

2013•2014
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Biological risk associated to bio-treatments: monitoring and modeling bacterial dispersion into the atmosphere in a soil bioremediation plant and in a wastewater treatment plant

Promotor :
Prof. dr. Jaak VANGRONSVELD

Promotor :
dr. ANDREA FRANZETTI

Olga Tarasiuk

Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.



Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt
Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek



Maastricht University

2013•2014
FACULTEIT GENEESKUNDE EN
LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Biological risk associated to bio-treatments: monitoring and modeling bacterial dispersion into the atmosphere in a soil bioremediation plant and in a wastewater treatment plant

Promotor :
Prof. dr. Jaak VANGRONSVELD

Promotor :
dr. ANDREA FRANZETTI

Olga Tarasiuk

Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen



Biological risk associated to bio-treatments: monitoring and modeling bacterial dispersion into the atmosphere in a soil bioremediation plant and in a wastewater treatment plant

Senior practical training

Centre for Environmental Science
Hasselt University, Belgium

Department of Environment, Territory and Earth Sciences
University of Milan, Bicocca, Italy
Period: 7/01/2014 – 31/07/ 2014

Tarasiuk Olga

2mas BMS-EH

Int. promoter: Prof. Dr. Jaco Vangronsveld

Promoter: Dr. Andrea Franzetti

Contents

1. Abbreviations	2
2. Acknowledgements	3
3. Abstract	4
4. Introduction	5
4.1. Wastewater treatment	5
4.2. Soil bioremediation.....	6
4.3. Sediments from channels of harbors	7
4.4. Airborne bacteria	8
4.5. Bacteria of interest	9
4.6. Hypervariable region of 16S rRNA gene of bacteria	10
4.7. The current research	11
4.8. Innovative elements.....	12
5. Materials & methods	13
5.1. Collection of the samples.....	13
5.2. Preparation of 16S rRNA gene libraries for Illumina sequencing	14
5.2.1. Extraction of bacterial DNA from the samples.....	14
5.2.2. Amplification of bacterial DNA by PCR	14
5.2.3. Illumina sequencing libraries preparation	15
5.2.4. Gel electrophoresis and extraction of amplified PCR product from the gel	15
5.2.5. DNA quantification by Qubit® dsDNA BR Assay Kit	15
5.3. Cultivation of bacteria with a plasmid vector pCR21	16
5.4. Real Time PCR	17
6. Results	18
6.1. Massively parallel Illumina sequencing	18
6.2. Quantitative RT-PCR.....	19
6.3. Relative abundance of the bacterial species	21
6.4. Statistical analysis	24
7. Discussion	26
7.1. Illumina sequencing	26
7.2. Quantification of bacterial copy numbers in the samples.....	27
7.2.1. Air filter samples	27
7.2.2. Soil samples	28
7.3. Relative abundance of the bacterial species	29
7.3.1. Air filter samples	29
7.3.2. Soil samples	30
7.3.3. Relative abundance of <i>E.coli</i> , <i>Enterococcus</i> spp., <i>C. perfringens</i>	31
7.4. Conclusion	31
7.5. Future perspectives	32
8. Appendix	33
9. References	37

1. Abbreviations

PM - particulate matter

E.coli - *Escherichia coli*

Enterococcus spp. - *Enterococcus* species

Salmonella spp. - *Salmonella* species

C. perfringens - *Clostridium perfringens*

COPD - Chronic obstructive pulmonary disease

ANOSIM - Analysis of Similarity

qPCR - Real-Time quantitative Polymerase Chain Reaction

DGGE - Gradient gel electrophoresis

TGGE - Temperature gradient gel electrophoresis

rep-PCR - Repetitive element Polymerase Chain Reaction

Sistemi ambientali S.r.l - Sistemi ambientali Società a responsabilità limitata

BBS gel loading dye - Bromphenol blue and sucrose gel loading dye

H₂O - water

MgCl₂ - Magnesium chloride

500bp - 500 base pairs

DNA - deoxyribonucleic acid

rRNA - ribosomal ribonucleic acid

dNTP's - deoxyribonucleotide triphosphates

dGTP - deoxyguanosine triphosphate

dATP - deoxyadenosine triphosphate

dCTP - deoxycytidine triphosphate

dTTP - deoxythymidine triphosphate

2. Acknowledgements

At the end of the senior internship I would like to convey my heartiest thanks to Dr. Andrea Franzetti and the University of Milan Bicocca for hosting me in their institution. Thank you very much for your collaboration, hospitality and the wonderful experience I was able to have in Milan. It has been a great pleasure for me to work in this research group. I would like to express my gratitude to Prof. Dr. Jaco Vangronsveld and Dr. Panagiotis Gkorezis for giving me the possibility to work and write my thesis at the University of Milan Bicocca. I am expressing my deep appreciation and thankfulness to my colleagues of microbiological labs, who taught me and contributed my work and were always available to share their knowledge with me. These seven months passed quickly, but this time we have spent all together I will remember forever. I thank all my friends, family and close ones for their support and understanding.

3. Abstract

Wastewater is a mixture of domestic, municipal and industrial waste dissolved in water. The biggest fraction of wastewater is sanitary sewer water. Before its release in rivers or sea, water must be cleaned and all harmful bacteria must be killed. Biosolids are nutrient-rich organic waste obtained following wastewater treatment and used beneficially as fertilizer. Routinely, biosolids are deposited in agricultural areas or incinerated. For this reason the level of microbial pathogens in the biosolids must be limited. Nowadays, while human population increases, the question of safety managing and disposal of biosolids is becoming more and more important. Traditional bio-engineering based methods of cleaning polluted water and soil are not always safe for human population and may involve some level of risk. Airborne particulate matter (PM) is a complex and dynamic mixture of organic and inorganic substances of different chemical, physical and biological origins in the atmosphere. The biggest fraction of airborne particulate matter is represented by airborne microorganisms as bacterial cells, endotoxins, fungal spores and viruses. Bioremediation plants contain a big amount of different microorganisms that can become aerosolized in the atmosphere during different steps of treatment procedures. These microorganisms cause different infections in plant workers and affect the population living in surrounding areas. Some bacterial species like *E.coli*, *Clostridium perfringens*, and *Enterococcus spp.* are quite pathogenic and are of particular interest since their concentration in drinking water, soil or air is an ideal indicator to test environmental samples for fecal contamination.

The main objective of this project was to quantify and characterize dynamics and pathogenicity of bacteria in the air, wastewater and contaminated soil present at the municipal wastewater treatment and soil bioremediation plants during different stages of the bioremediation processes. Another objective of this project was to estimate biological risks associated with working or living in areas close to a bioremediation plant.

The biodiversity of bacteria present in the air, sludge and water was analyzed quantitatively and qualitatively. The structure of microbial communities was described by massively parallel sequencing using Illumina sequencing technology. In this technique hypervariable regions of the 16S rRNA gene was analyzed. Total quantification of bacteria and quantification of *E.coli*, *Clostridium perfringens*, *Enterococcus spp.* in the samples were performed by using Real-Time quantitative PCR (qPCR) technique. This method allows to follow the exponential increase of amplicons and to quantify the number of copies of specific microbial genes present in the samples.

The results of the current research show that the total number of bacteria in the air are up to 10^4 - 10^8 #/m³ (#- number of ribosomal operons). The highest bacterial concentrations were found during the step of drying and aeration. For the soil samples the total number of bacteria in the samples was high during the drying and sieving step (about 10^{12} #/m³). It has been concluded that activity on the soil bioremediation plant lead to a significant increase of bacteria in the air. Statistical analysis also showed that this significant increase was due to the drying step of the treatment process.

4. Introduction

4.1. Wastewater treatment

Wastewater is a mixture of domestic, municipal and industrial wastes dissolved in water. Water from homes and factories goes to sewage collection systems includes water from baths, showers, kitchen, washing machines, and toilets. The biggest fraction of wastewater is sanitary sewer water, which is always contaminated with feces and urine. Wastewater contains 99% of water, ions, suspended solids and harmful bacteria. Before its release in rivers and sea, all harmful particles and bacteria must be destroyed and water must be cleaned. The treatment of wastewater takes place at the water treatment plants and occurs in three phases: pretreatment, primary treatment and secondary treatment.

During the first step of pretreatment all materials, large and heavy solids that can be taken by sewage stream, are removed by bar screening and are stored in particular storage places. These objects are mostly small garbage, tree branches, leaves and stones. For the screening of sewage metal bars spaced at 19 mm places across the influent channels are used.

After the screening process, wastewater flows to a grit chamber. Here the speed of waste flow is slows down and allows denser sand and other grit to sediment. Organic solids will not settle and will remain dissolved. The sedimented material is buried or disposed in the fields. During the primary treatment water remains still in large settling tanks in order to allow solids to sink on the bottom and fat particles to rise to the surface. Primary treatment consists in two steps. In the first step the wastewater is aerated by air pumped through perforated pipes at the bottom of the tanks. It is necessary to make the water less dense, causing the grit to settle out. The air also supplies oxygen for the aerobic bacteria. The grit is collected further and deposited. During the second step of the primary treatment water, flows slowly which allows suspended solids to sink to the bottom while fat particles and scum rise to the surface. The settle-able solids can be removed in the form of sludge and processed further on the sludge bioremediation plants, while oils float to the top and are skimmed off. The remaining liquid can be processed further into the secondary treatment (1). Figure 1 represents a schema of primary and secondary treatment processes of wastewater.

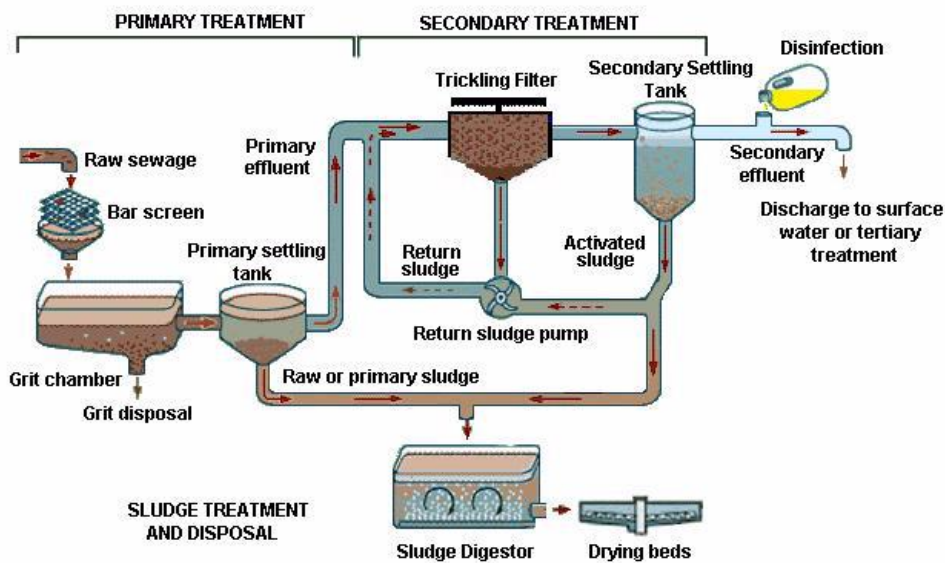


Fig.1. Wastewater treatment based on a trickling filter system (2).

During the secondary treatment, remaining sludge goes to "sludge digesters". The sludge in the "sludge digesters" is cleaned from chemicals by using microorganisms and as result it can be safely released into the environment. In "sludge digesters" the basic temperature is around 30–35°C, which is optimal for growth and activity of the anaerobic bacteria (environmental bacteria that do not need oxygen). The process of bioremediation takes usually 20–30 days. During this process the organic compounds are converted to carboxylic acids and further to methane and carbon dioxide. This gas can be collected and used as a fuel, also called "biogas". In the last step of treatment sludge has to be concentrated from 4 to 30% solid and landfilled. The important step is the thickening of sludge, which increases dry matter content. In the next steps, sludge is mechanically mixed and dried. After the second thickening the sludge is dewatered to a dry matter of approximately 25%.

The water, obtained from the primary treatment floats to the trickling filter for a further secondary treatment. The main system of treating sewage is using aerobic biological processes. One of the main systems is the fixed-film or attached growth systems. Here, the bacterial biomass grows on media in the bio-towers and rotating biological contactors spread the sewage by passing over its surface. The process allows the microorganisms consume nutrients and organic matter from the sewage. Oxygen is depleted within the biomass on top of the water and it makes inner layers of water in trickling filter anoxic or anaerobic.

After removing a major part of the suspended material from the wastewater, the liquid portion flows over to the secondary settling tank. The rest of the sludge precipitates in an activated sludge. The secondary settling tank is a sort of multi-chamber reactor unit, where aerobic microorganisms degrade organic materials in wastewater and produce a high-quality effluent. Oxygen is supplied constantly to maintain aerobic conditions for bacteria in the water. According to standardized protocols effluent water has to be chlorinated before being emitted into the environment (2).

4.2. Soil bioremediation

Nowadays soil pollution is a problem that appears regularly in the media. Contaminated soil often results from uncontrolled industrial activities of previous generations that resulted in a lot of environmental effects and disposition of chemical and other substances in the soil. During the last decades contaminated land has been admitted to be a potential threat to human health. It has become necessary to find remediation processes to reduce the risks of adverse health and environmental impacts caused by pollution.

Bioremediation is one of the possible techniques for soil purification that enables the elimination of hazardous soil contaminants using natural biological activity. Organic molecules are biologically degraded by microorganisms to transform the environmental contaminants into less toxic ones. This method is relatively cheap, does not require complicated technology and is generally accepted by people (3).

The presence of microorganisms in soil is essential for the bioremediation process. The degradation of soil pollutants depends on several environmental conditions as well as physical, chemical and biological interactions in the soil (4). The biodegradation processes of biological degradation and transformation of contaminants in soil and water where various mitigation processes such as natural attenuation, biostimulation, and bioaugmentation occur. Natural attenuation occurs without human intervention due to natural degradation by the naturally occurring soil microorganisms. Adding nutrients and other substances to the soil stimulates natural attenuation processes. Bioaugmentation is a process whereby exogenous microorganisms (sometimes genetically modified) outside the soil environment are added to detoxify a particular contaminant (5).

Heterotrophic bacteria use enzymes to break down the organic molecules through oxidation-reduction reactions and metabolize it to energy in order for microbes to live. During this natural process bacteria, fungi and plants consume contaminants for their normal life functions (6). Released metabolites are then less toxic. Generally, in case the more chemically similar the

structure of a contaminant is to a naturally occurring compound, the easier it will be degraded in the environment. However, microorganisms have some limits of tolerance for particular environmental conditions. Some factors like nutrient availability, moisture content, pH and temperature of the soil matrix have an important influence on the rate of microbial biodegradation. Inorganic nutrients like nitrogen and phosphorus are necessary for microbial activity and cell growth. Soil water content is also an important factor for microorganism's cell growth and function as it is important for diffusion of water and soluble nutrients in and out of microorganism cells. The oxygen concentration defines soil saturation and influences aerobic respiration of bacteria. Anaerobic respiration delivers less energy for the microorganisms and reduces biodegradation. Microbial activity is highly dependent on soil pH. Some microbial species can only survive within a certain pH range. Moreover, nutrient availability is also strongly related to soil pH. Temperature is another important factor that influences the rate of biodegradation. Enzymatic reactions are performed twice faster with each 10 °C rise in temperature. The knowledge about soil types, as it defines bioavailability of some contaminants, is essential for the bioremediation process (7).

The soil bioremediation with the biopiles method contains few steps in the process. Figure 2 presents the main steps of soil bioremediation at the soil treatment plant. First of all if the soil contains too much water, as for example sediment from water bodies, it has to be dried. In the next step soil is screened and large objects such as stones, tree branches and waste have to be removed from it. In some cases soil has to be pretreated by "soil washing". In the following treatment step oxygen is supplied by pumping air into the soil. Oxygen is important for aerobic bacterial processes. Soil is mixed with bacteria and additional nutrients and deposited in the biopiles for further natural remediation.

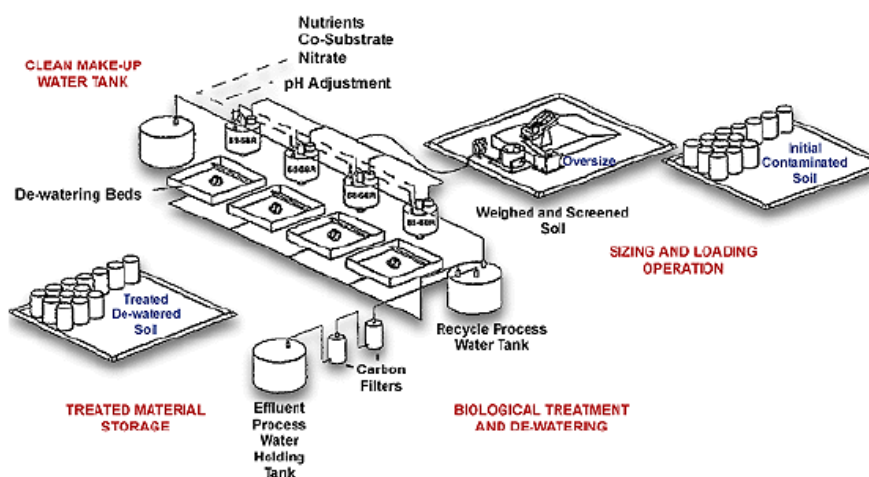


Fig.2. Soil bioremediation process (aec.army.mil)

Bioremediation processes are relatively new and always fully standardized method. In case the process is not occurring properly, dangerous bacteria remain in the treated soil and end up into the soil, water, air and people can easily get exposed to these airborne bacteria. It mainly concerns environmental contamination by fecal bacteria.

4.3. Sediments from channels of harbors

It is known that at many places close to the plants or industrial areas untreated sewage water and other industrial waste was and often still dumped in the harbor channels. As a result water and sediments in these channels are contaminated by high concentrations of biological and chemical contaminants. These sediments have shown high concentration of nutrients such as phosphor and nitrogen, but also other organic compounds indicating the high level of urban impact to the pollution. There exist two sources of sediment pollution in the harbor channels. One of the sources

is poorly treated effluent from the city sewage, combined sewer overflow, households, city water runoff, groundwater, the atmosphere, and rivers.

Emission of untreated sewage in the harbor channels leads to high concentrations of fecal bacteria in water and sediment. It has been shown that around 30% of the sewage dumped into the channel is deposited in the channel sediment. Sewage particles can be brought by water to the different distances in the channel. The speed of sediment aggregation is approximately 0.7 to 6.3 cm a year and is considered to be high (8).

Another type of contaminants in the harbor channels are those that are originating from shipping activities and industrial effluents. Toxic chemicals are mostly heavy metals, including cadmium, copper, lead, nickel, silver and organic priority pollutant compounds, including several kinds of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs).

Some studies demonstrated that the level of hydrocarbons pollution in the harbor channels is very high. The main reason for accumulation of high amount of hydrocarbons in the sediments is from oil fields, oil refineries and shipment activities. High concentration of metals in the sediments results from industrial and agricultural effluents from the surrounding factories. It is suggested that PAHs compounds in the harbor channel water originate from urban run-off made during incomplete combustion of organic matter such as fossil fuels, coal, waste incineration, industrial production of black carbon, asphalt and petroleum cracking.

Petroleum hydrocarbons are composed of a mixture of organic molecules. They originate from the fusion of benzene rings during incomplete agitation step of coal, oil, petrol and wood. Hydrocarbons getting dumped into the water and during low water flow are able to sink and accumulate at the bottom of harbor channels. Organic priority pollutant compounds and metals contained in the sediments are the result of decades of waste effluxion.

PAHs have the ability to bind to soot particles in the upper water layers, which makes the particles less soluble. When they reach the sediment-water interface, these PAH compounds have a smaller flux and are deposited on the bottom. It is believed that PAH are less absorbed by organisms and that explains their high concentration in sediment. It is known that PAH compounds possess carcinogenic behavior.

A high concentration of metals and organic molecules in the sediments decreases oxygen in the sediments. Due to the low level of oxygen at the bottom, the amount of living organisms decreases, including those that mix the sediments and release the contaminants.

High contamination of sediments is lethal for living organisms and plants. Some toxic contaminants absorbed by small organisms and flows through food chain, affecting the end higher consumers such as humans. Contaminated water can end up in the drinking water chain and cause serious epidemics (9).

4.4. Airborne bacteria

Airborne particulate matter (PM) is a complex and dynamic mixture of organic and inorganic substances of chemical, physical and biological origins present in the atmosphere. Its contents are made up of dust, dirt, soot, smoke and liquid droplets emitted into the air. Physical characteristics, such as size and molecular composition are important parameters which influence their transport, deposition, chemical interaction. They affect human health in particular.

PM can be in the range of PM₁₀ (aerodynamic diameter less than 10 μm), which are generally defined as coarse particles. PM_{2.5} ($\text{Ø} < 2.5 \mu\text{m}$) and PM₁ ($\text{Ø} < 1 \mu\text{m}$) are called fine particles. The size is an important parameter in triggering biological effects. PM structure and composition are complex and heterogeneous. They consist of organic and inorganic elements as well as a carbonaceous core. PM₁₀ contains a major part of mineral compounds and some adsorbed endotoxins (10).

The largest fraction of airborne particulate matter is represented by airborne microorganisms. Airborne biological particles are viruses, fungal spores, pollens, bacterial cells and their by-products such as endotoxins. Airborne biological particles have been more studied in recent years and have been identified as a key player to explain the negative effects of aerosols on biological systems (11).

Bioremediation plants contain a large amount of microorganisms. It has been shown that during the steps of aeration and mechanical mixing an important part of these microorganisms is aerosolized in the atmosphere. Depending on physical and meteorological conditions these aerosolized biological particles can be spread by the air for a big distances around treatment plants (12), (13). Airborne microorganisms generated during the bioremediation treatment may cause different infections in plant workers and affect the population living in surrounding areas (14). The plant workers can develop pulmonary diseases, gastrointestinal and skin problems, irritation of the eyes, allergies and headache. Important health hazards are microbial endotoxins. Endotoxins can be found in the cell wall of Gram-negative bacteria and are a cause of their pathogenicity. Recent research shows that, endotoxins activate several immune cells such as macrophages and epithelial cell lines and are partly responsible for pro-inflammatory cytokine response of immune cells. Even a small amount of endotoxins can lead to an increasing response of the immune system to PM (12). Exposure to airborne bacteria lead to overexpression of interleukins, production of pro-inflammatory mediators by the lung epithelium and is believed to play an important role in further development of allergic (asthma, allergic rhinitis) and nonallergic (chronic bronchitis and chronic pulmonary disease, COPD) conditions. Additionally, the expression of mediators entering the systemic circulation has an influence on the atherothrombotic disease development. Reaction of human body to bacterial exposure is very dependent on individual sensitivity of a person and the level of exposure.

Compared to chemical hazards, biological agents have the ability to multiply in the plant environment if the living conditions are suitable. Because infection risks for plant workers can be relatively high, the workplace regulations must be standardized and well followed (15). Although it has been known that endotoxins are responsible for inflammatory health symptoms, the endotoxin concentration at bioremediation plants is not well studied.

Several studies have demonstrated that the bacterial community composition can vary significantly according to the season, environmental and meteorological conditions. The highest bacterial concentration in the air is observed during autumn and spring. Bacterial cells represent 22% on average of the total near-surface aerosol particles $>0.5 \mu\text{m}$. Bacterial communities during spring, winter and the second half of the autumn periods are represented by bacterial taxa generally present in cold environments. A few studies suggest that bacteria are important component of the atmosphere, the abundance and composition of which adapts to seasonal shifts in atmospheric and local terrestrial environment conditions (16).

4.5. Bacteria of interest

Some bacterial species such as *Escherichia coli*, *Clostridium perfringens*, and *Enterococcus* spp. are pathogenic and therefore are of particular interest for this study. These bacteria are present in intestinal tracts and feces of humans and domestic animals. Their concentrations in drinking water, soil and air is an ideal indicator to test environmental samples for fecal contamination and is a perfect indicator for pollution.

E. coli is a Gram-negative, rod-shaped bacteria. It is a facultative anaerob. *E.coli* constitutes about 0.1% of the intestine flora of different organisms. The major route for its transmission is fecal-oral, which can cause serious food poisoning. *E.coli* bacteria in the environment can be responsible for waterborne gastrointestinal diseases (17).

Enterococcus spp. is a genus of lactic acid bacteria of the phylum *Firmicutes*. *Enterococci* belong to a part of the normal human intestine flora. The most frequent diseases caused by *Enterococcus*

spp. are gastrointestinal, urinary tract and blood stream infections. *Enterococci* are unable to form spores, and due to this they are very tolerant to extreme environmental conditions such as high temperature or pH. The limit concentration of *Enterococcus* spp. in water is approximately 35 colony-forming units per 100 ml of water (18).

Clostridium perfringens is a rod-shaped, anaerobic and spore-forming bacteria. This is an important type of bacteria that can be found in the intestinal tract of humans. *Clostridium perfringens* is differentiated in A, B, C, D and E types that produce many different toxins possibly involved in pathogenesis. Type A is a dominant strain and causes foodborne/non-foodborne illnesses and gas gangrene. *Clostridium perfringens* can also be found in some environmental samples such as soil and wastewater samples and therefore serves as a perfect indicator for environmental pollution (17).

It has been shown that some pathogenic fecal bacteria are very persistent and can survive for long time in different environmental conditions. Consequently, the quality of composted biosolids and treated wastewater needs to be carefully monitored in order to detect the presence of bacteria pathogens. Although it is known that some bacteria can survive wastewater treatment, not much information is available concerning the microflora of composted biosolids and treated wastewater. Therefore, monitoring bacterial pathogens present in proceeded biosolids and water, as well as estimating the release of human pathogenic bacteria into the environment are crucial process (19).

4.6. Hypervariable region of 16S rRNA gene of bacteria

The 16S rRNA gene has been chosen for analysis because it is the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy. Almost all bacteria possess this gene, which often exist as a multigene family or operons. 16S rRNA is a large gene of approximately 1,500 base pairs and serves for informatics purposes. The function of 16S rRNA gene has not changed much over time. The sequence of 16S rRNA gene helps to identify bacterial genus and species, its taxonomic relation and pathogenic characteristics even for bacteria that have difficult recognizable biochemical profiles (20), (21).

16S rRNA genes contain nine "hypervariable regions" (V1–V9) with a high diversity among bacterial species. Analyzing these "hypervariable regions" has become a standard for bacterial species identification. Molecular diagnostic methods, such as real-time PCR amplification use fluorescent probes that hybridize to relatively short amplicons. Universal primers for 16S rRNA hypervariable region sequences allow quantifying a single bacterial population in a pool of microorganisms (22), (23).

The 16S rRNA sequencing technique is a useful tool to identify isolated bacteria with unusual profiles. The benefit of using the sequence of 16S rRNA allows identify bacterial population that do not fit in any recognized biochemical profiles. Just like every other methods using 16S rRNA for species identification has its own difficulties. Gene and species identification requires understanding of novel taxa. Some of the possible complications are too few sequences deposited in nucleotide databases, species sharing similar 16S rRNA sequences and resolution problems of 16S rRNA gene sequencing. Moreover it has to be admitted that 16S rRNA gene sequencing has poor discriminatory power for some species, which has to be corrected by DNA relatedness studies to give absolute resolution to these taxonomic problems.

Although species identification happens to be imprecise, 16S rRNA sequence analysis is generally considered to be highly accurate, other identification techniques exist.

16S rRNA gene sequencing used for species identification has become a quite popular technique in clinical laboratories, and will develop to become a standard procedure. Moreover, applying of the knowledge from the 16S rRNA gene in microarray based technologies will lead to very strong and sensitive approaches for molecular species identification in the future (20).

4.7. The current research

The main objective of this project was to quantify and characterize the dynamics of bacteria in the air, wastewater and contaminated soil present at a municipal wastewater treatment plant and soil bioremediation plant and monitoring and modeling bacterial dispersion into the atmosphere during different stages of the bioremediation processes. Another aim of this project is to estimate the biological risks associated with working or living in areas close to a bioremediation plant.

In the current project the biodiversity of bacteria present in the air, soil and water was analyzed. Samples were described quantitatively and qualitatively in order to provide exhaustive information about the environmental matrices or material treated in the plants that potentially act as a source of bacterial release. The hypothesis was that during different steps of treatment bacteria have the ability to move from the soil or water that is treated into the air and create airborne bacterial communities. Sampling has been performed at different distances from the sources in both down- and up-wind position and at different times. Results from the samples were compared to the results of the control sample. An external upwind sampling point was located as background control.

The structure of microbial communities associated with air particulate matter and other matrices was described through the massively parallel sequencing using Illumina sequencing technology. Nowadays it is the most informative methods for the description of microbial community diversity. In this technique a hypervariable region of 16S rRNA gene is analyzed. This gene is present in almost all known bacteria and is the most common housekeeping genetic marker to study bacterial phylogeny and taxonomy. Template DNA is denatured, specific oligonucleotide primers bound and amplification cycles occur, which extend a complementary strand. This technique uses thermostable DNA polymerase. Illumina sequencing builds fragment libraries with thousands of sequences describing the structure of the community. Illumina sequencing is providing relative abundance of bacterial populations in the samples. In order to perform Illumina sequencing, libraries of hypervariable regions 16S rRNA gene of the samples, sequencing have been prepared. A specific sequence, consisting of an adaptor sequence, particular barcode sequence and forward or reverse primers for 16S rRNA hypervariable region have been attached to the bacterial hypervariable region of the 16S rRNA gene in each sample. The Illumina sequencing method delivers as a result a relative abundance of bacterial orders in the samples.

The total quantification of bacteria in the samples was performed using the Real-Time quantitative PCR (qPCR) technique. QPCR is the method used to determine the presence and abundance of a particular DNA sequence. This method allows following the exponential increase of amplicons and quantifying the number of copies of specific microbial genes present in the samples detected as the reaction progresses in "real time". Here the 16S rRNA gene has been targeted to induce the amplification. The amplification process can be monitored by fluorescent notification.

4.8. Innovative elements

For many years classical cultural microbiological methods have been used to study bacteria in soils, composts, air and water. Nowadays more rapid and sensitive culture-independent molecular technologies have replaced standard cultural methods. Other molecular approaches are now applied for rapid bacterial characterization, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), conventional PCR, amplified fragment length polymorphism, 16S–23S interspace fingerprinting, multilocus sequence typing, ribotyping and repetitive element PCR (rep-PCR) which allow classifying bacteria in the samples at the genetic level. Among others quantitative polymerase chain reaction (qPCR) is the most preferable method for quantification of target organisms (19). Real-time PCR can determine the number of target gene copies in extracted DNA and is an efficient method to determine bacteria in environmental

samples. Real-time PCR quantification is a cost-effective molecular approach, more precise and reproducible than the traditional culture-based methods (17).

The effect of particulate matter on human health as the result of air pollution is well known. Despite the presence of bacteria in the air, scientists pay only little attention to this fact. However, with the development of new powerful technologies, the analysis and quantification of bacteria has become possible. The innovative element of the on-going project concentrates on the biological fraction of particulate matter rather than on the general effect of it. Also, compared to previous studies on airborne bacteria in the on-going research used more informative molecular techniques, such as the massive parallel sequencing of DNA and real time quantitative PCR, which provided more precise information about bacterial communities in the samples.

The results of the project have national academic character and will be applicable in further research and monitoring of a sustainable environment. They are also important for academic research institutions, environmental and health organizations of Italy as for example The Italian National Health Service, The Institute for Environmental Protection and Research ISPRA or the National Institute of Urban. Moreover, the current research has direct importance for society, as it estimates the risk of pathogens present in the air for human health.

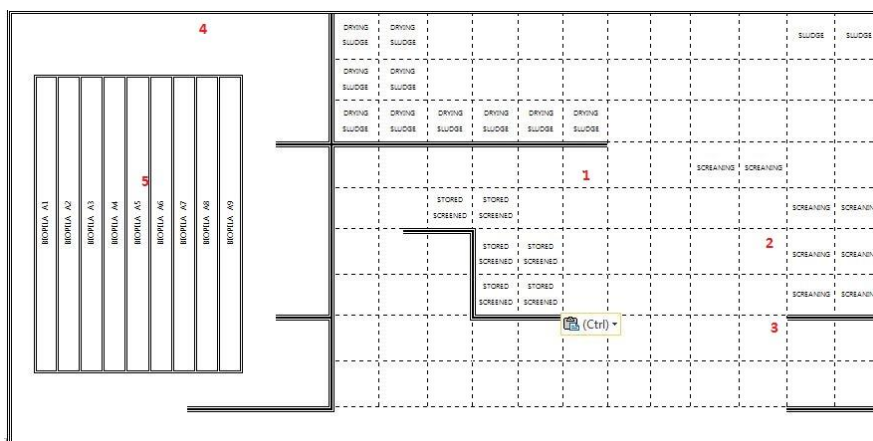


Fig.4. Map of soil bioremediation plant Calcinate with identification of sampling places. 1 drying of soil. 2 large screening, 3 fine screening, 4 near biopiles, 5 in between biopiles.

5.2. Preparing of 16S rRNA gene libraries for Illumina sequencing

5.2.1. Extraction of bacterial DNA from the samples

The first step of DNA fragment libraries preparation for Illumina sequencing technique is the extraction of bacterial DNA from the air filters, water and soil samples. Preparation of air filters for extraction requires to work under a laminar air flow hood. A fourth part of the air filter has been cut in small pieces and homogenized in the FastPrep machine. For extraction of bacteria from the water samples first water has been filtered through the filter. Bacteria stayed on the filter and afterwards it could be extracted. Soil samples did not required any special pretreatment. Further DNA has been extracted following protocol of FastDNA® Spin Kit (#38970, MP Biomedicals, IIRich, France), attached in Appendix I.

After the extraction of bacterial DNA from the samples, the presence of genomic DNA product was checked by gel electrophoresis. Control of extraction was performed only in soil and water samples. Gel electrophoresis of the bacterial DNA from air filters samples did not give visible results, as the amount of DNA was too small, therefore an amplification to increase the quantity of DNA product was performed. To identify the presence of genomic DNA 1% agarose (#9201207, Euroclone, Milan, Italy) gel were prepared. For performing gel electrophoresis 1µl of sample with 1µl of BBS gel loading dye (#37093, MP Biomedicals, IIRich, France) and 5µl of distilled water was loaded. The used ladder was EMRS814100 (#R8141302, Euroclone, Milan, Italy).

5.2.2. Amplification of bacterial DNA by PCR

Each sample was checked for the best dilution most suitable to work with for preparing the libraries for Illumina sequencing. PCR product of 500bp of 16S rRNA gene has been amplified with different dilution of the samples, namely it was checked with dilutions of 1:1 and 1:10. For soil samples it was checked with dilution of 1:100, as soil samples contained high concentration of bacteria. To perform a PCR amplification for the air filter samples the @Taq Hot start Taq polymerase mix (#0131305, Euroclone, Milan, Italy) was used. For each air filter sample the mix of 2,5 µl of 10X reaction buffer, 2µl of 50mM MgCl₂, 0,5µl of 10mM dNTP's (dGTP 100mM #26714101 U121A; dATP 100mM #25100110 U120A; dCTP 100mM #24987705 U122A; dTTP 100mM #26203604 U123A, Promega, Madison, USA), 2,5µl of 10µM forward primer 27F (#HA06120986, Sigma Aldrich, St. Louis, USA), 2,5µl of 10µM reverse primer 519R (5'_GWATTACCGCGGCKGCTG_3'; #HA03194508, Sigma Aldrich, St. Louis, USA), 0,5 µl of Taq, 9,5µl of H₂O and 5µl of DNA sample was prepared. To perform PCR amplification for the sludge or water samples GoTaq® Green Master Mix (#0000097989, Promega corporation, Madison, USA) was used. For each soil samples a mix of

10µl 2X GoTaq® Green Master Mix, 2,5µl of 10µM forward primer 27F (5'_AGAGTTTGCCTGGCTCAG_3'; #HA06120986, Sigma Aldrich, St. Louis, USA) , 2,5µl of 10µM reverse primer 519R (#HA03194508, Sigma Aldrich, St. Louis, USA), 4µl of H₂O and 1 µl of DNA sample was prepared. The PCR protocol used for amplification contained steps was 4s at 95°C followed by 29 cycles of 30s at 95°C, 45s at 55°C, 45s at 72°C, after closing the loop 5s at 72°C and store at 8°C. Presence of amplified DNA in the samples was checked by gel electrophoresis. 1% agarose (#9201207, Euroclone, Milan, Italy) gel was prepared. 1µl of sample was loaded with 1µl of dye (#37093, MP Biomedicals, IILrich, France) and 5µl of water. The gel was analyzed and the bands with high intensity represented concentration suitable for the procedure.

5.2.3. Illumina sequencing libraries preparation

The preparation of libraries for Illumina sequencing was performed in one step. A PCR product of a 500bp hypervariable region of 16S rRNA gene for each sample was prepared. By performing the PCR amplification technique a prepared sequence of primer, barcode and adaptor were attached to 16S rRNA gene in each sample. To perform PCR amplification for the air filter samples @Taq Hot start Taq polymerase mix (#0131305, Euroclone, Milan, Italy) was used. For each air filter sample the mix of 7,5 µl of 10X reaction buffer, 6µl of 50mM MgCl₂, 1,5µl of 10mM dNTP's (dGTP 100mM #26714101 U121A; dATP 100mM #25100110 U120A; dCTP 100mM #24987705 U122A; dTTP 100mM #26203604 U123A, Promega, Madison, USA), 7,5µl of 10µM forward primer 783F with attached barcode and adaptor sequence (5_TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCAGCCAGGATTAGATACCC_3;5_TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGAGCAGGATTAGATACCC_3;5_TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGATGCCAGGATTAGATACCC; #Eurofins Genomics, Germany), 7,5µl of 10µM reverse primer 1027R with attached barcode and adaptor (5_GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCATGCGACRRCCATGCANCACT_3; 5_GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGAGCCGACRRCCATGCANCACT_3; 5_GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGCAGCGACRRCCATGCANCACT_3; #Eurofins Genomics, Germany), 1,5 µl of Taq, 28,5µl H₂O and 15µl of DNA sample was prepared. To perform PCR amplification on the sludge and water samples GoTaq® Green Master Mix (#0000097989, Promega corporation, Madison , USA) was used. For each soil sample a mix of 37,5µl 2X GoTaq® Green Master Mix, 9µl of 10µM forward primer 783F with attached barcode and adaptor sequence (#Eurofins Genomics, Germany), 9µl of 10µM reverse primer 1027R with attached barcode and adaptor sequence (#Eurofins Genomics, Germany), 15µl of H₂O and 4,5 µl of DNA sample diluted 1:100 for soil sample and 1:10 for water sample was prepared. The PCR protocol used for amplification contained steps was 5s at 94°C followed by 29 cycles of 50s at 94°C, 30s at 47°C, 30s at 72°C, after closing the loop 5s at 72°C and store at 8°C.

5.2.4. Gel electrophoresis and extraction of amplified PCR product from the gel

After preparing amplified PCR products for Illumina sequencing with attached sequences of forward or reverse primers, barcode and adaptor, the PCR product was run by gel electrophoresis. For electrophoresis 1% agarose (#9201207, Euroclone, Milan, Italy) gel was prepared. Afterward the amplified DNA fragments were cut from the gel and extracted using Wizard® SV Gel and PCR Clean-Up System (#0000098042, Promega Corporation, Madison, USA) protocol attached in Appendix II. Extracted from the gel 16S rRNA fragment with attached forward or reverse primers on the both sides with barcodes and adaptors were completed for analysis by the Illumina sequencing technique.

5.2.5. Quantification of amount of DNA by Qubit® dsDNA BR Assay Kit

Once the libraries for Illumina sequencing were prepared, the amount of genomic product was quantified by Qubit® dsDNA BR Assay Kit (#1361019, Life technologies, Invitrogen, Oregon, USA) following the protocol attached in Appendix VII. The quantity of prepared libraries of 16S rRNA gene fragment in each sample had to be higher than 6µg/ml.

5.3. Cultivation of bacteria with a plasmid vector pCR21

In order to prepare the standard dilution curve for performing real time PCR standard samples with a known concentration of analyzed bacterial gene fragments were prepared. The preparations of standard samples, to obtain a standard dilutions curve for bacterial quantification in samples, was done in a few steps. First bacterial DNA was extracted from bacteria using ZR Soil Microbe DNA MiniPrep™ (#ZRC172167, Epigenetic, USA) following protocol attached in Appendix III. Afterwards DNA extraction was checked by performing PCR amplification of 16S rRNA for 1:1 and 1:10 diluted concentrations of extraction. In order to perform this amplification 16S rRNA gene was targeted by 27F and 519R primers. The following gel electrophoreses indicate whether or not the extracted fragment is bacterial DNA and also with which dilution of DNA is it most suitable to work. In the second step a fragment of the gene that had to be incorporated into the vector was amplified by PCR technique, ligated with a plasmid vector pCR21 and transformed into *E.coli* cells, following the protocol of TA Cloning® Kit with pCR®2.1 (#903734, Life technologies, Invitrogen, NY, USA) attached in the Appendix IV. For the quantification of the amount of *Enterococcus* spp. in the samples, a bacterial plasmid with inserted fragment of 23S rDNA gene targeted by forward primer ECST748F (5'_AGAAATCCAAACGAACCTTG_3'; #HA05979982, Sigma Aldrich, St. Louis, USA) and reverse primer ENC854R (5'_CAGTGCTCTACCTCCATCATT_3', #HA05979983, Sigma Aldrich, St. Louis, USA) was prepared (24). For quantification of *E.coli* bacteria in the samples a bacterial plasmid with inserted fragment of 16S rRNA gene targeted by forward primer 395F (5'_CATGCCGCGTGTATGAAGAA_3';#HA05979980, Sigma Aldrich, St. Louis, USA) and reverse primer 490R (5'_CGGGTAACGTCAATGAGCAA_3'; #HA05879981, Sigma Aldrich, St. Louis, USA) was prepared (25), (24).

Afterwards, the plasmid vector was inserted in *E.coli*. The bacteria containing the plasmid vector was then cultivated. Bacteria with an inserted plasmid were selected based on their resistance to ampicillin and by an X-gal blue-white screening technique. Afterwards, the incorporated vector was extracted following the protocol attached in the Appendix V and the PCR product of the incorporated vector with the gene fragment was prepared from cultivated bacteria. The PCR protocol used for amplification contained the following steps: 10s at 95°C followed by 29 cycles of 30s at 95°C, 45s at 55°C, 45s at 72°C, after closing the loop 5s at 72°C and stored at 8°C. In order to prepare the PCR product of the vector forward primer M13F (5'_GTAAAACGACGGCCAG_3'; #9630400, Sigma Aldrich, St. Louis, USA) and reverse primer M13R (5'_CAGGAAACAGCTATGAC_3'; #9630401, Sigma Aldrich, St. Louis, USA) were used. The presence of the vectors and the ligated products was checked by gel electrophoresis. Vector was extracted from the gel by using Wizard® SV Gel and PCR Clean-Up System (#0000098042, Promega Corporation, Madison, USA) protocol attached in Appendix II. The vector with an inserted fragment was sequenced by the microbiology group of the University of Florence.

Bacteria with inserted vectors were cultivated at 37°C overnight. The purification of plasmids from bacteria was performed by using PureYield™ Plasmid Miniprep System (#0000014698, Promega Corporation, Madison, USA) following protocol attached in Appendix VI. Quantification of prepared circular plasmids were performed by using Qubit® dsDNA BR Assay Kit (#1361019, Life technologies, Invitrogen, Oregon, USA). The values of this quantification were converted into the values of DNA copy numbers by using the online software endmemo.com.

5.4. Real Time PCR

The Real Time Polymerase chain reaction technique was applied to quantify the total amount of bacteria in the air, water and soil samples. In order to perform Real Time Polymerase chain reaction an Illumina Eco Real-Time Instrument (#EC-900-10 01, Illumina proprietary, California, USA) was used.

In order to analyze the concentration of bacteria in the samples, dilutions with known concentrations of analyzed gene was prepared. Namely the standard curve dilution includes concentrations 1/10.000, 1/100.000, 1/1.000.000, 1/10.000.000, 1/100.000.000, 1/1.000.000.000 for air filter samples and 1/10, 1/100, 1/1.000, 1/10.000, 1/100.000, 1/1.000.000 for soil and water samples. For each sample a mix of 5 μ l 2X Fluorocycle, 0,3 μ l of forward primer, 0,3 μ l of reverse primer, 3,9 μ l of water and 0,5 μ l sample DNA were prepared. For total quantification of bacteria in the sample 16S rRNA gene was targeted by 331F (5'_TCCTACGGGAGGCAGCAGT_3'; #HA03194510, Sigma Aldrich, St. Louis, USA) and 797R (5'_GGACTACCAGGGTATCTAATCCTGTT_3'; #SY130513014, Sigma Aldrich, St. Louis, USA) primers. 10 μ l of standard and analyzed samples were placed in the holes of a qPCR plate. One qPCR plate contained six different dilutions of standard curve, one blank control and three analyzed samples. Each sample was analyzed in triplicate, in its normal concentration, 1:10 and 1:100 diluted concentrations. The RT-PCR protocol used for amplification consisted of the following steps: of 4min at 95°C, 40 cycles of 15s at 95°C, 30s at 60°C and 30s at 72°C, 15s at 95°C, 15s at 60°C and 15s at 95°C. The mean value of the three triplicates was used as the final analyzed values for bacterial concentration of the samples.

6. Results

6.1. Massively parallel Illumina sequencing

Bacterial communities in soil and air samples from the bioremediation plant Calcinatè were described by using massively parallel Illumina sequencing technology. Stacked bar charts represent the percentage proportion of all detected bacterial sequences. Figure 5 represents bacterial community structures of the soil samples during the aeration, drying and sieving processes at the plant.

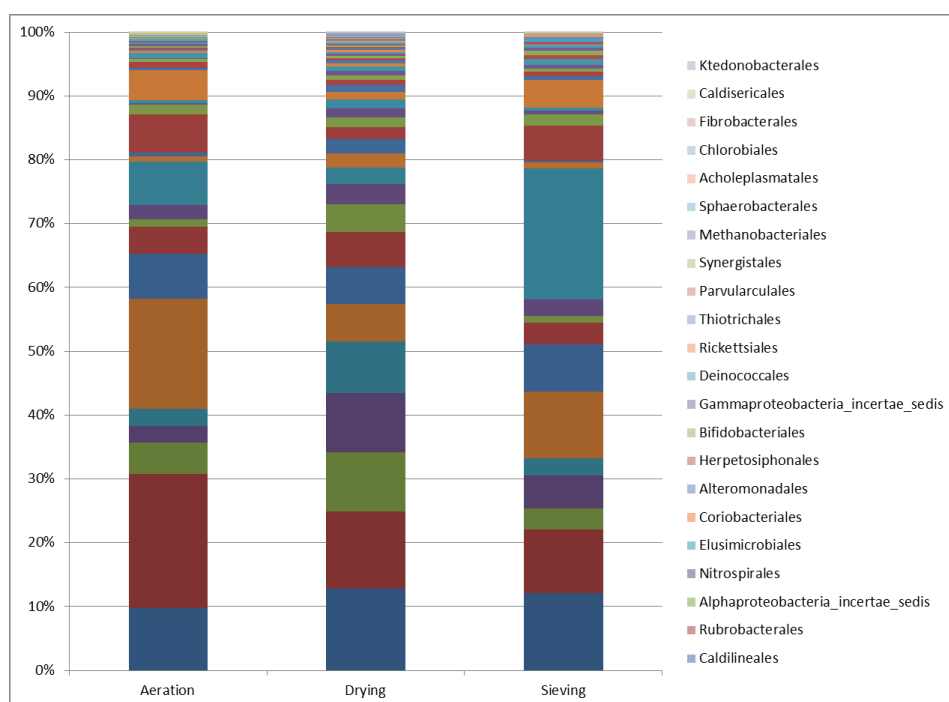


Fig.5. Bacterial community structures of the soil samples during aeration, drying and sieving processes at the Calcinatè plant.

Figure 5 illustrates that the main bacterial communities in the treated soil during steps of aeration, drying and sieving are *Burkholderiales*, *Xanthomonadales*, *Flavobacteriales*, *Rhodocyclales*, and *Actinomycetales*. The most abundant bacterial taxonomic groups during aeration step are *Xanthomonadales* and *Actinomycetales*. The most abundant bacterial group during the drying step is *Burkholderiales*. The most abundant bacterial species during the sieving step belong to the *Rhodocyclales*. During the step of aeration, the abundance of *Xanthomonadales* and *Actinomycetales* is much higher compared to the drying and sieving steps. The abundance of *Flavobacteriales*, *Rhodocyclales*, and *Clostridiales* increases during the drying step. The amount of *Actinomycetales* however decreases. The numbers of *Bacillales* and *Sphingomonadales* during the drying step are lower compared to the aeration and sieving steps. During the sieving step, the concentration of *Hydrogenophilales* and *Actinomycetales* become much higher, while the concentration of *Xanthomonadales* is much lower compared to the aeration step.

Figure 6 represents bacterial community structures of the air filter samples during no activity, treatment, aeration, drying and sieving processes at the plant.

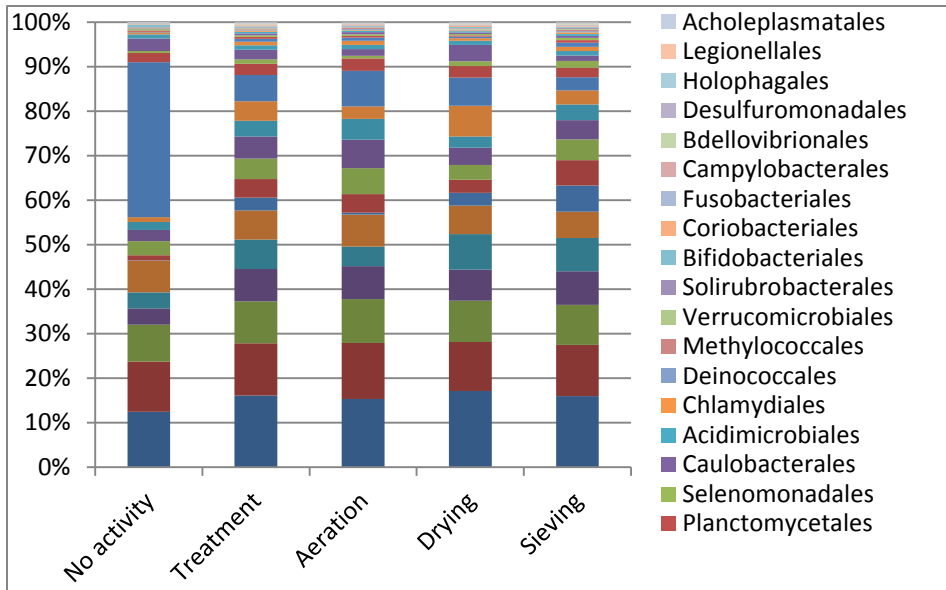


Fig.6. Bacterial community structures of the air filter samples during no activity, bioremediation treatment, aeration, drying and sieving processes at the Calcinat plant.

Figure 6 shows that the bacterial communities in the air filter samples are mostly containing the *Actinomycetales*, *Clostridiales*, *Bacillales*, *Burkholderiales*, *Pseudomonadales*, *Lactobacillales*, *Hydrogenophilales*, *Xanthomonadales* and *Rhizobiales* bacterial populations. The conclusion is that the relative abundance of these bacterial populations in the air filter samples is much lower in the control samples of no treatment activity compare to air filters of the aeration, drying and sieving steps. The relative abundance of *Actinomycetales*, *Clostridiales* and *Bacillales* is slightly lower when there is no activity. The abundance of *Burkholderiales*, *Pseudomonadales*, *Hydrogenophilales*, *Xanthomonadales*, and *Sphingobacteriales* are lower in the samples, which describe no activity compared to samples of the other treatment steps. The fact that the relative abundance of *Rhodobacteriales* is almost three times higher when there is no activity and is becoming very low during treatment steps is extremely interesting. *Hydrogenophilales* are not detected when there is no activity and is increasing in the other treatment steps especially during the drying and sieving steps. Relative abundance of *Flavobacteriales* is very low when there is no activity and is increasing during the treatment steps, especially during the drying.

6.2. Quantitative RT-PCR

The quantitative Real Time PCR technique was performed to estimate the numbers of total bacterial concentrations in the samples. Units are expressed in numbers of ribosomal operons per one cubic meter of air or numbers of ribosomal operons per gram of soil. Quantitative values of total numbers of bacteria, *C. perfringens*, *E.coli* and *Enterococcus* spp. in the air samples during no activity, drying, sieving and aeration processes at the plant are represented in Table 1. Table 2 represents quantitative values of total numbers of bacteria, *C. perfringens*, *E. coli* and *Enterococcus* spp. in the soil samples during no activity, drying, sieving and aeration processes at the plant. From the obtained results the relative abundances of three bacterial species of particular interest, namely *E.coli*, *Clostridium perfringens*, *Enterococcus* spp. have been translated in the actual numbers of bacterial copies in the samples.

Activity	Total #/m ³	<i>C. perfringens</i> #/m ³	<i>E. coli</i> #/m ³	<i>Enterococcus</i> #/m ³
No activity	8,22E+04	2,05E+00	4,31E+01	3,04E+02
No activity	7,87E+05	0,00E+00	3,92E+03	2,07E+03
No activity	1,57E+06	0,00E+00	3,44E+03	6,89E+03
Drying	1,46E+04	5,50E-01	9,07E+00	6,91E+01
Drying	1,74E+08	3,18E+04	4,96E+05	1,23E+06
Drying	5,10E+07	1,25E+04	3,17E+05	2,20E+05
Drying	2,97E+07	3,58E+03	2,28E+04	1,58E+05
Drying	7,47E+08	1,86E+04	4,64E+04	1,88E+06
Drying	6,14E+07	9,25E+03	2,67E+04	5,18E+05
Drying	2,07E+08	2,45E+04	1,09E+05	2,28E+06
Drying	8,04E+07	1,36E+04	3,97E+04	8,29E+05
Sieving	1,70E+06	1,73E+02	9,45E+01	2,72E+03
Sieving	7,63E+04	6,45E+00	1,10E+02	3,14E+02
Sieving	1,34E+04	0,00E+00	3,95E+00	5,40E+01
Sieving	3,23E+04	1,41E+00	1,41E+00	1,13E+02
Sieving	1,63E+07	1,11E+03	6,65E+03	3,32E+04
Sieving	9,29E+03	0,00E+00	1,12E+01	9,05E+01
Sieving	2,54E+09	8,40E+04	1,26E+05	8,44E+06
Sieving	7,63E+07	4,08E+03	4,81E+04	5,09E+05
Aeration	6,43E+05	1,07E+01	1,28E+02	2,58E+03
Aeration	9,00E+03	2,91E-01	8,43E+00	1,57E+01
Aeration	1,51E+08	3,60E+03	5,04E+04	8,01E+05
Aeration	3,84E+07	1,44E+03	8,30E+03	1,27E+05
Aeration	4,83E+08	1,82E+04	9,76E+04	2,84E+06
Aeration	1,23E+08	9,48E+03	5,21E+04	7,51E+05

Tab.1. Quantitative values of total numbers of bacteria, *C. perfringens*, *E.coli* and *Enterococcus* spp. in the air samples during no activity, drying, sieving and aeration processes at the plant.

Activity	Total #/g	<i>C. perfringens</i> #/g	<i>E. coli</i> #/g	<i>Enterococcus</i> #/g
Aeration	4,11E+09	2,10E+05	4,20E+05	7,07E+06
Aeration	7,41E+09	2,06E+05	0,00E+00	2,68E+06
Aeration	7,37E+09	2,16E+05	2,16E+05	1,52E+06
Aeration	2,99E+09	0,00E+00	3,97E+04	1,11E+06
Drying	2,03E+09	0,00E+00	0,00E+00	7,05E+05
Drying	3,44E+09	1,48E+05	0,00E+00	2,88E+06
Drying	3,14E+09	2,81E+05	2,01E+05	3,90E+06
Drying	3,69E+09	3,26E+05	0,00E+00	2,45E+06
Drying	1,20E+10	9,91E+05	3,97E+05	1,45E+07
Drying	7,78E+12	9,73E+08	0,00E+00	2,59E+09
Drying	3,33E+12	1,73E+08	0,00E+00	1,18E+10
Drying	4,89E+12	2,86E+08	0,00E+00	2,38E+10
Drying	2,75E+12	3,91E+07	3,91E+07	4,74E+09
Drying	7,95E+12	1,28E+09	0,00E+00	1,22E+10
Sieving	2,66E+11	0,00E+00	1,79E+07	1,14E+08
Sieving	4,15E+11	9,18E+07	3,44E+07	5,74E+06
Sieving	1,18E+12	5,27E+07	7,91E+07	2,11E+08
Sieving	7,29E+11	3,14E+07	1,57E+07	6,90E+08
Sieving	4,32E+11	9,99E+06	0,00E+00	2,10E+08
Sieving	1,04E+12	0,00E+00	0,00E+00	1,44E+08
Sieving	8,67E+11	0,00E+00	0,00E+00	1,97E+08

Tab.2. Quantitative values of total numbers of bacteria, *C. perfringens*, *E.coli* and *Enterococcus* spp. in the soil samples during no activity, drying, sieving and aeration processes at the plant.

Analyzing Table 1, which represents the air filter samples, it is possible to notice that the concentration of total bacteria, as well as *C. perfringens*, *E.coli* and *Enterococcus* spp., is much lower in the no treatment activity samples compared to the other treatment steps. The concentration of total bacteria, *C. perfringens*, *E. coli* and *Enterococcus* spp. is highest during the drying step. Also the step of aeration is leading to quite high concentrations of bacteria in the air filters.

From Table 2, which represents soil samples, it is clear that the bacterial concentrations are quite high during all the steps of treatment. During the steps of drying and sieving concentrations of bacteria are the highest.

6.3. Relative abundance of the bacterial species

Analyzing the relative abundance of the bacterial populations in the samples provides the information about the species that dominate the bacterial community in a particular ecosystem. It is usually measured as the large number of individuals found per sample. Analyzing the relative abundance in the air filter samples show that the most present bacterial taxonomic groups in the air during the different steps of treatment are *Caulobacterales*, *Flavobacteriales*, *Methylophilales*, *Parvularculales*, *Rhodobacterales* as shown in Figure 7.

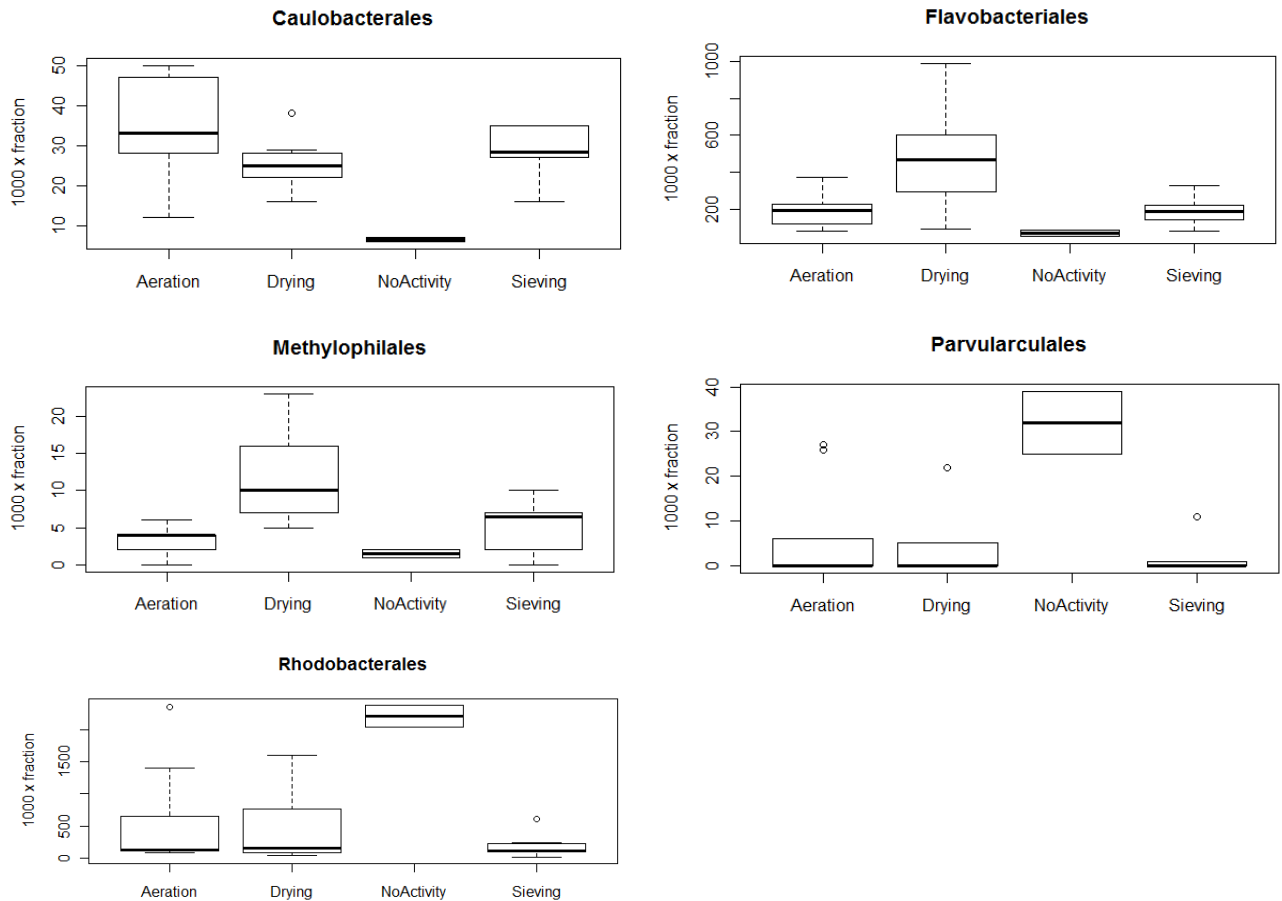


Fig.7. Relative abundance of *Caulobacteriales*, *Flavobacteriales*, *Methylophilales*, *Parvularculales*, *Rhodobacteriales* bacterial taxonomic groups in the air filter samples during no activity, aeration, drying and sieving steps of treatment.

From the plots it is possible to conclude that the concentration of *Caulobacteriales* is increasing during the treatment, especially during the aeration step, while *Flavobacteriales* and *Methylophilales* are showing more or less similar behavior during treatment steps and increase during the drying step. It is remarkable that the amounts of *Parvularculales* and *Rhodobacteriales* in the air are quite high when there is no activity and become very low during the treatment steps. *Parvularculales* and *Rhodobacteriales* are showing a very similar behavior pattern during the treatment steps.

Figure 8 presents the relative abundance in the soil samples. It has been shown that the most present bacteria in the soil during the different steps of treatment are *Actinomycetales* and *Bacillales*.

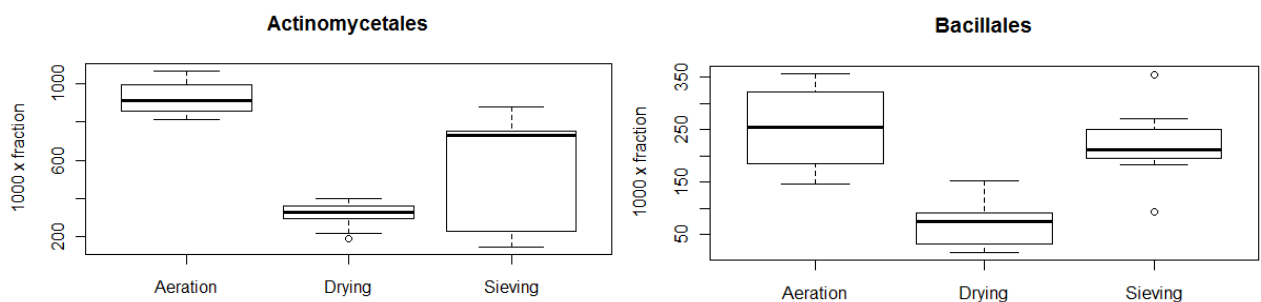


Fig.8. Relative abundance of *Actinomycetales* and *Bacillales* bacterial taxonomic groups in the soil samples during aeration, drying and sieving steps of treatment.

Box plots show that the behavior of *Actinomycetales* and *Bacillales* is very similar during different treatment steps. Their concentration is quite high during the aeration and sieving steps. The drying step contains the lowest concentration of these bacterial groups.

Figure 9 presents a comparison of *E.coli*, *Enterococcus* spp. and *C. perfringens* bacterial concentration in the air filter samples.

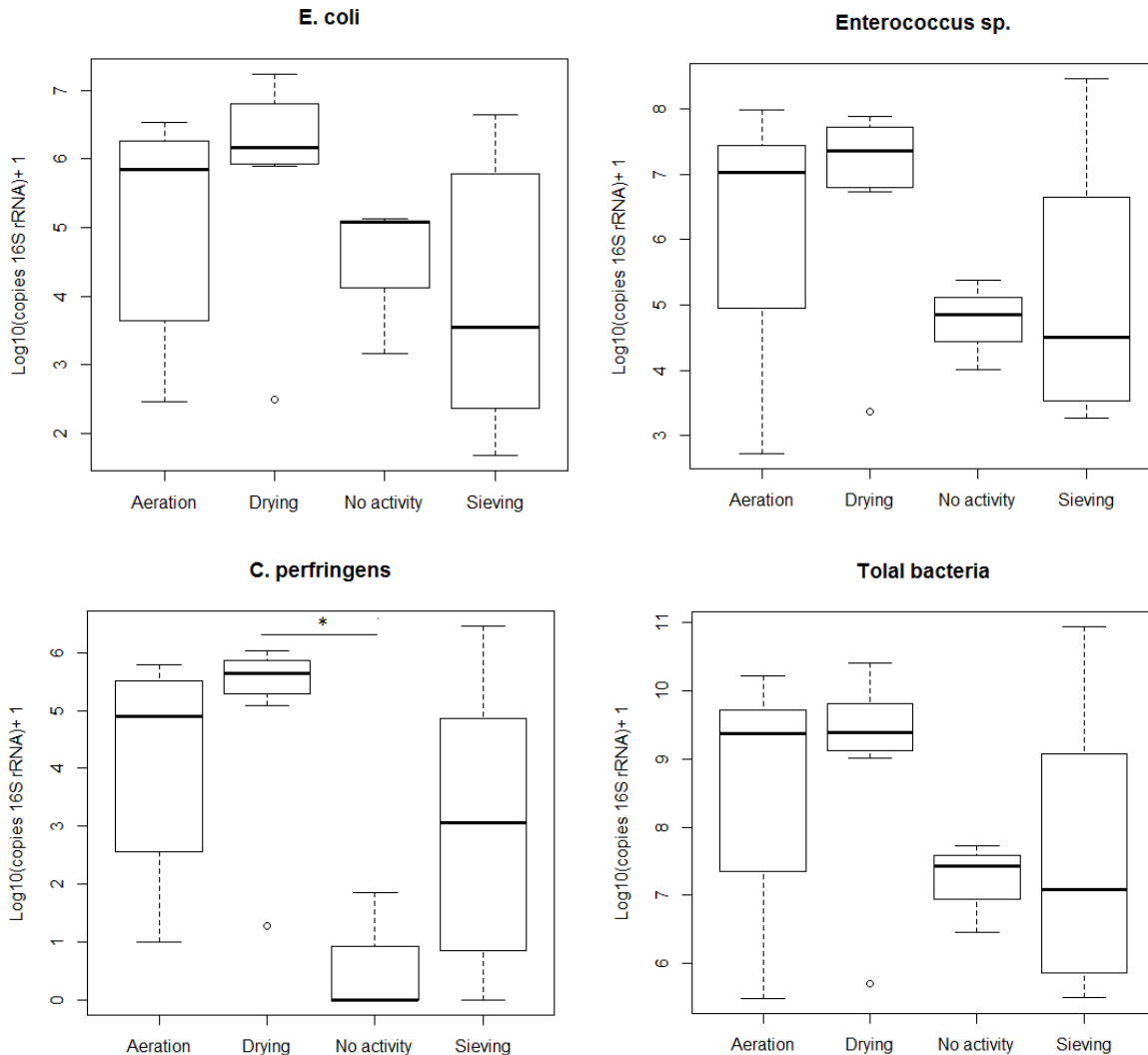


Fig.9. Comparison of means for bacterial species *E.coli*, *Enterococcus*, *C. perfringens* bacterial concentration in the air samples.

From the box plots of Figure 9 we conclude that the concentrations of *E.coli*, *Enterococcus* spp. and total bacteria in the air are higher during the steps of aeration and drying and lower during the step of sieving. The concentration of *C. perfringens* is also high during the sieving step. Concentrations of *C. perfringens* during the drying and no activity samples are significantly different. We conclude that the steps of aeration and drying contribute the most to the increase of amount of bacteria in the air.

Figure 10 presents the comparison of *E.coli*, *Enterococcus* spp., *C. perfringens* bacterial concentration in the soil samples.

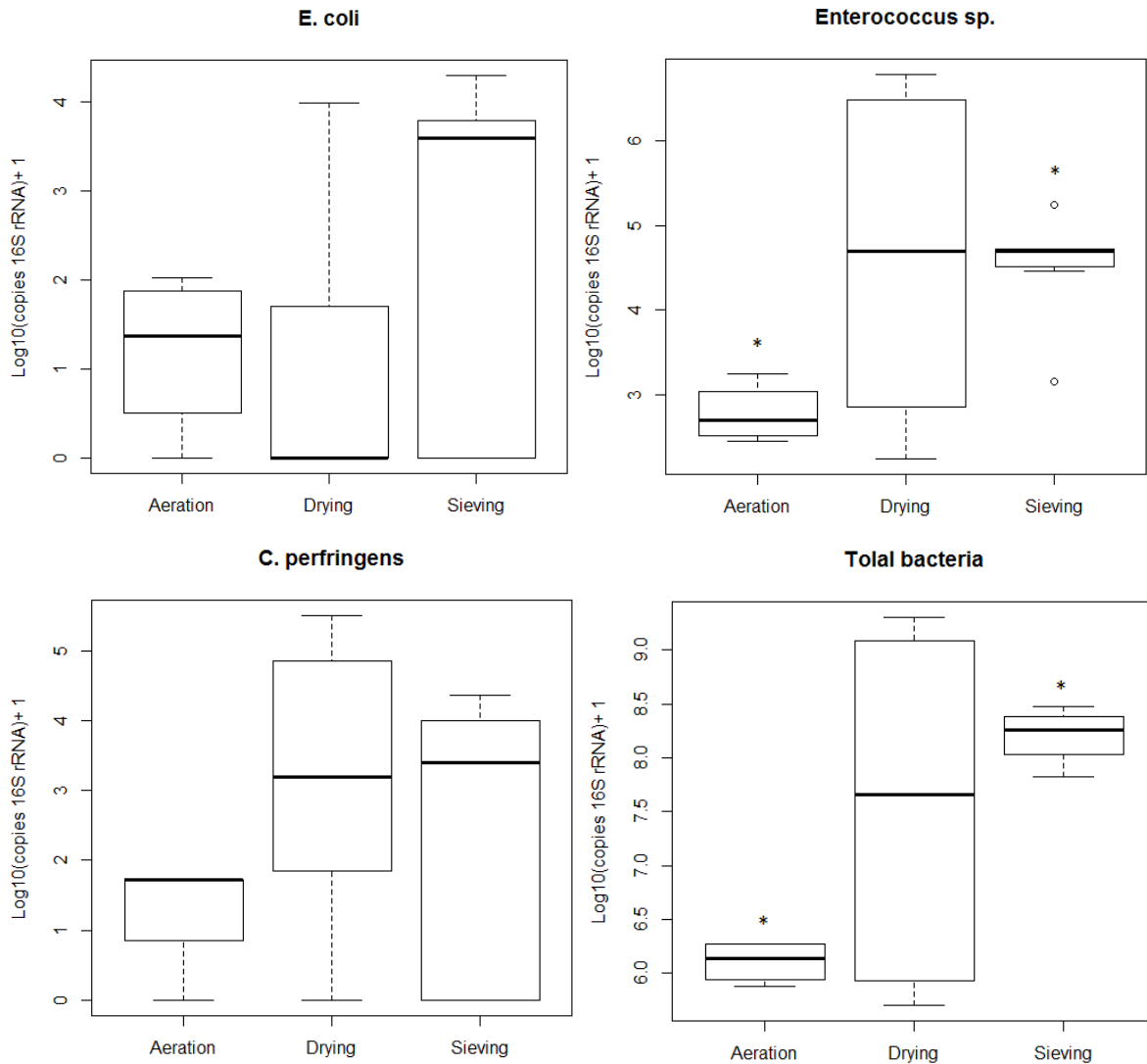


Fig.10. Comparison of means for bacterial species *E.coli*, *Enterococcus spp.*, *C. perfringens* bacterial concentration in the soil samples.

It is clear that the drying and sieving steps contribute the most to increasing of the bacteria in the soil samples. For the total amounts of bacteria and *Enterococcus spp.* the aeration and sieving processes are significantly different between each other.

6.4. Statistical analysis

To test the statistical significance of the differences between the analyzed groups of soil remediation activity and no activity for soil and air filter samples ANOVA tests have been applied. Furthermore, the multiple comparison Tukey test to evaluate the difference between soil remediation steps has been applied. Statistically the significant difference was set at $P < 0.05$.

For the air filter samples statistical ANOVA test showed significant change of concentration only for *C. perfringens* during soil remediation activity with $p = 0.014$ ($p < 0.05$). Furthermore, the Tukey test for multiple comparisons of means showed that this statistical significant difference is due to the significant difference between the no activity and drying steps of the process with $p = 0.012$ ($p < 0.05$). It can be concluded that the concentration of *C. perfringens* increases significantly during the step of drying compared to the no treatment activity step of the process.

For the soil samples the ANOVA test showed statistical significant difference of the total amount of bacteria for soil remediation activity with **p=0.035** ($p<0.05$). Furthermore the statistical Tukey test for multiple comparisons of means showed that this significance is due to the significant difference between the sieving and aeration steps with **p=0.028** ($p<0.05$). It can be concluded that the total concentration of bacteria in soil is much higher during the sieving step compared to the aeration step.

The test for relative abundance for the air filter samples showed that the bacterial species *Caulobacteriales*, *Flavobacteriales*, *Methylophilales*, *Parvularculales*, *Rhodobacteriales* are relative abundant in the air during soil remediation steps.

The Tukey test for multiple comparisons of means showed statistically significant differences of the *Rhodobacteriales* concentration between groups of no activity and aeration with **p=0.004** ($p<0.05$), groups of no activity and drying with **p=0.002** ($p<0.05$) and groups of no activity and sieving with **p=0.0004** ($p<0.05$). We can conclude that the concentration of *Rhodobacteriales* is much higher during the no activity compared to the aeration or sieving steps. The drying step contains significantly higher concentration of bacteria than the aeration step.

The Tukey test for multiple comparisons of means showed statistically significant differences of the *Flavobacteriales* concentration between groups of drying and aeration with **p=0.006** ($p<0.05$), groups of no activity and drying with **p=0.019** ($p<0.05$) and groups of sieving and drying with **p=0.004** ($p<0.05$). We conclude that the concentration of *Flavobacteriales* is significantly higher in the drying step compared to the aeration, sieving or no activity steps.

Tukey multiple comparisons of means showed statistically significant differences of the *Caulobacteriales* concentration between groups of no activity and aeration **p=0.001** ($p<0.05$), as well as groups of sieving and no activity **p=0.014** ($p<0.05$). It is possible to conclude that the concentration of *Caulobacteriales* is much higher during the aeration and sieving steps compared to the no activity step.

The Tukey test for multiple comparisons of means showed a statistically significant differences of the *Methylophilales* concentration between groups of drying and aeration with **p=0.001** ($p<0.05$), groups no activity and drying with **p=0.020** ($p<0.05$) and groups of sieving and drying with **p=0.013** ($p<0.05$). Thus, the concentration of *Methylophilales* is significantly higher in the step of drying compared to other groups.

The Tukey test for multiple comparisons of means showed a statistically significant differences of the *Parvularculales* concentration between groups of no activity and aeration with **p=0.002** ($p<0.05$), groups of no activity and drying with **p=0.001** ($p<0.05$) and groups of sieving and no activity with **p=0.0002** ($p<0.05$). It can be concluded that the concentration of *Parvularculales* in the no activity is much higher compared to other steps.

The test for relative abundance for the soil samples showed that the bacterial species *Actinomycetales* and *Bacillales* are relatively abundant in the soil during treatment.

The Tukey test for multiple comparisons of means showed a statistically significant differences of the *Actinomycetales* concentration between groups of drying and aeration **p=0.0001** ($p<0.05$), as well as the groups of sieving and aeration **p=0.012** ($p<0.05$). It is possible to conclude that the concentration of *Actinomycetales* is significantly higher in the aeration step compared to the other steps.

The Tukey test for multiple comparisons of means showed a statistically significant differences of the *Bacillales* concentration between groups of drying and aeration **p=0.0004** ($p<0.05$), as well as the groups of sieving and drying **p=0.0005** ($p<0.05$). It is possible to conclude that the aeration and sieving steps contain significantly higher concentration of *Bacillales* compared to the drying step.

7. Discussion

7.1. Illumina sequencing

From the bacteria phylogenetic groups that have been detected by Illumina sequencing technique, bacteria whose concentration is quite high in the samples belong to the Proteobacteria phylum. The Proteobacteria are a major bacterial phylum. Proteobacteria are Gram-negative bacteria and contain a lot of pathogenic species such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, and *Yersinia*. Most Proteobacteria are facultative or obligate anaerobic, chemoautotrophs, and heterotrophic. Some species are able to fixate nitrogen. The outer membrane has a lipopolysaccharides layer. Proteobacteria include six classes of bacteria, namely Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Acidithiobacillia.

From the bacteria that have been analyzed in Figure 6 *Pseudomonadales*, *Burkholderiales*, *Clostridiales*, *Rhizobiales*, *Xanthomonadales* which have been found in the air filter samples have been described as pathogenic bacteria. *Rhodocyclaceae* and *Sphingomonadaceae*, which have been found in the soil samples, are bacterial populations that play an important role in environmental remediation as they are able to degrade some aromatic compounds.

From Figure 5 it is clear that *Xanthomonadales*, *Actinomycetales*, *Burkholderiales*, *Rhodocyclales* are quite abundant in the soil samples.

The *Xanthomonadales* are a bacterial order from the class Gammaproteobacteria. *Xanthomonadales* are obligate aerobes and use oxygen as an electron acceptor. *Xanthomonadales* are Gram-negative bacteria and possess a flagellum that provides them motility. The cell wall is composed of a cytoplasmic layer, a peptidoglycan layer and an outer membrane layer. The outer membrane contains lipopolysaccharides, which are suggested to protect bacteria from antibiotics. Many *Xanthomonadales* are plant pathogens.

Burkholderiales are Gram-negative bacteria of the class Betaproteobacteria and include some pathogenic species. They are obligate aerobes. Only some species perform anaerobic respiration by using nitrates. They are able to grow in very poor nutritional conditions. A lot of *Burkholderiales* species are pathogenic for plants and animals. The most suitable environment for *Burkholderiales* grow is soil. *Burkholderiales* can be found in soil and water, and they contribute to the mineralization of carbon compounds in the environment.

Rhodocyclales are Gram-negative bacteria that belong to the class Betaproteobacteria. They are growing in anoxic conditions photoheterotrophically by using molecular hydrogen. The best growing temperature is 30°C. Normal habitats for these bacteria are freshwater ponds and sewage ditches (26).

From Figure 6 it can be seen that *Hydrogenophilales* are absent in the no treatment activity and are increasing in the other treatment steps especially during the drying and sieving steps. *Hydrogenophilales* are Gram-negative bacteria that belong to the class of Betaproteobacteria. *Hydrogenophilales* are very stable at high temperature up to 50°C and they consume hydrogen for energy. This can explain why *Hydrogenophilales* are absent in the no treatment activity and are increasing in the steps of drying and sieving. *Flavobacteriales* bacteria, as *Hydrogenophilales*, belong to the Betaproteobacteria and possibly for this reason they are showing a similar behavior pattern during the treatment steps.

7.2. Quantification of bacterial copy numbers in the samples

7.2.1. Air filter samples

Total bacterial quantification in the air filter samples collected during sampling campaigns performed at the soil bioremediation plant Calcinate, showed that concentration of bacteria in the air was up to 10^4 - 10^8 #/m³, (Table 1). The highest bacterial concentrations were found during the steps of drying and aeration (about 10^7 and 10^8 #/m³), while the lowest were observed in the no activity (control) samples (about 10^5 #/m³). In some control samples the concentration of bacteria was so low that it was impossible to detect it. These values allow it to be concluded that the concentration of the bacteria in the plant during the different steps of the process is quite high. Also, from the concentration values of the *C. perfringens*, *E.coli*, *Enterococcus* spp. we concluded that these bacteria in the air are increasing during the treatment steps twice as much as compared to the value of the control samples.

To estimate the risks of the plant workers exposed to the airborne bacteria during work it is important to have the control on the exposure grade of plant workers. Occupational Exposure Limits are established by the government on the threshold limit values of 1.0×10^5 , 2.0×10^4 , and 5.0×10^4 CFU/m³ for mesophilic bacteria, Gram-negative bacteria and fungi, respectively (27). Table 3 presents bacterial and fungi concentration values of CFU/ m³ defined by European Collaborative Action Report No. 12 (ECA 12) for non-industrial indoor environments.

	Mesophilic Bacteria (CFU/m ³)	Fungi (CFU/m ³)
Very low	< 50	< 25
Low	< 100	< 100
Intermediate	< 500	< 500
High	< 2000	< 2000

Tab.3. Bacterial and fungi limit concentration values in CFU/ m³ defined by the European Collaborative Action Report No. 12 (ECA 12) for non-industrial indoor environments (27, 28).

From the values mentioned in table 3 it is obvious that a concentration of bacteria up to 10^4 CFU/m³ in the air of can be considered as high. The bacterial concentrations in air samples taken at Calcinate are even higher. However it is not possible to draw definitive conclusions because the value of 10^4 CFU/m³ has been established for the identification of bacteria by the cultivation method. This limit value can give some general estimation about bacterial concentration in the air, but it can't be taken as a limit value for molecular methods of bacterial identification. Another important fact is that values, presented in Table 3 are established for non-industrial indoor environments, while the Calcinate plant is partly an outdoor plant.

However when estimating the exposure of the plant workers during their work time it must be taken into account that air on the bioremediation plant contains a complex mixture of different chemical and biological substances and plant workers are getting exposed not only to airborne bacteria but also to different bioaerosols, gasses, and VOCs.

Other important aspects that should be considered are the environmental conditions during the sampling. The factors such as higher temperatures during spring season in indoor facilities, ventilation, operational activities, the flow rate, characteristics of the wastewater, the time of day, the distance to the sampler and environmental conditions have to be taken into account as they may lead to high levels of microorganisms associated with increasing microbial growth rate.

Understanding how the bioaerosols are spreading around will allow estimating the potential health hazard zone to workers of a bioremediation treatment plant. It is crucial to reduce the risk of exposure to contaminants by applying efficient prevention measures at the workplace such as ventilation of the facilities, optimization of the process operations, work shifts and periodical changing of the working place as well as the use of equipment for personal protection such as working clothes and gloves.

Bioaerosols are in general associated with particulate matter in the upper layer of water or soil. Changing of preferable environmental conditions or the depletion of nutrients in the habitat environment of bacteria make them move in the air, which causes air to be a transfer medium for microorganisms, rather than a habitat.

The limiting factor for the bioaerosols dispersion in air are biotic factors, that limit the survival of the microorganisms, as well as the abiotic factors limiting release, transport, and dispersion of organisms. Physical characteristics such as size, density and shape but also environmental conditions such as air currents, relative humidity and temperature are important parameters.

A known fact is that bioaerosols originating from the water are different from bioaerosols originating from soil. Microorganisms originating from water have around them a thin layer of moisture or water droplets. Bioaerosols can also undergo physical movement, gravity, electrical forces, thermal gradients, electromagnetic radiation, turbulent diffusion, inertial forces, oxygen influence, and relative humidity. Depending on the physical properties of bioaerosols, these forces can have different effects on them. Due to gravitational settling and biological inactivation the concentration of bioaerosols decreases downwind. Another factor that describes bioaerosols movement is diffusion of bioaerosols as the gradient shifts from higher to lower concentration. One of the important factors of bioaerosols composition is the change of temperature. Its increase has a destroying influence on aerosolized organisms.

During different processes of the treatment sewage bubbles become aerated and burst in the air above the surface which produce large bioaerosols in the atmosphere. Concentration of bacteria in these drops can sometimes be 10–1000 times higher than those of the wastewater source, and it is also dependent on the drop size (27).

7.2.2. Soil samples

Analyzing the soil samples during different steps of the process (Table 2), we can notice that the total bacterial concentration in the samples is very high during the steps of drying and sieving (about 10^{12} # /m³) and it is getting lower at the step of aeration. If we remember that aeration is the last step of the treatment following drying and sieving, we may conclude that the process of bioremediation is efficient as we can notice that the amount of bacteria in the soil is decreasing. From Table 2 we can see that as the total concentrations of bacteria, the concentration of *C. perfringens*, *E.coli*, *Enterococcus* spp. are also decreasing in the aeration step. A hypothesis that can be made suggests that supply of oxygen to the soil partly destroys the bacterial community.

Many microorganisms are not able to tolerate oxygen because of its ability to destroy cells. Oxygen is able to attract electrons from other molecules, which is called "oxidation". Microorganisms that are strict anaerobes, also called "obligate anaerobes", can produce their energy from other sources such as fermentation or by anaerobic respiration and are very sensitive to oxygen and are not able to tolerate it. Such bacteria species are for example spirochetes and some *Clostridium* species. Other bacterial species can be facultative anaerobes, that is, they use oxygen for their growth but they can switch to another way of living in anaerobic conditions.

How oxygen influences microorganisms is not completely understood, but it is believed that an important role is played by the high lipid content of the cell walls of microorganisms which make them sensitive to oxygen. Oxygen is able to oxidize phospholipids and lipoproteins of the cellular envelopes and destroy cytoplasmic integrity in the microorganisms. In fungi, oxygen inhibits cell growth at certain stages.

It has been shown that Gram-negative and Gram-positive bacteria have distinctive levels of sensitivity to oxygen due to the different structure of their cell walls. Gram-negative bacteria possess a layer of membrane-lipopolysaccharide that makes it more resistant to extracellular oxygen. Oxygen can enter the cell and produce reactive by-products. Most Gram-positive bacteria do not have protective lipid layers and they are more sensitive. Microorganisms that are able to tolerate oxygen have enzymes, which can work against radicals, as H_2O_2 and O^{2-} . Another important protective factor is the presence of glutathione in the cell of the microorganism. Glutathione is able to detoxify reactive peroxides and free radicals in the cell. Bacterial species such as *E. coli* do not have glutathione and therefore are sensitive to oxygen supply (29).

7.3. Relative abundance of the bacterial species

7.3.1. Air filter samples

Bacterial species that have been shown to be relatively abundant in the air filter samples were *Caulobacterales*, *Flavobacteriales*, *Methylophilales*, *Parvularculales*, *Rhodobacterales* (Figure 7).

Flavobacteriales form a bacterial order under the phylum Bacteroidetes. *Flavobacteriales* are Gram-negative, strictly aerobic or facultative anaerobic chemoorganotrophs, environmental bacteria that are widely distributed in soil and freshwater. *Flavobacterium psychrophilum* cause bacterial cold water disease. *Methylophilales* are Gram-negative, aerobic bacteria that belong to the beta group of the phylum Proteobacteria. The abundance of *Flavobacteriales* and *Methylophilales* is decreasing as result of treatment. As can be noticed in Figure 6, it is higher in the first drying step and it decreases during the steps of aeration and sieving. Such behavior can be considered as expected, due to the fact that treatment is supposed to decrease bacterial concentration.

A quite unexpected result is that increase in abundance of the *Caulobacterales*. The concentration of these bacteria is increasing after drying in the sieving and aeration steps. *Caulobacterales* are Gram-negative bacteria that belong to the alpha subgroup of the phylum Proteobacteria. Hypothetically it can be explained that after the drying step, environmental conditions for these bacteria get more suitable for growing and reproducing in dry conditions. It has already been mentioned that dry conditions and atmospheric pressure play an important role in bacterial growth and reproduction.

Parvularculales are halophilic marine bacteria under the order of Alphaproteobacteria. *Parvularculales* is strictly aerobic, Gram-negative, chemoheterotrophs. Like the *Parvularculales* also *Rhodobacterales* belong to the group of Alphaproteobacteria. *Rhodobacterales* can be photoautotrophic or chemoorganotrophic, strictly aerobic or facultative anaerobic and require nutrients, hydrogen, sulfide or thiosulfate for growth. Figure 7 shows that the abundance of *Parvularculales* and *Rhodobacterales* is not very high in case of no activity and is almost absent during the treatment steps of drying, sieving and aeration. This might be explained by the fact that during the treatment salinity shifts and inappropriate conditions for bacterial growth make it disappear from the samples.

Salinity can have an important influence on a media density and plays an osmo regulatory function in microorganisms. Different studies show that bacterial community composition can be shifted due to changing salinity conditions. A good example is the domination of Betaproteobacteria in freshwater while Alphaproteobacteria are more present in water with a higher salinity. Alphaproteobacteria form a class of bacteria in the phylum Proteobacteria. Alphaproteobacteria are bacteria that are widely distributed in the marine environments. Marine plankton may contain over 10% of the open ocean microbial community. Bacteria in this class are mostly Gram-negative. Some species also lack peptidoglycan, several species are phototrophic and several genera metabolizing C1-compounds. Betaproteobacteria also belong to Proteobacteria. Betaproteobacteria are a Gram-negative group of aerobic or facultative anaerobic bacteria that are often highly

versatile in their degradation capacities. Some species are chemolithotrophic, ammonia-oxidizing or prototroph. Alphaproteobacteria is a very abundant group in oceans and salty water and their abundance decreases with lowering of water salinity. On the other hand, Betaproteobacteria are more present in freshwater lakes and decreases in abundance along the gradient of water salinity. Alpha- and Betaproteobacteria have quite different relationships with salinity but both show strong correlations between their abundance and water salinity (30).

It has not been clear why marine bacteria are present in the samples. Treated soil is coming from channels, where the water from bioremediation process is floating. The water that supplies the region is coming from the rivers that begin in the mountains and has no connection with the sea. Most likely due to the content of different nutrients in the channels' sediments the salinity of this environment is quite high and this allows marine bacteria to develop and reproduce. Unfortunately, due to unexpected results, the salinity of the ground was not investigated.

7.3.2. Soil samples

The relative abundance of *Actinomycetales* and *Bacillales* in the soil samples was high. *Actinomycetes* are soil microorganisms that belong to the group of Actinobacteria and are considered as a transitional form between bacteria and fungi, based on morphological structure and availability of mycelium like structure. Originally *Actinomycetes*, were classified as fungi. Later research let showed that *Actinomycetes* are more related to bacteria than to fungi. The *Actinomycetes* are able to utilize organic carbon compounds such as sugars, starch, hemicelluloses, proteins and other hardly decomposable compounds (26). Most *Actinomycetes* are aerobic, but a few species can grow under anaerobic conditions. *Bacillales* are gram-positive bacteria. They can be obligate aerobe or facultative anaerobes. Under stressful conditions or nutrient depletion they produce spores. *Actinomycetales* and *Bacillales* are bacterial groups that are normally present in the soil environment. Figure 8 represents the abundance of *Actinomycetales* and *Bacillales* in the soil samples from the remediation plant. *Actinomycetales* and *Bacillales* show a similar behavior pattern during the treatment. Concentration of these bacteria is low in the first step of drying but it increases during the following steps of aeration and sieving. Hypothetically it can be explained that as *Actinomycetales* and *Bacillales* are bacteria that normally are present in the soil environment, with the step of treatment sludge is becoming more like normal soil and a more normal soil flora begins to develop.

In order to draw and make conclusions about bacteria in the soil during and after the bioremediation treatment, it is necessary to have knowledge about which bacteria are normally present in the soil in its normal conditions. The biggest fraction of free-living microorganisms in soil is represented by bacteria. Even though the abundance of bacteria in soil is high, not much is known about individual bacterial species and the standard composition of the soil, its ecological micro-environments and interaction between each other. In order to understand the relationship of bacterial communities in the soil it is necessary to have some information on bacterial morphology and taxonomy structure, their environmental tolerances, their spatial distribution in the soil as well as their biotic affinity with other microorganisms.

It is known that a big fraction of the bacterial flora in a soil are *Actinomycetales*, which are counted by a factor of 10, the fungi, which are counted by a factor of 100 and eubacteria biomass that is present as much as *Actinomycetes*. The moisture content in the soil is an important factor for bacterial grow and interactions. Water is needed for bacterial metabolism and changes of the moisture content that make bacteria cannot longer keep sufficient water inside the cells. It has been shown that the amount of bacteria in the soil decreases with moisture tension of 3 atmospheres or higher. Because of this reason it is believed that drying of soil reduces the total number of bacteria in the soil. However, not all bacteria respond like this on the drying of soil. Some bacterial species are very resistant to harsh conditions and can sporulate in order to survive unsuitable circumstances. When the soil is getting again supplied by water bacteria can activate from the spores.

However if the soil is too wet, this environment can't be favorable for most bacteria living. Water simply takes the place in the pores of soil and decreases soil aeration, with lack of oxygen in the soil as a result. Appropriate environmental conditions make any bacterial species in soil rebuild its normal soil concentration and show zymogenous response. It has been shown that the most abundant bacterial species in the soil are small coccoid rods of variable morphology. Mostly they are described by bacterial genus *Arthrobacter*, in the family *Corynebacteriaceae* of the *Eubacteriales* and *Callumomonas* that are able to decompose cellulose. Very abundant bacteria in soil are the sporulating *Bacilli*. The species *B. cereus* and *B. subtilis* of the genus *Bacilli* are considered to be zymogenous soil bacteria. *Actinomyces* represent 70% of the total microbial count in soil. Sporulating *Bacilli* represent 25% of the total number of bacteria in the soil. Other bacterial species represented in the soil are *Pseudomonadaceae*, *Nitrobacteriaceae*, *Rhizobiaceae*, *Azotobacteriaceae*, *Achromobacteriaceae*, and *Micrococcaceae* (26).

7.3.3. Relative abundance of *E.coli*, *Enterococcus* spp., *C. perfringens*

Concentrations of *E.coli*, *Enterococcus* spp., *C. perfringens* in the air are getting higher during treatment steps as mechanical movement makes the dust from the dried soil rise to the air and bring with it bacteria in the air (Figure 9). The concentration of bacteria is the highest during the steps of aeration and sieving. An interesting fact is that during the step of sieving the concentration of bacteria is becoming even lower than when there is no activity. *E.coli*, *Enterococcus* spp., *C. perfringens* increase in the air when the soil is drying, so we may hypothesize that these bacteria move from soil into the air when water is evaporating from the soil. Sieving does not contribute much to the bacterial movement into the air, as the soil is dry. Aeration is another important step for bacterial movement into the air, probably due to the oxygen supply. It is possible that a high content of oxygen is not suitable for bacterial growth and reproduction in the soil, which makes them 'escapes' into the air environment. The concentration of *C. perfringens* in the air is very low in the no treatment and increases significantly during the treatment.

During the step of sieving the concentration of bacteria is very high (Figure 10), probably due to the fact that bacteria remain in the soil and are not moving to the air. The concentration of bacteria in the aeration step is not so high. If we compare the steps of aeration in Figure 9 for air filter samples with the step of aeration in Figure 10 for soil samples, we can see that during this step the concentration of bacteria in the soil is quite low, and in the air it is quite high, therefore it can be hypothesize again that the oxygen supply to the soil makes bacteria in the soil stop reproducing or maybe move from the soil to the air. Except for *E.coli* bacteria concentration of other bacteria is also quite high during the drying step, as it is the first step that contains the highest concentration of bacteria.

7.4. The main conclusion

We may conclude that activity on the soil bioremediation plant leads a significant increase of bacteria in the air. Statistical analysis also shows that this significant increase is mainly due to the drying step of the treatment process. The other data indicate that the step of aeration also highly contributes to the increase of bacteria in the air. From the bacterial concentrations in the soil samples we may conclude that the drying and sieving steps show quite high concentration of bacteria. In the aeration step, the concentration of bacteria in the soil decreases.

It might be that there exist two mechanisms for bacterial transfer from soil into the air. The first mechanism is bacterial transfer during evaporation with the droplets of water, for bacteria that prefer growing and reproducing in moisture conditions. The second mechanism of bacterial transfer into the air is during mechanical mixing for bacteria that prefer growing in dry conditions.

The abundant bacteria in the analyzed samples predominantly belong to the Alphaproteobacteria or Betaproteobacteria. Some phylogenetic groups of bacteria such as *Flavobacteriales*, *Hydrogenophilales* and *Caulobacterales* grow better in dry conditions, when the moisture content is decreasing. This behavior has often been observed in Betaproteobacteria. Bacteria that belong to the Alphaproteobacteria, as for example *Parvularculales*, *Rhodobacterales* are halophilic marine bacteria and salinity shifts in their environment can have an important influence on their osmotic regulatory function and by consequence on their growth.

Another important factor for bacterial growth is oxygen supply during the last step of bioremediation, as some bacterial species need oxygen for their respiration, while others are not able to tolerate it. For example, from the three bacteria of particular interest *E. coli* does not possess glutathione and therefore are sensitive to oxygen supply. *Clostridium perfringens* is a strictly anaerobic bacterium, while *Enterococcus* spp. are facultative anaerobe bacteria and have different respiration capacity.

The increase of some bacteria in the last step of treatment can be explained by the fact that in the course of treatment untreated soil resembles more the typical soil conditions with its normal bacterial environment. Some bacterial species such as *Burkholderiales*, *Rhodocyclaceae*, *Sphingomonadaceae*, *Actinomycetales*, *Bacillales* are bacterial groups that are normally present in soils.

7.5. Future perspectives

The current research provide an overview of the bioremediation processes on the soil bioremediation plant Calcinate and the bacterial composition in the soil and air during different steps of the treatment. Results of the current research can be used to understand if a plant is performing its bioremediation process within the allowed limits.

Moreover, data obtained in this project can be used to estimate the risk for the plant workers while being present and working at the plant. For this statistical analysis more data should be collected as well as information about the involvement of people in the treatment processes. It is also important to analyze the statistical risks of living in the area close to the plant, in order to estimate the patterns of spreading and migration of the bacteria in the atmosphere, which depend on the environment and weather conditions, such as wind and temperature.

Obtained data can be described statistically by Analysis of Similarity (ANOSIM). It is a statistical test to investigate a significant difference between two or more groups of sampling units. Microbial communities were also estimated by multivariate statistics. Regression models provide information about factors that can have an influence on the composition of the communities.

A further purpose of the current research was to analyze bacterial communities on other plants such as the waste water treatment plant Nosedo and agricultural breeding plants. The scientific approach for these plants will be the same as the one used for the Calcinate plant.

It can be also interesting to select some other bacteria of particular sanitary relevancy, which are considered to be markers of fecal bacteria environmental contamination, such as pathogenic strains of fecal coliforms and fecal streptococci, *Salmonella*, *Campylobacter*, *Bacteroides* and analyze their concentration in the environmental samples.

8. Appendix

I. Protocol Extraction DNA from the samples using FastDNA® Spin Kit

1. Cut the filter with ceramic scissors in for equal parts.
2. Add 90mg of CaCO₃ in each of the Lysing Matrix E tube.
3. Add a fourth part of the air filter to the Lysing Matrix E tubes.
4. Add 978 µl Sodium Phosphate Buffer to sample in Lysing Matrix E tube. Shake for 1 hour.
5. Add 122 µl MT Buffer.
6. Homogenize in the FastPrep® Instrument for 45 seconds at a speed setting of 6.5.
7. Centrifuge at 14,000 x g for 15 minutes to pellet debris.
8. Transfer supernatant to a clean 2.0 ml microcentrifuge tube. Add 250 µl PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.
9. Centrifuge at 14,000 x g for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 ml tube.
10. Resuspend Binding Matrix suspension and add 1.0 ml to supernatant.
11. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.
12. Remove and discard 500 µl of supernatant being careful to avoid settled Binding Matrix.
13. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µl of the mixture to a SPIN™ Filter and centrifuge at 14,000 x g for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.
14. Add 500 µl prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.
15. Centrifuge at 14,000 x g for 1 minute. Empty the catch tube and replace.
16. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minutes to “dry” the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.
17. Air dry the SPIN™ Filter for 5 minutes at room temperature.
18. Gently resuspend Binding Matrix (above the SPIN filter) in 50-100 µl of DES (DNase/Pyrogen-Free Water).
19. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or 4°C until use.

II. Wizard® SV Gel and PCR Clean-Up System

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.
3. Add an equal volume of Membrane Binding Solution to the PCR amplification.
4. Insert SV Minicolumn into Collection Tube.
5. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
6. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
7. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection.
8. Repeat Step 7 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.

9. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
10. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
11. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
12. Discard Minicolumn and store DNA at 4°C or –20°C.

III. ZR Soil Microbe DNA MiniPrep™

1. Centrifuge 1.5 ml of bacterial culture for 10min at 4,000rpf
2. Discard the supernatant.
3. To process a total of 3.0ml of culture, add an additional 1.5 ml of bacterial culture to the same tube. Repeat Steps 1 and 2.
4. Discard the supernatant and resuspend pellet in up to 200 µl of water or isotonic buffer (e.g., PBS). Add to a ZR BashingBead™ Lysis Tube.
5. Homogenize in the FastPrep® Instrument for 45 seconds at a speed setting of 5.5.
6. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.
7. Add 1,200 µl of Soil DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.
8. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
9. Discard the flow through from the Collection Tube and repeat step 8.
10. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute.
11. Add 500 µl Soil DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.
12. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 µl DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA.

IV. Molecular cloning in E.coli TOP 10 with TA Cloning® Kit with pCR®2.1

1. Amplification and purification of PCR product using Taq polymerase and primers for amplificative product (27F and 519R)
2. Ligate your PCR product into pCR21 Incubate the ligation reaction at 14°C for a minimum of 4h overnight.
 - a. Centrifuge one vial of pCR®2.1 to collect all the liquid in the bottom of the vial.
 - b. Determine the volume of PCR sample needed to reach the required amount of PCR product (see above). Use sterile water to dilute your PCR sample if necessary.
 - c. Set up the 10 µl ligation reaction as follows:
 - Fresh PCR product X µl
 - 10X Ligation Buffer 1 µl
 - pCR®2.1 vector (25 ng/µl) 2 µl
 - Sterile water to a total volume of 9 µl
 - T4 DNA Ligase (4.0 Weiss units) 1 µl
 - Final volume 10 µl
3. Transform your ligation into competent E.coli:
 - a. Pre-inoculum: 20 ml LD+E.coli TOP 10 colony (stirring 37°C overnight)
 - b. Inoculum: 19 ml fresh LD+1 ml pre-inoculum (stirring 37°C overnight)
 - c. OD culture: $0,3 < OD_{600} < 0,5$
 - d. Centrifuge the cells at 4000rpm for 10min (4°C)
 - e. Discard the supernatant and gently resuspend the pellet in 10 ml of MOPS1 (cooled in ice)(10mM MOPS pH7, 10mM RbCl)
 - f. Centrifuge at 4000rpm for 10min (4°C)

- g. Discard the supernatant and gently resuspend the pellet in 10 ml of MOPS2 (cooled in ice)(0,1M MOPS pH6,5, 50mM CaCl₂, 10mM RbCl)
- h. Incubate in ice for 15min
- i. Centrifuge at 4000rpm for 10min (4°C)
- j. Discard the supernatant and gently resuspend the pellet in 2 ml of MOPS2 (cooled in ice)(0,1M MOPS pH6,5, 50mM CaCl₂, 10mM RbCl)
- k. Pick up 200µl competent cells (other competent cells put at 4°C) and add up to 10 µl of ligation
- l. Incubate at ice for 60min
- m. Heat shock at 43°C for 30s
- n. Add 1ml LD
- o. Incubate at 37°C for 2/3 hours(vigorous stirring)
- p. Plate the cells on selective medium (LD+ amp 100µg/ml+ Xgal 40mg/ml)
- q. Incubate overnight at 37°C

V. Isolation of plasmid vector from E.coli bacteria colonies

1. Select and resuspend E.coli colonies with an inserted vector in 1ml of distilled water
2. Centrifuge for 2 min at 12000 rpf.
3. Take away the supernatant and resuspend pellet at 100 µl of distilled water
4. Prepare 10 µl Green Taq, 2,5 µl 1:20 forward primer M13F, 2,5 µl 1:20 reverse primer M13 and 5 µl of sample PCR mix for each PCR tube.
5. PCR product has to be run through the gel electrophoresis.
6. Vector extraction from the gel.
7. Analyze plasmid DNA for the presence of PCR product by sequencing.

VI. Purification of plasmid by PureYield™ Plasmid Miniprep System

1. Centrifuge 1.5 ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge.
2. Discard the supernatant.
3. To process a total of 3.0ml of culture, add an additional 1.5 ml of bacterial culture to the same tube. Repeat Steps 1 and 2.
4. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.
5. Add 100µl of Cell Lysis Buffer, and mix by inverting the tube 6 times.
6. The solution should change from opaque to clear blue, indicating complete lysis.
7. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting the tube. The sample will turn yellow when neutralization is complete, and a yellow precipitate will form. Invert the sample an additional 3 times to ensure complete neutralization.
8. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
9. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn.
10. Place the minicolumn into a PureYield™ Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
11. Discard the flowthrough, and place the minicolumn into the same PureYield™ Collection Tube.
12. Add 200µl of Endotoxin Removal Wash to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds. It is not necessary to empty the PureYield™ Collection Tube.
13. Add 400µl of Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.
14. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30µl of Elution Buffer directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
15. Centrifuge at maximum speed in a microcentrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.

VII. Qubit® dsDNA BR Assay Kits

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® dsDNA BR assay requires 2 standards.
2. Label the tube lids.
3. Make the Qubit® working solution by diluting the Qubit® dsDNA BR reagent 1:200 in Qubit® dsDNA BR buffer. Use a clean plastic tube each time you make the Qubit® working solution. Do not mix the working solution in a glass container.
4. Load 190 µL of Qubit® working solution into each of the tubes used for standards.
5. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing 2–3 seconds. Be careful not to create bubbles.
6. Load the Qubit® working solution into individual assay tubes so that the final volume in each tube after adding sample is 200 µL.
7. Add each of your samples to assay tubes containing the correct volume of Qubit® working solution (prepared in step 1.6), then mix by vortexing 2–3 seconds. The final volume in each tube should be 200 µL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. On the Home Screen of the Qubit® 2.0 Fluorometer, press DNA, then select dsDNA Broad Range as the assay type. The Standards Screen is displayed.
10. On the Standards Screen, select to run a new calibration or to use the last calibration:

9. References

1. G.Tortora, B.Funke, C.Case. Sewage (wastewater) treatment. *Microbiology*. 2010;16:1-6.
2. Australian, government. Health manual: wastewater treatment. In: defence Do, editor. 8 ed. Canberra ACT 2600: Defence Publishing Service; 2009.
3. M.Vidali. Bioremediation. An overview. *Pure Appl Chem*. 2001;73(7):1163–72.
4. N.Christofi, I.B.Ivshina. Microbial surfactants and their use in field studies of soil remediation. *Journal of Applied Microbiology*. 2002;93:915–29.
5. N.Kosaric. Biosurfactants and their application for soil bioremediation. *Food Technol Biotechnol*. 2001;39(4):295–304.
6. E.A. Perpetuo, C.B.Souza, C.A.Nascimento. Engineering bacteria for bioremediation. In *Tech*. 2011:605-30.
7. D.L.Donlon, J.W.Bauder. A General Essay on Bioremediation of Contaminated Soil. Montana State University. 2006:601-3.
8. J.M.Guerra-Garci, J.C.Garci'a-Go'mez. Assessing pollution levels in sediments of a harbour with two opposing entrances. Environmental implications. *Journal of Environmental Management*. 2005;77:1-11.
9. S.McCready, D.J.Sleea, G.F.Birch, S.E.Taylor. The distribution of polycyclic aromatic hydrocarbons in surficial sediments of Sydney Harbour, Australia. *Marine Pollution Bulletin*. 2000;40(11):999-1006.
10. E.Longhin, E.Pezzolato, P.Mantecca, J.A.Holme, A.Franzetti, M.Camatini. Season linked responses to fine and quasi-ultrafine Milan PM in cultured cells. *Toxicology in Vitro*. 2013;27:551–9.
11. I. Gandolfi, V.Bertolini, R.Ambrosini, G.Bestetti, A.Franzetti. Unravelling the bacterial diversity in the atmosphere. *Appl Microbiol Biotechnol*. 2013;97:4727–36.
12. S.Gangamma, R.S.Patil, S.Mukherji. Characterization and proinflammatory response of airborne biological particles from wastewater treatment plants. *Environ Sci Technol*. 2011;45:3282–7.
13. A.Sanchez-Monedero, M.A.Aguilar, M.I.Fenoll. Effect of the aeration system on the levels of airborne microorganisms generated at wastewater treatment plants. *Water Research*. 2008;42(14):3739–44.
14. H.Heinonen-Tanski, T. Reponen, J.Koivunen. Airborne enteric coliphages and bacteria in sewage treatment plants. *Water Research*. 2009;43(9):2558–66.
15. J.V.Teixeira, S.Miranda, A.R.Monteiro, V.S.Lopes, J.Madureira, G.V.Silva, et al. Assessment of indoor airborne contamination in a wastewater treatment plant. *Environ Monit Assess*. 2013;185:59–72.
16. R.M.Bowers, I.McCubbin, A.Hallar, N.Fierer. Seasonal variability in airborne bacterial communities at a high-elevation site. *Atmospheric Environment*. 2012;50:41-9.
17. A.F.Maheux, È.Bérubé, D.K.Boudreau, R.Villéger, P.Cantin, M.Boissinot, et al. Abilities of the mCP agar method and CRENAME Alpha Toxin- Specific Real-Time PCR Assay to detect *Clostridium perfringens* spores in drinking water. *Applied and Environmental Microbiology*. 2013;79(24):7654–61.
18. S.Sood, M.Malhotra, B.K.Das, A.Kapil. Enterococcal infections & antimicrobial resistance. *Indian J Med Res* 128,. 2008;August:111-21.
19. E.Karpowicz, A.Novinscak, F.B.Rlocher, M.Filion. qPCR quantification and genetic characterization of *Clostridium perfringens* populations in biosolids composted for 2 years. *Journal of Applied Microbiology*. 2010;108:571–81.
20. J.M.Janda, S.L.Abbott. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Clinical microbiology*. 2007;45(9):2761–4.

21. J.E.Clarridge. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*. 2004;17(4):840–62.
22. S.Chakravorty, D.Helb, M.Burday, N.Connell, D.Alland. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Microbiological Methods*. 2007;69:330–9.
23. P.H.Janssen. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and environmental microbiology*. 2006;72(3):719–1728.
24. J.Penders, C.Vink, C.Driessen, N.London, C.Thijs, E.E.Stobberingh. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiology Letters*. 2005;243:141–7.
25. G. M. Lunaa, A.Dell’Annoa, B.Pietrangelib, R.Danovaroa. A new molecular approach based on qPCR for the quantification of fecal bacteria in contaminated marine sediments. *Journal of Biotechnology*. 2012;157:446–53.
26. Burgers. A. *Soil Biology*. Elsevier, editor2012. 542 p.
27. E.Korzeniewska. Emission of bacteria and fungi in the air from wastewater treatment plants – a review. *Front Biosci*. 2011;1(3):393-407.
28. A.Cimmuto, F.D’Acunzo, L.Marinelli, M.Giusti, A.Boccia. Microbiological air quality in an urban solid waste selection plant. *JPH*. 2010;7(1):20-7.
29. T.A.Dahl, W.R.Midden, P.E.Hartman. Comparison of killing of gram-negative and gram-positive bacteria by pure singlet oxygen. *Journal of bacteriology* 1989;171(4):2188-94.
30. D.L.Kirchman, A.I.Dittel, R.R.Malmstrom, M.T.Cottrell. Biogeography of major bacterial groups in the Delaware Estuary. *Limnol Oceanogr*. 2005;50(5):1697–706.

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:

Biological risk associated to bio-treatments: monitoring and modeling bacterial dispersion into the atmosphere in a soil bioremediation plant and in a wastewater treatment plant

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**

Jaar: **2014**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Tarasiuk, Olga

Datum: **18/08/2014**