AMERICAN UNIVERSITY OF BEIRUT

# MUDDY RAIN TRANSPORTS LARGE NUMBERS OF DIVERSE MICROBES

by

GHIDA NOUHAD ITANI

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AMERICAN UNIVERSITY OF BEIRUT

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GHIDA NOUHAD ITANI

Approved by:

| | |
|---|---|
| Dr. Colin Smith, Associate Professor<br>Biology | Advisor |
| Dr. Imad Saoud, Professor<br>Biology | Member of Committee |
| Dr. Mike Osta, Assistant Professor<br>Biology | Member of Committee |

Date of thesis defense: June 19, 2014

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# AN ABSTRACT OF THE THESIS OF

Ghida Nouhad Itani for Master of Science

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Title: Muddy Rain Transports Large Numbers of Diverse Microbes

Muddy rain is frequently observed in Lebanon, yet its biological roles are unexamined, and there are no reports describing its microbial biodiversity. It is known that rain and snow can be nucleated by bacteria, that desert dust is transported globally, that human and crop pathogens are transported by dust, and that microbial exchange between distant habitats is rapid relative to climate changes and evolution. Muddy rain may be an important means by which microbial biodiversity is exchanged, because microbes in muddy rain are exposed to less inactivating solar radiation and are deposited directly into soils and other rich ecologies. One can imagine that microbes have not only adapted to dispersal in dust and rain, but that they may facilitate the formation of dust and rain. This study investigates the microbial biodiversity in Beirut muddy rains. During 2011 and 2012, 23 rainfalls were collected, of which, 18 were distinctly muddy and contained observable DNA. Rainfalls were backtracked by HYSPLIT model (NOAA), indicating 14 North African sources and 4 Arabian Peninsular sources. DGGE revealed microbial diversity of the samples. Plasmid libraries of bacterial 16S and fungal 18S rDNA were constructed using PCR amplification. Sequences of isolated plasmids were analyzed by database comparison and yielded a total of 168 bacterial sequences; comprising, in decreasing order of relative abundance, Betaproteobacteria, Alphaproteobacteria, Firmicutes, Actinobacteria, Gammaproteobacteria/Bacteroidetes, Cyanobacteria, Epsilonproteobacteria, and Deltaproteobacteria. Thirty-five fungal sequences were found; 20 of which were Basidiomycota, 11 were Ascomycota, and 4 were uncultured eukaryotic clones. These results show that diverse active microbial communities are carried and dispersed via muddy rain.

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# ABBREVIATIONS

| | |
|---|---|
| % | Percent |
| / | Per |
| µg | Microgram |
| µl | Microliter |
| µM | Micromolar |
| ° | Degrees |
| °C | Degrees Celsius |
| amp | Ampicillin |
| ATP | Adenosine Triphosphate |
| BC | Before Christ |
| bp | Base Pair |
| C | Cytosine |
| cm | Centimeter |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| EB | Extraction Buffer |
| EDTA | Ethylenediaminetetraacetic acid |
| et al. | Et allii (and other) |
| fmol | Femtomole |
| G | Guanine |
| GDAS | Global data Assimilation System |
| HYSPLIT | Hybrid Single Particle Lagrangian Integrated Trajectory |
| kg | Kilogram |
| km | Kilometer |
| $km^2$ | Squared Kilometer |
| kV | Kilovolts |
| l | Liter |
| LB | Luria Bertani |
| $m^2$ | Meter Squared |
| mA | Milliampere |
| mg | Milligram |
| min | Minute |
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |

| | |
|---|---|
| NASA | National Aeronautics and Space Administration |
| ng | Nanogram |
| NOAA | National Oceanic and Atmospheric Administration |
| PCR | Polymerase Chain Reaction |
| SDS | Sodium Dodecyl Sulfate |
| sec | Second |
| TAE | Tris-acetate-EDTA |
| TRFLP | Terminal Restriction Fragment Length Polymorphism |
| V | Volts |
| XRD | X-ray Diffraction |

# CHAPTER I

# INTRODUCTION

## A. Overview and Specific Aims

The phenomenon of muddy rain is frequent in Lebanon, where spring rain contains visible soil that is deposited onto surfaces (Figure 1). It is known that dust is transported globally with a high number of viable microbes and that these microbes can nucleate rain and snow. The existence of similar microbial ecologies in similar habitats worldwide and the adaptation of microbes to long distance travel via dust and muddy rain suggest a global exchange that is rapid relative to evolutionary, geological, and climatic change. Extensive studies have been made to describe the microbial biodiversity transported by desert dust; however, no similar studies have been made about the frequent phenomenon of muddy rain. This study investigates the biodiversity, the ecological roles, and the pathogenicity of muddy rain microbes. Because microbes in muddy rain are protected by the water droplet and are washed directly into soils and new ecologies, one can speculate that microbes have adapted to muddy rain to use it as an efficient and rapid means for global dispersal. One can even imagine that microbes facilitate the lofting of dust, the creation of dust storms, and the formation of muddy rain in order to maintain their survival by spreading worldwide.

Figure 1. The phenomenon of muddy rain is frequently observed in Lebanon.

The long-term objectives of this study are to understand the global dispersal and ecological exchange of muddy rain microorganisms. The specific aims and the approaches used to reach them are described below:

Specific Aim 1: Collect Samples of Muddy Rain: In order to generalize from local muddy rains, collection of every muddy rain is to be attempted during 2011 and 2012. Rain is collected on a clean 2.1 square meter rooftop tarpaulin. Preliminary processing of the rain water consists of filtering it through a 0.22 µm membrane, narrow enough to trap the smallest bacteria. Then, half membranes are to be stored in DNA extraction buffer for later manipulation of the DNA. A small part of the filter is to be used to prepare a bacterial glycerol stock and a small amount of the crude rain water is to be stored for later mineralogical analysis.

Specific Aim 2**:** Backtrack Rain Origins: To determine the origin of muddy rain and its associated microbes and to determine the path along which the microbes were picked up, wind trajectories will be traced backward toward their putative origin. This was achieved using the HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory) model from NOAA (National Oceanic and Atmospheric Administration).

Specific Aim 3: Corroborate Origins with Mineralogical Analysis: To support the backtracking results and to further ensure the origin of muddy rain microbes, mineralogical analysis of dried muddy rain solids is to be performed. The comparison between muddy rain minerals and Sahara desert (the putative origin identified by backtracking) minerals will corroborate the Saharan origin of muddy rain. Samples will be scanned by a Bruker D8 Discover X-ray diffractometer and analyzed using DIFFRAC.EVA software.

Specific Aim 4: Estimate Biodiversity by DGGE and TRFLP: Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) are two techniques that are to be used to estimate the microbial biodiversity of muddy rain. DGGE and TRFLP are methods that can separate DNA fragments based on sequence composition; thus, they are useful for mixed DNA sequences of similar sizes. These methods will allow the determination of the most diverse samples and the most abundant bacterial species.

Specific Aim 5: Amplify, Clone, and Sequence DNA: In an attempt to study the microbial community composition of muddy rain, a culture–independent approach known as the metagenomic approach is to be implemented. Total DNA is to be extracted from the microbe saturated filter by bead beating. A region of 16S rDNA will

be amplified by universal primers (Bacterial and Fungal). Amplified DNA is to be ligated to pcDNA3 plasmid followed by transformation into DH5α *Escherichia coli*. Microbial diversity is to be assessed by PCR screening of individual colonies. Plasmid DNA of desired colonies is to be extracted and sequenced by a service provider.

Specific Aim 6: Identify Genera and Species with Databases: Sequences from the muddy rain samples are blasted against and aligned with reference sequences of microbes. The percent composition of the different microbial phyla is calculated. Microbial biodiversity in muddy rain is compared to that of desert soil and dust reported in the literature.

Specific Aim 7: Cluster Sequences Based on their Phylogenetic Relationships: A phylogenetic tree is to be constructed. The tree indicates the phylogenetic affiliation of the bacterial muddy rain sequences and serves to cluster the sequences by phylotypes. Sequences are to be aligned, curated, and grouped based on the maximum-likelihood principle.

Specific Aim 8: Consider the Ecological Roles of Transported Microbes: The analysis of muddy rain microbes permits the prediction of their possible ecological roles. Each bacterial and fungal phylum is characterized based on pathogenicity, adaptation features, and environmental roles.

## B. Microbes are Dispersed Globally via Rain and Dust

### *1. History of Muddy Rain*

The phenomenon of muddy rain was noted in antiquity. The earliest record is found in Homer's *Iliad* written in the 8$^{th}$ century BC, were Zeus caused "a rain of blood". The full quotation is: "But Jove with dire confusion fill'd their ranks, who sent

from heav'n a shower of blood-stain'd rain, in sign of many a warrior's coming doom" (Stanley, 1865). The bloody rain has been explained by ancient authors as a sign of the gods' rage. The first natural explanation came from the Roman philosopher Marcus Tullius Cicero in 44 BC who suggested that the red bloody rain may be caused by "*ex aliqua contagion terrena*", that is "from some earthly contagion."

### *2. Three Important Global Dust Sources Are the Sahara Desert, the Gobi desert, and the Great Sandy Desert*

The three important global dust sources are the Sahara Desert (North Africa), the Gobi Desert (East Asia), and the Great Sandy Desert (Oceania) (Kellogg and Griffin, 2006).The dust from these deserts can move great intercontinental distances, and thus disperses globally. Figure 2 shows the patterns of global dispersal of dust and dust microbes from the three major dust sources. Annually, 3 billion tons of desert dust makes regional or global movements with altitudes reaching up to 8 km (Griffin, 2007; Griffin *et al.,* 2007). The Sahara and Sahel regions of North Africa are the main sources of dust, where they contribute 50 to 75% of the 3 billion tons but recent desertification and climate change have led to a noticeable increase in Asian dust activity (Griffin, 2007). Saharan dust moves north towards Europe, east towards the Middle East, and northwest towards North America and the Caribbean (Griffin *et al.,* 2003). These Saharan dust clouds can have major effects on downwind ecosystems. The interest of this study is the Saharan dust transported to and deposited in the Middle East, specifically in Beirut, Lebanon. According to Griffin *et al.* (2007), Saharan dust transported to the Middle East comes mainly from the arid regions of western Egypt, Libya, and eastern Algeria. One can imagine that in the related phenomenon of muddy rain, mud is picked by a storm into the clouds, but deposited directly in rain. In this

study, we use muddy rain as a tool to better understand global microbial dispersion and its potential ecological consequences.

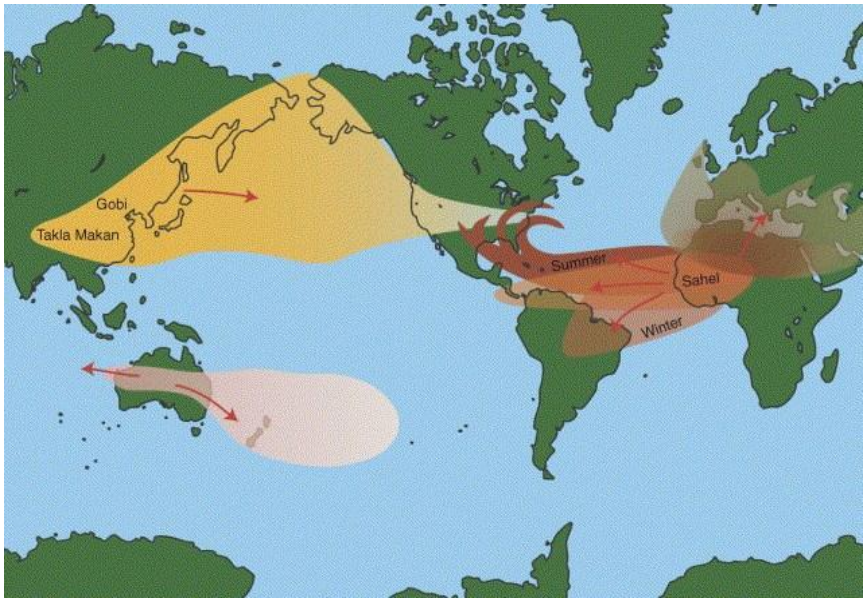



Figure 2. Global dispersal of microorganisms via dust. Figure is reproduced from Kellogg and Griffin (2006) and Garrison *et al.* (2003) with permission.

***3. Dust and Rain Contain Viable Microorganisms***

Typically, one gram of arid soil contains $10^9$ prokaryotes, $10^6$ fungi, and $10^8$ viral particles (Griffin, 2007) which can be aerosolized along with dust particles to which they are attached. Griffin *et al.* (2002a) roughly estimated the yearly amount of aerosolized sediment-borne bacteria to be big enough as to form a bridge between Earth and Jupiter. Similar to dust, rain and cloud droplets also harbor active microbial communities. Sattler *et al.* (2001) estimated the amount of bacteria in cloud droplets to be 1,500 per ml. One can imagine three categories of dust/rain associated microbes: metabolically inactive microbes, metabolically active microbes that do not reproduce, and metabolically active reproducing microbes (Womack *et al.*, 2010). Some might think that dust and rain cannot harbor live microorganisms. But studies provided evidence that aerosolized bacteria are able to metabolize glucose and go through

multiple growth generations (Dimmick *et al.*, 1975; Womack *et al.*, 2010). Similarly, rain bacteria are able to reproduce, degrade organic compounds, and perform cellular respiration (Sattler *et al.*, 2001; Hill *et al.*, 2007; Vaitilingom *et al.*, 2010). This suggests that dust and rain bacteria are viable and metabolically active.

***4. Microbes Disperse over Long Distances by Rain and Dust***

Long distance dispersal of microbes via dust was noted more than 200 years ago. The earliest observation recorded was made by the German biologist Christian Ehrenberg in the early 1800's, were he described "Infusoria" (a historical term meaning microorganisms) in African dust samples collected aboard the Beagle by Charles Darwin (Darwin, 1846; Griffin *et al.*, 2002a; Griffin *et al.*, 2003). In 1861, Louis Pasteur described living bacteria and fungi in air samples collected in the mountains and he made the observation that microbial concentrations in air differ according to their location (Pasteur, 1861; Griffin *et al.*, 2002a; Griffin *et al.*, 2003). Currently, numerous studies show that live microbes can be carried long intercontinental distances by dust clouds. For example, live bacteria and fungi were found in African dust clouds transported across the Atlantic to the Barbados Island in the Caribbean (Prospero *et al.*, 2005). The current study focuses on the huge masses of dust clouds that travel, with their associated microbial flora, from the Northern Sahara and deposit in the eastern Mediterranean in the form of spring muddy rain (Figure 3 and 4). Dust and rain associated microbes transported to the eastern Mediterranean are expected to be in a dynamic change dictated by the current climatic changes that alter the frequency and intensity of dust emission and induce changes to the ecosystem (Hervas *et al.*, 2009).

Microbes are able to remain viable throughout their long journey because the atmosphere is not a hostile environment and dust clouds/rain droplets provide a shield

for the microbes. The pH of rain water and clouds ranges from 5.5 to 7.5 (Khemani *et al.*, 1985), which is not hostile for bacteria that are adapted to tolerate more acidic or basic habitats (Womack *et al.*, 2010). Also, the various temperatures of atmospheric layers are within ranges tolerated by microbes (Womack *et al.*, 2010). Harmful UV radiation in the atmosphere is attenuated by more than 50% by dust clouds according to NASA research (Griffin, 2007). The above factors suggest that the atmosphere is a habitat in which microbes can remain active and Womack *et al.* (2010) claims that bacteria perform several rounds of division while suspended in the atmosphere.

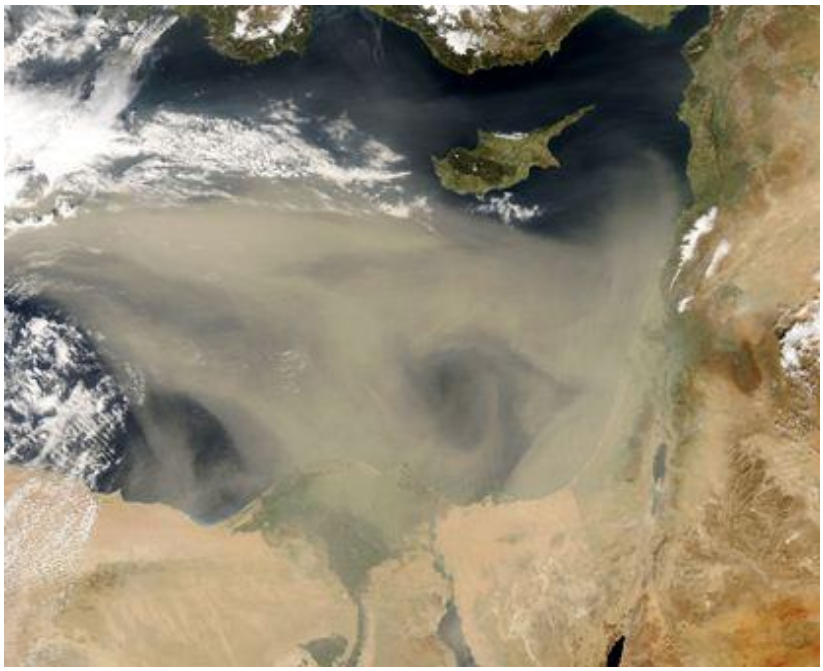


Figure 3. Sahara dust over the eastern Mediterranean on February 24, 2007. (earthobservatory.nasa.gov).

Figure 4. Dust storm in the eastern Mediterranean on October 19, 2002 (visibleearth.nasa.gov).

**C. Ecological Roles of Airborne Microorganisms**

***1. Microorganisms Nucleate Rain and Snow***

It is known that some bacteria and fungi act as biological nucleators for ice, rain, and cloud condensation (DeLeon-Rodriguez *et al.*, 2013). It is also proposed that bacteria participate in a sort of biological cycle of precipitation, thus seeding clouds to provoke rain formation (Morris *et al.*, 2004). The present study speculates that these microbes nucleate muddy rain and snow as a means to facilitate their global dispersal in a fast and safe manner. Biological nucleators, especially bacteria, have been shown to be the most active natural nucleators (Christner *et al.*, 2008) because they can nucleate ice at temperatures as high as -2°C which is much warmer than the nucleating temperature of non-biological molecules (DeLeon-Rodriguez *et al.*, 2013).

Several studies have attempted to quantify the biological nucleators in rain and snow. One study by Christner *et al.* (2008) showed that at temperatures warmer than -10°C, the concentration of biological ice nucleators ranged between 4 and 490 particles per liter of rain or snow. This study was performed in different regions of the world, suggesting the global presence of biological ice nucleators in the atmosphere. Only few species of bacteria are known to be efficient nucleators of rain and snow (Christner, 2010), those species include *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Pseudomonas fluorescens*, *Pantoea agglomerans*, and *Xanthomonas campestris* (Morris *et al.*, 2004). Among these species, *Pseudomonas* syringae, a plant pathogen causing necroses on fruits, stems and leaves, is the most common (Morris *et al.*, 2007; DeLeon-Rodriguez *et al.*, 2013), and it has the capacity to catalyze ice formation at relatively warm temperatures near -2°C (Christner, 2010).

Bacteria are able to serve as ice and rain nucleators because they express a protein on their surface that nucleates ice (Morris *et al.*, 2004). At temperatures near -2°C, the ice nucleating proteins aggregate and orient water molecules to form a small spherical ice surface which will in turn initiate the cascade of ice crystal formation (Morris *et al.*, 2004; Christner *et al.*, 2008; Christner, 2010). Christner (2010) proposed that plant associated microbes, such as the bacteria *Pseudomonas syringae*, developed the ability to nucleate ice as a survival advantage. The ice nucleating ability enables bacteria to endure freeze-thaw stress, facilitates the acquisition of nutrients leaked from frost damaged plant tissues, aids in infection, and protects the plant host by avoiding extreme supercooling (Christner, 2010). This project proposes another advantage for ice nucleation, which is the enablement of intercontinental microbial transport, spread, and exchange.

### *2. Similar Microbial Ecologies Exist in Distant Habitats*

The phenomenon of similar microbial ecologies in similar but distant habitats is well reported in various regions of the world. This phenomenon suggests the presence of global microbial spreading and exchange which allowed the existence of similar microbial ecologies in similar faraway habitats separated by geographic barriers. The phenomenon is common in various terrestrial and oceanic habitats. For example, similar bacterial genera were detected in two coral reef habitats 3,000 km apart (Rohwer *et al.*, 2002). But in this study, the interest is more associated with the soil bacteria recovered from similar but distant habitats. An example of which is the similarity in the *Pseudomonas* species recovered from soil samples 20,000 km apart (Cho and Tiedje, 2000; Martiny *et al.*, 2006) and in the nitrogen fixing bacteria species recovered from soil crusts samples 700 km apart (Yeager *et al.*, 2004; Martiny *et al.*, 2006). These examples support the hypothesis that microbes are able to survive long distant transport by dust and rain to colonize distant habitats.

### *3. Microorganisms Have Adaptations to Global Dispersal*

Previously, it has been assumed that microbes are not able to survive long distance dispersal because of the harmful exposure to UV radiation, desiccation, and unsuitable growth conditions (Kellogg and Griffin, 2006). This assumption is false because microbes have adapted to resist all of the above tribulations by the evolution of survival features such as pigmentation, UV resistance, and sporulation. Fahlgren *et al.* (2010) reported that 81% of air-borne bacteria recovered from their air samples were pigmented, thus shielding themselves from UV radiation (Kellogg and Griffin, 2006). Some members of the genus *Deinococcus* isolated from desert soil are pigmented and highly resistant to ionizing radiation (Yuan *et al.*, 2009). UV resistance can also be

acquired by the high GC content and enhanced DNA repair mechanisms that some bacteria possess (Griffin *et al.*, 2007). Sporulation is perhaps the most evident survival feature enabling bacteria and fungi of intercontinental transport. The best known spore-forming bacteria are members of the genera *Bacillus* and *Clostridium* that are commonly found in soil. Spores allow the bacterium to be dormant and protected from desiccation, chemicals, heat, radiation, and nutrient deficiency until it encounters suitable growth conditions permitting germination and outgrowth (Nicholson, 2002). These survival features are evidence that global microbial transport via rain and dust is not far-fetched.

### *4. Some Muddy Rain Microorganisms Are Disease Vectors*

On one hand, muddy rain is beneficial for its downwind environment. The dust in muddy rain is nutrient rich and its global redistribution serves as a nutrient supply to many terrestrial and oceanic habitats (Griffin *et al.*, 2003; Griffin and Kellogg, 2004). Nutrients such as nitrogen, phosphorus, and iron serve as ocean, lake, and soil fertilizers (Griffin and Kellogg, 2004; Hervas *et al.*, 2009). On the other hand, muddy rain microbes are a source of crop, coral, and human diseases. Crop diseases include sugar cane rust (*Puccinia melanocephala*), coffee rust (*Hemileia vastatrix*), banana leaf spot (*Mycospherella musicola*), and bacterial blotch in peaches (*Bacillus pumilus*) (Griffin *et al.*, 2002a; Kellogg and Griffin, 2006). Coral diseases are triggered mainly by the fungus *Aspergillus sydowii* the causative agent of sea fan aspergillosis (Griffin and Kellogg, 2004). This fungus is unable to reproduce in seawater, and thus its sole source is African desert dust transported great distances over the oceans (Kellogg and Griffin, 2006). Human diseases caused by muddy rain microbes are numerous. Few examples include anthrax (*Bacillus anthracis*), Meningitis (*Neisseria meningitides*), allergies from

dust mites, and metal toxicity by arsenic and mercury (Monteil, 2002; Griffin and Kellogg, 2004; Griffin, 2007). Opportunistic human pathogens found in desert dust include *Aspergillus fumigatus*, *Aspergillus niger*, *Staphylococcus gallinarum*, *Pseudomonas* species, and *Gordonia terrae* (Kellogg and Griffin, 2006).

## D. Experimental Approaches

### *1. The Metagenomic Approach: Advantages and Limitations*

The metagenomic approach is very useful in determining microbial biodiversity in a given environmental sample. This approach, as opposed to the culture dependent approach, is based on analyzing metagenomes recovered directly from the environmental sample. Total mixed DNA is extracted from the sample, DNA libraries are constructed by cloning specific genes (most commonly the 16S rDNA gene) into cloning vectors and host strains (most commonly *Escherichia coli*), and the library is screened for desired genes that can be sequenced for species identification (Figure 5).

The metagenomic approach has many advantages over the culture dependent approach. Since more than 99% of environmental microorganisms are not easily cultured (Streit and Schmitz, 2004), using culture based techniques largely underestimates the actual biodiversity in the environmental sample. Culturing conditions such as nutrients, temperature, pH, and density, vary among microbial species. Since we aim to culture a mixture of microbes, it is difficult to determine these suitable culturing conditions. Moreover, some microbes have a mutualistic relationship with plants (Soto *et al.*, 2009), insects (Currie *et al.*, 2003) and other microbes (West *et al.*, 2006); thus, they will not be viable when cultured alone. The metagenomic method however, allows us to extract DNA from all microbes in the sample irrespective of their growth requirements.

As all techniques, the metagenomic approach suffers some limitations. The most profound limitation is the bias imposed by the lysis process. Not all microbes are lysed with the same efficiency. Gram positive spore-forming bacteria are less likely to be lysed than gram negative bacteria. Cell wall composition varies significantly among fungal species (Adams, 2004), and thus various fungi respond differently to a lysis technique. Since the three domains of life have a variation of cell wall characteristics (Debono and Gordee, 1994; Kandler, 1995), the efficiency of a lysis method will vary among bacteria, archaea, and fungi. The bias towards microbes with a less rigid cell wall will be avoided by using a mechanical lysis technique called bead beating. This technique insures the lysis of the vast majority of microbes including spores and fungi (Smit *et al.*, 1999). Van Burik *et al.* (1998) tested 6 fungal lysis methods and bead beating resulted in the greatest DNA yield. Moreover, when compared to other lysis techniques such as sonication and grinding-freezing-thawing, bead beating yeilds the greatest bacterial diversity from environmental samples (de Lipthay *et al.*, 2004). Thus, using the bead beating method greatly reduces bias usually imposed by other lysis techniques.

Other challenges arise from the co-extraction of cloning inhibitors with the microbial DNA. These inhibitors originate from degraded organic matter in the environmental sample (Yeates *et al.*, 1998). The most common inhibitor is humic acid which hinders several enzymes required for cloning, two of which are DNA polymerases and restriction endonucleases (Braid *et al.*, 2003). The problems arising from the presence of DNA contaminants and inhibitors can be attenuated by using bead beating as a DNA extraction method. In an attempt to find the DNA extraction method yielding DNA with the highest purity, Yeates *et al.* (1998) compared absorbance

260/230 and 260/280 which are indicators of humic acid and protein contamination respectively. Out of four extraction techniques, bead beating yielded DNA with the lowest concentration of humic acids and proteins, thus reducing the effects of DNA inhibitors.

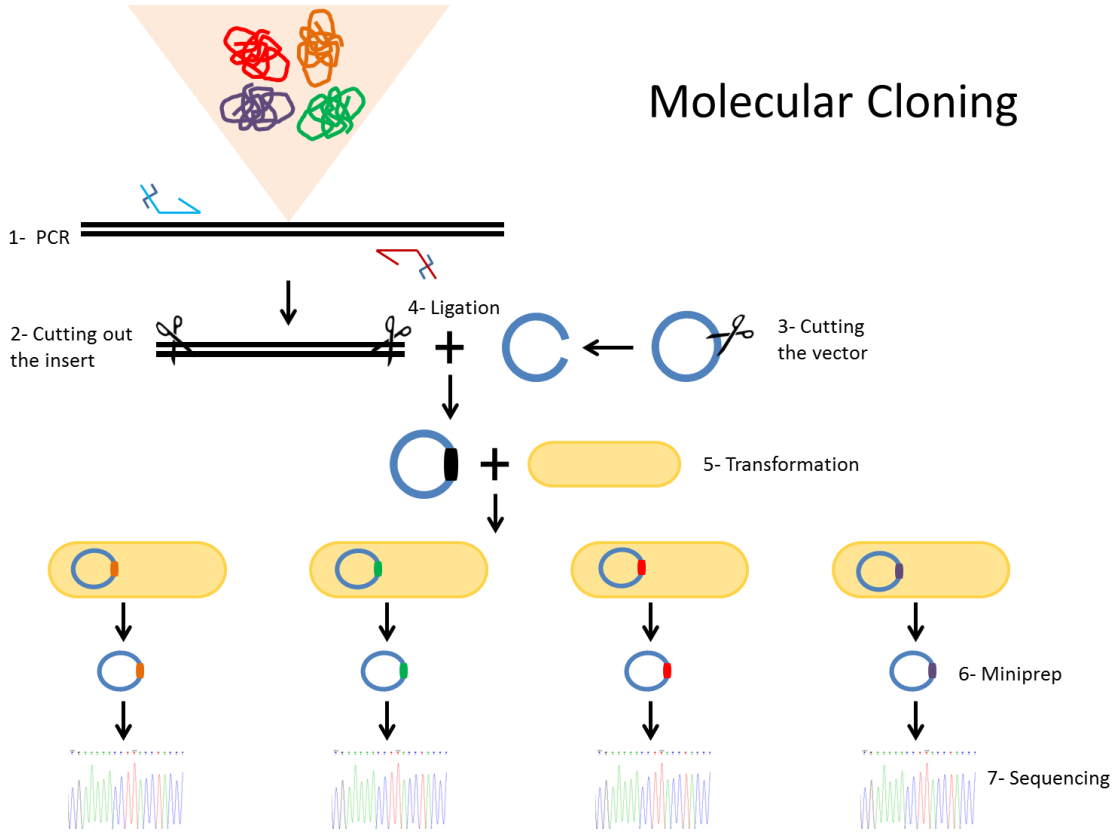



Figure 5. Molecular cloning.

***2. 16S rRNA Gene Analysis***

The microbial 16S rRNA gene is useful in identifying microbes, studying their evolution, and inferring their phylogenetic relationships. Several features of this gene made it convenient for such purposes. For example, the 16S rRNA gene is ubiquitous, conserved within a single species, and varies among species (Tringe and Hugenholtz, 2008). There are 9 variable regions in the 16S rDNA gene that represent species specific sequencing thus facilitating the identification of bacterial species (Figure 6). An additional advantage is that 16S rRNA is scarcely affected by horizontal gene transfer

(Pontes *et al.*, 2007). These features make this gene ideal for PCR and comparative DNA analysis. Currently, GenBank contains thousands of 16S rRNA gene sequences for cultured and uncultured prokaryotes (Riesenfeld *et al.*, 2004).

Few limitations exist for 16S rRNA sequence analysis. The low rate of evolution of the 16S rRNA gene makes the identification of ecotypes difficult (Pontes *et al.*, 2007). Thus, several strains of a bacterium might have identical 16S rRNA sequences. The second limitation is the PCR artifacts and amplification bias. PCR artifacts include chimeric sequences and false positives representing non-microbial species (Pontes *et al.*, 2007). Amplification bias can occur because of the preference of the universal primers towards specific sequences (Pontes *et al.*, 2007) or because genes with a high GC content are less likely to be denatured than those with a low GC content (Sueoka *et al.*, 1959). Less favored sequences and high GC content sequences will thus be misrepresented in the DNA library.

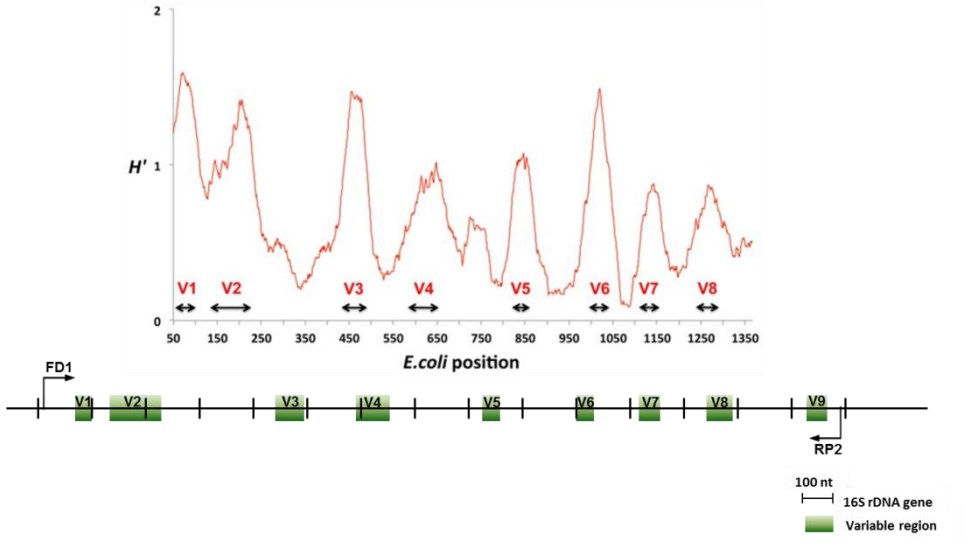



Figure 6. Bacterial 16S rRNA gene showing variable regions (V1→V9) and PCR primers annealing sites (Modified from Kuczynski *et al.*, 2012 and Vasileiadis *et al.*, 2012).

# CHAPTER II

# MATERIALS AND METHODS

## A. General

All plastic ware used throughout the project were from Sarstedt (Numbrecht, Germany). All primers were from TIB MOLBIOL (Berlin, Germany). Deoxynucleoside triphosphates were obtained from GE Healthcare (Little Chalfont, United Kingdom). EcoRI, HindIII, digestion buffer B, and PIPES were obtained from Roche (Mannheim, Germany). Tru9I, digestion buffer R and glycogen were from Fermentas (Massachusetts, USA). Agarose I for general application was obtained from Amresco (Ohio, USA). Luria Bertani agar and nutrient agar were obtained from HIMEDIA and Sisco Research Laboratories respectively (Mumbai, India). Reasner's 2A agar and glycerol were obtained from Sigma-Aldrich (Missouri, USA).

DNA extraction buffer (EB) is composed of the following: 20 mM Tris·HCl pH 7.8, 50 mM ethylenediamine tetraacetic acid (EDTA), and 20 mM NaCl. The 1X TAE buffer has the following components: 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0. Competent cells elixir solution has the following composition: 60 mM $CaCl_2$, 15% glycerol, and 10 mM PIPES (1, 4-piperazinediethanesulfonic acid), pH 7. The 4X glycerol solution is 65% glycerol, 0.1 M $MgSO_4$, and 0.02 M Tris·HCl pH 7.8.

All mentioned column and gel purifications were done using the GFX PCR DNA and gel band purification kit (GE Healthcare, United Kingdom).

**B. Muddy Rain Collection and Processing**

In order to generalize from local muddy rains, collection was attempted of every muddy rain since spring 2011. A new, clean, polyethylene tarpaulin in a 2.1 square meter frame (Figure 7) was placed on the southwest corner of the American University of Beirut Biology building roof before expected rains. Rain water was collected soon after falling and filtered through a 0.22 µm cellulose acetate, sterilizing, and low protein binding membrane with an area of 39 $cm^2$ (Corning, New York, USA). Half membranes were separately stored in 1 ml of DNA extraction buffer EB (composition mentioned in II.A) for later extraction. A small part of the filter was used to prepare a bacterial glycerol stock. Samples of some rains were stored for mineralogical analysis.

Figure 7. 2.1 $m^2$ tarpaulin for muddy rain collection.

**C. Rain Origin Determination**

***1. HYSPLIT Backtracking***

HYSPLIT (Hybrid Single Particle Lagrangian Integrated Trajectory) model from NOAA was used to trace backward trajectories of the wind using GDAS (Global Data Assimilation System) meteorological data. For each rainfall collected, wind back-trajectory was determined using the online HYSPLIT model (http://ready.arl.noaa.gov/HYSPLIT_traj.php). Input such as Beirut's latitude and

longitude (33.88 and 35.50), rainfall date, total run time (72 hrs) and altitude (1500 m) were entered. The output was a map with the trajectory of the wind starting from the day of rain fall and ending 72 hrs earlier.

### *2. XRD for Mineralogical Analysis*

In order to obtain dried muddy rain solids, mud was scraped off half the 0.22 µm filter which was stored in EB, using a sterile rubber scraper. Half the volume of the scraped mud was washed three times with water to remove the EB, and then placed in a small aluminum dish in a 70$^{o}$C oven to dry. The dry weight of the muddy rain solids was measured and the dry solids were mounted on a glass slide for X-ray diffraction (XRD) analysis.

Samples were analyzed on a Bruker D8 Discover X-ray diffractometer to identify the mineralogical composition of the dried muddy rain solids. The generator had the following settings: 40 kV, 40 mA, locked couple scan type, 2θ range 5.0°–80°, 0.1° step size and 0.5 seconds per step. DIFFRAC.EVA software was used to analyze the XRD data and to identify minerals in muddy rain solids.

## D. DNA Isolation and Amplification

### *1. DNA Extraction and Quantification*

Total DNA was extracted from muddy rain samples with bead beating. DNA extraction was done on the half membranes stored in 1 ml EB. Sodium dodecyl sulfate (SDS) and RNaseA were added to a final concentration of 3% and 10 µg/ml respectively. Spherical glass beads of 1mm diameter were added as to fill up three quarters of the extraction tube. The samples were beaten at maximum speed on a mini-bead beater (BioSpec Products, Oklahoma, USA) for 2 min then put on ice for 1 min.

This was repeated twice, and then a final beating for 1 min was performed. The samples were centrifuged at 14,000 rpm for 3 min at room temperature and the supernatant was collected. This step was repeated twice. To collect as much DNA as possible, an additional 500 µl of EB was added to the beads, the samples were beaten for 2 min, centrifuged as before, and the supernatant was combined with the previous one.

Phenol-chloroform-isoamyl alcohol extraction was performed to purify the collected DNA. Equal volume of phenol-chloroform-isoamyl alcohol mix (25:24:1) was mixed with the DNA sample then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new tube and the previous step was repeated. An equal volume of chloroform- isoamyl (24:1) was mixed with the DNA sample, and then the sample was centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new tube to which 1 µl of 20 mg/ml glycogen was added to help precipitate the DNA. Subsequently, 2.5 volume of absolute ethanol and 0.1 volume of 3 molar sodium acetate pH 5.2 were mixed with the DNA and the tube was stored at room temperature for 15 min then at -20°C for 30 min. The sample was then centrifuged at 15,000 rmp for 15 min at 4°C and the pellet was collected and washed with 500 µl of 75% ethanol. Finally, the ethanol was removed, the pellet was left to air dry, and was suspended in 30 µl sterile water.

To quantify the amount of extracted DNA, 2 µl of DNA extracted from each of the 19 muddy rain samples were loaded alongside a 1 kb ladder on 1% agarose in 1X TAE (composition mentioned in II.A). The concentration of DNA was inferred by comparing the intensity of the samples bands to those of ladder bands that have known concentration.

### *2. Polymerase chain reaction (PCR) Amplification with Bacterial and Fungal Primers*

Bacterial 16S and fungal 18S rRNA genes were amplified using universal bacterial and fungal primers (Figure 6). These primers have the following sequences: 5'-ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG-3' (FD1 forward bacterial primer) (Weisburg *et al.,* 1991), 5'-cccgggatccaagcttACGGCTACCTTGTTACGACTT-3' (RP2 reverse bacterial primer) (Weisburg *at al.,* 1991), 5'-ccgaattcgtcgaccTCCTCTAAATGACCAAGTTTG-3' (EF3 forward fungal primer) (Griffin *et al.,* 2002b), and 5'-cccgggatccaagcttGGAAGGGGTGTATTTATTAG-3' (EF4 reverse fungal primer) (Griffin *et al.,* 2002b). The upper case nucleotides represent the complement target while the lower case nucleotides represent linker sequences that have sites for the restriction enzymes EcoRI and HindIII. For each 50 μl reaction, the PCR amplification mixture contained: 5 μl of DNA template, 1X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.2 μM forward primer, 0.2 μM reverse primer, and 1.25 units of Taq polymerase. The PCR thermal profile was as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 52°C or at 47°C (for bacterial and fungal primers respectively) for 30 sec, and extension at 72°C for 90 sec. The final elongation step was extended to 20 min. Under these conditions, a single PCR product of 1.4 kb was obtained and subsequently gel purified.

## E. Sample Biodiversity

### *1. Diversity of Cultured Samples*

Glycerol stocks of the crude muddy rain filter were streaked on Reasoner´s 2A (R2A) and nutrient agar. The glycerol stocks of all the muddy rain crude samples were

diluted 100 and 10,000 fold before plating 5 µl on R2A plates using glass beads. From each plated sample, several bacterial and fungal single colonies were picked and re-streaked on nutrient agar plates. A single colony from each of the above re-streaks was then picked and PCR was performed to amplify the 16S rDNA from bacteria and the 18S rDNA from fungus. The PCR reaction was performed same as described in II.C.2; and the samples that showed good PCR products were gel purified and sequenced.

***2. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis***

DGGE was used to access the diversity of the microbial community using the approach described in Figure 8. For muddy rain samples, a part of the V3 variable region in the 16S rDNA gene was amplified using universal DGGE primers (Figure 9). These primers have the following sequences:
5'-cgcccgccgcgcgcggcgggcggggcgggggcacgggggCCTACGGGAGGCAGCAG-3' (P338FGC forward primer), and 5'-ATTACCGCGGCTGCTGG-3' (P518R reverse primer) (Muyzer *et al.*, 1993). The lower case nucleotides represent GC clamp which is a high melting GC-rich sequence that prevents the two DNA strands from complete dissociation. For each 50 µl reaction, the PCR amplification mixture contained: 5 µl of DNA template, 1X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.2 µM forward primer, 0.2 µM reverse primer, and 1.25 units of Taq polymerase. The PCR thermal profile was as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95$^{o}$C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 90 sec. The final elongation step was extended to 20 min. Under these conditions, a single PCR product of 233 bp was obtained and subsequently gel purified.

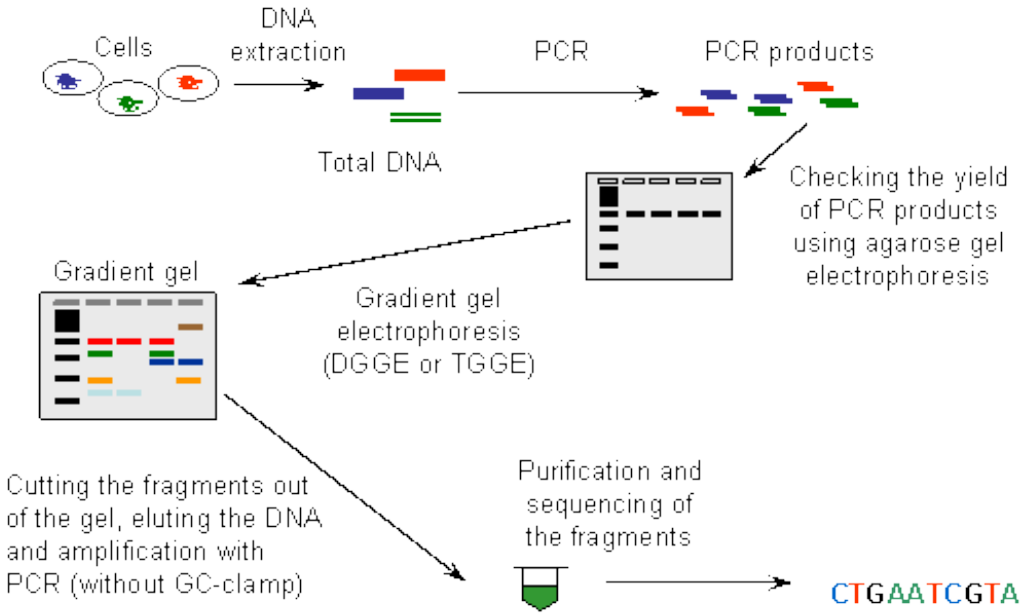



Figure 8. Denaturing gradient gel electrophoresis (http://wiki.biomine.skelleftea.se/biomine/molecular/index_11.htm).

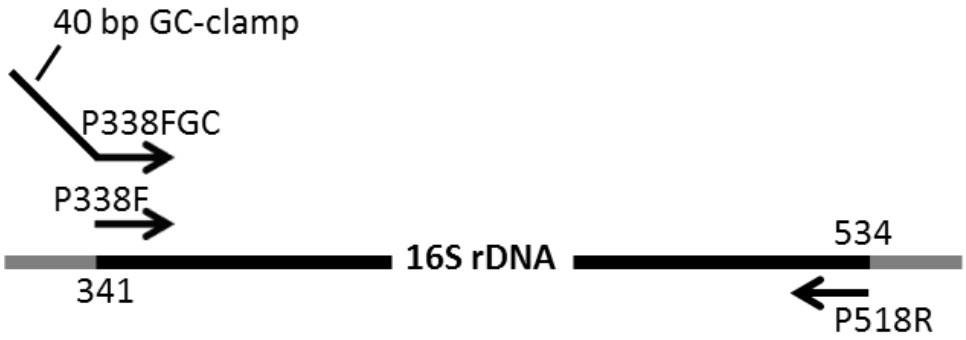



Figure 9. DGGE primer sites on the bacterial 16S rDNA gene (Modified from Muyzer *et al.*, 1993).

DGGE was performed using the DCode™ Universal Mutation Detection System (BioRad, Hercules, CA, USA). One mm thick vertical gels containing 10% (w/v) polyacrylamide and a linear gradient of denaturants (urea and formamide) were used. A denaturing gradient of 30–70% was applied to separate 16S rDNA fragments (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). The gels were prepared in 1X TAE buffer, which was also used as the electrophoresis buffer. Electrophoresis was run for 16 hours at 60°C, 75 V, and 50 mA.

PCR products (300 ng/µl) from muddy rain samples along with three DNA ladders were loaded. The DNA ladders had the following compositions of PCR products: *Bacillus cereus* and *Escherichia coli*; *Proteus vulgaris* and *Salmonella enterica*; and a mixture of the four mentioned PCR products. After electrophoresis, the gel was stained with Syber Green then destained in distilled water. Images were acquired using the Chemi Doc XRS (BioRad, Hercules, CA, USA).

### *3. Amplifying and Sequencing DGGE Bands*

The brightest bands from each lane on the DGGE gel were cut out and DNA was extracted from them by crushing the band with a flame-sealed blue tip and soaking it in 100 µl sterile water. The soaked gel was left rocking for 2 hours then spun to collect the extracted DNA, which was then amplified using the same PCR procedure in II.C.3.a. The PCR products were column purified, and sent for sequencing at Macrogen sequencing facility, South Korea.

### *4. Terminal Restriction Fragment Length Polymorphism TRFLP*

Biodiversity of the samples was also measured by another method called terminal restriction fragment length polymorphism or TRFLP. This method was used to identify different bacterial species based on the position of the Tru9I restriction site relative to the 6-carboxyfluorescein (6-FAM) labeled end of the PCR amplified 16S rDNA gene (Figure 10). To introduce 6-FAM to one end of the 16S rDNA gene, the gene was amplified with 6-FAM labeled FD1 forward primer (5'- 6-FAM ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG-3') and a non-labeled RP2 reverse primer (5'-cccgggatccaagcttACGGCTACCTTGTTACGACTT-3'). The PCR reaction and conditions are same as those in II.D.2. The PCR products where then gel purified

and the purified 6-FAM labeled PCR products were then cut by Tru9I restriction enzyme by the following 50 µl reaction: 30 µl PCR product, 1X digestion buffer R, and 20 units the restriction enzyme Tru9I. The digestion mix was incubated for 90 min at 65°C. The digested PCR product was then column purified and sent for florescence analysis at the University of Saint-Joseph (Beirut, Lebanon).

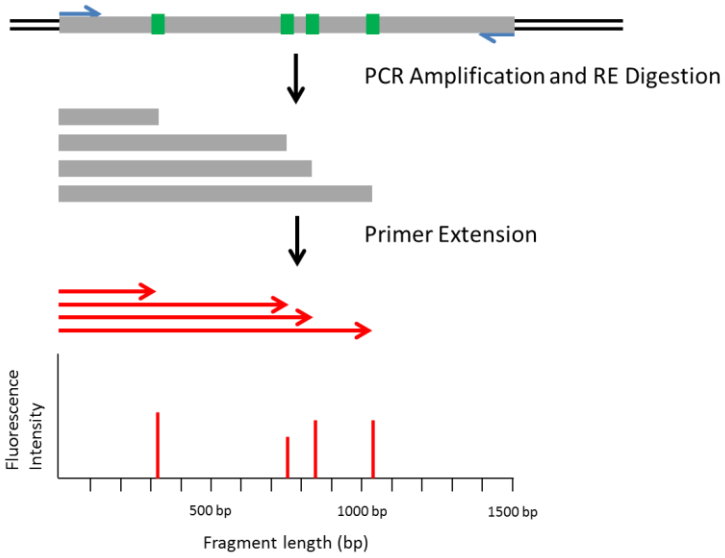



Figure 10. Terminal Restriction Fragment Length Polymorphism.

**F. Molecular Cloning**

***1. pcDNA3 Plasmid Preparation***

A pure culture (50 ml) of DH5α *E. coli* cells previously transformed with pcDNA3 was prepared. The culture was spun at 4,000 g and 4°C for 15 min and the pellet was resuspended in 1.5 ml of cold SET (20% sucrose, 50 mM EDTA, 50 mM Tris·HCl, pH 8.0). Subsequently, 3.5 ml of freshly prepared alkaline lysis buffer (2% of 10M NaOH and 5% of 20% sodium dodecyl sulfate) were mixed and the solution was left on ice for 5 min. Sodium acetate (2.5 ml of 3 M solution, pH 4.8) was added and the solution was left on ice for 10 min then centrifuged at 4,500 rpm and 4°C for 10 min. The supernatant was transferred to a new tube containing 6 ml of isopropanol and was

left at room temperature for 15 min then centrifuged at 10,000 rpm for 10 min at room temperature. The pellet was washed with 75% ethanol (5 ml) then spun at 15,000 rpm for 5 min at room temperature. The ethanol was decanted and the tube was left to air dry then resuspended in 400 µl sterile water. The solution was then incubated with 10 µl of 10 mg/ml RNaseA for 30 min at 34°C. To purify the extracted pcDNA3 plasmid, phenol-chloroform-isoamyl alcohol extraction was done similar to the procedure in II.C.1, and the final pellet was resuspended in 250 µl TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0). The concentration of the pcDNA3 plasmid was found to be approximately 230 ng/µl.

***2. PCR product and pcDNA3 Plasmid Restriction Enzyme Digestion***

The bacterial and fungal 16S rDNA PCR amplicoms from the muddy rain samples and the pcDNA3 plasmid were cut with the same set of restrictions enzymes which are EcoRI (cuts G/AATTC) and HindIII (cuts A/AGCTT). The digestion reaction contained, in addition to the plasmid or PCR product, 1 mM dithiothreitol (DTT), 1X digestion buffer B and 30 units of each of the two restriction enzymes EcoRI and HindIII. The digestion mix was incubated for two hours at 37°C. The digested PCR product was then column purified and the digested pcDNA3 plasmid was purified by phenol-chloroform-isoamyl extraction described in II.C.1. This EcoRI/HindIII cut plasmid will be used as the ligation backbone.

***3. Ligation***

The digested PCR products were ligated to the EcoRI/HindIII cut pcDNA3 plasmid using the following 20 µl ligation reaction: 30 fmol cut pcDNA3, 10 fmol cut PCR product, 1 mM adenosine triphosphate (ATP), 10 mM DTT, 1X ligase buffer, and 1 U of T4 DNA ligase (Roche, Mannheim, Germany). This ligation reaction was

incubated at room temperature overnight, then transformed into chemically competent cells as described below.

***4. Chemically Competent Cells Preparation***

From a pure culture (5 ml) of DH5α *E. coli* cells, 2 ml were inoculated into 200 ml Luria-Bertani (LB) broth and grown, at 37°C under continuous shaking at 250 rpm, to an OD600 of no more than 0.375. After a rapid cooling in ice water for about 10 min, the culture was split into pre-chilled 50 ml conical tubes and centrifuged at 4,000 g for 15 min at 4°C. The supernatant was decanted and the pellets were resuspended with 10 ml of ice-cold competent cells elixir solution (composition mentioned in II.A). The cells were centrifuged again for 5 min at 4,000 g and 4°C, and the supernatant was discarded. The pellets were resuspended a second time with 10 ml of ice-cold competent cells elixir solution and kept on ice for 30 min. After centrifugation at 4,000 g for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended with 2 ml ice-cold competent cells elixir. The cells were then aliquoted into pre-chilled 1.5 ml tubes and stored at -70°C. The efficiency of the chemically competent cells was 316,000 colonies per µg plasmid DNA.

***5. Transformation***

Chemically competent DH5α cells were thawed on ice and 50 µl cells were added to the ligations described in II.D.3, then placed on ice for 20 min. The cells were then heat-shocked at 37°C for 2 min then placed on ice for 5 min. Each transformation tube had 1 ml of fresh LB added, was outgrown for 1 hour at 37°C, and then spun for 10 sec at 14,000 rmp. All the supernatant was decanted except 50 µl, which were plated and grown on LB plates supplemented with ampicillin.

To construct clone pools of the bacterial and fungal muddy rain inserts, all the transformed colonies were collected from the LB agar plate by squirting 750 µl of LB broth and scrapping off all the colonies using a sterile spreader. The dissolved colonies are then transferred into a 1.5 ml tube and 250 µl of 4X glycerol (composition mentioned in II.A) is added to make glycerol stocks of the clone pools.

***6. Screening Clone Pools***

The clone pools mentioned above were screened for muddy rain inserts using PCR screening. The glycerol stocks from each transformation were separately streaked on LB/ampicillin (amp) agar plates and several random single colonies were picked from each agar plate. Each single colony was then re-streaked on LB/amp plates to pick a completely pure colony (Figure 11). One pure colony from each of the streaked colonies was then inoculated into 20 µl LB/amp broths. One µl of the 20 µl was used as a template for PCR screening and the rest were saved for subsequent plasmid extraction. The PCR reaction was done same as described in II.C.2 but with T7F (5'-TAATACGACTCACTATAGGG-3') as forward primer and BGHR (5'-TAGAAGGCACAGTCGAGG-3') as reverse primer. T7F and BGHR recognize sequences in the pcDNA3 plasmid close to the HindIII and EcoRI sites respectively.

PCR products were loaded on 1% agarose with 1X TAE alongside the following: 1 kb ladder, PCR product of pcDNA3 without any insert, PCR product of a short muddy rain insert, and a PCR product of a long muddy ran insert. Any PCR band longer than the band of the pcDNA3 with no insert, was considered as a muddy rain insert and was classified as a short or long insert by comparison to the PCR products loaded alongside. For each band that showed an insert, the remaining volume from corresponding 20 µl bacterial cultures was used to inoculate 3 ml of LB/amp broth and

these 3 ml cultures were grown at 37°C overnight. The pcDNA3 plasmid with insert was then extracted from each of the above cultures using a miniprep technique similar to the one in II.D.1 and the minipreps were subsequently column purified. The concentration of the plasmid DNA was between 250-300 ng/µl. These plasmid extractions were later used for sequencing the muddy rain inserts.

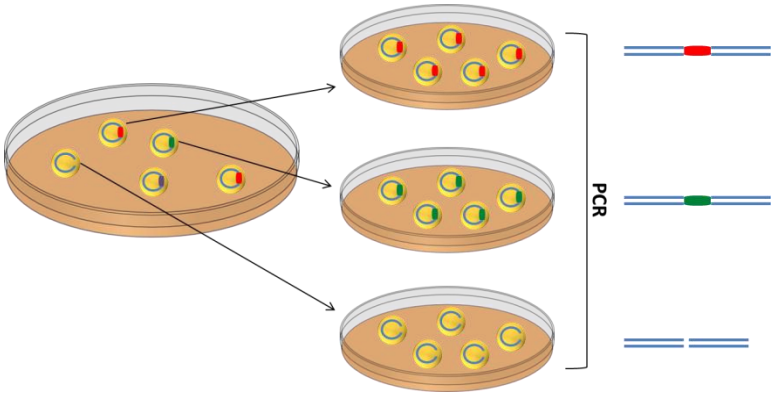



Figure 11. Screening muddy rain clone libraries inserts.

**G. Sequencing and Identification of Clones**

Purified plasmids or PCR products containing the muddy rain bacterial and fungal inserts were sent to Macrogen (Seoul, Korea) for sequencing. The sequencing primers were BGHR and 27F (same as FD1 but without restriction sites) for plasmid and PCR product respectively.

Molecular cloning was done by cutting the desired insert at EcoRI and HindIII restriction sites; thus, the sequences obtained were manually curated by removing the base pairs before EcoRI and after the HindIII sites. The curated sequences were then analyzed by Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared to sequences present in GenBank database. The sequences were assigned

to a certain taxon based on the highest percent sequence similarity between the obtained sequence and the sequence in GenBank.

**H. Phylogenetic Analysis**

Manually curated sequences were used to create a phylogenetic tree using Phylogeny.fr service (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi). The sequences were first aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation), and the alignment was then curated using the Gblocks program to eliminate poorly aligned positions and divergent regions. PhyML software was then used to create the phylogenetic tree based on the maximum-likelihood principle. Tree editing, graphics and annotations were done using TreeDyn graphical editor.

# CHAPTER III

# RESULTS

## A. Mud Varied Greatly in Collected Rainfalls

### *1. Amount of Mud in the Rain Samples Varies from 20 to 37000 mg/m$^2$*

During 2011 and 2012, a total of 23 rainfalls were collected, of which, 18 were distinctly muddy (Figure 12). The mass of mud in the collected rains varied significantly and ranged between 20 and 37,000 mg/m$^2$. Table 1 lists each muddy rain sample with the corresponding mass of mud per quarter filter and mass of mud per area.

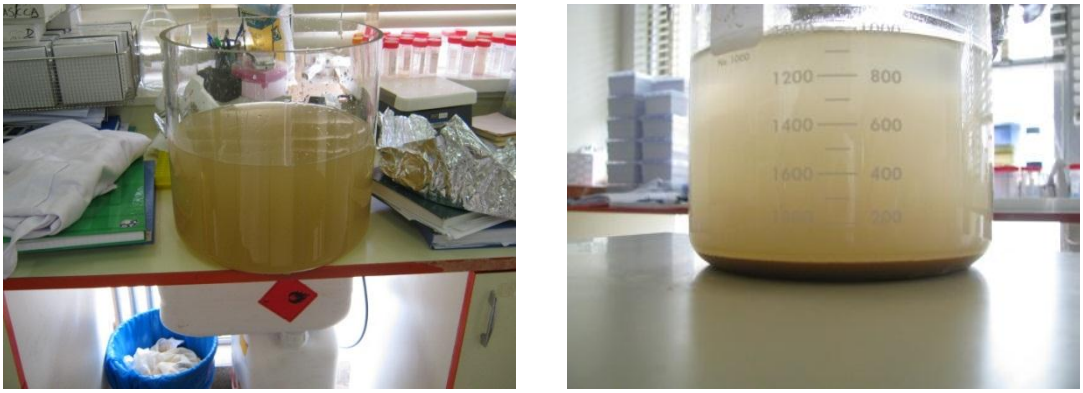



Figure Beaker. Collected muddy rain samples.

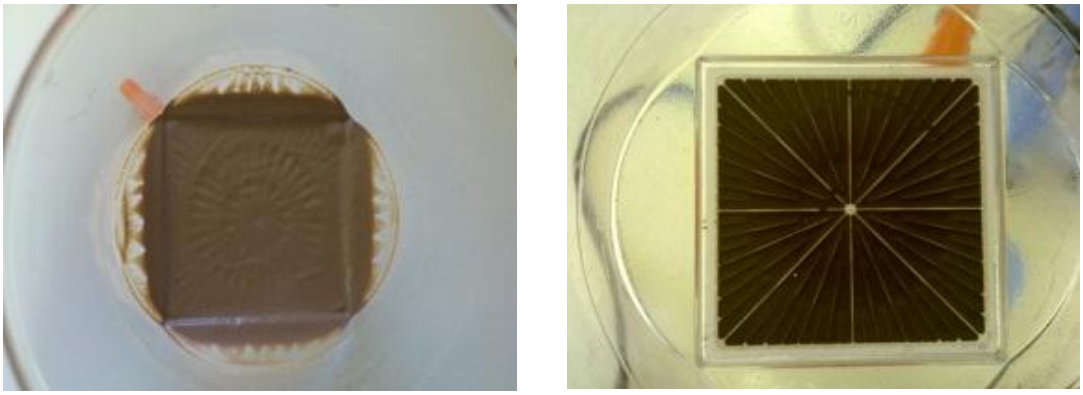


Figure 12. Filtration of muddy rain samples.

Table 1. Mass of mud (mg/¼ filter and mg/$m^2$) in each muddy rain sample

| Sample Name | Collection Date | Liters Filtered | Mass of mud per ¼ filter (mg) | Mass of mud (mg/$m^2$) |
|---|---|---|---|---|
| XIII-34-1 | 20110923 | 8.5 | 20 | 120 |
| XIII-36-1 | 20110924 | 0.7 | 10 | 20 |
| XIII-42-1 | 20111001 | 10 | 10 | 100 |
| XIII-49-2 | 20111025 | 7 | 20 | 270 |
| XIII-49-5 | 20111026 | 6.5 | 10 | 30 |
| XIII-50-1 | 20111103 | 17 | 13 | 80 |
| XIII-51-1 | 20111114 | 0.8 | 10 | 20 |
| XIII-52-2 | 20111115 | 4 | 2 | 190 |
| XIII-52-4 | 20111217 | 4 | 6 | 30 |
| XIII-53-2 | 20111224 | 4.5 | 42 | 270 |
| XIII-53-4 | 20120111 | 9 | 10 | 50 |
| XIII-54-2 | 20120113 | 9 | 1.5 | 20 |
| XIII-66-1 | 20120207 | 2.5 | 51 | 460 |
| XIII-72-1 | 20120216 | 1.5 | 19 | 270 |
| XIII-75-1 | 20120314 | 2.5 | 53 | 400 |
| XIII-78-1 | 20120401 | 2 | 160 | 300 |
| XIII-82-1 | 20120419 | 0.6 | 68 | 130 |
| XIII-82-4 | 20120430 | 0.07 | 260 | 500 |
| XIII-83-1 | 20120502 | 0.08 | 1460 | 37000 |
| XIII-83-7 | 20120529 | 1.2 | 40 | 80 |
| XIII-144-1 | 20121219 | 4 | 20 | 570 |
| XIII-145-1 | 20121221 | 13 | 8 | 40 |
| XIII-213-1 | 20130322 | 1.5 | 30 | 60 |

### *2. Mud Particle Size in Muddy Rain Is Larger than Dust Particle Size*

As observed under a dissection microscope, muddy rain particle size is significantly larger than Saharan dust particle size (typically 5-40 μm (Goudie and Middleton, 2001)). The larger size of muddy rain particles provides a larger attachment surface for the transported microbes. Crystals of various colors were observed indicating a diverse mineral composition of muddy rain (Figure 13). A more reliable mineral composition was later determined by XRD (see III.B.2).

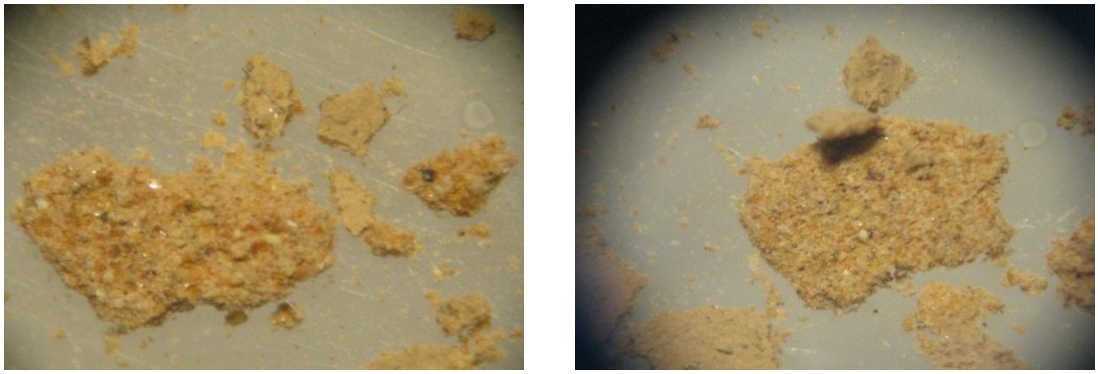


Figure 13. Microscopic images of dried muddy rain solids.

## B. Sahara Desert and Arabian Peninsula Are the Origins of Muddy Rain Solids

### *1. Wind Back-trajectories Determine Saharan and Arabian Peninsular Sources*

For each of the 23 rainfalls collected, wind back-trajectory was mapped using the online HYSPLIT model. Back-trajectories trace back the origin of the wind and rain carrying the muddy rain microbes. Wind back-trajectories allowed determining the path along which microbes were picked up and transported to Beirut (Figure 14). Among the 23 collected rains, 18 were distinctly muddy and contained observable DNA. Out of these 18 muddy rains, 14 originated from the Sahara desert and 4 from the Arabian Peninsula. The remaining 5 clean winter rains originated from Europe (Table 2).

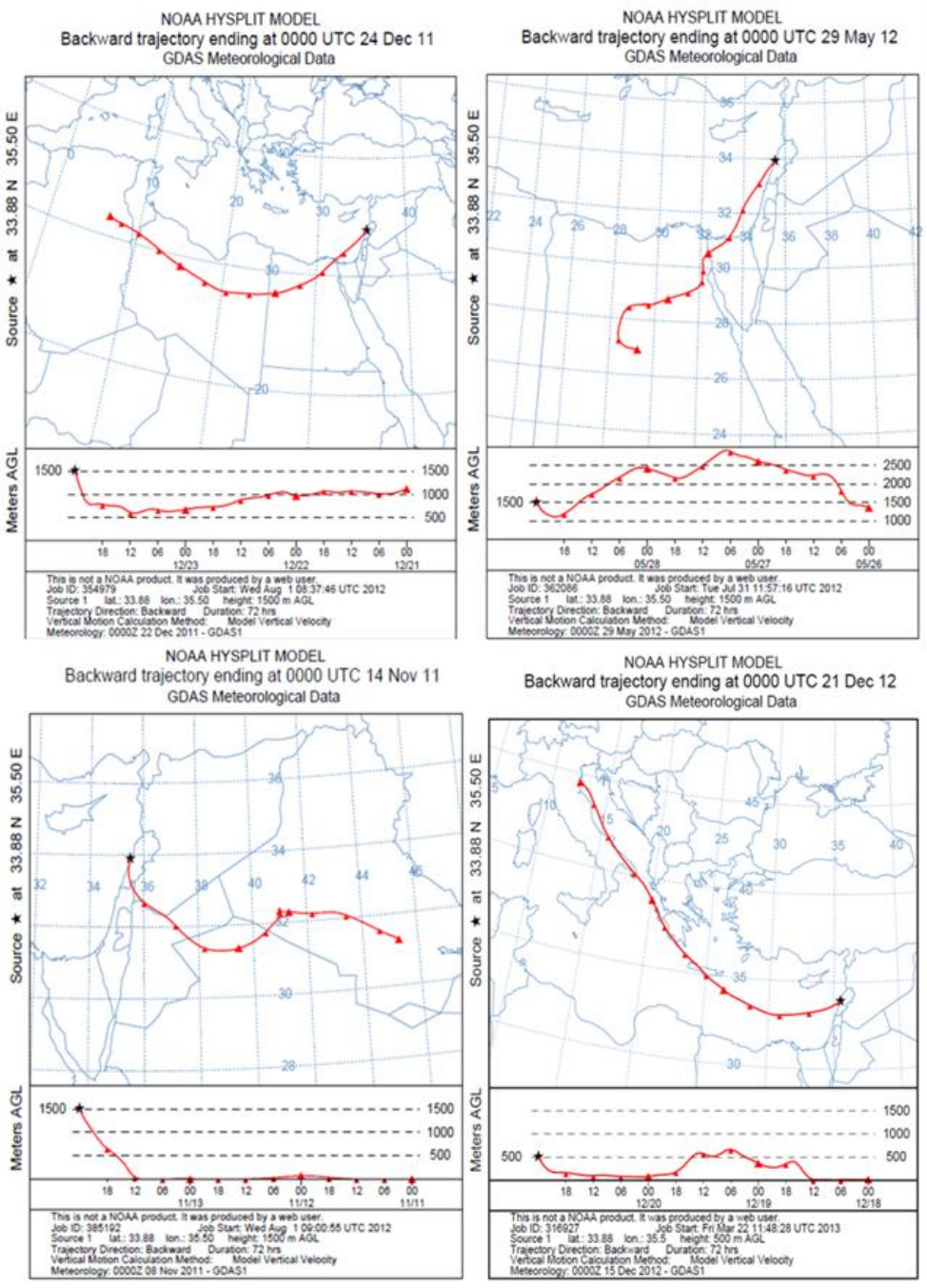



Figure 14. Samples of wind backward trajectories tracing back the origin of the microbe saturated dust transported via muddy rain.

Table 2. Backtracked origin of each muddy rain sample

| Description | Sample Name | Collection Date | Backtracked Source |
|---|---|---|---|
| Moderately muddy | XIII-34-1 | 20110923 | Sahara |
| Moderately muddy | XIII-36-1 | 20110924 | Sahara |
| Clean rain control | XIII-42-1 | 20111001 | Europe |
| Moderately muddy, dust in horizon | XIII-49-2 | 20111025 | Arabian peninsula |
| Muddy | XIII-49-5 | 20111026 | Sahara |
| Very clean rain control | XIII-50-1 | 20111103 | Europe |
| Very muddy | XIII-51-1 | 20111114 | Arabian peninsula |
| Hail rain | XIII-52-2 | 20111115 | Sahara |
| Clean winter rain | XIII-52-4 | 20111217 | Europe |
| Very muddy, full of particulates | XIII-53-2 | 20111224 | Sahara |
| Moderately muddy | XIII-53-4 | 20120111 | Sahara |
| Very clean rain control | XIII-54-2 | 20120113 | Europe |
| Extremely muddy, full of dust | XIII-66-1 | 20120207 | Sahara |
| Extremely muddy, full of dust | XIII-72-1 | 20120216 | Sahara |
| Very muddy, dust filled the air before rain | XIII-75-1 | 20120314 | Sahara |
| Very muddy, full of dust | XIII-78-1 | 20120401 | Sahara |
| Very muddy, full of dust | XIII-82-1 | 20120419 | Sahara |
| Extremely muddy, big droplets of mud | XIII-82-4 | 20120430 | Sahara |
| Very thick mud precipitated on cars | XIII-83-1 | 20120502 | Arabian peninsula |
| Muddy, dust filled the air before it rained | XIII-83-7 | 20120529 | Sahara |
| Hail rain | XIII-144-1 | 20121219 | Sahara |
| Clean winter rain that smelled like snow | XIII-145-1 | 20121221 | Europe |
| Dust in the air, washed by rain | XIII-213-1 | 20130322 | Arabian peninsula |

### *2. Muddy Rain Has Mineralogical Profiles Consistent with Saharan and Arabian Origins*

X-ray diffraction was used to identify muddy rain minerals based on the unique interplanar distance of each mineral lattice. Each mineral will thus have a unique X-ray diffraction pattern. The XRD profile of muddy rain solids originating from the Sahara desert and the Arabian Peninsula are represented in figures 15 and 16 respectively. The mineralogical analysis of those profiles allowed the determination of the mineral composition of muddy rain solids. A representative sample of muddy rain minerals from the Sahara desert and the Arabian Peninsula are listed in tables 3 and 4 respectively. The major minerals included calcite, quartz, and other oxides of Si, Al, Pb, Ca, Mg, Fe, K, Na, and P. This data is consistent with the mineralogical profile of Saharan dust found

in the literature (Guieu *et al.*, 2002; Hamdi-Aissa *et al.*, 2004; Linke *et al.*, 2006). The presence of calcite and quartz among muddy rain minerals was detected by the superimposition of their XRD profile with that of a muddy rain sample of Saharan origin as shown in figures 17 and 18 respectively.

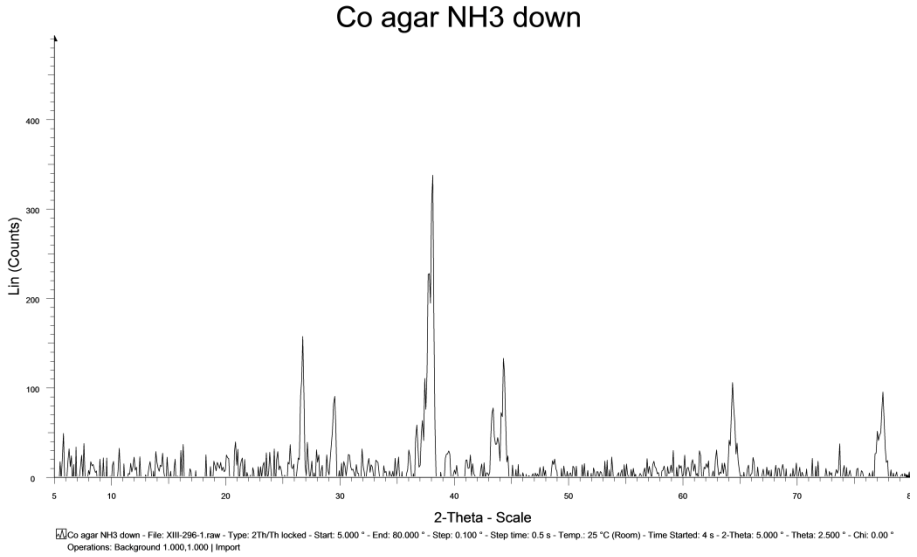



Figure 15. XRD profile of muddy rain solids originating from the Sahara desert.

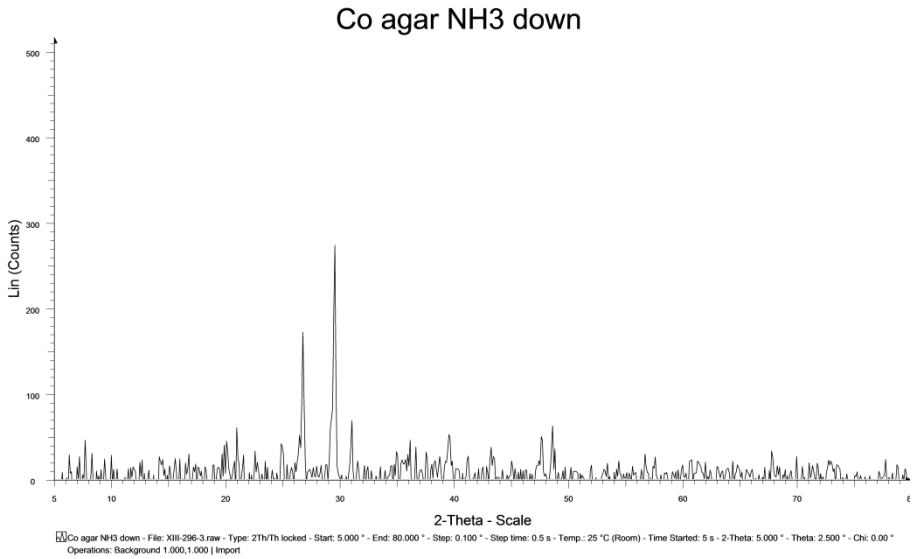



Figure 16. XRD profile of a muddy rain solids originating from the Arabian Peninsula.

Table 3. Some minerals in muddy rain samples from the Sahara desert

| **Compound Name** | **Formula** | **Compound Name** | **Formula** |
|---|---|---|---|
| Alamosite | $Pb(SiO_3)$ | Naujakasite | $Na_6FeAl_4Si_8O_{26}$ |
| Arsenic Cadmium Germanium | $Cd_2GeAs_4$ | Nickelhexahydrite | $NiSO_4(H_2O)_6$ |
| Calcite | $CaCO_3$ | Parabutlerite | $FeSO_4(OH)(H_2O)_2$ |
| Creedite | $Ca_3Al_2(OH)_{10}SO_4(H_2O)_2$ | Phosphide Oxide | $P_4O_{10}$ |
| Demesmaekerite | $Pb_2Cu_5(SeO_3)_6(UO_2)_2(OH)_6(H_2O)_2$ | Potassium Hydrogen | $KH_2PO_3$ |
| Elpidite | $Na_2ZrSi_6O_{15}(H_2O)_3$ | Quartz | $SiO_2$ |
| Fuloppite | $Pb_3Sb_8S_{15}$ | Quenstedtite | $Fe_2(SO_4)_3 \cdot 11H_2O$ |
| Julgoldite | $Ca_2(Fe.48Fe.52)(Fe)_2Si_3O_{10}.52(OH)_3.48$ | Sklodowskite | $MgU_2O_2(OH)_2(SiO_4)_2(H_2O)_4$ |
| Leucite | $KAlSi_2O_6$ | Ussingite | $Na_2AlSi_3O_8(OH)$ |
| Montgomeryite | $Al_4Ca_4Mg(PO_4)_6(OH)_4(H_2O)_{12}$ | Variscite | $AlPO_4(H_2O)_2$ |

Table 4. Some minerals in muddy rain samples from the Arabian Peninsula

| **Compound Name** | **Formula** | **Compound Name** | **Formula** |
|---|---|---|---|
| Aplowite | $Co(SO_4)(D_2O)_4$ | Kaolinite | $Al_2(Si_2O_5)(OD)_4$ |
| Brucite | $Mg(OD)_2$ | Krautite | $Mn(AsO_3(OD))(D_2O)$ |
| Chalcanthite | $CuSO_4(D_2O)_5$ | Sassolite | $D_3BO_3$ |
| Hibschite | $Ca_3Al_2(SiO_4)_2.3(OD)_2.8$ | Theophrastite | $NiD1.86H0.14O_2$ |
| Kafehydrocyanite | $K_4Fe(CN)_6(D_2O)_3$ | Tschermigite | $ND_4Al(SO_4)_2(D_2O)_{12}$ |

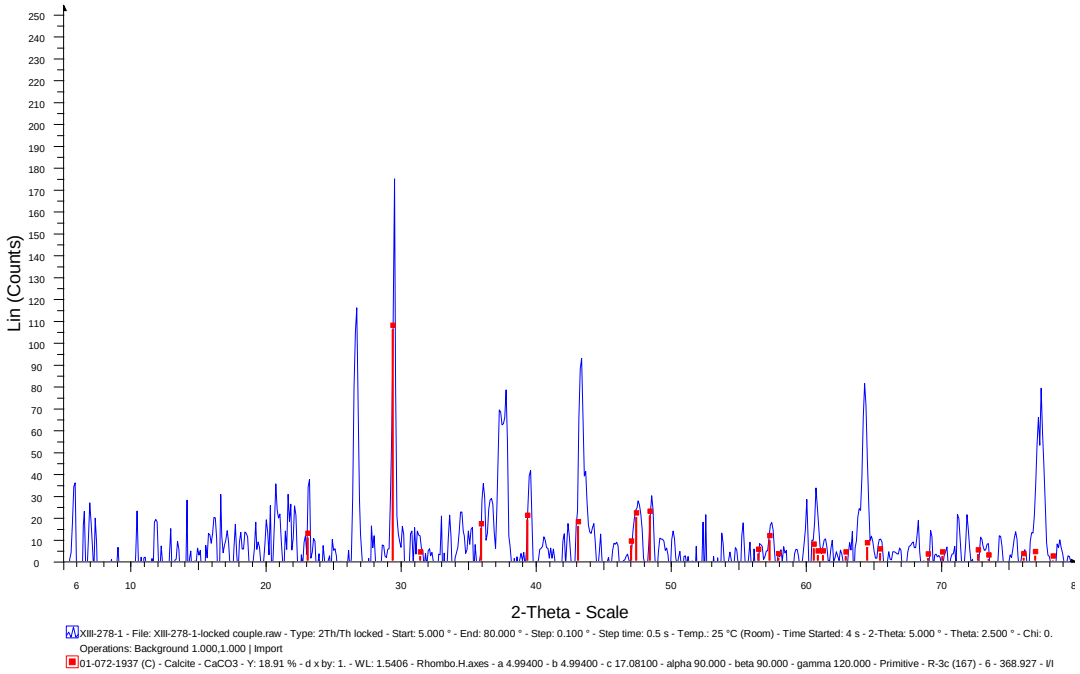



Figure 17. Superimposition of calcite XRD profile with that of a muddy rain sample of Saharan origin.

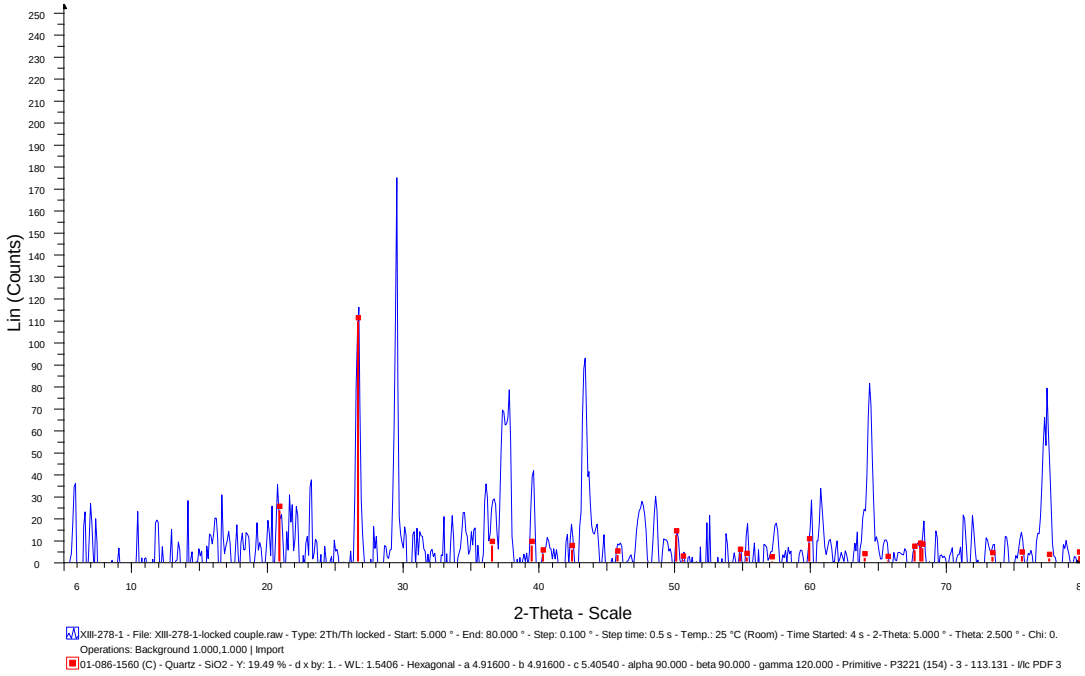



Figure 18. Superimposition of Quartz XRD profile with that of a muddy rain sample of Saharan origin.

**C. Diverse Viable Bacteria and Fungi Were Detected in Muddy Rain**

For determining microbial diversity in muddy rain, a culture based approach was performed in addition to the metagenomic approach. The culture based approach was based on sequencing the 16S rDNA gene from live microbial colonies grown on agar plates. Figure 19 show that microbes transported by muddy rain are live, abundant, diverse, and mostly pigmented. Various muddy rain samples had varying types and concentrations of culturable bacteria and fungi. A total of 98 bacterial and 27 fungal sequences were obtained. In general, the bacterial phyla detected by the culture based approach comprised, in decreasing order of relative abundance, Actinobacteria (35.71%), Firmicutes (23.47%), Betaproteobacteria (13.27%), Gammaproteobacteria (11.22%), Bacteroidetes (9.18%), Alphaproteobacteria (6.12%), and Deinococcus-Thermus (1.02%) (Figure 20). The fungal classes detected by the culture based approach comprised, in decreasing order of relative abundance, Dothideomycetes (62.96%), Eurotiomycetes (25.93%), and Sordariomycetes (11.11%) (Figure 21). The three classes belong to the phyla Ascomycota.

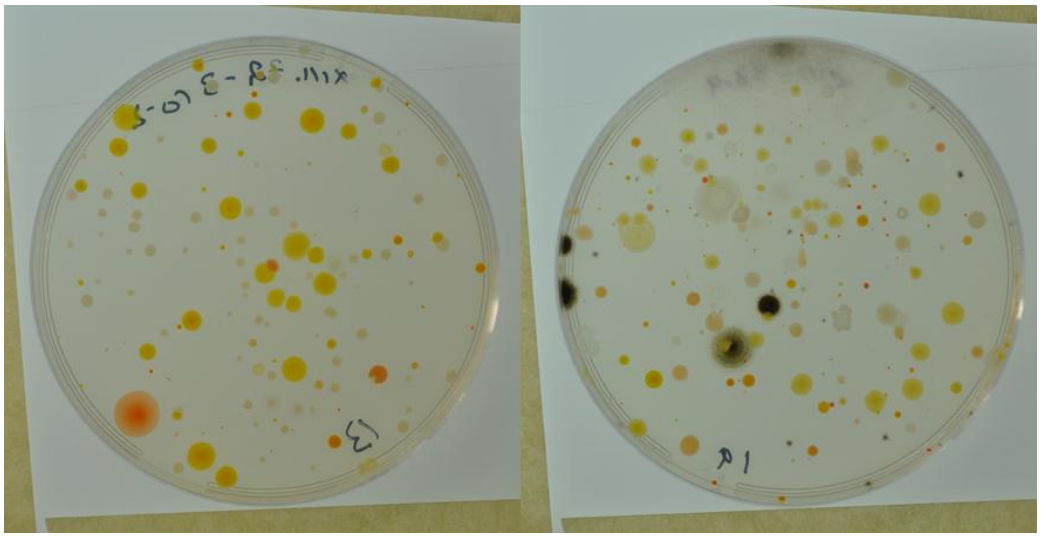


Figure 19. Muddy rain microbial growth on agar plates from samples diluted 100-fold.

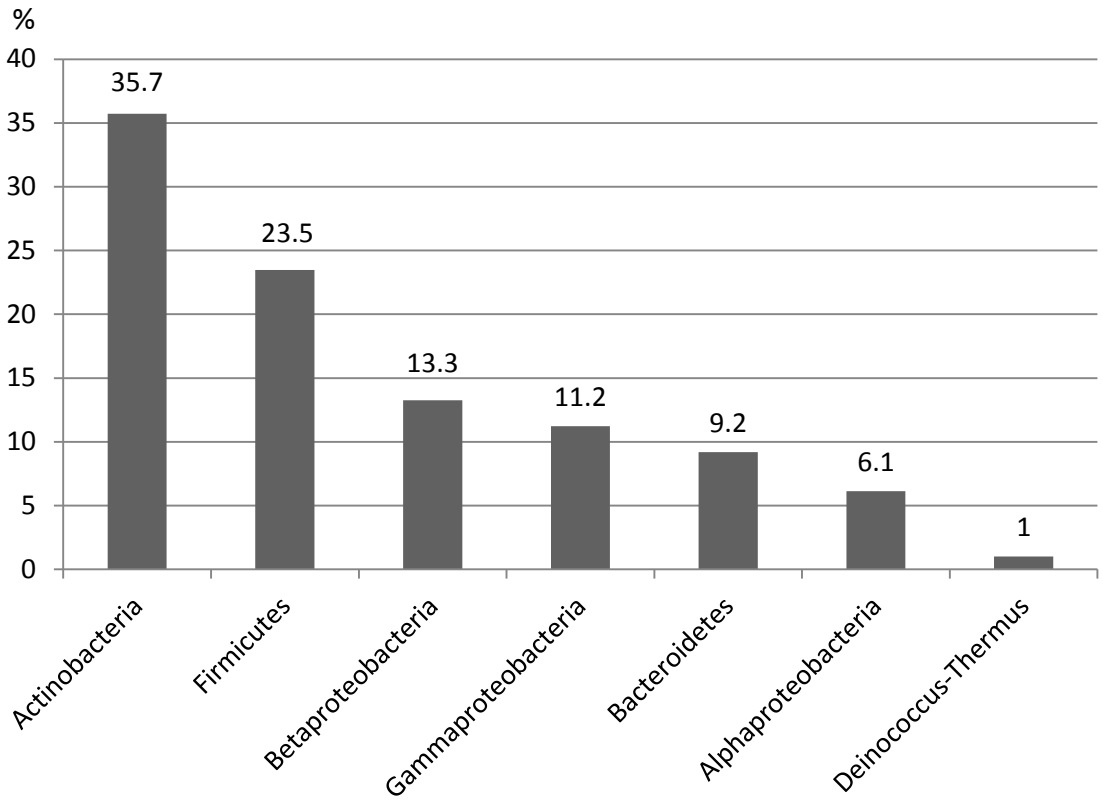



Figure 20. Muddy rain bacterial community composition from the culture based approach.

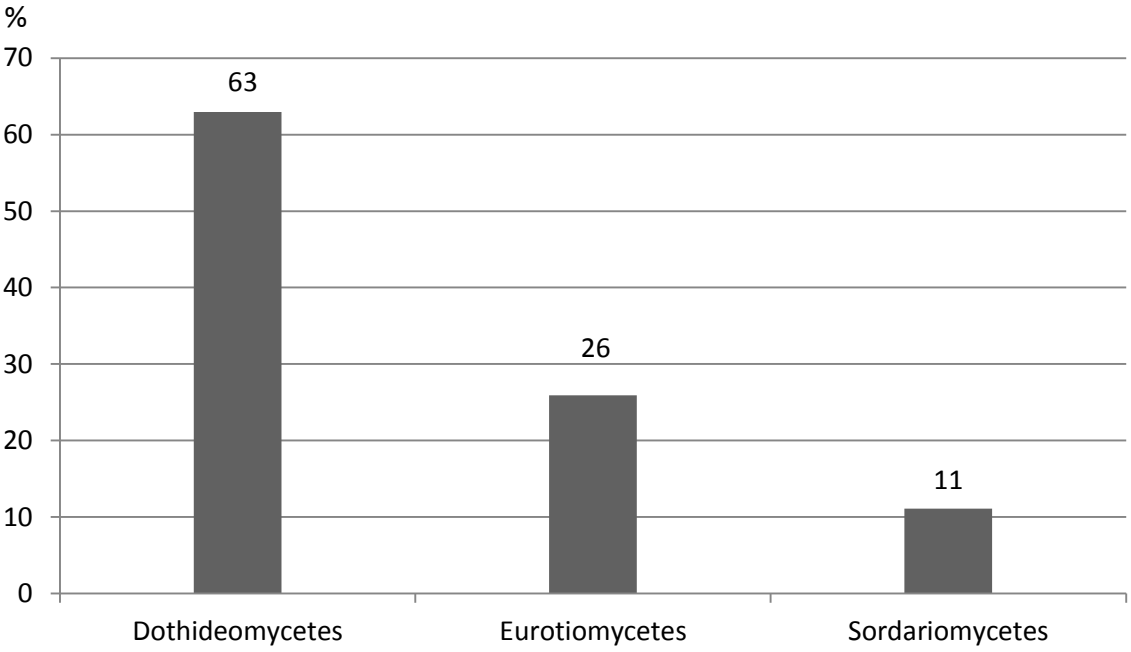



Figure 21. Muddy rain fungal community composition from the culture based approach.

## D. Muddy Rain Carries and Deposits Large Numbers of Diverse Microbes

### *1. Muddy Rain Samples Contained Various Amounts of Extractable DNA*

For each muddy rain sample, total bacterial and fungal DNA was extracted without bias by bead beating. DNA extractions of the 19 processed samples are shown in figure 22. All samples had a quantifiable amount of DNA, with clean rains having less DNA than muddy ones. The concentration of DNA ranged between 143 and 57,143 ng/$m^2$ with an approximate average of 8,000 ng/$m^2$ (Table 5). The difference in the DNA amounts among muddy rain samples shows that different samples have different amounts of microbes. PCR amplification of the 16S rDNA gene followed the extraction, and products were around 1500 bp (Figure 23).

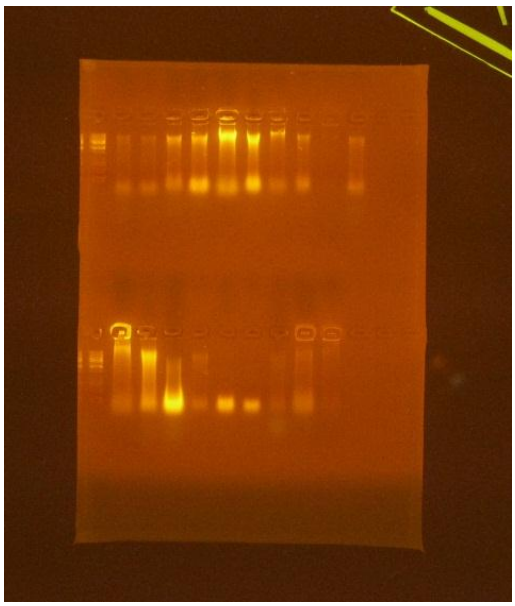


Figure 22. Total DNA extracted from all muddy rain samples. The first well of each lane is 0.5 µl of 1 kb ladder and the remaining wells are 2 µl of each the 19 processed samples loaded in the same order as listed in table 7.

Table 5. Mass of DNA (ng/µl and ng/$m^2$) in each muddy rain sample

| Description | Sample Name | Collection Date | Backtracked Source | Liters Filtered | Approximate Mass of DNA (ng/µl) | Approximate Mass of DNA (ng/$m^2$) |
|---|---|---|---|---|---|---|
| Moderately muddy | XIII-34-1 | 20110923 | Sahara | 8.5 | 10 | 870 |
| Moderately muddy | XIII-36-1 | 20110924 | Sahara | 0.7 | 10 | 290 |
| Clean rain control | XIII-42-1 | 20111001 | Europe | 10 | Not processed | Not processed |
| Moderately muddy, dust in horizon | XIII-49-2 | 20111025 | Arabian peninsula | 7 | 25 | 5100 |
| Muddy | XIII-49-5 | 20111026 | Sahara | 6.5 | 75 | 3300 |
| Very clean rain control | XIII-50-1 | 20111103 | Europe | 17 | 100 | 9200 |
| Very muddy | XIII-51-1 | 20111114 | Arabian peninsula | 0.8 | 200 | 6400 |
| Hail rain | XIII-52-2 | 20111115 | Sahara | 4 | 10 | 14000 |
| Clean winter rain | XIII-52-4 | 20111217 | Europe | 4 | Not processed | Not processed |
| Very muddy, full of particulates | XIII-53-2 | 20111224 | Sahara | 4.5 | 25 | 2400 |
| Moderately muddy | XIII-53-4 | 20120111 | Sahara | 9 | Not processed | Not processed |
| Very clean rain control | XIII-54-2 | 20120113 | Europe | 9 | 1 | 230 |
| Extremely muddy, full of dust | XIII-66-1 | 20120207 | Sahara | 2.5 | 20 | 2700 |
| Extremely muddy, full of dust | XIII-72-1 | 20120216 | Sahara | 1.5 | 50 | 10000 |
| Very muddy, dust filled the air before rain | XIII-75-1 | 20120314 | Sahara | 2.5 | 150 | 17000 |
| Very muddy, full of dust | XIII-78-1 | 20120401 | Sahara | 2 | 300 | 8600 |
| Very muddy, full of dust | XIII-82-1 | 20120419 | Sahara | 0.6 | 10 | 300 |
| Extremely muddy, big droplets of mud | XIII-82-4 | 20120430 | Sahara | 0.07 | 200 | 5700 |
| Very thick mud precipitated on cars | XIII-83-1 | 20120502 | Arabian peninsula | 0.08 | 150 | 57000 |
| Muddy, dust filled the air before it rained | XIII-83-7 | 20120529 | Sahara | 1.2 | 5 | 140 |
| Hail rain | XIII-144-1 | 20121219 | Sahara | 4 | 15 | 6500 |
| Clean winter rain that smelled like snow | XIII-145-1 | 20121221 | Europe | 13 | 5 | 330 |
| Dust in the air, washed by rain | XIII-213-1 | 20130322 | Arabian peninsula | 1.5 | Not processed | Not processed |

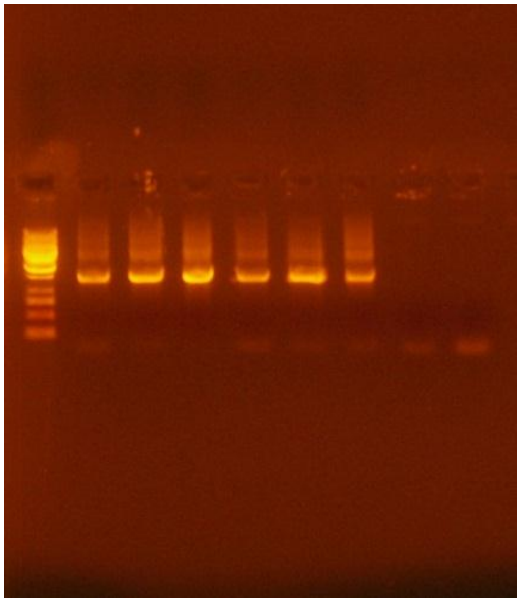


Figure 23. PCR amplification of the 16S rDNA gene from some muddy rain samples. The first well contains a 1 kb ladder and the rest are bacterial PCR products of about 1500 bp.

### *2. DGGE Analysis of 16S rDNA Shows Diversity of Microbial Communities in Different Muddy Rain Samples*

A part (233 bp) of the V3 variable region in the 16S rDNA gene was used for DGGE analysis of 13 muddy rain samples. Each sample had a unique DGGE profile demonstrating diversity of the samples composition, even among samples of the same origin. In general, the DGGE profiles were smeary with several dominant bands. The number and intensity of the dominant bands varied among the samples. The clean winter rains had few faint bands whereas muddy rains had several intense bands. The DGGE profiles are shown in figure 24 along with controls of known bacterial species. Analyzing these profiles allowed us to identify, based on sample diversity, the most interesting samples that we need to focus on by obtaining more sequences from them. These samples were XIII- 49-5, XIII-53-2, XIII-66-1 and XIII-78-1 from Sahara; XIII-49-2 from Arabian Peninsula; and XIII-52-2 as a hail sample.

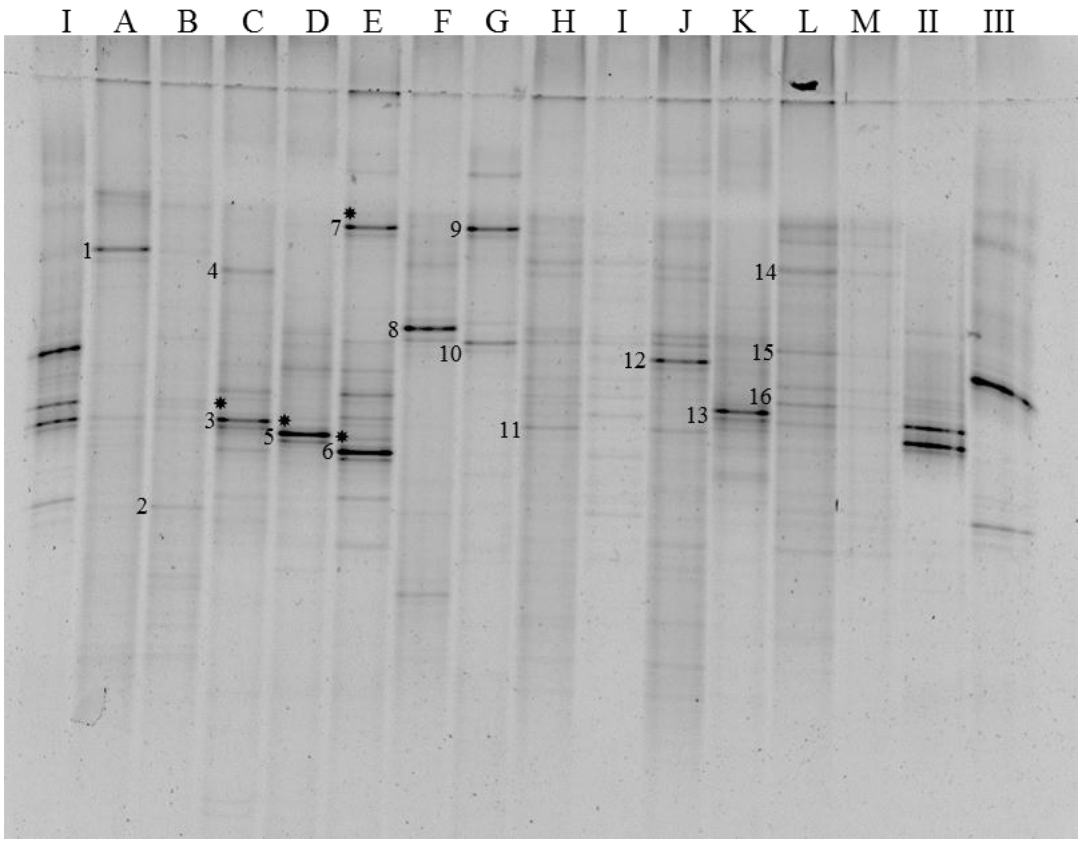



Figure 24. DGGE profiles of the 13 processed muddy rain samples. Lane I is a mix of *E. coli*, *B. cereus*, *P. vulgaris* and *S. enterica*; lane II is *E. coli* and *B. cereus*; Lane III is *P. vulgaris* and *S. enterica*; while Lanes A through M are XIII-34-1, 36-1, 49-2, 49-5, 50-1, 51-1, 52-2, 53-2, 54-1, 66-1, 78-1, 144-1 and 145-1 respectively. Bands 1 through 16 were cut out and sequenced. The bands which belonged to the same dominant genus identified by the metagenomic approach are labeled with an asterisk.

The dominant species in every sample was represented by the strongest band in the DGGE profile. The 16 strong bands labeled in figure 24, were cut out of the DGGE gel and sequenced. Some of these bands (labeled with an asterisk on figure 24) belonged to the same dominant genus identified by the metagenomic approach, further confirming that these genera are dominating the muddy rain bacterial community. The genera of the dominant bands are listed in table 6.

Table 6. The genera to which the DGGE dominant bands belong

| **Band Number** | Band 3 | Band 5 | Band 6 | Band 7 |
|---|---|---|---|---|
| **Genus** | *Methylibium* | *Aquabacterium* | *Ralstonia* | *Herbaspirillum* |

### *3. TRFLP Analysis Showed a Small Selection of Peaks*

Both DGGE and TRFLP techniques were performed to access sample biodiversity. However, TRFLP results were not as successful as the DGGE results. Very few fluorescent peaks appeared and thus the diversity could not be determined. Each of the *E. coli* and the *B. cereus* controls gave one band at 470 bp and at 615 bp respectively (Figure 25 and 26). This is very close to our expected results of a single band at 455 bp and 600 bp respectively, determined from the analysis of the Tru9I digestion of the 16S rDNA gene of these two bacteria. The two muddy rain samples however, did not show several peaks as expected. Instead, XIII-49-5 showed one big peak at 550 bp and one small peak at 590 bp; while XIII-53-2 showed several small peaks between 550 bp and 600 bp (Figure 26). Because of the weak results obtained from the TRFLP technique, microbial diversity was assessed using DGGE and the metagenomic approach.

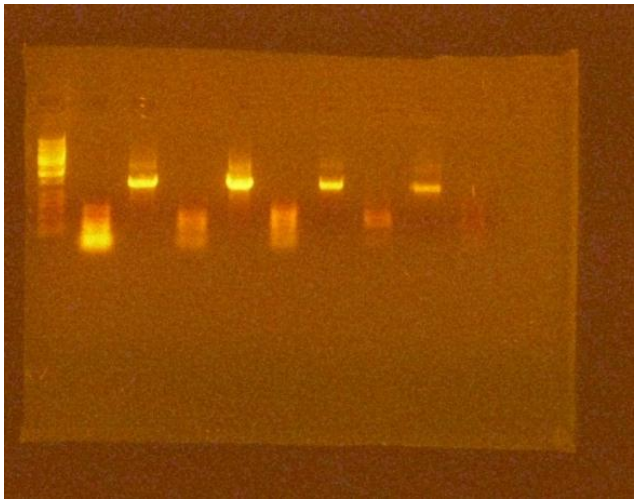


Figure 25. Tru9I digestion of the PCR products amplified with TRFLP primers. The first well is 0.5 µl of 1 kb ladder. Wells 2, 4, 6, and 8 are the uncut PCR products and wells 3, 5, 7, and 9 are the digested PCR products of *E.coli*, *B. cereus*, XIII-49-5 and XIII-53-2 respectively.

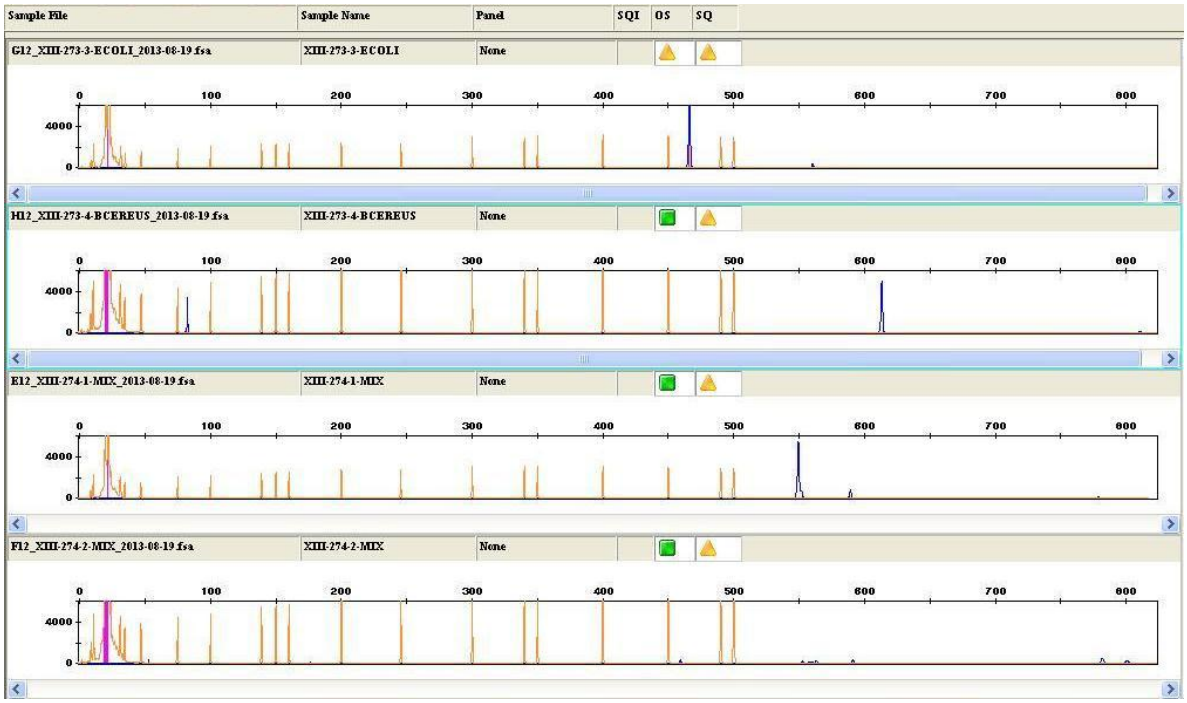



Figure 26. TRFLP profiles of two muddy rain samples. The top two panels are the TRFLP profiles of *E. coli* and *B. cereus* respectively. The bottom two panels show the profile of samples XIII-49-5 and XIII-53-2 respectively.

Table 7. Description and analysis of the collected muddy rain samples

| Description | Sample Name | Collection Date | Backtracked Source | Liters Filtered | Rain-fall (cm) | Mass of mud per ¼ filter (mg) | Mass of mud ($mg/m^2$) | Approxi-mate Mass of DNA (ng/µl) | Approxi-mate Mass of DNA ($ng/m^2$) | Number of Bacterial Sequences | Number of Fungal Sequences |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Moderately muddy | XIII-34-1 | 20110923 | Sahara | 8.5 | 1.2 | 20 | 120 | 10 | 870 | 4 | 1 |
| Moderately muddy | XIII-36-1 | 20110924 | Sahara | 0.7 | 0.03 | 10 | 20 | 10 | 290 | 3 | 2 |
| Clean rain control | XIII-42-1 | 20111001 | Europe | 10 | 2.3 | 10 | 100 | Not processed | Not processed | Not processed | Not processed |
| Moderately muddy, dust in horizon | XIII-49-2 | 20111025 | Arabian peninsula | 7 | 2.3 | 20 | 270 | 25 | 5100 | 28 | 3 |
| Muddy | XIII-49-5 | 20111026 | Sahara | 6.5 | 0.5 | 10 | 30 | 75 | 3300 | 40 | 2 |
| Very clean rain control | XIII-50-1 | 20111103 | Europe | 17 | 2.6 | 13 | 80 | 100 | 9200 | 5 | 3 |
| Very muddy | XIII-51-1 | 20111114 | Arabian peninsula | 0.8 | 0.04 | 10 | 20 | 200 | 6400 | 4 | 1 |
| Hail rain | XIII-52-2 | 20111115 | Sahara | 4 | 3.5 | 2 | 190 | 10 | 14000 | 28 | 4 |
| Clean winter rain | XIII-52-4 | 20111217 | Europe | 4 | 0.5 | 6 | 30 | Not processed | Not processed | Not processed | Not processed |
| Very muddy, full of particulates | XIII-53-2 | 20111224 | Sahara | 4.5 | 0.7 | 42 | 270 | 25 | 2400 | 33 | 3 |
| Moderately muddy | XIII-53-4 | 20120111 | Sahara | 9 | 1 | 10 | 50 | Not processed | Not processed | Not processed | Not processed |
| Very clean rain control | XIII-54-2 | 20120113 | Europe | 9 | 3.3 | 1.5 | 20 | 1 | 230 | 0 | 4 |

Table 7. Description and analysis of the collected muddy rain samples (Continued)

| Description | Sample Name | Collection Date | Backtracked Source | Liters Filtered | Rain Fall (cm) | Mass of mud per ¼ filter (mg) | Mass of mud ($mg/m^2$) | Approxi-mate Mass of DNA (ng/µl) | Approxi-mate Mass of DNA ($ng/m^2$) | Number of Bacterial Sequences | Number of Fungal Sequences |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Extremely muddy, full of dust | XIII-66-1 | 20120207 | Sahara | 2.5 | 0.4 | 51 | 460 | 20 | 2700 | 8 | 0 |
| Extremely muddy, full of dust | XIII-72-1 | 20120216 | Sahara | 1.5 | 0.5 | 19 | 270 | 50 | 10000 | 3 | DNA not amplifiable |
| Very muddy, dusty | XIII-75-1 | 20120314 | Sahara | 2.5 | 1.5 | 53 | 400 | 150 | 17000 | 3 | DNA not amplifiable |
| Very muddy, full of dust | XIII-78-1 | 20120401 | Sahara | 2 | 0.1 | 160 | 300 | 300 | 8600 | 9 | 2 |
| Very muddy, full of dust | XIII-82-1 | 20120419 | Sahara | 0.6 | 0.03 | 68 | 130 | 10 | 300 | DNA not amplifiable | 8 |
| Extremely muddy, big droplets of mud | XIII-82-4 | 20120430 | Sahara | 0.07 | 0.03 | 260 | 500 | 200 | 5700 | DNA not amplifiable | DNA not amplifiable |
| Very thick mud precipitated on cars | XIII-83-1 | 20120502 | Arabian peninsula | 0.08 | 0.1 | 1460 | 37000 | 150 | 57000 | DNA not amplifiable | DNA not amplifiable |
| Muddy, dusty | XIII-83-7 | 20120529 | Sahara | 1.2 | 0.1 | 40 | 80 | 5 | 140 | DNA not amplifiable | DNA not amplifiable |
| Hail rain | XIII-144-1 | 20121219 | Sahara | 4 | 1.6 | 20 | 570 | 15 | 6500 | 0 | 0 |
| Clean winter rain that smelled like snow | XIII-145-1 | 20121221 | Europe | 13 | 0.7 | 8 | 40 | 5 | 330 | 0 | 2 |
| Dust in the air, washed by rain | XIII-213-1 | 20130322 | Arabian peninsula | 1.5 | 0.03 | 30 | 60 | Not processed | Not processed | Not processed | Not processed |

## E. Saharan and Arabian Muddy Rains Contain Diverse Bacterial and Fungal Phyla

The metagenomic approach is the ideal way to determine the microbial biodiversity in a given environmental sample. This approach, as opposed to the culture dependent approach, allows us to determine, without bias, the comprehensive microbial profile of the environmental sample. After creating plasmid pools containing various inserts from each muddy rain sample, the pools were screened to identify the inserts suitable for sequencing. Inserts of the medium length or the full length 16S rDNA were picked and sequenced (Figure 27). The results of the metagenomic approach are presented below.

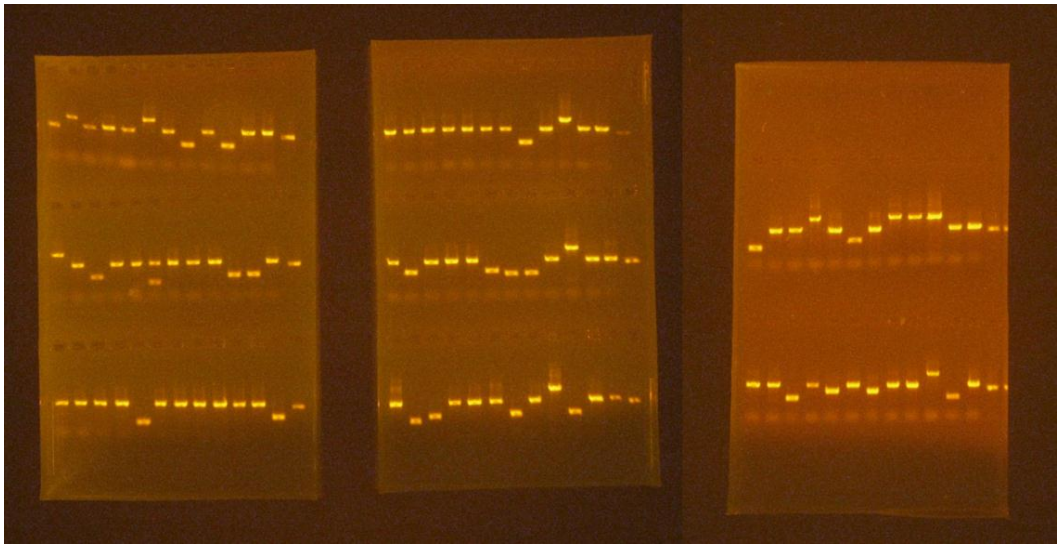


Figure 27. PCR pool screening for muddy rain inserts. The longest bands represent the full length muddy rain insert, the medium length bands represent a shorter muddy rain insert, and the shortest bands represent plasmids with no insert.

### *1. Muddy Rain Bacterial Community Composition Is Diverse and Consistent with Origin*

A total of 168 bacterial sequences were obtained from all samples. The attempted sequences were many more, but only 168 were readable and judged reliable. The distribution of these sequences among the muddy rain samples is presented in table

7. As shown in the table, the interesting samples identified in section III.D.2 have more sequences than other samples. The bacterial community in rains originating from the Sahara desert had a significantly different composition than those originating from the Arabian Peninsula. However, rains from both origins had Betaproteobacteria and Alphaproteobacteria as the two most dominant classes. Muddy rains from the Sahara had more variety of phyla and classes than rains from the Arabian Peninsula. The bacterial community profile of each muddy rain is presented in table 8. For rains originating from the Sahara desert, the bacterial phyla detected by the metagenomic approach comprised, in decreasing order of relative abundance, Betaproteobacteria (51.15%), Alphaproteobacteria (21.37%), Firmicutes (9.7%), Actinobacteria (4.58%), Gammaproteobacteria (4.58%), Bacteroidetes (4.58%), Epsilonproteobacteria (2.29 %), Cyanobacteria (0.76 %), and Deltaprotobacteria (0.76%) (Figure 28). For rains originating from the Arabian Peninsula, the bacterial phyla detected by the metagenomic approach contained, in decreasing order of relative abundance, Betaproteobacteria (71.88%), Alphaproteobacteria (15.63%), Cyanobacteria (9.38 %), and Actinobacteria (3.13%) (Figure 28). Out of a total of 26 described bacterial phyla, 5 were detected in muddy rain samples. The number of described bacterial genera is too many to count and thus it is difficult to compare them with the number of bacterial genera found in muddy rain samples.

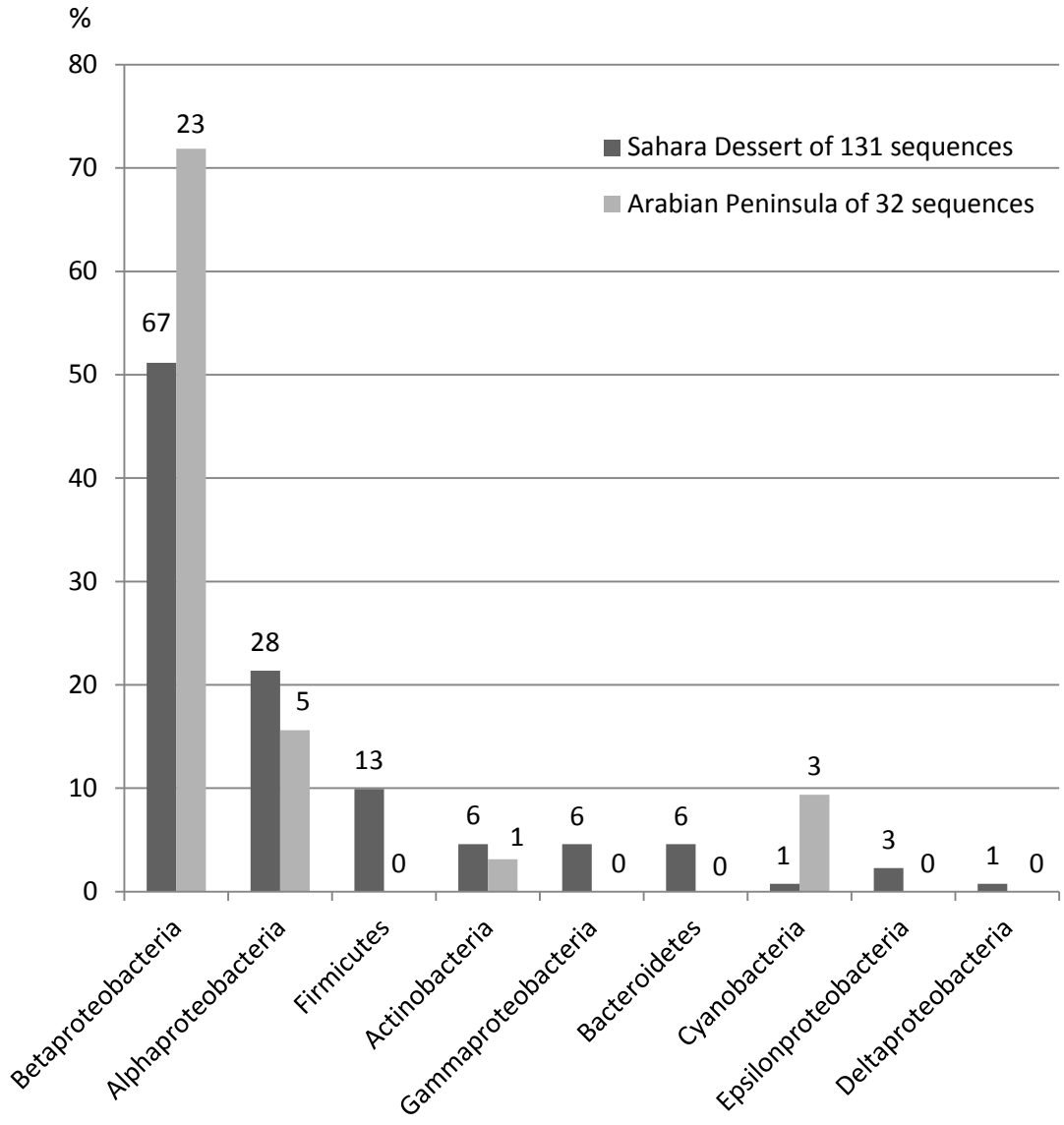



Figure 28. Muddy rain bacterial community composition from the metagenomic approach. The numbers above the bars represent the actual number of muddy rain sequences belonging to each phylum or class.

Table 8. Bacterial clone identities for each processed muddy rain sample (Alpha: Alphaproteobacteria, Beta: Betaproteobacteria, Gamma: Gammaproteobacteria, Delta: Deltaproteobacteria, Epsilon: Epsilonproteobacteria, Actino: Actinobacteria, Cyano: Cyanobacteria)

| Dirty rain sample | Date collected | Origin | Number of clones | Clone identity |
|---|---|---|---|---|
| XIII-34-1 | 20110923 | Sahara | 4 | 2 Gamma, 1 Alpha, 1 Cyano |
| XIII-36-1 | 20110924 | Sahara | 3 | 1 Alpha, 1 Beta, 1 Firmicutes |
| XIII-49-2 | 20111025 | Arabian peninsula | 28 | 23 Beta, 5 Alpha |
| XIII-49-5 | 20111026 | Sahara | 40 | 27 Beta, 12 Alpha, 1 Gamma |
| XIII-50-1 | 20111103 | Europe | 5 | 5 Beta |
| XIII-51-1 | 20111114 | Arabian peninsula | 4 | 3 Cyano, 1 Actino |
| XIII-52-2 | 20111115 | Sahara | 28 | 28 Beta |
| XIII-53-2 | 20111224 | Sahara | 33 | 13 Alpha, 6 Firmicutes, 4 Actino, 3 Epsilon, 2 Beta, 2 Gamma, 2 Bacteroidetes, 1 Delta |
| XIII-54-2 | 20120113 | Europe | 0 | Clean rain |
| XIII-66-1 | 20120207 | Sahara | 8 | 3 Firmicutes, 2 Beta, 1 Alpha, 1 Actino, 1 Bacteroidetes |
| XIII-72-1 | 20120216 | Sahara | 3 | 3 Bacteroidetes |
| XIII-75-1 | 20120314 | Sahara | 3 | 2 Beta, 1 Gamma |
| XIII-78-1 | 20120401 | Sahara | 9 | 5 Beta, 3 Firmicutes, 1 Actino |

### *2. Muddy Rain Fungal Community Composition Is Diverse and Consistent with Origin*

A total of 35 fungal sequences were obtained from all samples. The distribution of these sequences among the muddy rain samples is presented in table 7. The fungal community of rains originating from the Sahara desert had a similar composition to those originating from the Arabian Peninsula. Rains from both origins had Basidiomycota and Ascomycota as the two most dominant phyla. The fungal community profile of each muddy rain is presented in table 9. For rains originating from the Sahara desert, the fungal phyla detected by the metagenomic approach comprised, in decreasing order of relative abundance, Basidiomycota (54.55%), Ascomycotaa (36.36%), and Uncultured Eukaryotic Clone (9.09%) (Figure 29). For rains originating from the Arabian Peninsula, half the phyla belonged to Basidiomycota and the other half belonged to Ascomycota (Figure 29). Out of a total of 9 described fungal phyla, 2

were detected in muddy rain samples. These two fungal phyla (Ascomycota and Basidiomycota) are the only two phyla described in the Dikarya subkingdom.

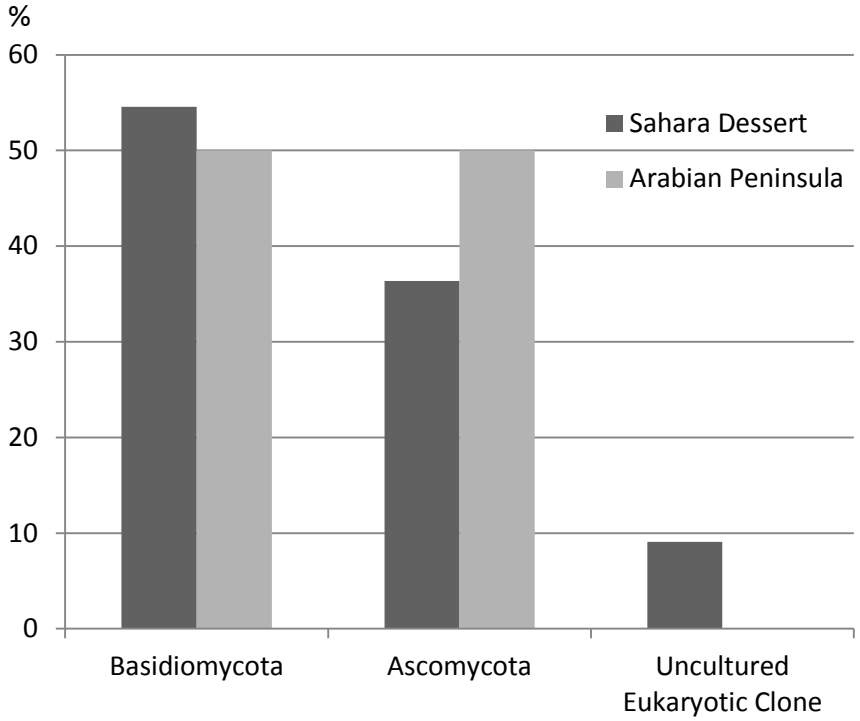



Figure 29. Muddy rain fungal community composition from the metagenomic approach.

Table 9. Fungal clone identities for each processed muddy rain sample

| Dirty rain sample | Date collected | Origin | Number of clones | Clone identity |
|---|---|---|---|---|
| XIII-34-1 | 20110923 | Sahara | 1 | 1 Ascomycota |
| XIII-36-1 | 20110924 | Sahara | 2 | 1 Ascomycota, 1 Basidiomycota |
| XIII-49-2 | 20111025 | Arabian peninsula | 3 | 1 Ascomycota, 2 Basidiomycota |
| XIII-49-5 | 20111026 | Sahara | 2 | 2 Basidiomycota |
| XIII-50-1 | 20111103 | Europe | 3 | 1 Ascomycota, 2 Uncultured Eukaryotic Clone |
| XIII-51-1 | 20111114 | Arabian peninsula | 1 | 1 Ascomycota |
| XIII-52-2 | 20111115 | Sahara | 4 | 1 Ascomycota, 1 Basidiomycota, 2 Uncultured Eukaryotic Clone |
| XIII-53-2 | 20111224 | Sahara | 3 | 2 Ascomycota, 1 Basidiomycota |
| XIII-54-2 | 20120113 | Europe | 4 | 4 Basidiomycota |
| XIII-66-1 | 20120207 | Sahara | 0 | No clones found |
| XIII-78-1 | 20120401 | Sahara | 2 | 2 Basidiomycota |
| XIII-82-1 | 20120419 | Sahara | 8 | 5 Basidiomycota, 3 Ascomycota |
| XIII-144-1 | 20121219 | Sahara | 0 | No clones found |
| XIII-145-1 | 20121221 | Europe | 2 | 2 Basidiomycota |

## F. Phylogenetic Analysis Shows the Sequence Similarity and Phylogenetic Affiliations of Muddy Rain Bacteria

The phylogenetic tree of 109 bacterial muddy rain sequences was constructed based on the maximum-likelihood principle. The tree was used as an unambiguous means to organize sequences without bias, by phylogenic relationships. The tree elucidates the phylogenetic affiliation of the bacterial muddy rain sequences and serves to group the sequences by phylotypes. *E. coli* and *B. cereus* were used as a reference for a gram negative and gram positive bacteria respectively. Some sequences were very closely related while others had more evident genetic variations; and this was the basis for the presence of several operational taxonomic units (Figure 30).

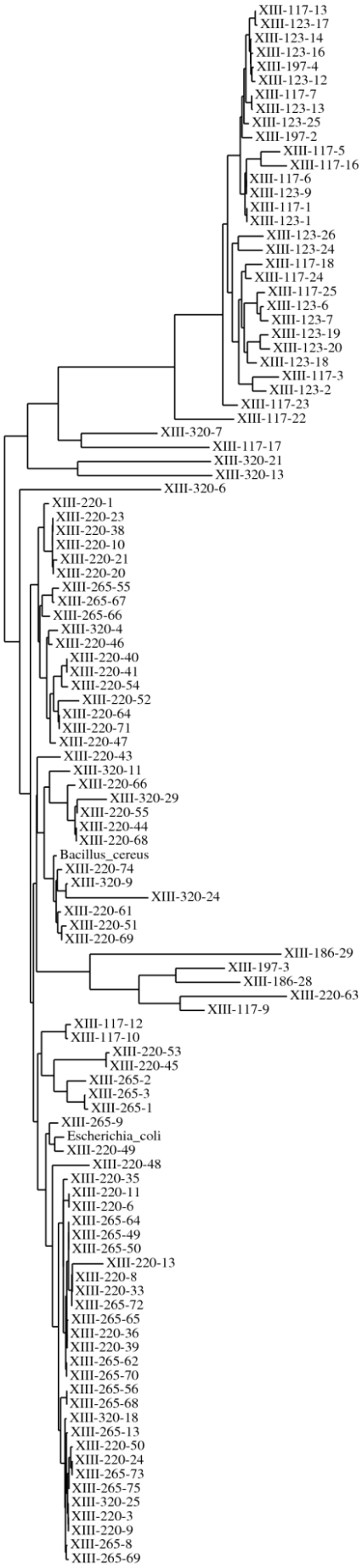



Figure 30. Phylogenetic tree of 109 muddy rain bacterial sequences

# CHAPTER IV

# DISCUSSION

## A. Spring Rains in Lebanon Contain Large Amounts of Mud Transported from the Sahara or Arabian Peninsula

Dust is known to be the major means of global microbial transport and exchange (Kellogg and Griffin, 2006). In Lebanon, spring muddy rains occur frequently yet remain undocumented and unexamined. The amount of mud in spring rain was surprisingly high, reaching up to 37,000 mg/m$^2$. The amount of mud varied greatly between individual rains, presumably depending on wind activity, sand lofting, soil friability, and weather patterns. Microbes would presumably be at an advantage when transported by muddy rain, rather than wind, because they would be less exposed to solar radiation, transported over larger distances, and deposited more rapidly into distant ecologies.

Backtracking muddy rain storms could indicate the origin of muddy rain solids and their microbial flora deposited in Lebanon. Wind back-trajectories revealed likely paths along which microbes are collected and transported to downwind ecosystems. These paths limit the composition of the microorganisms that are deposited with muddy rain, but do not indicate where along these long tracks mud originated. Backtracking indicated the Sahara desert as the major origin of muddy rain solids, as 14 out of 18 muddy rains traversed the Sahara, and the remaining 4 traversed the Arabian Peninsula. These origins of muddy rain solids are similar to those of Mediterranean dust intrusion events studied by Dayan *et al.* (1991). In contrast, all rains traversing only Europe and

the Mediterranean Sea were clean. These findings were expected, as it is known that the northern Sahara is the major dust source over the eastern Mediterranean in spring and summer (Griffin *et al.*, 2007).

Mineral composition found in muddy rain solids is consistent with the origins indicated by wind back-trajectories. Minerals typical of the Sahara desert were found in the muddy rain solids deposited in Lebanon. Silicon and calcium minerals, namely quartz, calcite and gypsum, are the most abundant in the Sahara; and of metal minerals, aluminum and iron containing minerals are the most abundant in the Sahara (Goudie and Middleton, 2001; Guieu *et al.*, 2002; Hamdi-Aissa *et al.*, 2004; Linke *et al.*, 2006). The XRD data of muddy rain samples showed similar results (Table 3), thus corroborating the Saharan origin.

**B. Muddy Rain Contains Large Amounts of Viable and Diverse Microbes**

A gram of arid soil may contain approximately $10^9$ prokaryotes (Griffin, 2007). The current study shows that muddy rain also carries large amounts of viable and diverse microbes. A wide range of DNA mass (143-57,000 ng/$m^2$) was found in muddy rain samples. Extracted DNA was amplifiable, which suggests that the microbes were still viable. This was corroborated by the successful culturing of microbes from muddy rain. Direct culturing of the stock muddy rain samples yielded confluent microbial growth of more than 2,000 colony forming units per microliter. In order to obtain growth of separated single colonies at least a 100-fold dilution of the original sample had to be done (Figure 19). If these numerous cultured colonies represent only 0.2-2 % of the total microbial community (Streit *et al.*, 2004), then it can be assumed that the total amount of microbes present in muddy rain is very high.

Culture-based and metagenomic approaches were used to determine the diversity of muddy rain microbes. Including all analyzed samples, 75 bacterial and 12 fungal genera were detected. The percent abundance of bacterial phyla identified by the culture based approach is represented in figure 20. The two most abundant phyla (Actinobacteria and Firmicutes) are gram positive and the rest are gram negative. This is not surprising considering that spore-forming bacteria are mainly gram positive, and spores would be expected to be more likely transported long distances by muddy rain because they can endure harsh environmental conditions. All the phyla identified by the culture based approach are known to be soil or water bacteria. One interesting phyla which had very low abundance is Deinococcus. This phylum is known to be extremely radiation resistant which explains its ability to travel long distances while exposed to UV light and ionizing radiation (de Groot *et al.*, 2005; Rainey *et al.*, 2005; Yuan *et al.*, 2009). Other phyla were also found by the metagenomic approach and will be described in a later section. Importantly, the percent abundance of bacterial and fungal phyla is not expected to be consistent with the results of the metagenomic approach, because only few microbes are culturable. Interestingly, all species of the two hail samples belonged to the genus *Herbaspirillum* and to the closely related *Duganella*. *Herbaspirillum* is a nitrogen- fixing root bacterium that is not commonly associated with precipitation (Baldani *et al.*, 1986; Olivares *et al.*, 1996).

**C. Implications Relevant to Ecology, Agriculture, and Climate Change**

Microbial transport by rain and dust imposes several implications relevant to ecology, forestry, agriculture, and climate change. Bacteria transported by muddy rain have various ecological roles in the soils of origin. Some of these roles include fixing nitrogen, oxidizing ammonia and methane, producing sulfur, degrading organic matter,

producing antibiotics, developing symbiotic relationships with plants and animals, and causing disease. The global microbial transport and exchange between distant habitats is rapid, based on the common ecologies found in similar, yet distant, habitats (Cho and Tiedje, 2000; Yeager *et al.*, 2004). Microbiomes of terrestrial soils are presumably in rapid equilibrium compared to the rate of climate change. Because this equilibrium has been present for hundreds of years, even thousands of years, it is reasonable to speculate that transport of microbes from regions of seasonal aridity and occasional drought contribute more microbes and affect down-wind ecologies than transport from deserts soils.

Deforestation might present new soil sources for muddy rain. The size of the lands being subjected to deforestation worldwide is alarming. A report by the Food and Agriculture Organization (FAO) of the United Nations estimated the net loss in forest area between 2000 and 2005 to be 73,000 $km^2$ (FAO of the United Nations, 2005). In North Africa, the region from where most of the muddy rains in Lebanon originate, 9,820 $km^2$ were subjected to deforestation during the same period (FAO of the United Nations, 2005). These new arid areas resulting from deforestation, in addition to poor agricultural practices which make the soil loftier, may contribute to the formation of muddy rain and the spread of its associated microbes.

Muddy ran might be a vector for the spread of antibiotic resistant bacteria. Livestock is being raised industrially in many areas that are a potential origin of muddy rain (Nordblom *et al.*, 1995) and antibiotics are constantly used for the treatment and prevention of bacterial diseases in livestock productions (Gustafson and Bowen, 1997). The excessive use of antibiotics is a major cause of drug resistance in microbes (Levy and Marshall, 2004). Thus, massive livestock production in countries from which

muddy rain originates may lead to the spread of antibiotic resistant bacteria via muddy rain. An example of other dangers is the case of toxin-expressing bacteria contaminating food, and one can imagine lofted dry manure facilitating its spread. A close example was when *E. Coli* O104:H4 caused an outbreak of foodborne disease in Germany during 2011 (The Local, 2011). Moreover, if genetically modified crops (GMCs) are grown, genetic exchange may occur between bacteria and GMCs. The transgenic bacteria will help spread transgenes by traveling long distances via muddy rain.

Implications relevant to climate change are also manifested by muddy rain. Climate change is what derived the formation of the main origin of muddy rain which is the Sahara desert. During the early Holocene Epoch, 10,000 years ago, the Sahara desert was thought to be a green and fertile land harboring a variety of terrestrial and aquatic ecosystems dominated by savannah grasses and lakes (Kropelin *et al.*, 2008). It was not until 4,300 years ago that rapid climatic change has caused the drying of the Sahara; and it was not until 3,700 years ago that enough sand dunes were formed for winds to start carrying and transporting dust particles (Kropelin *et al.*, 2008). This implies that muddy rain of Saharan origin started falling in Lebanon around 3,700 years ago. One can imagine that the range of areas receiving muddy rains and muddy rain microbes might be broadened if new arid places emerged as a result of the current climatic changes and desertification.

If climatic changes occurred abruptly, or a massive solar flare sterilizes the region, muddy rain would play important role in restoring the microbial flora of the destroyed habitats. For example, if climate change has led to the melting of ice glaciers and thus the exposition of lands from under the glaciers, it would not take long until the new land gets reseeded with microbial flora transported by muddy rain. A relevant

example is that of the Krakatoa Island which was eradicated in 1883 due to a massive volcanic eruption, and the new Anak Krakatoa Island that emerged in 1930 as a result of subsequent submarine eruptions (Thornton *et al.*, 1988). The plant flora of Krakatoa (Diamond, 1975) and of Anak Krakatoa (Partomihardjo *et al.*, 1992) were restored in a relatively short period of time, where it took only 8 years for 53 species of plants to recolonized Anak Krakatoa (Partomihardjo *et al.*, 1992). One can imagine that the microbial flora have been restored at an even faster rate. Winds, dust, and rains might have been vital tools for the accomplishment of such a fast restoration. Moreover, because muddy rain and the consequent exchange of bacteria have been establishing equilibrium between habitats for a long time, any change in rain and wind patterns caused by climate change would lead to alarming ecological changes. Thus, describing muddy rains and their associated microbes helps in monitoring ecological changes.

### D. Bacterial and Fungal Composition of Muddy Rain Is Diverse and Consistent with Putative Origins

Extensive studies have been done to describe the microbial communities transported by desert dust; however, no studies about the role of muddy rain in microbial transport could be found. Several features make muddy rain different from dust storms. For example, dust particles get irradiated if lofted to high altitudes while muddy rain is shielded from UV irradiation by clouds. Dust particles remain lofted in the atmosphere for longer periods of time while muddy rain is picked up and deposited soon after being lofted. Also, unlike dust, muddy rain is deposited in concentrated and localized places which make microbial transport more efficient. A comparison between muddy rain microbes and Saharan dust microbes that has been transported long distances is presented below, although muddy rain is different from dust. However, the similarity in the microbial composition of muddy rain and Saharan dust is consistent

with the conclusion that desert dust, along with its associated microbes, is carried via muddy rain.

The 9 bacterial phyla detected in muddy rain originating from the Sahara desert, were also detected, but with different abundance, in desert dust originating from different regions of the Sahara desert. Betaproteobacteria dominates the muddy rain bacterial flora reaching up to 51%. Similarly, Betaproteobacteria was found to be the dominating bacteria of Saharan dust deposited on high areas of the Pyrenees Mountains in Spain (Hervas *et al.*, 2009). However, Saharan dust deposited on Mount Blanc in France (Chuvochina *et al.*, 2011) and on Turkey's coastlines (Griffin *et al.*, 2007) contained minimal amounts of Betaproteobacteria and was dominated by Actinobacteia instead. Alphaproteobacteria ranks second among the dominant bacteria of muddy rain and has a similar high concentration among Saharan dust bacteria deposited on Mount Blanc and the Pyrenees Mountains (Hervas *et al.*, 2009; Chuvochina *et al.*, 2011).

The spore-forming Firmicutes is the third dominant phyla of muddy rain. This is not surprising given their strong survival feature that facilitates long distance travel. Firmicutes is the most dominant phyla in the Saharan dust of South Mali (Kellogg *et al.*, 2004) and many of them are thermophillic especially members of the genus *Geobacillus* (Perfumo and Marchant, 2010). Although, the muddy rain bacterial community had only 4.6% Actinobacteria, the latter phylum was dominant in Saharan dust collected in South Tunisia (Chanal *et al.*, 2006), Turkey (Griffin *et al.*, 2007), and Mont Blanc in France (Chuvochina *et al.*, 2011). Gammaproteobacteria had equal abundance as Actinobacteria in muddy rain; however, its abundance in Saharan dust varied among different deposition locations. Studies in the Spanish Pyrenees Mountains (Hervas *et al.*, 2009) and in South Tunisia (Chanal *et al.*, 2006) showed a fairly high amount of

Gammaproteobacteria while studies in Mont Balnc in France (Chuvochina *et al.*, 2011) and in South Mali (Kellogg *et al.*, 2004) showed a few amounts of Gammaproteobacteria.

The phylum Bacteroidetes have a high concentration in Saharan dust deposited on Mont Blanc according to Chuvochina *et al.* (2011) and a low concentration in Saharan dust deposited on the Pyrenees Mountains and Turkey (Hervas *et al.*, 2009; Griffin *et al.*, 2007). The three remaining phyla, Cyanobacteria, Deltaproteobacteria, and Epsilonproteobacteria, have very low abundance in muddy rain samples and this data is consistent with their low abundance in Saharan dust samples (Chanal *et al.*, 2006; Chuvochina *et al.*, 2011). As for fungal phyla, 54.5% of muddy rain fungus of Saharan origin belonged to the phylum Basidiomycota and 36.4% belonged to the phylum Ascomycota. In contrast, in studies conducted on Saharan dust, Ascomycota was the only phyla detected (Kellogg *et al.*, 2004; Griffin *et al.*, 2007).

The muddy rain microbial community contains a wide variety of bacteria and fungi with characteristics that enable them to survive long distance dispersal, survive in the new downwind ecosystem, and compete with its local microbial flora. Proteobacteria, the largest and most diverse phylum among the Bacterial domain (Kersters *et al.*, 2006), is the most dominant bacterial phyla in muddy rain, though its members lack features that protect them from desiccation, UV radiation, and nutrient scarcity such as spores and pigmentation. This implies that Proteobacteria and other non-sporulating muddy rain microbes such as Bacteriodetes need other forms of protection to survive the long distance journey. The attachment to a dust particle sheltered in a huge dust cloud, and being inside a water droplet may prevent desiccation and harm by radiation. Protebacteria are known to participate in several biochemical

processes such as the control of greenhouse gas emissions. For example, the methanotrophic Gammaproteobacteria consumes huge amount of methane present in soil and Alphaproteobacteria can deplete 30 million tons of atmospheric methane per year (Singh *et al.*, 2010). Members of Betaproteobacteria can oxidize ammonia into nitrate and nitrite which are subsequently converted into atmospheric nitrous oxide by microbial nitrification and denitrification (Singh *et al.*, 2010).

Some members of Proteobacteria are pathogens or opportunistic pathogens. Few genera of Alphaproteobacteria are pathogenic; these include *Rikettsia* which was not found in muddy rain and *Sphingomonas* (Ryan and Adley, 2010) which was abundantly detected among muddy rain sequences. The family Rhizobiaceae of Alphaproteobacteria is known to be a plant symbiont (Vigya *et al.*, 2013) and was found among the muddy rain bacterial flora. Muddy rain bacteria did not include any highly pathogenic Betaproteobacteria like *Neisseria gonorrhoeae* or *Neisseria meningitidis* but included many non-pathogenic *Neisseria* species. Many Gammaproteobacteria especially the family Enterobacteriaceae are highly pathogenic (Sanders and Sanders, 1997), but no members of the mentioned family was detected in muddy rain. However, opportunistic pathogens of the order Pseudomonadales were identified. Interestingly, sequences closely resembling ice nucleating Gammaproteobacteria were detected.

The gram positive muddy rain bacteria belonged to the phyla Actinobacteria and Firmicutes. Actinobacteria are known soil microbes with counts over 1 million per gram (Goodfellow and Williams, 1983). They are known for their ability to produce secondary metabolites with antibiotic activity (Selvameenal *et al.*, 2009) and their ability to decompose organic matter of plants and soil such as lignin and cellulose (Goodfellow and Williams, 1983). In muddy rain, several Actinobacteria of the genus

*Actinomyces* were found. The latter genus is a human pathogen that causes actinomycosis, a disease characterized by the formation of abscesses in the respiratory and gastrointestinal tract (Bowden, 1996). Firmicutes are a close relative of Actinobacteria and are known for their pathogenicity and ability to form endosporse. *Bacillus* and *Clostridium* are two genera of Firmicutes that are known to be pathogenic and were detected in muddy rain samples. *Bacillus* species transported by muddy rain might cause diseases such as anthrax, septicemia, endocarditis, and infections of the respiratory, urinary, and gastrointestinal tracts (Turnbull, 1996). *Clostridium* species transported by muddy rain might cause diseases such as gas gangrene, tetanus, botulism, and food poisoning (Wells and Wilkins, 1996).

Fungi transported by muddy rain also included a number of known pathogens. Among the muddy rain Basidiomycota, the *Cryptococcus* genus, which is able to cause diseases in people with compromised immunity, was detected (Cheng *et al.*, 2001). Among the phylum Ascomycota, the *Aspergillus* genus is known to be an air-borne plant, animal, and coral pathogen (Abarca *et al.*, 1994; Griffin *et al.*, 2002a; Beck *et al.*, 2014). This genus was not detected in muddy rain; however, several genera belonging to the same class as *Aspergillus* (Eurotiomycetes) were detected. In short, the muddy rain bacterial and fungal community is diverse, consistent with putative origins, and possesses various characteristics that ensure its fitness.

### E. How Microbes Exploit and Facilitate Dust Storms and Rain Formation as Means of Global Microbial Transport

Microbes have several adaptations for dispersal to distant habitats such as spore formation for survival in harsh environments, resistance to radiation, nucleation of rain, and lofting of dust. Conceivably, adaptations could exist that are specific for dispersal via muddy rain, which might include creation of dust and agglomeration to small

particles. Adaptation by spore formation is very evident in *Bacillus subtilis* where under extreme conditions, the bacteria sporulate to protect itself from heat, radiation and chemicals (Setlow, 2005). Microbes adapted to resist radiation either by pigmentation like the genus *Deinococcus* (Yaun *et al.*, 2009) or by sporulation like the genus *Bacillus* (Setlow, 2005). Biological ice, rain, and cloud condensation nuclei consist mostly of soil bacteria and nucleation is done most efficiently by members of the phylum Gammaproteobacteria (DeLeon-Rodriguez *et al.*, 2013). As an example of lofting, a recent study by Dressaire *et al.* (2013) showed that mushroom fungus manipulates its environment to create an air flow that facilitates the dispersal of its spores. Mushrooms modify the buoyancy of the surrounding air by certain techniques evolving evaporation and active cooling (Dressaire *et al.*, 2013). This sophisticated strategy of environment manipulation, lends credibility to speculation that microbes can manipulate dust, wind, rain, and snow to promote their global dispersal.

It is reasonable to speculate that microbes have evolved an ability to manipulate dust and rain for their dispersal. In this strategy, microbes facilitate the lofting of desert dust and the creation of dust storms by making the dust particles more friable as the soil habitat dries. Microbes would adhere to and hitchhike on moving dust particles to achieve altitude. To speed up their arrival and to protect themselves from radiation, microbes as well as their dust vehicles act as biological rain nucleators. Microbial transport in rain would be less mutagenic, more rapid, and more efficient than microbial transport in dust clouds. This is true because the water droplet will provide an additional shield to the dust particle, rain is deposited more rapidly than dust clouds, and it is more efficient for microbes to be washed into soil and seed new ecologies than settling on their surface.

To further diminish transport related hazards and to survive long distance dispersal via dust and rain, microbes are known to have evolved adaptation features such as pigmentation, sporulation, and UV resistance, the latter of which includes specialized DNA repair mechanisms (Minton, 1995; Moeller *et al.*, 2007). Perhaps the most significant ecological role filled by muddy rain could be the rapid global exchange of microbes which led to the current phenomenon of similar microbial ecologies in similar habitats world-wide. A study by Cho and Tiedje (2000) showed that soil samples from 4 continents (with an approximate distance of 20,000 km as estimated by Martiny *et al.* (2006)) exhibited very similar *Pseudomonas* genotypes. Another study by Yeager *et al.* (2004) showed that soil crusts samples 700 km apart (as estimated by Martiny *et al.* (2006)) harbored similar communities of nitrogen-fixing bacteria. The ability of microbes to survive long-distance transport made this global exchange feasible. Global exchange would ensure the spread of microbes into most habitats and various ecologies, thus making them less affected by habitat destruction and local competition.

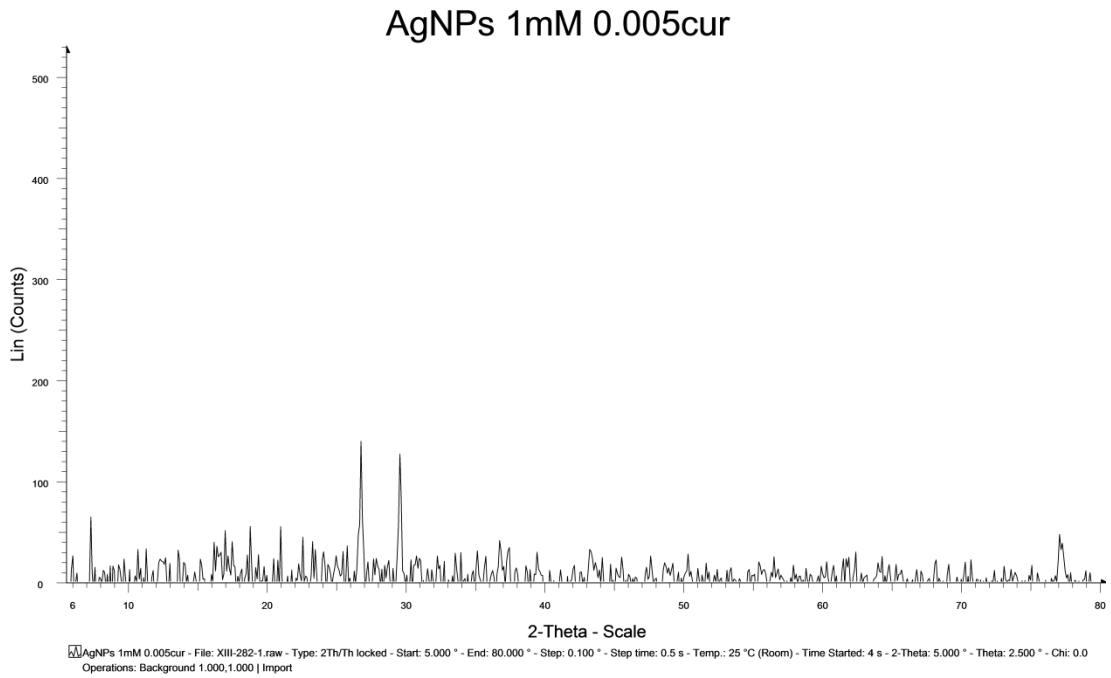
The phenomenon of muddy rain is intriguing with the unexpectedly large microbial community it harbors and the possible ecological roles it performs. Interestingly, microbial transport by muddy rain appears not passive but rather facilitated by the microbes themselves. Microbial colonization of new ecologies and their ability to out-compete locally adapted species is also intriguing. This study only begins to shed light on muddy rain and its associated microbes and many questions remain to challenge scientist's minds. What microbes are exploiting muddy rain to disperse? What adaptations exist? Do some species rely on others' adaptations? Do different species functionally cooperate to aid the formation of muddy rain? Do all microbes have the same chance of getting transported or have only some species

adapted? What other clever strategies do these single celled organisms use to ensure their fitness?

# APPENDICES

## I- XRD Profiles of Muddy Rain Samples

XIII-34-1



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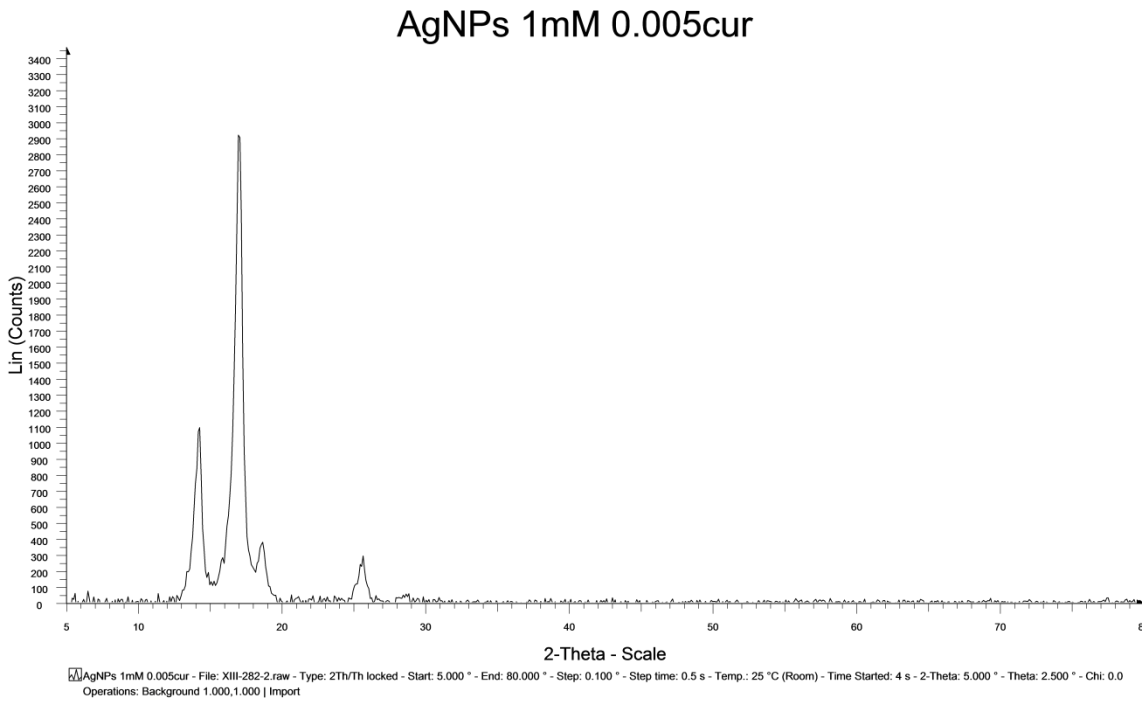


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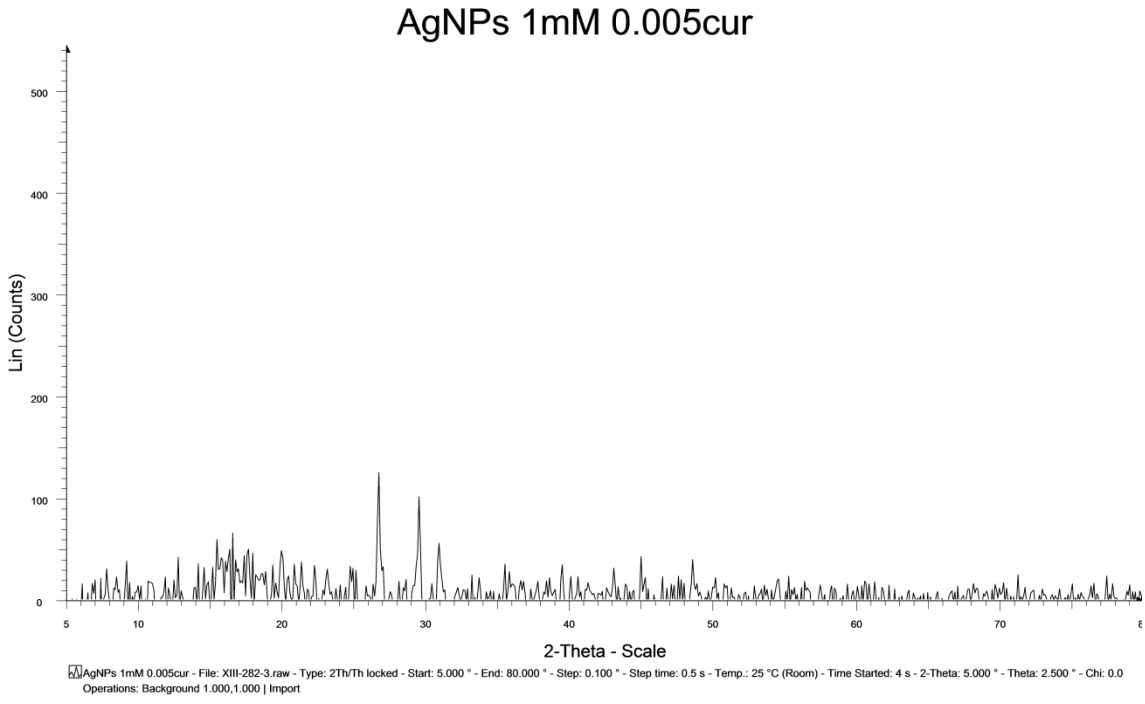

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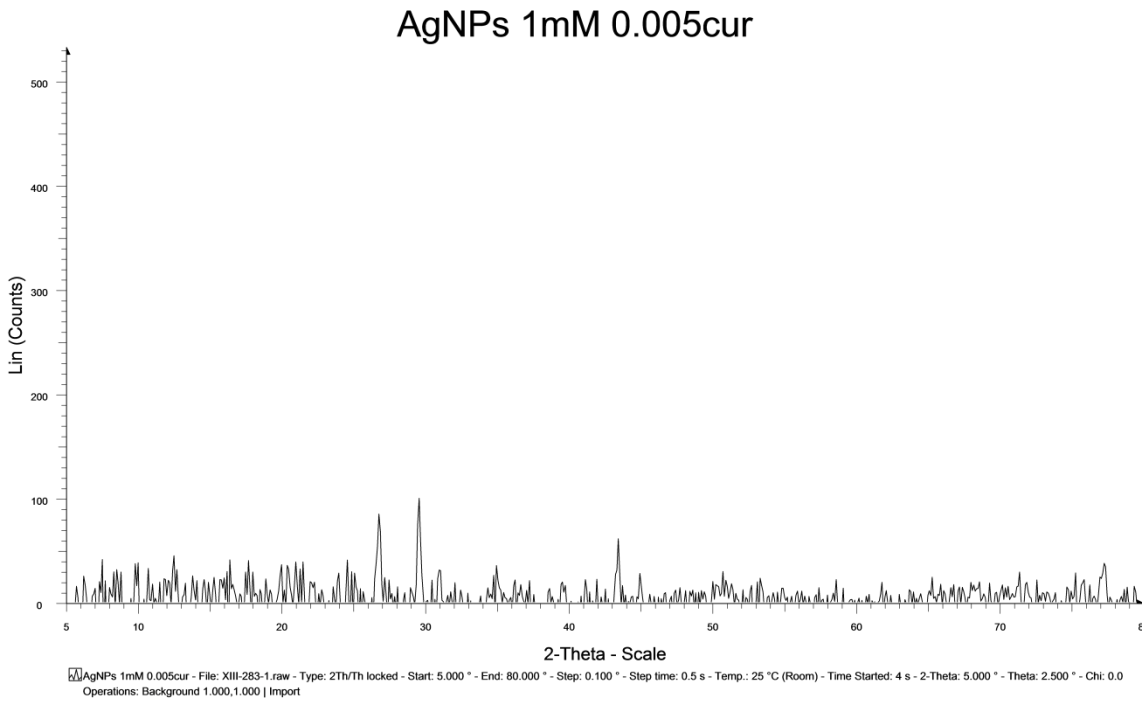


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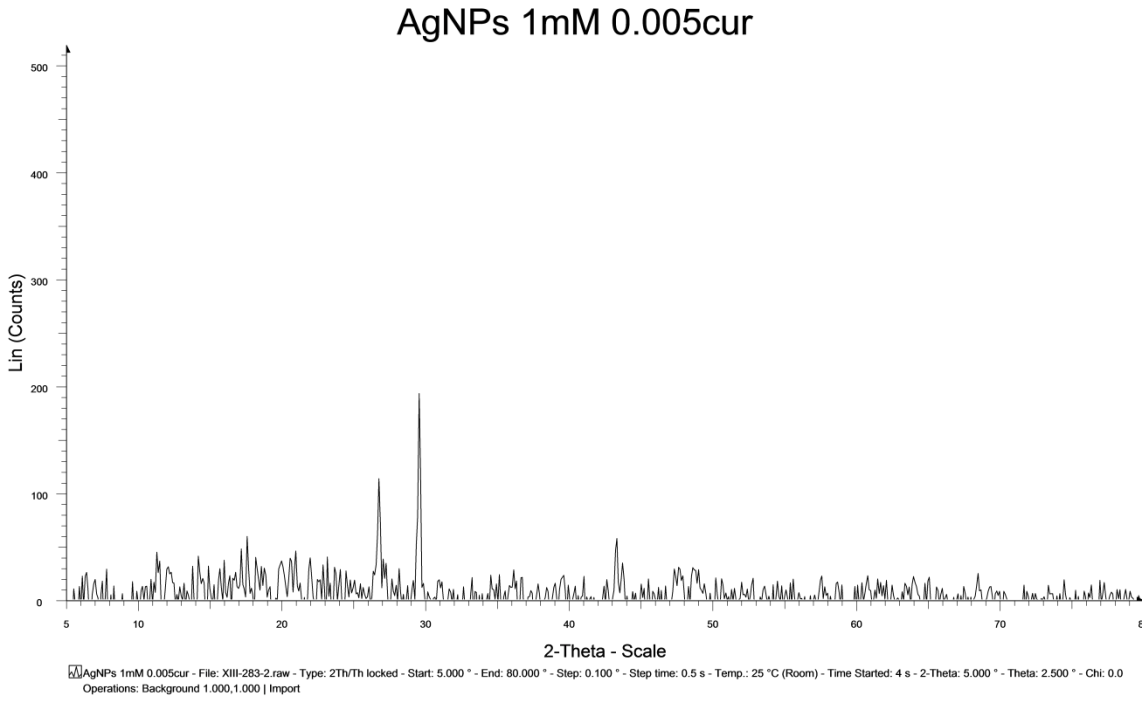

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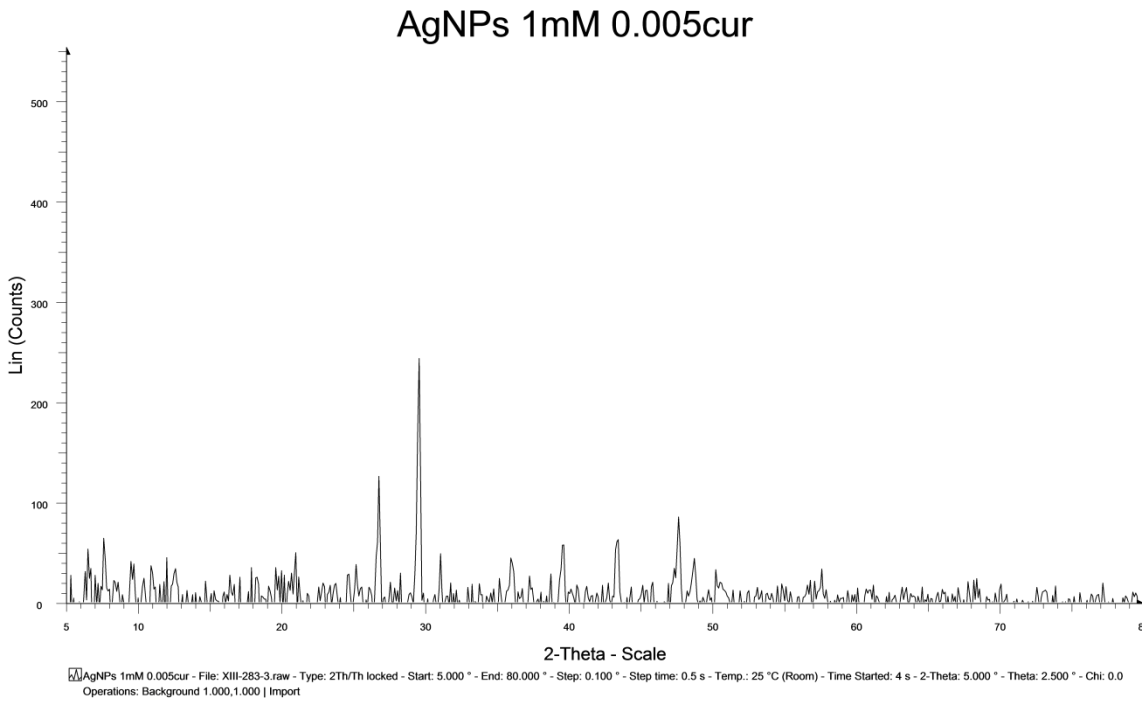
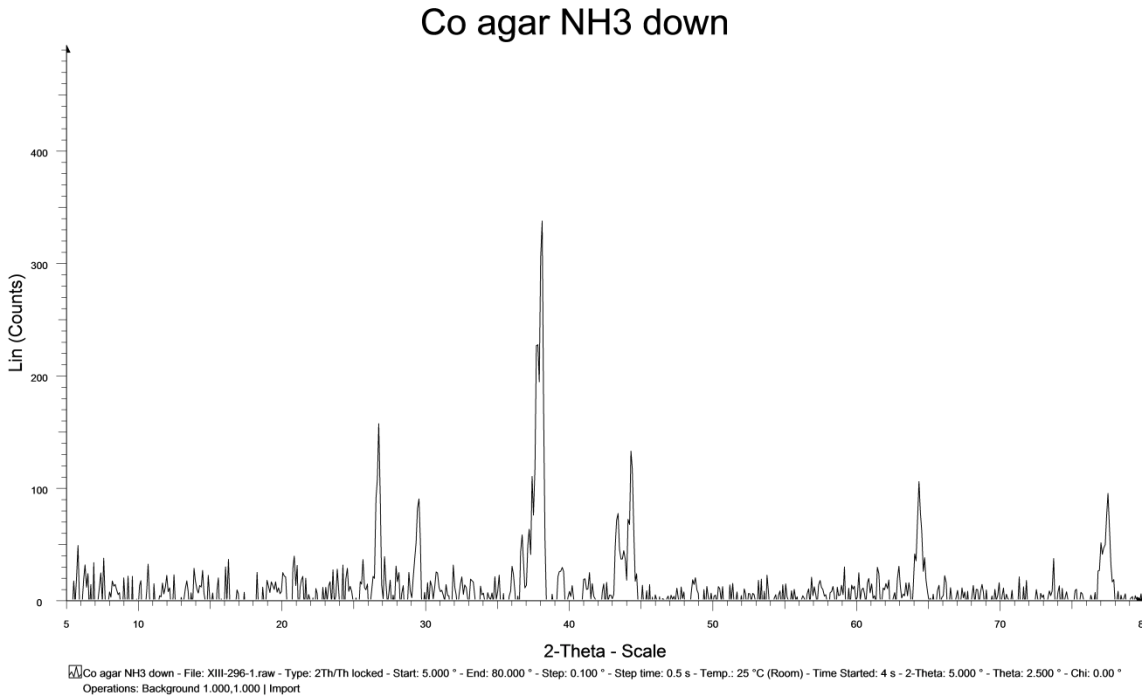
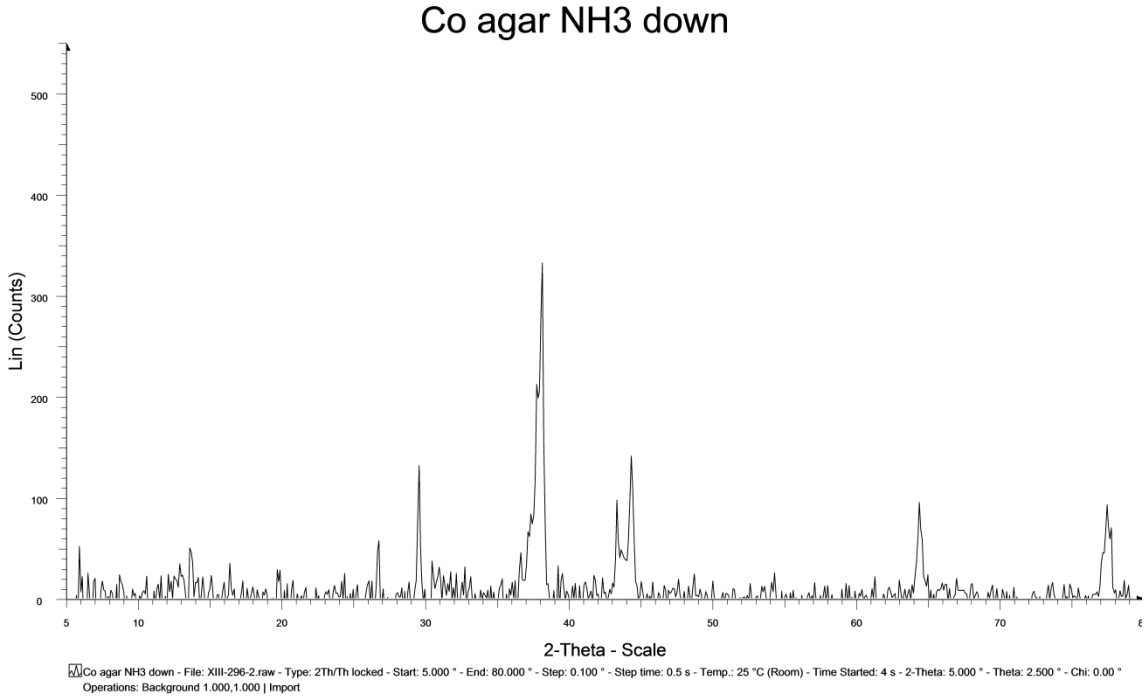


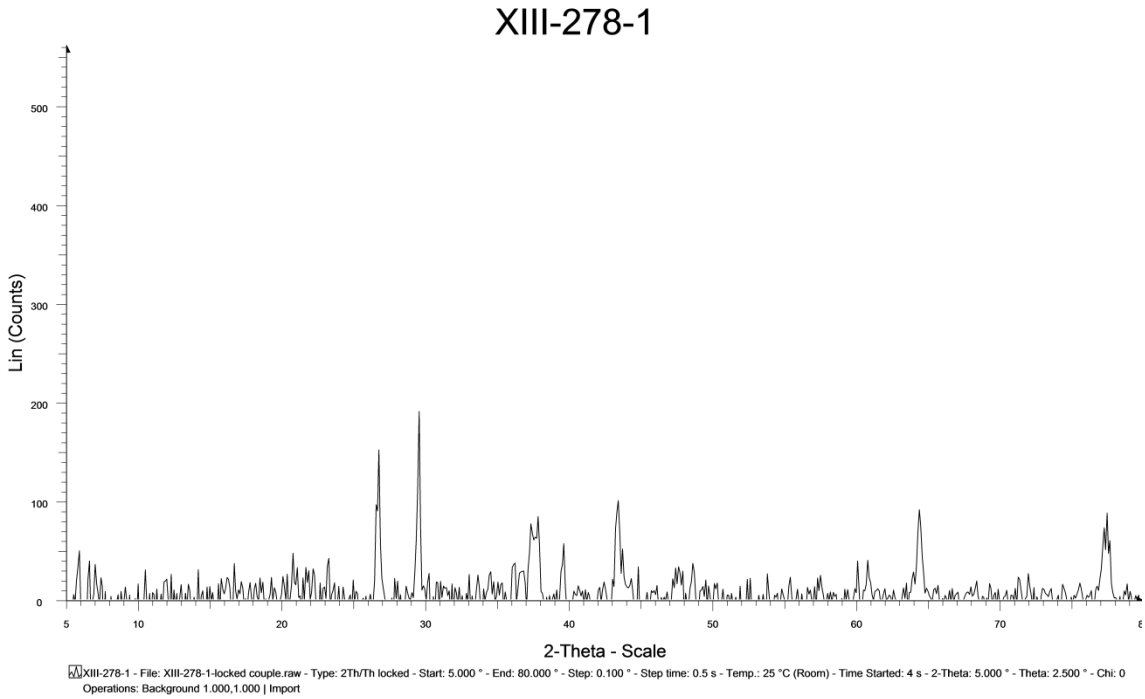

XIII-82-1



XIII-82-4




XIII-83-1



## II. List of some muddy rain microbial genera

| **Betaproteobacteria** | **Alphaproteobacteria** | **Gammaproteobacteria** | **Deltaproteobacteria** |
|---|---|---|---|
| *Acidovorax* | *Azospirillum* | *Acinetobacter* | *Bacteriovorax* |
| *Aquabacterium* | *Beijerinckia* | *Colwellia* | *Geothermobacter* |
| *Aquaspirillum* | *Caulobacter* | *Glaciecola* | *Myxococcus* |
| *Caldimonas* | *Ensifer* | *Oceanospirillaceae* | *Nannocystis* |
| *Cupriavidus* | *Loktanella* | *Pseudoalteromonas* | *Stigmatella* |
| *Duganella* | *Methylocystis* | | |
| *Flavobacterium* | *Oceanicola* | **Bacteroidetes** | **Firmicutes** |
| *Gallionella* | *Ochrobactrum* | *Cesiribacter* | *Bacillus* |
| *Herbaspirillum* | *Paracoccus* | *Chitinophaga* | *Carnobacterium* |
| *Herminiimonas* | *Pelagibaca* | *Flavobacterium* | *Clostridium* |
| *Hydrogenophaga* | *Phenylobacterium* | *Myroides* | *Planococcus* |
| *Janthinobacterium* | *Pseudoruegeria* | *Segetibacter* | *Trichococcus* |
| *Leptothrix* | *Roseovarius* | | |
| *Massilia* | *Rubellimicrobium* | **Actinobacteria** | **Cyanobacteria** |
| *Methylibium* | *Sinorhizobium* | *Actinomyces* | *Cyanothece* |
| *Mitsuaria* | *Skermanella* | *Arthrobacter* | *Halospirulina* |
| *Neisseria* | *Sphingomonas* | *Dietzia* | *Microcystis* |
| *Pelomonas* | *Thalassobius* | *Kocuria* | *Planktothricoides* |
| *Piscinibacter* | | *Nesterenkonia* | *Rubidibacter* |
| *Pseudacidovorax* | | | |
| *Pseudorhodoferax* | | **Basidiomycota** | **Ascomycota** |
| *Ralstonia* | | *Antrodia* | *Cucurbitaria* |
| *Rhodoferax* | | *Cryptococcus* | *Glyphium* |
| *Roseateles* | | *Filobasidium* | *Herpotrichia* |
| *Sideroxydans* | | *Malassezia* | *Knufia* |
| *Undibacterium* | | *Pseudozyma* | *Monodictys* |
| *Zoogloea* | | *Tilletiopsis* | *Phaeosphaeria* |

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