

ANOXIC NITROGEN CYCLING IN MARMARA SEA SEDIMENTS

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**MARMARA DENİZİ SEDİMENTLERİNDE
ANOKSİK AZOT DÖNGÜSÜ**

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Environmental Biotechnology

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ABBREVIATIONS

N	: Nitrogen
C	: Carbon
PCR	: Polymerase Chain Reaction
Q_PCR	: Real Time PCR
FISH	: Fluorescence in-situ Hybridization
DGGE	: Denaturing Gradient Gel Electrophoresis
ANAMMOX	: Anaerobic Ammonium Oxidation
DNRA	: Dissimilatory Nitrate Reduction to Ammonium
RD	: Respiratory Denitrification
OMZ	: Oxygen Minimal Zones
OLAND	: Oxygen-Limited Autotrophic Nitrification-Denitrification
DIN	: Dissolved Inorganic Nitrogen
AOB	: Ammonia-oxidizing Bacteria
NOB	: Nitrite-oxidizing Bacteria
FeS	: Iron Sulphide
WWTP	: Wastewater Treatment Plants
NO₃⁻	: Nitrate
NO₂⁻	: Nitrogen Dioxide
NH₄⁺	: Ammonium
PON	: Particulate Organic Nitrogen
TGGE	: Temperature Gradient Gel Electrophoresis
T-RFLP	: Terminal Restriction Fragment Length Polymorphism
SSCP	: Single Stranded Conformation Polymorphism
UV	: Ultraviolet
CCD	: Charged Coupled Devise
FRET	: Fluorescence Resonance Energy Transfer Generalized

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ANOXIC NITROGEN CYCLING IN MARMARA SEA SEDIMENTS

SUMMARY

Removal of nitrogen (N) in aquatic ecosystems gets great interest because excessive nitrate in groundwater and surface water becomes a growing problem. As a result of microbial activity in the environment, nitrate is either removed as N_2 gas by respiratory denitrification (RD) and anaerobic ammonium oxidation (ANAMMOX) or converted, via dissimilatory nitrate reduction to ammonium (DNRA), to biologically available NH_4^+ which is retained within the ecosystem. Assimilatory nitrate reduction (ANR) also results in preservation of NO_3^- -N in the system as a cell material.

How important are these pathways in marine N cycling? This is a particularly difficult question to answer, because relative contribution of these processes to marine N cycling is just beginning to be studied in detail.

Marmara Sea is a perfect candidate for investigation of these N cycling pathways, since the sediments have been extremely polluted with hydrocarbons and contained abnormally high concentrations of nitrate. Therefore, in this study, relative importance of the N cycle processes in Marmara Sea Sediments (MSS) were monitored in terms of quantity of the relevant bacterial groups by targeting the catabolic genes using quantitative real-time PCR (Q-PCR).

The surface sediment samples (15 cm below the sediment surface) were taken from the most polluted areas in the Marmara Sea. Sediments The results obtained in this study were correlated with other sediment properties which were previously characterized as a part of TUBITAK Project no 105Y307. The results demonstrated that anoxic microbial abundance was strongly related to the level of nitrate concentrations. Target genes were mainly dominant in Tuzla and Moda region where nitrate concentrations were high. Relative abundances of NrfA (targeting DNRA), nosZ (targeting RD) and hzoA (targeting ANAMMOX) were in the ranges of 5-30%, 5-15% and 2-5%.

Although the conditions promoting fermentative DNRA and RD are similar (in terms of available nitrate, and organic substrates), fermentative DNRA is thought to be favored in nitrate-limited environments rich in labile carbon, while RD would be favored under carbon-limited conditions (Kelso, 1997; Silver, 2001). Our results were in agreement with these previous findings.

Since dissimilative nitrate reducing community was very abundant (in the range of 5.40×10^8 and 3.83×10^{10} cells/cm³) and very efficiently use organic compounds as a carbon source, bioremediation strategy can be possible aiming to stimulate rate of hydrocarbons degradation under nitrate reducing conditions.

MARMARA DENİZİ SEDİMENTLERİNDE ANOKSİK AZOT DÖNGÜSÜ

ÖZET

Akuatik ekosistemlerde nitrojen giderimi çok ilgi görmektedir, çünkü yeraltı sularında ve yüzey sularında aşırı nitrat artan bir sorun olmaya başlamıştır. Çevredeki mikrobiyal aktivitelerin sonucu olarak nitrat, denitrifikasyon ve oksijensiz amonyak oksidasyon prosesleri ile N_2 gazı şeklinde giderilir ya da disimilatif nitrat indirgenmesi ile NH_4^+ 'a dönüştürülür ve bu şekilde sistemde kalır veya biyokütle bünyesine geçer.

Denizsel azot çevrimindeki bu prosesler ne kadar önemlidir? Bu cevaplanması zor bir sorudur, çünkü bu proseslerin denizsel azot çevrimine katkıları detaylı olarak son zamanlarda incelenmeye başlamıştır.

Marmara Denizinde bulunan sedimentlerde tipik seviyelerin üstünde bulunan nitrat seviyesi ve kronik hidrokarbon konsantrasyonu, bu bölgeyi azot döngüsü proseslerinin araştırılması için gayet uygun bir ortam haline getirmiştir.

Bu çalışmada, Marmara Denizi'nde kirliliğin en yoğun olduğu bölgelerden 2 sene boyunca yüzey sediment numuneleri alınmıştır. Anoksik nitrojen döngü prosesinde rol alan organizmaların (belirli bir bakteri grubuna ait olmadıklarından dolayı), sorumlu oldukları proseslere özgü spesifik genler hedeflenerek, Gerçek Zamanlı Polimeraz Zincir Reaksiyonu methoduyla miktar tayini yapılmıştır.

Bu çalışma kapsamında olmayan diğer sediment karakteristikleri Tubitak Projesinden alınmış ve koralizasyon analizinde hedef genlere hangi parametlerin etkilediğini belirlemek amacıyla kullanılmıştır. Sonuçlar, anoksik mikrobiyal kominiteyi hedefleyen genlerin miktarı ve nitrat konsantrasyonu arasında güçlü bir bağ olduğunu göstermiştir. Disimilatif nitrat indirgenmesi prosesine spesifik olan *nrfA* geni, her bölgede en fazla oranda bulunurken(%5-30), amonyum oksidasyonunun sorumlu *hzoA* geni en düşük oranda(%2-5) gözlenmiştir.

Nitrat konsantrasyonunun en yoğun olduğu Tuzla ve Moda sedimentlerinde tüm hedef genler en yüksek oranlarda bulunurken, en düşük konsantrasyonlara Haliç sedimentlerinde rastlanmıştır.

Denitrifikasyonu ve disimilatif nitrat indirgenmesinin görüldüğü durumlar benzer olsa da disimilatif nitrat indirgemesi, karbonun fazla ve nitratin az bulunduğu ortamlarda teşvik edilir. Bunun nedeni disimilatif nitrate indirgeyicilerin karbon kaynaklarını daha verimli şekilde kullanımlarından kaynaklanmaktadır. (Kelso, 1997, Silver 2001). Bizim bu çalışmada vardığımız sonuçlar, deniz ortamlarında bu prosesler hakkında edinilen daha önceki bilgilerle örtüşmektedir.

Marmara Denizi sedimentlerinin yüksek karbon içeriğine sahip olması disimilatif nitrat indirgenmesi prosesinden sorumlu organizmaların bu ortamda baskın hale gelmesine yol açmış olabilir. Bu organizmalar doğal ortamlarında situmule edilerek, hidrokarbon kirliliğinin giderilebilmesini amacıyla kullanılabilirler.

1. INTRODUCTION

Despite the inhospitality of subsurface sites, studies have revealed a previously unknown and diverse prokaryotic microbial community within this deep biosphere. Many 16S rRNA gene sequences retrieved from these sub-seafloor sediments which belong to previously unidentified and uncultured groups of organisms, some of which have no clear phylogenetic affiliation (Webster, 2004).

Sediment bacteria constitute a huge reservoir of genetic variability with a local diversity equal to soil ecosystems which means that it is possible to find new life forms buried within the sediments (Torsvik, 2002). However, the deep subseafloor biosphere is among the least-understood habitats on Earth regarding the organisms, their physiologies and their influence on surface environments (Inagaki, 2006). This is mainly due to the difficulties involved in enriching and isolating the representative deep sediment microorganisms (Toffin, 2004) and previous studies based on cultivation methods could not reveal the appropriate sedimentary microbial diversity.

Molecular based, culture independent techniques such as Real Time PCR (Q_PCR) Fluorescence in-situ Hybridization (FISH), Denaturing Gradient Gel Electrophoresis (DGGE), and 16S rDNA sequencing for investigating the prokaryotic diversity have given a more realistic picture of the community structure in marine sediments (Lysnes, 2004; Webster, 2004), and have been successfully employed to overcome the difficulties associated with culture dependent methods.

Marine coastal sediments are known to contain a rich diversity of microorganisms from different physiological and phylogenetic groups. The Marmara Sea seems to be a perfect candidate for further investigation of ecologically important microbial processes; since metabolically diverse communities existed within a very short distance from the sediment surfaces and chemical compositions of the sediments were unusual.

The Marmara Sea is a semi-enclosed water body connecting the Black Sea to the Aegean Sea. Its sub seafloor life has not been explored yet. Extensive pollution in

this region has led to 40% reduction in amount of viable marine species during the last 30 years (DSI, 2004). The main sources of pollution are the highly polluted Black Sea, spills from oil tankers (≈ 35 accidents/year), discharges during marine transportation (≈ 50000 tankers/year), municipal wastes (≈ 20 million people), industrial wastes (40% of the Turkish Industry), atmospheric deposition, and urban surface and river runoff (Bebek, 2000; Pekey, 2007; Unlu and Alpar, 2006). The most polluted regions are the Kucukcekmece Coast, Moda Coast, Halic Bay, Tuzla Bay, Gemlik Bay and Izmit Bay (Sur, 2003).

Microbial investigations on subsurface sediment layers had mainly targeted open-ocean and continental-margin sediments (Smith and D'Hondt, 2006). However, as the investigations started to pay more attention to subsurface coastal sediments, the number of publications is currently increasing (Wilms, 2006). The previous microbiological studies on coastal marine sediments focused on vertical distribution of microbial communities (Buhring, 2005; Wilms, 2006). Therefore, previous molecular studies focused on the general microbial community structure and the abundance and depth distribution of specific functional/phylogentic groups, mainly sulfate-reducing bacteria and methanogenic archaea (Webster, 2004). Little attention has been paid to marine benthic organisms responsible for Nitrogen (N) cycle.

Since the early 20th century, patterns and quantities of nitrogen discharged to the environment have changed dramatically, largely associated with increasing human activities and technical advancements (Herbert, 1999, Vitousek., 1997). So the removal of nitrogen in aquatic ecosystems get great interest because excessive nitrate in groundwater and surface water becomes a growing problem. High nitrate loading degrades water quality and is linked to eutrophication and harmful algal blooms, especially in coastal marine waters.

The rapid increase in knowledge of genes and molecular biology has had an enormous impact on our understanding of the N cycle by making it possible to study the ecological underpinnings and diversity of microorganisms involved in specific N cycle components.

As a result of microbial activity in the environment, nitrate is either removed as dinitrogen gas (N_2) gas by respiratory denitrification (RD) and anaerobic ammonium oxidation (ANAMMOX) or converted, via dissimilatory nitrate reduction to

ammonium (DNRA), to biologically available ammonium (NH_4^+) which is retained within the ecosystem.

In this study, relative importance of the anoxic N cycle processes in the sediments of most polluted regions in Marmara Sea were assessed by comparing abundance of the relevant microbial groups. Quantitative molecular biology techniques targeting catabolic genes for, denitrification, dissimilatory nitrate reduction, assimilatory nitrate reduction were well established (Smith, 2007, Cai and Jiao 2008, Dong, 2009). Although hydrazine-oxidizing enzyme (hzo) which oxidizes the unique anammox intermediate is known, ANAMMOX bacteria (AnAOB) have been quantified by targeting 16SrRNA gene of Planctomycetes (Li, 2009). In this study, hzo gene was targeted to quantify AnAOB.

2. POLLUTION OF THE MARMARA SEA

2.1 Description of the Marmara Sea

Being an intercontinental sea on a waterway between Mediterranean Sea and Black Sea, the sediments of the Sea of Marmara are believed to have been a rather sensitive recorder of climatic, biological and chemical changes and water-mass movements in the region (Cagatay, 1996).

The Marmara Sea is a small (size ~70 x 250 km) intercontinental basin connecting the Black Sea and the Mediterranean Sea (Figure 2.1) and has a volume of 3,380 km³, and consists of a complex morphology including shelves, slopes, ridges and deep basins (Algan, 2004). It has a relatively broad shelf (40 km) in the south and a narrow one (10 km) in the north. The Marmara Sea is connected to Black Sea in the northeast via the Istanbul Strait (Bosphorus) and to the Aegean Sea in the southwest via the Canakkale Strait (Dardanelles). The oceanographic features (chemical and biological) of the basin are influenced by the Black Sea and the Aegean Sea via the Bosphorus Strait and the Dardanelles, respectively. The waters of the Bosphorus are strongly stratified, with the upper layer comprising low salinity outflow from the Black Sea and the bottom denser layer generated by northerly, highly saline flow from the Mediterranean. Mixing between the two layers along the Bosphorus and the Bosphorus / Marmara junction, is strongly affected by the main features related to the physical oceanography of the area, namely the respective salinity of the approaching currents, the topography of the strait and the prevailing meteorological conditions which results in a permanent two-layer flow system with halocline at a depth of 20-25 m (Orhon, 1995). The stratification of the water column, together with the topographic restriction of the two straits, prevents the efficient circulation of the subhalocline layer. As a result, the dissolved oxygen content of the bottom waters decrease by microbial oxidation of organic matter from 7-10 mg/l near the Canakkale Strait towards east to about 1 mg/l above the deep basins, and 2.5 mg/l near the Istanbul Strait (Unluata and Ozsoy, 1986). The water coming from the

Mediterranean Sea is vitalizing to the Marmara Sea, because it has more oxygen and more salt (Algan, 2004). The significant feature of the hydrodynamics of the Bosphorus is the intense mixing of the deeper Marmara waters into the upper layer at the Bosphorus/Marmara junction (Orhon, 1995). The oxygen saturation of water below 25-30 meter depth from the surface varies between 20%-30% which is problematic for the mineralization of organic matter and coastal discharges from the Black Sea.

In terms of primary production, the Marmara Sea is intermediate between the Black Sea and the Aegean Sea, with values of 60-160 gcm²year (Yilmaz, 1986; Ergin , 1993), the highest values being located in the inner southern shelf.

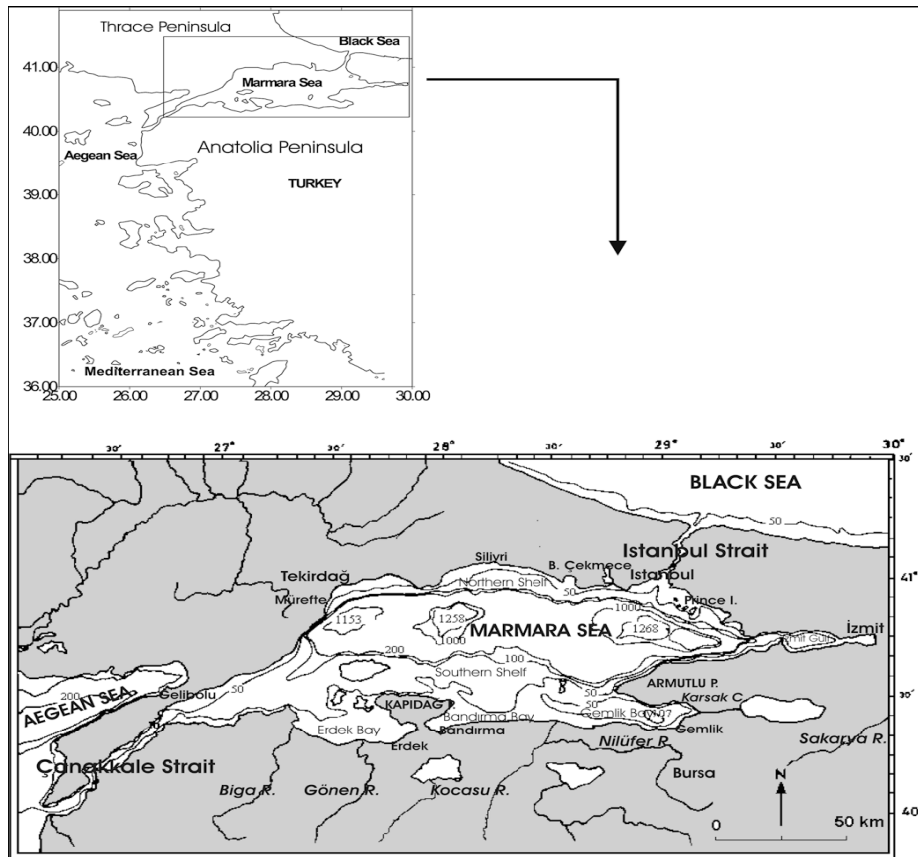


Figure 2.1 : Location of Marmara Sea adapted from Algan

2.2 Pollution Sources at the Marmara Sea

The Marmara Sea is now a critically polluted water body and the recipient of a large number of wastewater discharges from land based sources located along the coastal line, including the Istanbul metropolitan area (Orhon, 1995; Albayrak, 2006) and subject to several other anthropogenic activities that primarily cause severe

hydrocarbon and heavy metal pollution. The pollution of Marmara Sea is based on sewages, industries and vessels. The sewage pollution is considered to be the most important one among them.

The northern shelf of the Marmara Sea is more subjected to increasing human interferences in the form of industrial (metal, food, chemistry, textile) waste disposal, fisheries, dredging, recreation and dock activities, compared to the southern one.

It receives pollution both from various local land-based sources, but also from the heavily populated and industrialized Istanbul Metropolitan as well as from marine transportation. Istanbul is the most heavily populated and industrialized metropolitan area of Turkey. It has about 15% of the total population and 40% of the industrial activity of Turkey (Orhon and others 1994) and hence it is the largest contributor of various pollution in the Marmara Sea. In addition to industrial and domestic load coming from Istanbul Metropolitan, dissolved and particulate pollution loads from the Danube River are transported towards Istanbul Strait by alongshore currents (Sur and others 1994; Tugrul and Polat 1995). Therefore, the shelf area of the Marmara Sea is highly susceptible to the Marmara Sea turned into an open sewage, because there is not a purification system for sewages. Industrial pollution is mostly based on government-run factories (Algan, 2004).

The water quality measurements indicate severe signs of present and future eutrophication problems (Orhon, 1995). There are more than endangered species in the Marmara Sea over 50, such as monk seals, sturgeons, shrimps and crabs (Turkish Marine Research Foundation, 2004).

The Bosphorus, a strongly stratified natural channel between the Marmara Sea and the Black Sea, with significant mixing at the entrance to the Marmara Sea is also a major polluter for the Marmara basin, since it carries the highly polluted waters of the Black Sea (Orhon, 1995). The Marmara Sea receives via the natural exchange from the Black Sea roughly 15 times more organic matter than what is contained in the sewage discharges from Istanbul (Orhon, 1995). The basin receives a total of 1.9×10^6 tons of TOC (total organic carbon) and 2.7×10^5 tons of TN (total nitrogen) per year from the Black Sea inflow (Albayrak, 2006). Nutrient input from the Black Sea, however, is much more significant than coastal wastewater discharges according to the experimental evidence on the basis of extensive observations (Orhon, 1995).

In addition, tanker traffic of several thousand oil carrying vessels per day, via the Bosphorus Strait is a constant threat to the marine ecosystem. Tankers from oil exporting countries surrounding the Black Sea have only one exit to the Mediterranean Sea: via the Bosphorus Strait, the Sea of Marmara and the Dardanelle Strait. The Bosphorus and the Dardanelle's are typical narrow water channels and navigation route through the Marmara Sea. This route therefore increases the risk of collisions and running aground (Tan and Otay, 1999).

Many accidents of merchant ships and tankers occurred in the strait. Nine tanker accidents, which resulted in almost 193 tons oil spill, occurred in Bosphorus and the Marmara Sea between 1964-2002 (Güven, 2004). The two major accidents happened in 1979 and in 1994 by a tanker called *Independenta* and by a tanker called *Nassai*, respectively. In the *Independenta* at the exit of the Bosphorus to the Sea of Marmara in 1979 95 000 tons of crude oil was spilt and burnt (Etkin, 1997). In the *Nassai* accident which happened at the northern exit of the Bosphorus to the Black Sea in 1994, 13 500 tons of crude oil were spilt (Oguzulgen, 1995). M/V *GOTIA* sank into Bosphorus and 25 tons fuel oil was spilt and pollution spread out into a large area by winds (Güven, 2004).

Bilge water discharge is also a major problem for the Straits of Istanbul and Canakkale, and the Marmara Sea. Increase in petroleum hydrocarbon levels mainly from oil spills, sewage outfalls and ship bilge water, has been observed in the Marmara Sea- (Güven, 1997). The levels of pollution, particularly by the heavy metals, have increased dramatically due to large inputs from the Black Sea (Kut, 2000). At the same time, the Marmara Sea has been subject to very high levels of pollution due to industrial and municipal waste disposal.

2.3 Pollution of Tuzla and Moda

Tuzla is located on the Asian side, 60 km east of Istanbul, on the Sea of Marmara coast. Along the coast of Tuzla, there are agricultural lands and industrial plants (iron-steel plants, LPG plants, oil transfer docks, and cargo ship's ballasts water). Moda is located within the Kadikoy district in Istanbul, Turkey on the Northern coast of Marmara Sea. Moda is at the junction of Kurbagalidere which used to be an historical old rivulet surrounded by a recreational area connecting to Marmara Sea and a sanctuary for fisheries and boathouses.

Biogenic, diagenetic and anthropogenic components contribute to shelf sediments after their delivery to the marine environment. In coastal areas of densely populated large cities, the anthropogenic component of the sediments mostly exceeds the natural one. The surface sediments become a feeding source for biological life, a transporting agent for pollutants, and an ultimate sink for organic and inorganic settling matters (Algan, 2004). Marine sediments, particularly those in coastal areas, are commonly polluted with petroleum hydrocarbons (PHC) as a consequence of the extensive use of petroleum compounds by mankind (Miralles, 2007). In aquatic sediments, the depth of oxygen penetration through diffusion is controlled mainly by the consumption of degradable organic matter within the sediment and in coastal ecosystems rarely exceeds more than a few millimeters (Jorgensen, 1983). With the exception of the most superficial layer, the bulk of organic matter-rich marine sediments contaminated by PHC are assumed to be anoxic (Canfield, 1993). Consequently, microbial processes depending on the availability of free dissolved oxygen are constrained to the uppermost surface or, in deeper sediment layers, are coupled to irrigation and bioturbation processes of burrowing microorganisms (Freitag and Prosser, 2003).

During the last decade, studies have shown the potential of coastal marine sediments for anaerobic hydrocarbon degradation under sulphate-reducing conditions (Coates, 1997; Townsend, 2003). In marine reduced sediments, hydrocarbon degradation coupled to sulphate-reduction seems to be the most relevant among the different anaerobic processes, because sulphate is abundant in coastal and estuarine seawater, whereas nitrate concentrations are typically low and Fe(III) is often only sparsely available, especially in heavily contaminated sediments (Rothermich, 2002). Industrial activities, municipal wastewater, agricultural chemicals, oil pollution and airborne particles have been the main reasons for the pollution that has affected primarily the estuaries and bays of the Marmara Sea and has ultimately spread along the shoreline and continental shelf that constitutes 50% of its total area (Unlu, 2006).

Anthropic pollution trapped in bays, in particular, has created significant ecological damage resulting in the decrease or extinction of marine species (Unlu, 2006). The northern shelf of the Marmara Sea is more subjected to increasing human interferences in the form of industrial (metal, food, chemistry, and textile) waste disposal, fisheries, dredging, recreation and dock activities, than to the southern

shelf. It receives pollution not only from various local land-based sources, but also from the heavily populated and industrialized Istanbul Metropolitan and from maritime transportation (Algan, 2004). Because Marmara Region is an important coastal settlement in Turkey with rapidly increasing population and industrial activities, the Sea of Marmara and the Turkish Straits are subject to intensive navigation activity. With the recent increases in sea traffic, these waterways have become a prime site for oil spill pollution (Kazezyilmaz, 1998).

Tuzla has undergone heavy environmental stress due to expansion of the Istanbul Metropolitan City in terms of industrial and human settlement through this area over the past 25 years. Many buildings were built on the marshy rim of the Tuzla despite heavy criticism from environmentalists. Due to heavy industrial and agricultural activities in the region, the bay has the polluted coastal waters of Turkey. Therefore, mainly untreated agricultural municipal and industrial wastes affect the lagoon direct or indirectly. Moreover, on February 13th, 1997, a tanker named TPAO exploded in Tuzla shipyards located on the north-eastern coast of the Sea of Marmara. During the fire, an estimated amount of 215 tons of oil was spilled in to the Aydinlik Bay and 250 ton oil burnt (Kazezyilmaz, 1998; Unlu, 2000). The oil pollution was investigated and the pollution level was determined in seawater, sediments and mussels in Tuzla Bay after the TPAO tanker accident. The highest pollution was found as 33.2 mg/L in seawater and 423.0 µg/g in sediment on the first day after the accident (Unlu, 2000).

Moda is relatively considered as a less polluted area in comparison to Tuzla. However, Moda has been densely exposed to domestic wastewater discharges since the end of 1970s and has gone under amendment by ISKI since the early 2000. Based on the water quality monitoring projects, it has been showed that anoxic conditions have been occurred within the marine sediment samples taken from Moda region. Nevertheless, hydrocarbon rich wastewater discharge of cyanide containing wastewater has recently occurred in this region which was only exposed to pre-treatment.

2.4 Pollution of Kucukcekmece

Kucukcekmece is on the Marmara coast, on the eastern shore of an inlet of the Marmara called Kucukcekmece Golu (Kucukcekmece Lagoon). The inlet is connected to the Marmara Sea by a narrow channel, so the water is not salty. Until the 1950's Kucukcekmece was a popular weekend excursion, people would come by train from Istanbul to swim or to fish. The streams running into the inlet now carry industrial waste and the inlet is highly polluted but efforts are being made to get it clean again. There used be wildlife and many kinds of birds and efforts to get the wildlife back are taking effect slowly.

Due to geographical easiness to build any installation, the area has become an industrial region and crowded with huge housing projects. This development is still going on and is indeed accelerated as the TEM motorway to Europe passes through here now. The Ikitelli region in particular is very industrial and still more factories are being built. The Nuclear Energy Research center is located on the lake side.

The region is polluted heavily due to awry urbanization and intensive industrialization. The Kucukcekmece lagoon is subjected to take effluent of 2 million people at the year of 2000. Industrial sites are mainly composed of metal industry, textile and leather industry, medicine industry, paper industry, chemical industry, rubber and plastic industry. The control of discharges are not controlled or regulated by the government. These problems coupled with incomplete sewage system create huge impact on the region. Therefore a recreation place once becomes now a place with lots of buildings and eutrophicated lagoon. The sources of pollution are classified as point and nonpoint sources. Point sources composed of discharges from domestic and industrial sites. Waste loads of Nuclear Research Institution affect also rivers flowing to the lake. Nonpoint sources include drainage waters coming from runoff, groundwater including leachate and water coming from agricultural activities.

2.5 Pollution of Izmit Bay

Izmit Bay, the most important semi-enclosed body of water on the east side of the Sea of Marmara, about 50 km in length, 2–10 km in width and 310 km² in surface area. It consists of three district regions (western, central and eastern) connected through narrow openings.

The Bay is stratified throughout the year having a lower layer swept by highly saline (37–38.5 ppt) water from the Mediterranean and a brackish upper layer originating from the Black Sea. (Morkoc, 1996)

The commissioning of more than 140 large industrial plants since 1965 and, in particular, the consequent urbanisation of the coastal landscape have completely destroyed the previous serenity of Izmit Bay. Initially all solid and liquid wastes were discharged directly into the Bay. Though major industrial effluents are now treated, there has yet to be treatment of domestic waste (Okay, 1996). The renewal capacity and water Exchange within Izmit Bay is insufficient for compensation and equilibration (Morkoc, 2000). Eutrophication and deterioration of water quality have become serious problems (Morko, 2000).

Toxicity studies (Okay, 1996) of the dominant wastes have led most factories contributing to the wastes to construct biological treatment plants during the past 10 years. Sediments frequently contain higher concentrations of pollutants than are found in the water column and it is realised that, especially in turbulent waters, adsorption of pollutants by sediments scrubs the water column so that it appears relatively uncontaminated (Bauloubassi and Saliot, 1991), whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Adams, 1992). Sediment bioassays that measure the toxic effects of contaminated sediments on the test organisms have been recently developed and a large variety of bioassays is becoming available. They provide information on the toxicity of contaminated sediments that can be neither derived from chemical analysis nor from ecological surveys (Chapman and Long, 1983; Long and Chapman, 1985).

Izmit Bay is the centre of burgeoning industrial development accompanied naturally by a rapid growth of population. The consequent, continuing danger of pollution has been minimised by ongoing monitoring and subsequent mitigation of the eco toxicology of the bay, contributing to the wastes to construct biological treatment plants during the past 10 years. Nevertheless, the treatments are still insufficient to eliminate toxicity (Okay, 1998). Sediments frequently contain higher concentrations of pollutants than are found in the water column and it is realised that, especially in turbulent waters, adsorption of pollutants by sediments scrubs the water column so that it appears relatively uncontaminated whereas the sediment may become sufficiently polluted to disrupt natural biological communities (Adams, 1992).

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Testing procedures have included numerous techniques such as static tests, flow-through tests and elutriate tests (Burton and Scott, 1992; Schuytema, 1996; Mac, 1990).

2.6 Pollution of Gemlik

The Gemlik Bay emerges as a 31-km-long tectonic trough between two topographic heights, with an increasing width westward . It is 2–6 km wide in front of the Gemlik Town in the east of Tuzla Point and 12–24 km in the west between Trilye and Bozburun (Armutlu Town). The length of its coasts along the step Samanlidag Mountains in the north, alluvial plains and deltas in the east and small hills along the southern coasts is about 76 km.

The regional winds, mainly controlled by the surrounding mountains, blow from northwest in winter and mainly northeast for the rest of the year. They play a dominant role in the dynamics of this semi-enclosed sea. Gemlik Bay is open to the waves coming from the band between northwest and southwest. In winter, the dominant wave direction is from northwest with the significant wave heights less than 3 m. The dominant wave direction is from southwest in spring months with the significant wave height less than 2 m. The maximum hind casted significant wave height for Gemlik wave is 3 m for the duration of wind data 1994–1998.

The maximum depth is 107 m in the middle of a small northwest-trending elliptical central trough which is a fault-controlled depositional area (Yaltirak and Alpar, 2002). The southern coasts of the Gemlik pull-apart basin are controlled by the central strand of the North Anatolian fault. Holocene alluvial fans in the east disturb the symmetry of this marine depression which is separated from the Marmara Sea by a sill with an average depth of 50 m in the west .

With its 27600 km² drainage area and 158 m³/s average water flows, the Karasu River is the most important geographic element in the region. It carries 0.5– 5.5 tons of suspended solids daily into the sea depending on the climatic conditions (Yıldız, 2003).

2.7 Pollution of Halic

The Golden Horn is a highly used water body in Turkey. It was a famous recreational area at the time of Ottomans, when it also served as the most important port of the region. The Golden Horn suffered from heavy pollution due to extensive industrialization and rapid population growth in Istanbul in the twentieth century. This manuscript describes how metal pollution evolved in Golden Horn between 1912 and 1987, by analysing Pb slices of a 3-m long core collected close to Galata Bridge in 1989.

Estuaries are transitional zones between rivers and seas, and are important ecosystems typically rich in biodiversity. The Golden Horn Estuary supported thriving fisheries until the latter 20th century. It is a 7.5 km long, 200–900m wide horn shaped body of water that connects the Alibeykoy and Kagithane Rivers to the Bosphorus strait. Estuarine surface area covers 2.6 km² and maximum depth is 36m at the mouth, sloping to <1 m near tributary inflow. The shallow inner estuary, defined as the area north of the Valide Sultan/Old Galata Bridge, is more prone to anoxic conditions given that its depth abruptly slopes to <5m near the bridge. The estuary receives saline water from the highly stratified, two-layered Strait of Istanbul. The upper layer with 25 m thickness has 20 psu salinity and lower layer has 38 psu salinity, which is separated by a transition zone. This stratified structure disappears in mid-estuary where maximum depth is 12–13 m. In addition to these layers, a 2–3 m less saline permanent layer above the stratified waters of the estuary was reported due to the suspended sediment carried by local discharges and streams (Ozsoy, 1988).

The water column is a highly stratified product of the Mediterranean Sea , Black Sea, and freshwater urban runoff, precipitation, and a small fluvial contribution. Freshwater remains on the surface due to a greater rate of input (300,000m³ of freshwater enter the estuary annually) than evaporative loss (Ozturk, 1998; Alpar, 2005).

These three layers, separated by strong density gradients, effectively resist mixing of estuarine waters, and movement via tidal mixing or currents is negligible at <10 cm/s (Aksit, 1977). Low velocity surface winds and stable atmospheric conditions contribute to minimal air ventilation in the surrounding area (Incecik, 1986). Thus, both water and air circulation is severely hampered in and around the Golden Horn, which has led to a local environment extremely prone to lasting pollution problems. These conditions are compounded by steep hills lacking foliage, the presence of stone quarries, and the absence of drainage systems, all encouraging substantial erosion and estuarine sedimentation (Aksit, 1977).

3. NITROGEN CYCLE

3.1 Importance of Nitrogen

The element N is an essential nutrient for all organisms, and as a critical component of proteins, nucleic acids (DNA, RNA). N is fundamental to the structures and biochemical processes that define life. It is a constituent of vital importance for life on Earth, as all organisms, regardless of physiology, require N for growth and fix it in relatively consistent stoichiometric proportion to carbon (C) and other essential elements (Redfield, 1958). Also, forms of N are directly involved in the energetic metabolism of certain heterotrophic and autotrophic bacteria (Jorgensen and Gallardo, 1999; Seitzinger, 1988; Zehr and Ward, 2002). N is of such centrality that it has been suggested to be perhaps the best bio-signature for life on other planets (Capone, 2006). The largest reservoir of N on Earth is triple-bonded N_2 gas (78% of the atmosphere) and must be fixed by microorganisms before it is readily useable by other organisms.

N exists in multiple oxidation states and chemical forms (Table 3.1), and is rapidly converted by micro-organisms on land and in the sea. Most nitrogen in marine environments is present in five forms:

N_2 (dinitrogen gas): A quite stable molecule that requires specialized enzymatic systems to break and use;

NO_3^- (nitrate): The most oxidized form of nitrogen and the dominant biologically utilizable form of N within oxic environments;

NH_4^+ (ammonium): The most reduced natural form of N and the dominant biologically available form found in anoxic environments;

Particulate nitrogen: Predominant within sediments and primarily in the form of organic N,

Dissolved organic N (DON) : A complex mixture of compounds with a wide range of compositions.

Table 3.1 Nitrogen-containing compounds can be found in a multiplicity of forms at a wide range of oxidation states in marine environments.

Compound	Oxidation State
Nitrate (NO_3^-)	+ V
Nitrogen dioxide (NO_2)	+IV
Nitrite (NO_2^-)	III
Nitric oxide (NO)	II
Nitrous oxide (N_2O)	I
Di nitrogen (N_2)	0
Hydroxylamine(NH_2OH)	-I
Hydrazine (N_2H_4)	-II
Ammonium (NH_4^+)	-III
Amino Acids ($\text{R}-\text{NH}_2$)	-III
Urea (NH_2CONH_2)	-III

Nitrate, nitrite, ammonium, and organic nitrogen are typically grouped together as “fixed N” in discussions of nitrogen availability, although each form has a different level of reactivity. (Brandes, 2006)

3.2 The Main Processes in the Nitrogen Cycle

The nitrogen cycle is composed of multiple transformations of nitrogenous compounds, catalyzed primarily by microbes (Figure 3.1). The N cycle controls the availability of nitrogenous nutrients and biological productivity in marine systems (Ryther, 1971) and thus it is linked to the fixation of atmospheric carbon dioxide and export of carbon from the ocean’s surface (Falkowski, 1998). Human activities are influencing the N cycle even in the oceans (Vitousek, 1997), and some of the nitrogenous gaseous products of microbial metabolism are greenhouse gases that are potentially involved in controlling Earth’s climate.

The majority of processes in the extant global biogeochemical nitrogen cycle are facilitated by bacteria including: (1) N_2 fixation, (2) nitrification and (3) denitrification. A fourth process, anaerobic ammonia oxidation (anammox), is a more recently described bacterial contribution to the nitrogen cycle (Dalsgaard, 2005; Jetten, 2005). Specific enzymes catalyze many of these reactions, and the enzymes and genes are useful targets for studying microbial processes.

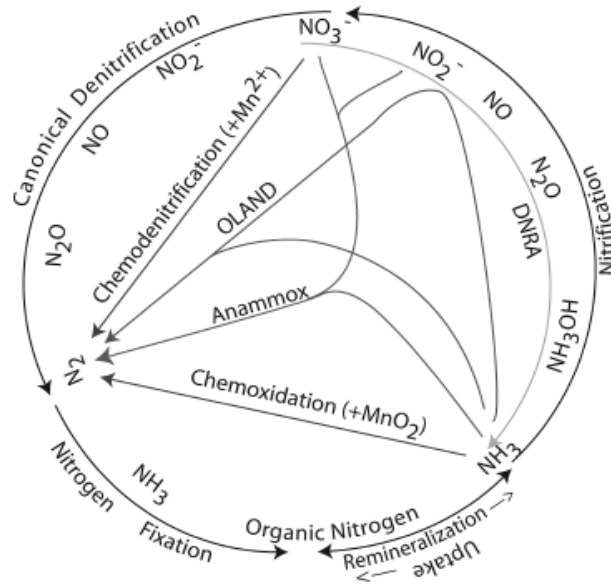


Figure 3.1: Diagram of the nitrogen cycle as it is understood today, adapted from Brandes (2006).

3.2.1 N₂ Fixation

The first comprehensive view of the N cycle in the surface ocean proposed that inorganic N was taken up by phytoplankton and that the N was subsequently recycled from phytoplankton cells by heterotrophs, both large grazers and microbial decomposers factors, liberated N in the form of dissolved organic N or ammonium, which was termed “regenerated” N (Dugdale, R., 1967). Nitrogen fixation, the assimilatory reduction of N₂, also produces biologically available or proteinaceous N (Carpenter, 1983; Howarth and Marino, 1988; Postgate, 1982). (Figure 3.2C).

Nitrogen is mineralized during the degradation of nitrogen-containing organic macromolecules such as proteins, polypeptides, dissolved free and dissolved combined amino acids. Mineralization products include ammonium, which may subsequently be oxidized through nitrification to nitrite and nitrate. (Figure 3.1) Nitrate and nitrite have the potential to be used as electron acceptors, preferentially when oxygen concentrations are low or in anoxic environments.

Nitrate, nitrite, and ammonium, called dissolved inorganic nitrogen (DIN), can be taken up (via membrane transporters) and assimilated by many microorganisms.

3.2.2 Nitrate Assimilation

The primary role of heterotrophic bacteria is classically considered to be the decomposition and mineralization of dissolved and particulate organic nitrogen (Richardson, 2001).

Bacterial NO_3^- assimilation is not a pathway currently considered in pelagic carbon and nitrogen cycle models. A recent review of freshwater and marine studies reported that bacteria may rely on both NH_4^+ and NO_3^- for growth and biomass synthesis (Figure 3.2A), (Kirchman, 2000)

Marine heterotrophic microorganisms that assimilate nitrate play an important role in nitrogen and carbon cycling in the water column. Nitrate utilization in aquatic communities is difficult to study by conventional tracer approaches, size fractionation does not allow for examination of nitrate uptake by attached bacteria or large cells caught in filters.

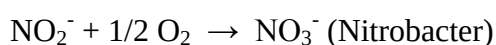
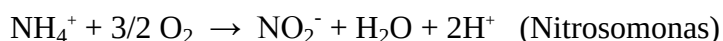
The detection of *nasA* genes in a variety of marine environments provided a basis for the hypothesis that the potential for NO_3^- utilization by heterotrophic bacteria is significant. The *nasA* gene, encoding the nitrate assimilation enzyme, was selected as a functional marker to examine the nitrate assimilation community.

3.2.3 Nitrification

Bacterial nitrification, proceeds by the sequential oxidation of ammonia to nitrite predominantly by ammonia-oxidizing bacteria (AOB) and of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) (Prosser, 1989). (Figure 3.1) This is an autotrophic process in which oxygen is the electron acceptor and ammonia is the electron donor.

Molecular phylogeny has supported this generalization, showing that both AOB and NOB belong to a small number of coherent groups (Teske, 1994). Respectively, two genera organisms are '*Nitrosomonas* and *Nitrobacter*.'

- Energy reaction:



3.2.4 Denitrification

Denitrification, the reduction of more oxidized forms of nitrogen, NO_3^- , NO_2^- , NO and N_2O to N_2 , is generally coupled to the oxidation of reduced C, Fe and S species (Jorgensen and Gallardo, 1999; Knowles, 1982; Payne, 1976; Seitzinger, 1988; Straub, 1996; Zumft, 1997). In this pathway, the predominant fate of the reduced nitrate may be determined by the ambient concentration of free sulfide, which is known to inhibit the final two reduction steps in the denitrification sequence.

Sulfide inhibition of these terminal steps may drive the reduction to ammonium rather than to N_2O and N_2 . On the other hand, metal-bound sulfides (eg iron sulfide, $[\text{FeS}]$), which are often abundant constituents of freshwater sediments (Holmer and Storkholm, 2001), can also be oxidized by such bacteria, but these compounds may not inhibit denitrification (Brunet and Garcia-Gil, 1996).

The ability of bacteria to couple the reduction of nitrate to the oxidation of sulfur has now been established in a number of taxa with diverse metabolic characteristics (Dannenberg, 1992; Bonin, 1996; Philippot and Hojberg 1999), including members of the genera *Thiobacillus*, *Thiomicrospora*, and *Thioploca* (Kelly and Wood, 2000).

Enzymes of denitrification are inhibited in the presence of oxygen, and recent investigations indicate inhibition at oxygen concentrations of only a few micromolar (Codispoti, 2001). Thus, denitrification is restricted to anoxic or nearly anoxic sediment horizons and anoxic/suboxic waters (Figure 3.2F) such as the Black Sea, the Cariaco Basin and the oxygen minimum zones of the eastern Tropical Pacific and the Arabian Sea. As mentioned in Chapter 3.2.3, nitrate was believed to be primarily by mixing, advection and diffusion from deep ocean water but in sediments with high rates of organic supply, oxygen consumption rates are generally high and oxygen penetration is narrow. In such environments, additional nitrate is typically obtained from the overlying water (Koike and Sorensen, 1988).

Denitrification is generally considered the major process removing nitrogen from the oceans (Christensen, 1987; Christensen, 1994; Devol, 1991; Seitzinger, 1988). It is also the main biological process responsible for the return of fixed nitrogen to the atmosphere. Bacteria capable of denitrification are frequently isolated from soil, sediment and aquatic environments (Gamble, 1997; Zumft, 1997).

The studies demonstrated that this denitrifying bacteria are the opposite of nitrifiers in many ways; denitrification ability is found in heterotrophic opportunists and in chemoautotrophs, is widespread among Bacteria and Archaea, and has even been reported in Eukarya (Zumft, 1997).

- **Denitrifying genes**

Denitrification consists of four reaction steps by which nitrate is reduced into dinitrogen gas by the metalloenzymes: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. These enzymes are usually induced sequentially under anaerobic conditions. During the last decade, important knowledge has been accumulated on the denitrifying genes and their regulatory mechanisms but for only few laboratory strains such as *Pseudomonas stutzeri* or *Paracoccus denitrificans*. Thus, for the genes encoding the catalytic subunit of the denitrifying reductases, e.g. *narG*, *napA*, *nirS*, *nirK*, *norB* and *nosZ*, the number of complete sequences is still limited and belongs to taxonomically related bacteria. However, a better knowledge of these functional genes is required to develop molecular approaches for environmental studies on the ecology of denitrifiers . (Bothe, 2000) The use of the functional genes for these molecular techniques is especially important in the case of denitrification. In addition to their role in the nitrogen cycle, the denitrifying genes also provide a good model for studying evolutionary relationship of functional genes because of the great diversity of bacteria capable of denitrification.

- **Nitrate Reductases**

Nitrate reduction is the first step in the pathway, although it is not unique for denitrification. Knowledge has recently emerged that bacteria can express two types of dissimilatory nitrate reductase, which differ in their locations: a membrane-bound (Nar) and a periplasmic-bound (Nap) nitrate reductase. Nitrate reduction is mediated by a diverse polyphyletic group of bacteria. Consequently, rRNA-based approaches are of limited value for understanding the structure and diversity of nitrate-reducing communities. in nitrate reduction have been exploited as molecular markers. The diversity of *NarG* encoding the membrane- bound nitrate reductase, has been widely studied in a variety of soil ecosystems and results have indicated that soil nitrate-reducing communities are dominated by diverse unknown nitrate reducers.

In contrast, there has been only one investigation of diversity of the NapA gene, which encodes the periplasmic nitrate reductase (R.A.Siddiqui,1993, F. Reyes,1998).

- **Nitrite Reductases**

The reduction of nitrite to nitric oxide, the key step in the dissimilative denitrification process, can be catalyzed by evolutionary unrelated enzymes that are different in terms of structure and the prosthetic metal: a copper- and a cytochrome cd1-nitrite reductase. The nirS gene and the nirK gene encode the cd1 and copper nitrite reductase, respectively. The nirS gene is part of a gene cluster containing several genes involved in the production of an active dissimilatory nitrite reductase. In contrast to the genes encoding the cd1 nitrite reductase, there is a lack of results concerning the characterization of the genes encoding the copper nitrite reductase. The copper nitrite (nir K) reductase was purified from several Gram negative bacteria, such as *Achromobacter cycloclastes* (H. Iwasaki, 1972), *Nitrosomonas europaea* and *R. shaeroides* (E. Sawada, 1978), Gram-positive bacteria (G.Denariáz, 1991), and even from the Archaeon *Haloferax denitrificans* and *Haloarcula marismortui*.

- **Nitric oxide reductases**

Nitric oxide reductase has been described as a complex of two subunits encoded by the norC and norB genes (H.Arai, 1995). The norC gene encoding the membrane-anchored c-type cytochrome is always located upstream from the norB gene, which encodes the cytochrome b subunit with 12 transmembrane regions. Evidence for a transcriptional unit of norC and norB was obtained from Northern blot analysis by Zumft et al. (W.G. Zumft,1995). Recently, a new nitric oxide reductase lacking the cytochrome c subunit was purified from *R. eutropha*.

- **Nitrous oxide reductase**

The final step in denitrification is the conversion of N_2O to N_2 . The only enzyme known to catalyze this reaction is nitrous oxide reductase (nosZ).

This step effectively closes the overall nitrogen cycle as N_2 gas may again be converted to soluble nitrogen oxides through the action of nitrogen-fixing and nitrifying organisms. The gene encoding nitrous oxide reductase is largely unique to denitrifying bacteria and has recently been used for detection of denitrifier specific DNA in environmental samples.

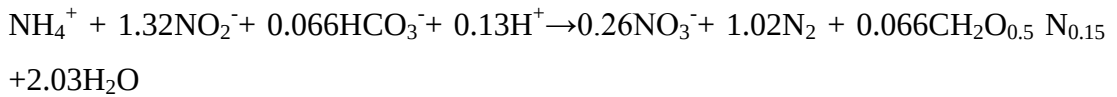
3.2.5 Anaerobic Ammonium Oxidation (ANAMMOX)

Anammox bacteria are chemolithoautotrophic microorganisms that oxidize ammonium with nitrite (or nitrate) as the electron acceptor to obtain energy to fix CO₂, thereby producing N₂ gas (Jetten, 2001).

Accumulation of NO₂⁻ is also possible during nitrification, when ammonium oxidation to nitrite is decoupled from further oxidation to nitrate (Sliekers, 2002).

Anaerobic ammonium oxidizers and aerobic nitrifying bacteria coexist under oxygen limiting conditions where nitrifiers oxidize ammonium to nitrite and deplete oxygen, while the anammox bacteria convert toxic nitrite and the remaining ammonium to N₂.

Overall Reaction is:



In both cases of anammox activity reported in the water column, the depth interval of the process was narrowly constrained to anoxic waters where nitrate and nitrite were present (Figure 3.3).

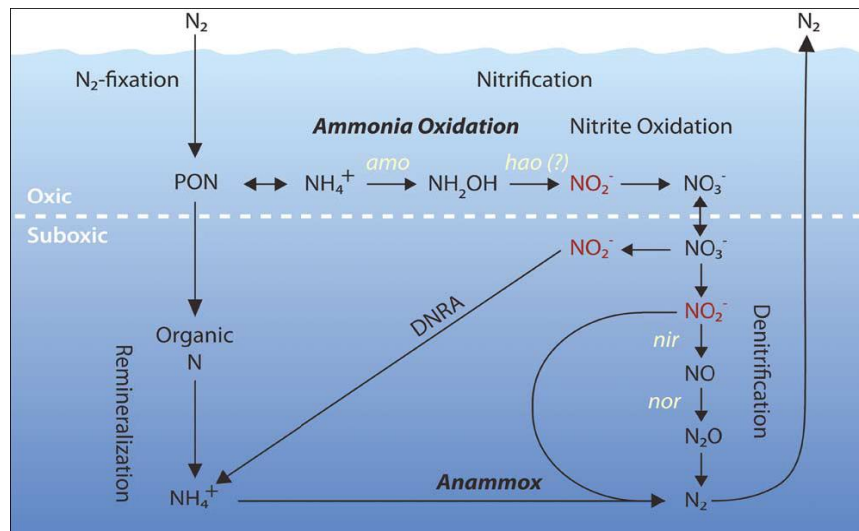


Figure 3.3: Microbial nitrogen transformations above, below and across an oxic/anoxic interface in the marine environment adapted from Francis, 2007

The first evidence ANAMMOX to N_2 gas was obtained from anoxic bioreactors of wastewater treatment plants (WWTPs) (Mulder, 1995), where it was eventually determined that novel organisms related to Planctomycetales were capable of oxidizing ammonium using nitrite (rather than O_2) as the electron acceptor (Strous, 1999).

Befitting micro-organisms capable of such a novel metabolism, these 'ANAMMOX' bacteria have a number of truly unique features, including the use of hydrazine (N_2H_4 , i.e., rocket fuel) as a free catabolic intermediate, the biosynthesis of ladderane lipids and the presence of an anammoxosome (intracytoplasmic compartment). Owing to their distinct metabolism and physiology, ANAMMOX bacteria received considerable attention in engineered systems, but were assumed to be minor players in the N cycle within natural ecosystems.

However, in 2002, Thamdrup and Dalsgaard found ANAMMOX to be responsible for 24–67% of N loss in marine sediments (Thamdrup and Dalsgaard, 2002), and in 2003, two parallel studies demonstrated that anammox was directly responsible for a substantial fraction of N loss in the ocean (Dalsgaard, 2003; Kuypers, 2003). In fact, 20–40% of N loss could be attributed to anammox in the suboxic water columns of the Black Sea and Golfo Dulce (Dalsgaard, 2003; Kuypers, 2003).

To date, ANAMMOX has been documented in marine, coastal and estuarine sediments (Risgaard-Petersen, 2004; Engstrom, 2005), anoxic basins (Dalsgaard, 2003; Kuypers, 2003), oxygen minimum zones (OMZs) off West Africa, Chile and Peru (Kuypers, 2005; Thamdrup, 2006; Hamersley, 2007), mangroves (Meyer, 2005), sea-ice (Rysgaard and Glud, 2004) and freshwater lakes .

Regardless of whether DNRA is performed by ANAMMOX or other (facultatively) anaerobic micro-organisms in situ, DNRA could provide NH_4^+ for ANAMMOX, and because this NH_4^+ is eventually lost as N_2 gas, the whole process is effectively concealed as denitrification in other words, an even larger fraction of N loss from anoxic systems may be driven by ANAMMOX organisms. In fact, evidence for DNRA has been detected in the Benguela upwelling system (Kartal, 2007a), where ANAMMOX bacteria actively remove massive amounts of N (Kuypers, 2005).

There is currently no established functional gene marker for analyzing anammox bacteria in the environment. However, functional genes encoding the most defining metabolic feature of anammox, hydrazine metabolism are beginning to be identified: candidate hydrazine hydrolase and hydrazine dehydrogenase genes have been identified in the *K. stuttgartiensis* genome (Strous, 2006), and a hydrazine-oxidizing enzyme, hzo, (Shimamura, 2007). Once these functional genes are definitively and specifically linked to ANAMMOX, analysis of ANAMMOX functional gene abundance and expression in the environment becomes a real possibility.

3.2.6 Dissimilatory Nitrate Reduction to Ammonium (DNRA)

The existence of DNRA has been widely recognized for at least the past 25 years, although its potential importance as a nitrate removal pathway on an ecosystem scale has generated increased interest within the past decade.

This microbially mediated pathway involves the dissimilatory transformation of nitrate to ammonium (NH_4^+), in contrast to assimilatory processes that incorporate N into cellular constituents. Compared to nitrate, the resultant ammonium is a more biologically available and less mobile form of inorganic N.

Little is known about the eventual fate of the nitrate that is converted to ammonium via DNRA pathways, but it is possible that, under appropriate conditions, the ammonium is converted back to nitrate via nitrification. The resultant ammonium may also be assimilated into plant or microbial biomass.

There are two recognized DNRA pathways: one involving fermentation and the other linked to sulfur oxidation. Early work on DNRA suggested that it was mainly carried out by fermentative bacteria (Tiedje 1988), though in recent years the existence of DNRA coupled to sulfur cycling has been documented in marine and freshwater ecosystems (Brettar and Rheinheimer 1991; Brunet and Garcia-Gil 1996). It is not known whether the two DNRA pathways are mutually exclusive. Fermentative DNRA couples electron flow from organic matter to the reduction of nitrate via fermentation reactions (Tiedje 1988; Megonigal, 2004). Many microbes perform fermentative DNRA, including species of *Clostridia*, *Desulfovibrio*, *Vibrio*, and *Pseudomonas*; these organisms can also carry out fermentation without using nitrate (Tiedje 1988).

For nitrate ammonification, *nrfA*, which encodes a periplasmic nitrite reductase catalyzing the conversion of nitrite to ammonia, can be used as a marker. Previous PCR based analysis of *nrfA* diversity in anammox and sulfate-reducing reactors showed that the majority of clones were most closely related to *nrfA* genes from *Bacteroides* spp.

4. ANOXIC MARINE SEDIMENTS AND ITS MICROBIOLOGY

4.1 Definition and Characteristics of Anoxic Marine Sediments

Sediments are a carbon and nutrient pool for aquatic environments. Processes for mineralization of organic matter mainly occur here by the benthic microbial communities (Aller, 1998). There are several studies about characterization of microbial communities involved carbon and nitrogen cycling in the benthic environments (Devereux, 1994; Gray and Herwig, 1996; and Teske, 1996b), however the studies about microbial populations in deep sea sediments are very poor. Coastal and shelf sediments are especially important in the remineralisation of organic matter. In those areas, an estimated 32 to 46% of the primary production settles to the sea floor. Prokaryotes reoxidize most part of the debris which is located in the sea sediments (Wollast, 1991).

Very little knowledge about diversity and structures of indigenous microbial populations within the polluted costal and shelf areas can be found in the literature. Some of them that are available for polluted marine sediments deal with main contaminants are polyaromatic hydrocarbons (Geiselbrecht, 1996; Gray and Herwig, 1996), heavy metals (Frischer, 2000; Gillan, 2004, Powell, 2003), and organic matter (McCaig, 1999; Stephen, 1996), hydrocarbons (Macnaughton, 1999; Roling, 2004; and Roling, 2002). The presence of hydrocarbon compounds and low oxygen level creates a suitable environment for the growth of anaerobic bacteria. Although anaerobic biodegradation processes are slower than aerobic biodegradation, anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen.

4.2 Microbial Life in the Anoxic Marine Sediments

Prokaryotic microorganisms are harder to identify at species level by their phenotypic character than eukaryotic ones. Their small size, the absence of distinguishing phenotypic characters, and the fact that nearly all of these organisms

cannot be cultured are most important factors that limit the evaluation of their biodiversity. (Pace, 1997; Torsvik and Ovreås, 2002)

It would estimate that only between 0.5% and 10% of prokaryote biodiversity has actually been identified. (Cases and de Lorenzo, 2002) The advent of culture-independent methods, such as molecular tools, has changed visualization of microbial diversity (Hugenholtz., 1998; Giovannoni and Rappe, 2000; Olsen, 1986; Amann., 1995a; Rossello-Mora and Amann, 2001).

4.2.1 The Importance of Microorganisms

Microbial communities in marine sediments are responsible for various important biochemical functions, including the degradation of pollutants, transformation and mineralization of organic matter, one of which is the most important in freshwater sediments (Urakawa, 2000; Schwarz, 2007). Sediment bacteria also play a significant ecological and biogeochemical role in marine ecosystems (Polymenakou, 2005) for they are instrumental in the marine food web, where they are the key players for recycling of nutrients and degradation of pollutants. This is largely a result of their high abundance relative to the overlaying water column and their key function in mediating and regulating the transformation and speciation of major bioactive elements (e.g. carbon, nitrogen, phosphorus, oxygen, and sulphur) in these environments (Polymenakou, 2005). Sediment bacteria also represent a major genetic variability with a local diversity equal to soil systems (Torsvik, 2002).

Since microorganisms have various important properties as mentioned above and may influence the maintenance of environment, amendment of polluted sites and the potential to serve as cleaner energy resources, microbial and intraterrestrial life is the center of interest for general social, professional and industrial motives.

Investigating the ecology of Archaea and bacteria is vital to understanding the functioning of global biogeochemical cycles. Thus the recognition of particular microbial groups that prevail under distinctive subseafloor environment is a significant step toward determining the role these communities play in Earth's essential biogeochemical processes (Inagaki, 2006).

The unknown microorganisms are the largest potential reservoir of useful genes for medicine and biotechnology and draw the attention of microbiologists for either their metabolisms or industrially interesting genes (Pedros-Alio, 2006).

Last but not least, knowledge of microbial diversity will provide essential information to understand evolution and create a catalogue of microbial diversity. There is a widespread interest, that has been triggered as a result of contamination of groundwater from surface and underground disposal sites, accidental spills, leakage and other human activities, in the possibilities of restoring contaminated underground sites with the help of autochthonous and/or allochthonous microorganisms (Heath, 1999).

Disposal of radioactive wastes and heavy metals in deep geological formations requires in-depth knowledge about the host rock environment and the effects of microbes in future repositories (Pedersen, 1997). Moreover, microbes living deep below the deposits are supposed to produce most of the methane (Waseda, 1998) found in methane gas hydrates that are enormous reservoirs of energy, possibly twice the amount of energy contained in known oil and gas reservoirs (Kvenvolden, 1995).

Another reason for the increased interest in subsurface life lies in the interest to find the origins of life. An increasing number of scientists propose an under-ground origin of life, possibly in the proximity of hydrothermal-vents that suggests life on other planets should be searched for underground rather than on the surface (Pedersen, 2000).

4.2.2 Bacterial Communities in Anoxic Sediments

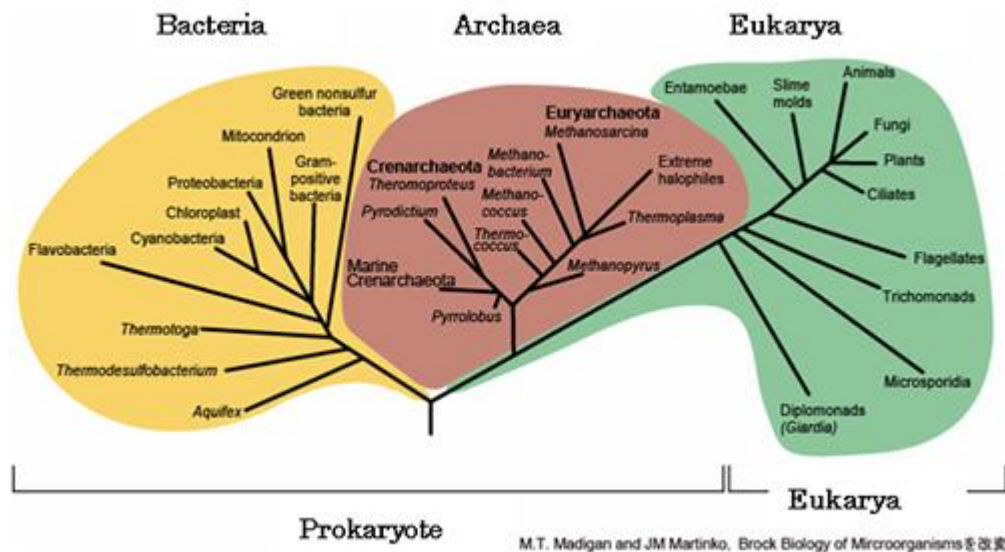


Figure 4. 1 : Universal phylogenetic tree adapted from Madigan , 2002

At least 17 major lineages of Bacteria (Figure 4.1) are known from the study of laboratory cultures, any many others have been identified from retrieval and sequencing of ribosomal RNA genes from Bacteria in natural habitats (Madigan, 2003).

The first phylum of bacteria is proteobacteria. This is the widest phylum of the bacteria. As a group these organisms are all gram-negative, show extreme metabolic diversity, and represent the majority of known gram-negative bacteria of medical, industrial, and agricultural significance. Proteobacteria has five major subdivisions:

Alpha

Beta

Gamma

Delta

Epsilon

One of the most important known groups of proteobacteria is purple phototrophic bacteria which carry out anoxygenic photosynthesis and contain chlorophyll pigments called bacteriochlorophylls with any variety of carotenoid pigments. The purple bacteria have different and spectacular colors, usually purple, red or brown. The most known of purple bacteria are purple sulfur bacteria and purple nonsulfur bacteria (Madigan, 2002).

The other known groups of proteobacteria are the nitrifying bacteria which are chemolithotrophs as Nitrosifiers and Nitrifiers, sulfur- and iron-oxidizing bacteria, hydrogen-oxidizing bacteria, methanotrophs and methylotrophs, Pseudomonas and the pseudomonads, acetic acid bacteria, free-living aerobic nitrogen-fixing bacteria, neisseria, chromobacterium and relatives, enteric bacteria, vibrio and photobacterium, rickettsia, spirilla, sheathed proteobacteria as sphaerotilus and leptothrix, budding and prosthecate/stalked bacteria, gliding myxobacteria, and finally sulfate- and sulfur-reducing bacteria (Madigan, 2002).

The second phylum of bacteria is gram-positive bacteria, gram-positive bacteria as lactic acid bacteria and relatives; endospore forming, low GC, gram-positive bacteria as Bacillus, Clostridium and relatives; cell wall-less, low GC, gram-positive bacteria as the Mycoplasmas; high GC, gram-positive bacteria as coryneform and propionic acid bacteria; high GC, gram-positive bacteria: Mycobacterium; and lastly filamentous, high GC, gram-positive bacteria as Streptomyces and other Actinomycetes (Madigan, 2002).

The other known phyla of the bacteria are cyanobacteria and prochlorophytes, Chlamydia, planctomyces/pirellula, verrucomicrobia, flavobacteria, cytophaga group, green sulfur bacteria, spirochetes, deinococci, green nonsulfur bacteria, deeply branching hyperthermophilic bacteria and finally nitrospira and defferibacter (Madigan, 2002).

4.2.3 Archaeal Communities in Anoxic Sediments

Archaea is one of the major phylogenetic groups. Even though they have similar characteristics to the bacteria, not only their phenotypical characteristics but also their phylogenetic characteristics are different. Some of the major features of the Archaea are below:

absence of peptidoglycan in cell walls

presence of ether-linked lipids in membrane

presence of the complex RNA polymerases

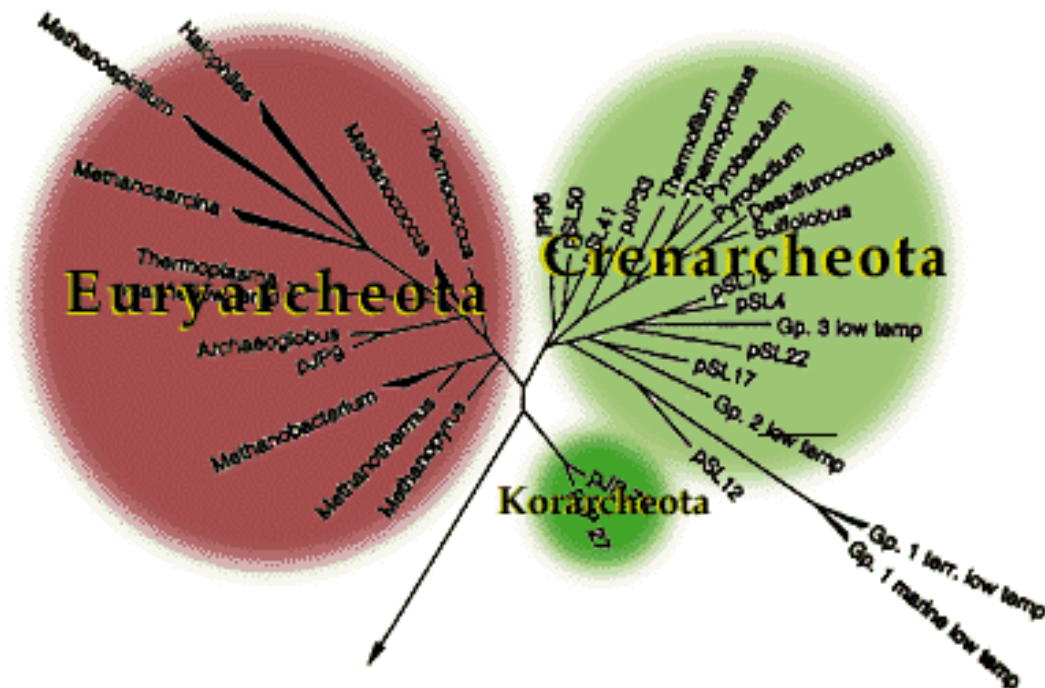


Figure 4.2 : A phylogenetic tree of *Archaea* adapted from Madigan.

The phylogenetic tree of Archaea is separated into two major phyla called the Crenarchaeota and Euryarchaeota. A third phylum is Korarchaeota which branches off close to the root (Figure 4.2).

The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of Archaea (Woese, 1990). It was believed to include only sulphur-dependent extreme thermophiles. Among cultured representatives, the Crenarchaeota contain mostly hyperthermophilic species including those able to grow at highest temperatures of all organisms. Most hyperthermophiles of crenarchaeota are chemolithotrophic autotrophs and primary producers in the harsh environments because of their habitats and devoid of photosynthetic life.

Hyperthermophilic crenarchaeota tend to cluster closely together and occupy short branches on the 16S rRNA-based tree of life because these organisms have slow evolutionary clocks and have evolved the least away from the hypothetical universal ancestor of life (Madigan, 2002).

The Euryarchaeota is a heterogeneous group comprising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens, and some extreme thermophiles so far (Madigan, 2002).

Moreover, a third archaeal kingdom has recently been discovered which is reported isolation of several archaeal sequences evolutionary distant from all Archaea known to date by Barns and co-workers in 1994 and then in 1996. The new group was placed on phylogenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota (Madigan, 2002).

4.3 Microbial Ecology Studies in Marine Sediments

Identification of dominant members of microbial communities and determination of their different roles are the key questions in microbial ecology (Purdy, 2003). Studying the genetic diversity and analyzing the members of mixed microbial populations are two of the most important steps in microbial community studies (Urakawa, 1999). Fundamental aspects of the vast sub seafloor ecosystem are poorly known. The phylogenetic composition of sub seafloor prokaryotic communities, their functional genes and the metabolic activities that allow prokaryotes to grow and survive in the subsurface are the main questions to be answered (Teske, 2006). Many studies were conducted to understand how microorganisms are distributed in the environment and how they influence geochemical processes (Leloup, 2007; D'Hondt, 2004). Numerous studies in the recent past have focused on the identification and community diversity of microorganisms based on 16S rDNA analysis of naturally occurring microbial communities (Hugenholtz and Pace, 1996).

Previous molecular studies focused on the general microbial community structure and the abundance and depth distribution of specific functional groups such as polymer degrading, sulphur-oxidizing or sulphate reducing bacteria (SRB), because the processes they catalyze can be measured directly (Sahm, 1999).

Among these studies, ODP Leg 201 being the first ODP expedition dedicated to the study deep beneath the seafloor (D'Hondt, 2003) combined geochemical analyses, cell counts, cultivations and molecular screening of subsurface microbial communities with the objective of a census of subsurface microbial life.

In the context of geochemical controls that shape microbial community ,taking part in N cycle, composition and activity (Teske, 2006)

Earlier, the N cycle in the shallow depths of the oceans has been investigated (Ward, 1996). The presence of deep-sea denitrifiers may indicate that the nitrogen cycle operates even in the depths of the deep sea. However, a nitrate supply would be required for the denitrifying growth of and the existence of nitrifiers is therefore suggested. Furthermore, several studies have been performed for investigating the respiratory systems in the deep sea (Tamegai, 1998, 2002, 2004, 2005). Thus, molecular studies of the nitrogen cycle could provide novel insights into the energy conservation systems in the deep sea.

The genes encoding the denitrifying reductases have been studied in only few species. Recent investigations have led to the identification of new classes of denitrifying reductases, indicating a more complex genetic basis of this process than previously recognized. The increasing number of genome sequencing projects has opened a new way to study the genetics of the denitrifying process in bacteria and Archaea. (Laurent, 2002).

5. MOLECULAR TECHNIQUES IN MICROBIAL ECOLOGY

5.1 The Need for Molecular Techniques

The development of molecular techniques using nucleic acids has led to many new findings in studies of microbial ecology (Amann, 1995). Microbial diversity studies were limited in the past by the lack of methodological tools, but the availability of the new molecular methods has made it possible to investigate the dynamics of the composition and structure of microbial populations and communities in defined environments, the phylogenetic relationships, and the impact of environmental or specific factors such as pollution by xenobiotics on microbial diversity (Morris, 2002), the origin and conservation of microbial biodiversity (Dorigo, 2005), allows enumeration, to study distribution and activity of microorganisms and detection of individual microbial taxa in natural habitats.

The classical approach for identification of viable microorganisms in environmental samples, including sediments, is plate counting on agar medium (Edlund and Jansson, 2006). It would appear that only between 0.5% and 10% prokaryote diversity has actually been identified due to the small size and the absence of distinguishing phenotypic characters of prokaryotic organisms, and the fact that most of these organisms cannot be cultured which are the most important factors that limit the evaluation of prokaryotic biodiversity (Torsvik, 2002).

There are many other short-comings and disadvantages of the past traditional microbiological techniques. Microscopy based and culture dependent techniques have only a limited use for classification and identification of microorganisms (Muyzer, 1999). Because many microorganisms are bound to sediment particles, they can not be detected by conventional microscopy. Unrecognized nutrient and growth conditions, the failure of selective enrichment cultures to mimic the environmental conditions required by particular microorganisms for proliferation in their natural habitat, fastidious nutritional and environmental requirements of anaerobes are the limits of culture dependent methods (Hofman-Bang, 2003).

Almost 99% of all microorganisms in nature cannot be isolated and classified based on physiological and biochemical features mainly due to the previously mentioned limitations of cultivation (Muyzer, 1999).

5.1.1 The 16S rRNA and its Importance

Since a great percentage of microorganisms cannot be cultured on laboratory conditions, an alternative approach was created. In this approach, a unique and distinct characteristic of each microorganism was used. From the microorganism(s) DNA was extracted and a data bank of specific genes was created. With these genes, microorganisms can be identified without cultivation. Mostly ribosomal RNA (rRNA) molecules (16S and 23S) were used for phylogenetic marker. The molecule was selected for analysis since ribosome is a well abundant (10³-10⁵) and obligatory component of each cell. Because ribosomes are directly taking part in protein production, its number gives also clue about cell volume and growth rate (Amann, 1995b; Alcamo, 1996).

The basic approach for molecular diversity analysis is shown in Figure 5.1. Briefly, isolates from a community can be subject to direct analysis, using in situ methods, or nucleic acid can be extracted for analysis using microarrays or dot-blot hybridization. A gene might also be amplified using the polymerase chain reaction (PCR) and evaluated using a range of techniques including pattern analysis, cloning and sequencing, probe hybridization and microarrays (Dahllöf, 2002).

The use of 16s rDNA or rRNA is currently the most common approach for community analysis (Dahllöf, 2002). Studies of subsurface communities have focused on 16S rRNA gene analyses, which have provided information on the diversity of prokaryotes in these environments (Parkes, 1994; D'Hondt, 2004).

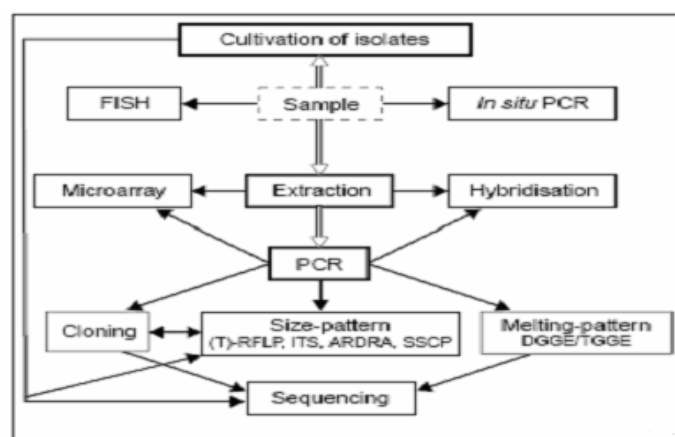


Figure 5.1 : Common approaches to the analysis of microbial diversity adapted from Dahllöf (2002)

5.1.2 The Variable Regions in 16S rRNA and its Importance

16S rRNA genes consist of highly conserved and highly variable regions (Lane, 1985). The amplification of this gene with suitable primers makes it possible to identify all microorganisms. The comparison of amplified genes with known sequences in database helps to build a phylogenetic classification system. With the developments in analysis of 16S rRNA, the detection and identification of microorganisms in nature enhances greatly. The 16S rRNA analysis also shows the truth of the suspicions about inefficiency of culture dependent techniques (Barns, 1994; Choi, 1994; DeLong, 1992; Liesack and Stackebrandt, 1992).

The rRNA is highly conserved in nucleotide sequence as well as in secondary structure since its function remains same through years of evolution. It has many variable regions in which random changes occur from time to time. These changes reflect evolutionary relationships of the organisms. Conserved regions functions as binding places for PCR primers or hybridization probes. Even data from this analysis is sufficient to compare statistically significant phylogenetic relations (Olsen, 1986). Among the variable regions, V3 region is mostly used in molecular analysis (Neefs, 1990; Ovreas, 1997).

5.2 Most Commonly Used PCR-Based Molecular Techniques

5.2.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) can be used to amplify DNA sequences from environmental samples. The PCR products can be analyzed by techniques such as DGGE (denaturation gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), or SSCP (single stranded conformation polymorphism), which have the potential to separate the PCR products originating from different DNA sequences representing populations in the original samples. The PCR products can also be cloned and subsequently sequenced to allow identification of population (Hofman-Bang ., 2003).

The development of synthetic DNA has spawned a new method for the rapid amplification of DNA in vitro, the polymerase chain reaction (PCR).

In some molecular tools including the ones which will be discussed below, the initial step relies on the using PCR to amplify a target sequence. PCR can multiply DNA molecules up to a billion fold in the test tube, yielding large amounts of specific genes for cloning, sequencing or mutagenesis purposes. PCR makes the use of the enzyme DNA polymerase, which copies DNA molecules (Madigan, 2003).

(1) The PCR technique requires that the nucleotide sequence of a portion of a desired gene to be known. This is necessary because short oligonucleotide primers complementary to sequences in the genes of interest must be available for PCR to work (Madigan, 2003).

(2) The choice of primers makes it possible to target the sequence at different taxonomic levels (strain, species, genus, etc.). The final PCR products obtained contain a mixture of the same fragment amplified at the chosen taxonomic level (strain, species, genus, etc.) (Dorigo, 2005).

(3) The PCR technique is that each cycle literally doubles the content of the original target DNA. The extension products of one primer can serve as a template for the other primer in the next cycle. In practice, 20-30 cycles are usually run, yielding a 10^6 to 10^9 fold increase in the target sequence.

The PCR amplification step is known to introduce biases, with or sometimes without irrespective of the gene targeted. All techniques that are based on PCR (cloning, pattern analysis and sequencing) will be affected by the biases introduced by PCR (Dahllof, 2002).

Factors that cause bias of PCR are:

Primer specificity is a major stumbling block, especially when attempting to quantify a mixture of homologous target sequences (Becker, 2000). “Universal” primers or other specific primers are designed based on sequence information available in databases (obtained from cultured organisms and clones) (Pace, 1996). However, primers targeting multiple groups of organisms may not amplify all target genes since the primer sites are not completely conserved (Hofman-Bang, 2003).

It was recently shown that the original sample template is amplified during the initial 5-6 cycles of the PCR reaction (Kurata, 2001), and that in the following cycles amplification occurs only on the PCR fragments produced earlier.

Even single mismatches in the middle of the primer can cause a preferential selection (Schafer, 2001). Degenerate primers could be used, but these are not suitable for techniques like DGGE as they produce multiple bands from one template, which gives the same problems when using heterogeneous genes (Dahllof, 2002).

An inappropriate annealing stringency, which results in amplification of genes that are not intended to be amplified, can also cause a bias (Ward, 1992). Lowering the annealing temperature allows for mismatches and increases the diversity in PCR products (Ishii and Fukui, 2001), while increasing the risk of unwanted by-products.

Contaminating sequences from chemicals and enzymes can be incorrectly included in the analysis (Hofman-Bang, 2003).

Imaginary sequences are often produced (Ward, 1992) due to the presence of partial fragments of rDNA in DNA extracts, partially reverse transcribed DNA when performing RT-PCR, or premature PCR products acting as primers in a subsequent PCR cycle (Amann, 1995).

5.2.2 Real Time PCR:

The advent of real-time PCR and real-time reverse transcription PCR (real-time RT-PCR) have dramatically changed the field of measuring gene expression. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (Franca, 2002).

Reactions are characterized by the point in time (or PCR cycle) where the target exponential amplification is first detected. This value is usually referred to as cycle threshold (C_t), (Figure 5.2.2.1.) the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower C_t (Maitra, 2004).

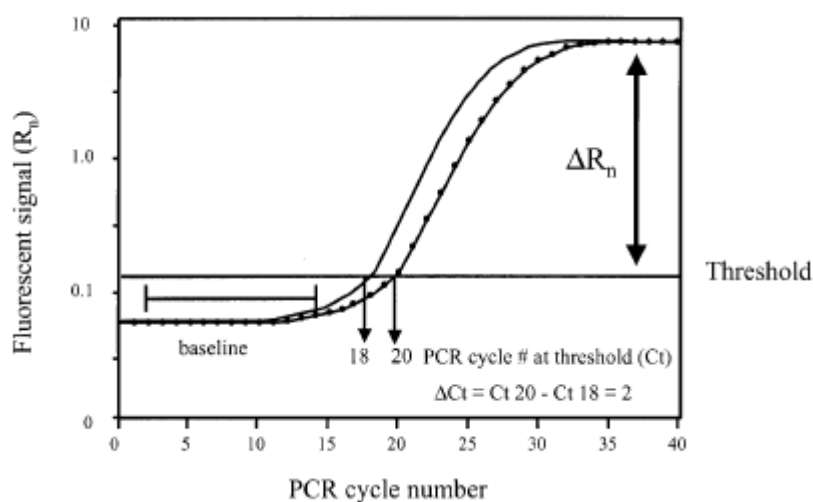


Figure 5.2 : A hypothetical amplification plot illustrating the nomenclature typically used in real-time Q-PCR experiments.

The real-time machine not only monitors DNA synthesis during the PCR, but also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short).

All PCR products for a particular primer pair should have the same melting temperature - unless there is contamination, mispriming (PCR products made due to annealing of the primers to complementary, or partially complementary sequences on non-target DNAs), primer- dimer artefacts (the primers can sometimes anneal to themselves and create small templates for PCR amplification) or some other problem.

After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. A primer-dimer artifact would give a peak with a lower melting temperature (because it is such a short DNA).

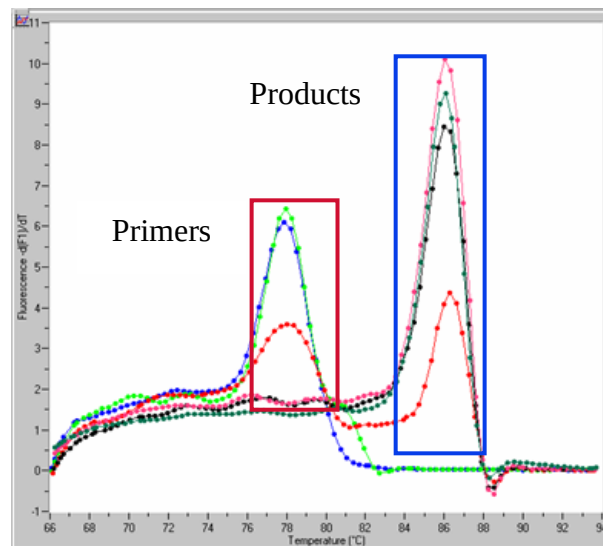


Figure 5.3 : Melting Temperatures of the products adapted from Gibson (2005)

There are many benefits of using real-time PCR over other methods to quantify gene expression. It can produce quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude (Schroell, 2003) and does not require post-amplification manipulation. Real-time PCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays (Newby, 2003), 1000-fold more sensitive than dot blot hybridization (Zhou, 2004), and can even detect a single copy of a specific transcript (Bengtsson, 2003). In addition, real-time PCR assays can reliably detect gene expression differences as small as 23% between samples (Gupta, 2004) and have lower coefficients of variation than end point assays such as band densitometry.

The major disadvantage to real-time PCR is that it requires expensive equipment and reagents. In addition, due to its extremely high sensitivity, sound experimental design and an in-depth understanding of normalization techniques are imperative for accurate conclusions.

5.2.2.1 Types Of Real Time Quantification

Relative Quantitation

During relative quantitation, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator (Bernard, 1990). When using a calibrator, the results are expressed as a target/reference ratio. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error (Harvey, 2004).

Absolute Quantitation

Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their Ct values (Maitra, 2004). This method assumes all standards and samples have approximately equal amplification efficiencies (Whitcombe, 1999). In addition, the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay.

Absolute quantitation is considered to be more labor-intensive than relative quantitation because of the necessity to create reliable standards for quantitation and include these standards in every PCR (Gundry, 2003). However, when performing relative quantitation, the data (Ct) used for comparison are arbitrary values and only applicable to the samples run within the same PCR. To compare samples between two different PCRs, it is necessary to include a reference control in every plate or run.

5.2.2.2 Detection Chemistries

The first reported method of real-time PCR, by Higuchi in 1993 (Higuchi, 1993), used ethidium bromide intercalation during the PCR process and a modified thermocycler to irradiate the samples with ultraviolet (UV) light and then detected the resulting fluorescent signal with a charged coupled device (CCD) camera. The fluorescent signal was plotted as a function of cycle number.

The resulting plot, now a very familiar sight, gave a good indication of the amount of PCR product that was generated during each cycle of PCR (except for those early cycles that are beneath the detection limits of the CCD camera). The primary drawback to this approach, other than the use of a carcinogen like ethidium bromide, is that nonspecific PCR products are equally detected and included in the total amount of fluorescent signal measured.

Today, the chemistries most commonly employed include 5' nuclease assays using TaqMan probes (Gibson, 1996), molecular beacons (Tyagi, 1996) and SYBR Green I intercalating dyes (Yin, 2001). Other methods have also been reported (Wilhelm, 2001, Nazarenko, 1997); however, in all cases a fluorescent signal is generated during the PCR process that is captured by any one of several different real-time instruments. Due to added specificity, the addition of a hybridization probe makes the Real- Time PCR assay much more robust.

- **DNA Binding Dyes**

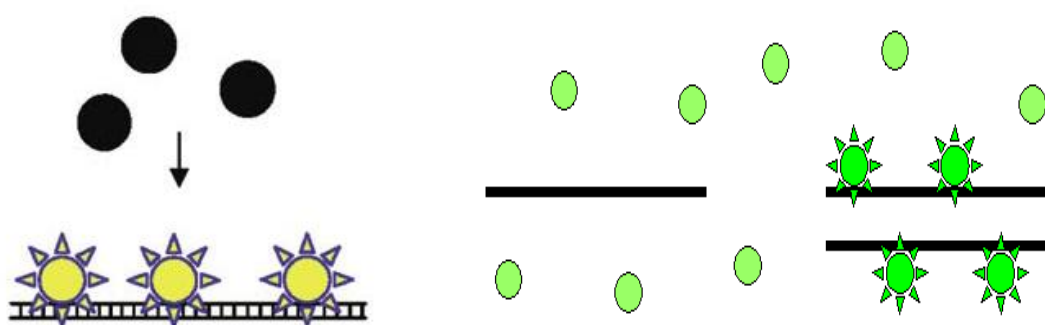


Figure 5.4: DNA Binding Dyes, SYBR Green adapted from Wong (2005)

DNA binding dyes emit fluorescence when bound to dsDNA (Figure 5.4). As the double-stranded PCR product accumulates during cycling, more dye can bind and emit fluorescence.

Thus, the fluorescence intensity increases proportionally to dsDNA concentration (Brandon, 2005). This technique is very flexible because one dye can be used for different gene assays.

Consequently, multiplexing reactions is not possible. Because DNA binding dyes do not bind in a sequence-specific manner, these assays are prone to false positives (Hodgson, 2001). Accurate results demand a specific PCR, which can be confirmed via dissociation curve analysis, where the presence of different PCR products is reflected in the number of first-derivative melting peaks (Hazbon, 2001) or gel analysis (Boniotto, 2004).

- **Hydrolysis Probes:**

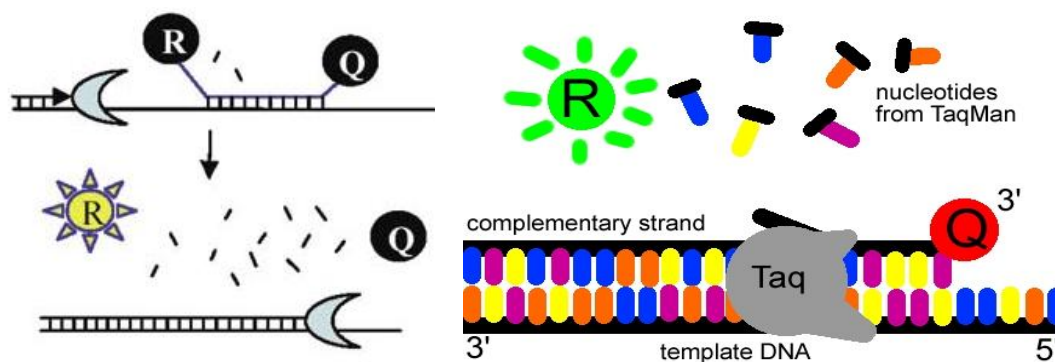


Figure 5.5 : Hydrolysis Probes, Taq Man

Hydrolysis probes, exemplified by the TaqMan chemistry, also known as 5' nuclease assay, fluoresce upon probe hydrolysis to detect PCR product accumulation (Figure 5.5). The sequence-specific probe is labeled with a reporter dye on the 5' end and a quencher dye on the 3' end, which allows the quencher to reduce the reporter fluorescence intensity by FRET (Fluorescence Resonance Energy Transfer) when the probe is intact (Germer, 2000). While both hydrolysis and hybridization probes rely on FRET to alter the intensity of fluorescence emission, the energy transfer works in opposite manners in these two chemistries. FRET reduces fluorescence intensity in hydrolysis probes and increases intensity in hybridization probes. When annealed to the target sequence, the bound and quenched probe will be degraded by the DNA polymerase's 5' nuclease ability during the extension step of the PCR. Probe degradation allows for separation of the reporter from the quencher dye, resulting in increased fluorescence emission (Maitra, 2004, Germer, 2003).

Hairpin Probes:

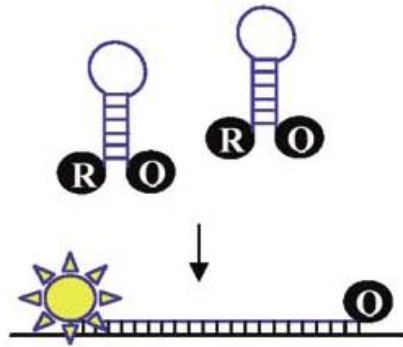


Figure 5.6 : Molecular beacons

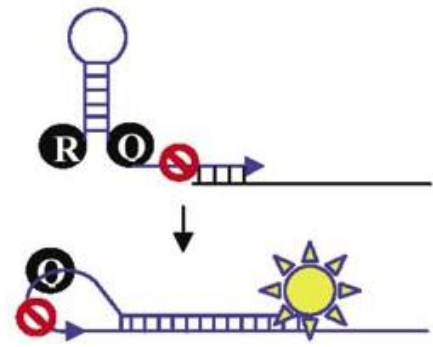


Figure 5.7 : Scorpions

Molecular beacons, Consisting of a sequence-specific region (loop region) flanked by two inverted repeats, molecular beacons are the simplest hairpin probe (Figure 5.6), (Payungporn, 2004). Reporter and quencher dyes are attached to each end of the molecule, causing a reduction in fluorescence emission via contact quenching (FRET) when the beacon is in hairpin formation (free in solution). When bound to the target, the quencher and reporter are separated, allowing reporter emission.

Scorpions, combine the detection probe with the upstream PCR primer (Figure 5.7) (Zhou, 2004) and consist of a fluorophore on the 5' end, followed by a complementary stem-loop structure (also containing the specific probe sequence), quencher dye, DNA polymerase blocker, and finally a PCR primer on the 3' end. The probe sequence contained within the hairpin allows the scorpion to anneal to the template strand, which separates the quencher for the fluorophore and results in increased fluorescence.

5.2.2.3 General Applications of Real Time PCR (Q_PCR)

Applications of real-time Q-PCR are numerous. They include, mRNA expression studies, DNA copy number measurements in genomic (Kariyazono, 2001) or viral DNAs (Desire , 2001), transgene copy number, allelic discrimination assays (Oliver, 2000, Walburger, 2001) and confirmation of microarray data (Rozzo, 2001).

Some of the most recent applications, which demonstrate the sensitivity of this technology when applied to expression analysis of limited samples, include expression analysis of specific splice variants of genes and laser capture of micro dissected material; paraffin embedded tissues (Specht, 2001); flow-sorted cells, including stem cells; or randomly amplified RNA from single cells (Taher , 2000).

The technology may be used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions.

Also, the technique is used in environmental microbiology, for example to quantify resistance genes in water samples. In this study DNA based Q_PCR is used for the quantification of the N cycle genes.

5.2.3 Pattern Analysis and Denaturing Gradient Gel Electrophoresis.

Pattern analysis or fingerprinting is often carried out by evaluating banding patterns of PCR products on gels (Dahllof, 2002). Several fingerprinting techniques, such as DGGE, TGGE, RFLP, and SSCP, have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure (e.g., trace one or more populations over time), to compare diversity and community characteristics in various samples and simply to identify differences between communities (Hofman-Bang, 2003; Dahllof, 2002). These techniques usually involve gel electrophoresis that can separate different DNA fragments of a community Rona library (Dahllof, 2002).

DGGE is now routinely used to assess the diversity of microbial communities, to monitor their dynamics (Muyzer, 1999; Muyzer and Smalla, 1998) and to screen clone libraries. This method can be used to obtain qualitative and semi-quantitative estimations of biodiversity.

The DGGE pattern obtained provides a rapid identification of the predominant species. In a DGGE gel the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of numerically dominant ribotypes in the sample (Boon, 2002).

The DGGE technique has been used to characterize the microbial diversity in different environments such as activated sludge (Curtis and Craine, 1998), sediments (Muyzer and De Wall, 1993), lake water (Ovreas, 1997), hot springs (Santegoeds, 1996), soils (Jensen, 1998), biofilm (Santegoeds, 1998).

DGGE has been used to monitor changes in complex communities (Santegoeds, 1996; Teske, 1996) and to identify microorganisms present in wall painting (Rolleke, 1995). It has recently been demonstrated that DGGE analysis of PCR products also works well in deep marine sediments and seafloor basalts (Lysnes, 2004). Denaturing gradient gel electrophoresis (DGGE) has been used extensively to profile prokaryotic community composition over both time and space in soils and aquatic environments (Schafer and Muyzer, 2001). It provides a quicker, less labor-intensive approach to comparing community composition in many different samples than sequencing of clone libraries. PCR amplification of the 16S rRNA gene utilizing conserved primers targeting either V3 or the V8 + V9 regions is normally used to produce a 300-500 bp fragment. Larger fragments are typically not used as the DGGE technique can not resolve these into distinct bands (Muyzer, 1993).

A big advantage of the technique is that they make it possible to obtain taxonomic information by excising, re-amplifying and sequencing specific DNA fragments or by hybridization analysis with taxon-specific oligonucleotides probes (Riemann and Winding, 2001). Another advantage of the technique is that it can resolve the microbial diversity of up to 15 different species by optimizing the DGGE in the gel. By using narrow gradients, rDNAs that differ in only one bp can be separated in DGGE (Muyzer, 1993). Bands from longer PCR products give better identification possibilities, although this must be weighed against separation efficiency. Creating a clone library which contains larger gene sequences is an alternative and thereby provides a more positive identification. However, a large number of clones have to be sequenced to get an appreciation of diversity, and clone libraries are victims of the same biases as other PCR methods (Hofman-Bang, 2003).

The main limitations are the choice of the primer set and the optimization of the gel running conditions before technique can be used to screen for sequence polymorphism of a particular gene (Muyzer, 1993) and the difficulty of comparing patterns across gels, when these patterns include numerous bands and its limited sensitivity of detection of rare community members (Vallaeys, 1997).

The banding patterns of highly diverse microbial communities, present in soils, activated sludges and sediments, are usually very complex when bacterial primers are used. Since only the major populations of the analyzed community are represented on these DGGE patterns and thus relatively less abundant but potentially very important species may not be detected by this molecular method (Heuer, 1997).

A drawback of the technique is that the reproducibility is not optimal; one DNA fragment may generate more than one band on the gel and a DNA sample analyzed on two different gels may not generate the same band pattern (Muyzer and Smalla, 1998). The possibility of a band in a DGGE gel to contain different sequences with similar denaturation characteristic may cause another bias to the technique (Hofman-Bang, 2003) because they may migrate together.

6. MATERIALS AND METHODS

6.1 Sampling and Preservation

Sampling locations were shown in Figure 6.1. and sampling depths and dates, and sample abbreviations were given in Table 6.1. The samples were taken using a Van Veen grab (volume of 3.5 L and penetration depth of 15 cm) on board of the RV Arar of Istanbul University (Figure 6.2), Institute of Marine Sciences during research cruises between the years 2005 and 2008. Samples were taken in three replicates from the top 10 cm of sediment from the grab using 50 ml sterile syringes, with top end removed. Samples were then subdivided for molecular analyses and sediment chemistry as 10 ml and 40 ml, respectively. Samples were then stored in sterile polypropylene tubes at -20°C.

All samples appeared visually similar possessing grayish-black colour(Figure 6.3) and had a noticeable odour of H₂S. Sediment grain size analysis was performed using a method adapted from Folk (1974) as described by Unlu and Alpar (2006). All of the sediments have fine-grained nature, being rich in mud (>90%) and poor in sand.



Figure 6.1: Sampling locations in Marmara Sea

Table 6. 1: Sampling locations, depths and dates, and sample abbreviations

Location	Coordinates			Sampling dates and sample abbreviations
	Latitude (N)	Longitude (E)	Depth (m)	
Tuzla	40°50.60'	29°13.60'	42	TUZAUG05, TUZNOV05, TUZFEB06, TUZNOV06, TUZFEB07, TUZMAY07, TUZAUG07
Kucukcekmece	40°58.24'	28°45.44'	22	KUCAUG05, KUCNOV05, KUCFEB06, KUCNOV06, KUCFEB07, KUCMAY07, KUCAUG07
Gemlik	40°33.17'	27°56.49'	87	GEMAUG05, GEMNOV05, GEMFEB06, GEMNOV06
Izmit	40°43.30'	29°37.00'	157	IZ17AUG05, IZ17NOV05, IZ17FEB06, IZ17FEB06
Izmit	40°44.00'	29°47.00'	30	IZ25AUG05, IZ25NOV05, IZ25FEB06, IZ25NOV06
Izmit	40°44.20'	29°53.50'	30	IZ30AUG05, IZ30NOV05, IZ30FEB06, IZ30NOV06
Moda	40°58.62'	29°01.49'	8	MODFEB06, MODNOV06, MODFEB07, MODMAY07, MODAUG07
Halic	41°19.38'	28°57.99'	6	HALVKNOV06, HALVKFEB07, HALVKMAY07, HALVKAUG07
Halic	41°24.24'	28°56.92'	6	HALEYNOV06, HALEYFEB07, HALEYMAY07, HALEYAUG07
Halic	41°33.66'	28°56.64'	2	HALASNOV06, HALASFEB07, HALASMAY07, HALASAUG07



Figure 6.2 : The research ship, ARAR, of Istanbul University and Van Ween grab Sampler

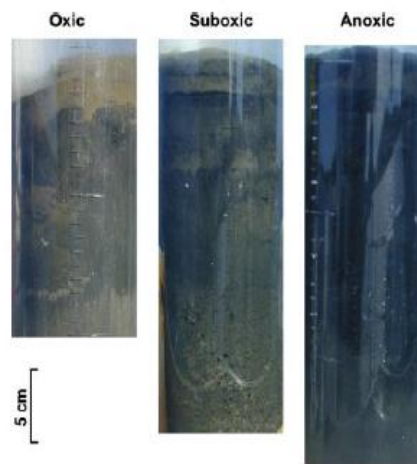


Figure 6.3: The oxic, suboxic and anoxic sediment samples adapted from (Virtasalo,2005)

6.2 Genomic DNA Extraction, Purification, Concentration

DNA was extracted from 0.5 g sample by using Fast DNA Spin Kit for Soil (Q-Biogene, Bio 101 Thermo Electron Corporation, Belgium) and a Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation, Belgium) according to the manufacturers' instructions.

The methodology of Genomic DNA extraction of by Fast DNA Spin Kit for Soil was as follows, approximately 0.5 g sediment was added up to lysing matrix tubes provided by the kit.

The tube contains mixture of ceramic and silica particles to lyse all microorganisms in sample. Then lysing matrix tubes were spinned in Ribolyser (Fast Prep™ FP120 Bio 101 Thermo Electron Corporation) for 30 seconds at a speed of 5.5 m/s. The tubes were then centrifuged at 14000xg for 30 seconds.. After centrifugation supernatants were transferred to clean 1,5 ml eppendorf tubes and added 250 µl PPS reagent.

To mix the composition tubes were shaken by hands for 30 seconds. After mixing the tubes centrifuged again at 14000xg for 5 minutes to pellet the precipitate. Supernatants were transferred to 2 ml eppendorf tubes and 1 ml of Binding Matrix Suspension was added to supernatant.

Tubes were inverted by hand for 2 minutes to allow binding of DNA to matrix. To settle the silica matrix tubes were incubated 3 minutes at room temperature. 500 µl of supernatant was removed carefully without disturbing settled silica matrix.

Then the binding matrix was resuspended in the remaining supernatant. All mixture was filtered and the filter was placed to a new tube.

Filter was washed by 500 µl SEWS-M wash solution. After washing, filter was dried by centrifugation at 14000xg for 2 minutes. The filter was removed to a new tube and 50 µl DES (DNase/Pyrogen free water) was added.

The filter with DES was vortexed and then centrifuged at 14000xg for 1 minute. Application-ready DNA was obtained in the tube.

To control the existence of extracted genomic DNA, 1/100 diluted genomic DNA was run on the %1 (w/v) agarose gel, prestained with Ethidium Bromide (EtBr) in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA; pH 8). Gel was visualized by using a gel documentation system, Mitsubishi 91.

The DNA concentration of each extract was determined using a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications. Quant-iT™ Working Solution was made by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer

All tubes was vortexed for 2–3 seconds and was incubated for 2 minutes at room temperature (15 minutes for the Quant-iT™ protein assay). Then, the samples to be quantified were prepared similarly using 1 ul of each sample combined with buffer and dye. All standards and samples are run in clear 500 ul tubes that are available from Invitrogen.

Tubes were read in Qubit™ fluorometer and it performed the multiplication by the dilution factor to determine concentration of our original sample.

Purity of the genomic DNA samples was controlled by using Spectrophotometer. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample.

In the analysis ddH₂O was used as a blank, and the values between 1.6 - 2.0 were used in the Q_PCR analysis.

6.3 Standards for Q_PCR:

Extracted GDNA's were used as templates, sequences of the genes ,taking part in the N cycle, were amplified by specific primers. The primers and their annealing temperatures were given in table 6.2.

Table 6.2 : PCR conditions used in the study

Primers	Target gen	Denaturation	Annealing
nosZ-F CGY TGT TCM TCG ACA GCC AG nosZ-R CAT GTG CAG NGC RTGGCAGAA (Kloos,2009)	Nos Z	94 ⁰ C	55 ⁰ C
nrfA-F GCN CCN TGY MGN TTY TGY GG nrfA-R RTG YTG RTT RAA NCC CAT NGT CCA (Smith, 2008)	Nrf A	94 ⁰ C	50 ⁰ C
hzoA-F TGT GCA TGG TCA ATT GAA AG hzoA-R ACC TCT TCW GCA GGT GCAT (Ran,2008)	Hzo A	95 ⁰ C	53 ⁰ C

Amplification was done in a 50 µl reaction volume containing 200 ng of DNA, 10 pmol of each primer, 10 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 5 µl of 10×Taq buffer and 4 U of Taq DNA polymerase (Fermentas, Latvia) by using a Techne TC-412 thermal cycler (Barloworld Scientific Ltd., U.K.).

An initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min.

Annealing for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min was done.

PCR products were visualized by electrophoresis (Thermo-Scientific Ltd., U.K.) on a 1% (w/v) agarose gel in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 7 V cm⁻¹ and gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France) after staining with ethidium bromide.

One of the positive PCR product result was chosen for cloning.

The initial step of the cloning procedure was the preparation of 6 µl reaction mix by adding 3 µl PCR product, 1 µl salt solution (1.2 M NaCl, 0.06 M MgCl₂), 1 µl TOPO vector and 1 µl Sterile Water. The solution was mixed gently and incubated at room temperature for 20 minutes. Following incubation, reaction mix was placed on ice before One Shot TOPO transformation step. After incubation, the tube was subjected to heat shock at 42° C for 30 seconds and transferred immediately to ice and 300 µl of SOC medium (2% Tyryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added. The solution was shaken horizontally for 60 minutes. Three LB plates containing 50 µg/ml kanamycin were warmed to room temperature and then 100 µl of solution was spread on plates using glass spreader. The plates were incubated overnight and white colonies were observed after incubation.

Colonies were picked from plate and transferred into 200 µl PCR tubes containing 50 µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). Colonies were boiled at 95° C for 5 minutes then frozen at -200° C overnight. Thawed solution was used as templates for PCR. The DNA fragments were isolated from vector by PCR with primers M13f-M13r (M13 Forward 5'-GTA AAA CGA CGG CCA G-3' / M13 Reverse 5'-CAG GAA ACA GCT ATG AC-3').

Other step of standard preparation is purification of M13 PCR products, which was done according to the Invitrogen PCR product purification Kit. according to the manufacturer's specifications. Purification with PureLink™ PCR Purification Kit, the yield of purified dsDNA has been estimated by agarose gel electrophoresis.

To estimate the yield, agarose gel electrophoresis of the purified PCR product and known quantities of DNA fragment of the same size was performed

The band intensity of the purified PCR product with the standard DNA fragments was compared. So the purified PCR product was used as Q_PCR standards. The standard concentrations were determined using a fluorometer (Qubit, Invitrogen) according to the manufacturer's specifications. Application ready standards were diluted in 1/100 ratio for Q_PCR experiments.

6.4 Q_PCR Experiment

PCR primer sets for the Q-PCR (Figure 6.3) assays were given in Table 6.3, 10^{3-7} copies of the standard sequences were used to obtain the calibration curves. Roche Light Cycler DNA Master SYBR Green I kit and Roche Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were utilized for all reactions. Reaction mixes contained 25 ng template DNA, 0.5 μ M of each primer and 2.5 μ M $MgCl_2$. Q-PCR conditions for the primer sets were described previously in Table 6.4. The following thermo cycling program was applied: 95°C, 10 min; 45 cycles of 10 s at 95°C, 5-10 s at primer dependent annealing temperature, 15 s at 72°C. A melt curve analysis was performed from 55°C to 95°C to determine if only one amplified product was generated during process. Q-PCR runs were analyzed using Roche Light Cycler Software 4.05. The efficiencies were between 1.8 and 2.0, and the correlation factors (r^2) were not lower than 0.97 in all reactions.

Table 6.3 : Q_PCR primer sets

Primer	Target Gene	Target Organism	Reference
nosZf	nosZ	Denitrifiers	Henry, 2006
nosZr	nosZ	Denitrifiers	Henry, 2006
nrfAf	nrfA	Dissimilatory Nitrate Reducers to NH_4^+	Smith, 2007
nrfAr	nrfA	Dissimilatory Nitrate Reducers to NH_4^+	Smith, 2007
hzoA_811f	hzoA	Anaerobic NH_4^+ oxidizers	This study
hzoA_1175r	hzoA	Anaerobic NH_4^+ oxidizers	This study



Figure 6.4 : General and capillary view of Q_PCR.

Statistical analyses were performed using the softwares MINITAB 15 (Minitab Ltd., England) and SPSS 17.0 (SPSS Inc., U.S.A.).

7. RESULTS AND DISCUSSION

7.1 Chemical Analysis Results

The results from the chemical analyses obtained from TUBITAK Project .105Y307, were given in Table 7.1 as ranges in which the concentrations fluctuate during the two years monitoring period. The results of the chemical analyses were used to determine the correlation of target gen abundance with chemical characteristics.

The heavy metals can be ranked for their potential adverse effects on benthic organisms in the MSS as $\text{Ni} > \text{Zn} > \text{Cu} > \text{Pb} > \text{Cr} > \text{Mn} > \text{Fe}$.

For elemental composition, TOC/N/P ratios of the MSS ($\approx 100/50/10$) were used and the results were very low compared to the Redfield Ratio (106/16/1) (Redfield, 1958) which indicates a perturbation of marine nutrient cycling by pollutants from anthropogenic sources.

The results for total petroleum hydrocarbon (TPH), contents of the MSS, were found to be in the similar ranges found in the studies of (Ahmed., 2006; Guerra-Garcia, 2003) which were performed for extremely polluted marine environments.

As anionic content, the NO_3^- and SO_4^{2-} levels were measured. The results showed that the concentrations in the MSS pore waters were considerably higher than typical seawater (10-50 μM), while the SO_4^{2-} levels were lower than the typical levels (2600-3200 mg/l) (Millero, 1996). Moreover, NO_3^- was more abundant compared to SO_4^{2-} in the TUZ and MOD sediments. This is an unusual case for marine sediments.

For physical characteristics, salinity, sediment grain size, pH and temperature were assessed. Also the activity analysis, targeting RNA and all the genes responsible for petroleum hydrocarbon degradation and sulfate reducing, nitrate reducing conditions were used for correlation analysis.

Table 7.1: Parameters for correlation analysis

ELEMENTAL COMPOSITION	C/N/P
HEAVY METALS	Ni>Zn>Cu>Pb>Cr>Mn>Fe
TOTAL CELL COUNT & ACTIVITY	DAPI and Q_PCR & RNA Level
ANIONIC CONTENT	SO ₄ ²⁻ , NO ₃ ⁻
PHYSICAL CHARACTERISTICS	Ph, Salinity, Temperature Sediment grain size
PETROLEUM HYDROCARBON	Aliphatics, Aromatics, Resene, Asphaltene
GENES / TRANSCRIPTS	Sulfate& nitrate reducing hydrocarbon degradation

7.2 Q_PCR and Correlation Analysis Results

To assess the organisms responsible for anoxic N cycle processes, it is more advantageous to target functional genes (*nrfA*, *nosZ*, *hzoA*) that code for enzymes involved in metabolic activity than targeting the rRNA gene in which function is implied by virtue of belonging to a group where members are known to have the ability to perform a function (Dhillon, 2003; Parkes, 2005; Webster, 2003). For example, denitrification is mediated by a diverse polyphyletic group of Bacteria (Zumft, 1997) and Archaea. rRNA-based approaches are of limited value for understanding the abundance of these communities (Smith, 2007). Therefore, in this study, functional genes were targeted to microbiologically characterize the Marmara Sea Sediments.

Correlation analysis results indicate that, there is a strong relation between *nrfA*, *hzoA*, *nosZ* and NO₃⁻ concentration. (Figure 7.1) Abundance of these genes did not correlate with other chemical and microbiological sediment characteristics. These correlation results imply that the main factor to govern the abundance of nitrate reducing community is the NO₃⁻ level.

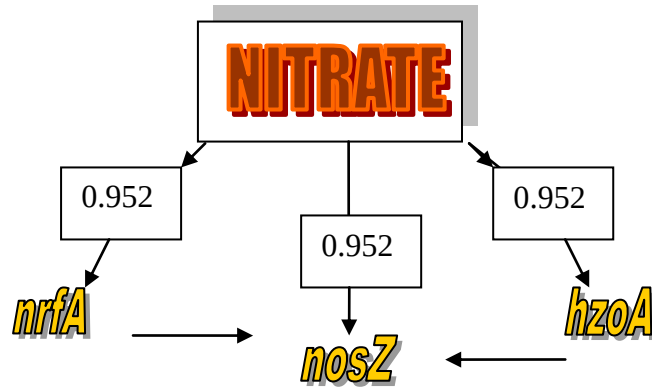


Figure 7.1 : Results of correlation analysis for target genes abundance

Relative abundance of the target genes in MSS was shown in Figure 7.2. This is calculated by, dividing gene abundance to number of total organisms. NrfA and nosZ were in the ranges of 5-30% and 5-15%, respectively, while hzoA was in the range of 2-5%. Thus, the dominance of the processes can ranked as DNRA, RD and ANAMMOX.

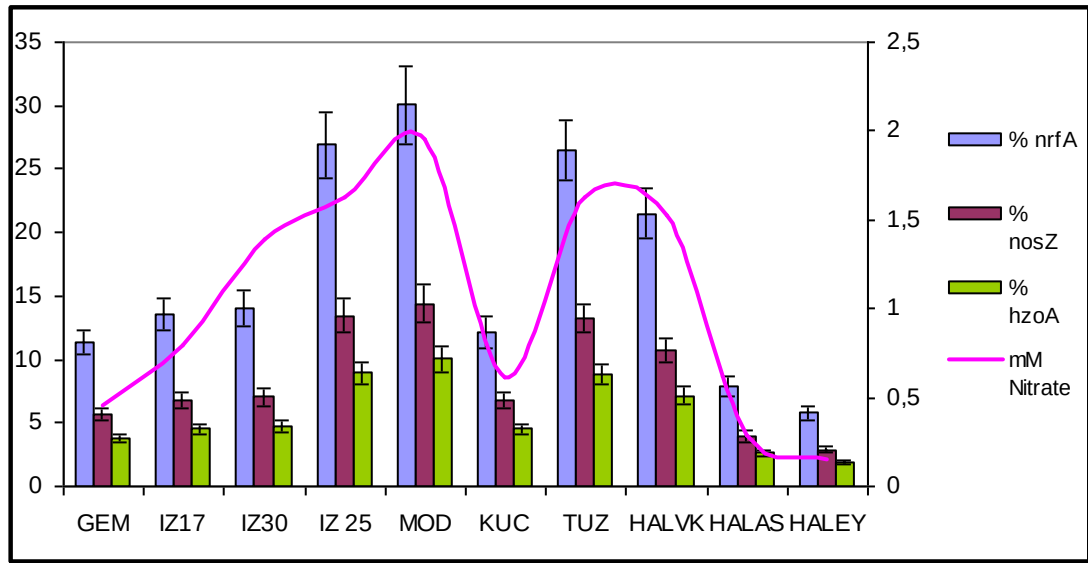


Figure 7.2 : Percentage of the target genes and nitrate concentration in MSS

From the correlation analysis results, the total cell abundance were strongly related to the N/P ratios and the N-P levels of the sediments while total cell activity (TUBITAK Project 105Y307) strongly related to the N and P levels of pore water in Marmara Sea.

Table 7.2 : Results of correlation analysis for total cell abundance

	<u>N Level in MSS</u>	<u>P Level in MSS</u>
TOTAL CELL ABUNDANCE	0,906	0,924

Total cell counts of the TUZ, MOD, HAL and KUC sediments were higher than the previously reported total cell count ranges ($10^8 - 10^{10}$ cells/cm³) for marine sediments (Schippers and Neretin, 2006; Smith and D'Hondt, 2006). Although the cell contents of the GEM and IZ sediments were considerably lower than the other sediments, their total cell counts were in the upper limit of the previously reported total cell counts (Figure 7.3).

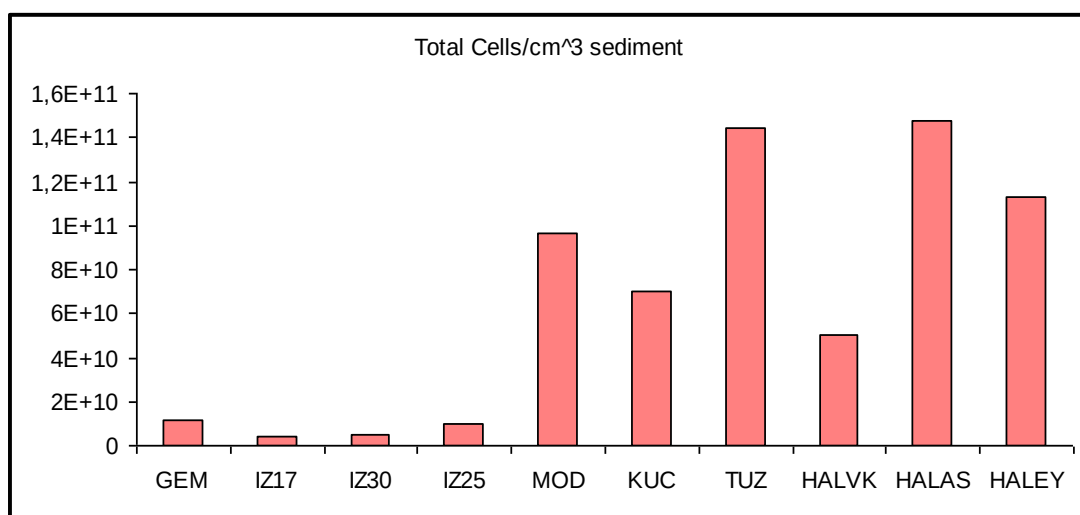


Figure 7.3 : Number of total cells per cm³- MSS

7.2.1 Q_PCR Results for Izmit Regions

In the Iz25, Iz20, Iz30 regions, the results indicate that *nrfA* was found to be dominant in every sampling period. The percentages of the genes in the IZ30 and IZ17 regions were nearly in the same range and lower than the Iz25 region.

This is because of the nitrate concentration in the IZ25 region which was in the range of 1,3 to 1,9 mM, while the Iz30 and Iz17 were in the ranges of 1.2 to 1.7 mM and 0,6 to 0,9mM, respectively. Also the number of total cells in the Iz25 was found to be higher than that of the Iz17 and Iz30. This is also correlated with the N and P concentrations in the Iz25sediments.

As can be seen from Figures 7.4 and Y, the target genes which belonged to the Iz30, Iz17 and Iz25 regions weren't influenced by the seasonality in the sediments physical and chemical characteristics.

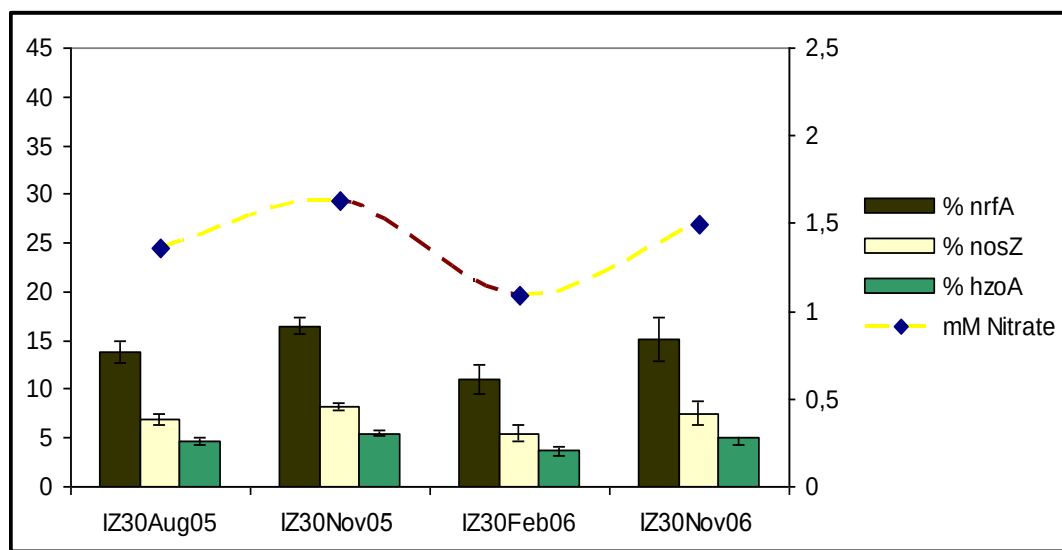


Figure 7.4 : The percentage of the target genes and nitrate concentrations in IZ30 Sediments

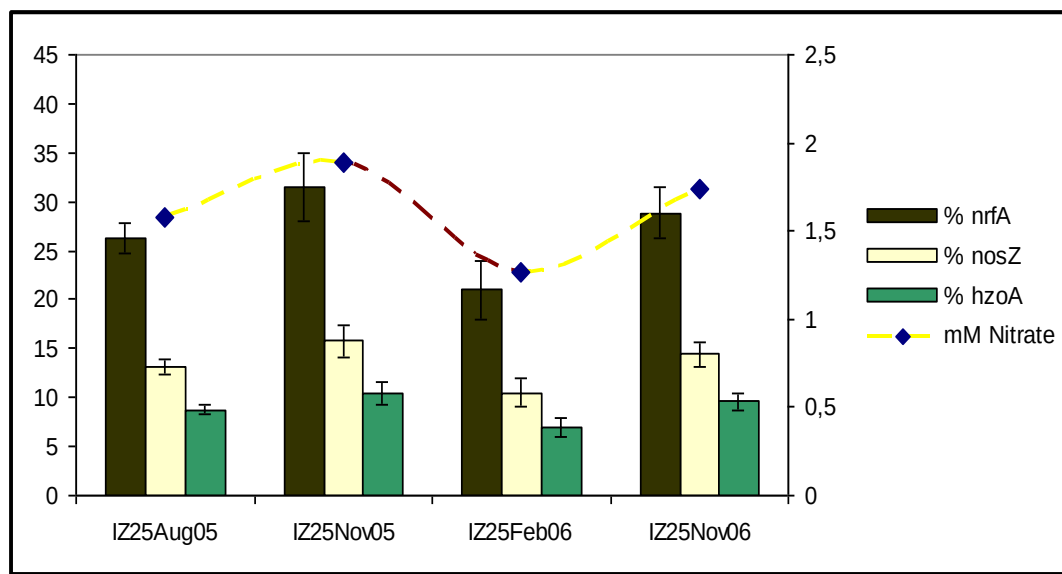


Figure 7.5 : The percentage of the target genes and nitrate concentrations in Iz25 Sediments

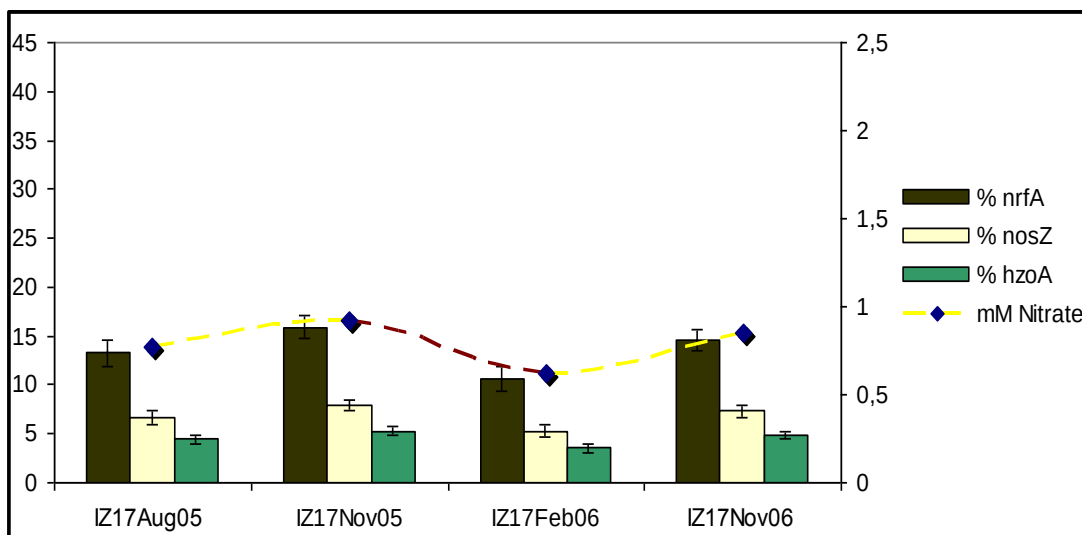


Figure 7.6 : Percentage of the target genes and nitrate concentrations in IZ17 Sediments

7.2.2 Q_PCR Results for Halic Regions

Considering all regions, *nrfA* was found to be the most dominant gene. Each target gene was abundant in the HALVK region, *nrfA* was in the range of 20- 25%, while *nosZ* and *hzoA* were in the ranges of 2,5-3% and 1,5-2%, respectively. In the HALAS region, the *nrfA* was in the range of 7-9% and in the HALEY region 5-7%.

This is because; the nitrate concentration in the HALVK region was the highest. In Aug and Feb sampling periods, there was a distinct increase in both N concentration and gene abundance.

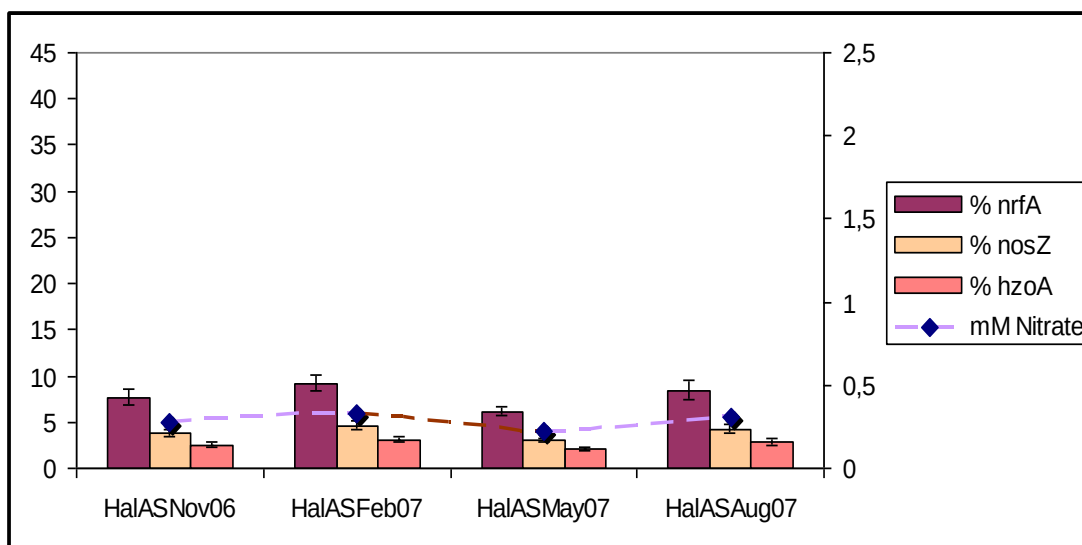


Figure 7.7 : The percentage of the target genes and nitrate concentrations in every sampling time for HalAS sediments

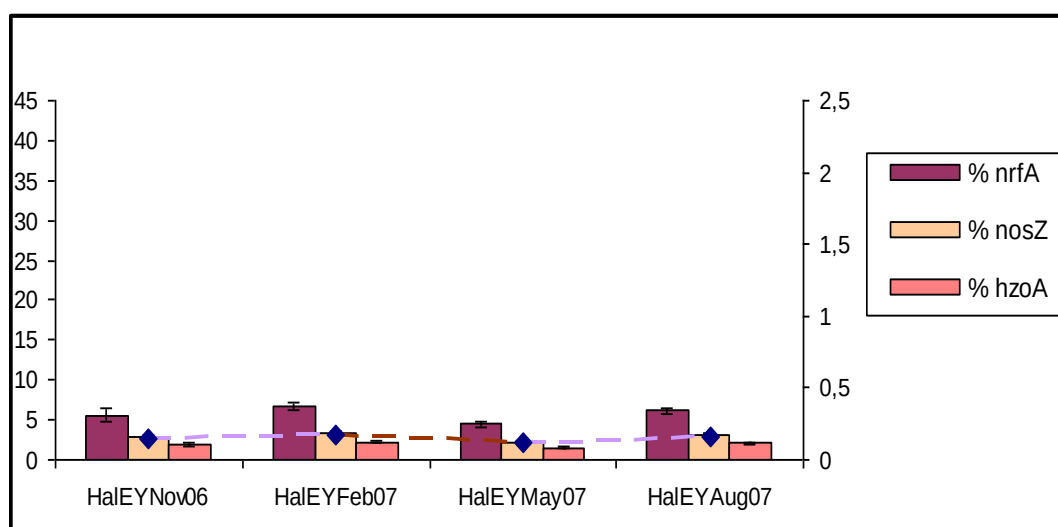


Figure 7.8 : The percentage of the target genes and nitrate concentrations in every sampling time for HalEY sediments

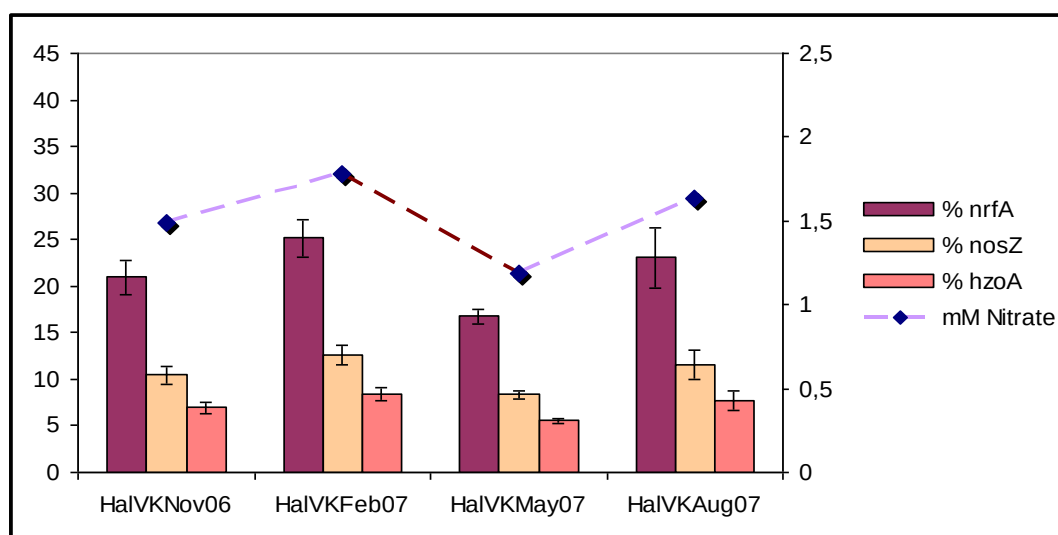


Figure 7.9 : The percentage of the target genes and nitrate concentrations in every sampling time for HalVK sediments

7.2.3 Q_PCR Results for Gemlik Region

In Gemlik location, since the *nrfA* was abundant, the DNRA was the most important process as can be seen in Figure 7.10. The abundance of the *nrfA* was again correlated with N concentration. The sampling periods didn't influence the abundance a lot, like in all sampling times in Nov05 and Nov06 (there was an increase in NO_3^- concentration so that the each gen abundance was higher).

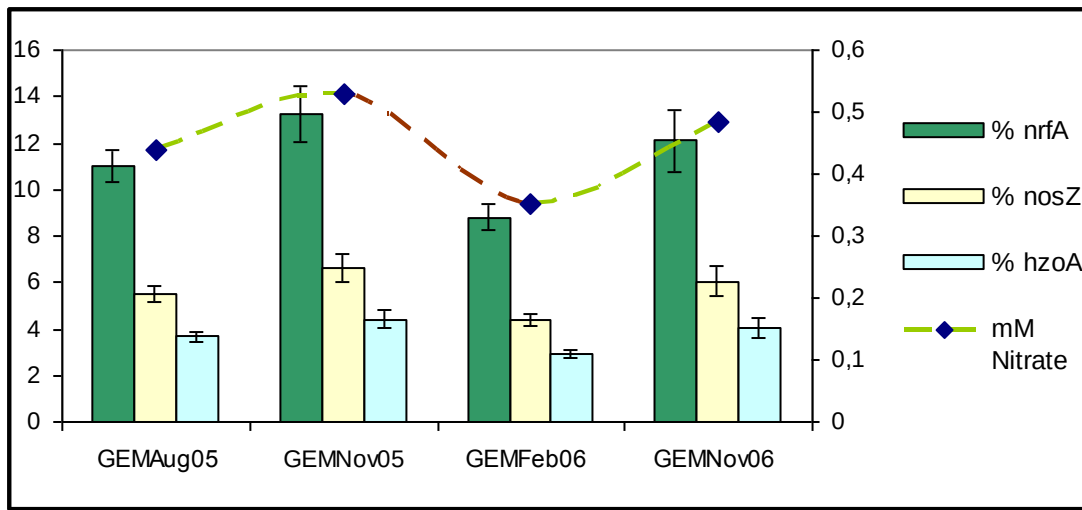


Figure 7.10 : The percentage of the target genes and nitrate concentrations in every sampling time for GEM sediments

7.2.4 Q_PCR Results for Kucuk Cekmece Region

In Kucuk Cekmece location, the *nrfA* was abundant so the DNRA was the most important process as can be seen in Figure 7.11. Since the nitrate concentration was near 0,5 mM, The gene abundance results were in lower ranges than other locations. The sampling periods didn't influence the abundance a lot. In Nov05, Nov06, May06 and Aug07 sampling times, there was an increase in NO_3^- concentration so that the each gen abundance was higher).

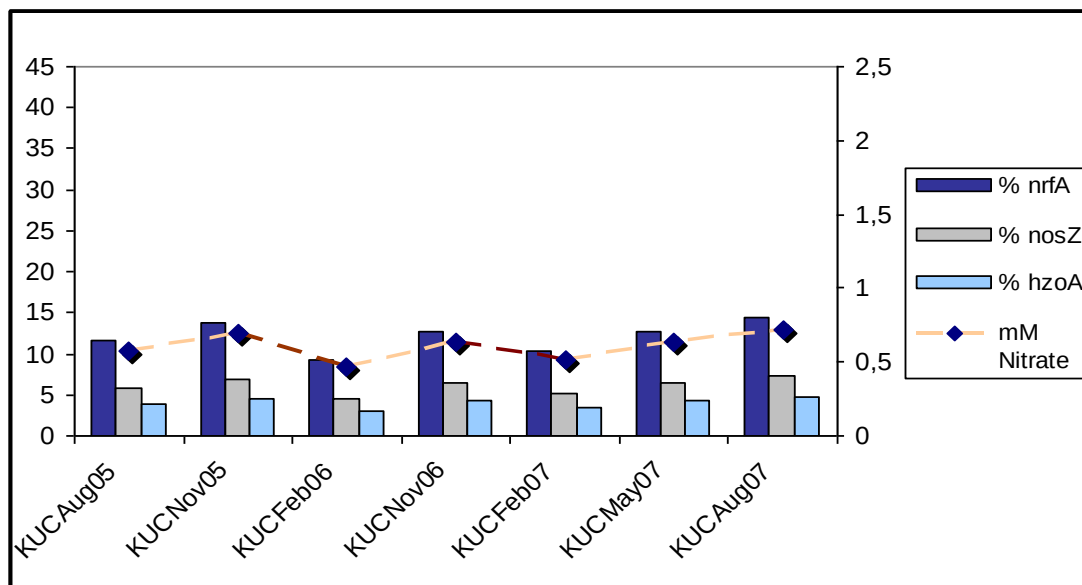


Figure 7.11: The percentage of the target genes and nitrate concentrations in every sampling time for KUC sediments

7.2.5 Q_PCR Results for Moda Region

The nitrate concentrations, nearly similar with Tuzla, were most abundant in Moda region among the all sampling locations. The results indicated that, Moda is important for the abundance of nitrate reducing community.

Like all sampling locations, *nrfA* gene was dominant and found in the highest ranges which were 25-30%, the abundance of *nosZ* gene was also higher, 12-15% and the *hzoA* was found in the ranges of 5-8%.

In Feb07 sampling time, the decrease in nitrate concentration positively affects the target gene abundance. However the following sampling times which are May07 and Aug07, nitrate concentration was nearly 2-2,5 mM in the sediments so this affected *nrfA*, *nosZ* and *hzoA* abundance positively.

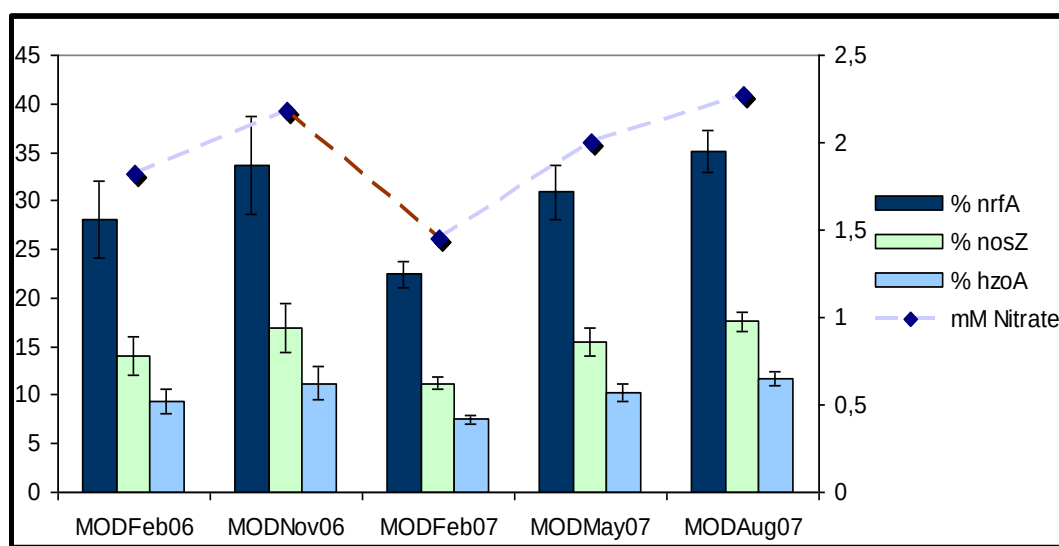


Figure 7.12 : Percentage of the target genes and nitrate concentrations in every sampling time for MOD sediments

7.2.6 Q_PCR Results for Tuzla Region:

The nitrate concentrations, 1,5- 2mM were most abundant in Tuzla region among the all sampling locations, which affected the gene abundance. Therefore, the abundance of the nitrate reducing was important in this region. The strong relation between the nitrate concentration and genes abundance was obvious as can be seen from Figure 7.13, the *nrfA* gen was in the range of 25-30% .the *nosZ* and the *hzoA* were 12-15%, 5-8%, respectively.

The *NrfA* gen was most abundant in Nov05, Nov06, May07, Aug 07 periods, because of the increase in the N concentration.

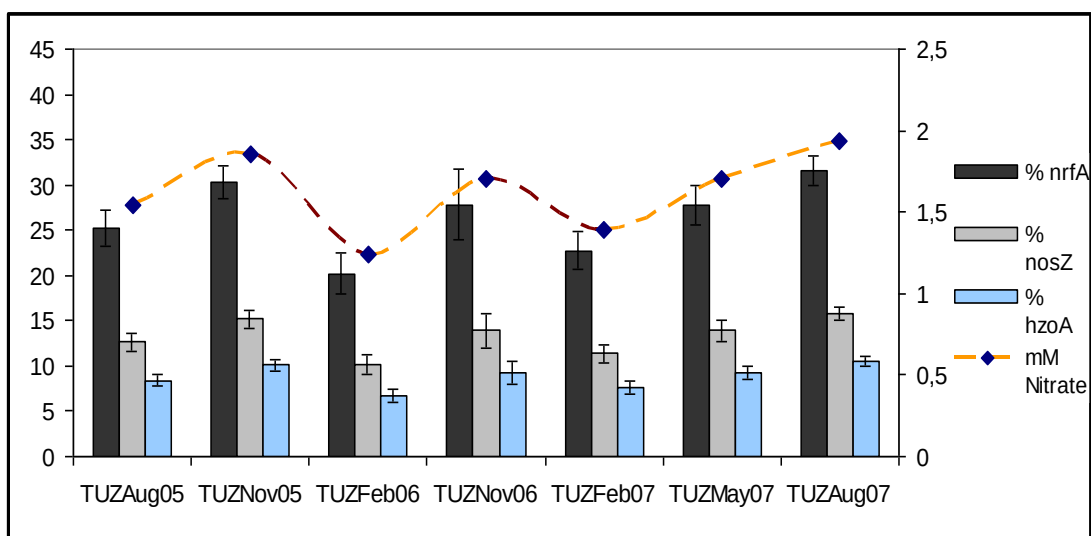


Figure 7.13 : The percentage of the target genes and nitrate concentrations in every sampling time for TUZ sediments

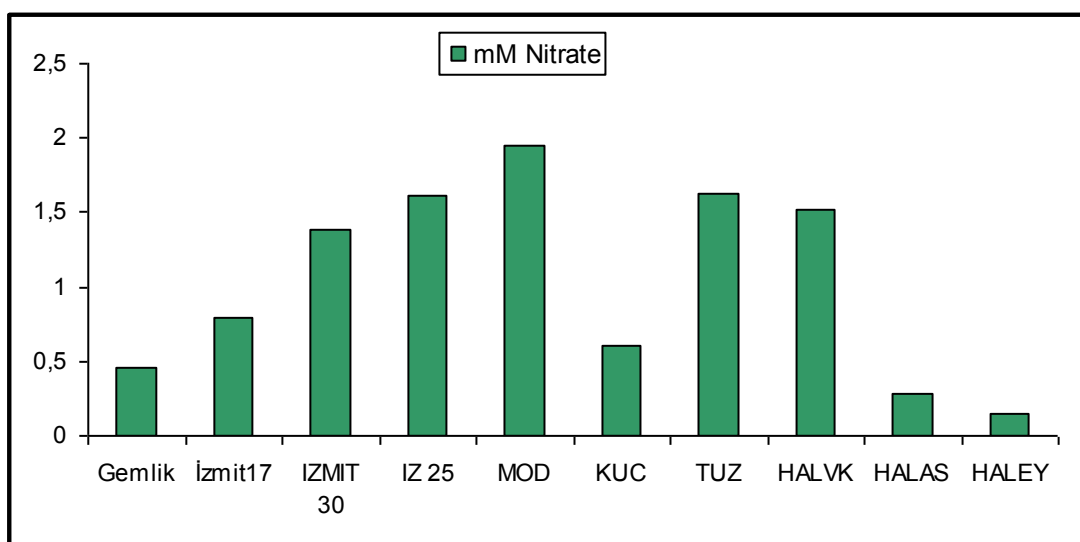


Figure 7.14 : Nitrate concentrations in all sampling locations of Marmara Sea

As can be seen Figure 7.14, by considering the results of all sampling regions, the abundances of the target genes were strongly related to the NO_3^- concentration. Since the *nrfA* was the dominant, the DNRA process was most important among all regions.

7.3 Relation between Microbial Abundance and Chemical Characteristics

Sulfate Reducing Bacteria and Methanogens are dominant in the sea sediments because sulfate is abundant in coastal and estuarine seawater, whereas nitrate concentrations are typically low (Jorgensen, 1982). However, in our case, in the MSS, the NO_3^- concentrations were found to be considerably higher than that of a typical seawater which is 10-50 μM , on the other hand the SO_4^{2-} levels in the MSS were found to be lower than the typical levels of SO_4^{2-} concentration (2600-3200 mg/l) (Millero, 1996). This is why the organisms responsible for anoxic N cycle processes became abundant in the MSS.

Correlation analysis results indicate that, *nrfA*, *hzoA*, *nosZ* and NO_3^- concentrations were positively correlated. Abundance of these genes did not correlate with other chemical and microbiological sediment characteristics. These correlation results imply that the main factor to govern the abundance of nitrate reducing community is the NO_3^- level. These conclusions are in agreement with chemical measurements (Kolukirik 2009) which showed that electron donors were not limited, instead, electron acceptors were limited in the MSS. In marine sediments, e^- -acceptors enter the sediment from the overlying water column. As the e^- -acceptors are reduced; their reduced products enter successively deeper redox zones. It is highly possible that heterotrophic microbial populations depleted electron acceptors quickly within a very short distance (15 cm) from the sediment surface which resulted in the succession of all the redox zones (Kolukirik,2009). This indicates that the scarcity of the electron acceptors determined abundance of the organisms responsible for the relevant terminal electron accepting processes.

The DNRA is the most important process in all regions. Although the conditions promoting fermentative DNRA and RD are similar (available nitrate, and organic substrates), fermentative DNRA is thought to be favored in nitrate-limited environments rich in labile carbon (carbon forms that are more easily broken down). This is because, the high labile carbon availability would favor organisms that used electron acceptors most efficiently; as seen in the figure 7.15 , DNRA transfers eight electrons per mole of nitrate reduced, whereas RD transfers five and ANAMMOX only transfers two. In MSS, high-carbon, low nitrate systems, the DNRA is more important.

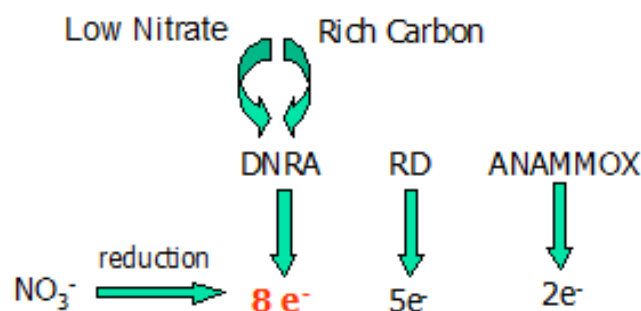


Figure 7.15 : Abundance of DNRA, RD and ANAMMOX Processes in low nitrate, rich carbon environments

The reason why organisms take role in anaerobic ammonia oxidation process in low ranges was that their growth rate was pretty slow compared to other nitrate reducing organisms. Their nutritional class belong to chemoautotrophs (Figure 7.16) which obtain energy by the dissimilatory oxidation of inorganic compounds and use it for the fixation of carbon dioxide as the carbon source.

The organisms containing *nrfA* gene plays a critical role for organic C degradation in the MSS since carbon content of the sediments were high (Kolukirik, 2009) and DNRA bacteria gain more energy from oxidation of organic compound compared to other nitrate reducing microbes.

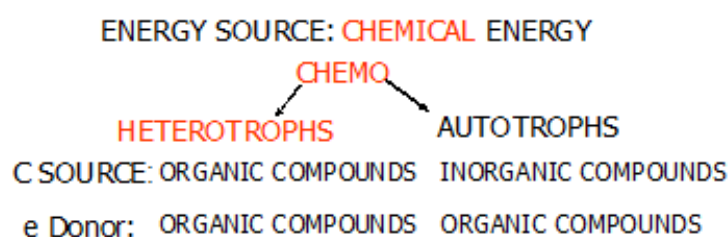


Figure 7.16: Nutritional classification of organisms

7.4 Bioremediation strategy

Bioremediation is a promising technology aiming to stimulate removal of pollutants. This strategy based on nature gets a great interest for cost effective treatment of the chronic hydrocarbon pollution in the MSS. As the Q_PCR technique enabled us to monitor abundance of anoxic N cycle processes, anoxic microbial processes could be an alternative for the chronic petroleum hydrocarbon degradation in the MSS.

Anoxic/anaerobic biodegradation processes are a significant component of natural attenuation owing to the abundance of anoxic electron acceptors relative to dissolved oxygen (Safinowski, 2006; Zwolinski, 2000). Furthermore, clean-up systems based on anaerobic biodegradation require less human intervention. Although anaerobic hydrocarbon degradation is likely to proceed at lower rates than aerobic hydrocarbon degradation, in situations where rapid clean-up is not required, or where aerobic treatments are not feasible (e.g. following long-term chronic hydrocarbon pollution in inaccessible anoxic marine sediments, such as occur in the Marmara Sea) it is likely to be of considerable environmental significance (Head and Swannell, 1999). Therefore, abundance of key organisms that play roles in biodegradation of petroleum hydrocarbons under anoxic nitrate reducing conditions is important for understanding, evaluating and developing in situ bioremediation strategies for marine environments.

From the results of this study, anoxic microbial processes could be an alternative for the chronic petroleum hydrocarbon degradation in the MSS.

In Tuzla, Moda sediments, the nitrate concentrations, thus the number target genes were abundant (Figure 7.17) and also the total cell concentrations were higher than other regions. (Figure 7.3)

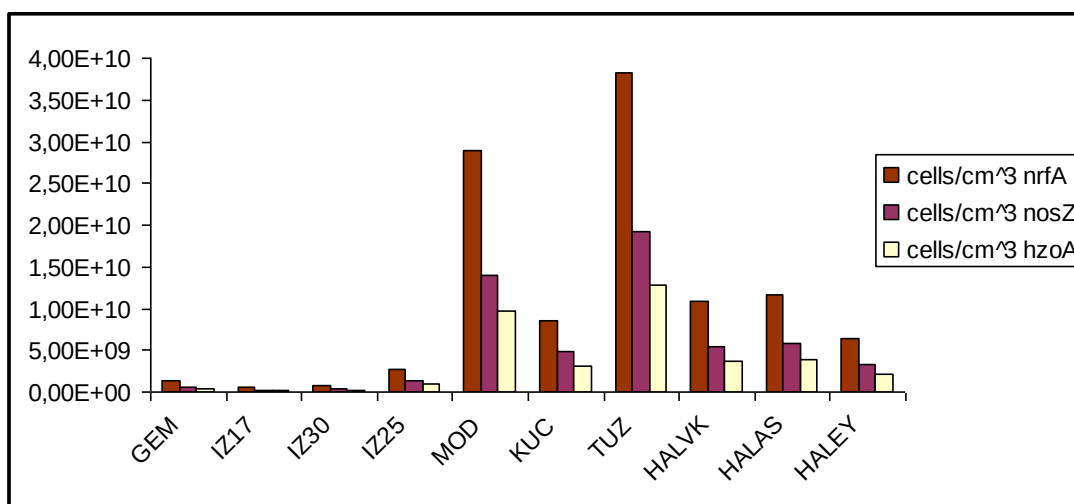


Figure 7.17 : Number of cells responsible for target processes per cm³ sediment in MSS

Therefore Tuzla or Moda sediments could be the first targets to assess petroleum hydrocarbon degradation coupled to the RD and DNRA in laboratory scale trials.

After this study was finished, microcosms seeded with the Tuzla sediments and fed with hydrocarbons were set up to investigate nutrient amendment on hydrocarbon degradation potential of the MSS. NO₃⁻ served both nitrogen and electron acceptors source which were both limiting in the pore waters of Marmara Sea. The results revealed that this potential can be increased $\approx 10\times$ which may imply that it is possible to remediate the Marmara Sea by stimulating the nitrate reducing communities in the sediments.

8. CONCLUSION AND RECOMMENDATIONS

Sediments which were taken from 10 different locations of the Marmara Sea contained abnormally high concentrations of nitrate (0.1-2 mM) and extremely polluted with hydrocarbons (2-20 g/kg) (Kolukirik, 2009). Therefore the Marmara Sea was a perfect candidate for investigating Anoxic Nitrogen Cycle processes. In the first 15 cm of the MSS were very rich in terms of hydrocarbon, nitrate, Ni and microbial cell content whereas N and P were limited in the pore waters (Kolukirik, 2009).

The genes targeting anoxic N cycle processes were useful tools for determining abundance of the organisms responsible for anoxic pathways. It was found that anoxic microbial abundance was dominated by organisms responsible for DNRA.

In the MSS, local and seasonal differences in the microbial abundance were strongly related to the changes in the chemical characteristics. Since there was chronic hydrocarbon pollution in the Marmara Sea, electron donors were not limited. Therefore microbial community composition was related to the scarcity of electron acceptors.

The results of the correlation analysis indicated that there was a strong correlation between nitrate concentrations and target genes responsible for anoxic N cycle processes. Since nitrate concentration was abundant in Tuzla and Moda sediments, target gene abundances were found in highest ranges.

Since *nrfA* gene was abundant in all sampling locations, DNRA process was the most important anoxic N cycle process in the MSS. The reason of this was high labile C and low NO_3^- environment which favoured the organisms responsible for DNRA.

Dissimilatory nitrate reducing organisms were using organic compounds as their energy source, thus they had an important role for the degradation of chronic hydrocarbon degradation.

The studies carried out for the TUBITAK Project 105Y307 showed that bioremediation of petroleum hydrocarbons could be possible under nitrate reducing conditions by NO_3^- and P addition which are limiting for biologic activity.

Since Tuzla and Moda sediments were abundant in terms of nitrate and total cell, petroleum hydrocarbon degradation coupled with denitrification and dissimilative nitrate reduction to ammonia could be stimulated by nutrient amendment.

After this study was finished, microcosms seeded with the Tuzla sediments and fed with hydrocarbons were set up to investigate nutrient amendment on hydrocarbon degradation potential of the MSS. NO_3^- served both nitrogen and electron acceptors source which were both limiting in the pore waters of Marmara Sea. The results revealed that this potential can be increased $\approx 10\times$ which may imply that it is possible to remediate the Marmara Sea by stimulating the nitrate reducing communities in the sediments.

It was concluded that the MSS provides an unique environment for both in-situ and ex-situ enrichment of hydrocarbon degrading microbial consortiums under nitrate reducing conditions which may in turn be used to overcome the chronic hydrocarbon pollution in the Marmara Sea.

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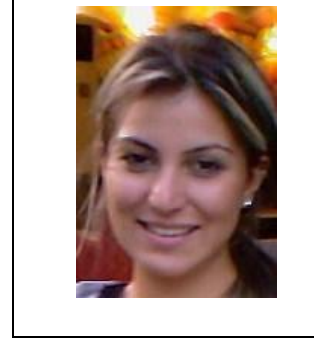
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