23.6	21.9-25.6	0.96	6.76	6.37-7.22	0.96	9.57	8.93-10.42	0.92
	Linear	24.6	1.53-2.05	0.98	6.22	4.54-9.84	0.96			0.9
	4-PL	127	very wide	0.97	-			11.25		0.92

Table A.1 Continue

		Cell-associated			Surface bound			Free ion		
Metal	Model	IC50	Confidence Range	R^2	IC50	Confidence Range	R^2	IC50	Confidence Range	R^2
Cu	Noncomp.	0.42	0.25-0.78	0.66	0.65	0.42-1.10	0.93	0.013	0.007-0.022	0.62
	Modified non.comp	0.72	0.46-1.21	0.93	0.65	0.42-1.09	0.93	0.009	0.004-0.025	0.89
	Linear	2.62	-5.06-23.7	0.59	2.52	-5.54-24.63	0.57	0.02	-93.5-3072	0.75
	4-PL	0.32	-9.20-9.06	0.93	0.17	-8.90-8.67	0.93	-	very wide	0.91
Cd	Noncomp.	4.01	2.93-5.40	0.87	1.33	0.86-1.97	0.77	2.64	2.05-3.39	0.84
	Modified non.comp	2.935	1.68-4.67	0.88	1.07	0.57- 1.76	0.81	2.84	2.51-3.20	0.93
	Linear	7.74	1.37-38.61	0.82	2.73	3.17-17.11	0.68	3.57	7.96-16.23	0.89
	4-PL	-	very wide	0.94	-	very wide	0.83	4.07	2.45-58.71	0.93
Ni	Noncomp.	0.91	0.76-1.09	0.91	0.65	0.53-0.79	0.9	1.72	1.45-2.03	0.92
	Modified non.comp	0.93	0.75-1.20	0.92	0.68	0.53-0.92	0.92	1.68	1.47-1.98	0.92
	Linear	1.14	0.56-?	0.77	0.86		0.74	1.77	-	0.95
	4-PL	0.44	0.28-∞	0.93	0.29		0.93	1.1	-8.97-8.88	0.95
Zn	Noncomp.	16.03	14.2-18.1	0.96	4.66	3.17-6.90	0.75	2.71	2.49-2.98	0.97
	Modified non.comp	15.08	14.1-16.2	0.98	4.82	2.43-10.0	0.79	2.71	2.49-2.98	0.96
	Linear	16.5	1.80-3.67	0.96	7.82	-0.49-8.31	0.73	2.86	15.3-19.7	
	4-PL	15.91	9.33-∞	0.98	2.76	3.10-18.8	0.89	-	very wide	0.96

6.2. APPENDIX B

Table B. 1 K_f values of heavy metal complexes

Species	Log K	Species	Log K	Species	Log K	Species	Log K
$Cd(OH)_4^{-2}$	-47,29	$Zn(CO_3)_2^{-2}$	7.30	$Ni(NH_3)_2^{+2}$	-13.61	$Cu(CO_3)_2^{-2}$	10...20
$Cd(OH)_3^-$	-33.30	$Zn(H_2BO_3)_2$ (aq)	-14.40	$Ni(NH_3)_3^{+2}$	-21.19	$Cu(H2BO_3)_2$ (aq)	-11..59
$Cd(NH3)_4^{+2}$	-30.26	$Zn(NH_3)_2^{+2}$	-13.99	$Ni(NH_3)_4^{+2}$	-29.31	$Cu(NH_3)_2^{+2}$	-11.09
$Cd(NH3)_3^{+2}$	-21.83	$Zn(NH_3)_3^{+2}$	-20.87	$Ni(NH_3)_5^{+2}$	-37.89	$Cu(NH_3)_3^{+2}$	-17.53
$Cd(OH)_2$ (aq)	-20.29	$Zn(NH_3)_4^{+2}$	-28.09	$Ni(NH_3)_6^{+2}$	-47.17	$Cu(NH3)_4^{+2}$	-24.68
$Cd(H_2BO3)2$ (aq)	-15.01	$Zn(NO_2)_2$ (aq)	1.20	$Ni(NO_2)_2$ (aq)	1.79	$Cu(NO_2)_2$ (aq)	3.03
$Cd(NH3)2^{+2}$	-13.93	$Zn(NO_3)_2$ (aq)	-0.30	$Ni(OH)_2$ (aq)	-18.99	$Cu(NO3)_2$ (aq)	-0.40
$CdOH^+$	-10.10	$Zn(OH)_2$ (aq)	-16.89	$Ni(OH)_3$-	-29.99	$Cu(OH)_2$ (aq)	-16.23
Cd_2OH^{+3}	-9.40	$Zn(OH)_3^-$	-28.39	$Ni(SO_4)_2^{-2}$	0.82	$Cu(OH)_3^-$	-26.64
$CdH_2BO_3^+$	-7.32	$Zn(OH)_4^{-2}$	-41.19	$NiCl^+$	-0.43	$Cu(OH)_4^{-2}$	-39.73
$CdNH_3^{+2}$	-6.69	$Zn(SO_4)_2^{-2}$	3.28	$NiCl_2$ (aq)	-1.89	$Cu_2(OH)_2^{+2}$	-10.49
$Cd(NO_3)_2$ (aq)	0.20	Zn_2OH^{+3}	-9.00	$NiCO_3$ (aq)	4.57	Cu_2OH^{+3}	-6.71
$CdNO3^+$	0.50	ZnCl+	0.46	$NiEDTA^{-2}$	20.11	$Cu_3(OH)_4^{+2}$	-20.79
$CdNO2^+$	1.95	$ZnCl_2$ (aq)	0.45	NiH_2EDTA (aq)	24.74	CuCl+	0.30
$CdCl^+$	1.98	$ZnCl_3$-	0.50	NiH_2PO_4+	20.50	$CuCl_2$ (aq)	-0.26
$CdSO_4$ (aq)	2.37	$ZnCl_4$-2	0.20	$NiHCO_3$+	12.42	$CuCl_3$-	-2.29
$CdCl_2$ (aq)	2.60	$ZnCO_3$ (aq)	4.76	NiHEDTA-	23.64	$CuCl_4$-2 1	-4.59
$Cd(SO_4)_2^{-2}$	3.50	ZnEDTA-2	18.00	$NiHPO_4$ (aq)	15.33	$CuCO_3$ (aq)	6.77
$Cd(NO_2)_2$ (aq)	3.54	ZnH_2BO_3+	-7.84	$NiNH_3^{+2}$	-6.52	$CuEDTA^{-2}$	20.49
$CdOHEDTA^{-3}$	4.29	ZnH_2EDTA (aq)	22.83	$NiNO_2$+	1.38	$CuH_2BO_3^+$	-5.26
$CdCO_3$ (aq)	4.37	$ZnHCO_3$+	11.83	$NiNO_3$+	0.40	CuH_2EDTA (aq)	26.23
$Cd(CO_3)_2^{-2}$	7.23	ZnHEDTA-	21.43	NiOH+	-9.90	$CuHCO_3^+$	12.13
$CdHCO_3^+$	11.83	$ZnHPO_4$ (aq)	15.69	$NiOHEDTA^{-3}$	7.56	$CuHEDTA^-$	24.02
						CuHPO4 (aq)	16.50
$CdHPO_4$ (aq)	16.08	$ZnNH_3$+2	-7.03	$NiSO_4$ (aq)	2.30	CuHSO4+	2.34
$CdEDTA^{-2}$	18.10	$ZnNO_2$+	0.78			CuNH3+2	-5.22
$CdHEDTA^-$	21.43	$ZnNO_3$+	0.40			CuNO2+	2.02
CdH_2EDTA (aq)	23.23	ZnOH+	-9.00			CuNO3+	0.50
		ZnOHEDTA-3	5.76			CuOH+	-7.50
		$ZnSO_4$ (aq)	2.34			CuOHEDTA-3	8.44
						CuSO4 (aq)	2.36

6.3. APPENDIX C

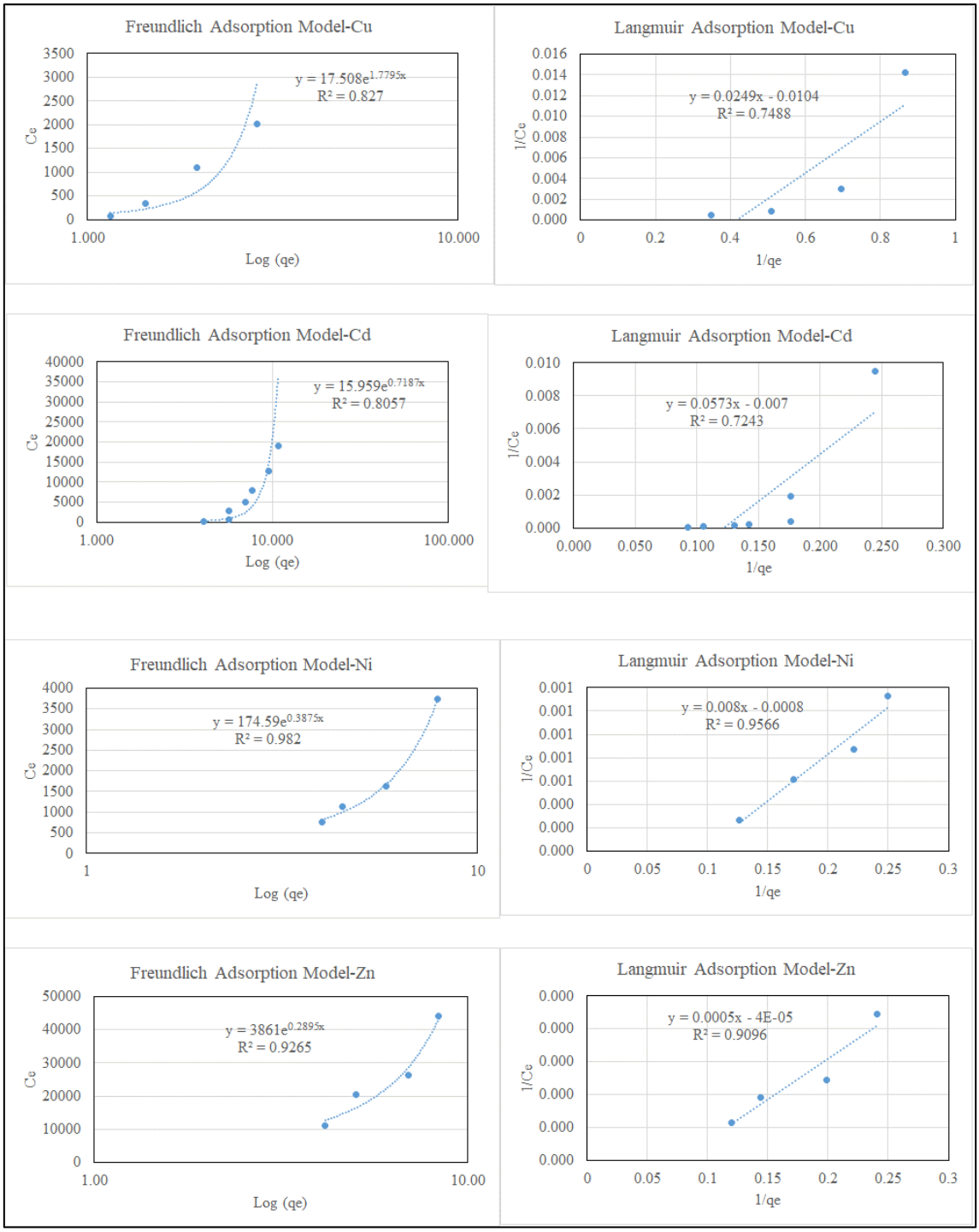


Figure C. 1 Applied isotherm models for each metal

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1. Identification of pathogens in wastewater treatment plant effluents using Real-Time PCR method, TÜBİTAK Project, 113Y304, Scholarship student, 1.08.2013-1.07.2015.

2. Examination of chemical risks of water reuse in the scenario of agricultural irrigation, Marmara University Scientific Research Projects Committee (BAPKO), FEN-C-DRP110913-0382, Researcher, 11.9.2013-11.09.2015

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List of Publications:

Papers in SCI journals

1. **Aktan C.K**, Uzunhasanoğlu E.A, Yapsakli K., "Speciation of nickel and zinc, its short-term inhibitory effect on Anammox, and the associated microbial community composition", Bioresource Technology, Volume 268, November 2018, Pages 558-567.

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1. **Aktan C.K.,** Uzunhasanoğlu E.A, Yapsakli K., Mertoglu B., Short-Term and Long-Term Inhibitory Effects of Copper on Anammox Process, 3rd EWAS International Conference, Lefkada Island, Greece, 27-30 June 2018.

2. **Aktan C.K.,** Yapsakli K., Mertoglu B.,, Evaluation of the Short and Long Term Effects of Cd on Anammox activity, 10th IWA Young Water Professionals Conference, Zagreb, Croatia, 7-12 May 2018.

3. Uzunhasanoğlu E.A.,**Aktan C.K.,** Yapsakli K., Mertoglu B., ICOCEE 2017 2nd International Conference of Civil and Environmental Engineering Conference "Long Term Effects of Cadmium on Anammox Process", Kapadokya, Turkey,8-10 Mayıs 2017.

4. **Aktan C.K.,** Yapsakli K., Mertoglu B., ICHE'2014 11th International Conference on Hydroscience & Engineering konferansı dahilinde,"Impacts of Reclaimed Water Irrigation on Heavy Metal Distribution in Soil Properties and Crop", Hamburg, Almanya 28 Eylül-2 Ekim 2014.

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6. **Kalkan C.,** Yapsakli K., Mertoglu B., International Sustainable Water and Wastewater Management Symposium konferansı dahilinde "International Sustainable Water and Wastewater Management Symposium" bildiri kitapçığındaki "Enrichment of Anammox bacteria in reactor packed with Kaldnes Rings", - pp., Konya,Türkiye 26-28 Ekim 2010.

7. K. Yapsakli, **C. Kalkan,** D. Tufan, B. Mertoglu, IWA World Water Congress and Exhibition konferansı dahilinde "IWA World Water Congress and Exhibition" bildiri kitapçığındaki "Tertiary treatment of Paşaköy Advanced Wastewater Treatment Plant by Biological Activated Carbon (BAC)", Manuscript Number: IWA-3359 pp., Montreal, Kanada, 19-24.Eylül.2010

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1. CHEM 101 General Chemistry I
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