ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DETERMINATION OF THE ROLE OF AMMONIA OXIDIZING ARCHAEA IN WASTEWATER TREATMENT PLANTS VIA MOLECULAR TECHNIQUES

Ph.D. THESIS

Halil KURT

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

AMONYAĞI OKSİTLEYEN ARKE LERİN ATIKSU ARITIMINDAKİ ROLÜNÜN MOLEKÜLER TEKNİKLERLE BELİRLENMESİ

DOKTORA TEZİ

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MART 2013

Halil KURT, **a Ph.D.** student of ITU **Graduate School of Science Engineering and Technology** student ID 521072034, successfully defended the **thesis** entitled "**Determination Of The Role Of Ammonia Oxidizing Archaea In Wastewater Treatment Plants Via Molecular Techniques**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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December 2012 Halil KURT
(Biologist)

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ABBREVIATIONS

amoA	**:** ammonia monooxygenase subunit A
AOA	**:** Ammonia-oxidizing archaea
AOB	**:** Ammonia-oxidizing bacteria
App	**:** Appendix
Blast	**:** Basic local alignment search tool
Ct	**:** Cycle threshold
DGGE	**:** Denaturing gradient gel electrophoresis
DNA	**:** Deoxyribonucleic acid
dNTPs	**:** Deoxynucleoside triphosphates
FISH	**:** Florescence in situ hybridization
HGT	**:** Horizontal gene transfer
IBEM	**:** Istanbul Teknik Universitesi, Biotechnology and Environmental Microbiology Group
ISTAC	**:** Istanbul cop sızıntı suyu atık arıtma tesisi
LACA	**:** Last archaeal common ancestor
LBA	**:** Long branch attraction
LSU	**:** Large small subunit
LUCA	**:** Last universal common ancestor
PAKM	**:** Pakmaya wastewater treatment plant
PCR	**:** Polymerase chain reaction
QPCR	**:** Quantitative Real-Time Polymerase chain reaction
RNA	**:** Ribonucleic acid
RNAP	**:** RNA polymerase
SSU rRNA	**:** Small subunit ribosomal RNA sequences
Tm	**:** Melting temperature

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DETERMINATION OF THE ROLE OF AMMONIA OXIDIZING ARCHAEA IN WASTEWATER TREATMENT PLANTS VIA MOLECULAR TECHNIQUES

SUMMARY

Ammonia is converted to nitrite by ammonia-oxidizing organisms and this process is often the rate-limiting step of nitrification. Ammonia monooxygenase (AMO) is one of the key enzymes responsible for ammonia oxidation and amoA gene is frequently used for DNA fingerprinting. It had been known that only ammonia oxidizing bacteria (AOB) and anaerobic ammonia oxidizing bacteria (anammox) oxidized the ammonia.

Recent studies showed that Ammonia oxidation is also performed by ammonia oxidizing archaea (AOA). Archaeal amoA gene is much more abundant and active than ammonia oxidizing bacteria (AOB) in various environmental samples. AOA might also play an important role in biological nitrogen removal reactors of waste water treatment plants.

Accordingly, in this study, activated sludge samples were collected from 16 different domestic and industrial wastewater treatment plants where nitrification was active, including petroleum and oil refineries; food, alcohol and chemical industries and landfill leachate treatment plants. The presence of AOA and AOB in engineered systems was investigated using two primer sets specific for archaeal and bacterial amoA gene sequences via quantitative real time PCR.

It was found that the comparative abundance of AOA and AOB amoA genes varied among the WWTPs with regard to the ammonium concentrations in the effluent wastewater of the WWTPs. Surprisingly in almost all of the samples, AOA occurrence has been showed within the 10^3 - 10^8 copy numbers. Significant numbers of AOA amoA genes occurred in all municipal WWTPs suggesting the potential role of AOA in autotrophic ammonia oxidation in WWTPs receiving low ammonium influent. It has been confirmed that conventional parameters such as NH3 concentration might be a good tool to predict AOA occurrence in engineered systems The finding of this study may change our understanding on nitrogen removal in WWTPs. AOA must also be considered as another microorganism potentially involved in nitrogen removal in WWTPs. Further studies should clarify the role of AOA and AOB in autotrophic oxidation of ammonia in WWTPs. This may be done by employing a combination of in situ activity investigations and molecular tools

As a result, AOA has been found in industrial engineered systems, whereas it may not be considered as a potential nitrifier in wastewater treatment plants.

AMONYAĞI OKSİTLEYEN ARKE LERİN ATIKSU ARITIMINDAKİ ROLÜNÜN MOLEKÜLER TEKNİKLERLE BELİRLENMESİ

ÖZET

Amonyak amonyağı-oksitleyen organizmalar tarafından nitrite dönüştürülür ve bu reaksiyon nitrifikasyonun hız belirleyen adımını oluşturur. Amonyak monooksijenaz (AMO) amonyak oksidasyonundan sorumlu enzimdir ve DNA dizilemesinde sıklıkla kullanılır. Yakın zamana kadar amonyağı sadece amonyak oksitleyen bakteriler ve anaerobik amonyak oksitleyen bakterilerin (anammox) oksitleyebildiği biliniyordu.

Son zamanlardaki çalışmalar, amonyağı oksitleyen Arkerin de amonyağın oksitlenmesinde rol aldıkları gösterilmiştir. Arkelere ait amoA geni birçok çevre örneklerinde amonyağı oksitleyen bakterilere göre daha fazla ve aktif olarak bulunmaktadır. Amonyak oksitleyen Arkeler (AOA) ayrıca atık su arıtma tesislerindeki biyolojik nitrojen gideriminde de önemli rol oynayabilmektedir.

Bu çalışmada, Petrol, yağ, gıda, alkol, kimyasal endüstri ve çöp sızıntı suyu atıksu arıtması gibi, onaltı farklı evsel ve sanayi atık su arıtma tesisinden örnekler toplanmıştır. AOA ve Amonyak oksitleyen bakterilerin (AOB) varlığı arkeal ve bakteriyel amoA genine özgü iki primer çifti kullanılarak kantitatif realtime PZR yöntemi ile çalışılmıştır. Ayrıca Arkeal Amo A geni klonlanarak dizilenmiş ve filogenetik ağaç elde edilmiştir.

Atıksu arıtma tesislerindeki amonyum konsantrasyonlarına bağlı olarak tesisler arasında AOA ve AOB miktarlarının farklılaştığı bulunmuştur.

DNA dizi analizi sonrası, AOA ların büyük bir kısmının deniz grubunu içeren (Grup 1.1a) kümesine dahil olduğu saptanmıştır. Küçük bir grup AOA ise toprak kümesine (Grup 1.1a) ait çıkmıştır. Diğer bir küme olan termofilik AOA kümesine ait Arkeler tespit edilememiştir.

Saf kültür çalışmaları, amonyak miktarı ve oksijen içeriğinin AOA varlığını ve kominite yapısını etkileyen en önemli iki faktör olduğunu göstermektedir. Fakat, bu iki substratı AOB'de kullandığından bu iki grup arasında atıksu arıtma tesislerinde oksijen ve amonyak için yarışma vardır.

Tüm örneklerde AOA varlığı 10^3 - 10^8 gen kopya sayısı arasında gösterilmiştir. Düşük amonya miktarlarına sahip evsel atık arıtma tesislerinde AOA amoA gen kopya sayısının yüksek çıkması bu tesislerde AOA'nın potansiyel ototrofik amonyak oksidasyonunda rol alabileceğini göstermektedir. NH_3 konsantrasyonu gibi konvansiyonel parametrelerin atıksu arıtma tesislerinde AOA varlığının tespitende kullanılabileceği bulunmuştur. Bu bulgu atıksu arıtma sistemlerinde AOA'nın nitrojen gideriminde rol alabileceğini düşündürmektedir.

Arkeal amoA gen kopyası hücre başına 1 kopya iken bakteriyel amoA gen kopyası 2,5' dur. Bu bilgi göz önünde bulundurulduğunda AOB kopya sayısı sadece amonyak konsantrasyonu en yüksek olan ISTAÇ ve Pakmaya atıksu arıtma

örneklerinde AOA kopya sayısını geçmektedir. Arkeal amonyak mono-oksigenaz amonyaga karşı bakteriyel enzinden daha yüksek afiniteye sahiptir. Genellikle AOA amonyak konsantrasyonunun düşük olduğu ortamlarda daha fazla bulunmaktadır. Bu durum bizim çalışmamızla da uyumludur.

AOB ve AOA tüm örneklerde bulunmasına rağmen, atıksu arıtma tesislerinde nitrifikasyona birlikte katkı sağlamaktadırlar. AOA düşük amonyak derişimine sahip tesislerde baskın türler iken AOB yüksek amaonyak derişimine sahip tesislerde baskındır. Bu sonuç yeni literatürle de uyumludur.

Düşük amonyak derişimine sahip evsel atıksu arıtma tesislerinde önemli miktarda AOA varlığı bu tesislerde potansiyel amonyak oksitleyen organizma olduğunu göstermektedir. Bu bulgu atıksu arıtma tesislerinde amonyak giderimi üzerindeki bilgilerimizi değiştirebilir. Doğada AOA varlığı ve aktif olarak biyojeokimyasal çevrime katkıda bulunduğu rapor edilmiştir. Bu bilgi ışığında, AOA'nın atıksu arıtmada amonyak gideriminde rol alabileceği düşünülmelidir. AOA amoA gen ifade miktarlarının tespit çalışmaları ileride nitrifikasyonda AOA katkısını daha iyi bir şekilde ortaya koyabilir. amonyak konsantrasyonu ile AOA ve AOB miktarları rasındaki ilişkide hala aydınlatılmamış noktalar bulunmaktadır.

Aşırı düşük çözünmüş oksijen konsantarsyonları atıksu arıtma sistemlerinde AOA büyümesini teşvik edebilir, bununla birlikte, tam ters sonuçlar da elde edilmiştir. Çözünmüş oksijen düzeyi 3.25 mgl−1 olan atıksu arıtma tesisinde yüksek miktarlarda AOA varlığı saptanmıştır. Bazı çalışmalarda, istatistiksel analizler sonucu atıksu arıtma tesisi havalandırma havuzunda çözünmüş oksijen miktarı ile AOA miktarı arasında korelasyon bulunamamıştır. Bazı çalışmalar ise çözünmüş oksijen miktarının AOA varlığı üzerine etki eden en önemli etkenlerden birisi olduğunu belirtilmektedir. Çalışmalardaki bu tutarsızlıklardan dolayı çözünmüş oksijen miktarının AOA ve AOB miktarları üzerine etkisi hala tartışmalıdır.

Bizim çalışmamızda, AOB amoA gen miktarı kimyasal oksijen ihtiyacı ile pozitif olarak korelasyon göstermiştir (iki yönlü parametrik olmayan Spearman’s rank korelasyon etkin değeri < 0.05). Fakat AOA amoA gen miktarı kimyasal oksijen ihtiyacı ile negatif olarak korelasyon göstermiştir (iki yönlü parametrik olmayan Spearman’s rank korelasyon etkin değeri < 0.05). AOB amoA gen miktarı amonyak miktarı ile pozitif olarak korelasyon göstermiştir (iki yönlü parametrik olmayan Spearman’s rank korelasyon etkin değeri < 0.05), bununla beraber AOA amoA gen miktarı amonyak miktarı ile korelasyon göstermemiştir (iki yönlü parametrik olmayan Spearman’s rank korelasyon etkin değeri < 0.1933). Bu sonuçlara göre AOA ekotipleri çok esnek olabilir ve bazı AOA türleri miksotrofik olabilir.

Mußmann ve arkadaşları tarafından yapılan çalışma atıksu arıtma sisteminde AOA aktivitesinin ilk ve tek kanıtı olabilir. Bu çalışmada elli iki tesisin sadece dördünde yüksek miktarlarda AOA tespit etmişlerdir. Bir tesisi derinlemesine araştırdıklarında AOB'den on bin kat daha fazla AOA tespit etmişlerdir. Fakat yapılan modelleme çaılışması sonucunda bu tesisteki amonyak miktarı tüm amonyak oksitleyen organizmalara yetemeyeceği tespit edilmiştir. Sistemde bulunan AOA'ların sadece % 1'ine yetecek miktarda amonyak bulunmaktadır. Bu sebepte sistemde bulunan AOA'ların enerjilerinin tümünü nitrifikasyondan elde edip etmedikleri tartışma konusudur. Floresans in situ hibridizasyon ve 14C-inorganik karbon izotopu kullanılarak yapılan mikro-otoradyografi çalışmasında AOB'nin aksine AOA'lar kemo-ototrofik olarak aktif olmadıkları gösterilmiştir. Bu sonuç sonunda bu tesiste AOA'ların amonyak oksitlemedikleri sonucuna varılmıştır. AOA'lar muhtemelen

diğer metabolik yolları kullanarak atıksuda bulunan karbon ve enerji kaynağını kullanmaktadırlar.

AOA ve AOB'lerin atıksu arıtma sistemlerinde amonya giderimindeki rollerinin belirlenebilmesi için daha ileri çalışmalar yapılmalıdır. Sonuç olarak, atıksu arıtma sistemlerinde AOA varlığı tespit edilmiş fakat bu grup atıksu arıtma sistemlerinde potansiyel nitrifierlar olarak düşünülemeyebilir.

1. INTRODUCTION

1.1 Purpose of Thesis

The aim of this study is to investigate the occurrence and abundance of ammonia oxidizing archaea (AOA) in wastewater treatment plants (WWTPs). Despite AOA has been found widely in nature; there are very few studies showing the potential role of AOA in engineered systems. Thus; activated sludge samples taken from nitrification reactors of different full scale WWTPs were studied for AOA occurrence and abundance using molecular tools both qualitatively and quantitatively. DNA from activated sludge samples were extracted, and polymerase chain reaction (PCR) is used to amplify archaeal amoA gene fragments.

1.2 Literature Review

Microorganisms are the most abundant and diverse forms of life on the earth. Most of naturally occurring microorganisms are not cultivable in laboratory conditions called viable but not culturable; therefore our understanding of microbial diversity has been incomplete. This technical problem has been mostly overcome by the improvements in molecular studies. Phylogenetic analysis has changed our view of classification and allowed a better understanding in diversity mapping based on evolutionary history for Prokaryotes.

Molecular phylogenetic analysis elucidated by comparison of orthologous gene`s sequences from different organisms. A phylogenetic tree is a graphical representation of genetically derived, evolutionary relationships between organisms or molecules. Accurate alignment of sequences is critical for a correct tree drawing, so conserved sequences and structures are important landmarks for better sequence alignments (Pace, 2009).

No single gene has sufficient resolution throughout the tree of life. The appropriate molecule for such studies should have following characteristics; the gene must occur

in all organisms, reflect the cellular line of descent, not have undergone lateral gene transfer between different genetic lineages and be extraordinarily conservative.

The rRNA genes seem to fit the criteria for developing the first outlines of a universal tree of life better than any other genes (Woese, 1987). Small subunit of prokaryotic ribosomal RNA, (16S rRNA) gene sequences have become the gold standard for microbial identification and evolutionary relationships. SSU (small subunit) rRNA genes occur in all cells and organelles, and are one of the most conservative large sequences in nature. The rRNA gene studies have shown that this gene have not undergone significant lateral transfer, and the structural properties of the rRNA provide for optimization of alignments. Moreover, the 16S rRNA databases are the main source of information on environmental microbial diversity. 16S rRNA sequences vary by 30 to 35% between the main bacterial phyla and ~50% between the domains (Pace, 2009).

SSU RNA (rRNA) like 16S and 18S rRNA genes are the most widely used molecule for phylogenetic analysis. Both prokaryote and eukaryote could be classified by rRNA sequence (Barns et al., 1996). Carl R. Woese proposed three domains of life, Archaea, Bacteria and Eucarya based upon rRNA sequence comparisons (Woese et al., 1990). These results also opened a new way to understand and classify microbial diversity in nature. Gene sequence comparisons provided the metric, natural and objective way to classify organisms based on sequence change.

SSU rRNA gene has some limitations. Because of the very conservative nature of SSU rRNA, sequences may not be useful for discrimination of close relatives at the strain or even species level. The size of the gene, 1,500 to 2,000 bp, is another limitation to be able to explain one of the most diverse lieanages of the tree of life. Also; only almost half of the gene is variable. There is no direct correspondence between the frequencies of rRNA genes and the frequencies of organisms. Different organisms contain different numbers of rRNA genes. rRNA genes can vary from one to 15 copies (Klappenbach et al., 2001; Pace, 2009). The sequences of multi copy of 16S rRNA gene are mostly or nearly identical. But, some studies indicated the variations of 16S rRNA gene sequence within a single bacteria. Therefore, other conserved single copy genes could be used alternative for 16S rRNA phylogeny (Rajendhran and Gunasekaran, 2011).

2. ARCHAEA

2.1 Overview of the Archaeal Domain

Archaeal domain was discovered by Woese while working on methanogens (Woese at al., 1978). The archaea are prokaryotes. They are metabolically diverse organisms and coexisting in both terrestrial and aquatic, including extreme and normal environment. The archaea can live extreme conditions like high or low pH, low or high temperature, high salinity or pressure (Rothschild et al., 2001). The Archaea are so far the sole organisms capable of methanogenesis (methane production from H_2 and CO_2) (Gribaldo, 2006).

They can be either heterotrophs or autotrophs and those whom are photosynthetic occur based on bacteriorhodopsin. Their membrane glycerolipids consist of ethers of glycerol and isoprenol. They do not have a murein in their cell wall; on the other hand it is present in most bacterial cell wall (Gribaldo, 2006).

The Archaea phylogeny is generally determined by sequence comparisons of their 16S rRNA genes. Sequence comparisons of 23S rRNAs and other proteins could give similar tree topologies (Huber et al., 2003). We used amoA gene sequence for phylogenetic analysis.

The Archaeal domain was divided into five major phyla, Euryarchaeota, Crenarchaeota, Korarchaeota, Nanoarchaeota and Thaumarchaeota (Figure 2.1). 'Aigarchaeota' and "TACK superphylum" are proposed as new phyla, but yet their status is unclear. Thus, current status of high level systematic in the Archeae is as shown in table 2.1.

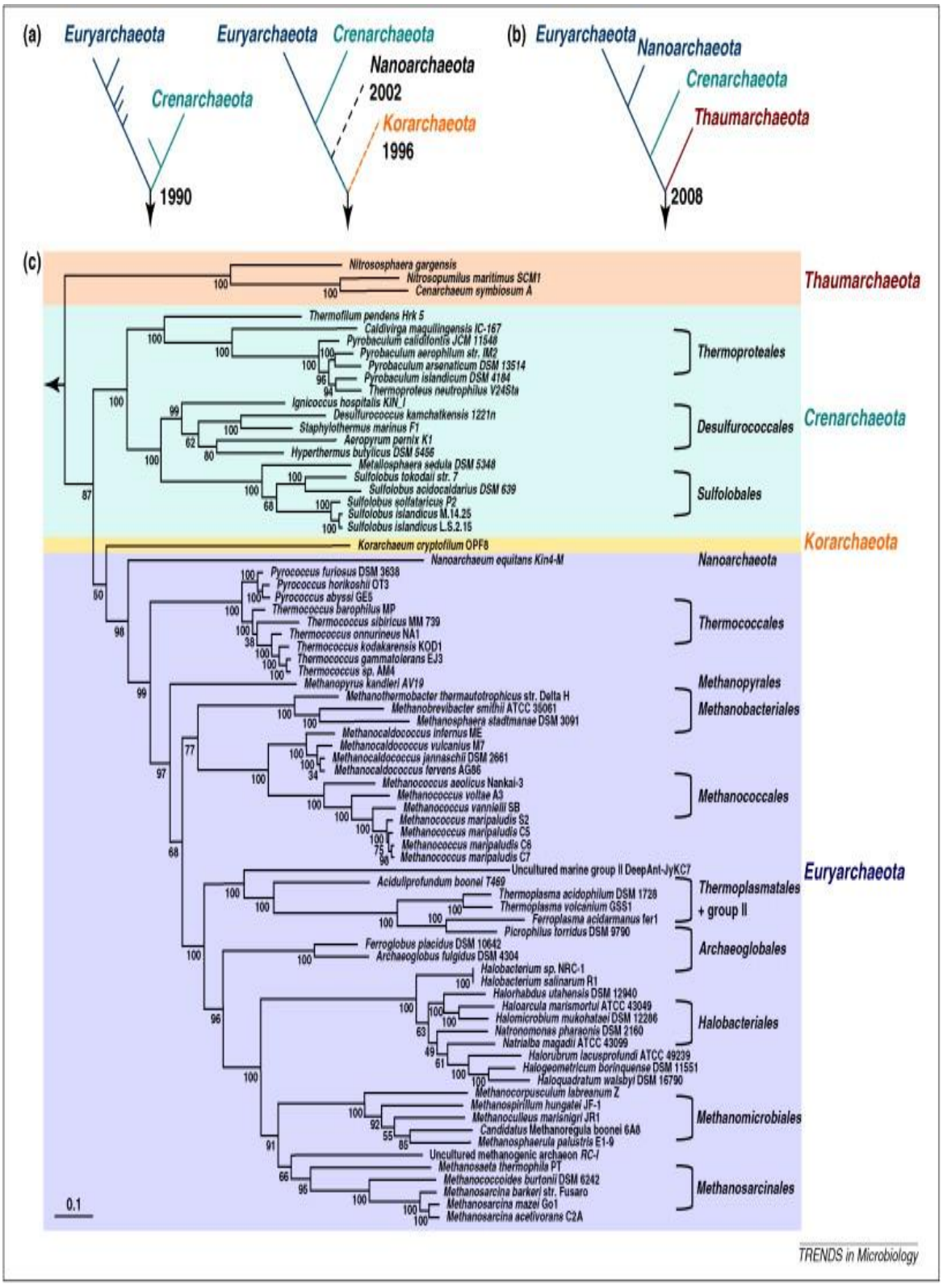


Figure 2.1 : Phylogeny of Archaea. (a) Schematic 16S rRNA trees demonstrating the view of archaeal phyla over the past two decades. (b) a concatenated data set of 53 ribosomal proteins (c) Rooted maximum likelihood tree of Archaea based on 53 concatenated ribosomal proteins (107 sequences, 4683 amino acid positions; eukaryotic sequences were used as an outgroup) (Spang et al., 2010).

Table 2.1 : Current status of high-level systematics in the Archaea (Gribaldo, 2012).

Phylum	Initial criteria	Number of representatives (isolates, enrichments, complete	Multigene phylogenies	Biological signature	Current status
Crenarchaeota (1990)	Phylogeny of 16S rRNA	>30	Distinct	Yes	Confirmed
Euryarchaeota (1990)	Phylogeny of 16S rRNA	>30	Distinct	Yes	Confirmed
Korarchaeota (1996)	Phylogeny of 16S rRNA	1	Distinct	Yes	Likely, more representatives are needed
Nanoarchaeota (2002)	Phylogenies of 16S rRNA ribosomal proteins	1	Within Euryarchaeota	Euryarchaeal	Euryarchaeal lineage
Thaumarchaeota (2008)	Biological signature, phylogeny of ribosomal proteins	3	Distinct	Yes	Confirmed
'Aigarchaeota' (2011)	Phylogenies of 16S rRNA, ribosomal proteins, and RNA polymerase subunits	1	Distinct	Similar to Thaumarchaeota	Unclear, more representatives are needed
TACK superphylum (2011)	Phylogeny of a few universal proteins		To be confirmed	No	Unclear

2.1.1 The Euryarchaeota & The Crenarchaeota

Formerly; Archaeal domain had splited into two main phyla based on 16S rRNA studies of Woese et al. (1990), the Euryarchaeota and the Crenarchaeota. The Euryarchaeota have morphologically, metabolicaly and physiologically more diverse species including thermoacidophiles, methanogens, extreme halophiles and hyperthermophiles; whereas the Crenarchaeota are less diverse including hyperthermophiles (Radhey et al., 2011).

The Euryarchaeota have been divided into nine orders, five orders for the methanogens (Methanobacteriales, Methanomicrobiales, Methanococcales, Methanosarcinales and Methanopyrales), one for the halophiles (Halobacteriales), one for the thermoacidophiles (Thermoplasmatales) and two for the hyperthermophiles (Thermococcales and Archaeoglobales) (Forterre et al., 2002).

2.1.2 The Thaumarchaeota

In 1992, new mesophilic sister group to hyperthermophilic Crenarcheoata was discovered from ocean surface waters (Fuhrman et al., 1992, Delong, 1992). This new group branched from Crenarchaeota and Euryarchaeota. Based on phylogenetic analysis, these organisms was proposed as the phylum Thaumarchaeota (Brochier-Armanet, 2008**)**. Thaumarchaeota, a mesophilic crenarchaeota, is widely distributed in oceans and soils; is an extremely diverse group considering crenarchaeota phylum (Ochsenreiter et al., 2003). Thaumarchaeota is the most abundant ammonia oxidizers in soil ecosystems and probably one of the most important participants in the global carbon and nitrogen cycles. (Schleper et al., 2005; Wuchter et al., 2006; Leininger et al., 2006) The first observation of archaeal nitrification reported by Konneke and colleagues shows that Candidatus *Nitrosopumilus maritimus*, a recently isolated mesophilic crenarchaeon, can grow chemolithoautotrophically by aerobically oxidizing ammonia to nitrite. (Konneke et al., 2005). Figure 2.2 showns the Phylogeny of ammonia-oxidizing Thaumarchaeota.

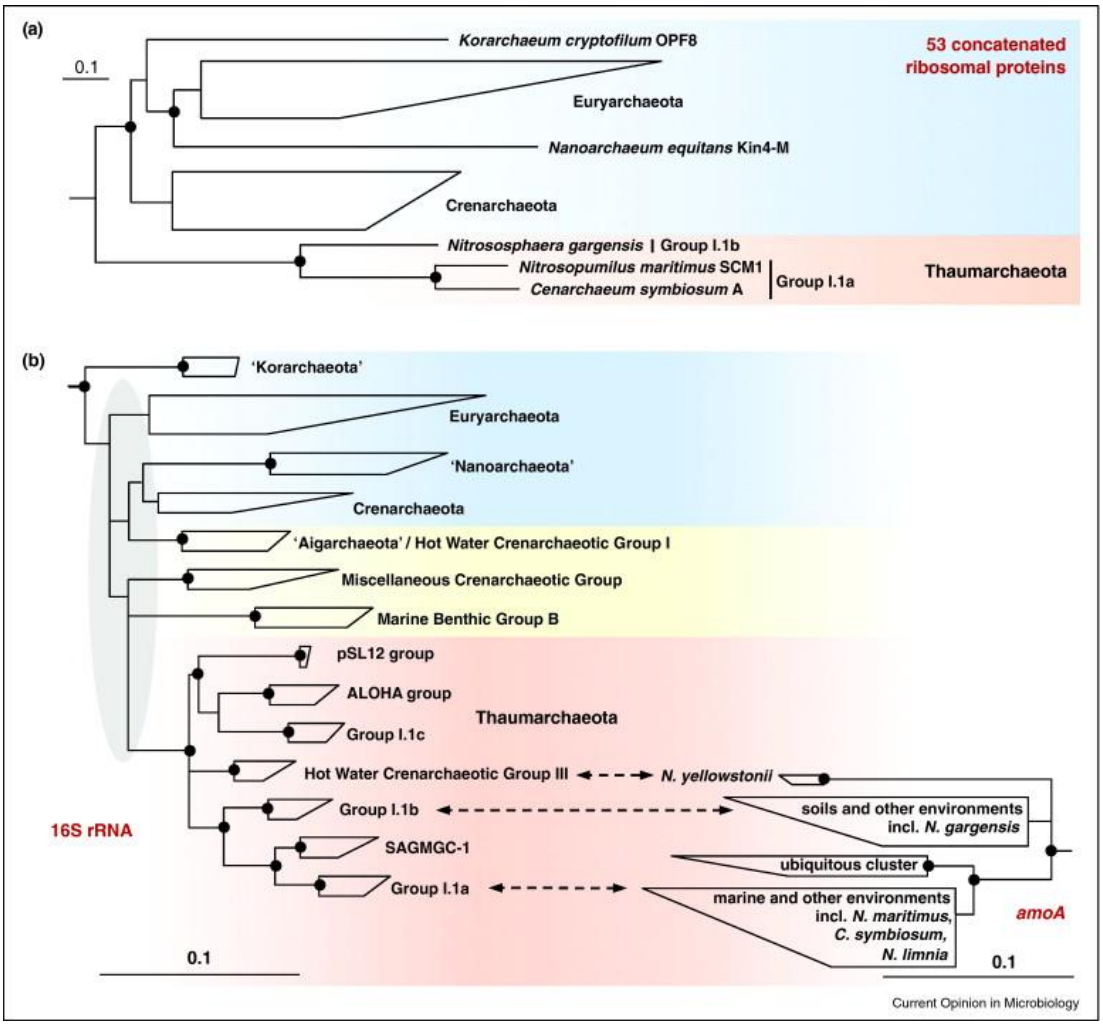


Figure 2.2 : Phylogeny of ammonia-oxidizing Thaumarchaeota. (a) Schematic phylogeny of Archaea redrawn after a rooted maximum likelihood tree of 53 concatenated ribosomal proteins of Archaea (4853 deduced amino acid positions), (b) Majority consensus trees based on the 16S rRNA gene (1067 nucleic acid positions conserved in >50% of all Archaea) and archaeal amoA gene (592 nucleic acid positions) as inferred by maximum likelihood, distance, and maximum parsimony methods. (Pester, 2011).

2.1.3 The Nanoarchaeota

This small archaea firstly isolated from marine hydrotermal system near Iceland which was one of the first member of novel archaeal phylum, called `the nanoarchaeota` (Huber et al., 2003)

Nanoarchaeum equitans was proposed to a new archaeal phylum based on trees that were produced using concatenated r-proteins (Waters et al., 2003) and SSU rRNA [Huber et al., 2002].

With a size of only 490,855 bp *N. equitans* is one of the smallest genomes today and has the smallest archaeal genome. *N. equitans* is the first parasitic archaea to be discovered, and can not survive without its host that belongs to the genus Ignicoccus [Huber et al., 2002].

Figure 2.3 shows the phylogenetic tree based on 16S RNA sequence comparisons, which was published by Huber and colleagues and it presents the Nanoarchaeota as a new phylum in archaeal domain [Huber et al., 2002].

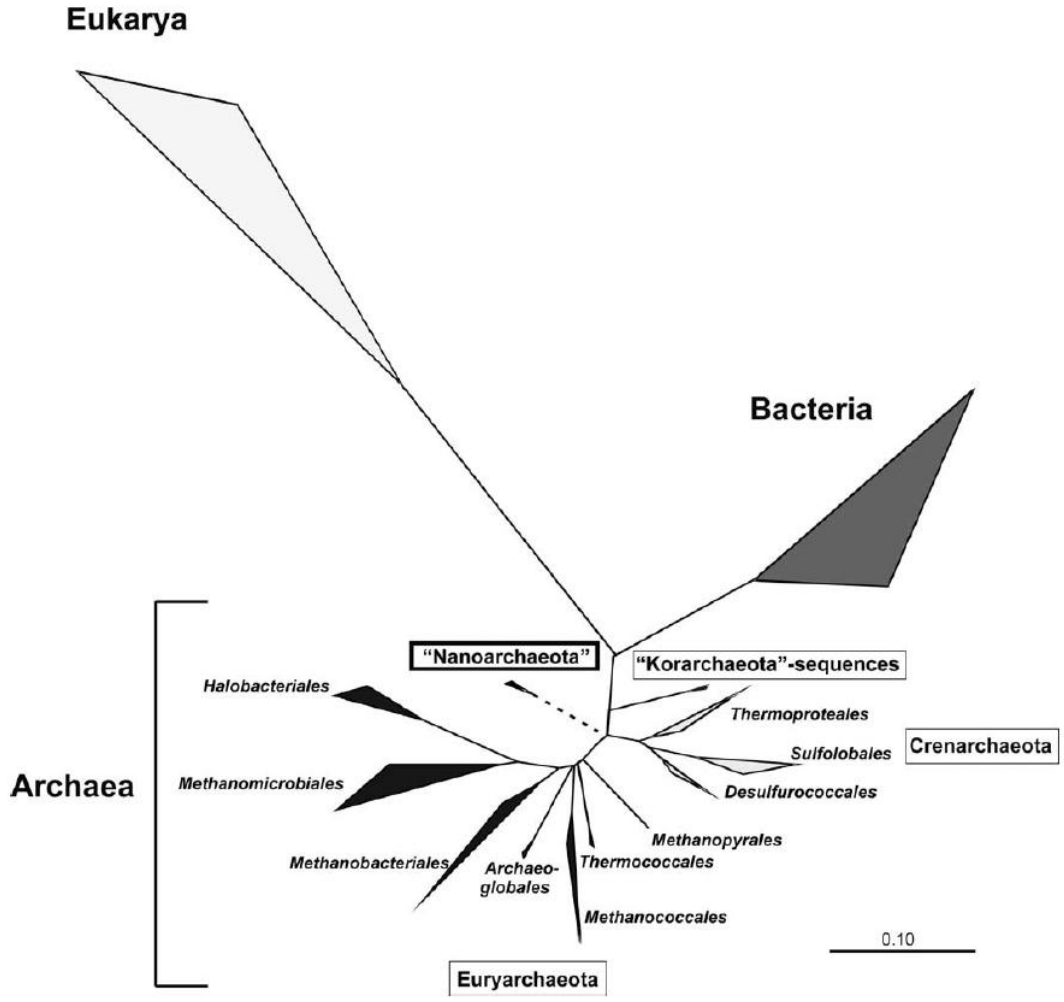


Figure 2.3 : Phylogenetic tree based on 16S rRNA sequence comparisons. Nanoarchaeota" outlines the uncertain position of the branching point (Huber et al., 2003).

2.1.4 The Korarchaeota

Korarchaeota is a recently proposed kingdom in Archaea (Barns et al., 1994, 1996). Korarchaeota has been detected in several geographically isolated terrestrial and marine thermal environments (Auchtung et al., 2006; Takai et al., 1999). A recent study from Schleper and colleagues also indicated the diversity and abundance of Korarcheota in terrestrial hot springs of Iceland and Kamchatka (Reigstad et al., 2010).Candidatus (Ca) *Korarchaeum cryptofilum* were enriched at 85°C by using a dilute organic medium and sediment samples from Obsidian Pool as an inoculum by Etkins et al., 2008. The organism was an ultrathin filament between 0.16 and 0.18 m in diameter and variable in length. They also obtained whole genome sequence of new species. Phylogenic tree of new phyla is shown Figure 2.4.

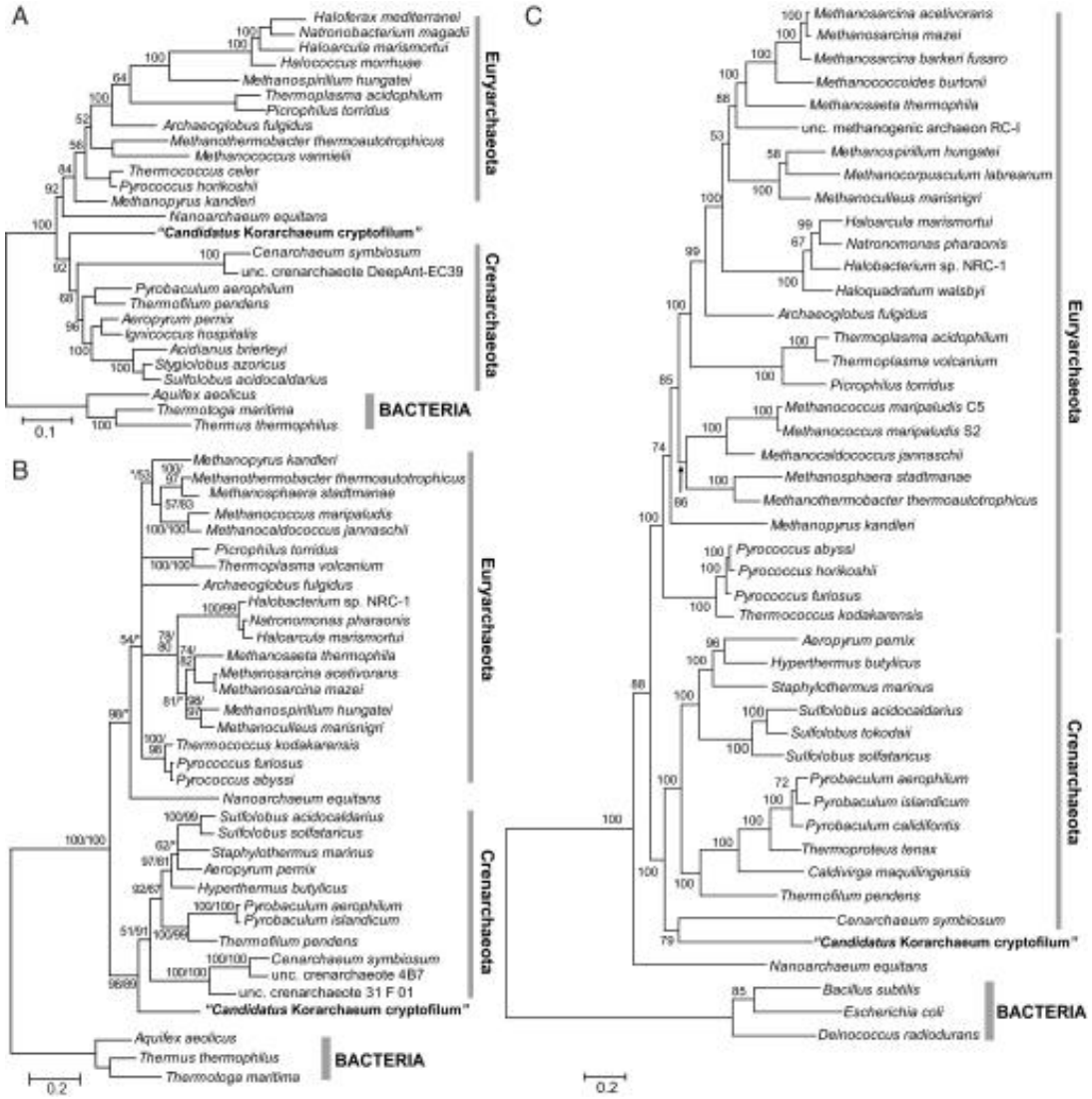


Figure 2.4 : Phylogenetic analysis of Ca. K cryptofilum. (A) Maximum-likelihood phylogenetic tree of combined (SSU + LSU) rRNAs rooted with corresponding bacterial sequences. (B) Archaeal phylogeny based on translation EF2 proteins rooted with bacterial homologs (C) Maximum-likelihood tree made from aligned sequences of 33 universally conserved ribosomal proteins and the three largest RNA polymerase subunits, RpoA, RpoB, and RpoD (Elkins, 2008).

2.2 NITROGEN CYCLE AND AMMONIA OXIDIZERS

Nitrogen is the fifth most abundant element in the earth and also essential for the synthesis of proteins and nucleic acids. Therefore, nitrogen is crucial for all living organisms. The biogeochemical cycle of nitrogen is dependent on oxidation - reduction (redox) reactions transformed primarily by microorganisms. Nitrogen takes nine different forms in soil corresponding to different oxidative states (Table 2.2).

Table 2.2 : Main Forms of Nitrogen in Soil and Their Oxidation States.

Name	Chemical formula	Oxidation state
Nitrate	NO_3^-	+5
Nitrogen dioxide (g)	NO_2	+4
Nitrite	NO_2^-	+3
Nitric oxide (g)	NO	+2
Nitrous oxide (g)	N_2O	+1
Dinitrogen (g)	N_2	0
Ammonia (g)	NH_3	-3
Ammonium	NH_4^+	-3
Organic	N_{RNH3}	-3

Nitrogen can be found in gas form (g) in the soil and atmosphere whereas dissolved in soil and water (Paul, 2007).

Dinitrogen gas (N_2) is the most abundant form of N in the biosphere however due to its inertness; it is unusable by most organisms, including plants. The nitrogen cycle can be divided into five steps: nitrogen fixation, assimilation, mineralization, nitrification and denitrification (Figure 2.5). The major biological nitrogen

transformation pathways and their associated enzymes (Table 2.3) are shown in Figure 2.6.

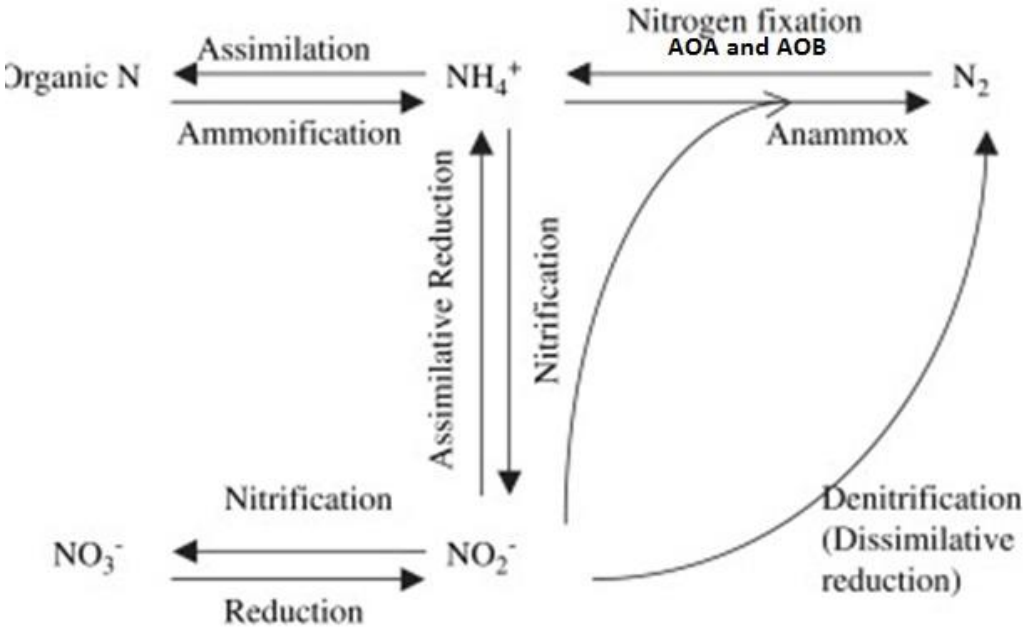


Figure 2.5 : Representation of of microbial nitrogen cycle (You et al. 2009).

Table 2.3 : Major enzymes involved in the nitrogen cycle and the reactions they catalyze (Jetten, 2009).

Process/enzyme	**Reaction**			**Equation number**	**$E^{0\prime}$ (V/e^-)**	**Location**
Nitritification						
Ammonia monooxygenase	$NH_4^+ + O_2 + H^+ + 2e^-$	→	$NH_2OH + H_2O$	1	0.73	Transmembrane
Hydroxylamine oxidoreductase	$NH_2OH + H_2O$	→	$NO_2^- + 5H^+ + 4e-$	2	−0.06	Periplasm
Nitratification/anammox						
Nitrite oxidoreductase	$NO_2^- + H_2O$	→	$NO_3^- + 2H^+ + 2e-$	3	−0.43	Membrane associated
Hydrazine hydrolase	$NH_4^+ + NO + 2H^+ + 3e^-$	→	$N_2H_4 + H_2O$	4	0.34	Anammoxosome
Hydrazine oxidoreductase	N_2H_4	→	$N_2 + 4H^+ + 4e-$	5	−0.75	Anammoxosome
Denitrification & dissimilatory nitrate reduction						
Nitrate reductase	$NO_3^- + 2H^+ + 2e^-$	→	$NO_2^- + H_2O$	6	0.43	Membrane associated, periplasm or cytoplasm
Nitrite reductase	$NO_2^- + 2H^+ + e^-$	→	$NO + H_2O$	7	0.34	Periplasm
Nitric oxide reductase	$2NO + 2H^+ + 2e^-$	→	$N_2O + H_2O$	8	1.17	Transmembrane
Nitrous oxide reductase	$N_2O + 2H^+ + 2e^-$	→	$N_2 + H_2O$	9	1.36	Periplasm
Dissimilatory nitrite reductase	$NO_2^- + 8H^+ + 6e^-$	→	$NH_4^+ + 2H_2O$	10	0.75	*

*Reported with the reducing activity both at the outside and inside of the cytoplasmic membrane.

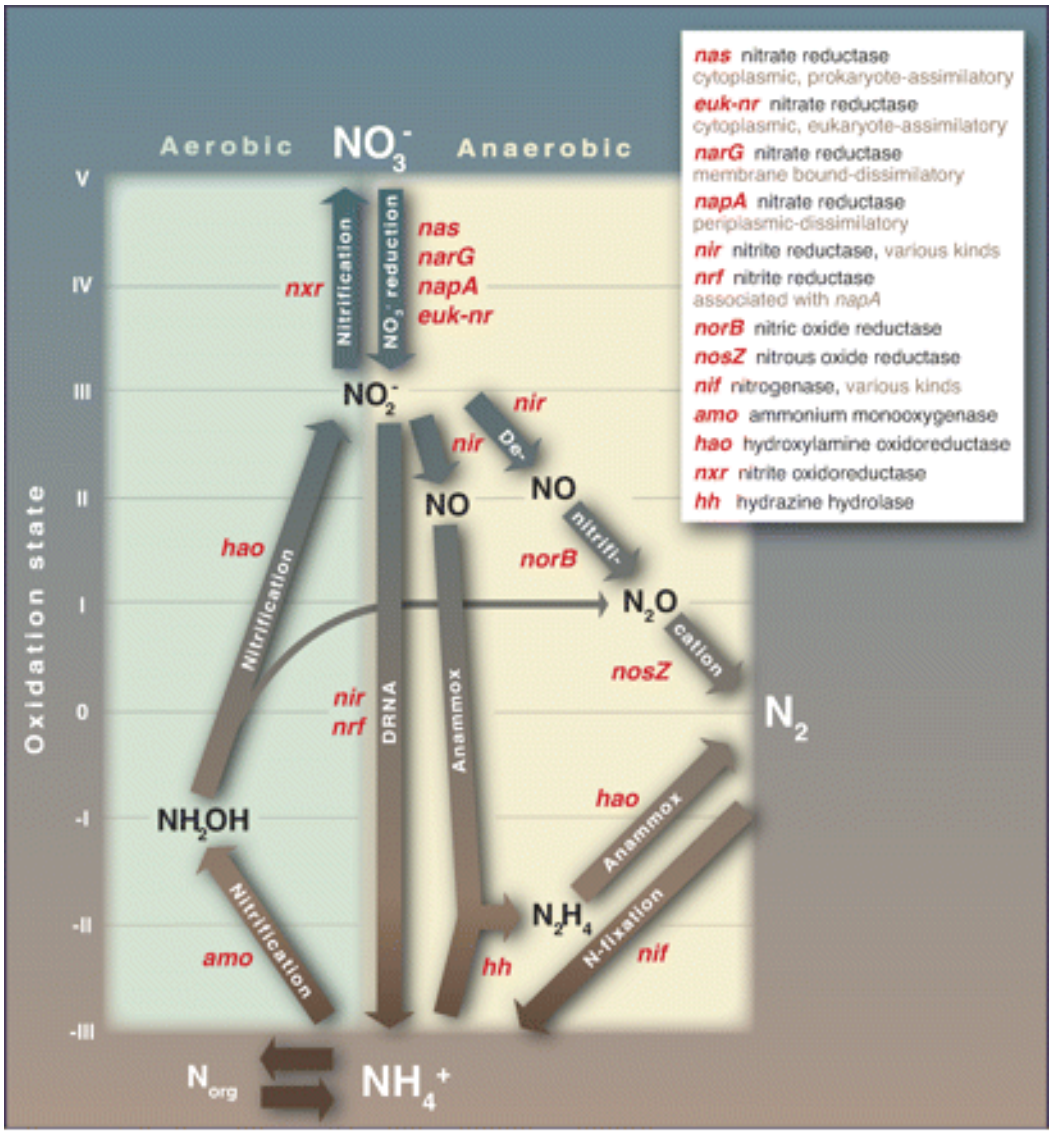


Figure 2.6 : The major biological nitrogen transformation pathways and their associated enzymes (Canfield et al., 2010).

2.2.1 Nitrogen Fixation

Nitrogen fixation is a process where mainly both bacterial and archaeal domains reduce nitrogen gas (N_2) to ammonium (Figure.2.1 and 2.2). Some eukaryotes (e.g., legumes and termites) also support nitrogen fixation, by symbiotic association with nitrogen-fixing prokaryotes. The reduction of N2 is an exergonic reaction, which is catalyzed by a nitrogenase enzyme with approximately16 adenosine triphosphate (ATP) molecules per molecule of N_2 fixed. Nitrogenase is an α2β2 tetramer, in which each of the two α subunits catalyzes the ATP-dependent reduction of N_2 to NH3. Nitrogenase subunits are highly conserved but are widely dispersed across many phyla of bacteria and archaea (Canfield et al., 2010).

2.2.1.1 Nitrogen-Fixing Microorganisms

Nonsymbiotic nitrogen-fixing microorganisms and symbiotic nitrogen-fixing microorganisms play major role in nitrogen fixation (Table 2.4). Azotobacter a gram-negative nonsymbiotic nitrogen-fixing microorganisms that forms cysts and fixes nitrogen in soils and other environments. Klebsiella, Clostridium (anaerobic, spore-forming bacteria active in sediments), and Cyanobacteria (e.g., Anabaena, Nostoc) are other commonly found nitrogen-fixing microoorganisms. The Cyanobacteria fix ten times more nitrogen than other free-nitrogen-fixing microorganisms in natural waters and soils. Cyanobacteria sometimes form associations with aquatic plants (e.g., the Anabaena–Azollae association). Symbiotic nitrogen-fixing microorganisms may enter in a symbiotic relationship with higher plants to fix nitrogen. An example of significant agronomic importance is the legume–Rhizobium association. Rhizobium form a nodule, which is the site of nitrogen fixation. Frankia and roots of woody perennial plants, and the association (with no nodule formation) between Azospirillum and the roots of maize and tropical grasses are other examples.

Table 2.4 : Nitrogen-Fixing Microorganisms (Bitton, 2005).

Category	Microorganisms
(A) Free-living nitrogen-fixing microorganisms	
Aerobes	Azotobacter
	Beijerinckia
Microaerophilic	Azospirillum
	Corynebacterium
Facultative anaerobes	Klebsiella
	Erwinia
Anaerobes	Clostridium
	Desulfovibrio
(B) Symbiotic associations	
Microbe – higher plants	Legume and Rhizobium
Cyanobacteria – aquatic weeds	Anabaena – Azolla
Others	Termites and enterobacteria

2.2.2 Nitrogen Assimilation

Heterotrophic and autotrophic microorganisms uptake and assimilate NH_4 and NO_3^{-2} after reduction to NH_4.

2.2.2.1 Nitrification

Nitrification involves the oxidation of ammonia to nitrate by two physiologically distinct group of organism: autotophicammonia- and nitrite oxidizers.(Nicol et al., 2006). The oxidation of ammonia is the rate limiting step of nitrification. Anaerobic ammonia oxidizers (Anammox) are also contributed nitrification. Figure 2.7 symbolizes Autotrophic ammonia oxidation during nitrification.

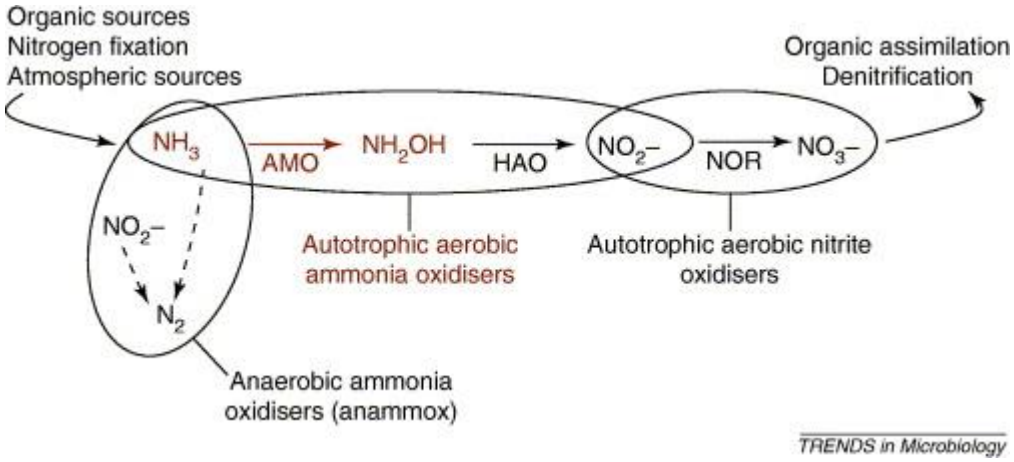


Figure 2.7 : Autotrophic ammonia oxidation during nitrification. (Nicol et al., 2006).

Ammonia-oxidising organisms convert ammonia to nitrite through hydroxylamine using ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). Autotrophic nitrite oxidisers convert nitrite to nitrate by using the enzyme nitrite oxidoreductase (NOR). In anaerobic environments, ammonia can be converted to molecular nitrogen by the 'anammox' process through several enzymatic steps (Nicol et al., 2006).

A wide variety of heterotrophic bacteria and also fungi have the capacity to oxidize NH_4. Heterotrophic bacteria such as *Arthrobacter globiformis, Aerobacter aerogenes, Thiosphaera pantotropha, Streptomyces grisens*, and various *Pseudomonas sp*. have been found active in nitrification process. *Aspergillus flavus* is one of the fungi species which is first isolated as a nitrifier (Paul, 2007)

The one of most important regulating factor of nitrification is ammonium supply. In the majority of soils or environments , nitrification rates will be high when ammonia is higher. Oxygen is another important regulator of nitrification; since nitrifiers are obligate aerobes.

Ammonia Oxidizing Bacteria (AOB)

Ammonium oxidation has been observed in many bacterial species. Ammonia is oxidized to hydroxylamine by ammonia monooxygenase and then oxidized to nitrite by hydroxylamine oxidoreductase enzyme (Figure 2.7). The aerobic chemolithoautotrophic ammonia-oxidizing bacteria are specialists that can grow on ammonia and carbon dioxide; which can be represented as :

$$NH_3 + O_2 + 2H^+ \xrightarrow[\text{Ammonia monoxygenase}]{} NH_2OH + H_2O \quad \textbf{(2.1)}$$

$$NH_2OH + H_2O \longrightarrow NO_2 + 5\,H^+ \quad \textbf{(2.2)}$$

Autotrophic ammonia oxidizing bacteria is commonly belongs to the ßeta (β) and Gammaproteobacteria (γ), including Nitrococcus (γ), Nitrosopira (β) and Nitrosomonas (Beta) (Figure 2.8) (Teske et al., 1994). Both Nitrosomonas and Nitrosospira species dominate the engineered and natural systems through the studies based on amoA gene sequences and 16S rDNA (Park etal., 2002).

Methanotrophs are capable of converting ammonia to hydroxylamine via the methane monooxygenase, but the ammonium monooxygenase can oxidize methane to carbon dioxide.

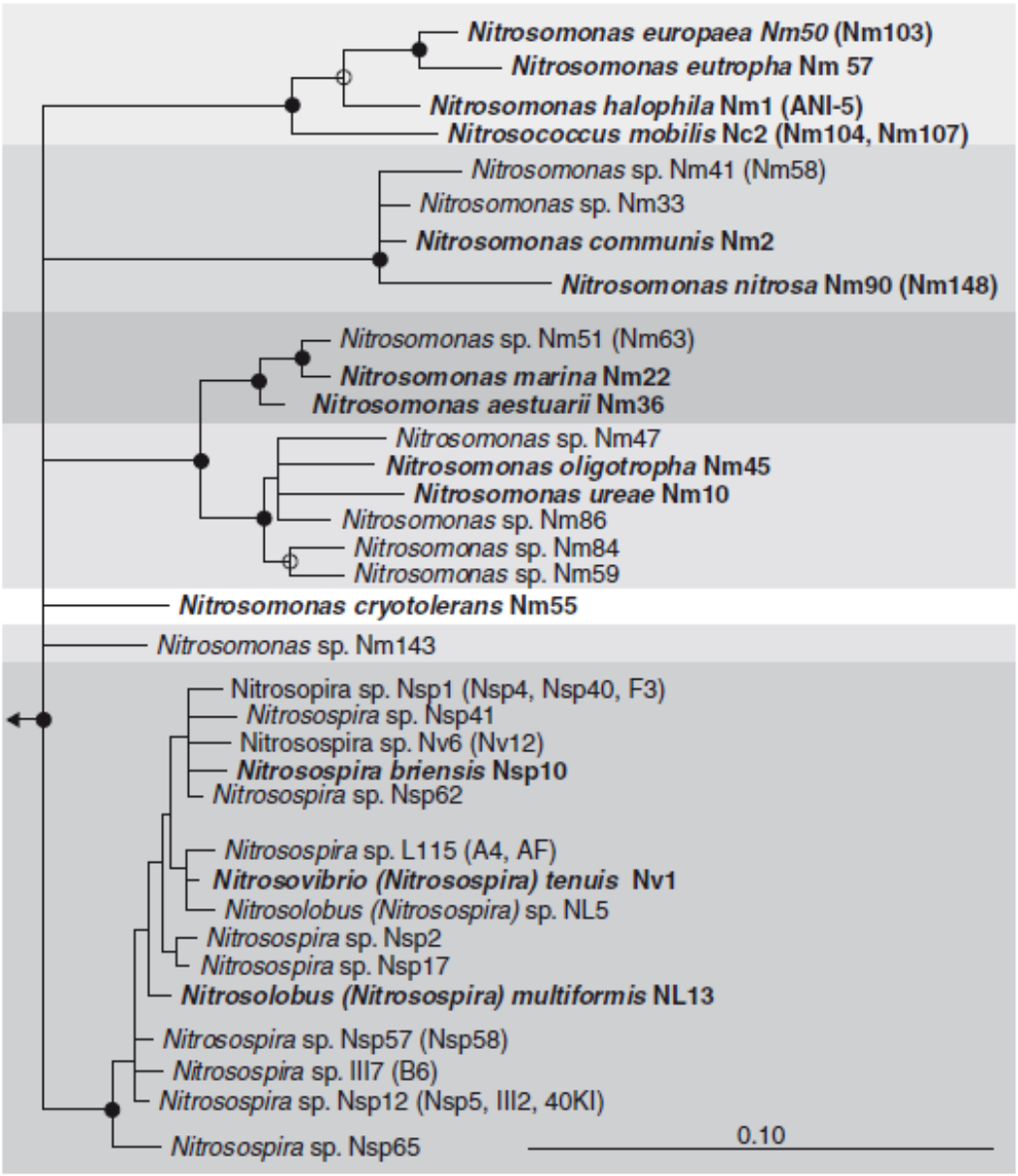


Figure 2.8 : 16S rRNA-based phylogenetic tree of the betaproteobacterial ammonia oxidizers (Paul, 2007).

Ammonia Oxidizing Archaea (AOA)

For more than hundred years it was believed that AOB were the only group responsible for the oxidation of ammonia. Further more anaerobic ammonium-oxidizing (Anammox) bacteria were purified from a laboratory enrichment systems by Strous et al., in 1999. AOA were firstly detected from the samples of the Sargasso Sea, a nutrient-limited open-ocean environment (Venter et al., 2004). *Nitrosopumilis maritimus* is the first strain of AOA isolated from the rocky substratum of a tropical marine aquarium tank (Könneke et al., 2005). Studies indicate that the archaeal

amoA gene is ubiquitous. The presence of the archaeal amoA gene was reported in many different environments such as coastal (Nakagawa et al., 2007) and marine waters (Wuchter et al., 2006, Agogue et al., 2008, Beman et al., 2008, Lam et al., 2007, Herfort et al., 2007, Francis et al., 2005, Coolen et al., 2007) in subterranean estuary (Santoro et al., 2008, Mincer et al., 2007, Sahan & Muyzer, 2008) in coastal, estuarine (Beman & Francis, 2006, Caffrey et al., 2007, Mosier & Francis, 2008) freshwater (Herrmann et al., 2008) and cold seep sediments (Park et al., 2008), in a subsurface of radioactive thermal spring (Reigstad et al., 2008) and neighboring biofilms, in the sediments (Weidler et al., 2007) and microbial mats/mud of hot springs (Le Roux et al., 2008, Hatzenpichler et al., 2008) and geothermal biofabrics (Spear et al., 2007), and in coral reefs (Beman et al., 2007, Siboni et al., 2008), in terrestrial systems both in sandy (Shen et al., 2008), agricultural (Tourna et al., 2008), semiarid and forest soils(Adair & Schwartz, 2008, Boyle-Yarwood et al., 2008, He et al., 2007) and grasslands and in the rhizosphere and in paddy soils (Leininger et al., 2006, Chen et al., 2008, Hansel et al., 2008, de la Torre et al., 2008, Treusch et al., 2005). Last but not the least, AOA has also been firstly reported in activated sludge bioreactors (Park et al., 2006). Based on the majority of the quantitative and qualitative analyses, it can be deduced that AOA are potentially important actors of the nitrogen cycle in many ecosystems (Erguder et al., 2009).

Most environmental factors like ammonia levels, temperature, salinity and pH affected the ecological niches of ammonia-oxidizing archaea. The archaeal or crenarchaeotal amoA genes were retrieved in low ammonium-containing environments such as open-ocean, marine water columns, sediments and hot springs (Wuchter et al., 2006, Coolen et al., 2007, Lam et al., 2007, Hatzenpichler et al., 2008, Herrmann et al., 2008, Reigstad et al., 2008). The culture studies showed that both soil and marine AOA could grow best around 1mM ammonium concentrations, whereas above 10mM ammonia concentrations was greatly retarded and above 20mM inhibited the AOA growth (Celik, 2010). *Nitrosopumilis maritimus* could be inhibited by organic ammonium substrates even at very low concentrations (Könneke et al., 2005). The nonthermophilic (i.e. *N. maritimus* and *C. symbiosum*) and thermophilic (i.e. *C. Nitrosocaldus yellowstonii* and *C. Nitrososphaera gargensis*) AOA were detected at sites with very low (down to 0.2 °C) to high (up to 97 °C) temperatures. Salinity is also shown to be a significant factor in determining the

diversity of AOA community structure (Francis et al., 2005; Mosier & Francis, 2008) and their spatial distribution (Sahan & Muyzer, 2008). One distinct phylogenetic cluster have discovered by Francis et al., (2005), thus, indicating a possible unique low-salinity AOA type. Some AOA species tolerant to the wide range of salinity conditions whereas other AOA ecotypes are specific for a narrow niche. Archaeal amoA genes were found, over a wide range of pH values going from 3.7 (He et al., 2007) to 8.65 (Wuchter et al., 2006, Shen et al., 2008, Urakawa et al., 2008). Thermophilic archaeal amoA genes were detected in sediments, microbial mats and mud of hot springs with predominantly alkaline (pH=8.0–9.0) or acidic (pH=2.5) conditions (de la Torre et al., 2008, Reigstad et al., 2008). AOA have a wide ecological and phylogenetic diversity (Erguder 2009).

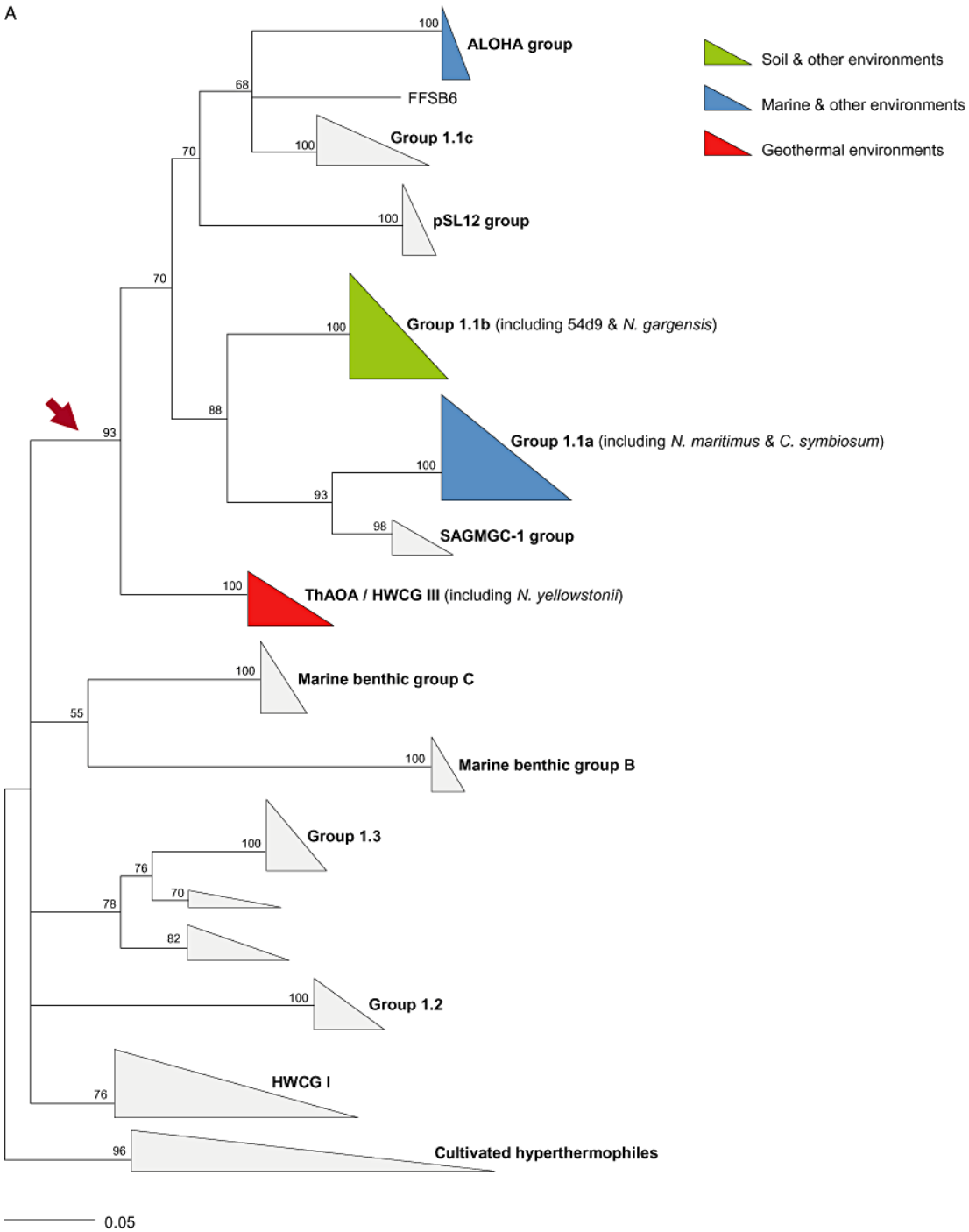


Figure 2.9a : Phylogenetic trees describing (A) major 16S rRNA gene- defined lineages within the kingdom Crenarchaeota (Prosser and Nicol, 2008).

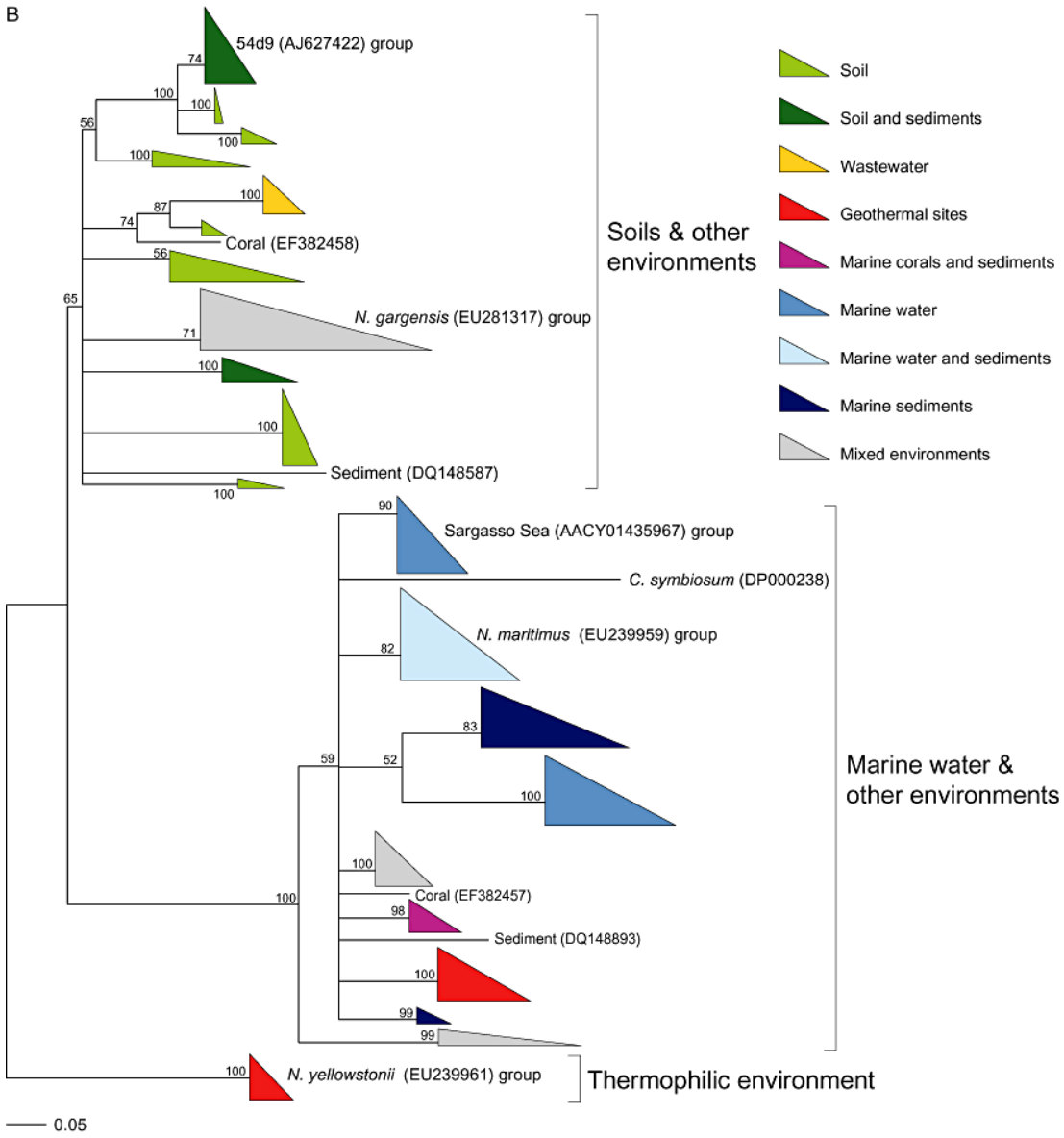


Figure 2.9b : Phylogenetic trees describing (B) amoA gene-defined lineages within the kingdom Crenarchaeota (Prosser and Nicol, 2008).

Kinetics and Stoichiometry of Ammonia Oxidation:

Stoichiometry **s**tudies using microrespirometry to measure oxygen and ammonia consumption relative to nitrite production showed that the overall stoichiometry of ammonia oxidation by *N. maritimus* is indistinguishable from that of AOB:

$$1NH_3 + 1.5O_2 \Rightarrow 1\ NO_2^{-2} + H_2O + H+ \qquad \textbf{(2.3)}$$

Ammonia oxidation kinetics are showed that *N. maritimus* is an extreme oligotroph, having an apparent half-saturation constant (Km) for total ammonia (ammoniumplus ammonia) of 132 nM (~3 nM NH3 at near-neutral pH). Furthermore, ammonia at concentrations more than 1 mM inhibits *N. maritimus* cell growth. Similar observations have been made with the moderately thermophilic AOA, *N. gargensis*. Km values of AOB have 200-fold higher than the Km value of *N. maritimus*. Nitrososphaera viennensis which is soil archeon, tolerates higher concentrations of ammonia than does *N. maritimus*. These culture-based observations showed that AOA populations grow over a wide range of ammonia concentrations in contrast to AOB that require significantly higher ammonia concentrations to initiate growth (Stahl and de la Torre, 2012).

The Biochemistry of Archaeal Ammonia Oxidation:

N. maritimus complete genome sequence revealed three major difference of ammonia oxidation and carbon fixation than bacterial system: (a) a role for copper (rather than iron) as the major redox active metal in electron transfer reactions, (b) the absence of any homolog to the bacterial oxidoreductase (hydroxylamine oxidoreductase, HAO) responsible for the oxidation of hydroxylamine to nitrite, and (c) a variant of the 3-hydroxypropionate/4-hydroxybutyrate cycle for CO_2 fixation. These data showed that marine and soil AOA derive energy and electrons primarily from the oxidation of ammonia but can supplement carbon from CO_2 fixation using a limited set of simple compounds that feed directly into central metabolism (Stahland de la Torre, 2012).

AOA have only AMO gene for nitrification pathway but other genes that found in bacterial counterpart are missing. Nitrosopumilus lacks a homolog of the bacterial HAO and the capacity for synthesis of c-type cytochromes. Bacterial c-type

cytochromes compose the redox-active centers of the HAO and mediate respiratory transfer of electrons from HAO to the terminal oxidase (Figure 2). As yet there is no evidence that the product of ammonia oxidation by the archaeal AMO is hydroxylamine. Nitroxyl (HNO) could be the product of the archaeal AMO (figure 2.10).

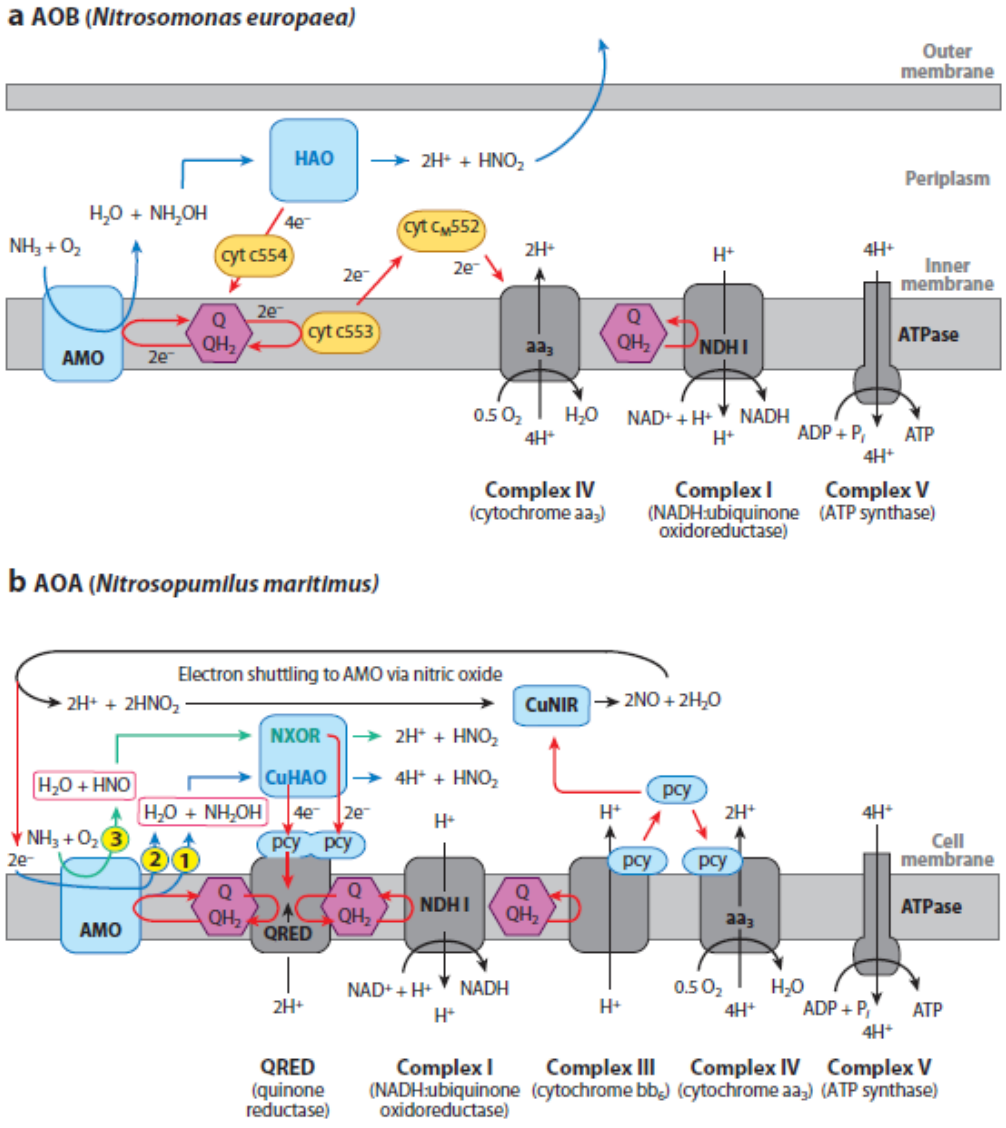


Figure 2.10 : Proposed respiratory pathways for ammonia oxidation in AOB and AOA. (a) Proposed pathway for ammonia oxidation in the AOB Nitrosomonas europaea. (b) Proposed pathway for ammonia oxidation in the AOA *Nitrosopumilus maritimus* (Stahland de la Torre, 2012).

AOB oxidize **a**mmonia to NH_2OH by the membrane enzyme complex AMO. Subsequently, hydroxylamine is oxidized to nitrite in the periplasm by HAO. Four electrons obtained forom these reactions are transferred to the quinone pool by

cytochrome c554. Two electrons from the reduced quinone pool return to AMO and are required to initiate ammonia oxidation. The remaining two electrons enter the electron transport chain via cytochrome c553 and cytochrome cM552 for ATP synthesis (Stahland de la Torre, 2012).

Three alternative pathways for AOA are proposed in this speculative diagram. Pathway 1 ; electrons are produced by the oxidation of hydroxylamine to nitrite by a presumed copper hydroxylamine oxidoreductase (CuHAO) and electrons are transferred to pcy electron carriers and then to the quinone pool by a membrane-associated quinone reductase (QRED). Two electrons would be recycled to AMO and the remaining two electrons would be transferred to the electron transport chain. Pathway 2 ; nitric oxide (NO), produced by the reduction of nitrite by a proposed copper-dependent nitrite reductase (CuNIR), is the source of electrons for AMO. Pathway 3; eliminate the requirement for electron recycling during the initial oxidation of ammonia. Subsequently, nitroxyl (HNO) would be oxidized to nitrite by a presumed putative nitroxyl oxidoreductase (NXOR). The two electrons extracted during this oxidation would be transferred to QRED (Stahland de la Torre, 2012).

Anammox Bacteria

Anaerobic ammonium-oxidizing (anammox) bacteria are one of the latest additions to the biogeochemical nitrogen cycle. Anammox pathway in the nitrogen cycles shown in Figure 2.1. These bacteria convert an ammonium and nitrite into dinitrogen gas in the complete absence of oxygen. These slowly growing microorganisms belong to the order Brocadiales and are affiliated to the Planctomycetes. Anammox bacteria are characterized by a compartmentalized cell architecture featuring a central cell compartment, the "anammoxosome". The worldwide presence of anammox bacteria has now been established in many oxygen-limited marine and freshwater systems, including oceans, estuaries, marshes, rivers and large lakes. In the marine environment over 50% of the N(2) gas released may be produced by anammox bacteria (Jetten, 2009) which has been changed our knowledge on 100 years old nitrogen cycle.

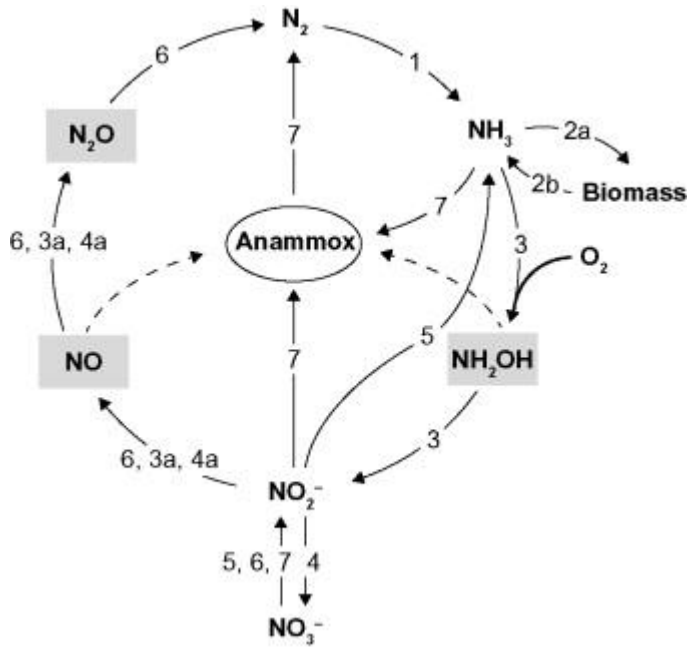


Figure 2.11 : Partial processes: (1), nitrogen fixation; (2a), ammonium assimilation; (2b), ammonification/mineralization, (3), aerobic ammonium oxidation; (4), nitrite oxidation; (3a) and (4b), anaerobic nitrification–denitrification by ammonium- and nitrite-oxidizing nitrifiers, respectively; (5), dissimilatory nitrite reduction to ammonium (DNRA); (6), anaerobic denitrification; (7), anammox. Bound intermediates in the pathways are marked by light gray boxes. The putative exchange of intermediates between different processes is represented by dashed lines (Kartal, 2012).

2.2.2.2 Denitrification

Denitrification is a respiratory process whereby nitrate is successfully reduced to nitrite, NO, N_2O and finally to N_2 (Figure 2.11). Denitrifiers and dissimilatory nitrate reducers use nitrate or nitrite as the electron acceptor for the oxidation of (in)organic substrates, thereby producing gaseous nitrogen compounds and ammonium, respectively. Many bacteria are at least facultative denitrifiers and denitrification is not limited to specific microbial phyla like nitrification.

2.3 WASTEWATER TREATMENT

The major contaminants found in wastewater are biodegradable organic compounds, volatile organic compounds, recalcitrant xenobiotics, toxic metals, suspended solids, nutrients (nitrogen and phosphorus), and microbial pathogens and parasites (Figure 2.12).

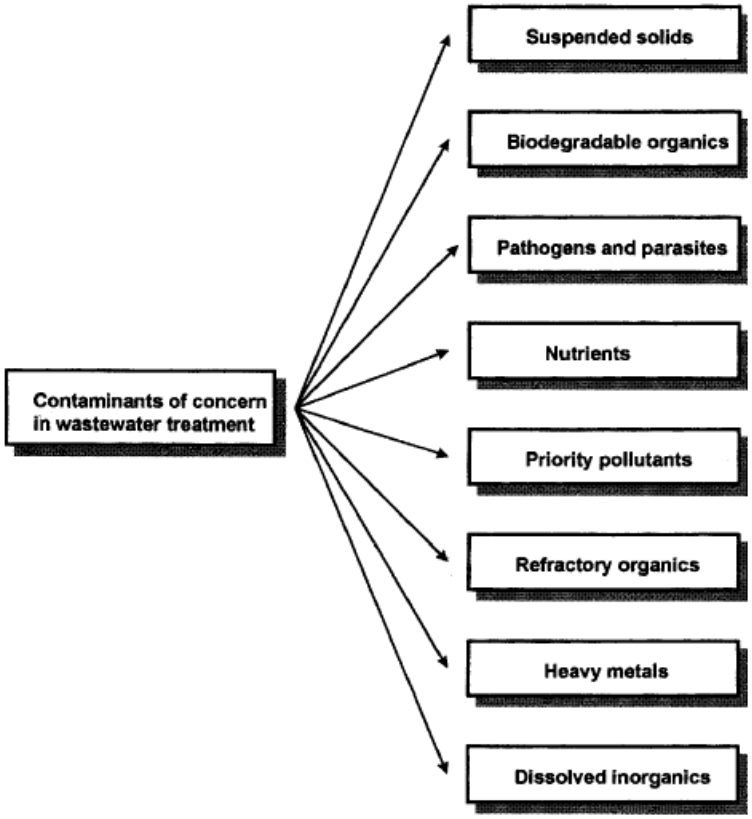


Figure 2.12 : The major contaminant in wastewater (Bitton, 2005).

There are several objectives of waste treatment processes:

1. Reduction of the organic content of wastewater (i.e., reduction of BOD).

2. Removal/reduction of trace organics that are recalcitrant to biodegradation and may be toxic or carcinogenic .

3. Removal/reduction of toxic metals.

4. Removal/reduction of nutrients (N, P) to reduce pollution of receiving surface waters or groundwater if the effluents are applied onto land.

5. Removal or inactivation of pathogenic microorganisms and parasites.

Domestic wastewater (Table 5) is a combination of human and animal excreta (feces and urine) and gray water resulting from washing, bathing, and cooking.

Table 2.5 : Typical Characteristics of Domestic Wastewater (Bitton, 2005).

	Concentration		
Parameter	Strong (mg/L)	Medium (mg/L)	Weak (mg/L)
BOD_5	400	220	110
COD	1000	500	250
Organic N	35	15	8
NH_3-N	50	25	12
Total N	85	40	20
Total P	15	8	4
Total solids	1200	720	350
Suspended solids	350	220	100

Chemical and biological processes drive the treatment of wastewater. Treatment methods that rely on physical forces are called unit operations; screening, sedimentation, filtration, or flotation. Treatment methods based on chemical and biological processes are called unit processes; Chemical unit processes disinfection, adsorption, or precipitation, biological unit processes; microbial activity, which is responsible for organic matter degradation and removal of nutrients (Bitton, 2005).

Wastewater treatment comprises the following four steps (Figure 2.13).

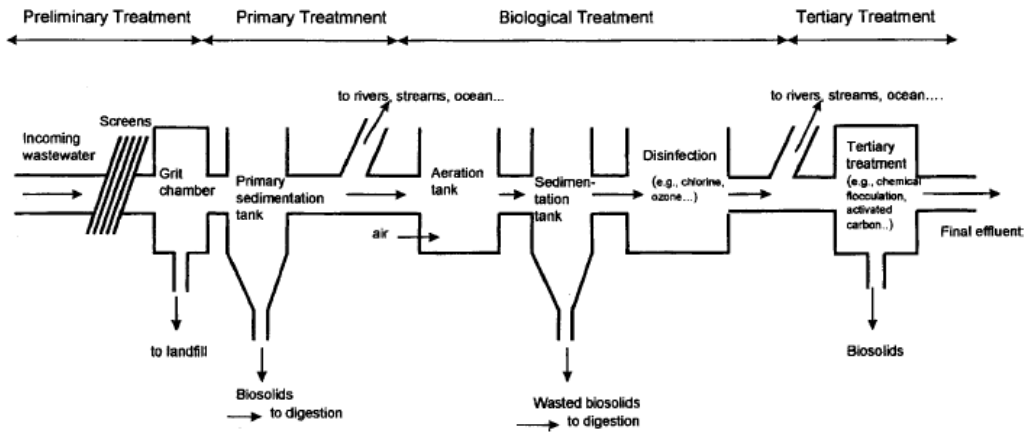


Figure 2.13 : Typical Wastewater treatment plants (Bitton, 2005).

1. Preliminary treatment. The objective of this operation is to remove debris and coarse materials that may clog equipment in the plant.

2. Primary treatment. Treatment is brought about by physical processes (unit operations) such as screening and sedimentation.

3. Secondary treatment. Biological (e.g., activated sludge, trickling filter, oxidation ponds) and chemical (e.g., disinfection) unit processes are used to treat wastewater. Nutrient removal also generally occurs during secondary treatment of wastewater.

4. Tertiary or advanced treatment. Unit operations and chemical unit processes are used to further remove BOD, nutrients, pathogens, and parasites, and sometimes toxic substances (Bitton, 2005).

2.4. ACTIVATED SLUDGE PROCESS

A conventional activated sludge process includes (Figure 2.14) aeration and sedimentation tanks.

Aeration tank: Aerobic oxidation of organic matter is carried out in this tank. Primary effluent is introduced and mixed with return activated sludge (RAS) to form the mixed liquor, which contains 1500–2500 mg/L of suspended solids. Aeration is provided by mechanical means. An important characteristic of the activated sludge process is the recycling of a large portion of the biomass. Sludge age is much greater than the hydraulic retention time. This practice helps maintain a large number of

microorganisms that effectively oxidize organic compounds in a relatively short time. The detention time in the aeration basin varies between 4 and 8 hours (Bitton, 2005).

Sedimentation tank: This tank is used for the sedimentation of microbial flocs (sludge) produced during the oxidation phase in the aeration tank. A portion of the sludge in the clarifier is recycled back to the aeration basin and the remainder is wasted to maintain a proper F/M (food to microorganisms ratio) (Bitton, 2005).

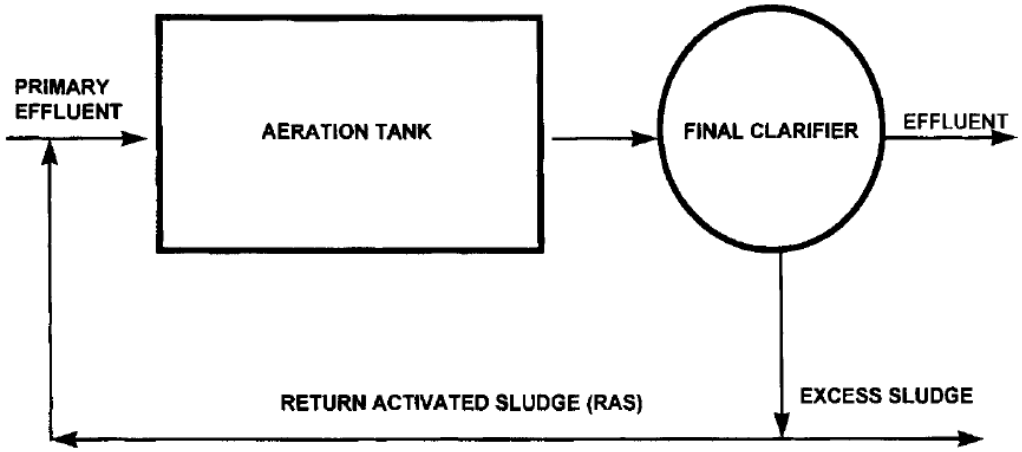


Figure 2.14 : Conventional activated sludge system (Bitton, 2005).

3. MATERIALS AND METHODS

3.1 Materials and equipment

3.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A

3.1.2 Buffers, reagents and enzymes

The compounds and enzymes used during this study are listed in Appendix B

3.2 Methods

Two parallel studies were performed during this study to investigate AOA occurrence in both WWTPs;(1) Activated sludge Samples from WWTPs reactors were screened for AOA occurrence ; (2) relative quantification of AOA and AOB in samples. In order to achieve the objectives of these studies various molecular biological methods were used (see below).

3.2.1 Sampling

3.2.1.1 Samples from WWTPs

Activated sludge samples were collected from 17 WWTPs and laboratuary scale reactors (Table 3.1). Duplicate or triplicate; samples (at least 20 ml) were taken from each plants, carried out to the lab at cold chain (ice box: $4^{o}C$ - $8^{o}C$) until DNA extractions were carried out within the same day of sampling. Out of seventeen plants; two of them(brewery and leachate treatment) has been chosen for detailed study due to their reachability. Thus samples were taken from each stage of the process of those two plants to analyze a detailed community structure for AOA occurrence. The detailed schematic structure of the treatment processes for both the baker's yeast wastewater treatment plant (Figure 3.1) and leachate treatment plant

(Figure 3.2) is shown below. 12 sampling points were chosen from a brewerybaker's yeast wastewater treatment plant whereas 8 sampling points were selected from a leachate treatment plant (Table 3.2).

Table 3.1 : Samples points which was screened for Archaeal amoA genes.

		Location	Reactor Sample Type
1	Paşaköy advanced biological treatment plant	Istanbul, Turkey	Activated sludge sample
2	Beer production	Istanbul Turkey	Activated sludge sample
3	Raki production	Tekirdag, Turkey	Activated sludge sample
4	Paper raw material	Istanbul Turkey	Activated sludge sample
5	Petrolium rafinery	Kirikkale, Turkey	Activated sludge sample
6	Beer production	Ankara,Turkey	Activated sludge sample
7	Iski digester	Istanbul, Turkey	Activated sludge sample
8	Gum	Istanbul, Turkey	Activated sludge sample
9	baker's yeast (Pakmaya)	Izmit, Turkey	All reactors*1 All reactors
10	Inorganic Chemicals	Yalova, Turkey	Activated sludge sample
11	Sunflower oil production	Edirne, Turkey	Activated sludge sample
12	Asat1	Antalya, Turkey	Activated sludge sample
13	Asat2	Antalya, Turkey	Activated sludge sample
14	İstaç Leachate wastewater treatment plant	Istanbul, Turkey	All reactors*2 All reactors
15	Eskişehir WWTP	Eskişehir, Turkey	Activated sludge sample
16	ESART A.Ş WWTP	Eskişehir, Turkey	Activated sludge sample
17	Tuzla wastewater treatment system	Istanbul, Turkey	Activated sludge sample

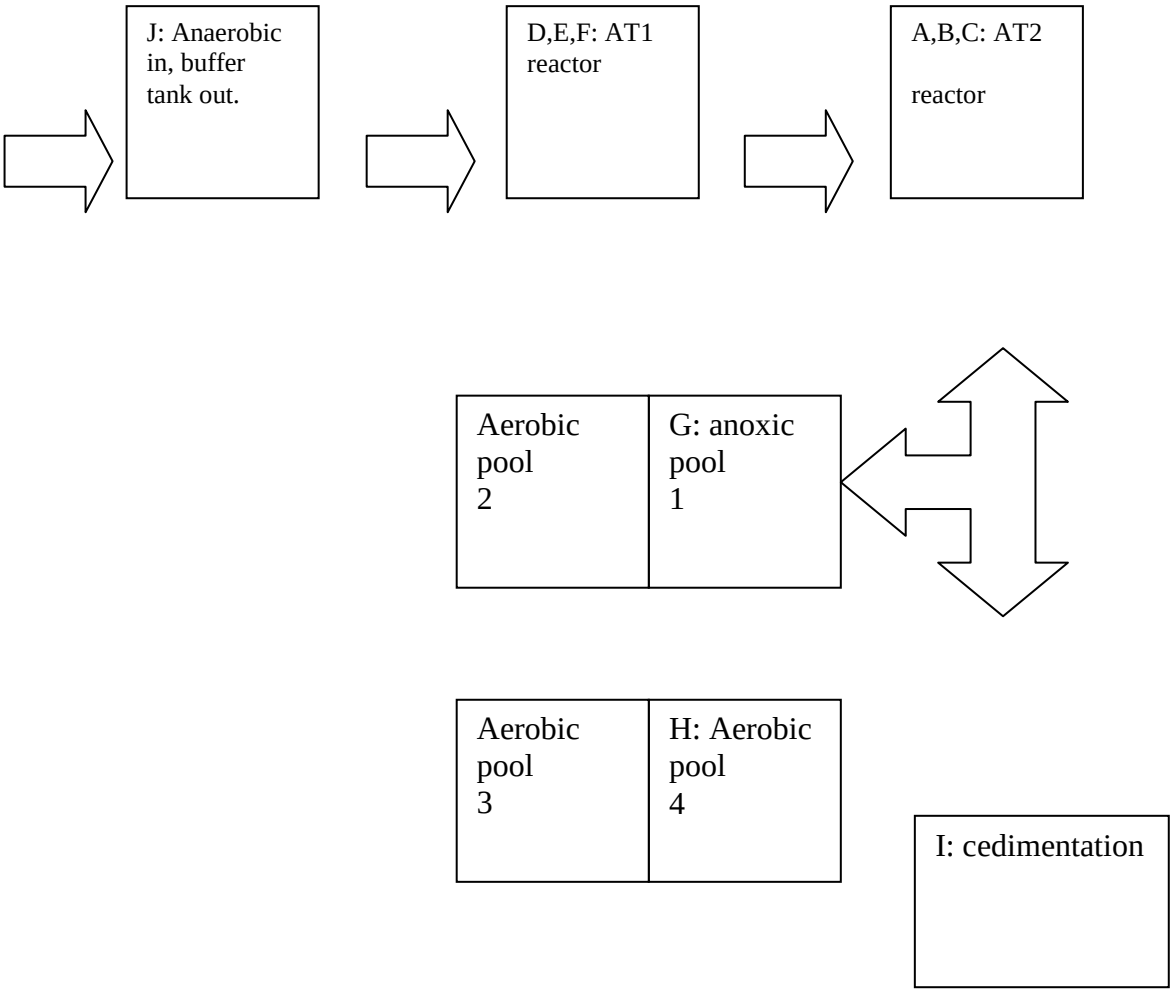


Figure 3.1 : Diagram of WWTS of PAKMAYA (A-J, samples points).

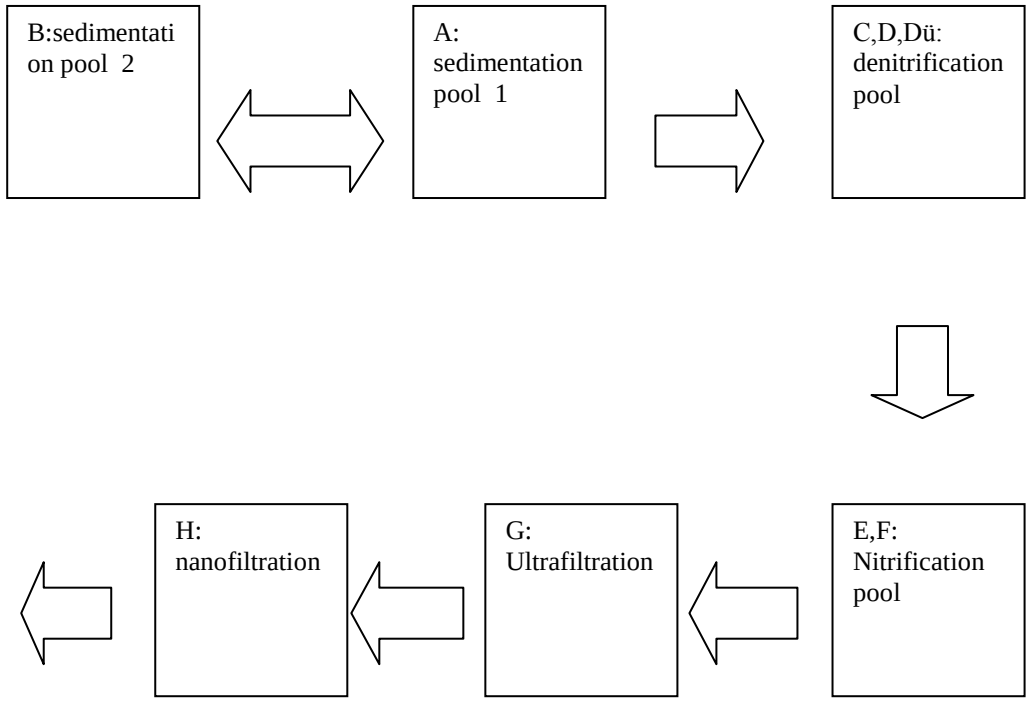


Figure 3.2 : Diagram of WWT of İSTAÇ (A-H, samples points).

Table 3.2a : Sampling points for İSTAÇ leachate treatment plant.

	Sampling Points	
Leachate Treatment Plant	A: Digester (pool 1)	E: Nitrification pool exit
	B: Digester (pool 2)	F: Nitrification pool
	C: Denitrification pool	G: Ultrafiltration exit valve (UF)
	D: Denitrification pool	H: Nanofiltration exit valve (NF)

Table 3.2b : Sampling points for PAKMAYA baker's yeast wastewater treatment plant .

	Sampling Points	
baker's yeast wastewater treatment plant	A: Reactor AT2 bottom exit valve	G: Denitrification pool (pool 1) valve (anaerobic reactor)
	B: Reactor AT2 middle exit valve	H: Denitrification pool exit valve (pool 4)
	C: Reactor AT2 top exit valve	I: Sedimentation- circulation tank
	D: Reactor AT1 bottom exit	J: Buffer tank exit valve
	E: Reactor AT1 middle exit	K:Raw waste
	F: Reactor AT1 top exit valve	L: Anaerobic reactor exit valve

3.2.2.1 DNA extractions from WWTPs activated sludge samples

DNA extractions from all WWTPs activated sludge samples were carried out within the day after sampling. DNA was extracted using Fastprep DNA extraction kit for soil (Qbiogene, Carlsbad, CA) The manufacturer's protocol was followed, starting with 0.5g sample material using a FastPrep instrument setting of 5.5 for 30s. DNA quantity is measured using Qubit fluorometer (invitrogen, Carlsbad, CA) and following quantification DNA is diluted to 10 ng/ml.

Samples were analyzed on agarose gels. The gels were prepared using 1% (w/v) agarose in 1XTAE buffer containing 0.5 μg/mL ethidium bromide. 3μl of DNA samples were mixed with 2x gel loading buffer. Electrophoresis was performed at 10V/cm and the gel was visualized under UV using Gel Doc (BIORAD, Ca, US3.2.3 Polymerase Chain Reaction (PCR)

3.2.2.2 Samples from Environments

Other part of this study over 50 environmental samples were screened for archaeal amoA gene occurrence, mainly pristine and agricultural soil samples (Celik 2010). In addition to the soil samples thermal, terrestrial hot spring, aquarium and fresh water

samples were also screened for AOA occurrence. DNA of some environmental samples was already extracted before this study,

3.2.3.1 PCR Overview

The polymerase chain reaction (PCR) is a primer-mediated enzymatic amplification of specifically cloned or genomic DNA sequences [Innes et., 1990]. A thermostable DNA polymerase such as Taq DNA polymerase catalyzes the buffered reaction in which an excess of an oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs)(Table 3.3) are used to make millions of copies of the target sequence.

Table 3.3 : Reagents used in PCR.

Reagents	Total Volume
ddH_2O	17.875 µL
10X PCR Buffer (with 18mM $MgCl_2$)	2.5 µL
Primer F	0.5 µL (0,2 pmol/µL)
Primer R	0.5 µL (0,2 pmol/µL)
dNTPs	2.5 µL (2,5 mM)
Taq Polimeraz	0,125 µL (5unit/µL)
Örnek	1 µL (10 ng)

3.2.3.2 Archaeal amoA gene

In order to study occurrence of AOA in WWTP samples, primers (Table 3.4a) targeting archaeal amoA gene was used; instead of AOA 16S rRNA coding gene. PCR conditions for such primer sets are listed at table 3.4b and used reagents are listed at Table 3.3.

Table 3.4a : Primers used for screening AOA.

Primer Name	Primer Sequence	Reference	Amplication size (bp)	Primer specific annealing T (^{o}C)
Arch_amoAF	5'-STAATGGTCTGGCTTAGACG-3'	Francis et al., 2005	635	56
Arch_amoAR	5'-GCGGCCATCCATCTGTATGT-3'	Francis et al., 2005		

Table 3.4b : PCR conditions for Archaeal amoA primer set.

cycles	**AOA amoA F-R**
1	95^{o}C 5 min
34	95^{o}C 30 sec
	55^{o}C 35 sec
	72^{o}C 50 sec
1	72^{o}C 10 min
1	4^{o}C oo

3.2.3.3 Archaeal 16S rDNA genes

Archaeal 16S rDNA primers are universal primers that are used to analyze the archaeal diversity (Table 3.5a). In this study archaeal 16S rDNA primers are mainly used to check population dynamics in terms of Archea as it was also checked for Eubacteria. All DNA templates that is positive for archaeal 16S rDNA genes used for screening AOA occurrence. In addition, archaeal 16S rDNA primers specifacally (Arch1017r/Arc46f) are used for cloning reactions. PCR conditions for those primer sets are listed at table 3.5b and also reagents are listed at Table 3.3.

Table 3.5a : 16S rRNA primers used for screening archaeal diversity.

Primer Name	Primer Sequence	Reference	Amplication size (bp)	Primer specific annealing T (oC)
Arch46f	5'-YTAAGCCATGCRAGT-3'	Ovreas et al., 1997	1000	57
Arch1017r	5'-GGCCATGCACCWCTCTC-3'	Barns et al., 1994		

Table 3.5b : PCR conditions for archaeal 16S rDNA primers.

cycles	**Arch 1017r-46f**
1	95^{o}C 5 min
34	95^{o}C 30 sec
	57^{o}C 45 sec
	72^{o}C 55 sec
1	72^{o}C 10 min
1	4^{o}C oo

3.2.3.4 Bacterial 16S rRNA genes

Bacterial 16S rRNA coding DNA primers are universal primers that are used to analyze the bacterial diversity (Table 3.6a). Vf / Vr primer set is used for screening inhibition of WWTPs samples. Pa / Ph primer set is used to produce PCR products that are used for cloning. PCR conditions for primer sets are listed at table 3.6b and also reagents are listed at Table 3.3.

Table 3.6a : Primers used for screening bacterial diversity.

Primer Name	Primer Sequence	Reference	Amplicatio n size (bp)	Primer specific annealing T (oC)
Vf	5'-GGCCTACGGGAGGCAGCA G-3'	Muyzer et al., 1993		
Vr	5'-ATTACCGCGGCTGCTGG-3'	Muyzer et al., 1993	180	55
pA	5'-AGAGTTTGATCCTGGCTC AG-3'	Edwards et al., 1989		
pH	5'-AGGGAGGTGATCCAG CCGCA-3'	Edwards et al., 1989	1500	59

Table 3.6b : PCR conditions for bacterial 16S rDNA primers.

cycles	**Vf-Vr**	**pA-pH**	
1	95°C 5 min	1	95°C 5 min
34	95°C 30 sec	34	95°C 30 sec
	55°C 45 sec		59°C 45 sec
	72°C 55 sec		72°C 55 sec
1	72°C 10 min	1	72°C 10 min
1	4°C ∞	1	4°C ∞

3.2.4 Denaturing gradient gel electrophoresis (DGGE)

3.2.4.1 DGGE overview

Denaturing gradient gel electrophoresis (DGGE) was introduced 20 years ago [Fischer et al., 1979] as a fingerprinting analysis to separate DNA fragments in one base pair accuracy. DGGE which uses both temperature and chemical gradient across the gel to differentiate microbial content in taxon level.

Different sequences partially melt at different positions in the gradient and therefore stop at different positions in the gel depending on the sequence's Tm.. Every seperate bands refere as different taxonomic unit (taxon) and give us clue about bacterial or archaeal diversity. DGGE was performed on a D-Code apparatus (Bio-Rad, Hercules, USA). The equal amounts of PCR products were loaded onto 10% (wt/vol) polyacrylamide gels (37.5:1; acrylamide: bisacrylamide) with a denaturing gradient ranging from 35 % to 65 % denaturant (100 % denaturant contains 40% [vol/vol] formamide and 7M urea in 1XTAE buffer). Electrophoresis was performed at constant 60$_o$C at 70V for 960 min in 1xTAE buffer. The gels were stained with SYBR Green I (1:5000) and visualized under UV using GelDoc imaging system (Bio-Rad, Dcode, USA).

GC-clamped forward primers listed at Table 3.9. Hot start Taq polymerase is (Qiagen, Germany) used for producing PCR products that will be examined in DGGE. In addition, final elongation time in PCRs kept longer to ensure GC-clamped products are successfully elongated. The PCR conditions for bacterial primers has been listed in Table 3.7. To get archaeal 16S rDNA products nested PCR is performed, archaeal 16S rDNA primers PCR conditions listed in Table 3.8.

Table 3.7 : GC-clamped forward primers used to produce PCR products that are analyzed in DGGE.

Primer Name	Primer Sequence	Reference
GC-Arch-344 F	5-GC**GACGGGGHGCAGCAGGCGCGA-3'	Raskin et al., 1994
GC-Vf	5'-GC**GGCCTACGGGAGGCAGCA G-3'	Muyzer et al., 1993
**GC-	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG G-3'	Muyzer et al., 1993

Table 3.8 : PCR conditions for bacterial 16S rDNA primers.

cycles	**Bacterial 16S rRNA (GC-VF, VR)**	**Archaeal Nested PCR (GC-344F-522R)**	
1	95°C 5 min	1	95°C 5 min
34	95°C 30 sec	34	95°C 30 sec
	55°C 45 sec		59°C 45 sec
	72°C 55 sec		72°C 55 sec
1	72°C 10 min	1	72°C 10 min
1	4°C oo	1	4°C oo

Preparation of DGGE Gel

10% (w/v) polyacrylamide [acrylamide-bisacrylamide (37.5:1)] solutions containing denaturing gradients of 0% and 100% were prepared (Table 3.11). Gel preparation steps are listed at Table 3.12. DGGE running and staining conditions for bacterial PCR products and archaeal PCR products are shown in table 3.11.

Table 3.9 : Preparation of the % (w/v) acrylamide/bisacyrlamide (37.5:1) solutions containing denaturing gradients of 0% and 100%.

	40% Acrylamide/Bis	50X TAE	Formamide	urea	dH2O	Total Volume
10% Gel 0% Denaturant	25 ml	2 ml	N	N	73 ml	100 ml
10% Gel 100% Denaturant	25 ml	2 ml%	40 ml	42 g	Final Vol.100 ml	100 ml

Table 3.10 : Preparation of DGGE Gels.

Clean glass plates carefully with ethanol. Assemble the gel sandwich according to the supplier's instructions.
From the stock solutions of acrylamide and denaturants, prepare two solutions of 16 ml each (for a 32-mL gel),
Add 160 µL of 10% ammonium persulfate and 16 µL of TEMED to each solution and mix by gently swirling.
Pour the two gel solutions into the gradient maker
Insert the comb gently and at an angle and let the gel polymerize for 30–45 min.
Remove the comb and immediately flush the wells carefully with 1X TAE buffer to remove unpolymerized acrylamide

Table 3.11 : DGGE conditions for archaeal and bacterial PCR products.

	Bacterial 16S rRNA (GC-VF, VR)	Archaeal 16S rRNA (GC-344F-522R)
Gel percentage	10%	10%
Denaturing Gradient	35-65	35-60
Running Volt	180 volt	180 volt
Running Temp.	60^{o}C	60^{o}C
Running time	330 min	360 min
Staining	15 min.	7 min.
De-staining	30 min.	15 min.
	Bacterial 16S rRNA (GC-VF, VR)	Archaeal 16S rRNA (GC-344F-522R)
Gel percentage	10%	10%
Denaturing Gradient	35-65	35-60
Running Volt	180 volt	180 volt
Running Temp.	60oC	60oC
Running time	330 min	360 min
Staining	15 min.	7 min.
De-staining	30 min.	15 min.

3.2.5 Cloning of PCR products

3.2.5.1 The TOPO TA cloning® kit for sequencing

The TOPO TA cloning® kit for sequencing (Invitrogen, USA) has been selected to clone our samples. It is a one-step cloning strategy (TOPO cloning) for sequencing. The lacZα- ccdB gene fusion is disrupted by ligation of a PCR product; selected via positive recombinants upon transformation in TOP10 cells.. Experimental outline for Topo TA cloning kit is as following: After PCR, mixing PCR products together with pCR ®4-TOPO® plasmid vector. Incubation at room temperature for 5 min (TOPO®- Cloning reaction), transforming into TOP10 E. coli cells, selection and analyzing colonies, isolation of plasmid DNA and sequencing.

Topo TA cloning kits are designed to clone Taq polymerase generated PCR products to ensure the presence of 3' A-overhangs. Hot start PCR strategy was used for producing DNA fragments. The PCR conditions are listed at table 3.12. PCR products are purified before performing TOPO cloning reaction in order to increase cloning efficiency by QIAquick PCR purification kit (QIAGEN, Germany).

Table 3.12 : Cloning PCR conditions.

cycles	Arc1384r-7f (Archaeal16S rDNA)	cycles	**pA-pH** (Bacterial 16SrDNA)
1	95°C 15 min	1	95°C 15 min
34	95°C 40 sec	34	95°C 30 sec
	55°C 45 sec		59°C 30 sec
	72°C 55 sec		72°C 45 sec
1	72°C 18 min	1	72°C 18 min
1	4°C oo	1	4°C oo

Chemically competent E. coli cells were used for the TOPO cloning reactions. 1 to 3 µl fresh PCR products, 0.5 µl of TOPO vector and 0.5 µl of salt solution are mixed. After cloning, pCR4-TOPO constructs heat-shocked into the competent E. coli cells. Soon after transformation, 500 µl of S.O.C medium is added to the cells and later 100 µl from each transformation is spread to prewarmed LB plates (containing 50 µg/ml kanamycin) for overnight incubation.

Fifty random colonies were selected for each sample, transferred to new LB broth incubate and incubated overnight. The plasmids were extracted using QIAGEN MiniPrep Plasmid isolation kit. (Qiagen, Inc., Valencia, CA).

Extracted DNA fragments are sequenced for nucleotide analyses. Big Dye terminator v3.1 premix (Applied Biosystems, CA, USA) was used for sequencing. Either M13 or T7 primer was used for one-strand amplification. In order to remove both the unincorpated dideoxynucleotides as well as salts from the reaction buffers the products are cleaned up prior to injection on to the sequencer (ABI, CA, USA).

3.2.6 Fingerprinting Analysis

BioNumerics software 5.1 was used to analyse DGGE patterns (Applied Maths, 2008). Similarity matrix and dendrogram of the DGGE profiles were generated based on the band based Dice correlation coefficient (band-based) and unweighted pair-group method arithmetic average (UPGMA).

3.2.7 Quantitative real time polymerase chain reaction (QPCR)

For each sample, duplicate sets of DNA samples were pooled and Q-PCR was carried out in triplicates with IQ Sybr green supermix. Archaeal amoA genes were quantified using the primers Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis et al., 2005). Quantitative- Real time PCR (Q-RT PCR) was performed with an iCycler iQ5 thermocycler and real-time detection system (Bio-Rad, Berkeley, CA). The PCR mixture contained 12.5 µl of the iQ Sybr green supermix (Bio-Rad, Berkeley, CA), 1 µl of each primer (0.4 µM), and 1 µl of each sample. PCR condition was 95 °C for 10 min, followed by 40 cycles of 60 s at 95 °C, 60 s at 56 °C, 30 s at 72 °C, with data capture for each cycle at 78 °C for 15 s. *N. maritimus* clones were used as standard controls (108 to 101 copies). Melting curve analysis for SYBR green assay was done

after amplification for the determination of nonspecific amplification if there were any. The quantification of bacterial amoA genes was performed using the primers amoA 1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA 2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe et al., 1997). The PCR mixture and conditions were identical to those used for the archaeal amoA genes. *Nitrosomonas sp*. clones were used as standard controls (10^8 to 10^1 copies). Melting curve analysis for SYBR green assay was done after amplification for the determination of nonspecific amplification if there were any.

3.2.8 Statistical analysis

The abundance of amoA genes of AOA and AOB in the WWTPs was assessed correlations to the plants' parameters including influent and effluent characteristics, removal efficiencies, and operational parameters. A calculation of nonparametric Spearman's rank correlation coefficient (rs) was performed with a two tailed p value, at p value of <0.05 being significant, using graphpad prism 5 software.

4. RESULTS AND DISCUSSION

Microorganisms play a crucial role in degradation and removal of pollutants in wastewater treatment systems. Microbial composition and its correlation with treatment efficiency is one of the most important and complex challenges on understanding of wastewater treatment systems where many scientists trying to answer. Many different microbial characterization methodologies have been developed to investigate wastewater treatment systems. However, due to unculturability of most of the microorganisms; molecular tools have been used to investigate microbial population and function in activated sludge systems. Microbial diversity is effected by the enviromental factors like: pH, salinity, temperature, dissolved oxygen, alkalinity and BOD. Conventional parameters are also important to understand community structure and composition. Since, molecular tools developed lately and complex relationships between microorganisms are still under investigation; those parameters have been used to engineer treatment systems for decades. Recent literature focuses more on the correlation of both microbial versus conventional data to be able to create more robust engineering of the systems.

4.1. Sampling

In this study, conventional parameters have been collected from chosen treatment plants where regulary controled, stable systems have been chosen. However, all the parameters have not been collected due to sampling time versus parameters chekced. Conventional parameters of various active sludge samples' are listed at table 4.3. Both, domestic and industrial wastewater systems have been chosen to investigate AOA occurence in different plant characteristics. Domestic wastewater ammonia loads were lower than the industrial wastewater as expected. Pakmaya and Istaç had highest amount of ammonia load.table 4.2-3 and figure 4.1-4 showed conventional parameters of baker's yeast (Pakmaya) and İSTAÇ.

Table 4.1 : Brewery wastewater treatment center conventional parameters for buffer tank, AT1 reactor, AT2 reactor, aerobic tank.

	Buffer tank		AT1 reactor		AT2 reactor		Aerobic tank (exit valve)				
Date	pH	COD (mg/l)	pH	VFA (mg/l)	pH	VFA (mg/l)	pH	COD (mg/l)	NH_4-N (mg/l)	NO_2-N(mg/l)	NO_3-N (mg/l)
Jan. 2009	8.31	8720	7.17	1512	7.38	956	7.7	920	6.4	2	86
Feb. 2009	8.73	4200	7.1	1194	n.d.	n.d.	7.67	760	6	20.7	24.4
Mar. 2009	6.95	7140	7.09	1992	7.35	1439	8.04	1230	25.4	8.4	5

Abbreviation: n.d., not determined

Table 4.2 : Leachate wastewater treatment plant conventional parameters for digester tank entrance valve and ultrafiltration entrance valve.

	Digester Tank						Ultrafiltration		
Date	pH	COD (mg/l)	NH_4-N (mg/l)	Nt (mg/l)	pT	T°C	Anoxic Oxyger	Anoxic	Aerobic
Dec. 2008	8.09	13790	2660.9	2840	23.3	28	0.06	8.58	8.54
Jan. 2009	8.1	13720	2829	3838	n.d.	24.5	0.09	8.55	8.48
Feb. 2009	8.4	10990	2931	3567	17.8	23.5	0.11	8.53	8.53
March 2009	8.23	14890	2263	2460	17.0	20.2	0.07	8.56	8.5

Abbreviation: n.d., not determined

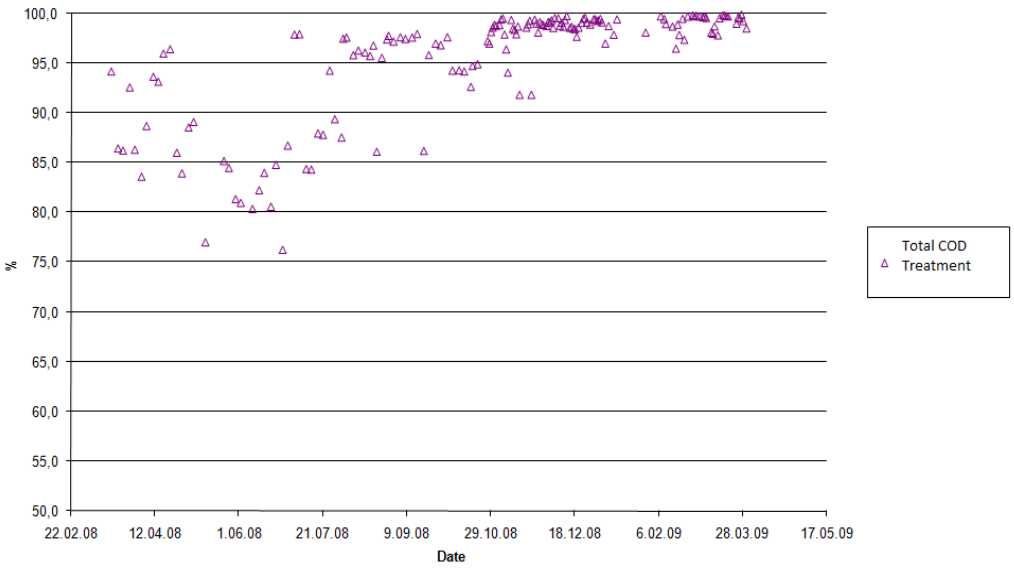


Figure 4.1 : Leachate wastewater treatment plant total COD treatment percentage.

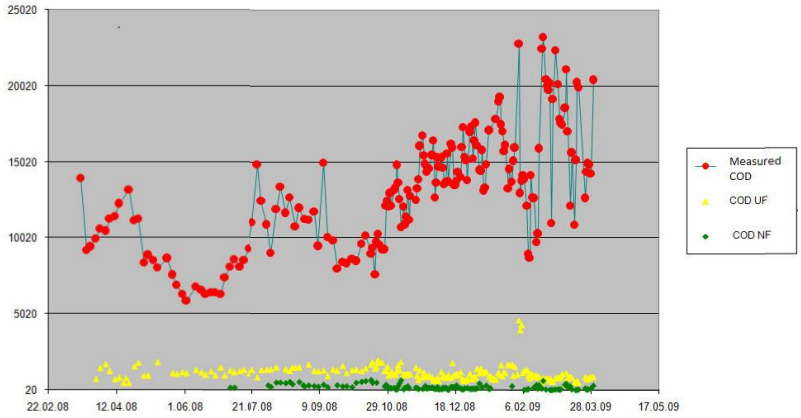


Figure 4.2 : Leachate wastewater treatment plant measured COD values.

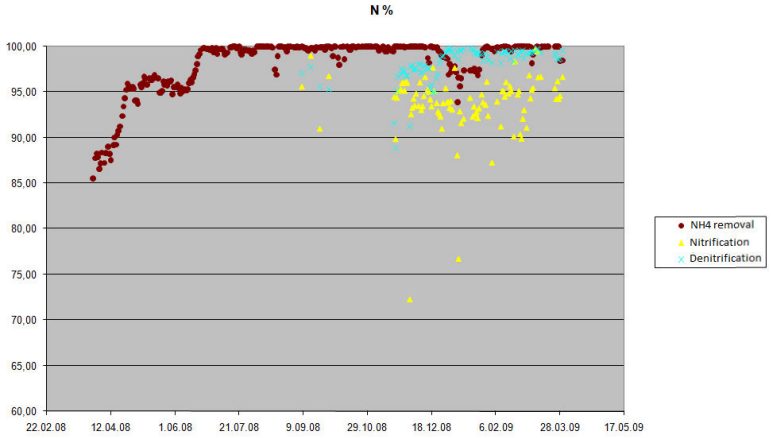


Figure 4.3 : Leachate wastewater treatment plant NH_4 removal, nitrification and denitrification percentage.

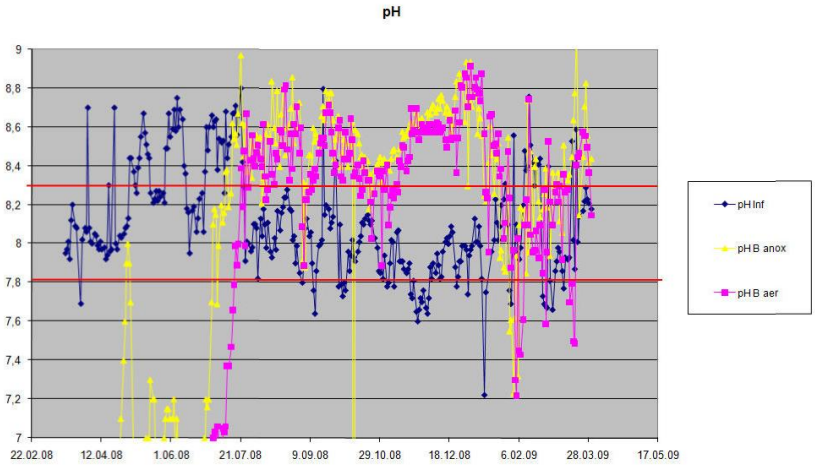


Figure 4.4 : Leachate wastewater treatment plant pH values of aerobic, anoxic and infiltration. pH values between 7.8 to 8.3 is the optimum conditions for nitrification.

4.2 DNA extraction results

DNA extraction of WWTPs active sludge samples were performed by Fastprep DNA extraction kit for soil (Qbiogene, Carlsbad, CA). DNA quantity and purity was checked both fluorometrically by Qubit (Invitrogen, MA) and also observed via running all the extracts on 1% agarose gel (100 volt, 30 minutes). DNA samples were diluted to 10ng using MilliQ water and stored at -20^{o}C for further experiments. Figure 4.5 shows the representative DNA extraction results from Leachate treatment plant samples. All DNA samples of Leachate treatment plant were intact .DNA samples stored at -20^{o}C for further analysis.

Figure 4.5 : DNA extraction of Leachate treatment plant electrophoresis results [A: Digester (pool 1) (32 ng/µl), Digester (pool 2) (110 ng/µl), C: Denitrification pool (66 ng/µl), D: Denitrification pool exit valve (68 ng/µl), E: Nitrification pool exit valve (62 ng/µl), F: Nitrification pool (72 ng/µl)].

Table 4.3 : Conventional parameters of WWTPs. Not: ND not determined.

	Samples	COD (mg/l)	BOD5 (mg/l)	NH_4N mg/L	pH	mM ammonia
1	baker's yeast (Pakmaya)	760	ND	1496	7,67	101,7 mM
2	ASAT 1(Antalya)	388	340	43	7.4	2.9 mM
3	ASAT 2(Antalya)	831	370	41	7.8	2.9 mM
4	İSTAÇ	22230	2900	1810	8.15	104,6 mM
5	Beer production Istanbul	2492	1744	1172	7,00	67.7 mM
6	Raki production	28000	13000	508	7,00	29.3 mM
7	Paper raw material production	500-1500	300-800	946	7,00	54.7 mM
8	Kırıkkale Petroleum refinery	452	236	5.98	ND	0.4 nM
9	Beer production Ankara	2000	1700	963	7	55.6 mM
10	İSKİ WWTP	600	300	723	7.3	41.8 mM
11	Gum production	ND	ND	NA	7,00	
12	Inorganic Chemicals Factory	266	200-300	409	7,00	23.6 mM
13	İzmit Petroleum refinery	ND	ND	207	8,00	12.2 mM
14	Eskişehir WWTP	1050	390	54	ND	3.6 mM
15	ESART A.Ş WWTP	2856	1400	45.5	7,6	3,1 mM
16	Paşaköy WWTP	670	268	41	ND	1.44 mM
17	Tuzla WWTP	840	295	230	7,68	0.63 mM
18	Sunflower oil production	ND	224	213	ND	14 mM

4.3 PCR results

4.3.1 Gradient PCR

Gradient PCR approach was used in order to determine optimal annealing temperature of PCR. Gradient PCR results of different PCR sets was shown Figure 4.6. PCR annnealing temperature for each reaction was determined according to gradient PCR results. Best annealing temperature and band length of PCR products were given at the methods section.

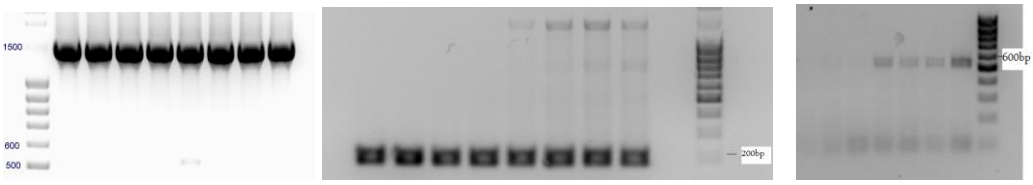


Figure 4.6a : Eubacterial Gradient PCR results (pA-pHr, Vf_GC-Vr, AOB amoA 1F-2R).

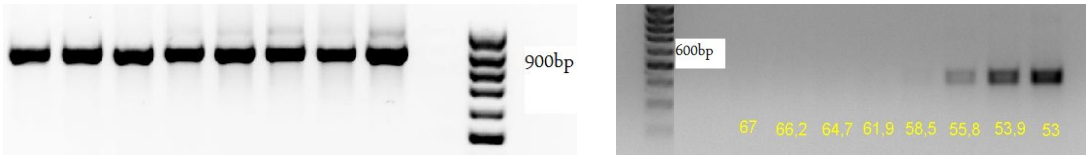


Figure 4.6b : AOB Gradient PCR results (16s rRNA1406R-519f, AOB amoA 1F_GC-2R).

Figure 4.6c : Archaeal Gradient PCR results (Arch 1017r-46f, ARCH-344F-GC-522r).

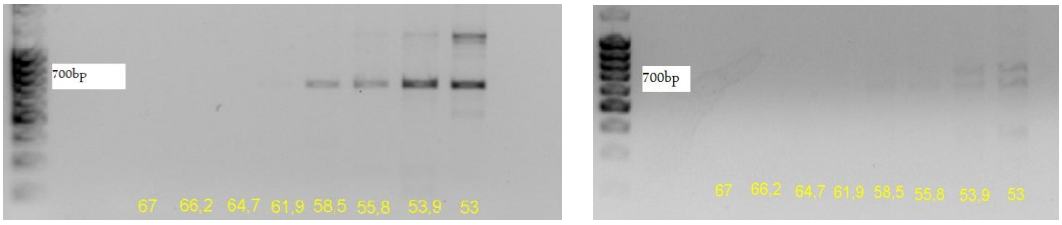


Figure 4.6d : AOA Gradient PCR electrophoresis results (AOA amoA F-R AOA amoA F_GC-R).

4.3.2 16S rRNA Archael and Bacterial gene PCR (Pakmaya WWTP)

Bacterial and archeal universal 16S rDNA primers are used to determine the bacterial and archaeal diversity of samples as mentioned in materials and methods section. (Figure 4.7-9).

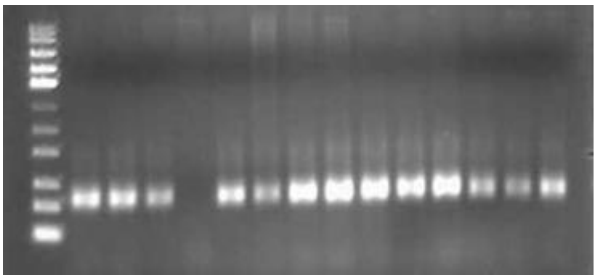


Figure 4.7 : Agarose gel electrophoresis of VfGc-Vr PCR results from Pakmaya Brewery wastewater treatment plant : Reactor AT1 bottom exit valve, Reactor AT1 middle exit valve ,Reactor AT1 top exit valve, Reactor AT2 bottom exit valve, Reactor AT2 middle exit valve, Reactor AT2 top exit valve, Nitrification pool, Denitrification pools exit valve, respectively.

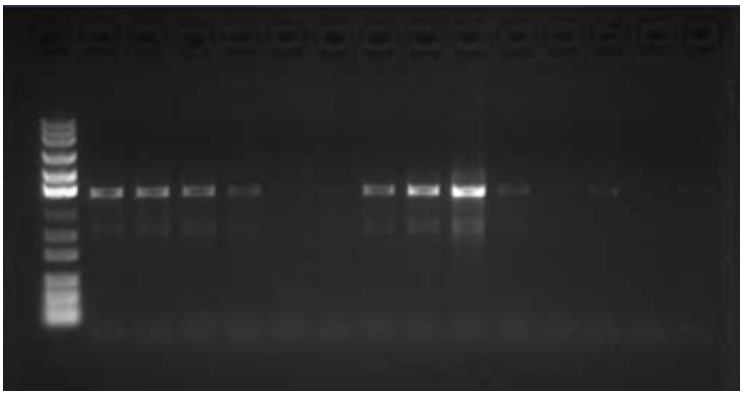


Figure 4.8 : Agarose gel electrophoresis photos of archaeal universal PCR results from Pakmaya Brewery wastewater treatment plant : Reactor AT1 bottom exit valve, Reactor AT1 middle exit valve ,Reactor AT1 top exit valve, Reactor AT2 bottom exit valve, Reactor AT2 middle exit valve, Reactor AT2 top exit valve, Nitrification pool, Denitrification pools exit valve, respectively.

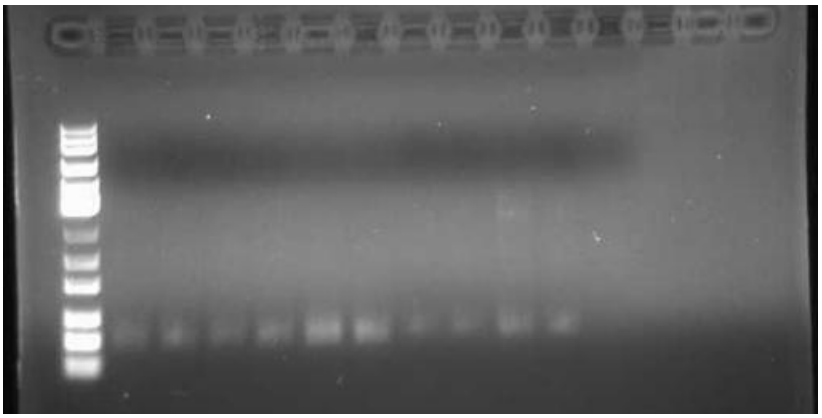


Figure 4.9 : Archaeal nested PCR results of Pakmaya. Reactor AT1 bottom exit valve, Reactor AT1 middle exit valve ,Reactor AT1 top exit valve, Reactor AT2 bottom exit valve, Reactor AT2 middle exit valve, Reactor AT2 top exit valve, Nitrification pool, Denitrification pools exit valve, respectively.

Bacterial and Archaeal PCR products with GC clamps of Pakmaya and Istaç samples were used in DGGE analysis for population dynamics study

4.3.3 AOB presence in WWTPs

Because of their key roles of in WWTPs, AOB presence has been investigated in samples. AOB_amoA_1F ve AOB_amoA_2R primers have been used to target functional AOB amoA gene and CTO primers used to target 16SrRNA gene for AOB. Figure 4.10-12 were shown AOB presence on the samples.

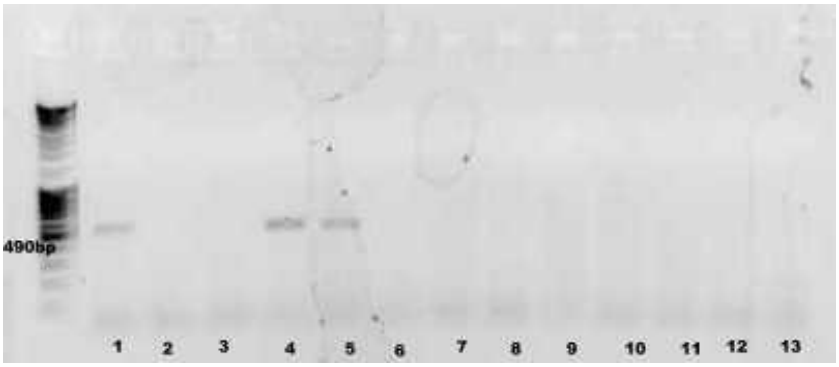


Figure 4.10 : AOB amoA PCR results samples 1-13. 1-Pakmaya, 2-ASAT 1(Antalya), 3-ASAT 2(Antalya),4-İSTAÇ denitrification reactors, 5-İSTAÇ nitrification reactors, 6-Beer production, 7-Raki production, 8-Pulp industry, 9-Kırıkkale Petrollium, 10-Ankara beer production, 11-İSKİ WWTP nitrification reactors, 12-Gum production, 13-Inorganic chemical plants.

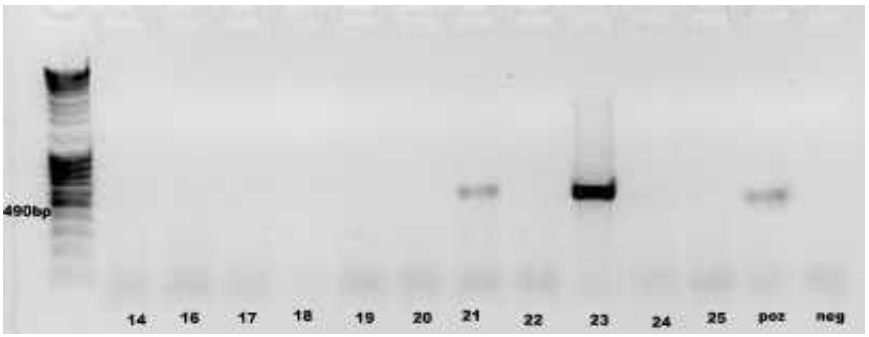


Figure 4.11 : AOB amoA PCR samples 14-25. 14-Oil production, 15-aquarium, 16-İzmit Petrollium refinary, 17-Eskişehir WWTP, 18-ESART A.Ş WWTP, 19-Paşaköy advanced biological treatment plant, 20-Marmara university BPR Fosforous remediation reactor, 21-Marmara university SBR reactor, 22-Marmara university biyofilm reactor, 23-Marmara university anammox reactor (system 1), 24-Marmara university anammox reactor (sistem 2), 25-Tuzla wastewater treatment system.

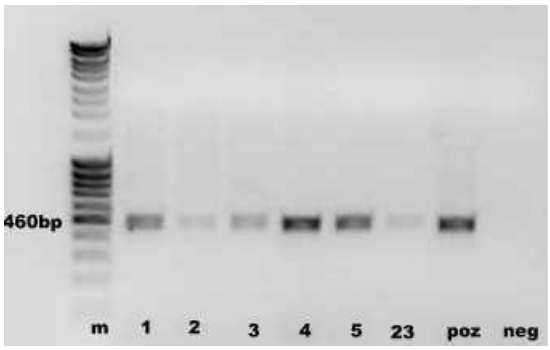


Figure 4.12 : CTO AOB 16S rRNA gene PCR results of some of the samples. 1-Pakmaya, 2-ASAT 1(Antalya), 3-ASAT 2(Antalya),4-İSTAÇ denitrification reactors, 5-İSTAÇ nitrification reactors, 23-Marmara university anammox reactor (system 1).

4.3.4 AOA presence in WWTPs and environmental samples

Two parallel studies were performed during this study to investigate AOA occurrence in both WWTPs and various environmental samples; (1) Activated sludge Samples from WWTPs reactors were screened for AOA occurrence; (2) Various environmental samples (soil/terrestrial ecosystems, thermal/hot springs, aquarium systems and freshwater/sediment bodies) were screened for AOA occurrence.

AOA abundance in environmental samples have been reported widely in literature whereas there are very few studies on AOA occurence in WWTPs (Park et al., 2006). One of the most interesting outcomes in this study was that of AOA presence found only in Antalya Lara domestic WWTP using conventional PCR approach (Figure 4.13); unlike wide occurence in the nature. Since there is no extensive literature found on AOA versus WWTPs; just one plant out of 19 was not a satisfactory result,

against wide distribution of AOA on nature samples. Therefore, PCR bias and low throughput has been taken into account and realtime PCR methodology was used to detect AOA amoA gene.

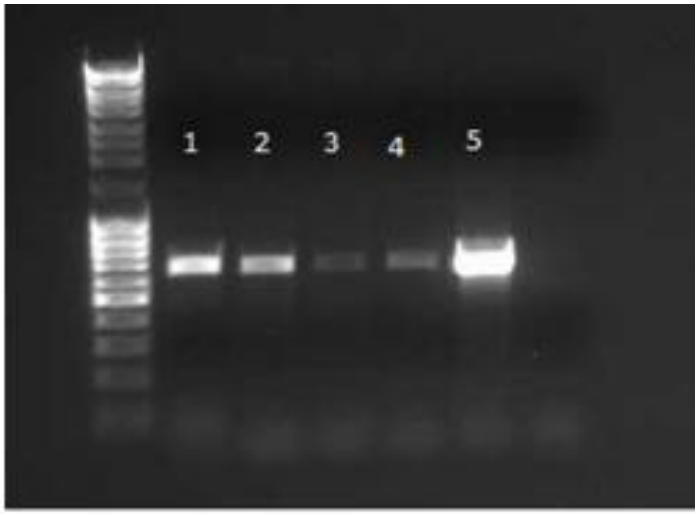
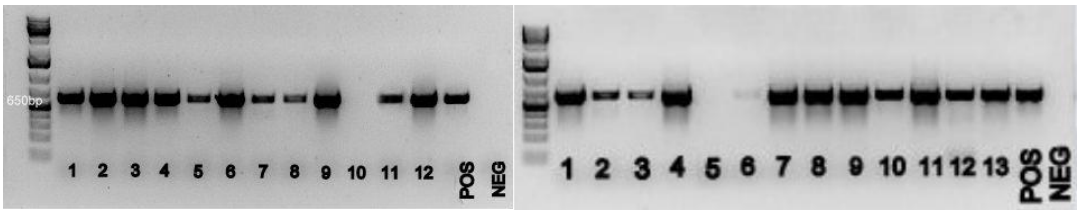


Figure 4.13 : AOA occurence in Antalya Lara WWTPs. 1-2 September 2009 aerobic pool samples, 3-4 December 2009 aerobic pool samples.

In parallel study over 50 environmental samples were screened for archaeal amoA gene occurrence, mainly pristine and agricultural soil samples. In addition to the soil samples thermal, terrestrial hot spring, aquarium and fresh water samples were also screened for AOA occurrence (Figure 4.14)



(a) (b)

Figure 4.14 : 1.5% Agarose gel electrophoresis photos of AOA amoA gene occurrence results. (a) soil samples (b) Hot/thermal springs samples.

4.4 Denaturant Gradient Gel Electrophoresis (DGGE)

DGGE analysis has been applied as mentioned in materials and methods section for both archeal and eubacterial microbial content of the samples. DGGE patterns were analyzed with BioNumerics software 5.1 [38]. Similarity matrix and dendrogram of the DGGE profiles were generated based on the band based Dice correlation coefficient (band-based) and unweighted pair-group method arithmetic average (UPGMA).

Pakmaya WWTP (figure 4.15) and İstaç Leachate treatment plant samples (figure 4.16) were further analysed via DGGE methodology. AT1 and AT2 reactor samples of Pakmaya WWTP (Figure 4.19-20.)were analysed via bionumerics software (Figure 21). Samples of this reactors branched separately which was shown that AT1 and AT2 have different microbial diversity. Archaeal and AOB DGGE results of these two plants were shown figure 4.15- 4.18.

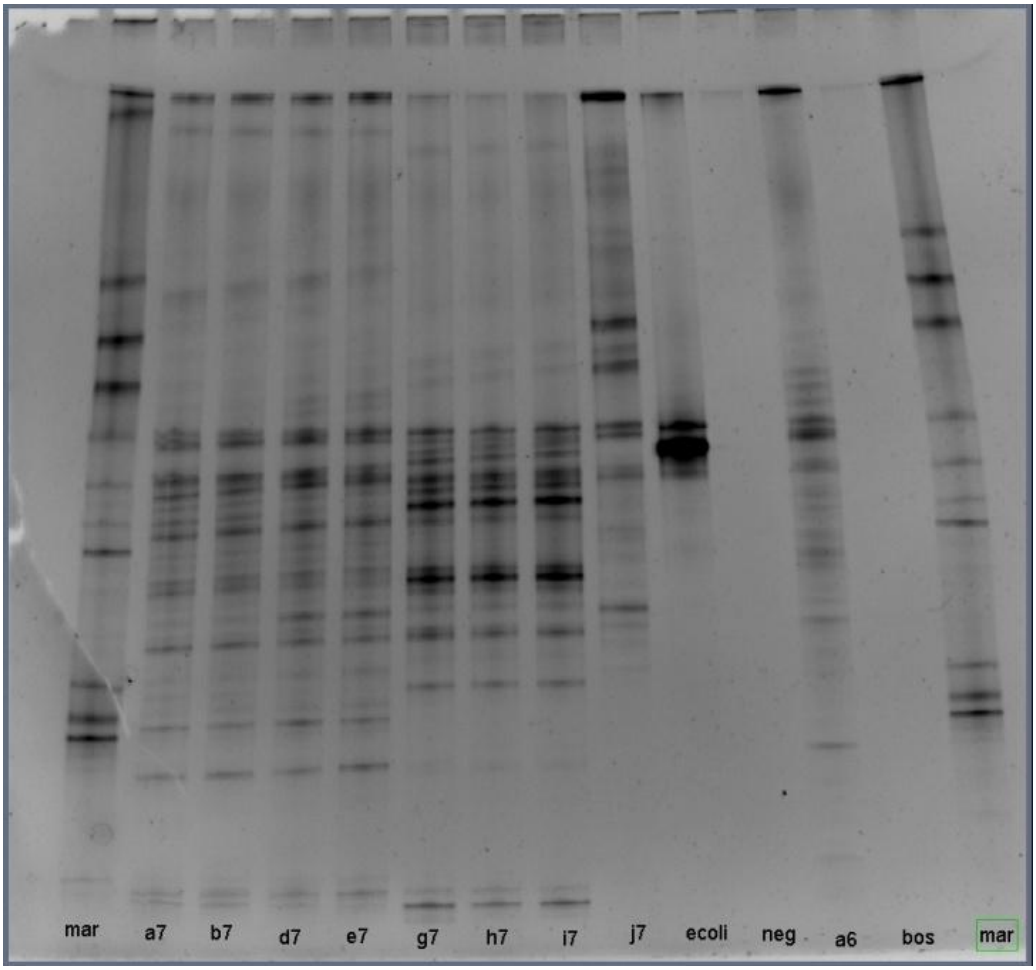


Figure 4.15 : Eubacterial DGGE (Vf_GC-Vr). AO6 series (İSTAÇ) gradient 35-65 %, gel 10%, 300 min., 180V . A: Digester (pool 1), B: Digester (pool 2), C: Denitrification pool, D: Denitrification pool, E: Nitrification pool exit, F: Nitrification pool, G: Ultrafiltration exit valve (UF), H: Nanofiltration exit valve (NF) , respectively.

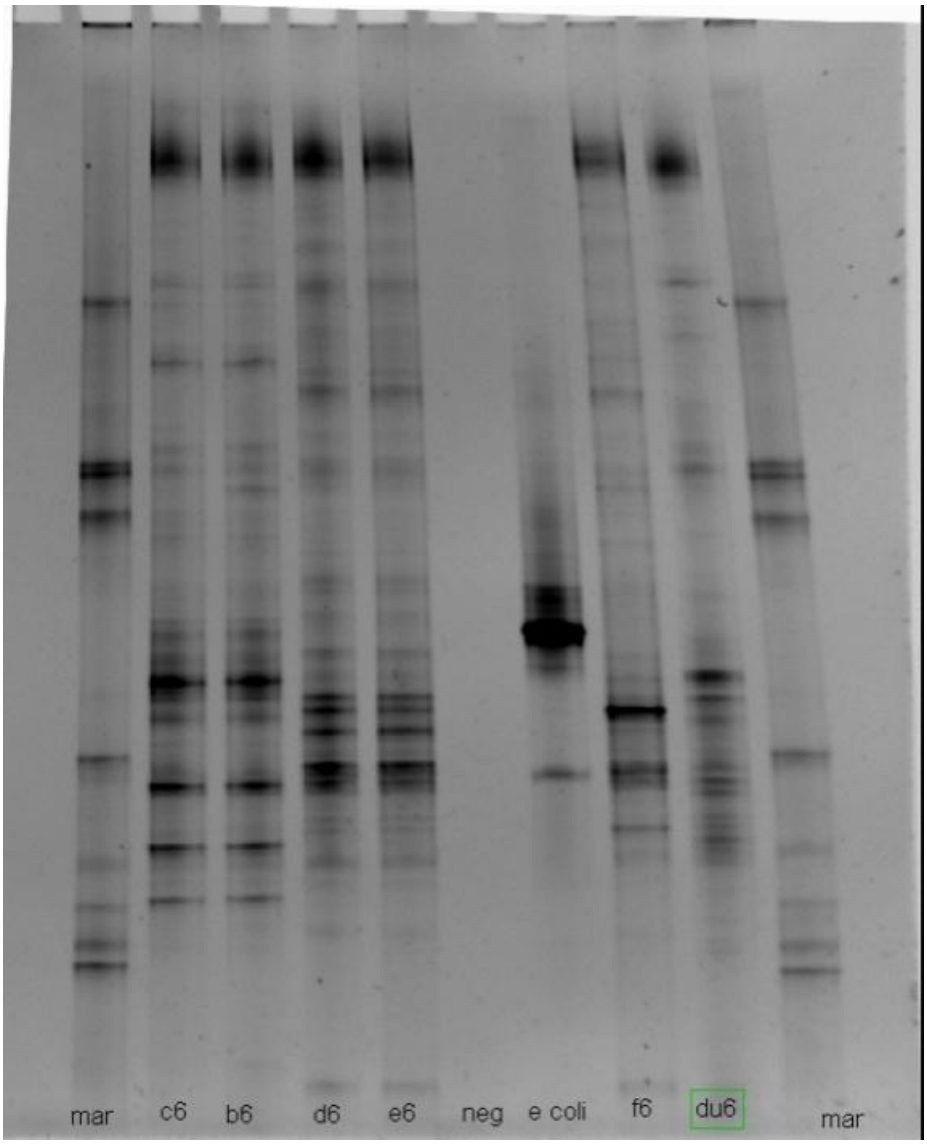


Figure 4.16 : Eubacterial DGGE of Vf_GC-Vr PCR products. AO7 series (PAKMAYA), denaturant gradient 35-65 %, gel 10%, 300 min., 180V A: Reactor AT2 bottom exit valve, B: Reactor AT2 middle exit valve, C: Reactor AT2 top exit valve, D: Reactor AT1 bottom exit, E: Reactor AT1 middle exit, F: Reactor AT1 top exit valve, G: Denitrification pool (pool 1) valve (anaerobic reactor), H: Denitrification pool exit valve (pool 4), I: Sedimentation- circulation tank, J: Buffer tank exit valve, K:Raw waste, L: Anaerobic reactor exit valve , respectively.

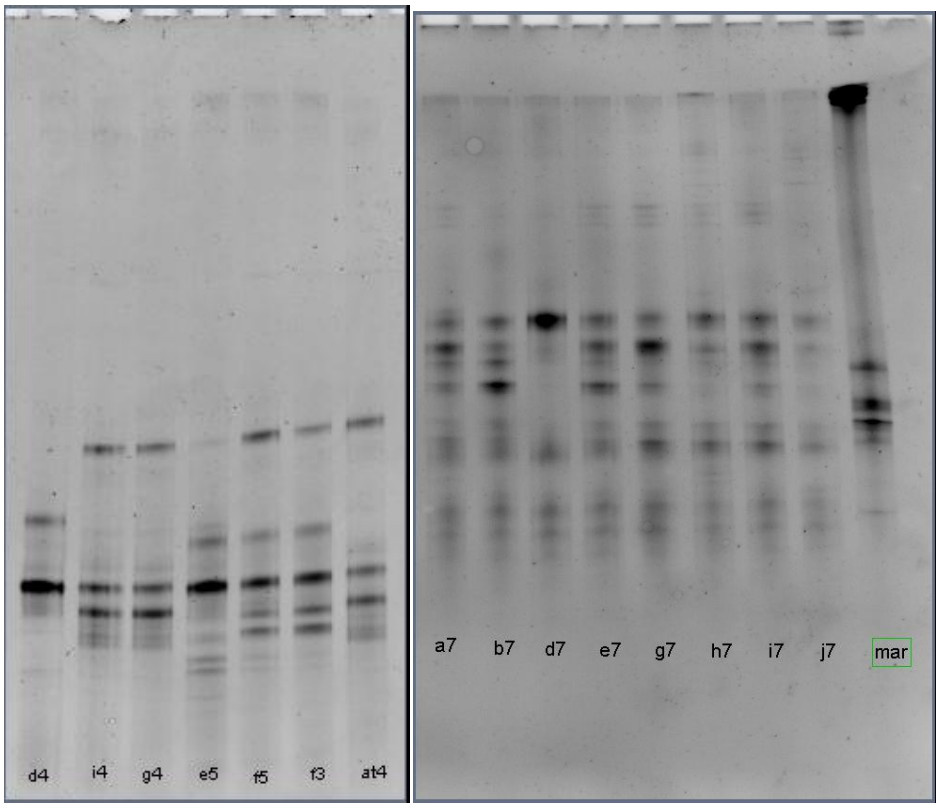


Figure 4.17 : Archeal DGGE (Arch344F_GC-Univ522) AO4 series (İstaç) and AO7 series (PAKMAYA) A: Reactor AT2 bottom exit valve, B: Reactor AT2 middle exit valve, C: Reactor AT2 top exit valve, D: Reactor AT1 bottom exit, E: Reactor AT1 middle exit, F: Reactor AT1 top exit valve, G: Denitrification pool (pool 1) valve (anaerobic reactor), H: Denitrification pool exit valve (pool 4), I: Sedimentation-circulation tank, J: Buffer tank exit valve, K:Raw waste, L: Anaerobic reactor exit valve A: Digester (pool 1), B: Digester (pool 2), C: Denitrification pool, D: Denitrification pool, E: Nitrification pool exit, F: Nitrification pool, G: Ultrafiltration exit valve (UF), H: Nanofiltration exit valve (NF), respectively.

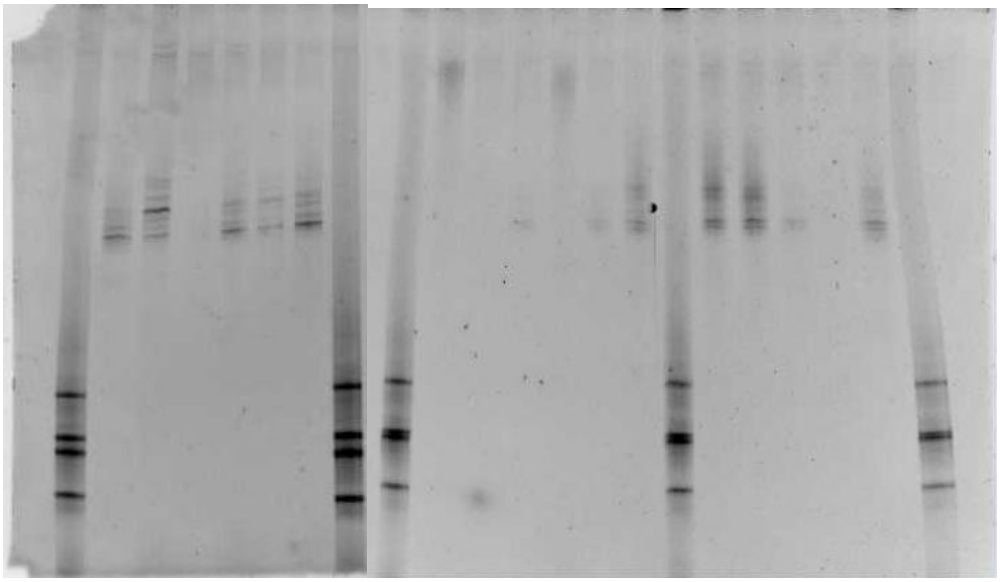


Figure 4.18 : AOB amoA DGGE of (1F_GC-2R) Denitrification pool (pool 1) valve (anaerobic reactor), H: Denitrification pool exit valve (pool 4), C: Denitrification pool, D: Denitrification pool, E: Nitrification pool exit, F: Nitrification pool, respectively.

Pakmaya WWTP (figure 4.10) and İstaç Leachate treatment plant samples (figure 4.11-12) were further analysed via DGGE methodology. DGGE of AOB amoA 1F_GC-2R PCR products were shown figure 4.13.

AT1(figure 4.14) and AT2 (figure 4.15) reactor samples of Pakmaya WWTP were analysed bionumerics software (Figure) and samples of this reactors branched separately. These data was shown that AT1 and AT2 have different micronbial diversity.

DGGE patterns were analyzed with BioNumerics software 5.1 [38]. Similarity matrix and dendrogram of the DGGE profiles were generated based on the band based Dice correlation coefficient (band-based) and unweighted pair-group method arithmetic average (UPGMA).

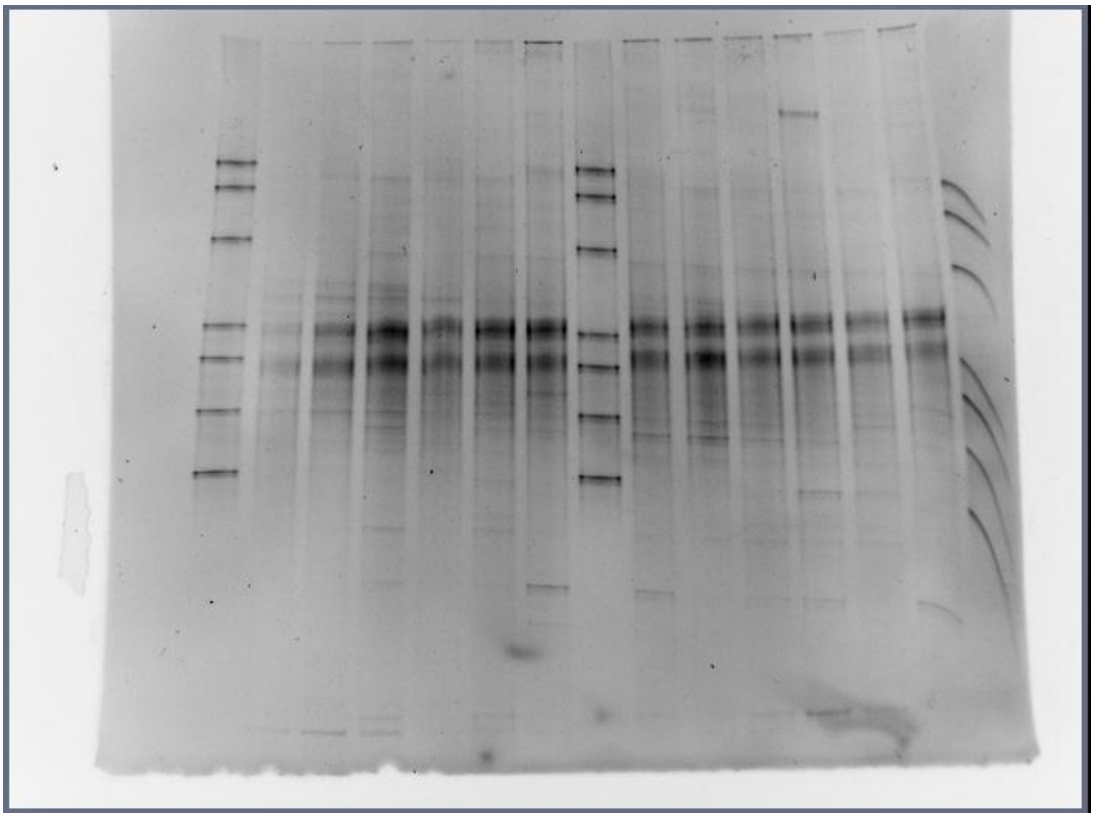


Figure 4.19a : Eubacterial (GCVf-Vr) DGGE result of Pakmaya AT1 Reactor figure 1.

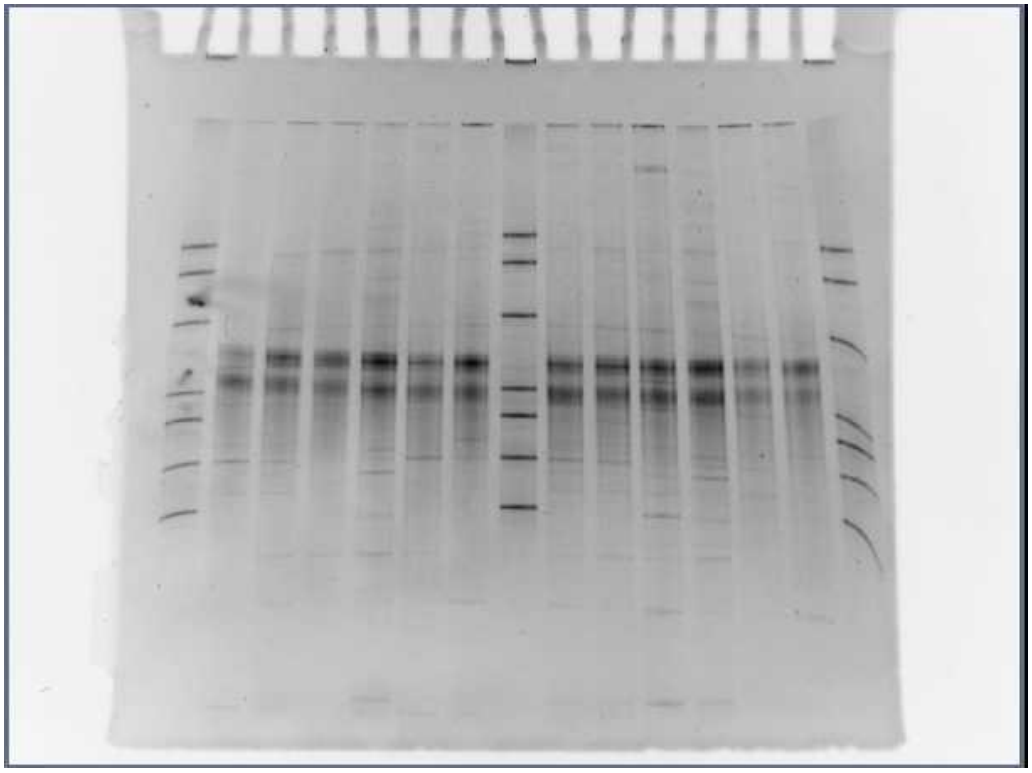


Figure 4.19b : Eubacterial (GCVf-Vr) DGGE result of Pakmaya AT1 Reactor figure 2.

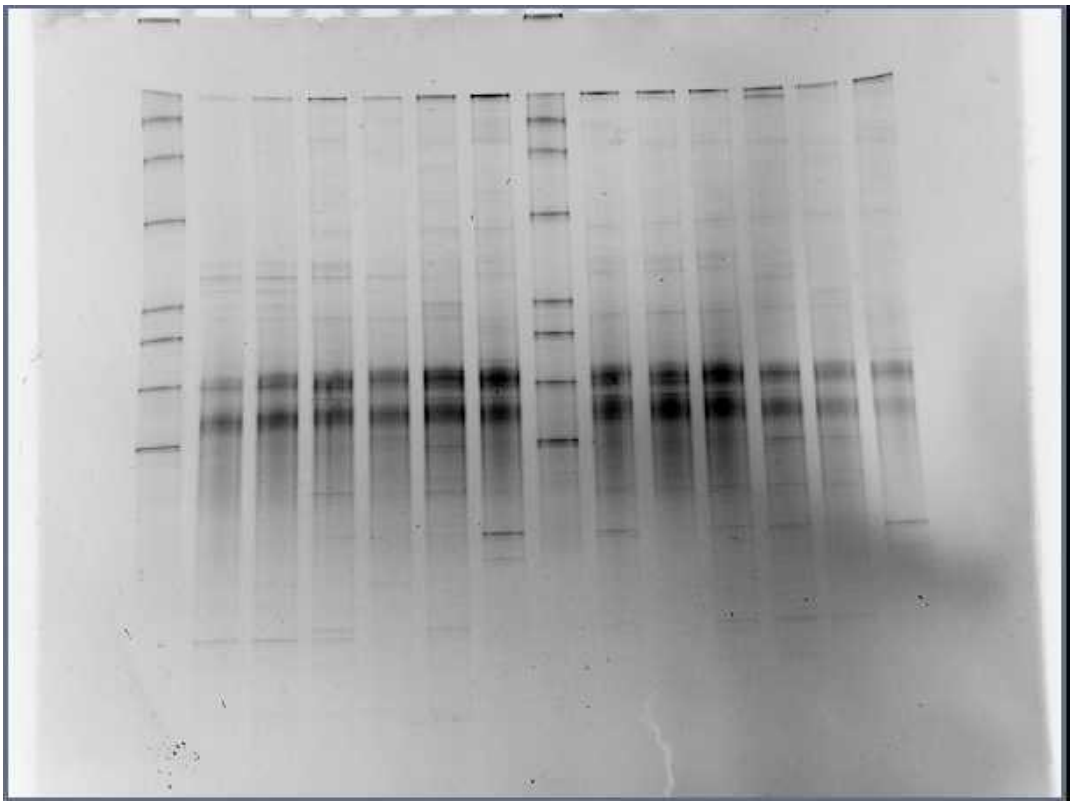


Figure 4.20a : Eubacterial (GCVf-Vr) DGGE result of Pakmaya AT2 Reactor figure 1.

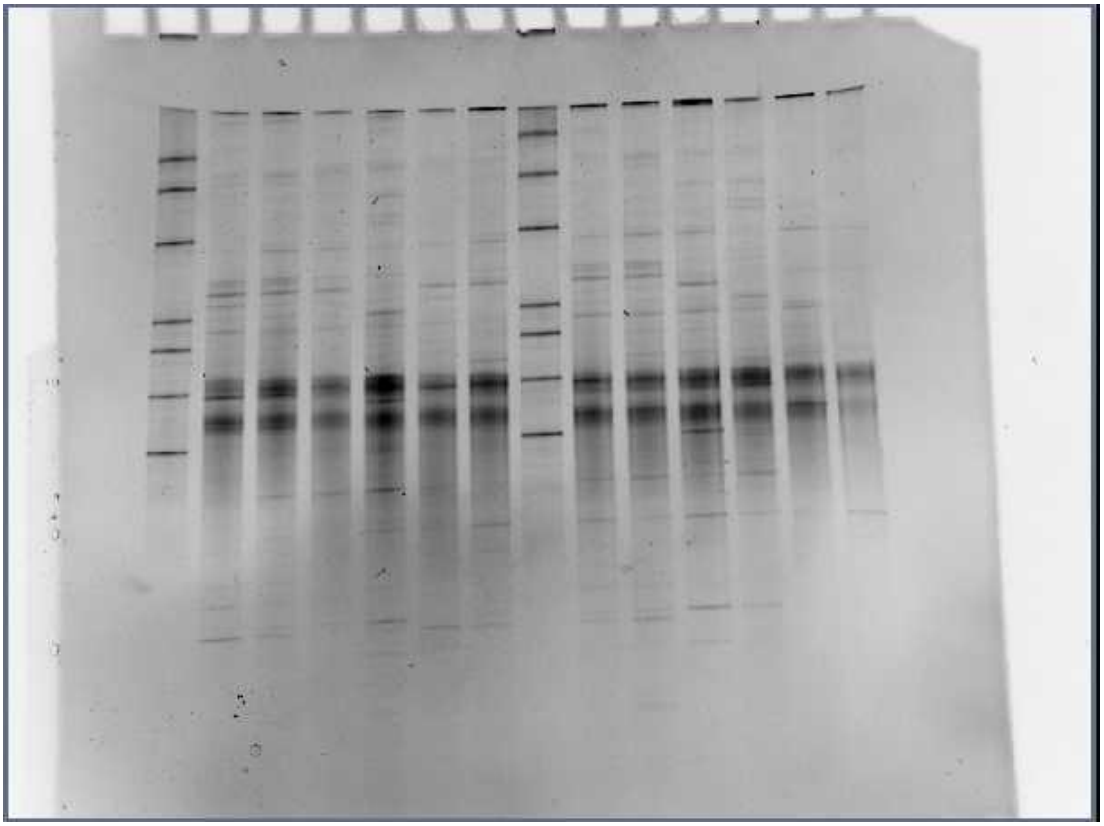


Figure 4.20b : Eubacterial (GCVf-Vr) DGGE result of Pakmaya AT2 Reactor figure 2.

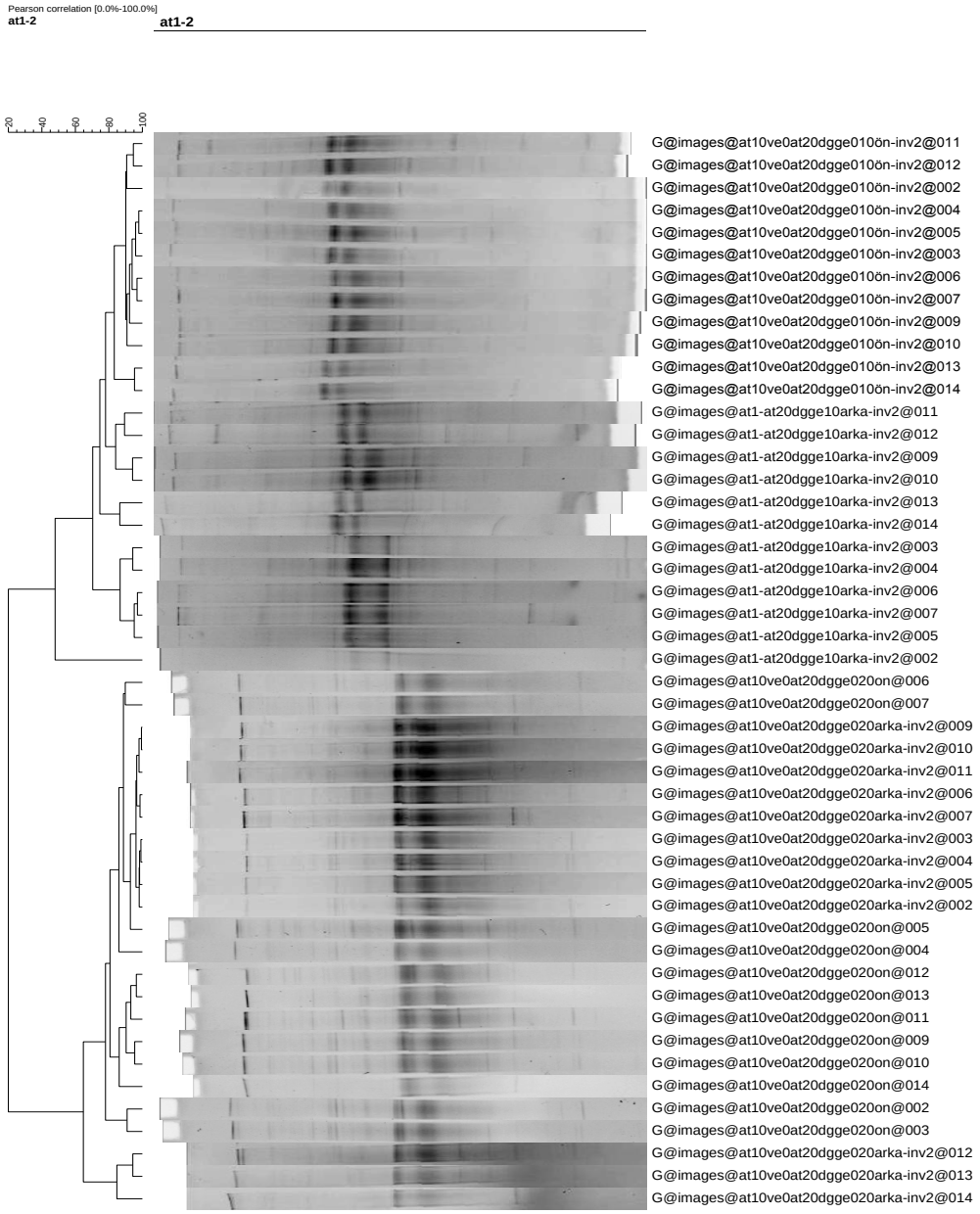


Figure 4.21 : Similarity of the DGGE banding patterns (Pearson correlation coefficient, UPGMA) for the bacterial communities for pakmaya AT1 and AT2 reactor samples.

According to bionumeric comparison AT1 and AT2 samples clustered distantly on the phylogenetic tree which means bacterial diversity of those reactor's samples were different. Both reactors also have different operational parameters and different operational functions.

4.5 Cloning

In order to determine bacterial and archaeal species in our samples, clone libraries were constructed by amplifying a certain region of genomic DNA specific to bacterial and archaeal domains. 16S rRNA gene serves as a molecular clock and is suitable for phylogenetic studies. Primers used to targetwider span to this gene named pA-pH and Arch 1017r-46f were used for PCR prior to clone library analysis. Topo TA Cloning reaction was performed as mentioned in the methods part. Following the cloning reactions, analysis of the transformants was accomplished via PCR using Vf-GC- vR primer set. PCR products were checked with DGGE analysis and as much clones as were picked for further sequencing analysis. DGGE results of cloned samples were shown at figure 4.22. In order to determine AOA species, AOA amoA-F-R primers which are used to target to this gene were used for PCR prior to clone library analysis.

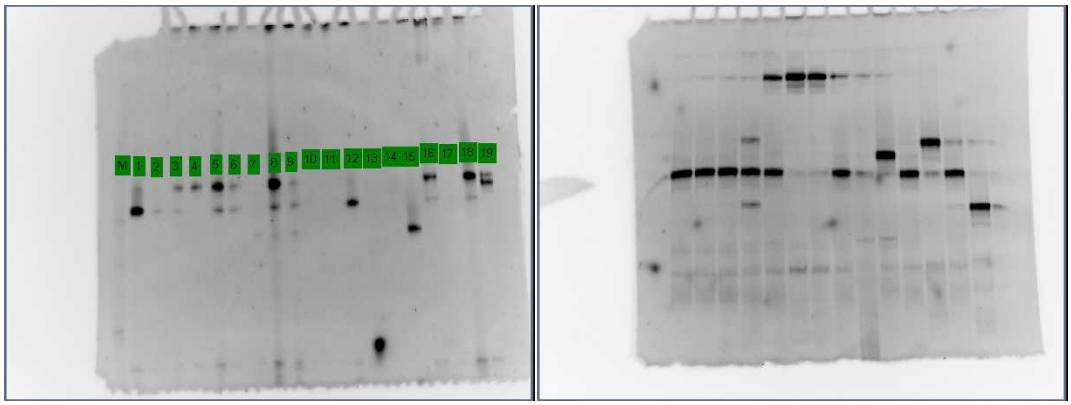


Figure 4.22 : DGGE pictures of transformants.

4.6 Sequencing PCR.

For detailed analysis of our samples in terms of functional comments and correlations, identification of affiliated phylotopes (sequence data) is necessary.. Sequence PCR procedure was used for this purpose according to kit manual as mentioned in methods section. T7 primer was used as a primer in this reaction. PCR products were purified using sodium acetate buffer and ethanol then dissolved in formamide and analysed in DNA sequence analyzer (ABI, USA). Sequence data was given in supplement 1.

Species found (Table 4.4).

The genus Thauera is Gram-negative bacteria in the family Rhodocyclaceae of the class b-Proteobacteria and comprises nine species. Most of them were known as nitrate-reducing bacteria Mao et al. 2012. Thauera uses aromatic hydrocarbons under anoxic conditions and contains unique pathways for the degradation of these aromatic compounds.

Thiobacter are gram-negative, motile and rod-shaped thermophilic aerobe. They are chemolithoautotrophicly reduced sulfur compounds as electron donors and with oxygen as an electron acceptor using CO_2 as a carbon source. Phylogenetically affiliated to the β-Proteobacteria (Hiramaya et al. 2005).

Nitrobacter hamburgensis is a gram-negative facultative chemolithoautotroph that conserves energy from the oxidation of nitrite to nitrate. Nitrobacter are belongs to alphaproteobacterium (Shawn et al. 2008). Comamonas could be a novel denitrifier isolated from a denitrifying reactor treating landfill leachate (Etchebehere et al. 2001). Rhodobacter sphaeroides is a group of bacteria that can obtain energy through photosynthesis.

Actinobacteria are one of the largest group within the domain Bacteria, The Actinobacteria are widely distributed in both terrestrial and aquatic ecosystems and have varied metabolic mechanisms. They are primarily chemoheterotrophs. They also can synthesize secondary metabolites.

Table 4.4 : Bacterial diversity of pakmaya aerobic pool sample.

Clone No	Closest RDP similarity	BLAST number	Clone number	RDP similarity percentage
clone 1	Thauera sp. MZ1T	CP001281	16	0.975
clone 5	Thiobacter subterraneus (T); C55	AB180657	2	0.770
clone 7	Peptostreptococcus sp. S1	AF044946	2	0.708
clone 12	Nitrobacter hamburgensis X14	CP000319	2	0.616
clone 13	Catabacter sp. YIT 12065	AB490809	2	0.700
clone 26	Comamonas sp. DJ-12	AY600616	8	0.759
clone 18	Alcaligenes sp. 3013	AM110970	3	0.799
clone 20	Rhodobacter sp. KYW73	FJ997595	1	0.876
clone 24	Ralstonia sp. HAB-01	AB051680	1	0.760
clone 29	actinobacterium CH9	FN554394	1	0.679
clone 35	Bacteroidetes bacterium ONB11	FN554384	1	0.705
clone 38	Ectothiorhodospira sp. 'Bogoria Red'; RB1	AF384207	1	0.671
clone 39	Acetobacterium sp. Mic42c02	AB546244	1	0.648

4.7 Phylogenetic analysis

Sequence data were analysed and homology searches were made using BLAST server of NCBI. Nucleotide sequences were queried against nucleotide sequence database (blastn). Bacterial diversity of pakmaya aerobic pool sample was listed in table 4.2. After determining the closest GenBank matches, phylogenetic analyses were conducted using MEGA version 4. A phylogenetic tree was constructed using neighbour analysis avalible on Ribosomal Database Project.(RDP) Constructed phylogenetic tree was shown on figure 4.23.

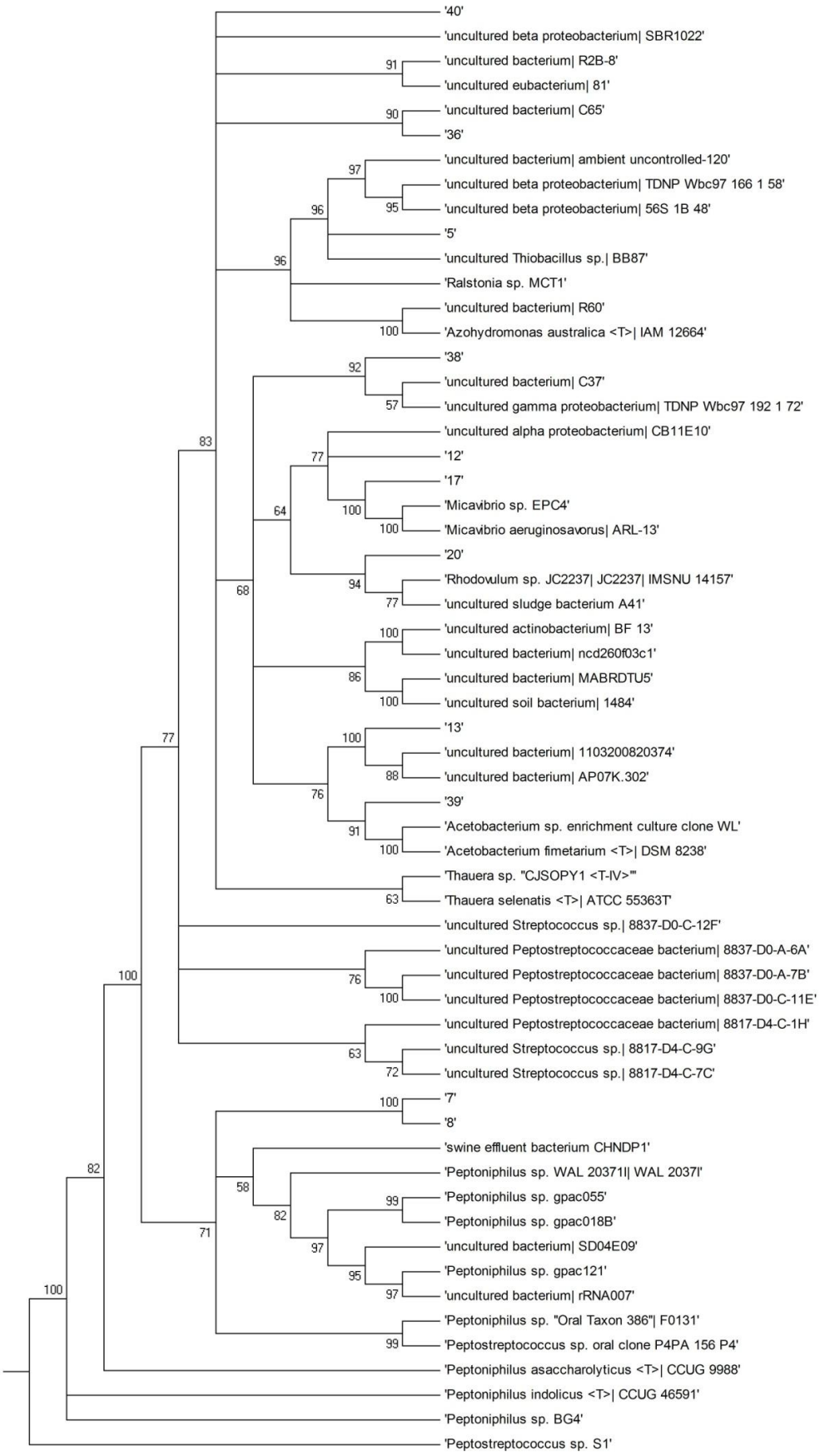


Figure 4.23 : Constructed phylogenetic tree of sequenced data (16S rRNA gene).

AOA AmoA phylogenetic tree:

Cloning library was constructed for AOA AmoA gene. Some clones were selected and sequenced. Sequence data were analysed and homology searches were made using BLAST server of NCBI. Nucleotide sequences were queried against nucleotide sequence database (blastn). After determining the closest GenBank matches, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4. A phylogenetic tree was constructed using weighbor analysis available on Ribosomal Database Project. Constructed phylogenetic tree was shown on figure 4.24.

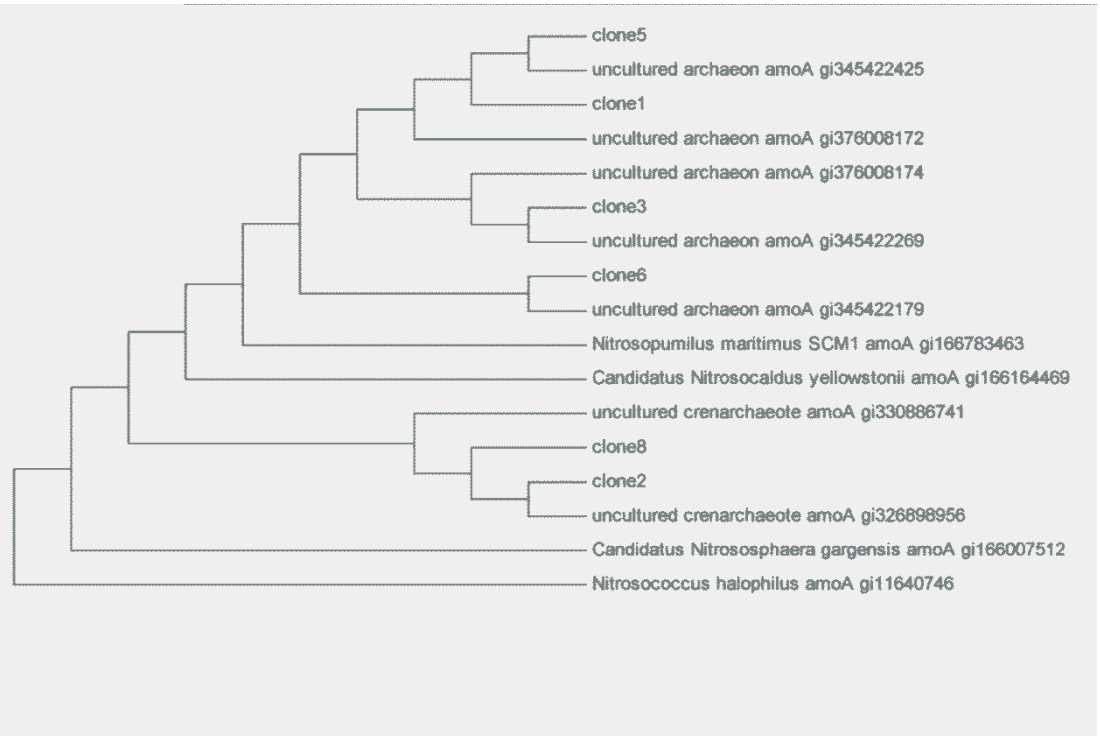


Figure 4.24 : ASAT Lara AOA amoA gene phylogenetic tree.

Archaeal amoA sequences showed 94–100% identity to the previously reported nucleotide sequences in the GenBank database. The MEGA program package (version 5.0; Tamura et all 2011 http://www.megasoftware.net/) was used for the phylogenetic analysis. Our nine AOA amoA sequences and reference sequences were aligned and calculated using three different methods (the distance matrix, maximum parsimony, and maximum likelihood),and the results show the same grouping of AOA clusters and tree topology as those computed using different approaches. The

phylogenetic tree was shown in figure 2. Most of the sequences retrieved fell into a marine lineage (Group 1.1a). Few sequences were found to belong to a soil lineage (Group 1.1a), but no sequence related to a thermophilic AOA lineage.

4.8 Quantitative-Real Time PCR

Archaeal amoA genes were quantified using the primers Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis et al., 2005). Quantitative- Real time PCR (Q-RT PCR) was performed with an iCycler iQ5 thermocycler and real-time detection system (Bio-Rad, Berkeley, CA)(figure 4.20). The PCR mixture for both reactions with a volume of 25 µl contained 12.5 µl of the iQ Sybr green supermix (Bio-Rad, Berkeley, CA), 1 µl of each primer (0.4 µM), and 1 µl of each sample. The PCR condition was 95 °C for 10 min, followed by 40 cycles of 60 s at 95 °C, 60 s at 56 °C, 30 s at 72 °C, with data capture for each cycle at 78 °C for 15 s. *N. maritimus* clones are used as standard controls (10^8 to 10^1 copies). Melting curve analysis for SYBR green assay was done after amplification for the determination of nonspecific amplification if there were any.

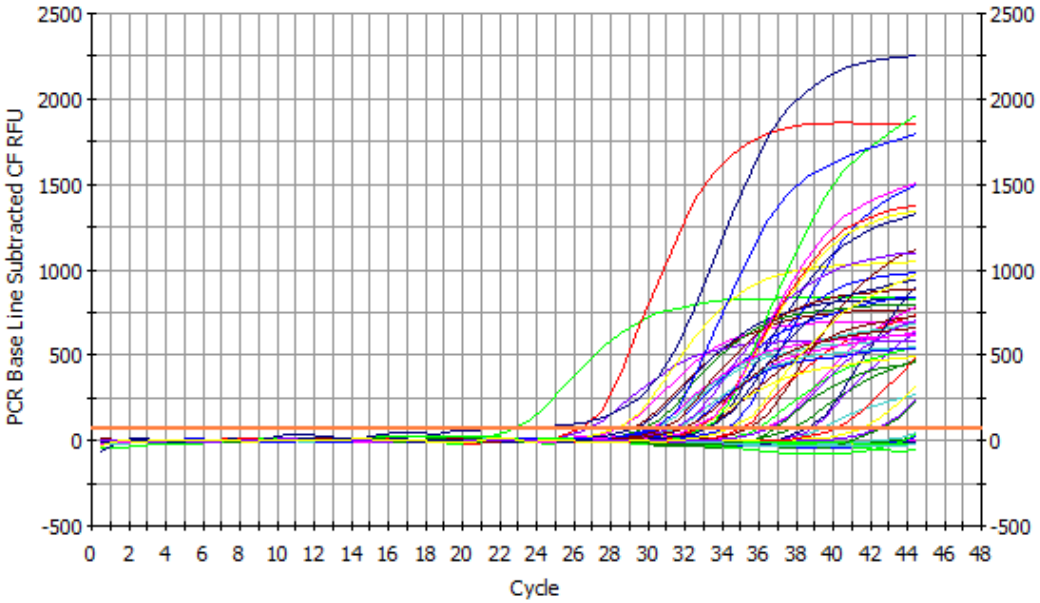


Figure 4.25 : Figure14. PCR Amp/Cycle Graph for AOA amoA gene.

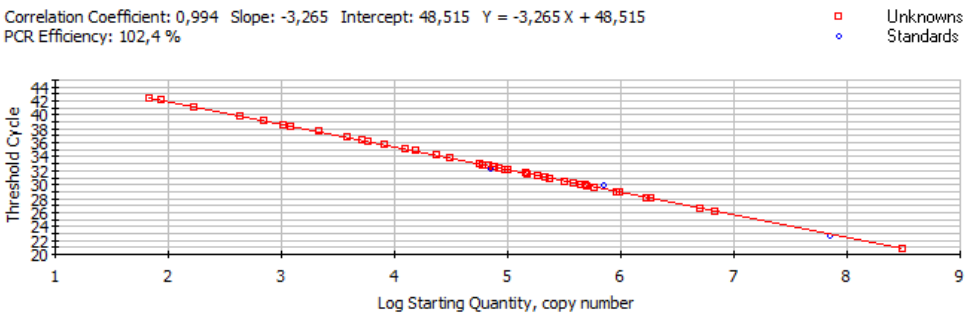


Figure 4.26 :. Standard Curve Graph for AOA amoA gene.

Archaeal amoA specific Q-RT PCR assay was very consistent, as shown by the strong inverse linear relationship between the threshold cycle numbers and the copy numbers of amoA gene (R^2 = 0.994). The amplification efficiencies were 102.4%, with slopes of -3.265 (figure 4.25-26).

The quantification of bacterial amoA genes was performed using the primers amoA 1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA 2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) (Rotthauwe et al., 1997). The PCR mixture and condition were identical to those used for the archaeal amoA genes. The PCR mixture was with a volume of 25 µl contained 12.5 µl of the iQ Sybr green supermix (Bio-Rad, Berkeley, CA), 1 µl of each primer (0.4 µM), and 1 µl of each sample. The PCR condition was 95 °C for 10 min, followed by 40 cycles of 60 s at 95 °C, 60 s at 56 °C, 30 s at 72 °C, with data capture for each cycle at 78 °C for 15 s (4.22). *Nitrosomonas spp*. clones are used as standard controls (10^8 to 10^1 copies). Melting curve analysis for SYBR green assay was done after amplification for the determination of nonspecific amplification if there were any.

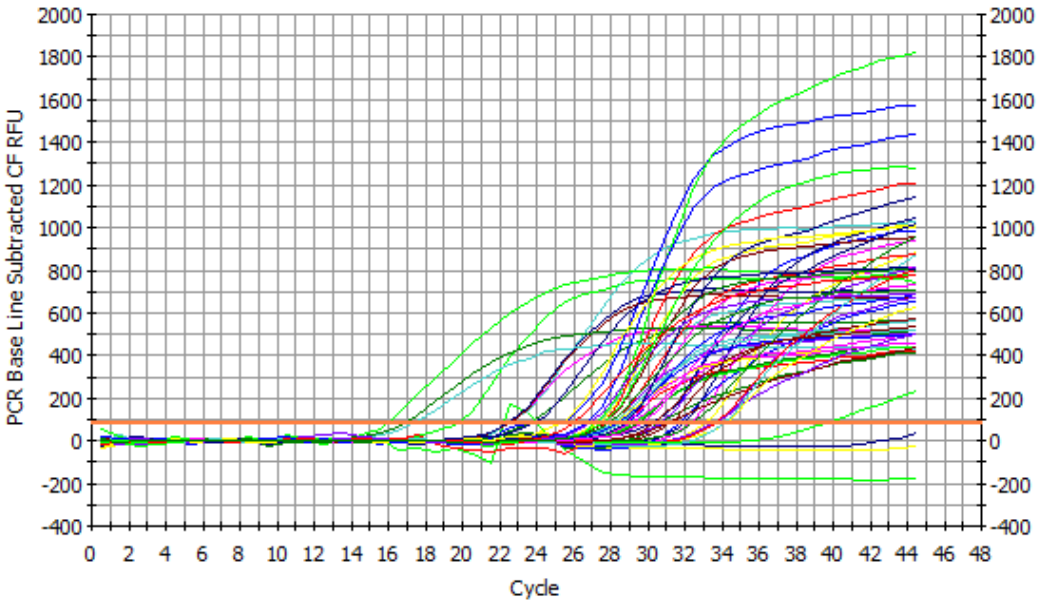


Figure 4.27 : PCR Amp/Cycle Graph for AOB amoA gene.

Bacterial amoA specific Q-RT PCR assay was very consistent, as shown by the strong inverse linear relationship between the threshold cycle numbers and the copy numbers of amoA gene (R^2 = 0.991). The amplification efficiencies were 104.0%, with slopes of -3.231 (Figure 4.27-28).

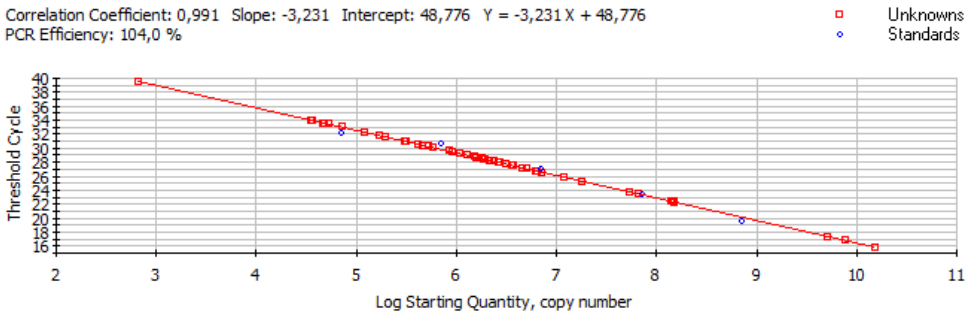


Figure 4.28 : Standard Curve Graph for AOB amoA gene.

The WWTPs in this study were selected wide range of different domestic and industrial characteristics. Six domestic and ten industrial engineered wastewater treatment systems were studied. Ammonium concentrations are between 6,7 - 104,6 mM in industrial WWTPs, whereas 0,94 – 1,44 mM in domestic WWTPs. Quantitative real time PCR showed that comparative abundance results of the AOA and AOB amoA genes varied among samples (figure 4.29 and table 4.5). ASAT 1 WWTP has the highest AOA amoA gene copy ($5.83X10^7 \pm 1.31X10^7$), whereas AOB amoA gene copy was $5.17X10^4 \pm 1.4X10^4$). ASAT 1 is a domestic WWTP with 1.44

mM ammonia concentration. ISTAC leachate WWTP, which is contained the higher ammonia level (104.6 mM), had AOA amoA gene copy ($1.39X10^{4}\pm9.89X10^{3}$) less than four order AOB amoA gene copy ($1.34X10^{8}\pm7.61X10^{7}$).

WWTPs, whose were contained the higher levels of ammonium (104,6 ,101,7) possessed the AOB amoA genes more than three orders of magnitude of the AOA amoA genes occurred. Industrial WWTPs, whose contained the medium levels of ammonium (67,7 and 54,7mM) possessed almost equal the AOB and AOA amoA gene copies. This result is contrary to those of the municipal WWTPs, whose contained lower levels of ammonium(0.35, 6.7mM) possessed the AOA amoA genes more than AOB amoA genes at least one magnitude. Other municipal and industrial WWTPs possessed equal amount of both groups.

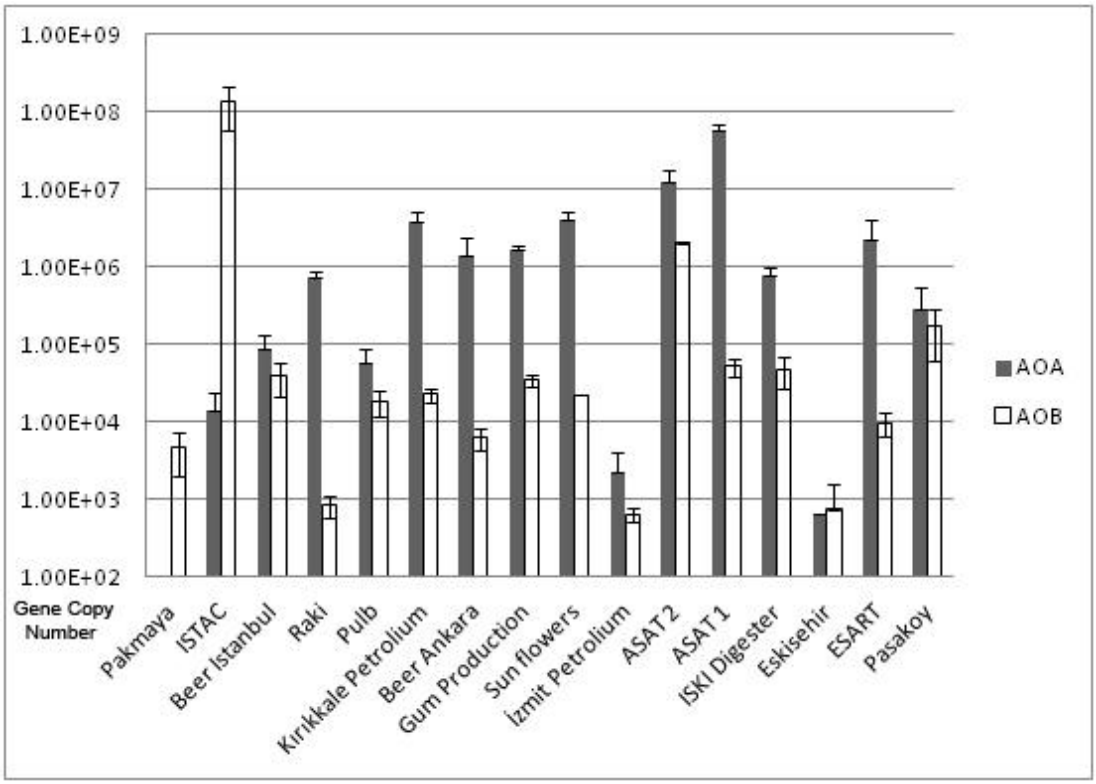


Figure 4.29 : AOA and AOB amaA gene copy numbers.

Table 4.5 : AOA and AOB amoA gene copy numbers.

	AOA gene copy numbers	AOB gene copy numbers	NH4N
Sample	**SQ Mean**	**SQ Mean**	mM
Pakmaya	N/A	4.71E+03	101.70
ISTAC	1.39E+04	1.34E+08	104.60
Beer Istanbul	8.98E+04	3.95E+04	67.70
Raki	7.46E+05	8.57E+02	29.30
Pulb	5.84E+04	1.81E+04	54.70
Kırıkkale Petrolium	3.74E+06	2.27E+04	N/A
Beer Ankara	1.44E+06	6.22E+03	55.60
Gum Production	1.67E+06	3.43E+04	N/A
Sun flowers	4.01E+06	2.25E+04	6.70
İzmit Petrolium	2.24E+03	6.47E+02	12.20
ASAT 2	1.24E+07	2.08E+06	1.51
ASAT 1	5.83E+07	5.17E+04	1.44
ISKI Digester	7.79E+05	4.86E+04	41.80
Eskisehir	6.70E+02	7.46E+02	0.94
ESART	2.26E+06	9.79E+03	N/A
Pasakoy	2.80E+05	1.73E+05	1.44

4.9 Statistical analysis

The abundance of amoA genes of AOA and AOB in the WWTPs was assessed correlations to the plants' parameters including influent and effluent characteristics, removal efficiencies, and operational parameters. A calculation of nonparametric Spearman's rank correlation coefficient (rs) was performed with a two tailed p value, at p value of <0.05 being significant, using graphpad prism 5 software.

Table 4.6 : Statistical results of AOA two tail nonparametric Spearman's rank. correlation coefficient value of < 0.05.

Parameter	NH4N mg/L	pH	mM ammonia	COD mg/L	BOD5 mg/L
Number of XY Pairs	14	11	14	12	12
Spearman r	-0.46205	-0.54899	-0.36964	-0.59441	-0.4965
95% confidence interval	-0.80353 to 0.10824	-0.86938 to 0.096386	-0.76015 to 0.21706	-0.87575 to -0.011649	-0.83890 to 0.12745
P value (two-tailed)	0.0962	0.0803	0.1933	0.0415	0.1006
P value summary	ns	ns	ns	*	ns
Exact or approximate P value?	Exact	Exact	Exact	Exact	Exact
Is the correlation significant? (alpha=0.05)	No	No	No	Yes	No

Table 4.7 : Statistical results of AOB two tail nonparametric Spearman's rank.

Parameter	NH4N mg/L	pH	mM ammonia	COD mg/L	BOD5 mg/L
Number of XY Pairs	15	12	15	13	12
Pearson r	0.5776	-0.3146	0.6899	0.5719	0.08471
95% confidence interval	0.09265 to 0.8411	-0.7527 to 0.3166	0.2746 to 0.8883	0.03045 to 0.8539	-0.5143 to 0.6282
P value (two-tailed)	0.0241	0.3193	0.0044	0.0411	0.7935
P value summary	*	ns	**	*	ns
Is the correlation significant? (alpha=0.05)	Yes	No	Yes	Yes	No
R squared	0.3336	0.09897	0.476	0.3271	0.007176

AOB amoA genes abundance was positively correlated to effluent COD (two tail nonparametric Spearman's rank correlation coefficient value of < 0.05). but AOA amoA genes abundance was negatively correlated to effluent COD (two tail

nonparametric Spearman's rank correlation coefficient value of < 0.05)(Table 4.6). AOB amoA genes abundance was positively correlated to ammonia concentration (two tail nonparametric Spearman's rank correlation coefficient value of < 0.05). however AOA amoA genes abundance was not correlated to ammonia concentration (two tail nonparametric Spearman's rank correlation coefficient value of < 0.1933) Table 4.7).

5. CONCLUSIONS

According to this study, activated sludge samples were collected from 16 different domestic and industrial wastewater treatment plants including petroleum and sunflower oil production; food, alcohol and chemical industries and landfill leachate treatment plants. The presence of AOA and AOB in engineered systems was investigated using two primer sets specific for archaeal and bacterial amoA gene sequences via quantitative real time PCR.

It was found that the comparative abundance of AOA and AOB amoA genes varied among the WWTPs with regard to the ammonium concentrations in the effluent of the wastewater treatment plants.

Most of the sequences retrieved from archaeal amoA fell into a marine lineage (Group 1.1a). Few sequences were found to belong to a soil lineage (Group 1.1a), but no sequence related to a thermophilic AOA lineage.

Pure culture studies showed that the ammonia and oxygen contents might be the two most crucial WWTP parameters likely shaping AOA abundance and community. However, these two substrates are co-utilized by AOB leading to the possibility that these two types of microorganisms compete with one another for the ammonia and oxygen in WWTPs.

Archaeal amoA gene copy number was 1 whereas bacterial counterparts was 2,5 (Konneke et al., 2005, Norton et al., 2002). When these copy number were taken into account, AOB amoA gene copy number was outnumbered only the highest ammonia contained wastewater treatment plants like İSTAÇ and Pakmaya. Archaeal ammonia mono-oxigenase had higher affinity to ammonia than bacterial enzyme. Generally AOA was more abundant in environments with low ammonia concentrations which were consistent with our results.

Although both AOB and AOA were present in all samples and contributed to nitrification simultaneously, AOA were dominant in the wastewater treatment plants with low ammonia concentration, whereas, AOB were the dominant nitrifiers in the

in the wastewater treatment plants with high ammonia concentration. These findings were consistent with very recent studies (Bai et al., 2011, Limpiyakorn et al., 2011).

Significant numbers of AOA amoA genes occurred in all municipal WWTPs suggesting that, there may a potential role of AOA in autotrophic ammonia oxidation in low ammonium influent receiving WWTPs (common characteristics of domestic WWTPs). The finding of this study may change our understanding on nitrogen removal in WWTPs. AOA abundance and active role on nitrogen biogeochemical cycle has been reported widely on nature. In the light of such information; AOA should be taken into consideration as potentially involved in nitrogen removal in WWTPs, specifically where ammonia levels low for its flourishment. Further studies, which more focuses on expression levels of AOA amoA genes will help to clarify the role of AOA better and its comparison with respect to AOB activity in autotrophic oxidation of ammonia in WWTPs. One reason for AOB amoA genes outnumbered AOA amoA genes in this study at endustrial wastewater treatment plants might be the relatively high influent ammonia concentration (6,7 - 104,6 mM in this study), which was in accordance with the situations in industrial bioreactors (Bai et al., 2012; Limpiyakorn et al., 2011) and several other studies (Ozdemir et al., 2011; Wells et al., 2009; Zhang et al., 2011).. However, there are some contrary samples, such as, in plant L1 with influent concentration as low as 8.5–9.7 mg 1, AOB amoA genes outnumbered AOA amoA genes (Kayee et al., 2011); The relationship between ammonia concentration and AOA and AOB amoA abundance remains unclear.

It was suggested that the extremely low DO concentrations in the systems might have facilitated the growth of AOA. A quantitative analysis, however, exhibited controversial results (Kayee et al., 2011): high numbers of amoA genes were even found in WWTPs with a dissolved oxygen concentration of 3.25 mgl−1. In addition, Spearman's rank correlation coefficients showed no correlation between the abundance of AEA amoA genes and dissolved oxygen concentrations in the aeration tanks of the WWTPs (Kayee et al., 2011). Related studies showed that low DO levels might be the most determinative parameters of the WWTPs where AOA amoA had been detected (Park et al., 2006). However, in the same study, AOA amoA could not be amplified from two Orbal plants with low DO level (plant American Bath and

Evesham). the comparisons above indicate that the effect of DO on the abundance of AOA and AOB remains in debate.

AOB amoA genes abundance was positively correlated to effluent COD (two tail nonparametric Spearman's rank correlation coefficient value of < 0.05). but AOA amoA genes abundance was negatively correlated to effluent COD (two tail nonparametric Spearman's rank correlation coefficient value of < 0.05). AOB amoA genes abundance was positively correlated to ammonia concentration (two tail nonparametric Spearman's rank correlation coefficient value of < 0.05). however AOA amoA genes abundance was not correlated to ammonia concentration (two tail nonparametric Spearman's rank correlation coefficient value of < 0.1933). Together with the studies of Mußmann (Mußmann et al., 2011), the SRCC results indicate that there may be versatile AOA ecotypes and some AOA might be mixotrophic.

The study performed by Mußmann et al., (2011) provided the sole direct evidence of in situ activity of AOA in a WWTP. In this study, only 4 out of 52 WWTPs showed a high abundance of AOA. When studying one refinery WWTP for in-depth analysis, study showed that it had up to a 10,000 times higher number of AOA than AOB., They revealed that the ammonia removal in the reactor would not be enough to support a population of autotrophic ammonia oxidizers by Using a nitrification model. It could support only 0.01–1 % of AOA found in the plant. Therefore, it was questionable as to whether the AEA in the plant gained most of their energy from chemoautotrophic ammonia oxidation. They revealed that, unlike AOB, AOA did not exhibit autotrophic activity in the presence of ammonia by using fluorescence in situ hybridization (FISH) in combination with microautoradiography (MAR) with 14C-inorganic carbon. With these results, they concluded that the AOA in this plant were not chemolithoautotrophic ammonia oxidizers. The AOA possibly had a heterotrophic metabolism and use some of the organic compounds in the wastewater as their carbon and energy sources.

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APPENDICES

APPENDIX A : Laboratory Equipment

APPENDIX B : Chemicals

APPENDIX C : Pakmaya WWTP aerobic pool sequencing results

APPENDIX A : Laboratory Equipment

MOBGAM, Istanbul Technical University
Pipettes Eppendorf 2.5 µl, 10 µl, 20 µl, 100 µl, 1000 µl
pH meter Mettler Toledo MP220
Pure water systems USF Elga UHQ-PS-MK3, Elgalabwater
Centrifuges Sigma 1-14
PCR Thermocycler BIORAD C1000 thermal cycler
Balances Precisa BJ610, OHAUS pioneer
Water Bath Memmert
DGGE system BIORAD DCODE universal mutation system
Electrophoresis system BIORAD mini sub cell GT
Gel documentation system BIORAD GELDOC
Vortex Heidolph reax top
Autoclave TOMY SX-700E
DRY heating thermostat block BIO TDB 100, BIOSAN
Power supply BIORAD power pac 300
Incubators Nüve EN120
Refrigerators Whirlpool +4oC, -20oC, Vestel -20oC; Haier -80oC
Laminar flow Faster BH-EN 2003
The FastPrep instrument Q-BIOgene, FP220A
Magnetic stirrer, heater Heidolph MR hei-standard
Microwave oven Vestel MD17
QPCR Thermocycler Eppendorf MP realplex
Microplate Spectrophotometer Infinite M200, Tecan AG
Electrophoresis system BIORAD mini sub cell GT
Power supply BIORAD power pac 300
Laminar flow Faster BH-EN 2003
PCR thermocycler Montreal Biotech inc, Tprofessional
Incubators Nüve EN120

APPENDIX B : Chemicals

LB Broth powder Sigma-Aldrich
SOC Broth BioChemika, for microbiology Fluka
40%ACRYLAMIDE/BIS SOLUTION 37.5:1 BIORAD
Formamide deionized solution Sigma-Aldrich
LB base Invitrogen
UREA Fluka
NaOH Reidel-de Haën
Sodium Acetate Anhydrous Sigma-Aldrich
Potassium Hydrogen phosphate J.T. Baker
Ethyl alcohol absolute Sigma-Aldrich
EDTA molecular biology reagent Sigma-Aldrich
TRIS-HCl Sigma-Aldrich
Sodium Phosphate, Monobasic Sigma-Aldrich
Ammonium Persulfate Sigma-Aldrich
Acetic acid extra pure %99.5 Sigma-Aldrich
taq polymerase INTRON
Hot start taq polymerase Qiagen
primers iontek
TEMED Biorad
SYBR green Super mix Biorad
InhibitEx Tablets Qiagen
$NaHCO_3$ Fluka
KH_2PO_4 Fluka
NaCl J.T. Baker
NaOH Fluka
BSA Sigma-Aldrich
Q-solution Qiagen
DMSO Sigma-Aldrich

APPENDIX C : Pakmaya WWTP aerobic pool sequencing results.

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

B3.**Halil Kurt**, Özgür melih Çelik, Mert Kumru, Alper Tunga Akarsubaşı The existence of ammonia-oxidizing archaea in wastewater treatment plants. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B11. Çelik M.Ö., **Kurt H**., Kumru M., Akarsubasi A.T. "Screening of Ammonia-oxidazing Archaea Abundance in Domestic and Industrial Wastewater Treatent Plants" 13th internatioanl symposium on microbial ecology, ISME13 Seattle, WA,USA, 2010

List of Publications and Patents:

A. <u>Uluslararası hakemli dergilerde yayımlanan makaleler :</u>

S. Teksoy Başaran, M. Aysel, **H. Kurt**, İ. Ergal, M. Kumru, A. Akarsubaşı, S. Sözen, D. Orhon Removal of readily biodegradable substrate in super fast membrane bioreactor. Journal of Membrane Science Volumes 423–424, 15 December 2012, Pages 477–486

B. <u>Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler :</u>

B1.**Halil Kurt**, Aslıhan Demircan, Mert Kumru, Alper Tunga Akarsubaşı Microbial community dynamics of İSTAÇ landfill leachate wastewater treatment plant in İstanbul, Turkey. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B2.**Halil Kurt**, Özgür melih Çelik, Mert Kumru, Alper Tunga Akarsubaşı The existence of ammonia-oxidizing archaea in wastewater treatment plants. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B3.Mehmet Sefa Ulutas*, Meltem Tatlı, Mert Kumru, **Halil Kurt**, Salih Süha Akan, Hakan Bermek, Alper Tunga Akarsubaşı Characterization and evolution of microbial communities in olive mill wastewater degrading microbial fuel cells. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B4.Ipek Ergal*, Mert Kumru, **Halil Kurt**, Senem Teksoy Başaran, Seval Sözen, Derin Orhon, Alper Tunga Akarsubaşı Effects of extremely short sludge retention time and short hydraulic retention time on microbial community in an external membrane bioreactor. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B5.Elif Esen, Duygu Dityapak, **Halil Kurt**, Mert Kumru, Bilge Alpaslan Kocamemi, Neslihan Semerci, Alper Tunga Akarsubaşı Molecular characterization of partial nitrification followed by anammox process. ISME14 - 14th International Symposium on Microbial Ecology THE POWER OF THE SMALL 19-24 August 2012, Copenhagen, Denmark

B6.Ulutaş, M.S., Kumru, M. **Kurt, H**., Akarsubaşı A.T., 2011. Characterization Microbial Communities In Olive Oil Wastewater Degrading Microbial Fuel Cells. [Poster, 2011 ICEST International Conference on Enzyme Science and Technology (İzmir/Turkey)].

B7. Budakoglu M., Kumru M., **Kurt H**., Karaman M., Kular A., Esen E., Akarsubasi A.T. "Halophilic Mcrobial Diversity of Alkaline Soda Lake Acigöl" Turkey. 20th International Symposium on Environmental Biogeochemistry, ISEB 2011, Istanbul, Turkey, 2011

B8. Budakoglu M., Karaman M., Kumral M., Akarsubasi A.T., Bulbul A., **Kurt H**., Kumru M., Karabel S.B. " Recent Sulfur Cycle of Lake Acıgöl (Denizli) Basin, SW Turkey". 20th International Symposium on Environmental Biogeochemistry, ISEB 2011, Istanbul, Turkey, 2011

B9. Akarsubasi A.T., **Kurt H**., Kumru M., Kular A., Esen E., Karaman M, Budakoğlu M. "Bacterial Diversity of Hypersaline Soda Lake Acigöl Turkey" 20th International Symposium on Environmental Biogeochemistry, ISEB 2011, Istanbul, Turkey

B10. Çelik M.Ö., **Kurt H.**, Kumru M., Akarsubasi A.T. "Screening of Ammonia-oxidazing Archaea Abundance in Domestic and Industrial Wastewater Treatent Plants" 13th internatioanl symposium on microbial ecology, ISME13 Seattle, WA,USA, 2010

B11. Cirakoglu A, Tarkan-Argüden Y, Yilmaz S, Kuru D, Deviren A, Guven GS, Pasalioglu E, Gürsel IM, Eren S, Arpag S, Aydogdu F, Birinci N, **Kurt H**, Hacihanefioglu S.Structural chromosome abnormalities in couples with recurrent fetal losses. 6th European Cytogenetic Conference (6th ECC). 7-10 July 2007, Istanbul, Turkey.

B12. Ayhan Deviren, Halil KURT, "lhan Onaran, Mehmet Güven, Gonul Kanigur, Seniha. Hacıhanefioğlu Assessment of hydroperoxides induced DNA damage by the comet assay and the micronucleus test. 2006 international forensic science symposium, Abb. Book. P234

B13.S. Yilmaz, Y.T. Arguden, A. Çırakoğlu, D. Kuru, G. Güven, **H. Kurt**, N. Birinci, A. Çınar, I. Öngören, Ü. Üre, I.İ. Güner A. Deviren, S. Hacıhanefioğlu Cytogenetic finding of adult ALL cases XXXth word congress of the international society of hematology Istanbul, Turkey, T. Journal of Haemotology , Suppl:22 Abs 413, p181-182, 2005.

B14. S. Yilmaz, Y.T. Arguden, A. Çırakoğlu, A. Deviren, D. Kuru, G. Güven, E.Y.Fenerci, **H. Kurt**, A.Yüksel, S. Hacıhanefioğlu: A case with partial trisomy 7p and monosomy 9p choromosome Cromosome Research, Vol 12 (Supl 1) PO:08:77, p145, 2004

E. <u>Ulusal bilimsel toplantılarda sunulan ve bildiri kitaplarında basılan bildiriler:</u>

E1. Şükriye Yılmaz, Ayşe Çırakoğlu, Başak Aslaneli, Emre Eşkazan, Dilhan Kuru, Cem Ar, Yelda Tarkan-Argüden, Şeniz Öngören, Sibel Penbe Yentür, **Halil Kurt**, Nazlı Birinci, Gülgün Güven, Aslı Silahtaroğlu, Ayhan Deviren, Zafer Başlar, Teoman Soysal, Burhan Ferhanoğlu, Yıldız Aydın, Seniha Hacıhanefioğlu, Asım Cenani. Hematolojik Kanserlerde Sitogenetik Çalışmalar**.**9. Ulusal Tıbbi Genetik Kongresi 1-5 Aralık 2010 İstanbul

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