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LONG-RANGE TRANSPORT OF BACTERIAL COMMUNITIES: IMPACT OF SAHARAN DUST STORMS ON LOCAL PARTICULATE MATTER AND MOUNTAIN ENVIRONMENTS

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Ai miei genitori, che mi hanno

donato la vita la prima volta,

a Giuseppe e Ginevra,

che me ne hanno regalata

una seconda.

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CHAPTER 1

INTRODUCTION

1.1 Microorganisms in the environment: ecological significance and biogeography

Microorganisms are the first living being to appear on Earth about three billion and half years ago. They were the only living forms on Earth before plants and animal evolution and for more than three billions of years they performed on their own, all biogeochemical processes. More complex organisms appeared about 0,6-0,7 billion years ago, but they didn't substitute microorganisms. Indeed, prokaryotes are the only living beings involved in fundamental biogeochemical processes. This is due to their metabolic versatility (provided by their long presence on Earth) and to their huge and quite unknown biodiversity; these qualities let them play a dominant role. Microorganisms have undeniable advantages about growth with respect to other living forms. First of all they are small and for that reason they present a high surface/volume ratio, this allow them to absorb efficiently nutrients from the environment; secondly they can grow very fast thanks to efficient absorption of nutrient, in this way their metabolism is faster than those of any other animal cell. Furthermore microorganisms show extraordinary capability of adaptation to adverse conditions such as aridity, high temperature, radiations, high osmotic pressure and some of them are able to grow without any input from other living organisms, using only inorganic material and solar light as energy and nutrient sources.

A central goal of ecology is to understand how biodiversity is generated and maintained. Spatial patterns of species diversity provide information about the mechanisms that regulate biodiversity and are important for setting conservation priorities. Although spatial patterns have been documented in many studies of plant and animal diversity, such patterns are not as well documented in microbial species (i.e. *Bacteria*, *Archaea*, and microscopic *Eukarya*). This is a serious omission given that microorganisms could comprise much of the biodiversity on Earth and have crucial roles in biogeochemical cycling and ecosystem functioning. Biogeography is the descriptive and explanatory study of spatial patterns and processes in the distribution of biodiversity. Nowadays is almost known that microorganisms are everywhere in the Earth's surface, but until recently microbial biogeography hasn't represented a field of study (O'Malley et al. 2008). Bacterial ubiquity represents one of their most striking ecological aspect. They can thrive in an extraordinary wide array of habitats, from those that are ideal for most living organisms to those too extreme to support most life forms (Horner-Devine et al. 2004). Furthermore, there is much of evidence in the literature about prokaryotes having a cosmopolitan distribution. At the domain level, it is known that

Bacteria and *Archea* are globally distributed; at the phylum level, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria* and *Bacteroidetes* showed a widespread distribution in both marine and terrestrial ecosystems. Deepening the look at the genus level, it is also possible to find prokaryotes that are distributed ubiquitously in extremely diverse regions of the planet. For instance, cosmopolitan bacteria may be found in many human, plant and animal pathogens such as *E. coli*, *S. pneumoniae* as well as spore-forming bacteria as *Bacillus mojavensis* and *B. subtilis* species. Alternatively, species with more specific ecological niches such as hyperthermophiles inhabiting hydrothermal vent materials or deep sediments, can also survive in dispersed forms along the open ocean, although dormant or metabolically inactive.

As reviewed by Ramette et al. (2007), the generation of biogeographic patterns is explained by the combination of speciation, extinction and dispersal. Within the first process, through the existence of genetic recombination the efficiency of vertical speciation is reduced. Natural selection is thought to subsequently act on the resulting pool of diversity by sorting out genotypes better adapted to the prevailing environmental conditions through periodic selection events. Recombination rates have been found to be low so that they may not alter the integrity of distinct adaptation in different ecotypic lineages. Hence, recombination may not hinder ecologically distinct populations to diverge even further. Although it is generally expected that speciation rates may be high in bacteria because of their large population sizes, the promiscuity of gene transfers, and the breadth of environmental conditions they encounter, little is known about the magnitude of those rates in nature. Dispersal is defined as the movement of populations away from their point of origin. This definition refers not only to a mere transport by a vector between two locations, but also to the colonization of new geographic locations by the emigration taxa. Transport of free-living prokaryotes over large geographic distances may be possible through the combination of favorable climatic factors (winds, storms), disseminating factors (ocean currents, dust, plant seeds, surfaces of human, birds, or insects migrating between regions), and sometimes the existence of survival or dormant stages in bacteria that would enhance their chance to endure long-distance transport. Even though it is universally accepted that microbial transport may be an ongoing process, little is known about the dispersal of free-living bacteria in the environment. About extinction, it is generally accepted that it may be rare in prokatyotes because of their large population sizes, high growth rates and/or the presence of survival or dormant states when facing harsh environmental conditions. However little evidence exist in the scientific literature about how common may such characteristics be in natural populations. Protection

against adverse environmental conditions has yet been observed in *Pseudomonas aeruginosa* that may undergo extensive self-generated diversification within its biofilms. In that process, induced pleiotropic effects could lead to the appearance of genotypes with increased dissemination abilities and greater resistance to environmental stresses. Such biological "insurance" may therefore contribute significantly to the reduction of the extinction risk, especially when colonies are transported further away from their original niches or when local resources have been depleted (Ramette et al. 2007).

In addition to their cosmopolitan distribution and biodiversity, bacteria on the Earth surface show an incredible abundance. As reviewed by Horner-Divine and colleagues (2004), the total number of bacteria on Earth may be as high as 4-6 10^{30}, with the largest proportion of bacterial cells possibly residing in the oceanic and terrestrial subsurfaces. Despite their modest size as individuals, as a group these organisms not only contribute to the flow of nutrients worldwide, but may also constitute a significant proportion of the nutrients in living biomass.

Bacterial biodiversity is unparalleled in the biosphere. It is influenced by environmental variables such as temperature, nutrient status, salinity, plant species present in the diverse habitats and contaminations due to the presence of chemical substances, i.e. pollutants. This habitat heterogeneity can be depending on two major variables: structural heterogeneity, such as discontinuity in space and time, and complexity in resources, conditions and/or interacting populations. To have a complete picture of the total biodiversity within a natural microbial community, it is necessary to determine not only the number and the relative frequency of all species, but also to evaluate the distribution of each individual within the species, which means identify its structure, the number and the relative frequency of strains belonging to the same species.

1.2 Microorganisms in the environment: investigation approaches

The concepts exposed above are undoubtedly general and pertinent to a variety of ecological contexts, not the least in the study of aerobiology, which is the main focus of the present PhD thesis. Nonetheless, in a relatively young subject of scientific research such as aeromicrobiology, it is of crucial importance to investigate in depth the density, diversity and activity of microbial populations.

Microbes play pivotal roles in natural processes, but their diversity and activity in natural environments is still relatively untapped to date, mainly due to the fact that until recently the only way to study them was trough cultivation-based methods. Cultivation-dependent approaches have been intensively used since the pioneering studies and, in spite of their limitations (ca. 1 to 5% of microbes in a given matrix are cultivable in laboratory conditions) they are still considered methods of choice for the isolation and metabolic/physiologic characterization of microbial strains. In addition, cultivation is fundamental for epidemiological and sanitary studies and when an accurate evaluation of microbial viability is needed (Gandolfi et al. 2013). On the contrary, the recent development of culture-independent analyses, based on molecular techniques, brought evident advantages for the study of microbial communities biodiversity.

Typical examples are nucleic acids-based approaches that often rely on the amplification of target DNA sequences by the use of polymerase chain reaction (PCR). The molecular marker most widely used for the assessment of microbial diversity is the gene coding for ribosomal RNA (16S and 18S rRNA for bacteria and fungi, respectively), that are considered like "molecular clock". These genes are characterized by both highly-conserved regions – in which universal primers can anneal – followed by ipervariable regions which provide evidence of taxonomic differentiation. The pool of PCR products can be either cloned and sequenced as such or can undergo further processing according to various genetic profiling methods, e.g. amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (tRFLP), denaturing gradient gel electrophoresis (DGGE) and many others reviewed extensively in Nocker and colleagues (Nocker et al. 2007).

In the last decade, the advent of next generation sequencing (NGS) have deeply revolutionized analysis of complex microbial communities in environmental samples/matrices. Traditional DNA-sequencing protocols (Sanger et al. 1977), in fact, can only sequence specimens individually and, therefore, it is inadequate for processing complex

environmental samples and it is not feasible for large-scale studies. Metagenomic DNA samples often contain mixtures of DNA from hundreds or thousands of individuals. Although conventional sequencing proved to be the most efficient method for the development of large DNA barcode reference libraries, the number of individuals in an environmental sample is beyond its capability. Recovering DNA sequences from the thousands of specimens present in an environmental bulk sample requires the ability to read DNA from multiple templates in parallel; something that next-generation sequencing technologies do effectively. NGS platforms allowed the direct recovery of DNA sequence data from environmental samples. Although available NGS technologies utilize quite diverse chemistry and base incorporation and detection tools, they share two main steps: library fragmentation or amplicon library preparation and detection of the incorporated nucleotides. By comparing sequences thus obtained with reference libraries stored in ever-expanding databases, nearly all taxa present in a given environmental sample can be thoroughly identified with high confidence. Hence, advanced computational methods allow to infer biodiversity measures across time and space by annotating and clustering DNA sequences using a combination of assignment and phylogenetic techniques. In this way, researchers have been able to observe the small changes in community structure that may occur following anthropogenic or natural environmental fluctuations. These alterations, although highly informative of ecosystem health and stability, are not discernible with less sensitive, traditional, molecular tools such as Sanger sequencing. (Shokralla et al. 2012).

1.3 Atmospheric particulate matter.

1.3.1 The Earth's atmosphere

In the most general terms, the atmosphere is divided into lower and upper regions. The lower atmosphere is considered to extend to the top of the stratosphere, up to an altitude of about 50 kilometers. The Earth's atmosphere is characterized by variations of temperature and pressure with height. In fact, the variation of the average temperature profile with altitude is the basis for distinguishing the layers of the atmosphere.

Troposphere is the lowest layer of the atmosphere spans from the Earth's surface up to the tropopause, which is at 10-15 km altitude depending on latitude and time of the year, and it is characterized by temperature decreasing with height. This is due to the strong heating effect at the hearth's surface from the absorption of solar radiation. Since hot air rises, strong vertical mixing occurs in the Troposphere, so that species emitted at the earth's surface can rise to the tropopause, the region separating the troposphere from the stratosphere; this ascent can occur in a few days or more rapidly, depending on the meteorological conditions. Essentially all of the water vapor, clouds, and precipitation are found in the troposphere, which provides an important mechanism for scavenging pollutants from the atmosphere.

Stratosphere extends from the tropopause to the stratopause (located at approximately 45 to 55 km altitude), and temperature generally increases with altitude in this region. Relatively little vertical mixing occurs in the stratosphere while no precipitation scavenging occurs. As a result, massive injections of particles, for example, from volcanic eruptions, can produce layers of particles in the stratosphere that persist for long periods.

Mesosphere spans from the stratopause to the mesopause (from ~ 80 to 90 km altitude); temperature decreases with altitude up to the mesopause, which is the coldest point in the whole atmosphere. Such temperature trend within the Mesosphere is due to the decrease in the ozone (0_3) concentration with altitude. However, rapid vertical mixing systematically occurs.

Thermosphere is the region of the atmosphere above the mesopause, characterized by high temperatures as a result of short-wavelength radiation absorption by N_2 and O_2; rapid vertical mixing. The ionosphere is a region of the upper mesosphere and lower thermosphere where ions are produced by photoionization.

Exosphere is the outermost region of the atmosphere (> 500 km altitude) where gas molecules with sufficient energy can escape from the Earth's gravitational attraction. Over the equator the average height of the tropopause is about 18 km; over the poles, about 8 km. The atmospheres of the Earth, as much as that of other terrestrial planets like Venus and Mars, are thought to have formed as a result of the release of trapped volatile compounds from the planet itself. The early atmosphere of the Earth is believed to have been a mixture of carbon dioxide (CO_2), nitrogen (N_2), and water vapor (H_2O), with trace amounts of hydrogen (H_2), a mixture similar to that emitted by present-day active volcanoes. The composition of the present atmosphere bears little resemblance to the composition of the early atmosphere. Most of the water vapor that outgassed from the Earth's interior condensed out of the atmosphere to form the oceans. The predominance of the CO_2 that outgassed formed sedimentary rocks (mainly from carbonate) after dissolution in the ocean. The early atmosphere of the Earth was a mildly reducing chemical mixture, whereas the present atmosphere is strongly oxidizing. The Earth's atmosphere is composed primarily of the gases N_2 (78%), O_2 (21%), and Ar (1%). Water vapor is the next most abundant constituent; it is found mainly in the lower atmosphere and its concentration is highly variable, reaching values as high as 3%. The remaining gaseous constituents, *i.e.* trace gases, represent less than 1% of the atmosphere, but they play crucial roles in the Earth's radiative balance and in the chemical properties of the atmosphere.

1.3.2 Aerosol

Aerosols are solid and/or liquid particles in the atmosphere, representing only a small fraction of atmosphere total mass. Nevertheless, they have a large impact on climate and biogeochemistry and therefore it is important to study processes in which aerosols are involved. Particles in the atmosphere can modify radiation in the short- and longwave and can also influenced cloud properties. (Mahowald et al. 2011). Aerosol sources are ubiquitous over the earth’s surface, spanning from deserts to oceans, forests and grasslands; alternatively, areosol can be produced by chemical reactions in the atmosphere or by suspension of soil materials and sea spray (Colbeck et al. 2010). Further factors contributing to aerosol composition are represented by emissions from industrial processes, vehicular traffic and other anthropic processes.

Atmospheric particles arise from both direct emissions and gas-to-particle conversion of vapor precursors. Aerosols can affect climate and stratospheric ozone concentrations and have been implicated in human morbidity and mortality in urban areas. The climatic role of atmospheric aerosols stems from their ability to reflect solar radiation back to space and from their role as cloud condensation nuclei. Tropospheric aerosols's properties such as mass and number concentrations and, particularly, their chemical composition and structure can affect human health, ecosystems, visibility and climate (Moroni et al. 2015).

Aersols vary in their impact on atmospheric and biochemical processes because of variability in size, composition and location in space and time. Particularly, aerosol's size is one of the most important characteristics, since it is deemed responsible for aerosol's impact and atmospheric lifetime. Particles size in the aerosol ranges from a diameter of 1 nm up to 100 μm; they can be categorized according to their size based on their observed modal distribution (Hinds 1999), the 50% cut-off diameter, or from dosimetric variables which are related to human exposure. In the latter case, the most common division is $PM_{2.5}$ and PM_{10} particles. PM10 is defined as PM which passes through a size-selective inlet with a 50% efficiency cut-off at 10 μm aerodynamic diameter, while the cut-off for PM2.5 is 2.5 μm. The division is related with the possibility of $PM_{2.5}$ particles penetrating to the lower parts of the human respiratory tract. In category 1, several subcategories can be observed:

- Nucleation mode: Particles with diameter <10 nm, which are formed through nucleation processes. The lower limit of this category is not very well defined but is dictated by instrument capability; an ion aerosol spectrometer can detect particles down to 1.5 nm, whereas the cut-off for a condensation particles counter is not below 3 nm.
- Aitken mode: Particles with diameter 10 nm<d<100 nm. They originate from vapor nucleation or growth of pre-existing particles due to condensation.
- Accumulation mode: Particles with diameter 0.1 μm <d<1 μm. The upper limit coincides with the minimum of the total particle distribution. Particles in this mode are formed with the coagulation of smaller particles or the condensation of vapor constituents. The size of particles is not increased over this category with condensational growth. Furthermore, the removal mechanisms of particles in this category are very slow and, as a result, there is an accumulation of particles.
- Ultrafine particles: Particles in the Aitkin and nucleation modes.
- Fine particles: Includes the nucleation, Aitkin, and accumulation modes, usually are formed in the atmosphere and comprise also anthropogenic aerosols

- Coarse particles: Particles with diameters between 1 and 10 μm. usually represent primary aerosols, meaning that they are directly emitted and often uplifted in the atmosphere by the wind streams.

Coarse particles are generally formed by mechanical processes such as dust resuspension. They are typically rich in the naturally occurring crustal material (Ca, Fe, and Si) as well as sea salt. The fine particles can be rich in carbon, nitrates, sulfates, and ammonium ions. They are generally formed by combustion or gas to particle conversion. The particles in the two modes not only differ in origin but also have different atmospheric fates (Colbeck et al. 2010).

1.3.3 Environmental and health effects of atmospheric particulate matter

As described above, particulate matter plays crucial roles in the atmosphere and affects numerous processes occurring in other receiving environmental compartments, such as for instance hydrosphere and lithosphere. Overall, climatic trends are primarily influenced by aerosol's spatial and temporal variability; for example, two aerosols differing in altitude and lifetimes will have different effects on climate. As for aerosol displacement from the atmosphere, particles in the atmosphere can be removed by wet and dry depositions. The first process is associated with precipitation, either rain, snow or hail, and it's very efficient at removing aerosols. The second one includes both the turbulent collisions of the aerosols with the surface and the gravitational settling of the aerosols. Because of the role of the latter mechanism, coarse particles are removed by dry deposition much more quickly than fine particles.

There are many uncertainties in aerosol removal processes, since it usually has a lifetime that vary from <1 day for very large particles (>10 μm) to 2–4 weeks for smaller particles far from precipitating regions; the uncertainties derive not from aerosol's lifetime, but from our poor understanding of aerosol-cloud interactions as well as from the paucity of high-quality deposition and vertical distribution observations. It's also important to consider that even if some particles are removed close to the source area, the majority of them are transported long distances before being removed. Once emitted into the atmosphere, aerosols mix into the atmospheric boundary layer (the lowest 1–3 km of the atmosphere, where mixing is strongest) and can be transported for long distances. When aerosols are deposited onto the earth's surface, they have an impact on both soil and aquatic environments. For

instance, aerosols deposition is known to decrease snow albedo by darkening glaciers surfaces and, besides, it provides a source of nutrients to both lands and oceans for thousands of years. In addition, deposition processes might harm terrestrial and aquatic biota by transferring a significant burden of acids or heavy metals to the receiving ecosystems. Thus, aerosols strongly interact with climate and biogeochemistry of the various environmental compartments (Mahowald et al. 2011).

The chemical composition of aerosol particles influence the type and rate of interactions with radiation and water vapor as well as their effects in lung tissues or on sensitive ecosystems. Aerosol particulate matter (PM) contributes significantly to the impacts of (or to phenomena such as) air pollution, acids and nutrients deposition, visibility reduction (haze), stratospheric ozone depletion or, more generally, climate change.

In particular, visibility reduction and occurrence of hazy conditions is primarily due to light scattering phenomena exerted by airborne PM. Atmospheric PM also plays a direct role in climate as seen before. Atmospheric aerosol particles, including cloud droplets, play a role in acids deposition by either scavenguing acid vapors formed by gas-phase atmospheric processes or by taking part in a number of heterogeneous reactions (for instance, when the key gaseous pollutants sulfur dioxide (SO_2) and nitrogen oxides (NO_2 and N_2O_5) are transformed into sulfuric, nitrous, and nitric acids). Wet and dry deposition delivers the resulting sulfate, nitrite, nitrate, and ammonium ions to lakes, rivers, and estuaries as well as farmland, forests, and grasslands. In well-buffered waters and soils, the deposited materials are nutrients and may fertilize either beneficial or harmful (e.g., eutrophication) growth, depending on prior nutrient levels and plant. In poorly buffered waters and soils, heavy deposition of nitrate and sulfate can cause dramatic acidification and serious harm to both aqueous and land biota. Aerosol particles also play a role of paramount importance in stratospheric ozone depletion. Both sulfuric acid particles in the Junge layer (a layer of microscopic aerosol particles between the tropopause and about 30 km altitude, also called *Stratospheric Aerosol Layer*) over low and mid-latitudes and nitric acid/sulfuric acid/water polar stratospheric cloud (PSC) particles at high latitudes convert relatively unreactive reservoir compounds, such as HCl, HBr, $ClONO_2$, and $BrONO_2$, into labile free radical precursors that lead to ozone destruction. (Kolb et al. 2012).

Due to the fact that fine aerosol particles can penetrate deeply into lungs they are responsible of the major adverse health effects and play a role in the increased incidence of pulmonary and cardiovascular diseases observed nowadays.

In this respect, epidemiological analyses correlating human health impacts with atmospheric PM exposure clearly indicate that aerosol PM exposure drives both human morbidity and mortality, although it must be pointed out that detailed health effects of specific PM components are not yet fully known.

As reported in recently published reviews (Colbeck et al. 2010), ambient air quality standards have been introduced at national and international levels with the aim to protect human health and the environment (The World Health Organization WHO, 2006). Legislation for PM_{10} concentrations has been implemented in the EU. In 1996, the EU adopted the Air Quality Framework Directive (96/62/EC), which in turn gave rise to a series of "daughter" directives. In 2008, a new Air Quality Directive (2008/50/EC) came into force (EU 2008). The new directive merges four existing directives into a single one concerning air quality. It sets new standards and target dates for reducing concentrations of $PM_{2.5}$ in addition to existing ones for PM_{10}. The directive recommends that measurements of fine PM at rural background locations should be made in order to understand better the impacts of this pollutant and to develop appropriate policies. It also acknowledges that there is currently non identifiable threshold below which $PM_{2.5}$ does not pose a risk to human health. The European Commission has had to start infringement proceedings against ten member states for failing to comply with the EU's air quality standard for PM_{10}.

1.4 Bioaerosol: the living component of particulate matter

In the 1860s a new area of biology was conceived based on the work of Louis Pasteur that showed that invisible, airborne particles were responsible for mysterious fermentative reactions in his experiments. Since the first years of the XX century, when the interest has been focused also on the study of life in the air, aerobiology has been defined by many as the study of the aerolosization, aerial transmission, and deposition of biological materials. Nowadays there's an ever expanding public concern regarding environmental issues and other related ecological problems. Microbes make up most of the biomass on Earth; almost all environments, including extreme ones characterized by severe conditions of heat, cold, radiation, pressure, salinity, acidity, and darkness, can virtually host microbes able to surve and thrive therein. The presence of these forms of life has been predominantly studied on terrestrial and aquatic environments. It has long been known that microorganisms are present also in the atmosphere, primarily associated with aerosol, hence the name "bioaerosol". However, the atmospheric environment has not been sufficiently appreciated as a biological entity so far, even if it is to be considered as alive as soil or water. (Polymenakou et al. 2008). With regard to bioaerosol composition as it is known so far, it encompasses bacteria as well as viruses, fungi (both mycelial propagules and spores), algae, protists, and plant-derived material such as pollen. These components range in size. Pollens from anemophilous plant have usually diameters of 17-58 μm; fungal spores are typically 1-30μm; bacteria range from 0.25-8μm and viruses are usually less than 0.3μm. it has been demonstrated that biological material, and especially bacteria, occur in the air associated with particles greater than 3 μm (Yoo et al. 2016).

Microorganisms can be transported in and through the continents with atmospheric currents at high altitude. Even though air provides a hostile environment for microorganisms, exposing them to UV light, paucity of water, decreasing temperature, oxygen concentration and pressure, there is a growing body of evidence about the abundance of bacteria in the atmosphere (Fahlgren et al. 2010).

The number of particles and microorganisms in the atmosphere can vary considerably depending on numerous meteorological and topographic factors such as climatic conditions (precipitations, wind's direction etc..), the source (natural or anthropic), period and day of the year, the pollution's level and the survivability of microorganisms. Transport of bioaerosol, as well as that of other components of PM, can be defined in terms of time and

distance. Sub-microscale transport involves short period of time, under 10 minutes, as well as relatively short distances, under 100m. Microscale transport ranges from 1 minutes to 1 hour and from 100m to 1 km and is the most common type of transport phenomenon. Mesoscale transport refers to transport in terms of day and distances up to 100km and in macroscale transport, the time and distance are extended even further. The growing interest on biological fraction of aerosol has been boosted by an increasing amount of evidence suggesting that this fraction may be responsible for the most of adverse health effects to both humans and wildlife. For example, it has been proved that airborne endotoxins are partly responsible for inflammation processes caused by aerosol inhalation, while some other biological particles, e.g. pollens and fungal spores, can cause asthma and allergies (Gandolfi et al. 2013). The general assumption of the atmosphere as a mere conduit for the dispersal of microorganisms rather than dynamic habitat itself has been recently questioned (Barberàn et al. 2015, Barberàn et al. 2014, Womack et al. 2010). Several studies strongly suggest that atmospheric microbes are viable and metabolically active. Microorganisms metabolize the organic matter in cloud water and potentially contribute to the biogeochemical cycles of earth. It is supposed that microbes affect atmospheric chemistry and the precipitation cycles of earth implicating airborne microbes in atmospheric processes, such as ice nucleation and cloud formation. The increase in cloud formation may potentially be responsible for earth's climate changes. (DeLeon-Rodriguez et al. 2013). Cultivation was the gold method used in the first aerobiological studies that aimed at investigating airborne microbial communities in outdoor environments. These studies provided a first description of airborne microorganisms by a taxonomic classification of a variable number of isolates from different environments such as urban, rural, forest, coastal and remote sites. Despite all its limit principally due by the fact that only a small fraction of microorganisms are cultivable (1%), traditional methods based on cultivations are the only way for the isolation of microbial strains, which is still the most direct method to obtain reliable information on physiologic and metabolic characteristics of bacteria. For example, cultivation-based studies revealed that a high percentage of colonies grown on plates are pigmented. This is a very common feature in airborne microorganisms, probably because it protects cells from atmospheric UV exposure and contributes to survival at low temperatures. Traditional methods are also preferred for asses if microorganisms are not only viable but also metabolically active.

However, as describe before, cultivation heavily underestimate both the number and the diversity of microorganisms in a given environment. The increasing awareness that the biases of this methods pose an obstacle to a deep and complete description of microbial

communities, forced microbiologists to turn to cultivation-independent methods based on metagenomic DNA also for aerobiology. Furthermore, the recent development of NGS allowed to obtain taxonomic profiles of multiple airborne communities by sequencing thousands of 16S rRNA gene amplicons per samples. Thus even rare bacterial populations could be detected and the robustness of statistical methods applied to the results increased. Notwithstanding that, few studies have appeared still now in literature using NGS on aerosol's samples (Gandolfi et al. 2013).

1.5 Impact of desert dust storms on particulate matter

Deserts, are the primary sources of mobilized top soil that move great distances each year. The study of desert dust, its entrainment, transport and deposition is an area of growing importance in investigations of global environmental change because dust storms have great significance for the physical environment and the world's human inhabitants. The dust storm is a common phenomenon that occurs with great frequency and magnitude in arid and semiarid areas. Desert dust include Asian and African dust, but it is worth of notice that the former affects primarily Asiatic regions such as China, Japan, Mongolia and others (Yamaguchi et al. 2014)**,** and the second one cross the Mediterrean reaching European latitudes (Griffin et al. 2007).

The reasons why dust storms are important is that dust loadings in the atmosphere are significant for climate. They affect air temperatures through the absorption and scattering of solar radiation. In addition, dust may affect climate through its influence on marine primary productivity; and there is some evidence that it may cause ocean cooling. Moreover, because of the thousands of kilometres over which the dust is transported, its influence extends as far a field as Northern Europe, Amazonia and the coral reefs of the Caribbean. Standard World Meteorological Organization (WMO) definitions for dust events that involve dust entrainment in the atmosphere are given by McTainsh and Pitblado (McTainsh et al. 1987). Dust storms are the result of turbulent winds raising large quantities of dust into the air and reducing visibility to less than 1000 m; blowing dust is raised by winds to moderate heights above the ground reducing visibility at eye level (1.8 m) but not to less than 1000 m; dust haze is produced by dust particles in suspended transport which have been raised from the ground by a dust storm prior to the time of observation; dust whirls (or dust devils) are whirling columns of dust moving with the wind and are usually less than 30 m high (but may extend to 300 m or more) and of narrow dimensions. There is some confusion in the literature between 'sand storms' and 'dust storms'. The former tend to be low altitude phenomena of limited areal extent, composed of predominantly sand-sized materials. Dust storms reach higher altitudes, travel longer distances and are mainly composed of silt and clay (Goudie et al. 2006).

As for any type of atmospheric aerosol particles, the environmental and climatic impacts of mineral dust depend on its physico-chemical properties, such as composition, shape, surface state, size, and mixing state of the particles. Mineral dust consists of irregular particles, often

aggregates of different composition, of sizes varying from tenths of nanometers to hundreds of microns. The size distribution evolves rapidly according to time after emission. Particle size is a fundamental parameter to understand and predict atmospheric lifetime, transport processes and impacts of mineral dust. The representation of the mineral dust size distribution remains a real challenge due to the large size spectrum. The number size distribution, dominated by submicron particles, controls the direct impact on radiation and on cloud processes.

Particle shape influences the aerosol optical properties underlying the direct effect. Deviation of dust grains from spherical shape can change light scattering by a factor of 2, depending on the scattering angle. Particle shape, besides size and density, also influences particle sedimentation. In general, sedimentation velocity decreases with increasing deviation from spherical shape. For example, flat particles (i.e., particles whose thickness is smaller than the width and length, such as spheroids) can be transported over longer distances than spherical ones. Composition is the third key property. Sokolik and Toon (Sokolik et al. 1999) demonstrated that the variable mineralogical composition (clays, quartz, carbonates, feldspars, sulphates, iron oxides) has to be incorporated into radiative models to estimate the dust optical and radiative properties. When first emitted, dust particles are often composed to a large extent of insoluble or low-solubility components. Referring to Koehler theory, mineral dust particles are considered almost CCN inactive (Formenti et al. 2011).

Some of the methods reviewed by Karanasiou (Karanasiou et al. 2012) for the identification of dust episodes are:

• by back-trajectory analysis (Hysplit model) using NRL, SKIRON and BSC-DREAM dust maps and satellite images provided by the NASA SeaWiFS (Tobías et al. 2011)

• by the combination of Lidar observations with operational models and the changes of the ratio PM_{10}/NO2. A PM_{10}/NO2 ratio larger than 0.6 revealed the intrusion of Sahara dust particles where PM_{10}/NO2 ratios≤0.6 were found to be characteristic of traffic air pollution (Mallone et al. 2011)

• by back-trajectory analysis in combination with a data driven criterion, based on the ratio of the PM_{10} concentration in the most remote fixed monitoring station to a centrally located one exceeding the median of this ratio during that year (Samoli et al. 2011)

• by the combination of air mass back-trajectories calculated using the FLEXTRA model and high hourly number concentration of coarse (diameter>1 μm) aerosol particles, considered to be a marker of dust transport from the Sahara desert (Sajani et al. 2011).

As explained above, the Saharan desert and its margins are the largest source of mineral dust on earth. Dust emission hot spots can be identified using visibility data, back trajectory analysis, satellite observations and isotopic composition studies. African and Asian dust consist primarily of clay soil minerals such as illite, quartz, kaolinite, chlorite, microcline, plagioclase and calcite, which may change chemically during aerosol transport. Some elements such as iron, manganese, scandium and cobalt occur on African dust particles in concentrations similar to average crustal abundance, while other elements i.e. mercury, selenium, lead accumulate at concentrations even three orders of magnitude grater then mean crustal abundance. (Garrison et al. 2003). During the transport, desert dust is enriched of sulphate and nitrate from sea surfaces, and of organic pollutants (e.g. PAH) from antropized areas. In addition to serving as a source of nutrients for aquatic and terrestrial microorganisms and plant life, dust storms can also deposit soil-associated pollutants (chemicals, metals, etc.) and microbes to downwind ecosystems (Griffin et al. 2007). Deserts dust have also a great importance for human health because the small particles can penetrate into lungs and cause adverse health effects via oxidative stress.

1.6 Desert dust storms and the microbiology of particulate matter

As explained in the previous section, dust storms originate mainly from the Sahara and Sahel regions of Africa and from the Gobi, Takla Makan, and Jaran deserts of Asia. Until recently, African dust has been the dominant source of dust in the atmosphere, but in the last decade, the contribution of Asian dust has largely increased. There are also other regions that contribute marginally to the total amount of the deserts storms such as the arid regions of United States, Central and South America, Central Australia and Middle East. Dusts coming from Africa reach Middle East, Europe, Asia, the Americas, and the Caribbean. On the contrary, dust originated from Asia can be dispersed universally in the Northern Hemisphere, even if seasonality of events generates a maximum level of emission in spring (Griffin et al. 2007).

Microorganisms associated with desert dust undergo long-range vertical and horizontal transport. This process, besides influencing chemical and physical processes in the atmosphere and climate changes as a whole, favors the expansion of microbial "hitch-hikers" biogeography, ultimately affecting human health in regions considerably far from the source (Yoo et al. 2016). A correlation between the exacerbation of asthma and other human pathologies in areas frequently impacted by desert intrusions has been observed (Griffin et al. 2007). However, little is known about the composition of microbial communities in the aerosol and how they change during desert dust intrusions. An accurate assessment of airborne microbial communities is difficult to achieve due to various interfering factors such as the choice of the sampler, the transport of fresh samples to the laboratory, DNA extraction and further processing steps.

1.6.1 Sampling methods

Nowadays, a wide variety of bioaerosol sampling methods are available, with the consequent development of numerous devices for an accurate sampling of PM. To date, there are three principal sampling methods for bioaerosol: filtration, impaction and liquid impingement.

The first, is the most common used for sampling bioaerosol. This method involves pumping air through a porous membrane filter to capture the air. Filtration can be used both for

culture- and non-culture-based studies, it has many advantages such as low cost, portability and high efficiency in trapping microorganisms larger than the pore size on the filter surface. The filters used for bioaerosol collection are commonly made of cellulose, nylon and glass fibers, all characterized by a porosity of 0.02 μm. The airflow rates of filtration usually range from 300 to 1140 L/min (Yoo et al. 2016).

Filtration allows the collection of bacteria, pollen, fungi and airborne viruses, that remain viable and can even grow on the filter by using nutrients and organic components present in particulate matter (Griffin et al. 2007).

Impaction involves the use of an air pump to capture air onto Petri dishes containing nutrient agar, even if tailor-made cassettes and stripes can be also used. The airflow is controlled by either slits or holes that convey and distribute the air stream evenly over the nutrient agar surface; in some cases, it is also possible to control the particle size ranges impacting the agar media. The airflow rates of impactors, normally range between 10 and 700 L/min. Impaction, as much as other approaches, presents both advantages and limitations; as for the former, the low cost, the ease of use, the portability and the possibility to assess the density of cultivable bacteria and fungi per volume of air make it a suitable technique. The disadvantages instead encompass the loss of microbial viability for the impact stress, the loss of recovery caused by the inability of some microorganisms to adhere to the agar surface and the analysis of relatively small sample volumes due to the low flow rates.

An alternative method to capture air-dispersed microorganisms is by means of impingement into a liquid matrix (via bubbling). One of the most widely used impinge is the AGI-30, which works with a low flow rate to bubble air through a liquid matrix. This instrument is efficient both for culture-dependent and independent analyses. Liquid impingers are advantageous because the liquid matrix can be divided for the different type of analyses: cultivation, direct count and all molecular assays. (Yoo et al. 2016). The drawbacks of this technique, are low capture rate of some low flow-rate impingers, high cost, loss of collected fluids due to evaporation phenomena and violent bubbling. The simplest method used to collect bioaerosol is gravity deposition. This approach involves the exposure of nutrient agar plates to an open-air environment for a distinct period of time; compared to other approaches it is extremely inexpensive, but it has been demonstrated that it suffers significant limitation with respect to other volumetric assays mentioned above. (Griffin et al. 2007).

Another variable of utmost importance for bioaerosol sampling is the choice of the size of particulate matter. Depending on the fraction of PM to be studied, it is possible to collect the whole load of total suspended solids (TSP) in a given air sample, or just a fraction of it (e.g.

2.5 and 10 μm). This separation of particles based on size-exclusion is generally obtained by means of specific cut-off samplers.

1.6.2 Bacterial quantification in desert dust-impacted PM

The bacterial abundance in PM has been considered of paramount importance for the description of airborne microorganisms. Traditionally and as for the past decades, quantification has been carried out by means of culture-dependent methods e.g. direct plate count. (Itani et al. 2016, Jeon et al. 2011, Schlesinger et al. 2006). However, traditional approaches are endowed with the methodological bias of viable-but-not-culturable (VBNC) microorganisms. Therefore, the use of PCR-based methods allowing the detection and quantification of a much greater diversity of microorganisms, has significantly increased in recent years. The main advantages of these techniques are better sensitivity, higher speed and above all the lack of any dependence from cell viability. Quantitative PCR (qPCR) is of particular interest because it allows for the measurement, in real-time, of the increasing amount of amplicons in PCR reactions using a DNA-binding, fluorogenic compound (SYBR Green, TaqMan Probes, etc.). Either total bacteria or a peculiar group of microorganisms can be quantified depending on the primer set used. (Gandolfi et al. 2013). For instance, Yamaguchi et al. 2012, 2016, used qPCR protocols for the assessment of the fluctuations of bacterial density in the aerosol, both in the presence and in the absence of desert dust intrusions. This said, it is always difficult to compare the results due to the different expression of output data and the fraction of particulate matter sampled/targeted (TSP; PM_{10}, $PM_{2.5}$). However, several authors (Yamaguchi et al. 2012, Yamaguchi et al. 2016 and Jeon et al. 2011) agreed that bacterial density in both TSP and PM_{10} was considerably higher during a desert storm event than in the absence of it, despite the use of different techniques (qPCR and plate counts, respectively). On the contrary, Smith et al. 2012, found that the concentration of microbes was relatively low in air samples collected in the free troposphere in concomitance with a long-range dust transportation event, i.e. air masses moving from Asia to the North America.

1.6.3. Microbial diversity in desert dust-impacted PM: Cultivation-based studies

Aerobiology studies that investigated the composition of microbial communities in desert dust intrusions were traditionally based on cultivation-dependent approaches. These studies provided a first description of airborne microorganisms and a taxonomic classification of a significant number of isolates. Some studies (Yamaguchi et al. 2016, Yamaguchi et al. 2014, Gourdarzi et al. 2014, Schlesinger et al. 2006) aimed at assessing the diversity and composition of culturable bacterial communities in Asian desert dusts, and the authors sampled TSP, PM_{10} and $PM_{2.5}$ nearby as well as far from the source. Dominant phyla during such dust events were *Proteobacteria* and *Firmicutes* besides a large number of *Actinobacteria* typically abundant in deserts environment (Gourdazi et al. 2014). On the contrary, other authors focused their attention on the investigation of cultivable bacteria present in PM drifting from Africa (Itani et al. 2016, Rosselli et al. 2015, Favet et al. 2013, Griffin et al. 2007, Prospero et al. 2005, Kellogg et al. 2004, Griffin et al. 2003, Griffin et al. 2001). In most of these studies TSP was the target matrix sampled, with the only exceptions of PM_{10} (Rosselli et al. 2015) and rain (Itani et al. 2016). Sampling occurred mostly far from the source of dust, with the exception of the work by Itani et al. (2016) that sampled in Israel, thus close to the Sahara/Sahel regions of Africa. Regardless of the difference between the studies reported above, the most abundant bacteria in the African dust were ascribed to the genera *Bacillus* and *Kocuria*, especially *B. simplex, B. mojavensis, B. subtilis and K. rosea* (Rosselli et al. 2015, Favet et al. 2013, Griffin et al. 2007).
However, cultivation-dependent methods are known to underestimate the diversity of microorganisms in a given environment. In addition, some studies reported large differences in the composition of airborne microbial communities assessed through culture-based or molecular methods (Rosselli et al. 2015, Yamaguchi et al. 2012, Cho and Hwang 2011), while others observed substantially overlapping results (Fahlgren et al. 2010). Despite these well-known limitations, cultivation is still the method of choice to study the physiologic and metabolic characteristics of bacteria. (Gandolfi et al. 2013).

1.6.4. Microbial diversity in desert dust-impacted PM: Cultivation-independent studies

The quick development of cultivation-independent technologies allowed a better assessment of the composition of PM-associated microbial communities, especially bacterial ones.

Indeed, with culture-dependent methods only a small fraction of the total biodiversity can be captured, as it is known that approximately 1 to 5% of the environmental bacteria are able to grow in laboratory conditions. As explained earlier, using molecular methods based on direct DNA extraction and the subsequent PCR amplification of specific genes (rRNA), it is possible to bypass cultivation, thus allowing to obtain a more complete picture of the total biodiversity. These techniques, revealed a greater diversity of airborne microorganisms compared to what it was previously described by using traditional methods (Gandolfi et al. 2013, Yoo et al. 2016,).

Low throughput molecular techniques, such as PCR-DGGE and cloning-sequencing of 16SrRNA genes, have been extensively used in past years to characterize Asian and African dust events, allowing to identify bacterial groups which are typically present in desert-originated particulate matter. As for the results, Polimenakou and co-workers (2008) sampled TSP during an African desert storm in the Easter Mediterrean sea, and taxa belonging to *Firmicutes* and *Actinobacteria* were shown to clearly dominate bioaerosol community. In a similar work, Sanchez de la Campa et al. (2013) sampled the TSP during an African desert storm in Spain, and they found *Firmicutes* and *Proteobacteri*a as predominant phyla. In both studies, however, the spore-forming *Bacillus* was the most abundant genus within *Firmicutes.* Likewise, Itani et al. (2016) sampling the rain in Israel during African storm, found *Proteobacteria* and *Actinobacteria* as predominant phyla; in particular, *Massilia*, a typical airborne bacterial genus, outnumbered other members of the same phylum. Shifting the attention on Asian dust intrusions, several authors (Yamaguchi et al. 2016, Yamaguchi et al. 2014, Maki et al. 2010, Lee et al. 2009) observed bacterial taxa closely related to those mentioned above. At the phylum level, *Actinobacteria* and, above all, *Firmicutes* dominated bacterial communities associated to Asian dusts. As for the latter phylum, *Bacillus* was again the most abundant genus.

Among the well-known limitations of the experimental approaches reported above, is the considerable amount of bench work to be done in order to create comprehensive libraries for any given sample. This problem is possibly overcome nowadays, thanks to the use of next-generation sequencing techniques, such as 454 pyrosequencing and Illumina MiSeq and HiSeq, that, together with the concomitant reduction of costs, enabled a substantial increase in read numbers from PCR amplicons. Recent improvements to the MiSeq and HiSeq platform can offer longer read lengths of up respectively to 2 × 250 bases with 8.5 Gb and 2 × 150 bases with approximately 200 Gb output. MiSeq and HiSeq sequencing have recently

been successfully used to characterize microbial communities and to investigate airborne pathogens in bioaerosol studies (Yoo et al.2016).

With the advent of NGS, it has been possible to obtain taxonomic profiles of multiple airborne communities by sequencing thousands of 16s rRNA gene amplicons per sample. Thus, even rare bacteria could be detected and the robustness of statistical methods applied to the results increased. (Gandolfi et al. 2013). Nevertheless, analyzing the scientific literature at present, there are relatively few works in which authors used NGS to investigate the bacterial community composition in desert dust (Mazar et al. 2016, Rosselli et al. 2015, Barberan et al. 2014, Katra et al. 2014, Favet et al. 2013).

Favet et al. (2013) analyzed desert sand from various locations in Chad and dust that had blown to the Cape Verde Islands. High throughput sequencing techniques showed that the samples contained a large variety of microbes well adapted to the harsh desert conditions. The most abundant bacterial grouped in four different phyla: *Firmicutes* (mainly *Bacillaceae*), *Actinobacteria* (mainly *Geodermatophilaceae, Nocardiodaceae* and *Solirubrobacteraceae*), *Proteobacteria* (mainly *Oxalobacteraceae, Rhizobiales* and *Sphingomonadaceae*), and *Bacteroidetes* (mainly *Cytophagaceae*).

Mazar et al. (2016), evaluated the impact of Saharan dust storms on the airborne microbiome, but in a city located in the Eastern part of the Mediterranean basin, sampling particles with diameter less than 10 μm during two spring seasons on both dusty and non dusty days. Illumina analysis showed the effect of dust events on the diversity of the atmospheric microbiome. The relative abundance of desert soil-associated bacteria increased during dust events, while the relative abundance of anthropogenic-influenced taxa decreased. Particularly, an high abundance of soil-associated classes, such as *Gemmatimonadetes* and *Rubrobacteria* together with *Pontibacter* and *Geodermatophilus*, which have been isolated from desert soil and the desiccation resistant *Arthrobacter.* On the contrary, human- and agriculture-associated families, such as *Dermabacteraceae* and *Lactobacillaceae*, displayed higher relative abundances during dust-free days.

On the contrary, Barberan et al. (2014), used pyrosequencing to characterize the microbial communities of atmospheric deposition collected in the Central Pyrenees (NE Spain) along three years. Additionally, bacteria from soils in Mauritania and from the air-water interface of high altitude Pyrenean lakes were also examined. The different habitats examined (soil, deposition, and air-water interface) harbored distinct microbial communities, although airborne samples collected in the Pyrenees during Saharan dust outbreaks were closer to Mauritian soil samples than those collected during no Saharan dust episodes. In particular,

the bacterial OTUs belonged mainly to *Alphaproteobacteria* (*Sphingomonadales* and *Rhizobiales*), *Actinobacteria* (*Actinobacteridae*), *Bacteroidetes* (*Sphingobacteriales*) and *Betaproteobacteria* (*Burkholderiales*).

Katra et al. (2014), determined the microbial diversities of dust from two storm events in southern Israel, by pyro-sequencing. They found that in the first event, most of the reads affiliated to *Betaproteobacteria*, consistent with prokaryotes of European origin; the second sample, on the other hand, originated in north-Africa, contained significantly more members of the *Actinobacteria*, a phylum that include typical desert bacteria (e.g. *Geodermatophilus* and *Kocuria*).

The most important study, relatively to what was the focus of our work, was conducted by Rosselli et al. (2015). The authors chose as sampling ground the island of Sardinia. Samples were collected in two opposite coastal sites and in two different weather conditions comparing dust-conveying winds from Africa with a control situation with winds from Europe. As for the results, in the non- dust samples they found background of bacteria already recognized as air-particles associated (e.g. *Propionibacterium*). On the other hand, as effect of dust-events, the authors detected an enrichment of specific bacterial populations like *Corynebacterium* (previously reported as desert-dust associated), and, differently from others authors, *Paracoccus* or *Brevundimonas*.

CHAPTER 2

AIM OF THE WORK AND THESIS OUTLINE

Air masses can move into the atmosphere microorganisms from one geographical location to another, expanding their biogeographical range. Deserts are one of the primary sources of mobilized top soils that move great distances through the atmosphere each year. African desert storms cross the eastern Mediterranean and inject a large pulse of microorganisms into the European air. Nevertheless, the impact of desert dust in the particulate matter present in anthropized areas may be difficult to study because of the complexity of the urban scenario. Further, while culture-based methods have been the only investigation approach for long time for different types of sample, including bioaerosol, culture-independent approaches based on molecular methods can provide a more complete picture of environmental microbial communities. Nevertheless, these methods have been rarely used to study the impact of air mass currents on the microbial communities of particulate matter. This PhD thesis aims to investigate the microbial ecology of particulate matter by studying the microbial diversity of bioaerosol and how this is impacted by different air masses, in particular those originating from the Saharan Desert and reaching our latitudes. A further aim was also to assess the impact of desert dust depositions on the microbial diversity of a remote environment, such as a mountain glacier. The project was carried on in the frame of collaboration between the Applied and Environmental Microbiology Lab (Dr. E. Federici) and the Atmospheric Chemistry and Physics Lab (Prof. D. Cappelletti) of the Department of Chemistry, Biology and Biotechnology at the University of Perugia.

Chapter 3 reports the characterization of the physical, chemical and microbiological composition of desert dust intrusions compared with local and other source currents. We chose a mid-elevation (approximately 1100masl), remote sampling site on the *Martani* mountain chain (Umbria, Central Italy) that is particularly well suited for monitoring long-range air mass intrusions. We collected several PM_{10} air samples during 2014 and 2015, representing all desert dust intrusion reaching our sampling site and currents coming from other part of Europe as well as regional air masses. Aerosol bacterial communities have been investigated using Illumina next generation sequencing (NGS) of amplified 16S rRNA gene fragments and related to physic-chemical characterization of the PM. Most of the studies on the microbiota present in desert dusts aerosol have been conducted in anthropized areas, where it may be difficult to discern the impact of desert dust with the local urban particulate matter. On the contrary, the remote sampling site of *Monte Martano*, being far from large

metropolitan areas and other relevant human activities, allowed us to clearly distinguish long-range desert intrusions from other currents and local air circulation.

Chapter 4 further develops the findings of Chapter 3 by focusing on particulate matter during an intense African desert dust storm that reached Central Italy on 30 November - 1st December of 2014. This was considered the strongest Saharan desert dust intrusion occurring in Europe in the last period. In this case, the culture-independent analysis, based on NGS of 16S rRNA genes, was coupled with a traditional culture-based approach to characterize, on the one hand, the total bacterial biodiversity, and, on the other hand, to investigate the viability and metabolic activity of the bacteria reaching our latitude after the long-range transport over the Mediterranean.

Chapter 5 follows-up the data presented and discussed in the previous two chapters that have clearly indicated that Saharan desert storms are able cross the Mediterranean and inject large pulse of microorganisms into Central Italy. In this part of the Thesis work, therefore, we aimed to investigate the impact of Saharan dust depositions on the microbiota of a mountain glacier environment. In particular, we collected and analyzed snow and water samples from the *Calderone* glacier that, being the southernmost in Europe, is often reached by African dust storms. As in the previous chapters, we employed culture-independent methods based on high-throughput Illumina sequencing of 16S rRNA genes to compare clean snow, Saharan-impacted snow and water samples from glacier.

CHAPTER 3

IMPACT OF AIR MASSES OF DIFFERENT ORIGINS ON MICROBIAL COMMUNITIES OF PARTICULATE MATTER

3.1 Introduction

The term aerosol defines an atmospheric suspension of solid and/or liquid particles in air, having different origins, natural and anthropic (both chemical and biological) as well as different physical and chemical properties (Putaud et al. 2004). Particles in the atmosphere can be produced by forest and grassland combustion or by chemical reactions in the atmosphere or by suspension of soil materials and sea spray (Colbeck et al. 2010). Further factors contributing to aerosol composition are represented by emissions from industrial processes, vehicular traffic and other anthropic contests.

The impact of long range transported aerosol on the particulate matter (PM) mass concentration and chemical speciation recorded on a site can vary substantially due to the source area, to synoptic/mesoscale meteorology and to local wind conditions (Moroni et al. 2015). Deserts constitute one of the most important sources of particulate matter because of the movement, for great distances, of billions metric tons of dust each year. Desert dust originates from both Asian and African continents, but it is worth noting that the former affects primarily Asiatic regions such as China, Japan, Mongolia (Yamagouchi et al. 2014), and the second one crosses the Mediterranean basin, thus reaching European latitudes. (Griffin et al. 2007). Progressive desertification and climate changes, as a consequence of atmospheric pollution, are increasing desert dust transport over the world. Many studies demonstrated that the transport of Saharan dust into European air has a clear seasonality being more frequent from February to June, and from late autumn to early winter, although dust events can be distributed throughout the year (Karanasiou et al. 2012). A significant fraction of desert dusts suspended particles (over 48%) consists of particles with an aerodynamic diameter $\leq$10 μm, indicated as PM_{10} (Goudie and Middleton, 2006), with suspension being the main mechanism of dust transport. Thus, silt is expected to be a dominant component, although clay and sand fractions can also be present, as dust deposits tend to get finer with increasing distance from their source regions. There has been a considerable amount of work aiming at the chemical characterization of African aerosols, mainly using major element variations (Moreno et al. 2006, Goudie and Middleton, 2006, Moroni et al. 2015), and on chemical variations linked to particle size fractionation. The chemical composition of desert dust is mostly dominated by SiO2 (60%), Al2O3 (14%), Fe2O3 (7%), CaO (4%), MgO (2.6%) and K2O (2.4%). As for the mineralogical

composition, quartz, magnetite/hematite and carbonates dominate in desert dusts. In the finer fractions, clay minerals such as palygorskite, illite and kaolinite can also be significant (Alastuey et al. 2005). Nowadays, it is possible to assess the arrival of desert intrusions by means of a combination of instruments, that analyze the number of particles in the air (OPC), and various software, that are able to recalculate the back trajectories.

In the last decade, a growing interest toward the study of the microbiological composition of desert dust along with its effects on downwind ecologies and human health has been observed (Griffin 2007, Gandolfi et al 2013, Yoo et al 2017). It's well known that airborne dust particles contain many microorganisms able to survive long-range transport. In fact, microorganisms in the aerosol can interact with each type of particle present in the aerosol, because they can utilize these particles as nutrient. A growing body of evidence highlights the possible adverse health effects of Sahara dust particles, with European regions being the target of numerous studies, reviewed by Karanasiou et al. (2012). It is now well established that microbial transport within desert dust originating from Saharan region of Africa is notable, since microorganism's concentrations during dust events are far higher than those observed in the absence of desert intrusions (Griffin et al. 2007). The increase in microbial density as a consequence of dust transport may have exceptional impacts on microbes biogeographical distribution. Microorganisms may be transported over short and long distances, they can influence the chemistry of the atmosphere and affect a number of processes such as cloud development, rain and snow precipitation. Another significant effect of the biotic fraction of aerosol is, without any doubt, on human health; in fact, particles with aerodynamic diameter <10μm can penetrate into lungs and those <2,5 μm can penetrate into deep lung and sub-epithelial tissues, where they can cause human pathologies or exacerbate pre-existing ones. It is exactly in the finest part of particulate matter (<10 μm) that bacteria and others biological components of aerosol (fungal spores, viruses) are included (Griffin 2007).

Regardless of their well-known limitations, culture-based microbiological methods have been widely used for a long time to investigate PM-associated microbial communities. For this reason, new technologies such as next generation sequencing (454, Illumina platform etc..) have been implemented in recent years, since they allow the assessment of the total microbial diversity (Franzetti et al. 2011, Bowers et al. 2013, Bertolini et al. 2013, Favèt et al. 2013, Barberàn et al. 2014). Despite this, only a limited number of studies are available to date in which authors exploited culture-independent techniques to investigate bacterial populations carried by African desert dust storms in the atmosphere (Sańchez de la Campa

et al 2013, Katra et al 2014, Rosselli et al 2015, Mazar et al 2016, Gat et al 2017). Many of these studies regarded the impact of long- and short-range Saharan intrusions on bioaerosol, but most the sampling sites were located within metropolitan areas or nearby big cities where, however, it may be difficult to discern the real effect of Saharan dust in PM due to many other PM-generating sources, e.g. pollution, anthropogenic activities, presence of vegetation. Further, many studies were based on samples collected in the coast of Middle East and thus could not appreciate the long-range transport (Katra et al 2014, Mazar et al 2016, Gat et al 2017). Only Sańchez de la Campa et al (2013) and Rosselli et al (2015) sampled in Europe but did not compare with PM other kind of intrusions.

In this Chapter, we aimed to characterize the physical, chemical and microbiological composition of desert dust intrusions compared with local and other source currents. We chose a mid-elevation (approximately 1100masl), remote sampling site on the *Martani* mountain chain (Umbria, Central Italy) that is particularly well suited for monitoring long-range air mass intrusions. We collected several PM_{10} air samples during 2014 and 2015, representing all desert dust intrusion reaching our sampling site and currents coming from other part of Europe as well as local background air masses. Aerosol bacterial communities have been investigated using Illumina next generation sequencing (NGS) of amplified 16S rRNA gene fragments and related to physic-chemical characterizations of PM_{10}.

3.2 Material and Methods

3.2.1. Sampling of particulate matter

Particulate matter (PM_{10}) was sampled at *Monte Martano* (MM), a medium altitude background site for air quality monitoring and atmospheric chemistry studies located in Central Italy, on the ridge of the Martani mountain chain (latitude 42°48′19″; longitude 12° 33′55″; altitude 1100 m asl).

Samples were collected on quartz fiber filters (Whatman QMA 20.3 x 25.4 cm) previously sterilized under UV lamp for 50 minutes on both sides, with a high-volume sampler (TISH, TE6001) operated at a flux of 1140 L min^{-1}. The sampling campaign was carried out in 2014-2015 and a total of twenty-two samples were collected.

Air mass back-trajectories were calculated using the HYSPLIT 4.9 (rev. 513, 2013-8-5) transport model (Draxler and Hess, 2003) using meteorological data fields supplied by the ARL (Air Resources Lab, http://ready.arl.noaa.gov/gdas1) archiving programs input data. In order to characterize the general behavior of air masses, 4-day backtrajectories (BT) were run every 6 h at an endpoint located 1000 agl over MM. Standard 1° latitude/longitude dataset output from the NCEP Global Data Assimilation System model (GDAS) on pressure surfaces, has been used. The ensemble of 1340 calculated BTs was characterized by the cluster analysis package included in the 4.9 version of HYSPLIT. Moreover, in depth calculations have been conducted for selected days at higher resolution. Specifically, we exploited a 3 hourly, global, 0.5 × 0.5 degree latitude/longitude dataset on hybrid sigma pressure surfaces.

3.2.2. Chemical analyses

The soluble ionic inorganic fraction was evaluated by ion chromatography (IC) (by means of a DIONEX 500 instrument) after extraction by ultrasonication in ultrapure water (18 MΩ) . The total concentration of alkali metals and many transition elements was determined by ICP-AES equipped with ultrasonic nebulizer (CETAC Technologies, U-5000AT), after acid digestion of the samples in a HNO3:H2O2 (3:1) mixture, assisted by a

microwave oven (MARS express, CEM). Suprapur® grade acids and multielement standard solutions from Merck (Darmstadt, Germany) were used. Reagent blanks and certified solutions (EnvironMAT, SCP Science) were employed to check precision and accuracy by replicated analyses. The elemental carbon (EC) and organic carbon (OC) mass concentrations were determined by thermal optical transmittance (TOT) technique (Sunset Carbon Analyzer) exploiting the high-temperature NIOSH protocol. (Moroni et al.2015)

3.2.3. Metagenomic DNA extraction

To perform the culture-independent assessment of the microbial communities in PM_{10} metagenomic DNA was extracted directly from the high-volume filters. Filters were handled in aseptic conditions, i.e. under a biological laminar flow. From the entire filter (20,3x25,4 cm), a 6x6 cm square was cut-off, suspended in40 mL of sterile water, shaken for 1h at maximum speed, centrifuged for 30' at 10000 x g and then at 11500 x g for 15' at 4C° to recover bacteria (Suarez et al. 2006, Radosevich et al. 2001). Supernatant was discarded and DNA was extracted from the pellet using the POWER SOIL DNA kit (MOBio) following the manufacturer's protocol.

3.2.4. 16S rRNA fragment amplification and Illumina sequencing

Bacterial communities were studied with a NGS approach by Illumina HISeq sequencing. The V5-V6 hypervariable regions of the 16S rRNA were amplified in 2 x 50 µL volume reactions with a Hot start Taq polymerase (Solis-Biodyne). The reaction included 1 µM each of primers 783F and 1027R (Huber et al, 2007, Wang and Qian, 2009). At the 5' end of 783F primer, one of 15 6-bp barcodes was also included to allow sample pooling and subsequent sequence sorting. The cycling conditions were: initial denaturation at 94°C for 5 min; 29 cycles of 94°C for 50 s, 47°C for 30 s, and 72°C for 30 s and final extension at 72°C for 5 min. The amplified products were purified with the Wizard SV PCR purification kit (Promega Corporation) and DNA quantity and purity were spectrophotometrically evaluated by Qbit (Invitrogen). Multiplexed samples were prepared with the Illumina Multiplexing Sample Preparation Oligonucleotide Kit, which provides 12 index oligos for pooling up to 12 samples per lane. Therefore, our customized tagging

system with 8 barcodes at the 5' end of the 783F primer allowed pooling up to 96 samples per lane. Multiplexed sequencing of all the pooled samples was performed on a single Illumina Hiseq 1000 lane, using a paired-end 2x100 base-pair protocol and the 4.0 sequencing chemistry, by *Parco Tecnologico Padano*.

3.2.5. Sequence analysis

Each sequence was assigned to its original sample according to its index oligos and barcode. After sorting the sequences, the reverse read of each paired-end sequence was reverse complemented and merged with the corresponding forward read. A quality cut-off was applied in order to remove the sequences that did not contain the barcode, those with an average base quality value (Q) lower than 30 and those that did not provide a perfect match in the overlapping part between the two pair-ends. The barcode was removed and sequences were sorted into Operational Taxonomic Units (OTUs) using the UPARSE-OTU algorithm. The minimum identity between each OTU member sequence and the representative sequence (*i.e.* the sequence that showed the minimum distance to all other sequences in the OTU) was set to 97%. The taxonomic classification of each OTU was carried on with the stand-alone version of RDP Bayesian Classifier, using a 50% confidence level.

3.2.6. Statistical analysis

Statistical analyses and graphical representations were carried out using the R framework and ggplot2 package (Wickham H. 2009; Oksanen *et al.* 2013). To visualize similarity among bacterial communities in samples, non-metric Multidimensional Scaling (nMDS) analysis was employed using Bray-Curtis dissimilarity matrix. The goal of nMDS is to reduce information from multiple variables into two dimensions using rank orders, so that they can be plotted and interpreted in the phase space. Principal-component analysis (PCA) was performed on the chemical data to correlate variables with the different samples.

3.3. Results

3.3.1 Particulate matter characterization

The particulate matter (PM_{10}) samples studied in this section of the thesis have been collected at the *Monte Martano* site in Central Italy (Fig. 3.1). *Monte Martano* is the highest mountain of the *Martani* mountain chain in the Umbria region, thus featuring a completely free horizon without any relevant orographic obstacles in its proximity. Due to its intermediate elevation (1100 m asl) and the low local background contamination, the latter granted by a limited vegetation and the absence of relevant anthropization, this site is ideal for air quality monitoring and atmospheric chemistry studies (Moroni et al. 2015). For these reasons, this site is particularly suited for catching Saharan dust advection, and more in general long ranged transported aerosol, in the Central Mediterranean area. Indeed, *Monte Martano* is one of the observational sites of the SDS-WAS (Sand and Dust Storm Advisory and Assessment System) network of WMO (*http://sds-was.aemet.es*) and since 2017 is also part of the European Monitoring and Evaluation Programme (EMEP) network (*http://www.emep.int*). The site is equipped with meteorological and atmospheric aerosol analysis instrumentations (Moroni et al, 2015).

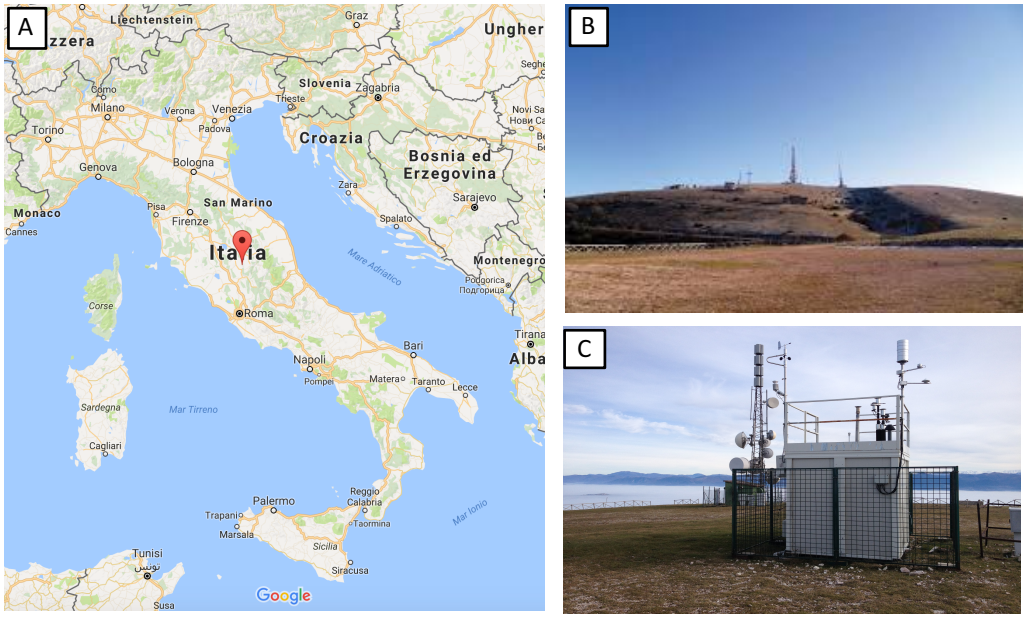

Figure 3.1. The *Monte Martano* sampling site: geographical localization (A), general view (B) and particulate matter sampling station (C).

A total of 22 PM_{10} samples have been collected during 2014 and 2015 (Table 3.1). These samples included all the desert dust intrusion recorded in Central Italy during the two years' campaign, as well as other air masses, namely those coming from other areas of Continental Europe and those of local origin. Our aim was to exploit these samples in order to investigate if desert dust intrusions significantly impacted the bacterial diversity of local particulate matter.

Table 3.1. Sample classification based on the geographical origin of the air mass intrusion and on the quantity of coarse fraction**.**

Sample Code	Sampling Date	Presumptive Classification	Geographical Origin[1]	Coarse Fraction[2]
SH_20140219	19/02/2014	**Saharan**	North Africa - Mediterrean	11.20
SH_20140404	04/04/2014	**Saharan**	North Africa – Mediterrean - Tyrrhenian	13.40
SH_20140522	22/05/2014	**Saharan**	North Africa – Mediterrean - Tyrrhenian	7.22
SH_20140624	24/06/2014	**Saharan**	North Africa - Mediterrean	3.90
SH_20141015	15/10/2014	**Saharan**	North Africa – Mediterrean - Tyrrhenian	15.70
SH_20141106	06/11/2014	**Saharan**	North Africa – Tyrrhenian	0.90
SH_20141130	30/11/2014	**Saharan**	North Africa – Mediterrean - Tyrrhenian	51.30
SH_20141201	01/12/2014	**Saharan**	North Africa – Tyrrhenian	55.70
SH_20150506	06/05/2015	**Saharan**	North Africa – Tyrrhenian	17.50
RG_20150315	15/03/2015	**Regional**	West - Tyrrhenian - Regional	4.80
RG_20150526	26/05/2015	**Regional**	Regional	0.30
RG_20150601	01/06/2015	**Regional**	Regional	5.00
RG_20150606	06/06/2015	**Regional**	North West – North East - Regional	2.00
RG_20151111	11/11/2015	**Regional**	Tyrrhenian - Regional	n.a.
RG_20151118	18/11/2015	**Regional**	North West - Regional	3.50
EU_20140310	10/03/2014	**European**	North East - East Europe	n.a.
EU_20140424	24/04/2014	**European**	North East Europe	0.90
EU_20141018	18/10/2014	**European**	West Europe - Tyrrhenian	n.a.
EU_20141022	22/10/2014	**European**	North – North West Europe	1.70
EU_20141029	29/10/2014	**European**	East Europe	2.10
EU_20141212	12/12/2014	**European**	North – North West Europe	1.10
EU_20150615	15/06/2015	**European**	West Europe – Tyrrhenian	4.20

[1] Geographical Origin as indicated by back-trajectories reported in Annex. 3.1.
[2] Coarse Fraction calculated as PM_{10}-$PM_{2.5}$ and expressed as $\mu g/m^3$

The presumptive classification of the air-masses origin was confirmed by analysis of the back-trajectories, reported in Annex. 3.1, as calculated with the HYSPLIT Trajectory Model (Stein et al., 2015). A further confirmation of the Saharan origin of the air masses

was provided by an increase in the concentration of the coarse fraction, i.e. the particles included between PM10 and PM2.5, that is indeed often associated with intrusions of African air-masses (Karanasiou et al. 2012, Moroni et al, 2015). It is possible to notice that the PM samples with the highest abundance of the coarse fraction were those collected during the intrusions of 30 November and 1st December, that were also characterized by air masses reaching the sampling site directly from North Africa and associated to very fast winds.

The chemical characterization of the samples is reported in Annex 3.2. To understand the chemical variables that best explained variation among PM_{10} samples, a principal component analysis was performed (Fig. 3.2). It is possible to remark that an increase of typical crustal metals, such as Ca, Fe, Mn and Ti, characterized the PM_{10} during the Saharan dust intrusions. Indeed, these chemical components are typical of arid soils and even if the dust composition changed depending on the origin area, such elements are always present (Moroni et al, 2015). Interestingly, on the 30 November and 1st December intrusions, the amount of dust typical elements was the highest.

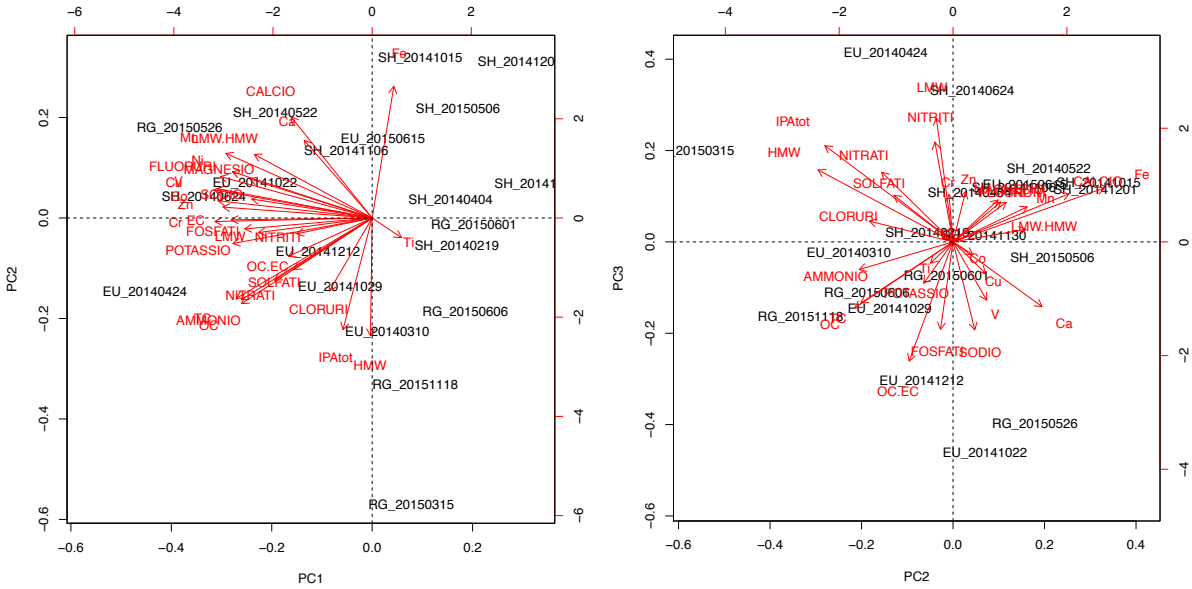

Figure 3.2. Principal-component analysis (PCA) of PM_{10} samples based on chemical variables. Each point represents a PM_{10} sample, with proximity of points in 2-D space indicative of relative similarities. PC1, PC2 and PC3 explained 42.9%, 12.2% and 9.4% of the variance, respectively.

3.3.2 Bacterial diversity in the particulate matter

Next generation sequencing (NGS) analysis of 16S rRNA gene fragments led to the recovery of 1286659 high-quality sequences, which clustered, across all samples, into a total of 10513 operational taxonomic units (OTUs) calculated applying a threshold of 97% sequence similarity. This cut-off is generally recognized as able to discriminate bacterial populations at the Genus – Species level. The average number of OTUs per sample was 2239 indicating that the 22 PM_{10} samples collected during the different air masses intrusions featured a great bacterial diversity.

To check the robustness of the experimental approach, we have performed technical replicates, namely analyzing three samples obtained by carrying on three independent extractions, 16S RNA gene amplifications and NGS analysis on the same PM_{10} filter sample. As an example, the result of one of these experiments (referred to the PM sample SH_20141201) is reported in Figure 3.3. It can be easily appreciated how the three technical replicates featured near identical OTU distribution profiles. This confirmed the robustness of our experimental approach and reassured that the structure of the bacterial community was not influenced by any of the experimental steps performed to obtain the bacterial diversity data.

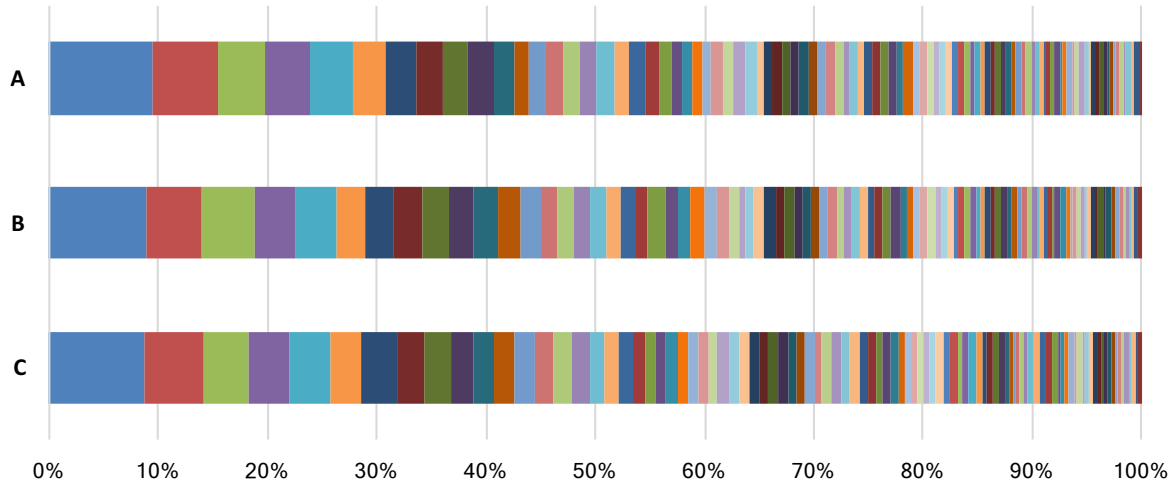

Figure 3.3. Distribution of the most abundant OTUs (average abundance higher than 0.2%) in three technical replicates (A, B, C) of one PM_{10} sample (namely SH_20141201).

To gain further insights into the identity and distribution of the bacterial populations present in the particulate matter, the sequences obtained from each sample, irrespective of their OTU assignment, were given a taxonomic attribution using the RDP Bayesian Classifier with a 50% confidence level. It should be, nevertheless, underlined that in this way a fraction of the sequence reads obtained in each sample may be ascribed, at each taxonomic level, to unknown bacterial taxa (Table 3.2). Consequently, this approach led to a loss of the diversity that could be further considered. For example, an average of 18.6% of the reads per sample, and thus of the community, could not be assigned with enough confidence at the *Genus* level. Nevertheless, this is the only way to draw proper considerations about the bacterial populations present in the particulate matter and to attempt the generation of hypothesis aimed to understand the ecological meanings of the possible impacts of the different air mass intrusions.

Table 3.2. Percentage of reads ascribed to unknown phylotypes in each *taxa*.

Sample	*Phylum*	*Class*	*Order*	*Family*	*Genus*
SH_20140219	0.83%	1.22%	1.35%	2.39%	4.97%
SH_20140404	1.54%	4.81%	5.15%	6.17%	10.71%
SH_20140522	4.05%	6.71%	6.99%	7.52%	11.24%
SH_20140624	2.85%	3.36%	3.68%	4.31%	8.38%
SH_20141015	16.13%	17.21%	17.86%	19.31%	24.20%
SH_20141106	2.95%	3.96%	4.61%	5.90%	12.25%
SH_20141130	3.24%	4.54%	5.50%	9.23%	19.71%
SH_20141201	2.63%	3.65%	4.83%	9.04%	22.46%
SH_20150506	3.72%	8.59%	10.01%	14.24%	21.06%
RG_20150315	0.66%	1.65%	2.53%	4.51%	7.93%
RG_20150526	7.75%	10.77%	12.02%	17.54%	20.09%
RG_20150601	8.90%	14.62%	15.95%	18.79%	27.14%
RG_20150606	6.19%	10.80%	12.01%	15.57%	24.18%
RG_20151111	4.98%	7.38%	8.35%	12.10%	23.24%
RG_20151118	9.85%	12.53%	14.56%	20.50%	28.47%
EU_20140310	0.55%	5.15%	5.70%	7.89%	11.49%
EU_20140424	3.62%	10.57%	12.55%	15.29%	20.94%
EU_20141018	21.44%	26.67%	31.43%	33.91%	39.74%
EU_20141022	4.69%	5.44%	5.96%	7.62%	14.20%
EU_20141029	0.60%	1.23%	1.46%	2.77%	6.52%
EU_20141212	7.48%	11.79%	13.85%	17.14%	23.85%
EU_20150615	16.59%	19.16%	20.61%	23.90%	27.37%

Figure 3.4 reports the *Phyla* distribution in all the 22 samples. The most abundant *phyla*, i.e. those with an abundance higher than 1% of the total reads, belonged to *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes.* The relative abundance of each phylotype showed significant changes among the different samples, suggesting a possible influence of the different air mass intrusions in composition of the particulate matter bacterial community.

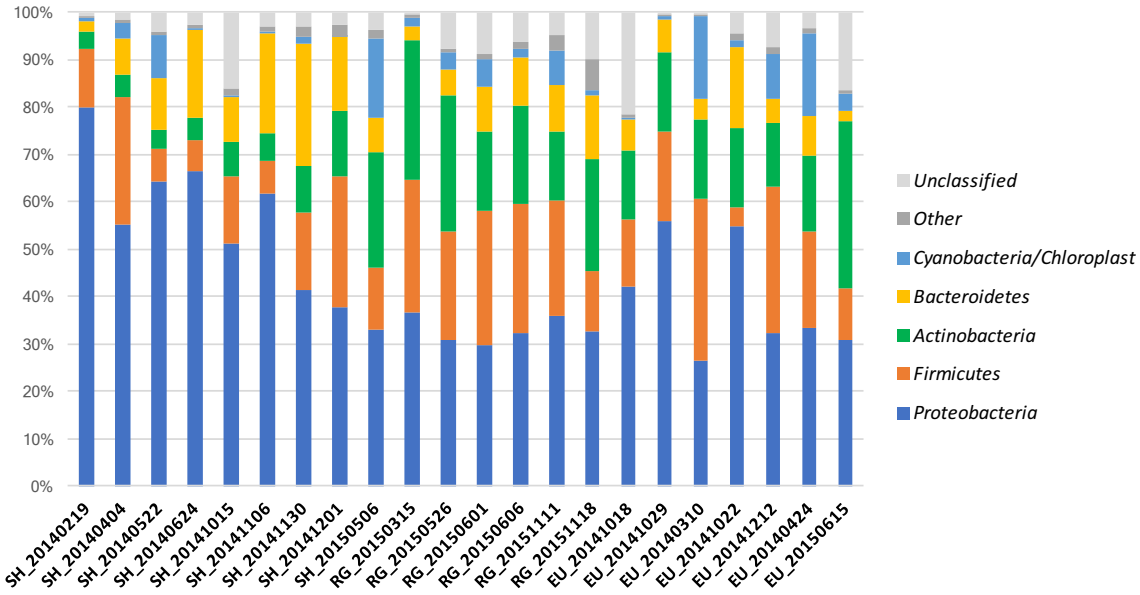

Figure 3.4. Distribution of the most abundant bacterial *phyla* (average abundance higher than 1%) in the PM_{10} samples.

Figure. 3.5 shows the distribution of the most abundant orders, i.e. those with an average relative abundance across all samples higher than 0.5%. Twenty-two orders overcame this abundance cut-off, with *Actinomycetales, Sphingomonadales, Burkholderiales, Bacillales, Clostridiales, Cytophagales* and *Rhizobiales* being the most abundant and representing, on average, almost 60% of all the community. As noticed at the level of phyla, also the order distribution was very different among all samples.

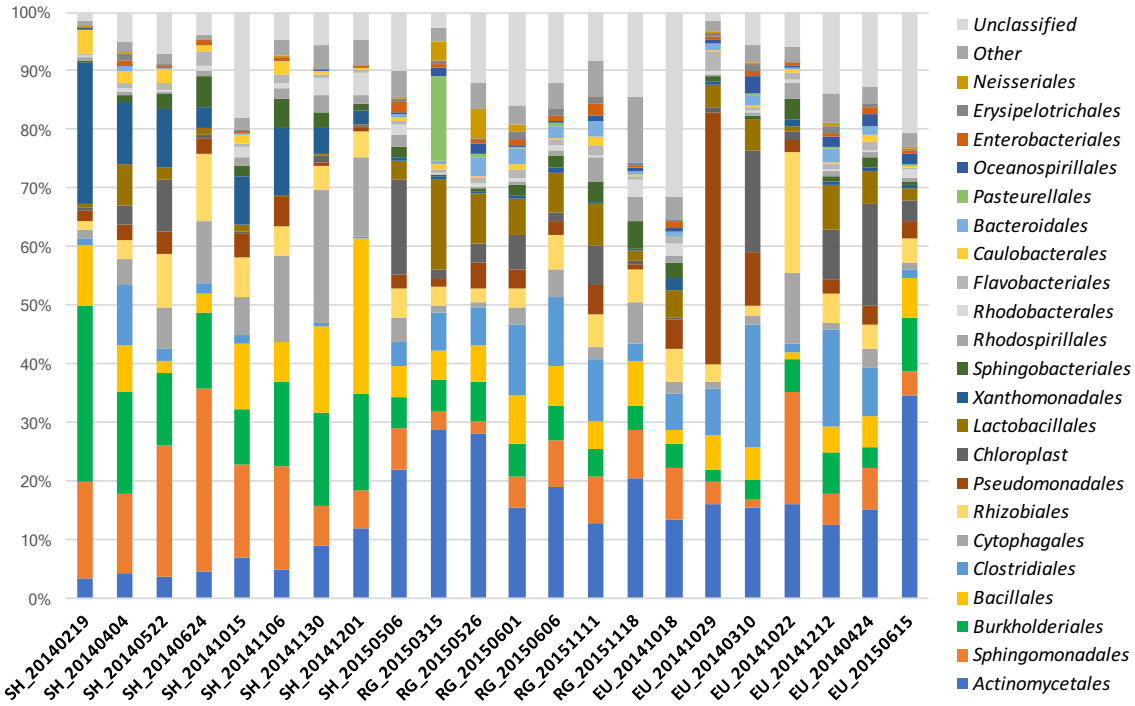

Figure 3.5. Distribution of the most abundant bacterial *orders* (average abundance higher than 0.5%) in the PM_{10} samples.

As stated before, gaining information on the taxonomic affiliation of the phylotypes found in the different samples is the best way to draw proper considerations about the bacterial populations in the particulate matter that were influenced by the air mass intrusions. In particular, analyzing the data at the genus level permits to fully appreciate the ecological meaning and implications of the bacterial biodiversity that characterized the PM_{10} samples analyzed in our study.

Despite a considerable fraction of the total biodiversity (18.6% on average) could not be described at this taxonomic depth, a total of 879 different genera were identified across all samples, indicating that complex bacterial communities were present in the bioaerosol. Those that could be considered as abundant, because showing a relative abundance higher than 0.5% in at least one sample, were 116. If only those with a relative abundance higher than 0.5% on average in all samples were considered, a total of 32 bacterial genera remained (Figure 3.6). Among these, the most abundant were *Sphingomonas* (8.47 %), followed by *Acidovorax* (3.89%), *Acinetobacter* (3.33%), *Stenotrophomonas* (3%), *Hymenobacter* (2.68%), *Methylobacterium* (2.57%), *Propionibacterium* (2.1%) and *Massilia* (1.85%).

At this taxonomic level, the distribution of the bacterial populations clearly showed differences due to the type of sample rather than other factors such as seasonality or the year of sampling. Interestingly, among all the PM samples, those collected during regional movements of air masses (codes RG) and, to a lesser extent, during European intrusions (codes EU), showed a high number of different genera with low abundance, indicating a highly diverse and even community. Conversely, PM_{10} during Saharan intrusions showed a lower number of genera but with higher abundances, indicating that these microbial communities were dominated by fewer phylotypes.

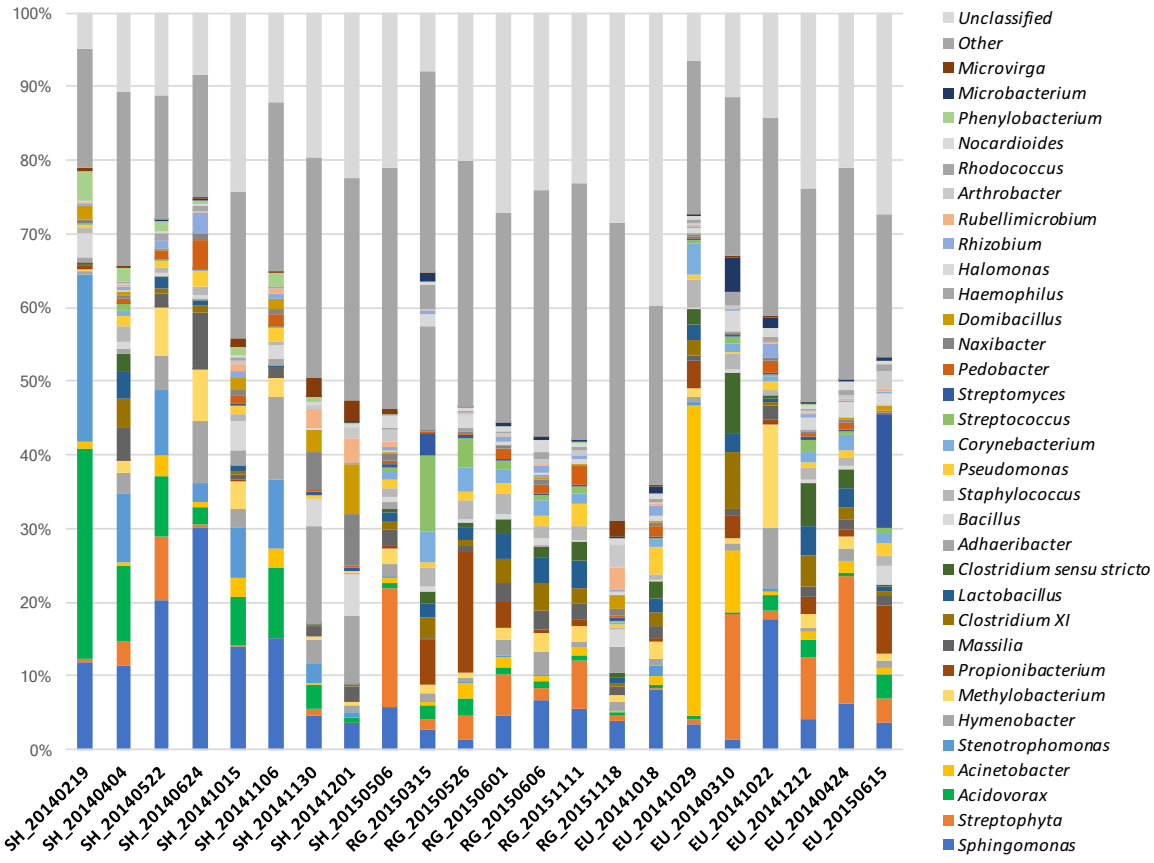

Figure 3.6. Distribution of the most abundant bacterial *genera* (average abundance higher than 0.5%) in the PM_{10} samples.

To gain further insights about similarities and differences among all the aerosol samples and to hypothesize possible effects of the air masses with different origins, a 2-D non-metric Multidimensional Scaling (NMDS) analysis was performed using the computation of Bray-Curtis distances between bacterial communities (Figure 3.7). The goal of NMDS is to find a configuration in a given number of dimensions which preserves rank-order

dissimilarities as closely as possible and thus provides "clusters" of samples that have a similar bacterial community structure.

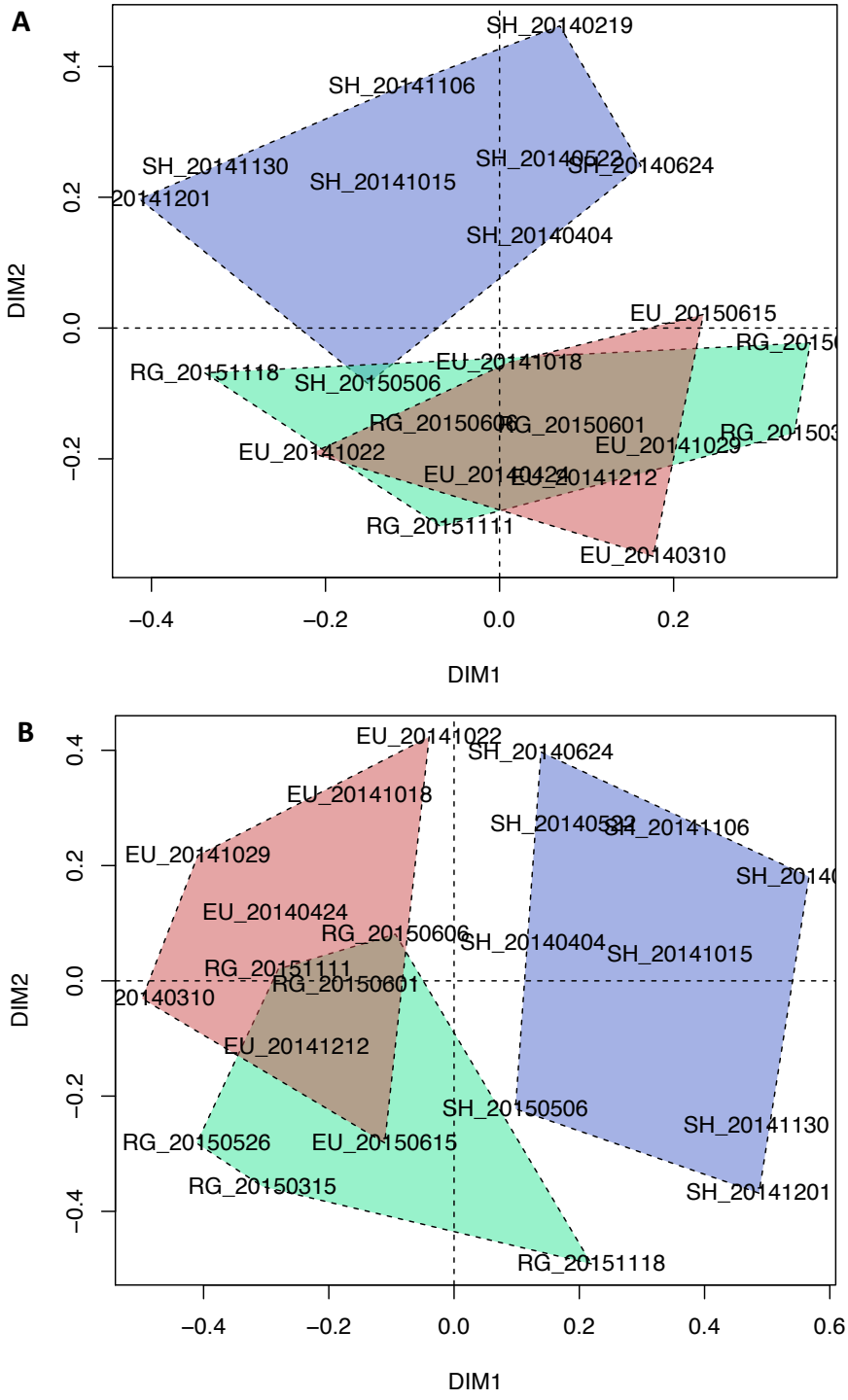

Figure 3.7. Non-metric Multidimensional Scaling (NMDS) ordination of Bray–Curtis distances between microbial communities of PM_{10} samples considering all genera (A) or only the most abundant (B). Each point represents an individual sample, with SH, RG and EU samples grouped in blue, green and red, respectively. 2D Stress values were equal to 0.23 and 0.20 for panel A and B, respectively.

Taking into consideration all the 879 different genera identified (Figure 3.7A), samples clustered into two well-separated groups: one included all the PM samples collected during the Saharan intrusions (codes SH, colored in blue) and one included the PM samples related to Regional and European air masses (codes RG colored in green and codes EU colored in red, respectively). When only the most abundant genera, namely those with a relative abundance higher than 0.5% on average in all samples, were considered for the NMDS analysis a further separation between the PM impacted by regional air masses and those collected during European intrusions could also be observed (Figure 3.7B). Similar indications were also provided by the 1-D dendrogram analysis reported in Figure 3.8.

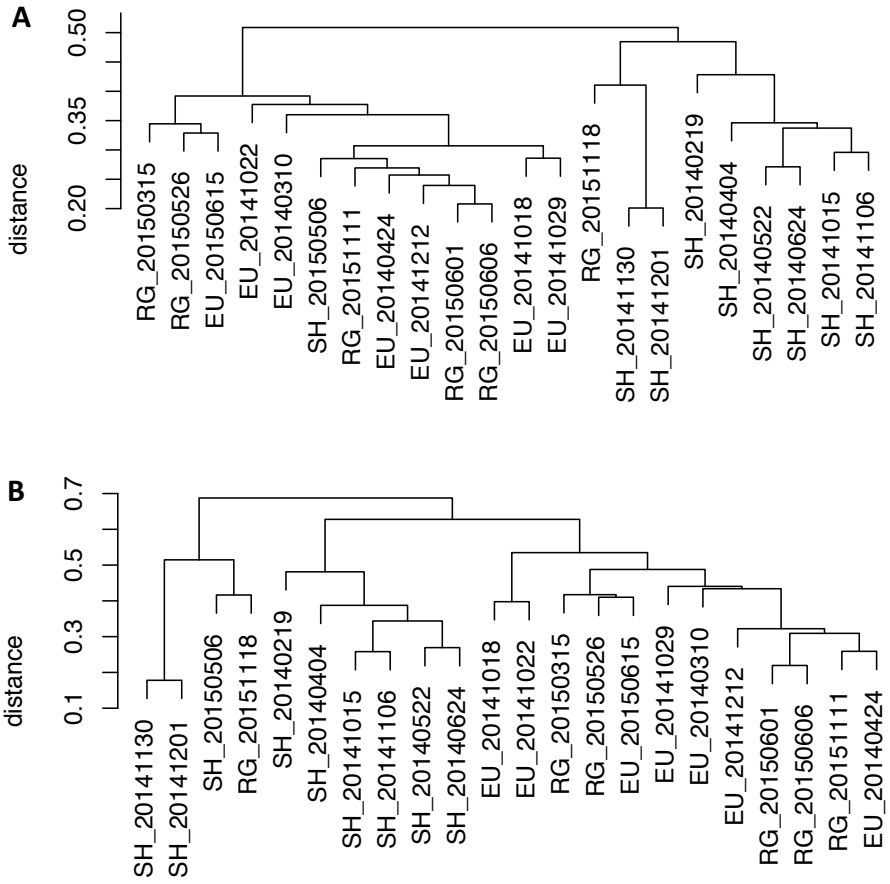

Figure 3.8. Dendrogram based on Bray–Curtis distances between microbial communities of PM_{10} samples considering all genera (A) or only the most abundant (B).

Taken together, both the 1-D dendrogram and 2-D NMDS analyses clearly indicated that the microbial communities of the PM_{10} sampled during Saharan intrusions were significantly different from those sampled when other air masses, either regional or

originated from different part of Europe, were present. These data strongly support our hypothesis that desert dust can impact the bacterial composition of the bioaerosol even at our latitudes.

As far as the β-diversity between the bacterial communities of the PM samples impacted by Saharan intrusions is concerned, it should be noticed that this was quite high. This can be easily deduced observing the average 1-D distances reported in the dendrogram (Figure 3.8), as well as by the 2-D area of the SH group in the NMDS graph (Figure 3.7). This suggests that the bacterial composition of the African dust, and consequently the impact on the local PM, were quite different and probably depended on other factors, such as, for example, the seasonality, the exact geographical origin of the air mass and the trajectory followed to reach our latitudes. Indeed, if we consider the bacterial composition and the chemical data related only to the Saharan-impacted samples, interesting correlations can be found. The PCA analysis reported in Figure 3.9, in fact, shows that three different groups can be drawn, even though they had their common origin in North Africa.

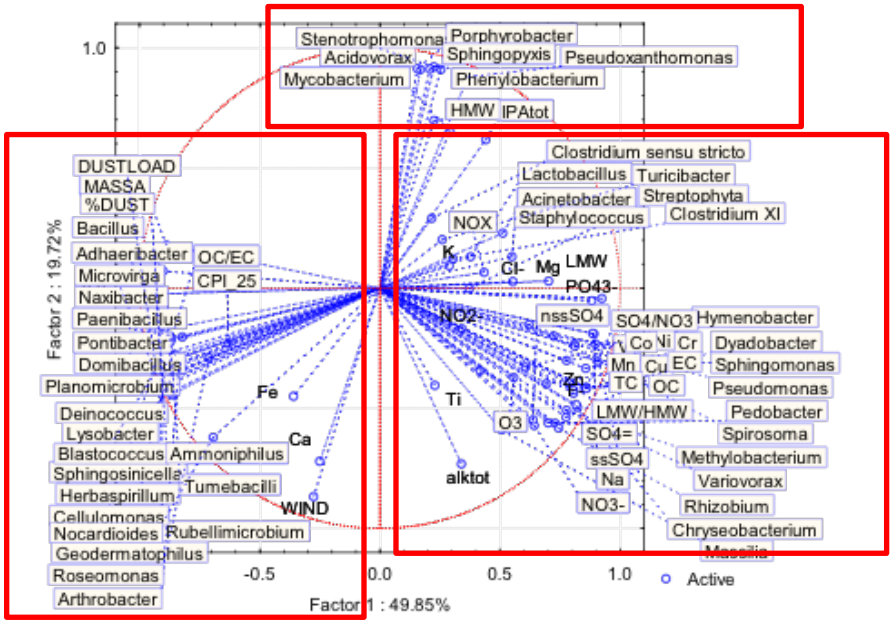

Figure 3.9. Principal-component analysis (PCA) of PM_{10} samples based on chemical variables and microbial communities.

Two samples, namely those relative to the intrusions of 30 November and 1st December 2014 made a cluster by their own. These samples, in fact, showed the most Saharan features based on back trajectory analysis, coarse fraction abundance (above 50 μg/m^3) and

chemical composition. As will be detailed in Chapter 4, these two samples were also characterized by the presence of many bacteria typical of arid environments. Another sample, namely that collected on 19 February 2014 was characterized by an high abundance of hydrocarbons that suggest a possible anthropogenic influence. The rest of the samples, beside featuring the presence of ubiquitous microorganisms and chemical elements, also included chemical and biological characteristics typical of marine environments. This could be explained by the trajectories followed by the air masses as well as their height above the sea level that may have caused the incorporation of marine and local bacterial populations and chemical elements.

3.3.3 Impact of Saharan intrusions on the bacterial diversity of particulate matter

With the aim of fully exploiting the information about the microbial composition of the bioaerosol impacted by Saharan intrusions and following-up on the indications revealed by the statistical analyses reported above, we herein present a comparison between the average bacterial profiles of the Sharan-impacted samples and those of the other two groups of samples, namely those impacted by regional and European currents.
Figures 3.10 and 3.11 report the most abundant *phyla* and orders, respectively.

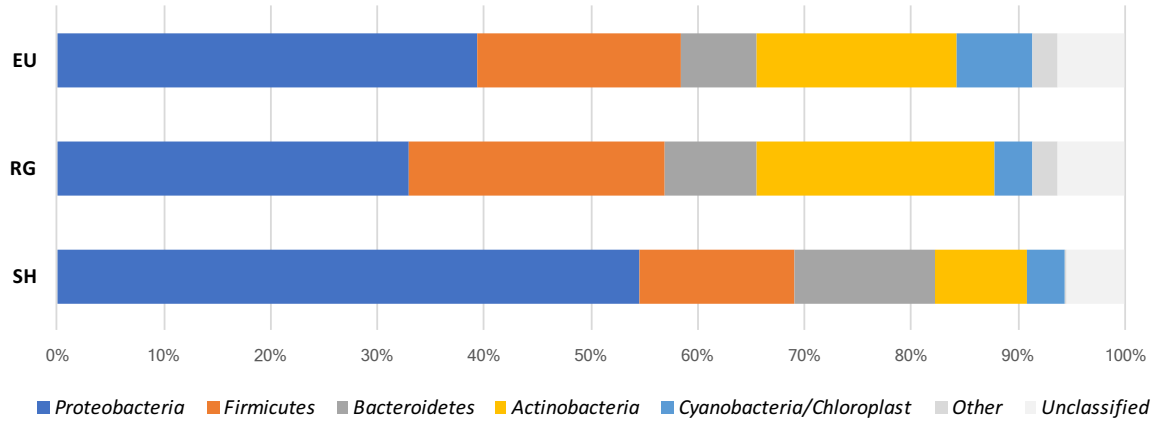

Figure 3.10. Average distribution of the most abundant bacterial *phyla* (more than 1% of total) in PM_{10} samples collected during intrusions of Saharan, Regional and European air masses.

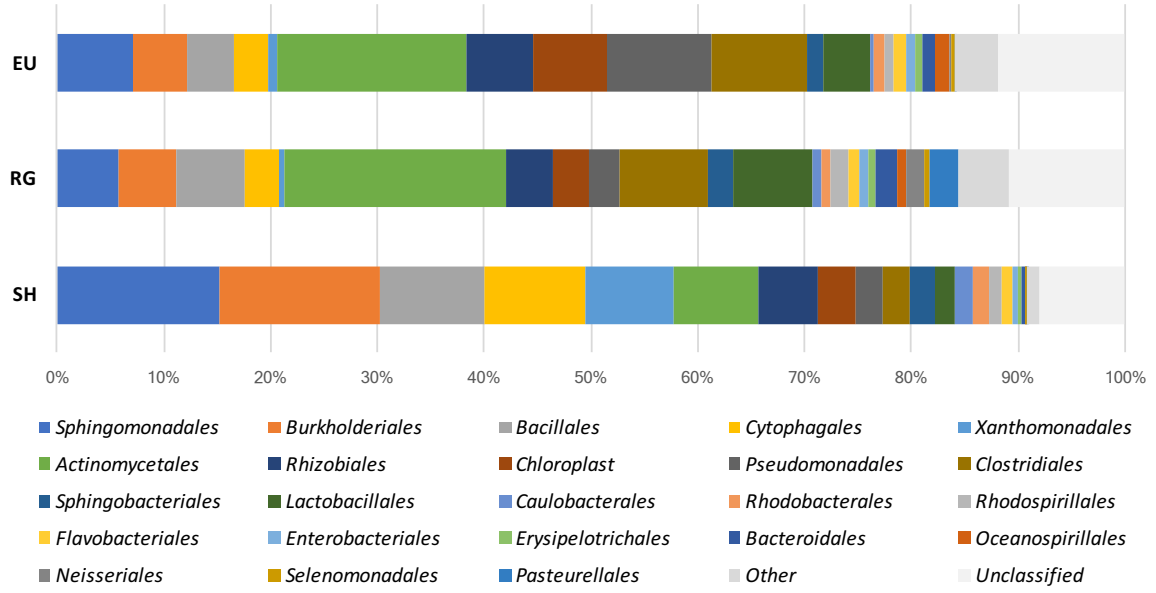

Figure 3.11. Average distribution of the most abundant bacterial *orders* (more than 0.5% of total) in PM_{10} samples collected during intrusions of Saharan, Regional and European air masses.

Samples with Saharan origin differed from all the others. Indeed, while *Firmicutes* and *Actinobacteria* decreased, *Proteobacteria* and *Bacteroidetes* showed an increment. At the order level, the Saharan-impacted PM samples showed, on average, a higher abundance of *Sphingomonadales, Burkholderiales, Bacillales, Cytophagales* and *Xanthomonadales* than both the other two groups of samples. On the contrary, *Actinomycetales, Clostridiales, Pseudomonadales, Rhizobiales* and *Lactobacillales* were higher in the samples impacted by European and/or regional currents.

The most abundant genera in the three groups of PM samples are reported in Figure 3.12.

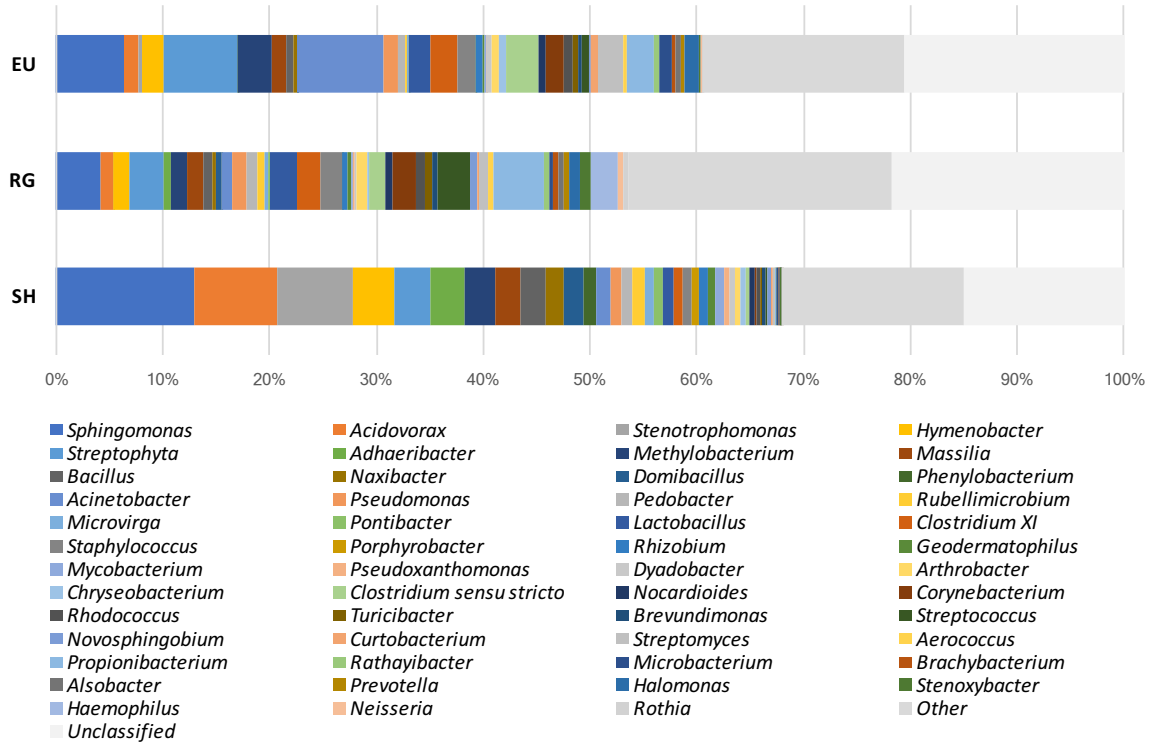

Figure 3.12. Average distribution of the most abundant bacterial *genera* (more than 0.5% of total) in PM_{10} samples collected during intrusions of Saharan, Regional and European air masses

At this taxonomic level, the differences between Saharan-impacted PM samples and those collected durum European and/or Regional ones appear striking. In order to underline the bacterial phylotypes that were significantly different in Sharan-impacted PM_{10} samples, the box-whiskers plot showed in Figures 3.13 and 3.14, report only the bacterial *genera* that were, respectively, significantly higher or lower in SH-impacted PM_{10} in comparison to either those impacted by Regional and/or European air masses. The dots in the plot show the relative abundances for each PM sample while boxes represent the abundances

following in the 75th percentile. Median and standard deviations together with p.-values are also shown. Saharan-impacted PM_{10} samples ware significantly enriched of *Sphingomonas, Acidovorax, Stenotrophomonas, Hymenobacter, Adheribacter, Bacillus, Domibacillus, Phenylobacterium, Microvirga, Porphyrobacter, Mycobacterium* and *Pseudoxanthomonas.* On the contrary, PM_{10} samples collected during Regional and/or European currents showed significantly higher abundances of *Acinetobacter, Propionibacterium, Streptococcus, Haemophilus, Lactobacillus, Corynebacterium, Staphylococcus, Halomonas, Microbacterium, Stenoxibacter, Novosphingobium, Methilobacterium, Turicibacter, Brchybacterium, Prevotella, Curtobacterium, Brevundimonas, Rathayibacter, Alsobacter* and *Aerococcus.*

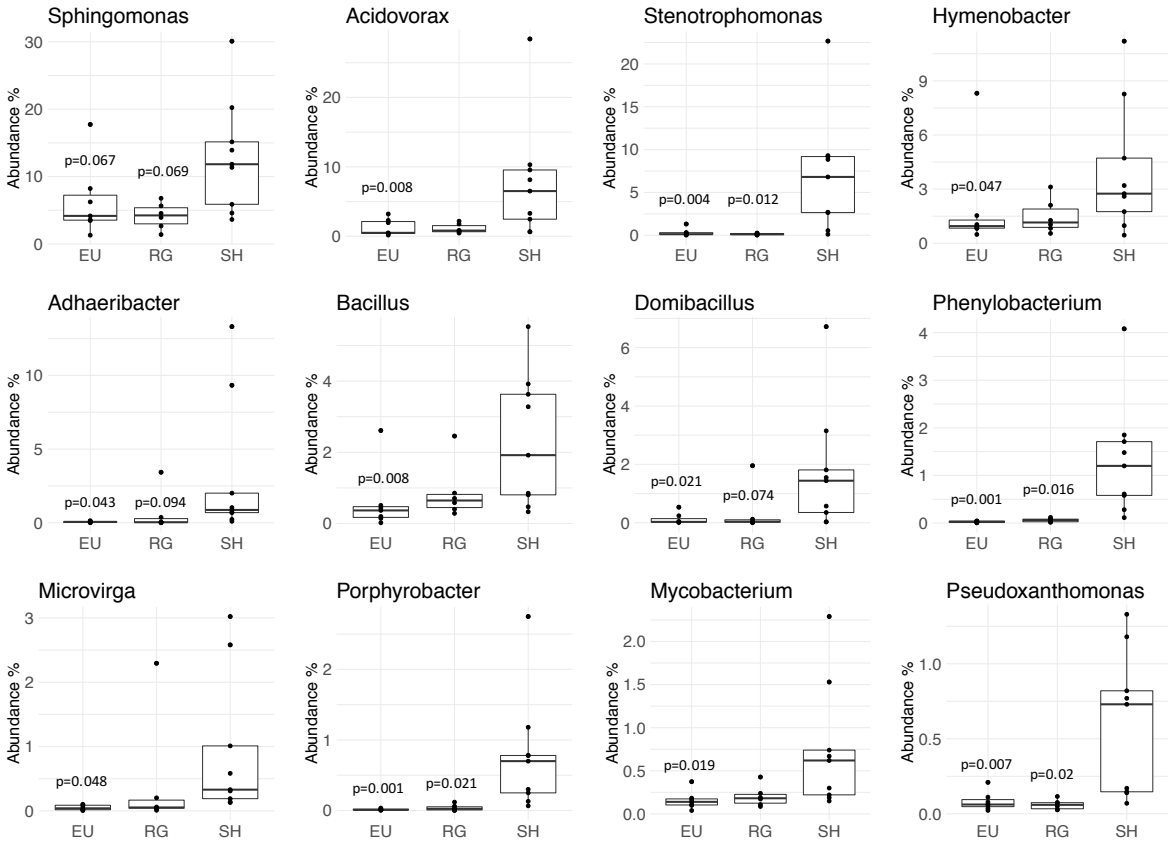

Figure 3.13. Box-whiskers plot depicting the relative abundances of the *genera* significantly higher in PM_{10} samples collected during Saharan intrusions (SH) compared to those related to Regional (RG) and/or European (EU) air masses. Box indicate the 75th percentile, error bars the SD and horizontal sign the median. p-values are reported only when values were significantly different.

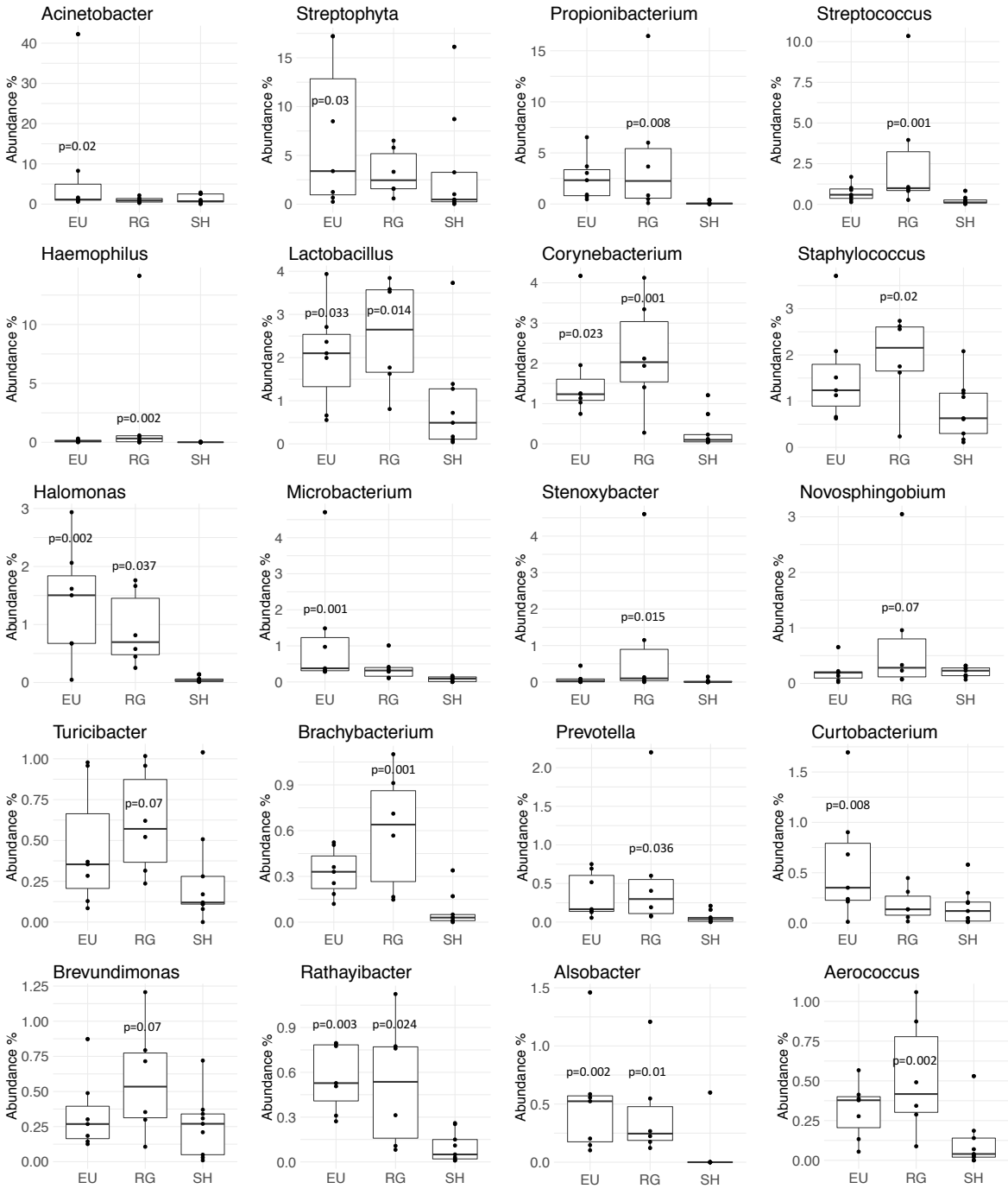

Figure 3.14. Box-whiskers plot depicting the relative abundances of the *genera* significantly lower in PM_{10} samples collected during Saharan intrusions (SH) compared to those related to Regional (RG) and/or European (EU) air masses. Box indicate the 75th percentile, error bars the SD and horizontal sign the median. p-values are reported only when values were significantly different.

3.4. Discussion

Although the microbial presence in atmospheric aerosols, has been recognized since the 19th century, it remained a scarcely investigated field of research until the last decade, maybe due to methodological limitations for describing microbial community composition, and because of its recently recognized links with climate change. Despite the probably key role played from long-range atmospheric dust transport for microbial dispersal, only recently, the interest is grown in investigation of the microbiological composition of bioaerosol through high-throughput technologies, as recently reviewed by Gandolfi et al. (2013) and Yoo et al (2017). Despite African desert dust is recognized as one of the most relevant source of particles in the atmosphere (Griffin 2007), few studies have, only recently, applied molecular based approaches to investigate the impact of African desert dust storms on particulate matter (Sańchez de la Campa et al 2013, Katra et al 2014, Rosselli et al 2015, Mazar et al 2016, Gat et al 2017). While the works of Katra et al. (2014), Mazar et al. (2016) and Gat et al (2017) were conducted in proximity of the dust sources, only Sańchez de la Campa et al (2013) and Rosselli et al. (2015) considered the long-range transport across the Mediterranean. Nevertheless, the former used a low-throughput approach (cloning and sequencing of 16S rRNA genes), the latter employed NGS but with few samples analyzed. Conversely, the study presented in this Chapter of the Thesis work is, as far as we know, the first one to investigate, using a high-throughput NGS approach, the microbial communities of PM_{10} impacted by different air masses, including Saharan dust, reaching Central Italy after long-range transport.

Back-trajectories and abundances of the coarse fraction of PM, allowed to first separate the PM_{10} samples into three group based on the impact of air masses of different origins (Goudi et al.2001): Saharan dust intrusions, Regional and European currents.

Saharan dust samples are known to be characterized for the majority by coarse particles (with a diameter >10μm). This substantial increment of the PM_{10} concentration in the sample coming from Africa, indicated that these intrusions impact the local aerosol bringing a high amount of particles that may have many effects on the chemical and microbiological composition of local aerosol. The high concentration of the typical desert elements such as Fe, Ca and Ti, reflected that the particulate matter collected had the

peculiar chemical characteristics of desert crust and provided another evidence that African air-masses can reach our latitudes. Furthermore, the samples coming from Europe and the local ones were characterized by the presence of the typical elements deriving from human and/or industrial activities. Nevertheless, within the same group of origins, and in particular in the case of those impacted by desert dust, the air-masses followed different trajectories to reach the sampling site, causing a variability among samples. The Saharan intrusions, for instance, even if originated from the same area (North Africa), could come straight to Italy (as those of 30 November and 1st December), or can turn over the Mediterranean basin and Southern Europe. This provided peculiar physic-chemical and microbiological characteristics.

Using a culture-independent approach based on Illumina NGS of 16S rRNA, we were able to deeply investigate the biodiversity of the microbial communities present in the PM_{10} samples. The results underlined that the diversity of the airborne microorganisms in the atmosphere was very high and similar to that of terrestrial and aquatic environments. Further, the analysis of the β diversity, i.e. that among the bacterial communities of all the PM samples, showed that the microbial communities of the PM_{10} sampled during Saharan intrusions were significantly different from those sampled when other air masses, either regional or originated from different part of Europe, were present, strongly supporting the hypothesis that desert dust can impact the bacterial composition of the bioaerosol even at our latitudes (Rosselli et al 2015, Mazar et al 2016, Gat et al 2017). On the contrary, European currents and Regional circulations showed more similar communities, probably because of the influence of similar environments in Italy as well as the rest of Europe, characterized by the presence of the sea as well as areas rich of vegetation or highly anthropized. Nevertheless, as observed for the chemical characteristics, even if similarities existed within the PM samples with the same origin, the differences were not negligible and suggested that each event was independent from the others. This was particularly true for the Saharan cluster, where each sample was well separated from the other. The only exceptions were the two samples of the 30 November and 1st December that were very similar and, indeed, represented two intrusions reaching the sampling site over a period of just two days.

As far as the bacterial diversity is concerned, our data provided interesting insights on the composition of dust-carried bacteria, that were, at least in part, similar to the findings of similar works (Polymenakou et al. 2008, Liu et al. 2009, Favet et al. 2013, Sańchez de la Campa et al 2013, Katra et al 2014, Rosselli et al 2015, Mazar et al 2016, Itani et al. 2016,

Gat et al 2017). Among the most abundant bacteria found in the Saharan –impacted samples, it is important to mention *Pseudomonas, Bacillus, Micobacterium* that include potential pathogen species but also many others that can usually found as soil-associated and often isolated from arid soils (Polymenakou et al. 2008, Favet et al. 2013, Itani et al. 2016, Liu et al. 2009). On the other hand, *Streptococcus, Propionibacterium, Haemophilus* and *Staphylococcus*, higher in the European or in Regional samples, include many human and/or animal pathogens. For instance, species of *Heamophilus* were found responsible for acute bacterial meningitis in infants and young children and for chronic pulmonary disease in adults. Species of *Streptococcus* can cause pneumonia, bacterial sinusitis, acute otitis and meningitis, members of *Propionibacterium* are suspected to induce pathologic reactions such as endocarditis (Polimenakou et al. 2008) and *Staphylococcus* species can cause bacteremic pneumonia. Even though the presence of these potential pathogens, there are no reports as yet of human infectious diseases related to long-range transport with desert dust (Kellog et al. 2006).

Among the most abundant desert typical bacteria found, *Sphingomonas, Acinetobacter, Acidovorax, Stenotrophomonas, Methylobacterium, Microvirga and Massilia* are member of the phylum *Proteobacteria,* that comprise one of the largest divisions within prokaryotes and account for the vast majority of the known Gram-negative bacteria. (Radhey et al. 2000). This phylum is frequently represented in soils; the beta-proteobacterium *Massilia* (oxalobacteriaceae), was found in many extreme environments impacted by desert dust (Chuvochina et al. 2011), indicating that it is very resilient bacteria and that it may survive to long range transport. *Methylobacterium i*s known to be resistant to desiccation and to γ radiation together with *Arthrobacter*, also abundant in our samples. (Favet et al. 2013). *Microvirga* is already found in desert-coming air-masses and some species of this genus can reduce nitrogen gas to ammonia (Favet et al. 2013). An interesting case is represented by *Acinetobacter*, that was present in the Saharan samples but showed a significant increment in European group. This is a bacterium recently proposed as indicator of airborne-transport from the Sahara, but that also includes contain opportunistic human pathogens; this group pf bacteria are ubiquitous in soil and water, easily transported by air (Barberàn et al.2014). Although *Sphingomonas* represent a quite ubiquitous genus, it is often found in desert-originated air samples (Griffin et al.2001, 2003). *Adhaeribacter,* and *Hymenobacter* belonged to *Bacteroidetes* that is a group of Gram-negative, non-spore-forming, anaerobic, and rod-shaped bacteria that are widely distributed in the environment, including in soil, sediments, and sea water. An important

characteristic for desert bacteria is pigmentation. Indeed, this, can protect microorganisms from the harsh conditions of deserts such as high (and low) temperature, (e.g. *Rubellimicrobium*), solar radiation, desiccation (*Arthrobacter*) and γ- radiation (*Hymenobacter*).

The results obtained in this part of the Thesis work, confirmed how new microbiological culture-independent approaches, such as NGS technologies, can improve our ability to investigate the variation in space and time of the bacteria present in particulate matter, allowing the identification of the environmental factors that determine this variability and the potential sources of bacteria in the atmosphere. The data obtained clearly showed that the bacteria carried by Saharan dust strongly affected the community structure of local particulate matter, making them very different from those found when either Regional or European air masses occurred. These evidences confirmed that the atmosphere, despite being an environment characterized by harsh conditions, features a high microbial biodiversity as a great variety of microorganisms can be transported with dust, thus expanding their bio-geographical range.

CHAPTER 4

BIODIVERSITY AND CHEMICAL SPECIATION OF ATMOSPHERIC PARTICULATE MATTER TRANSPORTED THROUGH THE MEDITERRANEAN BASIN DURING AN INTENSE AFRICAN DUST EVENT

4.1 Introduction

Bacterial migration is a natural phenomenon caused by ocean currents and, above all, atmospheric events such as desert dust. When a wind-sand stream occurs, dust particles and microbes can be lifted and transported over long distances. The major sources of desert dust is the Saharan region of Africa (Yamagouchi et al. 2014), but also the Gobi, Takla Makan, and Badain Jaran Deserts of Asia (Griffin 2007) The global atmospheric mobilization of dust has increased in the last decades due to persistent drought in desertic regions over the last 40 years, the onset of commercial agriculture, and the increase in land-use practices, that brought to a desiccation of large aquatic regions, decreasing of vegetation cover and increasing the frequency and intensity of dust storm (Barberan et al. 2014). There are both benefits and potential hazards related to soils moving through the planet's atmosphere. One of the benefits of the redistribution of soils through the atmosphere is that many terrestrial plants derive nutrients from dust-fallout in down-wind ecosystems. Negative impacts of dust movement include the transport of toxins such as pesticide and herbicide-laden soils and the long-range movement of pathogenic microorganisms (Griffin et al. 2003, 2001a).

It's well known, that airborne dust particles contain many microorganisms that can survive prolonged transport in the atmosphere and potentially they can even be metabolically active while aloft (Barberan et al. 2014). African desert storms cross the eastern Mediterranean and inject a large pulse of microorganisms into the European air, expanding their biogeographical range. Indeed, microorganisms can be transported in and through the continents with atmospheric currents at high altitude. Even though air provides a hostile environment for microorganisms, exposing them to UV light, paucity of water, decreasing temperature, oxygen concentration and pressure, there is a growing body of evidence about the abundance of bacteria in the atmosphere (Fahlgren et al. 2010)**.** While these atmospheric stresses may be lethal to a portion of the microbial populations moving with airborne dust, some organisms may resist thanks to peculiar features, such as pigmentation, high G + C nucleic content, enhanced DNA repair ability and UV resistance). Movement within dust clouds and over certain environments, such as water masses, can limit UV or humidity stress. In recent years, the investigation of microbial community's composition and the dynamics during their long-range transport with desert dust has been steadily increasing. Attention has been paid to assess whether this phenomenon is to be considered as a passive biomass

transportation or, at least in some cases, bacteria may be viable and metabolically active (Griffin et al. 2001a, Kellog et al. 2004, Favet et al. 2013, Prospero et al.2005, Rosselli et al. 2015). It is worth noting that the study of the biotic fraction of particulate matter is important because, beside the well-known negative effects exerted by the dust particles, the microorganisms associated with these particles may cause and/or exacerbate human pathologies (Griffin 2007). Cultivation has been the gold method used since the first aerobiological studies that aimed at investigating airborne microbial communities in outdoor environments. Indeed, despite all its limit principally due by the fact that only a small fraction of microorganisms is cultivable (1%), traditional methods based on cultivations are the only way for the isolation of microbial strains, which is still the most direct method to obtain reliable information on physiologic and metabolic characteristics of bacteria.

In this Chapter, we report the chemical and microbial characterization of particulate matter during an intense African desert dust storm that reached Central Italy on 30 November - 1st December of 2014. This was considered the strongest Saharan desert dust intrusion occurring in Italy in the last period. We coupled a culture-independent analysis (NGS of 16S rRNA genes) with traditional culture-based approaches to characterize, on the one hand, the total bacterial biodiversity, and, on the other hand, to investigate the viability and metabolic activity of the bacteria reaching our latitude after the long-range transport over the Mediterranean. Most of the studies on the cultivable fraction of bacteria present in Asian and African desert dusts have been conducted in anthropized areas, where it may be difficult to discern the impact of desert dust with the local urban particulate matter. On the contrary, we used the remote sampling site of *Monte Martano*. which, being far from large metropolitan areas and other relevant human activities, allowed us to clearly distinguish long-range desert intrusions from other currents and local air circulation, as demonstrated in Chapter 3 of this Thesis work.

4.2 Material and Methods

4.2.1. Sampling of particulate matter

Particulate matter (PM_{10}) was sampled at *Monte Martano* (MM), a medium altitude background site for air quality monitoring and atmospheric chemistry studies located in Central Italy, on the ridge of the *Martani* mountain chain (latitude 42°48′19″; longitude 12° 33′55″; altitude 1100 m asl).

Samples were collected on quartz fiber filters (Whatman QMA 20.3 x 25.4 cm) previously sterilized under UV lamp for 50 minutes on both sides, with a high-volume sampler (TISH, TE6001) operated at a flux of 1140 L min^{-1}. Sampling was carried out the 30th of November and 1st of December 2014 and two separate PM_{10} samples were collected.

Air mass back-trajectories were calculated using the HYSPLIT 4.9 (rev. 513, 2013-8-5) transport model (Draxler and Hess, 2003) using meteorological data fields supplied by the ARL (Air Resources Lab, http://ready.arl.noaa.gov/gdas1) archiving programs input data. In order to characterize the general behavior of air masses, 4-day backtrajectories (BT) were run every 6 h at an endpoint located 1000 agl over MM. Standard 1° latitude/longitude dataset output from the NCEP Global Data Assimilation System model (GDAS) on pressure surfaces, has been used. The ensemble of 1340 calculated BTs was characterized by the cluster analysis package included in the 4.9 version of HYSPLIT. Moreover, in depth calculations have been conducted for selected days at higher resolution. Specifically, we exploited a 3 hourly, global, 0.5 × 0.5 degree latitude/longitude dataset on hybrid sigma pressure surfaces.

4.2.2. Chemical analyses

The soluble ionic inorganic fraction was evaluated by ion chromatography (IC) (by means of a DIONEX 500 instrument) after extraction by ultrasonication in ultrapure water (18 MΩ). The total concentration of alkali metals and many transition elements was determined by ICP-AES equipped with ultrasonic nebulizer (CETAC Technologies, U-5000AT), after acid digestion of the samples in a HNO3:H2O2 (3:1) mixture, assisted by a microwave oven

(MARS express, CEM). Suprapur® grade acids and multielement standard solutions from Merck (Darmstadt, Germany) were used. Reagent blanks and certified solutions (EnvironMAT, SCP Science) were employed to check precision and accuracy by replicated analyses. The elemental carbon (EC) and organic carbon (OC) mass concentrations were determined by thermal optical transmittance (TOT) technique (Sunset Carbon Analyzer) exploiting the high-temperature NIOSH protocol (Moroni et al. 2015)

4.2.3. Culture-independent microbiological analyses

Culture-independent assessment of microbial communities in PM_{10}, was carried on with a NGS approach based on the Illumina HISeq platform.

Metagenomic DNA was extracted from a 6 cm^2 portion of the high-volume filters. In aseptic conditions, filters were suspended in40 mL of sterile water, shaken for 1h and centrifuged twice (30' at 10000 x g and 15' at 11500 x g) to recover bacteria (Suarez et al. 2006, Radosevich et al. 2002). DNA was extracted from the pellet using the POWER SOIL DNA kit (MOBio) following the manufacturer's protocol.

The V5-V6 hypervariable regions of the 16S rRNA were amplified in 2 x 50 µL volume reactions with a Hot start Taq polymerase (Solis-Biodyne). The reaction included 1 µM each of primers 783F and 1027R (Wang and Qian, 2009, Huber et al. 2007). At the 5' end of 783F primer, one of 15 6-bp barcodes was also included to allow sample pooling and subsequent sequence sorting. The cycling conditions were: initial denaturation at 94°C for 5 min; 29 cycles of 94°C for 50 s, 47°C for 30 s, and 72°C for 30 s and final extension at 72°C for 5 min. The amplified products were purified with the Wizard SV PCR purification kit (Promega Corporation) and amplicon quantity was evaluated by Qbit (Invitrogen).

Multiplexed samples were prepared with the Illumina Multiplexing Sample Preparation Oligonucleotide Kit, which provides 12 index oligos for pooling up to 12 samples per lane. Therefore, the tagging system with 8 barcodes at the 5' end of the 783F primer allowed pooling up to 96 samples per lane. Multiplexed sequencing of all the pooled samples was performed on a single Illumina Hiseq 1000 lane, using a paired-end 2x100 base-pair protocol and the 4.0 sequencing chemistry, by *Parco Tecnologico Padano*.

Each sequence was assigned to its original sample according to its index oligos and barcode. After sorting the sequences, the reverse read of each paired-end sequence was reverse

complemented and merged with the corresponding forward read. A quality cut-off was applied in order to remove the sequences that did not contain the barcode, those with an average base quality value (Q) lower than 30 and those that did not provide a perfect match in the overlapping part between the two pair-ends. The barcode was removed and sequences were sorted into Operational Taxonomic Units (OTUs) using the UPARSE-OTU algorithm. The minimum identity between each OTU member sequence and the representative sequence (*i.e.* the sequence that showed the minimum distance to all other sequences in the OTU) was set to 97%. The taxonomic classification of each OTU was carried on with the stand-alone version of RDP Bayesian Classifier, using a 50% confidence level.

4.2.4. Culture-dependent microbiological analyses

Viable bacterial populations were cultured from the PM by cutting-off a 6x6 square, then suspended in sterile water. Serial dilutions of the suspension were plated on R2A agar plates and incubated at three different temperature: 4 °C, 27°C and 50 °C with the aim of covering the highest biodiversity. After incubation colonies were counted and representative colonies were selected based on shape and color and isolated each at its own growth temperature.
To identify the bacterial isolate, DNA was extracted with the Ultraclean Microbial DNA isolation kit (MO BIO Laboratories). The 16S rRNA gene was amplified by PCR with the Hot start Taq polymerase (Fermentas) and the primers 8F and 1507R (AGAGTTTGATCMTGGCTCAG and TACCTTGTTACGACTT, respectively). PCR amplification was carried out as follow: initial denaturation at 95°C for 3 min; 34 cycles of 95°C for 60 s, 50°C for 45 s, and 72°C for 2 min and final extension at 72°C for 30 min. Five ul of 50ng/ul of PCR product were purified, added with 5ul of 5pmole/ul sequencing primer 8F and sequenced by Macrogene Europe. Taxonomic attribution of each sequence was carried out using Ribosomal Database Project classifier and BLAST, while the phylogenetic tree, including all the retrieved sequences was constructed with the software MEGA (http://www.megasoftware.net/index.html).
Enzymatic activities involved on the degradation of complex organic substrates were tested for each isolate to determine, together with their viability, also the metabolic potential. The following tests were performed at the respective growth temperature as well as 4°C in order to determine the possible activity at the low temperatures proper of the atmosphere.

Amylase activity was assessed by adding 2g/L of soluble amid to nutrient agar medium. After 24/48h of incubation with bacterial isolates, each plate was covered with 350ul of iodine solution (lugol solution), and the degradation was visible as a yellow halo in the blue medium.

Protease activity was assessed using a specific culture medium prepared by sterilizing water with agar (121 C° for 15') and adding after that, sterile skimmed milk heated previously at 80°C with the medium. After incubation, if the isolate plated showed protease activity an halo was present around the colony, indicating bacterial degradation.

Lipase producers were determined on agar plates by using tributyrin added to a sterilized medium composed by agar, peptone and yeast extract. After plating and incubation, if the bacterial isolate had lipase activity a halo was visible around the colony.

Esterase activity was carried out with a specific agar medium prepared with Agar, peptone, NaCl, $CaCl_2$ and Tween80 and sterilized by autoclaving. Isolates were plated and after incubation the presence of the enzymatic activity was revelaed by a degradation halo around the colony.

A test for assessing the ability to survive the exposure to UV was also performed on one strain for each of the most abundant species. Bacterial cell were grown overnight in Nutrient Broth (yeast extract 2gr/l, peptone 5gr/l, NaCl 5gr/l), at their isolation temperature (4°C, 27°C, 50 °C). After growth, serial dilutions were provided and 100µL of each dilution was plated on R2A agar Plates were expose to UV lamp for 1 and 5 minutes. Irradiated plates were incubated in the dark each one at its isolation temperature and the number of survived bacteria was determined as count of irradiated cells divided by those of the non-irradiated control cells (Sanchéz de la Campa et al. 2013).

4.3. Results

4.3.1 Chemical speciation of dust-impacted PM

An intense African desert dust storm reached Central Italy on 30 November - 1st December of 2014. This was considered the strongest Saharan desert dust intrusion occurring in Italy in the last period. As shown in Figure 4.1, the air-masse origin was confirmed by analysis of the back-trajectories, as calculated with the HYSPLIT Trajectory Model (Stein et al., 2015) and by the concentration of the coarse fraction, i.e. the particles included between PM10 and PM2.5 (Karanasiou et al. 2012, Moroni et al, 2015). It is possible to notice that the PM samples were related to sequential intrusions of desert dust from North Africa: dated 30 November and 1st December. Both were characterized by air masses reaching the sampling site directly from North Africa and associated to very fast winds.

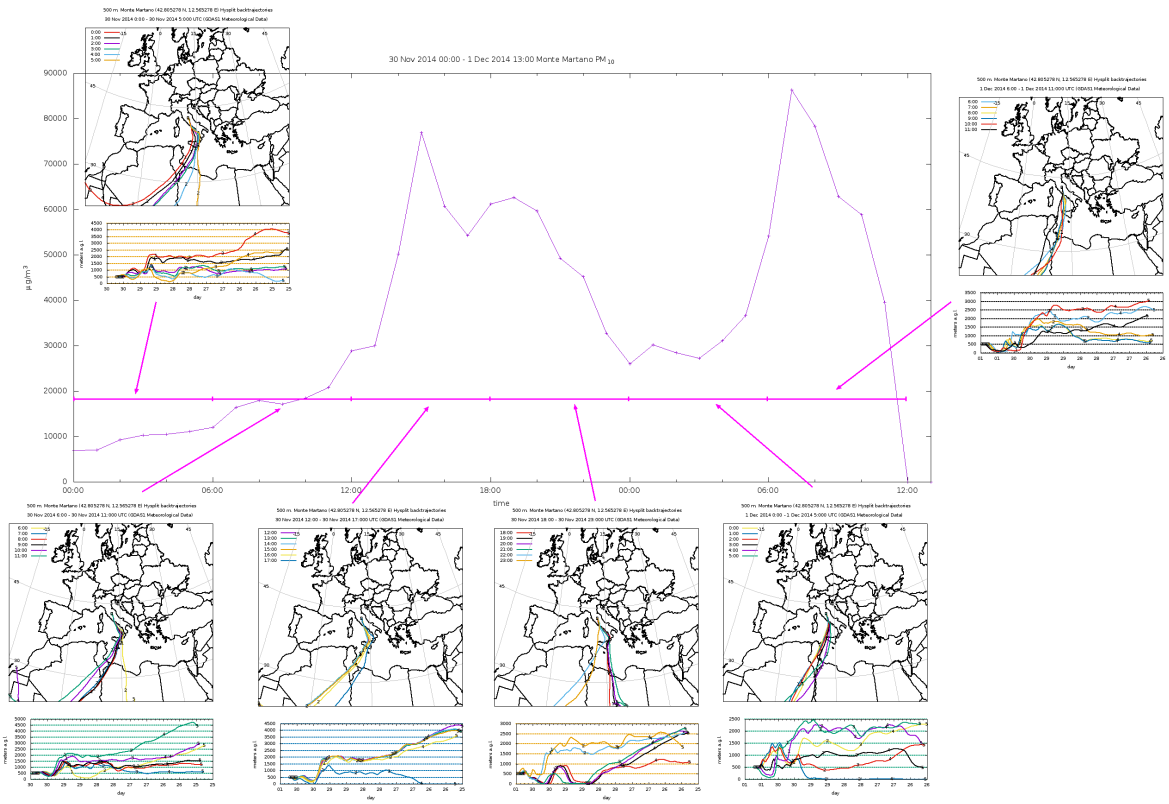

Figure 4.1. Bach trajectories and coarse fraction concentrations of the intrusions reaching the *Monte Martano* sampling site: 30 November and 1st December of 2014.

The concentration of the main inorganic ions and carbonaceous (EC, OC) components identified in the present Saharan dust samples is reported in Table 4.1, as μg m^{-3}, while oncentration of trace elements are reported in Table 4.2, expressed as ppm (weight/weight). Data are compared with 2014 year averages obtained by analysis of the routine HVS samplings at the sampling site.

Table 4.1. Concentration of major ions, expressed in μg m^{-3}. Data are compared with 2014 year averages (± SD) The relative species contribution to PM mass is reported in parentheses.

Ion	SH_30nov	SH_1dec	2014 average
Na^+	1.29	1.68	0.24 (0.09)
K^+	0.20	0.37	0.05 (0.02)
NH_4^+	0.22	0.14	0.46 (0.15)
Mg^{2+}	0.12	0.22	0.025 (0.08)
Ca^{2+}	1.32	4.29	0.26 (0.20)
Cl^-	0.76	1.30	0.091 (0.04)
NO_3^-	0.85	1.37	0.95 (0.6)
PO_4^{3-}	1.88	0.75	0.018 (0.02)
$ssSO_4^=$	0.32	0.42	0.06 (0.02)
$nssSO_4^=$	2.69	9.22	1.30 (0.3)
OC	2.89	3.73	2.83 (0.9)
EC	0.070	0.130	0.200 (0.07)
OM	4.9	6.3	4.8 (1.5)
PM10	83.6	86.9	12.7

Table 4.2. Concentration (w/w, ppm) of trace elements compared to 2014 year averages (±SD)

Element	SH_30nov	SH_1dec	2014 average
Zn	418	554	1100 (380)
Cr	274	353	86 (50)
Ni	154	183	70 (20)
Cu	531	610	115 (40)
V	220	287	54 (20)
Mn	359	1030	114 (40)
Ti	713	2190	133 (53)
Fe	22500	80500	3360 (1400)
Ca*	52800	125900	n.a.

The ionic species were nearly in balance in both samples even if a small shift from a slightly acidic aerosol the 30 of November sample (-0.009 μEq m^{-3}) towards a more basic condition the 1st of December (+0.04 μEq m^{-3}), is apparent. Many Saharan dust proxies show an increase of concentration with respect to the year average, noticeably Ca, Fe, Ti. The increase

was more marked for the 1st of December sample. The Ca/Fe ratios were in the range (>1) typical of Saharan dust intrusions in the Mediterranean basin (Scheuvens et al. 2013). On the other side ammonium and elemental carbon (EC) concentrations were lower in the Saharan samples with respect to the year average. The Mg/Na ratio for both samples were close to the year average and matches the expected ratio in sea-water (0.12) while the Cl/Na ratio (0.59 and 0.77, for the two samples respectively) was much less than expected for sea-water (1.8). Chlorine depletion is a well now process in long rang transported aerosol well documented in literature also for Saharan dust intrusions (Koçak et al. 2004). Interestingly the year average value (0.4) was even lower at MM, which typically experience long-range transported aerosol, particularly from Eastern Europe. The organic matter (OM) levels, in terms of mass per volume of air, were of the same order of magnitude for both samples and similar to the year average. By contrast, the mass-to-mass concentration of OM clearly decreased from nearly 40% of the year average to less than 10% in the present samples, due to the much higher concentration of mineral dust.

4.3.2 Bacterial diversity of dust-impacted PM

Next-generation sequencing of 16S rRNA gene fragments led to the retrieval of a total of 1439 bacterial OTUs, calculated applying a threshold of 97% sequence similarity, and allowed the computation of alpha-diversity indices for the bacterial communities present in the two PM_{10} samples (Table 4.3). Both samples were characterized by a high species richness and diversity, as indicated by the number of observed OTUs and by Shannon index values, respectively. Further, the high evenness values showed that both the communities were characterized by a quite homogenous distribution of the most abundant species. The coverage assessment, evaluated as the ratio between the observed and expected number of OTUs (S_{obs}/S_{Chao}), indicated that our analysis reached a good saturation of the expected bacterial diversity (nearly 70% for both samples, Table 4.3).

Table 4.3 Alpha-diversity indices based on OTUs distribution in the two PM_{10} samples

Sample	S_{obs}	S_{Chao}	H	E
30 Nov	1151	1695	5.696	0.808
1 Dec	1010	1464	5.483	0.793

S_{obs}, observed richness (number of OTUs); S_{Chao}, expected richness (Chao estimator); H, Shannon index; E, Evenness.

The number of OTUs was strongly reduced after removing singletons (OTUs with only one sequence read), namely from 1439 to 863. Among these 863 OTUs, 771 and 722 were recovered from the sample collected during the intrusion of 30 November and 1st December. Interestingly, 630 OTUs were shared across the two samples and represented, on average, more than 95% of all reads in each sample (Figure 4.2). Indeed, the OTUs that were exclusively present only in one of the two samples were only 141 and 92 for the 30 November and 1st December sample, respectively and represented only a minor part of all the reads in each sample (5.7 and 2.7 %).

To gain further insights into the identity and distribution of the bacterial populations in the two PM10 samples, the reads obtained from each one, irrespectively of their OTU assignment, were given a taxonomic attribution using the RDP Bayesian Classifier (http://rdp.cme.msu.edu), applying a threshold of at least 50% confidence.

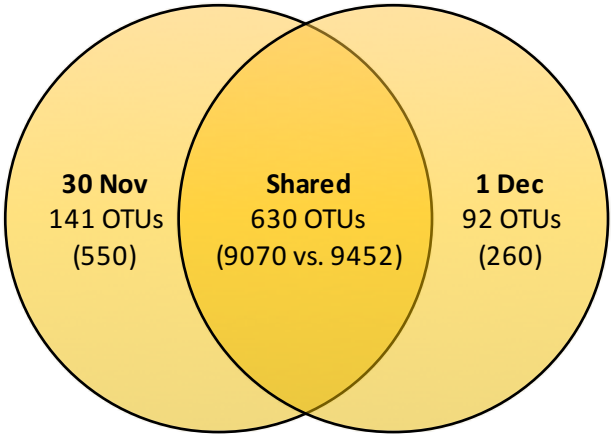

Figure 4.2. Venn diagram depicting the number of OTUs shared and exclusively present in the two PM samples impacted by Saharan intrusions reaching *Monte Martano* on 30 November and 1st December of 2014. Number in parenthesis are the sequence reads in each sample.

Excluding phylotypes represented by only one sequence and/or present in only one sample, we detected a total of 14 different phyla. Of these, only the 4 shown in Figure 4.3 were considered as dominant (i.e. with a relative average abundance higher than 1%): *Proteobacteria* (39% average abundance, mainly *Alpha-, Beta and Gamma-Proteobacteria*), *Firmicutes* (22% average abundance), *Bacteroidetes* (21% average abundance) and *Actinobacteri*a (12% average abundance). The distribution between the two samples were quite similar with only an increment of *Actinobacteria* and *Firmicutes* in the sample of 1st December.

Figure 4.3 also shows the most abundant orders found within each phylum. Between the two samples on average, nine most abundant orders were identified within *Proteobacteria*. Particularly abundant were *Burkholderiales* (with the 39% and 45% respectively in 30 November and 1st December), *Sphingomonadales* (with the 17% and 18% respectively in 30 November and 1st December) and *Rhizobiales* (with the 10% and 12% respectively in 30 November and 1st December). Within *Bacteroidetes*, only three different orders exceeded the relative abundance of 0.5%: *Cytophagales, Sphingobacteriales, Flavobacteriales*, with the predominance (88% in both samples) of the first one. Four orders were abundant for *Firmicutes: Bacillales, Clostridiales, Lactobacillales* and *Erysipelotrichales.* Within *Actinobacteria, Actinomycetales, Solirubrobacterales, Rubrobacterales* and *Acidimicrobiales* were abundant, with Actynomicetales the most abundant one (90-92%).

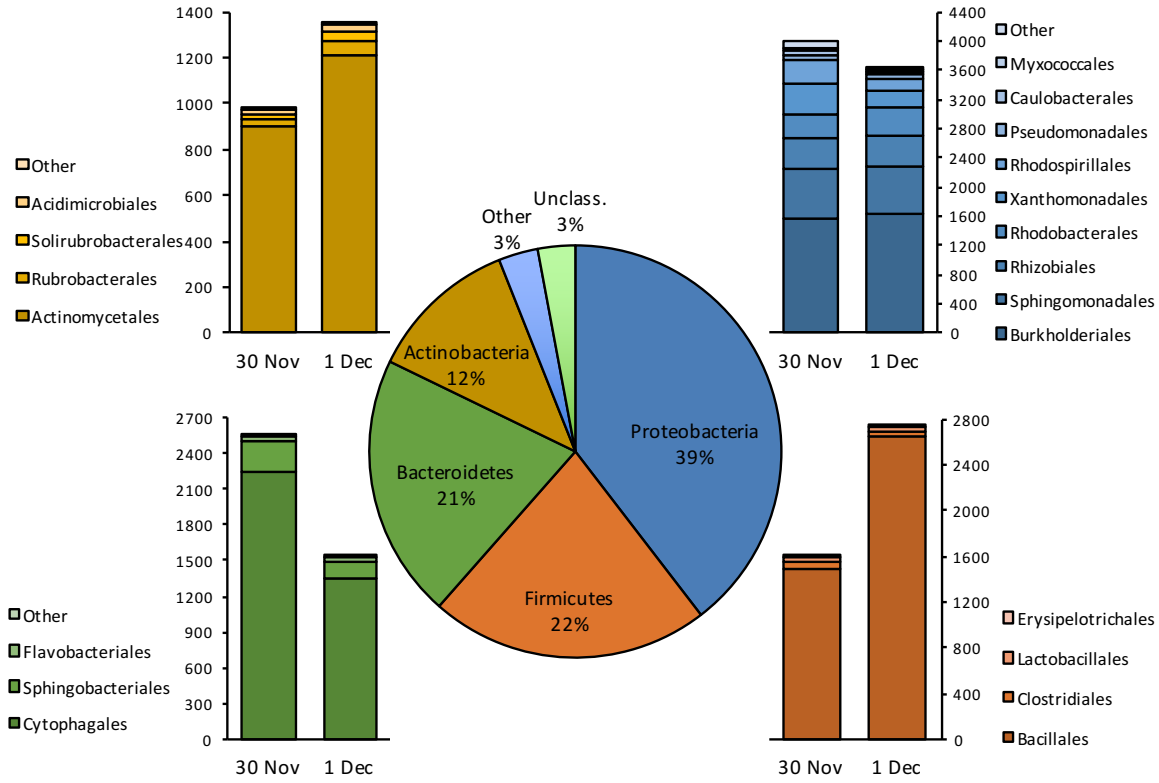

Figure 4.3. Distribution of the most abundant bacterial *phyla* (average abundance higher than 1%) and of the most abundant bacterial orders (abundance higher than 0.5%) in the two PM samples impacted by Saharan intrusions reaching *Monte Martano* on 30 November and 1st December 2014.

The distribution of the most abundant genera in the PM samples is reported in Figure 4.4. The most abundant genera belonged, on average, to *Adheribacter* (11.32%), *Naxibacter* (6.15%), *Domibacillus* (4.94%), *Bacillus* (4.58%), *Sphingomonas* (4.11%), *Pontibacter* (3.14%), *Rubellimicrobium* (3.07%), *Microvirga* (2.8%), *Hymenobacter* (2.09%), *Acidovorax* (1.99%) and *Massilia* (1.70%). It is possible to appreciate how the two communities showed very similar profiles suggesting that the two Saharan intrusions were very similar not only from a chemical point of view but also in respects of the dust-carried bacteria.

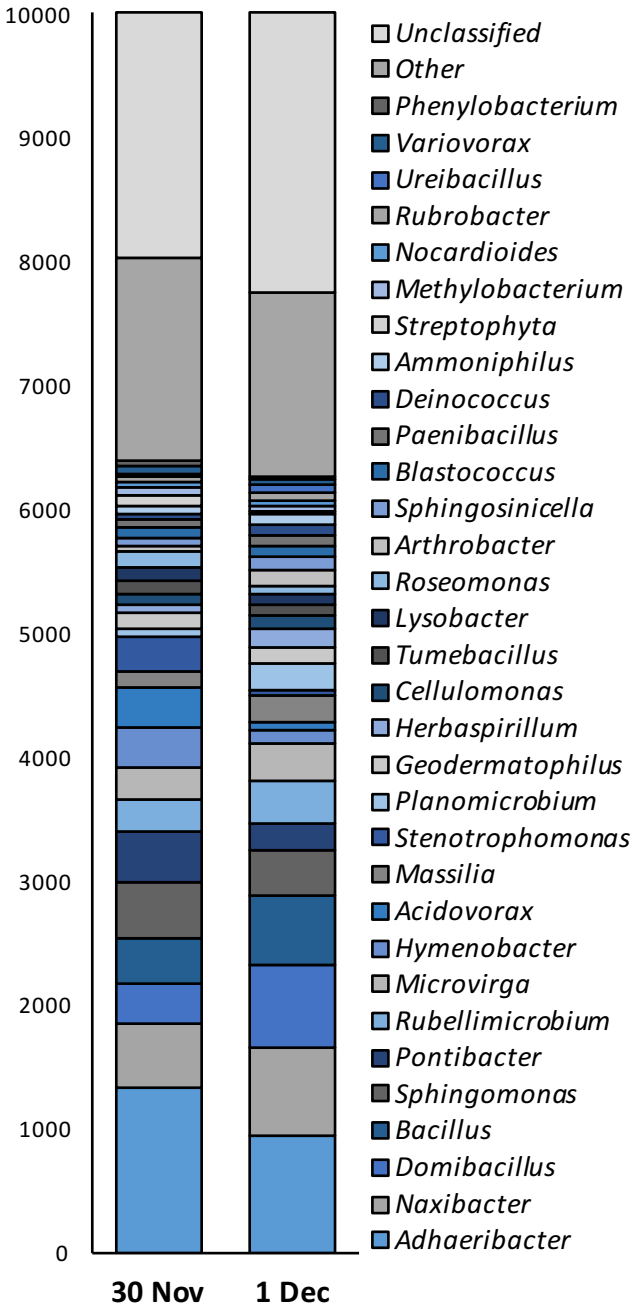

Figure 4.4. Distribution of the most abundant bacterial *genera* (average abundance higher than 0.5%) in the two PM samples impacted by Saharan intrusions reaching *Monte Martano* on 30 November and 1st December 2014.

4.3.3 Viable and metabolically active bacteria in dust-impacted PM

In order to investigate if the bacterial populations carried by the two dust storms and retrieved in PM samples were viable and metabolically active a collection of 92 isolates was established by culturing the PM samples on R2A agar plates at three different temperatures (4 °C, 25 °C ad 50 °C) to capture the widest diversity possible of the cultivable microbiota (Annex 4.1). The phylogenetic analysis reported in Figure 4.5 indicated that the isolates clustered into 49 different species (reported in Table 4.4), belonging to 16 different *genera* and four different *Phyla.*

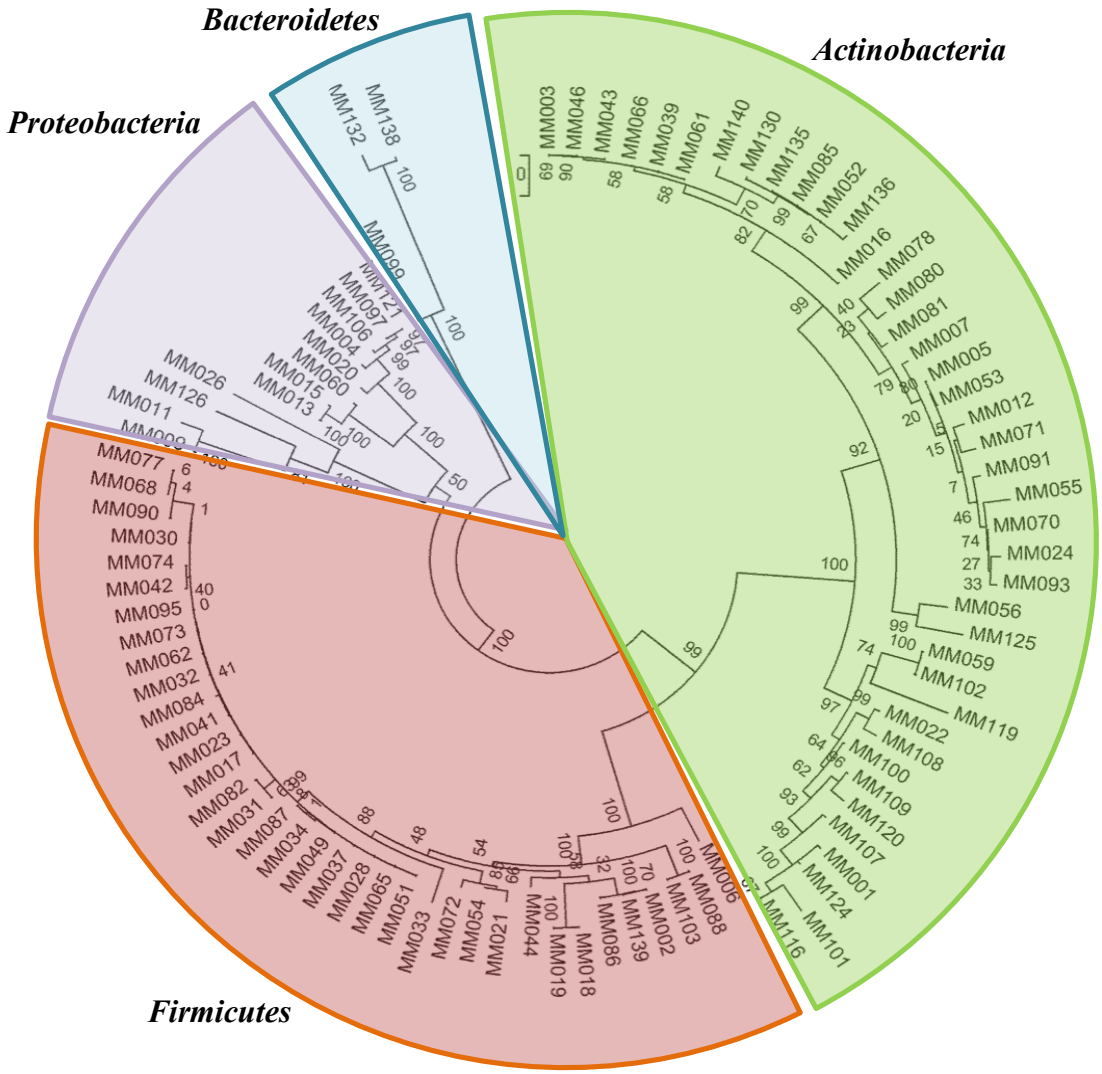

Figure 4.5. Phylogenetic tree of the isolates obtained from the two Saharan-impacted PM samples.

Table 4.4 Species isolated from the two Saharan-impacted PM samples, their growth temperature and enzymatic activities.

Species	Sample		Growth temp.			Amilase			Esterase			Lipase			Protease		
	30 Nov	1 Dec	4	25	50	4	25	50	4	25	50	4	25	50	4	25	50
Actinobacteria																	
Arthrobacter agilis	x	x	x	x			x		x			x	x		x		
Arthrobacter crystallopoietes	x		x														
Arthrobacter pascens	x		x														
Arthrobacter pityocampae	x		x						x								
Arthrobacter polychromogenes		x	x														
Frigoribacterium faeni	x	x	x	x					x	x		x				x	
Streptomyces roseolilacinus	x	x		x	x		x	x		x			x			x	x
Streptomyces mutabilis	x	x		x			x			x							
Streptomyces griseus	x			x			x			x							
Streptomyces niveoruber	x			x			x										
Streptomyces youssoufiensis	x			x			x			x							
Streptomyces atrovirens	x			x						x							
Streptomyces diastaticus		x		x			x			x						x	
Streptomyces flavoviridis		x			x												
Streptomyces fumanus		x			x			x									
Streptomyces glaucus		x			x			x									x
Streptomyces griseorubens		x			x												
Streptomyces kanamyceticus		x	x						x								
Streptomyces microflavus		x	x	x		x	x		x	x		x			x		
Streptomyces naganishii		x			x												
Streptomyces variabilis		x		x			x										
Streptomyces viridodiastaticus		x		x			x						x				
Saccharothrix xinjiangensis		x		x			x						x				
Sanguibacter keddieii		x	x			x											
Kocuria rosea	x		x	x						x			x			x	
Rhodococcus cerastii		x	x									x					

Table 4.4 continued. Species isolated from the two Saharan-impacted PM samples, their growth temperature and enzymatic activities

Species	Sample		Growth temp.			Amilase			Esterase			Lipase			Protease		
	30 Nov	1 Dec	4	25	50	4	25	50	4	25	50	4	25	50	4	25	50
Bacteroidetes																	
Nibribacter koreensis	x		x														
Pedobacter duraquae		x	x			x											
Pedobacter ginsengisoli		x	x			x									x		
Firmicutes																	
Bacillus endophyticus	x	x	x	x			x		x								
Bacillus halosaccharovorans		x		x			x			x			x				
Bacillus infantis	x				x												
Bacillus litoralis	x			x												x	
Bacillus mojavensis	x	x		x	x		x	x		x	x		x	x		x	x
Bacillus niabiensis		x			x												
Bacillus niacini	x			x			x			x			x			x	
Bacillus safensis	x				x									x			x
Bacillus simplex	x	x	x	x			x			x					x	x	
Bacillus subtilis	x	x			x						x			x			x
Bacillus vallismortis		x		x			x			x						x	
Paenibacillus xylanilyticus	x			x			x			x							
Proteobacteria																	
Citrobacter gillenii		x	x									x					
Massilia niastensis	x			x			x									x	
Microvirga aerilata		x		x													
Microvirga vignae	x			x			x			x							
Pseudomonas duriflava	x			x						x							
Sphingomonas aurantica	x			x			x			x							
Sphingomonas faeni	x		x														
Sphingomonas hankookensis	x			x						x						x	
Sphingomonas areolata	x	x	x									x					

The most abundant Phylum was *Actinobacteria* with the 45% of the total isolates followed by *Firmicutes* with 39% and then *Proteobacteria* and *Bacteroidetes* with 13% and 3% respectively. This result showed an opposite trend when compared with that obtained from the NGS analysis. There, indeed, *Actinobacteria* was the less abundant, while the highest number of sequences belonged to *Proteobacteria*. Only *Firmicutes* accounted about the same relative number of bacteria.

Of the total, 48 isolates were obtained from the 1st December sample and 44 from the 30 November sample (Figure 4.6). Further, most of the isolates (43) were obtained from plates cultured at 25°C while 32 isolates derived from the plates incubated at 4°C and 17 from those incubated at 50°C. Figure 4.6 also shows the *Phyla* distribution between the two samples and the three different cultivation temperatures. The only temperature at which, in both samples, it was possible to recover isolates belonging to all the 4 Phyla, was 4°C. On the contrary, only two different *Phyla* (*Actinobacteria* and *Firmicutes*) were found among the thermophilic bacteria growing at 50°C. Interestingly, this group of bacteria, especially those belonging to *Actinobacteria* was more abundant in the sample collected on 1st December, confirming the indication provided by the culture-independent analysis.

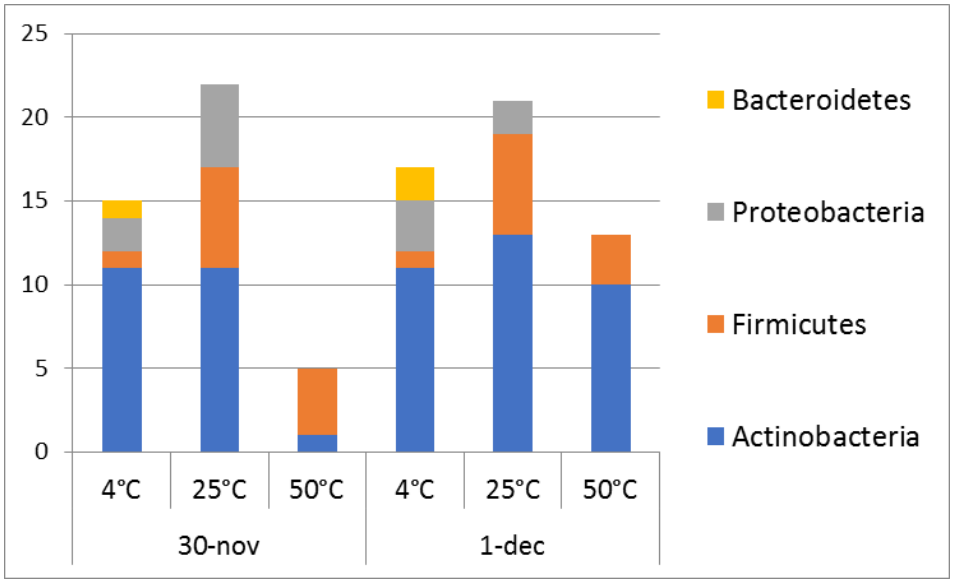

Figure 4.6. *Phyla* distribution of the isolates retrieved from the two Saharan-impacted PM samples and the three different cultivation temperatures.

Figure 4.7 reports the bacterial genera found in the two samples at the different temperature of growth. The bacteria distribution in the two samples shows a similar trend, with the highest biodiversity, as expected, recovered at psychrophilic (4°C) and mesophilic (25°C) temperatures. On average, the most abundant genus at 4°C was *Arthrobacter*, followed by S*treptomyces* and *Sphingomonas*. *Bacillus* and *Streptomyces* were predominant at 25 °, while at 50°C, only *Streptomyces* and *Bacillus* were able to grow.

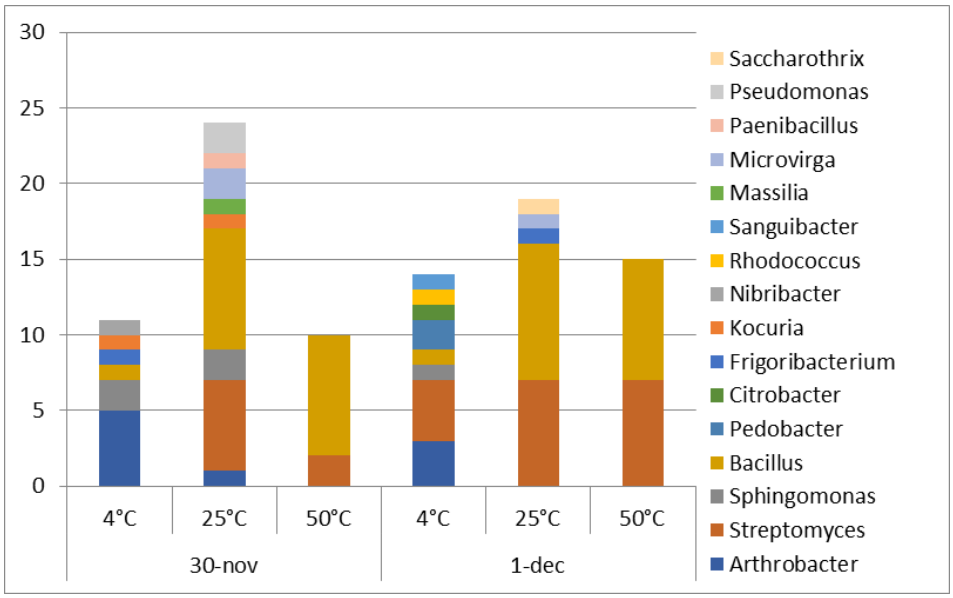

Figure 4.7. *Genus* distribution of the isolates retrieved from the two Saharan-impacted PM samples and the three different cultivation temperatures.

As well-known culture-based methods can catch only a part of the bacterial biodiversity. Indeed Figure 4.8 conforms this by comparing the *genera* diversity obtained in the two PM samples both by culture-independent (NGS) and culture-dependent approaches. As stated before Illumina-based NGS analyses allowed us to individuate 217 common genera between the two samples, with 67 exclusive of 30 Nov and 33 only present in 1 Dec samples. On the contrary, only 16 were obtained by culturing.

Few *genera* were shared between the NGS and culture-based data, namely *Sphingomonas, Bacillus*, *Arthrobacter, Massilia, Microvirga, Paenibacillus, Pseudomonas, Kocuria*, *Streptomyces* and *Pedobacter*. Interestingly some of them, namely *Arthrobacter, Bacillu* and *Streptomyces* showed an increase in relative abundance in the cultivable fractions, that made them account for most the genera recovered. On the contrary, many of the most abundant genera found through Illumina sequencing, such as for examples, *Adheribacter, Pontibacter, Domibacillus, Hymenobacter, Naxibacter, Rubellimicrobium* and *Stenotrophomonas* were not recovered by cultivation.

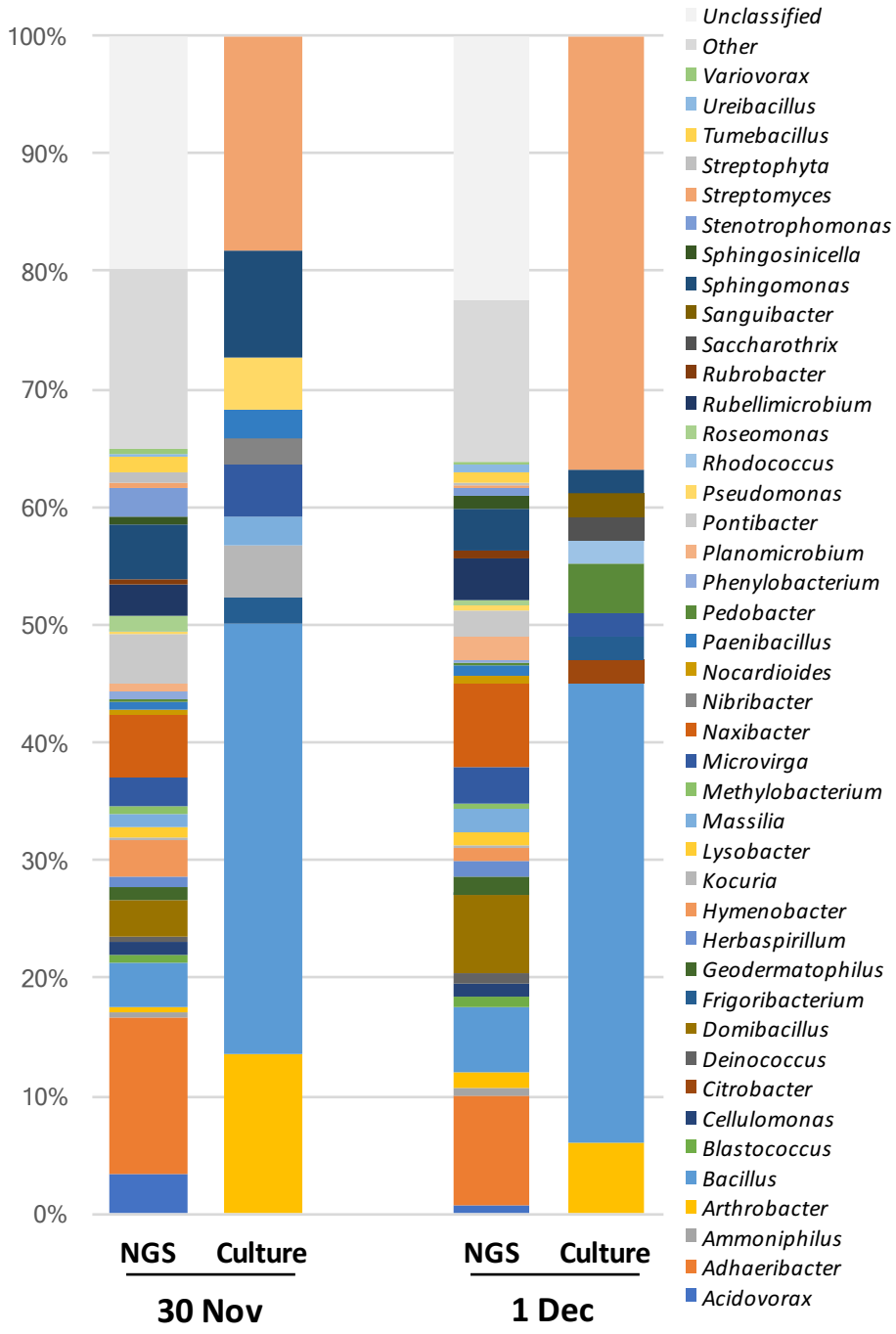

Figure 4.8. Distribution of the most abundant *genera* retrieved by culture-independent (NGS) and culture approaches from the two Saharan-impacted PM.

To further demonstrate that these isolates recovered from the PM impacted by the Saharan dust were metabolically active, we tested four different exocellular enzymatic activities (lipase, amylase, esterase and protease) related to the catabolism of complex substrates. The

overall results obtained from these experiments are reported in Table 4.4, while Figure 4.9 shows how most of the species isolated presented at least one enxymatic activity, and a large number of them was positive for more than one. In particular, *Bacillus mojavensis, Bacillus niacin* and *Streptomyces microflavus*, were positive for all the activities tested. *Kocuria Rosea, Streptomyces roseolilacinus, Streptomyces diastaticus, Bacillus halosaccharovorans, Bacillus simplex, Bacillus subtilis* and *Bacillus vallismortis,* were positive for three metabolic activities.

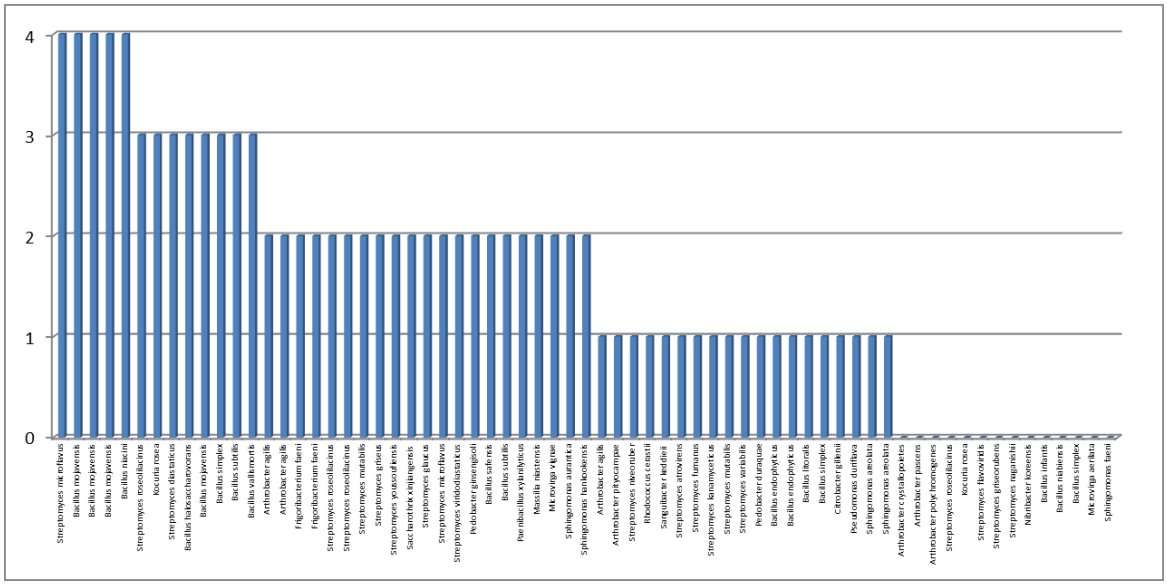

Figure 4.9. Number of enzymatic activities featured by each of the species isolated from the Saharan-impacted PM samples.

To gain further insights regarding the capability of dust-carried bacteria to resist to the adverse conditions during their transport in the atmosphere, we tested the species isolated from the two PM samples in respect of their capacity to survive after exposition to UV radiations (Figure 4.10). It is possible to notice that almost all isolates were able to resist after exposition to UV radiation for 1 minute, while only 5 isolates resulted resistant to 5 minutes of UV exposition, namely *Bacillus endophiticus, Rhodococcus cerastii, Citrobacter gilleni, Pedobacter duraquae*, *Saccharothrix xinjangensis*. Considering that the UV lamp that we used for our experiments featured a very strong UV intensity (as it is one of those used to decontaminate laminar flow cabinets), our results are remarkable and in agreement with those reported by Sańchez de la Campa et al (2013) and confirm the ability of at least

a part of the bacterial community carried by desert dust to resist to the cahallanging conditions, such a s radiations, that could be encountered during long-range transport.

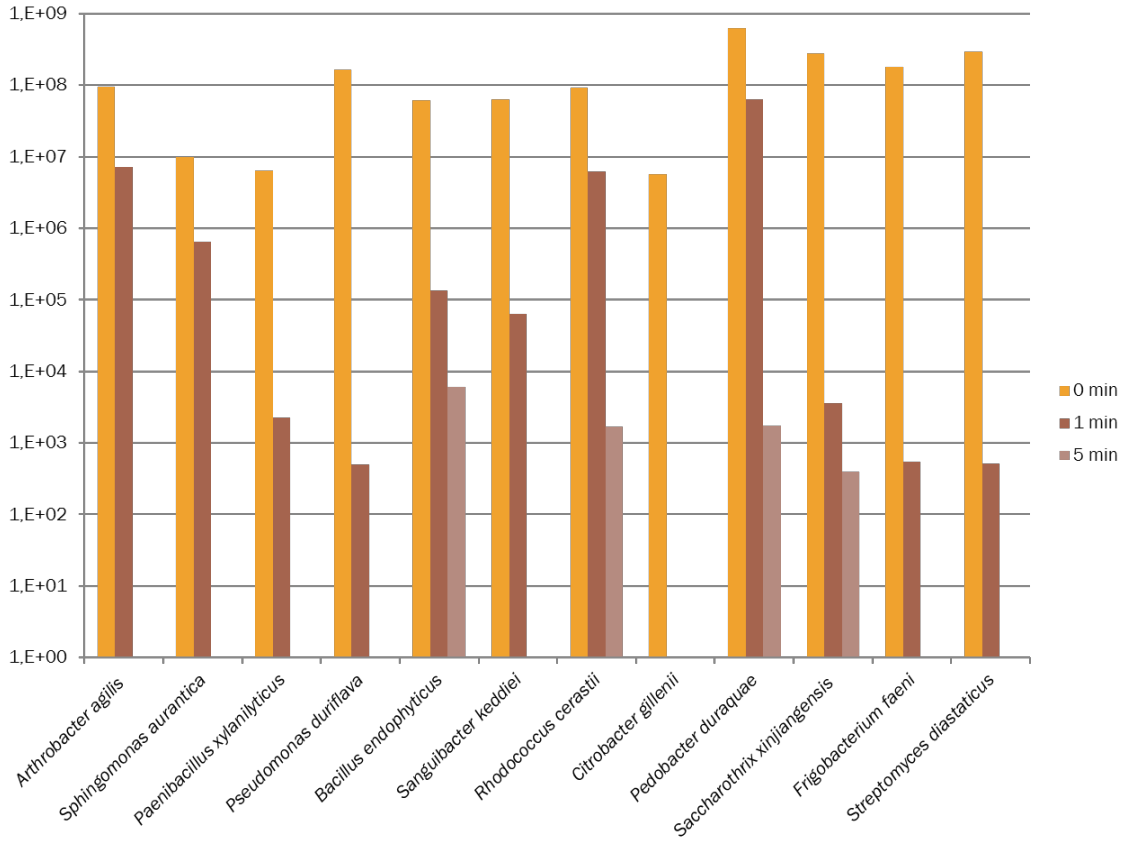

Figure 4.10. Survival at UV exposure of the species isolated from Saharan-impacted PM, indicated by the CFU counts in non-exposed samples (0 min) and in samples exposed to a laminar flow UV lamp for 1 and 5 minutes.

4.4. Discussion

In this part of the thesis work we follow-up on the data presented in the previous Chapter 3 that indicated how particulate matter is strongly impacted by Saharan dust intrusions, by reporting the chemical and microbial characterization of particulate matter during the most intense African desert dust storm that reached Central Italy in the last period and that occurred on 30 November and 1st December of 2014 by analysing and comparing PM_{10} samples taken in the two days.

Overall all the chemical data were consistent with a clear Saharan dust origin for both samples, even if with some differences between the two cases. Both were characterized by air masses reaching the sampling site directly from North Africa and associated to very fast winds. The coarse fraction of the PMs was also very high, as the concentrations of typical desert elements, noticeably Ca, Fe and Ti, confirming the intensity of the two episodes. Nevertheless, slight change in aerosol acidity together with higher values for EC, OC, K and Zn in the 1st December sample, indicated that the two were separate events. These evidences and in general the high values of nitrates and sulphates point to a possible mixing with aerosol resulting from industrial activities or by biomass burning along the African coasts for the 2 December sample (Rodriguez et al. 2011).

The present study, as far as we know, is one of the few that aimed to characterize the microbiota composition related to such a strong African desert dust intrusion reaching Europe, particularly applying and comparing both culture-independent and culture-dependent approaches. Most of the studies that focused on microbial composition of desert dust until present days, were conducted on the cultivable fraction (Kellog et al. 2006, Griffin et al. 2006, 2003, 2001). Indeed, despite they catch a very low biodiversity than molecular approaches, because it is known that only a small fraction of bacteria can grow in laboratory conditions, cultivation-based methods represent the gold method to investigate the microbial physiology (e.g. metabolic activities). Conversely, a few number of study were conducted far from the source of desert dust and compared the results of NGS-based methods with cultivation ones (Yamagouchi et al. 2012, Sańchez de la Campa et al. 2013). Further, most of the recent papers employing a NGS approach to study desert dust-carried bacteria have sampled PM on the coast of Middle East and thus could not appreciate the long-range transport (Katra et al 2014, Mazar et al 2016, Gat et al 2017). Only Sańchez de la Campa et al (2013) and Rosselli et al (2015) did a similar work in Europe.

As for the data presented in Chapter 3, the culture-independent analysis of the bacterial community in PM_{10} sampled during the two strong intrusions, allowed a deep characterization of the bacterial community indicating the occurrence of a high bacterial diversity with the presence of typical desert bacterial populations.

Proteobacteria, that comprise one of the largest divisions within prokaryotes, account for the vast majority of the known Gram-negative bacteria and are frequently found in dry soils (Gupta 2000) were very abundant. Within *this phylum,* the largest number of sequences were ascribed to the beta-proteobacteria *Massilia* and *Naxibacter*, both found in many extreme environments impacted by desert dust (Chuvochina et al. 2011).

Firmicutes were also abundant. This is a *phylum* where spore-forming bacteria are often reported as in bioaersol. Many *Firmicutes* produce endospores, which are resistant to desiccation and can survive extreme conditions. They are found in various environments, and the group includes some notable pathogens. At the genus level, interesting bacteria were *Planomicrobium, Domibacillus and Bacillus,* the first known to be psycrotolerant and typical of soil (Zhang et al. 2009), the second isolated before from an active layer soil of tundra (Alaska) (Gyeong et al. 2015) and the last known to be ubiquitous. Psycrotolerant bacteria could have an advantage to survive during their transport with dust in the atmosphere where the temperature undergoes a considerable decrease.

Bacteroidetes, widely distributed in the environment, including in soil, sediments, and sea water, were also present in our PM samples. Particularly interesting to mention within this *phylum* was the presence of *Cytophagales*, many members of which are marine bacteria and others are known to be able to digest plant material. Indeed, biodegradation is an important aspect of desert life, because it is an oligotrophic environment, so through biodegradation bacteria can obtain source of nutrient and energy (Ventura et al. 2007).

We also found many *Actinobacteria*, which have been previously shown to be abundant in desert bioaerosol (Yamaguchi et al. 2012, Yamaguchi et al. 2012). *Actinobacteria* are widely distributed in both terrestrial and aquatic (including marine) ecosystems, especially in soil, where they play a crucial role in the recycling of refractory biomaterials by decomposition (Ventura et al. 2007). They are known to inhabit extreme environments such as hypersaline lakes, thermal springs, and arid soils (Yamaguchi et al. 2012). Many members of this phylum are known to be thermophilic, and all are widespread in soils (Favet et al. 2013). One of the most abundant genus of this phylum belong to *Geodermatophylu*s, which has been isolated from stressful environments such as rock varnish in deserts (Ivanova et al. 2010) and frequently found associated with desert dust in PM (Mazar et al. 2016).

The presence in our samples of a high number of reads belonging to *Geodermatophylus, Pontibacter, Massilia, Rubellimicrobium, Microvirga, Naxibacter Stenotrophomonas*, *Lysobacter* and *Hymenobacter*, is in agreement with previous works that have already found these bacteria in desert soil, associated with dust in air or found in many extreme environments impacted by desert dust (Yamaguchi et al. 2007, Weon HY at al. 2010, Chuvochina et al. 2011, Rosselli et al. 2015, Mazar et al. 2016).

Our analysis of the cultivable fraction of bacteria in dust-impacted PM confirmed the difficulty of retrieving environmental isolates from bioaerosol (Yamagouchi et al. 2012, Sańchez de la Campa et al. 2013) and indeed provided a low bacterial diversity if compared to the culture-independent analyses. Nevertheless, this approach was useful to demonstrate that dust-carried bacteria in the PM_{10} were viable and metabolically active and thus could have the potential to colonize distant environments.

We could culture, even at thermophilic temperature, several different species of *Actinobacteria* and *Firmicutes*, in particular *Streptomyces* and *Bacillus,* respectively. Indeed, these bacteria are both gram positive, aerobic and spore-forming microorganisms, the reason why they can resist to high temperatures. Other *Actinobacteria* were *Saccharotrix*, a genus already found in Saharan soil (Zitouni et al. 2005), *Arhrobacter*, a genus known to be able to survive when exposed to desiccation for prolonged periods (Boylen 1973). Among *Proteobacteria*, we isolated several species of *Sphingomonas, Massilia* and *Microvirga,* that were frequently detected in desert dust samples (Katra et al. 2014, Favet et al. 2013). The vast majority of these isolates also showed at least one metabolic activity indicating their active state. Further, many of those belonging to *Streptomyces* and *Bacillus* were positive for all the tested activities. Many dust-carried bacterial populations were also able to resist to extreme conditions, such as UV radiations, one of the main factors that can reduce microbial survival in the atmosphere. In particular *Rhodococcus cerastii,* and *Citrobacter gilleni,* belong to genera for which this capability has been already characterized (Gabani et al. 2013, Urbano et al. 2013). On the contrary, *Pedobacter duraquae* and *Saccharothrix xinjangensis* belong to genera for which, as far as we know, we showed this feature for the first time.

In conclusion, our results regarding the chemical and microbiological analysis of the intense African dust intrusion recorded on 30 November and 1 December 2014, confirmed the findings presented in Chapter 3 that indicated how Saharan dust storms could cross the Mediterranean and inject into the European atmosphere, not only chemical particles, but also

a large variety of bacteria. Further, we demonstrated that these microbial populations, typical of desert and dry soil environments, could survive the long-range transport in the atmosphere reaching our latitudes still viable and metabolically active. Taken together, these evidences provide a clear indication on how bacterial populations associated with desert dust storms have the potential to impact and modify the endogenous bacterial community of bioaerosol and, eventually, of other local environments.

CHAPTER 5

EFFECTS OF SAHARAN DUST DEPOSITIONS ON THE MICROBIOTA OF A MOUNTAIN GLACIER ENVIRONMENT

5.1 Introduction.

Extreme environments are characterized by restricting conditions for organisms that inhabit them. 'Extreme' includes physical extremes, such as temperature, radiation, pressure, and geochemical extreme, for example desiccation, salinity, pH, depletion of oxygen or extreme redox potential. The investigation of these environments is important for the study of evolution relationships, emergence of new species and various ecological relations among organisms which compensate certain environmental conditions. Extreme habitats are populated by highly specialized organisms - extremophiles, which tolerate different stresses conditions (Oarga et al. 2009). Extremophiles were described as those organisms which thrive beyond 'normal' environmental parameters (Kristjansson et al. 1995). Especially, many species of bacteria, during evolution, developed unique adaptation capability that let them to colonize most extreme habitats. In such extreme environmental conditions, the structural simplicity of prokaryotes brings remarkable advantages.

In relation with the characteristics of the environmental niches required for their optimal growth, extremophiles microorganisms are divided in: extremophiles are psychrophiles (thrive at low temperatures), thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), piezophiles (under extremes of pressure), xerophiles (desiccation), and halophiles (salinity) (Rothschild et al. 2001, Magan 2007, Oarga et al. 2009). The question is if extremophiles love or rather tolerate extreme environments it's almost known that physiochemical properties of extreme environments are typically constant. Extremophilic organisms, as physiological response to the environment constancy, show optimal growth at such conditions and thus require them. Some environmental extremes e.g. radiation and vacuum include rather "tolerant" organisms than 'loving' organisms for such conditions. Microorganisms not only are able to grow in such conditions, but it is known that they play a crucial in the transformation of chemical components.

Often in literature, it has been affirmed that habitats with extreme environmental parameters showed a reduced species richness. For example, as assessed by Tobler et al. (2006), deep-sea hydrothermal vents possess a low species diversity due to extremes in temperature, hypoxia, sulfide, and heavy metals. However, a reduction in species richness may be due, for example, by the environment limited size, low productivity, or low spatial heterogeneity, and it is just been proved that in extreme habitats such as atmosphere, the

microbial diversity is rival that of other terrestrial or aquatic environments (Griffin et al. 2003).

Microbiology, have classified microorganisms into various thermal groups based on their optimum temperatures of growth. Of these groups, the cold-loving bacteria are usually referred to as psychrophiles, as organisms tolerating a range of temperatures from 0 °C (or even lower) to a maximum of about 20 °C, with an optimum for growth at about 15 °C (Morita, 1975). Psychrophiles are true extremophiles because they can be adapted not only to low temperatures but often also to further environmental limitations. The environments they inhabit are ubiquitous on Earth, indeed, a large number of areas in our planet exhibit temperatures lower than 15°C. They are present in alpine and arctic soils, high-latitude and deep ocean waters, polar ice, glaciers, and snowfields. Psychrophiles are protected from freezing and from the expansion of ice, if they cool-down slowly. Psychrophilic bacteria may continue to have some metabolic activity in the extracellular fluid at cold temperatures, and they remain viable once that the temperatures return to normal values. Psychrophiles can use a wide variety of metabolic pathways, including photosynthesis, chemoautotrophy (also sometimes known as lithotrophy), and heterotrophy. Many different microbial lineages of *Bacteria* and *Archea* show psychrophily. Among these, we can cite *Arthrobacter* sp., *Psychrobacter* sp. and members of the genera *Halomonas*, *Pseudomonas*, *Hyphomonas*, *Sphingomonas* and *Polaromonas* (Margesin and Miteva 2011).

In recent years, high mountain and polar glacier snow fields have been recognized as highly diverse microbial community habitats. Indeed, many microbial processes have been shown to occur in melting snow, such as heterotrophy, photosynthesis, and nutrient cycling (Xiang et al 2009, Maccario et al. 2015). It is already known that many microorganisms use wind-transport as a key strategy for colonization of new habitats, and especially the global dispersal of microbial pathogens, may constitutes a risk to environmental health. Dust particles can facilitate intercontinental movement by acting as vehicles for viable microorganisms. The Saharan/Sahel areas of Africa, are major sources of airborne dust that is frequently transported at high altitude over the Atlantic and the European continent (Griffin 2007, Smith et al 2011).

Desert environments, similarly to high-altitude atmosphere, are characterized by harsh conditions, such as limited availability of nutrients, high levels of radiation and extreme temperatures (Smith et al. 2011). The increasing frequency of dust storms, recently occurred, can intensify the intercontinental dispersal, causing a contamination of new

habitats by exogenous microorganisms. It has indeed been hypothesized that microorganisms that inhabit the desert can survive long-range transport in the atmosphere and potentially colonize mountain glaciers, that feature comparable challenging environments (Chuvochina et al. 2011, Meola et al 2015, Weil et al. 2017). The permanently frozen glacier environment harbour a variety of viable microorganisms that have been deposited over centuries, but little is known about their possible origin.

Most of the studies on Saharan deposited particles in mountain environments have focused on mineralogical, physic and chemical aspects, while microbiological analyses are poorly investigated. Indeed, to the best of our knowledge, only three studies have faced the culture-independent assessment of the microbial composition of Saharan dust-impacted snow and all were conducted in the European Alps (Chuvochina et al. 2011, Meola et al. 2015, Weil et al. 2017). All these authors showed how microorganisms associated with desert dust events can be deposited and persist as dust layers in the snowpack that, at the highest elevations, are preserved in the ice of glaciers.

The data presented and discussed in the previous two chapters of this Thesis work, have clearly indicated that Saharan desert storms are able cross the Mediterranean and inject large pulse of microorganisms into Central Italy. In this part of the Thesis work, therefore, we aimed to investigate the impact of Saharan dust depositions on the microbiota of a mountain glacier environment. In particular, we collected and analysed snow and water samples from the *Calderone* glacier that, being the southernmost in Europe, is often reached by African dust storms. As in the previous chapters, we employed culture-independent methods based on high-throughput Illumina sequencing of 16S rRNA genes to compare clean snow, Saharan-impacted snow and water samples from glacier.

5.2 Material and Methods.

5.2.1. Sampling

Snow samples were collected from February 2014 to September 2015 on the *Calderone* Glacier (*Gran Sasso d'Italia*, 2700 m asl), the southernmost in Europe and the only one in the Apennines. In order to sample snow impacted by desert dust depositions, a 1.20 meters-deep snow pit was excavated. Snow samples were taken with sterile single-use materials, stored in sterile bottles and transported in laboratory under refrigerated conditions. Once in the laboratory, samples were melted at room temperature under a biological laminar flow and filtered through sterile 0.2 μm filters, the latter stored at -20°C until DNA extraction.

Starting from September 2014, samples of water melting directly from the *Calderone* Glacier, together with water from a natural source located at the bottom of the mountain, were also collected. Water was filtered through sterile 0.2 μm filters, which were then stored at -20°C.

5.2.2. Microbial diversity analyses.

Culture-independent assessment of microbial communities in snow and water samples was carried on with a NGS approach based on the Illumina HISeq platform. Metagenomic DNA was extracted directly from the filters, prepared as described above, using the POWER SOIL DNA kit (MOBio) following the manufacturer's protocol.

The V5-V6 hypervariable regions of the 16S rRNA were amplified in 2 x 50 μL volume reactions with a Hot start Taq polymerase (Solis-Biodyne). The reaction included 1 μM each of primers 783F and 1027R (Huber et al. 2007, Wang and Qian, 2009). At the 5' end of 783F primer, one of 15 6-bp barcodes was also included to allow sample pooling and subsequent sequence sorting. The cycling conditions were: initial denaturation at 94°C for 5 min; 29 cycles of 94°C for 50 s, 47°C for 30 s, and 72°C for 30 s and final extension at 72°C for 5 min. The amplified products were purified with the Wizard SV PCR

purification kit (Promega Corporation) and amplicon quantity was evaluated by Qbit (Invitrogen).

Multiplexed samples were prepared with the Illumina Multiplexing Sample Preparation Oligonucleotide Kit, which provides 12 index oligos for pooling up to 12 samples per lane. Therefore, the tagging system with 8 barcodes at the 5' end of the 783F primer allowed pooling up to 96 samples per lane. Multiplexed sequencing of all the pooled samples was performed on a single Illumina Hiseq 1000 lane, using a paired-end 2x100 base-pair protocol and the 4.0 sequencing chemistry, by *Parco Tecnologico Padano*.

Each sequence was assigned to its original sample according to its index oligos and barcode. After sorting the sequences, the reverse read of each paired-end sequence was reverse complemented and merged with the corresponding forward read. A quality cut-off was applied in order to remove the sequences that did not contain the barcode, those with an average base quality value (Q) lower than 30 and those that did not provide a perfect match in the overlapping part between the two pair-ends. The barcode was removed and sequences were sorted into Operational Taxonomic Units (OTUs) using the UPARSE-OTU algorithm. The minimum identity between each OTU member sequence and the representative sequence (*i.e.* the sequence that showed the minimum distance to all other sequences in the OTU) was set to 97%. The taxonomic classification of each OTU was carried on with the stand-alone version of RDP Bayesian Classifier, using a 50% confidence level.

The similarity among bacterial communities in the different samples was displayed by 1-D dendrograms based on Bray-Curtis distances.

5.3. Results and Discussion.

5.3.1 Sampling campaign.

The sampling campaign was carried out on the *Calderone* Glacier (altitude 2630–2830 m a.s.l), that represents the southernmost glacier in Europe (42°28'15" N) and the only one in the Appennines mountain range. It is located within the *Gran Sasso d'Italia* mountain group (Abruzzese Appennines, Teramo), lying just beneath the *Corno Grande*, the highest peak in the Apennines (Figure 5.1). After the Last Glacial Maximum (about18 000 years ago), this glacier represents the residual presence of the past glaciarized area in the Apennines. The ice mass of the glacier, survives below the snow-line altitude thanks to its north-facing position, the shadow provided by the mountains surrounding the glacier and the effect of the supraglacial debris cover that insulate the ice below. All these factors, during the years have reduced the rates and quantity of the ice melting. (Branda et al. 2010).

Figure 5.1. The *Calderone* Glacier: geographical localization (A) and general view (B).

As demonstrated in the previous two chapters of this Thesis work, Saharan desert storms are able cross the Mediterranean and inject large pulse of microorganisms into the European air. Microorganisms that can survive the harsh conditions, e.g. limited nutrient availability, high radiation and extreme temperatures, that characterize deserts as well as the troposphere (Smith et al. 2011), can likely have the potential to colonize remote environments with comparable challenging conditions such as mountain glaciers. The *Calderone* Glacier, being the southernmost in Europe, is often reached by Saharan dust, leaving persistent layers that, when covered by successive snowfalls, remain sealed between dust-free layers preventing windblown contamination by material from the local environment (Figure 5.2). This clear separation of snow layers associated with Saharan dust deposition and with dust-free snowfall can represent the opportunity to investigate if bacteria transported from the Sahara are able to impact extreme environments, such as mountain glaciers.

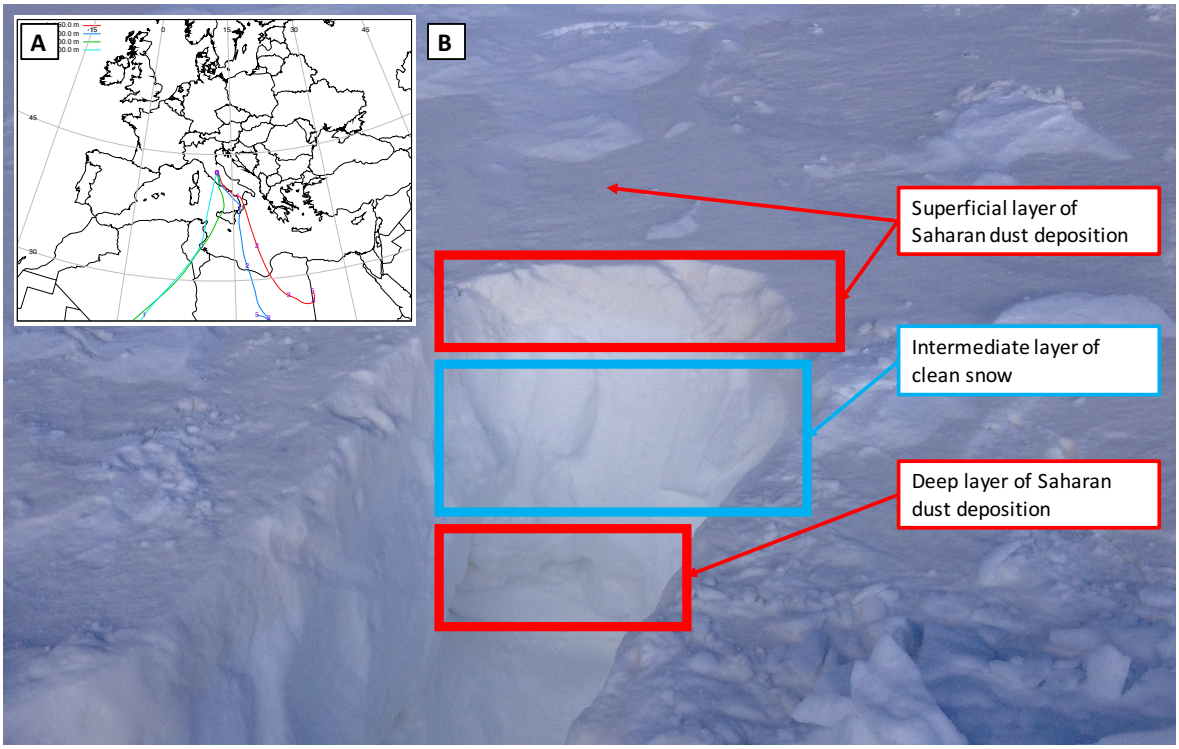

Figure 5.2. Sampling of dust-impacted and clean snow on the *Calderone* Glacier. Saharan dust intrusions reach the glacier (A) leaving dust layers on the snow (B).

Sampling campaigns were carried out in 2014 and 2015, generally in spring (June) and autumn (September) of every year, with the only exception of February 2014 when snow

samples were also taken (Table 5.1). When necessary, to collect Saharan dust-impacted snow samples and the nearby clean snow, a snow pit was excavated (Figure 5.2). Therefore, snow sample codes report letters s, m and d to indicate surface, median and deep layers of the snow pit, respectively (Table 5.1). The clean snow represented a control sample of the dust-impacted snow because recovered in the same time and close to the latter. During the two campaigns carried on in September, no dust-impacted snow was recovered. These samples have been nevertheless included in the study because they can represent a further control of the normal snow microbiota. Additionally, to test the contamination and colonization potential of the dust-carried bacteria deposited on the glacier, in September 2014, June 2015 and September 2015, samples of melting water were collected from the glacier (indicated with letter h in the sample codes), together with water samples from a source at the bottom of the mountain (letter l in the sample codes).

Table 5.1. Snow and water samples recovered during the different sampling campaigns**.**

Sample Code[1]	Sampling Campaign	Description of the sample
Sn_1402s	February 2014	Clean snow, superficial layer
Sn_1406m	June 2014	Clean snow, median layer, under ice
Sn_1409s	September 2014	Clean snow, superficial layer
Sn_1409m	September 2014	Clean snow, median layer
Sn_1409d	September 2014	Clean snow, deep layer
Sn_1506s	June 2015	Clean fresh snow, superficial layer
Sn_1506d	June 2015	Clean snow, deep layer
Sn_1509s	September 2015	Clean snow, superficial layer
SH_1402d	February 2014	Saharan dust-impacted snow, deep layer
SH_1402m	February 2014	Saharan dust-impacted snow, median layer
SH_1406s	June 2014	Saharan dust-impacted snow, superficial layer
SH_1506s	June 2015	Saharan dust-impacted snow, superficial layer
Wat_1409h	September 2014	Snow-melting water
Wat_1409l	September 2014	Source water
Wat_1506h	June 2015	Snow-melting water
Wat_1506l	June 2015	Source water
Wat_1509h	September 2015	Snow-melting water
Wat_1509l	September 2015	Source water

[1] Snow samples were collected on the surface (s), median (m) and deep (d) layer of the snow pit. Water samples were collected as water melting directly on the glacier (h) and from a source at the bottom of the mountain (l).

5.3.2 Bacterial diversity in the glacier environment.

Figure 5.3 shows the most abundant bacterial genera (more than 1% relative abundance in at least one sample) found in the clean snow samples. First of all, it is possible to underline the high variability of the community profiles, indicating how the clean snow, that we herein consider a kind of control sample set for our study, was characterized by a complex microbial community. Further, our data show that even an extreme environment, such as that of a glacier, can feature bacterial community dynamics. Indeed, as sampling was carried out during two years, it is possible to envisage community fluctuations due to seasonality, environmental changes, such as temperature and snow precipitation, or windblown depositions of aerosol microorganisms.

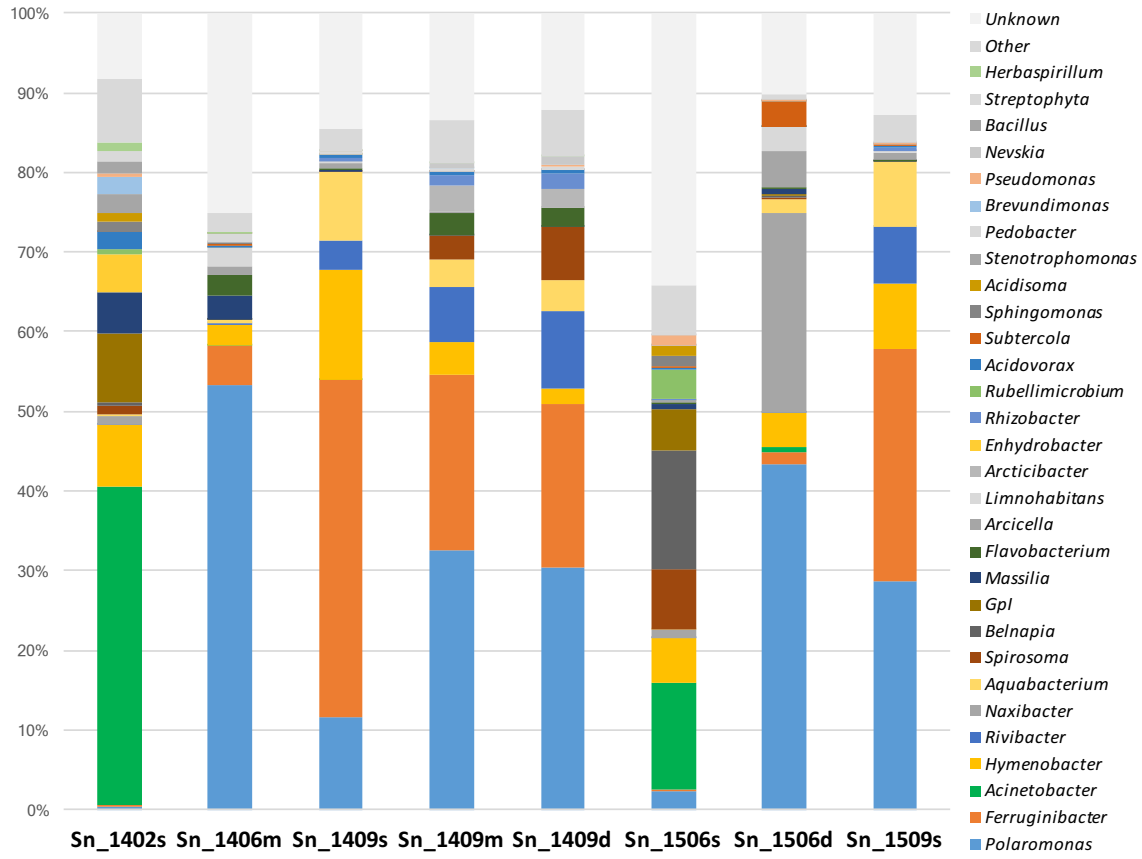

Figure 5.3. Distribution of the most abundant bacterial *genera* (relative abundance higher than 1%) in the clean snow samples. Snow samples were collected on the surface (s), median (m) and deep (d) layer of the snow pit.

Interestingly, the variability among samples taken at different depths in the same period (i.e. superficial layer, Sn_1409s; medium layer, Sn_1409m; deep layer, Sn_1409d) was lower with much more similarity in the genera distribution.

On average, the most abundant genera were *Polaromonas*, *Ferruginibacter*, *Acinetobacter* and *Hymenobacter*, bacterial groups that, as expected, were already found in cold environments (An et al. 2010, Liu et al. 2011, Franzetti et al. 2013, Peter and Sommaruga, 2016). Nevertheless, these groups of bacteria showed high variability among our snow samples. *Polaromonas*, for example, ranged from a minimum of 0,35% recorded on February 2014 to a maximum of 30,46% of September of the same year. Even more clearly, *Ferruginibacter,* ranged from a minimum of 0,02% on June 2015 to a maximum of 42,19% on September 2014. In the same way, *Hymenobacter* was a phylotype abundant across all samples, ranging from a minimum of 1.84% from a maximum of 13.82%. While most of the above mentioned phylotypes were abundant in almost all samples, *Acinetobacter* were abundant only in the superficial snow collected in February 2014 and June 2015 (39.79 and 13.44% of the total bacteria, respectively). Similarly, the *Naxibacter* genus was markedly abundant only in the deep snow layer sampled in June 2015.

The distribution of the most abundant genera found in the samples of snow impacted by Saharan dust is shown in Figure 5.4. The most abundant genera found were, on average, *Hymenobacter, Massilia, Acinetobacter, Polaromonas* and *Duganella.* As observed for the clean snow samples, also in this case the variability among samples was quite high. Only the *Hymenobacter* genus was abundant across all samples, ranging from a minimum of 4.33% of sequences in June 2015 to a maximum of 50.07% the same period of the previous year. On the contrary, *Massilia, Acinetobacter, Polaromonas* and *Duganella* showed very high abundances only in one out of the all samples (67.34% in June 2015, 40.38% in February 2014, 33.67% in June 2014 and 12.88 in June 2015, respectively).

As it will be further presented and discussed later in this Chapter, many of the bacteria that were present in the Saharan-impacted snow samples were also found by us in the Saharan dust collected in the *Monte Martano* sampling site, as well as reported by others in desert dust bioaerosol (Chuvochina et al. 2011, Meola et al. 2015, Weil et al. 2017). Nevertheless, it is interesting to note that the Saharan-impacted samples collected during the different campaigns showed different bacterial community profiles. The two samples collected in February 2014, for example, showed the highest number of abundant bacterial genera, with 12 and 17 different phylotypes with more than 1% relative abundance in the deep and median layer, respectively. On the contrary, the samples of June 2014 and June 2015 were

characterized by the presence of fewer abundant genera (4 and 8, respectively), but with a high number of sequences ascribed to each one. Indeed, in these samples more than 80% of the total bacteria belonged to only two different genera (*Hymenobacter* and *Polaromonas* in June 2014, *Massilia* and *Duganella* in June 2015).

This variability was already highlighted in the work of Chuvochina et al. (2011) that showed how in the snow containing four dust depositions over a 3-year period, only three phylotypes, out of 61, were detected in more than one sample. These findings can be explained by the fact that each Saharan-impacted snow derived from a different single desert dust intrusion, and that these, as demonstrated in Chapter 3, feature different physic-chemical and microbiological characteristics due to the different area of origin, season and trajectory followed to reach the glacier. All these factors may have thus contributed to shape the bacterial community of the particulate matter during the long-range transport and, consequently, that of the dust entrapped in the snow.

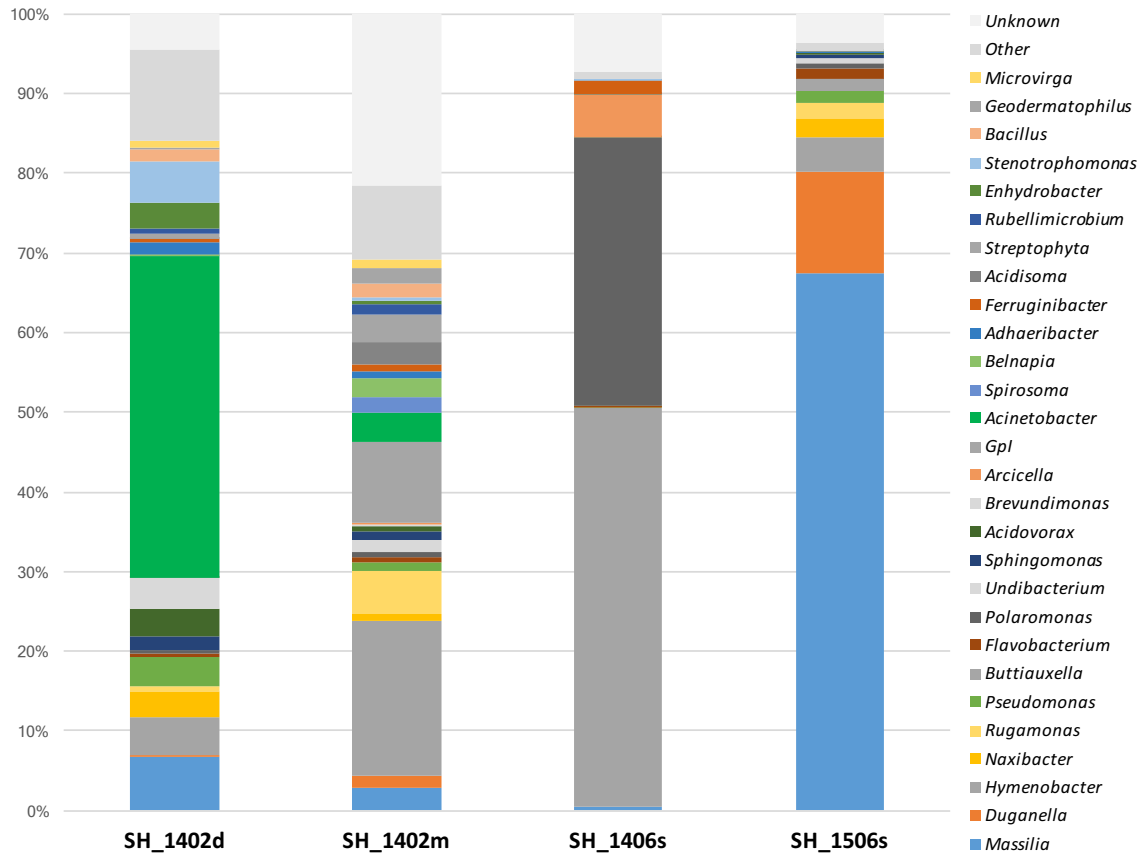

Figure 5.4. Distribution of the most abundant bacterial *genera* (relative abundance higher than 1%) in the Saharan-impacted snow samples. Snow samples were collected on the surface (s), median (m) and deep (d) layer of the snow pit.

The third group of samples analyzed was the melting water recovered directly on the glacier and the water from a natural source at the bottom of the mountain (Figure 5.5).

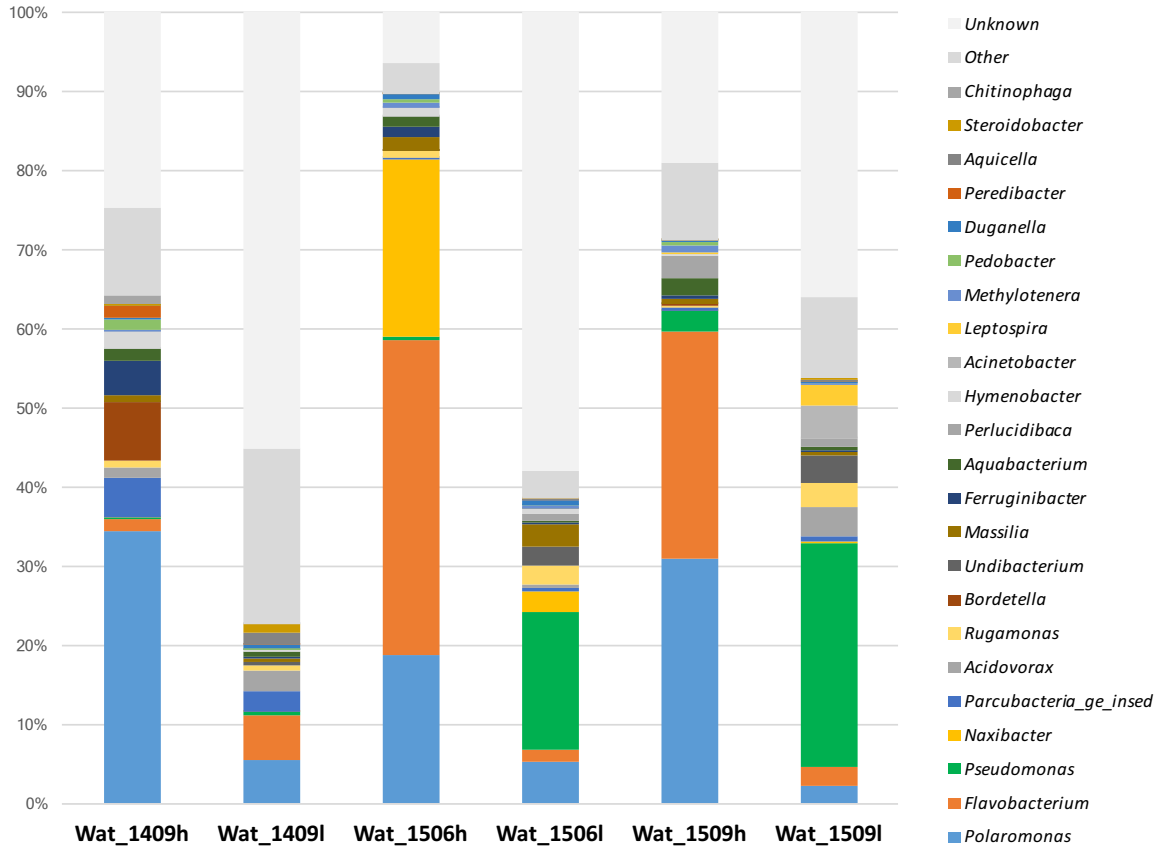

Figure 5.5. Distribution of the most abundant bacterial *genera* (relative abundance higher than 1%) in the water samples. Water samples were collected as water melting directly on the glacier (h) and from a source at the bottom of the mountain (l).

As expected, the analyses indicated that water from the *Calderone* glacier, both sampled directly on the glacier and from a natural source, featured complex bacterial communities with differences over time and between the two type of samples.

Polaromonas and *Flavobacterium* were the only genera abundant across all the samples, even though their average abundances were much higher in the melting water recovered directly from the glacier (28.05 and 23.33%, respectively), rather than in the natural source at the valley (4.3 and 3.24%%, respectively). Bacteria belonging to these genera are psychrophilic, being able to thrive at low temperatures and to survive and even maintain metabolic activity at sub-zero temperatures and have often been found in glaciers (Margesin and Miteva 2011, Franzetti et al 2013). The opposite trend was shown by, for

instance *Pseudomonas*, that were much higher in the source water (15.36%) than in the melting water. These differences are a direct consequence of the fact that despite both kind of water samples derive from the glacier, the one taken at the source is likely enriched by nutrient and microorganisms proper of rocks and soil that are crossed on its way to the valley.

The β-diversity among the bacterial communities in the all the samples is well represented by the 1-D dendrogram based on Bray–Curtis distances reported in Figure 5.6.

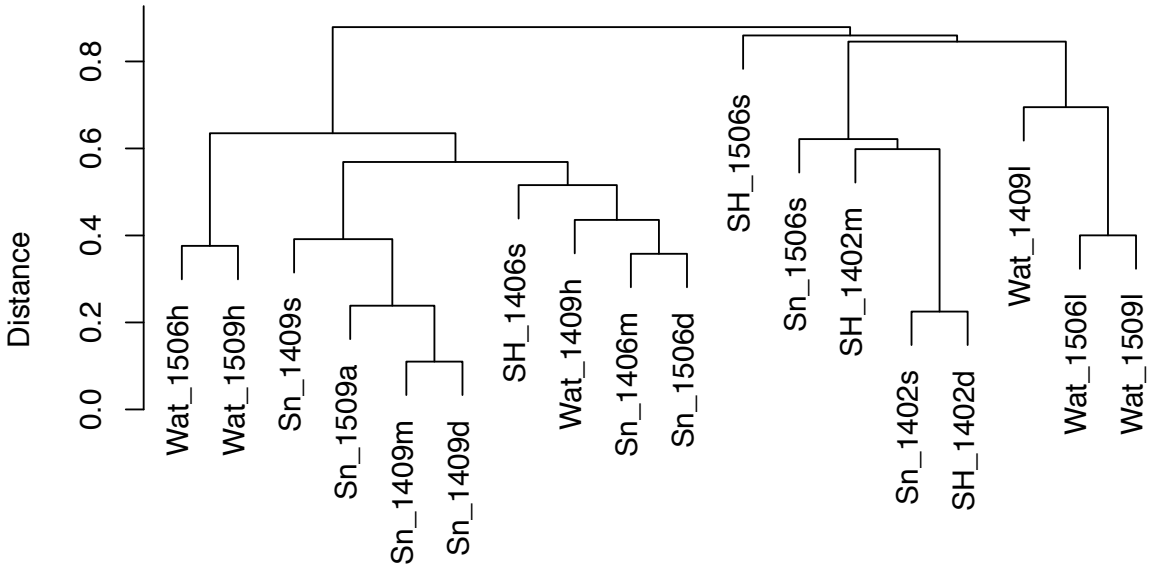

Figure 5.6. Dendrogram based on Bray–Curtis distances between microbial communities of Clean Snow (Sn), Saharan-impacted snow (SH) and water samples (Wat) recovered during the sampling campaigns. Snow samples were collected on the surface (s), median (m) and deep (d) layer of the snow pit. Water samples were collected as water melting directly on the glacier (h) and from a source at the bottom of the mountain (l).

Interestingly, all the Saharan-impacted snow samples, with the only exception of the one collected in June 2014, could be found in a separate cluster than most of the clean snow samples, suggesting that indeed the desert dust had a strong impact on the composition of the community. Nevertheless, two Saharan-impacted snow samples, those collected in February and June 2014 showed only a limited pair-wise distance with the clean snow sampled nearby and in the same period, indicating that a core microbiota of ice-typical bacteria may still occur. As expected, the samples of melting water recovered directly on the glacier showed high distances when compared to the water samples from the natural source. Further, the latter group formed a well-separated cluster on the 1-D dendrogram confirming the peculiarity, as well as the stability over time, of natural source water.

5.3.3 Impact of Saharan dust intrusions on the bacterial diversity of the glacier.

The results reported in the first part of this chapter, clearly suggested that Saharan dust deposited in the *Calderone* glacier impacted the bacterial diversity of the snow. Similar indications were reported in the few papers that, using a culture-independent microbiological approach, have previously studied bacterial diversity of snow with desert dust depositions (Chuvochina et al. 2011, Meola et al. 2015, Weil et al. 2017).

To further unravel the diversity of Saharan dust-impacted snow and to better visualize if this was different from the clean snow and the water originated from the glacier, Figure 5.7 shows the average distribution of most abundant genera (i.e. those over 0.5% in at least one group of samples) among the four different groups of samples, namely those of clean snow, Saharan-impacted snow, melting water from the glacier and source water. Figure 5.8 reports the cluster dendrogram based on the Bray-Curtis distances between the profiles of the average bacterial communities in each group.

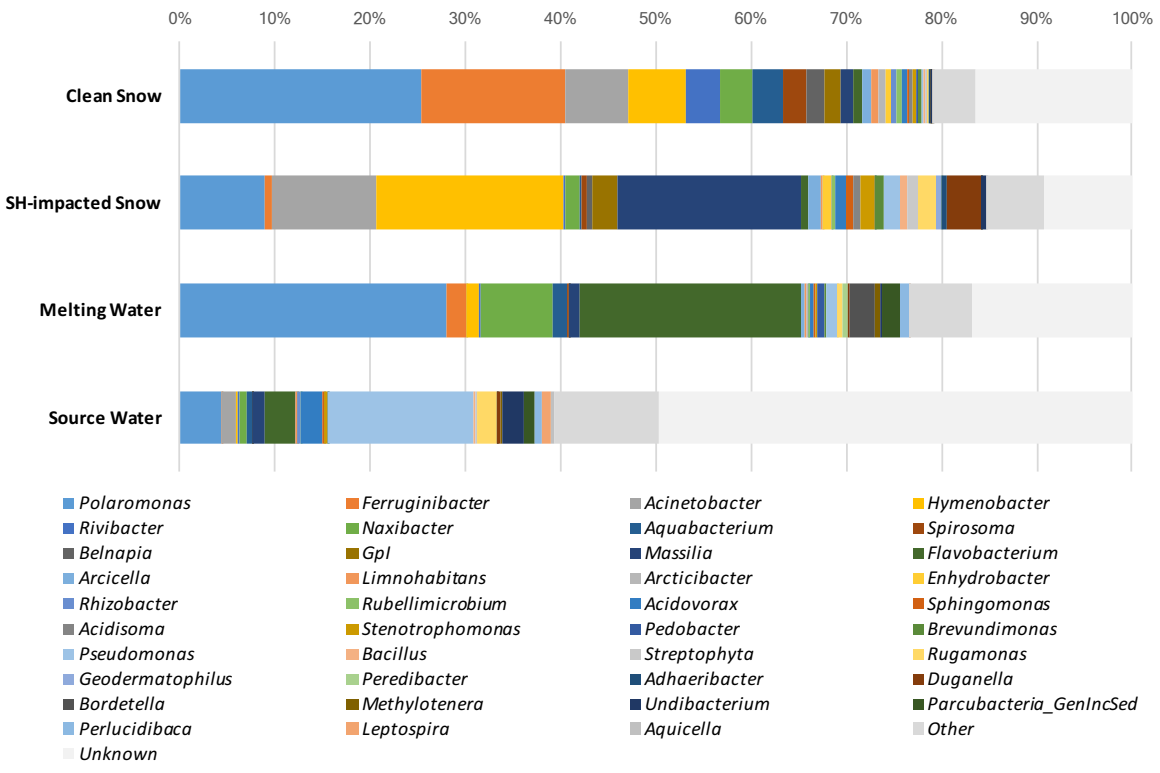

Figure 5.7. Average distribution of the most abundant bacterial *genera* (more than 0.5% of total) in the four different groups of samples (clean snow, Saharan-impacted snow, melting water from the glacier and source water).

Figure 5.8. Dendrogram based on Bray–Curtis distances between the average microbial communities of the four different groups of samples (clean snow, Saharan-impacted snow, melting water from the glacier and source water).

The two samples with the highest similarities were, as expected, those of the clean snow and the melting water harvested directly on the glacier. This is an evident consequence of the fact that the water melting from the glacier largely reflects the snow bacterial communities. Conversely, the most different communities were found in the water collected from the source at the bottom of the mountain, which is likely enriched with the microorganisms of the different environments crossed on its path. The most interesting data is indeed the confirmation that the Saharan-impacted snow samples were, on average, quite different from those of the clean snow.

Clean snow showed, in respect to dust-impacted snow, higher abundances of *Polaromonas, Ferruginibacter, Rivibacter, Naxibacter, Aquabacterium, Spirosoma* and *Flavobacterium*. Further many of these phylotypes were also abundant in the water from the glacier, indicating their predominance and relevance in the Calderone environment. Indeed, *Polaromonas* are ubiquitous bacteria in cold environments and often found in glaciers, especially those covered in debris (Franzetti et al. 2013). *Ferruginibacter* have been recently found as members of lake bacterial communities upon glacier retreat (Peter and Sommaruga, 2016), while *Aquabacterium* and *Flavobacterium* are known to be present in cold environments (Margesin and Miteva 2011).

Among the most abundant genera, the Saharan-impacted snow showed, in respect to the clean snow, much higher average abundances of bacteria belonging to *Acinetobacter, Hymenobacter* and *Massilia* (2, 3 and 15-fold increase, respectively). *Duganella, Rugamonas*, *Pseudomonas*, *Stenotrophomonas* and *Brevundimonas*, which were almost absent in the clean snow, resulted as abundant (average higher than 1% of total) in the Saharan-impacted samples. Other phylotypes, namely *Sphingomonas*, *Bacillus*, *Adhaeribacter*, *Geodermatophilus* and *Undibacterium*, despite being less abundant in the SH-impacetd snow (average between 0.5 and 1%) showed nevertheless an enrichment compared to the clean snow. Most of these genera have already been recovered in extreme environments such as the Antarctic, hot desert soils, rocks and monument surfaces. More importantly, some of them, namely *Hymenobacter, Sphingomonas*, *Bacillus* and *Geodermatophilus,* have already been showed to be enriched in dust impacted snow (Chuvochina et al. 2011, Meola et al. 2015, Weil et al. 2017). Conversely to our findings, the same authors showed that *Acinetobacter* and *Pseudomonas* were more abundant in the clean snow samples.

The enrichment of specific bacterial populations in Saharan-impacted snow may be due to either an input of exogenous microorganisms carried with the desert dust or simply to the amendment of the snow with mineral and nutrients that can stimulate the survival and growth of the indigenous, pre-existing glacial microbiota (Xiang et al. 2009). To unravel what was the portion of the bacterial community found in Saharan-impacted snow that could actually be ascribed to exogenous, dust-carried bacteria, Figure 5.9 reports the relative abundance data for only the most abundant genera (higher than the 1% of abundance) found in the particulate matter during Saharan intrusions, as showed and discussed in Chapter 3. These phylotypes are compared to those found in the snow samples (clean and Saharan-impacted) as well as the water samples (from melting ice and from the natural source) to assess their colonization potential. Considering that it is not easy to relate a dust deposition found in the snow with a precise Saharan dust intrusion, our approach of taking into consideration the data obtained in the occasion of the 9 strongest African dust events occurred in 2014 and 2015, aims to provide a broad picture of what can be consider a sort of "typical" bacterial community of Saharan intrusions (indicated as SH in Figure 5.9), despite the previously shown variability among different samples.

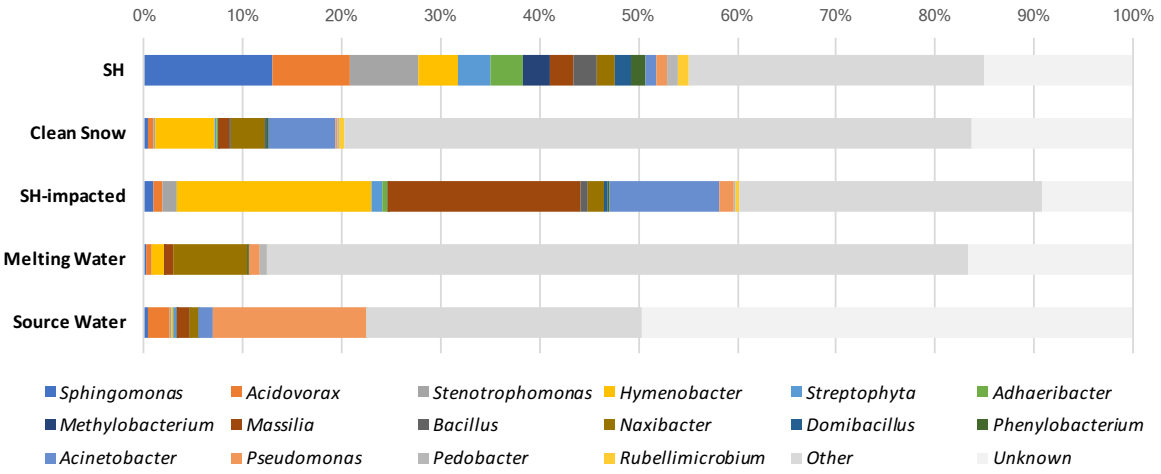

Figure 5.9. Average distribution of top-abundant bacterial *genera* (more than 1% of total) of particulate matter collected during Saharan intrusion (SH), in the four different groups of samples (clean snow, Saharan-impacted snow, melting water from the glacier and source water)

First, it is possible to notice that the 16 top-abundant bacterial genera of typical SH intrusions, that accounted on average for more than 55% of the total Saharan dust bacterial communities, represented also the majority (60.2%) of the community found on the SH-impacted snow, compared to a merely 20.3% of the clean snow. Further, among these 16 phylotypes, 8 were also abundant in the Saharan-impacted snow (*Hymenobacter*, *Massilia*, *Acinetobacter*, *Pseudomonas*, *Naxibacter*, *Stenotrophomonas*, *Acidovorax* and *Streptophyta*), while, only 4 were abundant in the clean snow and, nevertheless, three of the latter showed an enrichment in SH-impacted snow. These data provide a clear indication of the colonization potential of dust-carried bacteria.

The most striking examples of these colonizing bacteria are represented by *Hymenobacter*, *Massilia*, *Acinetobacter* and *Pseudomonas* that, besides being strongly enriched in SH-impacted snow, were also abundant in the water from the *Calderone* glacier, suggesting that their impact may be involving the entire mountain environment. *Hymenobacter* and *Massilia* have been previously detected among the most abundant bacteria in African dust-impacted snow in the Alps (Chuvochina et al. 2011, Meola et al. 2015) as well as in Guoqu and Zadang glaciers on the Tibetan Plateau covered with dust from Chinese deserts (Liu et al. 2009). Bacteria belonging to genus *Hymenobacter*, in particular, are known to be typical of desert environments (Griffin et al. 2003, Favet et al. 2013) and able to resist to high doses of ionizing radiation, thus providing them the advantages to survive during their

transport with dust and subsequent deposition on glacier snow and being distressed by harsh UV-radiation (Rainey et al 2005, Chuvochina et al. 2011). Even though previous studies have shown that dust-free snow was enriched, compared to the dust-impacted one, with bacteria belonging to *Acinetobacter* and *Pseudomonas* (Meola et al. 2015, Weil et al. 2016), our data showing their abundance in both impacted and clean snow layers, still suggest the possibility that these populations may have been dust-carried and have stably colonized the glacier environment.

5.4. Conclusions.

To the best of our knowledge, this work represents one of the few studies that have investigated the impact of African dust on the snow microbiota applying culture-independent approaches (Chuvochina et al. 2011, Meola et al. 2015, Weil et al. 2017). Further, these works have been carried on in the European Alps, while we chose the *Calderone* glacier in Central Italy because, being the southernmost in Europe, can be more easily reached by dust storms from the Sahara Desert. Using culture-independent methods based on high-throughput Illumina sequencing of 16S rRNA genes, we have analysed several Saharan-impacted and clean snow samples collected during several sampling campaigns carried on in 2014 and 2015. The striking differences observed between the bacterial communities of the two group of snow samples, clearly indicated that Saharan dust deposited in the *Calderone* glacier strongly impacted the bacterial diversity of the snow. Several of the bacterial populations that were enriched in the Saharan-impacted snow were also abundant in the particulate matter collected during African dust intrusions, thus confirming that these airborne, dust-carried bacteria were colonizing the glacier. Finally, the fact that some of these phylotypes were also retrieved in the water from the *Calderone* glacier, suggested that the bacteria transported over the Mediterranean from the Sahara can have the potential to impact the entire mountain environment.

CHAPTER 6

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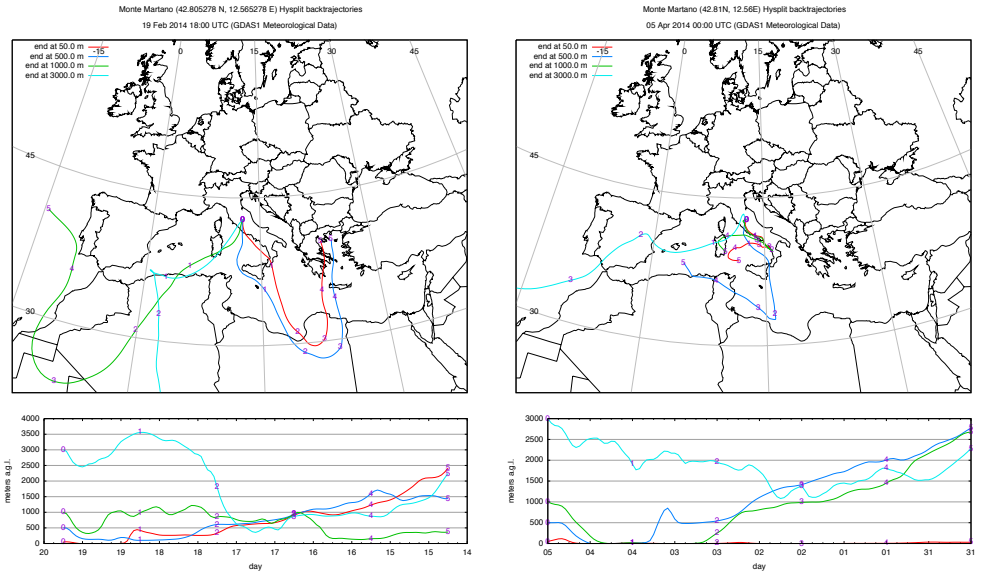
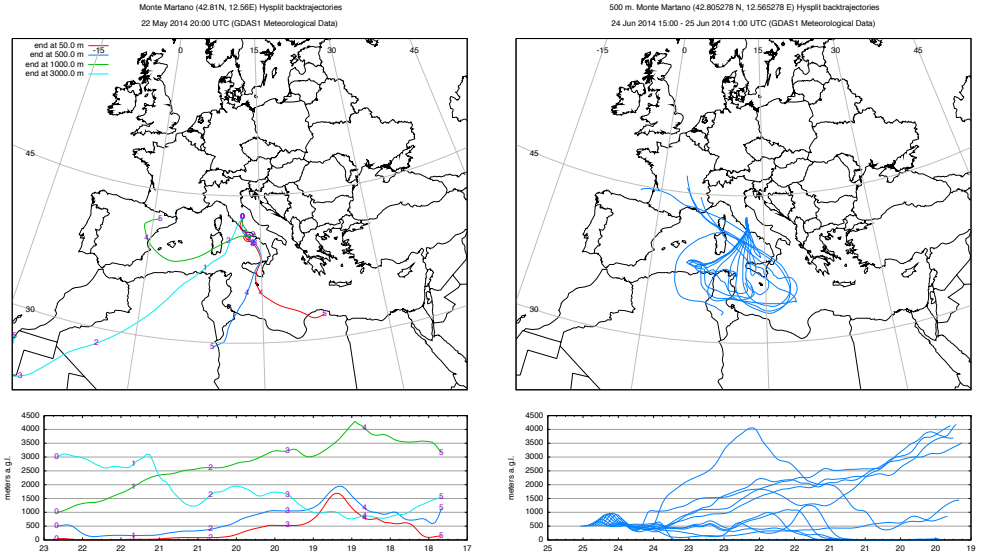
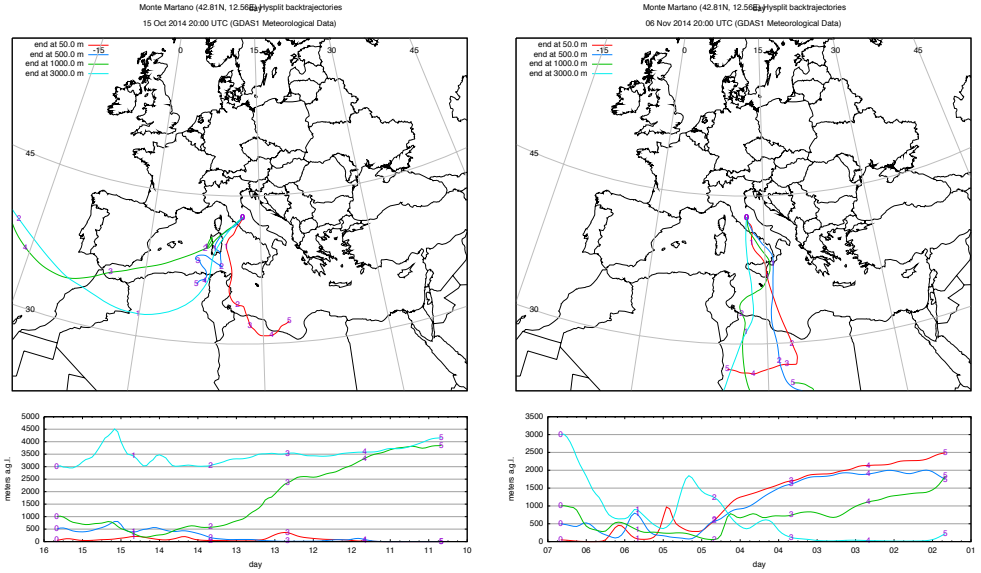
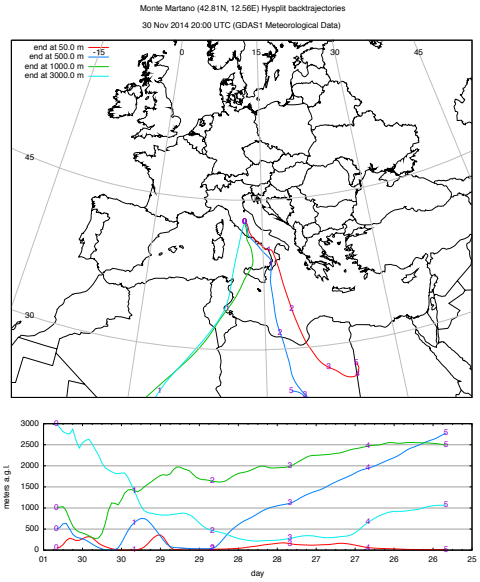
ANNEXES

Annex 3.1 Back-trajectories calculated with the HYSPLIT Trajectory Model of Saharan, Regional and European intrusions sampled in *Monte Martano*.








Monte Martano (42.81N, 12.56E) Hysplit backtrajectories
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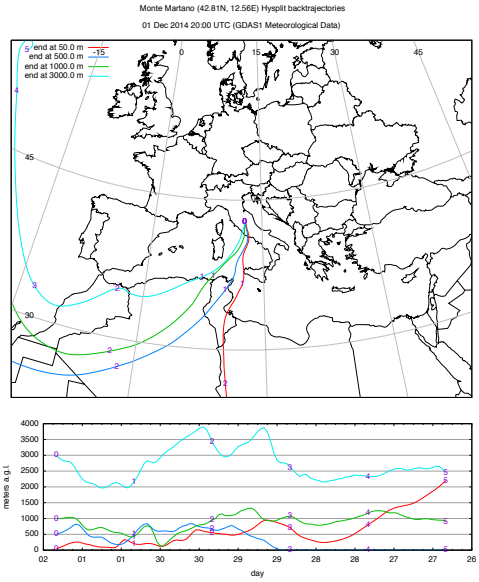
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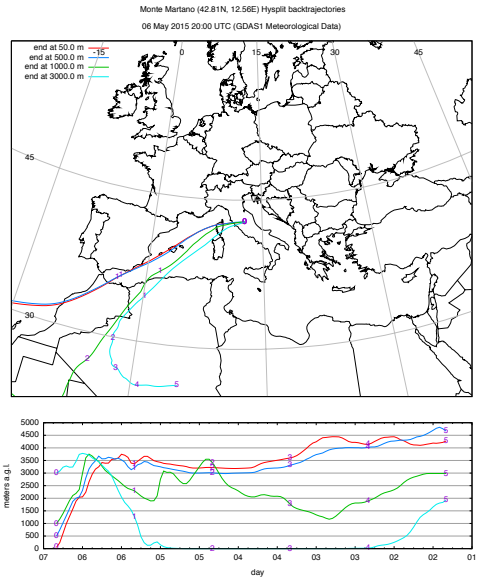
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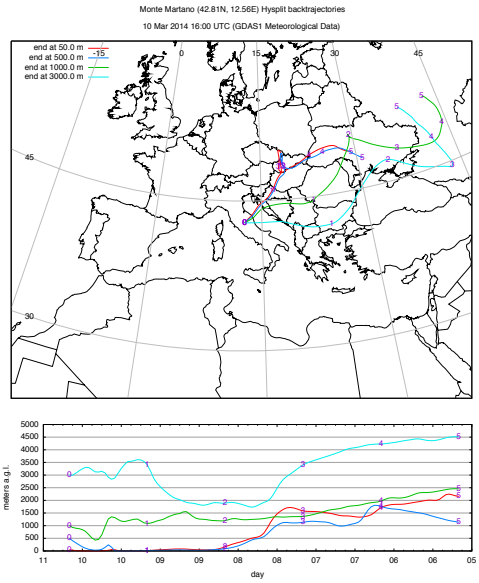
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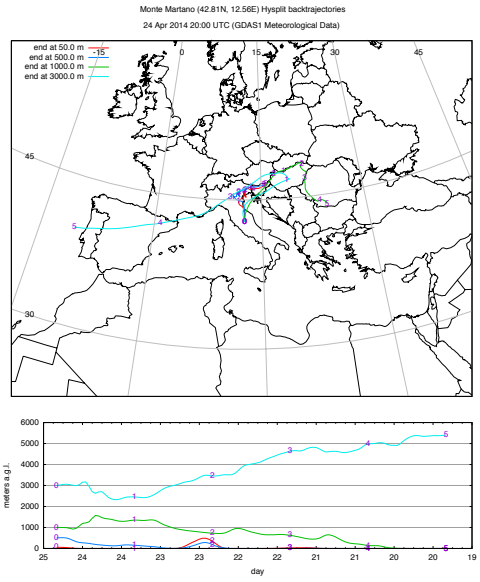
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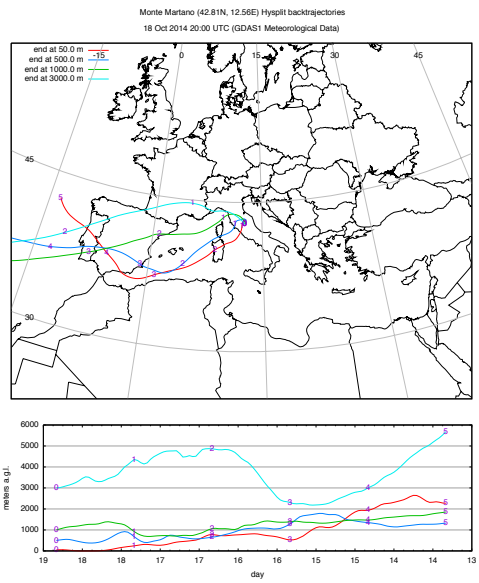
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day

Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

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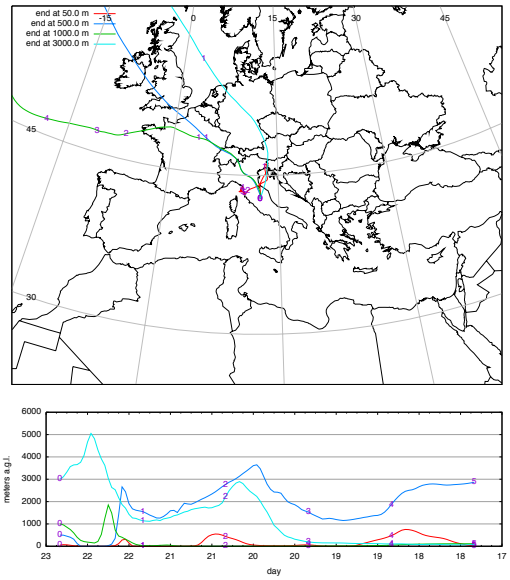

Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

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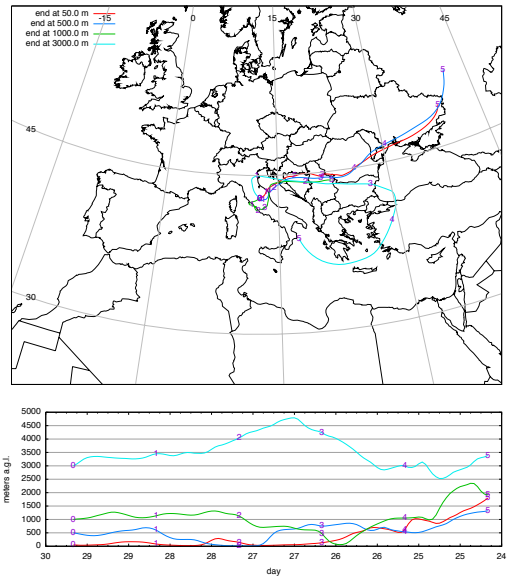

Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

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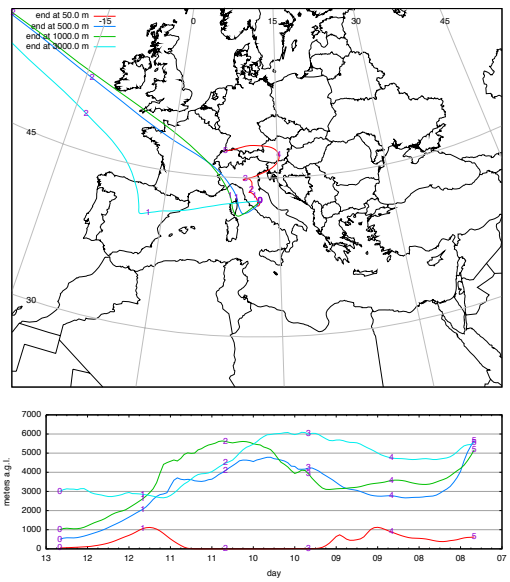

Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

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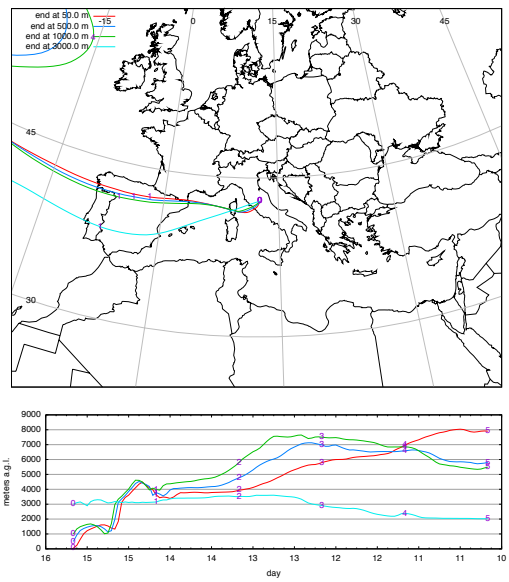

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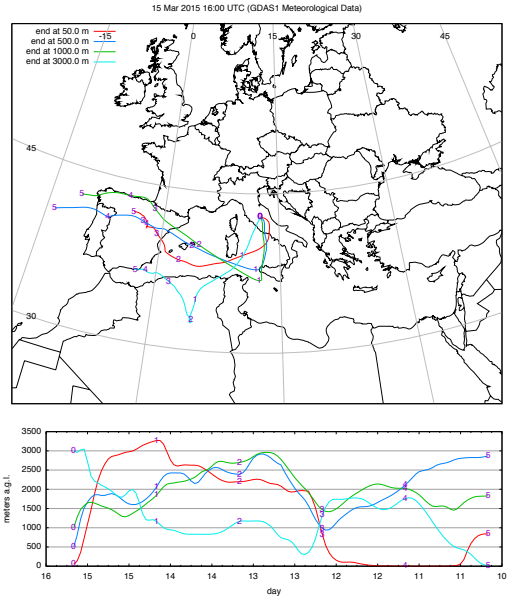
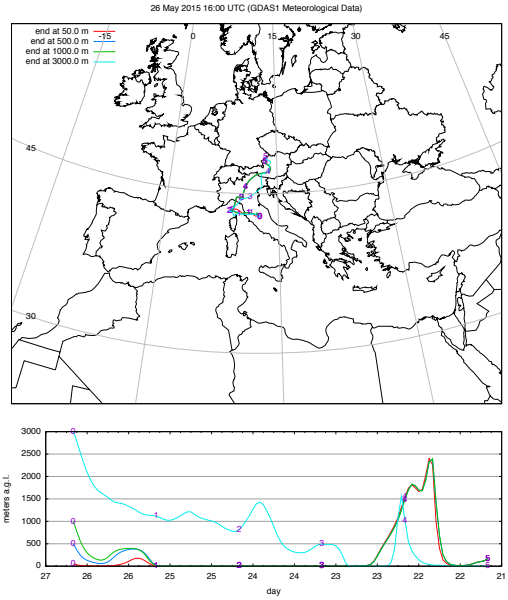
15 Mar 2015 16:00 UTC (GDAS1 Meteorological Data)



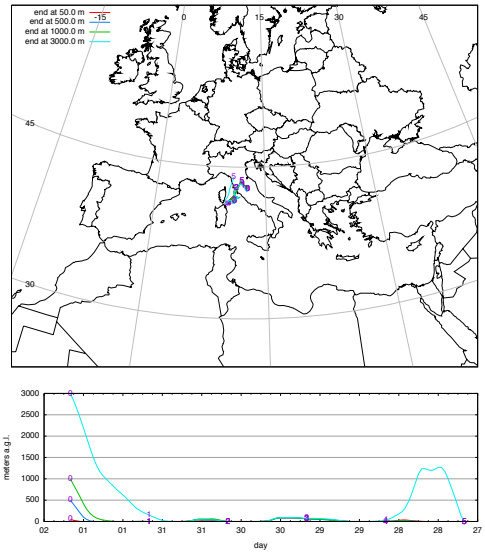
Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

26 May 2015 16:00 UTC (GDAS1 Meteorological Data)



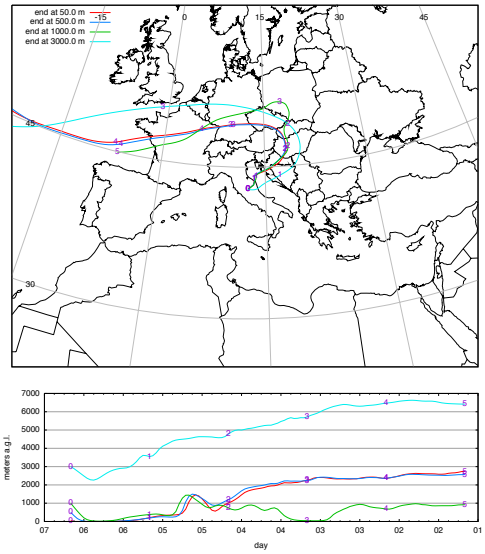
Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

01 Jun 2015 16:00 UTC (GDAS1 Meteorological Data)



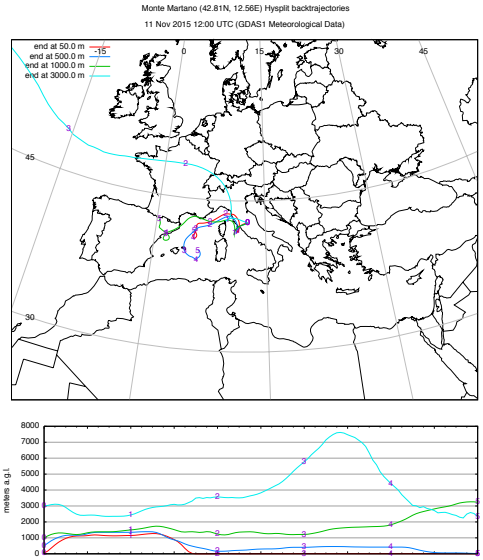
Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

06 Jun 2015 16:00 UTC (GDAS1 Meteorological Data)



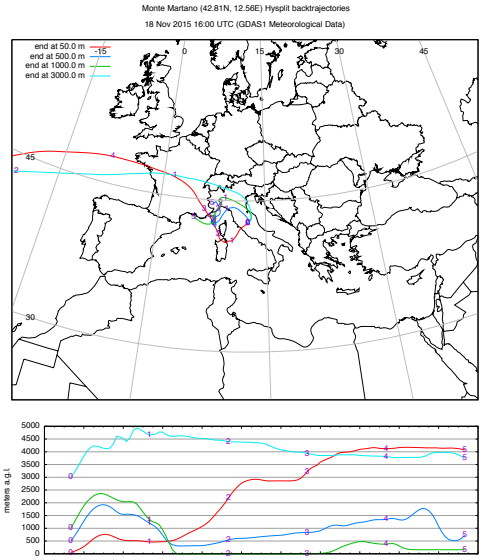
Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

11 Nov 2015 12:00 UTC (GDAS1 Meteorological Data)



Monte Martano (42.81N, 12.56E) Hysplit backtrajectories

18 Nov 2015 16:00 UTC (GDAS1 Meteorological Data)



Annex 3.2 Chemical characterization of the PM_{10} samples impacted by Saharan (SH), Regional (RG) and European (EU) air masses collected in the *Monte Martano* sampling site in 2014 and 2015.

Code	Zn [w/w]	Cr [w/w]	Ni [w/w]	Cu [w/w]	V [w/w]	Mn [w/w]	Ca [w/w]	Ti [w/w]	Fe [w/w]
SH_20140219	0,005	0,005	0,001	0,002	0,001	0,001	0,028	0,001	0,02
SH_20140404	0,006	0,002	0	0,002	0	0,001	0,046	0,001	0,024
SH_20140522	0,009	0,009	0,005	0,008	0,003	0,002	0,063	0,002	0,026
SH_20140624	0,021	0,009	0,005	0,007	0,002	0,002	0,084	0,002	0,035
SH_20141015	0,002	0,001	0,002	0,003	0,001	0,001	0,084	0,002	0,05
SH_20141106	0,004	0,005	0,005	0,007	0,003	0,002	0,067	0,003	0,049
SH_20141130	0,001	0	0	0,001	0	0	0,053	0,000713	0,022
SH_20141201	0,001	0	0	0,001	0	0,001	0,126	0,002	0,08
SH_20150506	0,002	0,000972	0,000412	0,00082	0,000397	0,000759	0,275	0,00571	0,06
RG_20150315	0,00209	0,00137	0,000728	0,00154	0,000695	0,000449	0,0265	0,000458	0,0116
RG_20150526	0,0245	0,00813	0,00333	0,00804	0,00504	0,00167	0,54	0,00195	0,0245
RG_20150601	0,000927	0,00105	0,000437	0,00106	0,000357	0,000332	0,0201	0,000347	0,0104
RG_20150606	0,00124	0,000992	0,000417	0,00118	0,000428	0,00045	0,0148	0,0351	0,0113
RG_20151118	0	0,000592	0,000086	0,00191	0,0000752	0,000126	0	0,000257	0,00793
EU_20141029	0,0028	0,00457	0,00194	0,00398	0,00156	0,000995	0,0452	0,00102	0,0146
EU_20140310	0,00524	0,00463	0,00131	0,00208	0,00152	0,000675	0,0257	0,000979	0,0179
EU_20141022	0,00646	0,00575	0,00236	0,0112	0,00388	0,00209	0,0904	0,00207	0,0322
EU_20141212	0,00375	0,00577	0,00209	0,00769	0,00349	0,00114	0,0747	0,00143	0,0151
EU_20140424	0,0314	0,017	0,00306	0,00954	0,00343	0,00232	0,103	0,00234	0,0259
EU_20150615	0,00386	0,00182	0,00144	0,00245	0,00155	0,00116	0,101	0,00166	0,0429

Code	PM10 [ug/m3]	PM2.5 [ug/m3]	FLUORURI [w/w]	CLORURI [w/w]	NITRITI [w/w]	NITRATI [w/w]	FOSFATI [w/w]	SOLFATI [w/w]
SH_20140219	18,9	7,7	0	0,003	0	0,045	0,026	0,034
SH_20140404	27,5	14,1	0,005	0,024	0,001	0,074	0,031	0,052
SH_20140522	18,7	11,5	0,031	0,014	0	0,148	0,036	0,11
SH_20140624	12,7	8,8	0,05	0,014	0,002	0,931	0,045	0,372
SH_20141015	28,4	12,7	0,009	0	0,004	0,035	0,019	0,014
SH_20141106	8,4	7,5	0,026	0,006	0	0,146	0,053	0,052
SH_20141130	83,6	32,3	0,0034	0,0011	0	0,01	0,0037	0,00629
SH_20141201	86,9	31,2	0,001	0	0	0,009	0,003	0,005
SH_20150506	30,1	12,6	0,00199	0,00365	0	0,0983	0,0166	0,0757
RG_20150315	19,6	14,8	0	0,0122	0	0,557	0,0245	0,156
RG_20150526	5,2	4,9	0,0365	0,00962	0	0,281	0,146	0,177
RG_20150601	15,3	10,3	0,00392	0,000654	0,000654	0,0366	0,015	0,154
RG_20150606	14,8	12,8	0	0,000752	0	0,0188	0,0195	0,222
RG_20151118	12,1	8,6	0	0,0934	0,00248	0,218	0,0455	0,0595
EU_20141029	11,5	9,4	0,00783	0,0113	0	0,156	0,0809	0,338
EU_20140310			0,0143	0,02	0	0,214	0,0217	0,259
EU_20141022	6,1	4,4	0,0115	0,0082	0	0,0623	0,0459	0,041
EU_20141212	6,3	5,2	0,0238	0,0111	0	0,252	0,0937	0,0349
EU_20140424	7,1	6,2	0,0324	0,0521	0,00986	0,297	0,069	0,231
EU_20150615	9,5	5,3	0,0126	0,0347	0	0,114	0	0,301

Code	SODIO [w/w]	AMMONIO [w/w]	POTASSIO [w/w]	MAGNESIO [w/w]	CALCIO [w/w]	OC	EC	TC
SH_20140219	0,023	0,006	0,007	0,002	0,012	0,17	0,01	0,18
SH_20140404	0,051	0,021	0,007	0,004	0,041	0,141	0,011	0,152
SH_20140522	0,055	0,042	0,021	0,006	0,049	0,226	0,037	0,264
SH_20140624	0,096	0,058	0,024	0,011	0,058	0,335	0,041	0,376
SH_20141015	0,103	0,006	0,012	0,011	0,067	0,121	0,008	0,129
SH_20141106	0,119	0,007	0,017	0,005	0,029	0,11	0,007	0,117
SH_20141130	0,0129	0,0021	0,00199	0,00119	0,013	0,0344	0,00083	0,0353
SH_20141201	0,016	0,001	0,004	0,002	0,041	0,043	0,001	0,045
SH_20150506	0,027	0,00857	0,0279	0,00283	0,0405	0,135	0,0171	0,152
RG_20150315	0,0385	0,0628	0,0173	0,00312	0,0183	0,308	0,0169	0,325
RG_20150526	0,143	0,0852	0,0281	0,00571	0,0521	0,378	0,035	0,413
RG_20150601	0,029	0,0272	0,00589	0,00272	0,0169	0,151	0,00756	0,159
RG_20150606	0,0232	0,0383	0,00662	0,0019	0,025	0,237	0,0107	0,248
RG_20151118	0,0683	0,0495	0,0206	0,00483	0,0256	0,34	0,0132	0,353
EU_20141029	0,11	0,0983	0,0317	0,0051	0,0303	0,239	0,0226	0,261
EU_20140310	0,0417	0,0737	0,019	0,00455	0,0179	0,294	0,0149	0,309
EU_20141022	0,303	0,0669	0,0267	0,011	0,0469	0,408	0,0154	0,423
EU_20141212	0,113	0,0503	0,0335	0,00405	0,021	0,273	0,0206	0,294
EU_20140424	0,127	0,105	0,0344	0,0111	0,0499	0,32	0,0213	0,342
EU_20150615	0,0498	0,0525	0,0131	0,0106	0,0764	0,168	0,016	0,184

Annex 4.1 Strains isolated from the two PM samples impacted by Saharan intrusions reaching *Monte Martano* on 30 November and 1st December 2014.

Isolate	Sample	Growth temp.	Closest match	Identity (%)	Accssion number	Sequence Lenght
MM001	30-nov	25°C	*Arthrobacter agilis*	99,9	X80748	736
MM101	30-nov	4°C	*Arthrobacter agilis*	96,1	X80748	806
MM124	01-dec	4°C	*Arthrobacter agilis*	99,6	KF924209	950
MM116	01-dec	4°C	*Arthrobacter agilis*	99,6	X80748	918
MM100	30-nov	4°C	*Arthrobacter crystallopoietes*	99,6	EU360469	1094
MM109	30-nov	4°C	*Arthrobacter pascens*	99,0	X80740	975
MM107	30-nov	4°C	*Arthrobacter pitocampae*	99,7	EU85574	997
MM120	01-dec	4°C	*Arthrobacter polychromogenes*	99,6	X80741	961
MM88	01-dec	25°C	*Bacillus endophyticus*	100,0	AF295302	871
MM103	30-nov	4°C	*Bacillus endophyticus*	100,0	AF295302	839
MM054	01-dec	25°C	*Bacillus halosaccharovorans*	98,3	HQ433447	831
MM044	30-nov	50°C	*Bacillus infantis*	99,6	AY904032	1139
MM021	30-nov	25°C	*Bacillus litoralis*	98,8	AY608605	899
MM017	30-nov	25°C	*Bacillus mojavensis*	99,9	AB021191	1189
MM028	30-nov	25°C	*Bacillus mojavensis*	99,8	AB021191	1119
MM023	30-nov	25°C	*Bacillus mojavensis*	99,8	AB021191	1161
MM030	30-nov	25°C	*Bacillus mojavensis*	100,0	AB021191	1123
MM037	30-nov	50°C	*Bacillus mojavensis*	99,7	AB021191	1140
MM032	30-nov	50°C	*Bacillus mojavensis*	99,8	AB021191	1125
MM034	30-nov	50°C	*Bacillus mojavensis*	99,5	AB021191	939
MM041	30-nov	50°C	*Bacillus mojavensis*	99,8	AB021191	1151
MM042	30-nov	50°C	*Bacillus mojavensis*	99,4	AB021191	874
MM051	01-dec	25°C	*Bacillus mojavensis*	99,7	AB021191	1126
MM049	01-dec	25°C	*Bacillus mojavensis*	99,7	AB021191	1158
MM095	01-dec	25°C	*Bacillus mojavensis*	99,9	AB021191	1133
MM087	01-dec	25°C	*Bacillus mojavensis*	99,8	AB021191	930
MM090	01-dec	25°C	*Bacillus mojavensis*	99,0	AB021191	985
MM073	01-dec	50°C	*Bacillus mojavensis*	99,8	AB021191	1089
MM062	01-dec	50°C	*Bacillus mojavensis*	99,8	AB021191	977
MM065	01-dec	50°C	*Bacillus mojavensis*	99,5	AB021191	996

MM068	01-dec	50°C	*Bacillus mojavensis*	99,6	AB021191	925
MM074	01-dec	50°C	*Bacillus mojavensis*	99,6	AB021191	923
MM077	01-dec	50°C	*Bacillus mojavensis*	99,3	AB021191	959
MM072	01-dec	50°C	*Bacillus niabiensis*	97,5	AY998119	1072
MM019	30-nov	25°C	*Bacillus niacini*	98,8	AB021194	1019
MM018	30-nov	25°C	*Bacillus niacini*	99,7	AB021194	967
MM033	30-nov	50°C	*Bacillus safensis*	99,1	AF234854	985
MM002	30-nov	25°C	*Bacillus simplex*	100,0	AJ439078	817
MM086	01-dec	25°C	*Bacillus simplex*	99,9	AJ439078	1164
MM139	01-dec	4°C	*Bacillus simplex*	100,0	AJ439078	879
MM031	30-nov	50°C	*Bacillus subtilis*	99,9	AF074970	809
MM082	01-dec	50°C	*Bacillus subtilis*	99,0	AF074970	949
MM084	01-dec	25°C	*Bacillus vallismortis*	99,7	AB021198	943
MM126	01-dec	4°C	*Citrobacter gillenii*	98,4	AF025367	1049
MM059	01-dec	25°C	*Frigoribacterium faeni*	99,4	Y18807	1130
MM102	30-nov	4°C	*Frigoribacterium faeni*	99,4	Y18807	1119
MM022	30-nov	25°C	*Kocuria rosea*	98,0	X87756	1028
MM108	30-nov	4°C	*Kocuria rosea*	98,4	X87756	948
MM026	30-nov	25°C	*Massilia niastensis*	98,6	EU808005	1102
MM060	01-dec	25°C	*Microvirga aerilata*	98,4	GQ421849	1068
MM015	30-nov	25°C	*Microvirga vignae*	98,4	JX504804	839
MM013	30-nov	25°C	*Microvirga vignae*	98,5	JX504804	839
MM099	30-nov	4°C	*Nibribacter koreensis*	96,5	JN607156	1018
MM006	30-nov	25°C	*Paenibacillus xylanilyticus*	99,5	AY427832	1158
MM132	01-dec	4°C	*Pedobacter duraquae*	99,5	KC494334	939
MM138	01-dec	4°C	*Pedobacter ginsengisoli*	98,5	AB245371	999
MM009	30-nov	25°C	*Pseudomonas duriflava*	99,8	EU046271	923
MM011	30-nov	25°C	*Pseudomonas duriflava*	95,7	EU046271	847
MM125	01-dec	4°C	*Rhodococcus cerastii*	99,3	FR714842	930
MM056	01-dec	25°C	*Saccharothrix xinjiangensis*	100,0	AY135693	839
MM119	01-dec	4°C	*Sanguibacter keddieii*	98,4	X79450	999
MM097	30-nov	4°C	*Sphingomonas areolata*	99,8	LN774415	987
MM121	01-dec	4°C	*Sphingomonas areolata*	99,7	AJ429240	919
MM004	30-nov	25°C	*Sphingomonas aurantica*	98,9	AJ429237	878

MM106	30-nov	4°C	*Sphingomonas faeni*	99,9	AJ429239	1050
MM020	30-nov	25°C	*Sphingomonas hankookensis*	98,1	FJ194436	1121
MM024	30-nov	25°C	*Streptomyces atrovirens*	99,2	DQ026672	803
MM091	01-dec	25°C	*Streptomyces diastaticus*	98,7	DQ026631	996
MM071	01-dec	50°C	*Streptomyces flavoviridis*	99,3	AB184842	993
MM080	01-dec	50°C	*Streptomyces fumanus*	97,4	AB184273	954
MM078	01-dec	50°C	*Streptomyces glaucus*	98,6	AB184665	1048
MM070	01-dec	50°C	*Streptomyces griseorubens*	99,2	AB184139	1055
MM007	30-nov	25°C	*Streptomyces griseochromogen*	99,3	AJ399491	777
MM140	01-dec	4°C	*Streptomyces kanamyceticus*	99,3	DQ442511	939
MM085	01-dec	25°C	*Streptomyces microflavus*	100	DQ445795	1137
MM052	01-dec	25°C	*Streptomyces microflavus*	99,9	DQ445795	999
MM136	01-dec	4°C	*Streptomyces microflavus*	99,3	DQ445795	957
MM130	01-dec	4°C	*Streptomyces microflavus*	98,9	DQ445795	946
MM135	01-dec	4°C	*Streptomyces microflavus*	100,0	DQ445795	928
MM005	30-nov	25°C	*Streptomyces mutabilis*	99,9	EF178679	1028
MM053	01-dec	25°C	*Streptomyces mutabilis*	99,8	EF178679	919
MM081	01-dec	50°C	*Streptomyces naganishii*	98,3	DQ442529	1020
MM012	30-nov	25°C	*Streptomyces naganishii*	98,2	DQ442529	1108
MM003	30-nov	25°C	*Streptomyces roseolilacinus*	99,2	AB184167	926
MM043	30-nov	50°C	*Streptomyces roseolilacinus*	100,0	AB184167	1181
MM046	01-dec	25°C	*Streptomyces roseolilacinus*	99,2	AB184167	924
MM061	01-dec	50°C	*Streptomyces roseolilacinus*	99,2	AB184167	1137
MM066	01-dec	50°C	*Streptomyces roseolilacinus*	99,2	AB184167	1064
MM039	30-nov	25°C	*Streptomyces roseolilacinus*	99,5	AB184167	1157
MM055	01-dec	25°C	*Streptomyces variabilis*	96,6	DQ442551	813
MM093	01-dec	25°C	*Streptomyces viridodiastaticus*	99,3	AY999852	976
MM016	30-nov	25°C	*Streptomyces youssoufiensis*	99,6	FN421338	1149

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