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CHARACTERIZATION OF BACTERIAL COMMUNITIES FROM SEAWATER SAMPLES: THE STUDY CASE OF KANDALAKSHA BAY, RUSSIA

(BIO/19)

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Preface

During my Ph-D work we studied two different bacterial communities. The first part of the thesis was dedicated to investigate possible applications of bacterial isolated from samples (water, sediments etc.) obtained from the East sector of the Tyrrhenian sea. In this context, ca. 90 isolates have been plate screened for their extracellular enzyme activities (preliminary work carried out before the Ph-D course beginning, Fenice et al. 2007). Among them, few strains have been characterized for specific production of high level of enzymes and/or for some peculiar metabolic properties. In particular, we studied the "Production of chitinolytic enzymes by a strain (BM17) of *Paenibacillus pabuli* isolated from crab shells samples collected in the East Sector of Central Tyrrhenian Sea" (Juarez-Jimenez et al, 2008) and the "Metabolic characterisation of a strain (BM90) *Delftia tsuruhatensis* showing highly diversified capacity to degrade low molecular weight phenols" (Juarez-Jimenez et al. 2010).

These works had not been included in this thesis but only enclosed as published papers.

Due to the huge amount of data and for better coherence, only the second part of the thesis work, concerning the characterization of the bacterial community of Kadalaksha Bay, White Sea, Russia, has been reported here.

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Abstract

In this work we studied the bacterial community obtained from water samples collected in Kandalaksha Bay (White Sea), Russia in order to obtain information both at the ecological level and for possible future application in biotechnology. The study has been carried out both on cultivable strains, isolated by traditional methods, and on the total bacterial (Eubacteria) community by molecular methods.

The isolates were preliminary investigated in order to understand their temperature preferences then their metabolic competences, including the production of extracellular enzyme activities, were tested for possible further biotechnological applications. Furthermore, a detailed taxonomical study was carried out both on cultivable and total bacterial population

Strain isolation and preliminary tests

Sea water was sampled in various areas of Kandalaksha Bay. The majority of samples were collected, at minimum tide level using sterile containers, in an intertidal zone pool and from the adjacent water surface. Others, from different offshore locations and depths (0.5, 2.5, 15, 70 m), were taken by scuba divers or boats using Niskin bottles. Water was filtered on membranes in order to obtain both bacteria pure cultures and DNA for molecular to studies. Pure cultures of isolates (ca. 500) were obtained by plate streak method. To discharge evident replicates of same isolates, preliminary tests were carried out considering strain morphological characteristics (shape, color and dimensions), Gram reaction and simple biochemical tests (catalase and oxidase production). This preliminary selection permitted to remove the majority of replicates and to keep 52 isolates.

Taxonomical identification of isolates by 16S rDNA

Bacterial genomic DNA was extracted from pure cultures and used for amplification of 16S rDNA, to allows taxonomical identification of each isolate.

Only 20 strains out of 52 (ca. 38%), showing high identity (99-100%) with a single known microorganism, were identified at species level. All other isolates were identified at the genus level only: for most of them, affiliation was not possible because 98-99% of identity was

recorded with various species of the same genus. Moreover 3 strains showed a very low percentage of identity (96-97%).

The majority of sequences were phylogenetically related to the genera *Pseudomonas* and *Serratia* that are well worldwide distributed. However, only a typical bacteria of cold marine environment *Shewanella baltica* (KB30) have been revealed.

When Blastn analyses supplied uncertain identification, sequences were aligned with highly similar 16S in the Genbank and phylogenetic analysis was performed. To get a confident branch-length, three different trees, *Pseudomonas*, *Serratia* and a group containing all the other genera, were inferred. Basing on the results some of the strains were identified with certainty at species level while some others, due to the branch length or the external position, probably belonged to new species. For all other strains identification was possible at genus level only. In many cases this could be due to the scarce informative power of the gene target used to discern the relations below the genus level.

Temperature growth profiles

The optimal temperatures for growth of the various strains were tested in the range 0-45°C on PCA plates (steps of 5 °C). Most of the strains (42% ca.) showed the optimum at 30°C. The lowest optimum was recorded at 15°C for one strain only (KB75), while the highest was at 40°C (KB22 and KB49). Most of the strains (56%) were able to growth at 0°C while only 25% grew at 45°C.In addition, the majority were able to grow in a rather broad range of temperature. The majority of the isolates (42%) seems to be psychrotrophics while no psychrophiles were detected. Almost all the strains could be considered as eurythermics, indicating adaptation to frequent and wide temperature variations such as those of Kandalaksha Bay. Moreover, it was evident that many KB strains had wider ranges of growth if compared with same species described in literature; optima were also different.

Extracellular enzyme activity

The strains where submitted to plate screening for the production of various extracellular enzyme activities (amylase, cellulase, chitinase, pectinase, phosphatase, protease, urease, lipases), at their optimal temperature, in order to obtain metabolic information and to find new microorganisms for possible applications.

Lipases, phosphatase and protease were common (ca. 54% and 44% of the isolates, respectively). Pectinase and amylase were present in about 32 % of the strains, while chitinase and urease were detected in a limited number of isolates (17% both); cellulase was not detected. Organisms producing large halos of activity were considered as possible high producers and could be further investigated for biotechnological application. However, none of the isolates produced all the tested activities and a rather large number of strains (17%) produced no activity at all. The results of the screening could be useful also at ecological level. In fact, the isolates producing a limited number of enzymes could be considered as specialized, while those with more diversified enzymatic competence, showing a higher eco-nutritional versatility, are probably advantaged in the harsh White Sea environment.

Metabolic competences by BIOLOG system

The BIOLOG system was used to test the metabolic competences of the strains by their ability to use different compounds as carbon sources. The system is able to detect the oxidation of 95 compounds (including sugars, fatty, organic amino and acids), used by microorganisms as sole carbon sources.In our case, amino acids were generally the preferred compounds: the most utilized carbon sources were L-Glutamic Acid and L-asparagine (96% of strains). Also use of sugars was, as expected, rather common, being α-D-Glucose oxidized by ca. 92%of strains. The information obtained by BIOLOG could be considered as an index regarding the strain metabolic complexity. A small number of strain apparently showed a rather simple metabolism being using a limited number of carbon sources and only few showed a very diversified metabolic competence. However, the majority of the bacteria tested

used about 30-50 compounds showing medium-high competence. In general, the strains able to use a wide array of carbon sources were also able to produce diversified extracellular enzyme patterns confirming their high eco-nutritional versatility.

Study on the total bacterial (Eubacteria) community

In order to have a complete overview of the bacterial community structure, by a cultivation-independent approach, total DNA was extracted from the filter-membranes. The bacteria biodiversity was studied by PCR-TGGE fingerprinting of partial 16S-rRNA gene amplicons.

TGGE band patterns were normalized, compared and clustered. showed The community structure was revealed by the cluster analysis of the fingerprints. Samples collected both from the intertidal zone and the nearby sea surface, grouped together (80% similarity). Samples from open sea clearly clustered away. This was particularly evident for the sample collected at -70 m, which branched away at only 40% similarity.

TGGE gel images allow the analysis of band patterns generated from the environmental samples representing the various species present in the community. A single species is identified by a single band and the relative abundance of a single species is determined by the band intensity. Among the KB samples, a total of 70 different banding positions (band classes) were detected. The average number of bands per sample was 26 with a maximum of 30 in the samples from open sea. Simpson's Diversity Index calculated for all the samples showed very high level of diversity. Based on the total number of bands in each TGGE pattern and the relative temperature gradient, range-weighted richness indexes (Rr) were calculated and indicated a very high community biodiversity. Relative bands intensities were also calculated and expressed as percentages of the total band intensity in each TGGE lane. To render a graphic representation of the bacterial communities evenness, Pareto-Lorenz distribution curves were drawn based on the intensities. The functional community redundancy (response to perturbing environmental conditions) is evaluated by the Functional organization index (Fo) considering curves slope. Results showed a balanced community with

medium *Fo* and a medium evenness. In other words, due to the elevated concentration of some species and the availability of many others, the community can potentially deal with environmental conditions changes thus preserving its functionality.

Prominent TGGE bands were excised from the gel, re-amplified and sequenced, to obtain the identities of the community predominant populations. Sequences phylogenetic analysis, showed a great presence of α-proteobacteria (16 sequences out of 27) with some γ-proteobacteria and some actinobacteria too. Some cyanobacteria were revealed also. Among α -proteobacteria strains can be affiliated mainly to the genus *Roseobacter* and one sequence was related to *Ruegeria*. All γ-proteobacteria showed highly similarity with the species of *Cobetia marina,* while all the cyanobacteria were unknown.

Taxonomic results obtained by the total community study were quite different from those gained by the pure cultures: the pure culture strains were completely different (genera and species) from those by 16S rDNA analysis. This could be explained by the limits of TGGE technique. In fact, due to the bias introduced by the PCR reaction, this methodology can only detect bacteria representing at least 1% of the total community. It is possible that some species, even if present in a very low percentage, had prevailed due to favorable culture conditions of the isolation procedures. However, it is known that a large number of marine bacteria are uncultivable.

Another important information was that some bands showed very low sequence identity if compared with sequences present in the database, suggesting the possible presence of unknown bacteria.

Study on Pseudomonas species present in the community

In the TGGE gel relative to the total bacterial community, no *Pseudomonas* species were revealed. By contrast, the majority (ca. 45%) of the cultivable KB strains were affiliated to this genus. In order to find possible explications to this apparent incongruity, further TGGE analyses have been carried out focusing on *Pseudomonas*. Thus, total DNA was used for

PCR-TGGE fingerprinting of partial 16S-rRNA gene amplicons using specific primers for the amplification of this Genus and different temperature gradients.

Again, prominent TGGE bands, excised from the gel, were re-amplified and sequenced. The phylogenetic analysis showed that only 6 sequences out of 22 can be affiliated to *Pseudomonas*. Although the primers used were specific for this genus, possible amplification of other similar 16S rDNA (in particular γ-proteobacteria) could occur. In fact, a big cluster, comprising the majority of the sequences, showed very low similarity with *Pseudomonas* but high similarity with clones of marine invertebrates symbiotic γ-proteobacteria. It is possible that some new genera and/or species were present in our samples.

TGGE patterns were normalized, compared and clustered as reported for the total bacteria community. Cluster analysis of the fingerprints was similar to that obtained for total community. However, in this case sample collected at -70 m, was even more clearly separated from all the others samples: similarity was only 20%.

Analysis of bands number allowed to detect a total of 26 different banding positions

All the statistical indexes, discussed for total bacteria and related to community diversity and organization, in this case are meaningless because they would refer only to a limited portion of the entire population.

To the best of our knowledge, this work represents the first and extensive study carried out on Kandalaksha Bay bacterial community.

Chapter 1

Introduction

The marine environment

Seas and oceans represent the largest expanses of water on the planet. In total, they cover 3.61 x 10^8 km^2 and contain 97% of all the water on earth (Austin, 1988).

A large proportion of all planet life exists in the oceans. Marine ecosystem is huge and it provides a lot of different habitats with a big variety of organisms adapted to its special conditions (Sumich and Morrissey, 2004).

The different habitats, present in sea environment, can be divided into the benthic realm, comprising organisms living on seafloor, and the pelagic realm, including organisms living in the water column (Ross, 1995) (Fig 1.1). The benthic and pelagic environments are subdivided into zones based on depth. As for coastal habitats, in the benthic environment, the shallowest zones are called intertidal and subtidal zones, while for the pelagic environment the shallowest zone is the photic or surface zone, that extends from the shore line to the edge of the continental shelf. Most of marine life has been found in these coastal habitats, even though the shelf area occupies only 7% of the total ocean area. However it is recently recognized that life is abundant in the deep oceans also.

The physical and chemical structure of the oceans and the variations of factors, such as temperature, light, pressure, salinity and nutrient concentration, create distinct conditions to which organisms are adapted (Figure 1.2).

Temperature has an important role in the organisms distribution in the sea, since various biological processes depends on this factor. Solar radiation leads to thermal stratification: while on sea surface temperature can reach 25-30°C (tropical seas), at 100-150 m depth there is a marked thermocline where temperature strongly decreases (10°C or less) reaching almost 4°C in the benthic environment (Munn, 2011).

Light is another factor limiting organisms presence in the sea especially those responsible for primary productivity: photosynthesis is restricted in the upper 150-200 m of the sea surface

(photic zone) and various adaptation to different light regimes are needed by photosynthetic organisms (Sumich and Morrissey, 2004).

Pressure at sea surface is ca. 1 atm and increases of 1 atm every 10 m, thus only organisms that evolved special adaptation can inhabits deepest water. The majority of organisms living in these areas are bacteria and Archea even if presence of some fishes and invertebrates is reported (Gage and Tyler, 1992).

Seawater is a mixture of inorganic and organic compounds, including nutrients, and dissolved gases (O_2, CO_2 etc.). Their concentration varies according to geographical and physical factors and is responsible for organisms distribution in sea environments. As for chemical elements, they are responsible for salinity that is on average 35‰. Salinity increases from coastal to open sea and with depth: this gradient affects organisms presence depending on their tolerance (Austin, 1988). Dissolved oxygen plays an important role in the surviving of organisms, since it is present at high concentration only in the top 10-20 m of water, while it decreases with depth until it reaches a minimum between 200 and 1000 m due to its consumption by aerobic bacteria. Below this depth it increases again because oxygen consumption rates are low in relation with the supply trough cold, oxygen-rich deep waters from polar regions (Munn, 2011). In addition, the high abyssal hydrostatic pressure supports increased oxygen solubilization. Seawater presents very low nutrients concentration and ocean primary production is limited by their availability, affecting all the food chain. Essential nutrients such as nitrogen and phosphorus are normally at low concentration and are often the principal limiting factors even if recent studies demonstrates that also iron could limit primary production (Moore et al, 2001; Speight and Henderson, 2010).

Marine organisms are a vast resource, providing food, medicine, and raw materials. They contribute to important biogeochemical cycles (i.e. oxygen and carbon) and they show peculiar adaptation to the huge variability of sea conditions.

Even if the role of marine organisms and the knowledge of their ecology is rapidly increasing, large areas beneath the oceans still remain actually unexplored.

Figure 1.1 Divisions of the marine environment as reported by Ross (1995)

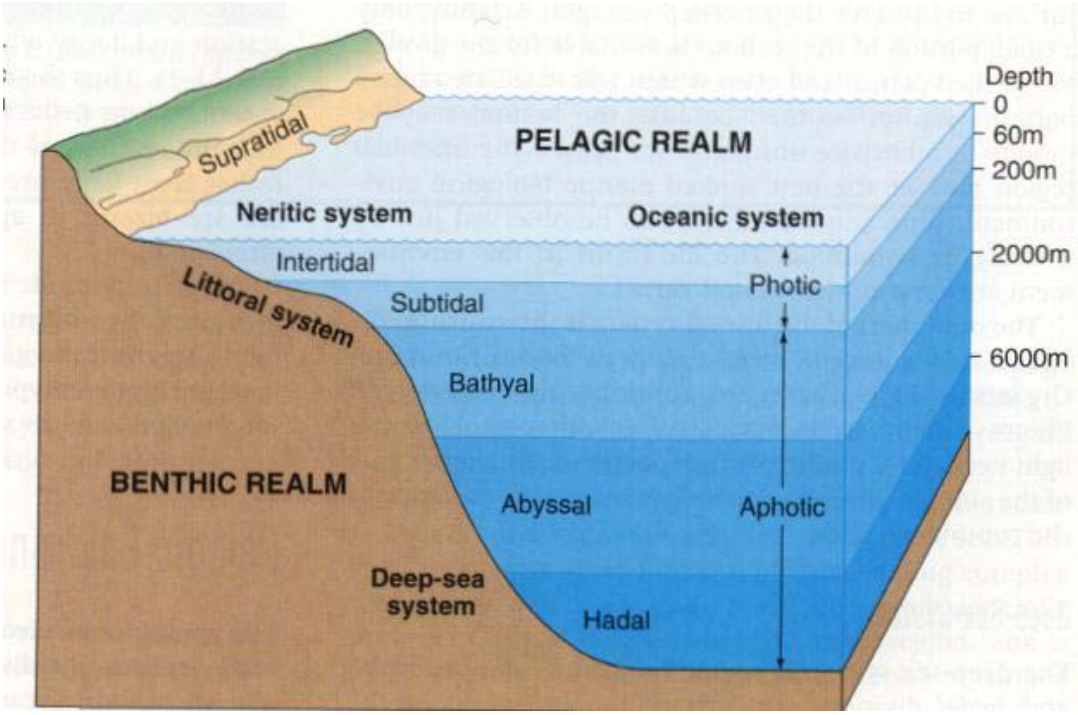
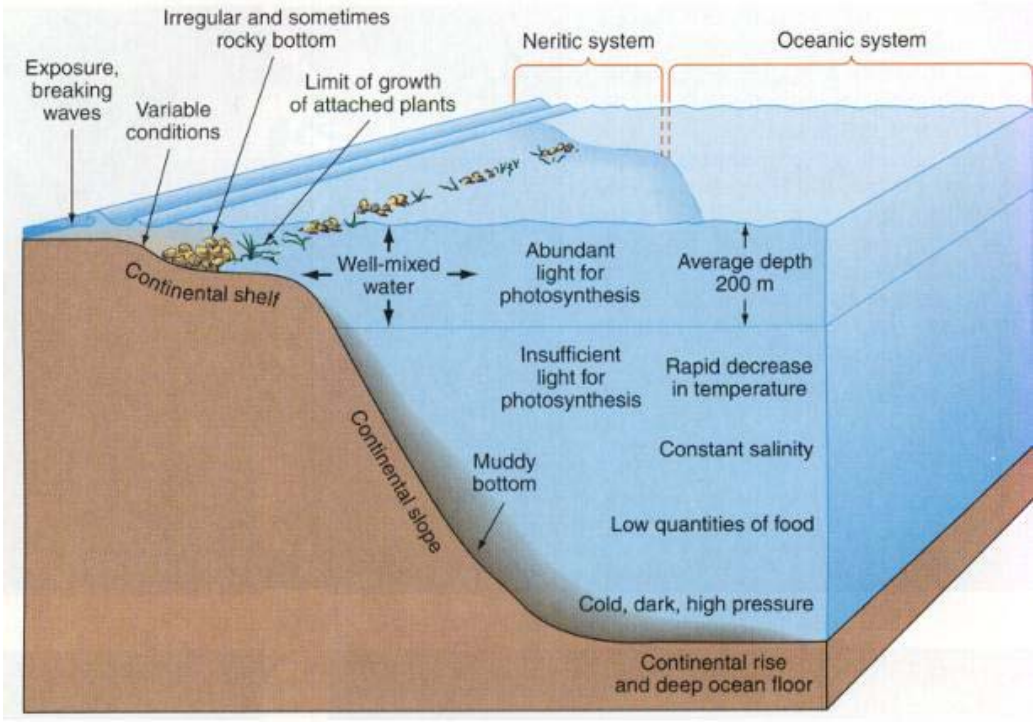


Figure 1.2 General characteristics of the marine environment as reported by Ross (1995)



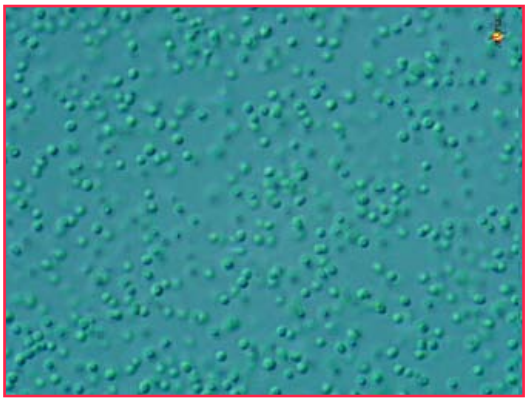
1.1 Marine microorganisms

Marine environment is a huge source of biological diversity and unexploited and/or unknown microorganisms. Nevertheless, investigation of seawater microorganisms for physiological, ecological and biotechnological studies is still scarce if compared with other environments (Pomponi,1999; Fenice et al., 2007).The main reasons for this lack of knowledge are probably the huge dimension of this environment, the difficulty of sampling in extreme habitats (i.e. ocean floor, hyper-thermophilic environments) and the problems to reproduce some microorganisms living conditions. Moreover, it is worth nothing that the majority of marine microorganisms, as occurs for other environments, cannot be isolated in pure cultures (Lewis, 2007). However, in the last decades, application of new technologies allowed a better knowledge of sea microorganisms. Powerful new tools in molecular biology, remote sensing and deep-sea explorations have led to important discoveries concerning the abundance and diversity of marine microbial life. Many distinct marine microbial ecosystems have been identified and studied. The most important microbial characteristic is the biodiversity and the ability to occupy habitats (i.e. polar or deep-seas and hydrothermal vents) generally inhospitable for any other organism. In fact, marine microorganisms growth occurs in all oceanic habitats from the seafloor to the top of ocean surface. Moreover, there is a growing awareness of the role of marine microorganisms in biogeochemical processes and their importance for the ocean food chain. For example, Cyanobacteria are major responsible of carbon organication and O_2 production. It has been calculated that the picocyanobacteria *Prochlorococcus* spp. and *Synechoccus* spp. contribute to ca. 50% of the photosynthetic primary production in oceans (McIntyre, 2010) (Figure 1.3). Then, microorganisms can carry out metabolic processes such as biotransformation and degradation of organic matter (including a wide array of pollutants and recalcitrant compounds) and recycling of nutrients that have a central role for maintenance and support of all other life forms (Fowler et al.,

1987).Furthermore, they are responsible for planetary processes such as changes in the atmosphere, water and soil composition (Riebesell et al., 2007)

For all these reasons a deeper understanding of the distributions of marine microorganisms and their activities in maintaining ecosystem functions is needed.

Figure 1.3 Microscope image of *Prochlorococcus marinus* (Image copyright: Bob Andersen and D. J. Patterson)



1.1.1 Microorganisms and marine habitats

As discussed, microorganisms are well represented in all the various marine habitats (Figures 1.4 and 1.5) as already reported by the early work of Sieburt (1979).

First of all, they constitute the major part of Plankton (small/microscopic organisms suspended in the water). Traditionally, plankton is divided in phyto- and zoo-plankton mainly taking into consideration animal and plants only. However, it is recognized that bacteria are an important part of plankton biomass. They have a basic role in planktonic marine microbial food webs and, being important agents of biogeochemical processes, their activity has a large impact on the entire ecosystem (Giovannoni et al., 1990; Gasol et al., 1999).

Microorganisms are also present on Neuston (the sea- air interface) that is rich in organic matter and, consequently, of microbial life. However, from some studies, carried out on coastal environments, there is no evidence of a stable an unique Neuston community, even

though it is possible that some very specialized bacteria, adapted to intense solar radiation, are prevalent (Maki, 2003, Agogue et al., 2005).

Surface of swimming animals(Nekton) as well as their digestive system (endobiotic environments)are widely colonized by microorganisms. Microbial biofilms occur on surfaces of all kind of animals and plants that, secreting organic compounds, provide nutrients. Many organisms seem to selectively enhance surface colonization by certain microorganisms and inhibit microbial growth or attachment of others trough the production of specific compounds (Jensen and Fenical 1994). Association among microorganisms and marine animals could be neutral or leading to mutual benefits (symbiosis) (Dubilier et al,. 2001, Goffredi et al., 2008). However, these microbial communities often strongly affect the health of marine animals and are important in fish spoilage (Banin et al., 2000; Gram and Dalgaard, 2002; Maloy et al., 2007).

The sea-sediment interface, including plants and animals, (Benthos) is widely colonized by microorganisms. The presence in the sediment of particulate organic debris and dissolved organic compounds lead to intense microbial activities. These activities are of paramount importance for the planetary carbon cycle but also for production/oxidation of methane and oxidation/reduction of sulfate (Iversen and Joergensen, 1985; Aller and Rude, 1988; Kirschner and Velimirov, 1999; Valentine, 2002). Concerning benthonic organisms, same consideration discussed for Nekton about biofilms formation and symbioses could be done.

Microorganisms are also present in Seston (particulate matter).A continue flow of matter, which falls through the water column (marine snow, Figure 1.6), consists of aggregates of inorganic particles, plankton cells (including bacteria), debris from dead or dying plankton and fecal material glued together by a polymeric matrix produced by bacteria and phytoplankton (Alldredge and Silver, 1988).Marine snow is mainly produced in the upper part of the water column (100-200 m); large particles can sink up to various hundred meters per day reaching the ocean bottom in matter of days. This is the main mechanism by which a part

of the primary production is transported from the surface layers to deeper waters and see floor (Munn, 2011).During particle sinking organic material is degraded by extracellular enzymes and active microorganisms (Karner and Herndl, 1992). Microbial aerobic respiration creates anoxic conditions; therefore aerobic and anaerobic microorganisms coexist in different niches within the snow particle. The great rate of solubilization, due to microorganism activities, leads to formation of dissolved nutrients that can be utilized by other organisms and planktonic bacteria. Thus the majority of carbon rate is re-mineralized during its descent. However, the remaining material reaches the ocean floor where it is consumed by benthic organisms or contributes to sediment formation (Munn, 2011).

All solid materials in water, including plants, animals, rocks and anthropic structures, are colonized by microorganisms forming complexes called biofilms (epibiotic habitats). These structures are constituted by sessile communities that develop morphological and physiological characteristics differentiated from free-living forms. These communities are attached to solid surfaces by secretion of an extracellular polymeric matrix entrapping organic and inorganic components. In this matrix, complex physiochemical processes lead to formation of microenvironments colonized by mixed microbial communities. Biofilms are particularly important in shallow and intertidal waters and are interesting for their complex ecological interactions (Donlan, 2002; Munn, 2011).

Sea microorganisms are important also for their ability to live in extreme habitats were life for other organisms is difficult and/or impossible.

Deep-sea environments present conditions of low temperatures, oligotrophy and high pressure, thus various efficient adaptation strategies are needed. The majority of microorganisms living in these areas are Bacteria and Archea. They are found to be doubly extremophiles being both piezophilic and, generally, psychrophiles (Kato et al., 1995, Somero, 1992). Synergistic adaptation strategies are necessary. One of the most important effect produced by high pressures is a change in protein conformation that become less

flexible and less subject to compression. Furthermore, piezophiles show control mechanisms of gene expression regulated by pressure. As for adaptation to nutrient low concentration, some bacteria (*Photobacterium profundum* and *Shewanella* sp.) produce high level of outer membrane proteins to increase transport providing a larger channel for easier nutrients assimilation (Bartlett, 1999, Munn, 2011). Another important adaptation, common both to pressures and to temperature, is the modified membrane composition. In fact, in all the microorganisms living in deep cold seas, the ratio of total unsaturated versus saturated fatty acids in the membrane lipids increased. This adaptation appear to be functional in maintaining membrane fluidity within a high range of pressures and at typical deep sea temperatures (Wirsen et al., 1986; Di Giulio, 2005).

In the ocean bottom a peculiar habitats is also constituted by hydrothermal systems (Figure 1.7). Hydrothermal vents are fissures in earth surface from which geothermally heated water springs. They are typical formations in the seafloor along the mid-ocean ridges, where two tectonic plates are diverging and new crust is being formed. The water emerging from hydrothermal vents consists mostly of sea water that permeated into the crust and had interacted with hot rocks or magma acquiring mineral elements. Two kind of vents has been discovered in the oceans up to ca. 5000 m of depth. The white smokers consist in warm water (20-23 °C) carrying low amount of minerals. Black smokers waters are very hot (350 °C) and transport lot of mineral elements such as metal sulfides, iron and manganese oxides and silicates that precipitate around the vent building a chimney structures. The gradient of temperatures and nutrients nearby the hydrothermal system create a wide array of water and sediments habitats colonized by microorganisms. Some of them are hyperthermophilic-chemosynthetic bacteria able to use sulfur compounds (i.e. hydrogen sulfide) to produce energyand obtain organic material by CO_2 fixation. This metabolism supports a small food chain of this area that is hence independent from photosynthetic processes. For these microorganisms adaptation to high temperatures is needed also. Peculiar conformation of

protein permits a better stability of enzymes, while membranes with monolayer of isoprene-like units (in Archea, only) increase their stability (Aller and Rude, 1988; Jørgensen et al., 1992; Martin et al., 2008).

Microorganisms have also an important role for food chain in sea ice of polar regions. Sea ice is an ecosystem in which both physical phenomena and biological activity help to shape the overall habitat. Ecological and biological structures in sea ice are essentially driven by the prevailing temperature and salinity that are temporally and spatially highly variable. The structure of sea ice provides a wide array of different microhabitats that can be colonized by mixed communities. Sea ice microbial populations have a major influence on various trophic levels of the oceanic food web. They are particularly abundant in surface layers, within ice floes, and concentrated in ice-seawater interface. Bacterial populations are tightly coupled to primary productivity levels in sea ice. Microalgae photosynthesis contribute significantly to carbon fixation, while mineralization of dissolved organic matter is carried out primarily through the action of heterotrophic bacteria (Grossmann and Dieckmann, 1994; Arrigo and Thomas, 2004).

Although lot of information is available concerning various marine peculiar habitats, to date, a wide array of marine environments are still unknown under the microbiological point of view.

Figure 1.4 Schematic representation of the habitats for marine microorganisms based on Sieburth (1979)

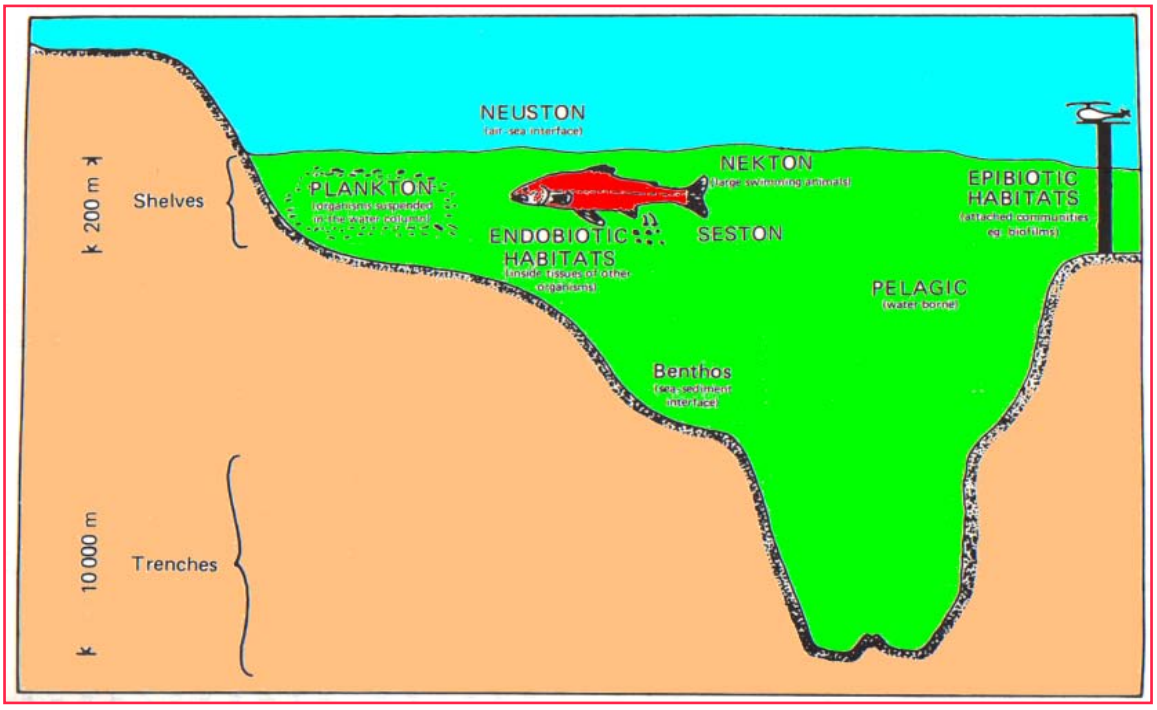


Figure 1.5 Schematic representation of the major ecological zones and habitats for marine microorganisms based on Munn (2011)

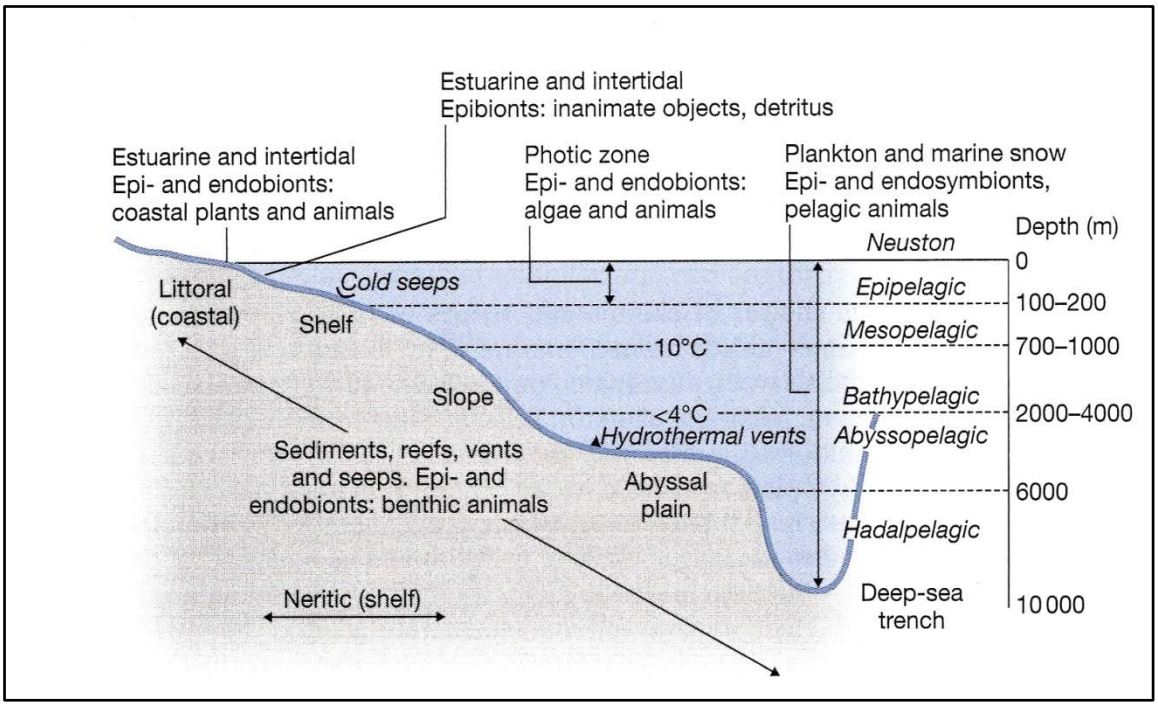


Figure 1.6 Schematic diagram of microbial processes during formation and fall of marine snow as reported by Munn (2011)

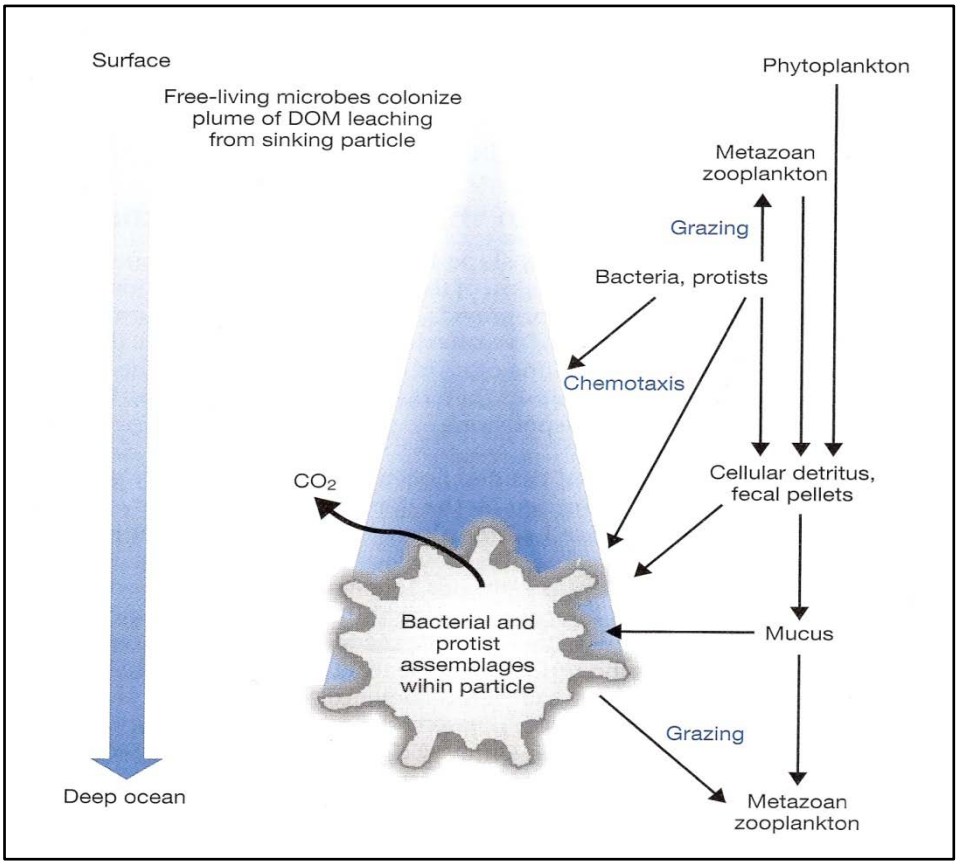
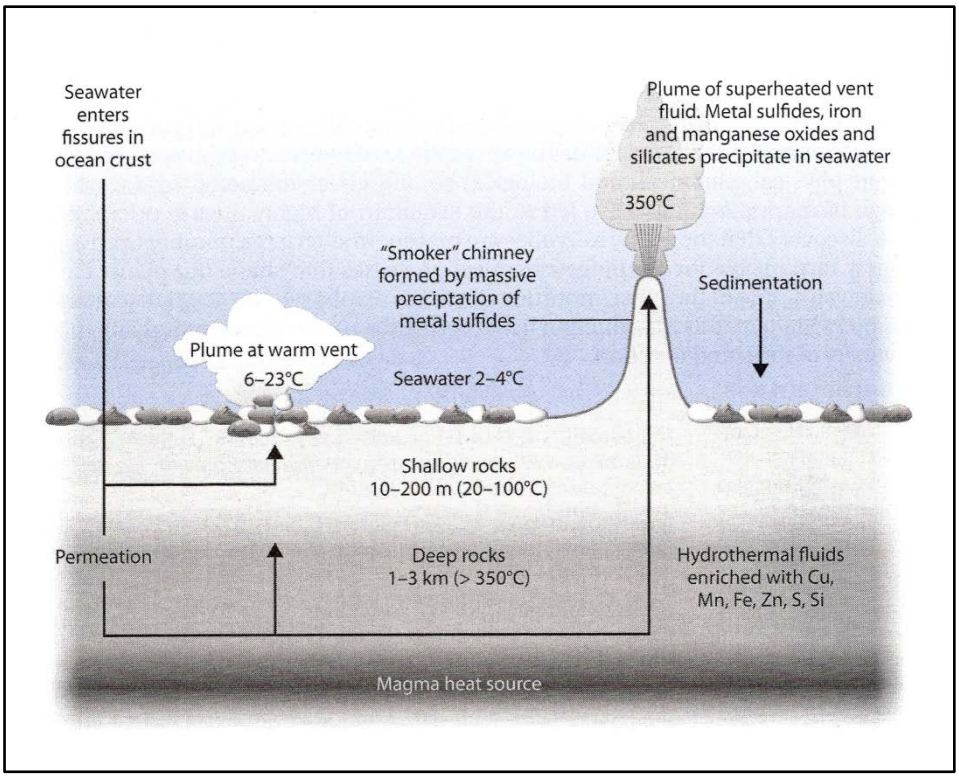


Figure 1.7 Processes occurring at hydrothermal vent system as reported by Munn (2011)



1.1.2 Marine microorganisms and biotechnology

Biotechnology is an applied discipline that can be defined as "*the integrated use of biochemistry, microbiology and engineering sciences in order to achieve the technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof*". Biotechnology find applications in major industrial areas, including health care, food production, agriculture, fine chemicals, biodegradable plastics, oils and biofuels, and for environmental uses.

Seas and oceans represent a pool of extremely interesting environments also for biotechnological studies. While much of the attention for this kind of investigations so far has been focused on others microbial ecosystems, marine environments remain largely unstudied and unexploited. Therefore, the enormous marine biodiversity still represents a source of microorganisms for a wide number of potential industrial and/or environmental applications (Fenice et al., 2007; Kennedy et al,. 2008).

Enzyme are among the most utilized compounds in biotechnology. The use of enzymes increased in the last 20 years, as well as their commercial value: total market for industrial enzymes reached $2.5 billion in the last few years reaching $6 billion in 2010 (Demain and Vaishnav, 2009; Munn, 2011). Strong increment of enzymes use was recorded not only in traditional fields (food and detergent industries), but in more emergent applications such as environmental depollution, pharmaceutical, bio-medical and chemical industries also (Fenice et al., 2007; Munn, 2011). Since various studies showed that marine bacteria are capable of producing unusual bioactive compounds, that are not observed in terrestrial sources, there is growing interest in obtaining higher value biochemicals from marine environments (Jha and Zi-rong, 2004, Debashish et al., 2005). The most commonly exploited enzymes are those that degrade polymers as proteins and carbohydrates. Marine bacteria can hydrolyze a wide array of polymers and particles using hydrolytic enzymes such as proteases, glucosidases, lipases, phosphatases, pectinase and chitinases (Hoppe, 2003;

Obayashi and Suzuki, 2008). Hence, oceans and seas can be valid alternatives to search microorganisms with high biodegradative properties. In particular, marine extreme environments represents a peculiar source of microorganisms showing activities of potential interest due to their adaptation to harsh conditions (Rothschild and Mancinelli, 2001; Bai et al., 2006; Srinivas et al., 2009). In fact, many successful biotechnological applications are due to enzymes from extremophiles (extremozymes). Many industrial processes often requires high temperatures, thus thermophilic enzymes can be useful in these processes due to their greater stability at these conditions. For example, thermo stable proteases and lipases are used in detergent formulation as stain removers. Their thermo-stability is often combined to resistance to the bleaching chemicals and surfactants present also in these products (Aaslyng et al., 1991, Munn, 2011). Enzymes from psychrophilic microorganisms, isolated from deep-sea sediments and/or polar regions, are useful in food-processing in which low temperatures are required to prevent spoilage and destruction of key ingredients as vitamins (van den Burg,, 2003).

Microbial bio-polymers found application in many industrial processes. For example, a wide variety of microorganisms are known to produce intracellular storage energy and carbon products in form of polyhydroxyalkanoates (PHA).The evaluation of PHA polyesters as natural, biodegradable, and biocompatible plastics for a wide range of possible applications, such as surgical sutures or packaging containers, was already reviewd by the early work of Brandl(1990).

Since global economy depends on oil for energy and chemicals, fossil sources of oil are subjected to overexploitation thus there is increasing interest in the use of renewable materials, such as land crops, for production of biofuels. However production costs and heavy ecological/social consequences make crops use unsustainable. Therefore, there is growing interest in use microalgae, including marine species, to produce biofuels. Microalgae are easy to cultivate, requiring only sunlight and simple nutrients, and they grow efficiently. Lipids

and fatty acids stored in the cell as energy source can be converted into a variety of compounds with similar properties of petroleum products including biodiesel (Chisti, 2007; Munn 2011)

Microbial products provide many compounds for medicine and health care. The pharmaceutical industry is constantly searching for new compounds and many of the most successful drugs (i.e. antibiotics) are bacterial secondary metabolites. Even if, traditionally, investigation have been carried out mainly on terrestrial environments, the huge biodiversity of marine microorganisms led to a recent focus on sea organisms as source of novel compounds. Many new bioactive products are cyanobacterial peptides presenting huge diversity in structures and activity in different strains permitting to obtain a great variety of compounds with different pharmaceutical properties (Welker and Von Döhren, 2006; Gerwick et al., 2008).

Marine microorganisms can find application also in biodegradation and bioremediation of many chemical pollutant including oil spills. Oil tankers pollution produce immediate damaging on marine life but also long-term disruption of ecosystem communities. There are various strategies to deal with accidental spills. However, naturally occurring microorganisms, most of all bacteria, are able to degrade most of oil components especially hydrocarbons. The metabolic processes used by bacteria for biodegradation are mainly aerobic, being oxygenases (mono- and di-oxygenases) the key enzymes for degradation of paraffins and cleavage of aromatics. Since petroleum provide only the carbon source (hydrocarbons), these processes can be increased by supplementation of nitrogen, phosphorus and other elements (Kanaly and Harayama, 2000; McKew et al., 2007)

Many marine microorganisms are also able to degrade recalcitrant organic pollutant such as DDT, polychlorinated biphenyls (PCB), dioxine and furans. These substances are very persistent and resistant to biological and chemical degradation. However, aerobic and

anaerobic microbial degradation of various pollutants has been reported (Borja et al., 2005; Begonja Kolar et al., 2007).

1.1.3 Study of marine microorganisms by molecular methods

Already from the very early studies, it was clear that only a small fraction of marine bacteria can be isolated and cultivated in pure cultures. In fact, big differences were recorded between microscopic observations and counts (Figure 1.8; Simidu et al., 1983). This concept has been extended to other environments and recent studies confirm that the majority (99%) of microorganisms in nature are uncultivable or fail to grow in laboratory media (Wayne et al., 1987; Ward et al., 1992; Lewis, 2007). Possible reason could be the difficulty to reproduce natural growth conditions in laboratory due to lack of knowledge on strain physiology and adaptation strategies to peculiar environments (Amman et al., 1995; Muyzer 1999; Munn 2001). In addition, most bacteria are subjected to dormancy or inanition and could be very difficult to revitalize them (Lewis, 2007). Moreover, various symbiotic microorganism could require presence of their host to grow (Fisher, 1990). Thus, to obtain a complete overview of presence, diversity and role of microorganisms in the ecosystem, other approaches are needed to complement traditional culture-based microbiological methods. The application of molecular biology techniques have revolutionized the knowledge of environmental microbial diversity, offering new opportunities to analyze structure and species composition of microbial communities. In particular, nucleic acid based methods, allowed the detection and identification of organisms based on their genetic sequence. These culture-independent molecular techniques are based on direct extraction of genetic material from the environment, without culturing. The most widely used tool for microbial diversity investigation is the study of ribosomal RNA genes. In fact, the rRNA molecules(16S for bacteria) in the small ribosomal subunit (SSU) are universal in organisms, due to the role of ribosomes in protein synthesis. Furthermore, rRNA can be considered as phylogenetic maker for its very slow mutations rate. The first steps in all nucleic acid investigations are genomic

DNA extraction and amplification of specific region in the target gene by polymerase chain reaction (PCR). In rRNA molecules, some parts are highly conserved and can be amplified using the so called universal primers. Other fragments, present among these regions, show high degree of variability and are proved to be species specific. Thus, they can be used to study the different species present in a given environment and to determine structure and diversity in a microbial community.PCR products can be analyzed by cloning or genetic fingerprints. In cloning techniques, PCR products can be cloned in *E. coli* to create a gene library. Characterization of cloned sequences enables assessment of the genetic diversity of a community and can reveal the phylogenetic affiliation of the community members (Muyzer and Ramsing, 1995; Ranjard et al., 2000). Identification of particular populations is obtained by genetic fingerprints also. However, these techniques are particularly useful to get information on structure and dynamics of a community, based on the number and rank abundance of numerically common organisms (Bent and Forney, 2008).

Figure 1.8 Discrepancy between microscopic count and viable count observed by Simidu et al. 1983

Table 3.1 *Bacterial populations in water of the South China Sea, as determined by direct counts, direct viable counts, i.e. involving use of nalidixic acid, and by a spread plate technique*

Depth at which sample was obtained (m)	Total microscopic count (/ml)	Direct viable count (/ml)	No. of aerobic heterotrophic bacteria by spread plating (/ml)
Station 1			
0	1.2×10^6	8.2×10^4	1.2×10^2
100	6.9×10^5	5.4×10^3	5.5×10^1
600	2.0×10^5	2.4×10^3	8.0×10^0
Station 2			
0	9.1×10^5	4.2×10^4	2.1×10^2
100	6.5×10^5	2.1×10^4	5.3×10^1
600	2.6×10^5	2.4×10^3	1.6×10^1

From Simidu *et al.* (1983).

1.1.3.1 Genetic fingerprint techniques

These techniques are based on the principle of resolving the diversity, in size or composition, of amplified sequences by differential electrophoretic migration on agarose or polyacrylamide gels (Ranjard et al., 2000). Genetic fingerprints provide complex band profiles which are representative of the genetic community structure. Providing a relatively comprehensive description of a given community, they have been widely adopted. In particular, these techniques are extremely suitable to compare microbial community compositions in different environments (Justé et al., 2008). The most important fingerprints techniques utilized in microbial ecology are discussed below. However, since PCR-DGGE/TGGE has been used in this study it will be discussed in a separate paragraph.

Single-strand conformation polymorphism (SSCP)

This method allows separation of different DNA fragments of similar length based on conformational differences of folded single-stranded products. After denaturation, single stranded DNA fragments are loaded on a non-denaturing acrylamide gel that allows the formation of a stable secondary structure which is mainly determined by the intra-molecular interactions depending on the nucleotide sequence. Based on the migration of these secondary structures, products with similar molecular weight can be separated and visualized. Major limitation of SSCP is the formation of several different stable conformations of a single stranded DNA fragment, resulting in multiple bands on gel (Tiedje et al., 1999). Furthermore, sometime high rate of re-annealing of DNA strands during electrophoresis was observed regenerating double strand DNA (Selvakumar et al., 1997).

Terminal restriction fragment length polymorphism(T-FRLP)

Target genes are amplified with fluorescent primers and treated with specific restriction enzymes. Fragments are separated and detected on an automated sequencer (Liu et al., 1997). Only terminal labeled restriction fragments (TRFs) are detected and their length heterogeneity indicates community complexity. An internal size standard, labeled with a different

fluorescent dye, allows precise length assignment with single-base pair resolution. Because the use of a single restriction enzyme often does not provide sufficient resolution (Marsh, 2005), multiple restriction enzymes are typically used, increasing the specificity and the reliability of the assay (Osborne et al., 2006). Despite the high resolution and sensitivity, T-RFLP is highly dependent on 16S rRNA amplification which is affected by the DNA extraction method, PCR biases and the choice of universal primers (Kirk et al., 2004). Different enzymes will produce different community fingerprints and incomplete digestion by the restriction enzymes may lead to an overestimation of diversity (Dunbar et al., 1999; Osborn et al., 2000).

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism, also known as amplified ribosomal DNA restriction analysis (ARDRA) is a relatively simple PCR-based fingerprinting technique centered on the digestion of amplified ribosomal community DNA followed by gel electrophoresis. RFLP can be used for microbial identification (Laguerre et al., 1994) or comparison of microbial communities and dynamics (Moyer et al.,1994). Differently from T-RFLP, all digested fragments are detected, increasing resolution. One single restriction enzyme generally does not provide sufficient resolution, and multiple restriction enzymes is needed. The main limitation of this method lies in the choice of restriction enzymes, which is crucial for obtaining optimal resolution (Ranjard et al., 2000).

Random amplified polymorphic DNA (RAPD)

The RAPD technique uses short random primers (about 10 bp) which anneal at different places on the genomic DNA, generating PCR products of various lengths further resolved on agarose or acrylamide gels. This technique is rapid and sensitive to reveal differences between similar complex prokaryotic genomes. Even if, some studies have evidenced possible lack of reproducibility due to its great sensitivity to PCR artifacts (Hadrys et al., 1992),sufficient reproducibility has been demonstrated to compare the genetic structure of complex bacterial

communities (Wikström et al., 1999). The main limitation is that it cannot provide phylogenetic information about the bacterial composition of the community (Ranjard, 2000).

Ribosomal intergenic spacer analysis (RISA)

RISA involves the analysis of the length polymorphism of the spacer between 16S and 23S genes. This intergenic spacer (IGS) region is species specific and successive sequencing of bands can allow taxonomic identification of definite populations within a community. The various amplified IGSs are directly separated on polyacrylamide gels by their size. To increase resolution, fluorescent primers can be used to further run RISA profiles on sequencing gel systems. Potential problems associated with RISA are the preferential amplification of shorter templates (Fisher and Triplett, 1999). Moreover, due to IGS length variation within a single genome, a single organism can contribute to more than one signal (Mateos and Markow, 2005).

1.1.3.2 PCR-DGGE/TGGE fingerprints analysis

Both denaturing gradient gel electrophoresis (DGGE) andtemperature gradient gel electrophoresis (TGGE) examine microbial diversity of small PCR-amplified DNA fragments (200−700 bp) by poly-acrylamide gel electrophoresis (PAGE) under denaturing gradient (Muyzer et al., 1993; Muyzer and Smalla, 1998). DGGE uses denaturing chemicals such as formamide and urea, while a temperature gradient is applied in TGGE. Both techniques allows the separation of DNA fragments having the same length but different composition/sequences. Although gradient conditions need optimization to resolve specific samples (Ogier et al., 2002), these methods have the theoretical potential to detect differences of as little as a single or a few base pairs.

In TGGE, separation is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules submitted to a linear temperature gradient. DNA fragments melting proceeds in discrete *"melting domains"*:stretches of base-pairs with identical melting temperature. When a domain reaches its melting temperature (*Tm*), at a particular position in

the temperature gradient gel, a transition from helical to a partially melted molecule occurs causing its migration stop. Sequence variations within such domains causes different melting temperatures, thus molecules with different sequences will stop at different positions. Generally, the melting behavior depends on the length of the product, its GC-content and nucleotide sequence.

By contrast, DGGE works at a fix temperature, thus, melting depends upon a chemical gradient.

For both these techniques, a sequence of guanines and cytosines (GC-clamp) is commonly added to the 5'end of one PCR primers, co-amplified and thus introduced into the amplified DNA fragments. The GC rich sequence is a high melting domain preventing the two DNA strands from complete dissociation into single strands (Sheffield et al., 1989).

Detection of correct size fragment of the 16S rRNA gene hypervariable regions usually requires a two-stage amplification (nested PCR). In fact, direct use of primers to amplify environmental DNA, often cause unspecific priming. Moreover, nested PCR improves amplification of scarcely represented species and yields richer band patterns (Nicolaisen and Ramsing, 2002; Ward and O'Mullan, 2002). For this reason, a first 16SrRNAgene PCR should be followed by a second PCR for the hypervariable regions. Furthermore, a PCR touch-down protocol during the second amplification is advisable (Watanabe et al., 2001). This technique consists in decreasing the annealing temperature, during PCR cycles, to avoid amplification of spurious DNA fragments (non-rRNA gene fragments and/or fragments with improper sizes).

Fragments separation generates samples profiles, representing the various species in the community being a single species revealed by a single band. Band intensities represent the relative abundance of each species. However, being PCR-based techniques, DGGE and TGGE detects only populations representing at least 1% of the total community targeted by

the primers (Muyzer, 1999). Therefore, the identified populations are only the most numerically predominant in the microbial community.

DGGE/TGGE are powerful tools to compare structural changes in microbial communities and for monitoring population dynamics (Sun et al., 2004; Camu et al., 2007). Several regions of the 16S rRNA gene have been used for DGGE/TGGE fingerprinting (Ercolini et al., 2003). However, length and species-specific heterogeneity of the V3 region within this gene make this fragment one of the better choices (Florez and Mayo, 2006). Furthermore, individual bands can be excised from the gel and identified by sequencing. However, reliable identification by sequencing may be hampered by the small fragment sizes of PCR products, which might not contain enough information for precise taxonomic classification (Øvreås, 2000). In addition, different sequences may have identical electrophoretic mobility, resulting in co-migration of different fragments (Sekiguchi et al., 2001). A major drawback often associated with DGGE/TGGE is lack of reproducibility. Actually, handling of big gels, primer–dimer formation and variable gel staining could affect reproducibility (Powell et al., 2005). However, these problems could be minimized by the inclusion of an internal standard, facilitating samples normalization within and between gels (Neufeld and Mohn, 2005).

1.2 The White Sea

The White Sea (Figure 1.9) is located in the north-west of Russia, in Artic Sea basin. It can be considered a semi-enclosed sea which occupies a long gulf located south-east of the Kola Peninsula. It is joined to the Barents Sea by a strait between Cape Kanin and the Kola peninsula. The White Sea covers approximately 95 000 km^2 and a total volume of 6.000 km^3 (Zubov et al., 1956; Dobrosovol'skii et al., 1982).

The northern part, 40% of the total area, presents three regions (the Voronka, the Gorlo, and Mezen' Bay) showing characteristic rather low depths (30–50 m). The southern part includes the Basin and three bays (Dvina, Onega, and Kandalaksha). A deep depression is located here with a maximum depth of 350 m; this occupies a small volume but plays a very important role in the White Sea ecosystem (Klenova, 1966).

The bays of the White Sea significantly differ in their morphometric properties. Mezen' Bay has a very limited depth (ca. 10 m). The maximal tides of the White Sea are observed in this region, causing great cyclic variations in depths and horizontal displacements of the coastline.

Dvina Bay is distinguished for having the most regular morphometric structure. The basin of the bay is open to the sea and here depths gradually increase reaching about 100 m.

In contrast, Onega Bay is characterized by a complex morphometric structure. The near-shore zone is shallow and has a typical skerry structure. Besides islands and "ludas" (small stony islands), there are many underwater and emerging rocks. The largest archipelago of the White Sea, the Solovets Archipelago, is located there.

Kandalaksha Bay notably differs from the other basin its form being more elongated and having a limited width. The coasts of the bay present numerous inlets resembling fjords caused by glacial erosion. The longest inlet of Kandalaksha Bay, Chupa Inlet, is 37 km long, while its mean width is 1–2 km and the depths of the depressions are approximately 65 m. Concerning the bottom topography, Kandalaksha Bay is divided into two parts. The inner part

is characterized by depth shallower than 50 m and an uneven relief, consisting of a complex alternation of depressions with depths gradually decreasing towards the apex of the bay (80 m, 50 m, and 30 m). The outer part of the bay is separated from the inner part by a sharp depth increase. The deepest point of the White Sea (335 m) is located there, off the Turii Peninsula (Pantyulin, 2003).

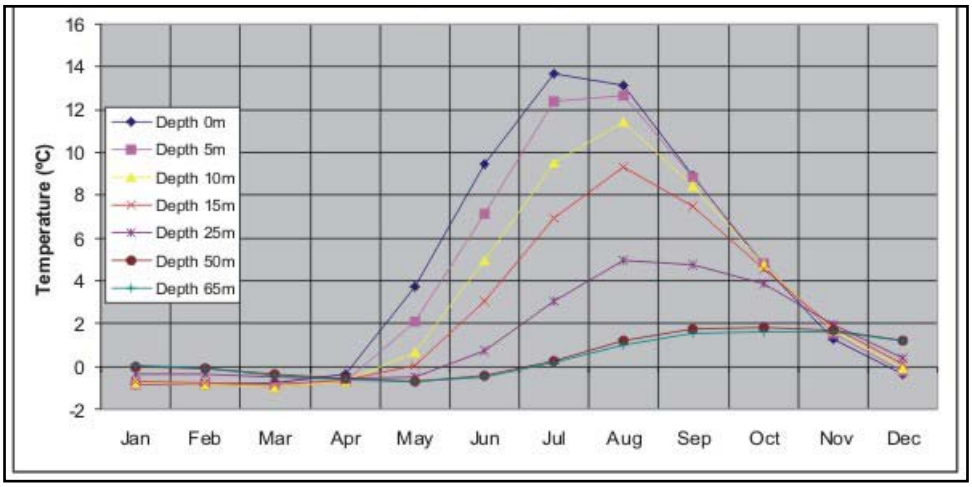
Figure 1.9 The White Sea

Temperature in the White Sea

The White Sea is located in the very north of the temperate climatic zone, but it shows subarctic climate characteristics. The climate is characterized by spatial variability and significant seasonal and inter-annual variations. However, Kandalaksha Bay, being located around the Arctic Polar Circle and above, could be considered as the most extreme area within White Sea.

Temperature is one of the crucial abiotic environmental factor determining the development and seasonal dynamic of many organisms: this has been widely demonstrated for White Sea also (Ushakova, 2003; Zubakha and Usov, 2004; Kuts and Ismailov, 2009). Temperature fluctuations (Figure 1.10) in White Sea and Kandalaksha Bay are quite broad and in the littoral zone they are even more evident (Savvichev et al., 2003). Winter is harsh and long (sea surface is covered with ice 6–7 months per year) and wheatear conditions could be very unpredictable. Temperature in winter may fall to -40°C, even if sometimes warm Atlantic air raises the temperature to ca. 5°C, while water temperature is about -1°C to -2°C. In summer, air could reach 30°C, albeit the average is in between 15-20°C, while sea water could reach 15°C on surface layers (Berger and Gorbushin, 2001; Shaporenko et al., 2005; Vershinin et al., 2006). In these extremely variable conditions, adaptation to wide temperature changes could be a winning surviving strategy as reported for microorganisms isolated in other extreme environments (Zucconi et al., 1996; Zhang et al., 2007; Srinivas et al., 2009).

Fig.1.10 Average annual temperature in White Sea



Freshwater contribution in the White Sea (Figure 1.11)

Freshwaters are transported to the sea mainly from river runoff and atmospheric precipitations. River runoff is the main input component of the freshwater budget in the White Sea (Pantyulin, 2003) and is largely due to three principal rivers Severnaya Dvina, Mezen', and Onega.

The White Sea rivers are predominantly fed by snow melting, with a characteristic regime of spring floods and winter low-water periods. However, they differ in inter-annual runoff distribution. During the long winters (October–April) the White Sea bays freeze over (Klenova, 1966) and freshwater runoff to White Sea is restricted. The May/June melt brings a short period of extreme flood, which slowly decreases to the winter values (Gordeev&Sidorov, 1993).

Annually, between 500 and 600 mm of precipitation falls over White Sea surface. The precipitation is unevenly distributed throughout the year. It is most abundant from June through October (280-300 mm), accounting for about the 60% of the annual total. Evaporation and precipitation practically compensate each other, only a small part of freshwater contribution can be addressed to precipitation (Pantyulin 2003, Filatov et al.,

2005).Thus, in the White Sea water balance an essential role is played by the river run-off delivering about 95% of net fresh water entering the sea.

White Sea salinity is considerably low. This is due to the remarkable fresh water runoff and limited water exchange with the Barents Sea. The salinity of the surface waters in the Basin and open parts of bays varies from 24 to 27‰ with a typical range of 22–26‰ for the Kandalaksha Bay (Derjugin, 1928, Howland et al.,1999; Vershinin et al., 2006). However, in the estuaries of large rivers it falls down to 5-8 or less. The mixed layer depth is 25 m across the whole sea and, in general, surface waters are considerably fresher than the bottom waters. Sharp seasonal variations of surface water salinity are typical for the White Sea, which can be explained by the dynamics of freshwater inflow. Freshening of surface water begins in winter when the sea is covered with ice. The most prominent decrease in salinity of the upper layer is observed in April-May just before and during the period of ice-melting (Babkov, 1982).

Tides and Tidal Energy in the Sea (Figure 1.11)

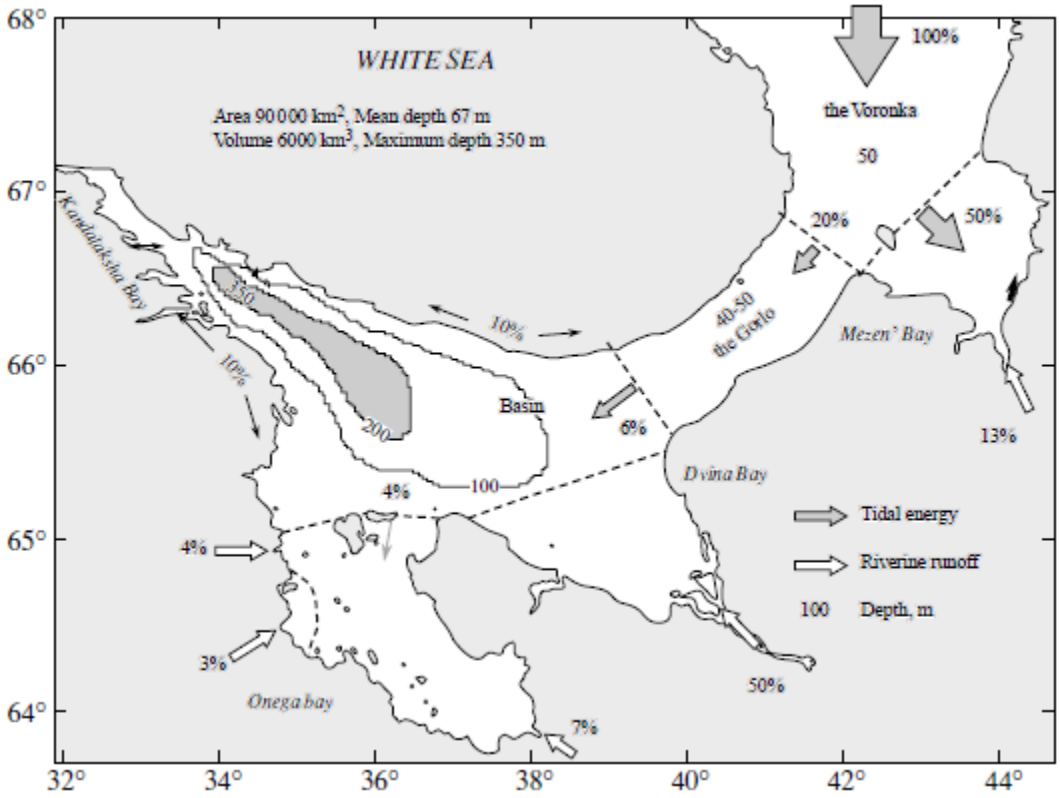
Tides are the most important factor forming the hydrodynamic White Sea regime. Tides propagate into White Sea from the Barents Sea as regular semidiurnal waves, with the elevation of the tide and the velocity of the currents decreasing along the wave front from East to West. The maximum tidal currents velocities over the entire White Sea (up to 250 cm/s) are observed at the boundary of the Voronka with Mezen' Bay, while the maximum tide height is located at the apex of Mezen' Bay (9.8 m). Waves propagation into the Gorlo and the Basin occurs with their partial reflection and diffraction. The deviations are related to the influence of morphometry and are better reflected in the currents. In the Basin, the tide height decreases to 1.5 m and the velocities of the currents do not exceed 20 cm/s. Approximately identical tides are observed in Dvina Bay, but with higher velocities. In Kandalaksha Bay, the topographic effect leads to an increase in tide height up to 2.5 m, but it shows its major influence in the tidal currents: in numerous inlets the velocities of the currents can reach 80–120 cm/s.

Kandalaksha Bay

Kandalaksha Bay is an estuarine system showing big sea level differences during tide cycles causing strong mixing of water (Melnikov et al., 2003; Savvichev et al., 2003). Estuaries are a dynamic ecosystem where seawater is diluted by the freshwater flowing from rivers and streams. Estuaries provide habitats for a large number of organisms and support very high productivity due mainly to phytoplankton (McLusky and Elliott, 2004). In Kandalaksha Bay, the seasonal extremes runoff of freshwater, due to the various rivers and intense precipitations, contributes to its very peculiar hydrodynamics (Howland et al., 1999; Dolotov et al., 2005).The coastal zone influenced by the tides (intertidal zone or littoral) is particularly interesting because all biogeochemical processes are most evident, especially if tidal phenomena are very pronounced as in Kandalaksha Bay. Organisms in this zone need adaptation to variable environmental conditions in which factors, such as water availability, temperature and salinity, change frequently (Savvichev et al., 2004).

Only a very limited number of studies is available on microorganisms from the White Sea and even less regards Kandalaksha Bay (Gorlenko et al., 1985; Dul'tseva et al., 1996; Rabold et al., 2006; Gorelova et al., 2009). In addition, most of these studies limit the investigation to the total number of bacteria in a determined environment (Savvichev et al., 2003; Savvichev et al., 2004; Kravchishina et al., 2008).

Fig. 1.11 Distribution of river runoff and tidal energy in the White Sea as reported by Pantyulin (2003).



Chapter 2

Materials and methods

2 Materials and methods

2.1 Microorganisms, media and culture conditions

2.1.1 Chemicals

Chemical compounds used were as follows:

Plate Count Agar (PCA, Difco, USA), Nutrient Broth (NB, BD, USA), Soluble starch (Carlo Erba, Italy), Agar (BD, USA), Peptone (BD, USA) , Phenol red (Sigma-Aldrich, USA), Phenolphthalein diphosphate (Sigma-Aldrich, USA), Tween 20, 60 and 80 (Sigma-Aldrich, USA), Yeast extract (BD, USA), Industrial pectin (Sigma-Aldrich, USA), Hexadecyltrimethylammonium bromide (Sigma-Aldrich, USA), Carboxymethyl cellulose (Sigma-Aldrich, USA), Cetrimonium bromide (Sigma-Aldrich, USA), Skim milk (Fluka, CH), N,N,N,N tetrametil-p-phenylenediamine in water (Sigma-Aldrich, USA). All other chemicals were of analytical grade. Colloidal chitin was prepared by treating chitin from crab shells (Sigma-Aldrich, USA) with sulfuric acid as follows: 50 g of chitin were dissolved in 500 ml of 50% sulfuric acid (BDH Prolabo, Belgium) and then placed in a tank containing 15 volumes of distilled water. Chitin was washed various times until pH reached the value of 5.0. After that, it was centrifuged at 11.000g for 15 minutes and autoclaved (121◦C, 20 min). Chitin concentration (solid content of the sterile chitin suspension) by dry weight was evaluated by dehydration in oven at 105°C for 12 hours.

2.1.2 Sampling, strain isolation and culture conditions

All the KB strains were obtained from water samples taken in various areas of Kandalaksha Bay, With Sea, Russia, during a 8 days sampling campaign in September 2008. The majority of samples were collected, at minimum tide level, in an intertidal zone pool and from the adjacent water surface using sterile containers. Others, from different offshore locations and depths (0.5, 2.5, 15, 70 m), were taken by scuba divers or boats using Niskin bottles sterilized by repeated washing with boiling sterile water and 70% (v/v) ethanol. Environmental

conditions, recorded during sampling, are reported in Table 2.1. Seawater was filtered on sterile membranes (0.22 µm, Millipore, USA) in order to obtain both pure cultures of bacteria and DNA to study the total bacterial community by molecular methods. Bacterial cultures were obtained placing the membranes on PCA plates and then incubating at 4, 15 and 25°C. Membranes to use for total DNA extraction, were maintained at 4°C in sterile tubes with silica-gel to avoid any contamination.

Pure cultures of isolates (ca. 500) were obtained on PCA by streak plate method. In order to discharge evident replicates of the same isolate, preliminary tests were carried out (see paragraph 2.2.1). Tests allowed to select 52 different isolates that were maintained on PCA slants at 4ºC and routinely sub-cultured.

2.1.3 Detection of growth temperature profiles.

Growth of the various isolates were tested in the range 0-45°C on PCA plates by steps of 5±0.5°C. Plates (diameter 90 mm) were inoculated in triplicate with inocula obtained pipetting 2 µl of bacterial suspension in sterile water (concentration was normalized spectrophotometrically at 600 nm). Plates were incubated at the different temperatures for 7 days and growth was monitored measuring the average increase of colony diameter. Plates were incubated for further 7 days to confirm the inability to grow at 0°C.

Table 2.1 Sampling description.

Sample number	Sampling site	Air temperature (°C)	Water temperature (°C)	Salinity (‰)	Weather conditions
1	P	11.9	10.1	23.0	Rainy, no wind
	M	11.9	10.1	23.7	
2	P	13.0	11.3	23.0	Sunny, moderate wind from W
	M	13.0	10.8	24.1	
3	P	11.7	10.3	23.8	Sunny, no wind
	M	11.7	10.2	23.3	
4	P	10.7	9.4	23.4	Cloudy after rain, wind from N
	M	10.7	9.1	23.5	
5	P	8.9	8.7	24.9	Partially cloudy , no wind
	M	8.9	8.4	24.4	
6	P	8.8	8.4	24.4	Partially cloudy, no wind
	M	8.8	8.7	24.2	
7	P	8.5	7.6	23.7	Cloudy, no wind
	M	8.5	7.5	24.0	
8	P	6.8	6.4	23.4	Sunny, no wind
	M	6.8	6.9	24.6	
9	N1	13.5	8.5	8.5	Sunny, moderate wind from W
	N3	10.5	6.6	6.6	
10	N2	13	8.0	8.0	Sunny, moderate wind from W
	N4	13	-1.0	6.0	Cloudy after rain, wind from N

Legend

Sampling sites:

P= intertidal zone pool, coordinates 66°33’15’’N; 33°05’47’’E; M= water surface nearby the intertidal zone pool, coordinates 66°33’15’’N; 33°05’47’’E; N1= water collected by scuba divers at – 2.5 m, coordinates: 66°32’29’’N, 33°14’94’’E; N3= water collected by scuba divers at – 15.5 m, coordinates: 66°32’30’’N, 33°14’94’’E; N2= water collected with boats at -0,5 m, coordinates: 66°32’21’’N, 33°14’77’’E; N4= water collected with boats at -70 m, coordinates: 66°32’21’’N, 33°14’77’’E

2.1.4 Detection of extracellular enzyme activities

The following activities were tested in triplicate: amylase (AMI), chitinase (CHI), cellulase (CEL), lipase on Tween 20, 60, 80 (T20, T60, T80), pectinase (PEC), phosphatase (PHO), protease (PRO), urease (URE). Plates for semi quantitative tests were prepared using previously reported techniques as follows: media for detection of chitinase, phosphatase, pectinase, lipase, cellulase and urease were prepared as previously reported Hankin and Anagnostakis (1975); media for detection of amylase and protease were prepared as reported by Paterson and Bridge (1994). Slight method modifications were carried out, when necessary, as described for each media. Where not specified, media were sterilized at 121°C for 20 minutes. Plates were inoculated with punctiform inocula using sterile needles from 1-2 day cultures grown on PCA plates. To avoid any interference from nearby colonies, only one isolate was inoculated onto each plate (60 mm diameter). The enzymatic activities were recorded measuring the diameter of the halo produced, after subtraction of colony diameter. For PHO, no activity halo is produced: positive colonies were recorded for their different color. The media for detection of extracellular enzyme activity were as follows:

Amilase

- Nutrient Broth 8.0 g/l
- Soluble starch 2.0 g/l
- Agar 20.0 g/l

Before sterilization, carried out at 0.75 atm for 30 minutes, pH was adjusted 7.0±0.2. After incubation plates were flooded with a iodine solution (Lugol) and amylolytic activity was indicated by the production of a yellow halo around the colony.

Urease

- Peptone 1.0 g/l
- Glucose 1.0 g/l

- NaCl 5.0 g/l
- KH_2PO_4 2.0 g/l
- Phenol red 0.0012 g/l
- Agar 20.0 g/l

Before sterilization, pH was adjusted to 7.0±0.2. After sterilization, medium was added with 110 ml per liter of a 20% urea solution previously sterilized by filtration. Urease activity was indicated by a pink halo around the colony.

Phosphatase

Autoclaved PCA (ca. 45%) was added with 0.096 g/l of phenolphthalein diphosphate, sterilized by filtration. After incubation plates were flooded with ammonium hydroxide. Colonies that turned pink to red were presumed to have degraded the substrate.

Lipase

- Peptone 10.0 g/l
- NaCl 5.0 g/l
- $CaCl_2(H_2O)_2$ 0.1 g/l
- Agar 20.0 g/l

Before sterilization, carried out at 0.75 atm for 30 minutes, pH was adjusted 7.0±0.2. Media were added with 10 ml per liter of one the following substrates: Tween 20, 60 or 80, previously autoclaved at 0.25 atm for 15 minutes. Lipolytic activity was indicated by the appearance of a visible precipitate, due to the formation of crystals of the calcium salt formed by the fatty acid liberated by the enzyme.

Pectinase

- Yeast extract 1.0 g/l
- Industrial pectin 5.0 g/l

- Agar 15.0 g/l

Reagents were dissolved to reach a final volume of 500 ml of distilled water and added with 500 ml of a mineral medium (Zucker and Hankin, 1970) containing:

- $(NH_4)_2SO_4$ 2.0 g/l
- KH_2PO_4 4.0 g/l
- Na_2HPO_4 6.0 g/l
- $FeSO_4(H_2O)_7$ 0.2 g/l
- $CaCl_2$ 50 µg/l
- H_3BO_3 10 µg/l
- $MnSO_4$ 10 µg/l
- $ZnSO_4$ 70 µg/l
- $CuSO_4$ 50 µg/l
- MoO_3 10 µg/l

The total volume of the medium was 1 liter. Before sterilization, pH was adjusted to 7.0±0.2. After incubation plates were flooded with 1% hexadecyltrimethylammonium bromide. The presence of the activity was indicated by a clarification halo around the colonies.

Cellulase

Following reagents were dissolved in 500 ml of distilled water and added to 500 ml of the mineral medium described for pectinase:

- Yeast extract 1.0 g/l
- Carboxymethyl cellulose 5.0 g/l
- Agar 10.0 g/l

Before sterilization, pH was adjusted to 7.0±0.2. After incubation, plates were flooded with 1% cetrimonium bromide. The presence of the activity was indicated by a clarification halo around the colonies.

Protease

- KH_2PO_4 1.0 g/l
- KCl 0.5 g/l
- $MgSO_4(H_2O)_7$ 0.2 g/l
- $CaCl_2(H_2O)_2$ 0.1 g/l
- Skim milk 3.75 g/l
- Glucose 10.0 g/l
- Agar 12,0 g/l

Before sterilization, carried out at 0.33 atm for 30 minutes, pH was adjusted 7.0±0.2. Protease activity was revealed by clarification halos in the opaque agar around the colonies.

Chitinase

The following reagents were dissolved in 500 ml of distilled water and added to 500 ml of the mineral medium described for pectinase:

- Yeast extract 0.1 g
- Colloidal chitin 12.0 g
- Agar 15.0 g

Before sterilization, pH was adjusted to 7.0±0.2. Chitinolitic activity was indicated by clarification halos in the opaque agar around the colonies.

2.2 Morphological, physiological and biochemical tests

2.2.1 Preliminary tests

Preliminary tests were carried out considering for each strain (colonies and/or cell) some morphological characteristics (shape, color, morphology, aspect and dimensions), simple biochemical tests (catalase and oxidase production) and Gram reaction. Gram staining was carried out using a commercial kit (Merck, Germany) following manufacturer's suggestion as reported in Table 2.2. Bacteria were measured on stained specimens using a Leitz Laborlux 11 microscope bearing a micrometric ocular calibrated with a micrometric slide (Leitz Wetzlar, Germany). Catalase and oxidase tests were performed as previously described (Kovacs, 1956; Whittenbury, 1964). Briefly: for oxidase activity, Kovacs reactive (1% of N,N,N,N tetrametil-p-phenylenediamine in water) was added to a fresh colony. After 60 seconds, develop of violet color means positive reaction. For catalase, H_2O_2 (3%) was added to a fresh colony: bubbles of O_2 production means positive reaction.

Table 2.2 Gram staining (accordingly to the manufacturer instructions)

Step	**Time**
Place fixed smears in crystal violet solution	1.5 min
Wash with water	30 sec
Lugol's solution	3 min
Wash with water	30 sec
Decolourizing solution	5 -10 sec
Wash with water	30 sec
Safranine solution	1 min
Wash with water	1 min

2.2.2 Metabolic characterization by BIOLOG

The BIOLOG system was used to test the metabolic profile of the strains by their ability to use 95 different compounds as sole carbon sources. Carbon sources available on the microplates can be divided, as previously reported by Zak et al. (1994), into six substrates groups: carbohydrates, carboxylic acids, amino acids, amines/amides, polymers and miscellaneous, including phosphorylated and aromatic compounds (Table 2.3).

The technology is based on redox reactions. The tetrazolium violet reduction is used as indicator to detect oxidation of the carbon sources (NADH formation). This reaction is revealed by spectrophotometric measurements (Garland and Mills, 1991; Odumeru et al., 1999; Juárez-Jiménez et al., 2010).

Strains were incubated on BUG (BIOLOG Universal Agar, Biolog, Hayward, CA, USA) plates for, at least, 24 h at their optimal grow temperature. Subsequently, BIOLOG GN2 and GP2 (for Gram-negative and Gram-positive bacteria respectively. Tables 2.4 and 2.5) microplates were inoculated with BIOLOG GP/GN Inoculation Fluid added with the appropriate amount of cells. Cells concentration, in the inoculation fluid, was determined with a turbidimeter according to the manufacturer suggestion (BIOLOG User Manual). Microplates were incubated for 24 hours. The 96-well microplates comprise the above mentioned substrate-containing wells and a control well with water. Substrates, dye and nutrients are supplied in each well in a dried-film form, which is reconstituted by addition of sample in microplates. Color development (Figure 2.1) was detected/recorded using the BIOLOG microplate reader at both 590 and 750 nm to measure growth and substrate oxidation, respectively. The BIOLOG software (BIOLOG Microlog 4.2) renders the capacity of strains to use a certain carbon source elaborating the information of the two readings. Results can only be -, ± or +, reporting the incapacity, the slight capacity and the full capacity to use a substrate, respectively.

If necessary, results could be also interpreted with the database and software to obtaining taxonomic information.

Figure 2.1 BIOLOG plate after 24 hours of incubation

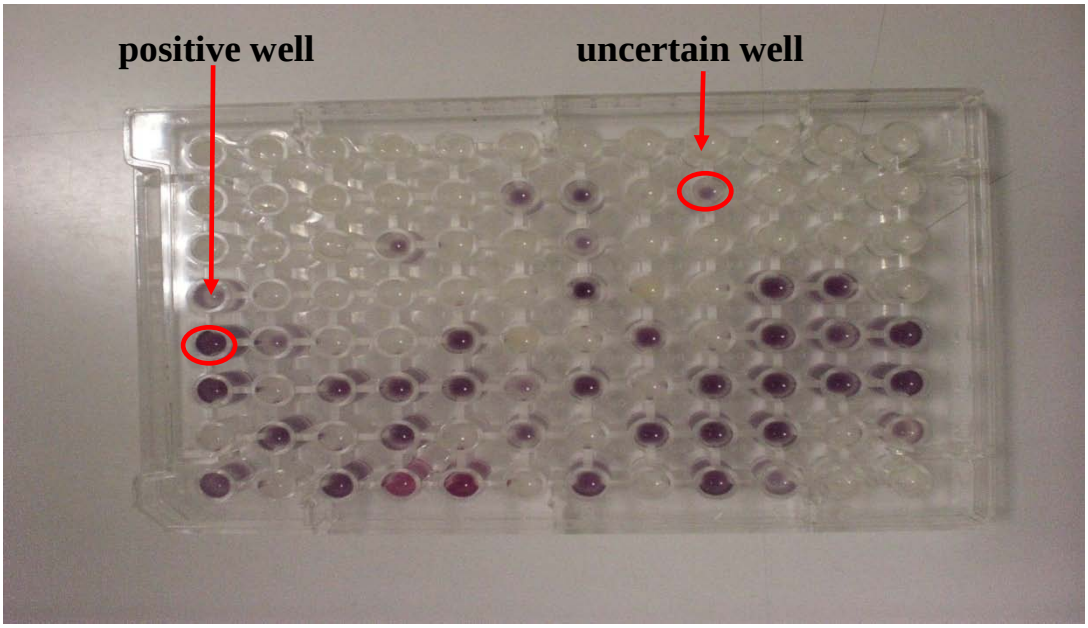


Table 2.3 Carbon Sources in substrates included in the Gram (-) and Gram (+) Biolog microplates categorized by substrate guilds by Zak et al 1994

Carbohydrates	**Carboxylic acids**	**Polymers**	**Amines/amides**	**Amino acids**	**Miscellaneous**
adonitol	acetic acid	a-cyclodextrin	2-amino-ethanol	D,L-camitine	2-deoxyadenosine
α-D-galactoside	α-hydroxybutyric ac.	p-cyclodextr.	alaninamide	D-alanine	2,3-butanediol
α-D-glucose	α-ketobutyric ac.	dextrin	glucuronamide	D-serine	adenosine
α-D-lactose	α-ketoglutaric ac.	glycogen	lactamide	γ-aminobutyric ac.	adenosine-5-monophosphate
α-methyl-D-glucoside	α-ketovaleric ac.	inulin	phenyl--ethylamine	glycyl-L-aspartic ac.	amygdalin
α-methyl-D-mannoside	β-hydroxybutyric ac.	tween 40	putrescine	glycyl-L-glutamic ac.	bromosuccinic acid
arbutin	*cis*-aconitic acid	tween 80	succinamic acid	hydroxy-L-proline	D,L-α-glycerolphosphate
β-methyl-D-galactoside	citric acid			L-alanine	D-lactic acid methyl ester
β-methyl-D-glucoside	D,L-lactic acid			L-alanyl-glycine	fructose-6-phosphate
cellobiose	D-galactonic ac. lactone			L-asparagine	glucose- 1-phosphate
D-arabitol	D-galacturonic acid			L-aspartic acid	glucose-6-phosphate
D-fructose	D-gluconic acid			L-glutamic acid	glycerol
D-galactose	D-glucosaminic acid			L-histidine	inosine
D-mannitol	D-glucuronic acid			L-leucine	salicin
D-mannose	D-malic acid			L-ornithine	thymidine
D-melezitose	D-saccharic acid			L-phenylalanine	thymidine-5-monophosphate
D-meliobiose	formic acid			L-proline	uridine
D-raffinose	γ-hydroxy-butyric ac.			L-pyroglutamic acid	uridine-5-monophosphate
D-ribose	itaconic acid			L-serine	Urocanic acid
D-psicose	L-lactic acid			L-threonine	
D-sorbitol	L-malic acid				
D-tagatose	malonic acid				
D-trehalose	N-acetyl-L-glutamic ac.				
D-xylose	*p*-hydroxy-phenylacetic ac.				

Carbohydrates	Carboxylic acids	Polymers	Amines/amides	Amino acids	Miscellaneous
gentiobiose	propionic acid				
i-erythritol	pyruvic acid				
L-arabinose	quinic acid				
L-fucose	sebacic acid				
L-rhamnose	succinic acid				
lactulose					
m-inositol					
maltose					
maltotriose					
mannan					
3-methyl glucose					
methyl pyruvate					
mono-methyl succinate					
N-acetyl-D-galactosamine					
N-acetyl-D-glucosamine					
N-acetyl-D-mannosamine					
palatinose					
sedoheptulosan					
stachyose					
sucrose					
turanose					
xylitol					

Table 2.4 Carbon Sources in GN2 MicroPlate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Water	α-Cyclodextrin	Dextrin	Glycogen	Tween 40	Tween 80	N-Acetyl-D-Galactosamine	N-Acetyl-D-Glucosamine	Adonitol	L-Arabinose	D-Arabitol	D-Cellobiose
B	i-Erythritol	D-Fructose	L- Fucose	D-Galactose	Gentiobiose	α-D-Glucose	m- Inositol	α-D-Lactose	Lactulose	Maltose	D-Mannitol	D-Mannose
C	D-Melibiose	β-Mehyl-D-Glucoside	D-Psicose	D-Raffinose	L-Rhamnose	D-Sorbitol	Sucrose	D-Trehalose	Turanose	Xylitol	Pyruvic Acid Methyl Ester	Succinic Ac.Mono Methyl Ester
D	Acetic Acid	Cis-Acotonic Acid	Citric Acid	Formic Acid	D- Galactonic Acid Lactone	D-Galacturonic Acid	D-Gluconic Acid	D-Glucosaminic Acid	D-Glucuronic Acid	α-Hydroxybutyric Acid	β-Hydroxybutyric Acid	γ-Hydroxybutyric Acid
E	p-Hydroxy Phenylacetic Acid	Itaconic Acid	α-Keto Butyric Acid	α-Keto Glutaric Acid	α-Keto-Valeric Acid	D,L-Lactic Acid	Malonic Acid	Propionic Acid	Quinic Acid	D-Saccharic Acid	Sebacic Acid	Succinic Acid
F	Bromosuccinic Acid	Succinamic Acid	Glucuronamide	L-Alaninamide	D-Alanine	L-Alanine	L-Alanyl glycine	L-Asparagine	L-Aspartic Acid	L-Glutamic Acid	Glycyl -L-Aspartic Acid	Glycyl –L-Glutamic Acid
G	L-Histidine	Hydroxy-L-Proline	L-Leucine	L-Ornithine	L-Phenylalanine	L-Proline	L-Pyroglutamic Acid	D-Serine	L-Serine	L-Threonine	D,L-Carnitine	γ-Amino Butyric Acid
H	Urocanic Acid	Inosine	Uridine	Thymidine	Pheniethyl-amine	Putrescine	2-Aminoethanol	2,3-Butanediol	Glycerol	D,L-α Glycerol Phosphate	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate

Table 2.5 Carbon Sources in GP2 MicroPlate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Water	α-Cyclodextrin	β-Cyclodextrin	Dextrin	Glycogen	Inulin	Mannan	Tween 40	Tween 80	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	Amygdalin
B	L-Arabinose	D-Arabitol	Arbutin	D-Cellobiose	D-Fructose	L-Fucose	D-Galactose	D-Galacturonic Acid	Gentiobiose	D-Gluconic Acid	α-D-Glucose	m-Inositol
C	α-D-Lactose	Lactulose	Maltose	Maltotriose	D-Mannitol	D-Mannose	D-Melezitose	D-Melibiose	α-Methyl-D-Galactoside	β-Methyl-D-Galactoside	3-MethylGlucose	α-Methyl-D-Glucoside
D	β-Methyl-D-Glucoside	α-Methyl-D-Mannoside	Palatinose	D-Psicose	D-Raffinose	L-Rhamnose	D-Ribose	Salicin	Sedoheptulosan	D-Sorbitol	Stachyose	Sucrose
E	D-Tagatose	D-Trehalose	Turanose	Xylitol	D-Xylose	Acetic Acid	α-HydroxybutyricAcid	β-HydroxybutyricAcid	γ-HydroxybutyricAcid	p-Hydroxy-Phenylacetic Acid	α-KetoglutaricAcid	α-Ketovaleric Acid
F	Lactamide	D-Lactic Acid Methyl Ester	L-Lactic Acid	D-Malic Acid	L-Malic Acid	Pyruvatic AcidMethyl Ester	Succinic Acid Mono-methyl Ester	PropionicAcid	Pyruvic Acid	Succinamic Acid	Succinic Acid	N-Acetyl-L-Glutamic Acid
G	L-Alaninamide	D-Alanine	L-Alanine	L-Alanyl-Glycine	L-Asparagine	L-Glutamic Acid	Glycyl- L-Glutamic Acid	L-Pyroglutamic Acid	L-Serine	Putrescine	2,3-Butanediol	Glycerol
H	Adenosine	2'-Deoxy Adenosine	Inosine	Thymidine	Uridine	Adenosine-5'-Monophosphate	Thymidine-5'-Monophosphate	Uridine-5'-Monophosphate	D-Fructose-6-Phosphate	α-D-Glucose-1-Phosphate	D-Glucose-6-Phosphate	D-L-α-Glycerol Phosphate

2.3 Strain identification and phylogeny

All the strains were identified by analysis of the sequences of the gene encoding for the 16S rRNA. Bacterial genomic DNA was extracted and used for amplification by polymerase chain reaction. Products of amplification were sequenced and data obtained were used for comparison with sequences present in databases.

2.3.1 DNA extraction

Isolates, grown for 24 h on PCA plates, were used for genomic DNA extraction by thermal shock as follows (Selbmann et al., 2010): a single colony suspension (in 14 µl of sterile deionized water) was heated at 100°C for 5 min, immediately cooled in ice and centrifuged at 4000*g* for 3 min. The supernatant was used for PCR reaction.

2.3.2 Polymerase Chain Reaction for amplification of the 16S rRNA gene

The amplifications were performed in a reaction mixture (final volume 25 µl) containing 2x BioMix (BioLine GmbH, Germany), 15-20 ng/µl of DNA template and 5 pmol/µl of the universal primers (Sigma-Aldrich, USA) reported in Table 2.6.

The amplification was carried out using a MiniCycler™ (MJ Research, USA) equipped with a heated lid as follows:

1. denaturation at 95°C for 5 min
2. denaturation at 95°C for 45 s
3. annealing at 55°C for 1 min
4. extension at 72°C for 90 s
5. final extension at 72°C for 5 min
6. cold-storage 4°C ∞

Step 2, 3 and 4 were repeated for 35 cycles.

The amplification products were visualized by electrophoresis on agarose gel (1.0%) prepared with 0.50 g of agarose (Starlab GmbH, Denmark) dissolved in 50 ml of TAE buffer 1X (40mM Tris-acetate, 1mM EDTA, pH 8.3, Brinkmann Instruments, Inc., USA) added with 5 µl of GelRed (10,000x, Biotium, USA). Loading was carried out by adding 1µl of Loading Dye (6x, New England Biolabs, USA) to 5µl of each sample. The DNA Ladder GeneRuler™ 100 bp (FERMENTAS, Lithuania) was used to quantify PCR products dimension by comparison. The products were purified using Nucleospin Extract kit (Macherey-Nagel, Germany). Sequencing reactions were performed by Macrogen sequencing service (Macrogen Inc., Korea). Sequence assembly was done using the software Chromas (version 1.5 2009, Technelysium Pty Ltd, Australia). Sequences with high similarity available in NCBI GenBank were identified using BLAST search (Altschul et al., 1997http://www.blast.ncbi.nml.nih.gov) and submitted to GenBank to obtain accession number.

Table 2.6 Primers used for the amplification of 16S r-RNA gene: fD1, rD1 and rP2 (Weisburg et al., 1991); 63f and 1389r (Hongoh et al., 2003)

Primer	**Sequence (from 5' to 3')**
fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCG
rD1	CCCGGGATCCAAGCTTAAGGAGGTGATCCAGC
1389r	ACGGGCGGTGTGTACAAG
63f	CAGGCCTAACACATGCAAGTC
rP2	TACGGCTACCTTGTTACGTCTT

2.3.3 Alignment and tree reconstruction

Automatic alignment was first carried out using CLUSTALX (Thompson et al. 1997), then exported to MEGA4 (Tamura et al., 2007) and improved manually. In order to get a confident branch-length, the whole a alignment was split and three different trees were generated for *Pseudomonas*, *Serratia* and the other genera analyzed, respectively. The final parts were cut off to compare fragments with the same length. Due to gaps necessary for alignment, the 16S domain spanned to 1452 positions for *Pseudomonas,* 1459 for *Serratia* and 1490 for all the other genera. For *Pseudomonas* and all other bacteria trees, alignments were then exported and the best-fit substitution model was determined using MrAIC.pl 1.4.3 (Nylander, 2004 program distributed by the author) estimated using PHYML (Guindon and Gascuel, 2003) through hierarchical likelihood ratio tests. MRAIC calculates the Akaike Information Criterion, corrected Akaike Information Criterion and Bayesian Information Criterion; Akaike weights for nucleotide substitution model and model uncertainty. All 56 models implemented in Model test were evaluated. Phylogenetic trees were reconstructed by Maximum Likelihood, using TREEFINDER (Jobb et al., 2004) and the resulting tree was displayed using TREEVIEW 1.6.6 (Page, 1996). The robustness of the phylogenetic inference was estimated using the bootstrap method (Felsenstein 1985) with 1,000 pseudoreplicates generated and analyzed with TREEFINDER. For *Serratia* tree, a p-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm (Saitou and Nei, 1987). The bootstrap test was conducted to infer the reliability of branch order (Felsenstein, 1985), with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.4 Statistical analysis of data

One-way analysis of variance (ANOVA) and pair-wise multiple comparisons procedure (Tukey test) were carried out using the software SigmaStat (Jandel Scientific, CA, USA). This statistical analysis were utilized for optimal growth temperature, extracellular enzymes

activities and for band analysis of TGGE gel (number of bands, diversity indexes and range-weighted indexes). In all others cases, statistical analysis was automatically performed by the software used.

2.5 Study of bacterial communities by temperature-gradient gel electrophoresis (TGGE) fingerprinting analysis

A cultivation independent approach based on PCR and temperature-gradient gel electrophoresis (TGGE) was chosen for the study of total eubacterial communities present in the Kandalaksha Bay area. After extraction, total DNA was used as template for 16S rRNA gene specific region (V3) amplification by PCR and products obtained were separated by TGGE. Predominant populations of bacteria in the TGGE profiles were phylogenetically identified by 16S rRNA gene sequencing and database comparison. Additionally, study of *Pseudomonas* species present in samples was also carried out using specific primers and TGGE program.

2.5.1 DNA extraction

Total community DNA was extracted from membrane filters. Each membrane was suspended in ca. 2 ml of sterile water and triturated grossly with a sterile pipette tip; tubes were then vigorously stirred (vorticated) by vortex (IKA, Germany). The suspension was transferred to a clean microcentrifuge tube and used for DNA extraction carried out with the commercial kit MasterPureTM Complete DNA and RNA Purification Kit (Epicentre® Biotechnologies, USA) accordingly to the manufacturer instructions as follows: each sample (ca. 25 µl) was added with a mixture of 1 µl of Proteinase K and 300 µl of Tissue and Cell Lysis Solution, mixing thoroughly. Samples were first incubated at 65°C for 15 minutes, mixing every 5 min, and cooled at 37°C, afterward they were added with 1 µl of 5 mg/ml RNase A and incubated again at 37°C for 30 minutes. Tubes were placed on ice for 3-5 minutes, then added with 150 µl of MPC Protein Precipitation Reagent and vorticated for 10 seconds. The pellet was

precipitated by centrifugation at 4°C for 10 minutes at 10,000 *g* and the supernatant was transferred to a clean sterile microcentrifuge tube. The recovered supernatant was added with 500 µl of isopropanol and the tube inverted 30-40 times. After centrifugation at 4°C for 10 minutes, the isopropanol was poured off and pellet was rinsed twice with 70% ethanol. All the residual ethanol was removed with a pipet and DNA was resuspended in 35 µl of TE Buffer. Subsequently, extracted DNA was visualized on agarose gel (0.8%) as previously reported (paragraph 2.3.2) and maintained at -20°C.

2.5.2 Study of total eubacterial community

A two-steps PCR (nested PCR) approach was selected for specific amplification of the V3 hypervariable region of the 16S rRNA gene, as previously described by other authors for TGGE or DGGE fingerprinting (Gray et al., 2002; Molina-Muñoz et al., 2009).

2.5.2.1 16S rRNA gene amplification (First amplification)

Extracted DNA was used as a template for PCR, using the universal primers fD1 and rD1 (Table 2.6) to amplify the 16S-rRNA gene (ca. 1.5-1.6Kb). PCR was carried out as reported by Vinuesa et al. (1998) with only slight modifications. The PCR reaction contained 1 µl of DNA template, 1x PCR Buffer Gold (Applied Biosystems, USA), 1.5 mM $MgCl_2$ (Applied Biosystems, USA), 200 µM deoxynucleotide triphosphates (Roche, Germany), 0.4 µM of each primer, 200 ng/µl of bovine serum albumin (New England Biolabs, USA), 2.5 U Ampli Taq Gold hot-start polymerase (Applied Biosystems), and 2.5 µl of Dimethyl Sulfoxide (Sigma-Aldrich, USA) in a final volume of 50 µl.

PCR was performed in a Perkin–Elmer 2400 DNA Thermo Cycler with the following conditions:

1. denaturation at 95°C for 7 min
2. denaturation at 94°C for 1 min and 10 s
3. annealing at 56°C for 40 s
4. extension at 72°C for 2 min

5. final extension at 72°C for 6 min and 10 s
6. cold-storage 4°C ∞

Step 2, 3 and 4 were repeated for 25 cycles.

After visualization on agarose gel (1%) 1 µl of the first PCR product was used as a template for a second PCR reaction.

2.5.2.2 Hypervariable region V3 amplification (Second amplification)

Samples from first PCR were submitted to a second reaction in order to amplify the hypervariable region V3 (corresponding to positions 341 to 534 in the *Escherichia coli* sequence, ca. 0.2 Kb) using the universal bacterial primers GC-341f and 534r reported in Table 2.7. The sequence of primer GC-341f incorporates a 40-bp GC clamp at its 5' end (Figure 2.2). The GC rich sequence acts as a high melting domain preventing the two DNA strands from complete dissociation into single strands due to the denaturation conditions of the TGGE gel (Muyzer and Smalla, 1998). The amplification was performed in a reaction mixture containing 1 µl of DNA template (first PCR product), 2x PCR Buffer Gold (Applied Biosystems, USA), 3 mM $MgCl_2$ (Applied Biosystems, USA), 400 µM deoxynucleotide triphosphates (Roche, Germany), 0.8 µM of each primer, 400 ng/µl of bovine serum albumin (New England Biolabs, USA), 5 U Ampli Taq Gold hot-start polymerase (Applied Biosystems), and 5 µl of Dimethyl Sulfoxide (Sigma-Aldrich, USA) in a final volume of 50 µl.

For this PCR reaction was employed a touchdown technique, as reported by Watanabe et al. (1998). PCR was performed in a Perkin–Elmer 2400 DNA Thermo Cycler with the following conditions:

1. denaturation at 94°C for 7 min
2. denaturation at 94°C for 1 min
3. touchdown primers annealing from 65°C to 55°C for 1 min decreasing 0.5°C per cycle
4. extension at 72°C for 2 min

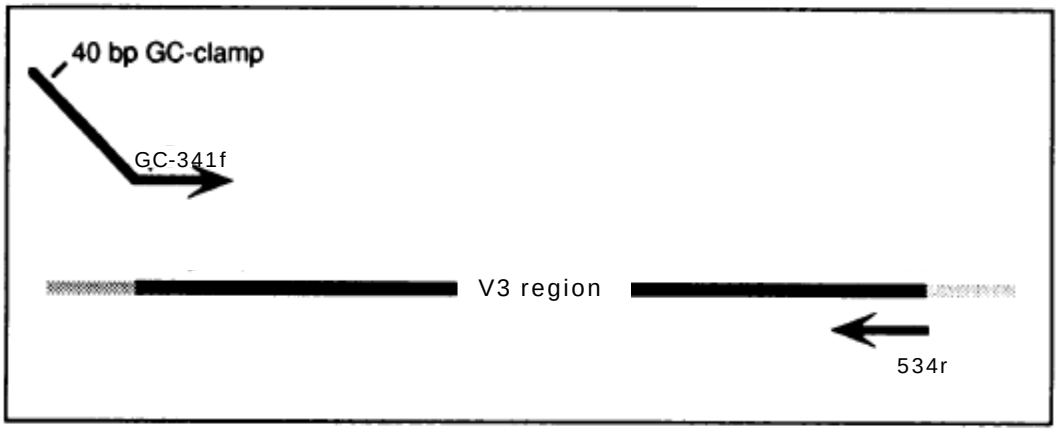
5. final extension at 72°C for 10 min
6. cold-storage 4°C ∞

Step 2, 3 and 4 were repeated for 20 cycles, plus 10 additional cycles keeping the annealing at 55°C. Subsequently, extracted DNA was visualized on agarose gel (1.5%) and maintained at -20°C.

Table 2.7 Primers used for the V3 region amplification (Muyzer et al. 1993)

Primer	Sequence (from 5' to 3')
GC-341f	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGATC CCTACGGGAGGCAGCAG
534r	ATTACCGCGGCTGCTGG

Figure 2.2 V3 region amplification mechanism



2.5.2.3 Concentration and purification of PCR products

All final PCR products, for each samples, were approximately concentrated 5 times, using the Microcon YM-100 (Millipore®) system accordingly to the manufacturer instructions as follows: sample (ca. 45 µl) was pipetted into sample reservoir, contained in a microtube, provided with a membrane (nominal cut-off 100.000 Da). Samples were then centrifuged at 500 *g* for 15 minutes to retain DNA. Subsequently, 15-20 µl of sterile MilliQ (Millipore, USA) water were added and the sample reservoir was placed upside down in a new vial then centrifuged 3 minutes at 1000 × g to transfer concentrate to vial. Final concentration was 60-100 ng/ µl. This process also allowed the purification of the DNA, eliminating salts and low molecular weight components.

2.5.2.4 Fragments separation by TGGE

Temperature-gradient gel electrophoresis was performed using a TGGE MAXI system (Biometra GmbH, Germany). For fragments separation, denaturing gel was made as reported in Table 2.8. All the reagents employed were of analytical grade (Sigma-Aldrich, USA). After solidification and polymerization (3 hours) gel was run with 2x Tris–acetate–EDTA buffer. PCR products (ca.8 µl) were added with 1 µl of 5x loading buffer (0.5% Triton X-100, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM EDTA and buffer 10x TAE) and then loaded in each well.

A six-species marker was included, made by amplification of V3 region from DNA of the culture collection strains reported in Table 2.9.

Table 2.8 Denaturing gel composition employed for TGGE technique

Reagent	**Concentration**
Acrylamide:bisacrylamide 37.5:1	6%
Deionized formamide	20%
Urea, molecular biology grade	8M
Glycerol	2%
TAE pH 8(Buffer tris-acetato40mM, EDTA 1mM)	1x
N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED)	0.06 %
Ammonium persulfate	0.06 %

Table 2.9 Six-species marker employed for TGGE

Species	**Strain**
Staphylococcus aureus	ATCC 25923
Pseudomonas putida	ATCC 8750
Acinetobacter calcoaceticus	ATCC 15308
Escherichia coli	DH5α
Nocardia corynebacterioides	ATCC 21253
Micrococcus luteus	ATCC 9341

The temperature gradient was optimized in the range 43-53°C and electrophoresis conditions were as follows:

1. Initial migration of samples:
- gradient initial temperature, L_0 = 20 ºC;
- gradient final temperature, L_{10} = 20 ºC;
- voltage, 300 V;
- duration, 18 minutes.
2. Temperature gradient stabilization :
- gradient initial temperature, L_0 = 43 ºC;
- gradient final temperature, L_{10} = 53 ºC;
- voltage, 0 V;
- duration 10 minutes.
3. Samples separation:
- gradient initial temperature, L_0 = 43 ºC;
- gradient final temperature, L_{10} = 53 ºC;
- voltage 125 V;
- duration, 18 hours

Gel was fixed in a solution containing 25 ml of glacial acetic acid, 200 ml of ethanol (final concentration 40%) and 275 ml of MilliQ water and then washed repeatedly (4 times) with MilliQ water. Bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce), following the manufacturer's recommendations as follows: gel was placed in the Silver Working Reagent for 30 minutes and then washed with bi-distilled water. Subsequently it was added with Reduction Reagent for 3-5 minutes and finally with stabilization reagent for 30 minutes.

2.5.2.5 TGGE fingerprints analysis

Cluster analysis was used to investigate the relationships between the TGGE profiles. Band patterns generated by TGGE were normalized, compared and clustered using the Gel Compar II v. 5.10 software (Applied Maths, Belgium).

For cluster analysis, TGGE profiles were compared both by the Pearson product–moment correlation and the Dice coefficient. The first is a band assignment-independent method, the second method is based on band presence/absence. The Pearson coefficient analysis uses the

whole densitometric curve, taking into consideration band intensity, and is affected much less than band-based similarity coefficients by the amount of PCR products loaded onto gel (Van Verseveld 2004). Band-matching (band assignment) and identification of band classes is performed automatically by the program GelCompar II. In band assignment, a 1% band position tolerance (relative to the total length of the gel) was applied, which indicates the maximum shift allowed for two bands in different TGGE lanes to be considered identical. Dendrograms relating band pattern similarities were automatically calculated with UPGMA algorithms (Unweighted pair group method with arithmetic mean). Significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients (Sokal, 1962). To test the reproducibility of the TGGE fingerprints, duplicate PCR reactions and TGGE runs were analyzed.

Bacterial bio-diversity of samples was measured using indexes based on the obtained band profiles. Statistics were calculated for each profile of a sample by using the number and intensity of bands in each profile as representations of the number and relative abundance of different species in a sample.

To evaluate diversity in the samples, Simpson's diversity index (Simpson 1949) was calculated. This index measures the probability that two individuals randomly selected from a sample will belong to the same species. It can be calculated by the following formula:

$$1\text{-}D = 1 - \Sigma(p_i)^2$$

where p is the proportion of an individual band intensity relative to the sum of all band intensities. The value of 1-D ranges between 0 and 1. With this index, 1 represents infinite diversity and 0, no diversity. The highest is the value of 1-D, the highest is the diversity, so diversity increases as richness increases.

Additionally, *range-weighted richness indexes* (Rr) (Marzorati et al. 2008) were calculated, based on the total number of bands in each TGGE pattern (N) and the temperature gradient (°C) comprised between the first and last band of each pattern (Tg):

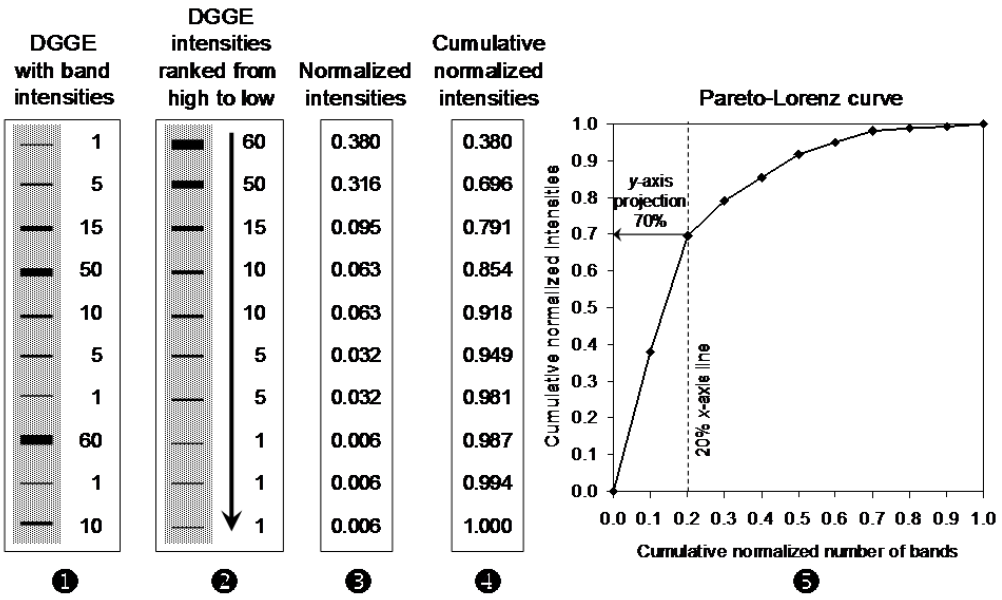
$$Rr = N^2 \times Tg$$

To calculate Tg, a template was used to divide the TGGE gel in discrete portions corresponding each to an increase of + 0.5 °C, and the initial (Ti) and final (Tf) temperatures required to describe the total diversity of a given sample were recorded. The resulting (Tf - Ti) values were divided by 100, with the aim of keeping an order of magnitude analogous to that of Rr index as described for DGGE by Marzorati et al. (2008):

$$Tg = (Tf - Ti)/100$$

Furthermore, to render a graphic representation of the evenness of the bacterial communities in the different sampling sites, Pareto-Lorenz distribution curves were drawn based on the TGGE fingerprints (Figure 2.3), as proposed for DGGE by Marzorati et al. (2008). The bands in each TGGE lane were ranked from high to low based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (Fo), given by the horizontal y-axis projection on the intercept with the vertical 20% x-axis line (Marzorati et al., 2008).

Figure 2.3 Construction of Pareto-Lorenz distribution curves



2.5.2.6 TGGE bands amplification and sequencing

Prominent TGGE bands were excised from the gel, re-amplified and sequenced, to obtain the identities of predominantly present populations within the total bacterial community. Portions of individual bands on silver-stained TGGE gel were picked up with sterile pipette tips, placed in 10 µl of filtered and autoclaved water and directly used for re-amplification with the appropriate primers following same procedure reported in paragraph 2.5.2.2. PCR products were purified with the QIAquick PCR Purification Kit (Quiagen, UK) following the manufacturer's recommendations as follows: 1 volume of the PCR sample was added to 5 volumes of Buffer PB and mixed, then it was transferred to a tube provided with a QIAquick column and centrifuged at 17,900 g for 30–60 s. After discarding flow-through, sample was washed adding 75 ml of Buffer PE to the QIAquick column and centrifuging 2 times for 30–60 s. The QIAquick column was then placed in a clean tube, added with 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) and centrifuged for 1 minute to elute DNA.

The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems) at the Intitute of Parasitology and Biomedicine "Lopez –Neyra" (Technological Park of Health Science of Granada, Granada, Spain).

2.5.2.7 Sequences analysis and phylogenetic tree

DNA sequences assembly was done using the software Chromas (version 1.5 2009, Technelysium Pty Ltd, Australia). Sequences were then analyzed using the biocomputing tools provided on-line by the European Bioinformatics Institute (http://www.ebi.ac.uk). The BLASTn program (Altschul et al., 1997) was used for preliminary sequence similarity analysis. The ClustalX v. 2.0.3 software (Jeanmougin et al., 1998) was used for the aligning of sequences. Phylogenetic analysis was conducted using MEGA version 4 (Kumar et al., 2008). A p-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm (Saitou and Nei, 1987). The bootstrap test was conducted to infer the reliability of branch order (Felsenstein, 1985), with a round of 1000 reassemblings. Bootstrap values below 50% are not shown in the tree.

2.5.3 Study of *Pseudomonas* commmunity

As reported for total bacterial community, nested PCR approach was used for the specific amplification of the V3 hypervariable region of the 16S rRNA gene of *Pseudomonas* spp.

All the steps for TGGE and subsequent studies, were the same as reported in paragraphs from 2.5.2.1 to 2.5.2.7 for total bacterial community. The following paragraphs report only modifications of the PCR and TGGE programs.

2.5.3.1 16S rRNA gene amplification (First amplification)

Extracted DNA was used as a template for the first PCR step, using the taxon-specific primers F311Ps and R1459Ps (Table 2.10) described by Milling et al. (2005) for the amplification of partial 16S rRNA genes. The PCR reaction, contained 1 µl of DNA template, 1x PCR Buffer Gold (Applied Biosystems, USA), 3.75 mM $MgCl_2$ (Applied Biosystems, USA), 200 µM deoxynucleotide triphosphates (Roche, Germany), 0.2 µM of each primer, 120 ng/µl of

bovine serum albumin (New England Biolabs, USA), 12.5 U Ampli Taq Gold hot-start polymerase (Applied Biosystems), and 3.5 µl of Dimethyl Sulfoxide (Sigma-Aldrich, USA) in a final volume of 50 µl.

PCR was performed, as reported by Milling et al. (2005) with slight modifications, in a Perkin–Elmer 2400 DNA Thermo Cycler with the following conditions:

1. denaturation at 94°C for 5 min
2. denaturation at 95°C for 1 min
3. annealing at 53°C for 1 min
4. extension at 72°C for 2 min
5. final extension at 72°C for 10 min
6. cold-storage 4°C ∞

Step 2, 3 and 4 were repeated for 35 cycles.

After visualization on agarose gel (1%) 1 µl of the first PCR product was used as a template for a second PCR reaction.

Table 2.10 Taxon-specific primers used for the amplification of *Pseudomonas* spp. partial 16S rRNA genes (Milling et al., 2005)

Primer	**Sequence (from 5' to 3')**
F311Ps	CTGGTCTGAGAGGATGATCAGT
R1459Ps	AATCACTCCGTGGTAACCGT

2.5.3.2 Hypervariable region V3 amplification (Second amplification)

To avoid amplification of fragments of others bacteria, template was diluted in order to obtain only most abundant species (theoretically species of *Pseudomonas*). Therefore, 1 µl of a 1/30 dilution of the first PCR product was used as a template for the nested PCR, using universal

primers targeting the V3 region (Muyzer et al., 1993). Reaction conditions, temperature profiles and cycles for this PCR were as reported in paragraph 2.5.2.2.

2.5.3.3 Fragments separation by TGGE

Temperature-gradient gel electrophoresis was carried out as reported in paragraph 2.5.2.4 except for temperature gradient that was optimized in the range 38-50°C.

2.5.3.4 TGGE fingerprints analysis

Cluster analysis was performed by Pearson and Dice coefficient as reported in paragraph 2.5.2.5. All the other statistical analysis (1-D and Rr), drawing of Pareto Lorenz curves and calculation of *Fo* where not performed.

TGGE bands amplification, sequencing, analysis of sequencing and phylogenetic tree were carried out as reported in 2.5.2.6 and 2.5.2.7 respectively.

Chapter 3

Results and discussion

3.1 Preliminary tests

The huge number of pure cultures obtained by the various samples evidently included various replicates of the same species. In order to remove them, all the pure cultures were submitted to preliminary tests, obtaining 52 isolates that were maintained and used for the further studies (Table 3.1). As expected, the majority of the bacteria (ca. 90 %) were Gram negative. In fact, already from the early studies of marine microbiology, it was evident that most of the prokaryotes (ca.95%) were Gram negative (Watson et al., 1977; Delille, 1992). However, recent studies demonstrate that the proportion of Gram positive in various marine habitats has been underestimated (Webster et al., 2001). Common explication of the predominance of Gram negative is related to their cell wall structure. Nutrients at low concentration can cross outer membrane, through porins, into periplasmic space due to specific proteins, having very high affinity for their substrates. Oligotrophic bacteria must have very efficient transport system to ensure that substances can enter the cell even at very low concentration(Munn, 2011). Thus Gram negative would be advantaged in an oligotrophic environment such as the sea water.

As for catalase and oxidase, positive strains were ca. 96 and 46%, respectively. All the isolates showed a more or less evident rod shape with a wide range of dimensions (1.1-4.0 µm length; 0.4-1.0 µm width).Bacteria isolated were substantially from the main sampling sites, the intertidal zone pool (P) and water surface (M) nearby it (ca. 80% of isolates).By contrast, all the samples taken offshore showed limited number of isolates. Samples from -70 m (N4) revealed minimum presence of isolates and none of them is exclusive of this sample site. Anyway, it is worth noting that strains KB68, KB72 and KB73, were found only at 15m and 70 m depth. Therefore, they seems to be typical of deeper water, probably because less influenced by the mixing of water caused by the tides. The elevated number of isolates in the intertidal zone could be probably caused by the biomass contribution due to freshwater coming from the land(Howland et al., 1999; Pantyulin, 2003; Cobelo-García, et al. 2006).

Table 3.1 Morphological and biochemical characteristics of the strains isolated from water samples collected in various sites of Kandalaksha Bay

Strain	Gram	Cat	Oxy	Dimensions[a] (µm)	Colony aspect[b] (colour/shape/edge/surface/elevation)	Sampling site[c]
KB1	-	+	-	0.7±0.1/1.2±0.1	R/C/E/W/U	M/N1/N2/N3
KB2	-	+	+	0.5±0.0/3.0±0.1	Y/C/U/P/F	P/M/N1/N3
KB3	+	+	+	0.4±0.2/1.5±0.2	O/C/C/G/R	P/M
KB4	-	+	+	0.5±0.0/4.0±0.1	B/C/F/P/F	P/M
KB5	-	+	+	0.8±0.2/1.3±0.0	Y/C/E/G/C	P/M/N1
KB6	-	+	+	0.8±0.0/4.0±0.3	W-G/I/U/S/R	P/M/N1/N3
KB7	-	+	+	0.8±0.2/1.8±0,2	W-G/C/U/S/C	P/M/N2/N3
KB10	-	+	-	1.0±0.2/2.5±0.1	W-G/I/U/R/R	P/M/N1/N3
KB11	-	+	+	1.0±0.1/2.0±0.0	W-O/C/E/W/C	P/M/N1/N2/N3
KB12	-	+	-	0.5±0.1/3.0±0.4	Y/I/L/S/R	M/N1
KB16	-	+	-	0.8±0.1/1.5±0.1	W/C/E/G/P	M/N3
KB17	-	+	-	0.8±0.3/1.6±0.2	W/C/E/G/P	P/M/N1/N2/N3
KB20	-	+	+	0.8±0.1/2.0±0.3	W/I/U/S/C	P/M/N3
KB21	-	+	+	0.8±0.0/1.5±0.0	W/C/E/S/C	P/M/N3
KB22	-	+	-	0.8±0.2/1.5±0.1	W/C/E/G/P	P/M
KB23	-	+	+	1.0±0.2/1.8±0.2	Y-G/I/U/S/C	P/M
KB24	-	+	+	0.8±0.1/2.0±0.1	W/C/E/S/C	P/M/N3/N4
KB25	-	+	-	1.0±0.1/1.5±0.1	W/C/E/G/P	P/M
KB30	-	-	-	0.8±0.3/1.2±0.1	W-O/I/U/G/C	P/M/N1/N3
KB31	-	+	+	0.8±0.1/1.5±0.2	Y/C/E/G/C	P/M
KB32	-	+	+	1.0±0.2/1.3±0.1	Y/C/E/G/P	M
KB33	-	+	+	0.9±0.3/1.2±0.0	Y/C/E/G/C	P/M/N3
KB36	-	+	+	0.8±0.1/2.0±0.0	W-G/C/L/R/R	M
KB37	-	+	+	1.0±0.0/2.0±0.1	W/C/U/G/C	P/M/N1/N3
KB38	-	+	-	0.8±0.1/1.5±0.0	Y-G/C/E/R/R	P/M/N3
KB39	-	+	-	0.8±0.0/1.8±0.2	W-O/I/U/S/R	M
KB40	-	+	-	1.0±0.2/2.0±0.4	W/I/U/G/C	P/M/N1/N3
KB42	-	+	-	1.0±0.3/2.5±0.2	W/C/U/R/R	P/M
KB43	-	-	-	0.5±0.0/2.0±0.0	Y/C/E/G/C	P/M
KB44	-	+	-	0.8±0.0/2.0±0.1	W-Y/I/E/S/R	P/M
KB45	-	+	-	1.0±0.1/3.0±0.6	W-Y/C/E/S/C	P/M/N3
KB46	-	+	+	1.0±0.2/2.0±0.3	Y/C/E/R/R	P/M
KB47	-	+	+	0.8±0.1/2.0±0.2	Y-G/I/U/R/R	P/M/N3
KB49	-	+	-	1.0±0.1/1.8±0.2	W/C/U/G/P	P/M/N3
KB50	-	+	+	0.5±0.1/2.0±0.0	Y-G/I/L/R/R	P/N3

Strain	Gram	Cat	Oxy	Dimensions[a] (µm)	Colony aspect[b] (colour/shape/edge/surface/elevation)	Sampling site[c]
KB51	-	+	+	1.0±0.1/2.0±0.3	V/I/L/W/R	P/M
KB52	-	+	-	0.8±0.2/1.1±0.1	W/I/L/R/R	P/M/N3
KB54	+	+	-	1.0±0.3/1.2±0.4	W-O/I/U/D/U	P/M/N3
KB56	-	+	-	1.0±0.0/1.8±0.0	W/C/U/G/P	P/M/N3
KB57	+	+	-	1.0±0.3/1.2±0.1	Y/C/E/G/R	P/M/N3
KB58	-	+	+	0.5±0.1/4.0±0.5	Y/C/U/P/F	P/M
KB59	+	+	-	0.5±0.2/1.1±0.3	Y/C/E/G/P	P/M/N3
KB61	-	+	-	1.0±0.3/2.0±0.0	W/C/U/G/P	P/N3
KB63	-	+	-	1.0±0.2/3.0±0.2	W-O/I/L/S/R	P/N3
KB64	-	+	-	1.0±0.4/3.0±0.7	Y/C/E/G/R	P/N3/N4
KB66	+	+	+	0.5±0.2/2.0±0.1	W/I/C/D/R	P
KB68	-	+	-	1.0±0.1/2.0±0.0	W/C/E/G/R	N3/N4
KB71	-	+	+	1.0±0.2/1.8±0.2	W-R/I/U/G/P	M
KB72	-	+	+	1.0±0.2/2.0±0.1	R/C/E/W/U	N1
KB73	-	+	-	1.0±0.0/2.0±0.6	W/I/L/G/R	N1
KB75	-	+	-	1.0±0.2/1.2±0.2	W-T/C/E/R/F	P
KB76	-	+	-	1.0±0.1/2.0±0.4	Y/I/L/R/F	P

Legend:

Cat = catalase; Oxy = oxidase

[a]Dimensions were average Data are mean of, at least, 10 measures; SD was less than 10%

[b]Colony aspect is described accordingly to the following parameters:

colour: B = brown G = green O = orange R = red T = transparent V = violet W = white Y = yellow; **shape:** C = circular I = irregular; **edge:** C = curled E = entire F = filamentous L = lobate U = undulate; **surface:** D = dry G = glistening P = powdery R = rough S = smooth W = wrinkled; **elevation:** C = convex F = flat P = pulvinate R = raised U = umbonate

[c]See Table 3.1for sampling sites

3.2 Strain identification

Isolates subjected to 16S rDNA sequence analysis were affiliated to the following genera: *Acinetobacter, Arthrobacter, Bacillus, Enterobacter, Exiguobacterium, Flavobacterium, Janthinobacterium, Microbacterium, Myroides, Pantoea, Pseudomonas, Rhodococcus, Serratia, Shewanella, Sphingobacterium and Stenotrophomonas*. All sequences matched with GenBank entries with similarities ranging from 96 to 100%.

Table 3.2 reports the GenBank accession number of isolates and their closest phylogenetic relatives. Only 20 strains out of 52 (ca.38%), showing 99-100% identity with a single known microorganism, were identified at species level. All other isolates were identified at the genus level only: for most of them, affiliation was not possible because 98-99% of identity was recorded with various species of the same genus. In addition, 3 strains showed a very low percentage of identity (96-97%).

Out of the 47 Gram-negative, 37 belonged to the phylum *Proteobacteria* being *Pseudomonas* and *Serratia*, both in the class γ-proteobacteria, the most predominant genera with 21 and 10 strains, respectively. Among *Pseudomonas*, only 7 strains were identified at species level and belonged to *P. fluorescens* (KB6, KB11, KB20, KB24 and KB36), *P. syringae* (KB10) and *P. putida* (KB12 and KB76). Among *Serratia*, strains were affiliated to *S. plymuthica* (KB1) and *S. proteamaculans* (KB22, KB49 and KB56). Other well represented classes, related to the phylum *Bacteroidetes*, are *Sphingobacteria* and *Flavobacteria*, with 5 and 4 strains respectively. Thus, in our samples, *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroidetes* group (CFB) seem to be dominant, as already reported for other cold environments (Bai et al., 2006; Amato et al., 2007; Srinivas et al., 2009). As said, *Pseudomonas* and *Serratia* were the most representative genera: this is not surprising since these are well-known ubiquitous bacteria. The only typical bacterium of cold marine environments was the gram negative rod *Shewanella baltica* (KB30, class γ-proteobacteria),

found in the Baltic Sea (Ziemke et al., 1998; Vogel et al., 2005). Moreover, this seems to be the only true marine species isolated in our study: all other strains are spread both in terrestrial and marine environments. This is an unexpected result since others works, carried out on samples from similar environments, demonstrated that marine bacteria were more recurrent (Bowman et al., 1997; Amato et al., 2007; Srinivas et al., 2009). Possible explication is the peculiar Kandalaksha Bay estuarine hydrodynamic characterized by strong contribution of freshwater carrying lot of microorganisms also from soil (Howland et al., 1999; Pantyulin, 2003; Cobelo-García et al., 2006). This is also confirmed by the fact that *Pseudomonas* and *Serratia* were recovered mainly in samples taken from the intertidal zone and water surface nearby it. On the other hand, the above considerations are valid for the microorganisms identified at species level: other marine bacteria could be present among the partially identified strains. Besides, *Flavobacterium* is well known as one of the most represented genera in marine environments being common also in sea ice (Hayes, 1963; Bowman et al., 1997).

As for Gram positive, the few strains present were affiliated to the phyla *Actinobacteria* (KB54, KB57 and KB59) and *Firmicutes* (KB3 and KB66).

Table 3.2 Identification of the Kandalaksha Bay strains based on 16S rDNA analysis

Strain	Accession number[a]	Closest phylogenetic relative	Identity
KB1	JF327440	*Serratia plymuthica*	99%
KB2	JF327441	*Flavobacterium* sp.	98%
KB3	JF327442	*Exiguobacterium oxidotolerans*	100%
KB4	JF327443	*Flavobacterium* sp.	97%
KB5	JF327444	*Sphingobacterium* sp.	99%
KB6	JF327445	*Pseudomonas fluorescens*	100%
KB7	JF327446	*Pseudomonas* sp.	99%
KB10	JF327447	*Pseudomonas syringae*	99%
KB11	JF327448	*Pseudomonas fluorescens*	99%
KB12	JF327449	*Pseudomonas putida*	99%
KB16	JF327450	*Serratia* sp.	100%
KB17	JF327451	*Serratia* sp.	99%
KB20	JF327452	*Pseudomonas fluorescens*	100%
KB21	JF327453	*Pseudomonas* sp.	98%
KB22	JF327454	*Serratia proteamaculans*	100%
KB23	JF327455	*Pseudomonas* sp.	99%
KB24	JF327456	*Pseudomonas fluorescens*	100%
KB25	JF327457	*Serratia* sp.	99%
KB30	JF327458	*Shewanella baltica*	100%
KB31	JF327459	*Myroides* sp.	96%
KB32	JF327460	*Sphingobacterium* sp.	99%
KB33	JF327461	*Sphingobacterium* sp.	99%
KB36	JF327462	*Pseudomonas fluorescens*	99%
KB37	JF327463	*Pseudomonas* sp.	99%
KB38	JF327464	*Pantoea agglomerans*	99%
KB39	JF327465	*Pseudomonas* sp.	99%

Strain	Accession number[a]	Closest phylogenetic relative	Identity
KB40	JF327466	*Pseudomonas* sp.	99%
KB42	JF327467	*Pseudomonas* sp.	99%
KB43	JF327468	*Stenotrophomonas* sp.	100%
KB44	JF327469	*Pseudomonas* sp.	99%
KB45	JF327470	*Sphingobacterium* sp.	100%
KB46	JF327471	*Sphingobacterium* sp.	99%
KB47	JF327472	*Pseudomonas* sp.	99%
KB49	JF327473	*Serratia proteamaculans*	99%
KB50	JF327474	*Pseudomonas* sp.	99%
KB51	JF327475	*Janthinobacterium lividum*	100%
KB52	JF327476	*Enterobacter* sp.	98%
KB54	JF327477	*Rhodococcus erythropolis*	100%
KB56	JF327478	*Serratia proteamaculans*	99%
KB57	JF327479	*Arthrobacter* sp.	100%
KB58	JF327480	*Flavobacterium* sp.	97%
KB59	JF327481	*Microbacterium oxydans*	100%
KB61	JF327482	*Serratia* sp.	100%
KB63	JF327483	*Pseudomonas* sp.	99%
KB64	JF327484	*Pantoea agglomerans*	100%
KB66	JF327485	*Bacillus pumilus*	100%
KB68	JF327486	*Pseudomonas* sp.	99%
KB71	JF327487	*Serratia* sp.	99%
KB72	JF327488	*Serratia* sp.	98%
KB73	JF327489	*Pseudomonas* sp.	99%
KB75	JF32749	*Acinetobacter* sp.	98%
KB76	JF327452	*Pseudomonas putida*	99%

a= accession numbers as assigned by GenBank

3.3 Phylogeny and tree reconstruction

Phylogenetic analysis, based on alignment with highly similar 16S sequences in the Genbank, was performed when comparison with Blastn analysis did not give a certain identification. In order to get a confident branch-length, the whole alignment was split and three different trees were generated for *Pseudomonas*, *Serratia* and the other analyzed genera, respectively.

Pseudomonas tree (Figure 3.1)

The tree was generated on the basis of the alignment of 58 sequences, including KB strains and out-group sequences, belonging to highly similar *Pseudomonas* species. The AICc selected as best model was GTRG, with base frequencies as follows: A= 0.255, T=0.210, G= 0.310, C= 0.223. The main topology of the tree obtained for this genus reflected the phylogeny obtained by Anzai et al., (2000). In particular, the groups *P. fluorescens*, *P. chlororaphis*, well supported with bootstrap values of 99.5% and 99.8% respectively, were clearly discernable, while *P. jessenii* and *P. brenneri* were in external positions. In the "*P. fluorescens* group" pooled many different species: *P. orientalis, P. antartica, P. meridiana, P. grimontii, P. rhodesiae, P. lurida* and *P. costantini*. In the "*P. chlororaphis* group" the species *P. fragi, P. psychrophila* and *P. lundensis* were included. However, within the groups the species were not well resolved:some groups were not well supported and some species were split in different clusters, as for *P. fluorescens* and *P. antarctica* in the "*P. fluorescens* group" or *P. fragi* in the "*P. chlororaphis* group". This apparent contradiction may be due to misidentifications in the GenBank, while the low bootstrap values supporting some clades were most probably a consequence of the inefficiency of the gene-target used. Some authors, in fact reported that the degree of resolution obtained with 16S rRNA gene sequence analysis is not sufficiently discriminatory to permit resolution of intrageneric relationships because of the its extremely slow evolution rate (Yamamoto et al., 2000, Ait Tayeb et al., 2005, Mulet et al., 2009).

Six KB strains pooled in the "*P. fluorescens* group". KB23 grouped confidently with *P. costantinii* and *P. lurida* EF111123. However, *P. lurida*EF111123 was most probably misidentified since all the strains belonging to this species grouped in a different position. For this reason we may conclude that strain KB23 can be assigned to the species *P. costantinii*.

KB50 and KB47 grouped together with an unidentified bacterium isolated from Antarctica (88.5% bootstrap) and could be considered sister of *P. orientalis*, *P. meridiana* and *P. antarctica*. It is worth noting that all the *P. meridiana* and *P. antarctica* were isolated in various Antarctic water bodies and could be somehow ecologically related to our strains.

The group where strain KB07 was pooling (bootstrap 74%) included *P. grimontii* and *P. rhodesiae*. The strain occupied an isolated position within the group and showed no clear relation with the two mentioned species.

Strain KB39 was in an isolated position and KB21 was on a long branch and grouped with any precise species. Basing on these results we may conclude that strains KB50, KB47,KB07, KB39 and KB21 can only be assigned to the "*P. fluorescens* group". In particular,KB21, with its long branch and low similarities in the GenBank most probably belonged to a still undescribed species.

Five KB strains pooled in the "*P. chlororaphis* group". KB44 and KB73, clustering with high bootstrap values whit two groups constituted only by the species *P. lundensis* and *P. fragi*, respectively, may be assigned to these species. KB42, KB63 and KB40 may be assigned to the "*P. chlororaphis* group" but not to specific species.

Strain KB37, grouping with *P. brenneri* with low bootstrap value, and strain KB68, pooling with an unidentified *Pseudomonas*, cannot be confidently assigned to any species.

Serratia tree (Figure 3.2)

The Neighbour-Joining tree was generated basing on the alignment of 27 sequences, including KB strains and outgroup sequences, belonging to highly similar *Serratia* species and rooted with *Enterobacter aerogenes* and *Pantoea agglomerans* ,which are strictly phylogenetically

related. The backbone of the tree reflected the uncertainty of the genus phylogeny with some low bootstrap values and scarce resolution for some groups. The species *S. grimesii, S. liquefaciens* and *S. proteamaculans*, for instance, cannot be fully resolved and grouped together with a bootstrap of 52% only. This group was previously indicated as *S. grimesii-liquefaciens-proteamaculans* Complex (Grimont et al., 1982) where *S. liquefaciens* was discerned on the basis of both DNA hybridization and biochemical data. Sequence analyses suggested that strains KB25 and KB17 were included in this group.KB25, in an isolated position, cannot be assigned to a precise species but at the Complex level only. Strain KB17, even if supported with a bootstrap of 65% only, pooled with a group purely constituted by *S. proteomaculans* and was assigned to this species. However, some species were resolved with good level of confidence and few isolates may be certainly ascribed at species level. Strains KB71 and KB72, for instance, grouped in a high supported group (bootstrap98%) represented by *S. plymuthica* and may be assigned to this species. However, KB 72 was located on a rather long branch probably due to the bad electropherogram obtained with the sequencing analyses. For this strain data must be confirmed and sequence verified possibly by cloning. KB16, in a 100% supported group with a single species, was assigned to *S. fonticola*. Strain KB61 clustered in a 100% supported group including strains of *Serratia* not identified at species level. One of these microorganisms, being isolated in sub-extreme environment such as the Sub-Antarctic region, could show ecological characteristics similar to those of KB strains.

Other genera tree (Figure 3.3)

The tree was generated on the basis of the alignment of 73 highly similar sequences, including KB strains and outgroup sequences, belonging to 7 different Orders and was rooted with the species *Deinococcus* sp. and *Synechococcus* sp. The AICc selected as best model was GTRG, with base frequencies as follows: A=0.260, T=0.213, G=0.302, C=0.222. All the genera analyzed clustered in well supported groups and species were separated in well discernable

sub-clusters. The only exception was the "*Sphingobacterium* group" where single species were scarcely resolved and appeared intermixed within the cluster.

In the genus *Arthrobacter* was included the strain KB57 only. The strain appeared to be strictly related with species from peculiar environments. *A.kerguelensis* was isolated in Kergueland Island in the Sub-Antarctic region and *A. psychrophenolicus* from an alpine ice cave. Strains *Arthrobacter* sp. AJ920001 and *Arthrobacter* sp. DQ173000 were isolated from Kerguelen Island coastal seawater and from the Tianshan Mountains alpine permafrost (Northwestern China), respectively. Although KB57 cannot be affiliated at species level basing on its phylogenetic position, phylogeny could help to predict its ecology. In fact, this strain grouped only with strains from cold regions.

Among the strains belonging to *Flavobacterium,* species clustered in well distinct groups. *F. johnsoniae* and *F. hercynium* while *F. aquidurense* and *F. frigidimaris* are closely related species (Cousin et al., 2007) and clustered together. StrainsKB02, KB04 and KB58 fell in this genus but showed low similarities in the GenBank (96-98%) and were located in isolated positions. Thus they most probably belong to undescribed species.

The genus *Myroides* included only strain KB31 showed very low similarities in the GenBank and had an external position in the tree. The highest similarity (96%) was with *Myroides* sp. GU253339 while lower matches (95%) were recorded with *M. odoratimimus* and *M. odoratus*. For these reasons, it may belong to a new species.

In the genus *Sphingobacterium* fell strains KB05, KB32, KB33,KB45 and KB46 showing similarities (98-99%) with different strains of *S. faecium*, *S. kitahiroshimense* and many unidentified *Sphingobacterium* species. Since no clear grouping was obtained for these strains, they can be assigned at genus level only. In this case, the target gene used was probably not informative enough to get a reliable phylogeny leading to the scarce resolution of the different species within the Genus.

The Genera *Pantoea* and *Enterobacter* are known to be strictly related (Gavini et al., 1989). In fact, they grouped with 100% bootstrap in the phylogenetic tree. However they were well discernable and formed two distinct cluster supported with bootstrap of 97.8% and 83.8%, respectively. Strain KB52was the only included in this group showing98% similarities with *E. aerogenes* and *E. amnigenus*. Basing on its phylogenetic position it may be assigned to the genus *Enterobacter* but its external position and low match in GenBank suggested that it could belong to a new species.

KB75 was the only strain included in the genus *Acinetobacter*. Basing on the very low similarities in GenBank(97-98%) and due to its external position in the phylogenetic tree it probably belongs to a new species.

Within the genus *Stenotrophomonas* the two species *S. rhizophila* and *S. maltophila* were well discernable and supported with good bootstrap values. Strain KB43 fell in the genus with 100% similarity with both the species and many other uncultured clones. The phylogeny allowed to resolve the strain position which can be certainly assigned to *S. rhizophila*.

In conclusion, phylogenetic analysis was useful to identify some strains at species level and to suppose that some others, due to isolated position or long branch, were probably belonging to new species even if further investigation are needed to support this hypothesis. For all other strains identification was possible at genus level only. In many cases this could be due to the scarce informative power of the gene target used to discern the relations below the genus level.

Figure 3.1

Molecular phylogeny of *Pseudomonas* species based on 16S rRNA gene sequences. ML tree, based on 58 sequences and 1452 positions, has been generated using GTR + I + G model and calculated using ML in MrAIC software. Bootstrap values from 1,000 re-sampled data sets are shown

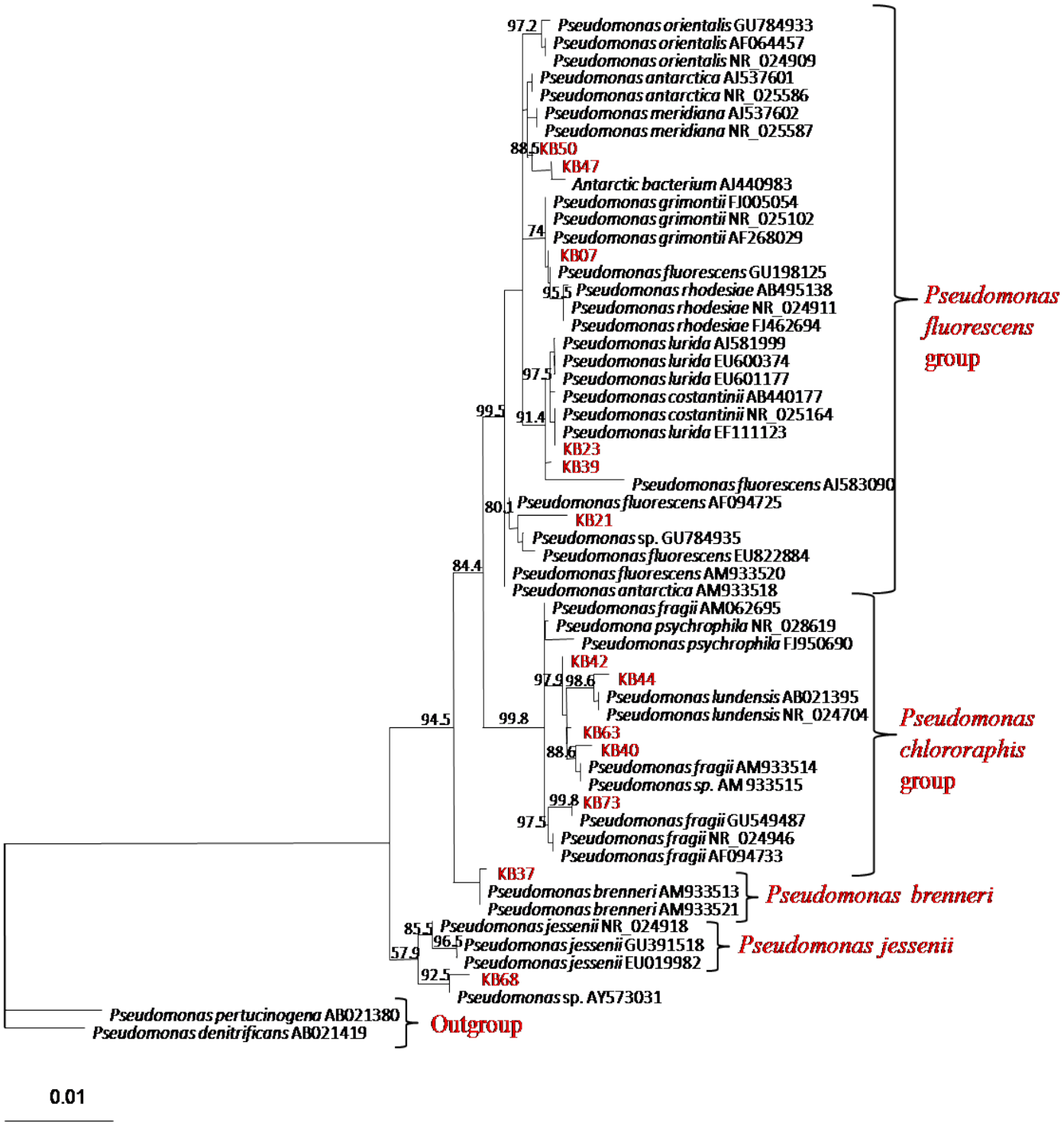


Figure 3.2

Phylogenetic tree of *Serratia* species. The tree was inferred using the Neighbour-Joining algorithm based on 27 sequences and ca. 1459 positions. Bootstrap values from 1,000 re-sampled data sets are shown: values below 50% are not shown in the tree.

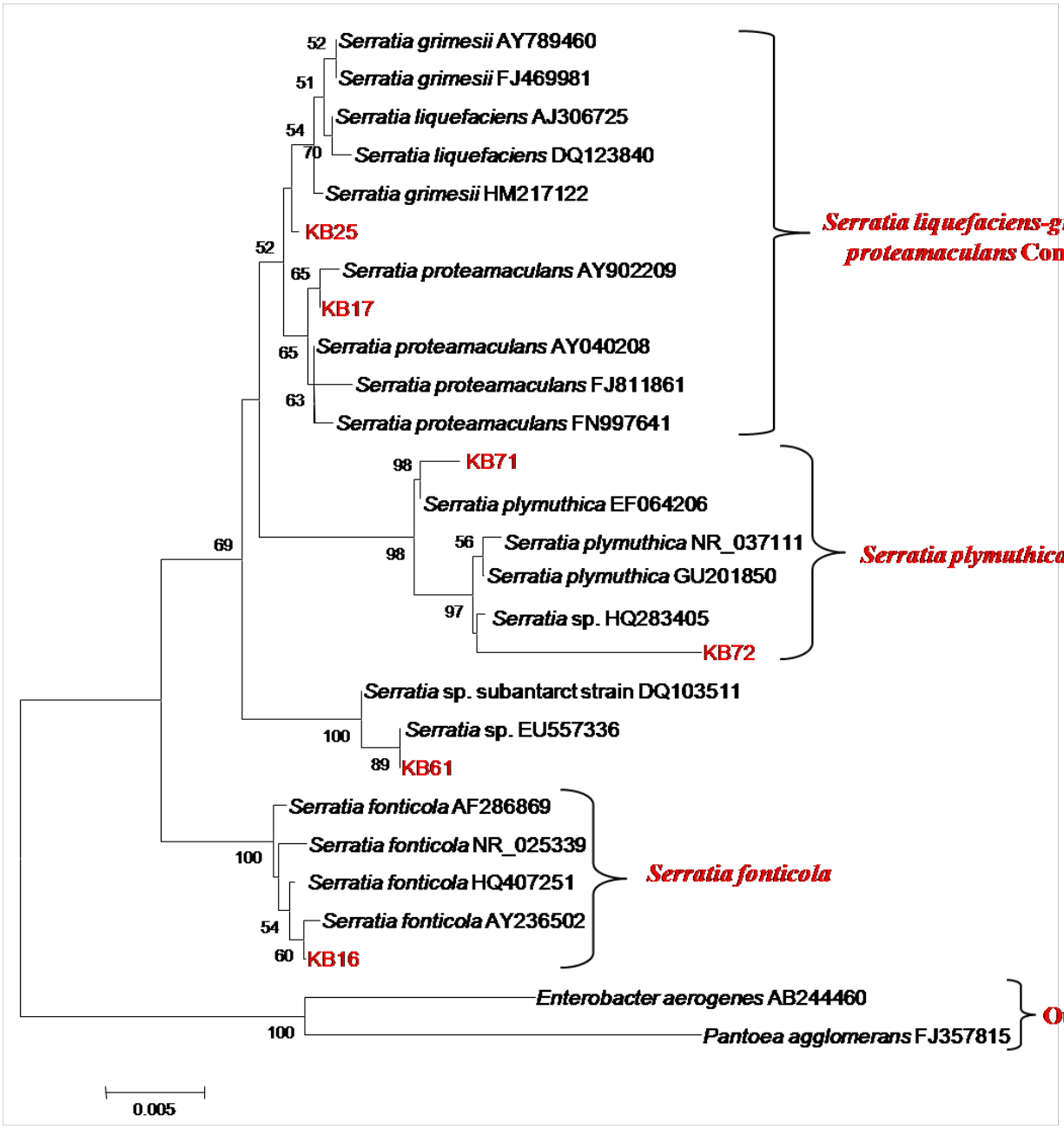
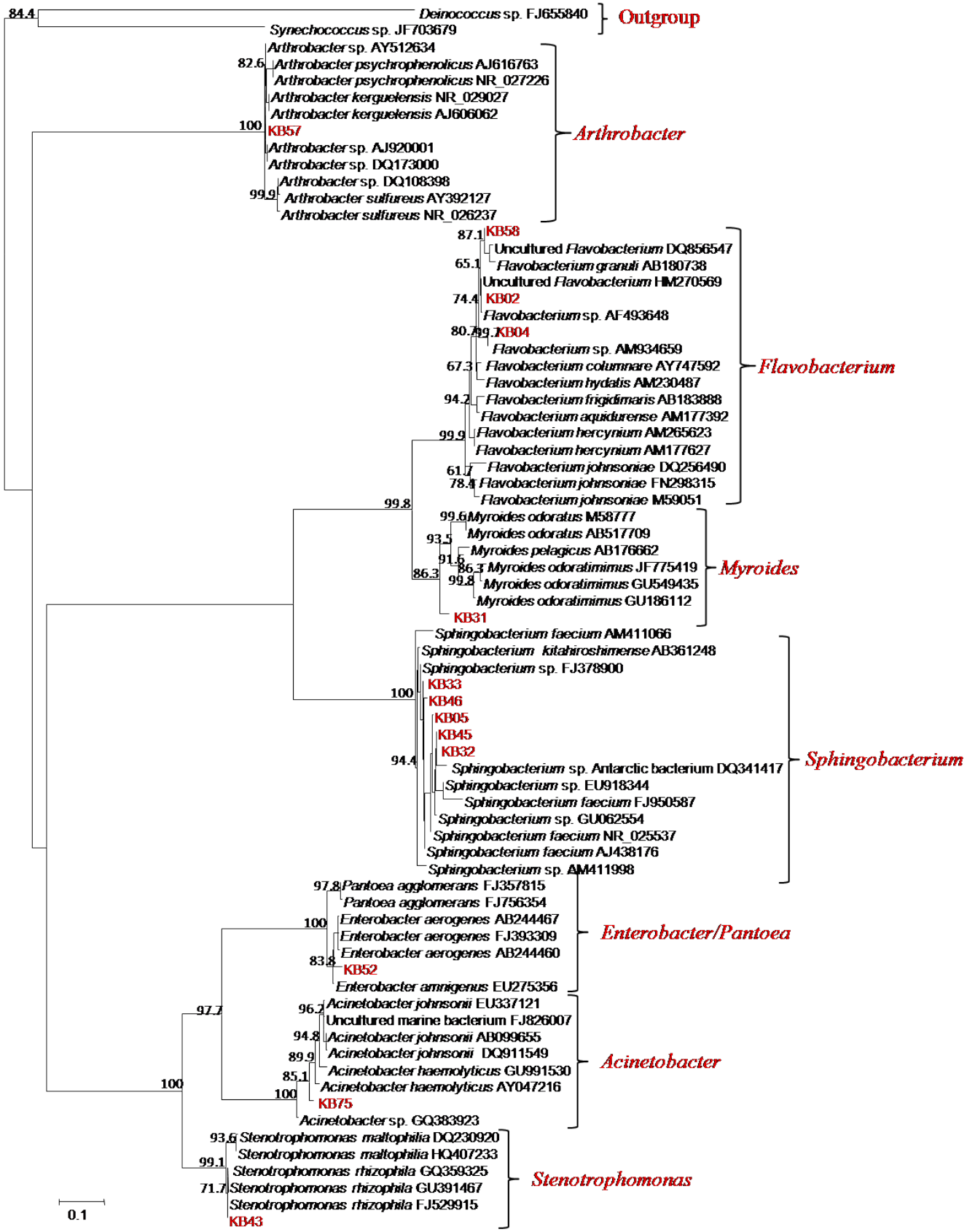


Figure 3.3 Molecular phylogeny of other bacterial species based on 16S rRNA gene sequences. The ML tree, based on 73 sequences and 1490 positions, has been generated using GTR + I + G model and calculated using ML in MrAIC software. Bootstrap values from 1,000 re-sampled data sets are shown



3.4 Determination of optimal growth temperature

Table 3.3 shows the growth range and optima of the various strains; comparison with literature, if available, is also reported for the identified species. Most of the strains (ca. 42%) showed the optimum at 30°C. In addition, the majority of them were able to grow in a rather broad range of temperature. The lowest optimum was recorded at 15°C for one strain only (*Acinetobacter* sp. KB75) while highest optimum was at 40°C (*Serratia proteamaculans* KB22 and KB49). Most of the strains (56%) was able to growth at 0°C while only 25% grew at 45°C. Figure 3.4 shows the temperature growth profile of the KB strains. Colony diameters at optima ranged between 6 and 70 mm.

As for temperature preferences, microorganisms are traditionally grouped according to their ranges and optima for growth (Wiegel, 1990). However, current definitions are not satisfactory to cover the huge microbial variability. As already reported by the early studies of Rouf and Rigney (1971), since the range of growth for certain species is from 0 to 55°C, simple consideration of minimal, optimal, and maximal growth temperatures may not be sufficient to classify them as psychrophiles or mesophiles. Due to the increased attention to extreme environments and consequent discovery of very peculiar microorganisms, showing uncommon adaptations to temperature variations, this question became progressively more crucial and has been largely debated (Gounot, 1991; Rothschild and Mancinelli, 2001; Helmke and Weyland, 2004). Nevertheless, the most accepted definition for cold adapted bacteria is still that of Morita (1975). Accordingly, psychrophiles were defined as those having an optimal temperature for growth at about 15°C, a maximum at 20°C and a minimum at 0°C or below. Microorganisms able to grow at about 0°C, but having maximum above 20°C, were considered as psychrotrophics: optimum is not mentioned (Morita, 1975; Gounot, 1986; Gounot, 1991; Dalluge et al., 1997). However, the term psychrotrophic had been recently replaced with the most significant “psychrotolerant” (Gounot, 1991; Rothschild and

Mancinelli, 2001; Helmke and Weyland 2004). Mesophilic microorganisms are often defined as growing in the range 10-50°C (Russell and Fukunaga, 1990; Wiegel, 1990) while optima are generally in the range of ca. 30-40°C. Lot of mesophilic microorganisms from cold environments, even if showing optima in the mesophilic range, grow well at about 0°C. These organisms had been defined as mesophilic-psychrotolerant and fall also in the broad group of psychrotolerant (Zucconi et al., 1996; Helmke and Weyland, 2004). Thus, the definition of psychrotolerant, that has in principal been proven to be useful, could be somehow too generic and nonspecific because it would combine microorganisms with very different optima. Therefore the cardinal temperatures for psychrotolerants should be reconsidered on the basis of increased numbers of cold adapted microorganisms isolated from various habitats and in the light of new ecological data (Helmke and Weyland, 2004). Even if strict definitions are never exhaustive, we would prefer to define as psychrotolerant those bacteria able to growth at 0°C and having their optima in the range >15-≤25°C. In consequence, we would consider as mesophilic-psychrotolerant those bacteria growing at 0°C with optima in the range >25-≤40°C.

It is also important to know what is the widest temperature range (temperature span) over which a single organism can grow. A wider temperature range makes an organism more versatile with regard to environmental changes and it enables the organism to utilize a wider array of ecological niches (Wiegel, 1990). Generally organisms able to growth in a wide range of temperature are defined as eurythermics.

In our case, accordingly to the above definitions, the majority of the strains were psychrotolerant (42%) or mesophilic-psychrotolerant (40%) and no real psychrophiles were detected (Table 3.3). Almost all the strains could be considered as eurythermics indicating adaptation to frequent and wide temperature variations such as those of Kandalaksha Bay (Howland et al., 1999; Pantyulin, 2003; Savvichev et al., 2004; Shaporenko et al., 2005). Actually, psychrophiles, with their narrow range of growth temperature, are typical of rather

stable environments (Zucconi et al., 1996; Bowman et al., 1997; Helmke and Weyland, 2004). Even strain *Acinetobacter* sp. KB75, having optimum at 15°C, cannot be strictly attributed to this group because it can grow up to 30°C. Anyway, presence of psychrotolerant microorganisms, rather than psychrophiles, in Arctic and Antarctic regions has been well documented (Zucconi et al., 1996; Ray et al., 1999; Shivaji et al., 2005). Finally, only KB66 showed a strictly mesophilic behavior.

Another important question to address is the growth curve profile. Normally, curves are characterized by an asymmetric shape: slope over the optimum is quite sharper than that below. In fact, a temperature rise of just 5°C above the optimum is often sufficient for strong growth inhibition and a further increase of 5°C could lead to death. By contrast, below the optimum, microorganisms could survive well even with a decrease of 30-40°C. Growth at the optimum is generally much more pronounced than that at lowest and highest limits.

Growth profiles of KB bacteria are very diversified (Figure 3.4 A-N) highlighting a composite community with different degrees of adaptation to environmental changing conditions (Berry and Foegeding, 1997). Some strain (ca. 33%) had the above described classic profile, others showed uncommon flat and wide curves: they presented a limited growth, even at the optimum, but temperature span was quite extended. For example, *Pseudomonas fluorescens* KB11 grew between 0 and 35°C but maximum colony diameter was ca. 7.5 mm only (Figure 3.4 B). In addition, few strains had a peculiar symmetric curve (e.g. *Acinetobacter* sp. KB75, Figure 4.1L) with apparent similar response to cold and warm temperatures. Some other bacteria, such as *Serratia* sp. KB61 and *Pseudomonas putida* KB76, showed an unusual sharper growth decrease below rather than above the optimum (Figure 3.4 I, 3.4 M). Apparently, they were more tolerant to warm than to cold temperatures. This phenomenon has never been reported before.

As said, temperature spans were, in general, quite broad (ca. 35 °C) and it was evident that many KB strains had wider ranges of growth if compared with same species described in

literature; optima were also different. However, it is worth noting, that studies concerning temperature preference of bacterial strains are not very common even for well-known species and few data were available.

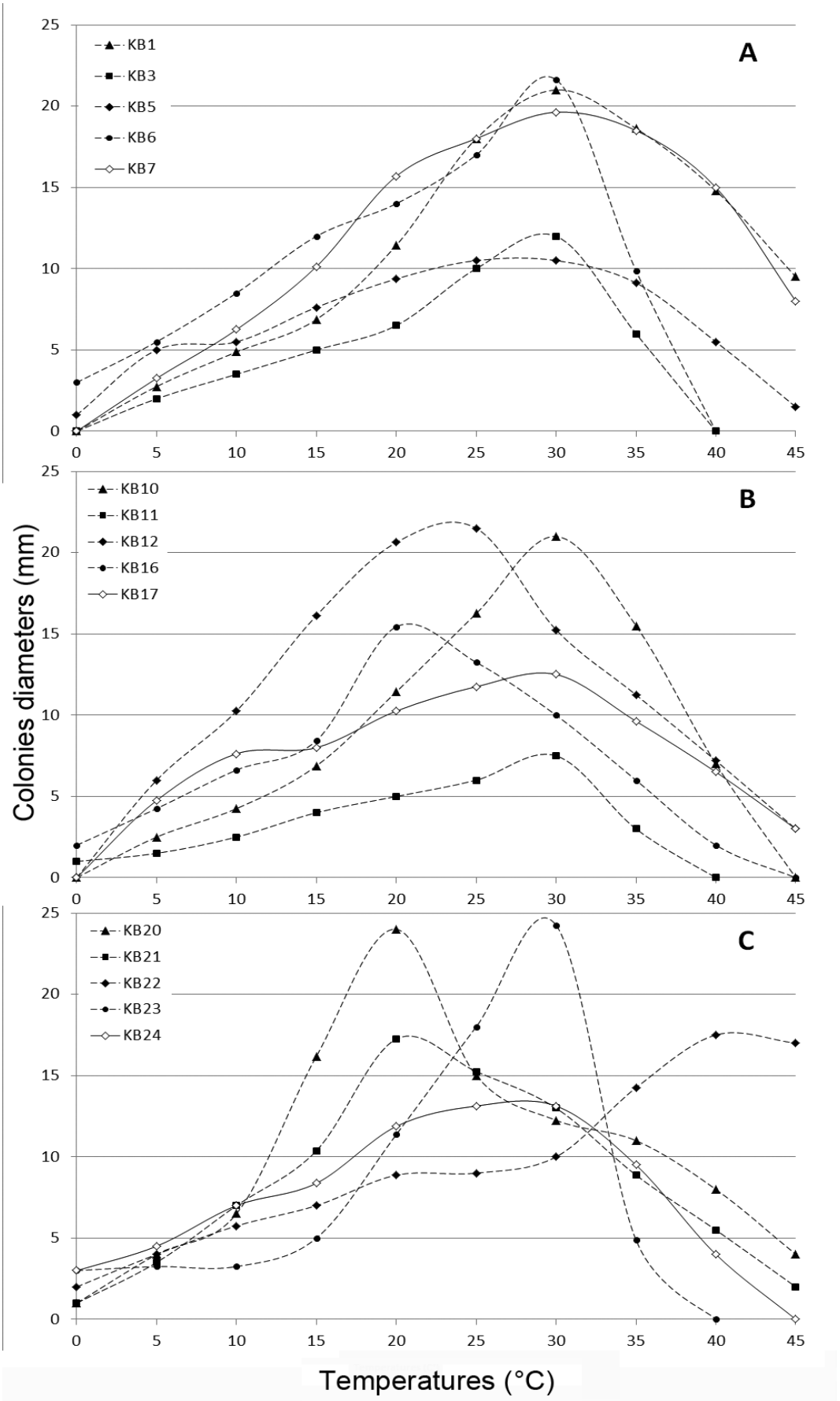
Table 3.3 Temperature preferences comparison between Kandalaksha Bay strains and strains of the same species reported in literature

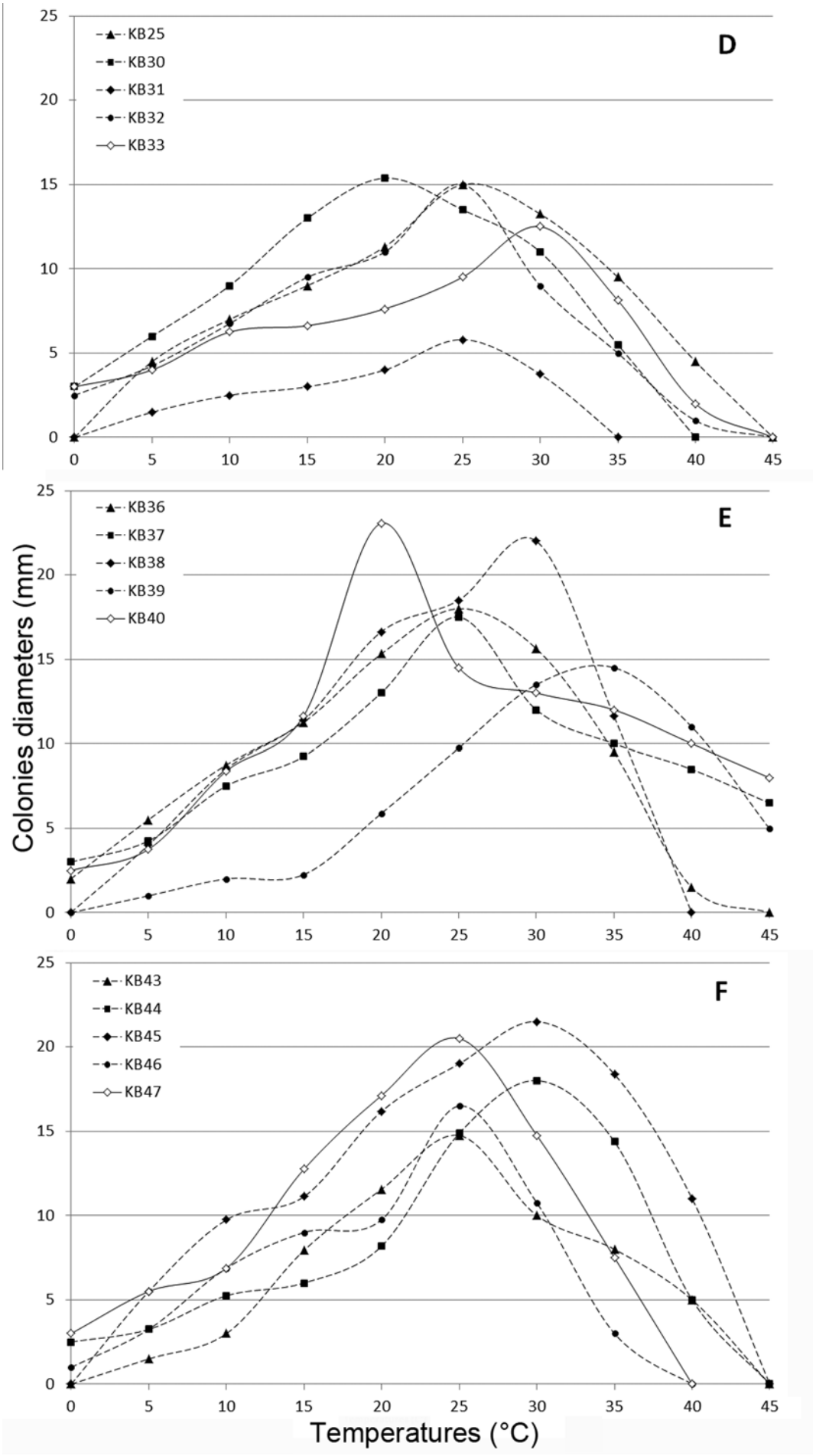
		KB strains		Literature		
Strain	**Closest phylogenetic relative**	**Range °C**	**Optimum °C**	**Range °C**	**Optimum °C**	**Citation**
KB1	*Serratia plymuthica*	5-45	30	10-40	30	(Breed et al., 1948; Pradhan and Ingle, 2007)
KB2	*Flavobacterium* sp.	0-30	20			
KB3	*Exiguobacterium oxidotolerans*	5-35	30	4-40	34	(Yumoto et al., 2004b)
KB4	*Flavobacterium* sp.	0-30	20			
KB5	*Sphingobacterium* sp.	0-45	25-30			
KB6	*Pseudomonas fluorescens*	0-35	30	0-37	30	(Gugi et al., 1991; Jaouen et al. 2004)
KB7	*Pseudomonas* sp.	5-45	30			
KB10	*Pseudomonas syringae*	5-40	30	4-30	28 22	(Young et al., 1977) (Sundareswaran et al., 2010)
KB11	*Pseudomonas fluorescens*	0-35	30	0-37	30	(Gugi et al., 1991; Jaouen et al. 2004)
KB12	*Pseudomonas putida*	5-45	25	0-30	30	(Palleroni, 1984; Kotturi et al., 1991)
KB16	*Serratia* sp.	0-40	20			
KB17	*Serratia* sp.	5-45	30			
KB20	*Pseudomonas fluorescens*	0-45	20	0-37	30	(Gugi et al., 1991; Jaouen et al., 2004)
KB21	*Pseudomonas* sp.	0-45	20			
KB22	*Serratia proteamaculans*	0-45	40	4-37	20-30	(Grimont et al., 1982; Old et al., 1983)
KB23	*Pseudomonas* sp.	0-35	30			
KB24	*Pseudomonas fluorescens*	0-40	25-30	0-37	30	(Gugi et al., 1991; Jaouen et al., 2004)
KB25	*Serratia* sp.	5-40	25			

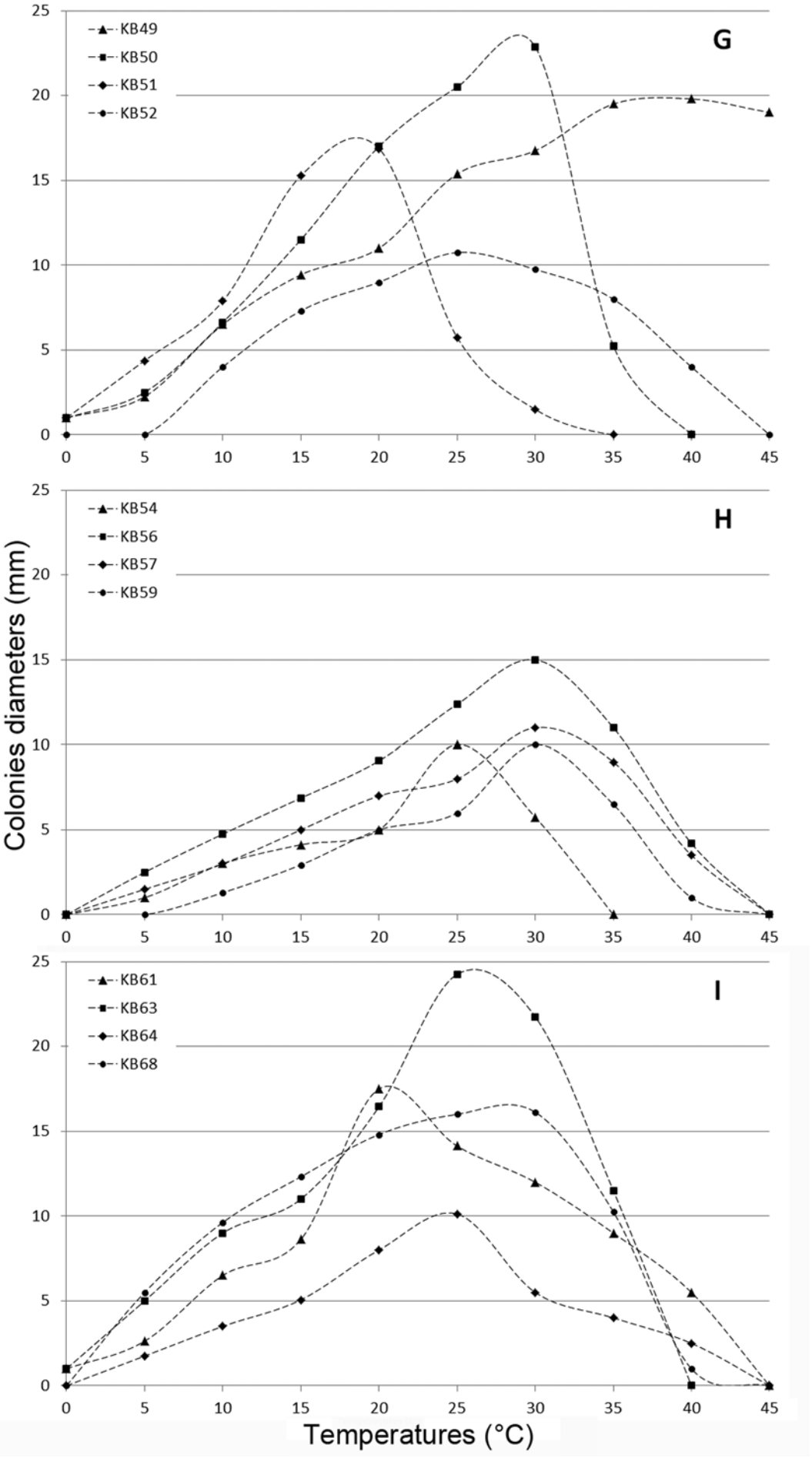
		KB strains		Literature		
Strain	**Closest phylogenetic relative**	**Range °C**	**Optimum °C**	**Range °C**	**Optimum °C**	**Citation**
KB30	*Shewanella baltica*	0-35	20	4-37	25	(Ziemke et al., 1998; Vogel et al., 2005)
KB31	*Myroides* sp.	5-30	25			
KB32	*Sphingobacterium* sp.	0-40	25			
KB33	*Sphingobacterium* sp.	0-40	30			
KB36	*Pseudomonas fluorescens*	0-40	25	0-37	30	(Gugi et al., 1991; Jaouen et al., 2004)
KB37	*Pseudomonas* sp.	0-45	25			
KB38	*Pantoea agglomerans*	5-35	30	5-45	30	(Gavini et al., 1989; Son et al,. 2006)
KB39	*Pseudomonas* sp.	5-45	35			
KB40	*Pseudomonas* sp.	0-45	20			
KB42	*Pseudomonas* sp.	0-35	30			
KB43	*Stenotrophomonas* sp.	5-40	25			
KB44	*Pseudomonas* sp.	0-40	30			
KB45	*Sphingobacterium* sp.	5-40	30			
KB46	*Sphingobacterium* sp.	0-35	25			
KB47	*Pseudomonas* sp.	0-35	25			
KB49	*Serratia proteamaculans*	0-45	40	4-37	20-30	(Grimont et al., 1982; Old et al., 1983)
KB50	*Pseudomonas* sp.	0-35	30			
KB51	*Janthinobacterium lividum*	0-30	20	4-30	25	(De Ley et al., 1978; Sneath, 1984)
KB52	*Enterobacter* sp.	10-40	25			
KB54	*Rhodococcus erythropolis*	5-30	25	10-40	25	(Tomioka et al., 1994)
KB56	*Serratia proteamaculans*	5-40	30	4-37	20-30	(Grimont et al., 1982; Old et al., 1983)

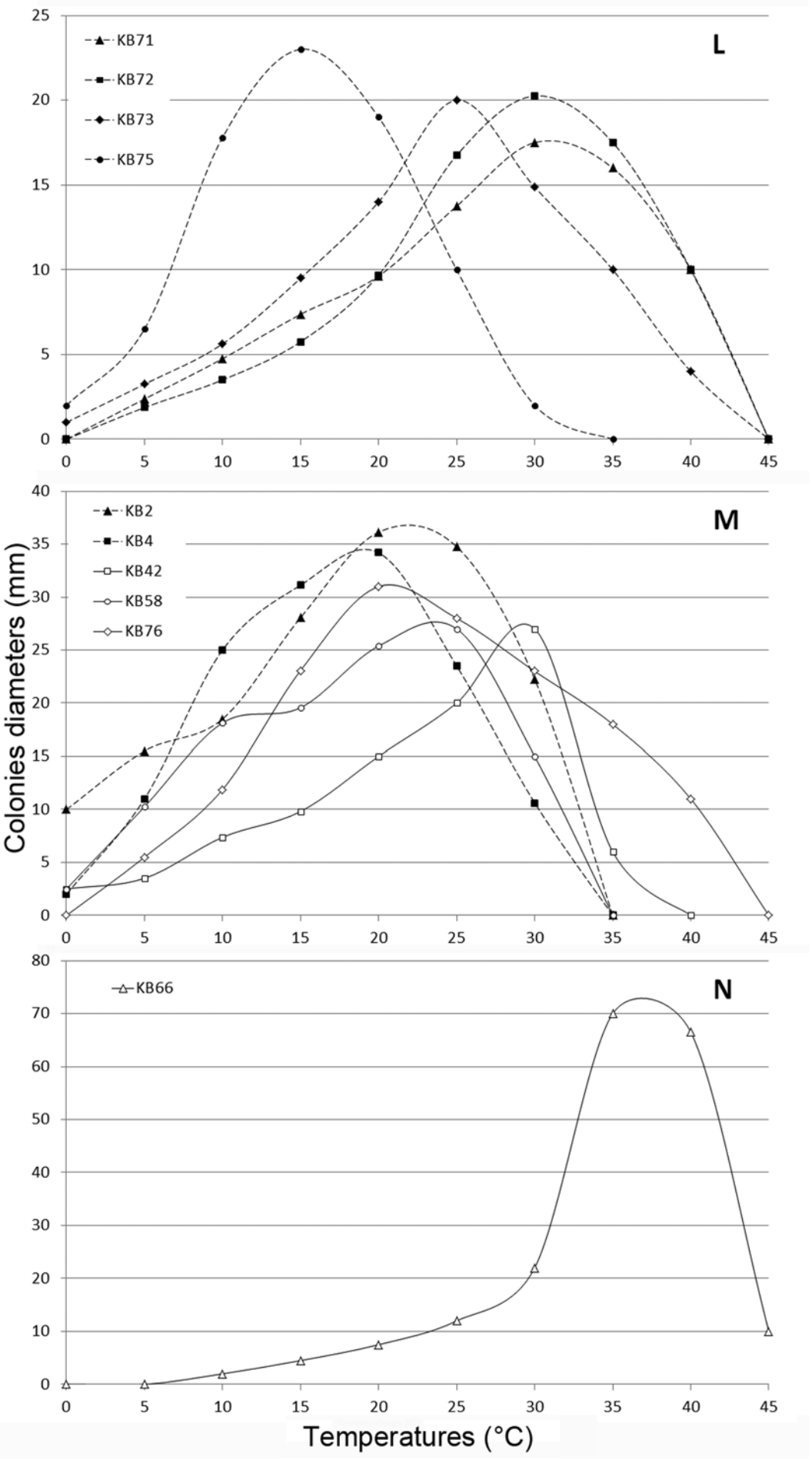
		KB strains		Literature		
Strain	**Closest phylogenetic relative**	**Range °C**	**Optimum °C**	**Range °C**	**Optimum °C**	**Citation**
KB57	*Arthrobacter* sp.	5-40	30			
KB58	*Flavobacterium* sp.	0-30	25			
KB59	*Microbacterium oxydans*	10-40	30		30	(Schumann et al., 1999)
KB61	*Serratia* sp.	0-40	20			
KB63	*Pseudomonas* sp.	0-35	25			
KB64	*Pantoea agglomerans*	5-40	25	5-45	30	(Gavini et al., 1989; Son et al., 2006)
KB66	*Bacillus pumilus*	10-45	35	10-55	37	(Battan et al., 2007)
KB68	*Pseudomonas* sp.	5-40	30			
KB71	*Serratia* sp.	5-40	30			
KB72	*Serratia* sp.	5-40	30			
KB73	*Pseudomonas* sp.	0-40	25			
KB75	*Acinetobacter* sp.	0-30	15			
KB76	*Pseudomonas putida*	5-40	20	0-30	30	(Palleroni, 1984; Kotturi et al., 1991)

Figure 3.4 Temperature profiles of KB strains cultivated in PCA in the range 0-45°C. Growth was measured as average increase of colony diameter. Data are mean of 3 replicates; SD was less than 10% .




D
KB25
KB30
KB31
KB32
KB33
E
KB36
KB37
KB38
KB39
KB40
F
KB43
KB44
KB45
KB46
KB47
Colonies diameters (mm)
Temperatures (°C)
0
5
10
15
20
25
30
35
40
45


G
KB49
KB50
KB51
KB52
H
KB54
KB56
KB57
KB59
I
KB61
KB63
KB64
KB68
Colonies diameters (mm)
Temperatures (°C)
0
5
10
15
20
25
30
35
40
45


L
KB71
KB72
KB73
KB75
M
KB2
KB4
KB42
KB58
KB76
N
KB66
Colonies diameters (mm)
Temperatures (°C)

3.5 Metabolic characterization

3.5.1 Screening for extracellular enzyme activity

All the isolates grew rather well on plates filled with the various media used for extracellular enzyme activities determination(data not shown). A certain growth was recorded also in case of no detection of the relative enzyme activity. The only exception was recorded on the chitinase medium (CHI):in this case, growth was recorded only for chitinase-positive strains.

The overall production of extracellular enzymes, by the tested strains, is shown in Table 3.4. Since no strain revealed cellulose activity, under the tested conditions, this data is not included in the table. Percent of strain, showing the various enzyme activities, is reported in Figure 3.4.

Enzymatic catalysis plays an important role in the flow of material and energy in marine ecosystem. Some substance transforming processes would not be completed without extracellular enzymes (Zheng et al, 2002). In fact, since only relatively small molecules can be readily transported across the bacterial membrane, larger substrates must initially be hydrolyzed outside the cell (Arnosti et al., 2005, Obayashi and Suzuki, 2008). Extracellular enzymes are generally used in biotechnology and they are easily produced at industrial level. However, due to the huge number of traditional and innovative enzyme applications, the research of new enzymes and/or new microorganisms could offer novel biocatalysts with peculiar properties such as high salt tolerance, hyperthermostability, barophilicity, cold adaptivity etc. (Debashish et al., 2005).

Lipases were the most diffuse activities and were present in ca. 54% of the isolates. This result is not surprising: it is well-know that lipase is one of the most spread activity in the sea (Davey et al., 2001). Tween 20 was the preferred substrate(hydrolyzed by ca. 50%)but various strains (25%) were able to use all the three substrates (Tween 20, 60 and 80). Relevant presence of lipolytic bacteria in seawater samples was already reported in other studies aimed

to characterize the production of extracellular enzymes by marine isolates (Zaccone et al., 2002, Fenice et al., 2007). High presence of this activity in seawater bacteria could be due to the degradation of some zooplankton components: lipids ,after proteins, are the most important zooplankton fraction (Zaccone et al., 2002). Result indicate that the majority of the strains showing this enzyme activity are related to the genus *Pseudomonas*. Production of lipase by *Pseudomonas* is known and in general *Pseudomonas* lipases are used in various industrial applications (Hasan et al., 2006). Moreover, a psychrotrophic *Pseudomonas* lipolitic strains has also been studied (Leonov et al., 2010).

Also protease and phosphatase were rather common, being produced by 44 % of the strains. The production of extracellular protease in marine bacteria could be activated in conditions of high competition between microorganisms and/or against pathogens. Furthermore, marine decay of particulate matter involves peptidase activity that, consequently, is rather common in this environment. (Than et al., 2004). Proteases were detected in ca. 40% of the KB *Pseudomonas* strains being *Pseudomonas fluorescens* KB6 and *Pseudomonas* sp.KB7, found in almost all the sampling sites, the best producers of this activity. Our data confirmed the importance of this enzyme in psychrotrophic species of this genus and in particular for *Pseudomonas fluorescens* (Gugi et al., 1991, Alanís et al., 1999). Moreover, virulence of pathogenic *Pseudomonas* spp. is correlated to the production of various extracellular proteases (Caballero et al; 2004). Phosphatases are key-enzymes in marine environment. They are typical of algae and bacteria and have an important role in the recycle of organic phosphate and to avoid phosphorus limitation in the sea (Hoppe, 2003). Although nitrogen is generally considered the main limiting factor in the oceans, various studies indicated phosphorus as an important limiting element in certain coastal and offshore regions (Cotner et al. 1997; Zohary and Robarts 1998; Benitez-Nelson and Karl 2002). In addition, phosphorus plays an important role in the regulation of biofilm formation (Monds et al, 2006) a fundamental process for marine bacteria ecology. At the applicative level, phosphatases find

use in molecular biology and immunology (Wright et al., 1996; Scanlan and Wilson, 1999). They are also used as indicators of marine dinoflagellates and cyanobacteria toxins production (Shi et al., 1999). As said, the production of this enzymes by the KB strains was rather relevant and particularly diffused in the *Pseudomonas* species. Phosphatases by psychrotrophic *P. fluorescens* strains have been widely studied (Burini et al. 1994; Guillou and Guespin-Michel 1996).

Amylase and pectinase were less common: they were detected in ca. 32% of strains. Generally pectin and starch are largely present, both in terrestrial and marine environments as result of plant degradation. As for amylase, enzyme production by bacteria of cold marine environment was studied by (Srinivas et al., 2009) showing similar diffusion rate. Amylase production by strain *Bacillus pumilus* KB66 was quite high, confirming the attitude of this genus, also employed at the industrial level, to produce these hydrolases (Demain, 2009).Concerning pectinase, degradation of this polymer by marine microorganism has been reported even if pectin is considered of secondary importance in marine environment (Cummings and Black, 1999). So far, only few pectinolytic psychrotolerant bacteria have been studied in some details most of them, including *Pseudomonas fluorescens,* are related to spoilage (Laurent et al., 2000; Truong et al., 2001).In our screening, various strains of *Pseudomonas*, including *Pseudomonas fluorescens*, were good producer of this activity but largest halos were detected in two *Flavobacterium* species.

Chitinase was not as common as the previously discussed activities (17% of the strains). Even if this data confirms other similar results (Fenice et al., 2007), reasons are not completely clear. Although, annual chitin turnover in marine environments is huge, much higher than those of other substrates, its concentration in water is almost negligible being only slighter higher in sediments. This was particularly noted in cold sea environments, such as Antarctica, as reported by Staley and Herwig (1993). Partial explication could be the very intense bacterial activity when chitin is present (i.e. krill booming) leading to its almost complete

depletion and consequent diminution of chitinolytic microorganisms. This could establish subsequent dominance of other populations. (Keyhani and Roseman, 1999; Ramaiah et al., 2000).

Two strains (KB49 and KB56),identified as *Serratia proteamaculans,*showed the highest halos for this activity that, however, was well diffused in the *Serratia* strains. Chitinolytic activity of this genus is well known already from the early work of Monreal and Reese (1969) and is related to its activity as insects and fungal pathogen (Grimont et al., 1979; Frankowski et al. 2001; Grimont and Grimont, 2006; Mehmood et al. 2009). Due to its very high chitinolytic activity this genus has been proposed for various applications such as the industrial production of chitinases, the biological control of pests (Ordentlich et al,1988) and the degradation of chitin-rich wastes (Giuliano Garisto Donzelli et al., 2003; Wang et al., 2008).

As for urease, this activity was detected only in a rather limited number of strains (17%) always showing quite large halos. Since these enzymes hydrolyze urea in carbon dioxide and ammonia, and activity detection is based on phenolred toning,big halos could be due to ammonia diffusion. Recent studies showed that bacterial ureases could find applications as antimycotic compounds and they can have a possible role in insect defense due to their proven insecticidal activity (Becker–Ritt et al., 2007, Carlini and Polacco, 2008).

New strains are continuously requested by industrial microbiology to be used in various applications. The aim to find producers of new microbial products and/or “high producers” of known substances could be achieved both trough the genetic improvement of known organisms or by screening processes. High producers are generally defined as those organisms able to produce high levels of certain compounds, possibly with scarce release of other products. This concept could be enlarged including the production of compounds having very peculiar characteristics. Generally screening procedure could be carried out using a two steps process. The preliminary semi-quantitative plate screening is aimed to select potential

high producers out of a large number of strains. The secondary quantitative screening, generally carried out using liquid cultures, is aimed to confirm plate-screening results and to quantify the actual production by instrumental determinations. Definition of high producers in plate-screenings is generally arbitrary and involve those organisms producing large halos of activity. In our case only those bacteria showing halos diameter ≥ 30 mm were considered as possible high producers (Table 3.4) and could be further investigated. In particular, *Flavobacterium* sp.KB58 showed the highest level of amylase and pectinase (34 and 48 mm respectively), while *Pseudomonas fluorescens* KB6 and *Serratia proteamaculans* KB56 were the best producers of protease(37 mm) and lipase (37 mm on Tween 80), respectively. Biggest halos were produced for urease: *Rhodococcus erythropolis* KB54 reached the highest level of this activity (95 mm).

Figure 3.5 shows the strains sorted by the number of extracellular enzyme activities detected, under the tested conditions. No strain was able to produce all the activities; the highest number of activities for a single strain (7 enzymes) was recorded only for *Serratia proteamaculans* KB17. By contrast, a rather large number of strains (17%) produced no activity at all. All other strains produced 1-5 enzymes. It is worth noting that plate screening could supply possible false-negatives. In some case, this could occur because enzymes could be located in the periplasm and not detected. Another factor contributing to negative results could be the catabolite repression exerted by some media component. In fact, most of the media contain a supplementary carbon source in addition to the compound used as enzyme target.

The results of the screening could be useful also at the ecological level. In fact, isolates producing a limited number of enzymes could be considered as rather specialized, while those with more diversified enzymatic competence could have higher eco-nutritional versatility (Figure 3.5).Specialization and eco-nutritional versatility of the strains will be better discussed in paragraph 4.5.2.

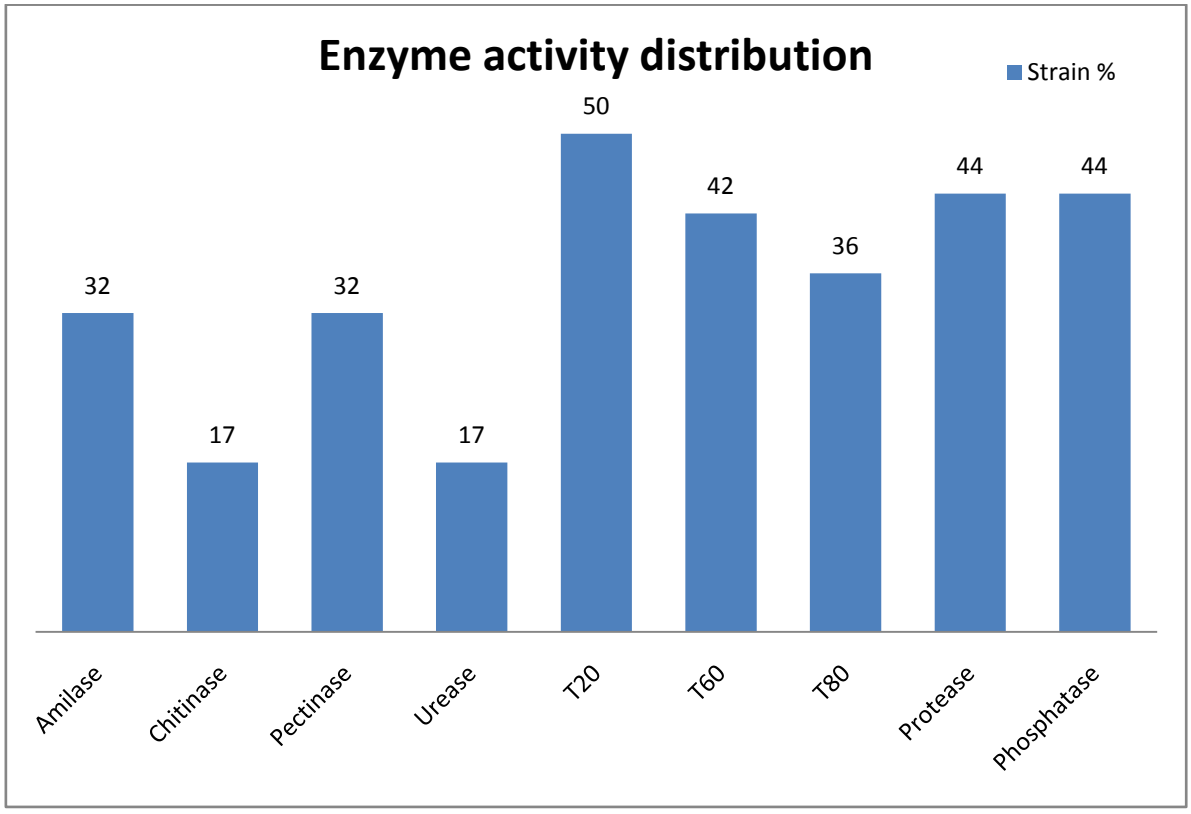
Table 3.4. Plate screening for extracellular enzyme activities by bacterial strains isolated from Kandalaksha Bay water samples.

Strain	AMI	CHI	PEC	PHO	URE	T20	T60	T80	PRO
KB1	-	5.0±0.2	-	-	-	8.1±0.5	-	13.1±0.3	28.3±0.8
KB2	**30.3±0.8**	-	-	+	-	-	-	-	-
KB3	18.0±0.5	-	-	-	-	-	-	-	11.2±0.5
KB4	**32.4±0.8**	-	**45.2±0.7**	+	-	-	-	-	-
KB5	-	-	**30.2±0.7**	-	**35.3±0.9**	3.0±0,5	15.1±0.6	12.1±0.6	-
KB6	15.0±0.1	-	**35.1±0.5**	+	-	-	-	-	**37.0±0.6**
KB7	13.1±0.2	-	**39.1±0.8**	-	-	-	-	-	**32.3±0.6**
KB10	12.2±0.4	-	17.0±0.4	-	-	26.0±0.5	15.2±0.2	14.2±0.8	-
KB11	-	-	13.0±0.1	+	21.2±0.8	14.2±0.8	23.1±0.6	23.1±0.6	-
KB12	-	-	21.3±0.6	-	-	-	-	-	-
KB16	-	-	23.1±0.8	-	-	20.3±0.6	18.5±0.5	22.4±0.5	-
KB17	-	8.0±0.2	23.1±0.5	+	-	25.0±0,.9	22.4±0.5	26.1±0.4	17.3±0.6
KB20	-	-	16.0±0.6	+	-	-	-	-	5.2±0.9
KB21	-	4.0±0.1	8.1±0.1	+	-	12.1±0.3	10.3±0.5	-	15.2±0.5
KB22	-	-	14.0 ±0.2	+	-	22.5±0,.5	23.1±0.5	28.0±0.3	17.0±0.7
KB23	-	-	21.2±0.5	-	-	11.0±0.9	16.1±0.4	-	-
KB24	-	-	-	-	-	-	-	-	10.1±0.5
KB25	-	8.0±0.3	-	-	-	26.4±0.7	27.0±00.3	24.0±0.7	16.0±0.8
KB30	-	-	-	+	-	12.2±0.3	14.1±0.3	11,.4±0.6	5.2±0.8
KB31	-	-	-	-	7.0±0.9	6.2±0.7	6,.0±0.3	-	-
KB32	16.2±0.3	-	27.1±0.2	-	**40.1±0.9**	-	13.1±0.5	10.1±0.5	-
KB33	16.0±0.4	-	-	-	**53.0±1.0**	10.0±0.9	10.0±0.9	14.0±0.5	-
KB38	-	-	-	-	-	7.0±0.5	10.0±0.5	-	-

Strain	AMI	CHI	PEC	PHO	URE	T20	T60	T80	PRO
KB39	-	-	-	-	-	-	-	-	3.2±0.3
KB40	-	-	-	+	17.1±0.7	-	-	-	-
KB43	-	2.0±0.7	-	-	-	17.3±0.4	17,.3±0.4	12.1±0.4	17.1±0.6
KB45	15.1±0.3	-	-	+	-	-	-	-	4,.0±0.4
KB46	15.2±0.3	-	20.2±0.2	+	**54.0±0.7**	-	17.2±0.3	6.0±0.5	-
KB47	-	-	-	+	-	9.1±0.3	-	-	15.4±0.4
KB49	-	-	-	+	-	24.2±0.7	19.1±0.5	27.2±0.3	18..3±0.8
KB50	14.0±0.4	-	-	+	-	16.1±0.3	11.1±0.3	-	25.0±0.8
KB54	-	-	-	+	**95.2±0.7**	-	-	-	-
KB56	-	9.0±0.4	-	+	-	12.2±0.7	26.4 ±0.5	**37.3±0.6**	16.1±0.5
KB58	**34.2±0.4**	-	**48.0±0.5**	+	-	15.1±0.5	-	-	-
KB59	16.1±0.4	-	-	-	-	-	-	-	15.4±0.6
KB61	-	6.1±0.6	-	+	-	29.5±0.6	26.2±0.5	**32.1±0.3**	22.0±0.8
KB66	**34.0±0.3**	-	-	+	-	9.4±0.8	17.2±0.3	-	-
KB68	22.1±0.2	-	-	+	-	23.3±0.6	-	23.2±10.5	6.2±0.6
KB71	-	7.0±0.4	-	-	-	11.1±0.5	-	11.5 ±0.8	20.0±0.9
KB72	12.1±0.4	5.0±0.,5	-	-	-	14.0±0.6	-	17.1±0.5	14.0±0.5
KB73	-	-	-	+	10.0±0.9	-	-	-	-
KB75	-	-	-	-	-	16.2±0.3	15.0±0.2	-	-
KB76	25.0±0.1	-	24.2±0.5	+	-	-	-	-	-

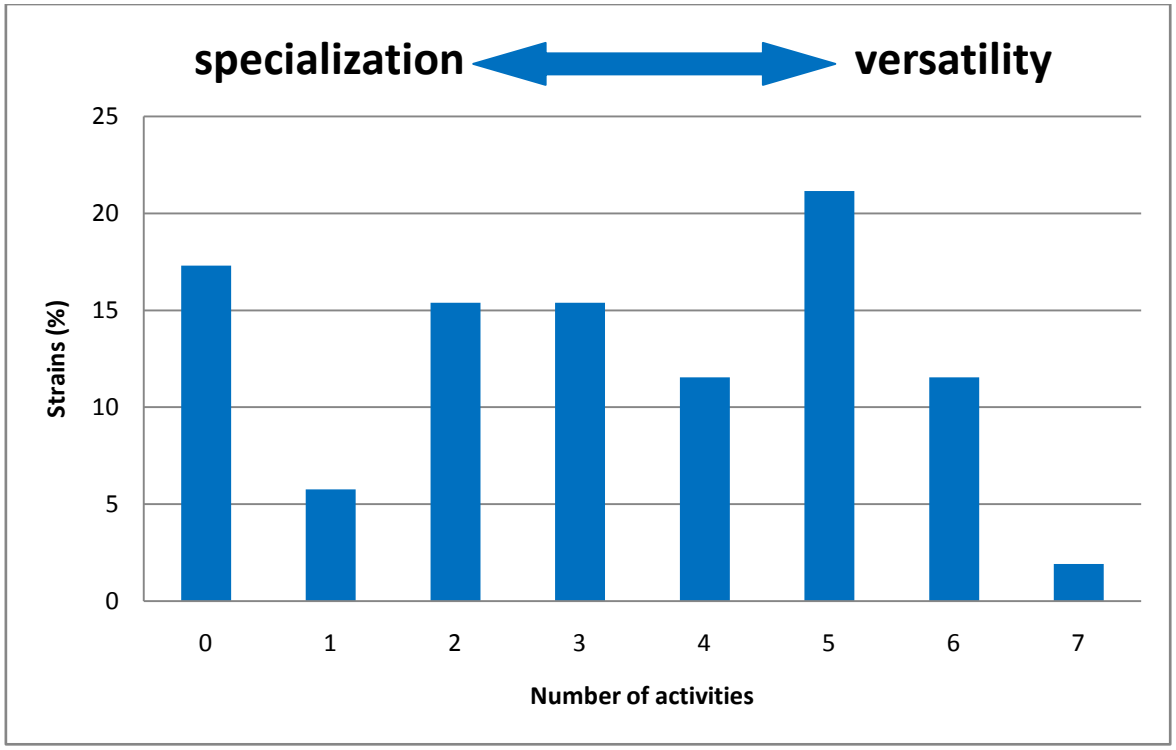
Legend:AMI=Amilase; CHI=Chitinase; PEC=Pectinase; Pho= phosphatase; URE=Urease; T20, T60, T80= Lipase on Tween 20,Tween 60 and Tween 80; PRO=Protease.Activity is expressed by halos diameters (mm). Isolates showingnoactivitiesare not reported. Potential high producers (activity halos ≥ 30 mm) are reported in bold. For phosphatase, positive strains are markedwith"+" (see M & M).Halos diameters have been calculated, after subtraction of colony diameter, as a mean of at least 3 measures± Standard Deviation.

Figure 3.4 Percent of strain, isolated from Kandalaksha bay water samples, showing the various enzyme activities



Legend: T20, T60, T80= Lipase on Tween 20, Tween 60 and Tween 80.

Figure 3.5 Percent of strain, isolated from Kandalaksha bay water samples, showing different numbers of enzyme activities



3.5.2 Metabolic profilesby BIOLOG

Even though many biochemical pathways are common in various microorganisms, different species could have diversified metabolism. This affect their ability to use specific organic substrates as carbon and energy sources. The various patterns of substrates used by a single species could be used both at taxonomical level and for its biochemical/metabolical characterization. In this context, various tests are available (i.e. API, Baxter MicroScan, BBL, IDS, GPI etc.). The information obtained by the BIOLOG system is the most detailed due to the use of 95 compounds. However, at taxonomical level, considering its rather limited database, it could be used only for certain microbial groups. BIOLOG is not very useful for environmental samples, in particular from peculiar/extreme environments, possibly showing a wide array of unknown microorganisms. Nevertheless, the biochemical information obtained could be helpful to defined the metabolic competences of unknown strains also.

In this work, BIOLOG was employed only in this context because the taxonomical identification was already carried out with molecular techniques.

Results are elaborated by the BIOLOG software computing information of growth and substrate oxidation (as OD measured at two wavelength). The system defines strain capacity of grow and oxidize a certain substrates in terms of inability (–),partial ability (±) and full ability(+).

Response of the Kandalaksha Bay Gram negative and positives strains to the BIOLOG are reported in Tables 3.5 and 3.6.

The various compounds have been grouped accordingly to the work of Zak et al. (1994). However, although this arbitrary subdivision is often used (Preston-Mafham et al., 2002; Ros et al., 2006), the different carbon sources categories gather compounds involved in very different metabolic pathways. Emblematic is the case of the carboxylic acids. This group includes both tricarboxylic and fatty acids.

It is difficult to compare the metabolic competence of different strains in function of such a big number of compounds. The question should be analyzed for a single strain or a single substrate. However, some very general considerations can be deduced from this huge amount of data that will be addressed separately for Gram positive and Gram negative strains. In addition, we will report some observations concerning the most abundant genera detected in our microbial community (*Pseudomonas* and *Serratia*)

Gram negative (Figure 3.6 A)

In general, all the strains showed well diversified metabolic competences being able to use a number of substrates ranging from a minimum of 19 (*Pseudomonas lundensis* KB44) to a maximum of 70 (*Serratia* sp. KB 61).

On average, amino acids were the preferred class of compounds (56% of the strain). In particular, considering each single substrate, L-Glutamic Acid, L-Asparagine and L-aspartic acid were preferred by 94% of the strains or more. These three metabolically correlated amino acids, have a key-role in the cell metabolism. Glutamic acid is precursor of several others amino acids and supplies the amino group for their bio-synthesis, being the transamination glutamate dependent on α-keto-acids the primary mechanism of this process. Aspartic acid is linked in the same processalso: aspartic and glutamic acids, synthetized by separate pathways, are coupled in the transamination reaction. They are also related to the TCA cycle: glutamic acid via a-ketoglutarate and aspartic acid via oxalacetate. Glutamic acid, precursor of glutamine, is also involved in the synthesis of purines and pyrimidines.

Amino acids metabolism is also interlaced concerning the various enzymes involved. For example, bacterial deamination of glutamine and asparagine could be carried out by enzymes having specificity for both of them (glutamine/asparagine-ase)(Stark et al., 1997). Finally, it is worth noting that the above mentioned compounds, could support the growth of a large number of marine microorganisms when used as sole carbon source(MacLeod, 1965).

All other amino acid included in the micro plates were widely used with the only exception of D,L-Carnitine and L-Phenylalanine that were oxidized by few strains only.

Sugars were less metabolized (42% of the strain, on average). Considering the single carbohydrate, only α-D-Glucose was used by almost all the strains (92%): this was an expected result since this sugar is the principal substrate to obtain pyruvate(by the Embden–Meyerhof–Parnas (EMP), Entner–Doudoroff (ED), and other pathways). Other well metabolized sugars were D-Trehalose (82%), D-Galactose(74%), L-Arabinose(72%) andD-Mannose (70%).

Similar results (average of 43%)were recorded for carboxylic acids, even with strong differences among them, possibly related to the use of different metabolic pathways. Compounds involved in the TCA cycle much more extensively used. Most of the strains were able to oxidize Succinic acid (80%), Citric acid (78%) and Cis-Aconitic Acid(76%). Lactic and D-Gluconic Acid were also widely utilized, while γ-Hydroxybutyric and Sebacic Acid, involved in the fatty acid metabolism, were oxidized by only few strains.

All other compounds were scarcely metabolized: only Glycerol was used by a considerable number of strains (78%).

Gram positives (Figure 3.6 B)

Generally, a very limited ability to use the substrates was evident in contrast with data recorded for Gram negative. In fact, various compounds were not metabolized and many others used by few strains. On the other hand, it must be considered that the number of Gram positive in our samples was definitely low (5 strains) with little statistical meaning of the results. In addition, some results appeared quite incomprehensible. Emblematic was the case of *Arthrobacter* sp. KB57 that was able to use only two carbon sources. Even if the test was repeated several times giving same result, this very limited biochemical competence seems quite strange.

In fact, arthrobacteria are nutritionally versatile, using a variety of substrates in their oxidative metabolism. Bacteria belonging to this genus are reported to have also a very peculiar metabolism, being able to utilize unusual compounds. For example, *Arthrobacter chlorophenolicus* can survive in high concentrations of toxic pollutants such as 4-chlorophenol (Nordin et al., 2005) while *Arthrobacter* sp., was able to use various pyridines and aromatics (O'Loughlin et al., 1999). However, also for Gram positives, amino acids were the most utilized carbon sources with the only exception of D-Alanine and L-Pyroglutamic Acid. Both simple and polymeric sugars were oxidized quite diffusely.

Differently from Gram negative, carboxylic acids oxidation was limited excepted for Succinic Acid Mono-methyl Ester.

Sugars were the most numerically abundant carbon sources tested (28 and 41 in GN and GP plates, respectively). These compounds, especially glucose, are often the ideal carbon/energy source for many bacteria with heterotrophic metabolism. Nevertheless, in our case amino acids seem to be the preferred substrate. Possible explanation is that various of the sugars assayed, (i.e. Gentiobiose, Palatinose or Stachyose) were very uncommon, while the tested amino acids were those generally present in proteins. Lot of microorganisms can synthesize all the needed amino acids; however, they could save metabolic energy if they are already present in the culture media. On the other hand, for some strains, amino acids could represent at the same time a source of carbon, nitrogen and energy, as already reported for marine bacteria already in early studies (Ostroff and Henry, 1939; MacLeod,1965), while those using sugars as carbon/energy source need nitrogen containing compounds also. It has been reported that dissolved free amino acids and dissolved DNA represent high potential contributors of carbon to heterotrophic bacteria. In particular dissolved free amino acids, have been calculated to sustain, even with big differences related to different environments, up to

about90 % of the bacterial carbon demand in sea water (Joergensen et al., 1993; Simon, 1991).

Pseudomonas (Figure 3.6 C)

Versatility of genus *Pseudomonas* is very well known (Jiménez et al., 2004). This genus has been extensively studied and many works deal with its degradation and metabolic capacities. It is able to degrade a wide array of different compounds including a number of xenobiotics (Sanin et al., 2003; Eyers et al., 2004; Griese et al., 2006). This was confirmed by our results, even with big differences among the various strains. In fact, *Pseudomonas lundensis* KB44 was able to use only 19 sources, while *Pseudomonas brenneri* KB37 oxidized 70 compounds. The average number of carbon sources oxidized by *Pseudomonas* KB species was of ca. 40, being amino and carboxylic acids the preferred substrates. All the other groups of compounds were significantly less used. The prevalence of amino acid instead of sugars for growth of cold-adapted *Pseudomonas* is reported by Meyer et al.(2004).

Serratia (Figure 3.6 D)

As for *Serratia*, the other most relevant genus in our study, a more uniform situation was recorded. The average number of carbon sources oxidized was ca. 60% (ranging from 50 to 69). Amino acids were againthe preferred sources but differences with other compounds groups were less evident.

Most of the results obtained in our studyconfirmeddata reported in the exhaustive work of Grimont and Grimont (2006) concerning this genus and obtained with different methods. In particular, the ability to use N-acetylglucosamine, Citrate, m-Inositol, Glycerol, Putrescine etc. and the limited or absent utilization of Phenylalanine and i-Erythritol seems to be characterizing for some *Serratia* species.

Nutritional versatility

The information obtained by the BIOLOG system can be useful to understand the strains metabolic complexity. In theory, a simple metabolism lead to reduced capability to use diversified substrates. By contrast, the use of a wide array of compounds indicates a very complex metabolism. Metabolic complexity is also related with the ability to produce extracellular enzymes. The substrates tested by BIOLOG are quite simple monomeric compounds; only few oligomers are present. The wide ability to use these substrates does not necessarily involve the strain ability to hydrolize complex environmental molecules that instead is indicated by production of extracellular enzymes.

Under the ecological point of view, simple metabolism could indicate high degree of specialization, while complex metabolism show high eco-nutritional versatility.

Specialized organisms could be confined in particular ecological niches and are scarcely adapted to face environmental changes. Eco-versatility consents colonization different niches and adaptation to environmental stress and diversified nutritional conditions (Fenice et al., 2007). In this context, microorganisms that, beside their wide capacity to use simple molecules, are able to produce a number of hydrolytic enzymes, are even more versatile.

A small number of KB strains showed apparent simple metabolism being able to use a limited number of carbon sources. However, the majority of the bacteria showed a medium high metabolic competence, being able to use about 30-50compounds. For only few bacteria a very diversified competence was recorded (Figure 3.7)

In general, the strains able to oxidize high number of substrates produced also a diversified extracellular enzyme pattern, as revealed by the plate screening, showing their high eco-nutritional versatility.

It is also possible that presence of organisms with different metabolic competences in the same environment could indicate complementary ecological roles (Fenice et al., 2007).

However, strains showing high eco-nutritional versatility would probably be advantaged in the harsh White Sea environment, showing frequent and dramatic variations (Howland et al., 1999; Pantyulin, 2003; Savvichev et al., 2003;Cobelo-García et al., 2006).

Table 3.5 Oxidation of carbon sources for the 47 Gram negative KB strains in GN2 MicroPlates

	Gram negative KB strains																																														
Carbon sources	**1**	**2**	**4**	**5**	**6**	**7**	**10**	**11**	**12**	**16**	**17**	**20**	**21**	**22**	**23**	**24**	**25**	**30**	**31**	**32**	**33**	**36**	**37**	**38**	**39**	**40**	**42**	**43**	**44**	**45**	**46**	**47**	**49**	**50**	**51**	**52**	**56**	**58**	**61**	**63**	**64**	**68**	**71**	**72**	**73**	**75**	**76**	**N2**
α-Cyclodextrin	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	**8**
Dextrin	+	+	+	+	±	-	-	-	-	+	+	-	±	+	-	±	+	+	-	+	+	±	-	+	±	±	±	+	±	-	+	-	+	±	+	+	-	+	+	-	+	±	+	+	-	-	-	**22**
Glycogen	-	+	+	+	-	-	-	-	-	+	±	-	-	-	-	±	-	±	±	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	±	+	+	-	-	-	**9**
Tween 40	+	+	+	+	±	-	-	+	-	-	+	±	±	+	-	+	+	±	+	-	-	+	+	±	-	-	-	±	-	-	+	±	+	+	+	-	+	+	+	-	-	-	+	+	-	+	-	**22**
Tween 80	+	+	+	+	±	-	-	+	-	-	+	±	±	+	+	+	+	+	±	-	±	+	+	+	-	-	+	±	-	-	+	±	+	+	-	-	+	+	+	±	+	±	+	+	-	+	-	**25**
N-Acetyl-DGalactosamine	+	+	+	+	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-	±	-	-	-	-	-	-	+	-	-	-	-	+	-	±	-	+	+	+	-	-	-	-	-	-	-	-	**14**
N-Acetyl-DGlucosamine	+	+	+	+	+	±	-	±	-	-	+	±	±	+	±	-	+	+	-	+	+	+	+	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	**26**
Adonitol	+	-	-	-	+	-	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	±	-	+	-	+	-	-	+	-	-	-	+	-	-	-	-	**12**
L-Arabinose	+	-	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	+	+	±	+	+	+	±	+	-	+	+	+	+	+	+	+	-	+	-	+	±	+	+	+	+	+	-	+	**34**
D-Arabitol	+	-	-	-	+	+	-	-	-	+	-	+	+	-	+	+	-	-	-	±	-	+	+	+	±	±	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	+	+	-	±	-	-	**17**
D-Cellobiose	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	-	±	±	-	+	-	+	-	+	+	-	-	-	**15**
i-Erythritol	-	-	-	-	±	-	-	-	-	-	+	-	-	-	-	±	-	-	-	-	-	±	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	**5**
D-Fructose	+	+	-	+	+	±	-	-	-	-	+	-	+	+	+	-	+	-	+	+	+	±	+	+	±	-	+	±	-	+	+	+	+	+	+	+	+	+	+	±	+	±	+	+	-	-	-	**28**
L-Fucose	+	-	-	±	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-	-	+	+	+	-	-	**14**
D-Galactose	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	±	-	-	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	**35**
Gentiobiose	+	+	+	+	-	-	-	-	-	+	+	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	±	+	+	-	-	-	+	+	-	-	-	**19**
α-D-Glucose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	**43**

Carbon sources	1	2	4	5	6	7	10	11	12	16	17	20	21	22	23	24	25	30	31	32	33	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	56	58	61	63	64	68	71	72	73	75	76	
m-Inositol	+	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	-	+	+	-	+	+	+	±	+	+	-	+	-	+	+	+	+	-	-	+	**30**
α-D-Lactose	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	±	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	**10**
Lactulose	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	±	-	-	-	**8**
Maltose	+	+	+	+	-	-	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+	-	-	+	-	-	-	+	-	-	+	-	+	-	+	+	+	+	+	-	+	-	+	+	-	-	-	**22**
D-Mannitol	+	-	-	-	+	+	+	-	-	-	+	±	+	+	+	+	+	-	-	-	-	+	+	+	+	±	-	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-	-	**27**
D-Mannose	+	+	+	+	+	±	+	-	-	+	+	±	±	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	-	**33**
D-Melibiose	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	+	-	+	-	-	-	+	+	-	-	-	**15**
β-A26Methyl-D-Glucoside	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	±	-	-	+	-	+	-	+	±	+	-	+	-	+	-	+	+	-	-	-	**16**
D-Psicose	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	+	-	+	+	-	-	-	**11**
D-Raffinose	+	-	-	+	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	±	+	+	-	+	-	-	-	+	+	-	-	-	**15**
L-Rhamnose	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	**10**
D-Sorbitol	+	-	-	±	+	+	-	-	-	+	+	-	+	+	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	+	+	±	-	-	-	**21**
Sucrose	+	-	-	+	-	±	+	+	-	+	+	-	-	+	+	-	+	+	±	+	+	-	-	+	-	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	-	**24**
D-Trehalose	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	**39**
Turanose	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	-	±	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	**11**
Xylitol	-	-	-	-	-	-	-	-	-	+	-	-	-	-	±	±	-	-	-	-	-	±	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	**5**
Pyruvic Acid Methyl Ester	+	-	-	+	+	+	+	-	±	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	±	+	+	+	+	-	+	±	+	+	+	±	+	+	+	+	+	±	+	+	+	+	+	**34**
Succinic Acid Mono-Methyl-Ester	+	+	±	+	±	±	-	-	-	+	±	-	±	+	+	+	-	±	+	-	±	+	+	+	±	±	+	+	-	-	-	-	-	+	-	-	+	+	-	±	±	+	±	±	+	-	**18**
Acetic Acid	+	+	+	+	+	-	+	-	-	+	+	-	+	+	±	+	+	+	±	+	+	+	+	-	±	±	+	+	-	-	+	-	+	+	+	±	±	+	+	-	+	+	+	+	±	+	-	**30**

Carbon sources	1	2	4	5	6	7	10	11	12	16	17	20	21	22	23	24	25	30	31	32	33	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	56	58	61	63	64	68	71	72	73	75	76	
Cis-Aconitic Acid	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	+	+	±	+	-	+	+	+	+	+	+	+	+	+	**36**
Citric Acid	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	**37**
Formic Acid	+	-	-	-	±	+	-	-	±	-	+	-	±	+	±	+	+	+	-	-	-	+	+	±	-	+	+	-	-	-	-	-	-	+	+	+	±	-	+	-	±	+	+	-	+	-	-	**18**
D-Galactonic Acid Lactone	-	-	-	-	+	±	-	-	-	-	-	+	±	+	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	+	-	-	**13**
D-Galacturonic Acid	+	+	-	-	+	+	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	+	-	+	-	-	+	+	+	-	+	±	+	+	-	-	+	**20**
D-Gluconic Acid	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	+	-	±	+	-	+	+	+	+	+	+	-	-	+	**35**
D-Glucosaminic Acid	+	-	-	-	+	+	+	-	-	-	-	±	+	+	+	+	-	-	-	-	-	±	+	-	±	-	-	-	-	±	-	-	-	+	-	±	-	±	+	-	-	+	+	-	-	-	-	**13**
D-Glucuronic Acid	+	-	-	±	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	+	+	-	-	-	+	-	+	-	+	-	±	+	-	+	-	+	-	+	+	+	-	+	**19**
α-Hydroxybutyric Acid	-	-	-	-	±	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	±	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	**5**
β-Hydroxybutyric Acid	-	-	-	-	+	+	+	+	±	-	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	-	+	-	+	+	-	-	-	+	-	-	+	-	-	+	+	-	**22**
γ-Hydroxybutyric Acid	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**
p-HydroxyPhenylacetic Acid	+	-	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-	+	-	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-	+	+	-	-	-	**19**
Itaconic Acid	-	-	-	-	+	-	-	-	-	-	-	-	±	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	±	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	**6**
α-KetoButyric Acid	-	-	-	+	±	-	-	-	-	-	-	-	-	-	±	±	-	-	+	-	+	±	+	-	-	-	-	+	-	-	+	-	-	±	-	-	-	+	-	-	-	-	-	-	-	-	-	**7**
α-KetoGlutaric Acid	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	-	±	+	+	±	±	+	-	±	-	+	+	+	-	-	+	±	-	+	-	-	+	+	+	**27**
α-KetoValeric Acid	-	-	-	+	±	-	-	-	-	+	-	-	±	-	±	-	-	-	±	+	+	±	+	-	-	-	-	±	-	-	+	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	**6**
D,L-Lactic Acid	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	±	+	+	+	+	+	+	+	+	+	+	±	-	+	-	±	+	-	+	+	+	+	+	+	+	+	**37**
Malonic Acid	-	-	-	-	+	+	+	±	+	+	-	±	±	-	+	+	-	-	-	-	-	+	+	-	-	-	-	+	-	±	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	**13**
Propionic Acid	+	-	-	+	+	+	+	+	+	-	-	±	+	-	+	+	-	+	-	-	-	+	+	-	+	+	+	+	+	±	-	±	-	+	±	-	-	-	-	+	-	+	-	-	+	+	+	**24**

Carbon sources	1	2	4	5	6	7	10	11	12	16	17	20	21	22	23	24	25	30	31	32	33	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	56	58	61	63	64	68	71	72	73	75	76	
Quinic Acid	+	-	-	-	+	+	+	+	+	+	-	±	+	-	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	±	-	+	-	-	-	-	-	+	+	+	+	+	+	-	+	23
D-Saccharic Acid	-	-	-	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	-	+	-	+	+	-	-	-	-	-	+	+	-	-	-	-	+	17
Sebacic Acid	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Succinic Acid	-	+	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	±	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	38
Bromosuccinic Acid	+	-	-	-	+	+	+	±	±	-	+	±	+	+	+	+	+	+	±	-	-	+	+	+	+	±	+	+	±	+	-	±	+	+	±	±	±	±	+	±	+	+	±	+	+	±	25
Succinamic Acid	-	-	-	-	+	+	+	-	-	+	-	+	±	-	+	+	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	±	-	-	+	-	-	12
Glucuronamide	-	-	-	±	±	±	-	-	-	+	-	-	±	-	+	-	-	-	-	-	-	-	+	+	-	±	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	7
L-Alaninamide	-	-	-	+	-	±	-	-	-	-	+	+	-	+	±	+	-	-	-	-	-	+	+	-	-	-	±	+	-	-	-	-	-	-	-	±	-	-	-	-	-	±	-	-	-	-	-	8
D-Alanine	+	-	-	+	+	+	+	+	±	+	+	±	±	+	±	+	+	-	-	-	-	+	+	+	-	+	+	+	+	-	-	±	+	+	+	±	+	-	+	±	+	+	+	+	±	+	+	29
L-Alanine	+	-	-	+	+	+	+	+	±	+	+	+	+	+	+	+	+	±	+	-	+	+	+	+	±	+	+	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	38
L-Alanylglycine	+	+	+	+	+	+	+	-	-	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	±	-	+	+	-	-	+	-	+	+	+	-	+	+	+	-	+	±	+	+	-	-	-	33
L-Asparagine	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	45
L-Aspartic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	44
L-Glutamic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	45
Glycyl-Laspartic Acid	-	+	+	+	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	+	-	-	+	-	-	-	+	±	-	+	+	-	+	-	+	-	-	-	-	18
Glycyl-Lglutamic Acid	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	29
L-Histidine	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	±	-	-	+	+	+	-	-	+	-	-	+	-	+	+	+	-	-	+	+	+	+	+	±	+	+	-	-	+	32
Hydroxy-LProline	-	-	-	±	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	-	+	-	+	-	-	-	23
L-Leucine	-	-	-	+	+	±	+	+	-	+	-	+	+	-	±	+	-	+	+	+	±	+	+	-	-	+	±	±	-	-	+	-	-	+	-	-	-	-	-	±	-	±	-	-	±	-	+	17

Carbon sources	1	2	4	5	6	7	10	11	12	16	17	20	21	22	23	24	25	30	31	32	33	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	56	58	61	63	64	68	71	72	73	75	76	
L-Ornithine	-	+	+	±	-	+	+	±	-	-	+	+	-	-	+	+	-	-	±	-	-	+	+	-	+	-	-	-	-	±	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	**13**
LPhenylalanine	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	**1**
L-Proline	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	±	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	**39**
L-Pyroglutamic Acid	-	-	-	-	+	+	+	+	-	+	-	+	+	-	+	+	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-	+	-	+	**20**
D-Serine	+	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	-	-	-	±	+	-	-	-	±	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	+	+	-	+	-	**15**
L-Serine	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	+	+	-	+	+	+	+	+	±	+	+	+	+	-	+	±	+	+	-	-	+	**37**
L-Threonine	-	+	+	+	+	+	-	-	-	-	+	-	-	±	+	±	±	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-	-	**19**
D,L-Carnitine	-	-	-	-	±	±	-	-	-	+	-	-	-	-	±	±	-	-	-	-	-	±	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	**4**
γ-Amino Butyric Acid	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	±	+	+	+	-	+	+	-	+	-	+	-	-	+	-	+	+	+	+	-	-	+	-	+	**27**
Urocanic Acid	+	-	-	±	+	+	+	-	-	-	+	+	+	+	+	+	+	-	±	-	-	+	+	+	+	-	-	-	±	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	-	-	-	**27**
Inosine	+	-	-	+	+	+	+	-	±	+	+	+	+	+	+	+	+	+	+	-	±	+	+	+	±	-	+	-	-	+	-	+	+	+	+	+	+	-	+	-	+	+	+	+	-	-	+	**32**
Uridine	+	-	-	+	+	+	-	-	-	+	+	-	-	+	±	+	+	+	+	+	±	±	+	-	-	-	±	-	-	±	+	±	-	+	+	+	±	+	+	-	±	-	+	+	-	-	+	**23**
Thymidine	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	**17**
Phenyethylamine	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**2**
Putrescine	+	-	-	-	+	±	+	+	+	+	+	+	-	+	±	-	+	-	-	-	-	±	+	-	-	+	±	-	-	-	-	-	+	-	-	-	+	-	+	±	-	±	+	±	+	-	+	**18**
2-Aminoethanol	-	-	-	-	+	+	+	±	±	+	-	±	±	-	±	+	-	-	-	-	-	+	+	-	-	-	+	-	±	±	-	±	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	**9**
2,3-Butanediol	-	-	-	±	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Glycerol	+	-	+	+	+	+	+	±	+	-	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	**37**
D,L-α-Glyc-Phosph	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+	±	+	+	-	-	-	-	-	-	-	±	+	±	+	-	+	+	+	-	+	-	+	+	-	-	-	**16**

Carbon sources	1	2	4	5	6	7	10	11	12	16	17	20	21	22	23	24	25	30	31	32	33	36	37	38	39	40	42	43	44	45	46	47	49	50	51	52	56	58	61	63	64	68	71	72	73	75	76	
α-D-Glucose-1-Phosphate	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	±	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	+	+	-	-	-	**12**
D-Glucose-6-Phosphate	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	+	+	-	-	-	**14**
N1	**65**	**28**	**25**	**50**	**53**	**47**	**44**	**28**	**20**	**55**	**61**	**34**	**40**	**65**	**54**	**54**	**56**	**33**	**20**	**28**	**36**	**50**	**70**	**46**	**32**	**27**	**32**	**33**	**19**	**32**	**37**	**31**	**52**	**61**	**40**	**31**	**50**	**34**	**69**	**21**	**48**	**36**	**67**	**55**	**32**	**20**	**32**	

Legend

‘‘+’’, ‘‘±’’ and ‘‘-’’indicate the full ability, the partial ability and the inability of the bacterium to grow and oxidize the various substrates

Writing in different colors refers to different carbon sources guilds as follows: **violet**= polymers; **red** = carbohydrates;**blue** = carboxylic acids; **black** = miscellaneous; **green** = amines/amides; **pink** = amino acids

N1 = number of positive wells for each strain;**N2**= number of positive strains for each carbon source

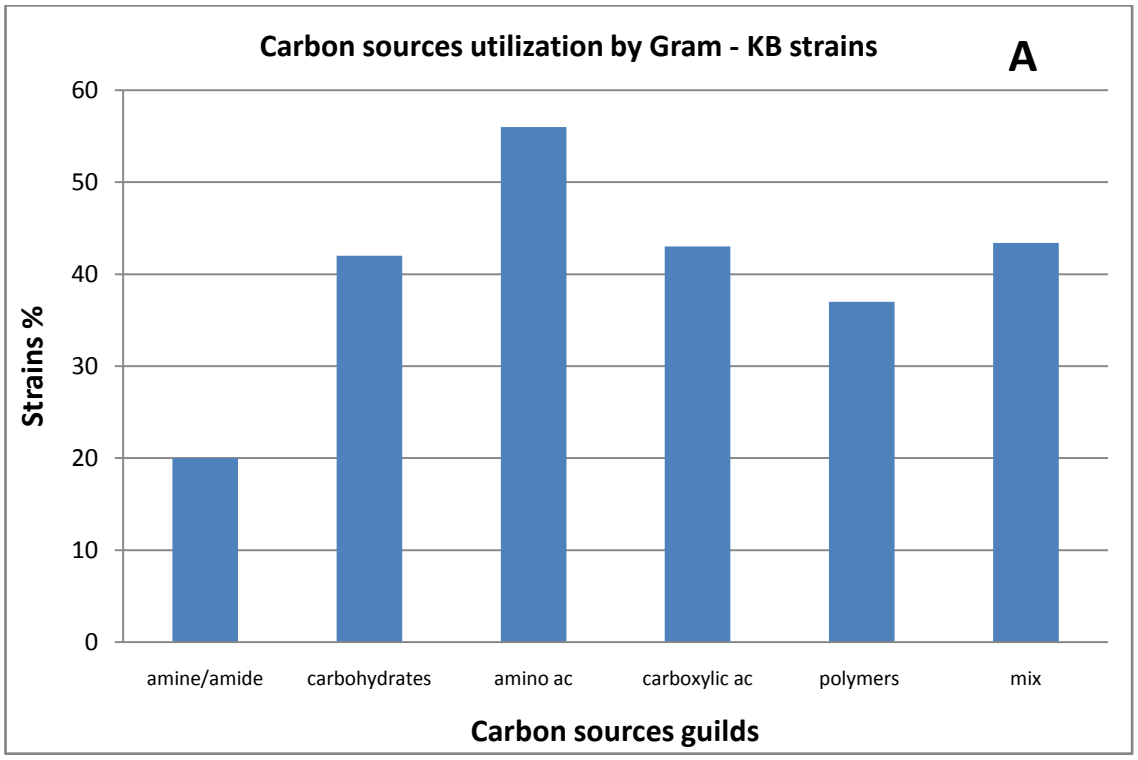
Table 3.6 Oxidation of carbon sources for Gram positive strains in GP2 MicroPlates

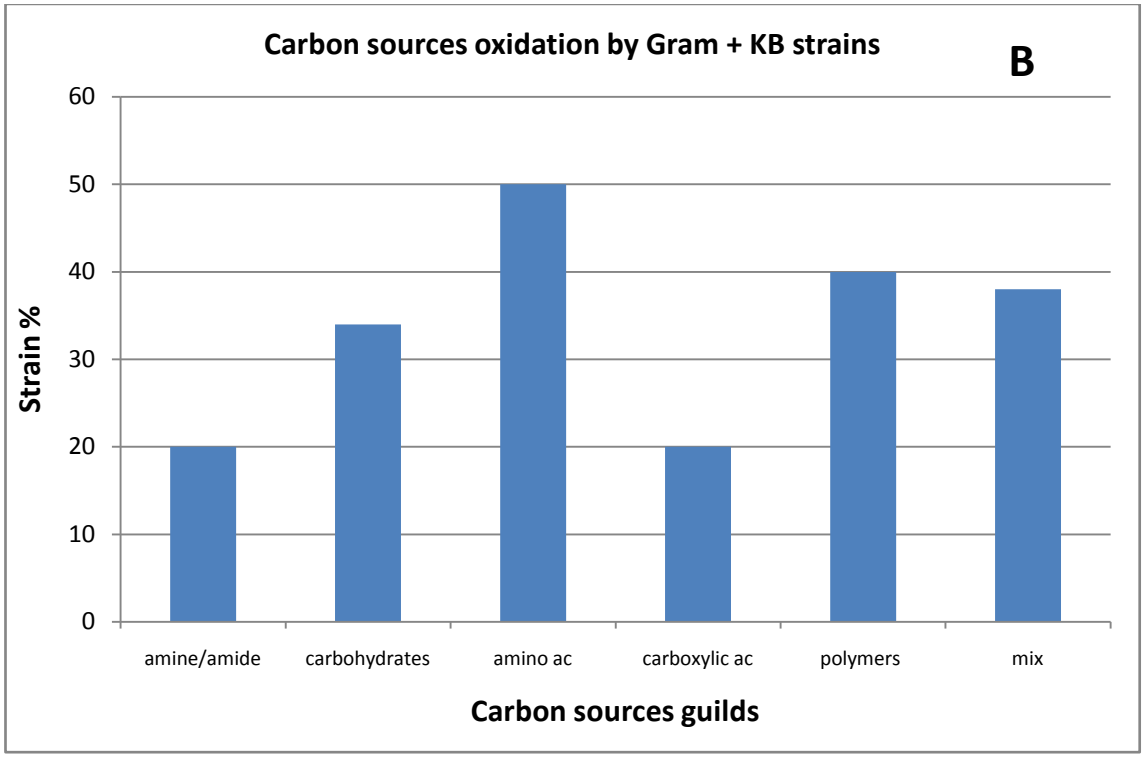
	Gram positive KB strains					N 2		Gram positive KB strains					N 2		Gram positive KB strains					N 2		Gram positive KB strains					N 2
Carbon sources	**3**	**54**	**57**	**59**	**66**		**Carbon sources**	**3**	**54**	**57**	**59**	**66**		**Carbon sources**	**3**	**54**	**57**	**59**	**66**		**Carbon sources**	**3**	**54**	**57**	**59**	**66**	
α-Cyclodextrin	+	±	-	-	+	**2**	Lactulose	-	-	-	-	-	**0**	D-Trehalose	-	-	-	-	-	**4**	D-Alanine	-	+	-	-	-	**1**
β-Cyclodextrin	-	+	-	-	+	**2**	Maltose	-	-	-	-	-	**3**	Turanose	+	+	-	+	+	**2**	L-Alanine	+	+	-	+	±	**3**
Dextrin	+	+	+	+	+	**5**	Maltotriose	+	±	-	+	+	**4**	Xylitol	-	+	-	+	±	**1**	L-Alanyl-Glycine	+	+	-	+	+	**4**
Glycogen	+	-	±	-	+	**2**	D-Mannitol	+	+	-	+	+	**3**	D-Xylose	-	+	-	-	-	**1**	L-Asparagine	-	+	-	+	+	**3**
Inulin	-	-	-	-	+	**1**	D-Mannose	+	+	-	+	-	**3**	Acetic Acid	-	±	-	-	+	**1**	L-Glutamic Acid	-	+	-	+	+	**3**
Mannan	-	±	-	-	±	**0**	D-Melezitose	+	+	-	+	-	**1**	α-Hydroxybutyric Acid	±	+	-	-	-	**1**	Glycyl- LglutamicAc.	+	+	-	-	+	**3**
Tween 40	-	+	-	-	-	**1**	D-Melibiose	-	±	-	+	-	**1**	β-Hydroxybutyric Acid	-	+	-	-	-	**1**	L-Pyroglutamic Acid	-	±	-	-	-	**0**
Tween 80	-	+	-	-	-	**1**	α-Methyl-DGalactoside	-	-	-	-	+	**0**	γ-Hydroxybutyric Acid	-	+	-	-	-	**1**	L-Serine	-	+	-	+	+	**3**
N-Acetyl-DGlucosamine	+	+	-	-	+	**3**	β-Methyl-DGalactoside	-	-	-	-	-	**0**	p-Hydroxy-Phenylacetic Acid	-	+	-	-	-	**0**	Putrescine	-	+	-	-	-	**1**
N-Acetyl-β-DMannosamine	-	+	-	-	-	**1**	3-Methyl Glucose	-	±	-	-	-	**1**	α-Ketoglutaric Acid	-	-	-	±	-	**0**	2,3-Butanediol	-	+	-	-	-	**1**
Amygdalin	-	±	-	-	±	**0**	α-Methyl-DGlucoside	-	±	-	+	-	**1**	α-Ketovaleric Acid	-	±	-	-	-	**2**	Glycerol	+	+	+	-	+	**4**
L-Arabinose	-	-	-	+	+	**2**	β-Methyl-DGlucoside	+	-	-	-	-	**2**	Lactamide	+	+	-	-	-	**1**	Adenosine	+	-	-	+	-	**2**
D-Arabitol	-	+	-	-	-	**1**	α-Methyl-Dmannoside	+	-	-	-	+	**0**	D-Lactic Acid Methyl Ester	-	+	-	-	-	**1**	2'-Deoxy Adenosine	+	-	-	+	-	**2**
Arbutin	-	-	-	+	+	**2**	Palatinose	-	-	-	-	-	**2**	L-Lactic Acid	-	+	-	-	-	**1**	Inosine	+	-	-	+	-	**2**
D-Cellobiose	-	+	-	+	+	**3**	D-Psicose	+	±	-	+	±	**2**	D-Malic Acid	-	+	-	-	-	**0**	Thymidine	+	+	-	+	-	**3**
D-Fructose	+	+	-	+	+	**4**	D-Raffinose	+	+	-	-	-	**3**	L-Malic Acid	-	-	±	-	-	**1**	Uridine	+	-	-	+	+	**3**
L-Fucose	-	-	-	-	-	**0**	L-Rhamnose	-	+	-	+	+	**0**	Pyruvatic Acid Methyl Ester	-	+	-	-	-	**2**	Adenosine-5'-Monophosphate	+	±	-	+	-	**2**
D-Galactose	-	-	-	+	+	**2**	D-Ribose	-	±	±	-	-	**3**	Succinic Acid Mono-methyl Ester	-	-	-	+	+	**3**	Thymidine-5'-Monophosphate	+	±	-	+	±	**2**
D-Galacturonic Acid	-	-	-	-	-	**0**	Salicin	+	+	-	+	±	**2**	Propionic Acid	+	+	-	-	+	**1**	Uridine-5'-Monophosphate	+	-	-	+	+	**3**
Gentiobiose	-	-	-	+	+	**2**	Sedoheptulosan	-	±	-	+	+	**1**	Pyruvic Acid	-	+	-	-	-	**2**	D-Fructose-6-Phosphate	-	-	-	-	+	**1**
D-Gluconic Acid	-	+	-	-	-	**1**	D-Sorbitol	-	±	-	+	-	**1**	Succinamic Acid	-	+	-	-	+	**0**	α-D-Glucose-1-Phosphate	-	-	-	-	+	**1**
α-D-Glucose	+	+	-	+	+	**4**	Stachyose	-	+	-	-	-	**1**	Succinic Acid	-	-	-	-	-	**1**	D-Glucose-6-Phosphate	-	-	-	-	+	**1**
m-Inositol	-	+	-	-	-	**1**	Sucrose	-	-	-	-	+	**4**	N-Acetyl-Lglutamic Acid	-	+	-	-	-	**0**	D-L-α-Glycerol Phosphate	-	+	-	-	+	**2**
α-D-Lactose	-	+	-	+	-	**0**	D-Tagatose	+	+	-	+	+	**0**	L-Alaninamide	-	-	-	-	-	**2**	**Total positives**	**31**	**49**	**2**	**37**	**38**	

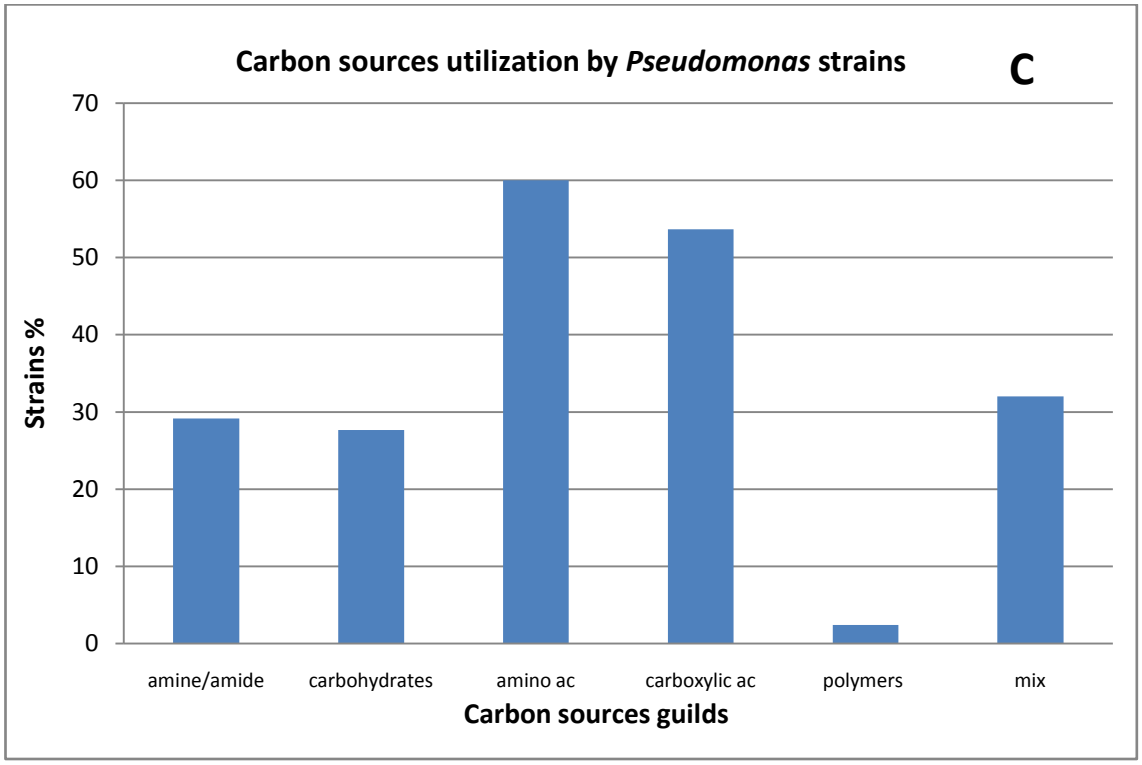
Legend

‘‘+’’, ‘‘±’’ and ‘‘-’’indicate the full ability, the partial ability and the inability of the bacterium to grow and oxidize the various substrates.
Writing in different colors refers to different carbon sources guilds as follows: **violet** = polymers; **red** = carbohydrates; **blue** = carboxylic acids; **black** = miscellaneous; **green** = amines/amides; **pink** = amino acids

Figure 3.6 Percentage of carbon sources utilization by Kandalaksha Bay strains






Carbon sources utilization by *Pseudomonas* strains
C
Strains %
70
60
50
40
30
20
10
0
amine/amide
carbohydrates
amino ac
carboxylic ac
polymers
mix
Carbon sources guilds

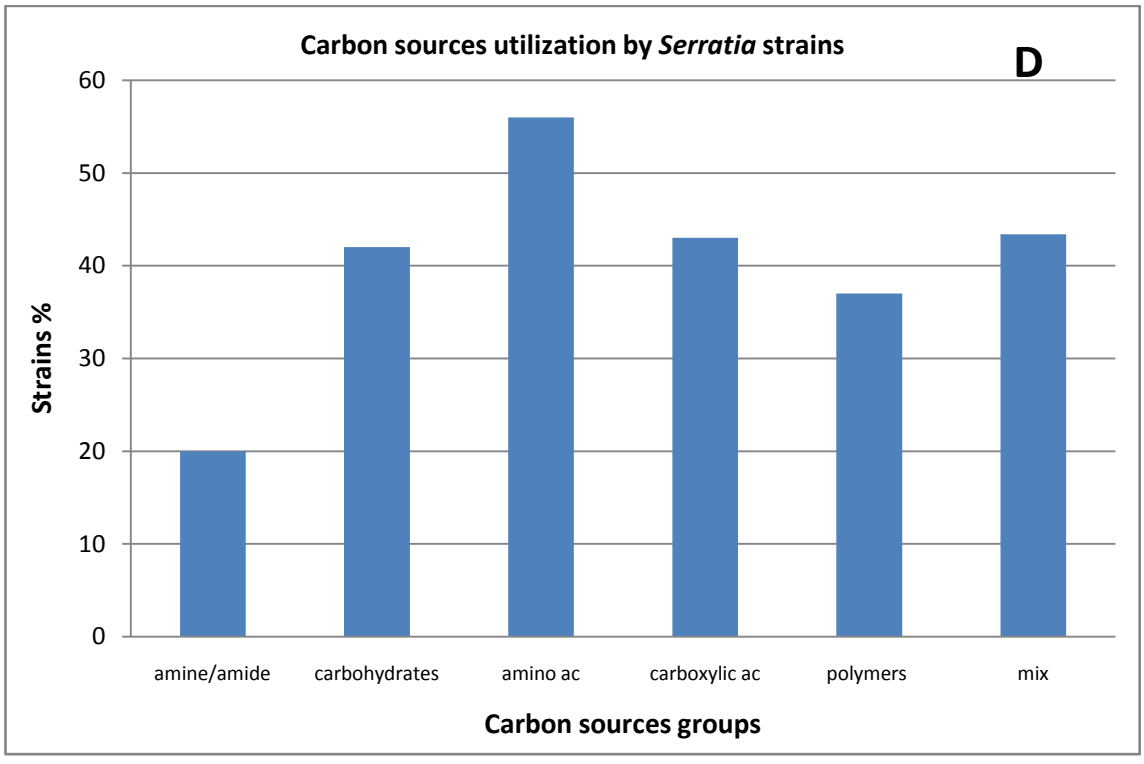
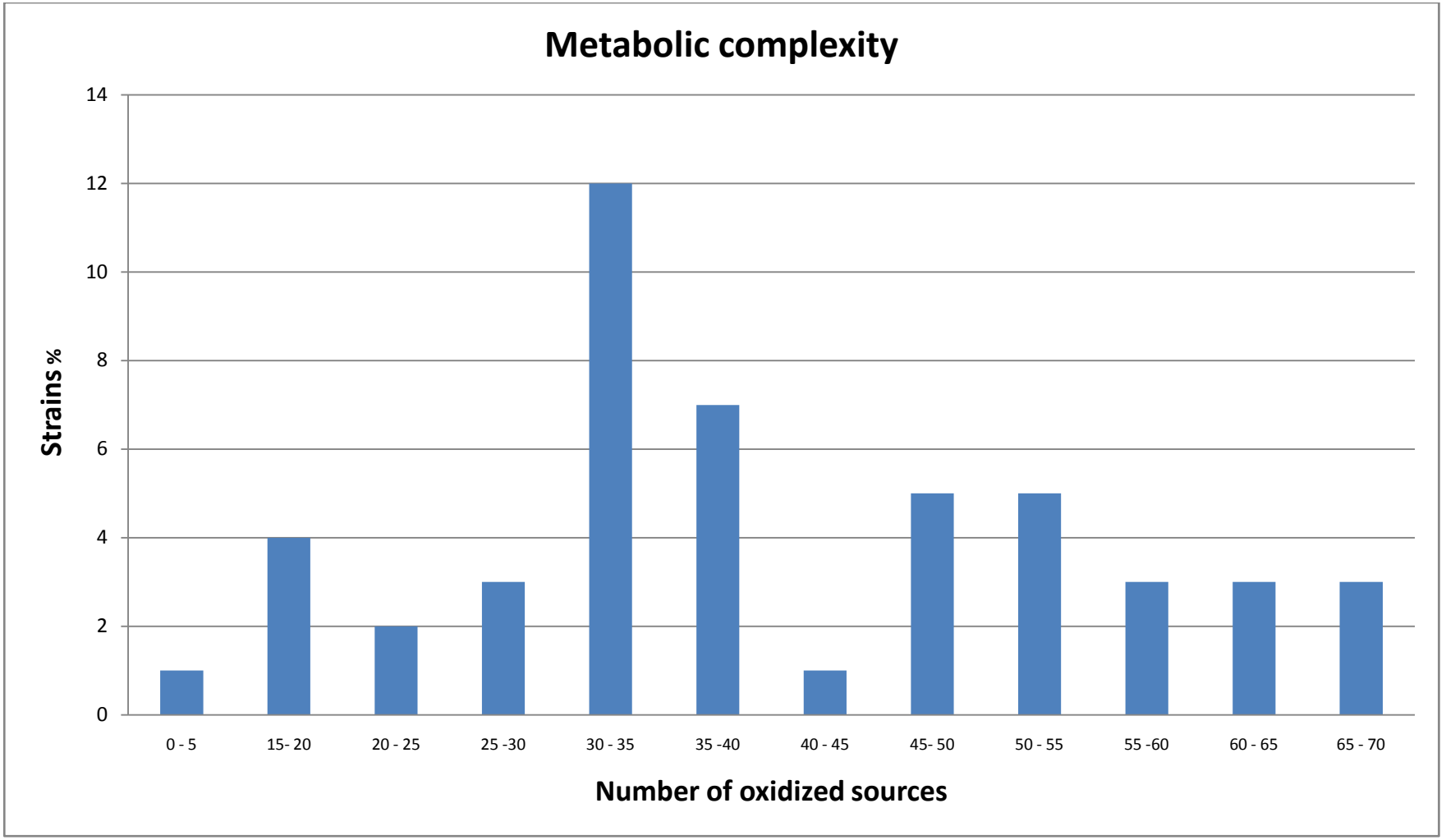

Carbon sources utilization by *Serratia* strains
D
Strains %
60
50
40
30
20
10
0
amine/amide
carbohydrates
amino ac
carboxylic ac
polymers
mix
Carbon sources groups

Figure 3.7 Metabolic complexity of Kandalaksha Bay bacteria as revealed by BIOLOG



3.6 Study of bacterial communities by PCR-TGGE fingerprinting analysis

The knowledge of microbial diversity and its role in nature is often inadequate, mainly because traditional microbiological techniques, such as use of microscopy and cultivation methods, have only a limited use to obtain information on microbial communities. The majority of microorganisms (ca.99%) in nature cannot be isolated in pure cultures, mainly due to lack of knowledge of the real conditions under which most of the bacteria are growing in their natural environment. Therefore, to better understand microbial diversity and its role in ecosystem maintenance, other techniques that complement the classical microbiological approach are necessary. In fact, comprehension of microbial diversity is essential to understand the relationship between environmental parameters and ecosystem function.(Hugenholtz et al., 1998; Webster et al., 2000; Lewis et al., 2007)

The application of molecular biological techniques to detect and identify microorganisms by molecular markers, such as 16S rRNA encoding gene, is frequently used to explore the microbial diversity.

Genetic fingerprinting techniques provide an useful pattern or profile to analyze the structure of microbial communities. The general strategy for these approaches consists in DNA extraction, amplification of 16S rRNA gene and analysis of PCR products. One of these techniques, commonly used in microbial ecology, is the *Denaturing gradient gel electrophoresis* (DGGE) of PCR amplified ribosomal DNA fragments and the related technique *Temperature gradient gel electrophoresis (TGGE).*

These are powerful tools whereby the diversity of PCR-amplified genes from a large number of samples can be compared in one gel to reveal community structure and biodiversity. They can also be used to obtain an overview of total bacterial communities or to study the microbial communities present in different sites. In addition, by using special amplification strategies

(i.e. specific primers or specific gradients) it is also possible to estimate the presence of specific groups within the bacterial community. Furthermore, information about the identity of community members can be obtained. In fact, amplification of excised DGGE/TGGE bands sequences allows the phylogenetic analysis of hypervariable regions of the 16s rRNA gene. Thus, application of these techniques can be useful to obtain interesting ecological information on environmental microbial communities (Muyzer and Smalla, 1998, Muyzer, 1999, Nicolaisen and Ramsing, 2002).

In this work, a cultivation independent approach based on PCR and Temperature-gradient gel electrophoresis (TGGE) was chosen for the study of bacterial communities present in the Kandalaksha Bay area.

3.6.1 Study of total eubacterial community

3.6.1.1 TGGE fingerprints analysis

The elaboration of TGGE gel image allowed the analysis of fingerprints (profiles) generated from the separation of the eubacterial community V3 hypervariable regions. To evaluate community structure, cluster analysis of the different samples fingerprints was carried out using different coefficients calculated by the "GelComparII"v. 5.10 software.

Cluster analysis with Pearson coefficient

Pearson coefficient clustering is based on the densitometric curves obtained for each sample. Thus, similarity among the samples is calculated on the band intensities (the relative abundance of each species) recorded in each profile. To test TGGE fingerprints reproducibility, duplicate PCR reactions of the same sample were run on the same TGGE gel and analyzed. Comparison of two samples (N3 and N4) in duplicate is displayed in Figure 3.8 A, showing that intrinsic variability of the method was rather good being below 15%.

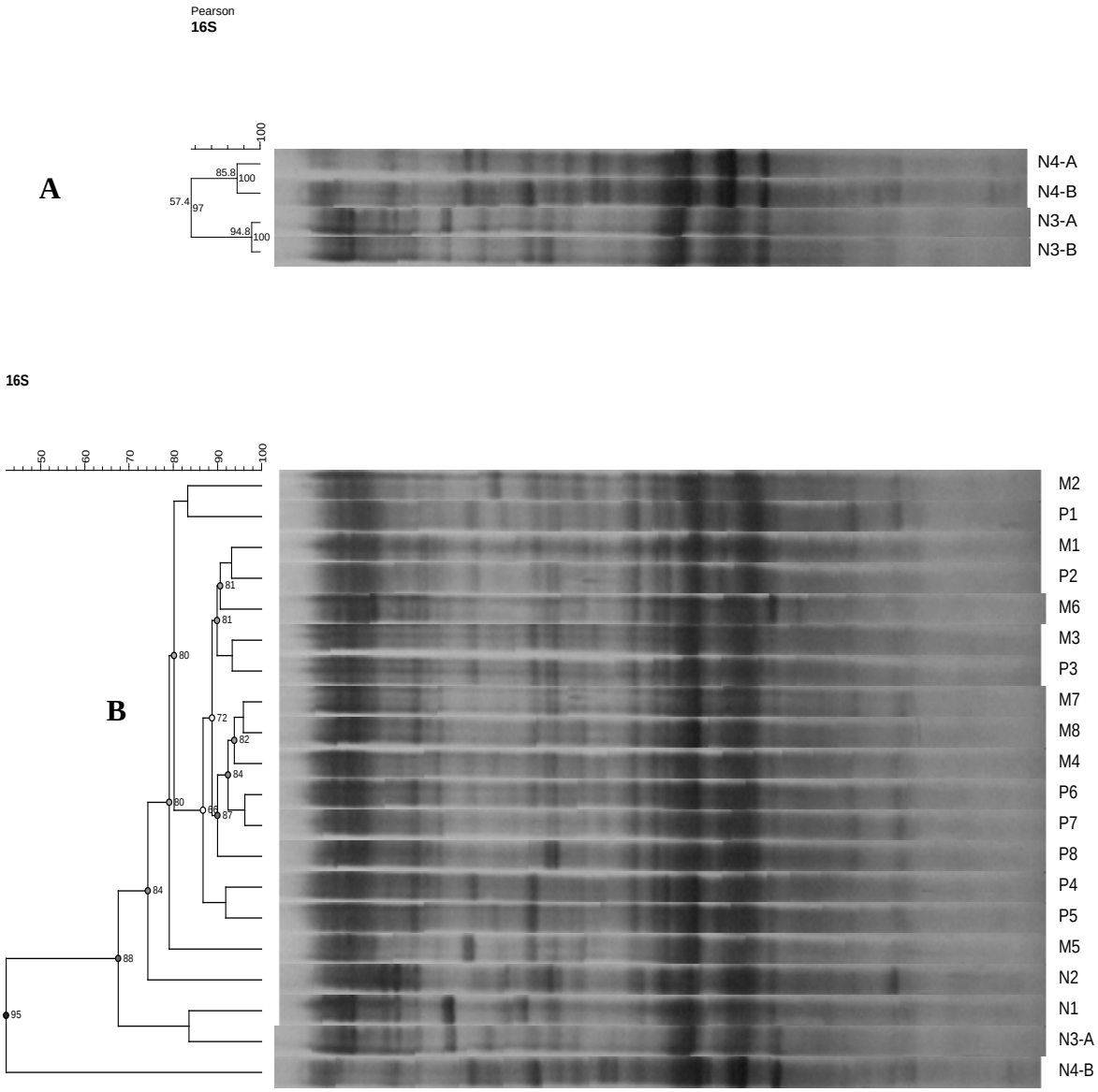
Results evidenced diversity in the communities structure of different sampling sites (Figure 3.8 B). In fact, fingerprinting groupings were evidently related to the various sampling locations. A big cluster included all the samples taken in the intertidal zone pool and the

nearby sea-surface (lanes from P1 to M8). The fingerprints related to these samples grouped together showing rather high similarity (80%). The only sample which clustered slightly away was M5:this was probably due to some contingent seawater variations caused by tide, precipitation or other environmental issues.

On the contrary, the other samples, taken in open sea at different depths, clearly clustered away. This was particularly evident for the sample collected at -70 m (N4), which branched at only 40% of similarity. Among the open sea profiles there was a certain variability but samples taken at 2.5 and 15 m depth (N1 and N3) were quite similar (ca 80%). Finally, the sample from open sea surface (N2) was similar to those from the coastal area (P and M samples). This was quite surprising since this sample was taken off-shore at a considerable distance from the coast.

However, considering the overall clustering profile, it was evident that all samples from water surface had similar community structure, while evident differences were detected in deepest water. It is possible that strong water mixing, typical of this area and mainly caused by the intense tides, was particularly active on superficial layers leading to a certain homogeneity of the microbial communities of almost the whole Kandalaksha Bay. On the contrary, community structure of deepest water changed proportionally to depth.

Figure 3.8. Kandalaksha Bay eubacterial community: Pearson coefficient-based analysis of band patterns generated by TGGE fingerprints



Legend:
A= reproducibility of the methods: variability is below 15%. N4-A and N4-B= duplicate PCR of sample N4; N3-A and N3-B = duplicate PCR of sample N3.
B= comparison of band patterns generated by TGGE fingerprints (see M&M for sample marks)

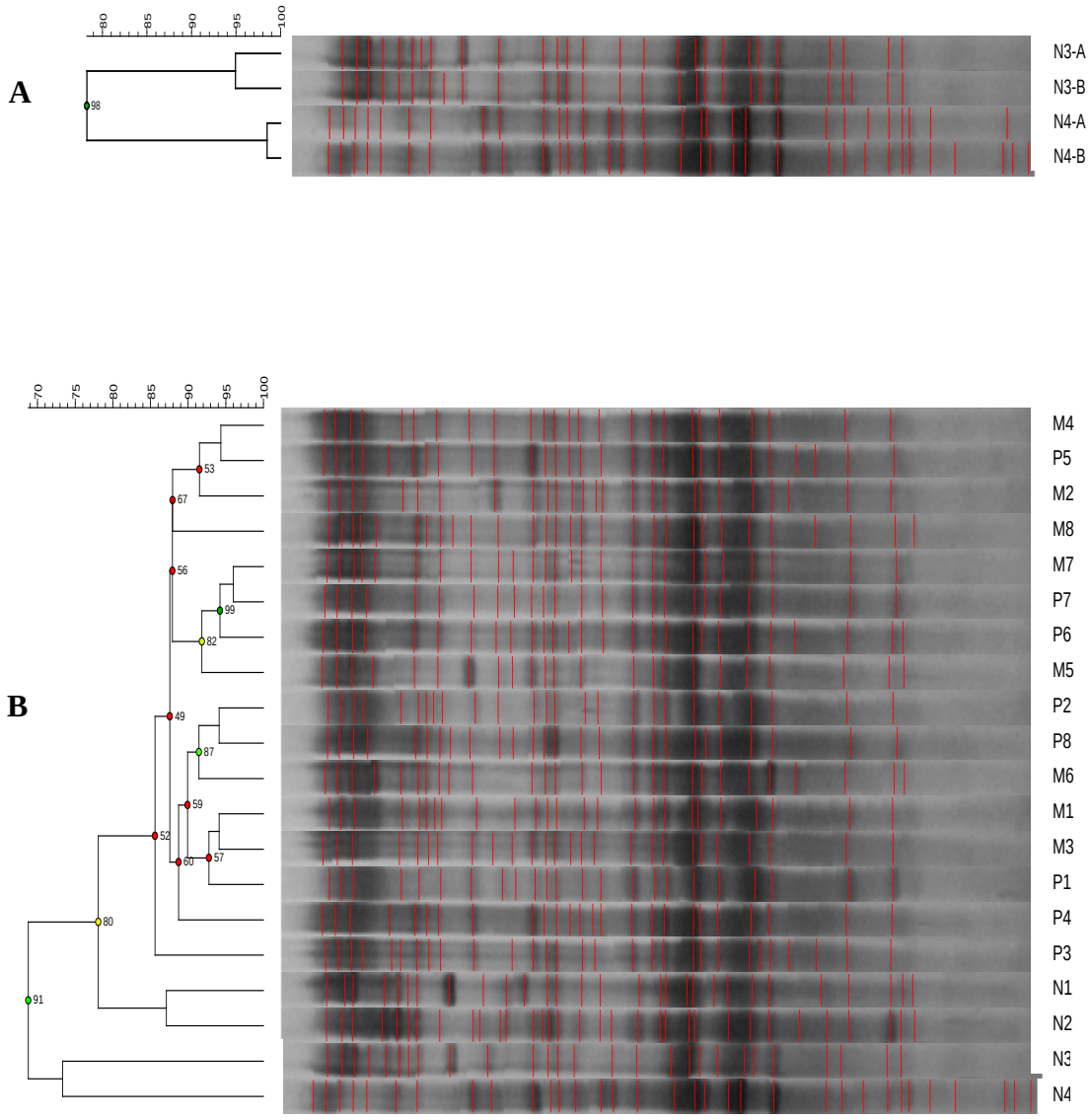
Cluster analysis with Dice coefficient

Clustering with Dice coefficient determined similarity among the samples based on the presence/absence of each band in the profiles. In this case clustering was carried out considering the presence and the position of each band only; band intensities were not considered. Thus information obtained was only related to the samples biodiversity. Reproducibility of the TGGE fingerprints showed that intrinsic variability of the method was even better than that of Pearson, being below 5% (Figure 3.9 A).

Also in this case, fingerprints' grouping was clearly related to the sampling sites (Figure 3.9 B). Profiles of the coastal zone samples (P and M samples) were even more similar (85%), showing presence of almost the same band categories. Among them, only P3 showed some differences: also in this case, this could be due to sea water variations. Open sea samples grouped in two different clusters, both showing low similarity with those from coastal zone (below 75%). However, in this case, samples from 0.5 and 2.5 m depth (N1 and N2) clustered together with high similarity (above 85%), while differences between -15 and -70 m samples (N3 and N4) were considerable (74%) and they clustered even more away from all the other (69 %).

In general, Dice coefficient clustering confirmed results obtained with Pearson coefficient. In other words, correlation among profiles and samples sites was significant also for sample biodiversity. Hence, presence of the various species was strongly depth-dependent.

Figure 3.9 Kandalaksha Bay eubacterial community: Dice coefficient-based analysis of band patterns generated by TGGE fingerprints



Legend:
A= reproducibility of the methods: variability is below 5%. N4-A and N4-B= duplicate PCR of sample N4; N3-A and N3-B = duplicate PCR of sample N3.
B= comparison of band patterns generated by TGGE fingerprints (see M&M for sample marks)

3.6.1.2 Number of bands and statistical indexes in TGGE profiles

Image analysis with Gel Compar II detected a total of 70 different band positions (band classes, Figure 3.10). Thus 70 different species were present in the totality of our samples as revealed by the method sensibility. For each lane, the program calculated relative band intensity also, giving information about the relative abundance of species.

Average number of bands (Table 3.7) per sample in the coastal zone (P and M samples) was ca.26, with a minimum of 23 for P1 and M5 and a maximum of 29 for P3 and M8. As for open sea (N samples), average number of bands was 30: minimum was detected in N3 (27 bands) and maximum in N2 and N4(33 bands).

Simpson diversity index

Species richness is a parameter measuring the number of species in a particular area (community): the number of individuals of each species is not considered. However, richness is not sufficient to describe the community biodiversity that depends on evenness also. Evenness compares the similarity of the population size for each species (number of individuals) thus measuring the relative abundance of different species contributing to the community richness. A community dominated by few abundant species is considered less diverse than one showing several species having less abundance.

Simpson's Diversity Index is a measure of diversity taking into account both richness and evenness.

In our case, as reported in Table 3.7, all the samples showed very high level of diversity. However, diversity was neither related to the sampling site nor to depth. In fact, samples from the coastal zone (P and M samples) showed similar diversity of those from open sea samples (N samples).

Range-weighted richness indexes (Rr)

Bands distribution within a single profile is correlated with the temperature gradient needed to represent the total diversity of a sample and indicates the environment characteristics concerning its ability to support microbial communities. In fact, since very hospitable environments could host many different microorganisms and genetic variability, wide gradients are needed to describe their total microbial diversity. On the other hand, inhospitable stressing environments could support less diverse microorganisms and a narrower gradient would be sufficient. "Carrying capacity" defines the number of individuals that the resources of an environment can support. Thus, the wider the carrying capacity is, the higher is the probability that the environment can host a high number of bands with a large GC variability both in terms of gradient width and positioning of the GC stretches within the 16S rDNA. This concept can be mathematically expressed by defining the range-weighted richness index (Rr) as the total number of bands multiplied by the Δ of the total gradient (temperature or chemical denaturing conditions) needed to describe sample total diversity (for TGGE $Rr=N^2 \times Tg$) (Marzorati et al., 2008). In other words, Rr provides an estimation of the level of microbial diversity. Marzorati et al. (2008) analyzed several DGGE/TGGE based studies targeting the bacterial domain of various environments and proposed thresholds of Rr. Thus, $Rr< 10$ and $Rr> 30$ defines low- and high-diversity communities, respectively.

In our case, all the sampling sites reached high level of Rr (Table 3.7). This was especially evident for samples taken in open sea (N samples) presenting an average Rr of ca. 69. Hence, even with differences related to the location, Kandalaksha Bay presented quite high levels of biodiversity. This environment showed a remarkable carrying capacity, being able to support a large number of individual and different species.

Functional organization (Fo)

The functional organization (functionality) of a microbial community is the result of the action of the most fitting microorganisms, to the current environmental/microbiological interactions, that tend to become dominant within the community structure (Gómez-Silván et al., 2010; Kim et al., 2011).

The relation between functionality and structure of a microbial community has been stated by Fernandez et al. (1999). These authors studied the behavior of different microbial communities under perturbed conditions and concluded that stability of functionality did not necessarily indicate stability of community structures. In addition, they suggested that conservation of a given functionality is ensured by a flexible community with minority members that may become dominant in a short period after a significant perturbation. This condition guarantees a fast recovery from a stress condition due to presence of functional redundancy and alternative ways to use the flow of energy (Fernandez et al., 1999).

Hence, it is possible to define *Fo* as the community ability to be organized in adequate distribution of dominant and resilient microorganisms. This condition should consent to counteract the effect of sudden stressing conditions (Marzorati et al., 2008)

The community functionality, as revealed by TGGE profiles, could be graphically represented by Pareto–Lorenz (PL) evenness curves (Lorenz, 1905). PL curves numerical interpretation could be obtained scoring the y-axis projection of their respective intercepts with the vertical 20% x-axis line (Wittebolle et al., 2008). *Fo* values are expressed as a percentage. Figure 3.11 reports a graphic example of various levels of functional organization. Perfect evenness, represented by the 45° diagonal, means that all the community species have the same number of individuals. Thus, the more the PL curve is close to the diagonal, the highest is the evenness. Low *Fo* values (i.e. 25%) means that the number of individuals in each species is quite similar and the community results scarcely functionally specialized. Since no species are present at higher concentrations, relatively long lag phase could be necessary after a stress

exposure. On the contrary, a medium value of *Fo* (i.e. 45%) is typical of a community with few species (20%) that are dominant and most fitting to environmental conditions (Marzorati et al., 2008). All the other species (80%) are numerically lower. However, due to the elevated concentration of some species and the availability of many others, the community can potentially deal with changing environmental conditions and preserve its functionality. Finally, high values of *Fo* (i.e 80%) represent a very specialized community in which a small amount of species is dominant and all the others are present in low numbers, with a large difference between the two groups. This community can be highly functionally organized; however, being vulnerable to external changes, long recovery times might be required after intense perturbing events.

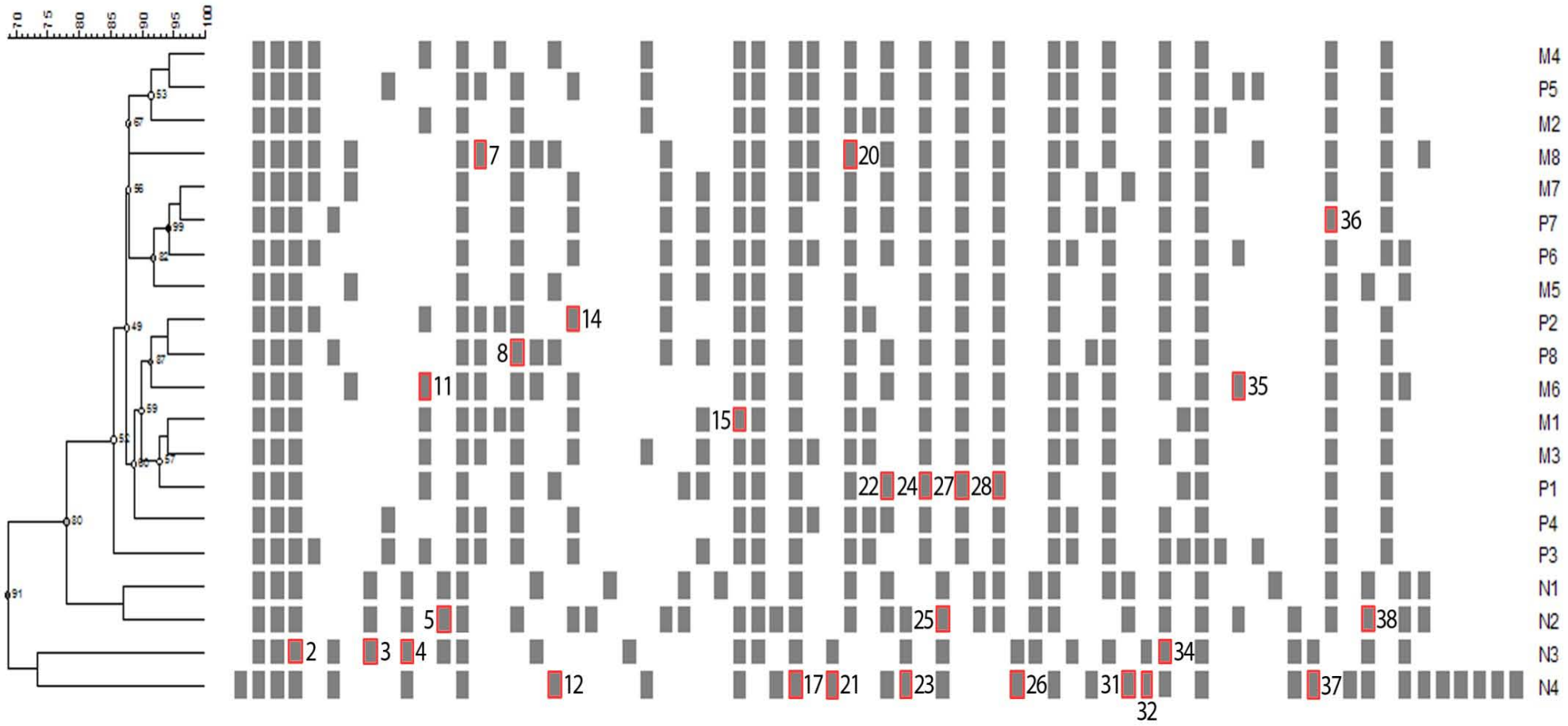
As for the Kandalaksha Bay total eubacterial communities studied here, Figure 3.12 reports the *Fo* values calculated for the various sampling sites that reached, on average, medium-high values (ca. 60%). The communities structure, in all the sampling sites, was quite balanced, showing the presence of some dominant specialized species and many other available at lower concentrations. In conclusion, these structures present good functionality and flexibility and the communities could rapidly react to changing conditions. These results seem to fit with the Kandalaksha Bay environmental conditions characterized by variability of temperature, fresh water and nutrient contribution and strong water mixing. Only a very adaptable community would be able to cope with such unstable environment.

Table 3.7 Average number of bands, diversity and range-weighted richness indexes calculated for different sampling sites

Sampling site	**N°**	**1-D**	**Rr**
P	25.9 ± 2^{a}	0.89 ± 0.02^{a}	45.9 ± 7.2^{a}
M	25.9 ± 1.7^{a}	0.88 ± 0.02^{a}	46 ± 6.5^{a}
N*	30.5 ± 3^{b}	0.92 ± 0.02^{a}	68.7 ± 17.5^{b}

Legend:
P= intertidal pool samples, **M=** water surface nearby the intertidal zone pool samples, **N=**open sea samples taken at various depths
N°=average number of bands, **1-D**= Simpson's diversity index; **Rr**= Range-weighted richness indexes
*****Although N samples were collected in different sites and depths, only averages are reported. In fact, 1-D values for the various depths were not statistically different. Moreover, Rr significant differences were recorded but single data are not necessary since high levels of biodiversity are defined by Rr> 30.
Values represent means ± standard deviation (SD).
Column values followed by the same superscript letter are not significantly different (Tuckey test, $p < 0.01$).

Figure 3.10 Dice coefficient-based analysis with matrix of band presence/absence as detected by Gel Compar II software for total eubacterial community



Legend:
Each rectangle represents a band.
Each column represents a class of bands.
Red surrounded rectangle represents all the excised and re-amplified bands

Figure 3.11 Typical Pareto-Lorenz curves of microbial communities showing different functional organizations (Fo) (Marzorati at al., 2008)

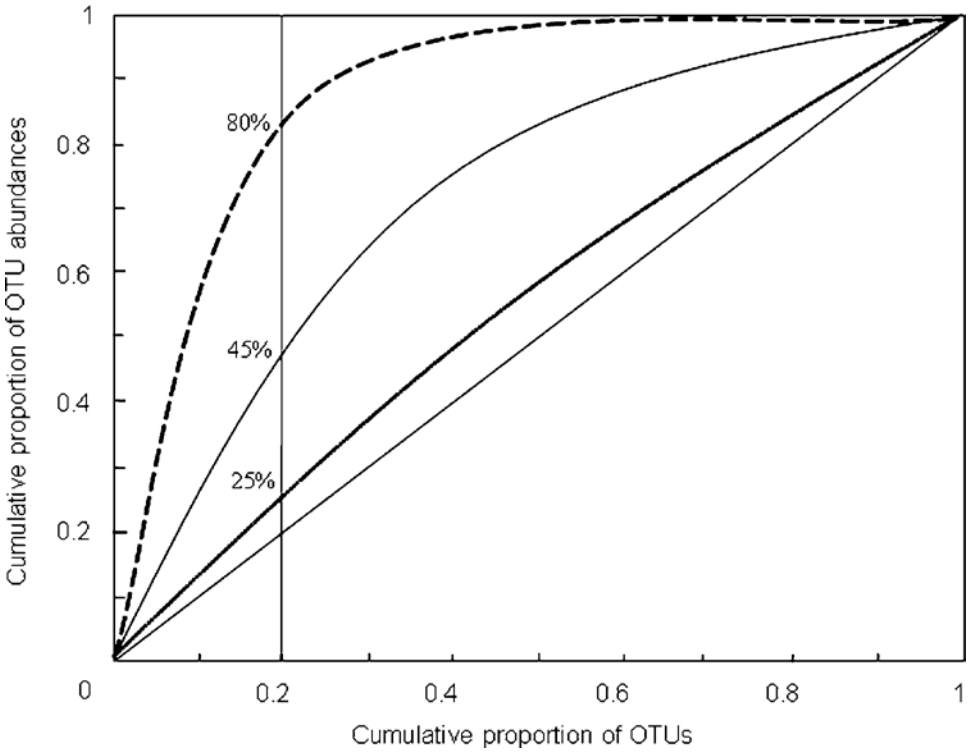
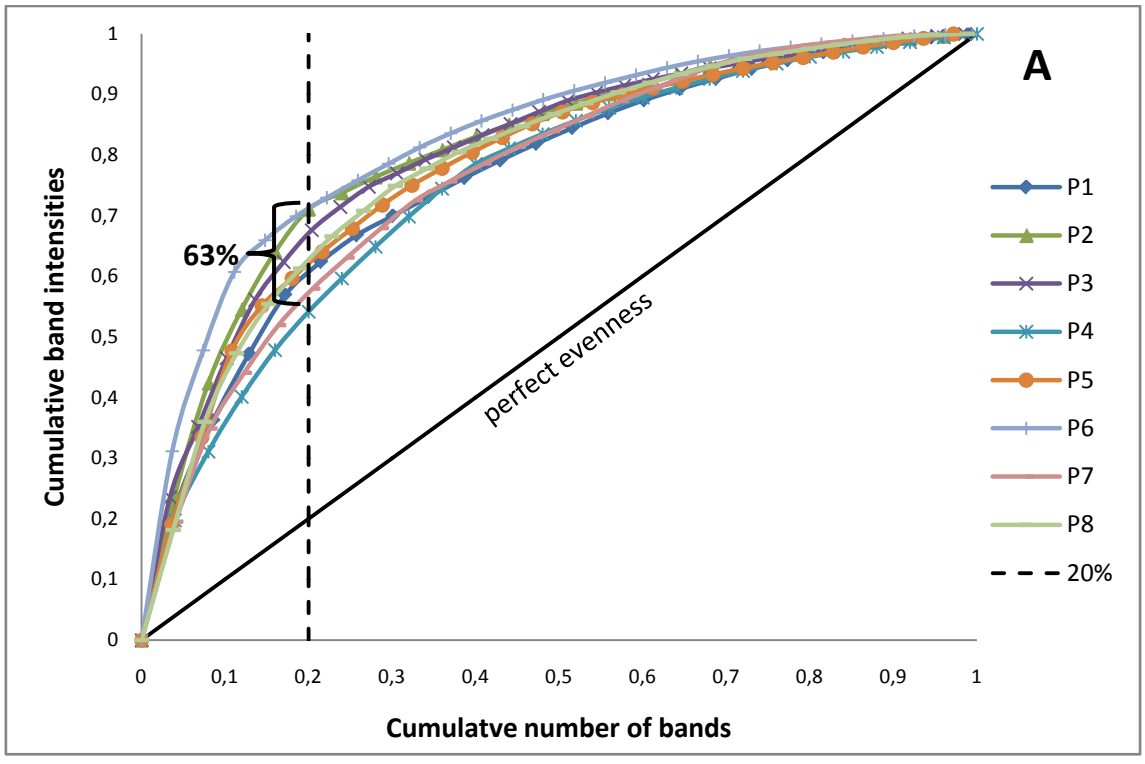
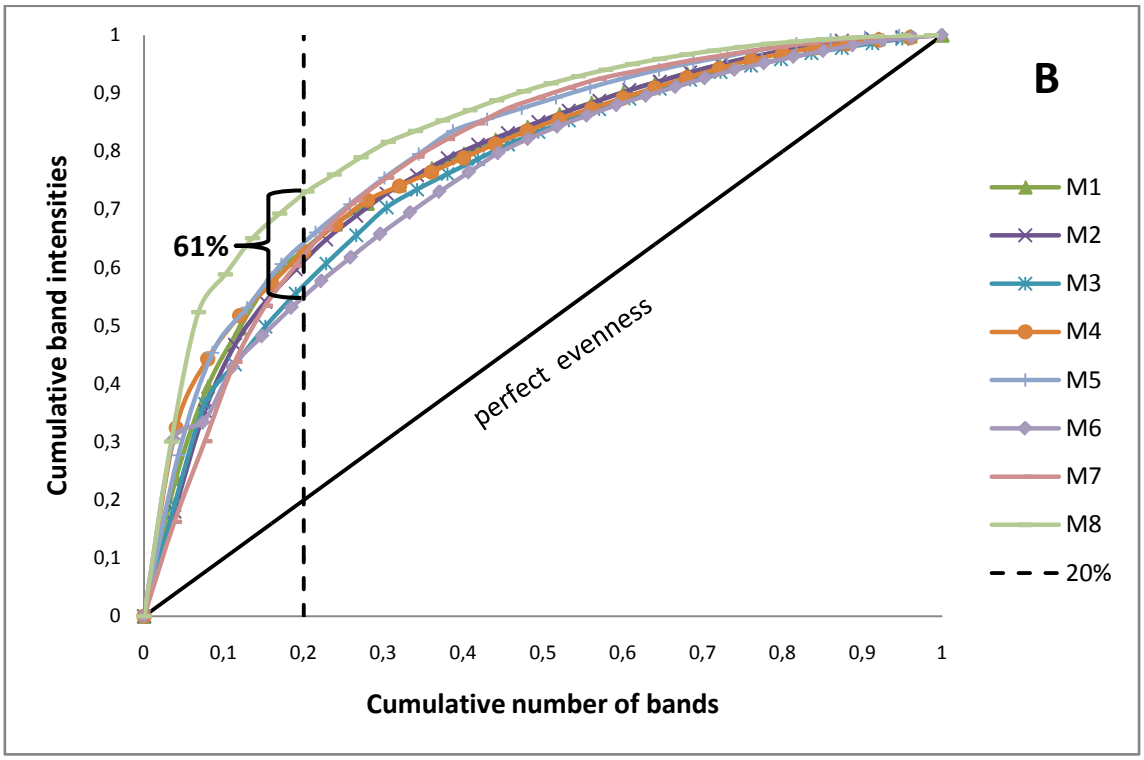
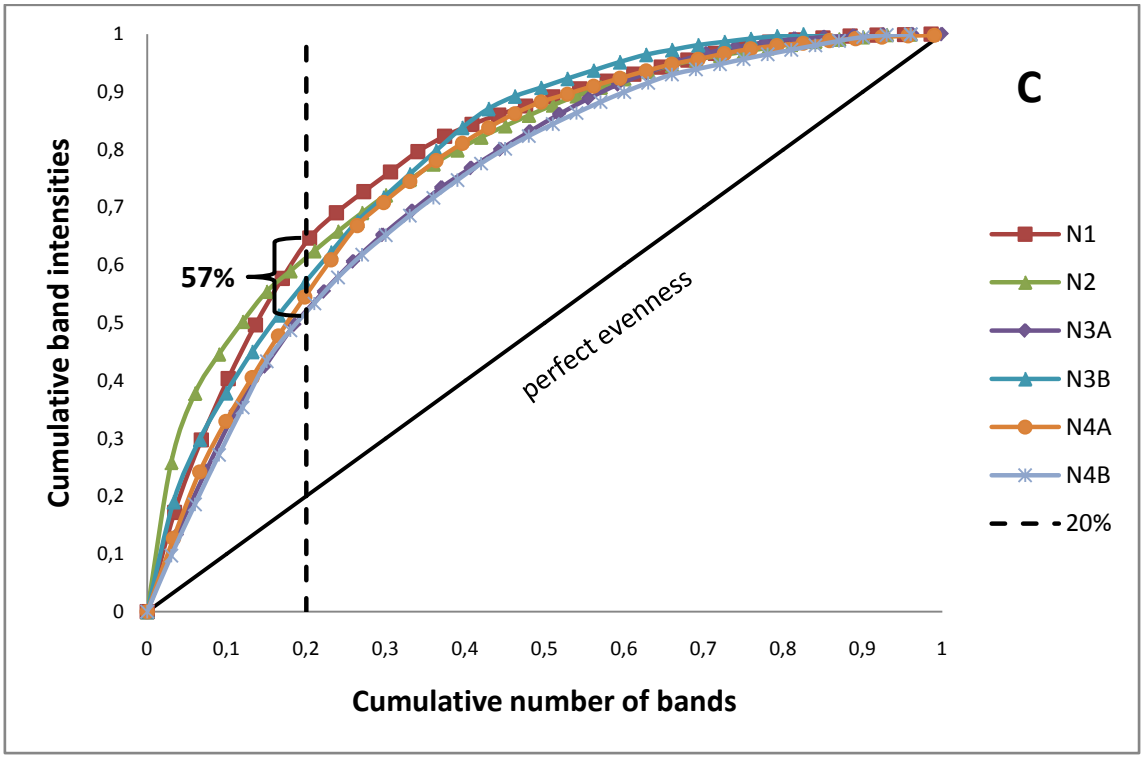


Figure 3.12 Pareto-Lorenz curves of Kandalaksha bay microbial communities for the various sampling sites







Legend:
A= PL curves of intertidal pool samples (P)
B= PL curves of water surface nearby the intertidal zone pool samples (M)
C= PL curves of open sea samples (N)
…..

3.6.1.3 Taxonomical affiliation and phylogenetic study

To obtain detailed overview concerning the predominant species of Kandalaksha Bay community, several TGGE bands were re-amplified and sequenced (Figures 3.10 and 3.13) to perform a phylogenetic study. A total of 27 bands were successfully re-amplified and yielded useful nucleotide sequence data. Sequences were compared to those filed in the EMBL database, and a phylogenetic tree was generated to visualize evolutive relationships between the 16S-rRNA gene sequences, retrieved from samples, and the sequences of the closest relatives affiliated to known bacterial lineages (Table 3.8 and Figure 3.14).

Sequences identified by this approach were mostly related to marine α-proteobacteria (16 out of 27, ca. 59%) belonging to the order *Rhodobacterales*. The sequences mainly grouped in the periphery of the genus *Roseobacter*. A couple of sequences of this cluster (band 25 and 26) were related to an unidentified marine sponge bacterium and an Arctic sea ice bacterium clone, respectively. Sequence 36 can be affiliated to *Ruegeria* sp., also related to the order of *Rhodobacterales*. The second important cluster, in terms of number of sequences affiliated, was the γ-proteobacteria group. In fact, 7 sequences were highly similar to the V3 region of *Cobetia marina*, a moderate halophilic γ-proteobacteria. One sequence (37) was related to actinobacteria. Finally, as primers used were universal for bacterial 16S rRNA gene, few unknown cyanobacteria and microalgae chloroplastic 16S rRNA sequences were revealed too. Even if many sequences were affiliated to known genera and species, some of the bands showed very low identity with those present in the database, suggesting possible presence of various unknown bacteria.

Results of our phylogenetic analysis showed the typical structure of marine environments taxonomic groups. In fact, presence of α- and γ-proteobacteria, as major groups in various seawater samples, was reported by Pommier et al. (2007) stating also that these groups were particularly important in ocean bacterioplankton.

Among α-proteobacteria, the *Roseobacter* lineage is a physiologically heterogeneous group comprising up to 25% of the known marine microbial communities, as revealed either by cultivation-dependent or -independent methods. The great majority of *Roseobacter* is of marine origin, however, several species have been isolated from saline and hypersaline lakes or saline soil (Labrenz et al., 2005; Martínez-Checa et al., 2005). They are well represented across diverse marine habitats, from coastal to open oceans and from sea ice to hydrothermal vents (Buchan et al., 2005; Wagner-Döbler and Biebl, 2006). Even if most *Roseobacter* species are mesophilic, some studies reported isolation of strains from extreme environments such as the polar sea ice (Brinkhoff et al., 2008). The broad distribution of these bacteria is confirmed also by our study. Although study was carried out mainly on coastal samples bands related to this group were present in almost all the profiles.

All the γ-proteobacteria sequences were related to species of *Cobetia marina*. This microorganism belongs to the family *Halomonadaceae,* that consists mostly of marine moderately halophilic bacteria found in temperate and Antarctic saline lakes and other marine environments (Arahal and Ventosa, 2006) including low-temperature hydrothermalflu ids (Kaye et al., 2004) and in/or marine organisms (mussels and algae) (Ivanova et al,. 2005). *Cobetia marina* is reported to be facultative psychrophilic (psychrotrophic) also (Arahal et al., 2002; Yumoto et al., 2004a). In our samples, the majority of *Cobetia marina* bands were retrieved from open sea profiles.

Cyanobacteria is one of the major divisions of the bacteria domain, occupies very diverse habitats both in terrestrial and aquatic environments and can live in extreme conditions of temperatures and salinity. In the oceans, were they contribute to the majority of earth primary production, their habitats include sea ice and sediments (Munn, 2011). In our case, the three cyanobacteria sequences were only related to the open sea samples.In the cyanobacteria cluster was also included the chloroplast sequence of the eukaryotic microalgae *Chlorella*. This is not surprising, in fact, many chloroplast genes are quite conserved and similar to those

of cyanobacteria and, consequently, cyanobacteria-targeted primers have been successfully used for identification of environmental isolates of microalgae (Burja et al., 2001).

The sequence affiliated to actinobacteria was from the sample taken at -70 m. Actinobacteria are abundant in soil and freshwater but they are also distributed in marine sediment and deep-waters (Bull and Stach, 2007; Munn, 2011).

The taxonomic results obtained for Kandalaksha Bay eubacterial community by PCR-TGGE were completely different from those gained by cultivation methods in terms of genera and species detected in the various samples. This could be explained by various factors. First of all, we have to consider the limits of TGGE technique. As already discussed, due to the bias introduced by PCR, this methodology can only detect species representing at least 1% of the total community. In addition, it is known that the majority (up to 99%) of environmental microorganisms could be uncultivable (Lewis, 2007). Then, minority species could prevail in relation to the conditions used for pure cultures isolation.

In our case, since the aim of our work on cultivable strains was the discovery of new microorganisms for possible applications, we selected an isolation medium (PCA) supporting the growth of the highest number of diversified strains. PCA is a rather rich medium and, possibly, unsuitable for those bacteria adapted to sea water oligotrophic conditions.

Figure 3.13 Band excised and reamplified from the TGGE gel

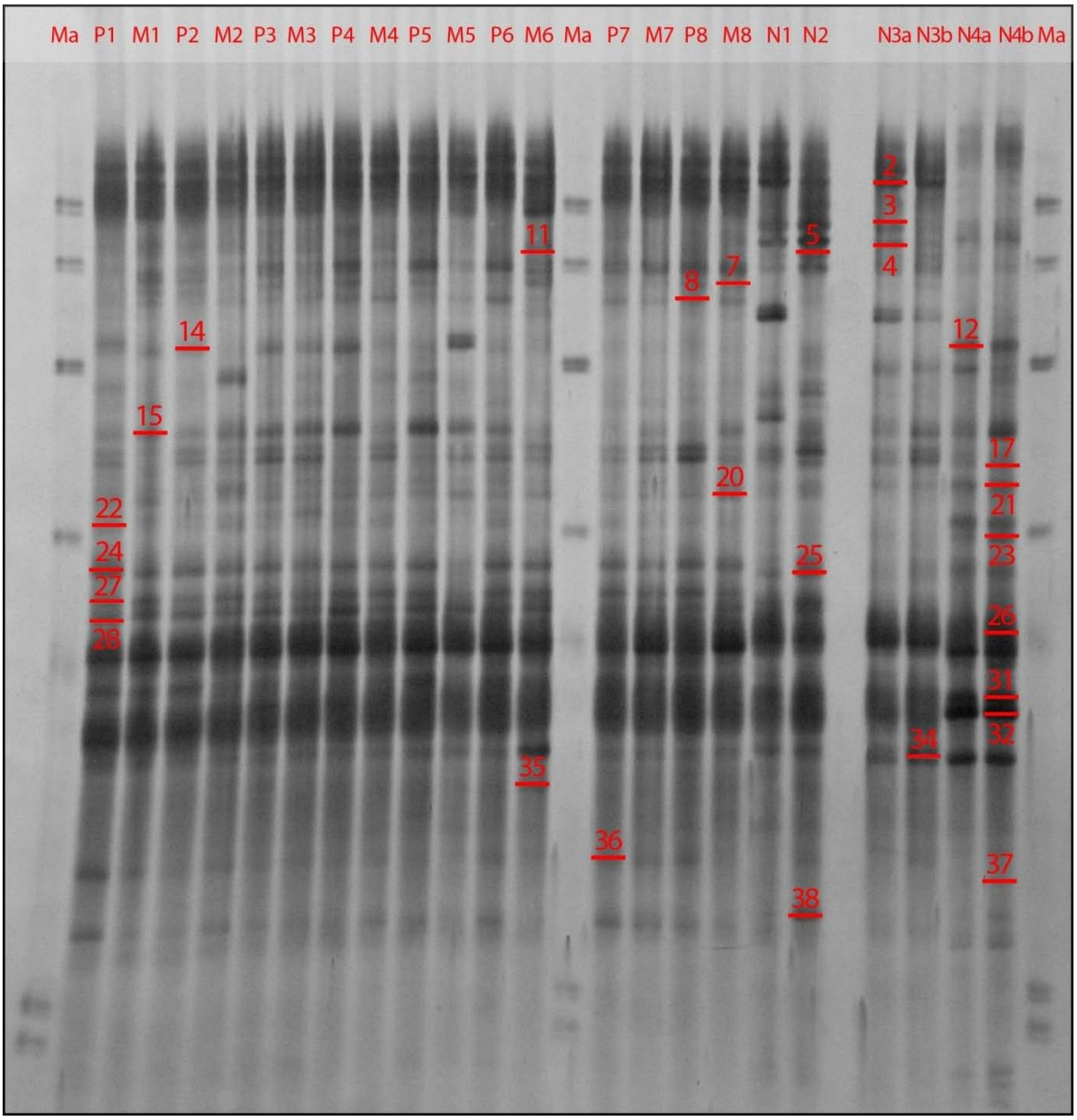


Table 3.8 Affiliation of prominent gel bands present in the TGGE gel

Band	Phylogenetic group	Closest phylogenetic relative	Accession number	Identity
02	α-proteobacteria	Uncultured α-proteobacterium	AM501844	94%
03	cyanobacteria	Uncultured cyanobacterium clone	EU780237	99%
04	cyanobacteria	Uncultured cyanobacterium clone	EU642172	98%
05	cyanobacteria	Uncultured cyanobacterium clone	EU642172	99%
07	α-proteobacteria	Marine sponge bacterium	EU346405	98%
08	α-proteobacteria	Marine sponge bacterium	EU346405	100%
11	α-proteobacteria	Uncultured *Roseobacter* sp.clone	AM501635	100%
12	γ-proteobacteria	*Halomonas* sp	FJ457270	100%
14	α-proteobacteria	Uncultured *Roseobacter* sp.	AM501635	100%
15	α-proteobacteria	Uncultured *Roseobacter* sp.	AM501635	100%
17	γ-proteobacteria	*Cobetia* sp.	DQ873738	98%
20	α-proteobacteria	Uncultured *Roseobacter* sp.	EU600644	98%
21	γ-proteobacteria	*Cobetia* sp.	DQ873738	100%
22	α-proteobacteria	Marine sponge bacterium	EU346508	97%
23	γ-proteobacteria	*Cobetia marina*	AY628696	99%
24	α-proteobacteria	Uncultured α-proteobacterium clone	EF471711	98%
25	α-proteobacteria	Marine sponge bacterium	EU346438	98%
26	α-proteobacteria	Uncultured *Sulfitobacter* sp. clone	EU375061	100%
27	α-proteobacteria	Marine sponge bacterium	EU346438	100%
28	α-proteobacteria	Uncultured *Roseovarius* sp. clone	EU167483	98%

Band	Phylogenetic group	Closest phylogenetic relative	Accession number	Identity
31	γ-proteobacteria	*Cobetia* sp. D49-PB-F05	DQ873738	100%
32	γ-proteobacteria	*Cobetia* sp. S2152	FJ457269	100%
34	γ-proteobacteria	*Cobetia* sp. D49-PB-F05	DQ873738	99%
35	α-proteobacteria	Marine sponge bacterium	EU346405	99%
36	α-proteobacteria	*Ruegeria* sp.	AM709692	97%
37	actinobacteria	Uncultured actinobacterium clone	AY711115	97%
38	α-proteobacteria	Uncultured hyphomicrobiaceae bacterium clone	AY711104	100%

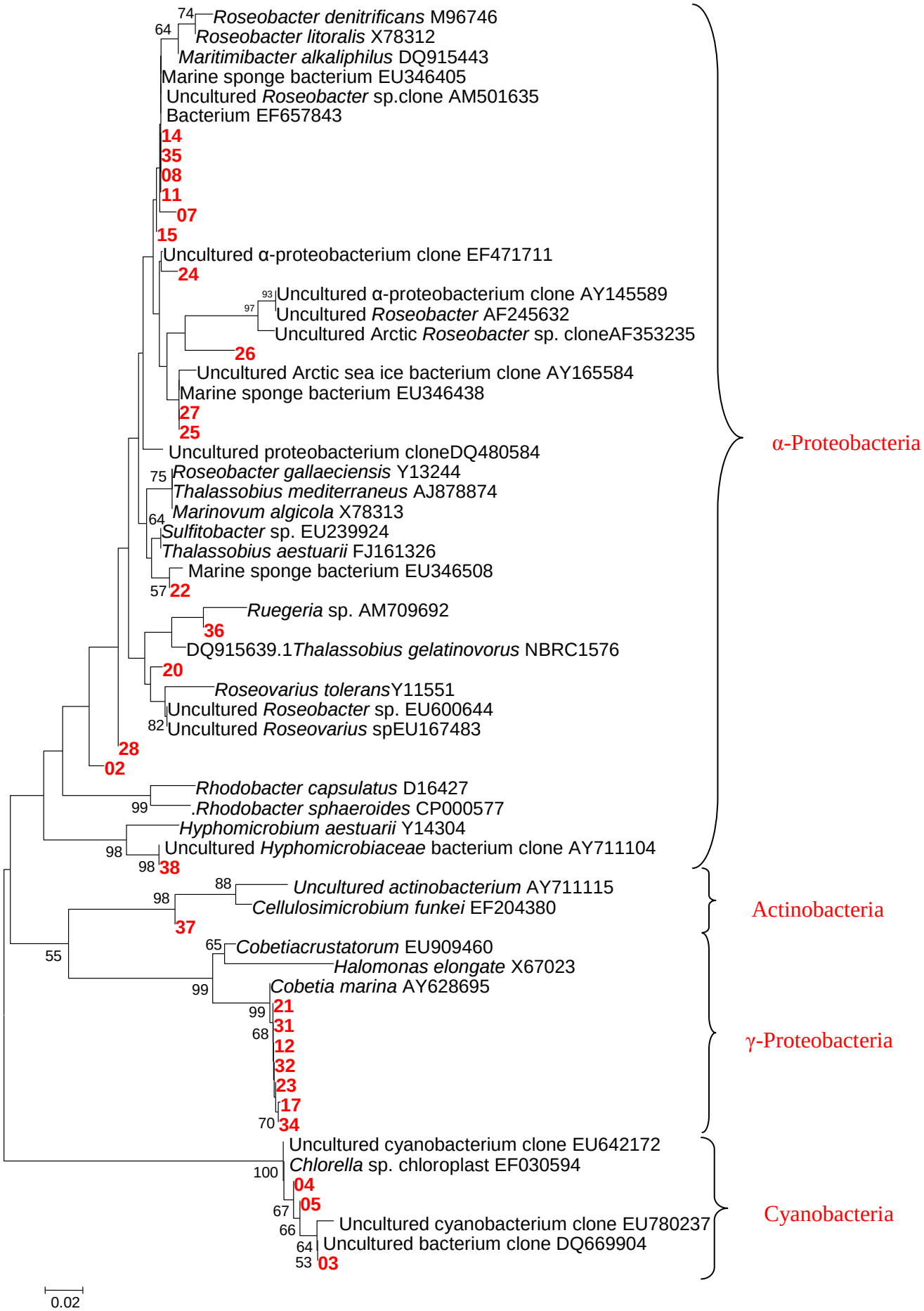


Figure 3.14 Phylogenetic tree of total bacterial community The tree was inferred using the Neighbour-Joining algorithm based on 64 sequences and ca.200 positions. Bootstrap values from 1,000 re-sampled data sets are shown: values below 50% are not shown in the tree.

3.6.2 Study of *Pseudomonas* community

In the TGGE gel, relative to the total eubacterial community, no *Pseudomonas* species were revealed. By contrast, the most abundant species among the pure cultures of KB strains were affiliated to this genus. In order to find possible explications to this apparent incongruity, further TGGE analyses have been carried out focusing on *Pseudomonas*. Thus, total DNA was used for PCR-TGGE fingerprinting of partial 16S-rRNA gene amplicons using specific primers and PCR conditions (Milling et al., 2005) for the amplification of this genus and a different temperature gradient. However, using the annealing temperature suggested by these authors (63°C) no amplification was obtained. Therefore, temperature was lowered and, after various trials, amplification was obtained at 53°C. In these conditions, possible amplification of species belonging to other genera closely related to *Pseudomonas* could also be obtained and separated by the subsequent TGGE. This was confirmed by the phylogenetic analysis as follows.

3.6.2.1 Taxonomical affiliation and phylogenetic study

As reported for the total community, several TGGE bands were re-amplified and sequenced (Figure 3.15) to perform the phylogenetic study. Twenty-two bands were successfully re-amplified from the gel. Sequences were compared to those filed in the EMBL database, and a phylogenetic tree was generated to visualize the evolutive relationships between the 16S-rRNA gene of our water samples and the sequences of the closest relatives affiliated to known species (Table 3.9 and Figure 3.16).

The phylogenetic analysis showed that only 6 sequences out of 22 could be affiliated to *Pseudomonas*. However, due to their external positions respect to the various branches, none of them could be affiliated to known species. In other words, the *Pseudomonas* species isolated in pure cultures were not revealed by the TGGE profiles.

A big cluster, comprising the majority of the sequences, showed very low similarity with *Pseudomonas* and included clones of various uncultured bacteria. However, also in this case

similarity was not very high. The highest level of identity (98%) was reached with some γ-proteobacteria known as ecto- or endo-symbiont of marine invertebrates. Association between various microorganisms and Kandalaksha Bay marine invertebrates has already been reported even if only microalgae and cyanobacteria were detected (Gorelova et al., 2009). This could explain the possible presence of symbiotic bacteria in our samples.

Finally, the only sequence showing 100% similarity to a validated species was that of band 59, evolutively close to *Marinomonas arenicola*.

Due to the general very low similarity of our sequences with those present in the database, very likely, new genera and/or species could be present in our samples.

Figure 3.15 Band excised and re-amplified from the *Pseudomonas* TGGE gel

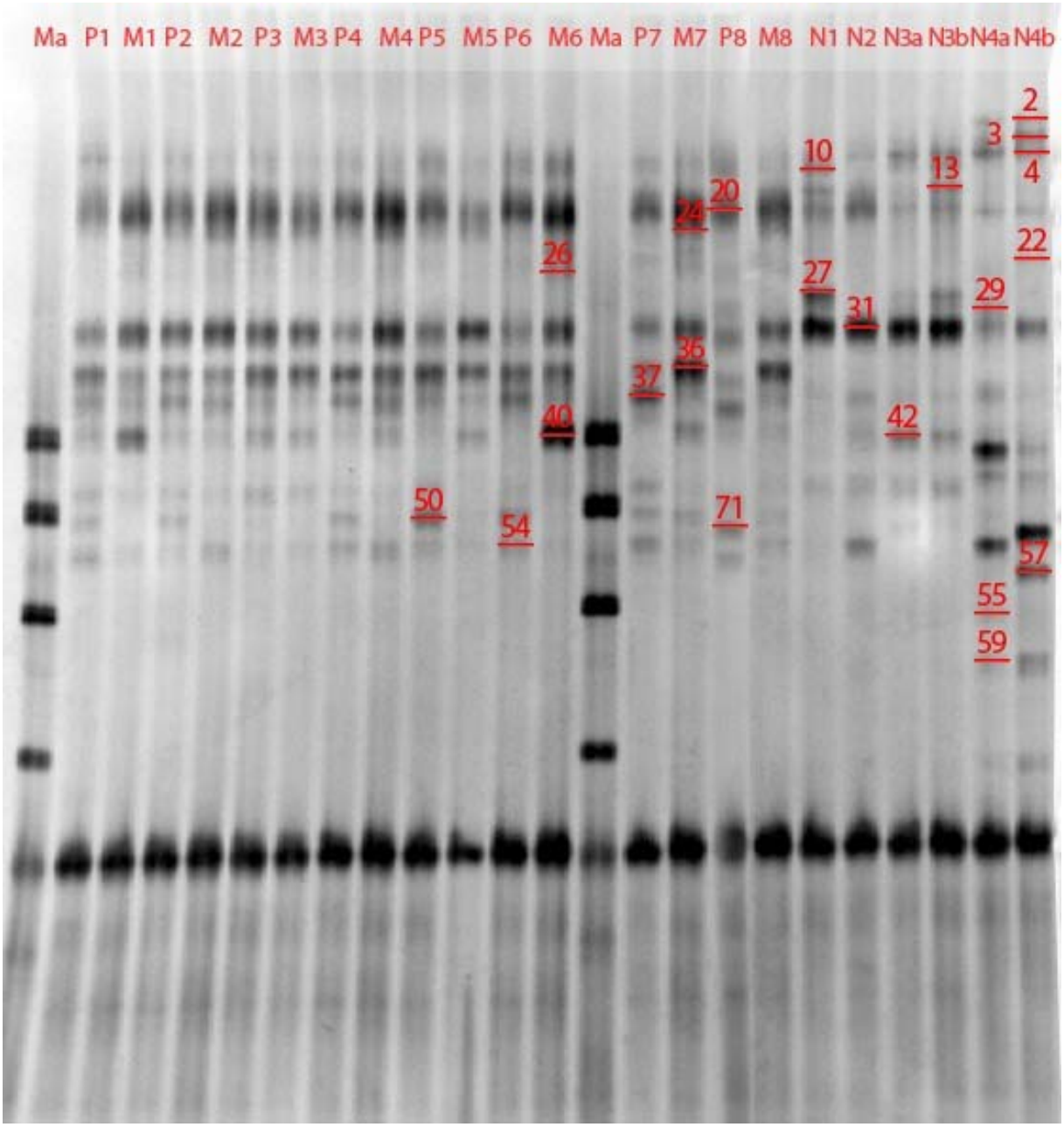


Table 3.9 *Pseudomonas* gel bands affiliation

Band	**Closest phylogenetic relative**	**Accession number**	**Identity**
02	*Pseudomonas putida*	AJ491835	99%
03	*Pseudomonas* sp. ARCTIC	AY573033	100%
04	*Pseudomonas fluorescens*	AJ581937	98%
10	*Maorithyashadalis* symbiont	AB238983	98%
13	Uncultured bacterium clone	AY354140	98%
20	Uncultured γ-proteobacterium clone	EF215819	100%
22	*Pseudomonas stutzeri*	PSU26262	97%
24	Uncultured bacterium clone	AY354140	95%
26	Uncultured bacterium clone	AY354140	94%
27	Uncultured γ-proteobacterium clone	EU265792	99%
29	Uncultured bacterium clone	AY354140	98%
31	Uncultured bacterium clone	AY354140	97%
36	Uncultured bacterium clone	AY354140	95%
37	Uncultured γ-proteobacterium clone	EF491292	94%
40	Uncultured bacterium clone	AY354140	97%
42	Uncultured bacterium clone	AB476255	95%
50	Uncultured bacterium clone	AB476179	97%
54	Uncultured γ-proteobacterium	EF491292	95%
55	*Pseudomonas* sp.	AY017062	100%
57	*Pseudomonas sabulinigri*	EU143352	97%
59	*Marinomonas arenicola*	AB467281	100%
71	Uncultured bacterium clone	AB476179	98%

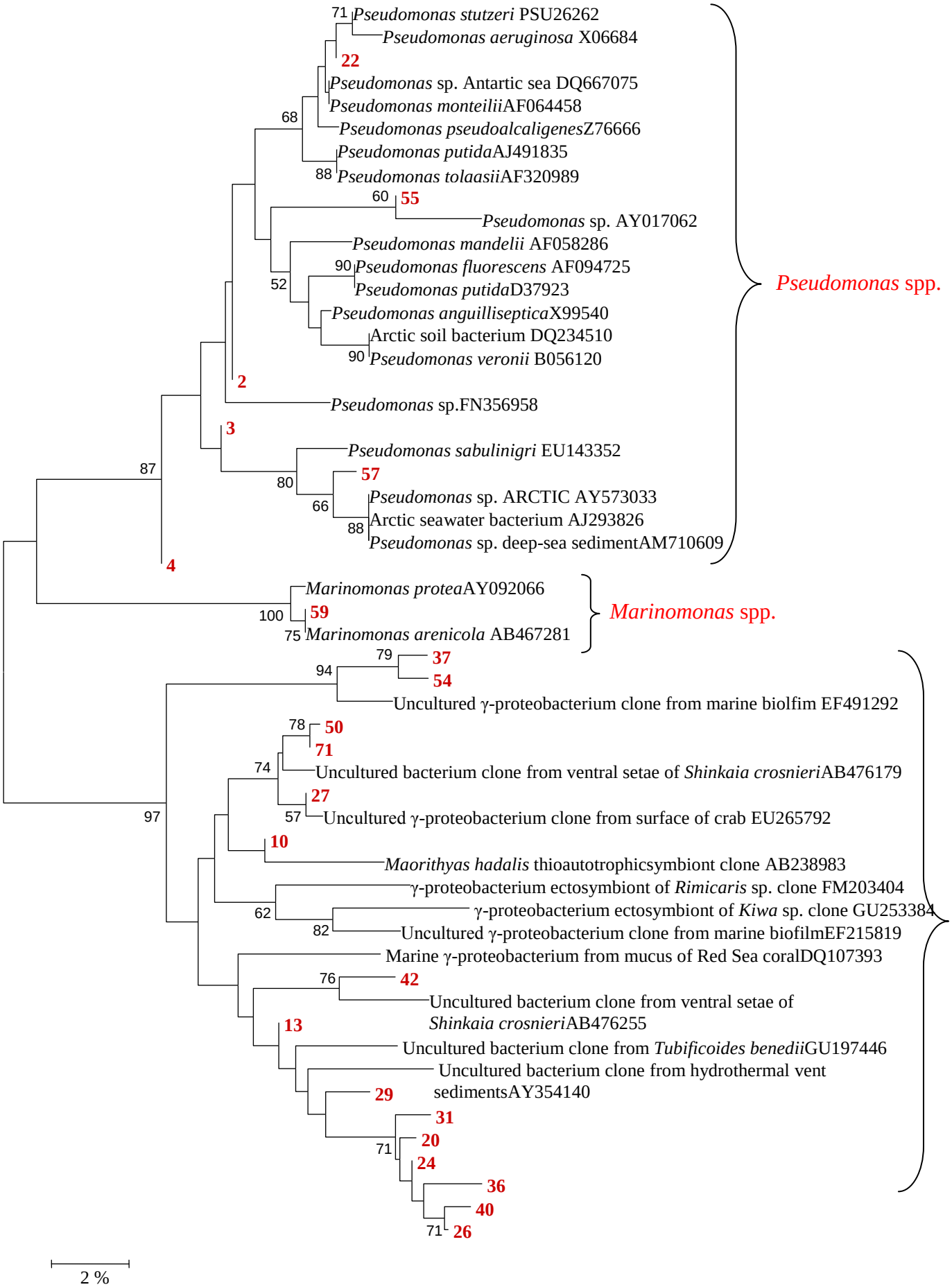


Figure 3.14 Phylogenetic tree of *Pseudomonas*–related communities The tree was inferred using the Neighbour-Joining algorithm based on 54 sequences and ca. 200 positions. Bootstrap values from 1,000 re-sampled data sets are shown: values below 50% are not shown in the tree.

3.6.2.2 TGGE fingerprints analysis

TGGE gel image allowed the analysis of fingerprints (profiles) generated from the separation of V3 hypervariable region of the 16S-rRNA gene, related to *Pseudomonas* species present in the community. To evaluate community structure, cluster analysis of the different fingerprints generated by the samples was carried out using different coefficients by the "GelCompar II" v. 5.10 software. To test the reproducibility of the TGGE fingerprints, duplicate PCR reactions and TGGE runs were analyzed.

Cluster analysis with Pearson coefficient

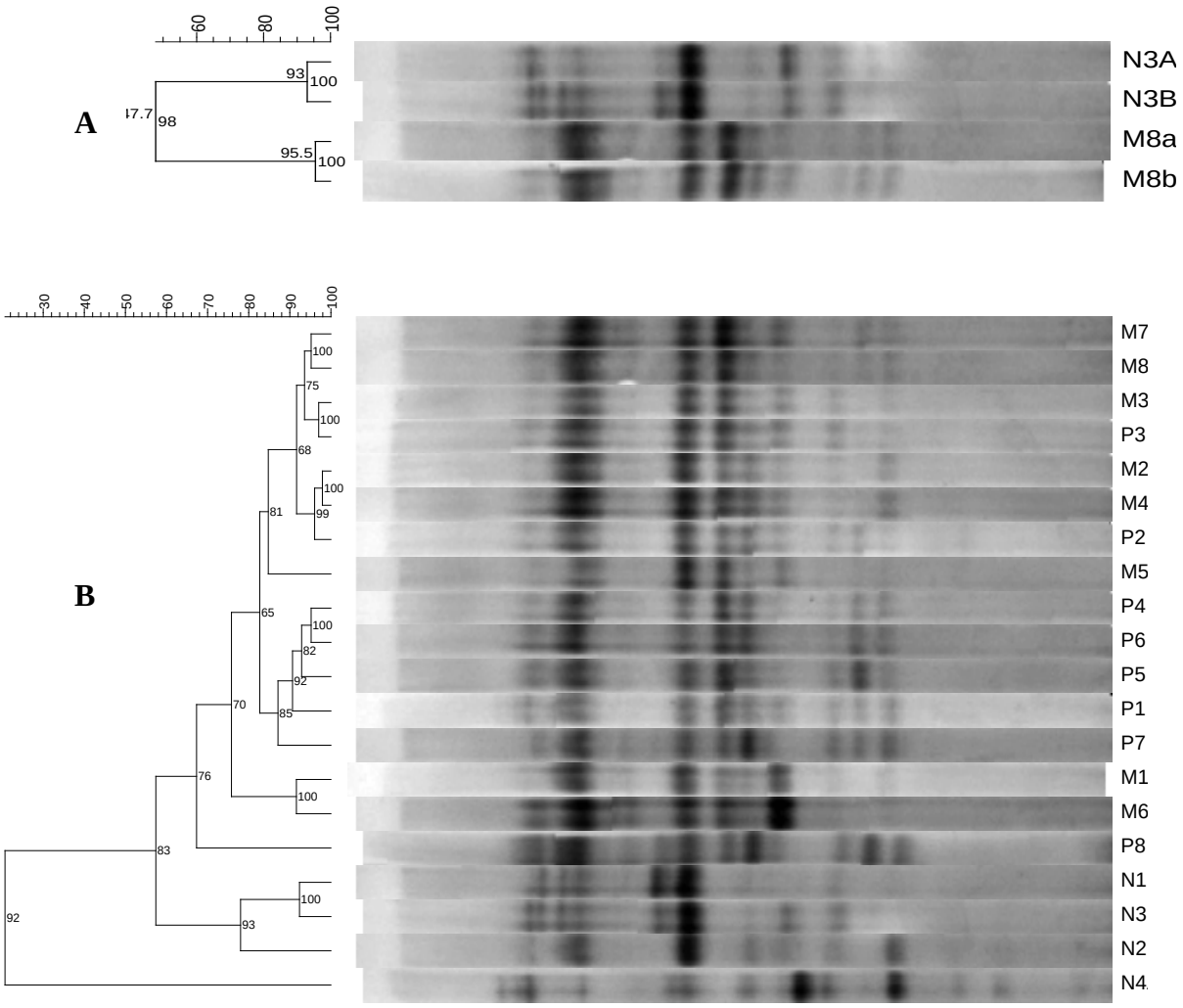
A comparison of two samples is displayed in Figure 3.17 A, showing that intrinsic variability of the method is below 10%. Results evidenced the same structure diversity as reported for the total eubacterial community. Also in this case, clustering was related to the different sampling sites even if in general variability was higher than that recorded for the total eubacterial community (Figure 3.17 B).

Again, a big cluster included all the samples taken in the intertidal zone pool and the nearby sea-surface (P and M samples). However, the fingerprints related to these samples grouped together: with similarity of ca. 70% only. The only profiles which branched rather away within the cluster were M1, M6 and P8. As reported for M5 in the eubacterial community gel, this is probably due to variability of environmental conditions.

Samples taken in open sea at different depths clearly clustered away. However, in this case sample collected at -70 m (N4), was even more separated from all the others samples with 20% of similarity only.

Very high similarity (ca. 95%) was recorded for samples taken at -2.5 and -15 m (N1 and N3).

Figure 3.17 Kandalaksha Bay *Pseudomonas*-related community: Pearson coefficient-based analysis of band patterns generated by TGGE fingerprints



Legend:
A= reproducibility of the methods: variability is below 10%. N3-A and N3-B = duplicate PCR of sample N3. M8-a and M8-b= duplicate PCR of sample M8.
B= comparison of band patterns generated by TGGE fingerprints (see M&M for sample marks

Cluster analysis with Dice coefficient

A comparison of two samples is displayed in Figure 3.18 A, showing that method show no significant variability.

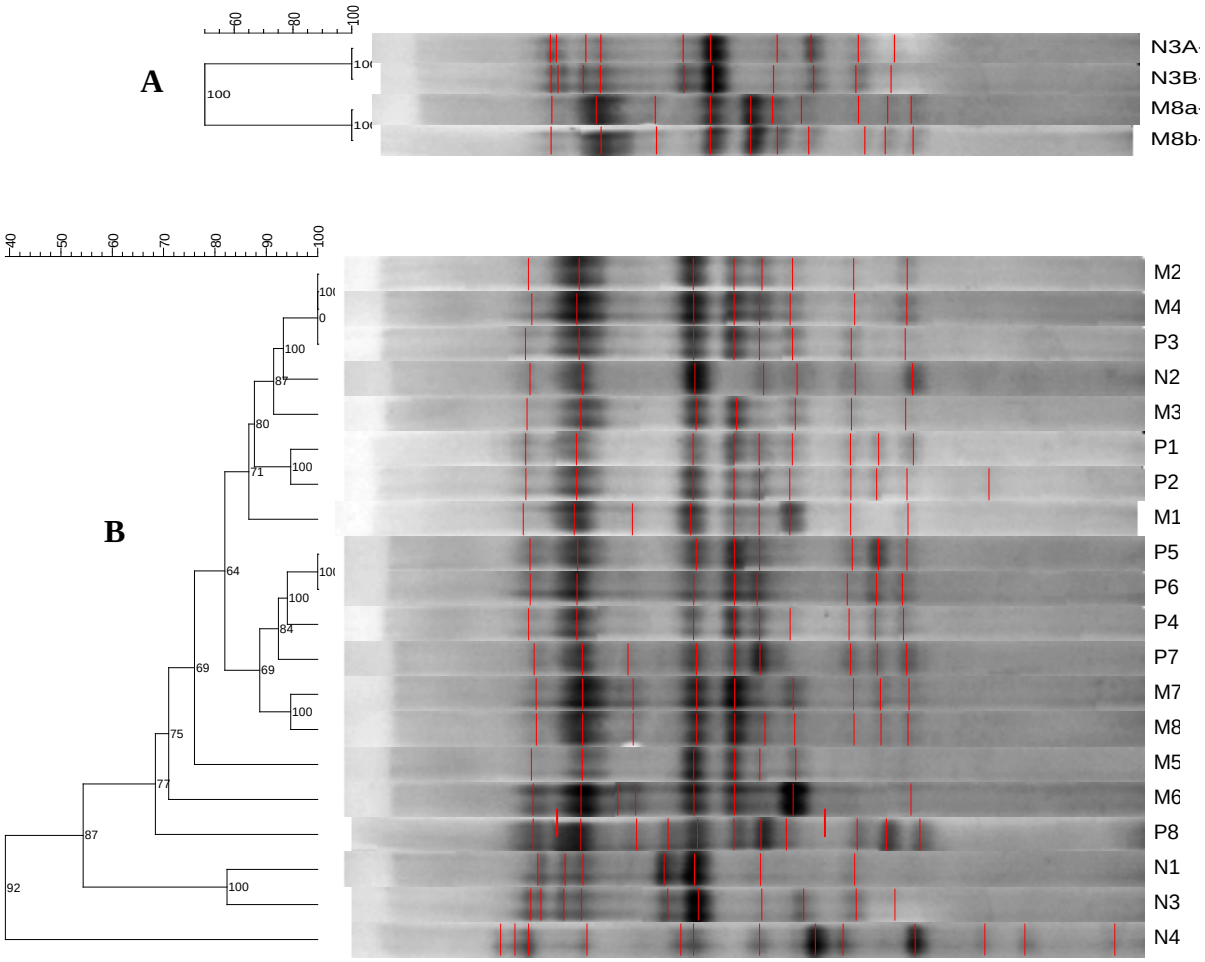
Also in this case, grouping of the fingerprintings was evidently related to the sampling sites, showing similar clustering of Pearson coefficient (Figure 3.18 B).

Profiles of the coastal zone were similar (ca. 70%), showing almost same categories of bands. As observed for Pearson coefficient, some profiles within this cluster (M5, M6 and P8) showed remarkable differences.

Again, open sea samples grouped in different clusters. In this case, sample from -2.5 (N1) and-15 m (N3) clustered together while -70 m (N4) sample clustered at ca. 40% of similarity. Unexpectedly sample N2, taken from open sea surface quite far off-shore, clustered together with coastal samples.

In conclusion, strong correlation between TGGE profiles and the samples sites was recorded for this gel also and species homogeneity depended on depth.

Figure 3.18 Kandalaksha Bay *Pseudomonas* community: Dice coefficient-based analysis of band patterns generated by TGGE fingerprints



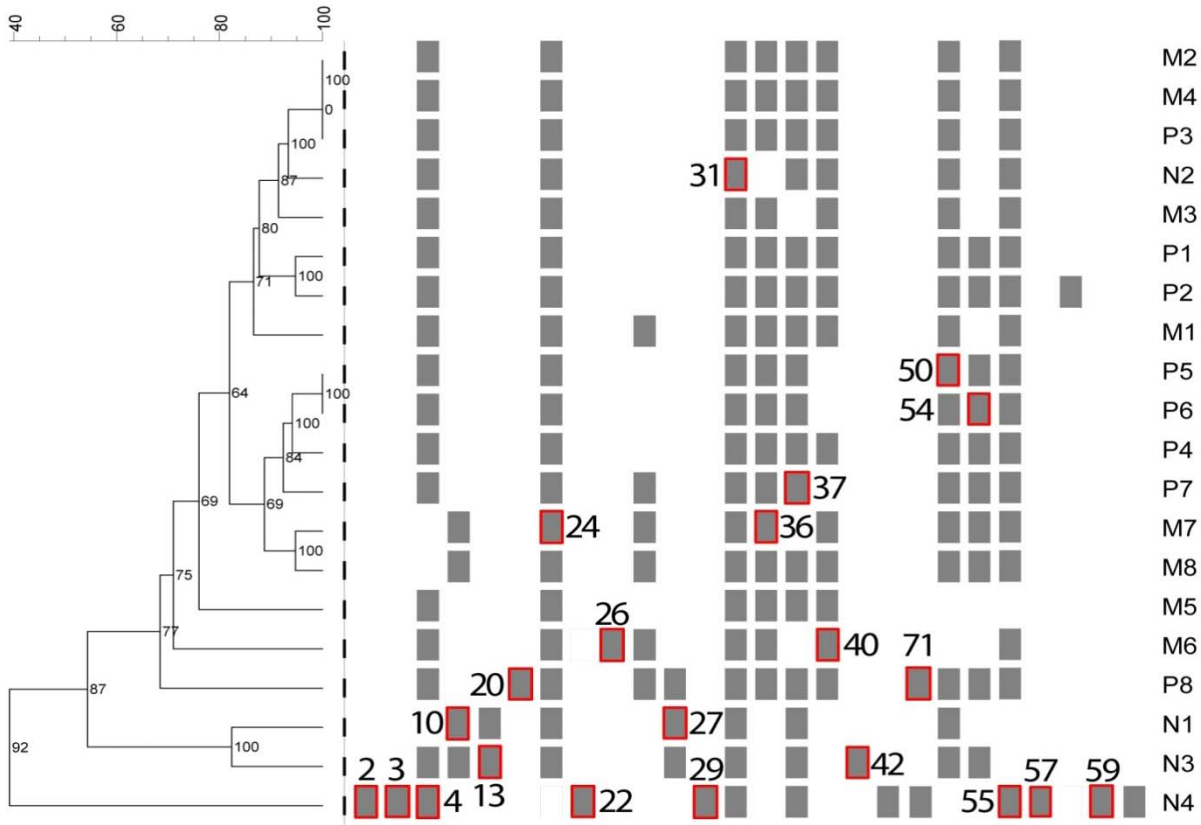
Legend:
A= reproducibility of the methods: variability is 0%. N3-A and N3-B = duplicate PCR of sample N3. M8-a and M8-b= duplicate PCR of sample M8.
B= comparison of band patterns generated by TGGE fingerprints (see M&M for sample mark

3.6.2.3 Analysis of the number of bands and statistical indexes in TGGE profiles

Image analysis with Gel Compar II detected a total of 26 different banding positions (band classes, Figure 3.19) among the samples analyzed. Average number of bands per sample in the coastal zone was 9, with a minimum of 6 for M5 and a maximum of 13 for P8. Also for open sea, average number of bands was 9: minimum was detected in N1 and N2 (7 bands) and maximum in N4 (13 bands).

All the statistical indexes discussed above for total eubacteria and related to community diversity and organization, in this case are meaningless because they would refer only to a limited portion of the entire population.

Figure 3.19 Dice coefficient-based analysis with matrix of band presence/absence as detected by Gel Compar II software for *Pseudomonas* community



Legend:
Each rectangle represents a band.
Each column represents a class of bands.
Red surrounded rectangle represents all the excised and re-amplified bands

Conclusions

A complete study of new and very articulated bacterial communities, involving both exhaustive taxonomical information and detailed metabolic characterization, requires years of study. This is particularly true when the investigated population regards extreme environments.

However, the level of characterization of the Kandalaksha Bay bacterial community, obtained in this work, is quite advanced and reports new and important features concerning this peculiar and scarcely studied sub-extreme environment.

Even if some of our results must be verified and implemented by further sampling campaigns in other season and locations, our data can delineate, with reasonably confidence, some of Kandalaksha Bay bacterial community characters.

The first remarkable outcome regarded the temperature preferences of the KB strains. Our results outlined never described microbial responses to temperature and possible uncommon adaptation strategies probably related to the frequent and wide Kandalaksha Bay temperature variations. The KB bacteria, in fact, showed unusual growth profiles and broad temperature ranges for growth. In addition, concerning their response to temperatures, KB species differ from same known species of other environments.

To the best of our knowledge, the metabolic characterization of KB strains, done in this work, represents the first extensive study carried out on such a big number of isolates, for a rather high number of enzyme activities and a huge variety of substrates. This data is principally important for further biotechnological applications. However, additional studies, concerning the biochemical pathways and genes involved in strains metabolism, are necessary. Moreover, to verify their actual potential, further works should be focused on specific applications.

Under the taxonomical point of view, the contribution of our work was very important. Although identification at the species level was possible for less than 50% of the strains, all

the KB bacteria were affiliated to known genera. Clear phylogenetic relationships among the various strains were established and possible new species were recognized.

Also, the total bacterial community was well characterized. The information gathered permitted to identify and relate the most abundant species to the community structure. In addition community response to environmental stress and changes was analyzed, showing high flexibility and fast recovery after perturbations. This represents good ecological feature of the studied population.

However, as already mentioned, a complete ecological overview of the Kandalaksha Bay microbial community needs further sampling campaigns to collect data about variations on a seasonal base and in different years. Moreover, other sampling sites must be investigated and more details are necessary concerning depth-related variations.

Our study yielded information only about a part of the complex microbial community that can be present in the samples. Further analysis, using also different molecular tools, would be necessary to study other microbial groups, such as Archea, Cyanobacteria, Fungi and Oomycetes. Some of these research activities are already in course.

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Appendix

Publications produced during Ph-D:

Full papers

JUAREZ-JIMENEZ B., RODELAS B., MARTINEZ-TOLEDO M.V., GONZALEZ-LOPEZ J., CROGNALE S., GALLO A.M., **PESCIAROLI C**., and FENICE M. (2008). Production of chitinolytic enzymes by a strain (BM17) of *Paenibacillus pabuli* isolated from crab shells samples collected in the East Sector of Central Tyrrhenian Sea. *Int. J. Biol. Macr*. 43 : 27-31 .

JUAREZ- JIMENEZ B., MANZANERA M., RODELAS B., MARTINEZ-TOLEDO MV., GONZALEZ-LOPEZ J., CROGNALE S., **PESCIAROLI C**. and FENICE M. (2010). Metabolic characterisation of a strain (BM90) *Delftia tsuruhatensis* showing highly diversified capacity to degrade low molecular weight phenols. *Biodegradation*. 21: 475-489.

CERRONE F., BARGHINI P., **PESCIAROLI C**. and FENICE M. (2011) "Efficient removal of pollutants from olive washing wastewater in bubble-column bioreactor by *Trametes versicolor*" *Chemosphere*. 84: 254-259.

PESCIAROLI C., CUPINI F., SELBMANN L., BARGHINI P. and FENICE M. "Temperature preferences of bacteria isolated from sea water collected in Kandalaksha Bay, White Sea, Russia." Submitted for publication to *Polar Biology*.

Posters and participation to conferences

PESCIAROLI C., JUAREZ-JIMENEZ B., CROGNALE S and FENICE M. "Metabolic competences of *Delftia tsuruhatensis* BM90, isolated from sea water samples, and its potentiality in polyphenols degradation". Bertinoro Meeting di Microbiologia Ambientale (BMMA), May 23-24, 2008.

PESCIAROLI C., JUÁREZ B., MARTINEZ-TOLEDO M.V., CUPINI F. and FENICE M. "Extracellular enzyme activity by bacterial strains isolated in the intertidal zone of Kandalaksha Bay, White Sea." National Congress Of the Italian Microbiology and Microbial Biotechnology Society, Spoleto, Italy, June 11-13, 2009.

JUÁREZ-JIMÉNEZ B., REBOLEIRO-RIVAS P., MARTINEZ-TOLEDO M.V., **PESCIAROLI C.** and FENICE M. "Proceso de formación de biopelículas por *Delftia*

tsuruhatensis en celulosas: degradación de compuestos fenólicos." Congress of the SEM (Spanish Microbiological Society), Almeria (Spain), September 21-24, 2009.

BARGHINI P.**,** CERRONE F., **PESCIAROLI C.** and FENICE M. Depollution of olive washing wastewater by Trametes versicolor in continuous process. BENA International Environmental Conference: Sustainable Development in Coastal Areas. June 29 - July 1, 2011. Joannina, Greece

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