

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**MOLECULAR PHYLOGENETIC ANALYSIS OF
ACTINOMYCETES ISOLATED FROM
SEDIMENTS OF AEGEAN SEA**

by
İlknur TUNÇER

December, 2014
İZMİR

**MOLECULAR PHYLOGENETIC ANALYSIS OF
ACTINOMYCETES ISOLATED FROM
SEDIMENTS OF AEGEAN SEA**

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Graduate School of Natural and Applied Sciences of Dokuz Eylül University
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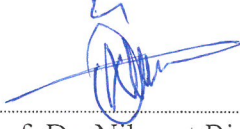
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December, 2014

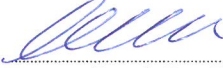
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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled “**MOLECULAR PHYLOGENETIC ANALYSIS OF ACTINOMYCETES ISOLATED FROM SEDIMENTS OF AEGEAN SEA**” completed by **İLKNUR TUNÇER** under supervision of **PROF. DR. NİHAYET BİZSEL** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.


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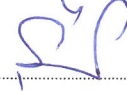
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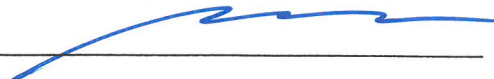
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MOLECULAR PHYLOGENETIC ANALYSIS OF ACTINOMYCETES ISOLATED FROM SEDIMENTS OF AEGEAN SEA

ABSTRACT

Actinomycetes are very important due to their high diversity and production of secondary metabolites. Since discovery from terrestrial actinomycetes has declined recently, marine environment has been opened to research area. Based on the need for phylogenetic research in Turkish marine environments, in this study, the isolation of actinomycetes from sediments of Aegean Sea was succeeded using seven sediment processing methods and two media with or without six antibiotics. Morphological characteristics and physiological tests showed that those thirteen strains were different from each other. Their partial 16S rRNA gene sequences were deposited to GenBank and accession numbers were obtained. According to phylogenetic analysis, it was shown that one of them belonged to the genus *Nocardiopsis* and the rest to the genus *Streptomyces* and also one of the *Streptomyces* strains was thought as the candidate of new taxa. Furthermore, the physicochemical data of the study area including temperature, salinity, density, pH, dissolved oxygen, total suspended solids, chlorophyll α , reactive silicon, nitrite, nitrate, phosphate of water column, total and organic carbon, nitrogen, phosphorus contents, grain size of sediments were analyzed and it was found that the study area showed the characteristics of Aegean Sea. When all biogeochemical data was combined and also compared to our present studies and previous research in Eastern Mediterranean Sea, it was indicated that the study area had optimum environmental conditions for high bacterial diversity rather than coastal sediments and deep-basins of Aegean Sea and also geographical and environmental effects on bacterial characteristics was supported by the enzymatic activities of actinomycetes.

Keywords: Actinomycetes diversity, 16S rRNA genes, enzymatic activities, biogeochemical parameters, Aegean Sea.

EGE DENİZİ SEDİMANLARINDAN İZOLE EDİLEN AKTİNOMİSETLERİN MOLEKÜLER FİLOGENETİK ANALİZİ

ÖZ

Aktinomisetler yüksek çeşitlilik ve sekonder metabolit üretimi bakımından önemli bakterilerdir. Karasal aktinomiset keşifleri son yıllarda azaldığından denizsel alanlar araştırmalara açılmıştır. Türkiye denizsel alanlardaki filogenetik araştırmalara ihtiyaca dayalı bu çalışmada, aktinomisetlerin Ege Denizi sedimanlarından izolasyonu yedi farklı sediman işleme metodu ve altı antibiyotikli veya antibiyotiksiz iki izolasyon ortamı kullanılarak başarılmıştır. Morfolojik özellikler ve fizyolojik testler onüç suşun birbirinden farklı olduğunu göstermiştir. Bunların 16S rRNA gen sekansları GenBank'a deposit edilmiş olup erişim numaraları elde edilmiştir. Filogenetik analizlere göre, bunlardan birinin *Nocardiopsis* ve diğerlerinin *Streptomyces* cinslerine ait olduğu ve ayrıca bir *Streptomyces* suşunun yeni tür olmaya aday olduğu gösterilmiştir. Ayrıca, çalışma alanının su kolonu sıcaklık, tuzluluk, yoğunluk, pH, çözünmüş oksijen, toplam askıda katı madde, klorofil a, reaktif silikon, nitrit, nitrat, fosfat ve sediman örneklerinin toplam ve organik karbon, azot, fosfor içeriği ile dane boyunu kapsayan fizikokimyasal verileri de analiz edilmiş ve Ege Denizi'nin karakteristik özelliklerini gösterdiği saptanmıştır. Bütün bu biyojeokimyasal data birleştirilip kendi yeni çalışmalarımız ve Doğu Akdeniz'de yapılmış olan önceki araştırmalarla karşılaştırıldığında bu çalışma alanının yüksek bakteri çeşitliliği için Ege Denizi'nin kıyısal ve dip sedimanlarına nazaran optimum çevresel koşullar taşıdığı gösterilmiş ve ayrıca bakteri karakteristiği üzerinde jeografik ve çevresel etkilerin olduğu aktinomisetlerin enzimatik aktiviteleri ile desteklenmiştir.

Anahtar kelimeler: Aktinomiset çeşitliliği, 16S rRNA geni, enzimatik aktivite, biyojeokimyasal parametreler, Ege Denizi.

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CHAPTER ONE

INTRODUCTION

1.1 Marine Bacteria

The oceans cover 71 % of the Earth's surface and the marine ecosystem is the largest as accounting over 90 % of the biosphere (Munn, 2004). It is estimated that 90 % of all prokaryotes live in the deep subsurface sediments of which 67 % occur beneath the deep seas (Whitman et al., 1998). For the seawater, bacteria dominate the top 150 m (photic zone) as about 90 % of all cells but decrease till 1000 m and then increase to 35 – 40 % of all cells below 1000 m (Karner et al, 2001).

The recent studies combining 16S rRNA database of all marine bacterial diversity research have indicated that *Proteobacteria* (one of the largest and most physiologically diverse group of bacteria), especially *Alphaproteobacteria* and then *Gammaproteobacteria* are dominant in overall marine environment and inverse is valid for culture-dependent studies (Giovannoni & Rappe, 2000; Hagström et al., 2002; McIntyre, 2010). *Alphaproteobacteria* play a critical role in nutrient cycling (carbon, nitrogen and sulfur) and accounts for approximately 25% of the biomass and 50% of the cell abundance in the ocean (McIntyre, 2010). Moreover, from this phylum, *Roseobacter* species are highly distributed in ocean waters and their association with algal blooms suggests their role as controlling bloom outbreaks (McIntyre, 2010). The most abundant photosynthetic bacteria are found as *Cyanobacteria* producing about 50% of the oxygen on Earth, especially *Prochlorococcus* and *Synechococcus* species contributing half of oceanic primary production (McIntyre, 2010).

In shallow and deep sediments, on the other hand, *Gammaproteobacteria* and *Firmicutes* (the genera *Bacillus* and *Clostridium*) become dominant (McIntyre, 2010). *Gammaproteobacteria* which are highly diverse bacteria play a crucial role in nutrient cycling and have pathogenic forms for humans and marine organisms (Munn, 2004). Similarly, in addition to being soil saprophytes, the genus *Bacillus*

have a large number of diverse species with some of them as pathogenic and *Clostridium* species (strict anearobes) play a major role in decomposition and nitrogen cycling in anoxic marine sediments (Munn, 2004). Furthermore, *Actinobacteria* form a smaller portion and occur in the photic zone and in sediments (Munn, 2004). Since they are highly abundant in soils, it is suggested that they are derived from terrestrial run-off but they are also found in deep basins. They play crucial role in decomposition and nutrient cycling by their extracellular enzymes which breakdown macromolecules as polysaccharides, proteins and lipids (Munn, 2004).

1.1.1 Nutrient Utilization of Marine Bacteria

Heterotrophic bacteria are key players in the processes of organic matter recycling, decomposition and mineralization in aquatic environments. Although only small and simple compounds can pass through cell walls with better permeability of Gram-positive bacteria rather than Gram-negative bacteria (Ghuysen & Hackenbeck, 1994), high molecular weight particulate or dissolved organic matter are utilized in aquatic environments (Cunha et al., 2010). Bacterial extracellular enzymatic activity as hydrolyzing process of complex molecules outside the cell into smaller size molecules (Arnosti, 2003) is regulated by environmental factors and enzyme-substrate interactions (Cunha et al., 2010).

Extracellular enzymes are mainly hydrolases (glycosidases, peptidases, esterases, etc.) cleaving C-O and C-N bonds and also catalyze oxidative reactions cleaving C-C and C-O bonds (Sinsabaugh, 1994). Extracellular enzymes are classified as ectoenzymes which are inserted in or spanning the plasma membrane, associated with the cell wall (Skujins, 1978) and as truly extracellular enzymes which occur in free form, are secreted by viable cells, released into the environment by viral lysis and more important in the decomposition of organic matter in the dark ocean (Baltar et al., 2010).

1.1.1.1 Extracellular Enzymes of Marine Bacteria

Hydrolytic enzymes such as proteases, glucosidases, chitinase, lipase and phosphatases are mainly responsible for the extracellular enzymatic activity in marine environment (Cunha et al., 2010) and their activity ranges in seawater are in the order aminopeptidase > phosphatase > β -glucosidase > chitinase > esterase > α -glucosidase (Hoppe et al., 2002). According to the hydrographic conditions in the ocean, polymer hydrolysis rates change horizontally and vertically between different water masses (Hoppe & Ulrich, 1999; Hoppe et al., 2002; Baltar et al., 2010) and temporal variation are also important for the activity rates of particular extracellular enzymes in coastal ecosystems (Cunha & Almeida, 2009).

β -glucosidase and leucine aminopeptidase are widely distributed in aquatic environments, mainly associated with heterotrophic bacteria and used as model ectoenzymes for studying bacterial degradation of natural polymeric compounds such as carbohydrates and proteins in the aquatic environment (Chrost, 1992). β -glucosidase is produced by heterotrophic bacteria in waters and sediments of marine environments and hydrolyzes β -linked disaccharides of glucose, cellobiose and carboxymethylcellulose (Barman, 1969). Leucine aminopeptidase hydrolyses a large number of peptides and amino acid amides of the L-configuration, with particular affinity to L-leucyl-peptides and L-leucyl-amides (Chrost, 1991).

Phosphatases are also widely studied enzymes in aquatic environments and are highly important especially in P-limited areas for phosphorus acquisition (Vidal et al., 2003). Phosphatases are the group of phosphohydrolases releasing phosphate in aquatic environments (Chrost & Siuda, 2002) and are a variety of enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid (Feder, 1973). These enzymes are characterized by different temperature and optimum pH (Hoppe, 2003). Alkaline phosphatase are a group of isoenzymes that react optimally in pH range 7.6 – 9.6 (Chrost & Siuda, 2002) and catalyzes the hydrolysis of a variety of phosphate esters, including esters of primary and secondary alcohols, sugar alcohols,

cyclic alcohols, phenols, and amines by releasing inorganic phosphate (Chrost, 1991).

For the assays of ectoenzyme activities, fluorophore-labeled artificial substrates are used in aquatic environments. Methyl coumarinyl- or methylumbelliferyl-substrates are non-toxic and yield highly fluorescent water-soluble products with optical properties significantly different from those of the substrate (Hoppe, 1993).

Extracellular enzymatic activity depends on organic matter input from sedimentation events (Fabiano & Danovaro, 1998) and decreases with depth in the sediment column (Meyer-Reil, 1987). In a study, activity rates of β -glucosidase, α -glucosidase, aminopeptidase, arylsulphatase and phosphatase are generally higher in the sediments of the vegetation banks than in uncolonized sediments and sediment types such as grain size and water content show significant relations with hydrolysis rates (Cunha et al., 2010).

1.1.1.2 Environmental Factors Regulating Extracellular Enzymatic Activity

Extracellular enzymes are produced when organic carbon in simple form is insufficient (Harder & Dijkhuizen, 1983). By the repression strategy, the majority of ectoenzymes are produced in the presence of utilizable dissolved organic matter, are controlled when utilizable substrates decrease below a critical level (Siuda, 1984; Hoppe et al., 1988) and are not produced by accumulation of end-products in aquatic environments (Chrost & Siuda, 2002; Francoeur & Wetzel, 2003). Extracellular enzymes can also be produced despite of substrate availability in the environment (Chrost, 1991). For instance, while alkaline phosphatase and β -glucosidase are synthesized by substrate addition (Chrost & Siuda, 2002), leucine aminopeptidase is reduced by the addition of dissolved inorganic nitrogen (Chrost, 1991; Francoeur et al., 2001; Sala et al., 2001), or not affected (Nausch & Nausch, 2000).

Environmental regulation might be the main factor for enzyme activity such that a positive correlation between aminopeptidase activity and N limitation has been

observed and interpreted as an indication of the utilization of organic N-sources for bacterial growth (Cunha & Almeida, 2009). Enzyme production is mainly regulated by environmental factors such as temperature (Insam, 1990). At low temperatures, the affinity of enzyme systems decreases (Zweifel, 1999; Pomeroy & Wiebe, 2001). The activity of protein- and polysaccharide-degrading bacterial extracellular enzymes of arctic isolates and in marine sediments increases with the temperature, showing optima well above the ambient environmental temperatures (King, 1986; Christian & Karl, 1995; Arnosti et al., 1998). The membrane-bound transporters of mesophilic and psychrotolerant marine bacteria respond with decreased substrate affinities to temperatures at the lower end of their specific temperature range (Wiebe, 1993; Nedwell & Rutter, 1994). Several studies have demonstrated that the temperature sensitivity of extracellular enzymes changes seasonally (Fenner, et al. 2005; Koch et al., 2007), which has been explained by the synthesis of different isoenzymes (enzymes with the same function but different structure) through time, produced either by different organisms or by a single species capable of producing multiple isoenzymes (Loveland et al., 1994). There is also some evidence for biogeographical patterns in enzyme temperature sensitivity. For example, many studies have observed that enzymes from microbes inhabiting cold environments have unusually low temperature optima (Feller, 2003).

Salinity seems to be only poorly correlated with the metabolic activity of bacterioplankton (Murrel et al., 1999). Analysis of the relation between the activity of leucine aminopeptidase and salinity in coastal systems has been shown to be positive in some environments (Murrel et al., 1999) and negative in others (Patel et al., 2000). In general, in low salinities bacterioplankton expresses higher levels of β -glucosidase activities, while in higher salinities bacterioplankton seems to be more adapted to protein degradation (Cunha et al., 2000). Information on the regulatory role of salinity on sediment microbial communities is less available (Cunha et al., 2010). Because more energy is required for the production of osmolites and less is used for the release of extracellular enzymes, decreased extracellular enzymatic activity can be detected at higher salinity sediments (Cunha et al., 2010). However, the inverse pattern has also been reported (Taylor et al., 2003).

Extracellular enzymes are directly affected by the pH of the environment, because changes in the concentration of hydrogen ions in the environment modify the ionization state of amino acids and the three-dimensional structure of the active site of the enzyme (Tipton & Dixon, 1979). Deviations from the optimal pH result in a decrease in enzyme activity rates (King, 1986; Münster, 1991; Chrost, 2001).

Trace metals and UV radiation are two ecologically relevant factors that inhibit the activity of alkaline phosphatase (Reuter, 1983). Free copper ions (trace metal) have been speculated to inhibit or totally block the direct utilization of selected dissolved organic phosphate compounds by natural microbial assemblages (Reuter, 1983). On the other hand, photodegradation of alkaline phosphatase activity may limit the ability of the cells to obtain inorganic phosphate, enhancing the effects of P limitation in well-lighted, near surface habitats (Garde & Gustavson, 1999). Experimental irradiation of natural marine bacterioplankton assemblages also caused a significant reduction in lipase and leucine aminopeptidase activities (Miiller-Niklas, 1995).

1.2 Marine Actinomycetes

Actinomycetes are aerobic, nonmotile, and Gram-positive bacteria with high guanosine and cytosine (G+C) content in their DNA as 70 – 80 %. The order *Actinomycetales* belonging to the phylum *Actinobacteria* has substrate hyphae, form aerial mycelia having greater diameter than the substrate mycelium and also form spores as resistance to extreme environmental conditions i.e. high temperature, dessication, low nutrients. Actinomycetes are phenotypically and phylogenetically highly diverse and can be found in adverse environments (Goodfellow & Williams, 1983). Among actinomycetes, the streptomycetes are the dominant and the non-streptomycetes are called rare actinomycetes, comprising approximately 100 genera (Manivasagan et al., 2013).

Due to their high abundance in soil, it was considered that actinomycetes were terrigenous bacteria and marine isolates were originated from land run-off spores

(Goodfellow & Williams, 1983). However, it is now clear that they not only exist in the marine environment but also the marine strains significantly contribute to diversity of actinomycetes taxa (Mincer et al., 2002; Manivasagan et al., 2013). They can be isolated from mainly sediments and then plankton, weeds, stones, algae, shellfish, mangroves, sea grass, coral reefs, fjords, and sponges (Munn, 2004; Manivasagan et al., 2013). Furthermore, it has been illustrated that actinomycetes account for about 10 % of total bacteria colonizing marine organic aggregates in marine snow (Grossart et al., 2004).

The first marine actinomycete species to be characterized is *Rhodococcus marinonascens* (Helmke & Weyland, 1984). Moreover, in recent culture-dependent studies, it has indicated that indigenous marine actinomycetes belong to members of the genera *Dietzia*, *Rhodococcus*, *Streptomyces*, *Salinispora*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris*, and *Verrucospora* were obtained (Stach et al., 2003; Bull et al., 2005; Magarvey et al., 2004; Jensen et al., 2005a, 2005b).

Among those indigenous marine actinomycetes, the most exciting finding is the discovery of the first obligate new marine actinomycete genus *Salinispora* (formerly known as *Salinospora*) is highly exciting since they are widely distributed in ocean sediments (Mincer et al., 2002, 2005), isolated from the marine sponge *Pseudoceratina clavata* (Kim et al., 2005) and also metabolically active in the natural marine environment (Mincer et al. 2005). In addition to the description of the genus *Salinispora*, three novel species *Salinispora arenicola*, *Salinispora pacifica* and *Salinispora tropica* have recently been published (Maldonado et al., 2005; Jensen & Mafnas, 2006).

1.2.1 Importance of Marine Actinomycetes

Actinomycetes are very important due to their high diversity and production of secondary metabolites. Since the discovery of actinomycin (Lechevalier, 1982), actinomycetes have been found to produce many commercially bioactive compounds

and antitumor agents in addition to enzymes of industrial interest (Tanaka & Omura, 1990). For instance, approximately two-third of naturally occurring antibiotics has been isolated from these organisms (Takaizawa et al., 1993) with mostly from *Streptomyces* (Goodfellow & O'Donnell, 1993). However, discovery from terrestrial actinomycetes has declined recently and thus marine environment has been opened to research area.

In recent studies, it has been indicated that novel compounds with biological activities have been isolated from marine actinomycetes (Magarvey et al., 2004; Fiedler et al., 2005; Jensen et al., 2005b), indicating that marine actinomycetes are an important source for the discovery of novel secondary metabolites. In fact, marine actinomycetes are the richest sources of secondary metabolites such that 67 % of marine natural products were provided by those isolated from Australia, the Caribbean, the Indian Ocean, Japan, the Mediterranean, and the Western Pacific Ocean sites (Manivasagan et al., 2013). Moreover, these natural products have been an extraordinary source for lead structures in the development of new drugs (Sivakumar et al., 2007).

1.2.1.1 In Biotechnological and Industrial Area

Marine actinomycetes produce potential source of novel bioactive compounds used in medicine, cosmetics, pharmacology, pharmaceutical industry, agriculture, veterinary, etc.

1.2.1.1.1 Antimicrobial and Anticancer Agents. Several antibiotics have been obtained from marine actinomycetes (Adinarayana et al., 2006; Bernan et al., 1994; Biabani et al., 1997; Buchanan et al., 2005; Charan et al., 20004; Maskey et al. 2002, 2003; Lam, 2006; Li et al., 2005; Lombo et al., 2006; Wo et al., 2002), even novel and entirely different compared to ones obtained from the terrestrial isolates due to the difference in environmental conditions (Meiying & Zhicheng, 1998). Since the discovery of streptomycin, a large number of antibiotics have been extracted from cultures of *Streptomyces* and *Streptoverticillium* (Yokota, 1997). However, isolation

of novel antibiotics has decreased and thus the research has moved to the genera of rare actinomycetes such as *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Kibdelosporangium*, *Microbispora*, *Micromonospora*, *Planobispora*, *Planomonospora* and *Streptosporangium* (Lazzarini et al., 2000). In addition to antibiotics, it has been reported that different types of anticancer agents, especially cytotoxic compounds have been obtained from marine actinomycetes (Georis et al., 2000; Jeong et al., 2006; Lang et al., 2004; Liu et al., 2005; Maskey et al., 2004; Manam et al., 2005; Soria-Merkado et al., 2005; Stritzke et al., 2004).

1.2.1.1.2 Enzymes. Marine actinomycetes have diverse enzymatic activities (Das et al., 2006). The genus *Streptomyces* is considered as a potential source of amylolytic enzymes which hydrolyze starch molecules and is biotechnologically important with applications in food, fermentation, textile, and paper industries (Pandey et al., 2005). Proteolytic enzymes, especially alkaline proteases used in leather, textile, organic synthesis, and wastewater treatment, accounting for 60 % of all industrial enzymes as used in detergents, cheese-making, baking, digestive aids, etc. are also synthesized by actinomycetes (Moorman et al., 1993; Abd El-Nasser, 1995; Bockle et al., 1995) i.e. a novel alkaline protease from *Nocardiopsis* species (Dixit & Pant, 2000). Cellulolytic enzymes including endoglucanases, exoglucanases, and β -glucosidases used in color extraction from juices, detergents causing color brightening and softening, biostoning of jeans, pretreatment of industrial wastes have mainly been isolated from the genera *Streptomyces* and *Thermoactinomyces* (Techapun et al., 2003). Again the genus *Streptomyces* is the best studied for chitinases hydrolyzing chitinous substrates as fungal cell walls, crab and shrimp shells (Miyashita et al., 1991; Mukherjee & Sen, 2006).

1.2.1.2 In Symbioses

Due to the production of secondary metabolites, actinomycetes are often components of symbiotic communities, especially in reef and deepwater sponges as sponge-specific symbionts such as the *Micromonospora* strains producing manzamines that have no terrestrial equivalents (Piel, 2006), the *Salinispora* strains

isolated from the Great Barrier Reef sponge *P. clavata* (Kim et al., 2006) high actinomycete diversity isolated from the Yellow Sea sponge *Hymeniacidon perleве* (*Actinoalloteichus*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, and *Streptomyces*) (Zang et al., 2006) and the greatest diversity in *Craniella australiensis*, with broad-spectrum of antibacterial activities (Li & Liu, 2006). Coral-associated actinomycetes have recently been reported in a culture-independent study of the recently discovered deep water Mediterranean corals (Yakimov et al., 2006) and a culture-based study of the symbionts of *Fungia scutaria* in Red Sea (Lampert et al., 2006).

1.2.2 Identification of Actinomycetes

There are various approaches for the identification of actinomycetes such as classical approach including morphological, physiological and biochemical classification and also molecular approach including 16S rRNA gene analysis.

1.2.2.1 Classical Approach

Morphological and biochemical characteristics are very helpful for identification of actinomycetes. The former described in Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974) includes the color of aerial mycelium (white, grey, red, green, blue and violet), reverse side pigments (pale yellow, olive or yellowish brown), soluble pigments (red, orange, green, yellow, blue and violet), melanoid pigments (greenish brown, brownish black or distinct brown), etc. The latter, on the other hand, recommended by International *Streptomyces* Project (Shirling & Gottlieb, 1966) includes the utilization tests of carbon sources (arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose), etc.

1.2.2.2 Molecular Approach

Using nucleic acids is the most powerful approach in taxonomical studies of bacteria (O'Donnell et al., 1993). For molecular phylogenetic analysis, the direct gene products or the genes themselves chosen to study must be universally distributed in the study group, functionally homolog in each organism, alignable and

proportional with evolutionary distance. Among those molecular chronometers, 16S ribosomal RNA (rRNA) genes (Figure 1.1) are the best for bacterial phylogeny, even rather than 5S and 23S rRNA genes. While the 16S rRNA gene provides a better phylogenetic resolution in addition to its rapid, trustworthy and simple usage, the 5S rRNA gene has insufficient sequence length for statistically significant comparison and the 23S rRNA gene has no primers for its sufficient amplification and has too long sequence resulting difficulty for the current technology (Soria-Mercado et al., 2012). Therefore, phylogenetic studies based on 16S rRNA gene sequences have increased in the systematics of bacteria, especially actinomycetes (Yokota, 1997). Analysis of 16S rRNA gene sequences of actinomycetes begins by isolating DNA (Hapwood, 1985) and amplifying the gene coding for the 16S rRNA using the polymerase chain reaction (PCR). Then, the purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample (Xu et al., 1999) and a computer is then used for studying the sequence for identification using phylogenetic analysis procedures.

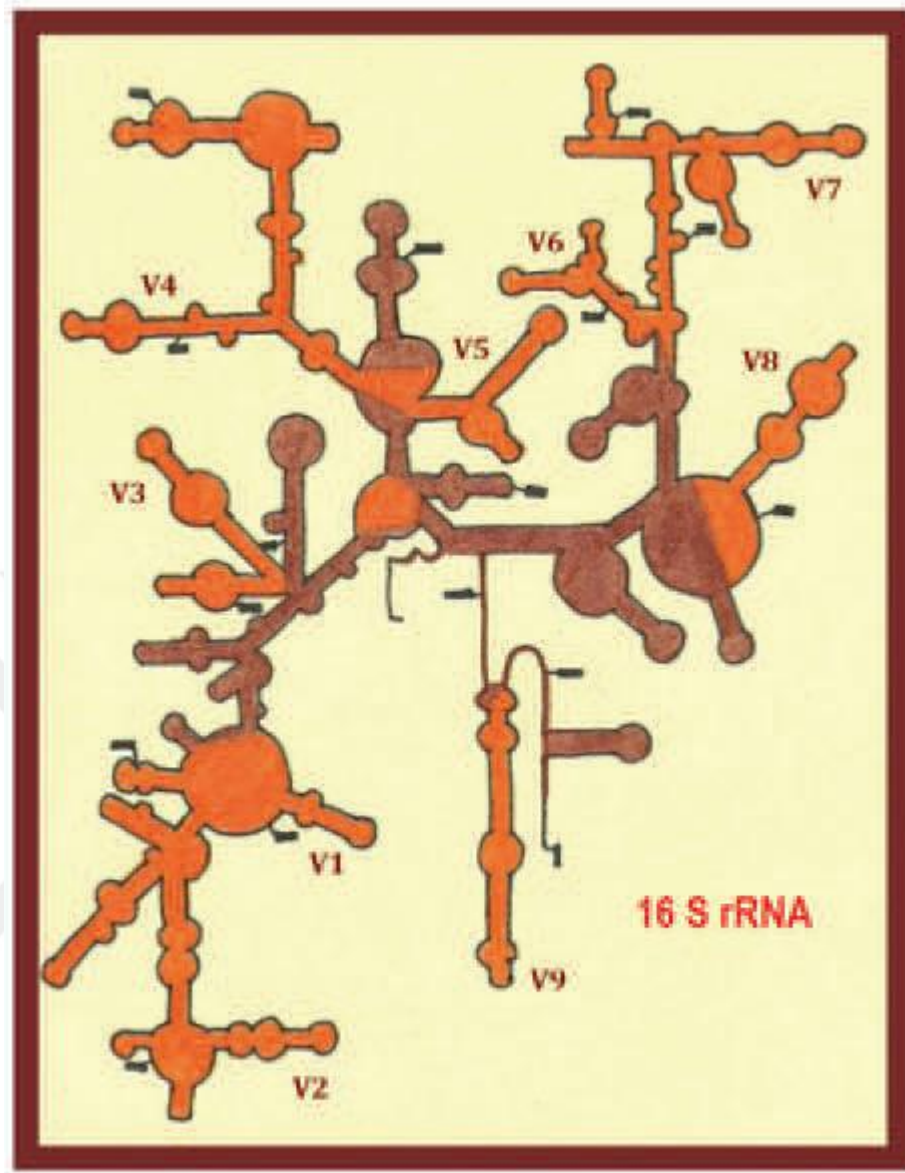


Illustration by Lianth Peres

Figure 1.1 The bacterial 16S rRNA structure with the variable regions (V1 – V9) used to characterize bacteria by species level in addition to the constant region used to correlate the genus (Soria-Mercado et al., 2012).

1.3 Eastern Mediterranean Sea

The Mediterranean is a semi-enclosed sea exchanging water with the North Atlantic Ocean through the Strait of Gibraltar and with the Black Sea through the Turkish Strait System as the Dardanelles, the Marmara Sea and the Bosphorus Strait. In addition to the fact that the net evaporation exceeds the precipitation, it is characterized by high salinities, temperatures, densities and low nutrient

concentrations (Tanhua et al., 2013). It is also a land-locked sea with antiestuarine circulation of water exiting through the Strait of Gibraltar at deep and entering from the Atlantic at the surface (Oguz & Su, 2004). It has the western and eastern basins separated with the Strait of Sicily and they have different hydrological features and nutrient regimes.

1.3.1 Hydrology

The Eastern Mediterranean Sea is connected to the Aegean Sea and the Black Sea which has small but significant inflow due to its low salinity. In contrast to relatively low salinity inflow of Atlantic Water into the Western Mediterranean Sea, there is high salinity at the surface of Eastern Mediterranean Sea (Tanhua et al., 2013). The separate deep water formation processes transporting water from the upper water column to the deep interior provides the differences of salinity and temperature in the deep and intermediate waters between the eastern and western basins of the Mediterranean Sea. In the western basin, the deep water formation primarily takes place in the Gulf of Lions such that during winter, dry and cold air mixes the Atlantic Water and the Winter Intermediate Water with the underlying, warmer and saltier Levantine Intermediate Water and then further heat loss leads to formation of Western Mediterranean Deep Water (Gascard, 1978; Killworth, 1983; Schott et al., 1993; Rhein, 1995; Tsimplis et al., 2006).

While the Adriatic Sea was previously the deep water formation area in the Eastern Mediterranean Sea, in the early 1990's the Aegean Sea became the main deep water formation area generating an event known as the Eastern Mediterranean Transient (Tanhua et al., 2013). It was suggested that the Eastern Mediterranean Transient was caused by very strong winters transporting high-salinity waters into the Aegean Sea (Josey, 2003; Gacic et al., 2013). The new deep water of Aegean Sea (below 1200 m depth) was characterized by higher temperature and salinity than previous deep water of Adriatic Sea. The circulation regime of the North Ionian Gyre (cyclonic or anticyclonic) determines a decadal variability in the thermohaline characteristics of the Levantine Intermediate Water and the switches between

Adriatic and Aegean as source regions for the Eastern Mediterranean deep water (Gacic et al., 2013).

1.3.2 Oxygen and Nutrients

The Mediterranean Sea is well oxygenated and oxygen utilization rate values are similar to other oceans in the upper water column but higher in the deep water layers (Roether & Well, 2001). The recently formed deep waters during the Eastern Mediterranean Transient containing labile dissolved organic carbon brought from the surface layer to the deep layer increased the oxygen consumption rate (Klein et al., 2003). The oxygen concentrations in the oxygen minimum layer, which is the results of the oxygen consumption due to the mineralization of the organic matter produced at surface and of the oxygen supply by vertical and lateral diffusion, are higher in the Eastern Mediterranean Sea than in the Western Mediterranean Sea (Tanhau et al., 2013). In contrast, the horizontal distribution of oxygen is more uniform in the western compared to the eastern basin since Eastern Mediterranean Sea has more complex morphology restricting the spread of new, dense and more oxygenated waters (Tanhau et al., 2013).

Due to the anti-estuarine circulation of the Mediterranean Sea as exporting deep water with higher nutrient values and importing low nutrient Atlantic Water, it has low nutrient concentrations (Huertas et al., 2012). The studies of the nutrient regime in Mediterranean Sea showed no significant seasonality (Pavlidou & Souvermezoglou, 2006). However, the influence of river discharge and coastal runoff is significant throughout spring due to snow melt (Migon et al., 2001). The increased oligotrophy towards the east in the Mediterranean Sea is influenced by the higher intermediate and deep water stratification in the eastern basin (Tanhua et al., 2013). The Eastern Mediterranean Sea has thicker nutrient depleted surface layer and deeper nutrient maximum layer than the Western Mediterranean (Tanhua et al., 2013).

In spite of N:P ratio in the world ocean is around 16 (Redfield et al., 1963), it is 24–27 in the deep eastern basin, and 20–22 in the deep western basin. The reason of

this anomaly is still questioning and possible suggestions are the Adriatic rivers (Civitarese et al., 1998), the atmospheric inputs (Krom et al., 1991) and the lack of denitrification in the generally well oxygenated Mediterranean waters and sediments (Krom et al., 2004 and 2005) as the possible sources of the N:P anomaly.

The Mediterranean, particularly the Eastern Mediterranean Sea, has been considered as phosphorus limited for biological growth (Krom et al., 1991, 2004), although recent work suggest N or N and P co-limitations (Thingstad et al., 2005). The phosphate and nitrate concentrations in deep waters increased in Western Mediterranean Sea from 1960 to 1994 (Bethoux et al., 1998 and 2002) in association with agricultural, industrial and urban activities since the 1960s (Bethoux et al., 2002) although the river outflows are generally the most significant source of phosphate supply and the atmosphere is for inorganic nitrogen (Bethoux et al., 1998; Moutin et al., 1998; Migon et al., 2001; Sanz et al., 2002). In contrast to this increase, the phosphate, silicate and nitrate concentrations have not dramatically changed in the Eastern Mediterranean Sea due to the differences in the residence times of deep waters in those western and eastern basins (Pavlidou & Souvermezoglou, 2006). Similarly, in April 2011 it was found that the concentrations of nitrate and phosphate in the deep layer were significantly lower in the Eastern Mediterranean compared to the Western Mediterranean unlike the similar the concentrations for silicate in the deep waters of both basins (Tanhau et al., 2013). One explanation for the lower nutrient concentrations in the eastern basin is the nutrient supply of the Eastern Mediterranean to the Western Mediterranean (Schroeder et al., 2010).

In contrast to oligotrophic system of the Mediterranean Sea, some coastal regions such as The Gulf of Lions (northwestern Mediterranean Sea), the Algerian coastal zone, the Northern Adriatic, the Nile Delta, and the northeastern part of the Levantine basin of the Eastern Mediterranean and the Northern Aegean Sea are identified as the most productive regions (Oguz & Su, 2004). The nutrient concentration in the Aegean Sea is twelve times lower than in Atlantic Ocean and eight times lower than in Alboran Sea (Pavlidou & Souvermezoglou, 2006). The

oxygen and nutrient data collected in the Eastern Mediterranean Sea during the past decade showed a depletion of nutrients in the following order : Levantine > Ionian > Aegean (Figure 1.2) (Stergiou et al., 1997; Pavlidou & Souvermezoglou, 2006).

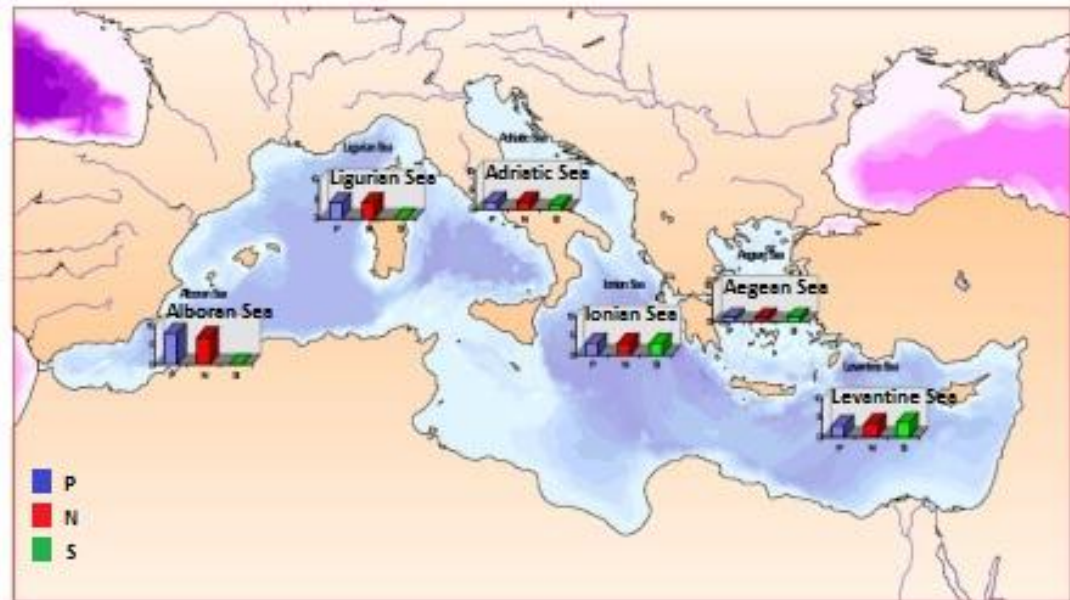


Figure 1.2 The relative concentrations of nutrients in the deep basins of the Mediterranean Sea (Pavlidou & Souvermezoglou, 2006).

In the Aegean Sea, there are coastal areas with eutrophication problems related to anthropogenic activities, agricultural runoff, sewage outfalls and river outflows. For instance, in the Gulf of Gera in Lesvos Island, the inorganic nitrogen flux from agricultural runoff is 40 – 60 % during the winter period and the oligotrophic water from the Aegean Sea enters into the gulf influencing the nutrient levels during the warm months of the year (April to October), in addition to the untreated domestic wastewater and effluents from the local industrial activities, especially olive oil processing by-products resulting to the development of eutrophication crises during the year (Archonditsis et al., 2000). In most of the polluted Hellenic coastal areas, the limiting factor is nitrogen (Pavlidou et al., 2005) as similar to Izmir Bay in Turkish coasts (Bizsel & Uslu, 2000; Kucuksezgin et al., 2006) in which high terrestrial inputs result to the increase of nutrient values (Dassenakis et al., 2000).

1.4 The Purpose of the Present Study

Actinomycetes are very important for the production of secondary metabolites used in especially industrial area and medical treatments. However, discovery from terrestrial actinomycetes has declined recently and thus marine environment has been opened to research area. Based on the need for phylogenetic research in Turkish marine environments and both the economical and biotechnological values of actinomycetes, the isolation of actinomycetes from sediments of Aegean Sea and their phylogenetic analysis are aimed.



CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Area

Totally 5 stations located between Lesvos Island and Karaburun in Aegean Sea were studied (Table 2.1, Figure 2.1) and the survey was performed on 31 March 2012 by RV/K Piri Reis research vessel of Institute of Marine Sciences and Technology, Dokuz Eylul University [IMST, DEU] (Figure 2.2).

Table 2.1 The coordinates of 5 stations in Aegean Sea.

Stations	Latitude (°N)	Longitude (°E)	Depth (m)
1	38° 44.00'	26° 32.10'	104
2	38° 46.58'	26° 26.44'	198
3	38° 45.66'	26° 31.28'	159
4	38° 48.23'	26° 30.00'	190
5	38° 46.58'	26° 33.55'	106

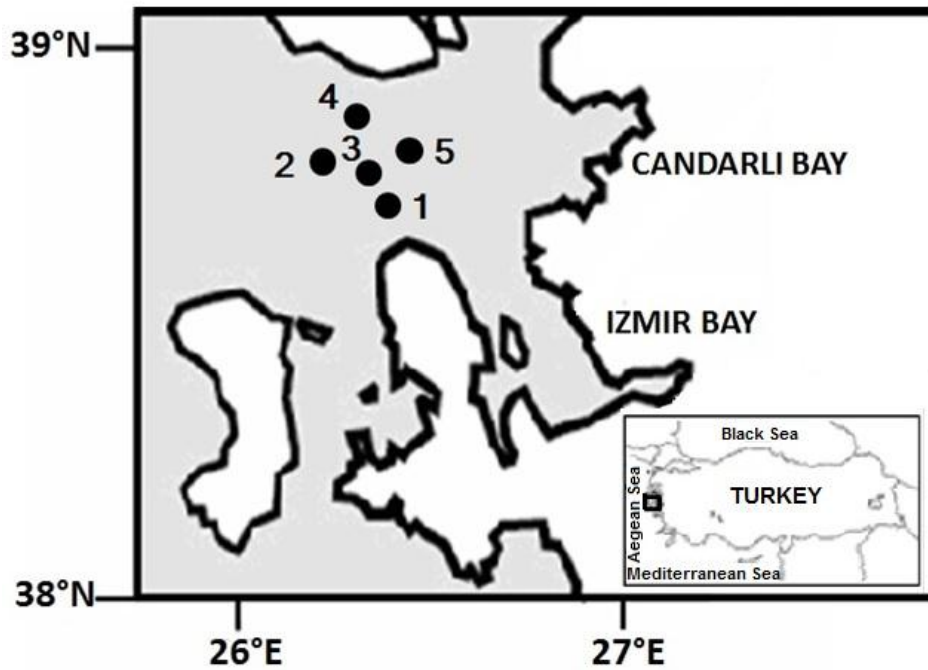


Figure 2.1 The study area including 5 stations in Aegean Sea.



Figure 2.2 RV/K Piri Reis of IMST, DEU.

2.2 Physicochemical Data Gathering and Analysis

Physicochemical data including temperature, salinity, density, pH, dissolved oxygen (DO), total suspended solids (TSS), chlorophyll α (Chl α), reactive silicate (Si), nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) of seawater samples and total carbon (TC), total organic carbon (TOC), total nitrogen (TN), total organic nitrogen (TON), total phosphorus (TP), total organic phosphorus (TOP) and also grain size of sediment samples were obtained from the survey.

Seawater samples were taken from GoFlo bottles of the SBE 19plus V2 (Version 2) Seacat Profiler CTD (Figure 2.3). Raw data of temperature, salinity and density for seawater samples were obtained from CTD, pH data by WTW and DO data with Winkler method during survey. After survey, reaching to the lab, the concentrations in filtered seawater samples for chlorophyll α (Lorenzen & Jeffrey, 1980) and nutrients as reactive Si, NO_2^- (Grasshoff et al., 1983; Strickland & Parson, 1972)

were determined by spectrophotometric analysis while nutrients as PO_4^{-3} , NO_3^- + NO_2^- (Grasshoff et al., 1983; Strickland & Parson, 1972) were obtained by autoanalyzer.



Figure 2.3 SBE 19plus V2 (Version 2) Seacat Profiler CTD on RV/K Piri Reis.

Sediment samples were obtained from Box-corer (Figure 2.4). For grain size distribution, standard test method for particle size of soils was used and the sieve analysis and the hydrometer method were performed for the larger sized and the finer particles, respectively (ASTM, 2007). TC/TOC and TN/TON percentages of sediment samples, on the other hand, were measured by Carlo Erba 2500 model CHN analyzer (Verardo et al., 1990) and TP/TOP contents were obtained from spectrophotometric analysis (Danovoro, 2009).

From all those data, the vertical graphs were drawn with Grapher 7 and the horizontal graphs were with Surfer 10 softwares according to user's manuals.

a)



b)



c)



Figure 2.4 Box-corer and sediment sampling on RV/K Piri Reis (a, b and c, respectively).

2.2.1 CTD in Water Column

Raw data for temperature, salinity, density and depth for water column were measured with the SBE 19plus V2 (Version 2) Seacat Profiler CTD on research vessel.

After survey, according to the manual revision 7.21h of Seasoft V2 software for SBE data processing, first, raw data were converted from CTD and stored in “.cnv” files. Second, data were averaged for each meter of depth with “bin average”. Third, those were saved as notepad file and then opened with Microsoft Office Excel 2003.

2.2.2 pH in Water Column

pH data of seawater samples taken from GoFlo bottles of the SBE 19plus V2 (Version 2) Seacat Profiler CTD were measured with WTW on the vessel.

2.2.3 DO in Water Column

Before survey, reagents were prepared in the laboratory of DEU IMST. Dissolved oxygen analysis of seawater samples taken from GoFlo bottles was performed during survey on the vessel (Grasshoff et al., 1983).

2.2.3.1 Before Survey

The reagents Winkler A and Winkler B, sodium thiosulfate, starch solution and potassium iodide solution were prepared and kept in brown glass bottles. Those solutions, concentrated sulfuric acid, stoppered BOD bottles, 250 ml Erlenmeyer flasks, Pasteur pipettes, dispenser and a dropper were taken to the research vessel.

2.2.3.1.1 Reagents. Winkler A: 450 g $\text{MnO}_4 \cdot 4\text{H}_2\text{O}$ was dissolved in 1 liter of distilled water. Winkler B: 500 g of NaOH and 135 g NaI were dissolved in 1 liter of distilled water. 3 % (w/v) starch solution: 3 g soluble starch was suspended in boiling

100 ml glycerin. 0.01 N sodium thiosulfate solution: 12.5 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was dissolved in 5 liter of boiled distilled water. 0.01 N potassium iodide solution: 0.3567 g of KIO_3 was dissolved in 1 liter of dissolved water.

2.2.3.2 During Survey

A stoppered BOD bottle was completely filled with seawater sample taken from GoFlo bottle of the SBE 19plus V2 (Version 2) Seacat Profiler CTD to get rid of air bubbles. 1 ml of manganese sulfate (Winkler A) and then 1 ml of alkali-iodide-azide reagent (Winkler B) were added to the sample carefully without air-bubble. The brownish orange cloud or the floc approved the presence of oxygen. It was left in a cool and dark container to settle down till a clear supernatant occurred. Then, 1ml of concentrated sulfuric acid was added to the sample and the bottle was turned upside down several times to mix it. It was transferred to an Erlenmeyer flask. When one drop of pale yellow starch solution was added, blue color appeared. Then, titration was applied with sodium thiosulfate till blue color disappeared, in other words, a clear solution occurred. Lastly, the titration volume was recorded.

2.2.3.2.1 *Standardization.* After mixing 1 ml of concentrated sulfuric acid, 1 ml of Winkler B, 1 ml of Winkler A, 25 ml of potassium iodide and 100 ml of distilled water in an Erlenmeyer flask and then waiting for 5 min, titration was performed in the same manner as applied to the sample.

2.2.3.3 After Survey

Dissolved oxygen contents in ml/l were calculated using volumes of standardization, of titration and of the BOD bottles according to the formulas below.

$$N = 25 \cdot 0.01 / V_{\text{standardization}} \quad (2.1)$$

$$\text{Dissolved Oxygen} = (5566 \cdot V_{\text{bottle}} \cdot N) / (V_{\text{titration}} - 2) \quad (2.2)$$

2.2.4 TSS in Water Column

The analysis of total suspended solids was done using 0.45 µm pore sized filter papers. Those filter papers were prepared before survey, seawater samples taken from GoFlo bottles were filtered during survey and then TSS contents were determined after survey

2.2.4.1 Before Survey

0.45 µm pore sized filter papers were dried at 105 °C overnight and they were put into desiccators for at least 3 h. Then, they were weighed and the weights were recorded as initial weights. Those filter papers and filtration apparatus were taken to the vessel.

2.2.4.2 During Survey

On the vessel, seawater samples taken from GoFlo bottles were filtered through the prepared filter papers. The filtration volumes, as at least 1.5 liters, were recorded. Then, those filter papers were placed onto aluminum folio and stored at + 4 °C.

2.2.4.3 After Survey

The filter papers used during the survey were dried at 105 °C overnight and they were put into desiccators for at least 3 h. Then, they were weighed and the weights were recorded as final weights. TSS contents in mg/l were calculated using filtration volume, initial and final weights of the filter papers according to the formula below.

$$\text{Total Suspended Solids} = (w_{\text{final}} - w_{\text{initial}}) / V_{\text{filtration}} \quad (2.3)$$

2.2.5 Chl α in Water Column

The analysis of chlorophyll α was performed using UV/Vis spectrophotometer at the lab after filtering seawater samples through GF/F filter papers on the vessel (Lorenzen & Jeffrey, 1980).

2.2.5.1 Before Survey

Magnesium carbonate solution was prepared. Then, this solution, filtration apparatus, GF/F filter papers with 47 mm diameter and about 0.7 μm pore size and also a mesh with 200 μm pore size were taken to the vessel.

2.2.5.1.1 Reagents. 1 % (w/v) magnesium carbonate solution: 1 g of MgCO_3 was dissolved in 100 ml of distilled water.

2.2.5.2 During Survey

On the vessel, seawater samples taken from GoFlo bottles were first filtered through the mesh with 200 μm pore size and then through GF/F filter papers with about 0.7 μm pore size. And just before the end of filtration, 2 ml of magnesium carbonate was added. The filtration volumes, as at least 1.5 liters, were recorded. Then, those filter papers were placed onto aluminum folio and stored at $-20\text{ }^\circ\text{C}$.

2.2.5.3 After Survey

At the lab, before spectrophotometric analysis, the filter papers were put into stoppered test tubes and 10 ml of 90 % acetone was added. Those were covered with aluminum folio and vortexed twice for 1 min in a dark room during the day and then stored at $+4\text{ }^\circ\text{C}$ over night. In the next day, if acetone was evaporated in the tubes, it was added as the final volume of 10 ml and then they were again vortexed for 1 min in a dark room. They were centrifuged for 5 min before analyzed spectrophotometrically. Absorbance values of the liquid phase at 630 nm, 647 nm and

665 nm were obtained from UV/Vis spectrophotometer and 90 % acetone was used as the blank.

2.2.5.3.1 Reagents. 90 % acetone solution: 900 ml of acetone and 100 ml of distilled water were mixed.

2.2.5.3.2 Calculations. Chlorophyll α concentrations in $\mu\text{g/l}$ were calculated using filtration volume of seawater sample, absorbances at 630 nm, 647 nm and 665 nm and also width of cuvette in cm used for spectrophotometric analysis according to formulas given below.

$$\text{TD}_{\text{Chl } \alpha} = (11.85 * \text{Abs}_{665}) - (1.54 * \text{Abs}_{647}) - (0.08 * \text{Abs}_{630}) \quad (2.4)$$

$$\text{Chlorophyll } \alpha = (\text{TD}_{\text{Chl } \alpha} * V_{\text{acetone}}) / (V_{\text{filtration}} * L_{\text{cuvette width}}) \quad (2.5)$$

2.2.6 Reactive Si in Water Column

Reactive Si concentrations were obtained by spectrophotometric analysis of filtered seawater samples kept in plastic bottles at $-20\text{ }^{\circ}\text{C}$ (Grasshoff et al., 1983; Strickland & Parson, 1972).

2.2.6.1 During Survey

Seawater samples taken from GoFlo bottles were filtered through the filter papers with $0.45\text{ }\mu\text{m}$ pore size and then 50 ml plastic bottles were filled with filtrate and those bottles were kept at $-20\text{ }^{\circ}\text{C}$ on the vessel.

2.2.6.2 After Survey

25 ml of filtered seawater samples were put into 50 ml plastic containers. 1 ml of mixed reagent was added and it was waited for 5 – 10 min. Then, first 0.5 ml of 2.8 % ascorbic acid and second 1 ml of 10 % oxalic acid were added and swirled. After

waiting for 30 – 60 min and blue color occurred, absorbance values at 810 nm were measured with UV/Vis spectrophotometer.

2.2.6.2.1 Reagents. 8.93 N sulfuric acid solution: 250 ml concentrated H_2SO_4 was added into the final volume of 1 liter distilled water. 12.67 % (w/v) ammonium heptamolybdate solution: 38 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ was dissolved in 300 ml of distilled water. Mixed reagent: 300 ml of 12.67 % ammonium heptamolybdate was added to 300 ml of 8.93 N sulfuric acid solution. 2.8 % (w/v) ascorbic acid solution: 2.8 g of $\text{C}_6\text{H}_8\text{O}_6$ was dissolved in 100 ml distilled water. 10 % (w/v) oxalic acid solution: 10 g of $(\text{COOH})_2\cdot 2\text{H}_2\text{O}$ was dissolved in 100 ml distilled water.

2.2.6.2.2 Standard Curve. By using silicate standard stock solution (SiO_2 in NaOH) whose Si concentration was 1000 mg/l, dilutions were prepared for the standard Si curve. Final concentrations of those dilutes were between 3 $\mu\text{g/l}$ and 180 $\mu\text{g/l}$. Then, the same procedure as applied to the seawater samples was followed.

2.2.6.2.3 Calculations. The concentrations of reactive Si in μM were calculated according to the formula given below.

$$\text{Reactive Si} = (\text{concentration at 810 nm}) / (\text{molecular weight of Si}) \quad (2.6)$$

2.2.7 NO_2^- in Water Column

NO_2^- concentrations were obtained by spectrophotometric analysis of filtered seawater samples kept in brown glass bottles at -20°C (Grasshoff et al., 1983; Strickland & Parson, 1972).

2.2.7.1 During Survey

Seawater samples taken from GoFlo bottles were filtered through the filter papers with 0.45 μm pore size and then 100 ml brown glass bottles were filled with filtrate and those bottles were kept at -20°C on the vessel.

2.2.7.2 After Survey

50 ml of filtered seawater samples were put into 250 ml glass Erlenmeyer flasks. 1 ml of mixed reagent was added and mixed. Then, 1 ml of 0.1 % NED solution was added and it was waited for 10 min. When pink color occurred, absorbance values at 543 nm were measured with UV/Vis spectrophotometer.

2.2.7.2.1 Reagents. Mixed reagent: 5 g of sulfanilamide ($C_6H_8N_2O_2S$) was dissolved in 50 ml of concentrated hydrochloric acid (HCl) to the final volume of 500 ml distilled water. 0.1 % (w/v) NED (N-1-naphthylethylenediamine dihydrochloride) solution: 0.5 g of $C_{12}H_{16}Cl_2N_2$ was dissolved in 500 ml of distilled water.

2.2.7.2.2 Standard Curve. By using nitrite standard stock solution ($NaNO_2$ in H_2O) whose NO_2^- concentration was 1000 mg/l, dilutions were prepared for the standard NO_2^- curve. And after dividing into molecular weight of NO_2^- , final concentrations of those dilutes were between 0.004 μM and 0.5 μM . Then, the same procedure as applied to the sea water samples was followed.

2.2.7.2.3 Calculations. The concentrations of NO_2^- in μM were directly given by UV/Vis spectrophotometer.

2.2.8 NO_3^- in Water Column

$(NO_3^- + NO_2^-)$ and PO_4^{3-} concentrations were obtained at the same time using the same filtered seawater samples kept in plastic bottles at $-20\text{ }^\circ C$ by autoanalyzer but with different reagents (Grasshoff et al., 1983; Strickland & Parson, 1972). NO_3^- concentrations were then found by subtracting the $(NO_3^- + NO_2^-)$ concentrations from NO_2^- concentrations obtained from spectrophotometric analysis.

2.2.8.1 During Survey

Seawater samples taken from GoFlo bottles were filtered through the filter papers with 0.45 μm pore size and then 50 ml plastic bottles were filled with filtrate and those bottles were kept at $-20\text{ }^{\circ}\text{C}$ on the vessel.

2.2.8.2 After Survey

3 – 5 ml of filtered seawater samples, wash solution and blank (distilled water) were put separately into polyethylene cups and those placed as 11 seawater sample cups, 1 wash sample cup and 1 blank cup in order onto the rotating plate of the sampler. Liquid sample and also reagents passed automatically through peristaltic multi channel pump after arranging the time intervals. After reduction of nitrate to nitrite in copper – cadmium reductor, the concentrations of their sum obtained from peak signals were then given by the computer of the autoanalyzer

2.2.8.2.1 Reagents. Buffer: 75 g of ammonium chloride (NH_4Cl) was dissolved in 5 liter distilled water with pH 8.5. Mixed reagent: 5 g of sulfanilamide ($\text{C}_6\text{H}_8\text{N}_2\text{O}_2\text{S}$) was dissolved in 50 ml of concentrated hydrochloric acid (HCl) to the final volume of 500 ml distilled water. 0.05 % (w/v) NED (N-1-naphthylethylenediamine dihydrochloride) solution: 0.5 g of $\text{C}_{12}\text{H}_{16}\text{Cl}_2\text{N}_2$ was dissolved in 1 liter distilled water.

2.2.8.2.2 Standard Curve. By using nitrate standard stock solution (KNO_3 in H_2O) whose NO_3^- concentration was 1000 mg/l, dilutions were prepared for the standard NO_3^- base. And after dividing into molecular weight of NO_3^- , final concentrations of those dilutes were between 0 μM and 20 μM .

2.2.8.2.3 Calculations. NO_3^- concentrations in μM were calculated by subtracting the $(\text{NO}_3^- + \text{NO}_2^-)$ concentrations obtained by autoanalyzer from NO_2 concentrations obtained by UV/Vis spectrophotometer.

2.2.9 PO_4^{-3} in Water Column

As mentioned above, PO_4^{-3} and $(\text{NO}_3^- + \text{NO}_2^-)$ concentrations were obtained at the same time using the same filtered seawater samples kept in plastic bottles at -20°C by autoanalyzer but with different reagents (Grasshoff et al., 1983; Strickland & Parson, 1972).

2.2.9.1 During Survey

Seawater samples taken from GoFlo bottles were filtered through the filter papers with $0.45\ \mu\text{m}$ pore size and then 50 ml plastic bottles were filled with filtrate and those bottles were kept at -20°C on the vessel.

2.2.9.2 After Survey

Since the same seawater samples were used for both PO_4^{-3} and $(\text{NO}_3^- + \text{NO}_2^-)$ concentrations by autoanalyzer, the procedure mention in section 2.1.8.2 was followed. Unlikely, the liquid sample passed together with air segment in a specific ratio through the peristaltic multi channel pump and copper – cadmium reductor was not used.

2.2.9.2.1 Reagents. 0.6 % (w/v) ascorbic acid solution: 6 g of $\text{C}_6\text{H}_8\text{O}_6$ was dissolved in 1 liter of distilled water. 2.3 % (w/v) potassium antimonyl tartarate solution: 23 g of $\text{K}(\text{SbO})\text{C}_6\text{H}_4\text{O}_6$ was dissolved in 1 liter of distilled water. Mixed reagent: 10 g of ammonium heptamolybdate tetrahydrate $((\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O})$, 20 ml potassium antimonyl tartarate solution, and 50 ml of concentrated H_2SO_4 was added into final volume of 1 liter distilled water.

2.2.9.2.2 Standard Curve. By using phosphate standard stock solution (KH_2PO_4 in H_2O) whose PO_4^{-3} concentration is 1000 mg/l, dilutions were prepared for the standard PO_4^{-3} base. And after dividing into molecular weight of PO_4^{-3} , final concentrations of those dilutes were between $0\ \mu\text{M}$ and $0.5\ \mu\text{M}$.

2.2.9.2.3 *Calculations.* The concentrations of PO_4^{-3} in μM were directly given by the autoanalyzer.

2.2.10 *TC-TOC and TN-TON in Sediment*

Sediment samples were collected from box-corer on the vessel and kept at -20°C . After reaching to the lab, the cores were sliced into 2-3 cm pieces and then total, inorganic and organic carbon and nitrogen content in all sediment samples were analyzed with Carlo Erba 2500 model CHN analyzer in Institute of Marine Sciences and Management, Istanbul University, Istanbul, Turkey (Verardo et al., 1990).

2.2.10.1 *After Survey*

After slicing a core sample, it was put into 40 ml glass container as done to a surface sediment sample. Then, it was dried in an oven at 50°C for 1-2 days. 1-10 mg dry subsample was weighed and recorded. It was put into a tin capsule for CHN analyzer. For organic carbon or nitrogen content, acidification was done with a few drops of 1 N hydrochloric acid. The sample was dried 1 h at 50°C and weighed again. For total carbon and nitrogen content, acidification was skipped. Then, the tin capsule was put into CHN analyzer and combusted at $1020-1050^\circ\text{C}$. Standards and blanks were treated in the same manner. Total and organic carbon and nitrogen concentrations were calculated using CHN analyzer software.

2.2.10.1.1 *Reagents.* 1 N hydrochloric acid solution: 83.3 ml of concentrated HCl was added into final concentration of 1 liter distilled water.

2.2.10.1.2 *Calculations.* Total and organic C or N percentages were calculated by CHN analyzer software and with those results, inorganic C or N percentages were calculated subtracting the organic values from total percents.

2.2.11 TP-TOP in Sediment

Sediment samples were collected from box-corer on the vessel and kept at -20°C . After reaching to the lab, the cores were sliced into 2-3 cm pieces and total, inorganic and organic phosphorus content in all sediment samples were analyzed with UV/Vis spectrophotometer (Danovaro, 2009).

2.2.11.1 After Survey

After slicing a core sample, it was put into 40 ml glass container as done to a surface sediment sample. Then, it was dried in an oven at 50°C for 1-2 days. For the determination of inorganic P, 0.3-0.5 g of dry subsample was weighed and recorded. For the determination of total P, again 0.3-0.5 g of dry subsample was weighed, combusted at 550°C for 2 h and then weighed and recorded again. After adding 50 ml of 1 N hydrochloric acid to each separately, it was shaken upside down and kept 16 h at room temperature. When the sediment was completely sink and the supernatant was clear, 2 ml of this clear liquid was taken. The final volume became 50 ml with distilled water after adding also 5 ml mixed reagent. It was kept 10 min at room temperature, blue color occurred and then its absorbance at 885 nm was measured.

2.2.11.1.1 Reagents. 1 N hydrochloric acid solution: 83.3 ml of concentrated HCl was added into final volume of 1 liter distilled water. 5 N sulfuric acid solution: 140 ml of concentrated H_2SO_4 was added into final volume of 1 liter distilled water. 4 % (w/v) ammonium heptamolybdate tetrahydrate solution: 40 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ was dissolved in 1 liter of distilled water. 0.3 % (w/v) potassium antimonyl tartarate solution: 3 g of $\text{K}(\text{SbO})\text{C}_6\text{H}_4\text{O}_6$ was dissolved in 1 liter of distilled water. 1.8 % (w/v) ascorbic acid solution: 18 g of $\text{C}_6\text{H}_8\text{O}_6$ was dissolved in 1 liter of distilled water. Mixed reagent: 50 ml of 5 N sulfuric acid, 15 ml of 4 % ammonium heptamolybdate, 30 ml of 1.8 % ascorbic acid and 5 ml of 0.3 % potassium antimonyl tartarate were mixed in order.

2.2.11.1.2 *Standard Curve.* By using phosphate standard stock solution (KH_2PO_4 in H_2O) whose PO_4^{-3} concentration is 1000 mg/l, dilutions were prepared for the standard P curve after calculating P concentrations by dividing into molecular weight of PO_4^{-3} and then multiplying with molecular weight of P. Final concentrations of those dilutes were between 0.0196 mg/l and 0.0782 mg/l. Then, the same procedure as applied to the supernatant of sediment samples was followed.

2.2.11.1.3 *Calculations.* Total, inorganic and organic P concentrations in ppm ($\mu\text{g/g}$) were calculated according to the formulas given below.

$$\text{Total Phosphorus} = \left(\frac{[\text{concentration measured in combusted sample}] * 25 * 30.97}{\text{weight of combusted sample}} \right) \quad (2.7)$$

$$\text{Inorganic Phosphorus} = \left(\frac{[\text{concentration measured in uncombusted sample}] * 25 * 30.97}{\text{weight of combusted sample}} \right) \quad (2.8)$$

$$\text{Organic Phosphorus} = (\text{Total Phosphorus}) - (\text{Inorganic Phosphorus}) \quad (2.9)$$

2.2.12 Grain Size of Sediment

To determine the percentages of different grain sizes of sediment samples taken from box corer on the vessel, ASTM D 422-63 standard test method for particle size analysis of soils was followed at lab after survey. The sieve analysis and the hydrometer method were performed for the larger sized and the finer particles, respectively.

2.2.12.1 After Survey

For the sieve analysis, sediment samples were air dried over night. Dry sediments were passed through the set of sieves with 38.1 mm, 19 mm, 9.51 mm, 4 mm, 2 mm, 1 mm, 0.5 mm, 0.25 mm, 0.125 mm and 0.075 mm mesh sizes, respectively on the sedimentation cylinder with a sieve shaker for 20 min. Then, sediment samples

retained on each sieve were weighed and recorded. Those were added one by one to find the mass arriving and in the end for the total mass of the sediment.

For the hydrometer analysis, the fine sediment retained on the bottom pan of the sieve set was put into a beaker. After adding 125 ml of sodium hexametaphosphate solution, it was waited over night. Then, this was put into a cylinder and 1 liter of distilled water was added. This was mixed with a mixer and immediately 152H hydrometer was put into the cylinder. The values at the junction of the hydrometer and the top level of the solution in the cylinder were recorded 1 min, 2 min, 5 min, 10 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min and 1440 min later. At the time period of 30 min, the hydrometer was removed slowly from the cylinder and put into a beaker full of water. Then, a few minutes before the time period of 60 min, the hydrometer was put slowly again into the cylinder and the value was recorded at that time. The same was performed for the rest of the time periods.

In the hydrometer analysis, there were values obtained such as zero correction, meniscus correction, k value, temperature correction and correction factor α . The zero correction was determined according to the reading at the top of meniscus formed by the hydrometer in the control solution which was the same mixture of 125 ml of sodium hexametaphosphate solution in 1 liter of distilled water but without sediment sample. The meniscus correction was determined as the difference between the top of the meniscus and the level of control solution in the cylinder according to the table of ASTM D 422 63 standard method. On the other hand, k value, temperature correction and correction factor α were obtained from tables of the same method according to the specific gravity of the sediment particles and the temperature as 2.70 g/cm^3 and 20°C , respectively.

In the end, percent passing sediment versus larger and finer particle diameter was drawn on the semilogarithmic sheet and the sediment sample was classified according to the calculated percent passing values.

2.2.12.1.1 *Reagents.* 4 % (w/v) sodium hexametaphosphate solution: 40 g of $\text{Na}_6\text{P}_6\text{O}_{18}$ was dissolved in 1 liter distilled water.

2.2.12.1.2 *Calculations.* For the sieve analysis, the percentages for retained and passing sediment for each sieve were calculated according to the formulas below.

$$\% \text{ Retained} = (\text{Mass retained} / \text{Total mass}) * 100 \quad (2.10)$$

$$\% \text{ Passing} = 100 - \% \text{ Retained} \quad (2.11)$$

For the hydrometer analysis, the corrected hydrometer reading (R_C) and meniscus corrected reading (R) were calculated according to the formulas below with the hydrometer reading (R_{ACTUAL}). Zero and meniscus corrections were determined as 3 and 1, respectively and also temperature correction (C_T) was obtained as 0.00 for 20 °C.

$$R_C = R_{\text{ACTUAL}} - \text{zero correction} + C_T \quad (2.12)$$

$$R = R_C + \text{meniscus correction} \quad (2.13)$$

According to the table of ASTM D 422 63 standard method, effective depth (L) in cm for 152H hydrometer was determined by using meniscus corrected reading. Then, by using elapsed time in min and k value determined as 0.01344, the equivalent particle diameter (D) in mm was calculated according to the formula below.

$$D = k * (L/t)^{1/2} \quad (2.14)$$

Adjusted percent passing finer particles was calculated according the below formula including % passing value from the sieve with 0.075 mm mesh size which was already calculated for sieve analysis.

$$P_A = R_C * \alpha * (\% \text{ passing from sieve with 0.075 mm mesh size}) \quad (2.15)$$

The percent passing values for particle diameters as 32 mm and 16 mm were obtained using the drawing of % passing versus diameter values for larger and finer particles on the semilogarithmic sheet. Then, the sediment sample classification was determined by using the calculated percent values of cobbles, coarse, medium and fine gravel, sand, silt and clay according to the formulas given below.

$$\% \text{ Cobbles} = 100 - \% \text{ passing from 32 mm mesh size} \quad (2.16)$$

$$\% \text{ Coarse Gravel} = \% \text{ passing from 32 mm} - \% \text{ passing from 16 mm} \quad (2.17)$$

$$\% \text{ Medium Gravel} = \% \text{ passing from 16 mm} - \% \text{ passing from 4 mm} \quad (2.18)$$

$$\% \text{ Fine Gravel} = \% \text{ passing from 4 mm} - \% \text{ passing from 2 mm} \quad (2.19)$$

$$\% \text{ Coarse Sand} = \% \text{ passing from 2 mm} - \% \text{ passing from 0.5 mm} \quad (2.20)$$

$$\% \text{ Medium Sand} = \% \text{ passing from 0.5 mm} - \% \text{ passing from 0.25 mm} \quad (2.21)$$

$$\% \text{ Fine Sand} = \% \text{ passing from 0.25 mm} - \% \text{ passing from 0.075 mm} \quad (2.22)$$

$$\begin{aligned} \% \text{ Coarse Silt} &= \% \text{ passing from 0.075 mm mesh size} - \\ &\quad \% \text{ having with max 0.0156 mm diameter} \end{aligned} \quad (2.23)$$

$$\begin{aligned} \% \text{ Medium Silt} &= \% \text{ having with max 0.0156 mm diameter} - \\ &\quad \% \text{ having with max 0.0078 mm diameter} \end{aligned} \quad (2.24)$$

$$\begin{aligned} \% \text{ Fine Silt} = \% \text{ having with max } 0.0078 \text{ mm diameter} - \\ \% \text{ having with max } 0.0039 \text{ mm diameter} \end{aligned} \quad (2.25)$$

$$\% \text{ Clay} = \% \text{ having with max } 0.0039 \text{ mm diameter} \quad (2.26)$$

2.3 Biological (Bacterial) Data Gathering and Analysis

For biological data, actinomycetes were isolated using 2 different isolation media (with or without 6 different antibiotics) and 7 different sediment processing methods (Galatenko and Terekhova, 1990; Gontag et al., 2007; Hayakawa et al., 1991; Mincer et al., 2002). Their morphological features were recorded and physiological characteristics were tested using commercial kits. After genomic DNA extraction using commercial kits and amplification of the 16S rRNA genes using 27f – 1492r universal primers, sequences of PCR products were analyzed using computer programmes. Accession numbers were obtained from NCBI GenBank and phylogenetic tree was constructed using softwares.

2.3.1 Isolation of Actinomycetes

Sediment samples were collected with sterile plastic containers and sterile plastic cores from 5 stations on 31 March 2012. Till processing in the lab, they were kept at – 20 °C.

After reaching to the lab, core samples were sliced into 3 cm pieces. In addition, seawater taken at 400 m depth of Aegean Sea was autoclaved and then filtered with 0.22 µm pore size. This sterilized sea water was kept at + 4 °C.

2 different isolation media (M1 and M7m) were used with or without 6 different antibiotics as cycloheximide (100 µg/ml), nystatin (50 µg/ml), polymixin B sulfate (5 µg/ml), rifampin (5 µg/ml), kanamycin sulfate (5 µg/ml), novobiocin (25 µg/ml) to isolate bacteria from the sediment samples and the agar media were prepared with sterile seawater. Agar plates contained only one antibiotic or not. The isolation media consisted of the following: M1, 18 g agar, 10 g starch, 4 g yeast extract, 2 g peptone,

1 liter sterile seawater; M7m (BD DifcoTM Actinomycetes Isolation Agar, modified), 22 g medium, 5 ml glycerol, 500 ml sterile seawater and 500 ml distilled water.

7 different sediment processing methods were performed. In the first processing method (a) (Mincer et al., 2002), 10 ml of wet sediment samples were aseptically placed on sterile aluminium foil and kept overnight under biosafety cabinet to dry. Then, the dried sediment was spread aseptically on the agar surface 35-40 times in a circular fashion with a triangular glass rod. In the dry spot method (b), over night dried sediment was taken with sterile sponge and put clockwise on the agar media. In the third method (c) (Gontag et al., 2007), 1 ml of wet sediment was added to 4 ml of sterile sea water and then heated for 6 min at 55 °C. The diluted sample was then vigorously shaken for 30 s by vortex and 75-100 µl of each dilution was inoculated by spreading aseptically with a triangular glass rod onto agar-based isolation media. In the fourth method (d) (Hayakawa et al., 1991), wet sediment was heated for 15 min at 70 °C and then spread aseptically on the agar surface 35-40 times in a circular fashion with a triangular glass rod. In the fifth method (e) (Galatenko and Terekhova, 1990), wet sediment was kept for 30 sec under UV and then spread aseptically on the agar surface 35-40 times in a circular fashion with a triangular glass rod. In the sixth method (f), 1 ml wet sediment sample was added to 1 ml of sterile seawater and then vigorously shaken for 30 s by vortex. The dilutions of 1:10 and 1:100 were also done. Then, 75-100 µl of each dilution was inoculated by spreading aseptically with a triangular glass rod onto agar-based isolation media. In the seventh method (g), wet sediment sample was directly taken and spread with a triangular glass rod onto agar-based isolation media. All plates were incubated at 26-28 °C upto 2 months in incubator Nuve ES110.

Actinomycetes seen on isolation media were subcultured aseptically on M1 or M7m media with cycloheximide and sterile seawater. Those subcultured bacteria were inoculated onto nutrient agar (23 g/l) prepared with sterile seawater (or distilled water and 4 % NaCl) by microwave oven and/or inoculated into nutrient broth (8 g/l) prepared with distilled water and 4 % NaCl in sterile glass test tubes having lids.

Those stock bacteria were incubated at 26-28 °C for 3 days to 2 weeks and then kept at + 4 °C till further use.

Bacteria seen on those agar plates were inoculated aseptically into autoclaved eppendorf tubes including brain-heart infusion (37 g/l) prepared with distilled water and 4 % NaCl. After autoclaved 50 % glycerol was added, they were incubated at 26-28 °C for 3 days to 2 weeks and then cryopreserved at – 20 °C.

2.3.2 Phenotypic Characters of Actinomycetes

Morphological features such as colony and spore colors of cultured actinomycetes were recorded after taking their photos on agar medium and getting their scanning electron microscope (SEM) images. Additional phenotypic characters such as enzymatic activities and carbon utilizations were obtained using commercial test kits.

2.3.2.1 Colony Images

The photos of the colonies on agar media (M1 and/or M7m) were taken by FinePix AV110 camera.

For SEM images, first, strains of each actinomycetes cultivated in 2 ml nutrient broth were filtered aseptically through 0.22 µm pore size by washing with 10 ml distilled water. Then, those filter papers were dried overnight at 50 °C and kept in desiccator for maximum one week till getting SEM images at IZTECH (Izmir Institute of Technology, Izmir, Turkey). The images were obtained using Phillips XL 30 FEG scanning electron microscope after coating the filter paper with gold to a depth of 30 nm.

2.3.2.2 Chemical Test Kits

Isolated actinomycetes were tested biochemically using two commercial kits following user's manuals. Fresh colonies of each actinomycetes strains cultivated on

M1 medium was used for biochemical tests. The first test kit included both fluorogenic and chromogenic substrates while the second one had only chromogenic substrates.

2.3.2.2.1 BD BBL Crystal™ GP ID Kit. Using aseptic technique, fresh colony of the strain was taken with a sterile cotton swab and suspended in a tube of BBL Crystal GP Inoculum Fluid (supplied with the kit). After vortexing for 10 – 15 s, it was poured into the target area of a base (supplied within the kit). The inoculum was rolled gently until all the wells were filled. Then, the base was covered with a lid (supplied within the kit) by pushing until the lid snapped into place with two “clicks”. All panels were placed in trays (supplied within the kit) and incubated face down at 26-28 °C for 1 – 2 days. Then, they were read according to the reaction chart (Figure 2.5) within 30 min after removing from incubator.

2.3.2.2.2 API ZYM Kit. Using aseptic technique, fresh colony of the strain was taken with a sterile cotton swab and suspended in a tube of API Suspension Medium (supplied within the kit). An incubation box (tray and lid supplied within the kit) was humidified by pouring 5 ml distilled water into honey-combed wells of the tray and then a strip (supplied within the kit) was placed onto the box. After inoculation by dispensing 65 µl of specimen into each cupule, the tray was covered with a plastic lid (supplied with the kit) and incubated at 26-28 °C for 4 – 4.5 h. After incubation, one drop of ZYM A and then one drop of ZYM B reagents (supplied within the kit) were added to each cupule. Then, it was waited till the color developed and interpreted according to the color reaction chart (Figure 2.6).

Panel Location	Test Feature	Code	Principle (Reference)
4A	fluorescent negative control	FCT	Control to standardize fluorescent substrate results.
2A	4MU-b-D-glucoside	FGC	Enzymatic hydrolysis of the amide or glycosidic bond results in the release of a fluorescent cuomarin derivative.
1A	L-valine-AMC	FVA	
4B	L-phenylalanine-AMC	FPH	
2B	4MU-a-D-glucoside	FGS	
1B	L-pyroglutamic acid-AMC	FPY	
4C	L-tryptophan-AMC	FTR	
2C	L-arginine-AMC	FAR	
1C	4MU-N-acetyl-b-D-glucosamide	FGA	
4D	4MU-phosphate	FHO	
2D	4MU-b-D-glucuronide	FGN	
1D	L-isoleucine-AMC	FIS	
4E	Trehalose	TRE	Utilization of carbohydrate results in lower pH and change in indicator (phenol red).
2E	Lactose	LAC	
1E	Methyl-a and b-glucoside	MAB	
4F	Sucrose	SUC	
2F	Mannitol	MNT	
1F	Maltotriose	MTT	
4G	Arabinose	ARA	
2G	Glycerol	GLR	
1G	Fructose	FRU	
4H	p-nitrophenyl-b-D-glucoside	BGL	Enzymatic hydrolysis of the colorless aryl substituted glycoside releases yellow p-nitrophenol.
2H	p-nitrophenyl-b-D-cellobioside	PCE	
1H	Proline and Leucine-p-nitroanilide	PLN	Enzymatic hydrolysis of the colorless aryl substituted glycoside releases yellow p-nitroaniline.
4I	p-nitrophenyl-phosphate	PHO	Enzymatic hydrolysis of the colorless aryl substituted glycoside releases yellow p-nitrophenol.
2I	p-nitrophenyl-a-D-maltoside	PAM	
1I	o-nitrophenyl-b-D-galactoside (ONPG) and p-nitrophenyl-a-D-galactoside	PGO	
4J	Urea	URE	Hydrolysis of urea and resulting ammonia change the pH indicator color (bromthymol blue).
2J	Esculin	ESC	Hydrolysis of esculin results in a black precipitate in the presence of ferric ion.
1J	Arginine	ARG	Utilization of arginine results in pH rise and change in color of indicator (bromcresol purple).

Figure 2.5 BD BBL Crystal™ GP ID Kit reaction chart.

Control					1
2-naphtyl phosphate					2
2-naphtyl butyrate					3
2-naphtyl caprylate					4
2-naphtyl myristate					5
L-leucyl-2-naphtylamide					6
L-vanyl-2-naphtylamide					7
L-cystyl-2-naphtylamide					8
N-benzoyl-DL-arginine-2-naphtylamide					9
N-glutaryl-phenyl-alanine-2-naphtylamide					10
2-naphtyl phosphate					11
naphthol AS-BI phosphate					12
6-Br-2-naphtyl-a-D-galacto pyranoside					13
2-naphtyl-b-D-galacto pyranoside					14
naphthol AS-BI b-D-glucuronate					15
2-naphtyl-a-D-glucopyranoside					16
6-Br-2-naphtyl-b-D-gluco pyranoside					17
1-naphtyl-N-acetyl-b-D-glucos aminide					18
6-Br-2-naphtyl-a-D-manno pyranoside					19
2-naphtyl-a-L-fucopyranoside					20

Figure 2.6 API ZYM Kit color reaction chart.

2.3.3 Genomic DNA Extraction from Actinomycetes

Genomic DNA of isolated actinomycetes was extracted under aseptic conditions with Invitrogen PureLink™ genomic DNA isolation kit according to the user's manual for Gram positive bacterial cell lysate. Then, those isolated DNA samples were illustrated on 2 % agarose gel with E-BOX VX2 gel documentation system.

2.3.3.1 Preparing Lysates

The cell pellet containing 2×10^9 Gram positive cells was suspended in a sterile 1.5 ml eppendorf tube containing 180 μ l lysozyme digestion buffer. After mixing well with brief vortexing, it was incubated at 37 °C for 30 min in a water bath. Then, 20 μ l of proteinase K (supplied within the kit) was added and mixed with brief vortexing. 200 μ l of genomic lysis/binding buffer (supplied within the kit) was also added and again mixed with brief vortexing. After incubation at 55 °C for 30 min in a water bath, 200 μ l of 96-100 % ethanol was added and vortexed for 5 sec.

2.3.3.1.1 Reagents. 20 mg/ml lysozyme digestion buffer: 0.5 ml of 1 M Tris-HCl, 0.0146 g of 292 g/mol EDTA, 0.2 ml of 1 % Triton X-100 and 0.4 g of lysozyme were mixed in the final volume of 20 ml distilled water.

2.3.3.2 Binding DNA

The prepared lysate (approximately 640 μ l) was transferred from the eppendorf tube to spin column in a collection tube (supplied within the kit). After centrifuging it at 10000 g for 1 min (or 6000 rpm for 2 min with microcentrifuge Hettich 220R) at room temperature, the collection tube was discarded and a clean one was replaced.

2.3.3.3 Washing DNA

Then, 500 μ l of wash buffer 1 (supplied within the kit) was added to the spin column. After centrifuging it at 10000 g for 1 min (or 6000 rpm for 2 min) at room temperature, the collection tube was discarded and a clean one was replaced. Then, 500 μ l of wash buffer 2 (supplied within the kit) was added to the spin column. After centrifuging it at maximum speed for 3 min (or 6000 rpm for 4 min) at room temperature, the collection tube was discarded and the spin column was placed into a sterile 1.5 ml eppendorf tube.

2.3.3.4 Eluting DNA

Then, 75 µl of genomic elution buffer (supplied within the kit) was added to the spin column and it was incubated at room temperature for 1 min. After centrifuging it at 10000 g for 1 min (or 6000 rpm for 2 min) at room temperature, the spin column was discarded. The DNA sample in the eppendorf tube was used for PCR or kept at – 20 °C.

2.3.4 Agarose Gel Illustration for Genomic DNA of Actinomycetes

2 % agarose gel was prepared with 1 gr agarose and 50 ml 1X TBE buffer. After boiling in a microwave oven, 5 µl of GelRed™ was added and swirled. A midi horizontal gel electrophoresis (Thermo EC300XL) tray was taped on both ends and comb(s) was placed on the tray. The solution was poured onto the tray and waited for the gel to solidify about half an hour. Then, tapes and the comb(s) were removed and the tray was placed into the tank which was filled with 1X TBE buffer up to the mark on the tank. The wells of the gel were visualized with dark stripes under the tank. 10 µl of DNA sample was mixed with 2 µl of 6X loading dye with a micropipette in a sterile eppendorf tube or on a parafilm and 10 µl was taken from this mixture and was loaded to the well of the gel on the tray. After loading all the samples, the external gel caster was put onto the tank and electrodes plugged into the electrophoresis power supply. It was run at 100mA and 100 V for 45 min. Then, DNA bands were seen under UV with E-BOX VX2 gel documentation system and the photo of the gel was taken.

2.3.5 PCR for 16S rRNA Gene of Actinomycetes

After seeing genomic DNA bands of actinomycetes on 2 % agarose gel, PCR was performed for 16S rRNA gene. First, 50 µl PCR mix was prepared for each extracted DNA sample. Second, PCR program was arranged for thermocycler. And last, those PCR products were illustrated on 2 % agarose gel with E-BOX VX2 gel documentation system.

2.3.5.1 PCR Mix

Totally 3 PCR mixes given below were tried with combination of 3 different PCR programs for polymerization of 16S rRNA gene with 27f (5'-AGAGTTTGATCCTGGCTCAG-3') – 1492r (5'-GGTTACCTTG TTACGACTT-3') universal primers. Before application of PCR, those forward and reverse primers were prepared as 100 pmol stock primers in sterile 1.5 ml eppendorf tubes by dissolving in autoclaved 1X TE buffer according to their concentrations. From those stock solutions, primers were prepared with nuclease free water for PCR mixes. For any of those PCR mixes, first, all ingredients except DNA sample were put into a sterile 1.5 ml eppendorf tube as much as their volumes multiplied with the total number of DNA samples. Then, 45 µl of total mixture was put into each sterile 0.2 ml PCR tube. Finally, 5 µl one of DNA samples was put into one of those sterile 0.2 ml PCR tubes to have 50 µl PCR mix. Moreover, all those including PCR mixes were prepared under ESCO Class II Biosafety cabin. The second PCR mix was also tried in Fundamental and Industrial Microbiology Division, Biology Department, Faculty of Science, Ege University, Izmir, Turkey.

	<u>PCR mix I</u>	<u>mix II</u>
Nuclease free water	27.5 µl	31.75 µl
10X <i>Taq</i> buffer (with (NH ₄) ₂ SO ₄ , Fermentas)	5 µl	5 µl
MgCl ₂ (25 mM, Fermentas)	5 µl	5 µl
dNTP (1 mM each, Fermentas)	5 µl	1 µl
Forward primer (5 pmol, Fermentas)	1 µl	1 µl
Reverse primer (5 pmol, Fermentas)	1 µl	1 µl
<i>Taq</i> DNA polymerase (5u/ µl 500U recombinant, Fermentas)	0.5 µl	0.25 µl
DNA sample	5 µl	5 µl

	<u>PCR mix III</u>
Nuclease free water	18 µl
One Taq Quick-Load 2X Master mix (with standard buffer, NED)	25 µl
Forward primer (10 pmol, Fermentas)	1 µl
Reverse primer (10 pmol, Fermentas)	1 µl
DNA sample	5 µl

2.3.5.2 PCR Program

After preparation of 50 µl PCR mix seen above, 3 different PCR program including denaturation, annealing and extension, respectively, given below were tried. The first and last ones were performed with thermocycler (Techne TC3000) having timer only as minute in DEU IMST and the second one, on the other hand, was applied with another thermocycler in Fundamental and Industrial Microbiology Division, Biology Department, Faculty of Science, Ege University.

PCR program I

94 °C for 5 min

30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min

72 °C for 7 min

PCR program II

94 °C for 5 min

30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec

72 °C for 7 min

PCR program III

94 °C for 5 min

30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min

72 °C for 7 min

2.3.6 Agarose Gel Illustration for PCR Product of Actinomycetes

The same procedure as mentioned in part 2.3.4 was also followed for PCR products. Instead of genomic DNA sample, 10 µl of PCR product was mixed with 2 µl of 6X loading dye. 5 µl of DNA marker (100-3000bp), 10 µl of each mixture and also 10 µl of the negative control mixture were all loaded to the wells, respectively.

2.3.7 Sequencing PCR Product of Actinomycetes

After obtaining PCR products whose bands were proven by agarose gel electrophoresis, those products and forward and reverse primers used were sent to IZTECH or REFGEN (Gene Research and Biotechnology, Ankara, Turkey) using ABI Prism 3100 DNA sequencer for sequencing service. Then, the chromatograms were obtained via e-mail.

2.3.8 Accession Numbers of Actinomycetes

Forward and reverse chromatograms of 16S rRNA gene of one strain were combined into one single sequence (5'-3') using Geneious (version R6.1, Biomatters Inc., USA) computer program. Then, all those sequences were entered to GenBank (www.ncbi.nlm.nih.gov/genbank/) using sequin program. Accession numbers of all actinomycetes are KC815756 – KC815765, KF366670, KF366671 and KF366674.

Those sequences were also searched for their similarities to both Basic Local Alignment Search Tool (BLAST) and Eztaxon databases.

2.3.9 Phylogenetic Analysis of Actinomycetes

Sequences of actinomycetes having accession numbers were phylogenetically analyzed using 1000 bootstrap neighbor-joining method with Mega 5.1 software (Tamura et al, 2011). In this method, maximum composite likelihood substitution model testing first, second, third codons and noncoding sites was used.

CHAPTER THREE

RESULTS

3.1 Physicochemical Results

Physicochemical data including temperature, salinity, density, pH, DO, TSS, Chl α , reactive Si, NO_2^- , NO_3^- , PO_4^{3-} of seawater samples and TC, TOC, TN, TON, TP, TOP and also grain size of sediment samples were analyzed and it was seen that the study area showed the characteristics of Aegean Sea.

3.1.1 Seawater Results

The water column of the study area gave narrow ranges for temperature, salinity, density and pH as 15.10 – 15.45 °C, 39.14 – 39.17 psu, 29.11 – 29.18 kg/m³ and 8.30 – 8.33, respectively. Unlike chlorophyll α (0.07 – 2.33 $\mu\text{g/l}$), the stations had much closer values for dissolved oxygen and total suspended solids as 5.47 – 5.81 ml/l and 0.20 – 1.60 mg/l, respectively. For nutrients, on the other hand, nitrate (BDL – 4.26 μM) and reactive Si (0.91 – 4.61 μM) had much broader ranges than nitrite and phosphate (BDL – 0.28 μM and BDL – 0.10 μM , respectively).

3.1.1.1 Temperature

Seawater temperature of the study area became lower through the station 5 at the surface layer but higher through the station 1 at the bottom (Figure 3.1). Although the temperature of the water column decreased in all stations from surface to bottom layers as expected (Figure 3.2), very close values for the stations and thus a narrow range were obtained for the study area as 15.10 – 15.45 °C.

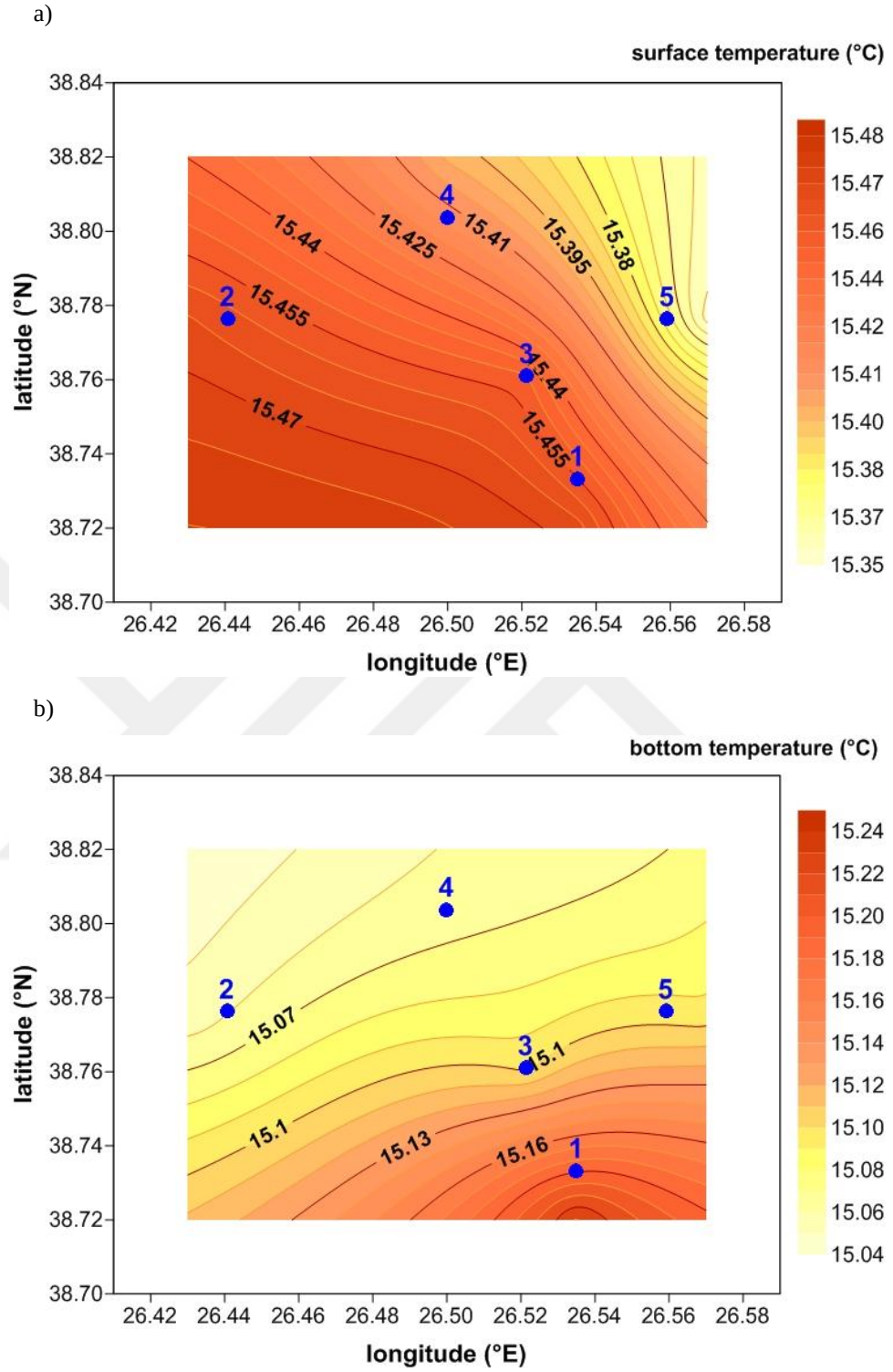


Figure 3.1 Seawater temperature of 5 stations, Aegean Sea a) surface, b) bottom layers.

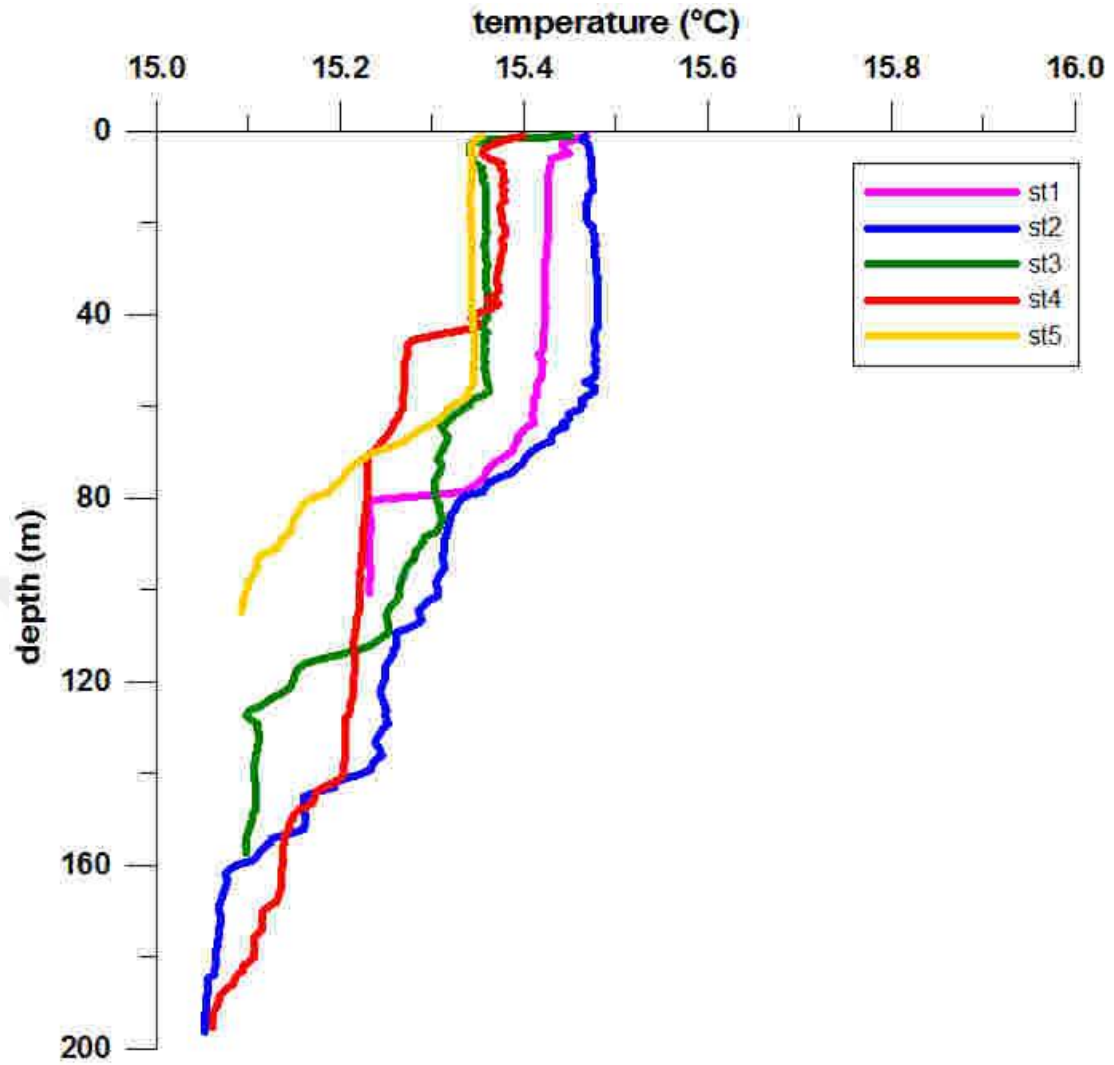


Figure 3.2 Vertical distribution of temperature at 5 stations, Aegean Sea.

3.1.1.2 Salinity

While the salinity decreased from station 1 to station 4 at the surface, it increased from station 1 to station 2 at the bottom (Figure 3.3). However, the salinity interval was 39.14 – 39.17 psu for the study area indicating close values for the water column of the stations as seen in Figure 3.4.

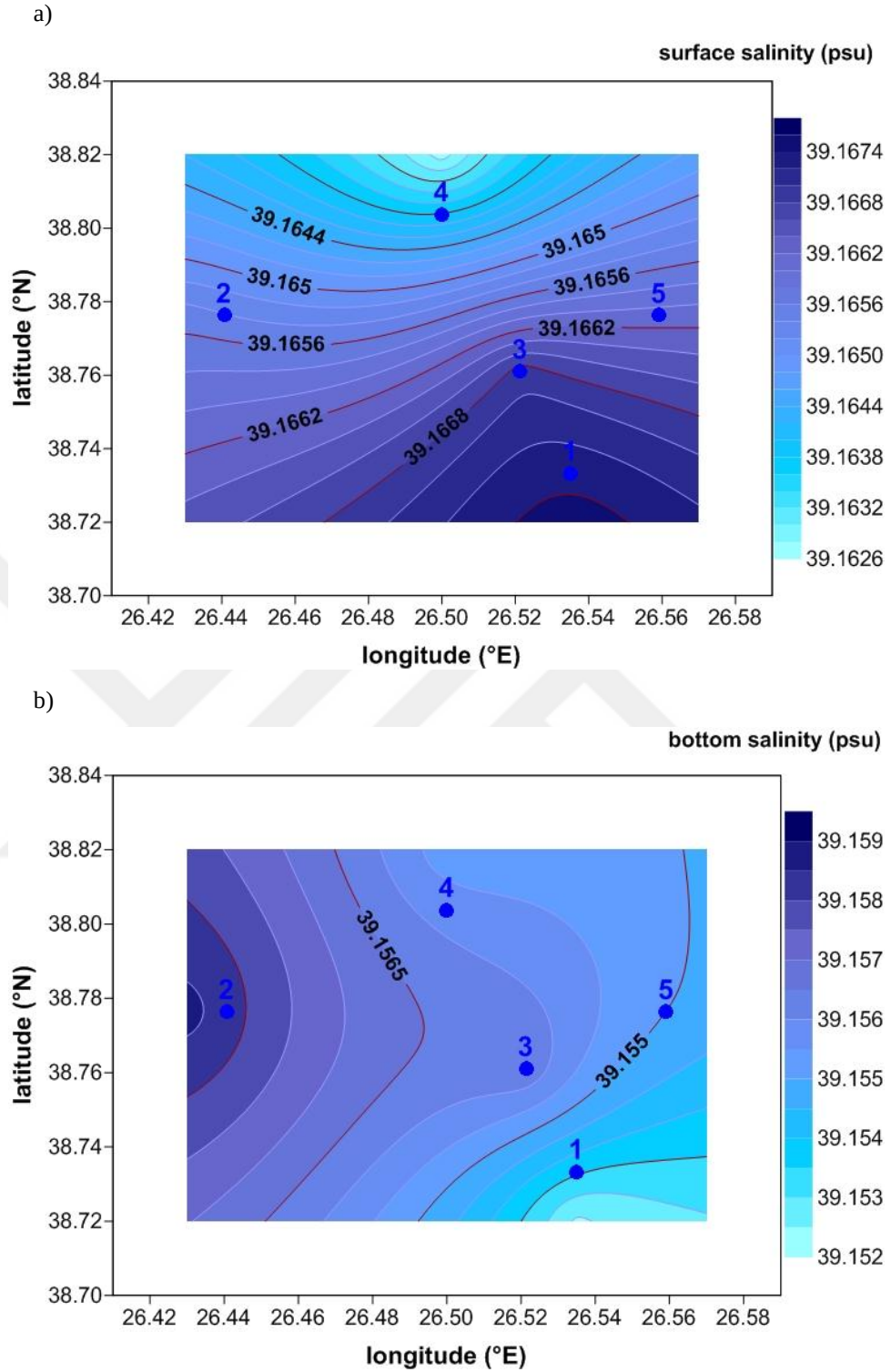


Figure 3.3 Seawater salinity of 5 stations, Aegean Sea a) surface, b) bottom layers.

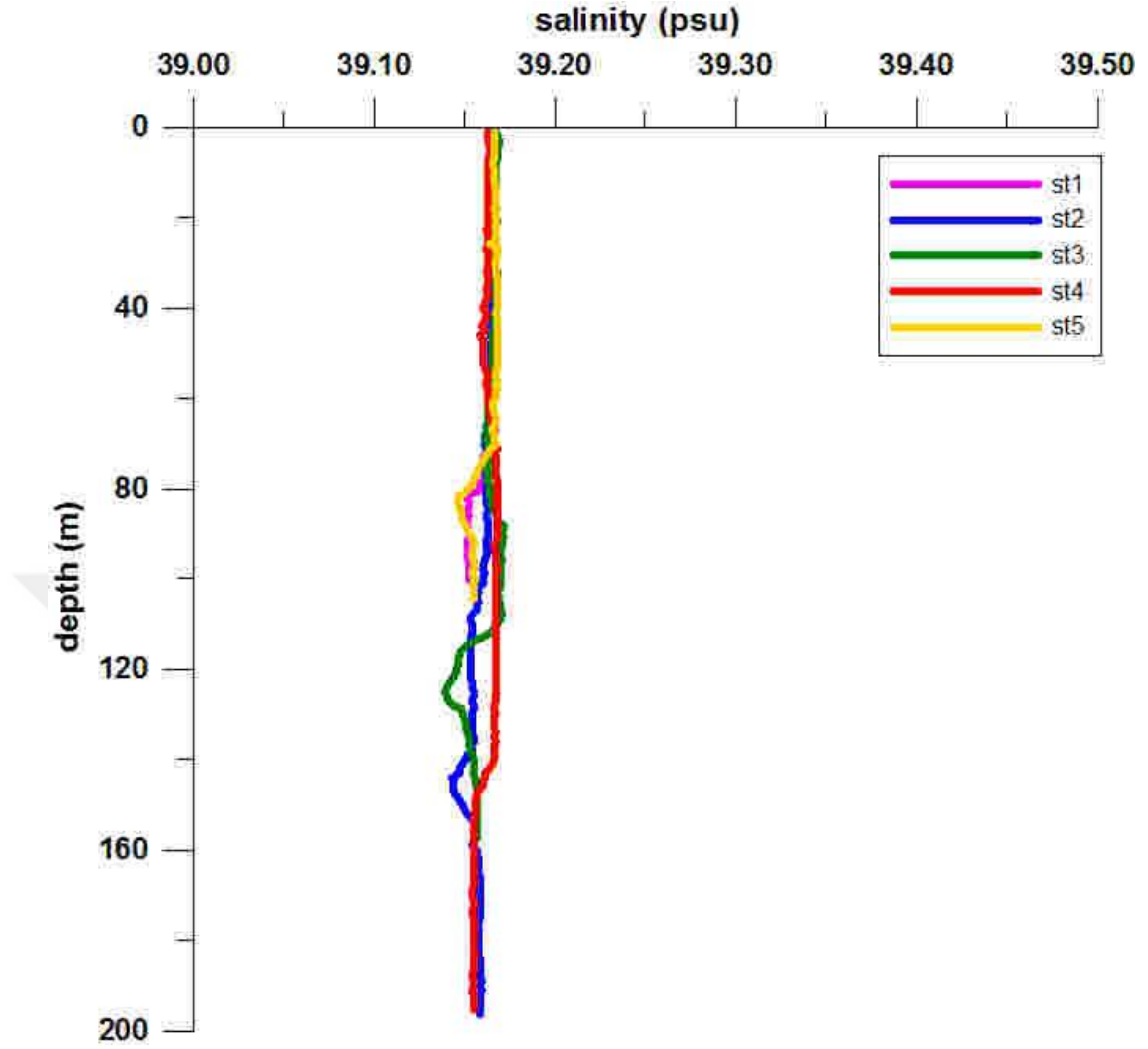


Figure 3.4 Vertical distribution of salinity at 5 stations, Aegean Sea.

3.1.1.3 Density

In contrast to temperature, density of the seawater increased from surface to bottom layers as expected (Figure 3.5). On the other hand, as similar to temperature and salinity intervals, a narrow range for the density of the water column was obtained in the study area as $29.08 - 29.18 \text{ kg/m}^3$.

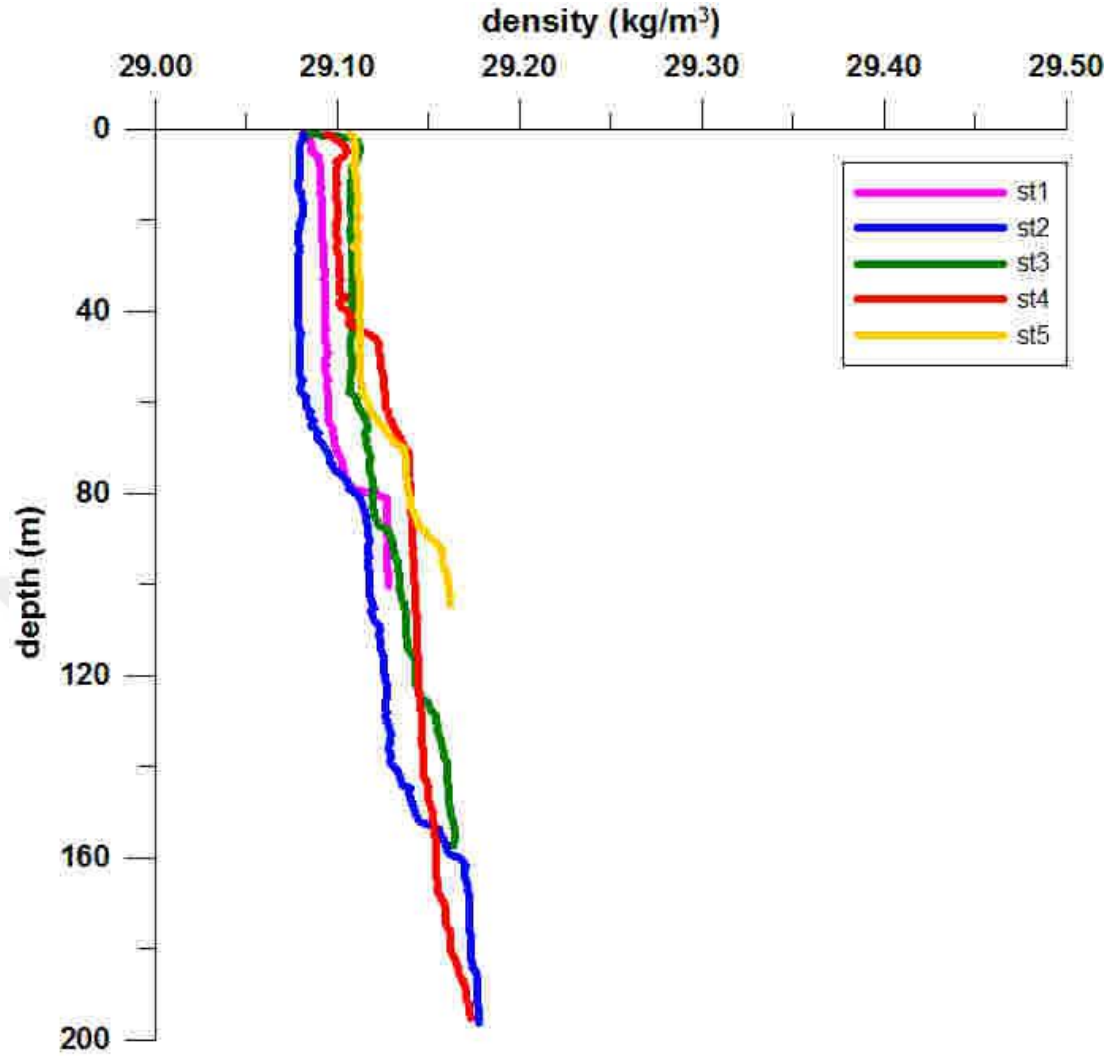


Figure 3.5 Vertical distribution of density at 5 stations, Aegean Sea.

3.1.1.4 Temperature Salinity and Density Relationship

When temperature, salinity and density data were combined (Figure 3.6), the water body was seen very homogenize underlying very close values of those parameters from the surface to the bottom layers of the study area (Figure 3.2, 3.4, 3.5).

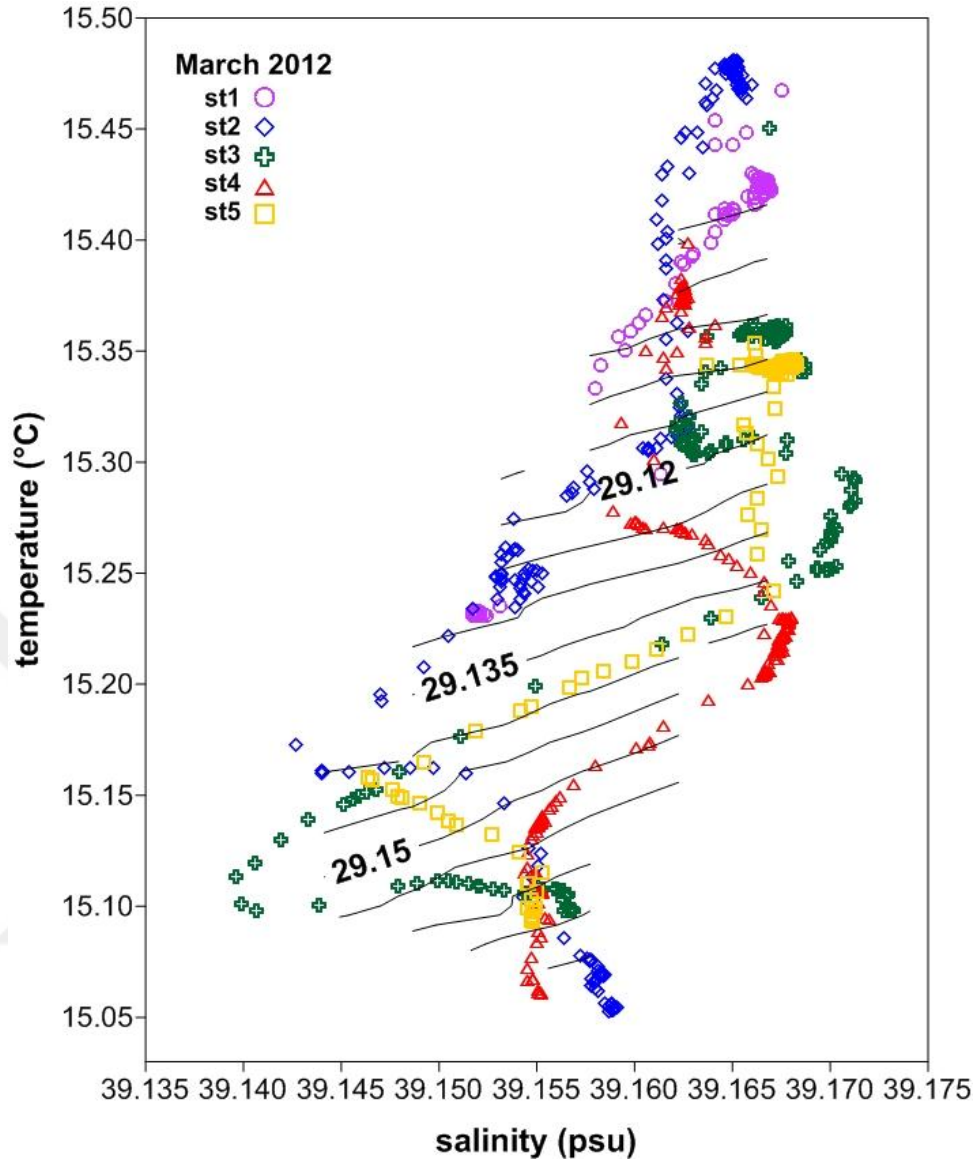


Figure 3.6 Seawater temperature, salinity and density change of 5 stations, Aegean Sea.

3.1.1.5 pH

Like the temperature, salinity and density of the water column, pH of the study area did not change so much with 8.30 – 8.33 interval as seen in Figure 3.7.

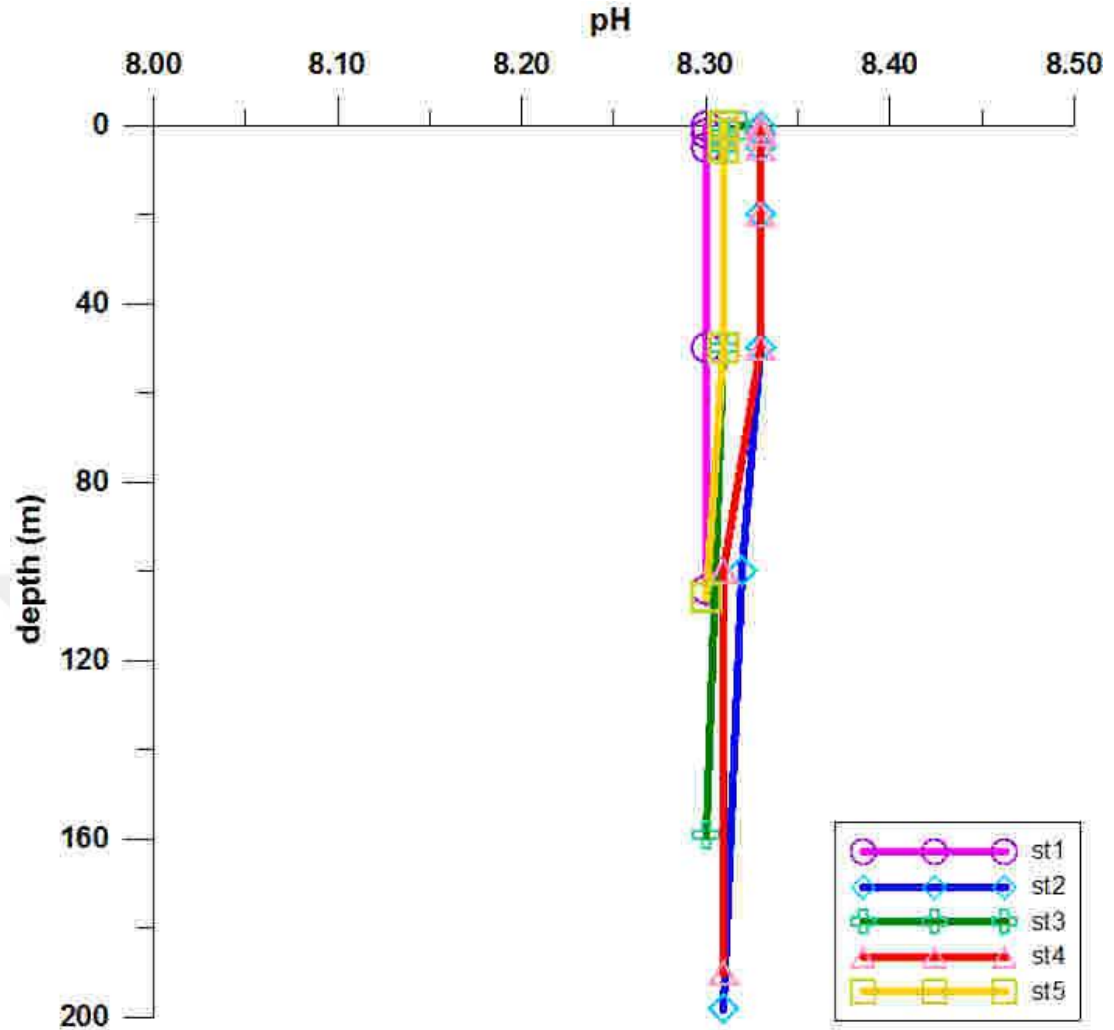


Figure 3.7 Vertical distribution of pH at 5 stations, Aegean Sea.

3.1.1.6 DO

Similar pattern for dissolved oxygen was clearly seen at the surface and bottom layers of the study area with the lowest values at station 3 (Figure 3.8). Although, there was a fluctuation at the first 5 m of the water body and then a slight decrease to the deepest parts for all stations (Figure 3.9), a narrow range was obtained for the area as 5.47 – 5.81 ml/l.

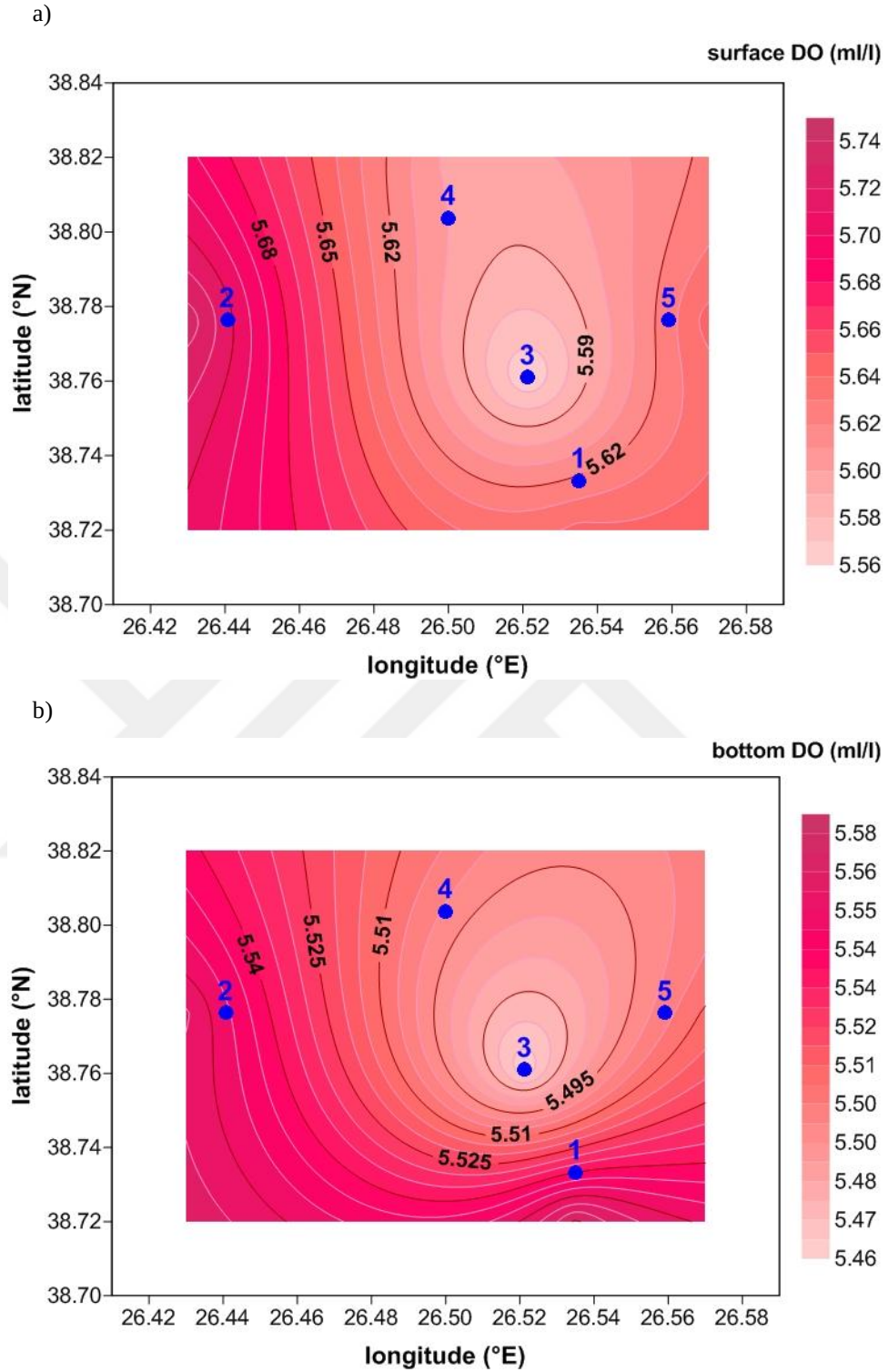


Figure 3.8 Seawater DO of 5 stations, Aegean Sea a) surface, b) bottom layers.

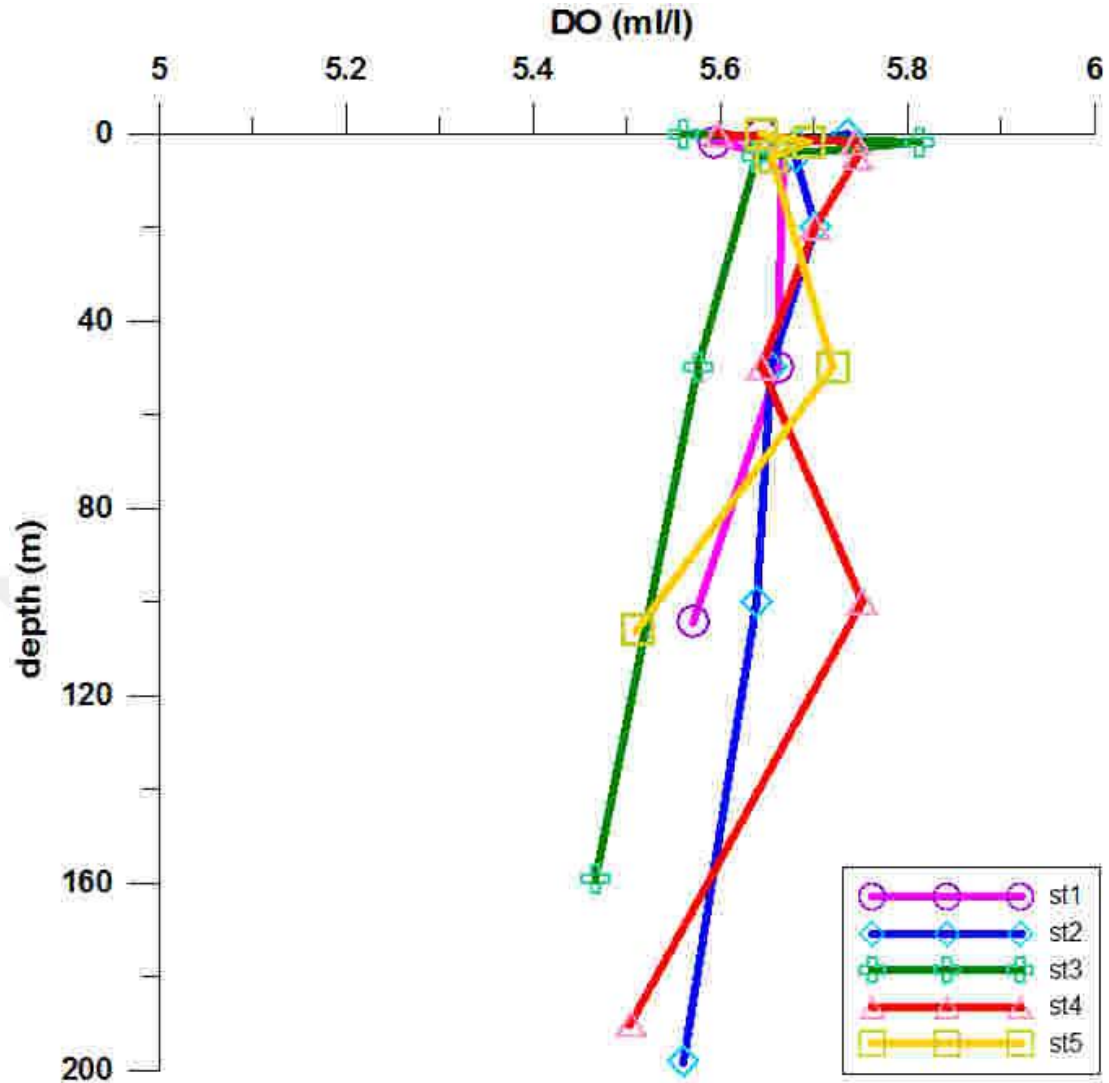


Figure 3.9 Vertical distribution of DO at 5 stations, Aegean Sea.

3.1.1.7 TSS

In the study area, the highest values of total suspended solids were seen at station 3 and 4 for surface and bottom layers, respectively (Figure 3.10). In fact, in addition to fluctuations especially at the surface layer, the highest values of all stations were obtained at the surface, except the station 4 (Figure 3.11). While the stations 1 and 5 had narrow range, the rest had the broader. The narrowest interval was 0.40 – 0.73 mg/l at station 5 and the broadest 0.20 – 1.40 mg/l was at station 4.

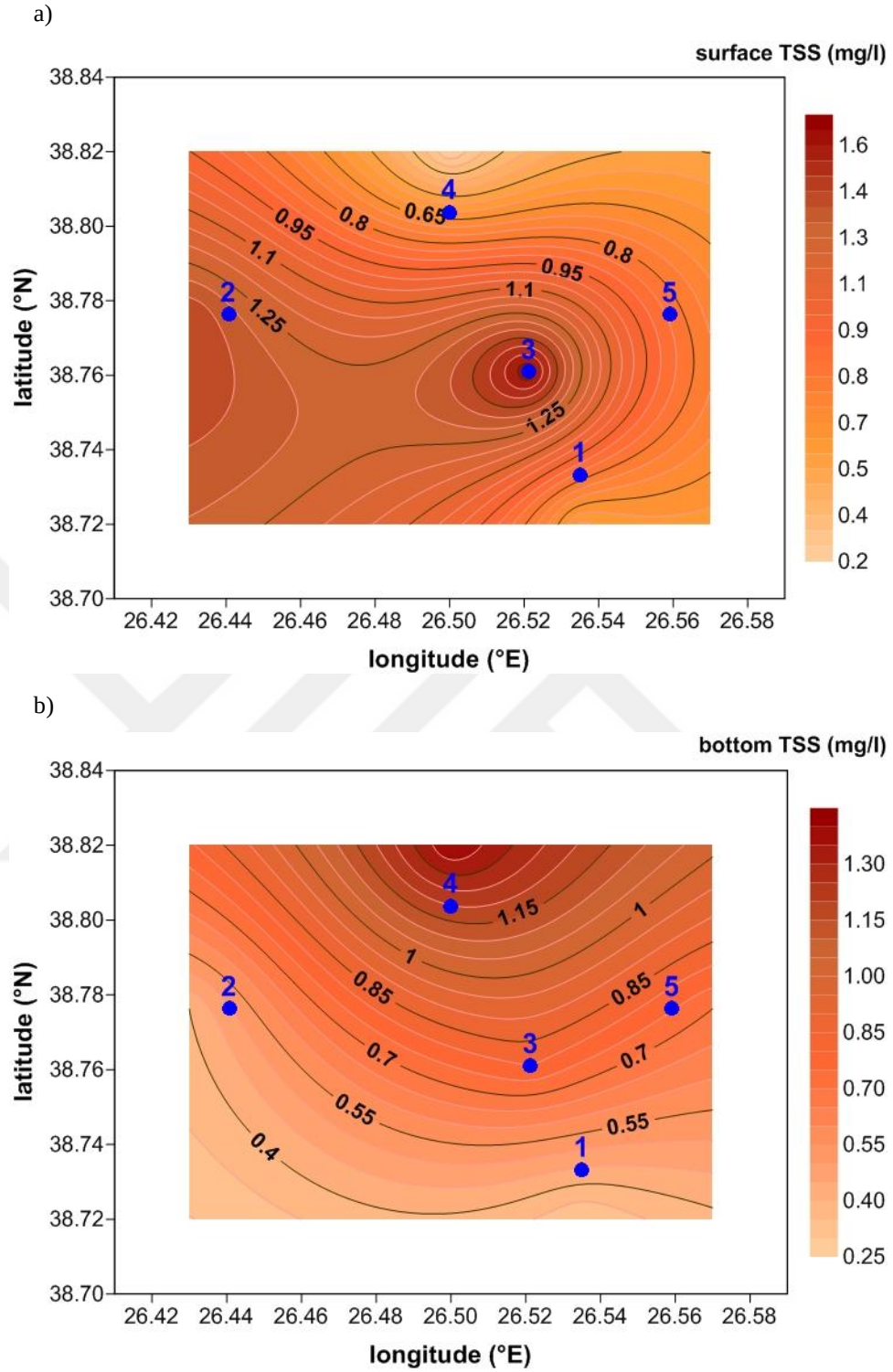


Figure 3.10 Seawater TSS of 5 stations, Aegean Sea a) surface, b) bottom layers.

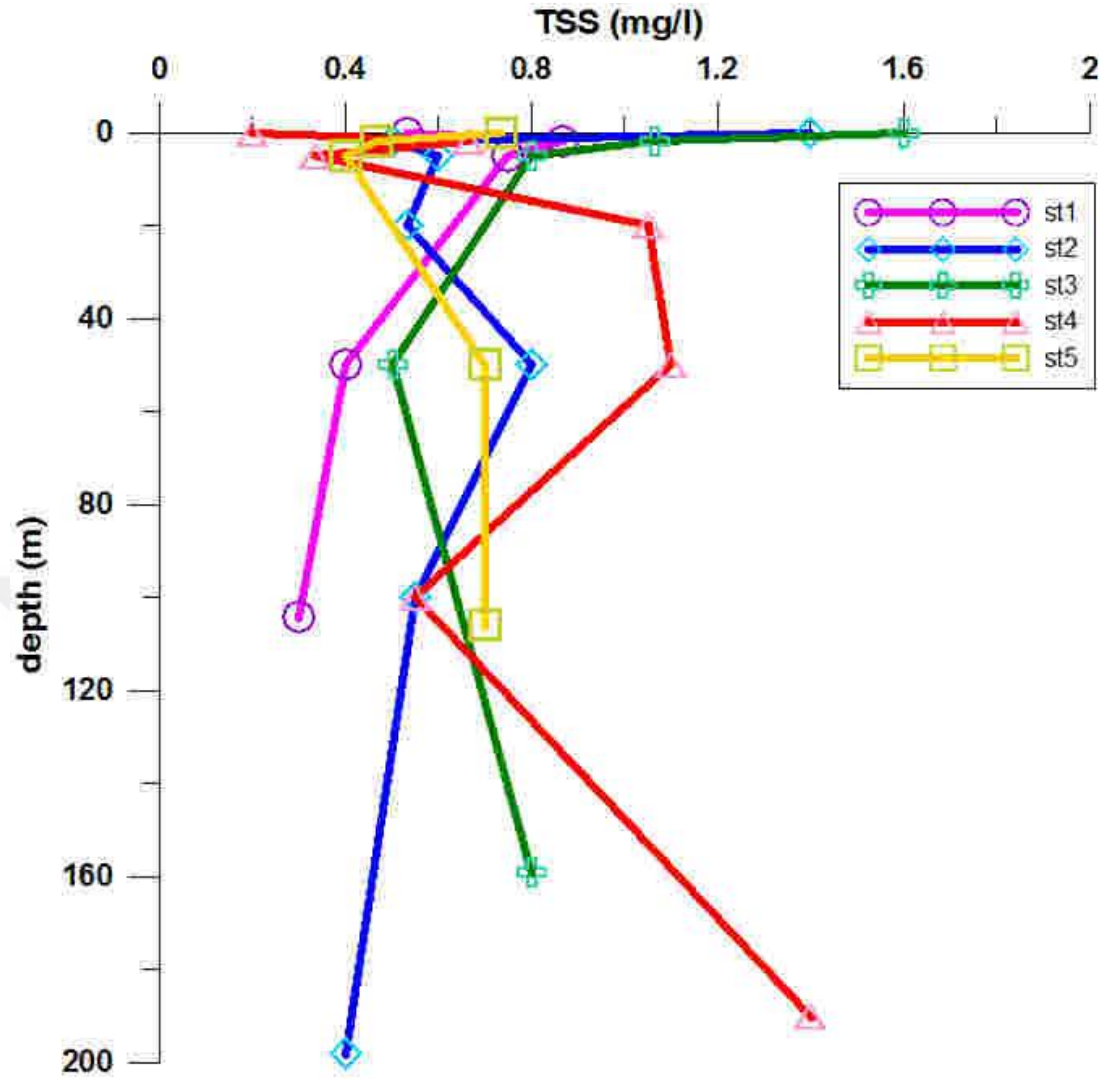


Figure 3.11 Vertical distribution of TSS at 5 stations, Aegean Sea.

3.1.1.8 Chl α

In the study area, the station 2 had the lowest chlorophyll α concentration at the surface and the highest at the bottom (Figure 3.12). In addition to fluctuations of all stations at the surface, stations 3 and 4 had higher values as 1.25 $\mu\text{g/l}$ at 50 m and 0.72 $\mu\text{g/l}$ at 100 m depths, respectively (Figure 3.13). The highest change, on the other hand, was obtained from station 2 (0.07 – 2.33 $\mu\text{g/l}$) and the lowest was from station 5 (0.22 – 0.45 $\mu\text{g/l}$).

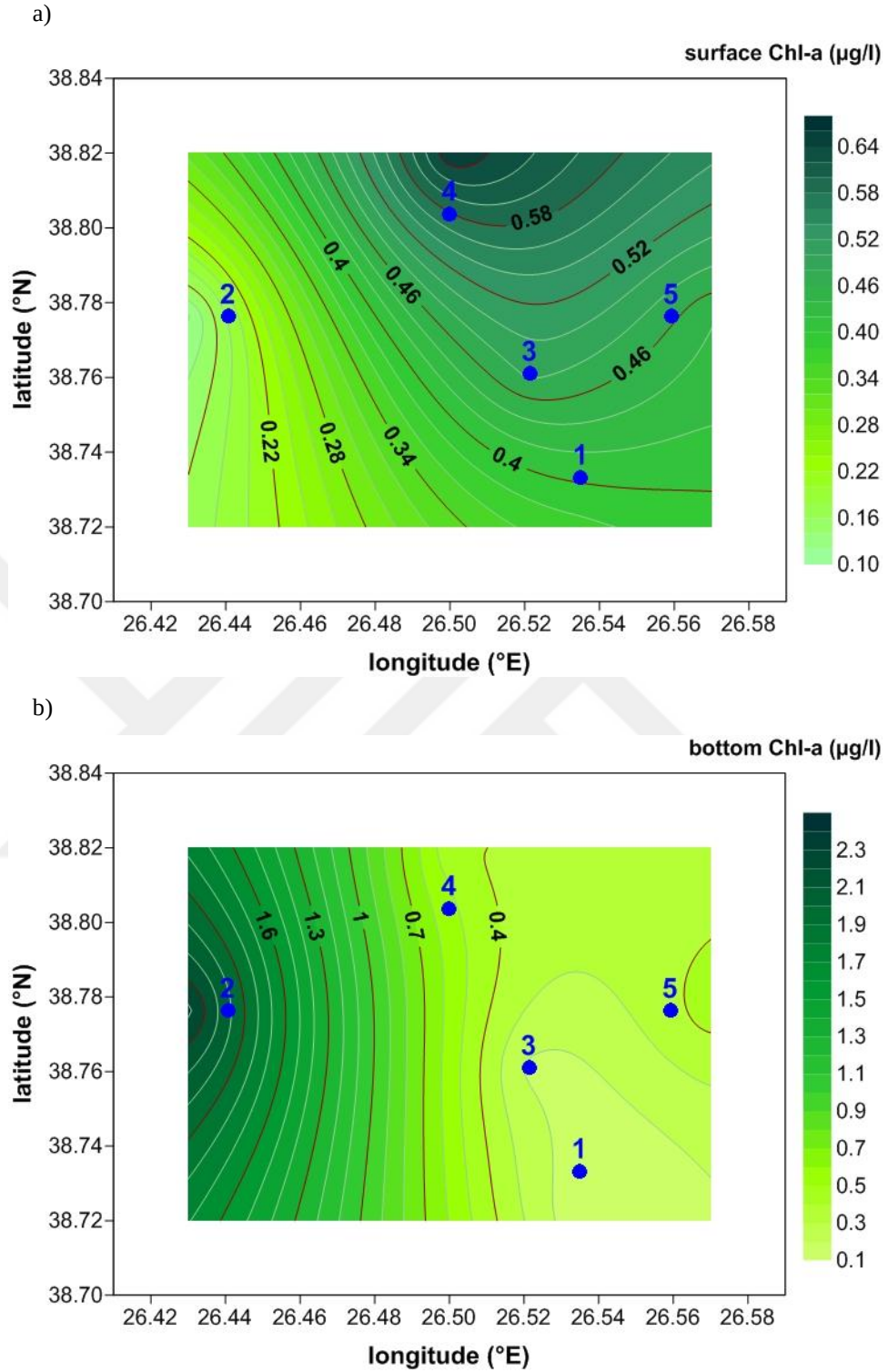


Figure 3.12 Seawater Chl α of 5 stations, Aegean Sea a) surface, b) bottom layers.

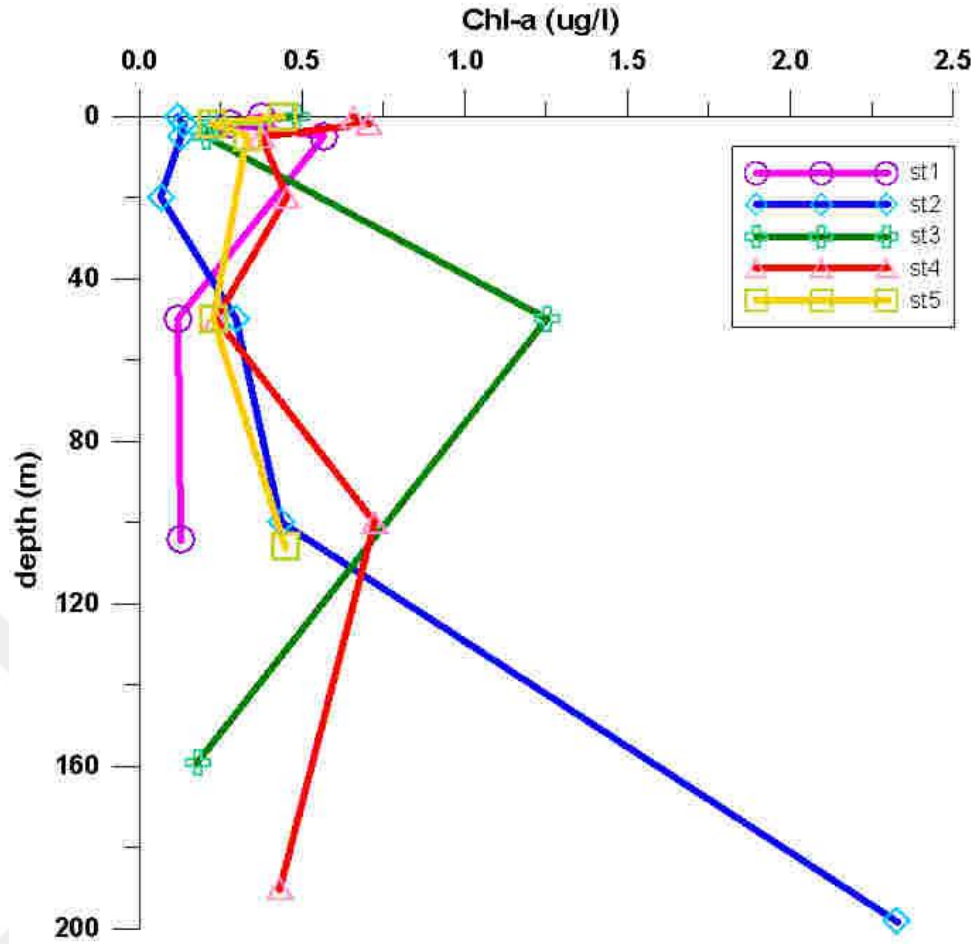


Figure 3.13 Vertical distribution of Chl α at 5 stations, Aegean Sea.

3.1.1.9 Reactive Si

Reactive Si concentrations were obtained higher for stations 1 and 4 at the surface of the water column whereas they were lower for stations 2 and 3 at the bottom (Figure 3.14). In addition to fluctuations of all stations at the surface, the highest and the lowest values were obtained at the station 4 as $4.60 \mu\text{M}$ at 50 m and at the station 2 as $0.91 \mu\text{M}$ at 198 m depths, respectively (Figure 3.15). Moreover, the highest change was seen again at station 4 ($1.25 - 4.60 \mu\text{M}$) and the lowest was at station 1 ($1.08 - 1.93 \mu\text{M}$).

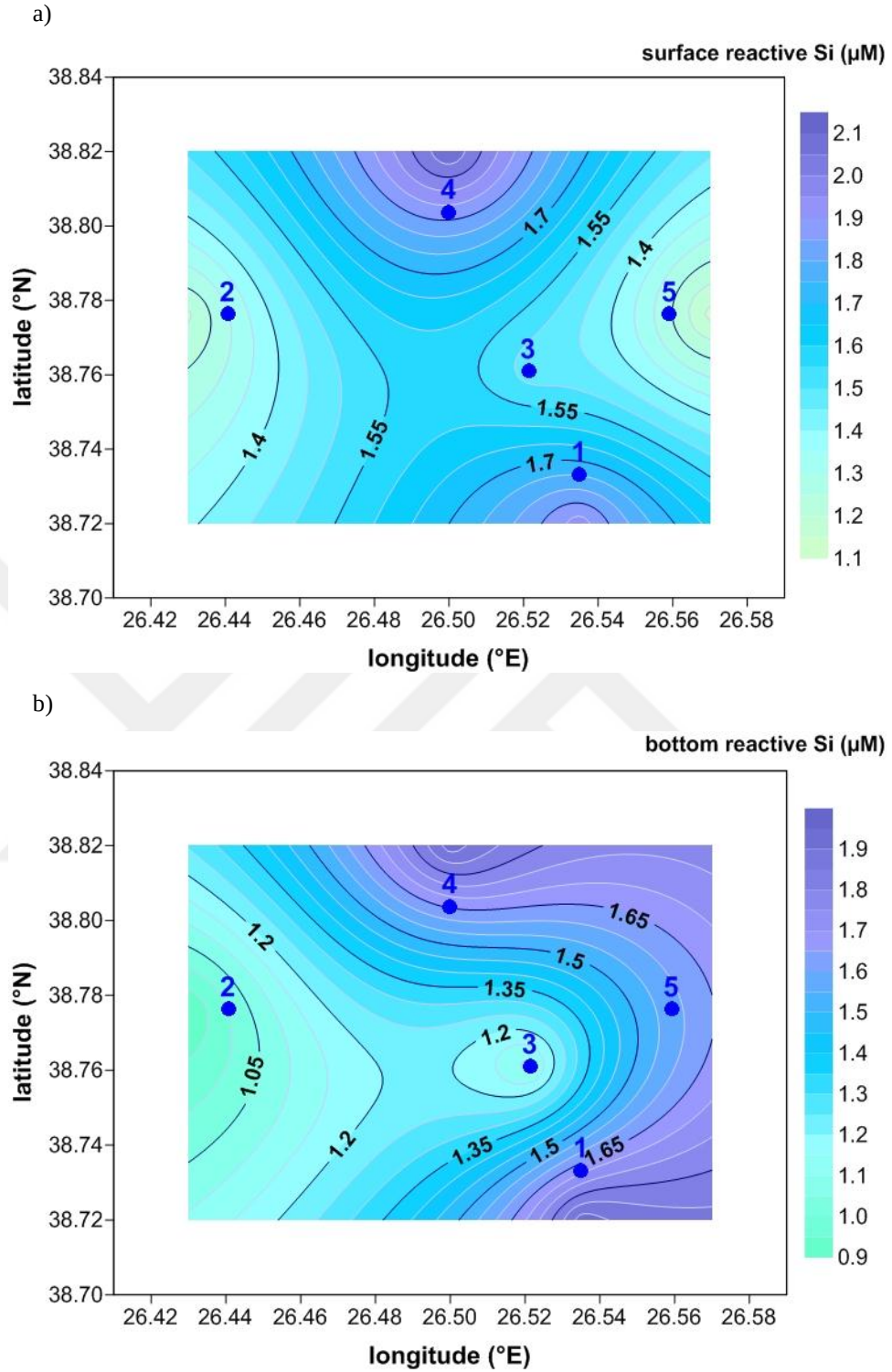


Figure 3.14 Seawater reactive Si of 5 stations, Aegean Sea a) surface, b) bottom layers.

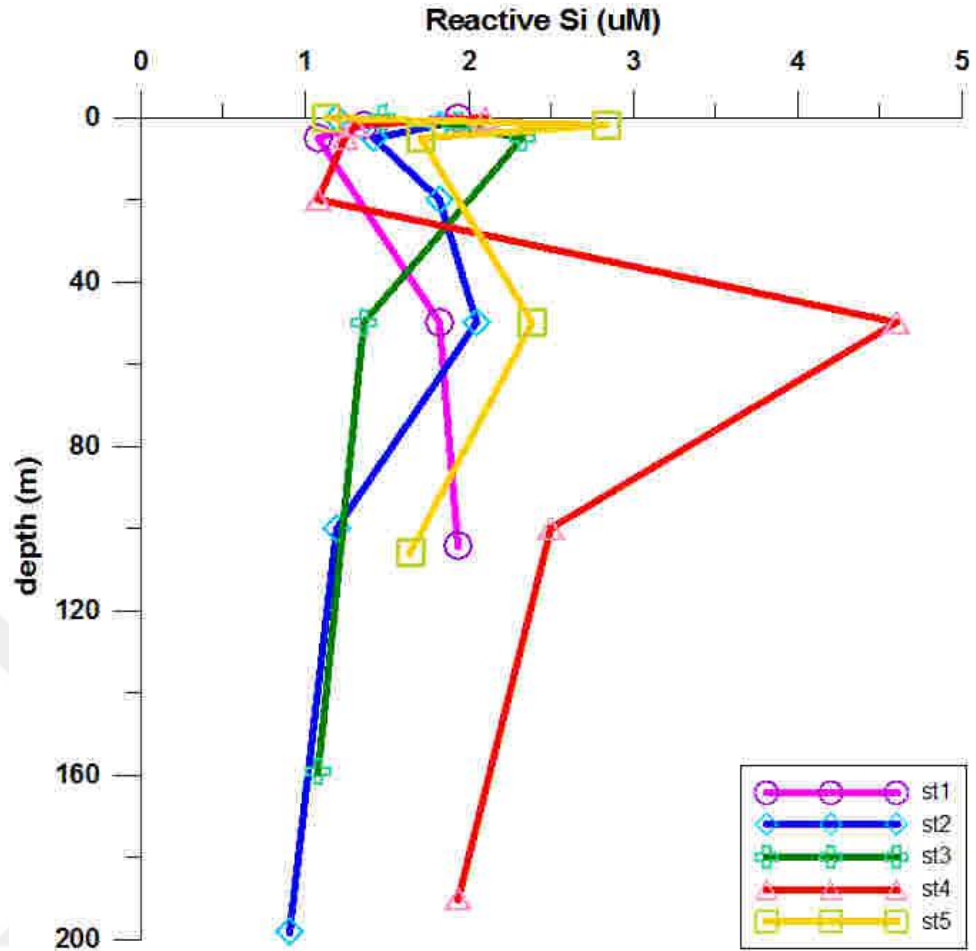


Figure 3.15 Vertical distribution of reactive Si at 5 stations, Aegean Sea.

3.1.1.10 NO_2^-

In the study area, the station 3 had the lowest nitrite concentration at the surface and the station 4 had at the bottom (Figure 3.16). In contrast to the pattern observed for dissolved oxygen values of the water body (Figure 3.9), relatively an increasing trend was seen through the deepest parts of all the stations (Figure 3.17). However, a narrow interval was obtained as BDL – 0.28 μM for the study area.

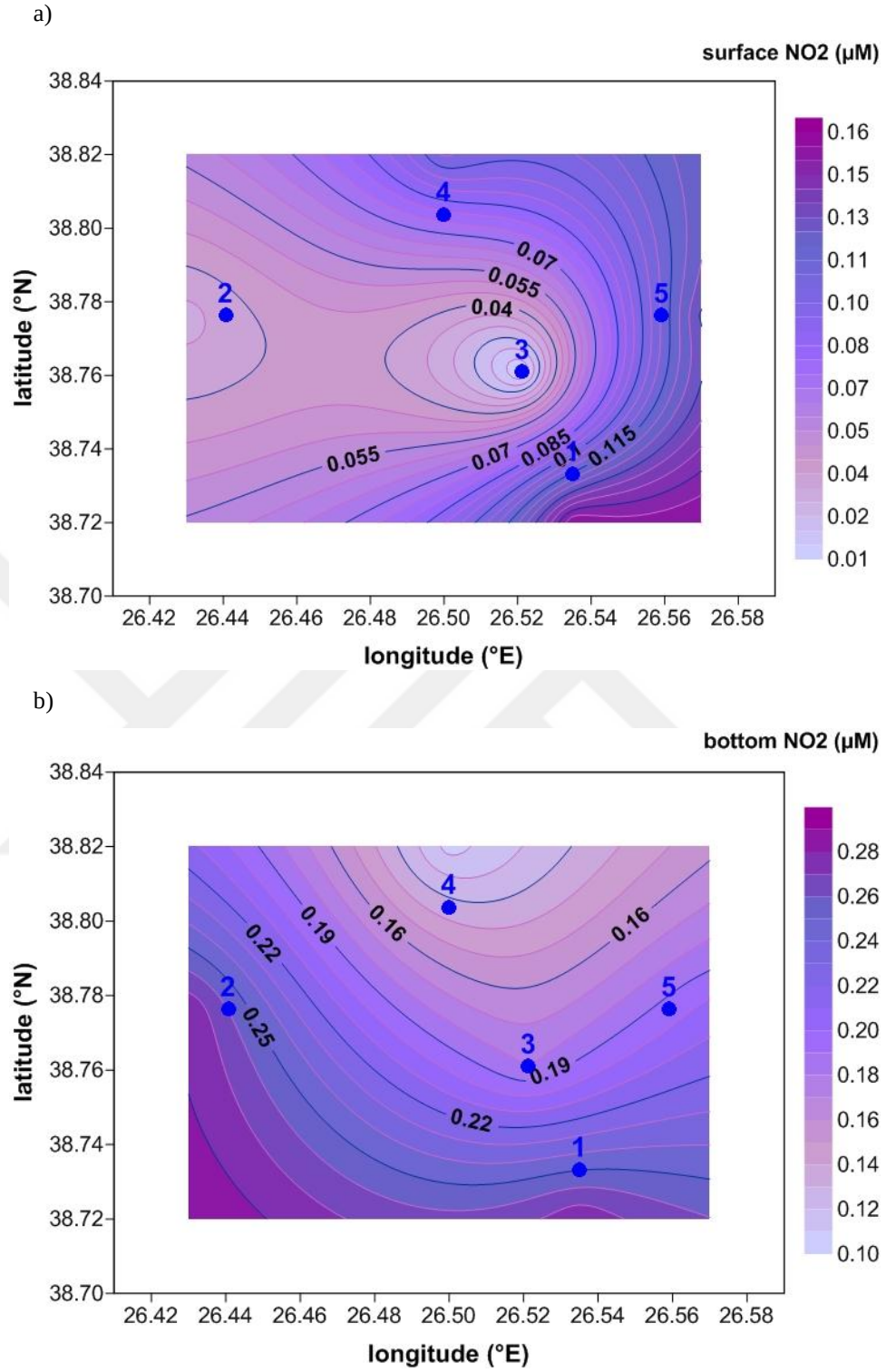


Figure 3.16 Seawater NO₂⁻ of 5 stations, Aegean Sea a) surface, b) bottom layers.

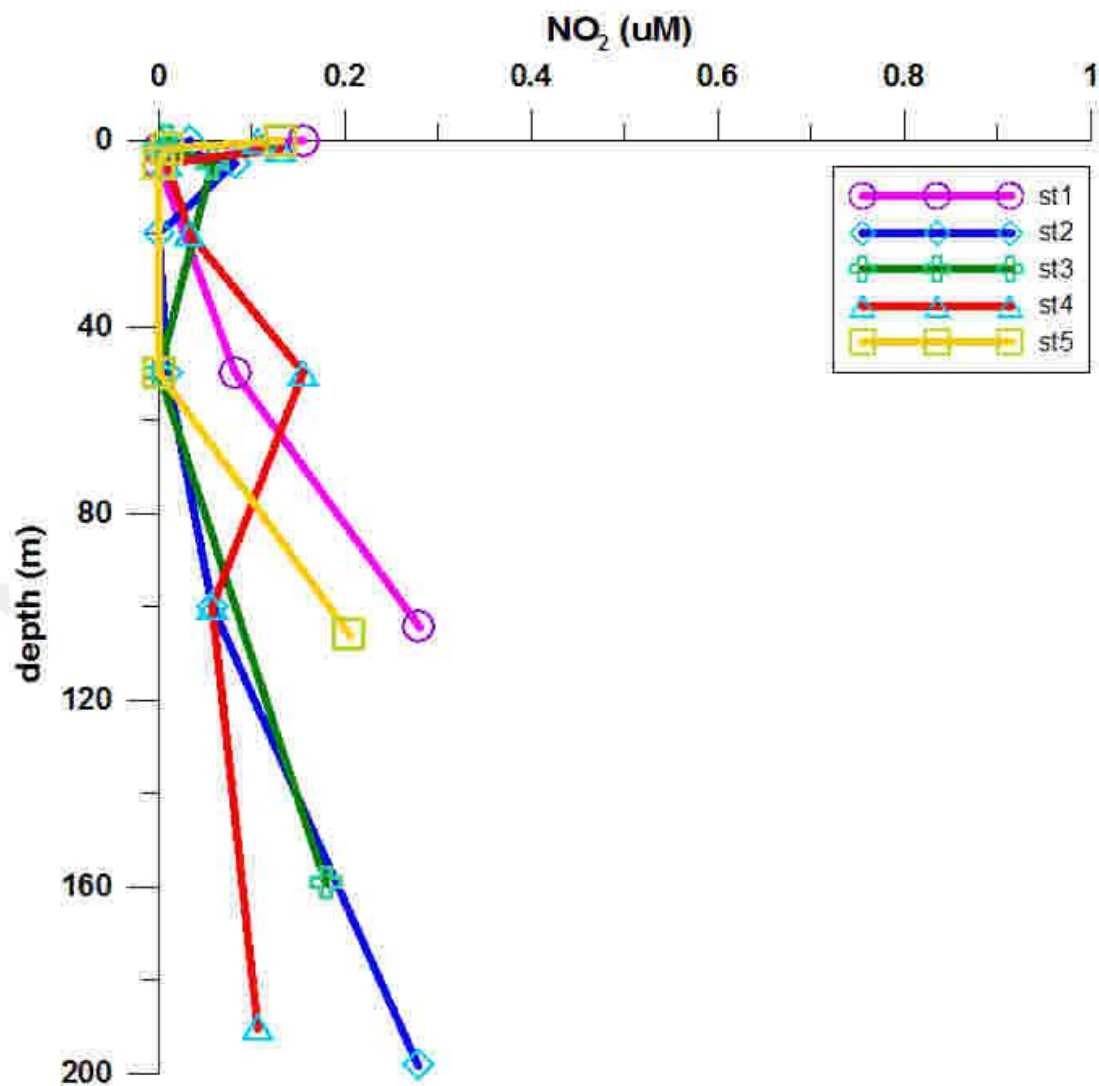


Figure 3.17 Vertical distribution of NO₂⁻ at 5 stations, Aegean Sea.

3.1.1.11 NO₃⁻

The nitrate concentrations showed opposite patterns as an increasing trend from station 1 to 4 at the surface and from station 4 to 1 at the bottom layers of the study area (Figure 3.18). In addition to fluctuations at the surface, the broadest change was observed at station 4 (0.46 – 4.26 μM) and the narrowest was at station 3 (0.80 – 1.51 μM) (Figure 3.19).

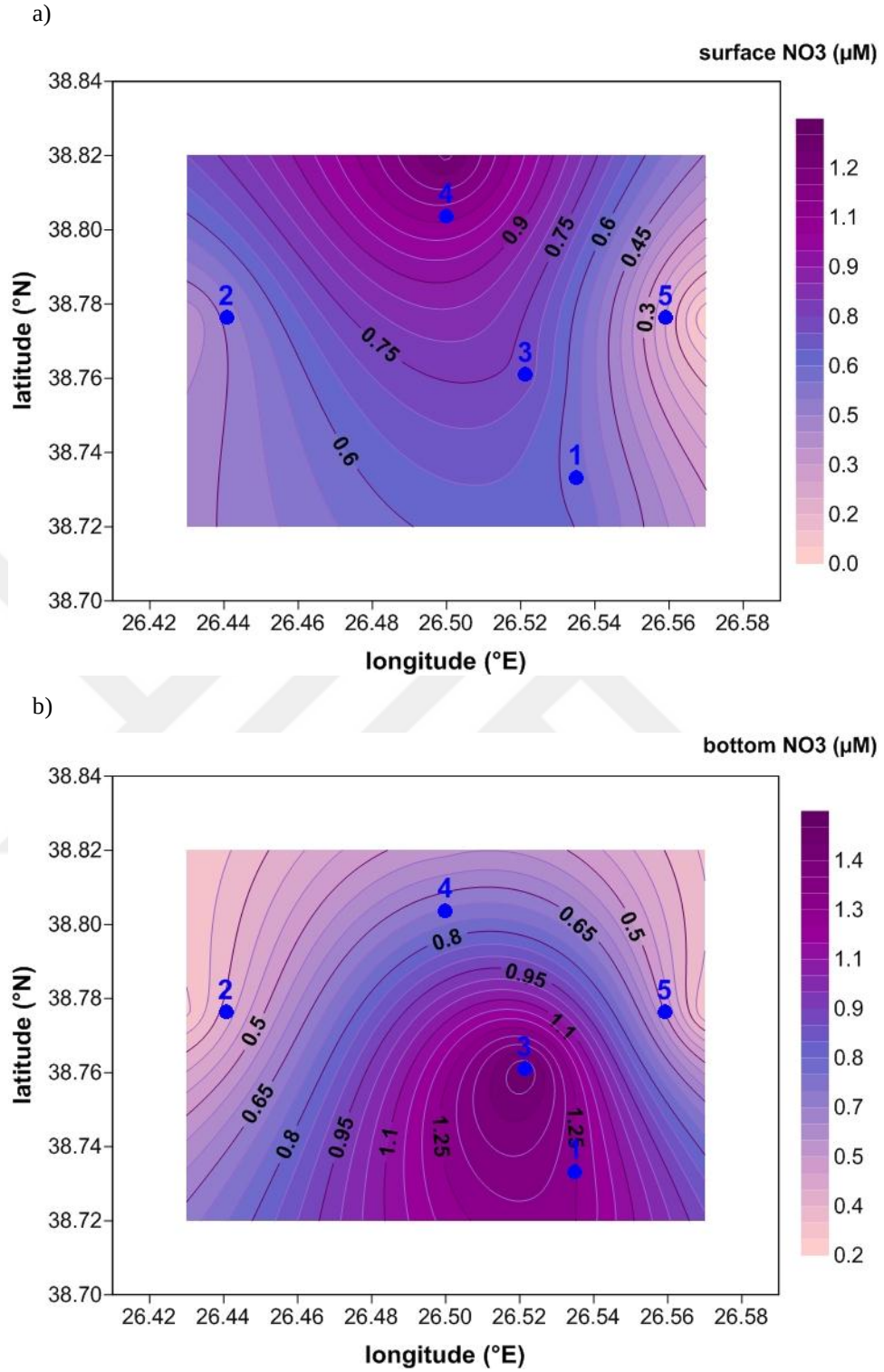


Figure 3.18 Seawater NO₃⁻ of 5 stations, Aegean Sea a) surface, b) bottom layers.

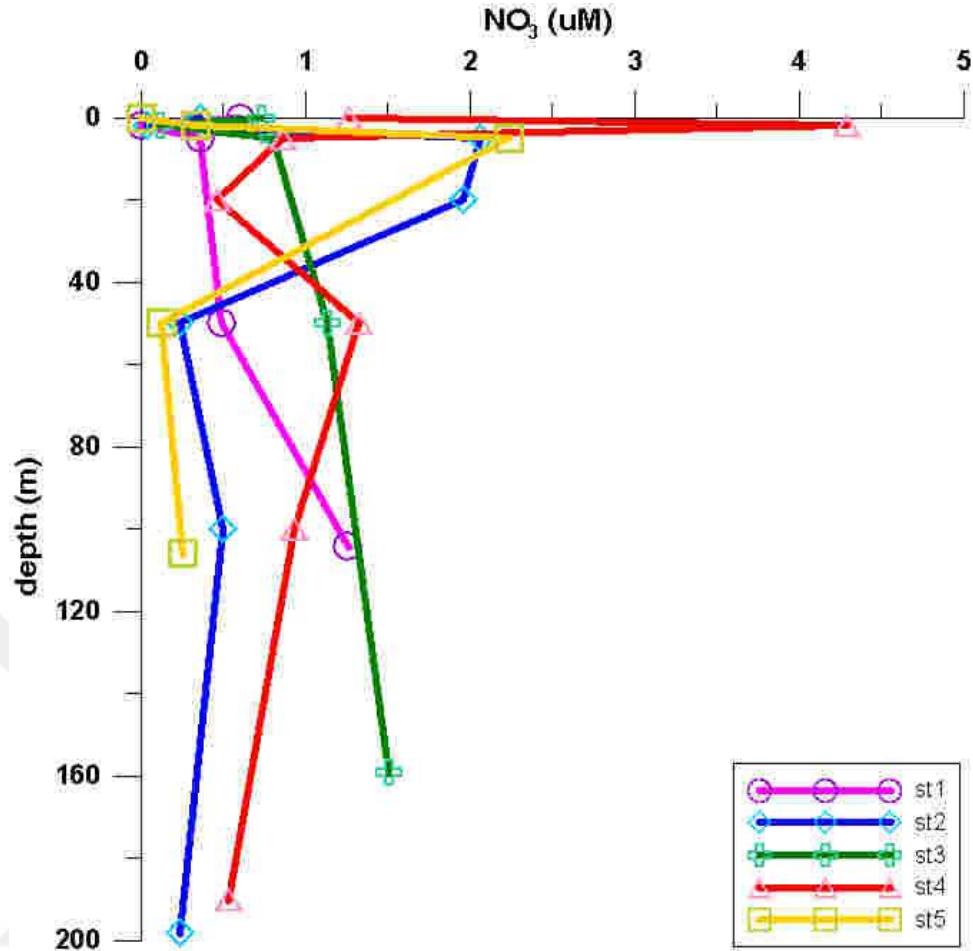


Figure 3.19 Vertical distribution of NO_3^- at 5 stations, Aegean Sea.

3.1.1.12 PO_4^{3-}

While the phosphate concentrations decreased through the stations 3 and 5 at the surface, it increased from the station 5 to 2 at the bottom (Figure 3.20). In contrast to the pattern of nitrite concentrations at the water column, a decreasing trend was seen through the deepest parts after fluctuations at the surface (Figure 3.21). However, a narrow interval was obtained as $\text{BDL} - 0.10 \mu\text{M}$ for study area.

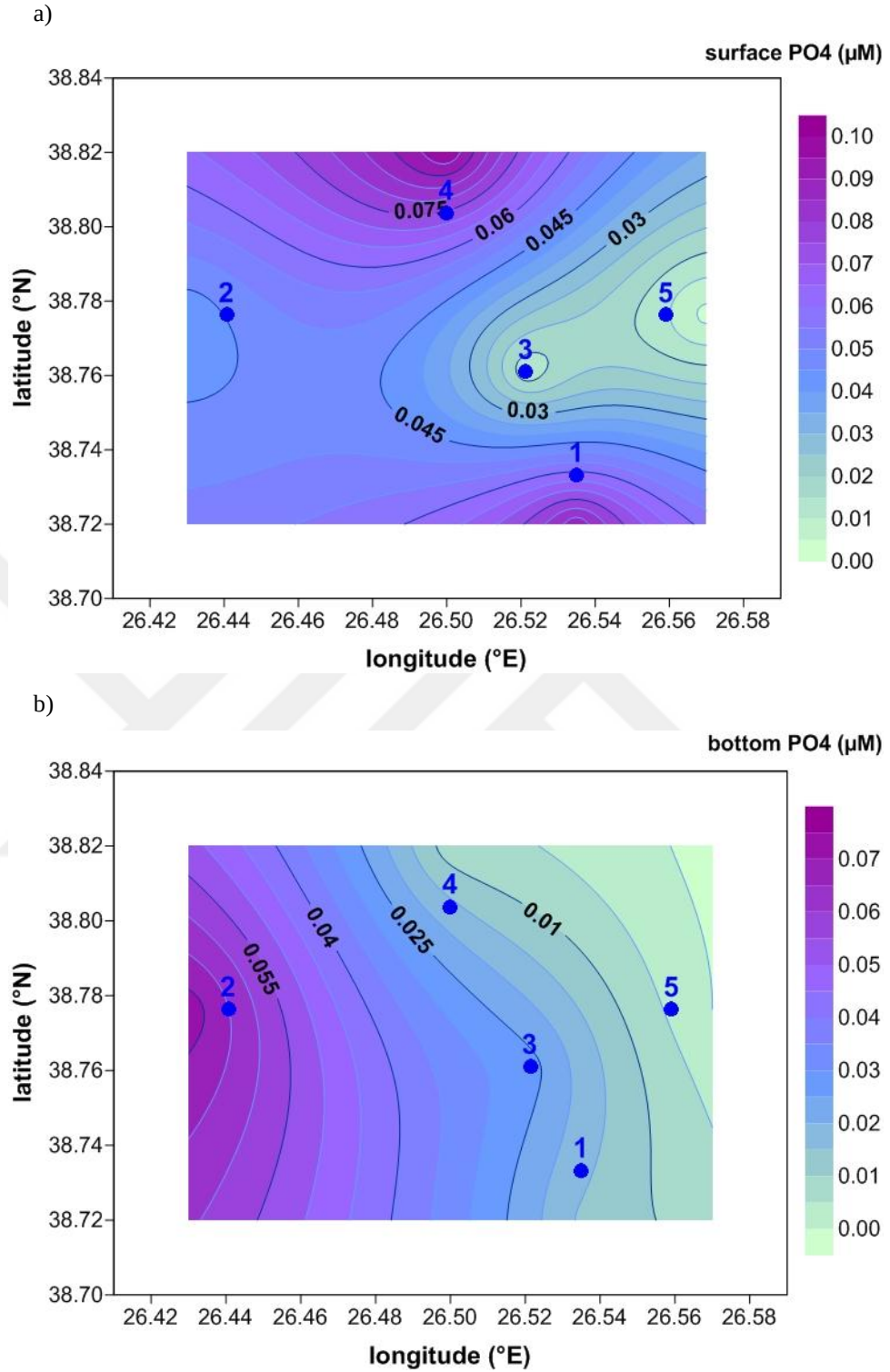


Figure 3.20 Seawater PO_4^{3-} of 5 stations, Aegean Sea a) surface, b) bottom layers.

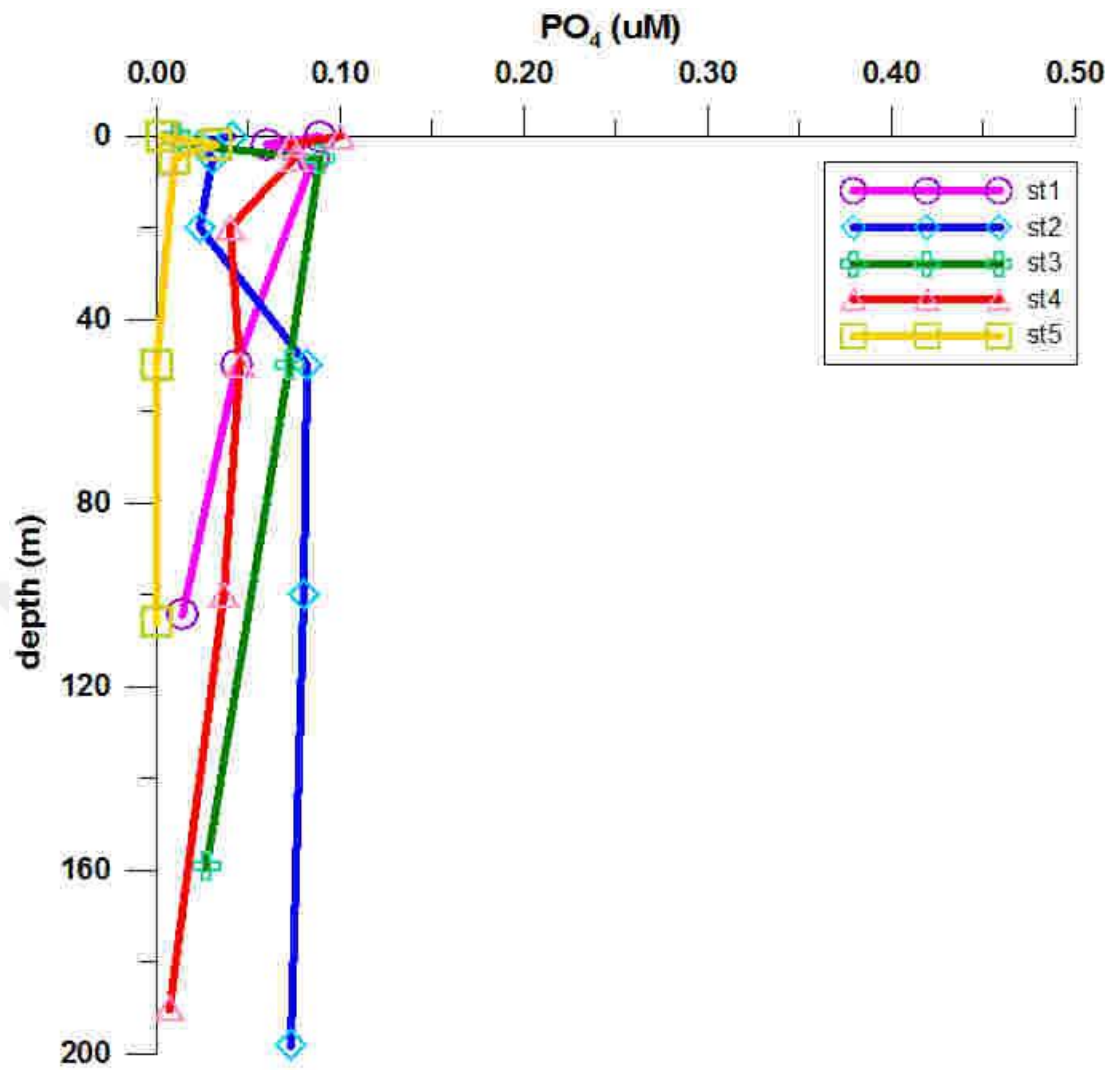


Figure 3.21 Vertical distribution of PO_4^{-3} at 5 stations, Aegean Sea.

3.1.2 Sediment Results

Sediments collected from approximately 100 – 200 m depths of the study area were composed of finer particles as expected. Moreover, their carbon and phosphorus contents were much homogeneous than nitrogen content. Total and organic carbon ranges were 5.33 – 9.61 % and 1.00 – 3.33 % whereas 0.032 – 0.053 % and 0.007 – 0.017 % for phosphorus, respectively. On the other hand, total and organic nitrogen percent intervals were 0.02 – 0.24 and BDL – 0.15, respectively.

3.1.2.1 Sediment Type

According to grain size analysis of sediment samples, it was obtained that the stations 1, 3 and 5 were sandy, the stations 2 and 4 were sandy-clayey silt (Table 3.1 and Figure 3.22).

Table 3.1 Grain size results of 5 stations, Aegean Sea.

	Station1	Station2	Station3	Station4	Station5
Sand (%)	73	26	72	34	83
Silt (%)	17	48	18	47	13
Clay (%)	10	26	10	19	4

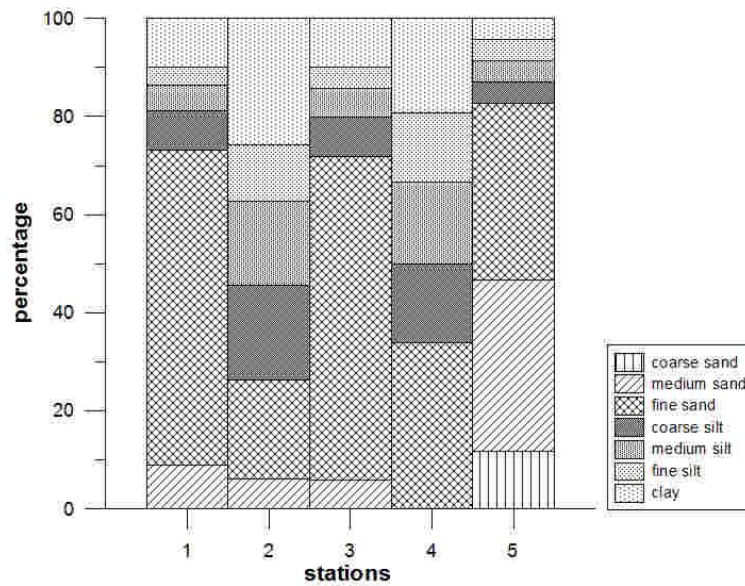


Figure 3.22 Grain size distributions of 5 stations, Aegean Sea.

3.1.2.2 Chemical Contents

As seen in Figure 3.23a and 3.23c, the higher TC and TP values were observed for stations 1 (9.61 % and 0.048 %, respectively) and 3 (6.42 % and 0.053 %, respectively). However, the stations 3 and 5 had the higher TOC values (2.82 % and 3.33 %, respectively) and the stations 1 and 2 had TOP values (0.015 % and 0.017 %, respectively). When TN and TON were considered, it was clearly seen in Figure 3.23 b that the stations 1, 3 and 4 had dramatically low values of TN and in fact TON contents of those including the station 2 were below detection limits. TN values of the stations 2 and 5 were 0.24 % and 0.20 %, respectively and TON value of the latter was 0.15 %.

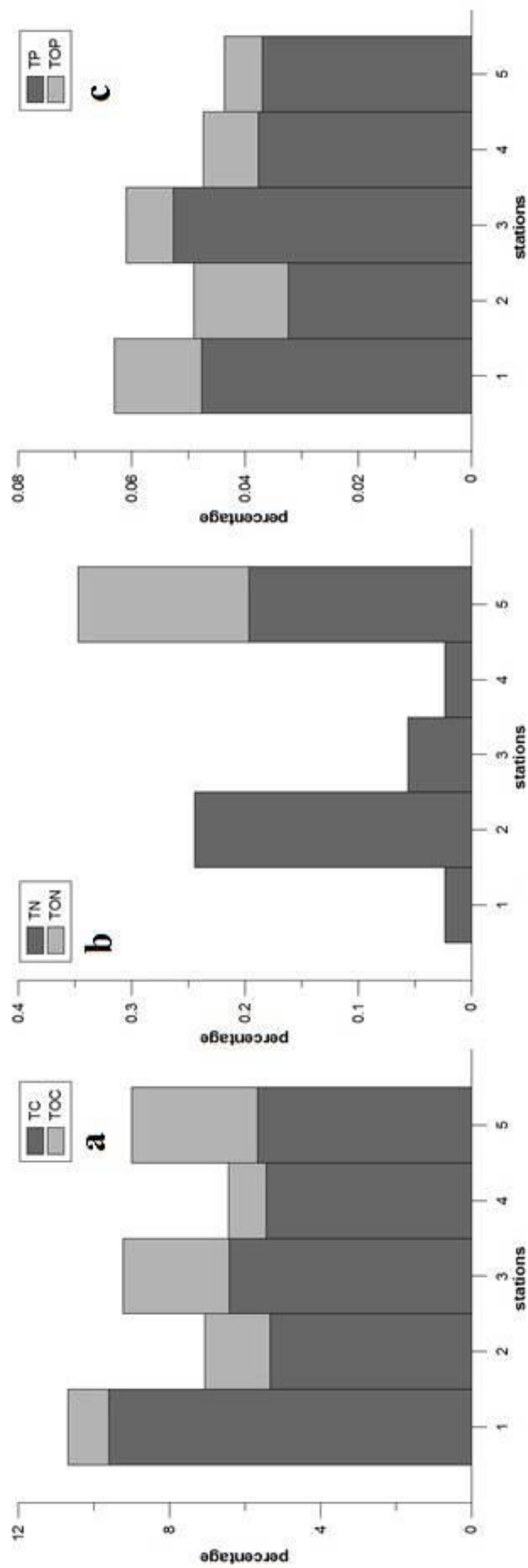


Figure 3.23 Chemical content of the surface sediment a) TC-TOC b) TN-TON c) TP-TOP percentages.

Biological Results

Using 7 sediment processing methods and 2 media with or without 6 antibiotics, totally 13 actinomycetes were isolated as 5 of them from the stations 1, 3 and 5, and 8 of them from the stations 2 and 4. Morphological characteristics and physiological tests showed that they were different from each other. In addition to the support of this fact by the phylogenetic analysis, it was also found that one of them belonged to the genus *Nocardiopsis* and the rest to the genus *Streptomyces*.

3.1.3 Growth Conditions

The actinomycetes were successfully grown at 26 – 28 °C and 2 – 4 % NaCl. Among 7 sediment processing methods, the strains were only obtained from the methods (a), (c) and (f). For those 3 methods, the first one which was the overnight dry of the wet sediment and then the direct spread on the agar plate was much more successful than the others.

The strains were also successfully isolated using the media M1 and M7m. The former was much better than the latter. Moreover, the antibiotics became very helpful to isolate the actinomycetes. Cycloheximide played crucial role to prevent growth of fungi on the agar plate. Kanamycin sulfate and polymyxin B sulfate made the media much preferable for Gram positives. However, the media became much more selective for the actinomycetes with the former. On the other hand, it was observed that rifampin had the strongest effect on the bacteria since there were no or only one or two colonies including actinomycetes on the agar media with rifampin.

3.1.4 Morphological Results

To understand the phenotypic characteristics of the isolates, colony morphology and physiological features were determined. It was found that they were phenotypically different from each other due to the variation of each isolate in analysis of both the colony morphology and physiological tests.

3.2.2.1 Colony Description

The colony morphology of each isolate was described in Table 3.2 and their photos on the agar plates and also SEM images were given in Figure 3.24 – 3.36. They generally had creamy white substrate mycelium and white aerial mycelium if existed, in addition to cream – yellow pigmentation on the agar media.

As given in Table 3.2, the strains 19BA4Y12, 23BA52Y12 and 25BA11Y12 isolated from the stations 4, 5 and 1, respectively were grown well on M1 isolation medium (Figure 3.31 – 3.33). The substrate and aerial mycelia of the strain 25BA11Y12 forming yellowish beige pigment on the agar plate were brownish violet and cream, respectively. Like the strain 23BA52Y12, as seen in Figure 3.27 the colors of the medium and also the aerial mycelium of the strain 7BA24Y12 were yellow and white, respectively.

Moreover, the strain 13BA2Y12 isolated without any antibiotics ad creamy beige substrate mycelium and white aerial mycelium as seen in Figure 3.30. Like this strain, the strain 3BA24Y12 having dark yellow aerial mycelium was also isolated from the station 2 (Figure 3.25).

While the strains 1BA43Y12, 5BA24Y12, 29BA45Y12, 41BA4Y12 and 43BA11Y12 (from the stations 1, 2 and 4) had creamy white – creamy beige substrate mycelia, no aerial mycelium was observed for those (Figure 3.24, 3.26, 3.34 – 3.36). On the other hand, the strains 9BA3Y12 and 11BA52Y12 isolated from the stations 3 and 5, respectively had white aerial mycelia with creamy white substrate mycelia but without pigmentation on M7m medium (Figure 3.28, 3.29).

Table 3.2 The colony morphology of the isolates grown at 26 – 28 °C using media prepared with sterile seawater .

Station	Representative isolate	Isolation medium (with antibiotics)	Growth	Color of substrate mycelium	Aerial mycelium	Pigment on the medium
1	25BA11Y12	M1 (rifampin)	good	brownish violet	sparse, cream	yellowish beige
	43BA11Y12	M1 (rifampin)	sparse	creamy white	none	none
2	3BA24Y12	M1 (kanamycin sulfate)	sparse	cream	sparse, dark yellow	none
	5BA24Y12	M1 (kanamycin sulfate)	sparse	creamy beige	none	beige
	7BA24Y12	M1 (polymixin B sulfate)	good	colorless	good, white	yellow
	13BA2Y12	M1 (without antibiotics)	sparse	creamy beige	sparse, white	creamy beige
3	9BA3Y12	M7m (cycloheximide)	sparse	creamy white	sparse, white	none
	1BA43Y12	M7m (cycloheximide)	sparse	creamy white	none	none
4	19BA4Y12	M1 (polymixin B sulfate)	good	cream	sparse, white	cream
	29BA45Y12	M1 (rifampin)	sparse	creamy white	none	none
	41BA4Y12	M1 (rifampin)	sparse	cream	none	cream
	11BA52Y12	M7m (cycloheximide)	sparse	creamy white	sparse, white	none
5	23BA52Y12	M1 (polymixin B sulfate)	good	colorless	good, white	yellow

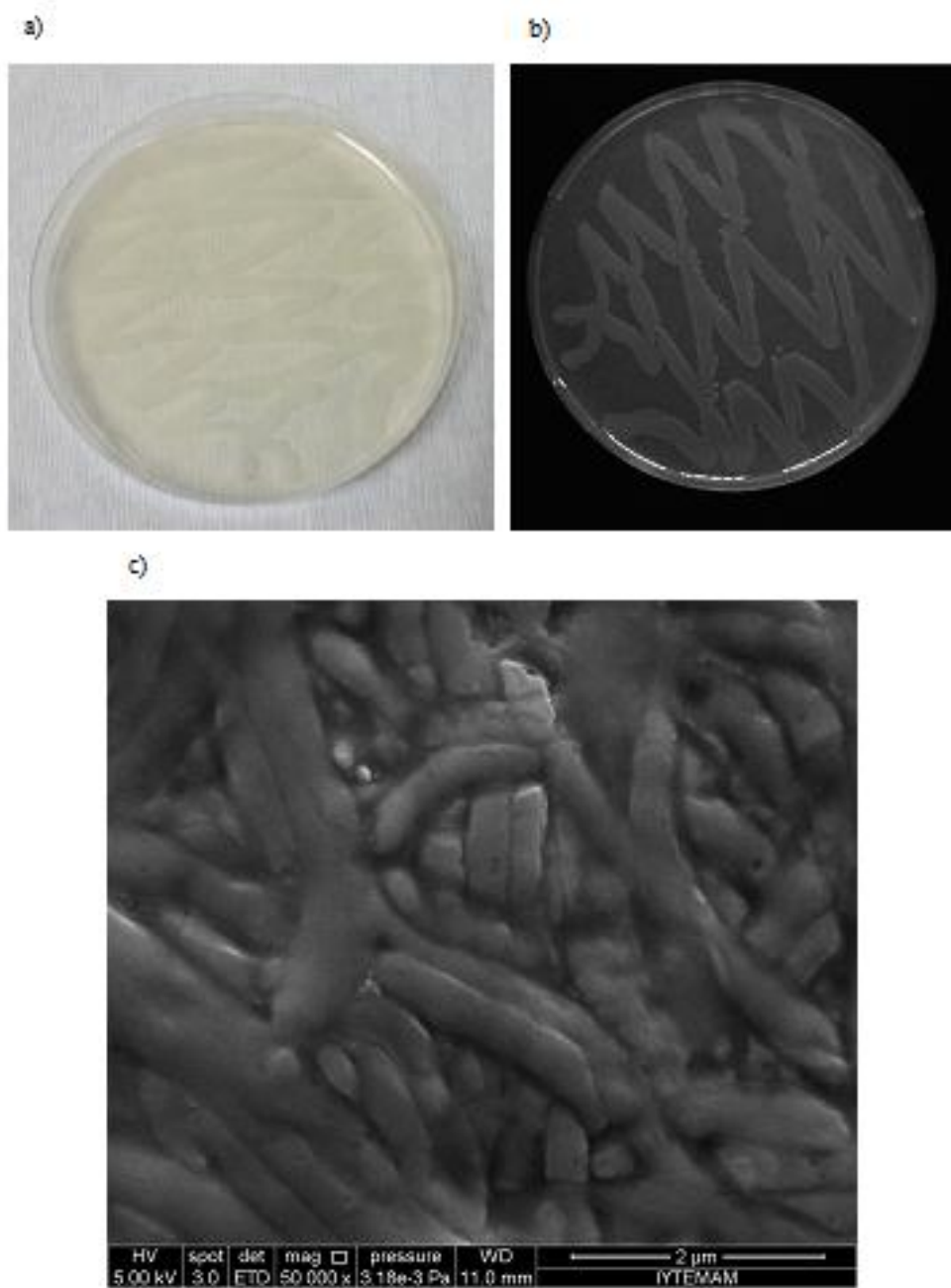
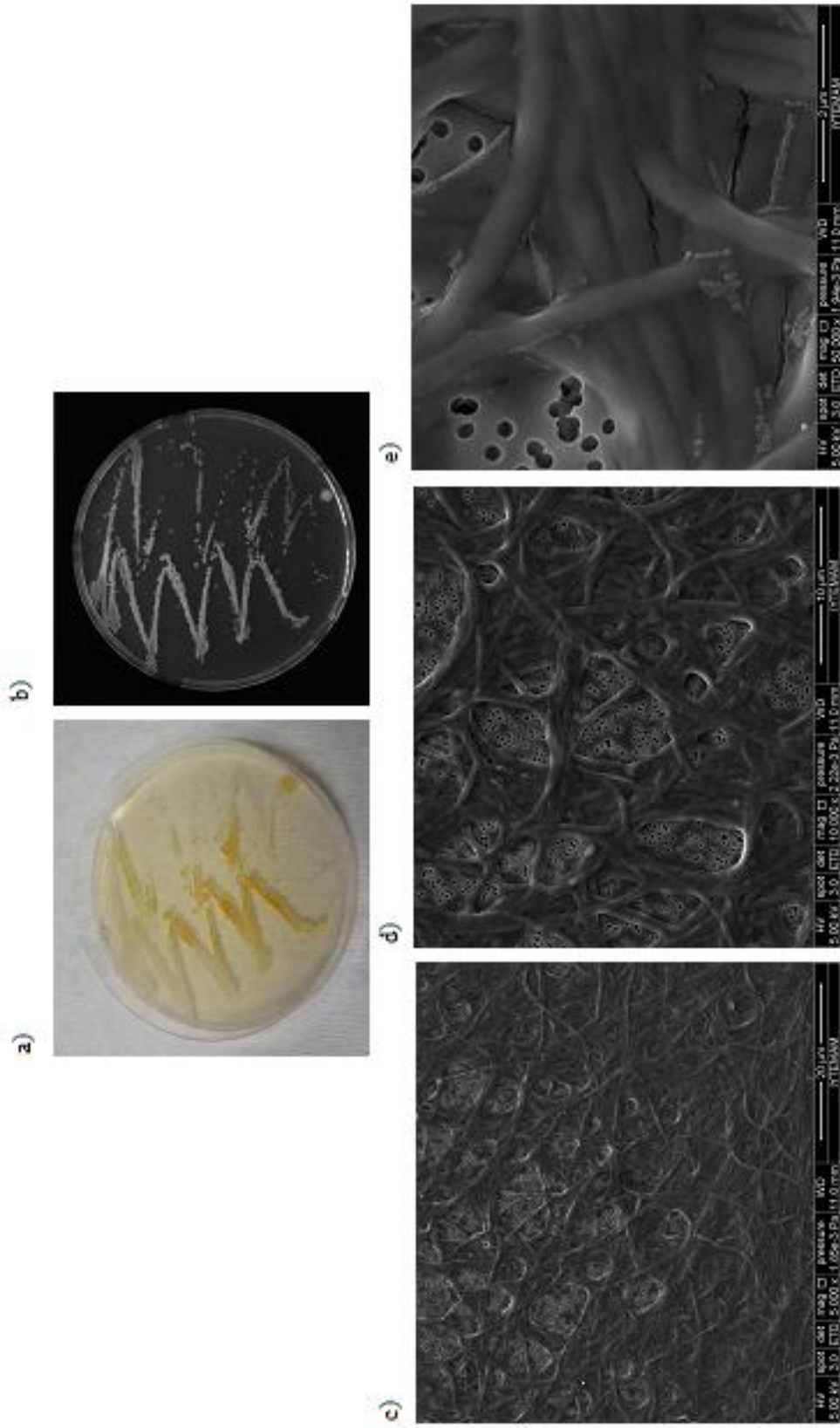
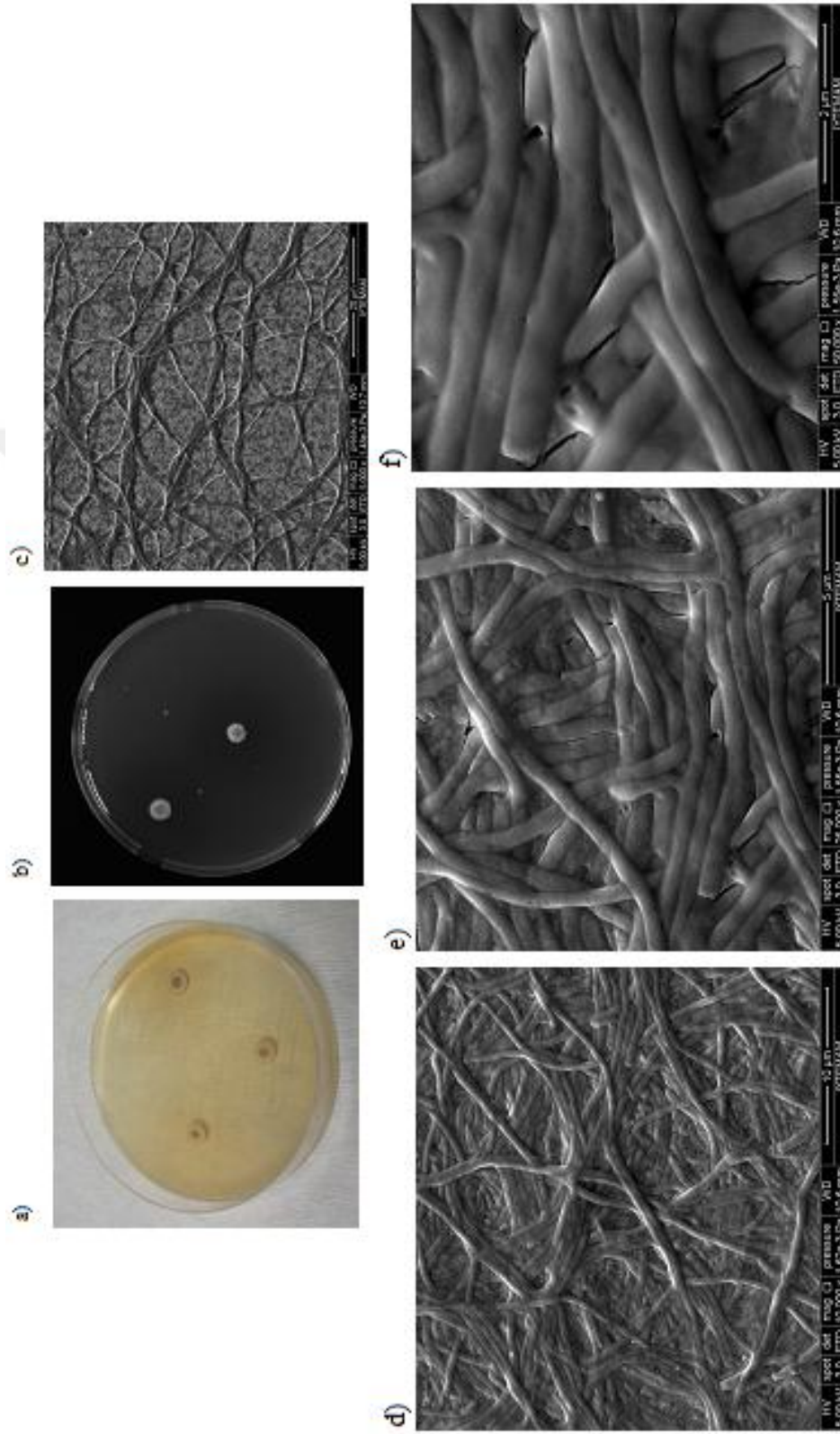


Figure 3.24 Morphology of the strain 1BA43Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 50000X SEM image.





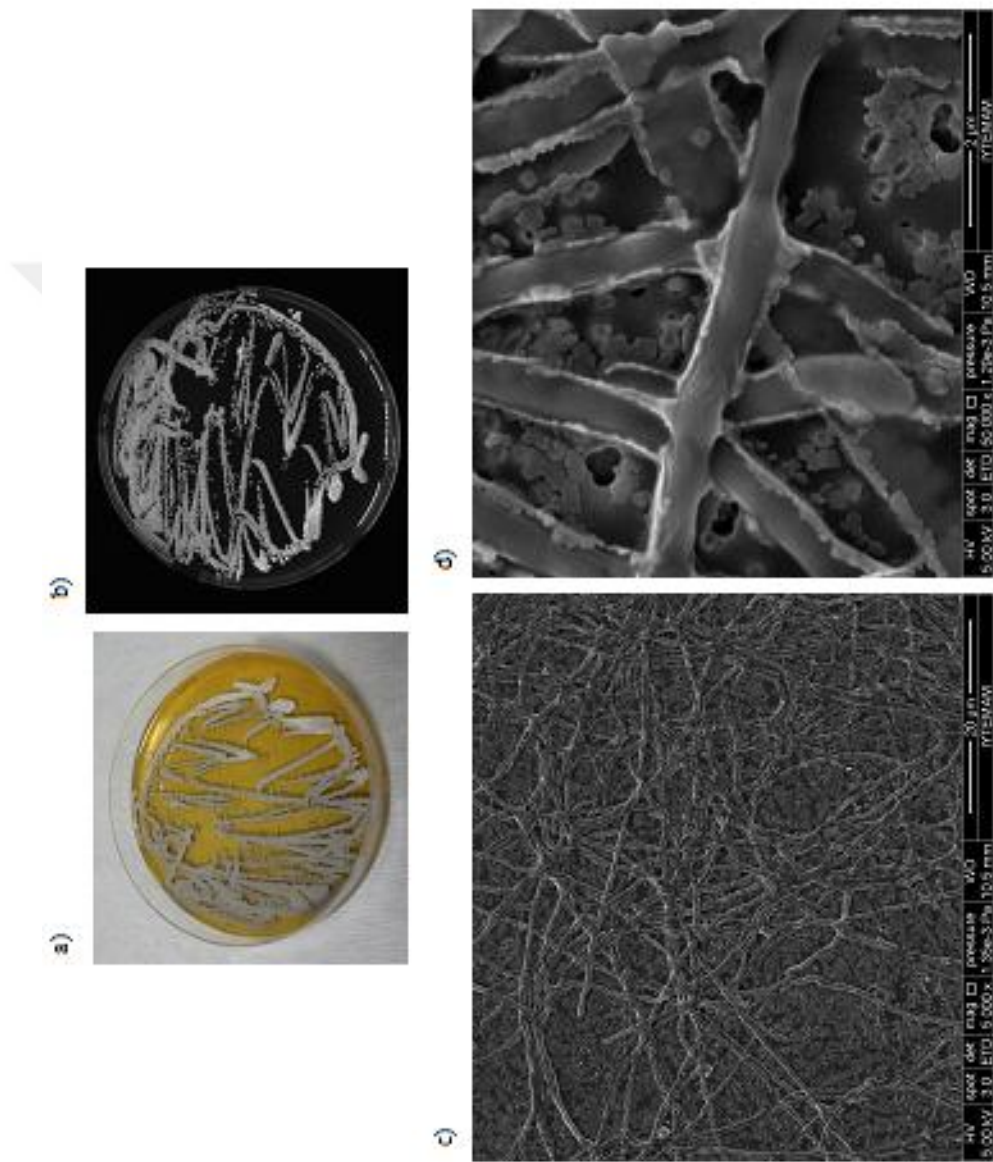
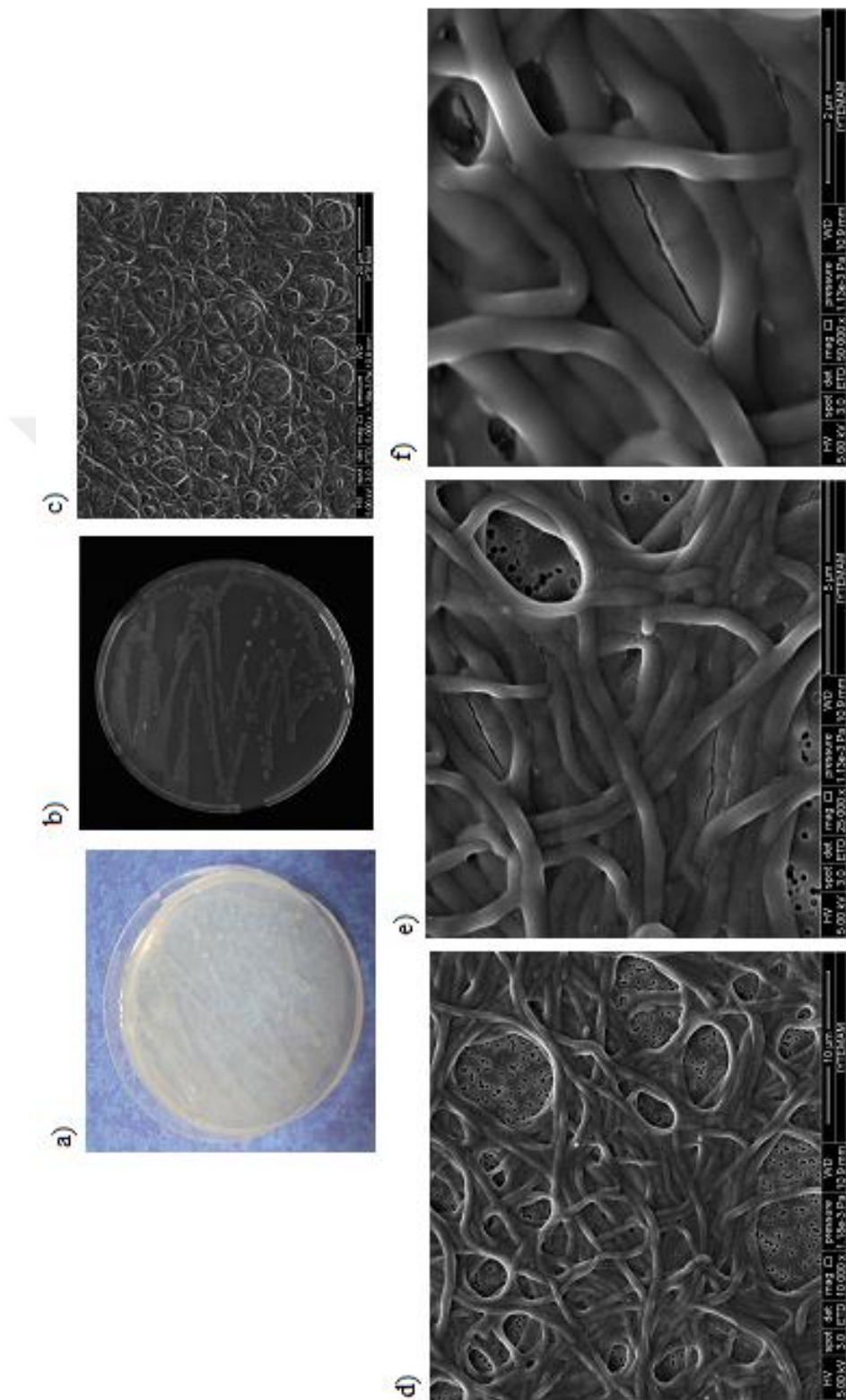


Figure 3.27 Morphology of the strain 7BA24Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 5000X SEM image, d) 50000X SEM image.



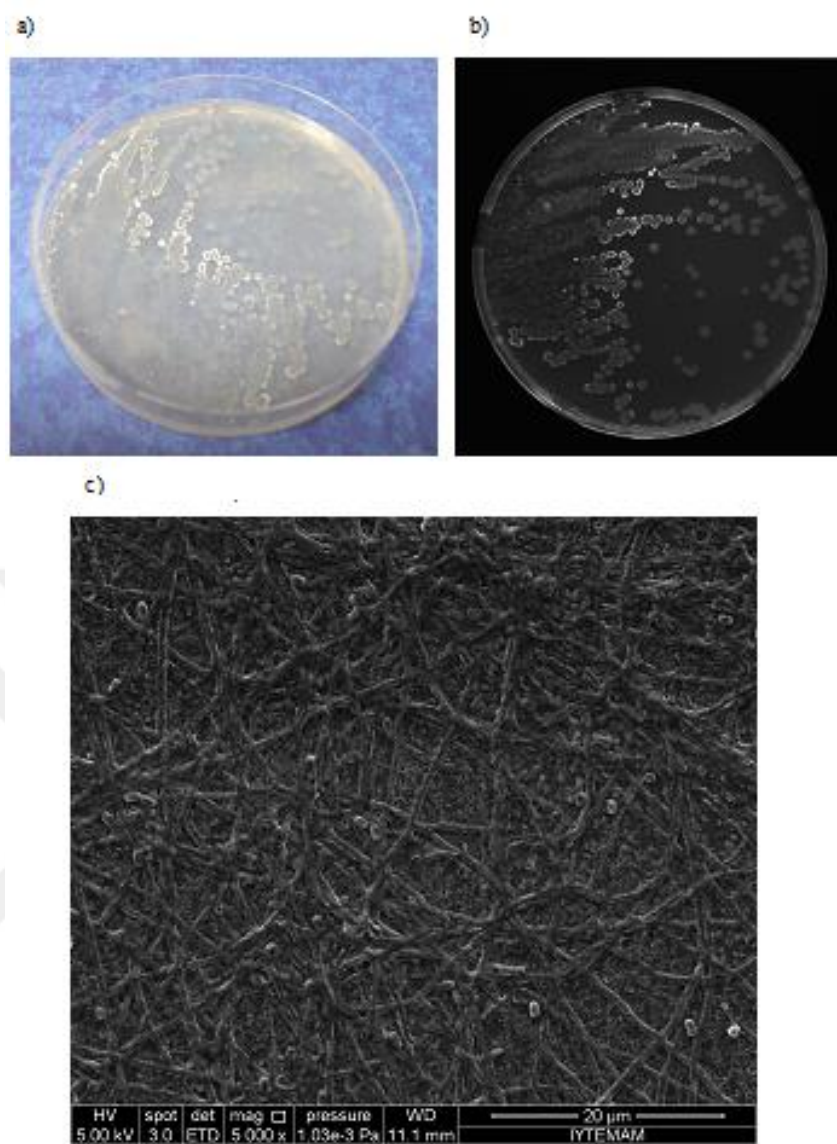


Figure 3.29 Morphology of the strain 11BA52Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 5000X SEM image.

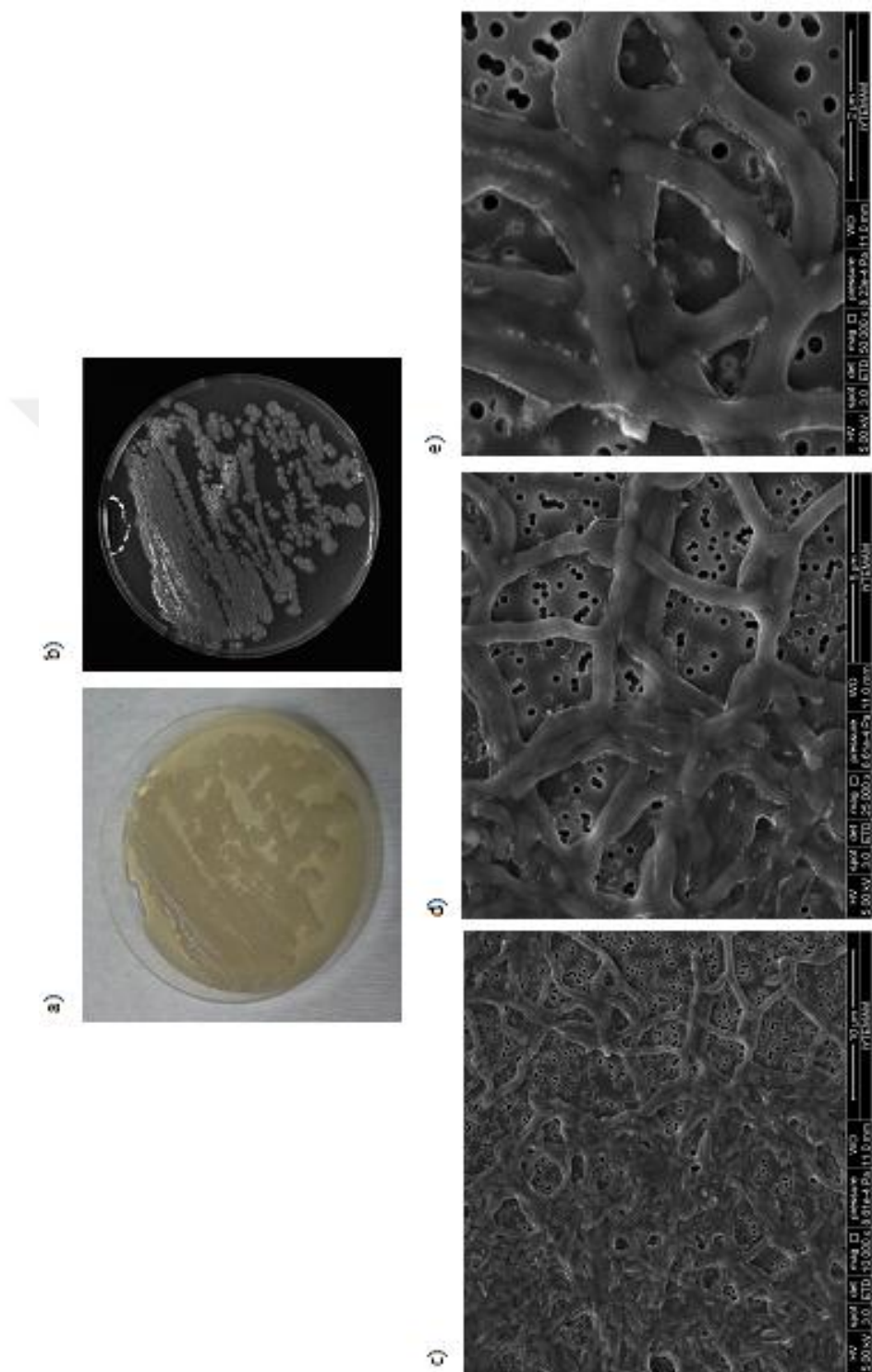


Figure 3.30 Morphology of the strain 13BA2Y12 a) petri photo taken by E-BOX VX2 camera, b) petri photo taken by FinePix AV110 camera, c) 10000X SEM image, d) 25000X SEM image, e) 50000X SEM image.

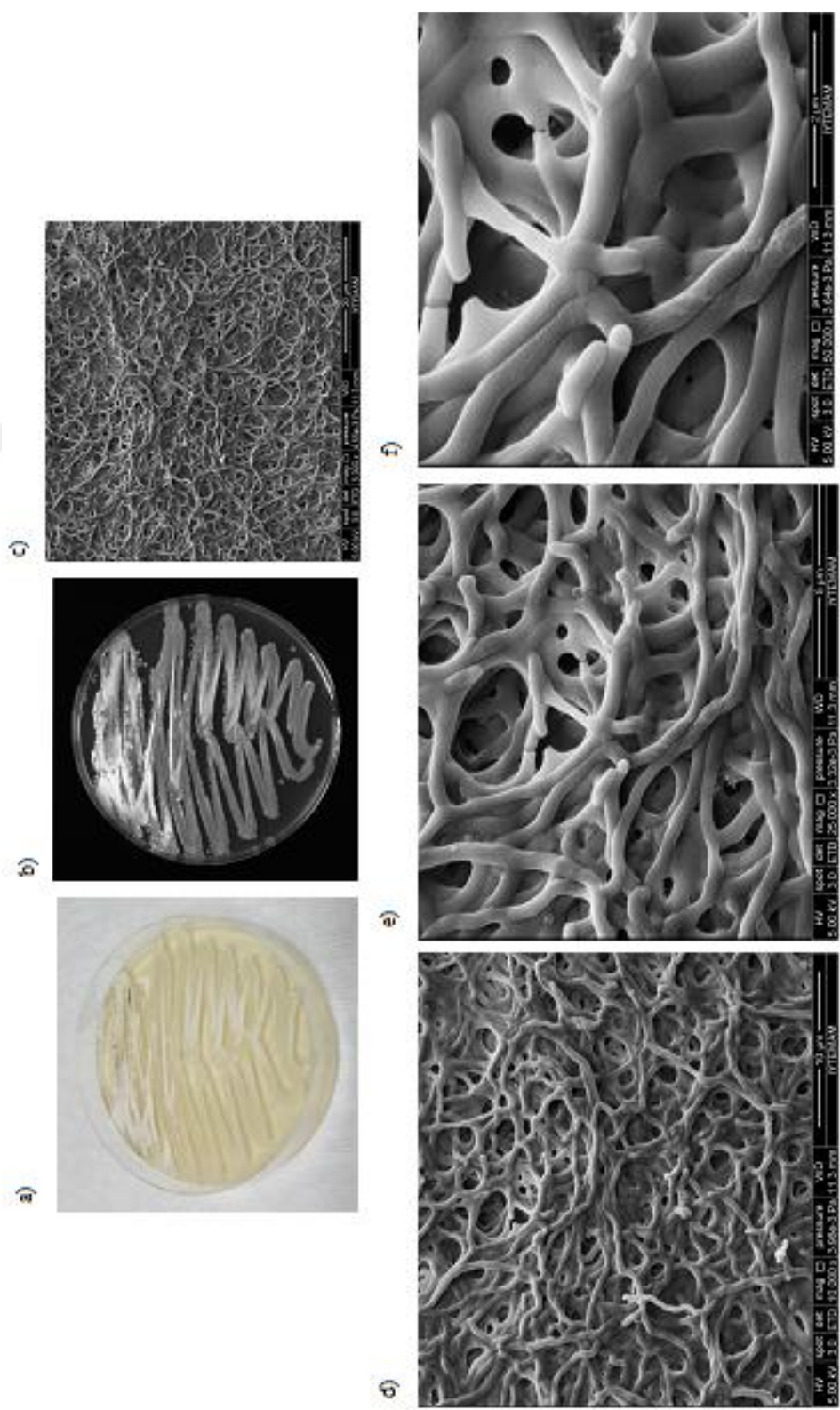


Figure 3.31 Morphology of the strain 19BA4Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 5000X SEM image, d) 10000X SEM image, e) 25000X SEM image, f) 50000X SEM image.

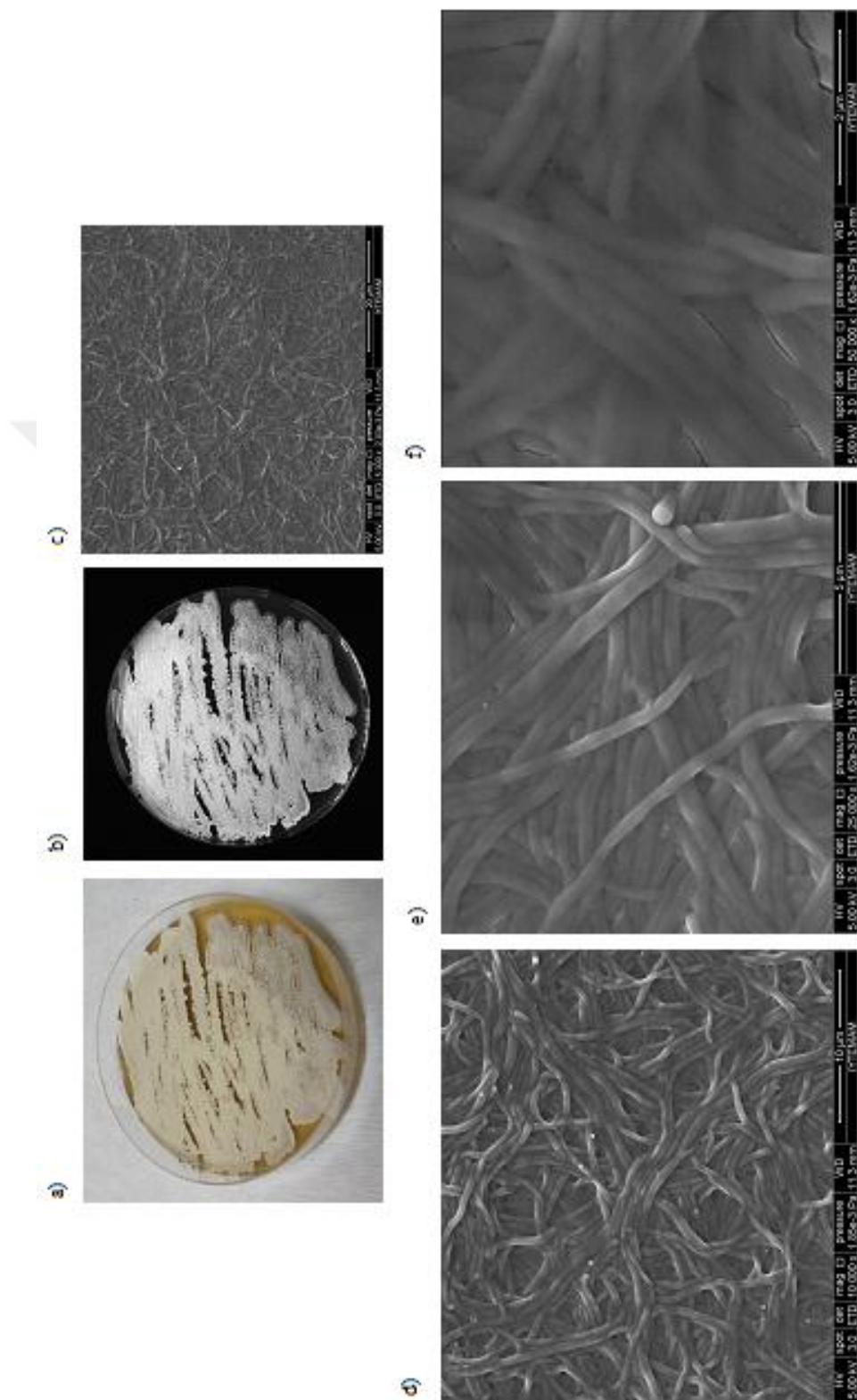


Figure 3.32 Morphology of the strain 23BA52Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 5000X SEM image, d) 10000X SEM image, e) 25000X SEM image, f) 50000X SEM image.

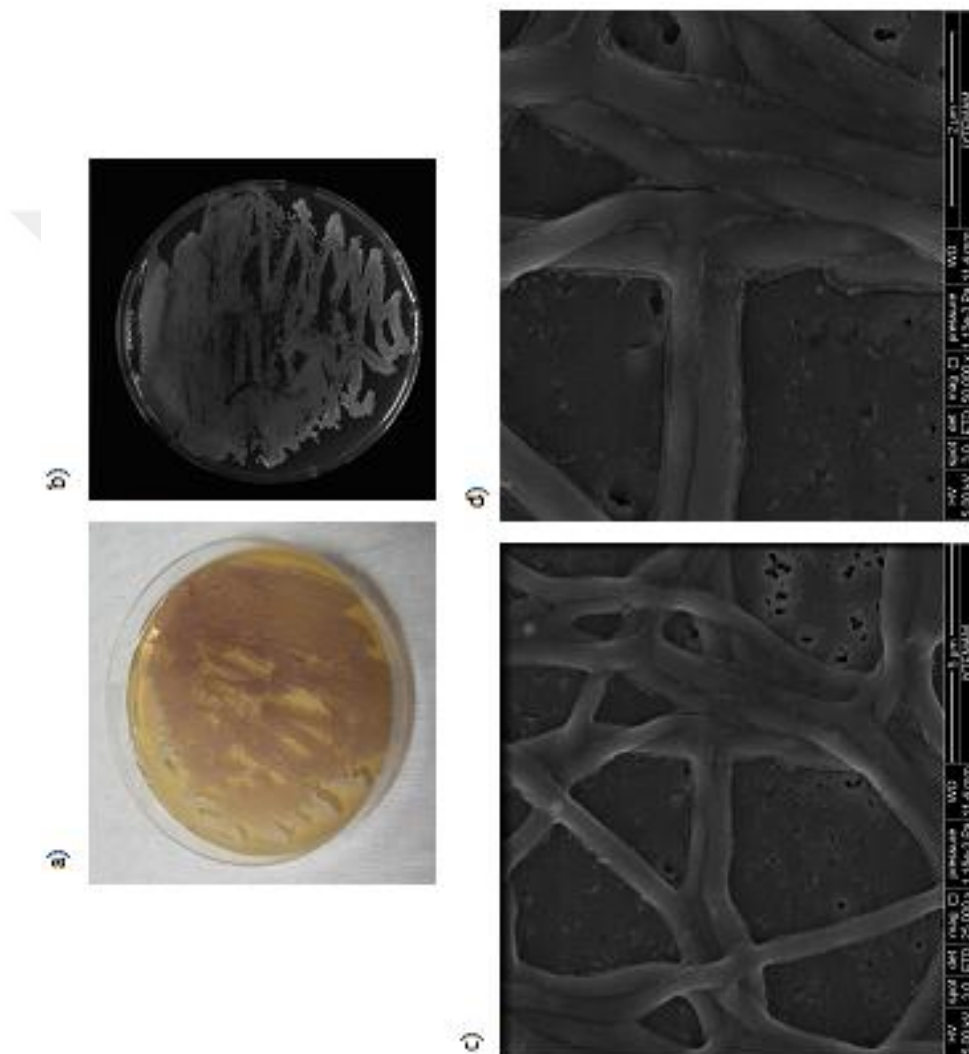


Figure 3.33 Morphology of the strain 25BA11Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 25000X SEM image, d) 50000X SEM image.

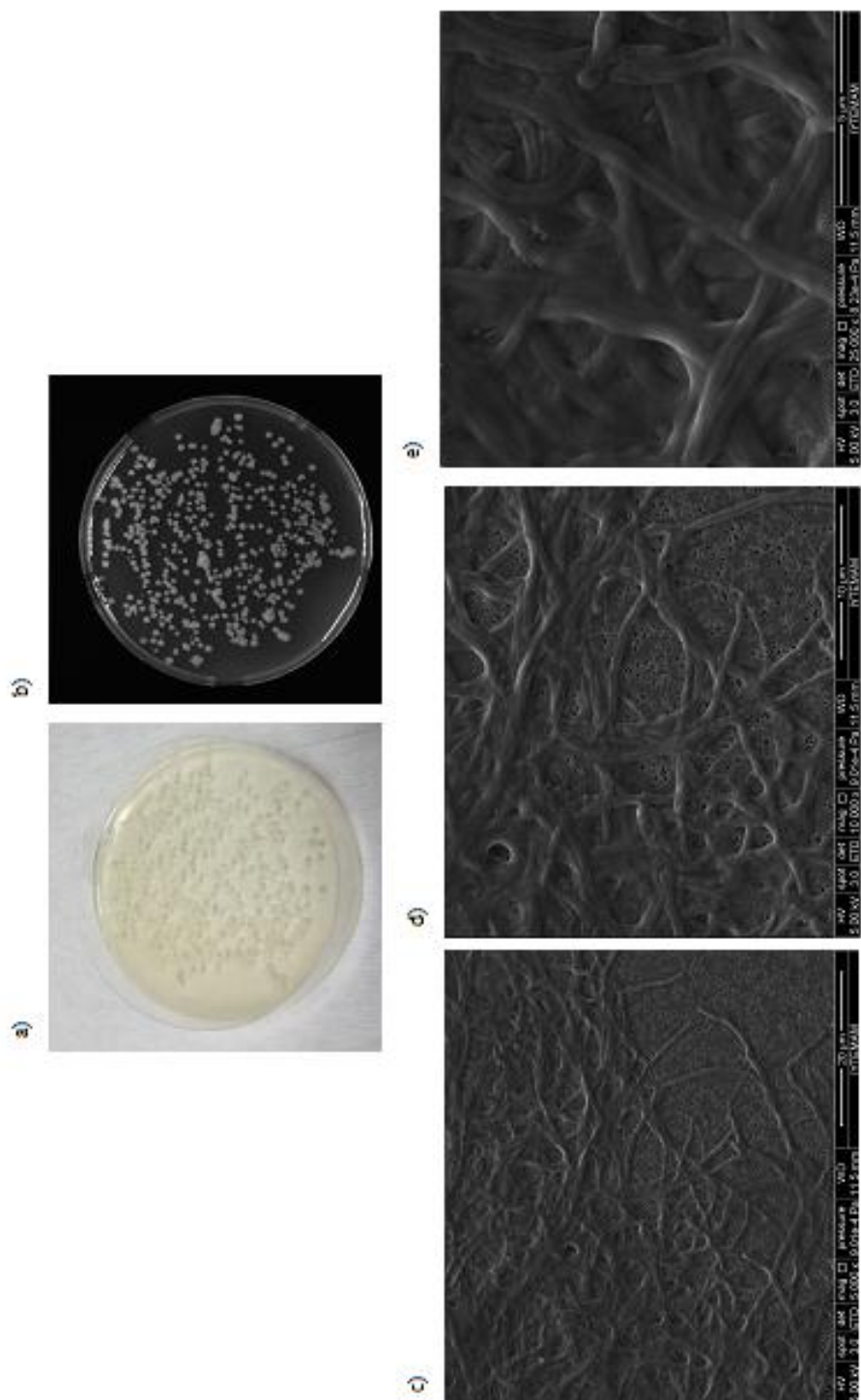


Figure 3.34 Morphology of the strain 29BA45Y12 a) petri photo taken by E-BOX VX2 camera, b) petri photo taken by FinePix AV110 camera, c) 5000X, d) 10000X, e) 25000X SEM image.

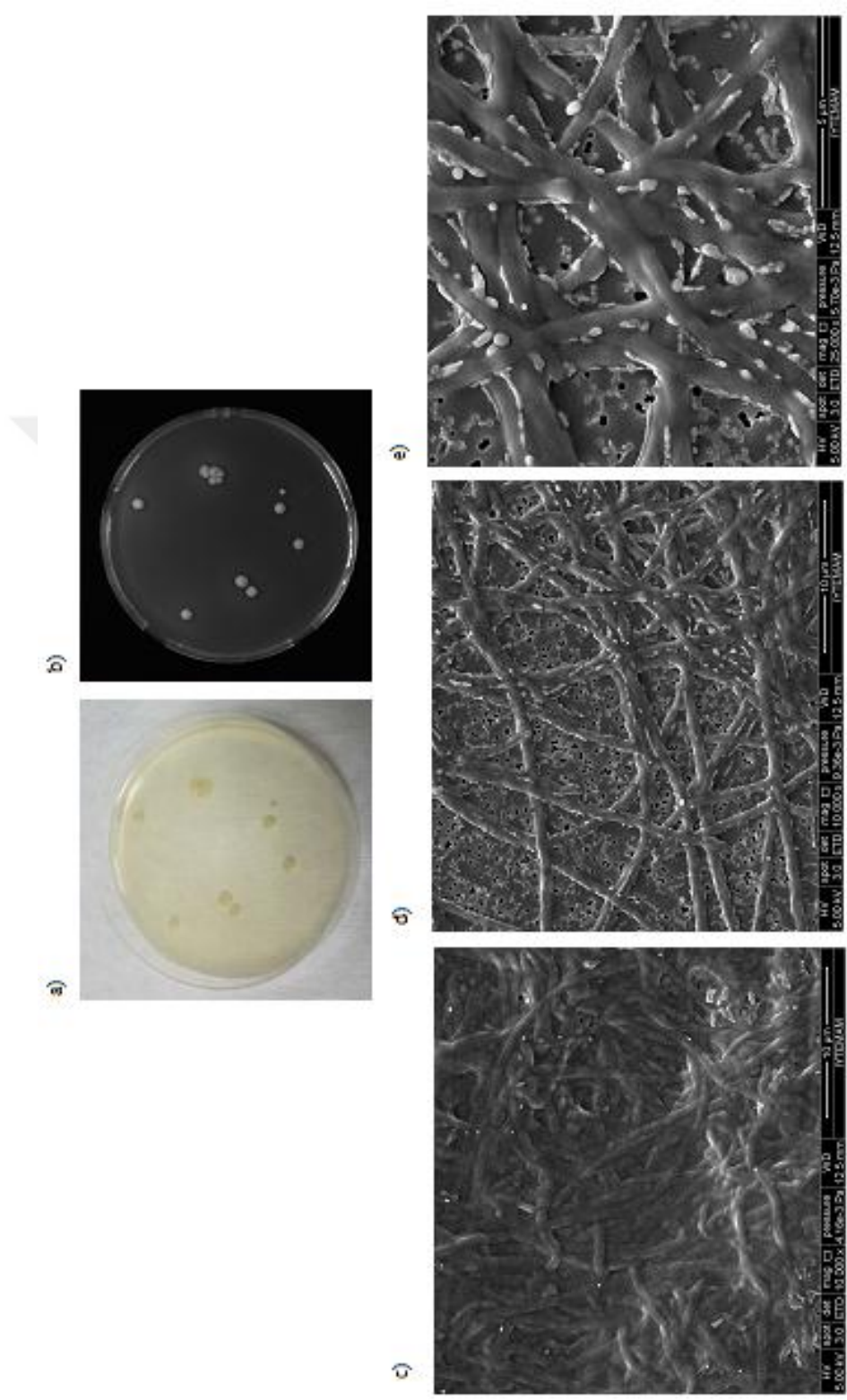


Figure 3.35 Morphology of the strain 41BA4Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 10000X SEM image, d) 10000X SEM image. e) 25000X SEM image.

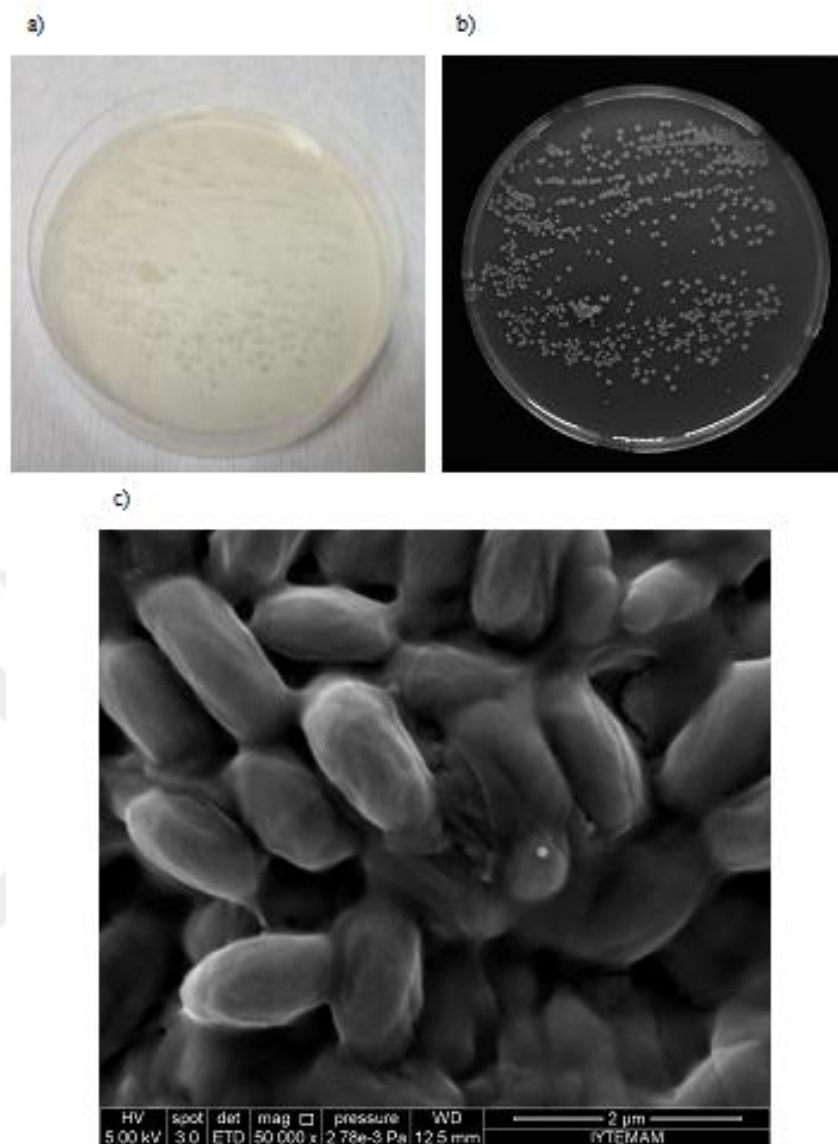


Figure 3.36 Morphology of the strain 43BA11Y12 a) petri photo taken by FinePix AV110 camera, b) petri photo taken by E-BOX VX2 camera, c) 50000X SEM image.

3.2.2.2 Physiological Tests

Similar to colony morphology, the isolates showed variation in physiological tests using commercial kits based on the formation of color or fluorescence as seen in Table 3.3 – 3.6. In spite of variation among isolates for extracellular enzyme synthesis, the results were similar when those two test kits were compared.

3.2.2.2.1 BBL GP ID kit Extracellular enzyme synthesis of isolates were very high for alkaline phosphatase, phenylalanine arylamidase, tryptophan arylamidase, arginine arylamidase, prolyl and leucine arylamidase, β glucosidase, N-acetyl- β -glucosaminidase, arginase unlike very low activity of pyrrolidonyl arylamidase and isoleucine arylamidase (Table 3.3 and 3.4). In contrast to high β glucosidase activity for p-nitrophenyl- β -D-glucoside, only one or two isolates showed activity of α maltosidase, urease and β glucosidase for esculin and p-nitrophenyl- β -D-cellobioside (Table 3.3 and 3.4). On the other hand, the isolates did not synthesize trehalase, lactase, α and β methyl glucosidase, sucrase, mannitol dehydrogenase, α amylase, arabinase, fructokinase, glycerol kinase and dehydrogenase (Table 3.3 and 3.4).

3.2.2.2.2 API ZYM kit Almost all isolates synthesized extracellular enzymes of alkaline and acid phosphatase, naphthol AS-BI phosphohydrolase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, N-acetyl- β -glucosaminidase (Table 3.5 and 3.6). While none of the isolates had α chymotrypsin activity, half of them showed trypsin and α glucosidase activity (Table 3.5 and 3.6). On the other hand, there was low activity of α and β galactosidase, β glucuronidase, α mannosidase, α fucosidase and β glucosidase for 6-Br-2-naphthyl- β -D-glucopyranoside (Table 3.5 and 3.6).

Table 3.3 BBL GP ID kit results of 6 isolates.

Panel	Test feature	Enzymes needed in cell for positive reaction	1BA43Y12	3BA24Y12	5BA24Y12	7BA24Y12	9BA3Y12	11BA52Y12
4A	fluorescent negative control							
2A	4MU- β -D-glucoside	β glucosidase	+	-	-	-	+	-
1A	L-valine-AMC	valine arylamidase	+	-	-	-	-	+
4B	L-phenylalanine-AMC	phenylalanine arylamidase	+	+	+	+	+	+
2B	4MU- α -D-glucoside	α glucosidase	+	+	-	-	-	+
1B	L-pyroglutamic acid-AMC	pyrrolidonyl arylamidase	-	-	-	-	-	-
4C	L-tryptophan-AMC	tryptophan arylamidase	+	+	+	+	+	+
2C	L-arginine-AMC	arginine arylamidase	+	+	+	+	+	+
1C	4MU-N-acetyl- β -D-glucosamide	N-acetyl- β -glucosaminidase	+	+	+	+	+	+
4D	4MU-phosphate	alkaline phosphatase	+	+	+	+	+	+
2D	4MU- β -D-glucuronide	β glucuronidase	-	-	-	-	-	-
1D	L-isoleucine-AMC	isoleucine arylamidase	+	-	-	-	+	-
4E	Trehalose	trehalase	-	-	-	-	-	-
2E	Lactose	lactase	-	-	-	-	-	-
1E	Methyl- α and β -glucoside	α and β methyl glucosidase	-	-	-	-	-	-
4F	Sucrose	sucrase	-	-	-	-	-	-
2F	Mannitol	mannitol dehydrogenase	-	-	-	-	-	-
1F	Maltotriose	α amylase	-	-	-	-	-	-
4G	Arabinose	arabinase	-	-	-	-	-	-
2G	Glycerol	glycerol kinase and glycerol dehydrogenase	-	-	-	-	-	-
1G	Fructose	fructokinase	-	-	-	-	-	-

Table 3.3 BBL GP ID kit results of 6 isolates. (Continued).

Panel	Test feature	Enzymes needed in cell for positive reaction	1BA43Y12	3BA24Y12	5BA24Y12	7BA24Y12	9BA3Y12	11BA52Y12
4H	p-nitrophenyl- β -D-glucoside	β glucosidase	+	+	+	+	+	+
2H	p-nitrophenyl- β -D-cellobioside	β glucosidase	-	-	-	-	+	-
1H	Proline and Leucine-p-nitroanilide	prolyl and leucine arylamidase	+	+	+	+	+	+
4I	p-nitrophenyl-phosphate	alkaline phosphatase	+	+	+	+	+	+
2I	p-nitrophenyl- α -D-maltoside	α maltosidase	-	-	-	-	-	+
1I	o-nitrophenyl- β -D-galactoside (ONPG) and p-nitrophenyl- α -D-galactoside	α and β galactosidase	-	+	+	+	-	+
4J	Urea	urease	+	-	-	-	-	+
2J	Esculin	β glucosidase	-	-	-	-	-	-
1J	Arginine	arginase	+	+	+	+	+	+

Table 3.4 BBL GP ID kit results of 7 isolates.

Panel	Test feature	Enzymes needed in cell for positive reaction	13BA2Y12	19BA4Y12	23BA52Y12	25BA11Y12	29BA45Y12	41BA4Y12	43BA11Y12
4A	fluorescent negative control								
2A	4MU- β -D-glucoside	β glucosidase	+	-	+	+	+	+	-
1A	L-valine-AMC	valine arylamidase	-	+	+	+	-	-	-
4B	L-phenylalanine-AMC	phenylalanine arylamidase	+	+	+	+	+	+	+
2B	4MU- α -D-glucoside	α glucosidase	+	+	+	-	+	-	+
1B	L-pyroglutamic acid-AMC	pyrrolidonyl arylamidase	+	+	-	-	-	-	-
4C	L-tryptophan-AMC	tryptophan arylamidase	+	+	+	+	-	-	-
2C	L-arginine-AMC	arginine arylamidase	+	+	+	+	+	-	+
	4MU-N-acetyl- β -D-glucosamide	N-acetyl- β -glucosaminidase	+	+	+	+	-	-	-
1C	glucosamide	glucosaminidase	+	+	+	+	+	+	+
4D	4MU-phosphate	alkaline phosphatase	+	+	+	+	+	+	+
2D	4MU- β -D-glucuronide	β glucuronidase	-	-	-	-	-	-	-
1D	L-isoleucine-AMC	isoleucine arylamidase	-	+	+	+	-	+	-
4E	Trehalose	trehalase	-	-	-	-	-	-	-
2E	Lactose	lactase	-	-	-	-	-	-	-
1E	Methyl- α and β -glucoside	α and β methyl glucosidase	-	-	-	-	-	-	-
4F	Sucrose	sucrase	-	-	-	-	-	-	-
2F	Mannitol	mannitol dehydrogenase	-	-	-	-	-	-	-
1F	Maltotriose	α amylase	-	-	-	-	-	-	-
4G	Arabinose	arabinase	-	-	-	-	-	-	-
2G	Glycerol	glycerol kinase and glycerol dehydrogenase	-	-	-	-	-	-	-
1G	Fructose	fructokinase	-	-	-	-	-	-	-

Table 3.4 BBL GP ID kit results of 7 isolates. (Continued).

Panel	Test feature	Enzymes needed in cell for positive reaction	13BA2Y12	19BA4Y12	23BA52Y12	25BA11Y12	29BA45Y12	41BA4Y12	43BA11Y12
4H	p-nitrophenyl- β -D-glucoside	β glucosidase	+	-	+	+	+	+	+
2H	p-nitrophenyl- β -D-cellobioside	β glucosidase	-	-	+	-	-	+	+
1H	Proline and Leucine-p-nitroanilide	prolyl and leucine arylamidase	+	+	+	-	+	-	-
4I	p-nitrophenyl-phosphate	alkaline phosphatase	+	+	+	+	-	-	+
2I	p-nitrophenyl- α -D-maltoside	α maltosidase	-	-	-	-	-	-	+
1I	o-nitrophenyl- β -D-galactoside (ONPG) and p-nitrophenyl- α -D-galactoside	α and β galactosidase	+	+	-	-	+	+	-
4J	Urea	urease	-	-	-	-	-	-	-
2J	Esculin	β glucosidase	-	-	-	-	-	-	+
1J	Arginine	arginase	+	+	+	+	+	-	+

Table 3.5 API ZYM kit results of 6 isolates.

Panel	Activity mark	Enzymes needed in cell for positive reaction	1BA43Y12	3BA24Y12	5BA24Y12	7BA24Y12	9BA3Y12	11BA52Y12
1	Control							
2	2-naphthyl phosphate	alkaline phosphatase	+	+	+	+	+	+
3	2-naphthyl butyrate	esterase (C4)	-	+	+	+	+	+
4	2-naphthyl caprylate	esterase lipase (C8)	+	+	+	+	+	+
5	2-naphthyl myristate	lipase (C14)	(+)	+	+	+	+	+
6	L-leucyl-2-naphthylamide	leucine arylamidase	+	+	+	+	+	+
7	L-valyl-2-naphthylamide	valine arylamidase	+	+	+	+	+	+
8	L-cystyl-2-naphthylamide	cystine arylamidase	(+)	(+)	(+)	(+)	(+)	(+)
9	N-benzoyl-DL-arginine-2-naphthylamide	trypsin	-	(+)	(+)	-	-	-
10	N-glutaryl-phenyl-alanine-2-naphthylamide	α chymotrypsin	-	-	-	-	-	-
11	2-naphthyl phosphate	acid phosphatase	(+)	(+)	(+)	(+)	(+)	(+)
12	naphthol AS-BI phosphate	naphthol AS-BI phosphohydrolase	+	+	+	+	+	+
13	6-Br-2-naphthyl- α -D-galactopyranoside	α galactosidase	-	-	-	-	-	-
14	2-naphthyl- β -D-galactopyranoside	β galactosidase	-	-	-	-	-	-
15	naphthol AS-BI β -D-glucuronate	β glucuronidase	-	-	(+)	-	-	-
16	2-naphthyl- α -D-glucopyranoside	α glucosidase	-	-	-	-	-	+
17	6-Br-2-naphthyl- β -D-glucopyranoside	β glucosidase	-	-	-	(+)	-	-
18	1-naphthyl-N-acetyl- β -D-glucosaminide	N-acetyl- β -glucosaminidase	-	+	+	+	(+)	-
19	6-Br-2-naphthyl- α -D-mannopyranoside	α mannosidase	-	-	(+)	(+)	-	-
20	2-naphthyl- α -L-fucopyranoside	α fucosidase	+	-	-	-	-	-

Table 3.6 API ZYM kit results of 7 isolates.

Panel	Activity mark	Enzymes needed in cell for positive rxn	13BA2Y12	19BA4Y12	23BA52Y12	25BA11Y12	29BA45Y12	41BA4Y12	43BA11Y12
1	Control								
2	2-naphthyl phosphate	alkaline phosphatase	+	+	+	(+)	-	-	(+)
3	2-naphthyl butyrate	esterase (C4)	+	+	+	(+)	-	(+)	+
4	2-naphthyl caprylate	esterase lipase (C8)	+	+	+	+	(+)	(+)	+
5	2-naphthyl myristate	lipase (C14)	-	-	+	+	-	-	-
6	L-leucyl-2-naphthylamide	leucine arylamidase	+	+	+	+	+	+	(+)
7	L-valyl-2-naphthylamide	valine arylamidase	+	+	+	+	+	+	+
8	L-cystyl-2-naphthylamide	cystine arylamidase	(+)	(+)	(+)	(+)	(+)	(+)	(+)
9	N-benzoyl-DL-arginine-2-naphthylamide	trypsin	-	(+)	(+)	(+)	(+)	-	-
10	N-glutaryl-phenyl-alanine-2-naphthylamide	α chymotrypsin	-	-	-	-	-	-	-
11	2-naphthyl phosphate	acid phosphatase	+	+	(+)	+	(+)	(+)	(+)
12	naphthol AS-BI phosphate	naphthol AS-BI phosphohydrolase	+	+	+	+	+	+	+
13	6-Br-2-naphthyl- α -D-galactopyranoside	α galactosidase	-	-	-	(+)	-	-	-
14	2-naphthyl- β -D-galactopyranoside	β galactosidase	+	-	-	(+)	+	-	(+)
15	naphthol AS-BI β -D-glucuronate	β glucuronidase	-	-	(+)	-	-	-	-
16	2-naphthyl- α -D-glucopyranoside	α glucosidase	(+)	(+)	-	(+)	(+)	-	(+)
17	6-Br-2-naphthyl- β -D-glucopyranoside	β glucosidase	-	(+)	(+)	-	-	-	-
18	1-naphthyl-N-acetyl- β -D-glucosaminide	N-acetyl- β -glucosaminidase	+	+	(+)	+	-	-	-
19	6-Br-2-naphthyl- α -D-mannopyranoside	α mannosidase	+	-	(+)	-	-	-	-
20	2-naphthyl- α -L-fucopyranoside	α fucosidase	-	-	(+)	+	(+)	(+)	-

3.1.5 Phylogenetical Results

The difference observed in the phenotypical features of the isolates was also obtained by the phylogenetic analysis. 12 strains were found as belonged to the family *Streptomycetaceae* with high affiliation to totally 8 different *Streptomyces* genera and 1 strain to the family *Nocardiopsaceae* with *Nocardiopsis* genus.

3.2.3.1 PCR Results

Among 3 PCR mixes tried with combination of 3 different PCR programs for polymerization of 16S rRNA gene with 27f – 1492r universal primers, PCR mix III together with PCR program III gave the best results as seen below in the photo of the PCR products on the agarose gel (Figure 3.37 and 3.38). PCR mix II and program II sometimes worked for the DNA samples of the isolates i.e. PCR mix II together with program II when laboratory equipments of Ege University were used but PCR mix II together with program III did not work in DEU IMST. On the other hand, PCR mix I and program I did not give PCR products. The reasons of those failures were thought as especially different Thermocyclers for the former and inappropriate annealing temperature for the latter.

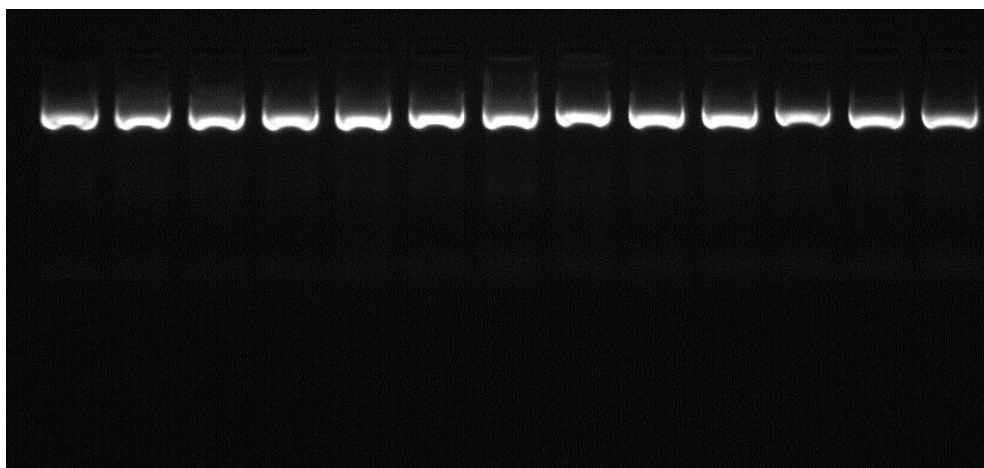


Figure 3.37 The photo of PCR products of 13 actinomycetes run on 2 % agarose gel electrophoresis.

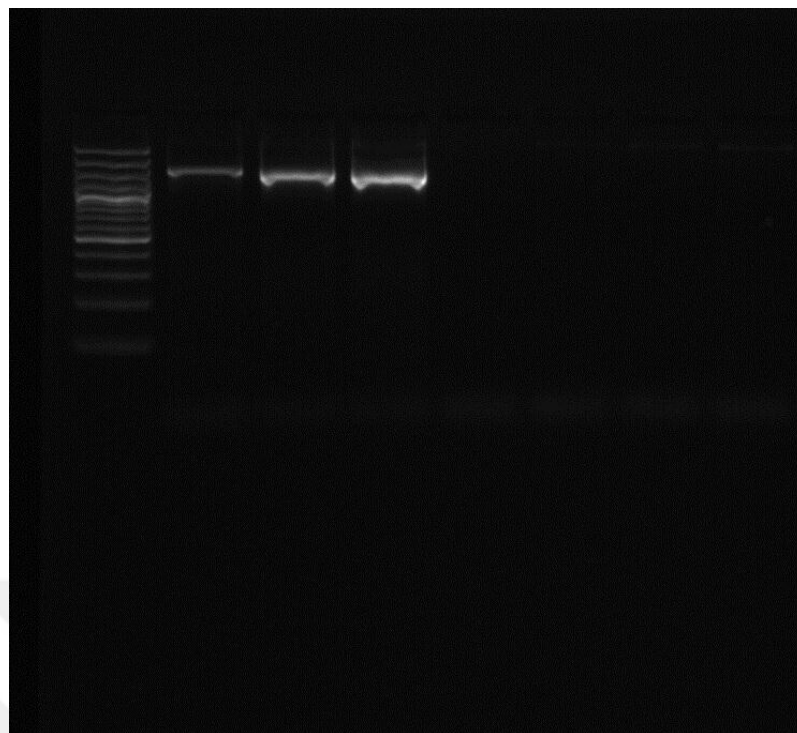


Figure 3.38 The photo of PCR products of actinomycete strains with DNA marker (100-3000bp).

3.2.3.2 Sequence Results

The forward and reverse chromatograms of the strain 23BA52Y12 were given as an example in Figure 3.39. 16S rRNA gene sequences of the isolates were from 1361 bp to 1393 bp long. When only those sequences were aligned, it was found that the major nucleotide differences were in between 15 – 30 bp, 110 – 140 bp, 890 – 910 bp, 980 – 1015 bp of the 3' end, in addition to extra 15 bp from 370 bp of the strain 11BA52Y12 unlike others and from 2 bp to 10 bp gaps for all in between 1000 – 1010 bp.

On the other hand, the strains 3BA24Y12 (1370 bp), 5BA24Y12 (1370 bp) and 7BA24Y12 (1375 bp) had exactly the same sequence in addition to extra 5 bp in the 3' end as “GAGCT” of the strain 7BA24Y12. They were all isolated from the station 2 and in fact, 3BA24Y12 and 5BA24Y12 from the same agar plate.

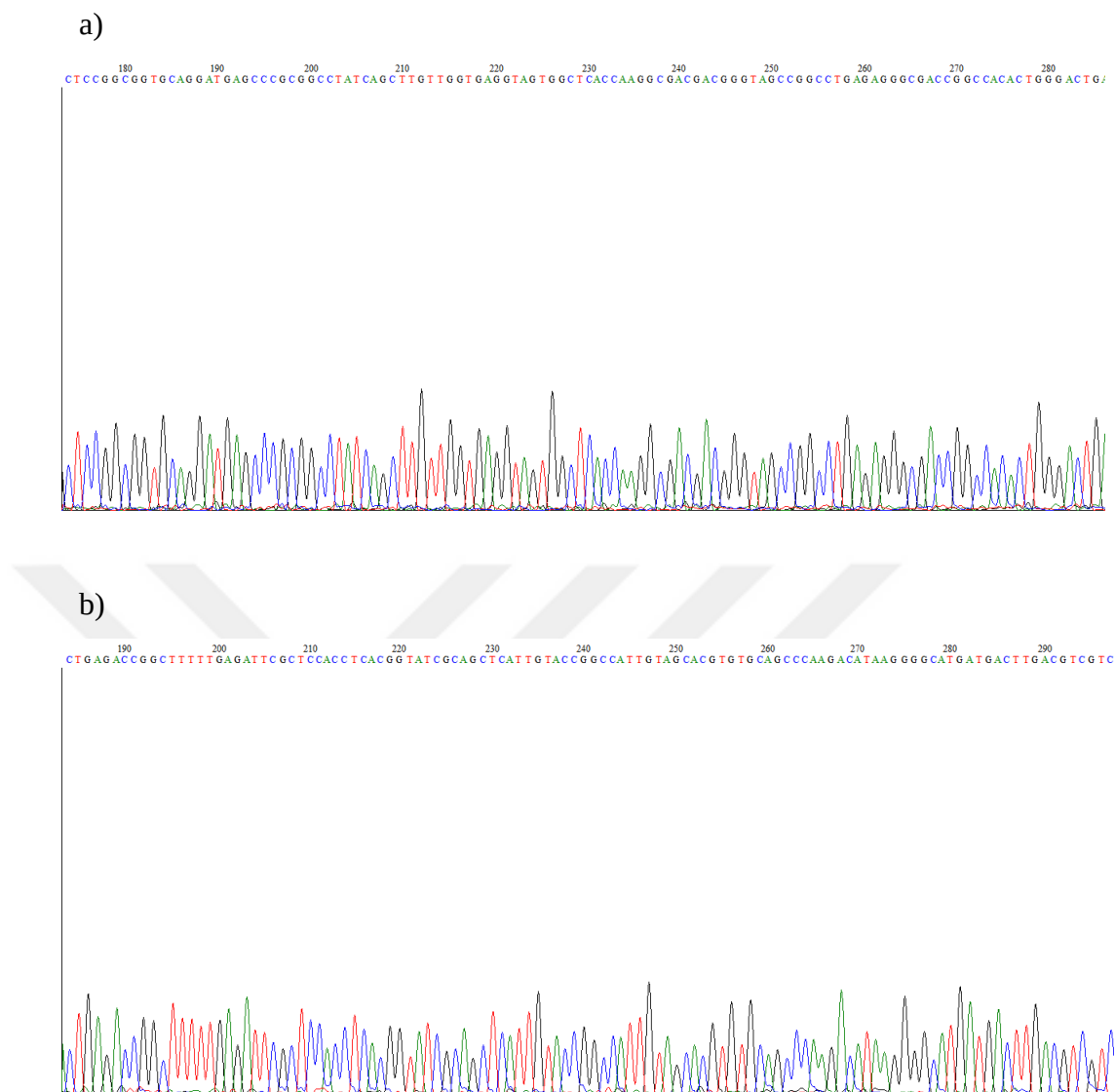


Figure 3.39 The forward and reverse chromatograms of the strain 23BA52Y12 (a and b, respectively).

Similarly, the strains 1BA43Y12 (1393 bp), 9BA3Y12 (1383 bp) and 23BA52Y12 (1383 bp) isolated from the station 4, 3 and 5, respectively had exactly the same sequence in addition to extra 10 bp in the 5' end as "TGCAAGTCGA" of the strain 1BA43Y12.

3.2.3.3 BLAST Results

According to phylogenetic analysis based on 16S rRNA gene sequences, it was found that 12 strains belonged to the family *Streptomycetaceae* and 1 strain to the

family *Nocardiopsaceae* (Table 3.7). Within the order *Actinomycetales*, the former shared a high phylogenetic affiliation with the genus *Streptomyces* and the latter to the genus *Nocardiopsis*. Except one strain, others shared $\geq 99\%$ 16S rRNA gene sequence identity with the members of those taxa.

The strain 11BA52Y12 isolated from the station 5 was closely related to *Nocardiopsis alba* with 100 % pairwise similarity. On the other hand, the *Streptomyces* strains were isolated from each station and a quarter of them (isolated from the stations 3 – 5) were closely related to *Streptomyces albidoflavus* and a quarter (isolated from the station 2) to *Streptomyces brevispora* with 99.71 % and 99.78 % sequence identity, respectively.

From the station 2, another strain 13BA2Y12 sharing 100 % pairwise similarity with *Streptomyces cellulosa* was also obtained. Similarly, the strains 25BA11Y12 and 43BA11Y12 isolated from the station 1 were closely related to *Streptomyces anulatus* (99.64 %) and *Streptomyces samsunensis* (99.93 %), respectively.

Like those isolates, the strains 19BA4Y12 and 29BA45Y12 from the station 4 shared 100 % sequence identity with *Streptomyces variabilis* and 99.35 % with *Streptomyces albogriseolus*. In contrast, the strain 41BA4Y12 isolated from the same station was related to *Streptomyces haliclone* with low pairwise similarity as 98.6 %.

If the pairwise similarity was lower than 98 % with their nearest type strains, it means that the strain has a high probability of representing new taxa. Although 13 actinomycete strains had pairwise similarity $> 98\%$ (Table 3.7), when a limit of 98.5 to 99 % was considered, as revised and proposed by Stackebrandt and Ebers (2006), the *Streptomyces* strain 41BA4Y12 had probability of representing new taxa.

3.2.3.4 Phylogenetic Tree

For the phylogenetic tree, the neighbor-joining method was used. The bootstrap values (in percent) calculated from 1000 resamplings were shown at the nodes for

values of $\geq 85\%$ (Figure 3.40). In spite of small changes in those values for the rest, the topology of the distance neighbor-joining tree was supported by UPGMA method.

With 1000 bootstrap neighbor-joining method, the phylogenetic tree constructed using nearly full 16S rRNA gene sequence of representative isolates for each nearest type strain clearly supported those families *Streptomycetaceae* and *Nocardiopsaceae* as forming 2 clades (Figure 3.40). While one of the clades was formed by the strain 11BA52Y12 with its nearest type strain *Nocardiopsis alba*, the majority of the strains formed a diverse clade having members belonging to the genus *Streptomyces*. Moreover, the branch formations supported the sequence similarities mentioned in previous section.

Table 3.7 The list of isolates representing the familia *Nocardiopsaceae* and *Streptomycetaceae* with the nearest type strains according to their 16S rRNA gene sequences.

Phylogenetic group (family)	Representative isolate (accession no.)	Sequence length (bp)	Nearest type strain (accession no.)	Pairwise similarity (%)	Source of nearest type strain
<i>Nocardiopsaceae</i>	11BA52Y12 (KF366671)	1392	<i>Nocardiopsis alba</i> (X97883)	100.00	water-damaged indoor buildings
<i>Streptomycetaceae</i>	9BA3Y12 (KC815759)	1383	<i>Streptomyces albidoflavus</i> (Z76676)	99.71	soil
<i>Streptomycetaceae</i>	23BA52Y12 (KC815762)	1383	<i>Streptomyces albidoflavus</i> (Z76676)	99.71	soil
<i>Streptomycetaceae</i>	29BA45Y12 (KC815763)	1391	<i>Streptomyces albogriseolus</i> (AJ494865)	99.35	soil
<i>Streptomycetaceae</i>	3BA24Y12 (KC815756)	1370	<i>Streptomyces brevispora</i> (FR692104)	99.78	soil
<i>Streptomycetaceae</i>	5BA24Y12 (KC815757)	1370	<i>Streptomyces brevispora</i> (FR692104)	99.78	soil
<i>Streptomycetaceae</i>	7BA24Y12 (KC815758)	1375	<i>Streptomyces brevispora</i> (FR692104)	99.78	soil
<i>Streptomycetaceae</i>	13BA2Y12 (KC815760)	1384	<i>Streptomyces cellulosae</i> (AB184265)	100.00	soil
<i>Streptomycetaceae</i>	41BA4Y12 (KC815764)	1361	<i>Streptomyces haliclonae</i> (AB473556)	98.60	<i>Haliclona</i> sp. homogenate
<i>Streptomycetaceae</i>	43BA11Y12 (KC815765)	1363	<i>Streptomyces samsunensis</i> (EU077190)	99.93	<i>Robinia pseudoacacia</i> rhizosphere
<i>Streptomycetaceae</i>	19BA4Y12 (KC815761)	1390	<i>Streptomyces variabilis</i> (AB184884)	100.00	soil
<i>Streptomycetaceae</i>	1BA43Y12 (KF366670)	1393	<i>Streptomyces albidoflavus</i> (Z76676)	99.71	soil
<i>Streptomycetaceae</i>	25BA11Y12 (KF366674)	1387	<i>Streptomyces anulatus</i> (DQ026637)	99.64	indoor air of moldy buildings

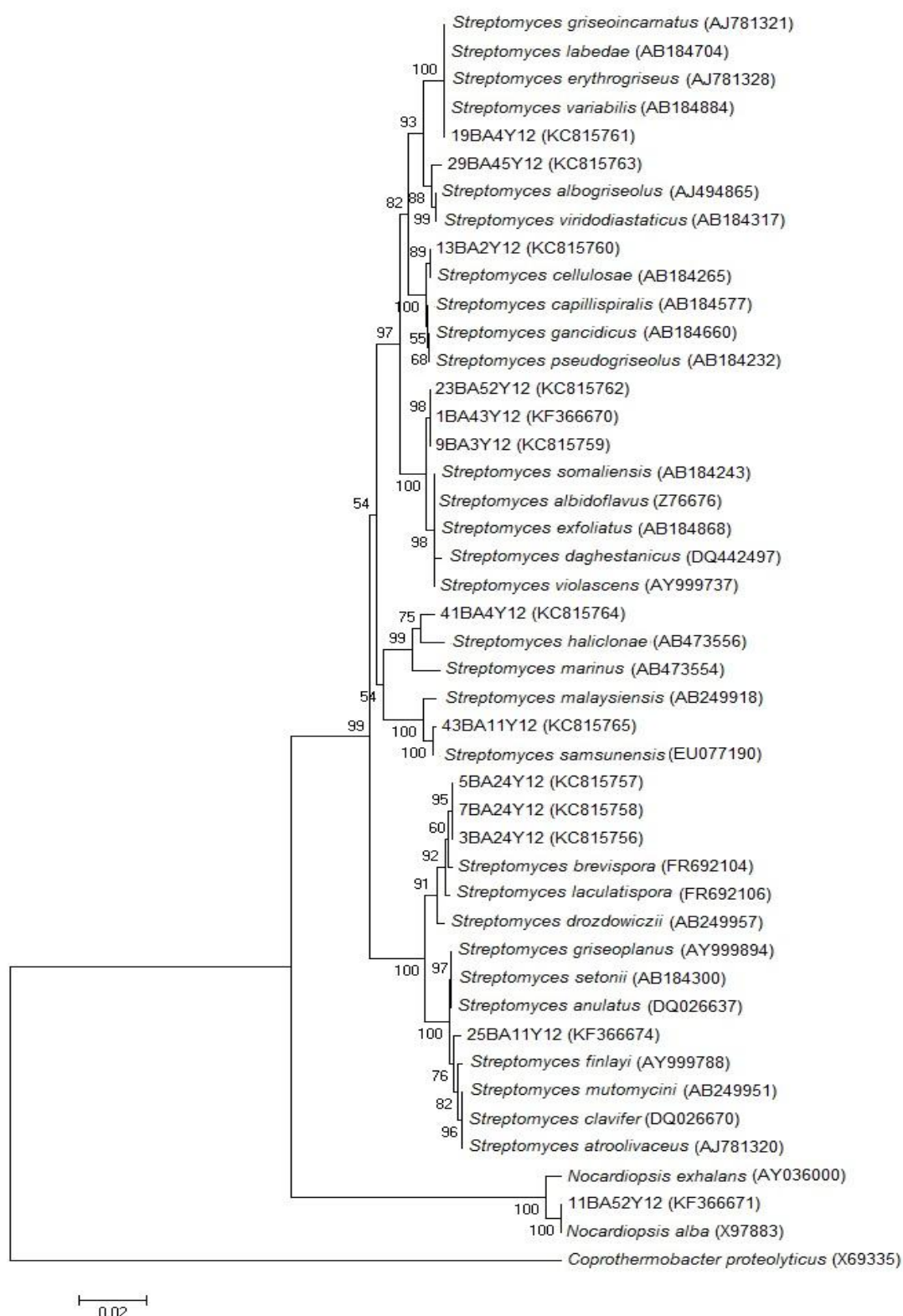


Figure 3.40 Neighbor-joining distance tree constructed in Mega 5.1 using 16S rRNA gene sequences. GenBank accession numbers were given in parentheses. Bootstrap values calculated from 1000 re-samplings were in percentage. *Coprothermobacter proteolyticus* was used to position the root.

CHAPTER FOUR

DISCUSSION

4.1 Comparison of Physicochemical Parameters

Overall seawater and sediment parameters of the study area were found in the range of Aegean Sea values when compared to the previous results of Eastern Mediterranean Sea. The study area also showed characteristics in between coastal and deep basins of Aegean Sea, supported by the sediment parameters of our present TUBITAK COST project no. 110Y193 and BAP project no. 2010.KB.FEN.014.

4.1.1 Seawater Parameters

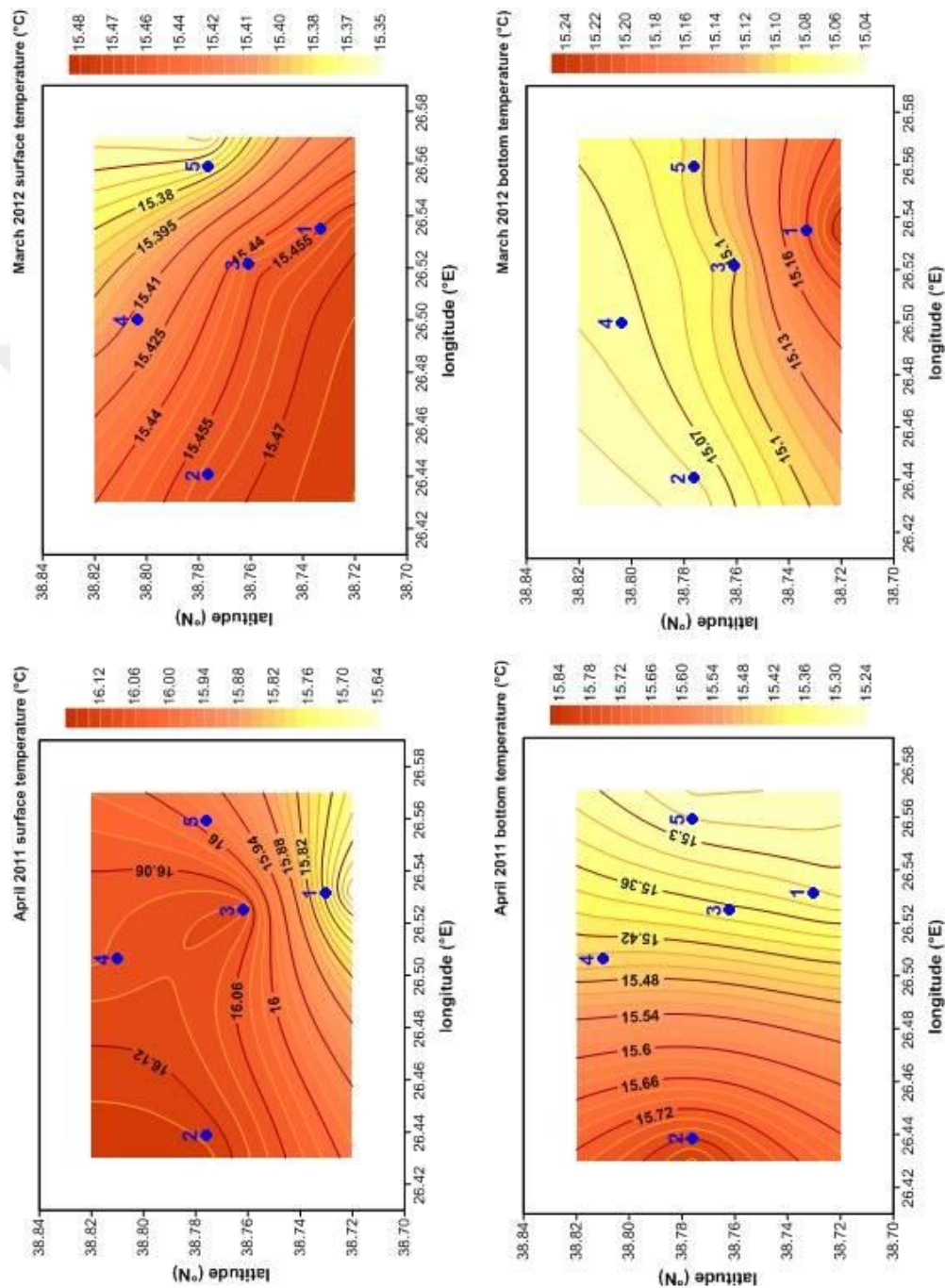
When compared to the previous sampling of the study area and of the other areas in Aegean Sea, it could be said that the seawater values of the study area were generally in the range of Aegean Sea values. In addition, especially nutrient concentrations were closer to the values of North Aegean Sea which is much eutrophic rather than the concentrations of oligotrophic South Aegean Sea.

4.1.1.1 Previous Sampling at the Study Area

The previous sampling was performed at the same study area in 25 April 2011. In addition to the close ranges, those previous parameters of the seawater samples as temperature, salinity, density, pH, dissolved oxygen, total suspended solids (15.26 – 16.21 °C, 39.13 – 39.18 psu, 28.92 – 29.10 kg/m³, 8.17 – 8.28 pH, 5.29 – 5.73 ml/l, 0.40 – 18.53 mg/l, respectively), except chlorophyll α (BDL – 0.93 μ g/l) and nutrients as nitrite, nitrate, phosphate, reactive Si (0.02 – 0.13 μ M, BDL – 0.16 μ M, BDL – 0.16 μ M, 0.63 – 2.53 μ M, respectively), were in slightly broader intervals with different spatial distribution at surface and bottom layers compared to the present ones (Table 4.1, Figure 4.1 – 4.12) (Tuncer & Bizsel, 2014c). In addition to this expected close values between two sampling periods, they were all in the range of Aegean Sea values as mentioned in the next section.

Table 4.1 The intervals of the seawater parameters at the study area in addition to pH range (8.17 – 8.33 and 8.30 – 8.33 for April 2011 and March 2012, respectively) (Tuncer & Bizsel, 2014c). The first row is for April 2011 and the second is for March 2012.

Station	Temperature (°C)	Salinity (psu)	Density (kg/m ³)	DO (ml/l)	TSS (mg/l)	Chl α (μ g/l)	NO ₂ ⁻ (μ M)	NO ₃ ⁻ (μ M)	PO ₄ ⁻³ (μ M)	Reactive Si (μ M)
1	15.31 - 15.64	39.14 - 39.15	29.03 - 29.10	5.50 - 5.66	0.40 - 13.00	0.28 - 0.64	0.04 - 0.08	BDL - 0.05	0.04 - 0.33	0.69 - 1.61
	15.23 - 15.47	39.15 - 39.17	29.08 - 29.13	5.57 - 5.67	0.30 - 0.87	0.12 - 0.57	BDL - 0.28	BDL - 1.25	0.01 - 0.09	1.08 - 1.93
2	15.82 - 16.21	39.17 - 39.18	28.92 - 29.01	5.29 - 5.61	1.23 - 18.53	0.25 - 0.93	0.06 - 0.13	BDL - 0.09	BDL - 0.06	1.05 - 2.10
	15.05 - 15.48	39.14 - 39.17	29.08 - 29.18	5.56 - 5.74	0.40 - 1.40	0.07 - 2.33	BDL - 0.28	0.04 - 2.06	0.02 - 0.09	0.91 - 2.04
3	15.36 - 16.11	39.15 - 39.17	28.93 - 29.09	5.35 - 5.73	0.81 - 2.69	BDL - 0.43	0.02 - 0.13	BDL - 0.16	0.05 - 0.09	0.63 - 2.40
	15.10 - 15.45	39.14 - 39.17	29.09 - 29.16	5.47 - 5.81	0.50 - 1.60	0.18 - 1.25	BDL - 0.18	0.80 - 1.51	0.01 - 0.09	1.08 - 2.33
4	15.44 - 16.08	39.13 - 39.18	28.95 - 29.07	5.37 - 5.73	0.53 - 2.83	BDL - 0.90	0.06 - 0.10	0.00 - 0.02	0.02 - 0.16	0.99 - 2.53
	15.06 - 15.40	39.15 - 39.17	29.09 - 29.17	5.50 - 5.75	0.20 - 1.40	0.24 - 0.70	0.01 - 0.16	0.46 - 4.26	0.01 - 0.10	1.25 - 4.60
5	15.26 - 15.96	39.13 - 39.18	28.98 - 29.10	5.40 - 5.59	3.72 - 7.44	0.18 - 0.88	0.06 - 0.10	0.00 - 0.12	0.02 - 0.04	0.75 - 1.97
	15.10 - 15.35	39.15 - 39.17	29.11 - 29.16	5.51 - 5.72	0.40 - 0.73	0.22 - 0.45	BDL - 0.20	0.00 - 2.24	BDL - 0.03	1.13 - 2.84



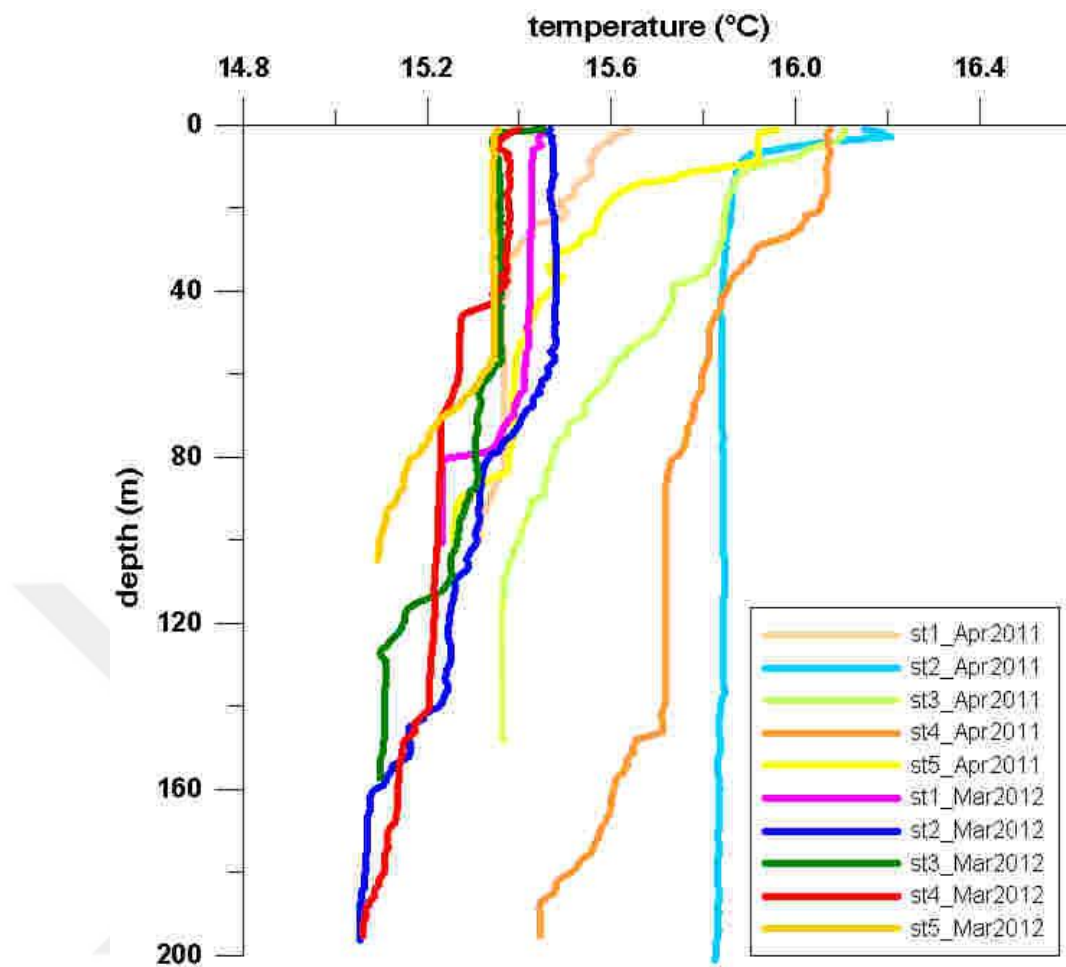


Figure 4.1 The comparison of seawater temperature in April 2011 and March 2012. (Continued).

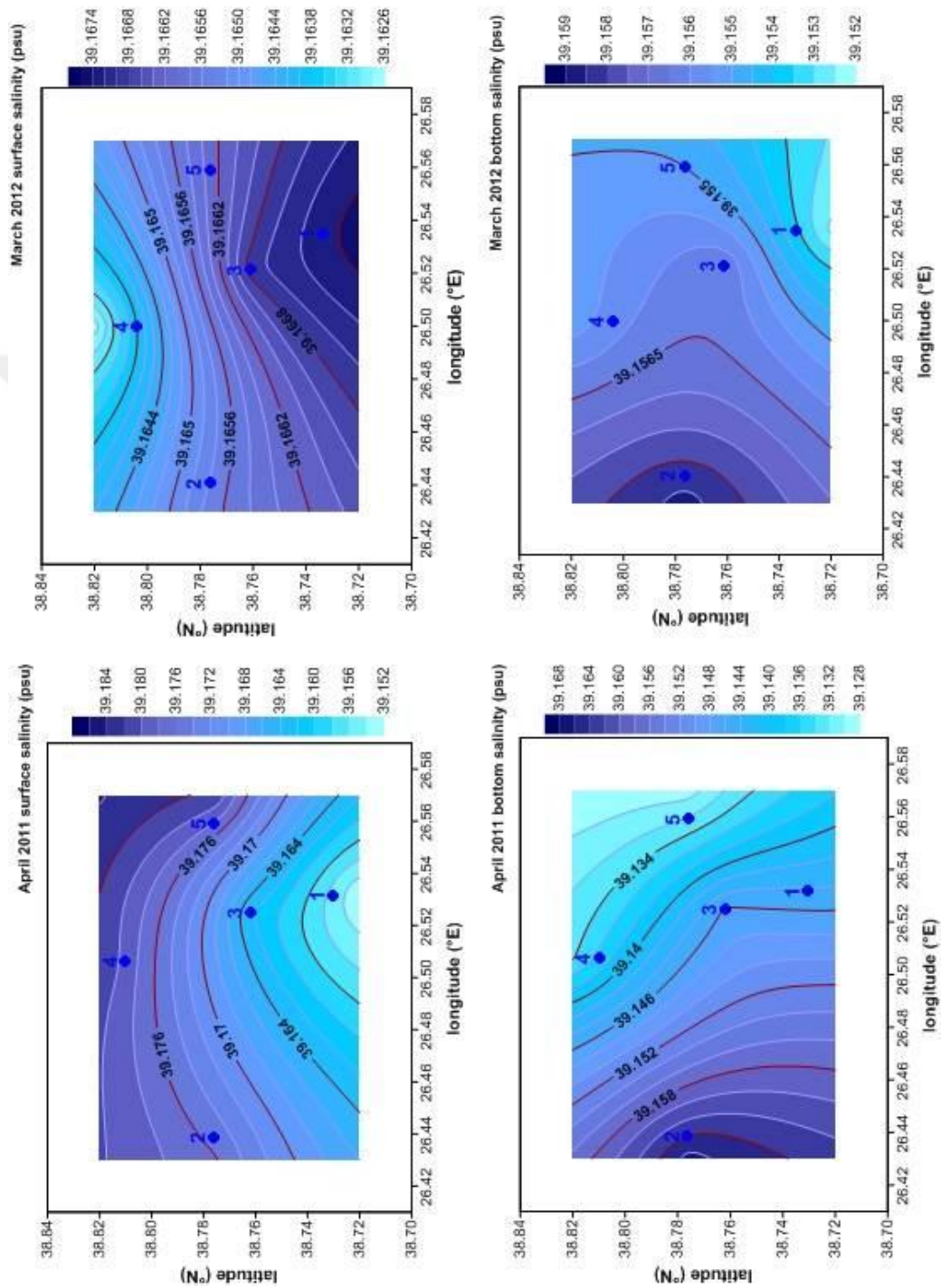


Figure 4.2 The comparison of seawater salinity in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

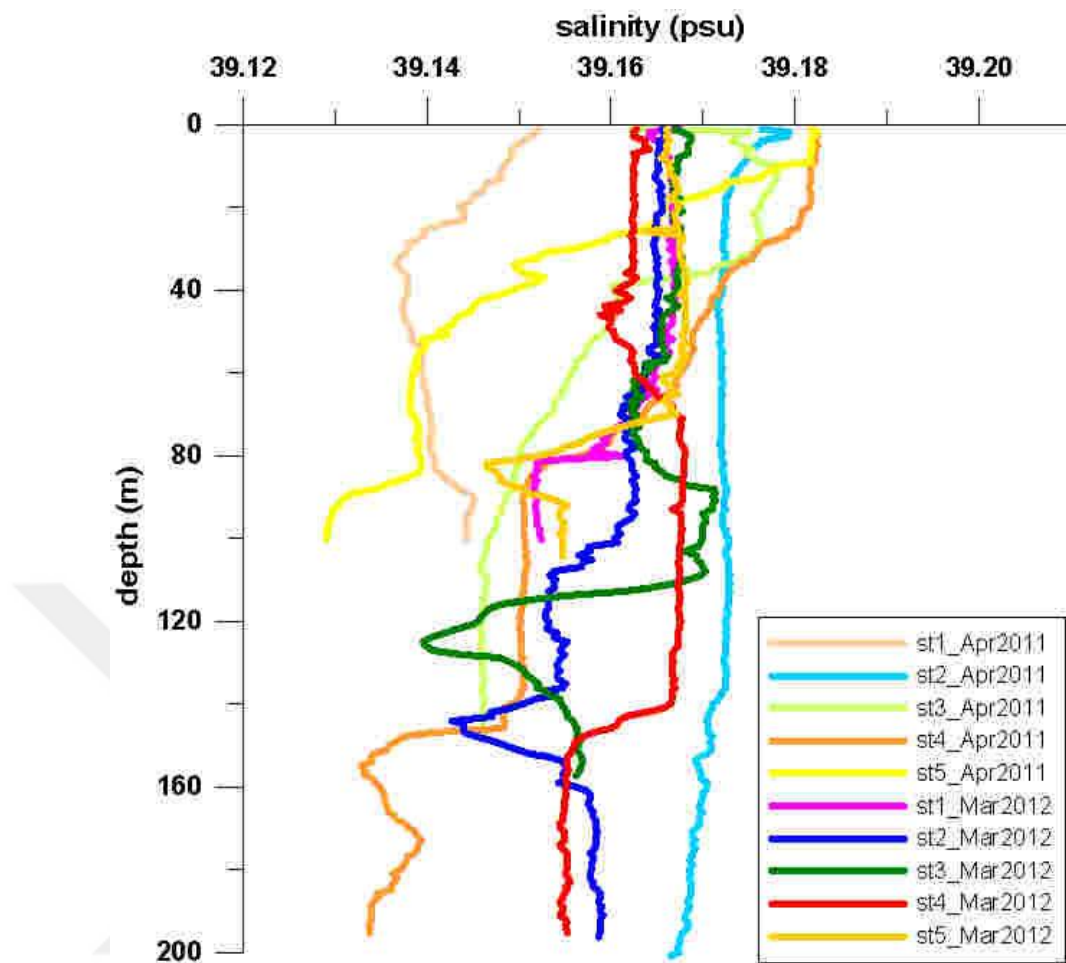


Figure 4.2 The comparison of seawater salinity in April 2011 and March 2012. (Continued).

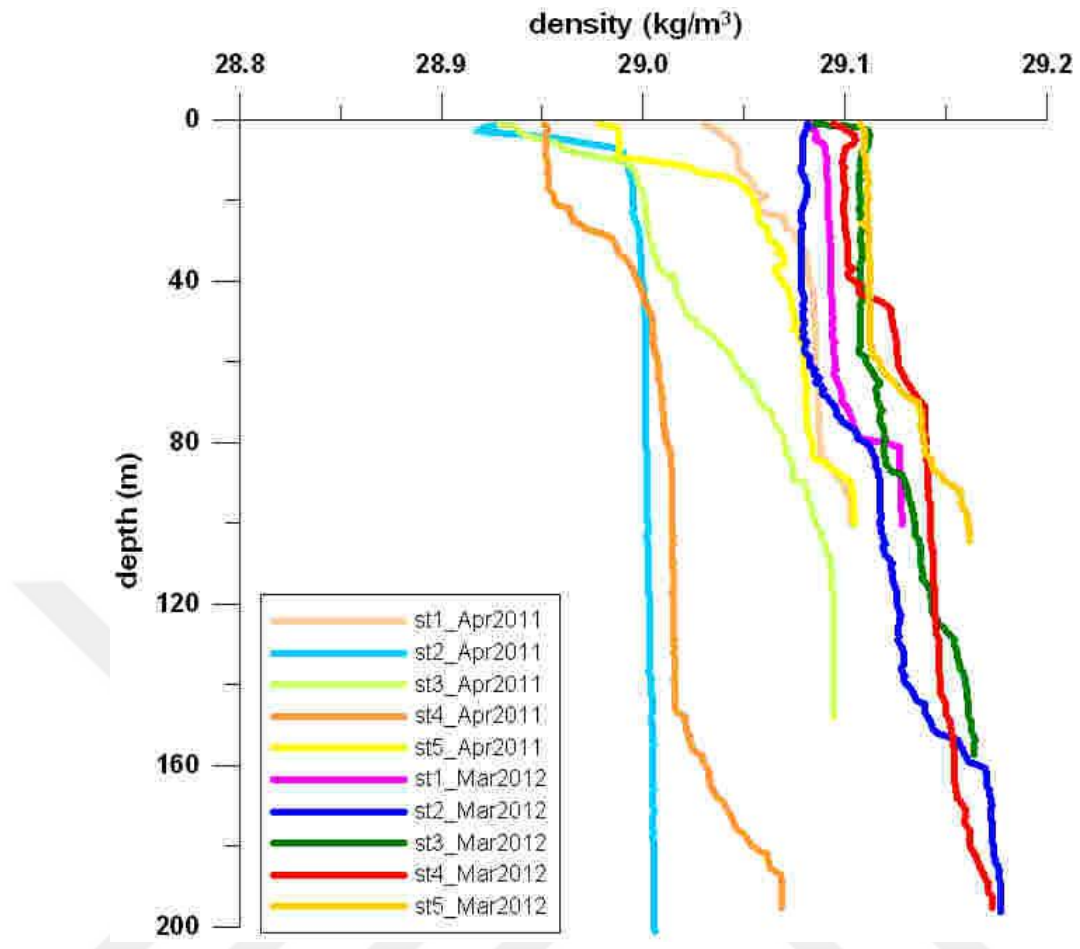


Figure 4.3 The comparison of seawater density in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

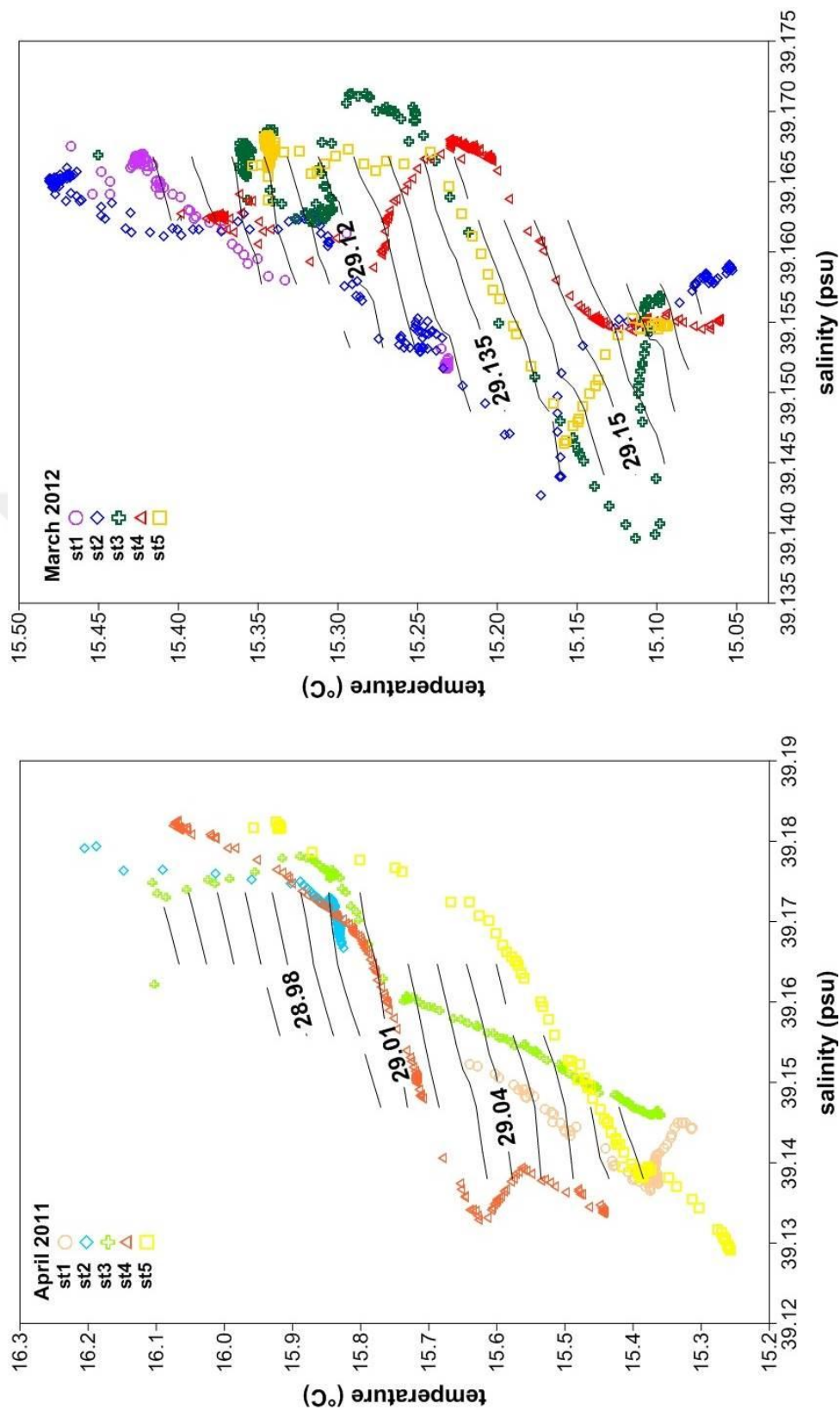


Figure 4.4 The comparison of seawater temperature, salinity and density in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

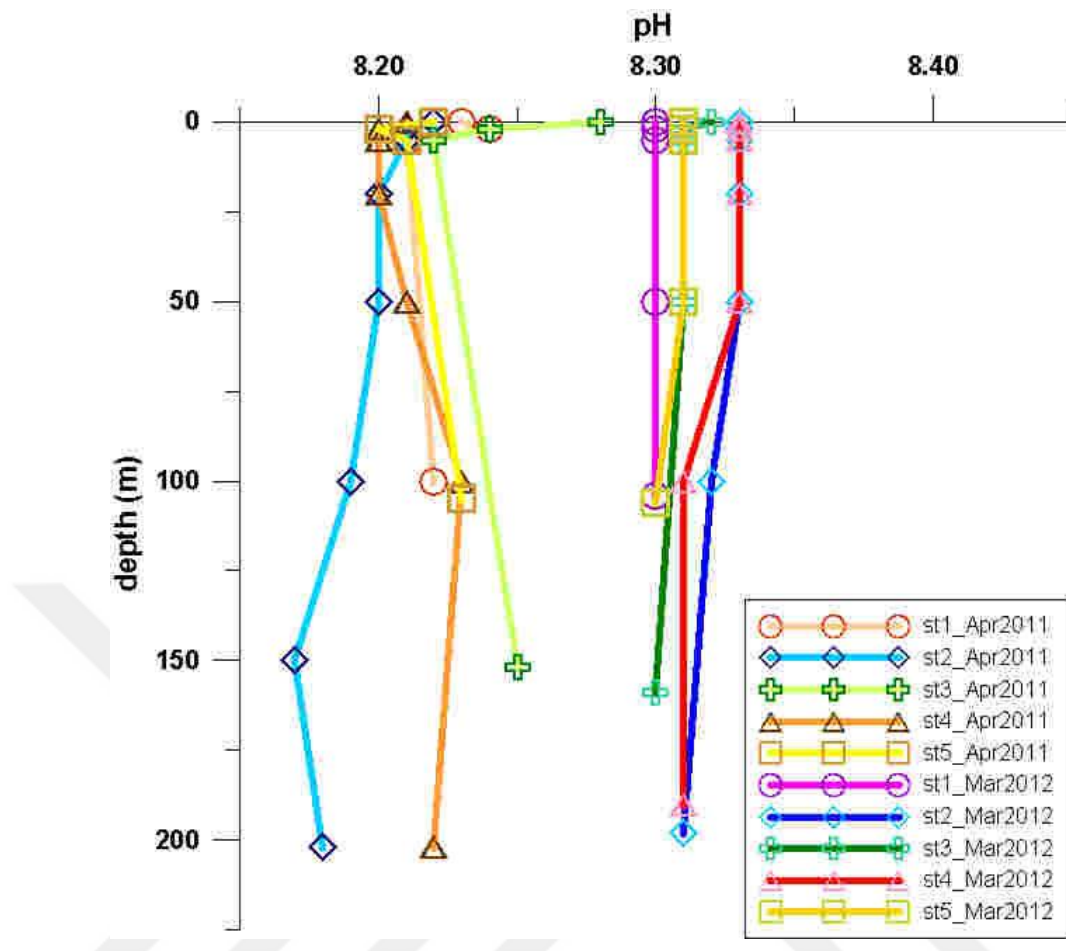


Figure 4.5 The comparison of seawater pH in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

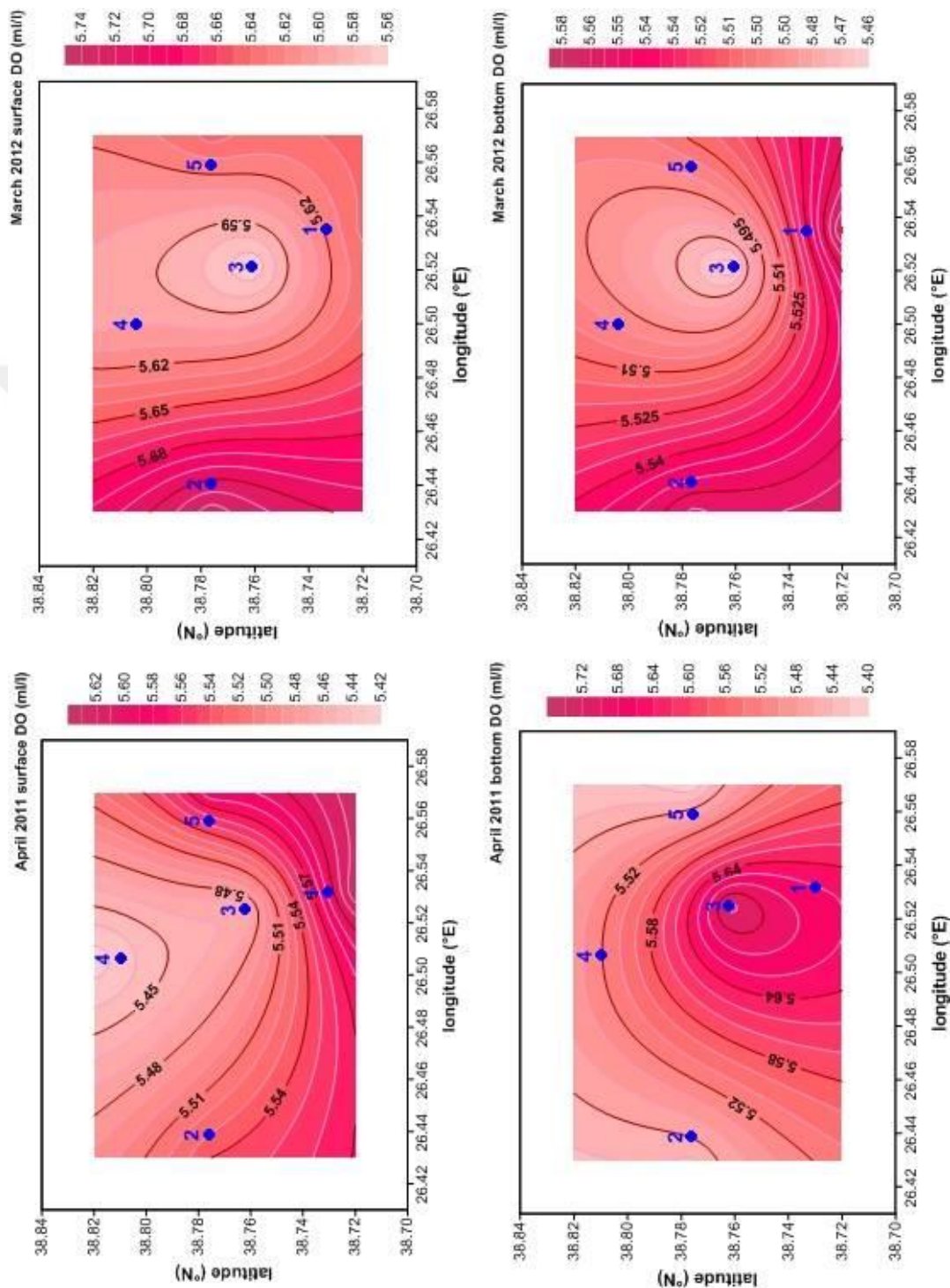


Figure 4.6 The comparison of seawater DO in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

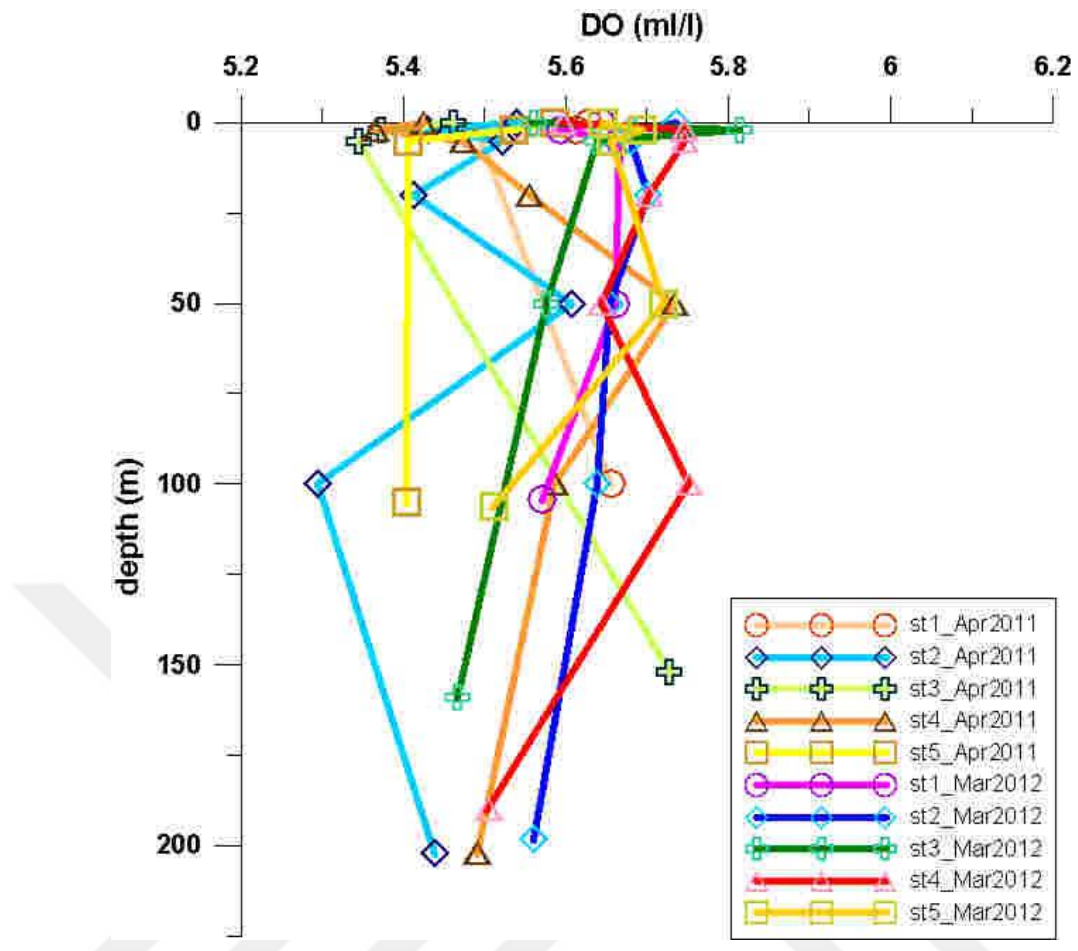


Figure 4.6 The comparison of seawater DO in April 2011 and March 2012. (Continued).

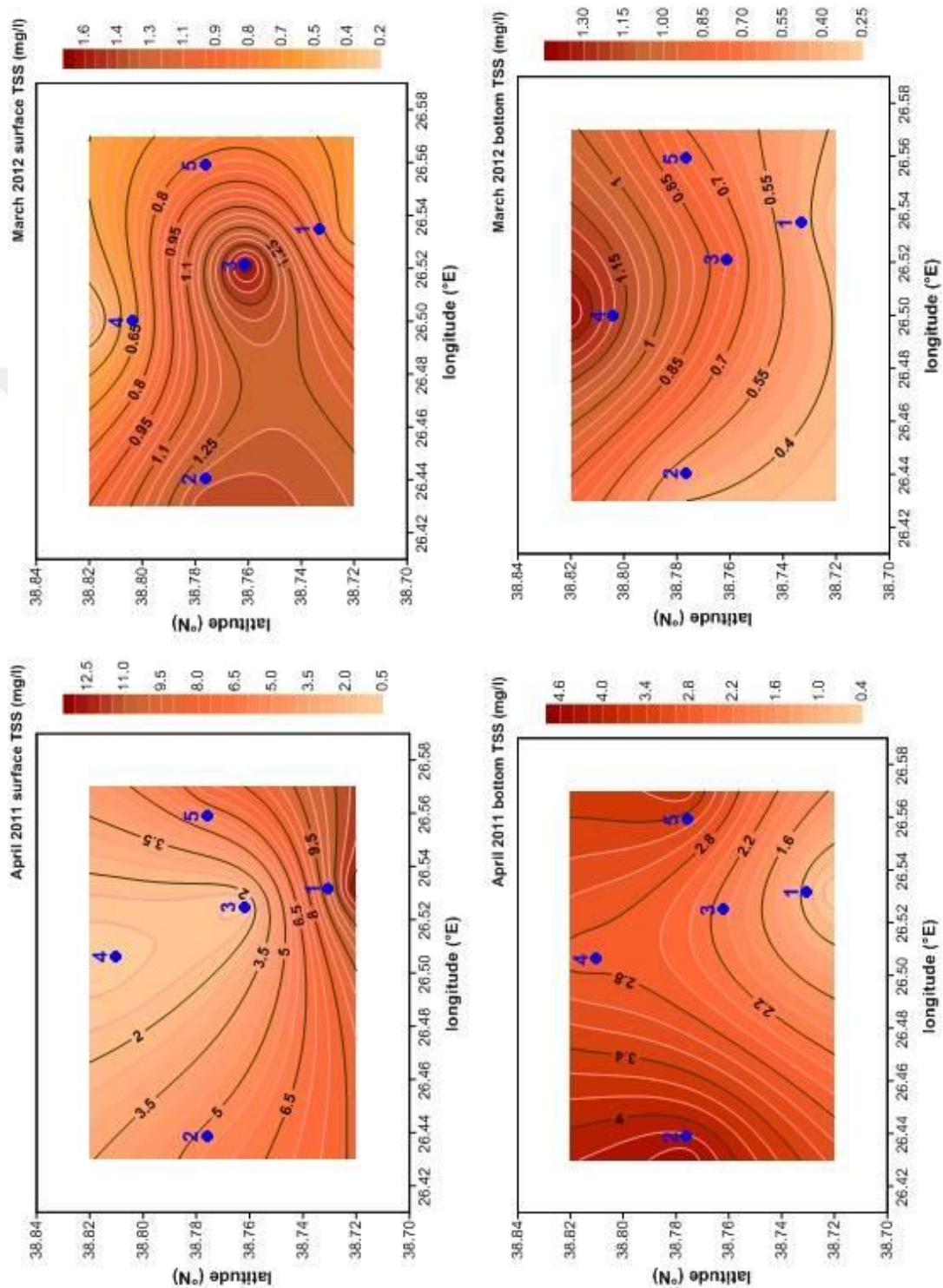


Figure 4.7 The comparison of seawater TSS in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

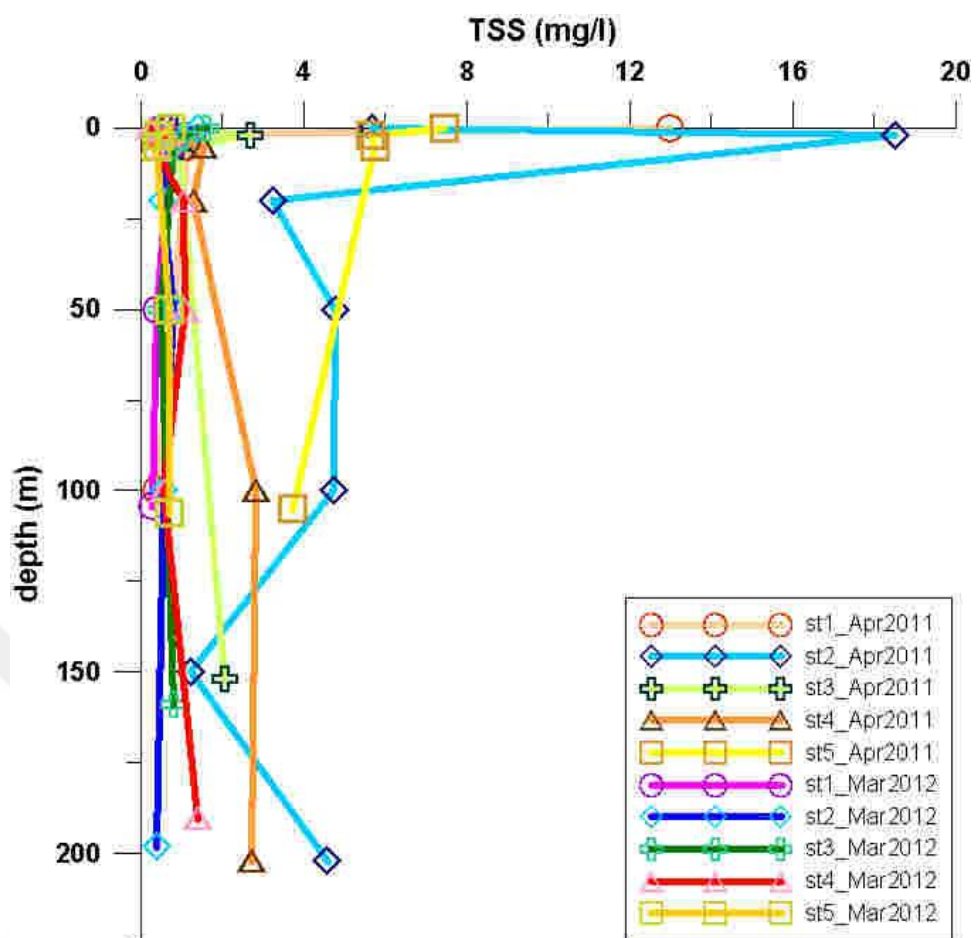


Figure 4.7 The comparison of seawater TSS in April 2011 and March 2012. (Continued).

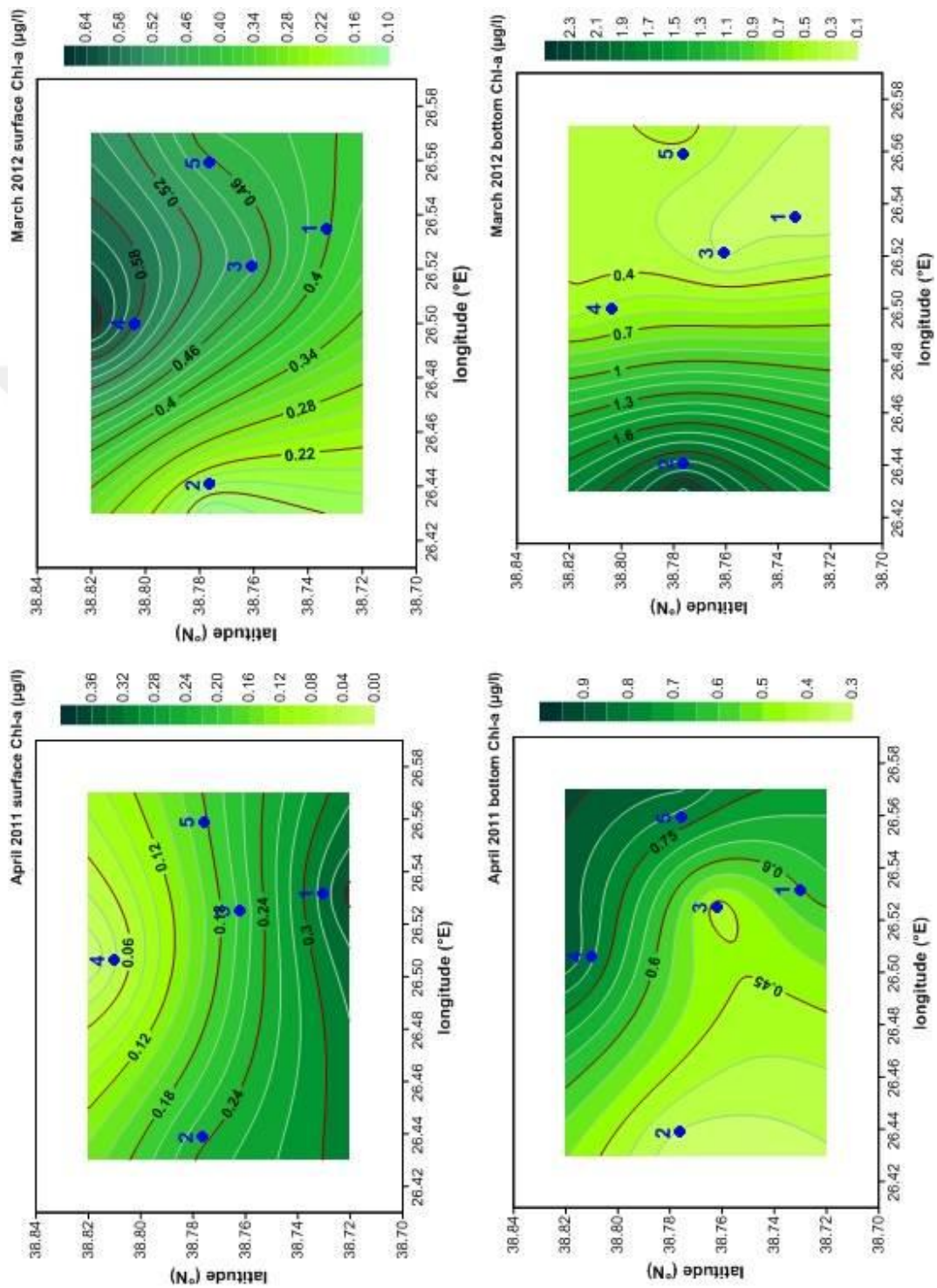


Figure 4.8 The comparison of seawater Chl α in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

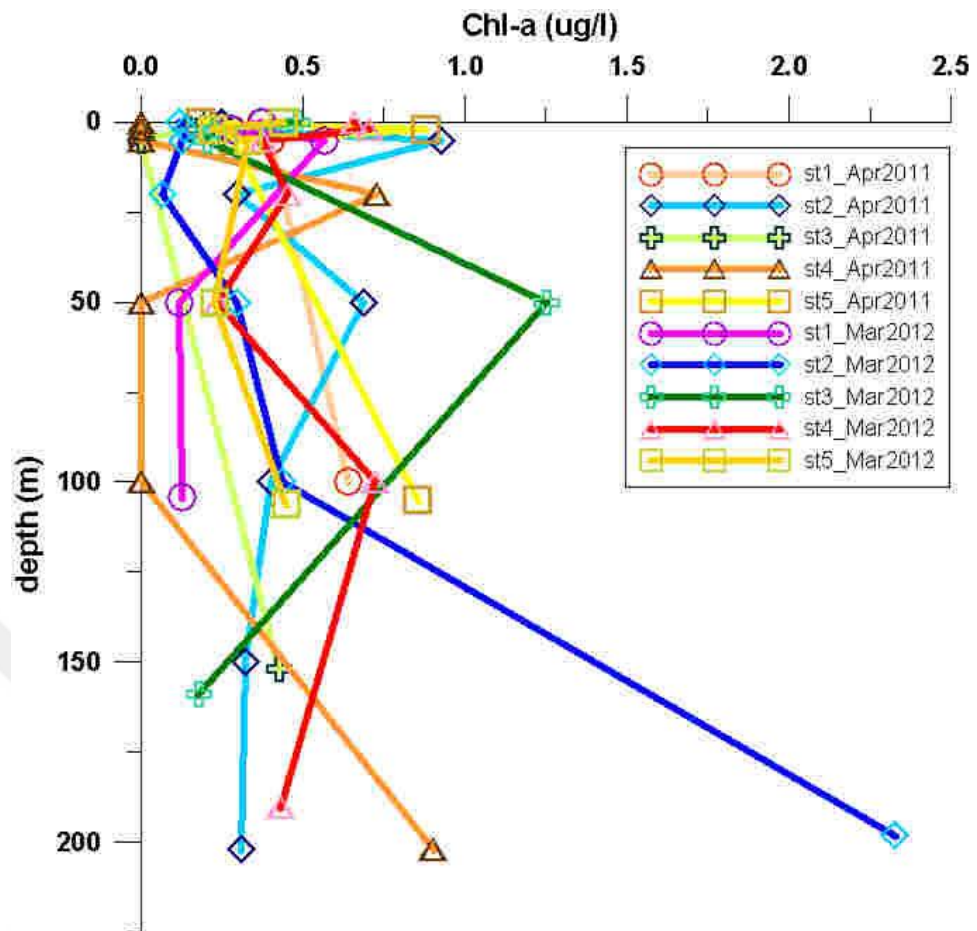


Figure 4.8 The comparison of seawater Chl α in April 2011 and March 2012. (Continued).

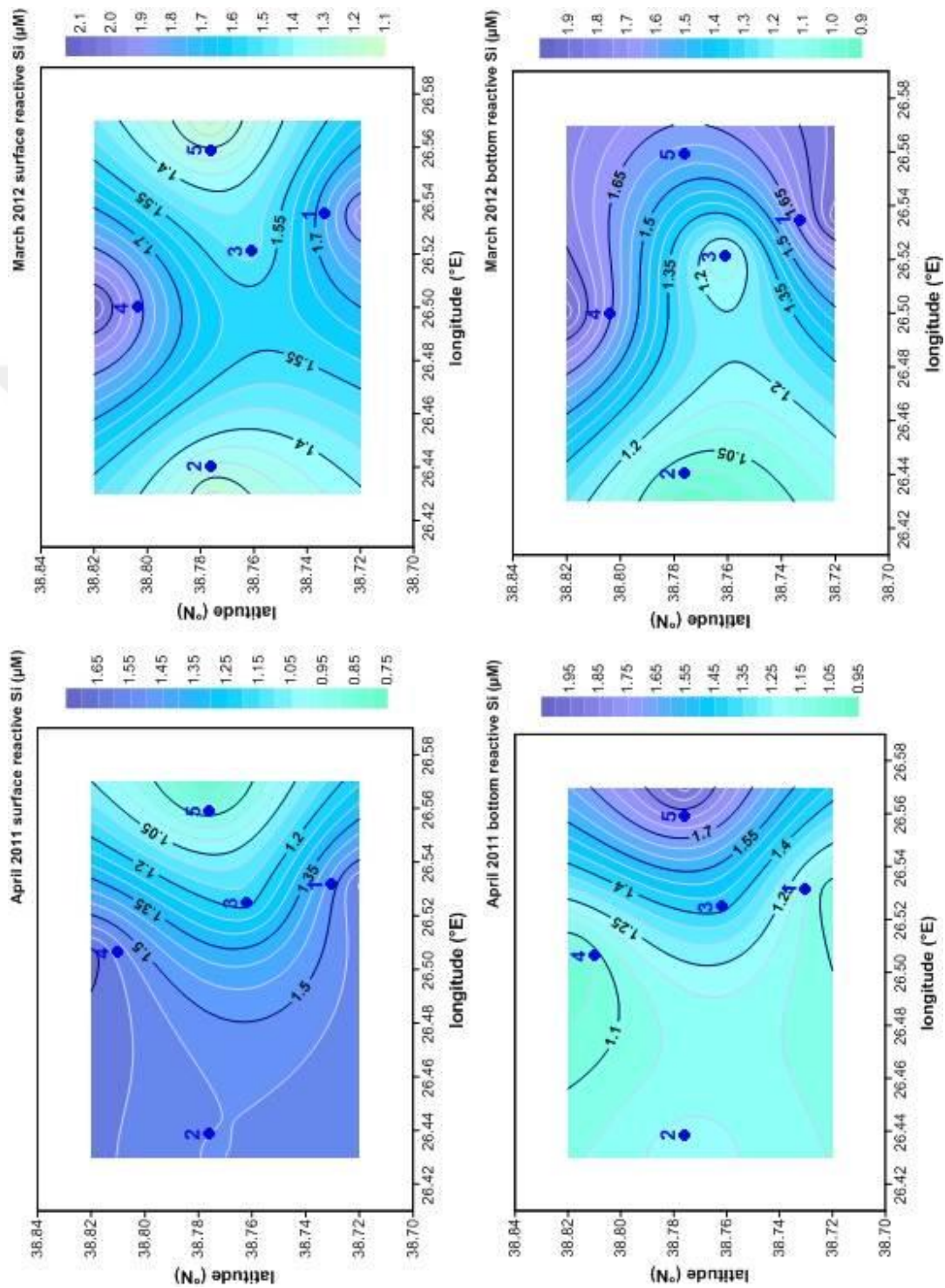


Figure 4.9 The comparison of seawater reactive Si in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

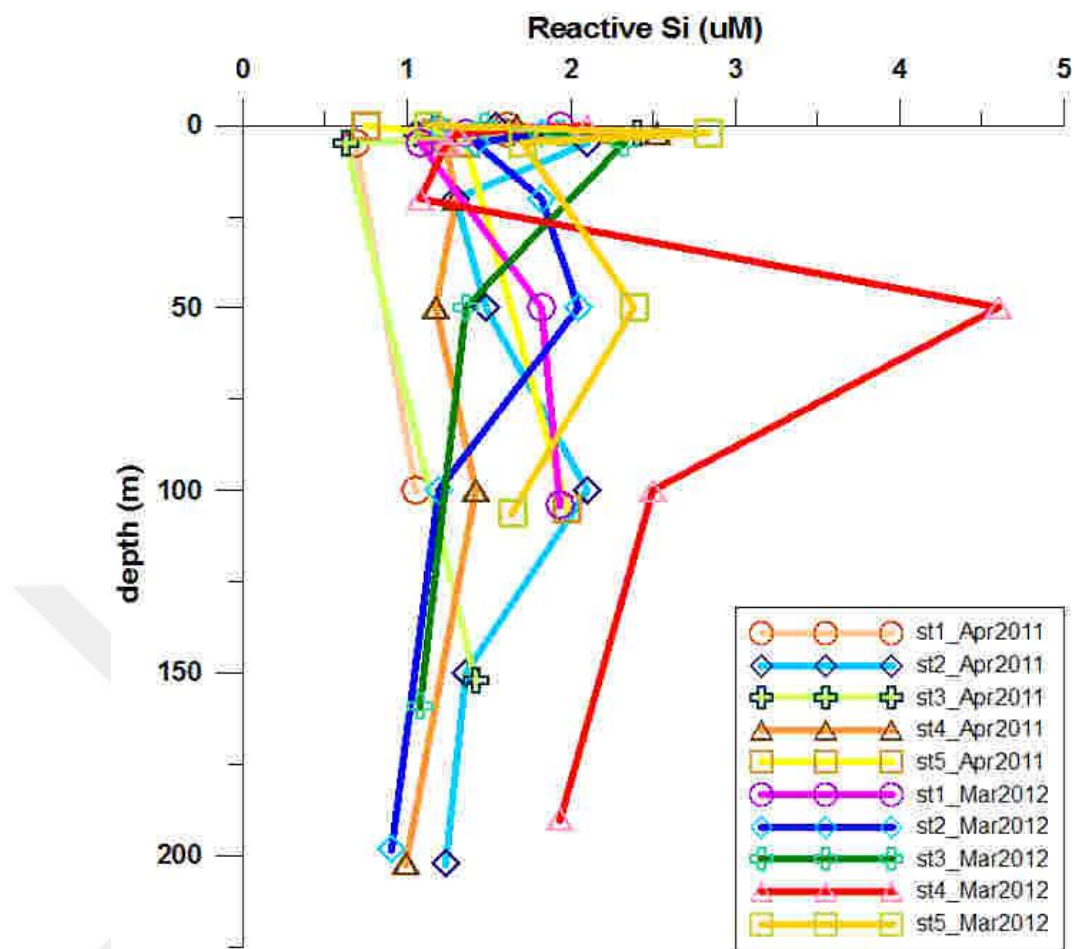


Figure 4.9 The comparison of seawater reactive Si in April 2011 and March 2012. (Continued).

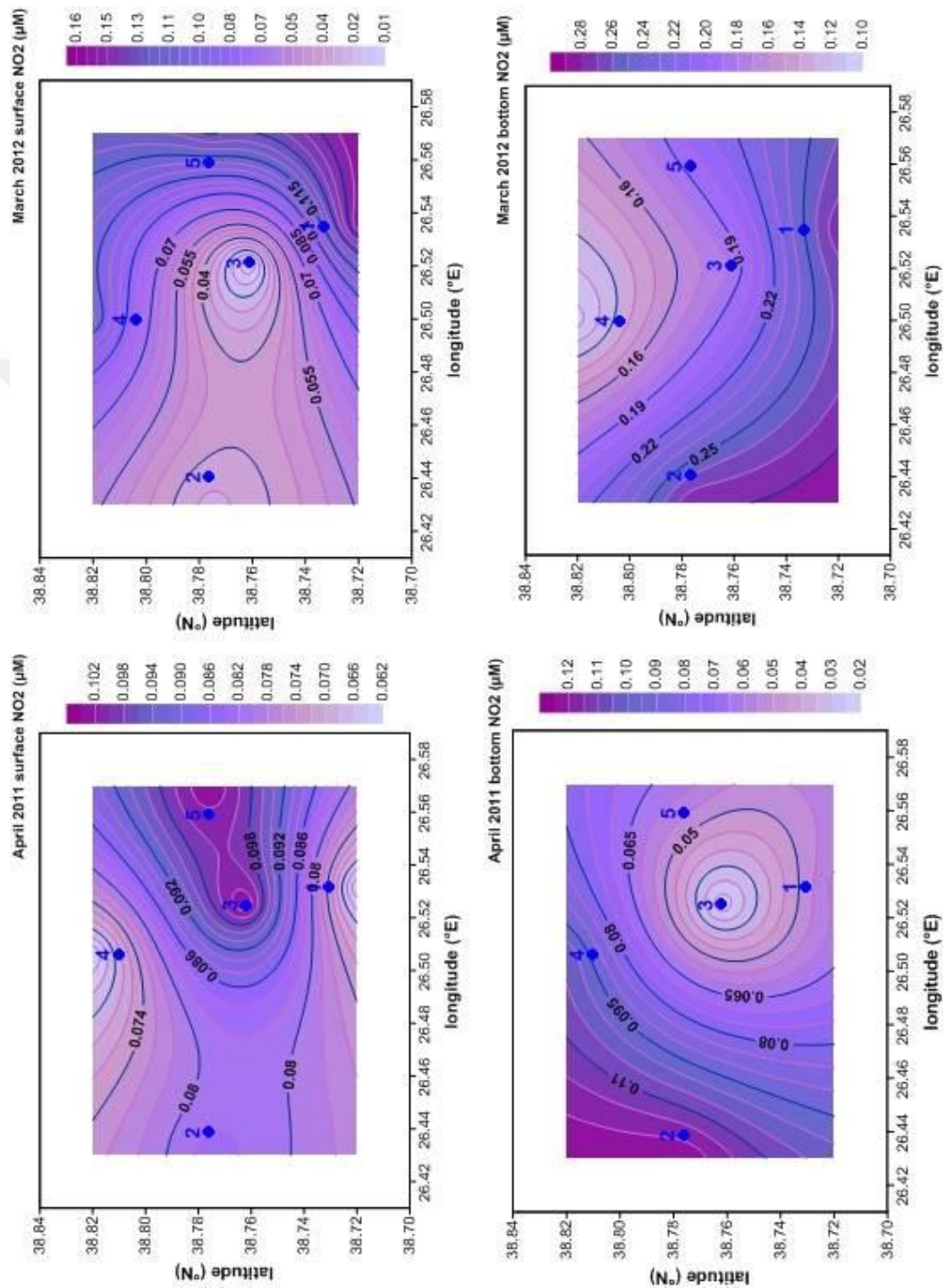


Figure 4.10 The comparison of seawater NO_2^- in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

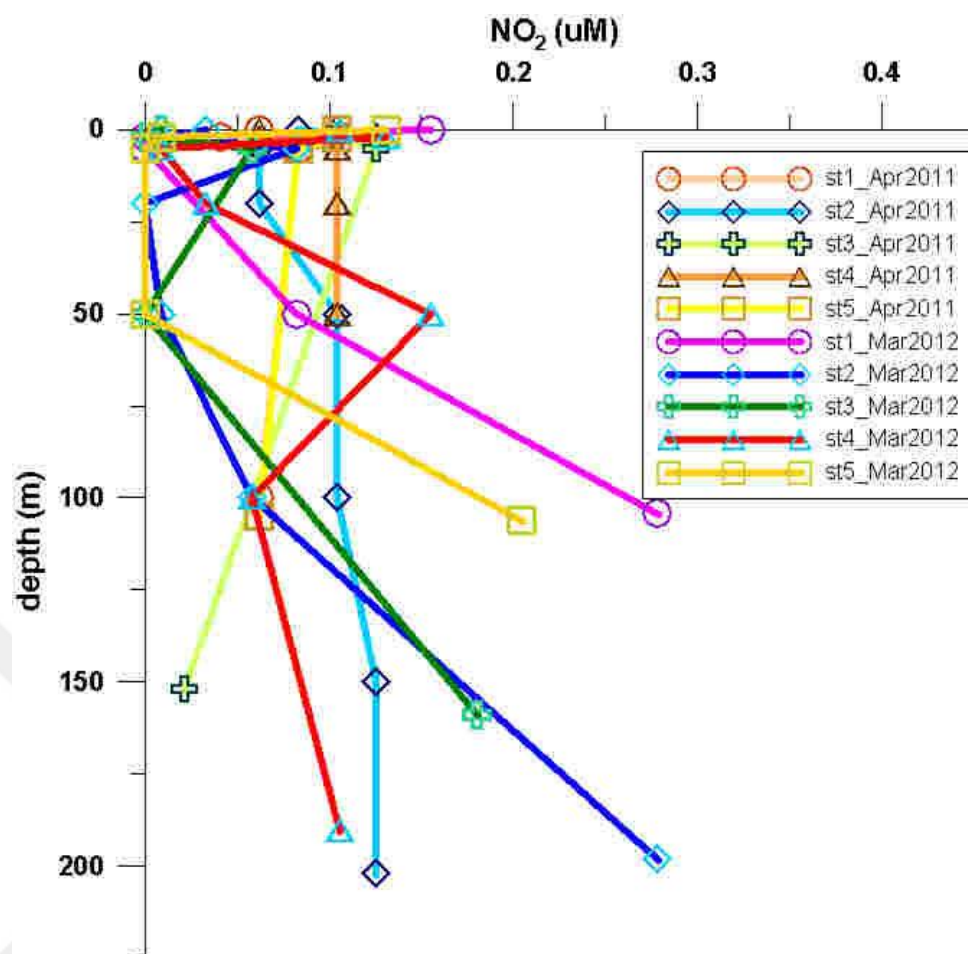


Figure 4.10 The comparison of seawater NO_2^- in April 2011 and March 2012. (Continued).

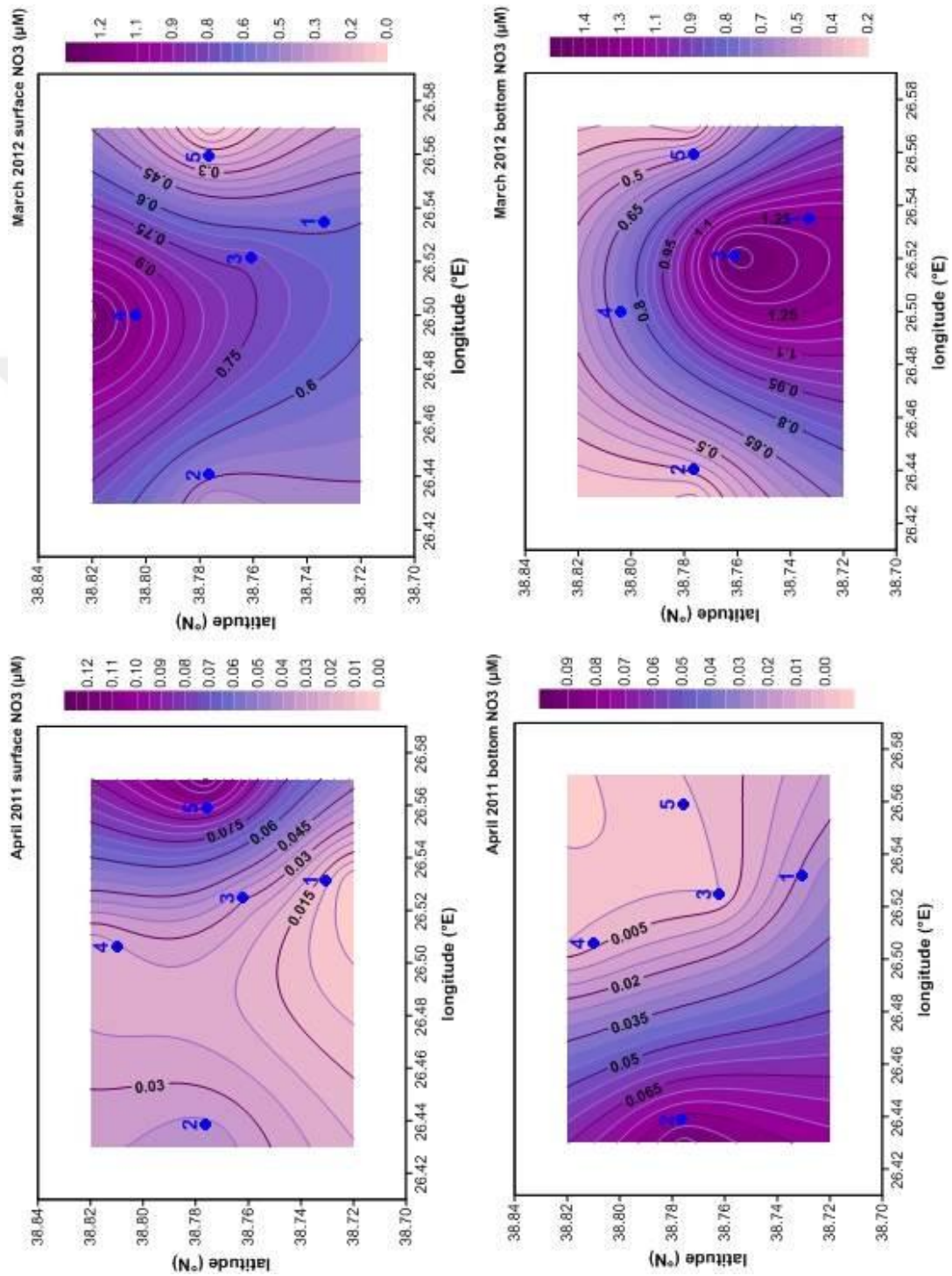


Figure 4.11 The comparison of seawater NO_3^- in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

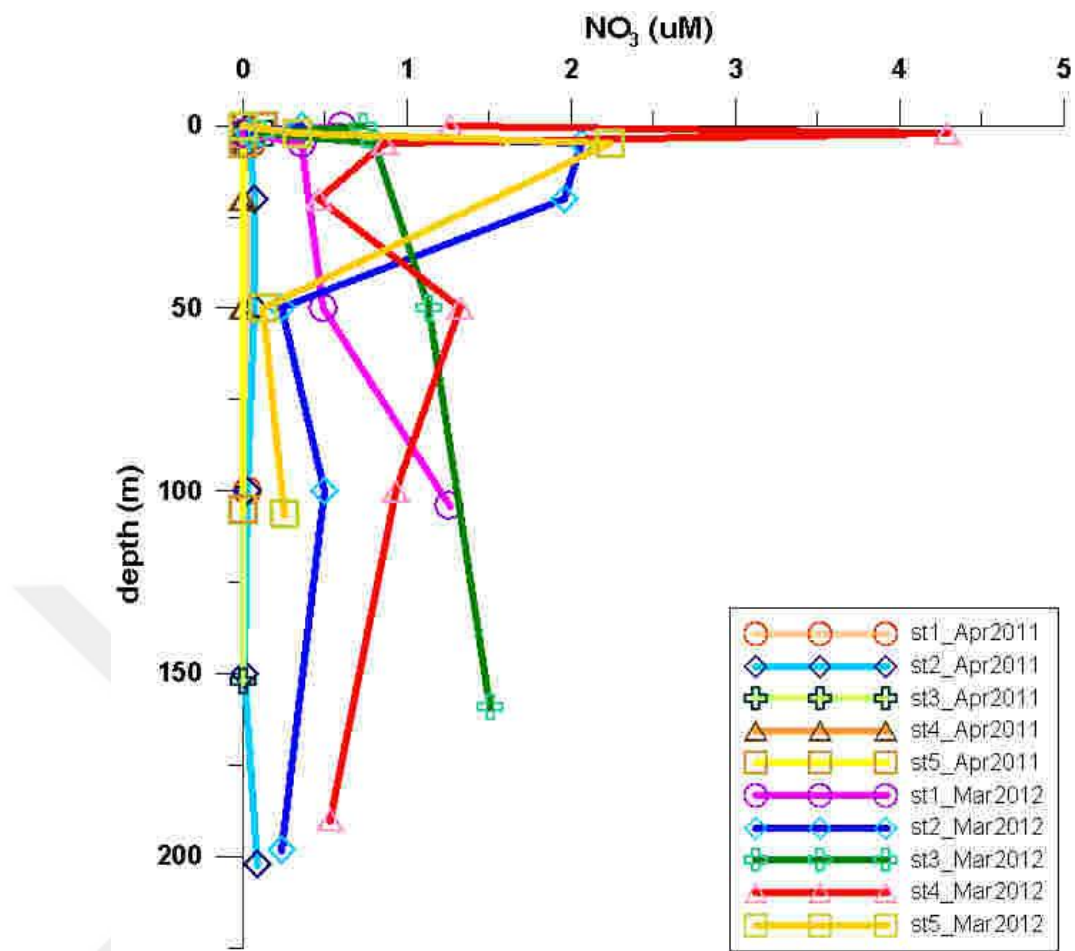


Figure 4.11 The comparison of seawater NO_3^- in April 2011 and March 2012. (Continued).

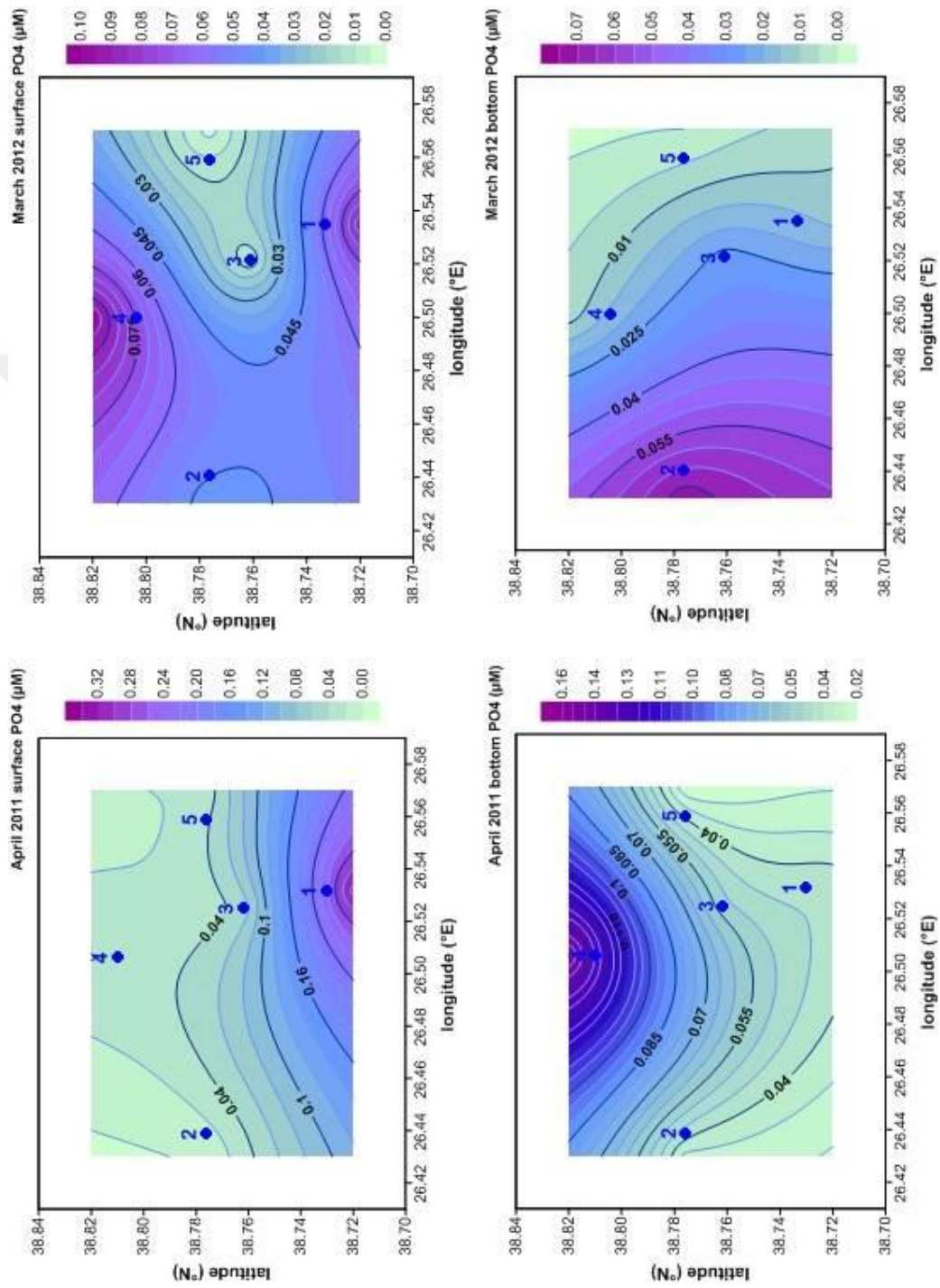


Figure 4.12 The comparison of seawater PO_4^{3-} in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

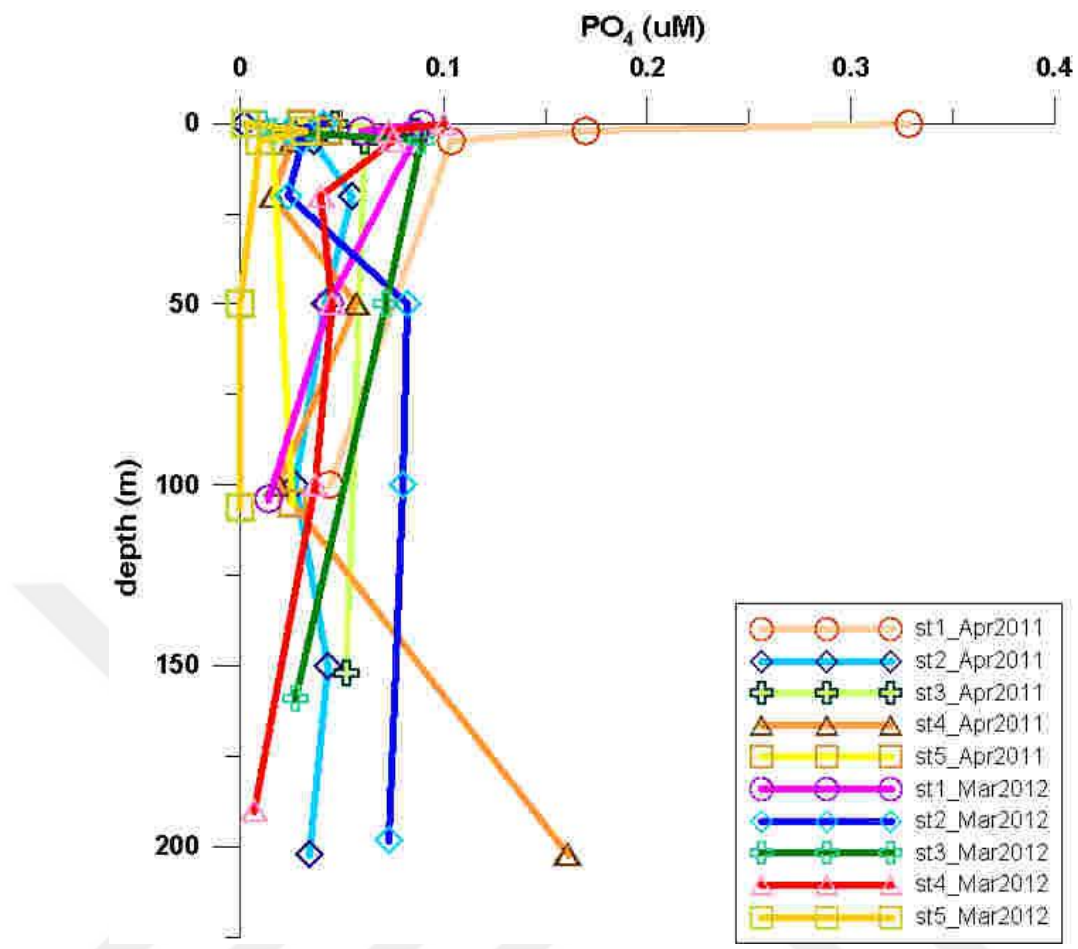


Figure 4.12 The comparison of seawater PO_4^{3-} in April 2011 and March 2012. (Continued).

4.1.1.2 Previous Studies in Aegean Sea

For spring 2011 and 2012 samplings at the study area (Tuncer & Bizsel, 2014c), as seen in Table 4.2, temperature, salinity and pH values measured as 15.05 – 16.21 °C, 39.13 – 39.18 psu, 8.17 – 8.33 were all in between the minima and maxima as 11.9 – 26.3 °C, 30.7 – 39.9 psu and 7.00 – 8.75 pH, respectively for the period of 1994 – 2009 in Aegean Sea water column including coastal areas (Ignatiades, 1998; Gotsis-Skretas et al., 1999; Tsikliras et al., 2001; Ignatiades et al., 2002; Zervoudaki et al., 2006; Meador et al., 2010; Aydin-Onen et al., 2012). Similarly, dissolved oxygen values as 5.29 – 5.81 ml/l were in the range of 2.29 – 7.00 ml/l for Aegean Sea from 1987 to 2012 (Souvermezoglou et al., 2000; Pavlidou et al., 2010; Aydin-Onen et al., 2012; IMST, 1996 – 2012) and, except the value 2.33 µg/l obtained at the bottom of the station 2 in March 2012, chlorophyll α values were in the range of 0.01 – 1.35 µg/l in 1992 – 2000 for Aegean Sea (Kucuksezgin et al., 1995; Ignatiades et al., 1995; Ignatiades, 1998; Tsikliras et al., 2001; Ignatiades et al., 2002; Zervoudaki et al., 2006).

For the nutrient concentrations in this study area (Tuncer & Bizsel, 2014c), as seen in Table 4.3, nitrite+nitrate (0.021 – 4.566 µM) were much closer to the range (0.010 – 4.355 µM) in North Aegean Sea (Kucuksezgin et al., 1995; Friligos & Krasakopoulou, 2001; Tsikliras et al., 2001; Ignatiades et al., 2002) rather than the range (0.395 – 0.881 µM) in South Aegean Sea (Ignatiades et al., 2002). Phosphate maxima (0.328 µM) obtained at the station 1 in April 2011, on the other hand, approximately doubled the values of both North Aegean Sea (0.002 – 0.170 µM) (Kucuksezgin et al., 1995; Friligos & Krasakopoulou, 2001; Tsikliras et al., 2001; Ignatiades et al., 2002; Pavlidou et al., 2010) and South Aegean Sea (0.010 – 0.140 µM) (Kucuksezgin et al., 1995; Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002). However, reactive Si concentrations (0.625 – 4.600 µM) were closer to both North Aegean Sea (0.170 – 5.657 µM) (Kucuksezgin et al., 1995, Friligos & Krasakopoulou, 2001; Tsikliras et al., 2001; Ignatiades et al., 2002; Pavlidou et al., 2010) and South Aegean Sea (0.300 – 4.700 µM) (Kucuksezgin et al., 1995; Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002).

Table 4.2 Comparison of the seawater ranges, except nutrients, at the study area with the previous results of Aegean Sea.

Variable	Values	Sampling area	Sampling period	Source
Temperature (°C)	14.3 - 22.5	South Aegean Sea	1994 - 1998	Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002; Meador et al., 2010
	12.1 - 22.6	North Aegean Sea	1997 - 2000	Ignatiades et al., 2002; Zervoudaki et al., 2006; Meador et al., 2010
	20.4 - 26.3	North Aegean Sea (coastal area)	1998	Tsikliras et al., 2001
	16.8 - 25.2	Kusadasi Bay	2008 - 2009	Aydin-Onen et al., 2012
	16.7 - 25.1	Candarli Bay	2008 - 2009	Aydin-Onen et al., 2012
	11.9 - 30.0	Izmir Bay	2008 - 2009	Aydin-Onen et al., 2012
	15.1 - 16.2	North Aegean Sea	2011 - 2012	Tuncer & Birzel, 2014c
Salinity (psu)	38.8 - 39.4	South Aegean Sea	1994 - 1998	Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002; Meador et al., 2010
	31.1 - 39.0	North Aegean Sea	1997 - 2000	Ignatiades et al., 2002; Zervoudaki et al., 2006; Meador et al., 2010
	30.7 - 37.6	North Aegean Sea (coastal area)	1998	Tsikliras et al., 2001
	38.1 - 39.8	Candarli Bay	2008 - 2009	Aydin-Onen et al., 2012
	36.0 - 39.9	Izmir Bay	2008 - 2009	Aydin-Onen et al., 2012
	38.1 - 39.6	Kusadasi Bay	2008 - 2009	Aydin-Onen et al., 2012
	39.1 - 39.2	North Aegean Sea	2011 - 2012	Tuncer & Birzel, 2014c
pH	8.09 - 8.39	Kusadasi Bay	2008 - 2009	Aydin-Onen et al., 2012
	8.19 - 8.47	Candarli Bay	2008 - 2009	Aydin-Onen et al., 2012
	7.00 - 8.75	Izmir Bay	2008 - 2009	Aydin-Onen et al., 2012
	8.17 - 8.33	North Aegean Sea	2011 - 2012	Tuncer & Birzel, 2014c

Table 4.2 Comparison of the seawater ranges, except nutrients, at the study area with the previous results of Aegean Sea. (Continued).

Variable	Values	Sampling area	Sampling period	Source
Dissolved oxygen (mL/l)	4.200 - 6.000	South Aegean Sea	1987 - 1997	Souvermeroglou et al., 2000
	2.505 - 2.894	North Aegean Sea	2008	Pavlidou et al., 2010
	4.786 - 6.143	Kusadasi Bay	2008 - 2009	Aydin-Onen et al., 2012
	4.643 - 5.643	Candarli Bay	2008 - 2009	Aydin-Onen et al., 2012
	2.286 - 7.000	Izmir Bay	2008 - 2009	Aydin-Onen et al., 2012
	3.420 - 6.000	Izmir Bay (outer bay)	1996 - 2012	IMST 1998 - 2012
	5.295 - 5.813	North Aegean Sea	2011 - 2012	Tuncer & Birsel, 2014c
Chl a (µg/l)	0.030 - 1.160	South Aegean Sea	1994 - 1998	Ignatiades et al., 1995; Ignatiades, 1998; Ignatiades et al., 2002
	0.030 - 0.700	Aegean Sea	1992 - 1994	Kucuksegin et al., 1995
	0.060 - 1.350	North Aegean Sea	1997 - 2000	Ignatiades et al., 2002; Zervoudaki et al., 2006
	0.010 - 0.550	North Aegean Sea (coastal area)	1998	Tsikliras et al., 2001
	0.010 - 11.000	Izmir Bay (outer bay)	1996 - 2012	IMST 1998 - 2012
	BDL - 2.326	North Aegean Sea	2011 - 2012	Tuncer & Birsel, 2014c

Table 4.2 Comparison of the seawater nutrient ranges at the study area with the previous results of Aegean Sea.

Variable	Values	Sampling area	Sampling period	Source
Nitrite + Nitrate (μM)	0.395 - 0.881	South Aegean Sea	1997 - 1998	Ignatiades et al., 2002
	0.036 - 4.355	North Aegean Sea	1992 - 1998	Kucuksezgin et al., 1995; Fnilgos & Krasakopoulou, 2001; Ignatiades et al., 2002
	0.010 - 0.420	North Aegean Sea (coastal area)	1998	Tsikiras et al., 2001
	0.100 - 8.300	Izmir Bay (outer bay)	1996 - 2012	IMST 1998 - 2012
Phosphate (μM)	0.021 - 4.566	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
	0.010 - 0.140	South Aegean Sea	1992 - 1998	Kucuksezgin et al., 1995; Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002
	0.019 - 0.032	North Aegean Sea	1992 - 1998	Kucuksezgin et al., 1995; Fnilgos & Krasakopoulou, 2001; Ignatiades et al., 2002
	0.002 - 0.077	North Aegean Sea	2008	Pavlidou et al., 2010
Reactive Si (μM)	0.010 - 0.170	North Aegean Sea (coastal area)	1998	Tsikiras et al., 2001
	0.010 - 11.000	Izmir Bay (outer bay)	1996 - 2012	IMST 1998 - 2012
	BDL - 0.328	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
	0.300 - 4.700	South Aegean Sea	1992 - 1998	Kucuksezgin et al., 1995; Ignatiades, 1998; Gotsis-Skretas et al., 1999; Ignatiades et al., 2002
	0.300 - 5.657	North Aegean Sea	1992 - 1998	Kucuksezgin et al., 1995; Fnilgos & Krasakopoulou, 2001; Ignatiades et al., 2002
	1.200 - 2.210	North Aegean Sea	2008	Pavlidou et al., 2010
	0.170 - 4.850	North Aegean Sea (coastal area)	1998	Tsikiras et al., 2001
	0.300 - 178.000	Izmir Bay (outer bay)	1996 - 2012	IMST 1998 - 2012
	0.625 - 4.600	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c

4.1.1.3 Present Studies in Aegean and Medierranean Seas

For 31 March 2012 sampling at the study area, satellite images of sea surface temperature and salinity in Aegean Sea (Figure 4.13 – 4.14) and surface chlorophyll α , nitrate and phosphate in Mediterranean Sea (Figure 4.15 – 4.17) indicated that they all covered the results of this study.

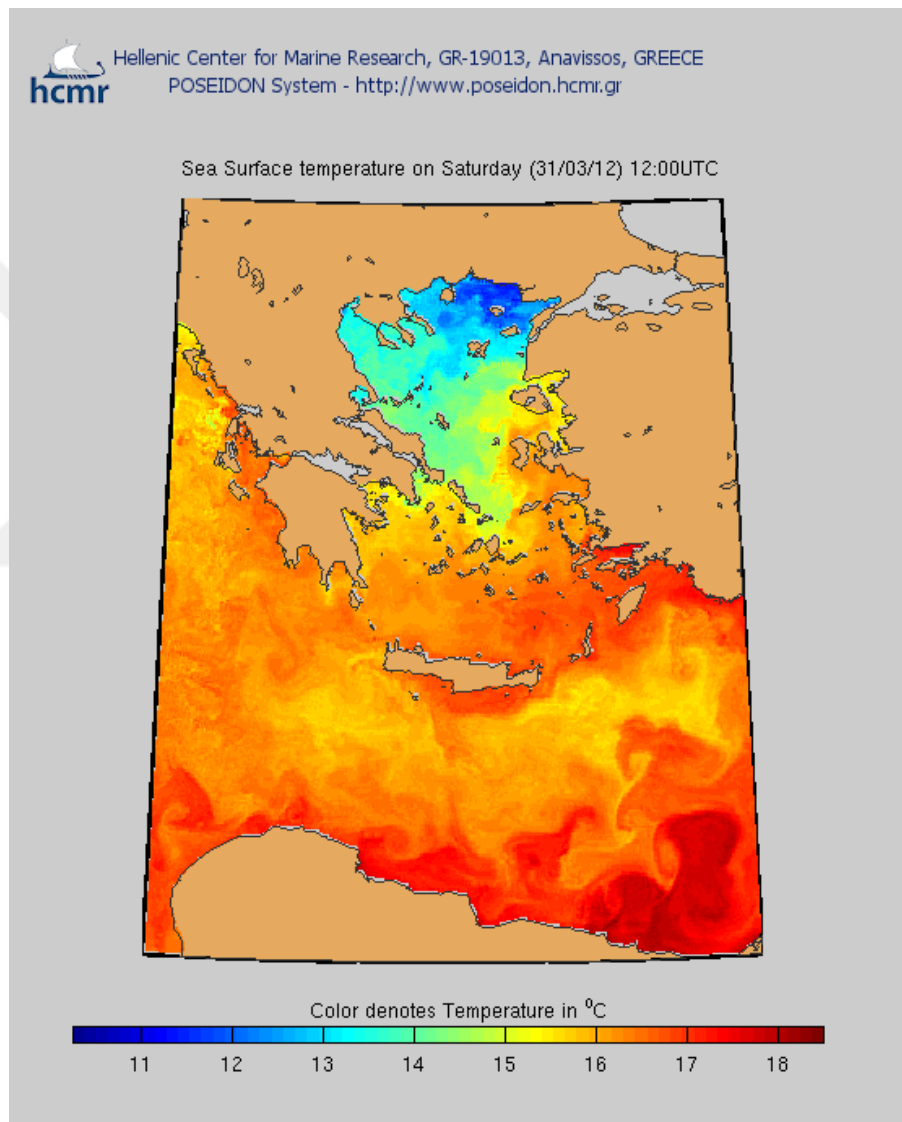


Figure 4.13 Sea surface temperature of Aegean Sea in 31 March 2012.

Sea Surface Salinity on Saturday (31/03/12) 12:00UTC

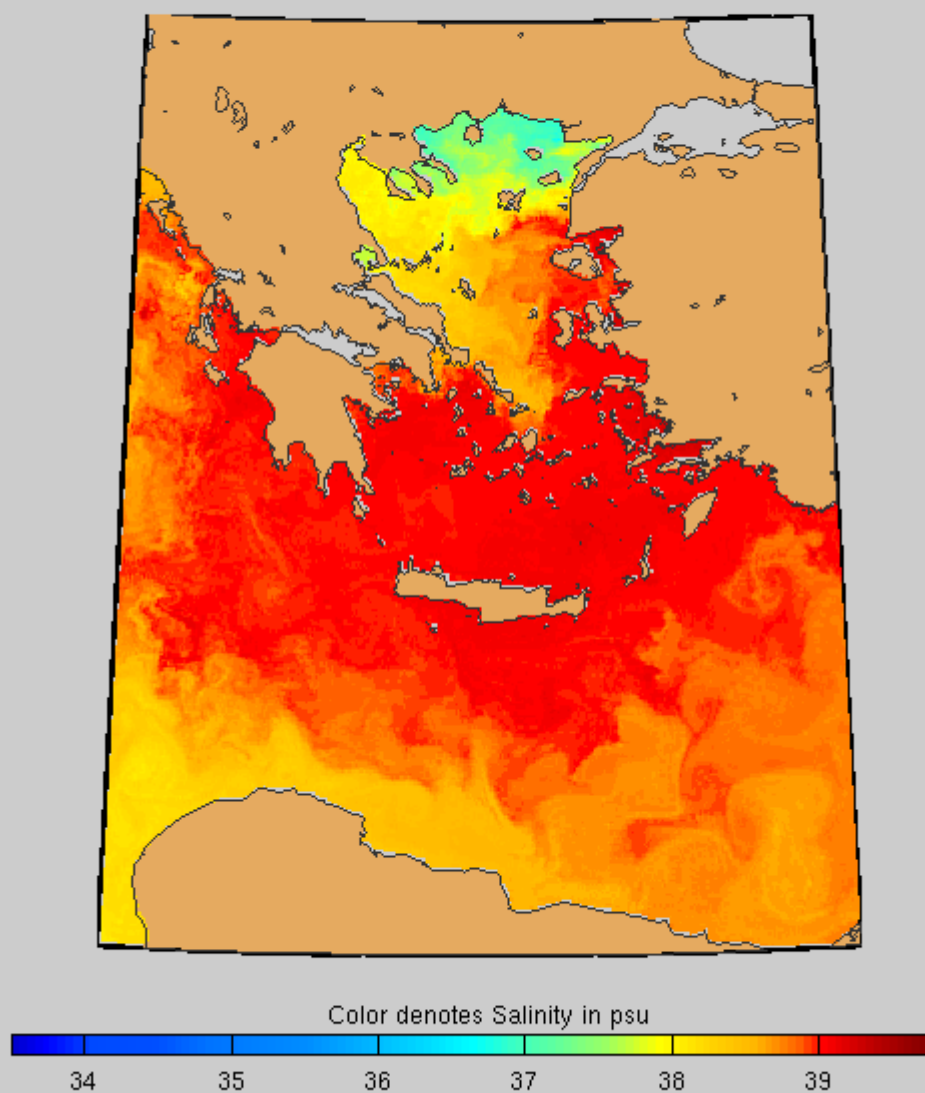


Figure 4.14 Sea surface salinity of Aegean Sea in 31 March 2012.

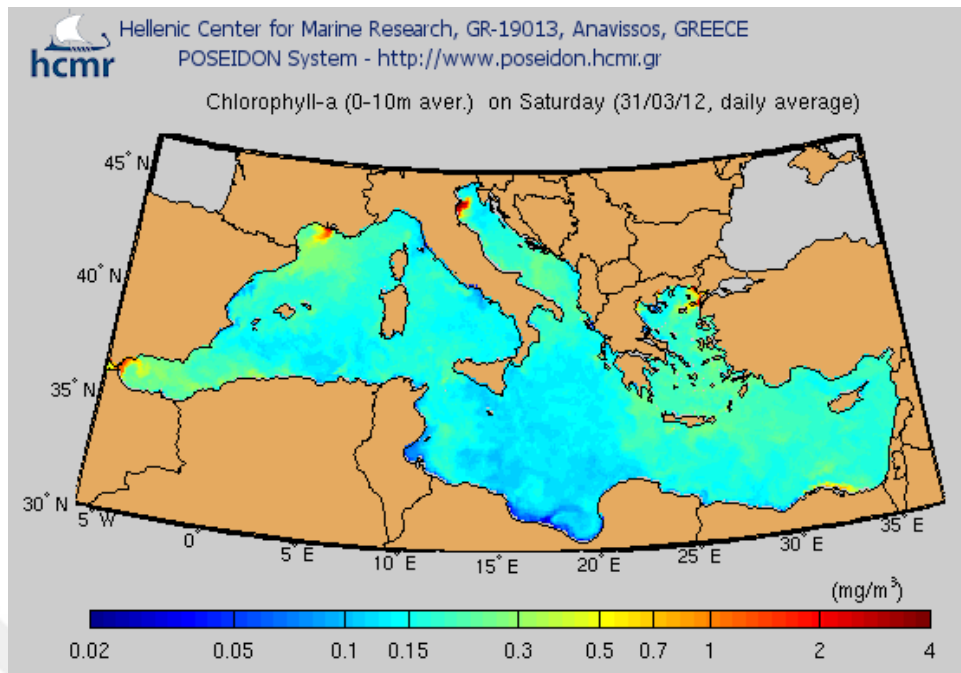


Figure 4.15 Sea surface chlorophyll α of Mediterranean Sea in 31 March 2012.

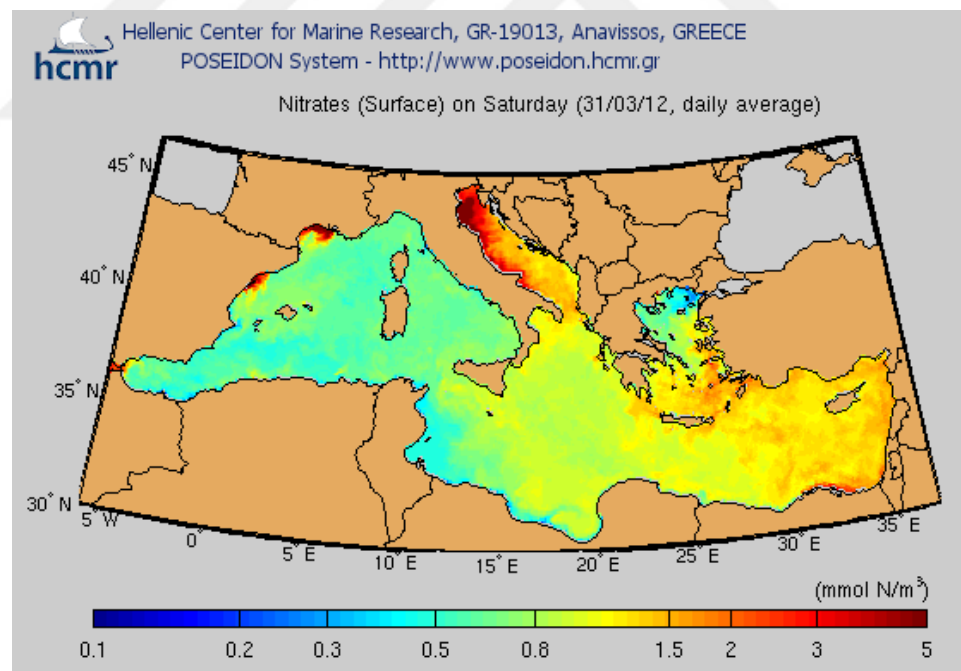


Figure 4.16 Sea surface nitrate of Mediterranean Sea in 31 March 2012.

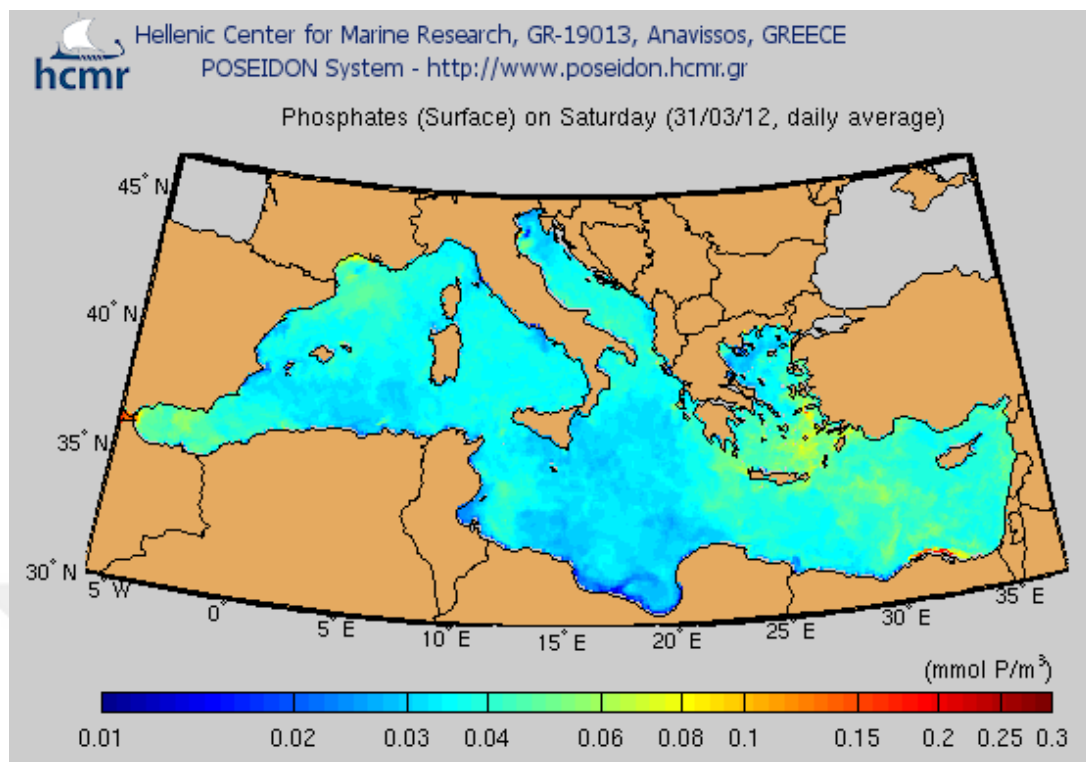


Figure 4.17 Sea surface phosphate of Mediterranean Sea in 31 March 2012.

4.1.2 Sediment Parameters

As similar to comparison results of seawater parameters mentioned above, the sediment values of the study area including the previous sampling were generally in the range of Aegean Sea values and also differentiated from the coastal regions and deep basins of Aegean Sea when compared to our present TUBITAK COST project no. 110Y193 and BAP project no. 2010.KB.FEN.014.

4.1.2.1 Previous Sampling in the Study Area

In the study area, the grain sizes decreased with depth as expected but the distribution was slightly different for April 2011 and March 2012 samplings due to non-exact coordinates of stations (Figure 4.18) (Tuncer & Bizsel, 2014c). However, it could be interpreted as such that approximately 100 – 150 m depths (stations 1, 3 and 5) were sandy while approximately 200 m depths (stations 2 and 4) were sand-silt-clay (Figure 4.18) (Tuncer & Bizsel, 2014c).

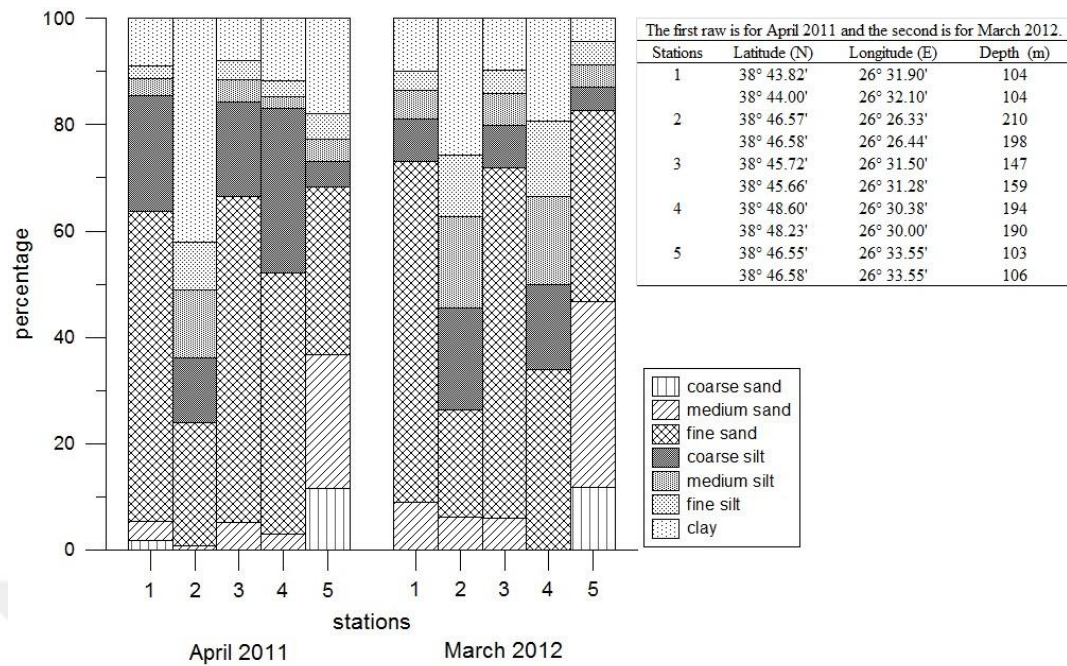


Figure 4.18 The comparison of sediment type in April 2011 and March 2012 (Tuncer & Bizsel, 2014c).

On the other hand, the chemical contents of the surface sediments had closer percent ranges for spring 2011 and 2012 (Table 4.4) (Tuncer & Bizsel, 2014c). While TC and TP percents did not change much among 5 stations, TOC and TOP showed spatial differences (Figure 4.19) (Tuncer & Bizsel, 2014c). Unlike carbon and phosphorus distribution, nitrogen contents of stations were completely different for those two sampling periods (Figure 4.19) (Tuncer & Bizsel, 2014c). Therefore, those underlined the spatial and temporal variability of organic contents and also the nitrogen playing much more dynamic role in sediments.

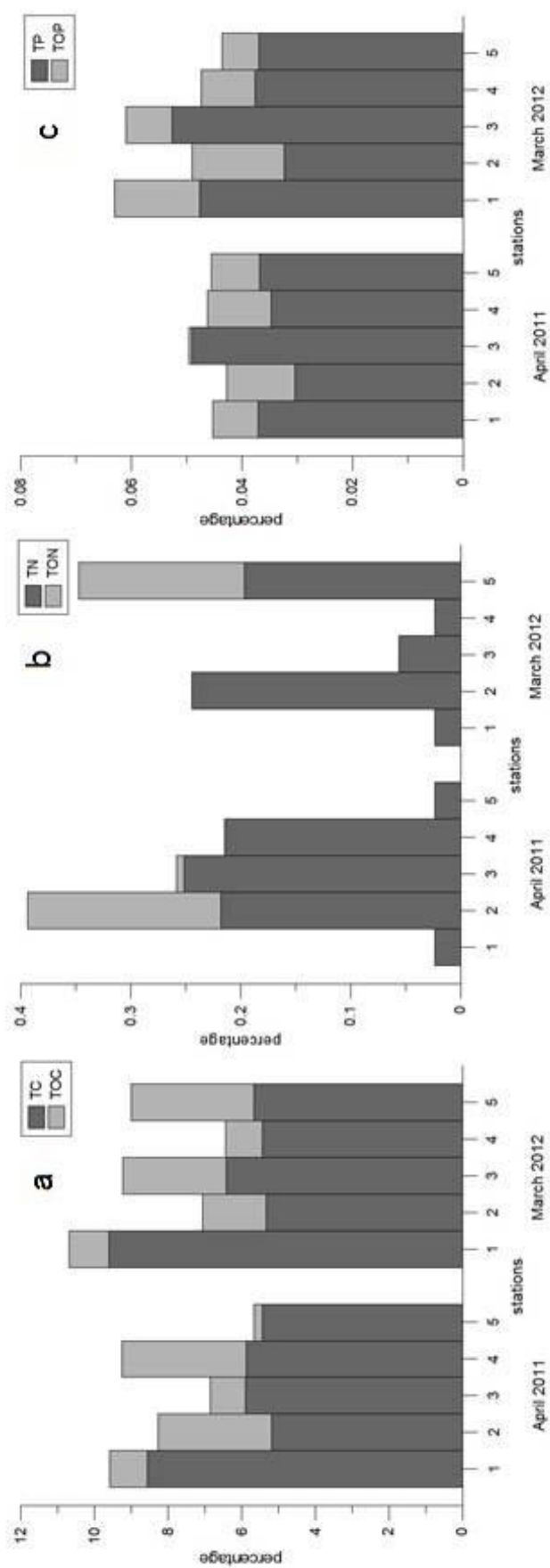


Figure 4.19 The comparison for chemical content of sediment in April 2011 and March 2012 (a) TC-TOC, (b) TN-TON, (c) TP-TOP percentages (Tuncer & Bizsel, 2014c).

Table 4.4 The surface sediment parameters at the study area. The first row is for April 2011 and the second is for March 2012 (Tuncer & Bizsel, 2014c).

Station	TC (%)	TOC (%)	TN (%)	TON (%)	TP (%)	TOP (%)
1	8.57	1.01	0.02	BDL	0.037	0.008
	9.61	1.09	0.02	BDL	0.048	0.015
2	5.19	3.09	0.22	0.18	0.030	0.012
	5.33	1.73	0.24	BDL	0.032	0.017
3	5.90	0.96	0.25	0.01	0.049	0.001
	6.42	2.82	0.06	BDL	0.053	0.008
4	5.88	3.36	0.21	BDL	0.035	0.012
	5.43	1.00	0.02	BDL	0.038	0.010
5	5.44	0.24	0.02	BDL	0.037	0.009
	5.67	3.33	0.20	0.15	0.037	0.007

4.1.2.2 Previous Studies in Aegean Sea

As seen in Table 4.5, total carbon contents of the study area (5.19 – 9.62 %) (Tuncer & Bizsel, 2014c) were in the ranges of both North Aegean Sea (0.33 – 15.63 %) (Friligos & Krasakopoulou, 2001; Sunlu et al., 2005) and South Aegean Sea (1.30 – 13.10 %) (Aydin & Sunlu, 2004) but much closer to coastal sediments of Candarli Bay (0.03 – 10.22 %) (Kaymakci-Basaran, 2004; Cagri-Tas et al., 2007). Similarly, organic carbon contents (0.24 – 3.36 %) (Tuncer & Bizsel, 2014c) were also in the range of North Aegean Sea (0.22 – 6.76 %) (Yasar, 1994; Hatzianestis et al., 1998; Friligos & Krasakopoulou, 2001; Polymenakou et al., 2005), in addition to values for the coastal sediments of Lesvos Island and Izmir Bay (2.50 – 4.10 and 0.24 – 5.08 %, respectively) (Kostopoulou et al., 2013). On the other hand, total nitrogen contents (0.02 – 0.25 %) (Tuncer & Bizsel, 2014c) were much higher than previous result (0.023 – 0.074 %) in North Aegean Sea (Friligos & Krasakopoulou, 2001) unlike total organic nitrogen values (BDL – 0.18 %) (Tuncer & Bizsel, 2014c) which were similar to previous result (0.04 – 0.10 %) in Cretan Sea (Polymenakou et al., 2009).

Table 4.5 Comparison of the sediment values at the study area with the previous results of Aegean Sea.

Variable	Values (mean range)	Sampling area	Sampling period	Source
TC (%)	1.30 - 13.10	South Aegean Sea		Aydin & Sunlu, 2004
	0.35 - 15.63	North Aegean Sea		Sunlu et al., 2005
	0.33 - 0.79	North Aegean Sea	1998	Friligos & Krasakopoulou, 2001
	0.54 - 2.28	Lesvos Island		Aloupi et al., 2007
	0.03 - 10.22	Candarli Bay		Kaymakci-Basaran, 2004
	0.20 - 1.36	Candarli Bay	2003 - 2004	Cagri-Tas et al., 2007
	0.87 - 1.60	Izmir Bay		Yaramaz et al., 1991
	1.25 - 2.10	Izmir Bay		Sunlu et al., 1999
	1.13 - 2.76	Izmir Bay		Atlgan & Egemen, 2001
	5.19 - 9.62	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
TOC (%)	0.70 - 1.10	South Aegean Sea	1990 - 1991	Yasar, 1994
	0.42 - 0.45	South Aegean Sea	2002	Polymenakou et al., 2005
	0.46 - 0.99	Cretan Sea	2006	Polymenakou et al., 2009
	0.80 - 1.20	North Aegean Sea	1990 - 1991	Yasar, 1994
	0.90 - 6.76	North Aegean Sea	1998	Friligos & Krasakopoulou, 2001
	0.22 - 0.78	North Aegean Sea	1996	Hatzianestis et al., 1998
	0.53 - 1.47	North Aegean Sea	2002	Polymenakou et al., 2005
	2.50 - 4.10	Mytilene Harbor (Lesvos Island)	2007	Kostopoulou et al., 2013
	0.24 - 5.08	Izmir Bay	2007	Kostopoulou et al., 2013
	0.24 - 3.36	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c

Table 4.5 Comparison of the sediment values at the study area with the previous results of Aegean Sea. (Continued).

Variable	Values (mean range)	Sampling area	Sampling period	Source
TN (%)	0.02 - 0.07	North Aegean Sea	1998	Frigos & Krasakopoulou, 2001
	0.02 - 0.25	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
TON (%)	0.04 - 0.10	Ctretan Sea	2006	Polymenakou et al., 2009
	BDL - 0.18	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
TP (%)	0.040 - 0.090	Izmir Bay	1994	Aksu et al., 1998
	0.030 - 0.053	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c
TOP (%)	0.001 - 0.015	North Aegean Sea	2011 - 2012	Tuncer & Bizsel, 2014c

4.1.2.3 Present Studies in Aegean Sea

The sediment results of the study area were also compared with intertidal zone of Izmir Bay (sampled in August 2010) and deep north – south line of Aegean Sea (sampled in March 2012) (Figure 4.20) (Tuncer & Bizsel, 2014d; unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)). As expected, the largest particles were obtained from the coastal area (Izmir Bay) as sandy gravel, the finer particles from the deeper parts of Aegean Sea as clayey silt (Figure 4.21) and the study area was in between (Figure 4.18).

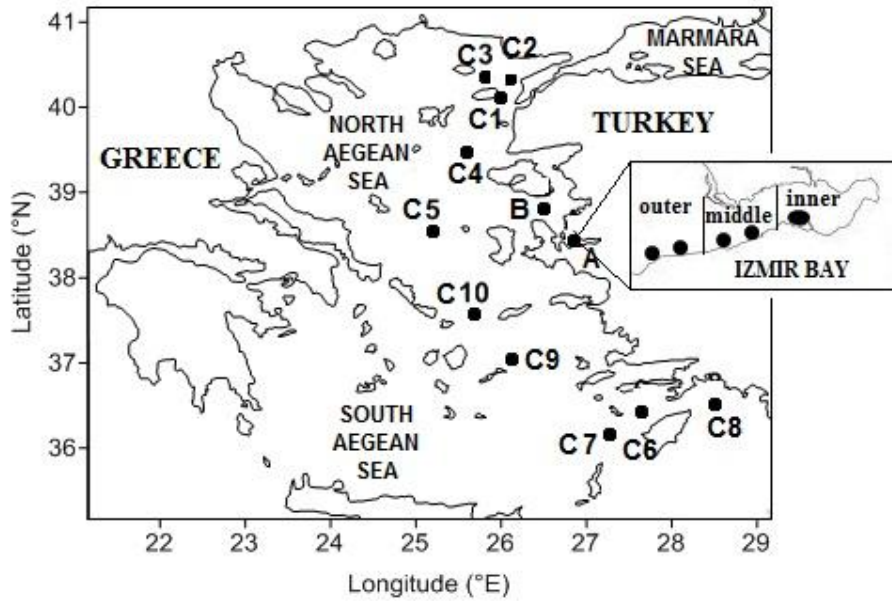


Figure 4.20 Map for stations A (9 stations at intertidal zone of Izmir Bay), B (5 stations of this PhD thesis) and C (10 stations) in Aegean Sea.

When the chemical content of the sediments including the study area were considered (Tuncer & Bizsel, 2014c, 2014d; unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)), the variations in the stations C1 – C3 in North Aegean Sea were seen in spite of very close coordinates and also the inner and the middle-outer bays of Izmir Bay showed clear separation (Figure 4.22). While the highest carbon content belonged to the station C3, the highest nitrogen was to C1 which in fact had the lowest values for the rest of the contents. In contrast, the lowest nitrogen values were obtained from the

station C2. Furthermore, the inner bay had higher carbon values (10.24 – 20.94 % for TC) but lower nitrogen values (0.11 – 0.22 % for TN). However, in spite of low carbon and nitrogen contents seen in the study area, its phosphorus content was higher together with the inner bay (0.032 – 0.046 % for TP) than the rest.

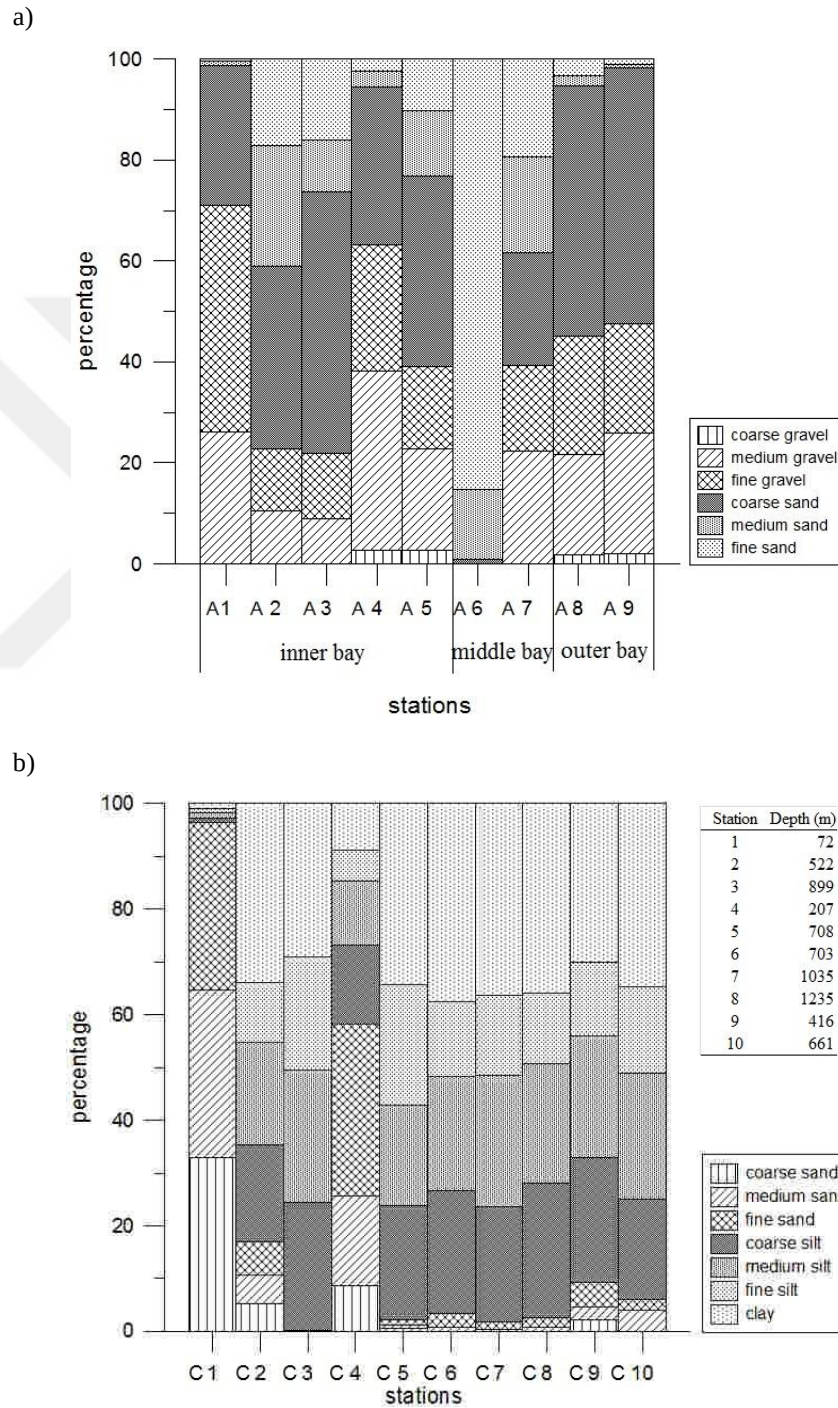


Figure 4.21 Sediment type. a) Izmir Bay, b) Aegean Sea (Tuncer & Bizsel, 2014d; unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)).

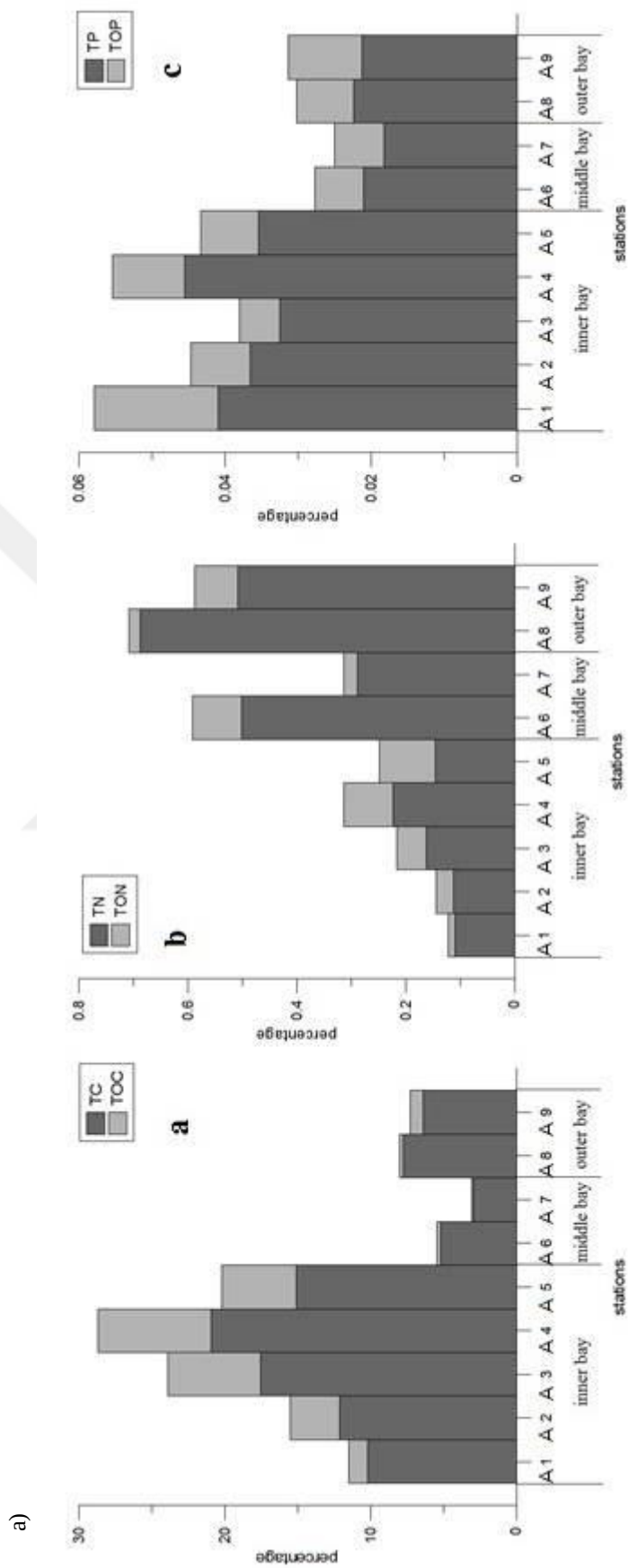


Figure 4.22 Chemical content of sediment as (a)TC-TOC, (b) TN-TON, (c) TP-TOP percentages. a) Izmir Bay and b) Aegean Sea (Tuncer & Bizsel, 2014d; unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)).

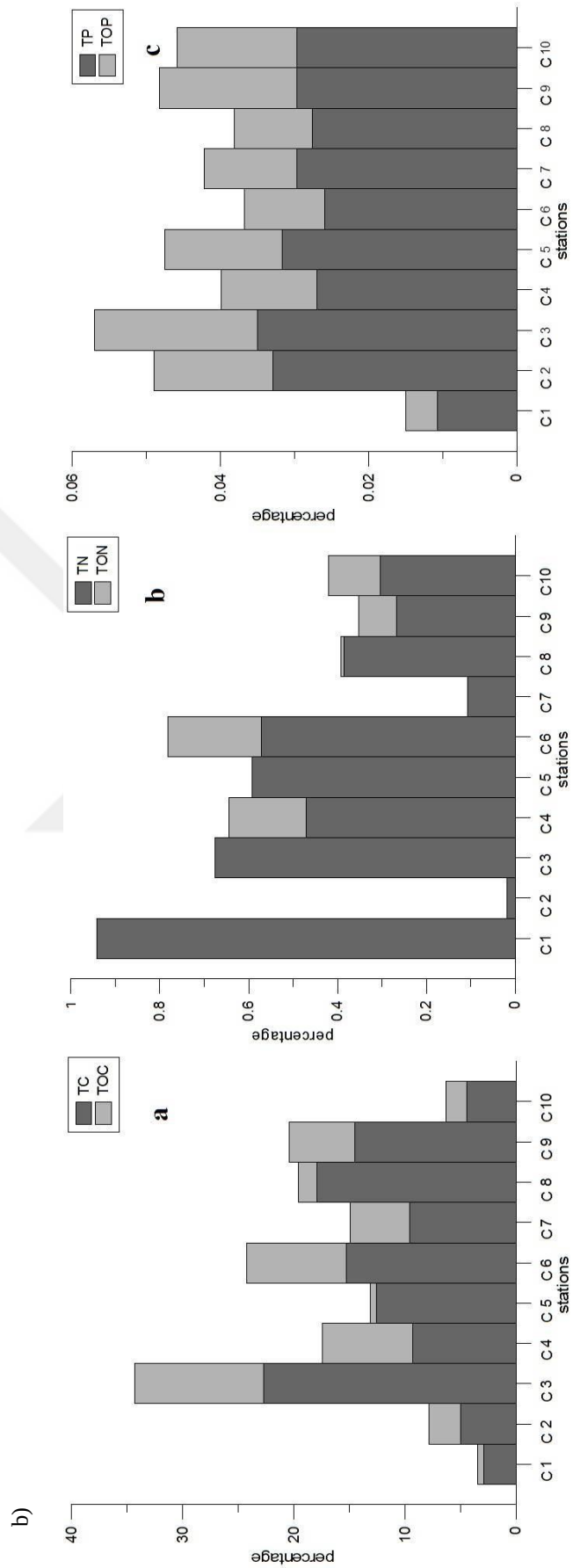


Figure 4.22 Chemical content of sediment as (a)TC-TOC, (b) TN-TON, (c) TP-TOP percentages. a) Izmir Bay and b) Aegean Sea. (Continued).

4.2 Comparison of Bacterial Diversity in Eastern Mediterranean Sea

In our present BAP project no. 2010.KB.FEN.014 and TUBIAK COST project no. 110Y193, 16S rRNA gene sequences of totally 185 isolates from sediments of Aegean Sea were deposited to NCBI GenBank and a highly diverse phylum *Firmicutes* was obtained in addition to the phyla *Actinobacteria* and *Gammaproteobacteria* with cultivation-based methods (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

In recent studies based on the comparative analysis of 16S rRNA clone libraries, it was reported that the dominance in deep sediments of Eastern Mediterranean Sea was constitute the *Acidobacteria* (Polymenakou et al. 2009; Kouridaki et al. 2010). On the other hand, according to 16S rRNA gene sequences, mainly the *Firmicutes* with *Bacillus* the most frequent genus and then *Actinobacteria* were isolated using cultivation-based methods from deep sediments of the Cretan Sea (Gärtner et al., 2011). Similarly, those phyla, especially the *Firmicutes*, were dominantly isolated from our present studies in sediments of Aegean Sea (Figure 4.20, 4.23 – 4.25) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)). Although they accounted minor part in 16S rRNA gene libraries of Mediterranean sediments (Heijs et al. 2008), especially for sediments of Eastern Mediterranean Sea as 3 % *Firmicutes* and 4 – 28 % *Actinobacteria* of total sequence (Polymenakou et al., 2009), they formed the majority in culture-dependent studies of sediments of Cretan Sea together with *Gammaproteobacteria* which became dominant under hydrostatic pressure incubation of those sediments (Gärtner et al., 2011). However, few isolation of *Gammaproteobacteria* were obtained mostly from our present studies in shallow sediments of Aegean Sea (Figure 4.25) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) supporting the frequent 16S rRNA gene clones of this phylum compared to the deeper sediments of Eastern Mediterranean Sea (Polymenakou et al., 2005).



Figure 4.23 The phyla *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria* isolated from stations A (9 stations at intertidal zone of Izmir Bay), B (5 stations of this PhD thesis) and 10 stations of Aegean Sea, neighbor-joining distance tree constructed in Mega 5.1 using 16S rRNA gene sequences (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

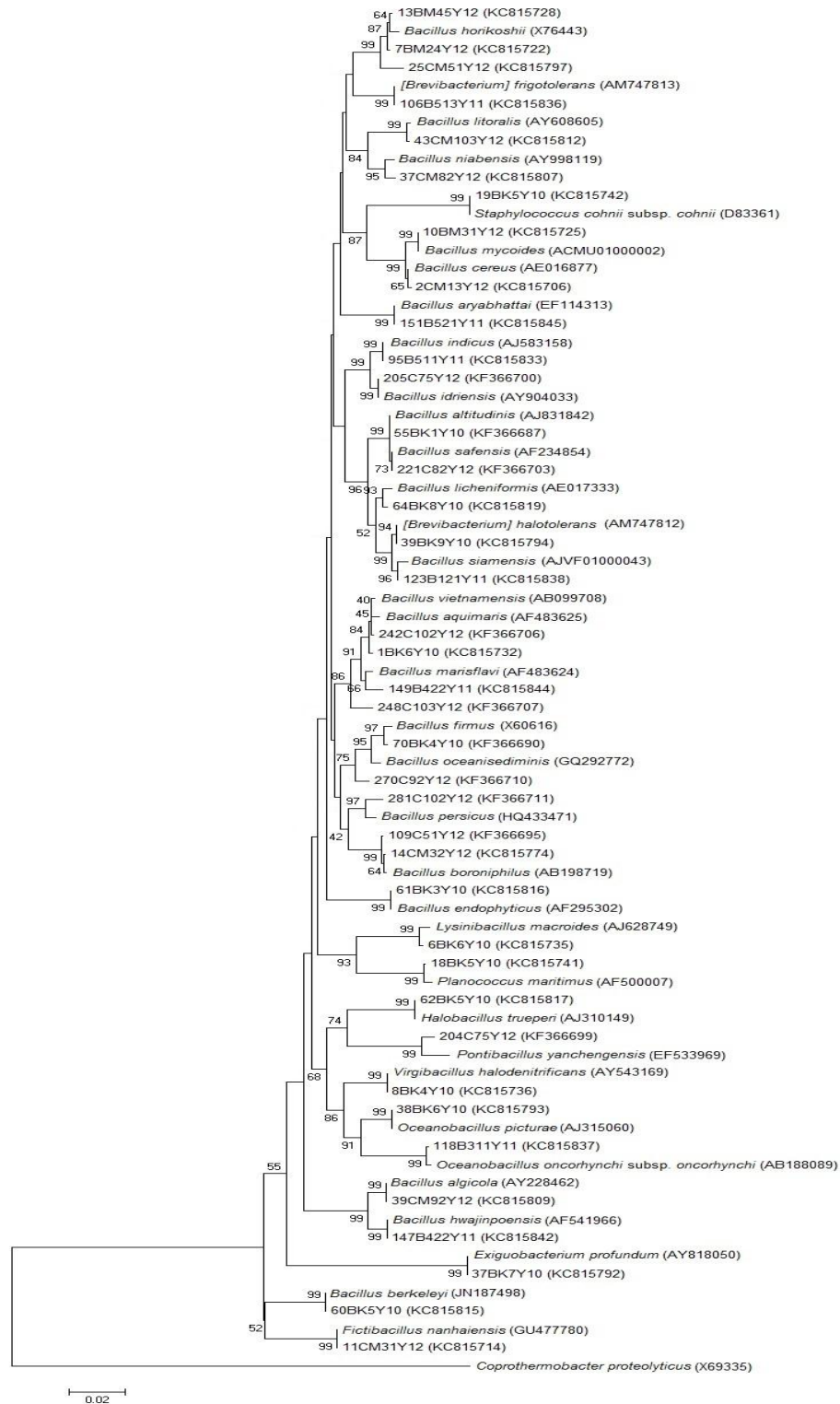


Figure 4.24 For the order *Bacillales* isolated from stations A (9 stations at intertidal zone of Izmir Bay), B (5 stations of this PhD thesis) and 10 stations of Aegean Sea, neighbor-joining distance tree constructed in Mega 5.1 using 16S rRNA gene sequences (Tuncer & Bizsel, 2014b, 2014c, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

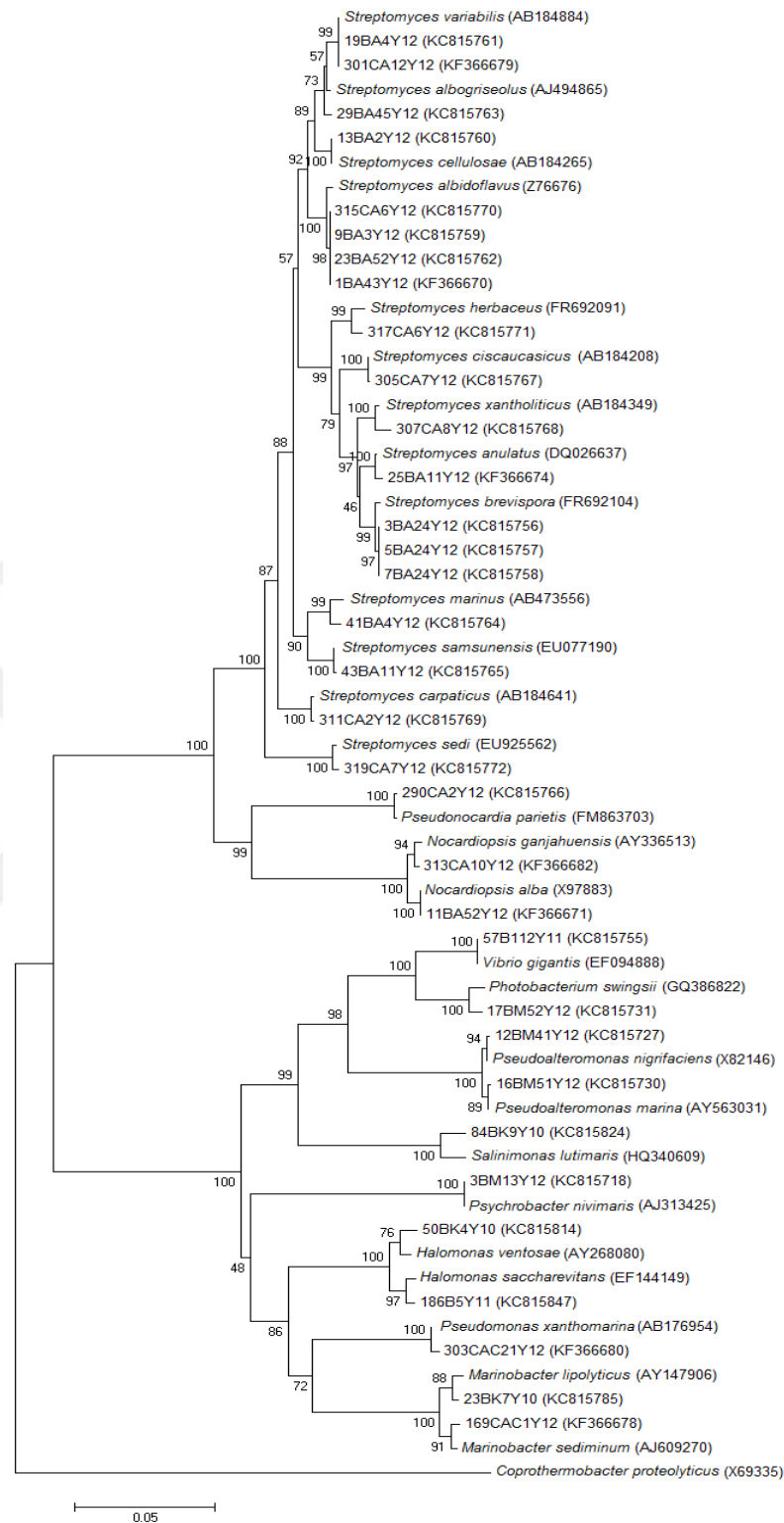


Figure 4.25 For the classes *Actinobacteria* and *Gammaproteobacteria* isolated from stations A (9 stations at intertidal zone of Izmir Bay), B (5 stations of this PhD thesis) and 10 stations of Aegean Sea, neighbor-joining distance tree constructed in Mega 5.1 using 16S rRNA gene sequences (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

Much diverse genera belonging to phyla *Firmicutes* and *Gammaproteobacteria* were obtained especially from our present studies in shallower stations A and B of Aegean Sea (Figure 4.20, 4.23 – 4.25) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) rather than the phylum *Actinobacteria* from previous study of Cretan Sea sediments (Gärtner et al., 2011). While a highly diverse clade with members belonging to the order *Bacillales* sharing a high phylogenetic affiliation with mostly *Bacillus* species in addition to the genera *Exiguobacterium*, *Fictibacillus*, *Halobacillus*, *Lysinibacillus*, *Oceanobacillus*, *Planococcus*, *Pontibacillus*, *Staphylococcus* and *Virgibacillus* was obtained from our present studies in sediments of Aegean Sea (Figure 4.24) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)), only 3 genera as *Bacillus*, *Halobacillus* and *Staphylococcus* were isolated from the sediments of Cretan Sea (Gärtner et al., 2011). Furthermore, for the phylum *Gammaproteobacteria*, 8 different genera as *Halomonas*, *Marinobacter*, *Psychrobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Photobacterium*, *Salinimonas* and *Vibrio* were obtained from our present studies in sediments of Aegean Sea (Figure 4.25) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) but 6 genera as *Alteromonas*, *Marinobacter*, *Microbulbifer*, *Pseudoalteromonas*, *Pseudomonas* and *Srenotrophomonas* were isolated from the sediments of Cretan Sea (Gärtner et al., 2011).

In our present studies, the *Actinobacteria* and *Gammaproteobacteria* classes and the genera (belonging to the order *Bacillales*) other than the genus *Bacillus* were mainly isolated from stations A and B, additionally few from stations C1 – C3 and C6 – C8 (Figure 4.20, 4.23 – 4.25) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)). However, the *Bacillus* strains, except [*Brevibacterium*] *frigorigerans* and *Bacillus algicola* were mainly isolated from stations B (Tuncer & Bizsel, 2014b, 2014c, 2014f). Furthermore, from stations A (Figure 4.20), the strains of [*Brevibacterium*] *halotolerans*, *Bacillus algicola*, *Bacillus aryabhattai* and *Bacillus subtilis* group were also obtained (Figure 4.24 (Tuncer & Bizsel, 2014a, 2014b, 2014e). For stations C

(Figure 4.20), the strains of *[Brevibacterium] frigoritolerans* and *Bacillus hwajinpoensis* were mainly isolated while *Bacillus algicola* and *Bacillus cereus* group were mostly from shallower stations as C1, C2, C4 and C9 (Figure 4.24) (unpublished TUBITAK data (COST project no. 110Y193)).

4.3 Comparison of Actinomycetes Diversity in Eastern Mediterranean Sea

In the study area and also in our present TUBIAK COST project no. 110Y193, actinomycete diversity was found lower compared to previous studies in Eastern Mediterranean Sea (Tuncer & Bizsel, 2014d). In the study of sediments in Cretan Sea, isolated actinomycetes were phylogenetically affiliated with the genera *Arthrobacter*, *Blastococcus*, *Corynebacterium*, *Micromonospora*, *Pseudonocardia*, *Streptomyces* (Gärtner et al. 2011). In addition to the isolation of alkaliphilic actinomycetes MA1-1 from sediments of Izmir Bay, Aegean Sea (Hames-Kocabas & Uzel, 2007), much more diverse *Streptomyces* strains and one *Nocardia alba* strain were seen among 66 antimicrobially active strains of 261 actinomycetes isolated from the sediments in Turkish coasts of Black Sea, Aegean Sea and Mediterranean Sea (Ozcan et al., 2013).

From our present studies in sediments of Aegean Sea, the actinomycete strains were found as closely related to the genera *Nocardiopsis*, *Pseudonocardia* and *Streptomyces* (Figure 4.20, Figure 4.25) (Tuncer & Bizsel, 2014d). The strains belonging to the genera other than genus *Streptomyces* were highly affiliated with *Nocardiopsis alba*, *Nocardiopsis ganjiahuensis* and *Pseudonocardia parietis*. In spite of high phylogenetic diversity in higher taxa in Gärtner et al. 2011, *Streptomyces* strains (closely related to *Streptomyces albidoflavus*, *Streptomyces albogriseolus*, *Streptomyces anulatus*, *Streptomyces brevispora*, *Streptomyces carpaticus*, *Streptomyces cellulosa*, *Streptomyces ciscaucasicus*, *Streptomyces haliclona*, *Streptomyces herbaceus*, *Streptomyces samsunensis*, *Streptomyces sedi*, *Streptomyces xantholiticus* *Streptomyces variabilis*) were found more diverse in our present studies of sediments in Aegean Sea (Figure 4.25) (Tuncer & Bizsel, 2014d). Those were isolated from the stations C1 and C2 in North Aegean Sea and from the

stations C6 – C8 in South Aegean Sea (Figure 4.20) in addition to the study area (stations B). Moreover, when a limit of 98.5 to 99 % is considered, as revised and proposed by Stackebrandt and Ebers (2006), the *Streptomyces* strain 41BA4Y12 and 317CA6Y12 isolated from the study area (stations B) and station C6 had probability of representing new taxa (Tuncer & Bizsel, 2014d).

4.4 Biogeochemical Comparison in Eastern Mediterranean Sea

Morphological, physiological and phylogenetical features of all bacterial isolates from our present studies in sediments of Aegean Sea (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) indicated high diversity and underlined the relations with environmental factors. When all data was combined in this present study, it was seen that they reflected the biogeochemical patterns in Aegean Sea.

In our present studies, it was found that there was a relation between enzymatic activities of actinomycetes and the trophic state of Eastern Mediterranean Sea (Tuncer & Bizsel, 2014d). Actinomycete strains isolated from stations B and C (Figure 4.20) showed high phosphatase, lipase, esterase, aminopeptidase activities (90%, 81% and 68% of all actinomycete strains, respectively), in contrast to low glucosidase or galactosidase activities (44% of isolates) and also 15% averaged activity against related substrates for glucosaminidase, maltosidase, urease, trehalase, lactase, methyl glucosidase, sucrase, mannitol dehydrogenase, amylase, arabinase, fructokinase, glycerol kinase, glucuronidase, mannosidase, fucosidase and dehydrogenase (Table 3.3 – 3.6; Tuncer & Bizsel, 2014d). In addition to a positive correlation between aminopeptidase activity and N limitation as an indication of the utilization of organic nitrogen for bacterial growth in the study of Cunha & Almeida (2009), it was also shown that bacterial biomass in the sediments of Aegean Sea displayed a positive correlation with the sedimentary protein concentration (Danovaro et al., 1999). It was also given that carbohydrates were a reservoir of non-utilised organic carbon in oligotrophic deep-sea sediments (Danovaro et al., 1999). Thus, in our present studies, enzymatic activity results (Table 3.3 – 3.6; Tuncer &

Bizsel, 2014d) and organic nitrogen depletions in the sediments as underlying N limitation (Figure 4.19, 4.22) supported this protein utilization rather than carbohydrates (Tuncer & Bizsel, 2014d). Those results also reflected the geochemical effects on bacterial characteristics in addition to the geographical separation such that the strains isolated from the station C10 showed the lowest activities whereas the highest glucosidase or galactosidase activities were obtained from stations C6 – C8 (Tuncer & Bizsel, 2014d). While the isolates from the station C2 gave the second lowest phosphatase activity, the station C1 had the lowest glucosidase or galactosidase activities (Tuncer & Bizsel, 2014d). Like this separation between closer stations C1 and C2, the isolates from deeper B stations showed lower activities compared to shallower B stations (Tuncer & Bizsel, 2014d). In fact, overall B stations had the highest aminopeptidase activity among all stations (Tuncer & Bizsel, 2014d).

Another indicator of environmental effects on bacterial characteristics was also shown as the variation in physiological features (Tuncer & Bizsel, 2014a, 2014b, 2014e, 2014f). In a previous study of Eastern Mediterranean Sea, the relation between antibiotic resistance and geographical differences was given as higher resistance in coastal areas of Syria than Turkey and Lebanon (48 %, 38 % and 31 % of total isolates, respectively) (Altug et al., 2010). Similarly, in our present studies, higher resistance for isolates from coastal sediments of Izmir Bay (stations A) than the ones from the sediments of the study area (stations B) (69 % and 50 %, respectively) and higher multiple antibiotic resistance (19 % and 14 %, respectively) in addition to different bacterial diversity of those two regions underlined the environmental separation of stations B from Izmir Bay influenced by river and sewage discharge, anthropogenic activities such as tourism-derived, industrial, agricultural and nautical activities (Tuncer & Bizsel, 2014a, 2014b, 2014e, 2014f). On the other hand, susceptibility levels in deep-basins were 40 – 67 % whereas stations C1 and C3 had the highest resistance values (67 % and 57 %, respectively) in addition to multiple resistance seen only in stations C1 – C4 and C8 (unpublished TUBITAK data (COST project no. 110Y193)). Furthermore, according to the statistical analysis of Pearson correlation (unpublished data (BAP project no.

2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)), a positive correlation was seen between antibiotic resistance and sediment types of stations A, B and C ($p<0.05$). This expected result supported the fact that coastal zones affected by terrestrial and anthropogenic inputs had higher resistance compared to deep-basins. There was also positive correlation between bacterial numbers (in both lower and higher taxa) and resistance and even multiple antibiotic resistance index ($p<0.05$).

When geochemical and bacterial parameters were statistically analyzed using Pearson correlation (unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)), it was indicated that for averaged values of stations B in spring period 2011 – 2012, a positive correlation between N contents of seawater (nitrate, nitrite) at the deepest layers and P contents of sediments (total, organic phosphorus) were found ($p<0.05$). However, there was no statistical correlation between chemical contents of the sediments and bacterial family and genus numbers in contrast to the positive correlation between sediment type and those bacterial numbers ($p<0.05$). Moreover, for stations A, B and C, there were a positive correlation between organic carbon and organic nitrogen ($p<0.05$) and a negative correlation between nitrogen and phosphorus contents of the sediments ($p<0.05$) in addition to the negative correlation between carbon content of the sediments and bacterial family and genus numbers ($p<0.05$).

The principle component analysis based on biogeochemical parameters (depth of stations, chemical contents and type of sediments, bacterial family and genus numbers, resistant family and genus numbers) was also followed for those A, B and C stations (unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)). It was found that the first two factors having eigenvalues higher than 1.0 accounted for approximately 82 % of the total variation (Figure 4.26a), in addition to the variation of the third factor as approximately 11 %. According to factor loadings (correlation values) greater than 0.5, the first factor had high values for TC, TOC, TP and TN percentages in the sediments while the second factor had high values for TON and TOP percentages in

the sediments (Table 4.6a). In the two-dimensional plot of principle component factor scores (Figure 4.26a), the separation of stations A, even with middle-outer bays separation from inner bay, was clearly seen in addition to the separation of geographically close stations C1 – C3. When chemical contents of sediments were removed from the analysis, the first factor had high values for bacterial family and genus numbers, resistant family and genus numbers in addition to a high value of the second factor for the sediment type (Table 4.6b). Moreover, the plot of this analysis (Figure 4.26b) distinguished stations B and C, compared to the separation of stations A seen in the first plot (Figure 4.26a).

Table 4.6 Factor matrix for biogeochemical parameters with and without chemical contents of sediments (a and b, respectively) obtained from principle component analysis.

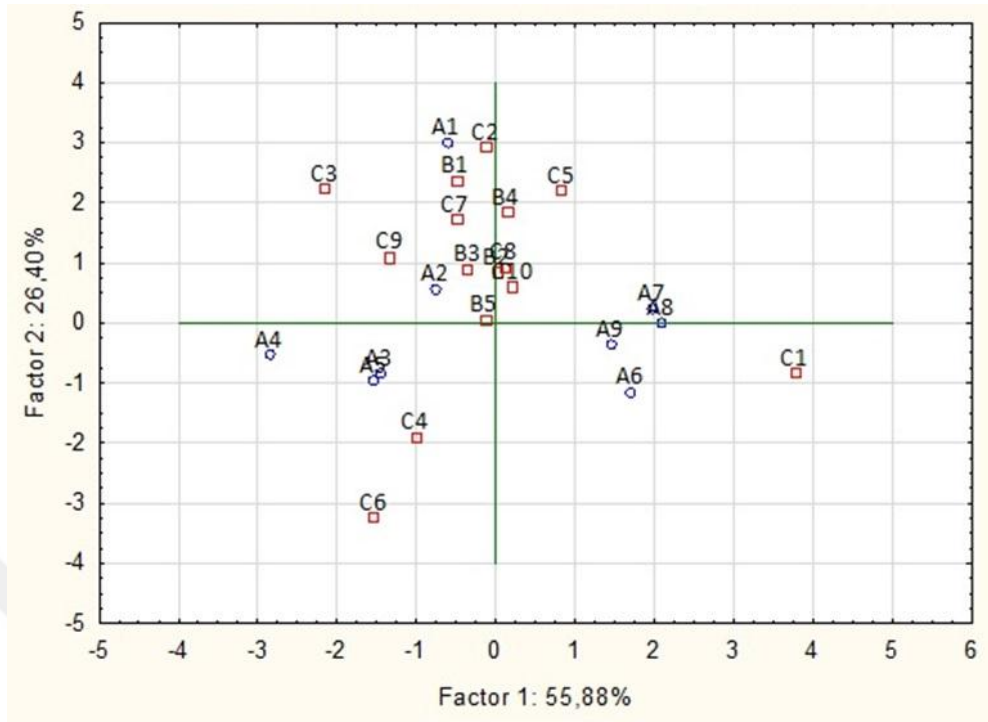
a)

Variables	Factor1	Factor2	Factor3
TC (%)	-0.95	-0.17	-0.02
TOC (%)	-0.93	-0.31	-0.10
TN (%)	0.76	-0.32	0.32
TON (%)	-0.33	-0.74	0.52
TP (%)	-0.93	0.31	0.10
TOP (%)	-0.19	0.84	0.50
Sediment type	-0.33	0.37	0.32
Bacterial family number	0.21	-0.49	0.24
Bacterial genus number	0.29	-0.47	0.23
Resistant family number	0.30	-0.49	0.34
Resistant genus number	0.45	-0.44	0.23

b)

Variables	Factor1	Factor2	Factor3
Sediment type	0.45	-0.89	0.09
Bacterial family number	-0.96	-0.25	-0.11
Bacterial genus number	-0.95	-0.22	-0.23
Resistant family number	-0.95	0.02	0.30
Resistant genus number	-0.99	0.03	0.08

a)



b)

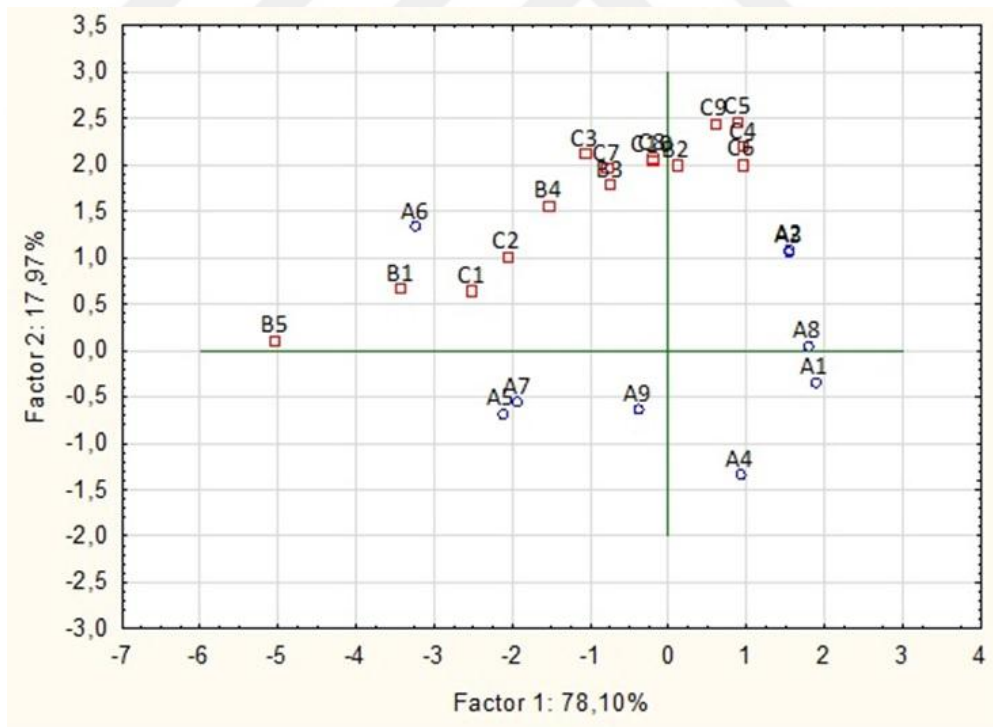


Figure 4.26 Principle component analysis plot based on the comparison of biogeochemical parameters with and without chemical contents of sediments (a and b, respectively) for stations A, B and C (unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK (COST project no. 110Y193)).

Like our present studies in sediments of Aegean Sea (Figure 4.20) (Tuncer & Bizsel, 2014a, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished BAP project no. 2010.KB.FEN.014; unpublished TUBITAK data (COST project no. 110Y193)), it was previously shown that environmental parameters together with geographical differences influenced the bacterial community composition in sediments of Eastern Mediterranean Sea (Danovoro et al., 1999; Polymenakou et al., 2005, 2009; Kouridaki et al., 2010). For instance, in a previous study, cluster analysis of bacterial compositions supported the environmental and geographical differences and especially gave the north-south latitudinal separation for bacterial diversity in sediments of Eastern Mediterranean Sea (Polymenakou et al., 2005). Furthermore, in another study, when 16S rRNA clone compositions for North East Pacific and Eastern Mediterranean sediments were compared, hierarchical cluster analysis again provided the geographical separation (Kouridaki et al., 2010). Similarly, in our present studies, the geochemical differences in addition to the variations of the geomorphologic, hydrographic and trophic states reflected on the phylogenetic diversity as supported in hierarchical cluster analysis of the isolates (Figure 4.27) (unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK data (COST project no. 110Y193)).

Although mainly the phylum *Firmicutes* with *Bacillus* the most frequent genus was obtained from geographically close stations C1 – C3 (with 72 m, 522 m and 899 m depths, respectively) in North Aegean Sea (Tuncer & Bizsel, 2014d; unpublished TUBITAK data (COST project no. 110Y193)), cluster analysis revealed their phylogenetic differences (Figure 4.27) supporting their geochemical and locational variations (Figure 4.26). Similarly, stations A and B in North Aegean Sea showed those differences in the dendrogram (Figure 4.27), even when they were grouped as Ai for inner bay, Aj for middle and outer bays, Bi for approximately 100 – 150 m depths (stations 1, 3 and 5 illustrated in Figure 2.1) and Bj for approximately 200 m depths (stations 2 and 4 illustrated in Figure 2.1) due to their geomorphological separation. In fact, those A and B stations had the highest phylogenetic diversity among all stations (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)). On the other hand, like

the stations C4, C5, C9 (with 207 m, 708 m and 416 m depths, respectively) (near to C10 with 661 m depth), the stations C6 – C8 (with 703 m, 1035 m and 1235 m depths, respectively) in South Aegean Sea formed a closer group including the families *Bacillaceae* and *Streptomycetaceae* in the dendrogram (Figure 4.27) underlying their geographical closeness (Figure 4.20).

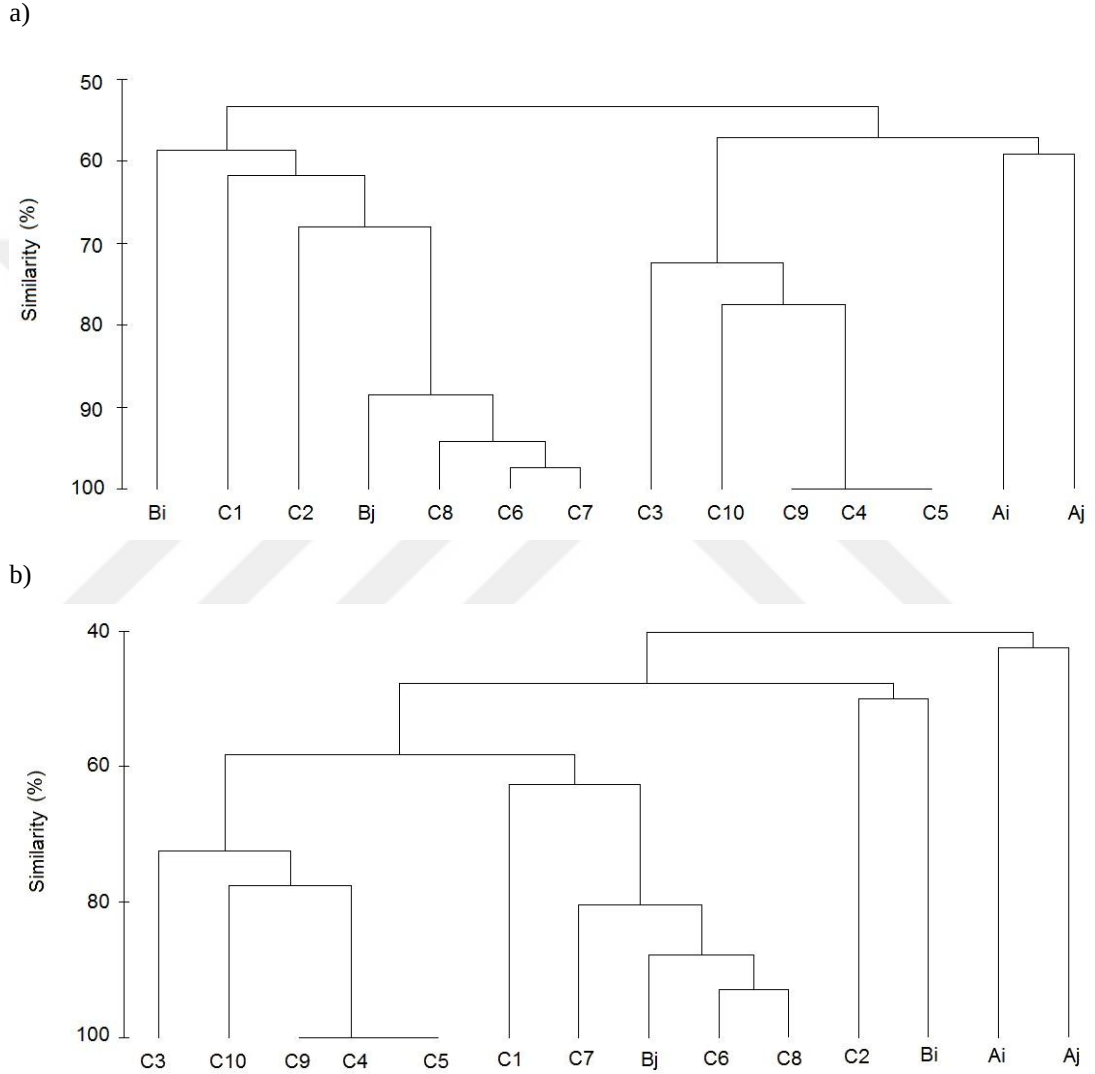


Figure 4.27 Cluster analysis dendrogram based on the comparison of bacterial community at the family and genus levels (a and b, respectively) from different sediment samples of stations A (Ai for inner bay; Aj for middle and outer bays), B (Bi for 100-150 m depths; Bj for 200 m depths) and C (unpublished data (BAP project no. 2010.KB.FEN.014); unpublished TUBITAK (COST project no. 110Y193)).

While the variation in chemical contents of the sediments had higher role in separation of coastal stations from deep-basins as seen in the first plot of principle component analysis (Figure 4.26a), the geographical differences such as sediment type provided additional separation among deeper stations as seen in the second plot (Figure 4.26b) which was supported by the hierarchical cluster analysis of bacterial diversity in the family level (Figure 4.27a) as giving similar variation in the distribution of stations A, B and C. Although latitudinal separation in bacterial diversity was obviously seen in hierarchical cluster analysis based on similarity matrices for bacterial community comparison at both family and genus levels (Figure 4.27), those physicochemical variables were not enough to explain the distribution seen in the dendrogram of bacterial diversity in the genus level (Figure 4.27b). Therefore, all environmental factors including the geomorphologic, hydrographic and trophic states should be considered for the phylogenetical and physiological differences in even much lower taxa.

The shallowest sediments belonging to stations A had the highest phylogenetic diversity in higher taxa (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) due to the dynamic environmental factors as continuous terrestrial and anthropogenic effects i.e. river and sewage input increasing the eutrophication. Furthermore, the higher influence of those inputs on the inner bay (stations Ai) was clearly seen as biogeochemical separation from the middle and outer bays (stations Aj) (Figure 4.22a, 4.26, 4.27) in addition to even higher diversity in stations Aj than Ai (Tuncer & Bizsel, 2014b, 2014e). In contrast, the deep and oligotrophic stations C9, C10 in North Aegean Sea and C6, C7 in South Aegean Sea (Figure 4.20) had higher diversity in lower taxa (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) due to high hydrostatic pressure, depletion of oxygen and nutrients so that bacterial community composition shifted according to hard environmental conditions. However, compared to those stations, diversity increased at the family level for especially station C1 in North Aegean Sea (Figure 4.20) (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)) due to lower depth and higher nutrient input from

Black Sea. On the other hand, stations B (Figure 4.20) showed the highest bacterial diversity in both lower and higher taxa indicating the optimum environmental conditions (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)). In addition to the location of stations B as geographically in between of coastal zone (stations A) and deep-basins (stations C), those optimum conditions were also supported by physicochemical results obtained in our present studies (Figure 4.1 – 4.12, 4.18, 4.19). Moreover, like biogeochemical separation seen in stations A as Ai and Aj (Figure 4.22a, 4.26, 4.27), the stations Bi and Bj showed differences i.e. sediment type, depth and bacterial community composition such that isolation and diversity of *Gammaproteobacteria* were higher in Bi and *Actinobacteria* in Bj (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014f).

CHAPTER FIVE

CONCLUSIONS

First of all, this PhD study provided actinomycete phylogeny, physiology, morphology together with physicochemical parameters of isolation area. This combination was very helpful to understand the biogeochemical structure of the area and also geographical and environmental effects on bacterial diversity and characteristics. Among actinomycete strains, possible new taxa was also obtained for further studies such as identification, secondary metabolite production, etc.

Including this PhD study, our present BAP project no. 2010.KB.FEN.014 and TUBITAK COST project no. 110Y193 provided isolation of highly diverse bacteria. The 16S rRNA gene sequences of totally 185 strains were deposited to the GenBank as a new supply especially when the scarcity of phylogenetic studies in Turkish marine environment was considered. Those would be not only important data for bacterial diversity of Aegean Sea but also for further biotechnological studies.

One of the most important outcomes of our present studies was to isolate strains with representing new taxa. The *Halomonas* strain 50BK4Y10 and the *Salinimonas* strain 84BK9Y10 isolated from the stations A and the *Pontibacillus* strain 204C75Y12 from station C7 have a high probability of representing new taxa due to pairwise similarity < 98 % with their nearest type strains (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)). When a limit of 98.5 to 99 % was considered, as revised and proposed by Stackebrandt & Ebers (2006), the number of new taxa increased as including the *Bacillus*, *Halomonas*, *Photobacterium* and *Streptomyces* strains (10CM31Y12, 149B422Y11, 17BM52Y12, 17CM41Y12, 186B5Y11, 235C101Y12, 248C103Y12, 270C92Y12, 317CA6Y12, 37CM82Y12, 41BA4Y12, 43CM103Y12 and 8BM24Y12 from stations B, C3, C4, C6, C8 – C10) (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

Our present studies also provided bacterial community comparisons since the strains were isolated from stations having geographical differences. In fact, the study area of this thesis (stations B) was found optimum for high bacterial diversity in both lower and higher taxa compared to coastal sediments (stations A) with higher diversity in higher taxa and deep-basins (stations C) with higher diversity in lower taxa (Tuncer & Bizsel, 2014b, 2014c, 2014d, 2014e, 2014f; unpublished TUBITAK data (COST project no. 110Y193)).

The physicochemical data was very important to verify the bacterial diversity differences among stations. In addition to the morphological characteristics and metabolic activity data of all isolates, enzymatic activity data of actinomycetes became very helpful to understand the biogeochemical structure of Aegean Sea. Finally, when the geographical and environmental effects were considered, it was concluded that all those data reflected the biogeochemical patterns in Aegean Sea and explained the reasons of variations among all stations. Moreover, it could be said that stations B were perfectly chosen as the study area for this PhD thesis to get the maximum efficiency for diversity by the help of their optimum environmental conditions.

As a result, our present studies including this PhD thesis are very crucial to understand the biogeochemical patterns in Aegean Sea with combination of the bacterial diversity and characteristics, to highlight the need of environmental information for bacterial studies and to provide the stocked isolates and new taxa for further biotechnological research.

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