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Optimization of ammonia removal from landfill leachate using activated sludge

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CONTENTS

1. Summary

Landfill leachate represents one of the most complex environmental challenges due to its highly pollutant impact. In fact, it is rich in ammonium nitrogen (NH_4^+-N), recalcitrant organic compounds and heavy metals. Traditional wastewater treatment plants (WWTPs) struggle to handle this pollutant, especially when leachate matures, standing for a long time in landfill, and becomes increasingly difficult to be biologically treated. This research aims to explore innovative solutions to this pressing issue by combining microbial engineering techniques with advanced biofilm-based technologies.

In the first phase, the present study analyzed the microbial composition of activated sludge derived from the Porto Sant'Elpidio, WWTP, located in central Italy. This plant annually collects and treats large volumes of landfill leachate from a large surrounding area.

Using metagenomic sequencing of the 16S rRNA gene, native microbial communities were identified and evaluated for their ability to tolerate high concentrations of ammonium, exceeding 350 mg/L. Through a selection process, called Repetitive Re-Inoculum Assay (RRIA), microbial diversity was significantly reduced, allowing the enrichment of specialized and highly efficient nitrogen-removing species.

The predominant bacterial families identified included Alcaligenaceae, Nitrosomonadaceae, Chitinophagaceae, and Comamonadaceae, which demonstrated ammonium removal rates of up to 85% in a leachate-based medium. Additionally, three genera – *Klebsiella* sp., *Castellaniella* sp., and *Acinetobacter* sp. were isolated and found capable of converting ammonium into gaseous nitrogen by combining heterotrophic nitrification and aerobic denitrification.

These coupled processes minimize the release of harmful intermediates such as nitrites and nitrates, further enhancing the efficiency and sustainability of the treatment.

The second phase of our study focused on applying these enriched microbial communities to develop a moving bed biofilm (MBB)

system. This approach leverages the capacity of biofilms to concentrate active biomass and improve the efficiency of biological treatment processes. The biofilms were cultivated on high-density polyethylene (HDPE) carriers, and their development was monitored using advanced techniques such as Crystal Violet staining and confocal microscopy. The results were remarkable: the MBBs achieved an ammonium removal rate of up to 80% within just 24 hours, with a nitrification efficiency ten times higher than conventional activated sludge where bacteria were in planktonic phase. Metagenomic analyses confirmed the critical role of bacterial families such as Chitinophagaceae, Comamonadaceae, Sphingomonadaceae, and Nitrosomonadaceae in driving nitrification and denitrification processes.

Moreover, MBBs exhibited exceptional robustness under varying environmental conditions, including low temperatures (as low as 10°C) and high salinity levels, making them a versatile solution for real-world applications.

The results so far achieved are very promising in the attempt to overcome the limitations of traditional WWTPs in handling highly polluted leachates. In fact, targeted microbial community selection, followed by bacteria integration into mobile biofilm systems, has proven to be a highly efficient and sustainable solution for landfill leachate treatment. The initial phase of this study provided a strong theoretical foundation to identify and select key microorganisms from the sludge in order to obtain an optimized mixture with elevated efficiency in ammonia removal. In the second phase, we used these selected bacterial communities to generate biofilms demonstrating the practical potential of MBBs to significantly enhance the performance of existing facilities. Our study represents an advancement in wastewater management, paving the way for large-scale application of the modern technologies as MBBs and offering an innovative and effective response to global environmental challenges.

2. Introduction

Environmental pollution is one of the most pressing global issues, driven by human activities such as industrialization, urbanization, and agriculture. It leads to the degradation of air, water, and soil quality, causing severe harm to ecosystems and public health.

Key pollutants include greenhouse gases, particulate matter, nitrogen compounds (such as ammonia), and heavy metals, all of which have direct and indirect effects on climate change, biodiversity, and human well-being. Air pollution is linked to respiratory diseases, while water pollution affects aquatic life and drinking water sources. Efforts to reduce pollution focus on stricter regulations, cleaner technologies, and sustainable practices in agriculture and waste management (WHO, 2023; EPA, 2022

2.1 Environmental pollution

In the recent years, ammonia pollution represents one of the most pressing environmental issues, especially concerning its presence in wastewater. Ammonia is primarily produced through agricultural practices, especially due to the large use of nitrogen-based fertilizers and manure management, but it also arises from industrial discharges and urban waste. Once released into the environment, ammonia is highly soluble in water, and it often contaminates surface and groundwater, leading to significant ecological and health risks. The toxicity of ammonia to aquatic ecosystems is well-established. In particular, ammonia is highly harmful to fish and other aquatic organisms. It interferes with their respiratory systems, as fish absorb oxygen through their gills. (D. J. Randall et al., 2002)

When ammonia concentrations exceed 0.5 mg/L, many fish species begin to experience respiratory distress, as ammonia disrupts osmosis and damages gill tissue, impairing their ability to exchange gases. (EPA, 2013) Research has shown that concentrations above 1 mg/L can cause direct mortality, while lower levels can reduce growth, reproduction, and immune function, making fish species more susceptible to diseases. For instance, studies on rainbow trout

(Oncorhynchus mykiss) have shown that even exposure to 0.6 mg/L of ammonia significantly impacts feeding behavior and growth. (H. Liu et al., 2023)

Moreover, ammonia is a significant driver of eutrophication, a process where, in general, excess of nitrogen stimulates an overgrowth of algae and aquatic plants.

This leads to a reduction in dissolved oxygen, creating “dead zones” where aquatic life cannot survive. (R. J. Diaz et al., 2008)

Eutrophication, exacerbated by ammonia, is a major environmental concern for freshwater ecosystems globally. The Environmental Protection Agency (EPA) has reported that more than 60% of the United States water bodies are affected by nutrient pollution, with ammonia being a key contributor to the acceleration of eutrophication (EPA, 2022). This overgrowth of algae does not only disrupt aquatic life but also degrades water quality, leading to increased treatment costs for drinking water.

Ammonia’s toxic effects extend beyond the immediate aquatic environment, affecting entire ecosystems. Invertebrates, which are vital for maintaining the food web in aquatic ecosystems, are also vulnerable to ammonia toxicity. The loss of these species can lead to cascading effects throughout the food chain, impacting higher trophic levels, including fish and birds that rely on aquatic ecosystems for food. (J. A. Camargo et al., 2006)

In addition to ecological impacts, ammonia pollution is a serious concern for wastewater treatment facilities. Ammonia is difficult to remove from wastewater, especially at higher concentrations.

Traditional methods, such as nitrification (the biological process where ammonia is converted to nitrites and then nitrates), are sensitive to ammonia’s concentration. When ammonia levels exceed 10 mg/L, nitrifying bacteria become inhibited, reducing the efficiency of biological wastewater treatment processes. As a result, wastewater treatment plants must use more energy, chemicals, and labor to address ammonia pollution. (Y. Liu et al., 2020).

Moreover, untreated or inadequately treated wastewater can lead to ammonia contamination in rivers, lakes, and oceans, further exacerbating the pollution problem. (S. Liu et al., 2024).

For wastewater treatment, several advanced technologies have been developed to address the challenges of ammonia removal. The Anammox process (anaerobic ammonium oxidation) has emerged as a promising solution for treating ammonia in wastewater, as it eliminates the need for oxygen while converting ammonia directly to nitrogen gas, reduces energy consumption. (H. Liu et al., 2023).

The increasing concentration of ammonia in wastewater and its ecological impact requires urgent attention. Several studies have documented how increasing ammonia levels impair aquatic ecosystems and increase treatment challenges. (Liu et al., 2020; Camargo & Alonso, 2006) Certainly, ammonia is not the only key factor contributing to the degradation of aquatic ecosystems, but it complicates the treatment of wastewater and increases the cost of water management. High concentrations of ammonia inhibit nitrifying bacteria, reducing treatment efficiency and raising operational costs. (Li et al., 2021; Xie et al., 2017)

It is essential to develop and implement efficient technologies for ammonia removal, as well as regulatory measures to limit ammonia emissions from agricultural and industrial sources. Advanced technologies such as Anammox, membrane processes, and novel adsorbent systems offer promising improvements in ammonia removal efficiency. (Deng et al., 2022) Regulatory efforts are particularly important given the substantial nitrogen loading from these sectors. (European Environment Agency, 2020)

Efforts to mitigate ammonia pollution must also focus on reducing emissions at the source. Sustainable agricultural practices, such as the optimization of fertilizer application and better management of manure, can significantly reduce the amount of ammonia released into the environment. Studies show that strategies like precision fertilization, manure treatment, and integrated nutrient management can lead to meaningful reductions in ammonia emissions from agriculture. (Zhang et al., 2018;)

Additionally, better wastewater management practices, combined with cutting-edge treatment technologies, can help limit the impact of ammonia on water quality and ecosystems.

In conclusion, ammonia is a potent pollutant with significant negative effects on water quality, aquatic life, and the efficiency of wastewater treatment systems. Addressing this issue requires integrated strategies, such as the development of advanced technologies, improved wastewater management, and the reduction of ammonia emissions from agricultural and industrial sources. Overcoming these challenges is essential to protecting aquatic ecosystems and ensuring better water resource quality.

2.2 Leachate Treatment and Nitrogen Removal Processes

Leachate is defined as a liquid effluent produced when rainwater percolates through waste materials, resulting in a complex mixture of contaminants, including significant levels of ammonia-nitrogen, heavy metals, chlorinated organic compounds, and inorganic salts (Nika et al., 2020).

The presence of these pollutant substances necessitates careful treatment prior to the discharge of leachate into natural water bodies, as untreated leachate can pose serious environmental risks and harm aquatic ecosystems. (Renou et al., 2008)

To ensure safe disposal, the removal of organic materials is typically measured by parameters such as chemical oxygen demand (COD), biological oxygen demand (BOD), and ammonium concentration. These metrics are critical in assessing the organic load and potential impact of leachate on receiving waters. (Kalčíková et al., 2022)

With the rise of industrial and commercial activities worldwide, there is an increasing demand for innovative technologies aimed at effective waste and leachate disposal.

Conventional treatment strategies encompass various approaches:

1. **Leachate Transfer**: This involves the recycling of leachate and its combined treatment with domestic wastewater to dilute the contaminant load and enhance treatment efficiency.
2. **Biodegradation**: This method includes both aerobic and anaerobic processes that utilize microorganisms to break down organic pollutants. Aerobic processes involve the presence of

oxygen, while anaerobic processes occur in oxygen-depleted environments, allowing for different pathways of degradation.

3. **Chemical and Physical Methods**: These techniques include chemical oxidation, which uses chemicals to break down contaminants; adsorption, where pollutants adhere to solid surfaces; chemical precipitation, which helps to remove dissolved substances; sedimentation/flotation to separate solids from liquids; and air stripping to remove volatile contaminants.

While landfill leachate is a challenging waste stream, it can also be seen as a potential source of carbonaceous nutrients that support microbial growth in activated sludge systems commonly used in wastewater treatment plants. However, the high concentration of ammonia present in leachate poses a significant challenge. Elevated ammonia levels can be toxic to the microorganisms responsible for treating wastewater, leading to reduced efficiency in biological treatment processes. (Spagni et al., 2019)

To mitigate these effects, strategies such as serial dilutions of leachate and the use of combined aerobic and anaerobic treatment systems are often employed.

The elevated concentrations of toxic nitrogen compounds, particularly free ammonia (FA), complicate the biological nitrogen removal processes. Typically, nitrogen is removed from wastewater through a two-step approach. This begins with the conversion of ammonia to nitrite/nitrate by aerobic autotrophic nitrifying bacteria, followed by the denitrification process, which is performed by anaerobic heterotrophic bacteria. (Z.Yuan, 2019)

However, autotrophic ammonia-oxidizing bacteria (AAOB) involved in the first stage of nitrogen removal are particularly sensitive to inhibition caused by high ammonia levels and organic matter concentrations. As a result, these bacteria often struggle to grow and function effectively in reactors treating high-ammonia leachate, like those from anaerobic incineration, where ammonia levels reach 1400-1800 mg/L. (X. Liu et al., 2021)

Conversely, heterotrophic nitrifying and aerobic denitrifying bacteria (HNADB) have shown greater resilience and adaptability in environments with high ammonia and organic matter. These bacteria can effectively remove nitrogen from wastewater by coupling heterotrophic nitrification with aerobic denitrification, utilizing organic matter as both a carbon and energy source. This dual capability enables them to thrive in challenging conditions and enhances nitrogen removal efficiency. (X. Liu et al., 2021)

The integration of heterotrophic nitrification and aerobic denitrification offers a promising avenue for the management of leachate and improving the overall performance of wastewater treatment systems. This approach not only addresses the nitrogen removal challenge but also capitalizes on the organic content of leachate as a nutrient source for microbial activity, thus enhancing the sustainability of treatment processes. Continued research and development in this field are essential for optimizing treatment strategies and addressing the growing challenges associated with waste management in an industrialized world.

2.3 Waste water

Wastewater is a combination of water and waste from various sources, including households and industries. Its composition is influenced by factors such as population size, climate, water availability, and the origin of the wastewater (Tchobanoglous et al., 2014).

In households, wastewater is primarily water (about 99.9%), along with dissolved or suspended organic, inorganic, and bacterial materials. It contains domestic waste such as food scraps, detergents, and human waste, which can include toxins (Metcalf & Eddy, 2014).

In industrial contexts, wastewater is generally more complex and hazardous. It contains various pollutants such as heavy metals, chemicals, solvents, and organic compounds that are harmful to human health and the environment (Al-Malack & Amin, 2001). Due to the large volume of wastewater discharged by industries, it is critical to monitor and evaluate the quality of the effluent to minimize its impact on public health and ecosystems. Industrial wastewater is often much more concentrated in pollutants compared to residential wastewater, requiring advanced treatment technologies (Kumar et al., 2017).

In Italy, the management of wastewater is primarily regulated by Legislative Decree 152/2006, which sets out the rules for water protection and waste management. **Legislative Decree 152/2006**, "Environmental Code", Italy. Specifically, this decree introduces the principles of precaution and producer responsibility, requiring industries and residential facilities to properly treat wastewater before discharging it into the environment. At the European level, Directive 91/271/EEC of the European Council sets regulations for the treatment of urban wastewater, requiring member states to ensure primary and secondary treatment of wastewater, with specific measures for industrial systems. **European Council**. (1991). *Directive 91/271/EEC concerning urban wastewater treatment*. Official Journal of the European Union. Italian regulations have been updated to reflect these European standards, incorporating new technologies and management practices, which include the reuse of wastewater for agricultural and industrial purposes, thereby contributing to sustainable water resource management. (Ministry of Ecological Transition, 2021)

2.4 Activated Sludge

Activated sludge is an active biomass water suspension, which plays an essential role in the biological treatment of wastewater.

The "activation" refers to the process by which microorganisms, including bacteria, fungi, and protozoa, are involved in the degradation of organic pollutants and removal of nutrients such as nitrogen and phosphorus from wastewater. (Xu et al., 2018). These microorganisms work synergistically, forming a dynamic ecosystem that facilitates the breakdown of complex organic compounds into simpler and less harmful substances. The sludge is generally composed of flocs, which are aggregates of microorganisms held together by extracellular polymers produced by bacteria. These flocs play a vital role in removing contaminants from wastewater, as the microorganisms within them metabolize organic matter and nutrients. (Neyens, 2004)

The composition and structure of flocs are heavily influenced by the microbial community present. Filamentous bacteria are a key component, forming long, thread-like structures that act as the framework onto which other bacteria, such as zoogloea (*Zooglea ramigera*), can attach. These bacteria contribute to the gelatinous nature of the floc. However, an overgrowth of filamentous bacteria can lead to the phenomenon known as "bulking."

Bulking occurs when excessive filamentous growth results in swollen, poorly settling flocs that hinder the effective separation of solids from the liquid during the sedimentation process. This can severely impact the efficiency of the wastewater treatment process, as the excess biomass cannot be removed efficiently.

Another significant issue in activated sludge systems is "rising," which occurs when anoxic conditions at the bottom of the sedimentation tank lead to the formation of gas bubbles. These bubbles drag the sludge upwards, preventing it from settling properly. This phenomenon can lead to the accumulation of sludge on the surface of the tank and further disrupt the treatment process. (Lu et al., 2023)

To address these issues, proper management of operational conditions is crucial. Factors such as aeration, temperature, and nutrient load must be carefully controlled to optimize the microbial community's performance and maintain effective treatment.

Aeration is particularly important because it supplies oxygen, which is essential for the bacteria to oxidize ammonia and other nitrogenous compounds in the wastewater. Ammonia oxidation is a critical step in nitrogen removal, as excessive nitrogen in treated wastewater can lead to eutrophication in receiving water bodies. Therefore, the aeration process not only supports the microbial degradation of organic materials but also helps reduce the environmental impact of treated effluents. Additionally, the progress in monitoring techniques, such as the use of advanced sensors and real-time data analysis, have helped improve the control of activated sludge systems, ensuring more efficient and reliable wastewater treatment. By fine-tuning operational parameters and better understanding the microbial interactions within the flocs, the overall performance of these systems can be optimized. (Mota et al., 2005)

2.5 Activated Sludge treatment and Biological Depuration Process

There are several purification processes available, whose application is based on the properties of the water to be treated and the level of purification required.

A classification of wastewater treatment methods concerns the nature of the processes employed.

Specifically, the following stand out:

- first treatments (mechanical) are focused on removing undesired suspended and floating particles from the water.
- secondary treatments (biological and non-biological) are based on the elimination of biodegradable and non-biodegradable organic material.
- tertiary treatments (generally chemical-physical) are necessary to further refine the wastewater.

The biological purification of activated sludge aims to remove certain types of pollutants, including viruses, enterobacteria, suspended and dissolved solids, nitrogen, and phosphorus.

The pollutants removed are concentrated in a bacterial biomass that can be separated from the water by sedimentation.

Native sludge can be subjected to biological processes to reduce its volume and putrescibility.

The performance of a biological purification system depends on the speed of the biological reaction, the bacterial biomass operating there, the contact time between the substrate in solution and the type of bacterial biomass.

Biological oxidation systems are divided into two main categories:

- suspended biomass systems, such as activated sludge systems, characterized by the presence of free flakes to move within the liquid mass;
- attached biomass systems, in which bacterial biomass grows while remaining attached to a surface.

The waste water is collected from the individual sewage systems and conveyed through collectors to the waste water treatment plant. Then, the sewage is conveyed to a coarse screening station where the solid and bulky parts, that could otherwise clog pipes and pumps, are automatically separated, washed, pressed and taken to the landfill. After grit and oil removal, the sewage is moved to the oxidation tanks, where it is subjected to intense aeration with consequent reduction of organic substances. This process is based on the metabolic action of microorganisms and bacteria that use organic substances and oxygen dissolved in sewage for their activity and reproduction. In this way, flakes are formed by colonies of bacteria that can be easily eliminated during the sedimentation phase where the activated sludge is separated. A scraper bridge collects the sedimented sludge of which, a part is recirculated in the aeration tank and the excess part is sent to the next treatment. The purified water then enters the chlorination tank where it is disinfected with a chlorine dosage. The sludge is sent to the digesters. In addition to mechanical and biological processes, other treatments are also needed to limit nutrients such as nitrogen and phosphorus in the final discharge, substances that can lead to eutrophication problems of rivers and lakes.

The removal of nitrogen occurs by biological processes through special bacteria in the oxidation tanks, while phosphorus elimination is generally achieved via a chemical process, which consists of adding a flocculant product (e.g., iron salts) during the purification process to precipitate and remove phosphorus from the wastewater.

2.6 Eco-Elpidiense plant

The biological treatment plant operated by Eco Elpidiense s.r.l., located in Porto Sant'Elpidio, represents a cutting-edge system designed for managing and treating liquid waste. This plant includes a biological treatment unit using activated sludge, authorized for a total capacity of 200 m^3 per day, specifically dedicated to the treatment of special non-hazardous wastewater. The management of the sludge generated during the process is a crucial aspect and includes stabilization, thickening, and dewatering phases, thus ensuring proper residue management and promoting a sustainable treatment cycle.

The main plant is organized in a modular way and includes two interconnected tanks that house the nitrification and denitrification processes, both essential for reducing the nitrogen content in the wastewater. These tanks are accompanied by two sedimentation tanks and one clarification tank, which ensure that the treated water can be safely discharged into the Tenna River in an environmentally friendly manner. The design of the plant takes into account not only technical needs but also environmental impact, aiming to minimize pressure on local water resources.

The operation of the plant is supported by good technologies and is based on a monitoring system that regulates the oxygen levels within the tanks. Installed probes continuously measure dissolved oxygen levels: if the levels drop below optimal, the aeration pumps are activated to add oxygen; when levels reach an adequate threshold, the pumps deactivate, thus allowing the denitrification process to activate. This dynamic and automated approach optimizes treatment efficiency and ensures that the treated water fully complies with the regulatory requirements established in Legislative Decree 152/2006, thus guaranteeing the protection of surface water quality.

In addition to the biological process, Eco Elpidiense also operates a chemical-physical plant (D9) located in Castellano, with a treatment capacity of up to 120 m^3 of leachate per day. This plant is designed to receive special non-hazardous waste from landfills and other sources in full compliance with current regulations. After acceptance, the leachate undergoes a complex process that includes fine screening, chemical oxidation, pH adjustment, coagulation, and precipitation. The resulting liquid solutions are then physically separated through sedimentation and dehydration of the sludges. This process is completed by a final treatment of the effluent, which may include advanced techniques such as ion exchange.

Below is a photo of the plant, which illustrates the organized setup and the good technologies employed. Fig.1.

In conclusion, Eco Elpidiense s.r.l. adopts an integrated and highly technical approach to wastewater treatment, providing specific solutions that ensure industrial activities can dispose of their waste in compliance with existing regulations. The synergy between biological and chemical-physical processes allows the plant to handle a wide range of contaminants, thereby reinforcing the company's commitment to environmental sustainability and the protection of the precious water resources in the area. This proactive approach not only improves the quality of the water returned to the environment but also contributes to fostering collective awareness of the importance of responsible waste.

Fig. 1. Eco-Elpidiense plant in the winter.

2.7 Enviromental Biofilm and Biofiltration

In nature, microorganisms form dynamic communities, known as biofilms, include a considerable quantity of extracellular polymer matrix (EPS) that ensures structure and mechanical stability.

Microbes that live in biofilms have a higher tolerance to adverse environmental conditions and toxic substances, making them suited for wastewater treatment. (Krishnan et al., 2023)

Biofiltration is process that uses microorganisms on biofilters to clean water by environmental pollutants.

Biofilters consist of filter media where the microorganisms attach themselves and colonization takes place. These microorganisms are responsible for the oxidation and removal of pollutants present in water. (Pachaiappan et al., 2022) The designed structure is used to immobilized bacteria on carrier materials such as sand, plastic, stones or ceramic beads (biocarriers).

This technique has the advantage that the bacteria are not washed-out, as happens in suspended growth systems.

There are new biofiltration technologies that allow for low-cost water recovery, one of which is moving bed biofilm reactor (MBBR).

2.8 Moving bed Biofilm

The moving bed biofilm reactor (MBBR) technology is a significant advancement in wastewater treatment. It was developed in the 1980s, and is now well recognized as a simple and adaptable wastewater treatment system. (Khaled Shahot, 2014)

MBBRs effectively remove nitrogen compounds, particularly ammonia, from mature landfill leachate combining the advantages of activated sludge and biofilm processes. (Feng et al., 2019)

MBBRs utilize biocarriers that support the growth of biofilms, which are essential for the degradation of nitrogen compounds. The biofilm comprises a diverse community of microorganisms, including ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). These microorganisms play crucial roles in the nitrification process, where ammonia is converted to nitrite and then further oxidized to nitrate. This sequential transformation is vital for reducing nitrogen levels in wastewater, minimizing its environmental impact.

Many studies indicate that MBBRs can achieve nitrification efficiencies exceeding 60% when treating mature landfill leachate. This high efficiency results from the synergistic interactions among the various microbial populations within the biofilm. The adaptability of the biofilm to fluctuations in ammonium concentrations enhances the system's resilience, allowing it to maintain stable performance even under varying operational conditions.

MBBR technology is characterized by a compact reactor design that allows for high biomass concentrations while minimizing the footprint required for installation. The biocarriers, often made from materials such as polyethylene or polypropylene, provide a large surface area for microbial attachment and growth. This design not only improves treatment efficiency but also facilitates easy integration into existing wastewater treatment facilities.

The operational parameters of MBBRs, including hydraulic retention time (HRT), aeration rates, and organic loading, are critical in determining overall treatment efficiency. Studies emphasize the importance of optimizing these parameters to maximize reactor performance. For instance, maintaining an appropriate HRT is essential for ensuring sufficient contact time between the wastewater and the biofilm, which is necessary for effective pollutant removal. Additionally, adequate aeration is required to support the aerobic conditions that favor the growth of nitrifying bacteria.

One of the key advantages of MBBR technology is its adaptability to varying operational conditions. MBBRs can effectively tolerate fluctuations in ammonium concentrations, which is particularly important when dealing with mature landfill leachate that often exhibits variable NH_3 strength and composition. The biofilm's ability to respond to these changes enables the system to maintain stable performance, ensuring compliance with regulatory standards for effluent quality.

Moreover, the compact design of MBBRs allows for straightforward upgrades to traditional systems, such as activated sludge processes, without the need of extensive modifications. This operational simplicity, combined with high treatment efficiency, makes MBBR technology a viable option for municipalities and industries seeking to enhance their wastewater treatment capabilities.

Despite the promising capabilities of MBBR technology, several challenges remain.

Continuous monitoring and optimization of operational parameters are necessary to maintain high treatment efficiencies. The potential for biofilm detachment and the impact of toxic substances on microbial communities require further investigation. Future research and development efforts should focus on optimizing MBBR performance in various wastewater treatment scenarios. This includes exploring innovative biocarrier materials, enhancing the understanding of microbial community dynamics within the biofilm, and developing strategies to mitigate the effects of toxic substances on treatment efficiency.

In conclusion, MBBR technology demonstrates significant potential for the removal of ammonia from mature landfill leachate. Its effectiveness is attributed to the synergistic interactions within the biofilm, the adaptability to varying operational conditions, and the compact design that allows for easy integration into existing treatment systems.

As wastewater treatment facilities encounter increasing challenges, related to nitrogen removal and environmental protection, MBBR technology offers a promising solution that combines efficiency with sustainability. Continued research and development will be essential to unlock the full capabilities of MBBR systems and optimize their performance in diverse wastewater treatment applications.

Through the integration of advanced technologies and a deeper understanding of microbial processes, MBBR systems can play a pivotal role in achieving sustainable wastewater treatment outcomes, ultimately contributing to the protection of water resources and of the environment.

2.9 Chemolithoautotrophic nitrifiers:

Ammonia oxidizers Nitrite oxidizers

The nitrogen cycle is considered as a significant biogeochemical cycle since all organisms require nitrogen. Two different categories of chemolithoautotrophic bacteria carry out the crucial nitrogen transformation process known as nitrification, which is the conversion of ammonia to nitrate.

In the first step, ammonia oxidizing bacteria (AOB) convert ammonia to nitrite, which is then converted into nitrate by nitrite oxidizing bacteria (NOB). (Jung et al., 2022)

As a result, the nitrification process was expanded to include additional groups such as anaerobic oxidants of ammonia (anammox) and oxidants of archeal ammonia (AOA). (Vijayan et al., 2021)

Nitrifers are chemolithoautotrophic organisms that obtain their energy from the oxidation of inorganic nitrogen molecules. They are also capable of obtaining all of their cellular carbon from the fixation of CO_2, which is mostly accomplished by the Calvin-Cycle.

Ammonia and nitrite oxidizer build very specialized groups, which are not closely phylogenetically related.

The biogeochemical N-cycle depends heavily on the nitrification process since only nitrifiers can change nitrogen from its most reduced form (NH_4^+) to its most oxidised form (NO_3^-). In both natural and artificial environments, both groups are widely dispersed. The nitrification process has a range of direct and indirect consequences on ecosystems, both natural and manmade.

On the one hand nitrification causes more nitrogen to be lost from the soil through nitrate leaching, which is why it is undesirable for agricultural soil. (Beeckman et al., 2018)

However, it is extremely desirable for wastewaters to be treated in order to effectively remove ammonium. (Luo et al., 2023)

Ammonia oxidizers

Phylogeny and distribution of AOM

Ammonia oxidizing bacteria and archea are responsible for the first step in nitrification, the conversion of ammonia to nitrite.

Based on phylogenetic research using comparative 16S rRNA and amoA gene sequences, all known AOB are located within two lineages within the *Proteobacteria β-* and *γ-class.* (Junier et al., 2010)

The genera *Nitrosomonas, Nitrosospira, Nitrosolobus* and *Nitrosovibrio* form a monophyletic group within the *β-Proteobacteria* (Qin et al., 2024). According to 16S rRNA phylogeny, the last three genera showed a very tight connection despite having different morphologies based on ultrastructure criteria. (Head et al., 1993)

Since they form a monophyletic clade, it has been suggested to combine them into a single genus. However, until now the proper phylogenetic classification of these three genera has not been definitively confirmed.

All identified AOA are *Thaumarcheota* and carry *amoA* genes, distantly related to those of AOB. (Pester et al., 2012)

Nitrosomonas is known to inhabit a variety of settings, including soil, sewage, and aquatic habitats.

These phylogenetic clusters of *Nitrosomonas* reflect species with distinct patterns of distribution. (Kikuchi et al., 2023) Since members of the *Nitrosospira* lineage have been frequently found in different soils (e.g. agricultural, turfgrass system, grassland), *Nitrosospira*-like bacteria are thought to be the dominant AOB in soil. (Aguilar-Rangel et al., 2024)

However, representatives of the four phylogenetic cluster have also been identified in aquatic habitats and sediments as well as occasionally in wastewaters. (Ward & O'Mullan, 2002)

Since their discovery, AOA have been found to be extensively dispersed throughout the world's seas and soils, and molecular surveys based on the archeal *amoA* gene have suggested that they may have a significant effect on ammonia oxidation in these environments.

Physiology of ammonia oxidation

The ammonia oxidation to nitrite is a two-step process catalyzed by two key enzymes, the membrane-bound ammonia monooxygenase (AMO) and the periplasmic hydroxylamine oxidoreductase (HAO).

In the first step the AMO oxidizes ammonia to the intermediate hydroxylamine (eq.1).

In the second step the HAO further oxidizes hydroxylamine to nitrite (eq.2).

$$NH_3 + O_2 + 2H^+ + 2e^- \; AMO \rightarrow \; NH_2OH \quad (\Delta G^{\circ\prime}\ -120\ \mathrm{kJ\ mol^{-1}}) \quad (1)$$

$$NH_2OH + H_2O \; HAO \rightarrow \; HNO_2 + 4H^+ + 4e^- \, (\Delta G^{\circ\prime}\ -114\ \mathrm{kJ\ mol^{-1}}) \quad (2)$$

The process by which ammonia is converted to nitrite and carbon via autotrophic fixation of CO_2 provides the energy for AOM. It has been demonstrated that these species have more metabolic versatility. It has been demonstrated that *Nitrosomonas* members are not limited to using CO_2 as their only carbon source.

Instead, heterotrophic growth has been observed with various organic substrates, such as fructose and amino acid (Hommes et al., 2003), as well as pyruvate as the only carbon source (Clark & Schmidt, 1967). Furthermore, it was shown for *Nitrosomonas eutropha* and *Nitrosomonas europaea* that they were able to simultaneously nitrify and denitrify under mixotrophic conditions (presence of ammonia and suitable organic substrates) and oxygen limitation by using nitrite in addition to oxygen as an electron acceptor.(Schmidt, 2009) Moreover, both species were capable to denitrify with H_2 as an electron donor and nitrite as an electron acceptor under anoxic condition. A further growth advantageouse fact for some *Nitrosomonas*-like AOB is the existence of ureases, which enable these bacteria to use urea as substrate by its hydrolyzation to ammonia and CO_2 .(Daims et al., 2016)

Nitrite oxidizers

Phylogeny and distribution of nitrite oxidizers

The second step of nitrification is the oxidation of nitrite to nitrate. This process is catalyzed by a group of microorganisms, named nitrite-oxidizing bacteria (NOB).

As one of the primary producers of nitrate, NOB are widespread in natural environments, such as in soil, ocean, freshwater, and hot springs.

NOB are a more heterogeneous group than the AOB, and are found in the α-, β-, δ-, and γ-proteobacteria.

All identified NOB belong to one of seven genera *Nitrobacter, Nitrospira*, *Nitrococcus*, *Candidatus*, *Nitrotoga*, *Nitrolancea* and *Nitrospina*.

They are all considered to be primarily chemoautotrophic, but most possess limited capability to assimilate a small range of simple organic compounds.

Owing to the biotechnological importance of nitrification and the ease of sampling activated sludge, WWTPs are often used as model systems to study NOB biology. (Daims et al., 2016)

The first described nitrite oxidizing bacterium was Nitrobacter, which species belong to the α-proteobacteria class.

Nitrobacter-like NOB are found in different acquatic and terrestrial enviroments including alkaline soda lake.

The genus *Nitrospira* has significant ecological and biotechnological value. It is not strictly chemolithoautotrophic.

Some *Nitrospira* species obtained from activated sludge or marine ecosystems are mixotrophic and can make use of simple organic carbon sources, such as formate, glycerol, and pyruvate, in addition to CO_2 and nitrite.

Furthermore the genus Nitrospira includes six phylogenetic lineages, only recently some members lineages I, II and IV are the main NOB in WWTPs. (Holmes et al., 2019)

However, *Nitrotoga* is another significant NOB group in designed systems that has just lately come to light. Remarkably, in certain WWTPs, populations of Nitrospira and Nitrotoga coexist. (Hu et al., 2024) The genera Nitrococcus and Nitrospina are members of the γ-Proteobacteria class, respectively. Both genera contain only few taxonomically described species, which were isolated from seawater and have never been detected outside of saline enviroments.

Physiology of nitrite oxidation

Nitrite oxidation is recognized as an essential process of biogeochemical nitrogen cycling in WWTP.

This process is catalyzed by the nitrite oxidoreductase (NXR), a membrane-bound enzyme that oxidizes nitrite to nitrate by using water as source of oxygen. (Daims et al., 2016)

$$NO_2^- + H_2O \; NXR \rightarrow \; NO_3^- + 2H^+ + 2e^- \; (\Delta G°' \; -76 \text{ kJ mol}^{-1}) \qquad (3)$$

The physiological diversity of NOB presents significant challenges in controlling their activity. NOB include various genera, such as *Nitrospira*, *Nitrobacter*, and *Candidatus Nitrotoga*, each of which displays unique metabolic characteristics that support their survival in different environmental niches. For example, *Nitrospira* are well-adapted to low-oxygen and low-nitrite conditions, exhibiting a high affinity for oxygen and the ability to grow in environments with limited resources. These traits allow *Nitrospira* to dominate in full-scale wastewater treatment plants where nitrite concentrations are typically low. On the other hand, *Nitrobacter* is an r-strategist that thrives in higher oxygen and nitrite concentrations, making it more competitive in environments with abundant substrates. Additionally, *Candidatus Nitrotoga* has adapted to cold environments and can become prevalent in WWTPs operating in colder climates.

One study focused on a full-scale duck wastewater treatment plant (Hu et al., 2024) identified novel strains of *Candidatus Nitrospira*, designated as *NOB01* and *NOB02*, that display a remarkable level of metabolic versatility. These strains, classified as members of a new phylogenetic lineage (lineage VII), demonstrated an ability to thrive in varying oxygen conditions across different parts of the treatment system. The study found that *NOB01* and *NOB02* were dominant in specific reactors, exhibiting a high metabolic plasticity that allowed them to persist in fluctuating oxygen and nutrient levels. This adaptability also enabled them to interact synergistically with ammonia-oxidizing and anammox bacteria, thus facilitating nitrogen removal through mutualistic interactions. For example, *Candidatus Nitrospira* strains in these reactors were observed to encode enzymes like urease and cyanate hydratase, which enabled them to degrade alternative nitrogen sources, providing additional ammonia that benefits coexisting nitrifying bacteria.

The ability of NOB, particularly *Nitrospira*, to occupy diverse ecological niches in WWTPs complicates efforts to control their activity. Strategies such as reducing dissolved oxygen (DO) concentrations or altering solid retention times (SRT) can suppress certain NOB, but many strains have shown resilience to these measures. For example, *Nitrospira* are well-suited to environments with low DO, which allows them to outcompete other NOB in systems designed to limit oxygen availability. Additionally, their high affinity for nitrite means they can thrive in continuous flow systems where nitrite levels are generally low. Biofilm formation is another survival strategy that enhances NOB resilience. Biofilms provide a stable environment that buffers NOB against environmental changes, allowing them to persist in wastewater systems and making them less susceptible to suppression strategies.

Efforts to mitigate NOB activity have led to a range of control strategies. For instance, intermittent aeration and transient anoxic conditions in sequencing batch reactors (SBRs) can create competitive disadvantages for NOB, favoring ammonia-oxidizing bacteria (AOB) instead. However, some NOB, like those observed in *Candidatus Nitrospira*, have adapted to persist in low-DO environments, which undermines the effectiveness of these approaches. Additionally, lowering DO levels below 0.5 mg/L, a common strategy for suppressing NOB, has not consistently prevented NOB from re-emerging, especially in systems where they have formed biofilms or adapted to survive under low oxygen levels. Shortening the SRT has also been explored as a way to reduce NOB populations, but some NOB strains with slow growth rates, such as *Nitrospira*, can still thrive in longer SRT conditions typical of many WWTPs.

From a broader perspective, the metabolic diversity of NOB, particularly *Nitrospira*, includes various pathways beyond nitrite oxidation. Some *Nitrospira* members, for instance, are capable of utilizing ammonia directly, a trait known as complete ammonia oxidation (comammox). This ability to perform both ammonia oxidation and nitrite oxidation within the same organism can make *Nitrospira* highly competitive in environments where ammonia is the primary nitrogen source. Additionally, *Nitrospira* can metabolize other compounds such as urea, cyanate, and hydrogen, further supporting their ability to adapt to diverse wastewater environments. The presence of such alternative metabolic pathways also allows *Nitrospira* to support mutualistic relationships with other bacteria, such as AOB, by providing ammonia through urea hydrolysis.

Understanding the genetic and metabolic foundations of NOB resilience is critical for advancing nitrogen removal technologies. Genomic and metagenomic analyses have shed light on the key genes and metabolic pathways that underpin NOB's survival in WWTPs. For example, genes encoding nitrite oxidoreductase (NXR), essential for nitrite oxidation, show variations across different NOB genera, with some strains having distinct adaptations that allow them to function efficiently in specific wastewater environments. By examining the genetic basis of NOB's ecological niches, researchers can better understand how these bacteria adapt to different suppression strategies and develop more targeted approaches to managing NOB populations. Additionally, the identification of novel NOB lineages, such as those described by Hu et al. (2024), highlights the potential for further discoveries that could provide new methods for controlling NOB activity in wastewater system s.

In conclusion, the findings from these studies underscore the complexity and resilience of NOB in wastewater treatment systems. The physiological adaptations and metabolic versatility of NOB, particularly *Nitrospira*, enable them to withstand a range of suppression efforts, making them formidable opponents to energy-efficient nitrogen removal strategies. To achieve effective NOB control, a deeper understanding of their ecology, physiology, and genetic diversity is essential. By leveraging genomic insights and developing nuanced suppression techniques, future WWTPs may be better equipped to mitigate NOB activity, supporting the transition toward more sustainable and carbon-neutral wastewater management practices.

2.10 Heterotrophic nitrification and aerobic denitrification process

The heterotrophic nitrification (HN) and aerobic denitrification (HNAD) process can simultaneously remove nitrogen and chemical oxygen demand under fully aerobic conditions. This coupled process has more advantages than the conventional nitrogen removal process in wastewater treatment due to their unique ability to oxidize ammonia using organic carbon sources.

This enables them to perform both nitrification and denitrification simultaneously, a process known as simultaneous nitrification and denitrification (SND). (Daims et al., 2016)

This capability allows heterotrophic nitrifying bacteria (HNB) to directly convert ammonia into nitrogen gas without the intermediate accumulation of nitrites or nitrates, providing a major advantage over traditional autotrophic processes that often result in partial nitrogen removal efficiency.

HNB possess distinctive physiological and biochemical traits that set them apart from conventional autotrophic nitrifying bacteria. They can thrive using organic substrates for energy, allowing them to grow and function effectively in environments with low oxygen concentrations. This makes them highly adaptable to various conditions, such as low temperatures, high salinity, and elevated ammonia concentrations, which can challenge the effectiveness of autotrophic bacteria.

The specific growth rates of HNB, ranging between 0.18–0.45 hr^{-1}, exceed those of typical autotrophic ammonia-oxidizing bacteria like *Nitrosomonas europaea*, making HNB advantageous for processes that require rapid microbial proliferation.　(Daims et al., 2016)

A key feature of HNB is their broad phylogenetic diversity, encompassing strains from various phyla, including Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. The genus *Pseudomonas* is particularly prominent, followed by *Bacillus*, *Acinetobacter*, and *Alcaligenes*, among others. This diversity indicates a wide range of potential applications in BNR systems, as different strains can be tailored to specific treatment needs, such as high-salinity environments or systems requiring robust performance under fluctuating temperature conditions.(Li et al., 2024)

Recent advancements in molecular biology and detection techniques, such as high-throughput sequencing and the use of specific genetic markers (e.g., *amoA* and *hao* genes), have deepened the understanding of HNB's role in BNR systems. These modern techniques have shown that traditional methods, which primarily monitored the accumulation of nitrite or nitrate, often underestimated the actual nitrification and denitrification capabilities of HNB. By leveraging these advanced detection tools, researchers have been able to capture a more accurate picture of the microbial community dynamics and the contribution of HNB within wastewater treatment systems.

Bioaugmentation strategies that incorporate pure or enriched HNB cultures have demonstrated significant improvements in nitrogen and chemical oxygen demand (COD) removal.

Examples of successful applications include sequencing batch reactors, biological aerated filters, and anaerobic-anoxic-aerobic systems. These systems have shown enhanced pollutant elimination efficiencies, with ammonia and total nitrogen removal rates often exceeding 85%, even under challenging conditions with lower carbon/nitrogen (C/N) ratios. The use of HNB in practical settings has also highlighted their adaptability and effectiveness across various types of waste-water, including domestic, industrial, and agricultural effluents. (Chen et al., 2015)

The advantages of integrating HNB into BNR processes extend beyond just improved nitrogen removal. Their ability to operate under diverse environmental conditions, such as low dissolved oxygen and high salinity, makes them valuable for achieving treatment stability and resilience. Moreover, HNB can reduce the overall operational costs and complexity by performing simultaneous nitrification and denitrification in a single step, thus minimizing the need for separate treatment phases. Future research is encouraged to address certain challenges, such as the long-term stability and sustainability of HNB in complex microbial communities. There is also a need to optimize operational parameters like the types of carbon sources and C/N ratios to maximize the efficiency of HNB-driven BNR processes. Understanding the genetic and enzymatic pathways underlying their dual nitrification and denitrification abilities will be essential for refining their application and enhancing the overall effectiveness of wastewater treatment systems. (Duan et al., 2022)

Unfortunately a limitation in optimizing Heterotrophic Nitrifying and Aerobic Denitrifying Bacteria (HNADB) for nitrogen removal is the lack of understanding regarding their physiology.

While numerous studies have confirmed that HNADB can oxidize ammonia into hydroxylamine and nitrite, data concerning the enzymes and genetic sequences involved remain scarce. Biochemical studies indicate that the enzymes used in these processes differ from those found in Autotrophic Ammonia-Oxidizing Bacteria (AAOB).

For instance, biochemical analyses reveal that the ammonia monooxygenase in *Paracoccus* consists solely of two subunits (AmoA and AmoB), unlike the three-subunit (AmoABC) ammonia monooxygenase associated with *Nitrosomonas*.(Moir et al., 1996)

Moreover, characterized hydroxylamine oxidases (HAO) in HNADB are reported to be monomeric non-heme iron proteins that utilize cytochrome c556 and pseudoazurin as electron acceptors, whereas *Nitrosomonas*'s HAO is a multimer with each subunit containing 7c-heme and P-460 heme. (Arciero & Hooper, 1993)

For this reason, further studies on biochemical pathways will be necessary to better optimize new nitrogen removal strategies by exploiting heterotrophic nitrifying bacteria.

2.11 Bacteria Biofilm in WWTP biofilter

Biofilm formation is a critical aspect of wastewater treatment, particularly in Moving Bed Biofilm Reactors (MBBRs), where it plays a vital role in the degradation of organic and inorganic pollutants, including nitrogen compounds. (Mahto & Das, 2022)

The biofilm consists of complex microbial communities that adhere to solid surfaces, creating a structured environment protected by a matrix of extracellular polymeric substances (EPS). This matrix provides protection against environmental stresses and promotes nutrient uptake and metabolic exchanges, allowing for optimal microbial growth and effective pollutant removal, including ammonia, nitrates, and organic compounds. The biofilm not only serves as a protective barrier but also stimulates the growth of specialized bacteria crucial for nitrification and denitrification, processes that are vital for reducing nitrogen levels in wastewater. Biofilm formation in MBBRs is influenced by several factors, including type of biocarrier material, flow conditions, and composition of the wastewater being treated. (Suarez et al., 2024) Materials like polyethylene and polypropylene are commonly used due to their favorable properties that promote microbial adhesion. Flow conditions, including shear forces and flow velocities, also affect biofilm thickness and structure, directly influencing pollutant removal efficiency. The microbial species within the biofilm adapt to changing environmental conditions so that biofilm structure allows the coexistence of diverse bacterial populations with complementary roles in pollutant degradation. The importance of biofilm in wastewater treatment extends to nitrogen management, particularly in MBBR systems, where biofilms facilitate nitrification and denitrification. In nitrification, bacteria oxidize ammonia to nitrite, while other bacteria convert nitrite to nitrate.

These processes are essential for reducing nitrogen content in effluent and mitigating the environmental impact of wastewater. Biofilms also support denitrification, which reduces nitrate to nitrogen gas, effectively removing excess nitrogen from the treated water. Due to its ability to create microenvironments with varying oxygen concentrations, the biofilm enables both nitrifying and denitrifying bacteria to coexist, enhancing the overall nitrogen removal efficiency of the system. Key operational parameters, such as Hydraulic Retention Time (HRT), aeration rates, and organic loading, are crucial in optimizing MBBR performance. The HRT determines the amount of time the wastewater is in contact with the biofilm, which is essential for effective pollutant removal. Studies have shown that longer HRT improves nitrification efficiency, especially in systems treating wastewater with high concentrations of pollutants. Aeration, which supplies oxygen for aerobic microbial processes, is critical for MBBR operation but must be balanced to prevent biofilm detachment. Excessive aeration can cause biofilm shedding, leading to a loss of microbial biomass and reduced treatment efficiency. (Kermani et al., 2008) Organic loading, referring to the amount of organic matter entering the reactor, must be regulated to prevent microbial competition that could inhibit the growth of nitrifying bacteria. Therefore, carefully managing organic loading is essential to maintain a healthy biofilm and optimize treatment efficiency. MBBR systems offer several advantages over traditional wastewater treatment methods. One of the main benefits is their compact design, which allows for high biomass concentrations in limited space, making them ideal for municipalities and industries with restricted space for treatment plants. Moreover, the ability of MBBRs to adapt to varying wastewater compositions makes them suitable for treating complex wastewater types, such as landfill leachates. The biofilms in MBBRs can respond to fluctuations in ammonium concentrations, ensuring stable performance and compliance with effluent quality standards. Another significant advantage of MBBR systems is their operational simplicity, as they can be easily integrated into existing treatment plants, enhancing traditional bioreactors such as activated sludge processes. Despite their numerous benefits, MBBRs also face challenges. One of the main issues is the potential detachment of the biofilm, which can compromise treatment efficiency. Additionally, the presence of toxic substances can negatively affect microbial communities, reducing their ability to degrade pollutants. Continuous monitoring of operational parameters is necessary to prevent biofilm loss, and strategies must be developed to address this issue. Future research should focus on optimizing MBBR performance in various wastewater treatment scenarios, exploring new biocarrier materials that improve biofilm formation and stability. Understanding the dynamics of microbial communities within the biofilm, as well as developing advanced monitoring techniques such as real-time sensors and automated control systems, will be crucial for optimizing operational performance and ensuring consistent and efficient treatment. (Maurya et al., 2023)

In conclusion, biofilm technology plays a vital role in the effectiveness of MBBRs for wastewater treatment, particularly in the removal of nitrogen compounds. The complex interactions between microbial populations within the biofilm enhance pollutant degradation, making MBBRs a promising solution for addressing wastewater treatment challenges. As the demand for sustainable and efficient wastewater treatment solutions increases, further research and development in biofilm technology will be essential to improve treatment outcomes, contributing to the protection of water resources and the environment.

microorganisms

Article

Selection, Identification and Functional Performance of Ammonia-Degrading Microbial Communities from an Activated Sludge for Landfill Leachate Treatment †

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† **Dedicated to the memory of Mauro De Angelis, a unique and ingenious man, founder of the Eco Elpidiense Company.**

Abstract: The increasing amounts of municipal solid waste and their management in landfills caused an increase in the production of leachate, a liquid formed by the percolation of rainwater through the waste. Leachate creates serious problems to municipal wastewater treatment plants; indeed, its high levels of ammonia are toxic for bacterial cells and drastically reduce the biological removal of nitrogen by activated sludge. In the present work, we studied, using a metagenomic approach based on next-generation sequencing (NGS), the microbial composition of sludge in the municipal wastewater treatment plant of Porto Sant'Elpidio (Italy). Through activated sludge enrichment experiments based on the Repetitive Re-Inoculum Assay, we were able to select and identify a minimal bacterial community capable of degrading high concentrations of ammonium (NH_4^+-N $\cong$ 350 mg/L) present in a leachate-based medium. The analysis of NGS data suggests that seven families of bacteria (Alcaligenaceae, Nitrosomonadaceae, Caulobacteraceae, Xanthomonadaceae, Rhodanobacteraceae, Comamonadaceae and Chitinophagaceae) are mainly responsible for ammonia oxidation. Furthermore, we isolated from the enriched sludge three genera (*Klebsiella* sp., *Castellaniella* sp. and *Acinetobacter* sp.) capable of heterotrophic nitrification coupled with aerobic denitrification. These bacteria released a trace amount of both nitrite and nitrate possibly transforming ammonia into gaseous nitrogen. Our findings represent the starting point to produce an optimized microorganisms's mixture for the biological removal of ammonia contained in leachate.

Keywords: biological nitrogen removal; nitrification-denitrification; landfill leachate; activated sludge; microbial community; metagenomics

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1. Introduction

The economic development and urbanization which occurred, during the last century, in Italy and in all developed countries, led to the production of increasing amounts of municipal solid waste (MSW) (reviewed in the Ref. [1]). Due to the Italian political choice to strongly promote differentiated collection, only a small fraction of MSW has been incinerated ([2] and references therein) and waste incineration plants are practically absent in central and southern Italy. Thus, because of low cost and easy management, MSW are mostly treated in landfill generating a very typical waste, the leachate.

Leachate is a dark-brown and bad-smelling liquid formed in landfills by rainwater percolation through solid wastes. Generally, landfill leachate is characterized by the presence of elevated concentrations of ammonia and organic compounds in addition to other toxic elements, such as heavy metals (reviewed in the Refs. [3–5]). The quality of landfill leachate frequently varies, being affected by many factors (i.e., composition of MSW solid, abundance of rainfall and climate), thus it is constantly monitored using such chemical parameters as pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), chloride ions (Cl^-) and ammonium nitrogen (NH_4^+-N). Currently, the most used classification of leachate is based on landfill age: young (less than 5 years old), middle (5–10 years old), and mature (more than 10 years old) (reviewed in the Refs. [5,6]). Young leachate shows high values of BOD and COD with a BOD/COD ratio ranging from 0.5 and 1. During aging of this pollution (older leachate), the BOD and COD parameters decline whereas huge amounts of ammonia accumulate (2000–4000 mg/L). This classification is mostly applicable for leachates produced in landfills located in warm temperature zones whereas leachates from equatorial and tropical regions are subjected to a very fast aging and more frequently contain recalcitrant compounds and inhibitors of biological reactions [7–9]. Because of the detrimental impact of ammoniacal nitrogen (NH_4^+), all the regulatory agencies request the industries, factories and municipals to treat their effluents for ammonia to meet the requirements. Italian Regulation No. 152/2006 established the limits for ammonia wastewater content discharged into shallow water $\leq$ 15 mg/L and into sewage network $\leq$ 30 mg/L. The limit for nitrite in the effluent is 0.6 mg/L whereas for nitrate it is 20 and 30 mg/L in shallow water and the sewage network, respectively.

Many physico–chemical methods involving adsorption, struvite precipitation, coagulation/flocculation, air-stripping and filtration were developed to treat landfill leachate [5,6,10–12]. However, the biological removal of complex organic matter and nitrogen by the microbial community forming the activated sludge remains the most widely used and somewhat the most promising approach [3–5,13]. This process occurs in conventional wastewater treatment systems, both in aerobic and anerobic conditions. Thus, nitrogen-fixing bacteria convert the nitrogen in the wastewater into ammonia (NH_3) which during the nitrification process is oxidized to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB). Subsequently, the NO_2^- is rapidly oxidized to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) and finally, in the denitrification process, NO_3^- is reduced to the gaseous nitrogen form which can directly escape into the atmosphere [14–18]. In particular, mature leachate creates serious disposal problems because of its high content of ammonia associated with a deficiency of biodegradable carbon sources utilized by bacteria of the activated sludge [19]. In fact, high levels of ammonia (NH_3) are toxic for bacterial cells and dramatically reduce, in municipal wastewater treatment plants (MWTPs), biological nitrogen removal by the activated sludge.

In the present study, we investigated by a metagenomic approach consisting of next-generation sequencing (NGS) [20] of 16S rRNA genes, the composition of sludge microbes in the municipal wastewater treatment plant (WWTP) of Porto Sant'Elpidio (FM, Italy) with the aim of increasing bioreactor performance. This WWTP, located in central Italy near the Adriatic coast and managed by the Eco Elpidiense s.r.l. company, serves about 50,000 inhabitants that can increase in summer for tourist arrivals. In addition, the Porto Sant'Elpidio WWTP collects and treats a huge quantity of leachate, after being pre-treated via physico–chemical processes, from numerous landfills in a large surrounding area. This implies a very big load in terms of ammonia. Thus, the structure of the microbial community of the native sludge from Porto Sant'Elpidio WWTP was compared to those selected after multiple ammonia stresses in leachate-based medium. This allowed us to identify those microorganisms which can survive at elevated NH_4^+ concentrations as those present in leachate and exhibit a high ammonium removal capacity.

Our results indicate that such a strong selection drastically reduced the number (usually more than 150) of different bacterial species, causing a considerable enrichment of certain genera as a function of ammonia stress prolongation. In particular, *Haliscomenobacter*, *Leadbetterella* and *Stenotrophomonas* were predominant (10–30%) after 10–15 days to be then replaced by *Rhodanobacter*, *Castellaniella* and *Nitrosomonas* (10–40%) in 75-day cultures. Therefore, three isolates, belonging to *Klebsiella* sp., *Castellaniella* sp. and *Acinetobacter* sp.

genera were further tested for their capability to perform combined heterotrophic nitrification and aerobic denitrification.

2. Materials and Methods

2.1. Description of Porto Sant'Elpidio Municipal Wastewater Treatment Plant

The municipal WWTP is placed about 500 meters from the Adriatic Sea coast on Porto Sant'Elpidio (FM, Italy). It has a maximum treatment capacity of a 62,000 population equivalent, and besides, Porto Sant'Elpidio serves some nearby towns. The WWTP, schematically represented in Figure 1, is composed of four different reaction basins of a volume of 1450 m^3. In these basins, nitrification and denitrification processes occur alternatively and they are controlled by specific probes which measure the quantity of oxygen dissolved in the active sludge. The overall depurative process includes three consecutive steps: (i) the physico treatment; (ii) the biological treatment; and (iii) the final chemical treatment. The wastewater reaches the plant and bar screens are used for removal of coarse solids. Next, sands are removed by gravity sedimentation. After that, the biological treatment occurs through the use of an activated sludge, which in anoxic conditions, favors the reduction of nitrogen and consequently the consumption of organic matter, thus greatly decreasing the nutrient contents in the wastewater. The resulting effluent displays a much lower organic and nitrogen content, with an average removal efficiency of about 95% from the initial values of both COD and nitrogen values. Before being discharged, the wastewater gets a chemical disinfection by a peracetic acid that produces wastewater with a reduced microbic population. In addition, this WWTP treats a large quantity (~100,000 tons per year) of leachate, coming from numerous landfills in a large area of central Italy, which results in about 12,000 equivalent people in terms of organic matter and nitrogen. The quality of four characteristic landfill leachates commonly treated in Porto Sant'Elpidio WWTP is reported in Supplementary Table S1. Initially, a mixture of these leachates is subjected to physico–chemical treatments in two separate plants, including coagulation/precipitation, filtration on active carbon and adsorption/ion exchange. These pre-treatments reduce the ammonia level from 1500–2000 mg/L to approx. 500 mg/L. Then, leachate after dilution (~1:100) with the urban wastewater is released into the plant where an additional biological reduction of ammonia occurs (Figure 1). The average composition of pre-treated leachates, used in our experiments, is shown in Table 1.

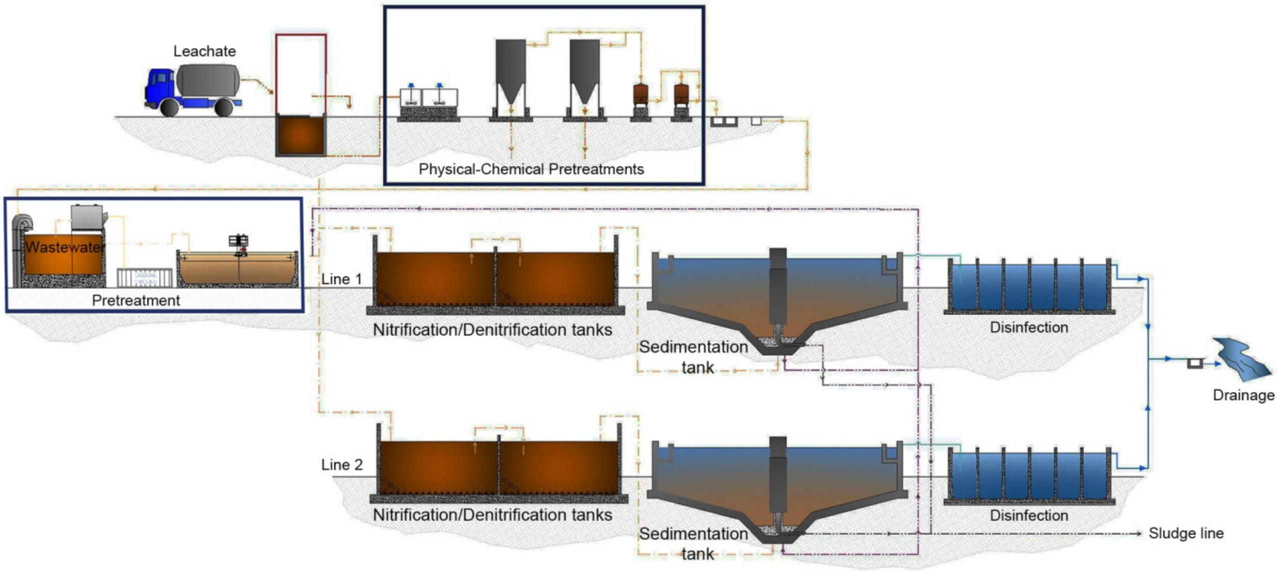

Figure 1. Flow chart of Porto Sant'Elpidio WWTP. A detailed description of the WWTP is provided in Section 2.1.

Table 1. Average composition of pre-treated leachates used in our experiments.

Parameter	**Value ± SD**
pH	7.6 ± 0.25
COD (mg/L)	4066 ± 830
BOD (mg/L O_2)	1207 ± 177
BOD/COD	0.30 ± 0.03
$NH_4{}^+$-N (mg/L)	1415 ± 166
$NO_3{}^-$ -N (mg/L)	1.5 ± 1.2
$NO_2{}^-$ -N (mg/L)	traces
Cl^- (mg/L)	3253 ± 472
Cu (mg/L)	0.061 ± 0.04
Pb (mg/L)	0.005 ± 0.01
Cr (mg/L)	1.04 ± 0.53
Ni (mg/L)	0.234 ± 0.12
Zn (mg/L)	0.106 ± 0.11

2.2. Media and Cell Growth Conditions

Aliquots of activated sludge, as indicated in *Figure Legends*, were diluted in Leachate Minimal Medium (LMM) and incubated in a rotary shaker at 28 °C. The LMM was prepared by combining 60 mL of leachate with 30 mL of MM medium and 90 mL of distilled water. The MM broth is a medium originally used to grow *N. europaea* (ATCC #2265) and is composed of three solutions to be mixed together. Solution 1: 4.95 g $(NH_4)_2SO_4$, 0.62 g KH_2PO_4, 0.27 g $MgSO_4$, 0.04 g $CaCl_2$, 0.5 mL $FeSO_4$ (30 mM in 50 mM EDTA at pH 7.0), 0.0002 g $CuSO_4$, 1.2 L distilled water. Solution 2: 8.2 g KH_2PO_4, 0.7 g NaH_2PO_4, 3 L distilled water, bring to pH 8.0. Solution 3: 0.6 g Na_2CO_3, 12 mL distilled water. The three solutions were sterilized by filtering and leachate by autoclave. The ammonia concentration of bacterial cultures was adjusted to the indicated values adding $(NH_4)_2SO_4$ (1 M). Agar plates were prepared using leachate minimal medium (LMM) supplemented with leachate and agar (1.7%) at a final concentration of $NH_4{}^+$ of 350 mg/L.

2.3. Analytical Methods

The ammonium concentration was estimated by Nessler's assay carried out in a total volume of 25 mL using 200 µL of each culture and 0.5 mL of Nessler's reagent. Ammonium rates were measured spectrophotometrically at a wavelength of 410 nm.

Nitrites and nitrate concentrations were determined by using a Dionex ICS-1100 (ThermoFisher Scientific, Waltham, MA, USA) ion chromatography system equipped with a DRS 600 suppressor and a conductivity detector. Anions were separated by a Dionex IonPac AS23 column and a Dionex IonPac AG23 guard column with a flow rate of 1 mL/min of a 0.45 M Na_2CO_3/0.08 M $NaHCO_3$ eluent. The determinations were conducted according to the APAT CNR IRSA 4020 Man. 29/2003 method, published by the Italian Environmental Protection Agency. The analytical procedure was conducted under UNI CEI EN ISO/IEC 17025:2018 standards; therefore, extensive method validation and the expanded uncertainty were available. Detection limits for nitrites (as $NO_2{}^-$) were 0.09 mg/L and 3.4 mg/L for nitrates (as $NO_3{}^-$).

Total nitrogen was determined by using the small-scale sealed-tube kit (LCK 138, Hach company, Loveland, CO, U.S.A.). Nitrogen compounds present in the samples were oxidized to nitrate according to the method EN ISO 11905-1:1998 which uses peroxodisulfate and a high temperature (120 °C for 30 min) for the digestion. Next, a solution of 2,6-Dimethylphenol was added to the sample, which reacts with the nitrates to form

2,6-Dimethyl-4-nitrophenol. The formed nitrophenol was then determined spectrophotometrically at a wavelength of 345 nm.

COD was determined by using the sealed-tube test method (ISO 15705:2002), while the BOD was determined by using a small-scale sealed-tube kit (LCK 555, Hach company, Loveland, CO, USA).

Chlorides were determined by titration with silver nitrate (APAT CNR IRSA 4090 Man 29:2003) and metals were determined after microwave-assisted aqua regia digestion (UNI EN ISO 15587-1:2002) with an ICP-MS (UNI EN ISO 17294-2:2016).

All the analytical procedures described were performed at the Eco Control Laboratorio Ascolano s.r.l. (Italy), (Certification UNI CEI EN ISO/IEC 17025:2018).

2.4. Analysis of Microbial Communities by 16S rRNA Gene Next-Generation Sequencing

Bacterial cells from native sludge and samples of Repetitive Re-Inoculum Assay (RRIA) (aliquots of 10–20 mL) were harvested by centrifugation (8000 rpm for 20 min). Chromosomal DNA was extracted by the E.Z.N.A. Kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the instructions given by the manufacturer.

DNA concentration was estimated by NanoDrop (ThermoFisher Scientific, Waltham, MA, USA). The V3-V4 hypervariable regions of 16S rDNA were amplified using universal primers (341F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAC GGGNGGCWGCAG-3′, 805R 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGA CTACHVGGGTATCTAATCC-3′) following the 16S Metagenomics Sequencing Library preparation protocol [21]. Libraries were sequenced using the MiSeq Illumina Platform (Illumina Inc., San Diego, CA, USA) with a 2 × 250 paired-end run. Poor quality reads were filtered with Trimmomatic [22]; paired-end reads were merged using FLASH [23] and processed with VSEARCH [24] to detect potential chimera sequences and to cluster merged amplicons in operational taxonomic units (OTUs), with a minimum pair-wise identity threshold of 97%. The NCBI 16S RefSeq database [25] was employed for taxonomic classification. Evaluation of microbial alpha (Alpha-diversity, Chao1, Simpson's and Shannon's diversity) and beta (UniFrac distances, Bray–Curtis dissimilarity) diversity measures were performed using an internal pipeline.

2.5. Isolates Identification by 16S rRNA Gene Sanger Sequencing

Cells from isolated colonies were picked up with a sterile loop and directly dissolved in the PCR reaction mix for 16S rRNA gene amplification using the forward primer #838F (5′-AGAGTTTGATCMTGGCTCAG-3′), reverse primer #839R (5′-TACGGYTACCTTGTTACGA CTT-3′) and the high-fidelity DNA polymerase (Ex Taq, Takara, Bio Inc., Shiga, Japan). PCR steps were: DNA denaturation at 94 °C for 30 s, primer annealing at 59 °C for 30 s and extension at 72 °C for 90 s (28 cycles). The amplification products were purified by the Real Genomics kit (RBC Bioscience Corp., New Taipei, Taiwan) and sequenced by the Sanger method at BMR Genomics (Padova, Italy). Finally, sequences were analyzed using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) accessed on 1 March 2022

3. Results and Discussion

3.1. Nitrogen Removal Rate by Activated Sludge under Ammonia and Salt Stresses

This study was promoted by the Eco Elpidiense s.r.l., a private company that manages the municipal wastewater treatment plant of Porto Sant'Elpidio (Figure 1). This WWTP, besides its routinely depuration activity, is overloaded by the disposal of large amounts of landfill leachate coming from a vast region of central Italy. Although after high dilutions, the spillage of big volumes of leachate into the municipal wastewater treatment plant represents a relevant risk. In fact, the reactor has to withstand peaks of ammonium (NH_4^+-N ≅ 100–150 mg/L) that could be toxic for bacteria of the sludge, thereby resulting in a slowing down of or even stopping the biological nitrogen removal. Even though this is a challenging condition, the Porto Sant'Elpidio WWTP has a mean nitrogen removal rate of about 95%, as determined by the routinely analyses carried out at the Eco Control

Lab, Ascolano (personal communication). This suggests that microorganisms of the active sludge adapted to these continuous NH_4^+ stresses make this plant very attractive for the analysis of its microbial community.

Thus, according to the Eco Elpidiense s.r.l. requirements, the primary aim of this study was to investigate and eventually to improve the WWTP performance in terms of the biological conversion of ammonia to nitrite, known also as partial nitrification or nitritation [26]. This reaction is certainly the critical one of the conventional nitrification–denitrification process by bacteria, and it is nevertheless the most energy-consuming step due to the aeration system which injects air into the plant. Thus, the microbial community taken from the Porto Sant'Elpidio WWTP was tested for its ability to tolerate ammonia stress in order to estimate a threshold value at which bacteria were still able to efficiently eliminate ammoniacal nitrogen. For this purpose, bacteria from the sludge were grown in minimal broth (LMM) supplemented with only leachate and containing increasing concentrations of ammonia. The results presented in Figure 2A,B revealed that good ammonia degradation was maintained for the two lower NH_4^+-N concentration curves (250 and 400 mg/L) where nitrogen removal rates were 70–85% and 50–75% at 3 and 8 days, respectively. Conversely, the nitrification process considerably slowed down and was dramatically inhibited for higher amounts of NH_4^+ (600–900 mg/L). Thus, this assay indicated that microorganisms of native sludge well-tolerate ammonium stress and retain high nitrification activity up to ~400 mg/L of NH_4^+-N. This finding was very promising, considering the experimental conditions used. In fact, to the best of our knowledge, most previous studies estimated the biological ammonia removal rate in wastewaters containing low NH_4^+-N ($\leq$100 mg/L). For this reason, data comparison is quite complicated.

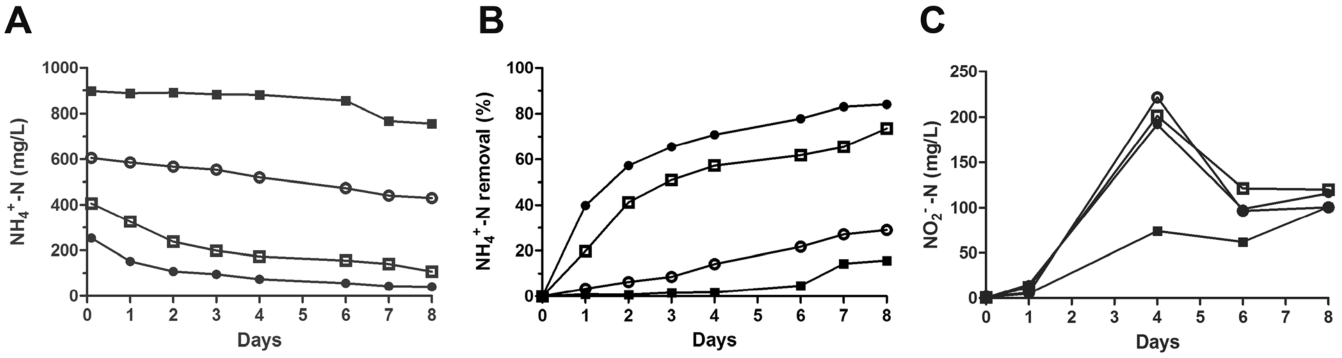

Figure 2. Effects of ammonia stress on NH_4^+ removal rate by the activated sludge. Aliquots (10 mL) of the activated sludge (150 mg, dry weight) were mixed with 20 mL of LMM and incubated at 28 °C. Increasing ammonium concentrations, expressed as NH_4^+-N, were adjusted in the four cultures to 250 (●), 400 (☐), 600 (○) and 900 (■) mg/L and monitored, using Nessler's test, as a function of time (**A**) and reported as removal percentage (**B**). Nitrite concentration, expressed as NO_2^--N, is reported (**C**). Data represent the average of three independent experiments and the standard deviation is ~10%.

Instead, Jiang et al. [27] used an elevated NH_4^+ concentration (300 mg/L) in a laboratory-scale reactor where the nitrification process reached about 94%. Differently from our experiments, carried out in common microbiology 150 mL flasks, this reactor was not a closed system, and to feed bacteria, the COD level was maintained constant (500–600 mg/L) for ~50 days by adding external organic compounds. Remarkably, the native microbial community of our sludge showed excellent nitrification performance (~85%) metabolizing, as only a carbon source, the organic materials in leachate without the need of extra nutrients. In addition, due to the fact that landfills, in the last decade, receive pre-treated waste (i.e., separate waste collection), the leachate generally contains low quantities of putrescible matter and is characterized by a low BOD/COD ratio. According to this, the leachate we used in our experiments had a COD/BOD ratio $\leq$ 0.3 (Table 1), thereby being poorly biodegradable and difficult to be treated by the conventional nitrification-denitrification process [28]. Under these experimental conditions and in the presence of oxygen, the nitrite was the main product of ammonia oxidation (Figure 2C), whereas the nitrate formation was negligible. In fact, the maximum concentration of NO_3^--N for the four different cultures ranged from 4 to 8 mg/L (data not shown).

Landfill leachate, managed by the WWTP of Porto Sant'Elpidio, contains high amounts of chloride ions (Cl^-) that, depending on the local climate (rainfall and temperature variations), range from 2 to 4 g/L as analytically determined by the Eco Control Laboratory (FM, Italy). In addition to ammonia, an elevated Cl^- content represents a further problem for leachate treatment in WWTPs. Thus, the effect of salt stress on the ability of bacteria to degrade ammonia was investigated. As seen in Figure 3, concentrations of Cl^- below 2 g/L slightly affected the NH_4^+ removal rate, whereas it was reduced to 50% at 4 g/L of chloride ions. For Cl^- values greater than 6 g/L, the pollutant degradation by the bacterial population of the sludge was strongly impaired.

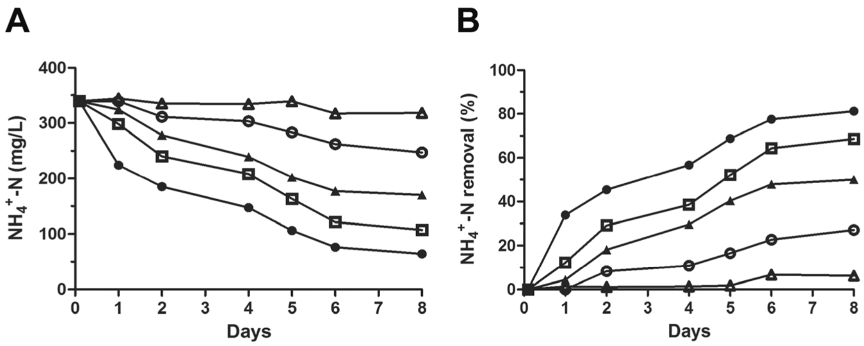

Figure 3. Effects of salt stress on NH_4^+ removal rate by the activated sludge. The activated sludge was incubated in LMM essentially as described in the legend of Figure 2. Chloride ion (Cl^-) concentrations were adjusted to 0.5 (●), 2 (□), 4 (▲), 6 (○) and 10 (Δ) g/L and NH_4^+-monitored, using Nessler's test, as a function of time (**A**) and expressed as a removal percentage (**B**). Data represent the average of three independent experiments and the standard deviation is ~10%.

3.2. Repetitive Re-Inoculum Assay

In this study, we devised a simple experiment, named the Repetitive Re-Inoculum Assay (RRIA), somewhat resembling the dilution to extinction approach [29,30] in which an uneven microbial community from environmental samples was subjected to serial dilutions. These previous studies [29,30] demonstrated that important changes, depending on dilution, were found in the community structure and in functional characteristics between the original and reformed communities.

RRIA consists of consecutive re-inocula/dilutions, of the same sludge culture, in leachate-based medium (LMM) with an elevated NH_4^+-N content. The primary aim of RRIA was to reduce the complexity of the bacterial population by selecting those species able to tolerate repeated ammonia stresses and characterized by high efficiency in nitrogen removal. Monitoring the ammonia level, this assay gives rise to a peaks-and-falls plot, as shown in Figure 4A. Basically, a representative RRIA was performed as follows: (i) bacteria from activated sludges were inoculated in a liquid minimal medium supplemented with only leachate and grown at 28 °C; (ii) ammonia concentration was estimated at the starting point (first inoculum) and at regular time intervals for the next 75 days; (iii) as bacteria started to degrade ammonia and its concentration dropped to about 50 mg/L (falls), about one-fourth of the culture volume was re-inoculated in leachate medium so that the initial value of NH_4^+-N (~350 mg/L) was restored (peaks); (iv) this step was performed over and over again (seven times in RRIA of Figure 4A) during the two months of the experiment, hence the name "Repetitive Re-Inoculum Assay"; (v) aliquots of the culture were withdrawn at different falls of the ammonia level curve (NH_4^+-N $\cong$ 50 mg/L in Figure 4A) and cells of bacterial communities were harvested to be investigated (see below). Comparable NH_4^+ curves were obtained in additional RRIAs where the ammoniacal nitrogen concentration of the starting point and of each re-inoculum was set to 150 and 220 mg/L (Supplementary Figure S1).

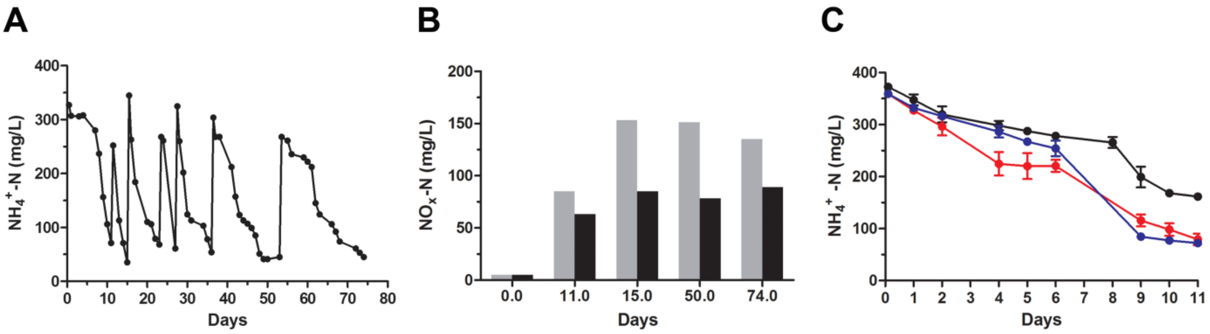

Figure 4. Repetitive re-inoculum assay. (**A**) The activated sludge (10 mL), corresponding to 150 mg dry weight, was mixed with 30 mL of LMM essentially as described in the legend of Figure 2. The culture was placed in a water bath shaker at 28 °C and ammonia concentrations were estimated using Nessler's test, at the starting point (indicated with day 0) and at regular intervals for the next 75 days. At each NH_4^+ fall, 10 mL of the culture was re-inoculated in fresh LMM (30 mL) and ammonia concentration was adjusted over again to ~350 mg/L (peaks). (**B**) Samples from RRIA were withdrawn at NH_4^+ falls to determine nitrite and nitrate and their values, expressed as NO_2^--N (grey bars) and NO_3^--N (black bars), respectively, have been plotted. Data represent the average of three independent experiments and the standard deviation is ~10%. (**C**) The curves of the NH_4^+ removal rate in LMM for sludge (●) and G5-2 (27 days, ●) and G5-4 (53 days, ●) communities are shown. The initial cell number of the cultures was ~5 × 10^6 cells/mL as determined by plating bacteria on Luria–Bertani solid medium after serial dilutions.

Unlike the NH_4^+ stress experiments (Figure 2) where the NO_3^- formation was negligible, a complete ammonia oxidation took place in RRIA, resulting in accumulation of both nitrites and nitrates (Figure 4B). This observation suggests that selection by RRIA modified the structure of the native bacterial community changing relative abundance, proliferation and nitrogen removal activity of nitrifying AOB, NOB and possibly complete ammonia oxidation (comammox) bacteria [31–33]. Notably, RRIA was prolonged for 75 days, ~10 times longer than the experiment of Figure 2, and it was not a closed system. In fact, the same culture was repeatedly inoculated in fresh leachate medium assuring adequate levels of organics, thus favoring, depending on nutritional requirements, the predominance of some species over others (see metagenomic analysis).

Two microbial communities corresponding to the 27th and 53th days of RRIA were compared with the native sludge for their efficiency to degrade ammonia in leachate minimal medium. As shown in Figure 4B, the G5-2 and G5-4 bacterial populations almost completely oxidized ammonia after 9–11 days, while within the same time interval, the control culture derived from the native sludge still contained 200 mg/L of NH_4^+-N. Notably, this experiment was carried out at a considerably high ammonium concentration (~370 mg/L) explaining the low removal rate observed (compare to Figure 2A at similar NH_4^+-N concentration).

3.3. Determination of Ammonia-Degrading Microbial Communities by 16S rRNA Amplicon NGS

Selected samples from RRIA, collected at 0, 10, 15 and 75 days, were analyzed through 16S rRNA NGS. The total number of raw and post-processed sequenced reads is reported in Table 2. The total number of obtained raw reads ranges from 98,151 to 18,060. While the samples collected at 0, 10 and 15 days were quite homogeneous regarding the sequencing/alignment metrics (Table 2), the sample collected at 75 days showed a quite small number of sequenced reads when compared to the other samples. Nonetheless, rarefaction curves (Supplementary Figure S2) demonstrated that the sample collected at 75 days also reached the maximum number of mappable OTUs. This is clearly attributable to the reduced microbial community selected at 75 days through the RRIA approach. Alpha-diversity metrics, reported in Table 3, show that the sample collected at 75 days displays, as expected, an important drop-down in all the alpha-diversity metrics. The analysis of beta-diversity, determined by Principal Coordinate Analysis (PCA) of both UniFrac distance and Bray–Curtis dissimilarity (Figure 5), further highlights the extremely different microbial composition of the sample collected at 75 days compared to the other three time-points.

Table 2. Sequencing read processing results. Sequencing and alignment metrics for the four selected sequenced samples are reported, from the number of initial raw reads to the number of reads not aligned to the reference NCBI 16S RefSeq database.

Sample	Raw Reads	Trimmed Reads	%_Trimmed_Reads_Over_Raw	Assembled Reads	%_Assembled Reads over Trimmed	Hit Reads	% Hit over Assembled	No Hit Reads
0 days	98,151	91,281	93	50,242	55	22,641	45	27,601
10 days	82,295	76,807	93	46,538	61	30,319	65	16,219
15 days	95,210	88,113	93	53,764	61	33,086	62	20,678
75 days	18,060	16,920	94	12,487	74	7861	63	4626

Table 3. Alpha-diversity metrics for the RRIA samples sequenced by 16S rRNA amplicon NGS. Alpha-diversity metrics (Shannon's, Simpson's, Chao1 and alpha-div) for the four selected sequenced samples are reported.

Sample	Shannon	Simpson	Chao1	α-Diversity
0 days	4.808	0.937	87.5	80
10 days	4.255	0.872	94.667	90
15 days	5.104	0.943	107.333	98
75 days	3.264	0.807	55.5	33

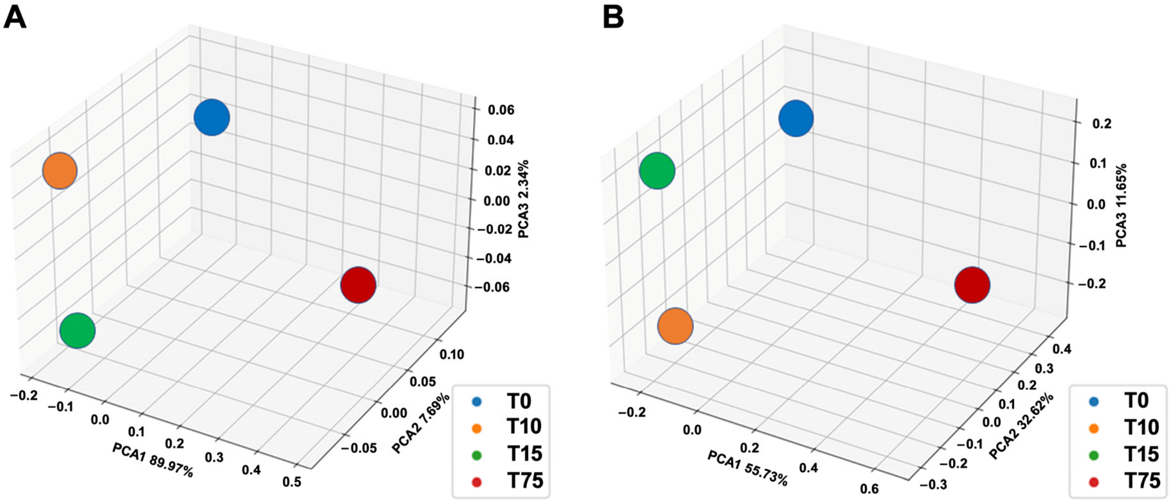

Figure 5. Principal coordinate analysis (PCA) of beta-diversity metrics for the RRIA samples sequenced by 16S rRNA amplicon NGS. (**A**) Unweighted Unifrac distance; (**B**) Bray–Curtis dissimilarity.

Conversely, seven different families (Alcaligenaceae, Nitrosomonadaceae, Caulobacteraceae, Xanthomonadaceae, Rhodanobacteraceae, Comamonadaceae and Chitinophagaceae) showed a progressive increase in frequency from time-point 0 to time-point 75 (Figure 6A). Notably, the Rhodanobacteraceae increased from 1% at time-point 0, to 40.2% at time-point 75. Some of the families (Cytophagaceae, Marinilabiliaceae, Sphingomonadaceae, Rhodobacteraceae, Haliscomenobacteraceae and Flavobacteriaceae) did not display a linear increasing/decreasing trend across the four time-points. However, the Haliscomenobacteraceae became the prevalent bacteria both at time-point 10 (32%) and at time-point 15 (18.1%), thus disappearing at time-point 75. Similarly, the Cytophagaceae became the second most prevalent bacteria at time-point 15 (15.8%) while disappearing at time-point 75.

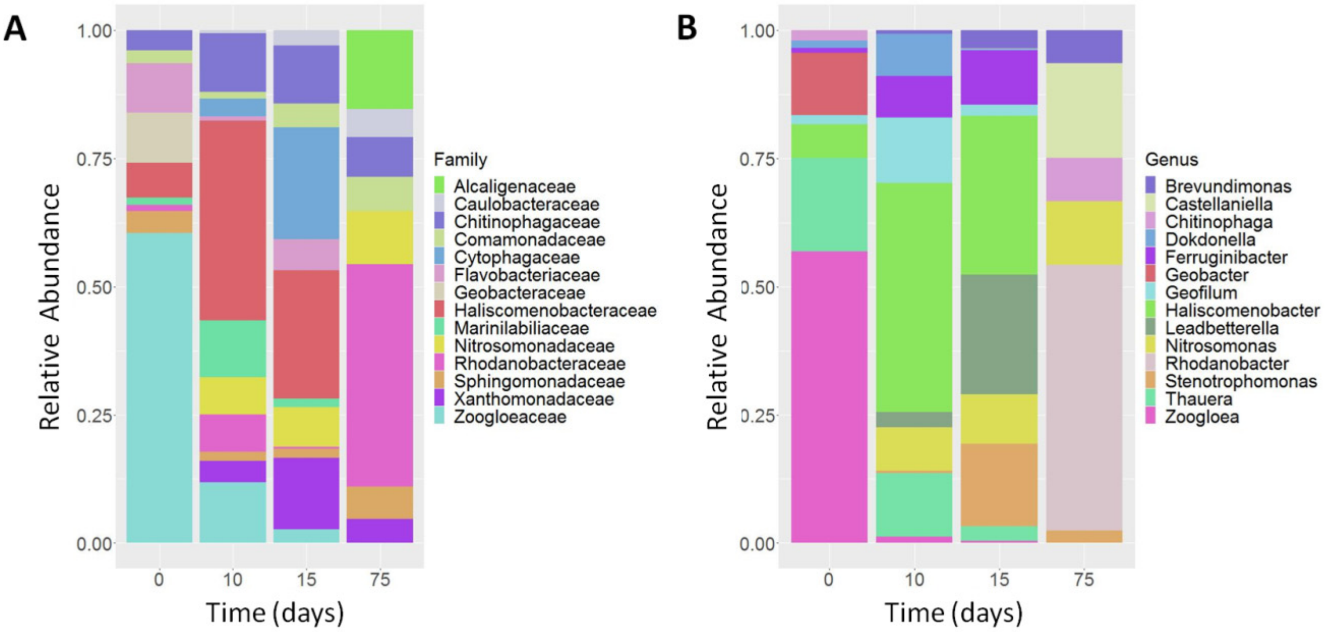

Figure 6. A 100% stacked bar-chart. Changes in the microbial composition, at family (**A**) and at genus (**B**) level of four times during the period of 75 days of RRIA (Figure 4A), as determined by sequencing of 16S rRNA.

When considering the composition of the microbial communities at the genus level, we identified 14 different genera with a cut-off of 5% in at least one sample (Figure 6B). *Brevundimonas* and *Nitrosomonas* showed a progressive increase across the four time-points, starting from being nearly absent at time-point 0, to peaking at 5% and 9.6%, respectively, at time-point 75. Conversely, *Geobacter*, *Thauera* and *Zoogloea* that represent the main bacteria at time-point 0 (6.4%, 9.5%, 29.8%, respectively), progressively disappeared across time-points 10, 15 and 75. The other genera did not show trends, although *Haliscomenobacter* represented the main genus at both time-points 10 (31.8%) and 15 (18%). It should be noted that *Leadbetterella* and *Stenotrophomonas* represented the second (13.6%) and the third (9.3%) most prevalent bacteria at time-point 15, respectively. Notably, *Rhodanobacter, Castellaniella* and *Nitrosomonas* became the predominant bacteria at time-point 75, with percentages of 40.2%, 14.3% and 9.6%, respectively.

The analysis of bacterial communities suggested that a concomitant autotrophic/heterotrophic nitrification took place during the Repetitive Re-Inoculum Assay. Our results are consistent, in terms of identified taxa, with those recently obtained by Wang et al. [34] who studied biological nitrogen removal in a pilot-scale reactor for six months. Thus, during the initial part of the RRIA (first 15 days), NH_4^+ removal was promoted by heterotrophic AOB mainly belonging to the Xanthomonadaceae, Cytophagaceae and Chitinophagaceae families. Heterotrophs do not oxidize ammonia as an energy source and by virtue of their ability to use the organic matter contained in leachate, they are characterized by a high growth rate and strong resistance to extreme NH_4^+ stress (≥300 mg/L) [35]. At genus level, the most abundant was *Haliscomenobacter* (~20–30%), a filamentous bacterium found in high ammonium-loaded activated sludge [36] and belonging to Haliscomenobacteraceae. Other genera as *Leadbetterella* (Cytophagaceae) and *Stenotrophomonas* (Xanthomonadaceae) were part of two largely represented families (~10–15%) as identified in Figure 6A. Chitinophagaceae was the third dominant AOB with a relatively high frequency (~8%), as previously reported by Wu et al. [37] analyzing the microbiota of a pilot sludge digester. In the final part of RRIA, possibly due to their slow growth, autotrophic AOB as *Nitrosomonas* accumulated over time, reaching ~10% in the two months of cultures. This genus includes some of the most important nitrifying species as *N. europaea and N. eutropha* that have been extensively investigated for their key role in wastewater treatment systems [38]. They convert ammonia to nitrite in two steps. In the first oxidation reaction, the NH_3 is transformed into hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO). Then, this intermediate product is converted to $NO^{-}{}_2$ by hydroxylamine oxidoreductase [39]. Notably, a dramatic increase was observed for *Rhodanobacter* (~40%). This genus was found to contribute to the denitrification process in a bench-scale reactor at a low pH [37]. Accordingly, during ammonia removal (NH_4^+ falls in RRIA) a gradual acidification of the medium occurred (pH decreased from 8 to 5), explaining the elevated abundance of this bacterial species. Therefore, the Comamonadaceae family, including many denitrifying species, was also increased [40], whereas comammox bacteria of the *Nitrospira* genus [31,32] were recovered at a very low frequency (≤1%).

3.4. Heterotrophic Nitrification and Aerobic Denitrification by Isolates

Aliquots of the culture at different times of the RRIA (53 and 75 days), after adequate dilutions, were plated on Petri dishes to isolate single colonies. These Agar plates contained the minimal medium supplemented with only leachate (LMM), ensuring further selection. Many isolates were inoculated in LMM (255 mg/L of NH_4^+-N) to test their ability to remove ammonia, and eventually, its oxidation products (NO_2^- and NO_3^-). Then, the most promising bacterial strains were identified at genus level by means of the following experimental approach: (i) PCR amplification of the 16S ribosomal RNA gene; (ii) Sanger sequence of 16S DNA; and (iii) nucleotide BLAST analysis.

The levels of the commonly used nitrogenous compounds were estimated during cell growth for the following identified bacteria: *Klebsiella* sp., *Castellaniella* sp., *Acinetobacter* sp. *Arthrobacter* sp., *Thermomonas* sp. and *Sphingomonas* sp. According to NGS analysis (Figure 6A), *Castellaniella* sp. (Alcaligenaceae), *Thermomonas* sp. (Xanthomonadaceae) and *Sphingomonas* sp. (Sphingomonadaceae) belong to families that were predominant at timepoint 75 of RRIA. All isolated bacteria displayed similar activity in nitrogen removal, and three genera are shown in Figure 7. The total nitrogen (TN) concentration, within the first four to six days of culture, rapidly decreased from 380 to 150 mg/L mostly reflecting the reduction (~50%) of ammonia content and, at lesser extent, the degradation of organic nitrogen compounds contained in leachate. Following the initial prompt decline, the total nitrogen showed a slight increase (10–12%) at the 10th day and its level closely accounted for the sum of the ammonia remained, not biodegradable chemical and cellular macromolecules. Notably, bacteria, albeit slowly, were growing in leachate broth with consequent accumulation of organic nitrogen (i.e., proteins) that was detected as TN, thus partially compensating for the progressive decrease of ammonia. These isolates showed a lower NH_4^+ removal rate (~70% after 12 days) than that observed for the native sludge at the same ammonia concentration (250 mg/L, Figure 2A,B), possibly because of the lack of reciprocal feeding interactions among diverse microorganisms. Unlike the complex bacterial population of the sludge (Figure 2C), the ammonia degradation by *Klebsiella* sp., *Castellaniella* sp. and *Acinetobacter* released negligible quantities of both nitrites and nitrates. This finding strongly suggests that these bacteria were able to go through with a combined heterotrophic nitrification and aerobic denitrification (HNADM), where NH_4^+ is oxidized to NO_2^- and then converted to gaseous nitrogen products (N_2O or N_2) that are finally released into the atmosphere [41,42]. However, we should acknowledge as a limitation the lack of determination of the changes in important physiochemical parameters such as BOD and COD related to the progressive removal of nitrogen compounds by bacteria.

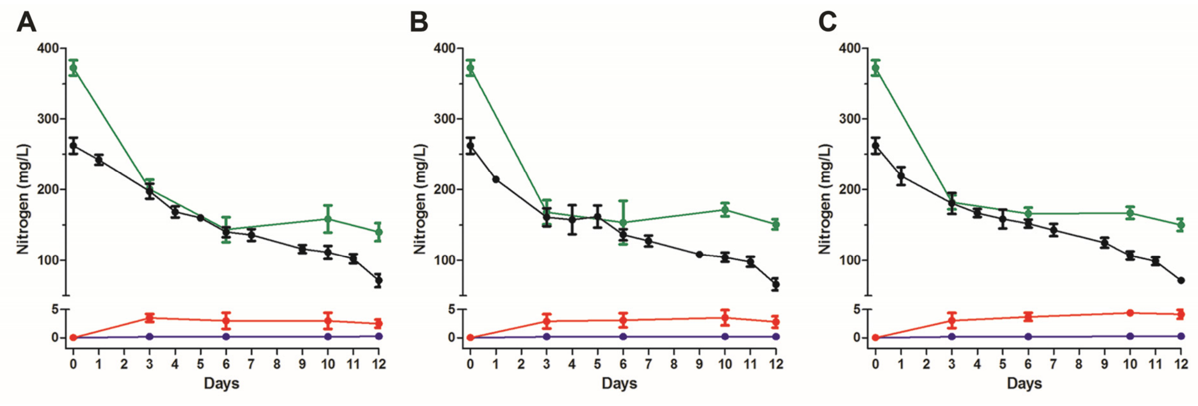

Figure 7. Change of nitrogen compounds' concentration of isolated bacteria. Bacterial strains were inoculated in LMM ($A_{600\ nm}$ = 0.1) and the levels of total nitrogen (🟢), ammonia (●), nitrite (🔵) and nitrate (🔴) monitored during the cell growth at 28 °C. Cultures were: *Klebsiella* sp. (**A**), *Castellaniella* sp. (**B**) and *Acinetobacter* sp. (**C**). Total nitrogen is the sum of ammonia, nitrites, nitrates and organic nitrogen compounds from leachate and cellular macromolecules. The optical density ($A_{600\ nm}$) of cultures was 0.3–0.4 at the 12th day.

During the last decade, heterotrophic nitrification and aerobic nitrite/nitrate denitrification under aerobic conditions was demonstrated for many genera of fungi and bacteria. HNADMs have been found in various environments retaining their nitrification/denitrification capabilities also under stress conditions as low pH, high ammonia and salty wastewater (reviewed in the Ref. [43]). According to previous studies [44–47], the genera *Acinetobacter* sp., *Klebsiella* sp. and *Arthrobacter* sp., isolated from the Porto Sant'Elpidio municipal WWTP exhibit considerable interest for their potential in nitrogen removal under aerobic conditions and are currently under further investigation in our laboratory.

4. Conclusions

The primary aim of this work was to investigate the biological degradation of inorganic nitrogen from landfill leachate. Initially, we evaluated the ammonia removal rate of the activated sludge from the WWTP of Porto Sant'Elpidio (Italy) identifying the upper limits, toxic for bacteria, of NH_4^+ (400 mg/L) and Cl^- (4 g/L). Then, the microbial composition of the native sludge, determined by NGS, was compared to that selected after repetitive ammonia stresses (RRIA). Differently from other studies, these experiments were carried out in leachate-based medium not containing an additional source of carbon. NGS analysis demonstrated that a significant enrichment of certain bacterial species takes place as a function of incubation time under ammonia stress. In particular, Rhodanobacter, Castellaniella and Nitrosomonas were the predominant genera after two months of RRIA experiments. Finally, the three isolated strains, *Klebsiella* sp., *Castellaniella* sp. and *Acinetobacter* sp. oxidized NH_4^+ to NO_2^- that was then transformed to gaseous nitrogen (N_2O or N_2), suggesting that these bacteria perform heterotrophic nitrification coupled with aerobic denitrification.

Our findings represent the starting point to produce an optimized microorganism's mixture for the biological removal of ammonia contained in leachate. This study could have practical implications possibly to increase, in a short time, the bioreactor performance. In fact, we are setting up a large-scale cultivation of this bacterial mixture to be introduced into a pilot reactor of 2000 L available at the Eco Elpidiense Company, thus enriching the native sludge with bacterial species able to resist and very active in degrading elevated NH_4^+ concentrations. These tests will permit to monitor changes in fundamental parameters as nitrogen compounds, pH, COD, and BOD with respect to the microbial population composition under experimental conditions that closely resemble those of a real wastewater treatment plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11020311/s1, Figure S1: Repetitive Re-Inoculum Assay; Table S1: Composition of different leachates; Figure S2: Rarefaction curves obtained from 16S rRNA amplicon NGS samples collected at time point 0, 10, 15 and 75.

Author Contributions: Conceptualization, R.P., K.P., A.F., G.P. and M.F.; Formal analysis, M.P. and V.N.; Investigation, R.P., A.F., A.C. and M.F.; Resources, K.P. and G.P.; Data curation, M.P. and V.N.; Writing—original draft preparation, A.F., V.N. and M.F.; Writing—review and editing, A.F., V.N. and M.F.; Visualization, A.F., V.N., M.P. and M.F.; Supervision, A.F. and M.F.; Funding acquisition, M.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no potential conflict of interest.

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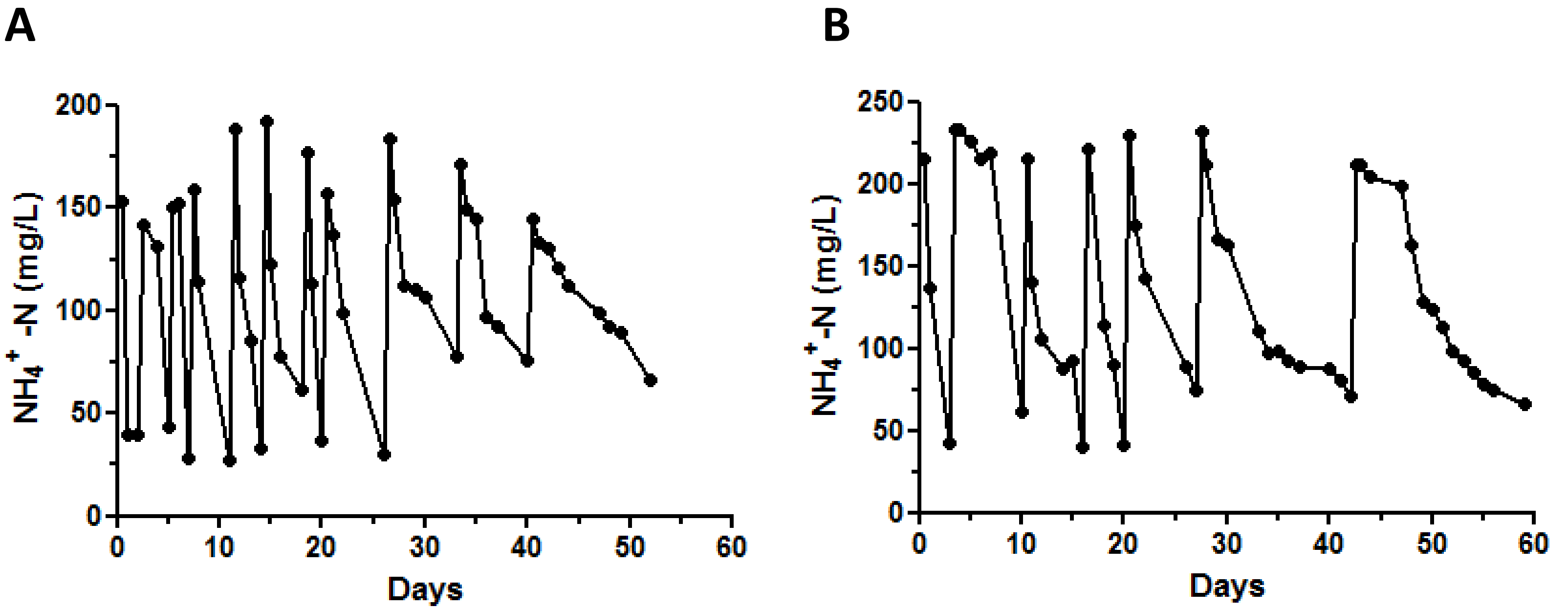
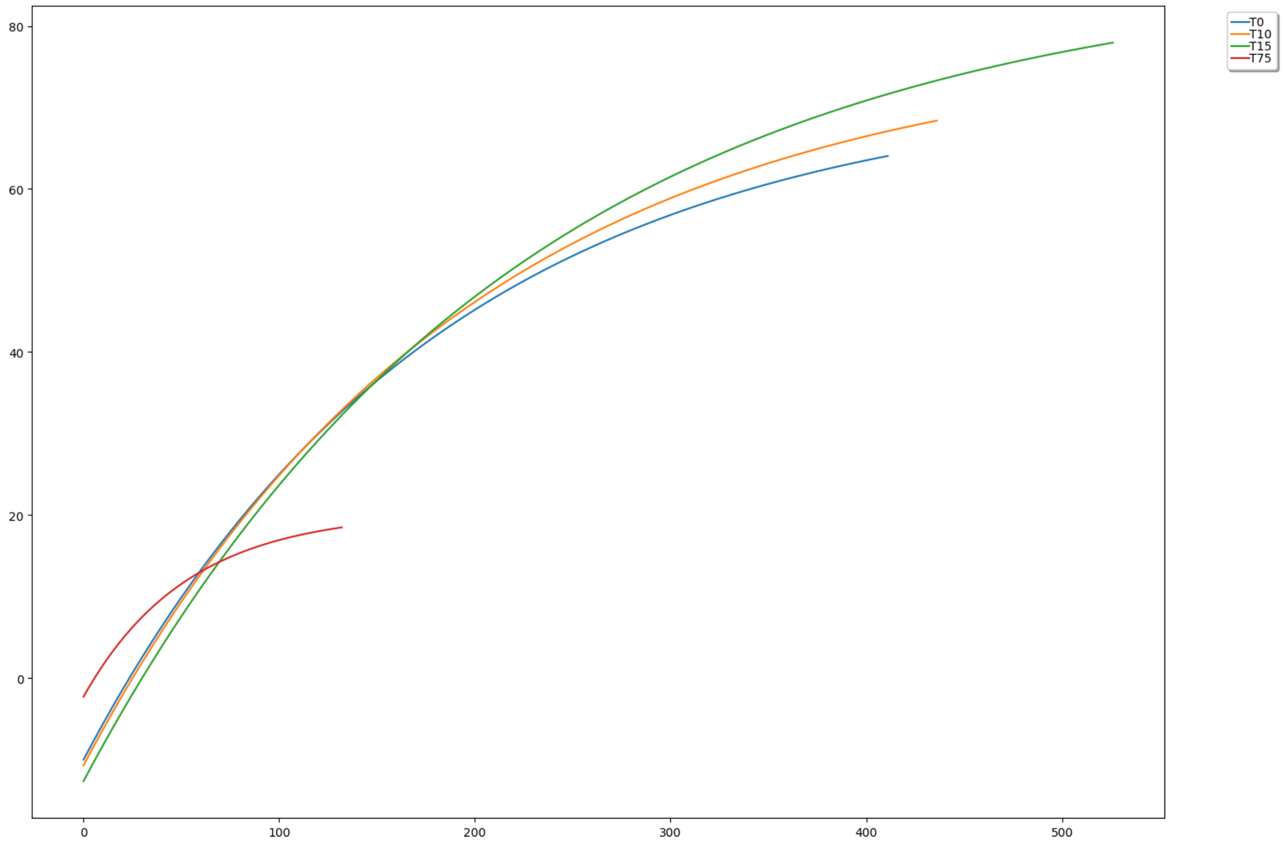
Parameters	**Leachate (A)**	**Leachate (B)**	**Leachate (C)**	**Leachate (D)**
Color	Dark brown	Dark brown	Dark brown	Dark brown
Smell	Strong	Strong	Strong	Very Strong
pH	8,28	8,40	6,00	8,89
NH_4^+-N (mg/L)	4427	2200	1140	1560
COD (mg/L)	5640	5000	2600	117000
BOD (mg/L)	640	1500	910	50370
BOD/COD	0,11	0,30	0,35	0,43
Cl^-	1806	3263	1430	3844

Supplementary Table S1.**Composition of different leachates.** The characteristics of four different leachates, such as (A) active landfill only special waste, (B) active landfill with pre-treated municipal waste, (C) post operational phase landfill and (D) landfill with high organic content waste, are shown. The nitrogen content in leachates is present almost only as ammoniacal nitrogen (NH_4^+-N).



Supplementary Figure S1. Repetitive Re-Inoculum Assay. RRIAs were carried out essentially as described in the text and in the legend of Figure 3. At the starting point (day 0), the ammonia concentrations, expressed as NH_4^+-N, were 150 (**A**) and 220 (**B**) mg/L . At each NH_4^+ fall, an aliquot of the culture was re-inoculated in fresh LMM medium and ammonia concentration was adjusted over again to the initial value (peaks). The two cultures subjected to RRIA were followed for about two months days.



Supplementary Figure S2. Rarefaction curves obtained from 16S rRNA amplicon NGS samples collected at time point 0, 10, 15 and 75. The number of different OTUs on the y axis is plotted vs. the number of sequences on the x axis.

microorganisms

Article

Development and Characterization of Ammonia Removal Moving Bed Biofilms for Landfill Leachate Treatment

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Abstract: Urbanization growth has intensified the challenge of managing and treating increasing amounts of municipal solid waste (MSW). Landfills are commonly utilized for MSW disposal because of their low construction and operation costs. However, this practice produces huge volumes of landfill leachate, a highly polluting liquid rich in ammoniacal nitrogen (NH_3-N), organic compounds, and various heavy metals, making it difficult to treat in conventional municipal wastewater treatment plants (WWTPs). In recent years, research has shown that microbial biofilms, developed on carriers of different materials and called "moving bed biofilm reactors" (MBBRs), may offer promising solutions for bioremediation. This study explored the biofilm development and the nitrification process of moving bed biofilms (MBBs) obtained from high ammonia-selected microbial communities. Using crystal violet staining and confocal laser-scanning microscopy, we followed the biofilm formation stages correlating nitrogen removal to metagenomic analyses. Our results indicate that MBBs unveiled a 10-fold more enhanced nitrification rate than the dispersed microbial community present in the native sludge of the Porto Sant'Elpidio (Italy) WWTP. Four bacterial families, Chitinophagaceae, Comamonadaceae, Sphingomonadaceae, and Nitrosomonadaceae, accumulate in structured biofilms and significantly contribute to the high ammonium removal rate of 80% in 24 h as estimated in leachate-containing wastewaters.

Keywords: biological nitrogen removal; moving bed biofilm (MBB); landfill leachate; microbial community; metagenomics

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1. Introduction

Urbanization and population growth have raised the issues of the production and, consequently, the need for appropriate treatment of increasing amounts of municipal solid waste (MSW). Because of low building and operation costs, landfills are widely used in Italy, as well as in developed countries, for MSW disposal. Inevitably, this generates huge volumes of landfill leachate, a highly pollutant liquid containing elevated concentrations of ammonium nitrogen (NH_4^+-N) and organic compounds in addition to a large spectrum of heavy metals [1–5]. Currently, most of the leachates from Italian landfills are classified as intermediate (5–10 years old) and mature ($\geq$15 years old) leachates [3]. They are characterized by a low content of biodegradable organics due to two major factors: (a) their long stay in landfills; and (b) the introduction of separate waste collection, which occurred in Italy about a decade ago. Thus, these leachates show a low biochemical oxygen demand/chemical oxygen demand ratio (BOD/COD $\leq$ 0.3) and elevated concentrations of ammonia (2000–4000 mg/L). Leachates with such a chemical composition are very hard to treat in conventional municipal wastewater treatment plants (WWTPs) where nitrogen removal is mainly promoted by the microbial community of the activated sludge [2,6]. In fact, the nitrification-denitrification process involves first the conversion, by autotrophic ammonia-oxidizing bacteria (AOB), of ammonium ion (NH_4^+) to nitrite (NO_2^-), which is further oxidized to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB). Finally, NO_3^- is reduced to gaseous nitrogen compounds by anoxic/anaerobic bacteria, which use organic carbon as an energy source [7].

In the last two decades, many studies have explained why and how planktonic cells start colonizing living and/or inert surfaces and move to a biofilm lifestyle. A biofilm displays a three-dimensional structure with a highly ordered and complex extracellular matrix [8,9]. The development of a mature biofilm confers to the cell population better fitness and protection against a broad range of environmental stress conditions including antimicrobial agents and sanitation processes of food and water. Thus, biofilm formation can generally increase the biological risk to human health [10–12]. On the other hand, bacterial biofilms are currently regarded as very promising, innovative, and powerful tools in bioremediation techniques. In fact, microbial communities organized into biofilms are much more active than planktonic cells in degrading harmful pollutants such as inorganic and organic compounds, plastic, and metals. According to this, microbial proliferation and biomass development are induced on carriers of different materials, which are subsequently placed into the reactor. This technology, called a moving bed biofilm reactor (MBBR), was first invented in Norway [13] and is now employed worldwide for the treatment of industrial and municipal wastewater. Biofilm reactors can operate in aerobic, anoxic, or anaerobic conditions, and it has been extensively proven that microbes attached to carriers and organized in multi-layered biofilms show an enhanced nitrification-denitrification activity as compared with dispersed biomass. In fact, stratified biofilms allow, through an oxygen gradient, the simultaneous growth of nitrifiers and denitrifiers for complete nitrogen removal, whereas organic matter is degraded by aerobic bacteria located mostly in the outer layers of biomass [14–16]. Thus, MBBRs are very efficient in ammonia removal in pollutants containing a low carbon/nitrogen (C/N) ratio, such as those in landfills [17–20]. Unlike suspended activated sludge reactors, the moving bed biofilm process also permits the volumetric capacity of the tank through the concentration of biomass to be fully exploited, thereby maximizing the plant's performance. In addition, MBBRs offer the advantages of reducing the clogging of bio-carriers and the number of head loss events because they do not require sludge recycling [21–23].

In a recent study, the bacterial population of the activated sludge derived from the WWTP of Porto Sant'Elpidio (FM, Italy) was subjected to repetitive ammonium stresses (NH_4^+-N $\cong$ 350 mg/L) in a minimal medium supplemented with leachate as only carbon source. These experiments, named the Repetitive Re-Inoculum Assay (RRIA) [24], allowed us to obtain an optimized microorganism mixture that can efficiently abate the high concentration of ammonia contained in mature landfill leachates. Herein, the formation of moving bed biofilms from these NH_4^+ selected microbial communities was investigated using crystal violet staining (CVS) and confocal laser-scanning microscopy (CLSM). Then, the nitrification process promoted by MBBs was estimated in a leachate-based medium and correlated to metagenomic analyses performed by 16S rRNA gene next-generation sequencing. Taken together, our results reveal that four bacterial families, Chitinophagaceae, Comamonadaceae, Sphingomonadaceae, and Nitrosomonadaceae, were predominant on structured biofilms derived from high NH_4^+ stress-selected bacterial consortia and largely contribute to the excellent ammonium removal rate (≅80% in 24 h) as estimated in wastewater–leachate mixtures.

2. Materials and Methods

2.1. Characteristics of Biofilm Carriers

The K1 moving bed biofilm carrier (Kaldnes, Sandefjord, Norway), used in Bio-RRIA experiments (see below), is characterized by polyethylene high density (HDPE) hollow cylinders with an internal cross and 18 external fins; the diameter is 9.1 mm, and the length is 7.2 mm. The density is 140 kg/m^3, and the efficient surface area is 500 m^2/m^3.

Limited to LIVE/DEAD staining and confocal laser-scanning microscopy analysis, the carriers were HDPE sheets that were 15-micron thick and 1 cm^2 in size.

2.2. Preparation of Bio-Carriers

Bacterial adhesion to K1 carriers and HDPE sheets was obtained using both selected bacteria populations (G1-2, G5-2, and G5-4) and native sludge. Aliquots of glycerol stock G1-2, G5-2, and G5-4 were plated on solid agar (1.7%) leachate minimal medium (LMM) (see below) at the final concentration of NH_4^+ of 350 mg/L. After 2 days of incubation at 30 °C, the bacterial lawns formed were collected with a sterile cotton swab and suspended in 30 mL of LMM. Then, 30 pieces of K1 carriers were immersed in the bacterial suspension. The initial optical density (OD_{600}) was ≅0.3, corresponding to ≅5 × 10 ^{7}cells/mL as determined by plating bacteria on Luria–Bertani (LB) solid medium after serial dilutions. The cultures were incubated in a rotary shaker at 28 °C for 10–15 days, and the decrease of ammonia (NH_3) was monitored with Nessler's test. Since ammonia degradation by bacteria caused acidification (pH about 6.5) of cultures, the initial pH = 8 of the LMM medium was restored by adding NaOH (5 N). The dissolved oxygen (DO) concentration was measured using the Edge DO meter (Hanna Instruments, Smithfield, RI, USA) and kept at 5.8 ± 0.5 mg/L with mild agitation. The composition, at the family level, of the selected bacterial populations G5-2 and G5-4 is reported in Supplementary Table S1. For sludge bio-carriers, 10 mL of the activated sludge (150 mg, dry weight) was mixed with 20 mL of the LMM and 30 pieces of K1 carriers and incubated at 28 °C following the above-described protocol. The optical density of this culture could not be measured for the high turbidity due to the addition of the sludge, and only the colony-forming units (CFUs) were determined (≅2 × 10^6 cells/mL).

The LMM was prepared by combining 60 mL of leachate with 30 mL of MM medium and 90 mL of distilled water. This step causes a 3-fold dilution of raw leachate. The MM broth was composed of three solutions that were mixed together. Solution 1 contained 4.95 g of $(NH_4)_2SO_4$, 0.62 g of KH_2PO_4, 0.27 g of $MgSO_4$, 0.04 g of $CaCl_2$, 0.5 mL of $FeSO_4$ (30 mM in 50 mM EDTA at pH 7.0), 0.0002 g of $CuSO_4$, and 1.2 L of distilled water. Solution 2 contained 8.2 g of KH_2PO_4, 0.7 g of NaH_2PO_4, and 3 L of distilled water, which was brought to pH 8.0. Solution 3 contained 0.6 g of Na_2CO_3 and 12 mL of distilled water. The three solutions were sterilized using filtering, and the leachate was sterilized using an autoclave. The ammonia concentration of bacterial cultures was adjusted to the values indicated in Figure Legends, adding 1 M of $(NH_4)_2SO_4$. The average compositions of pre-treated leachates used in our experiments are reported in Supplementary Table S2.

For determination of colony-forming units (CFUs) from bio-carriers, the HPDE supports were subjected to pulsed low-intensity sonication (three cycles of 45 s at 3.5 Watts) in 3 mL of ice-cold 0.9% NaCl solution to completely remove the bacterial biofilm. Then, the cells, after adequate dilutions, were plated on LB solid medium and incubated overnight at 28 °C.

2.3. Crystal Violet Staining

Crystal violet staining was carried out essentially as previously described [25,26]. Briefly, K1 bio-carriers were rinsed with 0.9% NaCl solution to eliminate medium residual and incubated with 0.1% (*w*/*v*) crystal violet solution in distilled H_2O. After 15 min at room temperature, the bio-carriers were rinsed four times with distilled H_2O to remove unbound dye. The crystal violet was washed out from the biofilms with 4 mL of 95% ethanol, and the color intensity was quantified spectrophotometrically at a wavelength of 600 nm [27].

2.4. LIVE/DEAD Staining and Confocal Laser-Scanning Microscopy (CLSM)

The HDPE sheets were fluorescence stained using the LIVE/DEAD®BacLightTM Bacterial Viability Kit according to manufacturer protocol (Invitrogen, Carlsbad, CA, USA). Briefly, the two dyes, SYTO9 green fluorescent and propidium iodide (PI) red fluorescent, were applied simultaneously as a 1:1000 dilution in distilled water and incubated for 15 min at room temperature in a dark room. After washing the stained carriers with distilled H_2O to remove the dyes in excess, the fluorescent signals from both live (green) and dead (red) bacteria were visualized simultaneously by a confocal laser-scanning microscopy CLSM (Nikon C2Si using the objective PLA APO λ 100× Oil, Nikon, Tokyo, Japan).

2.5. Analytical Methods

Concentration determinations of total nitrogen, ammonia, nitrite, nitrate, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) were carried out as previously reported [24].

2.6. Analysis of Microbial Communities by 16S rRNA Gene Next-Generation Sequencing

Bacterial cells from native sludge (aliquots of 10–20 mL) were harvested by centrifugation (8000 rpm for 20 min), whereas bio-carriers (4–6 tubes) were pulled out of culture medium, washed in 0.9% NaCl solution and subjected to a mild sonication for cells detachment as described above. Then, chromosomal DNA was extracted from all samples by the E.Z.N.A. Kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the instructions given by the manufacturer. DNA concentration was estimated by NanoDrop (ThermoFisher Scientific, Waltham, MA, USA), and the V3–V4 hypervariable regions of 16S rDNA were amplified by PCR using universal primers 341F 5′-TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGGNGGCWGCAG-3′ and 805R 5′-GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Then, 16S rRNA gene next-generation sequencing by MiSeq Illumina Platform (San Diego, CA, USA) was performed as previously reported [24]. Operational taxonomic units (OTUs) were defined by clustering at a minimum pair-wise identity threshold of 97%. The NCBI 16S RefSeq database was employed for taxonomic classification.

3. Results and Discussion

3.1. Monitoring and Quantification of Biofilm Formation

In a recent study, financed by the Eco Elpidiense s.r.l., a private company that manages the municipal wastewater treatment plant of Porto Sant'Elpidio (FM, Italy), we developed a bacteria selection procedure named the Repetitive Re-Inoculum Assay (RRIA). This assay, somewhat resembling the dilution to extinction approach [28], consists of consecutive re-inocula/dilutions (7–10 times) of the same bacterial community in a liquid leachate-based medium (LMM) at elevated NH_4^+-N content (≅350 mg/L). RRIA caused a drastic reduction (from ≅250 to ≅15) of bacterial species present in the activated sludge, causing a remarkable enrichment of those microorganisms characterized by elevated tolerance to recurring ammonia stresses coupled with high activity in nitrogen removal. Aliquots of RRIA cultures were taken at different times during the 2-month assay for bacterial species identification (Supplementary Table S1) using metagenomic analysis and cell storage at −80 °C [24].

In the present work, we first established whether our selected microbial communities were able to develop organized biofilms. To this end, K1 HDPE bio-carriers were prepared from both native sludges and RRIA-derived multi-species suspensions to verify the microbial adhesion to these plastic supports by the crystal violet staining (CVS). This colorimetric method is easy, quick to perform, and cost-effective, although it has the disadvantages of being poorly quantitative and not distinguishing viable and non-viable cells [29]. The result, shown in Supplementary Figure S1, was very promising and revealed that bacteria of all the processed samples stably colonized a large surface of K1 polyethylene supports, as visible from the intense blue color. In fact, G5-4, G1-2, and sludge bio-carriers, after ethanol wash and measure of related optical densities, displayed values of A_{600} nm about six times higher than that of controls (carriers not immersed in cell cultures), suggesting that this plastic material was a suitable carrier to be used in our planned MBBs experiments. In line with Irankhah et al. [30], who investigated MBB reactors from mixed cultures, we found comparable optical densities (A_{600} nm ≅ 2.0) in CVS assays in addition to the common observation of evident cell adhesion at the air–liquid interface of the walls of the flasks in all experiments.

Furthermore, biomass accumulation was also estimated as a function of time during biofilm formation, for G5-2 and native sludge HDPE carriers, using LIVE/DEAD staining and confocal laser-scanning microscopy (CLSM) (Figure 1). For image acquisitions, bio-carriers were taken out from bacterial suspensions in LMM broth on the 5th, the 10th, and the 20th days following the ammonia degradation. On the 20th day, the remaining bio-carriers were re-inoculated in a fresh medium (NH_4^+-N concentration ≅ 350 mg/L), and incubation was prolonged for an additional 10 days at 28 °C. The selective staining coupled with CLSM clearly visualized the predominance, across all samples, of green fluorescent–labeled cells (living ones), demonstrating that bacteria were abundant and, for a very large majority, vital; otherwise, red fluorescent cells (dead ones) were almost completely absent. According to the random acquisition of images, the cell number seemed to be slightly higher for G5-2 than in sludge MBBs in the first 20 days of biofilm development, whereas such difference disappeared for longer times (25th and 30th days) when more structured biofilms were formed. Colony-forming units (CFUs), determined by growing bacteria on LB agar plates after serial dilutions, supported the CLSM analysis and revealed that the cell number was similar for both biofilms, which was $\cong 4 \times 10^3$ per HPDE sheet on the 5th day and progressively increased up to $\cong 5 \times 10^4$ per HPDE sheet on the 30th day. Sludge and G5-2 MBBs also showed comparable partial nitrification activity. Thus, the NH_4^+-N concentration diminished from ≅350 to ≅60 mg/L in 6 days, corresponding to a removal rate of 83%, which was consistent with that estimated with K1 bio-carriers in the initial phase of the Bio-Repetitive Re-Inoculum Assay (see next paragraph).

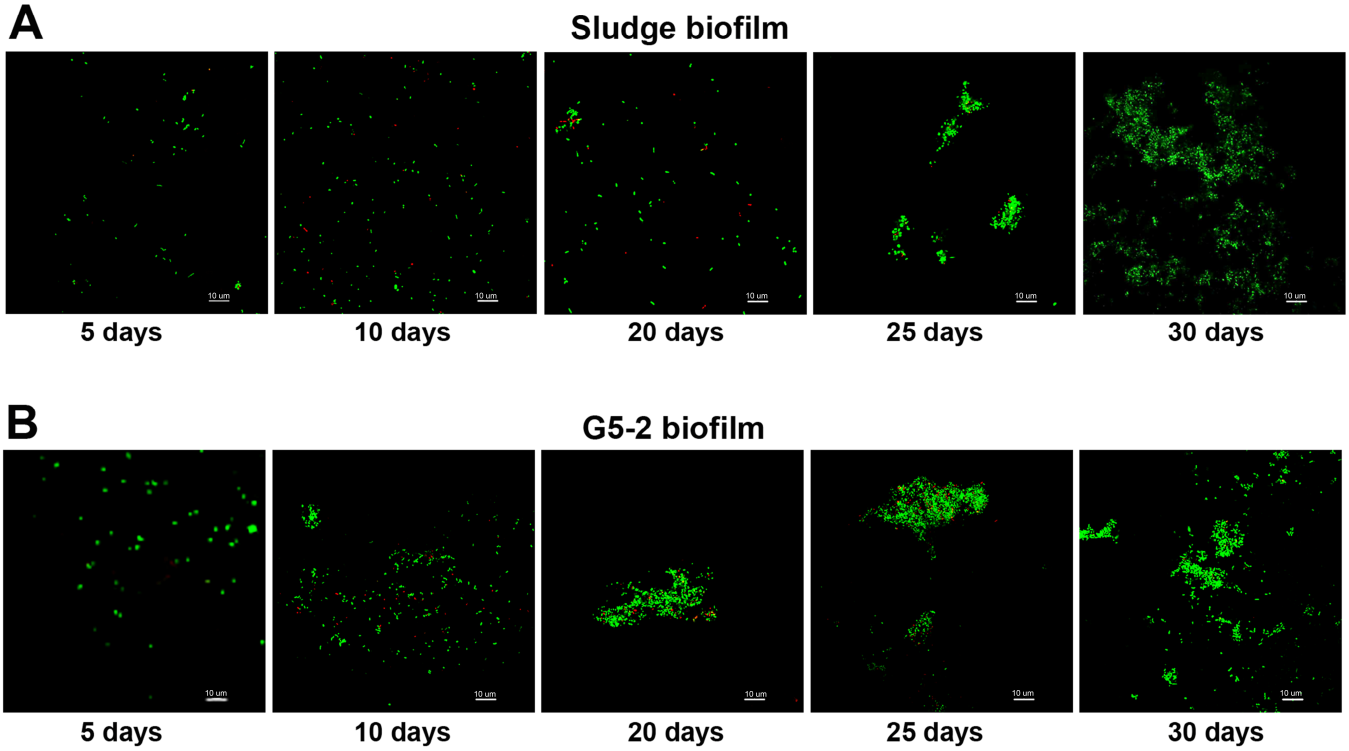

Figure 1. Analysis of biofilm formation using CLSM. Staining of sludge (**A**) and G5-2 (**B**) MBBs was carried out with SYTO9 and PI fluorescent dyes, followed by CLSM analysis as described in Section 2. Both dyes intercalate with nucleic acids but, while SYTO9 penetrates both living and dead cells, PI can only pass through damaged membranes and displaces SYTO9, allowing differentiation between live (green) and dead (red) cells [31,32]. Stacks of images were taken at random areas on the HPDE sheets, and each stack contained 10 images.

3.2. Ammonia Degradation by Biofilms Developed from Ammonia-Selected Bacterial Populations and Activated Sludges

Given that the biofilm-forming ability and biomass measurement were ascertained for our mixed cultures by different methods (Supplementary Figure S1 and Figure 1), G5-2 and G5-4 MBBs were tested for their capability to degrade ammonia in a minimal broth (LMM) supplemented with leachate as only carbon source (Figure 2). These bio-carriers were inoculated in LMM medium, and the ammonia concentration was estimated at the starting point (time 0) and at regular time intervals for the next 21 days. As bacterial biofilms began to oxidize ammonia and its concentration dropped to ≅80 mg/L (falls), bio-carriers were removed, washed, and transferred to a fresh leachate broth so that the initial value of NH_4^+-N (≅360 mg/L) was restored (peaks). This step was repeated 10 times. This experiment is essentially the Repetitive Re-Inoculum Assay (RRIA) as previously described by Petrilli et al. [24], with the exception that MBBs were used instead of planktonic cells as in the original assay. Thus, it was named Bio-RRIA. As seen in Figure 2A, the two different MBBs showed basically matching nitrogen removal patterns throughout the entire duration of the assay, characterized by an initial adaptation period in which a lower efficiency in ammonia degradation was observed. In fact, the NH_4^+ concentration was reduced from ≅360 to ≅80 mg/L (removal of 78%) in 5 days. From the 6th to the 15th days, the removal rate progressively increased and peaked, which identified the transfer of bio-carriers in a fresh leachate medium with consequent extra NH_3 stress, became closer and closer. Thus, after five re-inocula (on the 15th day), MBBs degraded the same amount of ammonia (78%) in only 24 h, exhibiting a 10-fold higher removal activity than the respective original microbial cultures. In fact, when G5-2 and G5-4 bacterial populations were grown in the planktonic phase, under the same experimental conditions, about 9–11 days were necessary to obtain ≅75% ammonia oxidation [24].

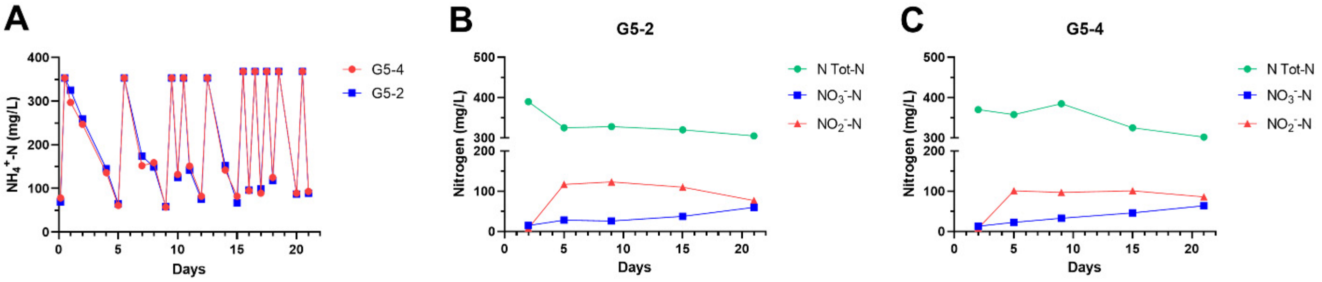

Figure 2. Bio-repetitive re-inoculum assay using G5-2 and G5-4 MBBs. Bio-carriers of G5-2 and G5-4 (30 pieces each) were incubated in 30 mL of LMM at 28 °C. The NH_4^+-N was adjusted to ≅350 mg/L and monitored, using Nessler's test, as a function of time for the whole duration of Bio-RRIA (**A**) (see body text). Samples from Bio-RRIA were withdrawn at indicated NH_4^+-N falls for total nitrogen, nitrite, and nitrate determinations of G5-2 (**B**) and G5-4 (**C**) biofilms. Total nitrogen is the sum of ammonia remaining, nitrites, nitrates, and organic nitrogen compounds from leachate. Leachate composition is reported in Supplementary Table S2.

During the last two decades, the performance of many biofilm-based reactors has been extensively investigated, and a bibliometric analysis reveals that ammonia removal varies approximately between 60 and 95% depending on the biofilm system adopted, the type of wastewater, ammonium strength, and bio-carried used. Most of these bioreactors operated with an initial ammonia concentration of less than 100 mg/L [18,33–38]. In this context, our results are promising, given that G5-2 and G5-4 MBBs show an excellent removal rate (≅80% in 24 h) with high-strength ammonium (≅360 mg/L). According to our standard protocol for the preparation of bio-carriers, the Bio-RRIA assays were highly reproducible with high ammonia-selected MBBs, and occasionally, small differences, limited to the length of the initial adaptation phase, were found. Another Bio-RRIA, in which the three biofilms, G5-2, G5-4, and G1-2, were compared, showed that, after the usual adaptation period, the one-day removal rate of 67% was achieved (Supplementary Figure S2). Nitrification was slightly lower due to the higher concentration of ammonia (≅425 mg/L) used in this Bio-RRIA.

In addition to NH_4^+, the levels of the most common nitrogenous compounds were estimated during the ammonia oxidation by the bacterial biofilms (Figure 2B,C). A progressive accumulation of NO_2^- was observed for both G5-2 and G5-4 MBBs, whereas appreciable amounts of NO_3^- ($\geq$50 mg/L) were produced only in the final part of the Bio-RRIA (after the 15th day). Consistently, the total nitrogen (NT) was only slightly reduced as a function of time, and its level closely accounted for the sum of the ammonia remaining, nitrites, nitrates, and non-biodegradable organic compounds from leachate. Under the experimental conditions used, leachate-based medium and presence of oxygen (DO ≅ 5.8 ± 0.5 mg/L), these findings indicate that MBBs mainly promoted nitrification due to the predominant carrier adhesion, selective growth, and action of autotrophic and heterotrophic nitrifying bacteria (see metagenomic analysis). Importantly, biofilm enables bacteria with different nutritional requirements to coexist by occupying distinct layers and niches within stratified biofilms where inorganic/organic carbon, oxygen, and various substrates can be exchanged by bacteria. In this context, multicomponent biofilms, as utilized in our experiments, are more efficient than single-species biofilms in bioremediation.

The municipal wastewater treatment plant (WWTP) of the Porto Sant’Elpidio town is located in central Italy near the Adriatic Sea coast where the climate is very mild and, particularly in recent years due to global warming, the temperature in winter rarely falls below 5–10 °C. A detailed description of this WWTP has been previously provided [24]. Thus, the effect of the temperature on the nitrification process by G1-2, G5-2, and G5-4 biofilms was investigated in the range of 10–30 °C (Figure 3). For this purpose, bio-carriers were taken from the final phase (≅after 20 days) of a preparative Bio-RRIA where biofilms were already formed and the ammonia removal rate was optimal (≅78% in 24 h). Then, MMBs were placed in a fresh leachate-based medium to start a new Bio-RRIA at four different temperatures. The nitrogen removal rate remained constant at 30 °C and 23 °C but slowed down by ≅ three times (78% in 72 h) at 18 °C for all the samples. In contrast, biofilm nitrification activity was considerably reduced at 10 °C (75% in 14 days). Importantly, the wastewater temperature in the two nitrification/denitrification tanks (1450 m^3 each) of the Porto Sant’Elpidio WWTP drops down 15–20 °C only in January and February, as monitored by the Eco Elpidiense (FM, Italy), the private company that manages this municipal wastewater bioreactor. Thus, the finding that G1-2, G5-2, and G5-4 MBBs retain their maximum performance at 23 °C with a limited loss of activity at 18 °C is very encouraging for their immediate application in the pilot reactor of 2000 L, which is already available at the Eco Elpidiense Company. Depending on the pilot reactor results, we are confident that our approach derived from MBB technology, implemented and transferred on a large scale, could be used to improve, in the near future, the Porto Sant’Elpidio municipal bioreactor performance by accelerating the biological conversion of ammonia to nitrite. This reaction is surely the most critical step of the entire nitrification-denitrification process by bacteria [39] and, importantly, needs a lot of energy to make the aeration system, which injects air into the tanks of the WWTP, operate.

Colorimetric assays and CFUs (Figure 1 and Supplementary Figure S1) showed that, in addition to high ammonia-selected cultures, also the bacterial community present in the native sludge from the Porto Sant’Elpidio MWWTP was able to adhere and form biofilm on HDPE supports. To obtain a representative picture, native sludges were sampled during the last 2 years, considering the chemical analyses performed by the Eco Control Laboratory (FM, Italy), to estimate the municipal bioreactor performance. In fact, the treatment capacity of a municipal plant can vary due to many factors (i.e., season, rainfall, change of equivalent people, etc.). However, in the specific case of the Porto Sant’Elpidio bioreactor, the treatment capacity also depends on the quantity and quality of landfill leachate introduced (GP and KP, personal communication). As compared with the high reproducibility of G5-2 and G5-4 MBBs, Bio-RRIA carried out with different sludge-derived biofilms exhibited quite variable ammonia degradation curves (Figure 4 and Supplementary Figure S3). In fact, the LS1 MBB plot was characterized by an initial ammonia removal rate of ≅60% in 10 days, a value more than two-fold lower than that of G5-2 and G5-4 MBBs. After this acclimatization period, a progressive increase of the NH_4^+ oxidation activity and consequent shortening in time was observed for G5-2 and G5-4 biofilms (Figure 2A). Diversely, SL4 MBB displayed a very poor nitrification capacity (NH_4^+ removal of ≅50% in 15 days) that remained low for the entire length of Bio-RRIA. Accordingly, this sludge sample was withdrawn when the Porto Sant'Elpidio WWTP was malfunctioning, and dense and persistent foams were well visible on the surface of wastewater contained in the two nitrification/denitrification tanks. As seen in Figure 4B,C, better NH_4^+ degradation patterns were obtained with SL2 and SL3 sludge-derived biofilms that approached the elevated ammonium removal rate of G5-2 and G5-4 MBBs. In fact, SL2 and SL3 biofilms lacked a pronounced early adaptation phase and were characterized by an average removal of ≅76% in 5–6 days that did not change for the duration of the Bio-RRIA experiment (30–40 days). Altogether, these results suggest that, unlike the high ammonia-selected G1-2, G5-2 and G5-4 MBBs, native sludge biofilms display a large variability and are much less reliable in producing biofilms with high efficiency in ammonia oxidation. As seen in Supplementary Figure S3, some sludge MBBs could be partially functional or even unsuccessful.

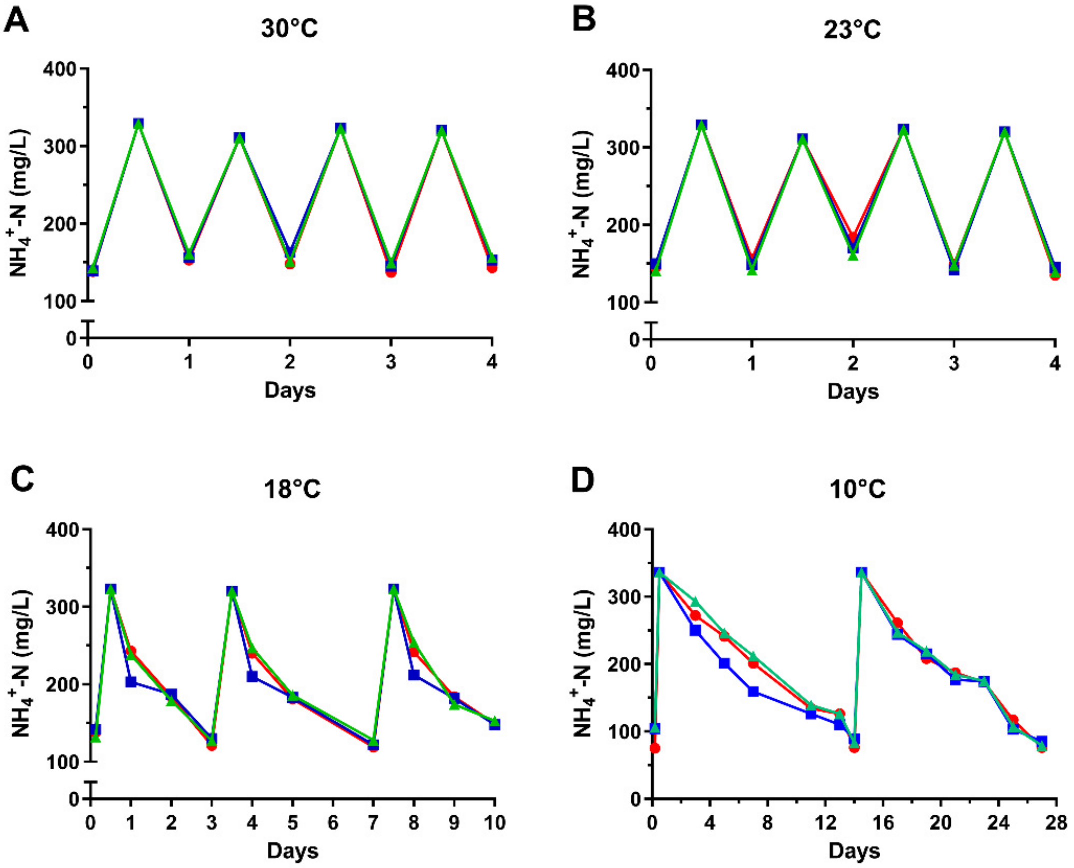

Figure 3. Effect of the temperature on Bio-RRIA by G1-2, G5-2 and G5-4 MBBs. The G1-2 (red circle), G5-2 (blue square), and G5-4 (green triangle) MBBs were incubated at 30 °C (**A**), 23 °C (**B**), 18 °C (**C**) and 10 °C (**D**). and then subjected to Bio-RRIA essentially as described in the body text and in the legend of Figure 2A.

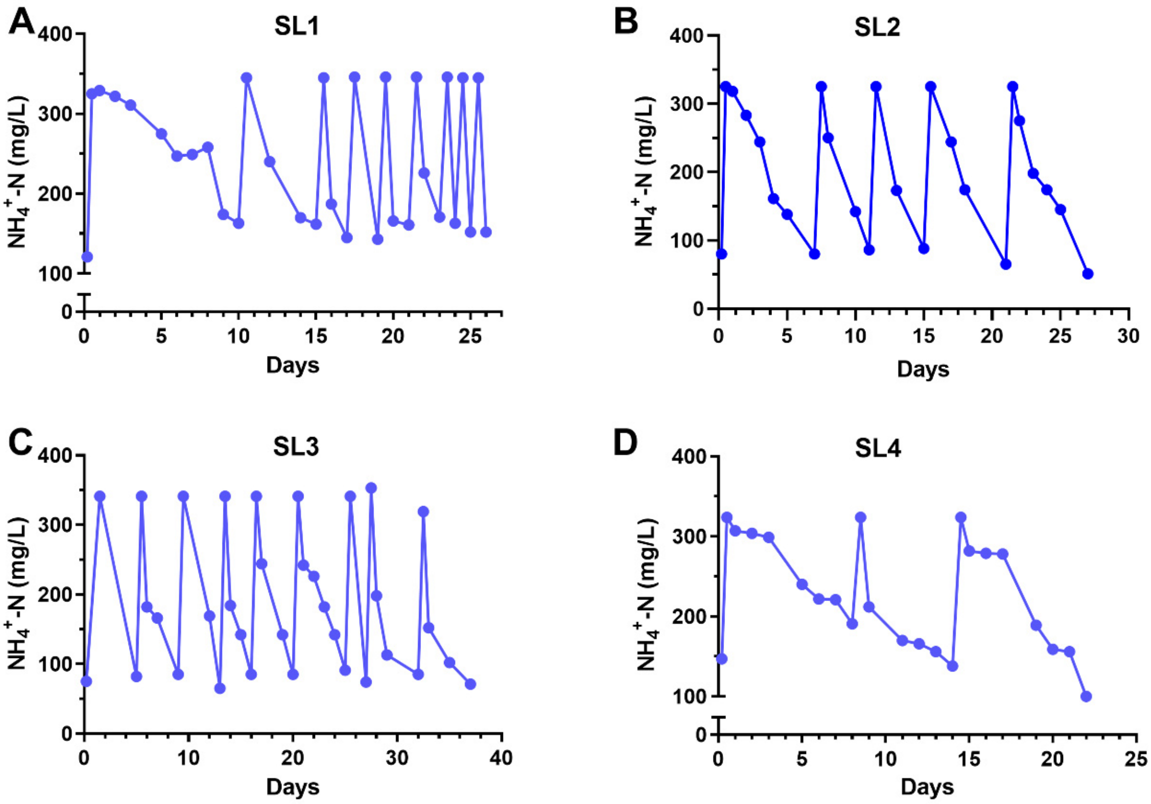

Figure 4. Bio-repetitive re-inoculum assay by sludge MBBs. Sludge samples were taken at different periods from the Porto Sant'Elpidio MWWTP and used for K1 bio-carriers preparation. Biofilms were investigated for their efficiency in NH_4^+-N removal in Bio-RRIA experiments performed as described in the body text and in the Legend of Figure 2A. Four representative sludges, indicated with SL1 (**A**), SL2 (**B**), SL3 (**C**) and SL4 (**D**), are shown.

3.3. Composition of Biofilms Formed from Ammonia-Selected Microbial Communities and Activated Sludges

The microbial composition of G5-2 and G5-4 biofilms, used in the Bio-RRIA experiments, was investigated as a function of time using 16S rRNA NGS analysis. According to the near-coincident NH_4^+ degradation curves (Figure 2A), the metagenomic analysis of these MBBs led to comparable results in terms of bacterial species identified, except for slight differences in their relative abundance. As seen in Figure 5A,B, at the family level, Chitinophagaceae, Comamonadaceae, Sphingomonadaceae, and Nitrosomonadaceae substantially increased over time, becoming the most prevalent in both G5-2 and G5-4 biofilms. In particular, Chitinophagaceae, which did not exceed 1.5% in liquid bacterial cultures and newly formed MBBs (the 1st day), reached 13% and 9% for G5-2 and 25% and 15% for G5-4 on the 10th and 15th day, respectively. Similarly, the Nitrosomonadaceae fraction, which was less than 1% in the first stages of biofilm development (on the 1st day), became 16–18% in both MBBs after 10 days. Comamonadaceae and Sphingomonadaceae changed to a lower extent in G5-2 and G5-4 samples, showing a two- to five-fold increment in structured biofilms (on the 10th and 15th days) as compared with planktonic growth cells and MMBs on the 1st day. By contrast, Alcaligenaceae and Xanthomonadaceae families, which were strongly predominant ($\cong$10–30% of all OTUs) in the early points of Bio-RRIA, dramatically decreased in MBBs for longer times (abundance $\leq$ 5% on the 10th and the 15th days). Notably, except for Chitinophagaceae, the bacterial families identified in our study were also found by Garcia et al. [40], who investigated in bench-scale MBB bioreactors how size and geometry of plastic supports affect biofilm structure, function, and relative microbial species abundance. Most of these families include many heterotrophic nitrifying bacteria, and Alcaligenaceae and Comamonadaceae, particularly, have been shown to perform simultaneous nitrification/denitrification in MBB reactors [41]. The biofilm composition at the genus level of G5-2 and G5-4 samples is reported in Supplementary Figure S4.

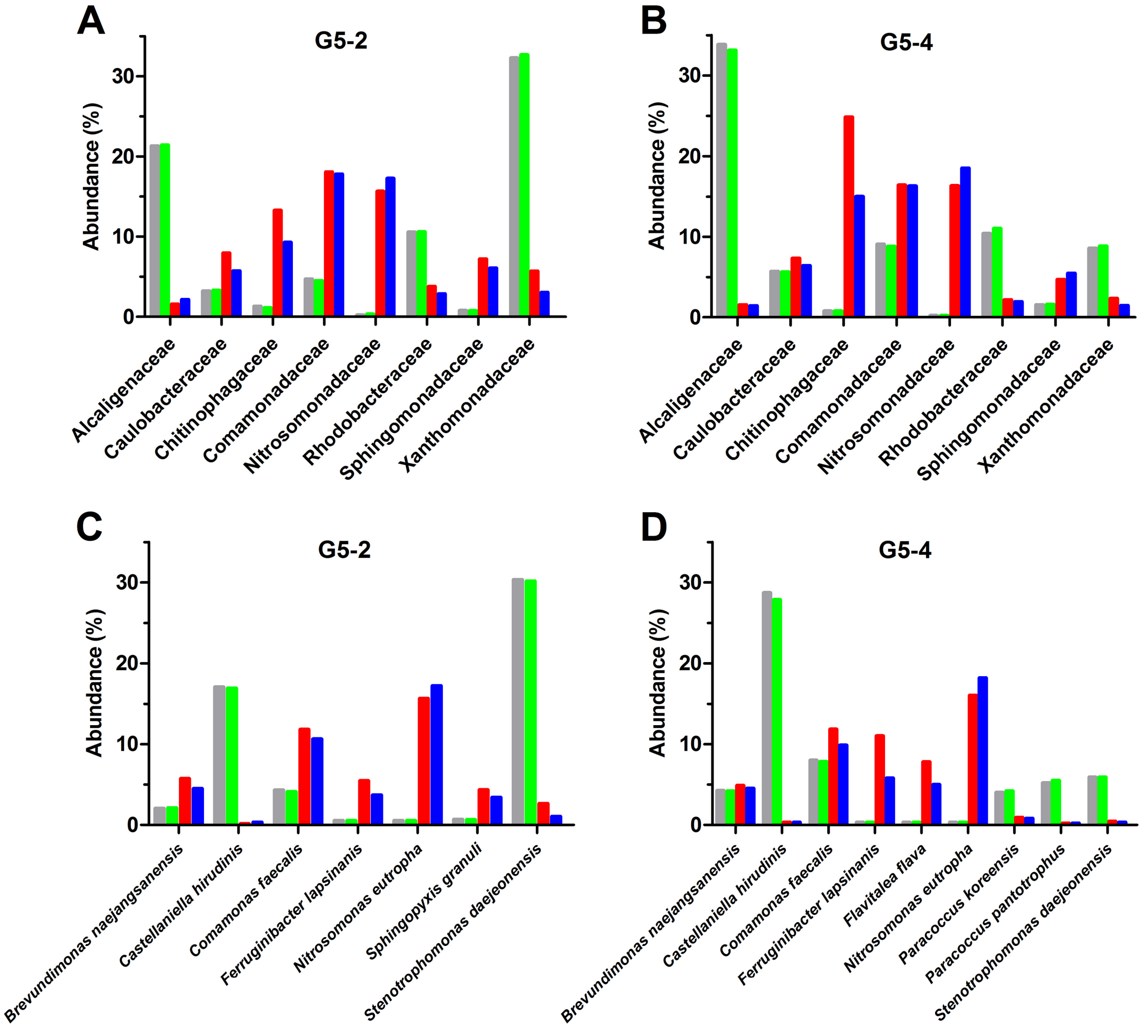

Figure 5. Change of biofilm population from selected microbial communities. Relative abundance, expressed as a percentage of total operational taxonomic units (OTUs), of the most prevalent families (**A**,**B**) and species (**C**,**D**) in the biofilms derived from G5-2 and G5-4 cultures are shown. Samples for species identification by 16S rRNA NGS were the bacterial cultures used for biofilm formation (gray bars) and MBBs taken on the 1st day (green bars), on the 10th day (red bars), and on the 15th day (dark blue bars) of Bio-RRIA reported in Figure 2A. The bar graph shows only families and species that contributed more than 4% to the total bacterial community in at least one point.

Consistent with the taxa distribution at family and genus levels, we identified three principal bacterial species that exhibited a huge increase in frequency at the more advanced stages of biofilm formation and were reasonably responsible for the progressive increment of the nitrification process (Figure 5C,D). Primarily, *Nitrosomonas eutropha*, one of the most investigated AOBs, initially at the limit of detection, reached 18% of all OTUs in both G5-2 and G5-4 MBBs on the 15th day. *Nitrosomonas* sp. was found to tolerate elevated NH_4^+ concentrations [42], and numerous studies indicated that this bacterium plays a key role in NH_4^+ degradation in different types of biofilm reactors for wastewater treatment [43–47]. Remarkably, *N. eutropha* is an autotrophic species that oxidizes ammonia in the presence of oxygen. In this respect, MBBs utilized in our Bio-RRIAs were operating in aerobiosis but in heterotrophic conditions due to the huge quantity of organic matter contained in leachate. Thus, the successful proliferation of *N. eutropha* can be explained by the fact that organized biofilms constitute a physicochemical barrier which, by allowing a selective penetration of nutrients (i.e., oxygen, inorganic carbon, and nitrogen compounds), creates an optimized micro-environment for growth and activity of this autotrophic nitrifier. Furthermore, a considerable biomass accumulation was observed on the 10th also for two genera of Chitinophagaceae. In fact, *Ferruginibacter lapsinanis* came up to 6% and 11% on G5-2 and G5-4, respectively, and *Flavitalea flava* to 8% on G5-4. Chitinophagaceae includes many heterotrophic AOB that oxidize ammonia using as an energy source the organic matter contained in leachate, and it was one of the most represented families identified by Wang et al. [48] studied for several months the nitrogen removal in a pilot-scale reactor. Altogether, our results suggest that, as discussed above, G5-2 and G5-4 biofilms, unlike planktonic cells, can provide multilevel metabolic environments that permit concomitant autotrophic/heterotrophic nitrification. Finally, *Comamonas faecalis*, although identified at all times, reached 12% on the biofilms after 10–15 days, possibly leading to the conversion of nitrate, first to nitrite, and then, although at a low extent, to gaseous nitrogen compounds. In fact, the denitrification activity of bacteria belonging to this genus has been known for a long time [49], and recently, it has been demonstrated in removing several pollutants that produce NO_2^- and NO_3^- as by-products of their degradation [50–53]. Unexpectedly, the *Nitrospira* and *Nitrobacter* genera, including canonical nitrite-oxidizing bacteria and generally prevalent in activated sludges from MWWTPs [54–56], were not identified across all MBBs analyzed in this study. The lack of these NOBs is possibly due to the unfavorable conditions (high concentrations of ammonium and organic compounds) used in biofilm formation and to the competition with heterotrophic species. Finally, other clades, such as *Castellaniella hirudinis*, *Paracoccus koreensis*, *Paracoccus pantotrophus*, and *Stenotrophomonas daejeonensis*, were considerably represented at the starting points, while became almost undetectable for longer times of Bio-RRIA. In particular, the drastic OTUs fall of *C. hirudinis* and *S. daejeonensis* accounted for the disappearance, at the family level, of Alcaligenaceae and Xanthomonadaceae, respectively (Figure 5).

It is well established that, in most cases, the poor performance of municipal bioreactors is caused by significant alterations of the microbial population of activated sludges in terms of bacterial species and their relative abundances. Thus, we correlated the ammonia degradation efficiency of SL2, SL3, and SL4 biofilms derived from the Porto Sant'Elpidio MWWTP sludge with their taxa distribution as determined using the metagenomic analysis. As evident in Figure 6, at the family level, SL3 MBB was quite dissimilar from the other two sludge biofilms. Specifically, SL3 MBB, which was very effective in NH_4^+ removal (Figure 4), exhibited the uncommon prevalence (≅16% of all the OTUs) of Zoogloeacea and the almost complete lack (<1%) of Nitrosomonadaceae. Zoogloeacea was not detected either in sludge SL2 and SL4 MBBs or in G5-2 and G5-4 MBBs formed from NH_4^+-selected microbial communities. Nevertheless, Zoogloeacea accounted for 2.6% of total bacterial communities of activated sludges, as shown by metagenomic analyses carried out in 14 MWWTPs in Asia and North America [57]. Notably, the two genera *Zoogloea* and *Thauera* represented the totality of members of this family in SL3 biofilm, being 12% and 4%, respectively, as estimated by metagenomic analysis. *Zoogloea* sp. is a heterotrophic/aerobic denitrifier that performs nitrogen removal when cultured in the presence of nitrates and nitrites. In addition, *Zoogloea*. sp. was also found to exhibit a considerable nitrification capacity (NH_4^+ removal rate of 44%) without nitrate and nitrite production when it was grown in a high ammonia-based medium [58]. Thus, the predominance of *Zoogloea* can account for the elevated ammonia oxidation potential of SL3 MBB in the absence of *Nitrosomonas* sp. The other two families, the Chitinophagaceae and Comamonadaceae, which accumulated in G5-2 and G5-4 biofilms, were identified at considerable levels, as well as in sludge MBBs. In particular, Chitinophagaceae, constituted by the only two genera *Ferruginibacter* and *Flavitalea*, became the most abundant family in SL2 (≅10%) and SL4 (≅16%) MBBs, whereas Comamonadaceae was equally represented (≅4–6%) across all sludge biofilms (Figure 6). The possible role of these taxa in bioremediation has already been discussed relative to G5-2 and G5-4 MBBs. As described in Section 3.2, the SL4 biofilm had a reduced efficiency in ammonia degradation (Figure 4D) according to the fact that sludge sampling was done when the Porto Sant'Elpidio WWTP was not well-performing, and an abundant foam was produced during the wastewater treatment. Notably, SL4 MBB contained considerable levels of two genera, *Gordonia* (4.5%) and *Rubinisphaera* (4.7%), which were not identified in the other sludge biofilms. While very little is known about *Rubinisphaera* (Planctomycetaceae), several members of *Gordonia* (Gordoniaceae) display a powerful catabolic activity in degrading hazardous pollutants such as organic substances, hydrocarbons, sulfur compounds, and rubber. The selection of this genus might have been caused by the peculiar and transient conditions related to the malfunctioning of the Porto Sant'Elpidio WWTP at the sampling time. Currently, *Gordonia* is emerging as a promising candidate, particularly in microbial consortia, to be used in targeted bioremediation techniques [59]. According to the SL4 sample, *Gordonia* strains were detected in stable foams formed in wastewater treatment plants [60,61]. Foams denote the suffering of the bioreactor and usually create, as observed for the Porto Sant'Elpidio WWTP, serious operating problems. In addition, SL4 MBB showed a lower content than SL2 MBB (6% vs. 9%) of Nitrosomonadaceae, which could be another reason that somewhat explains the malfunctioning of this sludge biofilm in ammonia oxidation. These findings provide further evidence that the bacterial composition of sludge biofilms strongly affects the nitrification process. Thus, monitoring the sludge microbial community can help to predict with a certain advance and eventually act to limit the possible malfunctioning of the bioreactor.

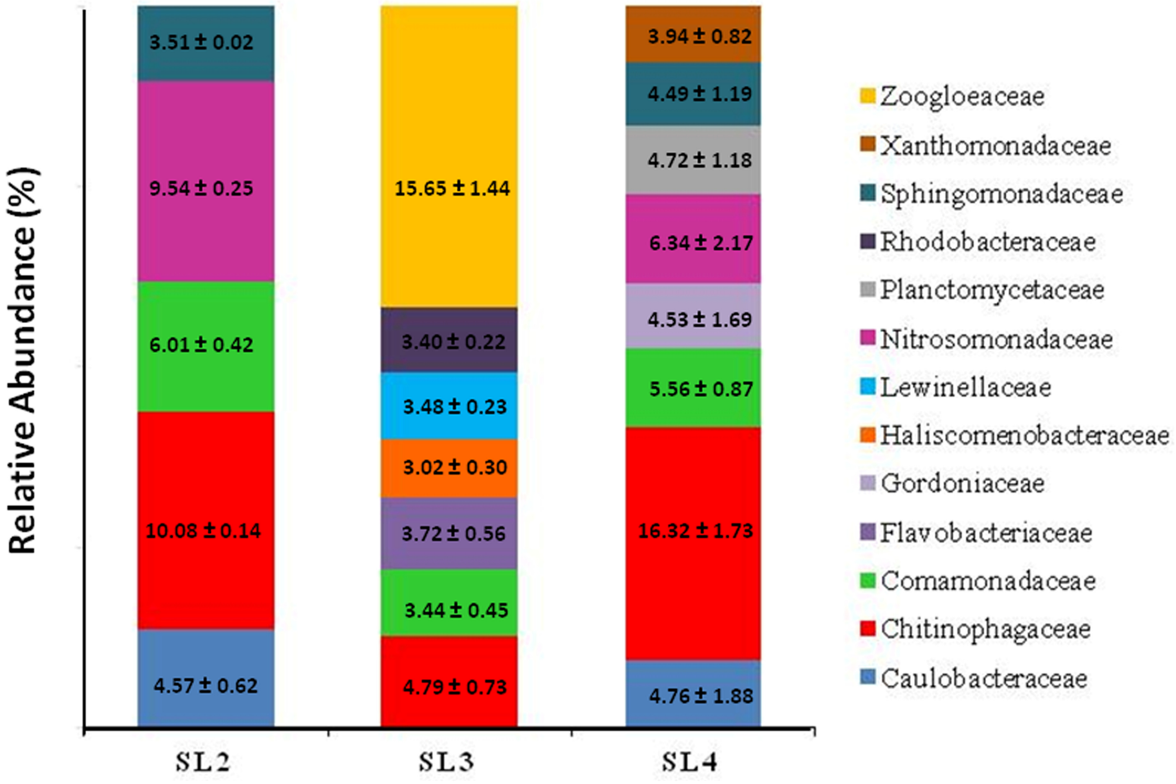

Figure 6. Stacked bar chart of MBBs from native sludges. Relative abundance, expressed as a percentage of total operational taxonomic units (OTUs), of the most prevalent families in biofilm-derived sludges SL2, SL3, and LSL4. Bacteria identification was carried out using 16S rRNA NGS, and values represent the average ± standard deviation of at least three points taken during Bio-RRIA experiments, as shown in Figure 4. Only families that contributed more than 3% to the total bacterial community are reported. Analysis of variance across the three samples shows a statistically significant difference in their microbial composition ($p < 0.05$).

4. Conclusions

The present study represents an advancement of our recent work [24] in which, through multiple ammonia stresses (Repetitive Re-Inoculum Assay), we strongly enriched the bacterial community of activated sludges to produce an optimized mixture of microorganisms retaining high efficiency in nitrogen removal from landfill leachate. Herein, we proved, using two different techniques (CVS and CLSM), that these high ammonia-selected microbial populations developed stable and organized biofilms on plastic carriers. Importantly, these bio-carriers (G1-2, G5-2, and G5-4 MBBs), after an initial adaptation phase, achieved a very good ammonia removal rate (≅80% in 24 h) in leachate-based medium with high-strength ammonium (≅360 mg/L). Metagenomic analysis revealed that four families, Chitinophagaceae (13–25% represented by the two genera *Ferruginibacter* and *Flavitalea*), Nitrosomonadaceae (16–18%, genus *Nitrosomonas*), Comamonadaceae (16–18%, genus *Comamonas*) and Sphingomonadaceae (5–7%, genus *Sphingopyxis*) accumulated in biofilms at advanced stages of development suggesting their crucial role in the nitrification process. Basically, MBBs exhibited a 10-fold higher activity in NH_4^+ degradation than respective planktonic growth bacteria due to the biofilm advantage of enabling a synchronous action of autotrophic and heterotrophic nitrifying bacteria.

Therefore, the performance of the Porto Sant'Elpidio MWWTP was monitored for more than 2 years, and MBBs were produced from the native sludge of this bioreactor. These biofilms evidenced huge variations in activity and reduced NH_4^+ degradation as compared with G1-2, G5-2, and G5-4 MBBs. Intriguingly, the most active sludge biofilm obtained (SL3) was characterized by an ammonia removal rate of 78% in 3 days and by a quite rare predominance (16%) of the Zoogloeaceae family. These results, particularly those achieved with the ammonia selection-derived MBBs, are very encouraging, and we are planning to quickly transfer this MBB-based technology initially to the pilot reactor (2000 L) available at the Eco Elpidiense Company and, subsequently, to the Porto Sant'Elpidio plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12122404/s1, Table S1. Microbial composition at family level of the high ammonia-selected bacterial populations G5-2 and G5-4; Table S2: Average composition of pre-treated leachates used in our experiments; Figure S1: Crystal Violet Staining; Figure S2: Bio-Repetitive Re-Inoculum Assay by G1-2 G5-2 and G5-4 MBBs; Figure S3: Bio-Repetitive Re-Inoculum Assay by sludge MBBs; Figure S4: Change of biofilm population from the ammonia-selected microbial communities G5-2 and G5-4.

Author Contributions: Conceptualization, R.P., A.F., K.P., G.P., V.N. and M.F.; funding acquisition, M.F.; investigation, R.P. and M.F.; methodology, R.P.; project administration, G.P. and M.F.; supervision, A.F., G.P. and M.F.; writing—review and editing, A.F., K.P., G.P., V.N. and M.F. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: Authors Kathleen Pucci and Graziella Pagliaretta were employed by the company Eco Elpidiense s.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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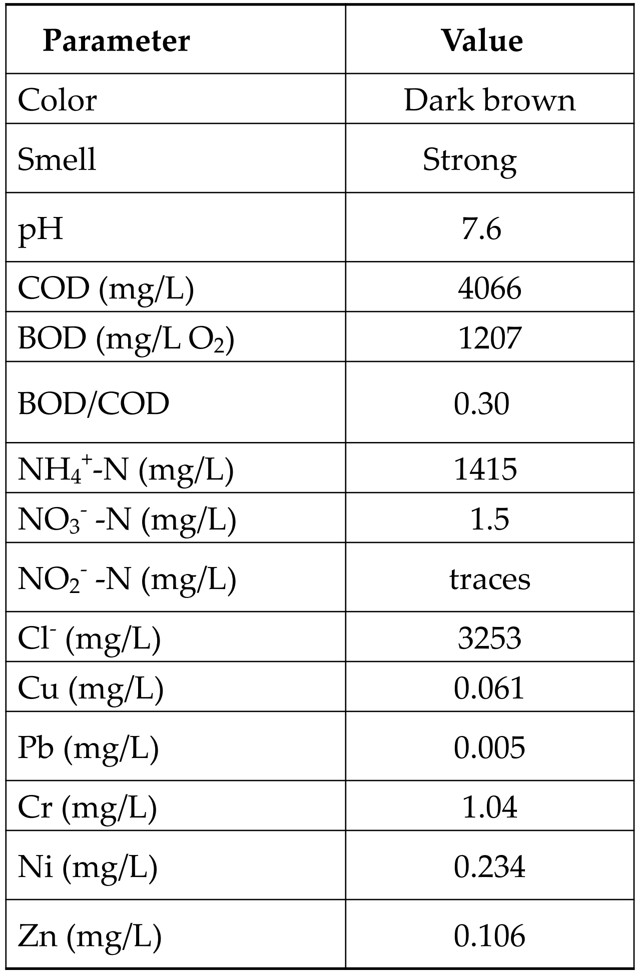
FAMILY	G5-2 (%)	G5-4 (%)
Xanthomonadaceae	32.30	8.54
Alcaligenaceae	21.31	33.81
Rhodobacteraceae	10.53	10.38
Bradyrhizobiaceae	6.96	6.16
Rhizobiaceae	4.75	4.34
Comamonadaceae	4.68	9.06
Brucellaceae	4.35	-
Caulobacteraceae	3.20	5.67
Phyllobacteriaceae	2.53	3.98
Hyphomicrobiaceae	2.50	6.61
Microbacteriaceae	1.98	4.00
Chitinophagaceae	1.28	0.78
Micrococcaceae	0.93	1.31
Sphingomonadaceae	0.75	1.52
Isosphaeraceae	0.49	-
Nitrosomonadaceae	0.2	0.2
Gordoniaceae	0.1	0.69
Chelatococcaceae	-	1.34
Nocardiaceae	-	0.29
Other	1.18	1.32

Table S1. Microbial composition at family level of the high ammonia-selected bacterial populations G5-2 and G5-4.



Parameter	Value
Color	Dark brown
Smell	Strong
pH	7.6
COD (mg/L)	4066
BOD (mg/L O_2)	1207
BOD/COD	0.30
NH_4^+-N (mg/L)	1415
NO_3^- -N (mg/L)	1.5
NO_2^- -N (mg/L)	traces
Cl^- (mg/L)	3253
Cu (mg/L)	0.061
Pb (mg/L)	0.005
Cr (mg/L)	1.04
Ni (mg/L)	0.234
Zn (mg/L)	0.106

Table S2. Average composition of pre-treated leachates used in our experiments.

C **G5-4** **G1-2** **SL**

0.3 ± 0.04 **1.9 ± 0.03** **1.7 ± 0.05** **1.9 ± 0.05**

Figure S1. Crystal Violet Staining. K-Kaldnes bio-carriers from control no cells (C), selected bacterial species (G5-4 and G1-2) and activated sludge (SL) were stained with crystal violet dye as described in *Materials and Methods.* The Optical density (OD_{600}) of the colored ethanol solution was measured for the four bio-carriers of each sample and the mean ± standard deviation are reported.

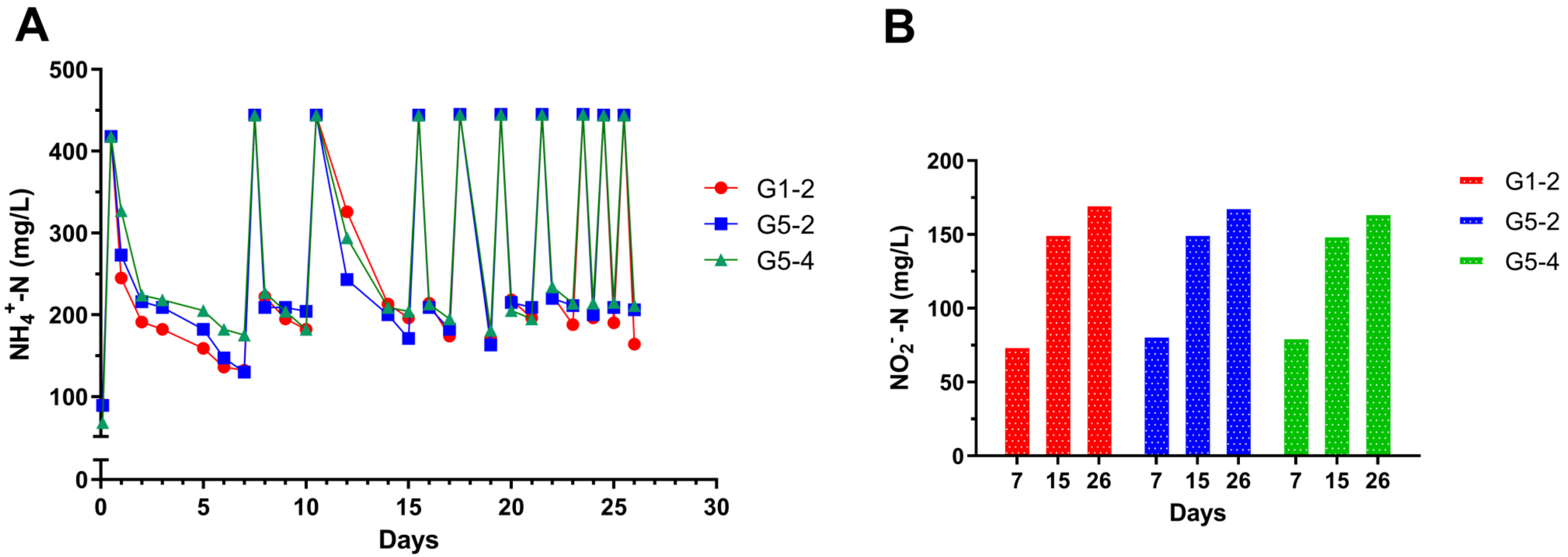


Figure S2. Bio-Repetitive Re-Inoculum Assay by G1-2 G5-2 and G5-4 MBBs. A. The experiment has been carried out as described in **Figure 3A** except for the higher NH_4^+-N concentration (425 mg/L). The three biofilms G1-2, G5-2 and G5-4 were tested. **B.** Samples from panel A were withdrawn at indicated times for NO_2^- determination.

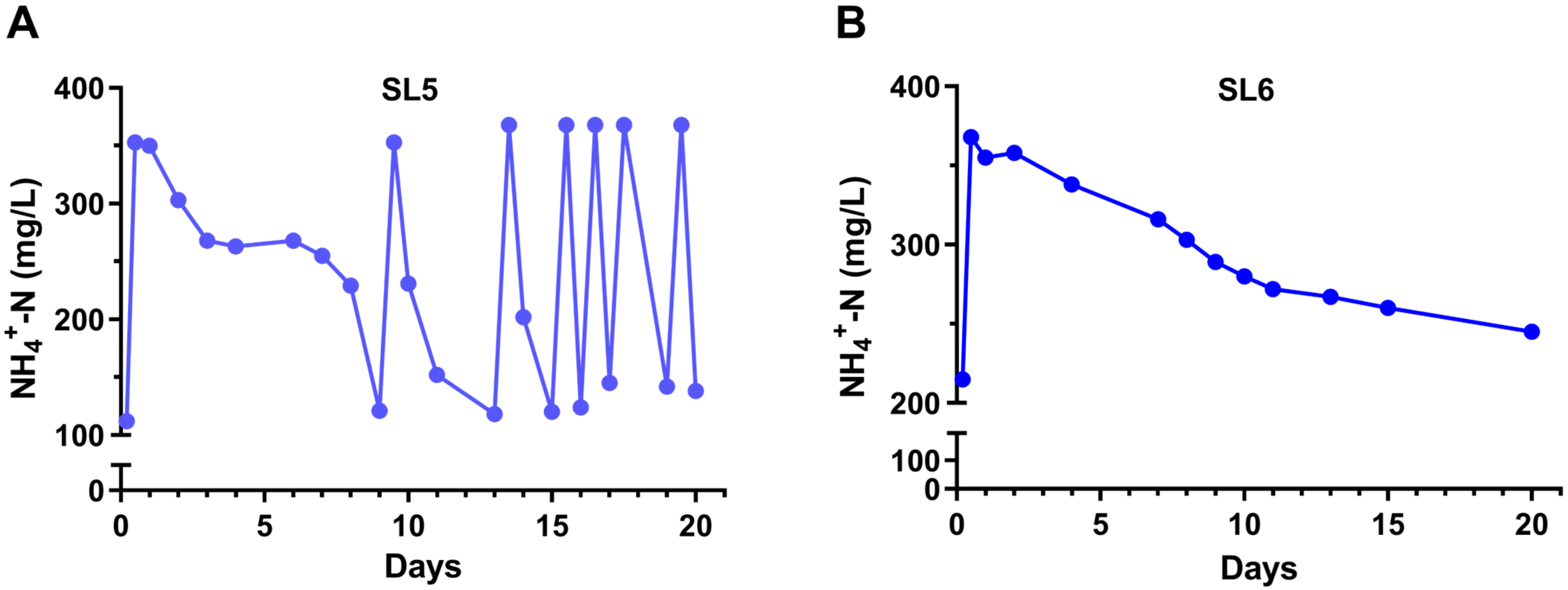


Figure S3. Bio-Repetitive Re-Inoculum Assay by sludge MBBs. Sludge samples (SL5 and SL6) were taken at different periods from the Porto Sant'Elpidio municipal WWTP for bio-carriers preparation. The ammonia removal by biofilms were investigated in Bio-RRIA experiments essentially as described in the legend of **Figure 5.**

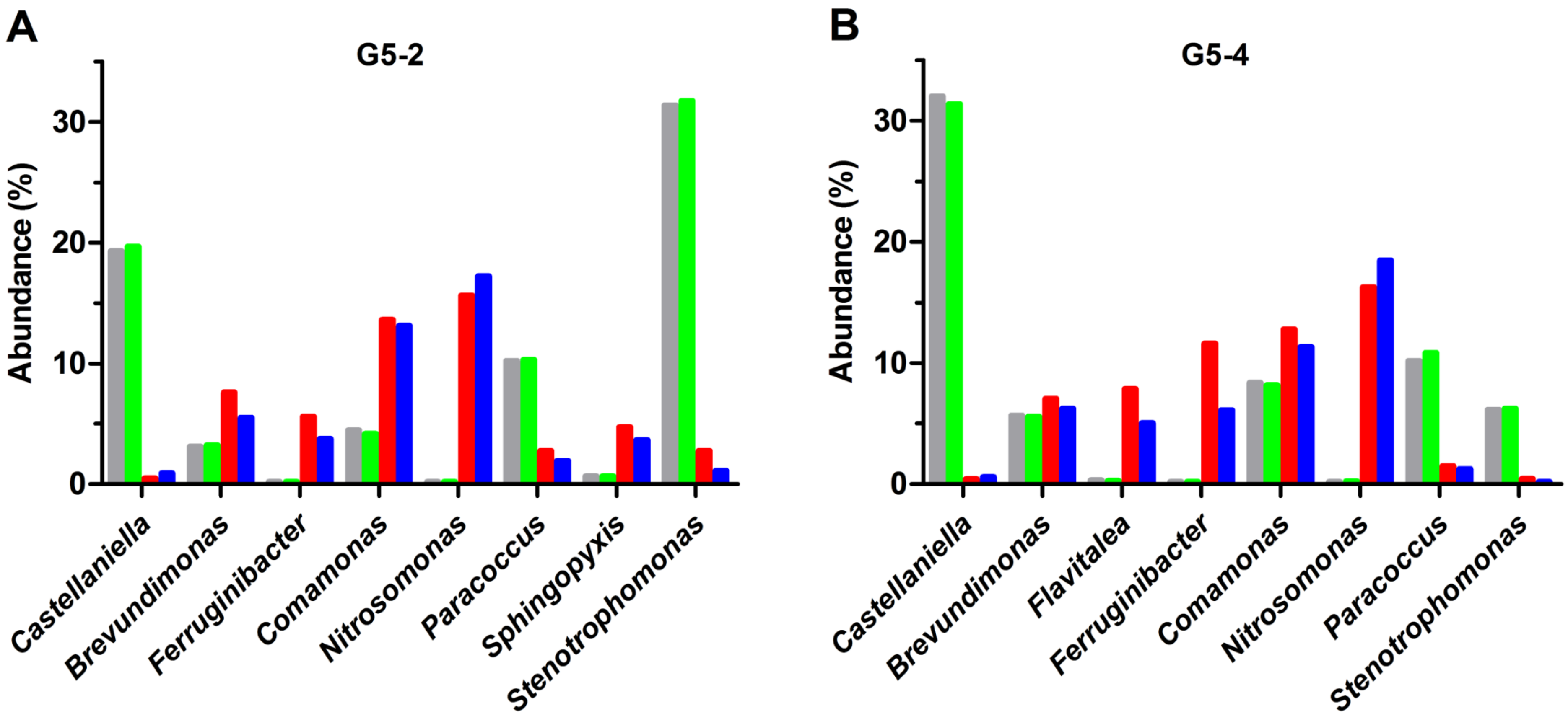


Figure S4. Change of biofilm population from the ammonia-selected microbial communities G5-2 and G5-4. Relative abundance (%) of the most prevalent genera in the biofilms derived from G5-2 (**A**) and G5-4 (**B**) essentially as described in the legend of **Figure 5**. Samples were the bacterial cultures used for biofilm formation (grey bars) and MBBs taken on the 1st day (green bars), on the 10th day (red bars) and on the 15th day (dark blue bars) of Bio-RRIA. The bar graph shows only genera that contributed more than 4% to the total OTUs in at least one point.

5. Analysis of Autotrophic Ammonium- and Nitrite-Oxidizing Bacteria in Activated Sludge Using Confocal Microscopy and Transmission Electron Microscopy (TEM)

An additional key step in this research project was selecting, analyzing, and monitoring the growth of autotrophic ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in three aliquots of activated sludge collected from the Porto Sant'Elpidio wastewater treatment plant.

The goal was to investigate the dynamics and ecological roles of these bacteria in a real-world setting, where they are crucial for the nitrogen cycle in wastewater treatment processes.

For this purpose, a combination of confocal microscopy and Transmission Electron Microscopy (TEM) was employed, enabling detailed imaging of both the spatial distribution and ultrastructural characteristics of the bacterial populations (Figure 2).

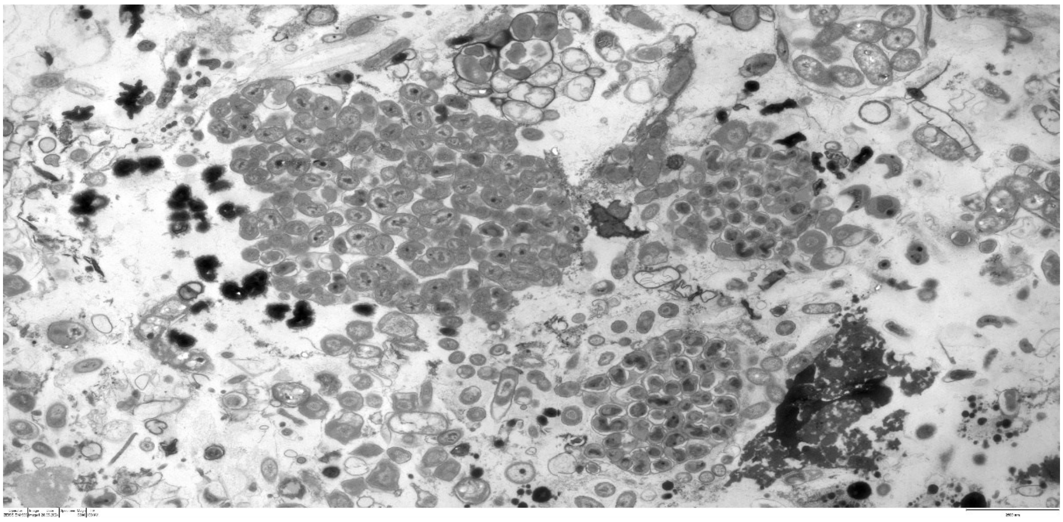


Figure 2. TEM images showing activated sludge map. Image taken show groups of ammonium-oxidizing and nitrite-oxidizing bacteria, characterized by their typical spiral shapes and by the presence of an increased number of internal membranes. However, in smaller quantities, there are filamentous environmental bacteria that are not involved in the nitrogen cycle. This parameter checks healthy and quality of the sludge.

Confocal microscopy was used to monitor the spatial arrangement and interactions of AOB and NOB within the activated sludge flocs. This technique provided three-dimensional images of microbial communities, allowing precise tracking of bacterial localization and their interactions within the complex microbial matrices (Figure 3). On the other hand, TEM offered high-resolution imaging at the nanoscale, providing insights into the cellular architecture of bacteria. This allowed for the observation of intracellular structures, such as specialized membranes and enzyme complexes involved in ammonia and nitrite oxidation (Figure 4). To ensure selective growth and enrichment of target microbial populations, specific selective media were used for AOB and NOB. AOB were enriched using media containing ammonium as the sole nitrogen source, promoting the growth of ammonia-oxidizing species.

Similarly, NOB were selectively grown on media containing nitrite as the primary electron acceptor, ensuring that only nitrite-oxidizing bacteria could thrive. The use of these selective media was essential for isolating and studying these two groups of autotrophic bacteria, ensuring that their metabolic activities could be accurately assessed and monitored under controlled conditions. By combining these techniques and selective growth conditions, a deeper understanding of the microbial dynamics within the wastewater treatment plant was gained, focusing on the role of AOB and NOB in the nitrification process. This research contributes to a better understanding of how these bacteria function in nitrogen removal and how operational parameters such as oxygen levels and nutrient availability can be optimized to improve the performance of wastewater treatment systems.

Additionally, the findings from this study have potential applications for improving the management and efficiency of wastewater treatment processes, especially in systems where nitrogen removal is a key concern.

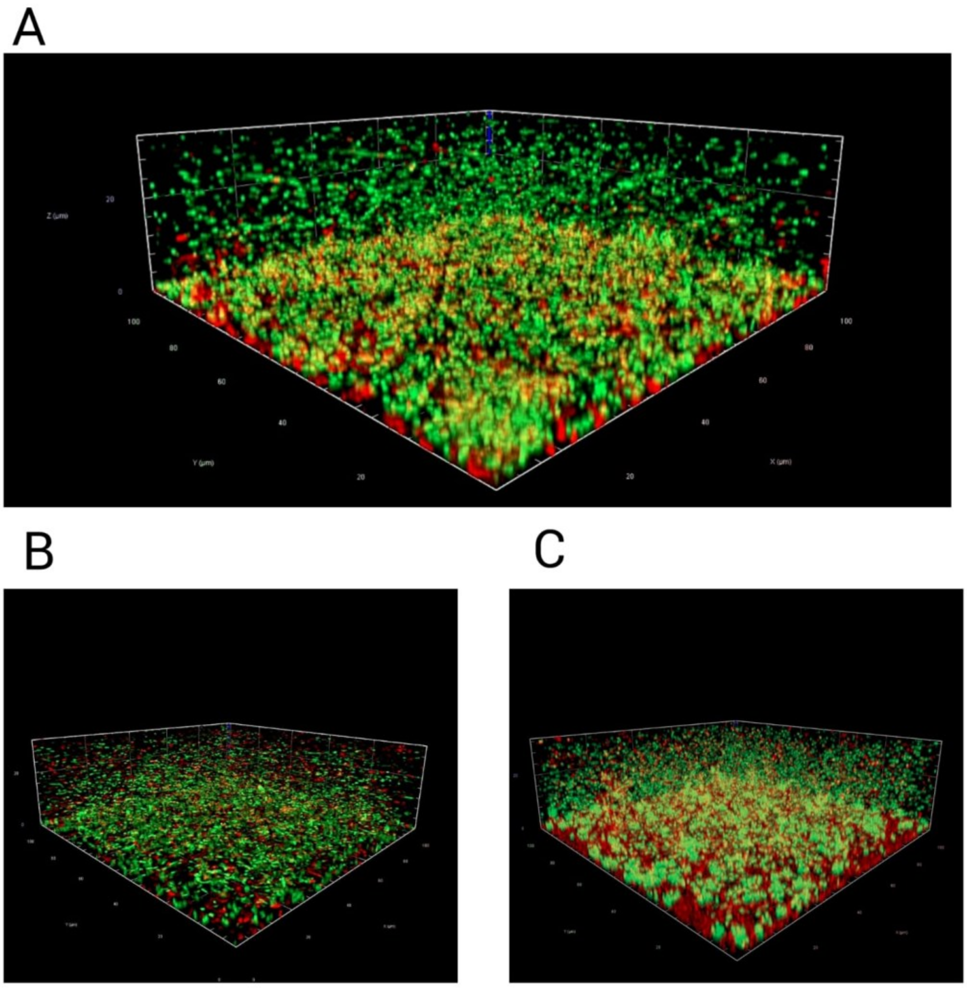


Figure 3. Confocal microscopy images. Photos show an activated sludge sample (A), AOB bacteria (B) and NOB bacteria (C). Both dyes intercalate with nucleic acids but, while SYTO9 penetrates both living and dead cells, PI can only pass through damaged membranes displaying SYTO9. This allows differentiation between live (green) and dead (red) cells.

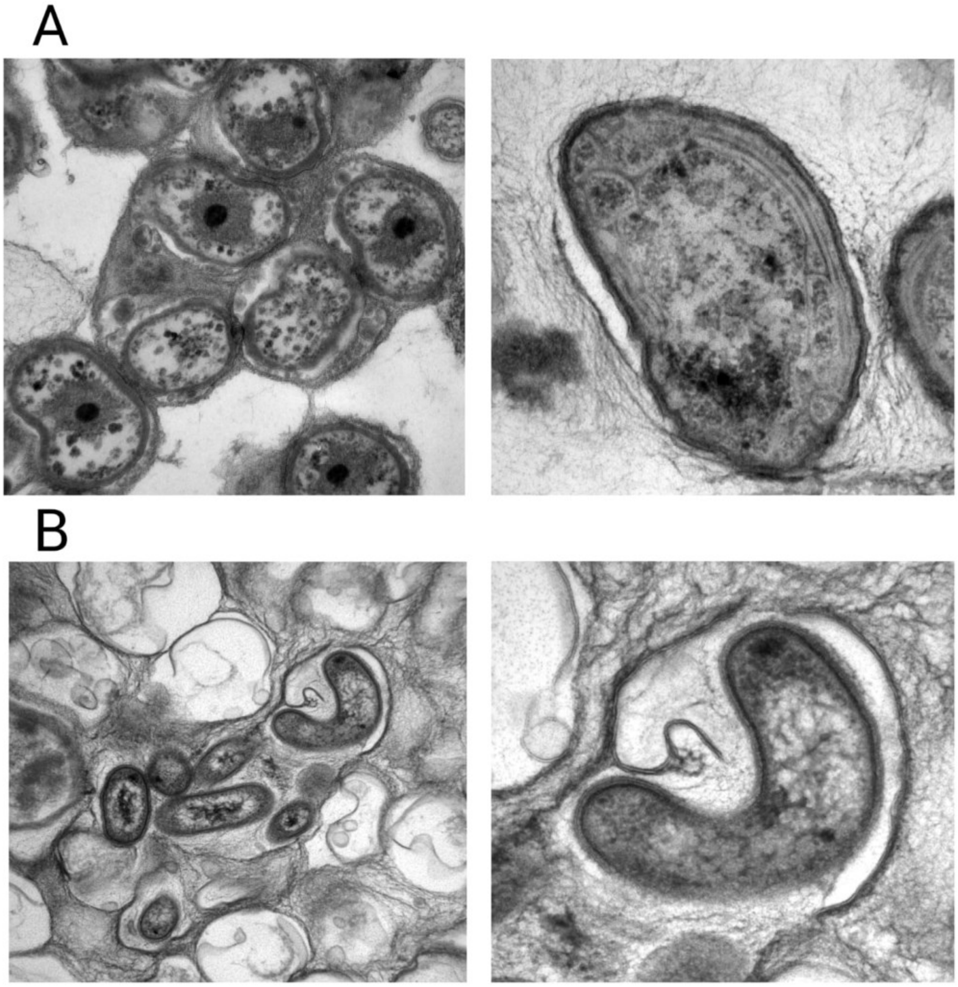


Figure 4. Electron micrographs of AOB (A) and NOB bacteria (B).

6. Discussion

This research addressed one of the main challenges in wastewater treatment: the effective and sustainable removal of ammonium, with a particular focus on complex effluents such as landfill leachates characterized by very high ammonium concentrations. This study adopted an innovative approach based on the selection of specific microbial communities from activated sludge and their subsequent fixation on K1 plastic carriers, combining advanced microbiological knowledge with cutting-edge engineering technologies.

In the first phase, activated sludge samples collected from the Porto Sant'Elpidio treatment plant were used as a starting point for identifying bacterial communities essential for nitrification and denitrification processes. Using an experimental procedure named "Repetitive Re-Inoculum Assay" (RRIA) that consists of multiple ammonia stress in a leachate-based medium, the original microbial community of the activated sludge was selectively enriched. This ensures that selected species are able to survive and to efficiently remove ammonium even under concentrations which typically inhibit biological processes in conventional activated sludge systems.

NGS data of RRIA-subjected population revealed that seven bacterial families (Alcaligenaceae, Nitrosomonadaceae, Caulobacteraceae, Xanthomonadaceae, Rhodanobacteraceae, Comamonadaceae and Chitinophagaceae) were able to grow in such leachate-medium and successfully oxidated ammonia.

Notably, we found that the bacterial species *Klebsiella sp.*, *Acinetobacter sp.*, and *Castellaniella sp.* demonstrated a strong ability to convert ammonium directly into nitrogen gas. This reduces the accumulation of intermediates such as nitrites and nitrates, often linked to negative environmental impacts.

The selected microbial communities were then integrated into a Moving Bed Biofilm (MBB) system, where the biofilm was formed on K1 plastic carriers-high-density polyethylene cylinders ensuring an extensive surface area for microbial growth. The stable fixation of bacteria on these carriers aimed to overcome one of the primary limitations of traditional biological systems: biomass washout.

In controlled laboratory conditions, selected MBBs demonstrated the ability to remove up to 80% of ammonium within just 24 hours, exhibiting a 10-fold higher activity in NH_4^+ degradation than respective planktonic growth bacteria. Metagenomic analysis indicated that four families, Chitinophagaceae, Nitrosomonadaceae, Comamonadaceae and Sphingomonadaceae accumulated in biofilms at advanced stages of development suggesting their crucial role in the nitrification process. In fact, a key factor of MBBs success was the stratified structure of the biofilm itself, which allowed for a synchronous action of autotrophic and heterotrophic nitrifying bacteria. This MBBs feature is particularly advantageous for treating pollutants with low carbon-to-nitrogen ratios, as is typical of landfill leachates.

The use of advanced techniques, such as confocal microscopy and transmission electron microscopy (TEM), further supported the research by providing detailed insights into the distribution and organization of microbial communities within structured biofilms.

7. Conclusions

In this study, we identified a multi-species (Chitinophagaceae, Nitrosomonadaceae, Comamonadaceae and Sphingomonadaceae) biofilm with a very high power in NH_4^+ degradation demonstrating that selected microbial communities forming MBBs represent a highly effective approach for ammonium removal from wastewater and in particular for landfill leachate treatment. In addition, our results confirmed the feasibility of overcoming the limitations of traditional methods, offering a sustainable and adaptable solution for diverse operational contexts. Beyond improving the management of ammonium toxicity in wastewater plants, this research lays the foundation for the development of more efficient systems capable of addressing complex environmental challenges.

8. Prospective Future

The future development of this work focuses on three main directions:

1. **Optimization of materials and technologies**:
   Research on new materials for biofilm carriers could further enhance the system's efficiency, providing improved stability and microbial adhesion properties.
2. **Deepening the understanding of microbial dynamics**:
   Metagenomic studies could provide a deeper understanding of interactions within a multicomponent biofilm, enabling the optimization of microbial community composition for specific operational needs.
3. **Industrial-scale implementation**:
   The technological transfer to pilot-scale systems would allow the validation of the proposed approach under real-world conditions in particular for leachate treatment. Additionally, assessing the economic and environmental impacts of the system on a larger scale would be crucial for its broader application.

With these advancements, the proposed system has the potential to become a reference solution for improving wastewater treatment, protecting the environment, and ensuring more sustainable water resource management.

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