ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

ANALYSIS OF METHANOGENIC ARCHAEL POPULATIONS IN ANOXIC SEDIMENTS OF THE MARMARA SEA USING FLUORESCENT IN SITU HYBRIDIZATION

M.Sc. Thesis by

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Department : Environmental Engineering

Programme: Environmental Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MARMARA DENİZİNİN ANOKSİK SEDİMENTLERİNDEKİ METANOJENİK ARKEAL POPULASYONUN FLORESANLI YERİNDE HİBRİTLEŞMEYLE ANALİZİ

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ÖZET

Bu çalışmada, Marmara Denizi'ndeki farklı noktalardan alınan (Gemlik Körfezi İzmit Körfezi ve Küçükçekmece) anoksik sedimentlerde metanojenik arkeal populasyonlar kültürden bağımsız moleküler metot, floresanli yerinde hibritleşme (FISH), kullanılarak sayıldı ve tanimlandi. Ayrıca sediment örneklerinin organik ve inorganik içerikleri araştırıldı ve sonuçlar sediment örneklerinin arkeal topluluk yapısıyla tarıtışıldı.

Deniz sedimentlerindeki mikroorganizmaların geçirgen hale getirilmesi zor olmasına rağmen, Gemlik Körfezi, Izmit Körfezi (Iz17), Izmit Körfezi (Iz25) ve Küçükçekmece'den alınan sedimentlerdeki DAPI ile boyanmış toplam mikroorganizmaların sırasıyla 67%±8%, 62%±5%, 77%±11% ve 80%±7%'i universal prob kullanılarak tespit edildi. FISH sonuçlarına göre Gemlik Körfezi, Izmit Körfezi (Iz17), Izmit Körfezi (Iz25) ve Küçükçekmece'den alınan sedimentlerdeki bakteriyal hücrelerin göreceli bolluğu sırasıyla 51%±9%, 73%±11%, 64%±8% ve 70%±12%'dir. Yine FISH sonuçlarına göre Gemlik Körfezi, Izmit Körfezi (Iz17), Izmit Körfezi (Iz25) ve Küçükçekmece'den alınan sedimentlerdeki arkeal hücrelerin göreceli bolluğu sırasıyla 32%±4%, 23%±3%, 33%±2% ve 29%±7%'dir.

Gemlik Körfezi, Izmit Körfezi (Iz17), Izmit Körfezi (Iz25) ve Küçükçekmece'den alınan anoksik sedimentlerdeki toplam metanojenlerin 19%, 21%, 23% ve 20%' si H_2 kullanan metanojenlerdir buna karsi bunlarin 7%, 9%, 10% ve 11%' i asetoklastik metanojenlerdir. Bu sonuçlar Marmara Denizi'ndeki anoksik sedimentlerde aktif bir arkeal populasyonun olduğunu ve baskın olarak H_2+CO2'ye bağımlı metanojenesisin önemli seviyelerde gerçekleştiğini göstermektir.

ANALYSIS OF METHANOGENIC ARCHAEL POPULATIONS IN ANOXIC SEDIMENTS OF THE MARMARA SEA USING FLUORESCENT IN SITU HYBRIDIZATION

ABSTRACT

In this study, methanogenic archaeal populations in anoxic sediments from different regions of the Marmara Sea (Gemlik Bay, Izmit Bay and Kucukcekmece) were identified and quantified using a cultivation independent molecular method, Fluorescent in situ hybridization (FISH). Organic and inorganic content of the sediment samples were also investigated and the results were discussed along with the archaeal community structure of the sediment samples.

Although it is difficult to permeabilize microorganisms in the marine sediments, 67%±8%, 62%±5%, 77%±11% and 80%±7% of DAPI stained total microorganisms in sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece respectively were detected using universal probe in this study. According to FISH results, relative abundances of bacterial cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 51%±9%, 73%±11%, 64%±8% and 70%±12% respectively. According to FISH results, relative abundances of archaeal cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 32%±4%, 23%±3%, 33%±2% and 29%±7% respectively.

19%, 20%, 21% and 23% of the total methanogens in anoxic sediments from Gemlik Bay, Kucukcekmece, Izmit Bay (Iz17) and Izmit Bay (Iz25) are H_2 utilizing methanogens whereas 7%, 11%, 9% and 10% of them are acetoclastic methanogens. . These results revealed that there are active archaeal populations within the the anoxic sediments from the Marmara Sea, and methanogenesis occurs in the sediments at important levels , dominantly H_2+CO_2 dependent.

1. INTRODUCTION

Microbially mediated production of methane in anoxic marine systems is a globally significant process since flux of methane from marine sediments to the atmosphere is one of the major environmental concern (Orphan, 2001). In addition, methane producing archaeal and sulfate reducing bacterial species removes most of the organic pollutants from the anoxic marine environments (Madigan et al., 2002). Therefore, identification and quantification of methanogenic species is a very important subject to understand and control biodegradation processes occurring in the anoxic marine environments.

The various processes of microbial carbon mineralization can be quantified by tracer techniques, and their importance for biogeochemical cycles in the marine environment is recognized; however, little is known about the microbial community responsible for them (Amann et al., 1999). Molecular biological approaches for studying microbial diversity have opened new perspectives for microbial ecology. Novel, cultivation-independent methods for studies of marine bacteria and archaea have revealed large numbers of unknown microorganisms, which appear to be largely unaffiliated with previous isolates from the same environment. Most of the research in marine molecular ecology has been directed toward microbial populations of the water column, marine sediments, arctic ice, and salt marsh sediments (Madrid et al., 2001). However, comprehensive descriptions of microbial communities in anoxic sediments have been studied poorly.

The Marmara Sea is a small intercontinental basin connecting the Black Sea and the Aegen Sea with the size of 70 x 250 km. The Marmara Sea is a critically polluted waterbody, subject to a multitude of wastewater discharges from major land-based sources located along the coastline, including the Istanbul metropolitan area and dispersion of petroleum hydrocarbons originated from various sources. Accumulation of

organic matters and petroleum hydrocarbons cause depletion of oxygen in the deep water. Therefore, some locations in the Marmara Sea have became anoxic.

Anaerobic processes can be a significant factor in removal of organic contaminants owing to the abundance of anaerobic electron acceptors relative to dissolved oxygen (Zwolinski et al., 2000) and are responsible for the removal of chronic hydrocarbon pollution in anoxic sediments. However, microbial diversity that are carrying out anaerobic processes in the anoxic sediments of the Marmara Sea is virtually unknown and have not studied until now. If the microbial diversity of the anoxic sediments can be defined, bioremediation strategies to overcome pollution problem can be made.

Thus, in this study, methanogenic archaeal populations in anoxic sediments from the Marmara Sea were identified and quantified using a cultivation independent molecular method, Fluorescent in situ hybridization (FISH). Organic and inorganic contents of the sediment samples were also investigated and the results were discussed along with the archaeal community structure of the sediment samples.

2. THE AIM OF THE STUDY

The Marmara Sea is a critically polluted waterbody, subject to a multitude of pollutant discharges from various sources. Accumulation of organic matters have been causing depletion of oxygen in the deep water, and some locations in the Marmara Sea have became anoxic. Methane producing archaeal and sulfate reducing bacterial species remove most of the organic pollutants from the anoxic marine environments (Madigan et al., 2002). Therefore, identification and quantification of methanogenic species is a very important subject to understand and control biodegradation processes occurring in the anoxic marine environments.

The aim of this study is to identify and quantify methanogenic archaeal populations in anoxic sediments from the Marmara Sea using a cultivation independent molecular method, Fluorescent in situ hybridization (FISH). Organic and inorganic content of the sediment samples were also investigated and the results were discussed along with the archaeal community structure of the sediment samples.

3. THE MARMARA SEA

3. 1. General Characteristics of The Marmara Sea

The Marmara Sea is a small intercontinental basin connecting the Black Sea and the Aegen Sea with the size of size ≈ 70 x 250 km. The deepest water of the sea is in an underwater through that extends 1300 meters below the surface. The basin has risk for earthquakes because it is a part of the North Anatolian fault. The coast of Istanbul is relatively straight with few natural harbors.

Black Sea and the Aegean Sea connected via the Bosphorus Strait and the Dardanelles affect the oceanographic features of the basin. The Marmara Sea is a critically polluted waterbody, subject to a multitude of wastewater discharges from major land-based sources located along the coastline, including the Istanbul metropolitan area. A great amount of wastewater is discharged to the Marmara Sea from different entering points. 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 enter to the basin from the Black Sea inflow. The major portion (40–65%) of the total anthropogenic discharges originates pollution loading from Istanbul, the biggest city of Turkey in population and industry, (Tuğrul and Polat, 1995). The impact of Istanbul's urban and industrial effluent is also apparent in the TOC. TOC content of sediment varied from 2.1 mg/g to 22 mg/g. Highest average TOC content value (12.5 mg/g) was detected at Büyükçekmece transect (near Istanbul) by Albayrak et al. (2006). The water quality measurements also indicate severe signs of present and future eutrophication problems (Orhon, 1995) due to the inputs from the Black Sea.

Anthropogenic activities in the coastal area of the north Marmara Sea including urban effluent, agricultural run off, summer resorts (untreated effluent discharged into the sea), sunflower oil factories, a big cement factory, fishing and shipping influence the

pollution of the Marmara Sea (Öztürk et al., 2000). Beside these, there is a tanker traffic on the straits and several thousand ton oil are carried by tankers. In the past, a lot of tanker accidents occurred in the Bosphorus Strait and in the Marmara Sea. Some examples of them are Volgoneft (1999), TPAO explosion (1997). The Bosphorus Strait is in a constant threat to the marine ecosystem in terms of tanker accidents and therefore oil dispersion. Another negative impact of shipping on the coastal benthic ecosystem seems to be threat the recent appearance of exotic species such as the manila clam Tapes philippinarum (Albayrak, 2005).

Currents and fish migrations make Istanbul a fishing center and therefore; the sea products are very rich in the area. Fishing is now limited to the Black Sea for different varieties found there and lobsters and shellfish off the coast of the Marmara. Both sources of fish seem to be dwindling due to the pollution of the water and over fishing through the years.

The benthic environment is a fundamental compartment of any aquatic ecosystem. Bottom sediments accumulate great amounts of organic matter affecting the oxygen content of the bottom water and they are the final sink for many anthropogenic contaminants (Venturini et al., 2004). There is a positive correlation between organic carbon and the contaminant level in coastal marine sediments. Content of organic carbon (TOC) in the sediment can be an indicator of pollution (Shine and Wallace, 2000; Hyland et al., 2005). There is a need for versatility in the use of indicators of biological change, in order to compensate for the effects of local variability in natural and anthropogenic sources of disturbance (Rees et al., 2005).

There are identified species typical for the Marmara Sea area. That is the community composition having elements from the rich Aegean Sea and the poor Black Sea faunas. The diversity and number of species reflects not only the type of substratum, sandy (11–23 species per 0.1 m^2) to muddy (4–24 species per 0.1 m^2) but also the impact of anthropogenic stress the sites receive (Albayrak et. al, 2006).

3. 2. Hydrography of the Marmara Sea

The Black Sea is connected with the Mediterranean Sea by the so-called Turkish Strait System: the Bosphorus Strait (length: ~30 km; width: ~0.7–3.5 km); the small basin of the Sea of Marmara (size: ~70 km x 250 km) and the Dardanelles (length: ~70 km; width: ~1.3–7.0 km), see in the Figure 3. 1.

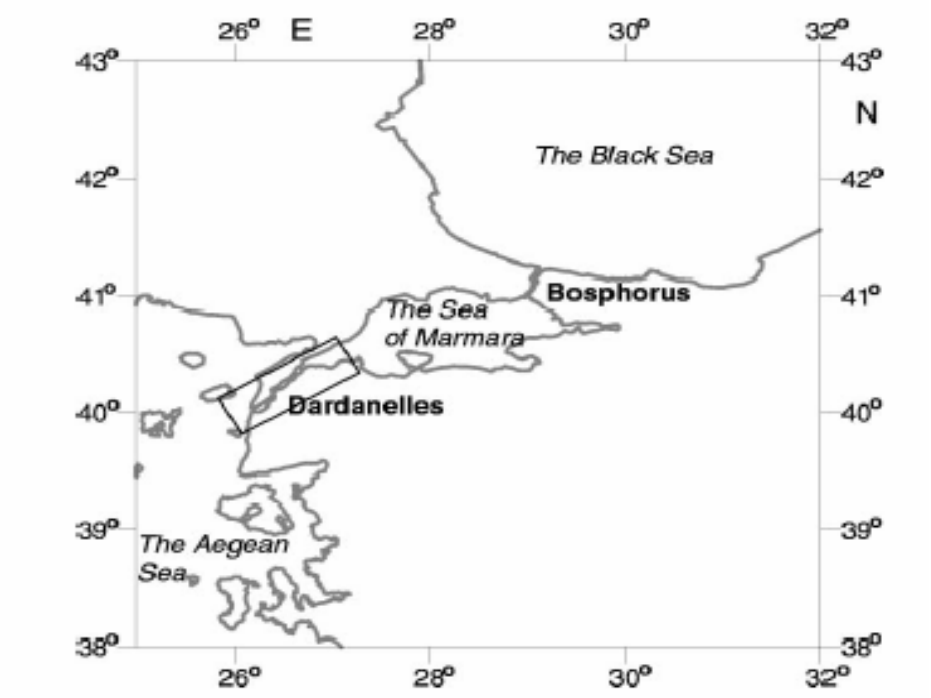

Figure 3. 1 The location map for the components of the Turkish Strait System

Flows through the Turkish Straits are driven by the density differences between the Black and the Aegean Seas and the maintained net level difference between these seas (Stashchuka, N., Hutter, K., 2001). The Black Sea waters flow to the Marmara Sea through the Bosphorus upper layer flow and exit the Marmara Sea from the Dardanelles Strait. Similarly, the Aegan water enter the Marmara Sea through the Dardanelles Strait lower layer flow, exit the Sea from the Bosphorus underflow and flows to the Black Sea (Besiktepe *et al*., 1994).

Fundamentally, the Black Sea water that has the salinity of ~17.8 ppt, flowing through the Bosphorus as a surface layer, enters the Sea of Marmara with ~19.4 ppt salinity. While it is crossing the Sea of Marmara, its salinity increases additionally by nearly 6 ppt. After increasing by yet another 4 ppt, it exits from the Dardanelles with a salinity of 29.6 ppt. On the other hand, the Aegean water has the salinity of 38.9 ppt when entering the Dardanelles. While flowing throug the strait, its salt content changes little. Based on the observations there is a nearly 2 ppt reduction in the salinity of this water within the Marmara basin. After a further dilution by another 2 ppt; while being transported through the Bosphorus, the Mediterranean waters enter the Black Sea with a salinity of nearly 35 ppt (Figure 3. 2) (Stashchuka, N., Hutter, K., 2001).

The Marmara Sea consists of two layers of water with either Black Sea or Mediterranean Sea origin, separated by a sharp interface. The upper layer volume is 230 km^3 and has an average renewal time of 4-5 months. The lower layer has a volume of 3378 km^3 and an average renewal time of 6-7 years (Besiktepe *et al*., 2000).

The strength of the surface jet entering from the Bosphorus (a result of the differences of density and barometric pressure and sea level in the adjacent seas) and the local wind stress distribution controls seasonally the circulation of the Marmara Sea. The surface circulation is mainly composed of a clockwise circulation. The dense water that enters from the Dardanelles Strait submerges to the depths of the Marmara Sea, reaching the bottom in winter and possibly transiting through shallower depths in other seasons, as a function of the initial density difference (Besiktepe *et al*., 2000).

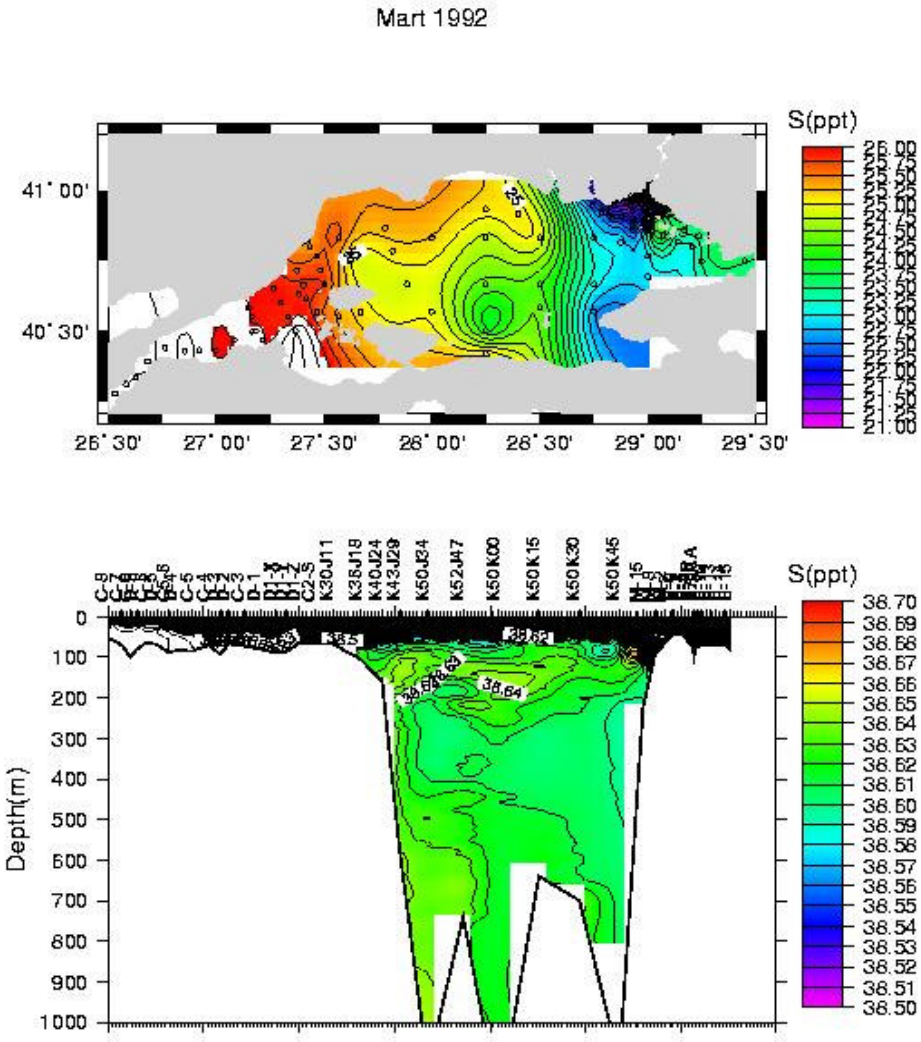

Figure 3. 2 In March 1992 (a) salinity distribution of the Marmara Sea at 5m depth and
(b) through main west-east axis

Mediterranean water, entering from the Dardanelles, supply the suhalocline layer. The negatively buoyant plume of well-oxygenated water is the only means of renewal of the deep waters, partially compensating for the oxygen consumed by the degradation of organic matter sinking from the upper layer into the lower layer. As a result of the internal balances of diffusion, advection and consumption, the subhalocline waters remain permanently deficient in oxygen. The depth to which the plume penetrates is a function of the seasonal characteristics of the inflow density (modified in the Strait) and the weak interior stratification. (Besiktepe *et al*., 1994)

The moderate wind climate of the Marmara region is strongly influenced by land topography. The entire region of low lying topography surrounding the Turkish Strait Systems is a passageway for cold wind system from the north, and for cyclones moving from the Aegean into the Black Sea. The topographies of the valleys of the Bosphorus and Izmit Bay locally influence wind direction. The daily average wind speed is 4 ms^{-1}. Strong wind events with typical ppeds of 8-25 ms^{-1} and durations of about 16 hours occur in winter. Moderate northeasterly and southeasterly winds are common during summer. The air temperature is coldest and precipitation highest during January. Warmest temperatures coincide with minimum precipitation during July. The sea surface temperature follows the air temperature with a lag of about one month reaching a minimum in February, and a maximum in August. (Besiktepe *et al*., 1994)

3. 3. Chracteristics of the Locations Studied

3. 3. 1. Izmit

Izmit Bay, located south of Istanbul on the southeast of the Marmara Sea (Figure 3. 3), is the centre of burgeoning industrial development accompanied naturally by a rapid growth of population (Tolun *et al.*, 2001). With its east–west elongated shape, Izmit Bay is located in northeastern Marmara Sea, latitudes 40º41´–40 º 47´ N, longitudes 29 º 21´–29 º 57´ E (Figure 3. 3). It is an important semi-enclosed embayment, and has been strongly affected by growing population and industrialization (Pekey, H., in press)

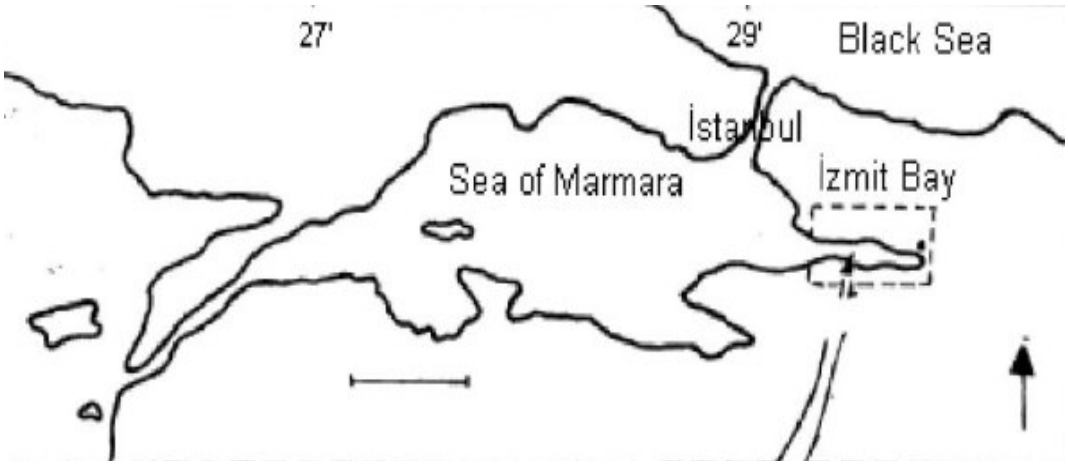

Figure 3. 3 The Sea of Marmara and İzmit Bay

Izmit Bay is an elongated semi-enclosed water body with a length of 50 km, width varying between 2 and 10 km and has an area of 310 km^2. The bathymetry of the Bay constitutes three subbasins separated by shallow sills from each other (Balkıs, N., 2003). It consists of three sections (western, central and eastern), connected to each other by narrow openings (Pekey, H., in press). The eastern basin is relatively shallow (at about 30 m) whereas the central basin has two small depressions with depths of 160 and 200 m. The western basin deepens westward from 150 to 300 m and connects the Bay to the Marmara Sea. Izmit Bay is oceanographically an extension of the Marmara Sea, having a permanent two-layered water system. The upper layer originates from less saline Black Sea waters (18.0–22.0 psu), whereas the lower layer originates from the Mediterranean Sea waters which are more saline (37.5–38.5 psu). A permanent stratification occurs at about 25 m in the Marmara Sea (Besiktepe et al., 1994), however it is highly variable in Izmit Bay. The thickness of the upper layer changes seasonally from 9 to 18 m spring and autumn, respectively. The upper layer enters into the Bay in spring and summer, corresponding to the freshwater inflow changes in the BlackSea, while the lower layer flows to the Marmara Sea from the Bay. However, the upper layer flows towards the Marmara Sea in autumn and winter Vertical mixing of the two layers is restricted and occurs at shallow depths. An intermediate layer develops throughout the year in the water column of the Bay with varying thickness. The upper layer of Izmit

Bay, in general, is saturated with dissolved oxygen (DO). DO concentrations in the lower layer of Izmit Bay have been found to be 2.5–3.0 mg l^{-1} in winter and spring periods and 0.7– 1.5 mg l^{-1} in summer, in previous studies. Minimum DO concentrations have been measured locally in the central basin (0.1–0.2 mg l^{-1}) and in the eastern basin (0.5 mg l^{-1}) during spring–summer period (Balkıs, N., 2003). Table 3.1 and Table 3.2 shows some physical and chemical features of the Izmit Bay.

Table 3. 1. Some physical features of the Izmit Bay (from Basturk et al., 1985)

Basin	Length (km)	Width (km)	Max. depth (m)	Surface area (km^2)	Volume (km^3)
Eastern	16	2–5	35	44	0·850
Central	20	3–10	180	166	12·420
Western	17	3–5·5	200	100	—

Table 3. 2. Some physical and chemical features of Izmit Bay (March, 1999-September, 2000).

	min.	max.	average
Temperature (°C)	3.5	30.0	18.4
pH	7.2	9.5	8.3
Salinity (psu)	13.0	28.0	21.3
NO_2+NO_3-N (µg l^{-1})	2.0 (Dec. 99)	40.9 (Mar. 99)	13.0
PO_4-P (µg l^{-1})	2.0 (Dec. 99, Mar. 00)	38.0 (May 99)	8.2
SiO_2 (µg l^{-1})	7.0 (Dec. 99)	430.0 (Mar. 00)	5.5
Suspended solids (mg l^{-1})	17.8 (Sep. 99)	32.4 (May 99)	22.6

There are no major rivers entering the Bay, with the exceptions of small streams from the east end and northern coasts. The estimated annual freshwater and solid inputs are 15 606 m^3 day^{-1} and 300 tonnes day^{-1}, respectively. The annual precipitation is 783 mm and the evaporation from the surface of the Bay is about 600 mm annually. The tidal amplitude is very low, ranging between 8 to 10 cm. Subtidal sea-level fluctuations in the Sea of Marmara occur dominantly at time scales greater than 10 days and are highly coherent for subtidal frequencies in the Aegean Sea. Most of sea-level changes are produced by the NE-SW wind. The regional and wind regimes influencing the Bay are dominated by easterlies in summer and autumn and south-easterlies in winter. Izmit Bay has a two-layer water system which is an extension of the Sea of Marmara where more saline Mediterranean waters (37.5–38.5, using the Practical Salinity Scale) and less saline Black Sea waters (18.0–22.0) form a permanently strong stratification. The sharp density interface driven mainly by the salinity difference between these two water types occurs at about 25 m. The oceanographic characteristics of the Sea of Marmara and meteorological conditions of the region influence the movement of water bodies in Izmit Bay. Less saline surface-water of Black Sea origin from the Sea of Marmara enters into the bay in spring and summer, corresponding to the freshwater inflow changes in the Black Sea. In autumn and winter, however, water of the upper layer flows towards the Sea of Marmara. Wind-induced vertical mixing between upper and lower layers is restricted and occurs only at shallow depth (Algana *et al*., 1999).

Several industries have been developing rather rapidly around the bay. In addition to untreated or partly treated domestic wastes originating from the increasing population, the substantial industrial development, the heavy maritime traffic and the agricultural activities in the surrounding areas have caused a considerable pollution burden. Furthermore, some factory and urban sewage systems were damaged by the earthquake of August, 1999. The bay ecosystem was strongly affected by the quake and subsequent refinery fire, as were the settlements and industrial regions (Aktan and Aykulu, 2005).

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. The renewal capacity and water exchange within Izmit Bay is insufficient for compensation and equilibration. Eutrophication and deterioration of water quality have become serious problems (Telli – Karakoc *et al*., 2002). Izmit Bay and its surroundings are one of the most industrialized and populated area of Turkey, receiving more than 300 industrial and domestic effluents. Industrial effluents discharges a total of 163,000 m^3/day wastewater, 24 tons/day BOD and 19.5 tons/day TSS to Izmit Bay. The eastern basin receives the highest inputs compare to other basins of the Bay. Based on previous studies, no DHS has been measured in Izmit Bay. Industrial loads have been reduced by treatment and waste minimization within the last 10 years, but domestic wastes have doubled, due to the increa the sing population in Bay. Therefore, the total (domestic +industrial) discharge load into the Bay during the last 10 years has not changed significantly. The dissolved oxygen content of Izmit Bay decreased dramatically from 1984 to 1999 and reached a minimum value at 20 m throughout the Bay (Balkıs, N., 2003).

The inner part, the eastern sector, is the most polluted portion of the bay. Petroleum industries are situated in the central bay. The western portion (the outerpart), which is the entrance to the Marmara Sea, has a better water exchange capability; hence it is the less polluted part compared to the other parts of the bay. The Izmit Bay ecosystem has been monitored for more than 15 years; therefore, the oceanographic characteristics and pollution level in terms of conventional parameters in discharges such as total organic carbon, total nitrogen, total phosphorous, total suspended solids, etc., are easily available. Additionally, in the last 5–6 years, ecotoxicological studies in the Izmit Bay system have progressed to include finding out the fate and effects of pollutants—such as polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCBs), heavy metals etc. All these previous data showed that the bay waters are eutrophic and the discharges that enter the bay are toxic. The last measurements and analysis taken just after the earthquake also showed that dissolved oxygen levels arevery

critical in the bottom waters and the sediment is polluted by PAHs. The highest concentrations of PAHs are known to be generall found around urban centers. They are aromatic substances, most of which are formed during incomplete combustion of organic material and are also components of crude oil and its refined products. Most of the PAHs are highly lipophilic and are therefore accumulated by mussels and tend to rapidly adsorb on particles which are deposited in the sediments. Since it is found in the shallowest part of the bay, PAH-contaminated sediment is a big problem especially for the inner bay.

The T-PAH levels of Izmit Bay, in all matrices especially in sediments and in mussels, were found to be much higher than those found in other marine systems. The western portion of the bay is the less polluted part of the system in terms of PAH pollution. The central sector has a special importance because of the petroleum industries. Although the earthquake and the subsequent fire in the refinery increased the PAH level of the system, the discharges of these industries appear to be significant contributors to the PAH pollution in the bay. Due to several reasons—being the shallowest part of the bay, having limited water circulation, etc.—the eastern part is also a risky region. Additionally, unstable oceanographic characteristics resulting from sudden changes in meteorological conditions enhance the risk of pollution in the system, as was seen in case of the upwelling of anoxic bottom waters and resuspension of polluted sediment, which was caused by strong wind. Therefore, polluted sediment and untreated or ineffectively treated waste waters will always be problem for the system (Okay et al., 2003).

3. 3. 2. Gemlik

The Gemlik Bay, which is the second most polluted hot spot in the Marmara Sea; a semi-enclosed sea connecting the Black Sea to the Aegean Sea via the Bosphorus and Dardanelles straits. The organic carbon content is relatively high within the bay. Contrary to low values in the outer basin, the organic carbon content is relatively high within the bay. Its highest values are distributed in the middle of the basin, inner Gemlik Port, Karasu River which is the most important fresh water discharge in the west, and a resort area Trilye. Highly-populated eastern and southern coasts are mainly influenced

by rapid ecotourism development, direct discharges from rivers, surface run-off and drainage from port areas, domestic and industrial effluent discharges through outfalls and various contaminants from ships. Bathymetric features and the associated hydrodynamic processes seem to play an important role in the enrichment of organic carbon. Organic carbon contents show consistency with the sediment textural characteristics and the oxygen deficiency observed in Gemlik Bay (Alpar et al., 2006).

Geographical setting of the Gemlik Bay is similar to Izmit Bay. The Gemlik Bay emerges as a 31- km-long tectonic trough between two topographic heights, with an increasing width westward (Figure 3.4). 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 steep Samanlıdag˘ Mountains in the north, alluvial plains and deltas in the east and small hills along the southern coasts is about 76 km (Alpar and Unlu, in press).

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 hindcasted significant wave height for Gemlik wave is 3 m for the duration of wind data 1994–1998 (Alpar and Unlu, in press).

The maximum depth is 107 m in the middle of a small northwest-trending elliptical central trough which is a- fault-controlled depositional area. 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 (Figure 3. 4). 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 (Alpar and Unlu, in press).

For the oceanographic conditions, no long-term current data is available to represent the water circulation in the bay which controls sediment transportation and deposition. Some short-term historical measurements revealed two distinct water layers; the upper Black Sea water and the dense lower Mediterranean Sea water. In normal weather conditions, the surface flow is clockwise with an average current speed of about 13– 17 cm/s at the entrance of Gemlik Bay and 2–6 cm/s in the central and inner parts (Figure 3.4). These figures increase in spring. The interface layer takes place at 20–30 m water depth depending on the seasonal variations. In addition to the main Mediterranean water in deep Marmara Sea basins, a relatively thinner plume of dense lower layer flows through the southern Marmara shelf and reaching Gemlik Bay (Besiktepe et al., 1994). The average speed of the lower layer is 9–10 cm/s at the entrance while they are 2.5– 4.5 cm/s in the central and inner parts of the bay, but with varying directions (Alpar and Unlu, in press).

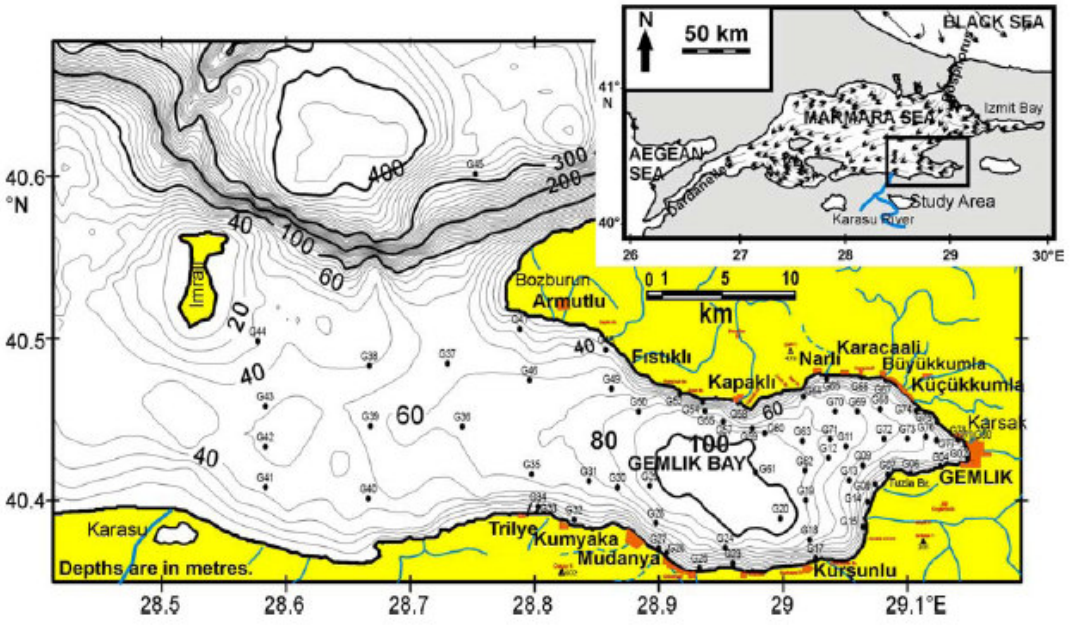

Figure 3. 4 Bathymetry is reconstructed from Yaltırak and Alpar (2002) and using the data obtained during the recent multibeam bathymetric survey of the Ifremer RV Le Suroit vessel (Rangin et al., 2001).

In the mean of environmental conditions; the bay, with a total surface area of 349 km^2, is most particularly subject to high anthropogenic pressure due to inputs from rivers, atmosphere, coastal shipping and industrial activities. The most important industrial towns along the bay are the Gemlik Town, Mudanya and Trilye (Figure 3. 4). Mudanya port is the export gate of the second biggest industrial city of Turkey, Bursa. As opposed to the industrialization along the southern coasts, Armutlu, Fıstıklı, Kapaklı, Narlı, Karacaali, Buyukkumla and Kucukkumla villages are tourism centers along the northern coasts. Total population exceeds 129000 and doubles in summers. The most densely populated towns, however, are Gemlik (80 000) and Mudanya (24 000). The total of domestic wastewater discharge into the bay is as much as 7.5 million m^3. Only Gemlik town and Kuckkumla have their own deep sea outfall discharge system. Other coastal settlements use creeks or simple outfalls for their wastewater discharge. New systems are ready to operate for Mudanya and Armutlu. (Alpar and Unlu, in press).

The easternmost part of the bay is subject to chronic severe contaminations, among which hydrocarbons play a major role. The main sources are ship traffic, fishery activities, domestic and industrial sewage waters and riverine inputs. The Karsak creek which discharges into the Gemlik port is the most important pollution source. Not only the discharges of a wide range of industrial plants in Gemlik town, but this creek also carries the waters of Lake Iznik, domestic and industrial wastewater discharges of Orhangazi town located 15 km in the west of the Gemlik Bay. The total load carried by Karsak River is therefore variable seasonally. The share of industrial waste water inputs is even higher, 13–20 million m^3/y. The total discharge of textile and chemistry plants is seemingly lower, but they introduce an important industrial pollution into the bay since they do not use treatment systems. The impact of such an anthropogenic pressure can be observed often in summer with the phenomenon of red waters, resulting from eutrophication and disequilibrium processes for the exploitation of natural resources (Alpar and Unlu, in press).

3. 3. 3. Kucukcekmece

Küçükçekmece is a large, crowded suburb on the European side of Istanbul, Turkey near Atatürk Airport. Küçükçekmece is on the Marmara coast and is the eastern shore (nearest they city) of an inlet of the Marmara called Küçükçekmece Gölü. The inlet is highly polluted but there are works to get it clean again. There used being wild life and many kind of birds on the life. The inlet is connected to the Marmara Sea by a very narrow channel so the water is not salty.

On December 29, 1999, the Volgoneft-248, a 25-year old Russian tanker, ran aground and split in two in close proximity to the southwest shores of Istanbul. More than 800 tons of the 4,300 tons of fuel oil on board spilled into the Marmara Sea, covering the coast of Marmara with fuel-oil and affecting about 5 square miles of the sea.

The amount of heavy fuel oil spilled from the Volgoneft-248 tanker to the Marmara Sea is estimated to be 1,290 tons. Approximately 1,000 tons of the remaining oil was discharged ashore, leaving another 2,000 tons in four tanks located in the sunken bow section. Field observations on the accident day evidenced that the spilled oil contaminated the shorelines between the grounded ship stern off the Menekşe Coast and the rock groin at Çiroz Park five kilometers to the East of the accident. Beaches, fishing ports, restaurants, recreation facilities, the Atatürk Pavilion, piers, groins and seawalls located in this area are directly affected.

Later field surveys associated with legal damage investigations were carried out to survey the state of pollution and the result of clean-up operations three to four months after the accident. It was found that the shorelines have been cleaned to a large extent except some minor leftover, and some fresh marks indicating a continuing contamination. The source for this recent pollution was found to be the oil leakage from the remaining oil in the sunken bow section of the tanker, which was later, recovered in summer, 2000 (Otay and Yenigün, 2000).

4. PHYLOGENETIC CLASSIFICATION

4. 1. The Archaea

Comparative analysis of ribosomal RNA (rRNA) sequences by Woese et al. (1990) indicated that all cellular life belonged to one of the three domains, Bacteria, Archaea and Eukarya (Head et al., 1998) (Figure 4.1.).

It is generally acceptecd that the Archaea diverged earliest from the common ancestor and are therefore regarded as the most primitive group of organisms. Many are highly adapted to cope with extreme chemical and/or physical environments (temperature, pH, salinity, etc.) and the group can conveniently be divided into hyperthermophiles, halophiles and methanogens. This suggests that the Archaea may be descendants of the earliest forms of cellular life which would have had to evolve under such conditions. Despite outward appearances however, the Archaea are more closely biochemically related to the Eukarya than to the Bacteria (Bullock, 2000)

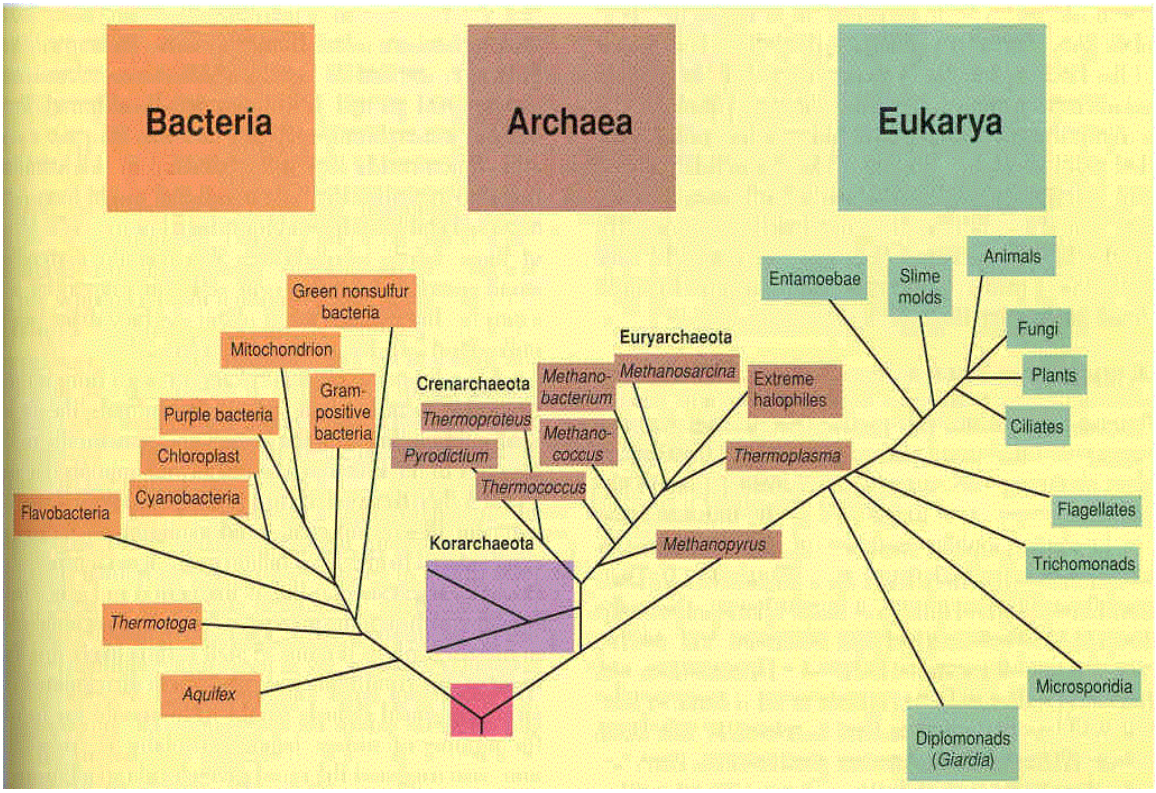

Figure 4. 1 Three major domains of new classification proposed by Woese et al., 1990

4. 1. 1. Kingdoms of the Archaea: Crenarchaeota, Euryarchaeota and Koarchaeota

Archaea is formally subdivided into the two kingdoms Euryarchaeota that encompasses the methanogens and their phenotypically diverse relatives and Crenarchaeota (Figure 4. 2). The kingdom Crenarchaeota comprises the relatively tight clustering of extremely thermophilic archaebacteria, whose general phenotype appears to resemble most the ancestral phenotype of the Archaea (Woese et al., 1990).

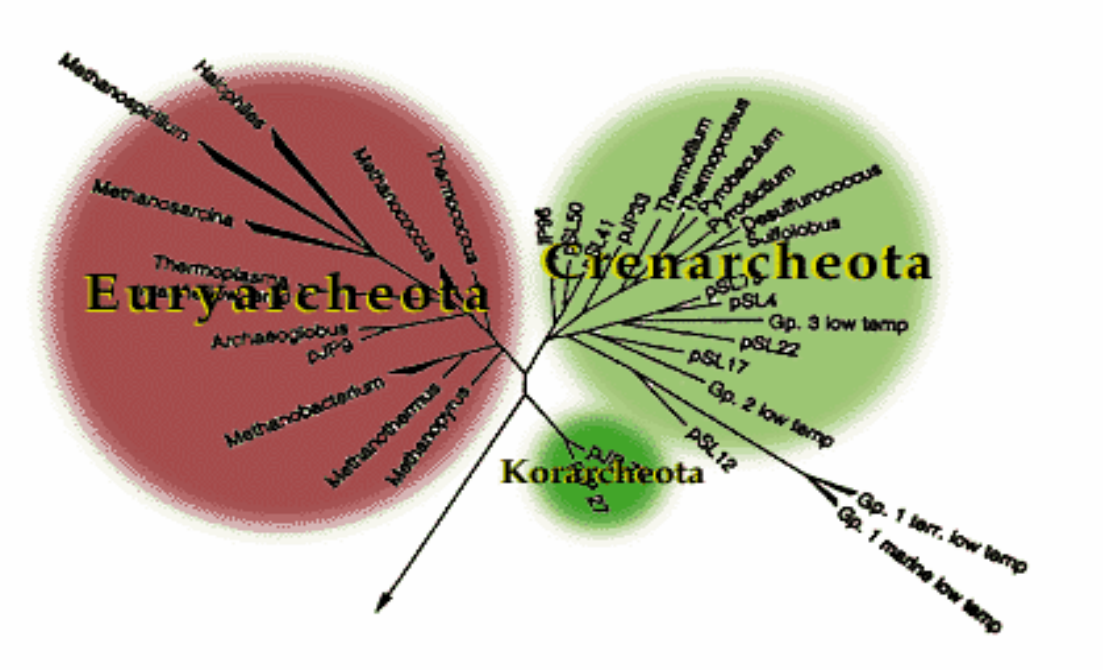

Figure 4. 2 Three kingdom of Archea

Crenarchaeota contains a single class, Thermoprotei, represented by the orders Thermoproteales, Desulfurococcales and Sulfolobales. The phylum Euryarchaeota consists of seven classes and nine orders, i.e., the classes Methanobacteria (comprising the order Methanobacteriales), Methanococci (orders Methanococcales, Methanomicrobiales and Methanosarcinales), Halobacteria (order Halobacteriales), Thermoplasmata (order Thermoplasmatales), Archaeoglobi (order Archaeoglobales), and Methanopyri (order Methanopyrales). Until now, all the three crenarchaeotic orders are exclusively hyperthermophilic or thermophilic. As for the phylum Euryarchaeota, hyperthermophilic archaea comprise both methanogenic and non-methanogenic archaeal orders. The former include Methanobacteriales, Methanococcales and Methanopyrales, while the latter Thermococcales and Archaeoglobales. Among the methanogenic orders, Methanobacteriales and Methanococtales contain both hyperthermophilic and mesophilic species (Itoh, 2003).

4. 1. 1. 1. Methanogens

Methanogens are strict anaerobes which share a complex biochemistry for methane synthesis as part of their energy metabolism. In natural anaerobic habitats which contain complex organic compounds and where light, sulfate, and nitrate are limiting, methanogens are linked to chemoheterotrophic bacteria for the degradation of organic substrates in a four-step process (Garcia et al., 2000):

(1) hydrolysis of polymers by hydrolytic microorganisms,

(2) acidogenesis from simple organic compounds by fermentative bacteria,

(3) acetogenesis from metabolites of fermentations by homoacetogenic or syntrophic bacteria,

(4) methanogenesis by methanogenic Archaea from H_2 + CO_2, acetate, simple methylated compounds or alcohols + CO_2 (Figure 4. 3)

Depending on physical-chemical conditions of temperature, pH, osmotic pressure and substrate composition, the species of bacteria implicated in the anaerobic digestion of organic matter may differ from one habitat to another. The activity of the primary microbial populations leads to accumulation of H_2 +CO_2 and volatile fatty acids. Formate is rapidly converted via the formic hydrogenlyase to H_2 and CO_2 and may not be detectable as it is rapidly metabolized. The ultimate formation of methane and CO_2 marks the last step in a series of dissimilatory reactions in which organic compounds are completely degraded. CH_4 is the most reduced form of carbon, and CO_2 the most oxidized form of carbon.

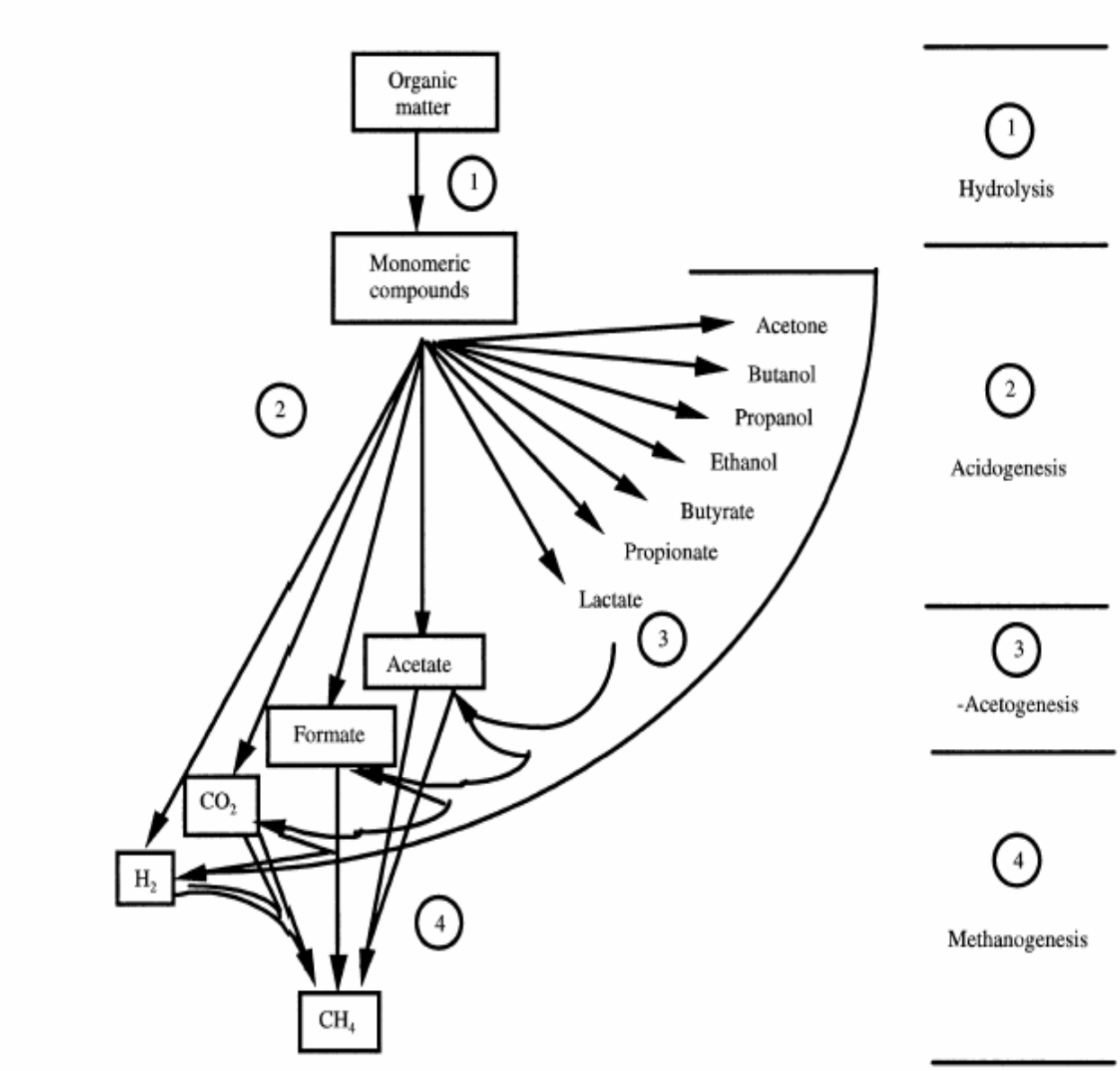

Figure 4. 3 Schematic diagram showing anaerobic degradation of organic matter

There are three different methanogenic ecosystems in nature (Garcia et al., 2000) (Figure 4. 4).

(a) In lacustrine and marine sediments, marshes, swamps, rice soils, sludge and digesters the organic matter is completed degraded (Figure 4. 4a).

(b) In case of ruminants and the intestinal tracts of almost all living creatures (e.g. humans, insects and termites), the process of mineralization is incomplete and most of

the intermediate products formed (e.g. volatile fatty acids) is resorbed into the bloodstream and serves as nutrition (Figure 4. 4b).

(c) In the absence of organic matter (e.g. hot springs) methanogenesis occurs only from geochemical hydrogen formed as part of the geological process (Figure 4. 4c).

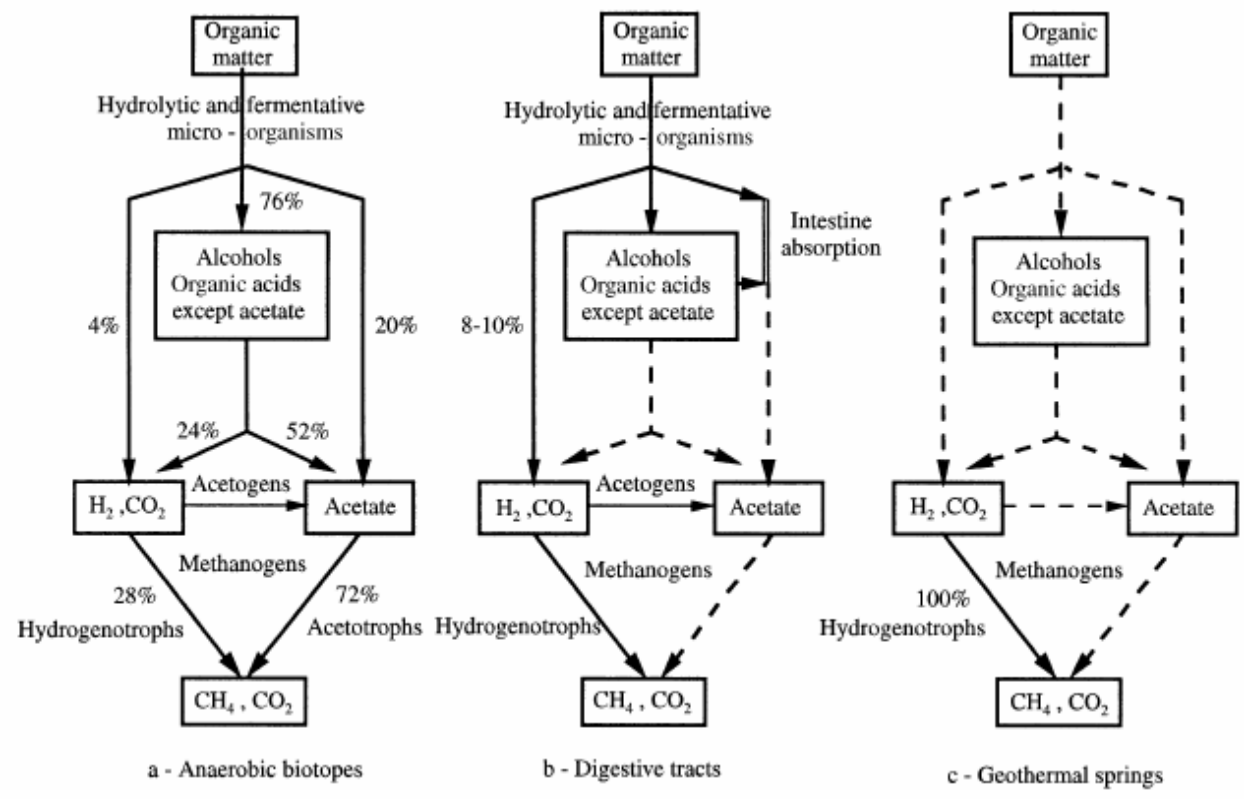

Figure 4. 4 Schematic diagram showing the three different methanogenic ecosystems operating in nature.

The distribution of known species of methanogens in natural environments is entirely dependent on their respective adaptation to various temperatures, pH and salinity ranges. The temperature range is quite broad, from 4 to 110 ^{o}C. Despite the fact that methanogenesis is operative at temperature near 4 ^{o}C, there are very few reports of psychrophilic methanogens. Most methanogens grow over a relative narrow pH range (6 to 8) although in vivo methanogenesis studies have shown that it does occur in acidic environments such as peat bogs. Tolerance to NaCl concentrations is variable according

the species examined. Marine methanogens range amongst the most halophilic, specially in the genera Methanococcus, Methanogenium, and Methanocalculus, and for most of the obligately methylotrophic methanogens, eg the genera Methanohalophilus, Methanohalobium, and Methanosalsus found in salt lagoons, salt lakes or saline oil field subsurface waters (Garcia et al., 2000).

4. 1. 1. 1. 1. Classification of Methanogens

Methanogens are classified into five orders that are the Methanobacteriales, the Methanococcales, the Methanosarcinales, the Methanopyrales, and the Methanomicrobiales. (Figure 4. 5)

The 83 species of methanogens described so far (including six synonymous) are separated into three main nutritional categories:

(1) 61 species (including five synonymous) of hydrogenotrophs oxidize H_2 and reduce CO_2 to form methane and among them 38 species (including three synonymous) of formatotrophs oxidize formate to form methane.

(2) Twenty species (including one synonymous) of methylotrophs use methyl compounds as methanol, methylamines, or dimethylsulfide and of which 13 species are obligate methylotrophs. It has been proposed that the metabolism of dimethylsulfide proceeds along a somewhat different route to that of methylamines and perhaps also to that of methanol.

(3) Nine species (including 1 synonymous) of aceticlastic (or acetotrophic) methanogens utilize acetate to produce methane, with two species in this group being obligate acetotrophs.

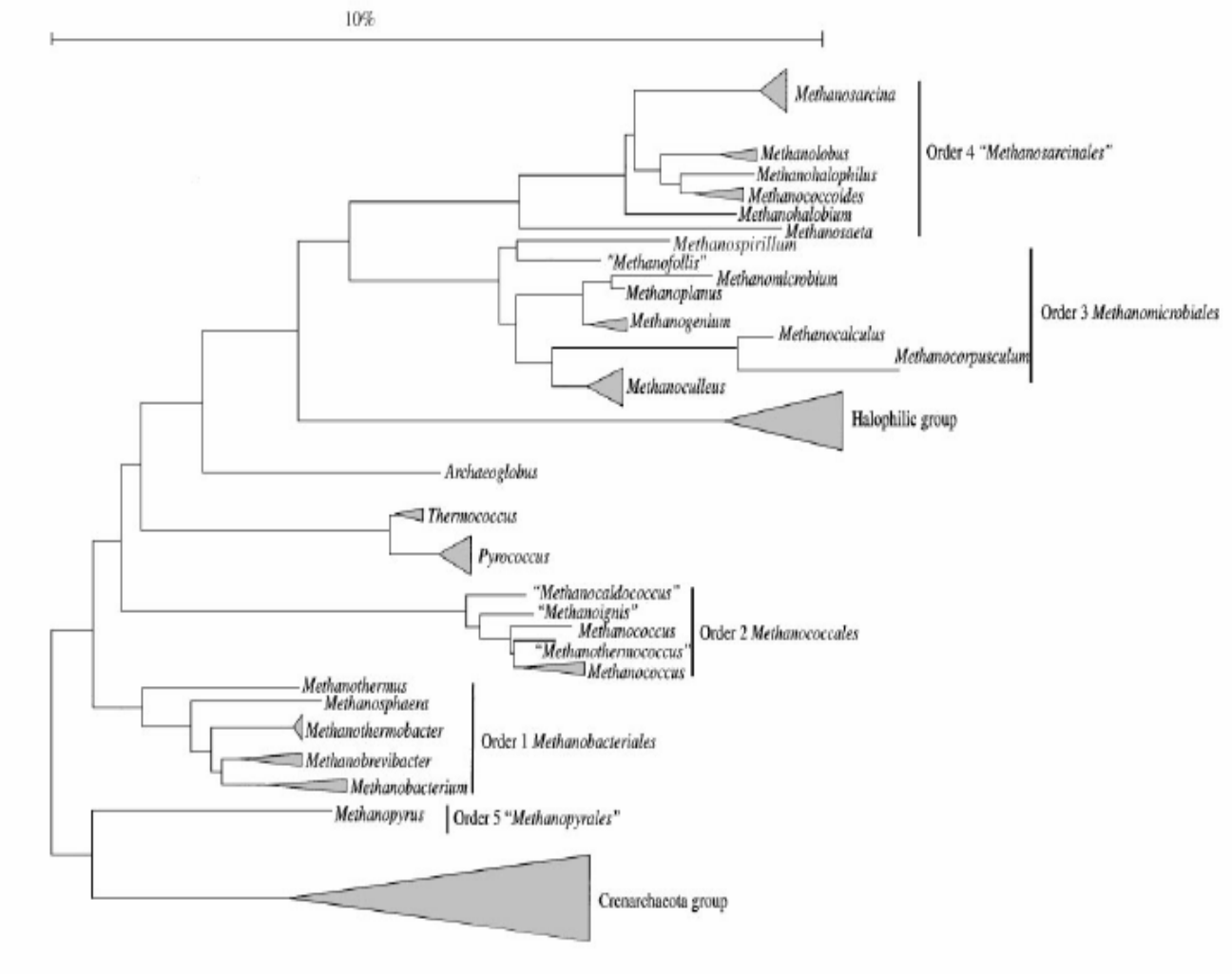

Figure 4. 5 Updated phylogeny of methanogens, domain Archaea. Non-methanogens are indicated by their group names (large triangles).

5. MOLECULAR TECHNIQUES

Among the disciplines devoted to studying the different life forms on our planet, microbiology was the last to be established. Its boundaries are very much defined as a consequence. Subtract from the pool of organisms those that can be studied by the classic techniques of botany and zoology, and the rest is left for microbiologists. What usually remains are small organisms that can be visualized only by using special equipment, microscopes. In contrast to animals and plants, the morphology of microorganisms is in general too simple to serve as a basis for a sound classification and to allow for reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined cocultures) followed by testing for multiple physiological and biochemical traits. Consequently, any approach to identify specific microbial populations without cultivation directly in their natural environments could be revolutionary, since it could change the character of microbiology and close the methodological gap which still exists in comparison with botany and zoology. (Amann et al., 1995).

5. 1. Culture Based Methods

Viable plate count or most-probable-number techniques have been, and frequently still are, used for quantification of active cells in environmental samples. However, because they select for certain organisms, these methods are inadequate to address this problem. Restrictions and potential biases of the established techniques were clearly stated by Brock, who strongly argued in favor of in situ studies. For oligotrophic to mesotrophic aquatic habitats, it has been frequently reported that direct microscopic counts exceed viable-cell counts by several orders of magnitude. The same is true for sediments and soils (Table 5. 1) (Amann et al., 1995).

Table 5. 1. Culturability determined as a percentage of culturable bacteria in comparison with total cell counts

Habitat	Culturability (%)[a]	Reference(s)
Seawater	0.001–0.1	48, 81, 82
Freshwater	0.25	75
Mesotrophic lake	0.1–1	150
Unpolluted estuarine waters	0.1–3	48
Activated sludge	1–15	160, 161
Sediments	0.25	75
Soil	0.3	153

[a] Culturable bacteria are measured as CFU.

The majority of microscopically visualized cells are viable but do not form visible colonies on plates. Two different types of cells contribute to this silent but active majority:

(i) known species for which the applied cultivation conditions are just not suitable or which have entered a nonculturable state and

(ii) unknown species that have never been cultured before for lack of suitable methods.

It has been well documented for pathogens like Salmonella enteritidis , Vibrio cholerae, and V. vulnificus that bacteria may quickly enter a nonculturable state upon exposure to salt water, freshwater, or low temperatures. (Amann et al., 1995). From the foregoing, it is obvious that the cultured species of the Bacteria and Archaea represent only a minor fraction of the existing diversity. Today, about 5,000 species have been described, with the Approved List of Bacterial Names currently containing less than 3,500 entries. Since the classic species concept for higher organisms applies only to organisms with sexual reproduction, it is very difficult to estimate the number of extant microbial species. (Amann et al., 1995).

Possible solution to the limitations of culture based methods is analysis of rRNA molecules. The first attempts to characterize environmental samples by studying rRNA began about a decade ago. An average bacterial 16S rRNA molecule, has a length of

1,500 nucleotides, contain sufficient information for reliable phylogenetic analyses (Amann et al., 1995). The rRNA is present in all organisms. Its function has not changed since the early days of evolution, therefore; rRNA is highly conserved in nucleotide sequence and also in secondary structure. It contains variable regions that reflect evolutionary relationships organisms. Conserved regions serve as convenient targets for the primers(for PCR) or hybridization probes. Analysis of rRNA molecules yield sufficient data for statistically significant phylogenetic comparisions (Olsen et al., 1986)

5. 2. Fluorescent in Situ Hybridization (FISH)

In situ identification and enumeration of microorganisms harboring a certain rRNA sequence require a technique in which the rRNA is specifically detected within morphologically intact cells, a technique here referred to as whole-cell hybridization. The more common term ‘‘in situ hybridization’’ means in which cells are detected in their natural microhabitat. Analysis at the single-cell level by in situ hybridization can obviously provide a more detailed picture. One can not only determine the cell morphology of an uncultured microorganism and its abundance but also analyze spatial distributions in situ. Quantification of the signal conferred by rRNA-targeted oligonucleotides should even allow the estimation of in situ growth rates of individual cells. (Amann et al., 1995).

The use of in situ hybridization for counting and identifying organisms had already been proposed by Olsen et al. The microscopic identification of single microbial cells with rRNA-targeted probes was first performed with radioactively labeled oligonucleotides. After hybridization of whole fixed cells, the 35S-labeled probe was visualized by microautoradiography. This step not only required extra time but also resulted in formation of silver grains up to several micrometers away from the target cell, thus preventing the exact in situ localization of small cells in clusters. Although this problem could be improved by using less energetic isotopic labels like 3H, the trade-off for a 1 mm-resolution would be an extension of the time required for autoradiography to several weeks or months. (Amann et al., 1995).

As demonstrated several years ago by the immunofluorescence approach, fluorescent probes yield superb spatial resolution and can be instantaneously detected by

epifluorescence microscopy. Fluorescently monolabeled, rRNA-targeted oligonucleotide probes were demonstrated to allow detection of individual cells. This made whole-cell hybridization with rRNA-targeted probes a suitable tool for determinative, phylogenetic, and environmental studies in microbiology. Like immunofluorescence, whole-cell hybridization with fluorescently labeled rRNA-targeted oligonucleotides can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations. (Amann et al., 1995).

Figure 5. 1 shows the different options of using rRNA-targeted nucleic acid probes to characterize an environmental sample by hybridization techniques.

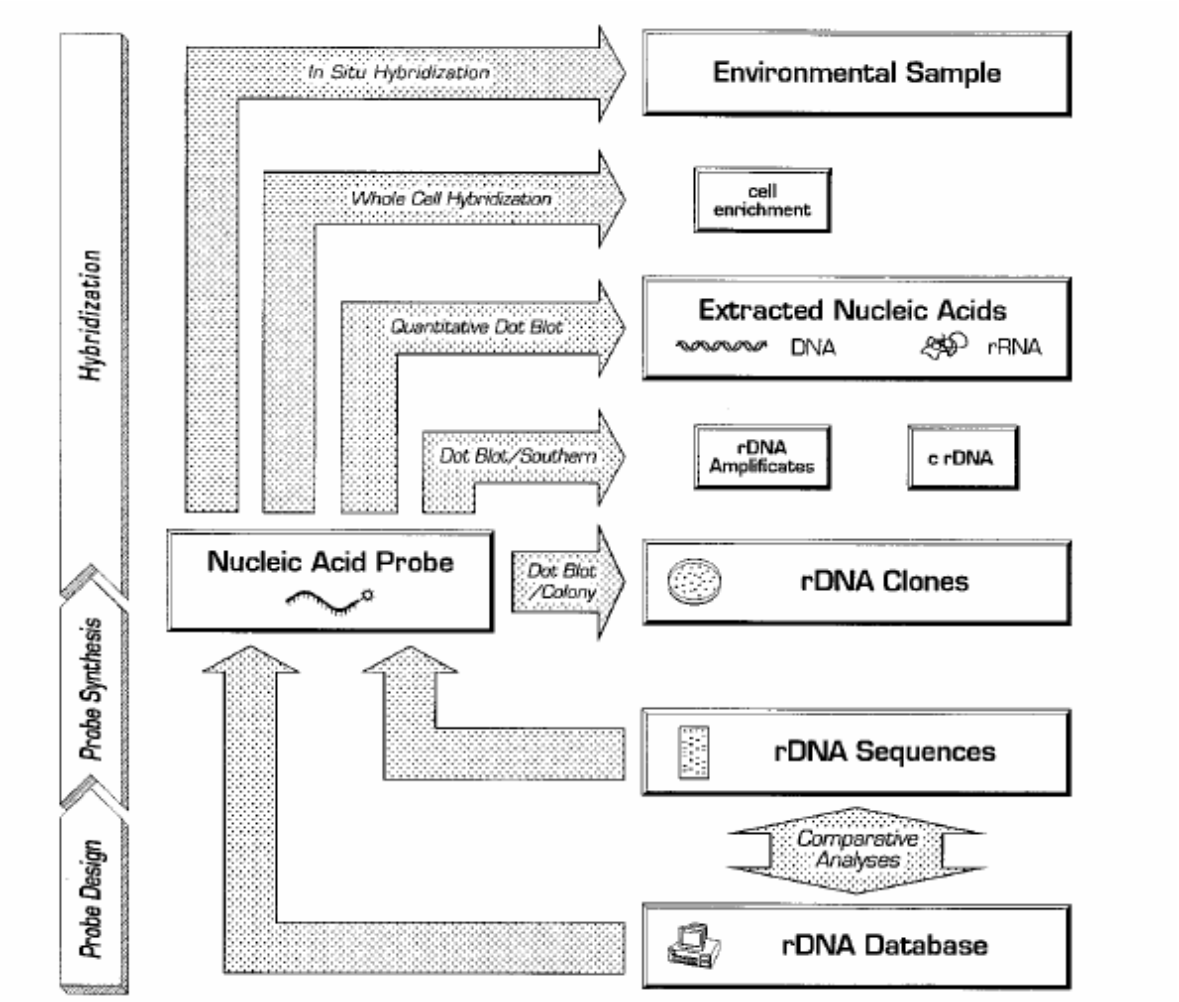

Figure 5. 1 Flow chart showing the different options of using rRNA-targeted nucleic acid probes to characterize an environmental sample by hybridization techniques.

5. 2. 1. Problems with Fluorescent In Situ Hybridization

5. 2. 1. 1. No cells, No signals

The first point in troubleshooting can be summarized in a simple line: ‘‘no cells, no signals.’’ Whole-cell hybridization can identify an individual cell; however, it is usually not trivial to bring this cell into the examined microscopic field(s).

5. 2. 1. 2. Low Signal Intensity

Low signal intensity after in situ hybridization is another frequently encountered problem. If nothing is wrong with the probes, this problem can be caused only by small numbers or insufficient accessibility of the target molecules, in our case the rRNA molecules. (Amann et al., 1995).

5. 2. 1. 3. Correlation Between Growth Rate and Cellular rRNA Content.

A direct correlation between rRNA content and growth rate of bacteria was first shown by Schaechter et al. in 1958 for Salmonella typhimurium. Accordingly, the signals conferred by fluorescently labeled rRNA-targeted probes to whole fixed E. coli cells were correlated to cellular rRNA contents and growth rates. This has two consequences:

(i) slowly growing cells are difficult to detect because of their low cellular rRNA content, and

(ii) the quantification of probe-conferred fluorescences should allow estimation of in situ growth rates of individual cells. (Amann et al., 1995).

5. 2. 1. 4. Cell Permeabilization

The important problem that must be overcome for in situ whole-cell hybridization to be successful is the entry of the probe into the cell. This is normally achieved by fixation with denaturants such as alcohols, or cross-linking reagents such as formaldehyde or paraformaldehyde. These fixation procedures not only aid in cell permeability, but also help maintain the cells’ morphological integrity during hybridization (Head et al., 1998).

5. 2. 1. 5. Target Accessibility

When a rapidly growing culture with presumably high cellular rRNA content exhibits low signal intensities, it has proven useful to distinguish between cases in which cell peripheries limit diffusion of rRNA-targeted probes into whole fixed cells and cases in which higher-order structures in the ribosomes, both RNA-RNA and RNA-protein, prevent probe hybridization (Amann et al., 1995). This problem can normally be detected by a strong hybridization signal being obtained with a universal probe that is known to target an accessible site on the rRNA molecule. If another probe does not give a hybridization signal in the same cell(s), this generally indicates poor accessibility of the target site (Head et al., 1998).

5. 2. 1. 6. Sensitivity

Sensitivity of in situ hybridization is also an issue. In general, probes containing a single labeled molecule give a strong signal only if cells are metabolically active and, hence, contain large numbers of ribosomes and target rRNA. A number of approaches have been taken to improve the sensitivity by using multiple singly labeled probe, multiply labeled probes, and enzyme-linked probes or detection systems that allow signal amplification. In addition, the development of highly sensitive cameras has improved the sensitivity of in situ hybridization assays (Head et al., 1998).

6. MICROBIOLOGY OF ANOXIC MARINE SEDIMENTS

The Archaea can be found in specialized environments, including those at high temperature, high salinity, and extremes of pH and in strictly anaerobic niches that permit methanogenesis (Vetriani et al., 1999). Archaeal abundance and distribution has been studied in deep and surface marine waters. In an earlier study, Fuhrman et al. (1993) found archaeal population maximum at 500 m below the sea surface. Moreover, Euryarchaea and Crenarchaea were found to occupy different zones of the water column with the former most abundant at the surface and the latter at the depth of 100 m (Massana et al., 1997). Fuhrman et al. reported the presence of the Archaea as deep as 3000 m in the Pasific Ocean in the year 1998.

The Archaea present in salt marsh sediment samples from a tidal creek and from an adjacent area of vegetative marshland, both of which showed active methanogenesis and sulfate reduction, were sampled by using 16S rRNA gene libraries created with Archaea-specific primers. None of the sequences were the same as reference sequences from cultured taxa, although some were closely related to sequences from methanogens previously isolated from marine sediments. A wide range of Euryarchaeota sequences were recovered, but no sequences from Methanococcus, Methanobacterium, or the Crenarchaeota were recovered. Clusters of closely related sequences were common and generally contained sequences from both sites, suggesting that some related organisms were present in both samples. Recovery of sequences closely related to those of methanogens such as Methanococcoides and Methanolobus, which can use substrates other than hydrogen, provides support for published hypotheses that such methanogens are probably important in sulfate-rich sediments and identifies some likely candidates. Sequences closely related to those of methanogens such as Methanoculleus and Methanogenium, which are capable of using hydrogen, were also discovered, in

agreement with previous inhibitor and process measurements suggesting that these taxa are present at low levels of activity (Munson et al., 1997).

Vetriani et al., (1999) investigated the relative abundance, vertical distribution, phylogenetic composition, and spatial variability of Archaea in deep-sea sediments collected from several stations in the Atlantic Ocean. Quantitative oligonucleotide hybridization experiments indicated that the relative abundance of archaeal 16S rRNA in deep-sea sediments (1500 m deep) ranged from about 2.5 to 8% of the total prokaryotic rRNA. Clone libraries of PCR-amplified archaeal rRNA genes (rDNA) were constructed from 10 depth intervals obtained from sediment cores collected at depths of 1,500, 2,600, and 4,500 m. Phylogenetic analysis of rDNA sequences revealed the presence of a complex archaeal population structure, whose members could be grouped into discrete phylogenetic lineages within the two kingdoms, Crenarchaeota and Euryarchaeota.

Anoxic sediments from Rotsee (Switzerland) were analyzed for the presence and diversity of methanogens by using molecular tools. After PCR-assisted sequence retrieval of the 16S rRNA genes (16S rDNA) from the anoxic sediment of Rotsee, cloning, and sequencing, a phylogenetic analysis identified two clusters of sequences and four separated clones. The sequences in cluster 1 grouped with those of Methanosaeta spp., whereas the sequences in cluster 2 comprised the methanogenic endosymbiont of Plagiopyla nasuta. Discriminative oligonucleotide probes were constructed against both clusters and two of the separated clones. These probes were used subsequently for the analysis of indigenous methanogens in a core of the sediment, in addition to domain-specific probes against members of the domains Bacteria and Archaea and the fluorescent stain 4´,6-diamidino-2-phenylindole (DAPI), by fluorescent in situ hybridization. Probes Eub338 and Arch915 detected on average 16 and 6% of the DAPI-stained cells as members of the domains Bacteria and Archaea, respectively. The spatial distribution of the two methanogenic populations corresponded well to the methane production rates determined by incubation with either [^{14}C]acetate or [^{14}C]bicarbonate. Methanogenesis from acetate accounted for almost all of the total methane production, which concurs with the predominance of acetoclastic Methanosaeta spp. that represented on average 91% of the archaeal population. Significant hydrogenotrophic methanogenesis was found only in the organically enriched upper 2

cm of the sediment, where the probably hydrogenotrophic relatives of the methanogenic endosymbiont of P. nasuta, accounting on average for 7% of the archaeal population, were also detected (Falz et al., 1999).

16S rDNA clone library analysis was used to examine the biodiversity and community structure within anoxic sediments of several marine-type salinity meromictic lakes and a coastal marine basin located in the Vestfolds Hills area of Eastern Antarctica. From 69 to 130 (555 total) 16S rDNA clones were analysed from each sediment sample, and restriction fragment length polymorphism (RFLP) and sequence analysis grouped the clones into 202 distinct phylotypes (a clone group with sequence similarity of > 0.98). A number of phylotypes and phylotype groups predominated in all libraries, with a group of 10 phylotypes (31%of clones) forming a novel deep branch within the low G+C Gram-positive division. Other abundant phylotypes detected in several different clone libraries grouped with Prochlorococcus cyanobacteria, diatom chloroplasts, delta proteobacteria (Desulfosarcina group, Syntrophus and Geobacter/Pelobacter/Desulphuromonas group), order Chlamydiales (Parachlamydiaceae) and Spirochaetales (wall-less Antarctic spirochaetes). Most archaeal clones detected (3.1% of clones) belonged to a highly diverged group of Euryarchaeota clustering with clones previously detected in rice soil, aquifer sediments and hydrothermal vent material (Bowman et al., 2000).

Fluorescence in situ hybridization (FISH) and rRNA slot blot hybridization with 16S rRNA-targeted oligonucleotide probes were used to investigate the phylogenetic composition of a marine Arctic sediment (Svalbard). FISH resulted in the detection of a large fraction of microbes living in the top 5 cm of the sediment. Up to 65.4 ± 7.5% of total DAPI (4´,6´-diamidino-2-phenylindole) cell counts hybridized to the bacterial probe EUB338, and up to 4.6 ± 1.5% hybridized to the archaeal probe ARCH915 (Ravenschlag et al., 2001).

Microbial communities in cores obtained from methane hydrate-bearing deep marine sediments (down to more than 300 m below the seafloor) in the forearc basin of the Nankai Trough near Japan was studied by Reed *et al* (2002). Characterization of 16S rRNA genes amplified from the sediments indicated that archaeal clones could be discretely grouped within the Euryarchaeota and Crenarchaeota domains. The bacterial

clones exhibited greater overall diversity than the archaeal clones, with sequences related to the Bacteroidetes, Planctomycetes, Actinobacteria, Proteobacteria, and green nonsulfur groups. The majority of the bacterial clones were either members of a novel lineage or most closely related to uncultured clones.

In situ identification of prokaryotic cells in subsurface sediments is hampered by the low cellular rRNA contents of the target organisms. Fluorescence in situ hybridization with catalyzed reporter deposition (CARD–FISH) has the potential to overcome this limitation, and was therefore optimized for a 40 cm deep sediment core sampled from a tidal sandy flat of the German Wadden Sea. Percentage of DAPI stained cells detected with the probe combination EUB(I-III), targeting nearly all the Bacteria, were comparable for CARD–FISH with a horseradish peroxidase (HRP)-labeled probe and FISH with a fluorescently monolabeled probe in the 2–3 cm depth interval (92% and 82%, respectively), but significantly higher with the HRP-labeled probe at 35–40 cm, the deepest layer sampled (63% with HRP vs. 26% with monolabeled probe). With CARD–FISH Alphaproteobacteria and the Desulfobulbaceae group of sulfate-reducing bacteria were detected only in the upper layers. In contrast, Desulfosarcinales, the Bacteroidetes group, Planctomycetes, Betaproteobacteria, and Gammaproteobacteria were found at all depths. Archaea were detectable with ARCH915-HRP after achromopeptidase treatment. Surprisingly, aggregates of Bacteria and Archaea were found, below 12 cm depth, that strongly resemble consortia involved in anoxic oxidation of methane that have previously been found in sediments near methane hydrate deposits (Ishii et al., 2004).

Newberry et al., (2004) studied diversity of Bacteria and Archaea in deep marine sediments by PCR amplification and sequence analysis of 16S rRNA and methyl co-enzyme M reductase (mcrA) genes. Samples analysed were from Ocean Drilling Program (ODP) Leg 190 deep subsurface sediments at three sites spanning the Nankai Trough in the Pacific Ocean off Shikoku Island, Japan. Phylogenetic analysis of clone libraries showed a wide variety of uncultured Bacteria and Archaea. Sequences of Bacteria were dominated by an uncultured and deeply branching ‘deep sediment group’ (53% of sequences). Archaeal 16S rRNA gene sequences were mainly within the uncultured clades of the Crenarchaeota. The mcrA gene analysis suggested limited

methanogen diversity with only three gene clusters identified within the Methanosarcinales and Methanobacteriales.

The Bacteria and Archaea from the meromictic Lake Pavin were analyzed in samples collected along a vertical profile in the anoxic monimolimnion and were compared to those in samples from the oxic mixolimnion. Nine targeted 16S rRNA oligonucleotide probes were used to assess the distribution of Bacteria and Archaea and to investigate the in situ occurrence of sulfate-reducing bacteria and methane-producing Archaea involved in the terminal steps of the anaerobic degradation of organic material. The diversity of the complex microbial communities was assessed from the 16S rRNA polymorphisms present in terminal restriction fragment (TRF) depth patterns. The densities of the microbial community increased in the anoxic layer, and Archaea detected with probe ARCH915 represented the largest microbial group in the water column, with a mean Archaea/ Eubacteria ratio of 1.5. Terminal restriction fragment length polymorphism (TRFLP) analysis revealed an elevated archaeal and bacterial phylotype richness in anoxic bottom-water samples. The structure of the Archaea community remained rather homogeneous, while TRFLP patterns for the eubacterial community revealed a heterogeneous distribution of eubacterial TRFs (Lehours et al., 2005).

Individual prokaryotic cells from two major anoxic basins, the Cariaco Basin- in the Caribbean Sea and the Black Sea, were enumerated throughout their water columns using fluorescence in situ hybridization (FISH) with the fluorochrome Cy3 or horseradish peroxidase-modified oligonucleotide probes. For both basins, significant differences in total prokaryotic abundance and phylogenetic composition were observed among oxic, anoxic, and transitional (redoxcline) waters. Epsilon-proteobacteria, Crenarchaeota, and Euryarchaeota were more prevalent in the redoxclines. Relative abundances of Archaea in both systems varied between 1% and 28% of total prokaryotes, depending on depth. The prokaryotic community composition varied between the two anoxic basins, consistent with distinct geochemical and physical conditions. In the Black Sea, the relative contributions of group I Crenarchaeota (median, 5.5%) to prokaryotic communities were significantly higher ($P < 0.001$; $n = 20$) than those of group II Euryarchaeota (median, 2.9%). In contrast, their proportions were nearly equivalent in

the Cariaco Basin. Beta-proteobacteria were unexpectedly common throughout the Cariaco Basin's water column, accounting for an average of 47% of 4´,6´-diamidino-2-phenylindole (DAPI)-stained cells. This group was below the detection limit (<1%) in the Black Sea samples. Compositional differences between basins may reflect temporal variability in microbial populations and/or systematic differences in environmental conditions and the populations for which they select (Lin et al., 2006).

The subsurface of a tidal-flat sediment was analyzed down to 360 cm in depth by molecular methods. A community structure analysis of all three domains of life was performed using domain-specific PCR followed by denaturing gradient gel electrophoresis analysis and sequencing of characteristic bands. The pore water profile was characterized by a subsurface sulfate peak at a depth of about 250 cm. Methane and sulfate profiles were opposed, showing increased methane concentrations in the sulfate-free layers. The availability of organic carbon appeared to have the most pronounced effect on the bacterial community composition in deeper sediment layers. In general, the bacterial community was dominated by fermenters and syntrophic bacteria. The depth distribution of methanogenic archaea correlated with the sulfate profile and could be explained by electron donor competition with sulfate-reducing bacteria. Sequences affiliated with the typically hydrogenotrophic Methanomicrobiales were present in sulfate-free layers. Archaea belonging to the Methanosarcinales that utilize noncompetitive substrates were found along the entire anoxic-sediment column (Wilms et al., 2006).

7. MATERIALS AND METHODS

7. 1. Study Sites

Anoxic sediment samples were collected from four stations- IZ-17(Izmit Bay), IZ-25 (Izmit Bay), MD-87(Gemlik Bay) and MKC (Kucukcekmece)- in the Marmara Sea. Figure 7.1 shows the stations that are studied.

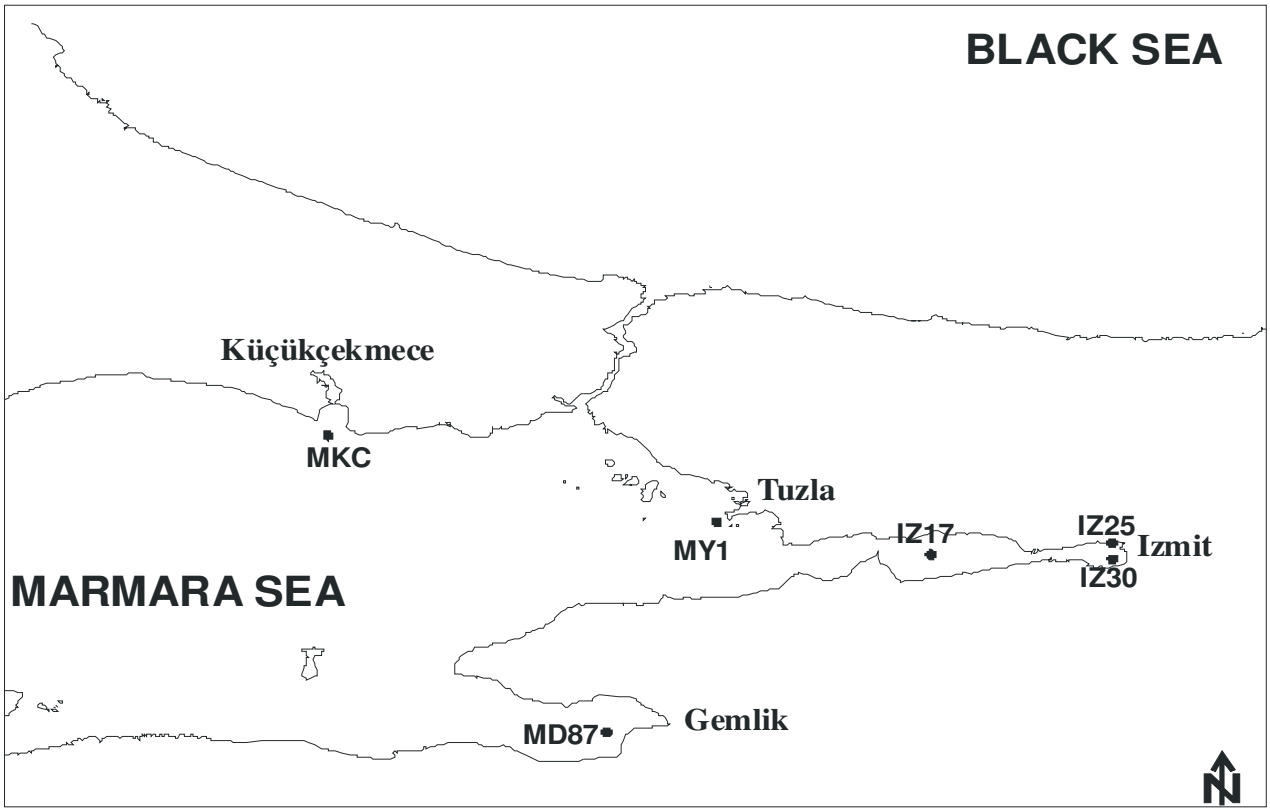

Figure 7. 1 Locations of the sediment samples collected for study.

7. 2. Characterististics of The Sediment Samples

7. 2. 1. IZ-17(Izmit Bay)

IZ-17 station was located in the middle of the Izmit Bay. Sampling depth of IZ-17 was 157 m. Total solid (TS) and total volatile solid (TVS) analysis were carried out. Total carbon (TC), total inorganic carbon (IC), total organic carbon (OC) contents and heavy metal concentrations of the sediment sample were also measured. All analysis were carried out according to the Standard Methods (APHA, 1995).

7. 2. 2. IZ-25 (Izmit Bay)

IZ-25 station was located on the coast of the Izmit Bay. Sampling depth of IZ-17 was approximately 30 m. Total solid (TS) and total volatile solid (TVS) analysis were carried out. Total carbon (TC), total inorganic carbon (IC), total organic carbon (OC) contents and heavy metal concentrations of the sediment sample were also measured. All analysis were carried out according to the Standard Methods (APHA, 1995).

7. 2. 3. MD-87 (Gemlik Bay)

MD-87 station was located close to the Gemlik Bay. This location has the depth of 87 m. Total solid (TS) and total volatile solid (TVS) analysis were carried out. Total carbon (TC), total inorganic carbon (IC), total organic carbon (OC) contents and heavy metal concentrations of the sediment sample were also measured. All analysis were carried out according to the Standard Methods (APHA, 1995).

7. 2. 4. MKC (Kucukcekmece)

MKC was located on the eastern shore of the Marmara Sea. Sampling depth of this location was 22 m. Total solid (TS) and total volatile solid (TVS) analysis were carried out. Total carbon (TC), total inorganic carbon (IC), total organic carbon (OC) contents and heavy metal concentrations of the sediment sample were also measured. All analysis were carried out according to the Standard Methods (APHA, 1995).

7. 3. Sediment Collection

For sampling event, the ship ARAR shown in the figure was used.

Figure 7. 2 The ship ARAR

Samples were taken using Van Ween Grab sampler (Hydro-bios, Germany). Samples were collected for FISH applications and sediment analysis.

50 ml 1:1 sample/ ethanol was taken for FISH application, fixed with ethanol and stored at -20 ^{0}C until FISH application.

50 ml sample was taken for sediment analysis and stored at -20 ^{0}C until extraction.

7. 4. Fluorescent in Situ Hybridization (FISH)

In FISH technique fluorescently mono-labelled rRNA –targeted oligonucleotide probes have been used. Each probe has optimum excitation and emission condition presented in Table 7. 1. These conditions for probes were obtained from literature (Amann et al., 1990a)

Table 7. 1. Fluorescent dyes using for FISH studies.

Fluorophore	**Color of Fluorescence**	**Excitation maximum(nm)**	**Emission maximum(nm)**
FLUOS(Fluorescein)	Green	494	518
TAMRA (Tetramethylrhodamine)	Orange	555	580
CY3	Red	550	565
DAPI (4´,6´-diamidino-2-phenylindole)	Green		

In this study, eight kind of Archaeal 16S rRNA –targeted oligonucleotide probes the were used. Arc915 was general Archaeal probe and the others were genus and family specific probes within these probes. Univ1392 is a general probe to the all known organisms and was used as a positive control of hybridization efficiency. Eubmix is a general bacterial probe complementary to the region of the 16S rRNA of the all bacteria. In addition to the positive control, the None-Eub probe was used used to assess nonspesific binding as a negative control. A well was also prepared as lacking a probe to monitor autoflorescences. Table 7. 2 shows names, target positions, sequences, and specifities of all probes that were used in this study.

All oligonucleotide probes were commercially provided from Qiagen Corporation. The probes were obtained as freeze-dried state and resuspended in TE buffer to obtain 500 ng/ml stock concentration. After suspension, the probes were stored at -20 ^{0}C.

Table 7. 2. Names, target positions, sequences of oligonucleotide probes (Raskin *et al*., 1994; Raskin *et al*., 1996 ; Pace *et al*., 1986 ; Manz *et al*., 1992)

Probe	Target Group	Target site	Probe Sequence (5´-3´)	Dye Type(5)
MC1109	Methanococcaceae	1128-1109	GCAACATAGGGCACGGGTCT	Cy3
MB310	Methanobacteriaceae	331-310	CTTGTCTCAGGTTCCATCTCCG	Cy3
MG1200	Methanomicrobiales	1220-1200	CGGATAATTCGGGGCATGCTG	Cy3
MS1414	Methanosarcina relatives	1434-1414	CTCACCCATACCTCACTCGGG	Cy3
MS821	Methanosarcina	844-821	CGCCATGCCTGACACCTAGCGAGC	Cy3
MX825	Methanosaeta	847-825	TCGCACCGTGGCCGACACCTAGC	TAMRA
ARC915	All Archaea	934-915	GTGCTCCCCCGCCAATTCCT	Cy3
EUBMIX	All Bacteria	338-355	GCTGCCTCCCGTAGGAGT	Cy3
UNIV1392	All Known Organisms	1407-1392	ACGGGCGGTGTGTAC	TAMRA
NONEUB	Non specific probe	355-338	ACTCCTACGGCAGGCAGC	TAMRA

The following steps were appied for FISH analysis: short-term fixation, ethanol fixation, hybridization, washing of excess probe, visualization of cells with epifluorescence microscope and processing&analysis of images with Image-Pro Plus 5 sofware.

7. 4. 1. Short Term Fixation

Samples were taken into sterile containers (50 ml 1:1 v/v sample-ethanol for each station) and stored at -20 ^{0}C during sampling.(September 2005-December 2005). Samples were transferred to the laboratory within ice boxes maintained at 4 ^{0}C and fixed with ethanol on 04 January 2005.

7. 4. 2. Ethanol Fixation

250 µl sediment sample-ethanol (1:1 v/v) was centrifuged at 13000 rpm for 3 minutes and supernatant was discarded. Then it was washed twice with 500 µl phosphate buffered saline (PBS) [130 mM NaCl p, 10 mM sodium phosphate, pH 7.2]. While washing, sample was pulled up and downed for 2 min using 2 ml syringe. After washing, it was suspended in 1 ml PBS-absolute ethanol (1:1 v/v) and stored at -20 ^{0}C.

7. 4. 3. Hybridization

25 µl of ethanol fixed sediment sample for each station was taken into ependorf tube and firstly washed twice with 500 µl PBS and then miliQ water. After washing, it was suspended in 1200 µl miliQ water. 5 µl sample for MD87 station, 10 µl sample for MKC station, 15 µl sample for IZ-25 station and 30 µl sample for IZ-17 station (amount of samples was experimentally optimized) was added on the teflon-coated slide and dried about 20 min at 46 ^{0}C in hybridizer. The slide was dehydrated at room temperature in increasing concentrations of ethanol (%50, %80 and %96 (v/v)) for 3 min each and ethanol was evaporated. 12 µl hybridization buffer (0.9M NaCl, 2mg/ml Ficoll, 2mg/ml Bovine Serum Albumen, 2mg/ml polyvinyl pyrrolidone, 5mM EDTA, pH 8.0, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) was added on dehydrated sample. Then 2µl of probe (50 ng μl^{-1}) was added to 12 µl hybridization buffer and incubated at 46 ^{0}C hybridization temperature for 3 hours. After incubation, 2 µl Dapi was added on each well and stained for 15 min. Then, the cells were washed with washing buffer containing 20 mmol l^{-1} Tris-HCl (pH 7.2), 0.01% SDS, 5 mmol l^{-1} EDTA and between 0.9 mol l^{-1} and 56 mmol l^{-1} NaCl according to the formula of Lathe (1985) for 15 min at the optimal washing temperature before final wash in miliQ water and air dried.

For each sample hybridization , two negative controls, one to assess nonspecific binding (Noneub) and the other to monitor autofluorescents (None), and one positive control (Univ1392), to assess success of hybridization and permeabilization of cells, were prepared . Optimum hybridization conditions for each probe are given in Table 7. 3.

Table 7. 3. Optimum hybridization conditions for oligonucleotide probes used in this study

PROBE	**Formamide Concentration** (%)	**NaCl Concentration** (mM)	**Hybridization Temperature** (^{0}C)	**Washing Temperature** (^{0}C)
ARC915	20%	225 mM	46 ^{0}C	48 ^{0}C
EUBMIX	10%	450 mM	46 ^{0}C	46 ^{0}C
MC1109	20%	225 mM	46 ^{0}C	48 ^{0}C
MB310	20%	225 mM	46 ^{0}C	48 ^{0}C
MG1200	20%	225 mM	46 ^{0}C	48 ^{0}C
MS1414	20%	225 mM	46 ^{0}C	48 ^{0}C
MS821	20%	225 mM	46 ^{0}C	48 ^{0}C
MX825	20%	225 mM	46 ^{0}C	48 ^{0}C
UNIV1392	0%	900 mM	37 ^{0}C	37 ^{0}C

7. 4. 4. Epifluorescence Microscopy

Hybridized cells on slide were examined with Olympus BX 50 epifluorescence microscope shown in the Figure 7. 3 equipped with a 100 W high pressure mercury lamp and a charged coupled device (CCD) camera. Spot 3.5 software were used while taking images. For each well, 10 random fields of view were obtained. In order to determine the relative abundance total microorganisms in each field of view, the images of dapi stained cells were taken with dapi filter. The hybridized microorganisms within total microorganisms determined with dapi staining were viewed from fluorescence images. In this study, Cy3 fluorocrome was used for all probes. Cy3 was excited by green light and emitted red fluorescence.

Figure 7. 3 Olympus BX 50 Epifluorescence Microscope

7. 4. 5. Image Processing and Analysis

The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.). Counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated.

8. RESULTS

8. 1. DAPI Staining

Samples were stained by DAPI to observe intact cell concentration in the anoxic sediments. Figure 8.1 shows the some examples of the DAPI stained cells.

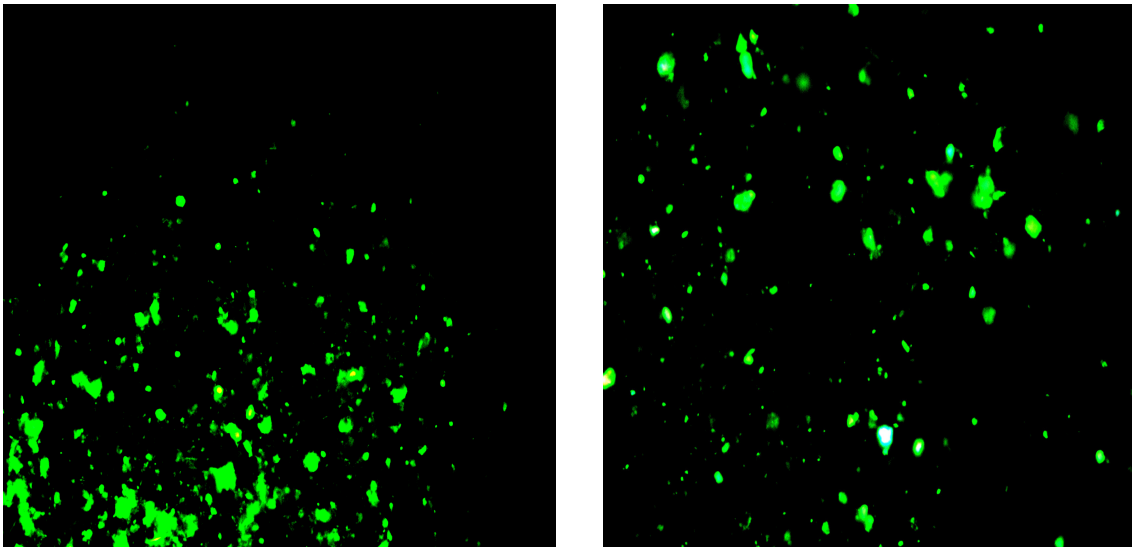

Figure 8. 1 Some examples of the DAPI staining results of the anoxic sediments

8. 2. Fluorescence *in situ* Hybridization (FISH) Results

The microbial community structure of anoxic sediments from four locations of the Marmara Sea were characterized using fluorescent rRNA targeted oligonucleotide probes specific for Bacteria, Archaea and phylogenetically defined groups of Methanogens. For each sample hybridization, two negative controls were prepared; one of these controls was used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was prepared to asses success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the four anoxic sediment locations were also stained using DNA stain DAPI to visualize intact cells in the samples.

8. 2. 1. FISH Results of the Gemlik Bay

Image examples of important microbial groups in the anoxic sediment of the Gemlik Bay were given in Figure 8. 2. Number and composition of methanogenic archaeal populations in anoxic sediments of the location were given in Table 8.1. Identified microbial species were grouped according to their trophic levels and the quantification results were rearranged in Table 8.2.

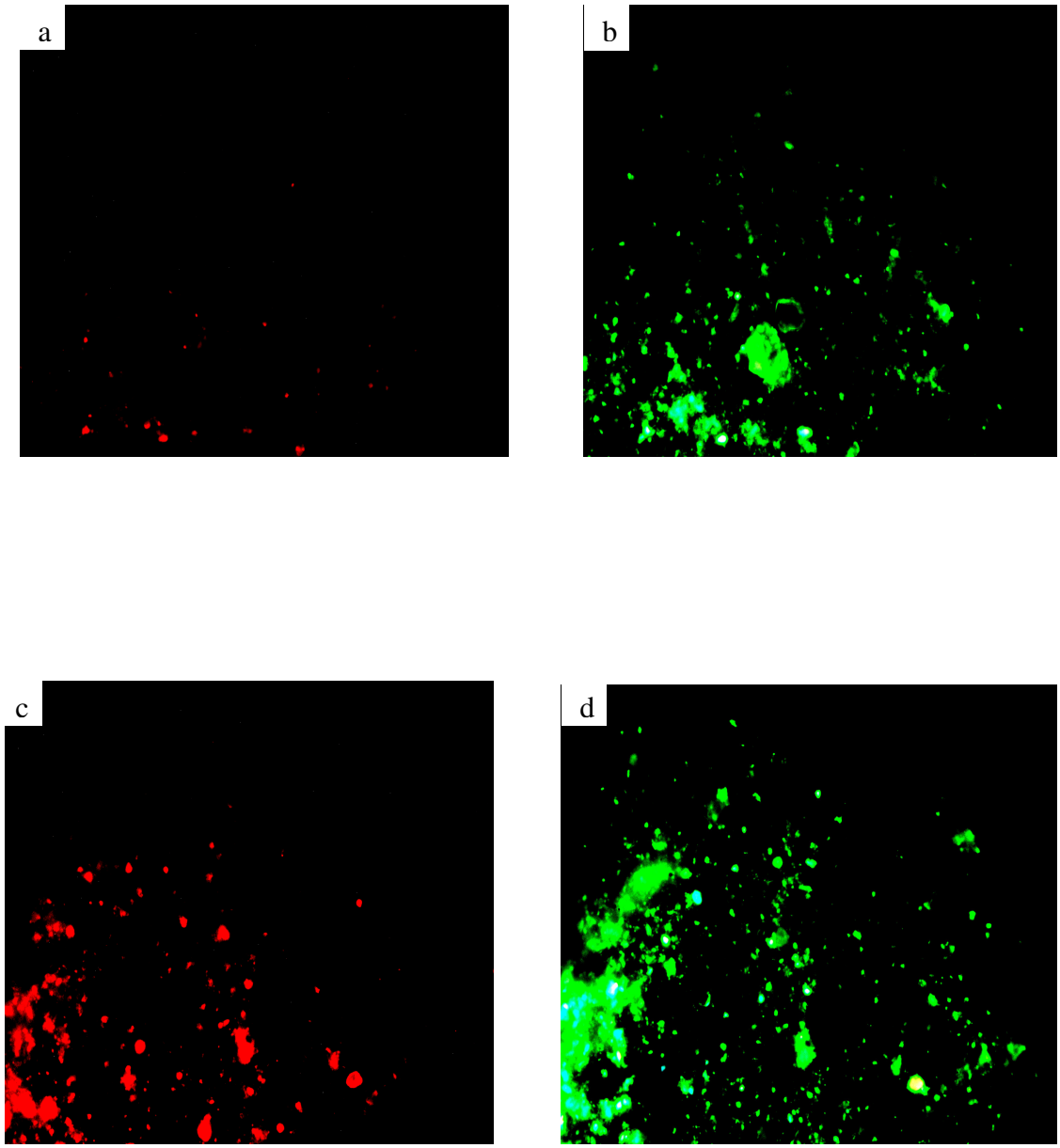

Figure 8. 2 **.** Image examples of important microbial groups in the sediment of Gemlik location a) Fluorescent view of hybridized cells with Arc915 (Archaea) probe b) DAPI view of the same area c) Fluorescent view of hybridized cells with MB310 (*Methanobacteriales*) probe d) DAPI view of the same area

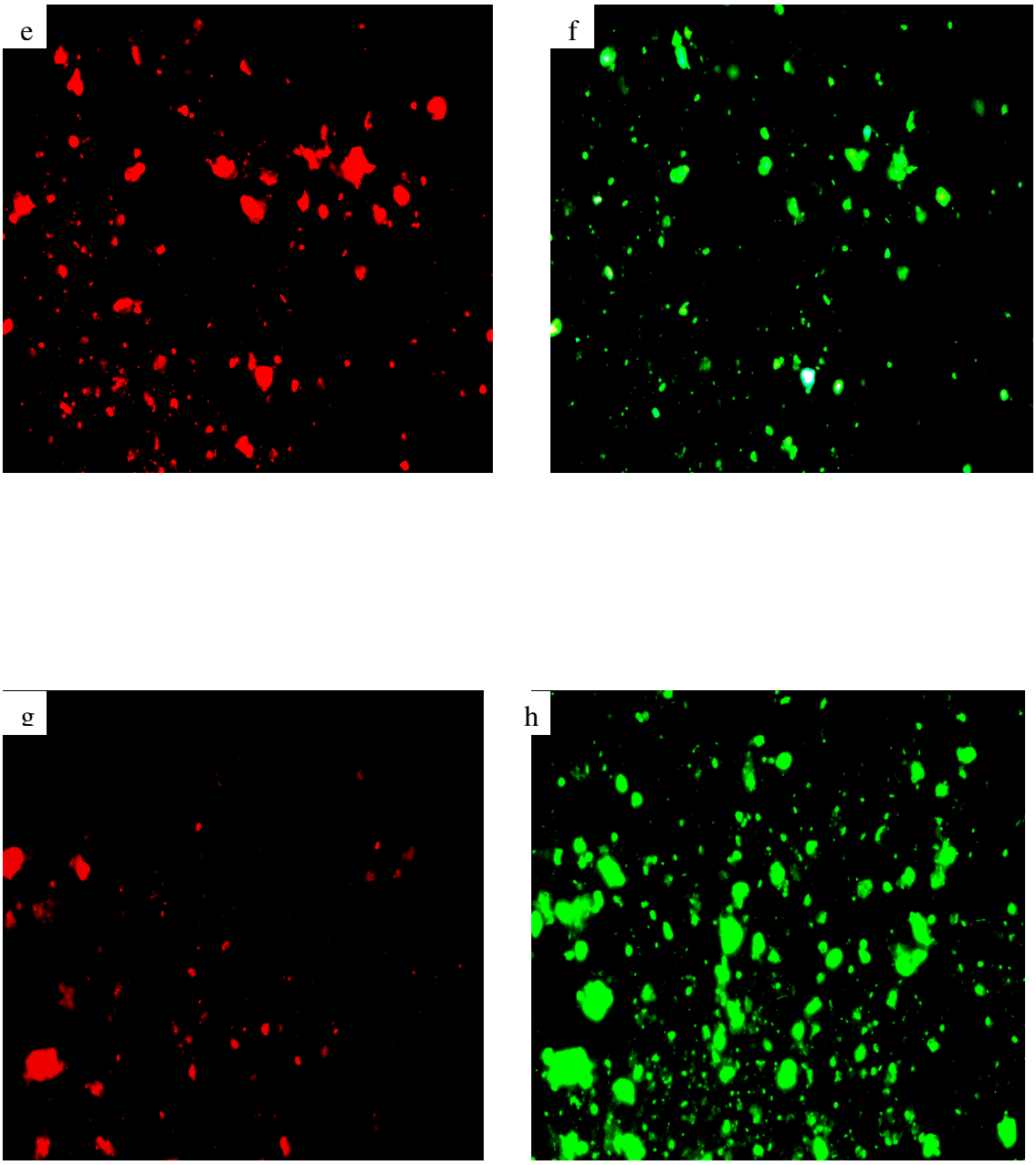

Figure 8. 2 (continued) e) Fluorescent view of hybridized cells with MC1109 (*Methanococcales*) probe f) DAPI view of the same area g) Fluorescent view of hybridized cells with MG1200 (Methanogenium relatives) probe h) DAPI view of the same area

67%±8% (mean ± standard deviation) of DAPI stained total microorganisms in anoxic sediment were detected using universal probe. Relative abundance of bacterial cells within the sediment is 51%±9% and relative abundance of archaeal cells is 32%±4% according to FISH results. The relative abundances of Methanosarcina relatives, *Methanococcales*, *Methanobacteriales,* *Methanogenium* relatives, *Methanosarcina, Methanosaeta are* 9%±1%, 8%±0%, 7%±0.3%, 4%±1%, 4%±0.5% and 3%±0.4% respectively. Total methanogens within the archael populations is 31%. Trophically, H_2 utilizing methanogens are the dominant groups with percent of 19. There are also 7% acetate utilizing methanogens in anoxic sediment of the location.

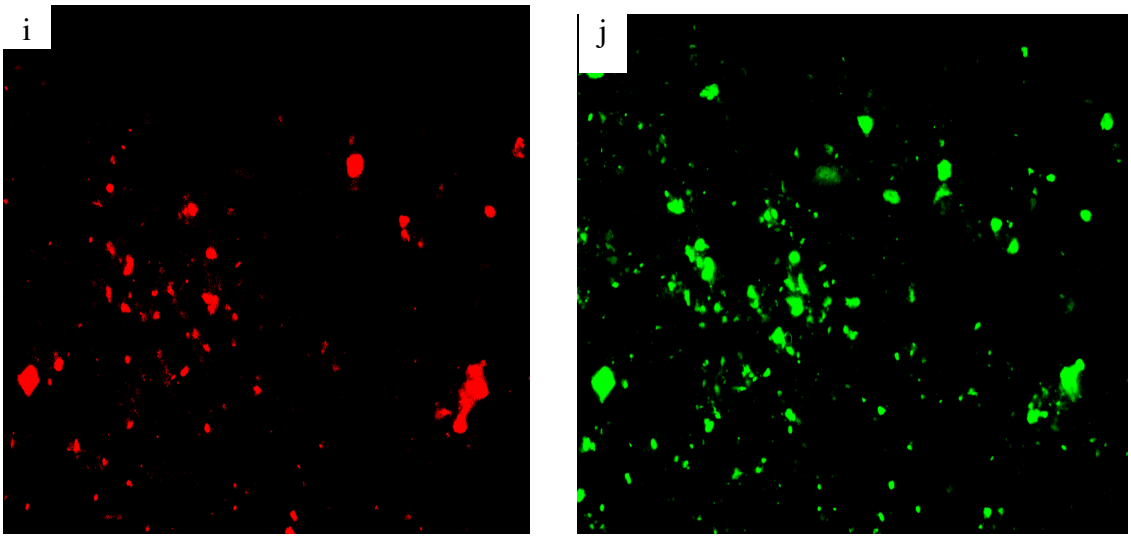

Figure 8. 2 (continued) i) Fluorescent view of hybridized cells with MS821 (Methanosarcina) probe j) DAPI view of the same area

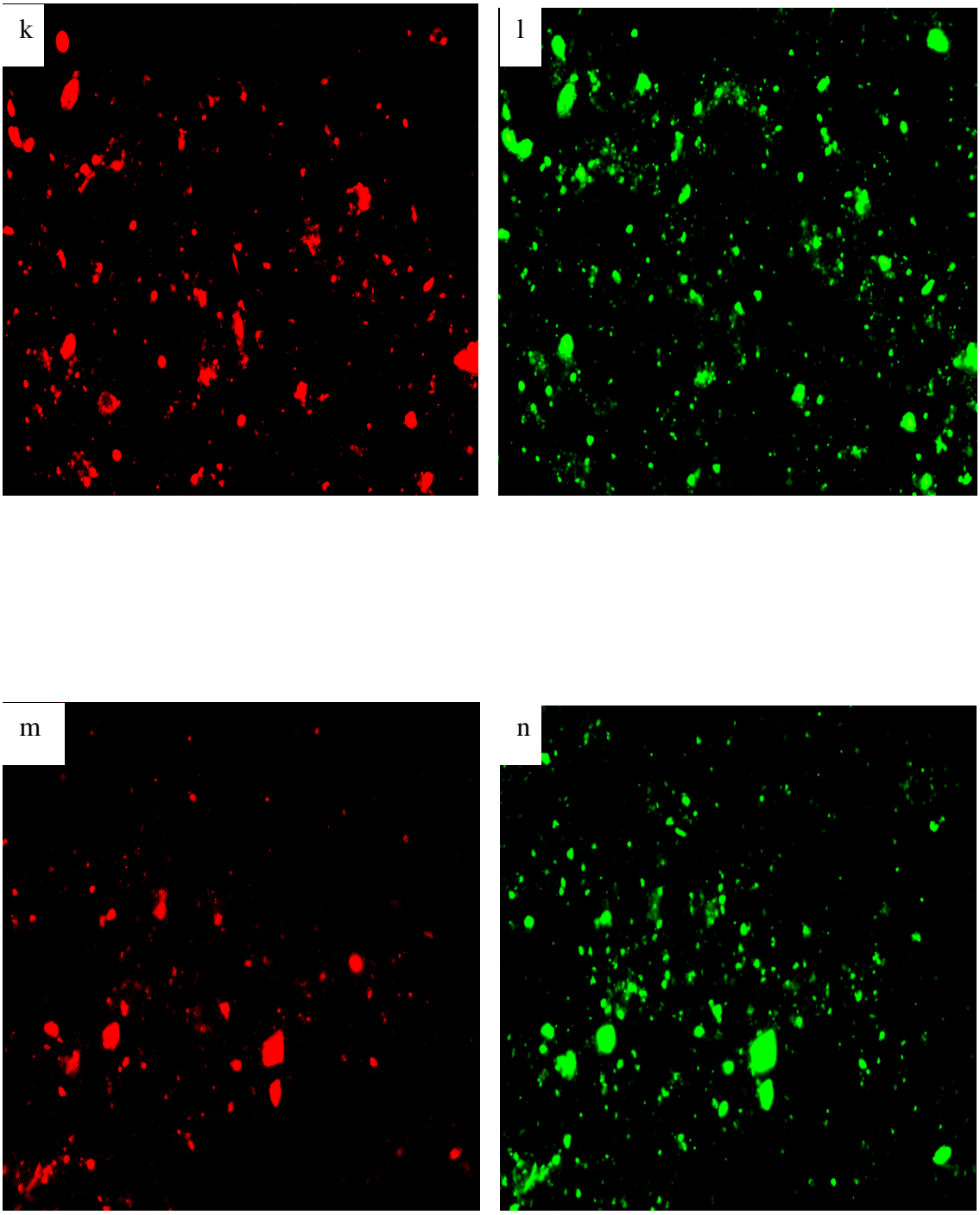

Figure 8. 2 (continued) k) Fluorescent view of hybridized cells with MS1414 (Methanosarcina + relatives) probe l) DAPI view of the same area m) Fluorescent view of hybridized cells with MX825 (Methanosaeta) n) DAPI view of the same area

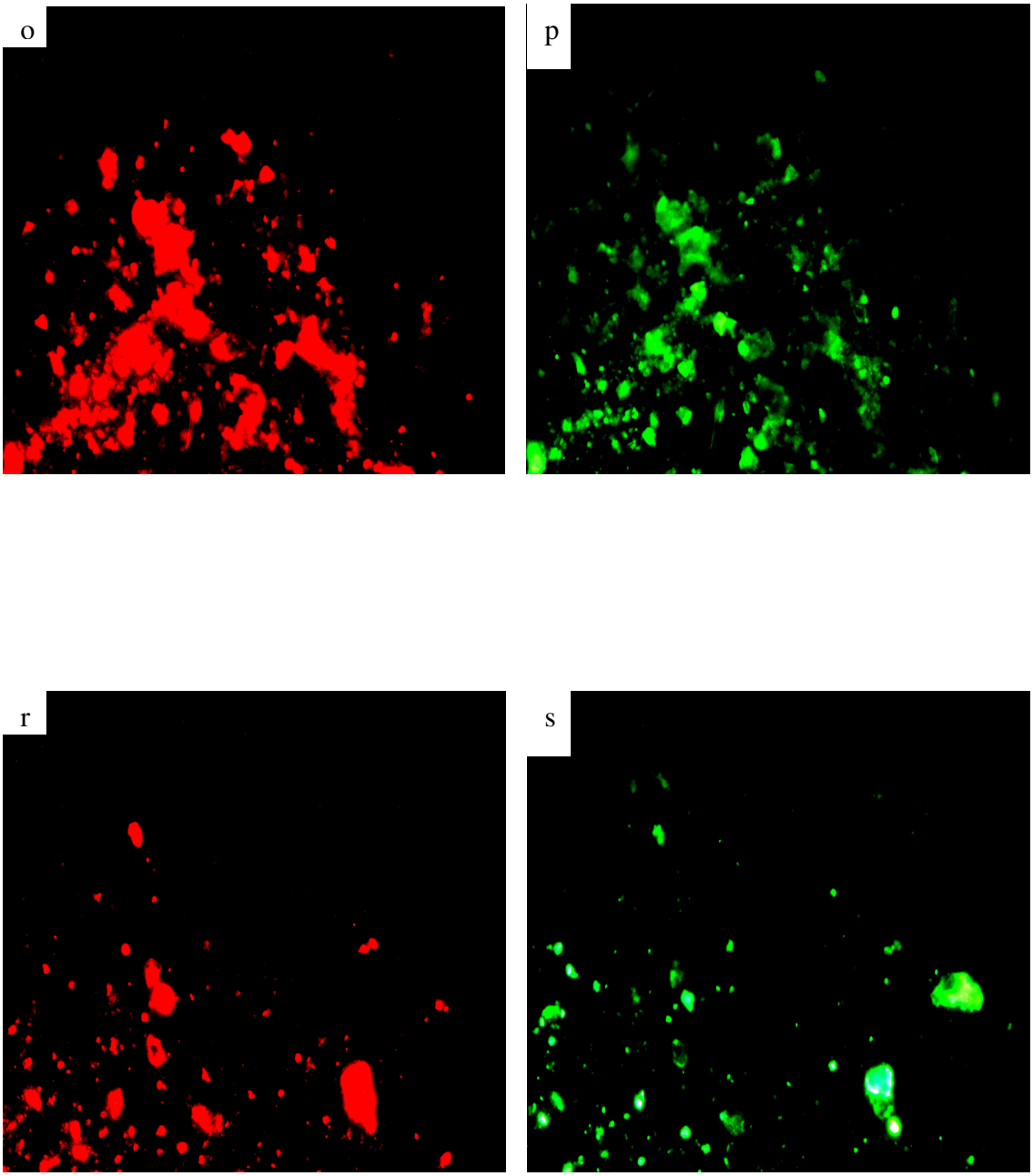

Figure 8. 2 (continued) o) Fluorescent view of hybridized cells with Eubmix (Eubacteria) probe p) DAPI view of the same area r) Fluorescent view of hybridized cells with UNIV1392 (Universal) probe s) DAPI view of the same area

8. 2. 2. FISH Results of the Izmit Bay (IZ17)

Image examples of important microbial groups in the anoxic sediment of the Izmit Bay (IZ17) were shown in Figure 8. 3. Ouantification results of methanogenic archaeal populations in anoxic sediments of Izmit Bay (IZ-17) were given in Table 8.1. Microbial species grouped according to their trophic levels and the quantification results were shown in Table 8.2.

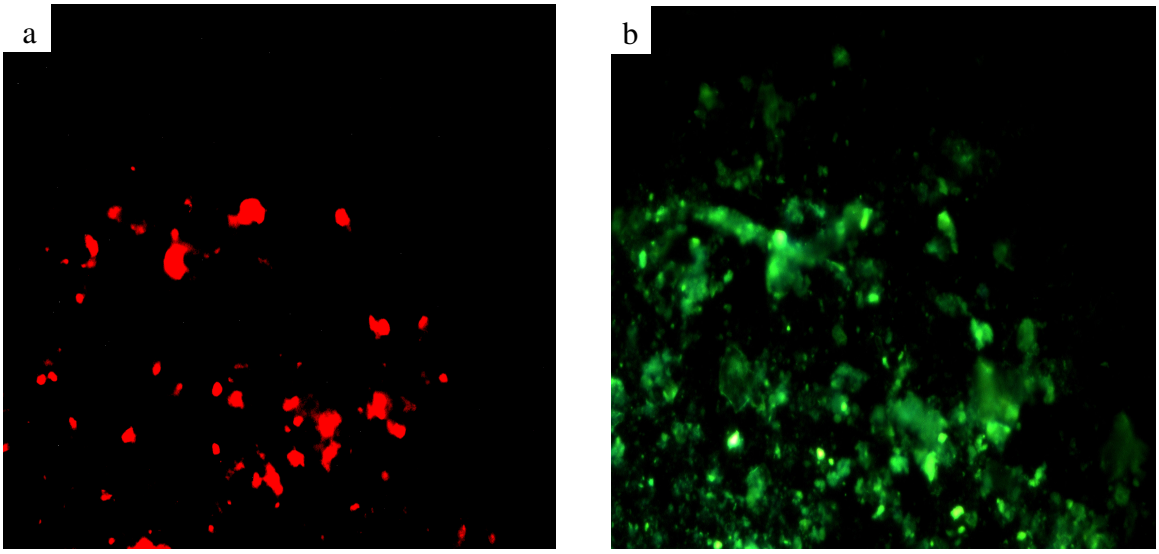

Figure 8. 3 **.** Image examples of important microbial groups in the sediment of Izmit Bay (IZ17) location a) Fluorescent view of hybridized cells with Arc915 (Archaea) probe b) DAPI view of the same area

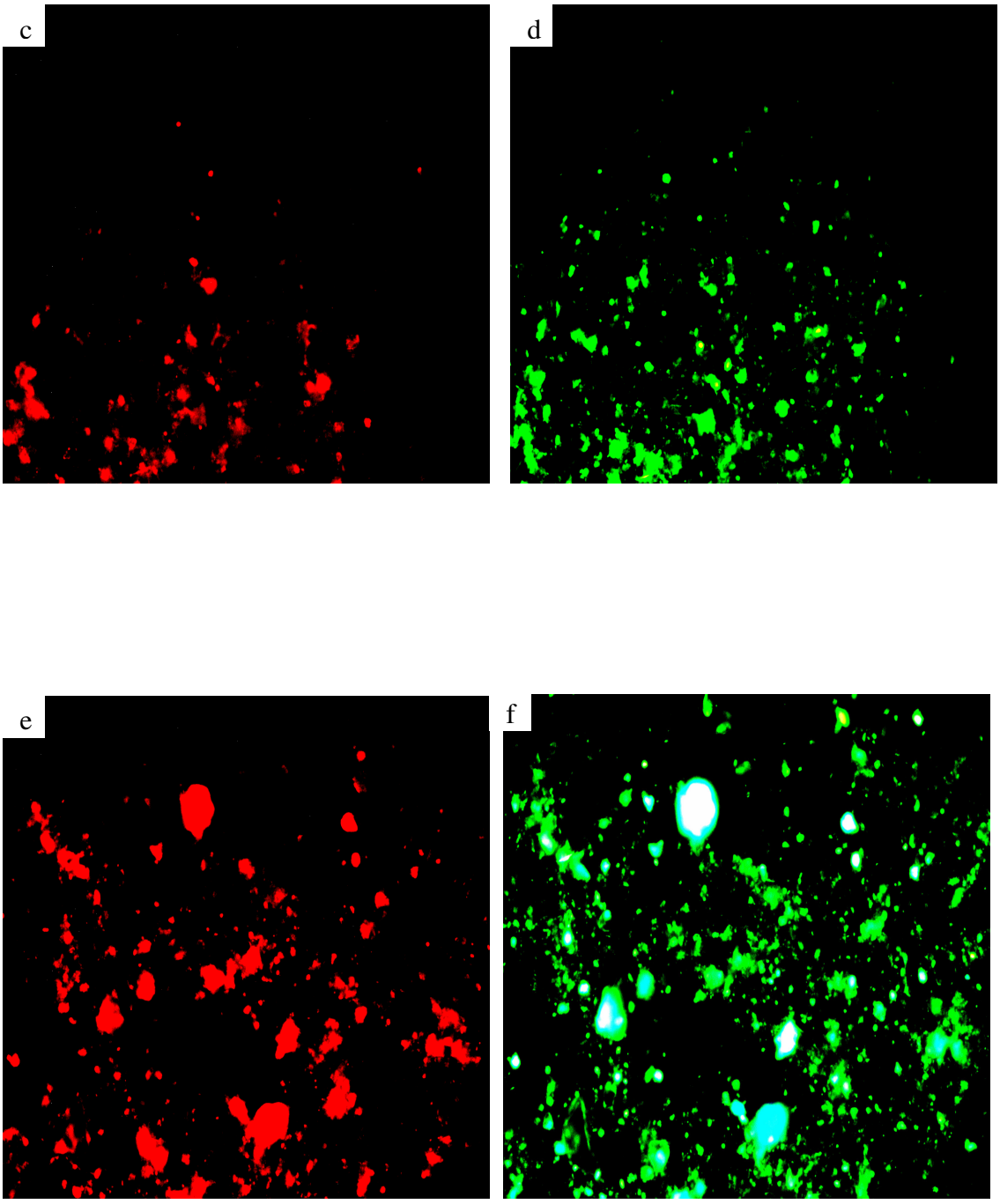

Figure 8. 3 (continued) c) Fluorescent view of hybridized cells with MB310 (*Methanobacteriales*) probe d) DAPI view of the same area e) Fluorescent view of hybridized cells with MC1109 (*Methanococcales*) probe f) DAPI view of the same area

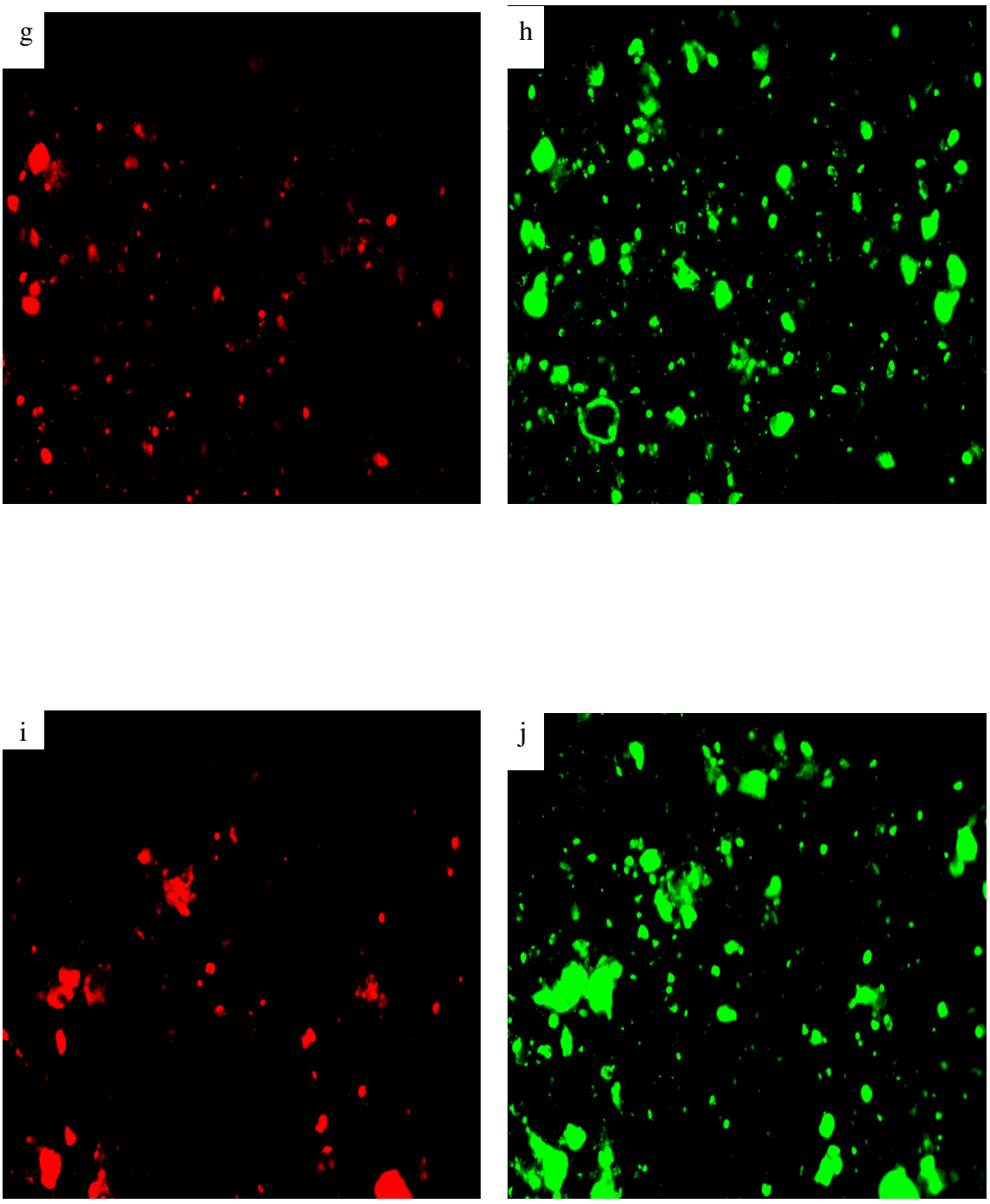

Figure 8. 3 (continued) g) Fluorescent view of hybridized cells with MG1200 (Methanogenium relatives) probe h) DAPI view of the same area i) Fluorescent view of hybridized cells with MS821 (Methanosarcina) probe j) DAPI view of the same are

62%±12% (mean ± standard deviation) of DAPI stained total microorganisms in anoxic sediment were determined using universal probe. Relative abundance of bacterial cells within the sediment is 73%±15% and relative abundance of archaeal cells is 23%±3% according to FISH results. The relative abundances of *Methanobacteriales,* Methanosarcina relatives, *Methanococcales*, *Methanosarcina, Methanogenium* relatives*, Methanosaeta are* 9%±0.3%, 8%±1%, 7%±0.5%, 6%±1.1%, 5%±1% and 3%±1% respectively. Total methanogens within the archael populations is 32%. Hydrogenotrophic methanogens are 21% as the dominant trophic group . The relative abundance of acetoclastic methanogens is 9%.

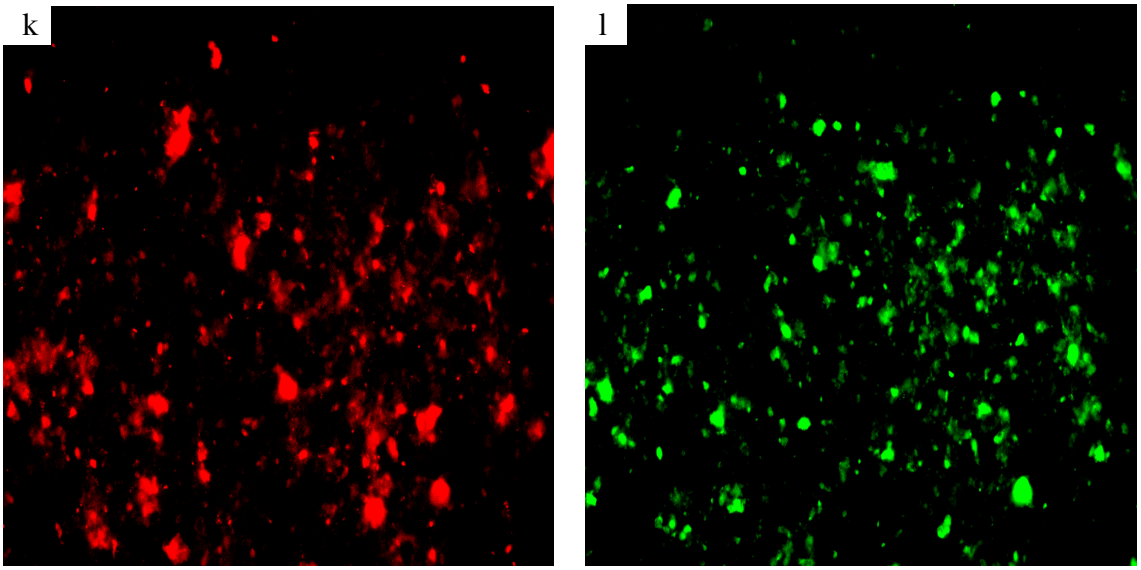

Figure 8. 3 (continued) k) Fluorescent view of hybridized cells with MS1414 (Methanosarcina + relatives) probe l) DAPI view of the same area

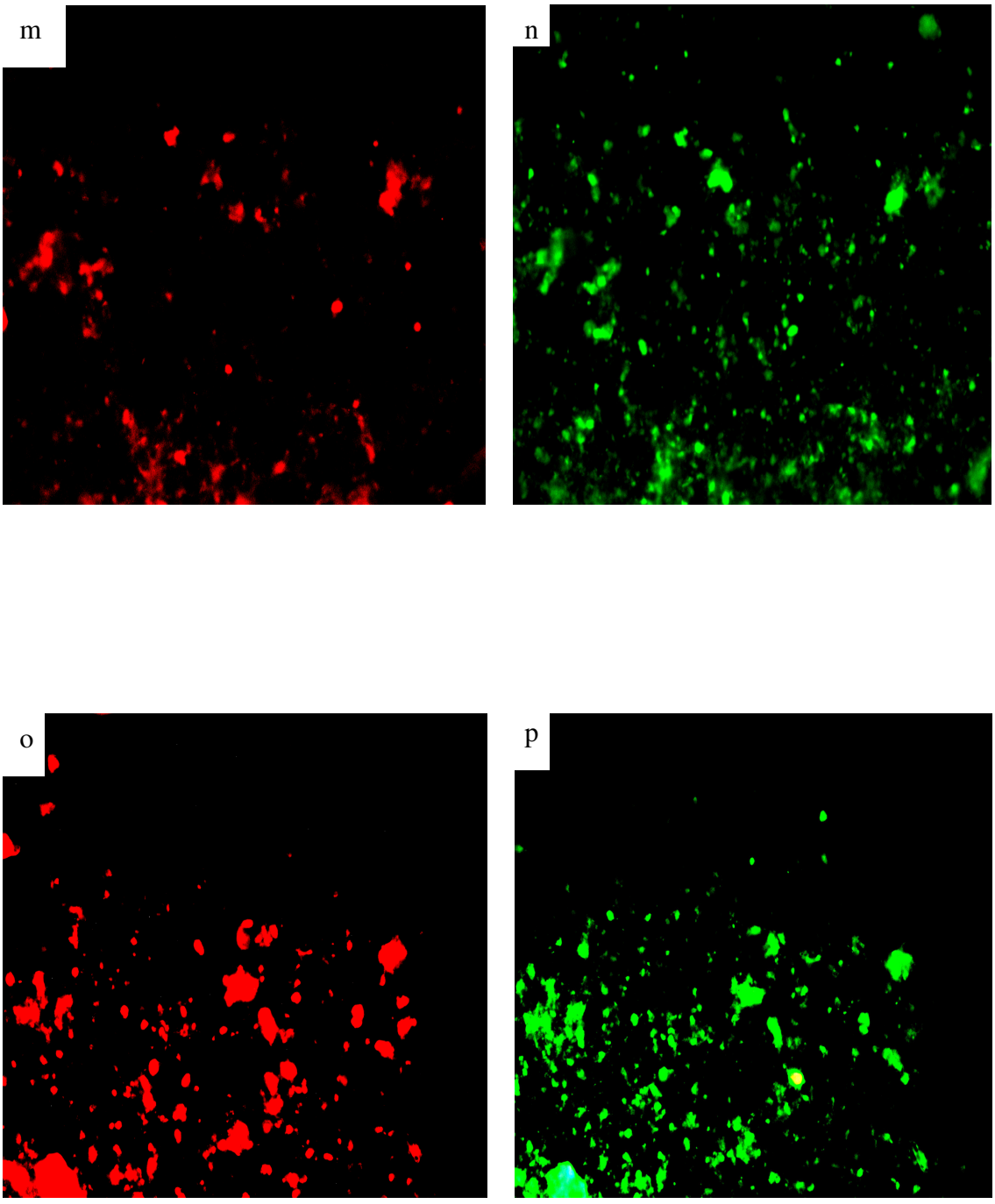

Figure 8. 3 (continued) m) Fluorescent view of hybridized cells with MX825 (Methanosaeta) n) DAPI view of the same area o) Fluorescent view of hybridized cells with Eubmix (Eubacteria) probe p) DAPI view of the same area

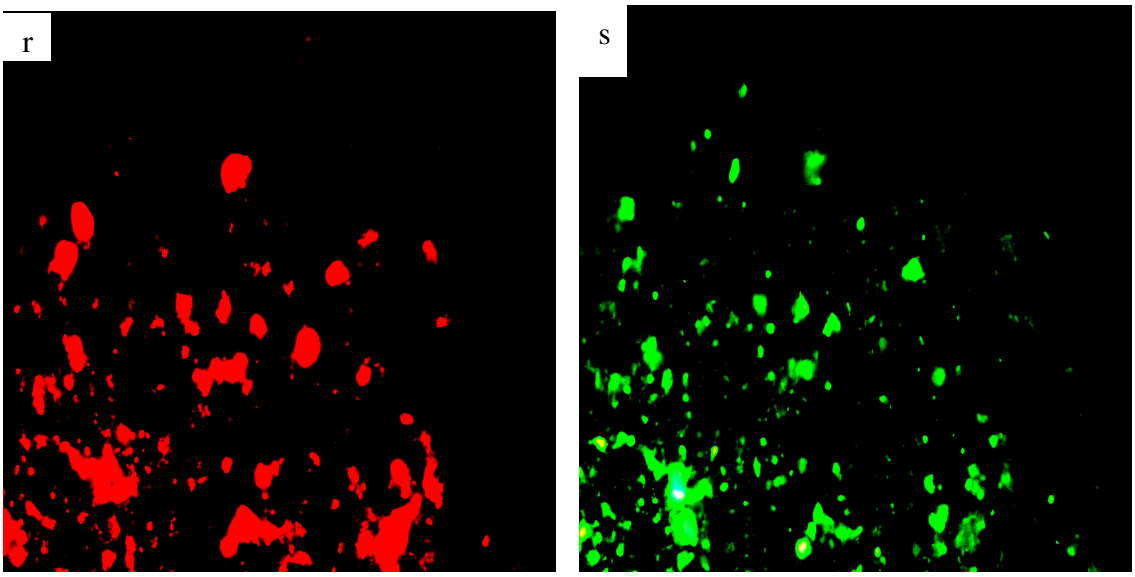

Figure 8. 3 (continued) r) Fluorescent view of hybridized cells with UNIV1392 (Universal) probe s) DAPI view of the same area

8. 2. 3. FISH Results of the Izmit Bay (IZ25)

Image examples of important microbial groups in the anoxic sediment of the Izmit Bay (IZ25) were shown in Figure 8. 4. The number of methanogenic archaeal populations in anoxic sediments of Izmit Bay (IZ-25) were given in Table 8.1. Quantification results of microbial groups according to their trophic levels were shown in Table 8.2.

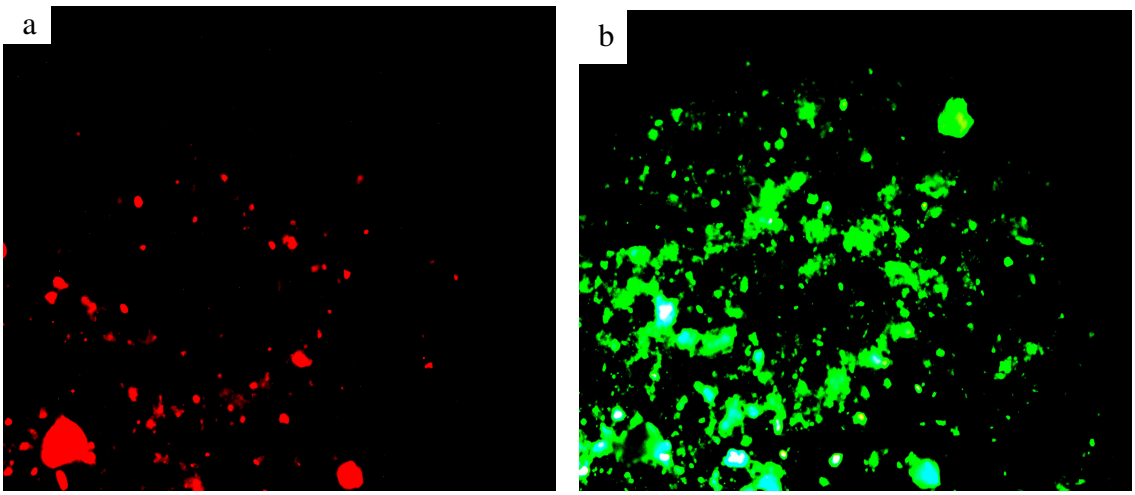

Figure 8. 4 . Image examples of important microbial groups in the sediment of Izmit Bay (IZ25) location a) Fluorescent view of hybridized cells with Arc915 (Archaea) probe b) DAPI view of the same area

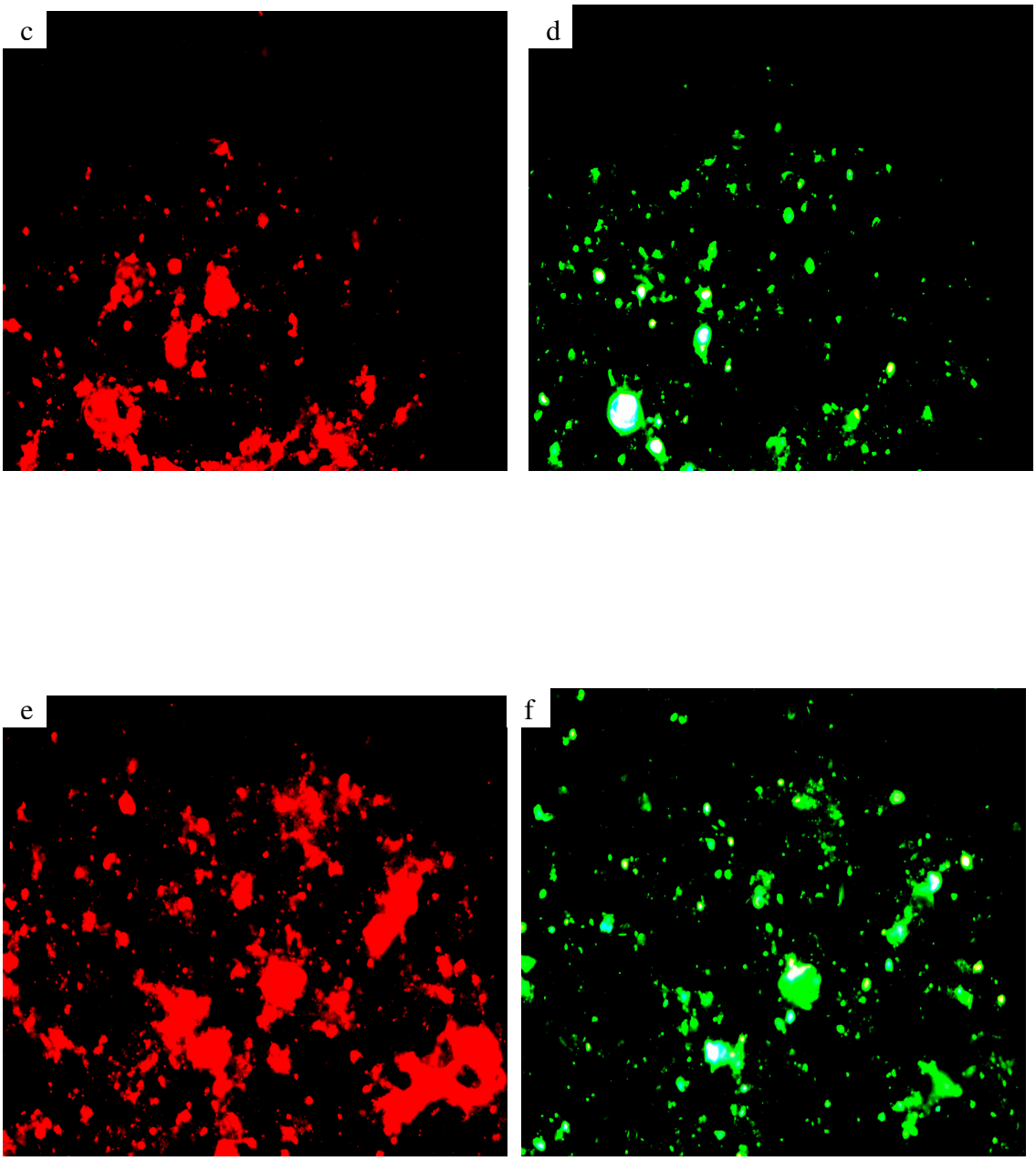

Figure 8. 4 (continued) c) Fluorescent view of hybridized cells with MB310 (*Methanobacteriales*) probe d) DAPI view of the same area e) Fluorescent view of hybridized cells with MC1109 (*Methanococcales*) probe f) DAPI view of the same area

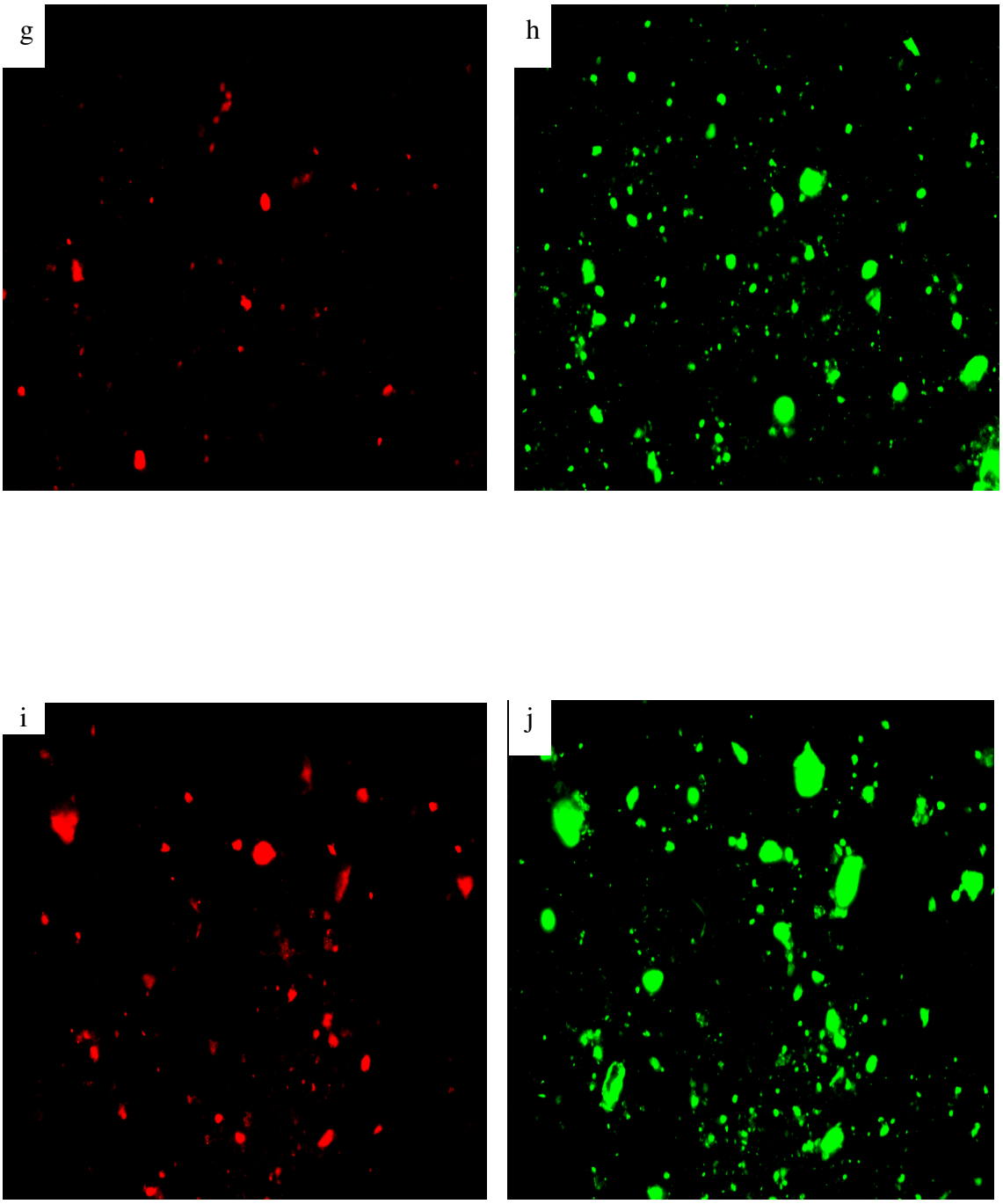

Figure 8. 4 (continued) g) Fluorescent view of hybridized cells with MG1200 (Methanogenium relatives) probe h) DAPI view of the same area i) Fluorescent view of hybridized cells with MS821 (Methanosarcina) probe j) DAPI view of the same are

77%±13% (mean ± standard deviation) of DAPI stained total microorganisms in anoxic sediment were determined using universal probe. Relative abundance of bacterial cells within the sediment is 64%±8% and relative abundance of archaeal cells is 33%±2% according to FISH results. *Methanococcales*, *Methanobacteriales,* Methanosarcina relatives, *Methanosarcina, Methanosaeta, Methanogenium* relatives *are* 10%±0.5%, 10%±1%, 8%±1%, 5%±0.1%, 5%±0.3% and 3%±0.4% respectively. Trophically, total archaeal methanogens is 36%. Hydrogenotrophic methanogens are 23% as the dominant trophic group . The relative abundance of acetoclastic methanogens is 10%.

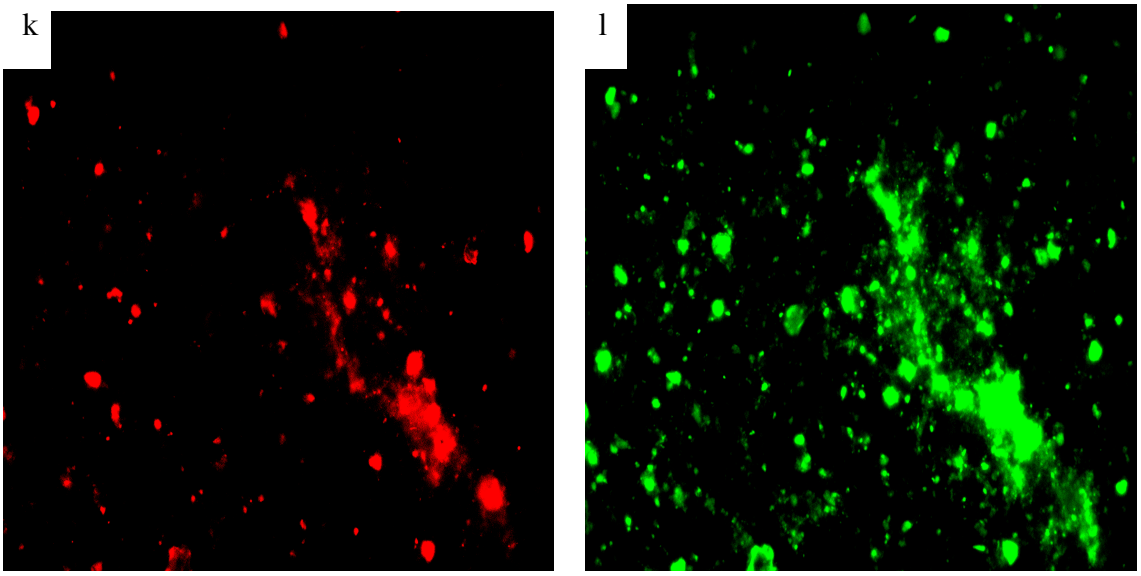

Figure 8. 4 (continued) k) Fluorescent view of hybridized cells with MS1414 (Methanosarcina + relatives) probe l) DAPI view of the same area

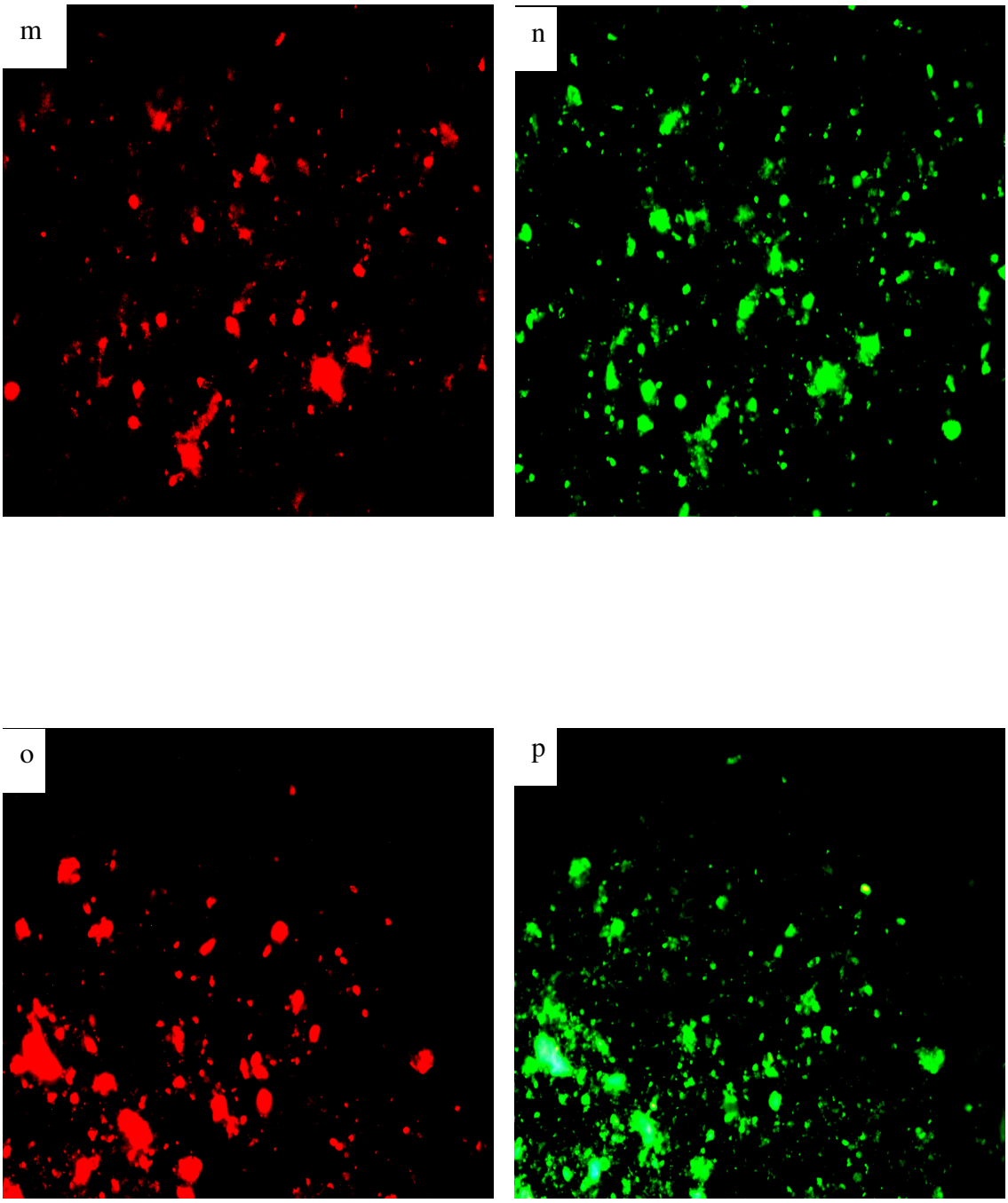

Figure 8. 4 (continued) m) Fluorescent view of hybridized cells with MS821 (Methanosarcina) probe n) DAPI view of the same area o) Fluorescent view of hybridized cells with Eubmix (Eubacteria) probe p) DAPI view of the same area

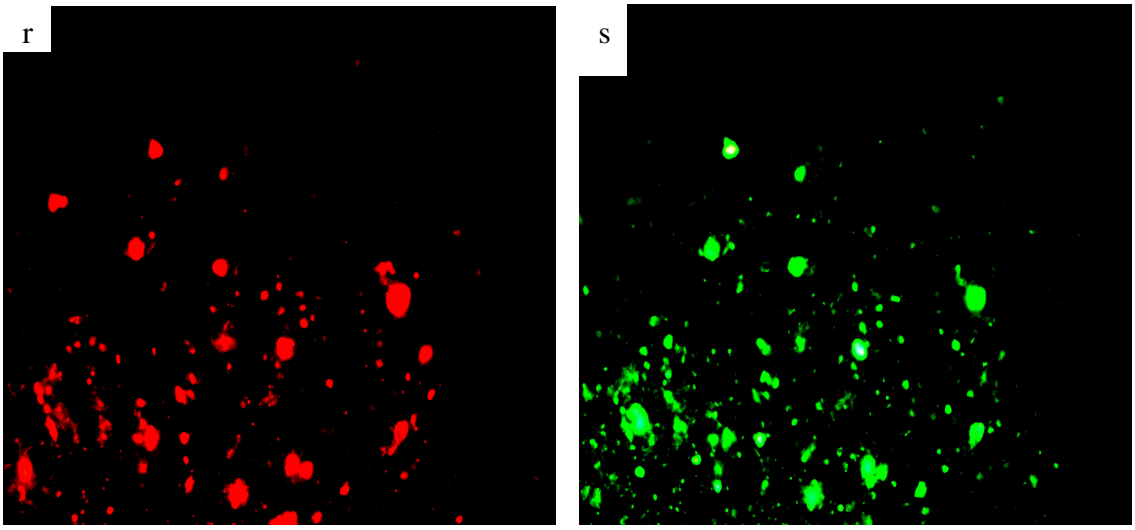

Figure 8. 4 (continued) r) Fluorescent view of hybridized cells with UNIV1392 (Universal) probe s) DAPI view of the same area

8. 2. 4. FISH Results of the Kucukcekmece

Image examples of important microbial groups in the anoxic sediment of the Izmit Bay (IZ25) were shown in Figure 8. 5. Number and composition of methanogenic archaeal populations in anoxic sediments of the location were given in Table 8.1. Identified microbial species were grouped according to their trophic levels and the quantification results were rearranged in Table 8.2.

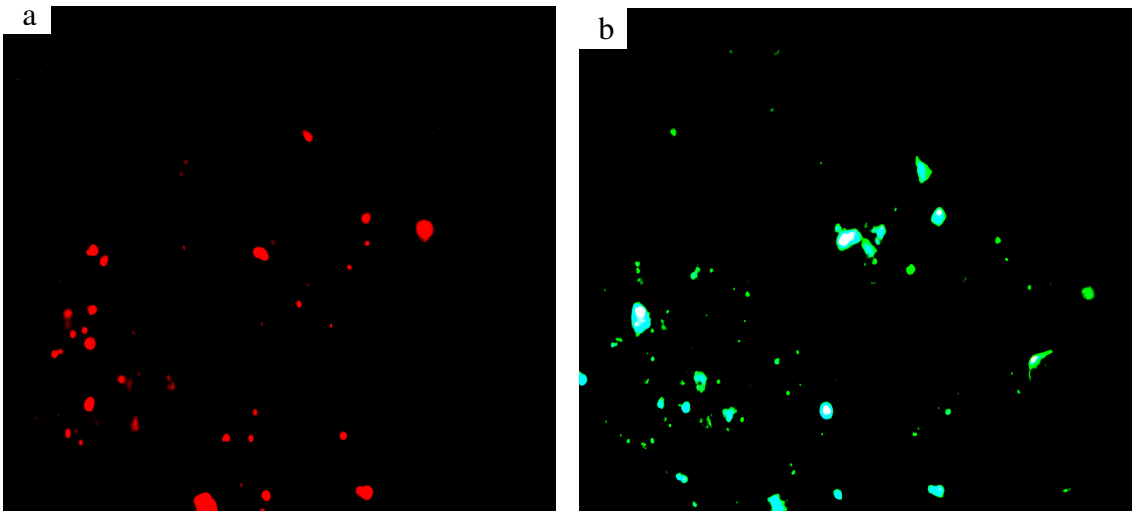

Figure 8. 5 . Image examples of important microbial groups in the sediment of Kucukcekmece location a) Fluorescent view of hybridized cells with Arc915 (Archaea) probe b) DAPI view of the same area

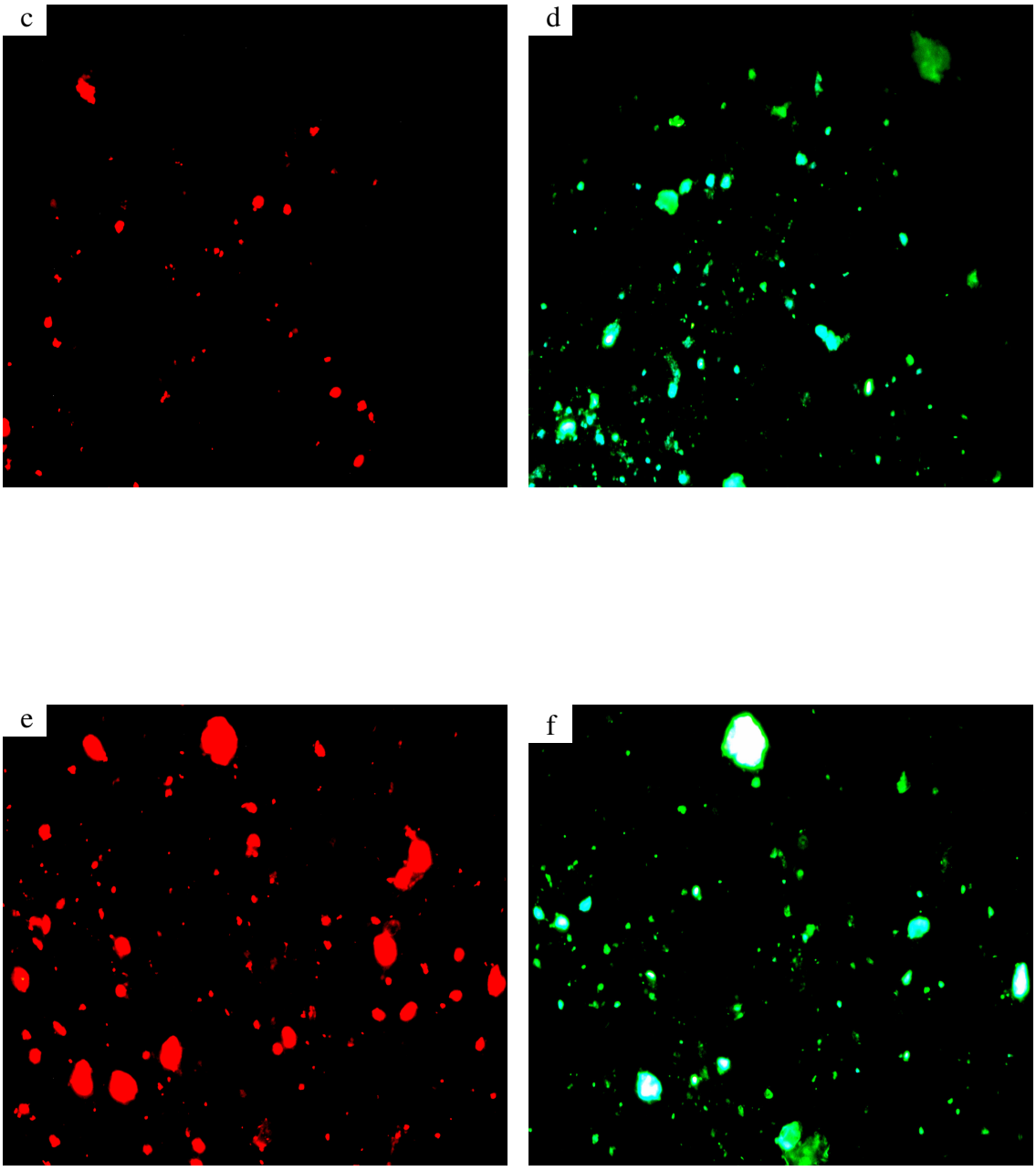

Figure 8. 5 (continued) c) Fluorescent view of hybridized cells with MB310 (*Methanobacteriales*) probe d) DAPI view of the same area e) Fluorescent view of hybridized cells with MC1109 (*Methanococcales*) probe f) DAPI view of the same area

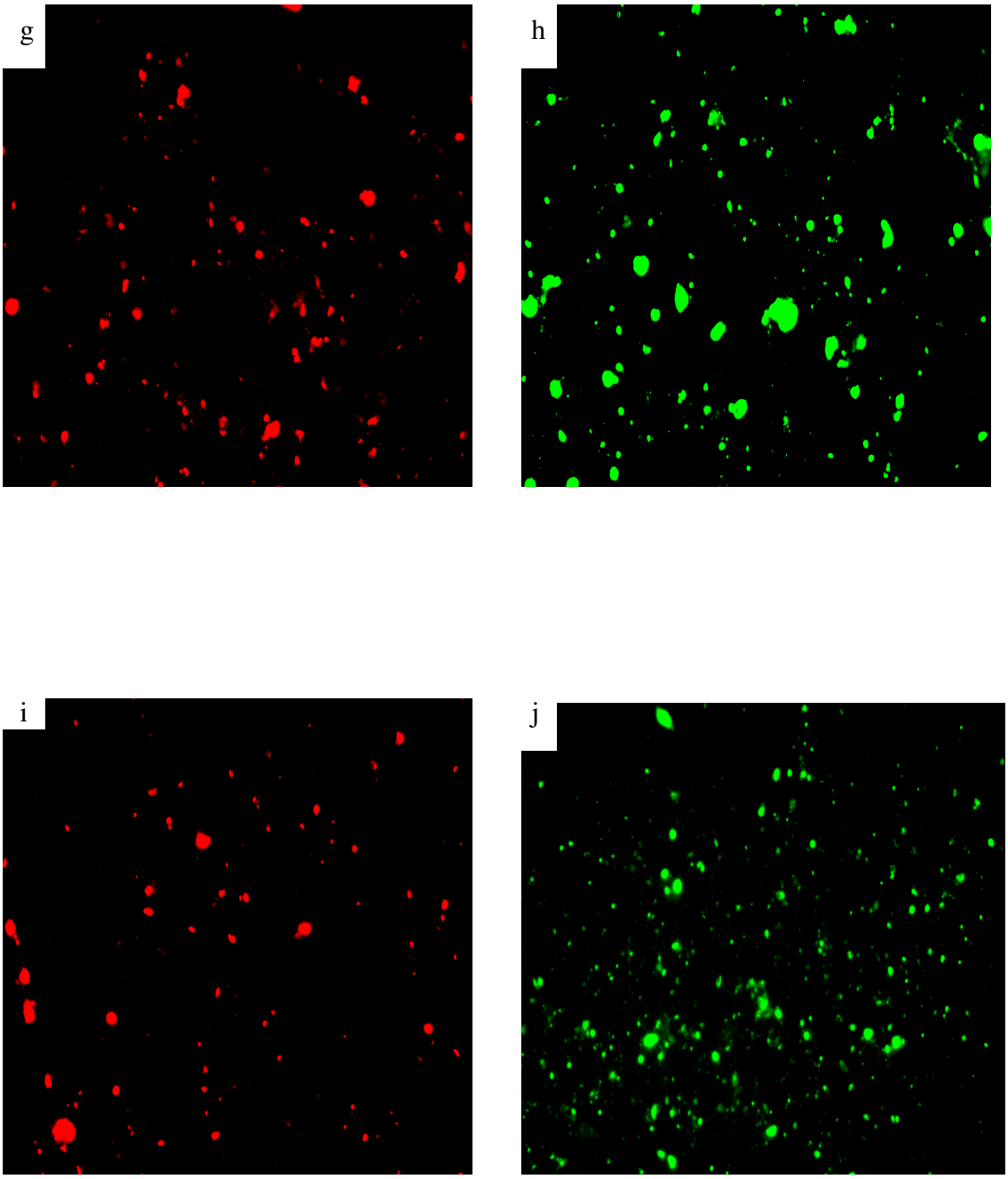

Figure 8. 5 (continued) g) Fluorescent view of hybridized cells with MG1200 (Methanogenium relatives) probe h) DAPI view of the same area i) Fluorescent view of hybridized cells with MS821 (Methanosarcina) probe j) DAPI view of the same are

80%±7% (mean ± standard deviation) of DAPI stained total microorganisms in anoxic sediment were determined using universal probe. Relative abundance of bacterial cells within the sediment is 70%±16% and relative abundance of archaeal cells is 29%±7% according to FISH results. The relative abundances of Methanosarcina relatives, *Methanococcales*, *Methanosaeta Methanobacteriales, Methanogenium* relatives, *Methanosarcina, are* 8%±0.2%, 8%±1%, 7%±0.4%, 6%±0.5%, 6%±1% and 4%±0.4% respectively. Total methanogens within the archael populations is 35%. Trophically, H_2 utilizing methanogens are the dominant groups with percent of 20. There are also 11% acetate utilizing methanogens in anoxic sediment of the location.

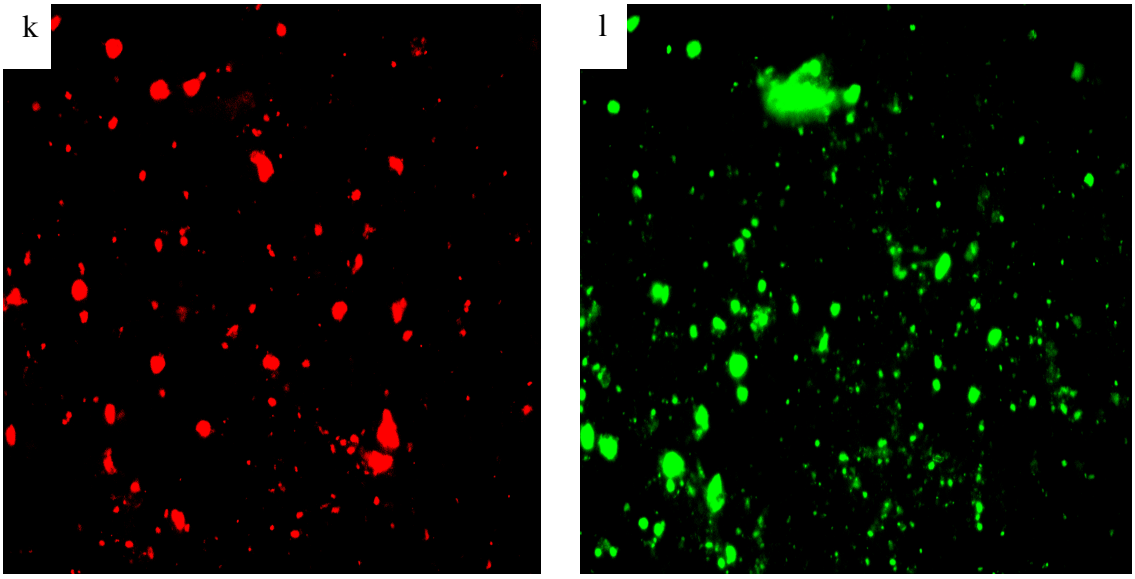

Figure 8. 5 (continued) k) Fluorescent view of hybridized cells with MS1414 (Methanosarcina + relatives) probe l) DAPI view of the same area

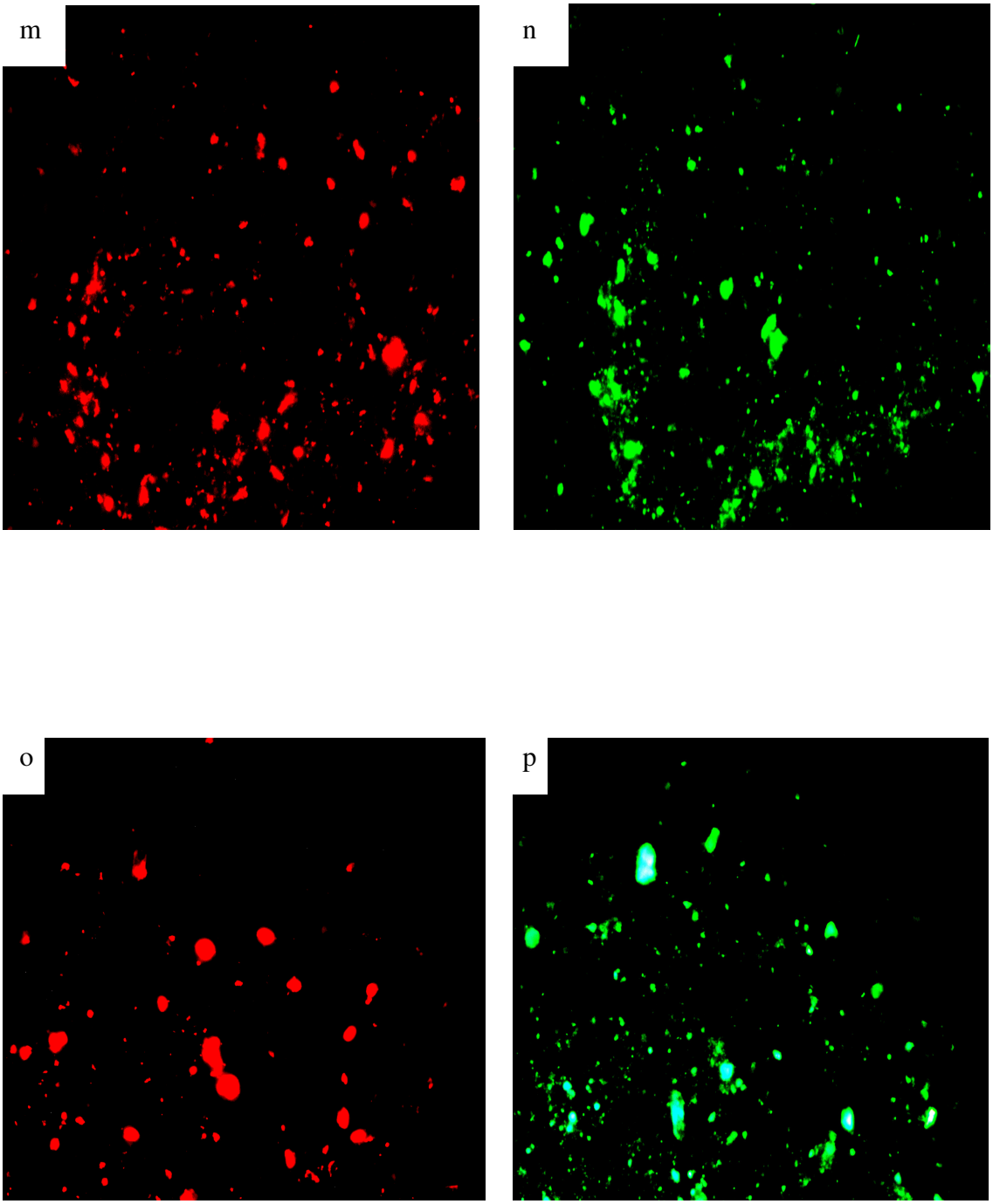

Figure 8. 5 (continued) m) Fluorescent view of hybridized cells with MX825 (Methanosaeta) n) DAPI view of the same area o) Fluorescent view of hybridized cells with Eubmix (Eubacteria) probe p) DAPI view of the same area

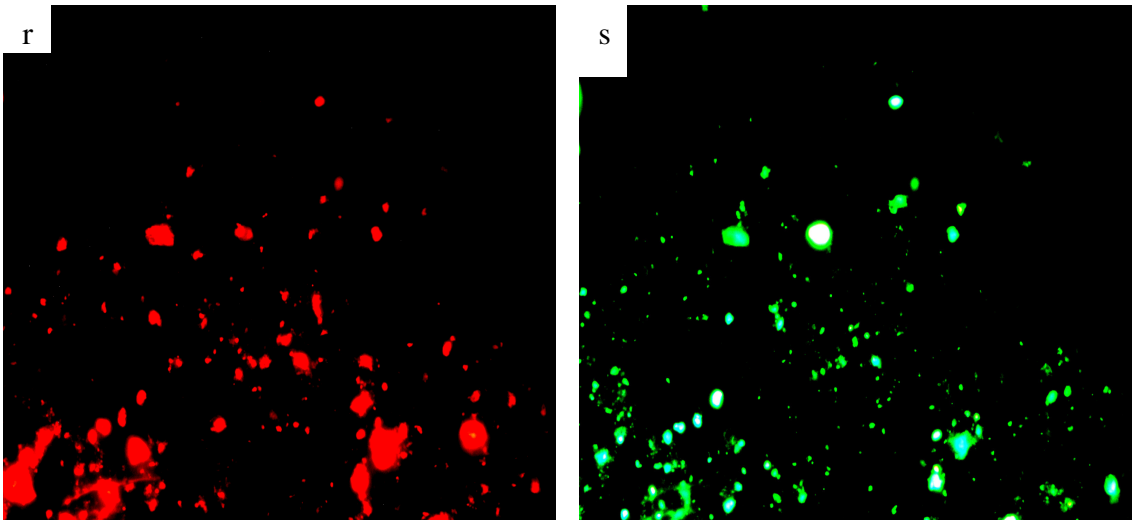

Figure 8. 5 (continued) r) Fluorescent view of hybridized cells with UNIV1392 (Universal) probe s) DAPI view of the same area

8. 3. Quantification Results

Although it is difficult to permeabilize microorganisms in the marine sediments, 67%±8%, 62%±5%, 77%±11% and 80%±7% of DAPI stained total microorganisms in sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece respectively were detected using universal probe in this study. According to FISH results, relative abundances of bacterial cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 51%±9%, 73%±11%, 64%±8% and 80%±12% respectively. According to FISH results, relative abundances of archaeal cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 32%±4%, 23%±3%, 33%±2% and 29%±7% respectively. Total archael methanogens in anoxic sediments of Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 31%, 32%, 36% and 35%.

These results revealed that there are active archaeal populations within the the anoxic sediments from the Marmara Sea, and methanogenesis may be occurring in the sediments at important levels.

Table 8. 1 Quantification Results of the Sediment Samples

Microbial group (Prob name)	**Relative abundance of microbial groups (%)**			
	Gemlik (MD87)	**Kucukcekmece (MKC)**	**Izmit (IZ-17)**	**Izmit (IZ-25)**
Universal (UNIV1392)	67±8	80±7	62±12	77±13
Eubacteria (Eubmix)	51±9	70±16	73±15	64±8
Archaea (ARC915)	32±4	29±7	23±3	33±2
Methanobacteriales (MB310)	7±0.3	6±0.5	9±0.3	10±1
Methanococcales (MC1109)	8±0	8±1	7±0.5	10±0.5
Methanogenium relatives (MG1200)	4±1	6±1	5±1	3±0.4
Methanosarcina (MS821)	4±0.5	4±0.4	6±1.1	5±0.3
Methanosarcina + relatives (MS1414)	9±1	8±0.2	8±1	8±1
Methanosaeta (MX825)	3±0.4	7±0.4	3±1	5±0.3

Table 8. 2.Grouping the identified microbial species according to their trophic levels

	Relative abundance of microbial groups (%)			
	Gemlik (MD87)	**Kucukcekmece (MKC)**	**Izmit (IZ-17)**	**Izmit (IZ-25)**
Total Methanogens (MB310+MC1109+ MG1200+MS1414+MX825)	31	35	32	36
H_2 utilizing methanogens (MB310+MC1109+MG1200)	19	20	21	23
Acetate utilizing methanogens (MS821+MX825)	7	11	9	10

8. 4. Results of the Sediment Analysis

Results of total solid (TS) and total volatile solid (TVS) analysis for the locations studied are given Table 8.3.

Table 8. 3. TS and TVS concentrations of the anoxic sediment samples from the Marmara Sea

Location of the Sample	**TS(mg/ml)**	**TVS(mg/ml)**
Küçükçekmece (MKC)	450	31
Gemlik (MD 87)	444	32
Izmit Bay (IZ 17)	569	29
Izmit Bay (IZ 25)	302	7

Heavy metal concentrations of the anoxic marine sediment samples are given in Table 8.4.

Table 8. 4. Heavy metal concentrations of the anoxic marine sediment samples studied

	Cr (mg/kg)	**Cu (mg/kg)**	**Ag (mg/kg)**	**Fe (mg/kg)**	**Cd (mg/kg)**	**Mn (mg/kg)**	**Pb (mg/kg)**	**Ni (mg/kg)**	**Zn (mg/kg)**
MD 87	55,15	20,75	< 25	28715	< 10	376	16,3	103,75	164
MKC	12,9	37,85	< 25	8725	< 10	162	15,5	34,4	177,5
IZ-17	20,9	18,95	< 25	14990	< 10	487,5	11,9	38,25	125
IZ-25	28,85	58,1	< 25	25345	< 10	243,5	32,15	45,55	255,5

Table 8.5. gives total carbon (TC), total inorganic carbon (IC) and total organic carbon (OC) contents of the sediment samples.

Table 8.5. Carbon contents of the anoxic marine sediment samples studied

SAMPLE	TC (mg/l)	IC (mg/l)	TOC(mg/L)
MKC	0,283	0,01	0,273
MD 87	0,199	0,028	0,171
IZ 17	0,239	0,046	0,193
IZ 25	0,461	0,044	0,417

9. DISCUSSION

Although it is difficult to permeabilize microorganisms in the marine sediments, 67%±8%, 62%±12%, 77%±13% and 80%±7% of DAPI stained total microorganisms in sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece respectively were detected using universal probe in this study. 10-30% of the cells stained with SYBR GreenI could not be detected in the sediment samples analyzed. It has been reported in a study, most bacteria in marine habitats are small, slow growing, or starving and the signal intensities of hybridized bacterioplankton cells were frequently below the detection limits or lost in high background fluorescence (Ince et al., 2006).

According to FISH results, relative abundances of bacterial cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 51%±9%, 73%±11%, 64%±8% and 70%±16% respectively. Relative abundances of archaeal cells within the sediments from Gemlik Bay, Izmit Bay (Iz17), Izmit Bay (Iz25) and Kucukcekmece are 32%±4%, 23%±3%, 33%±2% and 29%±7% respectively. In the study of Ravenschlag et al. (2001), up to 65.4 ± 7.5% of total DAPI (4´,6´-diamidino-2-phenylindole) cell counts hybridized to the bacterial probe EUB338, and up to 4.6 ± 1.5% hybridized to the archaeal probe ARCH915.

In this study, high abundance of Archaeal groups was observed in the anoxic sediments of the four locations of the Marmara Sea, Gemlik, Izmit Bay(IZ17), Izmit Bay(IZ25) and Kucukcekmece. The higher abundance of *Methanococcoles, Methanobacteriales* and *Methanogenium* relatives suggest that methanogenesis occurs in the anoxic sediments of the Marmara Sea, dominantly via H_2+CO_2. The study of Ince et al.(2006) showed that hydrogenotrophic methanogenesis primarily occurs in the sediments of the Black Sea. In another study based on the carbon and hydrogen isotopic analysis, most biogenic methane in marine sediments originates from H_2+CO_2 (Whiticar et al. 1986). In the anoxic sediments from Rotsee (Switzerland), significant

hydrogenotrophic methanogenesis was found in the organically enriched upper 2 cm of the sediment (Falz et al., 1999). High organic contents of the sediment samples also support that hydrogenotrophic methanogenesis occurs in the sediments (see in Table 8.5.).

Acetoclastic *Methanosaeta* and *Methanosarcina* was also found in the anoxic sediments but they are less abundant species than hydrogenotrophic species. This could be because of the presence of other acetate utilizer species.

The relative abundances of the total methanogens, hydrogenotrophic and acetoclastic methanogens demonstrate a similarity. It can be concluded that microbial community structure in anoxic sediments of the Marmara Sea shows a parallelism.

RECOMMENDATIONS

This study provides information about the diversity of methanogenic archael populations which are functioning in mineralising organic matter in anoxic sediments of the Marmara Sea using *in situ* hybridization with group-specific fluorescently labeled rRNA-targeted oligonucleotide probes. However, much more detailed study should be carried out in order to better define both the biodiversity of whole microbial structure in deep marine sediments and ecological cycling of organic material.

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