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ABBREVIATIONS

FISH	: Fluorescence *in situ* Hybridization
ISKI	: Water and Sewerage Administration of Istanbul
WWTP	: Waste Water Treatment Plant

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LIST OF SYMBOLS

μ	**:** Specific growth rate
$\hat{\mu}$	**:** Maximum specific growth rate
S	**:** Concentration of growth limiting substrate in solution
Ks	**:** Half velocity constant
Y	**:** Growth yield
q	**:** Substrate utilization rate
$\mathbf{k_d}$	**:** Endogenous decay rate coefficient

GERÇEK ÖLÇEKLİ AKTİF ÇAMUR TESİSİNDE NİTRİFİKASYON BAKTERİLERİNİN FLORESANLI YERİNDE HİBRİTLEŞME TEKNİĞİ (FISH) KULLANILARAK BELİRLENMESİ

ÖZET

Son yıllara kadar biyolojik arıtma karbon giderimi üzerine yoğunlaşmıştı ancak yapılan çalışmalar sadece organik madde gideriminin yeterli olmadığını; besi maddelerinin de su kalitesi ve canlı sağlığı açısından önemli olduğunu gösterdi. Bu nedenle en sık kullanılan biyolojik arıtma sistemi olan aktif çamur, kabonlu maddeler yanında besi maddesi gidermek üzere yeniden düzenlendi.

Deneyimler, arıtma sisteminin verimini sabit tutmak için sitemdeki mikroorganizma gruplarının iyi tanımlanmış olması gerektiğini göstermektedir. Bu amaçla, birçok mikrobiyolojik metod geliştirilmiş ve uzun zaman kullanılmıştır. Fakat, bu yöntemlerle elde edilen bilgiler yetersizdir. Çünkü, klasik mikrobiyolojik yöntemlerde genellikle mikroorganizmaların saf kültürlerinin elde edilmesi gereklidir. Özellikle nitrifikasyon bakterilerinin çoğalma sabitleri heterotrof organizmalara göre çok düşük olduğundan saf kültürlerini elde etmek zordur. 1950'lerde DNA ve RNA'nın yapısının çözümlenmesiyle klasik yöntemlerin dezavantajlarını ortadan kaldıran yeni moleküler yöntemler geliştirilmeye başlandı. Bu yöntemlerle, özellikle sistemdeki aktif mikroorganizmalar doğal ortamlarından kültive edilmeden tayin edilip, miktarı belirlenebilir. Özellikle hibridizasyon teknikleri, prosesten sorumlu mikroorganizma gruplarını belirleme ve sayımını yapmada kullanılmaktadır.

Bu çalışmada, en başarılı hibridizasyon yöntemlerinden olan Floresanlı yerinde Hibritleşme (FISH) yöntemi, İstanbul Su ve Kanalizasyon İdaresi, Paşaköy İleri Biyolojik Atıksu Arıtma Tesisi nitrifikasyon reaktöründen alınan numune üzerinde uygulanmıştır. Bu numune üzerinde amonyağı ve nitriti oksitleyici bakteri grupları ayrı ayrı tanımlanmıştır.

FISH ile elde edilen sonuçlar, amonyağı oksitleyicilerden *Nitrosospira briensis, Nitrosovibrio tenuis, Nitrosolobus multiformis, Nitrosomoas europaea, Nitrosomoas eutropha, Nitrosococcus mobilis, Nitrosomoas C56*; nitrit oksitleyicilerden *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi* ve *Nitrobacter sp.*'nin sistemde varolduğunu göstermiştir.

DETERMINATION OF NITRIFYING BACTERIA IN A FULL-SCALE ACTIVATED SLUDGE SYSTEM USING FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) TECHNIQUE

SUMMARY

Biological wastewater treatment was focused on the carbon removal until recent years, but the studies showed that it is not sufficient to remove solely organic matter. Nutrients are also important for the quality of receiving water bodies and organisms' health. The most commonly used biological wastewater treatment system, activated sludge, is therefore, modified to remove nitrogen beside carbonaceous matter.

Experiences indicate that microorganisms in activated sludge systems should be very well recognized to maintain the efficiency of the treatment systems. For this purpose, several microbiological methods were developed and used for a long time, but the information obtained by these methods, is not enough. Generally, these techniques require the cultivation of microorganisms to obtain pure cultures. Especially nitrifying bacteria is very difficult to obtain the pure cultures, because they have very low doubling times compared to heterotroph microorganisms. By solving the structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in 1950's, new molecular tools are developed to overcome the disadvantages of classical microbiological techniques. First of all, by molecular methods, active microorganisms in treatment systems can be detected and enumerated. Secondly, the microorganisms should not be extracted from their natural habitat to be examined. Especially hybridization techniques are very useful to detect and quantify the responsible microorganisms in short times.

In this study, one of the most successful molecular techniques, Fluorescent *in situ* Hybridization (FISH) technique is applied to the samples taken from nitrification reactor of Water and Sewerage Administration of Istanbul (ISKI), Pasakoy Advanced Wastewater Treatment Plant. Ammonia and nitrite oxidizing bacteria are determined by this technique.

Results obtained by FISH showed that *Nitrosospira briensis*, *Nitrosovibrio tenuis*, *Nitrosolobus multiformis*, *Nitrosomoas europaea*, *Nitrosomoas eutropha*, *Nitrosococcus mobilis*, *Nitrosomoas C56* as ammonia-oxidizing bacteria, and *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi* ve *Nitrobacter sp.* as nitrite-oxidizing bacteria are present within the reactor treating nitrogen.

CHAPTER 1 INTRODUCTION

Domestic and industrial wastewaters are treated almost for a century to overcome the harmful effects on the receiving water bodies and health. Since more pollutants are recognized and industries become more complex, advanced wastewater treatment techniques are required.

Conventionally, the treatment of carbonaceous material in wastewater was concerned sufficient until it is understood that the nitrogen and phosphorus contents are also important. These two inorganic compounds are nutrients. Therefore, their excess amount causes eutrophication in the receiving water. Especially, the treatment of nitrogenous components has become increasingly important because, ammonia is toxic to aquatic microorganisms and the form of nitrogen cause extra oxygen demand (up to 4.57 g O_2 / g NH_4^+-N) (Rittmann and McCarty, 2001). Nitrite is also toxic to most fish and other aquatic species even in low concentrations (Tchobanoglous and Burton, 1991).

Although some chemical and physical methods can be used to remove the nitrogen, biological treatment methods are concerned as the most successful method. Sequencial nitrification-denitrification processes are applied for the removal of nitrogen biologically. Nitrification is a two-step process. In first step, ammonia is oxidized to nitrite mostly by autotrophic *Nitrosomonas* species. In second step, nitrite is oxidized to nitrate mostly by autotrophic *Nitrobacter* and *Nitrospira* species. In denitrification process, nitrate is converted to dinitrogen gas by heterotrophic bacteria. Organic carbon is used as carbon and energy source, and nitrate is used as electron acceptor in anoxic conditions. In wastewaters, nitrogen generally exists as organic nitrogen or ammonium. These two forms of nitrogen should be oxidized to nitrite or nitrate before denitrification. Therefore, for the complete removal of nitrogen from wastewaters, denitrification is coupled to nitrification.

Denitrifiers are heterotrophic or autotrophic bacteria, which contain *Pseudomonas, Alcaligenes, Paracoccus,* and *Thiobacillus* as gram-negative. *Halobacterium* and gram-positive *Bacillus* can also denitrify. All denitrifiers are facultative aerobes; they shift NO_3^- or NO_2^- respiration when O_2 becomes limiting (Rittmann and McCarty,

2001). In denitrification systems, dissolved oxygen concentration is very important, because the presence of dissolved oxygen will suppress the enzyme system needed for denitrification and the metabolic activity is converted to aerobic respiration (Tchobanoglous and Burton, 1991).

Beside this nitrification-denitrification process, a newly developed pathway is used to remove ammonium from wastewaters. This process is called Anaerobic Ammonium Oxidation (ANAMMOX) during which NH_4^+-N is oxidized directly to dinitrogen gas anaerobically. In this process, ammonium is electron donor; nitrite is both electron acceptor and the nitrogen source. This reduces the oxygen demand. The yield and specific growth rate for these bacteria are very low (doubling time about 11 days), so sludge production is decreased compared to others.

Nitrifying bacteria also has a very low growth yield when compared to heterotrophs, and are very sensitive to environmental conditions and substrate fluctuations. Therefore, to design and operate the nitrification systems according to the characteristics of the nitrifiers increase the effect of the systems.

Many methods are applied to detect and enumerate the microorganisms responsible for nitrification such as; microscopic count, plating techniques, most probable number, etc. Although they can give some useful information about the nitrification bacteria, they have several limitations. Firstly, active biomass in the system can not be detected by classical microbiological methods. Secondly, they require the cultivation of these bacteria which are very difficult or impossible to obtain the pure cultures. The nitrifying bacteria have very long generation times and poor counting efficiencies (Mobarry *et al.* 1996). In recent years, molecular methods are applied successfully not only for determination of the microorganisms, but also to count them. In these methods, the genetic material of the organisms is used. The most important advantage of these techniques is that they do not require the cultivation of the bacteria from their natural environment. Hybridization techniques are one of the most useful molecular tools, because they give results about the active biomass in the system without cultivation. These results can be interpreted with the performance data of the system. Fluorescent *in situ* Hybridization (FISH) technique which is widely used to control the activated sludge system microbiologically is one of the most useful methods.

In recent years, there are several studies in which FISH technique is successfully applied for nitrifying biofilms (Mobarry *et al.*, 1996; Schramm *et al.*,1997; Wagner

et al., 1998; Juretschko *et al.*, 1998; Aoi *et al.*, 2000; Egli *et al.*, 2003). These studies provide precious information about the structure and the function of this group of bacteria

In this study, nitrification and the microorganisms, which are responsible for this process, are the main subject. Nitrification bacteria are determined by Fluorescent *in situ* Hybridization (FISH) technique in the samples taken from Water and Sewerage Administration of Istanbul (ISKI), Pasakoy Advanced Biological Wastewater Treatment Plant. Results are correlated with the operational and performance data of the plant. According to the overall results, precautions and new strategies for better operation of the nitrification reactors can be developed.

CHAPTER 2 RESEARCH AIM AND OBJECTIVES

Nitrification has not been fully understood yet because of several parameters, which may affect the process. Detection and quantification of nitrifying bacteria in these systems are tried to be obtained by a number of techniques, but nitrifying bacteria have relatively low growth rates and therefore, their cultivation from the natural environment is very difficult and time consuming. The disadvantages of classical techniques have limited the information obtained about the functional groups of bacteria and their distribution within a system. New molecular methods are developed to overcome the disadvantages of conventional microbiological techniques and they are applied successfully for better understanding of nitrification process and responsible organisms.

The aim of this study is to detect ammonia oxidizing and nitrite oxidizing bacteria in activated sludge systems using Fluorescent *in situ* Hybridization (FISH) technique as a molecular method. Since FISH can detect active microorganisms, the interaction between dominant bacterial groups and the efficiency of the system can be determined. In this study, this kind of interaction is set up for the Water and Sewerage Administration of Istanbul (ISKI), Pasakoy Advanced Wastewater Treatment Plant. The data, obtained by applying FISH technique, are correlated with the operation conditions of the plant, composition of wastewater, which comes to the system, and the efficiency of the treatment plant. The most important purpose of this study is to apply Fluorescent *in situ* Hybridization technique as a molecular tool for better understanding of the nitrification process especially in engineering systems.

CHAPTER 3 FUNDAMENTALS OF BIOLOGICAL NITROGEN REMOVAL

3.1 NITROGEN FORMS IN WASTEWATER

Nitrogen can occur in many forms in wastewater. These forms are organic nitrogen (amino acids, proteins), ammonia, nitrite, and nitrate. Table 3.1 lists the oxidation states of elemental nitrogen and the corresponding inorganic species.

Table 3.1 Simple forms of inorganic N and oxidation state of the N atom (Hagopian and Riley, 1998).

Species	Common name	N
NH_3	Anhydrous or free or un-ionized ammonia, ammonia gas	-3
NH_4^+	Ammonium, ammonium ion, ionized ammonia	-3
NH_4OH	Ammonium hyroxide	-3
N_2H_4	Hydrazine	-2
$N_2H_5^+$	Hydrazinium ion	-2
N_2H_5OH	Hydrazine hydrate	-2
NH_2OH	Hydroxylamine	-1
HN_3	Hydrogen azide, hydrazoic acid	-1/3
N_3^-	Azide ion	-1/3
N_2	Nitrogen, nitrogen gas, denitrogen gas	0
N_2O	Nitrous oxide, nitrous oxide gas, ‘laughing gas’	+1
$H_2N_2O_2$	Hyponitrous acid	+1
NO	Nitric oxide, nitric oxide gas	+2
NO_2^-	Nitrite ion	+3
HNO_2	Nitrous acid	+3
N_2O_3	Dinitrogen trioxide, nitrogen sesquioxide,anhydrous nitrous acid	+3
NO_2	Nitrogen dioxide	+4
N_2O_4	Denitrogen tetroxide, nitrogen peroxide	+4
NO_3^-	Nitrate ion	+5
HNO_3	Nitric acid	+5
N_2O_5	Dinitrogen pentoxide, anhdrous nitric acid	+5

Ammonia nitrogen exists as either the ammonium ion or ammonia, depending on the pH of the solution.

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \quad (3.1)$$

According to this reaction, at pH levels above 7, the equilibrium is shifted to the left, at levels below pH 7, the ammonium ion is predominant (Tchobanoglous and Burton, 1991).

Ammonium (about 60 %) and organic nitrogen (about 40 %) are the main nitrogen forms in domestic wastewater. TKN (Total Kjeldahl Nitrogen) analysis is used to measure the total free ammonium ion and the organic nitrogen concentrations.

Nitrite nitrogen is relatively unstable and easily oxidized to the nitrate form (Tchobanoglous and Burton, 1991). It is produced during the oxidation of ammonia nitrogen to nitrate nitrogen.

Nitrate nitrogen is the most highly oxidized form of nitrogen found in wastewaters. It has fatal effects on infants (Tchobanoglous and Burton, 1991). Its concentration may vary between 0-20 mg/l as N in wastewater effluents.

The transformations of nitrogen in biological treatment processes are shown in Figure 3.1.

3.2 THEORY OF NITRIFICATION

Nitrification, the first step of sequential nitrification-denitrification process, is the microbiological oxidation of NH_4-N and NO_3^-N (Rittmann and McCarty, 2001).

Nitrification is performed by a two-step process. The oxidation of ammonia to nitrite is the first step, and the oxidation of nitrite to nitrate is the second step of nitrification process.

The nitrifying bacteria are autotrophs, chemolithotrophs and obligate aeobes. Inorganic carbon is used as carbon source and inorganic nitrogen is used as energy source. Although some researches show that there are also heterotrophic microorganisms, which can perform nitrification, the nitrification potential of chemoautotrophs are greater than heterotrophs. Therefore, most researches are concentrated on autotrophic microorganisms (Grady and Lim, 1980).

Nitrosomonas is the most recognized genus responsible for the first step. *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* are also able to carry

out this step. Generally, these bacteria are known as ammonia-oxidizing bacteria (AOB).

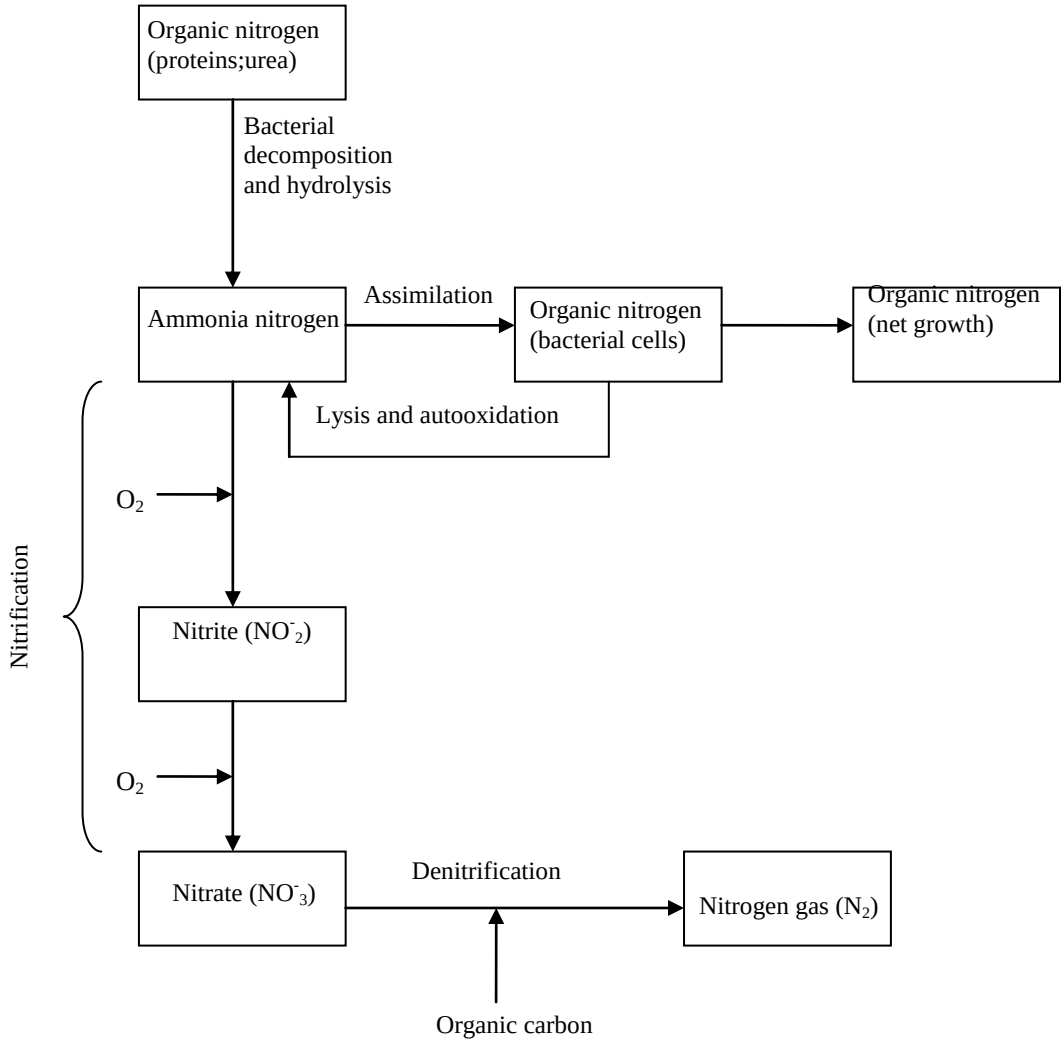


Figure 3.1 Nitrogen cycle in wastewater treatment.

Nitrosomonas is the most recognized genus responsible for the first step. *Nitrosococcus, Nitrosospira, Nitrosovibrio,* and *Nitrosolobus* are also able to carry out this step. Generally, these bacteria are known as ammonia-oxidizing bacteria (AOB).

Nitrospira, Nitrospina, Nitrobacter, Nitrosococcus, and *Nitrocystis* are generally known as nitrite-oxidizers. Although *Nitrobacter* is considered as the most famous genus of the NO_2^- oxidizers, recent findings using oligonucleotide probes targeted to the 16S rRNA of *Nitrobacter* indicate that it is not the most important nitrite-

oxidizing genus in most wastewater treatment processes, but *Nitrospira* is identified more often as the dominant nitrite-oxidizer (Rittmann and McCarty, 2001).

Since heterotrophic bacteria have a higher growth rate than autotrophic nitrifying bacteria, at first the heterotrophic bacteria occupied the outer part of the biofilm where concentrations of dissolved oxygen and substrate are sufficiently high for the bacteria to grow. In contrast, ammonia-oxidizing bacteria could exist only in the inner part of the biofilm due to their slow growth rate (Aoi *et al.*, 2000).

3.3 NITRIFICATION STOICHIOMETRY

Bacterial growth involves in two basic reactions: energy production and cellular synthesis. In nitrification reaction, ammonium serves as the electron donor and is oxidized to nitrate, oxygen is the electron acceptor since it is an aerobic reaction, and ammonium serves as the source of nitrogen for cell synthesis. In synthesis reactions, CO_2, CO_3^{2-} or HCO_3^- are the electron acceptors. The following reactions are the half-reactions for energy and cellular synthesis for the first and second step of nitrification.

First step:

Energy reaction:

$$\frac{1}{6}NH_4^+ + \frac{1}{3}H_2O \rightarrow \frac{1}{6}NO_2^- + \frac{4}{3}H^+ + e^- \qquad \Delta G = 7.852 kcal/e^- eq \quad (3.2)$$

$$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O \qquad \Delta G = -18.675 kcal/e^- eq \quad (3.3)$$

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \Delta G_r = -10.823 kcal/e^- eq \quad (3.4)$$

Synthesis reaction:

$$\frac{1}{6}NH_4^+ + \frac{1}{3}H_2O \rightarrow \frac{1}{6}NO_2^- + \frac{4}{3}H^+ + e^- \quad (3.5)$$

$$\frac{1}{4}CO_2 + \frac{1}{20}NH_3 + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{8}{20}H_2O \quad (3.6)$$

$$\frac{1}{6}NH_4^+ + \frac{1}{20}NH_3 + \frac{1}{4}CO_2 \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{1}{15}H_2O + \frac{1}{3}H^+ + \frac{1}{6}NO_2^- \quad (3.7)$$

Second step:

Energy reaction:

$$\frac{1}{2}NO_2^- + \frac{1}{2}H_2O \rightarrow \frac{1}{2}NO_3^- + H^+ + e^- \qquad \Delta G = 9.425 kcal/e^- eq \text{ (3.8)}$$

$$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O \qquad \Delta G = -18.675 kcal/e^- eq \text{ (3.9)}$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \qquad \Delta G_r = -9.250 kcal/e^- eq \text{ (3.10)}$$

Synthesis reaction:

$$\frac{1}{2}NO_2^- + \frac{1}{2}H_2O \rightarrow \frac{1}{2}NO_3^- + H^+ + e^- \text{ (3.11)}$$

$$\frac{1}{4}CO_2 + \frac{1}{20}NH_3 + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{8}{20}H_2O \text{ (3.12)}$$

$$\frac{1}{2}NO_2^- + \frac{1}{20}NH_3 + \frac{1}{4}CO_2 + \frac{1}{10}H_2O \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{1}{2}NO_3^- \text{ (3.13)}$$

By combining the above half-reactions, the overall reactions for energy and synthesis for nitrification are obtained:

Overall energy reaction:

$$\frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \rightarrow \frac{1}{8}NO_3^- + \frac{10}{8}H^+ + e^- \qquad \Delta G = 8.245 kcal/e^- eq \text{ (3.14)}$$

$$\frac{1}{4}O_2 + H^+ + e^- \rightarrow \frac{1}{2}H_2O \qquad \Delta G = -18.675 kcal/e^- eq \text{ (3.15)}$$

$$\frac{1}{8}NH_4^+ + \frac{1}{4}O_2 \rightarrow \frac{1}{8}NO_3^- + \frac{2}{8}H^+ + \frac{1}{8}H_2O \qquad \Delta G_r = -10.43 kcal/e^- eq \text{ (3.16)}$$

Overall synthesis reaction:

$$\frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \rightarrow \frac{1}{8}NO_3^- + \frac{10}{8}H^+ + e^- \quad (3.17)$$

$$\frac{1}{4}CO_2 + \frac{1}{20}NH_3 + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{8}{20}H_2O \quad (3.18)$$

$$\frac{1}{8}NH_4^+ + \frac{1}{4}CO_2 + \frac{1}{20}NH_3 \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{1}{8}NO_3^- + \frac{2}{8}H^+ + \frac{1}{40}H_2O \quad (3.19)$$

According to the overall reactions, in the ammonium conversion process, oxygen and alkalinity are required. 1 mg NH_4^+-N needs 4.57 mg O_2 to be oxidized. This value is 4.33 mg O_2/mg ammonium nitrogen in which cell synthesis is considered (US, EPA, 1975). Furthermore, 8.64 mg HCO_3^- are needed to oxidize 1 mg of ammonia-nitrogen to nitrate-nitrogen (Tchobanoglous and Burton, 1991).

3.4 NITRIFICATION KINETICS

Effective removal of pollutants is ensured by controlling the growth rate of the microorganisms. Monod equation is often used to define relationship between active biomass and primary substrate:

$$\mu = \hat{\mu}\,\frac{S}{K_S + S} \quad \text{where;} \quad (3.20)$$

μ : specific growth rate, time^{-1}

$\hat{\mu}$: maximum specific growth rate, time^{-1}

S: concentration of growth limiting substrate in solution, mass/volume

K_S: half velocity constant, substrate concentration at one-half the maximum growth rate, mass/unit volume.

K_S shows the affinity of microorganism for the growth limiting substrate. The lower the K_S values indicate the greater affinity of microorganism for the substrate. Organisms having the lower saturation coefficients will have a greater capacity to grow rapidly in an environment with low growth limiting substrate concentration. The maximum specific growth rate, μ_m also has an affect on the growth rate of different organisms competing for a single limiting substrate. If the food supply is in

excess, the outcome of the competition will be determined by μ_m, organisms having higher μ_m will be the successful competitor in this situation (Horan, 1991). Figure 3.2 shows the relationship between specific growth rate and growth limiting nutrient concentration.

The maximum specific growth rate, $\hat{\mu}$ also affects the growth rate of different organisms competing for a single limiting substrate.

The studies show that active biomass has an energy demand for maintenance, which includes cell functions such as motility, repair and resynthesis, osmotic regulation, transport, and heat loss. The cells supply this maintenance energy needs called as endogenous decay by oxidizing themselves (Rittmann and McCarty, 2001).

The net specific growth rate expression can be shown as following equation which includes endogenous decay rate:

$\mu = (Yxq) - k_d$ where; (3.21)

Y: growth yield

q: substrate utilization rate (mg/mg-day)

k_d: endogenous decay rate coefficient (day^{-1})

Cell yield represents the amount of biomass formed per unit amount of substrate removed. In wastewater, many organisms need energy for cell maintenance. The measured growth yield, Y must be corrected by considering the amount of cell decay during the declining phase of growth. This will give the true growth yield coefficient, which is lower than the measured yield (Tchobanoglous and Burton, 1991).

According to the energy and synthesis reactions, the oxidation of ammonia to nitrate require high amount of energy. Therefore, the energy produced during the reduction of oxygen is used for this purpose. Beside this, CO_2 needs energy to be reduced to pryuvate. Therefore, the energy, which can be used for cellular synthesis, is very low compared to aerobic heterotrophs. Due to this lower yield, the maximum specific growth rates are also low. This value is less than 1/d at 20^0C for both groups (Rittmann and McCarty, 2001). The maximum yield constant for *Nitrobacter* is lower than the one for *Nitrosomonas*. Thus, *Nitrosomonas* would be predicted to be

present at higher numbers than *Nitrobacter* in nitrifying environments (Ritmann, 1987). Reaction rate constants for nitrifying bacteria are listed in Table 3.2.

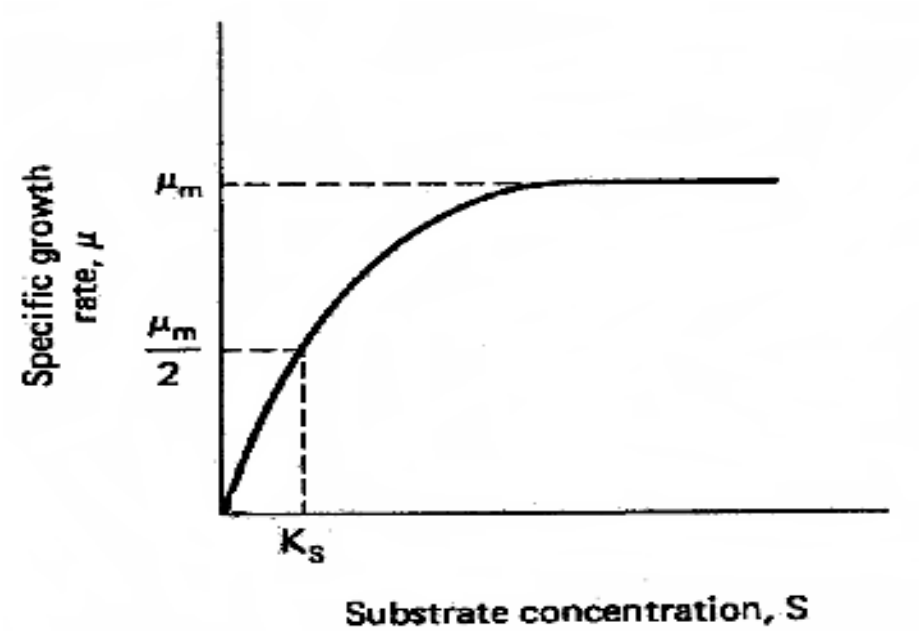


Figure 3.2 Relationship between specific growth rate and growth limiting nutrient concentration (Monod, 1949).

Table 3.2 Reaction rate constants for nitrification at 20°C (Henze *et al.*, 1995).

Parameters	Symbol	Unit	Nitrosomonas	Nitrobacter	Total process
Maximum specific growth rate	$\mu_{max,}$	d^{-1}	0.6 – 0.8	0.6 – 1.0	0.6 – 0.8
Saturation constant	$K_{S,NH4, and}$ $K_{S, NO2}$	g NH_4-N/m^3 gNO_2-N/m^3	0.3 – 0.7	0.8 – 1.2	0.3 – 0.7
Saturation constant	$K_{S, O2}$	g O_2/m^3	0.5 – 1.0	0.5 –1.5	0.5 – 1.0
Maximum yield constant	Y_{max}	g VSS/g N	0.10 – 0.12	0.05 – 0.07	0.15 – 0.20
Decay constant	k_d	d^{-1}	0.03 – 0.06	0.03 – 0.06	0.03 – 0.06

3.5 ENVIRONMENTAL FACTORS AFFECTING NITRIFICATION

Studies show that ammonia and nitrite concentrations, BOD_5/TKN ratio, dissolved oxygen (DO) concentration, temperature, and pH have a significant effect on the nitrification process (Tchobanoglous and Burton, 1991).

3.5.1 EFFECT OF AMMONIA AND NITRITE CONCENTRATIONS

Ammonia and nitrite concentration affect the maximum growth rate of nitrification bacteria. Monod equation which is explained previously can be used to determine this effect.

Since, it was found that nitrite oxidizing bacteria, *Nitrobacter*, have a greater growth rate than that of ammonia oxidizing bacteria, *Nitrosomonas*, the conversion of ammonia to nitrite is considered as the rate-limiting step (Tchobanoglous and Burton, 1991).

3.5.2 EFFECT OF BOD_5/TKN RATIO

High organic loadings decrease the growth of heterotrophic bacteria. This causes excess dissolved oxygen. Thus, any increase in the BOD_5/TKN ratio results in a decrease in the fraction of nitrifiers in the activated sludge system. Table 3.3 summarizes the relationship between the fraction of nitrifying organisms and BOD_5/TKN ratio.

Dincer and Kargi (2000) showed that increasing loading rates resulted in significant increases in nitrification-denitrification rates, especially at low levels of loading rates. However, the rates reached nearly constant levels at high loading rates.

Table 3.3. Relationship between the fraction of nitrifying organisms and the BOD_5/TKN ratio (Tchobanoglous and Burton, 1991).

BOD_5/TKN Ratio	Nitrifier Fraction	BOD_5/TKN Ratio	Nitrifier fraction
0.5	0.35	5	0.054
1	0.21	6	0.043
2	0.12	7	0.037
3	0.083	8	0.033
4	0.064	9	0.029

3.5.3 EFFECT OF DISSOLVED OXYGEN CONCENTRATION

Nitrifying bacteria need high quantities of oxygen to convert the ammonia to nitrate. The oxygen half saturation constant (K_{DO}) for heterotrophic organisms is relatively lower than that of nitrifiers. K_{DO} value is estimated 1 mg/L at 15^{o}C (Horan, 1991).

If enough DO is not provided to the system, the concentration of DO will control the rate of nitrification rather than the NH_4^+-N or NO_2^-N concentrations (Grady and Lim, 1980).

If relatively low levels of dissolved oxygen are supplied to the system, aeration tank detention time should be kept higher to ensure the complete nitrification.

3.5.4 EFFECT OF TEMPERATURE

Temperature has a significant effect on nitrification rate constants. Generally, nitrification rate decreases with decreasing temperature whereas increase up to about 30^{o}C increases the growth rates of ammonia and nitrite oxidizers. Mesophilic temperatures (30-38^{0}C) are optimum for nitrification.

3.5.5 EFFECT OF pH

The maximum rate of nitrification occurs between pH values of about 7.2 and 9.0. Nitrification rates decrease with decreasing pH below this range and the process stops at pH levels below about 5. (Winkler, 1981).

3.5.6 EFFECT OF SLUDGE AGE

Sludge age means cell residence time during which the sludge is kept in the activated sludge reactor. Different microorganisms require different sludge ages according to their growth kinetics at different temperatures. For nitrification, sludge age can not be less than $1/\mu_{max}$. Figure 3.3 shows the relationship between temperature and necessary aerobic sludge age for the achievement of nitrification in activated sludge treatment plants.

3.5.7 EFFECT OF INHIBITORS

The slow growth rate of nitrifiers makes them highly sensitive to chemical inhibition. Unionized NH_3 (at higher pH), surfactants, low pH, heavy metals, chlorinated organics, sulfides are some organic and inorganic inhibitors of nitrification process.

Organic substances in wastewaters cause a depletion of dissolved oxygen, because heterotrophic organisms besides nitrifying bacteria also use dissolved oxygen. So, organic matters are not directly inhibitory.

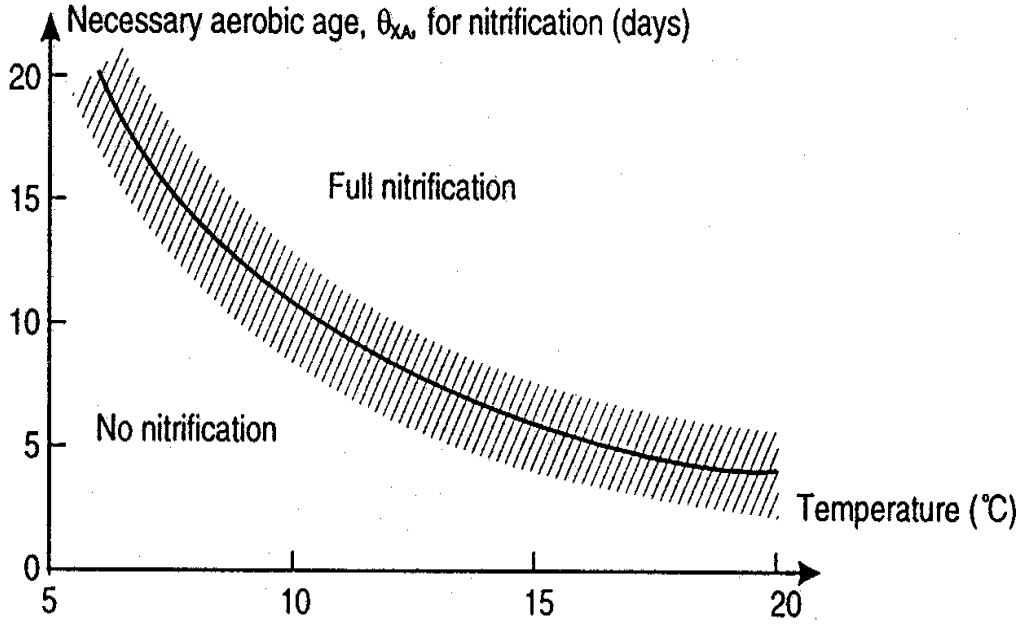


Figure 3.3 Relationship between temperature and necessary aerobic sludge age for the achievement of nitrification in activated sludge treatment plants

(Operating conditions: Oxygen concentration 2 g / m3) (Henze et.al., 1995).

Heavy metals are one of the most important groups of inorganic inhibitors. Copper, silver, mercury, nickel, chromium, and zinc can be counted as these heavy metals. It is also showed the fluoride inhibition of nitrification in suspended biomass system is higher than in immobilized biomass system (Carrera *et al*, 2003). They found that maximum nitrification rate decreased from 0.21 ± 0.01 mg N/ mg VSS-d to 0.06 ± 0.02 mg N/ mg VSS-d by the increase of fluoride concentration from 20 mg/l to 1500 mg/l. In another study, Wett and Rauch (2003) emphasized the difficulty of distinguishing the direct impact of a low pH value from bicarbonate limitation caused by a low pH.

Studies show that these compounds are more toxic to AOB than NOB (Tomlinson *et al.*, 1966). In their study, 52 compounds have been studied, 20 were found inhibitory to ammonia oxidation whilst only 3 inhibited nitrite oxidation.

Both *Nitrosomonas* and *Nitrobacter* are sensitive to high concentrations of their own substrate and also the substrate of each other. Unionized ammonia (NH_3) and unionized nitrous acid (HNO_2) are the most inhibitory forms of nitrogen substrates. The pH of the system and the temperature also affect the inhibition by these substrates.

3.6 MICROBIOLOGY OF NITRIFICATION

3.6.1 PHYLOGENY OF NITRIFYING BACTERIA

Phylogeny is a systematic classification of the species into larger groups based on genetic characteristics. These characteristics are encoded in the organisms' DNA (deoxyribonucleic acid), which contains hereditary material of cells, and RNA (ribonucleic acid) which is involved in protein synthesis. Figure 3.4 shows universal phylogenetic tree indicating all known life on earth. According to genetic characteristics, organisms are divided into 3 main domains: the Eukarya, the Archaea, and the Bacteria. Archaea and Bacteria comprise the prokaryotes, which do not contain their chromosomes inside a nucleus. The other domain, Eukarya has their chromosomes inside a nucleus and may be single cellular or multicellular.

Phylogenetic tree is determined by ribosomal RNA (rRNA) sequencing. RNA is a single-stranded molecule, which are used for protein synthesis. There are 3 basic forms of RNA: messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). mRNA carries the information for protein synthesis from the DNA whilst tRNA carries amino acids to the proper site on mRNA. rRNA functions as structural and catalytic components of the ribosomes which synthesize proteins. Therefore, all cells must have rRNA. The ribosome is composed of two subunits that are identified by their size as measured by sedimentation rate: 30S and 50S or prokaryotes. The 30S subunit is divided into 5S, 16S, and 23S components. In Eukaryotes there are 18S, which are similar to 16S. These 16S and 18S rRNAs are called as small system subunit (SSU). All SSU rRNAs have similar secondary structure, but their primary structure (the base sequences) differs. Similarities and differences in primary structures of 16S rRNAs show phylogenetic relationship between different organisms. Furthermore, they have 1500 base pairs, which provide enough genetic information to classify organisms. rRNAs have conserved and variable regions which are very useful to distinguish the organisms.

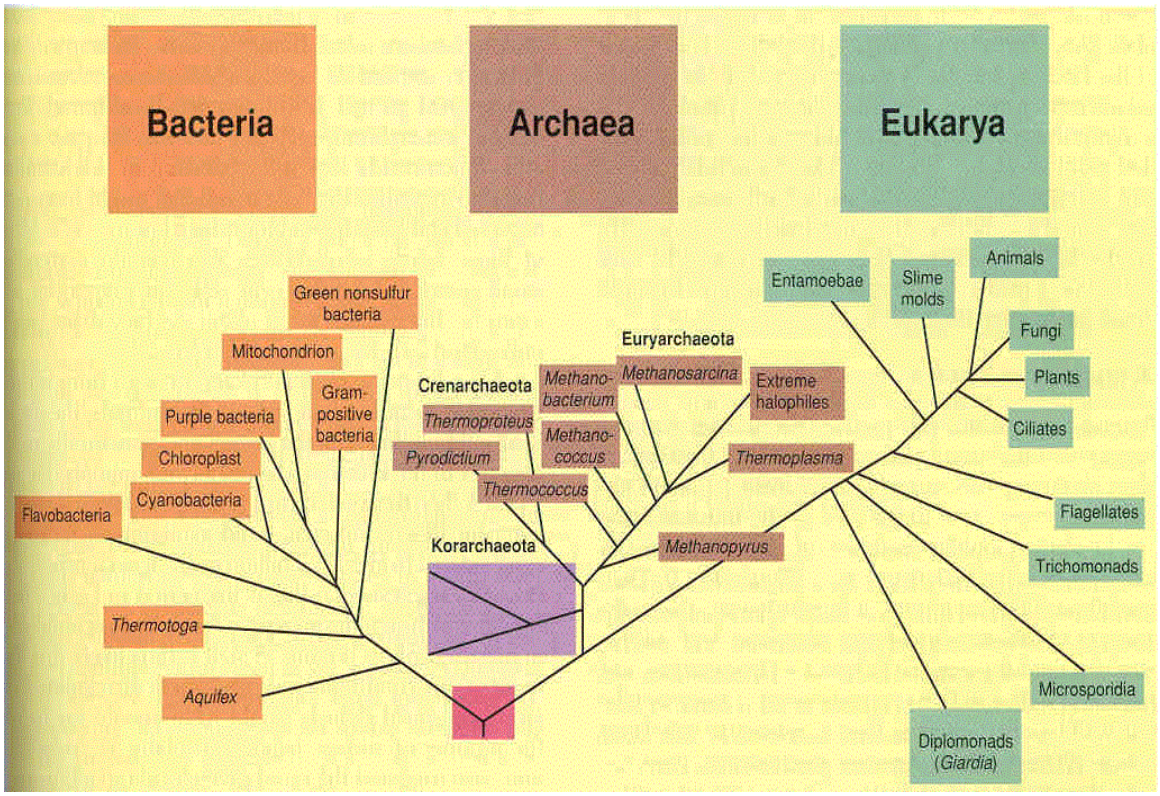


Figure 3.4 Universal phylogenetic tree (Madigan *et.al.*, 1997).

According to 16S rRNA sequences, the domain Bacteria has 12 kingdoms. The most diverse one, proteobacteria breakdowns into 5 groups because of the differences in their metabolism (alpha, beta, gamma, delta, epsilon).

The ammonium-oxidizing nitrifiers belong to the gamma and the beta subdivision of the proteobacteria. One group, *Nitrococcus oceanus* is identified in the gamma subdivision of the proteobacteria. The AOB in the beta proteobacteria can be divided into two branches: *Nitrosomonas* and *Nitrosospira* (Head *et al.*, 1993). The genus *Nitrosomonas* contains *Nitrosomonas europa*, *Nitrosomonas eutropha*, and *Nitrosomonas mobilis* wheras *Nitrosospira* genus contains *Nitrosospiramultiformis, Nitrosospira briensis*, and *Nitrosospira tenuis* (Head *et al*, 1993). *Nitrobacter, Nitrospira, Nitrospina, Nitrococcus*, and *Nitrocystis* are responsible for the second stage reaction. There are several subspecies within the *Nitrobacter* genus. They are related genetically within the alpha subdivision of the proteobacteria (Teske *et al*, 1994). *Nitrospina* is related with the delta subdivision whilst *Nitrococcus mobilis* belongs to the gamma subdivision of the proteobacteria. Figure 3.5 shows the phylogenetic tree of nitrifying bacteria.

Recent studies using oligonucleotide probes specific for *Nitrobacter* is not the most dominant nitrite-oxidizing genus in wastewater treatment systems. *Nitrospira* is indicated as the dominant group (Rittmann and McCarty, 2001).

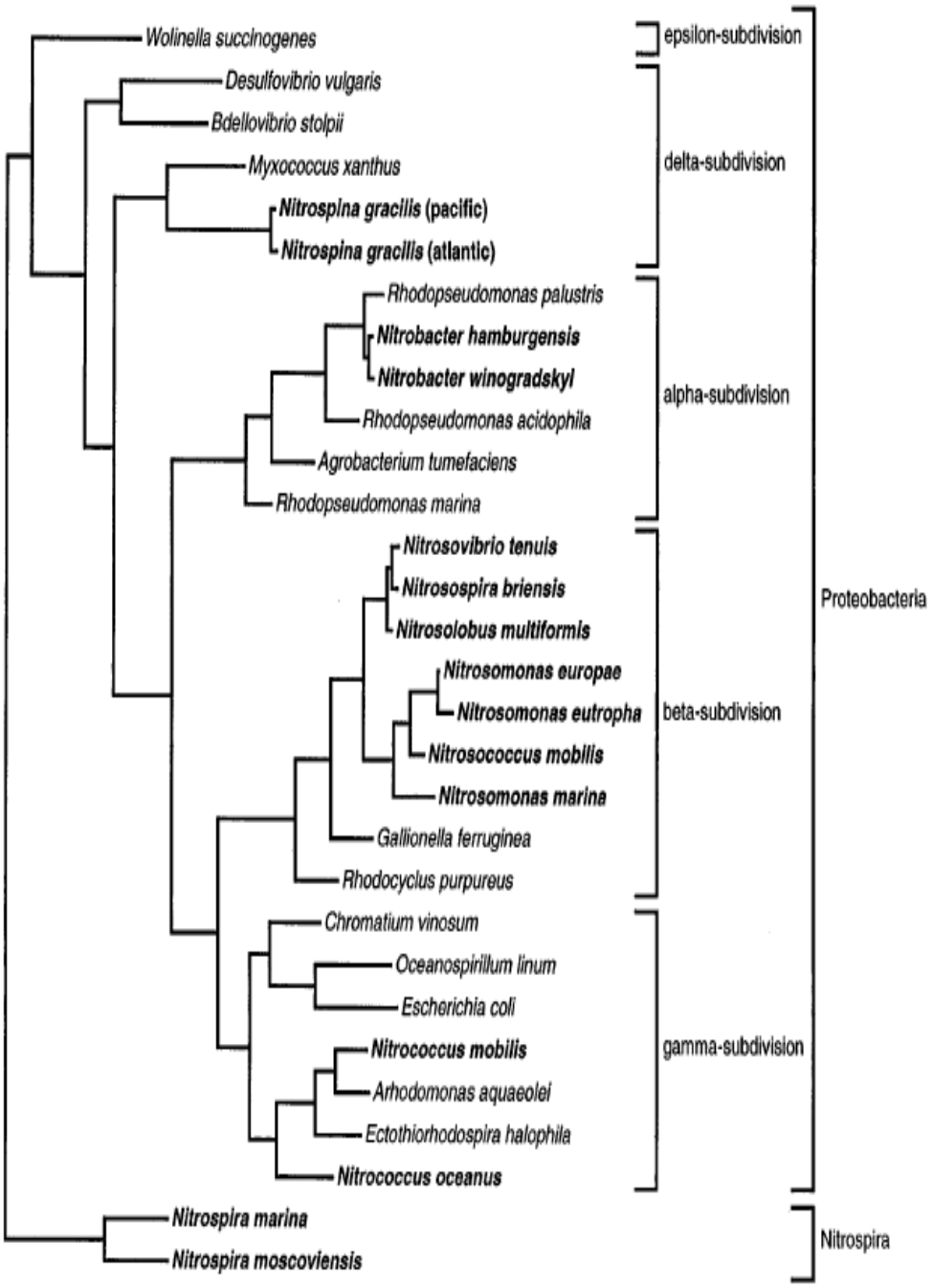


Figure 3.5 Phylogenetic tree of nitrifying bacteria

3.6.2 METHODS FOR IDENTIFICATION AND ENUMERATION OF NITRIFYING BACTERIA

3.6.2.1 TRADITIONAL METHODS

Microscopic technique has being used for enumeration of cells for a long time. Although microscopic observations can give an idea about the morphology and size of the microorganisms, for identification of different microorganisms, further investigations should be carried out. Selective plate method is one of these methods. Nitrifiers can be grown on plates which have appropriate medium and pH value about 7.8-8.0. Since it is difficult to distinguish the nitrifiers from contaminating colonies and to count them, this method is not generally used. Selective enrichment method can also be used for nitrifying bacteria by providing a medium rich in NH_4^+-N, dissolved oxygen, and bicarbonate, but lacking other electron donors. Successive dilutions lead to isolation of a single strain able to carry out ammonium oxidation. Although this technique gives valuable information, it has limitations. First of all, before applying this method, physiological properties of the microorganism should be known. Secondly, growth media which have high concentrations of electron donor (e.g. NH_4^+-N) or nutrients favor the growth of fast-growing microorganisms. The strain which is aimed to isolate may be oligotroph in its normal environment. Although the disadvantages, these methods are used to know the function of the community. For example, it can be determined whether nitrifying bacteria are present in the sample by measuring the production of NO_2^-N or NO_3^-N. The amount of biomass carrying out this process is also estimated by the reaction rates.

For counting microorganisms, Most Probable Number (MPN) technique is commonly used. MPN is not the absolute concentrations of organisms that are present but only a statistical estimate of that concentration (Tchobanoglous and Burton, 1991). The MPN of viable cells is determined by analysis of the number of positive and negative results obtained when testing multiple portions of equal volume and using the Poisson distribution. It is very difficult to estimate the number of nitrifiers by MPN technique because of media selectivity, particulate matter and long incubation times (Underhill, 1990; Gerhardt *et al*., 1994).

The fluorescent antibody (FA) technique has been developed to overcome the limitations of the MPN method (Abeliovich, 1992). This technique requires the production of antibodies for targeted organisms. This technique has also limitations. Firstly, uncultured bacteria can not be investigated because antibody production requires a pure culture of the target organisms. Non-specific binding of antibodies may cause high background fluorescence (Wagner *et. al.*, 1997). Many bacteria have not yet been cultured and such organisms can not be characterized by growth based methods. Beside the difficulty of cultivation, it is quite time consuming and the number of colony forming units is only a minor fraction (less than 1%) of the cell counts determined by direct microscopic technique (Amann *et. al.*, 1992).

The nitrifying bacteria are very difficult to study by cultivation techniques such as MPN and selective plating, because of their long generation times and poor counting efficiencies (Mobarry, 1990).

When it is aimed to determine the different strains carrying out a process, molecular tools should be used to overcome the biases of traditional enrichment culturing.

3.6.2.2 MOLECULAR METHODS

Molecular techniques allow us to study microbial diversity at the genetic level. Generally, comparative analysis of ribosomal RNA (rRNA) molecules (subunit 16S and 23S) are used as a phylogenetic marker. One of the most important reasons of this approach is that all living cells contain ribosomes whose numbers change between 10^3 and 10^5 (Amann, 1995). Secondly, RNAs have some nucleotide positions remained unchanged (universal sequences) and some regions differ among species of bacteria (Stahl, 1995). Furthermore, a correlation between the growth rate and the rRNA content of a species is observed. Since ribosomes work for the protein production, fluorescence intensity of the fluorescently labeled rRNA-targeted oligonucleotide (small, synthetic stretches of RNA or DNA) has a relationship with the cell volume and the growth rate (Amann, 1995; Alcamo, 1996).

During decades, many methods were developed to study the diversity and ecology of microorganisms such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and some hybridization techniques. Before giving brief explanations for these methods, it is intended to provide an overview about the structure of nucleic acids, RNA and DNA.

Structures of DNA and RNA:

Both DNA and RNA have three basic elements:

1. A five-carbon sugar: Ribose in RNA, Deoxyribose in DNA.
2. A series of phosphate groups derived from phosphoric acid molecules and containing phosphorus, oxygen and hydrogen.
3. Four different compounds containing nitrogen. These are known as nitrogenous bases because of their nitrogen content and basic qualities. In RNA, these bases are adenine, uracil, guanine, and cytosine; in DNA they are adenine, thymine, guanine, and cytosine. Adenine and guanine are called purines, and the others are called pyrimidines.

Each building block of a nucleic acid is known as nucleotides. A nucleotide of DNA is a combination of a deoxyribose molecule, phosphate group and a nitrogenous base. In early 1950's, the double-helix structure of DNA was suggested by Watson and Crick. Figure 3.6 shows this accepted structure.

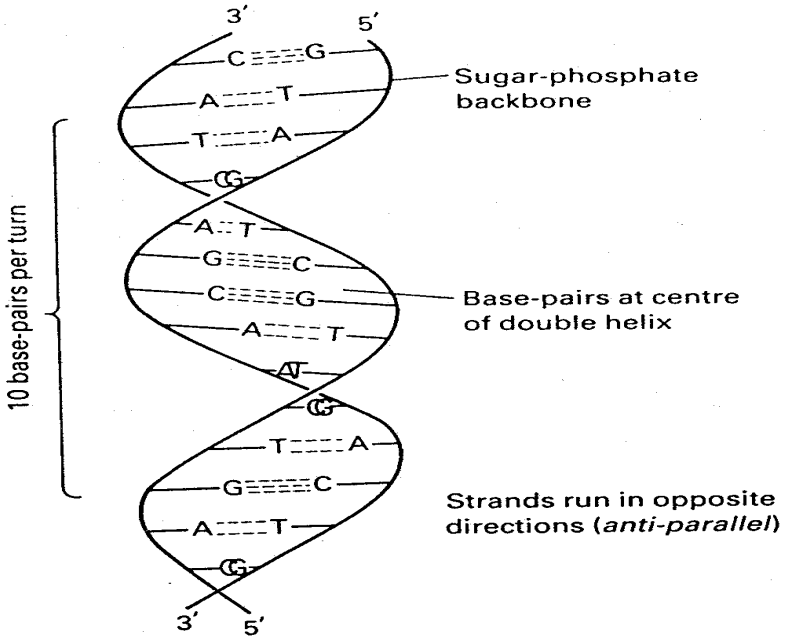


Figure 3.6 Structure of DNA double helix (Wilson and Goulding, 1986).

The sequence of the nitrogenous bases in the DNA molecule causes differences among species of organisms. Different sequences of bases in DNA specify different sequences of bases in RNA, and the sequence of bases in RNA specifies the sequence of aminoacids in proteins. At least 3 types of RNAs are constructed: messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA). rRNA combines with protein to form ribosomes where proteins are formed. As

explained in previous chapter, a ribosome has two subunits: a smaller subunit (30S) and a larger subunit (50S). Complete 80S ribosome is found in complex eukaryotic cells and the denser 70S ribosome of prokaryotic cells (Alcamo, 1996). Figure 3.7 shows the strucuture of RNA.

Ribosomal RNA analysis approach has many advantages to develop new molecular methods. For applying these new techniques, nucleic acids should be extracted in sufficient quality. The extracted DNA is subjected to Polymerase Chain Reaction (PCR) to provide sufficient amount of target DNA and amplify rRNA genes from a particular group of organisms. For achieving this, relatively small, single-stranded DNA molecules, called probe, are used. A DNA probe recognizes and binds to a complementary segment of DNA. In PCR machines, the target DNA is combined with primer DNA. Detection of specific groups of bacteria without cultivation can be detected by PCR, or oligonucleotide probes designed by comparing more variable regions of the molecule (Saunders, 1997).

The essential components of the PCR procedure are the DNA (double stranded) to be amplified in the sample, an excess amount of two oligonucleotide primers that are complementary to either end of the gene to be amplified, the DNA polymerase, a buffer containing magnesium and four nucleotide building blocks dATP, dCTP, dGTP and dTTP. In a PCR, the first step is the denaturation of DNA. This can be achieved by increasing the temperature to 95 ^{0}C at which double DNA strands are separated into single strands. After the DNA is denatured, the mixture is cooled to 50 ^{0}C to allow the primers to anneal to the complementary sequences at either end of the gene. Since primers are in excess relative to the target DNA, this ensures that most target strands will anneal to a primer and not to each other. This temperature also favors the hybridization of the primers to the desired DNA sequence. The temperature is again raised to 72 ^{0}C and the enzyme adds monomers to the end of the primers until a strand complementary to the original is produced. Two copies of the original target DNA are produced at the end of this process (Figure 3.8). Repetition of this process 20 to 30 times results in up to a 1 billion-fold increase in the number of the target gene. RNA can also be amplified with a process called RT-PCR. In both cases the result will be a mixture of DNA fragments obtained from the different community members.

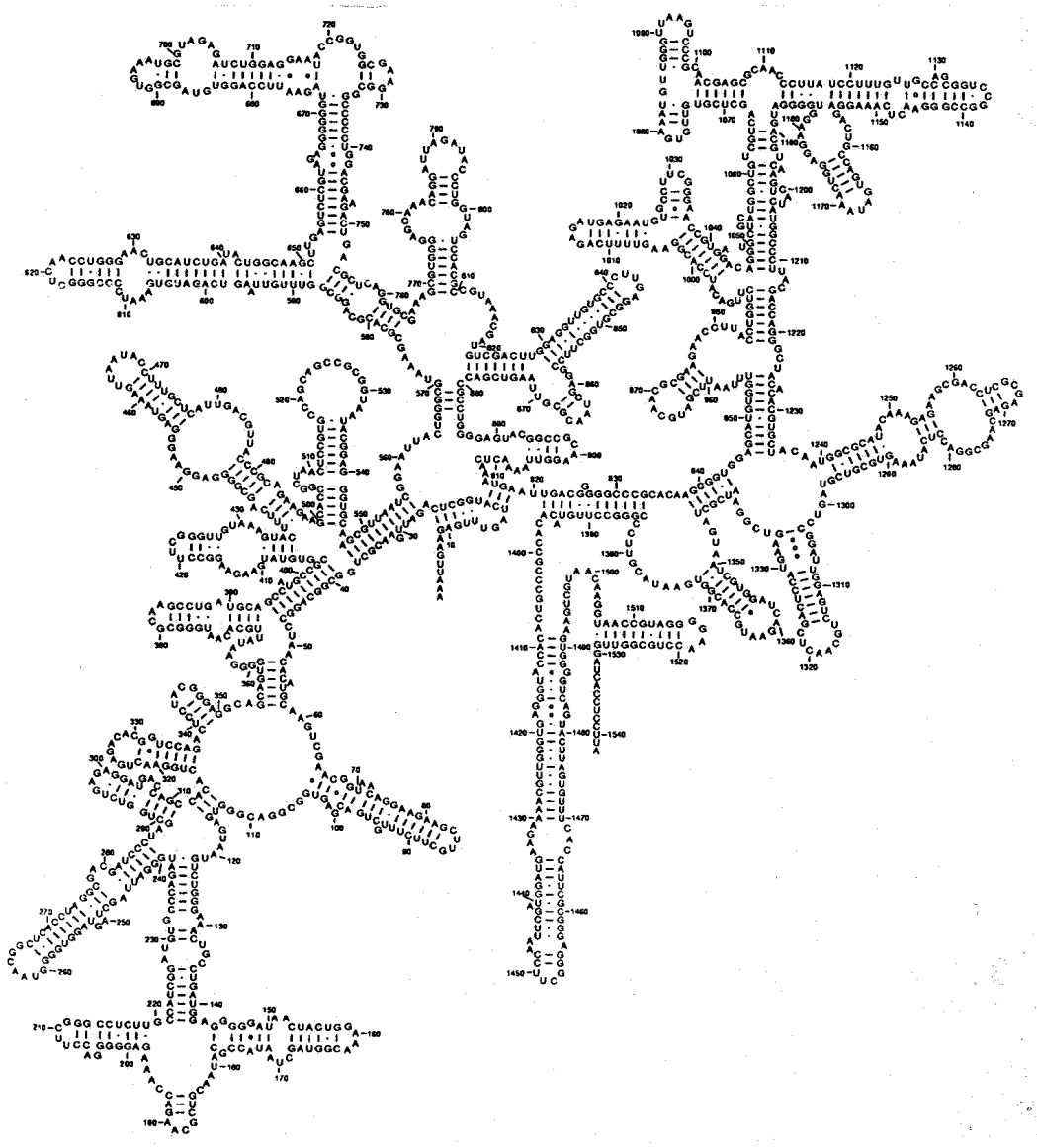


Figure 3.7 Primary and secondary structure of 16S ribosomal RNA (rRNA). This is the eubacterial form of *Escherichia coli* (Madigan *et al.*, 1997).

For analyzing of 16S rRNA genes from environmental samples and for separating of a heterogeneous mixture of PCR products, denaturing gradient gel electrophoresis (DGGE) method can be used. DNA fragments which have same length but different base-pair sequences can be separated electrophoretically. Molecules with different sequences have a different melting behavior, stop migrating at different positions and make several distinguishable bands in the polyacrylamide gels containing a linearly increasing gradient of denaturants which are the mixture of urea and formamide (Muyzer and Ramsing, 1995; Muyzer, 1999).

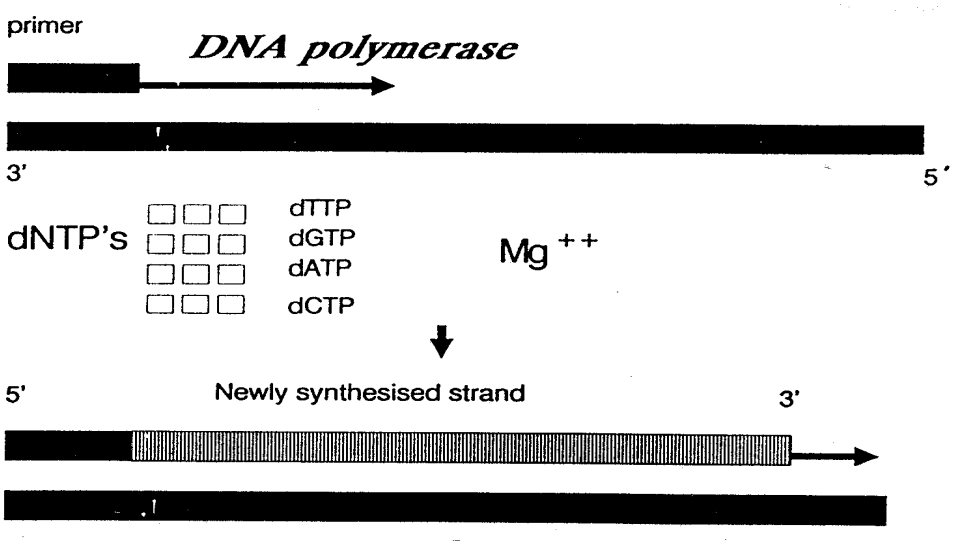


Figure 3.8 Primer extension. DNA polymerase extends a primer by using a complementary strand as a template (McPherson *et al.*, 1991).

CHAPTER 4 FLUORESCENT *IN SITU* HYBRIDIZATION TECHNIQUE

The determination of composition and number of bacteria without cultivation directly in their natural environment can be managed by rRNA-targeted oligonucleotide probes. Probe is a single strand of DNA or RNA intended to hybridize with a complementary sequence in order to detect that sequence. rRNA gene fragments was used as phylogenetic stains firstly in 1989 (DeLong *et al.*, 1989). Since that time, fluorescent *in situ* hybridization technique has become a common tool for identification of individual bacterial cells (Amann *et al.*, 2001).

Oligonucleotide probes are synthesized enzimatically or chemically. Fluorescently or radioactively labeled rRNA-targeted oligonucleotide probes are used as a complementary of a single-strand sequence to get a double-stranded nucleic acid. This process is called "hybridization". Hybridization has some advantages over classical techniques:

1. Probing does not introduce the selection biases inherent in culturing techniques,
2. 16S rRNA sequences provide phylogenetic information between organisms and can therefore distinguish between different populations independently of activity,
3. It does not depend on the amplified genes like PCR based methods.

There are four steps in a typical FISH protocol: Firstly, the sample should be fixed to permeable the cells while maintaining their morphological integrity. Alcohols or aldehydes are used for this purpose. Then, the sample is incubated in the hybridization buffer, which contains fluorescently labeled oligonucleotide probe. After hybridization step, the sample is washed by wash buffer to eliminate unbound probe. Finally, the sample, attached to a microscope slide is viewed by epifluorescent microscopy or confocal scanning laser microscope (CSLM). Figure 4.1 represents the base-pairing between a fluorescently labeled oligonucleotide probe and a target rRNA.

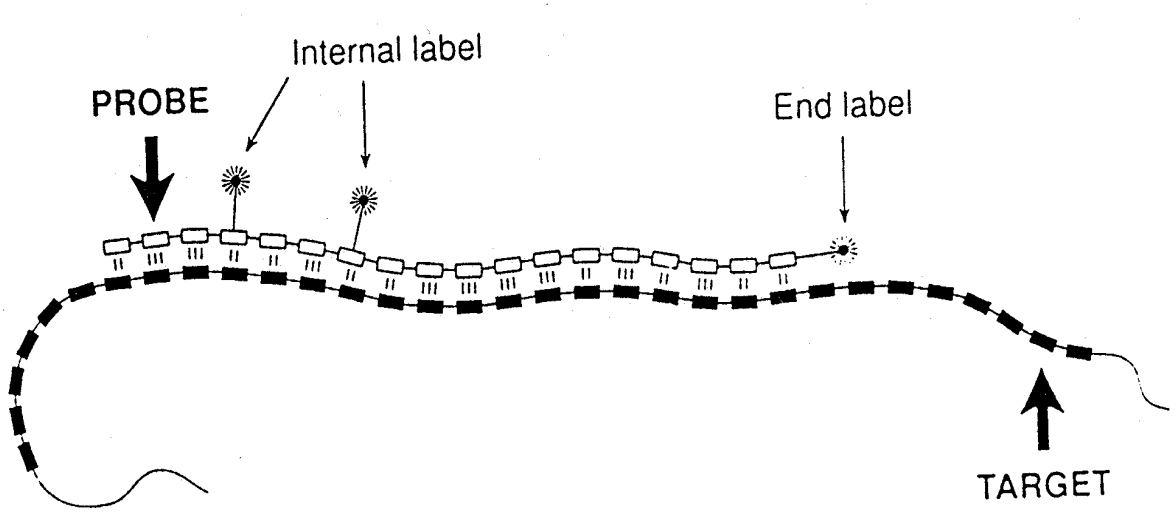


Figure 4.1 Base pairing between a fluorescently labeled oligonucleotide probe and a target rRNA (Stahl and Amann, 1991).

One of the most important points in FISH technique is to determine the optimum hybridization conditions for each probe. The optimal hybridization temperature should be determined empirically to avoid mismatching of the probe to rRNA sequences (Sounders, 1997). Different formamide concentrations are also applied at the same temperature to increase the stringency of hybridization. Addition of formamide decreases the excess probe-nontarget hybridization (Stahl and Amann, 1991). NaCl concentration in wash buffer is another factor affecting the stringency of the hybridization. Since increasing NaCl concentration decreases the specificity of hybridization, different NaCl concentration in wash buffer are tried to eliminate non-specific bindings. The melting temperature (Tm) also affects hybridization. The T_m is the temperature at which the DNA is 50% melted and can be related to the dissociation temperature (T_d) of oligonucleotide probes. Higher GC content will result with higher melting temperature since guanine-cytosine base pairs are stronger than adenine-thymine base pairs because of the three hydrogen bonds (Madigan *et.al.*, 1997). Figure 4.2 shows the effect of temperature on nucleic acids and how to determine melting temperature.

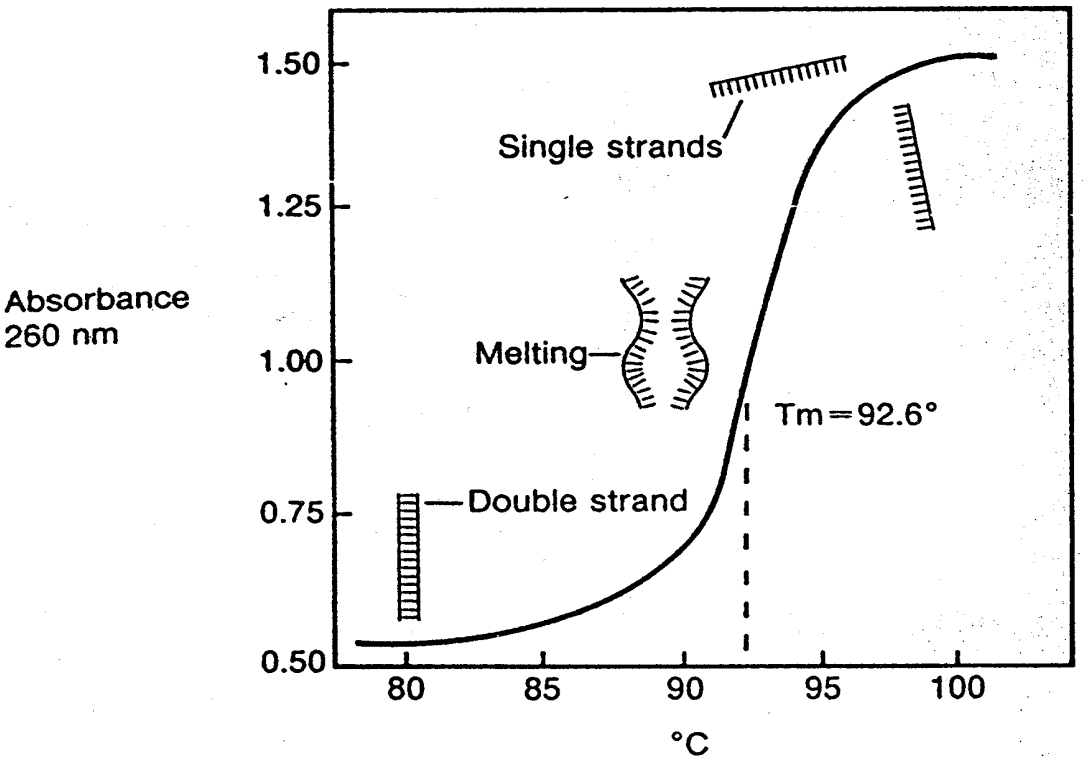


Figure 4.2 Effect of temperature on nucleic acids (Madigan *et.al.*, 1997).

There are some specific advantages of FISH technique:

1. FISH can give highly specific results with specific probes,
2. *in situ* growth rates may be estimated by measuring the fluorescence intensity,
3. It provides an opportunity for using multiple probes labeled by different dyes simultaneously within a single sample.

Beside these advantages, there are some problems to be overcome. Uneven cell penetration is one of them. The first step of FISH method, fixation, is applied to make the probes enter into the cell. Fixative solutions permeablize the cell walls of microorganisms. Although, permeabilization is managed, oligonucleotide probes may not permeate all cell types and find 16S rRNA target sequences (Muyzer and Ramsing, 1996). High stringency can be managed by increasing formamide concentration or decreasing salt concentration in hybridization buffer. Sometimes, low signal or no signal may be obtained after *in situ* hybridization. If there is not enough concentration of cells or RNA, no signal problem is experienced. It is reported that detection of less than 10^3 cells per cm^2 is very difficult (Amann *et al.*,

1995). Low or insufficient accessibility of the rRNA molecules may be the cause of low signal intensity. When the accessibility of target site is very low, no signals can be obtained with specific probes whilst strong signals with a universal probe. Accessibility of the cells can be increased by addition of formamide to the hybridization buffer (Amann *et al.*, 1992). High amount of background autofluorescence is another problem encountered frequently. Some microorganisms, such as archaea, may give more signals than the labeled ones. Combined application of multiple fluorescent oligonucleotide probes or the use of fluorescent dyes whose emission wavelengths do not coincide with the autofluorescence can solve this problem (De long *et al*., 1989). Positive and negative controls should be applied to avoid from the effect of this problem.

CHAPTER 5 CLASSIFICATION OF BIOLOGICAL NITRIFICATION PROCESSES

Nitrifying organisms are almost always present in biological treatment processes and competing with the heterotrophic bacteria for dissolved oxygen and space. To overcome the problems due to this competition, different process configurations are developed.

In one of these configurations, carbon oxidation and nitrification occur in the same reactor. This system is termed as "single-stage". In other type of configuration, which is called as "two-stage", carbon oxidation and nitrification are accomplished in separate reactors. Both systems have suspended or attached-growth reactor types. In suspended growth systems, the microorganisms responsible for the conversion of the organic matter or other constituents in the wastewater to gases and cell tissue are maintained in suspension within the liquid. In attached growth systems, microorganisms are attached to some inert medium such as rocks or specially designed ceramic or plastic materials (Tchobanoglous and Burton, 1991).

The ability of the activated sludge systems to nitrify has been correlated to the BOD_5/TKN ratio. It has been found that when the ratio is greater than about 5, the process can be classified as a combined carbon oxidation and nitrification process, and when the ratio is less than 3, it can be classified as a separate-stage nitrification process (U.S. EPA, 1975). Figure 5.1 shows a typical single-stage and separate-stage approaches (Tchobanoglous and Burton, 1991).

5.1 SINGLE-STAGE PROCESSES

Heterotrophic and nitrifying bacteria exist in same mixed liquor to remove carbon and nitrogen simultaneously. Conventional plug-flow, complete-mix, and extended aeration are the most commonly used processes. Trickling filter, rotating biological contactor, and packed-bed reactor are the three commonly used attached growth systems. These systems are resistant to shock loads, and they have low operating and

maintenance costs. On the contrary, attached growth systems are difficult to model accurately. Filter depth, hydraulic flow and organic loading rate, recirculation ratio, type of filter media, mass transfer of organic material and oxygen from the liquid layer to the attached slime layer, and the metabolism of the attached layer are the effective variables in attached growth processes (Horan, 1991).

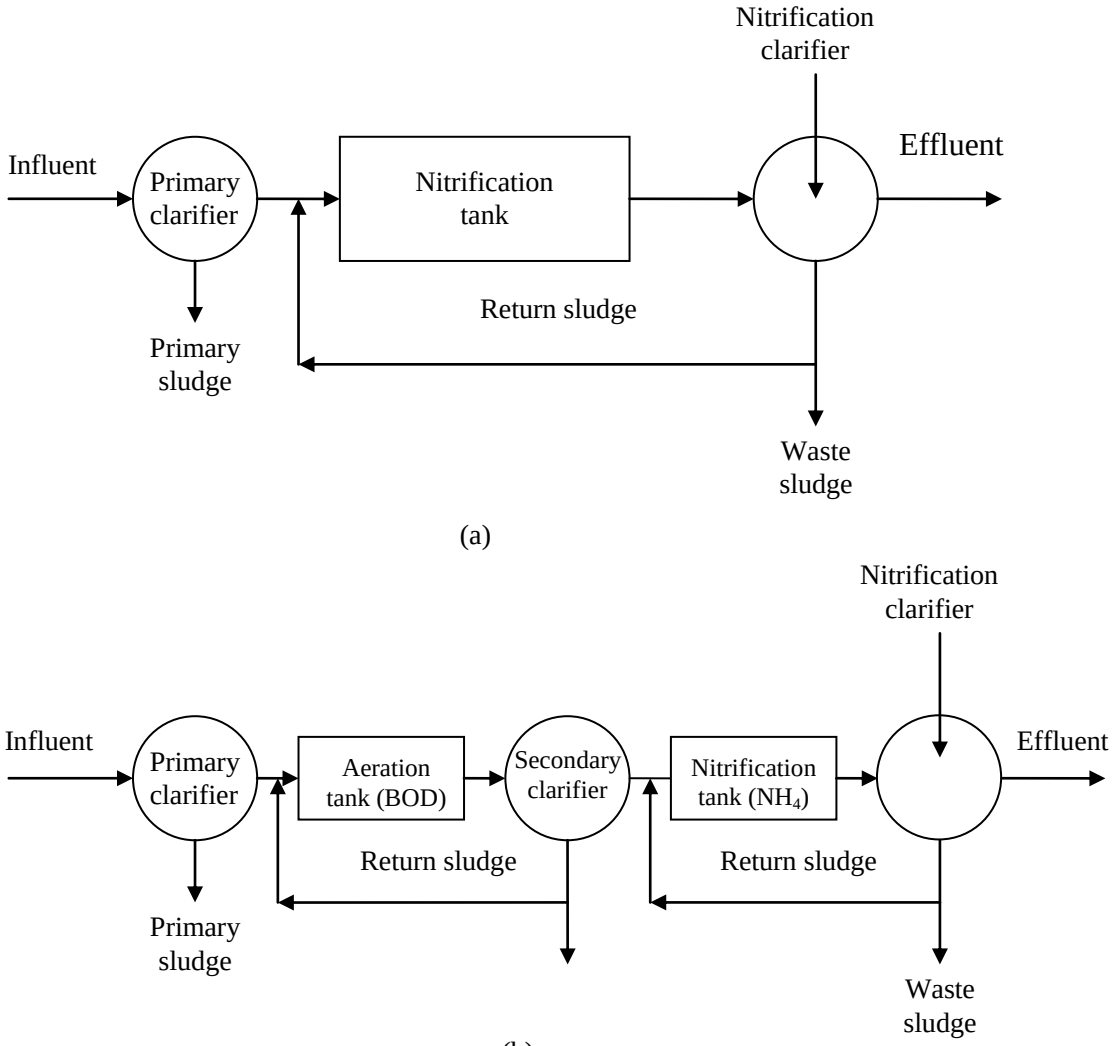


Figure 5.1 (a) single-stage (b) separate-stage treatment systems.

In trickling filters, there is a bed, which has a permeable medium to which microorganisms are attached and through which wastewater is percolated or trickled. The media generally has rock or plastic material. The microorganisms attached to the filter media degrade the organic material in wastewater. Since plastic medium have larger contact areas, higher organic loadings can be applied in trickling filters, which have plastic packing. In trickling filters, at high organic loadings the nitrifying

bacteria compete for oxygen with heterotrophic bacteria, which dominate the biofilm. 0,16-0,096 kg/m^3-day organic loadings are recommended to achieve 75-85% nitrification. A typical relationship between loading rates and ammonia removal rate in trickling filters is shown in Figure 5.2.

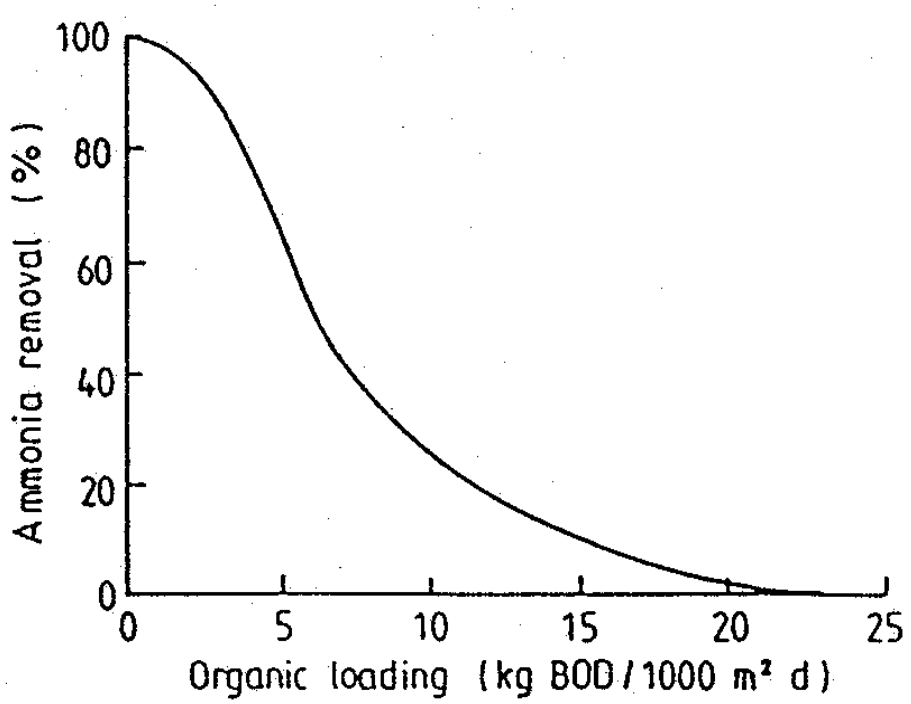


Figure 5.2 Relationships between loading rates and ammonia removal rate in trickling filters (Horan, 1991).

Rotating biological contactors have closely placed disks, which are submerged in wastewater and rotated slowly through it. Growth of microorganisms occurs on the surface of the disks. The rotation of the disks contacts the biomass with the organic material in the wastewater and then with the atmosphere for adsorption of oxygen. Disc rotation also removes the excess solids from the disks. Rotating biological contactors are very useful because of their large amount of biomass (Tchobanoglous and Burton, 1991).

Packed-bed reactors are used effectively as attached growth system for carbon and nitrogen removal. A packed-bed system consists of a reactor packed with a medium to which the microorganisms attach. Wastewater is introduced from the bottom and goes through an underdrain system or inlet chamber.

5.2 TWO-STAGE PROCESSES

In separate stage reactors, carbonaceous oxidation and nitrification processes are operated independently to achieve optimum performance. Potential toxic effects to nitrifying bacteria may be reduced during first stage.

Separate-stage suspended growth nitrification processes are similar in design to the activated sludge systems. In these systems, experimentally measured nitrification rates are used to design. Nitrification rates increase as the temperature increases and BOD_5/TKN ratio decreases. When high ammonia removal efficiency is required, complete mix staged flow or plug-flow reactors are favored.

Trickling filters and rotating biological contactors are generally used as separate-stage attached-growth processes. Two-stage trickling filter combination is effectively applied for carbon and nitrogen removal. In trickling filters, nitrogen removal efficiency decreases with increasing surface loading and decreasing wastewater temperature.

In rotating biological contactors, influent ammonia concentration is used to design the biological nitrification system. Significant nitrification will not occur in this system until the soluble BOD_5 concentration is reduced to 15 mg/l or less similar to single-stage attached-growth processes.

5.3 ISKI PASAKOY ADVANCED BIOLOGICAL WASTEWATER TREATMENT PLANT

The plant was built up on a 507.000 m^3 area to treat the wastewater in Omerli watershed as shown in Figure 5.3. It provides the removal of nitrogen and phosphorus contents beside carbon. Figure 5.4 shows the general view of the plant whilst Figure 5.5 shows the schematic diagram of it.

Wastewaters are directed to the phosphorus removal unit after primary treatment. This unit consists of 3 tanks, which have a total volume of 8800 m^3.

Aeration unit has 4 tanks; the first one is anoxic, second one is aerobic or anoxic according to the season (in winter it is aerobic and in summer it is anoxic), and two last ones are aerobic. Each of them has a volume of 10.000 m^3. In anoxic tank, denitrification; in aerobic tanks, nitrification occurs. Before denitrification,

wastewater is recirculated to anoxic tank to provide NO_3 rich wastewater in aerobic tanks. Figure 5.6 shows an aeration tank of the plant.

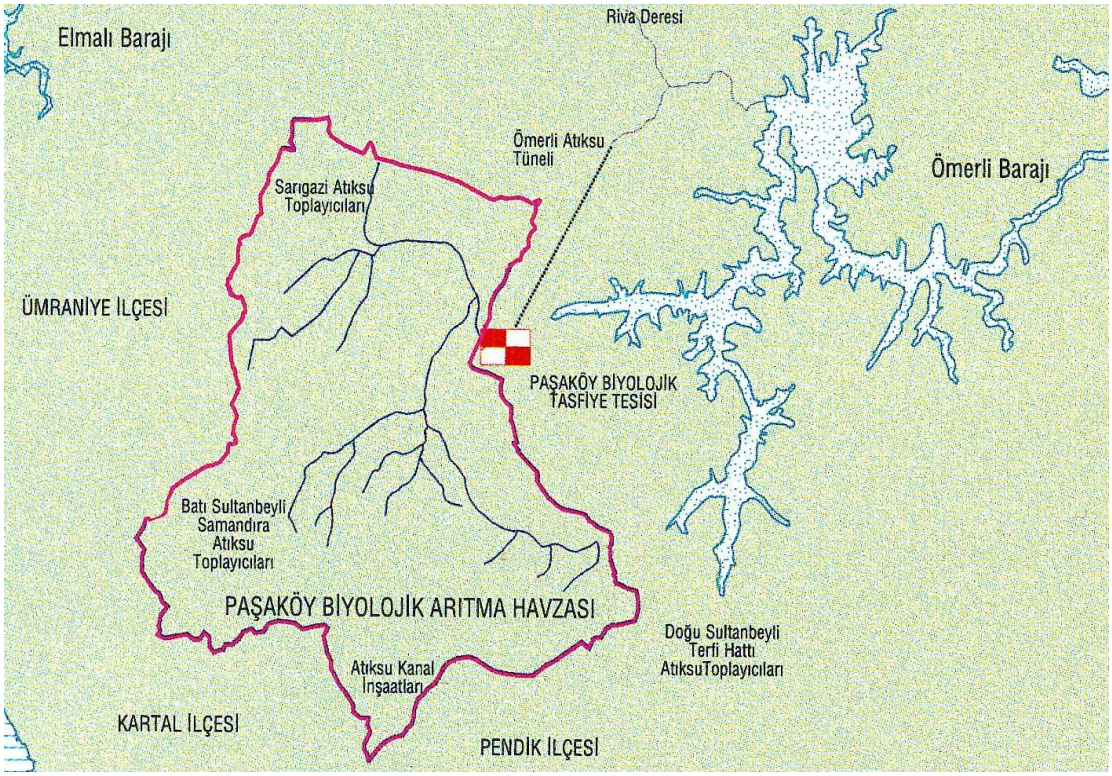


Figure 5.3 Placement of ISKI Pasakoy WWTP in Omerli watershed.

Figure 5.4 General view of treatment plant.

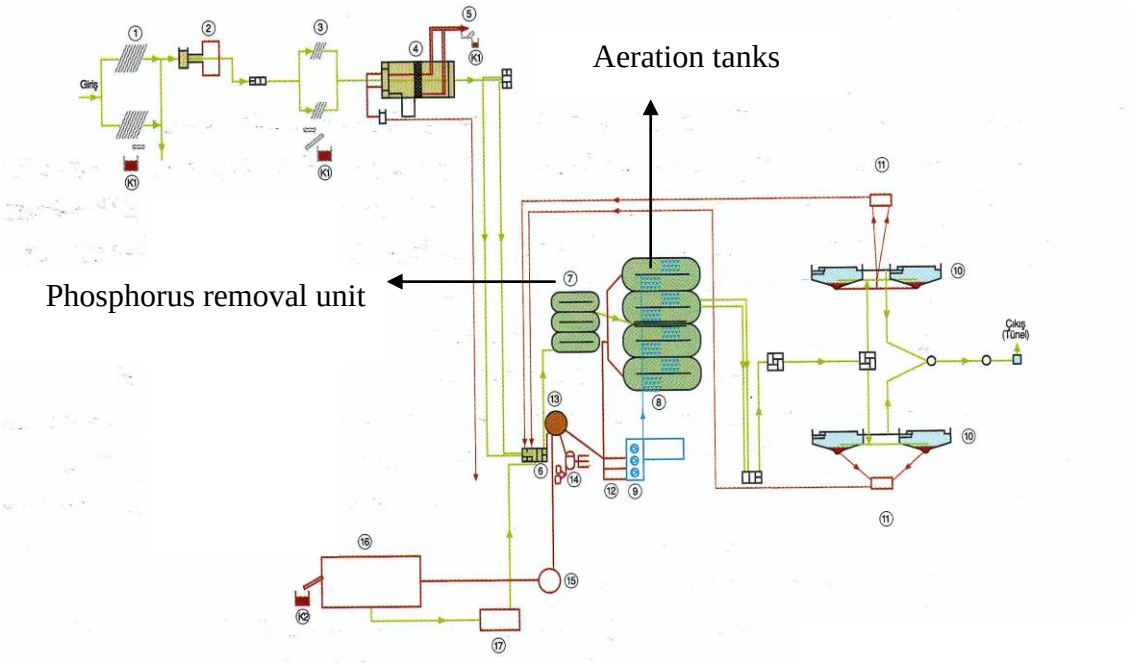


Figure 5.5 Schematic diagram of treatment plant.

Figure 5.6 Aeration tank of nitrification unit.

As shown in Figure 5.7 and Figure 5.8; BOD_5 value of the influent changes between 20-430 mg/l, in the effluent between 20-98 mg/l whereas TKN value of influent varies between 85-6 mg/l, effluent 0,4-21 mg/l.

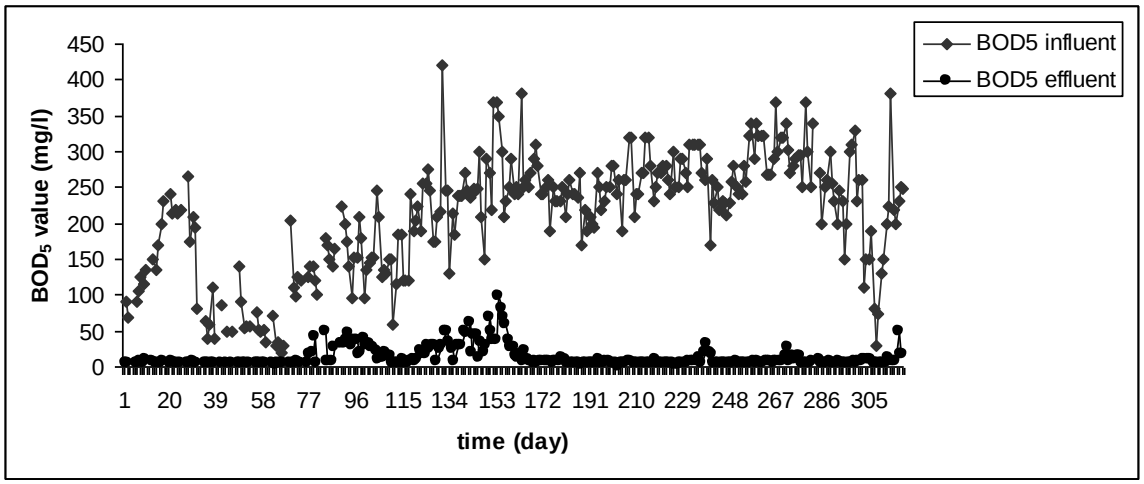


Figure 5.7 Influent and effluent BOD_5 values of system.

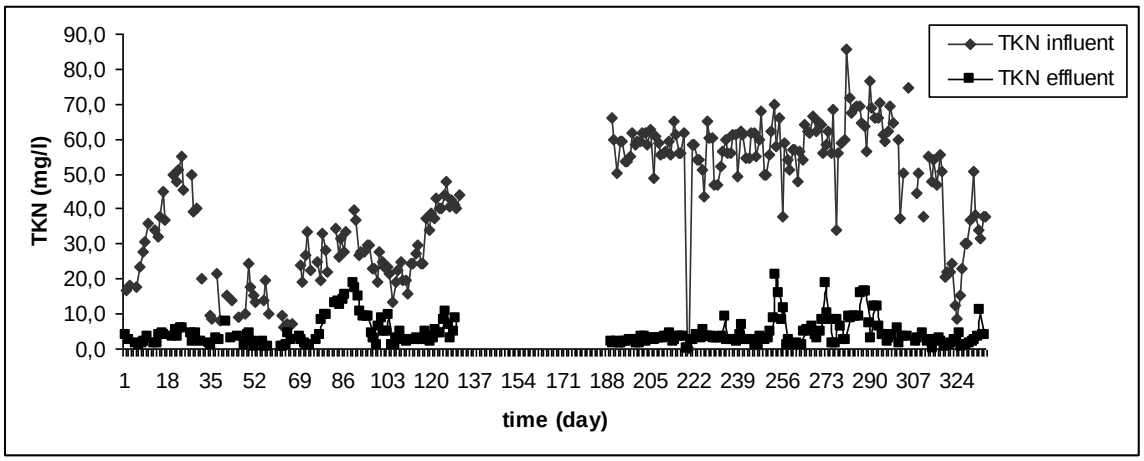


Figure 5.8 Influent and effluent TKN values of system.

CHAPTER 6 MATERIALS AND METHODS

6.1 ENVIRONMENTAL SAMPLING

Duplicate samples were taken from the nitrification reactor of ISKI Pasakoy Advanced Biological Wastewater Treatment Plant about which the details are given in Chapter 5. Necessary amount of sample was put into 50ml Falcon tubes where 1:1 ratio absolute ethanol added for molecular studies. The samples were transported to laboratory in cool box, stored at –20°C, and fixed immediately.

6.2 MATERIALS

SYBR Green I stain was used as a DNA stain before hybridization. Phosphate Buffered Saline (PBS), 0.2 μm polycarbonate isopore membrane filters (Millipore), filtration set were needed for this prodecure. For performing FISH technique, several materials are necessary. Hamilton adjustable volume pipettes (1 ml, 100 μl, 10 μl) and pipette tips (Hamilton Company, USA); micro centrifuge which has a maximum speed of 14000 rpm (Jouan, France); vortex (Scientific Industries, USA; Model G-560E); Eppendorf micro tubes were used. 50 ml falcon tubes, absolute ethanol, Phosphate Buffered Saline, Paraformaldehyde, 0.2 μm filter were used in fixation step. 60%, 80%, and 98% ethanol were necessary for dehydration step. Heat block (Grant Instruments Ltd., UK; Type QBT2), fluorescently labeled oligonucleotide probes, 4.5 M NaCl, 200 mMTris-HCl, 10% Sodium Dodecyl Sulphate, Deionized formamide, sterile distilled water were used in hybridization step. Seven 16S rRNA targeted oligonucleotide probes which have different target groups were used in this study. The probes were provided in a desalted and dry state from manufacturer (QIAGEN, Operon). They were resuspended in TE buffer which contains 10 mM Tris and 1 mM EDTA. These suspended stocks can be stored at -20^{o}C for several weeks. Table 6.1 indicates the names, sequences, and specificites of the probes used in this study.

Table 6.1 Names, sequences, and specifities of the probes.

Probe Name	Probe sequence	Specificity
S-*-Ntros-0190-a-A-19	5'-CGATCCCCTGCTTTTCTCC-3'	Ammonia – oxidizing β - *Proteobacteria*
S-*-Nsom-0156-a-A-19	5'-TATTAGCACATCTTTCGAT-3'	*Nitrosomonas* C - 56, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*
S-*-Nsv-0443-Sa-20	5'-CCGTGACCGTTTCGTTCCG-3'	*Nitrosolobus multiformis*, *Nitrosospira briensis*, and *Nitrosovibrio tenuis*
S-Neu-a-A-18	5'-CCCCTCTGCTGCACTCTA -3'	*Nitrosomonas europaea*, *Nitrosomonas eutropha* and *Nitrosococcus mobilis*
S-G-Nit1000-Sb-15	5'-TGCGACCGGTCATGG -3'	*Nitrobacter spp.*
S-G-Nbac-1035-a-A-18	5'-CCTGTGCTCCATGCTCCG -3'	*Nitrobacter* spp.
S-Univ1392-a-A-18	5'-ACGGGCGGTGTGTAC -3'	All organisms
S-D-Bact-0338-a-A-18	5'-GCTGCCTCCCGTAGGAGT-3'	All Bacteria
S-D-Bact-0338-a-S-18	5'-ACTCCTACGGGAGGCAGC-3'	No target (Non338)

In this research, 3 different dyes were used for labeling the oligonucleotide sequences. Fluorescein, Tetramethlyrhodamine (TAMRA) and cyanine (Cy3) dyes were added at the 3' or 5' ends of the sequences. Table 6.2 shows the fluorescent dyes, their wavelengths, and the probes to which they were bound.

In washing step, 0.5 M EDTA and Milli Q Water were also necessary beside other materials. For viewing, epifluorescent microscopy (Olympus, Model BX50) fitted with a 100 W high-pressure mercury lamp, Teflon coated slides, and an antifadent (Citifluor Ltd., Canterbury, UK) were used. Different filter sets were applied to the microscopic slides for viewing specific dyes. For Sybr Green I U-MWB (450-480 nm); for FITC, TAMRA, Cy3 için U-N61002 (350-750nm) filter sets were used. The images were taken and analyzed by the computer software SPOT Advance™.

Table 6.2 Names of dyes, wavelengths and probes they were binded.

Dye	Excitation wavelength	Emmision wavelength	Probe
FITC	494	517	Eub 338
TAMRA	542	568	Univ 1392, Nb 1000, NIT 3
Cy3	550	565	Nso 190, Nsm156, Nsv443, NEU
SYBR Green I	494	521	All cells

6.3 METHODS

Standard paraformaldehyde fixation and Hybridization method described by Manz *et al* (1992) were applied in FISH technique after SYBR Green 1 staining. BOD_5, COD, TKN, TP, SS analysis are carried out according to the Standard Methods (APHA, 1989) in İstanbul Technical University, Environmental Engineering laboratory.

CHAPTER 7 EXPERIMENTAL STUDY

7.1 SYBR GREEN I STAINING OF CELLS

SYBR Green I was used to view the whole cells active or inactive in the sample. SYBR Green I is a fluorescent dye which stains double stranded DNA and gives green signal when exposed by a light of a specific wavelength. According to the results, dilution factor were determined for hybridization experiments.

7.2 MAIN STEPS IN FISH

7.2.1 FIXATION

There are some fixation procedures which are applied successfully in the literature. Standard paraformaldehyde fixation is applied in this study. 250 µl of sample was centrifuged at 13000xg for 3 minutes and the supernatant was discarded. Then, Phosphate Buffered Saline (PBS) was added and the sample was mixed. The solution was centrifuged again for 3 minutes at 13000xg. Paraformaldehyde (PFA) fixative solution was added after discarding supernatant. This suspension was left to incubate at +4^{o}C for at least 3 hours.

After fixation, the cells were washed; PBS and absolute ethanol (1:1, v/v) solution was added, and mixed. This suspension can be stored up to 8 weeks at -20^{o}C, but it is not recommended to keep them for a long time because of decreasing rRNA content and hybridization signal (Wagner *et al.*, 1994a).

7.2.1.1 FIXATIVE SOLUTION

4% PFA fixative solution was prepared freshly for every time of use. 0.4 g PFA was dissolved in 10 ml PBS. This solution was sterilized by a 0.2 µm filter.

7.2.2 ALCOHOL DEHYDRATION

The cells were dehydrated to concentrate the rRNA by using increasing concentrations of ethanol in water. The fixed sample was centrifuged at 13000xg for 3 minutes. 1 ml of 60% ethanol in water (v/v) was added after decanting the supernatant. After mixing using a pipette, this sample was allowed to stand for a few

minutes, and centrifuged again at 13000xg. These steps were repeated with 80% and 100% ethanol.

7.2.3 HYBRIDIZATION

Different hybridization procedures are described in the literature (Davenport, 2000; Amann, 1990; Muyzer, 1992). According to the procedure described by Manz *et al.* (1992), the pellet was first pre-hybridized after adding the hybridization buffer so that the final volume with the probe will be 40 µl. After pre-hybridization, fluorescently labeled oligonucleotide probe in concentration of 50 ηg/µl was added. This solution was mixed using vortex and incubated overnight at the appropriate hybridization temperature. 6 probes for nitrifiers were used in this study. Probes Nso190 (s-Nso190Sb-19) targets all sequenced ammonia oxidizers (AOB) of the β subclass of proteobacteria. Nsm156 (S-Nsm156Sa-19) and Nsv443 (s-Nsv443Sa-20) were used to hybridize the different genera of AOB. First one identifies members of the genus *Nitrosomonas* and *Nitrococcus mobilis* while the second one identifies the *Nitrosovibrio, Nitrosolobus, Nitrosospira* groups. NEU probe (S-*-Nsm-061-a-A-18) targets most halophilic and halotolerant members of the genus *Nitrosomonas* (*Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis*). For identification of nitrite oxidizing bacteria, Nb1000 (S-G-Nit1000-Sb-15) and NIT3 (S-G-NBac-1035-a-A-18) were used as specific probes which target to members of the genus *Nitrobacter* (Mobarry, 1990). Figure 7.1 shows the distance tree for nitrifying bacteria. Beside these AOB and NOB specific probes, 3 other probes were used for positive and negative controls. Bact338 (S-D-Bact-0338-a-A-18) was used to hybridize the all bacteria. It is the positive control of the hybridization experiment. Univ 1392 probe was used as another positive control. This probe is specific to all known organisms. Furthermore, Non338 (S-D-Bact-038-a-A-18) was used as negative control and normally it is expected not to hybridize the rRNA of the bacteria. By using this probe it can be detected autofluorescent microorganisms if they are present in the sample. Hybridization procedures were also applied withour adding a probe as a negative control. Optimum hybridization conditions for each probe were determined experimentally by changing formamide percentages between 20%-60% in hybridization buffer. Other conditions were taken from literature (Mobarry *et al.*, 1996; Juretschko *et al.*, 1998; Wagner *et al.*,1998). Table 7.1 shows the optimum hybridization conditions for probes which were used in this research.

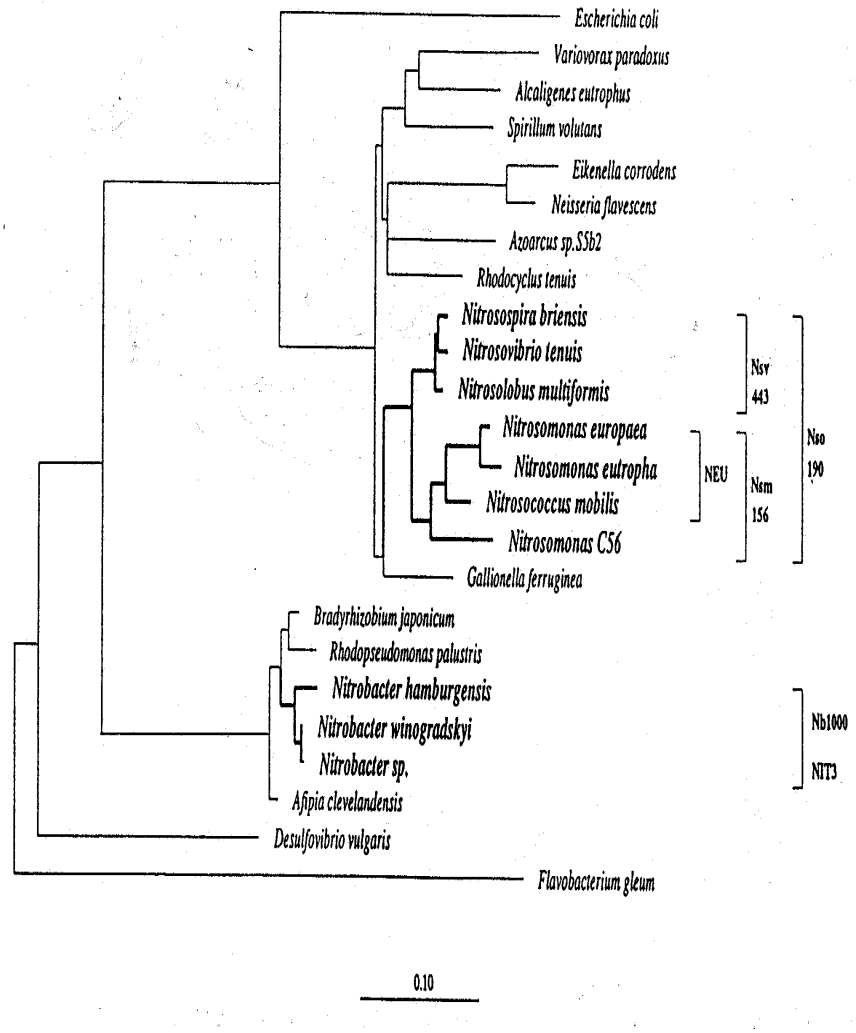


Figure 7.1 Distance tree for the Proteobacteria including the nitrifying isolates (Mobarry et.al., 1996). The bar represents 0.1 estimated changes per nucleotide.

7.2.3.1 HYBRIDIZATION BUFFER

1 ml solution containing different formamide concentrations (for instance 20% formamide) was prepared as follows (Manz *et al.*, 1992):

0.2 ml 4.5 M NaCl, 0.1 ml 200 mMTris-HCl, 10 µl 10% Sodium Dodecyl Sulphate (SDS), 0.2 ml Deionized formamide were mixed with 0.5 ml sterile distilled water. For higher formamide concentrations, the volume of deionized formamide and sterile distilled water were adjusted so that the final volume will be 0.45 ml.

Table 7.1 Optimum hybridization conditions for probes used

Probe	T_d (°C)	Formamide (%)	Washing Temp. (°C)	NaCl in WB (mM)
S-*-Ntros-0190-a-A-19	62	55	62	180
S-*-Nsom-0156-a-A-19	46	5	48	56
S-*-Ntros-0443-Sa-20	52	30	48	32
S-*-Neu-a-A-18	48	35	48	48
S-G-Nit1000-Sb-15	46	40	46	48
S-G-Nbac-1035-a-A-18	46	40	48	56
S-Univ1392-a-A-18	37	35	37	56
S-D-Bact-0338-a-A-18	37	30	37	180

7.2.4 WASHING

The purpose of this step is to remove the unbound or non-specific binded probe. The concentration of NaCl in the wash buffer is the critical point of this step, because increasing NaCl concentration decreases the specificity of the hybridization. According to the washing procedure described by Manz *et al.* (1992), the samples were centrifuged at 13000xg for 3 minutes and the supernatant was decanted. Freshly prepared wash buffer was added. The sample was mixed with this solution using a pipette and incubated at the appropriate washing temperature for 15 minutes. After incubation, the samples were pelleted by centrifugation and the supernatant was discarded, and 50 µl of Milli Q Water was added.

7.2.4.1 WASH BUFFER

20 ml buffer with 20% formamide was prepared by mixing 1 ml 4.5 M NaCl, 200 µl 0.5 M EDTA, 2 ml 200 mM Tris-HCl and 200 µl 10% SDS with 16.6 ml Milli Q Water to make up the total volume 20 ml (26).

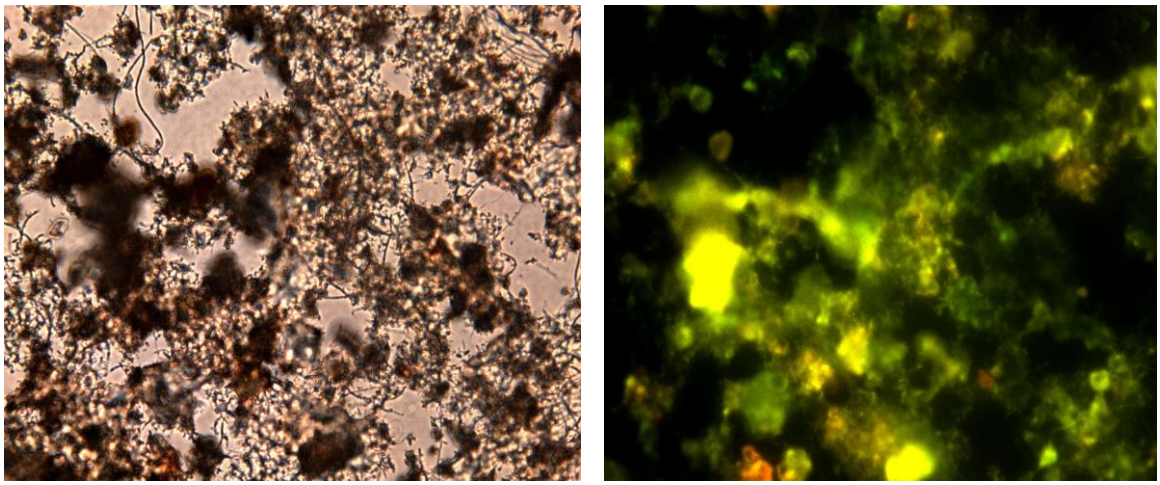
7.2.5 VIEWING WITH MICROSCOPY

The cells were observed and enumerated using Teflon-coated microscopic slides. A 10 µl sample was mounted onto a well of the slide. This was left to air dry in the dark. Afterwards, a drop of Citifluor was added as an antifadent which keeps the fluorescence from fading too quickly. This slide was ready to be observed by the epifluorescence microscope.

CHAPTER 8 RESULTS

8.1 SYBR GREEN 1 STAINING

Samples were stained by SYBR Green before hybridization. U-MWB filter was used to observe the whole cells. Figure 8.1. and 8.2 show the phase contrast wievs, and the SYBR Green 1 stained cells .

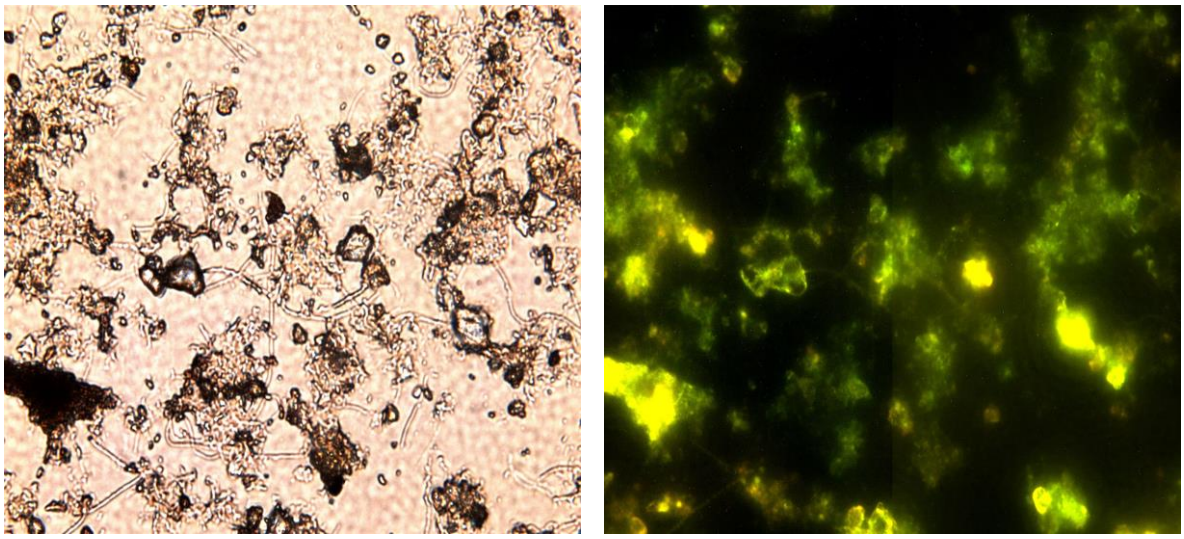


(a) (b)

Figure 8.1 Sybr Green 1 stained cells (a) Phase contrast view (b) U-MWB filter view (stained cells are yellow)

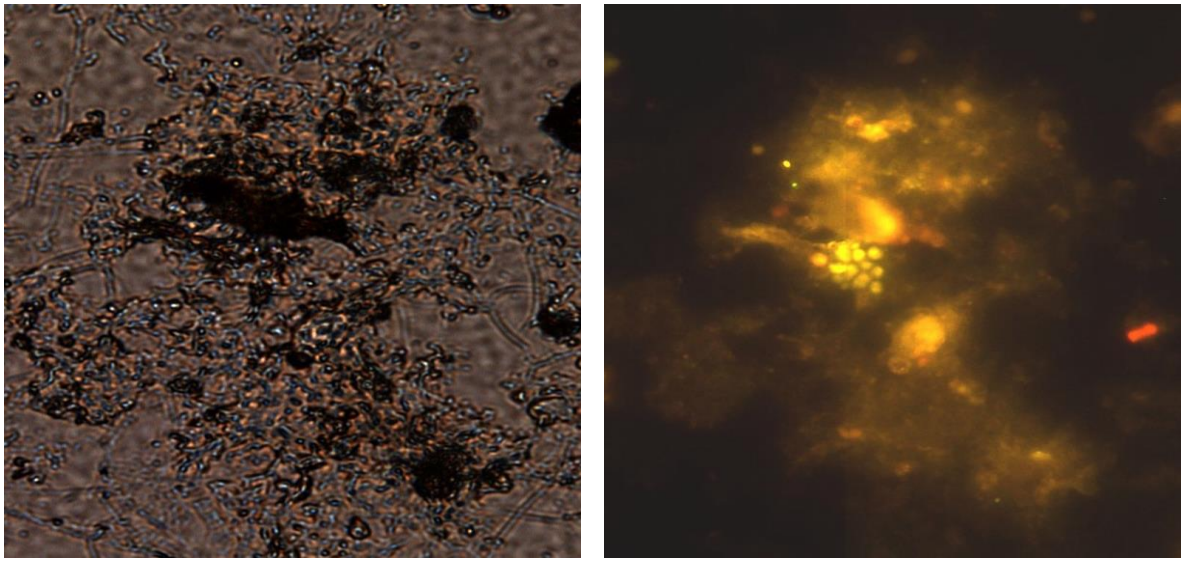
8.2 FISH RESULTS

Nso190, Nsv443, Nsm156 and NEU probes were used to determine the ammonia oxidizing bacteria whereas NIT3 and Nb1000 were used to determine the nitrite oxidizing bacteria within the nitrification reactor of ISKI Pasakoy Wastewater Treatment Plant. Positive and negative controls were applied simultaneously with hybridization experiments. Positive controls were with the probes Eub 338 and Univ 1392. Figure 8.3 (a) and Figure 8.4 (a) show the image of phase contrast; Figure 8.3 (b) and Figure 8.4 (b) show the image of the bacteria hybridized with Eub 338.



(a) (b)

Figure 8.2 Sybr Green 1 stained cells (a) Phase contrast view (b) U-MWB filter view (stained cells are yellow).

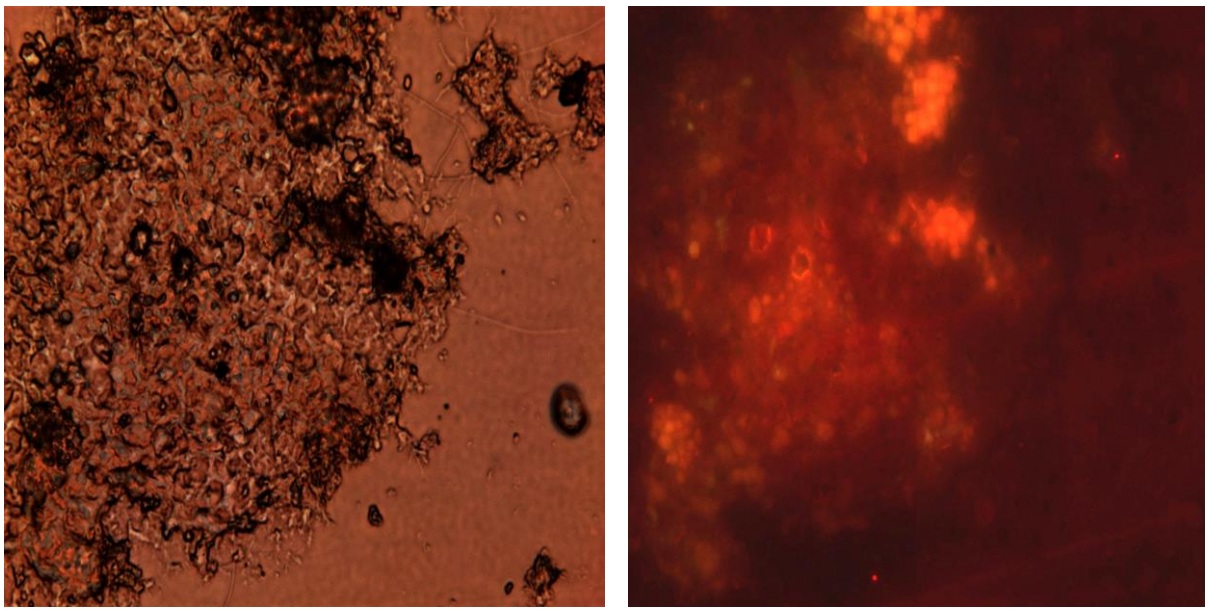


(a) (b)

Figure 8.3 Cells stained by probe Eub338 (a) Phase contrast view (b) U-N61002 filter view (FLUOS labeled cells seen yellow)

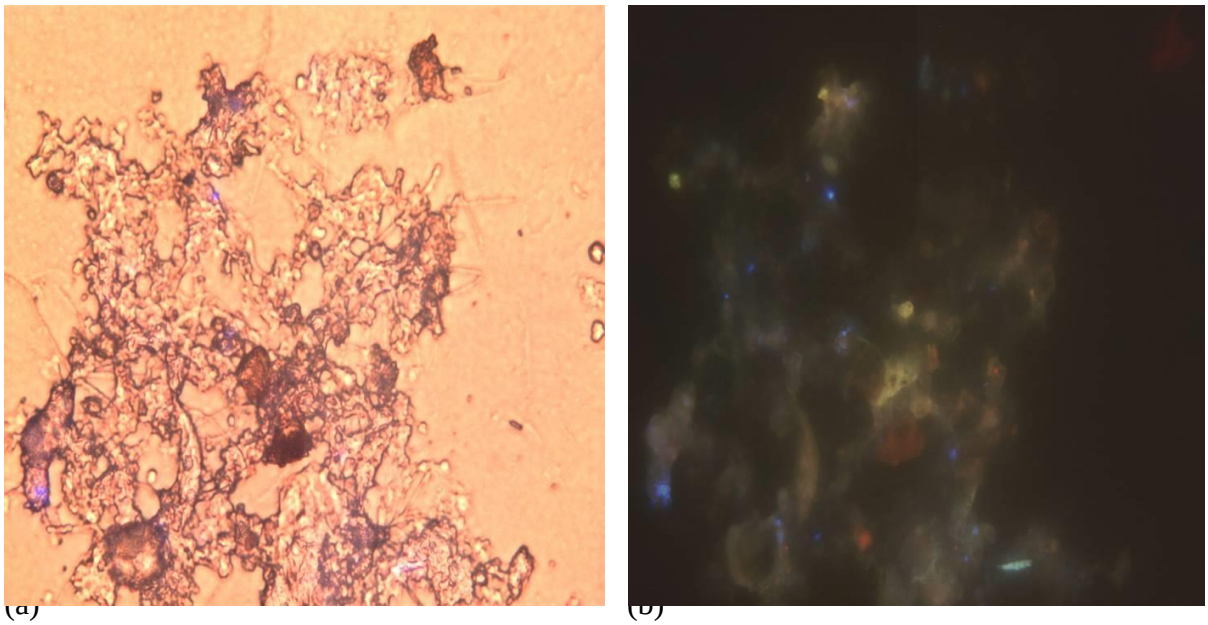
Univ 1392 probe was used as another positive control. Figure 8.4 shows the images of TAMRA labeled cells.

Non Eub probe was the negative control of these experiments. Figure 8.5 is the images of Cy3 labeled sample.



(a) (b)

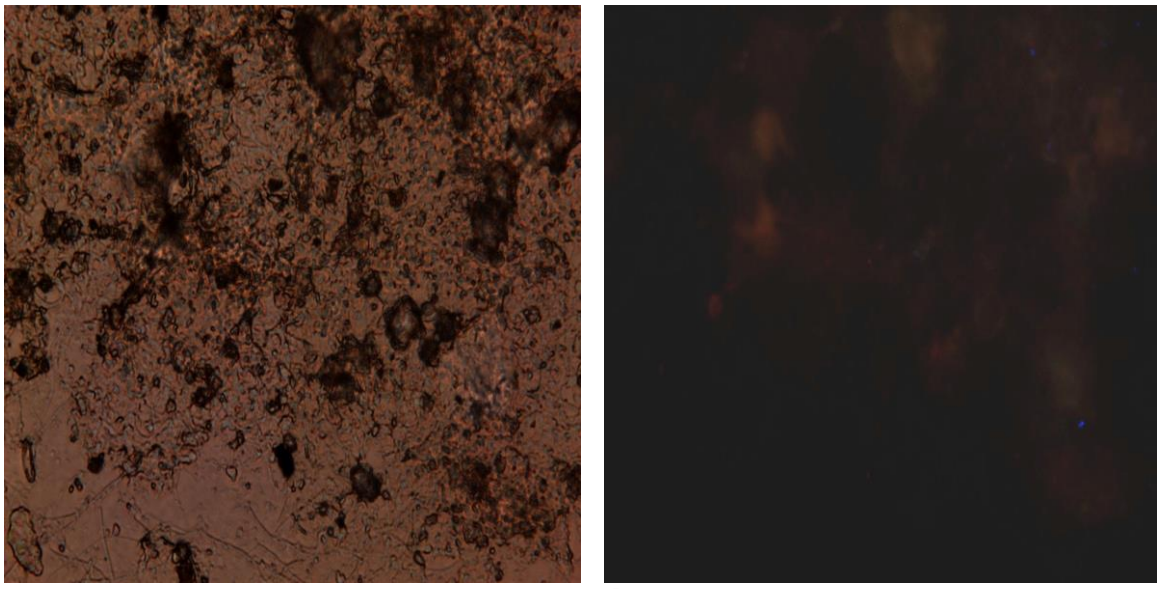
Figure 8.4 (a) Phase contrast view of Univ1392 (b) TAMRA labeled cells (red)



(a) (b)

Figure 8.5 (a) Phase contrast view of sample with NonEub probe (b) U-N61002 filter view.

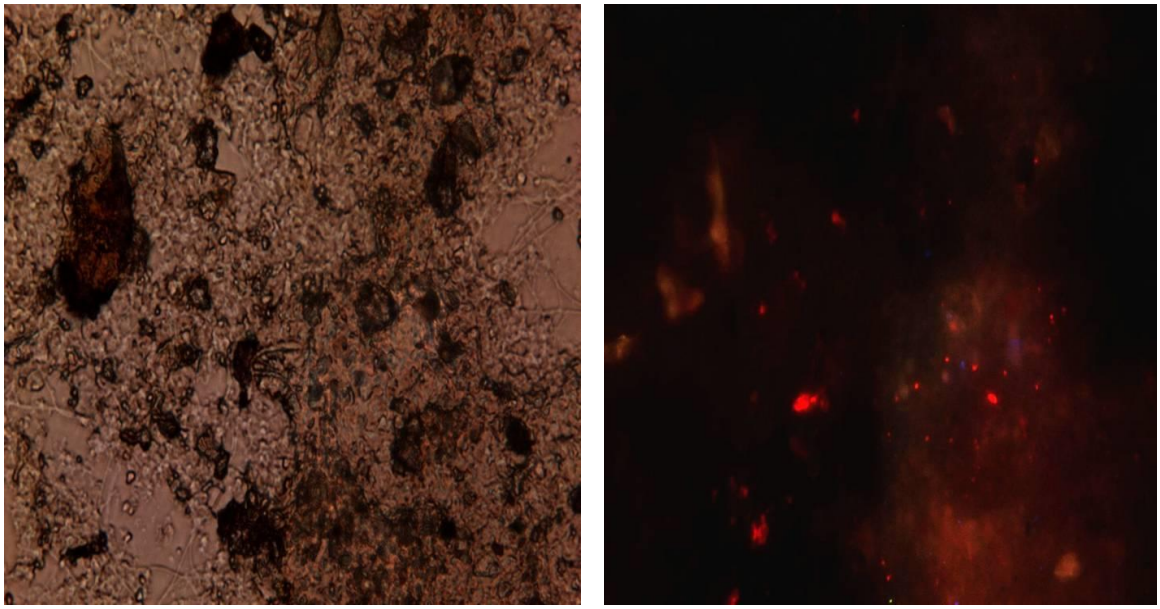
Hybridization without a probe was performed as another negative control. Figure 8.6 and shows these images.



(a) (b)

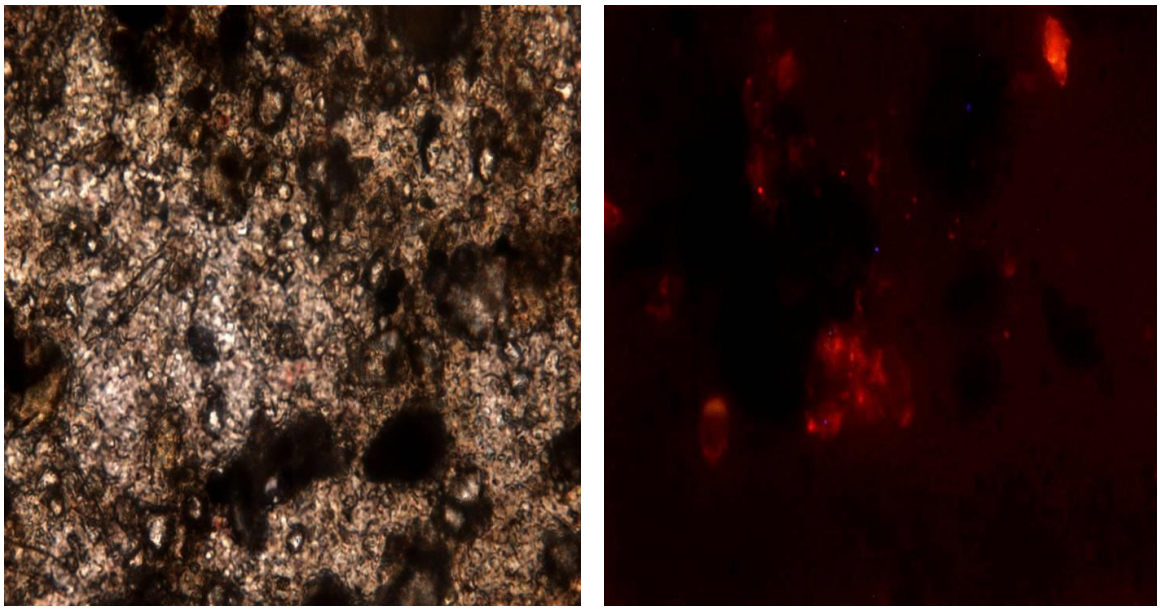
Figure 8.6 Phase contrast view of hybridization without a probe (b) U-N61002 filter view of the cells

For determining the ammonia oxidizing bacteria, Nso190 probe was firstly used. This probe is specific for the bacteria *Nitrosospira briensis, Nitrosovibrio tenuis, Nitrosolobus multiformis, Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis, Nitrosomonas C56*. Figure 8.7 and Figure 8.8 show images of the sample hybridized with Nso190.



(a) (b)

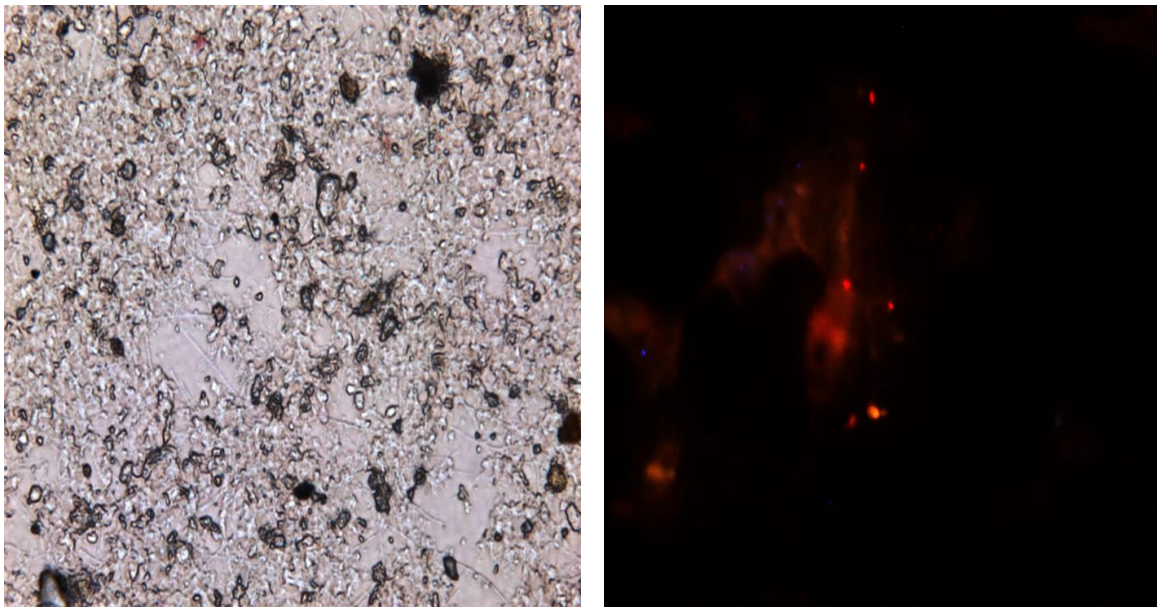
Figure 8.7 Images of hybridization with the probe Nso190 (a) Phase contrast (b) U-N61002 filter (Cy3 stained cells are red)



(a) (b)

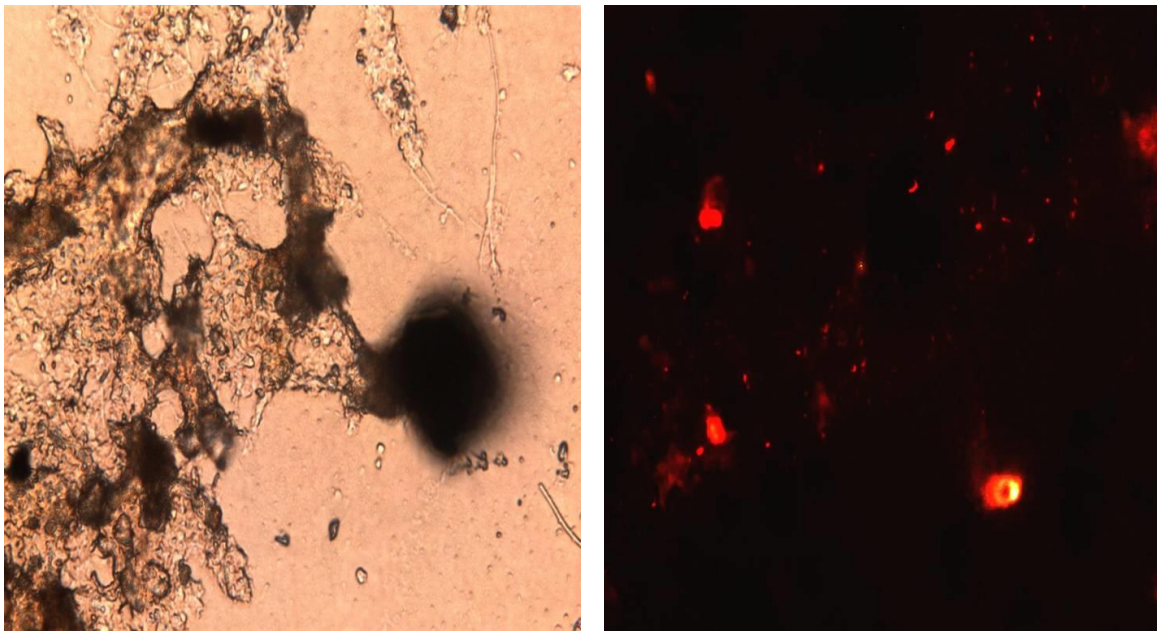
Figure 8.8 Images of hybridization with the probe Nso190 (a) Phase contrast (b) U-N61002 filter (stained cells are red).

Secondly, Nsv443 probe was used to determine *Nitrosospira briensis, Nitrosovibrio tenuis* ve *Nitrosolobus multiformis*. Figure 8.9 and 8.10 show the sample hybridized with Nsv443.



(a) (b)

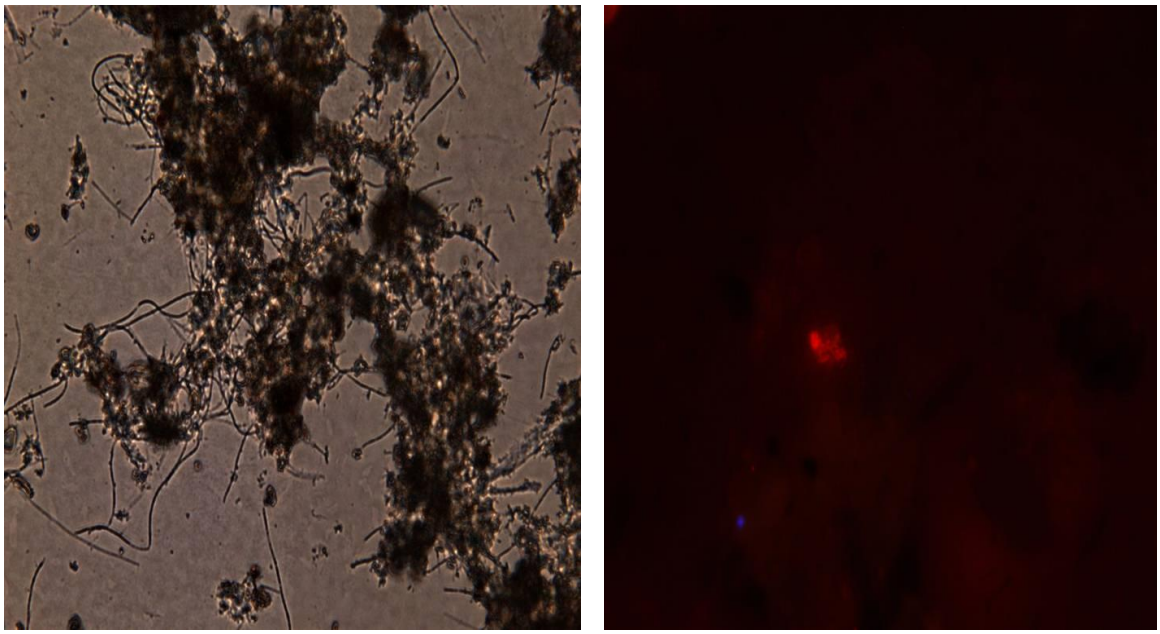
Figure 8.9 Nsv443 stained cells (a) Phase contrast view (b)U-N61002 filter view (Cy3 labeled cells seen red)



(a) (b)

Figure 8.10 Nsv443 stained cells (a) Phase contrast view (b) U-N61002 filter view (Cy3 labeled cells seen red).

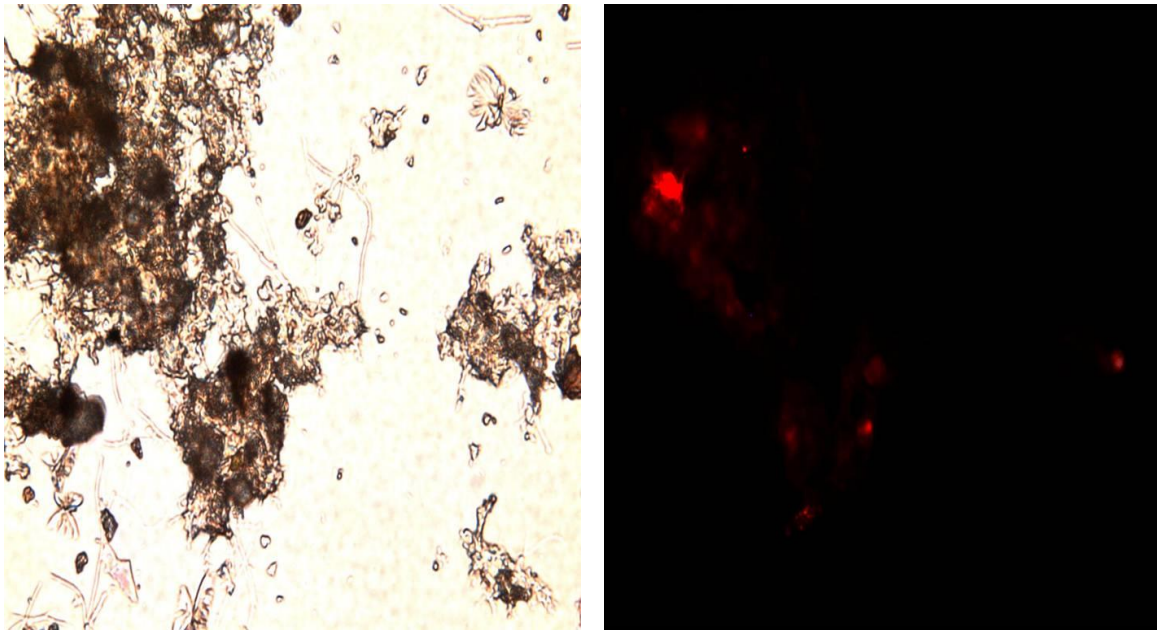
After Nsv443, the probe (Nsm156), specific for the bacteria *Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis, Nitrosomonas C56*, were applied. Figure 8.11 and 8.12 show the phase contrast images and the U-N61002 filter images of the cells.



(a) (b)

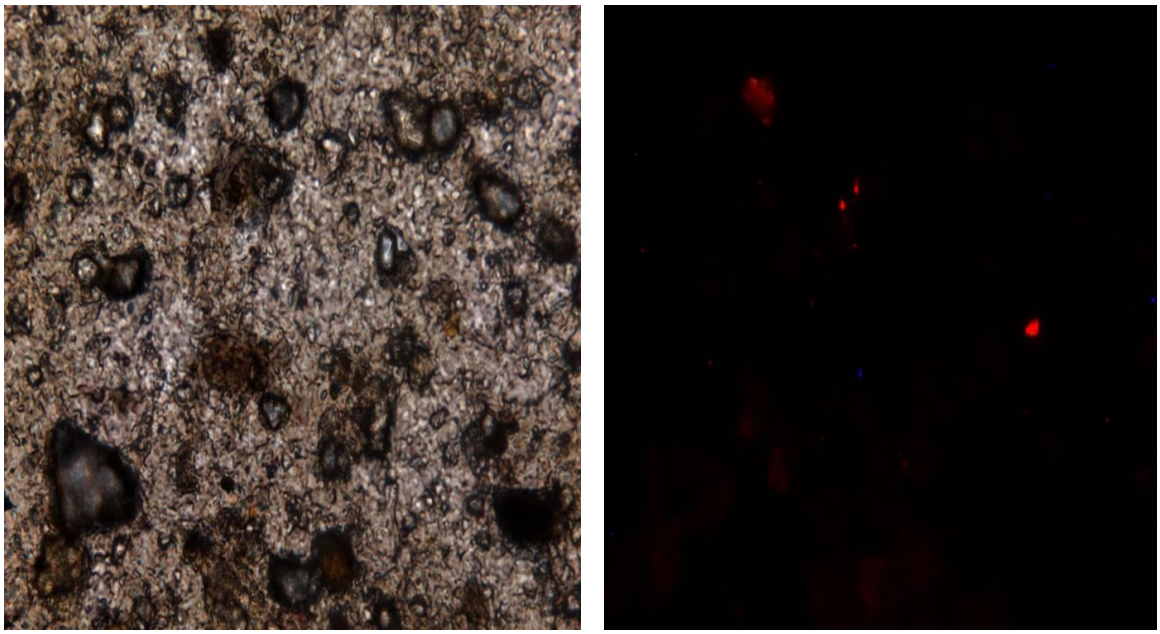
Figure 8.11 (a) Phase contrast view of hybridization with the probe Nsm156 (b) U-N61002 filter view (Cy3 labeled cells seen red)

NEU probe was used to determine the bacteria *Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis*. The phase contrast wievs of the sample are shown in Figure 8.13 (a) and 8.14 (a). The cells hybridized with this probe are shown in Figure 8.13 (b) and 8.14 (b).



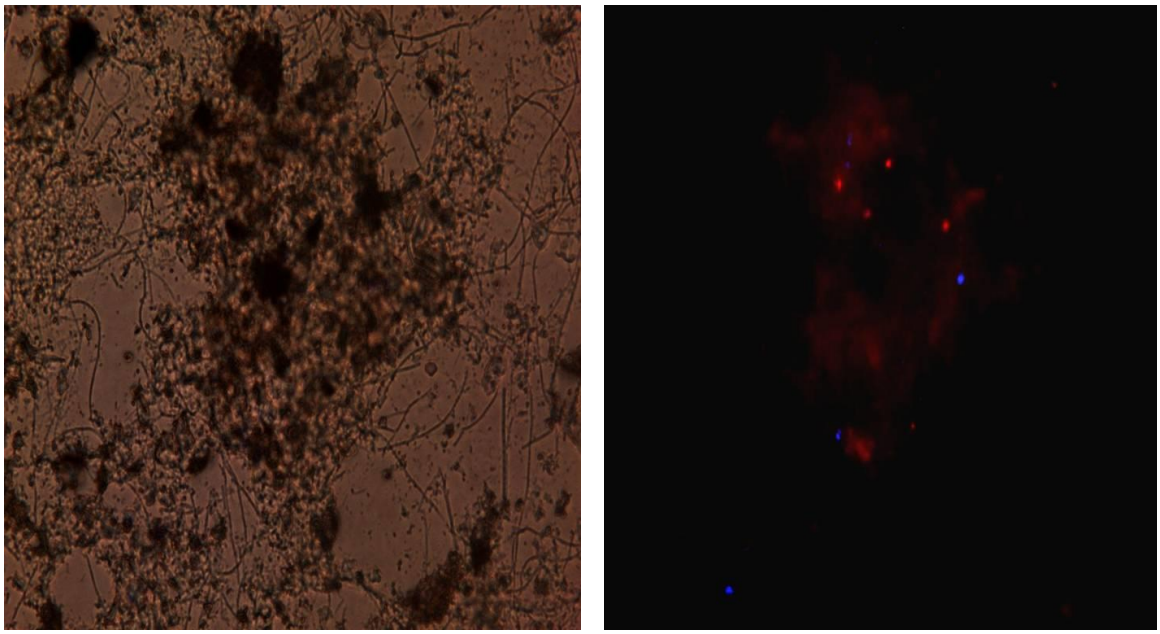
(a) (b)

Figure 8.12 (a) Phase contrast view of hybridization with the probe Nsm156 (b) U-N61002 filter view (Cy3 labeled cells seen red)



(a) (b)

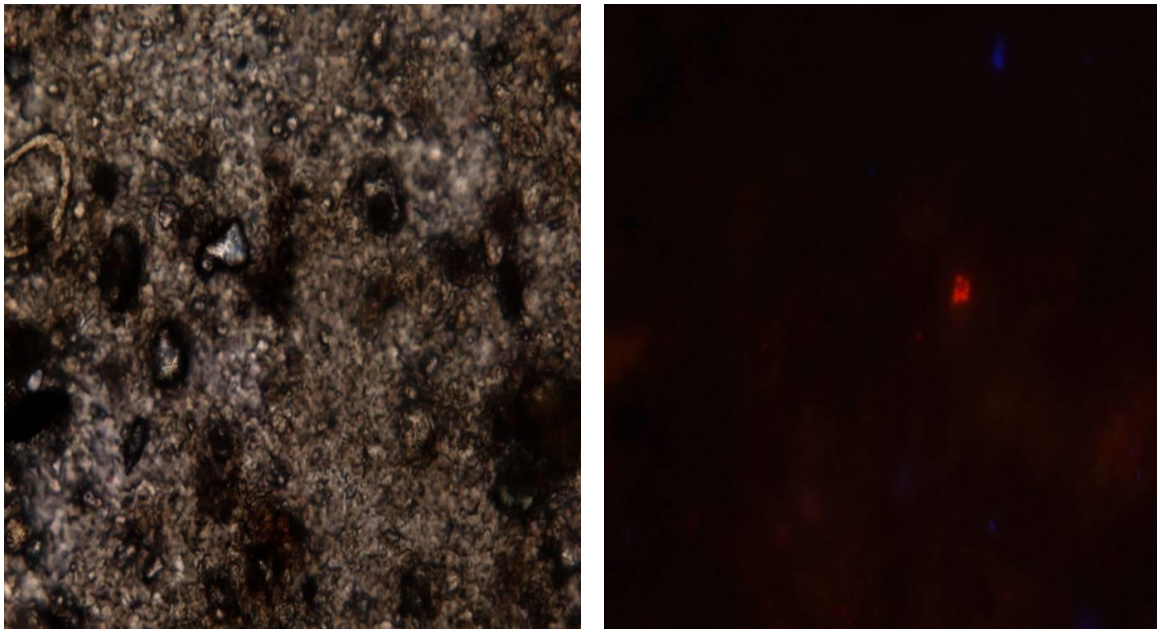
Figure 8.13 (a) Phase contrast view of hybridization with the probe NEU (b)U-N61002 filter view of hybridization with the probe NEU (Cy3 stained cells are red)



(a) (b)

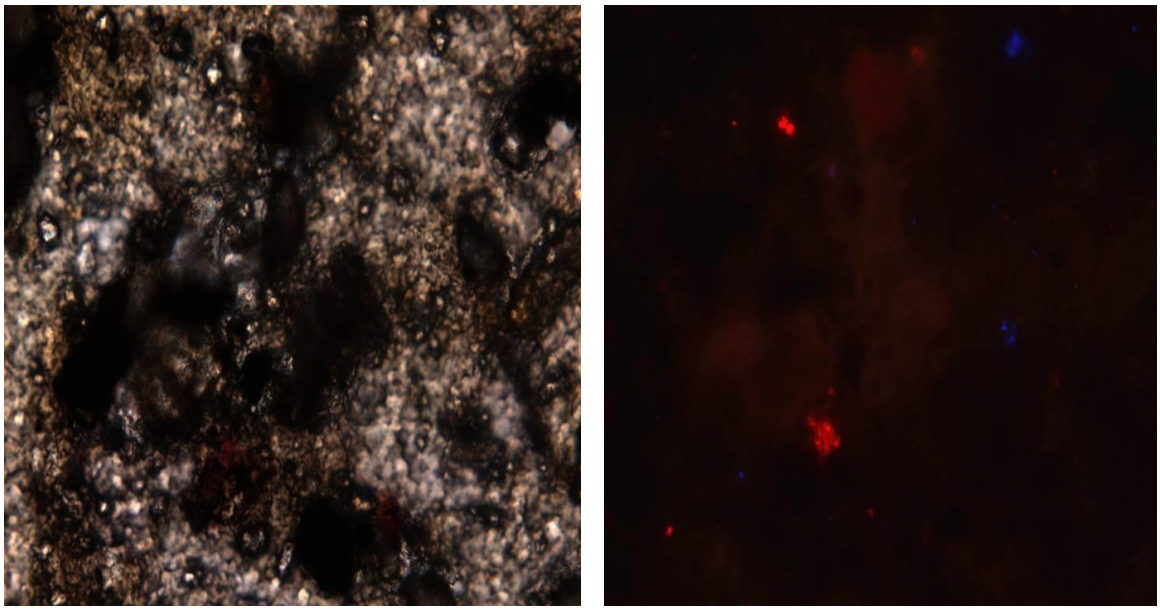
Figure 8.14 (a) Phase contrast view of hybridization with the probe NEU (b)N61002 filter view of hybridization with the probe NEU (Cy3 stained cells are red).

Experiments for determination of nitrite oxidizing bacteria were carried out with the probes NIT3 and Nb1000, which are specific to all *Nitrobacter* species (*Nitrobacter winogradskyi, Nitrobacter hamburgensis* ve *Nitrobacter sp.*). Figure 8.15 and 8.16 show the images of NIT3 whereas Figure 8.17 and 8.18 show the Nb1000 hybridized.



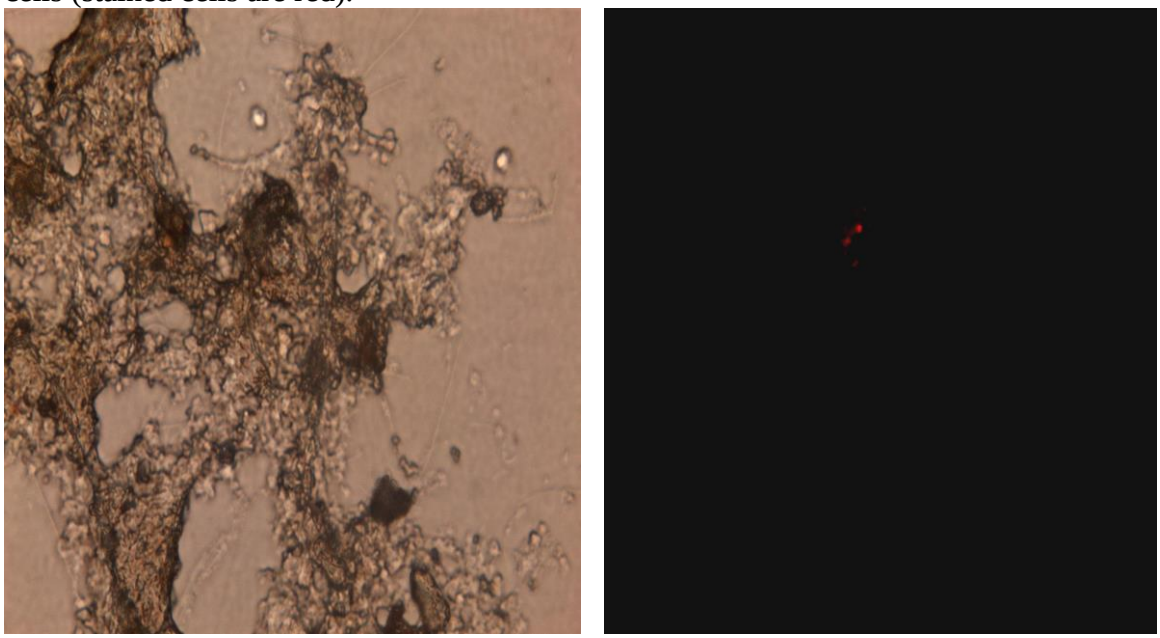
(a) (b)

Figure 8.15 (a) Phase contrast image of hybridized cells with NIT3 (b) TAMRA labeled cells (hybridized cells are red).



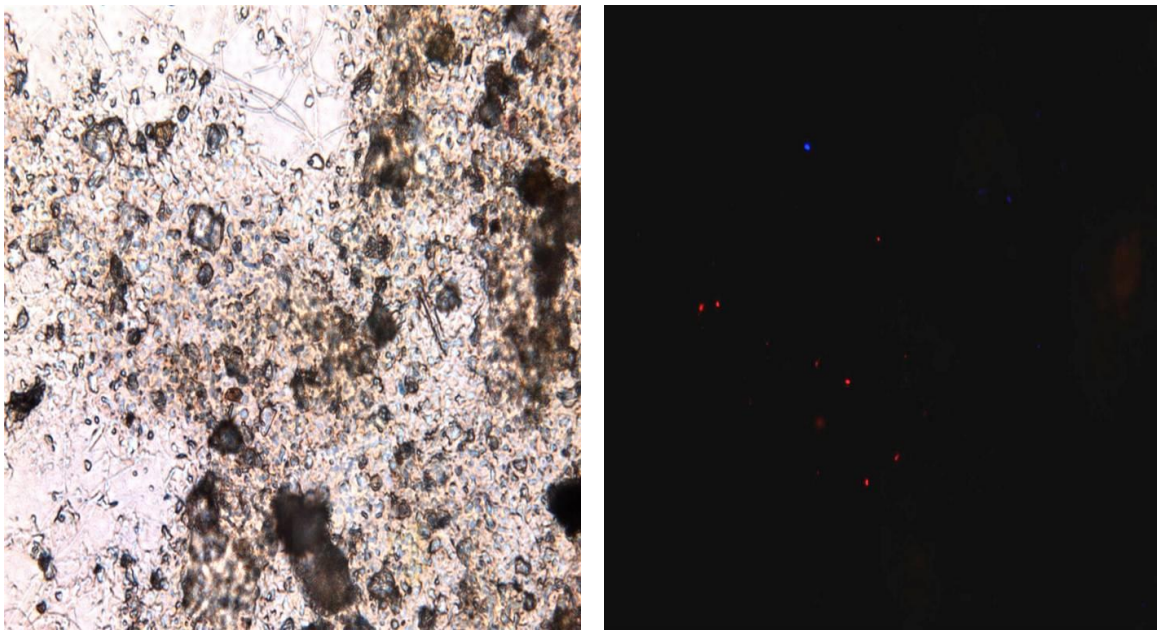
(a) (b)

Figure 8.16 (a) Phase contrast image of NIT3 hybridized cells (b) TAMRA labeled cells (stained cells are red).



(a) (b)

Figure 8.17 (a) Phase contrast image of hybridized cells with Nb1000 (b) U-N61002 Filter image of TAMRA labeled cells (hybridized cells are red).



(a) (b)

Figure 8.18 (a) Phase contrast image of hybridized cells with Nb1000 (b) U-N61002 Filter image of TAMRA labeled cells (hybridized cells are red).

8.3 WASTEWATER CHARACTERIZATION

Wastewater sample on which FISH technique was applied was also characterized according to the standard methods. This analysis showed that more than 50% TKN removal occured on the sampling day. The temperature was 22^0C and sludge age was 22 days. According to the wastewater analysis carried out in Istanbul Technical University, Environmental Engineering laboratory, COD values of the influent and effluent of the nitrification reactor were 400 mg/l and 80 mg/l, respectively. TKN values of influent and effluent samples of nitrification reactors were 31,5 mg/l and 15 mg/l. Volatile suspended solid analysis of reactor was also analysed. 2450 mg/l VSS value of the nitrification reactor was obtained.

CHAPTER 9 DISCUSSION

In this research, ammonia and nitrite oxidizing bacteria were determined in the nitrification reactor of ISKI Pasaköy Advanced Biological Wastewater Treatment Plant using the probes Nso190, Nsv443, Nsm156, NEU, NIT3 and Nb1000. It was shown that as ammonia-oxidizing bacteria *Nitrosospira briensis, Nitrosovibrio tenuis, Nitrosolobus multiformis, Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus mobilis, Nitrosomonas* C5, and as nitrite-oxidizing bacteria *Nitrobacter winogradskyi, Nitrobacter hamburgensis* and *Nitrobacter sp.* are present in the system. According to the wastewater characterization, on the day which the sample was taken, there were more than 50% TKN-nitrogen removal in the reactor. These results indicate that nitrogen removal was being performed via nitrification process by these groups of bacteria. These are also in agreement to the researchs which were carried out about nitrifying bacteria.

At least 10 microscopy images were taken from the slides of each probe. As a rough estimation, the fluorescence signals of Nsv443- and Nsm156- stainable populations are equal to the signal of Nso190. This result meets our expectations, because the target organisms of Nso190 comprehend the ones of Nsv443 and Nsm156. Fluorescence signal intensity of NIT3 and Nb1000 probes are less than the others. This suggests that the amount of nitrite oxidizing bacteria are less than ammonia oxidizing bacteria, or different organisms from the target groups are present within the system. Since nitrifying bacteria are grown as biofilm formation, nitrite oxidizing bacteria may be undetectable by fluorescence microscopy. According to the SYBR Green 1 results, it was decided to apply ½ dilution to the sample to obtain best view of AOB and NOB. Since AOB and NOB grow as biofilm structure, it is not recommended to destroy the floc. There are some studies about the biofilm structure of ammonia and nitrite oxidizing bacteria.

In one of these important researches, Juretschko *et al.* (1998) examined the ammonia-oxidizing and nitrite-oxidizing bacterial populations in the nitrifying activated sludge of an industrial wastewater treatment plant receiving sewage with high ammonia concentrations by FISH. The results revealed the dominance of *Nitrosococcus mobilis*-like bacteria and that *Nitrospira*-like bacteria were present in significant numbers (9% of total bacterial counts) and frequently occurred in the vicinity of *N.mobilis* microcolonies, which may reflect the syntrophic association between ammonia- and nitrite-oxidizing bacteria. Similarly, Schramm *et al.* (1997) found that ammonia-oxidizers are dominant within the first 100 μm of the biofilm over nitrite-oxidizers in the samples taken from the bottom part of a trickling filter .
In another study, Aoi *et al.* (2000) found that ammonia-oxidizing bacteria detected by the probes specific for halophilic and halotolerant members of the genus *Nitrosomonas* were observed as dominant populations over the entire biofilm. Furthermore, genus *Nitrosomonas* (together with *Nitrosococcus mobilis*) were the predominant ammonia-oxidizing bacteria in the fluidized bed reactor which was supplied with inorganic wastewater containing neither organic compounds nor other supplemented elements such as phosphate; in the same study, it is also found that only two types of ammonia-oxidizing bacteria were observed in the fluidized bed reactor which was fed with inorganic wastewater with low ammonia loading rate (0.3

$kgNH_4^+$-N/m^3.d). One type of ammonia-oxidizing bacterium (*Nitrosomonas* together with *N.mobilis*) has a higher activity at a concentration of ammonia and the other type (*Nitrosospira, Nitrosovibrio*) has a higher activity at a low concentration of ammonia. Therefore, ammonia-oxidizing bacterial ecology strongly depends on operational conditions such as ammonia-loading rate.

Another research also indicated this relationship between *Nitrosomonas* and *Nitrobacter spp.* According to the researchers, the toxicity of nitrite for ammonia oxidizers and the poor energy yield of nitrite oxidation may be the reasons for this syntrophic association. Ammonia-oxidizing bacteria may grow first as microcolonies, which produce nitrite and then are colonized by nitrite-consuming *Nitrobacter* aggregates (Mobarry *et al.,* 1996).

Wagner *et al.* (1998) found that although some studies have indicated the formation of microcolonies of *Nitrosomonas* and *Nitrobacter*, in many nitrifying sewage treatment plants, *Nitrobacter* numbers were negligible.

Schramm *et al.* (1997) also showed this relationship between the genera *Nitrosomonas* and *Nitrobacter*. According to the results, nitrifiers appeared as dense and dominating layer within the first 100 μm of the biofilm, where ammonia-oxidizers were present in higher numbers and formed larger aggregates than nitrite-oxidizers. According to these studies, fluorescence intensity of nitrite-oxidizing bacteria are expected less than ammonia-oxidizing bacteria. Our study also supports this conclusion.

FISH can also be succesfully utilized to determine the effect of environmental conditions on nitrifying populations. Thus, the system performance may be increased according to the wastewater characteristics. There are some quantitative FISH results obtained by studies about nitrification. In a research carried out with laboratory scale reactors operated at pH 7.5 at 25^{o}C; 7.5 at 30^{o}C; and 7 at 30^{o}C, it was found that pH is more important than temperature in selecting ammonia oxidizers determined by the probe specific to most halophilic and halotolerant members of *Nitrosomonas* species (Egli *et al.,* 2003). On the contrary, Schramm *et al.* (1997) found that short term changes in oxygen concentration do not influence the adapted and optimized biofilm structure. The temperature of nitrification reactor from which the sample was taken for this study, was 22^{0}C. This temperature is between the optimum temperature interval for nitrifying organisms.

The quantification of fluorescence intensity is used as a way of determining the dominant bacterial populations. According to this opinion, higher cellular ribosome content gives higher signal. As it can be seen on the microscopic images of Nsv443-stained population; their signal intensity is relatively higher compared to especially nitrite oxidizing populations. This result can give an idea about the quantification of bacterial groups according to one another; but the study carried out to examine the activity and stratification of nitrifying bacteria in a trickling filter biofilm revealed that the cellular ribosome content can not be used to monitor short-term changes of the physiological activity of nitrifers. It is also indicated that nitrifiers can keep their ribosome content in spite of their activity starts to decrease (Schramm *et al.*, 1997). Furthermore, background fluorescence of the biofilm and overlapping nitrifier clusters affect reversely the usage the fluorescence intensity for quantification. It was experienced also in this study. According to the images of negative control (with nonEub probe and without a probe), there are a lot of background signal caused by autofluorescence microorganisms and inorganic material. Anoxic tank before the nitrification reactors may be the source of autofluorescence microorganisms. Some autofluorescent population may survive in aerobic tank for a short time. Since the probes gives red and green-yellow signals, it could be distinquish easily nitrifying populations from the others. Dual hybridizations with universal probe and group specific probes can be used for discrimination of target groups from the others.

As a conclusion; FISH is a very important and useful tool for determination of bacterial populations without cultivation from their natural environment. Direct measurement of active biomass provides an oppotunity to operate the system more efficient according to the changing environmental conditions or substrate fluctuations. Quantitative FISH result is of great importance in this manner.

It is still known little about particularly the nitrification bacteria. This study is consequential for giving information about the active biomass in a full-scale nitrification reactor. Long term analysis of this reactor improves an opportunity for process stability. Characterization of different populations by FISH technique is worth studying more intensively for better understanding of natural or engineered systems.

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