Doctoral Dissertation
Doctoral Program in Chemical Engineering (32nd Cycle)

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Waste meets poor soils: perspectives on sewage sludge recycling

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June 30th, 2020

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Summary

Sewage sludge and nutrient depletion of soil are two themes strictly related to actual environmental problems, such as waste management and soil degradation. On one hand, sewage sludge (SS) is the main by-product generating from wastewater treatment, whose production has grown in the last twenty years; sewage sludge has different destinations, however a proper solution for a suitable reuse has not been found yet. On the other hand, soil nutrient depletion is a phenomenon increasingly occurring, with tremendous drawbacks directly on soil health and functions, and, to a broader extent, on economy and society.

Land application of sewage sludge is a practice carried out since many years, as SS can behave as valuable source of organic matter and nutrients under an agronomic point of view. Nevertheless, its long-term application may pose serious risk of soil contamination as SS still contained unwanted residues from wastewater treatment, such as organic and inorganic pollutants.

The aim of the present research is thus to reprise the land application practice of SS and to challenge it to demonstrate its effectiveness also on poor soils. More in detail, the research work started from a detailed characterisation of the waste used, which were anaerobic digestates from sewage sludge (SSAD). Then the attention was moved on the evaluation of fertilizing and phytotoxic effects of SSAD application on the growth of a vegetal model species (*i.e.* cucumber plants) in a controlled environment. Successively, particular interest was devoted the characterization of rhizosphere microbial communities of tomato plants grown on a poor soil and treated with SSAD, exploiting a molecular ecology approach. Finally, the last part of the work was dedicated to the implementation of an extraction protocol of an added-value part of SSAD organic matter, which are humic acids.

The results revealed that investigated SSADs had interesting contents in organic matter and nutrients (N, P). SSAD application on sandy soil induced an

improvement of different growth parameters of cucumber at an intermediate dosage of SSAD (170 kg N/ha). As concerns microbial communities, bacteria of tomato rhizosphere were influenced by treatment with SSAD, showing that its application induced a higher presence of Plant Growth Promoting Bacteria (PGPB). Finally, the extraction of humic acids turned out to be feasible and might help in getting rid of toxic molecules such as heavy metals.

The work conducted in the present thesis had a strong interdisciplinary vocation, ranging from chemical engineering to analytical chemistry, from agronomy to molecular ecology. Hence, this variety of topics could be covered thank to the productive collaborations with external institutions. Pot experiments for plant growth in climatic chamber and greenhouse where conducted in collaboration with Agroinnova, Centre of Competence for the innovation in the agro-environmental field. Extraction of soil DNA and molecular characterisation of soil microbial communities were conducted during the PhD period spent at Molecular Ecotoxicology and Microbiology Laboratories of Joint Research Centre in Ispra (VA). Extraction of humic acids from SSAD was performed during the PhD period spent at Escuela de Ingeniería Bioquímica of Pontificia Universidad Católica de Valparaíso (Chile). Hence, the present work aims to be pioneering in the exploitation of an effective interplay between different scientific branches to deal with relevant actual problems in a clever and integrated manner. The future research will rely even more on scientific interactions and, within this perspective, the engineering approach should wisely drive the next investigations and challenges.

Acknowledgment

I wish to thank sincerely my supervisor Prof. Debora Fino and my co-supervisor Dr. Tonia Tommasi for proposing me a very stimulating and interdisciplinary PhD project, for pushing me beyond my limits to do my best, and for giving me the chance to spend part of my PhD away from Politecnico, which have been occasion of great scientific and cultural growth.

I show gratitude to Prof. Massimo Pugliese from Agroinnova for his valuable technical and scientific support in all the experiments dealing with plant growth in climatic chamber and greenhouse.

I want to acknowledge also Dr. Teresa Lettieri of Joint Research Centre in Ispra (VA) for giving me the opportunity to become part of the temporary staff of Molecular Ecotoxicology and Microbiology Laboratories and to appreciate how the research works in institutions different from university.

I am really grateful to the Chilean Professors Maria Cristina Schiappacasse, Lorena Jorquera and Carminna Ottone for their fantastic support during all my period spent at Escuela de Ingeniería Bioquímica of Pontificia Universidad Católica de Valparaíso (Chile). Their hospitality was just amazing, and they contributed to make me spend one of the most beautiful and constructive periods of my entire life.

Furthermore, I want to express gratitude to all my colleagues, Ph.D. students and Post-docs for their encouragement during the research work. In particular, I thank my colleagues of XXXII Italian Cycle of Doctorate, who undertake with me this challenging experience, and Giulio, who turned out to be a precious companion in both work and everyday life.

Finally, I want to show gratitude to all my family and friends, and to my girlfriend Anna, who have always believed in me. Their moral support has always been fundamental during the most difficult times of the work!

Last but not the least, I am very grateful to Mr Umberto Rossi, who always believed and supported my research work, with the admirable ethical aim of lending

support to the peoples dwelling in areas facing relevant social and ecological issues such as poverty and desertification.

Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

Abs	Absorbance
AD	Anaerobic Digestion
ANOVA	Analysis of Variance
AOB	Ammonia Oxidizing Bacteria
ASV	Amplicon Sequence Variant
ATP	Adenosine Triphosphate
BH	Benjamini-Hochberg correction
BOD_5	Biological Oxygen Demand
CA	Conservation Agriculture
CCI	Chlorophyll Content Index
CEC	Cation Exchange Capacity
CHP	Combined Heat and Power (Cogeneration)
CLPP	Community Level Physiological Profile
CO	Carbon Monoxide
COD	Chemical Oxygen Demand
CRT	Cyclic Reversible Termination
DNA	Deoxyribonucleic Acid

dNTP	Deoxyribonucleotide triphosphate
EC	Electrical conductivity
EC_{50}	Half Maximal Effective Concentration
EDX	Energy-Dispersive X-Ray Spectroscopy
EEA	European Environment Agency
EIOnet	Environmental Information and Observation Network
EPS	Extracellular Polymeric Substance
FA	Fulvic Acids
FAAS	Atomic Absorption Spectroscopy
FAO	Food and Agriculture Organization of the United Nations
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier-transform infrared spectroscopy
GF-AAS	Graphite Furnace Atomic Absorption Spectroscopy
GI	Germination Index
HA	Humic Acids
HGAAS	Hydride Generation Atomic Absorption Spectroscopy
HS	Humic Substances
IAA	Indole Acetic Acid
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectroscopy
IRGA	Infrared Gas Analyser
IUSS	International Union of Soil Sciences
MCE	Mixed Cellulose Esters
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
OTU	Operational Taxonomic Units
PBS	Phosphate-buffered Saline
PCA	Principal Component Analysis

PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PCR-DGGE	PCR - Denaturing Gradient Gel Electrophoresis
PE	Paired End sequencing
PERMANOVA	Permutational Multivariate Analysis of Variance
PFC	Product Function Categories of EU Fertilising Products
PGPB	Plant Growth-Promoting Bacteria
PLFAs	Phospholipid-derived Fatty Acids
Qiime	Quantitative Insights into Molecular Ecology (software)
RDI	Root Development Index
RNA	Ribonucleic Acid
RSG	Relative Seed Germination
SBL	Sequencing by Ligation
SBS	Sequencing by Synthesis
SDGs	Sustainable Development Goals
SLM	Sustainable Land Management
SNA	Single-Nucleotide Addition
SOM	Soil Organic Matter
SS	Sewage Sludge
SSAD	Anaerobic Digestate from Sewage Sludge
TOC	Total Organic Carbon
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TS	Total Solids
TSS	Total Suspended Solids
Tukey HSD	Tukey's Honestly Significant Difference test - Post-hoc in ANOVA
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant

Chapter 1

Introduction

1.1 Environmental issues in the 2020 scenario

In 2015, United Nations adopted a rich agenda that includes 17 Sustainable Goals with the purpose of supporting the sustainable development in society, economy and environment with a fifteen years perspective. Just an integrated approach that takes into account not only the processes leading to their realisation but also the international cooperation and the policy making would lead to the concrete achievement of those aims (United Nations, 2015).

United Nation lesson meets the programmes of many countries and organisations. European Union is one of the most emblematic examples since it seems having perfectly shared and adapted the guidelines linked to environmental concerns like the need of clean water for everybody to the fight against climate change or the protection of the ecosystems of land and water. Within those areas of interest, European Environmental Agency (EEA) and European Environment Information and Observation Network (EIOnet) - institutions for the monitoring and surveillance of the environment and related issues - are contributing in developing, adopting, implementing and evaluating environmental policy and the general public (EEA, 2019). “Sustainability and well-being” and “Nature” are two of the more active research areas explored by the two institutions with particular attention oriented to two main topics: “Resource efficiency and waste” and “Soils”. Within these two broad areas of interest, sewage sludge and soil degradation are undoubtedly emerging issues.

1.1.1 Sewage sludge: production and destinations

1.1.1.1 Description of the waste and generation process

Sewage sludge (SS) is the principal by-product of wastewater treatment processes. Urban wastewater is an effluent water that contains a mixture of residues from human metabolic waste, domestic uses, outdoor run-off and industries. Its

main components are settleable solids, dissolved organic compounds, metals and microorganisms, whose concentrations vary considerably depending on season and location. Wastewater treatment plants (WWTPs) are engineered to remove bulky and suspended solid and to effectively removal contaminants in the effluent, so that the water is clean enough to be returned to natural water bodies. Specifically, the organic load of the wastewater must be strongly reduced and the effluent of WWTP must respect standards in terms of indicators such as chemical oxygen demand (COD), biological oxygen demand (BOD) and total suspended solid (TSS). For instance, Table 1 reports the requirements for discharges from urban wastewater treatment plants in the European Union (Council of the European Communities, 1991).

Table 1.1 Requirements of effluents from WWTP.

The values for concentration or for the percentage of reduction shall be applied. Values for phosphorous and nitrogen are only for the eutrophication-sensitive areas (Adapted from Council of the European Communities, 1991). p.e. population equivalent.

Parameter	Concentration	Minimum reduction in relation to the load of the influent (%)
Biochemical oxygen demand (BOD_5)	25 mg/l O_2	70-90 %
Chemical oxygen demand (COD)	125 mg/l O_2	75 %
Total suspended solids (TSS)	35 mg/l (more than 10000 p.e.)	90 %
	60 mg/l (2000 - 10000 p.e.)	70 %
Total phosphorus (P)	1 mg/l (more than 100000 p.e.)	80 %
	2 mg/l (10000 - 100000 p.e.)	
Total nitrogen (N)	10 mg/l (more than 100000 p.e.)	70 – 80 %
	15 mg/l (10000 - 100000 p.e.)	

To this aim, urban wastewater is commonly collected by sewerage systems and delivered to WWTP, where different treatment processes take place, according to the plant dimension and to the population served. In general, they can be resumed in:

- *Preliminary treatments:* removal of bulky solids, oil, soil and sand.
- *Primary treatment*: physical process for abatement of organic load. This sedimentation step may be improved by the presence of chemicals, enhancing particles agglomeration. BOD_5 and TSS are reduced at least by 20% and 50%, respectively.
- *Secondary treatment*: biological processes for further abatement of organic load. Microbial consortia of activated sludge (*i.e.* suspended growth processes) or immobilised on different support (*i.e.* attached growth processes) actively degrade organic substances.
- *Tertiary treatments*: advanced treatment for removal of residual solids (*e.g.* membrane processes) and nutrients, such as nitrogen (*e.g.* nitration-denitrification process) and phosphorous (*e.g.* chemical precipitation or

biological phosphorous removal), take place before the effluent turns back to groundwater bodies.

1.1.1.2 Sewage sludge processing

Sewage sludge is the principal by-product of wastewater treatment and originates from the primary and secondary treatments. It is a brownish and smelly slurry, with liquid to semisolid texture. Due to its richness in organic substances, it is a putrescible waste that can make SS even more malodorous as the degradation proceeds. In order to guarantee its proper management, water removal and stabilisation of sewage sludge are required.

Dewatering of SS ensures that humidity is reduced to the required levels for SS disposal or reuse. SS dewatering makes mobilisation easier and cheaper, allows SS thermal treatment and stops the decaying process (Campbell and Crescuolo, 1982). Mechanical dewatering is the most diffused technique and is usually performed with devices such as vacuum filters, pressure filter presses and centrifuges, which usually requires a preliminary conditioning step with polyelectrolyte to enhance agglomerating properties. The resulting SS is a shovellable solid with a dry matter content ranging between 15% and 30% (Gurjar and Tyagi, 2017). Further water removal can be obtained by thermal drying, to reach dry matter content up to 95%. This also functions as sterilisation and eliminates almost all viruses and pathogens. SS drying is usually performed by means of direct or indirect dryers or combined mode drying systems. Depending on the drying process used, dried sewage sludge can show different forms, such as granular, pelletized, powdery, and beads of defined and not-defined shape (Lowe 1995; Chen et al. 2002).

SS stabilisation answers simultaneously to two different needs: reduction of sludge odour and putrescence, and abatement of pathogens. At WWTP level, SS stabilisation can take place by means of chemical (*e.g.* lime stabilisation with quicklime or hydrated lime) or biological processes (Peirce et al. 2007). Biological processes are undoubtedly the most exploited ones and are generally classified in aerobic and anaerobic ones. Biological aerobic processes for SS stabilisation require oxygen and occur through aerobic digestion or composting.

Aerobic digestion takes place in digesters, where biodegradable matter is oxidised by microbial consortia, in water and carbon dioxide. Once the “feed” is used up, microorganisms eat the cell structures of other microorganisms; when the digestion is completed, cell tissues have degraded by 80%, where the remainder is composed of inert compounds and recalcitrant cell components, such as cellulose. The process efficiently reduces the SS volume and pathogens (Demirbas et al., 2017; Gurjar and Tyagi, 2017; Zhang et al., 2016). Composting is a process to stabilise sewage sludge as well as waste from agriculture, food, or gardens. This process takes place in piles or containers and the content is previously dewatered (50% dry matter) and mixed with a bulking agent to ensure proper ventilation. The composting process undergoes three phases (mesophilic, thermophilic and curing), eventually yielding an odourless product, rich in humic acids and with good soil conditioner properties (Stentiford and de Bertoldi, 2010).

On the other hand, the most known anaerobic stabilisation process is anaerobic digestion (AD). AD is the most common SS stabilisation strategy in larger WWTPs. The technology is often used to manage SS as well as other types of putrescible waste, such as biomasses from agriculture and food-processing industries, and the organic fraction of municipal solid waste. Despite the high initial investments and maintenance costs, this is currently the only technology that allows simultaneous stabilization and energy recovery through biogas production and combustion (*i.e.* combined generation of heat and electricity (CHP)). The AD process can be summarised in three phases:

- *Liquefaction*: extracellular enzymes are synthesized and secreted by hydrogenotrophic bacteria which decompose the organic matter and makes it more soluble.
- *Acidogenic phase*: soluble organic compounds are catabolised by anaerobic microbial consortium composed of acid forming microorganisms, which drop the pH to 5-6.
- *Methanogenic phase*: methane forming bacteria convert organic acids to methane. These anaerobe microorganisms are very sensitive to oxygen, pH and temperature variation.

Anaerobic digesters must ensure an anoxic environment and remain heated to activate different microorganisms living at moderate-warm temperatures (~35°C) in the case of mesophilic digestion, or microorganisms normally active at higher temperatures (~50°C) in the case of thermophilic digestion. The advantages of thermophilic digestion are higher digestion rates, biogas production, and pathogen removal, while the main drawback is a higher energy demand. In recent years, AD has gained lot of attention, not only in terms of enhancement of process efficiency, but also of improvement of product quality. In facts, many efforts have been made to develop the technologies of upgrading biogas to biomethane, a promising and high added-value fuel, injectable directly into the gas network and utilisable for automotive (Gurjar and Tyagi, 2017; Peirce et al., 2007).

Despite its classification as "product" of sewage treatment in Water Framework Directive (European Parliament and Council of the European Union, 2000), SS is treated and managed as a waste. Therefore, SS must be properly disposed with the aim of encouraging its recycling.

1.1.1.3 Sewage sludge in Europe: production and destinations

As the WWTPs became more efficient in returning cleaner effluent water to the natural hydrological systems (Council of the European Communities 1991), a rise in SS production has been assessed. For instance, between 1992 and 2005, European yearly production of sewage sludge has

Figure 1.1 Statistics of total sludge production, percentage of disposal and sewage sludge destination in European countries.

Data are referred the most recent data available (Eurostat, 2019)

Nation	Year	Total sludge production (kt)	Percentage of disposal
Albania	2015	92	100%
Austria	2014	239	100%
Belgium	2012	157	68%
Bosnia and Herzegovina	2013	1	100%
Bulgaria	2015	57	82%
Croatia	2015	18	96%
Cyprus	2015	7	100%
Czechia	2015	210	100%
Denmark	2010	141	81%
Estonia	2013	19	100%
Finland	2012	141	100%
France	2014	962	97%
Germany	2015	1821	99%
Greece	2014	116	100%
Hungary	2015	157	71%
Ireland	2015	58	100%
Italy	2010	1103	86%
Latvia	2013	23	91%
Lithuania	2015	43	87%
Luxembourg	2015	9	100%
Malta	2015	8	100%
Netherlands	2014	344	93%
Norway	2015	n.a.	n.a.
Poland	2015	568	100%
Portugal	2012	339	33%
Romania	2015	210	74%
Serbia	2015	11	100%
Slovakia	2015	56	100%
Slovenia	2015	29	100%
Spain	2012	1083	100%
Sweden	2014	198	93%
Switzerland	2013	195	100%
United Kingdom	2012	1137	95%

raised from 5.5 to around 9 million tonnes in dry matter (European Commission, 2019). Figure 1.1 gives a general overview of SS production and destination in some selected European countries. For what concerns yearly production, the amount of SS produced obtained in each country is directly proportional to the population. However, this "rule" is not ubiquitous and lower production of sewage sludge often indicates a less extended sewerage network, where other wastewater collection strategies are still adopted (*e.g.* septic tanks, cesspits, pit latrines, drywells), especially in those countries with a relevant rural population. In the case of Romania (45% of rural population), in 2018 total population connected to wastewater collecting systems was 52.7%, while 51.4% had WWTP treating wastewater from sewerage network (INS, 2019).

With regards to SS destinations in Europe (see Figure 1.1; Eurostat, 2019), they can be generally ascribable to five different categories: agricultural use, composting, landfill, incineration and other treatments.

- *Agricultural use*: SS shows interesting fertilizing properties and the agricultural reuse of SS has been allowed and encouraged as a cost-effective strategy to recycle nutrients and improve soil properties (Council of European Communities 1986). This practice is quite diffused in some

European countries (*e.g.* Ireland, Spain, Portugal) and it is regulated by national and international guidelines and regulations. Nevertheless, many nations are still very sceptic due to the presence of pollutants which can severely threaten soil health.

- *Composting*: besides being a stabilization strategy, composting can be also a destination of SS. SS composting mechanism has been described previously, and it can be performed even in presence of other feedstocks (namely *co-composting*). The purpose of the final product is similar to the above-mentioned agricultural use. More in detail, SS compost is mostly exploited in landscaping, parks and gardens (Christodoulou and Stamatelatou, 2016). Moreover, while other typologies of stabilized SS (*e.g.* SSAD, lime stabilized SS) are exploited as fertilizers, compost is mainly used as soil conditioner (Kacprzak et al., 2017). In Europe, countries like Finland, Estonia and Hungary, are the nations where composting is mostly adopted as SS destination.

- *Incineration*: thermal treatments are aimed basically to recover energy and reduce SS volume by means of different available technologies. Conventional incineration and co-incineration are heat treatment processes where SS is burn alone or in presence of other substances (coal, fuel oil or natural gas). By-products of the process are exhaust gases, slags and fly ashes. The last ones may be furtherly recovered to produce cementitious materials. Incineration is the (almost) unique SS destination in European countries such as the Netherlands and Switzerland (Cieślik et al., 2015).

- *Landfilling*: this practice is an old-fashioned approach of waste management where waste is stored in dedicated sites (landfills). Despite its easiness of application, this solution is the less suggested for its negative environmental impact (*e.g.* risk of contamination of adjacent areas and above-ground waters with leachate, CO_2 emissions)(Kacprzak et al., 2017). Moreover, landfilling is the less sustainable solution as it does not consider any kind of recycling and should be limited only where other recycling and reuses approaches are not feasible (Council of the European Union, 1999). However, it is still the principal disposal solution in some European countries (Serbia, Bosnia and Herzegovina, Croatia and Romania).

- *Other destinations*: other kinds of thermal treatment can be adopted, such as vitrification (more than 1000°C in presence of silica) and pyrolysis (absence of oxygen). These technologies are certainly very effective, but they still show the big drawback of the high cost (Cieślik et al., 2015). Ocean dumping is an old-fashioned SS destination, which has progressively been abandoned in recent years due to its tremendous effects on marine ecosystem: in 2015, no European country was practising this kind of SS disposal (Gurjar and Tyagi, 2017).

1.1.2 Soils "under threat"

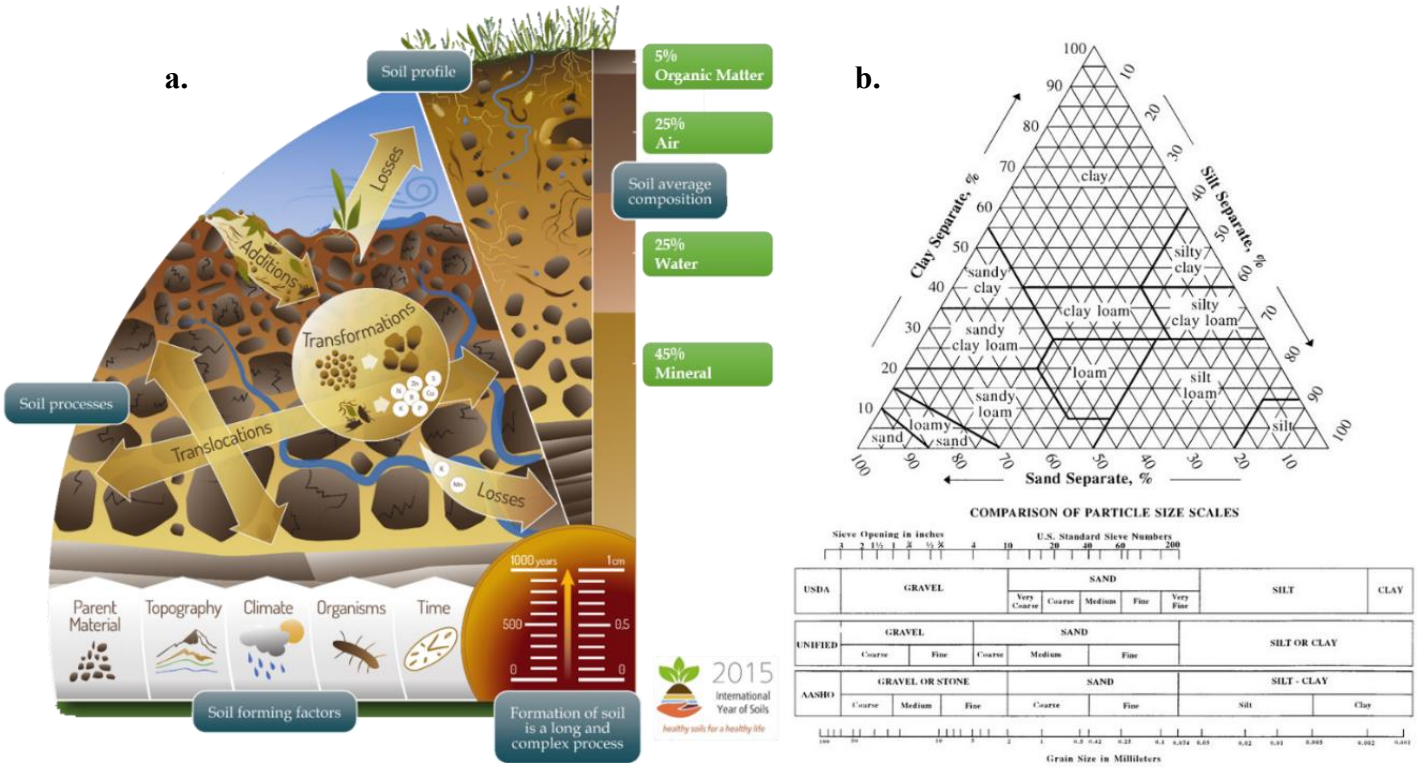
Soil is one of the fundamental components for supporting life on the planet. Soil can be defined as a mixture of rock particles, organic matter, air and water that occupies the uppermost few metres of the Earth's crust. Soil performs a number of key environmental, social and economic functions that are vital for life. The most evident relevance of soil is for plants and crops, which are strictly dependent on soil for the supply of water, nutrients and as a medium for growing. (European Commision, 2005).

1.1.2.1 Soil: properties and functions

Providing complete information about soils' properties and functions in few lines is quite a hard task. The focus of this short paragraph is to provide key concepts on soil to highlight its crucial role in the environment. Soil is composed of organic matter, air, water and mineral, which are combined to form soil aggregates, which can be considered the functional unit of the soil, where different dynamic processes (translocations, transformations, losses and additions) take place (Figure 1.2 a; FAO, 2015a). The formation of soil aggregates is a time-demanding process since around 1000 years are required for the formation of 1 cm of soil. The structured assembling of these aggregates together with the

Figure 1.2 Description of general soil peculiarities in terms of composition and dynamics.

a. Infographics from FAO summarizing various soil peculiarities (FAO, 2015a). b. Particle size distribution in different soil textures.



formation of different soil layers, named horizons, results in an organized soil profile, which is the optimal condition to support soil functions. Beyond this general definition, soil composition is extremely variable and is commonly used as parameter to classify different kinds of soil. The most common soil classification is based on the size distribution of soil particles (without considering gravel): according to their dimension, they are sand, silt or clay, whose percentage defines the soil texture (Figure 1.2 b). Other soil classification systems are as USDA soil taxonomy and the World Reference Base for Soil Resources, which use taxonomic criteria involving not only soil texture, but also soil chemical composition and horizon organization, clustering the different soils in defined hierarchical classes.

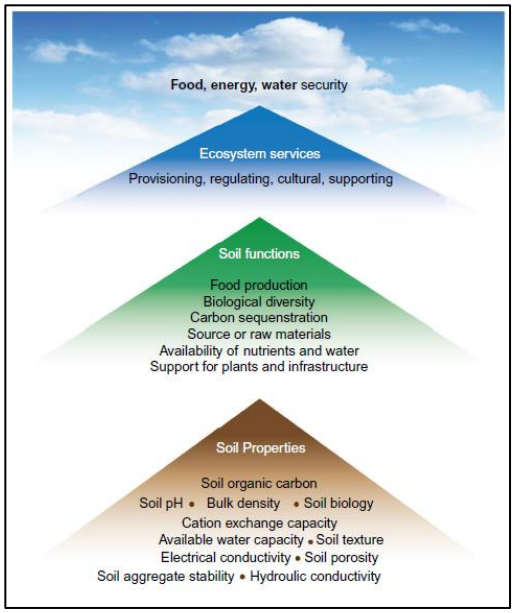
Soil properties are strictly connected with soil composition and they can be summarized in physical, chemical and biological properties. Physical properties include soil bulk density, hydraulic conductivity and available water capacity, soil porosity and soil aggregate stability; chemical properties are mostly related with organic carbon and nutrients presence, soil pH and electrical conductivity; biological properties are really broad and are related to the capacity of supporting life at different levels (microorganisms, fungi, insects and plants) (European Commision, 2005).

Good soil properties are the basis to support soil functions, which ranges from environmental to social and provisional issues:

- *Environmental*: carbon sequestration, climate regulation, nutrient cycling, habitat for organisms, water purification and soil contaminant reduction, flood regulation;
- *Social*: foundation for human infrastructure, cultural heritage;
- *Provisional*: food, fibre, fuel, medicinal products, genetic resources, construction materials.

Figure 1.3 Relationships in soil.

The figure resume the relationships between soil properties, soil functions and ecosystem services (Hatfield et al., 2017).



As explained in Figure 1.3, the soil functions are the mediator between soil properties and ecosystem services. Indeed, the ability of the soil to provide these functions depend obviously on the state of the soil properties; however, this ability is much more complex than a mere list of the soil properties. Our understanding of the linkage between soil properties, soil functions and the resultant ecosystem services is a very complex system and still a lot of research is needed to better understand the insights (Hatfield et al., 2017).

1.1.2.2 Degradation of soil

Although soils are undoubtedly a precious resource, approximately one-third of the planet's soils are degraded. But why? And what is the real meaning of degradation? Soil degradation was firstly defined as "the decline in soil quality caused through its misuse by humans." It is a broad and vague term and refers to a decline in the soil's productivity through adverse changes in nutrient status and soil organic matter and structural attributes. In other words, it refers to a diminution of the soil's current and/or potential capability to produce quantitative or qualitative goods or services, such as growing crops, as a result of one or more degradative processes. The concept seems to be rather simple, but quantifying degradation has been very challenging and the quantification of land degradation is still a debated issue (Hatfield et al., 2017; Lal and Stewart, 1990).

From a pedological point of view, soil degradation has been described by Lal and Stewart (1990)
in terms of physical, chemical and biological factors:

- Physical factors:
    - *Compaction and Hardsetting.* The suppression or decrease of structural pores increases soil bulk density, making soils prone to accelerated runoff and erosion.
    - *Soil Erosion and Sedimentation.* Erosion is usually linked to the weathering effects (wind and water) and, of course, to the anthropogenic action, resulting in loss of organic matter and colloidal fraction of soil. A direct consequence of wind erosion is desertification, known as the spreading of desert-like conditions.
- Chemical Degradation:
    - Nutrient depletion is a major cause of chemical degradation. In addition, excessive leaching of cations causes a drop in soil pH and a reduction in base saturation. Chemical degradation is also caused by the presence of toxic compounds (e.g. organic and inorganic pollutants) and by the elemental imbalance, disadvantageous for plant growth.
- Biological Degradation:
    - Reduction in soil organic matter content, decline in biomass carbon, and decrease in activity and diversity of soil fauna are all theme related with this kind of degradation. It is more diffused at the tropics

since high temperatures favour this process. Biological degradation can also be caused by the excessive use of chemicals and soil pollutants.

But why does soil degradation occur, and which are the consequences? Even in this case, the answer is not trivial. The Food and Agriculture Organization of United Nations is the principal international institution taking care of soil and soil degradation, and tried to approach the problem in an integrated and systematic way (Figure 1.4; FAO, 2015b). Soil degradation starts occurring for the effect of social, environmental and economical drivers and pressures, which have important negative consequences on soil, as described above. This soil status has tremendous drawbacks on society (poverty and insecurity), environment (water scarcity, climate change, reduction of ecosystem services) and population (migration, food and nutrition insecurity). Therefore, taking care of our soils is a really urgent issue.

Figure 1.4 Infographics from FAO summarizing drivers, types and consequences of soil degradation.

Besides the soil problems, also a general overview of sustainable soil management solutions is listed (FAO, 2015b).

1.1.2.3 Solutions to face soil degradation

Soil degradation is really problematic but, in many cases, is not an insurmountable problem. An interesting article on *The Lancet* affirms that only an integrated approach to the different soil degradation issues can bring effective solutions, involving a mix of scientific, local, and indigenous knowledge (The Lancet Planetary Health, 2018). To date, plenty of strategies have been proposed and adopted to combat land degradation, and some of them are summarized in Figure 1.4. However, three relevant initiatives by FAO deserves attention and will be here briefly reported.

The first example is the "Sustainable Land Management" (SLM), defined by UN as "the use of land resources, including soils, water, animals and plants, for the production of goods to meet changing human needs, while simultaneously ensuring the long-term productive potential of these resources and the maintenance of their environmental functions". It involves a holistic approach to achieving productive and healthy ecosystems by integrating social, economic, physical and biological needs and values, and it contributes to sustainable and rural development. SLM includes many practices to fight land degradation under different point of views, restoring degraded soils (management of forests, rainforests, pastoralism and rangeland), improving soil-water storage (*e.g.* rainwater harvesting, smallholder irrigation management) and promoting soil carbon sequestration (integrated soil fertility management, conservation agriculture), just to cite a few.

The second example is Conservation Agriculture (CA), that is a farming system that can prevent losses of arable land while regenerating degraded lands. CA principles are both universal, since they can be applied to all agricultural landscapes, and specific, since they can shape on locally adapted practices. Soil interventions (*e.g.* mechanical soil disturbance) are strongly reduced or even avoided, and agrochemicals and fertilizers are quantified and used optimally in order to not affect the biological processes. CA facilitates good agronomy and improves overall agriculture for rainfed and irrigated production. CA is a solid base for sustainable agriculture when exploited synergistically with other known good practices, including the use of quality seeds, and integrated pest, nutrient, weed and water management. It opens increased options for integration of production sectors, such as crop-livestock integration and the integration of trees and pastures into agricultural landscapes.

Conservation Agriculture is based on three main principles adapted to reflect local conditions and needs:

- *Minimum mechanical soil disturbance*: low soil-disturbing techniques such as no-tillage and direct seeding. The disturbed area must be less than 15 cm wide or less than 25% of the cropped area.
- *Permanent soil organic cover*: maintaining a protective layer of vegetation on the soil surface. It suppresses weeds, protects the soil from the impact of extreme weather patterns, helps to preserve soil moisture, and avoids compaction of the soil.

- *Species diversification*: crop rotation with at least three different crops promotes nutrient cycling and improved plant nutrition and helps to prevent pests and diseases.

Conservation Agriculture is 20 to 50% less labour intensive and thus contributes to reducing greenhouse gas emissions through lower energy inputs and improved nutrient use efficiency. At the same time, it stabilizes and protects soil from breaking down and releasing carbon to the atmosphere. Conservation Agriculture provides a number of advantages on global, regional, local and farm level such as sustainability, enhanced biodiversity, carbon sequestration, labour savings, healthier soils, increased yields and reduced costs. (Corsi, 2019)

The last example is more focused on the nutrient depletion issue and deals with the Global Soil Partnership (GSP). The mandate of the GSP is to improve governance of the limited soil resources of the planet in order to guarantee agriculturally productive soils for a food secure world, as well as support other essential ecosystem services. Within all the initiatives promoted by this programme, one of the most interesting is the adoption of the International Code of Conduct for the Sustainable Use and Management of Fertilizers, developed in response to the request to increase food safety and the safe use of fertilizers. The Fertilizer Code aims to address issues of global importance, thereby contributing to the implementation of the Sustainable Development Goals (SDGs). The Code aims to guarantee an effective and efficient use of fertilizers, putting an important milestone in the fight against soil nutrient depletion. In particular, several indications on fertilizers management are given with the scope of optimizing their effective and efficient use to meet agricultural demands while minimizing nutrient losses to the environment, preserving ecosystem services and minimizing environmental impacts from the use of fertilizers (air, soil and water pollution), promoting safe recycling of nutrients for agricultural and other land uses to reduce the environmental and human, animal and soil health impacts of excess nutrients in the biosphere, atmosphere and hydrosphere. (FAO, 2019)

1.2 The convergence of two concepts: land application of sewage sludge

Land application is one of the most common destinations of sewage sludge. In the following paragraphs some details on chemical nature of sewage sludge will be provided, trying not only to highlight its chemical features but also to link them with their agronomic function. Successively, some information on examples of direct land application of SS will be provided.

1.2.1 Chemical and physical features of sewage sludges, and their agronomic interest

Sewage sludge contains a varied quantity of different chemical substances: most of them are nutrients necessary for plant growth, but others (*e.g.* heavy metals) are phytotoxic. More in detail, it should be noted that all the nutrients are required

by plants in optimal dosages since an excess normally causes toxicity, while a deficiency usually damages metabolism and physiological functions of plants. In order to briefly summarize the elements present in SSAD, minimum, maximum and mean values of some elements contained in SSADs deriving from 10 WWTPs located in the Mediterranean area (Spain, Portugal, France and Greece) are presented in Table 1.2. The data derive from scientific works published in the last fifteen years (Alvarenga et al., 2007; Bourioug et al., 2015; Carbonell et al., 2009; De Andres et al., 2010; Ferreiro-Domínguez et al., 2011, 2012; Fuentes et al., 2004; Koutroubas et al., 2014; Tarrasón et al., 2008; Walter et al., 2006). Organic matter is the principal component of sewage sludge and covers also very important functions in all soil processes: it acts as a depository for nutrients, it reduces soil compaction, it enhances micro and macropores, it increases microbial population and activity, and it rises cation exchange capacity (CEC) and soil water retention. The positive effect on soil physical properties improves the plant root environment. In this way, plants are better able to utilize nutrients, to extract water and to tolerate dryness (Jones et al., 2005). Besides organic matter, important roles are played by other two categories of chemicals: plants macro- and micro-nutrients, and organic and inorganic toxic compounds.

1.2.1.1 Plant macro and micro-nutrients in sewage sludge

Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) are elements today considered macronutrients (Jones Jr., 2012). Nitrogen, in its different forms (*e.g.* ammonium (NH_4^+), nitrate (NO_3^-)), is involved in many essential plant processes and functions such as amino acid synthesis and, consequently, proteins formation. Furthermore, nitrogen is a fundamental compound of nitrogenous bases of nucleic acids (*e.g.* deoxyribonucleic acid (DNA), ribonucleic acid (RNA)) and it is present in chlorophyll and some vitamins (*e.g.* B vitamins). Nitrogen can be present in good concentrations in SS, which easily suggests its reuse as soil conditioner.

Phosphorus is present in sewage sludge in appreciable amounts. In plants, it is a basic compound of adenosine triphosphate (ATP), which is the most important metabolic “energetic carrier”. Besides, it is present in nucleic acids and in enzymes and proteins with post-translational modifications.

After nitrogen and phosphorus, potassium is the last of the mostly known “fertilizer elements” because of their high presence in commonly formulated chemical fertilizers (Jones Jr., 2012). Potassium is involved in the control of water balance in plants by changing cell turgor pressure and by opening or closing stomata leaf. Furthermore, it is essential for the accumulation and translocation of carbohydrates. Calcium plays important roles in cell wall structure, cell membrane phenomena (such as cell permeability), and signal transduction. Moreover, it improves pollen germination and growth, and it is responsible of the activation of the enzymes required for cell mitosis, division, and elongation.

Table 1.2 Minimum, maximum and mean values of SSAD chemical components.

O.M.: organic matter; d.m.b.: dry matter basis.

Parameters		Minimum value		Maximum value		Mean value
O.M.	(% d.m.b.)	37.4	(Koutroubas et al., 2014)	83.5	(Bourioug et al., 2015)	52.9
N	(% d.m.b.)	1.8	(Koutroubas et al., 2014)	7.2	(Bourioug et al., 2015)	3.7
P	(% d.m.b.)	0.25	(Koutroubas et al., 2014)	3.90	(Bourioug et al., 2015)	1.86
K	(% d.m.b.)	0.13	(Alvarenga et al., 2007)	1.54	(Koutroubas et al., 2014)	0.48
Ca	(g/kg d.m.b.)	3.2	(Ferreiro-Domínguez et al., 2011)	82.7	(Carbonell et al., 2009)	40.9
Mg	(g/kg d.m.b.)	3.4	(Alvarenga et al., 2007)	11.7	(De Andres et al., 2010)	7.9
Mn	(g/kg d.m.b.)	0.006	(Ferreiro-Domínguez et al., 2012)	0.359	(Ferreiro-Domínguez et al., 2011)	0.178
Fe	(g/kg d.m.b.)	13.9	(Ferreiro-Domínguez et al., 2011)	28.3	(Carbonell et al., 2009)	21.3
Cd	(mg/kg d.m.b.)	0.4	(Carbonell et al., 2009)	18.3	(Fuentes et al., 2004)	4.7
Cr	(mg/kg d.m.b.)	25.5	(Walter et al., 2006)	3809	(Fuentes et al., 2004)	559.3
Cu	(mg/kg d.m.b.)	142.7	(Ferreiro-Domínguez et al., 2012)	843.8	(Bourioug et al., 2015)	351.6
Hg	(mg/kg d.m.b.)	0.78	(Carbonell et al., 2009)	2.6	(Tarrasón et al., 2008)	1.7
Ni	(mg/kg d.m.b.)	14.7	(Carbonell et al., 2009)	142.8	(Ferreiro-Domínguez et al., 2011)	46.6
Pb	(mg/kg d.m.b.)	15.9	(Bourioug et al., 2015)	167	(Fuentes et al., 2004)	93.5
Zn	(mg/kg d.m.b.)	429.5	(Carbonell et al., 2009)	7620	(Alvarenga et al., 2007)	1557

In plant cell, magnesium ions have a specific role in the activation of enzymes involved in respiration, photosynthesis, and DNA and RNA synthesis. Furthermore, magnesium is a fundamental element in chlorophyll structure.

In plants, sulphur is present in two amino acids (cysteine and methionine) and it is a constituent of several compounds essential for the metabolism such as coenzymes (*e.g.* Coenzyme A) and vitamins (*e.g.* Vitamin B_1). None of the considered SS showed a sulphur quantification, but is assumed that this element is present many compounds of SS (Dewil et al., 2008).

Micronutrients boron (B), chlorine (Cl), molybdenum (Mo), iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) are found and required in relatively low concentrations in plants compared to the macronutrients. Usually they are not provided by most conventional chemical fertilizers (Lu et al., 2012). Generally, boron, chlorine and molybdenum are not quantified in scientific works on SS, but their medium to low concentrations in SSAD has already been assessed (Epstein, 2002). Nevertheless, boron presence in SSADs is certain and its role in plant deals with cell elongation and nucleic acid synthesis (Chu and Poon, 1999). Chlorine is necessary on a specific part of photosynthesis reaction. Molybdenum is a component of many enzymes such as nitrate reductase (a catalyst promoting cell assimilation through the reduction of nitrate to nitrite) and nitrogenase (responsible for molecular nitrogen formation) (Epstein, 2002; Nelson and Cox, 2013).

Manganese ions are cofactors of different enzymes families. For instance, manganese is required by decarboxylases and dehydrogenases taking part in tricarboxylic acid cycle (energetic metabolism), and superoxide dismutase, involved in protection from damage of reactive oxygen species. Last but not the least, manganese is part of a catalytic cluster present in the oxygen evolving-complex, which allows the water-splitting activity, that is the very first step of photosynthesis (Nelson and Cox, 2013).

Iron is a relevant component of prosthetic groups, such as heme groups and iron-sulphur clusters, of redox proteins like cytochromes and ferredoxin, respectively. More in detail, *cytochrome* b_6f complex and *ferredoxin* are involved in electron transfer of redox reactions occurring during the photosynthesis (Willows, 2006).

Copper and Zinc are considered both micronutrients, at low concentrations, and contaminants, when in excess. Similarly to iron, copper is associated to enzymes responsible of redox reactions, while zinc is necessary as cofactor of other several enzymes. Due to the high concentrations of these elements in sewage sludge, the European Community limited their concentration in sewage sludge for agricultural use (Council of the European Communities, 1986).

1.2.1.2 Organic and inorganic toxic compounds in sewage sludge

The main drawback of SS exploited as fertilizer is the presence of both organic and inorganic contaminants. Organic contaminants in SS are decisively diverse. To give an idea, a Chinese study of 2016 reported thirteen categories of organic pollutants in Chinese sewage sludges: phthalate esters, alkylphenol polyethoxylates, synthetic musks, antibiotics, polycyclic aromatic hydrocarbons, ultraviolet stabilizers, bisphenol analogs, organochlorine pesticides, polybrominated diphenyl ethers, pharmaceuticals, hormones, perfluorinated compounds, and polychlorinated biphenyls (Meng et al., 2016). Some of these compounds can be broken down with treatments, but some of them require a long time for degradation and (if SS is land applied) they can accumulate and generate adverse effects at various trophic levels (Díaz-Cruz et al., 2009; Harrison et al., 2006; Kolpin et al., 2002). Therefore, presence of other organic contaminants in SSADs and their effects on plants still require much more study (Kolpin et al., 2002).

Heavy metals are undoubtedly another "dark side of the moon" aspect concerning land application of SSAD. Normally, SSAD is analysed to reveal and quantify the presence of cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn). Mean heavy metals presence and concentration is summarized in Table 1.2. Taking into account the phytotoxicity (*e.g.* Pavel et al. 2013) and the possible environmental damages, the quantification of these chemical species in SSAD is mandatory prior to agricultural use (European Commission 1986). Moreover, their values must comply with the thresholds imposed by policy makers. Once applied, heavy metals have multiplex effects: firstly, they accumulate in soil, where they are more or less bioavailable depending on different conditions (*e.g.* soil pH, oxidation number, presence of other chemicals). Secondly, they can be absorbed by plants: at low levels, a simple bioaccumulation occurs, often with no specific evident effect. As the heavy metals' concentration increases, phytotoxic effects emerge, such as growth retard and inhibition of iron translocation (copper, nickel, zinc), and reduced root development (hexavalent chromium). To this end, a great deal of attention needs to be paid when plants grown in presence of SSAD are exploited for animal nourishment or human nutrition (Epstein, 2002).

1.2.2 An outlook on land use of sewage sludge

In order to meet proper conditions for agronomic use, SS must necessarily observe two requirements: undergoing to above-mentioned stabilization processes and comply to laws on SS land application (Lu et al., 2012). The objective of legislation is the environmental preservation from possible contamination derived from potential toxic materials. Generally, several countries (*e.g.* in Europe: Council Directive 86/278 (Council of the European Communities, 1986); in the USA: 40 CFR Part 503 (Environmental Protection Agency, 2005)) regulate the agricultural use of sewage sludge depending on: quantity of nutrients, heavy metals and organic contaminants contained in SS, characteristics of disposal area (*e.g.* physical and chemical characteristics of the soil), quantity of human pathogens in SS (*e.g.* *Salmonella* spp. and *Escherichia coli*) and performances of mandatory treatments of sewage sludge.

Depending on the degree of dewatering, SS can be land-applied in solid, semisolid or liquid form. Furthermore, treatment techniques can change modality of application. For these reasons, SSAD can be incorporated to the soil, injected below land surface or sprayed or spread onto land surface (Zain et al., 2002). In order to reduce smell, it is better to incorporate or inject SSAD below land surface. Several literature examples demonstrate that land application of SS increase plants biomass growth. Since from ancient times, fertilizing properties of animal manure and human metabolic wastes were known; in fact, these feedstocks were extensively exploited as source of nutrients to fertilize soils before the massive use of synthetic fertilizers (Adjei and Rechcigl, 2002). Many scientific works dealt with this topic, carrying out trials in greenhouse (Perez-Murcia et al., 2006) or in open field (Singh and Agrawal, 2010). First scientific work was published by Bartow and Hatfield in 1916 (Bartow and Hatfield, 1916): they compared the biomass yields of lettuce and radish grown on a SS amended soil with a not treated one. The results of this work demonstrated for the first time the fertilizing effect of SS. From this moment forward, the research on this topic have multiplied, investigating combination of types and dosages of SS, soils, plants species and many other variables. In last years, researches became more and more specific, even including many examples of SS usage, *e.g.* with barley (Antolín et al., 2005), kenaf (De Andres et al., 2010) and wheat (Koutroubas et al., 2014). Even in these cases, biomasses produced were increased when SS was used in an appropriate dosage.

However, it must be remarked that, as previously said, environment absorbs some quantities of potential toxic materials. Some of these materials are degraded over time, but others accumulate in soil, groundwater, and plants. Nevertheless, in some conditions minimize negative effects of SSAD can be minimized (such as toxicity) as well as positive ones can be maximized. Several reports support the use of SSAD on alkaline soils and low organic matter soils since the toxic effects of heavy metals can be minimized. In fact, Cu, Cd, Mn, Ni and Zn are less bioavailable for plant absorption at basic soil pH (Antolín et al., 2005; García-Gil et al., 2004; Healy et al., 2016; Navas et al., 1998). Organic matter can absorb many heavy metals derived from SSAD application but, subsequently, the gradual

mineralization of organic matter can release these metals into more soluble forms. In this way, the metals will be absorbed by plants causing a possible phytotoxicity. This effect is called "sludge time bomb" (Chang et al., 1997; McBride, 1995; Mosquera-Losada et al., 2017; Parat et al., 2005). Moreover, the SS application on a soil composed by clay minerals such as Na- or Ca- bentonite (or their subsequent addition to the soil) can reduce the bioavailability of heavy metals. This effect can be explained by the absorption of heavy metals on clay materials (Usman et al., 2005).

Reclamation and restoration of disturbed or contaminated lands can be another case of maximization of SSAD positive effects. Lands revegetation using SSAD on old mines (zinc, coal, copper etc.) was largely studied by Sopper (Sopper, 1993): he showed that SSAD application can be useful for a rapid reclamation of this places avoiding problems of human contamination with pathogens. In fact, SSAD improves the soil physical properties and releases all nutrients that are necessary to the establishment of plants. This fact is an advantage in degraded places, where revegetation and restoration are traditionally more difficult. Another interesting example of this kind of use was experimented in central Spain: different doses of SSAD were applied on a degraded semiarid land. After one year, total plant cover and total biomass yield were significantly increased (Walter et al., 2000).

Finally, it is possible to summarize that SS is actually considered a waste, but its use on lands could improve soils characteristics and plants growth. Furthermore, the benefits for its application can be maximized on poor, disturbed, alkaline and clay soils.

1.3 Microbial communities.

1.3.1 The concept of microbial community and its role in rhizosphere

A microbial community can be defined as a reunion of interacting bacteria present in a defined habitat. Despite the small size, microorganisms are the key elements for the biogeochemical dynamics on Earth because they are characterized by an incredible functional and genetic diversity that makes them excellent adaptors even to extreme environments (Pepper et al., 2014).

Among all the microbial communities present in the different environment, soil microbial communities have a relevant importance, and more in detail the ones of rhizosphere play keys roles. The word 'rhizosphere' was introduced by Lorenz Hiltner in 1904 to describe the influence of root exudates on the proliferation of soil microorganisms around and inside roots. Since then, much has been discovered about the interactions between soil microorganisms, rhizosphere colonists and plant hosts. Rhizodeposit nutrients and plant exudates provide a fascinating source of food for soil microorganisms, which join the rhizosphere realizing a profitable symbiosis with the plant. Thus, plants exert a great influence on the diversity of microorganisms of rhizosphere as well as they can influence the plants by releasing different compounds useful for plant growth. Hence, microbes dwelling the

rhizosphere are considered a well-developed external functional environment for plants and they are considered the second genome of plants. As a consequence, the further understanding the actual desirable functions of rhizosphere microbiome for plant health and growth is necessary (Hirsch and Mauchline, 2012; Igiehon and Babalola, 2018).

Within the different microorganisms composing the plants rhizosphere, the most interesting ones are the Plant Growth Promoting Bacteria (PGPBs), which are bacteria capable of sustain plant growth in different ways, as shown in Figure 1.5. More in detail, the roles of PGPBs can be practiced directly, boosting resources acquisition by plants and regulating plant phytohormones levels, and indirectly, acting as biocontrol agents with inhibitory effects of pathogens. Direct functions of PGPB include molecular nitrogen fixation and general sustainment of nitrogen cycle, solubilization of insoluble forms of phosphorus and sulphur, and iron sequestration through the production of siderophores. Moreover, PGPBs are responsible for production of phytohormones influencing the plant growth (cytokinins, gibberellins and auxins, like indole-3-acetic acid) or the plant conditions during biotic stress (ethylene).

Figure 1.5 Roles of Plant Growth Promoting Bacteria (PBPBs) at level of rhizosphere.

Biofertilization effects (green arrows) act at the nutrition level. Biostimulation effects (yellow arrows) are related to the action of phytohormones and signaling components. Biocontrol effects (blue arrows) are related to the antagonism with plant pathogens (Ferreira et al., 2019).

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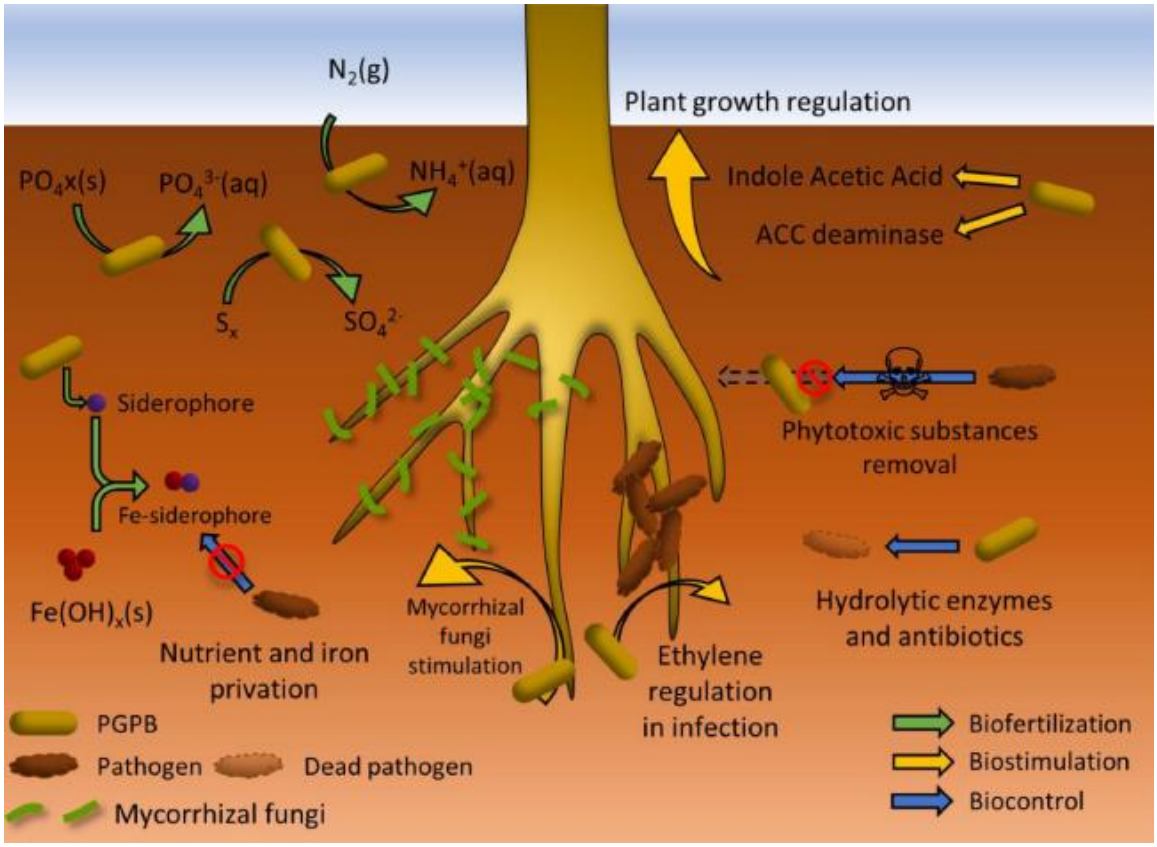


On the other hand, indirect functions include the production of antibiotics and lytic systems, competition and antagonisms with pathogens and induction of systemic resistance in plants. Amongst all the bacteria, some genera turned out to be the real protagonist of the plant-rhizosphere symbiosis, such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Pantoea*, *Pseudomonas* and *Rhizobium*. The growing relevance of these bacteria and their activity is not merely related to a scientific interest, but also to a business opportunity. Indeed, in recent years, some of these PGPBs have been introduced in the formulation of bio-based fertilizers present on the market. For the more extensive commercialization of PGPB strains, a number of issues need to be further investigated, such as the determination of the relevant traits of each strain and which strains are more suitable for the different plant, consistency among regulatory agencies in different countries regarding the release of bacteria in the environment, and a deeper knowledge of the potential interactions between PGPB and other interesting soil promoting microorganisms, such as mycorrhizae (Ferreira et al., 2019; Glick, 2012).

1.3.2 Generations of DNA sequencing strategies

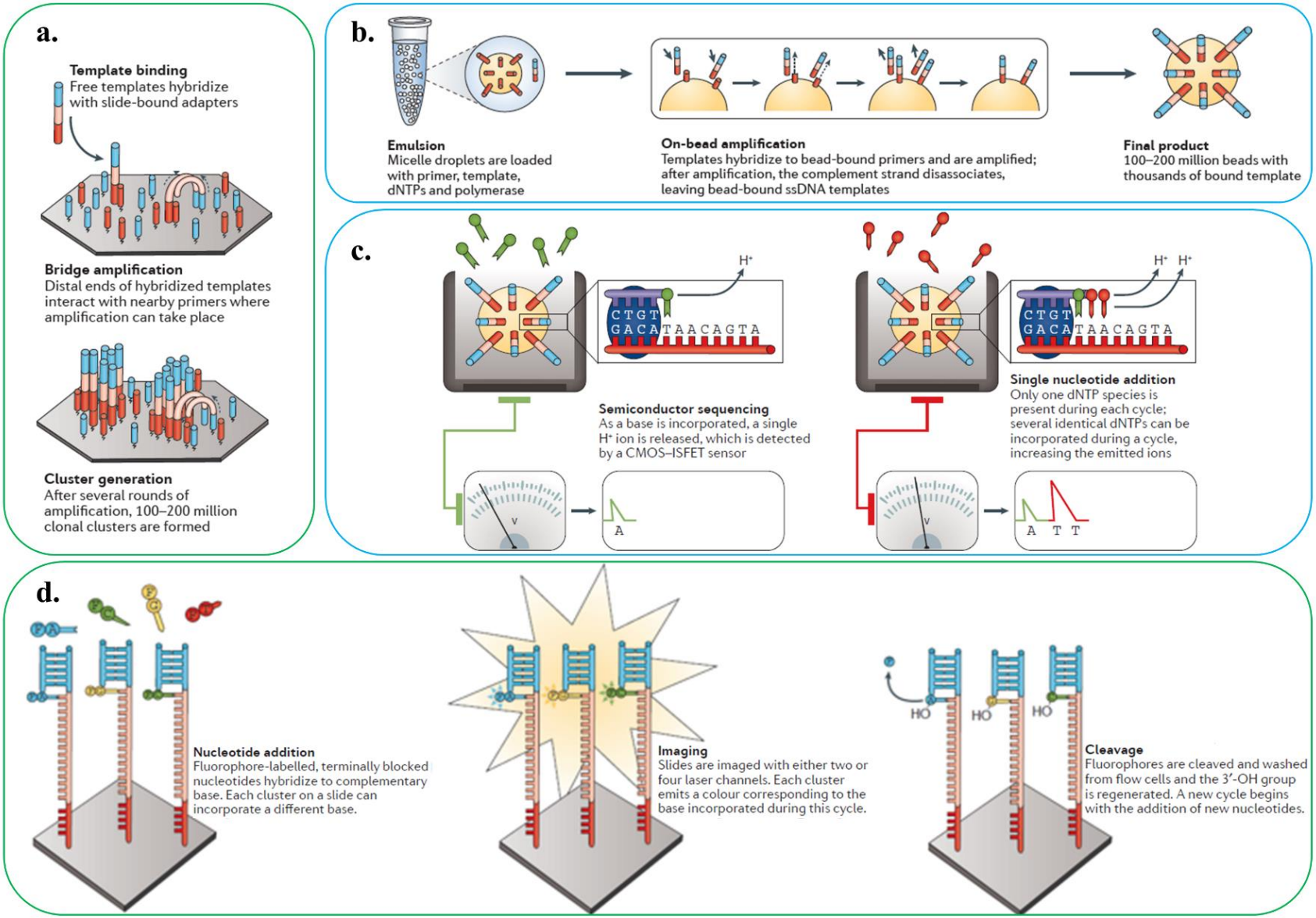
A promising technique for the investigation of the microbial communities is next generation sequencing (NGS), which is a molecular technique based on the reading of portions of the genetic code of the microorganisms belonging to the analysed samples. Hence, in this paragraph, few details about DNA and its sequencing are briefly provided.

The order of nucleic acids in polynucleotide chains contains the information for the hereditary and biochemical properties of terrestrial life. DNA is a polymer consisting of monomers called nucleotides characterized by the presence of a nitrogenous base in the structure. The single nucleotide is the basis of genetic information and the sequential reading of nucleotides is the key for the interpretation of this kind of information (Alberts et al., 2015).

The major breakthrough that forever altered the progress of DNA sequencing technology came in 1977, with the development of Sanger's ‘chain-termination’ or dideoxy technique by two Nobel laureates (Sanger et al., 1977). Sanger method has dominated the industry for almost two decades leading to a number of monumental achievement including the achievement of human genome sequence (Collins et al., 2004). However, Sanger sequencing is expensive and inefficient for larger-scale projects, such as the sequencing of an entire genome or metagenome. For similar tasks, new, large-scale sequencing techniques are faster and less expensive. The automated Sanger method is considered as a ‘first-generation’ technology, and newer methods are referred to as next-generation sequencing (NGS). NGS is based on a high-throughput approach and its major advantage is the ability to produce rapidly and cheaply an enormous volume of data, in some cases in excess of one billion short reads per instrument run, through a parallel sequencing strategy. The NGS’s technologies refers to two main categories that are already known as sequencing by ligation (SBL) and sequencing by synthesis (SBS)(Metzker, 2010).

Figure 1.6 Technical details of IonTorrent and Illumina NGS technologies.

The figure reports some details about template amplification strategies and signal detection from the sequencing by synthesis in IonTorrent (blue boxes) and Illumina (green boxes) platforms. IonTorrent exploits Emulsion PCR as amplification strategy (b.) and the sequence is read via single nucleotide addition by change in pH, detected by an integrated complementary metal-oxide semiconductor (CMOS) and an ion-sensitive field-effect transistor (ISFET) (c.). IonTorrent exploits solid-phase bridge amplification as amplification strategy (a.) and the signal of the cyclic reversible termination is detected via the fluorophore cleaved in each cycle from the inserted nucleotide. (Adapted from Goodwin et al., 2016).



The main difference between the SBL and SBS approach is that the first one is a DNA-ligase-dependent method in which a probe sequence is bound to a fluorophore that hybridizes to a DNA fragment and is ligated to an adjacent oligo-nucleotide for imaging, while the second one is a DNA-polymerase-dependent method in which a fluorophore or a change in ionic concentration identifies the incorporation of a nucleotide into an elongating strand. SBS approaches are classified either as single-nucleotide addition (SNA) and cyclic reversible termination (CRT). Example of technologies based on SNA are 454-Pyrosequencing and Ion Torrent, while Illumina is based on the CRT one (Goodwin et al., 2016). Figure 1.6 reports some technical details about the mechanisms of the last two technologies.

Pyrosequencing is a method of DNA sequencing based on the SNA principle, in which the sequencing is performed by detecting the nucleotide incorporated by a DNA polymerase. Pyrosequencing relies on light detection based on a chain reaction when pyrophosphate is released. Hence, the name pyrosequencing. In comparison to pyrosequencing, the Ion Torrent approach (Figure 1.6 b. and c.) detects the H^+ ion that are released as a single deoxynucleotide (dNTP) is incorporated in a strand. This causes a change in the pH that is proportional to the number of nucleotides incorporated. The signal is detected by a sensor made of a metal-oxide-semiconductor and an ion-sensitive transistor. The Ion Torrent was the first NGS platform without optical sensing (Goodwin et al., 2016; Rothberg et al., 2011).

CRT approaches are based on terminator molecules that are similar to that one used in Sanger sequencing in which the ribose 3´-OH group is blocked, thus preventing elongation (Guo et al., 2008; Ju et al., 2006). To begin the process, a DNA template is primed by a sequence that is complementary to an adapter region, which will initiate polymerase binding to this double-stranded DNA (dsDNA) region. During each cycle, a mixture of all four individually labelled and 3´-blocked dNTPs are added. After the incorporation of a single dNTP to each elongating complementary strand, unbound dNTPs are removed, and the surface is imaged to identify which dNTP was incorporated at each cluster. The fluorophore and blocking group can then be removed and a new cycle can begin. Illumina platform is actually the “golden standard” within CRT approaches (Figure 1.6 a. and d.) (Goodwin et al., 2016).

1.3.3 16S gene: the target of molecular investigation of microbial communities

Soil microorganisms carry out important processes, including support of plant growth and cycling of carbon and other nutrients. However, the majority of soil microbes have not yet been isolated, and their functions are largely unknown.

Microbes have traditionally been characterized by studying individual strains cultivated in the laboratory using various techniques such as traditional plate counting and direct counting method of fluorochrome stained cells under epifluorescent microscope (Kirchman et al., 1982; Nannipieri et al., 2017).

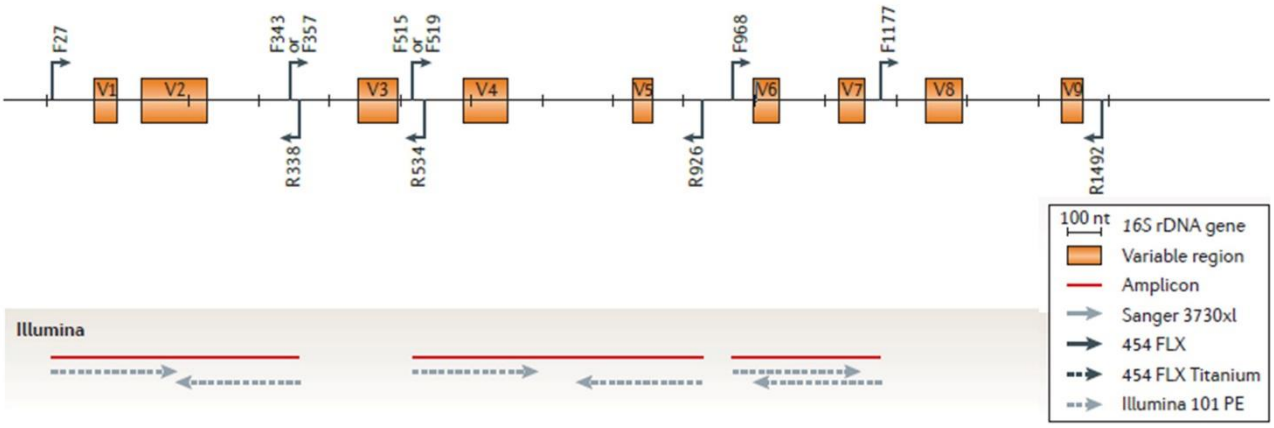
However, these methods are limited by slow-growing rate of the microorganisms, resistance to grow in conventional media and numerical irrelevance in their natural communities. For these reasons, molecular methods, such as the sequencing, have become increasingly useful as tools to better examine the soil bacteria and bacterial diversity.

Most of the methods are based on analysis of 16S rRNA gene, which is a preferred target gene for describing microbial diversity and for establishing phylogenetic relationships between unknown and uncultivated microorganisms. 16S ribosomal RNA is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16S rRNA gene and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene (Woese and Fox, 1977). The 16S rRNA gene is used for phylogenetic studies (Weisburg et al., 1991) as it is highly conserved between different species of bacteria and archaea (Coenye and Vandamme, 2003).

In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria (Kolbert and Persing, 1999; Pereira et al., 2010). As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification (Clarridge, 2004). Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species (Lu et al., 2009) or genera (Brett et al., 1998). It has also been used to describe new species that have never been successfully cultured (Gray and Herwig, 1996; Schmidt and Relman, 1994). With third-generation sequencing coming to many labs, simultaneous identification of thousands of 16S rRNA sequences is possible within hours, allowing metagenomic studies, not only for soil microbial communities but also for microorganisms relevant for human health, such as gut microbiota (Sanschagrin and Yergeau, 2014).

Figure 1.7 Structure of 16S RNA gene in prokaryotes.

The figure shows how the gene is structured with variable regions in red, flanked by conserved regions. The variable regions are the ones targeted to catch the phylogenetic and taxonomic information of species. On the other hand, the flanking regions are conserved and are exploited to design primers to read the variable regions. The bottom part of the figure reports an example of amplification for sequencing with the Illumina platform (Kuczynski et al., 2012).



The bacterial 16S gene (Figure 1.7; Kuczynski et al., 2012) contains nine hypervariable regions (V1–V9), ranging from about 30 to 100 base pairs long, that are involved in the secondary structure of the small ribosomal subunit (Gray et al., 1984). The degree of conservation varies widely between hypervariable regions, with more conserved regions correlating to higher-level taxonomy and less conserved regions to lower levels, such as genus and species. In this study the Illumina platform was used because it produces reads at rates 50-fold and 12,000-fold less expensive than pyrosequencing and Sanger sequencing strategy, respectively (Bartram et al., 2011). Full hypervariable regions can be assembled from a single Illumina run making this technology ideal for rapid microorganisms identification (Burke and Darling, 2016).

1.4 Helping soils with sustainable resources

1.4.1 Biostimulants, a new promising category of fertilizing products

As explained previously, some of problems related to soil degradation are nutrient imbalance and loss of organic carbon. Together with the adoption of wise land management strategies, a way to combat this problem is the use of substances helping soil health generally named "fertilizers".

Table 1.3 Product Function Categories according to European Union.

(source: European Parliament and Council of the European Union, 2019)

Product Function Categories (PFC)	Sub-categories	Definition
Fertiliser	Organic fertiliser Organo-mineral fertiliser Inorganic fertiliser	A fertiliser shall […] provide nutrients to plants or mushrooms.
Liming material	-	A liming material shall […] correct soil acidity.
Soil improver	Organic soil improver Inorganic soil improver	A soil improver shall […] maintain, improve or protect the physical or chemical properties, the structure or the biological activity of the soil to which it is added.
Growing medium	-	A growing medium shall […] product other than soil in situ, the function of which is for plants or mushrooms to grow in.
Inhibitor	Nitrification inhibitor Denitrification inhibitor Urease inhibitor	An inhibitor shall […] improve the nutrient release patterns of a product providing plants with nutrients by delaying or stopping the activity of specific groups of micro-organisms or enzymes.
Plant biostimulant	Microbial plant biostimulant Non-microbial plant biostimulant	A plant biostimulant shall […] stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving […] nutrient use efficiency, tolerance to abiotic stress, quality traits o availability of nutrients […].
Fertilising product blend	-	A fertilising product blend shall be […] composed of two or more EU fertilising products of PFC […].

However, the definition of fertilizing product is complex and diverse; for instance, Table 1.3 gathers the different "Product Function Categories" which specify the standards of each fertilizing product, according to the recently released regulation of European Union (source: European Parliament and Council of the European Union, 2019).

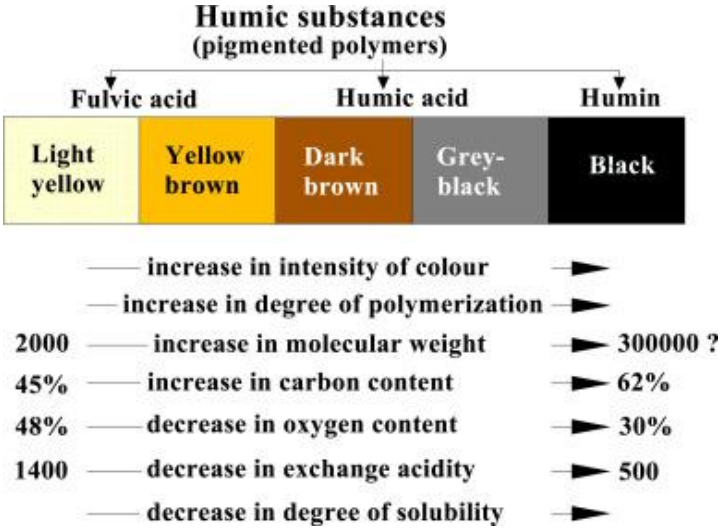
Within the different categories, plant biostimulants are acquiring even more interest by market and producers as well as by the scientific communities. Plant biostimulants were firstly defined in 1997 as "materials that promote plant growth in minute quantities"; this definition has been improved successively to distinguish them from other fertilizing products (*i.e.* fertilizers, soil improvers) used at higher dosages (Kauffman et al., 2007). Nowadays, the generally accepted definition of biostimulants is "any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content". Besides these features, other common traits of biostimulants are diversity of their nature, and effectiveness on different cellular mechanisms and physiological functions of the plant. Obviously, beneficial effects of biostimulants strictly depends on agricultural and environmental context and policies. Components usually considered biofertilizers include humic and fulvic acids, protein hydrolysates, seaweed extracts, chitosan, inorganic compounds and beneficial fungi and bacteria (du Jardin, 2015). Amongst the different kinds of biostimulants, humic substances are acquiring even more importance and their market has rapidly grown over the last twenty years in Europe (Germany, UK, Switzerland, Spain, Italy) and in North America (USA & Canada)(Metzger, 2010).

1.4.2 Definition, structure and composition of humic substances

Soil organic matter components are ascribable to three different categories: organic polymers of known structure, small organic compounds (*e.g.* sugars, amino acids, lipids and organic acids) and humic substances (HS). HS are recognised as natural components of the portion of soil organic matter called *humus*, deriving from chemical and biological degradation of animal, microbial, and plant residues. Due to the high heterogeneity of their composition, humic substances are quite difficult to define and classify. The first systematic classification of humic substances, relying on HS solubility, has been provided by Stevenson and it is still the one accepted by the International Humic Substances Society; according to Stevenson (see Figure1.8), HS can be ascribable to three categories: humins, humic acids (HA) and fulvic acids (FA). Humins are the non-soluble part of humic substances at any pH and is the portion mostly resistant to decomposition; humic acids are more oxidized than humins and are soluble at pH > 2, while fulvic acids are the most oxidized part of HS soluble in all pH conditions. According to this model, some peculiarities were attributed to each category in terms of in terms of colour, polymerization, molecular weight, carbon and oxygen content, acidity and solubility, as shown in Figure 1.8 (Liu et al., 2020; Stevenson, 1994).

Figure 1.8 Peculiarities of humic substances.

Characteristics of humic substances in terms of colour, degree of polymerisation, molecular wights, C and O content, exchange acidity and solubility (Liu et al, 2020).



Over years, many studies and works improved the information on these substances, to further shape their features, in terms of chemical composition and structure. The typical elemental composition of HS includes for 98-100% (not considering ashes) C, H, O, N, S and P; their mean concentrations are reported in Table 1.4, while P is usually present in very low amounts (0.1-1.0 %).

Table 1.4 Mean composition of humic and fulvic acids.

Mean elemental composition of C, O, H, N and S, on dry matter basis (Sparks, 2003).

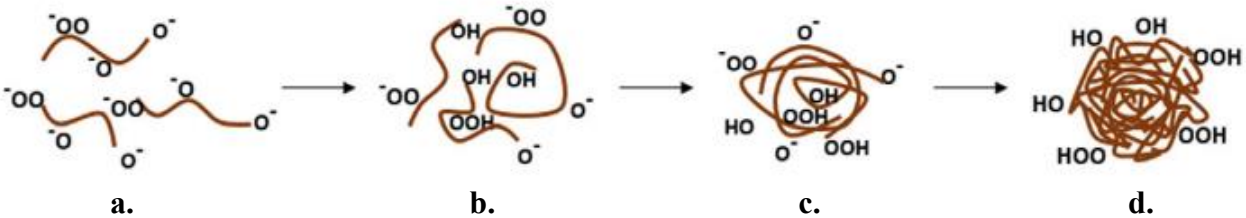
	Dry and de-ashed (%)	
Element	**Fulvic acids**	**Humic acids**
C	40-50	50-60
O	44-50	30-35
H	4-6	4-6
N	<1-3	2-6
S	0-2	0-2

Concerning the chemical structure of humic substances, the clear characterization is very difficult to obtain since it depends on different factors, such as geographical origin, age, climate and biological conditions. (de Melo et al., 2016). However, HA and FA have been extensively studied and plenty of information is today available to give a more detailed idea on these species.

Humic acids have a molecular weight typically included between 10 and 300 kDa and they include different functional groups, ranging from phenols, carboxylic acids, quinones and ethers to sugar and peptides, in some cases. This really varied composition strongly influences HA properties and chemical behaviour in terms of pH activity, solubility, amphiphilic character and metal chelation. Concerning the pH activity, HA show a slightly acidic behaviour mainly due to the two prevalent

Figure 1.9 Behaviour of HA molecules at different pH.

a. Alkaline pH and charge repulsion. b. Decreasing pH. c. Decreasing pH and intermolecular aggregation. d. Acidic pH and precipitation. (de Melo et al., 2016)



groups in HA structure, that are carboxylic and phenolic; in fact, the total acidity is calculated as the sum of phenolic + carboxylic group acidity. For instance, acidity of HA from different environmental samples was around 6 meq g^{-1} (de Melo et al., 2016). In terms of solubility, HA are completely soluble in basic conditions, while they are even less dissolved with decreasing pH. It has been observed that even at neutral pH, HA solubility is only partial, in some cases. As shown in Figure 1.9, this behaviour can be justified by the protonation forms of carboxylic and phenolic groups. In alkaline conditions, these moieties are deprotonated, and their repulsion induces a stretched conformation in HA structure. Moreover, these negatively charged groups offers a thermodynamically stable interaction with water molecules. As the pH decreases, the repulsion effects diminish and protonation begins to occur, inducing intra- and inter-molecular aggregation through hydrogen bonds; as a result, HA structure evolve in an even more compact conformation, which culminates with the precipitation at pH < 2 (de Melo et al., 2016; Nardi et al., 2017).

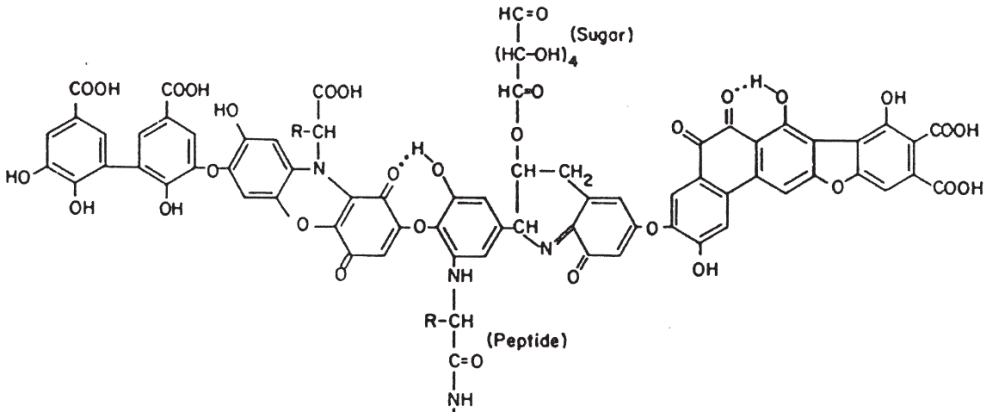
With regards to amphiphilic character of HA, this aspect relies newly on its composition, as in the general formula are included both hydrophilic (hydroxyl groups) and hydrophobic (aliphatic and aromatic groups) portions. These peculiarity makes HA a good alternative to surfactants or other solvent to solubilize nonpolar substances, especially those containing polycyclic aromatic hydrocarbons (Klavins and Purmalis, 2010). Metal chelation is another interesting feature of HA, finding plenty of applications in different fields. HA chelating potential is maximized when HA are deprotonated, being capable of forming pseudo-micelles to sequestrate metal cations, as described by von Wandruszka (2000): electrostatic interaction between HA and metals takes place, inducing a conformational change in HA structure, resulting in spherical HAs-metal complexes.

Fulvic acids are the portion of humic substances with lower molecular mass, which ranges to few hundred to few thousands Da (~ 10 kDa). Similarly to HA, they can be considered as an “arabesque” of different chemical groups, such as phenols and carboxylic groups. The most relevant differences of FA with HA are the lower molecular size, the higher hydrophile nature, the lower aromatic to total carbon ratio (25% versus 35-40% of HA), and higher negative charge and polarity (Stevenson, 1994; Varanini and Pinton, 1995).

For what concerns HA molecular structure, many models have been proposed, and Figure 1.10 reports an example (Flaig, 1960; Schnitzer and Khan, 1972; Schulten and Schnitzer, 1993; Stevenson, 1994). Despite all of these have tried to render the real complexity of HA, none of them was actually satisfying their real heterogeneous nature, as stated by Hayes (1991).

Figure 1.10 . Proposed structure for humic acids.

This structure highlights the high heterogeneity of chemical moieties involved in HA structure (de Melo et al., 2016).



Over the last thirty years, the efforts on interpretation of HA structure have moved basically in two different directions. On one side, particular attention has been devoted to characterising HA structures with on holistic approach, including techniques which range from spectroscopy techniques (*e.g.* infrared Fourier transform, nuclear magnetic resonance, electron paramagnetic resonance), to thermal behaviour (*e.g.* thermogravimetric analysis, differential thermal analysis), to surface potential (*e.g.* zeta potential), to microscopy (*e.g.* scanning electron microscopy) and elemental analysis (de Souza and Bragança, 2018; Muscolo et al., 2007; Varanini and Pinton, 1995). On the other hand, a change of perspective on humic substances has been proposed, moving from the vision of "large undefined polymers" to "supramolecular associations of small and different molecules". In this point of view, molecules, coming from degradation of biological materials, self-assemble and the resulting complex is stabilized not by covalent bonds, but by intermolecular forces such as hydrogen bonds and hydrophobic interactions. This theory may also justify the above-mentioned conformational elasticity of HA structure (Piccolo, 2001). To date, both the models are generally accepted, as humic substances are considered a cocktail of small to large molecules which are involved in association/dissociation phenomena in supra-molecular colloid; the next challenge, especially for soil humic substances, is to understand their complex dynamics and cross-talking with the other components of soil organic matter, soil components, microorganisms and plants (de Souza and Bragança, 2018; Nardi et al., 2017).

1.4.3 Functions and sources of humic substances and humic acids

HS find most of their applications in fields related to soil science and agriculture different fields, even if they are becoming even more relevant in pollution remediation (*e.g.* metal chelating effect), medicine (*e.g.* anti-inflammatory effect and cancer therapy) and pharmaceuticals and cosmetics (*e.g.* drug solubilizer and carriers) (de Melo et al., 2016). Concerning their main role, functions and desirable aspects of humic substances will be briefly analysed from a duplex point of view, considering the effects on soil fertility on one side and on plant growth on the other.

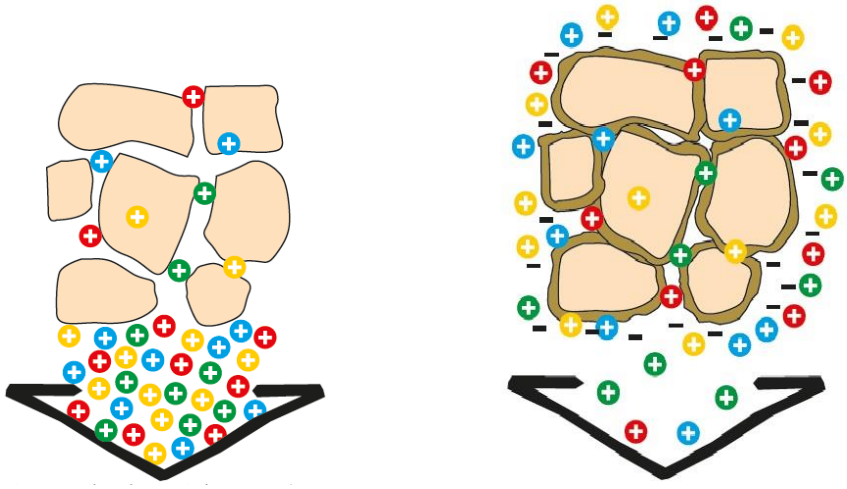
Roles of HS in soil are commonly related to the increase of fertility and can be classified in physical and chemical effects:

- *Physical effects on soil.* The principal function of HS is to prevent soil cracking and to stabilise the structured soil texture, since they are key components of soil aggregates. More in detail, HS behave as cement interacting with soil clay particles (through hydrogen bonds) and polyvalent cations (by coordination complexes). These aggregates induce the formation of a "crumb" structure in the topsoil, typical of tilth, rich in pores, guaranteeing an optimal interchange of air and water. Moreover, HS are good water adsorbent, improving soil water holding capacity and allowing a better resistance to drought.
- *Chemical effects on soil.* The main role of HS is related to nutrient availability, as they are source of organic carbon for soils microorganisms and they improve macro- and micro- nutrient availability. This effect derives from the improvement of soil cation exchange capacity (C.E.C.) given by HS; in fact, humified organic matter contributes for about 50% to soil cation exchange capacity and favours retention of cationic nutrients in an exchangeable form that can become available to plant nutrition. As a consequence, HS not only have a synergistic effect with commercial fertilizers, enhancing nutrient use efficiency, but form complexes with micronutrients acting as "reservoir". Another interesting function of HS is the chelation of heavy metals, promoting a desirable detoxification effect. Moreover, HS are involved also in pH regulation, behaving as buffer and neutralizing the deficiency or excess of hydrogen ions.

Beyond these general peculiarities, effects of HS strictly depend on the typology of soil on which they are applied. For instance, Humintech GmbH, one of the leading firms in production and commercialisation of HS, has studied the effect of the product Perlhumus® on a sandy and poor soil, with low content of organic matter. The greening effect provided by this product has been explained by the scheme in Figure 1.11. Humic acids form a layer all over the sand particles, increasing the cation exchange capacity (CEC). In this way water and nutrient are better retained by soil and more bioavailable to plants, without the risk of being leached out to groundwater (Humintech, 2015).

Figure 1.11 Effects of humic substances on sandy and low-textured soils.

Humic substances adhere on soil particles improving the retention of nutrients and electrolytes (Humintech, 2015).



The effects of HS on plant growth can be elucidated both from an agronomic perspective, which considers the macroscopic effects on plant, and a biological one, which take into accounts issues related to cell structure and metabolism. Starting from the agronomic effects, the most important is surely the relevant influence on the development of radical apparatus: HA and FA stimulates linear growth of roots, both in terms of initiation and elongation. Other interesting aspects on plant growth regards the positive influence on seed germination and development, and the improvement in plant foliage and fruits when applied as foliar sprays blended with micronutrients. The effect of sustained growth may be justified for the increase in both cationic and anionic nutrient uptake rates (*e.g.* K+ 30%), and for the so-called auxin effect. Auxins are plant growth hormone and HS are natural inhibitor of the enzymes degrading these substances, for instance the indole-3-acetic acid (IAA) oxidase.

From a cellular point of view, it has been proved that HS provide a general speed up of energetic metabolism of the cell, resulting in an increased production of adenosine triphosphate (ATP), considered the energy coin at cellular level. Moreover, humic acids (HA)s and fulvic acids (FAs) have effects on plant cell membranes, increasing their permeability, resulting in an improved translocation of mineral nutrients to sites of metabolic need.

As stated above, HS are naturally present in soil as humified part of soil organic matter. But which are the real sources which are actually exploited for the extraction and recovery of these added-value compounds? To date, the principal source of humic substances is the mineraloid leonardite, named after Arthur Gray Leonard, who firstly discovered and studied this interesting substance. Leonardite is a form of coal considered as an oxidation form of lignite and contains considerable amounts of humic substances which can reach up to 85% of its composition. Besides the appreciable concentrations, also the quality of HS contained is

excellent. In fact, differently from other HS sources, leonardite is extremely bioactive, reaching a biological activity up to five times higher than other humic matter. Thus, leonardite is currently exploited as soil conditioner as well as it is exploited as raw matter for HS extraction. Extraction of humic substances from leonardite ores has become a prominent area of study in recent years. While humic substance extraction from leonardite is generally carried out by chemical dissolution technique (leaching) in alkali medium, physical enrichment methods were also used in limited number of studies. However, removing inorganics found in leonardite would decrease dissolving reactive consumption and would also prevent unnecessary capacity use. This study investigates the effect of physical pre-enrichment processes on humic substance leaching (Canieren et al., 2017). The most important leonardite deposits on Earth crust are in North Dakota (USA), where firstly Leonard discovered this kind of organic matter, and, still today, American leonardite is currently considered the benchmark standard. Other relevant deposits are placed in Australia, Canada, Turkey, China and Russia.

Other mineral sources of humic substances are of course deriving from different oxidation forms of coal, such as lignite and peat: while the first one may be exploited as raw material, the second one is predominantly used as cultivation substrate largely exploited in greenhouse applications since it is rich not only in organic matter, but also in plant macronutrient. All the above-mentioned resources are the currently most exploited ones for production of commercial humic and fulvic acids, and all of them are non-renewable sources of carbon. In a low-carbon economy perspective, companies and researchers are on the way to find solutions for HA production from renewable and sustainable sources, such as compost and vermicompost (du Jardin, 2015). Recently, it was demonstrated that HAs could be produced by fermentation using the empty fruit bunch of palm trees as a substrate (Motta and Santana, 2013).

1.5 Aim of the PhD project

The whole work of the PhD project dealt with the topics presented above and it can be placed in the complex scenario of the sewage sludge recycling and fight to soils nutrient depletion. The general purpose of the research work was to deepen the knowledge about the controversial theme of sewage sludge land application. More precisely, the present study intended to challenge the fertilizing properties of sewage sludge anaerobic digestates, testing these features on poor soils, usually not devoted to agriculture. Thus, the final aim was to understand if this waste could be a possible solution to face nutrient depletion of soils, trying to minimise the risk of potential addition of toxic compounds.

Hence, the detailed targets of the research work were substantially four:

i) the characterization of four different anaerobic digestates from sewage sludge (SSADs), both with an analytical chemistry and an ecotoxicological approach in order to evaluate not only their potentialities as fertilizer (organic matter and nutrient content), but also the potentially toxicity they can induce to living organisms for the presence of contaminants (*e.g.* heavy metals).

ii) the evaluation of the fertilizing and phytotoxic effects of SSADs on the growth of cucumber plants on a nutrient-poor and sandy soil, by the means of pot experiments in a controlled environment (environmental chamber) and agronomic and physiological measurement to assess plant growth;

iii) the study of the soil bacterial communities of tomato plants grown in a poor soil and treated with SSAD, by the means of pot experiments in greenhouse, isolation of microbial communities from plant rhizosphere, purification of soil DNA, next generation sequencing of DNA with Illumina platform, and bioinformatic elaboration of the reads for the analysis of the taxonomic and ecological information;

iv) the implementation of a laboratory protocol for extraction and quantification of humic acids from SSAD, and the characterization of the extract by the means of membrane filtration processes and electron microscopy.

Given the strong interdisciplinarity, these topics were successfully explored thanks to the productive collaboration with external collaborations. Pot experiments for plant growth in climatic chamber and greenhouse where conducted in collaboration with Agroinnova, Centre of Competence for the innovation in the agro-environmental field. Extraction of soil DNA and molecular characterisation of soil microbial communities was conducted during the PhD period spent at Molecular Ecotoxicology and Microbiology Laboratories of Joint Research Centre in Ispra (VA, Italy). Extraction of humic acids from SSAD was performed during the PhD period spent at Escuela de Ingeniería Bioquímica of Pontificia Universidad Católica de Valparaíso (Chile).

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Chapter 2

Characterization of anaerobic digestates from sewage sludge

2.1 Introduction and aim of the study

The goal of the present chapter is to introduce one of the main topics of the whole work of this thesis: anaerobic digestates from sewage sludge (SSADs). This feedstock surely covered a key role in the different experimental campaigns conducted and the preliminary analyses of these wastes were fundamental to design the experiments. Hence, the aims are substantially two. The first is to give a brief description of the wastewater treatment plant (WWTP) where the SSADs were produced and to shortly describe the way in which these wastes were generated. The second is to describe in a more detailed manner the main peculiarities of the four SSADs used for the subsequent experiments, object of the Chapter 3 and Chapter 4.

In particular, the way in which SSADs were characterised deserves a little bit more attention. Indeed, two approaches were exploited to this aim: an analytical chemistry one, and an ecotoxicological one. Analytical chemistry provides powerful tools for the individuation of different chemical species present in complex matrices, such as SSADs, with the possibility to detect also molecules present at very low levels (ppm). This kind of characterization is aimed to measure features of different kind, from pH and electrical conductivity, to the concentration of organic matter and macro-elements, to the presence of inorganic and organic contaminants. Moreover, chemical analysis is mandatory for subsequent uses of SSAD, for instance for land applications, as specified in European (Council of the European Communities, 1986) and national laws (Italian Decree Law 99/1992). However, when working with these complex and diverse matrices of unknown contamination, some relevant difficulties have been recognized because analytical techniques answer to precise “queries”, without considering other aspects which, conversely, could be very important to assess environmental toxicity and risk. To

this aim, ecotoxicological test (or *bioassays*) are decisively helpful. Basically, these are biological experiments performed exposing a model organism to a potentially toxic environmental sample and measuring quantitatively a specific response. Hence these bioassays consider the complexity of the matrix studied and may be a powerful tool to better target the analytical strategies. However, ecotoxicological test cannot replace analytical methods, but give a different point of view to the problem.

Since the two approaches seemed to be complementary, both were used for the characterisation of the SSAD. In the case of chemical characterization, routinely analyses conducted on wastes and fertilizers were performed. As regards ecotoxicological tests, germination assays in Petri dishes with common cress (*Lepidium sativum* L.) were done. Results of the present characterizations have been recently published on Waste Management (Cristina et al., 2019).

2.2 Description of wastewater treatment plant and typologies of sludge produced

The sewage sludge came from the wastewater treatment plant (WWTP) serving the urban and metropolitan area of the city of Turin (north-western Italy). This WWTP is a large-scale plant (3,800,000 population equivalents) and it is designed to treat wastewater along four different steps (Figure 2.1): preliminary treatments (removal of coarse material, sand and oil), primary treatments (sedimentation processes), secondary treatments (biological oxidation, denitrification and sedimentation) and tertiary treatments (chemical phosphorus removal, chlorination and filtration). Sludges coming from primary and secondary processes are the main by-products and are stabilised by means of anaerobic digestion. Briefly, prior to fermentation, sludges are thickened and preheated for activation of thermophilic bacteria. Anaerobic digestion (AD) of primary and secondary sludge takes place in separate reactors and lasts up to 20 days. Besides biogas production, the main side products of AD are primary (P) and secondary (S) anaerobic digestates from sewage sludge (SSAD). P and S digestates are then

Figure 2.1 Scheme of the wastewater treatment plant of Turin.

Blue lines and icons indicate the water stream, while green ones the sludge

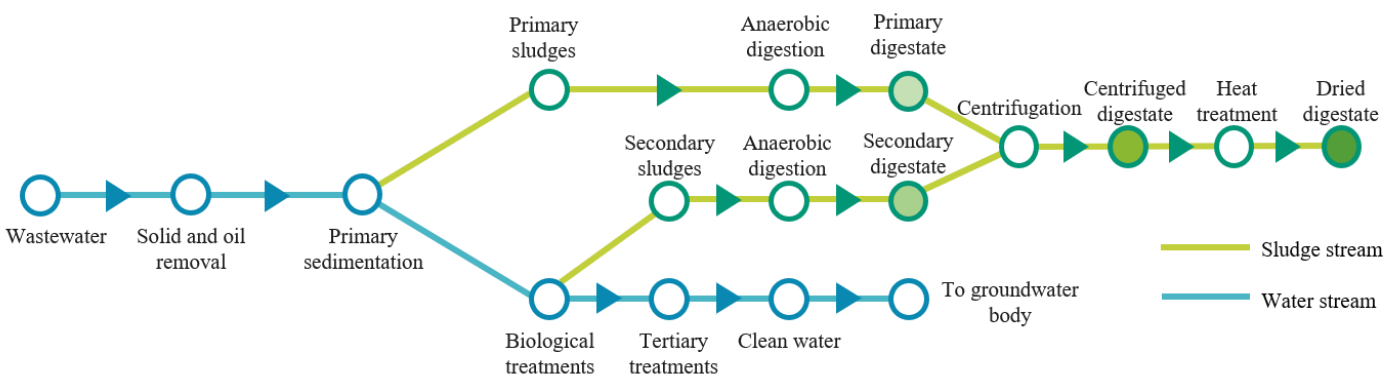

Table 2.1 Technical details of WWTP of Turin metropolitan area.

Water flow

Annual pollution removed from rivers

Treated water flow	215,000,000	m^3/year
Suspended solids removed	35,000,000	kg/year
Organic load removed (BOD_5)	40,000,000	kg/year
Surfactants removed	700,000	kg/year
Ammoniacal nitrogen oxidised	4,500,000	kg/year
Total phosphorus brought down	700,000	kg/year
Heavy metals removed	30,000	kg/year
Sludges disposed (dry solids)	30,000,000	kg/year

Average inlet flow

Average daily inlet flow	600,000	m^3/day
Average flow rate	25,000	m^3/h

Inlet and outlet sewage parameters

	Inlet	Outlet	
Total suspended solids	200	8	mg/l
BOD_5	220	5	mg/l
COD	380	30	mg/l
N	31	8.4	mg/l
P	4	0.7	mg/l

Primary sedimentation

Circular basins	8	n°
Total volume	59,440	m^3
Total surface	16,981	m^2
Average retention time	2.4	h

Denitrification

Rectangular basins	12	n°
Total volume	110,000	m^3
Maximum flow of returned mixed-liquor	133,000	m^3/h

Biological oxidation

Rectangular basins	24	n°
Total volume	210,000	m^3
Maximum flow rate per hour	37,500	m^3/h
Average retention time	5.1	h
Sludge recirculation rate	70	%
Maximum flow rate of returned sludge	25,000	m^3/h

Secondary sedimentation

Circular basins	24	n°
Total volume	175,600	m^3
Total surface	55,000	m^2

Final Filtration

Multilayer beds	27	n°
Maximum filtration speed	10	$m^3/m^2/h$
Filter surface	1,500	m^2

Sludge flow

Average daily flow (2% dry solids)	6,000	m^3/day
Maximum daily flow	12,000	m^3/day

Pre-thickening section

Covered circular basins	6	n°
Total capacity	7,890	m^3
Total area	2,300	m^2
Load	50	kg SS/m^2
Average retention time	6 ÷ 24	hours

Anaerobic digestion of sewage sludge

Digester tanks	6	n°
Diameter	26	m
Height	30	m
Total capacity	72,000	m^3
Average digester temperature	37 - 40	°C
Average retention time	15 ÷ 20	days

Digested sludge post-thickening

Covered circular basins	6	n°
Total capacity	7,980	m^3
Total area	2,300	m^2

Centrifuge dewatering

Centrifuges		
Drum diameter	725	mm
Rotation speed	2,800	rpm
Centrifugal acceleration	3,160	g
Capacity flow (each) for sludge at 2% dry solids	80	m^3/h
Dewatered sludge storage capacity	460	m^3

Sludge drying

Sludge drying plants	2	n°
Feeding (each)	5,000	kg/h
Inlet dry solids content	26	%
Dried sludge production (each)	1,428	kg/h
Outlet dry solids content	91	%
Outlet temperature	105	°C
Evaporation capacity (each)	3,572	kg H_2O/h
Thermal energy need (each)	2,636	kW
Thermal energy recovery (each)	1,750	kW
Natural gas consumption (each)	300	Nm^3/h
Electric power consumption (each)	160	kW
Process time	6	h
Dried sludge storage capacity	360	m^3
Total production per year (sludge 90% dry solids)	11,000	t

further thickened, mixed, and conveyed to dewatering processes, that are centrifugation and thermal drying. In the former, liquid SSAD is mixed with polyelectrolyte and then centrifuged, yielding a dark shovellable solid named centrifuged (C) SSAD. In the latter, C digestate is further heated to 200°C via paddle driers, resulting in the dried (D) SSAD, a dark powdery solid with high dry matter content. Technical details and operative parameters of the wastewater treatment plant are reported in Table 2.1.

2.3 Material and methods

2.3.1 Chemical and physical characterisation of SSADs

Primary, secondary, centrifuged and dried SSADs were sampled directly at WWTP and stored at 4°C until chemical characterisation and further uses. The chemical analyses were performed according to "Analytical Methods for Fertilizers" by the Italian Ministry of Agriculture and Forestry (M.P.A.A.F., 2006) and "Methods for Analysis of Sewage Sludge by Water Research Institute of National Council of Researches (IRSA-CNR, 1985), unless specified differently. pH and electrical conductivity were measured on distilled water extracts (1:10 m/v) by potentiometry and conductometry, respectively. Dry matter content and humidity were measured by gravimetry, drying the samples at 105°C until constant weight. Ashes were determined with calcination at 550°C for 5 hours.

Total organic carbon was evaluated as reported on "Official methods of soil analysis" by the Italian Ministry of Agriculture and Forestry (Italian Ministerial Decree, 1999), exploiting the Walkley-Black method: sample digestion with potassium dichromate and sulphuric acid is followed by titration with iron(II) sulphate heptahydrate. Organic matter content was calculated with the Van Bemmelen conversion factor (1.724) (Pribyl, 2010).

Total nitrogen (N_{Tot}) was measured with the Kjeldahl method, which allows to titrate both organic and inorganic forms of nitrogen. Ammonium nitrogen (NH_4^+) was evaluated through distillation with magnesium oxide followed by titration with sulphuric acid, while nitrates ($N\text{-}NO_3^-$) were determined by the means of ionic chromatography. Organic nitrogen (N_{Org}) was then calculated by subtraction: $N_{Org} = N_{Tot} - (N\text{-}NH_4^+)$. Other macronutrients (K and P), micronutrients (Ca, Mg, Na, Fe, Mn, B, Zn) and heavy metals (Pb, Cr, Ni, Cu) were extracted with mineral acid digestion and then analysed by the means of inductively coupled plasma optical emission spectrometry (ICP-OES). Other contaminants such as Cd and As were extracted with the same digestion protocol, but analysed with graphite furnace atomic absorption spectroscopy (GF-AAS). Hg was evaluated with hydride generation atomic absorption spectroscopy (HGAAS) after microwave mineralisation, while Cr^{6+} was determined by colorimetry after complexation with diphenylcarbazide.

2.3.2 Ecotoxicological assay: evaluation of toxicity on germination with garden cress (*Lepidium sativum*)

Evaluation of SSAD toxicity on germination was performed following the protocol of DIVAPRA et al., (1998), with some modifications. The assay was conducted only on liquid separates of Primary and Secondary SSADs, obtained by centrifugation (15 minutes, 4000 rpm). On the other hand, this test was not performed on Centrifuged and Dried SSADs, since it was not possible to adopt the same experimental approach, neither to obtain a suitable aqueous extract due to high water soaking by the solid digestates.

After centrifugation, the pure supernatant (100%) of P and S was diluted with distilled water at ten different concentrations (2.5, 5, 7.5, 10, 15, 20, 25, 50, 75, 100 %). The negative control (0%) was prepared with pure deionized water. Per each concentration, four replicates were set as follow: one Whatman n°1 filter paper was placed in a sterile plastic Petri dish (Ø 90 mm), where subsequently 5 ml of the abovementioned solutions were poured. In the meantime, garden cress (*Lepidium sativum* L.) seeds (Green Paradise Srl, Italy) were sterilized in sodium hypochlorite for 30 seconds and then rinsed twice with abundant deionized water. Afterwards, imbibition of seeds was performed by incubation in deionized water for one hour; before use, each seed was inspected and selected, discarding discoloured, damaged or abnormally small ones (Pavel et al., 2013). Finally, ten cress seeds were sown in each Petri dish, which was subsequently sealed with parafilm. Plates were incubated for 72 hours at 25°C in the dark. After 24, 48 and 72 hours germinated seeds were counted to calculate the relative seed germination (RSG) according to Bosker and colleagues (2019):

$$RSG = \left(\frac{G_t}{G_c}\right) \times 100$$

where G_t is the average number of germinated treated seeds and G_c is the average number of germinated seeds in the control. After 72 hours, roots length (root + hypocotyl + epicotyl) was measured according to Lencioni and colleagues (2016) and germination index (GI) was calculated as reported by Zucconi and colleagues (1981):

$$GI = \left(\frac{L_t \times G_t}{L_c \times G_c}\right) \times 100$$

where L_t is average root length of treated seeds, G_t is the average number of germinated treated seeds, L_c is the average root length of control seeds and G_c is the average number of germinated seeds in the control. Seeds were considered germinated when emerging roots were longer than seed diameter (Bae et al., 2014). Successively, EC_{50} was calculated on GI as the concentration value determining a reduction of 50% over untreated control.

2.3.3 Statistical analysis

In the germination tests on Petri dishes, the statistically significant differences between treated and untreated samples were identified with Student's *t* test, specifying the different levels of significance ($p\backslash 0.05$ = *, $p\backslash 0.01$ = **; $p\backslash 0.001$ = ***).

2.4 Results and discussion

2.4.1 Characterisation of SSADs

Results of characterization of the digestates are shown in Table 2.2. Dry matter content in liquid digestates was 4.4% and 4.8% (for P and S, respectively), while it reached 25.8% and 88.8% (for C and D, respectively) after dewatering processes. pH decreased throughout the different digestates from 7.7 to 6.8; total nitrogen levels ranged from 7.5% (S) to 5% (D), while NH_4^+ was up to six times higher in liquid than in solid SSADs. No consistent variation in organic matter levels was observed through the four digestates; as a consequence, C/N ratio increased from liquid to dewatered SSADs. Plant macronutrients such P and K had opposite behaviours: the first one showed appreciable concentrations, with a growing trend from liquid to solid digestates; the latter revealed highly low levels (<1%), with a slight decrease in C and D SSADs. Meso- and micronutrients (Ca, Mg, B, Zn) and some metals (Na, Cd, Ni, As) exhibited decreasing concentrations from liquid to solid digestates; the only metals which showed a diametrically opposed behaviour were Fe and Cu. No consistent difference in Pb, Cr and Hg concentrations was reported across the four digestates. All digestates showed interesting contents in macronutrients (N > 5% and P > 4%) as well as in meso- and micronutrients. Indeed, these values were even slightly higher than the mean ones published in other works (N_{Tot} = 3.6%; P_{Tot} = 2.5%). In the case of nitrogen, dewatering probably induced an immobilisation effect, remarked by the increasing levels of N_{Org}/N_{Tot}. On the contrary, despite K levels were a little bit low if compared to other studies (*e.g.* mean values of works in the references: K = 0.59 %), the applied dosage in this work was sufficient for the early growth stages (Adjei and Rechcigl, 2002; Alvarenga et al., 2016; Antonkiewicz et al., 2018; Asagi and Ueno, 2008; Belhaj et al., 2016; De Andres et al., 2010; Ferreiro-Domínguez et al., 2011; Hussein, 2009; Singh and Agrawal, 2008; Tarrasón et al., 2008; Wang et al., 2008). However, this aspect can negatively affect the proper potassium supply when SSAD is applied as fertilizer, especially in the phase of fruit maturation (Hawkesford et al., 2012). The main disadvantage of these digestates was the presence of heavy metals. Despite all the analysed ones complied with the limits imposed by the Italian Law on Sewage Sludge Land Application (Italian Decree Law 99/1992), in some cases (*i.e.* Zn, Cu and Ni) the thresholds imposed by Italian Discipline on Fertilizers (Italian Decree Law 75/2010) were overcome.

Table 2.2 Physicochemical properties of the four anaerobic digestates from sewage sludge used

Last three columns on right specify analysis methods for sewage sludge, Italian law limits for Land application of sewage sludges (Italian Decree Law 99/1992), and law limits for heavy metals in fertilizers (Italian Decree Law 75/2010). d.m.b., Dry matter basis.

Parameter	Unit of measure	Anaerobic digestates				Method of analysis	Technique	Italian Law Land application of sewage sludge (D. Lgs 99/92)	Italian Law Discipline on fertilizers (D.Lgs 75/2010)
		Primary (P)	Secondary (S)	Centrifuged (C)	Dried (D)				
Dry matter	%	4.4	4.8	25.8	88.8	Calculation	Calculation		
Humidity	%	95.6	95.2	74.2	11.2	M.P.A.A.F., 2006 Method III.1	Gravimetry		
Ashes	% d.m.b.	35.3	31.5	36.1	35.6	Calculation	Calculation		
pH (1:10)		7.7	7.5	7.3	6.8	M.P.A.A.F., 2006 Method III.3	Potentiometry		
E.C.	mS/cm	0.378	0.36	1.069	1.575	M.P.A.A.F., 2006 Method III.4	Conductometry		
Organic matter	% d.m.b.	64.7	68.5	63.9	64.4	Calculation	Calculation		
TOC	% d.m.b.	37.5	39.7	37.1	37.3	D.M. 13/09/99 GU 248 21/10/199 met. VII.3	Walkley & Black method	>20	
N - Tot	% d.m.b.	7.4	7.5	6.3	5	CNR IRSA 6 Q64 vol.3, 1985	Kjeldahl method	>1.5	
N - Org	% d.m.b.	5.84	6.16	5.33	4.75	M.P.A.A.F., 2006 Method IV.12	Calculation		
N - NO_3^-	% d.m.b.	<0.01	<0.01	<0.01	<0.01	M.P.A.A.F., 2006 Method IV.12	Ionic chromatography		
N - NH_4^+	% d.m.b.	1.56	1.34	0.97	0.25	M.P.A.A.F., 2006 Method IV.12	Distillation and titration		
N - org / N - Tot	%	79	82	84	94	Calculation	Calculation		
C/N		5.1	5.3	5.9	7.4	Calculation	Calculation		
P	% d.m.b.	4.16	5.75	6.74	6.26	M.P.A.A.F., 2006 Method VIII	Acid digestion + ICP-OES	>0.4	
K	% d.m.b.	0.55	0.69	0.39	0.18	M.P.A.A.F., 2006 Method VIII	Acid digestion + ICP-OES		
Ca	% d.m.b.	6.46	4.69	5.02	4.64	M.P.A.A.F., 2006 Method VIII	Acid digestion + ICP-OES		
Mg	% d.m.b.	1.78	1.53	1.45	1.16	M.P.A.A.F., 2006 Method VIII	Acid digestion + ICP-OES		
Na	% d.m.b.	1.05	1.03	0.34	0.19	M.P.A.A.F., 2006 Method VIII	Acid digestion + ICP-OES		
B	mg/kg d.m.b.	51	60	52	41	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES		
Zn	mg/kg d.m.b.	918	650	849	719	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES	2500	500
Fe	% d.m.b.	2.43	3.32	3.99	3.48	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES		
Mn	mg/kg d.m.b.	255	190	268	228	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES		
Cu	mg/kg d.m.b.	357	340	406	396	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES	1000	230
Pb	mg/kg d.m.b.	92	70	92	79	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES	750	140
Cr	mg/kg d.m.b.	245	210	245	217	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES	*<200**	
Cd	mg/kg d.m.b.	1	0.6	0.8	<0.1	M.P.A.A.F., 2006 Method IX	Acid digestion + GF-AAS	20	1.5
Ni	mg/kg d.m.b.	163	120	155	137	M.P.A.A.F., 2006 Method IX	Acid digestion + ICP-OES	300	100
As	mg/kg d.m.b.	2.8	2.1	0.9	<0.1	M.P.A.A.F., 2006 Method IX	Acid digestion + GF-AAS	*<20**	
Hg	mg/kg d.m.b.	<0.1	<0.1	<0.1	<0.1	Internal method	HGAAS	10	1.5
Cr^{6+}	mg/kg d.m.b.	<0.1	<0.1	<0.1	<0.1	CNR IRSA 16 Q64 vol.3, 1986	Colorimetry	*<2**	0.5

** Values introduced with Italian Law 130/2018*

Moreover, heavy metals concentrations were generally lower than those published elsewhere (Cu: 413 mg/kg, Zn: 922 mg/kg, Pb: 116 mg/kg, Cd: 3.9 mg/kg, As: 3.5 mg/kg), except for Cr and Ni (93 mg/kg and 72 mg/kg, respectively) (Adjei and Rechcigl, 2002; Alvarenga et al., 2016; Antonkiewicz et al., 2018; Asagi and Ueno, 2008; Belhaj et al., 2016; De Andres et al., 2010; Ferreiro-Domínguez et al., 2011; Hussein, 2009; Singh and Agrawal, 2008; Tarrasón et al., 2008; Wang et al., 2008).

2.4.2 Evaluation of toxicity on germination

Relative seed germination (RSG) was a parameter useful to trace a sort of time-course of the germination, without perturbing the seeds with length measurement of emerging roots. For this reason, Germination Index (GI) measurement was performed only at the third day. Table 2.3 reports the results of RSG measured on cress seeds exposed to Primary SSAD. In general, increasing RSG values were registered along the three days at same digestate concentrations. At 24h and 48h the first value significantly lower than control was the RSG at 7.5% (0.867), while at the third day it was the RSG at 25%. Interestingly, the RSG values at 10% (at 24h) and 15% (24h and 48h) were not significantly lower than untreated control. Hence the RSG at 7.5% concentration was likely not due to phytotoxic effects of SSAD, but rather to the fact that not all the seeds have the same germination timing; in fact, at 72h, the RSG value at 7.5% was no longer significantly lower. In all days, 50% concentration showed RSG values of 0. This likely indicated that concentrations higher than 25% sharply influenced RSG in cress. Moving to Secondary SSAD, results of RSG are showed in Table 2.4. Differently from Primary SSAD, no germination was assessed after the first day, which can be attributable more to a peculiarity of the stock of used seed than to phytotoxic effect, since also the control showed null germination. Increasing values of RSG at the different concentrations were found at 48h and 72h. Interestingly, in both cases, the first value significantly lower than untreated control was at the same concentration level (10%), likely indicating that at this point phytotoxic effects began to occur. Similarly to Primary SSAD, also in this case RSG value reached 0 at 50% concentration, suggesting that concentrations higher than 25% were negatively affecting cress germination in a more sustained manner.

Few other works in literature reported RSG characterisation of sewage sludge or its derivatives. In any case, comparison resulted not very reliable since different authors exploit different strategies to obtain liquid separates or extracts, which strictly influence RSG values. The most similar work was the one published by Mañas and De las Heras (2018), who exposed lettuce seeds to sewage sludge. RSG values ranged between 94% (at 1% concentration) and 28% (at 10% concentration); these values were decisively lower to the ones registered for Primary SSAD in present study, while they were quite comparable to the ones of Secondary SSAD. Other works reported information about aqueous extracts of sewage sludge which were obtained with a 1:10 weight/volume ratios, hence the obtained values could be reasonably comparable to the 10% dilution of this work. For instance, Fuentes

and co-workers (2004) reported RSG values on cress and barley between 70% and 85%, which were even comparable (with Secondary SSAD) or lower (with Primary SSAD) than the present study. Similar values were also reported by a work where tested extracts came from sewage sludge was blended with other wastes (washery waste, coal sludge) (Sobik-Szołtysek et al., 2017). Thus, considering these results, RSG values (after three days) found in the SSADs studied in the present work were in general comparable to slightly higher to the ones published in literature.

Table 2.3 Relative Seed Germination (RSG) on *Lepidium sativum* L. using Primary SSAD.

Observed significance levels (p-values) from Student's t test (p\0.05 = *, p\0.01 = **; p\0.001 = ***) comparing treated with untreated seeds. Error is expressed as standard deviation (SD).

	RSG on Primary SSAD								
	24 h			48h			72h		
% digestate	Mean RSG		SD	Mean RSG		SD	Mean RSG		SD
0%	1.000		0.000	1.000		0.000	1.000		0.000
2.5%	1.006		0.147	1.000		0.091	0.975		0.050
5%	0.947		0.061	1.000		0.000	1.000		0.000
7.5%	0.867	*	0.059	0.867	*	0.059	0.975		0.050
10%	0.981		0.156	1.006		0.147	1.000		0.000
15%	0.636	**	0.117	1.003		0.086	1.000		0.000
20%	0.292	**	0.111	0.367	**	0.128	0.900		0.082
25%	0.181	**	0.128	0.181	**	0.128	0.675	*	0.150
50%	0.000	***	0.000	0.000	***	0.000	0.000	***	0.000
75%	0.000	***	0.000	0.000	***	0.000	0.000	***	0.000
100%	0.000	***	0.000	0.000	***	0.000	0.000	***	0.000

Table 2.4 Relative Seed Germination (RSG) on *Lepidium sativum* L. using Secondary SSAD.

Observed significance levels (p-values) from Student's t test (p\0.05 = *, p\0.01 = **; p\0.001 = ***) comparing treated with untreated seeds. Error is expressed as standard deviation (SD).

	RSG on Secondary SSAD								
	24 h			48h			72h		
% digestate	Mean RSG		SD	Mean RSG		SD	Mean RSG		SD
0%	0.000		0.000	1.000		0.000	1.000		0.000
2.5%	0.000		0.000	0.944		0.111	0.950		0.100
5%	0.000		0.000	0.802		0.188	0.750		0.191
7.5%	0.000		0.000	0.760		0.247	0.800		0.163
10%	0.000		0.000	0.611	*	0.136	0.700	*	0.115
15%	0.000		0.000	0.378	**	0.129	0.650	*	0.129
20%	0.000		0.000	0.149	***	0.067	0.475	**	0.171
25%	0.000		0.000	0.028	***	0.056	0.250	**	0.208
50%	0.000		0.000	0.000	***	0.000	0.000	***	0.000
75%	0.000		0.000	0.000	***	0.000	0.000	***	0.000
100%	0.000		0.000	0.000	***	0.000	0.000	***	0.000

A more complete perspective on phytotoxicity was achieved with measurement of emerging roots and calculation of GI. Results of GI are shown in Table 2.5. In both P and S, GI in 50%, 75% and 100% concentrations were 0 due to absence germination. On Primary SSAD the highest GI was obtained at 2.5% concentration, with a gradual decrease at higher concentrations. The 10% concentration case deserved particular attention because it showed a significantly higher value than previous and following points. Moreover, the calculated EC50 was at 17.5%.

On Secondary SSAD, GI at 2.5%, 5% and 7.5% was slightly higher than control, but not enough to affirm that GI was significantly increased from control dosage. Then, the index decreased to 0% more rapidly than P for concentrations higher than 7.5%. However, this GI value was reached at the same concentration of P treatment (50%). The calculated EC50 was at 12.5%.

Germination of cress increased with dilution and this trend is confirmed elsewhere (Abdullahi et al., 2008). GI of Primary SSAD, at 2.5% concentration, showed a significative improvement compared to 0%. In order to determine a more precise GI trend in this range, the approach proposed by Lencioni and colleagues (2016) should be applied, exploring the interval 0% - 10%, with steps of 1%. Differently, results of GI of Secondary SSAD did not show any significative variation from not treated samples until the 7.5% concentration. At higher concentration rates, GI presents a sudden decrease. For sure, concentration of 15% for P and 10% for S were the highest ones with a germination index of at least of 60%, which is considered the GI threshold to support the absence of phytotoxic effects (Zucconi et al., 1985).

Table 2.5 Germination Index (GI) on *Lepidium sativum* L. using Primary and Secondary SSAD.

Observed significance levels (p-values) from Student's t test (p\0.05 = *, p\0.01 = **; p\0.001 = ***) comparing treated with untreated seeds. Error is expressed as standard deviation (SD).

	Liquid SSADs			
	Primary (P)		**Secondary (S)**	
Concentration (%)	**GI**	**SD**	**GI**	**SD**
0.0	1.000	0.000	1.000	0.000
2.5	1.144 **	0.061	1.067	0.379
5.0	1.017	0.195	1.049	0.279
7.5	0.781 **	0.096	1.036	0.409
10.0	0.815	0.188	0.640 **	0.164
15.0	0.620 ***	0.076	0.459 ***	0.051
20.0	0.245 ***	0.077	0.183 ***	0.134
25.0	0.093 ***	0.023	0.081 ***	0.106
50.0	0.000 ***	0.000	0.000 ***	0.000
75.0	0.000 ***	0.000	0.000 ***	0.000
100.0	0.000 ***	0.000	0.000 ***	0.000

Compared to other sewage sludges, the two digestates used in this study revealed higher GI values. For instance, in the work of Mañas and De las Heras (2018) a not-digested sewage sludge utilized at 10% concentration revealed a lower GI (1.4%) than the one obtained in the present work (81% on Primary SSAD and 64% on Secondary SSAD). Alburquerque and colleagues (2012) used twelve different kinds of anaerobic digestates from animal origin (obtained from co-digestion of different organic matrix) and only two of them showed a GI higher than 50% at concentration of 10%. Interesting results derived from the comparison of the GI of an anaerobic digestate from microalgae, and digestates from a co-

digestion of microalgae with primary sewage sludges: while the former showed a GI comparable to the one obtained with Primary SSAD of the present work (at concentration of 10%), the latter displayed a GI of nearly 100% for the same concentration (Solé-Bundó et al., 2017). Thus, the minor toxicity of primary co-digested sludge could be justified by the synergic effect of co-digestion, which has been demonstrated to be more advantageous than mono-digestion ones due to a dilution effect of inhibitory compounds, among other factors (Tritt, 1992). Hence, SS co-digestion could be a nice suggestion to elevate GI at higher digestate concentrations.

2.5 Conclusions

The present chapter introduced the typology of the waste studied, that is SSAD, and the different approaches exploited for its characterisation. The chemical analysis revealed interesting features for what concerns agronomic applications. Indeed, the SSADs presented appreciable concentrations of organic matter as well as element exploited as macro- (N, P) and micro-nutrients (Ca, Mg, Zn, Fe) by plants. Moreover, these values resulted even higher than the mean ones normally present in literature, except for K, which was slightly lower. Concentration of heavy metals was below law limits and, in most of cases, it was satisfying the requirements imposed to commercial fertilisers. The ecotoxicological assays revealed nice values of RSG as well as of GI, measured on Primary and Secondary SSAD. In fact, below certain concentrations, no phytotoxic effect was observed, suggesting that controlled use of these "feedstocks" could be feasible.

However, future work should include a broader ecotoxicological characterisation of these digestates, in term of both the material analysed and the techniques exploited. Indeed, also the dewatered sludges (Centrifuged and Dried) should be taken into consideration, implementing a strategy to obtain a proper eluate to be tested via bioassay. On the other hand, the spectrum of bioassays used should be amplified. Indeed, in the present work, only the effects on a plant, garden cress (L. sativum), were studied since the subsequent application of the digestates would have dealt with the evaluation of their fertilisation potential on other plants, in a pot experiment. Hence, other bioassays should be considered in order to explore the toxic effects also on different organisms, such as bacteria, algae, little invertebrates, earthworms and other plants. Concerning bacteria, it is possible to evaluate the growth inhibition through "photobioassays" of *Vibrio fischeri* (luminescence) and *Arthrobacter globiformis* (fluorescent assay with resazurin). Also in the case of algae, the parameter measured is growth inhibition and *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* are the most used species for these test. As regards little invertebrates, ecotoxicological assays measure the immobilisation of small planktonic crustaceans (*Daphnia magna*) or rotifers (*Brachionus plicatilis*) Earthworms are another important ecotoxicological indicator, especially to evaluate the soil health, which is even more pertinent in the case of agricultural application of SSADs; bioassays with worms usually consist of avoidance tests with *Eisenia fetida* and *Lumbriculus variegatus*. Finally, also other

plants can give useful preliminary ecotoxicological information on germination (*Avena sativa*) and root elongation (*Lactuca sativa*).

In general, the main outcome from the analyses presented in this chapter was the fact that the SSADs showed desirable agronomic peculiarities. Hence, their effectiveness had to be confirmed not only on common agricultural substrates, but also on more challenging soils, showing depletion of organic matter and nutrients, which are some of the aspects related with desertification and soil degradation.

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Chapter 3

Evaluation of fertilizing and phytotoxic effects of anaerobic digestates from sewage sludge (SSADs) on cucumber grown on a sandy and poor soil

3.1 General scenario and aim of the study

The sewage sludge is the principal by-product generating from wastewater treatment. In last thirty years, European Union devoted particular attention to this issue and adopted policies boosting wastewater treatment in order to give back cleaner water to groundwater bodies (Council of the European Communities, 1991a). As a result, sewage sludge production raised from 5.5 (European Commission, 2019) to nearly 10 Mtonnes of dry solid matter of sludge per year (Milieu Ltd., 2008) divided in 8.7 Mtonnes from EU-15 countries and 1.2 Mtonnes from EU-12 (Pellegrini et al., 2016). Common destinations of sewage sludge and its stabilised forms (*e.g.* anaerobic digestate from sewage sludge, SSAD) include landfilling, incineration, composting and land application (Eurostat, 2019). Land and agronomic application as soil improver is currently regulated in Europe by an obsolete directive (Council of the European Communities, 1986) and, until 2015, it was mostly diffused in countries like Portugal and Spain.

Coming to the scientific scenario, many papers on the soil application of the sewage sludge (McGrath et al., 1995) and its derivatives (Andrés et al., 2011; Tarrasón et al., 2008) have also been published: in general, the main focus has been the evaluation of fertilising effects depending on the dose, species and soil studied, with experiments scaling from pots in greenhouses (Perez-Murcia et al., 2006;

Wong et al., 1996) to pots in outdoor (Alvarenga et al., 2016; Singh and Agrawal, 2009), to open field applications (Hussein, 2009; Rigueiro-Rodríguez et al., 2010). On the other hand, many works have investigated the phytotoxic effects of SS and SSAD, which are principally related to the excess of heavy metals (Belhaj et al., 2016; Singh and Agrawal, 2007), organic pollutants (Erhardt and Prüeß, 2001) and ammonia nitrogen (Gulyás et al., 2012).

The aims of the present work were mainly three: i) the comprehension of the applicability of anaerobic digestates from sewage sludge (SSADs) with an agronomic approach, exploiting nitrogen dosages commonly used in field operations; ii) the evaluation of their effects on sandy and nutrient-poor soils; iii) the study of their phytotoxicity on cucumber plants (pot experiment with *Cucumis sativus* L.) to assess the correct range for their application as soil improvers.

The main outcome of this study was thus the understanding of the reuse dynamics of this waste of our society, which is increasing more and more over years. The use of SSAD as soil improver is already known in literature (Alvarenga et al., 2016). Nevertheless, to the best of our knowledge, this was the first example of comparison of the fertilizing effects between two liquid SSADs (derived from separated anaerobic digestion of primary and secondary sludges) and two dewatered ones (centrifuged and dried SSADs) deriving from the same WWTP. Moreover, another aspect of novelty was the study of physiological parameters of cucumber plants grown in presence of different type of SSADs, with particular focus on physiological parameters. To a broader extent, this work wanted to address the problem of soils depleted in terms of nutrient and organic matter, evaluating pros and cons of soil application of SSAD. The results of the present study have recently been published on Waste Management (Cristina et al., 2019).

3.2 Materials and methods

3.2.1 Soil sampling and analysis

Two different growth substrates were used: a sandy soil and a peat substrate.

The soil used in this study was sampled in Grugliasco (TO), Italy (45°03'58.4"N, 7°35'32.9"E). It was collected within 20 and 100 cm depth, sieved at 2 mm and not previously sterilized. Physical and chemical soil properties (Table 1) were measured before the application of treatments. This soil can be classified as sandy (IUSS, 94% sand, 3% silt, 3% clay), alkaline (pH 8.7), really poor in organic matter (0.2%), high in carbonates (36.9%) but low in terms of active carbonates (1.0%) and with normal salinity (E.C. 0.080 mS/cm).

Physical and chemical analysis were performed according to the official methods of soil analysis of Italian Ministry of Agriculture and Forestry (Italian Ministerial Decree, 1999) and United States Environmental Protection Agency (USEPA). Measure of pH, electrical conductivity, organic matter, nitrogen forms and phosphorous was conducted on an aqueous extract obtained following the Sonneveld method (Sonneveld & Voogt, 2009). Measure of cation exchange capacity (C.E.C.) and exchangeable bases (Na, K, Mg, Ca) was performed on an

extract obtained with ammonium acetate protocol (Italian Ministerial Decree, 1999. Method XIII.1). Extraction of soil metals was performed by means of microwave assisted acid digestion (USEPA, 2007). All the methods and techniques exploited for soil analysis are reported in Table 3.1.

Peat substrate was mixed with perlite and then sterilised before each application. Chemical characterization of peat substrate was performed on an aqueous extract 1:2 (v/v water/peat substrate) according to Sonneveld method (Sonneveld & van den Ende, 1971). The analytical methods for peat analysis were all internal methods, and all techniques exploited are specified in Table 3.1.

3.2.2 Phytotoxicity evaluation on cucumber (*Cucumis sativus*)

3.2.2.1 Description of experimental set-up

The experimental campaign overall lasted 30 days and took place in a climate chamber with controllable photoperiod and temperature, which were set at 28°C for 14 hours during the day (07:00 - 21:00) and to 20°C for 10 hours during the night (21:00 - 07:00). During the first week after sowing, shoots were irrigated from the top one time a day; after this time water level in flowerpot saucer was kept constantly between 1 and 3 cm for the purpose of guarantee always water availability.

Commercial plastic pots (pyramid frustum shaped) were used with a total volume of 1250 cm^3 and a surface area of 144 cm^2; consequently, each pot was filled with approximately 250 g of peat substrate and 2000 g of sandy soil. Ten not treated seeds of cucumber (*Cucumis sativus* L.), cv. Marketmore (Four company, Italy) were sown in each pot. Cucumber was selected since it is relatively easy to grow and manage in climate chamber, and it is a "model plant" with a large scientific literature. Moreover, it was important that the dimension of maturity leaves would be bigger enough (more than 4 cm^2) to use them for physiological tests.

The experimental trials lasted thirty days. The position of all plants in the cell was changed every week to minimize location effects. The cultivations on peat substrate and on sandy soil were performed by using the substrate mixed with different treatments: four types of anaerobic digestates from sewage sludge (P, S, C, D), one commercial fertilizer (M) (NPK 22-5-6 + 2MgO, "Osmocote Topdress", ICL, Israel) and one not treated control (T). All of them were tested at three increasing doses (85, 170, 255 kg N/ha and they will be called as mentioned above), with four replicates per each. The intermediate nitrogen dosage (170 kg N/ha) was selected according to the Nitrates Directive (Council of the European Communities, 1991b), and the lowest (85 kg N/ha) and highest (255 kg N/ha) ones were chosen to keep the same difference between the application rates.

3.2.2.2 Measures performed

Germination was evaluated counting germinated seeds after three to ten days; then, germination was calculated as:

$$Germination = \left(\frac{germinated\ seeds}{sown\ seeds}\right) \times 100$$

Assimilation (A_N), stomatal conductance (g_s) and CO_2 concentration in substomatal cavity (C_i) were recorded two days before the end of the experiment using an Infrared Gas Analyzer (IRGA, ADC, Hoddesdon, UK). The measurement was performed on three fully formed leaves of each sample treated with the 170 kg/ha dosage. The selected leaves were the second or the third from the top and they were the best developed and directly exposed to artificial light.

The day before the end of the test, Chlorophyll Content Index (CCI) was evaluated with SPAD 502 chlorophyll meter (CCM-200, Opti Sciences, Inc., Hudson, NH, USA), which measures the absorbance in the regions of red (650 nm) and near-infrared (940 nm). After the ordinary calibration, it was used on five different fully formed leaves per pot. CCI was used as an indicator of the healthy state and the photosynthetic potentiality of plants. With the purpose of evaluating CCI, SPAD (Minolta) and CCM (Opti-Science) meters can be exploited and the second one was utilised in our investigation; to compare values obtained with results of studies that used SPAD-meter, the equations proposed by Parry and colleagues (2014) were considered.

At the end of the experiment, all plants were cut and immediately weighed to measure the fresh biomass of single pots (replicates), and then stored at -20°C until further use. Determination of dry biomass was carried out weighing these samples after thermal treatment (105°C for 72 hours). In order to compare the yields of each treatment, dry biomass ratio was calculated as ratio between mean dry biomass of each treatment and control. Besides the related-to-control biomass values, even absolute dry biomasses were analysed and compared. Per each concentration, each treatment was compared to the other ones, including the control.

Root Development Index (RDI) was assigned with a proposed method for the evaluation of root apparatus. This index is based on the soil compactness and cohesion, and on the coverage intensity by the roots over the pot-shaped soil. A score between 0 (no developed) and 4 (very well developed) was given to the apparent root expansion, inspecting the upside-down soil contained in each pot.

3.2.2.3 Statistical analysis

All data about pot phytotoxicity experiment with cucumber were analysed by one-way ANOVA with a Tukey's post-hoc test ($P \leq 0.05$), after the assessment of the fundamental assumptions of ANOVA: the normality of distributions (Shapiro-Wilk test, p-value > 0.05) and the homogeneity of the variances of the residuals (Levene's test with P(>F) > 0.05). The statistical software R (version 3.5.1 - Feather Spray - 2018) was used for all statistical analysis.

3.3 Results and discussion

3.3.1 Soil features

Table 3.1 Chemical characterization of sandy soil and peat substrate.

C.E.C., Cation exchange capacity; FAAS, Flame atomic absorption spectroscopy; GF-AAS, Graphite furnace atomic absorption spectroscopy; ICP – OES, Inductively coupled plasma optical emission spectrometry.

Parameter	Unit of measure	Sandy soil			Peat	
		Value	Method	Technique	Value	Technique
Stones	-	absent	Method II.1	Sieving at 2 mm		
Sand (2.0 - 0.020 mm)	%	94%	Method II.6	Granulometry		
Silt (0.020 - 0.002 mm)	%	3%	Method II.6	Granulometry		
Clay (< 0.002 mm)	%	3%	Method II.6	Granulometry		
Texture	-	sandy	Method II.6	Granulometry		
pH	-	8.7	Method III.1	Potentiometry	6.2	Potentiometry
Electrical conductivity	mS/cm	0.08	Method IV.1	Conductometry	0.722	Conductometry
Total limestone	%	36.9	Method V.1	Calcimetry		
Active limestone	%	1	Method V.2	Titration		
Organic matter	%	0.2	Method VII.3	Walkley-Black method		
N - Tot (Kjeldahl)	%	0.021	Method XIV.3	Kjeldahl method	0.42	Kjeldahl
N - NO_2^-	mg/kg	<1.0	Method XIV.9	Ionic chromatography	<0.05	Colorimetry
N - NO_3^-	mg/kg	1.6	Method XIV.9	Ionic chromatography	30.4	Colorimetry
N - NH_4^+	mg/kg	22.3	Method XIV.7	Ion selective electrode	1.3	Colorimetry
N - Org	mg/kg	186	Calculation	Calculation	4000	Calculation
P	mg/kg	2	Method XV.3	Olsen method	8.1	Colorimetry
Fe	mg/kg	5.6	EPA 6010C 2007	ICP-OES	0.79	FAAS
Mn	mg/kg	4.2	EPA 6010C 2007	ICP-OES	0.15	FAAS
Ca	mg/kg	950	Method XIII.4	FAAS	36	FAAS
Mg	mg/kg	54	Method XIII.4	FAAS	28	FAAS
Na	mg/kg	20	Method XIII.4	FAAS	16	FAAS
K	mg/kg	53	Method XIII.4	FAAS	41.1	FAAS
C.E.C.	meq/100 g	5.48	Method XIII.1	Titration		
As	mg/kg	1.9	EPA 7010 2007	GF-AAS		
Cd	mg/kg	0.1	EPA 7010 2007	GF-AAS		
Cr	mg/kg	64.5	EPA 6010C 2007	ICP-OES		
Hg	mg/kg	<0.1	Internal method	Direct mercury analyser		
Ni	mg/kg	57.5	EPA 6010C 2007	ICP-OES		
Pb	mg/kg	8.5	EPA 6010C 2007	ICP-OES		
Cu	mg/kg	17	EPA 6010C 2007	ICP-OES	<0.03	FAAS
Zn	mg/kg	45.5	EPA 6010C 2007	ICP-OES	0.02	FAAS

The sandy soil was alkaline and carbonate-rich, with very low concentration of organic matter (0.2%) and nutrients (N: 0.021%; P: 2 mg/kg; K: 53 mg/kg). According to the information reported by Arpa Veneto (2007), this soil could be reasonably considered very poor for what concerns organic matter, macro-nutrients

(N, P, K) and micro-nutrient (Mg, Ca, Fe, Mn). Hence, the soil respected those requirements of nutrient depletion which wanted to be addressed in the present study. Several reports have shown that SS application in soils with these peculiarities can provide a good nutrient supply with a relatively small risk of pollution (Antolín et al., 2005; García-Gil et al., 2004; Navas et al., 1998).

On the other hand, analysis of peat substrate revealed really different values, indicating the richness in nutrients of this cultivation substrate widely used for scientific purposes as well as for gardening use.

3.3.2 Evaluation of fertilizing and phytotoxic effects

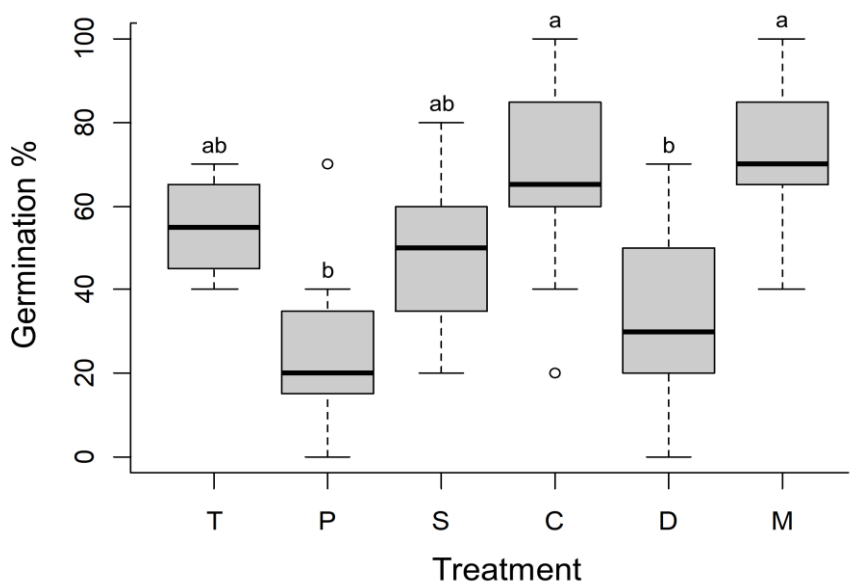
3.3.2.1 Germination

Germination on sandy soil revealed significative differences only at the third day after sowing. More in detail, these differences were found only between the typology of treatment, highlighting a greater germination on C and M than on P and D (Figure 3.1). This treatment was the only one displaying significative differences even between treatment concentrations, with D255 presenting the smallest germination value (16%). Overall germination (all germinated seeds to all sown seeds ratio) after 3 days was 43% on sandy soil, while at the end of experiment it reached 80% (data not shown).

On the other hand, no significative difference in germination on peat substrate emerged during the 10 days after sowing. Even in this case overall germination increased along the experiment, shifting from 83% at 3 days to 90% at 30 days after sowing.

Figure 3.1 Mean germination (%) after three days of *Cucumis sativus* grown on sandy soil under each treatment.

Different letters indicate differences between treatments that were significant at $P < 0.05$ (Tukey HSD).



The germination of cucumber seedlings grown on peat substrate was higher compared to sandy soil, which may be due to pH values of the growing substrate. Optimal pH conditions for cucumber germination are between 5.5 and 6.5 (Baudoin et al., 2017), that are values roughly similar to peat substrate, but far away from sandy soil ones (pH 8.7). Moreover, D255 induced a significatively low germination within all treatments applied on sandy soil. This effect could be explained by the high E.C. of D, which is 300% and 50 % higher than liquid and centrifuged digestates, respectively. Indeed, other authors (Eklind et al., 2001; Sánchez-Monedero et al., 2004) demonstrated a clear correlation between the E.C. increase in soil and germination decrease.

3.3.2.2 Dry biomass

On sandy soil, all treatments, except for P255, overcame the yields of the control: C255 and D255 were considerably higher than others doubling the control biomass. The increase of biomass production was proportional with the dosages of C and D digestates as well as M; the highest dosage of the last one did not seem to cause further increase. On the other hand, P and S digestates had the highest yields at intermediate dosages (P170 and S170), while dry biomasses at lowest dosages (P85 and S85) were comparable to the highest ones (P255 and S255) and were not significantly different from control (Figure 3.2).

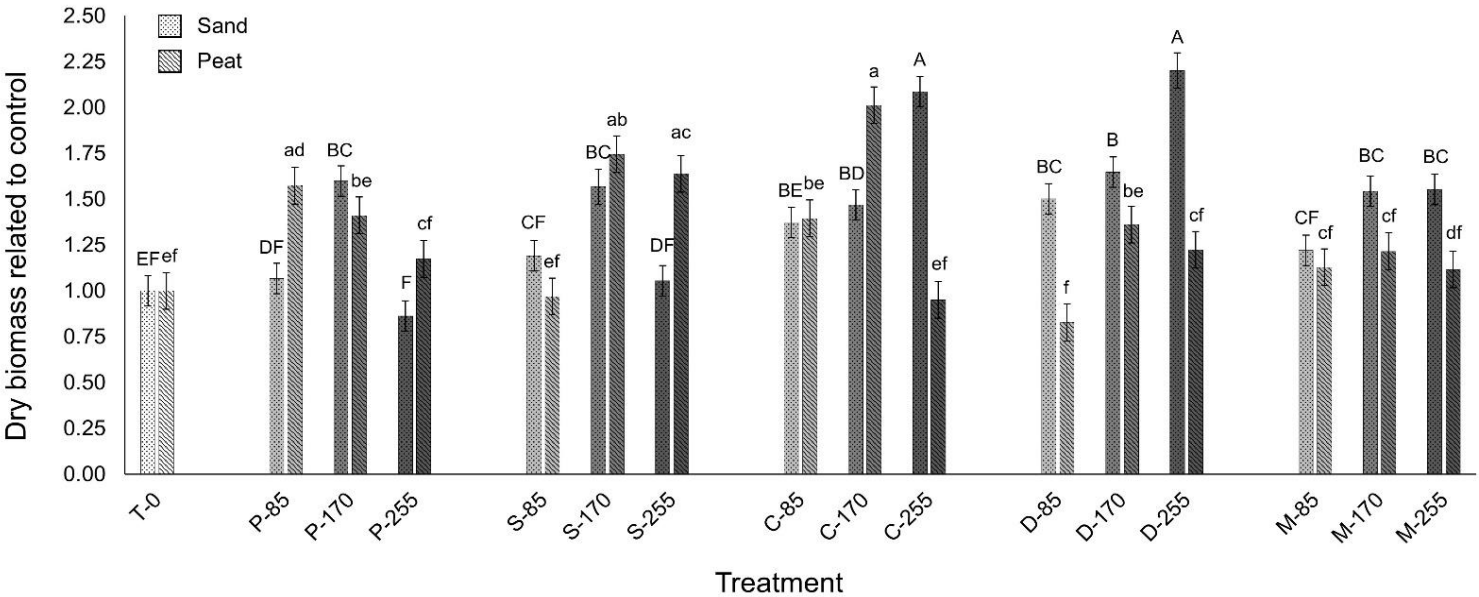
For what concerns the biomass yield on peat substrate, the common biomass trend showed an increase going from 85 to 170 kg N/ha dosages, and a decrease moving from 170 to 250 kg N/ha. However, P digestate was the only one displaying decreasing biomass values for higher application rate of treatment. The highest biomass yield was found in C170, even doubling the control one. Moreover, P85, S170 and S255 were the only ones showing a significantly higher biomass than control. (Figure 3.2). Very important differences were found in 170 kg N/ha treatments: all yielded significantly more biomass (1.10 to 1.21 g) than the control (0.75 g) on sandy soil (Figure 3.3); on peat substrate, P, S and C treatments provided more biomass (2.92 g, 3.61 g and 3.95 g, respectively) than control (2.07 g), with S and C showing the top production, while D (2.82 g) and M (2.51 g) behaved similarly to the control (Figure 3.4).

The fertilizing effects of the digestates on cucumber were studied in previous works. However, the ones dealing with sewage sludges and derived products were mostly focused on the toxic effects derived from organic and inorganic pollutants present in this waste (Waqas et al., 2014; Wyrwicka et al., 2014). In the present work, higher biomass yields were recorded for the plants grown on peat substrate than on sandy soil due to the richness in organic matter and macronutrients of the first one. Nevertheless, this aspect likely contributed to the lower degree of differences between control and treated samples; indeed, all treatments on sandy soil at 170 kg N ha^{-1} were significantly different from the control, while the same conditions on peat substrate revealed results, for D and M, slightly comparable to T. In general, it could be inferred that fertilizing effects occurred at different levels both in terms of soil and treatment concentration. In fact, dry biomass overcame the control in all cases except four (P255 on sandy soil; S85, C255 and D85 on peat

substrate). These biomass-promoting effects on cucumber grown on sandy soil have already been reported by Hussein (2009): despite the higher application rate (up to ten times greater, in terms of total nitrogen), the authors observed a crop yield improvement over control around 70%, which is in good agreement with the results of the present study. Moreover, cucumber was utilised to test the effects of sewage sludge compost applied on a sandy soil. Even in this case, the dry weight of shoot biomass almost doubled the control one (Xu et al., 2012), similarly to C255 and D255 conditions on sandy soil of the present work. Moving to a broader perspective, other works designed with a pot experiment approach assessed the fertilizing effect of sewage sludge on different species. Asagi and Ueno (2008) and Shaheen and co-workers (2014) reported examples of komatsuna (*Brassica rapa* L. var. perviridis) grown on sandy soil, and rocket (*Eruca sativa* Mill.), grown on calcareous soil, which quintupled and doubled their dry biomass yield, respectively. Furthermore, relevant outcomes have been described on sunflower (*Heliantus annuus* L.) (Belhaj et al., 2016) and kenaf (*Hibiscus cannabinus* L.) (De Andres et al., 2010) grown in presence of dewatered anaerobic digestates similar to C and D treatments, providing well comparable results with this study. Qasim and colleagues (2001) and Alvarenga and co-workers (2016) provided examples of cereal crops (maize and sorghum, respectively) fertilized with an unstabilized sewage sludge and a yield increase of 40% and 400%, respectively, over untreated control was reported. Even if it's difficult to compare the behaviour of different plants exposed to diversely treated sludges, it is conceivable that weaker performances of digestates of this study may be due not only to lower application rates, but also to the nitrogen fractionation.

Figure 3.2 Mean dry biomass related to control of *Cucumis sativus* grown on sandy soil and peat substrate.

Each data point represents mean of replicates to mean of control replicates ratio ± standard error; different letters indicate differences between treatments and concentrations of N that are significant at $P < 0.05$ (Tukey HSD); upper-case letters refer to samples from sandy soil and lower-case letters refer to samples from peat substrate.



In fact, in the present work, this was skewed in favour of organic nitrogen (N_{Org} / N_{Tot} ranging from 79% to 94%), with lower concentrations of "readily-available" nitrogen (*i.e.* NH_4^+ and NO_3^-).

Figure 3.4 Mean dry biomass of *Cucumis sativus* grown on sandy soil with 170 kg N/ha treatments.

Different letters indicate differences between treatments that are significant at P < 0.05 (Tukey HSD).

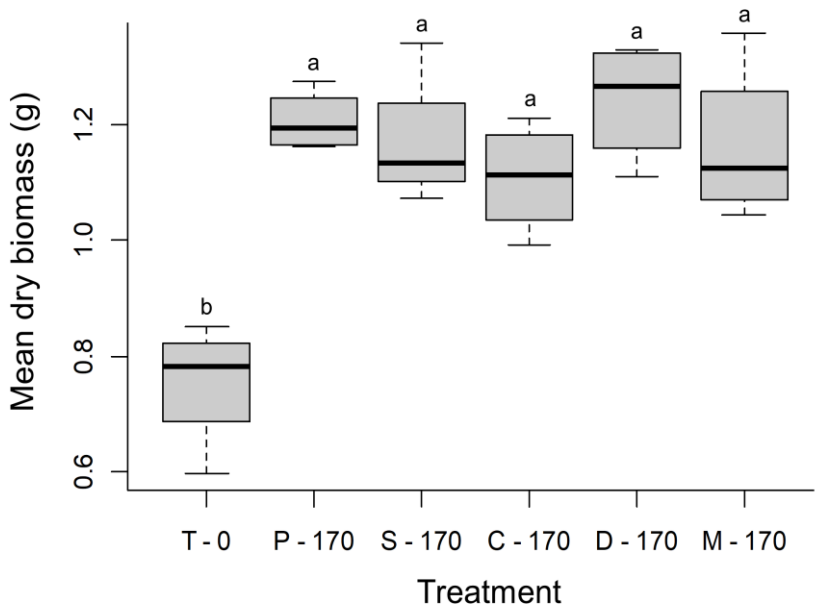
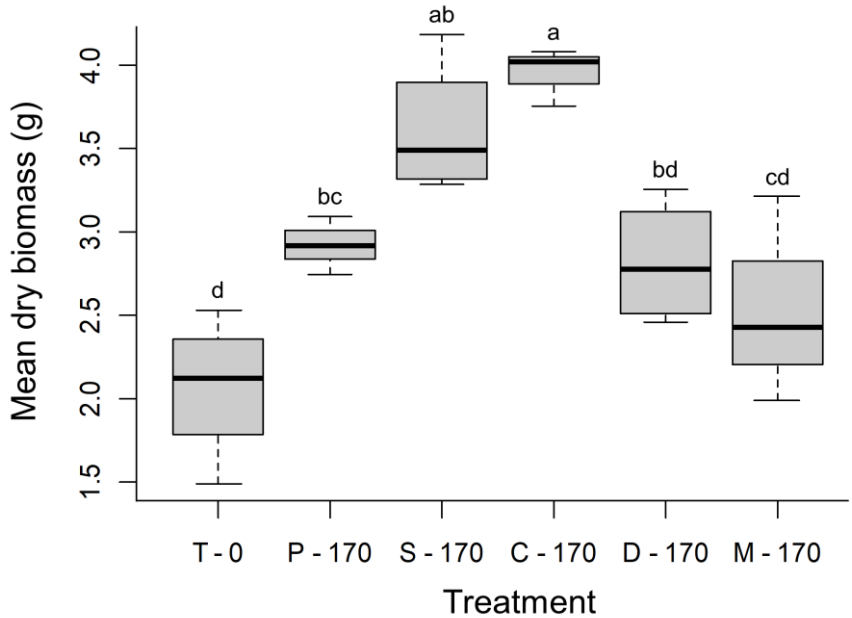


Figure 3.3 Mean dry biomass of *Cucumis sativus* grown on peat substrate with 170 kg N/ha treatments.

Different letters indicate differences between treatments that are significant at P < 0.05 (Tukey HSD).



Nevertheless, the main drawbacks of sewage sludge land application are the phytotoxic effects occurring at higher application rates, preventing the optimal growth of the plant. Indeed, this aspect has been deeply investigated as regards the presence of organic and inorganic pollutants, such as heavy metals. These ones can interfere with the biomass yield as widely reported in literature (Nagajyoti et al., 2010; Singh and Agrawal, 2007). In the present work, the decrease of dry weight with higher application rates was observed only in few cases (*e.g.* P255 and S255 on sandy soil, and P255, C255 and D255 on peat substrate). These reductions could be justified in part with the metal-derived toxicity, especially in the case of peat substrate. Its slightly acidic conditions maybe allowed a more sustained metal bioavailability, which was instead down-modulated by high pH in sandy soil (Belhaj et al., 2016; Sukreeyapongse et al., 2010). On the other hand, another conceivable hypothesis was the ammonia-connected toxicity occurring in alkaline conditions: increasing soil pH induces higher NH_3 percentage of total ammoniacal nitrogen (Masoni and Ercoli, 2010), according to the NH_4^+/NH_3 acid-base equilibrium (Gay and Knowlton, 2005). Thus, at the pH of sandy soil exploited in this work (8.7), around 20-25% of ammoniacal nitrogen was represented by NH_3, which could negatively affect the plant growth under different aspects as described by van der Eerden (1982). This aspect has been observed mainly on plants exposed to liquid digestates, which revealed ammonia-nitrogen concentrations up to six times higher than dewatered ones. On the contrary, dehydration of SSAD might have had a positive effect on the ammonia abatement, which resulted in an overall slighter phytotoxicity exhibited by solid SSADs (C and D, in this study). In this respect, this aspect is confirmed by (Alvarenga et al. (2016) and De Andres et al. (2010). Moreover, the latter work devoted particular attention to the treatment formulation (pelletization, in this case), which can be an aspect to take into account even for future work.

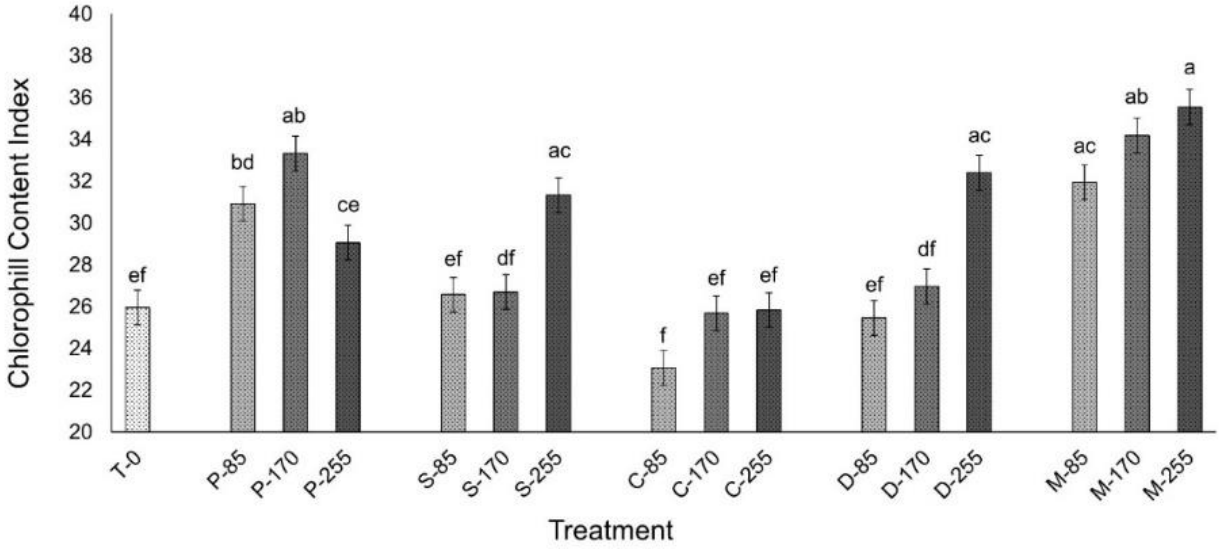
3.3.2.3 Chlorophyll content index

CCI control mean level of plants grown on sandy soil was 26.0 (Figure 3.5); the chlorophyll concentration significantly higher than control were obtained in M255 (35.5), M170 (34.2), P170 (33.3), D255 (32.4) and M85 (31.95), S255 (31.3) and P85 (30.9). Moreover, the chlorophyll content was higher with the increase of the SSAD application rate. However, this behaviour was not detected for P digestate, where the increase of treatment dosage was related firstly to a CCI increment in P170, then to a CCI reduction in P255 (29.1).

On peat substrate **(**Figure 3.6**),** control mean level of CCI (28.2) was higher than on sandy soil. Similarly to CCI of cucumber grown on sandy soil, mineral fertilizer in M255 (36.4) and M170 (33.3) gave high results and, together with C170 (37.2), were significantly higher than control. Moreover, C170 was significantly different from other dosages within same treatment, while no significative difference among concentrations was found on P, S and D treatments.

Figure 3.5 Chlorophyll Content Index (CCI) of leaf of *Cucumis sativus* grown on sandy soil.

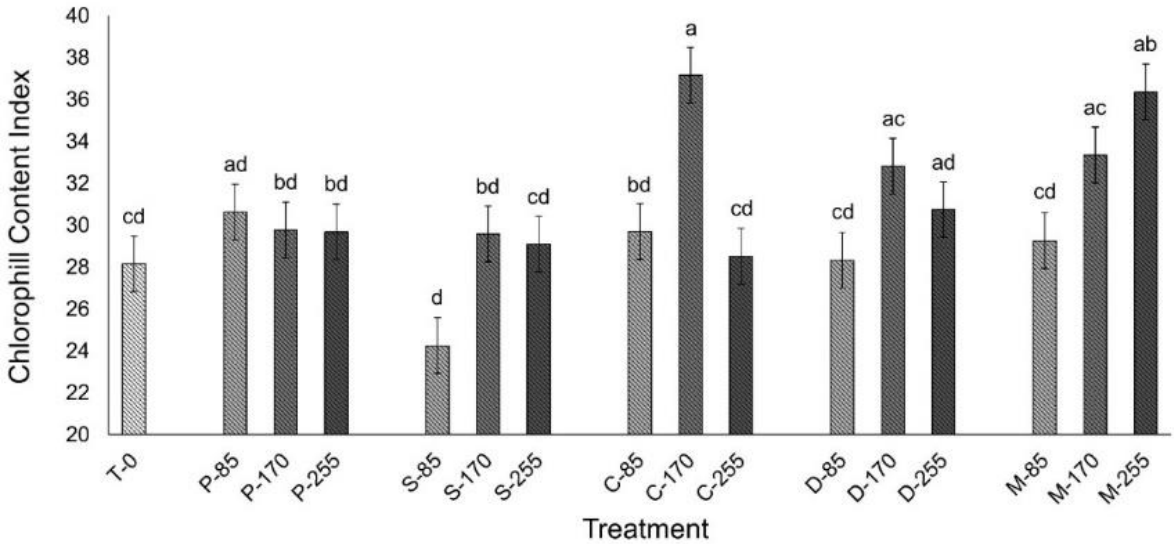
Different letters indicate differences between the treatments with the different concentrations of N at 85, 170 and 250 kg N/ha, that are significant at P < 0.05 (Tukey HSD).



Chlorophyll content can be strongly correlated to crop nitrogen content and can be sensitive to differential nitrogen nutrition in vegetable crops (Padilla et al., 2017). Nitrogen nutrition index (NNI) is an indicator of plant nitrogen status, and NNI = 1 values correspond to optimal N nutrition (Lemaire and Gastal, 1997); in the case of cucumber, it was matched to CCI values between 24 and 36.

Figure 3.6 Chlorophyll Content Index (CCI) of leaf of *Cucumis sativus* grown on peat substrate.

Different letters indicate differences between treatments and concentrations of N that are significant at P < 0.05 (Tukey HSD).



In the present work, the CCI values obtained on peat substrate were in this range, likely due to the better capacity of peat substrate to retain nutrients, while on sandy soil they were lower. These values were in agreement with the ones reported in other studies (Guler and Buyuk, 2007; Jahromi et al., 2012; Xu et al., 2012). Higher values of CCI did not coincide necessarily to higher biomass yields: in fact, on

sandy soil C255 had middle-low CCI, but its biomass yield was the highest. Latare and co-workers (2014) reported a similar behaviour for wheat and rice, in which yield increase was not accompanied by a significative rise in chlorophyll content values (measured with SPAD). Moreover, M255 showed the highest CCI value on sandy soil: this result was probably linked to the mineral fertilizer formulation which ensured a long-lasting nitrogen release. Considering the typologies of treatment, many works showed a general improvement of CCI values upon application of sewage sludge and its derivatives. Improvements of chlorophyll content compared to untreated controls have been recorded on cereals (Alvarenga et al., 2016; Koutroubas et al., 2014), edible plants (Asagi and Ueno, 2008) and trees (Han et al., 2004). This general behaviour indicated that sewage sludge provides a good amount of nutrients, which is an aspect that clearly emerged even in this work.

3.3.2.4 Infra-red gas analyser

Treated and control cucumber plants grown on sandy soil showed significative differences in net photosynthesis (A_N): control value (1.83 CO_2 $m^{-2}s^{-1}$) was lower than all other treatments, which however did not differ from each other (Figure 3.7.a). Therefore, it is worth underlining the value measured on P treatment (3.75 µmol CO_2 $m^{-2}s^{-1}$), which doubled control value. In order to stomatal conductance (g_s), all digestate treatments at least doubled the one of control thesis (0.098 mmol H_2O $m^{-2}s^{-1}$), while S even trebled this result (0.333 H_2O $m^{-2}s^{-1}$) (Figure 3.7.b). On the other hand, while M showed an intermediate behaviour between digestates and control as regards stomatal conductance, it reached the highest concentration of CO_2 (536 ppm) in substomatal cavity (C_i) (Figure 3.7.c).

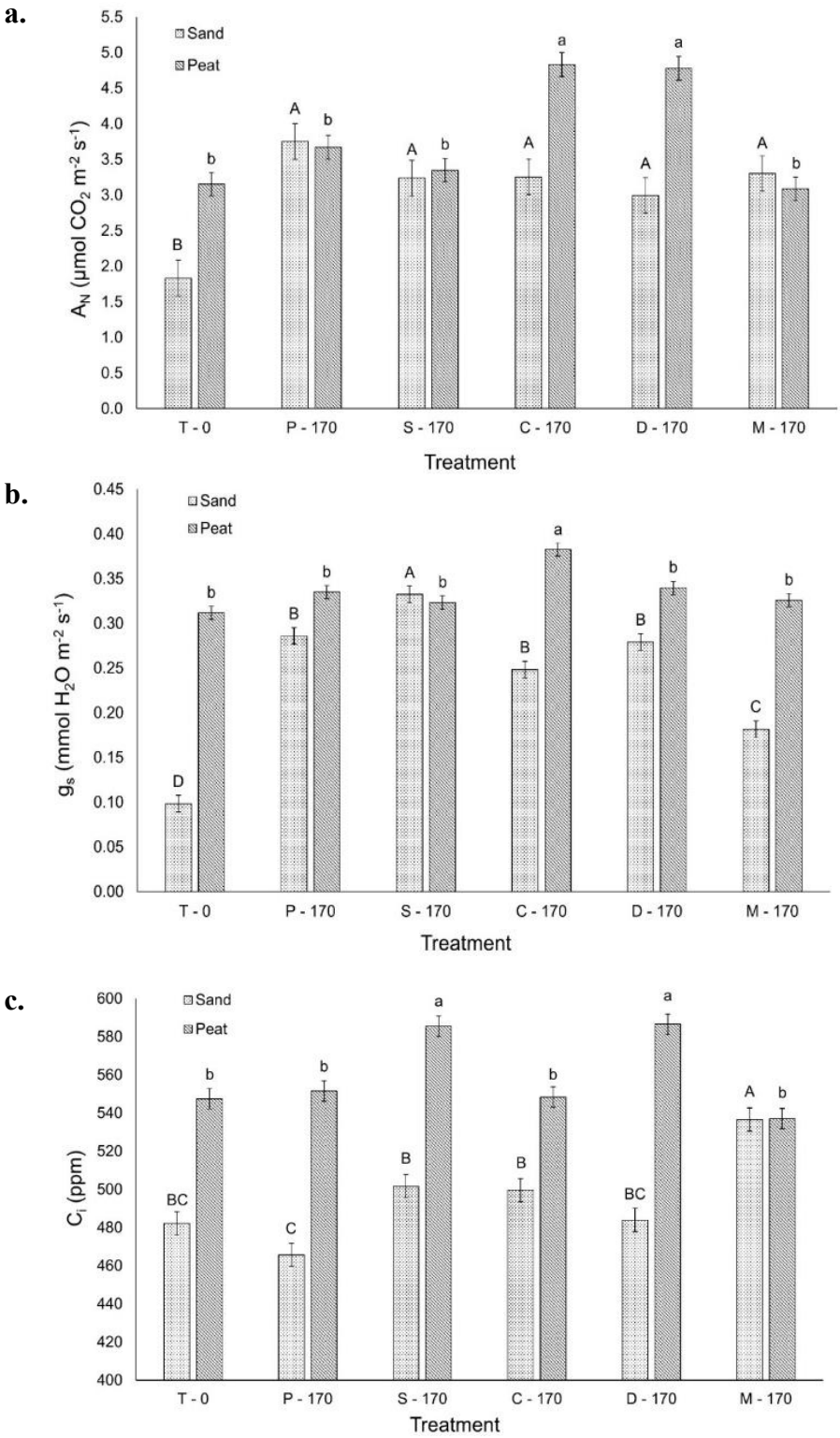
Moving to peat substrate, differences in net assimilation of CO_2 (Figure 3.7.a) between treatments and control were few: C (4.83 CO_2 $m^{-2}s^{-1}$) and D (4.78 CO_2 $m^{-2}s^{-1}$) had a higher A_N than all other treatments (including T). However, it is important to point up that only C (0.383 mmol H_2O $m^{-2}s^{-1}$) displayed also a significantly greater value in terms of stomatal conductance (Figure 3.7.b).

CO_2 concentration in substomatal cavity revealed two different groups: the first gathering the highest C_i values, S and D (586 ppm), and the second collecting all other treatments (T included), which showed lower results (Figure 3.7.c).

The results of gas analysis measurements were not directly comparable to other values in literature because these are strictly depending on environmental conditions (light, temperature, irrigation and phenological phase). On peat substrate, almost no difference was appreciable; just in C case, A_N and g_s values were higher than T; anyway, these differences reflect values obtained in biomasses and CCI measurements. To the best of our knowledge, no measurements of physiologic parameters and gas exchange have been performed on cucumber exposed to sewage sludge treatments with pot experiments.

Figure 3.7 IRGA measurements on *Cucumis sativus* grown on sandy soil and peat substrate with 170 kg N/ha treatments.

a. Net assimilation (A_N in µmol CO_2 m^{-2} s^{-1}) ± mean standard error, b. Stomatal conductance (g_s in mmol H_2O m^{-2} s^{-1}) ± mean standard error and c. CO_2 concentration in substomatal cavity (C_i in ppm) ± mean standard error. Different letters indicate differences between treatments that are significant at $P < 0.05$ (Tukey HSD); upper-case letters refer to sandy soil and lower-case letters refer to peat substrate



However, some comparisons can be done with studies on physiologic parameters of plants exposed to sewage sludge and studies on physiologic parameters of cucumber**.** Antolín et al. (2010) and Bourioug et al. (2015) carried out pot experiments with alfalfa (*Medicago sativa* L.) and European larch (*Larix decidua* L.), applying both sewage sludge rates like the ones of this study. The significative differences reported in the case of cucumber grown on sandy soil are in good agreement with A_N and g_s values of the first work, while in the second study only with A_N ones. Furthermore, similar results of A_N and g_s have been assessed using two different dosages of sewage sludge in field on rice crop (*Oryza sativa* L.) (Singh and Agrawal, 2010). On the other hand, studies with sewage sludge on beet (*Beta vulgaris* L.) (Singh and Agrawal, 2007) and okra (*Abelmoschus esculentus* L.) (Singh and Agrawal, 2009) showed lower results in terms of A_N and g_s, probably due to the higher SS doses, provoking phytotoxic effects.

Physiologic parameters of cucumber plants were studied mainly as regards metals stress, such as toxicity derived from copper (Alaoui-Sossé et al., 2004) and sodium (Chartzoulakis, 1994): their increasing concentration caused the decrease of the physiologic parameters. Anyway, in the present study, concentrations of copper and sodium were lower and, consequently, A_N and g_s values were higher. Moreover, an increase of stomatal conductance in presence of heavy metals was explained by (Singh and Agrawal, 2010), claiming that it may be due to high nutrient availability through SS amendment which nullified the heavy metal toxicity.

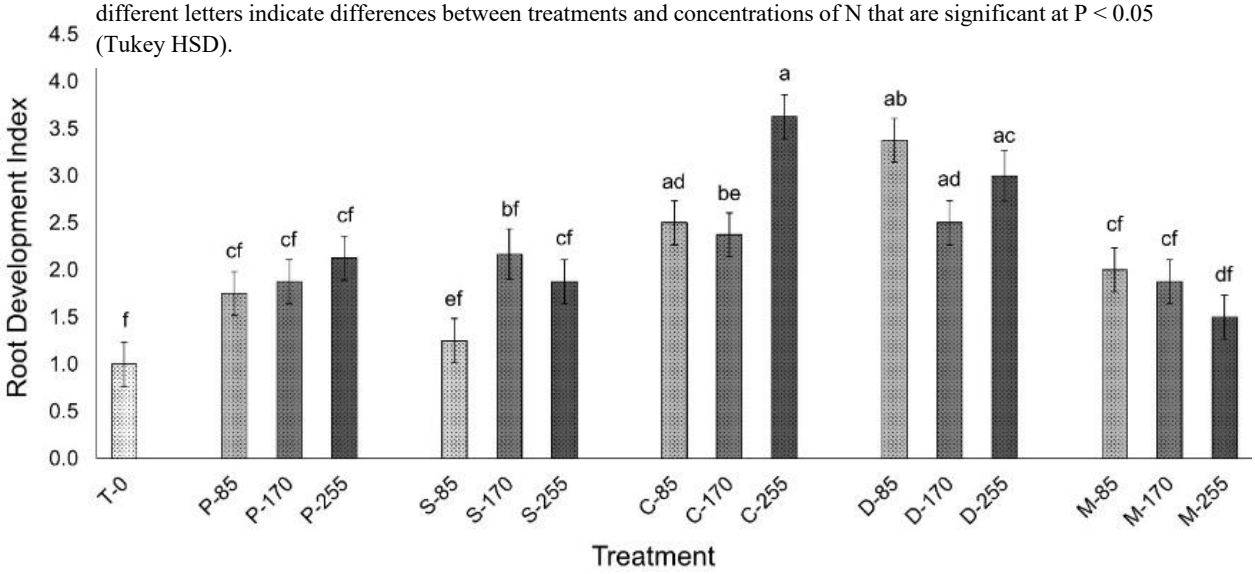
3.3.2.5 Root development index

Root apparatus was mostly developed in plants grown on C and D treatments on sandy soil (Figure 3.8). Indeed, C255 (3.625), D85 (3.375), D255 (3.000), D170 (2.500), C85 (2.500) and C170 (2.375) revealed an RDI significantly higher than control. Data on peat substrate did not respect the homogeneity of variances (P-value = 0.0449) (data not shown).

The trend of biomass production did not match always with a sustained root development (RDI). This mismatch between shoot and roots biomass in cucumber has been already reported in literature (Xu et al., 2012). Root development results clearly revealed that C and D gave best outcomes, with an RDI similar between them and higher than liquid digestates and M. Furthermore, these findings demonstrate that the kind of treatment had a greater effect on roots growth than the nitrogen amount (except for the case of C255). This observation is in contrast to the study of Gulyás and co-workers (2012), which described a root reduction in ryegrass (*Lolium perenne* L.) treated with same dosages of SSAD, probably due to excessive ammonium content. Despite comparable nitrogen application rate, root development was not inferior than control presumably because of a lower NH_4^+/N_{Tot} ratio of the SSADs used in the present work.

Figure 3.8 Mean Root Development Index of *Cucumis sativus* grown on sandy soil

Each data point represents mean of replicates to mean of control replicates ratio ± mean standard error; different letters indicate differences between treatments and concentrations of N that are significant at P < 0.05 (Tukey HSD).



3.4 Conclusions

Four different SSADs (two liquid and two dewatered) coming from the same WWTP were characterized and exploited as soil improver for promoting cucumber growth in pot experiments. Application of SSADs improved plant growth according to the exploitation of nitrogen dosages commonly used in field operations. Overall, an intermediate nitrogen dosage (170 kg N/ha) showed the best results in terms of biomass, chlorophyll content, net photosynthesis, stomatal conductance, and root development. All these results were much more evident for cucumber plants grown on an alkaline, sandy and poor (concerning organic matter and nutrients) soil than a more acid and rich cultivation substrate, such as peat substrate. However, in some cases phytotoxicity effects occurred probably due to an excessive addition of ammonia nitrogen or heavy metals.

More in general, this work contributed to deepen the knowledge about the agronomic recycling of SSAD. As far as we know, this was the first study conducting a systematic comparison of the fertilizing and phytotoxic effects of anaerobic digestates from primary, secondary, centrifuged, and dried sludges. The significant differences between the SSADs likely indicated that the ways in which the digestate was treated at WWTP level had an effect not only on its chemical peculiarities but also on its agronomic potential. Thus, these findings can pave the way for a wiser recycling of this waste, through its usage for the improvement of nutrient-deficient soils, representing a promising solution to combat this relevant environmental issue. Future work should include the study of long-term effects and of repeated applications consequences deriving from land application of these SSADs; on the other hand, strategies for contaminants abatement or recovery of valuable substances should be investigated.

Broadening the perspective, the present study may offer valuable cues within the topics of desertification and soil aridity, which are quite related with the nutrient depletion in soils. The information reported in the present chapter was obtained through experimental campaign in a controlled environment (*i.e.* climatic chamber), which was the most indicated to conduct a preliminary assay, since it allowed assessing the fertilizing effects of the treatments, which might have been hidden by the environmental ones. Thus, the logical continuation of this work should be the progressive "scale-up" of the scenario (greenhouse, open-field, etc.) in which the treatments are tested in even more challenging conditions in terms of plant fertilisation and soil amelioration. Another crucial point is the irrigation, the main issue related to aridity, which is an environmental parameter based on precipitations. During this study, enough water was constantly provided to let the plant grow, in line with the above-mentioned exclusion of factors which could have hindered the effects of digestates. Of course, in a real arid context, drought is the major issue and, likely, the application of SSADs (even the liquid ones) cannot satisfy *per se* the water demand for a proper plant growth. So, SSADs alone are not sufficient to fight soil aridity, but they could represent a nice element to combat desertification, improving soil fertility. A promising strategy to provide an integrated solution is represented by fertirrigation, which can supply simultaneously water and nutrients at slow rates (drip irrigation). This approach may ensure many advantages, such as total consumption of water provided to plants without leaching, and progressive improvement of soil features, such as organic matter enrichment. In this scenario, an attractive future perspective is the assessment of fertilizing effects using of fertirrigation using not only SSADs, but also the abovementioned valuable compounds which can be possibly recovered from them, excluding the potential toxic elements present in this waste.

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Chapter 4

Analysis of rhizosphere microbial communities of plants grown in presence of SSADs

4.1 General scenario and aim of the study

The soil application of sewage sludge has been protagonist of many published works in literature especially for the effects both on soil chemical properties and on plant growth. In recent years, the interest also on the microbiological aspect of this practice has really grown, with particular attention on the soil microbial communities. Soil addition of sewage sludge and its derivatives (anaerobic digestate, compost, etc.) has a duplex biological effect: on one side, many substances that elicits microbial activity are provided to the soil (*e.g.* organic matter, macronutrients), and on the other microorganisms deriving from sludge stabilization process are delivered to soil (Pascual et al., 2008).

Among all soil microbial communities, rhizosphere ones have gained relevant interest in the scientific scenario. Rhizosphere is defined as the soil part most proximal to plant roots and it is the arena of really important biotransformations which contributes to nutrient cycling, improving soil dynamics; as a consequence, the more rhizosphere microbial communities are structured, the better the plant grows (Igiehon and Babalola, 2018).

The technologies available for the study of soil microorganisms are manifold. In general, they can be divided in culture dependent and independent. The first ones rely in the isolation of the microorganisms, the cultivation in vitro and their phenotypic identification. The latter are the most widely diffused since the majority of soil microorganisms are not culturable. These technique are quite heterogeneous and include: i) evaluation of microbial biomass, respiration and activity (Antolín et al., 2005); ii) analysis of concentrations of phospholipid fatty acids (PLFAs) used as markers of defined microbiological taxa (Börjesson et al., 2012); iii) community

level physiological profile (CLPP), which provide general information on the taxa based on the consumed substrates (Calbrix et al., 2007); iv) molecular methods to target defined microbial DNA sequences such as terminal restriction fragment length polymorphism (T-RFLP) (Enwall et al., 2007) and denaturating gradient gel electrophoresis after amplifications of bacterial target DNA sequences (PCR-DGGE) (Ros et al., 2010). However, all these methods allow to provide a general description of the microbiological soil scenario, without the possibility of the simultaneous individuation of all the microorganisms involved. In recent years, the disruptive technology of Next Generation Sequencing (NGS) finally gave this possibility, allowing to detect simultaneously all the microorganisms taking part to a specific community. The possibility to read defined and highly variable bacterial sequences (*i.e.* the one coding for 16S RNA) permit to depict a quite reliable "family picture" of the involved bacteria (Knight et al., 2018).

The number of scientific works dealing with soil microbial communities' characterization via NGS is sharply increasing and it is surely a promising technique for the scientific community. However, to date, the works dealing with microbial characterization of plant rhizosphere are still few, and even more less the one dealing with sewage sludge application. To the best of our knowledge, this is the first study of characterisation of rhizosphere microbial communities of tomato plants on a nutrient-poor sandy soil and on a peat substrate treated with differently processed anaerobic digestate from sewage sludge (SSAD).

4.2 Materials and methods

4.2.1 Experimental set-up in greenhouse

The experimental campaign took place in greenhouse during spring-summer season lasting overall 60 days (30th May – 30th July). The greenhouse was equipped with both an automated irrigation system from the top (operating each day three cycles of 5 minutes of rain) and an automatic vent opener to help controlling climate and temperature within the greenhouse. Cultivations were set-up with two cultivation substrates (*i.e.* sandy soil and peat substrate; see characterization in *Chapter 3*) in commercial plastic pots located on gridded mobile benches. Cone frustum shaped pots were used with a total volume of 2500 cm^3 each and a surface area of 230 cm^2; consequently, each pot was filled with approximately 500 g of peat substrate and 4000 g of sandy soil. In order to prevent the sand spill from the holes at the pot bottom, sterile gauze pads were inserted, allowing at the same time sand retention and drainage of excessive water. Cultivation substrates were mixed with different treatments: four types of anaerobic digestates from sewage sludge (Primary, P; Secondary, S; Centrifuged, C; Dried, D), one commercial fertilizer (M) (NPK 22-5-6 + 2MgO, "Osmocote Topdress", ICL, Israel) and one not treated control (T). All of them were tested, with five replicates per each, only at 170 kg N/ha since it was the dosage showing the best results in terms of fertilization (see *Chapter 3*). Once the pots were filled with the treated cultivation substrates, three not treated seeds of tomato (*Solanum lycopersicum* L. var. beefsteak) (Furia

Sementi, Parma, Italy) were sown in each pot in order to ensure the germination of at least one plant in each pot. In order to guarantee an adequate plant development, thinning out was performed 20 days after sowing, leaving only one plant per pot. The position of all plants on the greenhouse bench was changed every week to minimize location effects. When plant height overcame 50 cm, the stems were tied loosely with a wire to a wooden stake inserted directly in the pot. In this way, the tomato plants were adequately supported and were able to keep on growing without sprawling.

4.2.2 Sampling of rhizosphere and Soil DNA

At the end of the experiment, all plants were cut and immediately weighed to measure the fresh biomass of the sixty tomato plants. After the evaluation of RDI, root apparatus was recovered by manual extraction from cultivation substrates and shacked to discard the bulk soil in excess. From this point to the end of isolation of rhizosphere, all manipulations were performed wearing nitrile laboratory gloves washed with denatured alcohol and using only sterile laboratory stuff to prevent contamination between samples. Isolation of the rhizosphere (defined as the soil layers adherent to root apparatus) was performed as reported by Lundberg and colleagues (2012), with some modifications. Portion of roots were chosen in the central part of root apparatus, not too close to the apex neither to epigeal part of the plant. The selected roots were placed in 50 ml falcon tubes filled with 20 ml of sterile phosphate buffered saline (PBS, pH 7.4). Afterwards, each falcon was vortexed at maximum speed to completely detach the soil from the roots; the washed roots were removed with a sterile tweezer from the falcons, which were then centrifuged at 14000 rpm for 15 minutes. The supernatant was discarded, while the soil pellet was stored fresh at 4 °C (not more than one month) until DNA extraction, as indicated by Urra and co-workers (2019).

Soil DNA extraction protocol was implemented by the means of two different commercial kits: DNeasy PowerSoil Kit (QIAGEN, The Netherlands) and NucleoSpin® Soil (Macherey-Nagel, Germany). Both kits relied on the same way of working: in the first part, sample was lysed to solubilise soil DNA through the synergic effect of chemical lysis (with patented reagents) and mechanical lysis (with glass/ceramic microbeads); in the second part, DNA was washed from contaminants by means of columns for microcentrifuge tubes; finally, DNA was eluted in 50-100 µl of PCR grade water, recovered in sterile Eppendorf tubes and stored at -20 °C. Both kits were used following manufacturers indications, with the only modification of the shacking system to perform sample lysis; in fact, TissueLyser bead mill (QIAGEN, The Netherlands) was used (30 min, 20 Hz) instead of simple vortexing to guarantee an appropriate sample agitation.

After the extraction protocol, NanoDrop® spectrophotometer (NanoDrop® ND-1000 – Marshall Scientific – New Hampshire, USA) was used to assess DNA yield and purity.

DNA yield was calculated with the formula:

$$c = \frac{(Abs_{260} \times e_{260})}{b}$$

where c is the nucleic acid concentration in ng/µl, Abs_{260} is the absorbance at 260 nm in AU, e is the extinction coefficient at 260 nm in ng·cm/µl (double-stranded DNA: 50 ng·cm/µl) and b is the path length in cm (path length: 1 mm). DNA purity was evaluated with Abs_{260}/Abs_{280} and Abs_{260}/Abs_{230} ratios: absorbance at 280 nm is mainly due to protein presence in the samples, while absorbance at 230 nm is due to contaminants presence, such as humic acids, which may affect further DNA manipulations (*e.g.* enzyme inhibition during polymerase chain reaction, PCR). Benchmark values of ~1.8, for Abs_{260}/Abs_{280}, and 2.0-2.2 are generally accepted as "pure" for DNA; excessively low ratios values stand for "dirty" sample.

A preliminary phase was dedicated to find out the best DNA extraction strategy for the cultivation substrates exploited in this study. After the screening, NucleoSpin® Soil kit showed best results in terms of DNA yield and purity. Table 4.1 reports all the technical details exploited for DNA extraction from the 60 samples of rhizosphere:

Table 4.1 Overview of technical details for soil DNA extraction protocol.

Rhizosphere	Commercial Kit	Amount of substrate	Lysis Buffer	Shacking	Final elution
Sandy soil	NucleoSpin® Soil	0.51 ÷ 0.56 g	SL1 (700 µl per sample)	TissueLyser (30', 20 Hz)	50 µl PCR grade water (80 °C)
Peat substrate	NucleoSpin® Soil	0.39 ÷ 0.42 g	SL2 (700 µl per sample)	TissueLyser (30', 20 Hz)	50 µl PCR grade water (80 °C)

After the first round of extractions, most of samples (22 out of 30) from sandy soil revealed really poor DNA yields (<20 ng/µl). Thus, three additional extractions per rhizosphere samples were performed and extracted DNA was pooled together to increase the final amount of DNA. In order to concentrate these samples and to improve DNA purity of all the samples with low with Abs_{260}/Abs_{280} and Abs_{260}/Abs_{230} ratios, DNA precipitation protocol was conducted. Briefly, each sample was mixed with 1/10 of its volume of sodium acetate 3M (pH 5.2) and with 2.5X of its volume of cold 100% ethanol. After 1 hour of incubation at -20°C, the sample was centrifuged for 15 minutes at 14000 rpm at 4°C and the supernatant was completely discarded. 250 µl of cold 70% ethanol were added to the sample, which was then thoroughly mixed and centrifuged for 5 minutes at 14000 rpm at 4°C, and the supernatant was completely discarded; this step was repeated twice. Samples were then put at 40° for 5 minutes (thermoblock) to let the DNA pellet dry from the remaining ethanol. Finally, the DNA pellet was resuspended in 50 µl of PCR grade water. After DNA precipitation, all 60 DNA samples showed a DNA concentration >40 ng/µl, and Abs_{260}/Abs_{280} and Abs_{260}/Abs_{230} higher than 1.5.

4.2.3 Evaluation of DNA amplifiability, library construction and next-generation sequencing (NGS)

Prior to sequencing, control PCR was performed on all the 60 DNA samples to assess their amplifiability. The rationale of this test was the simulation of the first (and most critical) step performed by the sequencer. PCR was performed exploiting universal primers Pro341F and Pro805R, purchased at Eurofins Genomics (Eurofins MWG Synthesis GmbH, Germany); primers features are resumed in Table 4.2. Taking into account that this step was performed only as a control passage, it is worth specifying that these primers were not "tailed" (*i.e.* primers with an over-hanging sequence which does not anneal directly on the target genome, but it is essential for the insertion of barcoding sequences which are required to perform the sequencing).

Table 4.2 Peculiarities of universal 16S primers for control PCR.

Sense	Sequence	Length (bp)	T_m (°C)	Molecular weight (g/mol)	GC-Content (%)	Region	Position on *Escherichia coli* genome	Reference
Forward	5' – CCTACGGGNBGCASCAG – 3'	17 bp	60.4	5199	71.5 %	V3 – V4	340	Takahashi et al., 2014
Reverse	5' – GACTACNVGGGTATCTAATCC – 3'	21 bp	58.2	6407	48.4 %	V3 – V4	784	Takahashi et al., 2014

PCR mixes were set-up in a dedicated room, under biohazard hood and using only sterile laboratory stuff to prevent contamination of the samples. Mixes were prepared in sterile Eppendorf PCR tubes, following the instructions of Taq DNA polymerase, as reported in Table 4.3:

Table 4.3 Control PCR Mix.

Reagents	Volume (µl)
PCR buffer 10X for *Taq* Polymerase (QIAGEN, The Netherlands)	5.00
dNTP Mix (10 mM each) (ThermoScientific, USA)	1.25
Primer FW (10 µM)	2.50
Primer RV (10 µM)	2.50
Taq DNA Polymerase (5 U/µL) (QIAGEN, The Netherlands)	0.50
Template DNA (5 ng/µL)	5.00
PCR grade H_2O (GIBCO - ThermoScientific, USA)	33.25
TOTAL	**50.00**

Once ready, the PCR mix was placed in the thermocycler (SureCycler 8800, Agilent Technologies, USA), whose steps were set as reported in Table 4.4:

Table 4.4 Settings of thermocycler for control PCR.

Step	Temperature & Time	Number of cycles
Initial denaturation	95°C, 3 minutes	1 cycle
Denaturation	95°C, 40 seconds	25 cycles
Annealing	55°C, 45 seconds	
Extension	72°C, 1 minute	
Final extension	72°C, 7 minutes	1 cycle
Hold	+4°C, indefinitely	-

In order to evaluate the presence of amplicons, PCR products were run on agarose gel (1%, stained with SYBR™ Safe 10000X). 5 µl of PCR product were mixed with 1 µl of Loading Dye 6X (ThermoScientific, USA) to allow the samples loading in the gel wells. Along with the samples, on agarose gel were loaded:

- Fast Ruler Middle Range DNA Ladder (5 µl) (ThermoScientific, USA), containing DNA strands of known length;
- A positive control, that is a PCR mix done with a DNA sample showing amplifiability in previous PCR and run in parallel with the other samples;
- A negative control, that is a PCR mix in which no DNA template was added (to asses absence of contamination in the PCR reagents) and run in parallel with the other samples.

The gel was run on a Horizontal Electrophoresis System (Bio-Rad Laboratories, USA) at 60V for 45 minutes (PowerPac™ Basic Power Supply, Bio-Rad Laboratories, USA); successively, it was exposed on a UV lamp visualization of DNA bands onto the gel.

Once the samples were checked for their amplifiability, libraries were prepared for 16S sequencing with the Illumina MiSeq platform. To this aim, the Nextera XT DNA Library Preparation Kit (Illumina, Inc. USA) was used and the following passages were followed:

- **Amplicon PCR**. PCR was performed with universal primers with overhanging sequences in order to both target the variable sequences of microbial genome and insert an extra sequence in the amplicons for the subsequent indexing process required for the NGS. Technical details of the primers used in this passage are reported in Table 4.5. PCR mixes (Table 4.6) and thermocycler (Table 4.7) were set according to manufacturer indications.

Table 4.5 Peculiarities of universal 16S "tailed" primers for Amplicon PCR.

Sense	Sequence	Length (bp)	T_m (°C)	Molecular weight (g/mol)	GC-Content (%)	Region	Position on *Escherichia coli* genome	Reference
Forward	5'- TCGTCGGCAGCGTC AGATGTGTATAAGAGA CAGCCTACGGGNBGCA SCAG - 3'	50 bp	80.2	15504	58.3 %	V3 – V4	340	Takahashi et al., 2014
Reverse	5'- GTCTCGTGGGCTCG GAGATGTGTATAAGAG ACAGGACTACNVGGGT ATCTAATCC - 3'	55 bp	78.5	17072	51.2 %	V3 – V4	784	Takahashi et al., 2014

Table 4.6 Amplicon PCR Mix.

Reagents	Volume (µl)
Microbial template DNA (5 ng/µl)	2.50
Amplicon PCR Forward Primer (1 µM)	5.00
Amplicon PCR Reverse Primer (1 µM)	5.00
2x KAPA HiFi HotStart ReadyMix	12.50
TOTAL	**25.00**

Table 4.7 Settings of thermocycler for Amplicon PCR.

Step	Temperature & Time	Number of cycles
Initial denaturation	95°C, 3 minutes	1 cycle
Denaturation	95°C, 30 seconds	25 cycles
Annealing	55°C, 30 seconds	
Extension	72°C, 30 seconds	
Final extension	72°C, 5 minutes	1 cycle
Hold	+4°C, indefinitely	-

- **PCR Clean-up**. This passage was performed with AMPure XP beads to remove primers and primers dimers from the previous PCR according to manufacturer indications.
- **Indexing PCR**. PCR was performed to introduce in the amplicons the dual indices and the sequencing adapters with Nextera Index Primers. These sequences are required to allow the DNA fragments to be univocally identified and to bind the flow cell in which sequencing process takes place. PCR mixes (Table 4.8) and thermocycler (Table 4.9) were set according to manufacturer indications.

Table 4.8 Indexing PCR Mix.

Reagents	Volume (µl)
DNA (from first PCR)	5.00
Nextera XT Index Primer 1 (N7xx)	5.00
Nextera XT Index Primer 2 (S5xx)	5.00
2x KAPA HiFi HotStart ReadyMix	25.00
PCR Grade water	10.00
TOTAL	**50.00**

Table 4.9 Settings of thermocycler for Indexing PCR.

Step	Temperature & Time	Number of cycles
Initial denaturation	95°C, 3 minutes	1 cycle
Denaturation	95°C, 30 seconds	8 cycles
Annealing	55°C, 30 seconds	
Extension	72°C, 30 seconds	
Final extension	72°C, 5 minutes	1 cycle
Hold	+4°C, indefinitely	-

- **PCR Clean-up 2.** This passage was performed again with AMPure XP beads to obtain the final libraries.

- **Library Quantification, Normalization, and Pooling**. The amount of the constructs was quantified fluorometrically with Qubit4 (Invitrogen, Thermo Fisher Scientific, USA). Libraries were diluted at 4 nM and libraries with unique indices were pooled together.

Libraries were denatured through NaOH application and heat treatment before being loaded for MiSeq Sequencing. Sequencing was performed in the 2X300 bp paired end reads (300 PE) mode.

4.2.4 Bioinformatic analysis of NGS data

4.2.4.1 Pre-processing of raw data

Bioinformatic tools and dedicated software were exploited to deeply analyse raw sequences obtained from MiSeq Paired End sequencing. Pre-processing of the data was aimed to elaborate data to make them usable by tools for molecular ecology analysis. All the bioinformatic analyses were performed with the software Qiime2 (Bolyen et al., 2019), unless differently specified. The tool Demux (Qiime2) was used to conduct demultiplexing (*i.e.* the assignment of the reads to the original samples by means of indexing sequences) and the first quality filtering of the reads to remove truncated sequences and sequences exceeding maximum ambiguous bases. The tools vsearch (Qiime2) and cutadapt v1.18 were used to merge paired reads and to remove primer sequences, respectively. The tool Deblur (Qiime2) was used to conduct the denoising procedure on the remaining sequences, which includes the quality trimming of the reads at 250 bp length, the dereplication for removal of singletons, the removal of the sequencing artifacts (*i.e.* the filtering of reads matching the sequences of PhiX or adapters), the multiple sequence alignment to remove sequences with indels, the running of the core deblurring algorithm (*i.e.* the progressive elimination of the error-derived reads based on a fixed probability of error occurring) and, finally, the removal of chimeric sequences. The final output of Deblur was the table of Amplicon Sequence Variances (ASVs) per each sample, where ASVs are clusters of sequences obtained from a process of error-modelling within the sequencer. The concept of ASV is gradually overcoming the original one of OTU (Operational Taxonomic Unit), that is a mere grouping of sequences imposed by an arbitrary fixed similarity (usually 97%); hence, the usage of OTUs is limiting since "fake" variants (*i.e.* errors introduced by sequencer) are grouped together with real variants, which are not distinguished one from another for slight variations (*e.g.* single nucleotide polymorphisms)(Callahan et al., 2017; Caruso et al., 2019). However, the concept of OTU is still widely diffused and this lettering is kept during different analyses. For this reason, the acronym OTU will be found many times across this chapter.

4.2.4.2 Taxonomic analysis

Taxonomic classification of bacterial sequences was performed with Naïve Bayesian classifier (Wang et al., 2007) present in Qiime2. The classifier is based on Bayes theorem and exploits models which guarantee an accuracy of ~80% in the taxonomic assignment of the sequences at the genus level (Knight et al., 2018). In the present work, the classifier was trained on the database Greengenes v13_8 (99% OTUs full-length) sequences. After taxonomy assignments, abundancy percentages were calculated at six different taxonomic ranks: phylum, class, order, family, genus and species.

Differences in bacterial composition were performed with ANCOM (Qiime2), and with DESeq2 in R environment. Differential abundancy tests were performed pairwise, comparing two samples from two different treatments within the same kind of cultivation substrate. The abundancy table was used collapsed at the genus level to guarantee more reliable results.

In the case of ANCOM (Mandal et al., 2015), three steps are usually run to test differential abundances across the treatments:

- the calculation of log-ratio of abundance of each genus to the abundance of all the remaining taxa one by one;
- the comparison of relative abundancies of genus through the log-ratios;
- the calculation of W statistic per each genus. The genus showing W statistic higher than a "W critic" value allowed to refuse null hypothesis (*i.e.* two groups with no significant differences).

In other words, ANCOM computes the empirical distribution of W statistic to express the final significance: the higher W of a genus, the more genus is significantly different across different samples.

DESeq2 (Love et al., 2014) is a software generally used to compare differential gene expressions, but it also utilized on data from metagenomic analyses. DESeq2 is based on a fitting routine conceived to deal with the variance heterogeneity typically found in sequencing data. This tool belongs to the scenario of the generalised linear modelling and exploits the negative binomial distribution as an error distribution to compare ASV abundance between replicated samples. Operatively, the Wald statistic test is used to determine ASVs significantly different between two-samples; Benjamini-Hochberg (BH) adjustment (Benjamini and Hochberg, 1995) is then applied to prevent the false discovery rate (FDR) occurring during multiple testing, with a significance at 5% level; as a results, a new (and higher) p-value is calculated. The general output of DESeq2 elaboration provides a list of significantly different ASVs and for each of them the significant log2FoldChange (log_2FC; *i.e.* the log_2 ratio of ASV abundance between two treatments). Based on the output, it is possible to calculate the sample-to-sample distance matrix, which can be exploited for further elaborations for the identification of stratification across samples, with hierarchical clustering, and the explanation of general variance across samples, with principal component analysis (PCA). Hierarchical clustering calculates Euclidean distances between samples,

which are visualized in a heatmap; PCA displays differences across samples in a PCA plot.

4.2.4.3 Molecular ecology statistics

Molecular ecology parameters were calculated directly from sequencing data in order to understand the internal diversification within single samples (α-diversity) and the distances between samples (β-diversity). Differently from differential abundance analysis, the goal was not to discover more or less populated taxa across samples; instead, the aim was to attribute indicators which can describe the system simply by attributing a score. This process can be performed through different approaches, which take into account different features: that's why many alpha and beta diversity metrics are available. Preliminary steps are usually required for ecology parameters calculation. The first is the generation of phylogenetic tree from ASV, necessary for the calculation of those metrics relying on phylogenetic distances. The second is the rarefaction analysis, which allows to understand if the samples are sufficiently deeply sequenced to provide reliable ecological parameters. This analysis is performed calculating α-diversity metrics on a progressively growing dataset randomly subsampled from each sample. Then the metric is plotted in function of the sequencing depth (*i.e.* the number of sequences in the subsample) and, if the curve reaches a plateau, it means not only that the sample was sufficiently sequenced to gather the effectively present taxa, but also that the computed metrics are correctly describing the samples. But why rarefaction? Because at this stage a sampling depth is chosen to "thin out" the sequences of different samples in a way that all samples are evaluated based on an equal number of sequences, which allows to conduct reliable comparisons between samples. In other words, the choice of a sampling depth is essentially a trade-off between the inclusion of more samples as possible on one side, and the guarantee of catching the maximum diversity within each sample. In the present study, sampling depth values were set at 81725 for sandy soil and to 75860 for peat substrate, and both alpha and beta diversity metrics were calculated on these values.

As stated above, alpha diversity metrics were aimed to describe the internal differences of samples. This was performed by evaluating species *richness* (*i.e.* number of different species in a sample), species *evenness* (*i.e.* the homogeneity and the numerical balance between species) and *phylogenetic proximity*. To this aim, six different metrics were calculated:

- Observed OTUs (DeSantis et al., 2006): it accounts for species richness, simply calculating number of distinct OTUs (which are more properly mentioned above as ASV).
- Chao1 index (Chao, 1984): it accounts for species richness. It is an extended version of Observed OTUs, which computes diversity from abundant data, estimating the number of rare taxa which are usually missed from random subsampling.
- Pielou's Evenness (Pielou, 1966): it accounts for species evenness by calculation from species relative abundance. It attributes a score ranging

from 0 (presence of dominating species) to 1 (perfect homogeneous distribution of individuals across the species).

- Faith's Phylogenetic Diversity (or PD whole tree) (Faith, 1992): it accounts for phylogenetic proximity. It measures biodiversity between species by summing the length of branches of phylogenetic tree.
- Shannon index (Shannon, 1948): it accounts for both species' richness and evenness. The index is calculated with the following formula:

$$H' = -\sum_{j=1}^{s} p_j \log_e p_j$$

where p_j is the proportion of j-th species.

- Simpson's diversity index (Simpson, 1949): it accounts for both species' richness and evenness. The index is the probability that, sampling two organisms within the samples, they belong to different species. It is calculated with the following formula:

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)}\right)$$

where n is the total number of organisms of a particular species and N the total number of organisms of all species. The value of the index ranges from 0 (no diversity) to 1 (total diversity).

The last two indices are double-edged sword since on one side they resume different features of a sample in a single number, but on the other hand it is impossible to affirm *a priori* which effect, richness or evenness, is prevailing. All alpha diversity metrics were calculated with the software Qiime2. In order to compare the alpha diversity values between treatments, the non-parametric test of Kruskal-Wallis was used: H statistics and p-values were calculated both for all groups and for each pairwise comparison. Moreover, p-values of pairwise comparison were further adjusted with the Benjamini-Hochberg correction to obtain q-values, used to evaluate the statistical significance of each comparison.

A similar rationale could be adopted to understand the utility of different beta diversity metrics, which were exploited to analyse the distances or dissimilarities between samples under different perspectives. In this case, beta diversity parameters were calculated to analyse first the distances of soil microbial communities within the same cultivation substrate to evaluate the only effect of the treatment. Successively, the same metrics were computed also to study the distances of the microbial communities from the two different substrates. The calculated beta diversities were the following ones:

- Bray-Curtis dissimilarity (Bray and Curtis, 1957): it is based on the species counts in each sample. Differently from the other beta metrics, it cannot be considered a "distance" since it does not fulfil the triangle inequality. It is calculated with the following formula:

$$BC_{ij} = 1 - \frac{2\,C_{ij}}{S_i + S_j}$$

Where C_{ij} is the sum of the lower values of occurrence for only the species shared by sample i and j, while S_i, S_j are the total number of individuals in each sample.

- Canberra distance (Lance and Williams, 1967): it is based on the species counts in each sample. It is calculated with the following formula:

$$d_{ij} = \sum_{k=1}^{n} \frac{|x_{ik} - x_{jk}|}{|x_{ik}| + |x_{jk}|}$$

where x_{ik} and x_{jk} are the abundancy of *k*th species in samples *i* and *j*.

- Euclidean distance (Legendre and De Cáceres, 2013): it is based on the species count to compute a species-by-species distance matrix. It is calculated with the following formula:

$$d_{ij} = \sum_{k=1}^{n} \sqrt{(x_{ik} - x_{jk})^2}$$

where x_{ik} and x_{jk} are the abundancy of *k*th species in samples *i* and *j*.

- Jaccard distance (Jaccard, 1908): it takes into accounts for the fraction of unique features, regardless of their abundancies. It is calculated with the following formula:

$$d_{ij} = \frac{q + r}{p + q + r}$$

where q is the number of the species occurring only in sample *i*, r is the number of the species occurring only in sample *j,* and p is the number of the species occurring only in both samples *i* and *j*.

- Unweighted UniFrac distance (Lozupone and Knight, 2005): differently from the other metrics, UniFrac metrics accounts for the fraction of unique branch length within the phylogenetic tree. The following formula is used to calculate this distance:

$$u_{AB} = \frac{\sum b_A \,\Delta\, b_B}{\sum b_A \cup b_B}$$

where b_A and b_B are the summation of branch lengths of the compared samples *A* and *B* in the phylogenetic tree, Δ is the symmetric difference between two sets, and ∪ is the union between two sets. In other words, the summation of the branch lengths unique in a sample is divided by the summation of the branch lengths shared by both samples (Wong et al., 2016).

- Weighted UniFrac distance (Lozupone et al., 2007): it shares the same rationale of Unweighted UniFrac distance, but it takes into account abundance. The following formula is used to calculate this distance:

$$w_{AB} = \frac{\sum_i^n \left(b_i \times \left|\frac{A_i}{A_T} - \frac{B_i}{B_T}\right|\right)}{\sum_i^n b_i}$$

where A and B are the compared samples, b is the set of branch lengths, and $\frac{A_i}{A_T}$ and $\frac{B_i}{B_T}$ are the proportional abundances of branch length b_i (Wong et al., 2016).

Once the different beta diversity metrics were calculated, statistical analysis was conducted with the non-parametric multivariate statistical test PERMANOVA (permutational analysis of variance). A fixed number of permutations (999) was performed randomly exchanging samples but keeping the calculated distances. If the distances within the samples from the same treatment are similar to the distances between samples of different treatments, centroids and dispersions of the samples are unvaried (and vice versa). PERMANOVA tests the null hypothesis of equivalence of centroids and dispersion of the different replicates of each treatment. If the null hypothesis is rejected, centroids and/or dispersion of each treatment are different. A further elaboration of beta diversity metrics was performed through multidimensional scaling, which is a form of statistical analysis allowing the graphical rendering of differences and similarities within a set of elements. In the present case, Principal Coordinate Analysis (PCoA), also known as classical multidimensional scaling, was used to translate information on distances of pairs of elements in a configuration of points in a 3D cartesian space. The algorithm exploits ordination techniques elaborating data contained in a distance matrix and positioning each element in a dimensional environment so that distances between objects are represented in the best way. PCoA plots were obtained through the EMPeror tool integrated in the Qiime2 software (Vázquez-Baeza et al., 2013); axes of PCoA plots stand for the explained variance, which is reported in the scree plots also for the not-visualisable axes.

4.3 Results and discussion

4.3.1 Assessment of extracted DNA

The preliminary phase of soil microbial community study began with the implementation of a protocol for soil DNA extraction from two different cultivation substrates, namely sandy soil and peat substrate. As a result, NucleoSpin® Soil turned out to be the most suitable kit for the purposes of the present study. Figures 4.1.a and 4.1.b show the UV-Vis spectra of extracted DNA from a sample of sandy soil and peat substrate, respectively. These profiles confirmed good yields and quality of purified DNA (evaluated with Abs 260/230 and Abs 260/280), which, however, were not displayed by all the samples. In particular, sandy soil samples tended to have low DNA yields (Figure 4.2, a.b.c.d), while peat substrate ones mostly showed high absorbances at 230 nm (Figure 4.3.a.), indicating presence of contaminants. In both cases, DNA precipitation protocol ensured a proper concentration and purification of the final products, with acceptable values in terms of purity ratios as shown in Figures 4.2.e and 4.2.b.

Figure 4.2 Nanodrop spectra of soil DNA.

a. Sandy soil. b. Peat substrate.

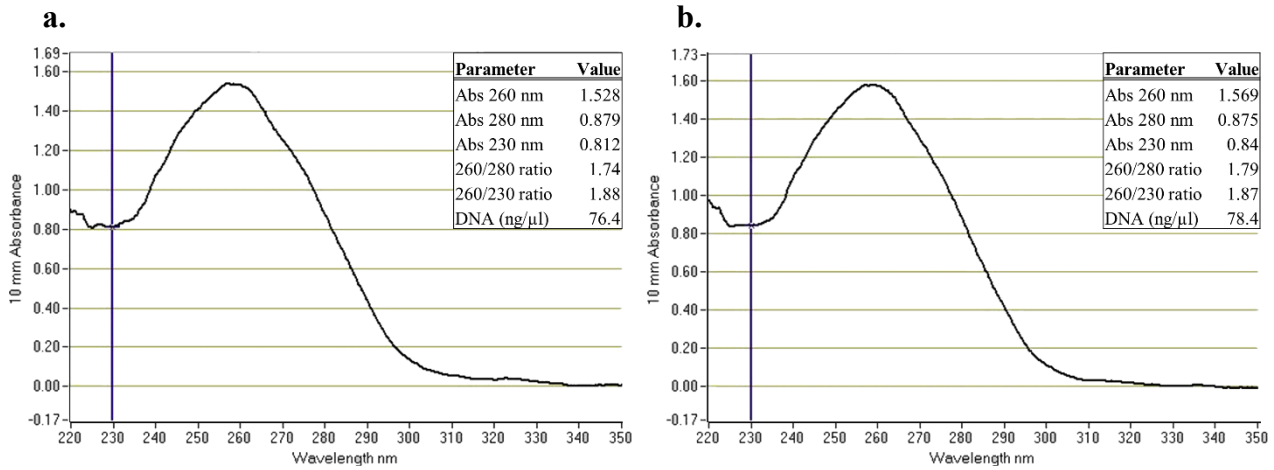
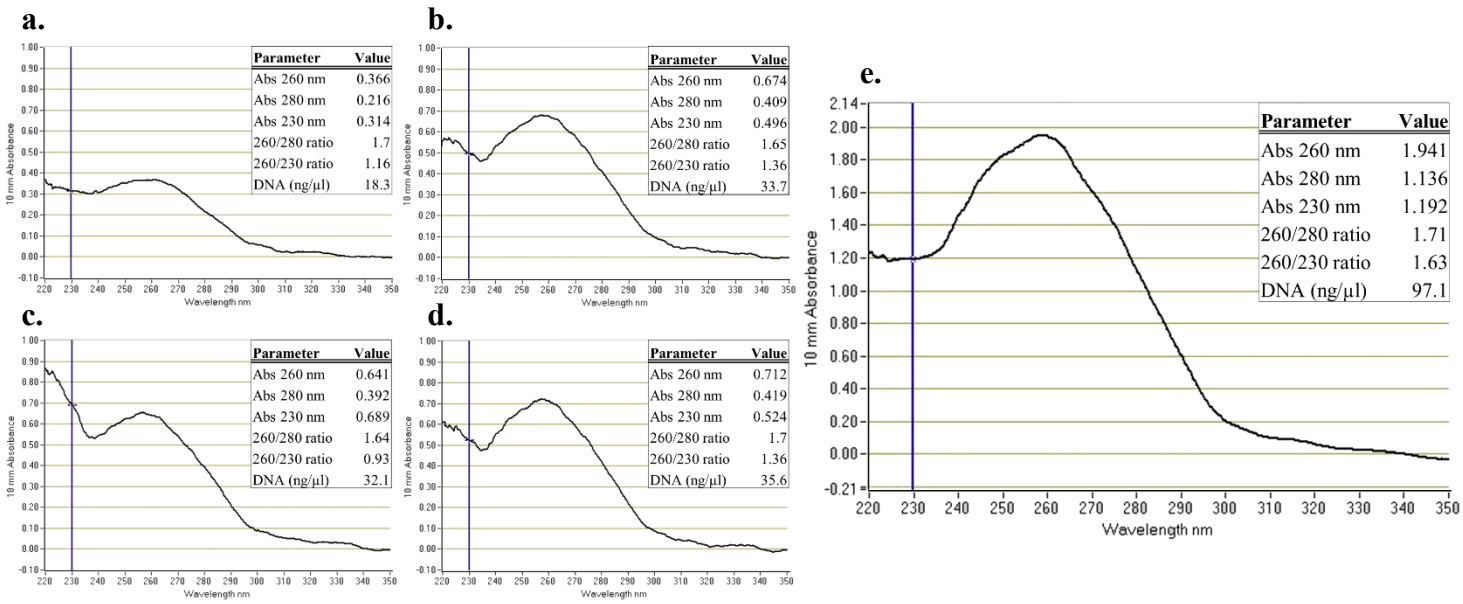


Figure 4.1 Nanodrop spectra of soil DNA from sandy soil.

a. b. c. d. Spectra of samples with low yield and quality. e. Spectra after pooling and DNA precipitation.



At this stage, DNA samples had to be checked in terms of "readability" prior to sequencing. PCR was performed with the same universal primers used for sequencing to check the amplifiability of DNA samples. Figure 4.4. shows the picture of agarose gel exposed at UV after electrophoresis of PCR products from sandy soil and peat substrate samples, respectively. Considering the annealing positions of the primer on bacterial genomes, band of amplicons was expected at ~440 bp. The presence of this band in the analysed samples and its absence in negative control (*i.e.* without DNA template, to ensure usage of DNA-free reagents) confirmed that all the soil DNA samples were suitable for sequencing.

Figure 4.4 Nanodrop spectra of soil DNA from peat substrate.

a. Spectra of samples with impurities. b. Spectra after DNA precipitation.

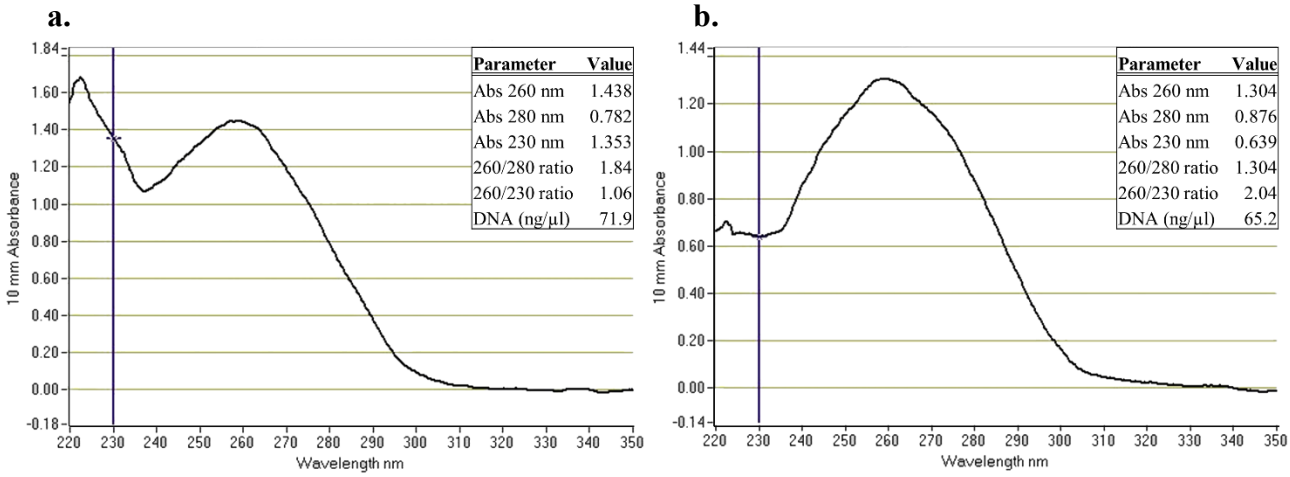
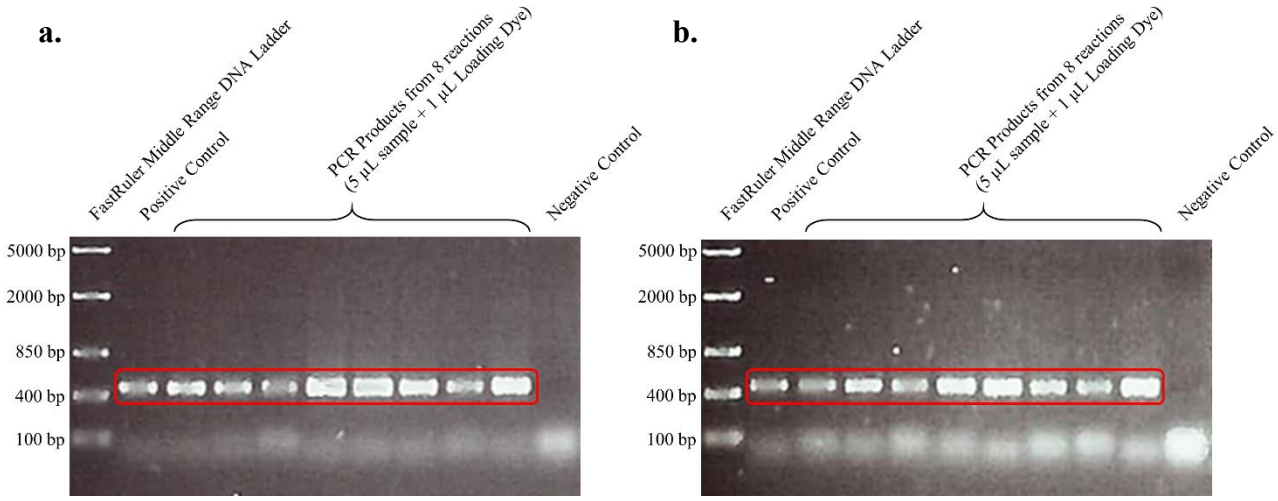


Figure 4.3 Agarose gel electrophoresis of amplicon of control PCR.

a. Sandy soil samples. b. Peat substrate samples. Red boxes highlight expected band at 440 bp.



4.3.2 Sequencing statistics

Illumina sequencing in paired-end mode of the 60 soil DNA samples produced 12,520,460 reads detecting 14,679 different amplicon sequence variants overall. Table 4.10 resumes statistics per each sample from the two cultivation substrates. Two samples (one not treated control from sandy soil and one secondary from peat substrate) were excluded from subsequent data analysis since they did not produce enough reads (3,759 and 2,709) to be considered reliable. Moreover, in Table 4.10 number of residual sequences after each quality filtering step is reported.

Table 4.10 Sequencing statistics of DNA samples from sandy soil and peat substrate.

For each sample are specified the reads produced by sequencing, the remaining sequences after quality filtering and the number of ASVs.

Sequences from sandy soil samples

Sample	Raw input sequences	After quality filtering	After dereplication and singleton removal	After deblurring	After chimera removal	Amplicon sequence variants (OTUs)
Control - 1	260926	260923	152125	115525	111754	3518
Control - 2	255029	255028	157808	117871	114375	3036
Control - 3	233932	233931	128013	94742	91137	3302
Control - 4	462390	462387	277939	202306	194855	4281
Primary - 1	257754	257754	161953	119055	113872	3571
Primary - 2	210163	210162	126334	92213	87863	3580
Primary - 3	245150	245150	150901	106708	101150	3079
Primary - 4	216523	216521	137569	96987	92908	2976
Primary - 5	217669	217669	136659	101358	97166	3068
Secondary - 1	276740	276739	166230	119939	116589	3318
Secondary - 2	239410	239410	148153	108561	105124	2898
Secondary - 3	166395	166393	104402	75176	71512	2566
Secondary - 4	257458	257456	156949	117186	114299	3189
Secondary - 5	224760	224760	141567	103443	100720	2608
Centrifuged - 1	244115	244115	159121	120428	116939	2722
Centrifuged - 2	235323	235320	152452	114254	109732	2775
Centrifuged - 3	222064	222062	149714	113023	110593	2089
Centrifuged - 4	238623	238623	151922	115392	112294	3020
Centrifuged - 5	203258	203256	128337	99548	97695	2802
Dried - 1	263442	263442	164315	121667	117612	3094
Dried - 2	237053	237049	155761	113375	108516	2493
Dried - 3	168057	168057	102674	75081	73329	1872
Dried - 4	262689	262688	180588	127327	124170	1992
Dried - 5	198891	198890	118970	88073	85529	2756
Mineral - 1	244066	244066	145031	109925	107367	2992
Mineral - 2	232421	232416	147726	111692	107542	2448
Mineral - 3	147166	147164	85643	64113	61831	2123
Mineral - 4	212904	212903	131113	99825	97531	2694
Mineral - 5	178914	178911	107386	84018	81729	2701
TOTAL	**6,813,285**	**6,813,245**	**4,227,355**	**3,128,811**	**3,025,733**	**9737**

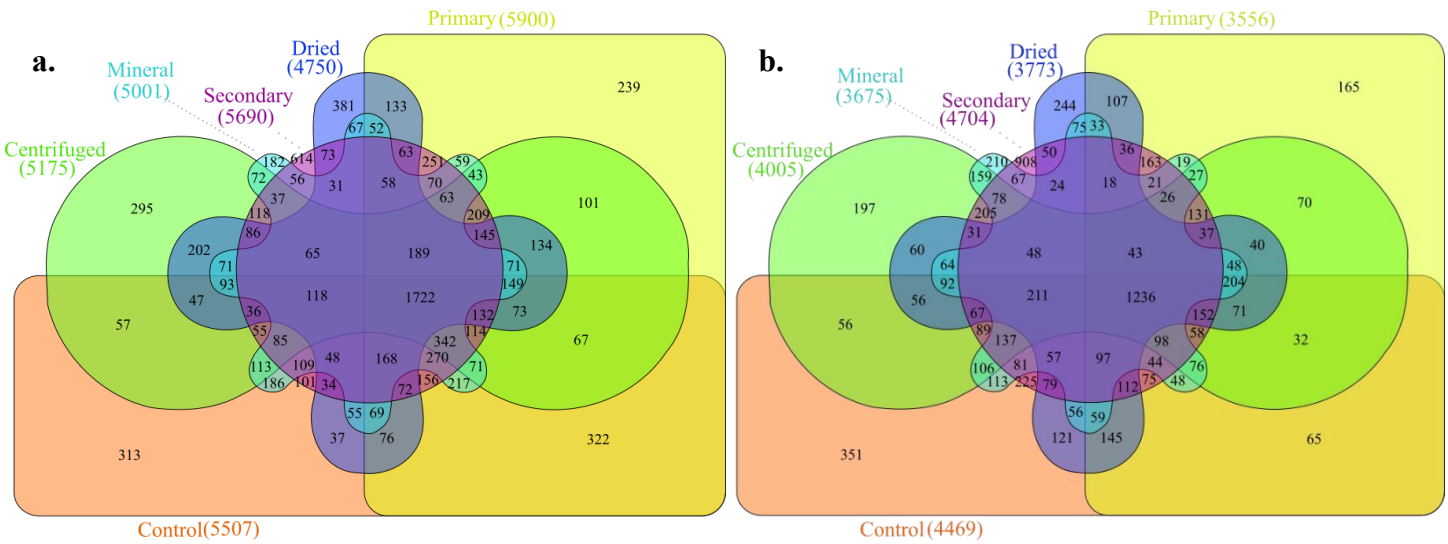
Sequences from peat substrate samples

Sample	Raw input sequences	After quality filtering	After dereplication and singleton removal	After deblurring	After chimera removal	Amplicon sequence variants (OTUs)
Control - 1	207193	207193	132699	103771	101759	2217
Control - 2	190531	190531	122256	94828	93093	2127
Control - 3	176204	176204	114581	90230	88150	2039
Control - 4	141768	141766	91202	74311	72748	1855
Control - 5	178539	178539	115904	90558	89053	1929
Primary - 1	202350	202349	130201	102326	100822	2145
Primary - 2	170027	170026	104488	81222	79985	2053
Primary - 3	235481	235478	152119	118350	116980	2381
Primary - 4	248034	248034	163057	124440	122727	2115
Primary - 5	164618	164617	107628	83470	82298	1899
Secondary - 1	415494	415490	273234	208711	204122	3309
Secondary - 2	173730	173728	104752	83239	81352	2562
Secondary - 3	230142	230140	149227	113777	111349	2498
Secondary - 4	192926	192926	126374	96442	94747	2241
Centrifuged - 1	228428	228428	150980	116684	115413	1904
Centrifuged - 2	173560	173558	110247	86366	85067	2286
Centrifuged - 3	204787	204787	128554	99842	98380	2225
Centrifuged - 4	223281	223278	147562	114600	113085	2210
Centrifuged - 5	204811	204809	133623	102905	101450	2293
Dried - 1	125364	125364	77853	62936	61507	2060
Dried - 2	173722	173721	109004	85398	83765	2124
Dried - 3	228234	228233	145441	110438	108710	2026
Dried - 4	228993	228991	153557	117690	115963	1772
Dried - 5	180307	180306	122883	93655	92514	1405
Mineral - 1	173226	173225	107180	83703	82086	2420
Mineral - 2	155610	155606	98420	77955	75870	2247
Mineral - 3	208886	208884	134071	105186	103880	1998
Mineral - 4	212725	212723	143820	112562	111202	1898
Mineral - 5	58204	58203	35065	28081	27736	1182
TOTAL	**5,707,175**	**5,707,137**	**3,685,982**	**2,863,676**	**2,815,813**	**7873**

Interestingly, number of the final sequences was more than (sandy soil) or almost (peat substrate) halved, highlighting the importance of this process, allowing to conduct elaboration on more reliable data and to obtain more robust results. Overall number of individuated ASV (or OTUs) per each soil is not equivalent to the sum of the ASV in each sample since this number considers the shared ASVs between samples. This aspect can be better understood with Figure 4.5, in which are reported how many ASVs were unique of each treatment and how many were shared with the other ones. At a first glance, it may be inferred that most of "species" were just shared by all the samples, however this information does not consider abundancies and ecological parameters which provided deeper knowledge, as explained below.

Figure 4.5 Venn diagrams of OTUs shared by the different treatments.

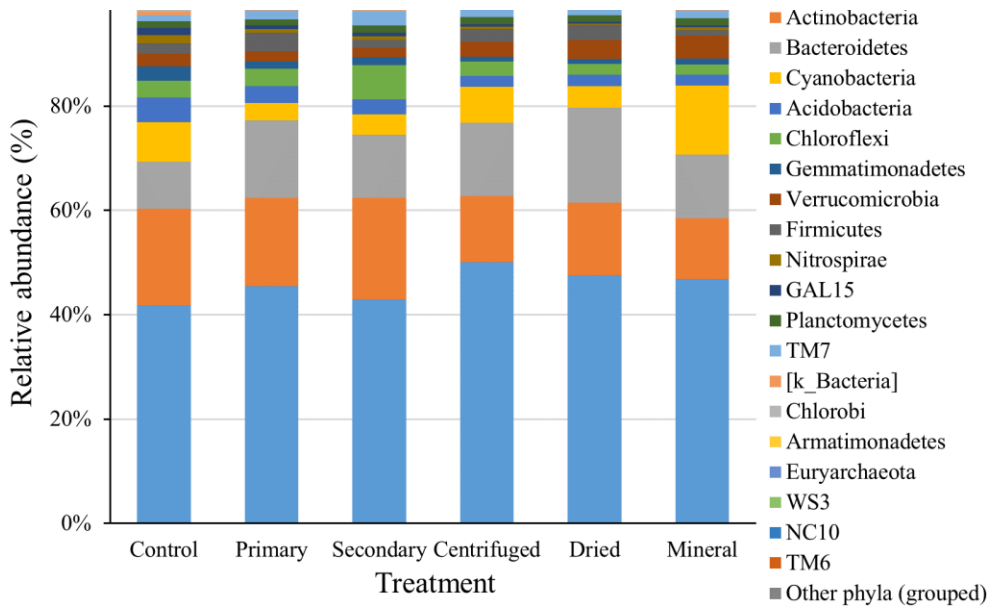
a. Sandy soil. b. Peat substrate.



4.3.3 Analysis of taxonomy and differential abundancy

Taxonomic analysis allowed to classify the bacterial population of sandy soil population in 38 phyla, 150 classes, 284 orders, 433 families and 706 genera. In the case of peat substrate, the bacterial population was classified in 42 phyla, 123 classes, 236 orders, 318 families and 706 genera. In general, taxonomy was investigated in terms of relative abundancies of the different taxa. It can be affirmed that the overall taxonomy was in line with the microbial composition of tomato rhizosphere (Li et al., 2014). Moreover, most of taxa, especially at phyla level, were similar between treated and untreated samples, indicating that sewage sludge did not induce a total displacement of original soil community, as already observed by Lloret and co-workers (2016), and confirming the general resilience of soil microbiome to external stress (Urra et al., 2019).

Figure 4.6 Taxonomy at phylum level of rhizosphere microbial communities from sandy soil.

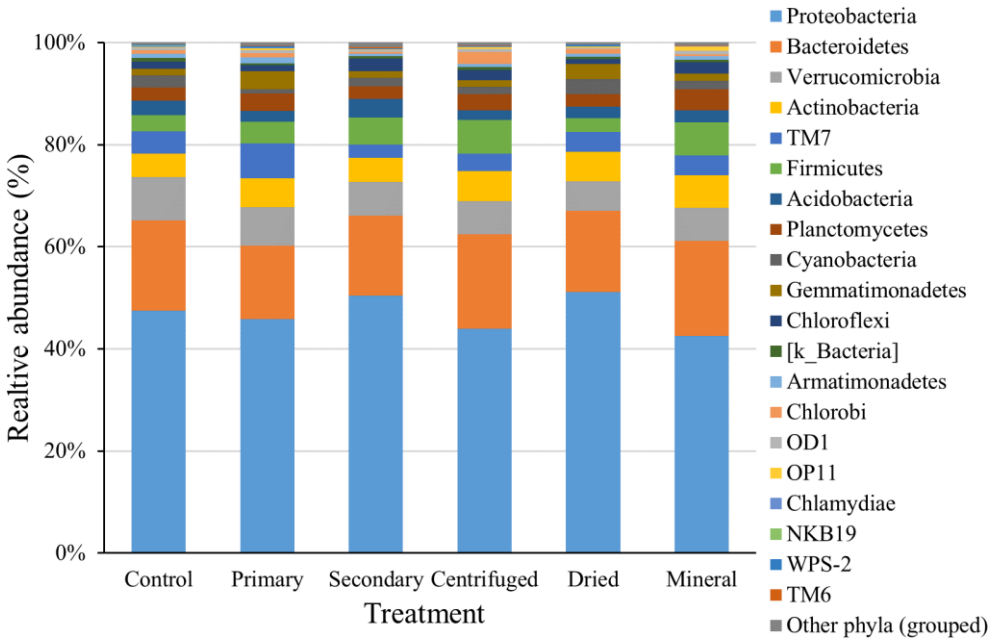


The taxonomy of sandy soil samples at the level of phyla is reported in Figure 4.6. In general, the ten most observed phyla were *Proteobacteria* (50.1% - 41.7%), *Actinobacteria* (19.6% - 11.5%), *Bacteroidetes* (18.2% - 8.9%), *Cyanobacteria* (13.2% - 3.4%), *Chloroflexi* (6.5% - 1.9%), *Verrucomicrobia* (4.3% - 1.9%), *Acidobacteria* (4.7% - 2.1%), *Firmicutes* (3.5% - 1.0%), *TM7* (2.7% - 1.2%) and *Gemmatimonadetes* (2.7% - 0.9%). Some of these taxa were slightly more abundant in soil treated with SSAD of all kinds (*Proteobacteria*: 50.1% - 43.0%; *Bacteroidetes*: 18.2% - 12.0%; *Firmicutes*: 3.5% - 1.5%) or only of one kind (Secondary: *Chloroflexi* 6.5%), with the mineral fertilizer (*Cyanobacteria*: 13.2%; *Verrucomicrobia*: 4.3%), or in the negative control (*Acidobacteria*: 4.7%; *Gemmatimonadetes* 2.7%).

The taxonomy of peat substrate samples at the level of phyla is reported in Figure 4.7. In general, the ten most observed phyla were *Proteobacteria* (51.2% - 42.6%), *Bacteroidetes* (18.6% - 14.3%), *Verrucomicrobia* (8.4% - 5.9%), *Actinobacteria* (6.3% - 4.6%), *Firmicutes* (6.5% - 2.6%), TM7 (6.9% - 3.4%), *Planctomycetes* (4.1% - 2.4%), *Acidobacteria* (3.8% - 2.1%), *Gemmatimonadetes* (3.5% - 1.2%). *Cyanobacteria* (2.9% - 0.9%). On peat substrate, less differences were detectable. Slightly more abundant taxa were TM7 (6.9%) and *Gemmatidomonadetes* (3.5%) with Primary SSAD, *Acidobacteria* (3.8%) with Secondary SSAD, *Planctomycetes* with mineral fertilizer (4.1%) and *Verrucomicrobia* in untreated control (8.4%).

Generally speaking about the microbial composition, the same dominating phyla have been already found in microbial communities of soil treated with organic and bio-based organic amendments (Ho et al., 2017).

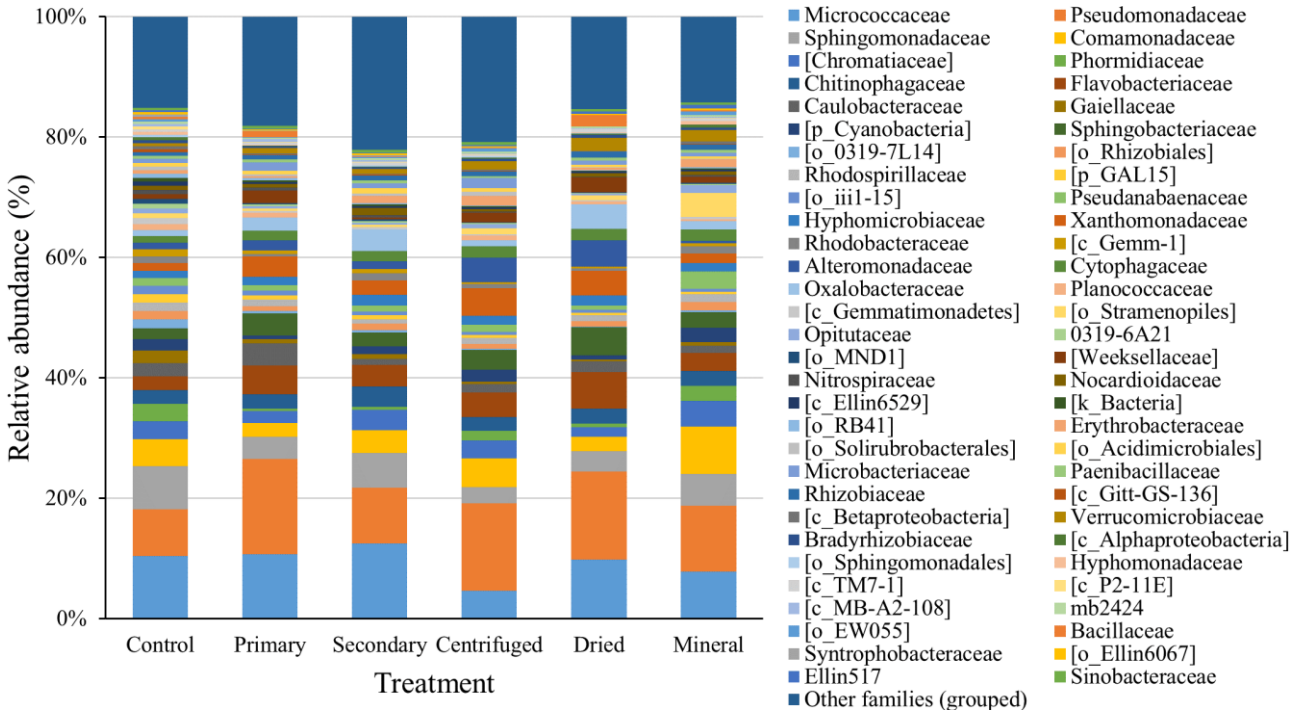
Figure 4.7 Taxonomy at phylum level of rhizosphere microbial communities from peat substrate.



Considering the singular microbial phyla, *Proteobacteria* include numerous genera and species involved in promotion of plant growth and biocontrol against diseases for tomato plants (Romero et al., 2014). *Proteobacteria* are widely recognised as copiotrophic bacteria, which grow rapidly under nutrient-rich conditions (good availability of C and N) (Li et al., 2014, 2019). So, the shift in *Proteobacteria* abundancy can be justified with the carbon enrichment due to SSAD addition, as already observed by Lloret and colleagues (2016). *Actinobacteria* include copiotrophic taxa as well, but usually less affected by nutrient changes, (Li et al., 2014, 2019); their relevance in sandy soil can be due to the fact that they have been demonstrated to be good degraders of organic matter and hydrocarbons (Ros et al., 2010), playing this role especially in sandy soils (Lloret et al., 2016). *Bacteroidetes* were more abundant in the case of sandy soil treated with SSAD, despite this phylum is usually less affected by soil nutrient state (Li et al., 2014). Conversely, *Acidobacteria* phylum generally hosts oligotrophic taxa, which metabolise nutrient-poor substrates: this is line with the observation that this phylum is a bit more expressed in the rhizosphere of the nude sandy and poor soil. Moreover, considering the present results, *Acidobacteria* accounted at most for 4.7% and 3.8% in sandy soil and in peat substrate, respectively, indicating that this phyla was generally poorly observed in tomato rhizosphere, in agreement with the work of Li and co-workers (2019). As regards *Firmicutes*, this phylum was more present in samples treated with SSAD in sandy soil. This is one of the major phyla in anaerobically stabilized sewage sludge due to its resistance to ammonium stress of anaerobic digestors (Little et al., 2020). Hence, given the fact that sandy soil was reasonably poorer in terms of microbial population, it depended more on the external inputs. In the case of peat, this phylum was probably already adequately populating this substrate before the treatment. *Chloroflexi* were found mostly in sandy soil samples treated with secondary SSAD; their importance in the microbial

network is usually related with the hydrolysation of carbohydrates and amino-acids, and transformation of chemical and biological contaminants (Bai et al., 2019). Probably, this phylum was more abundant in the secondary SSAD: the major effect on sandy soil could be due to its higher susceptibility to external inputs than the peat substrate. Interestingly, *Cyanobacteria* constituted a relevant part of phyla observed in sandy soil samples; their role was likely associated not only to nitrogen fixation (Rashid et al., 2015), but also to the turnover of nutrients and water retention in drylands and sandy soil (Chamizo et al., 2019). Concerning *Verrucomicrobia*, this phylum is rather less studied than the other ones, but its major relevance in samples from peat substrates than sandy soil maybe related to soil fertility issues (Navarrete et al., 2015).

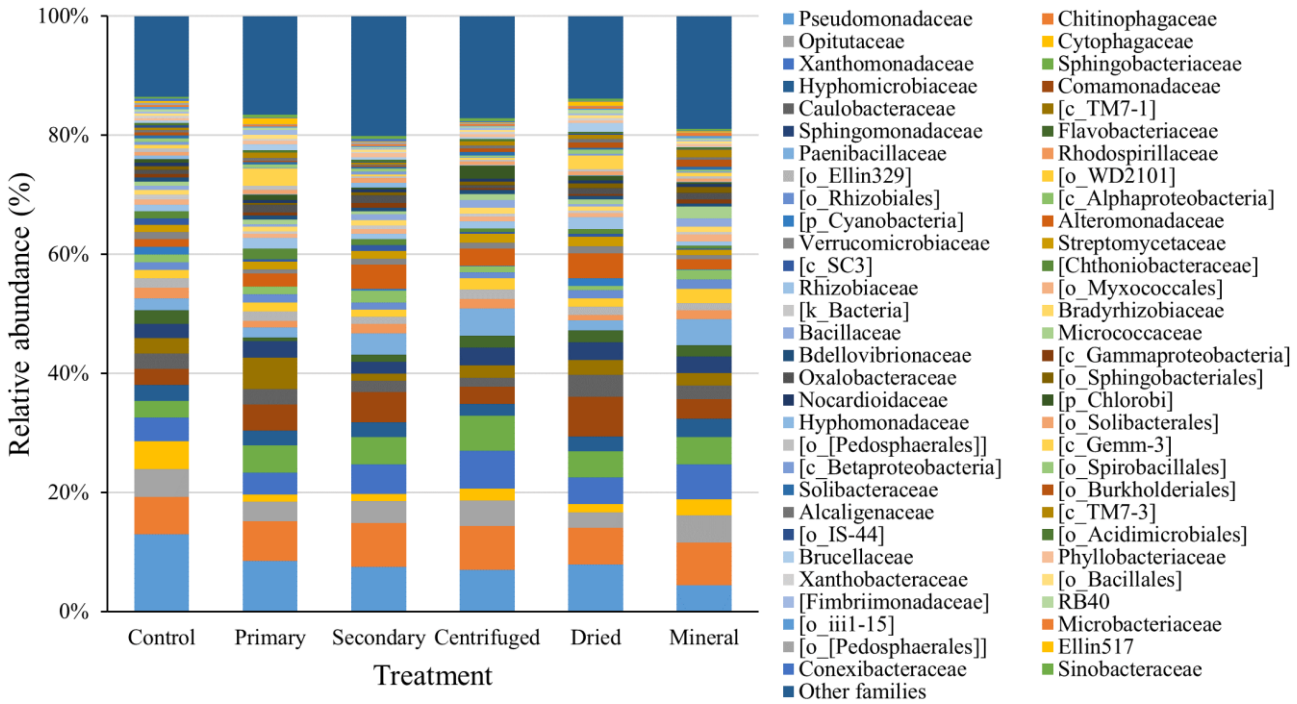
Figure 4.8 Taxonomy at family level of rhizosphere microbial communities from sandy soil.



Some additional comments on taxonomy can be drawn at family level. In the case of sandy soil (Figure 4.8), the ten most observed families were *Pseudomonadaceae* (15.8% - 7.8%), *Micrococcaceae* (12.5% - 7.8%), *Sphingomonadaceae* (7.1% - 2.7%), *Comamonadaceae* (4.7% - 2.4%), *Flavobacteriaceae* (6.1% - 2.3%), *Sphingobacteriaceae* (4.6% - 1.8%), *Xanthomonadaceae* (4.6% - 1.3%), *Chromatiaceae* (4.3% - 2.0%), *Chitinophagaceae* (3.3% - 2.3%), *Oxalobacteraceae* (4.2% - 1.0%). Some of these resulted more expressed in samples treated with SSAD such as *Pseudomonadaceae* (15.8% - 9.3%), *Flavobacteriaceae* (6.1% - 3.6%), *Sphingobacteriaceae* (4.6% - 2.4%) and *Xanthomonadaceae* (4.6% - 2.4%); moreover *Oxalobacteraceae* (4.2%) resulted more expressed in samples with Dried SSAD. Only *Comamonadaceae* and *Sphingomonadaceae* resulted slightly more expressed in mineral fertilizer (7.9%) and control (7.1%) respectively.

In the case of peat substrate (Figure 4.9), the ten most observed families were *Pseudomonadaceae* (13.0% - 4.4%), *Chitinophagaceae* (7.4% - 6.1%), *Xanthomonadaceae* (6.4% - 3.6%), *Sphingobacteriaceae* (4.6% - 1.8%), *Comamonadaceae* (6.7% - 2.7%), *Opitutaceae* (4.7% - 2.6%), *Paenibacillaceae* (4.6% - 1.7%), *Alteromonadaceae* (4.1% - 1.2%), *Sphingomonadaceae* (3.0% - 2.0%), *Hyphomicrobiaceae* (3.0% - 2.0%). In the case of peat less consistent differences were observed between treated and untreated samples, but the most evident aspect was that *Pseudomonadaceae* were more abundant (13.0%). Only *Alteromonadaceae* were more abundant than control (4.1% - 2.2%).

Figure 4.9 Taxonomy at family level of rhizosphere microbial communities from peat substrate.



The high percentages of *Pseudomonadaceae* in sandy soil samples treated with sewage sludge were likely correlated with the switch observed in the main classes of *Proteobacteria* phylum; in fact, untreated samples revealed abundances of 45% and 34% for *α*- and *γ-Proteobacteria*, respectively, while SSAD treated samples showed a mean composition of 29.5% and 50.5%, for *α*- and *γ-Proteobacteria*, respectively, with *Pseudomonadaceae* belonging to this last class. *Pseudomonadaceae* includes important genera of Plant Growth Promoting Bacteria (PGPB), such as *Pseudomonas*, which includes species promoting biofertilisation (nitrogen fixation, ammonia production, phosphorous solubilization), siderophore production (*e.g.* iron sequestration), biostimulation (production of phythormones as indolacetic acid (IAA)) and biocontrol (production of HCN, antagonism with plant pathogens such as *Fusarium oxysporum*, specifically for tomato)(Ferreira et al., 2019). Few works in literature investigated the trend of soil microbial families after application of sewage sludge. A comparison can be conducted with the work of Ho and colleagues (2017), which evaluated soil microbial communities of sandy soil

exposed to different organic amendments (including sewage sludge) in a litterbag assay. Similarly to the present results for sandy soil, the most abundant taxa involved were indicatively the same. Moreover, they detected more *Sphingomonadaceae* in control samples, while SSAD-exposed samples revealed higher concentrations in *Sphingobacteriaceae*, *Flavobacteriaceae* and, to a lesser extent, *Xanthomonadaceae*. Hence, development of these families was reasonably due to the treatment with SSAD. However, the most striking result was the absence of Pseudomonadaceae, which was, on the contrary, the most expressed family in the present work. The main hypothesis supporting this observation is the fact that this family is one of the main contributors of tomato rhizosphere; proof of this is the sustained presence of *Pseudomonadaceae* also in the rhizosphere of tomato samples grown on peat substrate. Another clue is suggested by the work of Li and colleagues (2014), who reported that *γ-Proteobacteria* (the class to which *Pseudomonadaceae* belong) as the most relevant class in healthy tomato rhizosphere. Influence of tomato rhizosphere can justify also the higher presence of *Comamonadaceae*, *Chitinophagaceae* and *Oxalobacteraceae*. Less comparisons can be performed in the case of peat substrate, apart from the above-mentioned issue on *Pseudomonadaceae*. The general composition was slightly different, meaning that the soil played a key factor in shaping the soil microbial community, aspect which is well known since many years (Bossio et al., 1998). A final comment on the work of Ho and colleagues (2017) is on the presence of *Opitutaceae* family in sewage sludge treated samples, attributing its contribute to the presence in the exploited sewage sludge. In contrast with this observation, in the present study, *Opitutaceae* were revealed only in peat substrate samples and no differences were appreciated between treatments and untreated control. Extra information on taxonomy and relative abundancies are available in Supplementary material, including the taxonomy at class (Figures S1 and S2), order (Figures S3 and S4) and genus (Figures S5 and S6).

In order to best capture differences in term of taxonomic composition, differential abundancy analysis was performed at the genus level with the softwares ANCOM and DeSeq2, comparing the genera abundancy between treatments (within the same soil) and providing a set of data which allowed to describe in general the effects and, more in detail, the significantly more abundant genera in each treatment. The first interesting results were provided by DeSeq2, providing the principal components analysis (PCA) and the heatmap of sample-to-sample Euclidean distance in the two analysed soils (Figure 4.10 a and b). In the case of sandy soil, PCA plot displayed an appreciable proximity of samples coming from the same treatment; thus, these consistent differences in term of general composition likely indicated that treatment had a remarkable effect. Furthermore, samples from control-mineral fertilizer were separated along the first principal component from the other ones, while SSAD treated ones were separated along the second principal component. This behaviour was confirmed by the heatmap of hierarchical clustering, which first clustered together samples per replicate, indicating high similarity between them, then clustered together samples treated with liquid and dewatered SSADs, and finally the SSAD treated samples clustered

with mineral fertilizer-control ones. A very interesting feature emerging from both the PCA and the clustering is the proximity of samples from untreated control-mineral fertilizer, Primary-Secondary SSAD (liquid SSADs) and Centrifuged-Dried SSAD (dewatered SSADs). This observation likely suggested that not only SSAD application affected soil microbial communities at level of genus, but also that the differential processing of SSAD had an effect on the microbiome of rhizosphere, corroborating the hypothesis of Lloret and colleagues (2016) and Mattana and colleagues (2014). In the case of samples from peat substrate (Figure 4.11 a and b), the interpretation of PCA plots and heatmap of hierarchical clustering was somewhat more complex. Samples from untreated control and Secondary SSAD showed the highest variance along the first principal component, suggesting that Secondary SSAD did not contribute to shape homogeneously the microbial community. On the other hand, Primary, Centrifuged and Dried (except for one sample), and mineral fertilizer showed a higher tendency to form cluster (even if less pronounced than the case of sandy soil). Moreover, the lesser difference impact of treatments emerges from the fact that, apart from Secondary SSAD, all of them were separated along the second principal component. Hierarchical clustering worked well for those samples already near in PCA. Moreover, it highlighted that, in terms of phylogenetic distance, samples treated with Dried SSAD and untreated

Figure 4.10 Outputs from data elaboration of differential abundancy of genera in sandy soil with DeSeq2 software.

a. Principal Component Analysis (PCA) plot. b. Heatmap of hierarchical clustering.

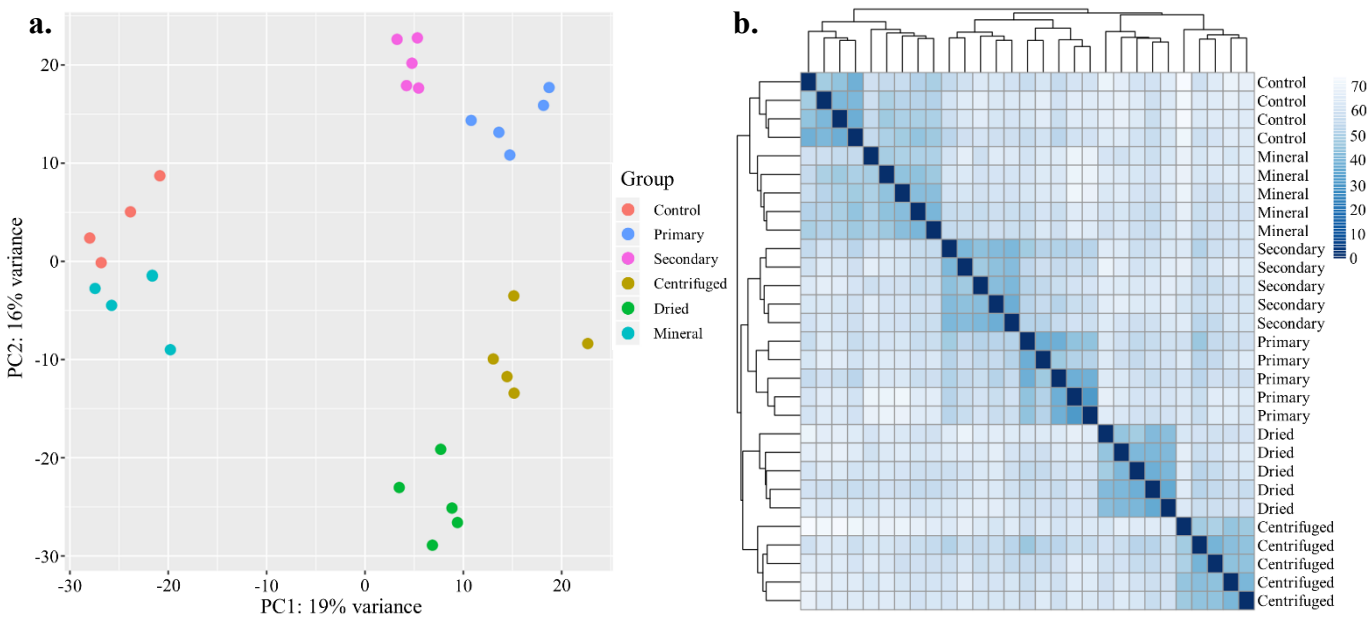
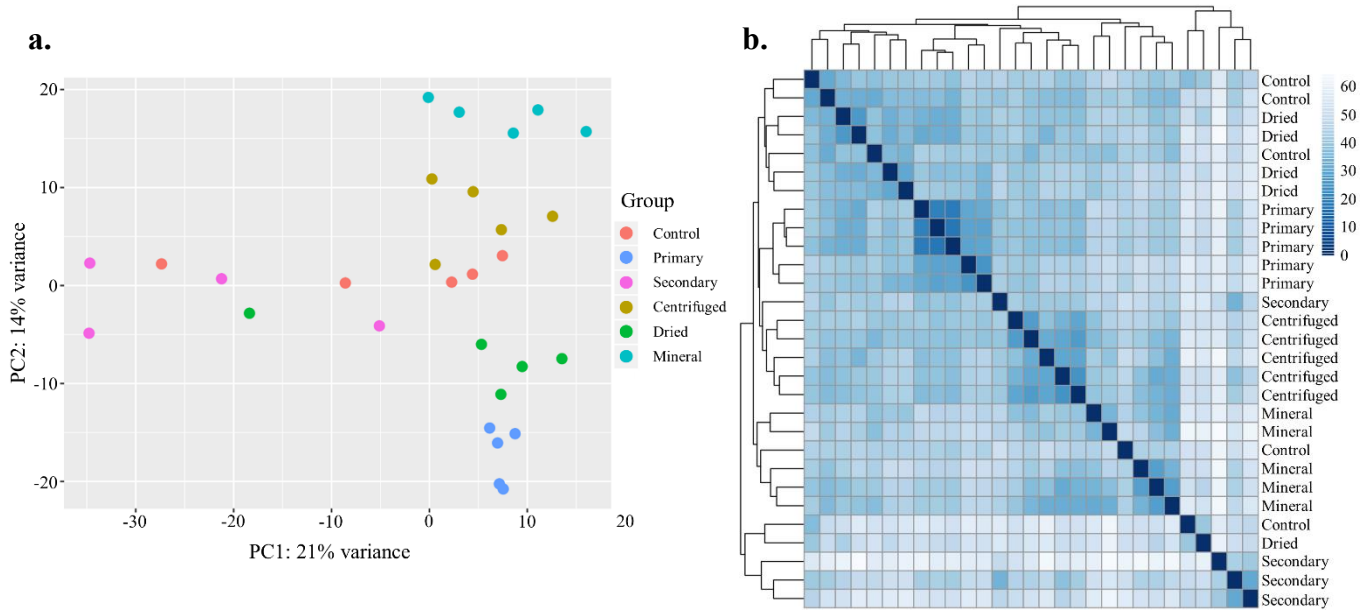


Figure 4.11 Outputs from data elaboration of differential abundancy of genera in peat substrate with DeSeq2 software.

a. Principal Component Analysis (PCA) plot. b. Heatmap of hierarchical clustering.



clustered together, indicating an overall similarity. Samples treated with Centrifuged and Primary SSADs, and mineral fertilizer tend to cluster together. Considering the hierarchy, samples from Primary and Centrifuged SSADs were more proximal to the cluster formed by control and Dried SSAD; the upper level of proximity was with samples from mineral fertilizer; lastly, samples with secondary treatment were the less proximal to the other ones. Interestingly, amongst the papers considered to compare the results obtained in the present work, none presented PCA and hierarchical clustering, underlining the novelty traits of this work in terms not only of areas of research, but also of the tools used for the investigation.

In order to deepen the issue about the differential abundancy, the software ANCOM was used to individuate the genera significantly more observed among the different treatments. This algorithm gave as a result a list of differently abundant genera, whose significance was validated with the W value above the computed W critic, which was 718 in the case of sandy soil and 610 for the peat substrate. Table 4.11 and 4.12 show the result of ANCOM analysis, reporting only the genera significantly more abundant. For each treatment is reported the number of reads associated per each genera; a conditional formatting was used to highlight the treatment(s) in which the genera were more abundant, and subsequently more abundant genera in the same treatment were manually clustered together to render the data in a heatmap fashion. Before moving forward on differential abundance, it must be specified that ANCOM elaboration (and also DeSeq2, as shown later) provides a bunch of results which resulted quite difficult to interpret. Hence a rationale had to be adopted in order to filter relevant information; the rationale adopted was the research of Plant Growth Promoting Bacteria (PGPB).

Table 4.11 Differential abundancy analysis of genera in sandy soil performed with ANCOM software.

The table resumes only genera significantly more abundant in each treatment (W > 718). The numbers in the treatments columns indicates the number of reads per each bacterial genus. For each genus, highest occurrences are highlighted in green.

Kingdom	Phylum	Class	Order	Family	Genus	W	Control	Primary	Secondary	Centrifuged	Dried	Mineral
Bacteria	Actinobacteria	Actinobacteria	Micrococcales			795	263	93	22	4	1	32
Bacteria	Proteobacteria	Deltaproteobacteria	NB1-j			790	47	217	45	62	1	17
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	796	1	63	1	1	1	1
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Virgibacillus	794	1	85	3	1	1	1
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herminiimonas	799	54	1878	495	10	6	286
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Dongia	796	4	231	29	115	9	1
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Nannocystis	796	33	550	37	444	53	5
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Myxococcaceae	Corallococcus	799	1	519	3	109	1	1
Bacteria	Acidobacteria	[Chloracidobacteria]	DS-100			784	34	27	44	36	14	13
Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae		754	40	18	50	34	3	1
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales			798	40	83	361	99	3	7
Bacteria	Chloroflexi	Anaerolineae	DRC31			757	1	20	74	1	1	1
Bacteria	Chloroflexi	Anaerolineae	Caldilineales	Caldilineaceae	Caldilinea	799	6	309	2115	38	30	28
Bacteria	Actinobacteria	KIST-JJY010				796	1	7	165	20	1	1
Bacteria	Chloroflexi	Anaerolineae				799	1	36	1042	1	1	1
Bacteria	Chloroflexi	Anaerolineae	SJA-15			761	1	1	5	1	1	1
Bacteria	Chloroflexi	Anaerolineae	SHA-20			734	1	1	5	1	1	1
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae	Candidatus Microthrix	748	1	17	46	46	1	1
Bacteria	WS5					744	1	1	20	28	1	1
Bacteria	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	Turicibacter	796	11	134	141	229	1	53
Bacteria	Firmicutes	Clostridia	Clostridiales			798	3	533	327	719	1	3
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		798	1	219	146	262	1	1
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Micromonospora	795	1	48	37	240	1	3
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae		799	1	143	11	549	1	4
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	798	1	1	1	1032	1	1
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	Actinocorallia	746	1	6	1	121	13	1
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Kaistia	798	1	89	3	467	279	4
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter	782	1	7	1	87	77	1
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae		799	4	64	3	894	581	2
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	753	40	197	13	1905	215	166
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Nocardia	771	10	125	58	458	493	10
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Nitrosomonas	720	22	56	9	87	103	41
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales			743	156	1	74	105	181	74
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	726	4	10	32	51	439	15
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia	774	18	42	1	12	163	1
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae		780	78	411	24	29	1414	17
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	778	5	167	10	5	969	1
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	768	15	38	20	9	530	19
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	Hyphomonas	743	830	10	7	7	10	1040

Table 4.12 Differential abundancy analysis of genera in peat substrate performed with ANCOM software.

The table resumes only genera significantly more abundant in each treatment (W > 610). The numbers in the treatments columns indicates the number of reads per each bacterial genus. For each genus, highest occurrences are highlighted in green.

Kingdom	Phylum	Class	Order	Family	Genus	W	Control	Primary	Secondary	Centrifuged	Dried	Mineral
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	681	277	384	2	3	195	11
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	677	1	4257	121	68	1	1
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		669	1	868	81	52	1	1
Bacteria	Firmicutes	Clostridia	Clostridiales			681	5	579	257	124	3	1
Bacteria	OP8	OP8_1	OPB95			639	3	81	109	13	1	1
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae		623	1	1	345	5	1	3
Bacteria	Firmicutes	Bacilli	Turicibacterales	Turicibacteraceae	Turicibacter	668	1	51	54	60	1	1
Bacteria	OP11	OP11-3				614	115	1	16	124	20	639
Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta	672	3	1	26	24	10	325
Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Ellin5301		653	1	1	1	1	1	3

PGPB are a wide group of bacteria of different genera characterized for their capability to ameliorate plant proliferation by the instauration of symbiotic relationships like the plant-microbes interaction in rhizosphere and the colonization of plant tissues by endophytes. PGPB exert their beneficial effect both directly and indirectly: in the former case, they are involved in acquisition of resources and modulation of plant phytohormones; in the latter, they induce plant systemic resistance and act as biocontrol agents, behaving as antagonists towards plant pathogens (Glick, 2012). A panel of 48 genera was elaborated based on the information reported in relevant published work on PGPBs (Dardanelli et al., 2010; Ferreira et al., 2019; Figueiredo et al., 2010; Glick, 2015, 2012; Hayat et al., 2010; Little et al., 2020; Ramakrishna et al., 2019; Rashid et al., 2015). On the basis of this filtration, in the case of samples from sandy soil (Table 4.11), some PGPBs were found in samples treated with Centrifuged and Dried SSADs. In the first case *Sporosarcina* and *Aminobacter* were detected. *Sporosarcina* usually lives at alkaline pH, likewise the present study, and is involved in nitrogen turnover (urease activity) and in detoxification from metals through reduction (Little et al., 2020; Shivaji et al., 2014). *Aminobacter* contributes to sustain nitrogen cycle as well, it is resistant to metal stress and it has the capability to decompose pollutants deriving from herbicides degradation (Rashid et al., 2015; Willems, 2014). In the second case, *Achromobacter* and genera of *Bacillaceae* family were detected. *Achromobacter* is a promoter of growth of tomato plants in drought conditions and, more widely, it can solubilize mineral phosphate compounds, making them bioavailable (Glick, 2015; Hayat et al., 2010). Genera of *Bacillaceae* (especially *Bacilli*) are within the "prima donnas" of PGPB, with functions ranging from biofertilisation (production of ammonia, solubilization of phosphorus), to production of siderophores and phytohormones (*e.g.* indolacetic acid and gibberellins), to biocontrol of pathogens (Ferreira et al., 2019). Moreover, Centrifuged and Dried SSADs shared three additional PGPBs within the significantly more abundant genera: *Nocardia, Rhodococcus* and *Nitrosomonas*. *Nocardia* and *Rhodococcus* are involved in the turnover of organic matter, in the production of phytohormones, siderophores and biotransforming enzymes, as well as in the bioremediation of azo-compounds (Ferreira et al., 2019; Goodfellow, 2014). *Nitrosomonas* belongs to the group of Ammonia Oxidizing Bacteria (AOB) and it is involved in the transformation of ammonia into nitrite (one of the rate limiting steps if nitrogen cycle), which is precursor of nitrate, a fundamental nitrogen source for plants (Enwall et al., 2007; Kowalchuk and Stephen, 2001). Besides these six genera, no other PGPBs were found nor in the control neither in other treatments. However other abundant genera are worthy of being mentioned for their environmental implications. In samples treated with Primary SSAD, presence of *Clostridium* (nitrogen fixing; Hayat et al., 2010), *Virgibacillus* (halotolerant; (Sánchez-Porro et al., 2014), *Herminiimonas* (detoxifying from arsenic; Baldani et al., 2014) and *Nannocystis* (halotolerant and degrading organic molecules) was appreciated. With regards to secondary SSAD treatment, genera of *Anaerolinae* class (*e.g. Caldilinea*) were found and they were likely associated to the population of anaerobic digestor, since particular conformations of pili allow

them a more advantageous adhesiveness to reactor wall (Xia et al., 2016); furthermore, this observation is accordance with the more copious presence of *Chloroflexi* phylum mentioned above. As concerns samples treated with Centrifuged SSAD, *Micromonospora* (degrader of organic matter and plant material, secondary metabolite producer, biocontrol agent; Trujillo et al., 2014) and *Kaistia* (degradation of organic matter, actor in bioremediation of CO and metal; Carareto Alves et al., 2014) were found, while *Massilia* (degrader of organic compounds; Baldani et al., 2014) was observed in Dried SSAD treated samples.

Shifting the focus on peat substrate (Table 4.12), significantly more abundant genera were consistently fewer and only one of them was classifiable as PGPB, that was *Agrobacterium* in control and Primary SSAD treated samples. *Agrobacterium* is involved in nitrogen fixation, siderophore production and acts as biocontrol agent (Cummings and Orr, 2010; Ferreira et al., 2019; Glick, 2015).

Analysis with ANCOM provided a general overview of most plentiful genera across the different treatments; however, this strategy was useful as first approach to have a general idea of relevant genera in each treatment. Nevertheless, it was not possible to observe the separated effects of the single treatments on soil. To this aim, outputs of pairwise comparisons elaborated by the tool DeSeq2 were exploited again and, more in detail, all the comparison between treated (with SSADs or mineral fertilizer) and untreated control were considered. Also in this case, the above-mentioned rationale of PGPBs was considered to highlight the presence of interesting genera enhancing plant proliferation. Table 4.13 shows the results of pairwise comparisons versus untreated control for each treatment, listing the found genera; numeric values indicate the $\log_2$ fold change and negative values mean that the genera was more abundant in control. In sandy soil (Table 4.13a), samples treated with Primary SSAD resulted significantly more abundant in *Nocardia*, *Agrobacterium*, *Aminobacter* (already described), *Chryseobacterium* (biocontrol agent; Glick, 2015), *Devosia* (nitrogen fixing; Little et al., 2020) and *Acinetobacter* (biocontrol agent specific for tomato; Glick, 2015); on the contrary control resulted significantly more abundant in *Cupriavidus* and in genera of families belonging to *Cyanobacteria* (*Nostocaceae*, *Pseudanabenaceae*) generally involved in fixation of molecular oxygen (Rashid et al., 2015). Concerning Secondary SSAD, samples amended with this digestate showed higher concentration in *Nocardia*, *Chryseobacterium*, *Devosia*, *Achromobacter* and some genera of *Pseudanabenaceae* (*Cyanobacteria*) (already described) than control, while control revealed higher concentrations in *Bacillus* and *Cupriavidus* (already described). As regards Centrifuged SSAD, some genera were already found during ANCOM computation, such as *Nocardia*, *Sporosarcina* and *Aminobacter*; *Achromobacter* was already considered but its prominence was attributed to Dried SSAD, however its presence in Centrifuged SSAD-treated samples resulted significantly higher than control. Other interesting PCPBs found more than untreated control were *Chryseobacterium*, *Devosia*, *Agrobacterium*, *Achromobacter*, *Acinetobacter* (already described) and *Shinella* (nitrogen fixing; Rashid et al., 2015).

Table 4.13 Differential abundancy analysis of genera performed with DeSeq2 software.

The table resumes only genera significantly more abundant in each treatment (q-value < 0.05) than control in the case of sandy soil (a.) and peat substrate (b.). For each genus, log2FoldChanges are reported. Negative values indicate higher occurrence in untreated control.

							log$_2$ Fold Change				
	Kingdom	Phylum	Class	Order	Family	Genus	Primary	Secondary	Centrifuged	Dried	Mineral
a. Sandy soil	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	n.s.	n.s.	-0.85	n.s.	n.s.
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Nocardia	4.23	2.25	6.28	6.40	n.s.
	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Chryseobacterium	7.14	4.34	5.00	6.20	n.s.
	Bacteria	Cyanobacteria	Chloroplast	Chlorophyta	Trebouxiophyceae	Chloroidium	n.s.	n.s.	n.s.	6.76	n.s.
	Bacteria	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Nostoc	n.s.	n.s.	n.s.	6.66	n.s.
	Bacteria	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae		-7.30	n.s.	n.s.	n.s.	n.s.
	Bacteria	Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	Phormidium	-3.18	n.s.	n.s.	n.s.	n.s.
	Bacteria	Cyanobacteria	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae	Arthronema	n.s.	n.s.	5.11	n.s.	6.40
	Bacteria	Cyanobacteria	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae		-15.60	8.77	n.s.	n.s.	n.s.
	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	n.s.	-2.32	-1.87	1.95	n.s.
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Bacillus	n.s.	n.s.	n.s.	8.94	n.s.
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	n.s.	n.s.	12.74	n.s.	n.s.
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	1.15	1.27	1.32	2.20	1.47
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter	3.67	n.s.	8.73	8.88	n.s.
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	8.31	n.s.	6.82	6.13	6.87
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	n.s.	n.s.	n.s.	-5.00	n.s.
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella	n.s.	n.s.	5.33	5.26	6.11
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	n.s.	3.59	5.68	8.75	n.s.
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus	-1.62	-1.47	-1.08	1.93	n.s.
	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	3.42	n.s.	5.59	n.s.	n.s.
b. Peat substrate	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	n.s.	n.s.	n.s.	n.s.	-0.80
	Bacteria	Cyanobacteria	Nostocophycideae	Nostocales	Nostocaceae	Dolichospermum	n.s.	-5.80	-4.91	n.s.	n.s.
	Bacteria	Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	Phormidium	-24.19	n.s.	n.s.	n.s.	n.s.
	Bacteria	Cyanobacteria	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae	Leptolyngbya	n.s.	n.s.	-6.79	n.s.	n.s.
	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	-1.53	n.s.	0.54	-0.74	0.71
	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	n.s.	n.s.	0.86	n.s.	1.16
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium	0.71	1.17	n.s.	n.s.	-0.85
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	n.s.	-9.05	-8.76	n.s.	-4.93
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	0.62	n.s.	n.s.	0.77	n.s.
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella	n.s.	n.s.	-3.19	n.s.	n.s.
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	-1.39	-1.95	n.s.	n.s.	n.s.
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	n.s.	n.s.	1.83	1.96	1.77
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus	1.08	-1.61	n.s.	1.36	n.s.
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	-5.42	n.s.	-5.66	n.s.	n.s.
	Bacteria	Proteobacteria	Betaproteobacteria	Hydrogenophilales	Hydrogenophilaceae	Thiobacillus	6.25	n.s.	n.s.	5.22	7.67
	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	n.s.	n.s.	6.91	n.s.	n.s.

Conversely, untreated control showed higher presence of *Bacillus*, *Cupriavidus* (already described) and *Arthrobacter* (phosphorous solubilizer, siderophore producer; Glick, 2015) than samples exposed to Centrifuged SSAD. Similar observation resulted in the case of Dried SSAD: some of the genera were the ones already revealed with ANCOM (*Nocardia*, *Bacillus*, *Achromobacter*), while other ones emerged in this phase, such as *Chryseobacterium*, *Devosia*, *Aminobacter*, *Agrobacterium*, *Shinella*, *Cupriavidus*, and two nitrogen fixing *Cyanobacteria* (*Nostoc* and *Chloroidium*). Conversely, in control *Rhizobium* (one of major players in plant growth enhancement in terms of biofertilisation, biostimulation and biocontrol, as reported by Ferreira and colleagues (2019)) resulted significantly more abundant than Dried SSAD-treated samples. Finally, concerning mineral fertilizer, *Devosia*, *Agrobacterium*, *Shinella* and genera of *Pseudanabenaceae* (already described) resulted significantly higher than untreated samples.

Moving to peat substrate, DeSeq2 elaboration (Table 4.13b), provided more insights in terms of differential abundances than ANCOM, allowing to reveal some genera more observed than control along the different treatments. Starting from Primary-SSAD samples, *Rhizobium*, *Cupriavidus* (already described), *Mesorhizobium* (nitrogen fixing; Glick, 2015) and *Thiobacillus* (enhancer of sulphate uptake through sulfur oxidation; Ferreira et al., 2019) were significantly more numerous than in control; on the other hand, *Bacillus*, *Achromobacter*, *Phormidium* (belonging to *Cyanobacteria*) and *Herbaspirillum* (nitrogen fixing, producer of phythormones and siderophores; Baldani et al., 2014; Ramakrishna et al., 2019) were more abundant in control. In the case of Secondary SSAD, only *Mesorhizobium* resulted more abundant than in control, while in this one *Agrobacterium*, *Achromobacter*, *Cupriavidus* and *Dolichospermum* (*Cyanobacteria*) were significantly more abundant. Centrifuged-SSAD samples showed the highest heterogeneity, showing both many genera significantly more and less expressed than control; the formers were *Bacillus*, *Acinetobacter* (already described), *Paenibacillus* (nitrogen fixing, producer of auxins, biocontrol agent; Ferreira et al., 2019; Little et al., 2020) and *Burkholderia* (phosphorous solubilizer, biocontrol agent; Ferreira et al., 2019); the latter were *Agrobacterium*, *Shinella* and *Herbaspirillum* (already described). In Dried SSAD samples, *Rhizobium*, *Burkholderia*, *Cupriavidus* and *Thiobacillus* (already described) resulted significantly more concentrated than in untreated control; on the contrary, only *Bacillus* resulted more present in control. Finally, *Bacillus*, *Paenibacillus*, *Burkholderia* and *Thiobacillus* (already described) resulted more abundant in samples minerally fertilized, while *Agrobacterium*, *Mesorhizobium* (already described) and *Streptomyces* (producer of phytohormones and siderophores, stimulator of mycorrhizal fungi; Ferreira et al., 2019) were significantly more numerous in control samples.

To conclude this paragraph on taxonomy and differential abundancies across samples, it can be affirmed that the application of different kinds of SSAD do not completely upset the general composition of soil microbial communities, as stated before, since the main phyla and families were generally conserved. However, it can be asserted that each treatment induced different effects on the bacterial

composition of tomato rhizosphere, especially at the level of genera, where the presence of interesting PGPBs was assessed. Furthermore, the detection of compositional differences was more pronounced in the case of sandy soil, which, considering its poorness in terms of nutrients and organic matter, was a less "cosy" environment. Thus, it was maybe more dependent on external inputs both in terms of chemical and biological features. Another interesting aspect was the lower difference between control and mineral fertilizer, especially on sandy soils (highlighted by PCA plots and ANCOM elaborations): this observation is in agreement with previous studies (Lloret et al., 2016), which affirmed that the boost on microbial community given by macro-elements is not powerful as the one provided by the addition of organic carbon, which is instead provided with SSAD. Indeed, the effect of mineral fertilizer was more similar to the one of SSADs on peat substrate, which is by "default" a carbon rich substrate; hence, the treatment was probably just a surplus to shape microbial communities. Lastly, differential abundancy analysis did not reveal difference of really important genera in plant growth enhancement, but it did not mean they were not present. Simply they were ubiquitously present. Indeed, *Pseudomonas* was one of the top genera in sandy soil taxonomy, while *Azospirillum* and *Rhizobium* (Ferreira et al., 2019) took part to tomato plant rhizosphere on peat substrate.

4.3.4 Molecular ecology parameters: α and β – diversity parameters

Ecological parameters are tools allowing to describe different ecosystems numerically. In this way, it is possible to "break free" from taxonomy, which is a double-edge sword since it provides decisively much information but pose the risk of get lost within all the taxa. So, ecological parameters allow to reduce the zoom on microbial communities and to have a more broad and comprehensive perspective. In this study, α- and β-diversity indices were evaluated respectively to understand richness and evenness of bacterial population within each treatment, and the distances and dissimilarities between the microbial communities from different treatments.

With regards to α-diversity, six different indices were calculated per each treatment from the sequencing data on the two cultivation substrates, sandy soil and peat substrate. The six indices accounted for species richness (Observed OTUs, Chao1), species evenness (Pielou), phylogenetic diversity (Faith PD), and mixed species richness and evenness (Shannon, Simpson). Rarefaction curves of these indices are reported for both cultivation substrates in Supplementary Material (Figures S7 and S8). For sandy soil, Table 4.14 shows the mean values of these indices across the different treatments, and in the lower part is reported the statistical analysis. Concerning species richness, values for observed OTUs and Chao1 were quite similar within each treatment, indicating that the contribution of rare species was minor.

Table 4.14 α-diversity indices measured on rhizosphere samples from sandy soil.

The upper part reports the mean value of each metric for each treatment. The lower part reports the results of statistical analysis with Kruskal-Wallis test, indicating values of H-statistic and experiment-wise p-value.

	Observed OTUs	Chao1 Index	Pielou's evenness	Faith PD	Shannon Index	Simpson Index
Control	3534.3	3573.4	0.714	130.68	8.376	0.979
Primary	3254.8	3292.7	0.684	127.15	7.959	0.977
Secondary	2915.8	2942.9	0.701	123.72	8.177	0.978
Centrifuged	2766.3	2793.5	0.707	122.69	8.085	0.984
Dried	2441.4	2468.6	0.673	110.15	7.524	0.975
Mineral	2591.6	2622.6	0.693	113.82	7.866	0.981
	Kruskal-Wallis test - Alpha diversity metrics					
H statistic	14.794	14.794	1.862	10.120	5.639	3.030
p-value	0.011	0.011	0.868	0.072	0.343	0.695

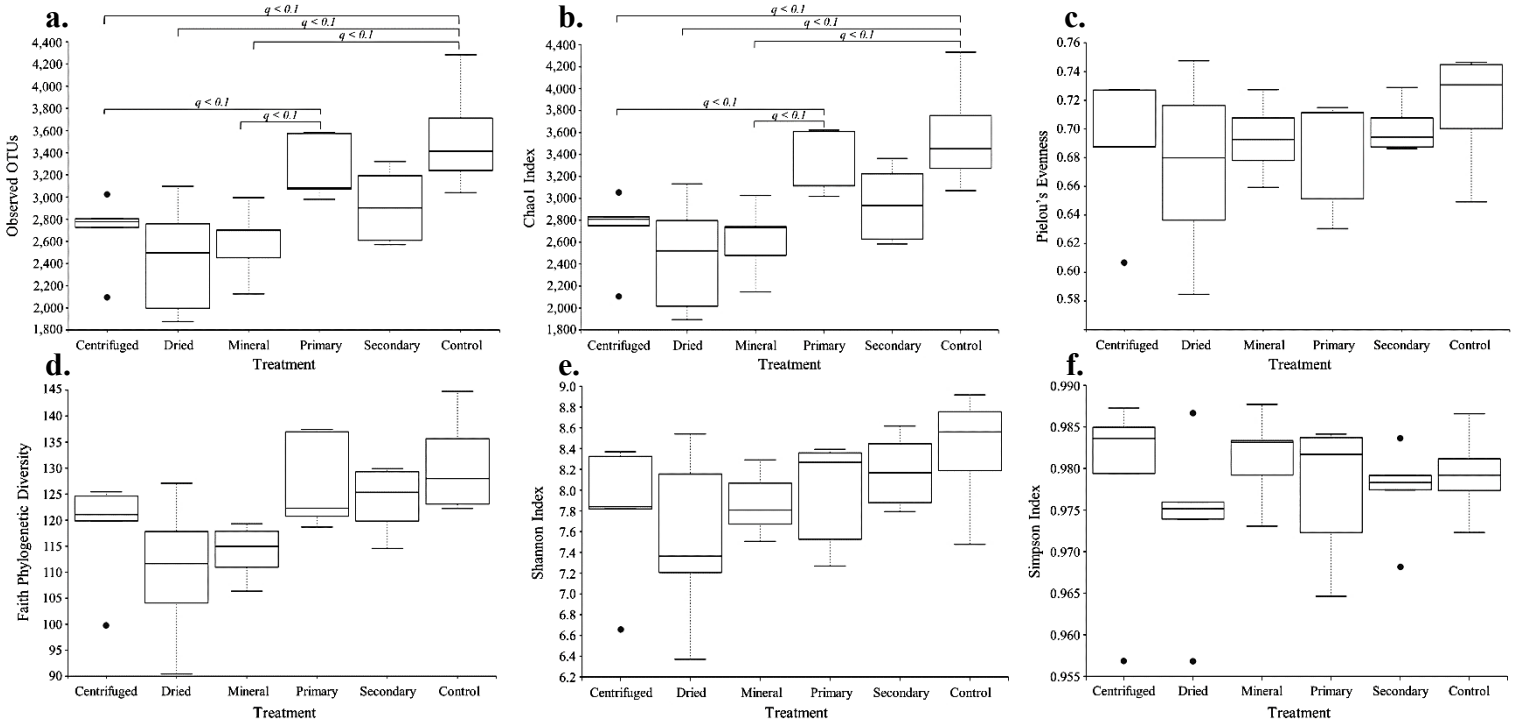
Species evenness ranged between 0.67 and 0.71, indicating that there was no predominance of few species, but a quite ubiquitous representation was present. Statistical analysis reported low experiment-wise *p*-values (< 5%) only for Chao1 and Observed OTUs indices, suggesting some differences in these metrics. Distribution of α-diversity indices is shown in Figure 4.12, where boxplots indicate the range of variation of these values and significant differences from pair-wise comparisons are specified. As can be seen, only few significant differences were found in Observed OTUs and Chao1: control was higher than Centrifuged and Dried SSAD and mineral fertilizer, while Primary was higher than Centrifuged SSAD and mineral fertilizer. It is worth specifying that in the case of pair-wise comparison, *p*-values were adjusted to *q*-values with the Benjamini-Hochberg correction, which allowed to discard false positives. The only differences revealed fulfilled only the largest significance value acceptable (q-value < 0.1), suggesting that significant differences were present, but they were not extreme. This indicated that application of SSAD based treatment in general poorly affected α-diversity indices.

Samples of peat substrate showed similar behaviour in terms of α-diversity (Table 4.15). Concerning species richness, also in this case Observed OTUs and Chao1 showed similar results; however, their values were slightly lower than the one observed on sandy soil. This fact may be justified considering that sandy soil is a real soil, used as is from the sampling site; on the other hand, the peat substrate is a cultivation substrate sterilized prior to use. This practice might have sensitively influenced the number of bacterial species present. Species evenness revealed values ranging from 0.733 to 0.828, even slightly more than samples from sandy soil, suggesting also in this case no species predominance.

The fact that these two aspects (richness and evenness) were counter-balanced between the two soils can be appreciated with Shannon and Simpson indexes, which were pretty similar.

Figure 4.12 Boxplots indicating variations and significant differences between α-diversity indices between different treatments in sandy soil.

a. Observed OTUs. b. Chao1. c. Pielou's evenness. d. Faith's Phylogenetic Diversity. e. Shannon Index. f. Simpson Index. Significant differences are indicated for significant pair-wise *q*-values (*p*-values corrected with Benjamini-Hochberg method).



Statistical analysis revealed significantly low experiment-wise *p*-values (<5%) for three α-diversity metrics: Observed OTUs, Chao1 and Faith's PD. However, these values were not confirmed by pair-wise comparisons, since none of them provided significant differences between samples (Figure 4.13). Also in this case, boxplots indicates values variation for each treatment in the different indices measured. Hence, it can be reasonably assessed that application of SSAD-based treatment had almost none effect on α-diversity of the microbial communities of the tomato rhizosphere in sandy soil and peat substrate.

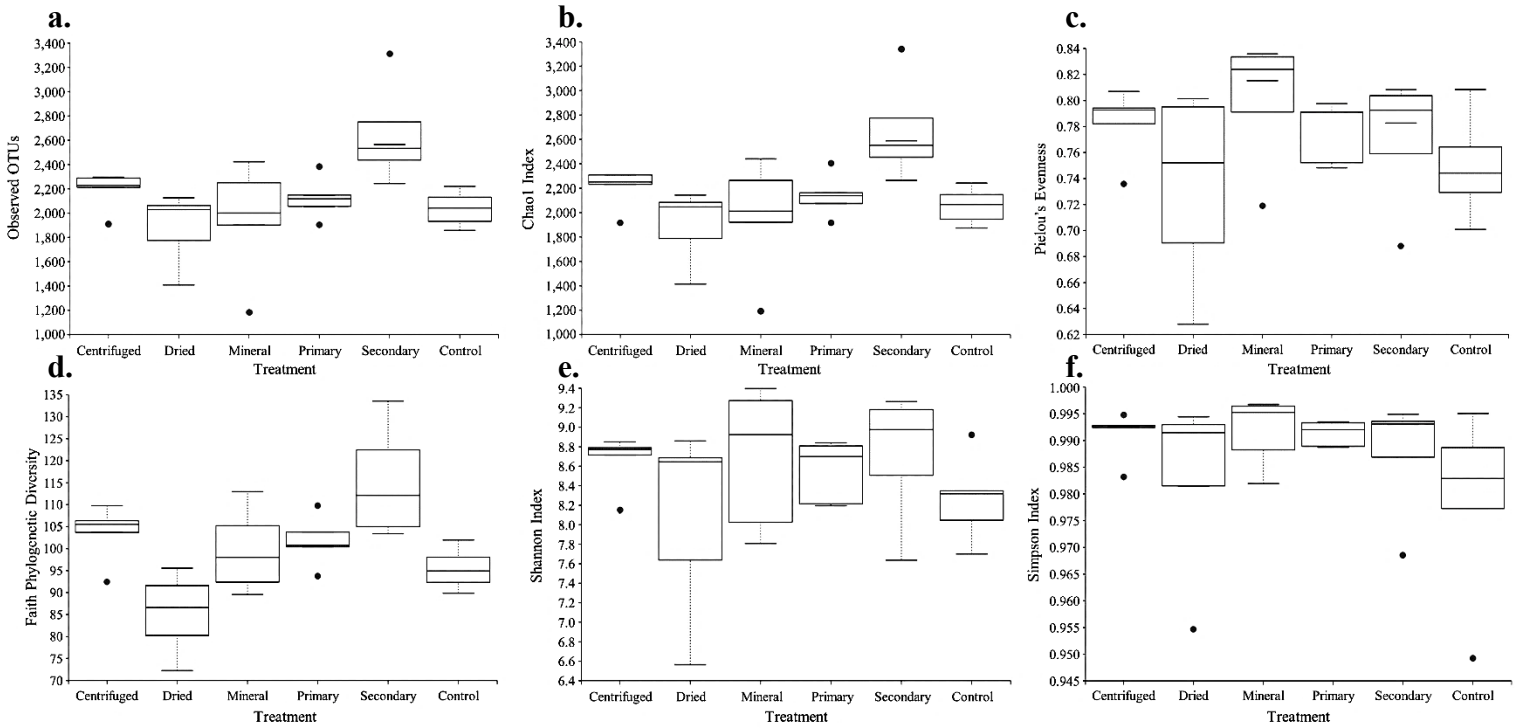
Table 4.15 α-diversity indices measured on rhizosphere samples from peat substrate.

The upper part reports the mean value of each metric for each treatment. The lower part reports the results of statistical analysis with Kruskal-Wallis test, indicating values of H-statistic and experiment-wise p-value.

	Observed OTUs	Chao1 Index	Pielou's evenness	Faith PD	Shannon Index	Simpson Index
Control	2033.4	2052.3	0.749	95.33	8.099	0.986
Primary	2104.3	2121.3	0.776	101.57	8.547	0.991
Secondary	2433.7	2453.5	0.797	115.23	8.708	0.994
Centrifuged	2253.5	2272.2	0.794	106.24	8.777	0.993
Dried	1877.4	1891.8	0.733	85.16	8.075	0.990
Mineral	2140.8	2156.4	0.828	99.54	8.682	0.992
	Kruskal-Wallis test - Alpha diversity metrics					
H statistic	11.802	11.598	4.450	12.730	3.683	4.448
p-value	0.038	0.041	0.487	0.026	0.596	0.487

Figure 4.13 Boxplots indicating variations of α-diversity indices between different treatments in peat substrate.

a. Observed OTUs. b. Chao1. c. Pielou's evenness. d. Faith's Phylogenetic Diversity. e. Shannon Index. f. Simpson Index. No significant difference was reported in pair-wise comparisons of the indices.



The obtained results were in good agreement with other works published in literature, where the general trend was the registration of α-indices values, of soil microbial communities after organic treatment, similar to lower than untreated control. Indeed, land application of sewage sludge has turned out to not affect bulk soil microbial communities in terms of α-indices such as Observed OTUs, Chao1, Shannon, Evenness and Phylogenetic Diversity (Chen et al., 2016; Li et al., 2019; Urra et al., 2019). Besides bulk soil, also microbial population of rhizosphere showed negligible differences not only after treatment of sewage sludge (Ondreičková et al., 2016), but also after external stresses, such as glyphosate application (Newman et al., 2016). Moreover, this aspect was found also in studies on tomato rhizosphere, which confirmed how tomato plants host a robust rhizosphere, whose α-diversity indices values did not change significantly upon external stresses, such as during organic amendments (Allard et al., 2016) or diseased state (Li et al., 2014). Furthermore, the importance of soil-plant interaction in terms of microbial community has been confirmed by Liu and colleagues (2019): they did not appreciate differences in species richness after field application of sewage sludge, but instead they observed an increased richness in those plots were plants were sown.

On the other hand, results of the present study were in contrast with the ones found in other works in literature dealing with soil application of different typologies of anaerobic digestates of sewage sludge, where Chao1, Faith's PD and

Shannon index resulted significantly lower than untreated soil (Lloret et al., 2016; Pascual et al., 2008). Probably, this effect could be due to the higher dosages of SSAD, which may have induced a selection effect on those bacteria able to use the specific substrates introduced or to better resist to adverse presence of heavy metals (Urra et al., 2019). Mattana and co-workers (2014) reported a study of microbial community characterization after soil application of fresh, thermally treated and composted sewage sludge; their Shannon index results on the use of the first two "feedstocks" were comparable to control (as the present study), while the use of compost induced a positive increase of this value. This feature was found also in the paper of Lavecchia and colleagues (2015), where treatment with green compost improved α-diversity indices of richness (Observed OTUs, Chao1), but not Evenness. Hence, this probably indicates that a major stabilisation of the organic treatment may be useful to improve α-diversity. In contrast with the results of this study, some works reported increased values of α-diversity indices of soil microbial communities; Mossa and colleagues (2017) showed that moderate application of sewage sludge increased Simpson Index of rhizosphere bacterial population thanks to the beneficial addition of organic matter and nutrient, but beyond certain dosages toxic effects prevail. Bai and co-workers (2019) reported that application at increasing dosages of sewage sludge amendment on coastal mudflat saline soil improved various α-diversity indices (as Chao1, Observed OTUs, Shannon and Simpson) of bacterial communities; however, this experiment was performed in absence of plants and, maybe, the presence of plants and their rhizosphere would have lessened this effect.

A last remark on α-diversity can be performed on those indices evaluated not on the basis of the taxonomy (as done in this study), but on the microbial activity in terms of respiration and enzymatic activity. Indeed, application of organic amendments of different kind such as cotton gin trash and composted poultry manure (Liu et al., 2007) and swine and dairy manure (Larkin et al., 2006) improved the parameters of richness and diversity. These observations confirmed that addition of organic amendments stimulated microbial activity. Moreover, a nice suggestion for future work is to carry out α-diversity analysis of taxonomy along with the one related to microbial biomass to achieve and depict a more complete scenario of the effects on microbial communities of SSAD application on soil.

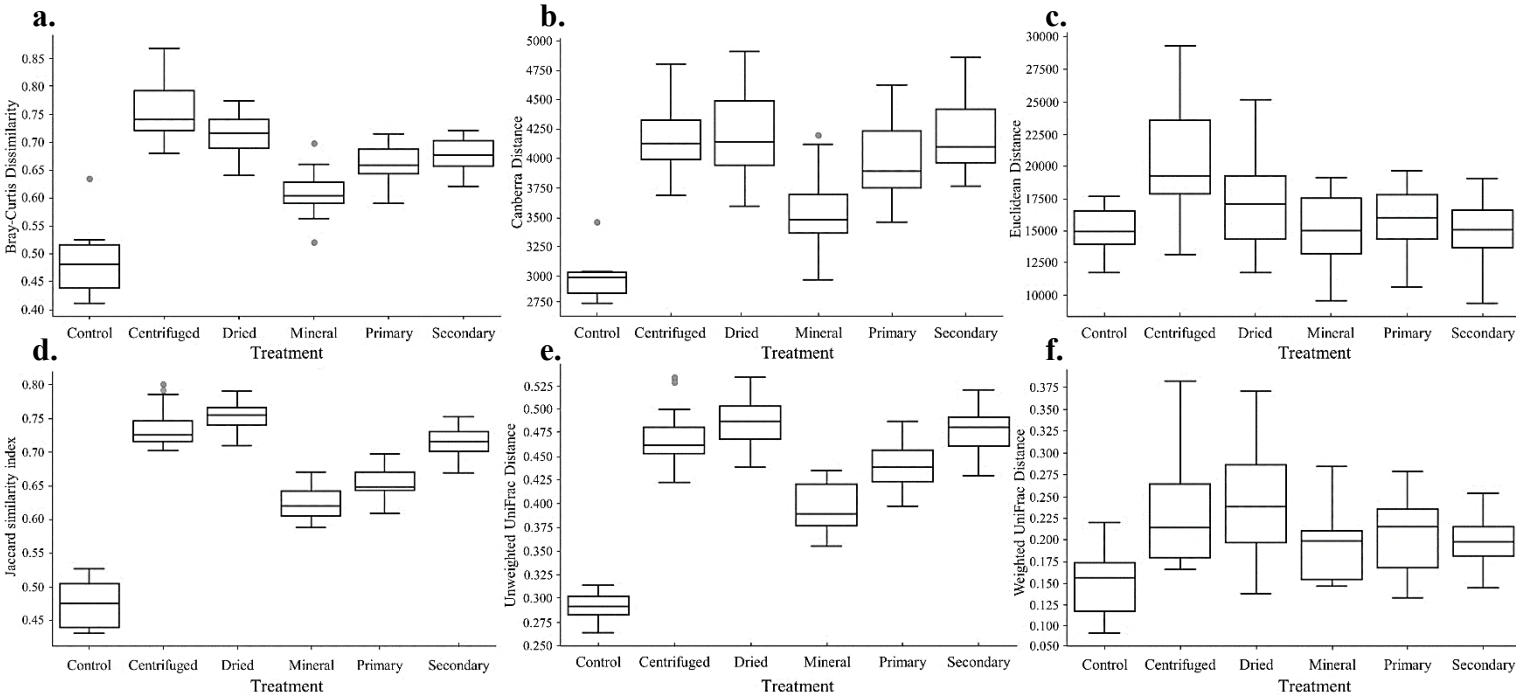
The second sets of ecological measurements were performed with regards to β-diversity parameters, which can be defined as "distances" or "dissimilarities" between microbial communities. In other words, β-diversity can provide synoptic information on how much microbial communities from different treatment are different or not. Six different β-diversity parameters were computed: Bray-Curtis dissimilarity, Canberra distance, Euclidean distance, Jaccard distance, Weighted and Unweighted UniFrac distances. These distances, or metrics, were calculated taking into account different features, as described in materials and methods, hence differences can result more pronounced for certain metrics than others. In particular, the target of this analysis was to evaluate if the intra-treatment distances (*i.e.* distances between replicates of the same treatment) were

Table 4.16 Statistical analysis of β-diversity metrics measured on rhizosphere samples from sandy soil.

	PERMANOVA - Beta diversity metrics					
	Bray-Curtis	**Canberra**	**Euclidean**	**Jaccard**	**Unweighted UniFrac**	**Weighted UniFrac**
Sample size	26	29	29	26	26	26
Groups	6	6	6	6	6	6
Test statistic	5.514	4.671	4.123	3.912	4.270	3.779
p-value	0.001	0.001	0.001	0.001	0.001	0.001
Permutations	999	999	999	999	999	999

Figure 4.14 Boxplots indicating variations of β-diversity distances between samples from control and treatments in sandy soil.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.

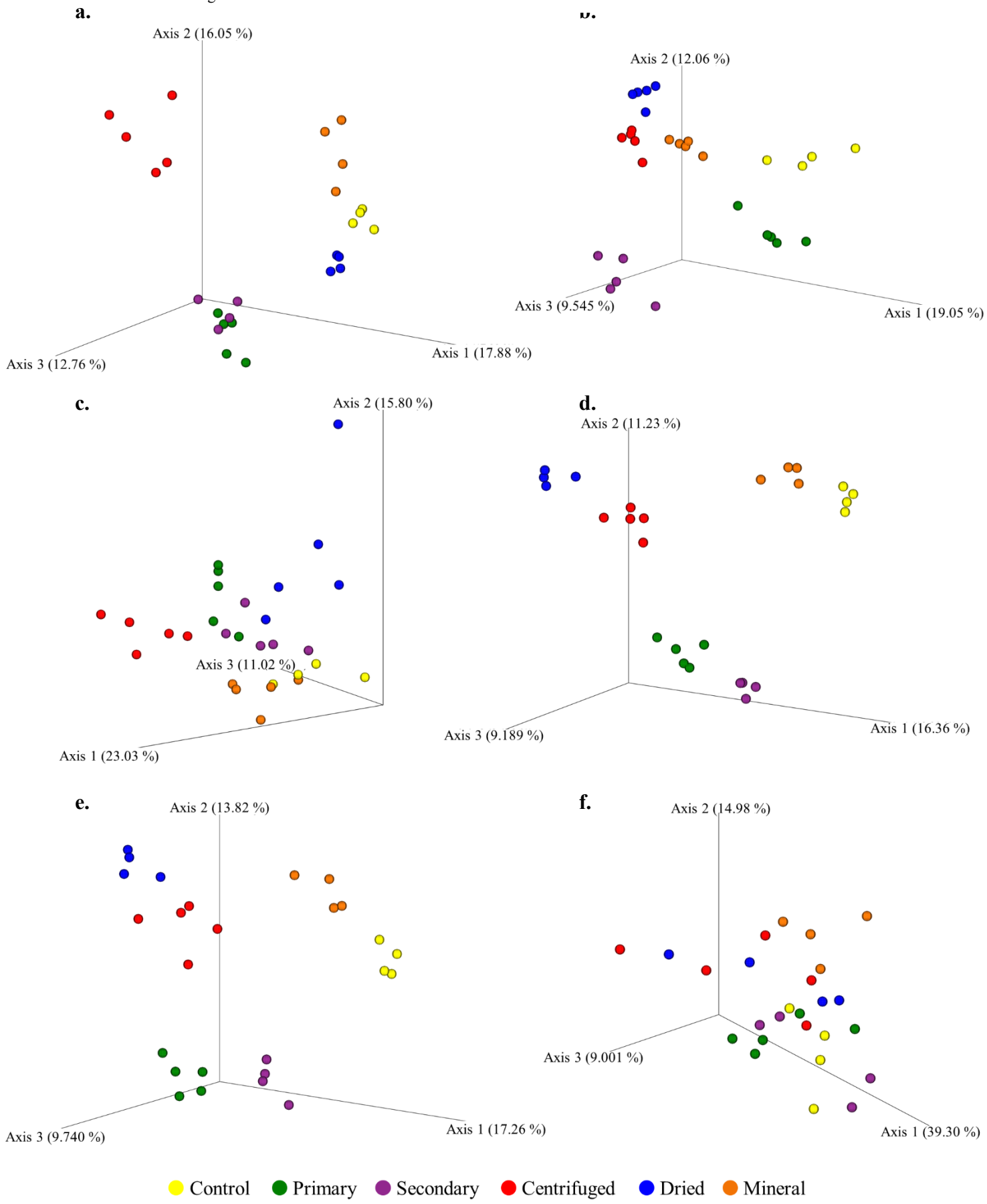


significantly more reduced than the inter-treatment ones (*i.e.* mean distances between replicates of different treatment). Hence after the calculation of these metrics, statistical validation through PERMANOVA test was performed. This computation was performed two-fold; firstly, distances were evaluated within the same cultivation substrate, to highlight the possible treatment effect; secondly distances were evaluated between the two cultivation substrates, to investigate the effect of the soil.

Starting from sandy soil, Table 4.16 and Figure 4.14 report the results of statistical validation with PERMANOVA and the mean distances with untreated control samples, respectively.

Figure 4.15 EMPeror plots of Principal Coordinates Analysis of β-diversity metrics in sandy soil.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.



The p-values (p = 0.001) between the groups indicated that for all the calculated metrics, there were significant differences, suggesting dissimilar microflora composition between rhizosphere samples. Centrifuged and Dried rhizosphere resulted to be the most distant from control, followed by Primary and Secondary ones; the rhizosphere of mineral fertilizer was the closest to control. These distances were more or less accentuated depending on the metric, for instance Bray-Curtis dissimilarity and Jaccard distance yielded more consistent distances than Euclidean and Weighted Unifrac distances. The representation with boxplots can provide information on the numerical values of each metric (and its variation); however, their interpretation becomes complex if all the distances between each treatment are represented in this way. Principal Coordinate Analysis (PCoA) allows to reach this goal providing a visual representation of microbial communities in a 3D space, permitting to appreciate simultaneously the distances between the communities. Figure 4.15 shows the results of PCoA analysis for the six different metrics as EMPeror plots; each dot represents the rhizosphere microbial community of each sample and the axes report the percentage of explained variance (Scree plots of explained variance in sandy soil is available in Supplementary material – Figure S9). In all metrics, except for Weighted UniFrac, the dots related to the same treatment tended to cluster together (more in the case of Bray-Curtis, Jaccard and Unweighted Unifrac, less for Canberra and Euclidean) and were clearly separated in the PCoA plots, suggesting the rhizosphere bacterial communities in sandy soil could be affected by the application of different treatment. Another aspect that emerged from these plots (and confirmed the data of boxplots) was that the dots of control and the one of mineral fertilizer were similar to each other. Moreover, these results were in good agreement with the cluster seen in the PCA Plot elaborated in differential abundancy analysis, confirming that addition of organic matter was a key factor in shaping microbial community in sandy soil more than macronutrients.

As concerns peat substrate, Table 4.17 and Figure 4.16 show the results of the PERMANOVA test and the mean distances with untreated control samples, respectively. Also in this case, the p-value (p = 0.001) indicated the presence of significantly higher distances between than within treatments. Differently from sandy soil, in this case the mean distances with control samples resulted more reduced and in general the variations of distances appeared broader. From these boxplots, it emerged that the treatment more distant from control was the Secondary (especially in Canberra, Jaccard and Unweighted UniFrac distance), while the other treatments resulted indicatively ubiquitously distant form untreated control. EMPeror plots display PCoA (Fig. 4.17) for β-diversity metrics of rhizosphere microbial communities on peat substrates (Scree plots of explained variance in peat substrate is available in Supplementary material – Figure S10). In the present case, Jaccard metric resulted to be the one which best clustered the dots of the same microbial communities and satisfying the higher distance of Secondary with respect to the other samples. Also PCoA of Canberra and Unweighted UniFrac distances managed to provide a good grouping of the dots from identical treatments, suggesting a good separation between the differently-treated samples. In the case

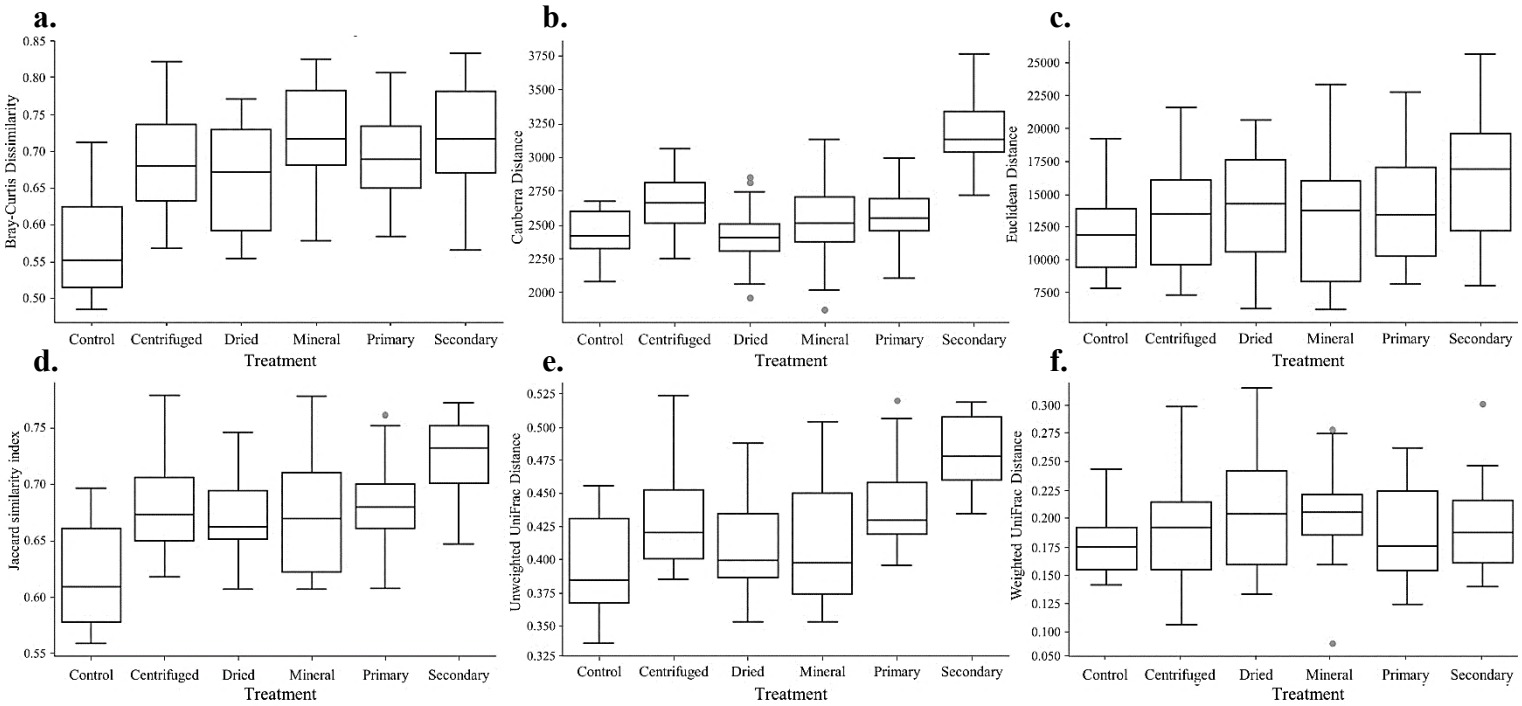
of Bray-Curtis dissimilarity, dots tended to be less separated, with samples from same treatment sparser (especially along second axis).

Table 4.17 Statistical analysis of β-diversity metrics measured on rhizosphere samples from peat substrate.

	PERMANOVA - Beta diversity metrics					
	Bray-Curtis	**Canberra**	**Euclidean**	**Jaccard**	**Unweighted Unifrac**	**Weighted Unifrac**
Sample size	26	29	29	26	26	26
Groups	6	6	6	6	6	6
Test statistic	4.572	4.118	2.537	4.093	4.561	2.513
p-value	0.001	0.001	0.001	0.001	0.001	0.001
Permutations	999	999	999	999	999	999

Figure 4.16 Boxplots indicating variations of β-diversity distances between samples from control and treatments in peat substrate.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.



Euclidean distance did not provide "dots clustering" for the different treatments, but the samples from control were considerably more distant from the other ones. Despite Weighted UniFrac distance was the one reporting the highest explained variance on Axis 1 (40.55%), it was the worst in providing information on microbial community differences from differently treated rhizospheres. In general, it can be asserted that the effect exerted by SSAD treatment on rhizosphere microbial communities of tomato in terms of β-diversity metrics was more intense on sandy soil than on peat substrate, and these results were consistent with the ones from PCA analysis and hierarchical filtering obtained with DeSeq2 during differential abundancy analysis.

Results of different works about multidimensional scaling of β-diversity distances of soil microbial communities (from soils treated with organic amendments) were in good agreement with the results of the present study. The papers of Li and colleagues (2019) and Lavecchia and co-workers (2015) demonstrated how soils treated respectively with sewage sludge and compost showed marked difference in terms of beta diversity (Bray-Curtis, and Weighted/Unweighted UniFrac respectively), with the samples belonging to the same treatments tending to form clusters. Moreover, likewise the present work, distances between control and minerally fertilized samples were lower. Another work corroborating the influence of organic treatments on β-diversity metrics of soil microbial communities was the one published by Lloret and colleagues (2016), who investigated the effects of sludge processing strategy The PCoA reported was computed from Weighted UniFrac distances and the overall representation of microbial communities structure showed different clusters associated with the differently processed sewage sludges. Moreover, an interesting aspect which influences β-diversity metrics is the presence or absence of plants on the soil treated with sewage sludge or organic amendments. Indeed, Liu and colleagues (2019) reported PCoA of Unweighted UniFrac distances, highlighting not only significant distances between soils treated or not with sewage sludge, but also between samples with plants or not. This observation lends support to the fact that the plant is crucial in shaping the structure of microbial communities; consequently, this permit to affirm the importance of soil rhizosphere rather than bulk soil for what concern microbial communities. Further confirms of this feature were brought by studies in which β-diversity metrics of microbial communities were not affected by the typology of treatment (organic amendments, glyphosate) but rather they were significantly influenced by the part of the plant (rhizosphere, phyllosphere, endosphere; Allard et al., 2016; Dong et al., 2019) and, to a broader extent, by the vegetal species (Newman et al., 2016).

Further analysis, concerning β-diversity, were performed to investigate whether also the typology of cultivation substrate behaved as important driver in determining distances between microbial communities. In this case, all the rhizosphere microbial communities were taken into account, from sandy soil and peat substrate samples. Firstly, all the distances were computed with the six different β-diversity metrics, and subsequently PERMANOVA test was run to validate differences significantly higher than intra-treatment distances. As shown in Table 4.18, the p-values ($p = 0.001$) indicated that for all the calculated metrics significant differences were present, suggesting dissimilar composition between rhizospheres isolated from the two different cultivation substrates samples.

Figure 4.17 EMPeror plots of Principal Coordinates Analysis of β-diversity metrics in peat substrate.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.

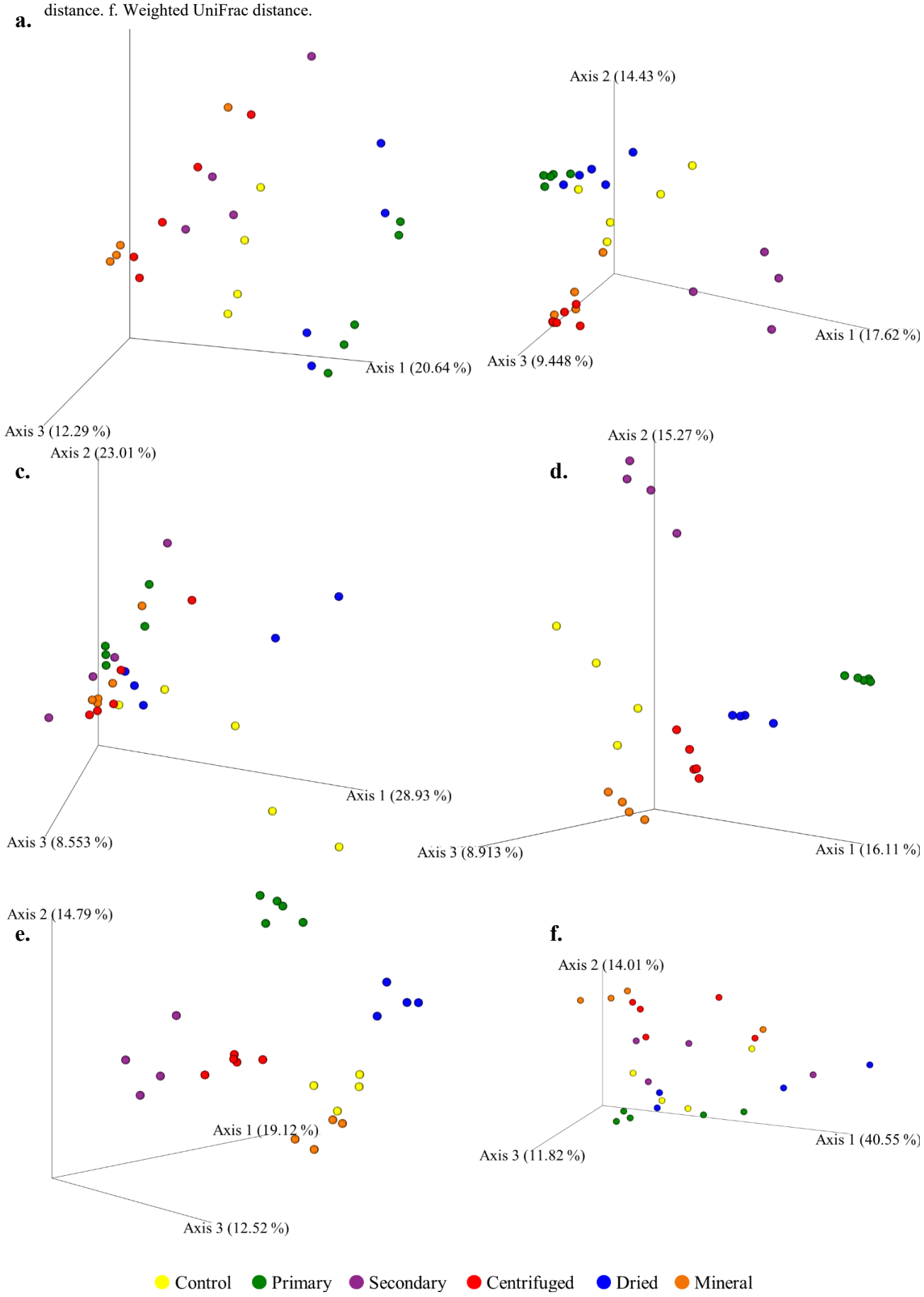


Table 4.18 Statistical analysis of β-diversity metrics measured between rhizospheres of sandy soil and peat substrate.

	PERMANOVA - Beta diversity metrics					
	Bray-Curtis	**Canberra**	**Euclidean**	**Jaccard**	**Unweighted Unifrac**	**Weighted Unifrac**
Sample size	52	58	58	52	52	52
Groups	12	12	12	12	12	12
Test statistic	9.151	9.899	5.289	7.770	10.607	6.835
p-value	0.001	0.001	0.001	0.001	0.001	0.001
Permutations	999	999	999	999	999	999

These clues were confirmed by distance to control sample and PCoA, shown in Figures 4.18 and 4.19; in each column is reported information relative to each measured metric: the top boxplot reports ranges of distances to control in sandy soil, the middle boxplot depicts ranges of distances to control in peat substrate and the bottom figure is the EMPeror plot. The most interesting results came from Bray-Curtis dissimilarity, Jaccard and Unweighted UniFrac distances. In all these cases it was possible to appreciate the sharp separation of microbial communities extracted from sandy soil and peat substrate and then, within the same soil, it could be observed the treatment driven clustering of the dots. This indication reinforced the theory of the strong influence of soil type on structure and shaping of soil microbial communities. Canberra and Weighted UniFrac distances showed the separation of microbial communities from the two different soils as well but, within the same soil, less clustering related to treatments was observed. Euclidean distance was the metric displaying less differences both numerically (boxplots with high variations) and visually, since the PCoA plot showed a general scattering of the dots.

Few examples in literature investigated the effects on β-diversity (of soil microbial communities) of the same organic treatments on two or more different soils. An interesting study was conducted by Ho and colleagues (2017), who evaluated the effects of different bio-based wastes (*e.g.* sewage sludge, green compost, aquatic plant material, etc.) used as soil amendments, on soil microbial communities in a litterbag assay. They exploited two different soils, a sandy loam and a clay soil. PCoA plot of Bray-Curtis dissimilarity was similar to the ones reported in the present study, since microbial communities formed firstly a soil-driven "master cluster" and then a series of "sub clusters" influenced by the treatment type. This behaviour strongly confirmed the theory that soil is the principal determinant of soil microbial communities, as stated by Bossio and colleagues twenty years ago (Bossio et al., 1998). Another interesting feature of the work of Ho and colleagues was the fact that β-diversity differences were more discrete on sandy loam soil than on clay soil. This was quite in accordance with the present study since the clearest differences were detected on sandy soil, which probably was a type of soil more prone to the shaping of microbial communities influenced by treatments application.

Figure 4.18 β-diversity distances between rhizospheres of sandy soil and peat substrate (I).

Top two rows show boxplots of variation of β-diversity distances to control samples of sandy soil and peat substrate, respectively (T: untreated control; C: Centrifuged SSAD; D: Dried SSAD; M: mineral fertilizer; P: Primary SSAD; S: Secondary SSAD). Bottom row shows EMPeror plots of Principal Coordinates Analysis of β-diversity metrics. a. d. g. Bray-Curtis dissimilarity. b. e. h. Canberra distance. c. f. i. Euclidean distance.

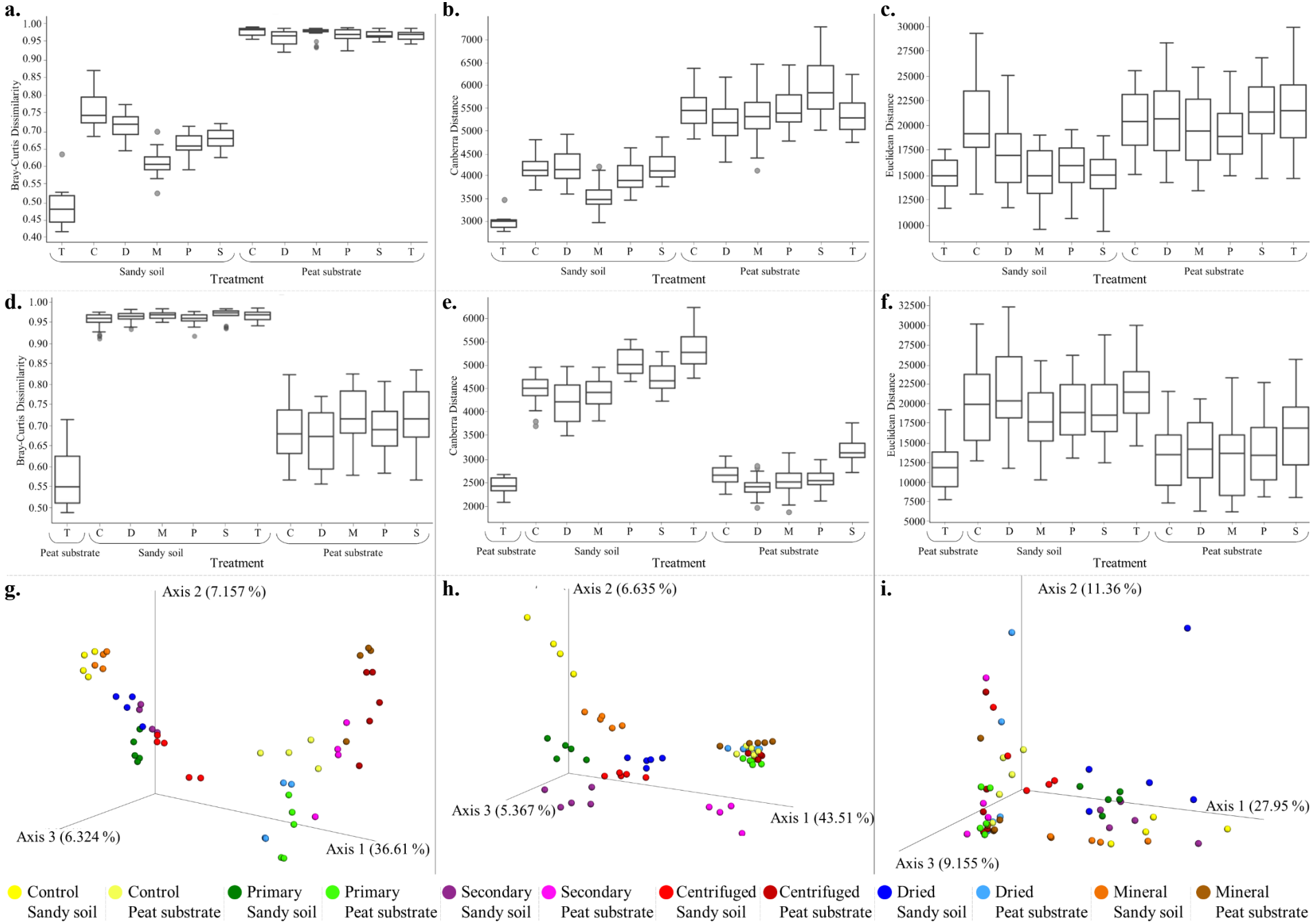
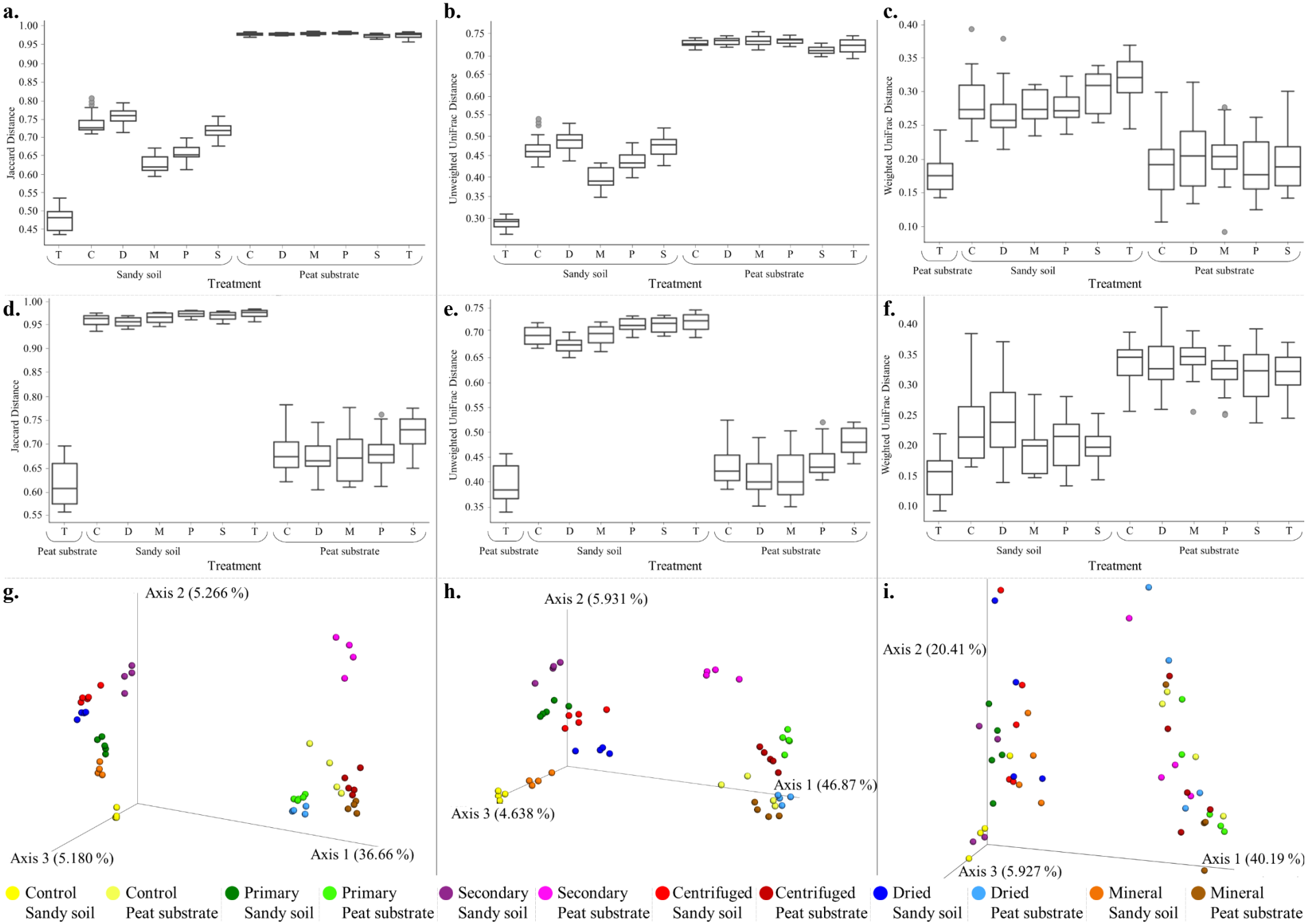


Figure 4.19 β-diversity distances between rhizospheres of sandy soil and peat substrate (II).

Top two rows show boxplots of variation of β-diversity distances to control samples of sandy soil and peat substrate, respectively (T: untreated control; C: Centrifuged SSAD; D: Dried SSAD; M: mineral fertilizer; P: Primary SSAD; S: Secondary SSAD). Bottom row shows EMPeror plots of Principal Coordinates Analysis of β-diversity metrics. a. d. g. Jaccard distance. b. e. h. Unweighted UniFrac distance. c. f. i. Weighted UniFrac distance.



Moreover, other soils proved to be more recalcitrant, such as the peat substrate in the present study and the clay soil in the paper of Ho, yielding more scattered PCoA plots. However, it must be highlighted that those comparisons were performed with laboratory treated soils, without a presence of a vegetal species, while in the present study the importance of rhizosphere role in microbial communities was stressed in numerous occasions. In this regard, this work can be considered pioneering in the perspective of the investigation on the combined effects which can contribute in the definition of rhizosphere bacterial communities.

4.4 Conclusions

The agricultural reuse of sewage sludge and SSAD shows relevant microbiological implications, as these nutrient-rich wastes produce a double effect: on one side they can stimulate microbial activity by the soil addition of organic matter, on the other their application means supplementation of external microbial inputs, including bacterial species enhancing plant growth (Little et al., 2020; Pascual et al., 2008). As a result, the impact on soil microbial communities is really important on microbial biomass (Calbrix et al., 2007), respiration and enzyme activity (Antolín et al., 2005), and of course on composition (Liu et al., 2007).

The beneficial effects on microbial activity of land application of organic amendments have been extensively described in literature, including compost and SSAD (Odlare et al., 2008). With regards to SSAD, it has been claimed that its organic matter composition shows more accessible organic carbon which can boost more microbial activity, with desirable results on the development of soil microflora (Pascual et al., 2008). A direct consequence of this aspect is the improvement in terms of production of crop biomass (Antolín et al., 2005). To a broader extent, other positive effects of the enhancement of microbial activity have been demonstrated on the increased capability of hydrocarbons degradation during contamination (Ros et al., 2010). Hence, a lot of data are available about evaluation of microbial activity of soil microorganisms after organic treatment, but complete information about the composition and taxonomy is still lacking.

In this scenario, the present work investigated the effects on the rhizosphere microbial communities of tomato plants on a nutrient-poor sandy soil and on a peat substrate treated with differently processed anaerobic digestate from sewage sludge (SSAD). The research was conducted exploiting a molecular ecology approach, utilising the technology of Next Generation Sequencing. The results showed that application of SSAD did not alter radically the microbiological composition of rhizosphere, as indicated by the taxonomic analysis at level of phylum and family. On the other hand, SSAD treatments had a significant influence on the occurrence of some bacterial genera as evidenced by the differential abundancy analysis. In this regard, a higher presence of bacteria with important implications in plant growth (PGPBs) and in other environmental issues was assessed. Moreover, estimation of ecological parameters was performed from sequencing data. α-diversity indices indicated that treatment did not significantly affect richness and evenness of microbial communities. On the contrary, β-diversity metrics showed

relevant distances between the microbial communities extracted from rhizosphere treated differently, indicating important effects in shaping bacterial populations especially on sandy soil. Moreover, β-diversity metrics confirmed the crucial role played by soil in the influencing the rhizosphere microbiome.

The results of the present research suggest that important factors should be considered for the future work, starting from the above-mentioned role of soil. Indeed, not only the type of soil is a principal determinant in shaping microbial communities (Bossio et al., 1998), but also some soil peculiarities are likely influencing microbial populations, such as total organic carbon and C/N ratio (Ho et al., 2017; Larkin et al., 2006). Broadening the perspective also to field experiments, some papers have recently evidenced that other factors may be correlated to tillage systems and NPK fertilisation (Bai et al., 2019; dos Santos Soares et al., 2019). Besides the "space" factor, also the "time" factor is worthy to be investigated. Indeed, the present work described the microbial communities in a defined moment, without considering what happened before and what would have occurred after. So, time-course experiments can be a powerful tool to switch from a "taking photo" to a "making movie" approach, allowing to follow the evolution of microbial communities over time. This kind of analysis can likely turn out to be a clever strategy not only to deepen the knowledge on characterisation of soil microbiome, but also a powerful tool for environmental monitoring, especially where SSAD is applied at fixed intervals. In this way, possible changes in soils features can be detected via biological indicators, which respond more rapidly to these variations, allowing to intervene more precociously.

In conclusion, SSAD application is an effective and feasible solution for vegetation restoration (Liu et al., 2019) and it can bring more benefits than drawbacks on chemical and biological properties of degraded semiarid soils (Bastida et al., 2008). Evaluation of bacterial population is a key factor to consider since it is a rapid indicator of changes in soil environment (Odlare et al., 2008), especially when working with SSAD, which can provide benefits, but also metal contamination in some cases (Kandeler et al., 1996; Mossa et al., 2017). Bacterial control turned out to be important also in another issue: pathogens. Indeed, not only SSAD soil application may result in addition of human pathogens, but some of these are natively present in soils and behave as PGPBs (Igiehon and Babalola, 2018), highlighting the importance of their monitoring. Speaking of PGPB, their role is crucial as extensively discussed in the present work, but their essentiality emerges especially for their capability to support plant growth in drought situations (Kaushal and Wani, 2016), which is a scenario highly connected to desertification issues, which in some way this research wants to address. Finally, all these issues related to soil monitoring stress, from different perspectives, the importance of preservation of biodiversity, which is fundamental for the conservation of soil functions and the resilience to external perturbations.

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Chapter 5

Extraction of humic acids from SSADs

5.1 General scenario and aim of the study

Humic acids (HA) are, together with humins and fulvic acids, one of the principal constituents of humic substances which, in turns, are one of the two principal macro-groups of molecules taking part to soil organic matter (SOM) (Stevenson, 1994). Humic acids play a crucial role in soil fertility under a physical, chemical and biological point of view, inducing beneficial effects on soil aeration, moisture retention and microbial activity, just to mention a few (Fernández et al., 2007). In recent years, this class of molecules has gained particular interest from the scientific communities not only for their widely known agricultural functions, but also for their applications in other fields such as medicine (anti-viral activity, cancer therapy), pharmaceutical and cosmetic areas (sunscreen and skin-care products) and nanoparticle production (De Melo et al., 2016).

As a consequence, the supply of humic acids is a very important issue. Unfortunately, the principal natural resources of humic acids are non-renewable, such as lignite, peat and leonardite. Overexploitation of these resources, of course, is not advisable and a solution becomes really necessary to make HA production sustainable. To this aim, different wastes and bio-based feedstocks have been individuated as potential HA sources, such as vermicompost (Promtov et al., 2016), municipal solid waste (Jindo et al., 2012), liquid swine manure (Brunetti et al., 2007), wood waste compost (Fukushima et al., 2009) and sewage sludge. Humic acids in sewage sludge and sewage sludge anaerobic digestate (SSAD) are present in the extracellular polymeric substances (EPS) and their content can reach up to 1.5%w/w of total solids. Many studies demonstrated that land use of sewage sludge proved to be effective in the increase of soil humic acids (Adani and Tambone, 2005; Fernández et al., 2008). However, long-term application on soil of sewage

sludge may pose environmental risks, such as heavy-metals contamination (McGrath et al., 1995).

In this scenario, the aims of the present study were substantially four: i) the implementation of a feasible extraction protocol of humic acids from SSAD, allowing on one side to obtain a solution enriched in HA, on the other to get rid of those contaminants normally present in sewage sludge, heavy metals amongst all; ii) the adaptation of a HA quantification protocol officially recognised valid by International Humic Science Society (Lamar et al., 2014) on SSAD; iii) the evaluation of the utility of membrane filtration process on the concentration and purification of extracted HA; iv) the morphological characterisation of extracted HA. Hence the general purpose of this work was to deepen the knowledge on HA extracted from renewable sources and to propose a sustainable method for HA provision to obtain a HA solution which can be used as is or as a base for more advanced formulations.

5.2 Materials and methods

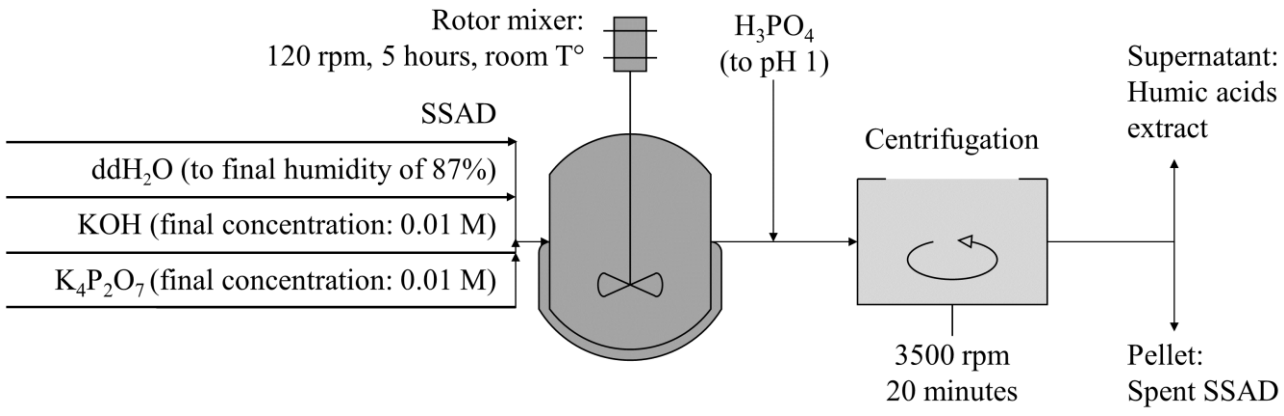
5.2.1 Sampling of anaerobic digestate from sewage sludge (SSAD)

The exploited SSAD was sampled from a large wastewater treatment plant (WWTP), located in Santiago, Chile (population equivalent: 3.7 million people; capacity 8.8 m^3/s). Sewage sludge from primary and secondary treatments was thickened, anaerobically digested, and dewatered through centrifugation to obtain a solid SSAD (26.13% dry matter). After sampling, SSAD was stored at 4°C, until further use. Heavy metal characterization was performed according to the methods described in *Chapter 2*, and revealed the following values: Cu: 410 ppm; Zn: 920 ppm; Pb: 39 ppm; Cr: 113 ppm; Cd: 1.8 ppm; Ni: 37 ppm; As: 2 ppm; Hg. Cr^{6+}: <0.1 ppm.

5.2.2 Experimental set-up

An extract of humic acids (HA), was obtained from SSAD adapting the protocol of humic substances (HS) alkaline extraction from soil (Stevenson, 1994). The scheme of the implemented process is reported in Figure 5.1. The solid SSAD was firstly mixed with water to reach a humidity of 87% in order to obtain an adequate liquefaction of the digestate. Successively, KOH and $K_4P_2O_7$ were added to reach a final concentration of 0.01 M each. Next, a steady and slow mixing was carried out for five hours at room temperature with a homogenizing mixer equipped with a paint mixing drill bit. Subsequently, the mixture was neutralized to pH 7 with H_3PO_4 6M. Finally, a centrifugation (40', 3500 g) was performed to separate humins (pellet) and the HA extract (supernatant). HA extract was stored at 4°C until further use.

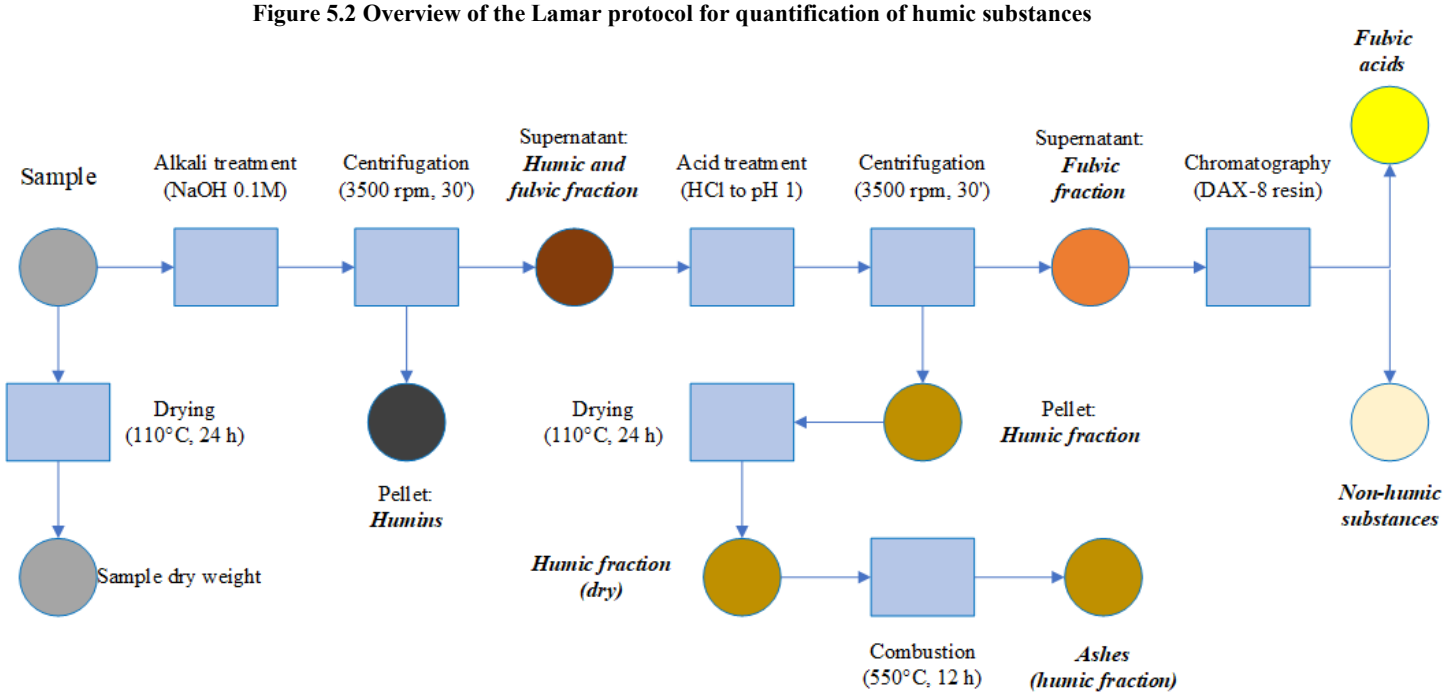
Figure 5.1 Scheme of the process for extraction of humic acids from SSAD.



5.2.3 Humic acids quantification

Quantification of humic acids was performed adapting the method proposed by Lamar and co-workers (Lamar et al., 2014) on the analysed matrixes, that are SSAD and HA extract. Quantification was carried out as well on a powder of commercial HA as benchmark reference. The process is described in Figure 5.2. Briefly, part of the sample was dried until constant weight for the determination of dry weight percentage, and part was treated with alkali. After centrifugation, humins were separated as pellet, while the supernatant included humic and fulvic fractions. The pH of the supernatant was then dropped to 1 with HCl to make the humic fraction precipitate. After centrifugation, the humic fraction was oven dried to get its dry weight. Humic acid amount and concentration was calculated as the

Figure 5.2 Overview of the Lamar protocol for quantification of humic substances



portion of the sample burnt after calcination (Humic fraction dry) minus ashes. This value was then related to starting samples to get the working concentrations.

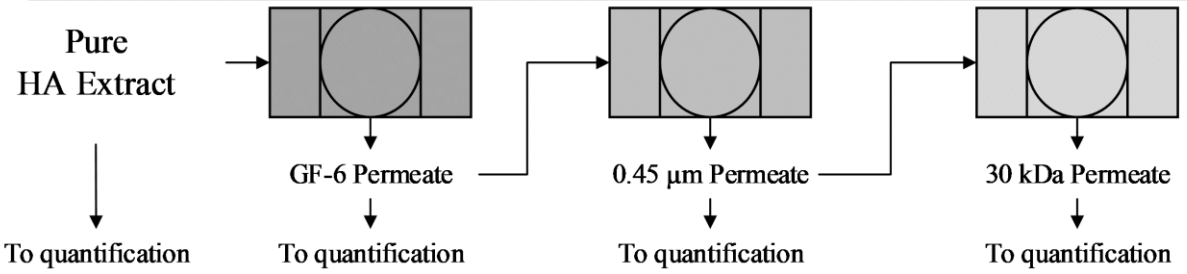
5.2.4 Membrane filtration for humic acids size characterization

Membrane filtration were exploited to study molecular size distribution of humic acids in the HA extract. To this aim, HA extract was submitted to three consecutive filtrations using membranes with a progressively smaller cut-off. The membrane filtration process was designed as reported in Figure 5.3, using three different modules: i) filtration set-up with Buchner flask (aspired with vacuum pump) and Buchner funnel equipped with GF-6 filter paper (Macherey-Nagel); ii) filtration set-up with Buchner flask (aspired with vacuum pump) and Buchner funnel equipped with 0.45 µm membrane (Membrane Solutions); iii) MilliporeSigma™ Amicon™ Bioseparations Stirred Cell (pressurized with N_2) equipped with Ultracel 30 kDa ultrafiltration disc (Merck Amicon Bioseparation). Full technical details of filter membranes are provided in Figure 5.3. After each filtration, filtrate was used partially for successive filtration, and partially for characterization of the filtrate. Characterization of the filtrate included total solid analysis, HA quantification and measurement of total organic carbon (TOC) with TOC analyser (TOC-VWS, Shimadzu Corporation).

Figure 5.3 Scheme of the process of the sequential filtrations of humic acids extract.

In the upper part, technical details of the exploited membranes are provided. In the lower part, the different permeates obtained and characterised are specified.

Commercial name	GF-6 Filter	0.45 µm	Ultracel Ultrafiltration Disc
Brand	Macherey-Nagel	Membrane Solutions	Merck-Millipore
Dimension	Ø 110 mm	Ø 47 mm	Ø 25 mm
Cut-off	~ 6 µm	0.45 µm	30 kDa
Material	Paper/Glass Fiber	Mixed cellulose esters (MCE)	Regenerated cellulose
Maximum flux	2.2 ml/min	0.5 ml/min	0.17 ml/min



5.2.5 Electron microscopy and Energy Dispersive X-Ray Spectroscopy

Structures of humic acids aggregates and presence of metal contaminants was evaluated through Field Emission Scanning Electron Microscopy (FESEM, Zeiss

MERLIN, Gemini-II column, Oberkochen, Germany) and Energy dispersive X-ray (EDX) analyses (AZTec, Oxford Instruments, Abingdon, UK). The EDX analysis was performed on a wide area (100 µm x 100 µm) in three different regions of the samples in order to have an average result of the elementary composition. The liquid HA extract was previously dewatered to be analysed by the means of FESEM. Therefore, lyophilization was performed instead of classical thermal drying in order not to compromise the structure of HA. Lyophilization was performed with an IlShin FD5518 Freeze Dryer with the following settings: temperature -60°C, pressure 5 mTorr, time 48 hours. As a result, a lyophilized SA extract with 82% in dry matter was obtained. Commercial HA were also analysed in order to get qualitative information on chemical composition and as a standard of comparison. Prior to FESEM analysis, samples were metalized with chromium.

5.3 Results and discussion

5.3.1 Mass balance of the process and humic acids quantification

Mass balance of the HA extraction process was carried out considering material inflows and outflows. Table 5.1 reports the amount of the different reagents used and products obtained, and the calculations for mass balance, which was closed at 98%. The 2% of losses were ascribable to the SSAD/reagents mix remaining on the walls of the container where extraction took place. In fact, this mix had a slurry consistence and its total recovery was impossible.

Table 5.1 Mass balance of humic acids extraction process.

Inflows			**Outflows**		
SSAD	6000	g	Extract	5390.0	g
H_2O	6060	g	SSAD spent	6500.0	g
KOH	6.766	g			
$K_4P_2O_7$	39.839	g			
H_3PO_4	33.7	g			
Total	12140.31	g	**Total**	11890.0	g
Loss	250.3	g			
Loss (%)	2.1%				
Balance	97.9%				

The application of Lamar method (Lamar et al., 2014) allowed to quantify the HA content in SSAD, HA extract and commercial humic acids. Results are summarized in Table 5.2. HA content in SSAD was 12.53 ± 1.60 % on dry matter basis (d.m.b.). This result was comparable to the results obtained in other works exploiting similar methods of analysis of SSADs, which revealed HA concentrations ranging between 7.33% (Li et al., 2017) and 13.22% (Tang et al., 2018). The HA content in the extract was 26.87 ± 0.35% d.m.b., indicating that the process contributed to an enrichment in humic acids of more than two-folds. The extraction recovery percentage of HA extraction process was calculated as:

$$HA\ Recovery\ (\%) = \frac{Grams\ of\ extracted\ HA}{Grams\ of\ HA\ present\ in\ SSAD} \times 100$$

This value was only 8%, indicating that large majority of the HA was still left in the spent SSAD. Ashes accounted for 0.5% and 40% on wet and dry matter basis, respectively; it has to be pointed out that the amount of ash is dependent on the conditions of extraction and purification (Bruckert et al., 1994). The quantification of HA in commercial HA powder revealed the highest content, estimated in 77.87 ± 1.46% d.m.b., in line with other commercial HA derived from leonardite (Zhang and Ervin, 2004). Despite the HA% of the extract was lower than commercial HA, it is important to underline that normally HA are extracted from non-renewable resources such as peat, lignite and leonardite, while in this case HA came from a waste.

Table 5.2 Characterisation of SSAD, humic acids extract and commercial humic acids.

For each material, dry matter, total solids, ashes and humic acids content are specified. Also, Percentage of HA recovery and HA enrichment are indicated for HA extract.

Parameter	Sewage sludge anaerobic digestate	Humic acids extract	Commercial humic acids
Dry matter (%)	26.13%	1.13%	83.95%
Total solids (g/L)	-	10.7	-
Ashes (%, dry bases)	-	40.00%	-
Ashes (%, wet basis)	-	0.50%	-
Humic acids (% dry matter)	12.60%	26.87%	77.10%
Humic acids (g/L)	-	3.01	-
Recovery of HA (%)	8.10%		
HA enrichment (on dry basis)	2.10		

In the scientific panorama, the extraction of humic acids from different sources following the conventional procedures (Schnitzer, 1982) is a quite common practice. In fact, there are numerous examples of HA extraction from non-renewable sources (*e.g.* coal; Proidakov, 2009) as well as from different feedstocks generating from wastes and bio-based by-products. Within these, successful HA extractions have been documented on vermicompost and sapropel slurry (Promtov et al., 2016), municipal solid waste (Jindo et al., 2012), liquid swine manure (Brunetti et al., 2007), wood waste compost (Fukushima et al., 2009) and of course sewage sludge, both fresh (Li et al., 2014a) and anaerobically digested (Li et al., 2014b). However, most of these processes focused more on the general obtainment of humic acids for subsequent applications than on the implementation of the extraction process. In fact, in most of cases humic acids were not quantified and, thus, this made difficult to find differences and analogies with other works. Moreover, the Lamar method is quite recent, and it is not exploited yet in many studies, making even more difficult a perfect comparison. For instance, one of the

few comparable studies was the one conducted on the extraction of humic acids from vermicompost, where the authors managed to recover 2.5 grams of HA from 1 kg of fresh vermicompost (Arancon et al., 2006). These results were quite in line with the ones obtained in the present study, where ~3 grams of HA were extracted from 1 kg of fresh dewatered SSAD. Furthermore, particular relevance was showed by the experimental campaigns conducted by Huan Li's group for the similar approach with the present study. They worked extensively on HA extraction from different kind of sewage sludge (as mentioned above) in order to obtain a liquid extract utilizable as biostimulant. Data reported in their studies showed higher HA recovery yields (~25%), which were may be due to higher reagent concentration (NaOH 0.1M)(Li et al., 2014b). Moreover, they registered a HA concentration of 1.5 g/L after extraction (Li et al., 2009), which was halved than the one measured in the present study. Also, they reported the results of HA concentration after an ultrafiltration step performed to obtain a more concentrated solution, whose values ranged between 2.97 g/L and 3.78 g/L. Interestingly, these values were fairly similar to the ones obtained in the present study in absence of ultrafiltration, but they anyway suggested that filtration with membranes was an aspect deserving attention to improve the peculiarities of the final product.

5.3.2 Membrane filtration processes

As regards membrane separation processes, it is worth specifying that this process was designed with a duplex aim. The first was the study of the molecular size distribution of the purified humic substances during extraction. The second was the individuation of one or more filters to separate HA from other components of the extract, with the purpose of increase purity and concentration of the final product, as discussed in the previous paragraph.

As reported by Steelink (2002), several techniques have been exploited to elucidate size and shape of HA, such as sedimentation, size exclusion chromatography (SEC), light scattering and many others; however each approach shows pros and cons, revealing a broad range of molecular weight values. Hence, the purpose of this study was not to investigate the theoretical molecular size distribution of humic acids and their chemical moieties, as described by Shin and co-workers (1999) on commercial humic acids. Instead, the goal was to adopt an engineering approach allowing to get a general characterization on one side, and to propose a potential process to get a purer and more concentrated product. For this reason, commercial membranes were used to design the filtration process. Results of membrane filtration are reported in Table 5.3, which shows the concentrations of total solids (TS), ashes, HA and TOC in the HA extract and the three permeates. In parallel, Table 5.4 shows the estimation of solid retention and permeation at each step, calculating the various contributes based on 1 litre of HA extract (10.7 grams of total solids).

The three consecutive filtrations showed retention effects over total solids, humic acid content and total organic carbon. Focusing on humic acids content, it emerged that roughly half of humic acids were retained already after first filtration

and the second half by the second one. This aspect should lead to the conclusion that humic acids tended to form very big complexes blocked by larger pores of GF-6 and medium sized ones of 0.45 µm membrane. As previously stated, information about molecular size of HA is not homogenous, but researchers commonly agree that their MW ranges between 2 and 1300 kDa (De Melo et al., 2016), and some studies estimated hydrodynamic radius of humic acids particles between 2 (Kawahigashi et al., 2005) and 110 nm (Wershaw et al., 1967), while other authors report the formation of HA aggregates with a mean particle diameter of 500 nm (Ghabbour and Davies, 2014). On the basis of these data, two different scenarios were possible. In the first case, the particles of extracted HA formed even bigger aggregates (up to 1,000 µm) as reported by (Klučáková, 2018). Another conceivable hypothesis was the effect derived from residual polyelectrolyte inside HA extract.

Table 5.3 Characterisation of HA Extract and of the three permeates after filtration.

For each sample, total solids, ashes, humic acids and total organic carbon are specified. Moreover, retention percentages are specified for each parameter with respect to the previous permeates obtained.

		Pure extract	**GF-6 permeate**	**0.45 µm permeate**	**30 kDa permeate**
	Dry matter (%)	1.13%	0.88%	0.64%	0.61%
	Total solids (TSS) (g/L)	10.7	8.32	6.03	5.74
TSS retention	vs pure extract	-	22.24%	43.64%	46.36%
	vs GF-6 permeate	-	-	27.52%	31.01%
	vs 45 µm permeate	-	-	-	4.81%
	Ashes (dry matter basis) (g/L)	4.52	4.50	4.39	4.30
Ashes retention	vs pure extract	-	0.54%	2.80%	4.87%
	vs GF-6 permeate	-	-	2.27%	4.35%
	vs 45 µm permeate	-	-	-	2.13%
	Humic acids (% dry matter)	26.87%	14.46%	n.a.	n.a.
	Humic acids (g/L)	3.01	1.52	0	0
Humic acids retention	vs pure extract	-	49.50%	100.00%	100.00%
	vs GF-6 permeate	-	-	100.00%	100.00%
	vs 45 µm permeate	-	-	-	-
	TOC (g/L)	3.32	2.02	0.81	0.71
TOC retention	vs pure extract	-	39.16%	75.60%	78.61%
	vs GF-6 permeate	-	-	59.90%	64.85%
	vs 45 µm permeate	-	-	-	12.35%

Table 5.4 Distribution of total solids in 1 liter of HA extract.

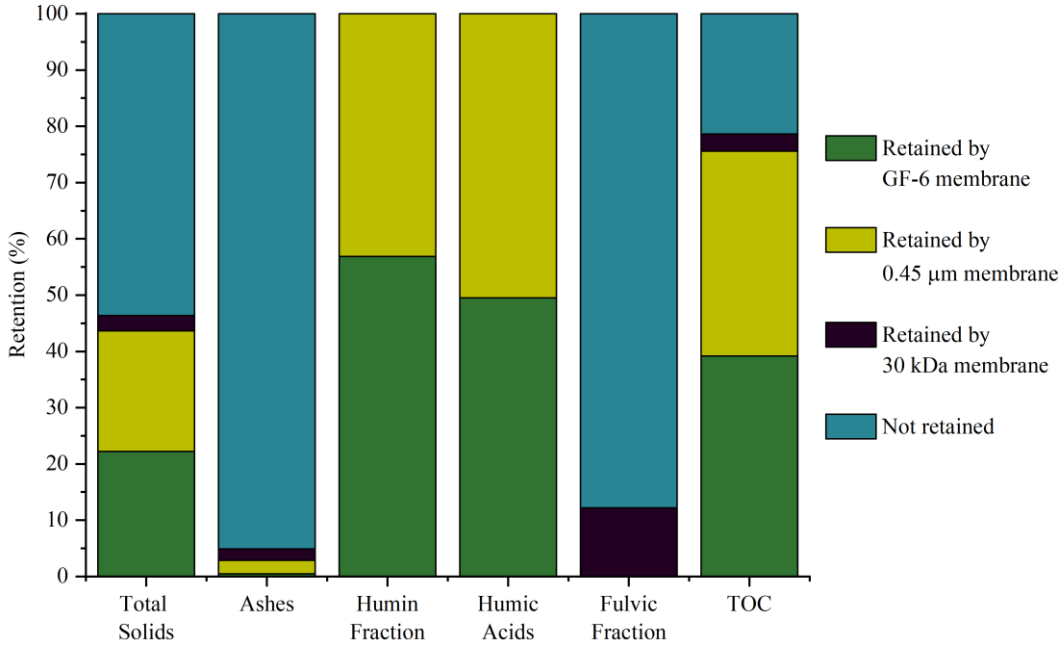
The table reprise the measured values of the different parameters in both HA Extract and the three permeates. Moreover, the values not available from experimental data were estimated by calculation and/or considering mean elemental composition of humic substances according to Steelink (1985) and (Sparks, 2003). Values in italic indicates the estimated values. *TOC value of 0.45 µm permeate was attributed totally to compounds belonging to fulvic fraction, which were considered not retained by the GF-6 and 0.45 µm membranes.

	Pure extract	GF-6 filtrate		0.45 µm filtrate		30 kDa filtrate	
		Retained	Permeate	Retained	Permeate	Retained	Permeate
Total solids (g)	10.70	*2.38*	8.32	*2.29*	6.03	*0.29*	5.74
Ashes (d.m.b.) (g)	4.52	*0.02*	4.50	*0.11*	4.39	*0.09*	4.30
Organic compounds (g)	*6.18*	*2.36*	*3.82*	*2.18*	*1.64*	*0.20*	*1.44*
HA (g)	3.01	*1.49*	1.52	*1.52*	0.00	*0.00*	0.00
Organic - non HA (g)	*3.17*	*0.87*	*2.30*	*0.66*	*1.64*	*0.20*	*1.44*
Humin fraction (g)	*1.53*	*0.87*	*0.66*	*0.66*	0.00	*0.00*	0.00
Fulvic fraction (g)	*1.64*	*0.00*	*1.64*	*0.00*	*1.64*	*0.20*	*1.44*
TOC (g)	3.32	*1.30*	2.02	*1.21*	0.81	*0.10*	0.71
TOC - HA (g)	*1.66*	*0.82*	*0.84*	*0.84*	*0*	*0*	0
TOC - non HA (g)	*1.66*	*0.48*	*1.18*	*0.37*	0.81	*0.10*	*0.71*
TOC - Humin (g)	*0.85*	*0.48*	*0.37*	*0.37*	*0.00*	*0.00*	0.00
TOC - Fulvic fraction (g)	0.81*	*0.00*	0.81*	*0.00*	0.81*	*0.10*	*0.71*

Cationic polyelectrolyte is a high MW polymer used at WWTP level to enhance SSAD dehydration via centrifugation. Interaction between humic acids and cationic polyelectrolyte has been already described by Kam and Gregory (2001), and it is quite plausible that it took place also in the present study, giving rise to a macromolecular network bulky enough to be retained even by GF-6 membrane. This hypothesis was furtherly supported by the experimental observation of the formation of a layer above the GF-6 and 0.45 µm membranes, with a jelly consistence (presumably formed by water molecules trapped inside polymer chains) and a brownish colour (presumably due to humic substances). Further considerations can be deduced from the trend of TOC along with the filtration steps. Starting from TOC of HA extract, it is possible to affirm that ~50% of TOC can be ascribable to humic acids, considering the mean elemental composition of HA (C ≅ 55%) according to Steelink (1985). Another interesting data was the TOC content in the 0.45 µm permeate (0.81 g/L), which was considered totally due to the fulvic fraction (also known as "acid soluble fraction", formed by fulvic acids and non-humic compounds (Chow et al., 2006)) still present in the HA extract. According to mean elemental composition of fulvic fraction (Steelink, 1985), it was possible to estimate its contribute in terms of total solid, that is 1.64 g (over 1 L of extract). Considering this result, there was still a quote of TOC to justify, which may be ascribable to residual humins present in the HA extract. HA extraction protocol did not reach extremely alkaline pH (9.5 – 10) to avoid potential deterioration of humic substances; on the other hand, this precaution may did not induced the complete precipitation of humins (insoluble at pH>13 (Tuhkanen and Ignatev, 2019)), negatively influencing the purity of final HA extract. Furthermore, it must be taken into account that, even when extraction conditions are kept more stringent, part of the non-humic organic material (*e.g.* cell material) is solubilized and is still present in the extract (Tuhkanen and Ignatev, 2019). In the present study, these contributes were included in the quote of humins and fulvic fraction. On the basis of this deduction, part of total solids in the extract was due to humins, which could be estimated from the calculated quote of humins-TOC (0.85 g/L) and from their mean elemental composition (according to Sparks (2003)). In this way, humins content should be likely 1.53 g (over 1 L of extract); hence, the sum of the contribute of all organic components (humins, humic acids and fulvic fraction) to TSS was likely around 6.18 g (over 1 L of extract). The reliability of this value was furtherly proved by the difference between measured values of total solids (10.7 g/L) and the ashes value (4.52 g/L) of the pure HA extract: the result was approximatively the same value obtained by calculation. With the same approach it has been possible to evaluate the contributions to TSS, organic compound and TOC also in all the other permeates. To sum up these calculations of mass balance, Figure 5.4 reports the percentage of retention of each component of the mixture per each step. Hence, it was possible to conclude that: i) humic acids were retained half by GF-6 and half by 0.45 µm membrane; ii) humins (usually considered a bunch of really heterogeneous group of partially degraded molecules including lignin, polysaccharides, carbohydrates, proteins and other macromolecules (Calace et al.,

Figure 5.4 Retention percentages of the different components in the mixture of HA extract.

Percentages of retention of total solids, ashes, humin fraction, humic acids, fulvic fraction and total organic carbon along the three different filtration steps.



2007)) were mostly retained by the first filter and less by the second one, probably because of their huge molecular size (Hayes et al., 2017; Pettit, 2004) or their entrapment in polyelectrolyte chains; iii) TOC contributions mostly came from HA and the humin fraction; iv) fulvic fraction was considered undisturbed by first and second filters, but partially retained by the third one; v) ashes value was not really affected by filtration process, indicating that they were mainly made of small inorganic molecules, for instance micro-elements, such as potassium (as K^+) and phosphorous (as PO_4^{3-}) already present in SSAD and further added during the extraction process, whose presence was confirmed by EDX analysis (see section 5.3.3). To a broader extent, the single passages of the described process did not improve HA purity of the extract as obtained from the extraction. However, within the perspective to obtain an added-value product, the 0.45 µm membrane could be a feasible solution for the HA concentration considering that ~100% of humic acids were retained in this step. In this context, particular attention should be deserved to the study of Li and co-workers (2014b), who adopted a homologous strategy to concentrate a HA extract. More in detail, they exploited an ultrafiltration membrane with a cut-off of 50 kDa, which was chosen according to molecular size distribution of HA in their extract. Probably, this could be due to the fact that they used higher alkali concentrations during the extraction (NaOH 0.1M), which one side can improve recovery of HA from SSAD (as mentioned above), but on the other it may induce more easily HA degradation.

Hence, future work should include a more precise characterization of the retentates to evaluate the effectiveness of the HA concentration increase. However, this solution should be applied after the implementation of the extraction procedure, which should find a trade-off between the achievement of higher yields in terms of

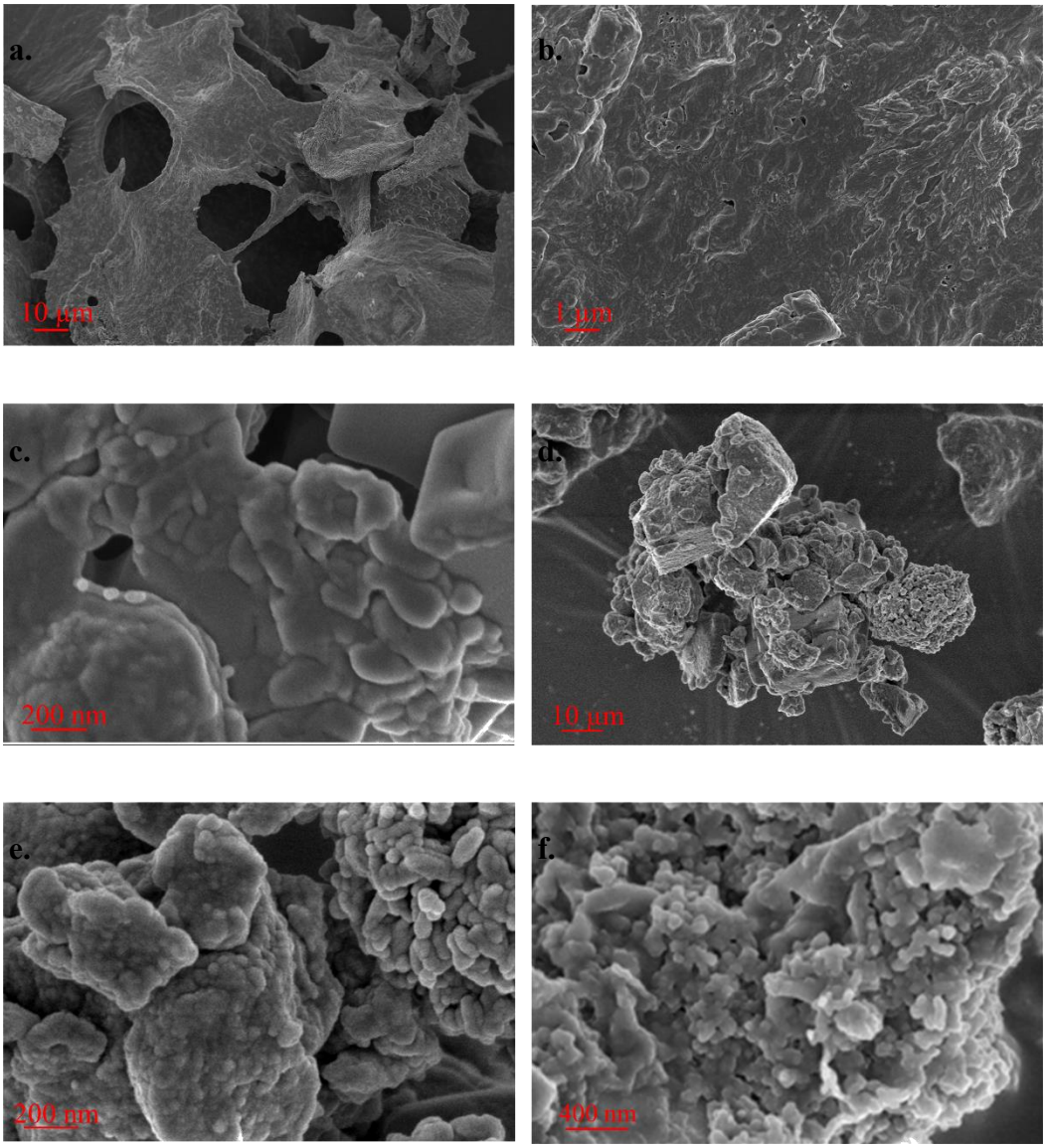
HA, the more efficient abatement of undesired humins and the preservation of HA structure.

5.3.3 Electron microscopy and X-ray spectroscopy

Structure of humic acids present in HA extract and commercial HA was evaluated with FESEM electron microscopy and results are reported in Figure 5.5.

Figure 5.5 Electron microscopy images of lyophilised HA extract, commercial humic acids and humic acids from literature.

a. b. c. FESEM Images of lyophilised HA extract at three different magnification levels (2.5K X, 25K X, 100K X). d. e. FESEM Images of commercial humic acids at two different magnification levels (25K X, 100K X). f. Images of humic acids from literature (Ghabbour and Davies, 2014).



Starting from lyophilised HA extract (Figure 5.5 a. b. c.), at lower magnification levels it can be observed that structures similar to perforated sheets with granular surfaces were present. At higher magnification, it emerged that the grained surface was due to the presence of grapes-like spherical structures, which tended to clump together forming bigger clusters. These observations were quite in

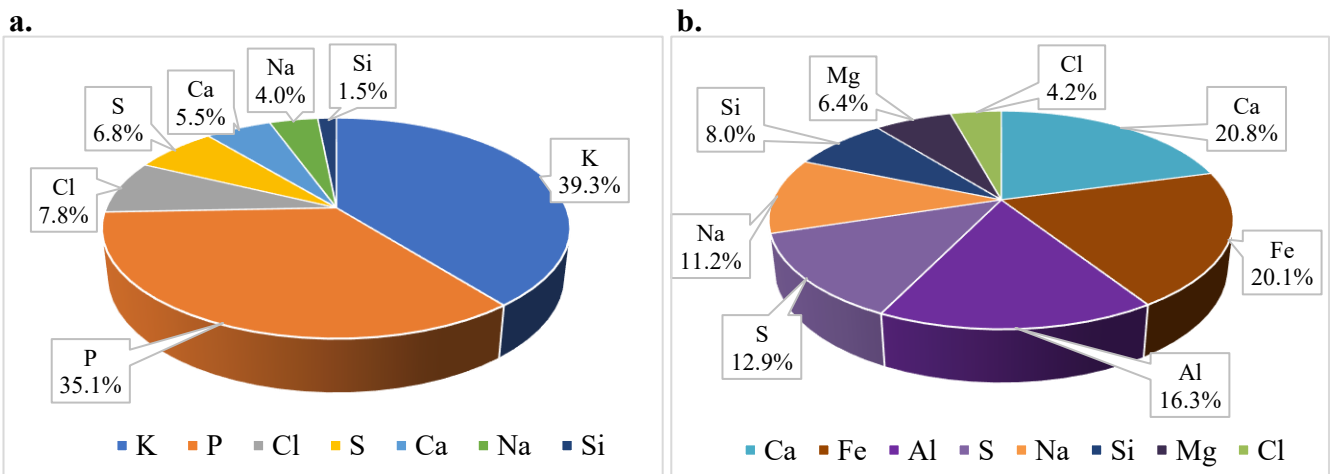
agreement with the studies of Tan (1985) on electron microscopy of humic acids. As regards commercial HA (Figure 5.5 d. e.), it appeared formed by fibrous spheroids at low magnification. This difference with HA extract may be justified by the different nature of the samples. Indeed, commercial HA were formulated as a powder likely obtained by extraction from leonardite and subsequent precipitation. On the other hand, HA extract was lyophilised for FESEM analysis. Tan and Lobartini explained in different works (Lobartini and Tan, 1988; Tan, 1985) that different factors influence the structure of HA, including strategy of sample preparation (drying, freezing) and pH.

As a result, HA can assume different forms and shapes, including spheroids, sheets interwoven by fibres, perforated sheets, bladed or flattened filaments, and solid shredded sheets. On the other hand, at higher magnifications, more similarities could be appreciated between the structures of commercial HA and HA extract, with the presence of the grapes-like structures coalescing together. As a further confirmation, these images were quite similar to the ones already present in published information on humic acids structure investigate via electron microscopy (Ghabbour and Davies, 2014). Further comparisons with other works in literature about humic acid structure with this technique resulted quite difficult since in recent times the main interests of the scientific community about HA shifted on other topics. In fact, the majority of papers of last years dealt with analysis of: i) configuration of soil particles combined with HA (Yang et al., 2020); ii) general characterisation of catalysts adsorbed on HA as support (Thangaraj et al., 2019), or HA adsorbed on inorganic support (Giasuddin et al., 2007); iii) detection of HA on devices for HA removal environmental samples (*e.g.* membrane filtration of water for removal of HA) (Law Yong et al., 2016).

The Energy Dispersive X-ray spectroscopy (EDX) analysis on lyophilised HA extract and commercial HA (Figure 5.6) was performed excluding the contribute of carbon, nitrogen and oxygen because of high errors associated with their low atomic weight. Taking into account that these three elements are commonly the principal components of humic substances, this analysis was carried out with the purpose of

Figure 5.6 EDX analysis of contaminants present in HA extract and commercial HA.

Pie diagrams indicates the distributions of weight percentages of elements detected with EDX, excluding C, H, O, N. a. HA extract. b. Commercial HA.



evaluate the contaminants present in each sample. As regards HA extract, K and P were the two elements with the highest concentration; their presence was likely attributable to the reactants used during the humic acids extraction procedure; minor amounts of Ca, Mg, S, Na and Si were registered. Conversely, no heavy metals were detected; in fact, Cu, Zn, Pb, Cr, Cd, Ni and As were present in the "raw material" (*i.e.* SSAD) used as input for the process. Hence, the extraction procedure probably helped in getting rid of them, maybe for the alkaline conditions applied, which negatively impact on metals solubility.

For what concerns commercial humic acids, the scenario of contaminants was a little bit more complex: Ca and Fe were the most concentrated, followed by Al, S and Na; lower concentrations in Si, Mg and Cl were found. In this case, the differences in the amounts of the different heavy metals were less pronounced; probably, the detected contaminants might be ascribable to the ones natively present in the raw material used production of HA (*i.e.* leonardite).

Thus, all the detected chemical species in the HA extract were more "plant-friendly" than the ones found in commercial HA, making it a good candidate for its utilisation as biostimulant. However, further study are still necessary to concentrate the extract get higher HA concentrations, useful for agronomic applications (~ 30 g/L; Li et al., 2009) and to assess the effective concentrations of potential toxic heavy metals by the means of more sensible techniques, such as inductively coupled plasma mass spectrometry (ICP-MS).

5.4 Conclusions

Humic acids are interesting compounds with various functions, behaving not only as well-known soil improvers in agricultural applications, but also as promising molecules in other sectors (De Melo et al., 2016). Natural sources of humic acids (*i.e.* lignite, perlite and leonardite) are non-renewable and their overexploitation will be no longer sustainable in the future. SSAD is a waste with nice contents in humic acids and represents a valuable source of these compounds (Liu et al., 2020). Many works in literature have dealt with HA extraction from this "feedstock", obtaining successful results (Li et al., 2014b). The present study reprised this approach and reported the achievement of a HA extract from SSAD, with a HA concentration higher or comparable to the ones reported in other works in literature. The HA extract turned out to be enriched in HA, showing a HA concentration (on dry matter basis) of ~27%, doubled with respect to the starting SSAD. However, percentages of recovery were low (8%) and are surely an aspect to improve in future work. Moreover, this work demonstrated also that the official protocol for humic substances quantification, implemented by Lamar and co-workers (2014), could be successfully adapted to SSAD, giving more reliable results than the traditional protocol and without the use of harsh chemicals. Furthermore, membrane filtration processes helped to understand the size distribution of the particles still present in the HA extract; an important information emerged from this experimental part was the effectiveness of the 0.45 μm membrane in total retention of humic acids. Hence, it may be exploited in future

work to increase the HA concentration and to reach peculiarities satisfying market requirements (~ 30 g/L; Li et al., 2009) for its use as is or as a "building block" for more advanced biostimulants formulations. Finally, electron microscopy analysis allowed to unravel the structure of purified humic acids, whose particles with globular surfaces were comparable to the ones published in other works; the concomitant EDX analysis did not reveal heavy metal presence, suggesting that the extraction process likely contributed to get rid of these toxic compounds.

As mentioned above, future work should include the improvement of process in order to increase recovery percentages of HA from SSAD, investigating the effects of humidity of the mix, timing, and reagents concentrations on the extraction. A possible work strategy is the one exploiting the design of experiments, in which the three above-mentioned parameters are investigated in a full factorial design. This should lead to the optimization of the values which can maximise recovery yields in this process. Moreover, higher attention should be devoted to engineering aspects, since the technical details of extraction apparatus have proven to have relevance on final yields (Promtov et al., 2016). In any case, complete characterization of the final product should be carried out to assess humic acids presence and morphology. To this aim, further analysis can be carried out to further elucidate their chemical structure, such as Fourier Transform Infrared Spectroscopy (FTIR) or Nuclear Magnetic Resonance (NMR). Another aspect to be considered in future work is related to the safe handling of carbonaceous compounds. In fact, self-combustion of coal is an example of potential flammability of these chemical species, if proper ventilation and stocking standards are not satisfied. Fortunately, the "raw material" used in this study (SSAD) is not flammable, but in any case these observation hint that more research is needed on this aspect for the humic acids obtained from the purification process, especially for the risk of dust explosion in those process for their formulation as powder.

In conclusion, these studies should pave the way for the future market of fertilizers and biostimulants, demonstrating that sustainable production of humic acids in a circular economy approach is possible and feasible.

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Chapter 6

Final remarks and future perspectives

The work illustrated in the present thesis addressed two main environmental issues of nowadays world, that are management of waste (*i.e.* sewage sludge) and the problem of depletion of organic matter and nutrients in degraded soils. A wise man said that "Solutions are problems, and problems are solution". This approach did not only sound as a *mantra*, but was also an effective strategy to come up with new ideas on these issues. Indeed, a common solution may be found: using the waste to ameliorate soils' peculiarities. Within this panorama, many researchers argued a lot about the benefits and the drawbacks of this practice. On one hand, some claimed that sewage sludge shows interesting agronomic peculiarities and its applications is a feasible solution to sustain the turnover of the elements, without the use of artificial fertilizers. On the other hand, others demonstrated that long-term application poses serious risks of soil accumulation of the organic and inorganic pollutants present in sewage sludge. Still others think that application of sewage sludge on degraded soil is a practice which, in the trade-off of pros and cons, can ameliorate soil qualities. Anyway, agricultural reuse of sewage sludge is a controversial theme and the debate is still open. An interesting perspective on use of sewage sludge has opened in recent times, which proposes a shift from the direct reuse to recycle. In other words, this is the exploitation of sewage sludge as raw matter for the extraction of added values compounds.

In this complex scenario, the work of this thesis was conducted with the purpose to further investigate "lights and shadows" of the effects deriving from soil application of sewage sludge under a chemical and biological profile. More in detail, the aims of the work were substantially four: i) the chemical characterization of an anaerobic digestate from sewage sludge (SSAD), to evaluate its potentialities as fertilizer; ii) the evaluation of the fertilizing and phytotoxic effects of SSAD on the growth of cucumber plants on a nutrient-poor soil; iii) the study of the bacterial communities dwelling in rhizosphere of tomato plants grown in a poor soil and

treated with SSAD; iv) the implementation of a protocol for extraction of humic acids from SSAD.

The results of these experimental targets brought interesting insights. As expected, SSAD revealed a really interesting composition from an agronomic point of view, with a marked richness in organic matter and macro-nutrients. Its application on sandy soil induced more benefits than harm on growth of cucumber plants, which displayed higher biomasses and better physiologic parameters than untreated ones. Moreover, these effects occurred at an intermediate dosage of SSAD (170 kg N/ha), suggesting that sustained use was not required. As concerns microbial communities, bacteria of tomato rhizosphere were influenced by treatment with SSAD, showing not only that differently processed SSADs had an effect on the shaping of soil microbiome as evidenced by the study of the taxonomy and ecological parameters, but also that its application induced a higher presence of Plant Growth Promoting Bacteria (PGPB). Another relevant aspect emerging from this analysis was the importance covered by the soil addition of organic matter in boosting microbial activity. This can be considered the linkage with the last part, that is the extraction of a valuable part of organic matter of sewage sludge, which were humic acids. The implemented process demonstrated that this practice is possible and may help in getting rid of toxic molecules such as heavy metals.

Considering these results, it can be reasonably affirmed that the outcomes are encouraging, but…we just scraped the surface. Indeed, future work should address different aspects. Firstly, a deeper characterization of SSAD should be carried out, especially with ecotoxicological assays which turned out to be very effective in the evaluation of toxicity of complex matrices. As regards the effects of SSAD use for fertilizing purposes, a systematic approach should be adopted to study the effects of SSAD on soil and plants. More precisely, soil characterization should be conducted before and after treatment application to highlight the effects of treatment, both under a chemical and microbiological profile. This strategy should lead to important findings, such as the effective soil accumulation of desirable and undesired substances, the understanding of releasing dynamics of different nutrient, and the relationship between chemical features and bacterial communities. Finally, the humic acid extraction should be further implemented to get higher recovery percentages. This could open different interesting scenarios such as the study of process scale-up to evaluate the production on industrial level, or the research on more technologically advanced formulations, ensuring blending with other nutrients and controlled release.

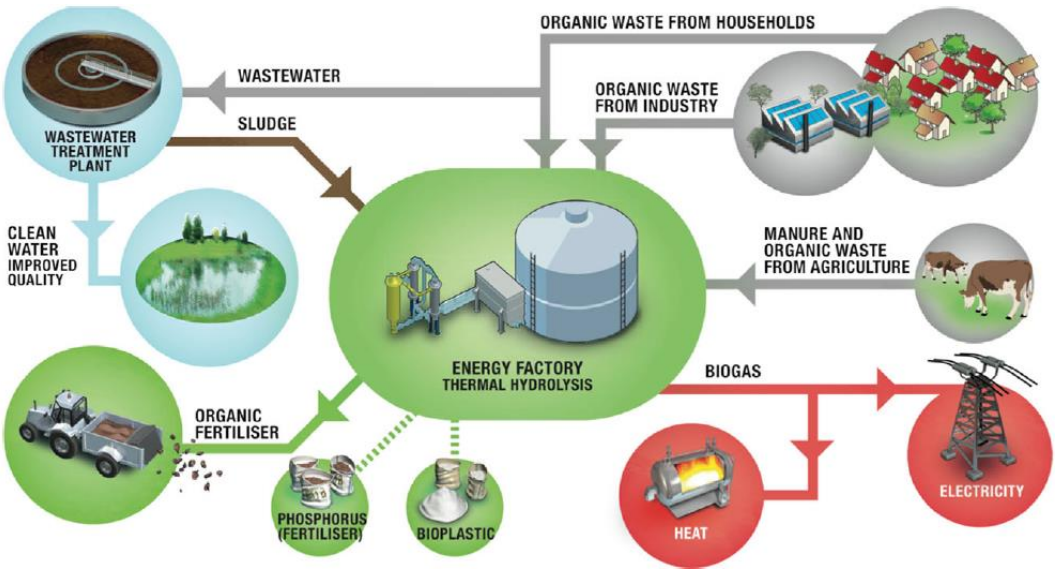
Future perspectives are quite diverse. With regards to sewage sludge, surely further studies are required to improve the sludge stabilisation processes to reduce its production. The land use of sewage sludge in agriculture will presumably be an increasingly less adopted practice since its application may pose long-term risks on chemical and biological soil features. Well, it must be highlighted that also unwise usage of common commercial fertilizers induces negative effect on soil as their intensive use is a driver of soil degradation. Nevertheless, the words “waste” and “toxicity” linked to sewage sludge sound bad to nowadays society, discouraging the agricultural exploitation. But, if one side pessimistic peoples only hear the

words “waste” and “toxicity”, forward-looking ones prefer to catch in sewage sludge “opportunity” and “resource”. “Opportunity” is referred to the possibility of sewage sludge use to boost initial fertility of poor soils, giving the chance to bring new life to soils unproductive and degraded. This make even more sense if we take into account all those contexts in which the supply of soil improvers is not affordable. Of course, this practice should be accompanied by clever policies facing the soil degradation problem in an integrated manner. These ones shall be aimed on one side to the safeguard of soils peculiarities (*e.g.* constant monitoring), on the other to the provision of feasible land use procedures directed to preserve health and biodiversity of soils (*e.g.* Conservation Agriculture). “Resource”, instead, suggests that sewage sludge should be considered, from here on out, a raw matter for the extraction of numerous added-value compounds. In this work, the extraction of humic acids was explored, but numerous studies demonstrated that is possible to recover other compounds, such as struvite, a mineral rich in phosphorous and nitrogen with fascinating agronomic characteristics.

The panorama described above is totally in line with two concepts which are driving many research interests of this century. The first is surely the “circular economy”, defined as a wise strategy to recover and reuse wastes to obtain valuable commercial products. The possibility to decline this concept also in the case of sewage sludge and, more in general, of stools is feasible and many ideas are coming up, not only related to soil application as described in this work. For instance, the journal *Nature* dealt recently with the topic of “The new economy of excrement”, highlighting how this waste can be a promising new business in the near future (Wald, 2017). On the other hand, another overwhelming concept is the one of “biorefinery”. Indeed, the function of the wastewater treatment plant in the future may evolve from the mere “waste management facility” to “a place where the by-

Figure 6.1 A valuable example of biorefinery concept.

The scheme represents the approach adopted in the case of Billund (DK). The conventional WWTP has been extensively evolved, being integrated in a more complex network to carry out different productive process, all relying on the valorisation of different kind of organic wastes (Nielsen, 2017).



products of a treatment process become the inputs of a productive line". This vision is not just an idea, but it is already a consolidated reality in some contexts, for instance in the case of Billund (Denmark), as reported in Figure 6.1, indicating that processes based on microbial biotechnology will likely cover a prominent position in the evolution of biorefinery concept (Nielsen, 2017). Hence, researchers and engineers are expected to play a key role to guarantee successful achievements in this field. In any case, important breakthroughs are required for the production of many commercial goods of the future, including fertilizers and soil improvers, which should adopt sustainable strategies within even smarter circular economy approaches.

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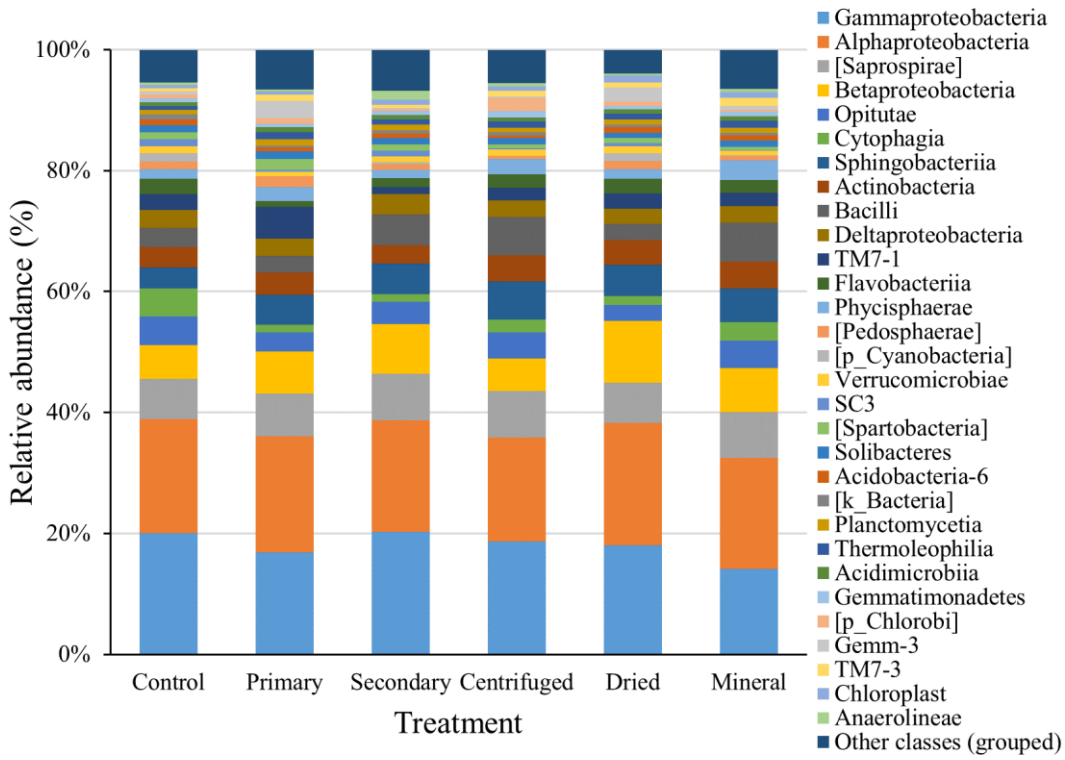
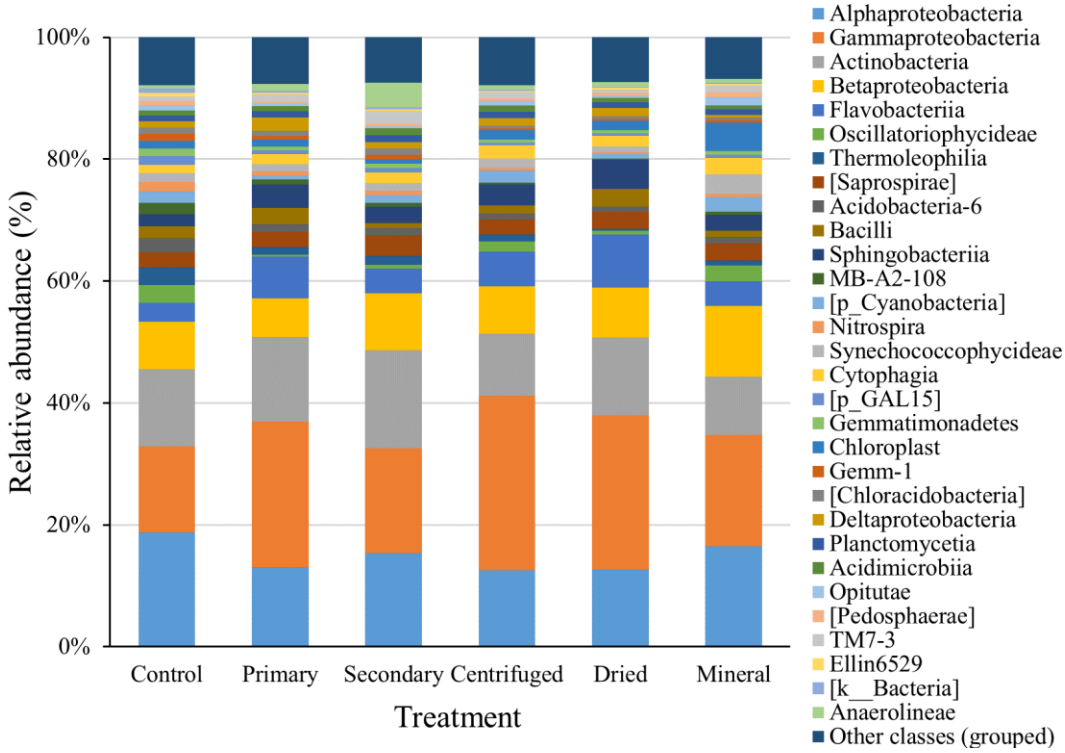
Supplementary material

This section is an appendix to Chapter 4, reporting some extra information about the bioinformatic analysis from the NGS data. In particular, this section contains:

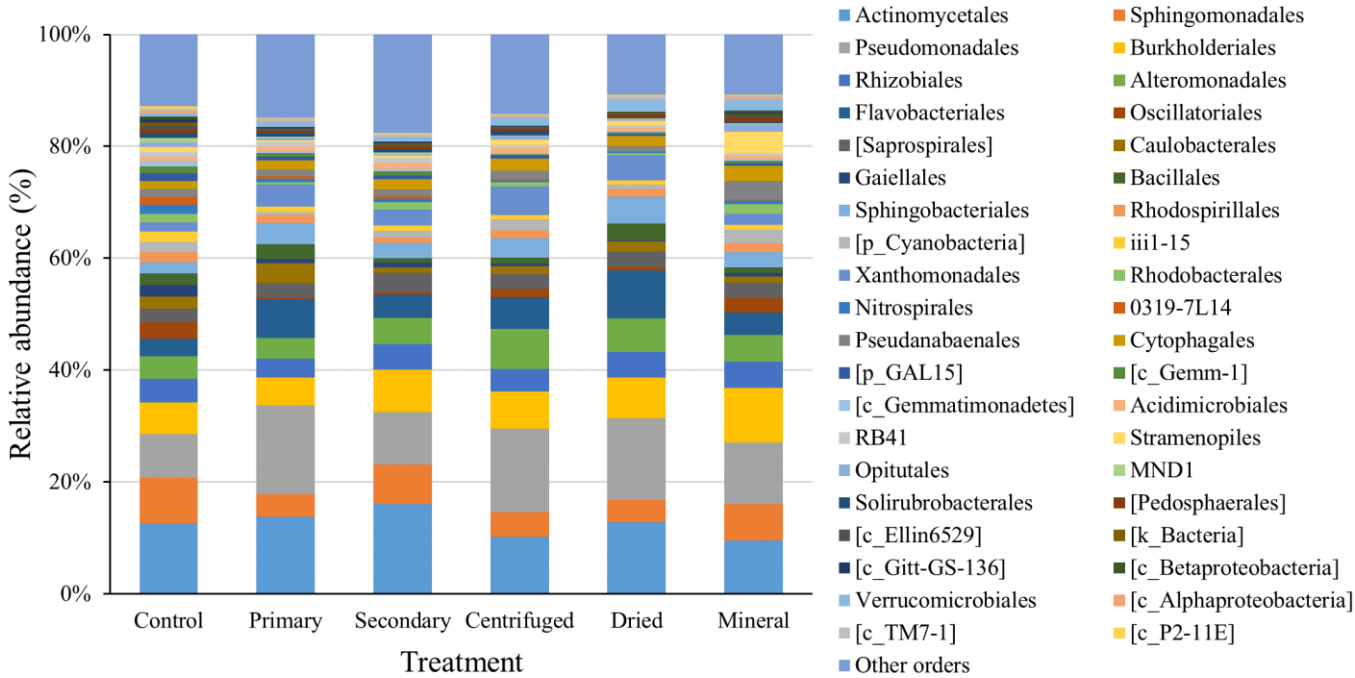
- Taxonomic analysis of the taxonomic levels not reported in Chapter 4 (class, order, and genus).
- Rarefaction curves of the α-diversity indices.
- Scree plots describing the explained variance per each axis in Principal Coordinate Analysis of β-diversity indices.



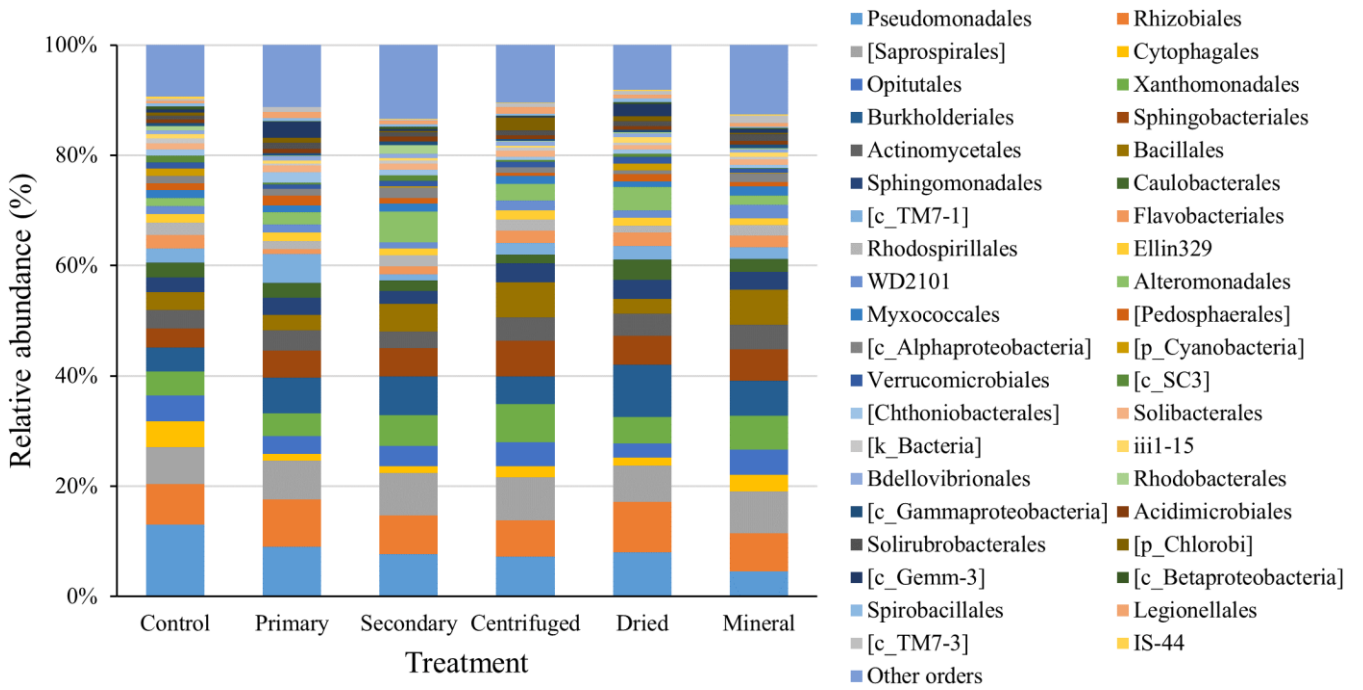
Supplementary Figure S1. Taxonomy at class level of rhizosphere microbial communities from sandy soil.



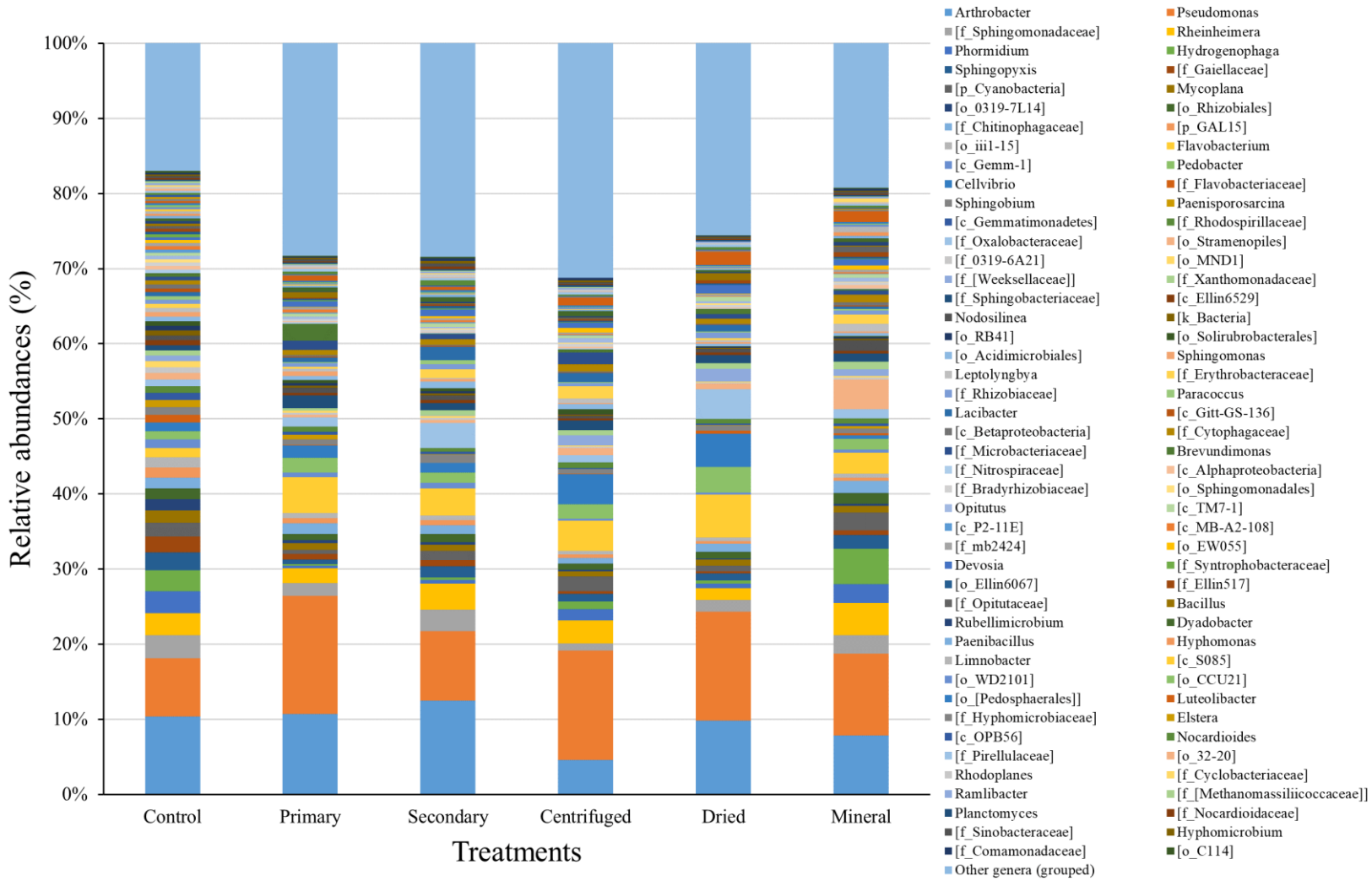
Supplementary Figure S2. Taxonomy at class level of rhizosphere microbial communities from peat substrate.



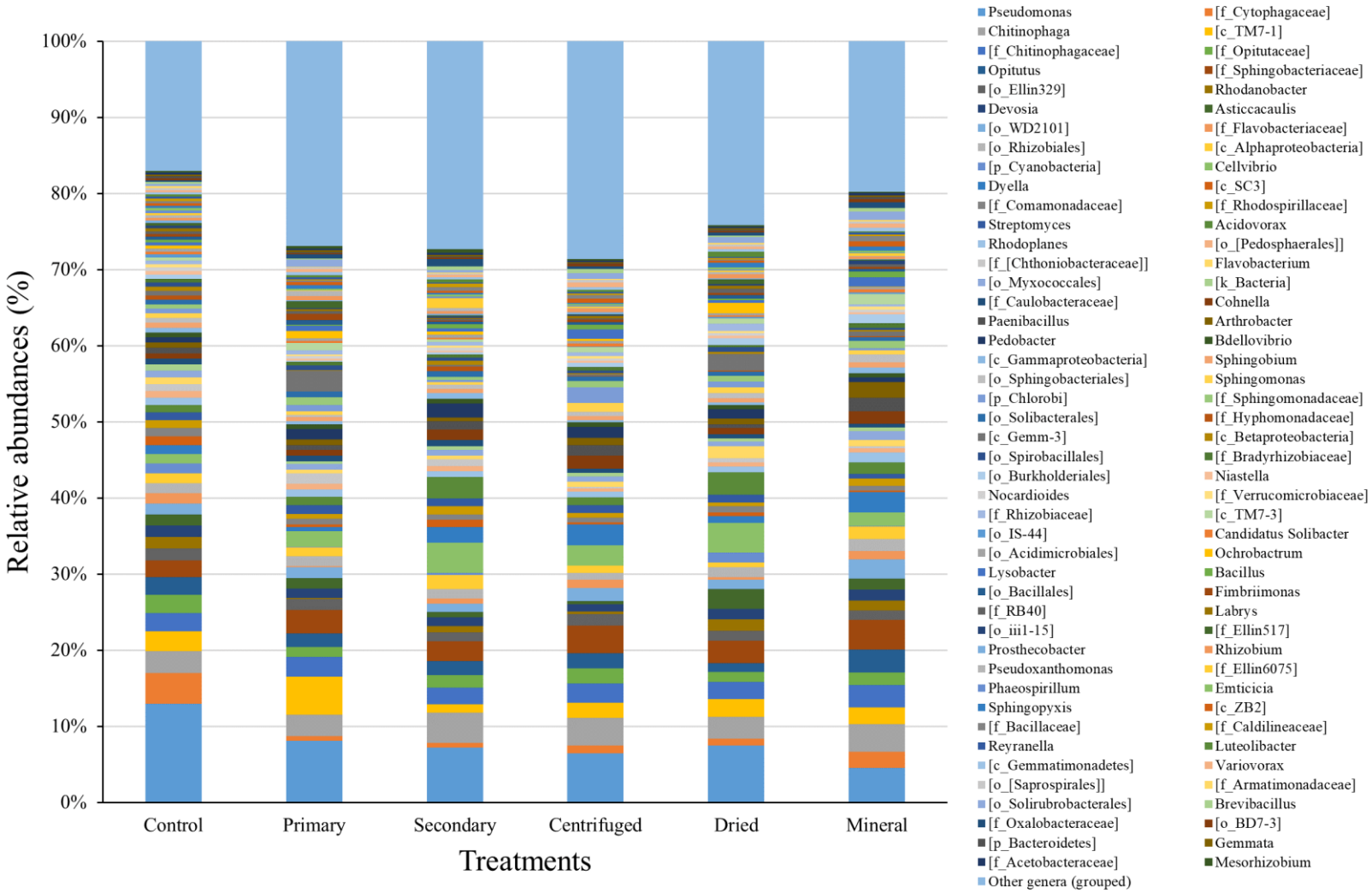
Supplementary Figure S3. Taxonomy at order level of rhizosphere microbial communities from sandy soil.



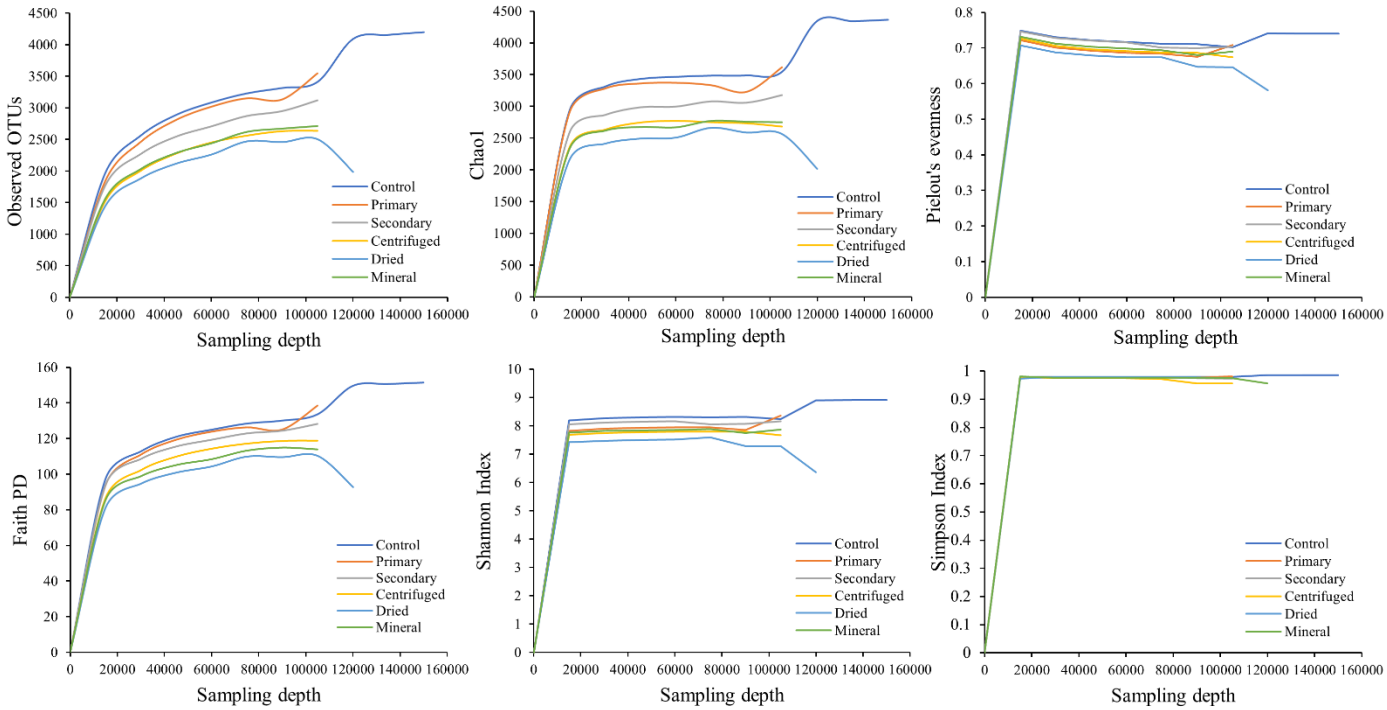
Supplementary Figure S4. Taxonomy at order level of rhizosphere microbial communities from peat substrate.



Supplementary Figure S5. Taxonomy at genus level of rhizosphere microbial communities from sandy soil.

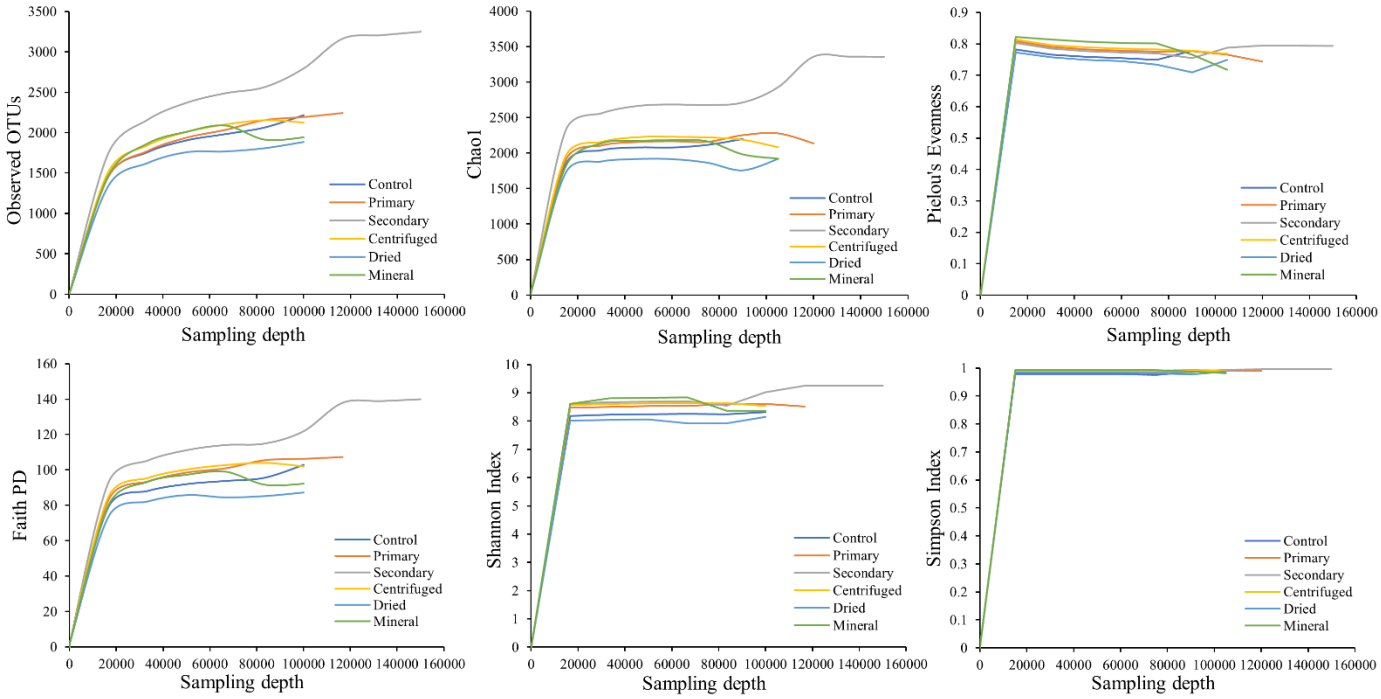


Supplementary Figure S6. Taxonomy at genus level of rhizosphere microbial communities from peat substrate.



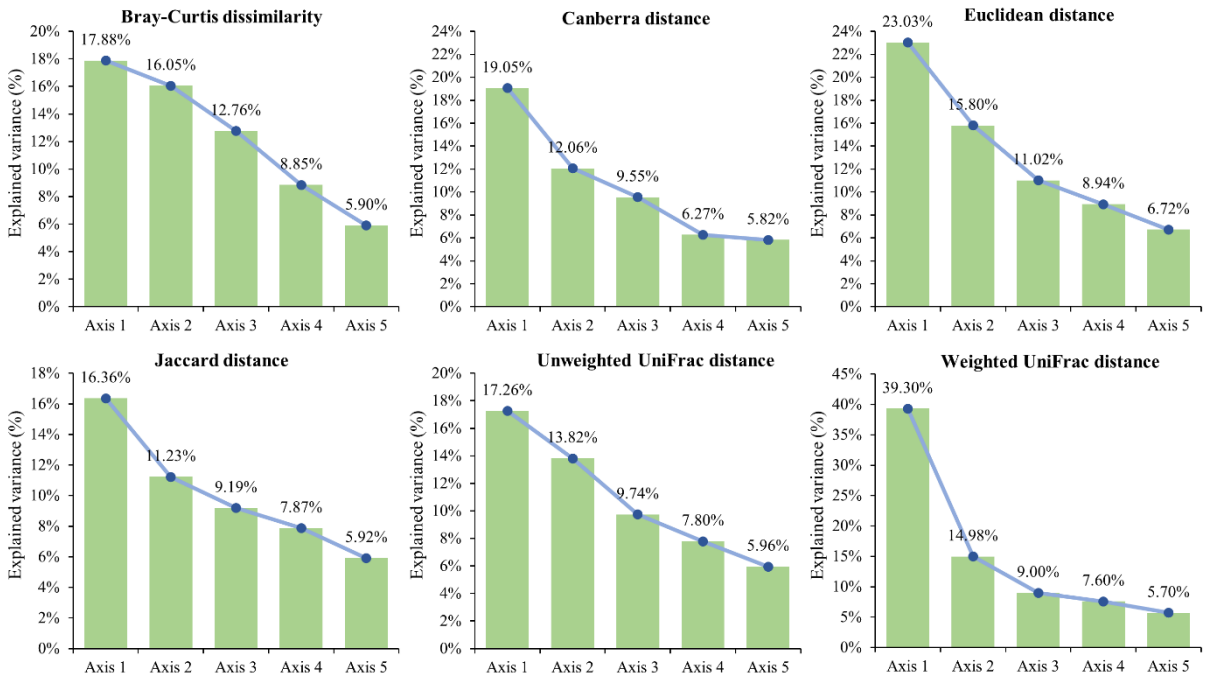
Supplementary Figure S7. Rarefaction curves for α-diversity analysis of rhizosphere microbial communities from sandy soil.

a. Observed OTUs. b. Chao1. c. Pielou's evenness. d. Faith's Phylogenetic Diversity. e. Shannon Index. f. Simpson Index.



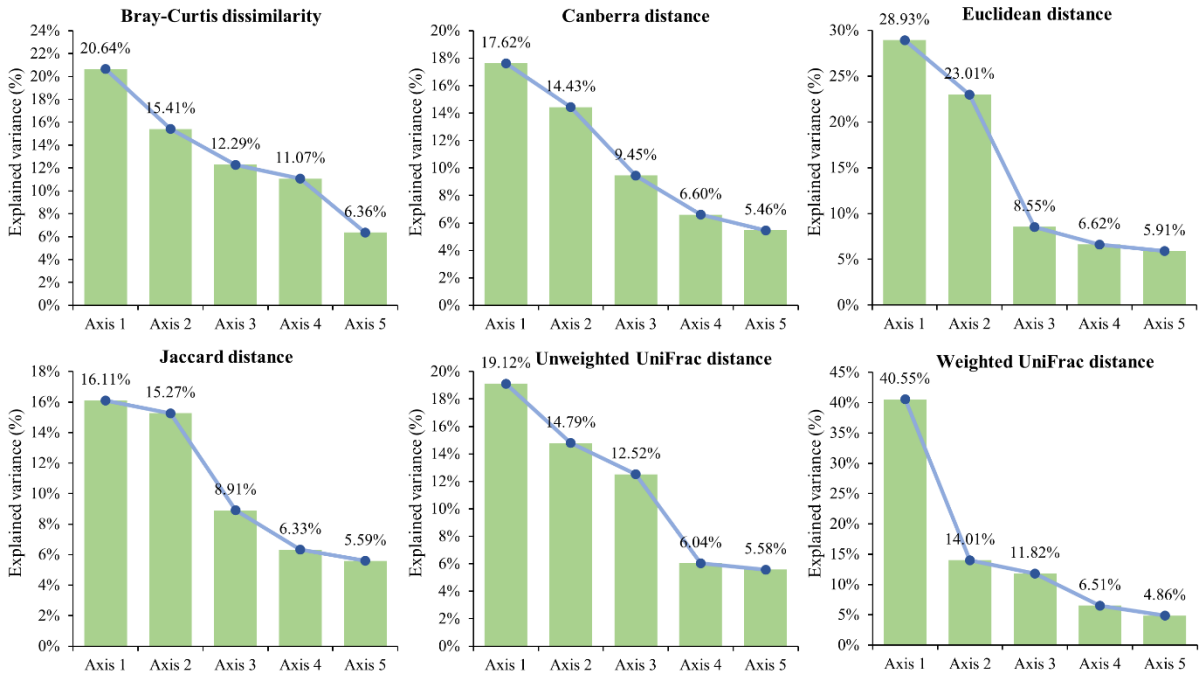
Supplementary Figure S8. Rarefaction curves for α-diversity analysis of rhizosphere microbial communities from peat substrate.

a. Observed OTUs. b. Chao1. c. Pielou's evenness. d. Faith's Phylogenetic Diversity. e. Shannon Index. f. Simpson Index.



Supplementary Figure S9. Scree plots indicating the explained variance per each axis in Principal Coordinate Analysis of β-diversity analysis of rhizosphere microbial communities from sandy soil.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.



Supplementary Figure S10. Scree plots indicating the explained variance per each axis in Principal Coordinate Analysis of β-diversity analysis of rhizosphere microbial communities from peat substrate.

a. Bray-Curtis dissimilarity. b. Canberra distance. c. Euclidean distance. d. Jaccard distance. e. Unweighted UniFrac distance. f. Weighted UniFrac distance.