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# Advanced Soil DNA Profiling by Next-Generation Sequencing (NGS) Technology

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*Agli affetti che mi hanno accompagnata fin qui.*

*A Kira ed Ettore che hanno visto solo l'inizio.*

*To my beloved ones who have been with me this far.*

*To Kira and Ettore who have only glimpsed the beginning.*

# TABLE OF CONTENTS

# GENERAL ABSTRACT

The establishment of soil science can be traced back to the 19th century. However, the study of soil, whose origins are intertwined with human history, began with the development of sedentary agriculture. Despite advancements in soil management techniques, recent estimations depict an alarming situation in which more than 60% of European soils are deemed unhealthy. The prevailing degradation processes appear to be linked to the loss of organic carbon (48%), the loss of biodiversity (37.5%), and the loss of solid phase due to erosion (32%). Hence, there is an urgent need for a multidisciplinary approach, employing the latest scientific techniques, to comprehend soil health. The microbiome, with its ability to influence nearly all soil processes, stands as one of the most crucial affecting the health of terrestrial ecosystems, albeit it is one of the most recent key players considered for soil evaluation.

The overarching goal of this thesis was to delve into the role of soil microbiomes within ecosystem services. A combination of physiochemical and molecular techniques has been employed to enhance the sustainability of current management practices. From a biodiversity conservation perspective, comprehending the structure and function of microbial communities influencing soil properties is crucial. In this work, the exploitation of contemporary molecular and bioinformatic techniques enabled taxonomical and functional classification of the bacterial species. The objective was the translation of the "soil microbial biodiversity" concept into practical, recognizable terms, associating it with taxonomic compositions that provide uniqueness and distinctive identification.

The first contribution included in this thesis presents a study focused on changes in the structure and functionality of the soil microbiome concerning the severe degradation of the matrix due to the use of the area as a municipal solid waste landfill and its subsequent restoration using a patented method aimed at rebuilding soil structure. The study reveals that, although soil restoration contributed to ecological diversity indices improvement, only prolonged observations over time could definitively ascertain whether the new microbial community structure stability and its functionality can be considered stable and permanent.

The second contribution presents a study that proposes molecular markers, including bacterial genera well known for their role as sugar beet (*Beta vulgaris* L.) growth promoters, to distinguish the effects of organic versus conventional soil management. The study reveals that the two managements do not significantly affect the overall microbiome structure. However, organic management is characterized by higher quantities of total soil

DNA and copies of 16S ribosomal RNA genes. Moreover, functional genes related to carbon and nitrogen biogeochemical cycles and bacterial growth-promoting genera are more abundant. Therefore, it can be inferred that organic management appears to favor the functionality of the agroecosystem.

The third contribution presents a study of microbial communities characterizing the active layer and permafrost along a chronosequence composed of three beaches in the Canadian High Arctic (Devon Island, Nunavut). These beaches are subjected to the same climatic and environmental factors but have emerged at different times between 2,360 and 8,410 years before the present (YBP). The results, in line with other scientific studies, identify specific chemical parameters, such as pH, organic carbon, and available phosphorus, as driving factors in shaping the structure of microbial communities. In detail, changes in chemical parameters along the soil profile correspond to variations in ecological diversity indices and the microbial community structure. However, it also emerges that the bacterial taxa composing the shared fraction of the microbial community across all horizons are also present in soils located in distinct ecosystems equally subject to significant environmental stresses. Finally, the substantial detectability of the bacterial 16S gene seems to be linked to a combination of specific environmental conditions on Devon Island, which are minimally impacted by human activity, and to the sporulation propensity of certain bacteria.

In conclusion, this thesis provides a tangible contribution to our understanding of soil microbial communities. The presented data are derived from the study of soils undergoing vastly different pedological developments and subjected to a wide range of management practices and land uses. The characterization of microbial community responses to various environmental stimuli is a significant outcome of this research, with implications for enhancing soil management sustainability and comprehending biosphere evolution in the context of anthropogenic stressors.

# RIASSUNTO GENERALE

La fondazione della scienza del suolo è collocata nel XIX° secolo ma, lo studio del suolo, le cui origini si fondono con la storia umana, inizia con lo svilupparsi dell'agricoltura stanziale. Nonostante il progresso delle tecniche impiegate nella gestione dei suoli, stime recenti riportano un'allarmante situazione attestante più del 60% dei suoli Europei come degradati. I processi degradativi più diffusi sembrano essere legati alla perdita di carbonio organico (48%), alla perdita di biodiversità (37.5%), e alla perdita di fase solida a causa dell'erosione (32%). Per comprendere lo stato di salute di un suolo quindi risulta necessario un approccio multidisciplinare che impieghi le più recenti metodologie d'indagine. Il microbioma, con la sua capacità di condizionare la quasi totalità dei processi che avvengono al suolo, è uno dei principali fattori che influenzano la salute degli ecosistemi terrestri, sebbene sia stato considerato, in relazione allo specifico campo di studi, solamente in tempi recenti.

Lo scopo generale di questa tesi è stato quello di approfondire il ruolo del microbioma del suolo all'interno dei servizi ecosistemici, utilizzando una combinazione di tecniche fisico-chimiche e molecolari, al fine di implementare la sostenibilità delle odierne pratiche di gestione. In un'ottica di conservazione della diversità biologica, è fondamentale conoscere la struttura e il funzionamento delle comunità microbiche in grado di influenzare le proprietà del suolo. Questo lavoro, attraverso l'impiego delle attuali tecniche molecolari e bioinformatiche, che permettono la classificazione tassonomica e funzionale delle specie batteriche, ha l'obiettivo di ricondurre a termini pratici ed identificabili il significato di "biodiversità microbica del suolo", associandolo a composizioni tassonomiche le cui specificità costituisce elemento di tipicità e unicità identificativa.

Il primo contributo incluso in questa tesi presenta uno studio focalizzato sui cambiamenti della struttura e della funzionalità del microbioma del suolo in relazione ad un forte deterioramento della matrice, dovuto all'impiego dell'area interessata come discarica di rifiuti solidi municipali, e al suo successivo recupero tramite un metodo brevettato che si prefigge di ricostruire la struttura del suolo. Dallo studio emerge che, sebbene la ristorazione del suolo abbia contribuito al miglioramento degli indici ecologici di diversità, solo un'osservazione prolungata nel tempo potrebbe affermare chiaramente se la stabilità della nuova struttura della comunità microbica e la sua funzionalità possano essere definite stabili e permanenti.

Nel secondo contributo è presentato uno studio che, in seguito all'analisi del microbioma del suolo, propone dei marcatori molecolari, tra i quali dei generi batterici conosciuti per il loro ruolo di promotori di crescita della barbabietola da zucchero (*Beta vulgaris* L.), per la distinzione degli effetti di una gestione organica di un suolo agrario comparata ad una gestione convenzionale. Ne emerge che le due gestioni non sembrano influenzare la struttura generale del microbioma in maniera significativa. La gestione organica, tuttavia, è caratterizzata da maggiori quantitativi di DNA totale del suolo e di copie geniche di RNA ribosomiale 16S. Inoltre, anche i geni funzionali legati ai cicli biogeochimici del carbonio e dell'azoto e i generi batterici promotori di crescita risultano più abbondanti. Si può dedurre, quindi, che la gestione organica sembri favorire la funzionalità dell'agroecosistema.

Il terzo contributo presenta lo studio delle comunità microbiche che caratterizzano sia lo strato attivo sia il permafrost lungo una cronosequenza composta da tre spiagge situate nell'alto Artico Canadese (isola di Devon, Nunavut), sottoposte agli stessi fattori climatici ed ambientali ma emerse con tempistiche diverse tra 2360 e 8410 anni prima del presente (*years before present* – YBP). I risultati, in accordo con altri studi scientifici, individuano alcuni parametri chimici, quali pH, carbonio organico, e fosforo disponibile, come fattori trainanti nel plasmare la struttura delle comunità microbiche. In particolare, alla variazione dei parametri chimici lungo il profilo pedologico, corrisponde una variazione degli indici ecologici di diversità e una variazione della struttura delle comunità microbiche. Tuttavia, emerge anche che i taxa batterici che compongono la frazione di comunità microbica comune a tutti gli orizzonti sono presenti anche in suoli situati in ecosistemi molto diversi ma ugualmente sottoposti ad importanti stress ambientali. Infine, la sostanziale rilevabilità del 16S batterico sembra essere legata ad una combinazione di condizioni ambientali specifiche dell'isola di Devon, che risulta essere minimamente impattata dall'attività umana, e di propensione alla sporulazione di alcuni batteri.

Questa tesi fornisce un contributo concreto alle conoscenze relative alle comunità microbiche dei suoli. I dati presentati provengono dallo studio di suoli provenienti da evoluzioni pedologiche molto dissimili e da un ampio range di gestioni e destinazioni d'uso. La caratterizzazione delle risposte delle comunità microbiche ai diversi stimoli ambientali è un risultato importante della presente ricerca per il miglioramento della sostenibilità della gestione dei suoli e per la comprensione dell'evoluzione della biosfera in relazione agli stress antropogenici.

# GENERAL INTRODUCTION

## SOIL DEFINITION AND KEY PEDOGENETIC PROCESSES

The quest for a universal definition of soil that encompasses its key characteristics, deemed essential for various categories of scientists, such as agronomists, engineers, and pedologists, emerged around the 1800s and resulted in the establishment of Soil Science (Hartemink, 2016). According to one of the most recent definitions provided by the USDA Soil Taxonomy, which is a globally recognised standard reference in pedology, the *soil is a natural body comprised of solids, liquid, and gases that occur on the land surface, occupies space and is characterized by one or both of the following: i) horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or ii) the ability to support rooted plants in a natural environment* (Soil Survey Staff, 2022).

The aforementioned description unveils a wide array of distinguishing factors, such as the presence of distinct horizons derived from the parent material, which play a pivotal role in soil characterisation. These factors can be attributed to the pedogenesis or soil formation process, which encompasses a complex series of events occurring concurrently or in sequence, all mutually interacting with one another (Huggett, 1998). In general, the events contributing to the development and evolution of a soil entail several key actions. These include the deposition of surface materials, such as animal and plant remnants and wind-transported particles, the decomposition of organic residues by the biotic compartment, the alteration of primary minerals and the subsequent formation of secondary minerals, and the colloidal constituents transfer facilitated by water movements (Jenny, 1994).

Among the several phenomena contributing to pedogenesis, calcification, gleization, laterization, podzolization, and salinization can be considered the central soil-forming processes, notably significant in shaping macro-scale patterns characterizing landscapes and ecosystems globally (Bockheim and Gennadiyev, 2000).

Calcification (Figure 1a), characterising arid and semi-arid soils presenting a parental material particularly enriched in carbonates, entails the accumulation of gypsum and secondary carbonates in the soil horizon (Buol, 1964). In soils characterised by higher moisture levels, calcification may occur as carbonates redistribution within the soil profile (Schaetzl et al., 1996).

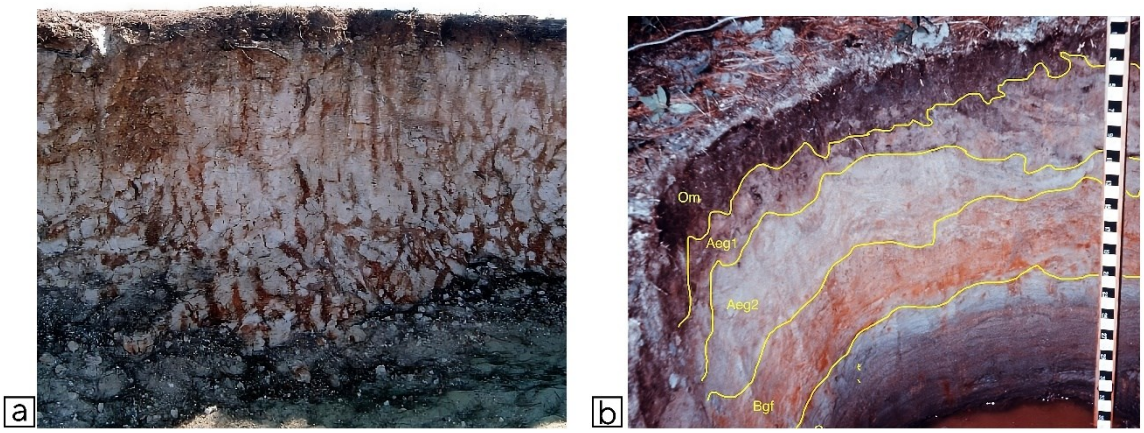


Figure 1. a) Luvic Calcisol profile from southern Turkey (European Soil Bureau Network, 2005), b) Gleysol profile from the Ontario region (Canadian Society of Soil Science, 2020).

Gleization (Figure 1b), alternatively known as hydromorphism, is a characteristic phenomenon of soils that remain consistently saturated with water. Due to anaerobic conditions, the reduction of ferric iron ($Fe^{3+}$) to ferrous iron ($Fe^{2+}$) enables the mobilization of the compounds containing the bivalent ion along the soil profile, resulting in the eventual formation of shallow horizons presenting a reddish mottling, and deeper horizons characterized by their typical dull grey or grey-greenish coloration (Bouma, 1983).

Laterization (Figure 2a), also known as ferralitization, is commonly observed in intertropical regions. This process causes the development of soil horizons distinguished by low silica (Si) content and a notable accumulation of Fe and aluminum (Al) oxides and hydroxides resulting from weathering processes of both primary and secondary minerals (Herbillion and Nahon, 1988, Righi et al., 1990).

Podzolization (Figure 2b), a complex series of processes, is characteristic of continental regions with a cold-humid climate. In general terms, podzolization comprises the translocation into the deeper soil horizons of compounds deriving from the alteration of Fe and Al minerals complexed with humic substances, particularly fulvic acids, and other polyaromatic compounds (Lundström et al., 2000).

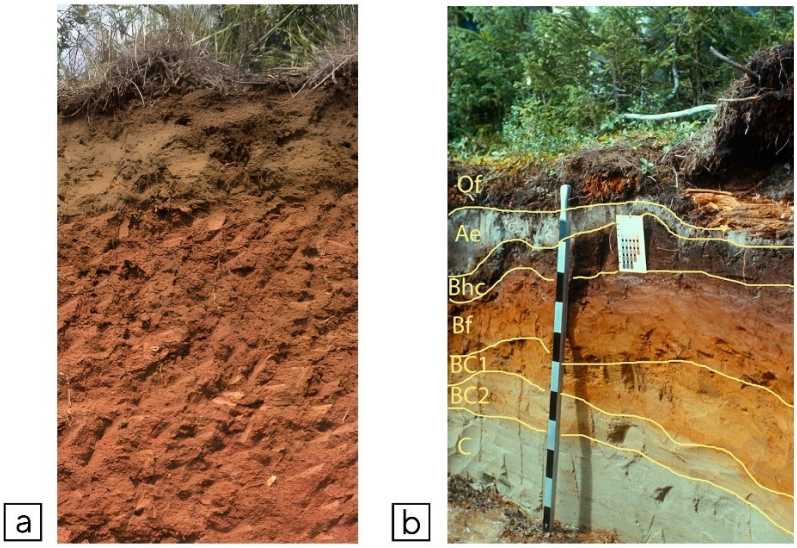


Figure 2. a) Ultisol profile from southern United States (USDA), b) Humic Podzol profile from Canada (Canadian Society of Soil Science, 2020).

Salinization (Figure 3) is a commonly observed process characterising arid and semi-arid regions. The high clay content of soils affected by this process prevents leaching, resulting in the concurrent accumulation of salts, mainly in the form of chlorides and sulphates, due to the evaporation of the circulating solution (Singh, 2021).

Figure 3. Aquisalid landscape from central Nevada (U.S.A.). Salt crusts are visible on the soil surface (Soil Science Society of America, 1993).

# SOIL ECOSYSTEM SERVICES

The expression “ecosystem services” (ES) encompasses the societal advantages conferred by natural ecosystems, including aspects such as biodiversity preservation and the safeguarding of ecological systems (Russo and Cirella, 2021). These services hold fundamental importance for the functioning of our society and assume a crucial role in the operation of several sectors. The concept of ES has undergone progressive refinement, assimilating perspectives from multiple disciplines and frameworks, such as the service-dominant logic framework, which underscores the collaborative generation of value by multiple stakeholders (Semeraro and Buccolieri, 2022).

Soil ES encompass the diverse benefits and functions provided by soils, which are essential for sustaining both human and environmental well-being. These services include a wide array of functions that can be primarily divided into ecological and non-ecological categories (Blum, 2005). According to the three prevailing classification schemes (De Groot et al., 2002, Kumar, 2010, M.E.A., 2005), soil ES can be systematically categorized into four distinct types, including production services, regulation services, support services, and cultural services. Within the realm of production services, which supply products with recognized market value, we can identify the production of food and animal feed, the raw materials supply for construction activities, and fuel production (Franzluebbers, 2015). In addition to these well-known services, there is also the genetic reservoir, which serves as a repository from which biotechnological applications can draw (Jónsson and Davíðsdóttir, 2016). Among the regulating services, which confer benefits by overseeing critical processes within several ecosystems, ecosystem protection and climate control are particularly relevant. Ecosystem protection is enabled by the soil organic matter's ability to immobilize several xenobiotic compounds (Bollag et al., 1992). The climate control function relies on the soil’s capacity for carbon dioxide ($CO_2$) sequestration (Lal, 2004). Soil serves as a carbon sink and contributes to offsetting greenhouse gas (GHG) emissions in the atmosphere. In the category of supporting services, which establish the foundation for ecosystem development and evolution, processes of soil formation, maintenance of the biogeochemical cycles’ functionality, and the provision of habitat for existing biodiversity are included (Ausseil et al., 2011, Ma et al., 2021). Eventually, cultural services comprise all the intangible benefits stemming from cognitive development.

Despite the abundance of publications in the current literature, it is evident that soil ES remain relatively understudied. Furthermore, although numerous systems for the

identification and classification of soil ES have been proposed, a consensus on the most efficient methodology is still lacking (Robinson and Lebron, 2010).

The urgency of drafting clear and comprehensive guidelines encompassing all parameters related to soil is not merely a transient demand of the scientific community; it is a concrete necessity, further underscored by the escalating threats of soil degradation and increasing anthropogenic pressures, which are nearing critical thresholds. In this regard, the European Union Soil Observatory (EUSO), in March 2023, developed a new survey system managed by the EU Commission's Joint Research Centre (JRC). This system has confirmed that 61% of European soils are categorized as unhealthy. Additionally, each year, 24 billion tons of fertile soil are lost due to climate change (AbdelRahman, 2023). Thus, the assessment of soil quality and the comprehensive understanding of the multifaceted nature of soil functions are imperative steps toward the enhancement and preservation of soil ecosystem services.

## SOIL MICROBIOME

Soil is not a single environment; it contains a wide range of environmental niches that can be mere micrometres to millimetres apart and can significantly differ in their abiotic characteristics (Fierer, 2017). Consequently, the soil harbours a wide array of microorganisms, selected based on the specific environmental conditions within these niches.

The intricacy of the environmental interactions and the collective genetic heritage of all microorganisms in each environment is collectively known as the microbiome. Within the soil, this microbiome comprises eubacteria, fungi, archaea, protists, and nematodes (Anandham and Sa, 2021, Santaella and Plancot, 2020, Shah et al., 2022, Wang et al., 2021).

The soil microbiome, controlling up to 90% of the soil processes, plays a major role in ecosystem functioning and, thus, regulates a multitude of essential functions crucial to our society (Nannipieri et al., 2003, Young and Crawford, 2004). Most of these functions fall within the realm of supportive services that serve as the foundational basis for the provision of all other services. Moreover, soil microbiome significantly influences major regulatory services (Guerra et al., 2021). In detail, the soil microbiome contribution can be ascribed to four main categories.

Soil aggregation, organic matter decomposition, and nutrient cycling: Soil microbiome contributes to the modification of soil structure and to the creation of ecological niches. Soil organic matter serves as a fundamental cornerstone for soil structure, playing a crucial role in soil aeration, water absorption, and nutrient retention (Hartmann and Six, 2023). Moreover, the soil microbiome is responsible for macro- and micronutrient cycling, as well as other essential elements, for plant and animal life (Ossowicki et al., 2021).

Carbon sequestration: Photo- and chemoautotrophic microorganisms undertake the $CO_2$ synthesis into organic compounds. Microbes, decomposing organic materials, release nutrients into the soil, and promote plant growth and development (Mason et al., 2023). Additionally, arbuscular mycorrhizal fungi aid in the transformation of carbon from labile to recalcitrant pools, significantly contributing to the sequestration of carbon in the soil (Ossowicki et al., 2021).

Biostimulation: One of the most studied advantages that soil microbiome provides to plants is their symbiotic relationships. Nitrogen-fixing bacteria, employing a complex enzymatic system known as nitrogenase, have the capacity to convert atmospheric nitrogen ($N_2$) into ammonium nitrogen ($NH_3$), which is subsequently made available for plants (Orr et al., 2011). Furthermore, phosphate-solubilizing bacteria (PSB) can make retrograde phosphorus biologically accessible (Li et al., 2023). On the other hand, arbuscular mycorrhizal (AM) fungi are biotrophic symbionts that establish close associations with nearly 80% of terrestrial plants (Wilkes, 2021). They supply soil-bound nutrients to the host plant through root cortical arbuscules while receiving photosynthetic. Additionally, other members of the soil microbiome produce plant growth-promoting hormones, such as indole-3-acetic acid (IAA), auxins, and gibberellins (Trivedi et al., 2017).

Bioremediation: Soil bacteria, and to a lesser extent fungi, have the capability to produce and release catabolic enzymes into the soil, which catalyse the degradation process of persistent organic pollutants (POPs), transforming complex molecules into simpler, lower molecular weight compounds. Soil microorganisms contribute to toxic organic compound detoxification by producing the oxidoreductase enzyme. This enzyme catalyses the transfer of electrons, aiding in the oxidation-reduction process. Finally, the metabolism of specific microorganisms, by altering the environmental pH and stimulating the release of chelating agents and organic acids, contributes to an increased complexation of heavy metals, thereby modifying their mobility (Ying and Wei, 2019).

The soil microbiome is a complex and dynamic system with a central role in ecosystem functionality and agriculture sustainability. Consequently, studying the soil microbiome and

comprehending its reactions to environmental shifts, including climate change, are imperative for anticipating and mitigating the consequences of these changes on soil fertility, productivity, and ecosystem resilience.

## APPROACHES TO THE STUDY OF SOIL MICROBIOME

The study of soil microbiome has been hampered for several years as only a few microorganisms can be cultivated using standard methods (Robe et al., 2003; Vester et al., 2015). In 1995 Amann et al. observed that only 0.001 to 0.3% of the bacteria coming from environmental samples can be cultivated using validated growth mediums in Petri dishes. The study of soil microbiome has evolved in recent years with the application of new methods and technologies (Wydro, 2022). High-throughput molecular approaches, based on the total soil DNA and RNA analysis, have replaced the conventional laboratory methods that relied on cultures and biochemical analyses, offering higher sensitivity and repeatability (Rantsiou and Cocolin, 2006, (Wang, 2023). The Next-Generation Sequencing (NGS) technique encompasses the analysis of small DNA or complementary DNA (cDNA) fragments and the subsequent whole genome computational reconstruction based on the comparison with a reference genome (Wang, 2021). The NGS techniques allow the identification and characterisation of the bacterial communities within a sample (DeBofsky, 2022).

The metagenomics technique involves the sequencing of the entire genetic material present in a sample, providing a comprehensive view of the microbial communities and allowing for the identification of both known and unknown organisms (Becker and Pushkareva, 2023). Metagenomic databases play a central role in data analysis, as they encompass information related to taxonomic classification as well as gene function annotations, sequence alignments, and phylogenetic tree inference (Shika et al., 2021).

16S metabarcoding, instead, uses the amplification of targeted hypervariable regions of the 16S ribosomal RNA gene. The 16S rRNA gene is a molecular marker commonly used for bacterial identification, whose sequence can be analysed to determine the genetic characteristics of different bacterial strains (Case et al., 2007). The nine hypervariable regions of the 16S rRNA gene, commonly targeted in microbiome studies for taxonomic identification and phylogenetic analysis, are gene portions containing a high level of genetic variation among different bacterial taxa (Rajeev et al., 2020, Yang et al., 2016). If 16S metabarcoding involves sequencing a single hypervariable region of the 16S rRNA gene,

typically either the V3 or V4 region, it is referred to as single-amplicon metabarcoding. Conversely, sequencing multiple hypervariable regions simultaneously is termed multi-amplicon metabarcoding. Single-amplicon 16S metabarcoding offers advantages in terms of sample throughput, cost-efficiency, and sensitivity. However, it is constrained by primer bias and lacks specificity (Schriefer et al., 2018). On the other hand, multi-amplicon 16S metabarcoding, which exhibits superior taxonomic classification compared to single-amplicon methods, along with higher positive predictive values and accuracy in estimating abundance, provides a more comprehensive perspective of microbial communities (Gonzalez et al., 2019).

The soil NGS data interpretation requires a multidisciplinary approach that integrates knowledge from biology, agronomy, pedology, and bioinformatics. Consequently, it becomes evident how a data validation step can provide greater confidence in the results interpretation process. The quantitative real-time PCR (qPCR) technique can be used for the amplification and quantification of target DNA or cDNA in real time. qPCR enables the quantification and analysis of the genetic target simultaneously with its amplification, using detection systems that employ melt curve analysis and/or fluorescent detection systems. The versatility of this technique allows gene expression analysis, allelic discrimination assays, and genotyping (Singh and Roy-Chowdhuri, 2016). Digital PCR (dPCR) is a reliable molecular technique with excellent biological sensitivity and reproducibility (Sanders et al., 2011), that allows for the detection of difficult-to-detect DNA amounts or rare taxa within a sample without requiring an external calibration curve. Unlikely qPCR, which carries out a single reaction in each well, dPCR divides the PCR solution into thousands of nanoliter-sized droplets, with a separate PCR reaction occurring in each one (Baker, 2012). Sample partitioning facilitates estimating the number of different molecules, based on the assumption that the molecules are distributed according to the Poisson distribution model, thereby considering the chance of several target molecules dwelling in one droplet (Duever et al., 2018). With Poisson's law of small numbers, it is possible to exactly approximate the concentration of target molecule within the sample, thereby enabling the quantification of the target in the PCR product without requiring an external calibration curve.

# RESEARCH OBJECTIVES

The overarching objective of this thesis is to advance the development of an integrated multidisciplinary approach that combines physicochemical and molecular techniques for the soil resource study. This approach seeks to deepen our comprehension of the role and impact of the soil microbiome on the soil ecosystem services. Ultimately, the aim is to bolster the sustainability of soil management practices.

The three specific objectives of this thesis are:

I. Investigation of soil microbiome and individual genes coding for the enzymes involved in the N biogeochemical cycle in soils treated with a novel technology, called soil reconstruction, which aims at creating a *de novo* soil structure from the original soil solid phase.

II. Identification of suitable markers from the soil microbiome that have the potential to display discernible variations in conventional versus organic farming practices.

III. Investigate the soil microbial communities characterizing both unglaciated soil and permafrost along a chronosequence represented by three beaches raised from 2,360 to 8,410 years before present (YBP) located in Devon Island, in the High Arctic Canadian region of Nunavut.

# STRUCTURE OF THE THESIS

The initial segment of this thesis delves into the background this research endeavor. The introduction provides an overview of soil and its associated environmental dynamics. In particular, it emphasizes the ecosystem services rendered by soil and underscores the pivotal role of the microbiome, which is responsible for governing up to 90% of soil processes, in terrestrial ecosystems functioning and development. Additionally, the introduction outlines both the general and specific objectives, as well as the overall structure of this thesis. The main body of this thesis comprises three scientific papers, referred to as contributions, and they are presented in chronological order (Table 1). Lastly, some general conclusions based on the three contributions are outlined in the last part of this thesis.

Table 1. Contributions included in this thesis.

| | Reference |
|---|---|
| Contribution I | **Maretto L**, Deb S, Ravi S, Chiodi C, Manfredi P, Squartini A, Concheri G, Renella G, Stevanato P, 2022. Microbial diversity of reconstituted, degraded and agricultural soils assessed by 16S rDNA multi-amplicon sequencing. Frontiers in Environmental Science, 9. https://doi.org/10.3389/fenvs.2021.807889 |
| Contribution II | **Maretto L**, Deb S, Ravi S, Della Lucia MC, Borella M, Campagna G, Squartini A, Concheri G, Nardi S, Stevanato P, 2023. 16S metabarcoding, total soil DNA content, and functional bacterial genes quantification to characterize soils under long-term organic and conventional farming systems. Chemical and Biological Technologies in Agriculture, 10, 78. https://doi.org/10.1186/s40538-023-00450-3 |
| Contribution III | **Maretto L**, Deb S, Squartini A, Concheri G, Stevanato P, Nardi S, Cocco S, Corti G. Evaluation of the soil microbial diversity of three raised beaches in the Devon Plateau, Devon Island. |

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# CONTRIBUTION I

# Microbial Diversity of Reconstituted, Degraded, and Agricultural Soils Assessed by 16S rDNA Multi-Amplicon Sequencing

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## Abstract

The microbial diversity is, among soil key factors, responsible for soil fertility and nutrient biogeochemical cycles, and can be modified upon changes in main soil physicochemical properties and soil pollution. Over the years, many restoration techniques have been applied to restore degraded soils. However, the effect of these approaches on soil microbial diversity is less understood and thus requires more investigation. In this study, we analyzed the impact, on soil microbial diversity of a patented novel technology, used to restore degraded soils. Soil samples were collected from three nearby sites located in Borgotrebbia, Piacenza, Italy, and categorized as reconstituted, degraded, and agricultural soils. After total soil DNA extraction, 16S rDNA multi-amplicon sequencing was carried out using an Ion GeneStudio S5 System to compare soils' bacterial community profiles. Sequenced reads were processed to assign taxonomy and then key microbial community differences were identified across the sampling sites. Species diversity featured significant abatement at all rank levels in the degraded soil when compared to the agricultural control. The 5-year restoration technique showed full recovery of this index at the genus level but not at the phylum level, displaying a rank- dependent gradient of restored richness. In parallel, the abundance of genes involved in the nitrogen (N) biogeochemical cycle was assessed using quantitative Real-Time PCR (qPCR). Total DNA content was significantly higher ($p < 0.05$) in degraded ($\mu = 12.69 \pm 2.58$ μg $g^{-1}$) and reconstituted ($\mu = 11.73 \pm 1.65$ μg $g^{-1}$) soil samples when compared to the agricultural soil samples ($\mu = 2.39 \pm 0.50$ μg $g^{-1}$). The taxonomic diversity of each soil site was significantly different, with some instances unique of the agricultural soil even at the phylum level. The analysis of N functional genes showed that the relative abundance of bacterial *amoA* ($p < 0.05$) and *nosZ* ($p < 0.01$) genes were significantly lower in the agricultural than in the reconstituted and degraded soils. We concluded that the application of the soil reconstitution technique appears to enhance the active microbial community, with distinct diversity and functionality towards genes involved in N biogeochemical cycle, as compared to both the degraded and the agricultural soil.

## Keywords

microbial diversity, next-generation sequencing, soil microbial activity, soil remediation, qPCR, soil microbial profile, 16S rdna multi-amplicon.

## Introduction

Soil originates from the weathering of parent materials under the combined action of climate, living organisms, and in function of the watershed relief and time (Jenny, 1946;

Hartemink, 2016). During pedogenesis soils form complex assemblages of clay minerals (hydr-)oxides and organic matter, that result in their ultimate structure. Soil structure is responsible for soil's physical and chemical functions in the environment such as water movement and retention, and mobility and bioavailability of nutrients and pollutants (Jastrow and Miller, 1991; Gregorich et al., 1997; Robinson et al., 2009; Bünemann et al., 2018). The biotic compartment of soil, composed of interrelated communities of fungi, bacteria, archaea, viruses, protists, and other microbial eukaryotes, is also defined as the soil microbiome (Jansson and Hofmockel, 2020).

It is estimated that soil microbiome controls up to 90% of soil processes, thus it plays a fundamental role in ecosystem functioning (Gregorich et al., 1997*;* Nannipieri et al., 2003; Young and Crawford, 2004). Moreover, the soil microbiome influences the biogeochemical cycles of nutrients, for example, acting as a source or sink of gasses, it contributes to nitrogen (N) and carbon (C) rates of fixation and oxidation, and it can degrade organic pollutants (Fierer, 2017). Therefore, though only a minor portion of the available soil space is colonized by the microbial communities (Young and Crawford, 2004), the stability and the resilience of the soil system are determined by the combination of soil physical structure, nutrient availability, microbial diversity and activity (Meuer et al., 2020). The soil microbiome is impacted by human activities like agriculture, soil sealing and industrial emissions that cause environmental pollution (Roose-Amsaleg et al., 2001; Maron et al., 2011) due to the changes that these activities induce in the soil structure. Since anthropogenic activities have decreased biodiversity in soils, the assessment of the soil microbiome can be a crucial indicator of soil quality (Lehmann et al., 2020; Vieira et al., 2022).

Soil is a non-renewable natural resource, and owing to the recent increased attention to its conservation, restoration of soil quality has become a key topic in science (Qilu et al., 2017; Yan et al., 2018; Xu et al., 2019). Several techniques have been used to form a porous structure in massive non-structured soils, and those based on the amendment with organic matter, revegetation or landfarming are among the most used (Sims and Sims, 2003). However, these techniques are primarily based on the mixing of soil with organic matter that improves their texture, mineralogy, pH value and cation exchange capacity, whereas the formation of a complex structure is slow and mainly due to the action of plant roots and soil microbes over relatively longer time periods. Techniques involving physical, chemical, and biological approaches have been also used to remove or transform harmful pollutants. Among these techniques, remediation using microbial consortia is well-established and widely used due to the lack of secondary pollution, potentially rapid degradation rates, and

low cost (Agamuthu et al., 2013; Hesnawi and Mogdami, 2013). However, little is known about the effect of these restoration techniques on soil microbial diversity in degraded soils that have undergone microbial biomass loss. While microbial activity can be significantly increased by soil restoration, a steady increase of diversity of mirobial communities in restored soils is more difficult to achieve, thus such techniques require a deeper investigation.

The study of soil microbiome has been constrained for a long time because only a minority of microorganisms can be cultivated using standard techniques (Robe et al., 2003; Vester et al., 2015). Amann et al. (1995) observed that the culturability of bacteria from environmental samples ranged between 0.001 and 0.3% depending on the characteristics of the matrix. High-throughput culture- unrelated techniques, like Next Generation Sequencing (NGS), have been established over time to bypass the underestimation of soil microbial diversity problem (Chiodi et al., 2020). 16S rDNA multi-amplicon metabarcoding, sequencing at the same time several hypervariable regions, can generate a substantial amount of sequences, providing crucial information for a deep characterization of the microbiome even of extremely complex natural matrices such as soils (Young et al., 2017).

In this study, combining 16S rDNA metabarcoding and qPCR analyses, we investigated soil microbiome and individual genes coding for the enzymes involved in the N biogeochemical cycle, on soils treated with a novel technology, termed soil reconstruction and patented by MCM Ecosistemi S. r. l, which aims at creating a *de novo* soil structure from the original soil solid phase.

Such in a way we aimed at filling what we perceived as a gap of knowledge. The degree of novelty of the present report being the combination of the patented novel technique and the multi- amplicon sequencing assessment of its effects on soil microbial communities.

## Materials and Methods

### Site Location and Soil Sampling

Soil samples were collected from three sites located in Borgotrebbia, Piacenza, Italy (4503′58″ N 0939′06″ E, Figure 1). Vegetation types were mainly annual terophytes, dominated by Scotch thistle (*Onopordum acanthium* L.) (Giupponi et al., 2013, Giupponi et al., 2015).


Figure 1. Aerial photograph of the studied area located along the hydrographic right bank of the Trebbia River. Geographical coordinates: 45°03'58" N 09°39'06" E. (i) Degraded sampling site, (ii) Reconstituted sampling site, (iii) Agricultural sampling site.

The degraded sampling site was a closed landfill made of municipal solid wastes. The landfill, which was active from 1972 to 1985 and that was covered with a 50 cm thick layer of backfill soil, covers a 20-ha area. The reconstituted sampling site corresponded to half of the landfill that underwent a reconstruction process, becoming a technosol, operated by MCM Ecosistemi S. r. l. with a patented novel technology (Manfredi et al., 2019). The agricultural sampling site was an adjacent agricultural field under conventional maize cultivation. Sampling was carried out with the linear transect technique (Brown, 1993). From each sampling site, 12 sub-samp samples were collected at a 20 cm depth using a manual auger. Sub-samples triplets were mixed to obtain four main samples for each site, referred to as: Reconstituted Soil (RS) (RS1, RS2, RS3, RS4), Degraded Soil (DS) (DS1, DS2, DS3, DS4), Agricultural Soil (AS) (AS1, AS2, AS3, AS4). Composite soil samples were air-dried at room temperature for 48 h, crushed, and sieved (Ø 0.5 mm) before the analysis.

**Soil Chemical Analyses**

Soil pH was measured potentiometrically in ultra-pure water (ratio soil/water 1:2.5 w/v) for each of the analyzed samples. Total carbon (C) and total nitrogen (N) content was determined by dry combustion using a CNS Vario Macro elemental analyzer (Elementar, Hanau, Germany), based on the Dumas combustion method (Dumas, 1831). The calibration curve was created using a certified sulphanilamide standard. The organic carbon content of each sample was tested using the Walkley- Black method (Walkley and

Black 1934), while the extractable phosphorus (P) was evaluated using the Olsen method (Olsen et al., 1954).

**Total Soil DNA Extraction, Multi-Amplicon 16S rDNA Sequencing, Analysis of Functional Genes of the N Biogeochemical Cycle**

Total soil DNA was extracted from 250 mg of air-dried soil using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), according to the manufacturer's instructions. Nucleic acid quantification was performed using the Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA) with Qubit DNA High Sensitivity Assay Kit (Thermo Fisher Scientific).

Library preparation was carried out using the 16S Ion Metagenomics Kit (Thermo Fisher Scientific) that contains two pools of primers targeting seven different hypervariable regions (V2-V4-V8 primer pool and V3-V6-V7-V9 primer pool). 16S rRNA multi-amplicon sequencing was performed using an Ion GeneStudio S5 System (Thermo Fisher Scientific). Raw reads were trimmed for 20 nucleotides on both ends to remove primers using the *cutadapt* utility and analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) v2020.08 (Bolyen et al., 2019) microbiome pipeline. Imported reads were first denoised and dereplicated using the "qiime dada2" plugin followed by taxonomic classification of Amplicon Sequence Variants (ASVs) by a "classify-consensus- blast" plugin using SILVA SSU v138.1 (Quast et al., 2012) as reference database. To check the quality of the achieved sequencing depth, alpha diversity rarefaction analysis was done using the "qiime alpha-diversity" plugin. The taxonomy abundance table at different taxonomic levels was further processed using the Calypso online suite (Zakrzewski et al., 2016) to Total Sum Scaling (TSS) normalized for library size differences. The resultant normalized table was filtered out by omitting taxa with less than the average of 10 reads across samples, and used for further diversity analysis and group comparison at different taxonomic levels. Principal component analysis was performed in Calypso using Bray-Curtis distances and the Shannon diversity index and Taxonomic Richness and community evenness were used for diversity comparisons.

The abundance of *amoA* (eubacterial, AOB), *nifH, nirK,* and *nosZ* bacterial genes was analyzed by quantitative Real-Time PCR (qPCR) using a QuantStudio 12K-Flex apparatus (Thermo Fisher Scientific). The 5 µL reaction mix was composed of 2.5 µL PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 0.15 µL each of forward and reverse primer (Table 1), 1.2 µL PCR-grade water, and 1 µL template DNA. A standard curve using known amounts of the target genes cloned in plasmids of known length (Chiodi et al., 2020) was

obtained and data were used to calculate the copy number of the gene targets based on the Ct value.

Data analysis of gene abundance was performed using SPSS Statistics v28.0.0.0 (190) (IBM, Armonk, NY). Significant differences among the mean values were evaluated with a one- way ANOVA followed by Duncan's post hoc test. Data were expressed as mean ± standard error of the mean. A Principal Component Analysis (PCA) based on Bray-Curtis distances was performed to display the core microbiome of the three soils.

Overall data analysis, including soil chemistry, was performed using SPSS Statistics v28.0.0.0 (190) (IBM, Armonk, NY). Significant differences among the mean values were evaluated with a one-way analysis of variance (one-way ANOVA) followed by post hoc analysis (S-N-K test). Data are expressed as mean ± standard error of the mean.

Table 1. Primer sequences and amplicon length.

| Primer | Sequence | Amplicon Length | Reference |
|---|---|---|---|
| *amoA* F | GGGGTTTCTACTGGTGGT | 500 | Rotthauwe *et al.,* 1997 |
| *amoA* R | CCCCTCKGSAAAGCCTTCTTC | | |
| *nifH* F | AAAGGYGGWATCGGYAARTCCACCAC | 432 | Rösch *et al.,* 2002 |
| *nifH* R | TTGTTSGCSGCRTACATSGCCATCAT | | |
| *nosZ* F | CGYTGTTCMTCGACAGCCAG | 706 | Rösch *et al.,* 2002 |
| *nosZ* R | CATGTGCAGNGCRTGGCAGAA | | |
| *nirK* F | ATYGGCGGVCAYGGCGA | 160 | Henry *et al.,* 2004 |
| *nirK* R | RGCCTCGATCAGRTTRTGGTT | | |

## Results

Soil chemical analyses results are summarized in Table 2.

The pH value of the RS was significantly lower ($p < 0.05$) than that of the DS and AS. The total C content of the AS was significantly lower ($p < 0.05$) than those of the RS and DS, whereas the organic C content was significantly higher ($p < 0.05$) in the RS when compared to DS and AS. Total N content was significantly higher ($p < 0.05$) in the following ranking order: RS > DS > AS. Extractable Olsen P was significantly lower ($p < 0.05$) in AS but no differences between DS and RS were observed.

Table 2. Results of the chemical analyses on the soil samples at the beginning of the experiment.

| Sampling Site | pH* | Total C* g·Kg$^{-1}$ | Organic C* g·Kg$^{-1}$ | Total N* g·Kg$^{-1}$ | Olsen P* g·Kg$^{-1}$ |
|---|---|---|---|---|---|
| Agricultural | 7.85±0.02 b | 29.98±0.38 c | 9.37±0.32 b | 1.69±0.25 c | 5.21±1.57 b |
| Degraded | 8.15±0.03 a | 41.11±3.46 b | 23.98±4.55 b | 3.07±0.19 b | 52.46±10.67 a |
| Reconstituted | 7.69±0.04 c | 66.94±4.11 a | 42.30±2.96 a | 3.98±0.19 a | 101.67±23.02 a |

Means with the same letter in the vertical comparison among the sampling sites are not significantly different at S-N-K test. *Significance level $p<0.05$.

Quantification of the total soil DNA showed a significantly ($p < 0.05$) higher amount of DNA in DS (μ=12.69 ± 2.58 μg·g$^{-1}$) and RS (μ=11.73 ± 1.65 μg·g$^{-1}$) soils compared to the AS soil (μ=2.39 ± 0.50 μg·g$^{-1}$).

Bacterial 16S rDNA metabarcoding on the 12 soil samples provided a total number of 6,926,539 single-end reads, with an average length of 234 nucleotides. A total amount of 9,348 ASVs were identified and finally classified into 717 taxa. The alpha diversity rarefaction plot, corresponding to the number of observed features within samples, showed the highest number of detected sequences in AS samples compared to DS and RS samples (Figure 2).

As regards the taxonomy depth achieved, 85.5% of the annotated sequences were classified at genus rank level, 92.7% at family level, 94.3% at order level, 95.9% at class level and 96.5% at phylum level.

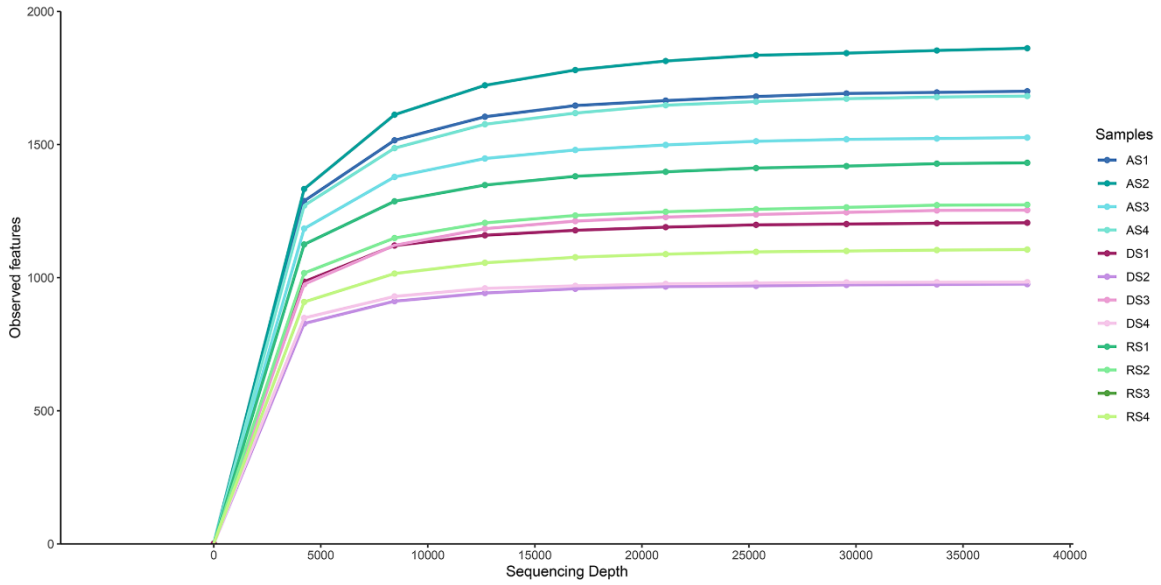


Figure 2. α-diversity rarefaction plot.

Upon splitting the output of the amplified sequence variants taxonomy table in subsets relative to the five different ranks of phylum, class, order, family, genus, and summing up the numbers of each in pivot tables, the consequent diversity within each level could be examined by calculating three ecological indexes assessing community richness, diversity and evenness and the results are shown in Figure 3.

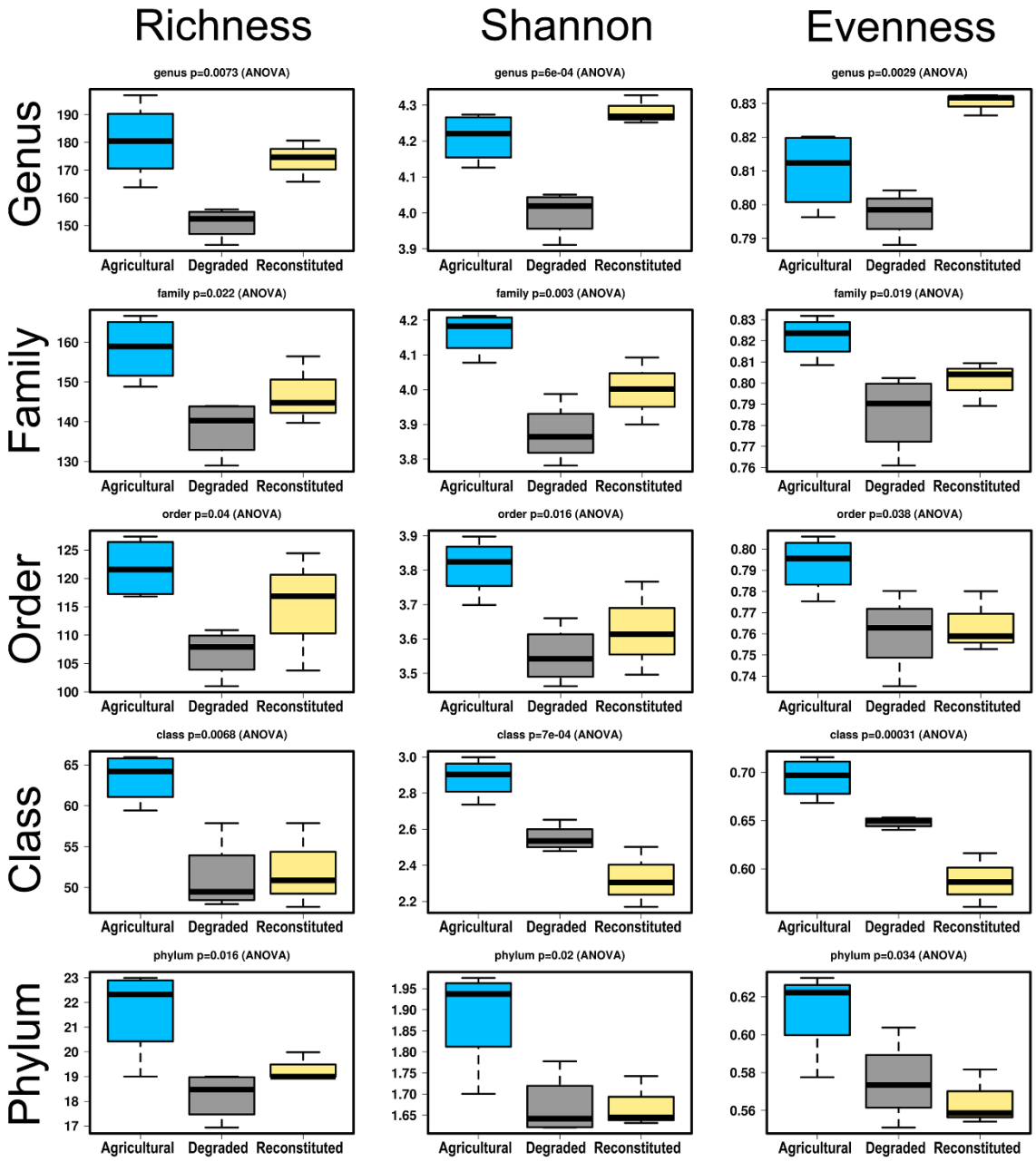


Figure 3. Boxplot comparisons of three ecological parameters (Taxa Richness, Shannon Index and Community Evenness) across five level of taxonomical ranks, for the bacterial communities resulting from the 16rDNA sequencing. Significance levels (ANOVA) are reported above each graph.

It can be seen that for all the three parameters, and in particular for those of diversity and richness, the agricultural control sampling site presents in most cases significantly higher

values than its compared degraded and reconstituted sampling sites as far as the broader systematics divisions are concerned. However, moving up to finer clades, starting from the order, and culminating in the most distinct level (genus), the rise of the values for the reconstituted sampling site is very evident and eventually yields means that become also higher than those of the agricultural sampling site. On the contrary, the values of the degraded sampling site tend to stay inferior to both other soils in almost all cases, with exceptions mainly at class level for the Shannon index and evenness values.

The relative difference of each community was further analyzed by cluster analysis and the results are shown in Figure 4. The communities coming from the three soil management types are indeed partitioned accordingly in three clustered groups. The distance between the group of the agricultural soil and that of the degraded soil is shorter than the one that separates both of them from the reconstituted soil. Consistently with its nature of a reconstituted soil, the hosted bacterial communities appear thereby more distinct from those of the other origin.

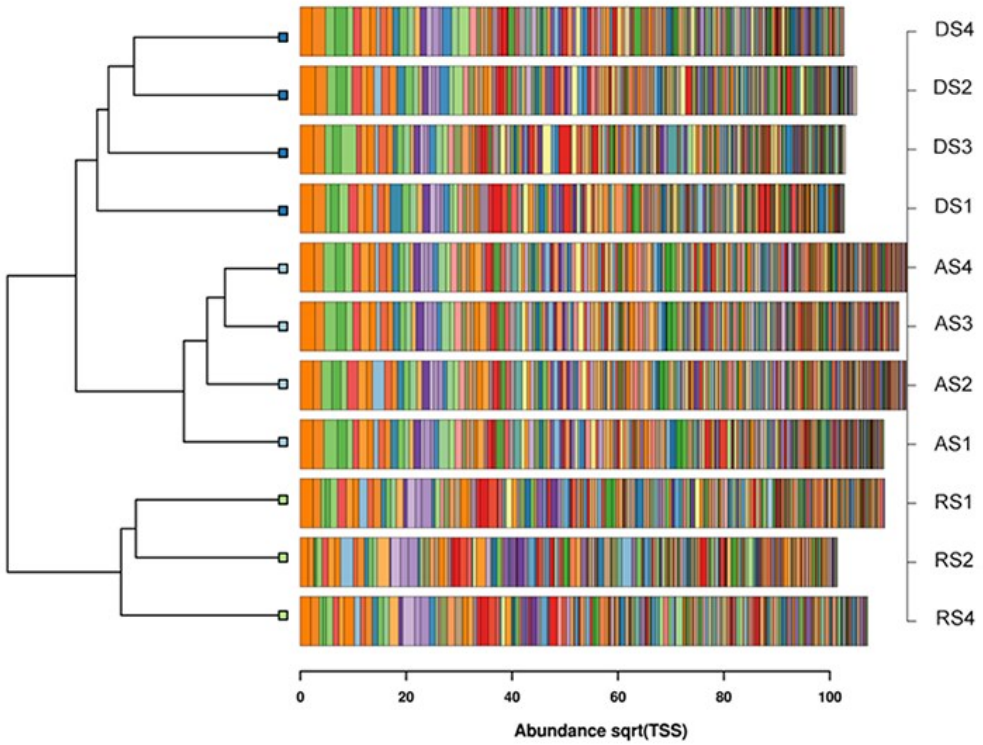


Figure 4. Clustered barchart dendrogram based on the Bray-Curtis distances of the first most abundant 250 taxa for each community.

Multivariate analyses were performed to further inspect the relative ordination of each of the communities and the consistency of the replicates within each group. Principal

Coordinates Analysis, Principal Component Analysis and PERMDISP2 were computed and the results are shown in Figure 5.

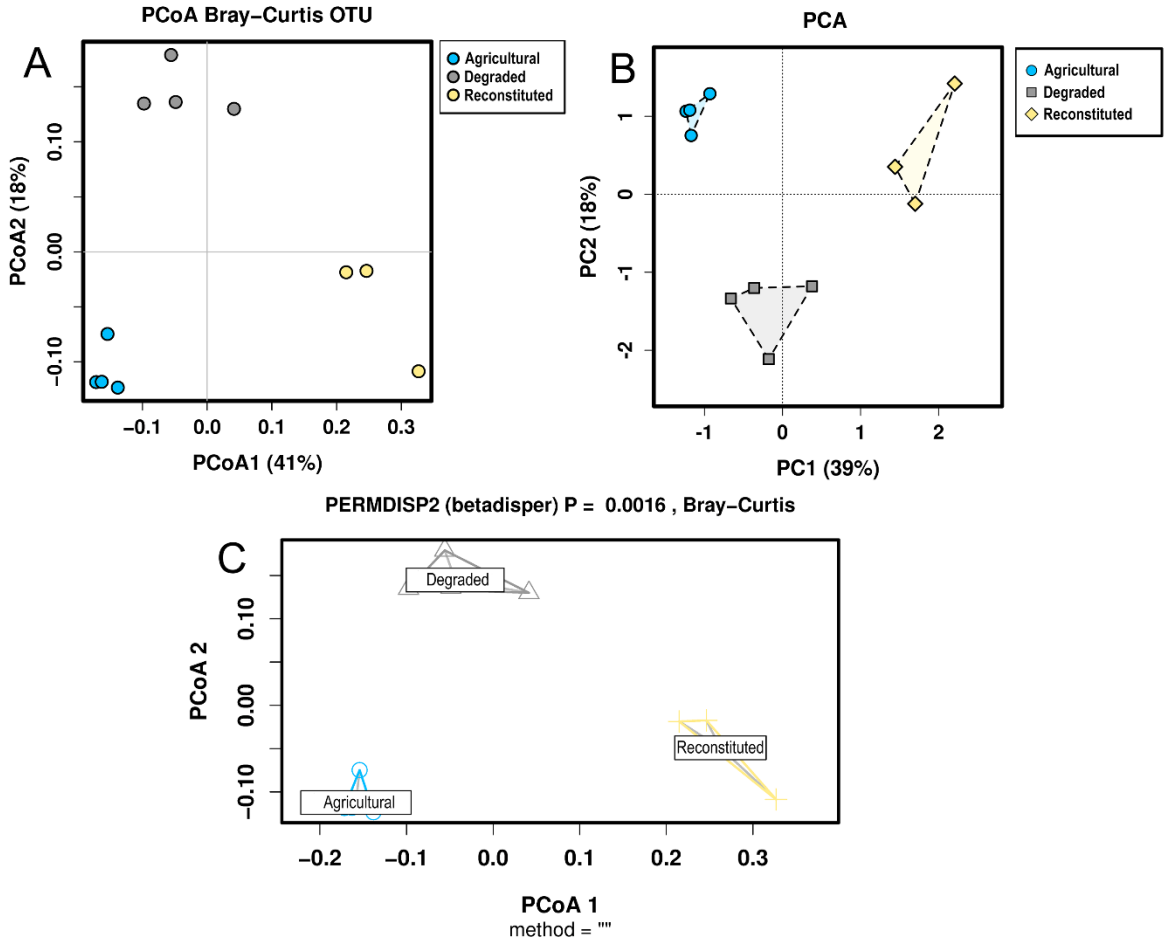


Figure 5. Multivariate analyses for the bacterial communities sequencing data. A) Principal Coordinate Analysis based on the Bray-Curtis distances, B) Principal Component Analysis, C) PERMDISP2, which visualizes the distances of each sample to the group centroid in a Principal Coordinate Analysis (PCoA) and provides a p-value for the significance of the treatments.

All approaches coherently separated each community on the basis of the soil management variable. The PCA showed that bacterial taxa of the three soils clustered separately with polygon's centroids significantly different (PERMANOVA $p < 0.05$).

Subsequently, on the five different rank-level data subsets, an analysis of the conserved core of shared taxa and of the unique ones was carried out, yielding the results shown in Figure 6.

This analysis of the core versus specific sets of the microbiomes (Shade and Handelsman, 2012) showed the extent of uniqueness of taxa occurring at different ranks in each of the three management types, with the agricultural sampling site retaining the highest degrees of specificity, followed by the reconstituted sampling site and with the least number at all

level the degraded sampling site. Additional information about top abundant unique taxa for each of the analysed soils, is reported in Supplementary Table S1 (Supplementary Material S1).

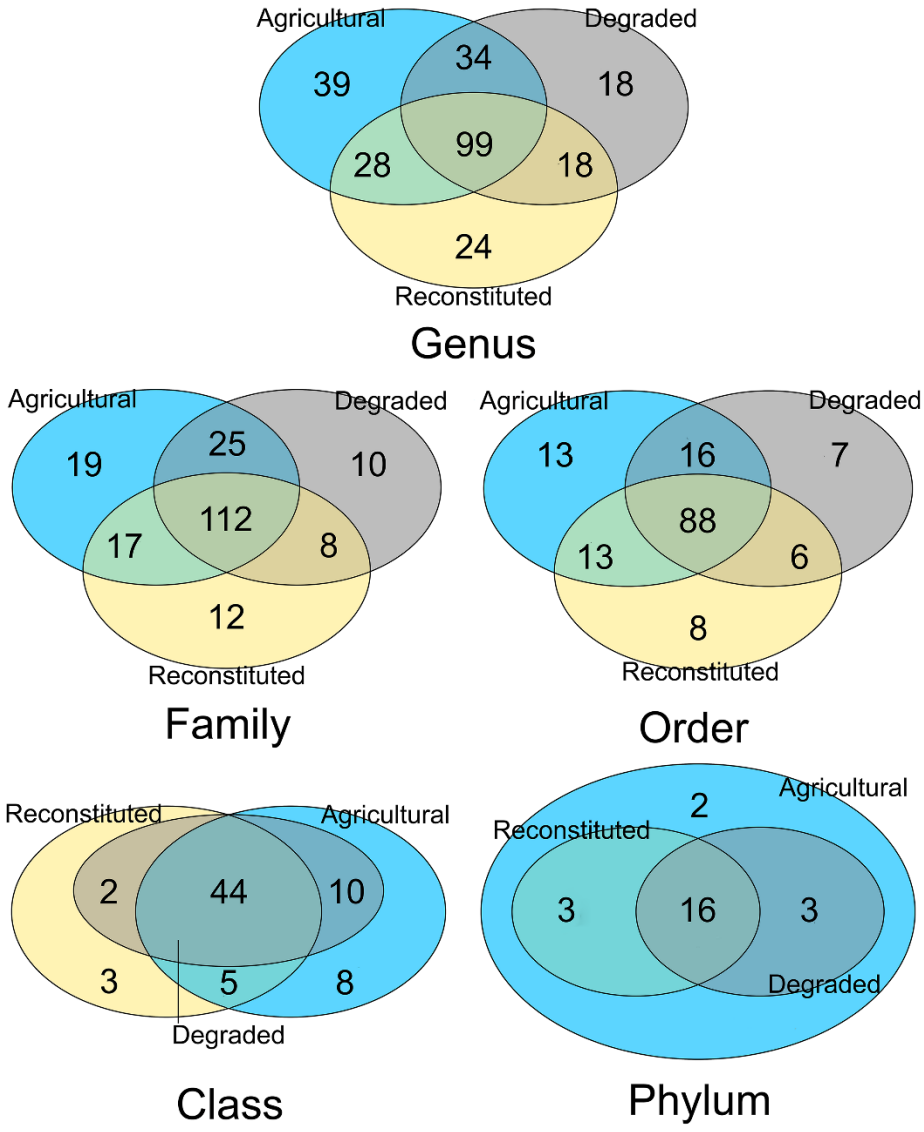


Figure 6. Venn diagrams showing the number of shared taxa (overlapping core and partials) and unique ones for each of the three types of soils, computed for each of the five taxonomy resolution layers. The stringency for unit's individuation abundance cutoff was set with the Relation-in-groups parameter = 0.40.

The qPCR analyses results of the *nifH*, the bacterial *amoA*, the *nosZ*, and the *nirK* genes are compared in Table 3. The RS samples yielded a higher content of the nifH gene copies when compared to the DS samples and to the AS samples. The bacterial *amoA* gene copies were significantly ($p < 0.05$) higher in the RS and in the DS samples that in AS samples.

RS samples showed the highest content of *nosZ* gene copies while DS samples showed a lower abundance and AS samples showed the lowest abundance.

The *nirK* gene copies showed a significantly lower abundance ($p < 0.01$) in AS samples than DS and RS samples.

Table 3. Gene copy numbers resulting from the qPCR analysis conducted targeting bacterial genes involved in the nitrogen biogeochemical cycle.

| Sampling Site | *amoA** | *nifH*** | *nosZ*** | *nirK*** |
|---|---|---|---|---|
| | Gene Copy number | Gene Copy number | Gene Copy number | Gene Copy number |
| Agricultural | $5.69\times10^{4}\pm5.20\times10^{3}$ b | $8.98\times10^{5}\pm8.37\times10^{4}$ c | $2.51\times10^{5}\pm4.76\times10^{4}$ c | $3.23\times10^{3}\pm2.02\times10^{2}$ b |
| Degraded | $7.91\times10^{4}\pm9.05\times10^{3}$ a | $9.22\times10^{6}\pm2.33\times10^{6}$ b | $1.51\times10^{6}\pm3.01\times10^{5}$ b | $1.17\times10^{4}\pm1.35\times10^{3}$ a |
| Reconstituted | $9.66\times10^{4}\pm8.31\times10^{3}$ a | $2.50\times10^{7}\pm6.50\times10^{6}$ a | $3.66\times10^{6}\pm4.07\times10^{5}$ a | $1.51\times10^{4}\pm1.86\times10^{3}$ a |

Means with the same letter in the vertical comparison among the sampling sites are not significantly different at Duncan's test. *Significance level $p<0.05$. **Significance level $p<0.01$.

## Discussion

Soils comprehend a wide range of variable conditions, including abiotic conditions, for instance, nitrogen availability and circulation, and biotic conditions that can affect the structure and the abundance of microbial communities (Islam et al., 2020). In addition, soil microbial communities are affected by anthropogenic activities like agriculture practices and environmental pollution (Fierer, 2017; Teng and Chen, 2019).

In this study we observed that, despite the lowest quantity of total soil DNA, the AS samples, under conventional management, showed higher *α*-diversity of the bacterial community when compared to the DS samples and to the RS samples. A lack of correlation between DNA yield and bacterial diversity was previously reported by Sagova-Mareckova et al., 2008. Total soil DNA is more related to soil microbial biomass, and it is generally related to the soil pH value, the clay and organic matter content, and the vegetation cover (Burgmann et al., 2001). Thus, more microbial diversity seems to be related to long term soil activity and it is not easily reproducible with human interventions (Strickland et al., 2009). Abiotic stresses, like the discontinuous

availability of nutrients and oxygen, and biotic stresses, like the presence and the abundance of predators, exert evolutionary pressure on soil microbial communities and help to select differences among the species without affecting soil functions (Hovatter et al., 2011; Jackson and Fahrig, 2014). The increase of microbial diversity at deeper taxonomic levels in RS samples when compared to DS samples might depend on the patented reconstruction technique that consists of a chemo-mechanical process. This reconstruction technique seems to implement particle aggregation and soil porosity enhancing the exchange of gasses and liquids (Manfredi et al., 2019), leading to a more suitable environment for soil microorganisms' proliferation. The PCA plot's underlying value

also confirms that the taxonomic features of each sampling site were significantly different ($p < 0.05$) to cluster the analyzed soil samples. The same was confirmed by the PCoA and by the Permdisp2 analyses.

As regards details from the core vs. specific/unique microbiome analysis, at phylum level two bacterial phyla, Latescibacterota and NB1-j, were uniquely present in AS samples, although at low frequencies (both <1%). Those phyla are reported to be present in several environments although their function is still unknown (Jimenez et al., 2020; Dries et al., 2021; Hamdan et al., 2008; Coelho et al., 2016). At order level Chtonomonadales, a bacterial order capable to utilize different carbohydrate substrates as carbon and energy sources (Wang et al., 2019), were almost unique in AS samples. Lactobacillales, lactic acid bacteria (Baureder and Hederstedt, 2013), and Bacteroidales, a bacterial order present in human and animal faeces (Levantesi et al., 2012), were encountered almost only in DS samples, although not in a dominant fashion. RS samples, instead featured Chlamydiales, a typical soil order reported being found in agricultural soils (Schmalenberger and Tebbe, 2002), but again not as prevailing members. In general however, it can be commented that, at each taxonomy level examined, the truly dominant members across all replicates of the three types of soils were the same, with the Proteobacteria (Gamma- and Alpha-) and Actinobacteria leading at Phylum/Class levels, the Rhizobiales and Burkholderiales at order level, the nitrifying Nitrosomonadaceae at family level. More peculiarities instead emerged at Family and even more at Genus levels. These phenomena, besides the Venn diagram comparisons shown in Figure 6, are also entailed in the rank-related shifts shown for the three ecological parameters in Figure 3. Interestingly, the superior biodiversity values displayed by the AS samples are mostly maintained at high ranks as phylum, order, class etc., but are progressively overcome by the reconstituted restored soil, that appears to “catch up” when examined levels are unclustered in deeper and deeper ranks, culminating at genus level. In the comparison between the degraded and reconstituted soils, it is worth noticing that, while at high ranks, and particularly at class level, the Shannon index and evenness means of the reconstituted soil were lower than those of the degraded one, such is not the case for richness, which is the only index of the three, whose formula is not linked to the number of individuals found. This suggests that in both these soils numbers of individuals have an impacting effect on the ecological outcome, irrespective of the number of taxa, as long as broad categories are considered. On the contrary, when finer taxonomy resolution is the metrics (genus), the reconstituted sampling site prevails and equates the agricultural sampling site. Such sampling site can be considered to be also under a disturbance (being cropped), but with a very long history of

adaptation to that predictable and recurring type of perturbation. In practice, Figure 3 shows that the agricultural soil is both phylum-rich and genus-rich, the degraded soil is phylum-poor and genus-poor, and the reconstituted soil is phylum-poor but genus-rich. This trend is consistent with data on really undisturbed controls as climaxing forest soils (Rösch et al., 2007), in those, phylum richness resulted even higher than that of cropped soils, but their established communities had relatively less genera and species, leading to what would be the fourth of these combinations (phylum-rich and genus-poor). In practice, the reconstruction of the degraded soils shows that in a few years, such degraded soil, which is the origin of the restored one, could be rescued up to a level of microbiodiversity that compares with that of the nearest agricultural soil control. Thus, the environmental carrying capacity K for possibly hosted species (Odum, 1953) and its imposed diversity ceiling appear to have been achieved by the soil reconstruction procedure, which can be seen as a-rather relevant ecological goal.

The interpretation of these trends suggests an intriguing picture. The short/unpredictable/non-cyclic type of human perturbation that led to the landfill conversion (the degraded soil DS) abated community structure and led to the loss of high- ranked taxonomical divisions (phyla), whose establishment involves time. The same soil, after 5 years of restoration (the RS soil) shows that, although this is too short a time to allow the return of lost phyla, it is nevertheless sufficient to drive a low- ranked diversification, leading to the recovery of diversity when measured by the genus metric. It can be also hypothesized that in fact the sudden absence of some previously present phyla, could even have left the available niches that would be then filled by the multiplying variants stemming from the remaining phyla. A scenario that, upon scaling-up of larger size and generation time, would comply to the “dinosaur-extinction/mammals radiation” model. The covariation of metabolic rate and body mass is in this sense well-demonstrated by Kleiber’s law (Kleiber, 1947). However, viewing the phenomena observed here as truly micro-evolutionary, would conflict with the notion of the 16S sequence being a molecular clock whose changes should require far longer timescales (Clark et al., 1999). Therefore, the rise of genus-level diversity in the reconstituted sampling site could be interpreted possibly as partly due to a physical recruitment (airborne immigration from other sites) and partly to a ‘technical’ recruitment, consisting in an increased detectability of reads in sequencing libraries as a consequence of the loss of other taxa that were otherwise quenching the counts of rare ones. The former mechanism (immigration) would not be sufficient by itself to explain why cells representative of missing genera should immigrate more easily than those of missing phyla and classes. But, since some phyla can encompass an extremely

high diversity, while some other could be represented by even a single known species, the difference can be accounted for.

As regards the qPCR analyses of the N cycle genes, assessing the abundance of bacterial functional genes involved in nitrogen circulation is a useful tool to evaluate soil health and quality. The key steps of the nitrogen biogeochemical cycle are nitrification, the process that converts ammonium firstly in nitrite and secondly in nitrate, and denitrification, the process that reduces nitrate to molecular nitrogen (Tang et al., 2019). qPCR validated the abundance of selected microbial targets by evaluating *nifH*, bacterial *amoA, nosZ*, and *nirK* genes involved in the nitrogen biogeochemical cycle within the sampling sites. The degraded and the reconstituted soil samples showed a higher abundance of all the nitrogen-circulating tested genes compared to the agricultural soil samples. These disparities in gene copy numbers might be related to soils nitrogen content. Anikwe and Nwobodo (2002) reported that nitrogen content in the superficial horizon was 646–750% higher in long-term municipal waste landfill sites compared to agricultural sites. In addition, our chemical analyses results, that are in accordance with previously published results by Manfredi et al., 2019, highlighted that the reconstruction patented technique increases soils' nitrogen content. Thus, the increased nitrogen inputs could have led to a higher nitrification and denitrification potential of degraded and restored soils. The fact that all these functional genes were found quantitatively in higher copies in the degraded and restored soil can be interpreted also in light of the above discussed result of the averagely six-fold higher content of total extractable soil DNA in both of them when compared to the agricultural cropped AS control soil. In interpreting both that difference and the ones resulting in these N-linked functional genes, it can be commented that the degraded and restored soils, being examples of recent and non-cyclic perturbations, turning over their previous nature, can be envisaged also as the equivalent of active construction worksites, in which the microbial populations would be engaged in multiplication, new nutrient flow interception, and a number of reorganizational responses in the shifted communities, that would explain the observed higher DNA values. Nevertheless, soil total DNA could be also contributed by fungi, protists, and by the remnant material from plants and animal origin. Therefore, the active state of bacteria could not necessarily be involving all of them but more likely, some funcrional groups as the ones we tested by qPCR.

In conclusion, several ecological hints arose from this comparative study. It is not easy to assess whether the differences acquired by the restored soil arose by the new chances open by the perturbation as such, which modified the environmental conditions, or were more specifically due to the restoration technique itself that requires the application of non-

sterile sludges coming from specific industrial processes. In addition, it is still unclear if the enhanced microbial diversity in RS samples, when compared to that of the DS samples, would be temporary or permanent. It can be also underlined that, while the restoration allowed the recovery of the ecological indexes of diversity, however the resulting community profile moved even farther away from the one of the agricultural soil, as shown in the cluster dendrogram of Figure 4, in which the degraded and agricultural soil bacteria appear closer to each other. The effect was therefore that of a shift to a novel assemblage, whose equilibrium and fate would have to be assessed in time. Of equal importance would be to determine whether the increased gene copies of the nitrogen cycling could entail some novel environmental concerns. In highly fertilized soils, considering that, in those cases, soil microbial activity is not able to metabolize the entire amount of nitrogen (Zilio et al., 2020), leading to nitrogen leaching through the vertical profile that can potentially reach subsurface water bodies. Their N enrichment is in fact one of the main causes of eutrophication. Such environmental syndrome, consisting of nutrients enrichment of water, culminates in the large production of biomasses related to algae proliferation. The degradation of these, once their short life cycle turns them into necrotic masses, results in hypoxia or anoxia situations and, also, in toxic bacterial emissions of methane, carbon dioxide and hydrogen sulphide (Le Moal et al., 2019). On the other hand, however, three reassuring issues can be put forward against these concerns. The first is the fact that a higher content of soil DNA is also reported in literature as a positive proxy for soil equilibrium (Fusaro et al., 2018). The second is that, since the gene copies detected by qPCR increased in all targeted genes, the phenomenon could be framed within that of the overall increase of soil DNA. The third is that, among the four PCR-targeted genetic determinants, the one that increased the most, and that did so in a statistically significant manner also in the comparison between degraded and restored soil, was *nifH*, i.e. one of the structural subunits of the nitrogenase protein, to which biological nitrogen fixation from either free- living or symbiotic prokaryotes is ultimately due. Being such metabolism the main gateway for nitrogen entrance into terrestrial as well as aquatic food chains, the enhancement of its key enzyme can be described as a positive premise in the pursuit of an improved environmental sustainability.

**Data Availability Statement**

The dataset generated for this study can be found in the European Nucleotide Archive, ID PRJEB48383.

## Authors' Contribution

The first two authors contributed equally to this work. LM, SD, and PS made the conception, design of the study. LM, SD, GC, GR, and PM carried out the sampling. LM, SD, and CC did the sequencing. LM, SD, and SR, performed analyses. LM, SD, and AS conducted statistical analyses. LM, SD, and PS wrote the paper. LM, SD, AS, GR, and PS contributed to critical writing and reviewing of the manuscript. All authors reviewed the manuscript and gave final approval for publication.


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## Supplementary Material

Table S1. Top most abundant unique microbial taxa for each of the three soil types, computed for each of the five taxonomy resolution layers.

| Agricultural Soil (AS) | Degraded Soil (DS) | Reconstituted Soil (RS) |
|---|---|---|
| | *Phylum* | |
| Latescibacterota | | Chlamydiae |
| | *Class* | |
| Chthonomonadetes<br>phylum Acidobacteriota, Subgroup 22<br>Coriobacteriia | | BD2-11 terrestrial group<br>Omnitrophia |
| | *Order* | |
| Chthonomonadales<br>Coriobacteriales | Bacteroidales<br>Lactobacillales<br>Brevibacillales<br>Ectothiorhodospirales<br>Lachnospirales | Chlamydiales<br>Ktedonobacterales<br>Micropepsales |
| | *Family* | |
| order Azospirillales, uncultured family | Kiloniellaceae<br>Geobacteraceae<br>Lachnospiraceae<br>Thioalkalispiraceae<br>Paludibacteraceae | Ktedonobacteraceae<br>phylum Bacteroidetes NS11-12 marine group<br>Micropepsaceae |
| | *Genus* | |
| *Candidatus* Alysiosphaera<br>*Candidatus* Ovatusbacter<br>order Azospirillales, uncultured genus<br>*Filomicrobium*<br>phylum Planctomycetes AKYG587<br>phylum Planctomycets, Pla3 lineage | *Actinoallomurus*<br>phylum Bacteroidetes WCHB1-32<br>*Laceyella*<br>*Rummeliibacillus*<br>*Crenothrix*<br>*Variovorax*<br>*Leuconostoc*<br>*Acinetobacter*<br>*Candidatus* Berkiella<br>*Brevibacillus* | *Methylocaldum*<br>phylum TM7 uncultured genus<br>*Bauldia*<br>*Flavitalea*<br>*Haloactinopolyspora*<br>*Terrabacter*<br>family Microtrichaceae, uncultured genus<br>*Niastella*<br>*Dokdonella* |

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Conflict of Interest: PM is employed by MCM Ecosistemi S. r. l.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# CONTRIBUTION II

# 16S metabarcoding, total soil DNA content, and functional bacterial genes quantification to characterize soils under long-term organic and conventional farming systems

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**Abstract**

Background

The threatening impact of conventional agriculture (CA) on soils could be due to the detrimental effects on soil microbial communities. Conversely, organic agriculture (OA) is envisaged as potentially enhancing helpful microbial communities and is proposed as environmentally sustainable. The soil microbiome influences soil health and quality, hence, it requires deeper investigation and understanding. In this study, applying 16S metabarcoding and qPCR techniques, we compared the microbial patterns of long-term organically and conventionally managed soils to explore their similarities and differences.

Results

Total DNA quantification showed an over twenty-fold higher amount of DNA in OA soils (mean=22.1±3.92 $\mu g \cdot g^{-1}$), compared to CA soils (mean=0.95±0.17 $\mu g \cdot g^{-1}$). While 16S metabarcoding evidenced the absence of significant differences among communities of the two farming systems in terms of ecological indices, the qPCR analyses targeting functional genes reported a significantly higher abundance of all considered targets in OA sites spanning up to four-fold log increases. While OA and CA did not appear to affect overall bacterial diversity or evenness *per se*, qPCR-based functional analysis in OA showed a consistently higher abundance of all the salient microbial genes tested, when compared to CA, underlying a potentially beneficial impact on soil fertility and sustainability.

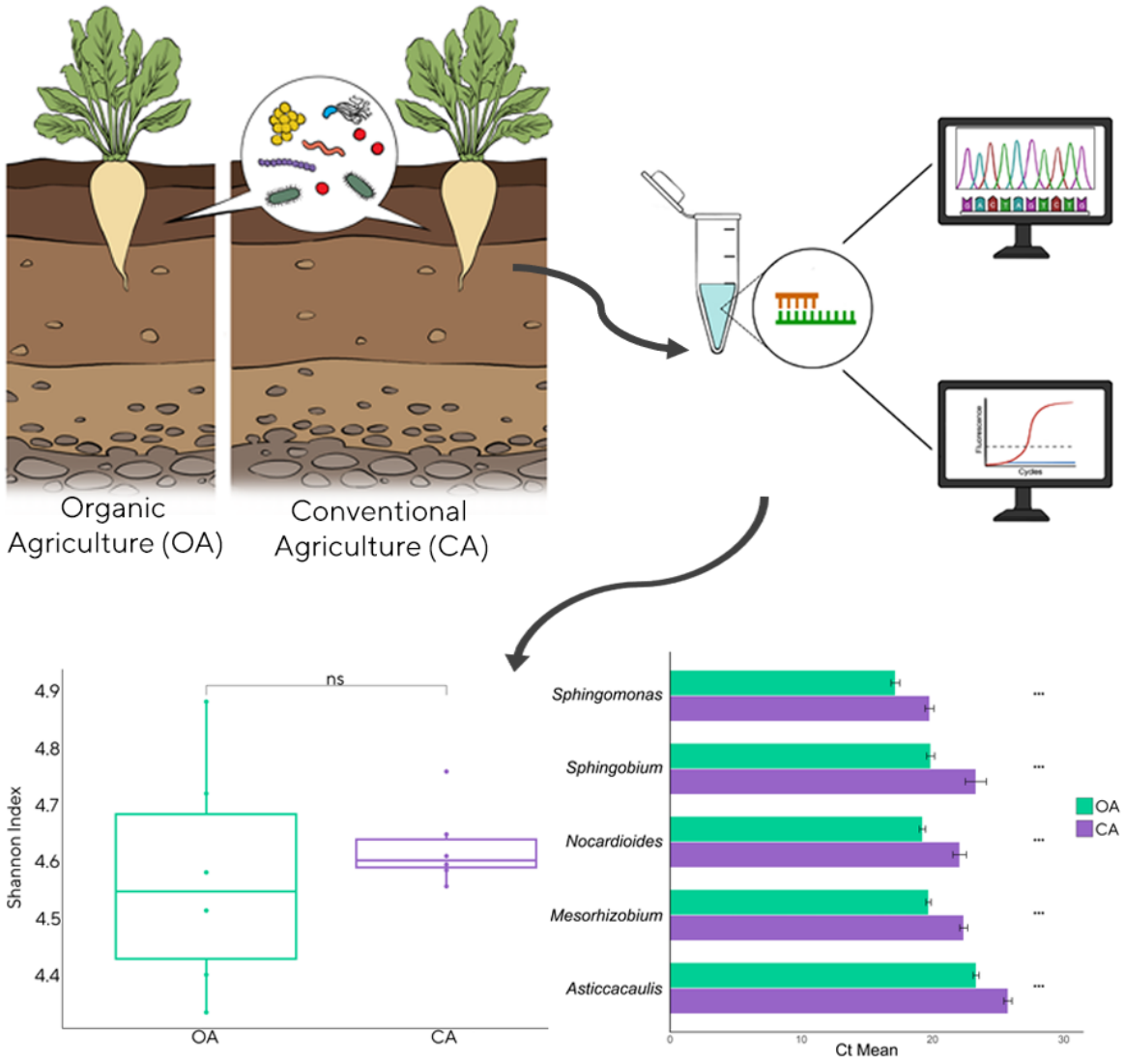
Conclusions

In essence, the sequencing-based analysis of absolute bacterial diversity could not differentiate the farming systems based on the amount of diversity but identified a unique set of taxa defining each. Hence, pairing this evaluation with the qPCR-based functional gene analyses can be a suitable approach to distinguish the exerted effects of CA or OA on soils.

**Keywords**

Organic agriculture, conventional agriculture, sustainable agriculture, 16S-metabarcoding, multi-amplicon sequencing, functional genes, soil profiling, plant-growth-promoting rhizobacteria, total soil DNA, sugar beet.

## Graphical Abstract




## Background

The global population, which is predicted to reach nine billion people by 2030 by the United Nations (2022), is demanding the modern agricultural sector to achieve several goals that need careful long-term planning (Pelletier and Tyedmers, 2010, Foley et al., 2011). Agriculture is expected to provide an increasing quantity of high-quality food to suppress hunger and malnutrition, but at the same time, prevent further impact on the surrounding environment (Godfray, 2010). The dilemma of choosing between nutritional versus environmental sustainability requires a thoughtful search for reliable solutions. Conventional agriculture has achieved several benefits for agricultural productivity, including increased yields and reduced crop losses, due to the utilization of external inputs

(Dunwell, 2010, Reganold and Wachter, 2013). However, these practices have led to an intensification of the environmental pressure (Gasteyer, 2008, Gomiero et al., 2008) and a diffuse detrimental impact on ecosystems across the world (Bommarco et al., 2013, Duru et al., 2015). By reducing the use of external inputs and integrating several practices considered more environmentally friendly, organic agriculture strives to address the challenge of limiting the anthropogenic factors that might cause the degradation of ecosystems (Heckman et al., 2015).

Sugar beet (*Beta vulgaris* L.), which serves as the primary source of approximately 20% to 30% of the annual global sugar production, constitutes an essential crop within the agricultural landscape of 52 countries situated in the temperate climate zone (Dohm et al., 2014, Nieberl et al., 2017, Stevanato et al., 2019). According to Eurostat (2020), EU-27 countries grew ~1.5 million ha of sugar beet in 2019 and harvested ~111.6 million t (74.4 $t \cdot ha^{-1}$ on average), which accounts for about half of the world's total production. The largest sugar beet producers in the EU-27 are France, Germany, and Poland, which together produce more than 60.0% of the EU-27's sugar beets (France 31.0%, Germany 22.8%, and Poland 12.1%). The demand for organic sugar is increasing in line with the growth of the organic market. In Europe, sugar production from organically grown beets is still embryonic with production reported only in Italy, Sweden, Denmark, and the Netherlands. One of the reasons for the low availability of organically produced sugar beet is the vulnerability to failure due to the management gap in organic farming. Moreover, organic sugar beet farming requires further improvements to achieve true multifunctionality that can assure a root yield comparable to the one identifying conventional farming along with the provision of several collateral ecological services that contribute to the preservation of the agroecosystem (Jordan et al., 2007, Chabert and Sarthou, 2020).

The preservation of the agroecosystem revolves around the concepts of protection of soil health and quality and implementation of sustainable soil management practices. Soil, in this respect, has been defined as an indispensable complex natural resource that provides ecological functions, such as gene reservoir, and non-ecological functions, like archaeological artifact protection (Blum, 2005). However, seeing soil as a granted commodity rather than the delicate result of the continuous delivery of plant organic compounds, and their microbially-mediated breakdown and re-synthesis risks shifting its understanding from its actual nature. The live components of soil, encompassed within the soil microbiome pool, have a crucial role in building and maintaining its structure, which in turn regulates water availability, hosts the transformation and cycling of nutrients, the

catabolism of toxins, mainly by microbes, that account for a sizeable portion of the global genetic diversity (Larkin, 2015). Therefore, it is crucial to deepen our knowledge of soil microbes and the extent of their active services in agricultural contexts, to protect soil ecosystems and assure human welfare.

Improved molecular biology and high-throughput sequencing-based methods have been used in recent years to better understand microbial communities over traditional microbiology methods. By employing culture-independent techniques, the limitations associated with obtaining comprehensive information from the challenging cultivation of microorganisms collected from the environment have been effectively overcome (Vester et al., 2015). DNA metabarcoding techniques using high-throughput sequencing, sequence alignment tools using bioinformatics methods, and databases of annotated microbes contribute to the determination of microbial species and knowledge of their genome functions in the soil. This is crucial in determining soil fertility and productivity with the goal of improving the agricultural system by relying more on microbial ecosystem services and less on the utilization of external input factors. Lately, multi-amplicon-based sequencing approaches provide a robust compositional structure of individual microbial community members and quantitative Real-Time PCR (qPCR) based approaches are used downstream for precise quantification of microbial targets (Dreier et al., 2022). Additionally, significant improvements in the algorithms and analysis methods of 16S analysis have been undertaken for community diversity estimation. Particularly, amplicon sequence variants (ASVs) based clustering by predicting sequencing errors and denoising the reads (Callahan et al., 2016) outperformed the operational taxonomical units (OTUs) based approach (Joos et al., 2020).

The hypothesis underlying this study posits that soil bioindicators have the potential to display discernible variations when comparing conventional and organic farming practices. By investigating these bioindicators, the study is aimed to identify suitable markers from the soil microbiome in differentiating the effects of conventional versus organic farming practices by testing: a) total soil DNA content, b) 16S bacterial community using metabarcoding, and c) specific functional gene quantitation via qPCR amplification.

## Methods

### Sites' location and soil sampling

Soil samples were collected in six agricultural sites located in the Po River valley, Northeast Italy (Figure 1). Among these sites, three were under organic agriculture (OA), and three

were under conventional agriculture (CA). The three organically-managed sites were located in Canal Dei Cuori farm (Loreo, RO, N 45°05'02'', E 12°10'55''), Le Barbarighe farm (San Martino di Venezze, RO, N 45°06'10'', E 11°53'03'') and Terre Emerse farm (Lozzo Atestino, PD, N 45°17'25'', E 11°35'00'') respectively. One of the three conventionally-managed sites was located in Marsilio farm (Pozzonovo, PD, N 45°10'51'', E 11°47'48''), and the other two were located in the Cooperativa Produttori Bieticoli (CoProB) farm (Minerbio, BO, CoProB 1 N 44°36'56'', E 11°31'0.4'; CoProB 2 N 44°38'12'', E 11°33'49'').

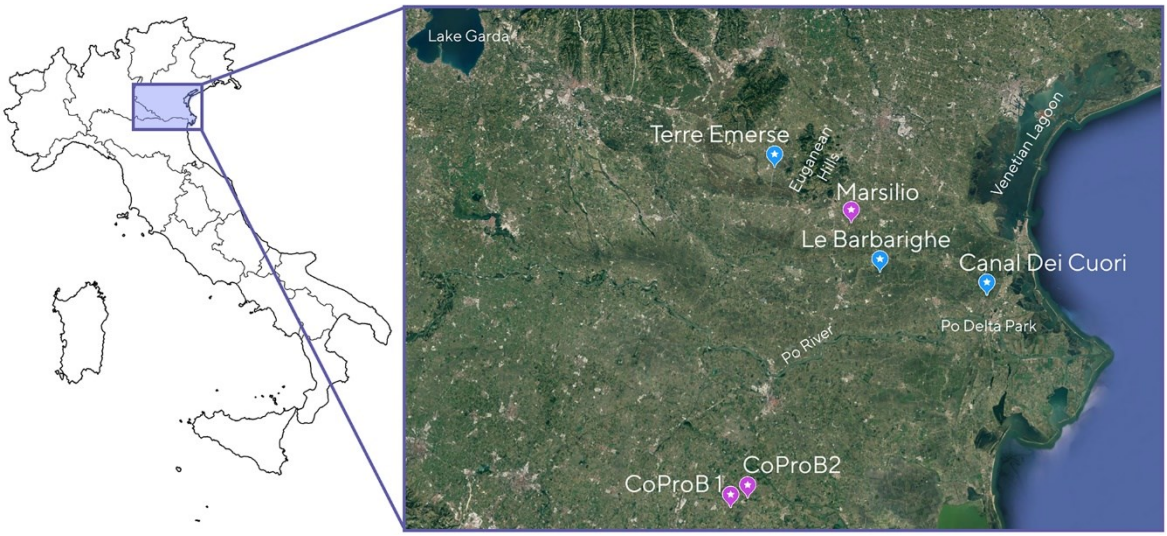



Figure 1. Left: Contour border line of Italy with the indication of the experimental zone; Right: Aerial photograph of the studied area located in the Po River valley. The OA sites are in blue, and the CA sites are in purple.

The soil classification, reported in Table 1, was based on the soil maps produced and published by the Regional Agency for Prevention and Environmental Protection of Veneto (ARPAV) (2020) and by the Emilia-Romagna Region (RER) (2021). Regarding the OA sites, Canal Dei Cuori and Le Barbarighe soils were classified as Endoaquolls (Soil Survey Staff, 2010) and Gleyic Phaeozem (IUSS Working Group, 2006), and Terre Emerse soil was classified as Haplustept (Soil Survey Staff, 2010) and Haplic Cambisol (IUSS Working Group, 2006). Concerning the CA sites, Marsilio soil was classified as Haplustept (Soil Survey Staff, 2010), and Fluvic Cambisol (IUSS Working Group, 2006), and CoProB soils were classified as Haplustepts (Soil Survey Staff, 2010), and Fluvic Cambisol (IUSS Working Group, 2006). All sampling sites, which have been applying their respective soil management for over 20 years, were under sugar beet cultivation at the sampling time, which occurred in the month of August 2020. At the sampling time, all sugar beets within the sampled fields had attained the harvestable size (BBCH-49) (Meier et al., 1993). Sugar beet yield traits such as root yield, sugar yield, and processing quality-related traits were evaluated in 2020 and 2021 (Supplementary Material, Table SM1). Soil sampling was performed by applying the grid sampling technique (Brown, 1993). A manual auger was

used to collect forty-eight sub-samples at a 15 cm depth from each sampling site. Sub-samples were, then, mixed to obtain two biological replicates from each farm, referred to as sample “a” and sample “b”. The composite samples were subjected to a 72-hour air-drying process at room temperature (Wang et al., 2021), followed by sieving through a nested 0.5 mm wire mesh and subsequent immediate physicochemical analyses and total soil DNA extraction.

**Soil physicochemical analyses**

Soil samples were profiled by applying physical and chemical analysis techniques. Particle size distribution, compiled in Table 1, was investigated for each sample using laser diffraction analysis (Mastersizer 2000, Malvern Panalytical, Malvern, United Kingdom) (Bittelli et al., 2022). Soil pH was measured potentiometrically by applying the soil/ultra-pure water ratio 1:2.5 w/v. A combustion analysis was performed to assess the concentration of total N, total C (Elementar Vario MACRO CNS, Elementar Analysensysteme GmbH, Hanau, Germany) and organic C (Skalar Primacs$^{SNC-100}$, Skalar Analytical BV, Breda, The Netherlands). The content of extractable phosphorus was determined using the Olsen P method (Olsen et al., 1954). Exchangeable cations assessment was performed using inductively coupled plasma optical emission spectrometry (ICP–OES) (Spectro Arcos MV, Spectro Ametek, Kleve, Germany).

Table 1. Results of the physical analysis to assess the particle size distribution characterizing each soil sample. The Soil taxonomy classification is based on the USDA and the WRB guidelines

| Sample | Sand % | Silt % | Clay % | USDA Soil Taxonomy | WRB Soil Taxonomy |
|---|---|---|---|---|---|
| CoProB 1 | 30.2 | 54.7 | 15.1 | Haplustept | Fluvic Cambisol |
| CoProB 2 | 20.1 | 56.0 | 23.9 | Haplustept | Fluvic Cambisol |
| Marsilio | 25.9 | 51.0 | 23.1 | Haplustept | Fluvic Cambisol |
| Canal Dei Cuori | 3.6 | 63.5 | 32.9 | Endoaquoll | Gleyic Phaeozem |
| Le Barbarighe | 4.2 | 56.8 | 39.0 | Endoaquoll | Gleyic Phaeozem |
| Terre Emerse | 20.5 | 50.3 | 29.2 | Haplustept | Haplic Cambisol |

**Total soil DNA extraction, multi-amplicon 16S rDNA sequencing, quantification of functional genes for ecosystem services and bacterial plant-growth-promoting traits**

Total soil DNA extraction from 250 mg of air-dried soil was performed using the DNeasy PowerSoil Pro Kit (Qiagen, Germany), in accordance with the manufacturer's instructions. Purified nucleic acid quantification was carried out using a Qubit Flex fluorometer (Thermo

Fisher Scientific, Carlsbad, CA) with a Qubit 1x dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Carlsbad, CA).

16S rDNA meta-barcoding library preparation was performed using the 16S Ion Metagenomics Kit (Thermo Fisher Scientific, Carlsbad, CA) that carries two sets of pooled primers targeting seven hypervariable regions of the 16S rRNA gene. The templating of 400 bp was carried out in an Ion One Touch 2 instrument using an Ion 520 chip and sequenced on the Ion GeneStudio S5 System (Thermo Fisher Scientific, Carlsbad, CA).

Raw reads processing was performed conforming to the pipeline published in Maretto et al. (2022). Demultiplexed uBAM files from the sequencer were converted into FASTQ format using samtools bamtofastq (v1.10) (Li et al., 2009). Raw reads were trimmed for 20 nucleotides on both ends to remove primers using cutadapt (v3.5) (Martin, 2011). A Quantitative Insights Into Microbial Ecology 2 (QIIME2) (v2020.08) (Bolyen et al., 2019) microbiome pipeline was further used to analyze the raw reads. Imported reads were first denoised and dereplicated using the “qiime dada2” plugin followed by taxonomic classification of ASVs by a “classify-consensus-blast” plugin using SILVA SSU (v138.1) (Quast et al., 2012) as the reference database. Alpha diversity rarefaction analysis was done using the “qiime alpha-diversity” plugin and corresponding results were plotted using ggplot2 R-package (Wickham, 2016). The feature abundance table and taxonomic assignment table were exported and further processed using RStudio (version R-4.2.2) (Posit Team, 2022, R Core Team, 2022) in conjunction with the tibble package (Müller and Wickham, 2023) and TaxaPhyloseq (McMurdie and Holmes, 2013). Partially classified taxonomic entries or entries with counts below 10 were excluded from the analysis. MicrobiotaProcess packages (Xu et al., 2023) in R were used to calculate the diversity indices, perform PCoA analysis and, counts normalization. All plots were generated using ggplot2 R-package.

The abundance of the 16S gene, and that of genes involved in the N biogeochemical cycle such as amoA (eubacterial, AOB and archaeal, AOA ammonia oxidase/nitrification), nifH (nitrogenase/nitrogen fixation), nirK (nitrite reductase/intermediate denitrification), and nosZ (nitrous oxide reductase/terminal denitrification), and the abundance of the gene coding for the gh48 cellulase enzyme, of the PKs (polyketides) and NRPs (non-ribosomal peptide) cluster genes that are thought to play a crucial role in the adaptation of bacteria to soil, and in plants' health and development (Dror et al., 2020), were analyzed by qPCR using a QuantStudio 12K-Flex apparatus (Thermo Fisher Scientific, Carlsbad, CA). Moreover, the presence of specific 16S rDNA from *Asticcacaulis*, *Mesorhizobium*,

*Nocardioides*, *Sphingobium* and *Sphingomonas*, which are known prominent sugar beet growth-promoting rhizobacteria (PGPR) (Okazaki et al., 2021), was assessed using the same qPCR approach. All primers sequences used are reported in Table 2. The assays targeting the five PGPR have been designed based on the sequences retrieved from the 16S metabarcoding data generated for this study. The reaction mix was composed of 2.5 µL PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Carlsbad, CA), 0.15 µL each of forward and reverse primer, 1.2 µL PCR-grade water, and 1 µL template DNA. The numbers of gene copies in soil for the targeted genes were calculated starting from the Ct values on the basis of calibration curves constructed using specific concentrations of *Escherichia coli* for the 16S gene or of the targeted functional genes cloned in plasmids of known length for the other amplicons (Zanardo et al., 2016).

Overall statistical analyses, including those performed on soil chemical properties, were performed using RStudio and the dplyr package (Wickham, 2023). The evaluation of significant differences between the mean values occurred with the non-parametric Wilcoxon Rank Sum test (Wilcoxon, 1945). Data are expressed as mean ± standard error.

Table 2. Sequences of the primers used in this project and targeted amplicon length. The NCBI accession number is provided for all the primers designed on the base of a reference sequence obtained from this work's metabarcoding

| Primer | Sequence | References |
|---|---|---|
| 16S F | GGGTTGCGCTCGTTGC | Johnson et al., 2016 |
| 16S R | ATGGYTGTCGTCAGCTCGTG | |
| Archaeal amoA F | STAATGGTCTGGCTTAGACG | Francis et al., 2005 |
| Archaeal amoA R | GCGGCCATCCATCTGTATGT | |
| Bacterial amoA F | GGGGTTTCTACTGGTGGT | Rotthauwe et al., 1997 |
| Bacterial amoA R | CCCCTCKGSAAAGCCTTCTTC | |
| nifH F | AAAGGYGGWATCGGYAARTCCACCAC | Rösch et al., 2002 |
| nifH R | TTGTTSGCSGCRTACATSGCCATCAT | |
| nosZ F | CGYTGTTCMTCGACAGCCAG | Rösch et al., 2002 |
| nosZ R | CATGTGCAGNGCRTGGCAGAA | |
| nirK F | ATYGGCGGVCAYGGCGA | Henry et al., 2004 |
| nirK R | RGCCTCGATCAGRTTRTGGTT | |
| gh48 8F | CGCCCCABGMSWWGTACCA | De Menezes et al., 2015 |
| gh48 5R | GCYTCCCAIATRTCCATC | |
| PKS I F | GGCAACGCCTACCACATGCANGGNYT | Amos et al., 2015 |
| PKS I R | GGTCCGCGGGACGTARTCNARRTC | |
| PKS II F | TSGCSTGCTTGGAYGCSATC | Wawrik et al., 2005 |
| PKS II R | TGGAANCCGCCGAABCCTCT | |

| | | |
|---|---|---|
| NRPS I F | CGCGCGCATGTACTGGACNGGNGAYYT | Amos et al., 2015 |
| NRPS I R | GGAGTGGCCGCCCARNYBRAARAA | |
| NRPS II F | GCSTACSYSATSTACACSTCSGG | Ayuso-Sacido and Genilloud 2005 |
| NRPS II R | SASGTCVCCSGTSCGGTAS | |

| Primer | Sequence | NCBI Accession Number |
|---|---|---|
| *Asticcacaulis* F | GCATTAAGCAATCCGCCTGG | NR_109665.1 |
| *Asticcacaulis* R | GGGATGTCCAGGCATGTCAA | |
| *Mesorhizobium* F | ATCCTGGCTCAGAACGAACG | NR_170463.1 |
| *Mesorhizobium* R | CCCGGAGTTGTTCCGTAGAG | |
| *Nocardioides F* | AATCTGCCCTTCACTTCGGG | FJ423762.1 |
| *Nocardioides* R | GAGCACATCCTCCACCGAAA | |
| *Sphingobium* F | CACTCGAAGGCGTTGAGCTA | NR_102886.2 |
| *Sphingobium* R | GCAGGTTCCCCTACGGCTA | |
| *Sphingomonas* F | GGCATGCCTAACACATGCAA | NR_132332.1 |
| *Sphingomonas* R | TATTCCGAACCCAAGGGCAG | |

**Results**

The analysis of the texture of the collected topsoils (Table 1), based on the size particle limits and abundance established by the United States Department of Agriculture (USDA) in 1951 (1951), showed that all CA samples are classified as “Silt Loam soils” since their silt percentage is higher than 50% and, those of sand and clay are in the range of 20–30% and 15–30% respectively. The samples from Canal Dei Cuori and Le Barbarighe farms are classified as “Silty Clay Loam soils” since they have less than the 20% of sand and clay content in the range of 27–40%. The samples collected in Terre Emerse farm are classified as “Clay Loam soils” since their percentages of clay and sand are higher than 27% and 20% respectively. All the soil chemical characterization results are compiled in Table 3. No significant differences were observed in soil pH, ranging between 7.60–8.11 in CA soils and 7.38–7.91 in OA soils. Total N content was not significantly different in the two farming systems. Both carbon forms explored in this study were significantly higher ($p$<0.01) in OA soils than in CA soils. In detail, the mean total C content in OA soils was 164% higher than in CA soils. Moreover, the organic C mean was 231% higher in OA soils when compared to CA soils. Olsen P did not show a significant difference between the two groups of soils. Among the exchangeable cations evaluated in this study, $Ca^{++}$ and $Mg^{++}$ were significantly

($p$<0.05) more abundant in OA soils than in CA soils. At the same time, $Na^+$ and $K^+$ showed no significant differences between OA and CA soils. The Principal Component Analysis (PCA) performed on the soil chemical analyses results showed (Figure 2) that the main parameters influencing dimension 1 are the exchangeable cations and the total N, and the main parameters influencing dimension 2 are total C and organic C.

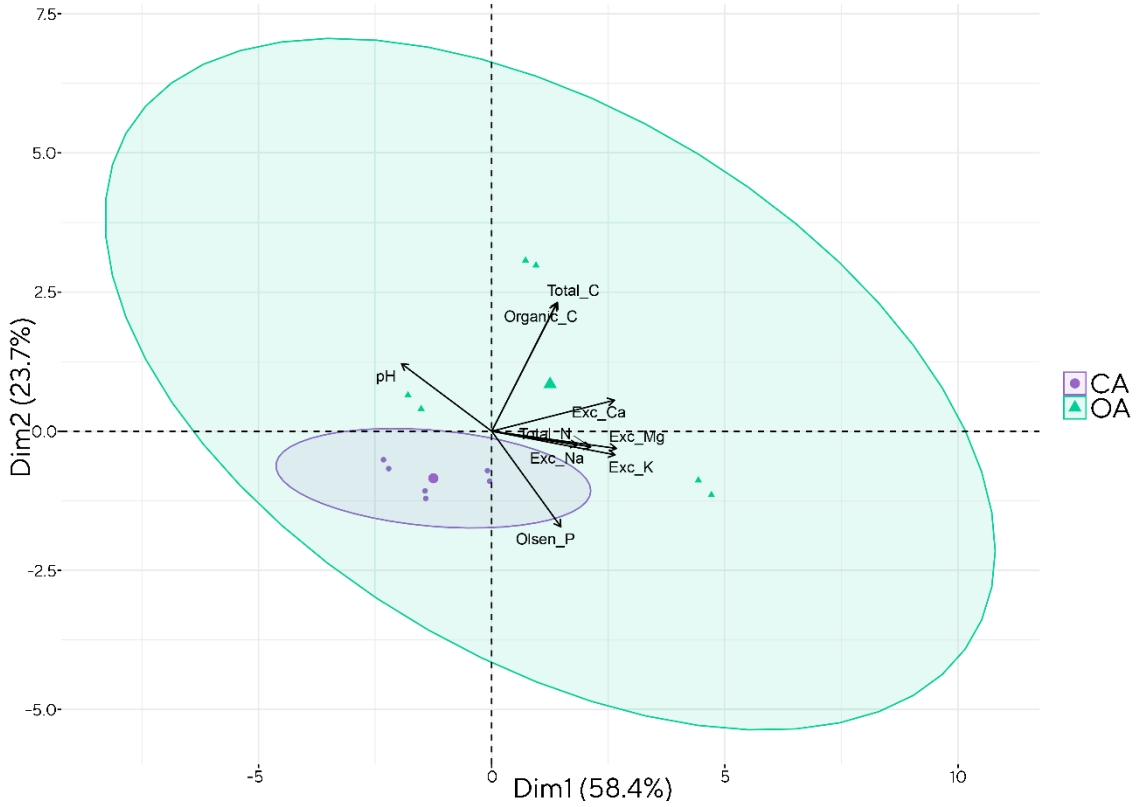



Figure 2. Principal Component Analysis (PCA) biplot showing the analyzed samples' spatial clustering based on their chemical properties. Samples on the same side of a given variable have a higher value for the same.

Total soil DNA quantification results highlighted that OA soils (mean=22.1±3.92 $\mu g \cdot g^{-1}$, individual farms: Canal Dei Cuori=33.45±0.75 $\mu g \cdot g^{-1}$, Le Barbarighe=20.61±0.27 $\mu g \cdot g^{-1}$, Terre Emerse=12.17±0.08 $\mu g \cdot g^{-1}$) held a significantly higher ($p$<0.01) amount of DNA than CA soils (mean=0.95±0.17 $\mu g \cdot g^{-1}$, individual farms: Marsilio=1.45±0.01 $\mu g \cdot g^{-1}$, CoProB 2=0.80±0.11 $\mu g \cdot g^{-1}$, CoProB 1=0.59±0.06 $\mu g \cdot g^{-1}$).

The metabarcoding analysis of the bacterial 16S gene on the soil samples provided 9,682,353 single-end reads with an average length of 217 nucleotides. The 32,725 identified ASVs were eventually classified into 980 database-featured taxa names. 91.1% of the annotated reads were classified at the phylum rank level, 90.3% at the class level, 88.7% at the order level, 87.0% at the family level, the 80.4% at the genus level.

Table 3. Results of the chemical analyses on the soil samples at the beginning of the experiment. All the analyses have been conducted on the dry matter (d.m.).

| Sample | pH | Total N g·Kg$^{-1}$ d.m. | Total C** g·Kg$^{-1}$ d.m. | Organic C** g·Kg$^{-1}$ d.m. | Olsen P mg·Kg$^{-1}$ d.m. |
|---|---|---|---|---|---|
| CoProB 1a | 7.99 | 1.96 | 29.54 | 11.86 | 71.53 |
| CoProB 1b | 8.11 | 2.02 | 29.89 | 11.77 | 72.44 |
| CoProB 2a | 7.61 | 1.56 | 28.30 | 11.16 | 58.09 |
| CoProB 2b | 7.73 | 1.63 | 28.47 | 11.20 | 60.31 |
| Marsilio a | 7.60 | 3.96 | 31.34 | 10.55 | 39.44 |
| Marsilio b | 7.66 | 4.02 | 32.15 | 11.26 | 40.58 |
| **CA** | **7.78±0.09 a** | **2.53±0.47 a** | **29.95±0.63 b** | **11.30±0.19 b** | **57.07±5.89 a** |
| Canal Dei Cuori a | 7.38 | 5.06 | 42.36 | 23.54 | 109.34 |
| Canal Dei Cuori b | 7.45 | 5.13 | 43.59 | 22.46 | 101.86 |
| Le Barbarighe a | 7.91 | 2.72 | 58.10 | 36.19 | 25.04 |
| Le Barbarighe b | 7.87 | 2.87 | 58.73 | 35.78 | 27.03 |
| Terre Emerse a | 7.86 | 3.42 | 37.17 | 17.77 | 26.55 |
| Terre Emerse b | 7.69 | 3.58 | 37.33 | 17.32 | 22.98 |
| **OA** | **7.69±0.09 a** | **3.80±0.43 a** | **46.21±4.00 a** | **25.51±3.46 a** | **52.13±16.95 a** |

| Sample | Exchangeable Ca* mg·Kg$^{-1}$ d.m. | Exchangeable Mg* mg·Kg$^{-1}$ d.m. | Exchangeable Na mg·Kg$^{-1}$ d.m. | Exchangeable K mg·Kg$^{-1}$ d.m. |
|---|---|---|---|---|
| CoProB 1a | 1734 | 140.30 | 65.90 | 64.12 |
| CoProB 1b | 1703 | 138.68 | 67.78 | 69.31 |
| CoProB 2a | 1886 | 166.97 | 91.09 | 84.67 |
| CoProB 2b | 1963 | 178.20 | 99.33 | 82.73 |
| Marsilio a | 3834 | 216.92 | 54.72 | 159.73 |
| Marsilio b | 4001 | 224.66 | 60.82 | 137.72 |
| **CA** | **2520±444 b** | **177.62±15.03 b** | **73.27±7.26 a** | **99.71±16.08 a** |
| Canal Dei Cuori a | 7004 | 472.80 | 118.20 | 267.92 |
| Canal Dei Cuori b | 7153 | 465.11 | 120.96 | 243.19 |
| Le Barbarighe a | 5122 | 244.53 | 93.47 | 121.28 |
| Le Barbarighe b | 5348 | 238.67 | 91.40 | 135.88 |
| Terre Emerse a | 2354 | 171.74 | 17.67 | 70.67 |
| Terre Emerse b | 2199 | 183.44 | 16.80 | 72.91 |
| **OA** | **4863±885 a** | **296.05±55.95 a** | **76.42±19.37 a** | **151.98±34.56 a** |

Means with the same letter in the vertical comparison are not significantly different at the Wilcoxon Rank Sum test. *Significance level $p<0.05$, **Significance level $p<0.01$.

The sequences rarefaction analysis conducted to assess whether the samples had been sequenced to a sufficient depth showed (Supplementary Material Figure SM2) that all the soils reached the plateau on the curve ranked from Canal Dei Cuori, the one with the

highest number of detected sequences, to CoProB 1, the one with the lowest. The alpha-diversity was examined within each taxonomical level by calculating three ecological indices such as Chao1, Shannon, and Simpson 1-D to evaluate community richness, diversity, and evenness, respectively. The results of the analyses conducted at the genus level, which is representative of all the higher taxonomic ranks, are reported in Figure 3. It is visible that, for all the considered indices, although some OA soils manifest the highest absolute values of richness, diversity and evenness, the means are even lower than those of the conventional soils and moreover, for none of the indices there was any significant difference between the two farming systems.

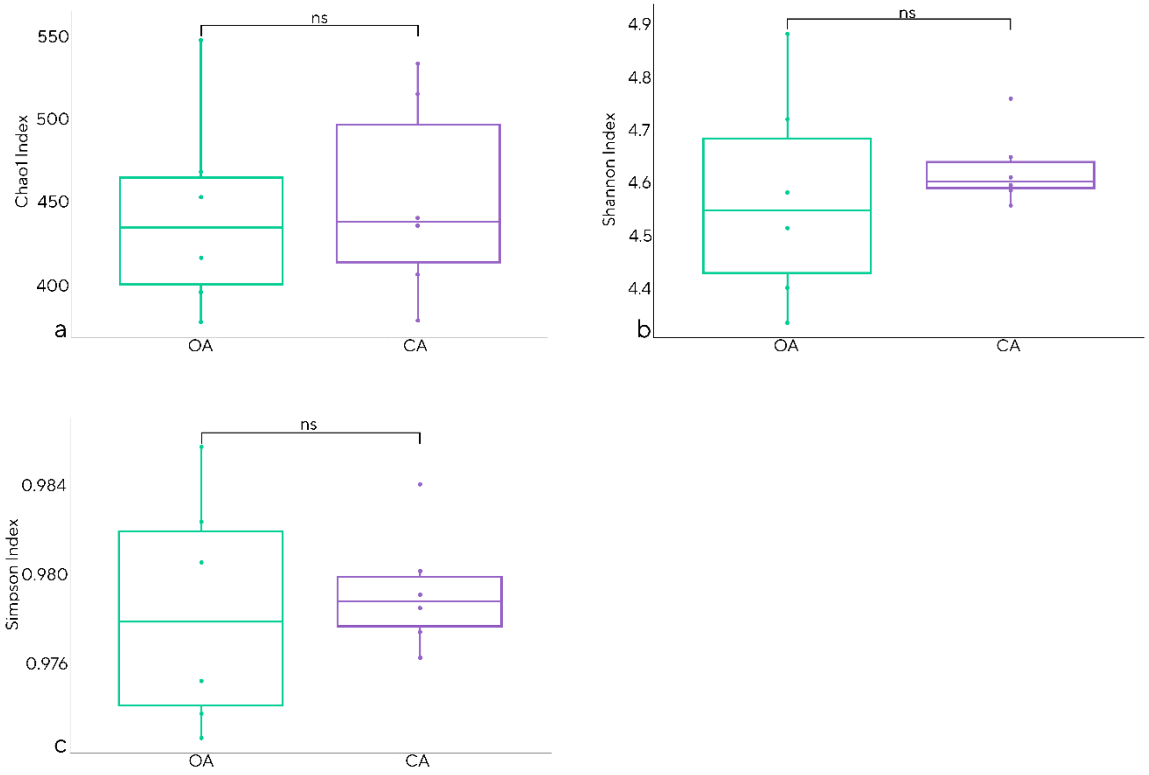



Figure 3. Boxplot comparisons of Taxa Richness (a), Shannon Index (b), and Simpson 1-D Index (c) at the genus taxonomic level to evaluate the alpha diversity in OA sites (green) and CA sites (purple).

In order to determine whether there could be any possible association between the multiple independent variables characterizing microbial communities, multivariate analyses were conducted. Figure 4 displays the results of the PCA, the Principal Coordinates Analysis (PCoA), and the Permutational Multivariate Analysis of Dispersion (PERMDISP2). Although PCA and PCoA approaches showed a partial overlap of the two clusters, it is noticeable that the OA samples clustered tightly compared to the CA samples. The

PERMDISP2 analysis, based on the Bray-Curtis dissimilarity matrix, indicated a significant ($p$=0.01035) separation of the ellipse's centroids. The Permutational Multivariate Analysis of Variance (PERMANOVA) returned a $p$-value=0.051 for 999 permutations, a $p$-value=0.0463 for 9999 permutations, a $p$-value=0.04703 for 99999 permutations, and the Analysis of Similarities (ANOSIM) reported an R value=0.2481 and a $p$-value=0.0177. The results of the analysis of the set of microbial taxa that are characteristic of the two farming systems and the analysis of the taxa shared among OA sites and CA sites are reported in Figure 5. The overall core microbiome analysis showed a higher abundance of detected taxa in CA soils compared to OA soils, however, for every considered taxonomical level, the analysis of the shared taxa reported that OA sites shared a higher number of taxa compared to CA sites. In detail, OA sites, from the phylum to the genus taxonomic level, shared 103%, 108%, 109%, 111% and 113% more taxa than CA sites.

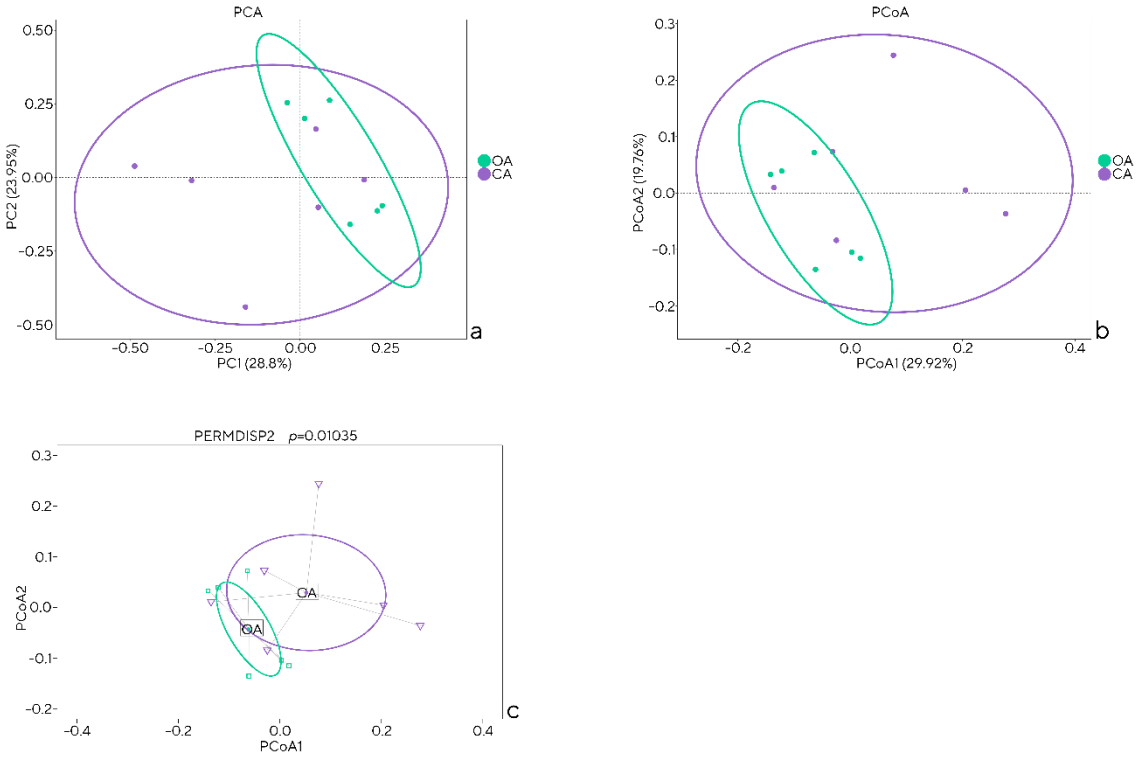



Figure 4. Multivariate analyses for the bacterial communities sequencing data at the genus taxonomic level to evaluate the beta diversity in OA sites (green) and CA sites (purple). (a) Principal Component Analysis (PCA), (b) Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity matrix, (c) Permutational Multivariate Analysis of Dispersion (PERMDISP2) showing the distance of each sample from the group's centroid.

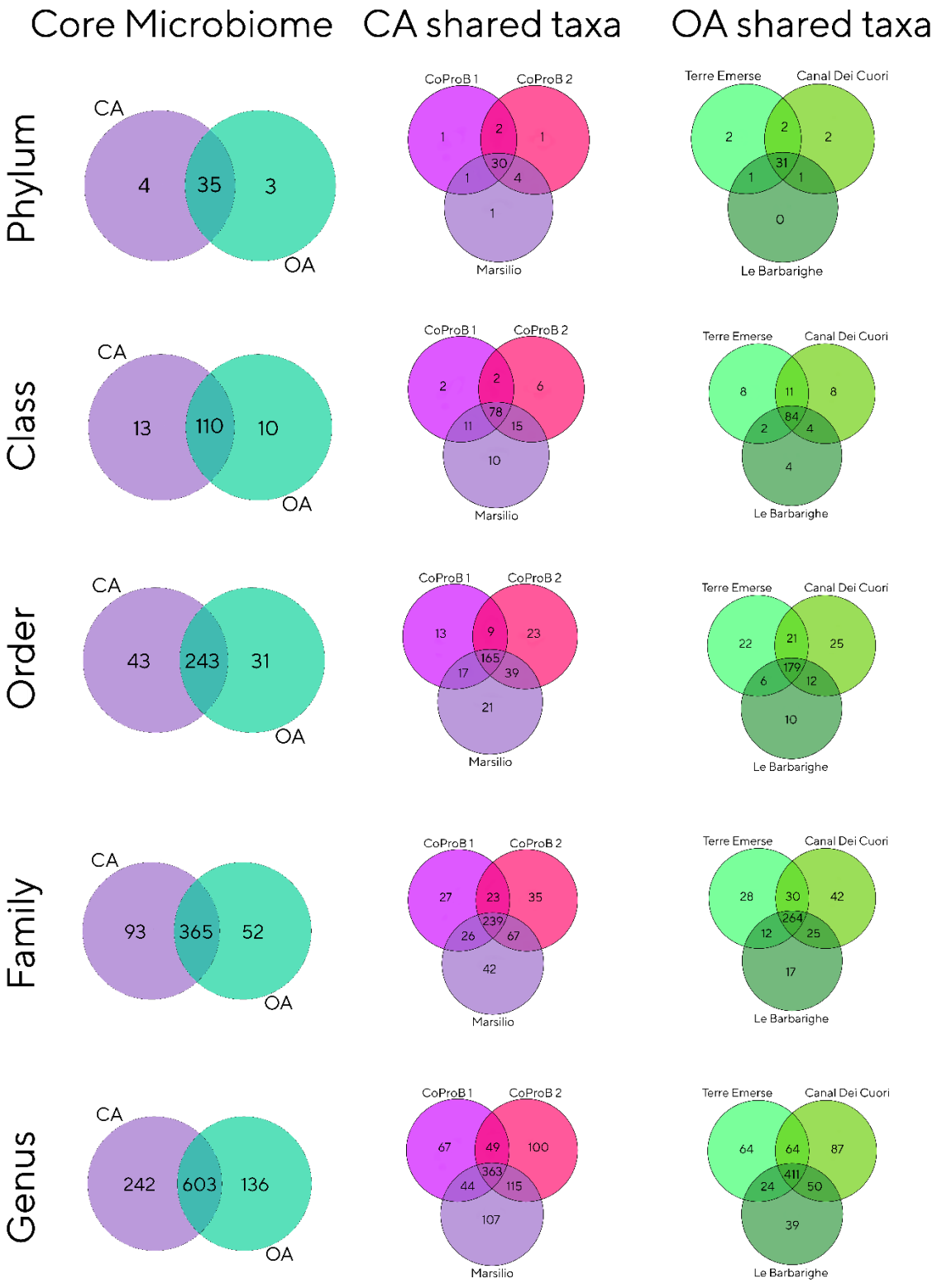



Figure 5. Venn diagrams showing the number of shared and unique taxa between the two soil farming systems (OA green, CA purple) and among the same soil management, compiled for each taxonomic level from phylum to genus.

The qPCR analysis results, reported in Table 4, depict that OA sites when compared to CA sites, have a significantly higher abundance of bacterial 16S gene ($p$<0.01) and a significantly higher number of copies of functionally relevant genes such as those involved in the N biogeochemical cycle (Archaeal-amoA $p$<0.05, Eubacterial-amoA $p$<0.001, nifH $p$<0.001, nosZ $p$<0.05, nirK $p$<0.01), the one coding for the gh48 enzyme ($p$<0.01) and those genes clusters coding for polyketides (PKs I $p$<0.01, PKs II $p$<0.001) and non-ribosomal peptides ($p$<0.05). The relative abundance of the sugar beet-growth-promoting rhizobacteria in OA and CA samples is portrayed in Figure 6. In this figure, the threshold cycle values (Ct) are shown (i.e. the lower the number the higher the abundance of the amplicon). All five bacterial genera were detected in all samples irrespective of the soil management but were significantly ($p$<0.001) more copious in OA sites.

Table 4. Gene copy numbers resulting from the qPCR analyses.

| Farming System | 16S** Gene Copy number | Archaeal amoA* Gene Copy number | Eubacterial amoA*** Gene Copy number | nifH*** Gene Copy number |
|---|---|---|---|---|
| CA | $1.36\times10^{8}\pm2.99\times10^{7}$ b | $2.43\times10^{3}\pm6.86\times10^{2}$ b | $5.36\times10^{3}\pm1.42\times10^{3}$ b | $5.40\times10^{3}\pm1.81\times10^{3}$ b |
| OA | $3.52\times10^{8}\pm3.30\times10^{7}$ a | $3.86\times10^{5}\pm1.34\times10^{5}$ a | $9.46\times10^{5}\pm2.22\times10^{5}$ a | $1.55\times10^{6}\pm4.02\times10^{5}$ a |
| Farming System | nosZ* Gene Copy number | nirK* Gene Copy number | gh48** Gene Copy number | NRPS I* Gene Copy number |
| CA | $1.13\times10^{3}\pm2.57\times10^{2}$ b | $5.29\times10^{5}\pm1.18\times10^{5}$ b | $1.12\times10^{4}\pm8.15\times10^{2}$ b | $6.11\times10^{1}\pm2.12\times10^{1}$ b |
| OA | $4.81\times10^{5}\pm1.89\times10^{5}$ a | $4.91\times10^{6}\pm1.34\times10^{6}$ a | $1.90\times10^{5}\pm2.52\times10^{4}$ a | $2.17\times10^{5}\pm1.08\times10^{5}$ a |
| Farming System | NRPS II* Gene Copy number | pks I*** Gene Copy number | pks II** Gene Copy number | |
| CA | $5.86\times10^{3}\pm1.09\times10^{3}$ b | $8.88\times10^{1}\pm2.62\times10^{1}$ b | $2.33\times10^{4}\pm6.87\times10^{3}$ b | |
| OA | $3.27\times10^{5}\pm1.26\times10^{5}$ a | $1.01\times10^{4}\pm1.33\times10^{3}$ a | $1.59\times10^{6}\pm5.23\times10^{5}$ a | |

Means with different letters in the vertical comparison are significantly different at the Wilcoxon Rank Sum test.

*Significance level $p$<0.05, **Significance level $p$<0.01, ***Significance level $p$<0.001.

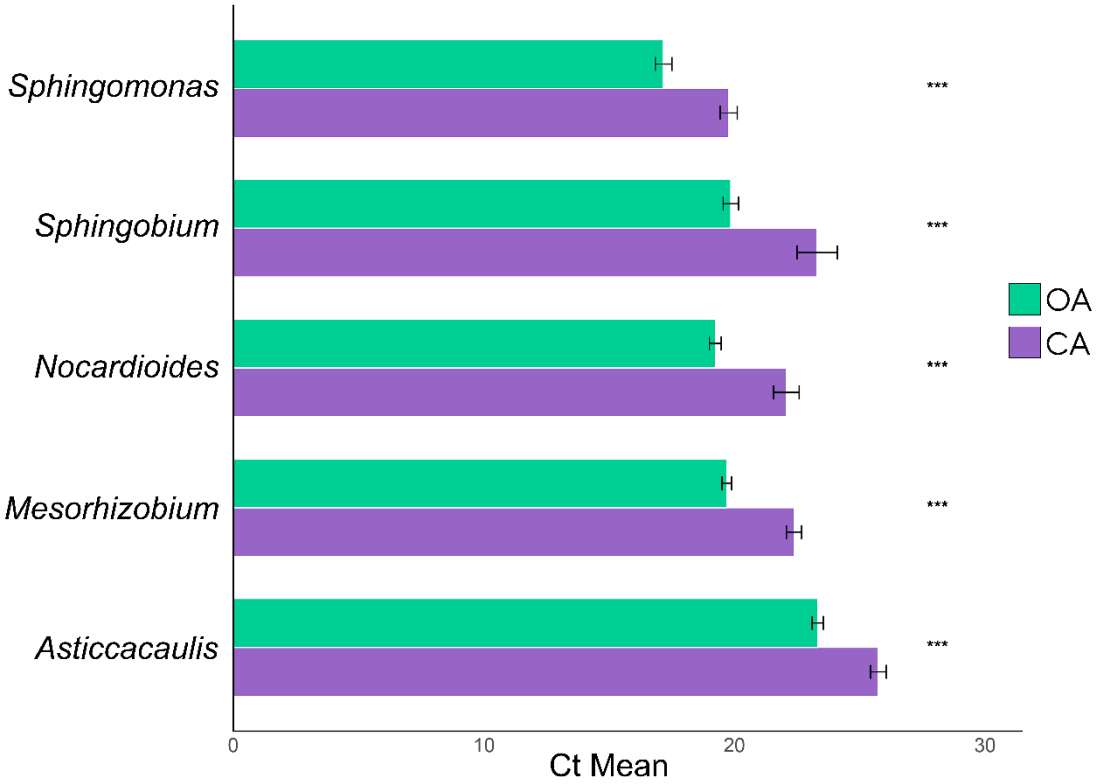



Figure 6. Relative abundance of five sugar beet growth-promoting rhizobacteria (PGPR) detected by qPCR. The plot shows the comparison between the Threshold Cycle (Ct) values for all the tested genera. Ct values are inversely related to the target abundance, meaning that a lower Ct value corresponds to a higher abundance of the investigated amplicon. ***Significance level $p<0.001$.

## Discussion

In this study, despite observing variations in several chemical soil characteristics and recording soil nutrients concentrations that, in accordance with Seufer et al. (2012), supported OA farms' production rate, as well as an over twenty-year-long difference in the organic versus conventional management regimes, the six studied sites exhibited similar alpha-diversity profiles for the total bacterial communities, as far as the 16S metabarcoding approach is concerned. The data confirm those from prior analyses of ours in a survey that included ten horticultural farms (Fusaro et al., 2018), as well as with the findings of a study covering 25 years of conversion from conventional to organic cropping in the Netherlands, in which authors concluded that the shift to organic management did not increase bacterial community diversity, and for fungi the increase occurred only in some soils (Van Rijssel et al., 2022).

The absence of significant differences in richness, diversity and evenness indices suggests that the bacterial community structures, in terms of absolute diversity *per se*, are determined by a combination of soil structure and chemistry (Girvan et al., 2003), and the

crop's rhizosphere (Turner et al., 2013, Botnen et al., 2014), or by a passive atmospheric discharge of cells (Rosselli et al., 2015) rather than by a deterministic selection brought about by each of the two farming systems. Despite the diverse soil management practices implemented over the past decades, the observed similarity in overall soil bacteria composition may be attributed to a long-lasting legacy effect of a much older common cropping regime before conversion to organic farming of part of the sites (Buckley and Schmidt, 2001). The beta-diversity analysis partially clustered the two farming systems separately. Although the PERMANOVA analysis, oscillating between significant and non-significant results, reports an uncertain level of differences in the overall community composition, PERMDISP2 analysis describes a significant difference in the dispersion of the samples, meaning that there is a somewhat detectable difference in the heterogeneity in the community composition (Heino et al., 2013, White et al., 2017). On the other hand, the ANOSIM analysis reports exiguous but still significant differences in the microbial communities' composition. The core microbiome evaluation reported an enhanced number of detected taxa within the CA soils compared to the OA soils.

The higher number of detected taxa can depend on the combination of environmental processes, for instance, the airborne immigration of foreign DNA from different sites as described by Rosselli et al. (2015), and the mechanical incorporation of that migrated DNA due to the frequent tillage the CA sites are subjected to. On the other hand, the qPCR results instead spotlight a remarkably different situation. Within a two-fold increase of the whole bacterial community shown by the 16S primers in OA soils, the single functional genes targets reported in Table 4 unravelled major changes. All these changes are pointed towards the same direction, as results from the ten-fold increase of the cellulase and nitrite reductase genes in OA soils, from the more than 100-fold increase of both nitrification genes, for the synthesis of the non-ribosomal peptide II and polyketide II, and for the terminal gene of the denitrification pathway *nosZ*, which eliminates nitrous oxide, which is one of the worst gases involved in global warming. Even more pronounced in OA soils as well, are the increases in the number of copies of other determinants as the nearly thousand-fold rise of the nitrogen fixation gene *nifH*, and of the polyketide synthesis I, and the almost 10000-fold over-representation of the non-ribosomal peptide I. At the same time the five genera *Asticcacaulis*, *Mesorhizobium*, *Nocardioides*, *Sphingobium* and *Sphingomonas*, which were all known as promoters of the sugar beet crop, that was in place at the sampling time, were found enriched in the OA fields. All the above-mentioned tested genes, all of which were significantly more abundant in the organic farms in comparison to the conventional ones, not only qualify as proxies to tell apart the effects of

the two farming systems but are also positive indicators of actively ongoing cycles for C and N and of the abundance of proficient plant-growth promoting guilds. As a general consideration, the 16S is a multicopy gene implying that some species may possess more copies of the gene than others. However, as always in this type of studies involving metabarcoding, it is important to note that the number of microbial gene copies does not accurately reflect the actual number of genomes in the system. This is due to the variable gene dosage, which introduces a certain level of overestimation (Větrovský and Baldrian, 2013, Kunin et al., 2010, Farrelly et al., 1995). Nonetheless, this situation is averagely occurring across various habitats, and there are no indications or reasons to suggest that either of our compared soil types (organic vs. conventional management) would unequivocally exhibit an overabundance of bacteria with higher numbers of 16S gene copies. Therefore, we hypothesize that any differences should balance each other out.

**Conclusions**

The novel findings presented herein are consistent with earlier studies that compared soil properties between conventional and organic cropping systems using various approaches, such as microbial biomass estimation and plate counts (Fraser et al., 1988), soil respiration and enzymatic assays (Gunapala and Scow, 1998), and phospholipid fatty acid (PLFA) profiles (Bossio et al., 1998). In the current report, the combination of the 23-fold more abundant total soil DNA, the 2.6 times more plentiful 16S gene, the significantly more copious amount of the considered functional genes in OA sites, and the consistently higher presence of all five PGPR taxa investigated, leads to the consideration that the OA management appears to have a positive effect on the functioning of the agroecosystem which is tightly connected to soil fertility.

As concerns the approaches employed, the metagenomics-based barcoding of the 16S via sequencing was extremely valuable in providing a detailed taxonomical view of the community structure and allowed to achieve the very important finding that conventional soils feature a bacterial alpha diversity which does not record signs of decline when compared with the one occurring in organic soils. However, the approach was not providing clues to identify the impacts of each management as it yielded non-significant differences in the bacterial biodiversity stemming from each of the indices. It appears that a high taxonomic richness, equally characterizing CA, and OA sites does not reveal elements of functional biodiversity that instead occur in the soil and can be unraveled by qPCR. The lack of concordance between metabarcoding and qPCR results strongly suggests that the analysis of the absolute microbial biodiversity cannot adequately differentiate agricultural

ecosystems as a function of their different managing systems. However, while the CA and OA soils show a similar level of diversity with the diversity indices, deeper investigation, as shown by Venn Diagrams in Figure 5, shows a set of taxa present uniquely in each soil type, which can perhaps lead to relevant functional attributes of the two soil types. Consequently, bacterial community sequencing, although extremely informative, is not a suitable stand-alone proxy to achieve the functional differentiation of the two farming systems. In this respect, it is necessary to state that this is not due to any limitation inherent to the technique, which remains one of the deepest tools for soil biota analysis, but rather on two distinct facts. The efficiency of taxa detection from environmental DNA strongly depends on the number of replicates, sequencing depth and quality, PCR conditions, and the characteristics of the environment itself. A lower number of biological replicates, as the one characterizing this project, can be sufficient for the assessment of the presence/absence of the main taxa but, at the same time, can lead to a biased analysis of rare taxa due to DNA degradation and sequencing errors. Moreover, soil as a whole is a poor reporter source of its ongoing true activity due to the way soils accumulate bacteria and preserve their DNA, i.e. to the fact that a vast majority of soil bacterial DNA is a relic or belongs to passive inmates, with no physiologically functional roles in the system. This limit however may be more specific to the prokaryotic component and likely, fungal ITS-based metabarcoding could be more suited to put in evidence function-related differences (Wang et al., 2023) as future studies on the same soil could reveal. In any event, the present data show that the microbial profiling of soils, constituting a fundamental step in the evaluation of their quality, health and sustainability, should consider pairing DNA sequencing, also with other highly informative analyses, such as qPCR targeting meaningful bio-indicator genes as the ones hereby covered along with functional biodiversity prediction from taxonomy data, and metatranscriptomics, to untangle the underlying mechanisms regulating the soil ecosystem functioning and its long-term stability and resilience.

## Supplementary Material

Table S1. Sugar beet productivity traits.

| Sample | Root yield $t \cdot ha^{-1}$ | Sugar yield $t \cdot ha^{-1}$ | Potassium meq% °S | Sodium meq% °S | Alfa-N meq% °S | Purity % |
|---|---|---|---|---|---|---|
| CoProB | 75.3±1.7 b | 11.5±1.3 b | 24.1±1.1 b | 8.72±0.09 b | 7.91±0.58 b | 92.8±2.3 a |
| Marsilio | 75.7±0.9 b | 11.4±1.4 b | 24.4±0.8 b | 8.07±0.13 b | 6.89±0.11 b | 92.9±1.6 a |
| Canal Dei Cuori | 74.3±1.4 b | 11.1±1.9 b | 23.9±1.7 b | 8.33±0.21 b | 8.25±0.32 b | 91.2±1.4 a |
| Le Barbarighe | 80.7±1.2 a | 12.3±1.1 a | 21.5±1.3 b | 7.73±0.27 b | 7.04±0.49 b | 93.3±2.2 a |
| Terre Emerse | 72.1±2.1 c | 10.9±1.6 c | 25.6±1.2 a | 9.24±0.16 a | 8.93±0.17 a | 89.6±1.9 b |

Significant differences among the mean values in the vertical comparison were evaluated with the Student t-test followed by post hoc analysis (Duncan's test). Significance was estimated at the $p<0.05$ level.

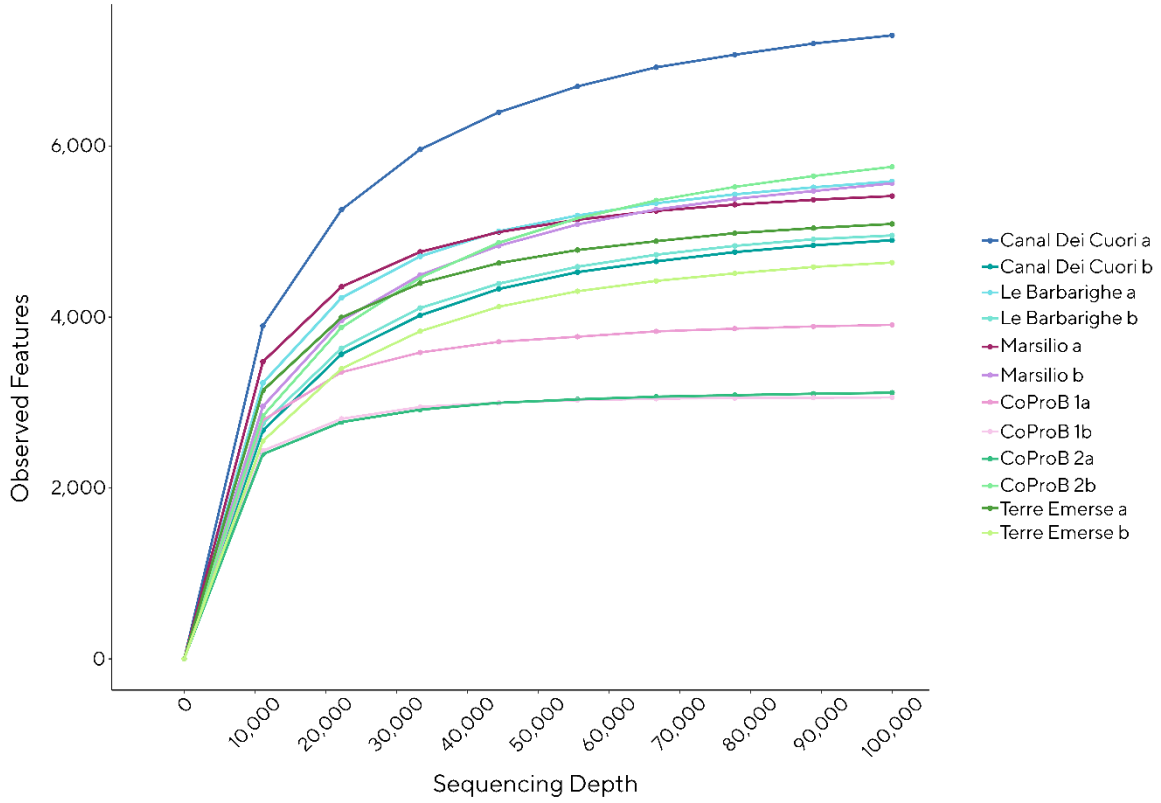



Figure S1. Alpha-diversity rarefaction plot. The curve is based on the number of observed amplicon sequence variants (ASVs) as a function of the sequencing depth.

## Declarations

Availability of data and material: The dataset generated for this study can be found in the EMBL Nucleotide Sequence Database (ENA), ID PRJEB60430.

Competing interests: All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Author Giovanni Campagna is employed by COPROB.

Funding: The study was carried out within the Agritech National Research Center and received funding from the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR)—MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022). Our study represents a position paper related to (1) Spoke 1 "Plant and animal genetic resources and adaptation to climate changes" and a baseline for the fulfillment of the milestones within task 1.2.4 titled "Profiling plant-

microbial associations and modulating these interactions by biostimulant treatments to enhance the ability of plants to cope with environmental stressors". (2) Spoke 7 "Integrated models for the development of marginal areas to promote multifunctional production systems enhancing agroecological and socio-economic sustainability" and a baseline for the fulfillment of the milestones within Task 7.1.2 titled "Strategies for development of the agricultural and forestry systems, plant and animal biodiversity enhancement also at landscape level in marginal areas". This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them. This study was also funded by Veneto Region in the framework of the PSR 2014–2020 (Project: "Implementation and validation of innovative plant protection methods to increase the environmental sustainability of organic and sugar beet production"). Authors SR and MB were supported by Cariparo Foundation and PON Research &. Competitiveness MIUR-CUP C93H20000320007, respectively. This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Authors' contribution: PS, GCa, and LM designed the study; LM, SD, and MCDL collected the samples; LM performed the analyses and collected the data; GCo classified the soil samples; LM, SD, SR, and MB analyzed the data; LM drafted the manuscript; all authors critically revised the manuscript.

**List of Abbreviations**

AOA – ammonia oxidising archaea

AOB – ammonia oxidising bacteria

ARPAV – Regional Agency for Prevention and Environmental Protection of Veneto

ASV – Amplicon Sequence Variant

CA – Conventional Agriculture

Ct – Cycle Threshold

NRPs – Non-ribosomal Peptide Synthetase

OA – Organic Agriculture

OTU – Operational Taxonomic Unit

PCA – Principal Component Analysis

PCoA – Principal Coordinates Analysis

PERMANOVA – Permutational Multivariate Analysis of Variance

PERMDISP2 – Permutational Multivariate Analysis of Dispersion

PGPR – Plant Growth-Promoting Rhizobacteria

PKs – Polyketide Synthase

QIIME2 – Quantitative Insight Into Microbial Ecology 2

qPCR – quantitative Real-Time Polymerase Chain Reaction

RER – Emilia-Romagna Region

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# CONTRIBUTION III

# Evaluation of the soil microbiome of three raised beaches in the Devon Plateau, Devon Island

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**Abstract**

The Arctic region, characterized by severe temperatures and a unique ecosystem, encompasses a peculiar microbiome in its soil. The soil microbiome has a crucial function in nutrient cycling, organic matter decomposition, greenhouse gas emissions, and the general functioning of the ecosystem. Increasing the knowledge about the composition, diversity, and dynamics characterizing the Arctic soils' microbiome is fundamental to better understanding the ecological processes within this threatened evolving environment. In this study, we compared the microbial profiles of three raised beaches in Devon Island (Nunavut, CA) to explore their similarities and differences. Samples were collected from each investigated site named AB1, AB2, and AB3, performing a vertical sampling from the active layer to the permafrost. Soil microbial DNA was isolated from each sample for total soil DNA and 16S gene copies quantification, 16S metabarcoding, and function prediction. The quantification of total DNA revealed a consistently comparable quantity of genetic material across the three beaches (AB1 μ= 4.71±2.35 $\mu g \cdot g^{-1}$, AB2 μ= 2.28±5.44 $\mu g \cdot g^{-1}$, AB3 μ= 5.44±2.91 $\mu g \cdot g^{-1}$), irrespective of the age of the investigated area (AB1=6,728 YBP, AB2=2,360 YBP, AB3=8,410 YBP). Regarding the 16S metabarcoding data analysis, it is noteworthy that the horizon clustering underscores how the chemical distinctions observed among the investigated horizons actively contribute to defining the composition and structure of microbial communities. In particular, the hierarchical cluster analysis based on the Bray-Curtis dissimilarity matrix vividly illustrates the distinct separation between surface and deep horizons. Eventually, the core microbiome analysis highlights that Actinobacteriota, Proteobacteria, and Firmicutes are the top three predominant phyla accounting for relative abundances of 42%, 22%, and 18%, respectively. From this study, we can infer that the physicochemical characteristics of soils exert a significant influence on the composition and clustering of microbial communities which show a set of unique taxa based on the horizon clustering. Nonetheless, the evolutionary convergence appears to favor the predominance of shared taxa resembling the core microbiome of highly disturbed environments such as hot deserts, grazed soils and soils disturbed by natural and wildfires. Eventually, the soil DNA is a relatively stable molecule that can be detected and analyzed after a substantial amount of time has elapsed.

**Keywords**

Soil chronosequence, soil microbiome profiling, arctic soils, 16S metabarcoding, functional diversity, digital PCR, raised beaches, Canadian High Arctic, Devon Island.

## Introduction

The Arctic region is a delicate ecosystem characterized by low temperatures, limited biodiversity, and extensive ice and snow coverage (Cusset et al., 2019, Hock et al., 2019, Koch et al., 2023). Due to the persistence of the permafrost layer in the High Arctic terrain, the active layer is subjected to severe frost-related processes, including cryoturbation. As a result, the soil typically exhibits fractured and uneven horizons, material injection, stone upheaval, and soil blending. Nonetheless, there are instances of non-cryoturbated soils characterized by a vertical arrangement of A–B–C horizons. Such soil profiles are commonly found in coastal regions (Ugolini et al., 2006). Because of its severe conditions, this area sustains only a restricted array of plant species, which is mainly composed of mosses and lichens.

Currently, the Arctic is undergoing rapid transformations because of climate change. Since 1979, temperatures in this region have been rising approximately fourfold faster than the global average (Rantanen et al., 2022). These alterations have extensive consequences which affect biological communities, biogeochemical processes, and the overall ecosystem's functioning (Hirawake et al., 2021). For instance, warmer temperatures in both air and water have changed the migration patterns of predators and their prey and have led to shifts in community composition as Arctic species are gradually replaced by southern counterparts (Solan et al., 2020).

Several studies have examined soil bacterial communities and their essential role in nutrient cycling (Jiao et al., 2019, Prasad et al., 2020, Yadav et al., 2021), soil fertility (Chaparro et al., 2012, Hartmann and Six, 2022) and ecosystem functioning (Gupta et al., 2017, Pérez-Valera et al., 2020, Wagg et al., 2019) has been widely documented by the scientific community. As with non-polar microbial communities, the Arctic soils' microbiome, despite the low activity rates due to environmental conditions, is responsible for organic matter decomposition (Tas et al., 2018), and essential nutrient cycling (Malard and Pearce, 2018, Poppeliers et al., 2022). Consequently, even minor alterations induced by global warming, such as augmented water availability and thawed active layer depth, might cause an increase in the methanogenic bacteria's abundance in soils, resulting in a boosted $CH_4$ production (Jansson and Hofmockel, 2020), which has a twenty-fold higher global warming potential (GWP) when compared to $CO_2$. Thus, a better understanding of the Arctic soils' microbial communities is a key step in predicting changes in soils' health and resilience across a wide range of latitudes.

This work aimed to investigate the soil microbial communities in both unglaciated soil and permafrost along a chronosequence represented by three beaches raised from 2360 to 8410 years before present (YBP). All the soils at raised beaches did not show signs of cryoturbation and displayed a surface with desert pavement with stones colored by desert varnish. This study employed a multidisciplinary approach, encompassing physicochemical analyses and molecular biology techniques such as total soil DNA assessment and 16S gene copies quantification, 16S multi-amplicon metabarcoding, and environmentally relevant functions prediction.


Figure 1. Aerial photograph of the study area located on Devon Island in the Canadian High Arctic region of Nunavut.

## Materials and Methods

### Study area

The study was carried out on three contiguous raised beaches (identified as AB1, AB2, and AB3) situated on Devon Island in the Canadian region of Nunavut (Figure 1).

Devon Island, part of the Queen Elizabeth Islands located in the High Arctic, has a surface of 56,000 $km^2$. Devon Island features a prevailing continental climatic condition due to the surrounding sea, which remains frozen for more than ten months each year, and due to the influence of a documented cold circumpolar vortex that blows from the west to the east (Courtin and Labine, 1977). Although the coastal and lower-lying regions exhibit biological and soil conditions resembling a wet tundra environment (Bliss, 1977), the remainder of

the island is characterized by an ice cap and a barren plateau. The annual mean temperature corresponds to -16.0 °C, and the annual mean precipitation is 185 mm (Courtin and Labine, 1977; Lev and King, 1999), as retrieved from the climatic records of Truelove, one of the coastal lowlands on Devon Island. Geologically, the island comprises Cambrian and Ordovician sedimentary rocks attributed to the Cass Fjord Formation (Ugolini et al., 2006). A glacial ice cap, which is still present in the northeast of the island, covered Devon Island. Approximatively 9,000 years ago, the deglaciation process began, resulting in the transformation of glacial deposits and the underlying bedrock into permafrost (Brown and Judge, 1977). Concurrently, the ice-cap retreat induced a glacio-isostatic crustal rebound phenomenon (Andrews et al., 1970) that originated the raised beaches in the coastal area. The raised beaches, nowadays, are located at some meter of elevation, host permafrost, and are mainly made of 80-90% rock fragments (particles>2mm). More often, these beaches encompass sub-rounded pebbles and blocks. The presence of fine earth fraction is limited, accounting for only about 5-10% of the beach's overall composition. This fine earth is typically found within silt/sand caps formed through illuviation on the surfaces of large blocks. Due to the predominantly skeletal nature of these soils, they exhibit exceptional drainage characteristics, preventing the active layer from experiencing waterlogging periods. This favorable drainage condition likely contributes to the absence of cryoturbation phenomena able to mix soil horizons. Consequently, the soils of the raised beaches maintain a vertical sequence characterized by A-B-C horizons formed through non-cryoturbated pedogenesis. Furthermore, it is noteworthy that the materials constituting both the permafrost and the soil overlaying it can be considered *in situ*. The primary distinction lies in the fact that all materials within the permafrost have remained unaltered since deglaciation. In contrast, the soil above it may have undergone translocations of organic and mineral materials due to processes such as illuviation, root growth, and animal activity.

The AB2 beach, which has an elevation of 3.22 meters above the sea level (masl) and has been radiocarbon dated to 2360 YBP, is characterized by a frosted table at a 60 cm depth, and by a surface covered for 50-60% by crusty lichens, and for 7-10% by vascular plants such as *Cerastium* spp., *Saxifraga* spp., and *Salix* spp., while the rest of the surface is barren and presents a poorly developed desert pavement. The AB1 beach, which has an elevation of 22.44 masl and has been dated 6728 YBP, presents a surface coverage made by crusty lichen for 30-40% and by vascular plants such as *Dryas* spp., *Saxifraga* spp., and *Salix* spp. for 7-10%. The rest of the surface (50-60%) is barren with a rather well-developed desert pavement. The frost table in AB1 beach can be found at a 70 cm depth.

The AB3 beach, the highest and oldest one, raises for 47.52 masl, has a frosted table at a 75 cm depth, and has been dated 8410 YBP. The surface coverage corresponds to crusty lichens for 10-20% and to vascular plants (*Dryas* spp., *Saxifraga* spp., and *Salix* spp.) for 7-10%. The majority of AB3's surface (70-80%) is barren and presents a well-developed desert pavement.

**Field operations and sample collection**

The study site selection was based on 6 excavations conducted within each raised beach, encompassing an area of approximately 500 $m^2$. At the chosen location, thorough identification of all surface vegetation was carried out, and two soil trenches were excavated to the frost table. The trenches were kept open for a span of three weeks, during which a camping gas lantern was placed inside to thaw the permafrost. This step was essential since scrutiny and collection of the near-surface permafrost is an integral aspect of this investigation.

These excavations yielded two biological replicates for each distinct soil sample. The soil morphology reported in Table 1 was described according to the guidelines defined by Soil Survey Staff (2014). Carbonates pendants and stains were assessed by testing with 3 M HCl solution. The presence of red, reddish, and purplish stains was quantified using the Munsell Color Chart (1954). Meanwhile, the extent of silt cap development was evaluated and categorized using a ranking system based on levels ranging from 1 to 5, relative to particle size ranges of 1-2, 2-4, 4-6, 6-8, and >8 mm. A pebble count was conducted in the field observing about 110 pebbles per horizon.

**Soil physicochemical analyses**

The soil samples underwent an air-drying process followed by subsequent sieving at a mesh size of 2 mm. This separation aimed to distinguish between the fine earth and skeleton components. In the context of particle-size analysis, coarse, medium, and fine sand portions were isolated through sequential sieving at mesh sizes of 0.5 mm, 0.25 mm, and 0.05 mm, respectively. Additionally, the clay component was separated from the silt through sedimentation in a suspension adjusted to a pH range of 8.8 to 8.9 using NaOH and maintained at a temperature of 20 °C. pH was determined potentiometrically on water suspensions with a solid-liquid ratio of 1:2.5. The estimation of the organic carbon (OC) content was performed following the Walkley–Black method (Walkley and Black, 1934). The determination of total Kjeldahl nitrogen (TKN) and micaceous nitrogen (MN) employed a modified approach published by Corti et al. (1999). The available phosphorus (P) quantification was carried out following the Olsen P method (Olsen et al., 1954).

Table 1. Summary table reporting the overrall raised beaches' characteristics

| Sample Name | Age | Area | Depth (cm) | Color[a] | Structure[b] | Consistence[c] | Roots[d] | Boundary[e] | Carbonates Pendent and Stains | Staining | Silt Caps | Pebble Count % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Crystalline | Dolomitic | Sandstone | Diabase | Breccia + Shales |
| AB1-A | 6726 YBP | AB1 | 0-2.5 | 5YR 2/2 | 1f cr | m fr, w ss, po | 3mi, vf, f | AB | ++ | 2.5YR 4/3 | - | 47.0 | 7.4 | 20.5 | 2.1 | 23.0 |
| AB1-E | | | 2.5-3.5 | 10YR 5/1 | sg | w so, po | 3mi, vf, f | AB | - | - | - | 53.6 | 10.2 | 9.6 | 0.0 | 26.6 |
| AB1-Bw1 | | | 3.5-9.0 | 10YR 3/4 | 1th pl → f cr | m vfr, w so, po | 3mi, vf, f, 2m | CS | ++++ | 2.5YR 3/2 | ++ | 54.5 | 39.3 | 4.7 | 0.0 | 1.5 |
| AB1-Bw2 | | | 9.0-14 | 10YR 1/2 | 1f cr | m vfr-fr, w so, po | 2mi, vf, f | CI | | | ++ | 51.0 | 36.6 | 2.4 | 0.6 | 9.4 |
| AB1-Bw3 | | | 14-24 | 10YR 4/3 | sg | w so, po | 2mi, vf, f | CW | ++++ | 2.5YR 3/3 | ++++ | 46.5 | 32.0 | 1.0 | 1.7 | 18.8 |
| AB1-BC1 | | | 24-45 | 10YR 4/2 | sg | w so, po | 1vf, f, m | CS | ++++ | 2.5YR 3/4 | +++++ | 51.2 | 41.5 | 1.3 | 2.4 | 3.6 |
| AB1-BC2 | | | 45-70 | 10YR 5/2 | sg + m | m vfr, w so, po | 0 | CS | - | - | - | 47.0 | 41.4 | 0.5 | 0.8 | 10.3 |
| AB1-BCf | | | 70-78 | 10YR 5/2 | sg | w so, po | 0 | - | - | - | - | 42.9 | 38.0 | 1.0 | 1.2 | 16.9 |

| Sample Name | Age | Area | Depth (cm) | Color[a] | Structure[b] | Consistence[c] | Roots[d] | Boundary[e] | Carbonates Pendent and Stains | Staining | Silt Caps | Pebble Count % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Crystalline | Dolomitic | Sandstone | Diabase | Breccia + Shales |
| AB2-A | 2360 YBP | AB2 | 0-2.0 | 10YR 3/2 | v1th pl →f cr | m vfr, w ss, po | 3mi, vf, f | CI | - | - | - | 35.5 | 56.3 | 6.2 | 0.0 | 2.0 |
| AB2-Bw1 | | | 2.0-18 | 10YR 4/4 | sg | w so, po | 3mi, vf, f, 1 co | CS | +++ | 2.5YR 3/2 | + | 23.7 | 73.4 | 1.3 | 0.0 | 1.6 |
| AB2-Bw2 | | | 18-40 | 10YR 5/5 | sg | w so, po | 2mi, vf, f | CS | ++++ | 2.5YR 3/4 | ++ | 19.7 | 76.9 | 2.9 | 0.0 | 0.5 |
| AB2-BC1 | | | 40-60 | 10YR 5/3 | sg | w so, po | 1mi, vf, f | CS | - | - | +++ | 21.0 | 70.3 | 7.7 | 0.0 | 1.0 |
| AB2-BCf | | | 60-78 | 10YR 5/4 | sg | w so, po | 0 | - | - | - | + | 21.8 | 69.0 | 5.5 | 0.0 | 3.7 |

| Sample Name | Age | Area | Depth (cm) | Color[a] | Structure[b] | Consistence[c] | Roots[d] | Boundary[e] | Carbonates Pendent and Stains | Staining | Silt Caps | Pebble Count % | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | Crystalline | Dolomitic | Sandstone | Diabase | Breccia + Shales |
| AB3-DV | 8410 YBP | AB3 | 4.0-0 | 7.5YR 6/4 | sg | w so, po | 0 | AI | | | | 84.9 | 12.5 | 1.0 | 0.0 | 1.6 |
| AB3-A | | | 0-8.0 | 2.5YR 3/2 | 1f cr | m fr, w ss, po-sp | 3mi, vf, f, 1co | AI | +++++ | 2.5YR 4/3 | - | 61.9 | 17.5 | 18.6 | 0.0 | 2.0 |
| AB3-Bw1 | | | 8.0-18 | 10YR 5/3 | sg | w so, po | 2mi, vf, f | AI | +++++ | 2.5YR 3/2 | +++++ | 68.3 | 26.4 | 3.3 | 0.0 | 2.0 |
| AB3-Bw2 | | | 18-47 | 10YR 5/3 | sg | w so, po | v1mi, f | CS | +++++ | 2.5YR 3/3 | +++++ | 68.0 | 29.0 | 0.0 | 0.0 | 3.0 |
| AB3-BC1 | | | 47-75 | 10YR 5/3 | sg | w so, po | 0 | - | - | - | ++++ | 61.8 | 22.1 | 9.1 | 5.1 | 1.9 |
| AB3-BCf1 | | | 75-85 | 10YR 5/2 | sg | w so, po | 0 | - | - | - | - | 61.6 | 31.6 | 1.1 | 5.0 | 0.7 |
| AB3-BCf2 | | | 85-106 | 10YR 5/2 | sg | w so, po | 0 | - | - | - | - | 59.5 | 31.0 | 3.9 | 3.8 | 1.8 |

[a]Classification accordin to Munsell Soil Color Chart (1954). [b]v1=very weak, 1=weak, 2=moderate, 3=strong; f=fine, th=thin, m=medium, c=coarse; sg=single grain, cr=crumb, abk=angular blocky, sbk=sub-angular blocky, pl=platy, m=massive. [c] m=moist, vfr=very friable, fr=friable, fi=firm; w=wet, so=nonsticky, ss=slightly sticky, po=nonplastic, sp=slightly plastic. [d]0=absent,1=few, 2=common, 3=many; mi=micro, vf=very fine, f=fine, m=medium, co=coarse. [e]A=abrupt, C=clear, G=gradual; B=broken, W=wavy, S=smooth, I=irregular.

**Total soil DNA extraction, 16S gene copies quantification, 16S metabarcoding, and function prediction**

The extraction of total soil DNA was carried out using the DNeasy PowerSoil Pro Kit (Qiagen GmbH, Hilden, Germany, DE), following the guidelines furnished by the manufacturer. The extracted and purified nucleic acids were quantified employing a Qubit Flex fluorometer (Thermo Fisher Scientific, Carlsbad, CA) paired with the Qubit 1x dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Carlsbad, CA).

The abundance of the 16S gene was quantified by digital PCR (dPCR) using a QIAcuity One, 5plex Device paired with QIAcuity Nanoplate 26k and QIAcuity EvaGreen (EG) PCR Kit (Qiagen GmbH, Hilden, Germany, DE). The reaction mix was composed of 13.3 µL of 3x EvaGreen PCR Master Mix (Qiagen GmbH, Hilden, Germany, DE), 1.6 µL each of forward and reverse primer (Johnson et al., 2016), 19.5 µL of PCR-grade water, and 4 µL of template DNA. The final number of 16S copies was calculated by multiplying the number of identified copies by the reaction volume and subsequently dividing it by the volume of the sample multiplied by the dilution factor.

The 16S rDNA multi-amplicon metabarcoding libraries were prepared using the 16S Ion Metagenomics Kit (Thermo Fisher Scientific, Carlsbad, CA) and sequenced on the Ion GeneStudio S5 System (Thermo Fisher Scientific, Carlsbad, CA) employing an Ion 520 chip.

The raw reads processing was performed according to the pipeline outlined by Maretto et al. (2022). The uBAM files sourced from the Ion GeneStudio platform were converted into FASTQ format using the samtools bamtofastq (v1.10) by Li et al. (2009). A 20-nucleotide trimming on both ends of the raw reads was performed to eliminate the sequencing primers using cutadapt (v3.5) (Martin, 2011). A “Quantitative Insights Into Microbial Ecology 2” (QIIME2) (v2020.08) (Bolyen et al., 2019) pipeline was subsequently used to analyze the trimmed raw reads. Within this process, imported reads were denoised and dereplicated using the “qiime dada2” plugin followed by taxonomic classification of Amplicon Sequence Variants (ASVs) by a “classify-consensus-blast” plugin using SILVA SSU (version 138.1) (Quast et al., 2012) as the reference database. Due to a low number of reads within each sample, the two AB2-BC1 biological replicates were merged using the “qiime feature-table group” plugin. Afterwards, the resulting feature abundance and taxonomic assignment tables were exported and further analyzed using RStudio (version R-4.2.2) (Posit Team, 2022; R Core Team, 2022) along with tibble (Müller, 2023) and TaxaPhyloseq (McMurdie and Holmes, 2013) R-packages. DESeq2 R-package (Love et al., 2014) was used for read

counts normalization, and MicrobiotaProcess packages (Xu et al., 2023) were used to calculate the diversity indices and perform beta-diversity analyses. All graphical visualizations were generated using the ggplot2 R-package (Wickham, 2016).

A function prediction analysis has been performed using the online database FAPROTAX (version 1.2.7) (Louca et al., 2016) to estimate the number of ecologically relevant functions within each sample.

**Statistical analyses**

The statistical analyses were performed using RStudio and the dplyr package (Wickham et al., 2023). The assessment of the ranking correlations among the biological parameters has been carried out employing the Spearman coefficient (Spearman, 1904). The evaluation of significant differences between the mean values calculated within the area clustering (AB1, AB2, AB3) and the horizon clustering (A, Bw, BC, F) occurred with the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952).

**Results**

**Soil physicochemical analyses**

The physicochemical analysis results are summarized in Table 2. The comparative analysis showed that the area clustering did not lead to any significant result except for the micaceous nitrogen. The AB3 beach showed a significantly higher ($p$<0.001) micaceous nitrogen content (9.36±0.66 mg·Kg$^{-1}$) when compared to AB1 and AB2 beaches (4.94±0.74 mg·Kg$^{-1}$ and 4.10±0.43 mg·Kg$^{-1}$ respectively). The horizon clustering (Figure 2), instead, highlighted several significant differences among the investigated parameters. The pH measurement showed a trend in which the A horizon has a significantly lower ($p$<0.001) pH value (7.61±0.06) compared to other horizons, the Bw horizon has an intermediate pH value (8.10±0.04), and the BC (8.26±0.04) and F (8.35±0.05) horizons have the highest pH values. The OC, the available P and the MN contents showed an opposite trend compared to the pH one. The OC content is significantly higher ($p$<0.001) in the A horizon (8.23±1.46 %), intermediate in the Bw horizon (0.927±0.20 %), and lower in the BC and F horizons (0.075±0.08 %, and 0.066±0.01 % respectively). In each considered horizon, a significant decrease ($p$<0.001) in the available P content is observed as the sampling depth increases. In detail, in the A horizon, the available P content corresponds to 7.1±0.64 mg·Kg$^{-1}$, in the Bw horizon is equal to 4.07±0.37 mg·Kg$^{-1}$, in the BC horizon corresponds to 2.0±0.33 mg·Kg$^{-1}$, and in the F horizon equals 0.38±0.18 mg·Kg$^{-1}$. The MN content is significantly higher ($p$≤0.05) in the A horizon (8.80±1.05 mg·Kg$^{-1}$) when compared to the BC (4.62±1.18

mg·Kg$^{-1}$) and to the F (4.38±1.19 mg·Kg$^{-1}$). The Bw horizon's MN content (6.50±0.59 mg·Kg$^{-1}$) does not significantly differ from the other horizons' content. Eventually, the TKN content showed a significant ($p$<0.05) enrichment in the A horizon (1.73±0.51 g·Kg$^{-1}$) when compared to Bw (0.23±0.08 g·Kg$^{-1}$), BC (0.33±0.11 g·Kg$^{-1}$), and F (0.16±0.03 g·Kg$^{-1}$) horizons.

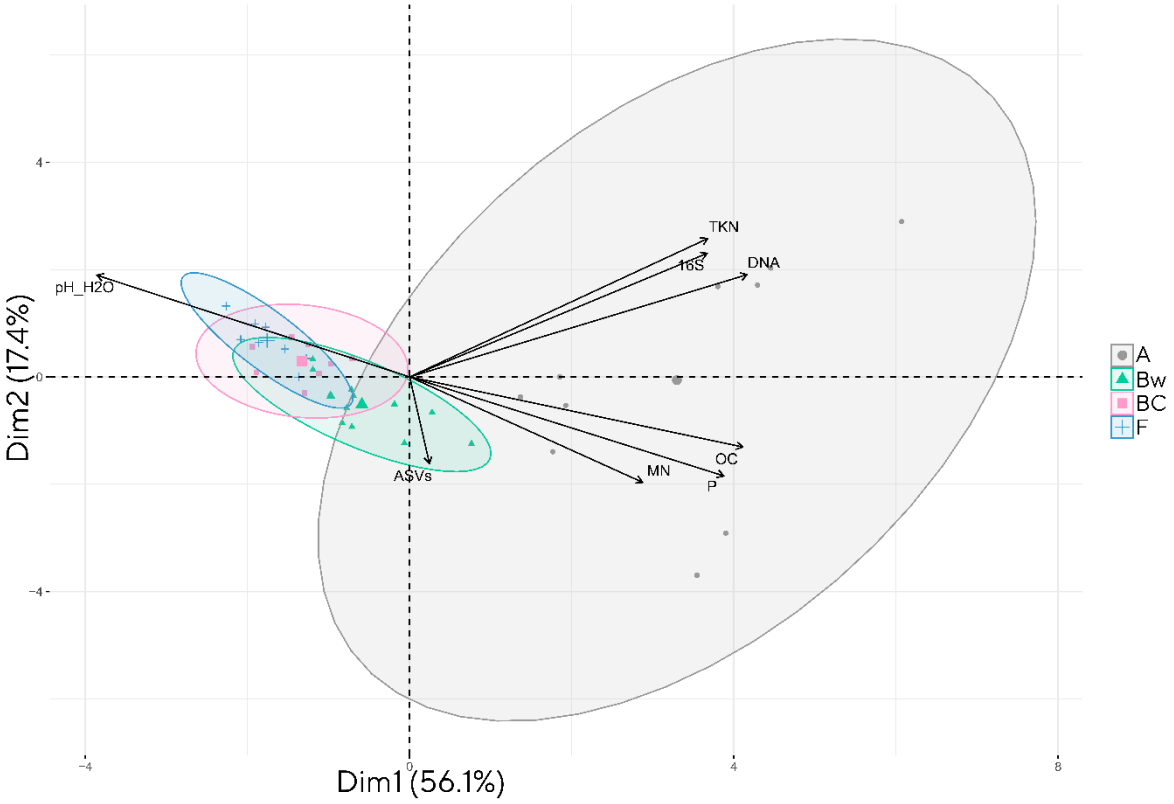


Figure 2. Principal Component Analysis (PCA) biplot illustrating the spatial clustering of the analysed samples based on their chemical and biological properties. Samples located on the same side of a particular variable exhibit higher values for that variable.

Tab. 2. Summary table reporting the results of the physico-chemical analyses performed on the soil samples

| Area | Skeleton | Fine Earth Fraction % | | | | | pH $H_2O$ | Organic C | Kjeldahl N | Micaceous N*** | Available P |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | Coarse Sand | Medium Sand | Fine Sand | Silt | Clay | | % | [g·Kg$^{-1}$] | [mg·Kg$^{-1}$] | [mg·Kg$^{-1}$] |
| AB1 | 65.98±4.49 | 82.27±1.31 | 6.61±0.40 | 8.53±0.85 | 2.59±0.47 | <0.1 | 8.08±0.07 a | 1.55±0.61 a | 0.67±0.25 a | 4.94±0.74 b | 4.44±0.70 a |
| AB2 | 80.96±1.49 | 69.28±4.19 | 6.77±0.83 | 16.91±2.99 | 7.04±1.03 | <0.1 | 8.02±0.08 a | 1.38±0.71 a | 0.26±0.05 a | 4.10±0.43 b | 2.50±0.37 a |
| AB3 | 71.99±4.09 | 81.91±1.90 | 8.24±0.69 | 6.03±0.78 | 4.47±0.95 | <0.1 | 8.06±0.11 a | 4.13±1.59 a | 0.80±0.37 a | 9.36±0.66 a | 3.64±0.89 a |
| Horizon | Skeleton | Fine Earth Fraction % | | | | | pH $H_2O$*** | Organic C*** | Kjeldahl N** | Micaceous N* | Available P*** |
| | % | Coarse Sand | Medium Sand | Fine Sand | Silt | Clay | | % | [g·Kg$^{-1}$] | [mg·Kg$^{-1}$] | [mg·Kg$^{-1}$] |
| A | 66.66±6.22 | 75.00±2.98 | 8.07±0.60 | 13.48±2.47 | 4.31±0.94 | <0.1 | 7.61±0.06 c | 8.23±1.46 a | 1.73±0.51 a | 8.80±1.05 a | 7.10±0.64 a |
| Bw | 69.21±4.00 | 74.71±2.26 | 7.44±0.38 | 12.03±1.64 | 5.83±1.03 | <0.1 | 8.10±0.04 b | 0.93±0.20 b | 0.23±0.08 b | 6.50±0.59 ab | 4.07±0.37 b |
| BC | 70.51±3.73 | 81.08±3.46 | 7.75±0.62 | 7.51±1.75 | 3.66±1.25 | <0.1 | 8.26±0.04 a | 0.08±0.08 c | 0.33±0.11 b | 4.62±1.18 b | 2.00±0.33 c |
| F | 84.18±3.55 | 88.91±2.10 | 5.24±1.26 | 3.34±0.72 | 2.51±0.30 | <0.1 | 8.35±0.05 a | 0.07±0.01 c | 0.16±0.03 b | 4.38±1.19 b | 0.38±0.18 d |

All the analyses have been performed on the dry matter. Means with the same letter in the vertical comparison are not significantly different at the Kruskal-Wallis test. Significance level: *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$

**Total soil DNA extraction, 16S gene copies quantification, 16S metabarcoding, and function prediction**

The assessed total soil DNA content (Table 3) was not significantly different among the three beaches. However, the horizon clustering showed that in the A horizon (17.0±3.32 $\mu g \cdot g^{-1}$) there is a significant ($p$<0.001) 64-fold remarkable enrichment in total soil DNA when compared to the Bw horizon (0.265±0.149 $\mu g \cdot g^{-1}$), and the enrichment spawns up to ~700-fold when the A horizon is compared to BC (0.024±0.001 $\mu g \cdot g^{-1}$) and F (0.022±0.001 $\mu g \cdot g^{-1}$) horizons. The observed trend in the number of 16S gene copies mirrored the one previously described for total soil DNA. Indeed, there are no significant differences in the number of 16S copies quantified within each area. However, it has been observed that the A horizon ($3.29\times10^7\pm9.96\times10^7$) encloses a number of 16S copies that are 164 times greater than those observed in the Bw horizon ($1.99\times10^5\pm1.56\times10^5$) and approximately 2500 times greater than those observed in the BC ($1.31\times10^4\pm2.05\times10^3$) and F ($1.35\times10^4\pm3.14\times10^3$) horizons. Despite the differences in the number of 16S copies and the quantity of total soil DNA, the number of identified ASVs is not significantly different, neither among the three beaches nor among the horizons.

Table 3. Summary table depicting the molecular biology analyses results performed on the soil samples

| Area | DNA Concentration [$\mu g \cdot g^{-1}$] | | *16S* gene copies | | ASVs | |
|---|---|---|---|---|---|---|
| AB1 | 4.71±2.35 | a | $8.59\times10^6\pm4.57\times10^6$ | a | 819,403±123,234 | a |
| AB2 | 2.28±1.51 | a | $5.82\times10^6\pm4.28\times10^6$ | a | 484,863±157,719 | a |
| AB3 | 5.44±2.91 | a | $9.72\times10^6\pm7.51\times10^6$ | a | 384,607±98,222 | a |
| **Horizon** | **DNA Concentration*** [$\mu g \cdot g^{-1}$]** | | ***16S* gene copies*** | | **ASVs** | |
| A | 17.0±3.32 | a | $3.29\times10^7\pm9.96\times10^6$ | a | 647,554±132,103 | a |
| Bw | 0.265±0.149 | b | $1.99\times10^5\pm1.56\times10^5$ | b | 706,962±138,942 | a |
| BC | 0.024±0.001 | c | $1.31\times10^4\pm2.05\times10^3$ | c | 553,579±210,493 | a |
| F | 0.022±0.001 | c | $1.35\times10^4\pm3.14\times10^3$ | c | 317,742±118,696 | a |

Means with the same letter in the vertical comparison are not significantly different at the Kruskal-Wallis test. Significance level: *p≤0.05, **p<0.01, ***p<0.001

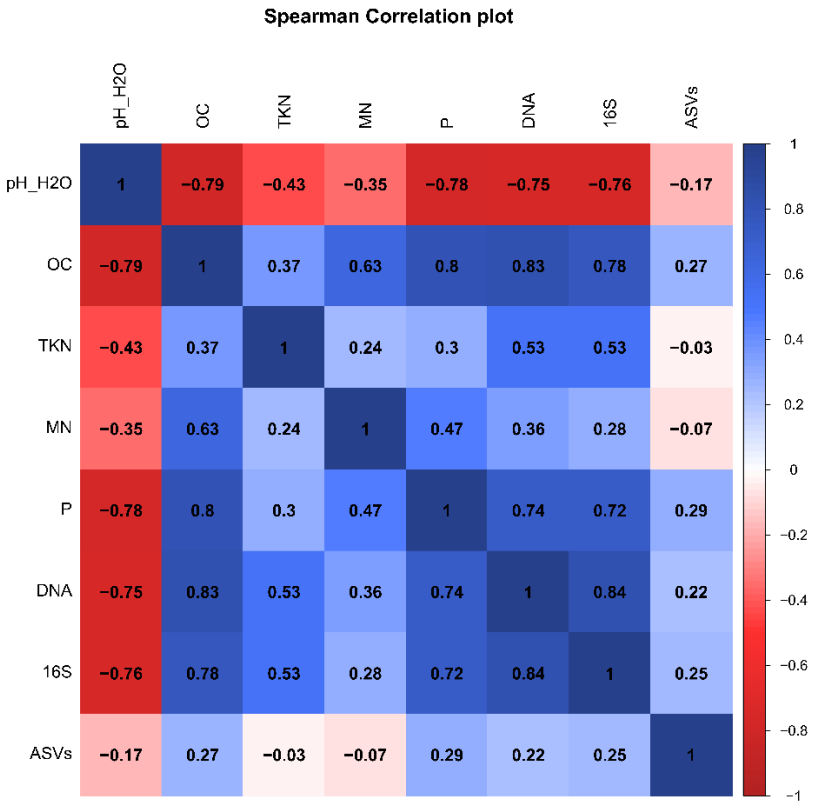


Figure 3. Spearman's rank correlation plot reporting the correlation coefficient ρ for alle the correlations among chemical and biological parameters. Blue and red colours indicate positive and negative correlation, respectively.

The correlation analysis among the biological and physicochemical parameters (Figure 3) revealed some key relationships. Notably, the quantity of total soil DNA (DNA) and the number of 16S gene copies showed a robust correlation (ρ=-0.84 $p \leq 0.05$). On the contrary, the number of ASVs showed no correlation with either the quantity of total soil DNA (ρ=0.22 $p<0.1$) or the number of 16S rRNA gene copies. (ρ=0.25 $p<0.1$).

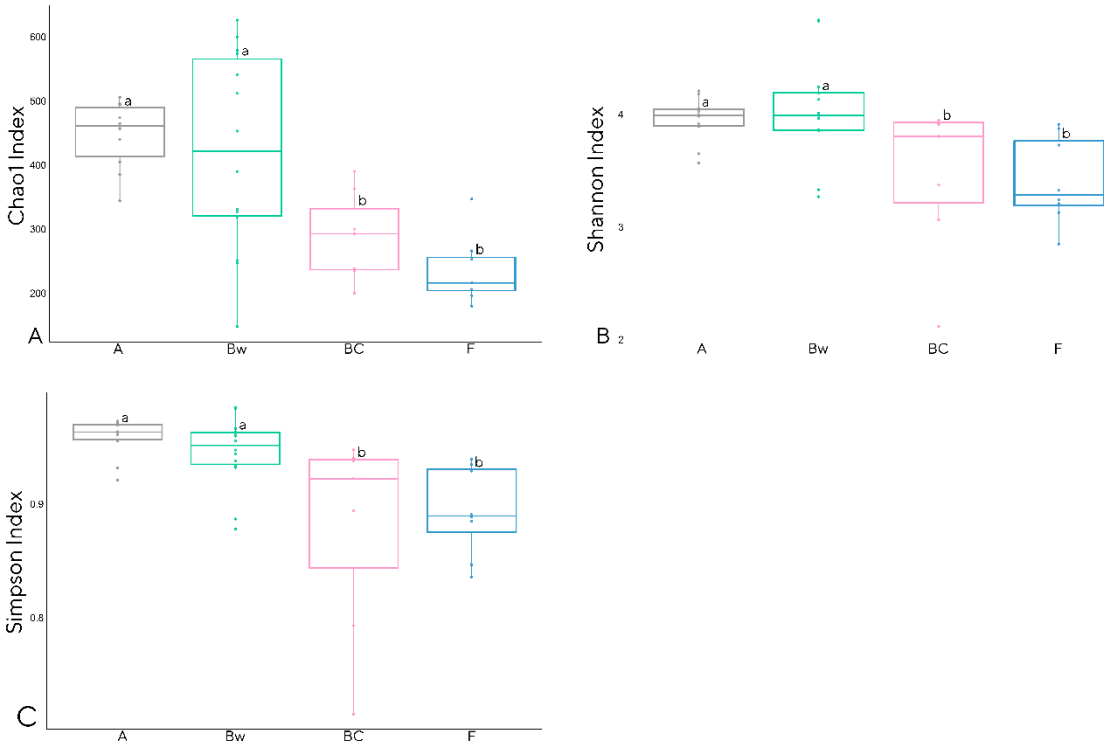


Figure 4. Boxplot comparison of three alpha-diversity indices calculated at the genus taxonomic level in the identified horizons (A horizon in grey, Bw horizon in green, BC horizon in pink, and F horizon in blue). (A) Taxa richness calculated using the Chao1 index, (B) Shannon index, (C) Simpson index.

The analysis of the 16S metabarcoding of the soil samples yielded 30,562,018 single-end reads with an average 239 nucleotide length. The 106,175 identified ASVs were classified into 1,425 taxa. At the phylum rank level, classification covered 76% of the annotated reads, while at the class level, it encompassed 75%. Further, at the order level, the classification covered 73% of the reads, and at the family and genus levels, it reached 72% and 68%, respectively. The findings of the metabarcoding data analyses are presented for the genus rank level, which serves as a representative for the higher taxonomic levels. The alpha diversity within every taxonomical level, from phylum to genus, was assessed through the computation of three ecological indices, specifically Chao1, Shannon and Simpson 1-D. These indices were employed to evaluate community richness and diversity. The area clustering (Supplementary Material, Figure S1) revealed that, in terms of the Chao1 index, AB1 raised beach has the highest absolute value of genus richness, while beach AB2 exhibits the broadest range of values. Conversely, for the Shannon and Simpson 1-D indices, AB2 raised beach shows the highest absolute diversity value, and beach AB3 displays the broadest range. These differences, however, are minimal and non-statistically significant. The horizon clustering (Figure 4) showed that, for all the considered indices,

the superficial horizons A and Bw have significantly ($p$<0.01) higher richness and diversity values when compared to the deeper BC and F horizons. This clustering contraposition of superficial vs. deep horizons has also been observed in the hierarchical cluster plot based on the Bray-Curtis dissimilarity matrix (Figure 5).

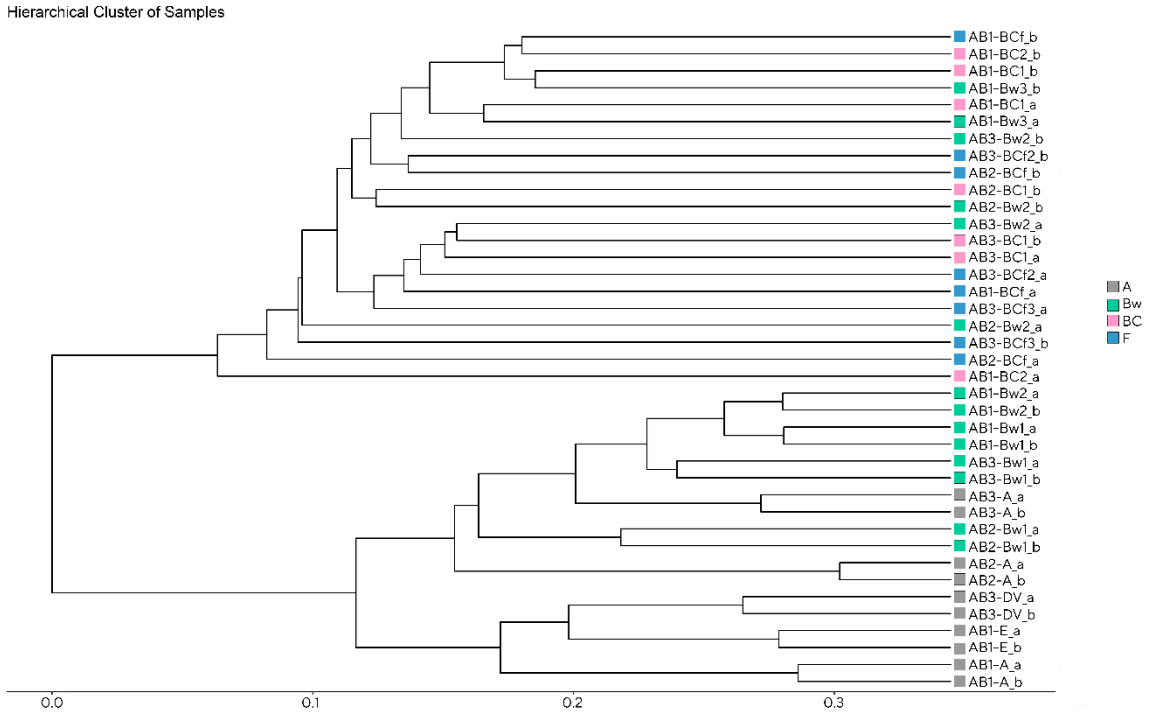


Figure 5. Hierarchical cluster dendrogram of samples based on the Bray-Curtis dissimilarity matrix. Soil samples displayed in grey belong to the A horizon, samples displayed in green belong to the Bw horizon, samples displayed in pink belong to the BC horizon, and samples displayed in blue belong to the F horizon.

A beta diversity analysis has been performed aiming to gain a deeper insight into the distinctions among the studied samples and to discern the relationship between regional and local diversity. The analysis, once more, showed that the area clustering did not feature any significant difference among the three raised beaches (Supplementary Material, Figure S2), yielding a Permutational Multivariate Analysis of Variance (PERMANOVA) $p$-value=0.648 for 999 permutations, and a Permutational Multivariate Analysis of Dispersion (PERMIDSP2) $p$-value=0.288 (999 permutations). The Analysis of Similarities (ANOSIM) returned an $R$ value=-0.02334 and a $p$-value=0.682. The horizon clustering (Figure 6), on the other hand, reported a significant ($p$≤0.001) PERMANOVA. Despite the absence of significant differences in the PERMDISP2, the ANOSIM rendered an $R$ value=0.5336 and a $p$-value=0.001.

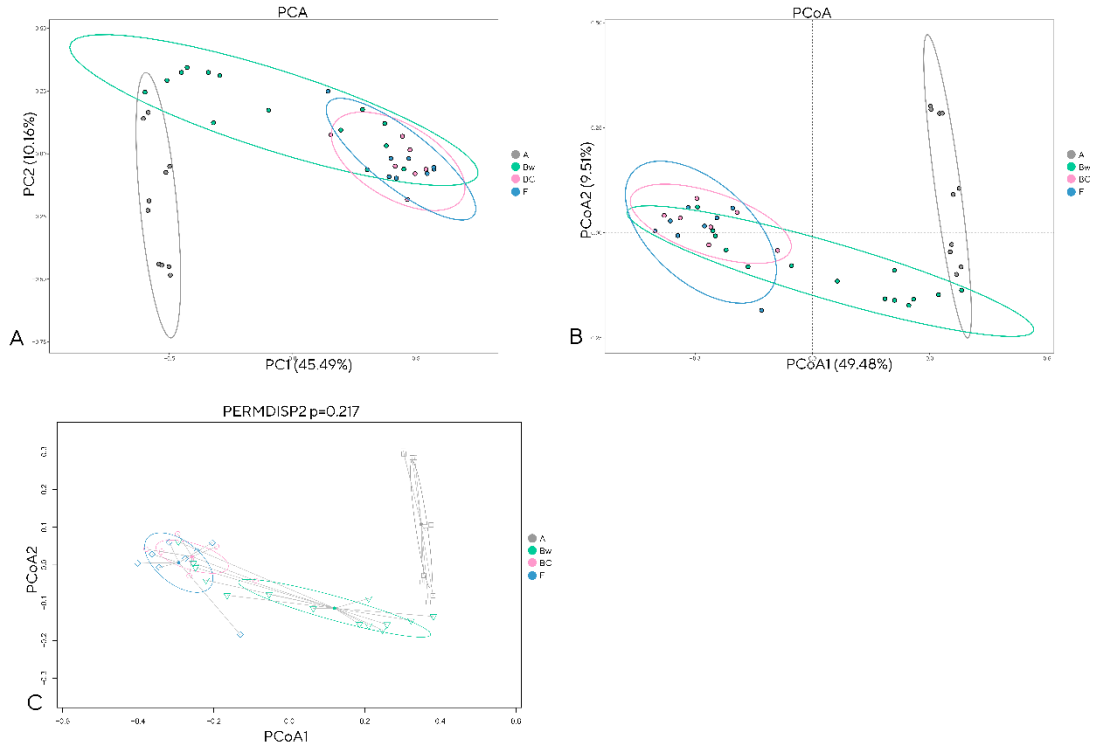


Figure 6. Multivariate analyses for the bacterial communities sequencing data at the genus taxonomic level to evaluate the beta diversity in the identified horizons (A horizon in grey, Bw horizon in green, BC horizon in pink, F horizon in blue). (A) Principal Component Analysis (PCA), (B) Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity matrix, (C) Permutational Multivariate Analysis of Dispersion (PERMDISP2) showing the distance of each sample from the group's centroid.

The analysis of shared and unique taxa (Figure 7) showed that, despite the homogeneity of the alpha and beta diversities among the investigated areas, each raised beach held a detectable number of unique genera. The horizon clustering, as well, revealed a substantial presence of unique genera characterizing each horizon. Once again, it is evident how the shallow horizons can be distinctly differentiated from the deep horizons in terms of the abundance of unique genera and microbial community's profile (Supplementary Material, Figure S3). It is necessary to emphasize that these differences, although notable, are associated with taxa that represent less than 1% of the total identified taxonomic composition. The core microbiome's major constituents, including the Actinobacteriota, Proteobacteria, and Firmicutes phyla, collectively account for 42%, 22%, and 18% of the total identified taxa, respectively.

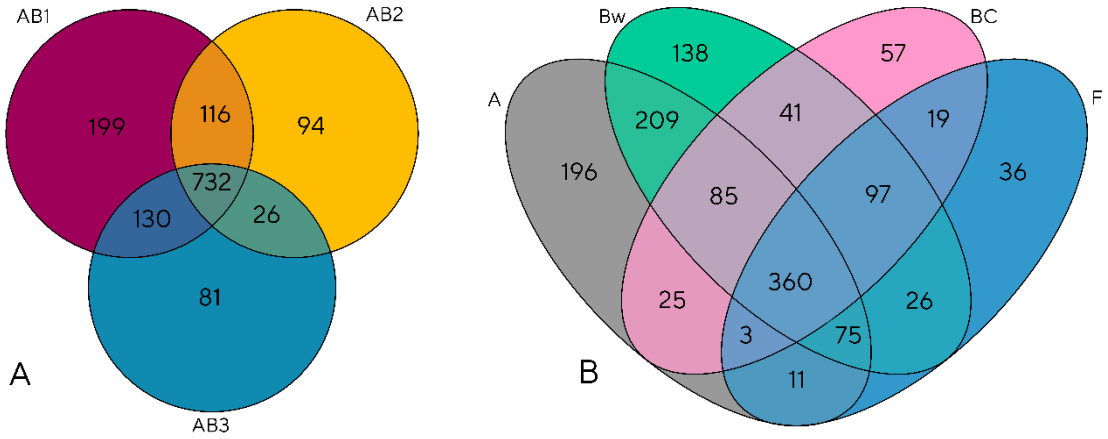


Figure 7. Venn diagrams reporting the shared and unique identified genera. (A) Core microbiome evaluated among the three raised beaches (AB1 in red, AB2 in yellow, and AB3 in blue), (B) core microbiome evaluated among the four horizons (A horizon in grey, Bw horizon in green, BC horizon in pink, and F horizon in blue).

The FAPROTAX online database identified a total of 57 functions. Among these functions, hydrogen oxidation stands out as unique to the AB1 raised beach. Meanwhile, the metabolic functions associated with dark iron oxidation and chlorate reduction, as well as the ecological function related to invertebrate parasitization, are distinctive to the AB2 raised beach. In contrast, the AB3 raised beach exclusively exhibits the ecological function linked to parasitization in fishes. The metabolic and ecological functions mentioned above were also found to be unique in the horizon clustering analysis. Notably, the A horizon is distinguished by the metabolic dark iron oxidation and chlorate reduction functions and by the ecological function of fishes' parasitization, while the invertebrates' parasitization characterizes the Bw horizon, and hydrogen oxidation metabolic function is a defining feature of the BC horizon. In general, the area clustering did not emphasize significant differences among the three raised beaches, whereas the horizon clustering drew attention to a higher number of identified functions in the superficial horizons (A and Bw). Simultaneously, the same horizons are characterized by a reduction in the even distribution of ASVs within each function (Supplementary Material, Figure S4).

## Discussion

In this study, the extensive analysis, encompassing physicochemical parameters and 16S metabarcoding data across the three raised beaches and the four considered soil horizons, offered intriguing insights into the ecological and functional diversity of soil bacterial communities in the Arctic environment.

While the MN enrichment in AB3 raised beach is indeed peculiar, however, probably driven by the non-deterministic variation in the mineral composition of the beach itself, the

consistent absence of significant differences among the investigated areas can be attributed to their common location within the Devon Plateau. This shared geographical setting exposes them to identical pedoclimatic conditions, which exert consistent ecological pressures on soil microbial communities. The quantifiable observed differences regarding unique taxa and predicted functions can be attributed to a passive enrichment in microbial biomass driven by wind action. As highlighted by Rosselli et al. (2015), events involving the transport of dust operated by the wind can lead to a noticeable increase in soil biodiversity. Concurrently, aeolian transportation is an utterly relevant phenomenon well-documented in the Canadian Arctic region (Fortier et al., 2006, Gregor et al., 1996, Lewkowicz and Young, 1991, Welch et al., 1991), and it has also been recorded in the Devon Plateau (Ugolini et al., 2006).

Extensive researches have provided substantial evidence regarding the pivotal role played by abiotic factors in shaping the composition and structure of soil bacterial communities (Fierer and Jackson, 2006, Islam et al., 2018, Malard and Pearce 2018). Furthermore, in arid environments, it has been consistently observed that edaphic niches exhibit remarkable heterogeneity, particularly concerning the wide array of physicochemical parameters (Choe et al., 2021, Pajares et al., 2016). In line with the existing literature, our results underscore how the physicochemical distinctions observed among the investigated horizons actively contribute to defining the composition and structure of microbial communities. In particular, the hierarchical cluster analysis based on the Bray-Curtis dissimilarity matrix in Figure 5 vividly illustrates the distinct separation between surface and deep horizons. The insights gleaned from the PCA biplot in Figure 2 and the corrplot in Figure 3 emphasize that pH, OC content, and available P content serve as the three primary controlling factors for the soil microbiome. Notably, pH is acknowledged as a key regulator of nutrient availability in soil, thus emerging as the primary influencer of bacterial community diversity, even within Arctic soils (Chu et al., 2010, Siciliano et al., 2014). This investigation unveils a robust negative correlation between soil pH and the levels of OC and available P. Simultaneously, it reveals a strong positive correlation linking total soil DNA content, 16S gene copies, and the content of OC and available P, in accordance with the results published by Tian et al. (2021) and Oliviero et al. (2020). The analysis of beta diversity, specifically employing PCA and PCoA coupled with PERMANOVA, reveals that the observed diversities in the average community composition within each horizon are statistically significant and not driven by stochastic effects. Additionally, ANOSIM further supports the validity of grouping the samples in the four identified horizons. The identification of a core microbiome comprising Actinobacteriota, Proteobacteria, and

Firmicutes aligns with prior research conducted in Arctic (Ganzert et al., 2014) and Antarctic (Krauze et al., 2021, Bajerski and Wagner, 2013) polar regions. These studies acknowledge these bacterial phyla as particularly well-adapted to endure the extreme climatic conditions prevalent in these regions. These phyla, which appear to play crucial roles in the circulation of carbon, nitrogen, phosphorus, and sulfur (Goodfellow and Williams, 1983; Hill et al., 2011; Holmalahti et al., 1994; Spain et al., 2009), are not confined to polar regions alone. They can also be found in highly disturbed environments, including hot deserts (Chilton et al., 2022; Makhalanyane et al., 2015), grazed soils (Vega-Cofre et al., 2023), and soils impacted by controlled and wildfires (Qin et al., 2022).

Eventually, the substantial detectability of 16S rDNA can be attributed to the specific environmental traits of the Devon Plateau. Devon Island has a slow pace of change, boasts minimal human impact, and features low precipitation levels. These factors, coupled with the propensity of certain bacteria to sporulate, collectively foster a conducive environment for DNA preservation. It is important to emphasize that DNA preservation and amplifiability, while crucial for the analysis process, are not sufficient on their own to ensure reliable results. The decision to employ dPCR, a robust molecular technique known for its high biological sensitivity and reproducibility (Sanders et al., 2011), enables the detection of challenging DNA quantities without the need for an external calibration curve (Devonshire et al., 2015, Whale et al., 2017).

The results presented in this manuscript contribute to expanding our knowledge concerning the microbial communities characterizing High Arctic soils and their responses to environmental conditions. Moreover, they provide potential evidence regarding the persistence and preservability of environmental DNA, which appears to be a relatively stable and detectable molecule. Finally, these findings enrich the available information for the scientific community, aiding in predicting how climate changes may influence the microbiome in the context of soil health and stability conservation.

## Supplementary Material

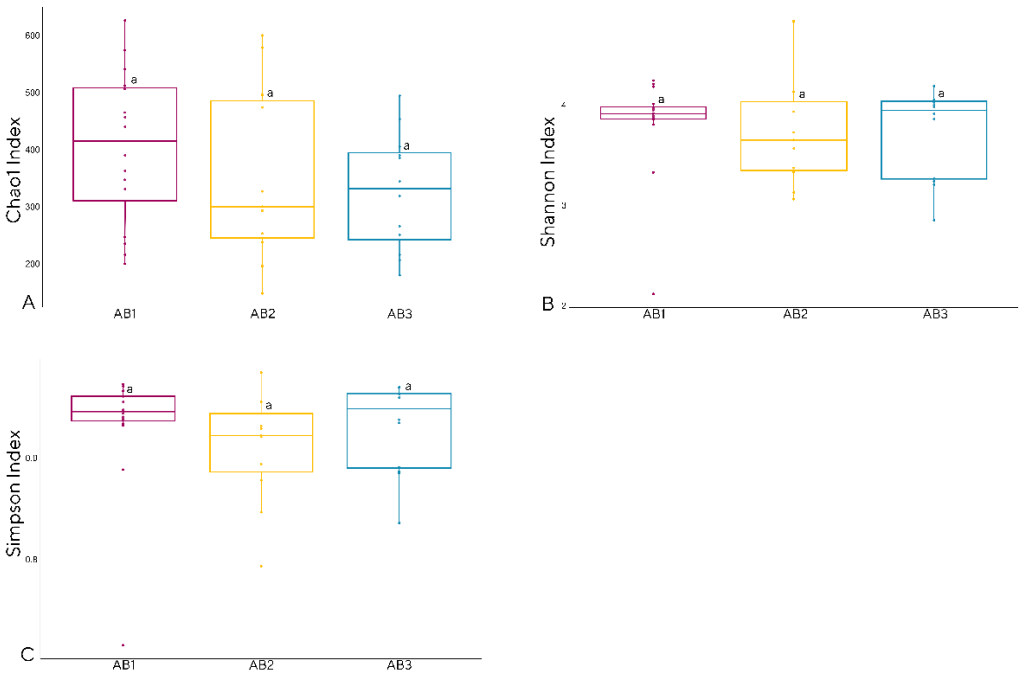


Figure S1. Boxplot comparison of three alpha-diversity indices calculated at the genus taxonomic level in the raised beaches (AB1 in red, AB2 in yellow, and AB3 in blue). (A) Taxa richness calculated using the Chao1 index, (B) Shannon index, (C) Simpson index.

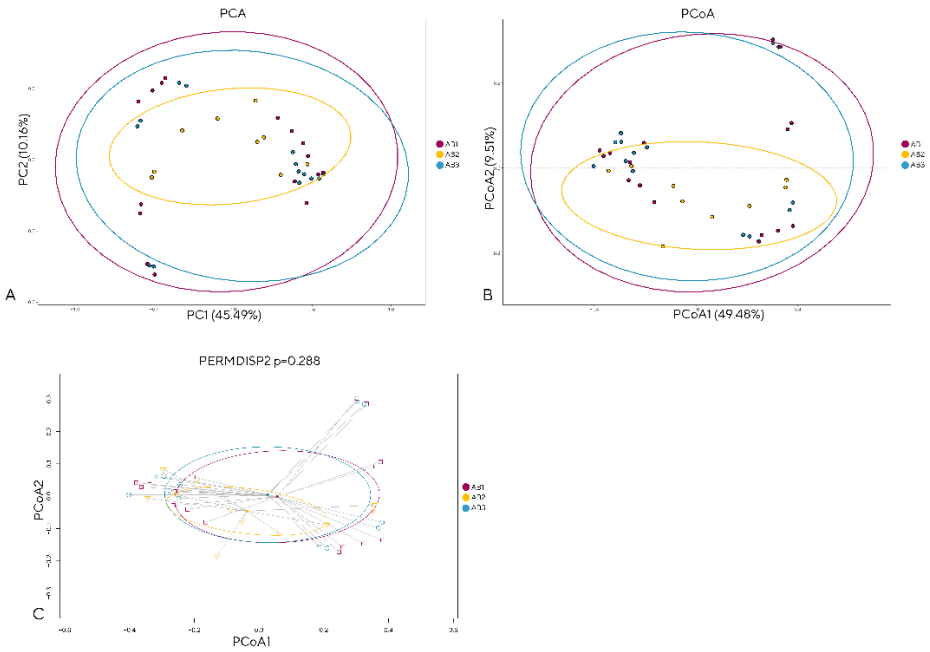


Figure S2. Multivariate analyses for the bacterial communities sequencing data at the genus taxonomic level to evaluate the beta diversity in the raised beaches (AB1 in red, AB2 in yellow, and AB3 in blue). (A) Principal Component Analysis (PCA), (B) Principal Coordinate Analysis (PCoA) based on the Bray-Curtis dissimilarity

matrix, (C) Permutational Multivariate Analysis of Dispersion (PERMDISP2) showing the distance of each sample from the group's centroid.

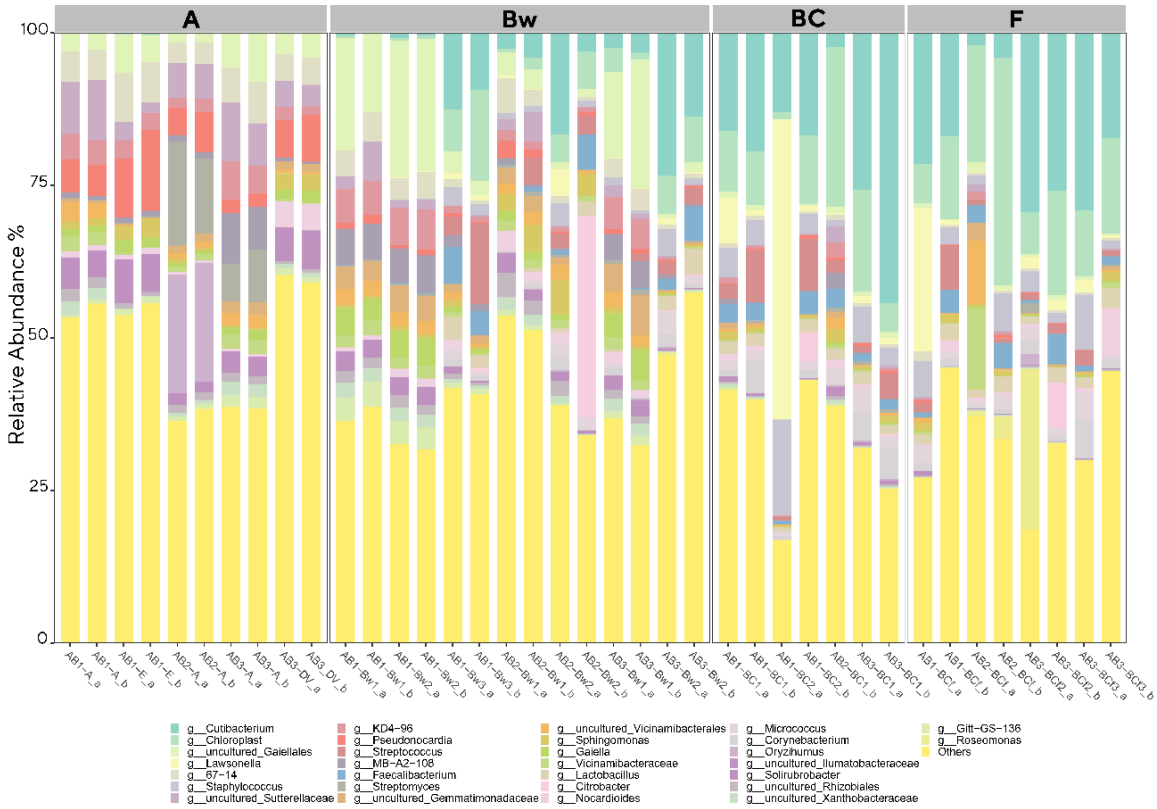


Figure S3. Relative sequence abundance of bacterial genera associated within each sample. The top 30 most abundant genera are displayed individually, the rest of the identified genera are marked as "Others".

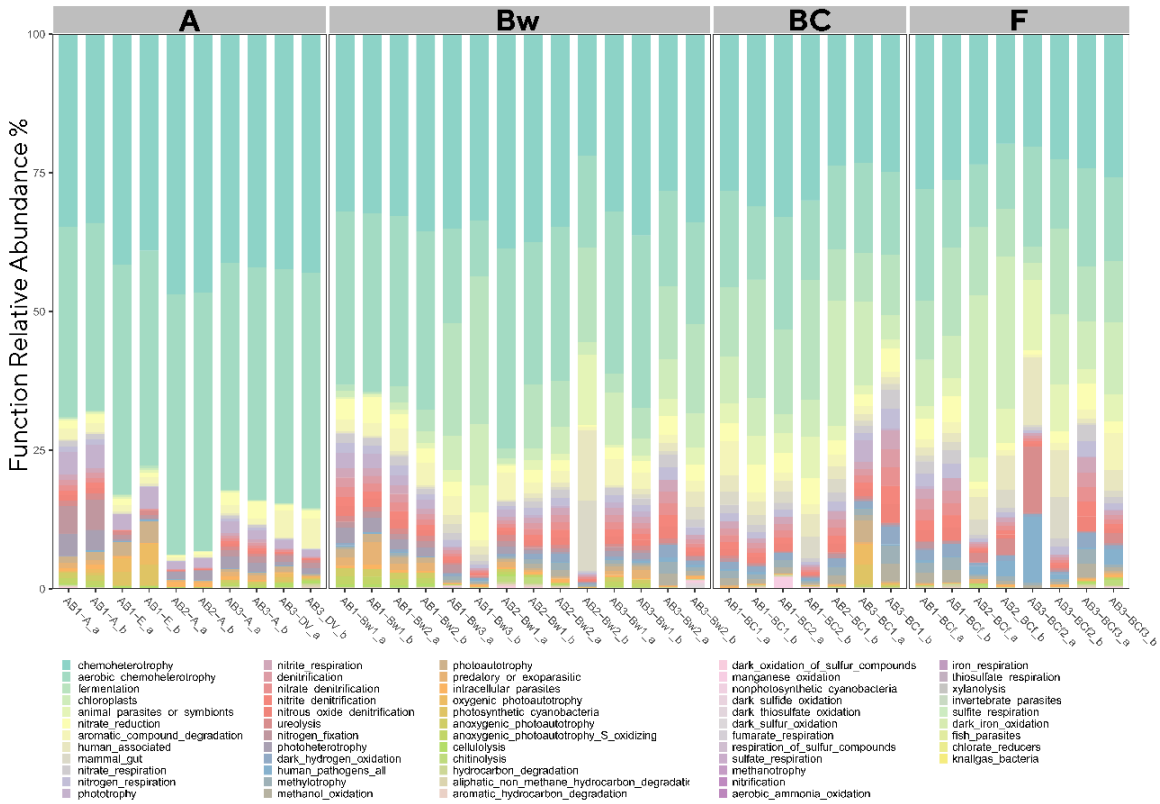


Figure S4. Relative abundance of the fifty-seven predicted functions identified within each soil sample by the FAPROTAX database.

## Authors' Contribution

LM drafted the manuscript, analyzed the samples, and performed the data analyses. SD performed the bioinformatic analysis. GCor and SC collected the samples. All the authors critically revised the paper.

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# GENERAL CONSLUSIONS

Soil is a vital resource for human existence, and its study plays a decisive role in sustainable development strategies. Soil profiling often relies on methodologies that approach each scientific discipline separately. This results in a loss of information and a diminished understanding of soil functioning within its ecosystem.

This thesis contributes to enriching the body of literature that delves into the topic of the soil microbiome related to environmental functionality.

The first contribution involves studying the effects of degradation and subsequent restoration of landfill soil. It proposes a methodological approach for soil analysis that combines a range of techniques, including physical, chemical, molecular, and bioinformatic methods. Moreover, the second contribution identifies specific molecular markers that offer valuable insights into soil productivity and fertility. The third contribution centered on the study of soils from the Canadian High Arctic, adds to our understanding of how the soil microbiome evolves in response to environmental factors that exert noticeable evolutionary pressures on these communities.

The outcome of this work offers a comprehensive insight into how biotic conditions, whether natural or influenced by human activities, impact soil functionality. Deciphering these intricate relationships that govern terrestrial ecosystems poses a significant challenge that the scientific community, with the support of institutions, has to address to effectively manage the future scenarios shaped by climate change.

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