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**Modulation of sewage sludge microbiota for enhancing the
transition from wastewater treatment plants into biorefineries
in the circular economy era**

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CHAPTER 1

Introduction

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ABSTRACT

Pollution demands urgent action, with waste reuse emerging as a critical strategy for sustainability. Wastewater treatment plants (WWTPs) play a key role in processing wastewater from residential, industrial, and commercial sources to ensure safe water discharge. However, WWTPs operate in a “linear” mode and that imply high energy and disposal costs. To address these challenges, the European Union promotes the principles of circular economy, envisioning WWTPs as biorefineries that recover valuable compounds from waste. For example, sewage sludge can yield volatile fatty acids (VFAs) and polyhydroxyalkanoates (PHAs), the latter being a sustainable alternative to petroleum-derived plastics. This study, part of the European “Achieving Wider Uptake of Water-Smart Solutions” project, examines how different operational conditions, such as pH, oxidizing agents like potassium permanganate, headspace volume, organic loading rate, and carbon-to-nitrogen ratio, affect microbial metabolism to enhance VFA and PHA production. The approach involves metagenomic DNA extraction, 16S rDNA sequencing, and gas chromatography analysis to assess microbial community changes and quantify VFA and PHA yields. Laboratory-scale batch reactors and a pilot plant on the University of Palermo campus simulate full-scale WWTP conditions, processing wastewater diverted from campus facilities. Metataxonomic analysis shows substantial shifts in sludge microbiota pre- and post-treatment, supporting improved VFA and PHA production. The sewage sludge microbiota primarily comprises Proteobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia, with minor Archaea presence. Increased VFA production was linked to higher Firmicutes levels, though some methanogenic archaea consumed these VFAs. PHA production was associated with a steady rise in Proteobacteria, particularly Betaproteobacteria and Rhodocyclaceae. Some isolated Proteobacteria strains demonstrated optimal PHA production, suggesting sewage sludge could yield bioplastic precursors. Treated wastewater (TWW), currently discharged into water bodies, could be reused for irrigation or for fertilization after enrichment of biochar and zeolite with nutrients. Further research examined the soil microbiota of plants irrigated using TWW added with wastewater recovered inorganic fertilizers, highlighting the presence of putative beneficial bacteria and the absence of possible pathogenic bacterial strains. One of the emergent contaminants to be considered in wastewater are antibiotic-resistant bacteria and DNA containing antibiotic resistance genes. This is consistent with the detection of resistance genes for vancomycin and tetracycline and 51.16% resistance to ampicillin and erythromycin of isolated bacteria.

Altogether these findings increase our understanding of the role of WWTP microbiome modulation under various operational conditions and highlight the potential of exploiting sewage sludge and water resources within a circular economy framework.

Introduction

1.1 Environmental pollution and wastewater treatment plants

Pollution, driven by urbanization and technological development, is a pressing global issue, endangering ecosystems and human health through different kinds of pollutants (Strokal et al., 2021; Sun et al., 2023). In particular, water pollution affects rivers, lakes, and aquifers through urban and industrial waste, agricultural runoff, plastics, and pharmaceuticals, with freshwater demand expected to rise by 55% by 2050, surpassing sustainable supplies (du Plessis, 2022; OECD, 2012). Climate change intensifies water scarcity via erratic rainfall and extreme weather, complicating access to drink water (WEF, 2020). Managing water use and improving wastewater treatment, which combines physical, chemical, and biological processes to ensure safe water discharge or reuse, is crucial to mitigate pollution impacts (Kassa, 2021). Untreated wastewater poses genotoxic and ecotoxic risks, highlighting the need for enhanced WWTP capabilities (Choudri et al., 2020).

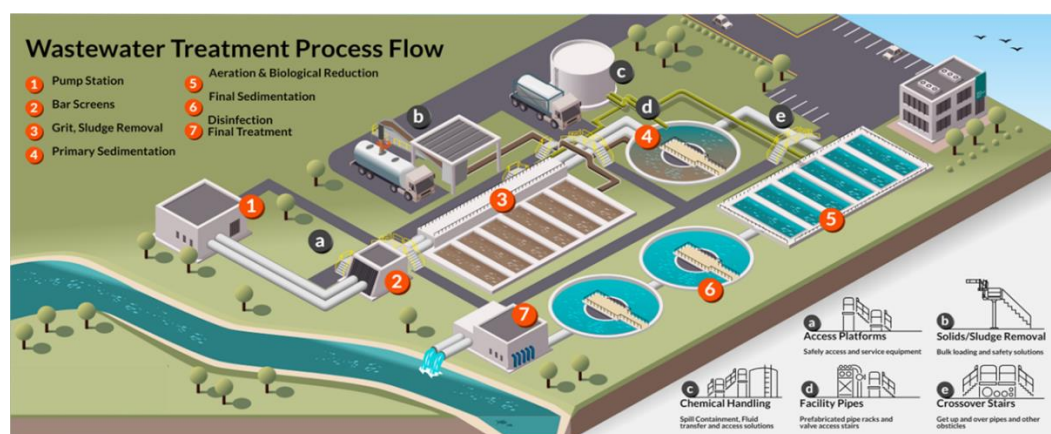


Figure 1.1. Scheme of wastewater treatment process flow (Image adapted from <https://www.saferack.com>).

The wastewater treatment process comprises several stages. Primary treatment involves screening to remove coarse particles and sedimentation to separate liquid from solids. Secondary treatment in wastewater treatment plants (WWTPs) utilizes biological processes and sedimentation to break down dissolved organic material, aided by bacteria that transform contaminants into harmless byproducts (Sravan et al., 2024). Tertiary treatment targets

nutrient removal, specifically nitrogen and phosphorus, to prevent eutrophication and includes filtration and disinfection using chlorine, ozone, or UV light (Crini et al., 2018). The microbiota residing wastewater supports aerobic, anaerobic and anoxic processes: aerobic bacteria decompose organic material with oxygen to carbon dioxide, while anaerobic bacteria produce different gasses like carbon dioxide, hydrogen and methane. Specialized bacteria remove nitrogen through nitrification and denitrification and reduce phosphorus by forming insoluble phosphates, helping to curb nutrient pollution (Delgadillo-Mirquez et al., 2016). Biological treatment is also cost-effective and environmentally sustainable compared to chemical methods (Tawfik et al., 2022).

1.2 The transition of wastewater treatment plant into water resource facility to recover valuable material from waste

Reusing resources from WWTPs is essential for meeting the increasing demand for water, reducing pollution, and fostering environmental and economic sustainability. Transitioning traditional WWTPs to biorefineries represents a practical approach to recover valuable compounds from waste. Sewage sludge (SS) contains a modifiable microbiota that can produce various valuable materials. This shift from a linear to a circular economy is critical for transforming WWTPs into water resource recovery facilities (WRRF), effectively acting as biorefineries for resource recovery (Mannina et al., 2021). The European Horizon 2020 project "Achieving Wide Uptake of Water-Smart Solutions" (WIDER-UPTAKE) aims to promote industrial symbiosis, enhance resource efficiency, reduce emissions, and develop sustainable water-smart solutions. The project aims to demonstrate how wastewater can be effectively reused through innovative technologies and governance strategies, thereby reducing social, regulatory, and economic barriers. These initiatives underscore the need to involve communities, policymakers, and utility managers in a collaborative framework, fostering trust and transparency around resource recovery processes and contributing to a broader cultural shift toward water-smart solutions in urban environments. The WIDER-UPTAKE research project employs a case study to provide practical insights into implementing these solutions across diverse socioeconomic, environmental, and climate contexts (Mannina et al., 2022). The project, which involves a multidisciplinary team of engineers, agronomists, microbiologists and lawyers, focuses on recovering resources from purified water and SS generated during wastewater treatment. The microbiota biomass of SS can be repurposed as compost, soil improvers, and bioplastic precursors. Meanwhile, purified water can serve as irrigation for green areas and experimental lands or as a source of fertilizers containing nutrients like nitrogen and phosphorus (Mannina et al., 2021). The

WIDER-UPTAKE project also aims at modernizing two municipal WWTPs in Sicily, located in Marineo and Corleone, which require upgrading (Figure 1.2 a-b). Due to the complexity of monitoring all process conditions in large-scale WWTP operations, pilot plants were developed and established at the Engineering Department of the University of Palermo (Figure 1.2 c). These pilot plants were realized in the within of the WIDER-UPTAKE project and collect wastewater from the campus canteen and guesthouse via a diversion line installed beneath the floor. Additionally, further experiments were conducted at the laboratory scale using batch fermentation reactors (Figure 1.2 d).

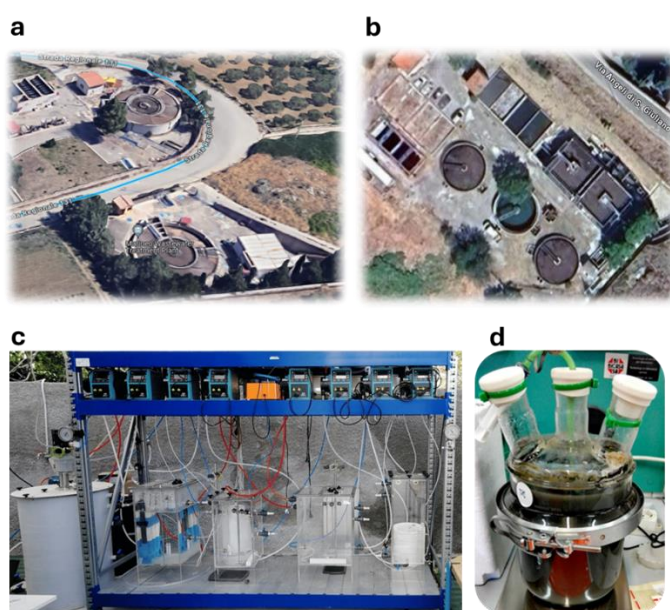


Figure 1.2. Panoramic view of WWTPs of Marineo (a) and Corleone (b)(<https://googlemaps>); panoramic view of pilot plant (c)(image adapted from Mannina et al., 2021); batch fermentation test reactor (d).

1.3 The exploitation of the capabilities of microbiota members to recover volatile fatty acids and polyhydroxyalkanoates from sewage sludge

SS microbiota members play a crucial role in wastewater management, aiding in organic matter breakdown, production of biomass to be converted to bioplastic precursors, and nutrient recovery, essential for environmental and agricultural sustainability (Annamalai et al., 2020). Specifically, bacteria in SS can produce volatile fatty acids (VFAs) through anaerobic fermentation, which serve to feed as building blocks specific bacterial fermentation reactors for polyhydroxyalkanoates (PHAs). PHAs are a sustainable alternative to petroleum-derived plastics (Varghese et al., 2022) and VFAs, which include acetic,

propionic, and butyric acids, are produced during the acidogenic fermentation process (Xu et al., 2021). Now a day, the *ad hoc* production of PHAs, especially polyhydroxybutyrate (PHB), in specific production plants are costly while utilizing SS as a carbon source can improve cost-effectiveness (Reddy et al., 2003; Li et al., 2016). The importance of WWTP operation conditions for resource recovery is exemplified by PHA production that can be achieved through a feast-famine strategy, stimulating microbes to store polymers. Enzymes crucial for this process are encoded within operons that enhance PHA biosynthesis (Huang et al., 2018; Zhou et al., 2023). Employing a selective-sequencing batch reactor (S-SBR) and manipulating organic loading rates (OLRs) and carbon/nitrogen ratios can further enhance microbial capacity for VFA and PHA production, supporting sustainable bioplastic development and resource recovery (Samrot et al., 2021).

1.4 The microbiota of soils irrigated with treated wastewater added with recovered fertilizer

TWW contains essential nutrients like nitrogen and phosphorus, which can reduce chemical fertilizer reliance. However, its high salt content—especially sodium and chloride—can hinder plant growth, contributing to low global reuse rates of less than 1% (Muscarella et al., 2024; Lahlou et al., 2021; Salgot & Folch, 2018). TWW includes bacteria belonging to the *Bacillus* genus, which produce and/or modulate plant growth-boosting phytohormones (Sarawaneeyaruk et al., 2019), and members of the genera *Desulfovibrio*, *Pseudomonas*, and *Rhodococcus*, which degrade harmful compounds and enhance soil health (Sharma et al., 2023). Actinobacteria, notably *Streptomyces*, are also beneficial in TWW, producing natural antibiotics and aiding in nutrient solubilization (Boufercha et al., 2022). As above mentioned, the WIDER UPTAKE project focuses on nutrient recovery from TWW, including ammonium and phosphate, with zeolite and biochar facilitating their extraction through ion exchange and adsorption (Muscarella et al., 2021, 2024). Experimental setups using biochar or zeolite-filled columns allow TWW flow via peristaltic pumps to enhance ammonium and phosphate recovery. The enriched absorbents are then applied to test soil types in greenhouse studies for tomato and fava bean growth, thereby assessing the impact of TWW on soil and plant health.

1.5 Wastewater treatment plants as reservoir of antibiotic resistance

Antibiotic resistance in wastewater presents a severe health risk globally, with evidence showing it as a leading cause of death worldwide (Sambaza et al., 2023). Wastewater from homes, hospitals, industries, and farms contains antibiotic residues and resistant bacteria,

primarily from drug use and products like antibacterial soaps. Hospitals and pharmaceutical manufacturers contribute significantly as antibiotics enter wastewater systems, while agricultural practices add to the load through animal waste runoff (Manaia et al., 2018). WWTPs cannot often entirely remove these contaminants, allowing antibiotics and resistant bacteria to persist and spread in the receiving reservoirs. Primary treatment can trap bacteria on organic matter in tanks, encouraging genetic exchanges that promote resistance, and biological treatment processes may also foster new resistant strains. Residual antibiotics in treated water continue to support resistant bacteria, contaminating water bodies, soil, and even crops irrigated with inadequately treated water, reintroducing resistant bacteria and antibiotic resistance genes into the food chain. This cycle complicates human infection treatment. Monitoring wastewater is crucial to control the spread of antibiotic resistance. In this study, bacterial strains from WWTPs were isolated and tested for resistance to Tetracycline, Chloramphenicol, and Gentamicin using the Kirby-Bauer disk diffusion method, while whole-genome sequencing (WGS) allows identifying resistance genes in the treated wastewater microbiome.

Objectives of the research project

This Ph. D. research project, paralleling the principles of a circular economy and the goals of the European WIDER UPTAKE project, aims to explore the relationships between modulation of SS microbiota structure and operating conditions in either batch fermentation or pilot plant reactors. Therefore, with the combination of DNA metagenomic Next Generation Sequencing (NGS) analyses – based on metataxonomic or whole genome shotgun approaches - and the monitoring of fermentation parameters, the study aspires to identify the best process performance to increase the production of valuable materials and enable the reuse of treated waste from a WWTP through the exploitation of microbial capabilities.

To optimize production of VFA, which can be used as precursors of bioplastic, batch fermentation experiments were conducted with modulations of parameters such as pH and the addition of potassium permanganate. Subsequently, headspace volume was evaluated in a pilot-scale plant, and the SS microbiota was analyzed pre- and post-fermentation to assess microbial dynamics.

To improve PHA production volumetric organic loading rate and C/N ratio were assessed in a pilot plant, with VFAs serving as the primary carbon source. A microbial community analysis was conducted using varying process parameters to identify the microorganisms selected and involved in PHA production. In addition to metagenomic analyses, this study exploits culture-dependent methods for isolating individual microbial strains, selecting excellent PHA-producer bacteria and analysing their in-vitro ability to produce PHAs in axenic culture.

A WWTP produces SS and purified water, typically released into water-receiving bodies but potentially reusable for crop irrigation. This purified water is also rich in nitrogen and phosphorus, which can be recovered as fertilizers through adsorption in columns containing zeolite and biochar, respectively. This study also aims to analyse the soil microbial community of seedlings grown in greenhouses, irrigated with TWW, and treated with biochar and zeolite enriched with phosphate and ammonium recovered from TWW filtration. The objective is to understand whether, despite the treatment with TWW and recovered fertilizer, plant growth-promoting bacteria (PGPB), bacteria involved in nutrient metabolism, and any pathogens harmful to humans may be present in the soil, given that the plants had parts edible properties.

It is known that WWTPs are also reservoirs of antibiotic-resistant bacteria and genes that confer resistance. Since these systems cannot completely eliminate antibiotic-resistant bacteria from the water, it is crucial to monitor TWWs not only through disk diffusion assays using the Kirby-Bauer method to identify resistant strains but also by employing a WGS approach to detect resistome and prevent their spread into the environment.

Such studies, in a multidisciplinary context in which the Agriculture and Engineering Departments are involved, provide the opportunity to optimize the functioning conditions and promote the enhancement of the products obtained in the treatment plants, to improve the transition of WWTP into WRRF, safeguarding human and the environment.

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CHAPTER 2

Initial pH Conditions Shape the Microbial Community Structure of Sewage Sludge in Batch Fermentations for the Improvement of Volatile Fatty Acid Production

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ABSTRACT

Conversion of wastewater treatment plants into biorefineries is a sustainable alternative for obtaining valuable compounds, thus reducing pollutants and costs and protecting the environment and human health. Under specific operating conditions, microbial fermentative products of sewage sludge are volatile fatty acids (VFA) that can be precursors of polyhydroxyalkanoate thermoplastic polyesters. The role of various operating parameters in VFA production has yet to be elucidated. This study aimed to correlate the levels of VFA yields with prokaryotic microbiota structures of sewage sludge in two sets of batch fermentations with an initial pH of 8 and 10. The sewage sludge used to inoculate the batch fermentations was collected from a Sicilian WWTP located in Marineo (Italy) as a case study. Gas chromatography analysis revealed that initial pH 10 stimulated chemical oxygen demands (sCOD) and VFA yields (2020 mg COD/L) in comparison with initial pH 8. Characterization of the sewage sludge prokaryotic community structures—analyzed by next-generation sequencing of 16S rRNA gene amplicons—demonstrated that the improved yield of VFA paralleled the increased abundance of fermenting bacteria belonging to Proteobacteria, Bacteroidetes, Chloroflexi, and Firmicutes phyla and, conversely, the reduced abundance of VFA-degrading strains, such as archaeal methanogens.

2.1 Introduction

Wastewater is the combination of domestic, industrial, urban, and agricultural water collected by urban drainage systems that flows into wastewater treatment plants (WWTPs). In a WWTP, the water undergoes various purification processes, both physical and microbiological, to get rid of organic and inorganic pollutants that can endanger human health and the environment by causing genotoxicity, cytotoxicity, tumorigenesis, and ecotoxicity (Choudri et al., 2020). Physical processes such as sandblasting (screening), deoiling (grease removal), and grilling (grit removal) represent the first stage of wastewater treatment to eliminate coarse particles. Afterwards, microorganisms with their metabolic capacities degrade the organic matter, producing the simplest compounds and biomass as a result of microbial growth. Clarification and decantation to remove the insoluble component from the water (i.e., sewage sludge including microbial cells) and disinfection represent the two final stages (Newhart et al., 2019). At the end of the treatment, the purified water is introduced into receiving water bodies, for example, lakes, rivers, and the sea. On the other hand, the produced sewage sludge is, in most cases, disposed of in solid-waste landfills or

incinerators. Although conventional WWTPs play an essential role in regulating the eutrophication of aquatic environments, they also cause environmental pollution because of the produced greenhouse gases (Hao et al., 2019) or the accumulation of sewage sludge disposals. In Italy, 395,000 tons of dry sewage sludge are produced annually from WWTPs. Various processes are applied to dispose of the sewage sludge (incineration, storage in specific landfills); only 9.9% is reused in agriculture (Mininni et al., 2019). Thus, it is a challenge to obtain materials and energy still present in the sewage sludge, transforming WWTPs into water resource recovery facilities (WRRFs) or wastewater biorefineries (WWBRs) to protect water quality, the environment, and thus human health and for the recovery of high-value compounds with the intent of sustaining circular economic processes (Mannina et al., 2021). In particular, sewage sludge can be a source of compounds, such as biopolymers or their precursors. The demand for biodegradable polymers has grown by 30%, while that for conventional polymers by 3% (Hejase et al., 2018). Polyhydroxyalkanoates (PHAs) are thermoplastic biodegradable polyesters with excellent properties. PHAs are environmentally and economically sustainable microbial fermentation products, suitable for replacing petroleum-derived plastics (Tarrahi et al., 2020). In a WWTP, microbial communities residing in the sewage sludge use organic matter, such as proteins, polysaccharides, and lipids, and produce VFA, H₂, and CO₂ (Sarkar et al., 2018; Sarkar et al., 2021). VFA such as acetic, butyric, propionic, and valeric acids can be excellent precursors to feed the production of PHAs that accumulate in bacterial cells in the form of granules as carbon and energy reserves (Shahid et al., 2021). It is known that the concentration of carbon, temperature, and variation in environmental pH are fundamental parameters that activate the production of PHAs (Zheng et al., 2021). Due to the importance of VFA production in the development of a PHA producing facility, an increasing number of studies have been carried out focusing on the prokaryotic (i.e., bacterial and archaeal) microbiota of sewage sludge that affect the production of VFA. Although bacterial and archaeal species have an essential role in VFA yields and many environmental factors influence microbial fermentation (Zhang et al., 2020), studies are now underway to better understand the effects of the microbiota structure on fermentation processes aimed at the production of VFA. Therefore, in order to optimize fermentation processes and the production of VFA, the relationships between different operating conditions leading to the improvement in VFA yields and their effects on microbial communities' structure need to be deeply analyzed. As an example, Chen et al. (2021) showed that different compositions of hydrothermal conversion wastewater led to the enrichment of different microbial communities, affecting the VFA yields. Other studies have aimed at optimizing the

production of VFA of one or more volatile fatty acids by exploiting axenic microbial cultures (Miguel et al., 2019) or by adding specific substances to enhance production (Zhang et al., 2021). Some studies have been conducted to test the effect of pH on the production of VFA from waste substances (wastewater, kitchen waste, and waste derived from industry and fishing). Many of these studies have found success in producing VFA at acidic pH (Fra-Vázquez et al., 2020). Another work showed that comparing acidic and basic conditions the best yields of VFA occur at alkaline pH (Garcia-Aguirre et al., 2017). To date, the main phyla identified at starting basic pH of waste fermentation are Bacteroidetes, Firmicutes, and Spirochaetes (Slezak et al., 2021). In a recent work (Presti et al., 2021), the positive influence of basic pH (i.e., pH 8) was revealed for stimulating the production of VFA. In another very recent study, preliminarily the importance of setting basic pH values at the initial condition to improve the production of VFA was assessed in batch fermentations, then the influence of pH on microbial community structures in continuous fermentation was evaluated comparing pH-controlled and uncontrolled conditions, where pH was set at 7.5–8 (Muhoraakeye et al., 2022). In this study, the sewage collected from a real Sicilian WWTP located in Marineo (Italy) as a case study (Mannina et al., 2022) was used to inoculate batch fermentations to set an optimized production process of VFA. In particular, the production of VFA was evaluated in respect of two initial pH (i.e., pH 8 and 10, respectively). Then, the different levels of VFA due to starting pH conditions were correlated with the structures of the prokaryotic microbiota residing in the sewage sludge in order to highlight the presence of specific prokaryotic members and bioinformatically inferred microbial metabolic capabilities with improved production of VFA.

2.2 Material and Methods

2.2.1 Experimental setup

The WWTP is located in Marineo, a town of 6438 inhabitants (ISTAT, 2019) in the metropolitan city of Palermo (Sicily). The plant is located at an altitude of 550 m above sea level and has an average daily flow rate of 2160 m³/d. The WWTP has a sewage sludge scheme, whereby the incoming wastewater is first subjected to sandblasting, deoiling, and mud screening, then subjected to the sewage sludge process (Mannina et al., 2022). In recent studies, it has been found that the basic pH 10 set at the start of batch fermentation favors the production of VFA (Presti et al., 2021); thus, two experiments in sequence were carried out (reported as conditions A and B, respectively) to evaluate the effect of pH 8 and pH 10,

respectively. The sewage sludge inoculum samples (ten liters each) for experiments A and B were collected 24 days apart from each other and were labeled T0-A and T0-B, respectively (Table 2.1). The two sewage sludge samples—collected from the recirculation circuit that carries the biomass from the settler to the biological sewage sludge reactor inside the plant—were transported to the laboratory at room temperature. The batch fermentation test reactors were assembled in anaerobiosis, on the same day of the sampling. For each tested pH value, 8 and 10, which are referred to as experiments A and B, respectively, two 1.1 L batch reactors were assembled in parallel in the following way: one having the pH adjusted by adding a saturated solution of NaOH 1N at the beginning of fermentation at the pH 8 (Experiment A) or at pH 10 (Experiment B), and the other one with unadjusted pH used as the control condition (Control A or Control B, respectively). Thus, a total of four batch reactor fermentations were performed (Table 2.1). The reactors are magnetic stirred glass bottles, equipped with two sampling ports for liquid and gas sampling and two electrode ports. pH, oxidation reduction potential (ORP) and temperature were continuously monitored using the relative probe connected to a Multi 3630 IDS WTW multimeter (Xylem brand). The fermentation processes were carried out until the soluble chemical oxygen demand (sCOD) and pH values tended to be almost stabilized. Fermentation took place at room temperature for 15 days. The reactors were constantly monitored.

Table 2.1. Details of the analysed samples.

Condition	Sample	Sampling date	Sample details
Condition A (pH 8)	T0-A	01/06/2021	Sewage sludge sample collected from Marineo WWTP and used as inoculum for Control A and Experiment A batch fermentations
	Control A	16/06/2021	Sewage sludge sample collected after batch fermentation without pH adjustment
	Experiment A	16/06/2021	Sewage sludge sample collected after batch fermentation with initial pH adjustment at 8
Condition B (pH 10)	T0-B	25/06/2021	Sewage sludge sample collected from Marineo WWTP and used as inoculum for control B and experiment B batch fermentations
	Control B	9/07/2021	Sewage sludge sample collected after fermentation without pH adjustment
	Experiment B	9/07/2021	Sewage sludge sample collected after fermentation with initial pH adjustment at 10

2.2.2 sCOD and VFA Analysis

Once the batch fermentation tests were assembled and equipped with a probe capable of detecting the pH, the soluble chemical oxygen demand (sCOD) was measured each day. The samples for which sCOD and pH were monitored were Control A, Control B, Experiment A and Experiment B. For these analyses, the sewage sludge sample was filtered each time and the analyses of sCOD were carried out on the supernatant. Sampling involved the use of a syringe through which 20 mL of each sample was collected from each batch fermentation reactor every day and placed in sterile 50 mL tubes. The samples were then subjected to centrifuge at 4000 x g for 5 min, then the supernatant was filtered with sterile 0.45 m Millipore filters. The filtrate was collected in new sterile tubes and subjected to analysis. Analyses of sCOD on the filtrate were carried out using a COD Cell Test—photometric 25–1500 mg/L kit (Supelco) following the manufacturer’s instructions. For the photometric reading, quartz cuvettes and a Merck spectrophotometer were used. On the peak day of the sCOD, the filtrate taken from the batch fermentation test was subjected to VFA analysis to evaluate the production. The VFA analyses were carried out in a gas chromatograph (Agilent Technologies 7820A, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a DB FFAA column (30 m x 0.25 x mm x 0.25 µm). The filtrate samples with 0.45 micrometer syringe filters (Sartorius Minisart) were prepared by adding dimethyl carbonate (DMC Reagent Plus, Sigma Aldrich, St. Louis, MI, USA). A rate of about 1 uL through a 10 uL Hamilton microsyringe was analyzed. Samples (200 µL) of the filtered fermentation mixture samples were transferred into gas chromatography (GC) vials and 800 µL of a stabilizing solution composed of HgCl₂ (0.5 g), phosphoric acid (5 mL, 100%) and hexanoic acid (0.54 mL, internal standard) was subsequently added. A gas chromatograph (GC) (Agilent Technologies 7820A), equipped with a flame ionization detector (FID) and a DB FFAA column (30 m x 0.25 x mm x 0.25 µm), was used for the detection of short-chain fatty acids (Presti et al., 2021).

2.2.3 Prokaryotic Microbiota Structure Analysis

Analysis of prokaryotic microbiota structure of sewage sludge was performed by metataxonomics based on NGS analysis of 16S rRNA gene amplicons obtained from metagenomic DNA. In particular, a total of six sewage sludge samples were analyzed, as indicated in Table 2.1: T0-A and T0-B corresponding to the sewage sludge collected in Marineo WWTP (Mannina et al., 2022); Control A and Control B corresponding to the sewage sludge of batch reactors in which pH was not adjusted at the beginning of the process; Experiment A and Experiment B corresponding to the sewage sludge of the batch reactors

in which the pH was set at the beginning of the fermentation at pH 8 and pH 10, respectively. The Control A, Control B, Experiment A, and Experiment B samples were collected at the end of the fermentation process. The analyses were performed on unfiltered and uncentrifuged samples. The procedures already described (Presti et al., 2021) were followed. The DNA extractions were evaluated by 1% (w/v) agarose gel electrophoresis analysis, with the addition of 0.5 µg/mL ethidium bromide for visualization using a UV lamp. The concentrations of the DNA, extracted from 1 g of sewage sludge samples and the corresponding tenfold serial dilutions were measured by reading absorbance at 260 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extracted DNA was evaluated by measuring the absorbance ratios (260/280 and 260/230 nm) to indicate contamination due to proteins and organic compounds or chaotropic agents, respectively. The following primers were used for PCR amplification of the V3-V4 regions of the 16S gene: PRO 341F CCTACGGGNNBGCASCAG and PRO 805R GACTACNVGGGTATCTAATCC. For PCR, DreamTaq DNA polymerases (ThermoFisherScientific) was used following the manufacturer's instructions. The thermal profile used was 95°C for 2 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds; final elongation 72°C for 5 minutes. Amplification products were sequenced in one 300 bp paired end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). The raw 16S rDNA data were processed by using the QIIME2 software (<https://qiime2.org/> (accessed on 30 January 2022)) as paired-end sequences. In the denoising approach, overlapping paired-end reads were processed with the plugin DADA2. Unique amplicon sequence variants (ASVs) were assigned and aligned to the Greengenes reference database at 99% sequence similarity (<https://greengenes.secondgenome.com/> (accessed on 30 January 2022)). For each sample, the number of ASVs and the percentages of relative abundances of phyla, orders, classes, and families were determined. Principal coordinate analysis (PCoA) was chosen as a multivariate statistical approach and was generated starting from the Bray–Curtis distance matrix using Emperor software. A heatmap was obtained through the online web server (<http://heatmapper.ca/expression/> (accessed on 8 January 2022)) based on genera. The heatmap was generated by a “complete linkage” calculation using Spearman rank correlation. METAGENassist (<http://www.metagenassist.ca> (accessed on 6 January 2022)) was used to distinguish the microbial species based on their metabolic activity (Arndt et al., 2012).

2.3 Results and Discussion

2.3.1 sCOD and pH Values in Batch Fermentations

The sewage sludge collected in the WWTP of Marineo had the following parameters: pH 7.2 and sCOD 790 mg/L (T0-A), and pH 7.2 and sCOD 237 mg/L (T0-B). Table 2.2 reports the data constantly monitored for “Control A” and “Experiment A” samples from the batch fermentations of Condition A. Table 2.3 reports the data for “Control B” and “Experiment B” samples from the batch fermentations of Condition B. Figure 2.1 shows graphs relating to the sCOD along with the incubation on batch reactors and highlights the peak per day. As shown in Figure 2.1, the sCOD peak was reached on the fifth day of incubation of Experiment A and Control A, on the sixth day for Control B, and on the seventh day for Experiment B. In addition, the amount of sCOD obtained in Experiment B was the highest measured, reaching 3000 mg/mL. The other samples reached approximately 1500–1800 mg/mL. The production of sCOD increased by 1.5-fold at pH 10 over pH 8 (2020 mg/L against 1391.71 mg/L). This result confirms the positive effect of basic pH at the beginning of fermentation on microbial species leading to high amounts of sCOD. VFA amount, determined by GC analysis, confirmed high levels on the peak day of the sCOD. Table 2.4 reports both the values of VFA and the respective VFA/sCOD ratios.

Table 2.2. Average sCOD and pH of the Control A and Experiment A batch fermentations of Condition A experiments.

Batch fermentation Condition A - initial pH 8					
Control A			Experiment A		
Day	sCOD mg/L	pH	Day	sCOD mg/L	pH
0	790	7.2	0	790	8
1	930	6.8	1	1186	6.8
2	1329	6.6	2	1377	6.7
3	1374	6.6	3	1587	6.6
4	1468.5	6.6	4	1705.5	6.6

5	1563	6.6	5	1824	6.6
8	1359	6.6	8	1540.5	6.8
9	1155	6.6	9	1257	6.8
10	1117.5	6.7	10	1188	6.7
11	1080	6.7	11	1119	6.7
12	1035	6.7	12	1089	6.8
15	825	6.7	15	813	6.8

Table 2.3. Average sCOD and pH of the Control B and Experiment B batch fermentations of Condition B experiments.

Batch fermentation Condition B - starting pH 10					
Control B			Experiment B		
Day	sCOD mg/L	pH	Day	sCOD mg/L	pH
0	237	7.2	0	237	10
1	556	6.8	1	2234	7.4
2	809	6.6	2	2413	6.7
3	1428	6.6	3	2568	6.6
6	1756	6.6	6	2733	6.7
7	1661	6.6	7	2916	6.8
8	1444	6.6	8	2838	6.8
9	1180	6.6	9	2610	6.8
10	1131	6.7	10	2418	6.8
13	1023	6.7	13	2070	6.8
14	1014	6.7	14	2025	6.8

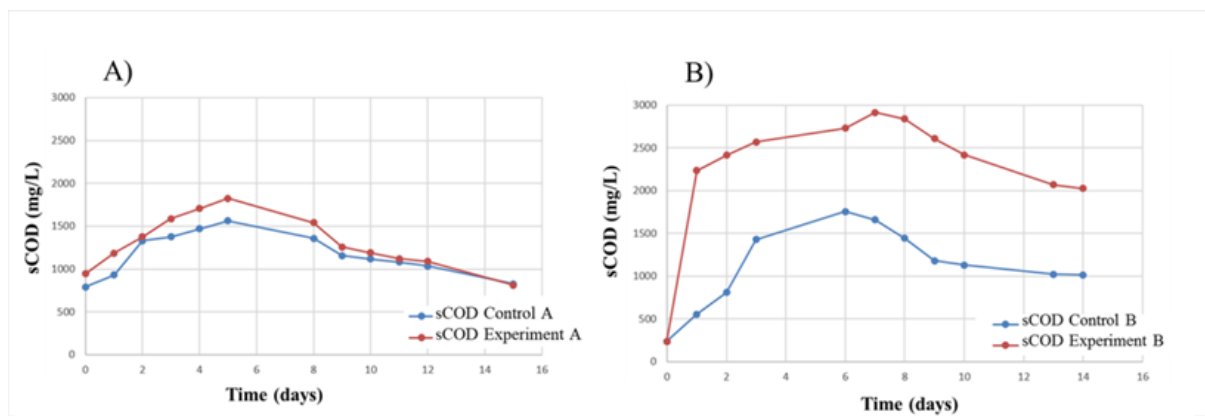


Figure 2.1. sCOD (mg/L) variation for 14–15 days in Condition A (A) and Condition B (B) batch fermentations. The red lines represent the controls (Control A and Control B), while the blue lines represent the experiments (Experiment A and Experiment B).

Table 2.4. Average VFA determination by GC assay and relative VFA/sCOD ratios of Condition A and Condition B batch fermentations.

Batch fermentation	Peak day of sCOD	VFA mgCOD/L	VFA/sCOD
Control A	5	1303.54	0.834
Experiment A	5	1391.71	0.763
Control B	6	1411.82	0.8
Experiment B	7	2020	0.69

As revealed in previous studies, sCOD and the production of VFA are affected by pH (Presti et al., 2021; Zhang et al., 2016; Atasoy et al., 2020). In particular, enhanced fermentation, and thus VFA production at pH 10 in a shorter time than at pH 8 was observed (Zhang et al., 2016). Comparing these results with other recent studies in which acid-forming batch fermentation was also performed at pH 10 for 15 days, such as in Atasoy et al. (2020) (i.e., 1942 mg COD/L), it is possible to note that the maximum VFA yield reported here (2020 mg COD/L) is higher. It is also interesting to note that despite the initial differences, the pH values are similar in all reactors during batch fermentations, starting from the second day onwards, similarly to the observed results of Atasoy et al. (2020) for mixed culture

fermentations. This could be due to the metabolic versatility of microbial communities in mixed cultivations, in which acidification processes could be counteracted by the metabolic activity of other microbial members acting as a metabolic consortium, as was also suggested by Wang et al. (2013).

2.3.2 Sequencing Output and Analysis

Metataxonomic analyses - based on NGS analysis of 16S rRNA gene amplicons obtained from metagenomic DNA of sewage sludge - were conducted using the samples collected from both sets of the two batch fermentation reactors. In total, 202,731 high quality reads were filtered from the 545,624 raw reads obtained. A total of 1683 ASVs were identified using the suite QIIME2, after the processes of filtration, denoising, and merging (Table 2.5). ASVs were classified at the genus level using a 99% sequence similarity threshold against the Greengenes database.

Table 2.5. Results of bioinformatic analysis of NGS reads obtained from metataxonomic analysis of sewage sludge samples collected from Condition A and Condition B batch fermentations.

Sample	Input	Filtered	Percentage of input passed filter (%)	Denoised	Merged	Percentage of input merged (%)	Non-chimeric	Percentage of input non-chimeric (%)
T0- A	74926	61752	82.42	56341	45171	60.29	28642	38.23
Control A	107349	88438	82.38	82197	66368	61.82	40084	37.34
Experiment A	101116	81999	81.09	76364	63186	62.49	37502	37.09
T0- B	117728	96462	81.94	89387	75002	63.71	46400	39.41
Control B	83492	67136	80.41	61455	51157	61.27	32491	38.92
Experiment B	61013	51049	83.67	46841	40321	66.09	18048	29.58

2.3.3 Structure of Prokaryotic Communities of Sewage Sludge

Most ASVs have been assigned to the Bacteria and only about 2% to the Archaea domains. Therefore, the prokaryotic composition of sewage sludge from conditions A and B was comparatively analyzed considering the relative abundances at the phyla level firstly and then at the level of family and finally at the level of genus. First of all, heatmap analysis (Figure 2.2) clearly showed that the T0-A and T0-B samples were more similar to each other than the others. In addition, while the Control A and Experiment A samples had a very similar microbial community and tended to cluster with Control B samples, Experiment B was separated from the other samples. This result clearly indicates a role of starting pH value in modulating microbial components, thus possibly positively influencing the production of VFA.

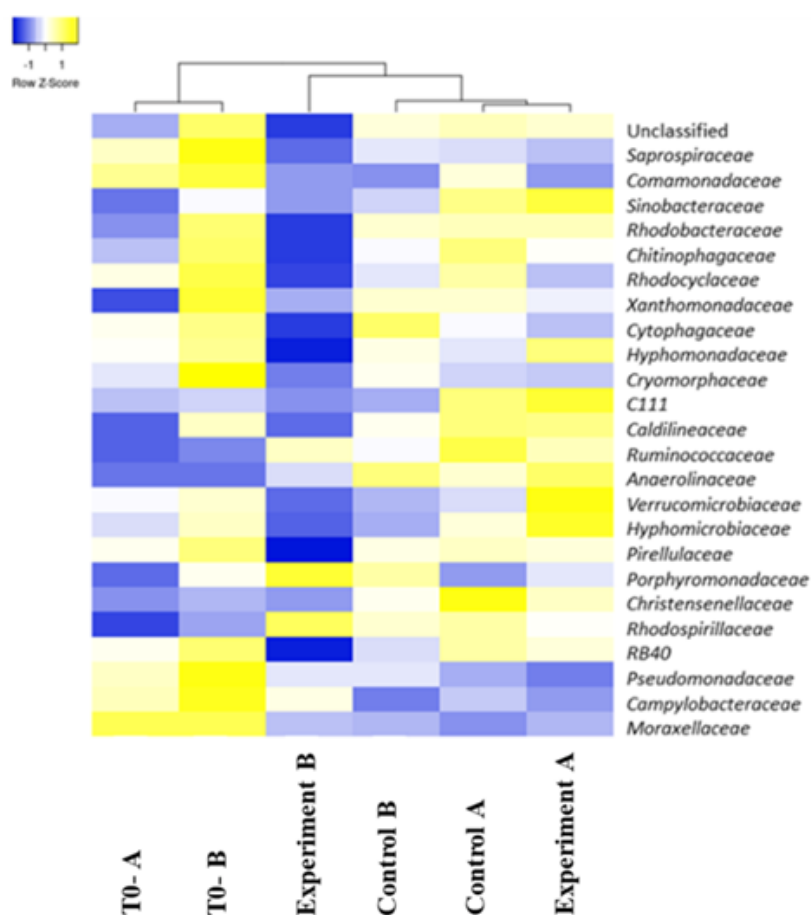


Figure 2.2. Heatmap of 25 most abundant prokaryotic families' composition profiles. The color intensity in each sample is normalized to represent its relative ratio in the two groups. Colors from blue to yellow indicate the relative values of microbiota.

The microbial composition of T0-A and T0-B showed no substantial difference at phyla or family level (Figure 2.3). The most represented phyla were Proteobacteria, Bacteroidetes, Chloroflexi, and Acidobacteriota, and Saprospiraceae, Comamonadaceae, and Rhodocyclaceae were the most represented families in all samples. The percentage abundances of the various phyla—more specifically, of the families that made up the T0-A and T0-B samples—were similar. In all samples of the four batch fermentations, the most dominant phyla were Proteobacteria and Bacteroidetes, followed by Chloroflexi and Firmicutes (Figure 2.4 A,B). The presence of Proteobacteria and Bacteroidetes as the main phyla agrees with the report of Guo et al. (2017), even if a very low representation of Firmicutes and no Chloroflexi were revealed in the latter. Interestingly, the majority of bacteria belonging to these phyla have been reported to be capable of degrading complex organic matters for the production of VFA (Luo et al., 2016; Zheng et al., 2013). During anaerobic fermentations, a crucial role was assigned to sewage sludge Proteobacteria and Bacteroidetes for propionate, butyrate, and acetate metabolism (Riviere et al., 2009). Interestingly, it has been observed that some species of Firmicutes, such as *Bacillus megaterium*, can assimilate glucose, acetic acid, butyric acid, and caproic acid and produce PHA with yields of 9–11% of the dry cell weight (Vu et al., 2021). Thus, the higher percentage of Firmicutes in the batch fermentation test at pH 10 could justify the highest VFA values. This is even more evident from the most abundant families that were detected by the analysis (Figure 2.4 C,D). The most abundant family was Saprospiraceae, followed by Comamonadaceae, Sinobacteraceae, and Rhodobacteraceae. There were no relevant family differences between the two batch fermentation tests. Saprospiraceae were more abundant in both T0 samples and gradually decreased in control and experiment samples in both batch fermentations and assumed an abundance profile similar to the Bacteroidetes phylum to which they belong. Members of the Saprospiraceae family were detected in sewage sludge samples, but their possible role in wastewater treatment is not well elucidated. Probably, Saprospiraceae members may be important in breaking down complex carbon sources. It has been suggested that their hydrolytic activity provides simple carbon substrates for other microorganisms involved in nutrient removal, and this could also be the limiting step for these processes (Johnston et al., 2019). For Comamonadaceae, there was a slight increase in relative percentage in the Experiment B sample compared to the corresponding control condition B (5.8% and 2.8%, respectively). This bacterial family not only has fermenting and PHA production capabilities but also its members are known denitrifying agents of sewage sludge microbial communities, as they are able to simultaneously remove nitrates and toxic organic contaminants from the wastewater (Wang et al., 2016). In order to

obtain a deep understanding of the relationships between sewage sludge microbial community structure and the production of VFA, bacterial and archaeal genera residing in sewage sludge microbiota were compared. Among the 25 most abundant genera, only bacterial members were highlighted, being archaeal genera scarcely represented (Figure 2.5). The main bacterial genera found in the highest percentage in all the analyzed samples were *Rhodobacter*, *Trachelomonas*, *Dechloromonas*, *Devosia*, and *Pseudomonas*. However, by examining the samples individually, it was possible to notice a different distribution of genera. For example, in Experiment B, characterized by the highest yield of VFA, the main bacterial genera present were *Acidovorax* (2.47%), *Paludibacter* (2.18%), *Fusibacter* (1.93%), *Arcobacter* (1.69%), *Comamonas* (1.45%), *Pseudomonas* (1.15%) and *Acinetobacter* (0.90%). These bacterial genera were also present in the other samples, but reached the highest percentage in Experiment B. Interestingly, previous studies demonstrated not only the ability of *Acidovorax* to survive in basic pH conditions till pH 12 but also that the basic pH creates a selective pressure in favor of this bacterial genus. At basic pH and anaerobic conditions, *Acidovorax* is an excellent producer of VFA (Yang et al., 2015). Strains belonging to the *Comamonas* (1.45%) genus are not known for fermentation capacity. However, in a previous study by Zakaria et al. (2009), it was found that *Comamonas* strains have excellent growth in the presence of VFA released by other bacterial strains, especially in the presence of acetic, propionic, and butyric acids. *Acidovorax* and *Comamonas* species are mainly involved in the storage process of PHAs, starting from volatile fatty acids (Pereira et al., 2020). Another strongly represented genus in the Experiment B sample was *Paludibacter* (2.18%). Strains belonging to this bacterial genus have the ability to use various sugars for the production of VFA, especially propionate and acetate. The strain has an optimum pH of 6.6–7 but also resists much higher pH, for example, pH 10 (Ueki et al., 2006).

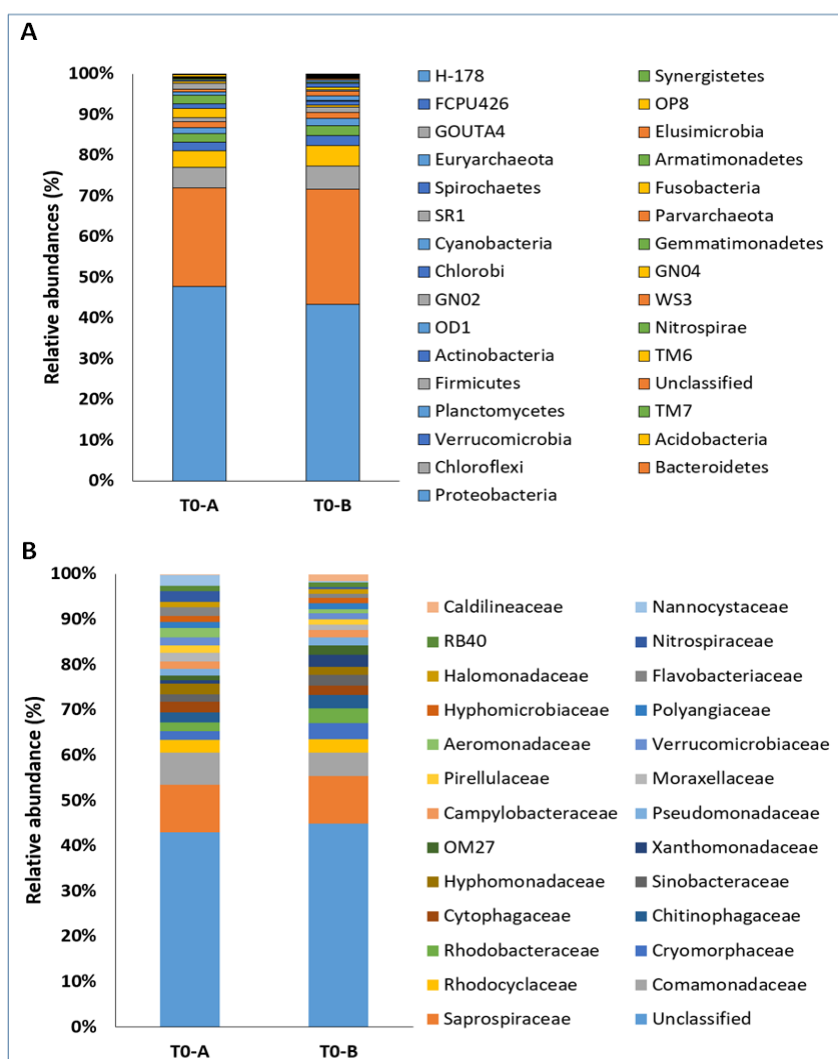


Figure 2.3. Prokaryotic community structures of T0-A and T0-B sewage sludge samples at level of phylum (A) and family (B). In panel B, the top 25 most abundant families are reported. Alphanumeric codes refer to candidate divisions.

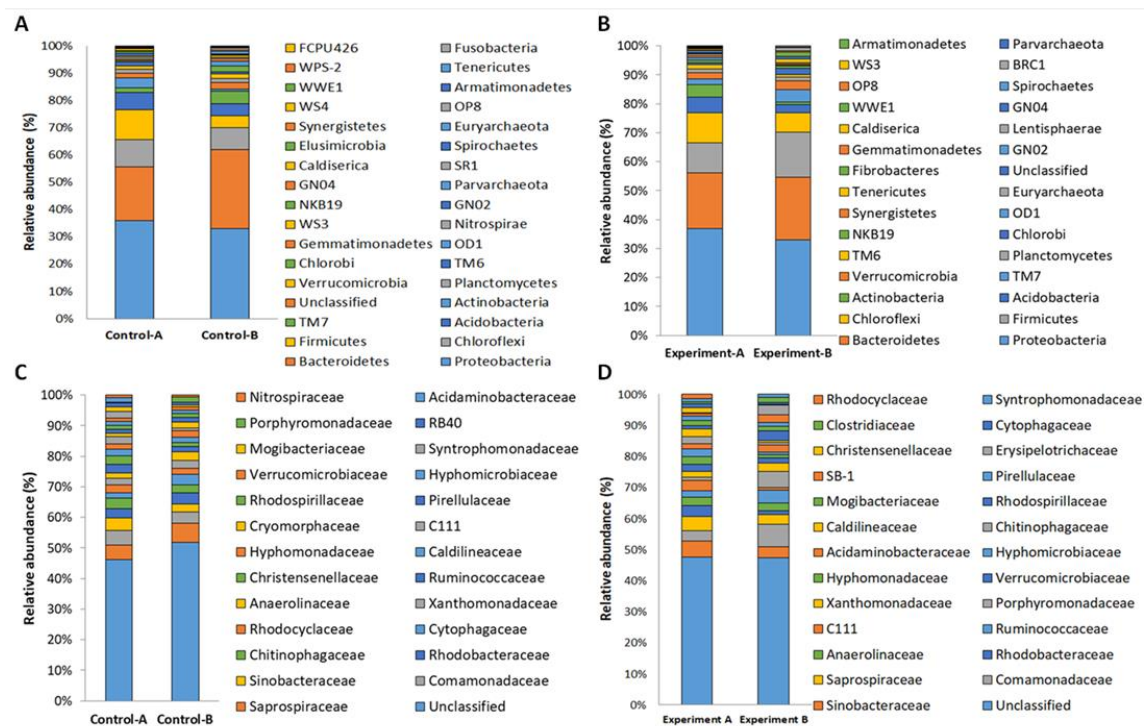


Figure 2.4. Prokaryotic community structures at the level of phyla (A,B) and family (C,D) of sewage sludge samples collected from Control A, Control B, Experiment A and Experiment B fermentations. In (C,D), the top 25 most abundant families are reported. Alphanumeric codes refer to candidate divisions.

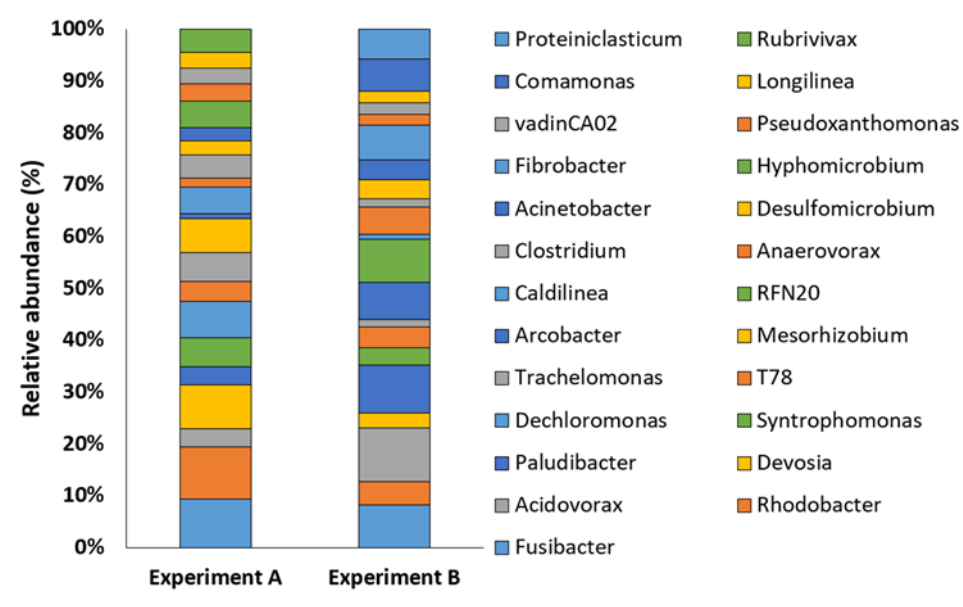


Figure 2.5. Prokaryotic community structures of sewage sludge samples collected from Experiment A and Experiment B fermentations reporting the top 25 taxa at the genus level. Alphanumeric codes refer to candidate divisions.

Other experiments that test the production of VFA at different pH conditions confirm that the condition in which the system is most performing is pH 10. At this pH, the genus *Pseudomonas* is likely to be the main producer of acetic and propionic acid starting from organic carbon in the sludge (Jie et al., 2014). In the Experiment B sample, the genus *Pseudomonas* is present in a modest percentage (1.15%) while it seems that in the T0-B the genus is more represented. *Fusibacter* present in Experiment B sample with a high percentage (1.93%) is an excellent producer of VFA in anaerobiosis (Hasegawa et al., 2014) as *Arcobacter* (1.69%), which simultaneously manages to degrade the biosolids of the sludge and fermenting the organic matter into VFA (Xin et al., 2021). Finally, *Acinetobacter* (0.90%) has fermentation capacity, especially in the production of acetic acid (Ramine et al., 2008). Thus, the initial pH 10 exerted selective pressure in favor of the genera *Acidovorax*, *Paludibacter*, *Fusibacter*, *Arcobacter*, *Comamonas*, *Pseudomonas*, and *Acinetobacter* in the Experiment B sample and are excellent producers of VFA. This suggests that pH 10 created the most performing conditions to produce VFA. This is also confirmed by the fact that in the Experiment A sample (pH 8), which gave lower yields of VFA (Figure 2.1); these genera were present in a reduced percentage or are not present at all. However, the genera *Rhodobacter* (1.51%), *Fusibacter* (1.41%), *Devosia* (1.27%), *Dechloromonas* (1.06%), and *Mesorhizobium* (0.98%) were present in the Experiment A sample. *Fusibacter* was present at both pH 10 (1.93%) and pH 8 (1.41%). However, another work demonstrated how controlled pH initially promotes the degradation of VFA by *Rhodobacter* for the production of H₂ (Hu et al., 2020). The genus *Devosia* (1.27%) is also involved in the H₂ production process, degrading the VFA for acetogenesis (Goud et al., 2017). Hence, *Devosia*, like *Rhodobacter*, is involved in the consumption of VFA produced by the other strains. *Dechloromonas* (1.06%) and *Mesorhizobium* (0.98%) are not only known to be capable of producing VFA but also of producing and accumulating PHA (Wang et al., 2016; Singh Saharan et al., 2014). Therefore, the lower yield of VFA relative to the Experiment A sample (pH 8) may be due to the selection of specific microbial genera that are able to produce VFA, while others degrade them. The control of operating parameters is an important aspect of a microbial fermentation process to achieve the product or perform the reaction of interest and the knowledge of the way in which operating parameters control and shape microbial community structure can be exploited for improving the process. In particular, the association between fermentation conditions leading to high levels of VFA and the presence of specific members of microbial community structure of sewage sludge can be exploited to drive reshaping of community structures in such a way to maximize and to inhibit the presence of microbial producers and consumers, respectively. This could be achieved in

different ways that, for example, involve the manipulation of sewage sludge used as inoculum. As an example, sewage sludge can be subjected to pretreatment procedures that affect the relative abundance or interfere with the metabolic activity of specific members, as described in the study of Muhorakeye et al. (2022) where sodium 2-bromoethane sulfonate was used to reduce the number of methanogen members that are known to be VFA consumers. Another possibility is to perform a pre-inoculum step in which bacterial growth is performed controlling operational parameters, such as temperature, oxygen (Kim et al., 2016), and pH (Muhorakeye et al., 2022) to obtain a restructured sewage sludge enriched with specific members to be then used as inoculum in fermentation reactors for production of VFA. In addition, *in silico* analysis of metabolic functions can be useful to highlight which metabolic processes can be stimulated or reduced. The following paragraph highlights the metabolic activities that can be positively or negatively associated with production of VFA based on the relative abundance of microbial taxa residing in the sewage sludge of batch fermentations.

2.3.4 Metabolism and Oxygen Requirement Analysis

Based on the interference of taxonomic-to-phenotypic mapping of metabolism, Bacteria and Archaea were classified on the basis of their oxygen demand and metabolism by (Arndt et al., 2012). The aim of the metabolic study was to identify whether there are microorganisms capable of producing PHA from VFA and analyze the diffusion of methanogenic strains, to correlate their activity with pH and the presence of VFA. In this study, all samples contained aerobic and anaerobic microbial strains. This is possible because first of all the sewage sludge samples come from an aerobiotic environment, and secondly because even if the fermentation takes place in anaerobiosis, when the batch fermentation reactor is assembled there is a quantity of oxygen that can be consumed by microbial cells as the fermentation proceeds, up to complete anaerobiosis. It was found that in the Experiment A samples 23% of prokaryotic species were aerobic and 16.2% were anaerobic. In the experiment B batch fermentation samples, there was a slight increase of both aerobic and anaerobic strains (25% and 19.6% respectively). On the basis of the type of metabolic features, all samples contained ammonia oxidizers, dehalogenating members, nitrite reducers, and sulfate reducers (Figure 2.6). The prokaryotic communities of the two conditions A and B have similar metabolic profiles, but in Experiment A, a higher representation of methanogenic microbial members and strains performing denitrification and chitin degradation, and, on the contrary, a lower representation of sulfur-reducing strains and strains degrading cellobiose and storing

polyhydroxybutyrate were found. On the basis of this result, the lower concentration of VFA in Experiment A may be due to the presence of methanogenic microbial strains. In fact, methanogens, that are prokaryotic microorganisms belonging to the Archaea domain, have an optimal pH range around neutrality and may consume VFA to produce CH₄ (Luo et al., 2019). In addition, the reduced amount of cellobiose degraders may be related to a reduced availability of carbohydrates since cellobiose is the product of microbial hydrolysis of cellulose, one of the most abundant sources of carbon in different ecosystems, including wastewater (Schellenberger et al., 2011; Ruiken et al., 2013). Thus, the analysis of prokaryotic community structures shaped by pH conditions revealed the positive and negative selection of VFA producers and consumers, respectively, as well as the in-silico prediction of metabolic functions-based on prokaryotic community structures-suggest that the development of fermentation conditions inhibiting methanogenesis and/or stimulating carbohydrate utilization can be a further practicable strategy to improve the production of VFA (Odriozola et al., 2019; Samanides et al., 2020).

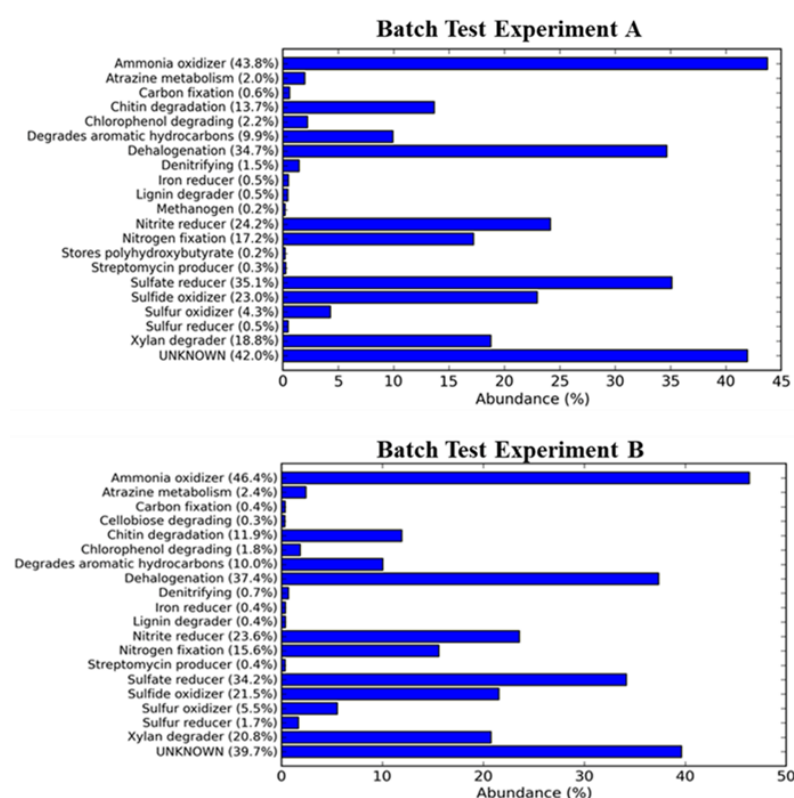


Figure 2.6. Putative metabolic requirements of the sewage sludge prokaryotic communities of Experiment A and Experiment B batch fermentation.

2.4 Conclusions

The investigation of the association between fermentation conditions and microbial community structure should be considered as the basis to promote the establishment of sustainable and feasible processes to get high-value products, such as VFA. In this work, two batch fermentation conditions, namely, Condition A and Condition B, were analyzed (Table 2.1): the former in which the starting pH was initially set at 8 and the latter in which the initial pH was set at 10 in comparison with unadjusted pH. In a previous study by Presti et al. (2021), it was shown that basic pH positively influences the production of VFA. In this study, it was further demonstrated comparatively that the higher basic is the starting pH, the higher production of VFA is observed. In addition, this finding parallels the restructure of the sewage sludge prokaryotic community residing and operating in batch fermentation reactors. Despite the pH difference being set at the beginning of batch fermentation, the production of VFA on the peak day of the sCOD was significantly higher in the Experiment B sample than in the others. The metataxonomic based on NGS analysis of the 16S rRNA gene showed that the sewage sludge samples collected from Marineo WWTP (Mannina et al., 2022) have similar prokaryotic community compositions, although they were collected within 24 h of each other. In addition, in the sewage sludge of Experiment B, in which the initial pH was set at 10, the microbial community composition evolved in a different way during batch fermentation in comparison with all of Experiment A, in which the initial pH was set at 8, and the unadjusted-pH control tests. Thus, it can be surmised that pH 10 shaped the microbial community structure favoring the fermenting microorganisms and thus increasing the production of VFA. The production of VFA by fermentation processes is positively correlated with the abundance of Firmicutes, Bacteroidetes and Chloroflexi in the microbial community, as observed in Experiment B. Accordingly, these three phyla have already been shown involved in the production of VFA from food waste at pH 10 by Khatami et al. (2021). Thus, it is evident that the initial pH 10 of the batch fermentation test promotes the growth of all those Bacteria and Archaea with fermentation capacity and a maximized production of VFA. Indeed, as it has been highlighted by the analysis of putative metabolic features of the sewage sludge prokaryotic communities of Experiment A and Experiment B, the starting pH 10 promotes a reduction of microbial members that use VFA to produce other substances (such as, methanogens that produce CH₄). Therefore, this work sheds light on the fact that the evolution and selection of the microbial community residing in sewage sludge of WWTP can be driven in favor of VFA producers by setting the starting parameters of operating conditions. In addition, these results suggest that the development of operating and

fermentation conditions affecting microbial metabolism can be a practicable strategy to improve the production of VFA. In perspective, this study suggests the possibility to scale up the process to maximize the production of VFA to sustain PHA production. Thus, the exploitation of a high-yielding microbial community coupled with the use of optimized fermentation conditions could be a strategy to favor the development of PHA-based bioplastics and start from waste for a “greener” world.

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CHAPTER 3

Enhancing volatile fatty acid production from sewage sludge in batch fermentation tests

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ABSTRACT

Volatile Fatty Acids (VFAs) derived from sewage sludge are a valuable resource recovered from wastewater treatment. This study evaluated sewage sludge treatment with potassium permanganate - KMnO_4 in batch reactors under acidogenic fermentation conditions. The results showed that using 0.1 g KMnO_4 /g Total Suspended Solids (TSS) when Volatile Suspended Solids (VSS) were 6 g/L can improve VFA production, yielding up to 452 mgCOD/g of volatile suspended solids. These findings were supported by metataxonomic analysis, which revealed shifts in the prokaryotic microbial communities of the sewage sludge across the various pre-treatments.

3.1 Introduction

Volatile fatty acids (VFA) represent a valuable resource that can be recovered from wastewater treatment processes, aligning with the principles of a circular economy. Bacteria produce VFA as metabolic byproducts during the anaerobic digestion of organic matter. The shift from a linear to a circular economic model has encouraged the development of new biotechnologies aimed at resource recovery from SS by exploiting microbial capabilities. The popularity of VFAs has grown in recent years, mainly due to their production through acidogenic fermentation of SS, making them one of the most valuable recoverable resources (Li et al., 2018). VFAs are essential building blocks for various microorganisms in producing biopolymers, such as polyhydroxyalkanoates (PHA) (Liu et al., 2020). However, producing VFAs under acidogenic conditions remains challenging due to several process limitations. Key obstacles include the modulation of process parameters such as the low biodegradability of substances in SS, the slow hydrolysis rate during fermentation, and the difficulty of separating VFAs from the fermentation broth (Bruni et al., 2021; Parchami et al., 2020). The hydrolysis rate during fermentation is particularly challenging, as it still requires optimization despite extensive research efforts (Valentino et al., 2021). Several operational factors, such as pH and temperature, are crucial for stimulating bacteria to speed up hydrolysis during fermentation. For instance, pH can significantly influence the activity of microorganisms (Wang et al., 2019). Recent studies have shown that hydrothermal treatment at 80°C leads to higher fermentation acid production than processes without sludge pretreatment (Penghe et al., 2020). Wu and colleagues explored the effects of pre-treating sludge under acidic or alkaline conditions, finding that acid pretreatment enhances VFA production (Wu et al., 2017). Guo and co-researchers used polyoxometalates as a pretreatment to boost VFA production from SS, achieving a 6.18-fold increase (Guo et al., 2021). Wang and co-workers investigated the use of coconut shell ash to enhance VFAs

production from algae fermentation, achieving a 1.4-fold increase in VFA concentration compared to untreated samples (Wang et al., 2021). Zheng and colleagues studied the impact of polystyrene microplastics on VFA production, finding that at 30 particles/g total solids, VFA production increased, reaching a peak of 1493 mg COD/L (Zheng et al., 2021). Chen and co-researchers used roxithromycin during SS anaerobic fermentation (Chen et al., 2020) to increase the VFA rate. More recently, Xu and colleagues explored the use of a potent green oxidant, potassium permanganate (KMnO_4), to enhance VFA production and fermentation quality using SS at a concentration of 14 g Volatile Suspended Solids (VSS)/L (Xu et al., 2021). Their research focused on determining the optimal KMnO_4 dosage, finding the best results with a dose of 0.1 mg KMnO_4 /g VSS as a treatment. Therefore, this study aims to evaluate the impact of the best treatment with the addition of KMnO_4 modulated based on VSS concentration on the production of VFA and, above all, the effect of the treatment on the composition of the SS microbial community, which plays a fundamental role in acidogenic fermentation to discover the mechanisms underlying the process.

3.2 Material and Methods

3.2.1 Batch fermentation tests and sewage sludge features

Batch fermentation tests were conducted in 1100 mL magnetically stirred glass bottles equipped with two sampling ports for liquid and two electrode ports at the Water Resource Recovery Lab of Palermo University (Mannina et al., 2021a). These activities are part of the EU Project Wider-Uptake and focus on, among other things, the production of polyhydroxyalkanoate from the fermentation of domestic sewage sludge (Mannina et al., 2021b; Mannina & Mineo, 2023). Table 3.1 provides details of the batch test treatments. In particular, three batch fermentation tests were conducted to assess the influence of KMnO_4 related to VSS on VFA production and on the modulation of the SS microbial community. Specifically, three different VSS levels were tested under the same KMnO_4 dosage (0.1 g KMnO_4 /g VSS): VSS 4 g/L-OT1, VSS 6 g/L-OT2, and VSS 8 g/L-OT3. All the tests were performed at room temperature. Throughout the tests, concentrations of soluble Chemical Oxygen Demand (sCOD), volatile fatty acids (VFA), ammonium, and phosphate were analyzed in the fermented liquid. pH, temperature, and redox potential were continuously monitored using a WiFi-enabled Multi 3630 IDS "WTW" device with related probes. The starting sample (inoculum) of sewage sludge was sourced from the sludge recycle line of a

municipal wastewater treatment plant in Marineo, Italy. Table 3.2 outlines the characteristics of the sewage sludge used and the details of the optimization batch tests.

Table 3.1. Details of the performed KMnO_4 treatment and VSS evaluation of batch fermentation tests.

Batch test	Treatment
OT1	0.1 g KMnO_4 /g TSS VSS: 4 g/L
OT2	0.1 g KMnO_4 /g TSS VSS: 6 g/L
OT3	0.1 g KMnO_4 /g TSS VSS: 8 g/L

Table 3.2. Sewage sludge features.

Parameters	Unit of measurement	Values
pH	-	7.88 ± 0.55
Total Suspended solids (TSS)	g/L	3.1 ± 0.01
Volatile Suspended solids (VSS)	g/L	1.58 ± 0.01
Total Chemical Oxygen Demand (TCOD)	mg/L	2760.00 ± 35.00
Soluble Chemical Oxygen Demand (sCOD)	mg/L	66.23 ± 4.21
Protein	mg/L	243.15 ± 7.69
Carbohydrate	mg/L	45.70 ± 2.91
Ammonium	mg/L	2.60 ± 0.15
Phosphate	mg/L	1.76 ± 0.23

3.2.2 Analytical methods

The analysis of COD, VSS, TSS, ammonium, and phosphate concentrations was conducted following standard methods (APHA, 1999). VFAs were measured using the VFA extraction method with dimethyl carbonate (DMC-OEI) as described in the literature (Ghidotti et al., 2018). Specifically, VFAs were analyzed with an Agilent Technologies 7820A gas chromatograph, equipped with a flame ionization detector (FID) and a DB-FFAA column (30 m x 0.25 mm x 0.25 μm). The VFAs measured included formic, acetic, propionic,

isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic, and n-heptanoic acids. The gas chromatography protocol used was based on that outlined in the literature (Montiel-Jarillo et al., 2017). VFA concentrations were converted to COD values using the conversion factors provided by Yuan et al. (2011). The soluble and bound extracellular polymeric substances (EPSs), including proteins and carbohydrates, were analyzed following the method described in the literature (Mannina et al., 2016).

3.2.3 Metagenomic analysis

Metagenomic analysis was performed using Next Generation Sequencing (NGS) according to the procedures described in the literature (Presti et al., 2021). The DNA extractions were evaluated by 1% agarose gel electrophoresis analysis, adding 0.5 µg/mL ethidium bromide for visualization using a UV lamp. The DNA concentrations, extracted from 1 g of activated sludge samples and the corresponding ten-fold serial dilutions were measured by reading absorbance at 260 nm with NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). The purity of the extracted DNA was evaluated by measuring the absorbance ratios (260/280 and 260/230 nm), and contamination was found to be due to proteins, organic compounds, or chaotropic agents. To perform a qualitative evaluation, metagenomic DNA and the diluted DNA aliquots were used as a template in a PCR protocol to amplify the region V3–V4 of the 16S rDNA gene (Taki and Kibayashi, 2014). Amplicons (464 bp) were visualized by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Amplification products were sequenced using a single 300-bp paired-end run on an Illumina MiSeq platform (BMR Genomics, Padova, Italy). Raw 16S rDNA data were processed with QIIME2 software, employing the DADA2 plug-in for denoising and handling overlapping paired-end reads. Unique Amplicon Sequence Variants (ASVs) were identified and matched to the Greengenes reference database at 99% sequence similarity. ASV counts and relative abundances at various taxonomic levels (phyla, orders, classes, families, and genera) were calculated for each sample.

3.3 Results and discussion

3.3.1 Organic matter solubilisation, VFA production, ammonium and phosphate concentrations

During the batch fermentation tests, an increase in sCOD concentration was observed across all the pretreatments used (Figure 3.1 a). Figure 3.1 b shows the maximum COD solubilization. All the pretreatments achieved their highest COD levels on the 17th day of fermentation. Specifically, the sCOD concentration reached 1200 mg/L for OT1, 2740 mg/L for OT2, and 1500 mg/L for OT3 (Figure 3.1 a). The COD solubilization percentages were 31.6% for OT1, 74.9% for OT2, and 28.4% for OT3 (Figure 3.1 b).

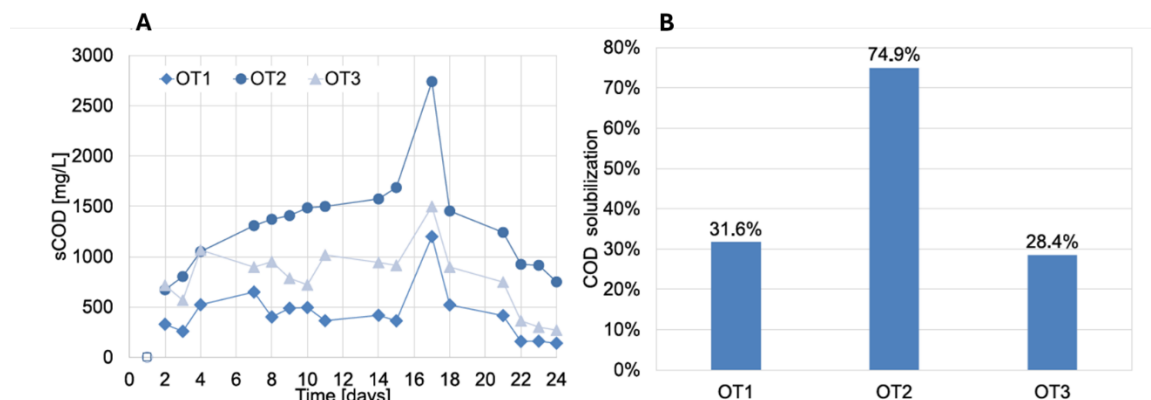


Figure 3.1. Trend of sCOD (a) and COD solubilization on day 17 (b).

COD solubilization increases with VSS concentration up to 6 gVSS/L. However, further increases in VSS lead to a reduction in COD solubilization. This outcome may be attributed to the rise in VSS, which increases the number of fermentation bacteria and the amount of organic material to be hydrolyzed (Xiong et al., 2012). With further increases in VSS (and consequently TSS), especially under high sludge retention time conditions, recalcitrant organics become more prevalent, requiring higher doses of KMnO_4 to achieve similar results. Figure 3.2 confirms that the hydrolysis process improved as VSS increased to 6 gVSS/L, after which it declined. On day 17, VFA production was 1172 mgCOD/L for OT1, 2713 mgCOD/L for OT2, and 1499 mgCOD/L for OT3, corresponding to VFA/sCOD percentage ratios of 99%, 97%, and 88%, respectively (Figure 3.2 a). Regarding VFA yield, 308.7, 452.3, and 187.5 mg COD/gVSS of VFA were produced during the OT1, OT2, and OT3 tests, respectively (Figure 3.2 b). Statistical analysis using ANOVA was performed to assess the reliability of the monitored data (Freni & Mannina, 2010). On day 17, the VFA composition showed acetic acid was dominant only in OT1 (Figure 3.2 c). Specifically, acetic acid accounted for 71% of VFA in OT1, 38% in OT2, and 34% in OT3 (Figure 3.2 c). In contrast, propionic acid was predominant in OT2 and OT3, making up 39% and 40%, respectively. Isovaleric acid was also present in OT2 and OT3 at 14% and 16%, respectively.

Additionally, n-heptanoic acid was detected only in OT3, at 3%. The VFA composition results confirm that as VSS increases, the presence of more complex organics also rises, producing more complex VFAs, such as n-heptanoic and isovaleric acids.

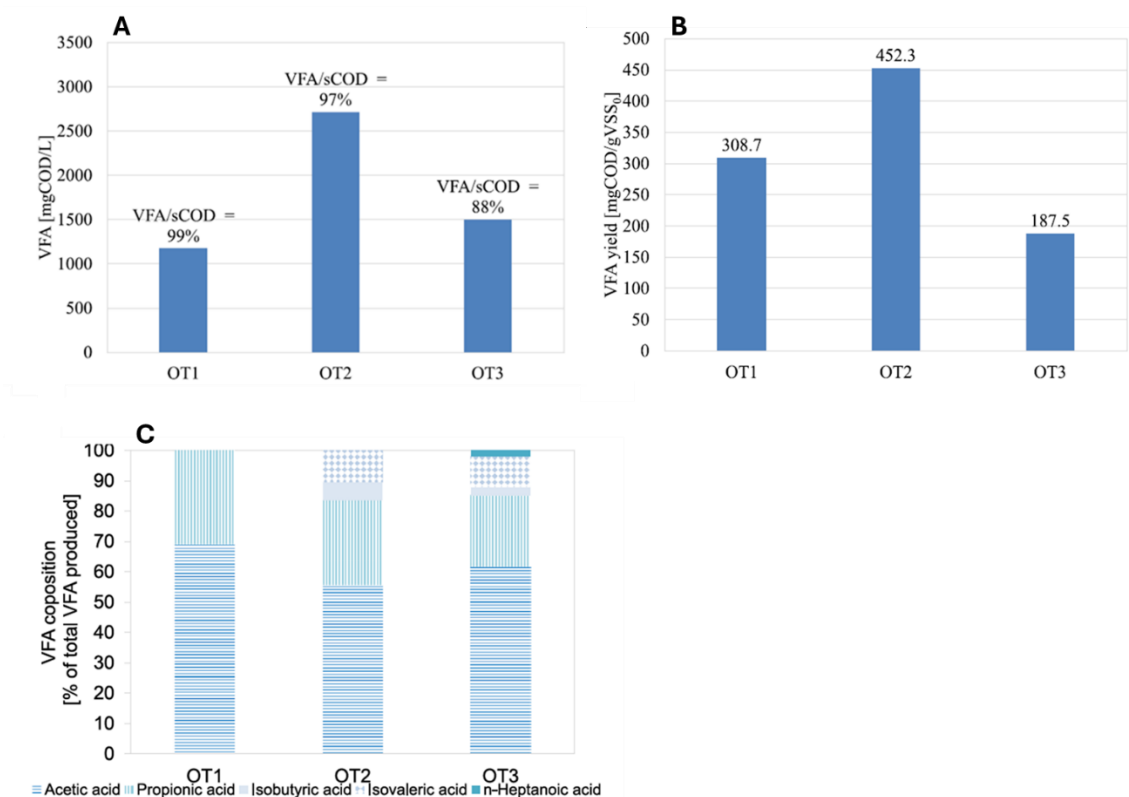


Figure 3.2. VFA production on day 17 (a), VFA yield on day 17 (b) and VFA composition on day 17 (c).

Regarding the $\text{NH}_4^+\text{-N}$ concentration measured during batch fermentation tests under VFA peak conditions (day 17), the $\text{NH}_4^+\text{-N}$ concentrations for OT1, OT2, and OT3 were 39.9, 119, and 85 mg/L, respectively. The highest ammonium release occurred in OT2 (VSS = 6 g/L). $\text{PO}_4^{3-}\text{-P}$ concentration increased throughout the batch tests, with the highest phosphate release observed in OT3 at 18.2 mg/L during the VFA peak. For OT2 and OT1, the peak $\text{PO}_4^{3-}\text{-P}$ concentrations were 8.3 mg/L and 8.1 mg/L, respectively. These results suggest that higher VSS concentrations increase $\text{PO}_4^{3-}\text{-P}$ concentration, unlike ammonium release.

3.3.2 Prokaryotic community structures of sewage sludge

A metataxonomic approach was used to characterise the possible influence of batch fermentation conditions on the microbial composition of the collected sewage sludge and used as fermentation inoculum. Metagenomic DNA was extracted from the sewage sludge

of the recycle line used as inoculum (T0) and from the sewage sludge of three different batch fermentation reactors (each indicated as OT1, OT2, and OT3, respectively) after 20 days of incubation. Preliminarily, the metagenomic DNA samples were quantified and analysed by PCR amplifying the V3–V4 region of the 16S rRNA encoding gene for a qualitative evaluation. Then, the bacterial community structures on the sewage samples were elucidated at phylum and family levels based on NGS analysis of the amplicons covering the V3–V4 region of the 16S rRNA encoding gene (Figure 3.3 and Table 3.S1). Looking at the microbial composition at the phylum level, represented in Figure 3.3 a, it is possible to appreciate that the microbial diversity reduces from the inoculum T0 to OT3 reactor in parallel with the concentrations of added potassium permanganate increasing from OT1 to OT3. The alpha-diversity (Table 3.S2) indices show, coinciding with the histograms of Figure 3.3, that there is an increase in diversity in OT1 (especially at the family level). At the same time, there is a gradual reduction in diversity in OT2 and OT3, as the concentration of added KMnO_4 . The most represented species are Proteobacteria, Bacteroidetes and Firmicutes. In particular, Proteobacteria are 40.32 % in T0, a slight increase in OT1 (55.71 %) and a decrease in OT2 and OT3 (32.72 and 18.36 %, respectively). Bacteroidetes are present at 25.55% in T0 and, contrarily, Proteobacteria decrease in OT1 (19.78%) and increase in OT2 and OT3 (39.31 and 70.22 %, respectively). The Firmicutes are 0.46%, 9.60%, 16.62% and 10.78 % in T0, OT1, OT2 and OT3, respectively. Furthermore, in T0, there is a high percentage of Chlamydiae (4.79 %), which increases in OT1, decreases in OT2 and even zeroes in OT3 (5.30, 1.64 and 0 %, respectively). The microbial composition is related to the different yields of VFA; in fact, Proteobacteria, Bacteroidetes and Firmicutes are excellent producers of VFA (She et al., 2020). Chlamydiae is a phylum whose abundance generally decreases as fermentation progresses (Qin et al., 2021). KMnO_4 has an excellent bactericidal effect against Chlamydiae at high concentrations, which justifies the reduction of the percentage abundance in OT2 and OT3 (Bommana & Polkinghorne, 2019). The leading families (Figure 3.3 b) present in the samples were Moraxellaceae, Porphyromonadaceae, Saprospiraceae, Cytophagaceae and Comamonadaceae. Moraxellaceae, which are present at 5.66% in T0, have a maximum growth in OT1 (19.42%), average in OT2 (12.4%) and minimum in OT3 (7.06%). Porphyromonadaceae are present in T0 in minimal percentages, less than 0%; however, they undergo a notable growth during fermentation, as they are present at 8.42% in OT1, at 20.91% in OT2 and 16.69% in OT3. Saprospiraceae are drastically reduced during fermentation, in fact, they are abundant at 6.34% in T0 while in OT1, OT2 and OT3 they are respectively abundant at 0.39%, 0.53% and 0%. As for Cytophagaceae and Comamonadaceae present respectively at 6.04% and 6.97% in T0, during fermentation and

after the addition of potassium permanganate, the percentages of both bacterial families are zero (0.00%). The first three families can ferment organic matter to produce VFA; therefore, the production of VFA can be delegated to these three families, especially Moraxellaceae and Porphyromonadaceae (Llamas et al., 2022). Comamonadaceae have a role in the degradation of VFAs to produce polyhydroxyalkanoates (Khan et al., 2002). The fact that adding different concentrations of potassium permanganate reduces the percentage abundance of this bacterial family can promote VFA accumulation.

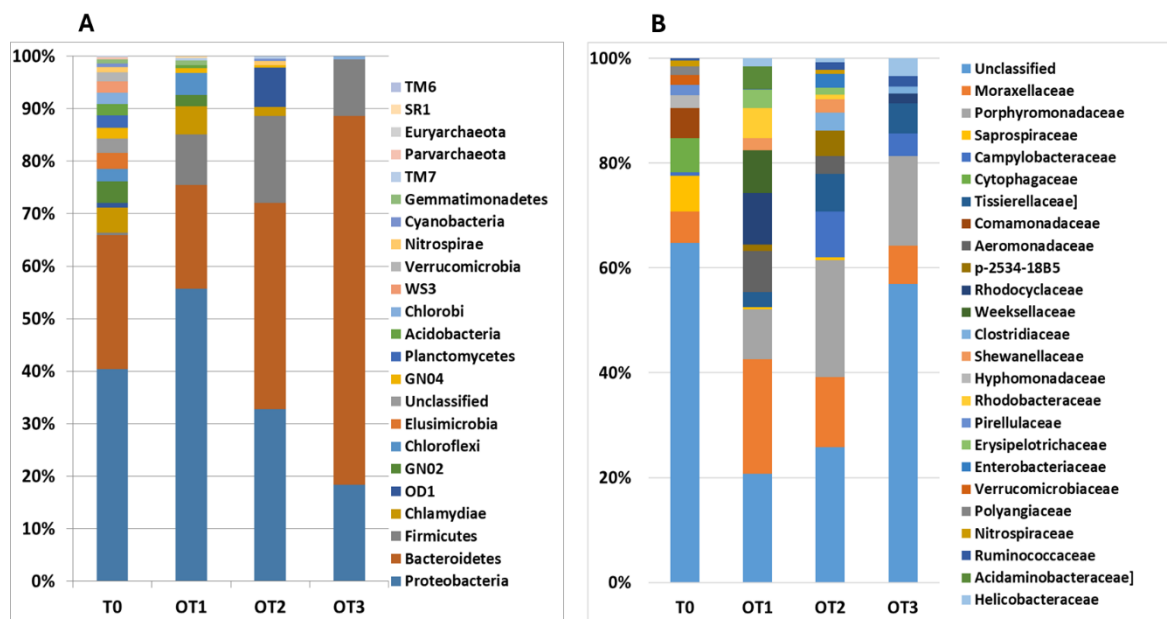


Figure 3.3. Structure of microbiota residing in the sewage sludge of T0, OT1, OT2 and OT3 reactors. Relative abundances of the top 25 bacterial phyla (a) and top 25 bacterial families (b) were identified by a metataxonomic approach based NGS analysis of the V3–V4 region of the gene encoding the 16S rRNA.

3.4 Conclusions

Batch fermentation tests were conducted to maximize VFA production from sewage sludge. It was established that a 0.1 mg $\text{KMnO}_4/\text{gVSS}$ dosage is an optimal condition for improving yields. Additional studies were carried out to investigate the role of VSS under KMnO_4 dosing to optimize the results further. The findings indicate that the optimal conditions for maximizing VFAs are achieved with 6 gVSS/L and a KMnO_4 dosage of 0.1 mg/gVSS. The primary bacterial phyla involved in the fermentation process were Proteobacteria, Bacteroidetes, and Firmicutes, while the main bacterial families affected were Moraxellaceae, Porphyromonadaceae, and Saprospiraceae. Importantly, the metataxonomic

analysis played a crucial role in providing valuable insights into the process performance and future outlook.

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3.6 Supplementary material

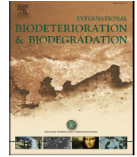
Table 3.S1. Microbial composition at the phylum level of the analysed samples. The numerical values refer to the percentage abundance.

Phylum	Percentage abundance T0 %	Percentage abundance OT1 %	Percentage abundance OT2 %	Percentage abundance OT3 %
Proteobacteria	40.32	55.71	32.72	18.36
Bacteroidetes	25.55	19.78	39.31	70.22
Firmicutes	0.46	9.60	16.62	10.78
Chlamydiae	4.79	5.30	1.64	0.00
OD1	0.87	0.00	7.46	0.00
GN02	4.15	2.21	0.00	0.00
Chloroflexi	2.41	4.21	0.00	0.00
Elusimicrobia	3.03	0.00	0.00	0.00
Unclassified	2.75	0.00	0.00	0.00
GN04	2.02	0.91	0.48	0.00
Planctomycetes	2.41	0.00	0.00	0.00
Acidobacteria	2.12	0.55	0.00	0.00
Chlorobi	2.20	0.00	0.00	0.64
WS3	2.13	0.00	0.00	0.00
Verrucomicrobia	1.70	0.00	0.00	0.00
Nitrospirae	1.03	0.00	0.77	0.00

Cyanobacteria	0.68	0.00	0.52	0.00
Gemmatimonadetes	0.76	0.85	0.00	0.00
TM7	0.00	0.51	0.48	0.00
Parvarchaeota	0.40	0.00	0.00	0.00
Euryarchaeota	0.14	0.00	0.00	0.00
SR1	0.00	0.36	0.00	0.00
TM6	0.09	0.00	0.00	0.00

Table 3.S2. ASV and ACE values and alpha-diversity indices (Shannon index and Simpson index) relative to samples T0, OT1, OT2, OT3.

Samples	KMnO₄	ASV	Ace	Shannon index	Simpson index
T0	0 g	437	437	6.54	0.92
OT1	4 g	163	163	6.64	0.98
OT2	6 g	231	231	5.99	0.96
OT3	8 g	27	27	3.8	0.89



CHAPTER 4

Enhancing volatile fatty acid production in batch test reactors by modulating microbial communities with potassium permanganate

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ABSTRACT

The shift from conventional wastewater treatment plants to biorefineries is one of the most environmentally and economically sustainable pathways for extracting valuable compounds from waste. Besides chemical-physical processes, microorganisms within sewage sludge utilize organic and inorganic pollutants in the wastewater as nutrients, leading to effective water purification. However, the remaining solid sludge residue, typically destined for specific landfills or incineration, could undergo microbial fermentation to produce volatile fatty acids (VFA), metabolic precursors for biopolymers. Increasing attention has been directed towards optimizing operational parameters to enhance VFA production during sewage sludge fermentation. This study examined the impact of potassium permanganate (PP) on microbial communities during the sewage sludge's acidogenic fermentation. The results highlighted the positive effect of PP treatment, which increased COD production and VFA yield up to 1263.5 mg/L and 664.2 mg COD/L, respectively. The presence of PP significantly enhances VFA yield promoting bacteria positively linked to VFA production.

4.1 Introduction

Wastewater comprises domestic, industrial, urban, and agricultural water collected through urban drainage systems, flowing into wastewater treatment plants (WWTP). Within WWTP, a series of purification processes, combining physical and microbiological procedures, are employed to eliminate organic and inorganic pollutants posing risks to human health and the environment, such as genotoxicity, cytotoxicity, tumorigenesis, and ecotoxicity (Choudri et al., 2020). The initial purification involves physical methods like sandblasting, de-oiling, and grilling to remove coarse particles, followed by microbial degradation of organic matter in the sludge. Clarification and decantation then separate solid components from the water, with subsequent disinfection marking the final stages (Newhart et al., 2019). Purified water is eventually discharged into receiving water bodies like lakes, rivers, and seas. However, sewage sludge produced in this process is typically disposed of in solid waste landfills or incinerators, contributing to environmental pollution alongside the greenhouse gas emissions produced by conventional WWTP (Hao et al., 2019). In Italy alone, 395,000 tons of dry sewage sludge are produced annually from WWTPs, with only 9.9% used in agricultural applications (Mininni et al., 2019). Thus, reclaiming and recycling valuable compounds (both materials and energy) remaining in the sludge poses a significant challenge, necessitating the transformation of traditional WWTP into Water Resource Recovery

Facilities or Wastewater BioRefineries. This shift aims to safeguard water quality, the environment, and human health (Mannina et al., 2021a,b). For instance, semiconductors can remove phenolic compounds from industrial discharges before the sewage sludge enters the WWTP. These semiconductors serve multiple purposes, including phenol removal from wastewater, CO₂ conversion, hydrogen generation from water splitting, and organic matter degradation (Al-Nayili and Alhaidry, 2023). Industrial wastewater contaminated by carcinogenic dyes can be effectively treated through adsorption using activated carbon, the most efficient, straightforward, and cost-effective natural treatment method for eliminating these organic compounds (Salman and Alshamsi, 2022). Undoubtedly, one potential application of organic matter usage is biopolymer production. Sewage sludge is particularly rich in compounds like biopolymers, or their precursors produced by microbial metabolism. In the last years, an increased demand for alternatives safer than plastics that can replace non-biodegradable plastic-based materials has surged (Gomaa et al., 2018; Piacenza et al., 2022). Plastics are more durable also in harsh environments and can spread antibiotic resistance bacterial strains (Sucato et al., 2021). Among biodegradable polymers, polyhydroxyalkanoates (PHAs) stand out as environmentally and economically sustainable thermoplastic biodegradable polyesters of microbial origin, suitable to replace petroleum-derived plastics (Tarrahi et al., 2020). Microorganisms play a significant role in breaking down and removing organic and xenobiotic contaminants to purify wastewater in WWTP and generate volatile fatty acids (VFA), H₂, and CO₂ (Sarkar et al., 2018). CO₂ and H₂ can be recovered from a wastewater treatment plant. H₂ is a valuable energy source to mitigate finite resource exploitation. The yield may vary based on the type of sewage sludge, concentration, and operating conditions and can be collected using different techniques (*e.g.*, gas separation, membrane technologies, bioreactors). CO₂ is a pollutant greenhouse gas that can be recovered using different types of activated carbon synthesized from phenol-formaldehyde resins or zeolites and exploiting different adsorption-desorption dynamics (Gil et al., 2015). VFAs serve as excellent precursors for PHAs, accumulated in granular form as an energy reserve (Shahid et al., 2021). The production of VFAs starting from sewage sludge is significant for several reasons. In fact, VFAs play a crucial role as substrates for microbial processes like anaerobic digestion or fermentation, leading to biopolymer generations. VFA production from sewage sludge allows waste valorisation into a valuable resource. Furthermore, VFAs enhance biological nutrient removal and overall treatment efficiency in wastewater treatment processes. Thus, VFA generation from sewage sludge offers economic advantages and advances sustainability. Thus, understanding the interplay between microbial communities and different WWTP operating conditions is crucial to improving fermentation

processes aimed to VFA production. Previous studies demonstrated the positive effect of KMnO_4 (potassium permanganate or PP), basic pH, and temperature on VFA production (Xu et al., 2021; Di Leto et al., 2022; Mineo et al., 2024). However, the impact of these conditions on microbial components remains relatively unexplored (Di Leto et al., 2022; Mineo et al., 2023). The Mn, contained in KMnO_4 , is a contaminating heavy metal. Following the positive effect of potassium permanganate on the sludge microbiota and subsequent recovery of VFAs through suitable filtration, the Mn released in the sewage sludge must be recovered. Leaching the sludge with HCl followed by hydrothermal treatment at 270 °C enables the retention of up to 98% of the Mn in the sewage sludge, ensuring environmental safeguarding against this heavy metal (Liu et al., 2021). This study aims to provide valuable insights into the microbial community shift during sewage sludge fermentation, especially when a chemical pretreatment is involved. Sewage sludge's acidogenic fermentation was investigated in batch tests, and sludge was sampled from two different WWTP sources pretreated with PP addition. A metataxonomic-based analysis was carried out on the sewage sludge microbiota in view to assess the microorganisms involved in the process, their metabolism, and diversity change after the oxidant treatment.

4.2 Material and Methods

4.2.1 Experimental set-up

Two experiments were conducted to assess the impact of PP on sewage sludge fermentation. Two sludge samples were collected from the pilot plant at the Water Resource Recovery Facility of Palermo University and a full-scale WWTP located in Marineo (Italy), indicated as A and B, respectively (Mannina et al., 2021, 2022). T0-A and T0-B sewage sludge were previously characterised (Mineo et al., 2023) in terms of pH, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), total Chemical Oxygen Demand (COD), soluble COD (sCOD), ammonium (NH_4^+ -N), and phosphate (PO_4^{3-} -P) concentrations (Table 4.1). sCOD, TCOD, VSS, TSS, NH_4^+ -N, and PO_4^{3-} -P concentrations were analysed according to standard methods suggested by APHA. Two fermentation tests were conducted for each sewage sludge sample: control tests (T18-A and T18-B) and PP dosing tests (T18PP-A and T18PP-B). The fermentation tests were executed in 1.1 L reactors at room temperature, with uncontrolled pH. PP was added at a concentration of 0.1 g per 1 g of TSS, as previously reported by Quixiang et al. (2021). The details of the batch fermentation tests are reported in Table 4.1. The tests were carried out for 18 days, during which the analysis of sCOD,

VFA, $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ were carried out. The VFAs were assayed by using gas chromatography (GC) after the extraction with dimethyl carbonate (DMC-OEI) as reported by Ghidotti et al., (2018). An Agilent Technologies 7820A GC with a flame ionisation detector (FID) and a DB FFAA column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) was used to analyse VFAs samples following the GC protocol described by Montiel-Jarillo et al. (2021). The VFA concentration (expressed as mg/L) was converted into COD concentration (mg COD/L) by applying the conversion factors reported in the literature (Yuan et al., 2011).

Table 4.1. Details of the fermentation batch tests.

Parameters	T0-A	T0-B	T18PP-A	T18PP-B
KMnO ₄ dosage (g/g TSS)	-	-	0.1	0.1
TSS (g/L)	4.7	4.8	4.7	4.8
VSS (g/L)	3.9	2.9	3.6	3.5
TCOD (mg/L)	5016.3	3329.4	4985.3	2972.4
sCOD (mg/L)	106.1	59.2	115.2	8.8
$\text{NH}_4^+\text{-N}$ (mg N/L)	4.7	2.3	3.7	1.9
$\text{PO}_4^{3-}\text{-P}$ (mg P/L)	2.3	3.6	8.5	2.1

4.2.2 Microbiota analysis

Six sewage sludge samples were analysed: T0-A and T0-B, corresponding to samples taken at the start of the fermentation from plant A and plant B, respectively; T18, representing samples collected after 18 days of the fermentation process, and T18PP, indicating samples collected from the test with PP addition. Samples were collected on the 18th day of fermentation, which aligned with the decline in sCOD values following the peak phase. The procedures outlined by Mineo et al. (2023) were used. Metagenomic DNA extraction was performed using 1 g of samples. DNA quality and quantity were assessed through 1% agarose gel electrophoresis analysis, supplemented with 0.5 $\mu\text{g/mL}$ ethidium bromide for visualization under a UV lamp, and by measuring absorbance ratios (260/280 and 260/230 nm) using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) to detect protein, organic compound, or chaotropic agent contamination. Amplification of the V3–V4 regions of the 16S gene was conducted using the primers PRO 341F CCTACGGGNNBGCASCAG and PRO 805R GACTACNVGGGTATCTAATCC, with DreamTaq DNA Polymerase (ThermoFisherScientific) following the manufacturer's protocol. The thermal profile comprised 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s; and a final elongation at

72 °C for 5 min. Amplification products were sequenced in a single-300-bp paired-end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). Raw 16S rDNA data were processed using QIIME2 software (<https://qiime2.org/>) for denoising, where overlapping paired-end reads were processed with the DADA2 plug-in. Unique Amplicon Sequence Variants (ASVs) were assigned and aligned to the Greengenes reference database at 99% sequence similarity (<https://greengenes.secondgenome.com/>). For each sample, the count of ASVs and the relative abundance percentages of phyla, orders, classes, families, and genera were determined. HeatMap generation was conducted via the online web server (<http://heatmapper.ca/expression/>), employing a “complete linkage” calculation based on Pearson correlation and focusing on families. METAGENassist (<http://www.metagenassist.ca> (accessed on March 5, 2024) was used to distinguish the microbial species based on their metabolic activity. The sequences were deposited under the number PRJNA1078840.

4.3 Results and discussion

4.3.1 Effect of potassium permanganate addition

The monitoring results carried out during the fermentation are reported in Table 4.2. The addition of PP significantly enhanced sCOD production of tests T18PP-A and T18PP-B by up to 1226 mg COD/L and 1263 mg COD/L, respectively, on days 12 and 7. For T18-A and T18-B, sCOD peak values were observed at 1119 mg COD/L and 284 mg COD/L on the 12th day, respectively. The comparison of the control tests with those treated with the oxidant addition showed that the treatment increased the sCOD concentration at its peak by + 9.6% for Plant A, while a higher increase of +344% was registered for Plant B. Additionally, T18PP-B achieved its peak of sCOD production five days earlier than T18PP-A, likely due to enhanced hydrolysis, as previously reported (Xu et al., 2019; Yu et al., 2008). PP considerably improved the breakdown of complex organic material, with a maximum effect observed in sludge from full-scale WWTP. The dosage of PP improved COD solubilization by nearly 30% for Plant B sludge and by only 2% for T18PP-A. Furthermore, PP favored the release of NH_4^+ to supernatant because of protein hydrolysis, as reported by the high ammonium concentrations on the sCOD production peak day for T18PP-B (87 mg N/L) compared to T18PP-A (78 mg N/L), conforming to sCOD trends. Peak day ammonium concentration in T18-A and T18PP-A showed no remarkable difference (70 mg N/L and 78 mg N/L, respectively). Furthermore, the addition of PP affected the concentration of

phosphates, whereby its concentration was higher due to the promotion of precipitation in an alkaline condition, as reported in Table 4.2 (Banister et al., 1998). The VFA production followed the sCOD production trend across all the trials, clearly showing an increment in VFA production, more prominently in T18PP-B, where the VFA concentration increased to 664 mg COD/L at day 7, composing around 55% of sCOD. The T18PP-A peak (474 mg COD/L at day 12) gave around 38% of sCOD. This result is thus evidence of PP effectiveness also in the acidogenic phase of fermentation, by a noticeable improvement in sCOD production, COD solubilization, and VFA yield in T18PP-B as reported in other studies (Li et al., 2015, 2020; Yu et al., 2013).

Table 4.2. sCOD, VFA, ammonium and phosphate concentration during the fermentation tests.

Parameters	Test	Time (days)					
		3	5	7	10	12	14
sCOD (mg/L)	T18-A	208.5	348.2	757.4	827.1	1119.2	880.9
	T18-B	10.2	50.3	120.1	150.8	284.4	90.2
	T18PP-A	667.1	768.2	954.8	1032.7	1226.4	1080.5
	T18PP-B	630.4	955.2	1263.5	922.7	890.2	758.3
VFA (mg COD/L)	T18-A	23.2	37.4	69.0	230.1	410.7	223.3
	T18-B	0.0	0.0	0.0	25.5	11.9	2.3
	T18PP-A	207.4	224.4	288.2	393.1	473.0	262.7
	T18PP-B	267.2	403.9	664.2	292.9	185.0	103.4
NH ₄ ⁺ -N (mg N/L)	T18-A	4.7	16.2	49.5	61.3	70.4	60.2
	T18-B	2.8	11.2	15.8	18.5	19.3	27.8
	T18PP-A	10.2	37.8	55.2	68.4	78.7	63.9
	T18PP-B	15.2	36.8	87.6	84.3	78.5	77.1
PO ₄ ³⁻ -P (mg P/L)	T18-A	9.9	16.9	26.1	30.5	31.3	28.1
	T18-B	7.8	10.2	12.3	14.1	16.7	15.4
	T18PP-A	10.3	9.2	13.3	14.9	22.1	21.3
	T18PP-B	2.7	3.9	5.7	7.4	7.7	6.8

As reported in Figure 4.1, acetic acid was the most prevailing VFA, accounting for 57% in T18-A, 38% in T18PP-A, 100% in T18-B, and 48% in T18PP-B. PP addition mainly increased the production of propionic, iso-butyric, and butyric acids in T18PP-A (26%, 10%, and 11%, respectively) and T18PP-B (35.8%, 10.1%, and 2.1%, respectively). The quantity of iso-valeric acid in T18PP-A was 12.2%, whereas other acids collectively contributed less than 4% in all tests. Regarding T18PP-B, butyric to n-heptanoic acid accounted for less than

7%. Generally, PP addition favored the production of different VFAs, suggesting a more efficient breakdown of organic materials, as reported above.

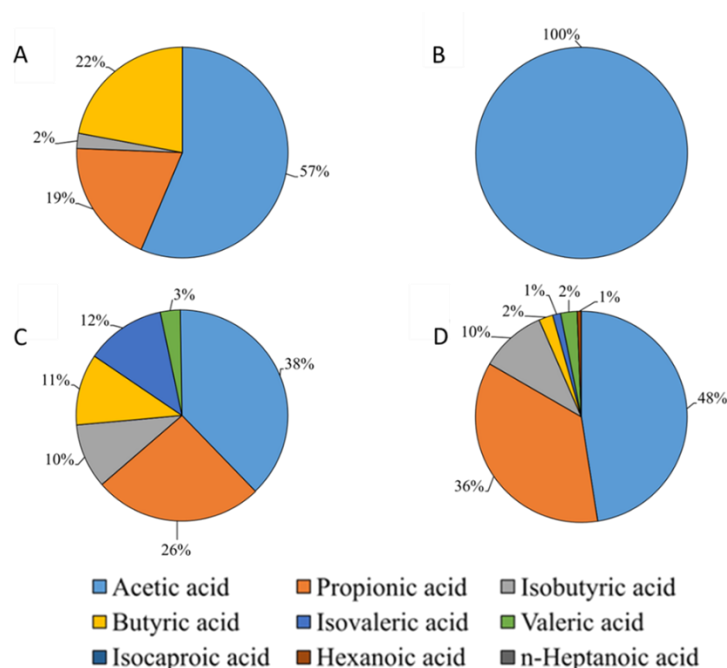


Figure 4.1. Average composition of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic, and n-heptanoic acids in T18-A (A), T18-B (B), T18PPA (C) and T18PP-B (D) samples after 18 days of fermentation.

4.3.2 Taxonomic composition of prokaryotic communities of sewage sludge at the phylum level

Microbiota analysis showed that both the sludge samples contained microorganisms belonged to the bacterial domain, while no Archaea was detected. Similar results were found in a few previous studies on sludge, in which bacteria represented almost the totality of the prokaryotic community of the sewage sludge (98.8%) to Archaea (only 1.2%) (Yu and Zhang, 2012; Zhang et al., 2012; Xia et al., 2010; McLellan et al., 2010; Mathur et al., 2023). The bacterial composition of the sludge samples from plants A and B was comparatively analysed considering the relative abundances at the phylum level. Proteobacteria represented the most abundant phylum accounting for 57.24% in T0-A and 53.84% in T0-B. In metropolitan WWTP, Proteobacteria (21–65%) represent the utmost prevalent phylum and are primarily responsible for nutrient and organic removal; subdominant phyla include Chloroflexi, Acidobacteria, and Bacteroidetes (Mathur et al., 2023; Wang et al., 2012). Apart from the similar percentages of Proteobacteria, comparing the microorganisms identified in T0-A and T0-B samples showed that the two samples contain different phyla or the same

phylum with different relative abundance percentages. As seen in Figure 4.2, the T0-B sample displayed a greater bacterial diversity than T0-A. In the T0-A sample, Bacteroidetes (32.01%) were predominant, followed by Verrucomicrobia (5.38%) and Firmicutes (1.86%). All the other phyla were present in a low percentage (less than 1.86%). In the T0-B sample, 15.60% of bacteria were unclassified, followed by Bacteroidetes (7.89%), Chloroflexi (4.28%), and Spirochaetes (3.41%). Also, a modest percentage of Nitrospirae (3.10%) and WS3 (2.77%) was found. This first result underscores the importance of bacterial composition that relies on the operational conditions but also on the initial bacterial load of the sewage sludge (Zhang et al., 2022). Despite the high variability of WWTP microbial communities that can contain members of various taxonomic, physiological (*e.g.*, aerobic, anaerobic, heterotrophic, phototrophic), and biochemical (*e.g.*, nitrification, N₂-fixation, sulfur oxidation, and denitrification) groups, some phyla are frequently found, such as Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia that can be considered as the core microbiota of the sludge samples, coherently with other reports (Yu and Zhang, 2012). Proteobacteria and Bacteroidetes are recognized for their pivotal roles in VFA production during fermentative processes (She et al., 2020). Verrucomicrobia are commonly found in wastewater and have recently been associated with VFA production via fermentation from substrates like glucose (Spring et al., 2016). Spirochaetes are also acknowledged for their proficiency in producing VFAs under alkaline conditions, around pH 8 (Slezak et al., 2021). Nitrospirae predominantly produce acetic and propionic acids (Yang et al., 2016), with an additional function of transforming nitrites into nitrates when VFA are present. Despite the WS3 phylum has been detected in Marineo sludge and polluted aquifers (Presti et al., 2021), their capability to produce VFA remains unproven. T18-A and T18PP-A exhibited a comparable microbial composition in terms of phyla, with four predominant phyla: Bacteroidetes (increased to 49.14% and 47.43%, respectively), Proteobacteria (decreased to 35.63% and 37.80%, respectively), Firmicutes (increased to 7.27% and 7.60%), and Verrucomicrobia (decreased to 2.54% and 0.43%, respectively). Thus, PP addition to T0-A did not substantially influence the sludge's bacterial composition. Conversely, the addition of PP dramatically impacted bacterial abundance percentages. T18-B closely resembled the T0-B sample regarding Proteobacteria, except for the increased of Bacteroidetes (14.35%) and decreased of the unclassified bacteria (6.46%). Particularly noteworthy, the high bacterial diversity observed in T0-B was significantly reduced in T18PP-B, leaving only three dominant phyla: Proteobacteria (slightly increased to 56.09%), Bacteroidetes, and Firmicutes (increased to 32.34% and 10.90%, respectively). Collectively, these three phyla represent 99.29% of the entire bacterial community.

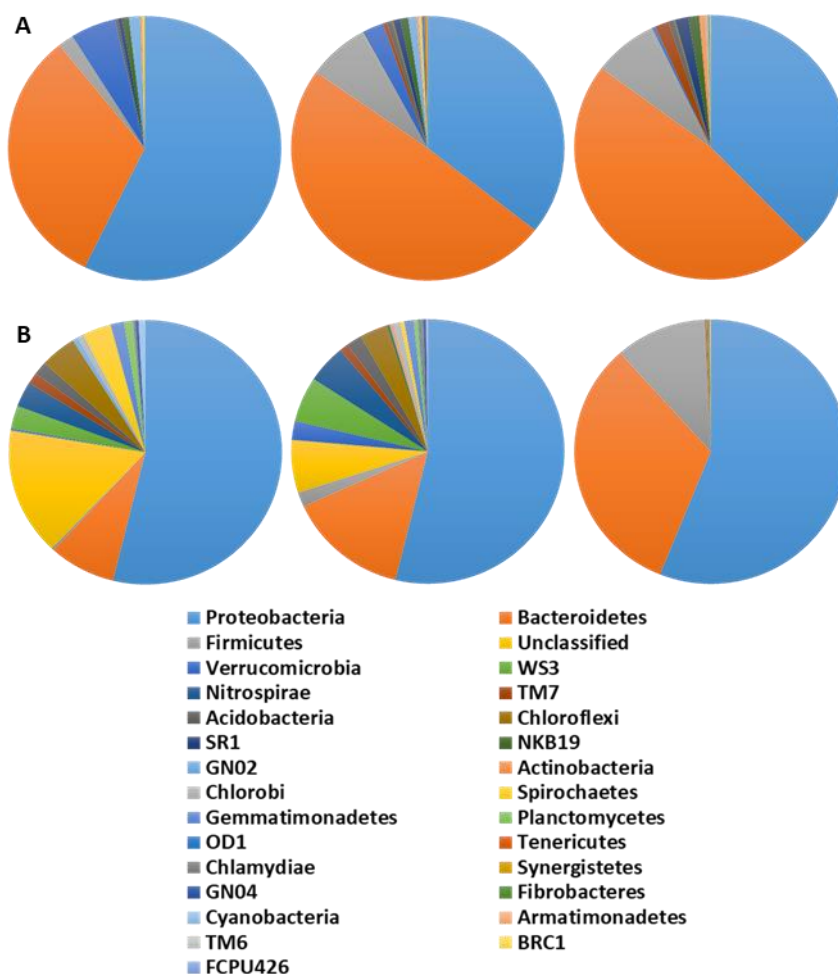


Figure 4.2. Pie charts reporting the taxonomic composition of samples T0-A, T18-A, T18PP-A (A) and T0-B, T18-B, T18PP-B (B). The relative abundance indicated the percentage of each phylum compared to all the identified phyla.

4.3.3 Clustering analysis of microbial community dynamics

According to the linkage of ASVs (Figure 4.3), the bacterial communities of the six samples were clustered into two large groups: Group I contained the sewage sludge inocula (T0-A and T0-B) and the control samples (T18-A and T18-B); Group II contained the two samples of sewage sludge treated with PP (T18PP-A and T18PP-B) indicating that, although the effect of PP on VFA is different, the impact on bacteria is similar. According to weighted Pearson distances, significant position differences occurred between the inocula samples and control samples of both experiments. The T18PP-A and T18PP-B were close in cluster analysis, showing similar bacterial diversity but different bacterial family abundance. PP treatment in sample T18PP-B resulted in a drastic decrease in the abundances of many

bacterial families, maintaining high percentages only of three families: Campylobacteraceae, Pseudomonadaceae, and p-2534-18B5 (indicated in yellow, Figure 4.3). We hypothesized that the increase of Gram-negative bacteria could rely on the development of resistance systems of these bacteria towards oxidizing agents such as PP (da Cruz Nizer et al., 2020, 2021).

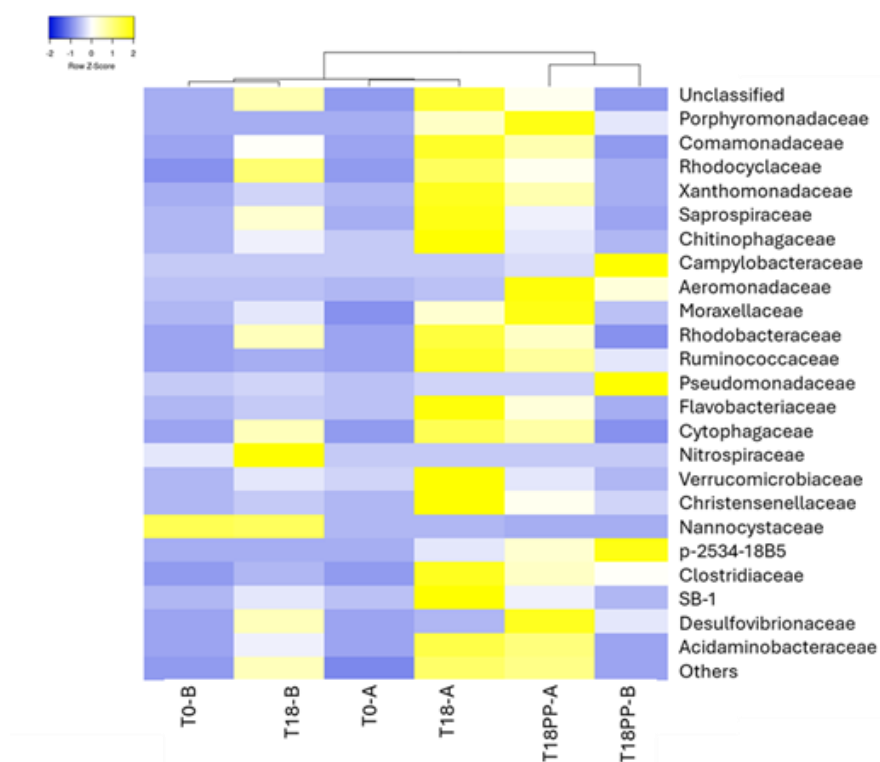


Figure 4.3. Heatmap of 25 most abundant prokaryotic families' composition profiles in T0-A, T18-A, T18PP-A, and T0-B, T18-B, T18PP-B samples as inferred by HeatMapper bioinformatic analysis. The colour intensity in each sample was normalized to represent its relative ratio in the two groups. Colours from blue to yellow indicated the relative values of microbiota.

4.3.4 Taxonomic composition of prokaryotic communities of sewage sludge at the family level

In bacterial families' analysis, a notable contrast between samples of Plant A and Plant B became even more pronounced (Figure 4.4). Sample T18PP-A was found enriched of Porphyromonadaceae (32.64%) towards 11.34% (T18-A). In T18PP-A, the modulation of unclassified bacteria (from 22.46% in T0-A and 29.21% in T18-A to 16.28% in T18PP-A) and the decrease of Moraxellaceae (from 5.94% of T0-A to 1.23% in T18-A and 3.26% in T18PP-A) was also evident. The percentage abundance of Saprospiraceae (7.80% in T0-A, 6.13% in T18-A and 1.99% in T18PP-A), Chitinophagaceae (7.40% in T0-A, 5.46% in T18-

A and 1.03% in T18PP A), and Campylobacteraceae (4.38% in T0- A, 0.12% in T18-A and 0.70% in T18PP-A) decreased after the fermentation process, mainly in presence of PP. In T18PP-B, there was a significant decrease in the presence of unclassified bacteria, from 39.43% of T0-B and 38.35% in T18-B to just 3.31%. The most abundant families in T18-PP-B were Campylobacteraceae (28.00% in T18PP-B towards 1.41% in T0-B and 0.48% in T18-B), Porphyromonadaceae (24.86% in T18PP-B towards 0.09% in T0-B and 0.12% T18-B), Pseudomonadaceae (12.27% in T18PP-B towards 0.97% in T0-B and 0.43% in T18-B) and Aeromonadaceae (8.19% in T18PP-B towards 1.44% in T0-B and 0.33% in T18-B). Contrarily, in T18PP-B Saprospiraceae were not present (0.00%) in respect to T0-B (4.17%) and T18-B (5.54%) and Comamonadaceae were very low (0.30%) in respect to T0-B (5.58%) and T18-B (7.01%).

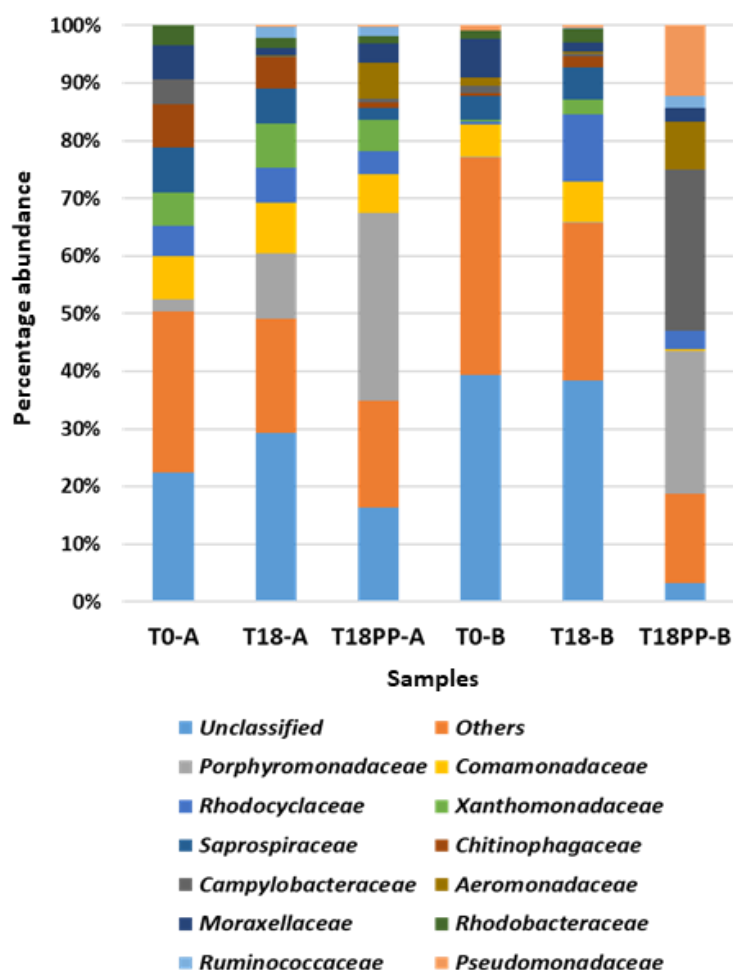


Figure 4.4. Histogram reporting the taxonomic composition of samples T0-A, T18-A, T18PP-A (A) and T0-B, T18-B, T18PP-B (B). The relative abundance was the percentage of each family related to all the identified families.

4.3.5 Predicting microbial metabolism

To analyse how PP can influence microorganisms capable of synthesizing VFA, bacteria were categorized based on their metabolic pathways. Metabolic profiling indicated the presence of dehalogenating bacteria, nitrite reducers, sulfate oxidizers, and ammonia oxidizers (Figure 4.5), as already reported in Di Leto et al. (2022). Although the prokaryotic communities in Plants A and B displayed similar metabolic profiles, some interesting differences can be highlighted. T18PP-B showed the abundance of ammonia oxidizers, sulfate reducers, sulfide oxidizers and nitrite reducers was higher than in T18PP-A. Bacteria capable of ammonium oxidation, referred to as nitrifying bacteria, can indirectly impact the production of VFA within wastewater systems. While nitrifying bacteria utilize ammonium as an energy source, they do not directly produce VFA as a metabolic byproduct (Luo et al., 2019). Sulfide oxidizer and sulfate reducer bacteria can influence the redox state of the environment and the availability of organic substrates for other bacterial species. For instance, sulfur oxidation can diminish the concentration of hydrogen sulfide, a compound toxic to numerous bacterial strains, thus fostering the proliferation of bacteria capable of VFA production during the fermentation of organic compounds within the wastewater (Odriozola et al., 2019; Samanides et al., 2020). Interestingly, an increase of anaerobic bacteria was registered after PP treatment in both plants, confirming the activation of the fermentation process leading to VFA production in both T18PP- A and T18PP-B.

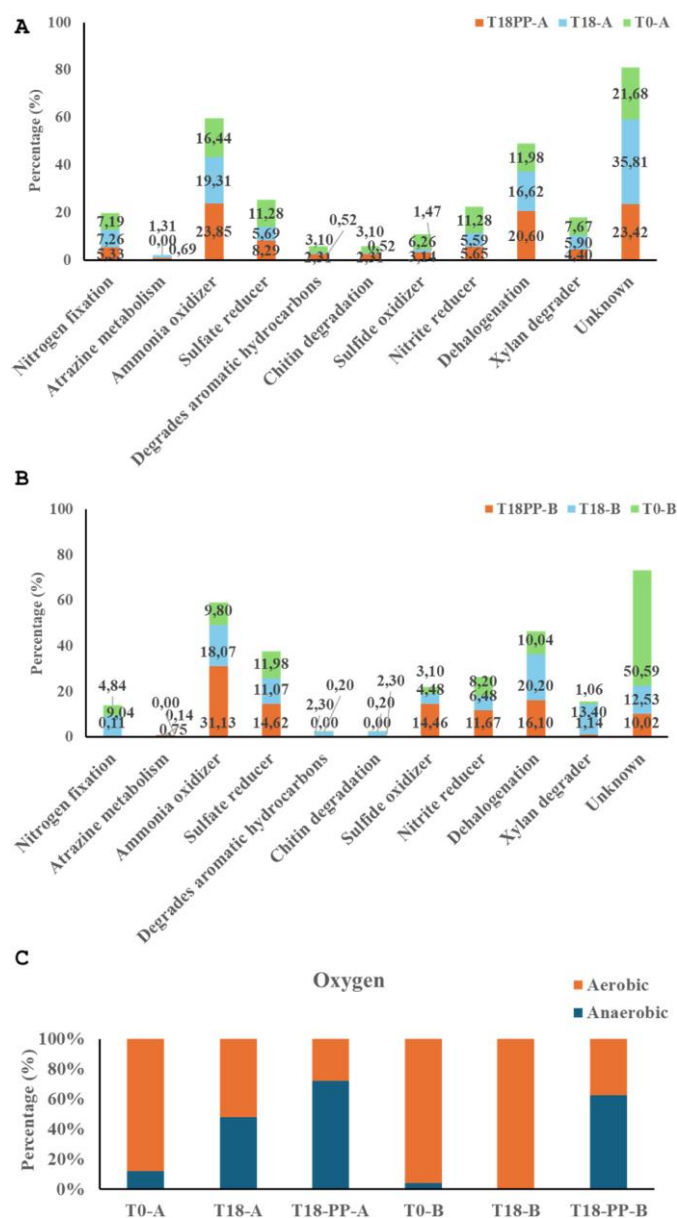


Figure 4.5. Putative metabolic requirements of the bacterial communities residing in the samples T0-A, T18-A, T18PP-A (A) and T0-B, T18-B, T18PP-B (B) as inferred by METAGENassist bioinformatic analysis. Oxygen requirements of bacterial communities (C). The abundance percentage of bacteria having a specific metabolism is indicated.

4.3.6 Bacterial signatures for VFA high production

When the microbial components were analysed for the presence of bacterial species, most bacteria belonged to unknown species; however, the species of *Macellibacteroides fermentans* was predominantly detected in samples PP-treated. After fermentation, the percentages of this species increased from almost zero in T0-A and T0-B, to 4.54% in T18-A, 29.30% in T18PP-A and 21.59% in T18PP-B, while it was reduced only in T18-B (0.04%)

Also, *Pseudomonas veronii* increased to 9.66% in the T18PP-B sample. Finally, *Proteocatella sphenisci* was present higher in T18PP-B (1.51%) than in other samples, where the percentage was approximately zero. *M. fermentans* is a species belonging to the *Porphyromonadaceae* family, isolated in recent years, which has the exceptional ability to degrade isosaccharinic acids (sugary acid typical of nuclear waste deposits). *M. fermentans* is capable of growing only in anaerobiosis, in the presence of sugars or the presence of isosaccharinic acid, it is capable of autonomously producing acetic acid, consequently causing a lowering of pH (Rout et al., 2017). It is also able to produce butyrate and isobutyrate (Jabari et al., 2012). *P. veronii*, belonging to the *Pseudomonas* genus, is among the most important producers of VFA. Previous studies confirmed that at any pH it can excellently produce VFA (Jie et al., 2014). An interesting point that deserves to be further investigated concerns the mechanisms that allow this specie to survive despite the oxidizing action of the latter. Other studies have shown the ineffectiveness of PP treatments administered at 0.015% in *Pseudomonas* genus and Gram-negative cultures (Hansson et Faergemann, 1995). As far as we know, there are no studies on the resistance of *Macellibacteroides* to PP or other antimicrobials. Being both Gram-negatives, we could speculate that these cells have developed resistance in the cell wall, which is the target of potassium permanganate (da Cruz Nizer et al., 2020; da Cruz Nizer et al., 2021).

4.3.7 Detection of potential harmful bacteria

Pathogenic bacteria can compete with other environmental bacteria for resources, such as organic substrates and nutrients. This competition can influence the growth and activity of bacteria involved in VFA production, thus altering the quantity and composition of VFA produced. Among the 108 bacterial genera, six are considered potentially harmful bacteria: *Pseudomonas* spp., *Acinetobacter* spp., *Clostridium* spp., *Desulfovibrio* spp., *Flavobacterium* spp., and *Lactococcus* spp. (Figure 4.6). All detected potential harmful bacteria genera except *Pseudomonas* spp. had lower relative abundance in the T18PP-B sample compared to the other samples. A greater abundance of pathogenic bacteria in Plant A, especially after PP treatment, may have caused the decreasing presence of bacteria capable of producing VFA. Some pathogenic bacteria can directly or indirectly use the organic compounds present in wastewater as a substrate for their metabolism and influence the structure and composition of the microbial community. Pathogenic bacteria that degrade organic matter can consume substrates that would otherwise be available to VFA-producing bacteria. This may reduce the availability of substrates for VFA production and thus affect

their production, as already found (Zhang et al., 2022; Guo et al., 2020; Liu et al., 2021). The presence of pathogenic bacteria can pose a risk to human and animal health (Vitale et al., 2019; Alduina 2020; Gargano et al., 2021; Gambino et al., 2022). Some pathogenic bacteria can cause disease if not treated properly, while others can produce toxins or harmful compounds. Therefore, the management and adequate treatment of wastewater are essential to mitigate the potential risks associated with the presence of pathogenic bacteria and to ensure the safety of water intended for human consumption and release into the environment.

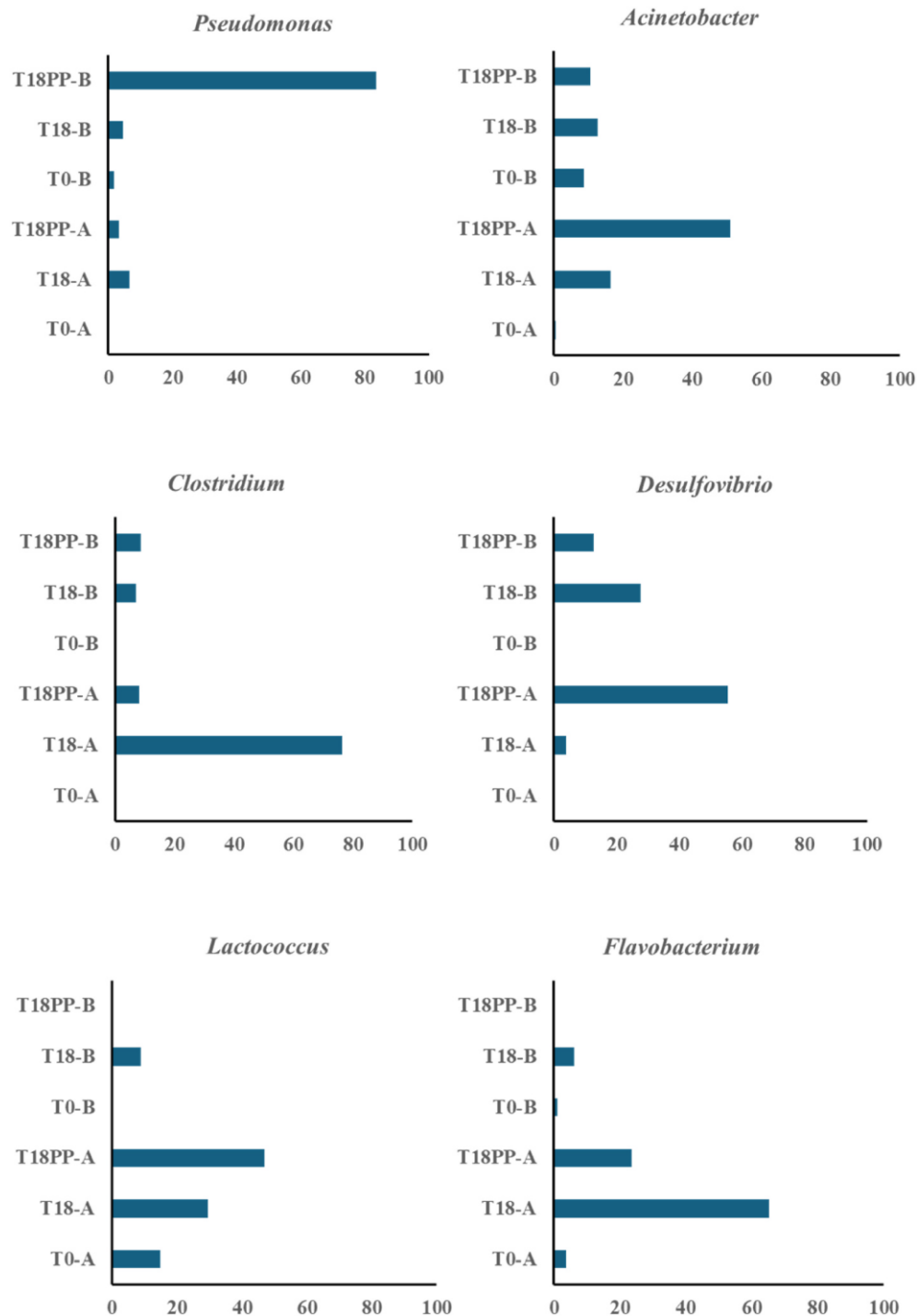


Figure 4.6. Detection of potential harmful bacteria (*Pseudomonas*, *Acinetobacter*, *Clostridium*, *Desulfovibrio*, *Lactococcus* and *Flavobacterium*) in T0-A, T18-A, T18PP-A, and T0-B, T18-B, T18PP-B wastewater samples. The percentage of relative abundance of each potential pathogenic genus was indicated.

4.4 Conclusions

This research represents a pioneering effort investigating how the microbial community in sewage sludge responds to potassium permanganate during fermentation to boost VFA production. Potassium permanganate, known for its antimicrobial properties, stimulated VFA yields by promoting the survival of VFA-producing bacterial species, belonging to Proteobacteria, Firmicutes, and Bacteroidetes phyla, and leading to a reduction of VFA degraders. Due to the complex system, further multidisciplinary studies are needed to define the bacterial signatures leading to specific production in a real WWTP.

4.5 References

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CHAPTER 5

The effects of headspace volume reactor on the microbial community structure during fermentation processes for volatile fatty acid production

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ABSTRACT

The transition from traditional wastewater treatment plants to biorefineries represents an environmentally and economically sustainable approach to extracting valuable compounds from waste. Sewage sludge produced from wastewater treatment is incinerated or disposed of in specific landfills. Repurposing this waste material to recover valuable resources could help lower disposal costs and reduce environmental impact by producing other beneficial polymers. Microorganisms present in the sewage sludge can ferment organic pollutants, producing volatile fatty acids (VFA), precursors for biopolymers that could be used as an alternative to petroleum-derived plastics. To boost VFA production during sewage sludge fermentation, it is necessary to understand the operating microbial community and its metabolic capacities in anaerobic conditions. This study presents the influence of the headspace volume on the microbial community and the VFA production to define the best operational parameters in a 225 L pilot plant fermenter. The wasted sewage sludge was withdrawn from an Oxidative Settling Anaerobic plant that collected wastewater from the canteen and dormitory of the UNIPA Campus (Palermo University, Italy) and incubated using a 40% and a 60% headspace volume. The microbial community was analysed before and after the fermentation process through metataxonomic analysis, and VFA yields were determined by gas-chromatography analysis. Our results showed that the 40% headspace volume induced a 10-fold higher VFA production than the 60% headspace volume, modulating the microbial community's efforts to establish a VFA-producing factory. Notably, at 40% headspace, the relative abundance of bacteria, like Proteobacteria, Firmicutes, Actinobacteria, and Chloroflexi, significantly increased, as well as the relative abundance of Bacteroidetes and Verrucomicrobia decreased during the fermentation process. This result is consistent with the selection of efficient VFA-producing bacteria that lead to increased VFA yields that are not obtained at 60% headspace. Thus, reducing headspace is a promising strategy that can be implemented, even in full-scale plants, to optimize the wastewater reuse process and maximize VFA production to produce bioplastics, like polyhydroxyalkanoate, for the transition from linear wastewater treatment plants to circular biorefineries.

5.1 Introduction

Sewage sludge (SS) production at wastewater treatment plants has significantly increased in recent years due to intensified industrial activity and enlarged population growth (Bianchini et al., 2016). Traditional methods of SS management, such as landfilling and incineration, have a high environmental impact and cost (Hao et al., 2020). The introduction of circular economy approaches can offer a sustainable alternative, treating SS as a resource rather than

waste (Mannina et al., 2021). SS can be further exploited to produce valuable nutrients and organic compounds through anaerobic digestion. This process involves the microbial decomposition of organic matter through stages such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis, ultimately yielding methane (CH_4) and carbon dioxide (CO_2). The production of high-value-added compounds such as volatile fatty acids (VFAs) can be increased by inhibiting certain stages, particularly acetogenesis and methanogenesis. VFAs are biochemical building blocks that microorganisms can use as substrates to produce several biopolymers. Despite the potential of this method, challenges persist, particularly in optimising VFA production and organic matter conversion. Various pre-treatments have been explored to assess factors like hydraulic retention time, temperature, pH, nutrient availability, and headspace pressure (HP), with studies indicating that lower HP promotes VFA production (Koch et al., 2015; García-Depraect et al., 2022; Darvekar et al., 2019; Li et al., 2019; Mineo et al., 2023). Although bacterial and archaeal species have an essential role in VFA yields and many environmental factors influence microbial fermentation (Zhang et al., 2020; Di Leto et al., 2022; Mineo et al., 2024; Di Leto et al., 2024), research on the influence of microbial communities on fermentation and VFA production is still in its early stages.

Previous studies have shown a positive correlation between reduced headspace volume and increased VFA production (Xu et al., 2020). Li et al. (2019) assessed how headspace affects VFA production from proteins and discovered that lower headspace pressure enhances VFA production. Headspace can have several impacts on anaerobic sewage sludge fermentation: reducing headspace can create an environment in which acidogenic bacteria can exploit H_2 and CO_2 to produce VFA (Yan et al., 2014). Headspace is also important to avoid pressure buildup during methane production, which reduces VFA production (Abubakar et al., 2024). Indeed, Mineo et al. 2023 demonstrated that a 40% headspace volume induced a 10-fold higher VFA production than a 60% headspace volume. However, none of these studies have prioritized the analysis of the sewage sludge microbiota structure, which is crucial in acidogenic fermentation and primarily responsible for VFA production. As far as the authors know, reports have yet to examine the microbial community of fermented sewage sludge under controlled headspace to implement VFA production. This study aims to fill this gap by examining the microbial community of fermentation sludge samples under different headspace volumes and comparing them with their initial compositions. The prokaryotic microbiota structure was examined using metataxonomic analyses to identify bacteria involved in acidogenic fermentation, focusing on controlling headspace volume to increase

VFA yields that can be used for the production of bioplastics, like polyhydroxyalkanoate during microbial fermentation (Di Leto et al., 2022).

5.2 Material and Methods

5.2.1 Experimental setup

The fermentation experiments were carried out at Palermo University, Italy's Water Resource Recovery Facility (Mannina et al., 2021). The fermentation unit is located in the polyhydroxyalkanoates (PHA) production line of the pilot plant hall and was previously described by Mineo et al. (2023). It consists of a continuous stirred tank fermenter (total volume of 225 L) fed with the sewage sludge produced by the wastewater treatment plant of the pilot plant hall and equipped with sampling ports for liquid and gas samples (Figure 5.1). The fermenter is linked to an ultra-filtration (UF) unit (total volume of 43 L) equipping a hollow fiber membrane (polyvinylidene, 0.03- μ m porosity and 1.4-m² surface area). Once the fermentation process was completed, the sewage sludge was pumped to the UF unit and filtered with a net flow rate of 13.2 L/h. The UF unit has a gas recycling pump (Gilian GilAir Plus, Recom Industriale), reducing membrane fouling during the sludge's filtration while keeping the anaerobic environment. The fermenter was operated with a hydraulic and sludge retention time of 5 days. The SS was collected in two seasons (F1, winter and F2, spring) from the wastewater treatment line within the pilot plant, which adopts an oxic-settling-anaerobic (OSA) configuration. Two fermentation experiments were started: fermentation 1, with 40% of the SS and 60% of the headspace, while fermentation 2, with 60% of the SS and 40% of the headspace. Throughout the fermentation, pH, temperature and Oxidation Reduction Potential (ORP) were continuously monitored inside the fermenter by using WiFi - Multi 3630 IDS "WTW" (Xylem brand) and related probes. Other parameters, such as total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), soluble proteins and carbohydrates, ammonium (NH₄-N), orthophosphate (PO₄-P), and volatile fatty acids (VFAs) were measured at the beginning and end of the fermentation process, as well as during the VFA peak day, following the procedure outlined in Table 5.1 and detailed in Mineo et al., 2023. Samples were collected at the start and end of the fermentation process to conduct metataxonomic analyses.

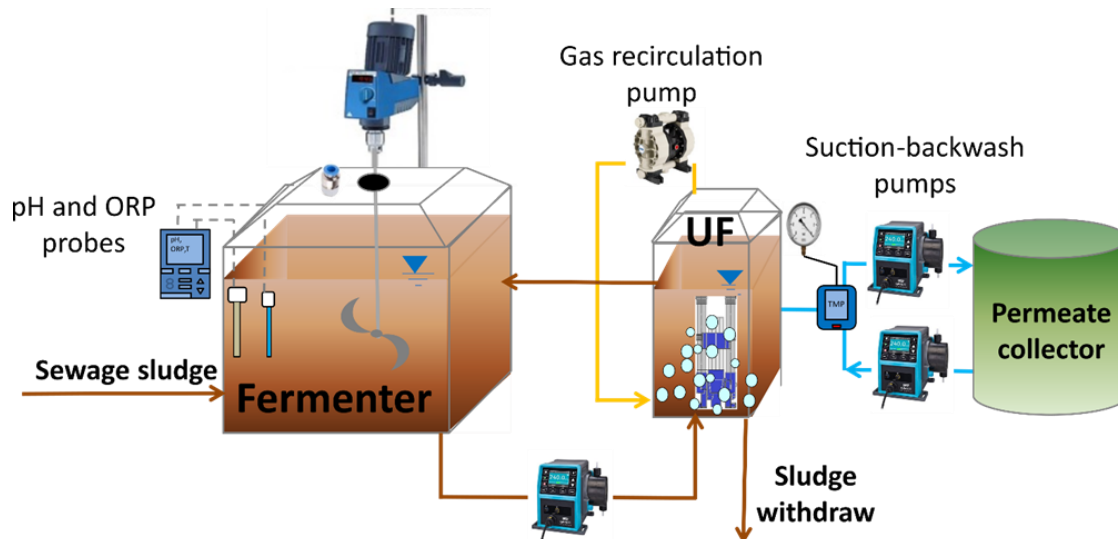


Figure 5.1. Schematic view of the fermentation unit. It consists of a fermenter, an ultrafiltration unit, and a tank for collecting the permeate. The scheme also shows the pH, temperature, ORP probes, the three connected pumps, and the gas recirculation pump.

5.2.2 Analytical methods

Standard methods were applied for sCOD, TSS, VSS, EPS, SMP, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$ analysis (APHA, 2012). VFAs were measured by treating filtered sludge samples used for sCOD analysis with dimethyl carbonate (DMC), according to Ghidotti et al. (2018). Briefly, SS samples were filtered using $0.45\ \mu\text{m}$ filters and mixed with 1 mL of DMC and 0.1 mL of a saturated potassium bisulfate (KHSO_4) solution. The mixture was then centrifuged at 1700 rcf for 10 minutes. An Agilent Technologies 7820A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a DB FFAA column ($30\ \text{m} \times 0.25\ \text{mm} \times 0.25\ \mu\text{m}$) was used to determine the VFA concentration (Montiel-Jarillo, 2021).

5.2.3 Prokaryotic Microbiota Structure Analysis

The prokaryotic microbiota structure of SS was analysed by metataxonomic analysis based on next-generation sequencing (NGS) of the 16S rDNA gene amplicons obtained from metagenomic DNA. In particular, four SS samples were analysed. T0-F1 and T0-F2 correspond to the starting SS of fermentation experiments 1 and 2, respectively (F1 and F2); TF-F1 and TF-F2 were the samples collected after fermentation of experiments 1 and 2, respectively. The analyses were performed on unfiltered and uncentrifuged samples. The procedures already described (Presti et al., 2021; Di Leto et al., 2024) were followed. The DNA extractions were evaluated by 1% (w/v) agarose gel electrophoresis analysis, adding $0.5\ \mu\text{g/mL}$ ethidium bromide for visualisation using a UV lamp. The concentrations of the DNA, extracted from 1 g of SS samples and the corresponding tenfold serial dilutions, were

measured by reading absorbance at 260 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extracted DNA was evaluated by measuring the absorbance ratios (260/280 and 260/230 nm) to indicate contamination due to proteins and organic compounds or chaotropic agents, respectively. PCR of the 16S rDNA gene was carried out with the parameters and primers described in Di Leto et al., 2024. Amplification products were sequenced in one 300 bp paired-end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). The raw 16S rDNA data were processed using the QIIME2 software (<https://qiime2.org/>) as paired-end sequences. The denoising approach processed overlapping paired-end reads with the plugin DADA2. Unique amplicon sequence variants (ASVs) were assigned and aligned to the Greengenes reference database at 99% sequence similarity (<https://greengenes.secondgenome.com/>). The number of ASVs and the percentages of relative abundances of phyla, orders, classes, and families were determined for each sample. In particular, 183804 high-quality reads filtered from the 257188 raw reads were obtained. HeatMap generation was conducted via the online web server (<http://heatmapper.ca/expression/>), employing a "complete linkage" calculation based on Pearson correlation and focusing on families. METAGENassist (<http://www.metagenassist.ca>), accessed on 5 March 2024, was used to distinguish the microbial species based on their metabolic activity.

5.3 Results and discussion

5.3.1 sCOD, nutrients, and VFA production

Two fermentation experiments were carried out at the Water Resource Recovery Facility at Palermo University in Italy using the SS collected in two different seasons (F1, winter and F2, spring) from the wastewater treatment line within the pilot plant, which adopts an oxic-settling-anaerobic (OSA) configuration. Fermentation 1 was filled up with 40% of the SS, leaving 60% of the headspace, while fermentation 2, with 60% of the SS, led 40% of the headspace. Samples were collected and analysed for chemical properties on the first day of the fermentation (T0), on the peak day of sCOD (TP) that was after 11 days, and on the final day (TF), after 20 days of fermentation (Table 5.1). Different headspace volumes provoked a different sCOD production being 4-fold higher sCOD production (615mg/L) in F2 than in F1 (146mg/L), although at the beginning of the fermentations (T0), the sCOD production was 2.6-fold higher in F1 (99mg/L) than in F2 (37mg/L). Previous studies (Zuo et al., 2020) state that sCOD and VFA generally assume the same trend after fermentation. In fact, in this work, during fermentation in F2, the increased sCOD value is associated with an increase in VFA yield. Ammonium was found 4, 13 and 11-fold higher in T0, TP, and TF of F2 than

F1, while orthophosphates were approximately the same in the two fermentations at T0 (6.1 mg P/L in F1 and 5.21 mg P/L in F2) and were found 1.18 and 1.13 -fold higher in TP and TF of F2 than F1. Typically, increased values of ammonium and orthophosphate in fermentation with increased VFA production are the result of accelerated degradation of proteins and other biomolecules containing nitrogen and phosphorus, likely to be due to microbial metabolic activity (Vijande et al., 2024). The two fermentations had a similar content of soluble proteins, and while carbohydrates proteins were found 1.16 and 1.37 -fold higher in T0 and TF of F2 than F1; carbohydrates were found 1.40 and 1.92 -fold higher in T0 and TF of F2 than F1. According to Mineo et al., 2023, these results suggest an effective higher protein hydrolysis during F2. In the TP, the VFA yield was 6.6-fold higher in the F2 experiment (197.76 mg COD/L) than in F1 (29.63 mg COD/L). Thus, headspace volume affected the fermentation process and the VFA rate.

Table 5.1. Features of the analysed SS samples in the T0 (starting day), TP (VFA peak day), and TF (final day): sCOD (soluble chemical oxygen demand), tCOD (total oxygen demand), chemical oxygen demand solubilisation, ammonium, orthophosphate, soluble proteins and carbohydrates, volatile fatty acid (VFA) and VFA/sCOD ratio.

Parameter	Unit	T0		TP		TF	
		F1	F2	F1	F2	F1	F2
sCOD	mg/L	99	37	146	615	133	475
tCOD	mg/L	7340	4801	-	-	5933	2948
COD solubilization	%	-	-	0.84	12.04	0.67	9.12
Ammonium	mg N/L	6.5	28.20	4.8	60.6	4.2	47.6
Orthophosphate	mg P/L	6.1	5.21	17.9	21.2	13.8	18.1
Soluble proteins	mg/g VSS	20.5	23.8	-	-	22.51	30.8
Soluble carbohydrates	mg/g VSS	5.13	7.19	-	-	6.3	12.1
VFA	mg COD/L	-	-	29.63	197.76	14.58	76.55
VFA/sCOD	%	-	-	20.29	32.16	10.96	16.12

5.3.2 Structure of Prokaryotic Communities of SS at the phylum level

All ASVs detected in this work were assigned to the Bacteria domain. Several earlier studies on sludge yielded comparable outcomes, showing that bacteria constituted nearly the entire prokaryotic population in SS (98.8%), with Archaea accounting for only 1.2% (Yu et al., 2012; Zhang et al., 2012; Xia et al., 2010; McLellan et al., 2010; Mathur et al., 2023). Bacteria are well-adapted to the sewage sludge environments, as many species can survive in aerobic and anaerobic conditions (e.g., through anaerobic respiration or fermentation). In contrast, Archaea are more commonly found in extreme environments, such as strictly anaerobic, very hot, acidic, or saline conditions, like swamps, deep ocean floors, or hot springs (Reed et al., 2013). Specifically, the microbiota structure of the SS samples from F1 and F2 was comparatively analysed considering the relative abundances at the phyla, family, and genus levels. Our analysis demonstrated the presence of a core microbial community and a small variable percentage, primarily considering the phyla level (Figure 5.2). These slight fluctuations in the composition of the starting SS sample can be justified considering that, although the starting SS samples were collected from the same Oxidic Settling Anaerobic reactor, the samplings were conducted approximately three months apart so the negligible differences can be attributable to the different environmental conditions and temperature, as previously indicated in other reports (Chen et al., 2017).

The Proteobacteria, Bacteroidetes, Firmicutes and Verrucomicrobia were identified as the core microbiota in the sludge starting samples (Figure 5.2), differently from other reports (Yu et al., 2012). In Yu et al., 2012, Actinobacteria were also part of the core microbiota in addition to these phyla. In another case, we detected Chloroflexi at a percentage of 5% (Di Leto et al., 2022) and Chlamydiae at a rate of 4.79% (Mineo et al., 2024), indicating the importance of the microbial analysis of the sewage sludge before the fermentation. The predominant components found in the sewage sludge samples at the beginning of fermentation processes (i.e. T0-F1 and T0-F2) belonged to the Proteobacteria, Bacteroidetes, Verrucomicrobia, Firmicutes, and Acidobacteria phyla.

Proteobacteria abundance was 52.11% and 48.64% in T0-F1 and T0-F2, Bacteroidetes 39.19% and 41.47%, respectively, Verrucomicrobia 3.34% and 3.59% respectively, and Firmicutes 1.59% and 0.81%. These four bacterial phyla are characteristic of the wastewater microbial community. Generally, Proteobacteria and Bacteroidetes are dominant phyla in wastewater treatment plant effluents (Do et al., 2019), and Verrucomicrobia phylum is predominant in soil. It can contribute to carbon cycling in terrestrial ecosystems (Nixon et

al., 2019). The Firmicutes, the Bacteroidetes, and Proteobacteria phyla constitute the faecal bacterial community of wastewaters (Scaccia et al., 2020).

In specific environmental and pH conditions, Proteobacteria, Bacteroidetes, and Firmicutes are known to be positively related to VFA production in reactors (Atasoy et al., 2022; Swiatkiewicz et al., 2021). High yields of VFA were analysed at a pH of 7.0 ± 0.3 (Swiatkiewicz et al., 2021), and in our study, the initial pH was 7.21 in F1 and 7.15 in F2 (Mineo et al., 2023). This suggests that Proteobacteria, Bacteroidetes, and Firmicutes had favourable conditions to produce VFA. Recent studies underlined the importance of the starting microbial community in influencing the VFA production mechanisms under precise fermentation conditions; it was also highlighted that the dominant phyla were Firmicutes, Bacteroidetes, and Proteobacteria. In particular, the samples yielding the maximum VFA during fermentation were initially composed of Proteobacteria, followed by Bacteroidetes and Firmicutes (Atasoy et al., 2019), as the starting samples of this study. As far as we know, the role of Verrucomicrobia in VFA production is unknown.

After fermentation, the most striking difference between the two experiments was the increase of Firmicutes from 0.81% in T0-F2 to 10.54% in TF-F2 compared to T0-F1 (1.59%) and TF-F1 (0.46%).

After the fermentation, it was possible to appreciate an increase of different phyla in the two conditions. Bacteroidetes (from 39.19% to 41.65%), Verrucomicrobia (from 3.34% to 4.36%), and Acidobacteria (from 0.75% to 2.26%) increased in TF-F1, whilst Proteobacteria (from 48.64% to 59.19%), and Actinobacteria (from 0.29% to 1.97 %) increased in TF- F2. Considering that condition F1 led to a VFA yield of 29.63 mg COD/L with acidogenic fermentation and condition F2 to a higher yield, equal to 197.76 mg COD/L, we can surmise that condition F2 selected VFA-producing microorganisms. In a previous work (Lim et al., 2020) that aimed to optimise the production of VFA from wastewater, the relevant involvement of Proteobacteria, Firmicutes, and Actinobacteria was observed. In particular, Firmicutes and Actinobacteria were found to be anaerobic microorganisms that tolerate the acidic conditions of fermentation to produce VFA (Lim et al., 2020). The mentioned groups can produce VFA as a product of their biochemical activity. Firmicutes are related to transforming simple sugars into organic acids, and Actinobacteria can produce propionic acid (Dworkin et al., 2006). It has been found (Qin et al., 2021) that Firmicutes and Actinobacteria phyla dominate in the anaerobic digestion of SS or, in any case, at low oxygen

concentrations. Therefore, the 40% headspace, where less oxygen is available, allowed for better adaptation and selection of VFA-producing bacteria.

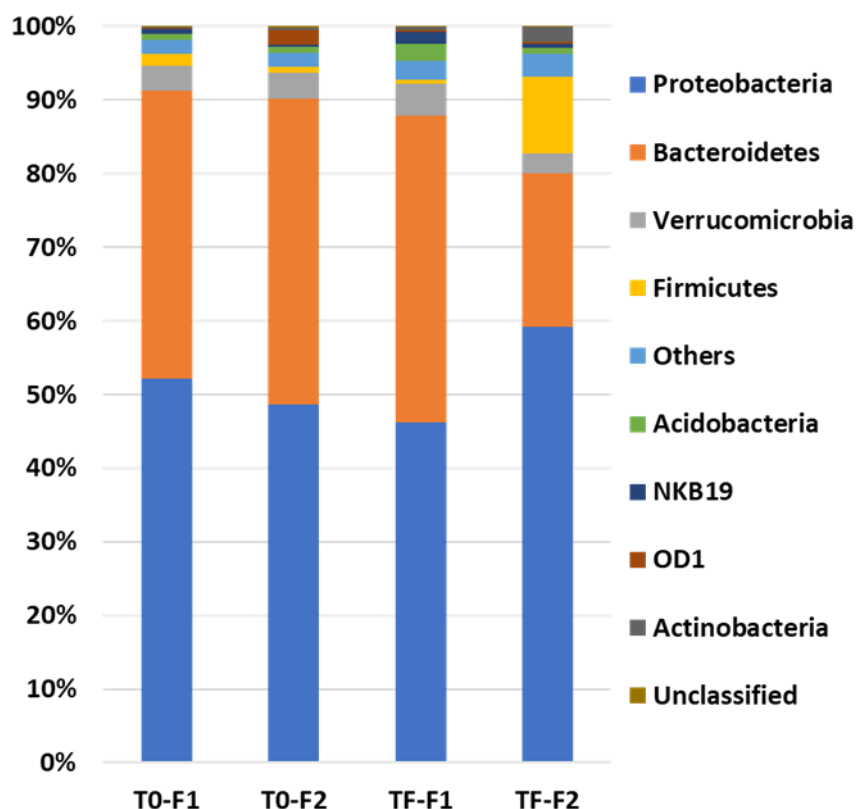


Figure 5.2. Histograms reporting the composition of samples T0 and TF at the taxonomic level of phyla (percentage abundance greater than 1%). The relative abundance indicated the percentage of each phylum compared to all the identified phyla.

5.3.3 Taxonomic composition of Proteobacteria at family level

The microbial composition within their respective phyla was studied at the taxonomic family level. Figure 5.3 illustrates the microbial composition of Proteobacteria, highlighting the predominant families. In samples T0-F1 and T0-F2, the most prevalent bacterial families were Comamonadaceae (26.78% and 21.10%, respectively), Rhodocyclaceae (18.17% and 14.96%, respectively), Rhodobacteraceae (8.97% and 7.03%, respectively), and Xanthomonadaceae (11.63% and 10.43%, respectively). Additionally, T0-F1 exhibited a notable abundance of Moraxellaceae (12.31%) compared to T0-F2 (5.83%), whereas T0-F2 showed notable abundance of Beijerinckiaceae (12.00%) absent in T0-F1. Notably, the Comamonadaceae family, known for its ability to degrade VFAs for synthesising polyhydroxyalkanoates, was prominent in both conditions (Strazzera et al., 2021). However,

their abundance was approximately constant after fermentation in F1 (26.78% in T0, and 24.42% in TF), while it dropped from 21.10% to 10.78% in F2. In a previous work that aimed to improve VFA production in batch tests, we found that Comamonadaceae abundance decreased in the sample with the best yield of VFA (Mineo et al., 2024). The reduction of Comamonadaceae, acting as VFA degraders, can explain the enhanced VFA yield in TF-F2. The Rhodocyclaceae family showed the same behaviour: constant in F1 (from 18.17% in T0 and 17.43% in TF) and mainly in TF-F2 (dropping from 14.96% of T0 to zero of TF). Generally, Rhodocyclaceae, being facultative anaerobes, can grow both in the presence and absence of oxygen. However, the greater availability of oxygen, such as in F1, where the headspace volume is higher (60%), favoured the growth of Rhodocyclaceae, compared to F2 (40% headspace). In mixed microbial communities, the response of Rhodocyclaceae to oxygen fluctuations can be complex and influenced by interactions with other microorganisms (Shi et al., 2021).

In TF-F1 and TF-F2, there was a notable increase in the Gram-negative acidotolerant family Rhodobacteraceae, increasing from 8.97% in T0-F1 to 13.42% in TF-F1 and from 7.03% in T0-F2 to 15.84% in TF-F2. Although their presence has been registered in other studies on waste fermentation for VFA production (Mirmohamadsadeghi et al., 2021), their role could have been better investigated. Also, the abundance of Xanthomonadaceae increased in both experiments: from 11.63% to 18.44% (T0-F1 to TF-F1), and from 10.43% to 16.82% (T0-F2 to TF-F2). This bacterial family was characterised by a metabolism associated with facultative anaerobic fermentation. A distinguishing factor between F1 and F2 is undoubtedly the presence of the Beijerinckiaceae family. In T0-F1 and TF-F1, this family was absent, whereas it constituted 12.00% of T0-F2 and significantly increased to 38.35% in TF-F2. The Beijerinckiaceae family is comprised of mesophilic bacteria with optimum growth at 20-40°C. Their absence in sample T0-F1, collected in winter, might be attributed to the colder winter temperatures compared to the mild conditions during the spring when sample T0-F2 was taken. While limited research confirms VFA production by Beijerinckiaceae, some family members have been identified in fermenters where VFA production occurred (Khan et al., 2020). This suggests they might have the required enzymatic machinery for fermentative metabolism and could generate VFAs or other organic acids as metabolic byproducts when degrading organic substrates. Finally, the abundance of Moraxellaceae decreased notably in both experiments during fermentation, dropping from 12.31% in T0-F1 to 1.85% in TF-F1, and from 5.83% in T0-F2 to 1.03% in TF-F2. The Moraxellaceae family was found to be related to less production of some VFAs, such as

propionate or butyrate (Miceli et al., 2016). Taking into account all these results and the role of bacteria in VFA biosynthesis or degradation, this study suggests that a microbial community characterised by the increase of the Beijerinckiaceae family and the decrease of the Comamonadaceae family can yield a higher VFA production, as we found in F2. However, more studies on the bacterial strains are necessary to investigate and exploit their role in VFA production from wastewater.

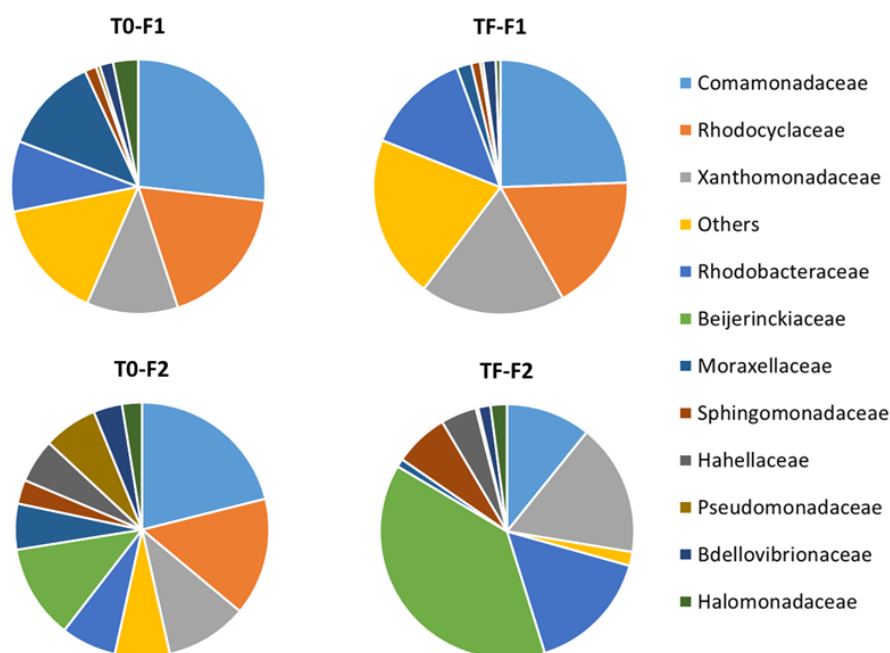


Figure 5.3. Pie charts reporting the microbiota structure of samples T0 and TF at the taxonomic level of the family belonging to Proteobacteria phyla. T0-F1 and T0-F2 represent sewage sludge microbiota structure at the beginning of the fermentation process in F1 and F2 conditions, respectively; TF-F1 and TF-F2 represent the sewage sludge microbiota structure after fermentation processes. The relative abundance indicated the percentage of each family compared to all the identified families belonging to the Proteobacteria phylum.

5.3.4 Taxonomic composition of Bacteroidetes at the family level

The predominant families within the Bacteroidetes phylum were Saprospiraceae and Chitinophagaceae, followed by Flavobacteriaceae and Cytophagaceae (Figure 5.4). Notably, the Cryomorphaceae family is more abundant in T0-F1 and the BA008 family in T0-F2. Saprospiraceae and Chitinophagaceae showed an increase in F1, increasing from 28.37% in T0-F1 to 32.73% in TF-F1 and from 29.36% in T0-F1 to 38.07% in TF-F1, respectively. In contrast, they decreased in F2 from 60.53% in T0-F2 to 56.30% in TF-F2 and were similar

in F2 (8.88% in T0 and 8.52% in F2). Flavobacteriaceae decreased in F1 (from 17.96% in T0-F1 to 14.22% in TF-F1) and F2 (from 9.68% in T0-F2 to 4.06% in TF-F2). Cytophagaceae declined in F1, dropping from 12.30% in T0-F1 to 8.17% in TF-F1, while they increased from 10.49% in T0-F2 to 26.01% in TF-F2. The BA008 family was absent in the initial and final samples of F1. In contrast, they were present in T0-F2 at 6.67% and slightly decreased in TF-F2 to 4.30%. Previous works determined a more significant percentage of Saprospiraceae and Chitinophagaceae in treatments with lower VFA yield (Di Leto et al., 2022). Arguably, members of the Saprospiraceae could significantly break down carbon sources, leading to beneficial carbon substrates for other microorganisms (Johnston et al., 2019). The Saprospiraceae family is commonly found in flocs and aggregates of activated sludge. However, their exact role in wastewater treatment must be better understood (Guo et al., 2016; Guo et al., 2017).

Flavobacteriaceae are known primarily as aerobic, Gram-negative bacteria. Although some members of the Flavobacteriaceae family can participate in organic matter degradation, they are not specifically VFA producers, and a decrease in both F1 and F2 samples was registered. The capacity of Cytophagaceae to produce VFA needs to be better understood; however, in our previous studies focused on optimising VFA yield from wastewater, we found Cytophagaceae to be associated with good VFA production (Mineo et al., 2024). Although little information is available on BA008, the only strain isolated so far belonging to this family has an anaerobic fermentative metabolism and could contribute to the production of butyrate (Sun et al. 2016). Altogether, this analysis suggests the main role of Cytophagaceae in VFA production in the tested conditions.

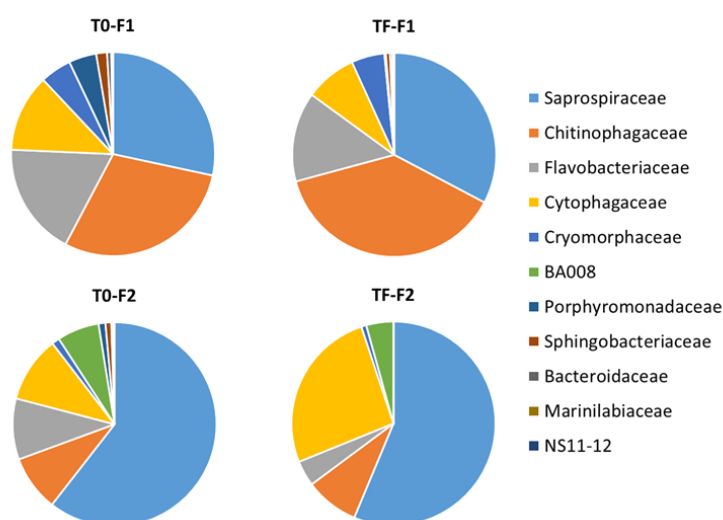


Figure 5.4. Pie charts report the microbiota structure of samples T0 and TF at the taxonomic level of the family belonging to the Bacteroidetes phylum. T0-F1 and T0-F2 represent

sewage sludge microbiota structure at the beginning of the fermentation process in F1 and F2 conditions, respectively; TF-F1 and TF-F2 represent the sewage sludge microbiota structure after fermentation processes. The relative abundance indicated the percentage of each family compared to all identified families belonging to the Bacteroidetes phylum.

5.3.5 Taxonomic composition of Verrucomicrobia, Firmicutes and Actinobacteria at family level

The most represented bacterial family for Verrucomicrobia was Verrucomicrobiaceae, which was equally abundant in both T0-F1 and T0-F2 (79.2%). However, during the fermentation process, the abundances shifted in opposite directions: in TF-F1, the abundance slightly decreased to 70.2%, whereas in TF-F2, it increased to 100% (Figure 5.5). Recent studies validated the role of Verrucomicrobiaceae in VFA production from xylan, confirming the fermentative capabilities of this microbial family (Takizawa et al., 2021).

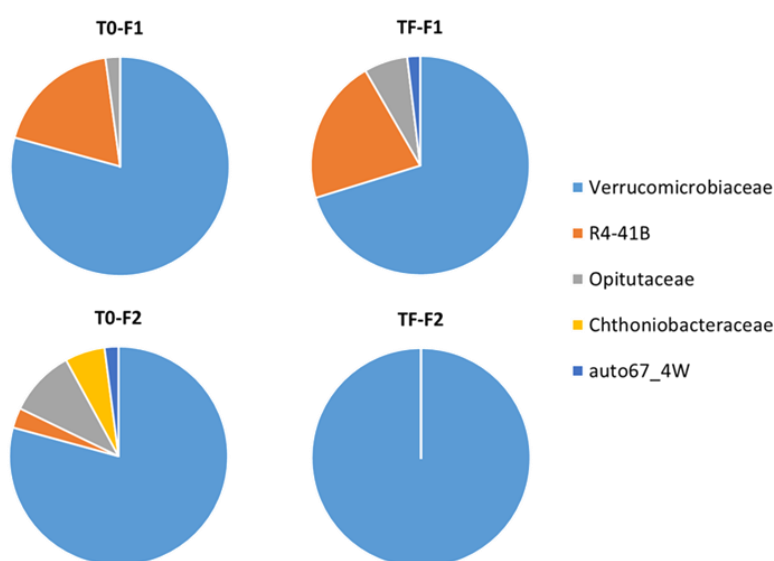


Figure 5.5. Pie charts reporting the taxonomic composition of samples T0 and TF at the taxonomic level of the family belonging to Verrucomicrobia phylum. T0-F1 and T0-F2 represent sewage sludge microbiota structure at the beginning of the fermentation process in F1 and F2 conditions, respectively; TF-F1 and TF-F2 represent the sewage sludge microbiota structure after fermentation processes. The relative abundance indicated the percentage of each family compared to all the identified families belonging to the Verrucomicrobia phylum.

In our previous studies, Firmicutes were the bacterial phylum most implicated in VFA production (Mineo et al., 2024 Di Leto et al., 2022). Carnobacteriaceae and Christensenellaceae mainly represented the Firmicutes phylum (Figure 5.6). Carnobacteriaceae increased from 46.53% in T0-F1 to 57.28% in TF-F1, absent in T0-F2 and TF-F2. So far, their role has yet to be deeply investigated. Christensenellaceae family was present at 21.15% in T0-F1 and reached 36.89% in TF-F1 while it started from 60.95% in T0-F2 and reached 73.04% in TF-F2. While the literature does not recognise the role of Christensenellaceae in wastewater, this bacterial family, found in the intestinal microbiota, is known as an excellent producer of acetate and, consequently, an excellent producer of VFA (Mackei et al., 2022). Other Firmicutes found were Ruminococcaceae and Acidaminobacteraceae, which decreased at fermentation.

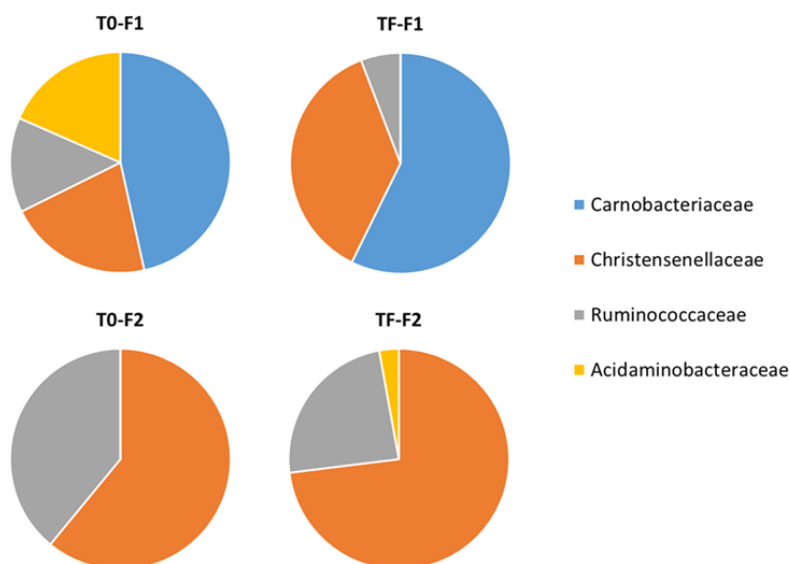


Figure 5.6. Pie charts reporting the taxonomic composition of samples T0 and TF at the taxonomic level of family belonging to Firmicutes phylum. T0-F1 and T0-F2 represent sewage sludge microbiota structure at the beginning of the fermentation process in F1 and F2 condition, respectively; TF-F1 and TF-F2 represent the sewage sludge microbiota structure after fermentation processes. The relative abundance indicated the percentage of each family compared to all the identified family belonging to Firmicutes phylum.

Finally, the most representative family belonging to the Actinobacteria phylum were Intrasporangiaceae, absent in F1 but 40.26% in T0-F2 and increased to 85.82% in TF-F2 (Figure 5.7). Intrasporangiaceae are involved in the biological removal of phosphates from wastewater and bioremediation processes. Some species are fermentative so they may be

involved in the decomposition of organic matter and potentially contribute to the production of VFA (Stackebrandt et al., 2014). The only family detected in both T0 and TF samples of F1 was C111, for which no information available was found.

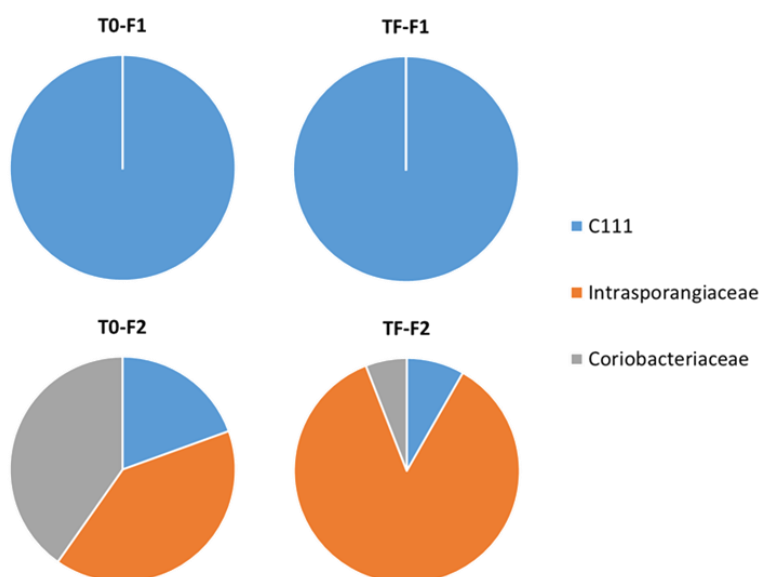


Figure 5.7. Pie charts reporting the taxonomic composition of samples T0 and TF at the taxonomic level of family belonging to Actinobacteria phylum. T0-F1 and T0-F2 represent sewage sludge microbiota structure at the beginning of the fermentation process in F1 and F2 condition, respectively; TF-F1 and TF-F2 represent the sewage sludge microbiota structure after fermentation processes. The relative abundance indicated the percentage of each family compared to all identified families belonging to the Actinobacteria phylum.

5.3.6 Predicting Microbial Metabolism and oxygen requirement

Understanding the metabolic functions of the microbiota of each sample can help discover how the headspace in the fermentation process favoured or penalised VFA production. As shown in Figure 5.8, the metabolic activities mainly represented were dehalogenation and ammonia oxidation, followed by sulfate and nitrite-reducing capabilities. Specifically, there was a high percentage of nitrite reducers in the TF-F2 sample, which gave the highest VFA yield (13.97%). In the T0-F1 and TF-F1 samples, there was a lower abundance of nitrite-reducers (6.91% and 7.13% respectively) and sulfate-reducers (13.32% and 12.34% respectively). Dehalogenating bacteria increased in TF-F2 compared to T0-F2 but remained at a lower percentage than T0-F1 and TF-F1 (18.80% and 18.83%, respectively). Sulfate-reducing bacteria can oxidize organic matter into acetate and carbon dioxide (Bertolino et

al., 2012). Some VFAs such as acetate, propionate, and butyrate are among the most relevant electron donors to sulfate-reducing bacteria. Indeed, sulfate-reducing bacteria can also use propionate and butyrate to synthesise acetate, which they then use as a donor for sulfate reduction (Grigoryan et al., 2008). This work detected acetate during day 8 of fermentation, and no propionic acid was measured (Mineo et al., 2023), demonstrating acetate production by sulfate-reducing bacteria. After the peak at day 11, methanogenic activity led to acetate consumption and CO₂ production operated by sulfate-reducing bacteria. VFA are also able to stimulate the biotransformation of nitrogen (Oguz et al., 2006) through the denitrification of nitric nitrogen to gaseous nitrogen, and this justifies the presence of metabolic activity of nitrite reducer marked in the TF-F2 sample in which the VFA concentration is higher. As regards dehalogenation, the relationship between VFA production and dehalogenation is not well known. What is known is that VFAs act as electron donors for the dechlorination of chloroethene processes (Lu et al., 2006). The fact that dehalogenating bacteria were lower in TF-F2 and higher in TF-F1 can justify the different yields of VFA. The produced VFA were probably used as electron donors by dehalogenating bacteria in greater abundance in TF-F1 (reducing VFA yields) compared to TF-F2, where there was a lower concentration of these bacteria. Therefore, it can be assumed that the increase in TF-F2 of the sulphate-reducers bacteria favours the rise of VFA, particularly acetate, on peak days. Instead, the increase of nitrite reducers bacteria is favoured by the presence of VFAs. The difference between sulfate-reducing and dehalogenating bacteria is that the first can produce VFA and then use it as an electron donor. At the same time, the second directly degrades the VFA produced by other bacteria as electron donors, reducing VFA yields.

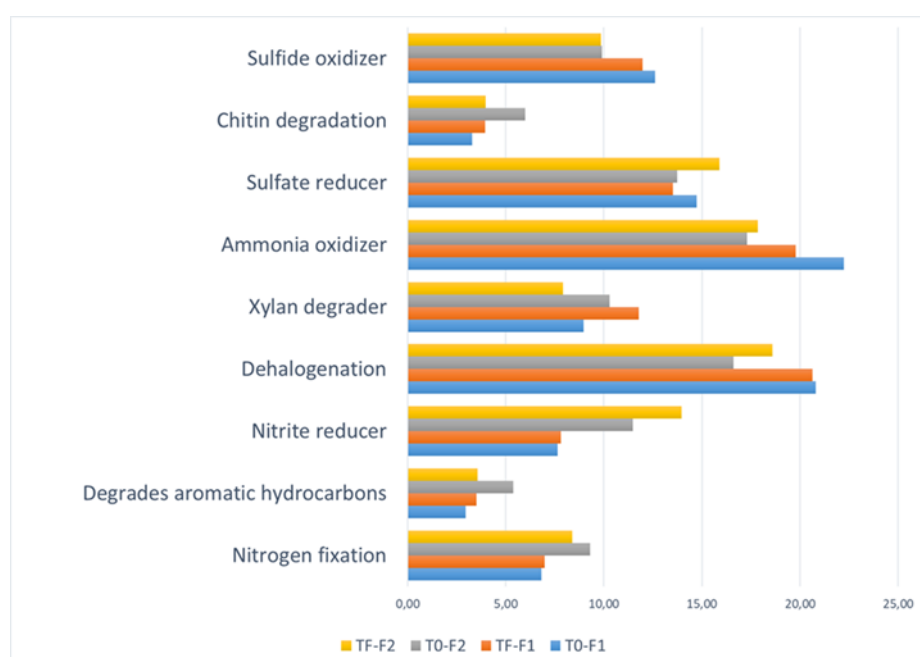


Figure 5.8. Putative metabolic requirements of the bacterial microbiota members residing in the samples T0-F1, TF-F1, T0-F2, TF-F2 as inferred by METAGENassist bioinformatic analysis. The abundance percentage of bacteria having a specific metabolism is indicated.

Considering the oxygen requirements (Figure 5.9) of the microbiota of the four analysed samples, variations were dependent on fermentation and the percentage of the headspace of each experiment. In the F1 experiment, at 60% headspace, there was a higher starting oxygen availability; in the F2 fermentation experiment, at 40% headspace, there was a lower starting oxygen availability. During the fermentation process, the starting oxygen rate was further reduced, causing an alteration in the aerobic/anaerobic microbiota ratio. From the beginning of fermentation onwards, there was a reduction in oxygen and an increase in CO₂ (Mineo et al., 2023). Condition F2 promoted anaerobic bacteria's growth and aerobic ones' reduction, thus establishing a VFA-producing factory characterised by acidogenic fermentation when the headspace was 40%.

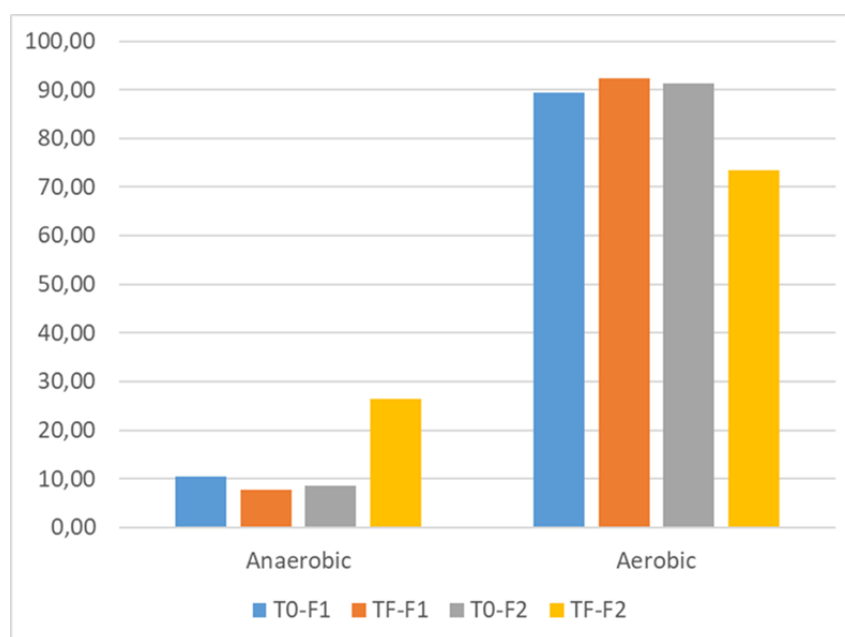


Figure 5.9. Oxygen requirements of the bacterial microbiota of T0-F1, TF-F1, T0-F2, TF-F2 samples. The abundance percentage of bacteria having a specific metabolism is indicated.

5.3.7 Structure of Prokaryotic Communities of SS at genera level

Heatmap analysis showed that a 60% headspace volume did not greatly influence the microbiota structure, as the two samples clustered together. Conversely, a 40% headspace

volume caused a great variation in the microbial community, as the two samples clustered in two different clades, and led to a higher VFA yield. This result underscores the impact of the headspace volume on the microbial fermentation carried out by bacteria in the sewage sludge.

The most representative genera revealed by the metataxonomic analysis are reported in Figure 5.10. Despite the high number of unclassified, *Rhodobacter* and *Thermomonas* were the most abundant genera in TF-F2 (reaching 4.74 and 4.05%, respectively). *Rhodobacter* was also abundant in TF-F1 (4.56%), while *Thermomonas* abundance was detected at a very low level in TF-F1 (1.29%). Both genera were less represented in the starting sewage sludge. Contrarily, *Flavobacterium* and *Dechloromonas* were close to zero in TF-F2.

Rhodobacter, *Thermomonas* and *Dechloromonas* belong to the Proteobacteria phylum and *Flavobacterium* to Bacteroidetes. It is noteworthy that different headspace volumes selected some bacterial genera that increased or decreased in abundance. In TF-F2, where the initial oxygen availability was lower (40% headspace), more anaerobic genera, such as *Rhodobacter* and *Thermomonas* increased. *Rhodobacter* is known for its ability to degrade VFAs and produce H₂ (Hu et al., 2020). *Thermomonas* is positively correlated with the acidogenic fermentation of sewage sludge and the production of VFA (Kim et al., 2010).

Although *Rhodobacter* and *Thermomonas*, known as VFA degraders, increased in TF-F2, the evidence that a better yield of VFA was obtained in TF-F2 could be due to pH. Indeed, Hu et al. (2020) demonstrated that the composition of VFAs produced by the mixed microbial community and a pH between 5.8 and 7.1 can determine the inhibition of the degradation effect of *Rhodobacter*. Given that the initial pH in F2 was 7.15 (Mineo et al., 2023), likely that *Rhodobacter* did not break down the VFAs produced by other bacterial genera. The other explanation could be the presence of VFA producers among the unclassified bacteria.

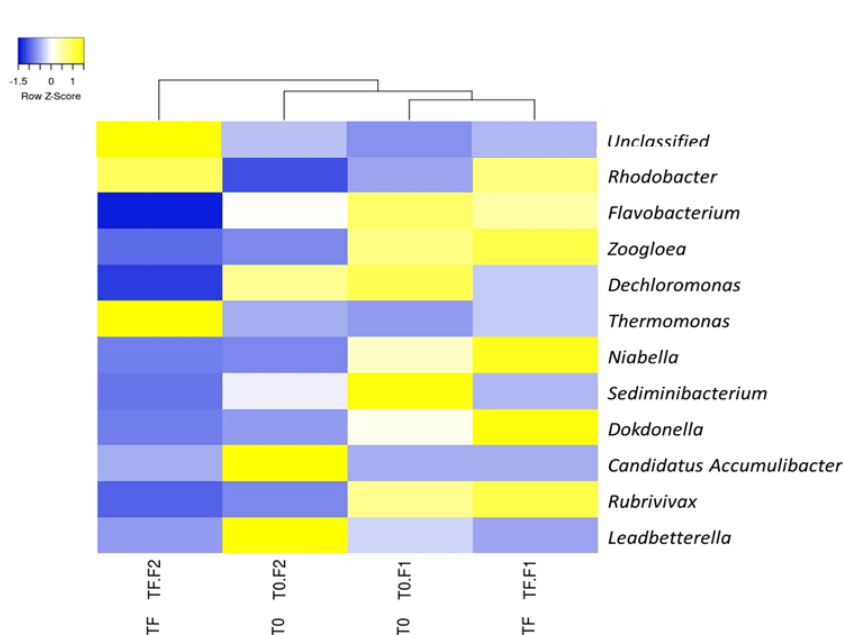


Figure 5.10. Heatmap of genus composition profiles. The colour intensity in each sample was normalised to represent its relative ratio in the two groups. A range of colours, from blue to yellow, indicated the relative values of microbiota.

5.4 Conclusions

SS microbiota subjected to acidogenic fermentation in a pilot plant reactor by modulating the headspace of the reactor was analysed to maximise VFA yield. Two different headspace conditions were evaluated: 60% and 40%. The best VFA yield was obtained after eleven days of fermentation at 40% headspace. Metataxonomic analyses revealed that the lower initial oxygen concentration (due to 40% of headspace) and acidic conditions (due to fermentation) favoured the selection of essentially Gram-negative, anaerobic, and good VFA-producing bacteria. At 60% headspace, there was greater initial oxygen availability, and a greater abundance of VFA-degrading microorganisms was identified. However, several bacterial families identified in this research as potential significant VFA producers were not previously explored for their metabolic capabilities in wastewater or other organic-rich environments. Consequently, this study should stimulate in-depth investigations into these bacteria and their role in acidogenic fermentation and VFA production, aiming towards biotechnological and industrial applications in the future. This work also demonstrates how the wastewater of Unipa Campus can be used, according to the idea of circular economy, to produce valuable material to improve bioplastic production and their precursor, reducing waste disposal and environmental impact. Thus, by optimizing the production of VFA to be used in the PHA production process, a transition from linear wastewater treatment to circular biorefineries can be promoted at the campus level. In addition, from a circular economy

perspective, the purified wastewater from the Unipa Campus could be repurposed to irrigate green spaces and clean campus streets.

5.5 References

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CHAPTER 6

The role of organic loading rate on sewage sludge microbiome for the production of polyhydroxyalkanoates in wastewater treatment plants

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ABSTRACT

The conversion of conventional wastewater treatment plants into biorefineries is an ecologically and economically sustainable methodology for the extraction of valuable compounds from waste. Microbial consortia within the sewage sludge facilitate the fermentation of organic contaminants, producing polyhydroxyalkanoates (PHA), precursors of bioplastics, to be used as alternatives to petroleum-based plastics. Enhancing volatile fatty acids (VFA) production during the anaerobic fermentation of sewage sludge necessitates a comprehensive understanding of the operational microbial community and its metabolic capabilities under anaerobic conditions. Understanding the impact of the operational parameters in PHA production is crucial to optimising the process and obtaining insights regarding the microbial role. In this study, different volumetric organic loading rates (vOLR) were applied in the enrichment reactor of a pilot plant scheme aimed at producing PHA from microbes present in the sewage sludge. Metataxonomic analysis revealed microbiota structure changes. A vOLR of $1.3 \text{ g COD L}^{-1} \text{ d}^{-1}$ determined an increase of PHA and producer bacteria, such as Proteobacteria.

6.1 Introduction

One of the most serious environmental problems is pollution from petroleum-derived plastics, compromising soil productivity, water quality, and ecosystems (Galitskaya et al., 2021). In recent decades the world has produced as much plastic as in the previous half-century, derived mainly from crude oil, used as chemical raw materials and a fuel source, causing health problems for humans, animals, and the environment (Rhodes, 2018). Plastic in the environment can be fragmented into smaller microplastics, enhancing its ability to absorb and concentrate organic pollutants. The direct human ingestion of microplastics through drinking water is a real possibility; even food can be contaminated by microplastics in the air. It is also surprising that microplastics have been detected in the soil, most likely transported by the wind. For these reasons, today, there is a growing emphasis on replacing petrochemical-derived plastic with bioplastics. Among the promising materials are thermoplastic biodegradable polyesters known as polyhydroxyalkanoates (PHAs). PHAs have recently gained attention as innovative biopolymers and are considered renewable alternatives to traditional petrochemical polymers. PHAs are natural polyesters that microorganisms accumulate as insoluble granules under specific environmental conditions. When bacteria starve from a readily available carbon source but still have access to other

essential nutrients such as nitrogen and phosphorus, they can accumulate PHAs as intracellular carbon storage compounds. This survival mechanism allows bacteria to store excess carbon for future energy use when primary carbon sources become scarce (Kovalcik et al., 2019). Volatile fatty acids (VFA) can be excellent precursors to feed the production of granules of PHAs that can reach up to 90% of the dry weight of the microbial mass (Shahid et al., 2021). The VFA conversion process into PHA typically is facilitated under conditions where other nutrients, such as nitrogen and phosphorus, are provided in sufficient amounts to support bacterial growth and metabolism. VFAs are intermediate products in the anaerobic digestion process of organic waste. The microorganisms, both in mixed microbial culture (MMC) and in axenic culture, are capable of producing VFA, such as acetic, propionic, butyric, and valeric acids. The different VFA production yields can be determined by the starting substrate type and the fermentation conditions (Di Leto et al., 2022; Mineo et al., 2024). Since the current production costs of PHA are not competitive with those of petroleum-based plastics due to the high costs of the starting organic material (Al Battashi et al., 2021), the current challenge is the use of raw material, such as wastewater for the production of biopolymers. The sewage sludge (SS) produced in WWTPs could become a resource rather than a waste, mainly because of the high value-added chemicals that can be recovered from it. Nowadays, SS is typically disposed of in solid waste landfills or incinerators. Conventional WWTPs are crucial for controlling water body eutrophication but contribute to environmental pollution through greenhouse gas emissions (Hao et al., 2019) and SS accumulation. During the last ten years, a fundamental shift in wastewater treatment plants WWTPs has become imperative for promoting environmentally sustainable development. However, integrating PHA production into existing WWTP infrastructures presents challenges. Indeed, efficient PHA production involves the delicate balance of organic carbon and nutrient removal within the legal parameters of wastewater treatment. A profound comprehension of the operational parameters influencing PHA production by MMC is crucial for process optimisation. This understanding provides valuable insights into the role of microbiota in the PHA production process, enabling the development of strategies to enhance efficiency and adhere to regulatory requirements. While bacterial and archaeal species have a pivotal impact on PHA production, current research focuses on understanding how the operational conditions affect microbiota and PHA production. In this study, a sewage sludge MMC was fed with a synthetic mixture of VFAs and subjected to a feast-famine (F/F) cycle in a selective-sequencing batch reactor (S-SBR) to enhance PHA production. During the F/F cycle, microorganisms alternate between periods of plenty (feast) and scarcity (famine) of the carbon source. An ample carbon substrate is provided in the

feast phase, allowing the microorganisms to grow and accumulate PHAs as storage granules. In the famine phase, the carbon source is limited, restricting cell growth and forcing the microorganisms to use the stored PHAs as an energy source. Experiments were conducted at two different volumetric organic loading rates (vOLR). Metataxonomic analysis of SS samples was performed before and after the enrichment selection process to understand the relationships between operational conditions, microbiomes, and the achievement of specific outcomes.

6.2 Material and Methods

6.2.1 Experimental Setup and analytical methods

The pilot plant used in this work is located at the pilot plant hall of the Water Resource Recovery Facility of the Palermo University Campus and collects wastewater from several buildings (Mannina et al., 2021a; b). The strategy adopted for selecting the biomass was aerobic dynamic feeding, which was used to compare two different vOLRs. A synthetic mixture of VFA (Tan et al., 2014) composed of 70% acetic acid and 30% propionic acid (Sigma-Aldrich) was used as a carbon source in the selective-sequencing batch reactor (S-SBR) to favour the growth of the PHA-storing organisms. The selected biomass was then used to increase the amount of PHA produced, as Isern-Cazorla et al. (2023) reported. Two different vOLRs were applied in the selection phase, 1.3 g COD L⁻¹ d⁻¹ for S-SBR A and 0.8 g COD L⁻¹ d⁻¹ for S-SBR B, respectively under a feast-famine regime (Mineo et al., 2023; Isern-Cazorla 2023). After about two months for each experiment (Isern-Cazorla et al., 2023), produced PHAs were extracted following the procedures reported by Mannina et al. (2019). The total concentration of PHAs was determined by summing the concentrations of poly(3-hydroxy) butyrate (PHB) and poly(3-hydroxy) valerate (PHV) monomers within the freeze-dried biomass. Following the procedure reported by Mineo et al. (2023), lyophilised samples were treated with butanol and hydrochloric acid and incubated at 100°C for 8–20 hours. Subsequently, hexane and MilliQ Grade water were added. Adding water and hexane effectively isolates PHAs from biomass, allowing their subsequent quantitative and qualitative analysis (Werker et al., 2008). After centrifugation (350 RCF, 10 min), the organic phase was extracted, filtered, and collected into gas chromatography (GC) vials. Sample GC analysis was conducted according to the procedure outlined by Montiel-Jarillo et al. (2017).

6.2.2 Prokaryotic Microbiota Structure Analysis

The prokaryotic microbiota structure in the SS was analysed through metataxonomic analysis using next-generation sequencing (NGS) of 16S rDNA gene amplicons derived from metagenomic DNA. Specifically, four SS samples were examined before and after the selection phase. T0-A and T0-B represent the initial sewage sludge used for the two experiments, A and B, respectively. At the same time, TF-A and TF-B are samples collected post-PHA production in the selection phase, subjected to a fast/famine (F/F) regime from experiments A and B, respectively. The analyses were conducted on unfiltered and uncentrifuged samples following established protocols (Presti et al., 2021; Di Leto et al., 2024). DNA extractions were verified through 1% (w/v) agarose gel electrophoresis, with ethidium bromide (0.5 µg/mL) aiding visualisation under UV light. DNA concentrations extracted from 1 g of SS samples and corresponding tenfold serial dilutions were measured by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) absorbance readings at 260 nm. Purity assessments of extracted DNA were determined by absorbance ratios (260/280 and 260/230 nm) to individuate contamination due to protein, organic compound, or chaotropic agent contamination. PCR targeting the 16S rDNA gene utilised parameters and primers detailed in Di Leto et al., 2024. Amplification products underwent sequencing via one 300 bp paired end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). Raw 16S rDNA data were processed using QIIME2 software (<https://qiime2.org/>) as paired-end sequences. DADA2 plugin was employed for denoising and processing overlapping paired end reads. Unique amplicon sequence variants (ASVs) were assigned and aligned to the Greengenes reference database at 99% sequence similarity (<https://greengenes.secondgenome.com/>). ASV numbers and relative abundances of phyla, orders, classes, and families were determined for each sample. HeatMap generation utilised the online web server (<http://heatmapper.ca/expression/>) with a "complete linkage" calculation based on Pearson correlation, focusing on families. METAGENassist (<http://www.metagenassist.ca>) distinguished microbial species based on metabolic activity.

6.3 Results and discussion

6.3.1 PHAs production

In this study, the vOLRs applied (1.3 and 0.8 g COD L⁻¹ d⁻¹) led to peak productivity values associated with the PHA biomass content of 20% (A) and 18% (B), respectively. Table 6.1 shows the F/F ratio (min/min) and the amount of PHA produced. The vOLR assumes a

pivotal role, significantly impacting both the peak productivity of PHAs and the ultimate PHA content within the biomass, as outlined by Mineo et al. (2023). In experiment, A, in which vOLR was 1.3 g COD L⁻¹ day⁻¹, the F/F ratio reached 0.16 min/min, while in experiment B (vOLR 0.8 g COD L⁻¹ day⁻¹), the F/F ratio was 0.23 min/min. Therefore, the vOLR and F/F ratio have an inversely proportional relationship. An increase in colour is associated with a decreased F/F ratio. This represents a more rapid consumption of available organic material by microorganisms. Conversely, a decrease in the vOLR results in less organic material leading to an increase in the F/F ratio as there is relatively more food available to microorganisms. Furthermore, the vOLR increase and the F/F ratio decrease led to a higher yield of PHAs (20% w/w in experiment A) compared to experiment B (18% w/w in experiment B) when the vOLR decreased and the F/F ratio increased. These results indicate that vOLR is a decisive factor in the selection step in enhancing the PHA production and storage ability of microorganisms and the productivity of microbial biomass and the maximum storage capacity of PHA increase with the vOLR, as previously reported (de Oliveira et al., 2019).

Table 6.1. Feast-famine ratio and amount of PHA produced in the selection reactors.

Experiment	vOLR	F/F ratio	PHA biomass contents
	g COD L ⁻¹ day ⁻¹	(min/min)	(% w/w)
A	1.3	0.16 ± 0.05	20
B	0.8	0.23 ± 0.11	18

6.3.2 Structure of Prokaryotic Communities of sewage sludge at phylum level

Bacterial abundance in the sewage sludge was determined by performing NGS analysis of 16S rDNA gene amplicons obtained from the metagenomic DNA using the SSof two PHA production experiments at the pilot plant different only for the vORL. All ASVs detected in this work have been assigned to the Bacteria domain (100%) and no microorganisms belonging to the Archaea domain have been identified. Several previous studies on SS produced similar results, indicating that bacteria made up nearly the entire prokaryotic population in SS (98.8%), while Archaea represented only 1.2% (Yu et al., 2012; Zhang et al., 2012; Xia et al., 2010; McLellan et al., 2010; Mathur et al., 2023). Our previous studies focused on metataxonomic analyses of SSexploited for VFA production, but we did not detect Archaea but only Bacteria (Di Leto et al., 2024). For each experiment, two samples were collected and analysed, the starting samples, T0-A and T0-B, and the final samples, TF-A and TF-B, collected two months apart. Although the samples were collected in two different seasons (winter and spring, respectively), and despite the variations in temperature

conditions, the T0 samples exhibited a similar microbial composition. The most represented phyla in both experiments were Proteobacteria, Bacteroidetes followed by Verrucomicrobia. These three bacterial phyla constitute the core community of SS, consisting of microbial species always found, regardless of seasonal, geographical, or environmental changes. On the other hand, each SS features variable microbial components. The existence of both a core and a variable community is crucial for the resilience and efficiency of SS treatment processes. The core community ensures the continuity of essential functions, while the variable community provides an adaptive response to variations in treatment conditions, as suggested by Saunders et al. 2016. In previous studies, such as Yu and Zhang (2020) and Scaccia et al. (2020), it has been confirmed that the core community of SS typically includes Proteobacteria, Bacteroidetes, and Verrucomicrobia, as well as Firmicutes and Actinobacteria. In our study, Proteobacteria, Bacteroidetes, and Verrucomicrobia were found at 54.52%, 37.36%, and 4.52% in T0-A, and at 48.64%, 41.47%, and 3.59% in T0-B, respectively (Figure 6.1). Firmicutes and Actinobacteria were present in low percentages, at 0.42% and 0.01% in T0-A, and 0.81% and 0.29% in T0-B. Compared to our previous studies, the microbial community consistently includes a core of Proteobacteria and Bacteroidetes, with some variations: in Di Leto et al. (2022), the initial sewage sludges also featured Firmicutes and Chloroflexi; in Mineo et al. (2024), Chlamydiae were also present; in Di Leto et al. (2024 a-b), along with Proteobacteria and Bacteroidetes, Firmicutes and Verrucomicrobia were also identified, similarly to the current findings. Therefore, the fluctuations in the core community could be due to environmental and anthropic conditions, microbial competitions and interactions (Zeng et al., 2022; Peces et al., 2022) or technical reasons (Crognale et al., 2022). Phyla not deeply studied were found in T0-A and T0-B; for example, the phylum GN02 was present at 1.45% in T0-A and 1.07% in T0-B. The phylum Acidobacteria was found at 0.42% in T0-A and 0.79% in T0-B.

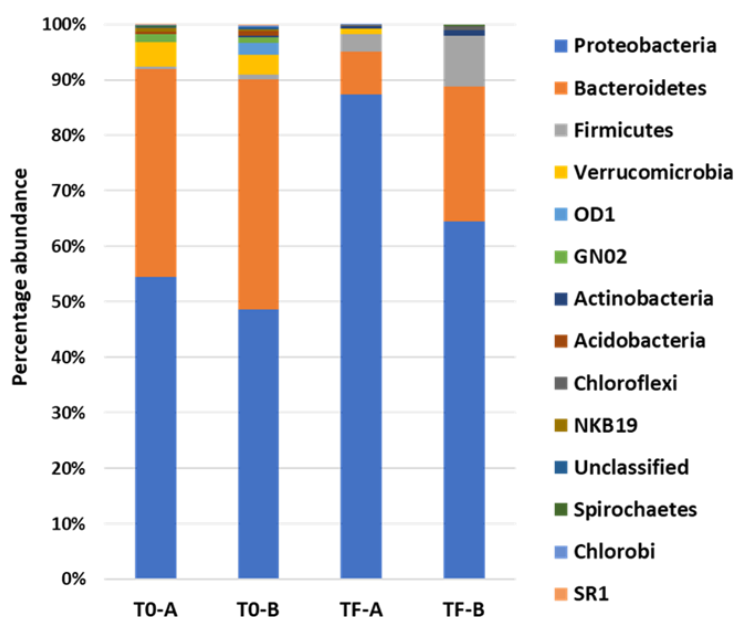


Figure 6.1. Histograms reporting the relative abundances of prokaryotic microbiota taxa of phyla of T0-A, TF-A, T0-B, and TF-B. The relative abundance indicated the percentage of each phylum compared to all the identified phyla.

By analysing the capabilities of these bacterial phyla in PHA production, Proteobacteria emerged as the best PHA producers. In a study on PHA production from acetate (Sruamsiri et al., 2020), metataxonomic analysis revealed the predominance of Proteobacteria, specifically Betaproteobacteria (51.37% of total sequences), Gammaproteobacteria (23.44% of total sequences) and Alphaproteobacteria (13.26% of total sequences). In our study, in T0-A Proteobacteria accounted for 54.52%, with 19.79% Betaproteobacteria, 15.32% Gammaproteobacteria, 10.52% Deltaproteobacteria, 6.88% Alphaproteobacteria, and 2.02% Epsilonproteobacteria (Figure 6.S1). In T0-B, Proteobacteria accounted for 48.64% with 18.47% Betaproteobacteria, 12.76% Gammaproteobacteria, 10.92% Alphaproteobacteria, 4.09% Deltaproteobacteria and finally 2.40% Epsilonproteobacteria.

In experiment A, where the highest yield of PHAs was achieved (20% w/w), Proteobacteria increased from 54.52% at T0-A to 87.41% at TF-A. Specifically, this population was constituted by 97.06% Betaproteobacteria and 1.28% Alphaproteobacteria, while Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria disappeared. In experiment B, where the yield of PHAs was 18% w/w, Proteobacteria also increased, but to a lesser extent than in experiment A. Their population grew from 48.64% at T0-B to 64.55% at TF-B, showing a 1.33-fold increase. This population of TF-B consisted of 34.10% Betaproteobacteria, 23.17% Alphaproteobacteria, 5.99% Gammaproteobacteria, 1.22% Deltaproteobacteria and finally, 0.07% Epsilonproteobacteria. Ultimately, a significant

increase in Betaproteobacteria was observed in TF-A (4.91-fold increase), accompanied by a reduction in all other classes of Proteobacteria. In TF-B, there was an increase in both Alphaproteobacteria (2.13-fold increase) and Betaproteobacteria (1.85-fold increase), while all other classes of Proteobacteria declined. As noted by Sruamsiri et al. (2020), Betaproteobacteria are primarily responsible for the production and accumulation of PHAs. Therefore, Betaproteobacteria make up 97.06% of all bacterial classes in the TF-A sample, which had the highest PHA yield, which can justify the higher PHA yield in experiment A compared to experiment B. For the other bacterial phyla observed, Bacteroidetes decreased from 37.36% at T0-A to 7.60% at TF-A, and from 41.47% at T0-B to 24.18% at TF-B. Verrucomicrobia declined from 4.52% at T0-A to 1.07% at TF-A, and from 3.59% at T0-B to undetectable in TF-B. The capabilities of Bacteroidetes and Verrucomicrobia in producing PHAs are not well-documented in the literature. While some species within these phyla may have the ability to produce PHAs (Sabapathy et al., 2020), they are not the major producers compared to groups like Betaproteobacteria. Thus, the reduction in their percentages during experiments A and B is unlikely to significantly affect the overall PHA production. Actinobacteria slightly increased from 0.01% at T0-A to 0.36% at TF-A, and from 0.29% at T0-B to 0.98% at TF-B. In contrast, Acidobacteria and GN02 decreased from 0.42% and 1.45% at T0-A to 0.09% and 0.00% at TF-A and 0.79% and 1.07% at T0-B to 0.00% at TF-B. Given the low percentages, it is not likely that they contribute significantly to PHA production. An interesting increase in the percentage abundance was given by Firmicutes which increased from T0-A (0.42%) to TF-A (3.17%) and from T0-B (0.81%) to TF-B (9.25%), increasing 7.93 times in A and 11.57 times in B. Firmicutes, which are good VFA producers (Di Leto et al. 2022; Di Leto et al., 2024; Mineo et al., 2024), can also produce PHAs, although they are not among the major producers. Some members of the Firmicutes are capable of degrading PHAs, as this group includes species with PHA depolymerase enzymes that hydrolyze PHAs into monomers, making them usable as a source of carbon and energy (Vogel et al., 2021). Therefore, considering these points, we surmise that most of the PHA production was carried out by Proteobacteria, particularly Betaproteobacteria, especially in TF-A where the maximum PHA yield was achieved and where Betaproteobacteria were 97.06% of total sequences. The other bacterial phyla contributed minimally to PHA production, and sometimes, as in the case of Firmicutes in TF-B, they may have contributed to their degradation. Proteobacteria/Bacteroidetes ratio was calculated as the proportion between the relative abundances of the two phyla. While the starting samples presented a bacterial signature with a Proteobacteria/Bacteroidetes ratio (Figure 6.2A) equal to 1.66 in the T0-A sample and with a Bacteroidetes/Proteobacteria ratio equal

to 1.04 in T0-B, in the final samples, a radical change occurred. In TF-A, the Proteobacteria/Bacteroidetes ratio significantly increased, reaching 546.06. In TF-B, the microbial composition was inverted as the Proteobacteria increased compared to the Bacteroidetes, unlike the respective sample starting point, reaching a Proteobacteria/Bacteroidetes ratio of 4.54.

6.3.3 Structure of Prokaryotic Communities of sewage sludge at family levels and bacterial signatures

The SS microbiota was also studied at the taxonomic level of families (Figure 6.2B) to better understand the changes and dynamics within the microbial community before and after two months. At family level, the primary families in the T0-A and T0-B samples were Rhodocyclaceae (6.36% and 5.78% respectively), Saprospiraceae (9.26% and 17.92%), Rhodobacteraceae (5.02% and 2.72%), Comamonadaceae (9.68% and 8.15%), Xanthomonadaceae (5.94% and 4.03%), Cytophagaceae (3.24% and 3.11%), Chitinophagaceae (7.93% and 2.63%), and Moraxellaceae (6.79% and 2.25%). Consistent with the abundances observed at the phylum level, the predominant component at the family level is proteobacteria. Specifically, Rhodobacteraceae belong to the Alphaproteobacteria class, Rhodocyclaceae and Comamonadaceae belong to the Betaproteobacteria class, and Xanthomonadaceae and Moraxellaceae belong to the Gammaproteobacteria. Following the PHAs production process, the bacteria were selected inside the reactor.

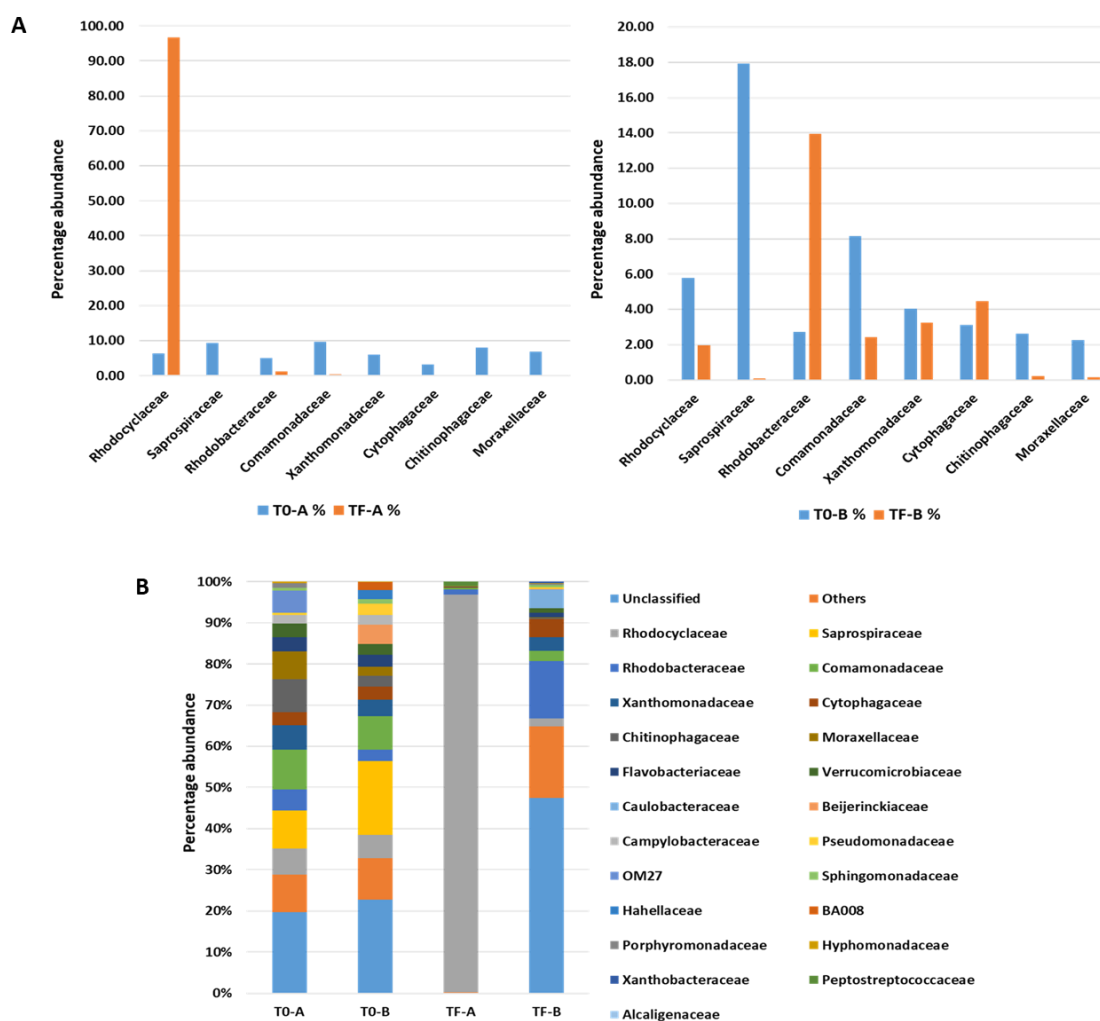


Figure 6.2. A) Histograms reporting the percentage abundances of the leading bacterial families (Proteobacteria/Bacteroidetes ratio) presented notable variations in percentage abundance between the different sewage sludge samples analyzed (T0-A compared to TF-A and T0-B to TF-B). The relative abundance indicated the percentage of each family compared to all the identified families. B) Histograms reporting the relative abundances of families of sewage sludge samples T0-A, TF-A, T0-B, and TF-B. The relative abundance indicated the percentage of each family compared to all the identified families. Taxa having percentage abundance lower than 1 were grouped as “Others”.

In TF-A, the only bacterial family that increased notably was the Rhodocyclaceae, which increased from 6.36% in T0-A to 96.62%; in the experiment B, Rhodobacteraceae rose from 2.72% in T0-B to 13.95%. In TF-B and Cytophagaceae, from 3.11% in T0-B reached 4.47%. All other bacterial families decreased. It is likely that in the TF-A sample, almost all of the PHAs were produced by Rhodocyclaceae (96.62% in TF-A compared to 1.97% in TF-B).

Recent studies have confirmed that, during the production of PHAs from industrial waste, 60-70% of the microbial community was made up of Rhodocyclaceae, which are positively correlated to the production of PHAs after the depletion of the carbon source (De Donno Novelli et al., 2021). Almeida and collaborators (2022) also confirmed that the increase in Rhodocyclaceae was concomitant with the increase in vOLR, which aligns perfectly with the results of this study. The Rhodobacteraceae (1.23% in TF-A and 13.95% in TF-B), being Proteobacteria, have good PHA production capabilities, accumulating over 84% of their weight in PHAs (Carvalho et al., 2022). There is little knowledge about the ability of Cytophagaceae to produce PHA. However, recent reports indicated a positive role of Cytophagaceae in PHA production (Perez-Zabaleta et al., 2021). Therefore, we surmise that the PHAs produced in TF-A can be attributed to Rhodocyclaceae, which represents the most abundant family after the production and selection process. As previously discussed, the microbial composition of the two initial samples, T0-A and T0-B, was similar. This was confirmed by the heatmap analysis (Figure 6.3), which showed the two T0 samples clustering into a single group. The TF-B sample was similar to the T0 samples. At the same time, the TF-A, which yielded the highest PHAs, exhibited a distinct microbial composition from the other samples and formed a separate cluster.

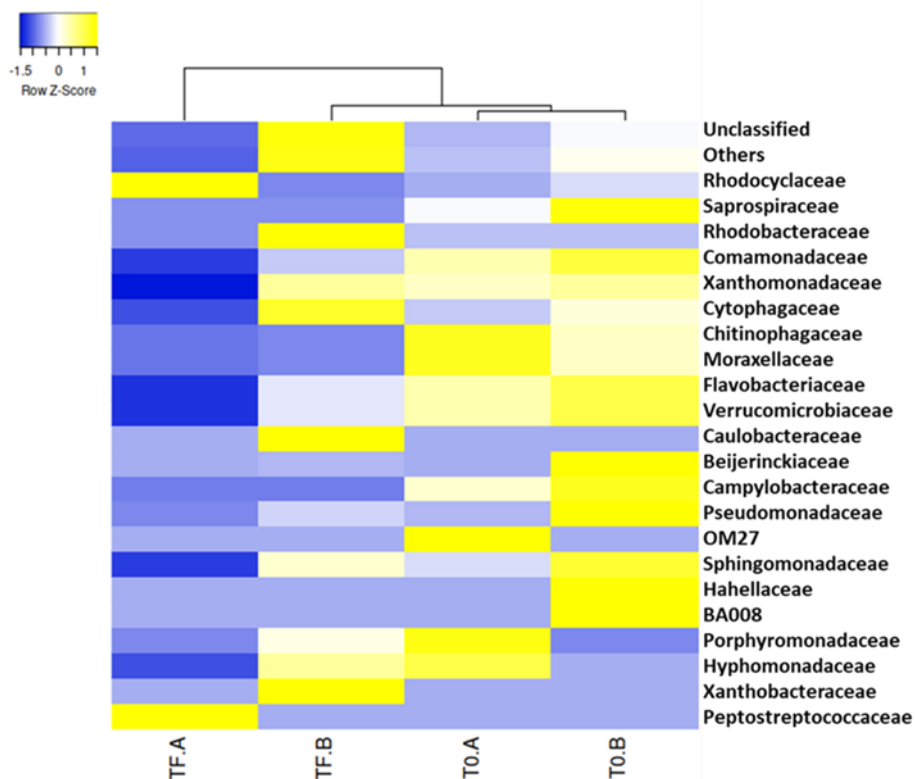


Figure 6.3. Heatmap of 24 most abundant prokaryotic family composition profiles in T0-A, TF-A, T0-B and TF-B samples as inferred by HeatMapper bioinformatic analysis. The color

intensity in each sample was normalized to represent its relative ratio in the two groups. Colors from blue to yellow indicated the relative values of microbiota.

6.3.4 Structure of Prokaryotic Communities of sewage sludge at genera levels

The most striking feature seen at the genera level was the predominance of *Azoarcus*, which, from 0.01% in T0-A, was present at 94.16% in TF-A (Figure 6.4-Table 6.S1). *Azoarcus* belongs to the Rhodocyclaceae family (Proteobacteria), the predominant family in the TF-A sample. It is known to accumulate PHA as a carbon and energy reserve, especially under stressful conditions (Carvalho et al., 2014).

Even the *Thauera* genus belonging to the Rhodocyclaceae family (Proteobacteria) was found to increase from 0.01% in T0-A to 2.46% in TF-A (Figure 6.4). It was recently demonstrated that *Thauera* is a good PHA producer (Andreolli et al., 2022). In this study, *Azoarcus* and *Thauera* comprise approximately 96% of the genera in this sample, strongly suggesting their predominant role in PHA production. On the contrary, in TF-A, some bacterial genera were present in a lower percentage concerning TF-B, like *Paracoccus* (1.23% in TF-A and 9.49% in TF-B), *Bdellovibrio* (0.00% in TF-A and 2.12% in TF-B), *Rhodobacter* (0.00% in TF-A and 3.85% in TF-B) and *Hydrogenophaga* (0.44% in TF-A and 1.95% in TF-B). Their implication in PHA production was previously investigated with *Paracoccus* able to use synthetic short and medium-chain carboxylic acids as the principal carbon sources in the growth medium, producing a higher PHA rate, about yields of 26% of cell dry mass (Szacherska et al., 2022). However, some genera of *Paracoccus* can activate pathways that reduce the production of PHAs. In gene regulation, phasins (PhaPs) are predominantly proteins associated with PHA granules that positively influence PHA synthesis. However, the *phaR* gene has been identified in *Paracoccus*, which codes for a negative regulator involved in the expression of *phaP*. The latter plays a role in regulating the expression of PhaP and, therefore, indirectly modulating the production of PHA (Maehara et al., 2002). *Bdellovibrio* has a very delicate role in the final yield of PHAs. Some species of *Bdellovibrio* genus are included among the "predatory bacteria", i.e., bacteria capable of preying on gram-negative species that produce and accumulate PHAs. After having lysed the cell of the prey bacterium, the predator hydrolyzes thanks to a depolymerase (*phaZ*) and consumes part of the PHA released into the extracellular environment. This process allows the hydrolysis of significant quantities of PHA granules. In genetic engineering, *phaZ*-inactivated strains have been created to improve the recovery of PHA but not hydrolysis (Martinez et al., 2016). The genus *Hydrogenophaga* is capable of producing PHA; in particular, previous studies (Povolo et al., 2013) have shown that it is not only capable of producing PHA-containing

comonomers such as 3-hydroxybutyrate, 3-hydroxyvalerate, and 4-hydroxybutyrate but also is capable of producing PHAs from waste resources. Even *Rhodobacter*, being a Protobacterium, is capable of producing PHAs. The photosynthetic bacterium *Rhodobacter* produces high PHA levels when an adequate carbon source is available. The three essential genes used in the PHA biosynthetic pathway are *phaA*, which encodes beta-keto thiolase; *phaB*, which encodes acetoacetyl coenzyme A reductase; and *phaC*, which encodes PHA synthase (Kranz et al., 1997). Therefore, although *Rhodobacter* and *Hydrogenophaga* are PHA producers, the TF-B sample shows the highest percentages at the genus level for *Paracoccus* and *Bdellovibrio*. These genera, which can reduce production or degrade PHA, likely contributed to the lower yield observed in TF-B.

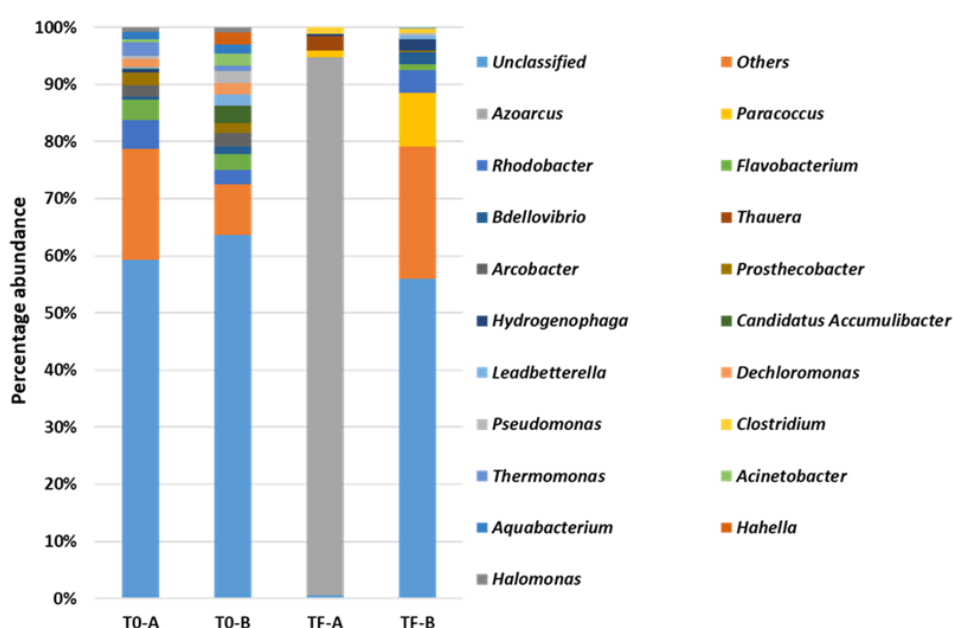


Figure 6.4. Histograms reporting the relative abundances of prokaryotic microbiota taxa of sewage sludge samples T0-A, TF-A, T0-B, and TF-B at the level of genera. The relative abundance indicated the percentage of each genus compared to all the identified genera. Taxa having percentage abundance lower than 1 are grouped as “Others”.

6.3.5 Predicting Microbial Metabolism

The metabolic activities mainly represented in the starting samples (Figure 6.S2) were dehalogenation (18.2% in T0-A and 15.5% in T0-B), sulfate reduction (15.5% in T0-A and 12.7% in T0-B) and ammonia oxidization (19.1% in T0-A and 15.8% in T0-B). These metabolic functions may be connected to the production of PHAs. Dehalogenating strains, for instance, remove halogen atoms (such as chlorine, bromine, or iodine) from organic compounds and use halogenated compounds as electron acceptors. These bacteria can

indirectly support the production of substrates for PHA synthesis by generating dehalogenated organic acids. However, toxic intermediate compounds can be formed during dehalogenation, inhibiting the growth of PHA-producing bacteria or disrupting the metabolic pathways involved in PHA synthesis. Additionally, these toxic products can negatively impact essential cofactors for PHA biosynthesis, such as NADH and ATP, thereby reducing production yields (Janssen et al., 2001). Sulfate-reducing bacteria can contribute to producing metabolites, such as volatile fatty acids like acetate and propionate, during the sulfate reduction process. These metabolites serve as precursors for the synthesis of PHAs by PHA-producing bacteria (Liu et al., 2015). Finally, some ammonia-oxidizing bacteria can also accumulate carbon intermediates that could be used for PHA synthesis. Fra-Vázquez and colleagues (2019) found that, in batch reactors, exposing a mixed microbial community to a feast/famine regime resulted in PHA yields reaching 60% of the bacterial mass by weight. Additionally, they observed the development of partial nitrification activity (oxidation of ammonia to nitrite), likely due to the inhibition by free ammonia, or the selected sludge retention time. After two months, following the PHA production process, the selection of the microbial community caused a reduction in the A experiment of dehalogenating bacteria (from 18.2% in T0-A to 1.4% in TF-A), sulfate reducers (from 15.5% in T0-A to 1.6% in TF-A) and ammonia-oxidizers (from 19.1% in T0-A to 1.6% in TF-A). In the B experiment, however, there was an increase in dehalogenating bacteria (from 15.5% in T0-B to 72.3% in TF-B), sulfate reducers (from 12.7% in T0-B to 68.8% in TF-B), and ammonia oxidizers (from 15.8% in T0-B to 27.3% in TF-B). Another interesting increase was seen in experiment B with xylan-degrading bacteria that increased from 9.8% in T0-B to 52.0% in TF-B. In contrast, in experiment A, they remained at low concentrations (from 8.8% in T0-A to 0.2% in TF-A). Bacteria can degrade and hydrolyze xylan and utilise xylan-derived xylose for growth and PHA production (Salamanca-Cardona et al., 2014). Finally, in experiment A, there was a substantial increase in nitrogen-fixing bacteria, rising from 6.3% in T0-A to 91.9% in TF-A, representing the highest metabolic capacity observed. Nitrogen-fixing bacteria carry out a crucial biological process in which atmospheric nitrogen (N_2) is converted into ammonia (NH_3). Nitrogen and carbon are primary elements for PHA production within a feast/famine regime. Specifically, nitrogen imposes significant selective pressure beneficial for microorganisms engaged in PHA storage (Correa-Galeote et al., 2022). As a result, the nitrogen-fixing bacteria played a crucial role, significantly enhancing PHA production in the TF-A sample and leading to the highest yield.

6.4 Conclusions

In this study, we examined the microbial community of sewadgesludge (SS) subjected to a feast/famine regime by adjusting the Organic Loading Rate (vOLR) in a selective-sequencing batch reactor to optimise the yield of polyhydroxyalkanoates (PHAs). The highest PHA yield was achieved after two months, using a vOLR of 1.3 g COD L⁻¹ day⁻¹. Metataxonomic analyses revealed that the predominant bacterial phylum was Proteobacteria, particularly Betaproteobacteria, known for their valuable PHA-producing capabilities. A distinct bacterial signature favouring Proteobacteria over Bacteroidetes was evident at the family level, particularly in the TF-A condition, which stimulated the Betaproteobacteria family of Rhodocyclaceae. Therefore, this research not only provides valuable insights into the role of bacteria in optimising PHA production, but also paves the way for future biotechnological and industrial applications and underscores the potential of utilising wastewater from the Unipa Campus within the framework of circular economy principles. By repurposing this waste stream to produce valuable materials like PHAs, waste disposal and environmental impact can be significantly reduced while enhancing bioplastic production.

6.5 References

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6.6 Supplementary material

Table 6.S1. Relative abundances in percentage of prokaryotic microbiota taxa of sewage sludge samples T0-A, TF-A, T0-B, and TF-B at the level of genera. The relative abundance indicated the percentage of each genus compared to all the identified genera.

Genus	T0-A (%)	TF-A (%)	T0-B (%)	TF-B (%)
Unclassified	59.33	0.59	63.59	56.00
Others	19.44	0.00	8.88	23.08
<i>Azoarcus</i>	0.00	94.16	0.00	0.00
<i>Paracoccus</i>	0.00	1.23	0.00	9.49
<i>Rhodobacter</i>	5.02	0.00	2.49	3.85
<i>Flavobacterium</i>	3.55	0.00	2.79	1.07
<i>Bdellovibrio</i>	0.50	0.00	1.37	2.12
<i>Thauera</i>	0.00	2.46	0.00	0.00
<i>Arcobacter</i>	2.02	0.00	2.34	0.00
<i>Prostheco bacter</i>	2.28	0.00	1.75	0.34
<i>Hydrogenophaga</i>	0.52	0.44	0.00	1.95
<i>Candidatus Accumulibacter</i>	0.06	0.00	3.01	0.00
<i>Leadbetterella</i>	0.27	0.00	1.99	0.69
<i>Dechloromonas</i>	1.47	0.00	2.08	0.00
<i>Pseudomonas</i>	0.57	0.00	1.99	0.39
<i>Clostridium</i>	0.00	1.12	0.00	0.71
<i>Thermomonas</i>	2.32	0.00	1.00	0.18
<i>Acinetobacter</i>	0.53	0.00	2.09	0.12
<i>Aquabacterium</i>	1.41	0.00	1.57	0.00
<i>Hahella</i>	0.00	0.00	2.10	0.00
<i>Halomonas</i>	0.71	0.00	0.96	0.00

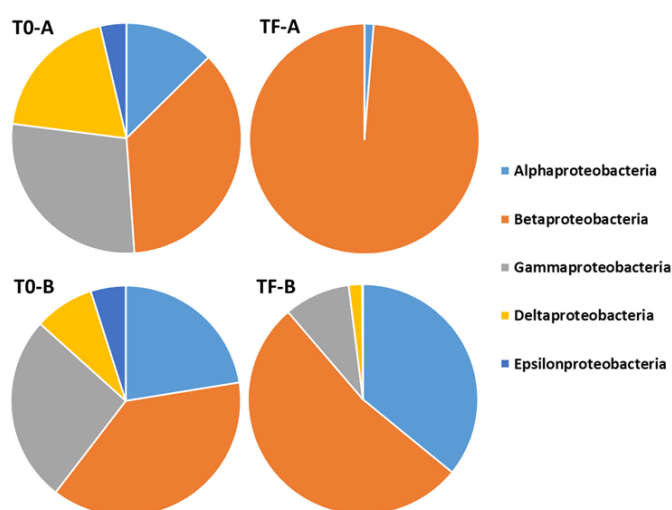


Figure 6.S1. Pie charts reporting the taxonomic composition of samples T0 and TF at the taxonomic level of class belonging to Proteobacteria phyla. The relative abundance represented by the pie charts indicated the percentage of each class compared to all the identified classes that belonged to the Proteobacteria phylum.

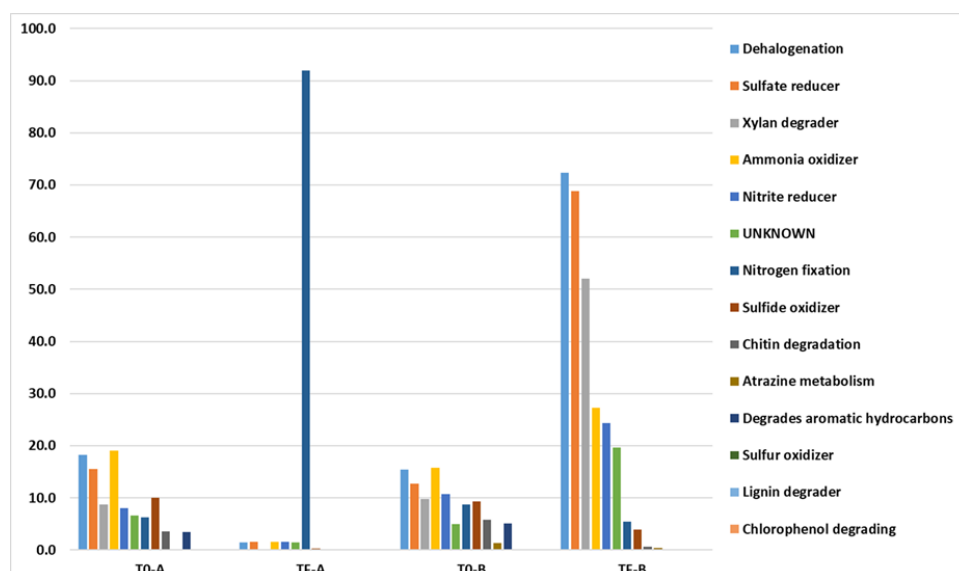


Figure 6.S2. Putative metabolic requirements of the bacterial communities residing in the samples T0-A, TF-A, T0-B, TF-B as inferred by METAGENassist bioinformatic analysis. The abundance percentage of bacteria having a specific metabolism is indicated.



CHAPTER 7

The role of carbon and nitrogen ratio on sewage sludge microbiota for producing polyhydroxyalkanoates

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ABSTRACT

The products of an advanced sewage sludge fermentation process can generate polyhydroxyalkanoates (PHA) biopolymers, precursors of bioplastics considered excellent candidates for replacing petroleum-derived plastics. The aerobic feast-anoxic famine cycling strategy has been proven to be an efficient method to enrich sewage sludge microbiota with PHA-producing microorganisms. This work evaluated the effect of different carbon and nitrogen ratios (C/N) of 3.5, 2, and 1 g COD/g N for modulating sewage sludge microbiota structure for improved PHA production. The study was executed on a pilot plant scale using sewage sludge as an organic carbon and nitrogen source deriving from an oxic-settling anaerobic plant that collects wastewater from different facilities at the University of Palermo (UNIPA) campus. The system performance of PHA production was monitored over the three experimental periods characterised by the different C/N values. The results show that a lower C/N value decreased the PHA production with 20, 24, and 26 % w/w of PHA for COD/N values of 1, 2, and 3.5 g COD/g N, respectively. In parallel, metataxonomic analysis revealed an increase of PHA producer microorganisms at the ratio of 3.5 g COD/g N, such as Proteobacteria, specifically Betaproteobacteria. In addition, the metataxonomic analysis showed an increase in fungal abundance and diversity with a decrease in C/N. Thus, metataxonomics proved helpful in highlighting relationships between operating conditions, microbiota structure and the achievement of specific outputs. The insights gained from this study demonstrated a positive correlation between C/N values, PHA-producer microorganisms and PHA yields.

7.1 Introduction

The possibility of exploiting waste to produce valuable materials represents an excellent opportunity to reuse natural sources from a circular economy perspective (Mannina et al., 2021 b). This approach emphasises the importance of minimising waste and maximising resources. By leveraging the metabolic capacities of microbial communities residing in sewage sludge (SS), we can effectively remove pollutants, thereby contributing to environmental protection and promoting the transformation of organic waste into valuable materials, such as specific microbial biomass containing bioplastic precursors, such as polyhydroxyalkanoates (PHA) (Thomas et al., 2023). Consequently, this innovative utilisation of waste aligns with sustainability principles, fostering a more resilient and resource-efficient economy. In line with the transition from wastewater treatment plants (WWTPs) to water resource recovery facilities (WRRF), PHA can be produced using mixed microbial cultures (MMCs) from SS microbiota (Nielsen et al., 2017). PHAs, a class of

thermoplastic biodegradable polyesters, offer a promising alternative to petroleum-derived plastics due to their environmentally friendly properties and versatility (Cabrera et al., 2021). PHA exhibits similar properties to conventional plastics, making them suitable for various applications, from packaging materials to medical devices, thus providing a versatile and eco-friendly alternative to traditional plastics (Acharjee et al., 2023). However, PHA's production cost is higher than fossil-based plastics (Zhou et al., 2023). Therefore, these biopolymers can be produced using biological processes, explicitly starting from precursors such as volatile fatty acids (VFAs) to reduce production costs. Interestingly, VFAs can be produced in WWTP reactors through an acidogenic fermentation process by the MMC obtained by shaping SS microbiota (Di Leto et al., 2023; Di Leto et al., 2024). The ability of MMCs to convert organic substrates into VFAs and, subsequently, into PHA presents a sustainable and renewable method of producing bioplastics, and it provides a viable solution for managing organic waste.

Different microbiota members in the MMCs are responsible for synthesising VFA and PHA, the latter accumulated as intracellular granules used for carbon and metabolic energy reservoirs (Berg et al., 2020). An efficient microbial PHA production involves the delicate balance of organic carbon and nutrient removal (Zhou et al., 2023). The so-called feast-famine cycling process has consistently proven to be the most effective among the various methods to shape SS microbiota to obtain MMCs suitable for PHA production. In particular, feast-famine cycling process alternates periods of nutrient abundance (feast) and limitation (famine) to exploit the metabolic behaviour of microorganisms. Thus, this method primarily supports the enrichment and turnover of PHA-producing bacteria within the microbial community while eliminating competitors (Huang et al., 2018). This cyclical pattern of nutrient availability exerts selective pressure on the SS microbiota members, shaping their metabolic activity and survival strategies (Correa-Galeote et al., 2022). During feast periods, bacteria strategically use abundant carbon sources to maximise growth. In contrast, during famine periods, they tactically switch to alternative metabolic pathways, often entering a state of metabolic slowdown or reduced activity. This adaptation includes decelerating growth, minimising energy-intensive processes, and optimising resource utilisation to survive until conditions improve. The scarcity of nutrients during famine intensifies competition among bacteria, driving shifts in metabolic activity as they compete for limited resources. During the feast phase, bacterial cells produce intracellular PHA granules that serve as an energy reserve, enabling the bacteria to survive periods of nutrient deficiency (Morya et al., 2023).

The carbon and nitrogen ratio (C/N) has an important impact on PHA production. Indeed, Zhou et al. (2022) performed tests to evaluate the impact of C/N associated with pH on the yield of PHA produced by MMC. Very little is known about the PHA production capabilities of fungal microbial communities. However, recent studies, such as those by Thu et al. (2023), have focused on utilising marine yeasts to maximise PHA yields. Therefore, this pioneering study aims to identify the interactions within the eukaryotic community, mainly focusing on fungi, concerning the three operational parameters established to maximise PHA yields. PHA production is performed through SS-MMC enriched by the aerobic feast-anoxic famine (AE/AN) cycling strategy in this work.

Several studies have examined the effect of varying C/N values using municipal solid waste (Sánchez Valencia et al., 2021) or high C/N values (Qinxue et al., 2010). However, this study's novelty lies in using a pilot scale treatment plant using waste activated sludge (WAS) to obtain different C/N values in fermented liquid waste to feed the PHA production process based on the AE/AN enrichment strategy. In addition, no previous study has conducted a comprehensive metagenomic analysis on SS microbiota to highlight microbial members of MMCs obtained using a similar setting. In particular, the influence of three different C/N values (3.5, 2 and 1 g COD/g N) in PHA production was evaluated using carbon and nitrogen sources obtained in a pilot plant at the University of Palermo (UniPA) Campus. Indeed, a profound comprehension of the operational parameters influencing PHA production by MMC is crucial for process optimisation. The objective of this study is to evaluate, through metataxonomic analyses, the change in the SS microbiota structure selected after the feast-famine cycling process carried out for a month and related to three C/N values tested. Metataxonomic analysis was carried out to highlight the relationships between the operating parameters and the SS microbial community structure.

7.2 Material and Methods

7.2.1 Pilot plant configuration

The experiments took place at the Water Resource Recovery Facility of Palermo University (Mannina et al., 2021 a and b). The PHA production line is situated at the pilot plant hall and operates as the side stream line for waste activated sludge (WAS) treatment and valorisation in view of a circular economy approach. This PHA line includes a 200 L fermenter equipped with an ultra-filtration (UF) unit, which feeds two sequencing batch reactors (SBR) operating in series. The nitrification SBR (N-SBR) is designed to produce a nitrite-rich effluent with a

working volume of 30 L and is fed with the fermented sludge's liquid. The selection SBR (S-SBR) enriches microbial cultures through a feast-famine cycle using the AE/AN strategy, also with a working volume of 30 L. At the beginning of the feast phase, the reactor is fed with the fermented sludge's liquid (aerobic phase). At the start of the famine phase, it is fed with the N-SBR effluent (anoxic phase) with a 50:50 proportion between the fermented liquid and the N-SBR effluent. After the enrichment process, the biomass undergoes batch-scale accumulation using custom-made software (Mineo et al., 2023). The experiments were conducted over three periods (45 days each) with varying C/N values (g COD/g N) in both the N-SBR and S-SBR: i) period I – 3.5; ii) period II – 2; iii) period III – 1.

The fermenter reactor feeds the entire PHA production line (Figure 7.1). The substrate used to carry out the acidogenic fermentation was the WAS of the water line in the pilot plant hall (Cosenza et al., 2023). The fermenter was operated in batch conditions by a hydraulic retention time (HRT) and sludge retention time (SRT) of 5 days. At the end of the fermentation process, the sludge was filtered by the UF equipped with a hollow fibre membrane of polyvinylidene fluoride with 0.03- μ m porosity and 1.4-m² surface area, as reported in the literature (Mannina and Mineo, 2023). The values of pH and temperature were monitored but not controlled during the acidogenic fermentation, and no pre-treatment was performed before the fermentation. The fermentations were carried out with average TSS and VSS of 3.3 ± 0.4 g/L and 1.6 ± 0.3 g/L, respectively. The pH accounted for 7.2 ± 0.3 and 6.4 ± 0.2 at the start and at the end of the fermentation, respectively, while the temperature was around 14.6 ± 2.5 °C.

The fermented liquid containing VFA, obtained at the end of the fermentation, was used to feed the N-SBR and S-SBR. N-SBR and S-SBR inoculum were also the WAS of the water line. The reactors were inoculated only once at the start of the experiments. Pseudo-steady-state conditions were achieved once the feast/famine ratio did not change significantly (within 5% standard deviation) for ten days (Isern-Cazorla et al., 2023). As reported in the literature (Basset et al., 2016; Frison et al., 2015), following the AE/AN enrichment, the N-SBR aims to produce nitrite from ammonia partial oxidation. The reactor effluent was then used to feed at the beginning of the anoxic famine phase in the S-SBR. In this reactor, VFA was consumed and PHA produced during the aerobic feast phase while, during the anoxic famine, PHA was used as a carbon source to denitrify the nitrites and nitrates produced by the N-SBR (Conca et al., 2020; Mannina and Mineo, 2024). The average fermented sludge liquid composition is reported in Table 7.1, while the average operational parameters for the

S-SBR and N-SBR are reported in Table 7.2. The different C/N values were obtained through ammonium chloride (NH_4Cl) dosage in the fermented sludge liquid.

Table 7.1. Average and standard deviation fermented SS liquid composition.

Parameter	U.M	Period I		Period II		Period III	
sCOD	mg/L	282.55	± 62.18	300.73	± 27.46	593.50	± 105.55
$\text{NH}_4^+\text{-N}$	mg N/L	82.13 ± 10.76		142.55	± 18.37	344.65	± 51.65
$\text{PO}_4^{3-}\text{-P}$	mg P/L	44.53 ± 22.91		28.74 ± 5.60		28.26 ± 5.06	
VFA	mg COD/L	106.71	± 47.26	100.19	± 68.51	262.14	± 22.71
Acetic acid	mgCOD _{AceticAcid} /L mg COD _{VFA} %	81.89 ± 6.27		66.46 ± 17.04		67.36 ± 9.46	
Propionic acid	mgCOD _{PropionicAcid} /L mg COD _{VFA} %	5.44 ± 3.24		27.10 ± 8.50		8.89 ± 4.62	
Butyric acid	mgCOD _{ButyricAcid} /L mg COD _{VFA} %	12.67 ± 10.42		6.44 ± 2.65		23.76 ± 13.17	

Table 7.2. Average and standard deviation operational parameters for S-SBR and N-SBR.

Parameter	U.M	S-SBR	N-SBR
F/M	kg BOD kg SS ⁻¹ d ⁻¹	0.14 ± 0.05	0.13 ± 0.02
OLR	kg COD m ⁻³ d ⁻¹	0.13 ± 0.07	0.10 ± 0.05
NLR	kg N m ⁻³ d ⁻¹	0.10 ± 0.08	0.09 ± 0.03
sCOD/NO _x	g COD g ⁻¹ NO _x -N	18.70 ± 4.35	-
HRT	Days	2	1
SRT	Days	7.98 ± 3.27	5.78 ± 0.53

F/M: food to microorganisms ratio; OLR: organic loading rate; NLR: nitrogen loading rate.

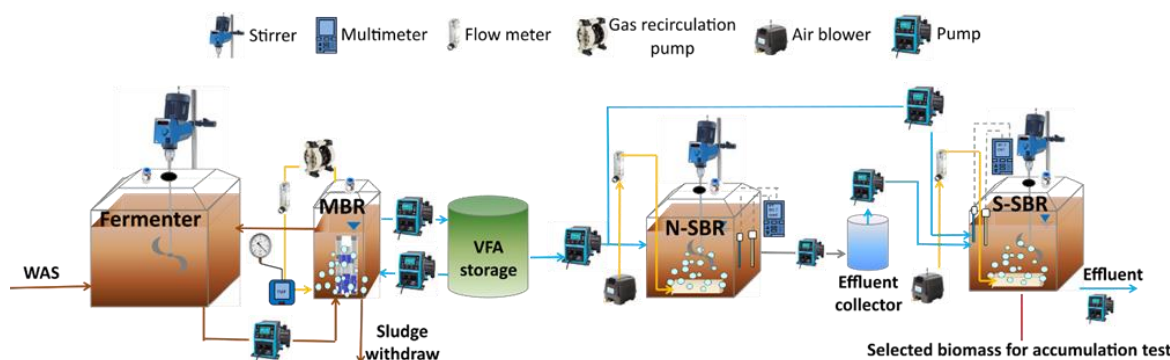


Figure 7.1. Diagram of the pilot plant used in this work consisting of a fermenter, an ultrafiltration unit, N-SBR and S-SBR. The graph also shows the probe related to S-SBR, the connected pumps and the gas and liquid sampling ports.

7.2.2 PHA accumulation batch fermentation tests

The biomass enriched at different C/N values was tested in batch fermentation accumulation tests. As reported in the literature (Mineo et al., 2023), 3 L of sludge is withdrawn from the S-SBR. 2 L of supernatant is removed after settling, and the remaining mixed liquor is washed twice with tap water, removing the supernatant each time to ensure that no external carbon source is left. The remaining biomass is left aerated overnight to achieve endogenous conditions to start accumulating without any stored PHA to compare the different periods. The fermented sludge liquid was used as feed (around 10 L per day), and the dissolved oxygen (DO) was monitored. The accumulation is automatically controlled by software, described by Mineo et al. (2023), based on DO variation after a substrate feed. The accumulation was performed in duplicate for ten days in two 2 L glass reactors for each experimental period.

7.2.3 Analytical methods

The fermented sludge liquid, the N-SBR and S-SBR effluents were monitored by analysing soluble chemical oxygen demand (sCOD), VFA, ammonium ($\text{NH}_4^+\text{-N}$), phosphate ($\text{PO}_4^{3-}\text{-P}$), nitrate ($\text{NO}_3^-\text{-N}$) and nitrite ($\text{NO}_2^-\text{-N}$) concentrations, according to the standard methods (Baird et al., 2017). Mixed liquor samples were taken from the accumulation batch tests to analyse PHA concentration and total and volatile suspended solids (TSS and VSS, respectively). VFA concentrations were measured, as reported by Montiel-Jarillo et al.

(2021), with a Gas Chromatograph (GC) (Agilent 8860) equipped with a flame ionisation detector (FID) and a DB FFAA column (30 m x 0.25 mm x 0.25 µm). PHA concentration, calculated as the sum of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) monomers (Sigma Aldrich standards) (Conca et al., 2020), was measured by a GC equipped with a FID detector and a Restek Stabilwax column (30 m × 0.53 mm x 1.00 µm), as reported by Mannina et al. (2019). The PHA concentration is expressed as weight percentage (g PHA/g VSS %), while the PHA productivity is expressed as the g of PHA produced over time (g PHA/day). The nitrite accumulation rate (NAR) was calculated as reported by Kowal et al. (2022) (Equation 1), while the nitrification and denitrification rate was calculated as reported by Mannina et al. (2016):

$$NAR (\%) = \frac{(NO_2 - N_{out})}{(NO_2 - N_{out} + NO_3 - N_{out})} * 100 \text{ (Equation 1)}$$

$$N_{nit}(\%) = \frac{(NH_4^+ - N_{in}) - (NH_4^+ - N_{out}) - (N_{metabolic})}{(NH_4^+ - N_{in}) - (N_{metabolic})} \text{ (Equation 2)}$$

$$N_{denit}(\%) = \frac{(NH_4^+ - N_{in}) + (NO_x - N_{in}) - (NH_4^+ - N_{out}) - (N_{metabolic}) - (NO_x - N_{out})}{(NH_4^+ - N_{in}) + (NO_x - N_{in}) - (N_{metabolic})} \text{ (Equation 3)}$$

where $N_{metabolic}$ accounts for 5% of the sCOD removed.

7.2.4 Metagenomic DNA analysis

The structure of the prokaryotic microbiota residing in SS of S-SBR was analysed using metataxonomic analysis based on next-generation sequencing (NGS) of 16S rDNA marker gene amplicons obtained from metagenomic DNA. This study aims to evaluate, through metataxonomic analyses, how the structure of the selected microbial communities varies with the feast-famine cycle process. It considers one month from the start of the experiment and compares three tested C/N values. The starting microbial communities are to be considered similar because a unique SS sample was used. Indeed, a metataxonomic characterisation of the microbial community structure of the starting SS sample was conducted. However, since the objective of this study was to assess how C/N influenced the SS microbiota structure, the metagenomic analyses focused on the comparison between 3.5 C/N (related to Period III), 2 C/N (related to Period II), and 1 C/N (related to Period I) samples from the S-SBR. The analysed samples were collected in S-SBR. Analyses were conducted on unfiltered and uncentrifuged samples, following procedures described by

Presti et al. (2021) and Di Leto et al. (2024). The quality of extracted DNA was preliminarily evaluated using 1% (w/v) agarose gel electrophoresis with 0.5 µg/mL ethidium bromide for visualisation under a UV lamp. The DNA concentrations from 1 g SS samples and their tenfold serial dilutions were measured with high precision by absorbance at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extracted DNA was also rigorously assessed by absorbance ratios at 260/280 nm and 260/230 nm, indicating potential contamination from proteins, organic compounds, or chaotropic agents. The PCR amplification of the 16S rDNA gene, a key step in this study, was conducted with the parameters and primers specified in Di Leto et al. (2024). The sequencing of the amplification products was performed in a single 300 bp paired-end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). The raw 16S rDNA data were then processed using the QIIME2 software (<https://qiime2.org/>), with overlapping paired-end reads denoised using the DADA2 plugin. Unique amplicon sequence variants (ASVs) were assigned and aligned to the Greengenes reference database at 99% sequence similarity (<https://greengenes.secondgenome.com/>). The number of ASVs and the relative abundances of phyla, orders, classes, and families were determined for each sample. HeatMap generation was conducted via the online web server (<http://heatmapper.ca/expression/>), using a "complete linkage" calculation based on Pearson correlation, focusing on genera.

The same DNA extracted for 16S rDNA analysis was used as a template for the amplification of ITS, using the following primers (5'-3'): ITS3_KYO2_NGS (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGAAGAACGYAGYRAA) and ITS4_univ (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC). Thermo Scientific DreamTaq DNA Polymerase was used following the manufacture instructions. The sequencing of the amplification products was also performed in a single 300 bp paired-end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy).

7.2.5 Bioinformatic analysis of prokaryotic and eukaryotic Microbiota Structure

Raw data from all samples were quality-filtered, and analysed using QIIME2 version 2024.5 (Bolyen et al., 2019). Sequences were filtered using the DADA2 software package to remove chimeric sequences and phiX reads and to perform the paired-end read joining (Callahan et al., 2016). Taxonomic assignment of ITS sequences was carried out using the UNITE

database (Abarenkov et al., 2010). Briefly the UNITE for eukaryotes version 10.0 (97% similarity) was used to train the taxonomic classifier using the fit-classifier-naive-bayes function of QIIME2 that was used to predict the taxonomic assignments using the classify-sklearn function. The phylogenetic tree of all taxa detected was performed using the QIIME2 phylogeny tool and visualised using TreeViewer (Bianchini and Sánchez-Baracaldo, 2024), while the PCoA was performed on the unweighted UniFrac distances (Lozupone et al., 2011). The analysis of the eukaryotic community is a pioneering study regarding the possible involvement of the latter in the production of PHA; therefore, the analyses were conducted in duplicate.

7.3 Results and discussion

7.3.1 System performance in contaminants removal

The C/N effect on system performance was evaluated by monitoring the S-SBR and N-SBR efficiencies. The monitoring results are reported in Tables 7.3 and 7.4 for S-SBR and N-SBR, respectively.

Table 7.3. Average and standard deviation of the S-SBR's efficiency over the three periods.

Parameter	U.M.	Period		
		I	II	III
sCOD influent	mg/L	298.7 ± 66.0	276.2 ± 133.5	610.3 ± 42.2
sCOD effluent	mg/L	17.1 ± 10.8	24.6 ± 8.1	72.1 ± 12.2
sCOD removal efficiency	%	93.9 ± 4.5	89.6 ± 5.4	88.2 ± 1.2
NH ₄ ⁺ -N influent	mg N/L	83.4 ± 7.5	127.1 ± 41.4	584.4 ± 20.0
NH ₄ ⁺ -N effluent	mg N/L	4.9 ± 2.3	14.7 ± 3.7	53.9 ± 8.2
NH ₄ ⁺ -N removal efficiency	%	94.1 ± 2.8	87.6 ± 5.4	90.2 ± 1.5
Denitrification efficiency	%	72.2 ± 5.8	80.7 ± 6.2	89.1 ± 1.4

Table 7.4. Average and standard deviation of the N-SBR's efficiency over the three periods.

Parameter	U.M	Period		
		I	II	III
sCOD influent	mg/L	272.9 ± 82.8	254.8 ± 135.0	546.3 ± 29.8
sCOD effluent	mg/L	12.8 ± 7.0	21.4 ± 3.9	64.0 ± 13.2
sCOD removal efficiency	%	94.8 ± 3.8	90.0 ± 4.5	88.3 ± 1.8
NH ₄ ⁺ -N influent	mg N/L	79.0 ± 9.5	119.0 ± 42.2	513.8 ± 19.6
NH ₄ ⁺ -N effluent	mg N/L	4.4 ± 3.1	8.1 ± 1.1	34.6 ± 17.7
NH ₄ ⁺ -N removal efficiency	%	94.0 ± 5.0	92.5 ± 2.8	93.2 ± 3.6
NAR	%	13.5 ± 9.6	16.6 ± 3.4	77.0 ± 3.2
Nitrification efficiency	%	92.9 ± 5.7	91.8 ± 3.0	92.9 ± 3.8

The first period was characterised by a high ammonium removal in the N-SBR and S-SBR, with nitrification efficiency in the N-SBR and moderate denitrification in the S-SBR. The C/N ratio adopted (3.5 g COD/g N) and the low sCOD influent concentration probably worsened the heterotrophic biomass activity, thus reducing the denitrification efficiency in the S-SBR (Wang et al., 2021). The NAR was lower than 15%, mainly because of the low influent ammonia concentration, which resulted in low free ammonia concentration inside the reactor, which worsened the Ammonia Oxidizing Bacteria (AOB) activity while favouring the Nitrite Oxiding Bacteria (NOB) (Conca et al., 2020 and Fu et al., 2009). Despite the lower C/N (2 g COD/g N) in period II, no significant difference was found in ammonia removal, NAR and nitrification efficiency compared to period I. The denitrification efficiency was the only parameter showing the positive effect of lower C/N, reaching an average of around 80% (Table 7.3). S-SBR denitrification was positively affected in period III by the high influent sCOD concentration, and the low C/N adopted (1 g COD/g N), similar to the NAR, which almost reached 77%. The high influent ammonia concentration created inhibiting conditions for the NOB in the N-SBR, thus favouring NO₂ production (Basset et al., 2016). Several studies indicated that in the PHA degradation process during the anoxic famine phase, there is no difference when NO₃ is used as an electron acceptor instead of NO₂, thus highlighting that the main focus of the N-SBR operation should be to achieve high nitrification efficiency, as reported in this work (Liu et al., 2023; Salehizadeh and Van Loosdrecht. 2004). Both N-SBR and S-SBR showed excellent biological activity in sCOD removal, maintaining a removal efficiency higher than 85% even during period III, characterised by high influent sCOD concentration and low C/N ratio.

7.3.2 System performance in PHA production

By analysing the PHA concentration and PHA productivity achieved for the three tests, it is possible to see a gradual decline in the yields of PHA produced, paralleling the reduction of the C/N value (Figure 7.2). Period I (C/N of 3.5 g COD/g N) reached the highest amount of PHA, reaching 36.42 ± 1.54 % w/w, with a PHV share of 3.36 ± 1.04 % w/w (Figure 7.2a). PHA productivity peaked on the first accumulation day (3.97 g PHA/day), achieving an average of 1.52 g PHA/day (Figure 7.2b). In period II, characterised by a C/N of 2 g COD/g N, PHA accounted for 24.12 ± 4.36 % w/w, with a PHV share of 6.23 ± 2.30 % w/w while the same productivity peak as period I was achieved. However, slightly higher average PHA

productivity was achieved in period II compared to period I (2.10 g PHA/day). The higher PHV production was due to the higher propionic acid concentration in the fermented liquid, as reported in Table 7.1 (Albuquerque et al., 2011; Jab Chung et al., 1997), while similar PHA productivity was achieved due to similar VFA concentration in the influent (Isern-Cazorla et al., 2023). In the last period, with a C/N of 1 g COD/g N, PHA accounted for 20.01 ± 3.37 % w/w on the 10th day of accumulation, achieving the lowest PHV share of 1.71 ± 0.36 % w/w. The highest PHA productivity peak was registered in period III (8.91 g PHA/day), mainly related to the higher VFA concentration of that period despite the lower C/N value. As reported in the literature (Cui et al., 2017), higher C/N favours PHA accumulation rather than biomass growth. Indeed, nitrogen deficiency is usually adopted to enhance the amount of PHA produced (Johnson et al., 2010). Ahmadi et al. (2020) implemented three strategies in a sequencing batch reactor (SBR), which included complete aerobic conditions with alternating abundance and starvation phases, decoupled carbon and nitrogen feeding regimes, and alternating anaerobic-aerobic conditions. They also assessed the impact of two different carbon sources, acetate and non-alcoholic beverage wastewater, on PHA production during the accumulation phase. Their findings revealed that the decoupled carbon and nitrogen feeding regime resulted in superior PHA accumulation compared to the other strategies, with significantly higher PHA levels when acetate was used as the carbon source compared to the non-alcoholic beverage wastewater (Ahmadi et al., 2020). Muangwong and collaborators (2016) evaluated the effects of the total organic carbon concentration and the mole ratio of carbon to nitrogen were investigated in batch cultivation starting from single culture and Novel PHA-producing strains were isolated and identified (Muangwong et al., 2016). Although they found new strains in pure culture-producing PHA, none of the previous works focused attention on the composition of MMC through metataxonomic analyses. Furthermore, the previous PHA production pilot studies in the literature did not investigate the effect of the shift of C/N over the microbial population responsible for biopolymer production. Other studies have evaluated the impact of the C/N value in SBR systems on enhancing PHA production. For instance, Sánchez Valencia and colleagues (2021) utilised VFAs derived from municipal solid waste as a carbon source, applying C/N values ranging from 13.3 to 42.1. They found that C/N of 13.3 and 23.3 notably promoted the accumulation of PHA, specifically in the form of polyhydroxybutyrate (PHB). Wen and collaborators (2010) investigated the effects of the C/P and C/N values using high values (20, 60, 100, 125, and 180) starting with SS biomass. They found that the highest yield of PHA was achieved at a C/N value of 125 during the nitrogen limitation experiment. This result aligns with the findings of this present work, as nitrogen limitation enhances PHA

yields. Compared to other studies adopting the same enrichment strategy, the amount of PHA produced in this study was higher only than the one obtained by Frison et al. (2015), which adopted co-fermented primary and waste-activated sludge, achieving around 20% w/w of PHA. The higher amount obtained in this work is mainly related to the longer accumulation time (10 days) compared to the one adopted by Frison et al. (< 1 day). Other studies achieved not less than 40 % w/w. Conca et al. (2020) obtained 44.1% w/w with the same substrate used by Frison et al. (2015). Tu et al. (2019) and Liu et al. (2023) adopted the single reactor AE/AN enrichment strategy, using the NO_x produced by the S-SBR during the feast phase or a synthetic source, and achieved 49.7% and 58-64% w/w of PHA, respectively. In view of this, it has to be reported that the substrate used during the accumulation in the above-cited papers had a C/N between 8 and 50 g COD/g N, thus explaining the higher amount of PHA produced.

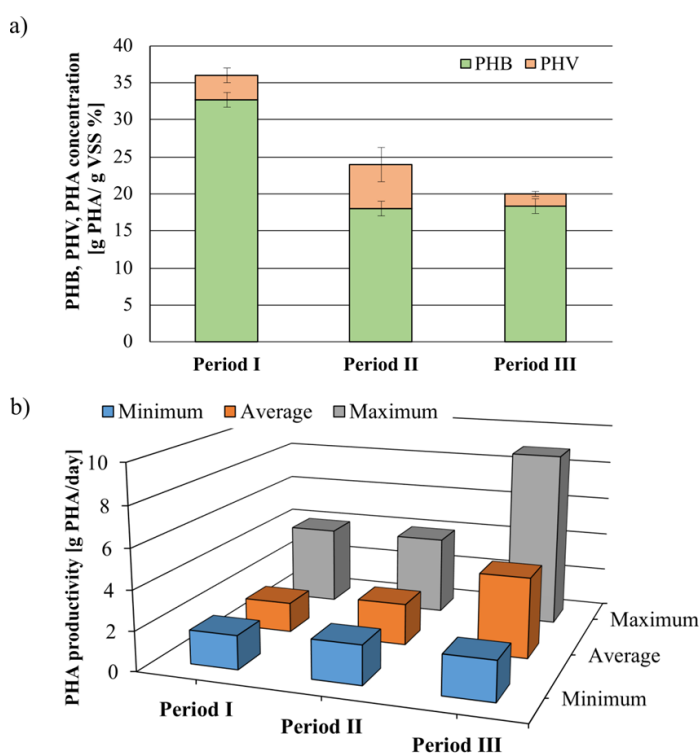


Figure 7.2. PHA accumulated at the 10th day of accumulation (a) and minimum, average and maximum PHA productivity achieved during the accumulation (b) for the three experimental periods.

7.3.3 Structure of prokaryotic microbiota of SS at the phylum level and bacterial signature

The metagenomic analyses focused on the comparison between 3.5 C/N (related to Period III), 2 C/N (related to Period II), and 1 C/N (related to Period I) SS samples collected from the S-SBR pilot plant. In particular, the samples were collected at the end of each experimental period (45 days) after the feast-famine cycle through the sampling port of the S-SBR reactor, when the monitored S-SBR parameters (i.e., sCOD and NH₄⁺-N removal efficiencies) remained stable (Table 7.3). Thus, the sampling occurring at the end of the selection process provided a snapshot of the microbial community structure shaped by the operational conditions. This sampling allowed the feast-famine cycle selection pressure to shape microbial populations with the most productive species represented by their relative abundances. Thus, sampling at this point ensures more consistent and reliable data that accurately represent the microbial community productive capacity.

For bacterial microbiota analysis, amplicon sequencing targeting the 16S rDNA gene, obtained starting from SS metagenomic DNA, was performed through NGS. Most ASVs identified in this study were assigned to the Bacteria domain. In the 3.5 C/N sample, 100% of the microorganisms belonged to the bacterial domain, and no Archaea were found. In sample 2 C/N, 99.96% of the organisms that formed the microbiota of the sample were Bacteria, and only 0.04% were Archaea. Finally, in the 1 C/N sample, 99.88% of the microorganisms belonged to the bacterial kingdom and 0.12% to the Archaea. It is not the first time that the predominant component in an SS microbiota is Bacteria, which prevails over Archaea. Previous studies, in line with our results, have shown Bacteria as the dominant prokaryotic population in SS (98.8%), with Archaea accounting for only 1.2% (Yu et al., 2012; Zhang et al., 2012; Xia et al., 2010; McLellan et al., 2010; Mathur et al., 2023). In our previous work, a preponderance of Bacteria compared to Archaea was found in the SS microbiota (Di Leto et al., 2024). The initial SS sample was subjected to a metataxonomic analysis of the prokaryotic microbial community structure (Figure 7.S1). The initial microbiota structure was composed primarily of Bacteria rather than Archaea. The predominant phyla were Proteobacteria (48.7%), Bacteroidetes (41.5%), Verrucomicrobia (3.6%), and OD1 (2.0%) (Figure 7.S1-A). The families mainly represented were Saprospiraceae (17.9%), Comamonadaceae (8.1%), Rhodocyclaceae (5.8%) and Beijerinckiaceae (4.7%) (Figure 7.S1-B). The structure of prokaryotic microbiota residing in the 3.5, 2 and 1 C/N samples was analysed by comparing the relative abundances at the phylum, family, and genus levels. This analysis revealed that Proteobacteria was the most abundant phyla, with the highest percentage abundance (74.91%) in the 3.5 C/N sample (Figure 7.3). The percentage abundance values in the other two samples were lower,

consisting of 69.05% and 73.20% in 2 and 1 C/N. The second most abundant phylum was Bacteroidetes, with the highest percentage of 24.85 % found in the 2 C/N and 19.11 and 18.68 % 1 and 3.5 C/N samples, respectively.

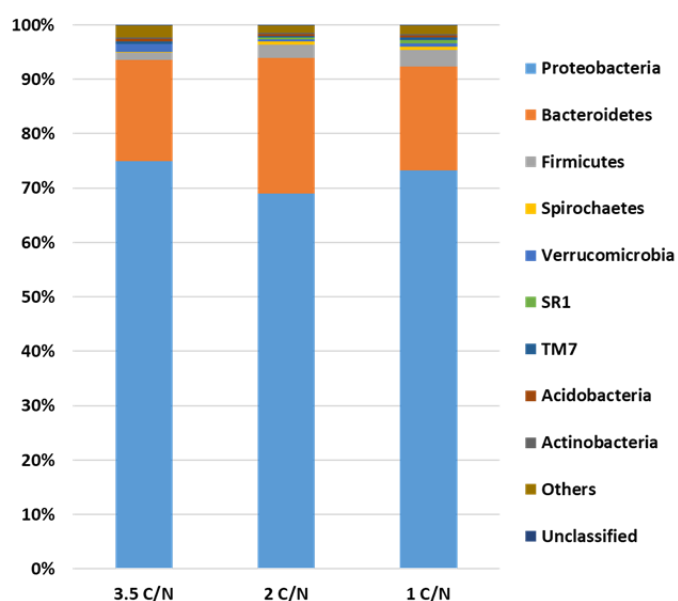


Figure 7.3. Histograms reporting the prokaryotic microbiota relative abundances of 3.5, 2 and 1 C/N condition samples at the level of phyla. The relative abundance indicated the percentage of each phylum compared to all the identified phyla. Taxa having percentage abundance lower than 1 are grouped as “Others”.

7.3.4 Bacterial signature

Understanding the bacterial signature of the relative abundance ratio between Proteobacteria and Bacteroidetes helps to study microbial relationships within an ecosystem. Indeed, these data reveal a preponderance of Proteobacteria compared to Bacteroidetes in the sample with the best PHA yield. Specifically, in this study, the relative abundance ratio between Proteobacteria and Bacteroidetes was 4.02, 2.78 and 3.84 in 3.5, 2 and 1 C/N SS samples. Interestingly, in all three final samples the increase in Proteobacteria phylum members, the best PHA producers (Sruamsiri et al., 2020), and a decrease in Bacteroidetes, Verrucomicrobia and OD1 phylum members was observed with respect to the initial SS microbiota. In addition, at the family level, Saprospiraceae and Beijerinckiaceae decreased in all C/N values with respect to the initial SS microbiota. At the same time, Comamonadaceae increased in 2 C/N and 1 C/N and decreased in 3.5 C/N in comparison to the initial SS sample microbiota. In contrast, Rhodocyclaceae, belonging to the Proteobacteria, increased in 3.5 C/N and decreased in the other two 2 and 1 C/N samples.

Proteobacteria are outstanding producers of PHA. Sruamsiri et al. (2020) identified them as the predominant phylum in PHA production. Additionally, Liu et al. (2013) investigated the impact of high COD/N levels (10-125) on maximising PHA yields from SS in an anaerobic/aerobic cycle. They recognised Proteobacteria as among the influential PHA producers. In this context, Sruamsiri et al. (2020) discovered that among the various classes of Proteobacteria, Betaproteobacteria (51.37%) were the ones contributing to the highest PHA yields, followed by Gammaproteobacteria (23.44%), and Alphaproteobacteria (13.26%). In line with these results, in this work, a high percentage of Betaproteobacteria was found in the sample, which gave the maximum yield of the PHA produced (Figure 7.S2). In fact, in the 3.5 C/N SS sample, the percentage of Betaproteobacteria was 90.23%, while in the 2 and 1 C/N SS samples, they were 59.62 and 65.15 %, respectively. Gammaproteobacteria (5.47, 33.67 and 28.71 % in 3.5, 2 and 1 C/N conditions, respectively) and Alphaproteobacteria (2.77%, 3.32 and 3.27 % in 3.5, 2 and 1 C/N conditions, respectively) were present in lower percentages than Betaproteobacteria. Deltaproteobacteria and Epsilonbacteria were present in minority concentrations. Bacteroidetes are generally considered consumers more than producers of PHA (Eronen-Rasmus et al., 2022). These bacteria are known for degrading complex organic materials and consuming PHA produced by other microorganisms rather than storing it as a carbon and energy reserve. Although they may possess some genes involved in the biosynthesis of PHA (Tyagi et al., 2021; Vincente et al., 2023), their primary role in the carbon cycle is often the decomposition of organic substances. From the perspective that Proteobacteria are seen as PHA producers and Bacteroidetes as potential PHA degraders, the bacterial signature linked to the relative abundance ratio between Proteobacteria and Bacteroidetes becomes significant since a higher ratio was observed in favour of Proteobacteria in the 3.5 C/N sample yielding the maximum PHA production (Figure 7.S2). However, there was also an interestingly high percentage of Firmicutes and Verrucomicrobia phyla. Firmicutes were less abundant in 3.5 C/N (1.34%) compared to 2 and 1 C/N, where they were more abundant (2.50% and 3.06%, respectively). Verrucomicrobia were most abundant in the 3.5 C/N sample (1.48%) compared to 2 and 1 C/N samples (0.32% and 0.68%, respectively). There is limited knowledge regarding the role of Verrucomicrobia in PHA production. However, Hanson et al. (2016) identified this bacterial phylum among the minority phyla (i.e. with a percentage abundance of 0.6-3%) in MMC involved in PHA production, suggesting a potentially positive role for this bacterial phylum. Firmicutes are generally considered good producers of PHA (Reddy et al., 2012). Several members of this phylum are known for their ability to produce and accumulate PHA as a carbon and energy reserve. These bacteria can

synthesise various types of PHA in response to stress conditions or excess carbon (Chou et al., 2023). However, their relative abundance is inversely correlated with the total PHA produced under the three C/N conditions assayed. The phyla Spirochaetes and Actinobacteria are present in very low percentages, but they show an increasing trend from 3.5 C/N (0.04% and 0.21%, respectively) to 2 C/N (0.60% and 0.25%, respectively) and 1 C/N sample (0.60% and 0.30%, respectively). Spirochaetes are not known to be PHA producers, while some bacterial genera belonging to the Actinobacteria, such as *Rhodococcus*, are excellent PHA producers (Trakunjae et al., 2021). However, their low percentage in the three analysed samples will have meant that they contributed minimally to the final yield of PHA obtained.

7.3.5 Structure of prokaryotic communities of SS at family level: Proteobacteria/Bacteroidetes subdivision

Examining the families provides deeper insights into the relationships within microbial communities concerning PHA production and different C/N conditions. At this taxonomic level, the significance of the bacterial signature previously discussed in the Proteobacteria/Bacteroidetes phyla context is even more pronounced. All the detected families (excluding unclassified and "others" with a percentage lower than 1%) with a considerable percentage abundance, exceeding 1%, belonged to the Proteobacteria or the Bacteroidetes, as shown in Figure 7.4. Considering the families belonging to the Proteobacteria phylum first, it is possible to note that the most abundant family in the 3.5 C/N sample is certainly the Rhodocyclaceae (9.16%), followed in a smaller percentage by Campylobacteraceae (1.27%) and Nitrosomonadaceae (1.31%). These families had a lower percentage abundance in the 2 C/N condition (4.44%, 0.95%, and 1.04%, respectively) and 1 C/N (3.49%, 0.80%, and 0.47%, respectively). On the other hand, samples from 2 and 1 C/N had higher percentages of Comamonadaceae (20.07% and 27.94%), Moraxellaceae (13.34% and 14.59%), and Pseudomonadaceae (5.90% and 6.30%), whereas in 3.5 C/N condition they were present in lower percentages (4.84%, 1.28%, and 0.69%, respectively). All the families mentioned above, belonging to Proteobacteria, are recognised for their excellent PHA production capabilities (Perez-Zabaleta et al., 2021). Still, the Campylobacteraceae and Nitrosomonadaceae families are less extensively documented for PHA production. Analysing the composition of the families belonging to the Bacteroidetes phylum, it is possible to note a marked percentage abundance of Flavobacteriaceae in 3.5 C/N condition (7.94%) compared to 2 and 1 C/N samples (2.63% and 2.62%, respectively). The scientific literature does not provide much information regarding the capabilities of

Flavobacterium; however, recent studies (Zhou et al., 2023) report that some genera belonging to the family mentioned above can exploit VFA to produce PHA to be used as their energy reserve. In 2 and 1 C/N conditions, there were also more significant percentages of Saprospiraceae (3.43% and 2.44 %, respectively), Chitinophagaceae (2.77% and 2.12 %, respectively), Porphyromonadaceae (2.83% and 1.96 %, respectively), and Weeksellaceae (2.24% and 1.62%, respectively) as compared to 3.5 C/N (2.53%, 1.24%, 0.58%, and 0.13% for Saprospiraceae, Chitinophagaceae, Porphyromonadaceae and Weeksellaceae, respectively). Interestingly, all of them are known to be PHA degraders (Lv et al., 2024; therefore, these findings paralleled the reduced PHA yields in the 2 and 1 C/N samples compared to the 3.5 C/N sample.

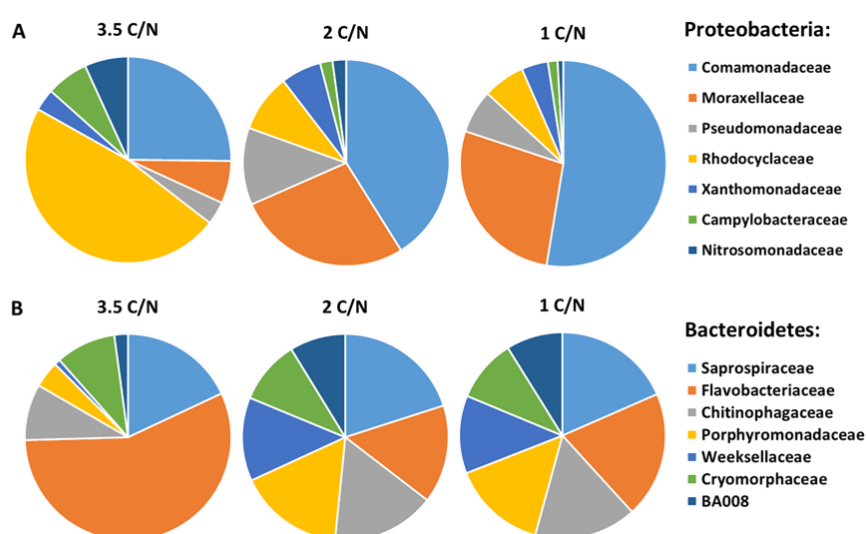


Figure 7.4. Pie charts reporting the microbiota taxonomic composition, at the level of family, of 3.5, 2 and 1 C/N condition samples, considering members belonging to Proteobacteria (A) and Bacteroidetes (B) phyla. The relative abundance represented by the pie charts indicated the percentage of each family compared to all the identified families that belonged to the Proteobacteria (A) or Bacteroidetes (B) phyla.

7.3.6 Structure of SS prokaryotic microbiota at the genus level

To better understand how the microbial community changed after the selection process due to the different C/N conditions, the bacterial genera that comprised the microbiota of the three different SS samples were also analysed (Figure 7.5). The 3.5 C/N sample, which produced the highest PHA yield, exhibited a higher percentage of *Flavobacterium* (7.97%)

at the genus level, along with smaller percentages of *Aquabacterium* (1.64%) and *Arcobacter* (1.25%). The percent abundance of these three bacterial genera was reduced in samples 2 C/N (2.61%, 1.13%, and 1.25%, respectively) and 1 C/N (2.63%, 0.94%, and 0.80 %, respectively). On the contrary, a greater percentage abundance of the genera *Comamonas*, *Acinetobacter*, *Pseudomonas*, and *Paludibacter* was identified in the samples 2 C/N (15.82%, 13.37%, 5.87% and 2.24% respectively) and 1 C/N (24.44%, 14.61%, 3.61%, and 1.41% respectively) compared to 3.5 C/N (0.00%, 0.37%, 0.00%, and 0.02% respectively). *Flavobacterium* and *Paludibacter* belong to the *Bacteroidetes* phylum, whereas all the other genera belong to the *Proteobacteria* phylum. At the genus level, as with phyla and families, the signature of *Proteobacteria/Bacteroidetes* is evident, while other phyla constitute a minority. Interestingly, *Flavobacterium*, the most abundant bacterial genera in the 3.5 C/N sample, is reported to possess PHA production capabilities. In particular, Wang et al. (2017) investigated how dissolved oxygen during the feast phase of the feast-famine cycle influences the selection of bacteria and yield of PHA production. They found that *Flavobacterium* was one of the primary bacterial genera involved, showing a preference for butyrate and valerate over acetate and propionate as substrates. Limited knowledge exists about *Aquabacterium* and *Arcobacter* and their PHA production capabilities. However, the first PHA-producing strain from the *Aquabacterium* genus was recently isolated and identified from soil (Feng et al., 2023). Based on its structural characteristics, the PHAs produced by this strain were determined to be poly(3-hydroxybutyrate) (PHB) (Feng et al., 2023). Concerning *Arcobacter*, this genus was mainly represented in reactors in which PHA production was maximised (Liu et al., 2023). *Comamonas* and *Pseudomonas* can synthesize PHAs as intracellular carbon and energy storage compounds (Correa-Galeote et al., 2022). Still, *Acinetobacter* and *Paludibacter* are mainly considered degraders, especially of PHBs (Cho et al., 2021). Therefore, given the preponderance of producing bacteria in all three samples analysed, it is clear that the greater PHA yield can be attributed, in the 3.5 C/N sample, to the greater percentage abundance of *Flavobacterium* and *Aquabacterium*, while the lower PHA yield in samples 2 and 1 C/N condition samples is to be attributed to a high percentage of *Acinetobacter* as it can also be surmised by heatmap representation of microbiota structure. This finding indicates that a 3.5 C/N effectively favoured the selection of PHA producers compared to the other two C/N conditions. Additionally, the clustering analysis supports this finding, as the 3.5 C/N sample forms a separate cluster, distinct from the other two samples, which instead cluster together.

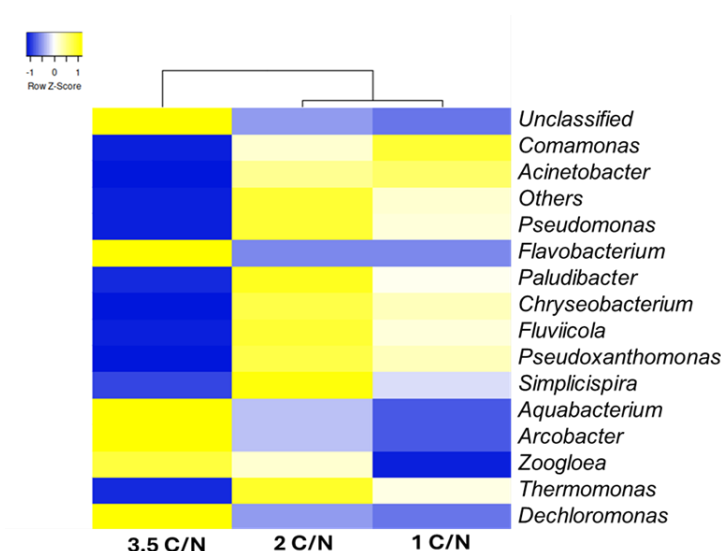


Figure 7.5. Heatmap of 16 most abundant prokaryotic genera in the microbiota of 3.5, 2 and 1 C/N condition samples as inferred by HeatMapper bioinformatic analysis. The color intensity in each sample was normalized to represent its relative ratio in the two groups. Colors from blue to yellow indicated the relative values of microbiota.

7.3.7 Structure of SS eukaryotic microbiota at kingdom level

Little is known about eukaryotes of MMCs from SS microbiota that produce PHA from SS. Most research has focused on bacteria (Fukala et al., 2024), while eukaryotes, including fungi, have yet to be studied in this context. Since the ITS region was used as a marker for analysis, all eukaryotic microorganisms in the different samples were screened at the kingdom level (Figure 7.6), while the focus at the phylum level was primarily on the fungal community (Figure 7.7). Figure 7.S3 shows a phylogenetic tree based on the ITS marker. Kingdoms were highlighted using colour code, while classes were shown for each taxon. Most clusters comprise the fungal community—considerable variance between the treatments 3.5 C/N and 1 C/N and between 2 C/N and 1 C/N. As highlighted in Figure 7.S4, the Principal Coordinate Analysis (PCoA) of the eukaryotic community based on the weighted UniFrac distance provides a visual representation of the analysed data, making it easier to identify that the trends obtained represent a high similarity between the two replicates of each treatment. The main kingdoms found were 5 (Figure 7.6), excluding a small percentage (0-0.09%) of eukaryotic *Incertae sedis*. Alveolata were present at an average rate of 97.17% in 3.5 C/N, 59.42% in 2C/N, and 22.09% in 1C/N, decreasing in proportion to the reduction in the C/N value. Fungi were present at 0.99% in 3.5 C/N, 7.24% in 2 C/N, and 66.30% in 1 C/N. Viridiplantae were present at 0.46% in 3.5 C/N, 21.49% in

2 C/N and 8.24% in 1 C/N. Stramenopila were present at 0.92% in 3.5 C/N, 9.92% 2 C/N and 1.90% in 1 C/N. Finally, Rhizaria were absent in 3.5 C/N, while in 2 C/N, they were 0.01% and 0.21% in 1 C/N. Many of the members of these groups, all characterised by the presence of the ITS region within their DNA, are microorganisms. Still, some also include macroscopic organisms visible to the naked eye. It is evident to note, as in the 3.5 C/N sample, which gave the maximum PHA yield, that the majority of the eukaryotes analysed are Alveolata. The Alveolata, a group of eukaryotes that includes dinoflagellates, apicomplexa, and ciliates, have not been recognised for their capability to produce PHA yet. Therefore, further investigations must be undertaken to explore their potential role in PHA production, especially considering their significant presence in the sample that yielded the highest PHA among those tested. Unfortunately, not even eukaryotes such as Viridiplantae (green algae and land plants), Stramenopila (diatoms, brown algae) and Rhizaria (foraminifera, radiolarians) are known for their ability to produce PHA. However, fungi are the only eukaryotes recognised for their capability to produce PHA, encompassing various genera of filamentous fungi and yeasts (Wu et al., 2017; Liu et al., 2021). However, some phyla of fungi are PHA degraders (Lee et al., 2005).

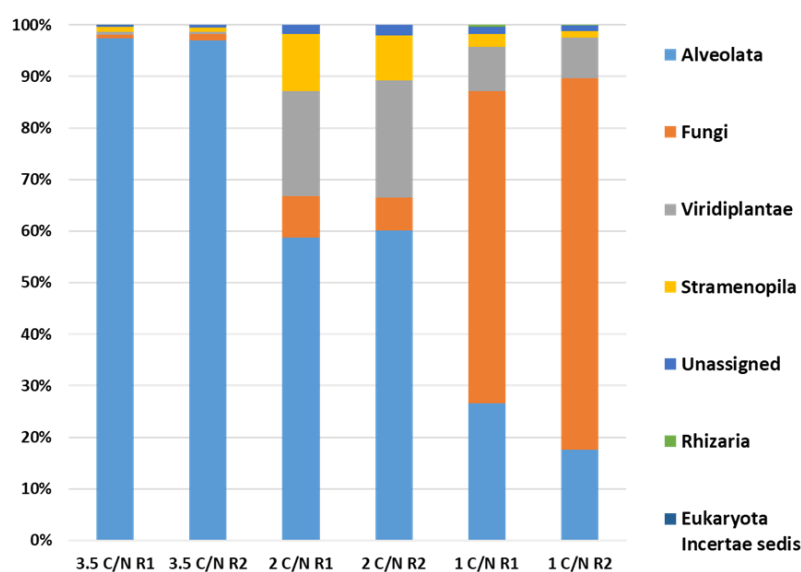


Figure 7.6. Histograms reporting the relative abundances of eukaryotic microbiota taxa at the level of kingdom of sewage sludge from 3.5, 2 and 1 C/N condition samples. The relative abundance indicated the percentage of each kingdom compared to all the identified kingdoms.

7.3.8 Structure of SS eukaryotic microbiota at the phylum level

To infer the ability to produce or degrade PHA, the fungal community was analysed at the taxonomic level of phylum (Figure 7.7). In the 3.5 C/N sample, the fungal community constitutes only 1.4% of the phyla identified (98.96% of others). This finding provides two significant insights: first, that PHA production in this sample is unlikely to originate from fungi; second, it suggests a potential involvement of Alveolata, the most abundant kingdom in the 3.5 C/N treatment, in PHA production. As the C/N value decreases, the fungal community has a corresponding increase. In the 2 C/N sample, 7.39% of the identified phyla were attributed to fungi, while 92.61% belonged to other organisms. Notably, a significant increase was observed in the Blastocladowmycota phylum, which reached 3.89% in the 2 C/N samples. Blastocladiomycetes are aquatic fungi that produce flagellated zoospores. They are known to be efficient decomposers, especially in degrading organic matter. It is documented that there is a correlation between the procariotic and eukaryotic microbiota members. In fact, in microbial community, Alveolata and bacteria members can establish symbiotic interactions that can determine concomitant changes of their abundance (Fokin and Serra, 2022) as well as fungi can interact with bacteria in different and even unpredictable ways, including competition and establishment of trophic chains, so that changes in bacterial and fungal community structures can parallel (Di Bella et al, 2023;Gralka et al., 2020).

Most studies on Blastocladowmycota focus on their function in the carbon and nitrogen cycle, emphasising the decomposition of plant organic matter and other organic materials in soil and aquatic environments (Watkinson et al., 2015). However, no evidence suggests that Blastocladowmycota can degrade or produce PHAs. Regarding the treatment with 1 C/N, the fungal community increased to 67.14% (others were 32.86%). The main phyla were Basidiomycota (49.03%), Ascomycota (11.04%), and Rozellomycota (6.25%). Basidiomycota, Ascomycota, and Rozellomycota are fungal phyla with diverse ecological roles, including decomposition of organic matter. While some fungi within these phyla are efficient decomposers of various organic compounds, including complex polymers like cellulose and lignin, there is limited evidence to suggest that they are natural degraders of PHA under normal environmental conditions. Zhou and collaborators (2022) noted that if microplastics or PHAs are added to a fungal community, mainly these phyla reduce its percentage abundance. Therefore, the fact that these three species are more abundant in 1 C/N sample may be dictated by the fact that there is the lowest concentration of PHA among the different treatments. These phyla may degrade PHA under specific conditions or in consortia with bacteria, but this capability is not well-documented compared to bacterial

degradation. Therefore, their role in PHA degradation remains an area for further research and exploration.

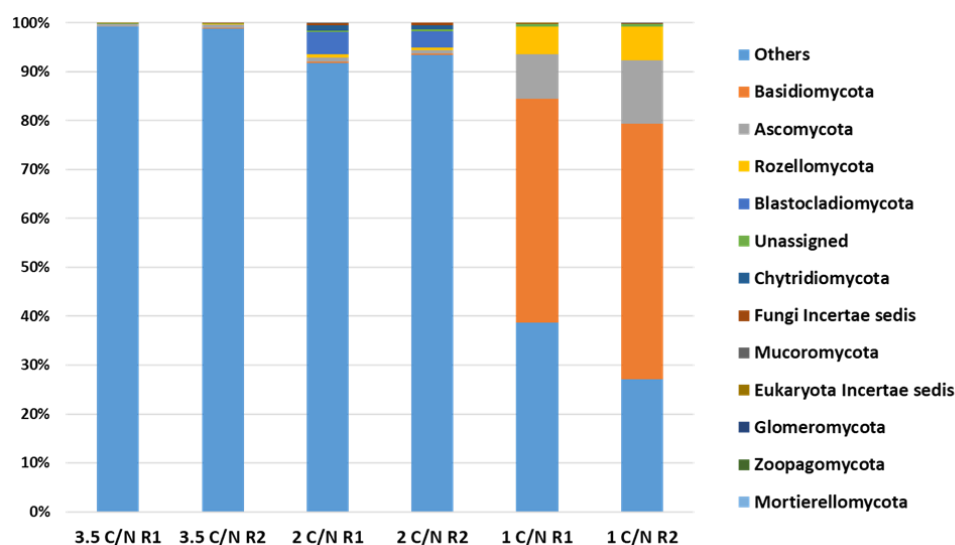


Figure 7.7. Histograms reporting the relative abundances of fungal microbiota at the level of phylum of sewage sludge from 3.5, 2 and 1 C/N condition samples. The relative abundance indicated the percentage of each phylum compared to all the identified phyla. Taxa belonging to eukaryote that that did not belong to the fungal community are grouped together like “Others”.

7.4 Conclusions

This study observed the SS microbiota structure modulation depending on three C/N settings during a feast-famine cycle regime to produce PHA in a selective-sequencing batch reactor. This experimental campaign allowed us to determine some criteria for optimising the yield of PHA production after a month. First of all, the C/N ratio, within a feast-famine cycle, allowed the selection of good PHA-producing bacteria such as Proteobacteria. In this case 3.5 C/N made possible a greater PHA yield. Therefore, by carefully applying the criterion of controlled feeding during the feast and famine phases, where the reactor receives liquid from the fermented sludge during the aerobic phase and effluent from the N-SBR during the anoxic phase in the pilot plant, we can achieve improved PHA yields. Indeed, the C/N value is a key parameter in optimising the PHA production by SS-MMC. Metataxonomic analyses revealed that the predominant bacterial phylum was Proteobacteria, particularly Betaproteobacteria, known for their valuable PHA-producing capabilities, followed by some PHA-producing genera in the Bacteroidetes phylum, such as *Flavobacterium*. A distinct

bacterial signature promoting Proteobacteria over Bacteroidetes was evident at the family level, particularly in the 3.5 C/N condition. At the level of the eukaryotic community, despite limited existing literature, certain phyla seem to play roles in various aspects of PHA production dynamics. This study could establish the basis for future investigations in this area. Hence, this study offers significant insights into the prokaryotic and eukaryotic. Optimised PHA production lays the groundwork for forthcoming biotechnological and industrial advancements for modulating MMC structure. In addition, it underscores the potential of exploiting wastewater from the UniPA Campus in circular economy practices. Converting this wastewater stream into valuable materials such as PHAs would substantially reduce waste disposal and environmental footprint while boosting bioplastic manufacturing in a sustainable small-scale WRRF.

7.5 References

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7.6 Supplementary material

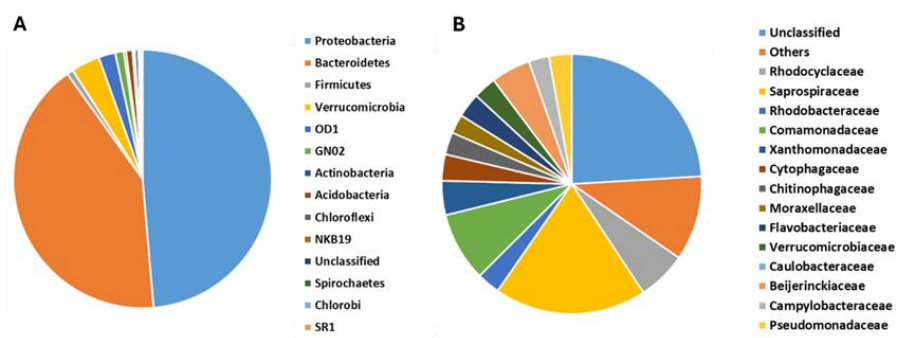


Figure 7.S1. Pie charts reporting the bacterial microbiota structure of SS inoculum used for the experimental setup at the taxonomic level of phylum and family. The relative abundances, represented by the pie charts, indicated the percentage of each taxon in the respect to all identified taxa for each taxonomic level.

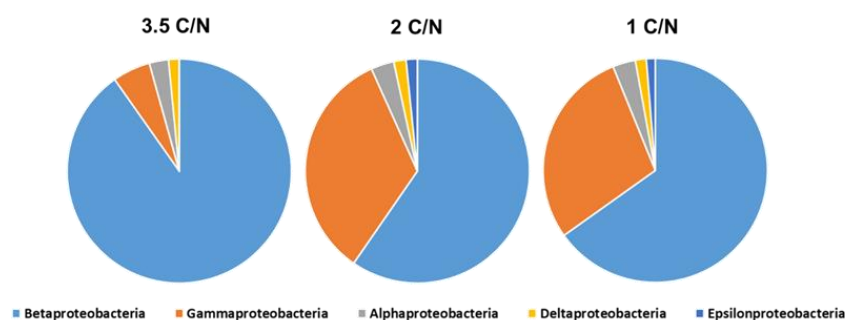


Figure 7.S2. Pie charts reporting the Proteobacteria composition at the taxonomic level of class of the microbiota of 3.5 C/N, 2 C/N, and 1 C/N S-SBR samples. The relative abundance represented by the pie charts indicated the percentage of each class compared to all the identified classes that belonged to the phylum.

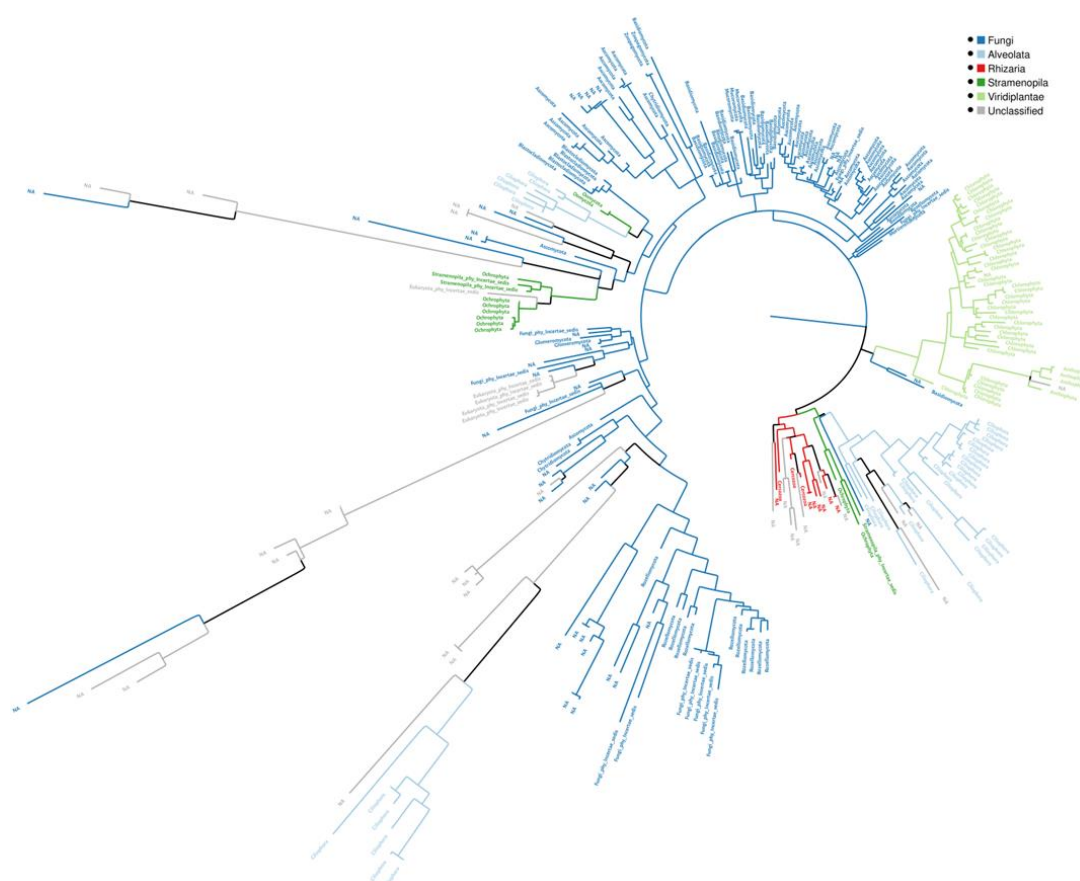


Figure 7.S3. Phylogenetic tree, based on the ITS marker, of the eukaryotic microbiota structures of the S-SBR samples. Kingdoms were highlighted using colour code (highlighted in the legend), while classes were shown for each taxon.

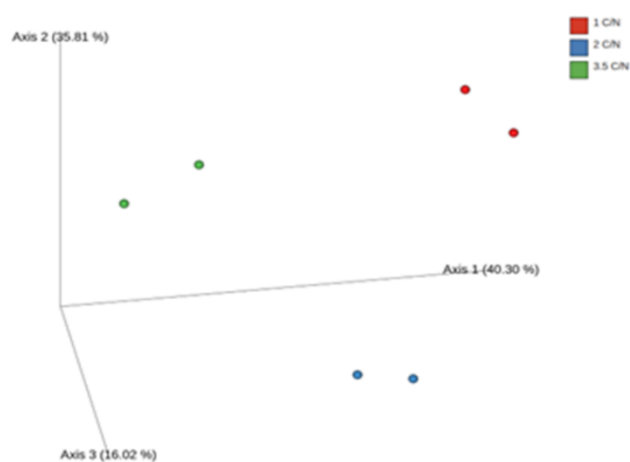


Figure 7.S4. Principal Coordinate Analysis (PCoA), based on the weighted UniFrac distance, of the eukaryotic microbiota structures of the S-SBR samples at the different C/N values.

CHAPTER 8

**Effects of treated wastewater reuse, enriched biochar,
and zeolite on microbial community structure,
soil fertility, crop growth**



**Water reuse of treated domestic wastewater in agriculture:
Effects on tomato plants, soil nutrient availability
and microbial community structure**

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Nutrient enriched biochar and zeolite as fertilizers counteract drawbacks of treated wastewater used for fava irrigation

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The effects of enriched biochar and zeolite and treated wastewater irrigation on soil fertility and tomato growth

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ABSTRACT

With rising global populations, the need for sustainable methods to boost soil fertility and crop productivity has grown, as reliance on chemical fertilizers presents economic and environmental challenges. Treated wastewater (TWW) emerges as a promising alternative, offering irrigation benefits and nutrient recovery, particularly when filtered with biochar and zeolite absorbent matrices that can be used as amendments. This work examines the reuse of TWW alone and alongside enriched biochar and zeolite, focusing on changes in the microbial community within the treated soil. Non-enriched biochar and zeolite were also used as controls. An experimental setup was designed to test the effects of TWW on tomato plants, comparing it with tap water (TAP) and tap water supplemented with fertilizer (IFW). Additionally, two setups were conducted to examine the effects of TWW combined with enriched biochar and zeolite on fava bean and tomato plants. In comparative tests with tap water (TAP) and an inorganic NPK solution (IFW), the TWW and IFW treatments significantly increased soil nitrogen, while only IFW boosted overall tomato biometric growth parameters. TWW and IFW also fostered a higher abundance of beneficial Proteobacteria, Acidobacteria, and genera such as *Balneimonas* and *Steroidobacter*. The results of the study also demonstrated that TWW irrigation increased soil nitrogen and phosphorus availability, thereby promoting crop growth. However, it also led to an increase in soil electrical conductivity, indicating heightened salinity. The application of enriched biochar and zeolite effectively mitigated these salinity effects, acting as slow-release fertilizers and enhancing biomass and nutrient uptake in fava and tomato plants. While TWW alone did not fully meet the nutrient requirements of tomato plants, it did encourage the growth of beneficial plant-growth-promoting bacteria (PGPB) like *Bacillus* and *Balneimonas*, particularly in soils treated with non-enriched biochar and zeolite. Notably, the microbial analysis showed that TWW irrigation had no adverse impact on the soil prokaryotic community structure, and the harvested pods and seeds were free from pathogenic bacteria, thereby confirming the safety of TWW in agriculture. The findings underscore the potential of TWW, along with enriched biochar and zeolite, to sustainably support crop productivity and soil health. This presents a viable, circular economy approach for resource recovery and sustainable agriculture.

8.1 Introduction

The rapid growth of the global population and associated challenges of water scarcity and deteriorating water quality pose critical threats to agricultural sustainability, food security, and environmental resilience. Estimates suggest that by 2025, 1.8 billion people will face

limited access to fresh water, a situation worsened by climate change, increased demand across sectors, and unsustainable water use (Hashem & Qi, 2021; Lahlou et al., 2021; Andrews et al., 2016). Agriculture, which accounts for 80% of freshwater consumption, is particularly vulnerable and requires innovative approaches for managing water and nutrients sustainably (Lahlou et al., 2021). Among these solutions, treated wastewater (TWW) represents a promising resource, addressing water scarcity and nutrient recovery, thereby reducing the reliance on chemical fertilizers and mitigating environmental pollution (Aziz & Farissi, 2014; Saliba et al., 2018; Urbano et al. 2017). Despite its potential, the global adoption of TWW remains low, primarily due to social, economic, and regulatory barriers (Salgot & Folch, 2018; Mannina et al., 2021a).

Adequately treated wastewater can be microbiologically safe for crop irrigation, aligning with circular economy principles by reducing effluent discharge and enhancing nutrient recycling (Elbana et al., 2019; Ofori et al., 2021; Lucia et al., 2022). TWW contains valuable nutrients like nitrogen (N) and phosphorus (P), essential for plant growth, yet additional fertilization may be required for nutrient-intensive crops. However, TWW can introduce high levels of salts and dissolved ions (e.g., sodium, potassium, magnesium, chloride), which may negatively impact soil health and microbial communities at elevated concentrations (Gao et al., 2021b; Zhang et al., 2018). Consequently, combining TWW irrigation with soil amendments that enhance nutrient retention and mitigate potential salt accumulation is an emerging focus.

Biochar and zeolite, two materials increasingly applied for nutrient capture in TWW, show promise as soil amendments. Biochar, a carbon-rich product of organic matter pyrolysis, is recognized for its high surface area and functional groups, allowing it to adsorb phosphate effectively (Dai et al., 2020). Biochar improves water retention, soil structure, and microbial activity, promoting overall plant health (Hardie et al., 2014; Brassard et al., 2019; Wang et al., 2022). Zeolite, a tectosilicate mineral with a crystalline structure and high cation exchange capacity, effectively adsorbs ammonium, supporting slow-release fertilization (Moshoeshoe et al., 2017). When used as soil amendments, enriched biochar, and zeolite improve soil fertility, enhance water retention, and foster microbial diversity, making them valuable for nutrient recovery and sustainable agricultural practices (Mannina et al., 2021b; Sengupta et al., 2015; Muscarella et al., 2023a, 2023b).

Soil microbiota, particularly plant-growth-promoting bacteria (PGPB), plays a vital role in nutrient cycling, soil health, and plant growth, producing bioactive compounds that enhance nutrient availability and improve resilience to environmental stressors (Putatunda et al.,

2024; Al-Tammar et al., 2022; Demissie et al., 2013). Biochar has been shown to modulate microbial diversity and increase enzymatic activity, although zeolite's impact on microbial communities remains less explored (Li et al., 2020; Pokharel et al., 2020; Bai et al., 2022; Ibrahim et al., 2023). Additionally, irrigation with TWW can alter soil microbiota structure, particularly in the short term, promoting bacterial phyla like Proteobacteria, which support nutrient dynamics and plant growth (Delitte et al., 2021; Frenk et al., 2018; Muscarella et al., 2024). However, the effects of combining TWW irrigation with nutrient-enriched biochar and zeolite on soil microbial communities and their implications for crop growth have not been extensively studied. First, the effect of TWW compared with TAP and IFW on tomato plants focuses on the microbiota of the treated soil was studied. Secondly, this research evaluates the impact of combining TWW irrigation with nutrient-enriched biochar and zeolite on soil fertility, microbial community composition, and plant growth. Using tomato (*Solanum lycopersicum L.*) and fava bean (*Vicia faba L.*) as model crops, this study focuses on N and P dynamics, microbial biomass, and plant growth parameters, providing insights into sustainable nutrient management practices and their implications for soil health and agricultural productivity (Mannina et al., 2021a; Mannina et al., 2022a; Wali et al., 2022; Faddetta et al., 2023).

8.2 Material and methods

8.2.1 Experimental setup to compare TWW, TAP and IFW in tomato plants

The experiment was conducted in a greenhouse on the University of Palermo campus (Palermo, Sicily, Italy). Tomato plants (*Solanum lycopersicum L.*) were selected as test subjects due to their tolerance to high temperatures. The experimental setup followed a completely randomized design, incorporating three irrigation treatments: tap water (TAP), treated wastewater (TWW), and inorganic fertilized water (IFW). The pots (8×8×15 cm) were filled with approximately 10 g of expanded clay and 600 g of S5 soil sieved to 4 mm. S5 soil was collected in the University Campus and characterized by a higher concentration of total organic C. The IFW treatment involved an aqueous solution of inorganic fertilizers with an N:P ratio of 20:10:20 at a concentration of 1 g/L, applied once per week. Throughout the experiment, each IFW pot received a total of 200 mg of nitrogen (50% as NH_4^+ and 50% as NO_3^-), 44 mg of phosphorus (as P_2O_5), and 166 mg of potassium (as K_2O). Manual watering was conducted three times a week, with applications at 11:00 and 18:00 to prevent water percolation. The total water volume provided in the experiment was 3.3 liters. The soil

used in the pots was collected from Unipa Campus. The tap water used in the experiment was sourced directly from the storage tank serving the greenhouse where the study was conducted (Mannina et al., 2021a). The treated wastewater was collected from the pilot wastewater treatment plant in the Engineering Department at the University of Palermo (Italy) as part of the Wider Uptake H2020 project (Mannina et al., 2021b). For sanitization, the wastewater was treated with sodium hypochlorite at a 5 mg/L concentration. Table 8.1 presents the key physical and chemical properties of both TAP and TWW. The nutrient levels supplied by the irrigation treatments (per 600 g of soil) were as follows: TAP provided 0.7 mg of nitrogen (N), 0.07 mg of phosphorus (P), and 3.3 mg of potassium (K); TWW supplied 2.3 mg of N, 0.7 mg of P, and 73 mg of K; while IFW delivered 200 mg of N, 44 mg of P, and 166 mg of K. After 56 days, plant height, root length, and both fresh and dry weights were measured to assess plant health. Soil from each pot was mixed, air-dried, and sieved to under 2 mm for analysis. Soil pH and electrical conductivity (EC) were measured using a pH meter and a conductometer, respectively. Ammonium and nitrate (NO_3^-) were quantified from soil extracts using colorimetric analysis, while phosphorus (P Olsen) was determined via the Olsen method with sodium bicarbonate extraction, both using a spectrophotometer. The results are obtained by statistical calculation of the replicates. A two-way analysis of variance (ANOVA) of the measured variables was performed with type of irrigation treatment (TAP, TWW and IFW) as main factors. Significant statistical differences were established by Tukey's test ($P < 0.05$).

Table 8.1 Chemical characteristics of the tap (TAP) and treated wastewater (TWW) used for tomato irrigation.

Water property	TAP	TWW
pH	7.5 ± 0.1	8.1 ± 1
EC (dS m^{-1})	0.10 ± 0.0	1.1 ± 0.1
Total Hardness ($^{\circ}\text{f}$)	8 ± 1	32 ± 3
N (as NH_4^+ ; mg/L)	0.1 ± 0.0	0.5 ± 0.0
N (as NO_3^- ; mg/L)	0.1 ± 0.0	0.2 ± 0.0

Total P (mg/L)	0.02 ± 0.00	0.21 ± 0.0
Fe (mg/L)	0.1 ± 0.0	3.0 ± 0.2
Mn (mg/L)	0.001 ± 0.000	0.041 ± 0.000
Ca (mg/L)	14 ± 2	69 ± 6
K (mg/L)	1.0 ± 0.2	22 ± 3
Mg (mg/L)	2.0 ± 0.2	19 ± 3
Na (mg/L)	3.0 ± 0.2	155 ± 27

8.2.2 Experimental setup to evaluate TWW, biochar and zeolite effect on fava bean plants

The experiment was conducted in a greenhouse at the University of Palermo, built as part of the Wider Uptake H2020 project (Palermo, Sicily, Italy) (Mannina et al., 2021a, 2021b). The study involved eight treatments, outlined in Table 8.2, which were classified based on the irrigation water used (tap water, TAP; treated wastewater, TWW) and the soil amendments applied (natural or enriched biochar, NB and EB, or natural or enriched zeolite, NZ and EZ). The experimental design was fully randomized, with each treatment replicated five times, resulting in forty pots.

Table 8.2. Experimental design. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively, or natural or enriched zeolite, NZ and EZ, respectively). The experimental design followed a complete randomization scheme with eight treatments, each replicated five times, for a total of forty pots.

Sample ID	Irrigation	Amendment
TAP		No
TAP – EB	Tap water	Enriched biochar
TAP – EZ		Enriched zeolite

TWW	No
TWW – EB	Enriched biochar
TWW – EZ	Treated Wastewater Enriched zeolite
TWW – NB	Natural biochar
TWW – NZ	Natural zeolite

TAP was drawn directly from the greenhouse storage tank (Mannina et al., 2021b), while the TWW used for irrigation came from a water recovery pilot plant at the University of Palermo, Italy (Mannina et al., 2021a). This plant utilizes an advanced membrane bioreactor for wastewater treatment (Mannina et al., 2022b; Mofatto et al., 2024). The wastewater was sanitized with 5 mg/L of sodium hypochlorite. The properties of TAP were pH 7.5, electrical conductivity 0.1 dS/m, NH_4^+ 0.1 mg/L, NO_3^- 0.1 mg/L, total P 0.02 mg/L, Fe 0.10 mg/L, Mn 0.001 mg/L, Ca 14.0 mg/L, K 1.0 mg/L, Mg 2.1 mg/L, and Na 3.0 mg/L. The properties of TWW were pH 8.4, electrical conductivity 0.9 dS/m, NH_4^+ 10.1 mg/L, NO_3^- 0.9 mg/L, total P 4.95 mg/L, Fe 0.12 mg/L, Mn 0.006 mg/L, Ca 127.5 mg/L, K 17.5 mg/L, Mg 11.9 mg/L, and Na 87.8 mg/L. Irrigation was done manually once daily to prevent water stress and avoid percolation. The biochar used in the study, provided by Nera Biochar s.r.l., was derived from pyrolyzed wood waste at 450°C, sieved to 2.0 mm, and had properties including a bulk density of 180 g/L, a pH of 9, and 75% total carbon. The natural zeolite was clinoptilolite (ZEOWATER ZN, Zeocel Italia), containing 85% clinoptilolite with a bulk density of 0.98 g cm⁻³ and a pH of 7.6. For the experiment, biochar, and zeolite were enriched with phosphate (PO_4^{3-}) and ammonium (NH_4^+), respectively, by passing treated wastewater through two columns filled with these materials in series, at a flow rate of 16.75 L h⁻¹ until saturation (Mannina et al., 2022a; Bulacio Fischer et al., 2024). The main characteristics of natural and enriched amendments were reported in Table 8.3.

Table 8.3. Main characteristics of natural and enriched biochar (NB and EB, respectively) and natural and enriched zeolite (NZ and EZ, respectively).

Parameters	Ca²⁺	K⁺	Na⁺	Mg²⁺	Total P	Total N
u.m.	(exchangeable cations) cmol(+) kg ⁻¹				mg g ⁻¹	mg g ⁻¹
NB	19.0 ± 0.9	7.0 ± 1.3	5.0 ± 0.6	4.3 ± 0.3	1.2 ± 0.1	n.d.

EB	42.7 ± 0.3	36.2 ± 1.0	16.8 ± 0.3	4.0 ± 1.1	2.7 ± 0.3	2.0 ± 0.1
NZ	53.0 ± 3.1	38.1 ± 2.9	8.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	n.d.
EZ	23.0 ± 1.0	20.9 ± 1.2	7.1 ± 1.9	5.3 ± 0.3	0.3 ± 0.1	13.1 ± 0.1

The soil for the experiment was collected from an orange orchard on the University of Palermo campus. It was air-dried and sieved to 4 mm, with a clay-loam texture, a pH of 7.9, electrical conductivity (EC) of 0.34 dS/m, total organic carbon (TOC) of 31 g/kg, total nitrogen (TN) of 1.4 g/kg, available phosphorus (P) of 129 mg/kg, and a cation exchange capacity of 7.98 mol/kg. After sieving, the soil was mixed with 10% perlite by volume to prevent pot compaction. Natural and enriched biochar and zeolite were incorporated into the soil at 10 g/kg, equivalent to 25 t ha⁻¹. Each pot (13 cm in diameter, 16 cm in height) was filled with 1.4 kg of soil, and two fava bean (*Vicia faba* L.) seeds were sown per pot. After sowing, the pots were irrigated with 300 mL of either TAP or TWW as per the experimental design.

8.2.3 Experimental setup to evaluate the TWW, biochar and zeolite effect on tomato plants

This study was conducted in the greenhouse at the Water Resource Recovery Facility of Palermo University (Palermo, Sicily, Italy), established as part of the Wider Uptake H2020 project (Mannina et al., 2021a, 2021b). Tomato plants (*Solanum lycopersicum* L.) were chosen for the trial due to their resilience to the high temperatures typical of Sicily during the experimental period. The study involved five treatment groups: control soil irrigated with tap water (TAP); soil amended with enriched biochar and enriched zeolite, irrigated with tap water (EB+EZ+TAP); soil irrigated with TWW; soil amended with enriched biochar and enriched zeolite, irrigated with TWW (EB+EZ+TWW); and soil amended with natural biochar and natural zeolite, irrigated with TWW (NB+NZ+TWW). The experimental design was fully randomized, using the same initial soil for all treatments, with five replicates for each treatment, resulting in twenty-five pots.

Tap water (TAP) was sourced directly from the storage tank supplying the greenhouse where the experiment occurred (Mannina et al., 2021b). TWW was obtained from a pilot wastewater treatment plant at the Engineering Department of the University of Palermo as part of the Wider Uptake H2020 project (Mannina et al., 2021a, 2021b). The characteristics of TAP were pH 7.5, EC 0.1 ds/m, NH₄⁺ 0.1 mg/L, NO₃⁻ 0.1 mg/L, total P 0.02 mg/L, Fe

0.10 mg/L, Mn 0.001 mg/L, Ca 14.0 mg/L, K 1.0 mg/L, Mg 2.1 mg/L, Na 3.0 mg/L. The characteristics of TWW were pH 8.4, EC 0.9 ds/m, NH_4^+ 10.1 mg/L, NO_3^- 0.9 mg/L, total P 4.95 mg/L, Fe 0.12 mg/L, Mn 0.006 mg/L, Ca 127.5 mg/L, K 17.5 mg/L, Mg 11.9 mg/L, Na 87.8 mg/L. Irrigation was manually performed once daily to prevent water stress on the plants and minimize excessive water percolation.

The biochar used in this study was supplied by Nera Biochar s.r.l. It was produced by pyrolyzing wood waste at 440°C and sieved to a particle size of 2.0 mm. The biochar had a bulk density of 180 g L⁻¹, a pH of 9, an electrical conductivity (EC) of 1.3 dS m⁻¹, a total carbon content of 75%, and a total limestone content of 5%. The zeolite used was Natural Clinoptilolite (ZEOWATER ZN) from Zeocel Italia by Dnd Biotech. Clinoptilolite belongs to the heulandite (HEU) group, with a tetrahedral structure consisting of SiO_4^{4-} and AlO_4^{5-} units. It is composed of 85% clinoptilolite, 8% cristobalite, 4% illite, and 3–4% plagioclase, with a Si/Al ratio of 4.8–5.5. The zeolite had a bulk density of 0.98 g cm⁻³, a surface area of 40 m² g⁻¹, a pH of 7.6, and K⁺ and Ca²⁺ as the predominant exchangeable cations. The zeolite was sieved to achieve particle sizes between 2.0 and 2.5 mm.

The experiment involved two plexiglass columns, each 0.1 m in diameter and 0.6 m in length. One column was filled with biochar, and the other with zeolite. These columns were arranged in series, with the treated wastewater first flowing through the biochar column, followed by the zeolite column, at a flow rate of 16.75 L h⁻¹ until saturation was reached (Bulacio Fischer et al., 2024). The main characteristics of the natural and enriched amendments are outlined in Table 8.4.

Table 8.4. Main characteristics of biochar natural and enriched (NB and EB, respectively) and zeolite natural and enriched (NZ and EZ, respectively). Reported values are means (n = 3) ± standard deviation.

Parameters	Ca ²⁺	K ⁺	Na ⁺	Mg ²⁺	Total P	Total N
u.m.	(exchangeable cations) mEq 100 g ⁻¹				mg g ⁻¹	mg g ⁻¹
NB	19.0 ± 0.9	7.0 ± 1.3	5.0 ± 0.6	4.3 ± 0.3	1.2 ± 0.1	n.d.
EB	42.7 ± 0.3	36.2 ± 1.0	16.8 ± 0.3	4.0 ± 1.1	2.7 ± 0.3	2.0±0.1
NZ	53.0 ± 3.1	38.1 ± 2.9	8.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	n.d.
EZ	23.0 ± 1.0	20.9 ± 1.2	7.1 ± 1.9	5.3 ± 0.3	0.3 ± 0.1	13.1±0.1

The soil used in the experiment was air-dried and sieved to 4 mm, with a sandy-loam texture. It was amended with 10% by weight of mature manure, which had 36% moisture and 39% total organic carbon. Key soil characteristics included a pH of 8.1, electrical conductivity (EC) of 0.91 dS m⁻¹ (1:2.5 w/v in water), 31 g kg⁻¹ of total organic carbon (TOC), 3.76 g kg⁻¹ of total nitrogen (TN), 129 mg kg⁻¹ of available phosphorus, and a cation exchange capacity (CEC) of 35 cmol/kg. Additionally, 10% perlite by volume was mixed in to prevent compaction. For the five replicates per treatment, approximately 15 kg of soil was used for the unamended treatments, irrigated with either TAP or TWW. For the amended treatments, 150 g each of enriched zeolite and biochar were added to 15 kg of soil (equivalent to 25 t ha⁻¹), while 75 g of non-enriched zeolite and biochar were added to 7.5 kg of soil (also equivalent to 25 t ha⁻¹). Each pot was filled with around 1.4 kg of soil according to the respective treatments, and one tomato seedling was planted per pot.

8.2.4 Post pot-experiment soil and plant analysis

After the growth periods (92 days for fava beans and 80 days for tomatoes), plants were harvested, and the soil from each pot was air-dried, manually homogenized, and sieved to 2 mm. Soil pH and electrical conductivity (EC) were measured using a pH meter (FiveEasy, Mettler Toledo Spa, Milan, Italy) and a conductometer (HI5321, Hanna Instruments Italia Srl, Padua, Italy), with a 1:2.5 w/v water ratio for tomatoes. Total nitrogen (TN) was determined by the Kjeldahl method (Bremner et al., 1996), and total organic carbon (TOC) was assessed using the Walkley-Black dichromate oxidation method (Nelson & Sommers, 1996). Available phosphorus (P) was quantified via the Olsen method, involving sodium bicarbonate extraction at pH 8.5 and subsequent spectrophotometric measurement after forming a blue complex (Olsen et al., 1954).

Plant biometric parameters, including height and fresh and dry weights, were evaluated. For fava beans, fresh and dry weights were taken to assess plant health, while for tomatoes, roots and shoots were separated and weighed, with roots being rinsed with distilled water before drying. Dried plant materials were then pulverized for further analysis. The TN content in both plant types was determined via the Kjeldahl method (Bremner et al., 1996). Total phosphorus (TP) was measured following wet acid digestion with nitric acid (HNO₃) and 30% hydrogen peroxide (H₂O₂) (Jones et al., 1990), with quantification using the Spectroquant® Phosphate spectrophotometer test to detect a yellow-orange complex. Total metal content (Ca, Mg, K, Na, Fe, Al, Cd, Ni, Pb, Zn) was analyzed in both plants by Microwave Plasma Atomic Emission Spectroscopy (MP-AES, Agilent 4210; Milan, Italy) following sample mineralization by wet digestion with HNO₃ and H₂O₂ (Jones & Case,

1990). The reported results represent the arithmetic means of five soil samples, expressed on a dry weight basis. Statistical analyses were conducted using one-way analysis of variance (ANOVA) for all treatments—eight for fava beans (TAP, TAP-EB, TAP-EZ, TWW, TWW-EB, TWW-EZ, TWW-NB, TWW-NZ) and five for tomatoes (TAP, EB+EZ+TAP, TWW, EB+EZ+TWW, NB+NZ+TWW). The Tukey test ($P < 0.05$) was used to identify significant differences among treatments. Data normality and homogeneity of variances were verified via Kolmogorov-Smirnov and Levene's tests prior to parametric analyses, which were conducted in SPSS 13.0. Additionally, for both fava bean and tomato samples, one-way ANOVA followed by Bonferroni and Holm methods was applied to metataxonomic data at the phylum and genus levels using an online tool (https://astatsa.com/OneWay_Anova_with_TukeyHSD/), with TAP as the control condition.

8.2.5 Prokaryotic metataxonomics

The structure of the prokaryotic microbiota in rhizospheric soil was analyzed through metataxonomic methods using next-generation sequencing (NGS) of 16S rDNA amplicons obtained from metagenomic DNA. DNA was extracted from soil microbiota using the DNeasy PowerSoil® Pro Kit (Qiagen), following the manufacturer's protocol. The extracted DNA was evaluated by 1% (w/v) agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide for visualization under UV light. DNA concentrations were measured at 260 nm absorbance using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purity was confirmed by absorbance ratios (260/280 and 260/230 nm) to detect potential contaminants like proteins, organic compounds, or chaotropic agents. The quality of metagenomic DNA was further validated by PCR analysis of the 16S rRNA gene, employing conditions specified by Di Leto et al. Amplified products underwent 300 bp paired-end sequencing on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). The raw 16S rDNA data were processed using QIIME2 software as paired-end sequences, with denoising and merging of overlapping reads via the DADA2 plugin. Unique amplicon sequence variants (ASVs) were aligned to the Greengenes reference database with 99% sequence similarity to classify taxonomic ranks (phyla, orders, classes, families, genera). ASV counts and relative abundances of each rank were determined for each sample. For fava plants, eight soil sample triplicates were analyzed (TAP, TAP-EB, TAP-EZ, TWW, TWW-EB, TWW-EZ, TWW-NB, TWW-NZ), collected after plant growth. For tomato plants, 15 soil samples, consisting of three replicates for each treatment, were analyzed after 80 days

of plant growth, with samples taken from soil surrounding the root system. In a separate set of water treatment tests (TAP, TWW, and IFW, each in duplicate), the V3-V4 regions of the 16S gene were amplified using the PRO 341F and PRO 805R primers and DreamTaq DNA polymerase (ThermoFisher Scientific) in a PCR protocol with thermal cycling conditions: 95°C for 2 minutes, 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 5 minutes. The ASV data processed through QIIME2 allowed for quantification of domains, phyla, orders, classes, families, and genera. In this study, the relative abundances of domains, phyla, and genera are highlighted and discussed. To evaluate the metabolic capabilities of soil microbiota following various irrigation treatments in the first setup (comparing TWW, TAP, and IFW), METAGENassist (<http://www.metagenassist.ca>) was utilized to analyze the predicted metabolic functions of the microbiota based on the abundances of resident prokaryotic genera (Figure 8.S1).

8.3 Results and discussion

8.3.1 Plant biometric data and effects of water irrigation on chemical soil properties of tomato plants irrigated with TWW, TAP and IFW

The type of water used for irrigating the tomato plants was the primary factor accounting for the most significant portion of variance (over 39%) in the biometric characteristics of the plants (Table 8.5). Plants irrigated with IFW showed the highest fresh and dry shoot and root weights, as well as the most significant height, followed by those irrigated with TWW and lastly with TAP water (Figure 8.1). These results corresponded to the nutrient levels provided by the irrigation treatments, in the order of IFW > TWW > TAP.

Table 8.5. Percentage of explained variance of the assessed biometric parameters of tomato irrigated with tap water (TAP), inorganic nutrient solution (IFW), and treated wastewater (TWW). *P<0.05; **P<0.01; ***P<0.001.

Biometric parameters	Soil	Water	Soil x Water
Plant height	29**	39***	NS
Root weight (dry)	15*	74***	NS
Root Weight (fresh)	30***	52***	NS

Shoot weight (fresh)	18*	73***	6*
Shoot weight (dry)	18*	64***	7*

Other researchers have reported similar positive outcomes with TWW. Leonel et al. (2022) found that TWW irrigation increased wheat grain yield nearly fivefold, likely due to higher nutrient content, such as nitrogen (N) and phosphorus (P), compared to conventional water. Zema et al. (2012) observed 26%, 87%, and 63% increases in lettuce plant height, leaf area, and biomass yield, respectively, with TWW versus conventional water. Aziz and Farissi (2014) also attributed improved plant growth and yield to better nutrient absorption. TWW contains more N and P than TAP water, with nitrogen provided as nitrate (NO_3^-) and ammonium (NH_4^+), which are more easily absorbed by plants, along with readily assimilable forms of phosphorus (orthophosphate) and potassium ions (Ofori et al., 2021). Finally, it is essential to note that although the tomato plants were exposed to varying water volumes - and therefore different amounts of sodium hypochlorite - during the experiment, this did not impact crop growth. This outcome is likely due to the low concentration of sodium hypochlorite (<5 mg/L) used for wastewater sanitization. Similarly, Lykogianni et al. (2023) found no adverse effects on growth, leaf gas exchange, fruit yield, or tissue mineral composition in tomatoes when using a nutrient-enriched solution treated with sodium hypochlorite at concentrations of 2.5, 5, and 7.5 mg/L. Previous research has indicated that higher residual sodium hypochlorite levels can cause more significant plant damage (Lonigro et al., 2017). Overall, the biometric response of the plants to the irrigation treatments indicates that TWW could not meet the whole nutrients requirement of tomato, thus nutrients integration by inorganic fertilization is required. However, using TWW for irrigation can still help reduce the need for external nutrient inputs during growth.

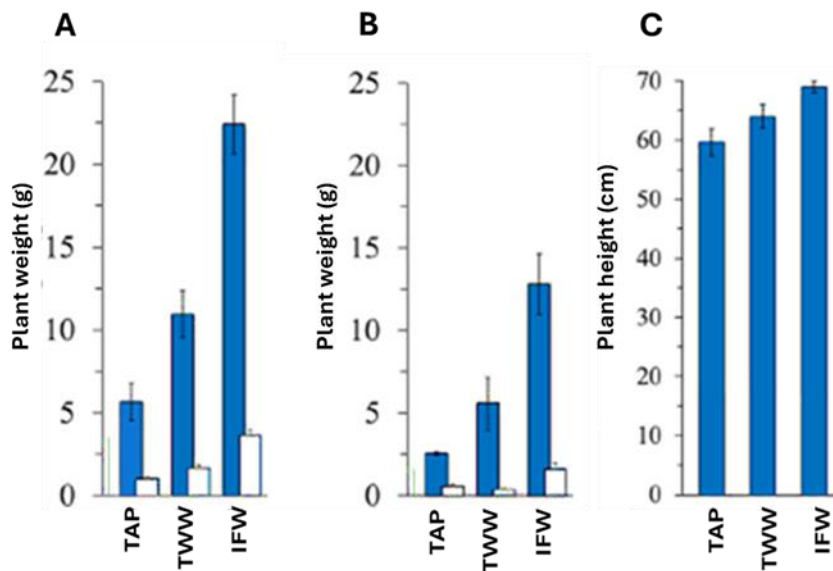


Figure 8.1. Fresh (A) and dry (B) weights of shoot (full histograms) and roots (empty histograms), and plant height (C) of tomato cultivated in pots after 56 days. The experimental factors tested were water type (TAP, tap water; IFW, inorganic nutrient solution; TWW, treated wastewater). Standard deviations are reported as vertical bars.

Irrigation treatments had varying effects on the chemical properties of the soil at the end of the tomato plant growth period. For the TAP treatment, the pH was 8.1, electrical conductivity was 210 $\mu\text{S}/\text{cm}$, available phosphorus (P) was 45 mg/kg, and mineral nitrogen (N) was 1.8 mg/kg. Under the TWW treatment, the pH remained at 8.1, electrical conductivity increased to 698 $\mu\text{S}/\text{cm}$, available P was 48 mg/kg, and mineral N was 9.7 mg/kg. Similarly, for the IFW treatment, the pH was 8.1, electrical conductivity was 604 $\mu\text{S}/\text{cm}$, available P reached 118 mg/kg, and mineral N was 11.2 mg/kg. The irrigation treatments did not affect pH levels. Still, they significantly influenced electrical conductivity, with the TWW and IFW treatments showing the highest EC values, likely due to the addition of soluble salts from the irrigation water (Romaneckas et al., 2023). The increase in soil salinity from TWW irrigation is a common concern raised by several authors (e.g., Chaganti et al., 2020; De las Heras and Mañas, 2020) since salt accumulation can negatively impact soil aggregation, permeability, and both hydraulic and conductivity (Leonel & Tonetti, 2021). However, in this study, soil EC values remained below the critical threshold of 2 dS/m at the end of the experiment, indicating that the soil remains suitable for cultivation (Weil & Brady, 2008). Since the study was conducted in pots, the increase in EC could be considered temporary. In open-field conditions, rainfall can help leach soluble salts. Field-grown tomato crops irrigated with treated wastewater have shown temporary increases in EC during the summer, which return to normal levels by the end of winter (Vergine et al., 2017). The

authors linked the temporary increase in EC to high irrigation levels and the absence of rainfall, which led to salt retention from TWW. The clayey soil type also contributed to higher EC values due to its ability to retain more water and soluble salts. Other studies have reported similar findings (Kallel et al., 2012; Klay et al., 2010; Shakir et al., 2017). Therefore, when using TWW for irrigation, selecting an appropriate soil type and monitoring EC levels to ensure they remain within safe limits for crops is essential. Irrigation treatments significantly influenced the availability of phosphorus (P), with the IFW treatment nearly doubling soil P levels compared to the TAP and TWW treatments. This is likely because tap and treated wastewater contain minimal total P, which may precipitate in soils with $\text{pH} \geq 7.0$ or be absorbed by plants, reducing the amount of P in the soil (Leonel et al., 2022). Mineral nitrogen ($\text{NH}_4^+ + \text{NO}_3^-$) was also mainly affected by irrigation, with the highest levels observed in the IFW treatment. Unlike P, mineral N was slightly higher in the TWW treatment than in TAP, likely due to the higher ammonium content in treated wastewater. Xu et al. (2010) reported increased nitrogen levels after long-term TWW use. The increase after 56 days in this work could be attributed to the pot experiment setup, which limited percolation.

8.3.2 Structure of prokaryotic communities of tomato plants soil irrigated with TWW, TAP and IFW

Most (85.7 %) of the ASVs have been assigned to the Bacteria domain while the remaining (14.3 %) to the Archaea domain. The prokaryotic composition of the soil was comparatively analysed considering the relative abundances at the phylum and genus levels (Figure 8.2 and 8.3, Tables 8.S1 and 8.S2). Metataxonomic analysis revealed that bacteria belonging to the Firmicutes, Proteobacteria, and Actinobacteria phyla are dominant in all irrigation treatments (Fig. 8.2). This result agrees with other studies, demonstrating the concurrent presence of these three bacterial phyla in both soil and TWW microbial communities (Becerra- Castro et al., 2015). Firmicutes, Proteobacteria, and Actinobacteria play crucial roles in plant growth and maintain constant soil parameters under stress conditions, especially in the case of tomatoes, as demonstrated by recent studies (Lee et al., 2021; Pokluda et al., 2021; Faddetta et al., 2023). Studies aimed at clarifying the role of the microbial community structure on the growth of tomato plants demonstrate that the soil microbiota exploits different metabolic capabilities to ensure the growth and health of tomato plants. In this regard, an increase in the abundance of Acidobacteria was observed in conjunction with a growth-promoting effect on tomato plants (Kalam et al., 2017). In fact, Acidobacteria can promote plant growth by solubilizing zinc, producing siderophores and enzymes useful for

plant growth and finally producing growth-regulating phytohormones that promote various phases of growth starting from germination and differentiation of tomato tissues (Kalam et al., 2017). In addition, it is known that Proteobacteria are capable of colonizing the root system of plants, improving the growth and health of the host organisms (Bruto et al., 2014). Resendiz-Nava et al. (2023) observed a close biological interaction between tomato plants and Proteobacteria that possess high genomic plasticity, easily adapting to stress and successfully colonizing plant niches. Furthermore, it has been shown that Proteobacteria promote processes beneficial to plants such as nitrogen fixation and phosphate solubilization. Indeed, the metataxonomic analysis (Figure 8.2A) showed that the relative abundance of Gram-negative bacteria, in particular those belonging to Proteobacteria and Acidobacteria phyla, increased in soils irrigated with TWW (29.32 % and 8.16 %, respectively) and IFW (28.85 % and 7.42 %) in comparison to those irrigated with TAP water (23.04 % and 6.32 %). This finding suggested that TWW can modulate soil microbiota. Such a result agreed with Wafula et al. (2015) report that found an increase in the relative abundance of Proteobacteria and Acidobacteria and a decrease of Actinobacteria in soil irrigated with TWW compared to not irrigated soil. Indeed, in this study, a decrease in Actinobacteria was observed in TWW with respect to IFW (Figure 8.2B). A strict correlation between Acidobacteria and Proteobacteria from the irrigated WW samples and total N concentration was already reported (Wafula et al., 2015). Some Proteobacteria, such as purple phototrophic bacteria, are considered microorganisms that can purify wastewater because their ability in degrading harmful substances in wastewater, such as hydrocarbons (Nhi-Cong et al., 2021). Unfortunately, the increase of Proteobacteria could represent a problem for human health, since it was previously reported that wastewater irrigation leads to an increased abundance of potentially harmful Proteobacteria, such as *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter* spp. (Broszat et al., 2014). In the soils analysed in this work, these bacteria were not found, but their presence in WW cannot be ruled out since it can be dependent on bacterial loading and quality of the irrigation water. Thus, the bacterial composition and, in particular, Proteobacteria and harmful bacteria should be carefully monitored in TWW before its use. The analyses conducted in this study revealed that there are not any bacterial genera present potentially dangerous to human health. However, to effectively mitigate the risk of their presence in TWWs and guarantee the safety of human health, disinfection methods, such as UV radiation, paracetic acid, ozonation, or the use of sodium hypochlorite could be exploited (Bonetta et al., 2021). Chlorination is the most used method for disinfecting wastewater, as it is effective and inexpensive. This treatment inactivates several pathogens but can cause an increase in substances such as total organic

halogens which are cytotoxic. Therefore, physical disinfection methods, such as UV radiation, that do not produce toxic by-products are increasingly used. However, it is known that some bacteria can have protective effects, reducing the microbial load of pathogenic bacteria (Thao et al., 2021). It has been shown (Frenk et al., 2014) that the soil microbiota is susceptible to irrigation treatments, especially in a short-term upon the use of TWW for irrigation; however, a return to a untreated state demonstrates that the soil community is resilient in the long term to the anthropic impact imposed by the quality of water for irrigation (Frenk et al., 2014). Furthermore, no change was detected in the predicted metabolic capability of the prokaryotic community due to the three treatments considered (Figure 8.S1). Indeed, these predicted metabolic activities were similar in all the treatments with a high percentage of unknown metabolic pathways (about 66 %), sulfate reducing bacteria (about 35 %), sulfide oxidizer (about 30 %) and nitrite reducer (about 30 %). Anyhow, it is not possible to exclude that the category Unknowns could probably include plant growth promoting capacities that can have a role in the stimulation of tomato plant growth in TWW in the respect of TAP. Some soil characteristics, such as salinity EC and pH may have modulated the soil microbial composition. EC is known to be able to prominently modulate soil bacterial community structure. In particular, in the short term, soils with high EC are characterized by lower bacterial diversity (Zhao et al., 2020). For example, it is reported that at high ECs (for example around 6 dS m⁻¹) Bacteroidetes, Gemmatimonadetes and Firmicutes can increase their abundance. In S5 soil, the pH is 8.1 for all the TAP, TWW and IFW treatments while the highest EC was obtained with the TWW treatment, but this difference is not consistent with a high EC value.

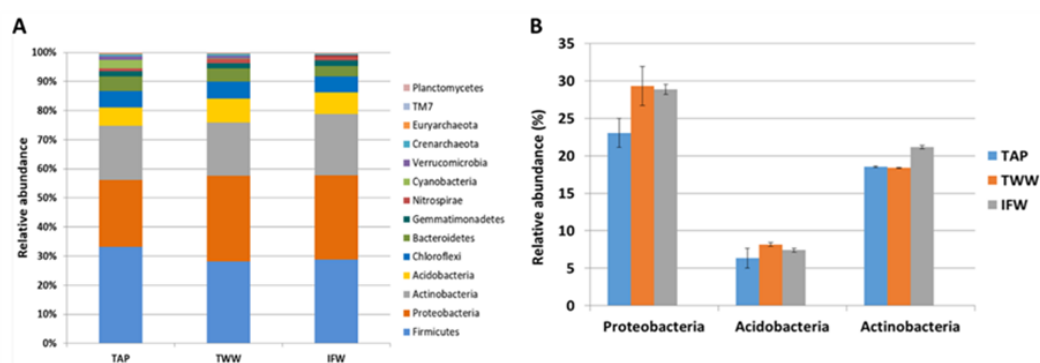


Figure 8.2. Histograms reporting the taxonomic composition of S5 soil samples irrigated with TAP (tap water), TWW (treated wastewater) and IFW (inorganic NPK solution) at the taxonomic level of phylum. (A) The relative abundance is the percentage of each phylum

with respect to all the identified phyla. (B) A magnification of the relative values of the three phyla Proteobacteria, Acidobacteria and Actinobacteria is reported.

Apart from the unclassified bacteria, at the genus level, 13 taxa showed an average percentage abundance higher than 1 % with *Bacillus* being the most abundant genus in all three irrigation treatments (Figure 8.3). It has been seen, in previous studies evaluating the role of the *Bacillus* genus in the wastewater that this bacterial genus is able to tolerate not only basic pH, but also high salinity level. Members of *Bacillus* can confer tolerance to abiotic stresses to plants, also generating symbiosis with microalgae, and have been proposed as agricultural biofertilizers, promoting plant growth (Bui-Xuan et al., 2022; Yong et al., 2021). Anyhow, also at the genus level, some variations of the relative abundances can be ascribed to the irrigation treatment. Among them, the most interesting abundance variations concern *Balneimonas*, *Rubrobacter*, and *Steroidobacter*. In particular, the genera *Balneimonas* and *Steroidobacter* increased in both TWW and IFW treatments compared to the TAP treatment, while *Rubrobacter* increased in IFW and decreased in TWW compared to TAP. In other studies, it is reported that bacteria belonging to *Balneimonas* and *Rubrobacter* genera are important for promoting plant growth and nutrient cycling. Their relative abundances decreased in soil under drought conditions while increasing in irrigated soils (Trivedi et al., 2021). Similarly, bacteria belonging to *Steroidobacter* halve drought and positively affect crop growth. Previous studies have reported that the growth of *Steroidobacter* in the soil is stimulated by the presence of nitrogen as it favours the synthesis of glycosides beneficial for plant growth. Thus, the increase of *Steroidobacter* relative abundance may be related to the high level of N and improved growth in TWW in comparison with TAP treatment. Therefore, the improved plant growth in soil irrigated with TWW compared to those irrigated with TAP, may be associated with a modulation of soil microbiota structure that for some bacterial taxa resembled that of IFW treatment which, however, showed a stronger plant-growth stimulating effect in comparison to TAP and TWW irrigations. These findings suggest a strong relationship between TWW irrigation, soil nutrient availability and soil microbiota structures and deserve further investigations to fully elucidate the key parameters controlling tomato plant-growth and soil microbiota structure.

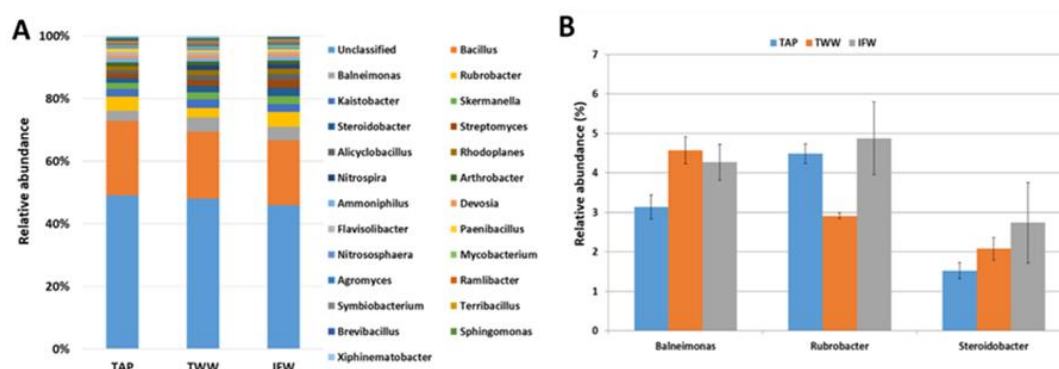


Figure 8.3. Histograms reporting the taxonomic composition of S5 soil samples irrigated with TAP (tap water), TWW (treated wastewater) and IFW (inorganic NPK solution) at the taxonomic level of genus. (A) The relative abundance is the percentage of each genus with respect to all the identified genera. (B) A magnification of the relative values of the three genera *Balneimonas*, *Rubrobacter* and *Steroidobacter* is reported.

8.3.3 Plants and soil analyses of fava bean treated with TWW, biochar and zeolite

Plants irrigated with TAP water and grown in unamended soil exhibited greater fresh weight and height than those irrigated with TWW (Figure 8.4 and Table 8.S1). Regardless of the irrigation water type, applying EB, EZ, and NB led to a 10% and 5% increase in fresh weight and height, respectively, compared to the TAP treatment. The reduced fresh weight and height observed in plants irrigated with TWW could be attributed to reduced water uptake, likely caused by the higher EC levels and elevated concentrations of Ca, Na, Mg, and K in TWW relative to TAP water.

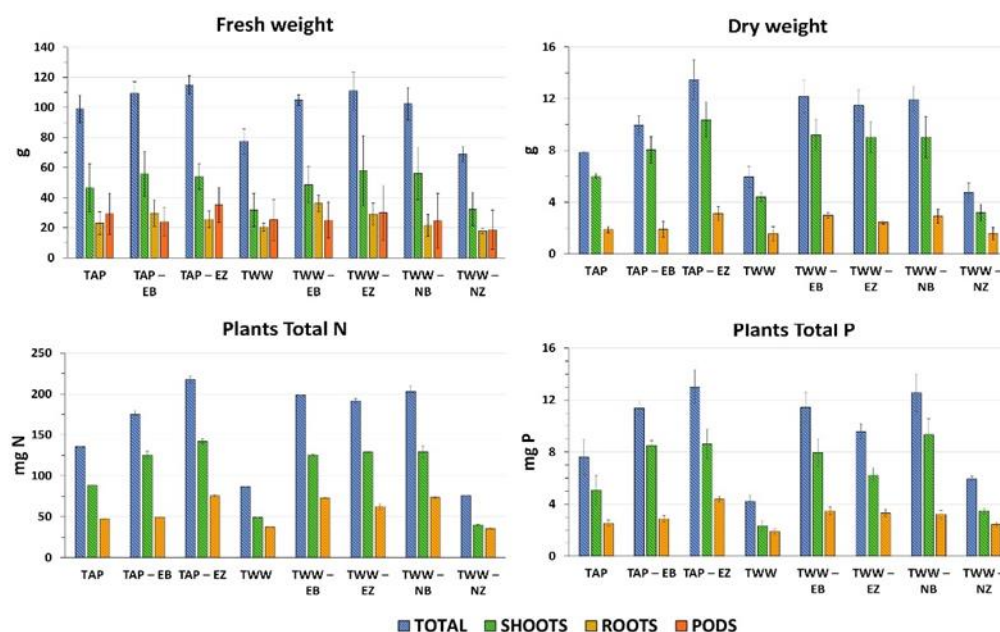


Figure 8.4. Fresh and dry weight, total N and P of plants subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively) or (natural or enriched zeolite, NZ and EZ, respectively).

This hypothesis is supported by soil EC data (Figure 8.5), which revealed significantly higher values in soils irrigated with TWW than those irrigated with TAP, alongside increased exchangeable Na^+ (Table 8.6). Previous studies have also shown that TWW irrigation raises soil salinity (Shakir et al., 2017; Ofori et al., 2021). In this study, daily TWW irrigation may have introduced excess soluble salts, elevating osmotic pressure in the rhizosphere and thereby restricting plant water potential (Djanaguiraman et al., 2014). This is consistent with findings from Heidari et al. (2012), who observed reduced plant growth in *Ocimum basilicum* due to osmotic stress from saline water. However, the negative impact of TWW was alleviated by the addition of EB, EZ, and NB, as biochar and zeolite can adsorb both cations and anions, mitigating the adverse effects of wastewater (Aiad et al., 2021; Kong et al., 2021). Additionally, enriched biochar and zeolite may serve as sources of Ca^{2+} and K^+ , helping to offset the high Na^+ concentrations.

Table 8.6. Exchangeable cations in soils subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively, or natural or enriched zeolite, NZ and EZ, respectively).

	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}
	cmol(+) kg^{-1}			
TAP	36.3 ± 2.6	1.1 ± 0.1	3.8 ± 0.3	1.6 ± 0.2
TAP – EB	34.3 ± 0.9	1.1 ± 0.1	3.6 ± 0.3	1.6 ± 0.0
TAP – EZ	34.7 ± 2.1	1.3 ± 0.2	3.2 ± 0.4	1.7 ± 0.3
TWW	36.1 ± 1.3	1.3 ± 0.1	3.5 ± 0.2	2.2 ± 0.1
TWW – EB	35.5 ± 0.8	1.1 ± 0.0	3.4 ± 0.1	2.0 ± 0.2
TWW – EZ	40.2 ± 0.9	1.5 ± 0.1	3.8 ± 0.1	2.3 ± 0.1
TWW – NB	35.4 ± 0.4	1.0 ± 0.1	3.2 ± 0.1	1.9 ± 0.3
TWW – NZ	34.0 ± 2.3	1.2 ± 0.1	3.0 ± 0.2	1.9 ± 0.2

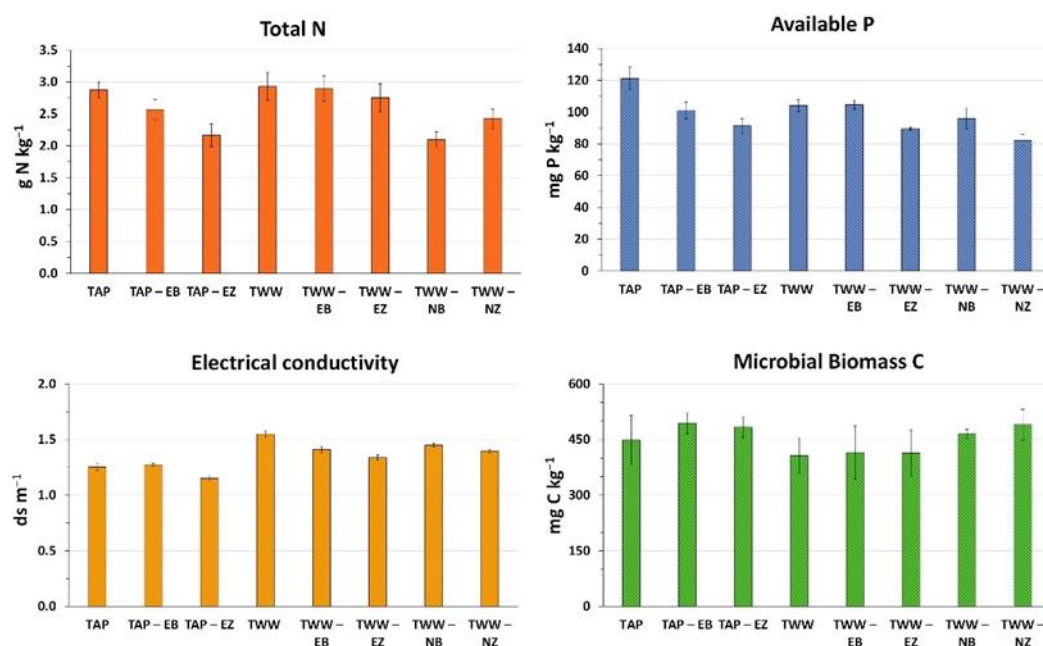


Figure 8.5. Total N, available P, electrical conductivity and microbial biomass carbon (MBC) in soils subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively) or (natural or enriched zeolite, NZ and EZ, respectively).

The dry weight of plants followed the same trend as fresh weight, with TWW treatments containing enriched biochar (EB), zeolite (EZ), and natural biochar (NB) nearly doubling dry weight compared to unamended soil and showing approximately 2.5 times the weight of the NZ (natural zeolite) treatment. Variations in fresh and dry weights occurred across irrigation methods and amendments, but no significant differences were found in shoot/root ratios. Plant TN uptake ranged from 76 to 218 mg, mirroring fresh and dry weight trends. TAP irrigation resulted in higher TN uptake compared to TWW, with EB and EZ additions enhancing TN uptake in both TAP and TWW treatments, except in TWW-NZ. No significant differences emerged among EB, EZ, and NB treatments. These results suggest that enriched biochar and zeolite may enhance plant growth by improving soil characteristics such as density and water retention (Ndede et al., 2022).

Natural zeolite reduced nitrogen uptake, likely due to its strong affinity for NH_4^+ , limiting N availability, consistent with Wiedenfeld (2003), who reported NH_4^+ immobilization by zeolite, thus reducing N availability and impeding crop growth. Similar results were found by MacKown and Tucker (1985), who observed an 11% decrease in soil nitrification due to NH_4^+ retention by clinoptilolite, hindering nitrification. TP uptake was influenced by irrigation type and amendment type, showing a pattern similar to TN, with TAP irrigation supporting higher TP uptake than TWW. Enriched and non-enriched amendments (except for TWW-NZ) increased TP compared to unamended soil. This suggests TWW may reduce P uptake due to its high EC and elevated Ca, Na, Mg, and K levels, likely causing plant osmotic stress. The high Ca and pH of 8.4 in TWW suggest a potential for $\text{Ca}_3(\text{PO}_4)_2$ precipitation, further limiting P availability (Weil & Brady, 2008).

Soil TN and available P were also affected by irrigation and amendments (Figure 8.5). EB and EZ added to TAP-irrigated soils reduced TN content by 12% and 33%, respectively, compared to unamended soils, possibly due to improved nitrogen absorption. TWW irrigation maintained soil N at levels comparable to TAP-unamended soil due to the additional N from TWW, EB, or EZ, compensating for plant uptake. NB and NZ, however, reduced soil TN by 38% and 20%, respectively, likely from increased nitrification and denitrification, leading to N volatilization losses (Liu et al., 2024; Szatanik-Kloc et al., 2021). No significant differences in TN were noted between NZ and EZ with TWW irrigation. In TAP-irrigated soils, TN and available P decreased as plant uptake increased. Available P showed similar patterns to TN, with enrichment reducing available P, which was inversely related to plant P uptake. TWW irrigation further reduced available P due to its high pH and Ca^{2+} content, potentially forming less-soluble calcium-phosphate compounds.

Metal concentrations in shoots and roots varied across treatments, except for Na in roots, which was higher in TWW-irrigated plants due to a continuous Na supply. Other metal concentrations remained stable, indicating minimal impact from TWW on metal uptake, regardless of amendments like zeolite or biochar.

Soil EC was primarily affected by irrigation, with TWW-irrigated soils showing increased EC due to added soluble salts, aligning with previous studies (Ofori et al., 2021; Muscarella et al., 2024) linking TWW to increased salinity, potentially affecting soil aggregation, permeability, and crop growth (Leonel & Tonetti, 2021). Nonetheless, soil EC stayed below the 2 dS m⁻¹ threshold, keeping conditions favorable for cultivation (Weil & Brady, 2008). Enriched and natural amendments helped mitigate TWW's negative impact by reducing EC, likely due to the cation adsorption properties of biochar and zeolite (Hedstrom, 2001), supported by studies from Doni et al. (2021) and Huang et al. (2019).

8.3.4 Structure of prokaryotic communities of fava bean soil treated with TWW, biochar and zeolite

NGS analysis of amplicons obtained by targeting the 16 rDNA regions using metagenomic DNA of the Fava plant rhizosphere led to the identification of 593629 high-quality reads filtered from the 1813386 raw reads. An average value of 99.18% of the ASVs detected in this work belonged to the Bacteria domain and an average value of 0.82% to the Archaea. Previous studies demonstrated that soil microbial communities predominantly comprise bacteria, with archaea less prevalent since they are typically found in more extreme environments (Catania et al., 2022). This study shows Proteobacteria as the predominant bacterial phylum (29-32%) and Crenarchaeota as the predominant archaeal phylum (0.21-1.07%). Their prevalence was also found in soil samples analysed in other reports (Catania et al., 2022). In addition, the TAP sample contained Bacteroidetes (26.14%), Acidobacteria (17.07%), Actinobacteria (7.91%), and Verrucomicrobia (5.04%). The other (less common) phyla were Firmicutes (2.77%), Chloroflexi (2.31%), Gemmatimonadetes (1.31%), Nitrospira (0.71%), and Planctomycetes (0.35%). Similar results were obtained in our previous work (Muscarella et al., 2024), in which we used the same soil, and Firmicutes, Proteobacteria, and Actinobacteria were among the most abundant phyla. Other phyla like Bacteroidetes and Acidobacteria were also present at high percentages. Specifically, Proteobacteria were present with an average percentage abundance ranging from 29 to 32% (Figure 8.6). Many members of the Proteobacteria phylum are known as Plant Growth-

Promoting Bacteria (PGPB). These bacteria are crucial in promoting growth through several mechanisms, including nitrification and phosphorus solubilization (Chauhan et al., 2023). Given that the distribution of Proteobacteria across different treatments was relatively homogeneous, it can be inferred that Proteobacteria are a predominant phylum of the soil used for plant cultivation, and this result agrees with Muscarella et al. (2024). Bacteroidetes, primarily known for breaking down soil organic matter, can participate in the nitrogen and phosphorus cycles, making these elements accessible to plants. Actinobacteria, the third most abundant phylum, contains the main PGPB (Faddetta et al., 2023). Actinobacteria, mostly soil bacteria, are involved in the nitrogen cycle and phosphate solubilization but are also significant producers of bioactive compounds, including antibiotics and plant hormones (Ibnouf et al., 2022). Acidobacteria had an average percentage abundance ranging from 8 to 20% (Figure 8.6). Acidobacteria play a role in phosphate solubilization while also being primary producers of ammonium and nitrate reducers (Kielak et al., 2016). Given that plants primarily absorb nitrate followed by ammonium, these bacteria supply ammonium for plant growth. Usually, soil samples contain less Bacteroidetes and more Actinobacteria than the percentages found in this study. Hartmann and Widmer (2006) indicated that Actinobacteria, Proteobacteria, Acidobacteria, and Verrucomicrobia constitute the four bacterial taxa important for agricultural soils. Similarly, in the present study, Actinobacteria were more abundant (35–39%) than Bacteroidetes (2–3%). The soil microbiota sample treated with TWW featured the same bacterial signature of TAP water-treated samples but a higher prevalence of Firmicutes and a lower abundance of Bacteroidetes. Bacteroidetes, a bacterial phylum crucial for organic matter degradation, plays a key role in nutrient cycling, especially for nitrogen and phosphorus and the changes in relative abundance can relay with a different availability of nutrient resources. The soil samples irrigated with TAP or TWW with enriched biochar or zeolite did not significantly differ from their relative controls. When the soil samples were irrigated with TWW and treated with enriched biochar and enriched zeolite, a higher abundance of Actinobacteria and Firmicutes was evident with a reduction of Acidobacteria and Bacteroidetes.

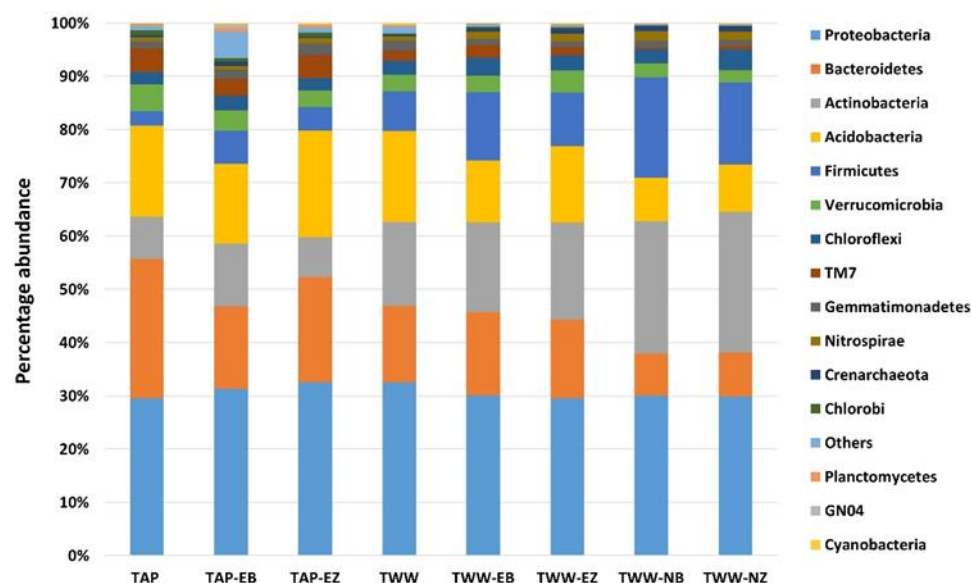


Figure 8.6. Histograms report the relative abundances of prokaryotic microbiota taxa, at the level of phyla, of soil subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively) or (natural or enriched zeolite, NZ and EZ, respectively). The relative abundance indicated the percentage of each phylum compared to all the identified phyla. Taxa having percentage abundance lower than 1 are groped as “Others”.

Although the highest fresh weight of the fava plants was found when TWW-EB and TWW-EZ were used, no significant difference in bacterial components was evident. We could surmise that the increase of Firmicutes in TWW-EB and TWW-EZ-treated soil samples had a beneficial effect on the fava plants since species like *Bacillus spp.* are also known as PGPB (Chauhan et al., 2023). The fact that Firmicutes are more abundant in the TWW-NB and TWW-NZ makes this result questionable, suggesting that different and complex relationships occur between the bacterial members residing in the soil and that a more profound analysis at the phylogenetical level was necessary. Thus, bacterial genera were investigated revealing more striking features (Figure 8.7). Specifically, it is noteworthy that TAP-EB and TAP-EZ did not affect the microbial components differently from all the treatments of the TWW. This result strongly suggests a greater possibility of modulating bacterial composition using different treatments and a strong resilience of the soil samples irrigated with TAP, TAP-EB, and TAP-EZ. The maximum effect was obtained with TWW-

NB and TWW-NZ, although the maximum fresh weight of the fava plants was obtained using TWW-EB and TWW-EZ. As reported in Figure 8.7, a great percentage of bacteria has not been classified yet, thus, correlating their effect of irrigation on plant growth is not straightforward, indicating that much more work is necessary to investigate the impact of the TWW deeply and to characterize those bacteria. Unclassified bacteria are reduced from more than 60% to 50% or less. The results suggest that the treatment using TWW positively influences the relative abundance of some bacteria, like *Bacillus*, *Rubrobacter*, *Balneimonas* and *Skermanella*, that could be important for plant growth, indeed, increasing their abundance in all the TWW treated samples, especially using also non-enriched biochar and non-enriched zeolite. Vlajkov et al. (2023) revealed that members of the *Bacillus* genus utilized biochar as a solid carrier, thus demonstrating that biochar not only provides an excellent environment for the growth of this kind of bacteria but also that the *Bacillus*-biochar system holds significant potential to produce enzymes and molecules that promote plant growth. In addition, the Vlajkov et al. (2023) study indicated that biochar is considered one of the most promising long-term solutions for enhancing soil quality, providing an ideal environment for immobilizing microorganisms. *Rubrobacter* is a predominant genus within the soil microbiota, and recent studies (Ajilogba et al., 2022) confirm its role in promoting plant growth, even in conditions where water resources are limited. Some studies refer that *Balneimonas* genus members have been shown to produce acid indol-3-acetic (Fernández-Bayo et al., 2017), to carry out nitrogen fixation (Grönemeyer et al., 2011), and to be associated with an increase enzymatic activity to degrade organic matter in tea plants (Tan et al., 2019). Interestingly, a recent study reported the use of biochar to promote chlorpyrifos and atrazine pesticide degradation in soil. As an effect, biochar reduced pesticide plant uptake, leading to an increase in the relative abundance of PGPB, such as *Balneimonas* and *Bacillus* (Singh et al., 2024). *Skermanella* genus has been reported as one of the most abundant bacteria in the soil of some crops of agricultural interest, such as cucumber and vineyard plantations (Gao et al., 2021a; Xue et al., 2022). Although this genus is phylogenetically close to such genera of diazotrophic bacteria (Körberl et al., 2016) and its relative abundance is reported to increase in soil amended using biochar (Zhang and Shen, 2022), its role in nitrogen fixation capacity is not clear it does not have the *anfG* gene, which codes the delta subunit of nitrogenase (Zhang et al., 2022).

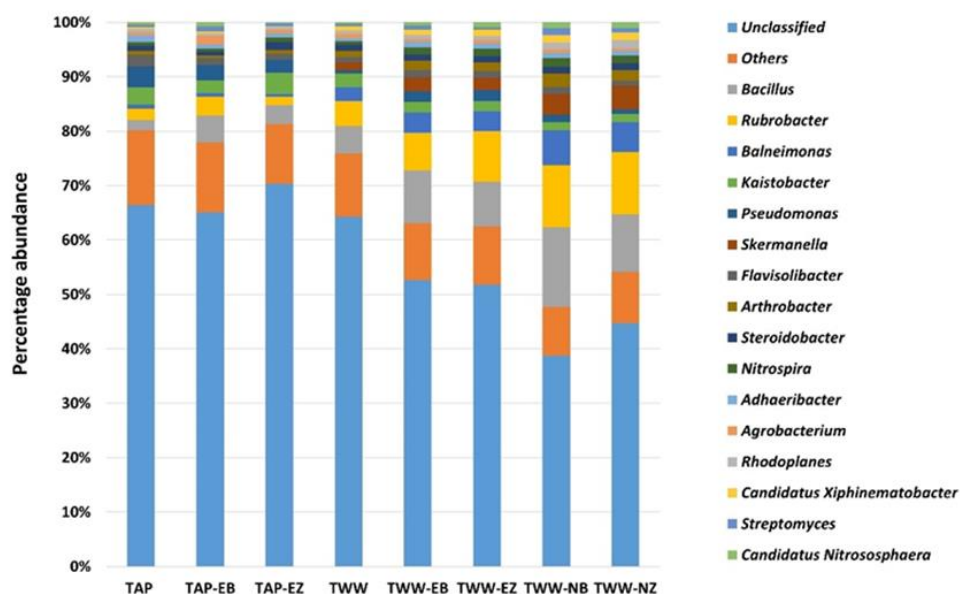


Figure 8.7. Histograms report the relative abundances of prokaryotic microbiota taxa, at the level of genus, of soil subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively) or (natural or enriched zeolite, NZ and EZ, respectively). The relative abundance indicated the percentage of each genus compared to all the identified genera. Taxa having percentage abundance lower than 1 are groped as “Others”.

8.3.5 Effects on plant and chemical soil properties of tomato treated with TWW, biochar and zeolite

The dry weight, total nitrogen (N), and phosphorus (P) content of plants irrigated with either tap water (TAP) or treated wastewater (TWW) and grown in soil amended or not amended with enriched (EB + EZ) or non-enriched (NB + NZ) biochar and zeolite, are shown in Figure 8.8. No significant differences in plant dry weight were observed across the treatments, although plants irrigated with TWW had slightly lower weights than those irrigated with tap water. In particular, the root weights of plants grown in soil irrigated with TWW and without biochar and zeolite amendments were 39% lower than those of the control group (TAP). This suggests a potential negative impact of TWW on plant growth, likely due to its higher electrical conductivity and soluble sodium (Na⁺) content, even though daily irrigation should have mitigated osmotic stress. This hypothesis is supported by the higher soil

electrical conductivity and exchangeable Na⁺ levels in TWW-irrigated soils compared to TAP-irrigated soils (Table 8.7).

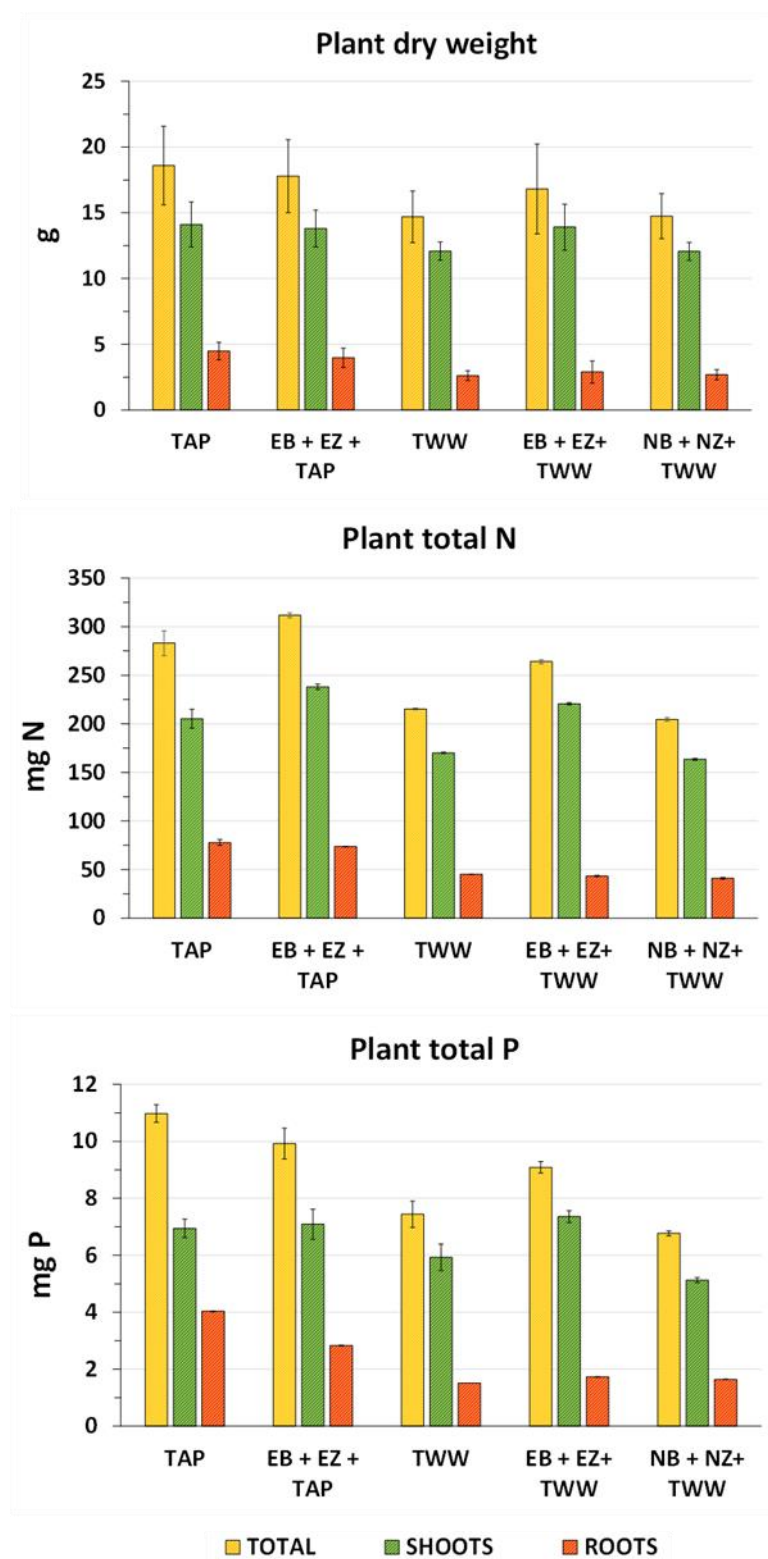


Figure 8.8. Dry weight, total N and P of plants subjected to different irrigation treatments (tap water, TAP; treated wastewater, TWW) and different soil amendments (natural biochar

and zeolite, NB + NZ, or enriched biochar and zeolite, EB + EZ). Error bars indicate standard deviation (n = 5).

Table 8.7. Chemical characteristics of soils subjected to different irrigation treatments (tap water, TAP; treated wastewater, TWW) and different soil amendments (natural biochar and zeolite, NB + NZ, or enriched biochar and zeolite, EB + EZ). Reported values are means (n = 5) $\pm$ standard deviation.

	pH	Electrical conductivity	Cation exchange capacity	Ca ²⁺	K ⁺	Mg ²⁺	Na ⁺
	-	dS m ⁻¹	mEq 100 g ⁻¹				
TAP	8.0 $\pm$ 0.1	1.31 $\pm$ 0.02	35.8 $\pm$ 1.3	29.5 $\pm$ 1.1	1.9 $\pm$ 0.1	2.9 $\pm$ 0.1	1.6 $\pm$ 0.0
EB + EZ + TAP	8.0 $\pm$ 0.1	1.41 $\pm$ 0.02	37.0 $\pm$ 0.5	30.3 $\pm$ 0.5	2.2 $\pm$ 0.1	2.8 $\pm$ 0.0	1.7 $\pm$ 0.0
TWW	8.1 $\pm$ 0.2	1.67 $\pm$ 0.01	36.5 $\pm$ 0.9	29.5 $\pm$ 0.7	2.2 $\pm$ 0.1	2.5 $\pm$ 0.1	2.2 $\pm$ 0.1
EB + EZ+ TWW	7.9 $\pm$ 0.1	1.60 $\pm$ 0.01	38.3 $\pm$ 1.9	31.2 $\pm$ 1.6	2.2 $\pm$ 0.2	2.6 $\pm$ 0.2	2.4 $\pm$ 0.1
NB + NZ+ TWW	8.0 $\pm$ 0.1	1.50 $\pm$ 0.05	35.4 $\pm$ 1.0	29.1 $\pm$ 1.0	1.9 $\pm$ 0.1	2.4 $\pm$ 0.1	2.1 $\pm$ 0.1

One issue associated with using TWW for irrigation, as highlighted by various researchers (Ofori et al., 2021), is the increasing soil salinity. For instance, Gao et al. (2021b) conducted a meta-analysis showing that TWW irrigation led to nearly a 60% rise in electrical conductivity and Na⁺ content in soils, resulting in a 6.5% reduction in crop yield. Similarly, Muscarella et al. (2024) reported a doubling of electrical conductivity in TWW-irrigated soils compared to controls, although they did not observe adverse effects on tomato plant growth. Monitoring soil salinity is crucial, as elevated exchangeable salts can increase osmotic pressure on plant roots, impairing water uptake and stunting plant growth. Furthermore, high salinity can deteriorate soil structure, reducing permeability and hydraulic

conductivity (Leonel & Tonetti, 2021). However, in this study, after 80 days of irrigation, the soil electrical conductivity remained below the threshold value of 2 dS m⁻¹, indicating that the soils were not saline (Weil & Brady, 2008).

A similar pattern in the dry weights of tomato plants can be used to infer their nitrogen (N) and phosphorus (P) uptake (Figure 8.8). Plants irrigated with tap water (TAP) exhibited an 11% increase in N uptake when amended with enriched biochar and zeolite (EB + EZ). However, while total P in the roots of TAP-irrigated plants amended with EB + EZ was lower than in the control group, no significant differences in P content were observed in the shoots. These findings suggest that the application of enriched biochar and zeolite acted as a slow-release fertilizer, enhancing N uptake in tomato plants. The reduced N and P uptake in plants irrigated with treated wastewater (TWW) can be attributed to increased soil salinity, often leading to nutrient deficiencies and imbalances. Salinity affects nutrient availability in the soil by inhibiting plant uptake and utilization (Ruiz et al., 1997), as Na⁺ and Cl⁻ ions in saline soils compete with essential nutrients such as K⁺, Ca²⁺, and NO₃⁻ (Nouri, 2017). This suggests that, despite nutrients in the soil, tomato plants irrigated with TWW struggle to absorb them effectively. These results align with those of Romero-Aranda et al. (2001), who found that saline water can reduce water uptake, transpiration, and the absorption and transport of nutrients in tomato plants, ultimately hindering their growth and water use efficiency. The total nitrogen (N) concentration in TAP-irrigated, non-amended soil was lower than in all other treatments. Specifically, soils amended with enriched biochar and zeolite (EB + EZ) and irrigated with either TWW or TAP and soils irrigated with TWW without amendment showed a 22% increase in total N compared to the control soil. However, the total N in soil irrigated with TWW and amended with natural biochar and zeolite (NB + NZ) was higher than in control but still lower than in the other treatments. The available phosphorus (P) pattern mirrored total N, except for soil irrigated with TWW and amended with NB + NZ, which showed lower P levels than the control (Figure 8.9).

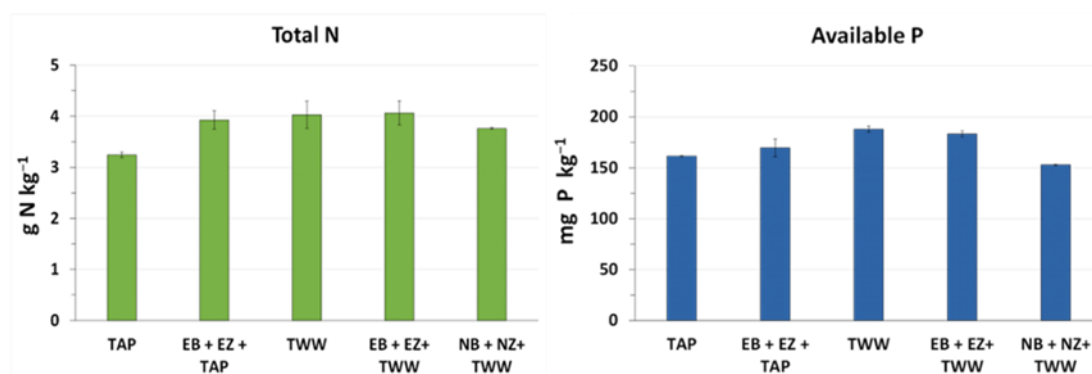


Figure 8.9. Total N and available P of soils subjected to different irrigation treatments (tap water, TAP; treated wastewater, TWW) and different soil amendments (natural biochar and zeolite, NB + NZ, or enriched biochar and zeolite, EB + EZ). Error bars indicate standard deviation (n = 5).

These findings indicate that enriched soil amendments and treated wastewater (TWW) enhance the availability of nitrogen (N) and phosphorus (P), which are crucial for plant growth. This is consistent with the work of Muscarella et al. (2024), who observed increased levels of inorganic N and available P in TWW-irrigated soils in a 56-day pot experiment. Similarly, Guaya et al. (2020) demonstrated the effectiveness of nutrient-enriched zeolite (containing NH_4^+ and PO_4^{3-}) recovered from municipal wastewater. Their study showed that adding nutrient-enriched zeolite to the soil-plant system boosted NH_4^+ and PO_4^{3-} concentrations by nearly tenfold and threefold, respectively, compared to control soils.

8.3.6 Structure of prokaryotic communities of tomato soil treated with TWW, biochar and zeolite

Bioinformatic analysis of the obtained sequences led to 388,866 high-quality reads filtered from 648,915 raw reads. ASVs analysis evidenced 98.96% of Bacteria and 1.04% of Archaea. In general, soil microbiota composition was not dramatically affected by the different irrigation waters.

Indeed, the core microbiota comprised Proteobacteria (20.20% in TWW, 24.30% in EB+EZ+TWW, 27.50% in EB+EZ+TAP, 31.29% in TAP and 33.86% in NB+NZ+TWW), Actinobacteria (12.17% in NB+NZ+TWW, 16.28% in EB+EZ+TAP, 21.11% in TAP, 23.43% in EB+EZ+TWW and 28.43% in TWW), Bacteroidetes (8.43% in TAP and TWW, 10.10% in EB+EZ+TWW, 13.49% in EB+EZ+TAP and 17.74% in NB+NZ+TWW), Firmicutes (2.48% in NB+NZ+TWW, 7.26% in EB+EZ+TAP, 13.57% in EB+EZ+TWW, 20.05% in TAP and 20.16% in TWW), and Chloroflexi (3.73% in NB+NZ+TWW, 5.05% in TWW, 6.07% in EB+EZ+TAP, 6.48% in TAP, 6.95% in EB+EZ+TWW).

Particularly, in the TAP treatment, there was 31.29% of Proteobacteria, 21.11% of Actinobacteria, 20.05% of Firmicutes, 8.43% of Bacteroidetes, and 6.48% of Chloroflexi, 4.95% of Acidobacteria and 0.78% of Verrucomicrobia. Catania et al. (2022) have studied European soils, discovering that the most abundant bacterial phyla were Proteobacteria (26.6%), Actinobacteria (24.2%), Acidobacteria (16.6%), Firmicutes (11.3%), Bacteroidetes

(5.5%), Chloroflexi (5.4%) and Verrucomicrobia (5.0%). Some bacteria belonging to these phyla, as discussed below, are known to be excellent PGPBs (; Faddetta et al., 2023).

Similar outcomes were observed in our previous studies (Muscarella et al., 2024), where we used the same initial soil and found that Firmicutes, Proteobacteria, and Actinobacteria were among the most abundant phyla. Both the TAP water and TWW samples exhibited the same bacterial composition. However, the most striking difference between the TAP and TWW irrigation was the increase from 21.11% in TAP, to 28.43% in TWW, of Actinobacteria and the decrease of Proteobacteria in TWW compared to TAP (31.29% vs. 20.20%). Proteobacteria increased in the soil amended with NB and EZ and more with NB and NZ; differently, Actinobacteria were reduced in the soil amended with NB and NZ. Another interesting feature was the reduction of Firmicutes and the increase of Acidobacteria following the application of enriched and natural amendments to the soil.

Soil bacterial community consists of different functional groups, including plant growth-promoting bacteria (PGPB), that can mitigate biotic and abiotic plant stress by increasing nutrient availability – i.e. by degrading organic matter, solubilizing inorganic phosphate, mineralizing organic phosphate, and participating in nitrogen cycles through nitrogen fixation or interconversion of nitrate and ammonium – and by producing bioactive metabolites – i.e. antibiotics and plant hormones (Odelade et al., 2019). Focusing attention on the bacterial phyla, particularly those involved in the nitrogen and phosphorus cycles having possible roles as PGPB according to scientific literature, it is notable that the primary phyla with statistically significant variations in percentage abundance, as determined by one-way ANOVA with Bonferroni and Holm method, were Acidobacteria, Chloroflexi, Verrucomicrobia, and TM7 (Figure 8.10). In particular, the Acidobacteria phylum was significantly more abundant in the EB+EZ+TAP treatment (14.82%) compared to the TAP condition used as a control (4.95%). A previous study focusing on reusing TWW for irrigation with the same initial soil (Muscarella et al., 2024) observed a slight increase in Acidobacteria abundance after TWW irrigation. Acidobacteria are classified among the recalcitrant bacteria because they are difficult to isolate and cultivate in the laboratory, with some species being uncultivable. However, Kalam et al. (2020) studied the genes involved in the metabolism of inorganic and organic nitrogen sources, showing that Acidobacteria possess enzymes involved in the phosphate cycle. Despite their fundamental ecological roles in plant-soil ecosystems, such as modulating biogeochemical cycles and influencing plant growth, there is still a lack of comprehensive information regarding the ecophysiological role of Acidobacteria. Another phylum showing significant variance was Chloroflexi, which

decreased the relative abundance in the EB+EZ+TAP treatment (6.07%) compared to the TAP control (6.48%). In other studies where soil was amended with biochar, changes in relative abundances of Chloroflexi were revealed during plant growth, peaking during germination (Tang et al., 2021). Chloroflexi are not typically known to be directly involved in the decomposition of nitrogen or phosphorus compounds as part of the main biogeochemical cycles, nor are they commonly recognized as PGPB. Anyhow, this evidence supports a possible direct or indirect relationship between Chloroflexi members and plant growth which deserves further and dedicated investigations. The phylum Verrucomicrobia also showed significant variance, increasing in the EB+EZ+TAP treatment (2.74%) compared to the TAP control (0.78%). Verrucomicrobia participate in phosphorus cycle through the mineralization of phosphate in the soil. In contrast, their role in nitrogen metabolism remains less well-defined, and they are not generally identified as PGPB. Despite this, Navarrete et al. (2015) investigated changes in Verrucomicrobia communities under varying soil nutrient conditions and found that members of Verrucomicrobia exert a significant impact on nitrogen availability. The last phylum whose statistically significant variance was TM7, which increased EB+EZ+TAP (1.79%) related to TAP control (0.68%). Very little is known about the TM7 phylum due to the difficulties of cultivating these bacteria in the laboratory. It is possible that they also colonize soil, and it has been discovered that they are excellent nitrifiers during the acidogenic nitrification phase of wastewater (Hanada et al., 2014). Additionally, they are involved in the biogeochemical cycles of phosphorus and carbon.

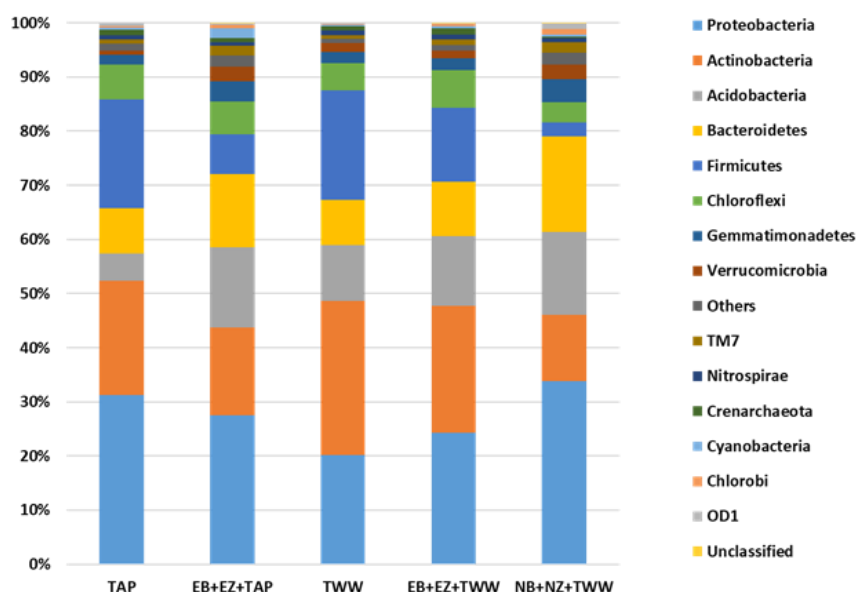


Figure 8.10. Histograms reporting the relative abundances of prokaryotic microbial taxa of soil subjected to different irrigation treatments (tap water, TAP; treated wastewater, TWW) and different soil amendments (natural biochar and zeolite, NB + NZ, or enriched biochar and zeolite, EB + EZ) at the taxonomic level of phyla. Values are reported as the mean of three independent samples. Taxa having a percentage abundance lower than 1 are grouped as “Others”.

The microbial composition was also examined at the genus level to better understand the effect of the different irrigation treatments on soil microbiota structure (Figure 8.11). Despite the high abundance of unclassified bacteria (61.71% in NB+NZ+TWW, 66.07% in EB+EZ+TAP, 56.20% in EB+EZ+TWW, 53.45% in TWW, and 45.40% in TAP), some striking bacterial differences can be highlighted to explain the effect of the treatments on soil microbiota. Some genera, including *Bacillus*, *Streptomyces*, and *Rubrobacter*, increased in TWW, and TWW amended with EB and EZ, likely due to their ability to thrive in harsh conditions, such as the high salinity in TWW. Indeed, TWW featured a tremendously high concentration of Na^+ and Ca^{2+} (87.8 mg L^{-1} and 127.5 mg L^{-1} , respectively). All three of these bacterial genera can tolerate high concentrations of Ca^{2+} and Na^+ (Yue et al., 2022; Wei et al., 2024). *Bacillus* was present in higher percentage in TWW treatment (14.94%) followed by EB+EZ+TWW (10.51%), TAP (6.20%), EB+EZ+TAP (5.28%) and, finally, NB+NZ+TWW (1.63%). *Bacillus* represents a significant group of bacteria known for enhancing plant growth and offers numerous advantages for sustainable agriculture. These strains have been shown to produce a wide range of phytohormones and other plant growth-promoting compounds. Consequently, it can affect several plant properties, such as biometric parameters, content of amino acids, proteins, fatty acids, and oxidative enzymes. Notably, a study with *Bacillus* (Dobrzyński et al., 2022) concluded that this bacterial species stimulates plant growth when the native microbiota of the inoculated soil is altered. *Rubrobacter* was another bacterial genus that increased with the TWW (11.16%) and EB+EZ+TWW (8.25%) treatments. The percentages were lower in the TAP, EB+EZ+TAP, and NB+NZ+TWW samples (8.33%, 5.43%, and 3.79%, respectively). *Rubrobacter* is an intriguing genus due to its resilience in extreme environments. Based on current scientific knowledge, it would not be considered a PGPB. However, it has been identified as one of the most abundant soil microorganisms of vineyard plants during different growth stages, suggesting a possible role in promoting plant growth (Ajilogba et al., 2022). *Streptomyces* was abundant mainly in EB+EZ+TWW (2.20%) followed by TWW (1.16%), NB+NZ+TWW (1.34%), TAP (0.92%)

and EB+EZ+TAP (0.89%). *Streptomyces* is a genus of Gram-positive bacteria known for its prolific production of antibiotics and ability to promote plant growth. *Streptomyces* plant growth-promoting capabilities include phytohormones, nutrient solubilization, nitrogen fixation, antibiotic production, and induction in the plant of systemic resistance (Sousa et al., 2016). These results confirm the positive impact of TWW, combined with enriched biochar and zeolite, on the soil microbial community, promoting PGPB.

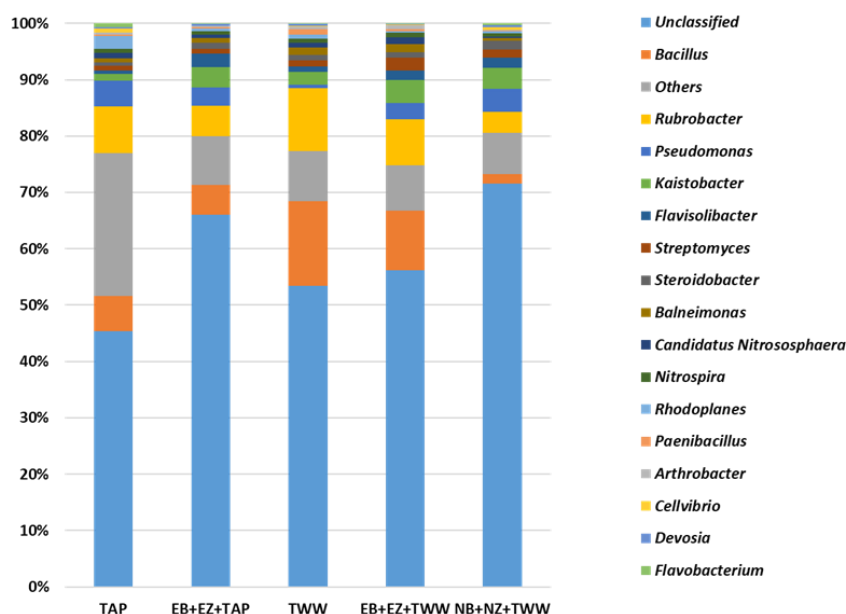


Figure 8.11. Histograms reporting the relative abundances of prokaryotic microbial taxa of soil subjected to different irrigation treatments (tap water, TAP; treated wastewater, TWW) and different soil amendments (natural biochar and zeolite, NB + NZ, or enriched biochar and zeolite, EB + EZ) at the taxonomic level of genus. Values are reported as the mean of three independent samples. Taxa having a percentage abundance lower than 1 are grouped as “Others”.

8.4 Conclusions

Reusing treated wastewater (TWW) for irrigation and integrating biochar and zeolite amendments demonstrates the potential for sustainable agriculture within a circular economy framework. Across three experimental studies—comparing TWW, TAP (tap water), and IFW (industrial freshwater) for irrigation; assessing TWW effects with biochar and zeolite on fava beans; and using these treatments on tomatoes—the findings suggest that TWW, when managed carefully, is a viable irrigation source. In tomato trials, TWW irrigation boosted

plant biomass due to increased phosphorus and nitrogen availability, though additional fertilization was required to meet nutritional needs fully. Sodium hypochlorite in TWW minimally affected plant growth and soil pH but raised soil EC; however, soils remained non-saline. The impact of sodium hypochlorite on soil microbes was moderated by soil alkalinity, with germicidal effects lessening as pH rose. Microbial communities remained stable, no pathogenic genera were detected, and beneficial bacteria were present, especially under TWW and IFW treatments.

TWW raised soil electrical conductivity (EC) due to its high salt and sodium content, risking increased salinity, which can hinder plant growth and nutrient uptake. However, enriched biochar and zeolite, acting as slow-release fertilizers, improved soil conditions, and nutrient availability, helping to counteract salinity effects and enhance crop growth, especially in tomatoes. Enriched biochar (phosphate) and zeolite (ammonium) were particularly effective in supporting beneficial microbial communities, especially with TAP irrigation. Additionally, microbial communities remained stable with TWW irrigation, showing TWW's viability without disrupting soil microbiota, while non-enriched biochar and zeolite fostered plant-growth-promoting bacteria (PGPB).

Enriched biochar and zeolite combined with TWW irrigation significantly improved nutrient uptake and overall plant health in fava beans. While TWW alone increased salinity and negatively impacted growth, the biochar and zeolite mitigated these effects through their cation exchange and adsorption properties, reducing soil EC and supporting better root development and nutrient absorption. Consequently, fava beans showed enhanced biomass and nutrient content, supporting sustainable nutrient management goals (Ndede et al., 2022). In summary, TWW can enhance sustainable agriculture as an additional water and nutrient source. When used with enriched biochar and zeolite, TWW irrigation decreases the need for synthetic fertilizers and fosters beneficial microbial communities while maintaining soil health. However, careful management is essential to control salinity and preserve soil fertility. This strategy underscores the potential of integrating waste resources into farming practices to promote nutrient sustainability and tackle water scarcity and soil health issues related to a growing global population.

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8.6 Supplementary material

Table 8.S1. Height (cm) of plants subjected to different irrigation treatments and soil amendments. Water used for crop irrigation (tap water, TAP; treated wastewater, TWW) and soil amendment (natural or enriched biochar, NB and EB, respectively) or (natural or enriched zeolite, NZ and EZ, respectively).

Treatments	Plant height (cm)
TAP	54.2 ± 3.6
TAP-EB	56.1 ± 7.8
TAP-EZ	59.9 ± 4.1
TWW	42.5 ± 7.0
TWW-EB	54.5 ± 5.1
TWW-EZ	59.4 ± 6.4

TWW-NB	53.4 ± 5.1
TWW-NZ	39.8 ± 2.4

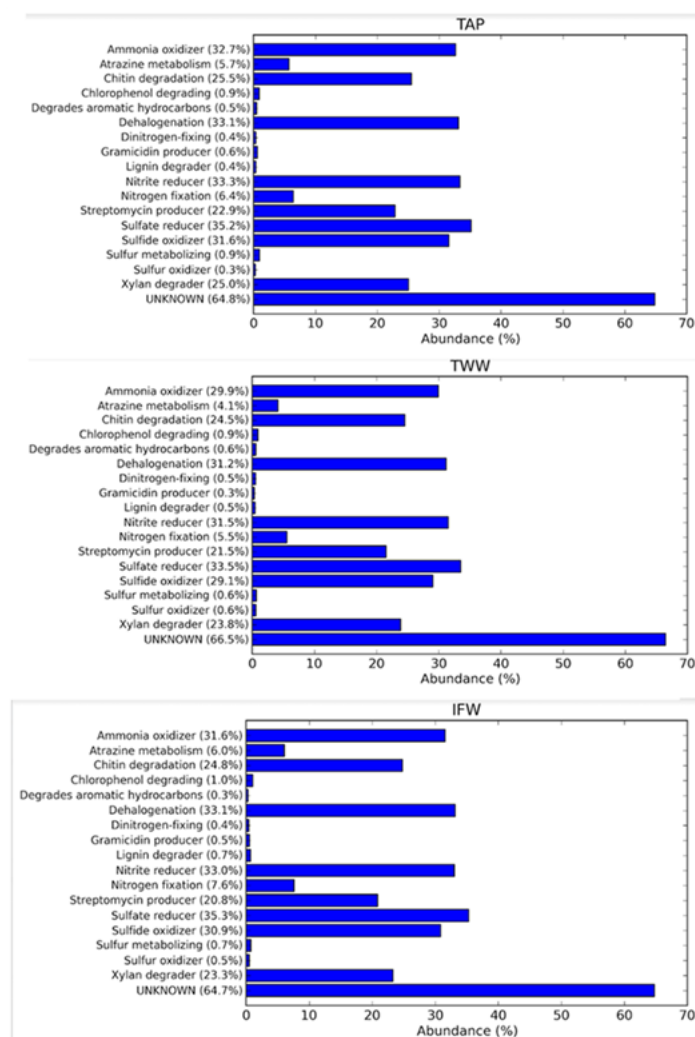


Figure 8.S1. Putative metabolic activities of the prokaryotic communities residing the TAP, TWW and IFW treated soils as inferred by METAGENassist bioinformatic analysis. The abundance percentage of Bacteria having a specific metabolism is indicated.

CHAPTER 9

Identification of sewage sludge bacterial strains and evaluation of PHA production capabilities

ABSTRACT

Reusing resources plays a crucial role in reducing environmental impact and promoting sustainability by supporting a circular economy. Wastewater treatment helps limit waste and pollution while reducing disposal costs. Although wastewater treatment typically yields purified water and nutrient-rich sewage sludge, much of the sludge is disposed of rather than reused. This sludge can be converted with appropriate treatment into agricultural fertilizers, biogas, or bioplastic precursors like polyhydroxyalkanoates (PHA), which are environmentally friendly alternatives to conventional plastics. This study focused on isolating 60 bacterial strains from sewage sludge microbiota and identifying two promising strains for PHA production from volatile fatty acids. These strains produced PHA, with yields verified through fluorescence and gas chromatography analysis.

9.1 Introduction

Pollution, driven primarily by human activities, poses a significant environmental threat, impacting ecosystems, human health, and climate (United Nations Environment Programme-UNEP). Wastewater Treatment Plants (WWTPs) are essential for treating water from domestic, industrial, and agricultural sources, but poor management can result in human and environmental danger (Di Leto et al., 2022). A significant byproduct of these plants, municipal sewage sludge, has increased in volume with urbanization and industrialization (Liang et al., 2021). Its disposal, often by incineration, faces challenges such as pollutant emissions and high costs, underscoring the need for alternative reuses. Sewage sludge, rich in organic matter and bacteria, plays a role in breaking down complex organic materials into simpler compounds, crucial for WWTP digestion processes that reduce waste volume and neutralize pathogens (Singh & Shyu, 2024). Research suggests that instead of disposal, sludge could be valorized by producing valuable bioproducts like polyhydroxyalkanoates (PHAs) using bacterial processes (Morgan-Sagastume et al., 2016). PHAs, fully biodegradable and thermoplastic, offer a sustainable alternative to conventional plastics, reducing persistent plastic pollution by utilizing VFAs from sewage sludge fermentation as substrates (Di Leto et al., 2024). This study, aligned with the European "Achieving Wider Uptake of Water-Smart Solutions" project, investigates using sewage sludge bacteria for PHA production. Sixty bacterial strains were isolated from sewage sludge at a municipal WWTP (Marineo) and a pilot plant (Palermo, Italy), identifying two strains capable of PHA synthesis from VFAs derived from campus wastewater (Lorini et al., 2022).

9.2 Material and Methods

9.2.1 Sample collection and bacterial strain isolation

Initial sludge samples were collected from two sources: the Marineo municipal wastewater plant in Palermo, Sicily, and a pilot plant at the University of Palermo's engineering department, which simulates real conditions by treating wastewater from the university's canteen and guesthouse (Mannina & Mineo, 2023). Sewage sludge from the municipal plant was sourced from the recirculation circuit, moving biomass from the sedimentation tank to the biological reactor. Pilot plant samples came from either the fermentation tank, which supports acidogenic fermentation for volatile fatty acid (VFA) production, or the selective-sequencing batch reactor (S-SBR) tank, which encourages PHA-producing bacteria under a feast-famine regime. Collected sludge samples were transported to the lab in sterile tubes at room temperature. Serial dilutions were conducted with a dilution factor from 10^{-1} to 10^{-6} , employing standard laboratory techniques to maintain precision. For culture growth, Starch Casein Agar (SCA) with 2.5 $\mu\text{g/mL}$ fungizone was used to inhibit fungi, along with Tryptone Soy Agar (TSA) as a nutrient-rich medium (Supplementary). Cultures were incubated at 30°C for 24 to 72 hours. Mixed microbial cultures were evaluated based on morphology to identify distinct colonies, from which pure cultures were isolated by transferring each colony to a new plate and confirming purity through additional streaking (Figure 9.1).

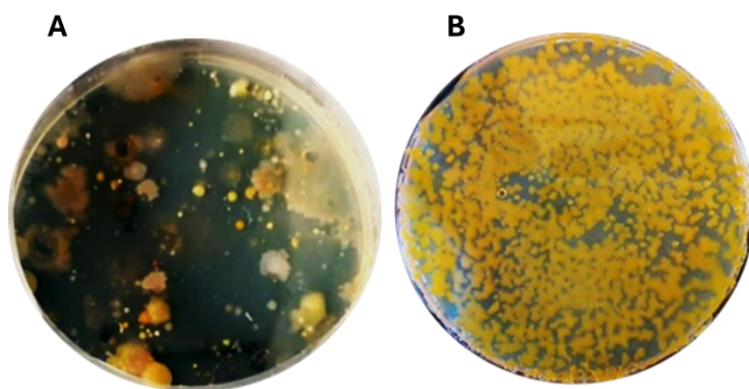


Figure 9.1 Comparison between mixed and pure cultures. Mixed microbial culture shows various morphologically distinct colonies representing multiple microbial species (A). The pure culture demonstrates the growth of a single, isolated microbial colony, confirming the successful separation and purification of the strain (B).

9.2.2 Molecular identification, VFA collection and characterization

From a single colony, DNA was extracted using the kit PureLink Genomic DNA Mini Kit (Invitrogen) according to the instructions of the manufacturer and following Capri et al.,

2024. An alignment tool (National Center for Biotechnology Information [NCBI] database; www.ncbi.nlm.nih.gov/BLAST) was used to identify the strains. VFAs were collected at the pilot plant on the Unipa Campus (Mannina & Mineo, 2023). The VFAs are generated in the fermentation unit of the PHA production line within the pilot plant facility, as Mineo et al. (2023a) described. The filtrate was collected in new sterile tubes for subsequent analysis. The soluble chemical oxygen demand (sCOD) of the filtrate was determined using a COD Cell Test—photometric kit (Supelco) following the manufacturer's instructions. Standard methods were applied for sCOD, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$ analysis (APHA, 2012). A Merck spectrophotometer was utilized for the photometric readings. On the peak day of sCOD, the filtrate from the batch fermentation test underwent VFA analysis to assess production levels following Mineo et al. (2024). An ion chromatographic analysis was conducted to assess the presence of ions in the VFA mixture. The concentrations of fluoride (F^-), chloride (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), sulfate (SO_4^{2-}), and phosphate (PO_4^{3-}) were measured using a Dionex Easion, Thermo Scientific ion chromatograph equipped with a Dionex IonPac AS22 (4×250 mm) column. The reagents used for anion determination included Dionex Anion Regenerant Concentrate (72 mN sulfuric acid) and Dionex AS22 Eluent Concentrate (4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate). For the determination of NH_4^+ , Ca^{2+} , Mg^{2+} , K^+ , and Na^+ concentrations, the Dionex Easion ion chromatograph (Thermo Scientific) was used with a Dionex IonPac CS12A (4×250 mm) column. The chemicals utilized included Thermo Scientific's Dionex cation regenerant solution (TBAOH, 100 mM), Dionex CS12A Eluent Concentrate, and Thermo Scientific methanesulfonic acid (20 mM).

9.2.3 PHA production assay

Before proceeding with the PHA production assay, growth curves of the two selected bacterial strains were studied. The growth was monitored at specific time intervals (T0, T2, T4, T6, T8, T24, T30, T48, T54, T72 hours). The optical density (OD) was determined using a spectrophotometer at 600 nm (OD600). After studying bacterial growth, the PHA production assay was subsequently carried out. A strain of *Streptomyces coelicolor*, known for its inability to produce PHA, was used as a negative control. Following pre-inoculation in a nutrient-rich TSB medium, the bacterial cultures were washed and transferred to a growth medium of 50% Bushnell-Haas minimal medium (BH) (Supplementary) and 50% VFA sterilized by filtration. Upon reaching the PHA production phase, as determined by the growth curve, the biomass was harvested and subjected to Nile Blue staining for analysis (Presentato et al., 2018). Images were collected using a Zeiss Axioscope microscope equipped with a 63 $\times$ objective. Finally, the produced PHAs were extracted following the protocol by

Mannina et al. (2019). PHA concentration was measured by Electron Capture Detector Gas-Chromatography (ECD-GC) as reported by Mineo et al. (2023b), and it was calculated as the sum of polyhydroxybutyrate (PHB) and polyhydroxy valerate (PHV) concentrations.

9.3 Results and discussion

9.3.1 Bacterial strain

This study reports isolating bacterial strains from sewage sludge using culture methods to investigate their biotechnological potential for PHA production. The culture media employed—initially for mixed microbial cultures and subsequently for pure cultures—actually demonstrate better growth of Actinobacteria on Starch Casein Agar and of the other bacterial phyla in TSA media. Table 9.1 reports the identification, through molecular analysis, of bacterial strains and the origin of the sewage sludge from which each strain was isolated.

Table 9.1 Bacterial isolates from sewage sludge of Marineo WWTP or Unipa Campus pilot plant and molecular identification based on 16S rDNA gene sequencing.

Genera	Best type strain	Nr. isolates	Origin of isolation
Kokuria	<i>Kokuria rhizophila</i>	5	Marineo sludge + KMnO ₄
Serratia	<i>Serratia marcescens</i>	1	S-SBR (pilot plant)
Pseudomonas	<i>Pseudomonas aeruginosa</i>	4	Marineo sludge + KMnO ₄
	<i>Pseudomonas veronii</i>	1	Marineo sludge + KMnO ₄
	<i>Pseudomonas simiae</i>	1	Marineo sludge + KMnO ₄
	<i>Pseudomonas sp.</i>	2	Marineo sludge + KMnO ₄ , Fermenter (pilot plant)
Comamonas	<i>Comamonas koreensis</i>	6	Marineo sludge + KMnO ₄ , Unipa campus
	<i>Comamonas piscis</i>	1	S-SBR (pilot plant)

	<i>Comamonas sediminis</i>	2	Unipa campus sewage sludge
Sphingomonas	<i>Sphingomonas sp.</i>	1	S-SBR (pilot plant)
Bacillus	<i>Bacillus altitudinis</i>	1	Marineo sludge + KMnO ₄
	<i>Bacillus subtilis</i>	4	Marineo sludge + KMnO ₄ , Unipa campus, Fermenter
	<i>Bacillus velezensis</i>	2	Fermenter (pilot plant)
	<i>Bacillus sp.</i>	1	Unipa campus sewage sludge
Microbacterium	<i>Microbacterium oxidans</i>	1	Marineo sludge + KMnO ₄
	<i>Microbacterium chocolatum</i>	1	Microbacterium chocolatum
	<i>Microbacterium sp.</i>	1	Unipa campus sewage sludge
Dietzia	<i>Dietzia maris</i>	2	Marineo sludge + KMnO ₄
Gordonia	<i>Gordonia sputi</i>	1	Marineo sludge + KMnO ₄
	<i>Gordonia sp.</i>	2	Marineo sludge + KMnO ₄
Rhodococcus	<i>Rhodococcus sp.</i>	1	Fermenter (pilot plant)
	<i>Rhodococcus qingshengii</i>	1	Fermenter (pilot plant)
Enterococcus	<i>Enterococcus faecium</i>	3	Fermenter, Unipa campus sewage sludge
Delftia	<i>Delftia tsuruhatensis</i>	6	S-SBR, Fermenter
Bosea	<i>Bosea vestrisii</i>	1	Fermenter (pilot plant)
Leucobacter	<i>Leucobacter sp.</i>	1	S-SBR (pilot plant)

Proteus	<i>Proteus penneri</i>	2	S-SBR (pilot plant)
Alcaligenes	<i>Alcaligenes aquatilis</i>	1	S-SBR (pilot plant)
	<i>Alcanigenes faecalis</i>	1	S-SBR (pilot plant)

The study revealed a nearly equal distribution of Gram-positive (48.39%) and Gram-negative (51.61%) bacteria, primarily within the phyla Proteobacteria (48.65%), Actinobacteria (24.32%), Firmicutes (18.92%), Bacteroidetes (5.41%), and Deinococcus-Thermus (2.70%). Proteobacteria, dominant in wastewater, include bacteria such as *Pseudomonas*, *Acinetobacter*, and *Serratia*, which contribute to organic compound degradation and nutrient removal (Fedeila et al., 2018). Actinobacteria are common in aerobic treatments and aid in breaking down complex organics (Boufercha et al., 2022). Firmicutes, found mainly in anaerobic settings, include *Bacillus* species known for organic substrate degradation (Qin et al., 2021). Although less common, Bacteroidetes and Deinococcus-Thermus also appear in wastewater, with the latter resistant to extreme conditions (Yang et al., 2023). Certain bacteria identified offer promising applications; for instance, *Delftia tsuruhatensis* may produce harmane, which inhibits malaria transmission by affecting *Plasmodium* parasites in mosquitoes (Huang et al., 2023). *Kocuria rhizophila* promotes plant growth by enhancing root interactions and nutrient uptake (Mauceri et al., 2024), while *Comamonas* species excel in denitrification, converting nitrates and nitrites to nitrogen gas, which prevents eutrophication in aquatic ecosystems (Gumaelius et al., 2001). Many of the identified strains produce PHA. This underscores the valuable potential of wastewater microbiota, which can be harnessed across various sectors by leveraging the metabolic capabilities of bacteria. Among the isolates, *Bacillus*, *Comamonas*, and *Gordonia* show promise as potential PHA producers. *Pseudomonas aeruginosa*, a Gram-negative bacterium, is recognized as one of the leading producers of PHA, particularly medium-chain polyhydroxyalkanoates. It can utilize diverse substrates, including waste and renewable materials, making it versatile for industrial applications (Oyewole et al., 2024). Conversely, *Rhodococcus*, a Gram-positive genus, is known primarily for producing PHB, short-chain PHA. *Rhodococcus* can utilize unusual and complex substrates, such as hydrocarbons, and exhibits an exceptional ability to degrade toxic compounds and pollutants (Trakunjae et al., 2021). Interestingly, PHA-producing strains were found in the S-SBR, the Fermenter, and the sewage sludge from Unipa and Marineo, showing their adaptation to different conditions. Therefore, given the capabilities of *Rhodococcus* and *Pseudomonas*, these two strains (a

strain of *Pseudomonas aeruginosa* and a strain of *Rhodococcus qingshengii*) were chosen to test the production of PHA.

9.3.2 PHA production

Before conducting the PHA production assay, the VFA composition were assayed. Growth curves of the two strains, *Pseudomonas aeruginosa* and *Rhodococcus qingshengii*, were also analyzed. PHA production in bacteria like *Rhodococcus* (Trakunjae et al., 2021) or *Pseudomonas* (Ren et al., 2010) is typically associated with the stationary phase or nutrient stress (e.g., nitrogen or phosphorus deficiency, with excess carbon). Monitoring bacterial growth helped identify when active growth has ceased, but carbon reserves were still being metabolized. The growth curve identified the optimal time of the stationary phase of both bacteria to collect samples for the Nile Blue assay when PHA accumulation peaked. Standard analyses of the VFA mixture in the pilot plant showed that during the peak phase, when the VFAs were collected, the sCOD reached 44,125 mg/L COD. The standard NH₄-N, PO₄-P analyses, and ion chromatograph results were consistent. Table 9.2 presents the concentrations of anions and cations in the mixture at the time of collection, as determined by ion chromatography.

Table 9.2. Composition of anions and cations in the VFA mixture, analyzed by ion chromatography. The results display the concentrations of key anions (such as F⁻, Cl⁻, NO₂⁻, NO₃⁻, SO₄²⁻, PO₄³⁻) and cations (such as Na⁺, NH₄⁺, Ca²⁺, Mg²⁺, K⁺) present in the mixture during the VFA collection phase in the pilot reactor.

ANIONS											
F ⁻		Cl ⁻		NO ₂ ⁻		NO ₃ ⁻		SO ₄ ²⁻		PO ₄ ³⁻	
Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)
n.d.	n.d.	n.d.	n.d.	0.0031	0.04	n.d.	n.d.	n.d.	n.d.	15.1399	199.68
CATIONS											
Na ⁺		NH ₄ ⁺		Ca ²⁺		Mg ²⁺		K ⁺			
Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)	Area	Concentration (mg/L)
n.d.	n.d.	n.d.	n.d.	4.1413	31.847	17.6539	86.386	n.d.	n.d.	n.d.	n.d.

The mixture was composed of PO₄³⁻ anions 199.68 mg/L and a minimal part of NO₂⁻ 0.04 mg/L. The cations present were Ca²⁺ 31.847 mg/L and Mg²⁺ 86.386 mg/L. The VFA

composition, as analyzed by GC-FID, showed that acetic acid accounted for 99.48%, propionic acid for 0.30%, isovaleric acid for 0.12%, and butyric acid for 0.10%. This composition effectively promotes PHA production by combining essential nutrients with a rich carbon source in volatile fatty acids while limiting the nutrients required for growth, thereby encouraging PHA accumulation in bacteria. Acetic acid, a key component, is an excellent carbon source and is easily converted into PHB by bacteria (Wang et al., 2019). The low nitrite (NO_2^-) indicates a potential nitrogen shortage, which can shift bacterial metabolism from growth to producing reserve materials such as PHA (Tu et al., 2019). Additionally, the presence of PO_4^{3-} , Ca^{2+} , and Mg^{2+} supports bacterial metabolism, as these ions are crucial for cellular functions, including the activity of enzymes involved in PHA biosynthesis. Therefore, the bacterial cells were stained with Nile Blue during the stationary phase and subsequently analyzed using fluorescence microscopy. No signal was detected for *Streptomyces coelicolor*. The analysis confirmed the presence of intracellular inclusion bodies in both *Pseudomonas aeruginosa* and *Rhodococcus qingshengii*. Specifically, Figure 9.2 highlights the bright orange fluorescence observed in Nile Blue-stained cells, making PHA accumulation within the cells visible. To perform a quantitative analysis of the yields of the two tested strains and the control, the PHAs accumulated within the cells were extracted and analyzed using gas chromatography. The results indicate that *Pseudomonas aeruginosa* produced 10.13 mg/ml of PHB and 0.96 mg/ml of PHV, while *Rhodococcus qingshengii* produced 7.54 mg/ml of PHB and 0.76 mg/ml of PHV. *Streptomyces coelicolor* yielded 2.06 mg/ml of PHB and 0.26 mg/ml of PHV. As confirmed in this study, *Pseudomonas aeruginosa* exhibited a higher capacity for PHA production, *Rhodococcus qingshengii* exhibited a moderate PHA production, while *Streptomyces coelicolor* produced a negligible amount. *Pseudomonas aeruginosa* produced 1.34-fold higher PHB and 1.26-fold higher PHV than *Rhodococcus qingshengii*. The production of 10.13 mg/ml of PHB by *Pseudomonas aeruginosa* is noteworthy, especially when considering waste materials, such as sewage sludge used as precursors. Agrawal and colleagues tested various *Pseudomonas putida* isolates on nutrient agar supplemented with glucose, reporting 6 to 14 mg/ml yields. Considering that glucose serves as a primary energy source and that its high cost would make PHA production from glucose economically challenging, the yield obtained in this study is very promising (Agrawal et al., 2015). Altaee and collaborators (2016) utilized *Rhodococcus equi* to produce PHB using waste materials, specifically 1% crude palm kernel oil as the carbon source, achieving a 1.43 mg/ml yield (Altaee et al., 2016). *Rhodococcus qingshengii*, on the other hand, is a relatively understudied species. No work has been reported on its PHA production, making this study potentially the first of its kind. When comparing the PHB

yields obtained in this research with those reported by Altaee et al. (2016), the results also appear highly promising, further underscoring the potential of *Rhodococcus qingshengii* for PHA production. In both strains tested, we had a lower production of PHV than PHB. Under typical conditions, bacteria tend to produce significantly more PHB than PHV. PHB is produced from acetyl-CoA, a central intermediate in many bacterial metabolic pathways. Because acetyl-CoA is more readily available from commonly used carbon sources, it is easier for bacteria to channel resources toward PHB production. PHV is synthesized from propionyl-CoA or other longer-chain carbon precursors, often less abundant or more difficult for bacteria to metabolize. The need for these specific precursors makes the production of PHV less efficient than PHB (Amabile et al., 2024).

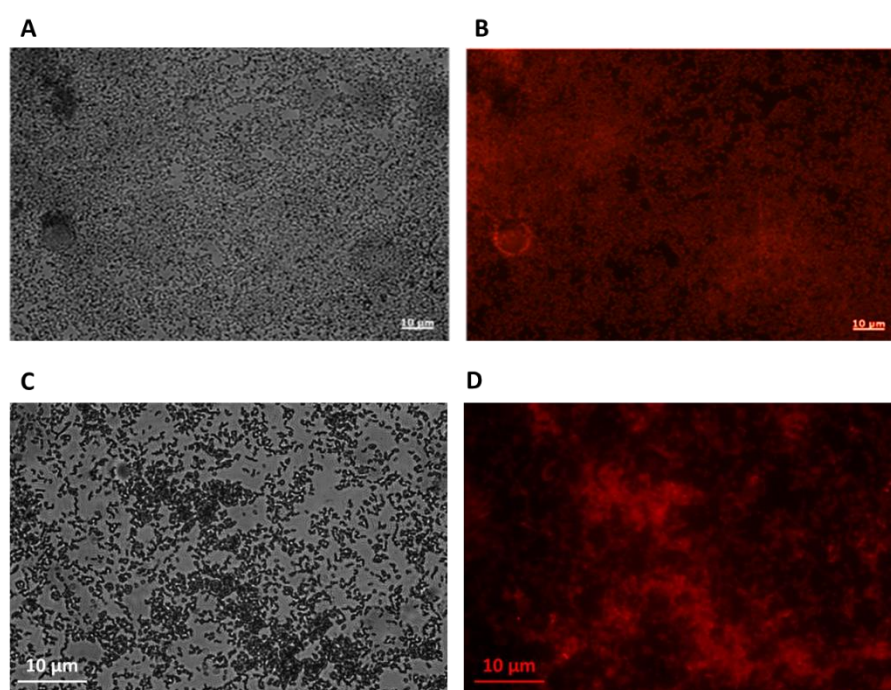


Figure 9.2 Fluorescence microscopy visualization of PHA within *Pseudomonas aeruginosa* and *Rhodococcus qingshengii* cells. Panels (A) and (B) show *Pseudomonas aeruginosa* cells under transmitted light and bright orange fluorescence emission, respectively, while panels (C) and (D) show *Rhodococcus qingshengii* cells under transmitted light and orange fluorescence, highlighting the bright fluorescence in Nile Blue-stained cells, making PHA accumulation within the cells visible.

9.4 Conclusions

This study assessed the capacity of two bacterial strains isolated from sewage sludge to produce PHA, specifically analyzing the yields of PHB and PHV through gas

chromatography. The two strains, *Pseudomonas aeruginosa* and *Rhodococcus qingshengii*, were selected from a collection of 60 isolates for their promising biotechnological potential. Both strains were fed a mixture of VFAs produced in a pilot plant, primarily consisting of acetic, propionic, isovaleric, and butyric acids, which served as precursors for PHA synthesis. Additionally, the presence of anions (PO_4^{3-} and NO_2^-) and cations (Ca^{2+} and Mg^{2+}) in the mixture supported microbial metabolism during PHA production. Fluorescence analysis, coupled with extraction and quantification through gas chromatography, confirmed the potential of these strains as biopolymer producers, demonstrating their ability to accumulate intracellular PHA as an energy reserve. Among the two, *P. aeruginosa* yielded the highest, while *R. qingshengii* produced a comparatively lower amount. The production of PHAs by microorganisms derived from waste environments using VFAs obtained from the fermentation of sewage sludge offers a sustainable approach to organic waste valorization and the production of biodegradable polymers. Nevertheless, further research is needed to optimize cultivation conditions and to gain deeper insights into the metabolic processes driving PHA accumulation, including exploring co-cultures to enhance yields. These findings present new opportunities for developing industrial-scale biotechnological processes with positive implications for both environmental sustainability and economic viability.

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9.6 Supplementary material

- STARCH CASEIN AGAR COMPOSITION

Soluble starch: 10 g/L

Casein (enzymatically hydrolyzed): 0.3 g/L

Sodium nitrate (NaNO_3): 2 g/L

Dipotassium phosphate (K_2HPO_4): 2 g/L

Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$): 0.05 g/L

Calcium chloride (CaCl_2): 0.02 g/L

Ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$): 0.01 g/L

Agar: 15 g/L

Distilled water

- TRYPTIC SOY AGAR COMPOSITION

Tryptone: 15 g/L

Soytone (enzymatic digest of soybean meal): 5 g/L

Sodium chloride (NaCl): 5 g/L

Agar: 15 g/L

Distilled water

- **BUSHNELL-HAAS AGAR COMPOSITION**

Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$): 0.2 g/L

Calcium chloride (CaCl_2): 0.02 g/L

Monopotassium phosphate (KH_2PO_4): 1 g/L

Dipotassium phosphate (K_2HPO_4): 1 g/L

Ammonium nitrate (NH_4NO_3): 1 g/L

Ferric chloride (FeCl_3): 0.05 g/L

Agar: 15 g/L

Distilled water

CHAPTER 10

**Spreading of metaresitome throught
sewage sludge microbiome of wastewater treatment pilot plant**

ABSTRACT

Antibiotic resistance is an escalating global health crisis, with wastewater treatment plants (WWTPs) serving as significant reservoirs for antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs). These enter WWTPs via human and animal waste, agricultural runoff, and pharmaceutical discharge, often persisting through treatment processes and spreading into natural water reservoirs. This persistence weakens antibiotic effectiveness, complicating infection treatment and increasing mortality. Reusing treated wastewater, especially in agriculture, raises concerns about introducing resistant pathogens to soils, crops, and aquifers, potentially exposing humans through the food chain. Given water scarcity and the necessity of water reuse, tracking metaresistome (i.e. antibiotic resistance genes from metagenomic DNA) in wastewater is essential. This study examined resistance in bacterial strains isolated from sewage sludge, revealing resistance primarily to Ampicillin and Erythromycin through cultural methods and whole genome shotgun (WGS) sequencing starting from metagenomic DNA of wastewater microbiota. Strengthened monitoring and regulations are critical to reducing the risks associated with antibiotic resistance in reclaimed water.

10.1 Introduction

Antibiotic resistance is a critical public health threat, primarily driven by bacterial acquisition of genetic resistance mechanisms (Salam et al., 2023). Bacteria employ several strategies to resist antibiotics, such as mutating molecular targets (e.g., ribosome proteins, DNA gyrase), producing inactivating enzymes (like β -lactamases), and activating efflux pumps that reduce drug concentrations inside cells (Reygaert, 2018). In Gram-negative bacteria, reduced membrane permeability through porin modification further limits antibiotic entry (Blair et al., 2014). Overuse of antibiotics in healthcare and agriculture accelerates resistance development, with clinical misuse (e.g., improper prescriptions, dosing errors) fostering selective pressure on microbes (Singh et al., 2024; Ahmed et al., 2024). This selective pressure allows resistant bacteria to survive and proliferate, increasing resistant strain prevalence. In agriculture, widespread antibiotic use in livestock also contributes to resistance, as it enables resistant strains to spread to humans through food, environment, and direct contact. Wastewater treatment plants (WWTPs) are important in waste management but also act as reservoirs of antibiotic-resistant bacteria (ARBs) and antibiotic-resistance genes (ARGs) (Gao et al., 2022). They process wastewater containing

antibiotics, metabolites, and bacteria from various sources. The treatment process can promote resistance, as ARBs can acquire genes via horizontal gene transfer (transduction, transformation, conjugation), enhancing resistance spread across species (Michaelis et al., 2023). Biofilm formation in WWTPs further promotes ARB survival and gene exchange, increasing ARB and ARG persistence in treated water, which then contaminates water resources, soils, and food chains. This contamination heightens the risk of difficult-to-treat infections, underscoring the need for monitoring and management strategies. Advanced WWTP technologies, such as ozonation, membrane filtration, and UV disinfection, are effective at reducing ARB and ARG presence (Alam et al., 2021). Routine surveillance of ARBs and ARGs in WWTPs and nearby waters is critical for assessing the resistance and developing interventions. This research evaluated antibiotic resistance using the disk diffusion (Kirby-Bauer) method on 60 bacterial isolates from sewage sludge, testing antibiotics from five major classes—ampicillin, tetracycline, gentamicin, chloramphenicol, and erythromycin—commonly resisted in WWTPs. In addition, Whole Genome Shotgun (WGS) analysis of metagenomic DNA from WWTP inlet and outlet microbiota samples characterized qualitatively and quantitatively ARGs.

These results aim at pioneering a comprehensive approach to monitor metaresistome (i.e. antibiotic resistance genes from metagenomic DNA) spreading through WWTPs as an important step for controlling the reuse of treated wastewater and protecting public health.

10.2 Material and Methods

10.2.1 Antimicrobial susceptibility by the disk diffusion method

Antimicrobial susceptibility testing using the disk diffusion method was performed on 60 bacterial isolates from wastewater at Marineo's municipal treatment plant and a pilot plant at the University of Palermo, which treats campus wastewater (Mannina et al., 2021). The Marineo plant is slated for upgrades to enhance material recovery from sewage sludge (Mannina et al., 2022), while the pilot plant replicates real conditions to optimize volatile fatty acid (VFA) and polyhydroxyalkanoates (PHA) production (Di Leto et al., 2022). Of the isolates, 45.61% came from Marineo sludge treated with KMnO_4 to promote VFA production, 21.05% from the pilot plant's selection-sequencing batch reactor for PHA production, 17.54% from the pilot fermenter under anaerobic conditions for VFA production, and 15.79% from untreated University of Palermo campus sludge. The antibiotic resistance profile was evaluated using the disk diffusion method (Kirby-Bauer) (Capri et al., 2024) on

Mueller-Hinton agar (MHA) (Supplementary). The antibiotics tested included ampicillin (Amp, 10 µg), gentamicin (Cn, 10 µg), erythromycin (E, 15 µg), chloramphenicol (C, 30 µg), and tetracycline (Te, 30 µg)(Oxoid, Thermo Fisher Scientific). Following incubation, the diameters of the inhibition zones (in mm) were measured in triplicate, averages were calculated, and bacterial isolates were classified as resistant (R), susceptible (S), or intermediate (I).

10.2.2 DNA extraction and whole genome sequencing

The sewage sludge samples that underwent the WGS were sampled at the Unipa pilot plant (Mannina et al., 2021). Specifically, duplicate aliquots were taken from both the first (Fermenter) and the last (S-SBR) tanks (fermentation and polyhydroxyalkanoates production tanks) of the system to assess how the distribution of antibiotic-resistance genes changes before and after treatment within the pilot system. The metagenomic DNA extraction procedure of the mixed microbial community was carried out according to Di Leto et al. (2024). For shotgun sequencing, the libraries were prepared and sequenced using the Illumina MiSeq platform in the 300 paired end (PE) format. This configuration allows for the generation of paired reads, each 300 base pairs long, which are used to cover longer sequences by overlapping the forward and reverse reads. The 300PE format provides a higher resolution of the genetic material, making it well-suited for metagenomic analyses and detailed assessments of microbial communities, including identifying antibiotic resistance genes or other functional elements.

10.2.3 Bioinformatic analyses and quality control

This study conducted a comprehensive bioinformatics analysis to process and interpret the sequencing data generated from the sewage sludge microbiome, focusing on taxonomic profiling and metagenomic assembly. The metaWRAP pipeline was employed to construct metagenome-assembled genomes (MAGs) from metagenomic reads. Initially, raw reads underwent quality control using FASTQC and TrimGalore via the READ_QC module. FASTQC was used to assess the quality of raw reads generated by the Illumina sequencing. This step evaluates parameters such as base quality scores, GC content, adapter contamination, and sequence duplication levels to ensure high-quality data for downstream analysis (Andrews, 2010).

10.2.4 Metagenome assembly

Metagenome analyses were conducted to provide a better overview of the microorganisms present and correlate them to the presence of genes that confer antibiotic resistance. To deeply characterize the most abundant taxa through genome assembly and functional characterization while incorporating less abundant taxa in the more inclusive assembly-free phase, assembly was performed using MEGAHIT default parameters to improve genome reconstruction and capture shared and rare genomic elements across samples (Li et al., 2015). After the assembly, contigs shorter than 300 base pairs were filtered out to improve quality. Paired-end reads were then mapped back to the contigs to verify accuracy and generate depth of coverage data, which was necessary for assessing assembly consistency and binning contigs into potential genomic units.

10.2.5 Genome binning, annotation of MAGs and assembly-free taxonomic analysis

Genome binning was used to reconstruct complete genomes from contigs by grouping those originating from the same genome based on characteristics like GC content, tetranucleotide frequencies, and coverage. This process is essential for generating MAGs that reveal the microbial community structure. The BINNING module utilized CONCOCT (Alneberg et al., 2014), MaxBin2 (Wu et al., 2016), and metaBAT2 (Kang et al., 2019) to bin the assembled contigs. MetaBAT2 uses probabilistic models to group contigs effectively, even in uneven environments. MaxBin2 leverages single-copy marker genes for precise genome reconstruction, while CONCOCT uses principal component analysis (PCA) to distinguish closely related species, especially in co-assembly scenarios. The BIN_REFINEMENT and BIN_REASSEMBLY modules were applied to consolidate and refine bins, using 50% completion and 10% contamination thresholds. Bin quantification was conducted using SALMON through the QUANT_BINS module. Gene prediction and annotation for each MAG was performed using Prokka (Seemann, 2014) while the taxonomic profile of MAGs was inferred using GTDB-Tk (Chaumeil et al., 2020). Finally, Antibiotic Resistance Genes (ARGs) were identified for each MAG using the Resistance Gene Identifier (RGI) and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2023). Metagenomic reads were classified using Kraken2 (Wood et al., 2019) with default parameters. Bracken was then used to calculate abundances for each taxonomical level from Kraken2 reports. Bracken results were visualized using the R package bracken_plot (Vill, 2023).

10.3 Results and discussion

10.3.1 Antimicrobial susceptibility of the isolated strains

The antibiotic resistance profile (Figure 10.1) reveals that the most concerning resistances were observed against ampicillin and erythromycin, each affecting 51.16% of the tested Bacteria. Conversely, the percentage of bacteria sensitive to ampicillin and erythromycin was 48.84%. In contrast, the results for tetracycline, gentamicin, and chloramphenicol appear less concerning. Specifically, 76.74% of the isolates are sensitive to tetracycline, 81.40% to gentamicin, and 76.74% to chloramphenicol. As a result, the resistance percentages are 23.26% for tetracycline, 18.60% for gentamicin, and 23.26% for chloramphenicol. We chose to test these antibiotics because wastewater generally contains Bacteria resistant to Ampicillin, Tetracycline, Gentamycin, Chloramphenicol, and Erythromycin (Fouz et al., 2020). Ampicillin is among the most widely used antibiotics in medical and veterinary practices. Since it is poorly metabolized by the body, a substantial portion is excreted through urine and feces, eventually reaching wastewater. It is also challenging to remove through conventional purification methods (Miri-Jahromi et al., 2022). Bacteria can develop resistance mechanisms to this antibiotic, which belongs to the penicillin class. Tetracycline, a drug commonly used in human and veterinary treatments, is one of the most prevalent antibiotic residues found in the environment. The lack of advanced treatment techniques in wastewater treatment plants to remove the residual from domestic and hospital wastewater poses a significant environmental risk (Gopal et al., 2020). Godinho and colleagues (2024) found that tetracycline and ampicillin were associated with the highest occurrence of resistant phenotypes among the antibiotics tested. They identified resistance to tetracycline and ampicillin in 32.5% and 25% of the strains isolated from wastewaters, respectively (Godinho et al., 2024). In our case, the situation seems to be less worrying for tetracycline, for which we found 23.26% antibiotic resistance, and more worrying for ampicillin, for which we found 51.16% resistance. The difference in resistance levels could be due to various factors, such as different sources of contamination, environmental conditions, or specific bacterial strains in our study area. In our case, the situation seems less worrying for tetracycline and more worrying for ampicillin, as this may reflect a more intensive use of ampicillin in the area, or a more rapid evolution of resistance mechanisms compared to tetracycline. Guo et al. (2015) confirmed the presence of erythromycin-resistant bacteria in both influent and treated wastewater (18%), with 70% of the resistant bacteria surviving post-treatment. When comparing these results with our findings, we observed a significantly higher and concerning resistance rate (51.16%) in our case. The discrepancy between the results of Guo et al. and our findings can be attributed to several factors. One key factor may be the different patterns of antibiotic use in the study areas. Higher usage of erythromycin or related antibiotics in our region could have exerted more significant

selective pressure on bacteria, promoting the emergence of resistant strains. A high percentage of sensitive bacteria regarding gentamicin and chloramphenicol was obtained. Aali and colleagues (2014) investigated the occurrence of antibiotic resistance in municipal and hospital wastewater separately by testing the most consumed antibiotics, including gentamicin and chloramphenicol. The result was that municipal wastewater showed a percentage of resistant bacteria between 0-20% for both, while hospital wastewater showed resistance percentages between 0-80% (Aali et al., 2014). Given that these values are based on an analysis conducted ten years ago, a comparison with our current data reveals that antibiotic resistance in municipal wastewater has unfortunately increased for these two antibiotics. Specifically, we observed resistance rates of 18.60% for gentamicin and 23.26% for chloramphenicol. In light of these findings, it is essential to implement effective strategies to curb the spread of antibiotic resistance and reduce the overuse of antibiotics in hospitals, agriculture, and domestic settings.

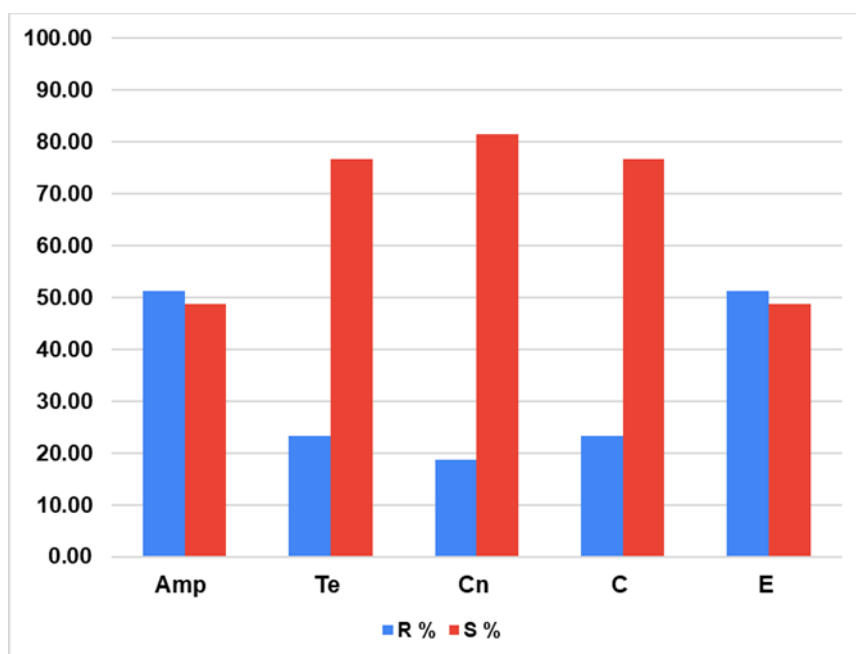


Figure 10.1 Antibiotic-resistance profiling of the 60 isolates analysed by Kirby-Bauer Assay testing the following antibiotics: Amp (Ampicillin); Te (Tetracycline); Cn (Gentamicin); C (Chloramphenicol); E (Erythromycin). The red bars indicate the percentage of susceptible isolates (S) and the blue bar the percentage of resistant isolates (R).

10.3.2 Read quality control

The assembly of bacterial metagenomes has achieved very satisfactory results, as demonstrated by several key parameters that attest to the quality and completeness of the dataset (Table 10.1). The N50 value, equal to 2826, indicates a good level of assembly continuity. A high N50 reflects the ability of the assembly to generate contigs of significant length, where half of the total assembled genome is contained in contigs of at least 2826 bp. This is a clear signal of the reconstructed high structural quality of the genome. In addition, the number of contigs longer than 10k bp is very high (10709 contigs), as is the number of contigs longer than 25k bp (1988 contigs) and those over 50k bp (456 contigs). This indicates not only the presence of very long and complete contigs but also a high distribution of contigs of significant size. This factor further contributes to the overall quality of the assembly. These data confirm that the coverage obtained is sufficient to represent the bacterial genome accurately. The quantity and length of contigs and the high N50 suggest that the dataset has adequate sequencing depth, reducing the risk of genome fragmentation and ensuring correct identification and annotation of genomic sequences. In conclusion, these results demonstrate that the assembly produced is of high quality, with sufficient coverage and continuity to robustly support subsequent bioinformatic analyses, allowing for in-depth characterization of the bacterial genome in question.

Table 10.1. Graphical representation of the number of contigs, illustrating the distribution and frequency of the contigs about the various parameters analyzed. The data shows significant variations and emerging trends, providing a clear picture of the relationships within the sample considered.

Statistics without reference	final_assembly
# contigs	442535
# contigs (≥ 0 bp)	442536
# contigs (≥ 1000 bp)	442535
# contigs (≥ 5000 bp)	33158
# contigs (≥ 10000 bp)	10709
# contigs (≥ 25000 bp)	1988
# contigs (≥ 50000 bp)	456
Largest contig	486966
Total length	1095566752
Total length (≥ 0 bp)	1095567075
Total length (≥ 1000 bp)	1095566752
Total length (≥ 5000 bp)	372914131

Total length (≥ 10000 bp)	220528786
Total length (≥ 25000 bp)	93487276
Total length (≥ 50000 bp)	42203424
N50	2826
N75	1535
L50	80990
L75	217419
GC (%)	56.76
Mismatches	
# N's	0
# N's per 100 kbp	0

10.3.3 Global overview of the sewage sludge microbiota structure

The taxonomic composition of the soil microbiome was examined using Kraken2 alongside Bracken. The findings, illustrated in Figure 10.2, display the relative abundance of bacterial taxa, offering insights into the overall microbial community. The sample-by-sample review of the reads reveals shared characteristics among the four samples and distinctions in composition between the Fermenter and S-SBR replicates. A general analysis at the domain level indicates that most microorganisms across all samples belong to the bacterial domain (96-97%), while the remaining organisms are classified within the eukaryotic domain (3-4%). The Bacterial domain, as illustrated by the Kronagram, was categorized into several groups: Proteobacteria comprised 59-60% of the Fermenter samples and 69-71% of the S-SBR samples; the Terrabacteria (a polyphyletic taxon comprising the Gram-positive phyla Actinomycetota and Bacillota as well as the phyla Chloroflexota and Deinococcota) represented 17-22% in the Fermenter samples and 15-16% in the S-SBR samples; the FCB (Fusobacteria-Cyanobacteria-Bacteroidetes) group accounted for 7% in the Fermenter samples and 3% in the S-SBR samples; while the PVC (Planctomycetes-Verrucomicrobia-Chlamydiae) group made up 2% of the Fermenter samples and 3% of the S-SBR samples. The phyla within the Terrabacteria group were subdivided as follows: Actinobacteria constituted 76-81% of the Fermenter samples and 86-87% of the S-SBR samples; Firmicutes represented 10-12% in the Fermenter samples, and 6% in the S-SBR samples; and Chloroflexi accounted for 5-7% in the Fermenter samples and 2-3% in the S-SBR samples. Additionally, some phyla, such as Cyanobacteria and Deinococci, contributed less than 1% to the Terrabacteria group. The FCB group consisted of 95% Bacteroidetes across all samples. In contrast, the PVC group comprised Planctomycetes (70% in the Fermenter

samples and 75% in the S-SBR samples) and Verrucomicrobia (30% in the Fermenter samples and 25% in the S-SBR samples). The differences in the composition of bacterial groups between the Fermenter and the S-SBR can be attributed to their distinct environmental conditions and the treatments they underwent. Similar microbial compositions have been reported in our previous studies, which utilized an NGS approach (Di Leto et al., 2022; Mineo et al., 2024; Di Leto et al., 2024). The fermenter, being the first reactor of the pilot plant, collects poorly treated sewage sludge, therefore showing greater microbial diversity. In this setting, Proteobacteria and the Terrabacteria group thrive due to their metabolic versatility (Nascimento et al., 2018), leading to a more varied composition of 7% from the FCB group. Conversely, the S-SBR, designed to select PHA-producing bacteria, predominates Proteobacteria (69-71%). This increase results from the active selection of bacterial strains effective at PHA production. The slight reduction in the Terrabacteria group suggests that some bacteria were not favoured in the S-SBR, where only the most suitable species were selected (Fernandes et al., 2020). Finally, the observed differences in bacterial composition reflect the specific environmental conditions and microbial selection strategies.

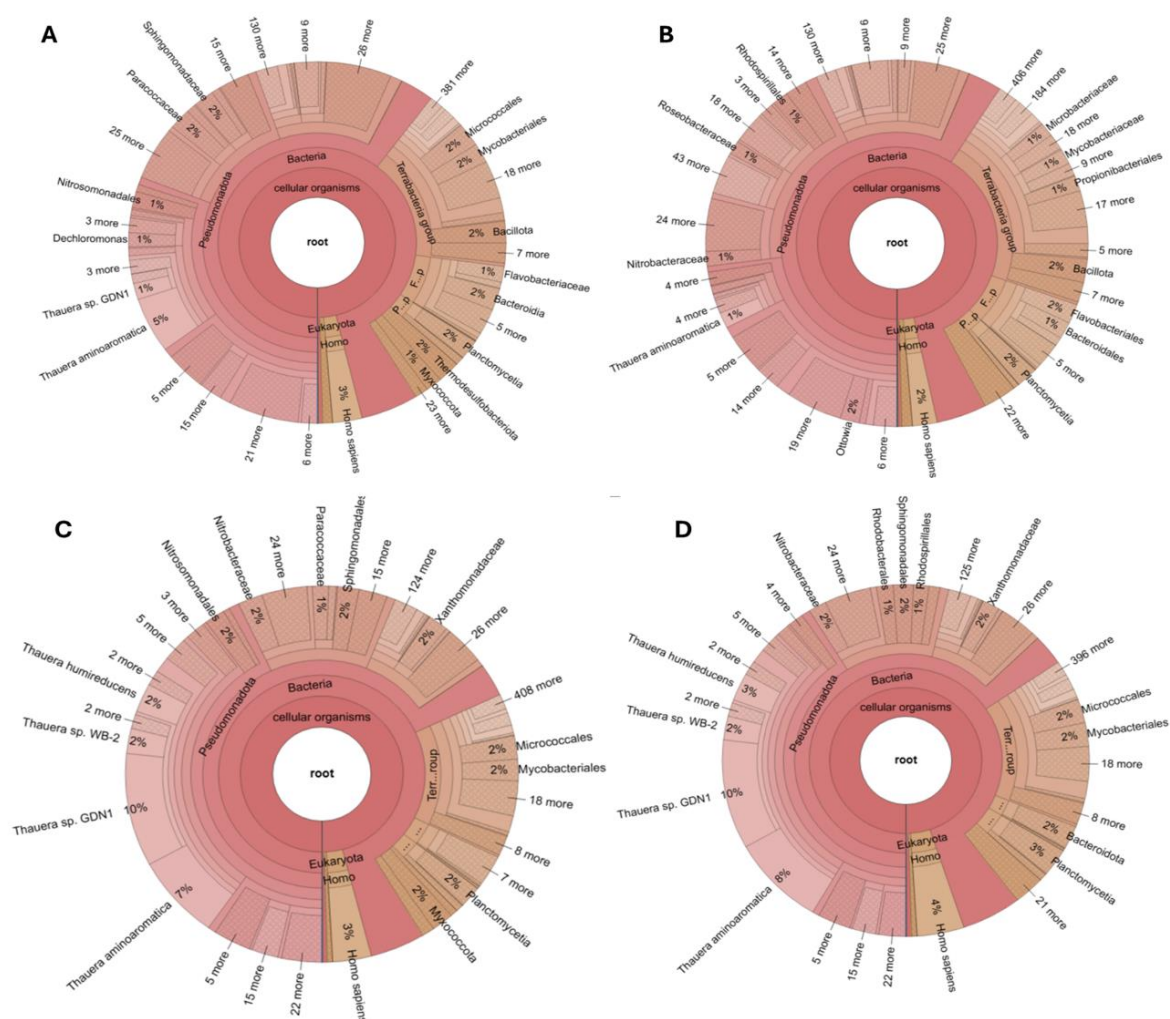


Figure 10.2 Kronagram highlighting all the taxonomy information at the bacterial family level from the co-assembly relating to the two biological replicates of sewage sludge aliquoted from the fermenter (A-B) and S-SBR (C-D).

10.3.4 Metagenome assembled bacterial genomes: metagenomic co-assembly and Bins quantification

The metagenomic binning process used multiple tools, including CONCOCT, MaxBin, MetaBat2, and MetaWRAP, with MetaWRAP integrating outputs to enhance bin completeness and reduce contamination. Performance was measured by bin completeness and contamination levels. This process yielded 136 high-quality genomes (over 50% complete and less than 10% contaminated). MetaWRAP stood out with near-complete genome bins and consistently low contamination, effectively leveraging the strengths of the other tools. In contrast, CONCOCT and MaxBin2 had high completeness for certain bins

but decreased in accuracy more quickly, while MetaBat2 showed lower overall completeness. After identifying the best assembly and binning method, we quantified the bin using Salmon. The abundance of each bin in each sample was estimated by mapping the sample reads, resulting in a relative abundance score. This score was then normalized to the commonly used Transcripts Per Million (TPM), allowing for direct comparison of bin abundances across samples. We identified two genome clusters using hierarchical clustering, each displaying distinct baseline abundance levels.

10.3.5 Antibiotic resistance genes identification

The RGI (Resistance Gene Identifier) CARD (Comprehensive Antibiotic Resistance Database) analysis is a powerful tool for identifying antibiotic resistance genes (ARGs) in genomic sequences, aiding in the understanding and mitigation of antimicrobial resistance. The CARD is a bioinformatic resource providing detailed information on ARGs, including data on resistant genes, proteins, mechanisms, and antimicrobial molecules. Regularly updated with the latest scientific research, it is a crucial reference for tracking resistance. The RGI is a tool developed to analyze genomic sequences and detect potential antibiotic-resistance genes by comparing them to those in the CARD database. It uses advanced algorithms and predictive models to categorize genes based on their resistance mechanisms.

The cluster analysis shows that antibiotic resistance was examined using duplicate samples at the phylum taxonomic level. Among the key findings from the RGI CARD analysis, 59.17% of microorganisms exhibited resistance to vancomycin, 21.30% to tetracycline, 7.1% to both erythromycin and metronidazole, 2.96% to benzalkonium chloride, and 1.77% to chloramphenicol. By comparing these results with those from the preliminary screening conducted via the antibiotic resistance assay, we can confirm a concerning and persistent antibiotic resistance to erythromycin. However, there is significant concern regarding antibiotic resistance, particularly vancomycin and tetracycline. This study represents one of the first investigations into antibiotic resistance utilizing the WGS approach and the RGI CARD analysis of such a complex microbial community. Nonetheless, recent studies focusing on individual bacterial strains or employing alternative methodologies have corroborated the rising trend of resistance to vancomycin (Brodrick et al., 2016; Sivaradjy et al., 2021) and tetracycline (LaPlante et al., 2022; Al-Mustapha et al., 2024). The increasing resistance to tetracycline and vancomycin in wastewater treatment plants is an escalating concern with multiple contributing factors. Antibiotic residues in wastewater—stemming

from extensive use in human and veterinary medicine and industrial applications—exert selective pressure on bacterial populations, promoting the proliferation of resistant strains. Additionally, WWTPs harbor a complex microbial community where bacteria can exchange resistance genes through various gene transfer mechanisms. Furthermore, many wastewater treatment processes, such as sedimentation and disinfection, may not effectively eliminate all resistant bacteria, allowing these organisms to persist and propagate within the environment (Sambaza et al., 2023).

Furthermore, this study detailed the percentages of resistance mechanisms in the examined microbial community. Specifically, 50.58% of the resistances were attributed to mechanisms conferred by antibiotic target alteration, 35.50% were due to antibiotic efflux, 3.55% resulted from antibiotic inactivation, and 2.37% stemmed from antibiotic target protection. The gene families associated with antibiotic resistance included the following: 31.95% were attributed to the resistance-nodulation-cell division antibiotic efflux pump; 29.58% to the glycopeptide resistance gene cluster *vanT*; 10.65% to the *vanY* and *vanW* glycopeptide resistance gene clusters; 6.50% to nitroimidazole reductase; 5.32% to the *vanH* glycopeptide resistance gene cluster; 1.27% to the small multidrug resistance antibiotic efflux pump; 1.18% to miscellaneous ABC-F subfamily ATP-binding cassette ribosomal protection proteins; and 0.6% to tetracycline-resistant ribosomal protection protein. In agreement with other works, the main resistance mechanisms are antibiotic target alteration and antibiotic efflux (Devi et al., 2024). Bacterial intrinsic resistance is marked by the continuous enhancement of resistance mechanisms through modifications in cell wall structure and other cellular characteristics. These resistance mechanisms include altered drug uptake, changes at the target site, mutations in target sites, and the action of efflux pumps. The efflux pump mechanism is particularly significant, enabling resistant bacteria to expel antibiotics from their cells into the external environment using transporter proteins. Drug-resistant bacterial cells also undergo natural mutations in their genes, significantly affecting the efficacy of antibiotics. Often, these mutational changes facilitate the maintenance of cellular homeostasis, which relies on the antibiotic's presence at the target site of action. Generally, the resistance mechanisms arising from mutations are attributed to modifications in antimicrobial action, including (i) reduced drug uptake, (ii) activation of efflux mechanisms, and (iii) alterations in metabolic pathways (Devi et al., 2024). This phenomenon causes the emergence of new antibiotic resistance mechanisms which can then spread throughout the environment.

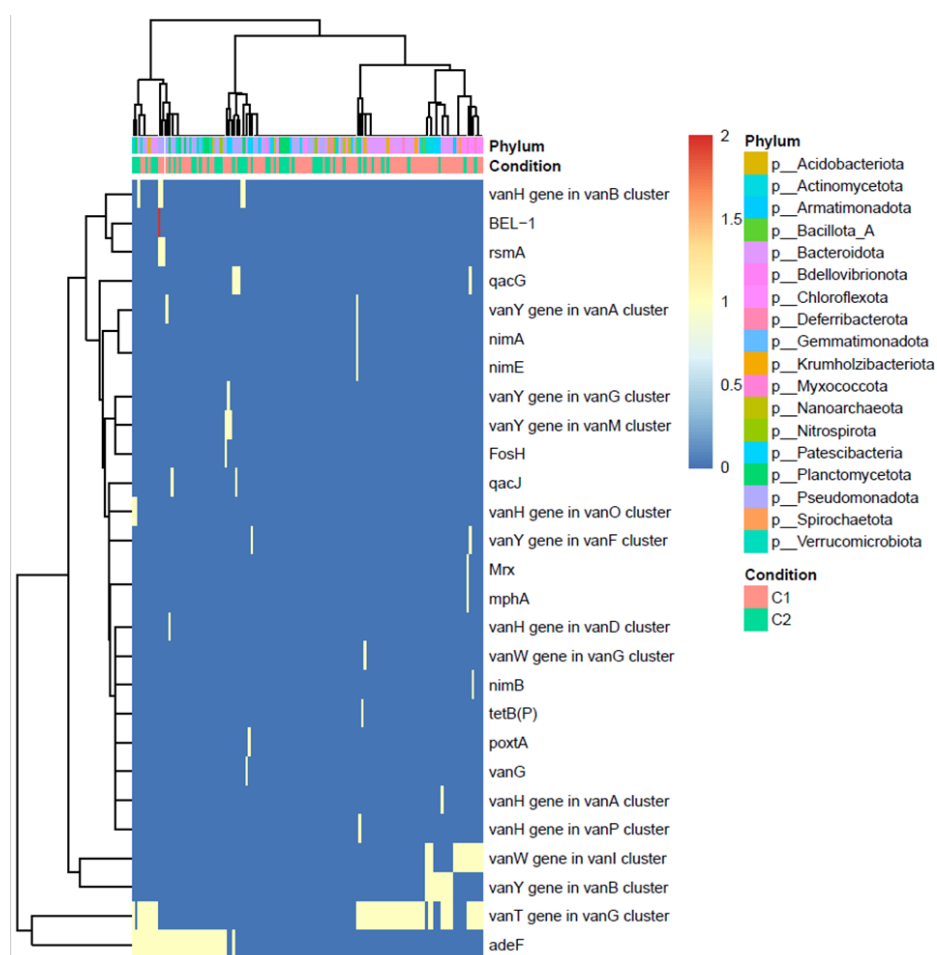


Figure 10.4 Heatmap showing the distribution of CARD RGI predicted resistance-encoding. C1 condition is related to Fermenter samples and C2 condition to S-SBR samples.

10.4 Conclusions

This study highlights significant findings regarding antibiotic resistance in wastewater. It is one of the first pioneering studies analyzing ARGs through WGS in complex microbial communities, such as those found in sewage sludge. The disk diffusion method reveals that the isolated strains were predominantly resistant to ampicillin and erythromycin. WGS analysis identified the most concerning antibiotic resistances, specifically vancomycin and tetracycline, primarily associated with Proteobacteria and Terrabacteria. These bacterial groups were the most prevalent at the overflow and outflow of the pilot plant from which the samples were collected. The study also found that the primary mechanisms underlying resistance were antibiotic target alteration and antibiotic efflux. In light of these results, it is evident that new strategies to mitigate antibiotic resistance in wastewater treatment must be developed urgently. Enhanced treatment methodologies, along with targeted interventions, are essential to address this growing public health concern and reduce the prevalence of antibiotic-resistant bacteria in the environment.

10.5 References

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10.6 Supplementary material

- MUELLER-HINTON AGAR COMPOSITION

Beef extract: 2.0 g/L

Acid hydrolysate of casein (peptone): 17.5 g/L

Starch: 1.5 g/L

Agar: 17.0 g/L

Final pH: 7.3 ± 0.1 at 25°C

Distilled water

CHAPTER 11

General Conclusions

General conclusions

Traditionally regarded as sources of pollutants and threats to human and environmental health, wastewater can be reevaluated as an inexhaustible resource of valuable materials from a circular economy perspective. This Ph. D. project aims to shed light in the relationship between the operating conditions and the structure of microbiota residing in different reactors of wastewater treatment plants (WWTP) to improve the exploitation of microbial capabilities in wastewater treatment and resource recovery. Indeed, the complexity and versatility of the sewage sludge (SS) microbiota members enable them to orchestrate biological processes essential for recovering valuable materials. Specifically, this research, conducted as part of WIDER-UPTAKE (<https://wider-uptake.eu/>), represents a pioneering effort to harness the potential of microbial communities in wastewater, sewage sludge, and treated water, to ensure their applicability in industrial and agricultural sectors. Several studies have been conducted, and various strategies have been implemented to optimize the process parameters. This research considered a municipal WWTP located in Marineo (Palermo, Sicily); a pilot plant, fully designed and constructed as part of the WIDER-UPTAKE project, situated at the Department of Engineering and fed with wastewater from the university campus; laboratory-scale batch reactors. Three sets of batch experiments were performed to improve VFA production; three experiments in a pilot plant one focused on enhancing VFA production and two on PHA production; three experimental campaigns in a greenhouse. To improve VFA production, the SS microbiota was utilized performing acidogenic fermentation, using carbon sources from the sewage sludge and by testing various process parameters to optimize yields. After VFA production and process optimization, these acids were employed as precursors for synthesizing PHA thermoplastic polymers, by leveraging the same microbiota and selecting strains based on their PHA accumulation capacity. Simultaneously, the purified water obtained from WWTPs was used for irrigation and as a nutrient source and applied to greenhouse plants. In this context, the microbial community of the treated and irrigated soil was analyzed to assess the beneficial impact of PGP bacteria on plant development. All the mentioned setups were based on leveraging the microbial potential of mixed microbial communities (MMC). Cultural isolations were also performed to identify bacterial strains in the sewage sludge and two strains were tested for PHA production in pure cultures. Additionally, given that WWTPs are known reservoirs of ARB and ARG, WGS analyses were conducted to assess the WWTP metagenome resistome profile. At the same time, specific investigations were carried out on individual isolated strains to identify ARBs. For the mixed microbial community, analyses were performed

using a NGS approach. By examining the starting microbial communities, it becomes evident that in all samples—whether sourced from a municipal plant or the university campus—the mixed microbial community of sewage sludge is predominantly composed of Bacteria, while Archaea are present in much smaller proportions, never exceeding 2% abundance. This observation can be attributed to the fact that wastewater is not considered an extreme environment, unlike high-temperature or high-salinity habitats. Instead, this environment provides a range of nutrients and non-extreme environmental conditions for microbial growth, resulting in a greater diversity of bacteria than archaea. Bacteria tend to proliferate more rapidly in nutrient-rich and variable conditions, whereas archaea, typically adapted to more extreme environments, may struggle to compete in wastewater settings. Additionally, despite the experiments being conducted across different seasons, the starting samples consistently exhibit a core microbial community alongside a small, variable community. The core community comprises Proteobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia.

Under the conditions that yielded the highest VFA production (pH 10; addition of KMnO_4 relative to an optimal level of volatile suspended solids (VSS) or total suspended solids (TSS); and a reduced headspace volume of 40%), metataxonomic analyses consistently indicated an increase in the percentage abundance of Firmicutes, known to be excellent VFA producer bacteria. Additionally, when treatments were conducted with KMnO_4 , there was a corresponding rise in Bacteroidetes and a decrease in Proteobacteria. KMnO_4 acts as an oxidizing agent that can affect microbial composition. Its addition promoted the growth of Firmicutes and Bacteroidetes, which are more resilient or better adapted to utilize the degradation products generated by KMnO_4 . In contrast, Proteobacteria, which encompasses many species sensitive to oxidizing agents, experienced a decline in their abundance. An increase of anaerobic bacteria was registered after KMnO_4 confirming the activation of the fermentation process leading to VFA production. The pH 10 increased both Chloroflexi and Firmicutes. However, when we examine microbial metabolism more closely, we observe a significant decrease in methanogens, consumers of VFAs. This decrease contributes to the higher yields of VFAs produced. The 40% volume headspace in the pilot plant favoured an increase in Proteobacteria and Firmicutes. The headspace volume likely created an environment that supported the growth of both Proteobacteria and Firmicutes through favourable metabolic interactions, nutrient availability, and optimal physical conditions.

Focusing on the optimal conditions in the pilot scale (organic loading rate of $1.3 \text{ g COD l}^{-1} \text{ d}^{-1}$ and a C/N ratio of 3.5), which led to the highest yields of PHA from VFAs, we observe

a clear and consistent selection of specific bacteria across both experimental setups. Notably, Proteobacteria is increasingly recognized as the best natural producers of PHA. Further evidence of their role in PHA production is seen in both setups, where the sample with the highest yield of PHA products contained a predominant family of Rhodocyclaceae (Betaproteobacteria). The eukaryotic community was also examined at a C/N ratio of 3.5, revealing that Alveolates were the most abundant in samples with the highest PHA yields. At the same time, Fungi predominated in samples with the lowest yields. This suggests that Fungi may have a negative impact on PHA production, whereas Alveolates may play a positive role. It is important to note that some Alveolata organisms can form symbiotic associations with bacteria, incorporating them within their cells. These interactions can be mutually beneficial: bacteria can supply nutrients or perform metabolic functions that the Alveolates cannot carry out independently, while the Alveolates provide a protective environment and a source of nutrients for the bacteria. Thus, it is plausible that Alveolates have established symbiotic relationships with PHA-producing bacteria. PHA production assays in single cultures revealed genera such as *Pseudomonas* and *Rhodococcus* can produce PHA from VFA under controlled laboratory conditions. The primary polymers produced were polyhydroxybutyrate and polyhydroxyvalerate.

By examining soils irrigated TWW and fertilizers recovered from it using absorbent matrices like biochar for phosphate and zeolite for ammonium, it was observed that TWW significantly enhances plant growth compared to tap water. However, its effect is not as pronounced as commercial fertilizers (which can be pollutants). Additionally, irrigation with TWW, in combination with enriched biochar and zeolite, helps mitigate the negative impacts of substances that could adversely affect plant growth. The soil microbial community was predominantly composed of Bacteria, with a lower presence of Archaea, including Actinobacteria, which are characteristic soil bacteria. Plant growth-promoting bacteria (PGPB) such as *Bacillus*, *Streptomyces*, *Kocuria* genera, and beneficial bacteria involved in nitrogen metabolism like *Nitrospira* genus members were identified. Notably, no pathogenic bacteria were detected.

Finally, the analysis of antibiotic resistance showed a predominant presence of ARBs, primarily resistant to ampicillin and erythromycin. WGS analyses confirmed a significant resistance to erythromycin and indicated concerning levels of resistance to vancomycin and tetracycline. WGS analyses explained the presence of specific ARGs. The main mechanisms contributing to the detected resistance involve antibiotic efflux and modification of the antibiotic target. The gene families associated with this resistance were primarily identified

as the resistance-nodulation-cell division antibiotic efflux pump and the glycopeptide resistance gene cluster (*vanY*, *vanW*, *vanH*, *vanT*). However, it has been observed that the abundance of ARBs tends to decrease throughout the pilot plant, with lower levels found in the final S-SBR. This indicates that the plant is already effective in reducing the load of antibiotic resistance that could be released into the environment. Nevertheless, careful monitoring may be desirable to prevent the spread ARBs and ARGs into the environment through the reuse of TWW.

In conclusion, the results of this study underscore the remarkable potential of treated wastewater and its associated microbial communities in promoting plant growth and enhancing the production of valuable biopolymers like PHA. The positive effects observed, particularly in the context of specific bacteria and their interactions, indicate that these resources can be effectively harnessed for agricultural and biotechnological applications by exploiting microbial metabolic capabilities improvement. This work has led to the understanding that the microbiota can be modulated to improve the production of valuable materials. For example, particular bacteria with discovered metabolic capacities could be introduced into the reactors to implement the yields of VFA and PHA or promote plant growth in the soil. However, it is equally important to acknowledge the potential risks associated with ARBs and ARGs in treated wastewater. Therefore, ongoing and rigorous monitoring will be essential to prevent the dissemination of these resistant strains into the environment, ensuring that the benefits of using treated wastewater are realized without compromising ecological safety. This thesis offers an essential contribution to human health and environmental protection. It highlights the crucial role of microorganisms in reusing raw materials starting from WWTPs and in promoting sustainable practices, paving the way for a future in which exploiting the capabilities of microorganisms favours sustainable strategies to improve the quality of the environment and human life.

GLOSSARY OF ACRONYMS

AD: anaerobic digestion

AMP: ampicillin

ARB: antibiotic-resistant bacteria

ARG: antibiotic resistance genes

ASV: Amplicon sequence variant

ASV: amplicon sequence variants

BH: Bushnell-Haas

C: chloramphenicol

CARD: Comprehensive Antibiotic Resistance Database

CN: gentamicin

COD: chemical oxygen demand

DMC: dimethyl carbonate

DNA: deoxyribonucleic acid

DO: dissolved oxygen

E: erythromycin

EB: enriched biochar

EC: electrical conductivity

ECD: Electron Capture Detector

EPS: extracellular polymeric substances

EZ: enriched zeolite

FCB: Fusobacteria-Cyanobacteria-Bacteroidetes

FID: flame ionization detector

GC: gas chromatograph

HP: headspace pressure

IFW: inorganic NPK solution

MAG: metagenome assembled genomes

MMC: mixed microbial community

NB: natural biochar

NGS: next generation sequencing

NPK: nitrogen-phosphorus-potassium

NZ: natural zeolite

OLR: organic loading rate

ORP : oxidation reduction potential

PCoA: Principal coordinate analysis

PCR: Polymerase chain reaction

PGPB: plant growth-promoting bacteria

PHA: polyhydroxyalkanoate

PHB: Polyhydroxybutyrate

PHBV: Poly-hydroxybutyrate-co-hydroxyvalerate

PHC: polyhydroxyhexanoate

PHD: polyhydroxydecanoate

PHV: Polyhydroxyvalerate

PP: potassium permanganate

PVC: Planctomycetes-Verrucomicrobia-Chlamydiae

RGI: Resistance Gene Identifier

RNA: ribonucleic acid

SCA: starch casein agar

sCOD: soluble chemical oxygen demand

SS: sewage sludge

S-SBR: selective-sequencing batch reactor

TAP: tap water

TCOD: total chemical oxygen demand

Te: tetracycline

TN: total nitrogen

TN: total nitrogen

TOC: total organic carbon

TP: total phosphorus

TSA: Tryptone Soy Agar

TSB: Tryptone Soy Broth

TSS: total suspended solid

TWW: treated wastewater

VFA: volatile fatty acid

VSS: volatile suspended solid

WAS: waste activated sludge

WGS: whole-genome shotgun

WRRF: water resource recovery facilities

WW: wastewater

WWBR: wastewater biorefineries

WWTP: wastewater treatment plant

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