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Phylogenetic diversity and metabolic potential of prokaryotic communities in permafrost and brine pockets of perennially frozen Antarctic lakes (Northern Victoria Land)

Tesi di Dottorato
Dott.ssa Antonella Conte

Il Coordinatore
Prof. Salvatore Cuzzocrea

Il Tutore
Prof.ssa Nunziacarla Spanò

Il Cotutore
Dott.ssa Angelina Lo Giudice

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Sede Amministrativa: Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali-
Università degli Studi di Messina

Viale Ferdinando Stagno d'Alcontres, 31-98166 Messina

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Abstract

Permafrost can be defined soil material which lies at temperature below 0 °C for at least two years due to a continuously frozen state. The depth of permafrost table is variable and changes depending on the seasonally temperature. Permafrost table should be not considered as a unique layer, as it is stratified in active layer, talik and brine. Active layer is the portion of soil above the permafrost table. It is called active because its physic status is seasonally modified by thawing and freezing changes, thus becoming active in interconnection with the atmosphere. This layer plays an important role in cold regions because most ecological, hydrological, biogeochemical activities take place within it. This depth varies during different seasons and locations, from 2 cm in the coldest area to 100 cm in the warmer area. Talik is a layer of unfrozen ground in a permafrost area. Talik may have temperatures above 0 °C or below 0 °C. Brines are amounts of liquid water, which is a salt water basin within permafrost table formed during winter season. The brine formation depends on the increase of salt concentration in groundwaters under or behind permafrost, combined to evaporation and halite dissolution processes that generate the formation of veins and pockets of salt liquid that does not frozen under 0 °C. Permafrost is considered as an extreme environment due to its physiochemical features, namely low temperature, oligotrophic nature of sediments and water availability. Microorganisms that live in this environment are generally psychrophiles, which had successfully colonized all cold environments adapting their enzymes to permit them to survive and function in extreme habitats. The study of the permafrost and brine is therefore important to understand microorganism adaptations to extreme environments. For these reasons, the aim of the present Thesis was the study of structure and functions of the prokaryotic communities inhabiting Antarctic permafrost and brines. Permafrost samples were collected during different Antarctic campaigns from three sites (i.e. Edmonson Point, Boulder Clay and Dry Valleys) at different depths, while brine samples

derived from lakes at Tarn Flat and Boulder Clay (in the inland and on the coast, respectively). Culture-independent (e.g. NGS sequencing of 16S rRNA genes and CARD-FISH) and culture-dependent (e.g. evaluation of best isolation method using basal and rich media at different concentrations of nutrient, identification and phenotypic characterization of bacterial isolates) approaches were used to investigate for the first time the composition, activity and adaptation of microbial assemblages in permafrost samples collected in Antarctica. The study was divided in two different chapters: the first one dealing with permafrost samples and the second one on brine samples. The first objective of this work was the analysis of the prokaryotic community inhabiting permafrost samples and the comparison of the different kind of samples among the communities. For this purpose, permafrost samples of different geomorphological eras were selected, from active layer to the oldest samples to ice permafrost. The samples were collected at Edmonson Point (active layer, EP), Boulder Clay (BC-1, BC-2 and BC-3) and Dry Valleys (oldest permafrost, DY). The culture-independent approach was applied for the sole active layer (EP). Such layer was chosen because it is continually in inter-connection with the atmosphere and changes seasonally due to the alternation between the thawed and frozen states, becoming an ecological niche colonized by diverse and functionally cold-adapted microbial assemblages, which adapt themselves to the seasonally changes of temperature, water availability and ice presence. The results showed a total of 330 OTUs distributed in 10 different bacterial phyla, with the predominance of *Proteobacteria* and *Actinobacteria*, followed by the *Acidobacteria*, *Nitrospirae*, *Chloroflexi*, *Firmicutes* and *Bacteroidetes*. The *Gemmatimonadetes*, *Chlorobi* and *Cyanobacteria* constituted a minor component in the sample EP. Differences in the relative abundances were observed for sequences affiliated to proteobacterial classes as they were mainly referred to the *Alpha-, Beta-*, and *Gammaproteobacteria*, whereas the *Delta-* and *Epsilonproteobacteria* were less represented. A number of sequences were not identified at phylum level. At genus level,

Lactobacillus (among *Firmicutes*), *Nitrospira* (among *Nitrospirae*), *Marmoricola* and *Propionibacterium* (among *Actinobacteria*), were the more represented. The culture-dependent approach was used with two strategies of cultivation, direct plating of diluted cell suspensions on agar media and enrichment in liquid media before plating. All the isolates were phylogenetically affiliated. Enumeration of cultivable heterotrophic bacteria showed an order of 10^3 CFU g^{-1} by direct plating, while after enrichment in TSB at different strengths, viable counts were three to six orders of magnitude higher than those obtained by direct plating. Overall, the cultivable bacteria were distributed within five different taxa, with the predominance of *Firmicutes*, followed by *Actinobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*. Members in these two latter groups were isolated only after enrichment, with the *Alphaproteobacteria* that were obtained only from BC samples and *Betaproteobacteria* only from sample EP. At genus level, the strains were affiliated mainly to *Bacillus*, *Arthrobacter* and *Sporosarcina*. The cultivation on R2 Agar medium, an oligotrophic medium, was the best method for the recovery of bacteria from Antarctic permafrost samples. Diluted media (i.e. TSA_{50} and TSA_1) generally yielded lower numbers of bacterial colonies than full strength media (i.e. TSA_{100} and R2A). The molecular approaches were useful to recovery a high number of sequences. The phyla retrieved were typical of soil, and some sequences were related to unknown or unclassified bacteria. This could suggest that they are unique to Antarctic soils or that similar environments globally have not been microbiologically well characterized.

The second part of work was the analysis of prokaryotic communities inhabiting brines using culture-independent and culture-dependent approaches. The samples were collected from two Boulder Clay lakes (BC1, BC2 and BC3) and in a Tarn Flat lake (TF4 and TF5), at different depths. TF4 and TF5 brines were collected from the same borehole, while BC1 and BC2 from the same lake and BC3 in a second lake in the Boulder Clay site. The abundance and diversity, using microscopy, was detected using DAPI staining

and CARD-FISH methods and was in the range of 10^6-10^7 cells mL^{-1}. The CARD-FISH analysis showed that *Proteobacteria* and *Bacteroidetes* were predominant in similar percentage, except for BC2 that presented *Bacteroidetes* as the only predominant phylum. The NGS sequencing method was used for the analysis of the total bacterial community, the active community (BC1, BC2 and BC3 only) and the Archaeal community. For the total community, TF5 was the highest diverse sample (Shannon index of 1.981) followed by BC1 (1.783), whereas BC2 was the lowest diverse sample (1.087). Total bacterial community composition was similar in phyla percentage and was represented by *Proteobacteria* followed by *Bacteroidetes* and *Actinobacteria.* At genus level, *Flavobacterium* and *Algoriphagus* were best represented in Boulder Clay samples, while *Ulvibacter*, *Marichromatium*, *Marinobacter* and *Shewanella* were best represented in Tarn Flat samples. The active bacterial community was represented by *Bacteroidetes*, followed by *Proteobacteria, Actinobacteria* and *Firmicutes*. At genus level, *Caldimicrobium* (among *Thermodesulfobacteria*), *Flavobacterium* and *Algoriphagus* (among *Bacteroidetes*) and *Lebetimonas* (among *Epsilonproteobacteria*) were predominant. The Archaeal community structure was highest in BC1 (Shannon index 1.178) al lowest in BC2 (0.4084) and was represented by *Euryarchaeota*, followed by *Crenarchaeota* and *Ancient_Archaeal_Group.* The predominant order were *Methanopyrales*, *Methanobacteriales* and *Methanosarcinales,* anaerobic methanogens often isolated from hydrothermal vents. The statistical analysis showed a similarity between TF4 and TF5 and BC2 and BC3, while BC1 was totally distant from the other samples. The culture-dependent methods showed a CFU abundance of 10^3 and 223 isolates that were shared between Boulder Clay and Tarn Flat samples. The phylogenetic affiliation of isolates showed a high presence of *Proteobacteria* followed by *Actinobacteria* and *Firmicutes.* The genera mainly represented were *Pseudomonas*, *Psychrobacter*, *Marinobacter*, *Leifsonia*, *Carnobacterium* and *Sporosarcina*. These strains were assayed for different capabilities and potentials, different growth conditions

(pH, NaCl percentage, temperature), production of extracellular enzymes (catalase, oxidase, hemolysis, DNAse), hydrolysis of complex substances (agarase, lipase, gelatinase, amylase, chitinase), antibiotic susceptibility, pollutant tolerance and biotechnological potential (EPS production, pollutant degradation, inhibitory activity). Numerous isolates grew in the presence of NaCl 19 %, mainly affiliated to *Psychrobacter*, all strains were able to growth between 6-7 to 9 pH range and all strains were able to growth at 4 and 15 °C (only *Marinobacter* sp. at 4 °C), 38 strains from TF and all strains from BC were able to grow at 25 °C. Few strains were able to tolerate antibiotics, in particular ampicillin was the best tolerated, while for heavy metals nickel and copper were the best tolerated metals, instead cadmium were no tolerated. EPS production was showed by seven strains, mainly belonging to *Pseudomonas* sp., while ten strains were able to degrade Aroclor 1242 at 4 and 15 °C (mainly belonging to *Rhodobacter* sp.). Two strains were further assayed for biodegradative efficiency and about 80-90 % of congeners were removed.

One aim of this work was to analyze the viable bacterial community in brine samples in order to understand the life in such extreme and harsh environment, which has been poorly studied. In conclusion, this study allowed to compare extreme habitats, such as Antarctic permafrost and brines, using culture-dependent and culture-independent approaches. The use of different approaches was helpful to have a more complete profile of the present situation in these environments and to understand the past matters through more detailed analysis. These environments represent the terrestrial model of such exobiological niches with their unique halotolerant and aerobic psychrophilic community, that is a possible model for extraterrestrial life.

Summary

1. Introduction

1.1 Main features of permafrost

Permafrost can be defined as soil material which lies at temperature below 0 °C for at least two years due a continuously frozen state. Permafrost is synonymous with "perennially frozen ground" (Black, 1976). It is the result of long periods of continuous cold climate as occurred frequently over the Pleistocene (about 2 million years). Permafrost occurrence, distribution and thickness are expected to increase during periods of colder temperatures and decrease during warm intervals. The term "cryotic" has recently became accepted as definition of permafrost environment because of the temperature below 0 °C (Gascoyne, 2000). Permafrost generally occurs in extreme latitudes where yearly temperatures permit continuous freezing, although permafrost is not limited to these places. Permafrost also frequently occurs in high altitude areas, such as major mountain ranges, where temperatures permit. Permafrost can also form underneath oceans, and is called "subsea permafrost". It is formed when the balance between net heat lost to the atmosphere at the surface of the Earth and heat received at the surface from sources within the Earth produces negative ground temperature below the base of the active layer. The temperature balance at the ground surface is function of specific permafrost thermal regimen and can change between two different places that had different regimens, sometimes places that are nearby present at diverse thermal regimens. The factors that influence permafrost characteristics are numerous, including: solar radiation received or transmitted from the surface heat lost to the air or gained by convection or conduction; heat lost or gained by evaporation or condensation of surface moisture (Carlson, 1952; Brown, 1965). The thickness of permafrost is determined by geothermal gradient and by the mean annual ground surface temperature (Terzaghi,

1952). The depth of permafrost table is variable and changes especially when the ratio of cold temperature in winter exceeds the warm temperature in summer, because the lower temperature extend downward the permafrost table. If the factors controlling the heat balance at the ground surface remain unchanged, equilibrium between the heat lost to the atmosphere and that gained from sources within the Earth will be formed and the permafrost will be constant (Williams, 1970). In Figure 1.1 a typical temperature profile through permafrost, from the ground surface to the base of the permafrost, is shown. Higher temperatures are to the right and lower to the left; 0 °C is represented as a dashed vertical line. The heavier curves show normal conditions. The summer profile is to the right and indicates that above-freezing temperatures near the ground surface.

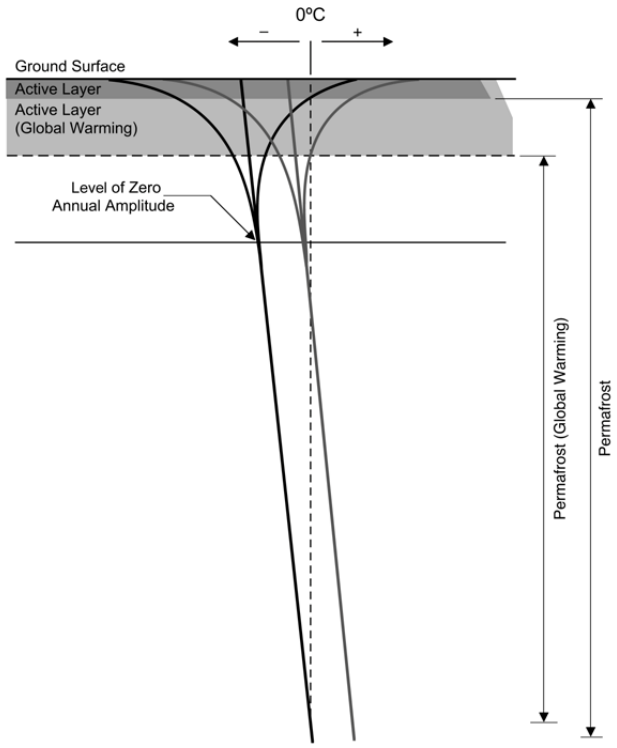

Figure 1.1 Ground temperature profile within permafrost (Permafrost Task Force report, 2003)

The winter profile to the left indicates the lowest temperature to the surface and the higher temperatures deeper in the permafrost. The summer and winter profiles intersect at depth were the external factors that don't influence the ground.

Another characteristic inside the permafrost is the water content. It may contain small amounts of liquid water whose quantities vary depending on the temperature, pressure, surface area of the soil or sediment particles, the mineralogy, chemical composition and packing arrangement of the soil particles, and the concentration, density and composition of the unfrozen fluid (Hivon and Sego, 1995). This fluid is adsorbed onto the surfaces of soil, or sediment particles, and is liquid even when the temperature is well below the initial freezing point. In non-saline soils, the amount of unfrozen water is a function of surface area and grain arrangement, while in saline soils particles are smaller and groundwater freezes and rejects dissolved salts into the remaining unfrozen liquid. This ground gradually becomes more saline and continues to decrease the freezing point until the eutectic composition is reached, at about 240 g L^{-1} and -22 °C (in the case of NaCl) (Biggar and Sego, 1993).

Permafrost composition

Permafrost table should be not considered as a unique layer. In fact, it is stratified in different strata with different characteristics and functions (Figure 1.2).

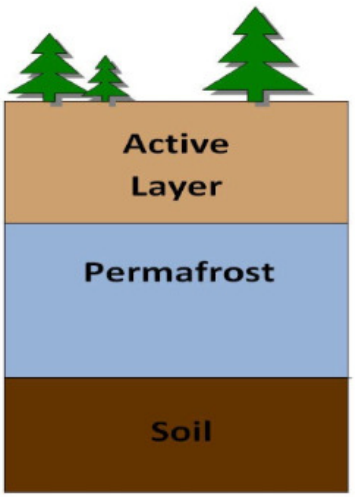

Figure 1.2 Permafrost layers diversification. It is possible to subdivide permafrost table in active layer (the first layer), permafrost and soil bottom (talik, cryopegs, etc.). (http://thetundra.yolasite.com/soil-profile-and-succession.php)

Active layer

The active layer is the portion of soil above the permafrost table. It is called "**active**" because its physic status is seasonally modified by thawing and freezing changes, thus becoming active in interconnection with the atmosphere. This layer plays an important role in cold regions because most ecological, hydrological, biogeochemical activities take place within it (Kane *et al*., 1991; Hinzman *et al*., 2003). Active layer thickness is affected by many factors, including surface temperature, physical and thermal properties of the surface cover and substrate, vegetation, soil moisture, snow cover duration and thickness (Brown *et al*., 2000; Frauenfeld *et al*., 2004; Zhang *et al*., 2005), and varies during the different seasons. When the other conditions remain constant, changes in active layer thickness could be expected to increase in response to climate warming, especially in summer. In spring, the active layer changes in physico-chemical composition and thickness due the thaw effect from few centimetres to meters. During summer the soil changes in humidity and stratification, even in unprecipitation area, because there is a separation between permafrost table and active layer, and therefore no connections are present in these two strata. Between these two layers could be another layer (**Talik**) that is imbalance index between permafrost and climate. The active layer is 30-50 cm in Arctic region and 1-3 m (maximum 5 m) in discontinuous or sporadic permafrost. The connection between active layer and permafrost table (due to temperature variation, water content and thermal imbalance) generates different geomorphological processes. In particular, in winter, the permafrost table will be frozen and does not create interconnection with the active layer.

Talik

Talik (or *cryopegs*) is a layer, body or channel of unfrozen ground in a permafrost area. Taliks may have temperatures above 0 °C (*non-cryotic*) or below 0 °C (*cryotic*, forming

part of the permafrost). Several types of taliks can be distinguished on the basis of their relationship to the permafrost (closed, open, lateral, isolated and transient taliks), and on the basis of the mechanisms responsible for their unfrozen condition (hydrochemical, hydrothermal and thermal taliks).

- The closed talik is a non cryotic talik occupying a depression in the permafrost table below a lake or a river, its temperature remains above 0 °C because of the heat storage effect of the surface water;
- The hydrochemical talik is a cryotic talik in which freezing is prevented by mineralized groundwater flowing through the talik;
- The isolated talik is a talik entirely surrounded by perennially frozen ground;
- The lateral talik is a talik overlain and underlined by perennially frozen ground and can be cryotic or non-cryotic;
- The open talik is a talik that penetrates the permafrost completely, it may be non-cryotic or cryotic.

Brines

Permafrost may contain small amounts of liquid water such as brine, which is a salt water basin within permafrost table formed during the winter season. The brine formation depends on the increase of carbonate, sulphate, sodium, magnesium and calcium concentration in groundwaters found under permafrost, combined to evaporation and halite dissolution processes (Alexeev and Alexeeva, 2002, 2003; Shouakar-Stash *et al*., 2007). When temperature comes down, frozen soil releases solutes in water, thus increasing the salinity of the brines. The formation of brine liquid pockets is due to the formation of veins into the permafrost and to its release of liquid and salt during thawing processes, thus creating an open network which provides viable aqueous habitats for microbes (Price, 2000; Mader *et al*., 2006). The extrusion of salts from sediments in a flat

topography may develop significant hydrostatic pressure which can result in the formation of pingos, froust mound and other structures where the surface is disrupted. For example, these features are generated from penetration by drilling (Mackay, 1978). The brine pockets represent also an oasis for the extant biota because they would have a high osmolality. Organisms inhabiting brine pockets must be able to withstand the osmotic pressure, generating peculiar substances that can protect organisms from desiccation (Brown, 1976; Yancey *et al*., 1982).

In Antarctica, two lakes (i.e. Lake Vanda and Lake Bonney) have been intensively studied for their salinity and unusual biology (Goldman *et al*., 1967). The east lobe of Lake Bonney contains a brine at 32.5 m depth with salinity of almost 300 g L^{-1} (Burton, 1981), previously attributed to the evaporative loss and capture of airborne aerosols and seaspray. The predominance of $CaCl_2$ waters in some lakes suggested that rock weathering and ion exchange are also important, combined with the loss of NaCl by precipitation at temperatures below –36 °C. Shvetsov (1941) was one of the first to draw attention to the association of saline groundwaters and brines with permafrost formation in polar regions. Since then, the abundance of saline waters in association with permafrost has been noted by several Russian scientists. Other study of saline waters in permafrost areas of the northern Russia include the existence of Mg-Ca-Cl brines, up to 100 g L^{-1}, in northeastern Siberia (Fotiev, 1983) where the depth of cryogenic beds is up to 1500 m. In addition, Chizhov (1980) described the existence and location of cryopegs in the Arctic and northern Siberia, discovered during exploration for minerals, oil and gas.

Moreover, most recently lenses of NaCl water brines with constant temperatures of -9 to -11 °C and mineralization of 170-300 g L^{-1} inside 100-120 thousand years-old marine permafrost have been described in the Arctic region (Gilichinsky *et al*., 2003). The temperature and salt presence could inhibit microorganism activity, but on the other hand they could be stimulated to develop peculiar adaptations to these habitats. The interest in

brines has increased because this extreme and cold environment is similar to environmental conditions retrieved on Mars, because they are both cold and dry climates and the development of soil is important in salt presence as brines (Wentworth *et al*., 2005; Marchant *et al*., 2009). As well as brines, permafrost is considered as an example of what life could be like on cold planets, including Mars (Jansson *et al*., 2014).

Types of permafrost

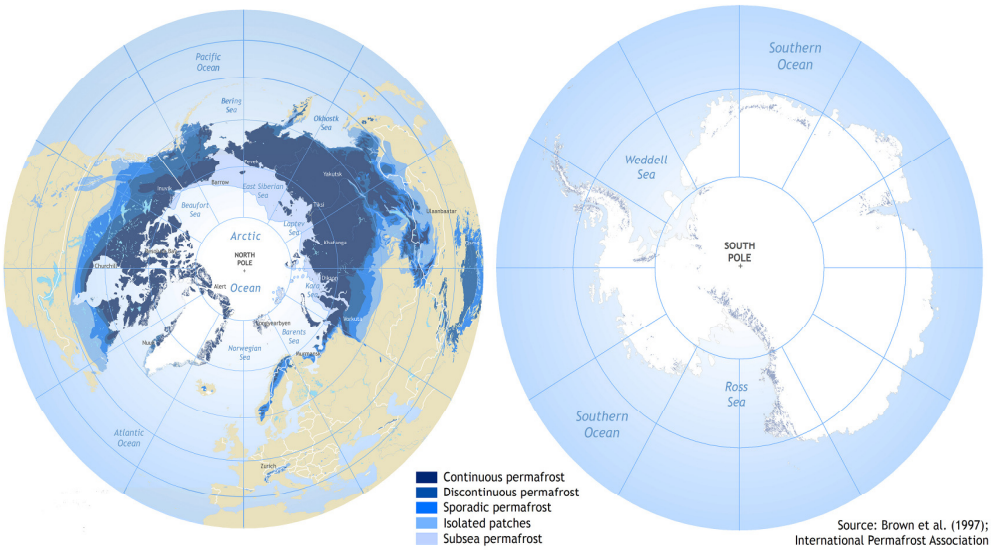

Figure 1.3 Permafrost global distribution and difiversification (Brown *et al*., 1997)

There are different types of permafrost:

- Continuous: it forms in areas with mean annual air temperature of -4 °C, independently from aspect and topographical influences;
- Discontinuous: it forms in areas with a mean annual air temperature of less than -2 °C, but more than -4 °C. As the air temperature alone is not enough to cause permafrost formation, this type requires some forms of topographical shading, usually mountains around the area that sheltered the spot. These features allow

the temperature to become constant and the wind to flow in this area with the characteristics required to form permafrost without low temperature;

- Sporadic: it forms in areas with a mean annual air temperature of less than 0 °C, but more than -2 °C. Sporadic permafrost forms as pockets of ice within peat and under existing ice. This sort of permafrost may also occur in high altitude mountain environments;
- Isolated patches: they form in areas that provide temperatures cold enough for ice production. These patches are often found in higher altitudes that exhibit variability in mean annual air temperature, but ultimately support ice retention in certain areas.

1.2 Antarctic permafrost

The Antarctic continent is the largest continent (14 million km^2) on Earth and in this area permafrost soil cover 49.000 km^2, about 0.35 % of the entire continent (Fox and Copper, 1994). The continent is circular and there are two massive ice sheets that dominate the topography: the *East Antarctic Ice Sheet*, with an average elevation of around 3.000 m, and the *West Antarctic Ice Sheet*, with an average elevation of around 1.500 m. The largest ice-free area is in the Transantarctic Mountains (23.000 km^2 estimate), which includes the Dry Valley region. The climate is severe with low mean annual temperatures, poor precipitation and rare presence of lichens and mosses. The exposed landscapes are dominated by glacial valleys with land surfaces and deposits that show the influence of glacial activity, which has extended from the Late Pleistocene to earlier than Miocene times (Denton *et al*., 1993; Marchant *et al*., 1993). Even if the physiochemical and meteorological characteristics are severe and extreme, in Antarctica there is a large biological diversity in soils and permafrost.

Temperature

There is a cold climate in Antarctica due the lower radiation (16 % respect the equatorial regions) and because there are same places that have an elevation near 4.000 m. The lowest temperature that was recorded is -89 °C at Vostok (3.488 m) and -49 °C at the South Pole. This air temperature increases nearest the coastal area: -25 °C at Mt. Fleming, -18 °C at McMurdo Station on Ross Island, -20 °C in the Dry Valley, -15 °C at Hallet Station. Obviously, the air temperature can influence the permafrost properties, with the variation of active layer thickness that ranges from 80 to 100 cm in the warmer coastal area and on average 2 cm in the high-elevation sites in the northern region. This wide variation depends on temperature, which is higher in summer and allow the thaw of the first layer of permafrost table with increase of the active layer thickness, while in winter the temperature is under 0 °C and the active layer thawed is little. The temperature can influence other factors in the permafrost formation, such as the thaw period, the number of thaw days during summer, the number of freeze/thaw cycles that occur and the time that the soil may be continuously above freezing. Two examples can explain this phenomenon. The first one is Marble Point, a site on the sea level (ca. 70 m above) that had the thaw period extended over 70 days, with 34 freeze-thaw cycles and 16 days with temperature continuously above 0 °C and, in contrast, Mt. Fleming, (2,000 m above the sea level) that had a thaw period over 31 days but only 6 days in which soil temperature is briefly above 0 °C.

Precipitation patterns and soil characteristics

The precipitation in Antarctica varies between the inland area and coastal area. In general, the mean annual precipitation is around 50 mm *per* year, with least falling inland and most in coastal locations. The precipitation normally falls as snow, and little is available for direct soil moistening because of evaporation. The quantification of falls

depends on the position of the sites, in the driest area the mean annual precipitation is 13 mm (McMurdo Dry Valleys), while near Lake Vanda (on the valley floor) the precipitation is 100 mm *per* year. In Molodezhnaya Station the precipitation is very high (650 mm *per* year; MacNamara, 1973).

Surface radiation balance is a characteristic that can influence the permafrost formation and properties, because it can change the soil with gains and losses of radiations, which vary during the seasons. In particular, the soil color is caused by albedo values, for example at Scott Base, in which albedo is approximately 5 %, the soil is dark-colored while in a site with a higher albedo the soil is light-colored.

Antarctica is formed from Precambrian (4600 million years) to Lower Paleozoic (251 million years) and is constituted by sandstone, siltstone and tillites, with some zones that are characterized by volcanic rocks. The permafrost and, in particular, the Antarctic permafrost is formed by weathering processes extend back to Miocene or earlier. In these areas glacial events play a role in till deposition. Two regions separated by Transantarctic Mountains constitute Antarctica, one is East Antarctic Ice Sheet and one is West Antarctic ice Sheet, which had different origins and stable period. The West Antarctic is affected by sea level and glacier changes that modify the composition of soils. The Antarctic soil biological environment is known from many studies (Gressitt, 1967; Cameron, 1971; Holdgate, 1977; Friedmann, 1982; Powers *et al*., 1995; Broady, 1996; Vishniac, 1996; Green *et al*., 1999) and its biodiversity is very low and can change due to climatic conditions. The community compositions depend on weather, temperature, light and water and these characteristics are more evident in coastal environments than within inland. Permafrost characteristics, that change from regions, alter the biodiversity during the year. The physical properties of Antarctic soils and permafrost are known from numerous studies since the 1960s, but principally from those of Ugolini (1964), Claridge (1965), Campbell and Claridge (1975; 1987; 2006), Claridge and Campbell (1977), Bockheim (1979), Blume *et al*. (1997) and Campbell *et al*. (1998). Antarctic soils are

coarse-textured, with coarse particles > 2 mm, and the color of soils diminishes in intensity with increasing depth because the lithology textural changes or because there is a salt accumulation. Furthermore, the shape of clasts varies between a younger sediment respects an old one, and the structure may depend on weathering and salt concentration becoming cohesive or structure-less. The Antarctic soils are everywhere underlined by permafrost, which can divided into ice-cemented or ice-bonded. This diversity changes the active layer depth in accordance to the mean annual temperature, from 1 m (in warmer area) to < 2 cm (in coldest area). Another type of permafrost is the dry permafrost that is distinguished by very low water content in both the active layer and the permafrost, which is non-cohesive. Permafrost often presents salty hallows and the associated soils are high saline. In summer months active layer contains brine, usually at a temperature several degrees below 0 °C, while the soil is characterized by abundant efflorescence of soluble salts. The distribution of this kind of permafrost is connected to weather. In fact, ice-bonded permafrost is generally found in coastal regions, whereas dry permafrost in inland, for example in the Dry Valleys. The permafrost soils showed some chemical characteristics that are bonded to the texture of permafrost, for example soils with very small particles are the most chemically reactive. The nature of minerals inside the soils can change the pH of the sediments. Near the coast (young soil), where the winds from the sea carry ocean-derived salts, the soil is constituted by chlorides and sulphates of sodium and the soil is alkaline (up to pH 9, in some cases). Further, in inland (old) soils the salts are accumulated in horizons and there are some chemical processes that made a low pH value. In other cases, weathering comprises the breakdown of the ferromagnesian that releases iron and cations causing the reddish staining of soil for the oxidation. For all these conditions the weathering processes play an important role in terrestrial ecosystem making it extremely fragile. Chemical contaminations may also persist in the absence of significant leaching and permafrost is altered when physical disturbances take place.

2. Permafrost as a microbial habitat

Permafrost is considered as an extreme environment due to its physiochemical features, namely low temperature, oligotrophic nature of sediments and water availability. Microorganisms that live in this environment are generally psychrophilic, which had successfully colonized all cold environments. These organisms, based on their optimum temperature of growth, can also be psychrotolerant or psychrotroph (Morita, 1975). Their psychro-nature allows psychrophilic microorganisms to have success in performing biochemical reactions and growing at temperatures around the freezing point. Even if this environment is extreme, it presents a lot of microbial abundance and diversity. Furthermore, even if permafrost environment is considered as an extreme and inhospitable habitat a significant number of viable ancient microorganisms are known to be present within. They have been isolated from both polar regions and cores up to 400 m deep and ground temperatures of -27 °C. Microorganisms are the only life forms known to have retained viability over geological times. Thawing of the permafrost renews their physiological activity and exposes ancient life to modern ecosystems (Margesin, 2007). Thus, the permafrost represents a stable and unique physiochemical complex, which maintains like incomparably longer than any other known habitat. If we take into account the depth of the permafrost layers, it is easy to conclude that they contain a total microbial biomass several orders of magnitude higher than that of the soil cover. This great mass of viable matter is peculiar to permafrost only. The ability of microorganisms, the most ancient life forms on the Earth, to live in a variety of natural environments continually forces us to redefine the limits of life in the biosphere. Microorganisms not only have adapted to the cold and populate the main ecological niches, but also survive under conditions that seem absolutely unsuitable for life in large populations and a high diversity.

Many studies have been carried out to understand this particular microbial habitat. Recently the regeneration of ancient giant DNA from viruses (Legendre *et al*., 2014) and moss species (Roads *et al*., 2014) from permafrost was investigated. These examples highlight the recognized potential for the preservation of microbial life (that is, Bacteria, Archaea and Fungi) in a dormant state in permafrost (Gilichinsky *et al*., 2008). The heterogeneity of permafrost, in horizontal, vertical soil or sediment texture, gives a differentiation between this and other low-temperature environments. Probably the closest correlation is to deep marine sediments, which are cold and vertically stratified with respect to oxygen and carbon content. The potential of life in permafrost must suggest an evolutionary perspective.

According to the 'Snowball Earth' hypothesis, the Earth has been completely frozen one or more times as recently as 650 million years ago (Kirschvink *et al*., 1992), so microorganisms would have had to be able to adapt to sub-zero temperatures to continue to live on our planet. In general, there is considerable variability in the diversity and composition of microorganisms that are found in permafrost.

2.1 Limits for microbial life in permafrost

The continual sub-zero temperatures and the main physical-chemical parameters of permafrost ensure the formation of microbial communities that adapt themselves to the abiotic parameters of the habitats and virtually provides invariable DNA conservation for million of years. It may be suggested that the mechanisms of such adaptation are universal and operate within the broad limits of modification for natural ecosystems on and beyond the planet Earth. In particular, temperature plays an important role as regulator of all physicochemical reactions and biological processes (Herbert, 1986). Cold temperatures (below 0 °C) allow the preservation of biological systems contributing to long-term survival of cells. In this way, sub-zero temperatures become a stabilizing factor that supports the viability of microorganisms rather than creates limits to life. For

example, in the Dry Valleys the maximal mean annual permafrost temperature is -18 °C near the coast. This temperature decreases when moving to inland and higher altitudes, reaching the lowest temperature on Earth (i.e. -24 to -27 °C). With these temperature ranges permafrost is characterized by a multiphase state of water, which plays a dual role from a biological point of view (Gilichinsky *et al*., 1992). The sub-zero temperature transforms water in ice and the percentage is 92-97 % of total water volume in permafrost. Ice can be considered as a cryo-conservant. The small percentage of water in liquid phase is 3-8 % and often it constitutes a film or it is conserved in brine pockets within permafrost (Anderson, 1967). Among other parameters the unfrozen water plays the leading role in the preservation of microorganisms (Gilichinsky *et al*., 1992). The validity of unfrozen water as a main ecological niche can be demonstrated by comparison between the numbers of viable cells recovered from permafrost and ice cores. In contrast to frozen soils with an abundance of microorganisms (Gilichinsky *et al*., 1995b; Vorobyova *et al*., 1997; Khlebnikova *et al*., 2000), the viable cells recovered from cores of the pure ice of the Antarctic Ice Sheet taken at the Vostok station (Abyzov, 1993) is an order of a few dozens per 1 mL of thawed water and increases with increasing concentrations of dust particles in the core (Abyzov, 1998). Another physic-chemical characteristic is the composition of the gases that are different from the atmospheric air. The pore space of frozen strata is occupied by oxygen, nitrogen, methane, carbon dioxide, etc. The oxygen and nitrogen is not more different from air respect to methane and carbon dioxide that are higher than atmosphere.

Freezing tolerance is often accompanied by tolerance to dehydration caused by a lack of free water at low temperatures or high salinities. Dehydration, caused by the high brine salinities, is a major stressor for ice-trapped organisms, which may experience salinities three times higher than in seawater. In contrast, when the ice melts the released organisms will be suddenly exposed to hyposaline conditions, close to freshwater values.

Even ultraviolet radiations make a limits to life, since the 1980s significant research efforts have been devoted to study the effects of increased ultraviolet radiation exposure occurring during the seasonal thinning of the ozone layer (Vincent, 1993; Prézelin, 1998). This increase of UV radiations could induce damage to RNA transcription and DNA replication. Organic compound contents in permafrost soils are heterogeneous. Soils in Antarctica are generally oligotrophic, though soils in close proximity to available water, coastal soils have higher-organic C content. Studies about permafrost involve the investigations of surface soils and report on the amount of microbial biomass that are similar to those in temperate soils (i.e. 10^6-10^8 cells g^{-1} wet soil) (Cowan *et al*., 2002; Lee *et al*., 2012). If there is a comparison between Antarctic permafrost and Arctic permafrost, it appears that relatively low amount of microbial biomass are present in Antarctic permafrost. The cells present in permafrost depend on the ability to adapt themselves to these limits with the adoption of adaptation strategy.

Microbial adaptation

"Cryophiles" is a new definition of microorganisms isolated from permafrost that are capable of growth and reproduction at low temperatures, typically ranging from -17 °C to +10 °C (Feller *et al*., 2003). Panikov *et al*. (2006) reported the lowest temperature limit for bacterial activity in permafrost soils as -17 °C. So, temperature and water availability are the most important factors to determine the biomass levels, bacterial diversity and community structure in low-temperature environments in general (Pointing *et al*., 2009; Yergeau *et al*., 2009; Zeglin *et al*., 2009). Microorganisms have evolved several strategies to survive at sub-zero temperatures, for example the regulation of membrane fluidity, the production of cold-adapted and antifreeze proteins or the cold-shock and cold-acclimation proteins. The adaptations to cold temperature allow to facilitate the motions of essential membrane proteins and not lose the membrane integrity. Also the proteins may allow to minimize the formation of secondary structure avoiding to

compromise translation efficiency and regulation of transcription during the low temperature periods. Furthermore some proteins lowering the freezing point of water inhibits the formation of large ice crystals, thus protecting the bacterium from freeze-damage. In particular:

- Membrane fluidity: The low temperature makes changes in physical properties and functions of membranes. One of the most features that is affected during lowing temperature is the reduction in membrane fluidity, gel-phase transition and a loss of functions. The lipid composition governs the physical properties of membranes and, in general, lower growth temperatures produce a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids. This altered composition is thought to have a key role in increasing membrane fluidity by introducing steric constraints that change the packing order or reduce the number of interactions in the membrane.
- Transcription and translation: Activity of transcriptional and translation enzymes are reduced during freezing status at low temperature generating the formation of stabilization features of DNA and RNA secondary structures. The adaptation is the modification of the structure of polymerase or other enzymes. For example, in *Escherichia coli* the CspA-related proteins are important for the destabilization of DNA and RNA secondary structure and are also overexpressed at low temperature in psychrophiles (Berger *et al*., 1996; Lim *et al*., 2000).
- Cold-shock responses: The production of cold-shock proteins (Csps) is involved in various cellular processes such as transcription, translation, protein folding and the regulation of membrane fluidity (Phadtare, 2004). In psychrophiles these proteins are constitutively expressed respect to mesophiles.
- Antifreeze proteins and cryoprotectants: Antifreeze proteins (AFPs) have the ability to bind the ice crystals outside the cells. In this way they lower the freezing point of ice formation allowing the inhibition of recrystallization and

thereby create thermal hysteresis, lowering the temperature at which an organism can grow (Jia and Davies, 2002). AFPs have been detected in Antarctic lake bacteria (Gilbert *et al*., 2004). Production of exopolysaccharides (EPSs) might also have an important role in cryoprotection in psychrophiles. High concentrations of EPSs have been found in Antarctic marine bacteria (Nichols *et al*., 2005) and in Arctic winter sea ice bacteria (Krembs *et al*., 2002). They modify the physico-chemical environment of bacterial cells, participate in cell adhesion to surfaces and retention of water, favor the sequestration and concentration of nutrients, retain and protect extracellular enzymes against cold denaturation and also act as cyoprotectants (Mancuso-Nichols *et al*., 2005).

- Cold-adapted enzymes: Bacteria adapted to cold temperature produce enzymes that have high specific activities, often up to an order of magnitude higher than those observed for their mesophilic counterparts (Russell, 2000; Feller and Gerday, 2003; Georlette *et al*., 2004). The psychrophilic enzymes increase the flexibility of their structure to compensate for the "freezing effect" of cold habitats (Johns and Somero, 2004). This increase in flexibility might concern the entire protein or might be restricted to parts of the structure especially those implicated in catalysis, and it is probably also responsible for the generally observed low stability of cold-adapted proteins (Collins *et al*., 2003: D'Amico *et al*., 2003).

2.2 Microbial biodiversity in Permafrost

Previous studies on permafrost

In 1911, Omelyansky carried out the first investigation on the presence of viable microorganisms in permafrost. Kris (1940) found low numbers of microorganisms below the interface between the active layer of soil and the permafrost, and fewer or no microorganisms at deeper levels in the Russian Arctic on Koluchin and Wrangel Islands. Subsequently, from the 1950s to the 1970s, a significant amount of work was done on the microbial flora in Arctic soils and in permafrost soil. The studies on permafrost have first focused to determine if viable microorganisms could be isolated (James and Sutherland, 1942; Boyd and Boyd, 1964; Horowitz *et al*., 1972; Cameron and Morelli, 1974). These pioneering investigations revealed that both significant numbers and varieties of viable microorganisms were present in permafrost from several Arctic and Antarctic sites. Isolated organisms included aerobic heterotrophs, anaerobic bacteria, nitrogen-fixing bacteria, sulphur-oxidizing bacteria and sulphur-reducing bacteria. Unfortunately, these first studies had some methodological and technical failures, and the results have never been confirmed. Early Russian studies reported aerobic heterotrophic plate counts in 87 % of samples from nine Siberian permafrost collected in bore holes (Gilichinsky *et al*., 1989). In the 1980s, Russian scientists began developing sampling methods using fluid-less drilling and bacterial tracers to ensure that samples were uncontaminated (Khlebnikova *et al*., 1990; Shi *et al*., 1997). Gilichinsky *et al*. (1988; 1989; 1990a; 1992; 1993a) analysed the north-eastern part of Siberia where permafrost have persisted for several millions of years. Even if permafrost is commonly thought to be unfavorable for maintaining the viability of organisms, their studies showed that samples contain not only organic matter but a large numbers of viable microbes (100 million *per* gram of soil), probably preserved for millions of years. In 1993 there were the first reviews of these

studies published by Zvyagintsev (1993) and Gilichinsky (1993b) reporting on viable microorganisms that were isolated from permafrost throughout the cold region of the earth. Different morphological and physiological groups were isolated including fungi, yeasts and actinomycetes. The biodiversity and number of viable microorganisms were independent on the depth and the permafrost microorganisms were not in a state of cryoanabiosis (frozen resting state) and maintained vitality in a frozen state. Isolation of organisms from cryo-environments has proven technically challenging. In the year the technologies had evolved, Juck *et al*. (2005) developed a new methodology to verify the uncontaminated drill samples. This methodology is in two steps using fluorescent microspheres during drilling and a green-fluorescent protein-marked *Pseudomonas* strain to culture-dependent and independent analyses. In this way it is possible to detect contaminated nucleic acids in core samples. The microorganisms isolated from permafrost are extremophiles, in particular they tend to be psychrotrophic rather than psychrophilic. This characteristic allows to describe them as "a community of survivors" (Friedmann *et al*., 1994), which have overcame the combined action of extremely cold temperature, desiccation and starvation. Under these conditions, the starvation-survival lifestyle is the normal physiological state (Morita, 1997). According to their growth temperature (Morita, 1975), the microbial community, even after its long-term existence within permafrost, is not composed of psychrophilic bacteria (Gounot, 1986). However, most of the isolates cells did not grow at temperatures higher than +30 °C, but were often capable of growth at sub-zero temperatures as low -10 °C in the presence of cryoprotectants such as glycerol (Gilichinsky, 1995a). It is interesting to note that Arctic and Antarctic sediments contain about the same number of microorganisms (estimated by direct counts using epifluorescence microscopy; Vorobyova *et al*., 1997), although the Arctic sediments are rich in organic carbon, whereas in the Antarctic ones the content of carbon and unfrozen water is close to zero. The number of microbial cells is relatively constant from modern to the oldest frozen layer. Overall, a number of different

morphological and physiological groups of microorganisms (spore forming and spore-less, aerobic and anaerobic, prokaryotic and eukaryotic) have been found. Morphologically, they are coccoid, coryneforming, nocardia and rod-like Gram-positive or Gram-negative bacteria. The ancient permafrost microbial community is predominantly bacterial, as the community in the depths of Antarctic Ice Sheet (Abyzov, 1993). This is in contrast with modern soils, where the fungal mass is much greater than the bacterial mass. Eukaryotic cells, while present, seem less able to survive to long-term cryopreservation (Soina *et al*., 1995). In Antarctic permafrost spore-formers dominate the habitats, thanks to the resistance of stressful factors, and recently some phototrophic microorganisms were isolated including cyanobacteria that use light of almost all wavelengths to synthesize organic material, green algae and mosses representing the lower plants, and even Protozoa from buried Arctic soils. Furthermore, gas presence in permafrost soils allowed the growth of anaerobic microorganism and the ratio between aerobic and anaerobic microorganisms is different in different geological facies. Rivkina and collaborators (1998) analysed the anaerobic presence (methanogens, denitrifyiers, sulphate-reducers and iron-reducers) in swamp and lagoon soils and their number was several orders of magnitude higher than aerobes. These data are related to permafrost age. For the aerobic microorganisms, the ratio of culturable bacteria is determined by the extent and duration of exposure to sub-zero temperature (Vorobyova *et al*., 1997). The recoveries of cells were inhibited by stress of thawing or exposure to oxygen rendering cells died. These disadvantages allowed the development of techniques for the yield of viable microorganisms (Vishnivetskaya *et al*., 2000). The phylogenetic diversity of the permafrost community has been addressed only recently by using DNA-based techniques. The first molecular data were reported in 1997 when Shi *et al*. (1997) obtained viable microorganisms from ancient permafrost samples collected in Kolyma-Indigirka lowland located in Siberia. Phylogenetic analysis revealed that the isolates felt into four phyla or classes: high-GC Gram-positive bacteria, *Betaproteobacteria*, *Gammaproteobacteria* and

low-GC Gram-positive bacteria. Shi *et al*. (1997) revealed that the microbial community structure depended on the permafrost age. In that study, high-GC Gram-positive bacteria, *Betaproteobacteria* and *Gammaproteobacteria* were retrieved from sample with an estimated age of 1.8-3.0 million years, while most low-GC Gram-positive bacteria came from sample with an estimated age of 5,000-8,000 years. Culture-independent methods were increasingly used in environmental microbiology bypassing the culturable studies by extracting and analysing total nucleic acids and theoretically representing the entire microbial population from environmental samples (Spiegelman *et al*., 2005). Steven *et al*. (2007a) used 16S rRNA gene clone library analyses to determine the microbial diversity present within a geographically distinct high Arctic permafrost sample. The use of clone libraries allowed to have a larger framework of microbial community inhabiting permafrost samples. In recent years, data based on high-throughput sequencing of 16S rRNA genes is becoming increasingly available for a range of permafrost and active-layer samples (Steven *et al*., 2007b; Steven *et al*., 2009; Yergeau *et al*., 2010; Mackelprang *et al*., 2011; Taş *et al*., 2014). The resulting information about the composition of the microbial community reflects the unique and extreme conditions of the permafrost environment. The following paragraphs will describe the main results from the studies cited above.

Abundance and diversity of prokaryotes in permafrost

Direct counts of total prokaryotic counts as determined by epifluorescence microscopy tend to be similar between most permafrost samples, ranging from 10^7 to 10^9 cells g^{-1}. Comparing Antarctic and Siberian permafrost from different studies it is possible to note some similarities/differences (Vorobyova *et al*., 1997; Shi *et al*., 1997; Spirina *et al*., 2003; Vishnivetskaya *et al*., 2005; Gilichinsky *et al*., 2007). Abundance and diversity of microbes inhabiting permafrost are very high. The total cell abundance counted by epifluorescence microscopy was 10^5-10^6 cells g^{-1} dry mass in Antarctica (Gilichinsky *et*

al., 2007) and 10^7-10^8 cells g^{-1} dry mass in Siberian (Vorobyova *et al*., 1997) permafrost. The number of bacterial cells that grew on nutrient media was < 0.1 % (Antarctica) and 0.1-1 % (Siberia) of the abundance counted by epifluorescence microscopy. Microscopic investigations of microorganisms *in situ* showed the presence of degraded cells or empty cells (Dmitriev *et al*., 2000; Soina *et al*., 2004), due to the constant sub-zero temperature that compromise microbial cell structure and integrity (Steven *et al*., 2009). Intact cells in permafrost showed an altered ultrastructure such as a non homogenous cytoplasm with numerous aggregates (Soina *et al*., 1995, 2004). Hansen *et al*. (2007) analysed Spitsbergen permafrost using different staining and microscopy methods and found that 74 % of microbial community presented compromised cell walls and were considered non-viable. Electron microscopy investigation in Arctic and Antarctic permafrost showed a wide range of morphologies, cocci and rods, and Gram-positive and Gram-negative bacteria. In terms of size, permafrost samples present cell population less than 1 μm in size (Dmitriev *et al*., 2000; Soina *et al*., 2004). That small cell size is predominant in cryo-environments, including permafrost (Steven *et al*., 2006).

Culturable microorganisms typically represent less than 1 % of the total microbial community in soils. This percentage is lowering in Antarctic permafrost samples, becoming only 0.001-0.01 % of viable cell recovery (Vorobyova *et al*., 1997). The ability to recover viable cells from permafrost seems to be independent on permafrost temperature or depth, but depends on the age of permafrost (Steven *et al*., 2008). With increasing age, both number and diversity of bacterial isolates decrease, with an increase in the number of sterile samples (Gilichinsky *et al*., 1989, 1992; Khlebnikova *et al*., 1990). Obviously, the presence of ice and salt brine can change the amount of viable microorganisms in permafrost sample, with a number that is often reduced. The recovery of isolates near ice system has been rarely reported (Gilichinsky *et al*., 1995b; Gilichinsky *et al*., 2002b). Therefore the origin, age and physicochemical characteristics of permafrost can determine the presence of abundance of a viable microbial community.

Several studies investigated the viable anaerobic and aerobic bacteria, spore-less and spore-forming bacteria, halo-tolerant and halo-philic and psychrophilic and psychrotrophic microorganisms inhabiting permafrost. For heterotrophic bacteria the range is 10^3 cells g^{-1} in High Canadian Arctic permafrost (Steven *et al*., 2004) and Antarctic permafrost (Horowitz *et al*., 1972; Cowan *et al*., 2002), 10^8 cells g^{-1} in Siberian permafrost (Gilichinsky, 2002b), and 10^6 cells mL^{-1} in cryopegs water and Antarctic lakes (Gilichinsky *et al*., 2003; Cowan and Ah Tow, 2004). The Siberian permafrost is the most studied site respect to Antarctic permafrost. Viable microorganisms were previously harvested from deep permafrost (Gilichinsky *et al*., 1989; Zvyagintsev *et al*., 1990) and probably their abundance and diversity depended on the type of soil and age of permafrost below zone of annual melting (Zvyagintsev *et al*., 1985). Rivkina *et al*. (1998) analysed the viable community of Siberian permafrost using media allowing to counts different groups of anaerobic bacteria. In particular methanogens, sulphate reducing bacteria, denitrifiers and ferric reducers bacteria were targeted. The range of viable counts was 10^3-10^5 CFU g^{-1} for denitrifiers and 10^7 CFU g^{-1} for methanogens. These data demonstrated that in permafrost there are organic carbon and other compounds trapped inside and these microorganisms retain viability in extreme conditions, increasing biological interest as potential source of greenhouse gas. In Antarctic permafrost viable cells of methanogens were 10 CFU g^{-1} of sediments less than in Arctic samples (Gilichinsky *et al*., 2007). This finding was due to the different soil composition that is more nutrient rich in the Arctic than in Antarctica. In general, the isolation of viable microorganisms from extreme environments provides a technical challenge. The culturable methods play a stronger role in the selection of organisms and it is important to identify the isolation protocols that optimize the recovery of genetically diverse bacterial lineages. Vishnivetskaya and collaborators (2000) tested three different plating methods and different concentrations of nutrients in the media for the recovery of microorganisms: direct plating of diluted cell suspensions on agar media, enrichment in liquid media

before plating and enrichment of natural permafrost sediments (NPS). The results showed a higher plate counts in diluted media than full-strength media; although bacterial yield was higher in less-diluted media, morphological diversity resulted greater in rich media, suggesting that medium composition could affected the recovery of microorganisms. NPS enrichment appeared to be the best method for the recovery of morphological diverse bacteria.

Phylogenetically, these isolates came from Antarctic and Arctic permafrost and include at least 70 genera (Shi *et al*., 1997; Vorobyova *et al*., 1997; Bakermans *et al*., 2003; Bai *et al*., 2006; Vishnivetskaya *et al*., 2006; Gilichinsky *et al*., 2007; Katayama *et al*., 2007; Steven *et al*., 2007a,b; Zhang *et al*., 2007). Both Gram-positive and Gram-negative cells are represented, and spore-forming bacteria are also commonly isolated. Until now, our knowledge on viable bacteria diversity is poor for Antarctic environments. In general, culturing permafrost bacteria on low-nutrient media has yielded the highest number of isolates (Goordial and Whyte, 2014). The isolate diversity is better investigated in Arctic permafrost rather than in the Antarctic one. Bacterial genera such as *Bacillus*, *Arthrobacter*, *Micrococcus*, *Cellulomonas*, *Rhodococcus*, *Flavobacterium*, *Pseudomonas*, *Aeromonas*, *Myxococcus*, *Exiguobacterium*, *Nitrobacter*, *Nitrosomonas*, *Nitrosospira*, and *Streptomyces* have been isolated from Arctic permafrost (Gilichinsky *et al.*, 1995b; Shi *et al*., 1997; Vorobyova *et al*., 1997), while *Arthrobacter*, *Bacillus* and *Streptomyces* were predominant in Antarctic permafrost (Vorobyova *et al*., 1997). Most permafrost isolates belonging to *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* have been isolated. The predominance of these isolates is due to their capability to survive in extreme environments and respond to stress by adapting themselves. *Firmicutes* produce spore that are resistant in cold environments and for this reason they are highly represented in culture (Steven *et al*., 2008; Niederberger *et al*., 2009). In addition, soil *Actinobacteria* have a high GC content that reduces DNA damage by temperature and high salinity stresses (Johnson *et al*., 2007).

The depth or temperature do not influence the strain recovery but influence the bacterial diversity. A variety of aerobic, anaerobic, spore-forming and non-spore forming, methanogens, acethogens, denitrifiers, sulphur reducing, psychrophilic and psychrotrophic bacteria have been isolated from permafrost and several recent studies report on their isolation from 120.000 years-old permafrost. An aerobic *Modestobacter multiseptatus*, belonging to *Actinobacteria* and able to grow on succinate or malate, was isolated from Antarctica at 1600 m depth. Such strain had an optimum temperature for growth between 19-22 °C (Katayama *et al*., 2009). An *Exiguobacterium* strain, belonging to *Firmicutes*, was isolated from old permafrost samples (3 million years) in Kolyma Lowland (Russia) at 43.6 m depth (Vishnivetskaya *et al*., 2005). In another study, six *Carnobacterium* strains were isolated from Siberian permafrost (6000-8000 years old) and showed to grow at 0 °C under low pressure and CO_2 anoxic conditions (Nicholson *et al*., 2013).

With regard to archaeal diversity, studies remain mostly limited to Arctic permafrost and only a few deals with Antarctic permafrost. Differently from bacteria, methanogenic Archaea occurred at low numbers and not in all samples (Rivkina *et al*., 1998, 2002). The focus study, in the last decade, has moved the attention on the global change and methane emissions. Permafrost is a reservoir of ancient CH_4 that is released, with other compounds, during seasonally thawing. With the methane presence methane-producing Archaea that are preserved in the permafrost were found (Rivkina *et al*., 1998). Biogenic methane is formed by biochemical reactions of a group of strictly anaerobic methanogenic Archaea (Ferry, 1993, 2001; Zinder, 1993). Carbone dioxide formation was found in Antarctic Dry Valley permafrost and may be attributed to microbial activity (Gilichinsky *et al*., 2007).

The extreme characteristics of permafrost samples allow to isolate microorganisms, like as bacteria, archaea, cyanobacteria, eukaryote, using nutrient poor-media (Gilichinsky *et al*., 1989; Bai *et al*., 2006; Steven *et al*., 2007a). Ice formation, salt concentration, gases,

chemical compounds, low temperature, and water availability could influence the viability and composition of organisms. Low temperature, rather than other characteristics, influences microbial life and different studies focused on microbial activity under temperature below 0 °C. The introduction of new cultivation procedures allowed focusing studies on low temperature activity, using cryo-protectants, artificial anti-freezers (i.e. ethanol) and incorporation of labelled precursors into culture to assay microbial vitality and activity. The lowest temperature at which microbial reproduction was demonstrated was -12 °C in an Arctic sea ice *Psychromonas ingrahamii* (Breezee *et al*., 2004). From another point of view, permafrost is an ideal environment for studying bacteria capable of sub-zero temperature growth because of the low temperature condition remains constant for a long geological time. Microbial growth or metabolic activity have been reported in permafrost bacteria at -10 °C (Gilichinsky *et al*., 1995) and in the Antarctic cryptoendolothic microbial community at temperature between -5 and -10 °C (Vestal *et al*., 1988; Friedmann *et al*., 1993). Using resazurin Bakermans *et al*. (2003) demonstrated that there was activity at -10 °C, by non-spore forming isolates, through cell division. Another study demonstrated that there was activity at temperatures from 5 to -20 °C with microbes that were able to incorporate ^{14}C-labeled acetate into lipids (Rivkina *et al*., 2000).

Many microorganisms represent potentially novel microbial species or genera (Bakermans *et al*., 2003; Ponder *et al*., 2005; Bai *et al*., 2006; Rivkina *et al*., 2007; Steven *et al*., 2007a, 2008a,b) and understanding their activity under low temperature, nutrient and other extreme parameters could allow us to know the adaptation mechanisms involved in survival strategies under physical limitations of terrestrial life compared to the extra-terrestrial one.

2.3 Phenotypic characteristics of permafrost isolates

As stated above, the recovery of viable cells from Arctic and Antarctic permafrost samples is generally facilitated by using nutrient-poor media (Gilichinsky *et al*., 1989; Bai *et al*., 2006; Steven *et al*., 2007a), suggesting that permafrost communities are primarily oligotrophic. However, organic carbon content appears to be particularly abundant in Arctic permafrost (Vishnivetskaya *et al*., 2000; Gilichinsky, 2002a; Steven *et al*., 2006).

Soil physical features, as porosity, organic carbon content and movement of liquid water, could affect microbial abundance, activity and physiological characteristics. The pore sizes of soil generate a water flux increasing availability of organic compounds (Kaiser and Bollag, 1990), while the ice formation limits the carbon, selecting oligotrophic microbial populations.

Ice formation and brine presence generate an increase in salt concentration, which might cause in turn the increase of halotolerant microorganisms in permafrost rather than organisms from active soil layer (Gilichinsky, 2002a; Steven *et al*., 2008). This is a survival strategy in environments with low water activity where low amount of water is bioavailable (Franks *et al*., 2003). Microorganisms inhabiting permafrost are capable of growth at sub-zero temperatures and have been isolated (Ponder *et al*., 2005; Bai *et al*., 2006; Steven *et al*., 2007a, 2008), this suggests the potential for growth and metabolism at the ambient sub-zero temperatures in permafrost.

Interestingly, stress could generate bacterial response combined to mobile genetic elements (Margesin *et al*., 2011) in relation to antibiotic resistance. Metagenomic analysis of ancient DNA from 30.000-years-old Beringian permafrost demonstrated the presence of a highly diverse collection of genes encoding for resistance to Beta-lactam, tetracycline and glycopeptide antibiotics (D'Costa *et al*., 2011).

2.4 Biotechnological potential of cold-adapted microbes

Microorganisms isolated from cold environments adapt their enzymes (amylase, protease, lipases, pectinase, cellulase, etc.) to permit them to survive and function in extreme habitats. Despite their high potential of applications in fundamental and applied fields, microorganisms isolated from cold environments have received little attention especially in comparison to thermophiles. Advantages about the biotechnological potential in ecological and economical values may not yet have been realized sufficiently. The application of cold-adapted microorganisms offers numerous advantages. For example high microbial growth rates as well as high enzymatic activities and catalytic efficiencies in the temperature range 0-20 °C prevent the risk of microbial contamination and quality of products is not affected by a low or moderate temperature treatment. Cold enzymes have high catalytic efficiency at low and moderate temperatures, low activation energy and high activity in biosynthetic processes. The low temperature stability of cold-active enzymes has been regarded as the most important characteristics for use in the industry. In few years, the interest has changed and more researchers are fascinated to focus the use of cold-adapted organisms in many fields, such as clinical, medicinal and analytic chemistry, as well as their widespread biotechnological and industrial applications such as food processing, additive in detergents and food industries, wastewater treatment, environmental bioremediation in cold climates, biotransformation and molecular biology applications.

Biotechnologies in industry

The biotechnological application includes the food processing, that uses low temperature to minimize undesirable chemical reactions and bacterial contamination. In the milk industry, beta-galactosidase is used at low temperature to reduce the amount of lactose, responsible for severe induced intolerances in approximately two thirds of the world's

population; in the fruit juice industry the use of pectinases helps the juice extraction process, reduces the viscosity and helps to clarify the final product; in the meat industry, proteases help to tenderize the meat.

Proteases have found extensive application also in the textile industry. Cold-adapted cellulases is useful for biopolishing, stone-washing process and reduces the pill-formation and increases the durability and softness of the tissues. Cleaning applications use enzymes to hydrolyse substrates, including laundry and dishwaters (Aehle, 2006), and are useful to improve efficacy and environmental sustainability (Laugesen, 2010; McCoy, 2011). Enzymes from psychrophiles, such as proteases from *Serratia rubidaea* and *Stenotrophomonas maltophilia* (Doddapaneni *et al*., 2007; Kuddus and Ramteke, 2009) and an amylase identified by metagenomic screening of glacial water (Sharma *et al*., 2010), are the types of enzymes that have potential to extend the effectiveness of enzyme-based, low-temperature cleaning formulations. A cold-active lipase, obtained by cloning a lipolitic gene from an Arctic bacterium (*Rhodococcus* sp. AW25M09), has been found to retain its activity at high pH, organic solvents and salt concentration. Thus it appears to be an interesting candidate for industrial applications (De Santi *et al*., 2014). A number of cold-adapted strains of *Pseudomonas* are known to produce lipases, which have high activity at moderate or low temperatures. Other molecules of bacterial origin are the Antifreeze Proteins (AFP), produced for the lowering of the freezing point in the water surrounding the bacteria, and used in biotechnologies for the food industries, such as in the ice-cream production, in order to avoid the ice crystal formation, or in medicine, for the transportation of organs and tissues.

Antimicrobial potential

In the last years the disproportionate use of antibiotics against pathogens caused the development of resistance to the most commonly used antibiotics. For this reason, the researcher attention has been focused on the detection of alternative antibiotic sources.

Bacteria isolated from polar regions are considered potential candidates as new antimicrobial compound producers. Bacteria isolated from Antarctic sponges were analysed for their ability to produce antimicrobial compounds active against cystic fibrosis opportunistic pathogens belonging to the *Burkholderia cepacia* complex (Bcc) (Papaleo *et al*., 2012). Antimicrobial activities are influenced by the concentration of high number of bacteria in a small niches, as Mangano *et al*. (2009) reported by analysing bacterial interactions between strains isolated from Antarctic sponges. The percentage of active bacteria was higher than that reported by Lo Giudice *et al*. (2007a,b) for bacteria isolated from seawater. Further studies focused on the volatile organic compounds (VOCs) produced by Antarctic bacteria. Solid phase micro extraction gas-chromatography mass-spectrometry (SPME-GC-MS) analysis revealed that Antarctic bacteria constitutively synthesize a large set of mVOCs (Romoli *et al*., 2011). More interestingly, the activity of the mVOCs appeared to be more effective in inhibiting the growth of Bcc bacteria than most of the commonly used antibiotics. A metabolomic approach applied to one of these strains, i.e. *P. haloplanktis* TB41 (Papaleo *et al*., 2013; Bosi *et al.,* 2015) allowed the detection of 30 compounds, some of which were sulfur-containing and presumably responsible for the inhibition of Bcc strains (Romoli *et al*., 2014). Further similar analyses performed on bacterial strains affiliated to different species and genera and isolated from seawater, sediments and sponges revealed that the mVOCs profiles were highly similar, if not identical (Papaleo *et al*., 2013; Maida *et al*., 2014), suggesting that the synthesis of these mVOCs is a common trait of Antarctic bacteria, whose biological significance in still unknown.

Tolerance to antibiotics

The introduction of antibiotics in the environment from anthropogenic sources (human health, treating animals, plant infections and animal farming) (Martinez, 2009) has produced the phenomenon of bacterial resistance, and modified the micro-biosphere and

the bacterial structure. In biotechnology, this aspect was focused by medical researchers because the widespread use of antibiotics generated a multidrug-resistant bacteria that probably transferred motile genetic elements one to each other. The acquisition of antibiotic resistance produces a metabolic burden (Andersson and Levin, 1999; Morosini *et al*., 2000; Andersson, 2006), and it was predicted that in the absence of selective pressure, resistance would disappear. The high presence of mobile antimicrobial genes it is considered a pollutant (Martinez, 2009). Therefore, in biotechnology it is important to study and find new molecular compounds that inhibit the resistant bacteria. Antarctica offers the best chance to analyse and develop new methodologists for relevant resistance mechanisms and facilitate the design of effective new drugs. With regard to the Ross Sea, antibiotic resistance has been the subject of two main studies (Lo Giudice *et al*., 2013; Mangano *et al*., 2014) and the majority of isolates were resistant to at least one antibiotic. Another example is the Antarctic marine waters that contain copious antibiotic-resistant bacteria, including many isolates that are resistant to ampicillin (De Souza *et al*., 2006).

EPS production

Exopolysaccharides (EPSs) are high molecular weight carbohydratic extracellular polymers that surround most microbial cells. EPSs are abundant in Antarctica because allow microorganisms to survive at extreme environmental conditions of temperature, salinity and nutrient availability. The industrial interest in EPSs was evaluated recently and it is still poor. The function of EPSs is related to their structure. The EPSs are produced by cold-adapted microorganisms to protect themselves, which sometimes are combined in biofilm matrix with a high production of extracellular EPSs that remove heavy metals or other organic pollutants. The EPSs bind heavy metals with some proteins and complex them (Spaeth *et al*., 1998). Cryoprotection is an important role of EPSs, as suggested by several studies (Krembs *et al*., 2002; Nichols *et al*., 2005a,b) that have shown the correlations between bacterial abundance and exopolymer production, and the

activity against freezing temperature and ice crystal formation. The species-specific structural heterogeneity and the many roles of EPSs in the natural environment are reflected in the numerous existing and potential applications of them (Weiner, 1997). The EPS is used to make a type of wound dressing for patients with burns, chronic ulcers, or extensive tissue loss (Sutherland, 1998). Several *Agrobacterium* and *Rhizobium* species produce curdlan, which improves the texture of tofu, bean jelly, and fish pastes in Japan (Sutherland, 1998). EPS bacterial producers are numerous in Antarctic environments (Helmke and Weyland, 1995; Krembs and Engel, 2001; Krembs *et al*., 2002; Mancuso Nichols *et al*., 2004, 2005). *Pseudoalteromonas antarctica* NF3 produces an exopolymeric compound of glycoprotein character that displays the ability to coat liposomes and provides protection against surfactants (Cocera *et al*., 2000, 2001). A study by Mancuso Nichols *et al*. (2005) showed that, even among closely related strains, EPSs produced by Antarctic bacteria commonly found in the marine environment were diverse.

Hydrocarbon-oxidizing bacteria

The accumulation of waste from human activities, even in polar regions, poses an insidious threat to the environment. Today, there is an increasing sensitivity to environmental pollution. Bioremediation is a biotechnological activity that transform pollutants into less hazardous products, which could be integrated into the biogeochemical cycle. Biodegradation in high temperature environments is associated with some advantages, not available to the bacteria in the cold environments. However, improvement of pollutant biodegradation shouldn't consider only mesophiles microorganisms, by considering that isolation of bacteria having degradation capacities from polar region soil has been frequently reported. Cold-adapted microorganisms are particularly interesting for waste treatment at ambient temperature in cold and temperate climates (Margesin and Schinner, 2001). Furthermore, the remediation of pollutant-affected soils could be realized by *in situ*-cleaning with the help of cold-adapted

microorganisms. This process occurs normally at temperatures below 15 °C, and temperature cannot be corrected by external factors. The hydrocarbon input in the polar regions is due to leakage events from vehicles, aircraft and storage tanks. A wide range of marine Antarctic bacteria from Ross Sea have been proven to possess the metabolic ability to utilize aliphatic and aromatic hydrocarbons as the sole carbon and energy source at low temperatures, suggesting that they very likely might play a key role in the *in situ* biodegradation of hydrocarbons (Lo Giudice *et al*., 2010).

Two strains affiliated to *Arthrobacter* and *Rhodococcus* genera isolated from Antarctic seawater were analysed for their capability to degrade hydrocarbons at low temperature by using their psychrotrophic ability, and resulted useful in biotechnological processes (Michaud *et al*., 2004). Other studies were focused on diesel oil degradation of Antarctic bacteria mediated by biosurfactant production or by membrane modification for a better hydrocarbon uptake (Pini *et al*., 2007). An Antarctic strain of *Pseudomonas* was found to grow optimally in presence of ammonium sulphate, nitrate and 3.5 % diesel oil at pH 7.0 within 10 to 20 °C (Shukor *et al*., 2009). Beside crude oil products, other types of environmental contaminants (e.g. heavy metals) also occur in Antarctic soil because of the anthropogenic activities (Claridge *et al*., 1995; Lohan *et al*., 2001; Chaparro *et al*., 2007). Despite the bacterial degradation of a wide range of hydrocarbons occurs in cold climate, some pollutants cannot be degraded, becoming recalcitrant in nature and resisting to microbial degradation.

PCB-oxidizing bacteria

Polychlorinated biphenyls (PCBs), widely used for industrial and commercial purpose, also occur in the polar regions because of atmospheric transportation. PCBs belong to a large group of persistent organic pollutants, which are released in the environment through a variety of human activities and have negative effects on the health of ecosystems. The PCB family is constituted by 209 congeners that differ for the

chlorination rate, so that higher chlorinated compounds are hardly biodegraded and persist for long periods in the environment. The occurrence of PCBs has been reported for several Antarctic abiotic matrices in the Ross Sea area (e.g., Risebrough *et al*., 1990; Larsson *et al*., 1992; Kennicutt *et al*., 1995; Fuoco *et al*., 1996, 1999, 2005; Crockett and White, 2003; Gambaro *et al*., 2005; Negri *et al*., 2006; Kennicutt *et al*., 2010). The biodegradation of these compounds is related to seasonal temperature, in summer the degradation depends on bacteria capability, in winter the darkness and low temperature slow down the PCB degradation and the molecules may be entrapped in pack ice and snow for a long time. Despite the recalcitrant nature, PCBs can be transformed into other chemical substances by anaerobic and aerobic microorganisms, possessing the genes and metabolic pathways for their biodegradation. Researches focusing on cold-adapted bacteria able to degrading PCBs in seawater and sediments at Terra Nova Bay (Yakimov *et al*., 1999; De Domenico *et al*., 2004; Michaud *et al*., 2007; Lo Giudice *et al*., 2013) and Arctic regions (Master and Mohn, 1998; Papale *et al*., 2017), and most of these bacteria were isolated in presence of Aroclor 1242 as carbon source. Michaud *et al*. (2007) reported the biodegradation of Aroclor 1242 by three psychrotolerant bacterial strains (*Pseudoalteromonas* sp., *Psychrobacter* sp. and *Arthrobacter* sp.) from Terra Nova Bay.

Heavy metal tolerant bacteria

Heavy metal pollution results by many activities, largely industrial. These pollutants are discharged or transported into the atmosphere and the aquatic system, than accumulated in sediments by generating considerable impact on the environment. The microbial communities are able to biotransform these pollutants in less recalcitrant molecules and accumulate them in the ecosystem. Metal depositions have great importance in biogeochemical cycles and fossil formations. The processes through which heavy metal reduction occurs are three: biosorption by bacteria and removal from the environment; microbial metal transformation by oxidation, reduction and methylation, with a decrease

of toxicity; metal binding proteins and peptides, for examples the production of metallothioneins.

A bacterial isolate obtained from the South Shetlands Islands, Antarctica, tentatively identified as *Pseudomonas* sp., was found to convert sodium molybdate or Mo^{6+} to molybdenum blue. Thus, the strain was suitable for bioremediation in both cold and temperate regions (Ahmad *et al*., 2013). Some bacteria isolated from soil of East Antarctica were tested for heavy metal tolerance coupled with enzyme presence and antibiotic resistance, which is developed by their adaptations in these habitats, probably with the presence of overproduce multidrug resistance efflux pumps (Pages *et al*., 2008; Lo Giudice *et al*., 2013). Metal resistance of the studied Antarctic bacteria associated with their ability to produce hydrolytic enzymes makes them promising for applications in bioremediation of heavy metal polluted sites (Tomova *et al*., 2014).

Other applications

As new biotechnological applications, the use of cold-enzymes as biofuels was recently proposed. As the human population continues to grow and exert demands on the fossil fuel industry, this leads to search for a new and alternative source of energy. In molecular biology cold-adapted alkaline phosphatase from Antarctic bacteria (Rina *et al*., 2000) was used in a variety of applications, as the removal of 5' phosphoryl groups from nucleic acids, thus preparing templates for 5' end labelling and preventing fragments from self-ligating. Many microorganisms produce both enzymes.

All these biotechnological potentials sometimes appear realistic to be used in bioremediation. The new technologies have implemented the use of molecular tools to develop new methods for extending the biotechnology. As an example of the technology applied for research purpose, the use of competent cells as *Escherichia coli* is useful for the clonation of gene fragments encoding for enzymes able to improve the pollutant degradation (Reddy *et al*., 2016). These allow to have engineered microorganisms that

may be used in natural environment, thus providing interesting tools in the environmental bioremediation strategies. The principal problem is that the introduction of microorganisms in Antarctica is forbidden by the Antarctic Treaty 1959 (Hanessian, 1960) because they can modified the natural assessment of environments. For this reason, the research on biotechnological applications will continue as long as they can really be used in the environment without any contraindication.

The modern biotechnology has the potential to revolutionize various sectors, from food industries to agriculture and from cosmetic industries to human health. Obviously, the biotechnological potential was improved in medicine and pharmaceutical sectors with numerous advances in compound production and commercialization in few years. With biotechnologies, the researchers improved their methodologies in order to understand more deeply the numerous pathways involved in the capability to produce biocompounds or the degradation ability highlighted those psychrophilic bacteria as useful potential. The studies on cold-adapted bacteria and on their metabolic pathways are important for an advantageous exploitation of biotechnological potentialities, also through the development of new methodologies for the commercial use of compounds of biological origin.

3. Microbial diversity using culture-independent methods

In the past decades, the application of molecular biological approaches has provided the development of knowledge into the uncultured microbial communities of soils, waters and extreme environments. In this way the microbiologists became motivated to screen the largely unexplored Antarctic and Arctic continents. These new approaches into the culture-independent methods allowed to examine a microbial diversity not necessarily reflected by results of culturing studies.

In the 1977 Sanger and collaborators improved a new method for determining nucleotide sequences in DNA. This method is based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase in vitro replication. This ddNTPs may be radioactively or fluorescently labelled for detection in automated sequencing machines and the fluorescent dyes emit light at different wavelengths. In this way a chromatogram with different peak heights that correspond to a specific base result. The set of the peaks set up a complete sequences of one single part of DNA of each sample.

The traditional DNA-sequencing method (Sanger *et al*., 1977) sequences DNA from individual species and is inadequate for studying the total microbial composition of environmental samples. Mixtures of DNA from hundreds or thousands of individuals constitute the natural environment communities. Although conventional sequencing has provided the most efficient method for the development of large DNA barcode reference libraries, the number of individuals in an environmental sample is beyond the scope of its ability (Hajibabaei *et al*., 2011). The analysis of DNA fragments from thousands of microorganisms inside the natural samples requires the possibility to read DNA from multiple templates in parallel; this approach has been achieved with the development of the new sequencing methods (next-generation sequencing).

The next-generation sequencing (NGS) platforms have made it possible to recover DNA sequence data directly from environmental samples (Sogin *et al*., 2006). These data have been used in a variety of applications, including the study of ancient DNA (Haile *et al*., 2009; Sønstebøet *et al*., 2010; Boessenkool *et al*., 2011), analysis of biodiversity in ecosystem, healthy versus diseased individuals (Andersson *et al*., 2008; Zhang *et al*., 2009), etc. The sequences resulting from these analyses should be compared with the reference library of known organisms in order to be identified with high confidence. The platforms for NGS are numerous and sometimes the choice of the appropriate NGS platform is difficult and dependent on the ecological research. This evolution is reflected in several sequencing systems (Shokralla *et al*., 2012), which have occurred over time. In the 1990s DNA sequences production has developed a semi-automated implementation of the Sanger sequencing method. The throughput is based on the 16S rDNA clone libraries (von Wintzingerode *et al*., 1997), an approach which combines culture-dependent and culture-independent methods. This technique picks randomly fragmented DNA from environmental samples and clone them into a high copy number plasmids, then transformed in *Escherichia coli* that will be spreaded on agar medium. The colonies are randomly picked and amplified, and the resulting sequences are than sequenced. This technique developed after the Sanger sequencing allow to sequence up to 1 kb from 96 individual organisms at the same time.

The NGS technologies can potentially generate several hundred thousands to tens of millions sequencing reads in parallel. This massively parallel throughput sequencing capacity can generate sequence reads from fragmented libraries of a specific genome; from a pool of cDNA library fragments generated through reverse transcription of RNA molecules; or from a pool of PCR-amplified molecules. In all cases, sequences are generated without the need of a convectional, vector-based cloning procedure that is typically used to amplify and separate DNA templates.

Since their introduction in 2005, high-throughput NGS technologies improved their methodologies by surpassing several challenges. One important challenge is the sequencing output and the read length accuracy. The second challenge has been the total output of the sequencing experiments in relation to the cost and the labour expended. The third challenge is related to the amplification step prior to sequencing. This final challenge includes different sources of PCR bias, formation of chimeric sequences and secondary structure-related issues (Mardis, 2008a; Shendure and Ji, 2008). New technologies promise to fundamentally change the nature of genomics-based studies, especially when coupled with the computational algorithms necessary to analyse their vast sequencing output (Mardis, 2008b). Although available NGS technologies utilize quite diverse chemistry and base incorporation/detection tools, they share two main steps: library fragmentation/amplicon library preparation and detection of the incorporated nucleotides (Glenn, 2011; Zhang *et al*., 2011). NGS techniques can be divided in two categories. One based on PCR (e.g. Roche 454 Genome Sequencer, AB SOLID^{TM} and Ion Personal Genome Machine) and another that is not based on PCR and without an amplification step first (HeliScope and PacBio RS SMRT system). The different technologies are:

- Roche 454 genome sequencer: it was introduced in 2005. This platform uses the pyrosequencing system. The pyrophosphate is released when the nucleotide is incorporated inside DNA-by-DNA polymerase. This step generates a light due by the enzyme luciferase. The library fragments are linked to beads and then amplified by emulsion PCR thermal cycling into a mixture of water and oil with PCR ingredients. Each library fragment is amplified and generates a billions of copies. The beads are recovered from emulsion oil and the single standed are annealed to a specific sequencing primer. Then the beads are set on picoliter plate, inside a well, with pyrosequencing enzyme beads. The plates are sequenced

and a camera records the emitted light from each bead and then the machine give the results.

- Illumina sequencer: it was introduced in 2007. This platform utilizes a sequencing-by-synthesis approach coupled with bridge amplification on the surface of a flow cell. Each flow cell is separated in eight lanes. Inside the flow cells oligos complementary to specific adapters that are ligated onto the library fragments are covalently attached. The hybridization between DNA and oligos are set up with cold and heat reactions and then incubated with amplification reactants and an isothermal polymerase that generates millions of clusters of the library fragments. In the sequencing step, each cluster is supplied with polymerase and four labelled fluorescent nucleotides with 3'-OH inactivated. After incorporation, an excitation followed an imaging step takes place to identify the nucleotide, followed by a chemical reaction that removes the fluorescent group and allows other incorporation.
- SOLiD sequencer: In 2007 SOLiD was introduced as NGS technologies that use a sequencing-by-oligo ligation. It is characterized by using in the process of sequencing of a ligase and not of a polymerase. In fact, octamers labelled with fluorochrome are used, which bind selectively to the sequence mold and whose first nucleotide is bound to the trigger. The light emission is recorded and subsequently a cut is performed between the fifth and the sixth nucleotide, which eliminates the fluorescent marker and allows the insertion of a new octamer.
- Ion Torrent sequencer: This sequencer will be discussed in the chapter below as it was the sequencer chosen for the total prokaryotic community analysis.

3.1 Ion Torrent Sequencing system

Figure 3.1 Ion Torrent Sequencing machine

Life Technologies introduced ion Personal Genome Machine (PGM) in 2010. The basic concept of PGM is performing sequencing-by-synthesis, with electrochemical detection of synthesis, and each reaction is coupled to its sensors which are in turn organized into a massively parallel sensor array on a chip. The chemical fundamental is that the synthesis by polymerase incorporation releases pyrophosphate and a hydrogen ion (H^+) from the 3'-OH incorporation site on the growing strand. The sensor is capable to detecting either pyrophosphate or H^+ of directly incorporation. The protocol provides Ion Torrent the clonal amplification of the sample through emulsion PCR. The latter consists in the

preparation of an emulsion of water and oil in which the microbubbles of water perform the action of microreactors for the PCR reactions. All reagents required for the amplification reaction are previously dispensed in aqueous solution together with the marbles support (beads) on which to bind the DNA fragments. When the reagents are present in balanced amounts, between them and with respect to the volumes of water and oil, according to the law of the Poisson distribution, they will be distributed in such a way that each microreactor contains: a fragment of DNA, the polymerase, the necessary reagents for the amplification reaction and a ball support. Subject to the cyclic changes of temperature, as normally happens in a PCR reaction, microreactors will give rise to the production of thousand of fragments clonal that, thanks to the specific sequences complementary to sequences of the court adherent at marbles, adhere to the entire surface (Figure 3.2).

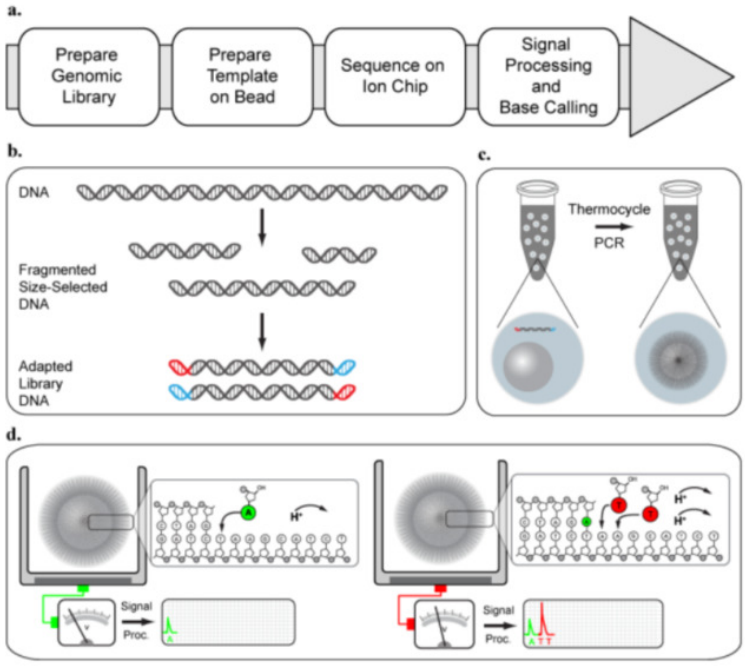

Figure 3.2 Ion Torrent workflow

The Ion torrent sequencing has two essential aspects that characterize than other technologies: 1) the use of semiconductors as a structural element of the media in which

the sample is dispensed for sequencing (Ion Chips) and 2) the use a detection system not based on luminescent reactions but on changes in electrochemical potential. Central nucleus of the sequencing system is the Ion Chip. It consists of a surface layer, in which are formed the wells specially structured to accommodate the beads and reagents for the sequencing. This rests in a second layer consisting of semiconductors, which allows the transmission of signals to the layer again below. The chip is structured as a plate of sensors that have the ability to record small changes in pH that occur inside the sump during the sequencing, transforming them into potential difference, or, in the data digitals. Sequencing process is characterized by a cyclical sequence of washes and streams on the chip surface (flows) of solutions containing each of the four different nucleotides. In this way, when it is dispensed the nucleotide complementary to the first free base mold on the fragment, it is incorporated into the nascent sequence. Since the bead on which occurred the amplification clonal presents on its surface fragments that are all equal, the link of the new base occurs simultaneously on thousands of fragments. The formation of any new phosphodiester bond determines the hydrolysis of the nucleotide triphosphate group entering the net release of a proton. The release of protons due to the lowering of the pH of the solution present inside the chip. This variation is transmitted and recorded by the sensor below and digitized. During each flow, the pH variation recorded is directly proportional to the number of bases incorporated into the sequence rising, and in the moment in which the mold has an area of homopolymeric sequence, the intensity of the potential variation is recorded as much superior as many are the bases consecutive identical. This data is represented by a graph, called ionogram, which displays the number of bases incorporated than flows performed by the device. The total process of generation and signal measurement lasts just over four seconds. Subsequently, through the flow of a washing solution, necessary to eliminate reagents from the wells already used, the chip is prepared for a subsequent flow that results in the incorporation of a new nucleotide. This process is repeated cyclically for 500 times allowing the reading of long

reads approximately 200bp. Different chips can be used in the process of sequencing Ion Torrent. They differ primarily in the duration time of the run and for the size of the outputs, as it is shown in Table 3.1.

Table 3.1 Differences between three different Ion Chips.

Chip name	Run time		Reads output	
Ion 314™ Chip v2	2.3 hr	3.7 hr	30–50 Mb	60–100 Mb
Ion 316™ Chip v2	3.0 hr	4.9 hr	300–500 Mb	600 Mb–1 Gb
Ion 318™ Chip v2	4.4 hr	7.3 hr	600 Mb–1	1.2–2 Gb

Two additional instruments are used in the system Ion Technology: Ion OneTouch DL, which performs the task automatically performing the emulsion PCR, and the One Touch ES, which is used for the enrichment/purification of the sample after the clonal amplification reaction.

3.2 Biological applications of NGS

The increase in the development of the new technologies in the studies on ecology and biodiversity of environmental samples has led to using these mass sequencing to reduce the costs and the longer time of culturable methods. The advent of NGS technologies have facilitated analysis of natural samples from different ecosystem as freshwater, seawater, soil, terrestrial, including Arctic and Antarctic environments. The researchers can analyse the changes in microbial community composition due to the anthropogenic or natural environmental fluctuations (Leininger *et al*., 2006; Fierer *et al*., 2007). Numerous researchers had used the 454 pyrosequencing platform for the study on microbial composition. This choice not was in relation to the ecosystem studied or the specific ecological question asked, but because give longer sequence read lengths. Several studies

have analysed soil bacterial diversity by examining 16S rDNA amplicons (Roesch *et al*., 2007; Rousk *et al*., 2010; Nacke *et al*., 2011). Other studies have focused on soil fungal diversity in both forest and agricultural setting by analysing ITS amplicons (Acosta-Martínez *et al*., 2008; Buée *et al*., 2009; Jumpponen *et al*., 2010; Rousk *et al*., 2010). An alternate approach has been used to target all soil microbiota, from Archaea to fungi, using either total RNA (Fierer *et al*., 2007) or selected functional gene amplicons (Leininger *et al*., 2006). Also, marine environment has been studied by NGS technologies. Analyses of marine bacterial communities have been conducted using 18S rDNA (Huber *et al*., 2007) and 16S rDNA amplicons (Sogin *et al*., 2006). Frias-Lopez *et al*. (2008) studied microbial community gene expression in ocean surface waters through transcriptomic sequencing analysis of cDNA libraries. Marine eukaryotic microbiota were investigated through NGS analysis of 18S rDNA amplicons (Stoeck *et al*., 2010). Recent articles used NGS for freshwater environmental samples. Next-generation technology has also been employed in recent research into terrestrial environmental samples, both ancient and modern. Haile *et al*. (2009) utilized both 454 pyrosequencing and conventional Sanger sequencing methods in the analysis of ancient DNA recovered from Arctic permafrost cores. Finally, Sønstebøet *et al*. (2010) analysed permafrost samples to identify ancient plant species.

3.3 Microbial community composition in Permafrost

Permafrost as extreme environment is particularly interesting to be studied as extreme conditions are generally unfavourable for microbial colonization, due to the frequent freeze-thaw and wet-dry cycles, low and transient precipitation reduced humidity, rapid drainage and limited organic nutrients (Wynn-Williams, 1990; Convey, 1996). But the studying of microbial composition is intriguing to understand the adapted organism and

their variability under extreme conditions. Only 1-3 % of Antarctic and sub-Antarctic land surface area is free of ice (Vishniac, 1993), with much of this area representing cold rock deserts. Despite the typical desert habitats, some area, especially maritime areas, are relatively well vegetated (Smith, 1984). Decreasing biodiversity with latitude is one of ecology's most fundamental patterns (Willig *et al*., 2003) and relatively little is known about patterns of microbial diversity across the Antarctic, except for specific local studies. The studies on Antarctic terrestrial sites revealed no connection between diversity and latitude (Lawley *et al*., 2004). While extreme environments with stressful conditions could cause the selection of organisms, the increasingly stressful environment conditions have been reported to act as strong selection factors, limiting biodiversity of soil organisms. NGS approaches, therefore, can produce an advance in high-throughput sequences that allows to offer new strategies for exploring the microbial universe at a resolution that was unimaginable. Application of these technologies is useful to simultaneously sequence samples.

Traditionally, active layer, permafrost and brine microbial communities have been studied using culture-dependent approaches (Vorobyova *et al*., 1997; Vishnivetskaya *et al*., 2000; Ozerskaya *et al*., 2009; Steven *et al*., 2009). With the general acceptance that the bulk of microorganisms in an environment are unculturable (Alexander, 1997), DNA-based assays have become routinely used in microbial ecology and environmental studies. Within the last few years, the application of molecular biology and genomics techniques has also been extended to permafrost ecosystem (Vishnivetskaya *et al*., 2006; Yergeau *et al*., 2010; Mackelprang *et al*., 2011). Attempts in applying metagenomic approaches to soils and sediments have been hampered by technical challenges as highlighted recently by Hazen *et al*. (2013). These include the extraction of representatives, with a differentially accessible of DNA, as well as the inherent DNA heterogeneity and uneven spatial distribution of microorganisms within soils (Torsvik *et al*., 1990; Curtis *et al*., 2002).

In general, the bacterial and archaeal community composition inhabiting cryo-environment has been studied principally using clone libraries and other molecular tools (e.g. T-FRLP; DGGE; ARISA). Molecular methods showed a high bacterial diversity in different cold terrestrial environments such the Arctic region (Hansen *et al*., 2007; Steven *et al*., 2007a; Liebner *et al*., 2008; Schutte *et al*., 2009; Frank-Fahle *et al*., 2014), the Antarctic continent (Aislabie *et al*., 2006; Smith *et al*., 2006; Yergeau *et al*., 2007; Niederberger *et al*., 2008; Ganzert *et al*., 2011; Bajerski and Wagner, 2013) or high alpine zones (Sigler *et al*., 2002; Bai *et al*., 2006; Costello & Schmidt, 2006; Schmidt *et al*., 2009; Zinger *et al*., 2009). The main groups that could be identified belonged to the phyla *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi* and *Verrucomicrobia*. Using culture-dependent approaches, mainly *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were isolated from high Arctic soils (Hansen *et al*., 2007; Steven *et al*., 2007a), with similar results reported for high alpine (Bai *et al*., 2006; Zhang *et al*., 2007) and Antarctic regions (Shivaji *et al*., 2004; Aislabie *et al*., 2006). The current knowledge on the bacterial community and diversity of permafrost is little and limited to metagenomic community composition (Ganzert *et al*., 2007; Yergeau *et al*., 2010; Barbier *et al*., 2012; Liebner *et al*., 2015), methanotrophic bacteria (Liebner *et al*., 2007, 2008), and brine community (Murray *et al*., 2012; Chih-Ying *et al*., 2012). Most investigations have target microbial community in soil habitats (Aislabie *et al*., 2006; Tian *et al*., 2009; Hinsa-Leasure *et al*., 2010; Wilhelm *et al*., 2011) and microbial activity (Yergeau *et al*., 2010; Blanco *et al*., 2012).

The Arctic and Antarctic permafrost are similar for some aspects, for example the thermal and climate characteristics, but they show some diversities. Bacterial communities from both Siberian and Antarctic permafrost samples were precisely characterized by culture-dependent and culture-independent methods. Both methods revealed the presence of *Gammaproteobacteria* and Gram-positive bacteria. *Arthrobacter*, *Bacillus* and *Pseudomonas* were detected from both methods. By a culture-independent approach it

was shown the dominance of *Gammaproteobacteria*, especially *Xanthomonadaceae* (75-84 %) and *Actinobacteria* (39-57 %) in Siberian permafrost (Vishnivetskaya *et al*., 2006), and Gram-positives (up to 45 %) and *Proteobacteria* (up to 25 %) in Antarctic permafrost (Spirina *et al*., 2003).

Culture-independent methods, which use molecular-based tools to analyse DNA extracted directly from permafrost (Spiegelman *et al*., 2005), bypass the need from the culturing and have increased the number of phylogenetic groups of Bacteria and Archaea associated with permafrost. For example, the culturable microbial community in a Canadian high Arctic permafrost sample was dominated by *Firmicutes*-related isolates, whereas *Actinobacteria* and *Proteobacteria*-related sequences were predominant by a culture-independent analysis, with the phyla *Gemmatimonadetes*, *CFB* and *Planctomyces* identified by the culture-independent survey but not among the isolates (Steven *et al*., 2007a). Antarctic Dry Valleys permafrost 16S rRNA gene clone libraries were composed of the phylogenetic groups *Proteobacteria* and *Actinobacteria*, with *Arthrobacter*, *Bacillus*, and *Pseudomonas* detected in all of the Antarctic permafrost clone libraries (Gilichinsky *et al*., 2007). To date, very few studies have described the Archaea communities in permafrost using culture-independent methodologies. Ochsenreiter *et al*. (2003) reported the detection of 16S rRNA genes related to the *Crenarchaeota* in Chinese alpine permafrost. Although methanogens have been isolated from Antarctic and Siberian permafrost (Rivkina *et al*., 1998; Gilichinsky *et al*., 2007), 16S rRNA gene sequences related to methanogenic Archaea were not detected in Canadian high Arctic permafrost, with the exception of a single sequence detected in a massive ground ice deposit (Steven *et al*., 2008). An interesting result regards halophilic Archaea inhabiting Canadian high Arctic permafrost, where a significant number of sequences were found, although the salinity was moderate (Steven *et al*., 2007a, 2008). This is possible due to the presence of saline liquid brines that surround soil particles and can influence the community composition of soil (Prince, 2007). The detection of DNA in permafrost or brine sample

do not give an evidence on the possible activity inside microbial community, but it can demonstrate that DNA is preserved at constant sub-zero temperature (Willerslev *et al*., 2003, 2004a; Steven *et al*., 2009). Thus, developing novel methods will be essential to determine if microorganisms identified by culture-independent surveys exist as viable cells or are the microbial equivalent to mammoths, frozen in time in the permafrost environment (Steven *et al*., 2009).

4. Aim of this Thesis

The aim of the present Thesis was the study of structure and functions of the prokaryotic communities inhabiting Antarctic permafrost and brines.

Permafrost samples were collected during different Antarctic campaigns from three sites (i.e. Edmonson Point, Boulder Clay and Dry Valleys) at different depths, while brine samples derived from lakes at Tarn Flat and Boulder Clay (in the inland and on the coast, respectively). Samples were analyzed by culture-dependent and culture-independent methods, and the following tasks were addressed:

1) Analysis of the prokaryotic community composition by culture-independent methods (e.g. NGS sequencing of 16S rRNA genes and CARD-FISH);
2) Evaluation of the best method for the recovery of cultivable bacteria from permafrost and brine samples;
3) Isolation, identification and phenotypic characterization of bacterial isolates;
4) Screening of bacterial isolates from brines for biotechnological potential (production of antibiotics, cold-adapted enzymes and esopolysaccharides, utilization of pollutants as the sole carbon and energy source);
5) Screening of bacterial isolates for tolerance to environmental stressors (i.e. antibiotics and heavy metals).

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5. Study of permafrost samples

The first objective of this work was the analysis of the prokaryotic community inhabiting permafrost samples and the comparison of the different kinds of samples among the communities. For this purpose, permafrost samples of different geomorphological eras were selected, so that the prokaryotic community of one active layer in contraposition with permafrost samples and an ice samples were analyzed.

5.1 Site description and characteristics of permafrost

Samples were collected from two sites in the Northern Victoria Land (Boulder Clay and Edmonson Point), close to the Italian Antarctic research station "Mario Zucchelli" (MZS), and one site in the Upper Victoria Valley within the McMurdo Dry Valleys.

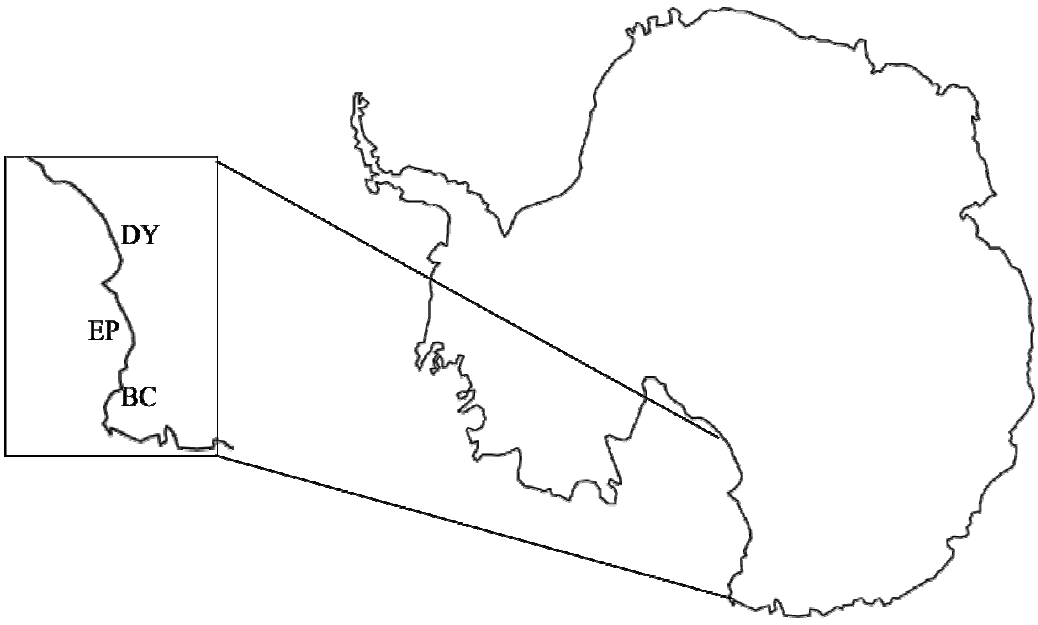

Figure 5.1 Sampling site location in Antarctica

Edmonson Point (EP)

Edmonson Point (74°20' S, 165°07' E) is a volcanic area located on the western coast of the Ross Sea, in the Northern Victoria Land under Mt. Melbourne. It is the most non-mountainous area and ice-free site in Northern Victoria Land. EP was modified by glacial and periglacial activity, due the formation of a mosaic of hills (up to 300 m high), knolls and moraines, separated by small valleys with several small melt-water streams, ponds and lakes. Even if there is some water-area, it is extremely dry with the ground covered by salt encrustations. The sediments are constituted by volcanic materials (lavas, pumice, scoria) and the ground is dark colored which is originated from past volcanic activity of Mt. Melbourne. The soil is fine gravel or coarse sand with a low portion of silt and clay. It is general poor in nutrient but due the breeding birds and penguin colonies, concentration of nitrogen and phosphate could be very high. The temperature ranged between -2 to -5 °C in January and to -26 to -30 °C in August, there is low humidity and low precipitation rate (100-200 mm), but there are the katabatic winds that give milder climate than the neighboring areas with summer temperature between -15 to +5 °C (Harris and Grant, 2003; Smykla *et al.*, 2010). Edmonson Point is characterised by overlying continuous permafrost and soils, these are quite rich in organic content (0.5-1.6 % C org) (Cannone *et al.,* 2008; Cannone and Guglielmin, 2009).

Boulder Clay (BC)

The Boulder Clay site (74°44' S, 164°01' E) is located in Northern Victoria Land close to the Italian Antarctic Research Station "Mario Zucchelli". This area is ice-free and located about 6 km south of the Italian station in a slope with south-eastern exposure. Surface features include perennially ice-covered ponds with icing blisters and frost mound (French and Guglielmin, 2000; Guglielmin and French, 2004), frost-fissure polygons and debris island (French and Guglielmin, 1999). The soils here are mainly glacic haplorthels

where no evidence of cryoturbation has been observed, and scattered mosses and epilithic lichens constitute the vegetation types according to Cannone *et al*. (2008). This site is exposed to katabatic winds from inland areas that produce numerous snow drifts. The surface of Boulder Clay shows debris and scattered boulders and the sediments, likely of glacio-marine origin, are dated to the Late Pleistocene (Orombelli *et al*., 1991). The mean annual temperature is -13.8 °C and the mean annual ground temperature is -16.5 °C at the surface and -16.5 °C at the permafrost level, while in the deepest monitor layer (3.6 m) the mean temperature is -17 °C (Guglielmin and Cannone, 2012; Guglielmin *et al*., 2014). The active layer, in Boulder Clay site, is thermally defined at about 18-20 cm, it is monitored in the adjacent CALM (Circumarctic Active Layer Monitoring) grid which ranges between 0 to around 90 cm and is continuously thickening (Guglielmin *et al*., 2014).

Dry Valleys (DY)

Dry Valleys are ice-free valleys located in South Victoria Land. This region is a cold desert area of over 2500 km^2 where the ground surface is practically free of snow and ice throughout the year. The area comprises three major valleys (the Taylor, Victoria and Wright Valleys) separated by mountain ranging up to 2400 m in elevation. The ground in this area is subject to sublimation and evaporation losses that exceed precipitation. Meteorological data from Lake Vanda indicate a mean annual precipitation of between 7 and 82 mm of snow (Thompson, 1973), which could be equivalent to about 0.7-0.8 mm of water (Chirm, 1980). The katabatic winds influence the meteorological characteristics of the area, with an increase of winds during winter. Mean annual temperature at valley floor levels is about -20 °C. Summer temperature is up to +8 °C warmer than the other areas of equivalent elevation. Soil surface during summer can vary from -15 to +27 °C in 3 hours (Cameron and Morelli, 1974). DY soils are considered to be the oldest (thousands to million of years) with the lowest organic carbon content (0.03 wt %) and biological

activity of any soils on Earth (Burkins *et al*., 2001). The DY landscape could be divided in three basic zones: coastal, intermediate and interior (Marchant and Denton, 1996).

5.2 Collection and *in situ* treatment of samples

Samples from BC (coordinates: 74°4404500 S-164°0101700 E) were obtained from three distinct permafrost cores collected from the Boulder Clay site in November 2003, as follows: BC-1 (60-74 cm depth), BC-2 (265-275 cm depth) and BC-3 (BC-Frost Mound 533-543 cm depth). Sample BC-1 was characterized by the first 70 cm of pure lake ice that was very rich in bubbles overlying frozen icy silt sediments and representing the lacustrine sediments on the bottom of the pond. Here gas exchanges with atmosphere were still possible. BC-2 was drilled immediately outside of the frozen lake and this core revealed an upper part (94 cm thick) characterized by glacial frozen sediments overlying 296 cm of massive buried glacier ice quite rich of debris, followed by a massive pure glacier ice unit. The sample BC-2 was a sample of debris of rich glacier ice and virtually had no gas exchanges. BC-3 core was drilled on the side of the frost mound (Guglielmin *et al*., 2009; Abramovich *et al*., 2012) located in the same frozen pond of the BC-1 core. The BC-3 core had a more complex permafrost stratigraphy, underlying below 42 cm of lake ice. Below that lake permafrost is constituted mainly by silty-sand with intrusive ice lenses occurred followed by 1.25 m of the same unit of buried glacier ice rich in debris of BC-2 sample. Below there was a talik with some hyper-saline brines (thick 50 cm) overlaid almost 2 m of pure glacier ice.

Sample EP (35 cm layer) was collected directly from the unfrozen part of the active layer at Edmonson Point (coordinates: 74°19’ S 165°7’ E) in early January 2014. Temperature was measured along depth, from surface to 35 cm (just above the permafrost layer). In this sample, water-atmosphere exchanges occur.

Sample DY was collected from an 11 m deep permafrost core achieved in November 2003 close to the Eastern side of the Upper Victoria Lake (77°2000300 S - 161°3702300

E). The core was characterised by quite homogeneous frozen sediments mainly composed by sand and silty-sand sediments with little fresh-water ice and water (Guglielmin *et al*., 2011; Zucconi *et al*., 2012). The main characteristics of the permafrost samples are reported in Table 5.1.

Until in lab, the frozen core samples were fractured in a hood with sterile knife and only internal fragments were taken by sterile forceps and placed in sterile containers to be processed according to standard techniques developed for sediments.

Table 5.1 Characteristics of permafrost samples collected in Antarctica. Temperature range and possible interconnection with atmosphere or other cryo-environment, such as cryopeges and brines.

Location	*Sample*	*Depth horizon*	*Age*	*T amplitude (°C)*	*T range (°C)*	*Possible interconnection*	*Ice*
Edmonson Point	EP	Surface	Modern	49	-30.3/ 18.6 (*)	Surface water atmosphere	Pore ice
Boulder Clay Lake	BC-1	60-74	<1020±70 BP (1)	32.6	-31.1/-1.5	Surface water atmosphere	Lake ice
Boulder Clay	BC-2	265-275	>17000 (2)	18.2	-24.9/-6.7	No	Relict buried glacier ice
Boulder Clay Frost Mound	BC-3	533-543	>17000 (2)	9.6	-21.5/- 12.1	Saline brine	Relict buried glacier ice
Upper Victoria Dry Valley	DY	355-380	>15-20000 (3) -113-120 Ka (4)	14.5	-11.5/- 26.5	No	Segregated ice

(*) Cannone and Guglielmin, 2009; 1) Abramovich *et al*., 2012; 2) Orombelli, 1991; 3) Zucconi *et al*., 2012; 4) Bockheim and McLeod, 2013.

5.3 Analysis of prokaryotic community composition by NGS

Edmonson Point samples (EP) were analyzed for prokaryotic community composition by a culture-independent method. The active layer was chosen because it is a cryo-environment that is continually in inter-connection with the atmosphere and changes seasonally due to the alternation between the thawed and frozen state. This layer is an ecological niche colonized by diverse and functionally cold-adapted microbial assemblages, which adapt themselves to the seasonally changes of temperature, water availability and ice presence.

5.3.1 DNA extraction and PCR amplification

DNA was extracted in duplicate from 5 g of each soil sample by employing the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentrations and purity were quantified by using a NanoDrop ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, USA).

5.3.2 16S rRNA gene amplicon sequencing

The V1-V2 region of the 16S rRNA genes was amplified by PCR. In order to reduce bias in massive sequencing, the two-step PCR protocol was applied, consisting in a first step of 30 PCR cycles with conventional PCR primers and then using 0.5 µL of first reaction amplicon for 6 cycles PCR with barcoded primers for Ion Torrent sequencing. Triplicate PCR reactions were set up at 0 °C under a PCR cabin by using 0.4 µL of Phusion High-Fidelity DNA polymerase (2U μL^{-1}), 8 µL of Phusion buffer (10 X), 1 µL of each dNTP (10 mM), 1 µL of Syber, 1 µL of each primer (10 µM). The universal primers 27f (5'-

AGAGTTTGATCCTGGCTCAG-3 ') and 338r (5'-GCTGCCTCCCGTAGGAGT -3') were used.

The amplification was performed according to the program described below: 30 sec at 98 °C; 30 cycles of 10 sec at 98 °C, 30 sec at 53 °C and 60 sec at 72 °C, 10 min at 72 °C. Amplified products were visualized by electrophoresis agarose gel (1.5 %, w/v), using ethidium bromide (EtBr) (1 mg mL^{-1}).

The three reactions were pooled and used for the second PCR under the same conditions. To 0.5 µL of pre-amplified DNA the components of the PCR mixture were added to a final volume of 20 µL: 0.2 µL of Phusion polymerase (2U μL^{-1}), 4 µL of Phusion buffer, 0.5 µL dNTPs (10 mM), 0.5 µL of Syber, 0.5 µL of each barcoded primer (10 µM). The reaction was carried out according to the program described below: 30 sec at 98 °C; 6 cycles of 10 sec at 98 °C, 30 sec at 53 °C and 60 sec at 72 °C, 10 min at 72 °C. Amplified products were visualized by gel electrophoresis as described above. PCR products were purified using the Agencourt AMPure XP (Beckman Coulter, Inc.) kit, according to the manufacturer's instructions, and then quantified using the Qubit fluorometer 2.0 (Thermofisher Scientific). Using the TapeStation 2200 (Agilent), samples were brought to a final concentration of 13 pM. Then, 25 µL of each solution were subjected to clonal PCR amplification. Sequencing was performed on an Ion Torrent Personal Genome Machine™ using the Ion Xpress™ Template Kit and the Ion 316™ chip following manufacturer's protocols.

5.3.3 Bioinformatics analyses

The raw data generated by the Ion Torrent machine were then analysed using the bioinformatics analysis software Mothur to determine the quality criteria of the reads. The bioinformatics analysis consisted in the trimming off the adapters, barcodes and primers using the default parameters, and to remove sequences

containing ambiguous "N" or shorter than 150 bp. Selected reads were denoised using the "pre.cluster" command in Mothur platform (Schloss *et al*., 2009) to remove sequences that were likely due to pyrosequencing errors and assemble reads which differed only by 2bp. Chimeric sequences were identified and removed. The sequences were grouped into operational taxonomic units (OTUs) at 97 % pairwise identities, and the phylogenetic affiliation with the on-line database Silva was carried out (Quast, 2013).

5.4 Prokaryotic community composition using culture-dependent methods

5.4.1 Enumeration of cultivable heterotrophic bacteria in permafrost samples

Two strategies were used to cultivate the permafrost microorganisms: 1) direct plating of diluted cell suspensions on agar media; 2) enrichment in liquid media before plating (Vishnivetskaya *et al*., 2000), as specified below.

If not otherwise specified, cultures in liquid and solidified media were incubated in the dark at +4 °C for 8 weeks, and colony forming units (CFU) *per* gram of wet soil samples (BC-1, BC-3, EP and DY), or mL of melted ice (samples BC-2) were calculated as averages of duplicate plates.

For direct plating of soil samples, material (1 g) from the centre of the core was diluted in cold filter-sterilized phosphate-buffered saline (PBS) 1X (10 mL). The suspension was shaken for 2 minute, and serial tenfold dilutions until 10^{-4} were made. Each dilution (100 µL) was spread-plated in duplicate on Triptic Soy Agar (TSA_{100}; Oxoid) and R2A Agar (Difco) a full strength. All plates were incubated aerobically at +4 °C for 4 weeks, and

colony-forming units (CFU) were counted. Plates were rexamined weekly for the appearance of new colonies.

For broth enrichments, 50 µL of soil suspension (as described above for sample EP, DY, BC-1 and BC-3) were added in duplicate to 2.5 mL of Triptic Soy Broth (Oxoid) that was used at full (TSB_{100}), 1/2 (TSB_{50}), and 1/10 (TSB_1) strength in 50 mL-polypropylene tubes. The cultures were monitored using spectrophotometer until good growth. After incubation, enrichments were then diluted and aliquots (100 µL) plated on the same solidified (1.5 % agar, w/v) TSB used for the enrichment (TSA_{100}, TSA_{50} or TSA_1) and R2A. CFUs were determined as described before.

The ice sample BC-2 was allowed to thaw at 4 °C for 24-48 h and then directly used (10 %) from enrichment by inoculation in duplicate in TSB_{100}, TSB_{50} and TSB_1, as described above for soil samples. The enrichments were incubated at 4 °C for 4 weeks, then diluted, and aliquots (100 µL) plated on the same solidified TSB used for the enrichment, and R2A.

Colonies were randomly selected from agar plates used for CFU counts, picked and sub-cultured almost three times under the same conditions. CFUs were determined as described.

5.4.2 16S rRNA gene PCR amplification of bacterial isolates

PCR-amplification of 16S rRNA genes from bacterial isolates was carried out under the conditions described earlier (Michaud *et al*., 2004). Briefly, a single colony of each strain was lysed by heating at 95 °C for 10 min. Amplification of 16S rRNA gene was performed with an ABI 9600 thermocycler (PE, Applied Biosystems) using the domain Bacteria-specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (spanning positions from 8 to 27 in *E. coli* rRNA coordinates) and 1492R (5'-CTACGGCTACCTTGTTACGA-3') (spanning positions from 1492 to 1513 in *E. coli*).

The reaction mixtures were assembled at 0 °C and contained 1-10 ng DNA, 10 X buffer, 1.5 mM $MgCl_2$, 150 ng of each forward and reverse primer (MWG, Germany), 250 μM dNTP (Polymed, Italy), 0.5 units of PolyTaq polymerase (5 PRIME) and sterile distilled water to a final volume of 25 μL. Negative controls for DNA extraction and PCR setup (reaction mixture without a DNA template) were also used in every PCR run. The PCR program was as follows: 3 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C and a final extension step of 10 min at 72 °C. The expected size of the PCR product was approximately 1.4 kb. The results of the amplification reactions were analysed by agarose gel electrophoresis (1 %, w/v) in TAE buffer (0.04 M Tris-acetate, 0.02 M acetic acid, 0.001 M EDTA), containing 1 μg mL^{-1} of ethidium bromide.

5.4.3 Sequencing and analysis of 16S rRNA gene

Automated sequencing of 16S rRNA gene from isolates was carried out by cycle sequencing using the dye terminator method. Sequencing was carried out at the Sequencing Service of the Macrogen Laboratory (The Netherlands). The closest relatives of isolates were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST (Altschul *et al*., 1997), and the "Seqmatch" and "Classifier" programs of the Ribosomal Database Project II (http://rdp.cme.msu.edu/). Sequences were further aligned using the program Clustal W (Thompson *et al*., 1994) to the most similar orthologous sequences retrieved from database. Each alignment was checked manually, corrected and then analysed using the Neighbour-Joining method (Saitou and Nei, 1987) according to the model of Jukes-Cantor distances. Phylogenetic tree was constructed using the MEGA 7 (Molecular Evolutionary Genetics Analysis) software (Kumar *et al*., 1993). The robustness of the inferred trees was evaluated by 500 bootstrap re-samplings. Isolates are part of the Italian

Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA, www.mna.it) “Felice Ippolito” kept at the University of Messina. They are currently maintained on TSA slopes at 4 °C and routinely streaked on agar plates from tubes every six months to control purity and viability. Antarctic strains are also preserved by freezing cell suspensions at – 80 °C in TSB to which 20 % (v/v) glycerol is added.

Study of permafrost samples: RESULTS

5.5 Analysis of prokaryotic community composition by NGS

Based on the OTU table that was generated by sequencing, the analysis of the different phylogenetic groups found within the Edmonson Point sample was carried out. The prokaryotic community composition was evaluated only for a sample of permafrost active layer. This environment was selected for a microbiological study due to the possible influence deriving from the occurrence of interconnections with atmosphere and seasonally changes. The analysis showed a total of 330 OTUs distributed in 10 different bacterial phyla, with the predominance of *Proteobacteria* and *Actinobacteria* (34.0 and 19.7 % of total sequences, respectively), followed by the *Acidobacteria*, *Nitrospirae*, *Chloroflexi*, *Firmicutes* and *Bacteroidetes*, which ranged between 4.9 and 7.5 % of total sequences (Figure 5.2). The *Gemmatimonadetes* (2.6 %), *Chlorobi* (0.7 %) and *Cyanobacteria* (1.3 %) constituted a minor component in the sample EP. Differences in relative abundances were observed for sequences affiliated to proteobacterial classes as they were mainly referred to the *Alpha-* (11.3 %), *Beta-* (11.1 %), and *Gammaproteobacteria* (9.4 %), whereas the *Delta-* (1.5 %) and *Epsilonproteobacteria* (0.5 %) were less represented. A number of sequences (9.5 % of total sequences) were not identified at phylum level.

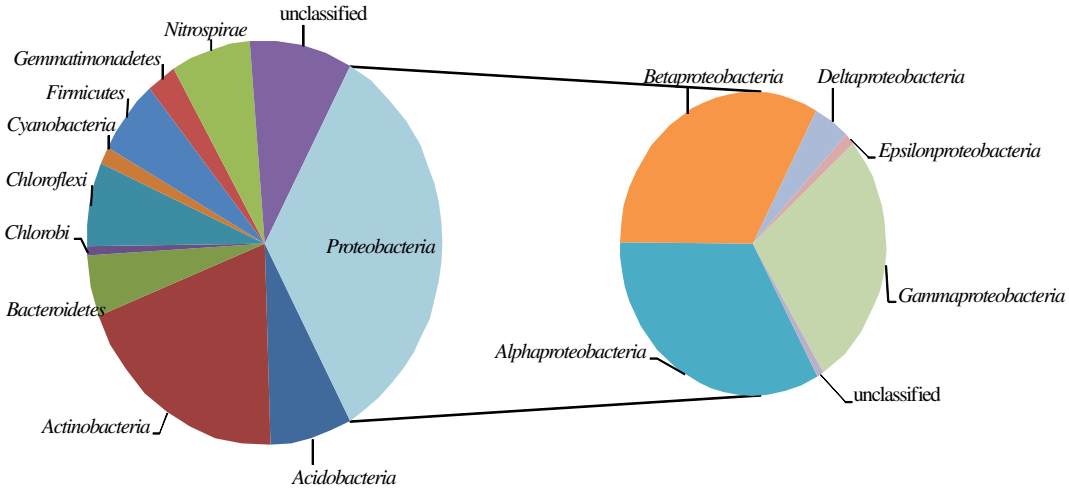

Figure 5.2 Phylogenetic affiliations of Antarctic bacteria as determined by Ion Torrent analysis

In details, 67 % of sequences were not classified at genus level. A total of 58 genera were resolved from the rest, ranging from 0.1 to 5.4 % of total sequences (Table 5.2). Only four genera occurred at ≥1 % of the total bacterial sequences and were related to the *Firmicutes*, *Actinobacteria*, and *Nitrospirae*.

Table 5.2 Bacterial genera retrieved in permafrost active layer at Edmonson Point.

Phylum or Class	Genus
Alphaproteobacteria	*Acidiphilium*
	Candidatus *Alysiosphaera*
	Anderseniella
	Pseudolabrys
	Candidatus *Pelagibacter*
	Rhodobium
	Sandarakinorhabdus
	Sphingomonas
Betaproteobacteria	*Aquincola*
	Burkholderia
	Dechloromonas
	Nitrosospira
	Candidatus *Nitrotoga*
	Polynucleobacter
	Rhizobacter
Deltaproteobacteria	*Syntrophus*
Epsilonproteobacteria	*Sulfurovum*
Gammaproteobacteria	*Acinetobacter*
	Arenimonas
	Pseudomonas
Bacteroidetes	*Ferruginibacter*
	Flaviramulus
	Gillisia
	Hymenobacter
	Lutimonas
	Niastella
	Polaribacter
	Schleiferia
	Winogradskyella
Acidobacteria	*Blastocatella*
	Bryobacter
	Granulicella
	Candidatus *Solibacter*
Actinobacteria	*Actinoplanes*
	Corynebacterium
	Crossiella
	Cryobacterium
	Demequina
	Friedmanniella
	Gaiella
	Micrococcus
	Modestobacter
	Marmoricola
	Mycobacterium
	Nocardioides
	Propionibacterium
	Sediminibacterium
	Solirubrobacter
Firmicutes	*Bacillus*
	Lactobacillus
	Streptococcus
	Clostridium
Gemmatimonadetes	*Gemmatimonas*
Nitrospirae	*Nitrospira*
Cyanobacteria	*Synechococcus*
	Microcoleus
	Tychonema
	Anabaena

0.1-0.5%
0.6-0.9%
1-5%
>5%

Within the class of the *Proteobacteria*, the *Alphaproteobacteria* were the most abundant and mainly represented by the *Candidatus Pelagibacter*, and the genera *Sphingomonas* and *Acidiphilium*. The *Betaproteobacteria* were represented by seven genera (e.g. *Aquincola*, *Nitrosospira*, *Polynucleobacter*) with percentages in the range 0.1-0.5 % of total sequences. The *Deltaproteobacteria* and *Epsilonproteobacteria* were represented by one genus each (*Syntrophus* and *Sulfurovum*, respectively). Within the

Gammaproteobacteria the genus *Acinetobacter* was the most represented, followed by *Pseudomonas* and *Arenimonas*. The phylum *Bacteroidetes* presented nine genera, in particular *Ferruginibacter* was the most represented, followed by *Flaviramulus*, *Gillisia*, *Lutimonas*, *Polaribacter* and *Winogradskyella*. Fifteen genera were observed within the *Actinobacteria* with *Marmoricola* and *Propinonibacterium* that resulted at higher percentages (4.4 and 3.2 % of sequences, respectively), followed by *Actinoplanes*, *Crossiella*, and *Gaiella* in a range of 0.6-0.9 % of total sequences. Four genera were obtained within the *Firmicutes*, with the genus *Lactobacillus* that was the most abundant (5.4 %). The *Nitrospirae* were represented only by the genus *Nitrospira* (3.5 %). Finally, the *Cyanobacteria* were represented by four genera, ranging from 0.1 to 0.9 % of total sequences.

5.6 Enumeration of cultivable heterotrophic bacteria

Soil samples yielded bacterial colonies following direct plating on R2A (range 0.6-27.5 x 10^3 CFU g^{-1} of wet soil, EP and BC-3 respectively) and TSA_{100} (range 0.7-20.4 x 10^3 CFU g^{-1} of wet soil, BC-1 and EP respectively) after incubation at 4 °C (Table 5.3). Exception was the oldest sample DY that yielded no colonies on R2A plates. By direct plating the range of colony number was in the order of 10^3. After enrichment in TSB at different strengths viable counts were three to six orders of magnitude higher than those obtained by direct plating. Bacterial colonies generally grew on both R2A [between 7.5 x 10^5 and 75.0 x 10^9 CFU g^{-1} of wet soil, DY (TSB_1) and DY (TSB_{50}), respectively; exceptions were BC-1 and BC-3 after enrichment in TSB_{50} that yielded no colonies] and TSA plates [between 1.8 x 10^6 and 2.8 x 10^9 CFU g^{-1} of wet soil, BC-3 (TSB_{50}) and BC-1 and BC-3 (TSB_{100}), respectively; with the exception of DY after enrichment in TSB_{50}, that yielded no colonies]. Pre-enrichments in TSB_{50} were in the order of 10^6-10^7. After enrichment in TSB_1 bacterial colonies grew on TSA_1 only from sample EP (i.e. 6.9 x 10^7 CFU g^{-1}). In the case of sample BC-2, the icy sample, after enrichment viable counts resulted quite similar on R2A (range 2.8- 3.0 x 10^3 CFU mL^{-1} of melted ice) and TSA (range 1.5-2.9 x 10^3 CFU mL^{-1} of melted ice) plates. The range of CFUs in melted ice was six orders lower than the soil sample (Table 5.3).

Table 5.3 Viable counts in permafrost samples. R2A and TSA media were used for direct plating and enrichment.

Sample *	Viable counts on ** ($CFUg^{-1}$ of wet soil)			
	R2A	TSA_{100}	TSA_{50}	TSA_1
EP	0.6×10^3	20.4×10^3		
EP (TSB_1)	64.0×10^9			6.9×10^7
EP (TSB_{50})	7.5×10^7		7.2×10^7	
EP (TSB_{100})	2.5×10^9	4.1×10^8		
BC-1	1.0×10^3			
BC-1 (TSB_1)	1.9×10^6	0.7×10^3		0
BC-1 (TSB_{50})	0		2.0×10^6	
BC-1 (TSB_{100})	75.0×10^6			
BC-3	27.5×10^3	3.3×10^3		
BC-3 (TSB_1)	5.0×10^6			0
BC-3 (TSB_{50})	0		1.8×10^6	
BC-3 (TSB_{100})	2.8×10^9	2.8×10^9		
DY	0	13.1×10^3		
DY (TSB_1)	7.5×10^5			0
DY (TSB_{50})	75.0×10^9		0	
DY (TSB_{100})	4.1×10^9	2.8×10^9		

Sample *	Viable counts on ** ($CFUg^{-1}$ of wet soil)			
	R2A	TSA_{100}	TSA_{50}	TSA_1
BC-2 (TSB_1)	2.9×10^3			
BC-2 (TSB_{50})	3.0×10^3		2.9×10^3	1.5×10^3
BC-2 (TSB_{100})	2.8×10^3	2.7×10^3		

*** EP**, **BC-1**, **BC-3** and **DY**: natural samples, not enriched; **EP (TSB_1)**, **BC-1(TSB_1), BC-2 (TSB_1)**, **BC-3 (TSB_1)**, **DY (TSB_1)**: samples enriched in TSB at 1 % strength; **EP (TSB_{50})**, **BC-1 (TSB_{50})**, **BC-2 (TSB_{50})**, **BC-3 (TSB_{50})**, **DY (TSB_{50})**: samples enriched in TSB at 50 % strength; **EP (TSB_{100})**, **BC-1 (TSB_{100})**, **BC-2 (TSB_{100})**, **BC-3 (TSB_{100})**, **DY (TSB_{100})**: samples enriched in TSB at 100 % strength.

** TSA_{100}, TSA_{50} and TSA_1: plates of TSA at full, 50 % and 1 % strength respectively.

5.7 Isolation of bacterial strains

Overall, a total of 673 strains were isolated from permafrost samples. Among them, 101 strains were isolated by the direct plating approach (47 and 54 from R2A and TSA_{100} plates, respectively). In details, a total of 19 and 6 strains were isolated on R2A and TSA_{100}, respectively, from BC-1, while 12 and 17 strains were achieved on R2A and TSA_{100}, respectively, from BC-3. In the case of EP, 16 and 10 strains were isolated on R2A and TSA_{100}, respectively, while 47 and 54 were obtained from DY on R2A and TSA_{100}, respectively.

A total of 572 strains were isolated by the enrichment culture approach. In particular, 289 strains were isolated from R2A enrichments. Enrichment in TSB_{100} and TSA_{50} allowed obtaining the highest and lowest number of isolates, respectively, from BC-1. No isolates were obtained after enrichment on TSA_{50} from BC-3, whereas for such sample the highest number of isolates (30 isolates) were obtained on TSA_{100}. A total of 30 and 140 strains were isolated on TSA_{100} from EP and DY enrichment, respectively.

The remaining 283 strains were isolated from TSB enrichments on TSA media. Isolates were obtained from all samples, with 82, 25 and 40 that derived from BC-1, BC-3 and EP, respectively. The natural enrichment of BC-2 allowed the recovery of 50 and 95 strains from R2A and TSA enrichments, respectively.

Based on colony morphology (e.g. color, margin, profile, surface), a total of 196 bacterial isolates were selected for further analyses (Table 5.4).

Table 5.4 Number of isolates obtained from direct planting and enrichments *per* sample and isolation medium.

Treatment	BC-1				BC-2				BC-3			
	R2A*	TSA_1	TSA_{50}	TSA_{100}	R2A*	TSA_1	TSA_{50}	TSA_{100}	R2A*	TSA_1	TSA_{50}	TSA_{100}
Direct plating	9	nt	nt	3	nt	nt	nt	nt	2	nt	nt	9
Enrichment	23	4	9	15	13	1	7	3	9	0	1	2
				63				**24**				**23**

Treatment	EP				DY					
	R2A*	TSA_1	TSA_{50}	TSA_{100}	R2A*	TSA_1	TSA_{50}	TSA_{100}		
Direct plating	4	10	nt	1	0	nt	nt	2		
Enrichment	33	2	9	10	7	5	0	3		
				68				**17**	***Total***	***196***

*R2A: isolates from R2A plates derived from different enrichments

**: not tested (see materials and methods for details)

Among them, 40 isolates (mainly from EP and BC-3; 15 and 12 isolates, respectively) were obtained by direct plating (TSA_{100} and R2A) that yielded the same number of isolates (15 isolates each). Conversely, the enrichment procedure allowed to obtain 156 isolates (mainly from BC-1, 51 isolates). In this case, they were mainly retrieved from R2A agar plates (85 isolates), followed by TSA_{100} (33 isolates), TSA_{50} (26 isolates) and TSA_1 (12 isolates). In general, the highest number of isolates (68) were selected from EP.

5.8 Sequencing and analysis of 16S rRNA gene

As stated above, a total of 196 strains were selected and their 16S rRNA gene was sequenced to achieve their phylogenetic affiliation. The sequences of bacterial strains were compared with database online after the correction with ChromasPro software. Overall, the cultivable bacteria were distributed within five different taxa, with the predominance of *Firmicutes* (119 isolates), followed by *Actinobacteria* (44 isolates), *Gammaproteobacteria* (23 isolates), *Alphaproteobacteria* (8 isolates) and *Betaproteobacteria* (2 isolates). Members in these two latter groups were isolated only after enrichment, with the *Alphaproteobacteria* that were obtained only from BC samples

and *Betaproteobacteria* only from sample EP. The distribution of detected phylogenetic groups in individual samples is shown in Figure 5.3.

All sequences with similarity ≥97 % were considered to represent one phylotype and were grouped in Operational Taxonomic Units (OTUs). A total of 22 OTUs were obtained, in addition to seven sequences that were obtained from one isolate each (isolates C3, A2, N6, P2, D16 and S11) (Table 5.5). The majority of OTUs were retrieved from single samples, whereas few OTUs were shared between different sites and/or samples. It was the case of OTUs 5, 3 and 18 that were shared between BC samples, and OTUs 1, 2, 8, 9, 10 and 11 that BC samples shared with EP and/or DY samples (Table 5.5).

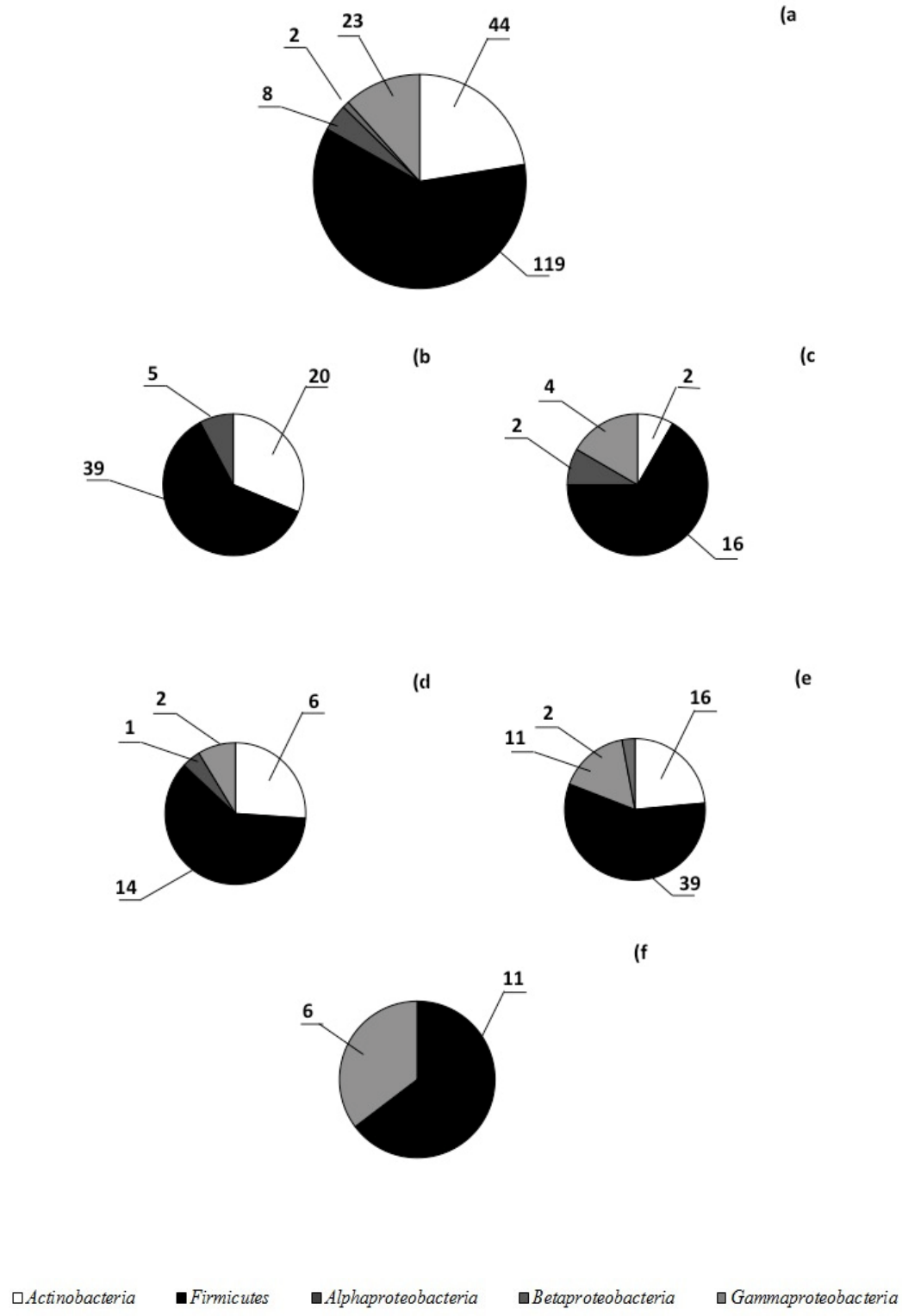

Figure 5.3 Phylogenetic affiliation of isolates from the permafrost samples. a: Total diversity; b: BC-1; c: BC-2; d: BC-3; e: EP; f: DY

Table 5.5 16S rRNA gene sequence affiliation to their closest phylogenetic neighbors of Antarctic isolates.

Next relative by GenBank alignment (AN[a], organism)	RI[b]	AN[a]	OTU[c]	Isolation medium	Treatment	Hom[e] (%)	Origin of next relative organism	Sample				
								BC-1	BC-2	BC-3	EP	DY
Alphaproteobacteria												
AM292064, *Brevundimonas bullata* isolate zf-69-I	L2	**KT965158**	5	TSA_1	TSB_1	99	East Rongbuk Glacier, Mt. Everest	5	2	1	-	-
Betaproteobacteria												
KC433619, *Polaromonas* sp. L19.9	B23	**KT965159**	6	TSA_1	TSB_1	98	Antarctica	-	-	-	2	-
Gammaproteobacteria												
KJ575020, *Pseudomonas stutzeri* strain NIOT-Ba-28	C3	**KT965171**	na	R2A	DP	97	Deep-sea sediment, Bay of Bengal	-	-	1	-	-
FN377723, *Pseudomonas* sp. VI-1	S21	**KT965160**	7	TSA_1	TSB_1	99	Kongsfjord, Svalbard Islands	-	-	-	-	3
JQ684240, *Psychrobacter cryohalolentis* strain HWG-A17	F25	**KT965161**	8	TSA_{50}	TSB_{50}	100	Permafrost soil, Tibet Plateau	-	4	-	6	-
JX005876, *Stenotrophomonas* sp. SOZ3-5081	S27	**KT965162**	9	TSA_1	TSB_1	99	Antarctic soil	-	-	1	-	3
Actinobacteria												
FN377733, *Arthrobacter* sp. SH-61B	P3	**KT965163**	10	TSA_{100}	DP	99	Marine sediment, Svalbard	3	-	-	1	-
GU176043, *Arthrobacter* sp. HY11	A2	**KT965170**	na	R2A	DP	97	Antarctic seawater	-	-	-	1	-
JN662538, *Arthrobacter sulfonivorans*	A5	**KT965164**	11	R2A	DP	99	Soils, Tianshan Glacier	2	-	-	11	-
DQ108399, *Citrococcus* sp. Tibet-ITa3	D9	**KT965166**	13	TSA	DP	99	Permafrost, Tibet plateau	-	-	6	-	-
GU733465, *Cryobacterium* sp. Lc30-4	L11	**KT965167**	14	TSA_1	TSB_1	99	Lake sediment, Antarctica	5	-	-	-	-
KC478079, *Leifsonia* sp. FO17	N10	**KT965169**	16	R2A	DP	100	Ice-covered Antarctic lake	3	-	-	-	-
KJ475136, *Marisediminicola antarctica* PAMC 27228	N26	**KT965165**	12	R2A	DP	99	Antarctic marine sediment, Ross Sea	5	-	-	-	-
KR007639, *Microbacterium* sp. SPO40	A29	**KT965172**	22	R2A	TSA_1	100	Polar oceanic water	-	-	-	3	-
GU082325, *Microbacterium* sp. clone ts0625T	E10	**KT965168**	15	R2A	TSB_{100}	99	Surface snow	-	2	-	-	-
AB362249, *Microcella alkaliphila*	N6	**KT965173**	na	R2A	DP	98	Sediment, Shimokita Peninsula	1	-	-	-	-
KF928904, *Tessaracoccus* sp. T18	P2	**KT965181**	na	TSA_{100}	DP	99	Antarctic lakes	1	-	-	-	-
Firmicutes												
LN774311, *Bacillus nealsonii* isolate 0511TES26Z1	D16	**KT965174**	na	TSA_{50}	TSB_{50}	98	Soil	-	-	1	-	-
KF818647, *Bacillus simplex* strain 265XG8	D26	**KT965154**	1	TSA_{100}	TSB_{100}	100	Alpine grassland	15	-	10	39	9
EF523244, *Bacillus thuringiensis* strain Dg-1018	D1	**KT965155**	2	TSA_{100}	DP	100	Russian soil	1	-	3	-	1
AY660701, *Bacillus* sp. cryopeg_9	L52	**KT965157**	4	TSA_{100}	TSB_{100}	97	Siberian permafrost	12	-	-	-	-
AY660700, *Bacillus* sp. cryopeg_4b	L35	**KT965156**	3	TSA_{50}	TSB_{50}	99	Siberian permafrost	3	1	-	-	-
KC355292, *Paenibacillus agarexedens* KUDC1785	S11	**KT965175**	na	TSA_{100}	DP	99	Rhizosphere	-	-	-	-	1
FJ932656, *Paenibacillus* sp. MC5-1	P13	**KT965176**	17	TSA_{100}	TSB_{100}	99	Antarctic snow, Dome A	2	-	-	-	-
JX195130, *Paenibacillus* sp. AA1 isolate EXG-12	E12	**KT965177**	18	R2A	TSB_{50}	100	Antarctic snow, Dome C	2	2	-	-	-
KF026330, *Sporosarcina globispora* strain HEN5	L46	**KT965178**	19	TSA_{100}	TSB_{100}	99	Antarctic soil	4	-	-	-	-
JF778686, *Sporosarcina* sp. DRB15	E41	**KT965179**	20	R2A	TSB_1	99	Permafrost active layer, Tibet plateau	-	5	-	-	-
KP745566, *Sporosarcina aquimarina* strain 43Ls.3	F18	**KT965180**	21	TSA_{50}	TSB_{50}	100	Antarctic rocks	-	8	-	-	-

[a]AN: Accession Number; [b] RI: representative isolate; [c] na: not assigned; [d] Treatment: DP, direct plating; [e]Hom: sequence homology

As it was shown in the Table 5.5, the *Firmicutes* contained 11 OTUs distributed among three genera of the phylum, as follows: *Bacillus* (95 isolates in five OTUs), *Sporosarcina* (17 isolates in three OTUs) and *Paenibacillus* (seven isolates in three OTUs). In EP 39 strains were found to belong to the genus *Bacillus* (OTU1), followed by BC-1 with 31 strains distributed in all five OTUs, while BC-2 only one isolate belonging to OTU3 was recovered. The 11 OTUs within the Actinobacteria formed two distinct clusters: the first one included *Arthrobacter* spp. (OTUs 10 and 11, and isolate A2, with 17 strains distributed in EP and BC-1) and *Citricoccus* members (OTU13, present only in BC-3 with six strains), while the second cluster branched in several sub-clusters that included, among others, the genera *Cryobacterium* (five isolates within the OTU14 in sample BC-1) and *Marisediminicola* (five isolates within the OTU12 in sample BC-1) as the dominant representatives, while the genera *Microbacterium* was includes in two OTUs (OTU22 and OTU15, three strains in EP and two strains in BC-2, respectively). The six OTUs belonging to the Proteobacteria phylum felt into three of the five classical classes: *Alpha-*, *Beta-* and *Gammaproteobacteria*. The *Gammaproteobacteria* (four phylotypes) clustered in two OTUs: the first one was composed by a single OTU (OTU8 including ten isolates, six in EP and four in BC-2) which was strongly related to the genus *Psychrobacter*, whereas the second included members in the genera *Pseudomonas* (three isolates within the OTU7 and isolate C3, in DY and BC-3 respectively) and *Strenotrophomonas* (four isolates within the OTU9, three in DY and one in BC-3). Finally, only eight and two isolates were affiliated to the *Alpha-* (genus *Brevundimonas*; OTU5, distributed in BC samples) and *Betaproteobacteria* (genus *Polaromonas*; OTU6 in EP), respectively. The strains were related to known sequences that were recovered by Arctic and Antarctic habitats, generally recovered in snow or sediments, other in water or lakes. In particular OTU13, OTU4, OTU3 and OTU20 were related to bacteria isolated from permafrost habitats.

In the following figures (Figure 5.4, 5.5, 5.6) the phylogenetic trees of strains compared with known sequences in database are shown. The *Actinobacteria* formed two branches: the first branch is represented by one isolate (P2) affiliated to *Tessaracoccus flavescens* that was away from other isolates; the second one included isolates affiliated to the genera *Arthrobacter* and *Citrococcus*. Members in the genus *Arthrobacter* were represented by 13 isolates within the OTU11, in addition to isolate A2, that were mainly related to *A. siccitolerans* and *A. psychrochitiniphilus*, and by four strains within the OTU10 related to *A. flavus*. The genus *Citrococcus* was represented by six strains in OTU13 and were related to *C. muralis* and *C. zhacaiensis*. The third branch was divided in three different ramifications, the first one was represented by two strains in OTU15, and three strains within the OTU22 affiliated to the genus *Microbacterium*. The second ramification was represented by five strains in OTU14 related to *Cryobacterium psychrophilum*. Finally, isolate N6 (from BC-1) which was related to *Microcella alkaliphila*, three isolates in OTU16 which were affiliated to *Leifsonia rubra*, and five isolates in OTU12 that were related to *Marisediminicola antartica*, constitute the third ramification (Figure 5.4).

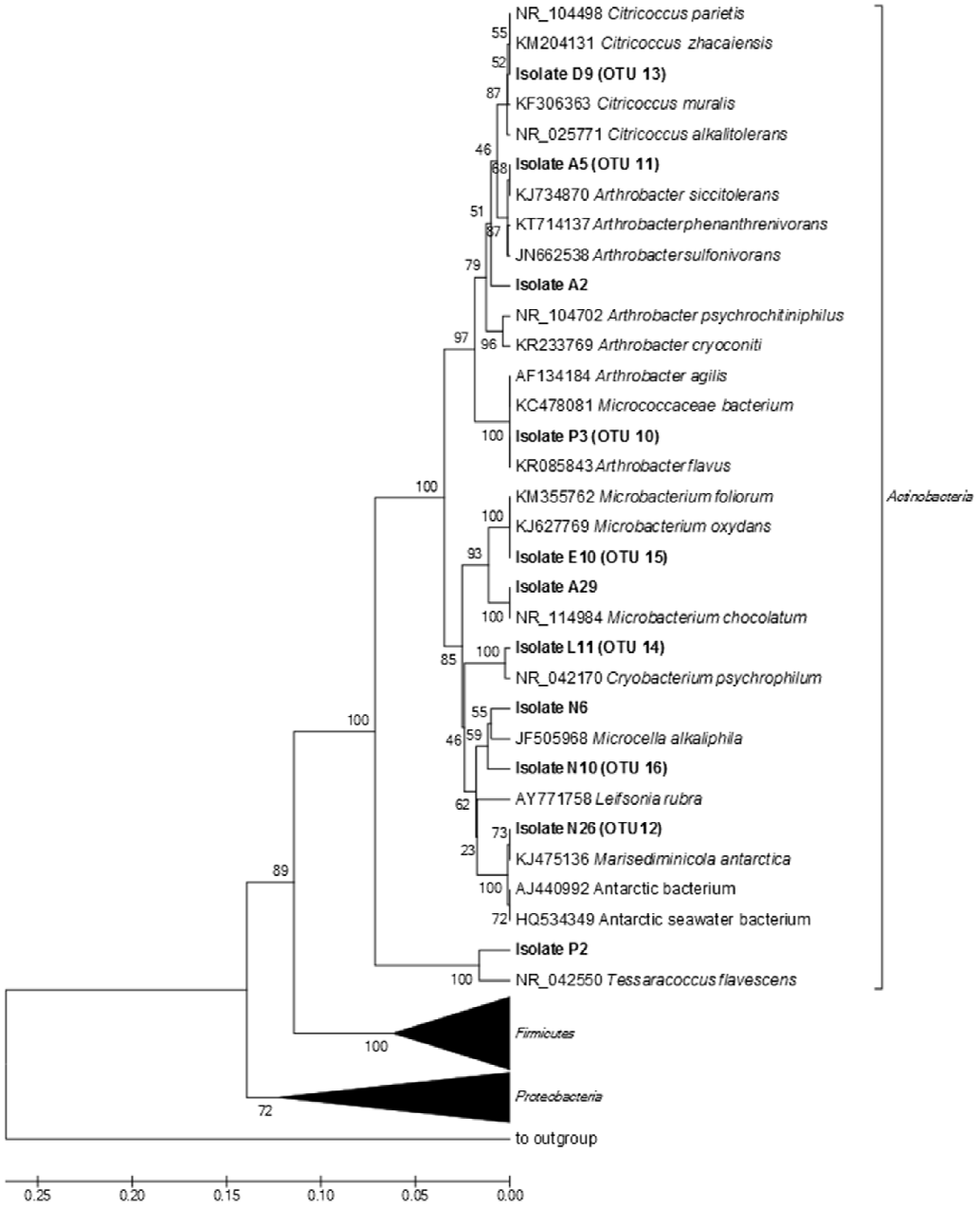

Figure 5.4 Actinobacteria Phylogenetic Tree

Two different branches formed the phylogenetic tree of *Firmicutes*. The first one was constituted by *Paenibacillus* strains, with four strains in OTU18, two strains in OTU17 and isolate S11. The second branch was the *Bacillus* ramification, mainly represented by two OTUs (OTU 1 and 2) and one isolate (D16). The OTU1 was constituted by 73 strains well distributed in the samples and related to *B. simplex*. Five strains were within the

OTU2 and related to *B. thuringiensis*, while the isolate D16 was recovery in BC-3 sample and related to *B. nealsonii*. A second ramification of the same branch was the biggest, with four sub-ramifications inside. OTU3 constituted the first sub-ramification with four strains affiliated to *Bacillus psychrodurans,* the second sub-ramification was formed by 12 strains in OTU4 related to *Bacillus* sp. and the third sub-ramification was represented by OTU20 (five strains) related to *Paenisporosarcina macmurdoensis*. The fourth sub-ramification was formed by OTU21 (eight strains) related to *Sporosarcina aquimarina* and the last sub-ramification was formed by OTU19 (four strains) related to *Sporosarcina globispora* (Figure 5.5).

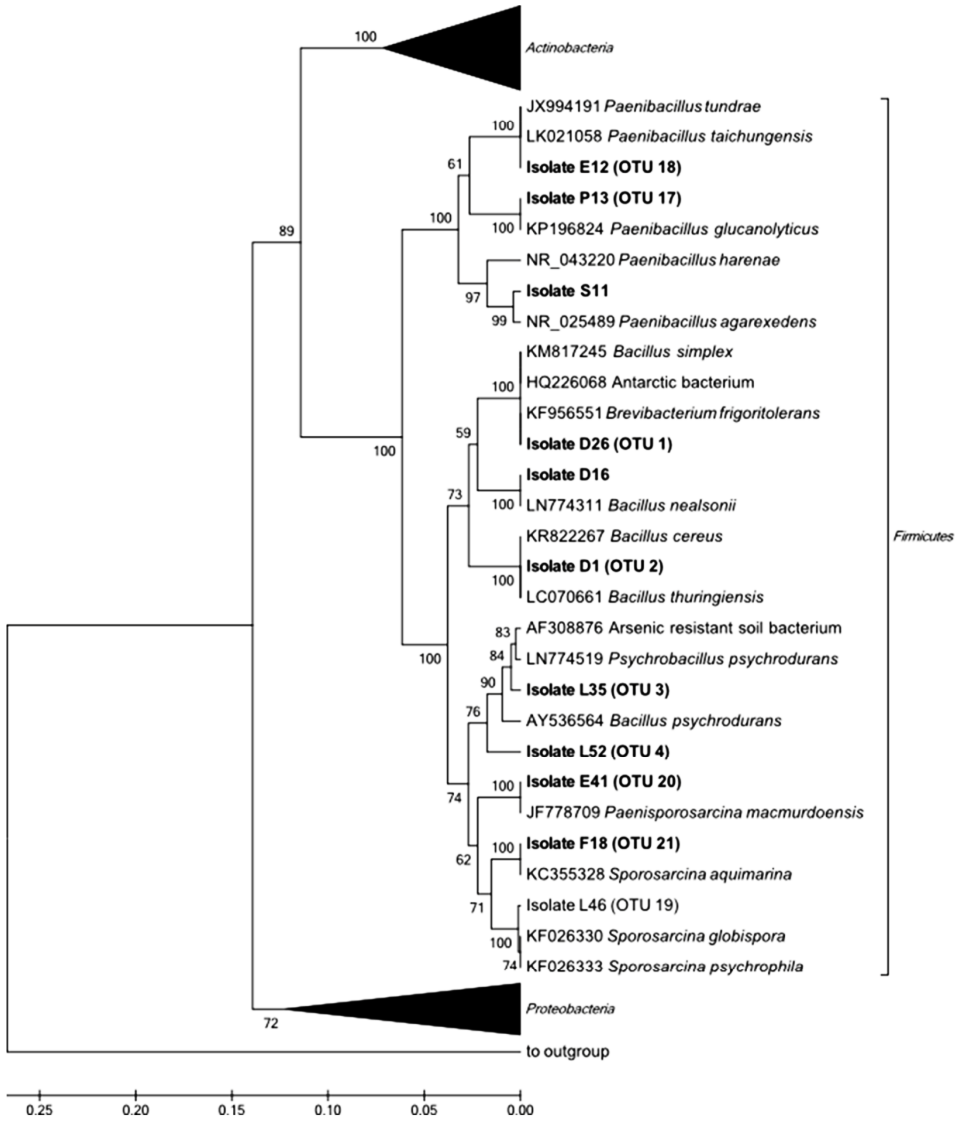

Figure 5.5 Phylogenetic Tree of Firmicutes

The *Proteobacteria* tree was constituted by three sub-phyla: *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. *Alphaproteobacteria* branch was constituted by OTU5 (eight strains in BC samples) related to *Brevundimonas bullata*. The *Betaproteobacteria* branch was represented by OTU6 (two strains recovered in EP sample). The *Gammaproteobacteria* was the widest branch, with three ramifications, one constituted by ten strains in OTU8 affiliated to *Psychrobacter cryohalolentis* (recovered

in EP and BC-2 samples), the second ramification that was constituted by four strains recovered in DY and BC-3 belonging to OTU9 and affiliated to *Stenotrophomonas rhizophila*, and the last ramification that was formed by isolate C3 recovered in samples BC-3 and related to *Pseudomonas stutzeri*, in addition to three strains (recovered from DY) belonging to OTU7 related to *Pseudomonas brenneri* (Figure 5.6).

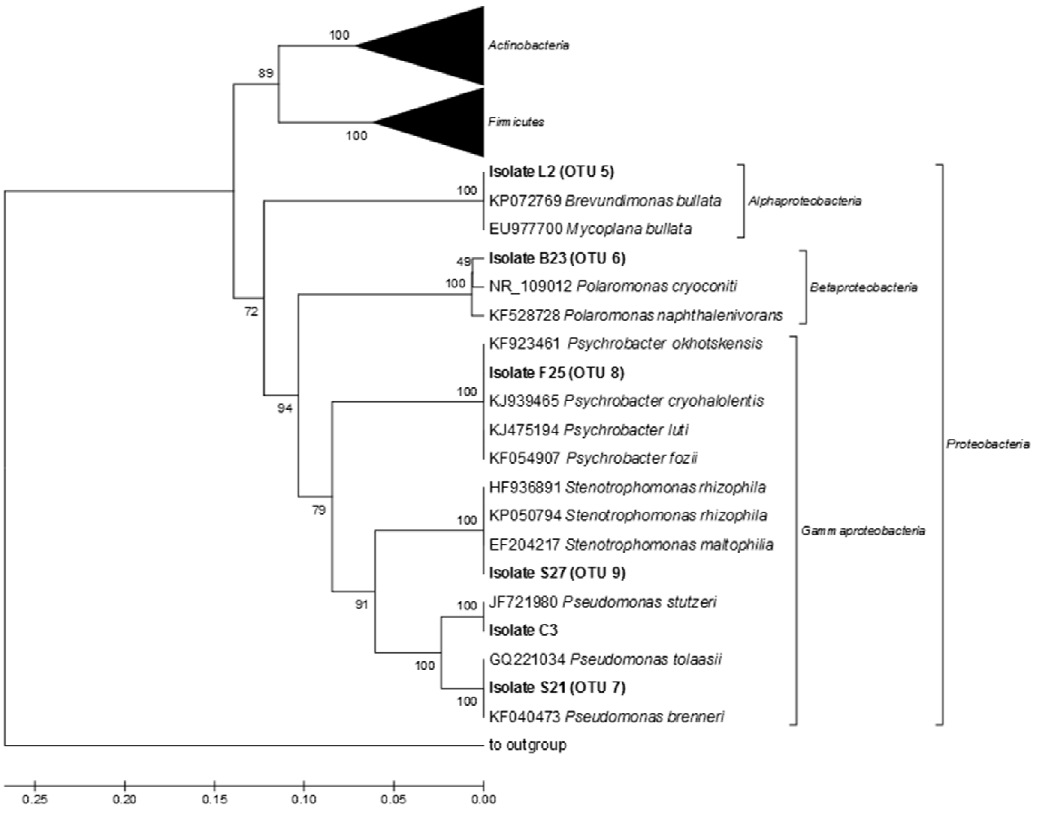

Figure 5.6 Phylogenetic Tree of Proteobacteria

Study of permafrost samples**: DISCUSSION**

The higher biodiversity in Antarctic permafrost can be understood in terms of where the microorganisms in subsurface frozen sediments originated. One aim of this study was to analyse the prokaryotic community composition inhabiting the permafrost active layer using a Next Generation Sequencing (NGS) approach. This layer is a particular cryo-environment continuously exposed to atmosphere during geological time. This feature is responsible for a seasonally variation in this habitat that is constantly subjected to weather conditions. The prokaryotic community, therefore, varies during summer and during winter time. Even, the microbial community undergoes to weather changes, the cold temperature, water available and poor nutrient presence, and survive in these ecological niches. The molecular approaches used to estimate the microbial communities were useful to recovery a high number of sequences, despite the harsh conditions of cryosoil (Whilhelm *et al*., 2011). Furthermore, the soil bacterial communities have relatively low diversity compared to temperate soils and may be dominated by few bacterial phylotypes. Clones retrieved from the soils typically group with the phyla *Acidobacteria*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Cyanobacteria* (Aislabie *et al*., 2007). A high portion of phylotypes observed in culture-independent analyses of Antarctic soil is from unknown or unclassified bacteria. This could suggest that they are unique to Antarctic soils or that similar environments globally have not been microbiologically well characterized (Vincent, 2000).

The phylum *Cyanobacteria* is commonly reported for Antarctic soil (Buckley and Schmidt, 2002). The phyla *Actinobacteria* and *Bacteroidetes* appear to be widespread in Antarctica, while some genera are not dispersed in the continent (e.g. *Acidobacteria*). The phylum *Firmicutes* is more prevalent in ornithogenic soil in Antarctica, as this site that is near penguin rookeries.

The phylum of *Proteobacteria*, in general, dominated the soil sequences, which frequent members of the *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria* classes (Yergeau *et al*., 2007), and was the best represented in this active layer sample as previously reported by Zhou *et al*. (1997), Costello and Schmidt (2006), Dedysh *et al*. (2006). Several phyla were significantly influenced by soil physicochemical factors, as the proportion of *Chloroflexi* and *Betaproteobacteria* were negatively correlated with pH, while the proportion of *Firmicutes* was positively correlated with pH (Yergeau *et al*., 2009). Vegetation has a pronounced influence on bacterial community characteristics in the Antarctic Peninsula. Edmonson point is characterised by moss vegetation and the nearness to penguin rookeries. The *Bacteroidetes* presence could be due to the mosses, in that this phylum was found in enriched moss covered soils (Ganzert *et al*., 2011). Representatives of the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria* generally dominate the rhizophere soils. The high representation of *Firmicutes* in rhizosphere soils is notable as this group is not found to make up a substantial proportion of the bacterial communities in unvegetated soils of the region (Yergeau *et al*., 2007; Teixeira *et al*., 2010). Edmonson Point soil is affected by penguins. This ornithogenic soil contains levels of bacterial diversity that are similar to those of mineral soils in the same region, but differ in abundance and community composition (Aislabie *et al*., 2009). The *Bacteroidetes* occurrence was not surprising because these microorganisms are known to include several psychrophilic members (Shivja *et al*., 1992), commonly found in permafrost-affected soil ecosystem (Steven *et al*., 2008; Wagner *et al*., 2009), while the *Actinobacteria* also made-up an important part of the bacterial community and their successful colonization of the entire active layer likely being supported by their ability to metabolize a wide range of substrates as sole carbon source and their adaptation to low temperature (Wynn-Williams, 1996; Aislabie *et al*., 2006). The *Chloroflexi* phylum is common in sediments, especially in those located in zones that share similarities with fluvial aquifers worldwide (Hug *et al.,* 2013). Several studies have postulated a link

between GIF9 *Chloroflexi* and organic-rich sediments (Teske *et al.,* 2011; Takeuchi *et al.,* 2009; Harrison *et al.,* 2009), but currently their role within these communities is unknown. Teske *et al.* (2011) reported *Gammaproteobacteria* clones as dominant in superficial sediment samples in disaccord with this study, in contraposition with *Deltaproteobacteria* and *Acidobacteria* that occurred at low percentages in surface sediments.

The prokaryotic community inhabiting permafrost active layer in Edmonson Point, Antarctica, resulted various and diverse. In particular, different phylogenetic phyla were retrieved and the sequences were well distributed in each phylum. Five sub-classes of bacteria represented the *Proteobacteria* with predominance of *Alpha-*, *Beta-* and *Gammaproteobacteria*, typical of Antarctic soil. This metagenomic study could be used as a starter point to analyse and study the microbial community composition in permafrost active layer, contributing to our knowledge on how global ecological changes may impact communities, as it continuously undergoes to atmospheric events.

The present study focuses on the opportunity to find viable microorganisms inside these habitats and study their physiological characteristics and biotechnological potential. The interest in permafrost microorganisms was first dictated by the need to improve protocols to recovery bacteria in ancient frozen sediments. The different protocols involve diverse adaptive strategies by microorganisms. The aim of the first objective was to study the best method to recovery microorganisms from different types (different lithology, vegetation and age) of permafrost samples, from active layer (EP) to deeper sample (DY). The cultivation on R2 Agar medium, an oligotrophic medium, was the best method for the recovery of bacteria from Antarctic permafrost samples. Diluted media (i.e. TSA 50 % and TSA 1 %) generally yielded lower numbers of bacterial colonies than full strength media (i.e. TSA 100 % and R2A). In line with previous observations (Vishnivetskaya *et al*., 2000), these showed also great colony diversity. The preliminary liquid enrichment of samples resulted in a pronounced increase in the viable counts and, in turn, it allowed

obtaining highest numbers of bacterial isolates. The direct plating showed an amount of 10^3 CFU g^{-1} on R2A and TSA media in accordance to Soina *et al*. (2004) in Antarctic permafrost samples. The enrichment method was the best method to recovery colonies because showed an amount of colonies of 10^5-10^9 CFU g^{-1} both in rich and oligo-medium. The obtained number of colonies was strongly dependent on the enrichment method as media at different concentration were used. These results are in accordance to Siberian (Rivkina *et al*., 1998; Gilichinsky *et al*., 2002) and Arctic permafrost and are similar to tundra soils. The natural enrichment of ice showed a low recovery of colonies (10^3 CFU g^{-1}). Isolates from ice are generally low respect to water and soil samples and ice often presents a reduced colony recovery. In fact, obtained results were similar to Miteva *et al*. (2004) in Greenland glacier ice within permafrost. Viable bacteria are rarely recovered from pure ice system such as ice widges (Gilichinsky *et al*., 1995; Gilichinsky *et al*., 2002a) or massive ground ice formations. Furthermore, in some samples the natural enrichment treatment facilitated a recovery of cells from a viable but non-culturable state (Steven *et al*., 2006).

Significant numbers of viable bacteria (10^2 to 10^8 cells g^{-1}) are known to be present in permafrost that is 1 to 3 million years old in the Arctic (Gilichinsky *et al*., 1995; Rivkina *et al*., 1998; Vestal *et al*., 1988; Vorobyova *et al.*, 1997) and in permafrost that is probably older in Antarctica (Wilson *et al*., 1998; Friedmann *et al*., 1996). Preliminary incubation with cryo-protectants such as salts, alcohols and/or sugars could be incorporated in culture media to enhance cellular survival and recovery. The cryo-protectant could be used to lower the freezing point of culture media to simulate permafrost temperatures and recovery some colonies that are non-culturable at higher temperature (4 °C) than environmental temperatures.

Based on the bacterial identification by the 16S rRNA gene sequencing, liquid enrichment and direct plating of samples generally resulted in the isolation of no-overlapping lineages, suggesting that the application of different treatments can facilitate

the recovery of genetically distinct bacteria (Vishnivetskaya *et al*., 2000). Isolates were grouped in 28 distinct phylotypes. Although some strains/phylotypes were isolates on all media, others were obtained only from full strength R2A, TSA_{100} and TSA_{50} or TSA_1. In particular, the use of diluted media allowed the isolation of almost all members within OTU5 (*Brevundimonas* sp.) among the *Alphaproteobacteria*, OTU6 (*Polaromonas* sp.) among the *Betaproteobacteria*, OTUs 7 and 9 (*Pseudomonas* sp. and *Stenotrophomonas* sp., respectively) among the *Gammaproteobacteria*, OTU11 (*Cryobacterium* sp.) and *Microbacterium* sp. (OTU22) among the *Actinobacteria*, and OTU20 (*Sporosarcina* sp.). Among them, the genus *Polaromonas* is widely distributed worldwide, probably depending on air-dispersion process, and generally dominated in polar and high-elevation environments, mainly in glacial ice and sediment samples (Darcy *et al*., 2011). Interestingly, its presence has been reported particularly in recently deglaciated substrates, as it was the case of active EP sample from which members of OTU6 were isolated (Frey *et al*., 2010; Darcy *et al*., 2011; Michaud *et al*., 2012; Franzetti *et al*., 2013). All the remaining genera cited above have been previously found in several freshwater and terrestrial habitats from Antarctica, also in association with plants and microbial mats (Suzuki *et al*., 1997; Pearce *et al*., 2003; Saul *et al*., 2005; Michaud *et al*., 2012; Vasileva-Tonkoba *et al*., 2014). At this purpose, the phylogenetic analysis allowed assigning sequences to their nearest phylogenetic neighbour that was generally associated to cold habitats (e.g. permafrost soils and glaciers), also located in polar areas, thus suggesting a bipolar distribution of isolates. Detected phylotypes were differently distributed among samples. In particular, the surface active sample BC-1 (including 15 distinct phylotypes) and the ancient DY (including five phylotypes) resulted more and less diverse than other samples, respectively. Surface soil at BC-1 (and EP) is probably more prone to exchanges with atmosphere than the deeper samples analysed in this study. In particular, the majority of phylotypes were isolated from individual samples, thus highlighting a different viable bacterial community composition, even among samples

from the same site (i.e. BC). The different sample lithology and features may account for some differences in bacterial community composition. Only OTU1 (*Bacillus* sp.) resulted to be ubiquitous as it was detected at all investigated sites. Results on the phylogenetic affiliation of Antarctic isolates are in line with those obtained by several authors for Arctic and Antarctic permafrost (e.g. Steven *et al*., 2007a; Gilichinsky *et al*., 2007). The predominance of Gram-positive bacteria (i.e. *Actinobacteria* and *Firmicutes*; 122 out of 196 isolates), mainly achieved from full strength media, is not surprising as they are generally thought to be associated with soil habitats, also in Antarctica (Jansson and Tas, 2014). The predominance of the *Firmicutes* has been previously reported for Antarctic permafrost (Dry Valleys) (Gilichinsky *et al*., 2007), whereas the *Actinobacteria* generally dominate in Arctic permafrost (Bakermans *et al*., 2014 and references therein). In particular, the 119 Firmicutes related to spore-forming bacterial genera (*Bacillus*, *Paenibacillus* and *Sporosarcina*), suggesting that the viable microbial community is surviving as spores rather than vegetative cells. On the other side, the *Actinobacteria* are known to enter a metabolically quiescent but viable state to adapt to stress (Jansson and Tas, 2014). These features make representatives of the *Firmicutes* and *Actinobacteria* cold survivers that are capable to revival and growth on culture media (Jansson and Tas, 2014). Among the *Actinobacteria*, *Arthrobacter* spp. are commonly identified in various terrestrial and aquatic habitats in the Antarctic (Ganzert *et al*., 2011; Dsouza *et al*., 2015). Among the 23 Gram-negative isolates, members in the OTU8 were strongly related to *Psychrobacter cryohalolentis*, a new species from Siberian permafrost which was first described by Bakermans *et al*. (2006). *Psychrobacter* species are cold-adapted and halo-tolerant, and they have been isolated from a variety of low-temperature marine environments (including Antarctic sea ice, ornithogenic soils and sediments, invertebrates, seawater). Other bacteria that are generally associated with the marine environment were detected, thus indicating a probable external input by atmospheric deposition (aerosols). This was the case of members in the OTU12 (isolated from BC-1),

which were strongly related to *Marisediminicola antarctica*, a recently described 646 species from sandy intertidal Antarctic sediment sample, East Antarctica (Li *et al*., 2010), and OTU21 (isolated from BC-2), which was strongly related to *Sporosarcina aquimarina* isolated from seawater (Yoon *et al*., 2001). Marine microorganisms could find a favorable environment for their growth in permafrost as metabolism at sub-zero temperatures occurs within salted brines (microorganisms living in permafrost are typically tolerant to high salt concentrations) (Bakermans *et al*., 2014).

6 Study of Antarctic brine samples

The second main aim of this work was to explore the prokaryotic community in saline brine samples collected in Antarctica. A brine is a liquid water that remains in this state below 0 °C and this main feature is interesting to investigate. The analyses were carried out using both culture-independent (next generation sequencing and CARD-FISH) and culture-dependent methodologies to examine the prokaryotic community composition occurring in brine samples from two geographically distinct lakes, one inland and one coastal site. The epifluorescence microscopy technique and the Next-generation sequencing approaches were chosen as culture-independent methods to investigate the prokaryotic community, while different isolation culture media and investigation on the biotechnological potential of brine isolates were chosen as culture-dependent methods.

6.1 Site description and characteristics of brines

Brine samples were collected from two sites in the Northern Victoria Land (Boulder Clay and Tarn Flat), close to the Italian Antarctic research station “Mario Zucchelli” (Figure 6.1) (MZS).

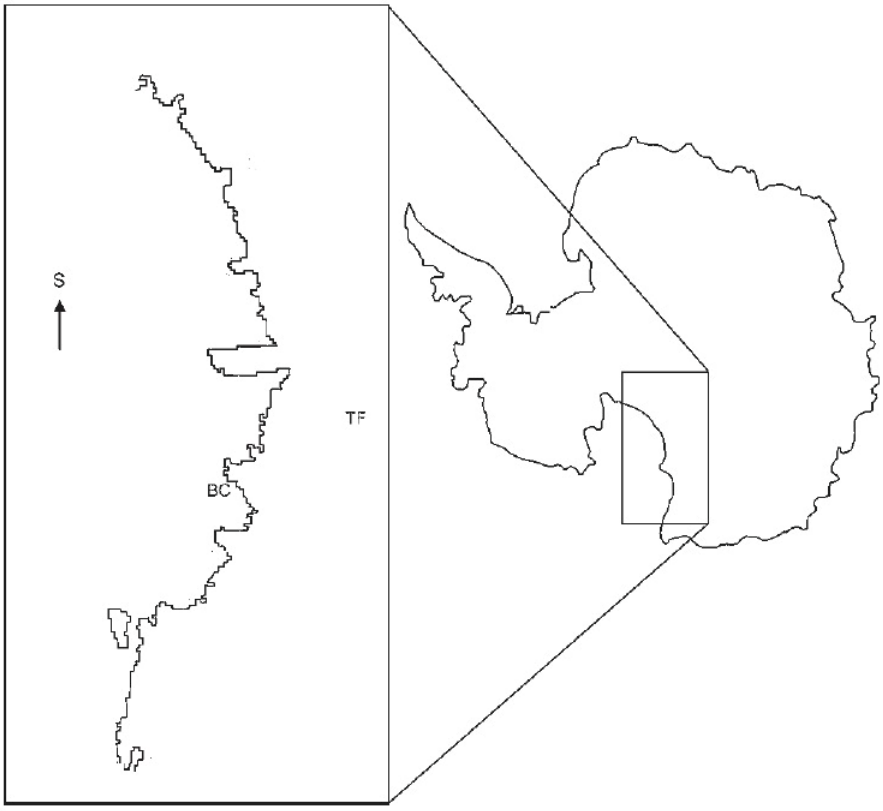

Figure 6.1 Sampling sites were located in Antarctica. The brines were collected in Boulder Clay (BC) and in Tarn Flat (TF) (Cannone *et al.*, 2008).

Boulder Clay (BC)

The BC site has been described above (§ 5.1.2).

Tarn Flat (TF)

The Tarn Flat (75°00' S, 162°30' E) area is located in north of the McMurdo Dry Valley in Victoria Land (Antarctica) and is the largest ice-free area (ca. 11 x 9 km) in the Antarctic continent, not far from the Italian Antarctica Research Station (Mario Zucchelli Sation, MZS). This ice-free area is characterized by Late Wisconsin glacial deposits, with some eskers at lower altitudes, and by Ordovician granites overlaid by sparse erratic boulders (Carmignani *et al.*, 1989; Baroni *et al.,* 1996). The study area presents a very large number of lakes and ponds (64). In general, the lakes located at lower altitudes are

partially or completed melted during the summer, whereas those at higher elevations are perennially frozen or only partially melted at their margins during warmer summers (Forte *et al*., 2016). It is a freshwater deglaciated area bounded to the north by Windowmaker and Reeves glacier, to the east by Nansen's ice shelf, to the south Backstairs Passage's glacier, and to the west by Larsen's glacier. Snow-free area is due to the katabatic winds, formed inland, that channelled through the valley. The deglacier area is an irregular landscape that is high up from 300 m to 980 m in the Mt. Gerlache. The snow is transported by the winds settles and is due to the glacier formation (Meneghel, 1990). The climate is cold and arid. The mean monthly air temperature ranges between -26 °C and 0 °C, with a mean annual temperature of -13.9 °C at Terra Nova Bay station (Frezzotti *et al*., 2001). Precipitation (rain and snowfall equivalent) is less than 200 mm *per* year (Caprioli *et al*., 1997). Permafrost is continuous. The site is located at the southern limit of the Reeves Glacier on a steep slope composed of fine-grained granite surrounded by a till characterized by loose coarse debris. Bedrock joints are several and variously oriented. A reddish weathering rind is widespread both on the bedrock and on till blocks. Flaking is also very common on both horizontal and vertical surfaces. There is no evidence of grus deposition. The rock scales are very large, reaching more than 10 cm in diameter and more than 3 mm in thickness. There are striations on Tarn Flat bedrock, while the granite bedrock is widespread in adjacent terrain near Mt. Gerlache. The striated surface is modified by weathering features probably during glaciation, but it is probably that in small places weathering has occurred subsequent the Terra Nova glaciation (Quat, 1991). Vegetation is very sparse and is mainly composed of epilithic lichens (Guglielmin *et al*., 2005).

6.2 Collection and *in situ* treatment of samples

Brine samples were collected during the Italian expedition 2014/2015 near the ice blisters in two different lakes in Boulder Clay (74°44' S, 164°01' E) and near the frost mound of one lake in Tarn Flat (75°00' S, 162°30' E).

A ProEx (Dual Channel configuration) Ground penetrating radar (GPR) was used to identify brines within permafrost in the Tarn Flat area. The sampling was carried out through a borehole (51 mm diameter) until 5.68 m of depth, in the centre of the frost mound using a semi-portable core auger. During the coring, the first brine pocket was found at 378 cm and was collected using a peristaltic pump and sterile tubing. After collecting the first brine, the coring was continued for the following 12 cm and then re-inserted a new sterile tube to collect the second brine, which was located between 410 cm and 494 cm of depth. The samples were named TF4 (first brine pocket) and TF5 (second brine pocket).

The fluids in Boulder Clay were previously identified with ground penetrating radar surveys and collected in one site at 2.5 m of depth and in another lake at 1 m and 2 m of depth (2 different points of sampling). The samples were named BC-1 (2.5 m), BC-2 (1 m) and BC-3 (2 m). The brines were collected and placed in sterile containers to be processed. In Table 6.1 the physical characterization of samples is reported.

Table 6.1 Physical characteristicsof samples at sampling time.

Location	*Sample ID*	*Coordinates*	*Depth (m)*	*Salinity (mS cm^{-1})*	*pH*
Boulder Clay	BC1	74°44' S, 164°01' E	2.5	0.2	8.17
Boulder Clay	BC2	74°44' S, 164°01' E	1	0.2	7.6
Boulder Clay	BC3	74°44' S, 164°01' E	2	3.6	8.76
Tarn Flat	TF4	75°00' S, 162°30' E	3.9	19.9	7.22
Tarn Flat	TF5	75°00' S, 162°30' E	4.5	16.6	6.74

6.3 Prokaryotic community composition using culture-independent method: TARN FLAT and BOULDER CLAY BRINES.

Prokaryotic community composition by epifluorescence microscopy

The prokaryotic composition in brine samples was investigated by using epifluorescence microscopy, more specifically the DAPI-staining for the total abundance and CARD-FISH for the enumeration of main phyla.

6.3.1 Total cell counts

Brine samples were fixed with 4 % (final concentration) formaldehyde, filtered and stored at 4 °C before staining and processing. Subsamples (1 mL) of each sample were stained with DAPI (4'-6'-diamidino-2-phenylidole; 1 μg μL^{-1} final concentration), according to the procedure reported by Porter and Feig (1980), directly on 0.22 μm black polycarbonate filter (Millipore) for 10 min, then filtered and mounted on slides. Cell count was performed with Olympus epifluorescence microscope equipped with UV lamp.

6.3.2 Catalysed reported deposition-Fluorescence in situ Hybridisation (CARD-FISH)

The CARD-FISH (Catalysed reported deposition-Fluorescence in situ Hybridisation) is a technique that allows to have a general overview about microbial diversity occurring in a sample. Brine samples were fixed with formaldehyde (4 % final concentration) and an aliquots (10 mL) were filtered on 0.22 μm white polycarbonate membrane filter (Millipore). CARD-FISH was performed in different steps, as follows.

The first step, called *embedding*, is important to prevent the cells loss possibly caused by the several washing steps. The filters were dipped with 0.2 % low melting point agarose solution and dried in an incubator at 37 °C for 20 min. The agarose-embedded filters where then incubated with a lysozyme solution (10 mg lysozyme in TE Buffer: 100 µL 0.5 M EDTA [pH 8.0], 100 µL 1 M Tris-HCl [pH 8.0], 800 µL of sterilized water), and were placed in a sealed Petri dish at 37 °C for 45 min. The lysozyme passage is called *permeabilization* step, and allows the probe penetrating into the cells trough pores made by the lysozyme effect. After a washing step, the filters were incubated with a 0.01 M HCl solution for 20 min a RT for inhibiting the endogenous peroxidases. Fifteen probes, which target for dominant permafrost prokaryotic groups, were used to quantify the relative abundance of various phylogenetic groups in the samples (Table 6.2). For the in situ hybridization, the filters were cut into small pieces, named, and incubated in 300 µL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8, 10 % (w/v) dextran sulphate, 2 % (w/v) blocking reagent (Roche, Germany), 0.1 % (w/v) sodium dodecyl sulphate, and formammide (with a final concentration depending on the probe used, as reported in Table 8.1) and 1 µL of Horseradish peroxidase-labeled probe solution (50 ng μL^{-1}). The stock solution of the blocking reagent (10 %, w/v) was prepared in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). The *hybrization* step was set up with the dip filter placed face-up onto parafilm covered glass slide positioned in tube kept in horizontal position at 46 °C for 3 h. This step allows the probe to hybridize with the cells. After the hybridization step, the filter pieces were washed with the washing solution (12.5 mM EDTA pH 8, 20 mM Tris-HCl pH 8, NaCl depending on the probes, 0.01 % SDS (20 % w/v) and Milli-Q Water until 50 mL) at 48 °C for 15 min to eliminate the excess of probe solution. For the CARD steps the filters were incubated in amplification buffer (1 X PBS pH 7.6, 0.1 % Blocking Reagent, initial concentration 10 % w/v, 2 M NaCl, 10 % dextran sulphate and Milli-Q water until 40 mL; 1 % of H_2O_2, 0.15 % initial concentration freshly prepared and 0.1 % of tyramide labelled) in the dark at 46 °C for 30

minutes. The CARD step amplified the hybridization step, because the tyramide labelled fluorochrome is more quantity than the probes and it is active if the probe hybridizes the cells. After these steps, it is important to counterstaining the CARD-FISH reaction using DAPI staining (1 μg mL^{-1} working solution) or mounted, on glass slide, directly the filters using an anti-fading reagent (Citiflour (Citiflour, UK), Vectashield (Vector Laboratories, USA) 4:1) containing μg mL^{-1}.

Table 6.2 Probes and hybridization conditions used for CARD-FISH.

Probe names	**Target**	**Sequence (5'-3')**	**FA***
EUB-338 I-III	*Eubacteria*	I: GCTGCCTCCCGTAGGAGT II: GCAGCCACCCGTAGGTGT III: GCTGCCACCCGTAGGTGT	35%
Arch915	*Archaea*	GTG CTC CCC CGC CAA TTC CT	20%
Alf968	*Alphaproteobacteria*	GGT AAG GTT CTG CGC GTT	20%
Bet42a	*Betaproteobacteria*	GCC TTC CCA CTT CGT TT	35%
Gam42a	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	35%
Delta495 mix	*Deltaproteobacteria*	a: AGT TAG CCG GTG CTT CCT b: AGT TAG CCG GCG CTT CKT c: AAT TAG CCG GTG CTT CCT	35%
Epsy914	*Epsilonproteobacteria*	GGT CCC CGT CTA TTC CTT	35%
CF319a	*CFB°*	TGG TCC GTG TCT CAG TAC	25%
ANME1-350	*Methanotrophic Archaea*	AGTTTTCGCGCCTGATGC	40%
Pla46	*Planctomycetes*	GAC TTG CAT GCC TAA TCC	20%
HoAc1402	*Acidobacteria*	CTT TCG TGA TGT GAC GGG	15%
Cya664	*Cyanobacteria*	GGA ATT CCC TCT GCC CC	50-55%
Euk516	*Eukarya*	ACC AGA CTT GCC CTC C	0%

*Formamide concentration

° CFB = Cytophaga-Flavobacteria-Bacteroidetes

Prokaryotic community composition using Next-Generation sequencing

Analysis of prokaryotic community was carried out using DNA and RNA extractions of brine samples. The RNA was transcripted to cDNA and the samples extracted were sent to the Macrogen Company (Korea) for the ION Torrent analysis.

6.3.3 Analysis of the prokaryotic community

Both DNA and RNA were taken into consideration for the analysis of the prokaryotic community composition in brine samples. Cells for DNA/RNA extraction were collected on 0.22 μm-pore size polycarbonate filters (diameter 47 mm, Millipore) by filtration of 150 to 600 mL of liquid. The brine color changed when at room temperature and at air with brines from TF4 and TF5 that became dark brown and dark grey, respectively, from light yellow.

6.3.4 DNA extraction

Brine samples were collected from the area of Boulder Clay and Tarn Flat. The brine samples were taken from two different points in Boulder Clay near the ice blisters in two different lakes in Boulder Clay. The fluids in Boulder Clay were previously identified with ground penetrating radar surveys and collected in one site at 2.5 m of depth twice and in another lake at 1 m and 2 m of depth (2 different sampling points).

The brines collected from Tarn Flat, near the frost mound, were taken in the same point at two different depths (3.9 and 4.5 m). The brine were collected and placed at -20 °C in sterile containers to be processed. For DNA extraction, were used beating lysis using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA). After extraction, DNA was quantified using a Nanodrop spectrophotometer (Thermofisher).

6.3.5 RNA extraction and cDNA isolation

Total RNA was extracted from membranes. To avoid contamination all instruments were sterilized by UV-C radiation under laminar flow hood and washed with RNasy away. Total RNA was extracted using RNeasy Mini Kit (Qiagen) with a pre-modification. A Lysozyme solution (5 mg mL^{-1}) in TE buffer 1 X pH 8 (10 mM Tris-HCl; 1 mM EDTA) was prepared and used for the release of RNA from inside the cells. The samples were incubated for 15 min at 37 °C prior to use the Kit following the manufacture's procedure. The synthesis of cDNA was carried out from total RNA (4 µL) with RT-PCR reaction using SuperScript III First-Stand Synthesis System (Invitrogen). Two reaction mixes were prepared: the MIX 1 (RNA 4 µL; Random hexamers 50 ng μL^{-1}; dNTP mix 10 mM, RNasy free water until 10 µL), and the MIX 2 (RT-buffer 10 X; $MgCl_2$ 25 mM; DTT 0.1 M; RnaseOUT 40 U μL^{-1}; SuperScript III RT 200 U μL^{-1}). Mastermix and samples were then added to each tube (20 µL total volume) and the RT step, which involved incubation at 25 °C for 10 min followed by 50 min at 50 °C and 5 min at 85 °C, was performed. Until this step the samples were incubated for few seconds in ice and 1 µL of RNase H was added, followed by 20 min at 37 °C. After this, cDNA was isolated and it was possible to pass to the PCR step. The reaction mixtures were assembled at 0 °C and contained 1-10 ng DNA, 5 X Phusion buffer, 150 ng of each 27 forward and 1492 reverse primer (MWG, Germany), 0.1 % of BSA, 10 mM of dNTP (Polymed, Italy), 0.2 units of *Taq* polymerase (Phusion) and sterile distilled water to a final volume of 40 µL. Negative controls for cDNA extraction and PCR setup (reaction mixture without a cDNA template) were also used in every PCR run. The PCR program was as follows: 95 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min. The results of the amplification reactions were analysed by agarose gel electrophoresis (1 %, w/v) in TAE buffer (0.04 M Tris-acetate,

0.02 M acetic acid, 0.001 M EDTA), containing 1 μg mL^{-1} of ethidium bromide. The cDNA was quantified using Nanodrop (Thermofisher).

6.4 Cultivable heterotrophic bacteria

6.4.1 Enumeration, isolation and phylogenetic characterization of cultivable heterotrophic bacteria

An aliquot (200 μL) of each brine sample was spread-plated in duplicate on different solidified media: **Triptic Soy Agar** at full (TSA_{100}; Oxoid), 1/2 (TSA_{50}), and 1/10 (TSA_1) strength; **R2A agar** (Difco) at 1/5 ($R2A_{20}$) and 1/10 ($R2A_{10}$) strength; **AcidAgar** (composition per liter: NaCl, 0.813 g; glucose, 1.0 g; K_2HPO_4* 3 H_2O, 0.86 g; $MgSO_4$ * 7 H_2O, 0.5 g; Yeast Extract, 1.0 g; Agar, 15.0 g; pH 4); **DSMZ 97** (composition per liter: NaCl, 250.0 g; $MgSO_4$ * 7 H_2O, 20.0 g; KCl, 2.0 g; Sodium Citrate, 3.0 g; Casaminic acid, 7.5 g; Yeast Extract, 1.0 g; $FeSO_4$, 0.00023 g; Agar, 15.0 g; Polimixin B, 50 mg mL^{-1}, 0.75 g; pH 7.35) e DSMZ 371 (composition per liter: KH_2PO_4, 1.0 g; KCl, 1.0 g; NH_4Cl, 1.0 g; $MgSO_4$ * 7 H_2O, 0.24 g; $CaSO_4$ * 2 H2O, 0.17 g; SL-10 solution; NaCl, 200.0 g; Na2-glutammate, 1.0 g; Yeast Extract, 5.0 g; Casaminic acid, 5.0 g; Na_2CO_3, 5.0 g; pH 6.5).

Agar plates were incubated in the dark at +4 °C for 8 weeks, and colony forming units (CFUs) per mL of brine samples were calculated as averages of duplicate plates.

Colonies were randomly selected from agar plates used for CFU counts, picked and sub-cultured almost three times under the same conditions.

Bacterial isolates were phylogenetic identified by the 16S rRNA gene sequencing as previously described (§5.4) for isolates from permafrost.

6.4.2 Characterization of bacterial isolates from brines

Bacterial isolates were phenotypically characterized by studying their morphological, physiological and biochemical features, as described in the following paragraphs.

6.4.2.1 Morphological tests

Bacterial colonies were morphologically described in terms of shape, pigmentation, elevation, margin and surface characteristics. These features were checked after good growth occurred on TSA plates incubated at 4 °C.

6.4.2.2 Bacterial growth conditions

Bacterial growth was assayed at different temperatures in Nutrient Broth (NB; Oxoid) after incubation at +4 °C, +15 °C and +25 °C for up to 4 weeks.

The pH range for growth was determined in NB with pH values of separate batches of the medium adjusted to 4, 5, 6, 7, 8 and 9 by the addition of HCl and NaOH (0.01 g L^{-1}, 0.1 g L^{-1} and 1 g L^{-1} solutions) and incubated at 4 °C for 30 days.

Salt tolerance tests were performed on Nutrient Agar (NA; Oxoid) with NaCl concentration ranging from 0 to 19 % (w/v). Plates were incubated at 4 °C and growth was monitored up to 30 days.

6.4.2.3 Production of extracellular enzymes

6.4.2.3.1 Catalase test

Catalase is an enzyme produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites, such as H_2O_2. This enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects bacterial cells. Catalase mediates the breakdown of hydrogen peroxide into oxygen and water. To test the

occurrence of catalase small inoculums of bacterial colonies were mixed into hydrogen peroxide solution (3 %, v/v), The rapid production of oxygen bubbles were recorded as a positive response.

6.4.2.3.2 Oxidase Test

Oxidase test detects the presence of a cytochrome oxidase system that catalyses the transport of electrons between electron donors in the bacteria and a redox dye tetra-methyl-p-phenylenediamine dihydrochloride. The dye is reduced to deep purple color. The colony to be tested was picked up with a loop and smeared over the moist area of strips (Dry Slide, DIFCO Laboratories). A positive reaction was indicated by an intense deep-purple hue, appearing within 5-10 seconds, a "delayed positive" reaction by coloration in 10-60 seconds, and a negative reaction by absence of coloration or by coloration later than 60 seconds.

6.4.2.3.3 Hemolysis test

Hemolysis test detects the ability of some bacteria to produce exoenxymes that lyse red blood cells and degrade hemoglobin. Bacteria can produce different types of hemolysis. Beta-hemolysis breaks down the red blood cells and hemoglobin completely. This leaves a clear zone around the bacterial growth. Alpha-hemolysis partially breaks down the red blood cells and leaves a greenish color behind. The test was performed by using the specific Blood Agar medium (Oxoid), containing 5 % of sheep blood. The strains were streaked on solidified medium and incubated for 15 days at +15 °C. After incubation, plates were checked and positive results were indicated by the presence of halos

6.4.2.3.4 DNAse test

DNA hydrolysis test or Deoxyribonuclease (DNase) test is used to determine the ability of an organism to hydrolize DNA and utilize it as a source of carbon and energy for

growth. An agar medium, DNase agar (Oxoid), a differential medium, is used to test the ability of an organism to produce this enzyme. The strains were streaked on agar medium and incubated for 15 days at 15 °C. The presence of DNAse was assayed using an indicator, hydrochloric acid 1 N, after bacterial growth occurred. Bacteria resulted positive if they showed a halo around their colonies. *Staphylococcus aureus* was used as a positive control.

6.4.2.3.5 Hydrolysis of complex substrata

The hydrolysis of complex substrata was checked after incubation at 4°C for 30 days, even if otherwise specified.

To perform the test, 2 g of chitin was mixed with H_2SO_4 (50 %, v/v) and incubated for 2 h. The obtained suspension was washed twice with cold distilled water and neutralized with NaOH until pH 5. The suspension was sterilized and added (0.1 %) to the medium suggested by Brisou *et al*. (1964) (Yeast extract, 0.1 % w/v; Peptone, 0.5 % w/v; Agar, 1.5 % w/v; Potassium monophosphate, trace). Hydrolysis of chitin was tested by detecting the presence of a halo around colonies after growth on the medium.

Agarolitic activity was tested on the medium suggested by Vera *et al*. (1998). The medium composition was: Casein Hydrolysate, 0.25 % w/v; Yeast Extract, 0.05 % w/v; Peptone, 0.5 % w/v; NaCl, 3.0 % w/v; NaH_2PO_4, 0.06 % w/v; $MgSO_4$, 0.5 % w/v; F_3SO_4 * 7 H_2O, 0.002 % w/v; $CaCl_2$, 0.01 % w/v; Agar, 1.5 % w/v. The strains were streaked perpendicularly and the positive hydrolization was checked by halo formation.

Lipolitic activity was determined adding Tween 80 (1 %, v/v) in the Sierra (1957) medium (composition *per* liter: Seawater, 750 mL; Distilled water, 250 mL; Peptone, 10.0 g; $CaCl_2$ * H_2O, 0.1 g; Agar, 20.0 g). The colonies were inoculated by stabbing and incubated at 4 °C for 30 days.

Gelatinase activity was tested using a medium containing (per liter of distilled water): Gelatine, 120.0 g; Yeast extract, 1.0 g; Peptone, 6.0 g. The medium was dispensed in

tubes (3 mL aliquot). The strains were inoculated by stabbing and incubated at 4 °C for 30 days. Positive strains produced the fluidization of the medium.

The presence of amylase was tested using a medium containing per liter: Tryptone, 1 % w/v, Yeast extract, 1 % w/v, KH_2PO_4, 0.5 % w/v, Soluble starch, 0.3 % w/v; Agar, 1.5 % w/v. After incubation the positive response was checked by flooding Lugol solution on the medium surface. The degradation of starch was showed by the formation of a halo around bacterial colonies.

6.4.2.4 Antibiotic susceptibility

Antibiotic susceptibility was determined on TSA agar medium supplemented with ampicillin, kanamycin, streptomycin, chloramphenicol, gentamycin and polimixin B. Different concentration were tested for each antibiotic: polimixin B and chloramphenicol (50 and 100 ppm), streptomycin and gentamicin (25, 250 and 350 ppm), kanamycin and ampicillin (50 and 100 ppm). These concentrations were chosen on the base of previous results on antibiotic tolerance of Antarctic bacteria (De Souza *et al*., 2006; Lo Giudice *et al*., 2013; Mangano *et al*., 2014).

A stock solution of each antibiotic was prepared and added to sterile TSA at 50 °C to avoid damages of the molecules. Plates were inoculated by streaking and incubated +4 °C for 30 days.

6.4.2.5 Heavy metal resistance

Bacterial strains were tested for tolerance to five different heavy metals (HMs) on TSA supplemented with the heavy metal salts $CdCl_2$, $CuCl_2$, $HgCl_2$, $CoCl_2$ and $NiCl_2$ at both concentration of 500 and 1000 ppm. A stock solution at 10000 or 20000 ppm in Phosphate-buffered saline (PBS) 1 X was prepared for each heavy metal salt and added

before sterilization to the medium to final concentration of 500 or 1000 ppm. Isolates were streaked on TSA plates plus HMs and incubated at +4 °C for 30 days. Strains that were able to grow until 1000 ppm of a certain heavy metal were streaked on TSA medium amended with the same metal until a concentration of 10000 ppm.

6.5 Biotechnological applications

Microorganisms living in extreme environments have adapted their metabolic processes to their habitat in order to allow them to survive and function correctly. In the course of evolution, adaptation of organisms to extreme environments led to the development of halophilic, osmophilic, barophilic, acidophilic, alkalophilic, thermophilic and psychrophilic organisms. Since relatively few organisms possess these adaptations, diversity in extreme environments is generally low. The extremophilic microorganisms successful faced the adverse environmental effects by evolving new features including productions of cold-proteins, maintenance of membrane fluidity, production of cryoprotectants (such as exopolysaccharides), resistance to organic pollutants and production of antimicrobial polymers. The strains isolated from these samples were collected in one of the most extreme environments on Earth as permafrost and brines in Antarctica, whose unique and extreme conditions could be reflected in a great biotechnological potential. On this regard, the enzymes and bio-compounds obtained from cold-tolerant organisms, named “cold-enzymes”, are of immense biotechnological importance. Cold-adapted bacteria capable to degrade hydrocarbons, or other pollutants, or produce exopolysaccharides and antimicrobial compounds are useful in biotechnological applications.

6.5.1 Screening for exopolysaccharide production

A preliminary screening of exopolysaccharides (EPSs) production was performed to select the producer strains. The strains were streaked on TSA medium added with a glucose solution (3 % v/v, final concentration) and incubated for 1 months at 4 °C. The EPS production was monitored by observing the texture of bacterial colonies (mucoid texture was considered to be positive).

Positive strains, considered as most promising were subjected to a test of *slime* highlighting according to the method described by Christensen *et al.* (1985), modified by Maugeri *et al.* (2002). Bacteria were tested in Tryptone Soy broth added plus Glucose (2 %, v/v, final concentration) by using 50-mL polypropylene tube. EPS production was checked by staining the tubes with safranin, as follows. The tubes were emptied from the culture broth, rinsed twice with distilled water and stained with safranin. The *slime* presence was confirmed by the formation of a visible film that covered the tube walls (Christensen G.D. *et al*., 1985 modified by Maugeri *et al*., 2002).

6.5.1.1 EPS production

A further analysis of EPS quantification was performed on selected positive strains. A bacterial pre-culture (10 %, v/v) in the exponential phase was used to inoculate 300 mL of a minimal medium, which contained (*per* liter of Väätänen nine-salt solution, VNSS): Peptone, 0.5 g; Yeast extract, 0.1 g and Glucose (2 %, w/v, final concentration). The VNNS solution contained (*per* liter of distilled water): NaCl, 17.6 g; Na_2SO_4,1.47 g; $NaHCO_3$,0.08 g; KCl, 0.25 g; KBr 0.04 g; $MgCl_2$ x$6H_2O$, 1.87 g; $CaCl_2$ x $2H_2O$, 0.41 g; SrCl x $6H_2O$, 0.008 g; H_3BO_3,0.008 g (pH 7). Cultures were incubated at 15 °C for one month. After incubation time, the cultures were centrifuged at 8000 rpm for 10 minutes and the cell-free suspension were sampled from the culture broth to evaluate the EPS

production by applying the phenol-sulphuric acid method on *cell free* broth, with glucose that was used as a standard Dubois *et al*. (1956).

6.5.2 Inhibitory activity against indicator organisms

Brine isolates were screened for their ability to inhibit the growth of the terrestrial indicator organisms. The indicator microorganisms used throughout this study were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Salmonella enterica*, *Photobacterium damselae damselae*, *Vibrio cholerae*. Experiment was performed on TSA at 1.5 % of NaCl. Antibacterial activity was detected by the cross-streak method as previously described by Lo Giudice *et al*. (2007). Antarctic bacteria were streaked across one-third of an agar plate and incubated at +15 °C (due the psychrotrophic nature of the isolates). After that growth was obtained generally in 7-10 days (depending on growth of the isolates), indicator organisms were streaked perpendicular to the initial streak, and plates were further incubated at +15 °C for 72/120 h at +37 °C and checked afterwards for inhibiting zones. The antagonistic effect was indicated by the failure of the target strain to grow in the confluence area.

6.5.3 Growth in the presence of organic pollutants

The bacterial ability to grow in the presence of organic pollutants as the only carbon source and energy was tested for solid (i.e. naphthalene, phenanthrene, pyrene, biphenyl) and liquid hydrocarbons (i.e crude oil, diesel oil, toluene, tetradecane, heptane, octane, dodecane). Strains were streaked on the mineral medium Bushnell Haas added with 3 % of NaCl (w/v) and Agar 1.5 % (w/v) (BH, DIFCO Laboratories) and hydrocarbons were placed on the lid of the Petri Dish. In the case of liquid hydrocarbons, a cellulose pad was

soaked with an aliquot (ca. 100 µL) of the substrate. Plates were incubated for one month at +4 °C.

In addition, strains able to grow on plate in the presence of diesel oil and crude oil were further tested in liquid cultures. Sterile crude oil or diesel oil (final concentration, 2.0 %, v/v) as the sole carbon and energy source were added to liquid BH supplemented with 3 % NaCl (w/v). The medium was inoculated with 10 % of a freshly prepared bacterial suspension in 3 % (w/v) NaCl supplemented BH medium. Tubes were incubated at +4 °C for 30 days. The ability to use hydrocarbons as growth substrates was evaluated according to the degree of turbidity or the appearance of cellular flocs in the test tubes. Uninoculated medium was incubated in parallel as a negative control.

6.5.4 Screening for growth on Aroclor 1242

Aerobic PCB degradation by strains isolated from plates amended with biphenyl was screened in liquid BH supplemented with NaCl (3 %, w/v) and amended with a n Aroclor 1242 solution (100 ppm in dichloromethane) as sole carbon and energy source (final concentration 0.1 %, v/v) (De Domenico *et al.,* 2004; Michaud *et al.,* 2007; Lo Giudice *et al.,* 2013). Aroclor 1242 is a mixture of PCB congeners (ranging from dichloro- to hexachlorobiphenyls) made of twelve carbon atoms in the biphenyl molecule and containing 42 % chlorine by weight. Before adding the bacterial inoculum, 10 µL of Aroclor solution were transferred to 100 mL flasks containing 9.5 mL of BH and left in a cabinet until CH_2Cl_2 completely evaporated.

The medium was inoculated with 0.5 mL of a freshly prepared bacterial suspension (5 %, v/v) in 3 % (w/v) NaCl supplemented filter-sterilized distilled water. The optical density (OD) of the suspension was adjusted to 1.0 at 600 nm. Cultures were incubated at 4 °C for 3 weeks on a rotary shaker operated at 100 rpm. The ability to use PCBs as growth substrates was evaluated according to the degree of turbidity or the appearance of cellular

flocs in the test tubes. Uninoculated medium was incubated in parallel as negative control (Lo Giudice *et al.,* 2013).

6.5.5 Screening for the presence of the functional gene *bphA*

6.5.5.1 Design of specific primers for the amplification of the A portion of the BPH gene

Burkholderia xenovorans (DSM 17367), a well known PCB-degrader, was used as positive control for the detection of BPH gene and preliminary tested for the amplification of the BPH gene by using primer and annealing protocol reported in Table 6.3. In particular, there search was addressed to the *bph*A gene associated with the aerobic biodegradation of PCBs (Lehtinen *et al.,* 2013). We set up a PCR protocol using MyTaq (Bioline) and the result was analysed by agarose gel electrophoresis to check the right position of DNA bands at ≈ 900 bp.

Table 6.3 List of primers used for the amplification of the *bphA* gene.

Primer	***Sequence Written 5' to 3'***	***Primer annealing temperature***
2BPHAREV1	ADV-CCS-CGB-GCC-GCB-TCH-TCG	65-60 °C
2BPHAFWD1	GGC-TGG-GCC-TAC-GAC-ANC-GC	60 °C

6.5.5.1 Amplification of the *bph*A gene

Strains showing the ability to grow in the presence of Aroclor 1242, as sole carbon and energy source, were screened for the presence of the catabolic gene *bph*A involved in PCB degradation (Master and Mohn, 2001). The presence of genes was highlighted by PCR, using the primers cited above. The reaction mixtures were assembled at 0 °C and

contained 2 µL of DNA, 5 X of MyTaq Buffer MIX, 10 µM of each of the two primers, 5U μL^{-1} of *Taq* polymerase MyTaq (Bioline), and sterile Milli-Q water to a final volume of 25 µL. Negative controls for DNA extraction and PCR setup (reaction mixture without a DNA template) were also used in every PCR run. DNA from *Burkholderia xenovorans* (DSM 17367) was used as positive control. The PCR program was as follows: 1 min at 95 °C; 35 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C; 10 min at 72 °C (Lehtinen *et al.,* 2013). The results of the amplification reactions were analyzed by agarose gel electrophoresis (2 %, w/v) in TAE buffer (0.04 M Tris-acetate, 0.02 M acetic acid, 0.001 M EDTA), containing 1 µg mL^{-1} of ethidium bromide.

6.5.6 Degradation efficiency test

Based on the screening for the *bph*A gene, most promising strains were analysed for their ability to degrade Aroclor 1242. According to Michaud and collaborator (2007), isolates were grown in 200 mL of Aroclor 1242 supplemented (1 % w/v) BH, and incubated at 4 and 15 °C for 2 weeks. Uninoculated controls were incubated in parallel to monitor abiotic losses of the substrates. After incubation, biodegradation activity was stopped by acidifying cultures with HCl 10 M to achieve pH 2. Before PCB extraction, the cultures were centrifuged at 4 °C for 20 minutes at 8000 rpm to remove the excess of cellular material. Then the cultures were separated in two aliquots of 100 mL to replicate the extraction, after 10 µL of octachloronaphthalene (OCN) solution (5 mg mL^{-1} in CH_2Cl_2) was added to each aliquot to monitor substrate losses during the extraction procedure. Cultures were extracted three times with 10 mL of CH_2Cl_2 in a separatory funnels to isolate the organochlorine molecules from the enrichment cultures. Funnels were vigorously shaken for 15 min during each extraction. The three organic phases were pooled and the solvent evaporated to dryness.

The composition of PCBs and their concentration were determined by high resolution gas chromatography-mass spectrometry (GC-MS), by using a Perkin-Elmer TurboMS

AutoSystem XL GC, equipped with a DB-TPH capillary column (30 m with a 0.322 mm i.d. and 0.25 µm film thickness, J & W Scientific). Helium (5 bar) was used as carrier gas (1.8 mL min^{-1}). The injector was kept at 300 °C; the column oven was initially kept at a temperature of 200 °C for 4 min, and then heated up to 300 °C at a rate of 8 °C min^{-1}. Two microliters portion of each sample, containing 79.3 ng of heptamethyl nonane as internal standard (to account for injection errors and other variable encountered during the chromatographic analysis), was analyzed by means of the splitless injection technique (50 mL min^{-1}). Finally, the percentage reduction of Aroclor was determined according to the following equation:

$$\%R = 100 - [(H_{PCB\ sample}/H_{PCBcontrol}) \times 100]$$

where $H_{PCB\ sample}$ and $_{HPCB\ control}$ are the height of a PCB peak in the sample and the control, respectively

Antarctic Brines: RESULTS

6.6 Microbial community composition using culture-independent methods: TARN FLAT and BOULDER CLAY BRINES.

Prokaryotic community analysis using epifluorescence microscopy

6.6.1 Total cell counts

The prokaryotic cell abundances detected using 4',6-diamidino-2-phenylindole (DAPI) staining were reported in Figure 6.2. In Tarn Flat (TF) brines, prokaryotic cell abundances ranged between 1.96 x 10^6 and 2.12 x 10^6 cells mL^{-1} (in TF4 and TF5, respectively). In Boulder Clay (BC) the prokaryotic cell numbers ranged between 2.54 x 10^7 (BC1) to 1.14 x 10^6 (BC3) cells mL^{-1}. Brines from BC1 and Tarn Flat showed higher abundances than BC-3. Total prokaryotic cell abundance in BC1 brine was an order of magnitude higher than in the other samples (Figure 6.2).

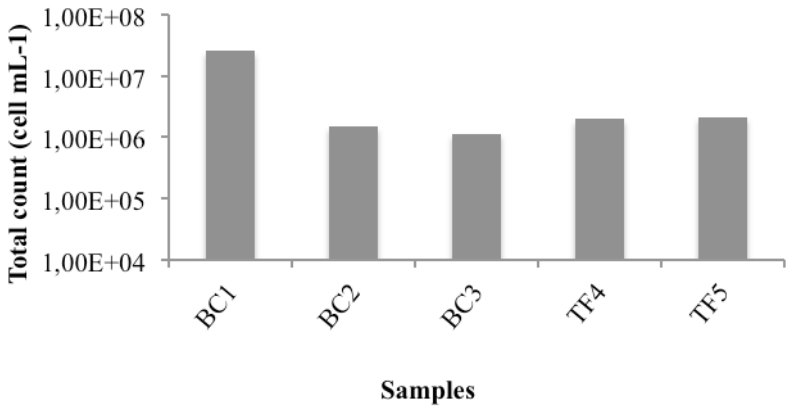

Figure 6.2 Total counts after DAPI staining

6.6.2 Catalysed reported deposition-fluorescent in situ hybridization (CARD-FISH)

Brine samples were analysed for microbial community composition using the CARD-FISH technique. A total of 13 probes were used, i.e. nine probes to target Bacteria (*EUB338 I-III*, *Alf968*, *Bet42a*, *Gam42a*, *Delta995mix*, *Epsy914*, *CFB319a*, *Pla46* and *HoAc1402*), one probe for Cyanobacteria (*CYA664*), two probes for Archaea (*Arch915* and *ANME1-350*) and one probe for Eukaryotes (*EUK516*).

Overall, the CARD-FISH carried out on brines collected in Antarctica indicated that *Eubacteria* (probe *EUB338 I-III*) accounted for between 69.7-86.04 % (TF5 and BC1, respectively), *Archaea* for 0.04-1.2 % (BC1 and TF4 and TF5, respectively) and *Eukaryote* for 0.03-1.3 % (BC1 and BC3, respectively) of total DAPI-stained cells.

Tarn Flat. Among *Eubacteria*, the *Proteobacteria* accounted for 69.1 and 70.7 % of EUB-targeted cells (in TF4 and TF5, respectively). In detail, they were mainly represented by *Alpha-*, *Beta-* and *Gammaproteobacteria* which accounted for 7.9-4.8 %, 42.0-39.4 % and 16.6-24 % of EUB-targeted cells in TF4 and TF5, respectively. Conversely, *Delta-* and *Epsilonproteobacteria* were less represented, accounting for 1.1-0.9 % and 1.5-1.6 % of EUB338-stained cells, in TF4 and TF5, respectively. Among the Eubacteria, the CF319 stained cells (*Cytophaga-Flavobacterium-Bacteroidetes* group) accounted for 16.7-23.8 % in TF4 and TF5, respectively, while autotrophic *Cyanobacteria* were absent in TF5 and less present in TF4 (0.35 % of EUB-targeted cells). *Planctomycetes* accounted for 0.94 and 1.1 % in TF4 and TF5, respectively. The *Acidobacteria* were absent (Figure 6.3).

Among *Archaea*, *Anaerobic methane-oxidizers* accounted for 38.5 % of Arch-target cells, in TF4 while were absent in TF5. Eukaryote accounted for 0.3-0.5 % of DAPI-staining, TF4 and TF5, respectively.

Boulder Clay. Among *Eubacteria*, the *Proteobacteria* were between 13.65 and 49.6 % (in BC2 and BC3, respectively) of EUB-targeted cells. In detail, *Alpha-*, *Beta-* and

Gammaproteobacteria were between 1.7 and 21.6 % (BC2 and BC1, respectively), 9.4 and 26.8 % (BC2 and BC3, respectively), and between 0.7 and 17.0 % (BC2 and BC3, respectively), respectively. *Epsilonproteobacteria* were detected only in BC1 and BC2 samples, ranging between 0.46 and 1.85 % of EUB338 stained cells. The *CFB* were in the range 47.2-95.3 % (BC1 and BC2, respectively), while *Planctomycetes* ranged between 0.02 and 0.58 % of *EUB338* stained cells (BC1 and BC3, respectively) (Figure 6.3). The *Acidobacteria* and autotrophic *Cyanobacteria* were absent in samples from BC.

Archaea accounted 0.04 % of DAPI-staining, no ANME were found, while *Eukaryotes* ranged between 0.03-1.3 % of DAPI-staining.

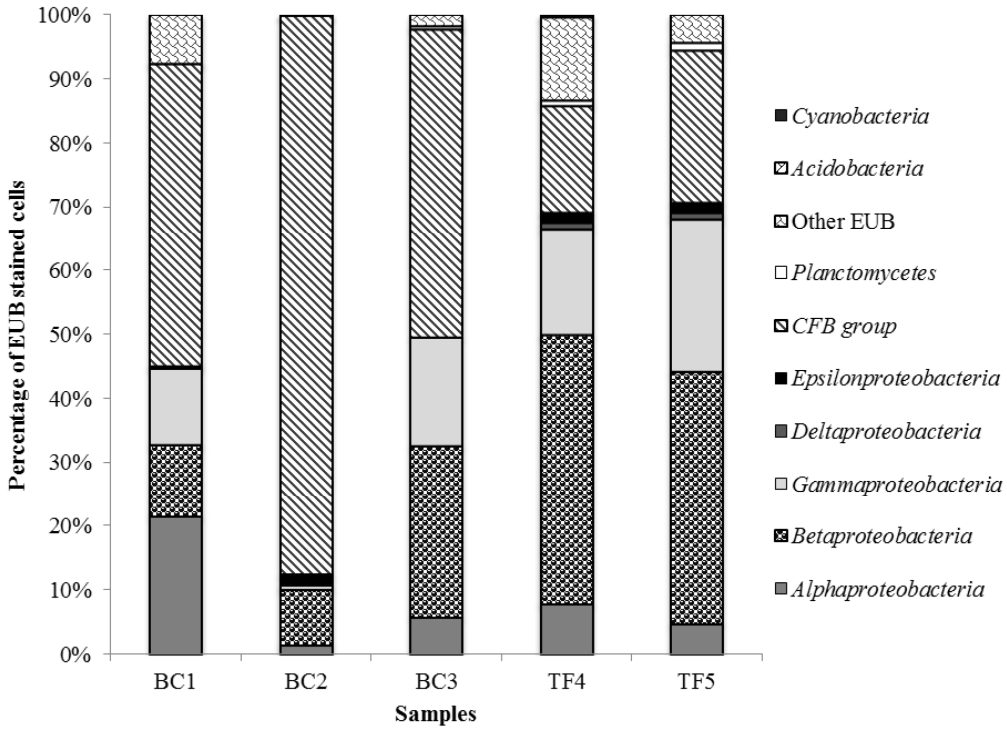

Figure 6.3 Relative percentages of Eubacteria as determined by CARD-FISH

6.7 DNA and cDNA extraction

The samples were filtered and DNA extracted using MoBio Isolation DNA kit and then the DNA was quantified using Nanodrop spectrophotometers (Thermofisher) (Table 6.4). Two duplicates were extracted from each sample and then used to sequence *Archaea* and *Bacteria* total communities. Furthermore, RNA was extracted from the samples to amplify cDNA and sequence the active community inhabiting Antarctic brines.

Table 6.4 In this table extracted DNA concentrations in brine samples are reported. DNA was used to sequence total Archaea and Bacteria and active Bacteria.

Site	***Samples***	***DNA concentration ng μL^{-1}***
BOULDER CLAY	*BC-1 Archaea*	25.8
	BC-1 Bacteria	27.8
	BC-1 cDNA	1016.4
	BC1 bis Archaea	14.4
	BC1 bis Bacteria	6.5
	BC1 bis cDNA	1071.9
	BC-2 Archaea	15.1
	BC-2 Bacteria	7.9
	BC-2 cDNA	983.5
	BC-3 Archaea	15.1
	BC-3 Bacteria	13
	BC-3 cDNA	991.2
TARN FLAT	*TF4 Archaea*	15.1
	TF4 Bacteria	13.0
	TF4 cDNA	1038.2
	TF4 Archaea	15.8
	TF4 Bacteria	6.9
	TF4 cDNA	1095.5

6.7.1 Ion Torrent analysis and phylogenetic affiliation

Overall, a preliminary analysis showed a total of 2,984,522 reads. The density of ion Sphere Particles (ISP), represented by a color image that indicates the percentage of loading of the chip, was equal to 73 %. Another preliminary data was the percentage of the usable sequences, namely sequences that showed a good level of quality, which were equal to the 65 %. The histogram that can be observed in the last frame of the summary showed the preliminary average length measured in base pairs of all reads, equal to 177 bp, after the appropriate bioinformatics corrections reached the length of about 320-350 bp (Figure 6.4).

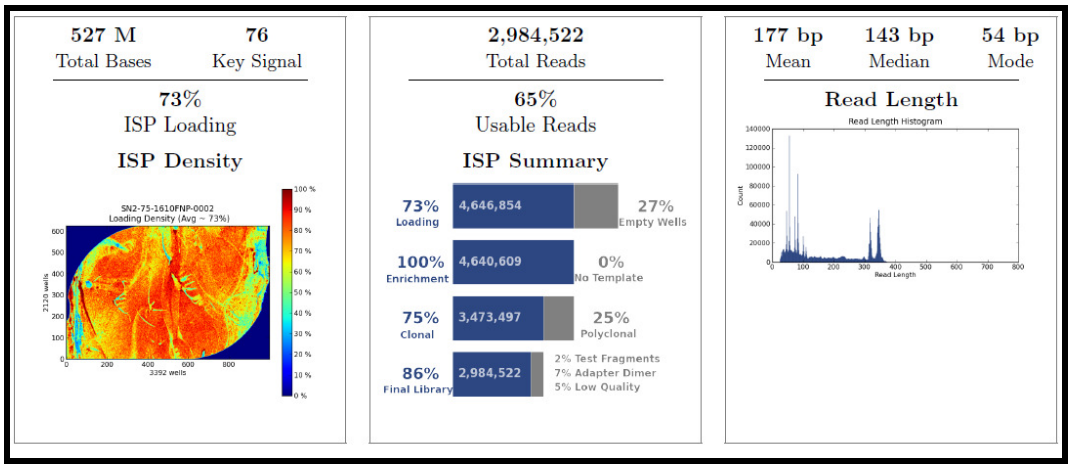

Figure 6.4 Ion Torrent preliminary run report

Starting from the OTU table generated after sequencing, the analysis of different phylogenetic groups found within the different samples in the different sampling sites was performed. The microbial community composition was evaluated for brine samples collected in Boulder Clay and Tarn Flat. The results here reported refer to brine samples active prokaryotic community (BC1; BC2 and BC3) and to Bacterial and Archaea total community (BC1, BC2, BC3, TF4 and TF5). The Shannon diversity index was analyzed

for the samples within the total prokaryotic diversity. This ranged between 1.485-2.056 in BC2-BC1 in BC samples, and 1.951-2.059 TF4-TF5 in TF samples. The Evenness indices were 0.1919-0.2895 in BC2-BC3 and 0.2514-0.2902 in TF4 and TF5.

Total bacterial community

The Ion Torrent analysis was used to study the bacterial community composition inhabiting brine samples. The community will be described *per* sampling site, i.e. Boulder Clay followed by Tarn Flat.

The Shannon_H index was the highest in BC1 (1.783) and the lowest in BC2 (1.087) for BC samples, whereas in TF5 (1.981) it was higher than TF4 (1.79) for TF sample. The Evenness_e^H index was highest in BC1 (0.2705) within BC samples and in TF5 (0.3294) in TF samples.

BOULDER CLAY

A total of 131,619 reads resulted from Ion Torrent sequencing analysis that were used for the bioinformatics analysis. After this step the number of reads decreased of 72.9 % with a final total number of 35,632. In the Table 6.5 the read numbers for each sample than grouped in OTUs (Operational Taxonomic Units) are reported.

Table 6.5 Read numbers for each sample and the relative OTU number. The percentage of affiliation to known genera is also reported.

Sample ID	*Reads number*	*OTU*	*% Affiliation to genera*
BC1	11,135	2,139	65
BC2	13,920	7,393	97
BC3	10,577	1,930	88

The analysis showed a predominance of *Bacteroidetes* (19.75-33.9 %, BC2 and BC3, respectively), followed by *Proteobacteria* (19.75-36.1 %, BC2 and BC1, respectively), *Actinobacteria* (1.43-12.36 %, BC1 and BC3, respectively), whereas a lower percentage

was obtained for *Cyanobacteria* (0.3-6.5 %, BC2 and BC3 respectively) and *Firmicutes* (0.36-0.96 %, BC3 and BC2, respectively). Other groups were less represented (< 1 %), i.e. *Acidobacteria*, *Aquificae*. *Chlamydiae*, *Chloroflexi*, *Fusobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Thermotogae*, and they did not occur in all samples (Figure 6.5). The bacterial community composition presented different percentages among the samples. BC1 was the more diversified samples with high percentage of Other groups, within there are different phyla that were less diversified in BC2 and BC3. Furthermore, sequences related to *CF groups* of *Bacteroidetes* were more abundant in BC2 than other samples.

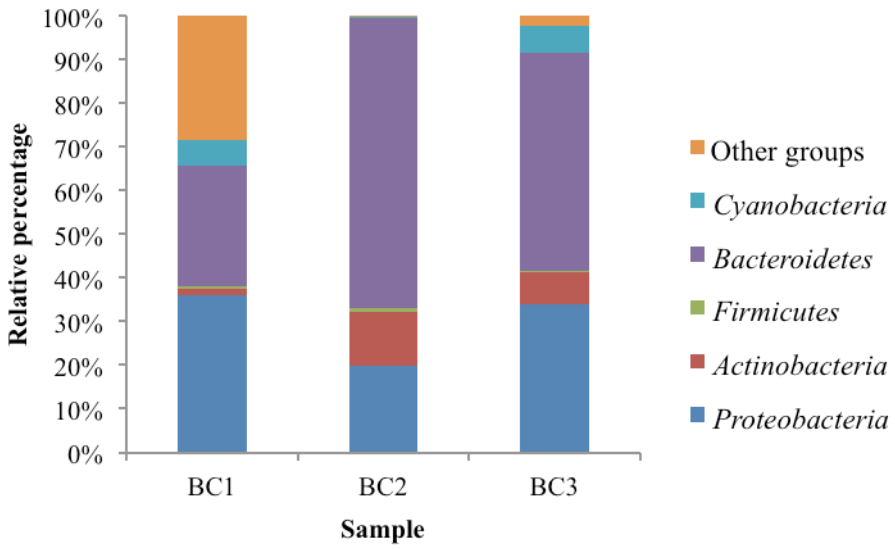

Figure 6.5 Bacterial community structure in brine samples in Boulder Clay

The *Proteobacteria* phylum was represented by five major classes, *Alpha-*, *Beta-*, *Gamma-*, *Delta-* and *Epsilon*proteobacteria. The *Gammaproteobacteria* were the predominant sub-class (13.1-18.3 % in BC1 and BC3, respectively), followed by *Betaproteobacteria* (3.4-17.7 % in BC1 and BC3, respectively) and *Deltaproteobacteria* (1.1-2.55 % in BC2 and BC3, respectively), whereas the *Epsilonproteobacteria* were retrieved at low percentage in BC1 (0.8 %) and were absent in the other samples (Figure 6.6).

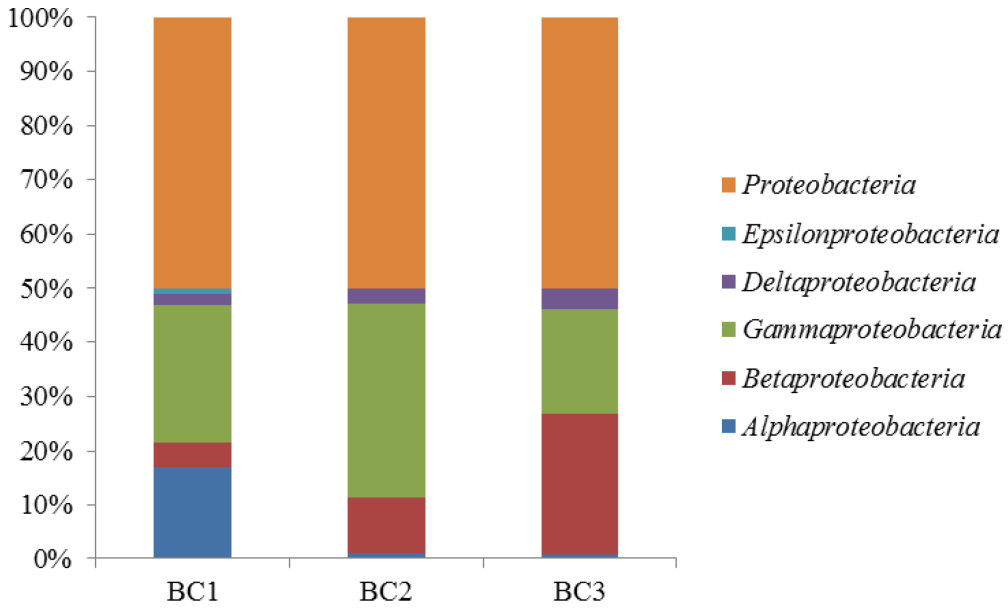

Figure 6.6 Distribution of Proteobacteria subclasses in brine samples from Boulder Clay

BC1. The NGS analysis reported 46,106 reads before the software analysis with Mothur and after the chimera-check the reads became 11,135 that were grouped in 2,139 OTUs. The bacterial community composition was distributed in different phyla, with the predominance of *Proteobacteria* (36.1 %) followed by *Bacteroidetes* (27.5 %) and *Cyanobacteria* (5.9 %). Minor phyla, which occurred at low percentages, were grouped in a unique group (27.5 %) that included *Chloroflexi* (0.1 %), *Planctomycetes* (0.1 %) and *Chlamydiae* (0.1 %), whereas the other had percentage lower than 0.1 % (*Acidobacteria*, *Aquificae*, *Fusobacteria*, *Lentisphaerae*, *Nitrospirae*, *Synergistetes*, *Thermodesulfobacteria*, *Thermotogae*, *Verrucomicrobia*). The *Proteobacteria* were distributed in all five classes, with the predominance of *Gammaproteobacteria* (18.3 %), followed by *Alphaproteobacteria* (12.3 %), *Betaproteobacteria* (3.4 %), and *Deltaproteobacteria* (1.3 %), while *Epsilonproteobacteria* were only 0.8 % of total reads. Of the total high-quality DNA bacterial sequences from the BC1 brine, about 35 % were not classified at genus level. A total of 176 genera were resolved from the rest, ranging from 0.01 to 13.5 % of total sequences. Only few genera occurred at ≥0.1 % of the total

bacterial sequences, as reported in Table 6.6. In particular, the genera *Flavobacterium* (13.5 % of total sequences), *Marichromatium* (11.4 %), *Psychroserpens* (9.1 %) and *Sulfitobacter* (7.6 %) were the most frequent. Finally, 4.2 % of total sequences were related to cyanobacterial chloroplasts.

The genera were well distributed in each class of *Proteobacteria*, in particular the *Alphaproteobacteria* were represented by five genera, among which *Methylobacterium* and *Sulfitobacter* were better represented. *Betaproteobacteria* were represented by three genera and the reads were mainly affiliated to *Rhodoferax* spp.. *Gammaproteobacteria* presented different genera with sequences well distributed between them, with a presence of *Escherichia*, *Pseudomonas* and *Marichromatium*. *Deltaproteobacteria* and *Epsilonproteobacteria* were represented by one genus each, *Desulfobacterium* and *Arcobacter*, respectively. The other phyla presented one genera each except for *Bacteroidetes* with six genera well distributed, such as *Algoriphagus*, *Flavobacterium* and *Psychroserpens*.

Table 6.6. Genera retrieved within the total bacterial community in the BC1 brine.

Phylum or Class	Genus
Alphaproteobacteria	*Paracoccus*
	Rhodobacter
	Sphingomonas
	Methylobacterium
	Sulfitobacter
Betaproteobacteria	*Polaromonas*
	Roseatales
	Rhodoferax
Gammaproteobacteria	*Glaciecola*
	Citrobacter
	Vibrio
	Escherichia
	Pseudomonas
	Marichromatium
Deltaproteobacteria	*Desulfobacterium*
Epsilonproteobacteria	*Arcobacter*
Actinobacteria	*Propionibacterium*
	Candidatus_*Aquiluna*
Bacteroidetes	*Cellulophaga*
	Lutibacter
	Ulvibacter
	Algoriphagus
	Flavobacterium
	Psychroserpens
Chloroflexi	*Roseiflexus*
Cyanobacteria	*Symploca*
	Cylindrospermum

0.1-0.9%
1.0-5.0%
≥5.0%

BC2. After the NGS analysis the Ion Torrent recovered 41,611 reads. After Mothur software analysis the reads were reduced until 13,920 reads grouped in 7,393 OTUs. The bacterial community showed a predominance of *Bacteroidetes* (66.4 %) followed by *Proteobacteria* (19.75 %) and *Actinobacteria* (12.4 %). The other phyla were retrieved below 1 %, as *Firmicutes* (0.96 %) and *Cyanobacteria* (0.3 %). The other phyla were grouped in 0.2 % of total reads (i.e. *Aquificae*, *Chloroflexi*, *Cyanobacteria*, *Fusobacteria*, *Lentisphaerae*, *Synergistetes*, *Thermotogae* and *Verrumicrobia*).

The *Proteobacteria* were represented by the five classes with the predominance of *Gammaproteobacteria* (14.25 %), followed by *Betaproteobacteria* (4.03 %) and *Deltaproteobacteria* (1.06 %). The other two sub-phyla were represented by reads with

percentage below 1 %, as *Alphaproteobacteria* (0.4 %) and *Epsilonproteobacteria* (0.01 %).

Of the total high-quality bacterial sequences from the BC2 brine, only <3 % were not classified at genus level. A total of 114 genera were resolved from the rest, ranging from 0.01 to 64.2 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.7, with the genus *Flavobacterium* and *Marichromatium* that accounted for 64.2 and 13.7 % of total sequences, respectively.

The better represented genera were distributed in the four classes of *Proteobacteria*, i.e. *Methylobacterium* and *Sphingomonas* within *Alphaproteobacteria*, *Rhodoferax*, *Hydrogenophaga* and *Polaromonas* within *Betaproteobacteria*, *Marichromatium* and *Escherichia* within *Gammaproteobacteria*, *Desulfobacterium* within *Deltaproteobacteria*. The *Bacteroidetes* were mainly represented by *Flavobacterium* and *Pedobacter* genera, while *Actinobacteria* were represented by *Agreia*, *Microcella* e *Yonghaparkia*.

Table 6.7 Genera retrieved within the total bacterial community in the BC2 brine.

Phylum or Class	Genus
Alphaproteobacteria	*Methylobacterium*
	Mesorhizobium
	Sphingomonas
Betaproteobacteria	*Brachymonas*
	Hydrogenophaga
	Malikia
	Polaromonas
	Pseudacidovorax
	Rhodoferax
Deltaproteobacteria	*Desulfobatcerium*
Gammaproteobacteria	*Acinetobacter*
	Escherichia
	Marichromatium
Firmicutes	*Atopobacter*
	Megasphaera
	Selenomonas
Bacteroidetes	*Algoriphagus*
	Flavobacterium
	Hymenobacter
	Myroides
	Sporocytophaga
	Pedobacter
Actinobacteria	*Salinibacterium*
	Agreia
	Microcella
	Yonghaparkia
Cyanobacteria	*Symploca*

0.1-0.9%
1.0-9.0 %
≥10.0 %

BC3. The number of reads after Ion Torrent sequencing was 38,295 that was reduced to 10,577 in 1,930 OTUs after bioinformatics analysis. *Bacteroidetes* (49.7 %) was the predominant phylum followed by *Proteobacteria* (33.9 %), *Actinobacteria* (7.4 %) and *Cyanobacteria* (6.5 %). The phylum of *Firmicutes* was retrieved at low percentage (0.4 %) and the other phyla were grouped in one group with a total percentage of 2.1 %. The *Proteobacteria* were distributed in four principal classes: the *Betaproteobacteria* (17.7 %) was the sub-classes better represented followed by *Gammaproteobacteria* (13.1 %)

and *Deltaproteobacteria* (2.55 %). *Alphaproteobacteria* showed a low percentage (0.55 %), while *Epsilonproteobacteria* were absent.

Of the total high-quality bacterial sequences from the BC3 brine, about 12 % were not classified at genus level. A total of 168 genera were resolved from the rest, ranging from 0.01 to 35.7 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.8. In particular, the genera *Flavobacterium*, *Marichromatium* and *Algoriphagus* accounted for 35.7, 12.1 and 11.5 %, respectively. Further, the 4.9 % of total sequences were assigned to cyanobacterial chloroplasts.

The reads were mainly distributed in four classes of *Proteobacteria* with a predominance of *Gammaproteobacteria* with *Marichromatium*, *Alphaproteobacteria* with *Rhodoferax* and *Deltaproteobacteria* with *Desulfobacterium*. Among the *Bacteroidetes* two principal genera were mainly represented, i.e. *Flavobacterium* and *Algoriphagus* with a percentage >10 %.

Table 6.8 Genera within the total bacterial community retrieved from the BC3 brine.

Phylum or Class	Genus
Alphaproteobacteria	*Mesorhizobium*
	Rhodoferax
Betaproteobacteria	*Brachymonas*
	Hydrogenophaga
	Malikia
	Pseudacidovorax
	Denitratisoma
Deltaproteobacteria	*Myxococcus*
	Desulfobacterium
Gammaproteobateria	*Acinetobacter*
	Marichromatium
Actinobacteria	Candidatus_*Aquiluna*
	Yonghaparkia
	Agreia
	Microcella
Bacteroidetes	*Cellulophaga*
	Flavobaterium
	Algoriphagus
	Persicivirga
	Hymenobacter
	Sporocytophaga
	Pedobacter
	Polaromonas
Cyanobacteria	*Aphanothece*
	Pseudanabaena
	Symploca
	Calothrix
	Nostochopsis

0.1-0.9%
1.0- 9.0%
≥10%

TARN FLAT

The number of reads before the bioinformatics analysis was 75,727 that was reduced of 67.3 % during chimera and cleaning analysis with Mothur software and became 24,740 than grouped in OTUs in each sample. The read number for each samples and the OTUs

number were reported in the Table below. In the last column the percentages of reads that were affiliated to the genera are reported (Table 6.9).

Table 6.9 Read number *per* samples and the relative OTU number.

Sample ID	*Reads number*	*OTU*	*% Affiliation to genera*
TF4	12,731	8,309	86
TF5	12,009	5,434	82

The analysis showed a predominance of *Proteobacteria* (65.9-41.1 %, TF5 and TF4, respectively), followed by *Bacteroidetes* (9.4-28.6 %, TF5 and TF4, respectively) and *Actinobacteria* (13.7-17.2 %, TF5 and TF4, respectively), whereas the *Firmicutes* (0.6-1.5 %, TF5 and TF4, respectively), *Spirochaetes* (0.82-2.1 %, TF5 and TF4, respectively) and *Planctomycetes* (0.14-1.55 % TF5 and TF4, respectively) were less abundant in the total bacterial community. The other phyla were less than 1 % (i.e. *Acidobacteria*, *Aquificae*, *Chloroflexi*, *Deinococcus-Thermus*, *Gemmatimonadates*, *Lentisphaerae*, *Verrucomicrobia*) and were grouped in Other groups with a percentage of 7.6-8.9 % (TF4 and TF5, respectively) (Figure 6.7).

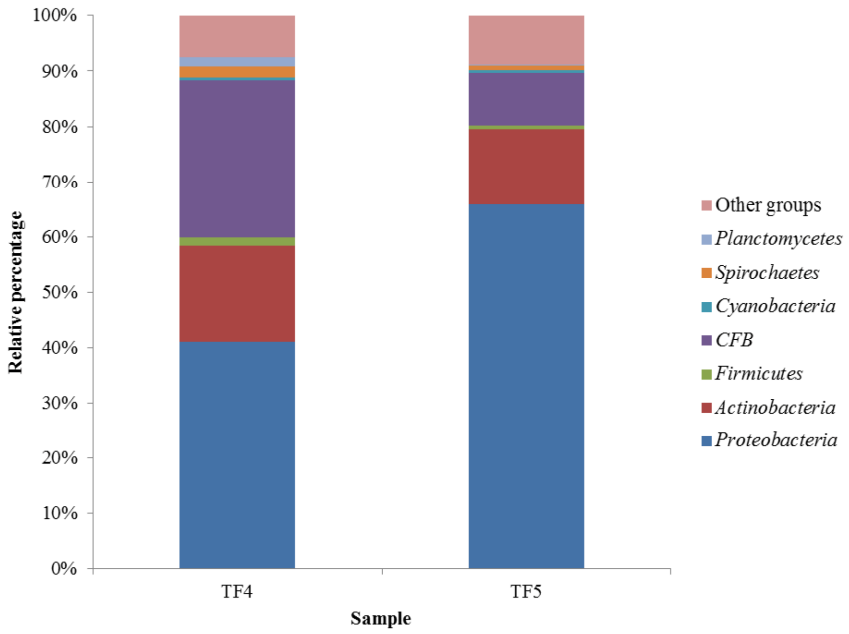

Figure 6.7 Bacterial community structure of brine samples from Tarn Flat

The Proteobacteria reads were distributed in five classes: *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, *Epsilonproteobacteria*. The *Gammaproteobacteria* were the most abundant sub-class (41.1-65.9 %, TF4 and TF5 respectively), followed by *Betaproteobacteria* (2.9-4.6 %, TF4 and TF5, respectively), *Deltaproteobacteria* (1.6-22.0 %, TF4 and TF5 respectively) *Epsilonproteobacteria* (1.36-5.4 %, TF4 and TF5 respectively) and *Alphaproteobacteria* (1.85-4.3 %, TF4 and TF5, respectively) (Figure 6.8). TF4 presented a higher percentage of *Gammaproteobacteria* than TF5, which in turne had higher percentage of *Deltaproteobacteria* than TF4.

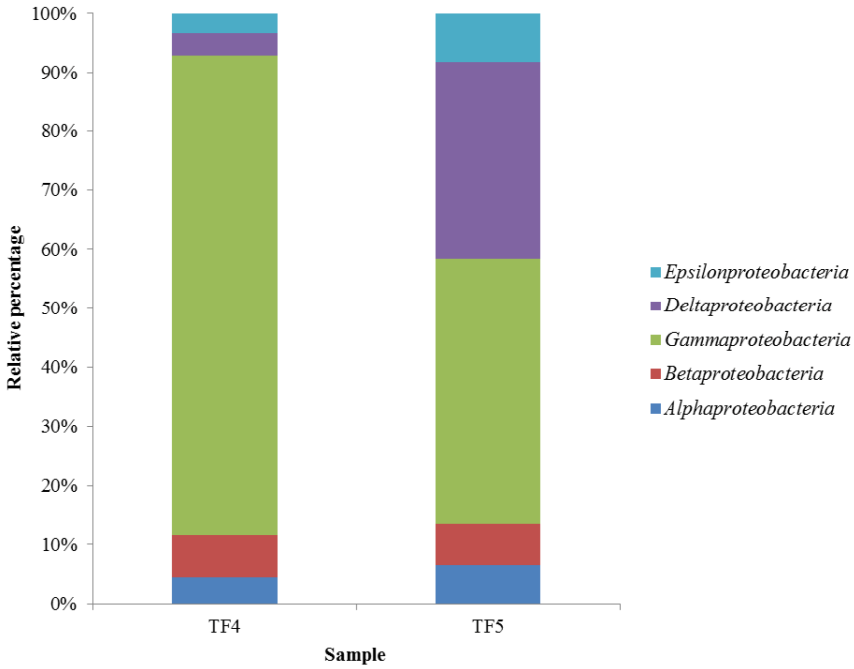

Figure 6.8 *Proteobacteria* community structure in brine samples from Tarn Flat

TF4. A total of 37,583 reads were retrieved from Ion Torrent sequencing analysis. This number decreases to 12,731 grouped in 8,309 OTUs after bioinformatics analysis using Mothur software. The OTUs were distributed in 21 different phyla at different percentages. The predominant phylum was *Proteobacteria* (41.1 %), followed by

Bacteroidetes (28.6 %), *Actinobacteria* (17.2 %), *Spirochaetes* (2.1 %), *Firmicutes* (1.5 %) and *Planctomycetes* (1.5 %). The other phyla presented percentages lower than 1 % (i.e. *Aquificae* 0.2 %, *Cyanobacteria* 0.35 %, *Verrumicrobia* 0.48 %, *Acidobacteria*). The *Proteobacteria* were distributed in five classes with a predominance of *Gammaproteobacteria* (33.4 %), followed by *Betaproteobacteria* (2.88 %), *Alphaproteobacteria* (1.85 %), *Deltaproteobacteria* (1.6 %) and *Epsilonproteobacteria* (1.36 %).

Of the total high-quality bacterial sequences from the TF4 brine, about 14 % was not classified at genus level. A total of 268 genera were resolved from the rest, ranging from 0.01 to 13.7 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.10. In particular, the genera *Ulvibacter* (13.7 % of total sequences), *Marichromatium* (10.5 %), *Thiomicrospira* (8.8 %) and *Marinobacter* (5.7 %) were the most frequent. The TF4 samples were represented by different genera with a percentage ranging between 0.1-0.9 % and by few genera with a percentage of reads higher than 5 %. Three classes of *Proteobacteria* were well represented with different genera. *Alphaproteobacteria* reads were affiliated to *Sulfitobacter*, *Parococcus* and *Devosia*, *Betaproteobacteria* with *Polaromonas* and *Rhodoferax*, whereas *Gammaproteobacteria* presented the higher number of sequenced mainly distributed in *Marinobacter*, *Thiomicrospira*, *Marichromatium* genera. *Deltaproteobacteria* and *Epsilonproteobacteria* were related to one genus each, Candidatus_*Thiobios* and *Sulfurimonas*, respectively. The other phyla were distributed among different genera, i.e. *Actinobacteria* were mainly related to *Illumatobacter*, *Leifsonia*, Candidatus_*Aquiluna*, while *Bacteroidetes* with *Gaetbulibacter* and *Ulvibacter*, *Spirochaetes* with *Cloacamonas*. The other phyla presented genera with few affiliated reads.

Table 6.10 Bacterial genera of total bacteria retrieved from the TF4 brine.

Phylum or Class	Genus	Phylum or Class	Genus
Alphaproteobacteria	*Devosia*	Bacteroidetes	*Lutimonas*
	Paracoccus		*Bizionia*
	Sphingopyxis		*Polaribacter*
	Sulfitobacter		*Cyclobacterium*
	Roseovarius		*Fluviicola*
Betaproteobacteria	*Caenimonas*		*Cellulophaga*
	Polaromonas		*Lutibacter*
	Rhodoferax		*Sediminicola*
Gammaproteobacteria	*Haliea*		*Psychroserpens*
	Modicisalibacter		*Dyadobacterium*
	Pseudospirillum		*Arenibacter*
	Paraferrimonas		*Flavobacterium*
	Candidatus_*Ruthia*		*Gaetbulibacter*
	Marinobacter		*Psychroflexus*
	Thiomicrospira		*Algoriphagus*
	Marichromatium		*Ulvibacter*
Deltaproteobacteria	Candidatus_*Thiobios*	Spirochaetes	*Cloacamonas*
Epsilonproteobacteria	*Sulfurimonas*	Firmicutes	*Alkalibacter*
Actinobacteria	*Yonghaparkia*		*Filifactor*
	Nitriliruptor	Planctomycetes	*Schlesneria*
	Salinibacterium		Candidatus_*Kuenenia*
	Ornithinicoccus	Cyanobacteria	*Symploca*
	Coriobacterium		
	Microcella		
	Lamia		
	Okibacterium		
	Physiococcus		
	Illumatobacter		
	Leifsonia		
	Candidatus_*Aquiluna*		
Aquificae	*Hydrogenobaculum*		

0.1-0.9%
1.0-5.0%
≥5.0%

TF5. The samples were analysed for the prokaryotic community composition using Ion Torrent analysis. The output gave a 38,144 read numbers that was reduced to 12,009 reads distributed in 5434 OTUs. The OTUs were distributed in different bacterial phyla with a predominance of *Proteobacteria* (65.9 %), followed by *Actinobacteria* (13.7 %) and *Bacteroidetes* (9.4 %). The other phyla were less abundant with a lower percentage

under 1 % of total reads. For example, the phyla *Spirochaetes* (0.8 %), *Firmicutes* (0.6 %) and *Cyanobacteria* (0.6 %) were retrieved but at less percentage.

Of the total high-quality bacterial sequences from the BC1 brine, about 18 % were not classified at genus level. A total of 269 genera were resolved from the rest, ranging from 0.01 to 14.4 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.11. In particular, the genera *Geopsychrobacter* (14.4 % of total sequences), *Thiomicrospira* (11.9 %), *Shewanella* (10.9 %) and *Sulfiromonas* (5.3 %) were the most frequent. TF5 showed a high numbers of genera with a percentage above 1 % of reads affiliated. In particular the class of *Proteobacteria* had the sequences well distributed among them, with predominance of *Roseovarius* within *Alphaproteobacteria*, *Pseudoacidovorax* within *Betaproteobacteria*, *Shewanella* and *Thiomicrospira* within *Gammaproteobacteria*, *Desulfobacterium*, *Desulforhopalus* and *Geopsychrobacter* within *Deltaproteobacteria*. *Sulfurimonas* was the only one genus retrieved within *Epsilonproteobacteria*. *Actinobacteria* were represented by *Leifsonia*, Candidatus_*Aquiluna* and *Okibacterium*, whereas *Bacteroidetes* were represented by *Arenibacter*, *Algoriphagus* and *Gaetbulibacter* genera.

Table 6.11 Genera retrieved within the total bacterial community in the TF5 brine.

Phylum or Class	Genus	Phylum or Class	Genus
Alphaproteobacteria	*Hoeflea*	Actinobacteria	*Kytococcus*
	Sneathiella		*Microcella*
	Sphingopyxis		*Salinibacterium*
	Parvibaculum		*Illumatobacter*
	Catellibacterium		*Leifsonia*
	Pelagibus		Candidatus_*Aquiluna*
	Devosia		*Okibacterium*
	Paracoccus	Bacteroidetes	*Crocinitomix*
	Roseovarius		*Flavobacterium*
Betaproteobacteria	*Aquamonas*		*Psychroflexus*
	Polaromonas		*Arenibacter*
	Pseudacidovorax		*Algoriphagus*
Gammaproteobacteria	*Balneatrix*		*Gaetbulibacter*
	Citrobacter	Firmicutes	*Anaerovorax*
	Haliea	Spirochaetes	*Spirochaete*
	Colwellia	Cyanobacteria	*Geovibrio*
	Shewanella		
	Thiomicrospira		
Deltaproteobacteria	*Desulfobulbus*		
	Desulfobacula		
	Desulfocapsa		
	Desulfobacterium		
	Desulforhopalus		
	Geopsychrobacter		
Epsilonproteobacteria	*Sulfurimonas*		

0.1-0.9%
1.0-5.0%
≥5.0%

Active bacterial community

The analysis was performed only on BC sample due the low reads presented in TF samples, which were not analysed. In the table below (Table 6.12) the reads retrieved and in the samples and their distribution in OTUs are shown.

Table 6.12 Read number *per* sample and relative OTU number.

Sample ID	*Reads number*	*OTU*	*% Affiliation to genera*
BC1	672	419	40
BC2	238	155	40
BC3	8,485	3,894	80

The active bacterial reads were related to the total bacterial reads (reported in the paragraphs above) to evaluate the percentage of active bacteria within the community. 6.0, 1.7 % and 80.2 % of the bacterial community resulted active in BC1, BC2 and BC3 brines, respectively.

In Boulder Clay, the analysis showed the predominance of CF group of *Bacteroidetes* (range 8.8-57.7 %, BC1 and BC3 respectively), followed by *Proteobacteria* (16.4-22.7 %, BC2 and BC3, respectively), *Actinobacteria* (5.0-9.2 %, BC2 and BC3 respectively) and *Firmicutes* (0.5-10.1 %, BC3 and BC1 respectively). Minor groups (i.e *Acidobacteria*, *Aquificae*, *Cyanobacteria*, *Fusobacteria*, *Deinococcus-Thermus*, *Planctomycetes*, *Verrucomicrobia*, *Fusobacteria*) occurred at percentages below 2 % and 0.1 % were observed only in certain samples. The unclassified reads ranged between 42.1 and 46.2 % (BC1 and BC2, respectively). The BC1 and BC2 samples were well diversified with similar percentages of phyla, while BC3 presented a predominance of *CF group* of *Bacteroidetes* and low percentage of the other major phyla (Figure 6.9).

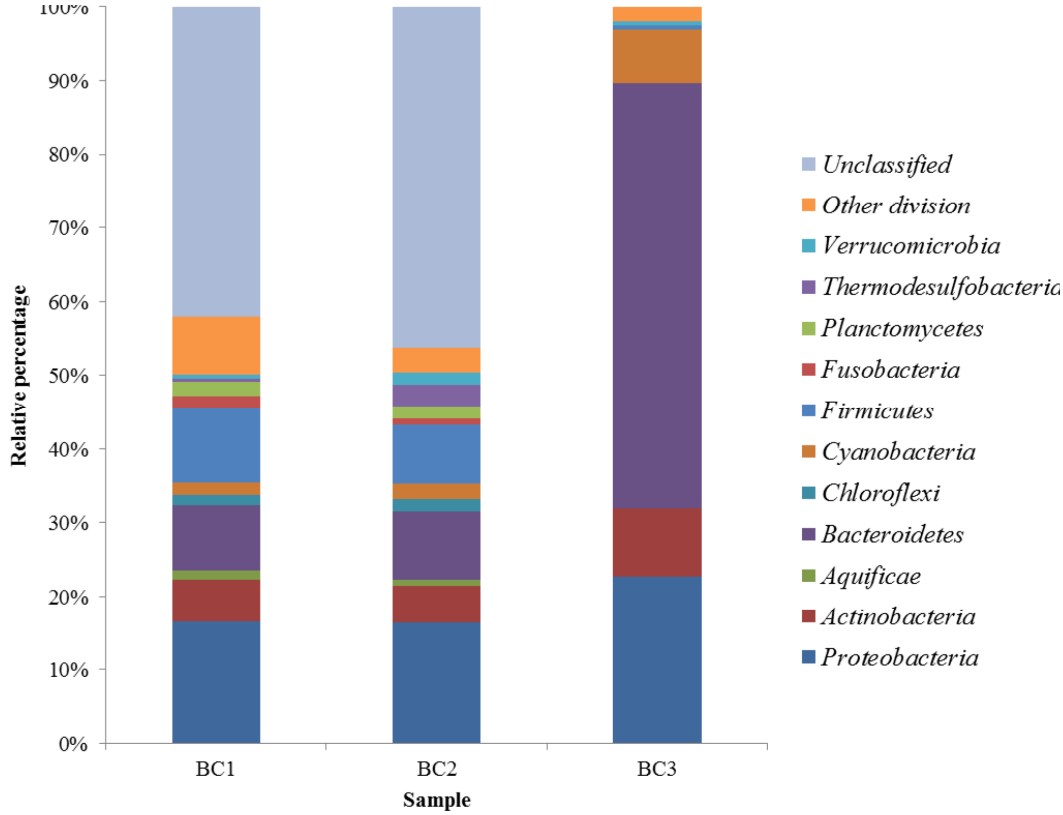

Figure 6.9 Active microbial community structure in brines sample collected from Boulder Clay

The *Proteobacteria* were represented by five classes (*Alpha-*, *Beta-*, *Gamma-*, *Delta-* and *Epsilon-*), as follows: *Betaproteobacteria* (1.9-21.0 %, BC1 and BC3 respectively), followed by *Alphaproteobacteria* (0.4-5.9 %, BC3 and BC2 respectively) and *Gammaproteobacteria* (1.1-4.0 %, BC3 and BC1 respectively), whereas the *Deltaproteobacteria* (0.2-3.6 %, BC3 and BC1 respectively) and *Epsilonproteobacteria* (0.4-2.1 %, BC2 and BC1 respectively) were found at lower abundances and only in some samples (Figure 6.10). The sub-classes of *Proteobacteria* were constant in percentage in the BC1 and BC2 samples, while BC3 presented a higher percentage of *Bacteroidetes*.

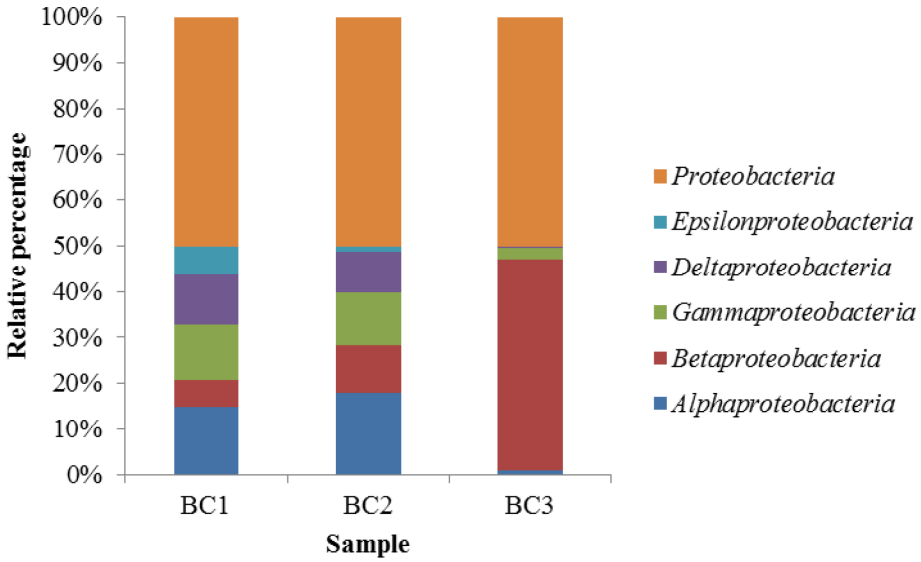

Figure 6.10 Active Proteobacteria structure in BC samples

BC1. After the quality check with Mothur and chimera removal, a total of 672 reads were retained and grouped in 419 OTUs. The bacterial community in BC1 was mainly constituted by *Proteobacteria* (16.5 %), followed by *Firmicutes* (10.1 %), CF group of *Bacteroidetes* (8.8 %) and *Actinobacteria* (5.8 %), whereas the phylum *Planctomycetes* (1.9 %) and *Cyanobacteria* (1.6 %) were less abundant. The other phyla were detected at low percentages, for example *Fusobacteria* (1.6 %), *Chloroflexi* (1.5 %) and *Aquificae* (1.2 %), whereas minor groups occurred below 1 % (i.e. *Verrucomicrobia*, *Spirochaetes*, *Nitrospirae*, *Lentisphaerae*, *Deinococcus-Thermus*, *Thermodesulfobacteria*, *Gemmatimonadetes*, *Chlorobi*, *Thermotogae*, *Deferribacteres*, *Acidobacteria*). The *Proteobacteria* were distributed in five classes, with a higher percentage for *Alphaproteobacteria* (4.9 %), followed by *Gammaproteobacteria* (4.0 %) and *Deltaproteobacteria* (3.6 %), while the sub-classes of *Epsilonproteobacteria* (2.1 %) and *Betaproteobacteria* (1.9 %) were the less abundant.

Of the total high-quality bacterial sequences from the BC1 brine, about 60 % were not classified at genus level. A total of 214 genera were resolved from the rest, ranging from

0.1 to 0.9 % of total sequences. Only few genera occurred at ≥0.3 % of the total bacterial sequences, as reported in Table 6.13. The active bacterial community were distributed among few genera mainly represented by *Rhodobacter* among *Alphaproteobacteria*, *Lebetimonas* in *Epsilonproteobacteria*, *Streptomyces* and *Rubrobacter* in *Actinobacteria* and *Streptobacillus* among *Fusobacteria*.

Table 6.13 Genera of active bacteria retrieved from the BC1 brine.

Phylum or Class	Genus	Phylum or Class	Genus
Alphaproteobacteria	*Rhodobacter*	Fusobacteria	*Cetobacterium*
	Sphingomonas		*Streptobacillus*
Betaproteobacteria	*Petrobacter*	Spirochaetes	*Treponema*
	Silvimonas	Thermodesulfobacteria	*Thermodesulfobacterium*
Gammaproteobacteria	Candidatus_*Carsonella*	Firmicutes	*Oceanobacillus*
	Oleiphilus		*Salsuginibacillus*
Deltaproteobacteria	Candidatus_*Entotheonella*		*Dolosicoccus*
Epsilonproteobacteria	*Sulfurimonas*		*Clostridium*
	Lebetimonas		*Acetitomaculum*
Actinobacteria	*Glycomyces*		*Johnsonella*
	Propionibacterium		*Dehalobacter*
	Streptomyces		*Syntrophomonas*
	Gardnerella		*Megasphaera*
	Rubrobacter	Chloroflexi	*Roseiflexus*
Aquificae	*Aquifex*	Cyanobacteria	*Anabaena*
	Thermovibrio		*Gloeothece*
Bacteroidetes	*Prevotella*		
	Saprospira		
	Pseudosphingobacterium		
	Hymenobacter		

0.3-0.5%
≥0.6%

BC2. After quality check with Mothur and chimera removal the reads were 238 distributed in 155 OTUs. The bacterial community was constituted by *Proteobacteria* (16.4 %) followed by *Bacteroidetes* (9.2 %), *Firmicutes* (8 %) and *Cyanobacteria* (2.1 %), whereas the phylum of *Thermodesulfobacteria* (1.7 %), *Chloroflexi* (1.7 %) and *Planctomycetes* (1.7 %) were less abundant. The other phyla occurred at less than 1 % of reads. The phylum of *Proteobacteria* was represented by five classes and the reads were distributed mainly in *Alphaproteobacteria* (5.9 %), followed by *Gammaproteobacteria*

(3.8 %), *Betaproteobacteria* (3.4 %) and *Deltaproteobacteria* (2.9 %), while *Epsilonproteobacteria* were the less abundant sub-class (0.4 %).

Of the total high-quality bacterial sequences from the BC2 brine, about 60 % were not classified at genus level. A total of 77 genera were resolved from the rest, ranging from 0.4 to 2.9 % of total sequences. Only few genera occurred at ≥0.8 % of the total bacterial sequences, as reported in Table 6.14, with the genus *Caldimicrobium* that accounted for the 2.9 % of total sequences. BC2 active genera was represented by few reads related to *Desulfonema*, *Caldimicrobium* and *Niabella* distributed in *Deltaproteobacteria*, *Thermodesulfobacteria* and *Bacteroidetes*, respectively.

Table 6.14 Genera of active bacteria retrieved from the BC2 brine.

Phylum or Class	Genus
Alphaproteobacteria	*Bosea*
Betaproteobacteria	*Polaromonas*
Deltaproteobacteria	*Desulfonema*
Synergistetes	*Anaerobaculum*
Thermodesulfobacteria	*Caldimicrobium*
Firmicutes	*Mahella*
Bacteroidetes	*Niabella*
	Reichenbachiella
	Rhodothermus
Actinobacteria	*Rubrobacter*

0.8%
≥1.3

BC3. After the quality check with Mothur and chimera removal reads were 8485 distributed in 3894 OTUs. The bacterial community was mainly constituted of *Bacteroidetes* (57.7 %), followed by *Proteobacteria* (22.7 %), *Actinobacteria* (9.2 %) and *Cyanobacteria* (7.2 %). Minor groups were represented only by *Firmicutes* (0.5 %) and *Verrucomicrobia* (0.5 %). The *Proteobacteria* were mainly represented by

Betaproteobacteria (21 %), whereas *Gammaproteobacteria* (1.1 %), *Alphaproteobacteria* (1.1 %) and *Deltaproteobacteria* (0.2 %) were less represented.

Of the total high-quality bacterial sequences from the BC3 brine, about 20 % were not classified at genus level. A total of 165 genera were resolved from the rest, ranging from 0.01 to 41.9 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.15. In particular, the genera *Flavobacterium* and *Algoriphagus* accounted for 41.9 and 13.6 %, respectively. Further, the 6 % of total sequences were assigned to cyanobacterial chloroplasts.

Table 6.15 Bacterial genera of active bacteria retrieved from the BC3 brine.

Phylum or Class	Genus
Betaproteobacteria	*Hydrogenophaga*
	Ideonella
	Malikia
	Brachymonas
	Pseudacidovorax
	Polaromonas
Actinobacteria	Candidatus_*Aquiluna*
	Salinibacterium
	Yonghaparkia
	Agreia
	Microcella
Bacteroidetes	*Cellulophaga*
	Persicivirga
	Hymenobacter
	Flavobacterium
	Algoriphagus
Cyanobacteria	*Aphanothece*
	Calothrix
	Cylindrospermum
	Nostochopsis

0.1-0.9%
1.0- 9.0%
≥10%

Archaeal community structure

Brine samples were analyzed by Ion Torrent sequencing for studying the Archaeal community structure. Results will be reported in two different paragraphs, one for BC and one for TF. The diversity indices were analyzed. The Shannon_H index was highest in BC1 samples (1.178), while was lowest in BC2 (0.4084), whereas in TF samples the index was similar between 4m and 5m. The Eveness_e^H index was higher in BC3 sample (0.5762), the lowest value was recorded for BC2 sample (0.4084).

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A total of 136,185 reads in all three samples resulted from the Ion Torrent analysis. This number decreased after Mothur analysis of 71.5 % with a overall number of 38,745 reads. The number of retained sequences and the number of OTUs for each sample are reported in Table 6.16.

Table 6.16 Read number *per* sample and relative OTU number.

Sample ID	***Reads number***	***OTU***	***% Affiliation to genera***
BC1	9,752	4,146	35
BC2	17,234	7,106	46
BC3	11,759	4,590	0.1

The Archaeal community structures were constituted by a predominance of *Euryarchaeota* (45.5-62.5 %, BC3 and BC2 respectively) followed by *Crenarchaeota* (5.7-18.7 %, BC3 and BC1 respectively) and *Ancient_Archaeal_Group* (1.6-13.4 %, BC2 and BC3, respectively), while the other group were distributed at low percentages with *Korarchaeota* (0.09-0.18 %, BC3 and BC1, respectively) and *Marine_Hydrothermal_Vent_Group_1* (0.05-0.12 %, BC3 and BC1 respectively). The percentage of reads that were unclassified ranged between 27.5-31.47 %, BC1 and BC3, respectively (Figure 6.11).

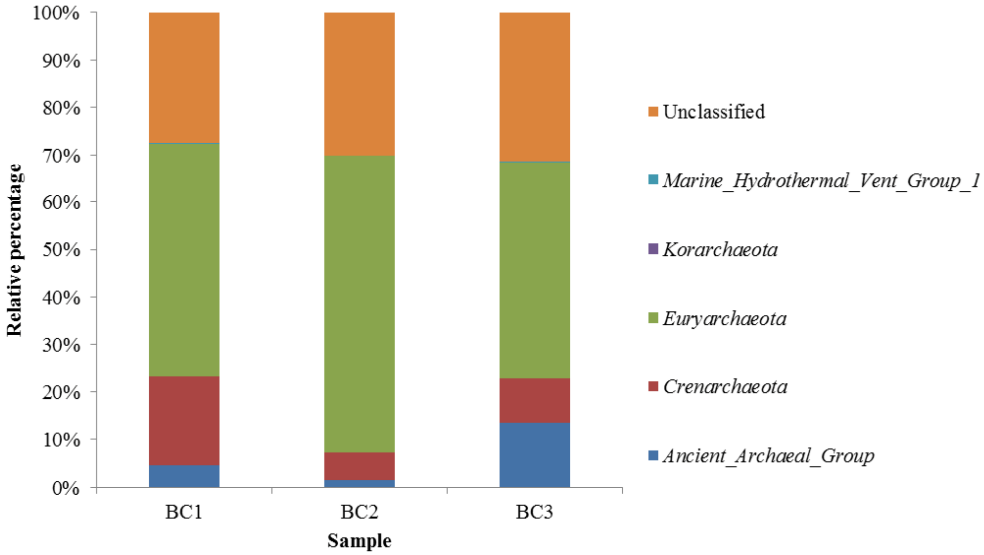

Figure 6.11 Archeaal community structure in Boulder Clay brine samples

The percentages of sequences affiliated to an order are shown in the table below (Table 6.17). The 48.0, 57.9 and 49.6 % of total sequences were assigned to an order in BC1, BC2 and BC3, respectively. In particular *Methanosarcinales* and *Methanopyrales* were predominant in BC samples. BC1 differred from the other two brines as it was characterized by a lower number of sequences related to a order and by the absence of *Methanobacteriales*, *Methanococcales* and *Methanocellales*.

Table 6.17 Distribution of Archaeal sequences in known orders. The number of sequences assigned to an order is in brackets. Percentages are referred to the number of order-assigned sequences for each sample.

Phylum or Class	Order	BC1 (4680)	BC2 (9979)	BC3 (5827)
Crenarchaeota	Acidilobales	5.2	1.9	3.9
	Desulfurococcales	2.4	1.3	3.1
	Sulfolobales	17.7	1.2	6.1
	Thermoproteales	1.5	1.1	2.0
Euryarchaeota	Archaeoglobales	2.2	2.2	2.2
	Halobacteriales	11.5	16.1	11.0
	Methanobacteriales	-	23.7	39.7
	Methanococcales	-	0.1	0.3
	Methanocellales	-	0.04	0.1
	Methanomicrobiales	1.1	0.8	3.8
	Methanosarcinales	18.1	13.6	14.5
	Methanopyrales	29.6	33.1	5.7
	Thermococcales	2.4	0.4	0.9
	Thermoplasmatales	8.2	4.4	6.8

BC1. Retained reads after Mothur analysis were 9,752 (out of 49,197) and distributed in 4,146 OTUs. The Archaeal community were represented mainly by *Euryarchaeota* (49.0 %) followed by *Crenarchaeota* (18.7 %) and *Ancient_Archael_Group* (4.54 %). The unclassified reads were 27.5 % of total reads while low percentage of reads were affiliated to *Korarchaeota* and *Marine_Hydrothermal_Vent_Group_1* (0.18 and 0.12 %, respectively).

Of the total high-quality archaeal sequences from the BC1 brine, about 65 % were not classified at genus level. A total of 75 genera were resolved from the rest, ranging from 0.01 to 14.2 % of total sequences. Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.18. In particular, the genera *Methanopyrus* (14.2 %), *Methanothermus* (9.3 %) and *Sulfurisphaera* (7.6 % of total sequences) were the most frequent.

In particular, the sequenced affiliated to genera were mainly among *Euryarchaeota* with a predominance of *Methanothermus* and *Methanopyrus* while in *Crenarchaeota* the sequences were mainly affiliated to *Acidilobus*, *Methanoplanus*, *Methanosalsum* and *Sulfurispaera*.

Table 6.18 Genera retrieved within the total archaeal community in the BC1 brine.

Phylum or Class	Genus
Crenarchaeota	*Ignicoccus*
	Sulfophobococcus
	Sulfolobus
	Caldivirga
	Thermocladium
	Caldisphaera
	Acidilobus
	Methanoplanus
	Methanosalsum
	Sulfurisphaera
Euryarchaeota	*Ferroglobus*
	Halostagnicola
	Haloterrigena
	Methanobrevibacter
	Methanococcus
	Methanimicrococcus
	Pyrococcus
	Thermococcus
	Thermoplasma
	Methanothermus
	Methanopyrus
Korarchaeota	Candidatus_*Korarchaeum*

0.1-0.9%
1.0-4.9%
≥5.0%

BC2. A total 17,234 (out of 42,195) sequences were retained after the analysis by Mothur and were distributed in 7,106 OTUs.

The samples BC2 presented a major reads affiliated to *Euryarchaeota* (62.5 %) followed by *Crenarchaeota* (5.7 %), the number of reads that did not have affiliation were 30.1 %, whereas the other groups were represented by low percentages, i.e. *Ancient_Archaeal_Group* (1.57 %), *Korarchaeota* (0.13 %), *Marine_Hydrothermal_Vent_Group_1* (0.05 %).

Of the total high-quality archaeal sequences from the BC1 brine about 54 % were not classified at genus level. A total of 73 genera were resolved from the rest, ranging from 0.01 to 19.2 % of total sequences. Only few genera occurred at ≥0.1 % of the total

bacterial sequences, as reported in Table 6.19. In particular, the genera *Methanopyrus* (19.2 % of total sequences). *Methanothermus* (13.6 %) and *Methanosalsum* (7.5 %) were the most frequent. The reads affiliated to a genera were among the *Crenarchaeota* and *Euryarchaeota* with a predominance of *Acidilobus* and *Ferroglobus*, *Methanotermus*, *Methanosalsum*, *Methanopyrus* respectively.

Table 6.19 Genera retrieved within the total archaeal community in the BC2 brine.

Phylum or Class	Genus
Crenarchaeota	Candidatus_*Nitrosocaldus*
	Caldisphaera
	Ignicoccus
	Sulfophobococcus
	Sulfurisphaera
	Pyrobaculum
	Thermocladium
	Acidilobus
Euryarchaeota	*Haloplanus*
	Methanoplanus
	Methanosphaerula
	Methanimicrococcus
	Pyrococcus
	Thermococcus
	Ferroplasma
	Picrophilus
	Ferroglobus
	Methanothermus
	Methanosalsum
	Methanopyrus

0.1-0.9%
1.0-4.9%
≥5.0%

BC3. A total of 11,759 reads (out of 44,793) were retained and were distributed in 4590 OTUs. The archaeal community was divided in five different phyla with a predominance of *Euryarchaeota* (45.5 %), followed by *Ancient_Archaeal_Group* (13.4 %) and *Crenarchaeota* (9.4 %). The other phyla were diversified in low percentage under 1 % (i.e. *Korarchaeota* 0.09 % and *Marine_Hydrothermal_Vent_Group_1* 0.11 %). 31.5 % of reads were unclassified.

Of the total high-quality archaeal sequences from the BC1 brine, about 60 % were not classified at genus level. A total of 71 genera were resolved from the rest ranging from 0.01 to 19.5 % of total sequences.

Only few genera occurred at ≥0.1 % of the total bacterial sequences, as reported in Table 6.20. In particular, the genera *Methanothermus* (19.5 %) and *Methanosalsum* (6.2 %) were the most frequent. The reads were affiliated to genera present in the *Crenarchaeota* and *Euryarchaeota* phyla, in particular the *Acidilobus*, *Sulfurisphaera*, *Ignicoccus* and *Thermocladium* were among the *Crenarchaeota* while *Ferroglobus*, *Methanoplanus*, *Methanopyrus*, *Methanothermus* and *Methanosalsum* were related to *Euryarchaeota*.

Table 6.20 Genera retrieved within the total archaeal community in the BC3 brine.

Phylum or Class	Genus
Crenarchaeota	*Ignicoccus*
	Sulfophobococcus
	Pyrodictium
	Sulfolobus
	Caldivirga
	Thermocladium
	Acidilobus
	Sulfurisphaera
Euryarchaeota	*Natronomonas*
	Methanocaldococcus
	Methanosaeta
	Methanimicrococcus
	Pyrococcus
	Thermococcus
	Methanosarcina
	Ferroglobus
	Methanoplanus
	Methanopyrus
	Methanothermus
	Methanosalsum

0.1-0.9%
1.0-4.9%
≥5.0%

TARN FLAT

Brine samples from Tarn Flat lake were analyzed for the archaeal community structure using Next Generation Sequencing system. A total of 76,562 reads were the output of the sequencing analysis. These reads were used for a bioinformatics analysis that allow to reduce the numbers of gaps or chimeras and produce a final total read number of 25,138. The number of reads *per* sample and OTU number are reported in Table 6.21.

Table 6.21 Read number per sample and relative OTU number.

Sample ID	*Reads number*	*OTU*	*% Affiliation to genera*
TF4	13,425	4,542	36
TF5	11,713	3,635	44

In general, the Archaeal community was constituted by *Euryarchaeota* (55.8-57.1 %, TF4 and TF5, respectively), *Crenarchaeota* (7.8 %, similar in all samples) and *Ancient_Archaeal_Group* (1.6-1.8 %, TF4 and TF5, respectively), whereas the *Korarchaeota* (0.02-0.05 %) and *Marine_Hydrothermal_Vent_Group_1* (0.13-0.19 %) were less represented. A total of 33-1-34.6 % of reads were unclassified (Figure 6.12).

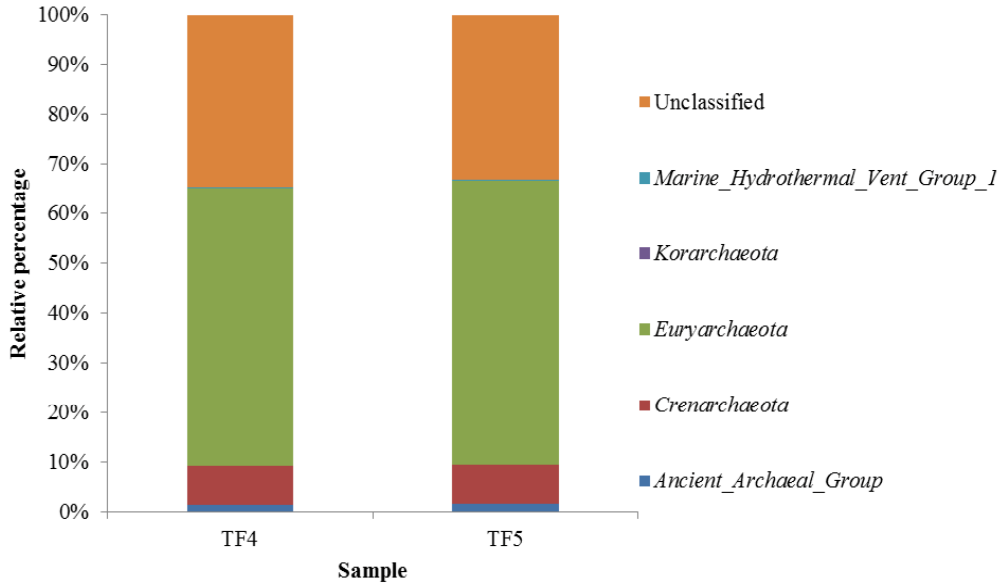

Figure 6.12 Archaeal community structure in brine samples collected in Tarn Flat

The distribution of the orders is reported in Table 6.22. The 43.0 % of sequences were related to an order in TF4 and TF5, respectively.

Table 6.22 Distribution of archeaal sequences in known orders. The number of sequences assigned to an order is in brackets. Percentages are referred to the number of order-assigned sequences for each sample.

		TF4 (5038)	TF5 (5635)
Crenarchaeota	Acidilobales	3.3	2.1
	Desulfurococcales	1.4	1.6
	Sulfolobales	3.2	2.1
	Thermoproteales	-	2.9
Euryarchaeota	Archaeoglobales	-	1.1
	Halobacteriales	3.4	3.9
	Methanobacteriales	51.4	36.1
	Methanococcales	-	1.2
	Methanosarcinales	-	28.3
	Methanocellales	0.4	-
	Methanosarcinales	8.1	5.3
	Methanopyrales	26.1	9.9
	Thermococcales	0.5	0.4
	Thermoplasmatales	1.9	4.9

TF4. A total of 39,623 reads came out from Ion Torrent analysis. This number was reduced by bioinformatics analysis to 13,425 distributed in 4,542 OTUs. The Archaeal community was mainly represented by *Euryarchaeota* (55.8 %), followed by *Crenarchaeota* (7.8 %) and *Ancient_Archaeal_Group* (1.56 %). The other groups were below 1 %, therefore their presence was trascurable (*Marine_Hydrotermal_Vent_Group_1*, 0.19 % and *Korarchaeota*, 0.05 %).

Of the total high-quality archaeal sequences from the TF4 brine, about 64 % were not classified at genus level. A total of 41 genera were resolved from the rest, ranging from 0.01 to 19.1 % of total sequences. Only few genera occurred at ≥0.1 % of the total archaeal sequences, as reported in Table 6.23. In particular, the genera *Methanothermus* (19.1 %), *Methanosalsum* (9.7 %) and *Methermicoccus* (2.1 % of total sequences) were the most frequent.

Table 6.23 Genera retrieved within the total archaeal community in the TF4 brine.

Phylum or Class	Genus
Crenarchaeota	*Acidilobus*
	Aeropyrum
	Ignicoccus
	Acidianus
	Sulfolobus
Euryarchaeota	*Haloarcula*
	Halobaculum
	Halorhabdus
	Methanobrevibacter
	Methanothermus
	Methanomicrobium
	Methanoplanus
	Methanimicrococcus
	Methanosalsum
	Methermicoccus
	Thermococcus

0.1-0.9%
1.0-4.9%
≥5.0%

TF5. A total of 36,939 reads were resulted from sequencing analysis. After bioinformatics analysis a total of 11,713 reads distributed in 3,635 OTUs resulted.

The archeal community structure were represented mainly by *Euryarcheaota* (57.14 %), followed *Creanarchaeota* (7.8 %) and *Ancient_Archeal_Group* (1.76 %). The *Korarchaeota* (0.02 %) and *Marine_Hydrotermal_Vent_Group_1* (0.13 %) were less abundant. The unclassified reads accounted for 33.14 % of total reads.

Of the total high-quality archaeal sequences from the TF5 brine, about 56 % were not classified at genus level. A total of 53 genera were resolved from the rest, ranging from 0.01 to 17.4 % of total sequences. Only few genera occurred at ≥0.1 % of the total archaeal sequences, as reported in Table 6.24. In particular, the genera *Methanothermus* (17.4 %), *Methanoplanus* (13.1 %) and *Methanopyrus* (4.8 % of total sequences) were the most frequent.

Table 6.24 Genera retrieved within the total archaeal community in the TF5 brine.

Phylum or Class	Genus
Crenarchaeota	*Acidilobus*
	Caldisphaera
	Aeropyrum
	Ignicoccus
	Sulfurisphaera
	Caldivirga
	Pyrobaculum
	Thermoproteus
	Vulcanisaeta
Euryarchaeota	*Ferroglobus*
	Halobacterium
	Halorhabdus
	Methanobacterium
	Methanothermobacter
	Methanothermus
	Methanoplanus
	Methanosaeta
	Methanimicrococcus
	Methanosalsum
	Methanopyrus
	Thermococcus
	Picrophilus

0.1-0.9%
1.0-4.9%
≥5.0%

Statistical analysis

Results from the Ion Torrent Sequencing were statistically analysed to evaluate the differences between prokaryotic community composition among TF and BC brines. NMDS analysis on DNA data is reported in Figure 6.13.

To compare the bacterial community compositions across groups of samples, the Bray-Curtis similarity analysis was performed and similarity matrices were used to obtain dendrograms and nonmetric multidimensional scaling (NMDS) plots. Samples TF4 and

TF5 showed a similar diversity, but they were different fron BC brines. In particular, BC1 was totally different from BC2 and BC3, even if BC1 and BC2 were sampled in the same lake.

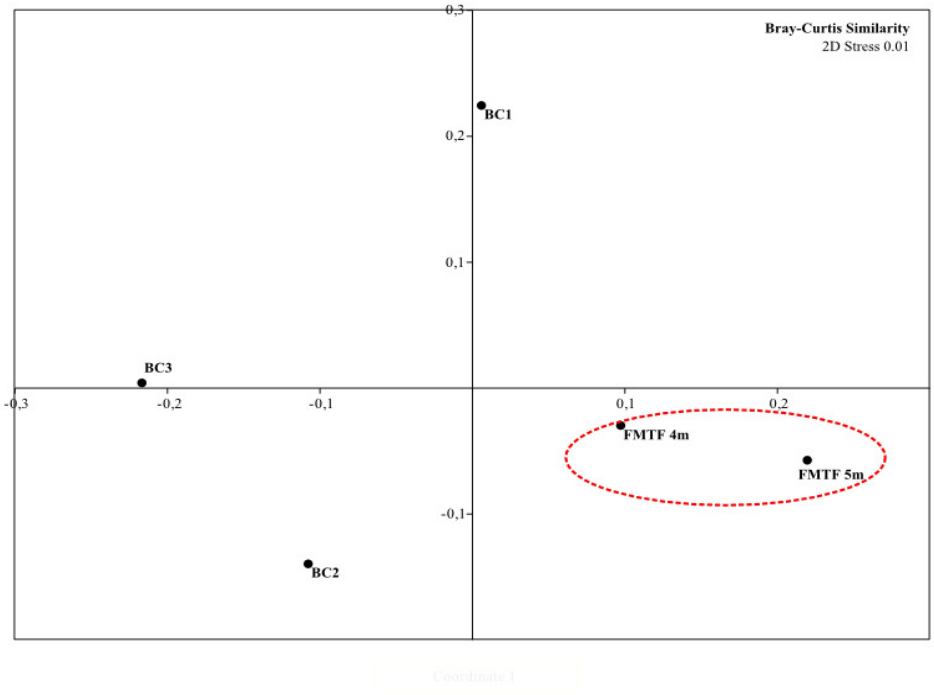

Figure 6.13 Nonmetric multidimensional scaling (NMDS) diagram representing the bacterial community composition in five Antarctic brines

Furthermore, the difference between active bacterial community and total bacterial community was analysed by NMDS analysis. The active bacterial community was named BC1c, BC2c and BC3c, while the total bacterial community was named with the sample name. The active bacterial community from BC3c was dissimilar from the BC1c and BC2c. Conversely it was similar to the total community from BC3 and BC2. The site BC1 was dissimilar from the other samples and clusterized far from the others (Figure 6.14).

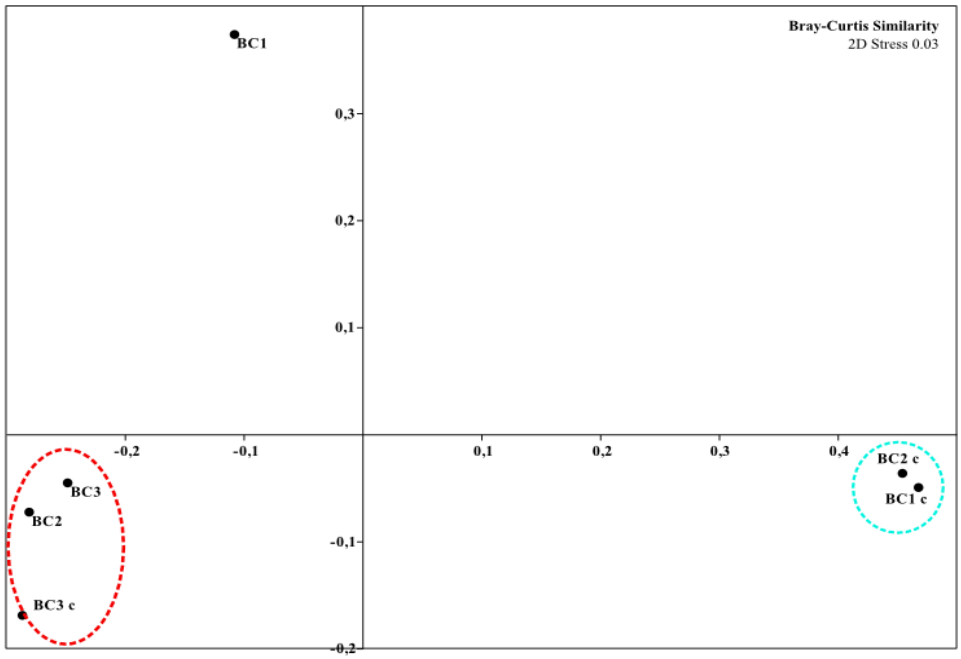

Figure 6.14 Nonmetric multidimensional scaling (NMDS) diagram representing the bacterial community composition of sampling stations BC1, BC2, BC3 from Antarctic brines

A cluster analysis was carried out to compare the similarity among samples. For Archaea it was possible to identify a high similarity between BC3 and BC1 samples and a dissimilarity from BC2 (that was more similar to TF samples than BC ones). For Bacteria, a similarity between TF and BC occurred and BC1 was dissimilar from BC2 and BC3, that clusterized together (Figure 6.15).

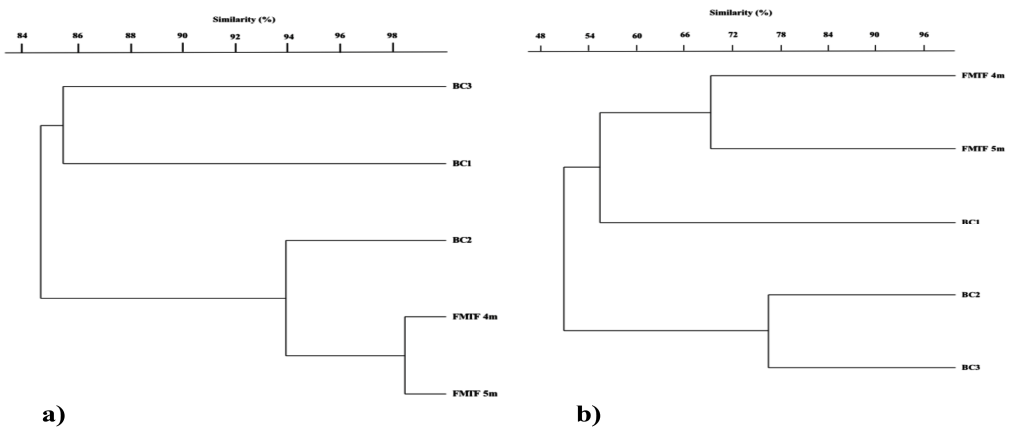

Figure 6.15 Cluster analysis to compare the similarity between the samples. a) cluster analysis on Archaeal community; b) cluster on Bacterial community

6.8 Enumeration, isolation and phylogenetic characterization of cultivable heterotrophic bacteria

All Tarn Flat samples yielded colonies on TSA (different concentration of nutrients) and $R2A_{10}$ medium. Viable counts in TF brines on TSA medium ranged between 3.1 x 10^3 and 4.7 x 10^3 CFU mL^{-1} in TF4 (on TSA_1 and TSA_{50}, respectively), and between 5.3 x 10^3 and 7.2 x 10^3 CFU mL^{-1} in TF5 (on TSA_{100} and TSA_1, respectively). $R2A_{10}$ yielded colonies only in TF samples (about 60 CFU mL^{-1}). The results are reported in Table 6.25. No strains were yielded from the other media.

Table 6.25 Microbial abundances in Tarn Flat brine samples**.**

Sample	Viable counts on ** (CFU mL^{-1} x 10^3 of brines)			
	$R2A_{10}$	TSA_{100}	TSA_{50}	TSA_1
TF4	0.1 ± 0.0	3.2 ± 0.1	4.7 ± 0.9	3.1 ± 0.8
TF5	0.1 ± 0.0	5.5 ± 0.8	5.5 ± 0.0	7.2 ± 0.6

** TSA_{100}, TSA_{50} and TSA_1: plates of TSA at full, 50 % and 1 % strength respectively.

A total of 151 strains were isolates from TF brine samples, 72 from 4 m and 79 from 5 m. In particular, 58 strains were yielded from TSA_1 (16 and 43, from TF4 and TF5, respectively); 31 strains were isolated from TSA_{50} (17 and 14 from 4m and 5m, respectively); 51 from TSA_{100} (33 and 18 from 4m and 5m, respectively). Finally, 11 strains were isolated from $R2A_{10}$ (six and four from TF4 and TF5, respectively) (Table 6.26).

Table 6.26 Number of isolates obtained from direct plating *per* sample and isolation medium.

	Sample	
Medium	**TF4**	**TF5**
TSA_1	*16*	*43*
TSA_{50}	*17*	*14*
TSA_{100}	*33*	*18*
$R2A_{10}$	*6*	*4*
	72	*79*
Total	***151***	

6.8.1 Sequencing and analysis of the 16S rRNA gene

The 151 (72 from TF4 and 79 from TF5) sequences from Tarn Flat brines were distributed across three bacterial phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*). Sequences affiliated to the *Proteobacteria* were divided in two sub-classes, i.e. *Alphaproteobacteria* (21 sequences, 13.9 %) and *Gammaproteobacteria* (74 sequences, 49.0 %), followed by *Actinobacteria* (29 sequences, 19.2 %) and *Firmicutes* (16 sequences, 10.6 %).

In particular, in **TF4**, 49 strains were related to the *Proteobacteria* (68.0 %) and were affiliated to the *Alpha-* (2 strains; 2.8 %) and *Gammaproteobacteria* (47 strains; 65.3 %). Other eight and nine strains were related to the *Actinobacteria* (11.1 %) and *Firmicutes* (12.5 %), respectively. In **TF5**, 46 sequences were found to be affiliated to the *Proteobacteria* (58.2 %), with representatives of the *Alpha-* (19 strains; 24.1 %) and *Gammaproteobacteria* (27 strains; 32.2 %), 21 strains were affiliated to the *Actinobacteria* (21.6 %) and seven to the *Firmicutes* (8.9 %).

A total of 11 strains (7.3 %) were not identified due to failed PCR reactions, despite repeated attempts (Figure 6.16 and Table 6.27).

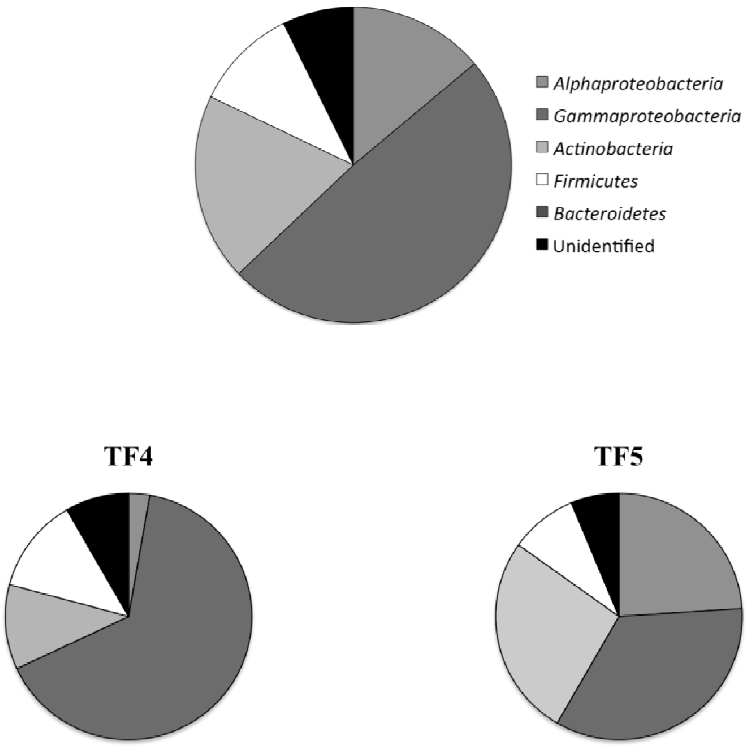

Figure 6.16 Viable bacterial community composition in Tarn Flat brines. The first graphic shows the viable diversity from both samples

Isolates from TF4 were affiliated to the genera *Rhodobacter* (two strains; 2.8 %), *Psychrobacter* (34 strains; 47.2 %), *Marinobacter* (seven strains; 9.7 %), *Pseudomonas* (6 strains; 8.33 %), *Leifsonia* (six strains; 8.3 %), *Kocuria* (one strain; 1.4 %), *Aeromicrobium* (one strain; 1.4 %), *Sporosarcina* (four strains; 5.6 %), *Carnobacterium* (three strains; 4.2 %), *Planococcus* (one strain; 1.4 %), and *Alkalibacterium* (one strain; 1.4 %).

Isolates from TF5 were affiliated to the genera *Rhodobacter* (15 strains; 19 %), *Devosia* (four strains; 5 %), *Pseudomonas* (19 strains; 24 %), *Marinobacter* (eight strains; 10.1 %), *Leifsonia* (14 strains; 17.8 %), *Rothia* (one strain; 1.3 %), *Cryobacterium* (one strain; 1.3 %), *Aeromicrobium* (four strains; 5 %), *Sporosarcina* (seven strains; 8.9 %) (Figure 6.17). Some genera occurred only in one sample, such as *Kocuria* and *Planococcus*

(which were isolated only from TF4) or *Rothia*, *Devosia* and *Rhodoglobus* (which were isolated only from TF5).

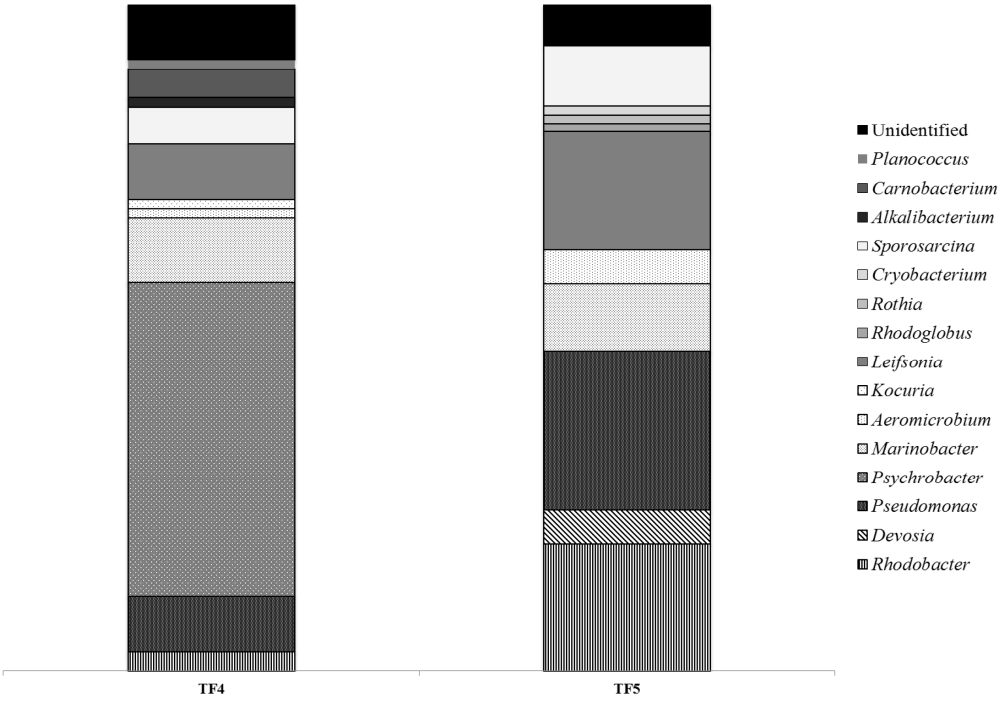

Figure 6.17 Bacterial genera retrieved form Tarn flat brines

All sequences with similarity of ≥97 % were considered to represent one phylotype and were grouped into Operational Taxonomic Units (OTUs) (Table 6.26). At this regard, OTU-sharing among TF brines was seldom observed with six OTUs that were common to both brine samples, i.e. *Aeromicrobium* sp. TF4-24 (OTU1), *Leifsonia* sp. TF5-105B (OTU4), *Marinobacter* sp. TF4-237 (OTU6), *Pseudomonas* sp. TF4-182 (OTU9), *Rhodobacter* sp. TF5-149 (OTU13) and *Sporosarcina* sp. TF4-168 (OTU15). Among them, members in the genera *Rhodobacter*, *Pseudomonas* and *Leifsonia* were more abundant in TF5 than TF4. All remaining OTUs/phylotypes were obtained from individual samples (seven and five form TF4 and TF5, respectively). In particular, *Psychrobacter* isolates (32 isolates within OTU10 and *Psychrobacter* sp. TF4-164) were

retrieved exclusively in TF4. Interestingly, the phylogenetic analysis allowed assigning the majority of sequences to nearest phylogenetic neighbors that were generally from the cold areas, including perennially Antarctic ice-covered lakes, glaciers and brines. Based on the sequences results a phylogenetic tree was constructed (Figure 6.18). The tree shows two main branches, which the first one that included *Proteobacteria* and a second one represented by *Actinobacteria* and *Firmicutes*.

The Proteobacteria were subdivided into two sub-clusters, *Gammaproteobacteria* and *Alphaproteobacteria*. The *Gammaproteobacteria* included three groups, the two bigger ones were represented by *Psychrobacter* sp. (32 isolates within OTU10 and isolate TF4-164) and *Pseudomonas* sp. (22 isolates within OTU9 and four isolates within OTU8), and the third one represented 15 isolates related to *Marinobacter* sp. within OTU6. The *Alphaproteobacteria* were represented by a single isolate (TF5-35A), which was closely related to *Parvibaculum hydrocarboniclasticum*, and two OTUs that comprised 17 isolates within OTU13, related to *Rhodobacter* sp., and four isolates within OTU3 related to *Devosia* sp. Two other branches were represented by the *Actinobacteria* and *Firmicutes*, which were composed by different ramifications. The *Actinobacteria* clusterized in six different ramifications: the biggest one was related to *Leifsonia* sp. (19 strains within OTU4), the second one was represented by *Aeromicrobium* sp. (five isolates within OTU1). All other ramifications were represented by single isolates closely related to *Rothia* sp. (Isolate TF5-35B), *Kocuria* sp. (Isolate TF4-15) and one isolate closely to *Cryobacterium mesophilum* (Isolate TF5-151). The *Firmicutes* branch was constituted by four ramifications. The biggest was represented by *Sporosarcina* sp. (11 strains within OTU15), the second one was related to *Carnobacterium* sp. (three isolates within OTU2), while the other two ramifications were constituted by two single isolates TF4-239 and TF4-125 closely affiliated to *Alkalibacterium* sp. and *Planococcus* sp., respectively.

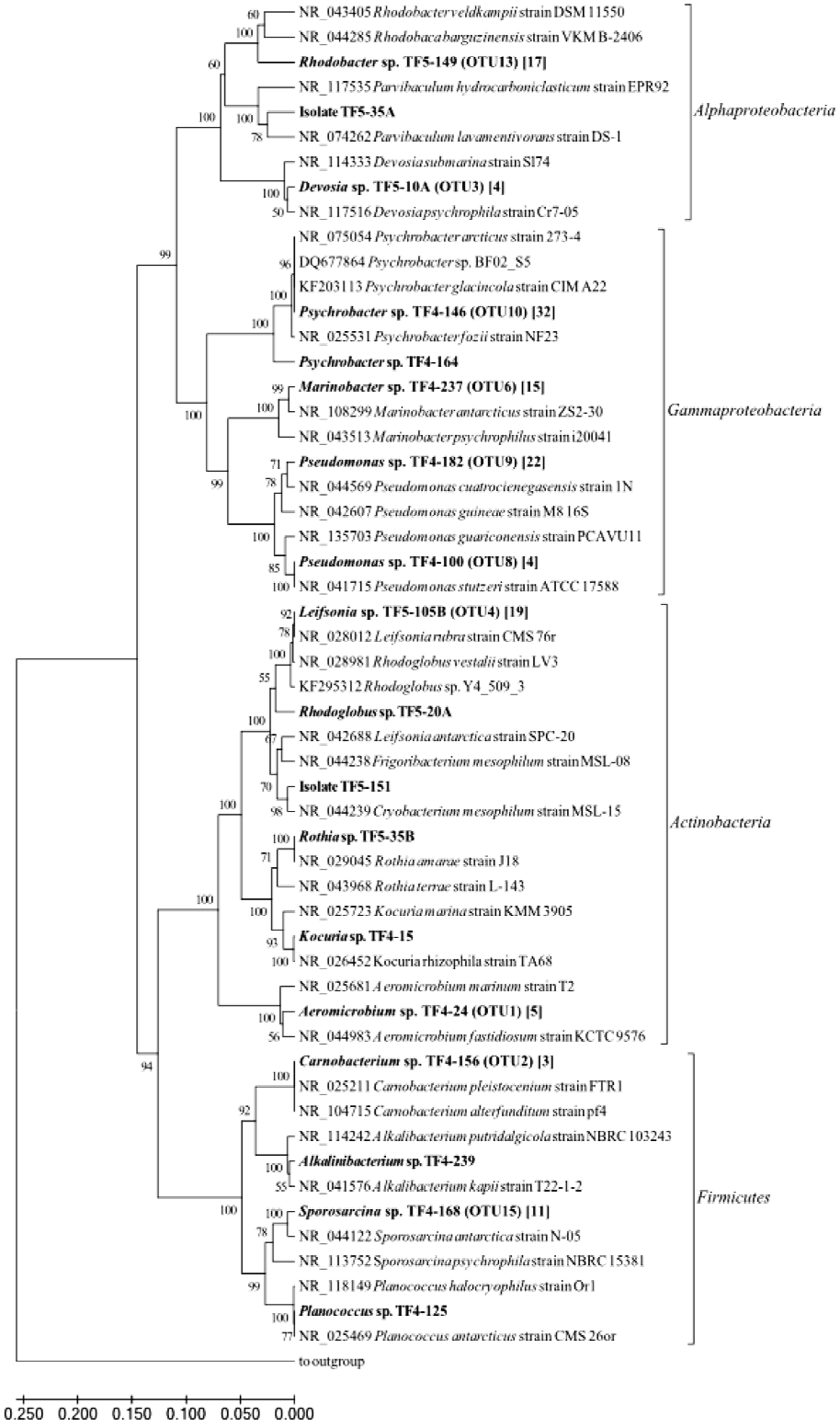

Figure 6.18 Phylogenetic tree based on 16S rRNA genes of OTU representatives

Table 6.27 16S rRNA gene sequence affiliation to their closest phylogenetic neighbors of Antarctic isolates.

					Isolates (n°)		Isolation medium [d]	
Next relative by GenBank alignment (AN[a], organism)	RI[b]	OTU[c]	Hom[e] (%)	Origin of next relative organism [e]	TF4	TF5	TF4	TF5
Alphaproteobacteria								
KR140230, *Devosia psychrophila* strain NJES-30 16S	TF5-10A	3	99	Antarctic krill	0	4		TSA_1
AF513400, *Rhodobacter* sp. 1-5	TF5-149	13	99	Arctic sea ice	2	15	TSA_{50} (1), TSA_{100} (1)	TSA_1 (1), TSA_{50} (8), TSA_{100} (6)
EU369117, Uncultured bacterium clone MBIOS-01	TF5-35A	na	96	Oyster shell	0	1		TSA_1
Gammaproteobacteria								
KF384120, *Marinobacter* sp. LV10R510-5	TF4-237	6	99	Antarctic brine (Lake Vida)	7	8	$R2A_{10}$ (5), TSA_{50} (2)	$R2A_{10}$ (4), TSA_{50} (1), TSA_{100} (3)
KU749990, *Pseudomonas stutzeri* strain 1005	TF4-100	8	99	Nr	4	0	TSA_{50}	
DQ677869, *Pseudomonas* sp. BF02_S14	TF4-182	9	99	Taylor glacier (Antarctica)	3	19	TSA_1 (2), TSA_{100} (1)	TSA_1 (16), TSA_{50} (1), TSA_{100} (2)
KX417186, *Psychrobacter fozii* strain 9.22	TF4-146	10	100	Arctic Kongsfjorden	32	0	TSA_1 (3), TSA_{50} (8), TSA_{100} (21)	
DQ677864, *Psychrobacter* sp. BF02_S5	TF4-164	na	96	Taylor glacier (Antarctica)	1	0	TSA_{100}	
Actinobacteria								
FJ196003, *Aeromicrobium* sp. ZS1-19	TF4-24	1	99	Antarctic sediment	1	4		
KM507649, *Kocuria* sp. FXJ8.237	TF4-15	na	99	Indian Ocean	1	0	TSA_1	
KC478079, *Leifsonia* sp. FO17	TF5-105B	4	99	ice-covered Antarctic lake	6	13	TSA_1	TSA_1 (10), TSA_{100} (3)
KF295312, *Rhodoglobus* sp. Y4_509_3	TF5-20A	na	96	Tibetan ice core	0	1		TSA_1
AM183255, *Rothia* sp. BBH4	TF5-35B	na	99	Marine sediment	0	1		TSA_1
DQ521554, uncultured bacterium ANTLV9_C09	TF5-151	na	99	Antarctic Lake Vida	0	1		TSA_{100}
Firmicutes								
KF151857, *Alkalibacterium kapii* strain MGR70	TF4-239	na	97	Nr	1	0	TSA_{50} (1)	
FR691465, *Planococcus antarcticus* strain R-36948	TF4-125	na	99	Antarctic lake	1	0	TSA_{100}	
HM224487, *Sporosarcina* sp. TPD39	TF4-168	15	99	Urumqi River	4	7	TSA_1 (1), TSA_{50} (1), TSA_{100} (2)	TSA_1 (2), TSA_{50} (4), TSA_{100} (1)
LC145583, *Carnobacterium alterfunditum* JCM 12498	TF4-156	2	99	Nr	3	0	TSA_{100}	

[a] **AN**: Accession Number; [b]**RI**: representative isolate; [c]**na**: not assigned; [d] **Hom**: sequence homology; [e]**nr**: not reported; [d], **TSA_{100}**, **TSA_{50}** and **TSA_1**: plates of TSA at full, 50 % and 1 % strength, respectively; **$R2A_{10\%}$**: plates of R2A at 20 % strength. Numbers in brackets indicate the number of isolates *per* medium.

6.8.2 Characterization of bacterial isolates

A total of 151 isolates were assayed for different capabilities and for the presence or absence of some features, such as the production of extracellular enzymes.

6.8.2.1 Morphological tests

Bacterial colonies were described (not reported) using a common criteria and classified based on morphological features such as shape, color, elevation, margin and surface. These morphological characterizations were carried out on agar plates after good growth of each isolate.

6.8.2.2 Bacterial growth conditions

Bacterial isolates were mainly Gram-negative motile rods. With few exceptions (six isolates growing only at 4 °C, mainly belonging to *Marinobacter* sp.), isolates were able to growth at both 4 and 15 °C. Among the isolates, 38 strains (12 from TF4 and 26 from TF5, mainly belonging to the genera *Rhodobacter* and *Leifsonia*) did not grow at 25 °C. Regarding the pH, the majority of isolates grew well within a pH range from 6-7 to 9 (80 isolates). All isolates were able to grow in the absence of NaCl with the majority of them that grew in a wide salinity range and tolerated up to 15 % (w/v) NaCl. A total of 22 isolates (14.6 %, mainly *Gammaproteobacteria* in the genus *Psychrobacter*) were able to grow up to NaCl 17-19 %. In particular, the isolates from TF4 were more salt tolerant than isolates from TF5. A total of 37 isolates (24.5 %; 21 and 16 from TF5 and TF4, respectively) were able to tolerate NaCl up to 7. No strains from TF5 were able to tolerate 17-19 % of NaCl.

The Tables below summarizes the salinity range for the growth of strains from TF brines (Table 6.28 and Table 6.29).

Table 6.28 Salinity range for growth of isolates from TF4.

Phylum or class	Genus	Salinity (%)			
		0-3	5-9	11-15	17-19
Alphaproteobacteria	*Rhodobacter*			1	
Gammaproteobacteria	*Psychrobacter*	1	7	7	18
	Pseudomonas		5		1
	Marinobacter		5	1	1
Actinobacteria	*Leifsonia*		5		
	Kocuria			1	
	Aeromicrobium	1			
Firmicutes	*Sporosarcina*	3		1	
	Carnobacterium	1	2		
	Alkalibacterium		1		
Unidentified	-	3	1	1	2
		9	26	12	22

Table 6.29 Salinity range for growth of isolates from TF5.

Phylum or class	Genus	Salinity (%)			
		0-3	5-9	11-15	17-19
Alphaproteobacteria	*Rhodobacter*		8	5	
	Devosia	2	2		
Gammaproteobacteria	*Pseudomonas*	9	8	1	
	Marinobacter	1	5	1	
Actinobacteria	*Leifsonia*	3	5	6	
	Aeromicrobium	2	1	1	
	Rhodoglobus		1		
	Cryobacterium		1		
	Rothia	1			
Firmicutes	*Sporosarcina*		7	1	
Unidentified	-	1	2	1	
		19	40	16	0

6.8.2.3 Production of extracellular enzymes

6.8.2.3.1 Catalase test

TF4. 59 strains (81.4 %) were catalase positive and were distributed in different phylogenetic genera; two strains belonging to *Rhodobacter* sp. (OTU13), seven strains belonging to *Marinobacter* sp. (OTU6), four strains belonging to *Pseudomonas* sp. (OTU8), 33 strains belonging to *Psychrobacter* spp. (OTU10 and TF4-164 strain), strain TF4-24 belonging to *Aeromicrobium* sp. (OTU1), strain TF4-15 belonging to *Kocuria* sp., six strains belonging to *Leifsonia* sp. (OTU4), four strains belonging to *Sporosarcina* sp. (OTU15) and strain TF4-125 belonging to *Planococcus* sp. were catalase positive.

TF5. 54 strains (69.3 %) were catalase positive: 15 strains belonging to *Rhodobacter* sp. (OTU13), eight strains belonging to *Marinobacter* sp. (OTU6), six strains belonging to *Sporosarcina* sp. (OTU15), four strains belonging to *Devosia* sp. (OTU3), 19 strains belonging to *Pseudomonas* sp. (OTU9) and the strains TF5-20A belonging to *Rhodoglobus* sp. and TF5-35B belonging to *Rothia* sp. (Table 6.30).

6.8.2.3.2 Oxidase test

TF4. A total of 53 strains (73.6 %) were oxidase positive. The positive strains were affiliated to *Rhodobacter* sp. (two strains, OTU13), *Marinobacter* sp. (seven strains, OTU6), *Pseudomonas* spp. (four strains in OTU8 and tree in OTU9), *Psychrobacter* spp. (32 strains in OTU10 and isolate TF4-164), *Sporosarcina* sp. (four strains in OTU15).

TF5. A total of 54 strains (68.3 %) were oxidase positive. The strains were affiliated to *Rhodobacter* sp. (15 strains, OTU13), *Devosia* sp. (four strains, OTU3), *Marinobacter* sp. (eight strains, OTU6), *Pseudomonas* spp. (19 strains, OTU9), *Sporosarcina* sp. (seven strains in OTU15) and *Rhodoglobus* sp. (TF5-20A) (Table 6.30).

Table 6.30 Catalase and Oxidase response by isolates from Tarn Flat brine pockets.

Phylum or class	*Affiliation*	*OTU or isolate*	*Catalase*	*Oxidase*
Alphaproteobacteria	*Rhodobacter* sp.	OTU13	+	+
	Devosia sp.	OTU3	-	+
Gammaproteobacteria	*Marinobacter* sp.	OTU6	+	+
	Pseudomonas sp.	OTU8	+	+
	Pseudomonas sp.	OTU9	-	+
	Psychrobacter sp.	OTU10	+	+
	Psychrobacter sp.	TF4-164	+	+
Actinobacteria	*Aeromicrobium* sp.	OTU1	+	-
	Kocuria sp.	TF4-15	+	-
	Leifsonia sp.	OTU 4	+	-
	Rhodoglobus sp.	TF5-20A	+	+
	Rothia sp.	TF5-35B	-	-
	Uncultured bacterium	TF5-151	+	+
Firmicutes	*Sporosarcina* sp.	OTU15	+	+
	Alkalibacterium sp.	TF4-229	-	-
	Planococcus sp.	TF4-125	+	-
	Carnobacterium sp.	OTU2	-	-

6.8.2.3.3 Hemolysis test

Only 4 strains (2.7 %) isolated from TF4 sample were able to breakdown red blood cells. *Kocuria* sp. TF4-15 showed beta hemolysis, sometimes called complete hemolysis, as it is a complete lysis of red cells in the medium that appears transparent and lightened around the colonies on agar plates. Three strains belonging to *Carnobacterium* sp. (TF4-156, TF4-157, TF4-163), within the OTU2, showed an alpha hemolysis with the agar under the colony that became dark and greenish. It is a non-complete hemolysis caused by hydrogen peroxide produced by the bacterium, that oxidizes hemoglobin to green methemoglobin (Figure 6.19).

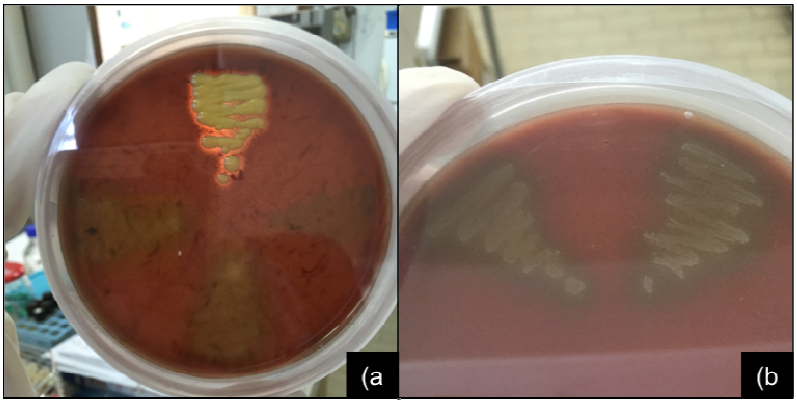

Figure 6.19 Example of Beta (isolate *Kocuria* sp.) and alpha (isolate *Carnobacterium*) haemolysis

6.8.2.3.4 DNAse test

A total of 40 strains (26.5 %) isolated from Tarn Flat samples were able to utilize DNA as a carbon source. Positive strains (25 isolates) from **TF4** mainly belonged to the genera *Psychrobacter* (14 strains), following by *Sporosarcina* (four strains) and *Kocuria*, *Aeromicrobium, Leifsonia*, *Planococcus* and *Marinobacter* (one strain each) and two unidentified. 15 strains from **TF5** were DNAse positive and belonged to *Pseudomonas* and *Sporosarcina* (four strains), followed by *Leifsonia*, *Rhodobacter* (three strains) and *Rothia* (one strain).

6.8.2.4 Hydrolysis of complex substances

No strain showed agarolytic activity, whereas only one strain (*Psychrobacter* sp. TF4-146) showed lipolytic activity on Tween80. The presence of gelatinase occurred in 11 strains (7.3 %). Among them, four were from TF4 and belonged to the genera *Sporosarcina* (two strains), *Aeromicrobium* and *Planococcus* (one strain each). Other seven strains were from TF5 and were related to the genera *Pseudomonas* (four strains), *Sporosarcina*, *Cryobacterium* and *Rhodobacter* (1 strain each). Amylase occurred in eight strains (5.3 %) of which six were from TF4 and two from TF5. Amylase positive

strains were mainly affiliated to the genera *Pseudomonas* and *Sporosarcina* (Figure 6.20). Chitinase activity was detected in seven strains from TF5 were mainly affiliated to the genera *Pseudomonas* and *Leifsonia*.

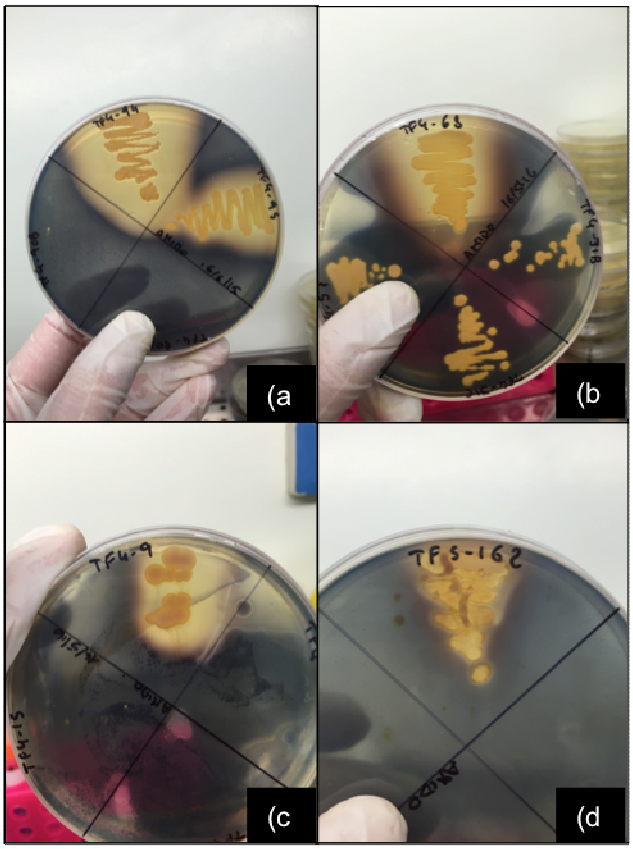

Figure 6.20 Example of *Pseudomonas* (a) and *Sporosarcina* (b, c, d) amylase positive isolates

6.8.3 Antibiotic susceptibility

A total of 151 identified isolates were subjected to antibiotic susceptibility test by using TSA medium supplemented with antibiotics [chloramphenicol (C), streptomycin (S), ampicillin (A), gentamycin (G), kanamycin (K), polimixin B (P)] at different concentrations. In general, four strains (2.7 %) were resistant to kanamycin, eight strains (5.3 %) to streptomycin, five strains (3.3 %) to gentamycin, 12 strains (7.9 %) to

polimixin B and 18 strains (11.9 %) to ampicillin. The relative resistance of antibiotics decreased in the order of *A>P>S>G>K*.

TF4. The data are shown in Table 6.31. Few strains were able to growth in presence of antibiotics. Ampicillin was the best tolerated antibiotic, followed by polimixin B. Four strains tolerated streptomycin until 25 ppm (*Sporosarcina* sp. TF4-9, *Psychrobacter* sp..TF4-164, *Carnobacterium* sp. TF4-163 and *Leifsonia* sp. TF4-181), while *Sporosarcina* sp. TF4-68 was able to tolerate streptomycin until 350 ppm. *Leifsonia* sp. TF4-14 tolerated kanamycin until 350 ppm. Five strains were able to tolerate ampicillin until 100 ppm (*Psychrobacter* sp. TF4-69, *Planococcus* sp. TF4-125, *Leifsonia* sp. TF4-181 and *Rhodobacter* sp. TF4-242), while only two strains were able to tolerate chloramphenicol up to 100 ppm (*Marinobacter* sp. TF4-233 and one unidentified isolate). Few strains tolerated the other antibiotics at low concentrations. Six strains were able to tolerate two different antibiotics and two were resistant to three antibiotics.

TF5. Few strains were able to tolerate the antibiotics. Multi-resistance was particularly evident for two *Leifsonia* isolates (TF5-105A and TF5-105B) that tolerated five and four antibiotics, respectively. Three strains, related to *Leifsonia* sp., tolerate streptomycin until 350 ppm, while two strains (**TF5-105A** and **TF5-105B**) related to *Leifsonia* sp. tolerated polimixin B until 350 ppm. Only three strains tolerated chloramphenicol, while no strains tolerated gentamycin.

In general, isolates from TF4 were more tolerant to antibiotics than isolates from TF5. Ampicillin was the best tolerated antibiotic, while gentamycin was the worst tolerated and only up to 25 ppm. The genera *Psychrobacter* and *Leifsonia* tolerated a higher number of antibiotics than other genera (Table 6.31).

Table 6.31 Antibiotic resistance by isolates from Tarn Flat.

TF4	Antibiotics concentration (ppm)									
Genus	Streptomycin		Kanamycin		Ampicillin		Polimixin B	Chloramphenicol		Gentamycin
	25	350	25	350	50	100	25	50	100	25
Rhodobacter						1				1
Psychrobacter	1				5	1	7	1		1
Pseudomonas							1			
Marinobacter									1	
Leifsonia	1			1	2	1	1			2
Sporosarcina	1	1	1		1			1		1
Planococcus						1				
Carnobacterium	1									
Unidentified						1	1		1	
Total	5		2		13		10	4		5

TF5	Antibiotics concentration (ppm)									
Genus	Streptomycin		Kanamycin		Ampicillin		Polimixin B	Chloramphenicol		Gentamycin
	25	350	25	350	50	100	350	50	100	25
Rhodobacter					1			1		
Aeromicrobium					1					
Leifsonia		3	1		3		2		2	
Sporosarcina			1							
Unidentified										
Total	3		2		5		2	3		0

6.8.4 Heavy metal tolerance

A total of 151 isolates were first screened for heavy metal tolerance towards five different metals added to the culture medium (mercury, $HgCl_2$; cadmium, $CdCl_2$; copper, $CuCl_2$; cobalt, $CoCl_2$; nickel, $NiCl_2$) at two different concentrations (500 and 1000 ppm). Tolerant isolates were then assayed in a medium amended with heavy metal salts up to 10000 ppm. The relative resistance of the metals decreased in the order of *Ni>Cu>Co>Hg>Cd*. Cd was tolerated by any strain. The genus *Psychrobacter* was the only strain able to tolerate almost all metals.

Strains from TF4 resulted more tolerant than those from TF5. A total of 13 strains (8.6 %) were able to grow up to 500 ppm of Hg and 11 strains (7.3 %) tolerated Hg up to 1000 ppm; 23 strains (15.3 %) were able to tolerate Co until 500 ppm; 40 strains (26.5 %) tolerated Cu up to 500 ppm, while 33 strains (21.8 %) tolerated Cu up to 1000 ppm; 47 strains (31.1 %) were able to tolerate Ni up to 500 ppm, while 33 (21.8 %) tolerated Ni up

to 1000 ppm, seven (4.6 %) tolerate Ni up to 2500 ppm and one strain (0.66 %) was able to tolerate Ni up to 5000 ppm.

TF4 brine. In particular, 12 strains affiliated to *Psychrobacter* (eight strains), *Rhodobacter*, *Sporosarcina*, *Marinobacter* and one unidentified (one strain each) were able to tolerate Hg up to 500 ppm, while 11 strains affiliated to *Pseudomonas* (four strains) and *Psychrobacter* (five strains), *Sporosarcina* and *Planococcus* (one strain each) tolerated Hg up to 1000 ppm. Co was tolerated by 18 strains including 13 strains affiliated to *Psychrobacter*, two strains affiliated to *Carnobacterium*, one strain each for *Sporosarcina* and *Pseudomonas* and an unidentified, but up to 500 ppm. Twelve strains affiliated to *Psychrobacter* (six strains), *Marinobacter* (two strains), *Rhodobacter*, *Sporosarcina*, *Leifsonia*, and an unidentified (one strain each) were able to tolerate Cu up to 500 ppm. 32 strains were able to grow in the presence of Cu up to 1000 ppm and were affiliated to *Psychrobacter* (22 strains), *Pseudomonas* (five strains), *Leifsonia*, *Planococcus*, *Sporosarcina* (one strain each), while other two remained unidentified. Ni was the most tolerated metal (up to 5000 ppm). Seven strains tolerated Ni up to 500 ppm and were affiliated to *Psychrobacter* (two strains), *Sporosarcina*, *Pseudomonas,* *Planococcus*, *Carnobacterium* and *Marinobacter* (one strain each). 31 strains were able to tolerate Ni up to 1000 ppm and were affiliated to *Psychrobacter* (24 strains), *Sporosarcina* and *Pseudomonas* (two strains each), *Rhodobacter* (one strain each) and two strains unidentified (TF4-153 and TF4-226). Six strains tolerated Ni up to 2500 ppm and were affiliated to *Psychrobacter* (four strains), *Leifsonia* (one strain) and one unidentified (TF4-171). *Leifsonia* sp. TF4-181 was able to tolerate Ni up to 5000 ppm. In general 17, 22 and three strains, mainly belonging to *Psychrobacter,* were able to tolerate two, three and four different metals, respectively.

TF5 brine. One strain affiliated to *Rhodobacter* was able to tolerate Hg up to 500 ppm, while five strains mainly affiliated to *Pseudomonas* were able to tolerate Co up to 500 ppm. Cu was tolerated only up to 500 ppm by 28 strains, mainly affiliated to

Pseudomonas (16 strains). Nickel was tolerated up to 500 ppm by 40 strains, mainly affiliated to *Pseudomonas* (17 strains), *Leifsonia* (nine strains) and *Rhodobacter* (six strains), while Ni was tolerated up to 1000 ppm and 2500 ppm by four strains and one strain, respectively. A total of 26 strains, mainly belonging to *Pseudomonas* (16 strains), were able to tolerate two different metals, while two strains were able to tolerate three metals (Table 6.32).

Table 6.32 Heavy metal tolerant isolates**.**

TF4 Genus	**Heavy metal concentrations (ppm)**								
	$HgCl_2$		**$CoCl_2$**	**$CuCl_2$**			**$NiCl_2$**		
	500	**1000**	**500**	**500**	**1000**	**500**	**1000**	**2500**	**5000**
Rhodobacter	1			1			1		
Psychrobacter	8	4	11	6	22	2	24	4	
Pseudomonas		5	1		4	1	2		
Marinobacter	1			2		1			
Leifsonia				1	1			1	1
Sporosarcina	1	1	1	1	1	1	2		
Planococcus		1			1	1			
Carnobacterium			2		1	1			
Unidentified	1		3	1	2		2	1	
	12	11	18	12	32	7	29	6	1

TF5 Genus	**Heavy metal concentrations (ppm)**						
	$HgCl_2$	**$CuCl_2$**	**$CoCl_2$**		**$NiCl_2$**		
	500	**500**	**500**	**1000**	**500**	**1000**	**2500**
Rhodobacter	1	3			6		
Devosia		1			1		
Pseudomonas		16	3		17		
Marinobacter		1			2		
Leifsonia		4	1		10	1	
Aeromicrobium			1			1	**1**
Sporosarcina		2			3	1	
Unidentified		1		1	1	1	
	1	28	5	1	40	4	1

6.9 Enumeration, isolation and phylogenetic characterization of bacterial strains

All Boulder Clay samples yielded colonies on TSA media. Viable counts in BC brines on TSA media ranged between 17.5 and 1.6 x 10^4 CFU mL^{-1} on TSA_1 (BC3 and BC1 respectively); from 5.3 x 10^2 to 5.35 x 10^2 CFU mL^{-1} on TSA_{50} (BC2 and BC1 respectively); from 87.5 to 5.3 x10^3 CFU mL^{-1} on TSA_{100} (BC2 and BC1 respectively). BC1 and BC3 showed growth on DSMZ 97 medium (3.1 x 10^3 and 60 CFU mL^{-1}, respectively), but no colonies were yielded (Table 6.33).

Table 6.33 Boulder Clay sample viable count using different media.

Sample	Viable counts on ** (CFU mL^{-1} x 10^3 of brines)			
	DSMZ 97	TSA_{100}	TSA_{50}	TSA_1
BC1	3.1 ± 0.4	5.3 ± 0.8	4.3 ± 0.9	15.5 ± 0.7
BC2	0.0 ± 0.0	0.08 ± 0.8	0.5 ± 0.3	0.0 ± 0.0
BC3	0.06 ± 0.0	2.3 ± 0.3	0.9 ± 0.3	0.01 ± 0.0

** TSA_{100}, TSA_{50} and TSA_1: plates of TSA at full, 50 % and 1 % strength respectively.

A total of 74 strains were isolated from BC brine samples: 64 from BC1, two from BC2 and eight from BC3. In particular, 40 strains were yielded from TSA_1 (38 from BC1, two from BC3); 21 strains from TSA_{50} (15 from BC1, one from BC2 and five from BC3); 13 strains were yielded from TSA_{100} (11 from BC1, one from BC2 and one from BC3) (Table 6.34).

Table 6.34 Number of isolates obtained from direct plating *per* sample and isolation medium.

	Samples		
Medium	**BC1**	**BC2**	**BC3**
TSA_1	*38*	*0*	*2*
TSA_{50}	*15*	*1*	*5*
TSA_{100}	*11*	*1*	*1*
	64	*2*	*8*
Total		*74*	

6.9.1 Sequencing and analysis of 16S rRNA gene

A total of 73 sequences (63 from BC1, two from BC2 and eight from BC3) were distributed across four bacterial phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*). Overall, 51 sequences were affiliated to the *Gammaproteobacteria* (69.9 %), followed by the *Firmicutes* (10 sequences, 13.7 %), *Bacteroidetes* (5 sequences, 6.85 %) and *Actinobacteria* (one sequences, 1.4 %). Six sequences were not affiliated (8.2 %) (Figure 6.21).

In BC1 samples, 45 strains were affiliated to the *Gammaproteobacteria* (71.4 %), ten strains were affiliated to *Firmicutes* (15.9 %), three strains to *Bacteroidetes* (4.8 %), one strain was affiliated to *Actinobacteria* (1.6 %) and four strains were unidentified. In BC2 were retrieved two sequences and were affiliated to the *Bacteroidetes*. In BC3, six strains were affiliated to the *Gammaproteobacteria* (75 %) and 2 strains were unidentified.

The 73 identified strains were grouped in OTUs (Operational Taxonomic Units) according to their similarity (≥ 97 %). Three OTUs (24, 27, 28) were affiliated to the *Gammaproteobacteria*, two OTUs (30 and 31) and isolate BC1-72A were affiliated to the *CF* group of *Bacteroidetes*, isolate BC1 bis-11 was affiliated to the *Actinobacteria*, and OTU22 and isolate BC1 bis-71 were related to the *Firmicutes* (Figure 6.21 and Table 6.34). In particular, isolates from Boulder Clay were mainly related to sequences of isolates from cold environments, e.g. Antarctic lakes and sediments.

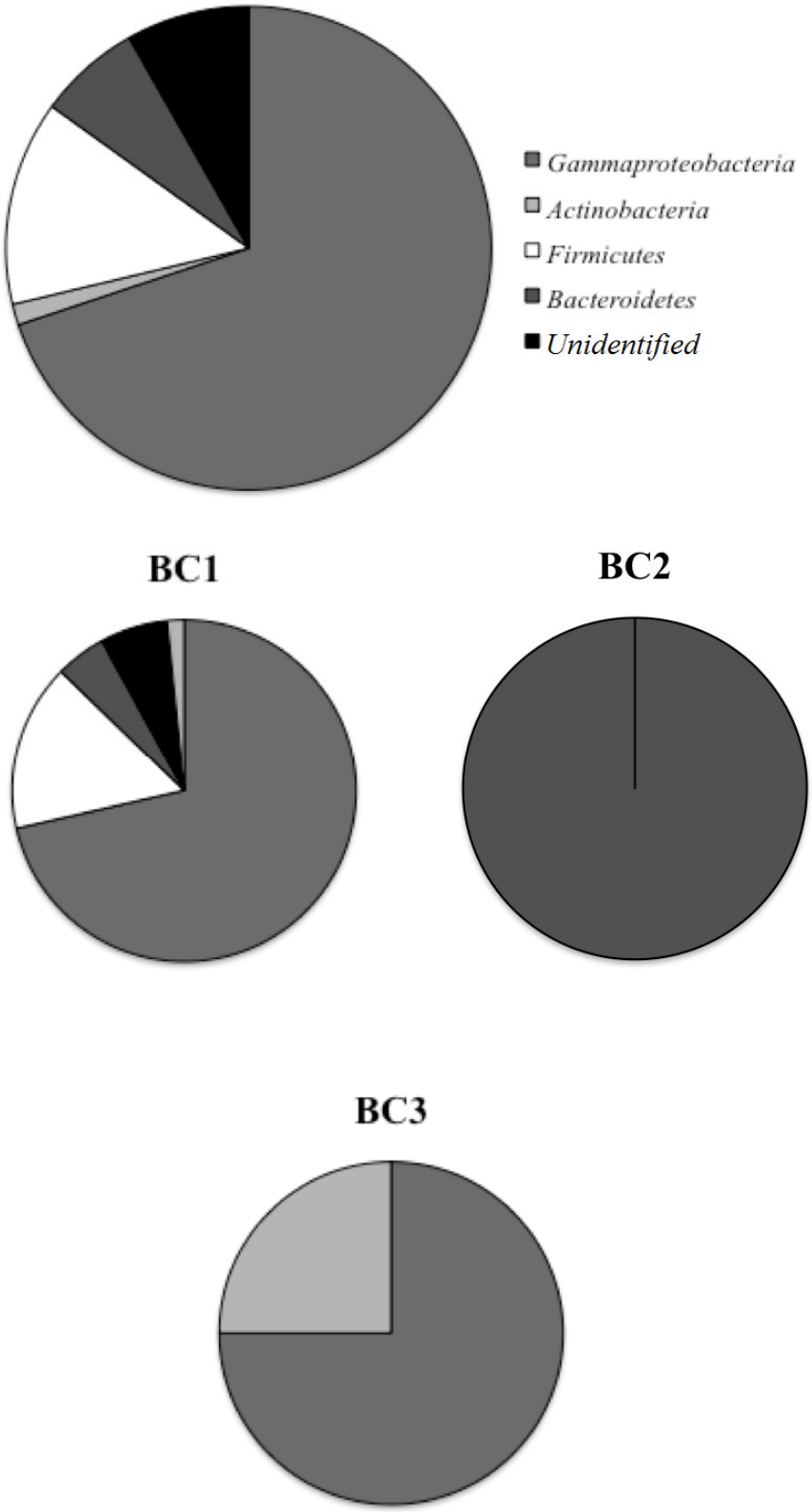

Figure 6.21 Phylogenetic affiliation of isolates from Boulder Clay

In BC1, 63 strains were affiliated to the genera *Pseudomonas* (41 strains, 65.1 %), *Shewanella* (three strains, 4.8 %), *Psychrobacter* (one strain, 1.6 %), *Carnobacterium* (9 strains, 14.3 %), *Gelidibacter* (2 strains, 3.2 %), *Actinobacterium* (one strain, 1.6 %), *Staphylococcus* (one strain, 1.6 %), *Bacteroidetes* bacterium (one strain, 1.6 %) and 4 strains (6.35 %) weren't affiliated. In BC2, the two sequences were both affiliated to the genus *Flavobacterium*. In BC3, a total of eight strains were related to the genera *Psychrobacter* (five strains, 62.5 %), *Pseudomonas* (one strain, 12.5 %) and two strains were unidentified (Figure 6.22 and Table 6.35).

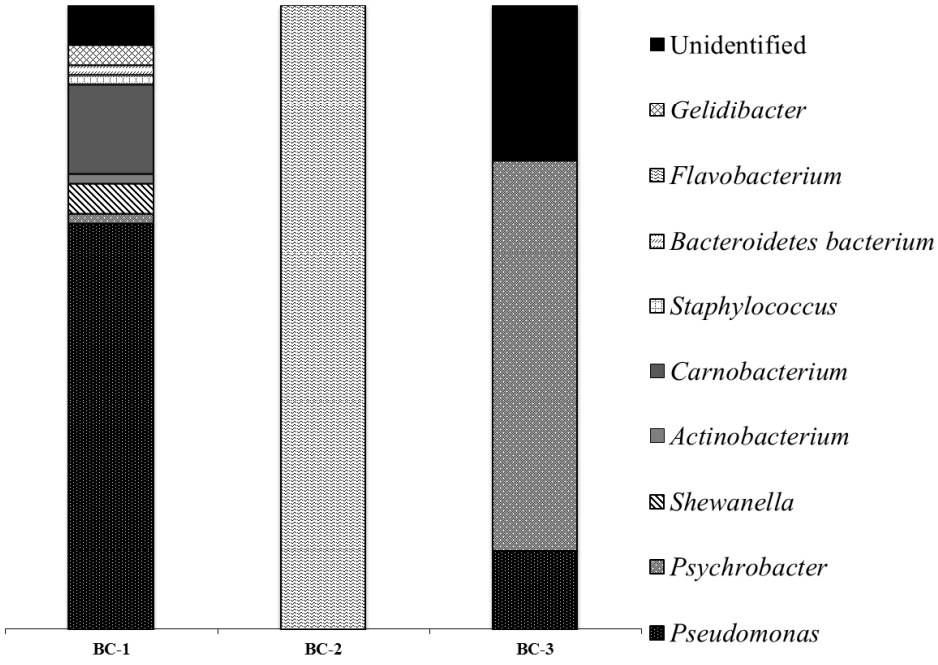

Figure 6.22 Phylogenetic affiliation (genera) of strains isolated from Boulder Clay brines

The phylogenetic tree based on the 16S rRNA genes of OTU representatives is shown in Figure 6.23. Two main branches constitute the tree: the first one included two ramifications, one affiliated to the *Gammaproteobacteria* and another one affiliated to the *Actinobacteria* and *Firmicutes*. The second main branch was related to the *Bacteroidetes*. The *Gammaproteobacteria* ramification was constituted by six strains related to OTU27

affiliated to *Psychrobacter* sp., three strains related to OTU28 affiliated to *Shewanella* sp. and 42 strains related to OTU24 affiliated to *Pseudomonas* sp. The *Actinobacteria* ramification included one isolate (BC1 bis-11) related to *Leifsonia rubra*. The *Firmicutes* ramification was constituted by nine strains related to OTU22 affiliated to *Carnobacterium* sp. and *Staphylococcus* sp. BC1 bis-71. The last branch was constituted by the *Bacteroidetes* with two strains in OTU31 affiliated to *Flavobacterium* sp., *Gelidibacter* sp. BC1-72A and two strains within OTU30 affiliated to *Gelidibacter algens*.

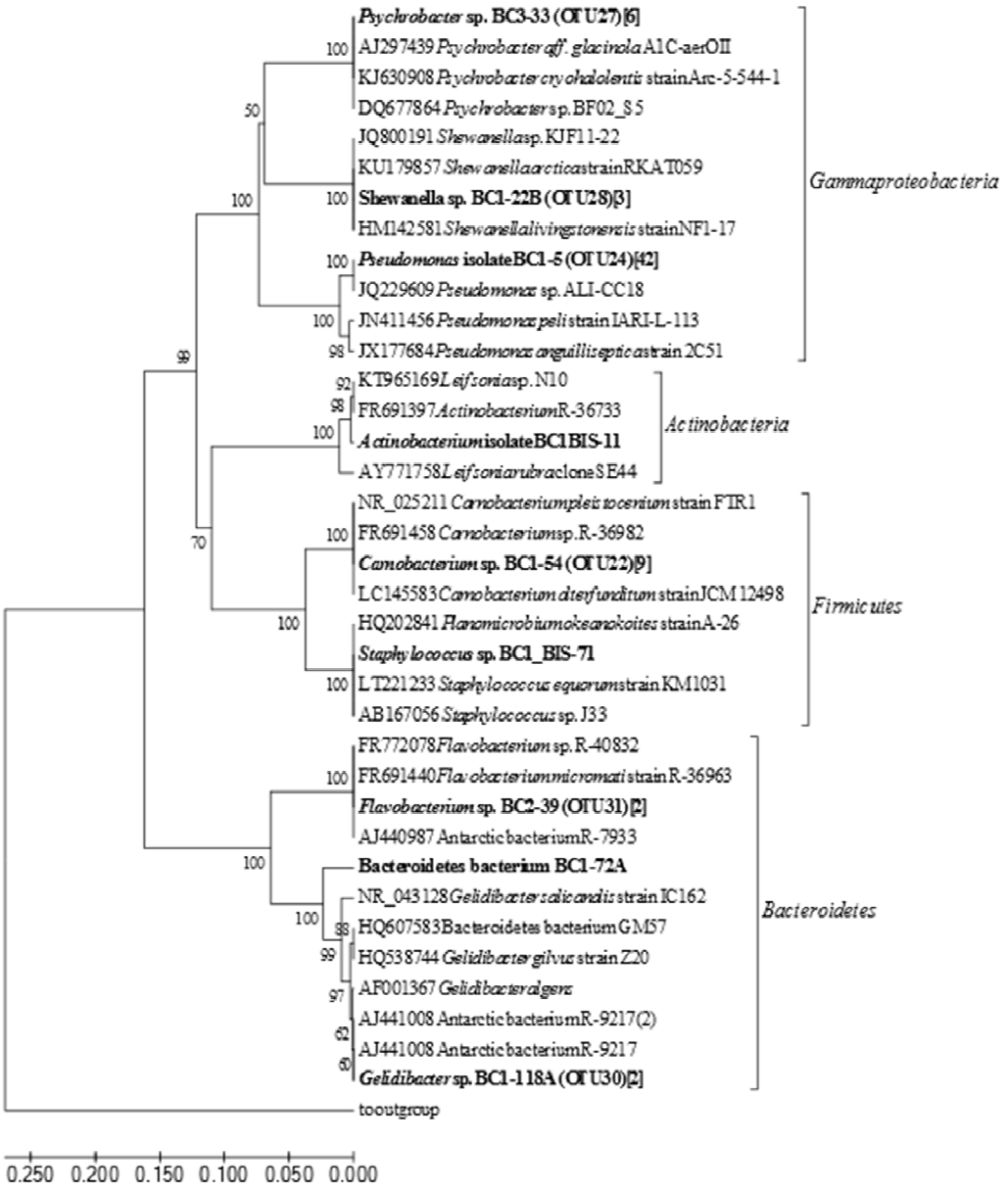

Figure 6.23 Phylogenetic tree based on 16S rRNA genes of OTU representatives

Table 6.35 16S rRNA gene sequence affiliation to their closest phylogenetic neighbors of Antarctic isolates from Boulder Clay.

Next relative by GenBank alignment (AN[a], organism)	RI[b]	OTU[c]	Hom[e] (%)	Origin of next relative organism [e]	Isolates (n°)			Isolation medium [d]		
					BC1	BC2	BC3	BC1	BC2	BC3
Gammaproteobacteria										
JQ229609, *Pseudomonas* sp. ALI-CC18	BC1-5	24	99	Antarctic lake	41		1	TSA_1 (32), TSA_{50} (9)		TSA_{100} (1)
DQ677864, *Psychrobacter* sp. BF02_S5	BC3-33	27	99	Taylor Glacier, Antarctica	1		5	TSA_{50} (1)		TSA_{50} (5)
KU179857, *Shewanella arctica*RKAT059	BC1-22B	28	99	Arctic sediment	3			TSA_1 (3)		
CF group of *Bacteroidetes*										
HQ607583, Bacteroidetes bacterium	BC1-72A	na	96	Seawater	1			TSA_{100} (1)		
AF001367, *Gelidibacter algens*	BC1-118A	30	98	Antarctic seaice	2			TSA_{100} (2)		
FR772078, *Flavobacterium* sp. R-40832	BC2-39	31	99	Antarctica		2			TSA_{50} (1), TSA_{100} (1)	
Actinobacteria										
FR691397, Actinobacterium R-36733	BC1bis-11	na	98	Antarctic lake	1			TSA_1 (1)		
Firmicutes										
LC145583, *Carnobacterium* JCM 12498	BC1-54	22	99	nr	9			TSA_{50} (3), TSA_{100} (6)		
LT221233, *Staphylococcus* LK1HaP1	BC1bis-71	na	99	Rhizosphere	1			TSA_{100} (1)		

[a] **AN**: Accession Number; [b]**RI**: representative isolate; [c]**na**: not assigned; [d] **Hom**: sequence homology; [e]**nr**: not reported; [d], **TSA_{100}**, **TSA_{50}** and **TSA_1**: plates of TSA at full, 50 % and 1 % strength, respectively. Numbers in brackets indicate the number of isolates *per* medium.

6.9.2 Characterization of bacterial isolates

Isolates were assayed for different capabilities and for the presence or absence of some features, such as the production of extracellular enzymes.

6.9.2.1 Morphological tests

Bacterial colonies were described (not reported) using a common criteria and classified based on morphological features such as shape, color, elevation, margin and surface. These morphological characterization were carried out on agar plates after good growth of each isolate.

6.9.2.2 Bacterial growth conditions

Isolates were able to growth at 4, 15 and 25 °C, with few exceptions (one isolate growing only at 4 °C, belonging to the genus *Carnobacterium*, and three *Pseudomonas* isolates that did not grow at 25 °C). Regarding the pH, the majority of isolates grew well within a pH range from 6-7 to 9. All isolates were able to grow in the absence of NaCl with the majority of them that grew in a salinity range up to 3 % (w/v) NaCl (25 isolates; 32.9 %). A total of four isolates (5.4 %; mainly *Gammaproteobacteria* in the genus *Psychrobacter*) were able to grow up to 17-19 (%) NaCl. In particular, the isolates from BC1 were more salt tolerant than isolates from the other samples. Ten isolates (13.5 %) from BC1 were able to tolerate 7 % of NaCl (Table 6.36).

Table 6.36 Range of NaCl concentration for growth of isolates from BC1 and BC3.

Phylum	Genera	Salinity (%)			
		0-3	5-9	11-15	17-19
Gammaproteobacteria	*Psychrobacter*			1	
	Pseudomonas	27/ 1*	12	1	3*
	Shewanella		2	1	
Actinobacteria	Actinobacterium	1			
Firmicutes	*Carnobacterium*	1	5	3	
	Staphylococcus				1
Bacteroidetes	*Gelidibacter*	2			
	Bacteroidetes bacterium		1		
	Unidentified	2*			
		31/35*	20	6	1/4*

* Isolates from BC3

6.9.2.3 Production of extracellular enzymes

6.9.2.3.1 Catalase test

The majority of isolates were catalase negative (56 out of 73; 76.7 %). All nine *Carnobacterium* isolates (OTU22) were catalase negative. Among the *Gammaproteobacteria*, *Pseudomonas* (OTU24, 42 from BC1 and one from BC3) and *Shewanella* isolates (three strains in OTU28) resulted catalase negative, whereas all *Psychrobacter* isolates (OTU27, one from BC1 and five from BC3) were catalase positive. Within *Bacteroidetes*, the unidentified isolate BC1-72A was catalase positive, while the two *Gelidibacter* isolates (in BC1 within the OTU31) was catalase negative.

6.9.2.3.2 Oxidase test

A total of 50 isolates out of 73 (68.5 %) were oxidase positive. All *Carnobacterium* isolates were oxidase negative. The *Pseudomonas* strains (OTU24) were oxidase positive as all *Psychrobacter* (OTU27), *Shewanella* (OTU28) and *Actinobacteria* strains (BC1

bis-11). Within the *Bacteroidetes*, *Gelidibacter* strains (OTU31) and the isolate BC1-72A were oxidase negative (Table 6.37).

Table 6.37 Catalase and Oxidase response by isolates from Boulder Clay in relation to OTU.

Phylum	*Affiliation*	*OTU o isolate*	*Catalase*	*Oxidase*
Gammaproteobacteria	*Pseudomonas sp.*	OTU24	-	+
	Psychrobacter sp.	OTU27	+	+
	Shewanella sp.	OTU28	-	+
Actinobacteria	Actinobacterium	BC1 bis-11	-	+
Firmicutes	*Carnobacterium sp.*	OTU22	-	-
Bacteroidetes	Bacteroidetes bacterium	BC1-72A	+	-
	Gelidibacter sp.	OTU31	-	-

6.9.2.2.3 Hemolysis test

Eleven strains (15.1 %) from BC1 were the only able to breakdown the red blood cells. All isolates showed alpha hemolysis making the agar around the colony dark and greenwash. It was a non-complete hemolysis, caused by hydrogen peroxide produced by bacterium that oxidized hemoglobin to green methemoglobin. These strains were related to *Carnobacterium* (8 strains in OTU22), one *Psychrobacter* and two unidentified.

6.9.2.2.4 DNAse test

16 strains (21.9 %) from BC were able to hydrolase DNA and utilize it as a source of carbon and energy for growth. They belonged to the genera *Pseudomonas* (eight strains in OTU24), *Carnobacterium* (four strains in OTU22), *Staphylococcus* (BC1 bis-71) and one unidentified (BC1-119) from BC1. Only two *Psychrobacter* from BC3 were DNAse positive (Figure 6.24).

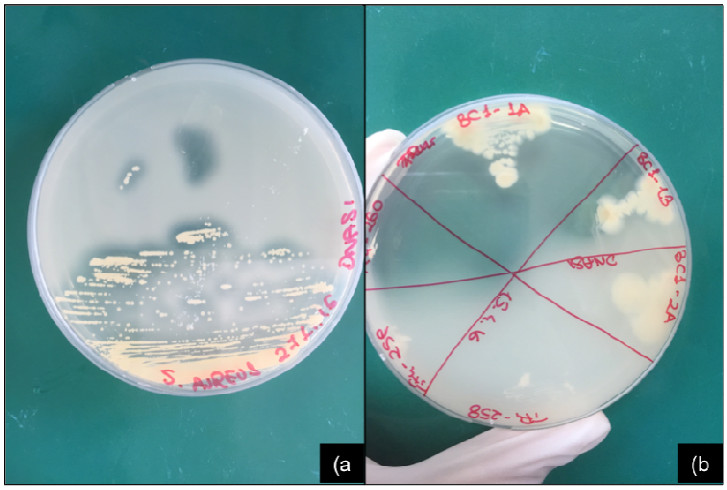

Figure 6.24 DNase test. a) Positive response from *S. aureus* (positive control); b) example of positive isolate from BC1

6.9.2.2.5 Hydrolysis of complex substrata

No strain showed agarolytic activity. Ten strains showed lipolytic activity (13.7 %; nine from BC1 and one from BC3) and were related to the genus *Pseudomonas*. The presence of gelatinase occurred in 11 strains from BC1 (15.1 %; three unidentified and two belonging to *Gelidibacter* sp., five related to *Pseudomonas* sp. and one to *Staphylococcus* sp.). Three strains (4.1 %) belonging to *Psychrobacter* sp. (one strain) and *Carnobacterium* sp. (two strains) from BC1 were amylase positive, while two strains (2.7 %) related to *Pseudomonas* sp. and *Shewanella* sp. were chitinase positive.

6.9.3 Antibiotic susceptibility

Isolates were subjected to antibiotic susceptibility testing using TSA medium added with antibiotics at different concentration. Three strains (4.1 %) were able to tolerate both chloramphenicol and kanamycin; four strains (5.5 %) were resistant to ampicillin; five strains (6.8 %) tolerated gentamycin and polimixin B each; six strains (8.2 %) tolerated streptomycin. The tolerance decreasing in order of *S>A>P>C>G>K,* but few strains were able to growth in antibiotics presence.

Two strains from BC1 belonging to the genus *Gelidibacter* were multi-resistant and tolerated ampicillin up to 50 ppm, chloramphenicol up to 100 ppm and polimixin up to 25 ppm and up to 350 ppm for gentamycin. Streptomycin was tolerated up to 25 ppm by five

strains related to *Carnobacterium* sp. while BC1-23 (*Shewanella* sp.) was able to growth up to 250 ppm of streptomycin. Kanamycin was tolerated up to 150 ppm by one isolates unidentified, while ampicillin was tolerated up to 50 ppm by two *Gelidibacter* sp. Five strains tolerated polimixin up to 25 ppm and were related to *Gelidibacter* sp., *Pseudomonas* sp. and *Staphylococcus* sp. Gentamycin was tolerated by two strains (BC1-31 unidentified and *Pseudomonas* sp. BC1 bis-7).

In BC3, three strains (two *Psychrobacter* and one *Pseudomonas*) were able to tolerate ampicillin up to 50 ppm and one *Psychrobacter* was able to tolerate kanamycin up to 25 ppm (Table 6.38). Bacterial strains from Boulder Clay did not give a great response about tolerance to antibiotics. Only few strains were able to tolerate antibiotics (on average three strains *per* antibiotic). Strains belonging to *Gelidibacter* were able to tolerate four different antibiotics at high concentrations (e.g. chloramphenicol up to 100 ppm and gentamycin up to 350 ppm). The only *Staphylococcus* isolate was able to tolerate three antibiotics, but at low concentration.

Table 6.38 Antibiotic susceptibility test. Isolates from BC1 and BC3 were grouped in a unique table. Isolates from BC3 were marked.

Genera	Antibiotic concentrations (ppm)								
	Streptomycin		Kanamycin		Ampicillin	Polimixin	Chloramphenicol	Gentamycin	
	25	250	25	150	50	25	100	25	350
Pseudomonas					1*	1		1	
Shewanella		1							
Psychrobacter				1*	1*			1*	
Carnobacterium	4								
Staphylococcus			1			1	1		
Gelidibacter					2	2	2		2
Unidentified	1			1				1	
	5	1	1	1/2*	2/4*	4	3	2/3*	2

* Isolates from BC3

6.9.4 Heavy metal tolerance

Isolates from Boulder Clay brines were first screened for heavy metal tolerance towards five different metals (mercury, $HgCl_2$; cadmium, $CdCl_2$; copper, $CuCl_2$; cobalt, $CoCl_2$; nickel, $NiCl_2$) at two different concentrations (500 and 1000 ppm). Tolerant isolates were then assayed in a medium amended with heavy metal salts up to 10000 ppm. The relative resistance of the metals decreases in the order of *Ni>Cu> Hg>Co> Cd*. Cd was tolerated by any strain. Overall, nine strains (12.3 %) tolerated Hg, ten strains (13.7 %) tolerated Co, 43 strains (58.9 %) tolerated Cu up to 500 ppm, five strains (6.8 %) tolerated Cu up to 1000 ppm, 44 strains (60.3 %) tolerated Ni up to 500 ppm, 14 strains (19.2 %) tolerated Ni up to 1000 ppm and four strains (5.5 %) tolerated Ni up to 2500 ppm.

BC1. Three strains (affiliated to *Carnobacterium* sp., *Psychrobacter* sp. and *Staphylococcus* sp.) were able to tolerate Hg up to 1000 ppm while five strains were able to tolerate Co until 500 ppm (four were affiliated to *Carnobacterium* sp. and one was not identified). 44 strains were able to tolerate Cu up to 500 ppm and were related to *Pseudomonas* sp. (33 strains), *Carnobacterium* sp. (four strains), *Shewanella* sp. (three strains), *Actinobacterium* (BC1 bis-11), *Staphylococcus* sp. (BC1 bis-71) and two unidentified. 45 strains were able to tolerate Ni up to 500 ppm and were affiliated to *Pseudomonas* sp. (35 strains), *Carnobacterium* sp. (tree strains), *Shewanella* sp. (3 strains), *Gelidibacter* sp. (1 strain), *Actinobacterium* (one strain) and two unidentified; Ni was tolerated up to 1000 ppm by 12 strains and were affiliated to *Pseudomonas* sp. (7 strains), *Carnobacterium* sp. (3 strains), *Psychrobacter* sp. (1 strain) and unidentified (1 strain). *Staphylococcus* (BC1 bis-71) and *Gelidibacter* strains (BC1-118B) were able to tolerate Ni up to 2500 ppm. (Table 6.39). In BC1, 33 strains related to the genus *Pseudomonas*, three *Shewanella* strains, 4 *Carnobacterium* and one *Actinobacterium* (BC1 bis-11) were able to tolerate two different metals, while two *Carnobacterium* were

able to tolerate three different metals. One *Staphylococcus* strain was able to tolerate four metals.

BC3. Six strains were able to tolerate Hg up to 1000 ppm and were affiliated to *Psychrobacter* sp. (5 strains) and *Pseudomonas* sp. (BC3-46); five *Psychrobacter* isolates were resistant to Cu up to 1000 ppm and Co up to 500 ppm; three *Psychrobacter* strains were able to tolerate Ni up to 1000 ppm and two were able to tolerate Ni up to 2500 ppm (Table 6.39). In BC3, five *Psychrobacter* isolates were able to tolerate four different metals. In general *Pseudomonas* strains appeared to be more tolerant than other isolates.

Table 6.39 Heavy metal tolerance of isolates from Boulder Clay bines.

BC1 Genera	**Heavy metals concentrations (ppm)**					
	$HgCl_2$	**$CoCl_2$**	**$CuCl_2$**		**$NiCl_2$**	
	1000	**500**	**500**	**500**	**1000**	**2500**
Pseudomonas			32	34	7	
Psychrobacter	1				1	
Shewanella			3	3		
Actinobacterium			1	1		
Gelidibacter				1	2	1
Carnobacterium	1	4	4	4		
Staphylococcus	1		1			1
Unidentified		1	2	1	1	
	3	5	43	44	11	2

BC3 Genera	**Heavy metals concentrations (ppm)**				
	$HgCl_2$	**$CoCl_2$**	**$CuCl_2$**	**$NiCl_2$**	
	1000	**500**	**1000**	**1000**	**2500**
Pseudomonas	5	5	5	3	2
Psychrobacter	1				
	6	5	5	3	2

6.10 Biotechnological application: RESULTS

Biotechnological potential was analyzed for a total of 223 strains from Antarctic brines.

6.10.1 Screening for exopolysaccharide production

Isolates were screened for exopolysaccharide (EPS) production using a preliminary test using TSA plates amended with glucose in order to select bacterial isolates whose colonies appeared mucoid or viscous. Overall, 18.8 % of the strains showed a mucoid appearance on plate. To confirm the plate assay, 42 strains were further inoculated in TSB *plus* glucose to ensure that they were EPS producers. Three positive strains (2 %) from Tarn Flat belonged to the genera *Psychrobacter* (isolates **TF4-71**, **TF4-72** from TF4) and *Pseudomonas* (isolate **TF5-192A** from TF5). Four strains (5.4 % from BC1) from Boulder Clay were able to produce exopolysaccharides and were affiliated to the genus *Pseudomonas* (**BC1-139**, **BC1 bis-18**, **BC1 bis-19**, **BC1 bis-22**).

Based on the preliminary screening, four strains with different phylogenetic affiliation and from different samples (i.e. TF4-72, TF5-192A, BC1-139 and BC1 bis-18) were selected for further analyses. Isolates were inoculated on Väätänen nine-salt solution added with Glucose solution to extract the produced EPS. Extracted EPSs were characterized using a colorimetric assay that analysed the total carbohydrate in the culture medium. Glucose was used as a standard (OD490). This method allows to calculate the carbohydrate amount produced by the tested strains. The carbohydrate quantification using the Dubois method is shown in Figure 6.25. Isolate **BC1-139** was the best carbohydrate producer with a concentration of 170.1 µg EPS mL^{-1}.

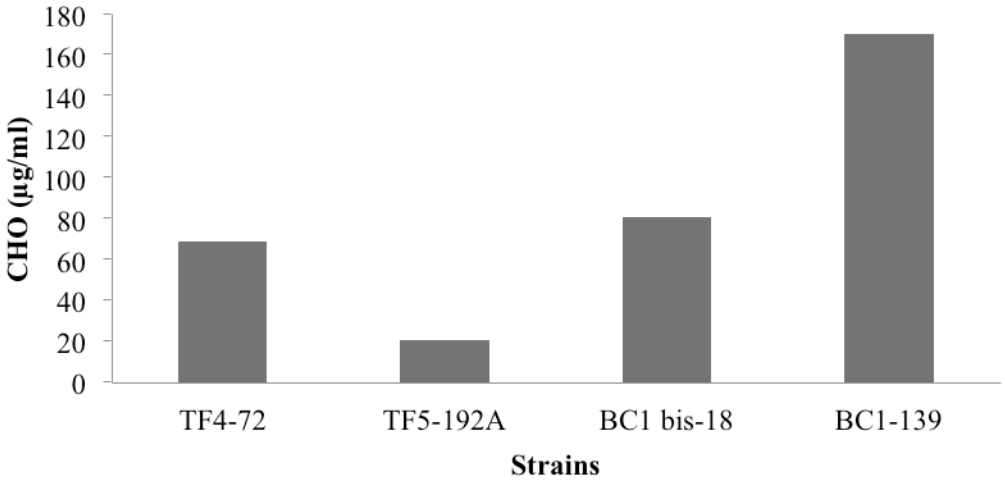

Figure 6.25 Carbohydrate quantification

6.10.2 Inhibitory activity against indicator organisms

None of the tested 223 strains isolated from brines showed inhibitory activity against *E. coli*, *M. luteus*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, *S. enterica*, *P. damselae damselae*, and *V. cholera*.

6.10.3 Growth in the presence of hydrocarbons

The ability to growth in the presence of different hydrocarbons was tested on agar plates incubated at 4 °C.

Tarn Flat. Isolates from TF4 were able to growth in the presence of almost all hydrocarbons. The most used substrate was diesel oil with 26 strains (17.2 %), mainly affiliated to *Psychrobacter* spp. (20 strains, 76.9 %). Isolates from TF5 mainly utilized naphthalene with positive strains that were mainly affiliated to the genus *Rhodobacter* (14 strains; 9.3 %). Biphenyl was used by 21 strains (13.9 %), mainly affiliated to the genera *Leifsonia* (5 strains) and *Rhodobacter* (5 strains).

Boulder Clay. No growth occurred in the presence of naphthalene and pyrene as the sole carbon source. Isolates from BC3, mainly belonging to *Psychrobacter* spp., were able to grow in the presence of crude oil, heptane and octane, while one *Psychrobacter* strain (BC3-97) was able to use also diesel oil and phenanthrene. *Staphylococcus* strain BC1 bis-71 from BC1 was able to use diesel oil. Biphenyl was used by ten (13.5 %) strains affiliated to *Pseudomonas* spp. (8 isolates from BC1), *Staphylococcus* (one isolate from BC1) and *Psychrobacter* (one isolate from BC3).

6.10.4 Screening on hydrocarbons

Isolates grown on diesel oil and crude oil (39 and 21, respectively) amended agar plates were screened for growth also in liquid culture. Among them, eight strains (6.8 %; TF4-31C, TF4-69, TF4-119, TF4-164, TF4-166, TF4-233, BC3-8, BC3-97) resulted positive (Table 6.40). Except for TF4-233 (affiliated to the genus *Marinobacter,* OTU6), all isolates belonged to the genus *Psychrobacter* (OTU10, OTU27 and isolate TF4-164). Diesel oil was better utilized than crude oil. In particular, *Psychrobacter* isolate TF4-31C (OTU10) better growth in the culture broth, even if no growth occurred after plating.

Table 6.40 Growth of hydrocarbon-oxidizing isolates in crude oil/diesel oil amended liquid cultures (na=not assigned).

Strain	OTU	Carbon source
Psychrobacter sp. (TF4-31C)	OTU10	Diesel oil
Psychrobacter sp. (TF4-69)	OTU10	Diesel oil
Psychrobacter sp. (TF4-119)	OTU10	Diesel oil
Psychrobacter sp. (TF4-164)	na	Crude oil
Psychrobacter sp. (TF4-166)	OTU10	Crude oil
Marinobacter sp. (TF4-233)	OTU6	Diesel oil
Psychrobacter sp. (BC3-8)	OTU27	Crude oil
Psychrobacter sp. (BC3-97)	OTU27	Diesel oil

6.10.5 Screening on Aroclor 1242

A total of 33 isolates (out of 223; 14.8 %) from Antarctic brines were able to growth in the presence of biphenyl. Among them, only ten isolates (10.8 %) from Tarn Flat and two

from Boulder Clay grew in the presence of Aroclor 1242 at 4 and 15°C. Their phylogenetic affiliation is given in Table 6.41. All strains related to *Rhodobacter* sp. were represented by OTU13, while the strains related to *Sporosarcina* sp. were represented by the OTU15.

Table 6.41 Phylogenetic affiliation of bacterial isolates able to growth in the presence of the PCB mixture Aroclor 1242 (na=not assigned).

Strain	OTU	Aroclor screening	
		4 °C	15 °C
Sporosarcina sp. (TF5-66)	OTU15	+	+
Sporosarcina sp. (TF5-76)	OTU15	+/-	+
Rhodobacter sp. (TF5-131)	OTU13	+	+
Rhodobacter sp. (TF5-135)	OTU13	+	+
Rhodobacter sp. (TF5-137)	OTU13	+	+
Unidentified (TF5-140)	na	+	+
Rhodobacter sp. (TF5-148)	OTU13	+	+
Rhodobacter sp. (TF5-224)	OTU13	+	+
Staphylococcus sp. (BC1 bis-71)	na	+	++
Psychrobacter sp. (BC3-97)	OTU27	+	+/-

Screening for the presence of the functional gene *bph*A

The Figure 6.26 reports the agarose electrophoresis gel control of *Burkholderia xenovorans* (DSM 17367), a positive control for *bph*A gene using the BPHA primer set.

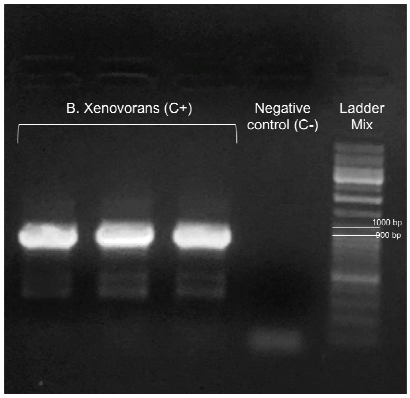

Figure 6.26 Screening of BPH gene using *B. xenovorans* as positive control for the presence of bphA portion of the gene using 2BPHFWD1 (5' ADVCCSCGBGCCGCBTCHTCG 3') and 2BPHREV1 (5' ADVCCSCGBGCCGCBTCHTCG 3') primer

The *bph*A gene fragment was screened for the ten positive strains that were able to grow in the presence of Aroclor 1242. The strains were TF5-66, TF5-76, TF5-131, TF5-135, TF5-137, TF5-140, TF5-148, TF5-224, BC1 bis-71 and BC3-97. The amplification of the *bphA* gene fragment was found in four strains from TF5 (33.3 % of positive strains on Aroclor 1242; i.e. TF5-131, TF5-135, TF5-148 and TF5-224), all affiliated to the genus *Rhodobacter* (OTU13) (Figure 6.27, Table 6.42).

Among them, two strains (BC1 bis-71 and BC3-97) were selected for degradation efficiency even if they did not harbour the *bph*A gene.

Table 6.42 Screening for bphA fragment using 2BPHAfwd1 and 2BPHrev1.

Strain	*bph*A
Sporosarcina sp. (TF5-66)	-
Sporosarcina sp. (TF5-76)	-
Rhodobacter sp. (TF5-131)	+
Rhodobacter sp. (TF5-135)	+
Rhodobacter sp. (TF5-137)	-
Unidentified (TF5-140)	-
Rhodobacter sp. (TF5-148)	+
Rhodobacter sp. (TF5-224)	+
Staphylococcus sp. (BC1 bis-71)	-
Psychrobacter sp. (BC3-97)	-

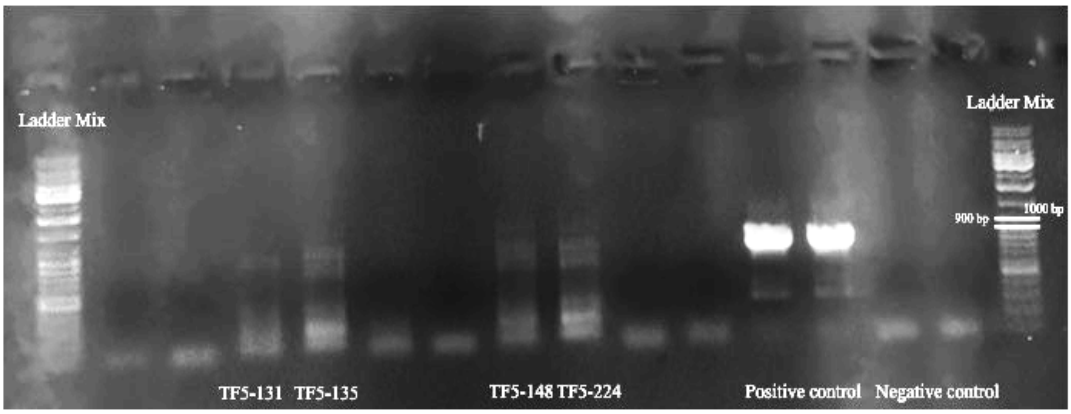

Figure 6.27 Gel electrophoresis (1 %, agarose gel) of *bph*A gene amplification. Positive control was *B. xenovorans*, negative control was the mixture without DNA. Isolates TF5-131, TF5-135, TF5-148 and TF5-224 were positive to the amplification

Degradation efficiency test

The biodegradation efficiency on Aroclor 1242 was tested for two strains, *Psychrobacter* sp. BC3-97 and *Staphylococcus* BC1 bis-71 sp. The strains were able to degrade noticeably the 1,1'-Biphenyl, 2,2'-dichloro among the Aroclor 1242 congeners, and reduced by more than 50 % the corresponding chromatographic peaks. The degradation efficiency resulted higher during incubation at 15 °C rather than at 4 °C, with a removing activity of the substrate until a percentage of 80.8 % and 96.95 % for *Psychrobacter* sp. BC3-97 and *Staphylococcus* BC1 bis-71 sp., respectively (Fig. 6.28). The representative peaks of the other congeners resulted not easily detectable, and were not considered.

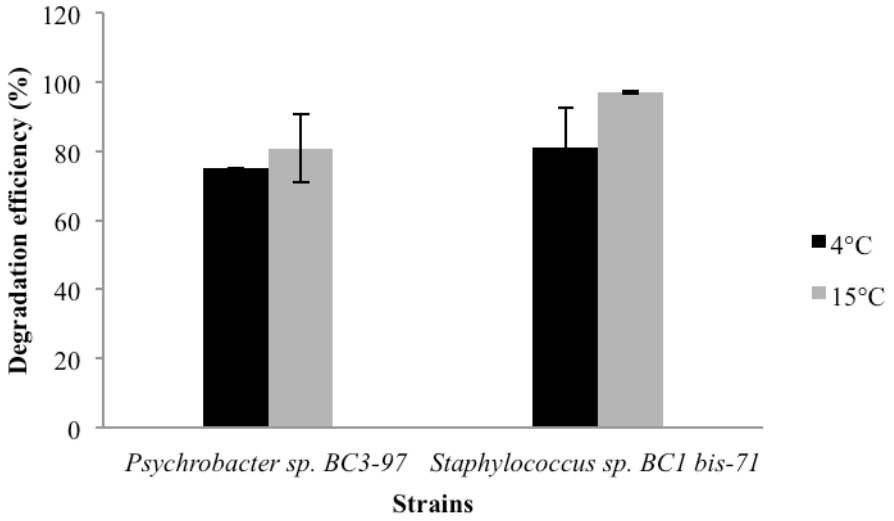

Figure 6.28 Degradation efficiency of two strains isolated from Antarctic brine samples. The percentage of degradation on 1,1'-Biphenyl, 2,2'-dichlorophenyl is reported

7. Brines: DISCUSSION

This study is one of the first report on the prokaryotic communities of brines within permafrost in Antarctica. This unique environment is used as a test to study the physiologic adaptations of isolate to extreme environments. In addition, Antarctica is seen as an analogue of extraterrestrial environments (Pyne, 2007). The application of a combination of two different approaches resulted in a more complete characterization of the prokaryotic diversity existing within brine environments and lead us to a better understanding of how microorganisms function and survive in such extreme cryo-environments.

The culture-independent approach was carried out using microscopy techniques, as DAPI-staining and CARD-FISH, and Next Generation Sequencing analysis using Ion Torrent.

Bacterial densities estimated by direct epifluorescence microscopy are in line with previous reports for the Lake Vida brines in Antarctica (10^6 cells mL^{-1}, Murray *et al*., 2012) and Antarctic permafrost (10^5-10^6 cells g^{-1}, Horowitz *et al.,* 1972; Cowan *et al*., 2002). The discrepancies between viable count number and total counts are explained by the expected uncultivable capability of some microorganisms that prefer different conditions.

Before the NGS analysis a preliminary analysis on *Bacteria*, *Archaea* and *Eukaryota* phyla in brines sample was carried out by using the CARD-FISH enumeration methods. A total of 13 probes were used, nine for *Eubacteria*, two for *Archaea* and one universal probe for *Eukaryotes.* Such probes were chosen to analyze the general microbial community structure using different targets which are potentially dominant soil microbial groups (Lauber *et al*., 2009) and water system. Bacterial, archaeal and eukaryotic cells were enumerated. Bacteria accounted for 66.4-86 % of DAPI-stained cells, while *Archaea* and *Eukaryote* were at very low percentages (below 2 %). The community

structure in Tarn Flat and Boulder Clay was similar, with high percentage of *Bacteroidetes* that were generally followed by *Betaproteobacteria*, and *Gammaproteobacteria*. BC1 samples differed from the other samples both for abundance of DAPI stained cells (10^7), respect the others, which showed a lower order of magnitude (10^6), and for a higher percentage of *Alphaproteobacteria* (21.6 % *vs* 4-7 % of other samples). The analyses reported a major presence of *Betaproteobacteria* and *Gammaproteobacteria*, followed by *CF* group of *Bacteroidetes*. TF4 and TF5 were quite similar, suggesting that the brines could stay in communication between the two layers (Forte *et al*., 2016). The heterogeneity of diversity could be due by dynamics factors (fluid flux, gas concentrations and diffusion, seasonal effects etc.). The community presented numerous phyla and was similar to that of sediment samples. The higher percentage of *Betaproteobacteria* is in accordance with previous studies and it has been recently reported for alpine snowpack and sub-glacial systems (Alfreider *et al*., 1996; Foght *et al.,* 2004). *Betaproteobacteria* were reported as dominant in volcanic lakes in Iceland (Gaidos *et al*., 2004) and in glaciers in Canada and Alaska (Skidmore *et al*., 2005). The predominance of *Betaproteobacteria* is in disagreement with previous studies (inland waters; Glockner *et al*., 1999; Bockelmann *et al*., 2000; Brummer *et al.,* 2004) on dynamic saline systems (del Giorgio and Bouvier, 2002; Bouvier and del Giorgio, 2003; Cottrell and Kirchmann, 2003; Kirchman *et al*., 2005; Zhang *et al*., 2006) because it wasn't influenced by increase in salinity. In fact, the high percentage of *Betaproteobacteria* was observed in Tarn Flat site (higher salinity) and in Boulder Clay (very low salinity, less than 1 psu). Langenheder *et al*. (2003) reported the abundance of *Alpha-*, *Beta-* and *Gammaproteobacteria* under freshwater conditions. This was in disaccordance with our study because brines from Tarn Flat had a high salinity, whereas it was in line with Boulder Clay that was similar to freshwaters. *Proteobacteria*, *CF* group of *Bacteroidetes* and psychrophilic *Eukaryotes* and few *Archaea* were retrieved in Antarctic glaciers (Simon *et al*., 2009). Anyhow, molecular analyeis reported the

dominance of various classes of *Proteobacteria* (Yang *et al*., 2008). Most diversity studies of worldwide polar and non-polar glaciers at 16S rRNA gene resolution have consistently detected representatives of similar genera of *Actinobacteria*, *Proteobacteria* and *Cytophaga-Flavobacteria*, suggesting that members of these genera likely possess similar survival mechanisms (Christner *et al*., 2008b).

Ion Torrent analysis

To improve the knowledge about brine samples the prokaryotic community composition was analysed by next generation sequencing analysis using Ion Torrent. The prokaryotic community was evaluated with the analysis of cDNA, active fraction of bacteria, and DNA for Bacteria and Archaea. As stated above, samples were collected from two different sites, one inland (TF) and one coastal site (BC). TF samples were collected from the same borehole at 3.9 and 4.5 m depth, while in BC samples were collected from two different lake (BC1 and BC2 from the same lake; BC3 in a second lake). The community composition was explored through active bacterial community and total prokaryotic community (Bacteria and Archaea) analysis. The cDNA (active community) was analyzed only in Boulder Clay samples because the TF samples presented low sequencing quality after file and chimera cleaning.

The community inhabiting permafrost and brine samples could be peculiar and thanks to low temperature, glacial ice and permafrost it might contain sequences from the oldest nucleic acid and microbial cells on Earth, which could prove key to reconstructing past ecosystems. Willerslev *et al*. (2004) discussed on the long-term survival DNA and/or RNA molecules in ice and permafrost. Within permafrost there were some DNA molecules that remain trapped inside for a long geological era. Several authors claim to have isolated ancient DNA and RNA molecules from ice cores up to 100 thousand years (kyr) old and from permafrost-cores up to 2-3 million years (MY) old (Catranis *et al.,* 1991; Abyzov *et al*., 1993; Shi *et al*., 1997; Vorobyova *et al*., 1997; Priscu *et al.,* 1999).

This discovery makes permafrost and brine preserved DNA potentially important in the study of past ecosystems. To analyze the prokaryotic community and the difference between active and total DNA Ion Torrent Sequencing was used. The active community was evaluated only on BC samples that present a total of 9,395 sequences distributed in each sample, in particular the sample BC3 produced the higher numbers of reads (8,485). The active community were 6.03 % of total reads in BC1, whereas in BC2 the percentage was 1.71 %. The BC3 percentage of active bacteria was the highest with 80.22 % of total reads. This different percentage could be explained due to the temperature and the seasonal cycles. In fact, total DNA remains trapped inside the permafrost and ice that melted during the spring and summer, allowing the release in liquid brines. Probably the microorganisms remain quiescent until the different seasonal changes. Furthermore, the low temperature brings the DNA or RNA to remain uncontaminated for long time and stored inside the permafrost and brines. This could explain the high read number in total prokaryotic community in contraposition with the active community.

The active community was represented by numerous phyla, such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* at high percentage, while other groups (i.e. *Acidobacteria*, *Aquificae*, *Chlamydiae*, *Chlorobi*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus-Thermus*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*) occurred at low percentage and were not represented in all BC active samples. Through the NMDS analysis it was showed the high similarity between BC2 active and BC1 active communities, while BC3 was very far from the other two samples. This difference could be explained because the BC3 site was the less deep sample and remains in continually active state because it does not maintain the temperature under 0 °C for geological eras. This is for such reason that 80 % of sequences of total reads are compared to active reads. Another explanation could be that such sample was collected in a different lake with different chemical-physical characteristics respect to the other lake from which BC1 and BC2 derived.

In particular, the BC1 sample, that was the deepest, was characterized by a high diversity within the active fraction of the bacterial community with the presence of several phyla, even if at low percentage. In particular, the sequences were affiliated mainly to *Firmicutes* (10.1 %) and *Bacteroidetes* (8.8 %). The high percentage of *Firmicutes* could be explained by their ability to form spores to cope with adverse environmental conditions, thus remaining quiescent for a long time. In particular, the *Firmicutes* were represented by different genera of facultative anaerobes (i.e. *Oceanobacillus*, *Dolosicoccus*, *Salsuginibacillus*, *Clostridium*), suggesting that brines may experience period of lack of oxygen. These conditions are also supported by the presence of the main representatives of *Epsilonproteobacteria*, *Labetimonas* and *Sulfurimonas* genera, which were retrieved near the hydrothermal vents, sulphur-oxidizing bacteria in the Baltic Sea (Grote *et al*., 2007), and by a facultative anaerobic *Streptomyces*, belonging to *Actinobacteria*, isolated from an Antarctic lake (Xiao, *et al*., 2005). BC2 active was quite similar to BC1 but with a lower number of genera, one for each phyla, with high percentages of *Desulfonema* and *Caldimicrobium* that are strictly anaerobic and sulphur-oxidizing in organic rich sediments and microbial mats (Fukui *et al*., 1999; Miroshnichenko *et al.,* 2009). Results were quite similar between the different brines.

Conversely, in the BC3 active (collected from another lake) the abundance of *Bacteroidetes* (57.7 %) was higher and frequently identified in glacial environments such as cryoconite holes (Edwards *et al.,* 2011; Cameron *et al*., 2012), glacier-fed streams (Wilhelmet *et al*., 2013), fjords and polar waters influenced by glacial meltwater (Zeng *et al*., 2009; 2013; Piquet *et al*., 2010; 2011) and *Cyanobacteria* (7.2 %). These phyla are often abundant in ice and areas with high dissolved organic matter (Brakstad *et al*., 2008; Coolen *et al*., 2011). The other phyla were present at low percentages. Furthermore, the high percentage of *Flavobacterium* genus indicates a possible presence of organic matters and low salinity water, as it is generally isolated from polar lakes (Van Trappen *et al*., 2002) and from streams, rivers, lakes and other cold environments, and therefore play a

role in mineralizing various types of organic matter in aquatic systems. The high occurrence of *Betaproteobacteria* (21 %) may be explained by the low salinity of the lake and brine system, because such subclass is often dominant in freshwater lakes (Methe *et al*., 1998) and represents a substantial proportion of the microbial communities in various types of rivers.

The total prokaryotic community structure was analyzed for all samples. A higher diversity was determined in BC1 (Shannon_H index is 1.783), which was far from the other four samples in the NMDS plot. In particular TF4 and TF5 clusterized together while BC2 and BC3 were distant one from each other and from BC. In Boulder Clay site the general diversity was constituted by *Bacteroidetes*, followed by *Proteobacteria* and *Actinobacteria*. The other phyla changed in percentage from sample to sample.

In particular, BC1 was represented even by numerous phyla, for example *Planctomycetes* (0.12 %), *Cyanobacteria* (6 %) and other groups (27.9 %). The *Cyanobacteria* are cosmopolitan and adapt theirselves to develop efficient systems to harvest the light of the sun in the permafrost table. It is possible that the DNA from *Cyanobacteria* is old DNA (Zhumur *et al*., 1999, Boyd, 2001). The *Proteobacteria* were the most abundant phylum, with a predominance of *Gammaproteobacteria*, followed by *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria*. Normally the *Gammaproteobacteria* are marine bacteria isolated from superficial sediments (Teske *et al*., 2011) and pelagic water (Choe and Giovannoni, 2004). Their presence suggests that there could be some influences from the sea (which is next to the BC lakes), maybe aerosol, or influences that date them back to a different geological era. This is also suggested by the presence of *Marichromatium* that prefer anoxic marine sediments and stagnant waters containing hydrogen sulphide (Imhoff *et al.*, 1998). The ocean influencers maybe exist in this brine samples because sequences related to *Psychroserpens* and *Algoriphagus*. Both genera have been frequently retrieved in Antarctic marine lake (Bowman *et al*., 1997; Nedashkovskaya *et al*., 2004). Within the

Gammaproteobacteria there was the predominance of *Pseudomonas*, a frequent genus with versatile metabolism, always found in Antarctic lakes (Shivaji *et al*., 1989). In BC2 there was a high percentage of *Bacteroidetes* (66.4 %) and *Proteobacteria* (19.75 %). The genus that was mainly represented was *Flavobacterium* which is very frequent in cold environments and halotolerant (Bowman et al., 1998; McCammon and Bowman, 2000). Other abundant genera were *Marichromatium, Rhodoferax* and *Pedobacter*. The first two were related to stagnant aquatic systems exposed to light (Imhoff *et al*., 1998; 2006), the last were reported in cold environments, such as cryoconite holes (Takeuchi *et al*., 2001; Margesin *et al.,* 2000; Margesin *et al*., 2003). In particular, the genus *Rhodoferax* was retrieved in saline ponds in Cape Royds in Antarctica (Madigan *et al*., 2000). The sample BC2 probably was collected during a period that brine erupted from the frost mound. This peculiar stagnant community may be explained in this way. BC3 samples was very similar to BC2 with respect to total bacterial community and this was confirmed by the the cluster analysis that presents the two samples in the same ramifications. Therefore, the community structure of BC3 sample was similar in old DNA, with genera influenced by marine sedimentary environments.

Tarn Flat is an inland site far from seawater. The samples were collected from a borehole under the permafrost carrots performed within the lake. The sample TF4 was collected at 3.9 m while TF5 was collected at 4.5 m of depth. The statistical analysis allows to demonstrate that the two samples are quite similar for the diversity, in fact they present a similar Shannon_H index (1.951 in TF4 and 2.059 in TF5). The total bacterial community was distributed mainly within *Proteobacteria*, followed by *Bacteroidetes*, *Spirochaetes* and *Planctomycetes*, while the other phyla were less represented. In particular, *Spirochaetes* phylum was widely distributed in sediments, muds, lakes, saline solar lakes and in deep-sea hydrothermal vents (Henry *et al*., 1978; Canale-Parola, 1981; Harwood *et al*., 1982). Furthermore, they could be anaerobic or facultative anaerobic, could have the capacity to persist in the environments and adapt theirselves to particular conditions.

Franzmann *et al*. (1996) found well-less spirochete in a Antarctic saline lake and they probably colonized this environment. Instead the *Planctomycetes* were retrieved in lakes (Hirsch and Muller, 1985; Griepenburg *et al*., 1999; Schlesner *et al*., 2004), marine habitats (Bauld and Staley, 1976), often in permanently cold marine sediments (Ravenschlag *et al*., 1999), marine snow (Rath *et al*., 1998), psychrophilic environments (Antarctic lake water) (Glatz *et al*., 2006), Lake Vida ice cover in Antarctica (Mosier *et al*., 2007), and seasonal Arctic snow and meltwater from Svalbard Norway (Larose *et al*., 2010). At genus level numerous genera were retrieved and were well distributed among phyla, such as *Paracoccus* that was isolated from permafrost in China (Zhu *et al*., 2013), *Rhodoferax* genera that were retrieved in saline ponds in Cape Royds in Antarctica (Madigan *et al*., 2000). In particular, the sequences were mainly affiliated to genera that were isolated from marine environments, such as *Marinobacter* (Antarctic sea ice, Yu *et al*., 2009), *Thiomicrospira* (marine arctic sediments, Knittel *et al*., 2005), *Marichromatium*, *Ulvibacter* and *Polaribacter* (Choi *et al*., 2007, the last two are isolated from polar environments, both Arctic and Antarctic, such as ice and coastal marine waters). Probably these isolates retrieved in total prokaryotic community structure may be dependent on the possibility that the sediments inside the permafrost had an marine origin before the permafrost formation. Therefore, during the seasonally temperature cycles, less water and salt were released from sediments to brines that take the old DNA before retained by the soil. Obviously, the temperature under 0 °C allow the adaptation of some organisms that are able to stay in these particular habitats. In fact, the sequences found were associated to psychrophilic genera isolated from Antarctica, in particular *Psychroflexus* (Bowman *et al*., 1998), *Algoriphagus* (Van Trappen, 2004), *Polaribacter* (Brinkmeyer *et al*., 2003). The deepest sample (TF5) was similar to TF4 for the ratio of total bacterial community, but differed for genera composition. In particular the sequences were mainly affiliated to *Geopsychrobacter* (14.4 %), retrieved in Antarctic

glacier (Mikucki and Priscu, 2007), *Thiomicrospira* (11.9 %) and *Shewanella* (10.9 %) retrieved in Antarctic coastal area (Bozal *et al.,* 2002).

Among the classified *Epsilon-* and *Deltaproteobacteria*, members related to the sulfur and nitrogen cycles were retrieved, such as *Sulfiromonas*, *Desulfobulbus*, *Desulfocapsa* and *Desulfophropalus*.

In conclusion, the active and total prokaryotic community revealed how the communities can change during the time and how they could be influenced by the external factors and, in particular, how the marine environments could influence the community. It is not known the mechanism that allows this influence, maybe the permafrost near the brine could have a marine origin.

The Archaeal community would deserve a separate discussion. Using a cluster analysis it was possible to separate the Archaeal community in each sample. BC3 and BC1 "clusterized" in the same ramifications, while TF4 and TF5 "clusterized" together. The sample with a higher Shannon_H index was BC3 (1.241), while the lower was obtained in BC2 (0.8963).

The Archaeal community, which is difficult to cultivate in laboratory, structure was analyzed using Ion Torrent. The Archaeal diversity was highest in BC1 sample (Shannon_H index= 1.178) and lowest in BC2 (0.8963). The cluster analysis distributed BC2 in the same TF ramifications, while BC1 and BC3 were together. This was a different behavior between Bacteria and Archaea, especially in BC3 sample.

Very few studies have described the Archaea communities in permafrost using culture-independent methodologies. A report detected 16S rRNA genes related to the *Crenarchaeota* in Chinese alpine permafrost (Ochsenreiter *et al*., 2003). Another study revealed the presence of *Euryarchaeota* and *Crenarchaeota* in Canadian permafrost, with sequences numerically dominant within *Euryarchaeota* (Steven *et al*. 2007a,b).

In our study, the sequences were distributed in different groups with a different percentage among the samples, but with a similar trend. *Euryarchaeota* were mainly

represented, followed by *Crenarchaeota* and *Ancient_Archaeal_Group*, with low percentage *Korarchaeota* and *Marine_Hydrothermal_Vent_Group_1*. Numerous orders were retrieved in brine samples, similar between TF and BC samples. The order *Archaeoglobus* (Archaea sulphate reducers, using sulphate or sulphite as electron acceptors), inhabiting aquatic and terrestrial sediments, live in anoxic condition isolated initially in anoxic shallow and abyssal submarine hydrothermal vents of the coast of Italy (Stetter *et al.,* 1987). This is strictly anaerobic and hyperthermophilic. The *Halobacteriales* order is aerobic or facultative anaerobic and ubiquitarious where salt concentration is high, like salt lakes (Youssef *et al*., 2012), soda lakes (Feng *et al*., 2005; Jones *et al.,* 1998) and subterranean salt deposits. The methanogenic group was represented by *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* orders. Their presence could be related to the probable presence of methane inside permafrost and ice that remains stored during geological era. The minimum seasonally changes bring to a release of methane as a greenhouse gas. The presence of these methanogenic *Archaea* is an important source in the global methane budget. The methane, thus, could be oxidised by a consortium of methane-oxidising Archaea and sulphate-reducing bacteria (Hoehler *et al*., 1994; Hinrichs *et al*., 1999; Boetius *et al*., 2000; Pancost *et al*., 2000), and probably the bacterial genera retrieved in brine samples and the *Archaea* related to methane cycles could work together for the carbon balance during the seasonally time. The explanation of the presence of phylotypes affiliated with methanogenic orders would be means that there are a lot of carbon inside both TF and BC samples. The DNA sequences did not explain the possibility that the methane cycle is active or not because in this study new approaches were not developed for working with *Archaea*. Methanogenic Archaea are strictly anaerobic that carry out methanogenesis, a metabolitic process. The principal orders retrieved were: *Methanosarcinales* which is halotolerant and produces methane from methyl group containing compounds, with many species that can reduce CO_2; *Methanosalsum* is a moderate halophile, requires bicarbonate and produces methane from

methylamines, methanol or dimethylsulfide, prefers an alkaline pH; *Methanopyrales* grow well at high temperature, are obligate anaerobic and hyperthermophilic, use CO_2 to formed methane, are able to reduce sulphur; while *Methnobacteriales* are striclty anaerobic and grow oxidixing H_2, Recently they have been found in Arctic permafrost with *Methanothermus* genera (Shcherbakova *et al*., 2011). *Methanococcales* contain mainly isolates from marine and estuarine sediments. *Methanomicrobiales* colonize freshwater and less saline marine environments. The genera mainly represented in all samples were *Methanothermus* and *Methanopyrus*, with this latter that was retrieved in warm environments such as submarine high temperature environment (Huber *et al*., 1990). *Methanosalsum* was isolated from hypersaline soda lakes (Sorokin *et al*., 2015). In BC1 sequences of *Methanococcales* and *Methanocellales* were not represented, probably because they are extremely related to marine environments. *Methanobacteriales* were less abundant in BC2 and BC1, while they were abundant in TF4, TF5 and BC3 probably due to the salinity that was higher in these samples rather than in BC1 and BC2.

The Crenarchaeota were mainly represented by the *Sulfolobales* order which is made of extreme acidophilic members isolated from acidic terrestrial and marine solfataras. In particular, the genus that occurred in all samples was *Sulfurisphaera*, an extremely thermophilic acidophile (Kurosawa *et al.*, 1998) isolated from acidic hot springs.

The sample TF4 was less order-represented than TF5. *Thermoproteales*, *Archeoglobales*, *Methanococcales*, *Methanosarcinales* were not present probably because such orders prefer acid environments, and the pH of TF5 was lower than TF4. In particular, it is possible that down the TF5 there was an acidophilic springs, as a possibility for all of these genera.

This particular Archaeal community constituted by methanogens, sulphur-oxidiser, acidophilic and halotolerant Archaea is interesting because it seems to not reflect the extreme cold environments that we have studied. In particular, the thermophilic Archaea retrieved during these analyses could lead to consider the peculiar environments from a

different prospective as the detection of these sequences were related to DNA that is preserved in the brine and permafrost (Willerslev *et al*., 2003, 2004). This DNA may appear as fossil record that could belong to microorganisms that were present geological time ago before the formation of permafrost and brine. A huge deposit of methane and sulphur in permafrost is a potential source of ancient methane. This methane occurs in discrete layers and in discontinuities distribution that favours anaerobic microbial activity and indicates that methane is present in a bound non diffusible form (Rivkina *et al*., 2002). Beside methane, these microorganisms are also preserved in the permafrost (Rivkina *et al*., 1998). In conclusion, permafrost and brine are a huge reservoir of biogenic methane and other compounds that are probably excluded from biogeochemical circulation. Furthermore, one can expect that upon permafrost thawing, the paleomicrobial community will be actively reinvolved in present-day biogeochemical processes. This also includes production of greenhouse gases due to accessibility of organic matter or oxidation of buried methane by CH_4-oxidizing bacteria. Further investigations are clearly needed in this regard.

Culturable bacteria

Tarn Flat

The brine system is an extreme environment, both for the physical characteristics (e.g. temperature, salinity, pH) and for absence of free water. This cryogenic habitat is similar to Mars system for dry and cold characteristics and for high salinity. One aim of this work was to isolate heterotrophic bacterial strains able to live in this cryo-environment and analyze the viable bacteria community to understand the life in extreme environment, that is poor studied, and investigate the biotechnological potential of cold-adapted enzymes in brine samples. The samples were collected from Tarn Flat frost mound within

a borehole. The brine samples were collected at 3.95 and 4.5 m of depth. Tarn Flat is an inland lake, about 35 km from sea, located in a basin and in the proximity of Mount Gerlache. This lake is behind two glaciers, is an ice-free area rich in shallow lakes and ponds. This is influenced by strong katabatic wind events, that blowing seaward from the plateau well-mix the unfrozen lakes during summer (Cremisini *et al*., 1991a, 1991b; Gragnani and Torcini, 1992; Gasparon *et al*., 2001). Abollino *et al*. (1996) analyzed the mineral component of a Tarn Flat Lake and found the high presence of Sodium (Na), Calcium (Ca) and Potassium (K) and presence of Nickel (Ni), Copper (Cu) and Iron (Fe), metals that are essential for life and could be absorbed by algae or other organisms. The Cadmium (Cd) concentration was very low (< 0.02 mg kg^{-1}) and this indicates the absence of anthropogenic contamination (Malandrino *et al*., 2009). The high presence of other heavy metals indicates that the lake sediments are a collector of snow meltwater, which transport the particles from the leached area. Sodium partly derived from a marine source and this element is present at concentration higher than typical values for soils also in Tarn Flat, which is relatively far from Ross Sea (Malandrino *et al*., 2009), and this is probably confirmed by the reached marine salt to the soils (Claridge and Campbell, 1977) and to the sodium that might reach the polar plateau from the tropical oceans and part of the global air circulation (Malandrino *et al*., 2009). The Tarn Flat lake is a closed system, due the winds that leave the sea-spray particle due to this proximity. The temperatures that are present in Antarctic area allow the formation of permafrost sediments, in which liquid brine pocket could be formed due the thaw and frozen of sediments. The formed liquid is constituted of metals and minerals extruded from sediment during the frozen state. Although Tarn Flat is an inland area, due to the frost mound formation, this system is an open system and the brine pockets could be influenced by the permafrost soils and the bottom sediments. The sediments in the carrots were silty-sand with same organic material and a layer rich of debris. The salinity between the two brine pockets was diversified. The first brine was saltier than the second brine and the pH was also different.

To recovery microorganisms and evaluate the differences existing between the two different layers in terms of microbial viable bacteria, two different isolation media were used: TSA rich medium (containing peptone, yeast extract) at different concentrations (from high to low nutrient presence) and a R2A basal -medium at low concentration (10 % and 20 %). The different medium concentration was used to highlight the possible variation about phylogenetic diversity of strains and for the abundance of microorganisms. The rich medium was the best method for recovery microorganisms and for the diversity of strains, while the basal medium was able to recovery few isolates, only belonging to a single genus. The CFU range was 10^3 CFU mL^{-1} on TSA medium in each concentration, while the CFU range in R2A medium was 10^1. This CFU range was found, even, in Greenland Glacier ice (10^2; Miteva *et al*., 2004), in High Arctic Glacier Ice (10^2-10^3; Skidmore *et al*., 2000) and in Glacier cryoconite ice (10^4; Christner *et al*., 2003). In brine samples Gilichinsky *et al*. (2003) counts CFU abundance in range as 10^2-10^5 CFU mL^{-1} (Siberian brine), while in mineral medium with glucose as carbon source the counts was 10^2 CFU mL^{-1} (Gilichinsky *et al*., 2005). This low abundance of microorganisms could be due to the insoluble nutrient particles that remain trapped into sediments or in ice. Furthermore, the cultivation methods are to be considered difficult from this environment and is it not surprising that obtaining isolates by direct plating from frozen samples has been especially challenging. This very low cultivability has been attributed to the extreme conditions in these environments, where cells may be dormant, damaged or simply not capable of growth on the media used. In accordance with our results, reports of successful recovery of isolates from frozen environments indicate that most researchers used typically low nutrient media such as R2A or diluted rich media, as well as several months long incubations at relatively low temperatures (Christner, 2002a; Christner *et al*., 2003b; Zhang *et al*., 2002; Miteva *et al*., 2004; Xiang *et al*., 2005). TSA_1 was the best method for the recovery of isolates from TF5, while TSA_{50} was the best medium for TF4. However, the diluted rich medium allows to recovery isolates

phylogenetically diversified respect to the basal medium and concentrated rich medium. Some genera were recovered only from TSA_1 (e.g. *Kocuria*, *Leifsonia*, *Aeromicrobium* and *Devosia*), while *Rhodobacter* was exclusive for TSA_{50} and TSA_{100}. The genus *Marinobacter* preferred the oligo-medium ($R2A_{10}$). The phyla represented were *Proteobacteria*, with *Alpha-* and *Gamma-*, followed by *Actinobacteria* and *Firmicutes*. The percentage of phyla changes between TF4 and TF5. In the first sample the *Gammaproteobacteria* was followed by *Firmicutes*, *Actinobacteria* and *Alpha-*, while in the second one *Gammaproteobacteria* was followed by *Actinobacteria*, *Alpha-* and *Firmicutes*. The diversity of strains was different from permafrost samples, which presented *Actinobacteria* and *Firmicutes*, followed by *Proteobacteria*. The members of *Gammaproteobacteria* are temperature-adapted (Scott *et al*., 2006). A total of 79 strains were isolated from the sample TF5 and they mainly belonged to *Gammaproteobacteria, Actinobacteria* and *Alphaproteobacteria*, while in TF4 the *Gammaproteobacteria* were predominant and followed by *Firmicutes* and *Actinobacteria*. Most isolates from permafrost were *Firmicutes*, *Actinobacteria, Bacteroidetes* and *Proteobacteria*. Their percentage in brine samples is different because there is a predominance of *Proteobacteria* compared to the permafrost soils. The occurrence of these phyla suggests that they are equipped for cold survival and that are also capable of revival and growth in culture media. The ability of many members of *Firmicutes* to form spores may be one reason for the high representation of this phylum in culture (Steven *et al*., 2008; Niederberger *et al*., 2009). In addition, some members of the *Actinobacteria* are known to adapt to stress by entering a dormant, metabolically quiescent but viable state (Groves *et al*., 2010). Furthermore, *Actinobacteria* have a high GC content (65-69 %) that may help to reduce DNA damage to cold temperature and to high salt concentration (Johnson *et al*., 2007), while the sub-phylum *Gammaproteobacteria* is predominant in sediment samples (Arnosti *et al*., 2008).

The strains were subdivided into 18 distinct phylotypes, mainly isolated from diluted medium TSA_1 and TSA_{50}. Diluted media allowed isolating *Pseudomonas* sp. (OTU9) among *Gammaproteobacteria*, *Devosiapsychrophila* (OTU3) among *Alphaproteobacteria* and *Kocuria* sp. (isolate TF4-15), *Rhodoglobus* sp. (isolate TF5-20A) and *Rothia* sp. (isolate TF5-35B) among *Actinobacteria*. *Marinobacter* sp. (OTU6) among *Gammaproteobacteria* was isolated from R2A.

Among the TF4 isolates, the predominant genus was *Psychrobacter.* This genus was isolated from cold and warm regions and are strictly aerobic and probably for this it was better represented in brine samples. Sequences were isolated from sea-ice and icy coastal seawater (Bowman *et al*., 1997a; Bowman *et al*., 1997b; Bozal *et al*., 2003; Brinkmeyer *et al*., 2003; Yumoto *et al*., 2003), and have also isolated from ancient glacial and ice sheet cores (Christner *et al*., 2003), in super cooled water brine lenses in permafrost derived from ancient marine layers of the Artic Ocean (Vishnivetskaya *et al*., 2000; Gilichinsky *et al*., 2003), and accreted ice at the base of deep ice cores above seawater (Bowman *et al*., 1997a). In these apparently totally frozen locations, liquid can still occur at temperatures down to –10 to –15 °C owing to the high salt (as high as 170–300 practical salinity units [psu]) concentrated by freezing. Carbon sources have been shown to be metabolized at –15 °C in such places, and thus these exceptionally extreme environments have been suggested as useful models for astrobiology (Gilichinsky *et al*., 2003). These genera can tolerate a wide range of salt concentrations. *Marinobacter* spp. and *Psychrobacter* spp. affiliation were similar to strains previously reported from Lake Vida brine (Murray *et al*., 2012; Mondino *et al*., 2009), in which these genera have been repeatedly cultivated. Further, these genera are well known for inhabiting cold and salty environments (Gilichinsky *et al*., 2005; Zhang *et al*., 2008; Ayala-del-Rio *et al*., 2010).

The species *Carnobacterium alterfunditum* (OTU2) were found only in TF4. This bacterium was isolated from anaerobic monimolimnion of Ace Lake in Antarctica (Franzmann *et al*., 1991). *Carnobacterium* spp. appear to have both the temperate and

polar aquatic environments as habitats including Antarctic lakes (Franzmann *et al*., 1991; Bratina *et al*., 1998), and Arctic and Antarctic seawater as well as the deep sea (Galkin *et al*., 1999; Groudieva *et al*., 2004; Newberry *et al*., 2004; Toffin *et al*., 2004; Lauro *et al*., 2007). Furthermore it was found in terrestrial environments, including permafrost ice (Pikuta *et al*., 2005). *Kocuria* sp. was isolated from Antarctic environments (Kuhn *et al*., 2014) and in Canadian High Arctic permafrost (Steven *et al*., 2007a). This aerobic bacterium exhibits tolerance to high salt concentrations (Stackebrandt *et al*., 1995; Kovàcs *et al*., 1999). The isolate **TF4-125** was affiliated to *Planococcus antarcticus* and was found just in TF4. This bacterium is aerobic and it was isolated from cyanobacterial mat samples in Antarctica. This presence could be due to frozen algae present in the upper layer of TF4.

In TF5 there were isolates belonging to three different genera detected only in this brine and on diluted TSA medium. The sequences were related to *Devosia psychrophila* (OTU3) among *Alphaproteobacteria*, *Rhodoglobus* sp. (isolate **TF5-20A**) and *Rothia* sp. (**TF5-35B**) among *Actinobacteria*. The genus *Devosia* (aerobic bacteria) was isolated from other extreme environments, such as alpine glacier cryoconite (Zhang *et al*., 2011) and digestive tracts of Antarctic krills. The genus *Rhodoglobus* had a polar distribution as it has been found in Wright Valley, Antarctica (Reddy *et al*., 2003), in Antarctic sea ice and in the Arctic (Steven *et al.,* 2008). The genus *Rothia* was isolated from deep ocean and from air samples in Russia space station Mir (Li *et al*., 2004), another extreme and cold environment. In conclusion, probably the diluted medium could be useful to detect more phylogenetic groups, while the rich medium could be useful for the recovery of more abundant bacteria but less diversified. Furthermore, all isolates were closely related to sequences from Antarctic and Arctic environments, like glaciers or sediments. This means that there was less contamination during collecting samples and that brine pockets, in Tarn Flat area, remain unaltered respect the possibly interconnection with atmosphere and with other permafrost layer during upwelling of brines layer during sampling.

The isolates were screened for physiological analyses (temperature, pH and salinity ranges), capability to hydrolyse some substrates, ability to tolerate heavy metals and antibiotics and degrading ability towards pollutants.

These abilities could be important by a biotechnological point of view. Antarctic bacteria growing in cold and extreme environments improve their adaptations to these habitats and survive with other organisms under poor nutrient and water availability. The adaptation could be made to produce some compounds, e.g. EPS, as cryo-protectants, or antibiotic molecules, useful in biotechnology.

Brine isolates were primarily cold-adapted and only few representatives were mesophilic or thermophilic (Steven *et al*., 2006; Steven *et al*., 2009; Gilichinsky *et al*., 2002). Growth at 37 °C was rarely reported (Hinsa-Leisure *et al*., 2010). Steven *et al*. (2007b) reported a tree-fold higher amount of viable cells growing at 5 °C compared to viable counts at 25 °C. In our study, the microorganisms are able to grow at 4, 15 and 25 °C. The growth was better at 4 than 15 °C and only a *Marinobacter* genera was able to grow only at 4 °C.

Permafrost and brine microorganisms tend to be more halo-tolerant than organisms from the overlaying active layer (Steven *et al*., 2009; Steven, 2008), which is seen as a microbial survival strategy in environments with low water activity, such permafrost, where little water is bioavailable (Franks *et al*., 2003). Tolerance to 7 % NaCl (Steven *et al*., 2007a) or 8 % NaCl (Hinsa-Leisure *et al*., 2010) has been reported. In our study salt tolerance was diversified between the two brine samples. In the FTMF 4m (higher salty brine) most isolates were able to growth up to 19 % NaCl (affiliated to *Psychrobacter*, more halo-tolerant bacteria) and 7 % NaCl (Steven *et al*., 2007a). In TF5, the isolates were able to grow in range between 3 to 7 % NaCl, thus the 4m isolates were more halo-tolerant respect the 5m isolates. Permafrost bacteria are resistant to a wide range of antibiotics combined to the presence of mobile genetic elements (Petrova *et al*., 2011), which might be part of generalized bacterial response to stress conditions (Margesin *et*

al., 2011). Metagenomic analysis of ancient DNA from 30,000 years old Beringian permafrost sediments demonstrated the presence of highly diverse collection of genes encoding resistance to Beta-lactam, tetracycline and glycopeptide antibiotics (D'Costa *et al*., 2011). The strains investigated in our study were not resistant to all antibiotics, only few of them were resistant to kanamycin (aminoglycoside bacteriocidal antibiotic, 350 ppm), most of them were resistant to ampicillin (β-lactam antibiotic, 100 ppm) and two strains affiliated to *Leifsonia* were able to tolerate five antibiotics. In general, the strains demonstrated a diversified sensitivity toward antibiotics. Segawa *et al*. (2012) reported that bacteria isolated from the Antarctic region have low resistance to antibiotics. In a similar study on bacteria isolated from the bottom sediments in the Wijdefjorden, Spitsbergen, Konieczna *et al*. (2011) observed that most of the bacterial isolates were sensitive to antibiotics such as cefoperazone, piperacillin, gentamycin, tobramycin, ciprofloxacin and ofloxacin. The antibiotic resistance in uncontaminated environments, such as Antarctic habitats, could be attributed to airborne bacteria and migration birds that allow the genomic transfer to antibiotic resistance (Segawa *et al*., 2012; Sjolund *et al*., 2008).

Strains isolated from brine samples were tested for their ability to oxidize hydrocarbons using a first preliminary test with solid and liquid hydrocarbons. Most of them were able to grow in mineral agar medium with crude oil and diesel oil as a carbon source. The test was carried out at 4 °C for evaluating this ability at low temperature. The biodegradation of many compounds of petroleum has been reported in a variety of terrestrial and marine cold ecosystems, including Arctic (Braddock *et al*., 1997), alpine (Margesin, 2000) and Antarctic soils (Aislabie *et al*., 1998). Cold bacteria adapted theirselves rapidly to the contamination. This screening was carried out for analysing the possible biotechnological role of bacteria that live in extreme habitats. In TF4 26 and 12 strains were able to use diesel oil and crude oil, respectively, and they were mainly affiliated to *Psychrobacter* sp. within OTU10. In TF5 only 12 and five were able to grow in the presence of diesel oil

and crude oil, respectively, and they were mainly affiliated to *Rhodobacter* sp., and grouped in the same OTU. Anthropogenic-related factors may also influence the distribution of *Psychrobacter*. For example, *Psychrobacter* enrichment has been noted in aquatic environments contaminated with hydrocarbons and they have emerged as hydrocarbon-degrading bacteria in Antarctic, Arctic and Indonesia seawater (Harwati *et al*., 2007; Prabagaran *et al*., 2007; Lo Giudice *et al*., 2010; Gerdes *et al*., 2005). Deppe *et al*. (2005) investigated on crude oil degradation from psychrotolerant bacteria, and the genus *Psychrobacter* was able to degrade in consortia with other microorganisms.

Aerobic degradation of polychlorinated biphenyls (PCBs) was investigated and eight strains from TF5 were able to use Aroclor 1242 as carbon source at 4 and 15 °C. The pre-screening was carried out on all strains from 4m and 5m using biphenyls as carbon source. Positive strains from this screening were used for a batch culture at two temperatures to evaluate the degrading capability on a mixtures of PCBs as carbon source. To confirm the biodegradation capability of our isolates, analyses were carried out at genetic level. The *bph* gene is organized as an operon that presents, in the order, *bphA1A2A3A4-bphB-bphC-bphD*. This internal organization is maintained essentially identical in many strains, although it can have deletions of some parts (Hayase *et al*., 1990). There are also microorganisms with gene cluster *bph* dissimilar, for organization, number of genes and sequences (Peloquin and Greer, 1993; Arai *et al*., 1999; Mouz *et al*., 1999). The *bph*A gene portion encodes for the first fundamental step in the biphenyl upper pathway, in which biphenyl is converted to dihydrodiol and further to CBA. The presence of the bacterial *bph*A gene, confirming the ability to aerobically degrade PCBs, was tested by PCR with a previously published primer set (Master and Mohn, 2001). The *bph*A gene was detected in four strains (out of 223; 1.8 %) related to *Rhodobacter* sp., thus indicating the possible low PCB presence in the brines samples. The biodegradation of certain, usually less chlorinated, PCB congeners has been reported for several microorganisms elsewhere in the world. However cold-adapted PCB-degraders have been

rarely isolated from cold environments, such as Arctic soils (Mohn *et al*., 1997; Master and Mohn, 1998; Papale *et al.,* 2017) and Antarctic seawater and sediments (Yakimov *et al*., 1999, Michaud *et al.*, 2007; Lo Giudice *et al*., 2013). Lo Giudice *et al*. (2012) found *Psychrobacter* species strongly related to PCB-degrading bacteria that were isolated from Antarctic seawater (Yakimov *et al*., 1999; Michaud *et al*., 2007), this bacteria could be autochthonous and shared between water and sediments. The ability to degrade PCBs could be due to the possibile air transport. The deposition of pollutants probably odccurred during time in sediments and they were includes in brines during the thaw/frozen cycles.

Also heavy metals (HMs) constitute a serious threat to natural communities and ecosystems, because of their toxicity to most organisms (Martins *et al*., 2014). High concentrations of HMs are always toxic but bacteria have been able to evolve different resistance mechanisms, and thus persist in contaminated environments (Martins *et al*., 2014). Many HMs are physiologically necessary for bacterial life, but it depends on their concentrations. HMs are commonly released into the environment over manifold human activities. In our study the tolerance was high towards nickel and copper, low towards mercury and cobalt, and absent towards cadmium. In TF4 the strains were able to tolerate nickel up to 5000 ppm, even if the majority of isolates tolerated such metal up to 1000 ppm. Tolerant strains were mainly affiliated to *Psychrobacter* spp. In general *Psychrobacter* genus was able to tolerate more HMs (copper, nickel, cobalt and mercury). In TF5 the HMs were less tolerated. In fact, the maximum tolerated concentration was 500 ppm for nickel and copper and few strains tolerated nickel up to 1000 ppm. The strains were mainly affiliated to *Pseudomonas* spp. and *Rhodobacter* spp. Tarn Flat area is not influenced by sea, and the anthropogenic impact is limited in this isolated area. The heavy metal occurrence could be explained for the presence of rock and sediments, containing high concentrations of copper, nickel or uranium (e.g. fluorite, muscovite and biotite), which are more easily weathered. The nickel and copper tolerance by bacteria

inhabiting brines could be explained by the presence of these heavy metals in sediments, as reported before, that are released in brines. Thus bacteria, being in continuous contact with them, could became able to tolerate them. The cadmium assay gave a negative response. This is probably dependent of the absence of this metal in Tarn Flat sediments.

With regard to bacteria involved in the biodegradation of organic pollutants, they often belonged to the genera *Pseudomonas*, *Comamonas* or *Acinetobacter* (Kupka and Sevcik, 1995; Barberio and Fani, 1998; Lyamlouli *et al*., 2011), all of these being Gram-negative bacteria. However, in environments contaminated not only with organic pollutants but also with heavy metals, species diversity and metabolic activities of the microorganisms are reduced, and the metal-tolerant bacterial populations are developed (Knotek *et al*., 2003) with species of *Pseudomonas* and/or acidophilic bacteria that become predominant (Babich and Stotzky, 1985; Dopson *et al*., 2003).

Exopolysaccharide (EPS) production was tested for 151 strains isolated from Tarn Flat brines. Three strains from Tarn Flat (two from 4m and one from 5m, affiliated to *Psychrobacter* and *Pseudomonas*, respectively) resulted positive to the preliminary screening. The positive isolates were grown in batch culture to analyse the EPS characteristics. In extreme environments, under cold temperatures, bacteria could produce some exopolysaccharide compounds that can have a cryo-protective role, or to protect theirselves from pollutants or other bacteria. The production of exopolysaccharide (EPS) by bacteria in natural systems has been described as a strategy for growth (Costerton, 1999). Numerous studies provide the EPS production by bacteria from Antarctic seawater and from ice (Nichols *et al*., 2004; Krembs and Engel, 2001; Krembs *et al*., 2002). These authors suggest that high concentrations of EPS in the brine channels may provide buffering against harsh winter conditions and high salinity and cryo-protect the microbes living there against ice-crystal formation. Biotechnological uses for microbial produced EPS include environmental, clinical, nutritional and cosmetic applications, to name a few (Gutnick and Bach, 2000; Sutherland, 2001). Increased knowledge of the role of

Antarctic bacterial EPS will also provide insight into possible commercial uses for these novel polymers. The EPS production by *Psychrobacter* and *Pseudomonas* is binds to their halotolerant characteristics and for the ability to grow and adapt theirselves to cold temperature.

The analyses carried out on these strains inhabiting permafrost and brine samples and living in extreme conditions (e.g. temperature) allow the adaptation of bacteria and their enzyme that could be extracted and used for biotechnological potential. The potential of new biocompounds from Antarctic brine and permafrost bacteria could be useful also to understand the extraterrestrial potential from Mars. Furthermore, new compounds could be used in medicine or food industry for new applications.

Boulder Clay

Boulder clay site is located near the Italian Antarctic Research Station "Mario Zucchelli" on the Terra Bay coasts. This site is ice-free and with south-eastern exposure. Surface features include perennially ice-covered ponds with ice blisters and frost mounds (Guglielmin *et al*. 1997; French and Guglielmin, 1999; 2000). The vegetation is very scarce and the borehole is devoid by vegetation. The sediments of Boulder Clay probably have a glacio-marine origin dated to Late Pleistocene (Orombelli *et al*., 1991). Knowledge of microbial life existing in ice does not only improve our understanding of the taxonomic diversity, richness and biogeography of cold-adapted microorganisms, but also assists in evaluating the metabolic requirements for survival and proliferation of life in the cryosphere, and in defining the actual limits of life. Boulder Clay have been studied for their isotopic composition, mechanisms of ice distribution, geological formation bacterial composition (Guglielmin *et al*., 1997; Gragnani *et al*., 1998; French and Guglielmin, 1999; Guglielmin and French, 2004; Abramovich *et al*., 2012). However, it is unknown for bacterial activity and composition on Boulder Clay brines. The samples were collected through a borehole at 2.5 m and 2.0 m, the salinity was very low and the

pH was between 7.6 and 8.7, neutral-alkaline brines. A rich media diluted in nutrient composition, an oligo-medium diluted and a selective media (DMSZ 97) were used. The rich medium (TSA) allows to yielded colonies, while no colonies were recovered from the oligo-medium (R2A). TSA medium was used at different nutrient concentrations, from 100 % to 1 % of nutrient, and the colony forming units (CFU) were higher in diluted medium than rich medium. BC1 was the sample from which the maximum number of colonies grew, while BC2 was the sample that allowed the recovery of the lowest number of colonies (two). BC1 allowed to isolate more colonies respect the other samples, mainly from diluted medium. Most bacteria isolated were closely related to species retrieved in Antarctic and Arctic environments. The consistent isolation of related microbes from such geographically diverse frozen environments suggests that these species may indeed have features that confer resistance to freezing and extended survival under frozen conditions.

Gammaproteobacteria, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* composed the viable community of Boulder Clay. BC1 was more heterogeneous than BC3 with genera affiliated to *Pseudomonas*, *Carnobacterium*, *Gelidibacter* and *Staphylococcus*. Furthermore, the samples were different in number of isolates. Only two strains were recovered from BC2. They were enable to growth in agar medium and are affiliated to *Flavobacterium* sp. (belonging to *Bacteroidetes*). Only eight strains, mainly affiliated to *Psychrobacter*, one *Pseudomonas* and two unidentified with a green morphological aspect were recovered from BC3.

Gammaproteobacteria were the predominant phylum. They were also abundant in several other polar areas, including the Arctic (Junge *et al*., 2002; Brinkmeyer *et al*., 2003) and Antarctic sea ice (Bowman *et al*., 1997; Brinkmeyer *et al*., 2003) and the water column of Lake Bonney (Glatz *et al*., 2006). These data suggest that certain *Gammaproteobacteria* (e.g. *Colwellia* and *Marinobacter*) are common among these habitats. Furthermore, this

phylum was retrieved in diluted media. Probably, its members have adapted to resist to extreme environment and low nutrient concentrations.

Firmicutes retrieved in brine samples selected this environment for their adaptive characteristics. *Firmicutes* are sometimes able to produce spores to resist under permanently frozen conditions. Conversely, *Actinobacteria* isolates were retrieved in cryo-extreme environment, normally they are at higher percentages in active layer than permafrost table and often dominate such habitats thanks to the G-C high percentage that allows them to resist under low temperature and water availability conditions. Isolates were found in ice formation (Antarctic soil, Siberian permafrost, Greenland glaciers, cryoconite holes, sea ice, ice cover, etc.) ecosystems. Some show metabolic activity at sub-zero temperatures (Christner, 2002; Katayama *et al*., 2009) and also may be protected in the permafrost environment by cyst-like resting forms as observed in *Arthrobacter* and *Micrococcus* species (Soina *et al*., 2004).

Bacteroidetes were found in BC2 and BC1 samples. Their presence could be inputed to the remineralisation processes in ice system. This phylum is correlated to organic matter presence, probably due to low or absent salinity of the brines or to the presence of carbon that could be inside.

In particular, retrieved isolates were closely related to bacterial species associated with sub-glacial environments. For example a high number of *Pseudomonas* sp. from BC1 were found to be closely related to *Pseudomonas* recovered from Taylor Glacier in Antarctica (Mikucki and Priscu, 2007). In BC1 *Carnobacterium* sp. (belonging to *Firmicutes*), *Shewanella antarctica* (*Gammaproteobacteria*) and *Gelidibacter* sp. (*Bacteroidetes*) were retrieved. All of these strains were closely related to Antarctic and Arctic isolates.

73 strains isolated from Boulder Clay brines were tested for physiological characterization, as growth temperature and salinity, and for biochemical activity and biotechnological potential. As it is typical for permafrost isolates, none of the isolates was

psychrophiles as they were able to grow until +25 °C. They are probably cold-adapted microorganisms which may possibly be metabolically active at permafrost temperature, under 0 °C. Only half of the characterized isolates also showed a high salt tolerance, another common feature among permafrost isolates (Gilichinsky *et al*., 2002a, b; Bakermans et al., 2003). This result is indicative of adaptation to low water activity, in permafrost environments, where small amounts of salty, unfrozen water are present along mineral surfaces, which provides a path for substrates and product diffusion (Rodrigues and Tiedje, 2008).

The strains recovered from Boulder Clay were unable to oxidize pollutants. Only four *Psychrobacter* isolates from BC3 were able to growth in crude oil presence on agar plate but not in liquid enrichment. Strains were able to grow in nickel and copper presence (up to 500 ppm). These metals are essential for life, but probably within the brines there are algae or other organisms that unabsorbed HMs and avoid the accumulation in the natural environment. In BC there isn't more vegetation and are most rocks or permafrost.

The PCB degradation was observed only in two strains belonging to *Psychrobacter* sp. (BC3-97) and *Staphylococcus* sp. (BC1 bis-71) isolated from BC1 and BC3, respectively. These genera have been already reported as PCB degraders (Leaeset *et al*., 2006; Michaud *et al*., 2007). Low PCB degradation could be explained by the low presence of such pollutants in the environments. The strains related to *Staphylococcus* sp. (BC1 bis-71) and *Psychrobacter* sp. (BC3-97) had high biodegradation potential, even if they did not seem to possess the catabolic gene. The gene or cluster of genes coding enzymes responsible for the PCB degradation activity is probably plasmid-harbored or present on transposons (Furukawa *et al*., 2004). These strains were tested for the degradation efficiency ability and were able to degrade at higher percentage (up to 70 %) the congener 1,1'biphenil 2,2'-dichloro both 4 and 15 °C. This molecule was the simplest of the Aroclor1242 mixture, with two chlorides that are simple to eliminate. The degradation was better at 15 °C, *Staphylococcus* and *Psychrobacter* strains probably present the

degradation pathway, even if not present the gene encoding for the first portion of *bph* gene. Another explanation could be that gene are plasmid-harboured.

Four strains (BC1 bis-18, BC1 bis-19, BC1 bis-22 and BC1-139) seems to be able to produce exopolysaccharide (EPSs). In particular BC1-139 related to *Pseudomonas* sp. was assayed for carbohydrate contents in EPS production and this strain resulted as a possible EPS producer. Production of extracellular polymeric substance (EPS), primarily composed of polysaccharides, is generally a survival mechanism for many mesophiles as well as Antarctic marine bacteria (Nichols *et al*., 2004). Bacteria produce EPS as either capsules, biofilms or mucoid secretions in the extracellular environment, helping them to cope with adverse environmental conditions (Looijesteijn *et al*., 2001; Nichols *et al*., 2005a,b,c; Omar *et al*., 1983; O'Neill *et al*., 1986; Steinmetz *et al*., 2000; Uhlinger and White, 1983). Panicker *et al*. (2006) reported the presence and the nature of EPS in an Antarctic soil bacterium, *Pseudomonas* sp. 30-3, at various temperatures. Also, the secretion of EPS leading to an increased aggregation of cells in response to cold temperatures may help to understand physiological changes involved in the adaptation of this microorganism to the extremely cold and dry Antarctic environment. In particular, the strains BC1-139 seems to produce high amount of carbohydrate respect the other strains, maybe because it is better adapted to the cold temperature than the others.

Conclusions

This thesis allowed to compare extreme habitats, such as Antarctic permafrost and brines, using culture-dependent and culture-independent approaches. The use of different approaches was helpful to have a more complete profile of the present situation in these environments and to understand the past matters through more detailed analyses. The interest has shifted in such cold environment to understand if it is possible to find microorganisms in extreme environments that have evolved specific characteristics, which are of potential interest for biotechnology. Obviously, the total community analysis was useful to try to understand the possible implications that had led at these final situations and how the microorganisms changes according to the environment.

These extreme environments select the prokaryotic community. Overall, the prokaryotic community structure varies from the environments. Two sites were compared, one inland and one coastal site, among brine samples, which have different characteristics with respect to lithology age and atmospheric characteristics. The community contains culturable, non-culturable, viable but not culturable and dead cells. The community of viable cells was affected by low temperature (under 0 °C) because it could made degraded cells or part cells. This did not compromise the total community structure, rather, it preserved the DNA which contributed to the total microbial studies. Different factors influence the successful recovery of microorganisms, such as low temperature enrichment strategies and media composition. The natural enrichment at 4 °C resulted in enhanced recovery of permafrost bacteria with high diversity and high abundance of bacteria. Anyhow the bacterial diversity depends from rich or oligo media and type of enrichments. These extreme environments, such as permafrost or brines pockets, could be useful to contribute to information to better understand how global ecological changes may impact communities. All this, also in relation to the presence of brine veins, suggests the possibility that there are living microorganisms in this peculiar niche. In particular,

the detection of viable microorganisms provides new information to search life in other cold habitats, in order to understand the possible life strategies for adaptation in cold environments. In addition, the study of phylogenetic affiliations in permafrost provide a specific targets to astrobiology for analyze in deeper the biotechnological potential of bacteria recovered from permafrost and brines. The culturable bacterial numbers were different among the sites. In particular, the deepest samples recovery most isolated than the others samples. The total community composition was different between the two sites, in particular TF4 and TF5 were quite similar maybe because they were collected in the same borehole with similar physico-chemical characteristics, whereas in BC the site reflected the different sampling situations: BC1, which was the deepest among BC samples, varied in total community assemblage from TF and the other site BC; BC2 was the shallowest sample and BC3 was collected in another lake, thus they are no well comparable respect TF samples that are similar in composition regarding Bacteria and Archaea.

For the culturable strains, even there the TF5 (the deepest) recovered 79 isolates, quite similar to TF4 (72), while only two isolates were recovered from BC2 (the shallowest sample), 63 strains from BC1 and only eight from BC3. The phylogenetic affiliation was different among the sites. These could be explained because the characteristics of the samples in the deepest remain constant respect the shallowest that continually undergoes the atmospheric agents. Inside the genera, the OTU representatives of such genera were quite different between Tarn Flat and Boulder Clay. In fact different OTUs were retrieved such as OTU6, OTU13 and OTU3 that were recovered only in Tarn Flat samples, while the isolate from the other phylogenetic genera were similar in both samples. For example, isolates from OTU9 (from Tarn Flat) were similar to isolates from OTU24, affiliated to *Pseudomonas* sp. with a similarity of 99 %. These sequences were phylogenetic similar to isolates from Taylor glacier in Antarctica. Instead, isolates from OTU10 (from Tarn Flat) and OTU27 (from Boulder Clay) affiliated to *Psychrobacter* spp. had a similarity over 98

% and sequences were also obtained for Arctic fjord. Finally, other isolates with high similarity were within OTU2 (Tarn Flat) and OTU22 (Boulder Clay) affiliated to *Carnobacterium* sp. The sequences were shared between the two sites. This could be possible because these extreme environments select the organisms due to the low temperature, water and nutrient availability. Furthermore, these isolates were the most abundant in the samples, such as *Psychrobacter* and *Carnobacterium* genera, and probably the more adapted. The antibiotics and heavy metals tolerance response was similar in the samples with low tolerance for all antibiotics and high tolerance for HMs, such as nickel until high concentrations. Another similar response was the hemolysis that was positive in *Carnobacterium* sp. retrieved from both samples. The comparable response were explained for the similarity of extreme environments, especially for the depth of samples and for the similar physics characteristics. Despite the similar isolates in the samples they responded in different way to the physiological tests. For example, the highest salinity value that was tolerated by Tarn Flat isolates was 5-9 % of NaCl, whereas it was 0-3 % of NaCl for Boulder Clay. The high tolerance to NaCl in Tarn Flat, respect to Boulder Clay, is explained for the different salinity of the samples. In fact, the isolates adapt theirselves to higher salinity (16.6-19.9 mS cm^{-1}) in TF than BC (0.2-3.6 mS cm^{-1}).

The strains isolated from these peculiar environments are not able to produce particular molecules useful in biotechnological applications. In particular the strains isolated from BC1 were generally more efficient than TF strains. This particular contraposition may be due to the open system in BC1 that allows bacteria to adapt theirselves to the continuous changes, respect to TF strains that are probably under the same conditions for long time.

In conclusion, the brines within permafrost make up the only habitat on Earth that is characterized by under zero temperature, high salinity, and low external influence in geological time. These environments represent the terrestrial model of exobiological

niches with their unique halotolerant and aerobic psychrophilic community, which is a possible model for extraterrestrial life.

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