The impact of lead pollution and *Azospirillum brasilense* Sp7 inoculation in lemongrass cultivation on soil nitrogen cycling and microbial communities

（鉛汚染及び *Azospirillum brasilense* Sp7 株の接種を伴う
レモングラスの植栽が土壌中の窒素循環と微生物群集に及ぼす影響）

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The impact of lead pollution and *Azospirillum brasilense* Sp7 inoculation in lemongrass cultivation on soil nitrogen cycling and microbial communities

By

Akira Nagata

Soil heavy metal contamination is a serious environmental issue observed worldwide. It accumulates in the environment through various pathways, including mining activities, industrial waste incineration, exhaust gas emissions, disposal of mining waste, and excessive use of chemical fertilizers and pesticides. Heavy metals such as lead (Pb) are non-biodegradable, lack biological functions, and exhibit high biotoxicity. Additionally, heavy metal-contaminated soil not only leads to a decline in ecosystem functions and loss of vegetation but also poses health hazards due to the dispersal of contaminated topsoil. Therefore, in order to remediate heavy metal-contaminated soil, it is essential to improve soil fertility while simultaneously removing heavy metals. The nitrogen cycle is an ecosystem function that is closely related to soil fertility. Due to its high sensitivity, it has also been used as an indicator for assessing environmental impacts caused by heavy metals. However, when evaluating the nitrogen cycle under heavy metal contamination, there are only a limited number of studies that have assessed both the levels of inorganic nitrogen compounds and the abundance of functional genes. Moreover, many studies have focused on specific reactions or genes, leaving uncertainties regarding how the overall nitrogen cycle is affected simultaneously. Recently, studies combining the inoculation of plant growth-promoting microorganisms (PGPMs) and heavy metal-tolerant plants have gained attention as a low-cost and environmentally friendly remediation strategy for heavy metal contamination. However, the types of microorganisms used for inoculation and the inoculation methods vary across studies, making it necessary to identify the most effective inoculation methods tailored to specific plant species. Furthermore, while many studies focus on plant growth, it has been emphasized that their impact on the soil environment and ecosystem functions should also be evaluated. Lemongrass is gaining

research attention as a heavy metal-tolerant plant with high economic value and growth potential. However, compared to well-studied heavy metal-tolerant plants such as those in the Brassicaceae and Asteraceae families, information on lemongrass remains limited, and studies on microbial inoculation under heavy metal contamination are scarce. Based on these issues, this study aims to: (1) Conducting a comprehensive assessment of the nitrogen cycle under Pb contamination by measuring both inorganic nitrogen levels and the abundance of functional genes. (2) Evaluating the effects of microbial inoculation and lemongrass cultivation on the nitrogen cycle under Pb contamination. (3) Assessing how different inoculation methods influence the nitrogen cycle and lemongrass growth. In the first experiment, a soil incubation study was conducted to investigate the temporal changes in denitrification activity and microbial communities in response to rapid Pb exposure. Four Pb treatment levels (0, 200, 400, 1600 mg Pb/kg soil) were applied, with or without nitrate addition. The results suggested that 1600 mg Pb/kg soil inhibited NO_3^- and NO_2^- reduction and increased N_3O emissions. The *narG* gene, which encodes NO_3^- reductase, significantly decreased under 1600 mg Pb/kg soil, whereas the *nirS* gene, which encodes NO_2^- reductase, showed no clear correlation with Pb contamination. Following Pb exposure, 16S rRNA gene abundance tended to decrease. However, the Shannon diversity index showed an increasing trend. In addition, exposure to 1600 mg Pb/kg soil resulted in a rapid decline of the dominant Proteobacteria. Furthermore, Pb contamination exceeding 400 mg Pb/kg soil led to a rapid reduction of Azotobacter to less than 50% of the control. In the send experiment, to evaluate the impact of microbial inoculation on the nitrogen cycle under Pb contamination, an eight-week cultivation experiment was conducted using *Azospirillum brasilense* Sp7 as the PGPM and lemongrass as the heavy metal-tolerant plant. Three inoculation methods were applied: seed inoculation, root inoculation, and a combination of both, to compare the effects of different inoculation strategies. After eight weeks of cultivation, it was suggested that the combined seed and root inoculation method resulted in the highest leaf and root growth. In contrast, root-only inoculation appeared to suppress leaf and root growth. Additionally, by the fourth week of cultivation, the combined seed and root inoculation led to the highest abundance of *nxrB*, *amoA, nirK*, *nirS*, *norB*, *nosZ*, and 16S rRNA genes compared to other treatments, with some genes showing significantly higher levels than the control.

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Chapter 1: General Introduction

1.1Heavy metal pollution

Heavy metal contamination is an environmental issue observed worldwide, posing severe adverse effects on both biological organisms and the natural environment. Consequently, prompt remediation efforts are required. Heavy metals enter the environment through various pathways, including mining activities, industrial waste incineration, exhaust gas emissions, disposal of mining waste, and excessive use of chemical fertilizers and pesticides (Defarge, Spiroux de Vendômois, and Séralini 2018; C. Li et al. 2019). Elements classified as heavy metals include essential elements such as iron (Fe), copper (Cu), and Manganese (Mn) and non-essential elements such as lead (Pb), cadmium (Cd), and mercury (Hg). Essential elements function as crucial cofactors in metalloproteins and enzymes, and their deficiency can lead to a decline in metabolic activity. However, at excessive concentration, they can inhibit vital functional groups, replace other metal ions, and alter the three-dimensional structures of biomolecules, resulting in toxic effects. In contrast, non-essential heavy metals play no biological role and instead compete with other elements, binding to enzymes and proteins and impairing their functionality. Furthermore, as heavy metals are non-biodegradable, they persist both in the natural environment and within organisms, exhibiting long-term toxicity. Due to this characteristic, they are prone to bioaccumulation, posing significant risks to the health of plants and animals through the food chain (Gong, Zhao, and Wang 2018; R. Sun et al. 2020). Additionally, heavy metal contamination in water resources can lead to the ingestion of these toxic elements through drinking water and food, further increasing the risk of health hazards. Heavy metals also have negative effects on organisms and plants near the surface of the soil. Plants play a vital role in ecosystems by producing oxygen and organic matter through photosynthesis, maintaining soil structure through root systems, preserving soil moisture, providing habitats for various organisms, and serving as resources for food, medicine, and construction materials. These functions are not only crucial for sustaining ecosystems and the natural environment but are also closely linked to human livelihoods and economic activities. However, when exposed to heavy metal contamination, plants experience disruptions in biochemical and physiological processes, resulting in reduced productivity, decreased biomass, and tissue necrosis (Kapusta-Duch et al. 2011; A. Yan et al. 2020; Prosekov

2021). Furthermore, the loss of vegetation due to heavy metal toxicity destabilizes soil structure, leading to the dispersal of contaminated topsoil. This process not only causes direct health hazards to humans and animals through inhalation and ingestion but also results in nutrient loss and soil degradation. Additionally, heavy metals are known to have various adverse effects on soil microorganisms, as demonstrated by many previous studies. Soil microorganisms are deeply involved in nutrient cycling and soil fertility due to their diverse chemical reactivity. Some soil microorganisms, regardless of symbiotic associations, play a crucial role in promoting plant growth through various mechanisms, such as nitrogen fixation, phosphate solubilization, protection against plant pathogens, and the production of phytohormones. However, exposure of soil microbial communities to heavy metal contamination has been reported to lead to reduced enzymatic activity, increased oxidative stress, decreased biomass and diversity, and shifts in community composition due to varying sensitivities to heavy metals (Frey et al. 2006; F. Wang et al. 2010; Thavamani et al. 2012). These changes hinder nutrient cycling and reduce soil fertility, ultimately leading to declines in agricultural productivity and losses of ecosystem services. As described above, heavy metal contamination not only poses a serious threat to human health but also exerts severe adverse effects on plants and soil microorganisms, which are closely linked to ecosystem functions and services. Therefore, immediate remediation efforts are essential. To restore heavy metal-contaminated soils, it is crucial to enhance soil fertility while simultaneously facilitating the removal of heavy metals. Addressing these challenges effectively requires leveraging the functional capacities of both soil microorganisms and plants.

1.1.1 Lead (Pb) metal pollution

Pb (lead) which is one of the most abundant heavy metals may negatively affect the organism and the natural environment due to their non-biodegradability(Ghosh and Singh 2005). As with other heavy metal contamination, most Pb contamination is caused by anthropogenic activities such as chemical manufacturing, refining, and pesticide use. For these reasons, Pb has become a major pollutant, especially in intense mining activities and urban areas. Pb exists in various oxidation states (0, I, II, IV) in soil, and the Pb^{2+} or Pb hydroxy complex state is the most stable form. Pb^{2+} has the highest reactivity and mobility among these types, and it forms various oxides and

hydroxides by bonding with various substances including inorganic components such as carbonate ions, sulfate ions, and chloride ions (Sammut et al. 2010). In addition, because of its high affinity for organic substances, Pb forms compounds with organic ligands such as amino acids and fumaric acid. Therefore, it has been reported that the mobility of Pb is reduced in soils rich in organic matter (Waroszewski, Kabala, and Szopka 2009). In addition, Pb competes with divalent metals such as Mn^{2+} and Zn^{2+} for uptake pathways in vivo and exhibits toxic effects such as protein structural changes and inhibition of enzyme activity (Bruins, Kapil, and Oehme 2000). The availability of Pb is also closely related to soil pH, and it has been reported that Pb forms carbonates and phosphates in alkaline soils, while it exists as a free ion in acidic soils (Sauvé, McBride, and Hendershot 1998).

1.2 Nitrogen cycle

Nitrogen is an essential element for all living organisms, as it is a fundamental component of proteins and nucleic acids and plays a crucial role in metabolism and energy production. Nitrogen sources can be broadly categorized into inert atmospheric nitrogen (N_2) and reactive nitrogen, which is involved in biological metabolism and growth. The transformation between these two forms is primarily mediated by microbial processes, including nitrogen fixation, nitrification, and denitrification. In degraded soils, such as those contaminated with heavy metals, physical and chemical properties, as well as biological functions mediated by plants and soil microorganisms, are significantly impaired, leading to reduced soil fertility. Given that nitrogen is a critical nutrient for plant and microbial growth, understanding its cycling and associated microbial functions is essential for developing effective nitrogen management strategies. Proper nitrogen management is crucial for restoring degraded soils and enhancing ecosystem functionality, ultimately supporting sustainable land use and environmental conservation.

1.2.1 Nitrogen fixation

Nitrogen fixation is a biochemical process in which atmospheric dinitrogen (N_2) is converted into ammonia nitrogen (NH_3-N), a biologically available form of nitrogen. This reaction is exclusively performed by certain prokaryotic organisms, commonly

referred to as nitrogen-fixing bacteria. Given that dinitrogen is the most abundant form of nitrogen in the biosphere yet remains largely inaccessible to most organisms due to its chemical stability, the specificity and ecological significance of this process are remarkable. Nitrogen fixation is carried out by nitrogen-fixing bacteria, which possess the enzyme nitrogenase. This enzyme complex consists of two types of proteins: an iron (Fe) protein and a molybdenum-iron (Mo-Fe) protein, which are encoded by the *nifH, nifD*, and *nifK* genes, respectively (Dixon and Kahn 2004). The process of nitrogen fixation requires a substantial energy input to break the strong triple bond of N_2, consuming 16 molecules of ATP and 8 electrons per nitrogenase molecule in the reaction. Nitrogen-fixing bacteria can be broadly classified into two types: symbiotic nitrogen-fixing bacteria (e.g., Rhizobia and Frankia) and free-living nitrogen-fixing bacteria. Symbiotic nitrogen-fixing bacteria establish mutualistic relationships with host plants by forming root nodules, wherein they supply fixed nitrogen in exchange for organic carbon compounds derived from plant photosynthesis. Although these bacteria exhibit higher nitrogen-fixing efficiency compared to their free-living counterparts, their symbiotic relationships are restricted to specific host plant species. In contrast, free-living nitrogen-fixing bacteria do not form root nodules but are often found densely colonizing plant root surfaces and the rhizosphere. The rhizosphere is a highly dynamic environment where the availability of oxygen, carbon, phosphorus, and other nutrients fluctuates significantly. Free-living nitrogen-fixing bacteria exhibit remarkable adaptability to such environmental variations. Although their nitrogen-fixing efficiency is generally lower than that of symbiotic nitrogen-fixing bacteria, they have the distinct advantage of enhancing plant growth without being restricted to specific plant species. Both types of nitrogen-fixing bacteria play an essential role in plant growth and development by supplying nitrogen, a crucial macronutrient. Their ability to facilitate nitrogen availability highlights their ecological and agricultural importance in sustaining plant productivity. When biologically available nitrogen sources are abundant, such as following nitrogen fertilizer application, the abundance of nitrogen-fixing bacteria decreases, and the symbiotic relationship between plants and nitrogen-fixing bacteria is disrupted due to the reduction of root nodules(Yanyan Liu et al. 2011; Smercina et al. 2019; Zheng et al. 2019) . This phenomenon occurs because nitrogen fixation is an energetically expensive process, and under nitrogen-rich conditions, microorganisms preferentially utilize externally available nitrogen sources rather than fixing atmospheric nitrogen. In addition to nitrogen fixation, some nitrogen-fixing bacteria contribute to

plant growth by producing plant hormones such as auxins, cytokinin-like substances, gibberellin-like substances, and gibberellic acid (Tien, Gaskins, and Hubbell 1979; Pedraza 2008). Auxins play a crucial role in cell elongation and root development, cytokinins promote cell division and photosynthetic activity, and gibberellins enhance stem elongation, collectively facilitating plant growth across different developmental stages and tissues. Furthermore, ethylene, a plant hormone, is known to inhibit plant growth, and certain nitrogen-fixing bacteria possess 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that hydrolyzes ACC, the precursor of ethylene. By reducing ethylene levels, these bacteria can alleviate growth inhibition and further stimulate plant development (Döbereiner , Day, and Dart 1972). Additionally, some nitrogen-fixing bacteria have been reported to enhance the uptake of essential nutrients, including phosphorus (P), nitrogen (N), potassium (K), and various micronutrients, thereby further promoting plant growth (Dobbelaere and and Okon 2003)

1.2.2 Nitrification

Nitrification is an oxidative process within the nitrogen cycle, in which ammonia (NH_4^+) is sequentially oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). The first step of this process, ammonia oxidation, is not only the rate-limiting step but also plays a central role in the global nitrogen cycle (Leininger et al. 2006). Traditionally, the conversion of ammonia to nitrite was thought to be carried out exclusively by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), while the oxidation of nitrite to nitrate was attributed to nitrite-oxidizing bacteria (NOB), which belong to phylogenetically distinct groups. However, the existence of microorganisms capable of performing both oxidation steps in the nitrification process was later proposed. Indeed, certain bacteria of the genus *Nitrospira* possess the ability to carry out complete ammonia oxidation (comammox), oxidizing ammonia to nitrate independently(Koch, van Kessel, and Lücker 2018; S. Xu et al. 2020). The key enzyme involved in ammonia oxidation, ammonia monooxygenase (AMO), is a membrane-bound trimeric protein consisting of α, β, and γ subunits, which are encoded by the *amoA*, *amoB*, and *amoC* genes, respectively(Klotz, Alzerreca, and Norton 1997) . The oxidation of nitrite to nitrate is performed by NOB and is catalyzed by nitrite oxidoreductase, which is encoded by the *nxrB* gene (Brochier-Armanet et al.

2008; Ge et al. 2015). Nitrification plays a crucial role in regulating ammonium (NH_4^+) levels in the soil. Ammonium, once taken up by plants, is directly assimilated into amino acids such as glutamine and glutamate, making it an energetically efficient nitrogen source. However, at high concentrations, ammonium can exhibit toxicity, leading to excessive elongation of stems, inhibited root growth, chlorosis and necrosis of leaves, delayed flowering and fruiting, and disruptions in osmotic balance(Clarke and Baldwin 2002). Nitrate (NO_3^-), on the other hand, requires energy for conversion to NH_4^+ within plants, yet it has lower salinity toxicity and higher water solubility than ammonium, making it a more efficient and safer nitrogen source for plant uptake. Nitrification is highly sensitive to heavy metal contamination, and its activity has been shown to be significantly inhibited under such conditions. Lu et al. (2022) demonstrated that nitrification is more susceptible to heavy metals than denitrification, as evidenced by a significant decrease in the abundance of amoA and nxrB genes in contaminated soils compared to uncontaminated soils. Furthermore, the oxidation of NH_4^+ to NO_2^- was reduced to approximately one-fourth of that observed in non-contaminated soils. Similarly, Thavamani et al. (2012) reported that NH_4^+ oxidation was significantly suppressed in heavy metal-contaminated soils. Additionally, studies have revealed that under heavy metal stress, the transcription levels of the *amoA* genes of both AOB and AOA are reduced (Ruyters et al. 2010). The conversion of NO_2^- to NO_3^- is also inhibited by heavy metal contamination (J. Yan, Quan, and Ding 2013). Notably, the *amoA* gene of AOA exhibits higher tolerance to heavy metals than that of AOB (Xiaofang Li et al. 2009; Ollivier et al. 2012; M. Zhang et al. 2017). This increased tolerance is likely due to the stronger metal ion reduction capability and greater cell membrane robustness of AOA. Given that the dynamics of nitrate are critical for assessing soil fertility, further research is required to enhance the understanding of nitrification in heavy metal-contaminated soils. Future studies should integrate microbial inoculation and phytoremediation approaches to mitigate the adverse effects of heavy metal contamination on nitrification and soil health.

1.2.3 Denitrification

Denitrification is a reductive process within the nitrogen cycle, consisting of four sequential reactions: the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitrite to nitric oxide (NO), nitric oxide to nitrous oxide (N_2O), and finally, nitrous oxide to

dinitrogen gas (N_2). Each of these reduction steps is catalyzed by specific enzymes: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos). These enzymes are encoded by the following genes: *narG* or *napA* for Nar, *nirK* or *nirS* for Nir, *norB* for Nor, and *nosZ* for Nos. The gaseous nitrogen compounds produced through denitrification represent a major pathway of nitrogen loss from ecosystems. Notably, N_2O, an intermediate product in this process, exhibits a global warming potential approximately 300 times greater than that of carbon dioxide (CO_2), making it a significant contributor to climate change (Wang et al. 2011). Conversely, similar to nitrification, denitrification serves an important ecological role in preventing excessive nitrogen accumulation, thereby mitigating the negative effects of nitrogen overload, such as plant growth inhibition, eutrophication of aquatic ecosystems, and groundwater contamination. This function is also of practical importance for human activities, as denitrifying bacteria are widely utilized in wastewater treatment and industrial effluent management to remove excess nitrates from water bodies. Denitrification occurs most efficiently under anaerobic conditions and within a pH range of 6.6 to 8.3 (ŠImek and Cooper 2002). Additionally, it has been reported that as soil pH decreases, the reduction of N_2O to N_2 is inhibited, resulting in a greater proportion of N_2O being emitted as the final product (Mørkved, Dörsch, and Bakken 2007; Van Den Heuvel et al. 2011). Like nitrogen fixation and nitrification, denitrification is also significantly inhibited in heavy metal-contaminated soils. One of the most critical concerns is the incomplete progression of denitrification, where the conversion of N_2O to N_2 is inhibited, leading to increased emissions of N_2O, a potent greenhouse gas. Numerous studies have reported this phenomenon(C. Zhang et al. 2016; T. Zhou et al. 2014; Bollag and Barabasz 1979) . Furthermore, heavy metals can lower soil pH, which in turn enhances N_2O emissions(Čuhel et al. 2010). Several studies analyzing inorganic nitrogen levels and enzyme activity have demonstrated that heavy metal contamination inhibits not only the reduction of NO_3^- to NO_2^- (Aiken et al. 2003) but also the subsequent reduction of NO_2^- (Chen et al. 2016). It has been shown that metals such as Cd, Cu, and Zn inhibit both NO_3^- reduction to N_2O and N_2O reduction to N_2 (Holtan-Hartwig et al. 2002). Conversely, some studies have reported a positive correlation between increasing heavy metal concentrations and higher N_2O emissions (Bollag and Barabasz 1979; ŠImek and Cooper 2002; Felgate et al. 2012). It has been reported that heavy metal contamination leads to a reduction in the abundance of key denitrification functional genes, including *narG, nirS, norB,* and *nosZ* (Feng et

al. 2023; Yang et al. 2024). However, contradictory findings exist; some studies have observed an increase in denitrification gene abundance under heavy metal contamination (M. Wang et al. 2023; Hu et al. 2025). The discrepancy between inorganic nitrogen levels and denitrification gene abundance in heavy metal-contaminated soils may be attributed to differences in the sensitivity of each denitrification reaction to heavy metals and the duration of heavy metal exposure. Therefore, to accurately assess denitrification activity in heavy metal-contaminated soils, it is essential to conduct longitudinal studies that integrate both functional gene analysis and inorganic nitrogen measurements over time.

1.3 Technique for the remediation of heavy metal contamination

Given the adverse effects of heavy metals discussed thus far, extensive research has been conducted to develop technologies for their removal from contaminated soils. Remediation strategies for heavy metal-contaminated soils can be broadly classified into physicochemical methods and biological methods. Examples of physicochemical methods include excavation and replacement with uncontaminated soil, electrokinetic remediation, thermal treatment, vitrification, and chemical extraction or immobilization using specific reagents (L. Xu et al. 2024). These approaches offer rapid and effective solutions, particularly for heavily contaminated soils. Furthermore, chemical treatments allow for selective removal of specific heavy metals, providing a targeted remediation strategy. However, these methods also have notable drawbacks. They are generally highly costly in terms of both implementation and post-treatment management. Additionally, they can significantly alter soil structure and properties, making them unsuitable for agricultural applications. In contrast, biological methods such as bioremediation, which utilizes microbial functions, and phytoremediation, which employs plant-based mechanisms, have emerged as promising alternatives. Unlike conventional physicochemical approaches, these methods leverage natural processes, resulting in lower economic costs and reduced environmental impact while preserving soil structure (Boopathy 2000; Vidali 2001; Hadi and Bano 2010; Beškoski et al. 2011).

1.3.1 Bioremediation

Bioremediation is a remediation technology that utilizes the heavy metal resistance mechanisms of microorganisms to immobilize and adsorb heavy metals. While microorganisms are adversely affected by heavy metal contamination, they have also developed unique mechanisms to mitigate metal toxicity. These mechanisms include active efflux pumps for metal expulsion, the release of chelating agents to inactivate metals, exclusion through permeable barriers (modulation of cell membrane permeability), alteration of soil pH, and methylation reactions (Gadd 2000; Kidd et al. 2009; Mani Rajkumar et al. 2010; Pavel et al. 2013) . These processes are facilitated by microbial secretions such as siderophores, organic acids, biosurfactants, polymeric substances, and glycoproteins, which enhance heavy metal mobility and solubility, ultimately increasing metal uptake by plants (M. Rajkumar et al. 2012). Additionally, microorganisms can modify the bioavailability of heavy metals through redox reactions and directly uptake metals through biosorption (Joshi and Juwarkar 2009; Y. Ma et al. 2011). Due to their microscopic size and vast population densities, microorganisms possess a high surface area-to-volume ratio, making them highly effective for the removal of heavy metal contamination. As a result, bioremediation has been recognized as a promising strategy for mitigating heavy metal pollution in contaminated soils.

1.3.2 Phytoremediation

Phytoremediation is a remediation technology that utilizes plants to absorb and immobilize heavy metals in the soil, thereby purifying contaminated soil. Among plants, those capable of absorbing and accumulating high concentrations of specific heavy metals are referred to as hyperaccumulators. To date, approximately 500 plant species across 45 families, including *Brassicaceae*, *Fabaceae* (legumes), *Euphorbiaceae*, *Asteraceae*, *Lamiaceae,* and *Scrophulariaceae* have been identified as hyperaccumulators (Ghosh and Singh 2005) . The underlying reason why plants perform metal hyperaccumulation remains a topic of scientific investigation, with the most widely accepted hypothesis suggesting that metal accumulation serves as a defense mechanism against herbivores by making leaves unpalatable or toxic and pathogens (Meharg 2005; Dipu, Kumar, and Thanga 2012). However, plants with exceptionally high metal accumulation capacities often exhibit slow growth rates, shallow root

systems, and low biomass production, which pose challenges for large-scale phytoremediation applications (Brewer et al. 1999; Krämer 2005; Słomka et al. 2012). Therefore, in selecting plants most suitable for phytoremediation, factors such as heavy metal tolerance, high aboveground biomass, rapid growth, and competitive ability must be considered (Y. Ma et al. 2011; Glick 2010; Ribeiro de Souza et al. 2012). Additionally, one of the major limitations of phytoremediation is its relatively long duration compared to physicochemical removal techniques, as plants require time to grow and accumulate heavy metals. To address this challenge, ongoing research focuses on selecting fast-growing, high-biomass heavy metal-tolerant plant species, as well as integrating microbial inoculation with phytoremediation plants to enhance remediation efficiency.

1.3.3 Lemongrass (Cymbopogon citratus)

Additionally, it has been observed that essential oil production in lemongrass increases under heavy metal contamination (Lermen, Mohr, and Alberton 2015; Sete da Cruz et al. 2024), and the heavy metal concentration in the essential oil remains within safe limits Pandey et al. 2020). Like other Poaceae species, lemongrass possesses a well-developed root system, rapid growth rate, and high aboveground biomass production, which are advantageous traits for phytoremediation. These characteristics have led to growing interest in lemongrass as a potential plant for heavy metal phytoremediation. However, compared to well-established phytoremediation plants such as *Thlaspi caerulescens* and *Brassica juncea* from the Brassicaceae family, and *Helianthus annuus* from the Asteraceae family, research on lemongrass remains relatively limited. Moreover, the impact of microbial inoculation in combination with lemongrass phytoremediation remains unclear, and field-scale applications in heavy metal-contaminated environments are still scarce. Therefore, further studies are necessary to accumulate knowledge on the applicability of lemongrass in heavy metal phytoremediation, including its interactions with microbial inoculants and its performance under real-world contamination conditions.

1.4 Microbial inoculation of Plant Growth Promoting Microbes

Microbial inoculation is a technique that involves the application of beneficial

microorganisms to plants and soil to enhance plant growth, regulate soil functionality, and manage nutrient cycling. Microbial inoculants are generally classified into three major categories: biofertilizers, phytostimulants, and biocontrol inoculants. Due to their diverse plant growth-promoting effects, these inoculants are collectively referred to as PGPMs. Biofertilizers enhance nutrient availability and uptake in plants, typically including nitrogen-fixing bacteria and phosphate-solubilizing bacteria (I. R. Kennedy and Islam 2001). Representative inoculants include *Azospirillum* and *Rhizobium*. Phytostimulants promote plant growth by producing phytohormones such as indole-3-acetic acid (IAA), gibberellic acid (GA_3), cytokinins, abscisic acid (ABA), and salicylic acid. These hormones enhance cell elongation, root development, and stress tolerance, thereby improving overall plant growth. IAA and cytokinins stimulate cell expansion and root growth, increasing water and nutrient uptake efficiency, while ABA, ethylene, and salicylic acid enhance tolerance to biotic and abiotic stressors. Representative inoculants include *Azospirillum* and *Bacillus*. Biocontrol inoculants protect plants from pathogenic microbes and pests, playing a crucial role in plant disease management. Common examples include *Trichoderma*, *Pseudomonas*, and *Bacillus* (Peighami-Ashnaei et al. 2009; Dawar et al. 2010; Mohiddin et al. 2010). Given their broad range of beneficial effects, microbial inoculants are increasingly recognized as sustainable alternatives to conventional agrochemicals, offering enhanced plant productivity while minimizing environmental impact.

1.4.1 Azospirillum

Azospirillum is a well-known PGPM that was first identified as a nitrogen-fixing bacterium by Beijerinck in 1925 (DÖBEREINER, DAY, and DART 1972) . Studies have demonstrated that *Azospirillum* species exhibit non-species-specific plant growth-promoting effects across a wide range of host plants (Okon and Kapulnik 1986; Perrig et al. 2007). The five major species include: *A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferens*, and *A. irakense*. The plant growth-promoting effects of *Azospirillum* are primarily attributed to biological nitrogen fixation and phytohormone production(Steenhoudt and Vanderleyden 2000) . Additionally, certain strains have been found to solubilize inorganic phosphorus in soil, enhancing plant phosphorus uptake and increasing crop yield (Turan et al. 2012). Studies have also demonstrated that *Azospirillum* exhibits tolerance to heavy metal contamination, and strains have been

successfully isolated from heavy metal-contaminated soils (Moreira 2008). Moreover, inoculation with *Azospirillum* has been reported to enhance plant growth and yield in heavy metal-polluted environments. El-Ballat et al. (2023) found that *A. brasilense* EMCC1454 exhibited Cr resistance up to 260 µM while displaying multiple plant growth-promoting (PGP) activities, including nitrogen fixation, phosphate solubilization, siderophore production, trehalose accumulation, exopolysaccharide production, ACC deaminase activity, IAA production, and hydrolytic enzyme secretion. These traits not only improved chickpea growth under Cr stress but also upregulated the expression of multiple stress tolerance genes. Vazquez et al. (2021) reported that inoculation with *A. brasilense* Az39 promoted root system development in wheat while simultaneously reducing Cd stress. Phytohormones play a crucial role in limiting heavy metal uptake and accumulation, thereby enhancing plant tolerance and mitigating contamination risks in the food chain (Saini, Kaur, and Pati 2021). Interestingly, some studies have shown that *Azospirillum* inoculation can enhance heavy metal uptake in plants, contributing to phytoremediation efforts. Muratova et al. (2022) demonstrated that inoculation with *A. brasilense* Sp7 (IBPPM 150), *A. brasilense* Cd (IBPPM 288), and *A. baldaniorum* Sp245 reduced Cu toxicity in wheat while simultaneously increasing Cu accumulation in roots and shoots. Arora et al. (2016) found that co-inoculation with *Azospirillum* and arbuscular mycorrhizal fungi (AMF) promoted switchgrass growth under Pb and Cu contamination, while also enhancing Pb and Cu accumulation in roots. The findings from these studies indicate that *Azospirillum* is capable of promoting plant growth even in heavy metal-contaminated environments, improving tolerance to environmental stressors, and enhancing heavy metal uptake and detoxification mechanisms. Moving forward, further research should focus on expanding the application of *Azospirillum* to a broader range of phytoremediation plant species to optimize its potential in heavy metal remediation and sustainable agriculture.

1.4.1.1 Azospirillum brasilense Sp7

Azospirillum brasilense strain Sp7 was originally isolated from tropical grasses native to Brazil and has been reported to colonize the surface of plant roots through a two-step process involving adsorption and anchoring *Azospirillum brasilense* strain Sp7 was originally isolated from tropical grasses native to Brazil and has been reported to colonize the surface of plant roots through a two-step process involving adsorption and

anchoring (Michiels, Croes, and Vanderleyden 1991). Like the Sp245 strain, Sp7 is widely used as one of the standard strains of *Azospirillum brasilense* in scientific research. However, unlike Sp245, which is endophytic (Baldani et al. 1986), Sp7 is characterized as an epiphytic strain (Baldani et al. 1986) and exhibits high sensitivity to environmental stressors and pollutants, including heavy metals (Kamnev et al. 2012). Due to these characteristics, Sp7 is considered to have high potential for protecting plants against soilborne pathogens and abiotic stresses (Pereg 2015). Furthermore, Sp7 is a free-living plant growth-promoting microorganism (PGPM) capable of nitrogen fixation, and numerous studies have investigated its inoculation into various plant species and soils. These studies have reported improvements in nitrogen uptake, enhanced plant growth through phytohormone production, and increased tolerance to environmental stresses (Lade et al. 2018). In particular, inoculation with *A. brasilense* Sp7 has demonstrated beneficial effects even under heavy metal stress—one of the major abiotic stresses—by promoting plant growth and enhancing heavy metal tolerance (Muratova et al. 2022). However, most of these studies have been conducted on major crop species, and only limited research has explored the inoculation of Sp7 into heavy metal-tolerant plants such as lemongrass. Therefore, further studies are needed to accumulate knowledge on the interaction between *A. brasilense* Sp7 and phytoremediation-relevant plant species, in order to support the development of novel phytoremediation strategies. Like the Sp245 strain, Sp7 is widely used as one of the standard strains of *Azospirillum brasilense* in scientific research. However, unlike Sp245, Sp7 is characterized as an epiphytic strain (Baldani et al. 1986) and exhibits high sensitivity to environmental stressors and pollutants, including heavy metals (Kamnev et al. 2012). Due to these characteristics, Sp7 is considered to have high potential for protecting plants against soilborne pathogens and abiotic stresses (Pereg 2015). Furthermore, Sp7 is a free-living plant growth-promoting microorganism (PGPM) capable of nitrogen fixation, and numerous studies have investigated its inoculation into various plant species and soils. These studies have reported improvements in nitrogen uptake, enhanced plant growth through phytohormone production, and increased tolerance to environmental stresses (Lade et al. 2018; Ajinde, Dayo-Olagbende, and Akpor 2024). In particular, inoculation with *A. brasilense* Sp7 has demonstrated beneficial effects even under heavy metal stress by promoting plant growth and enhancing heavy metal tolerance (Muratova et al. 2022). However, most of these studies have been conducted on major crop species, and only limited research has

explored the inoculation of Sp7 into heavy metal-tolerant plants such as lemongrass. Therefore, further studies are needed to accumulate knowledge on the interaction between *A. brasilense* Sp7 and phytoremediation-relevant plant species, in order to support the development of novel phytoremediation strategies.

1.4.1.2 Application of microbes for the remediation of heavy metal contaminated soil

Many studies have investigated the effects of PGPM inoculation in heavy metal-contaminated soils. For instance, inoculation with *Azotobacter chroococcum* and *Rhizobium leguminosarum* significantly enhanced the growth of maize under Pb-contaminated conditions while also improving Pb uptake capacity (Hadi and Bano 2010). Similarly, inoculation with the heavy metal-resistant nitrogen-fixing bacterium *Burkholderia* sp. J62 in the rhizosphere of tomato and maize resulted in substantially increased Pb and Cd absorption compared to non-inoculated soils (Jiang et al. 2008). Among PGPMs, arbuscular mycorrhizal fungi (AMF) have also been shown to promote plant growth in heavy metal-contaminated environments, making them valuable for phytoremediation applications. However, several studies have reported that AMF reduce heavy metal accumulation in host plants (Göhre and Paszkowski 2006; Audet and Charest 2007; Hildebrandt, Regvar, and Bothe 2007). This reduction in heavy metal accumulation is attributed to the sequestration of metals within fungal spores and hyphae, thereby decreasing metal translocation to plant tissues (Kaldorf et al. 1999; Weiersbye, Straker, and Przybylowicz 1999). A review by Wood et al. (2016) on PGPM inoculation in heavy metal-contaminated soils highlighted that increased biomass production following PGPM inoculation contributes to enhanced heavy metal uptake. Additionally, PGPMs have been reported to increase the concentration of bioactive compounds related to plant stress tolerance, thereby reducing heavy metal accumulation in plant tissues while simultaneously enhancing biomass production (Q. Xu et al. 2018; Cui et al. 2023). Collectively, these findings indicate that various PGPMs can significantly enhance plant growth and phytoremediation potential under heavy metal stress. However, the effects of inoculation vary depending on the plant species, microbial strains, heavy metal type, and inoculation method. Therefore, future research should focus on evaluating different combinations of these factors to optimize microbial inoculation strategies for effective phytoremediation in heavy metal-contaminated

environments.

1.4.2 Practical example with Lemongrass

In recent years, research on microbial inoculation in lemongrass has been increasing, regardless of the presence or absence of heavy metals in the soil. Most studies have focused on inoculating lemongrass with AMF, either alone or in combination with PGPM such as *Azospirillum brasilense*. For instance, inoculation with AMF alone enhanced lemongrass growth while also increasing the antibacterial activity of its essential oil (Eke et al. 2020). Similarly, studies that applied AMF and A. brasilense either individually or in combination demonstrated that lemongrass growth, mycorrhizal colonization, and essential oil production were significantly enhanced (da Cruz et al. 2020; de Souza et al. 2022). Moreover, total sugar, reducing sugar, flavonoid content, and total phenol levels in the essential oil were also found to increase following microbial inoculation. These changes in essential oil composition may contribute to the alterations in antibacterial activity observed in previous studies. Furthermore, Cruz et al. (2024) evaluated the effects of *A. brasilense* inoculation on lemongrass growth and essential oil composition in Pb-contaminated soils. Their findings indicated that essential oil content was influenced by soil Pb concentration, reaching its highest level at 500 mg Pb/kg soil, the highest Pb concentration tested in the study. This result aligns with previous findings that essential oil concentration in lemongrass increases under heavy metal stress (Lermen, Mohr, and Alberton 2015; Sete da Cruz et al. 2024). However, when *A. brasilense* was inoculated, no significant increase in essential oil content was observed under Pb contamination. The researchers suggested that this may be due to the ability of *A. brasilense* inoculation to alleviate Pb-induced stress in lemongrass. Despite the growing interest in microbial inoculation for lemongrass, there are very few studies investigating its impact on heavy metal removal efficiency. Lermen et al. (2015) reported that AMF inoculation reduced Pb uptake in lemongrass, a trend commonly observed in AMF studies with other plants. However, little research has been conducted on the effects of inoculating PGPMs such as Azospirillum on heavy metal uptake capacity. Although microbial inoculation studies on lemongrass have primarily focused on plant growth and essential oil composition, the majority of these studies have been conducted with AMF. In contrast, there has been little assessment of soil chemical properties, soil fertility, or microbial functionality following microbial

inoculation. As discussed in the previous section, introducing high concentrations of external microorganisms through inoculation can potentially alternative soil microbial communities and their functional roles. Therefore, future research on microbial inoculation in lemongrass should also include comprehensive evaluations of changes in soil chemistry and microbial functionality to better understand its broader ecological impact.

1.4.3 Effects on Nitrogen cycle

As discussed previously, microbial inoculation has been widely studied for its potential to enhance plant growth through various mechanisms. In recent years, research has also focused on managing soil microbial communities through microbial inoculation to improve biological nitrogen fixation and nitrogen retention in soil. Currently, BNF in agricultural ecosystems supplies approximately 50–70 teragrams (Tg) of nitrogen annually (Herridge, Peoples, and Boddey 2008), accounting for about 25–33% of the total nitrogen input from human activities, which is estimated at 210 Tg/year (Fowler et al. 2013). The use of microbial inoculants containing nitrogen-fixing bacteria as an alternative or supplement to synthetic nitrogen fertilizers is expected to reduce both economic costs and environmental impacts. Nitrogen fixed by inoculated bacteria can be absorbed by plants, accumulated in the soil, or lost through nitrification and denitrification, leading to gaseous emissions. However, the exact pathways through which nitrogen derived from inoculated nitrogen-fixing bacteria moves and influences these processes remain largely unknown. One of the key aspects in evaluating the effects of microbial inoculation on nitrogen cycling is its influence on nitrous oxide (N_2O) emissions, a potent greenhouse gas. Both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) contribute to N_2O production; however, AOA generally produce less N_2O per mole of ammonia oxidized than AOB (Hink et al. 2017; Stein 2019). Furthermore, AOB predominantly generate N_2O under high soil ammonium (NH_4^+) concentrations, whereas AOA produce N_2O only under low NH_4^+ conditions (Kozlowski et al. 2016; Hink et al. 2017; 2018). Additionally, a decrease in soil pH has been shown to increase the AOA/AOB ratio. Since the AOA/AOB ratio serves as an indicator of N_2O production potential, it is essential to assess how microbial inoculation affects nitrogen availability and soil pH, thereby influencing AOA/AOB ratios. Denitrification is also closely related to N_2O emissions, as it includes

the stepwise reduction of NO_3^- to N_2O and subsequently to N_2. Therefore, the activities of nitrate reduction to N_2O and N_2O reduction to N_2, along with their association with microbial communities possessing the *nosZ* gene, should be evaluated when assessing the impact of microbial inoculation on nitrogen cycling. Excessive accumulation or leaching of nitrate (NO_3^-) can pollute soil and water resources and negatively affect plant growth. Therefore, evaluating the impact of microbial inoculation on nitrification and denitrification processes is crucial. Thus, in addition to measuring NO_3^- concentrations, it is essential to assess the abundance and expression levels of genes involved in NO_3^- metabolism, such as *nxrB* (nitrite oxidoreductase gene) for nitrification and *narG* (nitrate reductase gene) for denitrification. Future research should integrate both biochemical and molecular approaches to provide a comprehensive understanding of how microbial inoculation influences nitrogen cycling in soils, particularly in relation to nitrogen retention, greenhouse gas emissions, and ecosystem sustainability.

1.4.4 Key challenges of microbial inoculation

Considering the various previous research, the benefits of microbial inoculation have been extensively discussed. However, microbial inoculation does not always enhance plant growth and, in some cases, can even have negative effects. Since this technology involves introducing exogenous microbial strains into native soil microbial communities, microbial competition may arise, potentially disrupting existing microbial populations and their functional roles. For example, Kozdrój et al. (2004) reported that inoculation with *Pseudomonas corrugata* IDV1 and *P. putida* RA2 successfully established populations in the rhizosphere of maize. However, the inoculated strains altered the indigenous microbial community in the maize rhizosphere and slightly inhibited maize growth. This result is particularly noteworthy because the introduced bacteria not only successfully colonized the root zone but also exerted negative effects despite their establishment. Similarly, studies on co-inoculation of PGPMs in different plant species, including tomato (Felici et al. 2008), Italian stone pine (Probanza et al. 2002), and common bean (Trabelsi et al. 2011), have shown that the effects of co-inoculation were lower than those of single inoculations. These findings suggest that competition between co-inoculated microbes and native microbial communities may have influenced the observed results. Conversely, some studies have suggested that

interactions among soil microbes, plants, and soil ecosystems may help stabilize microbial community structures, reducing potential disruptions caused by inoculation (A. C. Kennedy 1999). Furthermore, research on functional redundancy in microbial ecosystems—the concept that different microbial species can perform the same ecological function—suggests that ecosystem functionality can be maintained despite shifts in microbial community composition (A. C. Kennedy 1999; Nannipieri et al. 2017). Given these findings, microbial inoculation should not only be evaluated based on its impact on plant growth but also in terms of its effects on native microbial communities and their functional roles. This consideration is particularly critical for heavy metal-contaminated soils, as such soils are typically more vulnerable than uncontaminated soils, often exhibiting reduced microbial diversity, lower functionality, and decreased biomass. Therefore, the need to assess the impact of microbial inoculation on multiple ecological factors is even greater in heavy metal-contaminated environments. In addition to microbial competition, the method of inoculation can also influence the outcomes of microbial application. Generally, microbial inoculation methods can be classified into three major types. Seed inoculation is the most commonly used microbial inoculation method, in which seeds are immersed in a microbial suspension prior to sowing (Lopes et al. 2018). During seed germination, seed exudates, including carbohydrates and amino acids, are released, providing nutrients that facilitate the proliferation and colonization of inoculated microorganisms on the developing roots(Ammor, Michaelidis, and Nychas 2008). Due to the high accessibility of microbes to the rhizosphere immediately after germination, seed inoculation has been reported to require approximately 1,000 times less microbial biomass to achieve the desired inoculation effect compared to soil inoculation (Wilson and Jackson 2013). Therefore, seed inoculation is considered a cost-effective and environmentally friendly approach to microbial inoculation. Root inoculation involves immersing plant roots in a microbial suspension before transplanting the seedlings into the soil. This method enables direct contact between the inoculated microorganisms and the host plant's roots, thereby facilitating more efficient colonization of the root system (Ahemad and Kibret 2014). Soil inoculation refers to the direct application of plant growth-promoting rhizobacteria (PGPR) into the soil, using techniques such as soil irrigation, soil mixing, or microencapsulation (Hernández-Montiel et al. 2017; Domenico 2020). These approaches ensure that microbial inoculants are introduced in close proximity to the plant roots, optimizing their potential for colonization and interaction with the host

plant. Although microbial inoculation has been widely reported to positively influence plant growth, the effectiveness of inoculation varies depending on the method used. For instance, Ciccillo et al. (2002) found that inoculating maize seeds with *Burkholderia ambifaria* MCI 7 enhanced seedling growth, whereas direct soil inoculation reduced maize growth. Conversely, Lopes et al. (2018) reported that seed inoculation with *Burkholderia pyrrocinia* and *Pseudomonas fluorescens* in *Brachiaria brizantha* had no significant effect, while soil inoculation 14 days after germination promoted plant growth. These differences suggest that microbial establishment and function depend on the inoculation method, likely due to differences in microbial colonization efficiency, adaptation, and interactions with native soil microbes. Additionally, factors such as light, temperature, moisture, pH, and nutrient availability may also influence microbial behavior and plant-microbe interactions. Considering the findings from previous studies, it is essential to select the most appropriate inoculation method for each plant species based on microbial compatibility, plant characteristics, and soil conditions. This is particularly critical in heavy metal-contaminated soils, where microbial and plant growth, as well as ecosystem functionality, are often compromised. Therefore, future research should aim to compare different inoculation methods systematically, considering plant species, microbial strains, soil properties, and environmental conditions, to optimize microbial inoculation strategies for improved phytoremediation and sustainable agriculture.

1.5 Research needs

Heavy metal contamination, including Pb pollution, is a severe environmental issue observed worldwide. It not only poses risks to biological health but also leads to declines in soil fertility, crop productivity, and ecosystem functionality. Consequently, the accumulation of knowledge and the development of remediation technologies are urgently required. Effective remediation of heavy metal-contaminated soil necessitates both the improvement of soil fertility and the removal of heavy metals, with a preference for cost-effective and environmentally sustainable approaches. The nitrogen cycle is a critical ecosystem function closely associated with soil fertility, involving a diverse range of microorganisms. Moreover, due to its high sensitivity to heavy metal contamination, the nitrogen cycle has been widely employed as an indicator for assessing the environmental impact of heavy metals. Understanding nitrogen cycling

and the associated microbial communities under heavy metal contamination is thus essential for the restoration of degraded soils. In particular, nitrogen fixation and denitrification are intimately linked to soil fertility enhancement and degradation, respectively, highlighting the necessity of accumulating data on their dynamics. However, most studies evaluating nitrogen cycling have focused either on changes in inorganic nitrogen concentrations or the abundance of functional genes. Few studies have integrated both aspects to provide a comprehensive evaluation of inorganic nitrogen sources and functional gene dynamics. Based on these challenges, the first study was designed to assess denitrification activity in Pb-contaminated soil by adding NO_3^- as a nitrogen source. Soil samples were collected at weekly intervals from the start of the incubation, and temporal changes in inorganic nitrogen concentrations (NO_3^-, NO_2^-, N_2O) as well as the abundance of denitrification-related genes (*narG, nirK, nirS, norB, nosZ*) were analyzed. Furthermore, the total abundance of 16S rRNA, Shannon diversity index, and microbial community structure at the phylum and genus levels were examined to evaluate the effects of Pb contamination on microbial communities. As a remediation strategy for heavy metal-contaminated soil, increasing attention has been given to cost-effective and environmentally sustainable approaches that utilize the functions of heavy metal-tolerant microorganisms and plants, often in combination. Lemongrass has recently been studied as a promising candidate for phytoremediation due to its high heavy metal tolerance and uptake capacity, rapid growth, substantial aboveground biomass, and economic value across various industries. However, compared to other phytoremediation plants, studies investigating microbial inoculation of lemongrass remain limited. Moreover, most research has focused on the growth and essential oil composition of lemongrass, while relatively few studies have evaluated its effects on soil properties and microbial communities. Microbial inoculation offers several advantages, including enhanced plant growth, increased resistance to heavy metal stress, and improved heavy metal uptake. Consequently, considerable research has been conducted to assess these benefits. However, the introduction of high concentrations of exogenous microbes may also have negative impacts on native soil microbial communities and their functionality, necessitating thorough evaluation. Additionally, the effects of microbial inoculation have varied across studies due to differences in plant species, inoculation methods, and soil characteristics. To select an appropriate inoculation strategy for specific plant-soil systems, comparative evaluations of different inoculation methods and conditions are required. In light of these

challenges, the second study was designed to evaluate the effectiveness of microbial inoculation in Pb-contaminated soil using lemongrass as a heavy metal-tolerant plant and *A. brasilense* as the inoculum source. In this study, *A. brasilense* was inoculated onto lemongrass seeds, roots, or both, and the effects on lemongrass growth were compared to determine the most effective inoculation strategy under Pb contamination. Additionally, temporal changes in soil chemical properties, including inorganic nitrogen concentrations (NH_4^+, NO_3^-), soil pH, and water-soluble carbon content, were assessed to evaluate the impact of *A. brasilense* inoculation. Furthermore, quantitative analyses of nitrogen fixation genes (*nifH*), nitrification genes (*amoA, nxrB*), denitrification genes (*narG, nirK, nirS, norB, nosZ*), and 16S rRNA were performed. These analyses aimed to assess the effects of *A. brasilense* inoculation on the abundance of nitrogen-cycling soil microorganisms and the overall soil microbial community in Pb-contaminated soil. In this study, we particularly focus on nitrogen cycling to assess the impact of heavy metals on soil fertility. Additionally, we provide insights into heavy metal bioremediation using *Azospirillum brasilense* Sp7 and lemongrass as model organisms. The following chapters will explore these key topics in further detail.

Chapter 2:Impact of lead contamination on denitrification after nitrate addition to soil: An interaction between nitrogen substrates, denitrification genes, and microbial community structures

2.1 Abstract

Lead (Pb) contamination is a worldwide environmental issue that also affects microbial cycling of nitrogen, a crucial plant nutrient, and environmental health. This study performed a 4-week soil incubation experiment with four levels of Pb (0, 200, 400, and 1600 mg Pb/kg soil), with and without nitrate (NO_3^-) addition, to assess the impact of Pb on denitrification and microbial communities. Reductions of NO_3^- and nitrite (NO_2^-) were inhibited at 1600 mg Pb/kg soil, with nitrate reductase (*narG*) gene abundance decreasing with increasing Pb, however nitrite reductase (*nirS)* gene abundance showed no correlation to Pb. The increase in nitrous oxide (N_2O) at 1600 mg Pb/kg soil may have been related to a decrease in genes encoding N_2O reductase (*nosZ*) and the increase in genes encoding nitric oxide reductase (*norB*). Following exposure to Pb, 16S rRNA abundance decreased, while the Shannon diversity index increased. *Proteobacteria* initially dominated but decreased at 1600 mg Pb/kg soil, whereas *Actinobacteria*, *Chloroflexi*, and *Nitrospirae* increased with Pb at all levels. *Azotobacter* decreased by 50% at > 400 mg Pb/kg soil, while *Bacillus* demonstrated high Pb resistance. The microbial communities were initially influenced by Pb but shifted toward the Control over time.

2.2 Introduction

Heavy metal pollution in soil, primarily caused by human activities such as pesticide use, mining operations, industrial wastewater discharge, sewage sludge disposal, and chemical manufacturing (Facchinelli, Sacchi, and Mallen 2001), is a significant environmental concern found in numerous global regions. Lead (Pb) is among the most prevalent forms of heavy metal pollution. Similar to other heavy metals, it is non-biodegradable, posing a substantial risk to organisms and the environment (Ghosh 2005). Furthermore, Pb competes with the uptake pathways of

essential divalent metals such as Mn^{2+} and Zn^{2+} in the body, leading to toxicity manifested as structural changes in proteins and inhibition of enzyme activity (Bruins, Kapil, and Oehme 2000). Consequently, Pb contamination of soil can impact microbial enzyme activities, leading to a decline in soil health (Khan et al. 2009; Pan et al. 2020). The soil N-cycle warrants a more detailed study with respect to the effect of Pb on microbial enzyme activities. This is because N is a critical nutrient for plant growth and is associated with the generation of reactive N compounds that contribute to eutrophication (e.g. nitrate (NO_3^-)) and greenhouse gas emissions (e.g. nitrous oxide (N_2O)). Within the N-cycle, denitrification is a key microbial process that removes NO_3^- from soils and which, ultimately, releases N back into the atmosphere as dinitrogen (N_2). Denitrification sequentially reduces NO_3^- to nitrite (NO_2^-), to nitric oxide (NO), to N_2O and finally N_2. The N_2O molecule is a precursor to N_2 formation, and this may also be released into the atmosphere if denitrification does not go to completion. Each stage of denitrification is associated with different reductive enzymes, classified as NO_3^- reductase (NAR), NO_2^- reductase (NIR), NO reductase (NOR), and N_2O reductase (NOS). Heavy metal pollution has been reported to negatively affect denitrification activity. The *narG*, *nirS* and *nirK*, *norB*, and *nosZ* genes are involved in the expression of NAR, NOR, NOR, and NOS, respectively. The presence of NO_3^- has been shown to be positively correlated with the level of expression of *narG* (Li et al. 2019), as well as with increased expression of the *nirS* and *norB* genes (Saleh-Lakha et al. 2008; 2009). In addition, it has been reported to increase the expression of *nosZ* in various denitrifying bacteria (Olaya-Abril et al. 2018; Wasai-Hara et al. 2023). Metals such as Cd, Cu, and Zn have been found to inhibit both the reduction of NO_3^- to N_2O, and the reduction of N_2O (Holtan-Hartwig et al. 2002). Conversely, some studies have reported an increase in N_2O emissions with heavy metal concentrations (Bollag and Barabasz 1979; ŠImek and Cooper 2002; Felgate et al. 2012). Heavy metal pollution has been shown to have negative effects on denitrifying bacterial communities. For instance, the diversity of the community containing the *nirK* gene was observed to decrease with increasing Pb concentration (Sobolev and Begonia 2008). Heavy metals (Cd, Pb, Zn, and Cu) have also been associated with a reduction in the abundance of the *nirK* and *nosZ* genes (Liu et al. 2016). Conversely, heavy metal pollution has been linked to an increase in the activities of denitrification genes. Specifically, Hg was found to stimulate denitrification activity and significantly enhance the diversity of the *nirS* gene while leaving the *nosZ* gene unaffected (Zhou et al. 2012). Therefore, the influence of

heavy metals on denitrification can affect the genes involved in denitrification differently, necessitating further research in this field. Only a handful of studies have thoroughly assessed the temporal effects of heavy metal pollution on inorganic N transformations, the abundance of denitrification genes, and the structure of the microbial community in soil. Moreover, while numerous prior studies have identified a positive correlation between different denitrification stages and the abundance of gene expression that propels them, none have scrutinized whether this correlation persists under Pb-contaminated soil. A comprehensive examination of the relationship between denitrification and functional genes under Pb contamination is crucial for devising strategies to rehabilitate contaminated soil using plants and other resources. Consequently, we conducted an experiment using soil contaminated with Pb, and supplemented with NO_3^-, to explore the denitrification processes under Pb. We then assessed the fate of the NO_3^- and alterations in the abundances of functional genes related to the denitrification process. Additionally, we examined the bacterial taxa resistant to Pb based on the analyses of the 16S rRNA-based bacterial community structure to discern potential shifts in microbes responsible for denitrification under Pb contamination. We hypothesized that the sensitivity of denitrification to Pb would vary with the stage of denitrification, and that the correlation between the expression of denitrification functional genes and substrate changes would vary with the extent of Pb contamination.

2.3 Material and Methods

2.3.1 Incubation set up

In this study, we used an Arakida soil, a horticultural soil classified as a Fluvisol according to the FAO World Reference Base (WRB) for Soil Resources. The soil was collected in Honjo City, Saitama Prefecture, Japan. The sampling site was located in an alluvial plain with a humid temperate climate (Cfa) and was previously used as a paddy field. A 25 g sample of dry soil (characteristics detailed in Table 1) sealed in a 50-ml Falcon tube comprised a treatment replicate. Eight treatments (four Pb levels × two N levels (with or without N addition)), outlined in Supplementary Table 1, were implemented in triplicate. The Pb levels were 0, 200, 400, and 1600 mg/kg soil, hereafter referred to as control, low, mid and high Pb concentrations, respectively. Lead

acetate ($Pb(CH3COO)_2$) was used to elevate the soil Pb concentrations. To neutralize the effect of varying rates of carbon addition, due to the different amounts of $Pb(CH3COO)_2$ added, a potassium acetate (CH3COOK) solution was also incorporated into each soil replicate to standardize the abundance of acetate ions (as described in Supplementary Table 2). Potassium nitrate (KNO_3) was introduced to the soil as the N source at a concentration of 500 mg NO_3^--N/kg soil. The Pb and NO3− compounds were thoroughly mixed into the soils, and adjustments were made to the soil's moisture and bulk density to achieve a water-filled pore space of 75%, and a soil bulk density of 1.25 g/cm3. Following the N application and moisture adjustment, the soil was incubated at room temperature (25°C) for 4 weeks, during which time soil samples were collected five times: at 2 days (equivalent to week 0), 1st week, 2nd week, 3rd week, and 4th week after the start of the incubation. At these times, soil samples were collected from the same Falcon tube allocated to each replicate. Throughout the incubation period, Milli Q water was periodically added to maintain a constant soil moisture content.

Table 2-1. Chemical and physical characteristics of the Arakida soil

Factors	
pH	6.8 ± 0.1
Bulk density	1.1 ± 0.01
CEC (meq /kg soil)	155 ± 2.0
CWSC (g C/ kg soil)	17.1 ± 7.0
HWSC (g C/ kg soil)	134.8 ± 27.1
Moisture (%)	11.1 ± 0.46

CEC= Cation exchange capacity, CWSC = Cold water-soluble carbon
HWSC = Hot water-soluble carbon

2.3.2 Measurement of N_2O gas emission

Nitrous oxide sampling was conducted during the 1st, 2nd, 3rd, and 4th weeks after the start of incubation. Each 50 ml Falcon tube was relocated to a 900 cm^3 glass bottle, and the headspace air was replaced with ambient air for a duration of 2 min. The

bottle was then promptly sealed with a lid equipped with a septum for gas sampling. Gases within the bottle were sampled at 0, 2, and 4 h post-sealing, and the collected gases were transferred into pre-evacuated 30 ml vials. These gas samples were subsequently analysed using a gas chromatograph (GC-2014, Shimadzu Co., Kyoto, Japan) fitted with an electron capture detector (Nishimura et al. 2005; Shimbo and Uchiyama 2022), a Porapak Q column, and N_2 as the carrier gas. Any water vapour in the gas sample was separated from the gas of interest in the pre-column and exhausted through the choke column. The N_2, O_2 and CO_2 gases were also subsequently separated and exhausted through another choke column. Subsequently, the N_2O concentrations at 0, 2, and 4 hours post-sealing was used to calculate the N_2O flux. using the trapezoidal method and accumulated weekly. During the measurement of N_2O emissions, C_2H_2 gas was not injected. As a result, the inhibition of N_2O reduction by acetylene did not occur, and the observed N_2O emissions were potentially underestimated due to the potential for the conversion of N_2O to N_2.

2.3.3 Measurement of NO_3^--N content, NO_2^--N content and soil pH

For the assessment of soil NO_3^--N and NO_2^--N, 10 ml of a 2 M KCl solution was added to 1 g of the sampled soil and shaken for 30 minutes. The extracts were then filtered using filter paper (Grade 5C, <5 mm; Advantec, Tokyo, Japan) prior to the measurement of inorganic N content. The extracted solution was preserved at 4°C until the time of measurement. The concentrations of inorganic N (NO_3^--N and NO_2^--N) in the soil were determined using a flow injection analyser (FIA, AQLA-700; Aqualab Co., Ltd., Tokyo, Japan) via colorimetric methods (Saltzman 1954). With the FIA, the NO_3^--N was identified by reducing it to NO_2^--N through a cadmium column. The composition of the solutions employed for the measurement of inorganic N sources is described in Supplementary Table 3. The soil pH was measured using the KCl extract and a pH meter (AS800; AS ONE Corporation, Osaka, Japan) after calibration with standard solutions of pH 4, 7, and 9.

Table 2-2. Reagents used for NO_3^--N and NO_2^--N measurement

Solution	Reagent	Volume
Carrier solution	EDRA-4Na	0.8 g
	Ammonium Chloride	3.0 g
	Milli Q	Up to 1000 ml
R1	Sulfanilamide	1.0 g
	N-1-Naphthylethylenediamine	0.10 g
	Hydrochloric Acid	30 ml
	Milli Q	Up to 1000 ml

2.3.4 Soil DNA extraction and qPCR

Soil DNA extraction was conducted using NucleoSpin® Soil (Takara Bio, Inc., Shiga, Japan) using the supplier's protocol. The SL2 buffer solution was utilized during the DNA extraction process. During DNA extraction, 0.2 g of skim milk was incorporated to counteract the strong adhesion of DNA to the Andosol (Takada Hoshino and Matsumoto 2005). The DNA extracts were preserved in a freezer at −20°C until subsequent analysis. To determine the abundance of denitrification functional genes (*narG*, *nirS*, *norB*, and *nosZ*) in the soil (Fig. 1), quantitative real-time PCR was executed using a CFX96 Touch Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mix for qPCR analysis was prepared by incorporating 10.4 μL of the Kapa SYBR Fast Master Mix (2×) from the Kapa SYBR Fast qPCR Kit (Agilent Technologies, Palo Alto, CA, USA), 0.5 μL of the forward primer, and 0.5 μL of the reverse primer for each functional gene (Table 2). The final volume was adjusted to 20 μL using nuclease-free water. The cycle conditions corresponding to each functional gene are depicted in Table 2 (Philippot et al. 2002; Braker, Fesefeldt, and Witzel 1998; Sun et al. 2017; Henry et al. 2006; Caporaso et al. 2011).

Table 2-3. Target, primers, sequences and PCR cycle information for denitrification genes and 16S rRNA

Target	Primers	Sequence	PCR cycle	References
narG	**narG 1960F**	TAYGTSGGSCARGARAA	[1 cycle] 95 °C for 5 min 94 °C for 30 sec 60 °C for 30 sec 72 °C for 30 sec [8 cycles] 94 °C for 30 sec 59 °C for 30 sec (decreased by 0.5°C/cycle) 72 °C for 30 sec [30 cycles] 94 °C for 30 sec 55 °C for 30 sec 72 °C for 1 min and a final elongation at 72 °C for 6 min	(Philippot et al. 2002)
	narG 2650R	TTYTCRTACCABGTBGC		
nirS	**nirS 1F**	CCTAYTGGCCGCCRCART	[1 cycle] 96 °C for 3 min [40 cycles] 95 °C for 30 sec 65 °C for 1 min 72 °C for 1 min and a final elongation at 72 °C for 1 min	(Braker, Fesefeldt, and Witzel 1998)
	nirS 3R	GCCGCCGTCRTGVAGGAA		
norB	**qnorB 2F**	GGNCAYCARGGNTAYGA	[1 cycle] 96 °C for 3min [40 cycles] 95 °C for 30 sec 56 °C for 90 sec 72 °C for 1 min and a final elongation at 72 °C for 1 min	(Sun et al. 2017)
	qnorB 5R	CAKRTGCAKSGCRTGGCAGAA		
nosZ	**nosZ 2F**	CGCRACGGCAASAAGGTSMSSGT	[1 cycle] 95 °C for 10min [40 cycles] 95 °C for 15 sec 60 °C for 30 sec 72 °C for 30 sec and a final elongation at 72 °C for 1min	(S. Henry et al. 2006)
	nosZ 2R	CAKRTGCAKSGCRTGGCAGAA		
16S rRNA	**16S 515F**	GTGCCAGCMGCCGCGGTAA	[1 cycle] 94 °C for 3 min [35 cycles] 94 °C for 45 sec 50 °C for 60 sec 72 °C for 90 sec and a final elongation at 72 °C for 10 min	(Caporaso et al. 2011)
	16S 806R	GGACTACHVGGGTWTCTAAT		

2.3.5 The analysis of soil prokaryotic community structures using 16S rRNA gene amplicon sequencing

The soil samples used for the 16S rRNA experiments were collected from the soil treatments at five-time points: 0, 1, 2, 3 and 4 weeks after the incubation started. These samples were subsequently used for DNA extraction and downstream analyses. To examine the bacterial community structure, the extracted soil DNA, which was also used for the qPCR analyses as mentioned above, was amplified for the V4 region of the 16S rRNA gene using a primer set (amplicon size 250 bp; forward primer 515F: 5'-GGCCAGCMGCCGCGGTAA-3' and reverse primer 806R: 5'-GGACTACHVGGGTWTCTAAT-3'). The PCR mixture comprised of 12.5 µL of AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.5 µL each of forward and reverse primers, and 1.25 µL of the soil DNA, supplemented with nuclease-free water to achieve a total volume of 25 µL The PCR cycling conditions entailed an initial phase at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 sec and a final elongation at 72°C for 10 min. The annealing temperature employed was determined from the Tm values of the utilized primers. The amplification of the region of interest was confirmed through agarose gel electrophoresis, and the PCR products were quantified using a Quantus fluorometer (Promega Corporation, Madison, WI, USA). The extracted DNA was purified using the Agencourt AMPure XP Kit (Beckman Coulter, Inc., Brea, CA, USA) as per the manufacturer's guidelines. The DNA samples were then linked to an Ion Xpress P1 adaptor and each Ion Xpress Barcode (Thermo Fisher Scientific) to create specific Ion Torrent sequencing samples. The PCR samples were formulated with 12.5 µL of AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific), 0.5 µL Ion Xpress P1 Adaptor, 1.0 µL Ion Xpress Barcode, and 1.25 µL of DNA sample, supplemented with nuclease-free water to achieve a total volume of 25 µL. The libraries were purified using the Agencourt AMPure XP Kit (Beckman Coulter). The final length and concentration of the amplicon were verified using a Sensitivity DNA Kit (Agilent Technologies). The library was subsequently diluted to 50 pM, and 25 µL of the target dilution library was loaded onto the Ion 318 Chip (Thermo Fisher Scientific). Emulsion PCR was executed using the Ion Personal Genome Machine (PGM) Hi-Q View Chef Kit (Thermo Fisher Scientific) by the manufacturer's instructions. Following the

collection and enrichment of the ionic spheres, sequencing was performed using the Ion PGM Hi-Q View Sequencing Kit and Ion PGM (Thermo Fisher Scientific). The obtained sequence data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) software package version 2019.7. Sequence quality control was accomplished using the DADA2 pipeline embedded in QIIME2. The Shannon diversity index was computed in QIIME2.

2.3.6 Statistical analysis

All statistical analyses were performed using R ver 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria), and the significance level was set at $p < 0.05$ for all tests except for Dunn's test, where significance was determined at $p < 0.025$. The Shapiro-Wilk test was performed on all obtained data to assess normality. The datas were considered normally distributed if $p > 0.05$, whereas they were considered non-normally distributed if $p \leq 0.05$. For data that followed normal distribution, a Repeated Measures Two-Way Analysis of Variance (RMT-ANOVA) was performed to assess the effects of inoculation treatment and cultivation period. If significant main effects or interactions were observed, multiple comparisons were subsequently conducted using Tukey's HSD test. For data that did not follow a normal distribution, a non-parametric Aligned Rank Transform Analysis of Variance (ART-ANOVA) was employed to evaluate the effects of inoculation treatment and cultivation period. If significant main effects or interactions were detected in the ART-ANOVA, a Kruskal-Wallis test was conducted. If the Kruskal-Wallis test was significant ($p < 0.05$), multiple comparisons were performed using the Dunn test with Benjamini-Hochberg correction.

2.4 Results

2.4.1 Soil pH

The changes in the soil pH over time are shown in Fig. 2-1, and the numerical data and statistical results are presented in Table 2-4a, 2-4b, 2-4c, 2-4d, 2-4e, and 2-4f. The Shapiro-Wilk test indicated that soil pH did not follow a normal distribution, regardless of N treatment. At week 0, regardless of N treatment, the Control and Low Pb treatments had higher pH values compared to the Mid and High Pb treatments. A clear trend for soil pH to decrease over time with increasing Pb concentration was

observed. Significant differences were only observed when N was added, with the Control and Mid Pb treatments showing notably higher pH values than the High Pb treatment in the 1st week. From the 2nd week, a general decreasing trend in pH was observed across all treatments, although no significant differences occurred. An increasing trend in soil pH was observed between the 3rd week and 4th week but only in the Low Pb treatment, regardless of N addition. However, by the 4th week, pH values in all treatments had decreased when compared to week 0.

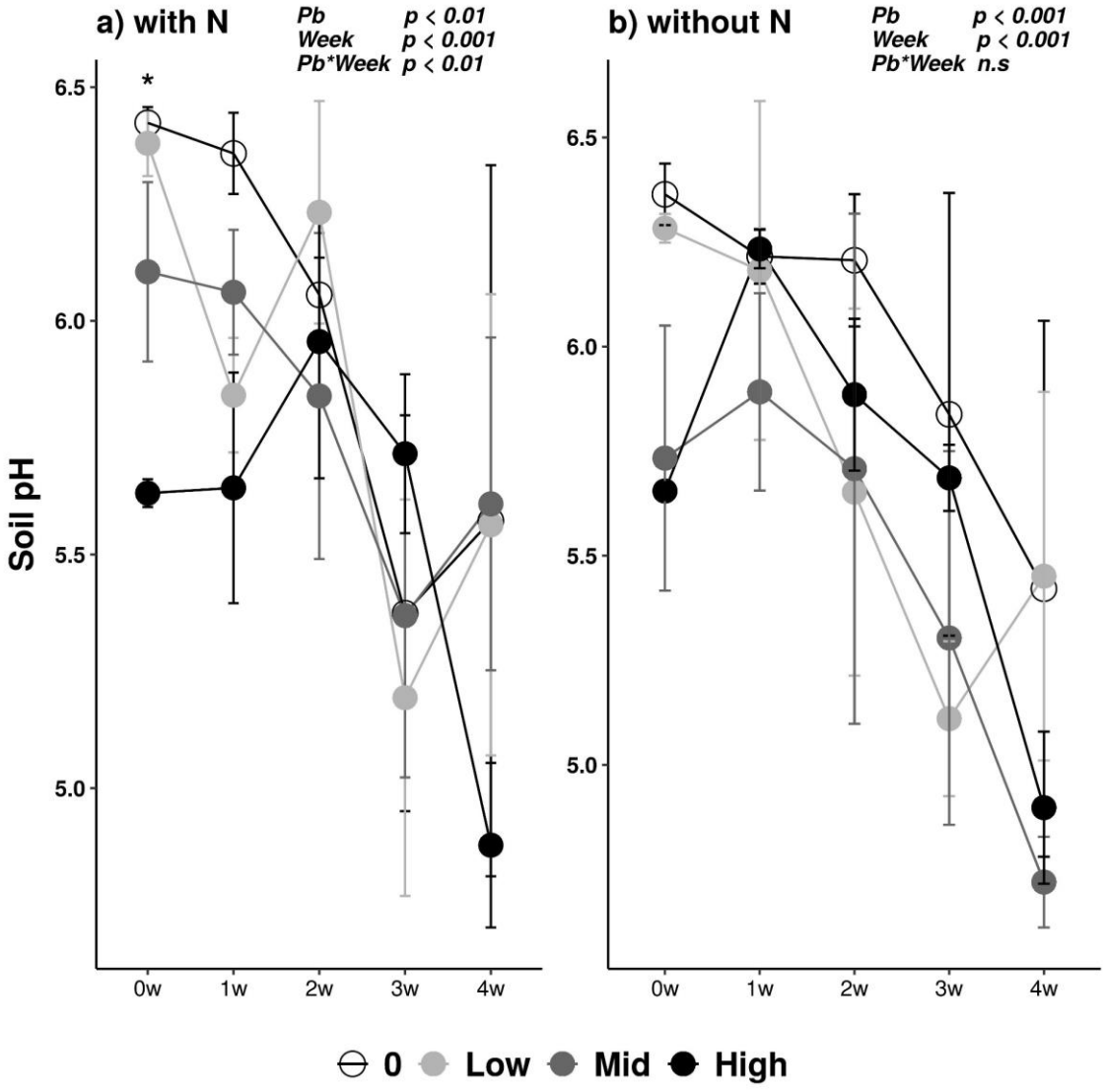

Figure 2-1. The changes in soil pH in soil samples collected each week. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2-4a. The temporal changes in the soil pH (mean ± SD) in the cultured soil of each treatment from the week 0 to the 4th week (n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Soil pH	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	6.42 ± 0.03	6.36 ± 0.09	6.06 ± 0.08	5.37 ± 0.42	5.57 ± 0.76
Low + NO_3^-	6.38 ± 0.07	5.84 ± 0.12	6.23 ± 0.24	5.19 ± 0.42	5.56 ± 0.49
Mid + NO_3^-	6.11 ± 0.19	6.06 ± 0.13	5.84 ± 0.35	5.37 ± 0.35	5.61 ± 0.36
High + NO_3^-	5.63 ± 0.03	5.64 ± 0.25	5.96 ± 0.29	5.72 ± 0.17	4.88 ± 0.18
Control	6.36 ± 0.07	6.22 ± 0.07	6.21 ± 0.16	5.84 ± 0.53	5.42 ± 0.64
Low	6.28 ± 0.03	6.18 ± 0.41	5.65 ± 0.44	5.11 ± 0.19	5.45 ± 0.44
Mid	5.73 ± 0.32	5.89 ± 0.24	5.71 ± 0.61	5.3 ± 0.45	4.72 ± 0.11
High	5.65 ± 0.02	6.23 ± 0.05	5.89 ± 0.18	5.69 ± 0.08	4.9 ± 0.18

Table 2-4b. The results of the Shapiro-Wilk test for the soil pH: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
Soil pH with N	0.00436
Soil pH without N	0.00180

Table 2-4c. The results of the Kruskal-Wallis test for non-normally distributed soil pH: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

Shannon diversity index	p-value (With N)	p-value (Without N)
0w	0.0287*	0.0329*
1w	0.0273*	0.1916
2w	0.4329	0.2479
3w	0.3125	0.1718
4w	0.2044	0.1234

Table 2-4d. The results of Dunn's test for non-normally distributed soil pH with N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

Soil pH with N at 0w	Control	Low	Mid	High
Control	-			
Low	0.2853	-		
Mid	0.1000	0.1687	-	
High	0.0164*	0.0405	0.1925	-

Table 2-4e. The results of Dunn's test for non-normally distributed soil pH with N addition at 1w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

Soil pH with N at 1w	Control	Low	Mid	High
Control	-			
Low	0.3727	-		
Mid	0.3650	0.3388	-	
High	0.3253	0.3863	0.3904	-

Table 2-4f. The results of Dunn's test for non-normally distributed soil pH without N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

Soil pH without N at 0w	Control	Low	Mid	High
Control	-			
Low	0.3428	-		
Mid	0.0472	0.0847	-	
High	0.0382	0.0542	0.3670	-

2.4.2 Inorganic -N dynamics and N_2O fluxes

The changes in the concentrations of NO_3^--N, NO_2^--N, and accumulative N_2O-N over time are shown in Fig. 2-2, and the numerical data and statistical results are presented in Table 2-5a, 2-5b, 2-5c, 2-5d, 2-5e, 2-5f, and 2-5g. The Shapiro-Wilk test indicated that the NO_3^--N content did not follow a normal distribution, regardless of nitrogen addition. In the week 0, the High Pb treatment exhibited the highest NO_3^- concentration, irrespective of the N treatment. With the addition of N, the Low Pb treatment demonstrated the highest NO_3^- concentration throughout the experiment, followed by the High Pb treatment (Fig. 2-2a). In the absence of N the Low Pb treatment displayed the highest NO_3^- concentrations in the 3rd and 4th weeks. NO_2^--N amounts also did not follow a normal distribution with or without N addition, as indicated by the Shapiro-Wilk test. With the addition of N peak NO_2^- concentrations occurred after the 1st week for the High Pb treatment, while the peak occurred in the week 0 for all other treatments. Additionally, from the 1st to the 4th week, with N, the NO_2^- levels were highest for the High Pb treatment (Fig. 2-2c). Without N, the High Pb treatment exhibited the highest NO_2^- levels at the onset of the incubation period. However, from the 1st to the 4th week, all treatments exhibited a similar decreasing trend, the NO_2^- concentrations was < 1 mg N/kg soil at the 4th week (Fig. 2-2d). The Shapiro-Wilk test indicated that the N_2O-N content did not follow a normal distribution, regardless of nitrogen addition. In terms of N_2O emissions, with the addition of N, the High Pb treatment displayed the highest cumulative N_2O values, with emissions approximately doubling from the 1st to the 4th week (Fig. 2-2e). Without N, no significant changes were observed in the cumulative N_2O values from the 1st week to the 4th week in the Control, Low, and Mid Pb treatments. In the High Pb treatment without N, a substantial increase in N_2O emissions was noted from the 2nd to the 3rd week, reaching levels comparable to the Control (Fig. 2-2f).

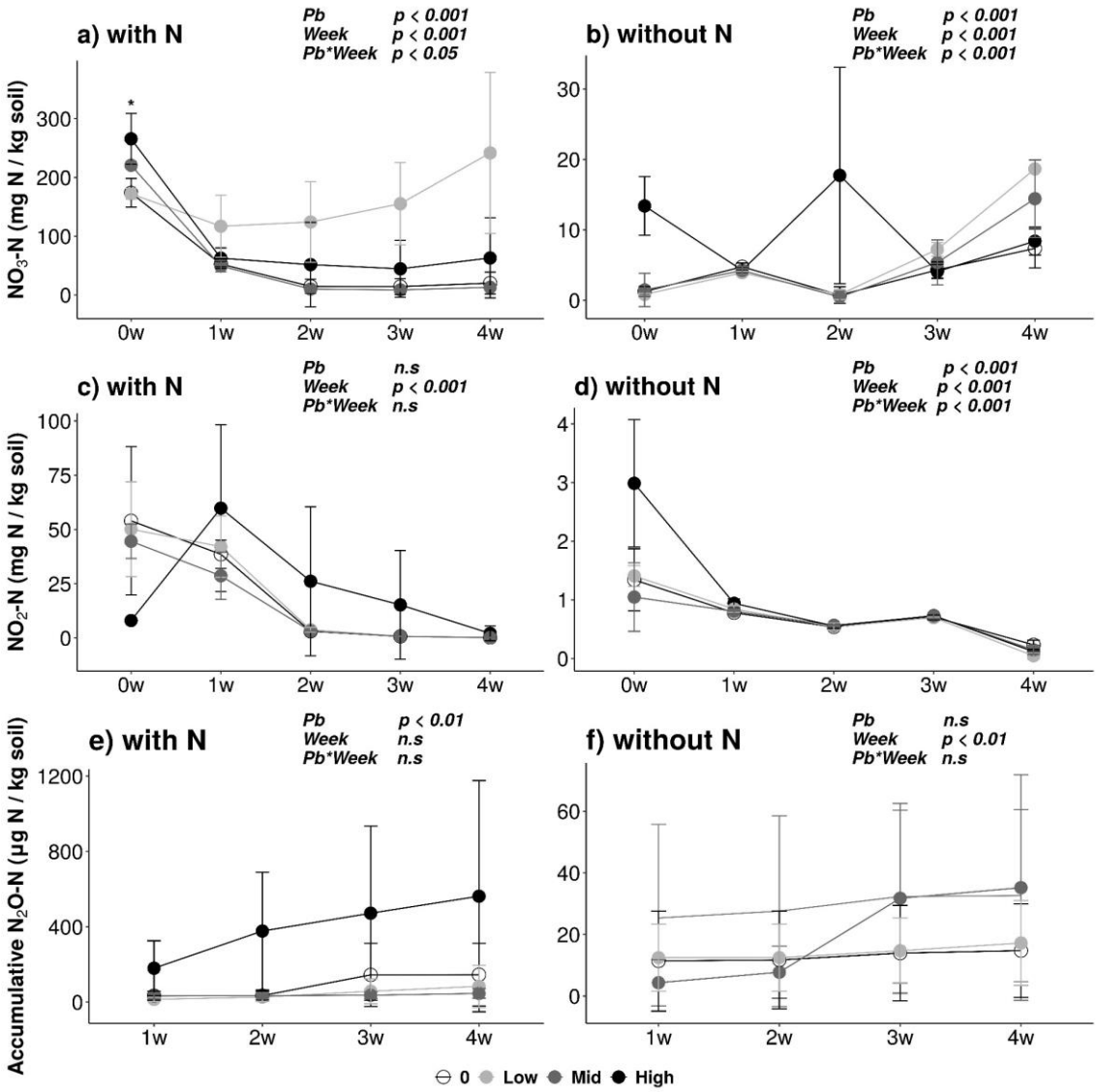

Figure 2-2. The changes in the contents of NO_3^--N, NO_2^--N, and N_2O-N in soil over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2-5a. The temporal changes in the contents of NO_3^--N, NO_2^--N and N_2O emission (mean ± SD) in the cultured soil of each treatment from the week 0 to the 4th week (n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment. ND = No data

NO_3^--N (mg N/ kg soil)	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	173.8 ± 24.4	52.5 ± 4.7	14.7 ± 12	14.3 ± 13.6	20.2 ± 18.6
Low + NO_3^-	172.2 ± 9.4	117 ± 52.7	124.2 ± 68.6	155.3 ± 69.9	241.7 ± 137
Mid + NO_3^-	220.7 ± 6.3	49.2 ± 9.7	10.1 ± 6.2	8.6 ± 2.2	13.1 ± 2.2
High + NO_3^-	265.8 ± 43.4	62.6 ± 17.5	51.6 ± 71.8	44.5 ± 48.4	63 ± 68.2
Control	1.3 ± 0.7	4.8 ± 0.5	0.7 ± 1.2	4.3 ± 1.1	7.4 ± 2.8
Low	0.8 ± 0.2	3.9 ± 0.2	0.8 ± 0.7	7.2 ± 1.0	18.7 ± 0.2
Mid	1.5 ± 2.4	4.2 ± 0.6	0.5 ± 0.8	5.4 ± 3.2	14.4 ± 5.5
High	13.4 ± 4.2	4.3 ± 0.4	17.7 ± 15.4	4.1 ± 1.0	8.4 ± 2.0
NO_2^--N (mg N/ kg soil)	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	53.98 ± 34.18	38.55 ± 6.54	3.02 ± 0.08	0.70 ± 0.04	0.10 ± 0.04
Low + NO_3^-	50.10 ± 21.83	42.09 ± 14.12	3.74 ± 1.15	0.71 ± 0.05	0.05 ± 0.05
Mid + NO_3^-	44.54 ± 7.93	28.64 ± 10.83	3.13 ± 0.05	0.72 ± 0.01	0.17 ± 0.02
High + NO_3^-	8.00 ± 1.03	59.79 ± 38.42	26.12 ± 34.35	15.22 ± 25.05	2.08 ± 3.42
Control	1.34 ± 0.53	0.78 ± 0.01	0.54 ± 0.06	0.71 ± 0.05	0.23 ± 0.08
Low	1.41 ± 0.18	0.84 ± 0.06	0.54 ± 0.06	0.70 ± 0.02	0.05 ± 0.06
Mid	1.05 ± 0.58	0.80 ± 0.05	0.57 ± 0.01	0.72 ± 0.05	0.15 ± 0.08
High	2.99 ± 1.09	0.94 ± 0.08	0.55 ± 0.04	0.73 ± 0.00	0.12 ± 0.01
N_2O-N (μg N/ kg soil)	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	ND	34.1 ± 23.4	34.5 ± 22.8	144.7 ± 167.4	145.6 ± 166.6
Low + NO_3^-	ND	14.6 ± 11.1	26.8 ± 23.9	57.5 ± 66.6	84 ± 112.3
Mid + NO_3^-	ND	32.5 ± 16.5	34.2 ± 18	36.6 ± 18.4	46 ± 25.5
High + NO_3^-	ND	180.1 ± 145.5	377.4 ± 312.6	472.1 ± 462.3	562.4 ± 613.7
Control	ND	25.4 ± 30.4	27.6 ± 31	32.3 ± 28	32.6 ± 27.9
Low	ND	11.4 ± 16.2	11.7 ± 15.9	13.9 ± 15.5	14.7 ± 15.2
Mid	ND	12.5 ± 10.9	12.5 ± 10.9	14.7 ± 10.6	17.2 ± 13.7
High	ND	4.3 ± 7.5	7.7 ± 8.5	31.7 ± 30.8	35.2 ± 36.6

Table 2-5b. The results of the Shapiro-Wilk test for inorganic nitrogen content: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
NO_3^--N with N	6.55×10^{-6}
NO_3^--N without N	2.28×10^{-7}
NO_2^--N with N	2.61×10^{-8}
NO_2^--N without N	4.04×10^{-9}
N_2O-N with N	2.09×10^{-11}
N_2O-N without N	1.46×10^{-11}

Table 2-5c. The results of the Kruskal-Wallis test for non-normally distributed inorganic nitrogen content: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

NO_3^--N	p-value (With N)	p-value (Without N)
0w	0.0345*	0.0752
1w	0.2479	0.3467
2w	0.1349	0.6167
3w	0.1176	0.2479
4w	0.1129	0.0378*
NO_2^--N	p-value (With N)	p-value (Without N)
0w	0.0345*	0.07524
1w	0.2479	0.3467
2w	0.1349	0.6167
3w	0.1176	0.2479
4w	0.1129	0.0378*
N_2O-N	p-value (With N)	p-value (Without N)
0w	-	-
1w	0.0656	0.4577
2w	0.7016	0.1940
3w	0.5719	0.8496
4w	0.9473	0.8803

Table 2-5d. The results of Dunn's test for non-normally distributed NO_3^--N content with N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

NO_3^--N with N at 0w	Control	Low	Mid	High
Control	-			
Low	0.4549	-		
Mid	0.0671	0.0700	-	
High	0.0353	0.0523	0.3428	-

Table 2-5e. The results of Dunn's test for non-normally distributed NO_3^--N content without N addition at 4w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

NO_3^--N without N at 4w	Control	Low	Mid	High
Control	-			
Low	0.0706	-		
Mid	0.0542	0.4405	-	
High	0.4549	0.0472	0.0525	-

Table 2-5f. The results of Dunn's test for non-normally distributed NO_2^--N content with N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

NO_2^--N with N at 0w	Control	Low	Mid	High
Control	-			
Low	0.4549	-		
Mid	0.0671	0.0700	-	
High	0.0353	0.0523	0.3428	-

Table 2-5g. The results of Dunn's test for non-normally distributed NO_2^--N content without N addition at 4w : If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

NO_2^--N without N at 4w	Control	Low	Mid	High
Control	-			
Low	0.0706	-		
Mid	0.0542	0.4405	-	
High	0.4549	0.0472	0.0525	-

2.4.3 The abundance of denitrification genes

The changes in the log copy number of *narG*, *nirS*, *norB* and *nosZ* genes over time are shown in Fig. 2-3, and the numerical data and statistical results are presented in Table 2-6a, 2-6b, 2-6c, 2-6d, 2-6e, 2-6f, 2-6g, 2-6h, 2-7a, 2-7b, 2-7c, 2-7d, and 2-7e. For the denitrification functional genes, significant impacts of the Pb treatments on their abundances were observed, although the relationships were not linear. The Shapiro-Wilk test revealed that the abundance of the *narG* gene did not follow a normal distribution with N addition but followed a normal distribution only in the absence of N addition. The *narG* gene abundance in the High Pb treatment demonstrated the lowest abundance in the week 0 regardless of the N addition (Fig. 2-3a, 2-3b). The High Pb treatment with N addition exhibited the lowest *narG* abundance among all treatments at the week 0 and 1st week. The *narG* abundance in the Control treatment increased in the 4th week. Without N, the Control treatment exhibited significantly higher values of *narG* abundance than all other Pb-contaminated treatments at the 4th week (Fig. 2-3b). The Shapiro-Wilk test indicated that the *nirS* abundance did not follow a normal distribution, regardless of N addition. For the *nirS* gene, the Mid Pb treatment exhibited the highest abundance during the 0th, 2nd, and 3rd week with N addition (Fig. 2-3c). In contrast, there was a different trend from these at the 1st week, with the Control being significantly higher than the Mid Pb treatments. The entire amount of *nirS* abundance without N addition was less changed than with the addition of a nitrogen source (Fig. 2-3d). The abundance of *norB* gene also did not follow a normal distribution with or without N addition, as indicated by the Shapiro-Wilk test. The abundance of the *norB* gene increased over time with the addition of N in all Pb treatments (Fig. 2-3e). In the Mid and High Pb treatments, the abundance of the *norB* gene was relatively low in the 1st week but increased from the 2nd to the 4th week. Furthermore, in the 2nd and 3rd weeks, all Pb treatments exhibited higher *norB* abundance than the Control. The Low Pb treatments had significantly higher *norB* abundance than the Control in the 3rd week. Conversely, without N, the High Pb treatment demonstrated the highest *norB* abundance throughout the entire experimental period, although no significant differences were observed (Fig. 2-3f). The Shapiro-Wilk test indicated that the *nosZ* abundance followed a normal distribution with and without N addition. With N, the relationship between *nosZ* abundance and Pb concentration varied with time (Fig. 2-3g). Initially, the Control showed the highest *nosZ* abundance and the High Pb treatment

showed the lowest. However, in the 3rd and 4th weeks, all Pb treatments exhibited higher *nosZ* abundance than the Control. Furthermore, the Mid and High Pb treatments were significantly higher than the Control at the 3rd week. Without N, there was no significant difference in *nosZ* abundance throughout the entire experimental period (Fig. 2-3h).

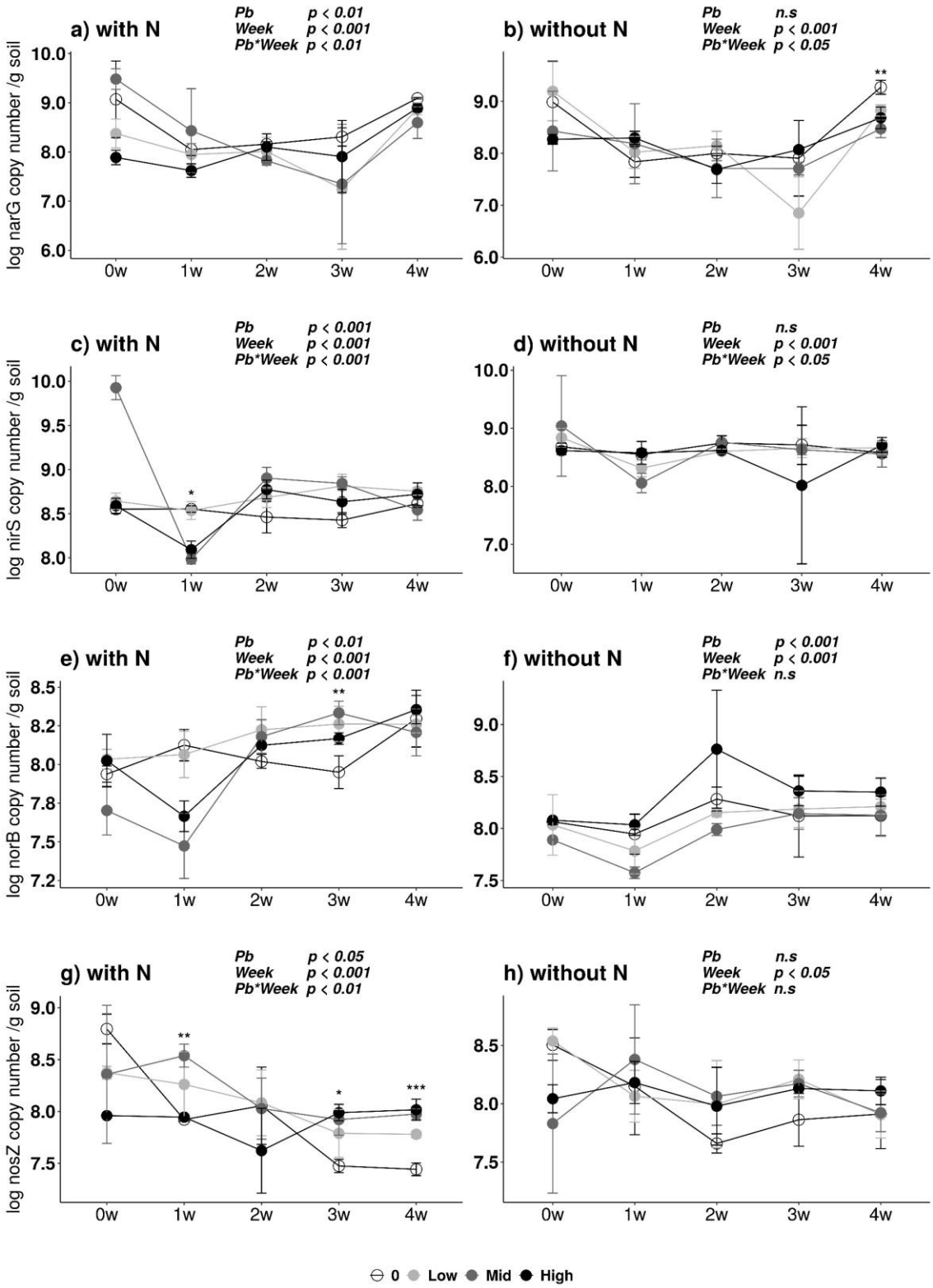

Figure 2-3. The changes in the log copy numbers of denitrification genes, *narG*, *nirS*, *norB*, and *nosZ* in soil over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2-6a. The temporal changes in the log copy number of *narG* gene and *nirS* gene (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 4th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

narG **(log copy number / g soil)**	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	9.07 ± 0.78	8.05 ± 0.12	8.16 ± 0.05	8.31 ± 0.19	9.09 ± 0.01
Low + NO_3^-	8.37 ± 0.29	7.95 ± 0.21	8.01 ± 0.29	7.25 ± 1.23	8.88 ± 0.12
Mid + NO_3^-	9.48 ± 0.21	8.43 ± 0.86	7.82 ± 0.08	7.35 ± 1.21	8.60 ± 0.32
High + NO_3^-	7.89 ± 0.15	7.62 ± 0.14	8.10 ± 0.27	7.91 ± 0.73	8.90 ± 0.08
Control	8.99 ± 0.79	7.84 ± 0.3	8.00 ± 0.14	7.91 ± 0.73	9.27 ± 0.14 **b**
Low	9.19 ± 0.57	8.02 ± 0.31	8.15 ± 0.28	6.85 ± 0.70	8.83 ± 0.11 **a**
Mid	8.43 ± 0.77	8.19 ± 0.77	7.71 ± 0.56	7.71 ± 0.12	8.47 ± 0.17 **a**
High	8.27 ± 0.09	8.30 ± 0.13	7.69 ± 0.27	8.07 ± 0.05	8.68 ± 0.21 **a**
nirS **(log copy number / g soil)**	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	8.55 ± 0.05	8.55 ± 0.04	8.46 ± 0.18	8.43 ± 0.08	8.61 ± 0.04
Low + NO_3^-	8.64 ± 0.09	8.54 ± 0.10	8.68 ± 0.11	8.81 ± 0.13	8.75 ± 0.04
Mid + NO_3^-	9.93 ± 0.14	7.99 ± 0.05	8.90 ± 0.12	8.84 ± 0.08	8.54 ± 0.12
High + NO_3^-	8.59 ± 0.08	8.09 ± 0.10	8.77 ± 0.11	8.64 ± 0.14	8.72 ± 0.13
Control	8.68 ± 0.07	8.55 ± 0.09	8.55 ± 0.09	8.55 ± 0.09	8.55 ± 0.09
Low	8.84 ± 0.16	8.31 ± 0.15	8.31 ± 0.15	8.31 ± 0.15	8.31 ± 0.15
Mid	9.04 ± 0.87	8.06 ± 0.17	8.06 ± 0.17	8.06 ± 0.17	8.06 ± 0.17
High	8.61 ± 0.07	8.58 ± 0.20	8.58 ± 0.20	8.58 ± 0.20	8.58 ± 0.20

Table 2-6b. The results of the Shapiro-Wilk test for denitrification genes: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
narG with N	0.00891
narG without N	0.11432
nirS with N	4.80×10^{-7}
nirS without N	4.16×10^{-10}

Table 2-6c. The results of the Kruskal-Wallis test for non-normally distributed denitrification genes: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

narG	p-value (With N)	p-value (Without N)
0w	0.0418*	-
1w	0.1834	-
2w	0.3595	-
3w	0.3611	-
4w	0.0416*	-
nirS	p-value (With N)	p-value (Without N)
0w	0.062	0.3379
1w	0.02998*	0.05467
2w	0.04148*	0.0719
3w	0.04148*	0.9883
4w	0.1916	0.4925

Table 2-6d. The results of Dunn's test for non-normally distributed *narG* abundance with N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

narG with N at 0w	Control	Low	Mid	High
Control	-			
Low	0.1849	-		
Mid	0.3670	0.1742	-	
High	0.0353	0.1597	0.0276	-

Table 2-6e. The results of Dunn's test for non-normally distributed *narG* abundance with N addition at 4w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

narG with N at 4w	Control	Low	Mid	High
Control	-			
Low	0.1173	-		
Mid	0.0135*	0.1682	-	
High	0.1250	0.4101	0.1435	-

Table 2-6f. The results of Dunn's test for non-normally distributed *nirS* abundance with N addition at 1w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

nirS with N at 1w	Control	Low	Mid	High
Control	-			
Low	0.2311	-		
Mid	0.0197*	0.0894	-	
High	0.0814	0.2190	0.2140	-

Table 2-6g. The results of Dunn's test for non-normally distributed *nirS* abundance with N addition at 2w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

nirS with N at 2w	Control	Low	Mid	High
Control	-			
Low	0.1410	-		
Mid	0.3904	0.1627	-	
High	0.2311	0.3253	0.2115	-

Table 2-6h. The results of Dunn's test for non-normally distributed *nirS* abundance with N addition at 3w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

nirS with N at 3w	Control	Low	Mid	High
Control	-			
Low	0.0261	-		
Mid	0.0523	0.5000	-	
High	0.2190	0.1058	0.1410	-

T**able 2-7a.** The temporal changes in the log copy number of *norB* gene and *nosZ* gene (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 4th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

norB **(log copy number / g soil)**	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	7.94 ± 0.05	8.12 ± 0.10	8.02 ± 0.05	7.95 ± 0.11	8.30 ± 0.18
Low + NO_3^-	8.03 ± 0.06	8.06 ± 0.15	8.22 ± 0.15	8.26 ± 0.11	8.26 ± 0.06
Mid + NO_3^-	7.70 ± 0.16	7.47 ± 0.21	8.18 ± 0.11	8.33 ± 0.08	8.21 ± 0.15
High + NO_3^-	8.02 ± 0.17	7.67 ± 0.10	8.12 ± 0.02	8.17 ± 0.04	8.35 ± 0.09
Control	8.06 ± 0.03	7.95 ± 0.19	8.28 ± 0.12	8.12 ± 0.40	8.12 ± 0.19
Low	8.03 ± 0.29	7.78 ± 0.22	8.15 ± 0.04	8.19 ± 0.18	8.21 ± 0.07
Mid	7.89 ± 0.004	7.57 ± 0.06	7.99 ± 0.06	8.14 ± 0.15	8.13 ± 0.20
High	8.08 ± 0.01	8.03 ± 0.10	8.76 ± 0.57	8.36 ± 0.14	8.35 ± 0.13
nosZ **(log copy number / g soil)**	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	8.80 ± 0.14	7.92 ± 0.02 **a**	8.05 ± 0.37	7.47 ± 0.06 **a**	7.44 ± 0.06 **a**
Low + NO_3^-	8.37 ± 0.06	8.26 ± 0.31 **ab**	8.08 ± 0.32	7.79 ± 0.23 **ab**	7.78 ± 0.03 **b**
Mid + NO_3^-	8.36 ± 0.67	8.54 ± 0.11 **b**	8.03 ± 0.30	7.92 ± 0.15 **b**	7.97 ± 0.05 **c**
High + NO_3^-	7.96 ± 0.03	7.95 ± 0.003 **a**	7.62 ± 0.41	7.99 ± 0.08 **b**	8.02 ± 0.10 **c**
Control	8.50 ± 0.13	8.15 ± 0.42	7.66 ± 0.08	7.86 ± 0.23	7.91 ± 0.30
Low	8.54 ± 0.11	8.06 ± 0.22	8.00 ± 0.37	8.21 ± 0.17	7.91 ± 0.20
Mid	7.83 ± 0.60	8.38 ± 0.47	8.06 ± 0.25	8.17 ± 0.11	7.92 ± 0.16
High	8.04 ± 0.12	8.18 ± 0.18	7.98 ± 0.33	8.13 ± 0.02	8.11 ± 0.12

Table 2-7b. The results of the Shapiro-Wilk test for *norB* gene and *nosZ gene*: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
norB with N	0.00392
norB without N	0.00155
nosZ with N	0.08339
nosZ without N	0.90472

Table 2-7c. The results of the Kruskal-Wallis test for non-normally distributed *norB* genes: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

norB	p-value (With N)	p-value (Without N)
0w	0.062	0.1834
1w	0.02607*	0.07486
2w	0.0683	0.06271
3w	0.03781*	0.516
4w	0.536	0.3466

Table 2-7d. The results of Dunn's test for non-normally distributed *norB* abundance with N addition at 1w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

norB with N at 1w	Control	Low	Mid	High
Control	-			
Low	0.0321	-		
Mid	0.0807	0.3196	-	
High	0.1000	0.2731	0.3884	-

Table 2-7e. The results of Dunn's test for non-normally distributed *norB* abundance with N addition at 3w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

norB with N at 3w	Control	Low	Mid	High
Control	-			
Low	0.0472	-		
Mid	0.0197*	0.2856	-	
High	0.1597	0.2190	0.1410	-

2.4.4 The abundance of 16S rRNA

The changes in the log copy number of 16S rRNA over time are shown in Fig. 2-4, and the numerical data and statistical results are presented in Table 2-8a, 2-8b, 2-8c, and 2-8d. The Shapiro-Wilk test indicated that the 16S rRNA abundance followed a normal distribution with and without N addition. With the addition of N, the abundance of 16S rRNA was relatively higher in the Control and Low Pb treatments, compared to the Mid and High Pb treatments at the week 0 (Fig. 2-4a). However, this trend reversed, and the highest abundance of 16S rRNA was observed in the High Pb treatment from the 2nd week to the 4th week. When averaged across the Pb treatments, the abundance of 16S rRNA rapidly declined over the incubation period. Without N, trends were similar to those observed with N addition, where the High Pb treatment displayed the lowest 16S rRNA abundance at the week 0 (Fig. 2-4b) and the highest abundance of 16S rRNA from the 3rd to the 4th week.

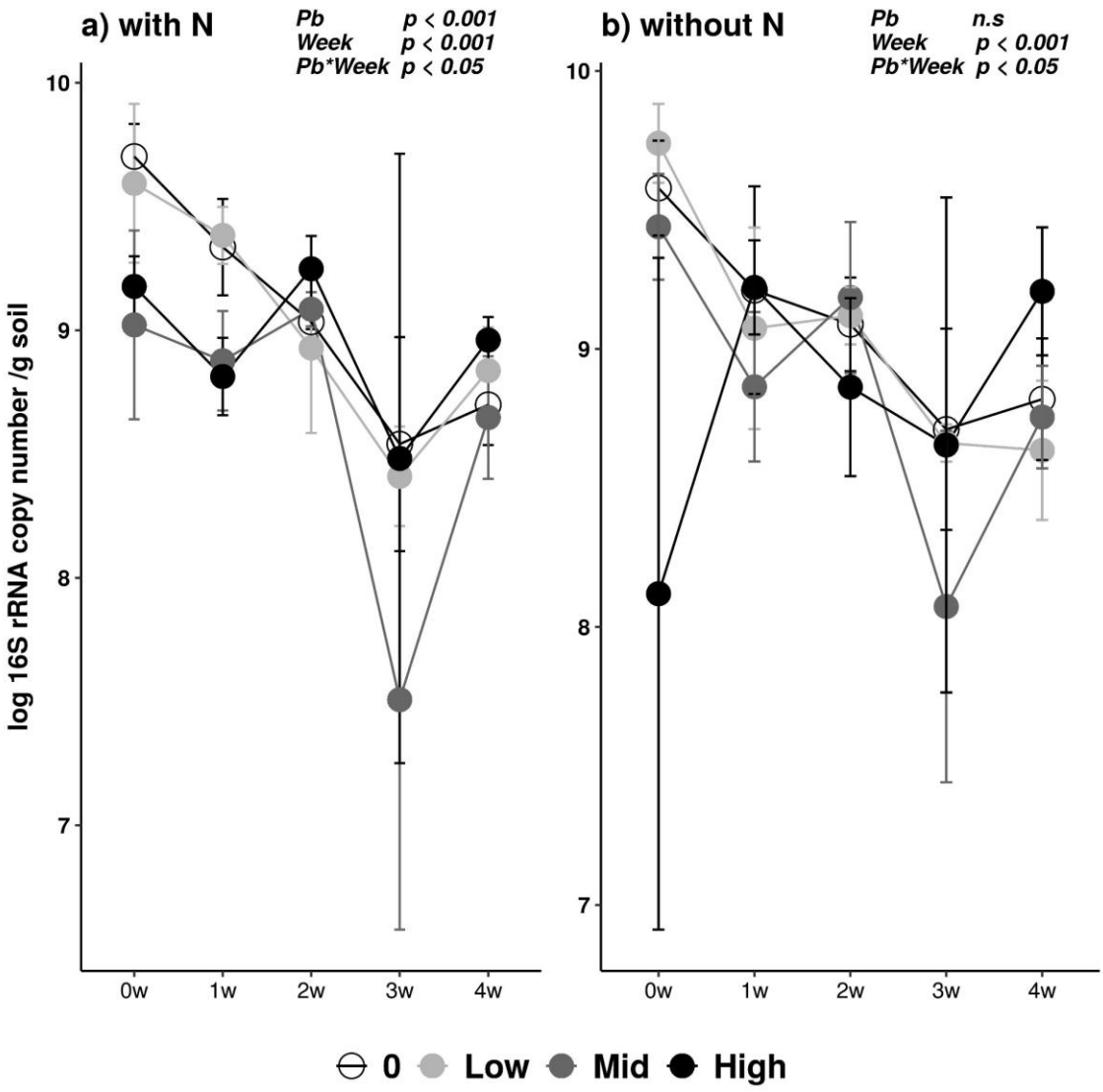

Figure 2-4. The changes in the log copy numbers of 16S rRNA in soil over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2-8a. The temporal changes in the log copy number of 16S rRNA (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 4th week(n=3). Alphabets were shown in the table Only when the Tukey-Kramer analysis determined significant differences between each treatment.

16S rRNA (log copy number / g soil)	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	9.70 ± 0.13	9.34 ± 0.19	9.03 ± 0.03	8.54 ± 0.43	8.70 ± 0.16
Low + NO_3^-	9.59 ± 0.32	9.38 ± 0.12	8.93 ± 0.34	8.41 ± 0.20	8.84 ± 0.18
Mid + NO_3^-	9.02 ± 0.38	8.88 ± 0.20	9.09 ± 0.07	7.51 ± 0.93	8.65 ± 0.25
High + NO_3^-	9.18 ± 0.12	8.81 ± 0.16	9.25 ± 0.13	8.48 ± 1.23	8.96 ± 0.09
Control	9.58 ± 0.17	9.21 ± 0.37	9.09 ± 0.17	8.71 ± 0.36	8.82 ± 0.22
Low	9.74 ± 0.14	9.07 ± 0.36	9.12 ± 0.1	8.66 ± 0.07	8.64 ± 0.25
Mid	9.44 ± 0.19	8.86 ± 0.27	9.19 ± 0.27	8.07 ± 0.63	8.76 ± 0.18
High	8.12 ± 1.21	9.22 ± 0.17	8.86 ± 0.32	8.65 ± 0.89	9.21 ± 0.23

Table 2-8b. The results of the Shapiro-Wilk test for the abundance of 16S rRNA: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
16S rRNA with N	5.16×10^{-6}
16S rRNA without N	0.0004

Table 2-8c. The results of the Kruskal-Wallis test for non-normally distributed 16S rRNA: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

16S rRNA	p-value (With N)	p-value (Without N)
0w	0.05343	0.07028
1w	0.0396*	0.4593
2w	0.2072	0.6676
3w	0.3916	0.345
4w	0.2669	0.06564

Table 2-8d. The results of Dunn's test for non-normally distributed 16S rRNA abundance with N addition at 1w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

16S rRNA with N at 1w	Control	Low	Mid	High
Control	-			
Low	0.4773	-		
Mid	0.3655	0.4275	-	
High	0.1036	0.1825	0.1913	-

2.4.5 Shannon diversity index

The changes in the Shannon diversity index over time are shown in Fig. 2-5, and the numerical data and statistical results are presented in Table 2-9a, 2-9b, 2-9c, and 2-9d. The Shapiro-Wilk test indicated that the shannon diversity index followed a normal distribution with and without N addition. For the Shannon diversity index without N addition, the values escalated with increasing Pb levels in the week 0 (Fig. 2-5). With N, although not significant, differences were observed, all Pb treatments displayed higher Shannon diversity index values compared to the Control in the week 0 (Fig. 2-5a). Without N, all Pb treatments similarly exhibited higher values than the Control in the week 0. Moreover, there was an increase in the Shannon diversity index with increasing Pb levels, with the High Pb treatment significantly surpassing the Control (Fig. 2-5b). During the latter stage of the incubation period, Pb had no impact on the treatments without N. However, for the index with N, the value was smallest for the Low Pb level. In contrast, the Mid and High Pb treatments demonstrated values similar to the Control during the 3rd and 4th weeks, with N.

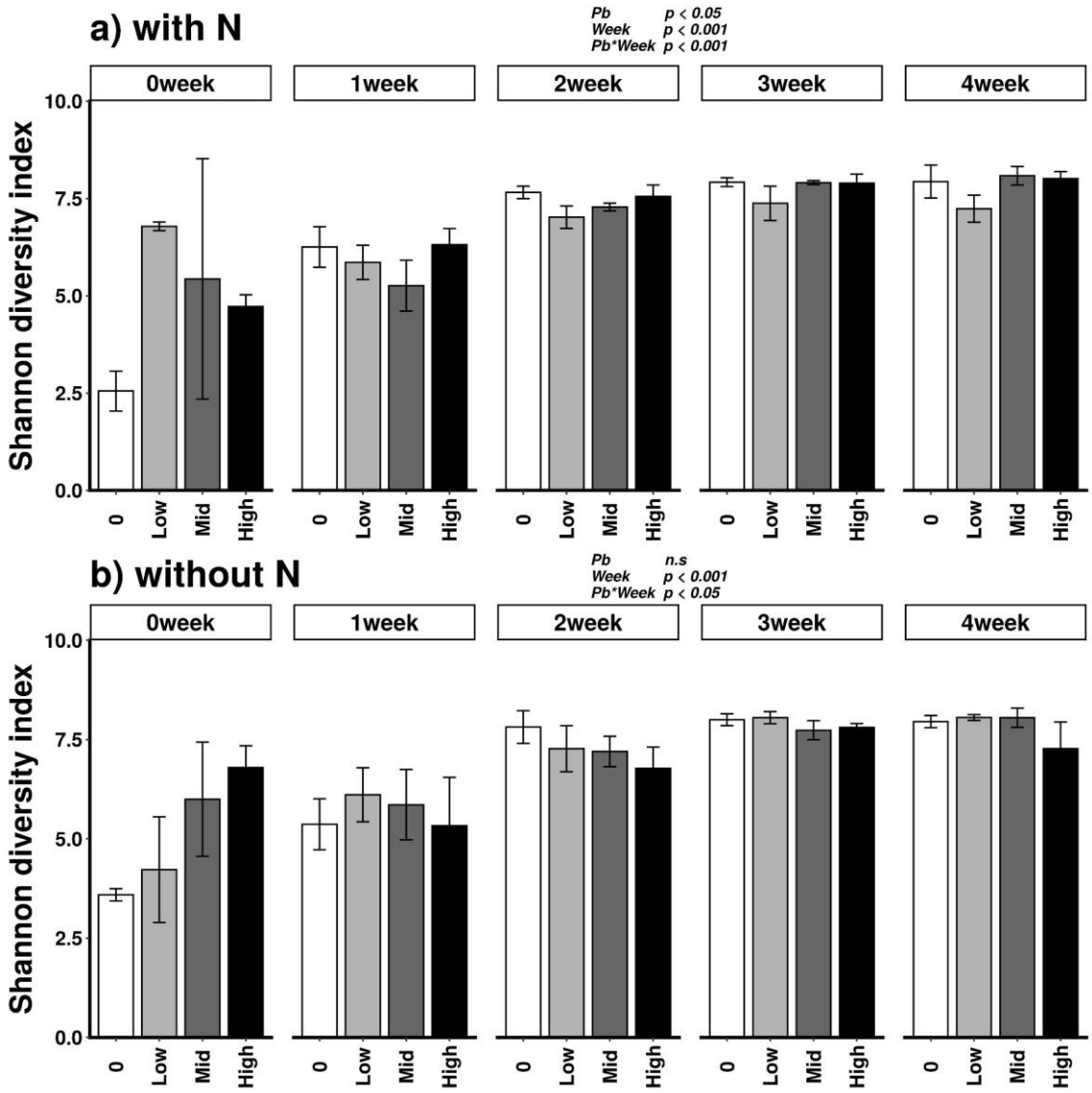

Figure 2-5. The changes in the shannon diversity index in soil samples collected each week. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2-9a. The temporal changes in the Shannon diversity (mean ± SD) in the cultured soil of each treatment from the week 0 to the 4th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Shannon diversity index	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	2.6 ± 0.5	6.3 ± 0.5	7.7 ± 0.2	7.9 ± 0.1	7.9 ± 0.4
Low + NO_3^-	6.8 ± 0.1	5.9 ± 0.4	7 ± 0.3	7.4 ± 0.4	7.2 ± 0.4
Mid + NO_3^-	5.4 ± 3.1	5.3 ± 0.7	7.3 ± 0.1	7.9 ± 0.1	8.1 ± 0.2
High + NO_3^-	4.7 ± 0.3	6.3 ± 0.4	7.6 ± 0.3	7.9 ± 0.2	8.0 ± 0.2
Control	3.6 ± 0.2	5.4 ± 0.6	7.8 ± 0.4	8 ± 0.1	8.0 ± 0.2
Low	4.2 ± 1.3	6.1 ± 0.7	7.3 ± 0.6	8.1 ± 0.2	8.1 ± 0.1
Mid	6.0 ± 1.4	5.9 ± 0.9	7.2 ± 0.4	7.7 ± 0.2	8.1 ± 0.2
High	6.8 ± 0.5	5.3 ± 1.2	6.8 ± 0.5	7.8 ± 0.1	7.3 ± 0.7

Table 2-9b. The results of the Shapiro-Wilk test for Shannon diversity index: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
Shannon diversity index with N	1.69×10^{-7}
Shannon diversity index without N	5.57×10^{-6}

Table 2-9c. The results of the Kruskal-Wallis test for non-normally distributed shannon diversity index: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

Shannon diversity index	p-value (With N)	p-value (Without N)
0w	0.1834	0.0499*
1w	0.1079	0.4593
2w	0.0824	0.1574
3w	0.1473	0.1009
4w	0.1129	0.0752

Table 2-9d. The results of Dunn's test for non-normally distributed shannon diversity index without N addition at 0w: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

Shannon diversity index without N at 0w	Control	Low	Mid	High
Control	-			
Low	0.3253	-		
Mid	0.0542	0.1058	-	
High	0.0523	0.0814	0.3904	-

2.4.6 Community structure at the phylum level

The taxonomic characteristics of soil microbial communities at the phylum level, for the four levels of Pb and five distinct incubation periods, are depicted in Fig. 2-6. These were divided into conditions of non-N addition and NO_3^- addition. In the absence of N addition, 41 phyla were identified from all read sequences, while 45 phyla were identified with NO_3^- addition. The top 10 phyla are presented in Table 2-10a, 2-10b, 2-10c, and 2-10d. Among these top 10 phyla, 9 phyla were common to both N addition treatments, while *Verrucomicrobia* was only confirmed in the non-N addition, and *Bacteroidetes* was only confirmed in the NO_3^- addition. In the week 0, the microbial community structure underwent significant change due to Pb. Proteobacteria displayed the lowest abundance in the High Pb treatment, being significantly lower in the High Pb treatment compared to the Control, particularly with NO_3^- addition. In contrast, *Actinobacteria*, *Chloroflexi*, *Nitrospirae*, *Gemmatimonadetes*, and *Latescibacteria* exhibited significantly higher abundances in the High Pb treatment compared to the Control. Additionally, *Planctomycetes* and *Verrucomicrobia* demonstrated significantly higher abundances in the High Pb treatment compared to the Control, but only without N. On the other hand, *Acidobacteria* and *Firmicutes* showed significantly higher abundances in the High Pb treatment compared to the Control, but only with N. In the 4th week, notable differences in microbial community structure were not observed, except for *Firmicutes*. *Firmicutes* displayed the highest abundance in the High Pb treatment, regardless of N addition, and a significantly higher abundance compared to the Control, particularly without N addition. Conversely, *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, *Planctomycetes*, *Latescibacteria*, and *Verrucomicrobia* demonstrated the lowest abundances in the High Pb treatment, regardless of N addition. Specifically, *Planctomycetes* and *Verrucomicrobia* without N and *Latesbacteria* with N showed significantly lower abundances in the High Pb treatment compared to the Control.

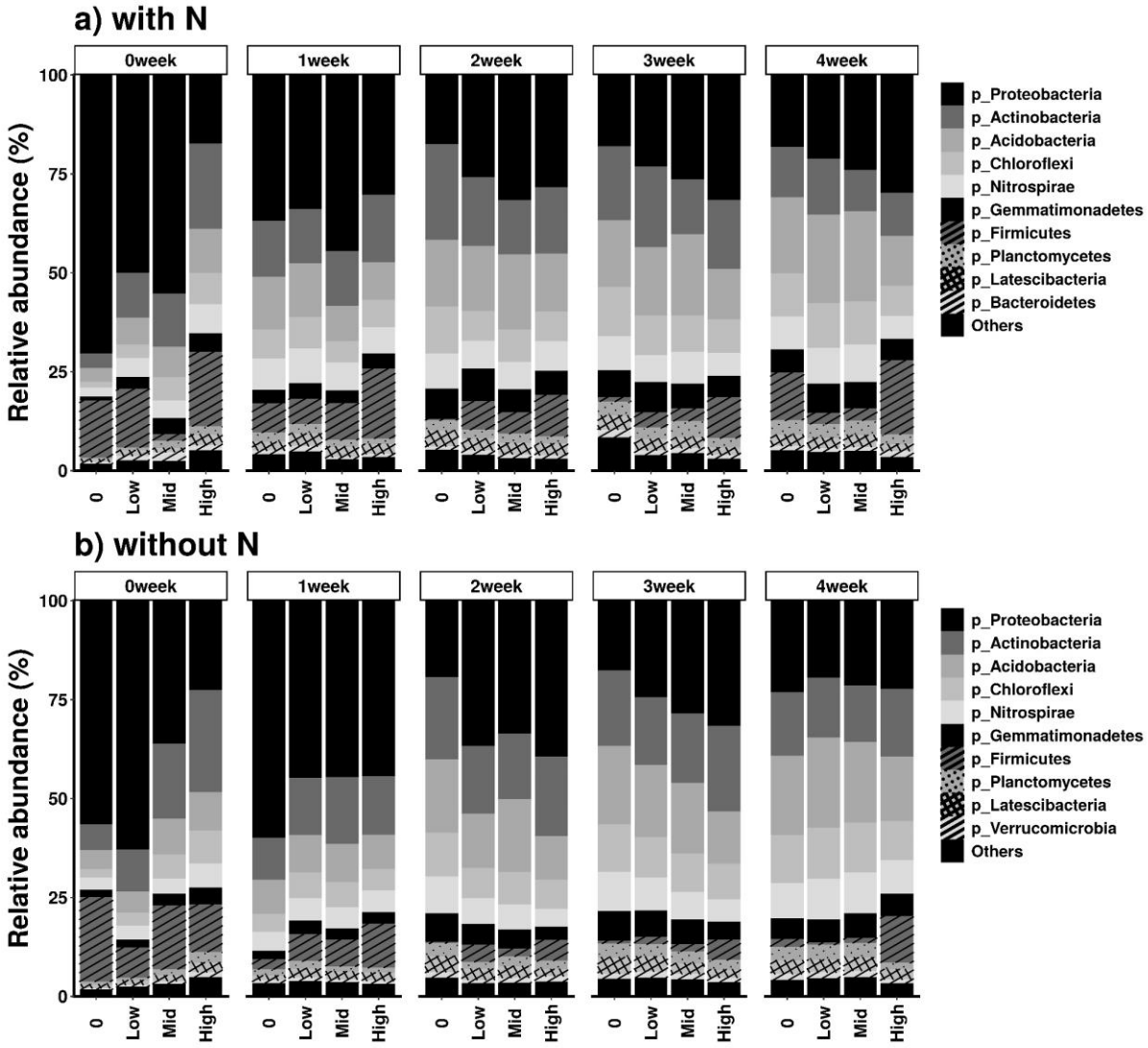

Figure 2-6. The changes in microbial community structure at the phylum level over time with non-nitrogen addition and NO_3^- addition. "Others" indicates the relative abundance of all other phyla observed, excluding the top ten phyla.

Table 2-10a. The temporal changes in the relative abundances (%) of *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* in the cultured soil of each treatment from the week 0 to the 4th week (mean ± SD). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Proteobacteria	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	70.3 ± 10.7 **b**	36.8 ± 5.6	17.4 ± 0.7 **a**	18 ± 1.7 **a**	18.1 ± 2.8
Low + NO_3^-	50 ± 2.2 **ab**	33.9 ± 7.2	25.8 ± 3.1 **b**	23.2 ± 2.3 **ab**	21.2 ± 3.6
Mid + NO_3^-	55.3 ± 24.9 **b**	44.4 ± 12.8	31.6 ± 3.6 **b**	26.4 ± 2.2 **bc**	24 ± 1.0
High + NO_3^-	17.3 ± 3.8 **a**	30.2 ± 6.8	28.4 ± 0.7 **b**	31.5 ± 4.2 **c**	29.8 ± 8.2
Control	56.4 ± 18.9	59.9 ± 10.5	19.3 ± 1.8	17.6 ± 1.4 **a**	23.1 ± 2.4
Low	62.9 ± 15.4	44.8 ± 11.8	36.6 ± 10	24.3 ± 4.5 **ab**	19.4 ± 2.5
Mid	36 ± 17.7	44.5 ± 17.5	33.6 ± 9.4	28.5 ± 6.0 **ab**	21.3 ± 1.9
High	22.6 ± 6.7	44.3 ± 19.4	39.4 ± 9.4	31.6 ± 5.6 **b**	22.3 ± 1.0
Actinobacteria	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	3.7 ± 1.3 **a**	14.2 ± 3.3	24.2 ± 1.9 **b**	18.8 ± 2.2	12.8 ± 3.1
Low + NO_3^-	11.3 ± 1.1 **ab**	13.7 ± 2.6	17.4 ± 3.7 **a**	20.3 ± 0.9	14.1 ± 3.2
Mid + NO_3^-	13.4 ± 8.7 **ab**	13.9 ± 2.7	13.7 ± 0.8 **a**	13.8 ± 4.3	10.4 ± 1.2
High + NO_3^-	21.5 ± 2.1 **b**	17.1 ± 1.5	16.7 ± 1.4 **a**	17.5 ± 2.3	10.8 ± 4.1
Control	6.5 ± 1.1 **a**	10.6 ± 3.4	20.8 ± 1.9	19.1 ± 2.7 **ab**	16 ± 4.2
Low	10.6 ± 4.3 **a**	14.3 ± 1.6	17.2 ± 2.5	17.1 ± 4.0 **a**	15.2 ± 0.2
Mid	19 ± 9.6 **ab**	16.9 ± 5.0	16.5 ± 3.5	17.5 ± 1.2 **ab**	14.3 ± 0.9
High	25.8 ± 4.1 **b**	14.8 ± 5.4	20 ± 8.1	21.5 ± 2.3 **b**	17.1 ± 1.2
Acidobacteria	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	3.5 ± 1.2 **a**	13.2 ± 2.7	16.9 ± 1.2	16.8 ± 1.9	19.2 ± 4.0 **ab**
Low + NO_3^-	6.7 ± 1.2 **ab**	13.6 ± 1.6	16.3 ± 1.1	17.4 ± 1.5	22.3 ± 1.8 **b**
Mid + NO_3^-	7.7 ± 4.5 **ab**	9.0 ± 1.6	18.9 ± 2.2	20.5 ± 2.5	22.8 ± 0.3 **b**
High + NO_3^-	11.2 ± 0.4 **b**	9.4 ± 1.8	14.6 ± 2.3	12.8 ± 5.0	12.6 ± 4.8 **a**
Control	4.8 ± 0.5	8.6 ± 1.7	18.5 ± 1.8 **b**	19.7 ± 2.0 **b**	20 ± 2.5 **ab**
Low	5.4 ± 2.1	9.5 ± 2.0	13.7 ± 2.5 **a**	18.2 ± 2.2 **ab**	22.7 ± 1.0 **b**
Mid	9.0 ± 3.1	9.6 ± 2.3	18.5 ± 0.9 **b**	17.9 ± 3.1 **ab**	20.4 ± 1.3 **ab**
High	9.6 ± 0.8	8.7 ± 3.0	11.1 ± 0.7 **a**	13.3 ± 1.6 **a**	16.2 ± 1.8 **a**

Table 2-10b. The temporal changes in the relative abundances (%) of *Chloroflexi*, *Nitrospirae,* and *Gemmatimonadetes* in the cultured soil of each treatment from the week 0 to the 4th week (mean ± SD). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Chloroflexi	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	1.5 ± 0.7 **a**	7.4 ± 1.6	12 ± 0.9 **b**	12.5 ± 1.1 **b**	10.9 ± 3.1
Low + NO_3^-	3.4 ± 0.9 **ab**	7.9 ± 1.7	7.6 ± 1.8 **a**	10.0 ± 0.6 **a**	11.3 ± 1.6
Mid + NO_3^-	5.8 ± 3.7 **ab**	5.4 ± 1.7	8.2 ± 0.6 **a**	9.2 ± 0.9 **a**	10.9 ± 0.7
High + NO_3^-	7.9 ± 0.8 **b**	7.0 ± 0.8	7.6 ± 0.7 **a**	8.4 ± 1.0 **a**	7.7 ± 0.6
Control	2.2 ± 0.6 **a**	4.6 ± 1.2	11.1 ± 0.8	12.1 ± 0.1 **b**	12.1 ± 1.6 **ab**
Low	3.2 ± 1.6 **a**	6.5 ± 1.7	7.7 ± 1.2	10.2 ± 0.5 **ab**	13.0 ± 0.2 **b**
Mid	6.1 ± 3.4 **ab**	6.4 ± 1.4	8.2 ± 1.5	9.7 ± 0.6 **a**	12.6 ± 0.3 **b**
High	8.4 ± 0.9 **b**	5.3 ± 2.3	7.4 ± 2.2	9.0 ± 1.4 **a**	10.0 ± 0.9 **a**
Nitrospirae	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	2.2 ± 1.1 **a**	8.0 ± 1.1	8.8 ± 1.0 **b**	8.7 ± 1.2 **b**	8.3 ± 1.4 **ab**
Low + NO_3^-	4.8 ± 1.0 **ab**	8.8 ± 1.5	7.0 ± 0.1 **ab**	6.8 ± 0.7 **ab**	9.1 ± 1.0 **b**
Mid + NO_3^-	4.5 ± 2.3 **ab**	7.0 ± 1.7	7.0 ± 0.8 **a**	8.1 ± 1.3 **ab**	9.5 ± 0.6 **b**
High + NO_3^-	7.3 ± 0.9 **b**	6.6 ± 1.7	7.5 ± 0.4 **ab**	5.8 ± 0.6 **a**	5.7 ± 1.1 **a**
Control	1.8 ± 0.2 **a**	3.4 ± 1	4.8 ± 0.3 **b**	4.5 ± 0.6 **ab**	4.1 ± 0.4
Low	2.4 ± 1.5 **a**	3.9 ± 2	3.4 ± 1.2 **a**	4.8 ± 0.5 **b**	4.6 ± 0.3
Mid	3.2 ± 0.9 **ab**	3.6 ± 1.1	3.5 ± 0.6 **a**	4.3 ± 1.2 **a**b	4.9 ± 0.7
High	4.9 ± 1.0 **b**	3.2 ± 0.7	3.7 ± 1.2 **a**	3.6 ± 0.3 **a**	3.3 ± 0.7
Gemmatimonadetes	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	1.0 ± 0.6 **a**	3.4 ± 0.4	7.7 ± 1.1 **bc**	6.8 ± 0.9	5.8 ± 1.6
Low + NO_3^-	3.0 ± 0.4 **ab**	3.9 ± 0.3	8.2 ± 0.4 **c**	7.7 ± 0.9	7.3 ± 0.3
Mid + NO_3^-	4.0 ± 2.6 **ab**	3.2 ± 0.4	5.8 ± 0.3 **a**	6.2 ± 0.9	6.6 ± 0.2
High + NO_3^-	4.7 ± 0.2 **b**	3.8 ± 0.6	6.1 ± 0.6 **ab**	5.4 ± 1.0	5.4 ± 1.5
Control	1.8 ± 0.3 **a**	2.2 ± 0.4	7.2 ± 0.4 **b**	7.4 ± 0.3 **b**	5.3 ± 0.2
Low	2.0 ± 0.9 **a**	3.4 ± 0.3	5.3 ± 1.2 **ab**	6.7 ± 1.1 **b**	5.8 ± 0.2
Mid	3.0 ± 0.9 **ab**	2.8 ± 0.9	4.9 ± 0.9 **ab**	6.1 ± 0.6 **ab**	6.3 ± 0.7
High	4.2 ± 0.5 **b**	2.9 ± 1.1	3.4 ± 1.2 **a**	4.5 ± 0.6 **a**	5.6 ± 0.3

Table 2-10c. The temporal changes in the relative abundances (%) of *Firmicutes, Planctomycetes, Verrucomicrobia*, and *Bacteroidetes* in the cultured soil of each treatment from the week 0 to the 4th week (mean ± SD). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Firmicutes	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	14.5 ± 11.7 **b**	7.3 ± 3.3 **a**	0.3 ± 0.1 **a**	1.1 ± 0.5 **a**	12.1 ± 17.6
Low + NO_3^-	14.9 ± 2.5 **ab**	6.4 ± 2.2 **a**	7.3 ± 4.0 **ab**	3.8 ± 2.1 **ab**	2.9 ± 1.9
Mid + NO_3^-	1.7 ± 1.0 **a**	9.3 ± 3.6 **a**	5.3 ± 1.3 **ab**	3.2 ± 0.8 **ab**	3.1 ± 1.1
High + NO_3^-	18.9 ± 5.0 **b**	17.7 ± 1.5 **b**	10.5 ± 4.4 **b**	10.4 ± 9.3 **b**	18.7 ± 12.7
Control	21.4 ± 21.2	2.5 ± 0.4	0.3 ± 0.1	0.8 ± 0.6 **a**	1.9 ± 0.4 **a**
Low	7.6 ± 3.0	6.7 ± 3.3	4.3 ± 2.0	1.8 ± 0.8 **a**	0.6 ± 0.3 **a**
Mid	16.1 ± 15	6.8 ± 6.0	1.9 ± 1.0	1.9 ± 0.9 **a**	1.2 ± 0.5 **a**
High	12 ± 1.4	11.1 ± 3.2	5.2 ± 7.2	5.2 ± 1.8 **b**	11.8 ± 3.3 **b**
Planctomycetes	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.4 ± 0.3	2.4 ± 0.8	2.7 ± 0.4	3.6 ± 1.1	3.8 ± 1.2
Low + NO_3^-	0.7 ± 0.3	2.5 ± 1.0	2.3 ± 0.4	3.0 ± 0.3	3.5 ± 0.3
Mid + NO_3^-	1.9 ± 1.4	1.7 ± 0.5	2.5 ± 0.4	4.2 ± 0.5	3.7 ± 0.7
High + NO_3^-	2.1 ± 0.7	1.4 ± 0.2	1.8 ± 0.4	2.4 ± 0.5	2.4 ± 0.2
Control	0.7 ± 0.4 **a**	1.6 ± 0.9	3.3 ± 0.5	3.5 ± 0.6	3.5 ± 0.7 **b**
Low	0.6 ± 0.5 **a**	2.0 ± 1.2	1.8 ± 0.2	3.2 ± 0.2	3.8 ± 0.5 **b**
Mid	1.6 ± 1.1 **ab**	1.3 ± 0.2	2.5 ± 0.8	3.0 ± 0.7	3.7 ± 0.7 **b**
High	2.7 ± 0.6 **b**	1.3 ± 0.3	2.2 ± 1.1	2.4 ± 0.2	1.7 ± 0.6 **a**
Verrucomicrobia	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control	0.3 ± 0.01 **a**	0.7 ± 0.3	1.4 ± 0.1	1.3 ± 0.1 **ab**	1.5 ± 0.1 **b**
Low	0.5 ± 0.4 **a**	0.9 ± 0.2	0.8 ± 0.2	1.5 ± 0.1 **b**	1.5 ± 0.2 **b**
Mid	0.8 ± 0.2 **ab**	0.8 ± 0.2	1.3 ± 0.1	1.4 ± 0.2 **ab**	1.6 ± 0.1 **b**
High	1.2 ± 0.03 **b**	0.7 ± 0.3	1.4 ± 1.0	1.1 ± 0.1 **a**	1.0 ± 0.04 **a**
Bacteroidetes	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.5 ± 0.3	1.0 ± 0.1 **ab**	0.9 ± 0.2	2.0 ± 1.4	1.1 ± 0.1
Low + NO_3^-	1.1 ± 0.1	1.4 ± 0.3 **b**	1.1 ± 0.1	1.3 ± 0.6	0.9 ± 0.1
Mid + NO_3^-	2.3 ± 1.8	0.6 ± 0.1 **a**	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
High + NO_3^-	1.3 ± 0.2	0.9 ± 0.02 **a**	1.0 ± 0.3	1.0 ± 0.1	1.5 ± 0.8

Table 2-10d. The temporal changes in the relative abundances (%) of Others in the cultured soil of each treatment from the week 0 to the 4th week (mean ± SD). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Others	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	1.7 ± 0.8	4.1 ± 1.2	5.3 ± 0.6	8.4 ± 4.9	5.2 ± 1.2
Low + NO_3^-	2.6 ± 0.6	4.9 ± 1.3	4.0 ± 1.2	4.0 ± 0.5	4.7 ± 0.2
Mid + NO_3^-	2.4 ± 1.4	2.9 ± 0.6	3.2 ± 0.4	4.4 ± 0.2	5.0 ± 0.2
High + NO_3^-	5.2 ± 0.3	3.4 ± 0.5	3.0 ± 0.3	3.0 ± 0.7	3.4 ± 0.9
Control	3.0 ± 0.7	4.7 ± 1.4	9.3 ± 0.2	9.9 ± 1.4	8.9 ± 1.0
Low	3.6 ± 1.5	5.7 ± 1.3	6.4 ± 1.3	8.4 ± 1.8	10.2 ± 0.9
Mid	3.8 ± 0.8	5.4 ± 1.1	6.3 ± 0.7	7.0 ± 1.2	10.2 ± 0.8
High	6.2 ± 0.4	5.5 ± 2.1	4.5 ± 1.0	5.7 ± 0.9	8.4 ± 1.0

2.4.7 Community structure at the genus level

The taxonomic characteristics of soil microbial communities at the genus level for the four levels of Pb and five sample times are depicted in Fig. 2-7. The abundances of the 15 most abundant genera are presented in Table 2-11a, 2-11b, 2-11c, 2-11d, 2-11e, and 2-11f. In the week 0, *Azotobacter* and *Bacillus* were dominant, and microbial community structures varied significantly with Pb concentration. The abundance of *Azotobacter* decreased to less than half of the Control's under Pb levels exceeding 400 mg Pb/kg soil. Additionally, the abundance of *Azotobacter* in the Control was significantly higher than in the High Pb treatment with N. *Gemmatimonadaceae*, *Gaiellales*, *Nitrospirales_0319-6A21*, and *Latescibacteria* exhibited significantly higher abundance in the High Pb treatment compared to the Control, regardless of N addition. Similar trends were observed with *Nocardiodaceae* without N and with *Nocardioides*, *Holophagae_Subgroup7*, and *Choloflexi_KD4-96* with N. In the 2nd week, *Gemmatimonadaceae* and *Acidobacteria_Subgroup4* displayed significantly higher abundance in the Control compared to the High Pb treatment. Similarly, in the 3rd week, *Nitrospirales_0319-6A21* and *Latescibacteria* demonstrated significantly higher abundance in the Control than in the High Pb treatment. In the 4th week, similar microbial community structures were observed across all treatments, both with and without N addition, unlike in the week 0. Specifically, the relative abundances of *Azotobacter* and *Bacillus*, which were dominant in the week 0, decreased to below 1% and 3%, respectively.

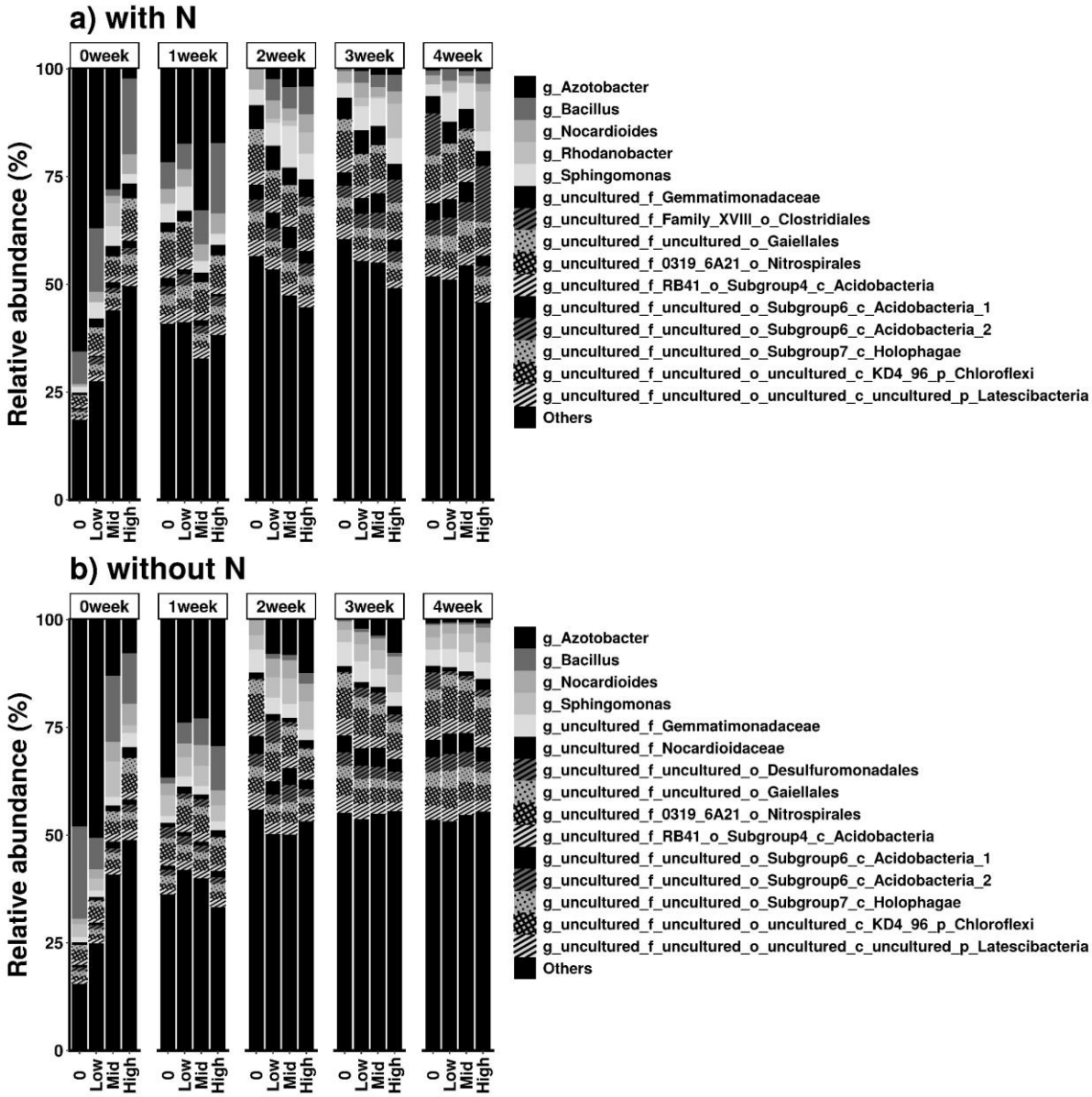

Figure 2-7. The changes in microbial community structure at the genus level over time at the phylum level with non-nitrogen addition and NO_3^- addition. ”Others” indicates the relative abundance of all other phyla observed except for the top fifteen genera.

Table 2-11a. The temporal changes in the relative abundances (%) of *Azotobacter, Bacillus*, and *Nocardioides* in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Azotobacter	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	65.6 ± 10.8 **b**	21.7 ± 6.1	0 ± 0	0.1 ± 0.1	0.4 ± 0.2
Low + NO_3^-	37 ± 3.1 **ab**	17.4 ± 5.5	2.3 ± 0.5	0.5 ± 0.1	0.03 ± 0.03
Mid + NO_3^-	28 ± 46.1 **ab**	32.8 ± 13.8	4.2 ± 4.1	1.4 ± 0.6	0.5 ± 0.3
High + NO_3^-	2.2 ± 2.8 **a**	17.3 ± 5.9	4.1 ± 1.0	1.4 ± 1.7	0.5 ± 0.7
Control	48 ± 18.1	36.6 ± 10.8	0 ± 0	0.04 ± 0.1	0.9 ± 0.7
Low	50.6 ± 18.3	23.9 ± 12.5	7.9 ± 8.8	2.1 ± 0.4	0.7 ± 0.6
Mid	13 ± 20.9	22.9 ± 20.8	8.2 ± 6.5	3.7 ± 5.6	0.6 ± 0.8
High	7.8 ± 7.9	29.4 ± 22.2	12.4 ± 4.3	7.6 ± 3.2	0.9 ± 0.4
Bacillus	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	7.6 ± 2.2 **b**	6.2 ± 2.7 **a**	0.2 ± 0.04 **a**	0.5 ± 0.3	1.2 ± 0.4
Low + NO_3^-	14.8 ± 2.5 **c**	5.9 ± 1.9 **a**	5.0 ± 2.5 **b**	2.8 ± 1.8	2.7 ± 1.9
Mid + NO_3^-	1.4 ± 1.2 **a**	7.9 ± 3.4 **a**	5.0 ± 1.2 **b**	2.8 ± 0.6	1.1 ± 0.1
High + NO_3^-	17.6 ± 2.9 **c**	16.3 ± 1.5 **b**	6.5 ± 1.4 **b**	3.8 ± 2.4	2.9 ± 2.2
Control	21.4 ± 21.2	1.3 ± 0.4	0.2 ± 0.1	0.4 ± 0.3	0.3 ± 0.1 **a**
Low	7.4 ± 2.9	4.8 ± 1.6	1.2 ± 0.3	0.8 ± 0.2	0.3 ± 0.1 **a**
Mid	15.4 ± 14.9	6.2 ± 6.5	1.3 ± 0.9	0.6 ± 0.2	0.5 ± 0.2 **ab**
High	11.7 ± 1.5	10.2 ± 3.6	2.4 ± 3.6	0.9 ± 0.2	0.9 ± 0.3 **b**
Nocardioides	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.6 ± 0.2 **a**	3.3 ± 0.9	4.6 ± 0.6 **b**	2.6 ± 0.7	2.0 ± 0.3
Low + NO_3^-	2.4 ± 0.04 **b**	4.0 ± 1.2	4.1 ± 0.2 **b**	3.5 ± 0.6	2.3 ± 0.5
Mid + NO_3^-	1.8 ± 0.9 **ab**	3.7 ± 1.0	2.7 ± 0.3 **a**	2.3 ± 0.8	1.6 ± 0.3
High + NO_3^-	4.6 ± 0.8 **c**	4.6 ± 0.3	4.1 ± 0.6 **b**	2.9 ± 0.7	1.8 ± 0.7
Control	1.4 ± 0.4	2.7 ± 1.2	3.5 ± 0.3	2.0 ± 1.0	2.9 ± 0.8
Low	2.1 ± 0.7	3.2 ± 0.3	4.3 ± 0.8	2.8 ± 0.7	2.2 ± 0.2
Mid	4.5 ± 2.6	4.8 ± 1.4	4.2 ± 0.8	2.7 ± 0.6	2.1 ± 0.6
High	5.0 ± 1.4	3.5 ± 1.1	4.1 ± 2.2	4.2 ± 1.1	3.5 ± 1.2

Table 2-11b. The temporal changes in the relative abundances (%) of *Sphingomonas, Gemmatimonadaceae*, *Nocardioidaceae,* and *Desulfuromonadales* in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Sphingomonas	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	1.3 ± 0.4	4.2 ± 1.8 **ab**	3.6 ± 0.5 **a**	3.3 ± 0.7	2.7 ± 0.7 **a**
Low + NO_3^-	3.8 ± 0.8	5.5 ± 1.2 **b**	5.4 ± 0.8 **a**	5.6 ± 0.8	6.7 ± 2.2 **b**
Mid + NO_3^-	4.6 ± 3.8	2.5 ± 0.4 **a**	9.7 ± 1.0 **b**	6.5 ± 0.4	6.0 ± 0.9 **ab**
High + NO_3^-	2.2 ± 0.6	2.5 ± 0.2 **a**	5.9 ± 2.2 **a**	6.0 ± 4.2	4.6 ± 1.6 **ab**
Control	2.9 ± 0.5 **a**	4.9 ± 1.8	3.3 ± 0.6	2.8 ± 1.0	2.8 ± 0.9
Low	2.8 ± 0.5 **a**	4.4 ± 1.6	4.8 ± 1.7	3.9 ± 0.5	3.6 ± 0.9
Mid	8.2 ± 1.1 **b**	4.6 ± 2.5	6.0 ± 1.7	4.3 ± 0.9	4.2 ± 0.9
High	1.8 ± 0.1 **a**	3.7 ± 0.8	6.6 ± 0.8	4.1 ± 0.1	4.6 ± 0.5
Gemmatimonadaceae	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.6 ± 0.4 **a**	2.3 ± 0.2	5.7 ± 0.8 **b**	4.9 ± 0.9	4.0 ± 1.0
Low + NO_3^-	2.1 ± 0.4 **ab**	2.5 ± 0.2	5.7 ± 0.3 **b**	5.4 ± 0.9	5.1 ± 0.4
Mid + NO_3^-	2.5 ± 1.6 **ab**	2.2 ± 0.5	4.1 ± 0.1 **a**	4.4 ± 0.6	4.6 ± 0.2
High + NO_3^-	3.4 ± 0.1 **b**	2.5 ± 0.3	4.1 ± 0.4 **a**	3.7 ± 0.5	3.5 ± 0.8
Control	1.3 ± 0.3 **a**	1.6 ± 0.3	5.4 ± 0.4 **b**	5.6 ± 0.1 **b**	3.8 ± 0.2
Low	1.5 ± 0.7 **a**	2.4 ± 0.1	3.8 ± 1.0 **ab**	4.9 ± 1.0 **b**	4.2 ± 0.1
Mid	2.1 ± 0.5 **ab**	1.9 ± 0.7	3.2 ± 0.6 **a**	4.3 ± 0.4 **ab**	4.5 ± 0.6
High	3.3 ± 0.5 **b**	2.1 ± 0.9	2.4 ± 0.7 **a**	3.2 ± 0.4 **a**	3.8 ± 0.4
Nocardioidaceae	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control	0.6 ± 0.1 **a**	1.0 ± 0.4	1.5 ± 0.3	1.4 ± 0.5	1.6 ± 0.4 **ab**
Low	0.6 ± 0.1 **a**	1.5 ± 0.5	1.5 ± 0.6	1.3 ± 0.4	1.0 ± 0.3 **a**
Mid	1.3 ± 0.7 **a**	1.2 ± 0.1	0.9 ± 0.7	1.4 ± 0.5	1.0 ± 0.5 **a**
High	2.5 ± 0.1 **b**	1.5 ± 0.7	1.9 ± 1.1	2.0 ± 0.4	2.6 ± 0.8 **b**
Desulfuromonadales	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control	0.1 ± 0.1	2.5 ± 0.6	0.3 ± 0.1	0.3 ± 0.04	3.9 ± 1.2 **b**
Low	0.3 ± 0.1	1.2 ± 1.1	5.1 ± 4.4	2.2 ± 3.1	0.8 ± 1.0 **a**
Mid	0.1 ± 0.1	1.6 ± 1.9	1.0 ± 1.0	2.5 ± 3.1	1.0 ± 0.9 **a**
High	0.2 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	1.7 ± 1.4	1.5 ± 0.9 **ab**

Table 2-11c. The temporal changes in the relative abundances (%) of *Clostridiales, Rhodanobacter*, *Gaiellaless,* and *Nitrospirales_0319_6A21* in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Clostridiales	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.1	9.7 ± 16.8
Low + NO_3^-	0.0004 ± 0.007	0.01 ± 0.01	0.003 ± 0.01	0.2 ± 0.1	0.001 ± 0.001
Mid + NO_3^-	0 ± 0	0.05 ± 0.1	0 ± 0	0 ± 0	0.5 ± 0.4
High + NO_3^-	0 ± 0	0.03 ± 0.03	2.1 ± 2.0	5.3 ± 8.4	13 ± 13.7
Rhodanobacter	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control	0 ± 0	0.3 ± 0.1	0.02 ± 0.02 **a**	0.3 ± 0.3	0.1 ± 0.1
Low	0.02 ± 0.03	0.1 ± 0.1	1.0 ± 1.2 **a**	2.0 ± 1.7	0.5 ± 0.7
Mid	5.3 ± 7.1	0.3 ± 0.05	1.3 ± 0.1 **a**	0.3 ± 0.1	0.1 ± 0.1
High	0.01 ± 0.02	0.2 ± 0.1	5.0 ± 1.9 **b**	8.0 ± 6.6	9.3 ± 9.1
Gaiellaless	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.5 ± 0.2 **a**	1.8 ± 0.4	3.7 ± 0.6 **b**	2.8 ± 0.4	2.2 ± 0.9
Low + NO_3^-	1.6 ± 0.3 **ab**	1.4 ± 0.3	1.8 ± 0.9 **a**	2.4 ± 0.4	2.3 ± 0.3
Mid + NO_3^-	1.1 ± 0.5 **a**	1.8 ± 0.3	1.9 ± 0.2 **a**	2.0 ± 0.7	1.9 ± 0.2
High + NO_3^-	2.6 ± 0.8 **b**	2.3 ± 0.8	1.9 ± 0.3 **a**	2.2 ± 0.2	1.5 ± 0.1
Control	1.2 ± 0.1 **a**	1.4 ± 0.5	3.3 ± 0.7	3.5 ± 1.0	2.5 ± 0.6
Low	1.4 ± 1.1 **a**	1.8 ± 0.1	2.2 ± 0.3	2.6 ± 0.3	2.8 ± 0.2
Mid	2.4 ± 0.6 **ab**	2.0 ± 0.5	2.3 ± 0.5	2.6 ± 0.4	2.6 ± 0.2
High	3.5 ± 0.9 **b**	2.0 ± 1.1	1.9 ± 0.7	2.6 ± 0.2	2.7 ± 0.5
Nitrospirales_0319_6A21	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	1.8 ± 0.9 **a**	5.8 ± 0.9	5.9 ± 1.1	6.2 ± 0.7 **b**	5.8 ± 1.0 **ab**
Low + NO_3^-	3.8 ± 0.8 **ab**	6.9 ± 1.0	5.0 ± 0.4	4.8 ± 0.5 **ab**	6.6 ± 0.7 **b**
Mid + NO_3^-	3.4 ± 1.7 **ab**	5.2 ± 1.4	5.1 ± 0.5	5.8 ± 1.0 **ab**	6.8 ± 0.5 **b**
High + NO_3^-	5.2 ± 0.9 **b**	5.1 ± 1.2	5.7 ± 0.3	4.4 ± 0.4 **a**	4.2 ± 0.9 **a**
Control	2.3 ± 0.5 **a**	3.5 ± 1.1	6.4 ± 0.3 **b**	6.9 ± 1.1 **b**	6.0 ± 1.0
Low	2.7 ± 1.2 **ab**	4.1 ± 1.0	4.4 ± 1.0 **a**	6.0 ± 1.4 **ab**	7.3 ± 0.6
Mid	2.7 ± 0.5 **ab**	3.9 ± 0.8	4.6 ± 0.4 **a**	5.0 ± 1.0 **ab**	7.0 ± 0.4
High	4.2 ± 0.04 **b**	4.2 ± 1.6	3.3 ± 0.8 **a**	3.8 ± 0.6 **a**	6.1 ± 0.9

Table 2-11d. The temporal changes in the relative abundances (%) of *Acidobacteria_Subgroup4, Acidobacteria_Subgroup6_1*, and *Acidobacteria_Subgroup6_2* in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Acidobacteria_Subgroup4	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.7 ± 0.4	2.9 ± 0.7 **b**	3.2 ± 0.6 **b**	3.2 ± 0.3	3.2 ± 0.9
Low + NO_3^-	1.3 ± 0.4	2.8 ± 0.2 **b**	3.0 ± 0.3 **ab**	3.0 ± 0.3	3.9 ± 0.6
Mid + NO_3^-	1.4 ± 0.8	1.7 ± 0.2 **a**	2.6 ± 0.3 **b**	3.4 ± 0.5	3.1 ± 0.02
High + NO_3^-	1.9 ± 0.2	1.6 ± 0.3 **a**	2.8 ± 0.6 **ab**	2.0 ± 0.7	2.1 ± 1.0
Control	1.1 ± 0.3	1.5 ± 0.7	3.3 ± 0.5 **b**	4.0 ± 0.2 **b**	3.1 ± 0.1 **ab**
Low	0.9 ± 0.5	1.8 ± 0.4	2.3 ± 0.6 **ab**	3.3 ± 0.9 **ab**	3.6 ± 0.3 **b**
Mid	1.8 ± 0.5	2.1 ± 0.3	2.8 ± 0.3 **ab**	2.6 ± 0.5 **a**	2.6 ± 0.2 **a**
High	1.8 ± 0.3	1.5 ± 0.9	1.9 ± 0.2 **a**	2.1 ± 0.3 **a**	2.8 ± 0.5 **ab**
Acidobacteria_Subgroup6_1	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.4 ± 0.4	2.0 ± 0.3	3.5 ± 0.1 **a**	3.1 ± 0.5	4.0 ± 1.1
Low + NO_3^-	0.4 ± 0.6	1.1 ± 1.0	3.7 ± 0.3 **a**	3.7 ± 0.7	4.3 ± 0.01
Mid + NO_3^-	1.5 ± 1.3	1.4 ± 0.4	4.9 ± 0.6 **b**	4.6 ± 0.7	4.7 ± 0.4
High + NO_3^-	1.7 ± 0.1	0.6 ± 0.6	2.9 ± 0.6 **a**	2.7 ± 1.2	2.5 ± 0.9
Control	0.5 ± 0.1	1.0 ± 0.4	4.2 ± 1.1	4.0 ± 0.4	4.1 ± 0.7
Low	0.6 ± 0.6	0.9 ± 0.7	3.1 ± 1.0	4.0 ± 0.4	4.8 ± 0.1
Mid	1.6 ± 0.9	1.0 ± 0.7	3.9 ± 0.3	4.4 ± 0.9	4.4 ± 0.7
High	1.3 ± 0.4	1.4 ± 0.3	2.3 ± 0.2	3.0 ± 0.3	3.4 ± 0.4
Acidobacteria_Subgroup6_2	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.6 ± 0.2 **a**	3.3 ± 0.9	4.6 ± 0.6 **b**	2.6 ± 0.7	2.0 ± 0.3
Low + NO_3^-	2.4 ± 0.04 **b**	4.0 ± 1.2	4.1 ± 0.2 **b**	3.5 ± 0.6	2.3 ± 0.5
Mid + NO_3^-	1.8 ± 0.9 **ab**	3.7 ± 1.0	2.7 ± 0.3 **a**	2.3 ± 0.8	1.6 ± 0.3
High + NO_3^-	4.6 ± 0.8 **c**	4.6 ± 0.3	4.1 ± 0.6 **b**	2.9 ± 0.7	1.8 ± 0.7
Control	0.9 ± 0.2	1.4 ± 0.6	2.9 ± 0.6 **bc**	3.1 ± 0.7	3.4 ± 0.4
Low	0.9 ± 0.3	2.0 ± 1.5	2.3 ± 0.3 **ab**	3.1 ± 0.3	3.9 ± 0.2
Mid	1.0 ± 0.8	1.5 ± 0.7	4.0 ± 0.4 **c**	2.8 ± 0.4	3.5 ± 0.2
High	1.6 ± 0.4	1.4 ± 0.8	1.7 ± 0.3 **a**	2.1 ± 0.3	2.5 ± 1.0

Table 2-11e. The temporal changes in the relative abundances (%) of *Holophagae_Subgroup7, Chloroflexi_KD4_96*, and *Latescibacteria* in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3) Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Holophagae_Subgroup7	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.9 ± 0.3 **a**	2.7 ± 0.7 **ab**	2.6 ± 0.4	2.4 ± 0.1	3.2 ± 0.5 **ab**
Low + NO_3^-	1.5 ± 0.3 **a**	3.2 ± 0.5 **b**	1.6 ± 0.3	2.1 ± 0.3	3.8 ± 0.2 **a**
Mid + NO_3^-	1.3 ± 0.6 **a**	1.8 ± 0.4 **a**	2.4 ± 0.4	2.5 ± 0.2	3.5 ± 0.2 **b**
High + NO_3^-	2.6 ± 0.1 **b**	2.1 ± 0.2 **ab**	2.3 ± 0.4	1.9 ± 0.6	2.2 ± 0.7 **a**
Control	1.3 ± 0.2	1.9 ± 0.5	2.6 ± 0.3	2.9 ± 0.5	3.6 ± 0.6 **ab**
Low	1.3 ± 0.4	1.9 ± 0.3	1.8 ± 0.4	2.4 ± 0.2	4.2 ± 0.2 **b**
Mid	1.7 ± 0.2	1.9 ± 0.4	2.4 ± 0.5	2.3 ± 0.5	3.6 ± 0.3 **ab**
High	1.9 ± 0.4	2.0 ± 0.8	1.6 ± 0.4	1.9 ± 0.5	3.0 ± 0.4 **a**
Chloroflexi_KD4_96	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.3 ± 0.3 **a**	2.0 ± 0.2	3.9 ± 0.4 **b**	3.7 ± 0.7	3.5 ± 1.2
Low + NO_3^-	1.0 ± 0.3 **b**	2.2 ± 0.6	1.9 ± 0.6 **a**	2.8 ± 0.1	3.7 ± 0.7
Mid + NO_3^-	1.7 ± 0.9 **ab**	1.4 ± 0.4	2.6 ± 0.2 **a**	2.6 ± 0.7	3.8 ± 0.2
High + NO_3^-	2.2 ± 0.2 **b**	2.1 ± 0.5	2.1 ± 0.1 **a**	2.5 ± 0.5	2.2 ± 0.2
Control	0.7 ± 0.2	1.2 ± 0.2	3.5 ± 0.2	3.8 ± 0.4	4.1 ± 0.7
Low	0.9 ± 0.3	1.7 ± 0.4	2.4 ± 0.7	3.2 ± 0.1	4.4 ± 0.2
Mid	2.0 ± 1.3	2.3 ± 0.9	2.3 ± 0.5	3.3 ± 0.1	4.2 ± 0.1
High	2.1 ± 0.3	1.5 ± 0.5	2.4 ± 1.2	3.0 ± 0.8	3.5 ± 0.5
Latescibacteria	**0 week**	**1 week**	**2 week**	**3 week**	**4 week**
Control + NO_3^-	0.7 ± 0.3 **a**	2.1 ± 0.7	3.9 ± 0.2 **b**	3.5 ± 0.3 **b**	2.7 ± 0.1 **ab**
Low + NO_3^-	1.4 ± 0.4 **a**	3.1 ± 0.04	3.0 ± 0.6 **ab**	2.7 ± 0.5 **ab**	2.7 ± 0.7 **ab**
Mid + NO_3^-	1.0 ± 0.4 **a**	2.6 ± 0.6	2.8 ± 0.3 **a**	2.7 ± 0.5 **ab**	2.9 ± 0.2 **b**
High + NO_3^-	2.7 ± 0.2 **b**	2.5 ± 0.3	2.9 ± 0.3 **ab**	1.8 ± 0.3 **a**	1.8 ± 0.1 **a**
Control	1.0 ± 0.2 **a**	1.2 ± 0.4	3.9 ± 0.3 **b**	4.1 ± 0.4 **b**	3.4 ± 0.7
Low	1.1 ± 0.7 **a**	2.2 ± 0.6	2.7 ± 0.6 **ab**	3.7 ± 1.3 **ab**	3.2 ± 0.3
Mid	1.4 ± 0.6 **ab**	2.0 ± 0.4	2.8 ± 0.6 **ab**	2.7 ± 0.3 **ab**	3.3 ± 0.3
High	2.4 ± 0.2 **b**	2.0 ± 1.0	1.8 ± 0.2 **a**	2.0 ± 0.4 **a**	2.6 ± 0.4

Table 2-11f. The temporal changes in the relative abundances (%) of Others in the cultured soil of each experimental plot from the week 0 to the 4th week (mean ± SD, n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Others	0 week	1 week	2 week	3 week	4 week
Control + NO_3^-	18.6 ± 13.8	40.9 ± 5.6	56.5 ± 1.2	60.5 ± 5.6	51.9 ± 8.6
Low + NO_3^-	27.5 ± 2.6	41.3 ± 2.6	53.5 ± 3.3	55.5 ± 1.9	51.1 ± 2.4
Mid + NO_3^-	44 ± 31.2	32.8 ± 6.0	47.5 ± 1.6	55.1 ± 1.2	54.5 ± 1.0
High + NO_3^-	49.6 ± 2.2	38.2 ± 3.4	44.7 ± 1.0	49.1 ± 4.8	45.8 ± 3.4
Control	15.5 ± 2.6	36.3 ± 5.4	55.9 ± 1.4	55.2 ± 3.0	53.6 ± 4.0
Low	24.9 ± 9.5	42 ± 8.7	50.2 ± 5.2	53.7 ± 1.7	53.2 ± 0.7
Mid	40.9 ± 17.9	39.9 ± 9.2	50.1 ± 5.7	54.9 ± 3.3	54.7 ± 2.5
High	48.9 ± 6.0	33.2 ± 8.6	53.2 ± 8.1	55.6 ± 4.1	55.5 ± 3.0

2.5 Discussion

2.5.1 NO_3^- reduction to NO_2^-

The reduction of NO_3^- to NO_2^-, the initial step in denitrification, was found to be adversely affected by Pb at 1600 mg Pb/kg soil. The presence of Pb^{2+} ions has previously been reported to negatively affect nitrate reductase activity, with a reduction in activity of approximately 90% observed at a Pb concentration of 10 μM (Aiken et al. 2003). Others have also observed reductions of 50% and 99% with soil Pb concentrations of 2.5 μmol/kg and 25 μmol/kg, respectively(Fu and Tabatabai 1989). The Pb concentrations utilized in these previous studies were significantly lower than those in the current study. These findings suggest that Pb can negatively impact nitrate reductase enzyme function even at lower concentrations, corroborating the results of this experiment. In the present study, the abundance of *narG* genes, which are involved in the expression of nitrate reductase enzymes, also diminished in the High Pb treatment. The Pb concentration threshold where denitrification is affected may be soil type dependent. Yang et al. (2024) found that the abundance of the *narG* gene declined in Cd contaminated soils with greater reductions in acidic soils when compared to neutral soils. The Pb contaminated soils in the current study were also acidic (Fig. 2-1) which may have contributed to the significant decrease in *narG* gene abundance (Fig. 2-3).

2.5.2 NO_2^- reduction

In the present study, the reduction of NO_2^- was impacted by Pb at 1600 mg Pb/kg soil. Similarly, previous studies have reported the activity of NO_2^- reductase to be inhibited by Cd, 25μM~100μM $CdCl_2$, (Dinakar et al. 2009) and 2 mM Pb (Chen et al. 2016). Based on these studies, the high NO_2^- levels observed in the High Pb treatment in the current study may be attributed to Pb decreasing the activity of NO_2^- reductase. Furthermore, since NO_2^- is a toxic substance, the high NO_2^- levels in the High Pb treatment may have exerted further negative effects on other functionalities and community structures in the soil. The abundance of the *nirS* gene, which is implicated in the expression of nitrite reductase, did not exhibit a correlation with NO_2^- concentration (Fig. 2-1). Indeed, prior studies have reported both a decrease and an

increase in the abundance of the *nirS* gene due to heavy metal contamination. The *nirS* gene abundance has been reported to significantly decrease due to various factors, including Cu concentrations ranging from 0 to 159 μg Cu/g soil (Magalhães et al. 2011), complex heavy metal contamination involving Pb, Zn, Cd, and Cu (Tang et al. 2023), and contamination with 500 to 1500 mg Pb/kg soil (Feng et al. 2023). In contrast, Wang et al. (2023) found that the abundance of the *nirS* gene exhibited higher values in response to Pb contamination. Therefore, while NO_2^- reduction reactions are typically inhibited by heavy metal contamination, the abundance of the *nirS* gene does not necessarily decline. This discrepancy may be related to the relative abundance of the *nirK* and *nirS* genes. Bowen et al. (2020) reported that denitrification activity is strongly correlated with the abundance of the *nirS* gene under high pH conditions, but at low pH, a correlation was observed with the *nirK* gene. Indeed, Yang et al. (2024) observed that *nirS* gene abundance was reduced in acidic Cd-contaminated soils, while *nirK* gene abundance increased, when compared to Cd-contaminated soil with neutral pH. In the current study, with acidic Pb-contaminated soil, the abundance of the *nirK* gene was not quantified. The fact that *nirS* gene abundance could not explain the decrease in NO_2^- reduction activity, in the current study, may have been due to *nirK* playing a dominant role. Future assessment of denitrification activity in heavy metal-contaminated soils requires the quantification of both *nirS* and *nirK* genes, along with soil pH measurements.

2.5.3 N_2O emission and gene abundance

The increase in N_2O emissions, observed in the High Pb treatment during the 3rd week without N addition, may have been correlated with the increased level of the *norB* gene. Similarly, the abundance of *norB* gene was reported to be higher in soils severely contaminated with heavy metals than in lightly contaminated soils (Hu et al. 2025), and higher in the Pb-treated plots than in control plots (M. Wang et al. 2023). The increase in the abundance of the *norB* gene during the later stages of incubation may have been attributed to the microbial communities, possessing the *norB* gene, developing resistance to Pb contamination over time. Conversely, the high N_2O emissions in the High Pb treatment with NO_3^- addition may have been ascribed to the inhibition of N_2O reduction by Pb, since the abundance of the *nosZ* gene in the High Pb treatment with NO_3^- addition was the lowest from the week 0 to the 2nd week. This

outcome suggested that Pb at 1600 mg Pb/kg soil inhibited the activity of the N_2O reductase enzyme. The increase in N_2O emissions due to the adverse effects on N_2O reduction by heavy metal contamination has been confirmed in several studies (Zhang et al. 2016;Zhou et al. 2014; Bollag and Barabasz 1979). Holtan-Hartwig et al. (2002) also claimed that N_2O reduction was more sensitive to heavy metal contamination, including Cd, Cu, and Zn, than N_2O production in contaminated soils. Previous studies have reported that the *nosZ* gene decreases due to heavy metal contamination (Liu et al. 2016; Liu et al. 2018). Based on these preceding studies, the decrease in the abundance of the *nosZ* gene abundance and the activity of the N_2O reductase enzyme is likely the result of Pb contamination at 1600 mg Pb/kg soil, particularly when N was added to the soil.

2.5.4 Effects of Pb contamination on soil microbial abundance and diversity

The abundance of 16S rRNA was found to be lower in the Mid and High Pb treatments when compared to the Control, irrespective of N addition. The High Pb treatment was significantly lower than the Control at both the week 0 and the 1st week (Fig. 2-4). These findings indicate that Pb at levels surpassing 400 mg Pb/kg soil exerts a negative impact on the abundance of 16S rRNA. Former studies have reported abundance of bacteria and fungi decreased in soils containing Pb and other heavy metals (Gołębiewski et al. 2014; Deng et al. 2015). Additionally, Igalavithana et al. (2017) demonstrated that the introduction of biochar, which suppresses the bioavailability of heavy metals in soil, led to an increase in the abundance of microorganisms in heavy metal-contaminated soil. The observed reduction in 16S rRNA abundance during the early stages of the incubation, in the current study, indicate a correlation between heavy metal contamination of soil and microbial abundance. The observed decline in the abundance of 16S rRNA over time, with and without N addition, may be due to the acidity of the incubated soil. Yang et al. (2024) reported that the abundance of 16S rRNA in acidic Cd-contaminated soil was lower than that in a neutral soil. In terms of prokaryotic diversity, as indicated by Shannon indices, all Pb-contaminated treatments exhibited higher diversity indices than the Control. This suggests that Pb acts as a disruptive factor to the original community structure and potentially leads to an increase in Pb-tolerant microorganisms. However, the shift in Shannon diversity with increasing Pb concentration varied depending on the presence of NO_3^- addition. These results imply that not only resistance to Pb but also responses to the availability of NO_3^- may

have influenced the diversity of soil microbial communities. In general, heavy metal contamination has been reported to reduce microbial Shannon diversity (Gołębiewski et al. 2014; Deng et al. 2015). However, Li et al. (2024) reported significant increases in alpha and beta diversity with Cd contamination. It has also been reported that a decrease in the availability of heavy metals in the soil promotes an increase in microbial diversity (Ren et al., 2024). Considering these previous studies, microbial diversity under heavy metal contamination is clearly affected by both soil properties and heavy metal species. Thus, increases in diversity may have been the result of the acidic soil in conjunction with Pb contamination. The Shannon diversity index of all treatment groups converged to approximately 7.5 at the 4th week, irrespective of the presence of N. Furthermore, the analysis of community structures at the phylum and genus levels revealed that all Pb treatment groups displayed structures similar to the Control at the 4th week, except for some genera. These results suggest that following short-term exposure to Pb, microbial species diversity and population numbers transition toward a stable community structure over time.

2.5.5 Relationship between denitrification activity and abundance of denitrification genes under Pb pollution.

The levels of inorganic N and the abundance of denitrification functional genes were adversely affected by the presence of 1600 mg Pb/kg soil. However, some of the observed decline in denitrification activity could not be accounted for by alterations in the abundance of denitrification functional genes. It is generally reported that the abundance of denitrification functional genes exerts an influence on the activity of these denitrification enzymes (J. Ma et al. 2014; Lindemann et al. 2015; Barrena et al. 2017; R. Zhang et al. 2022). In contrast, certain studies have discovered that the abundance of denitrification genes does not correlate with the activity of denitrification enzymes or the quantity of inorganic N under heavy metal pollution (Yuan Liu et al. 2016; L. Lu et al. 2022). But this is not always the case, for example, Lu et al. (2022) noted that in heavy metal contaminated soils, including Pb, there was a positive correlation between N_2O emissions, and *nosZ* gene abundance. Conversely, Liu et al. (2016) found paddy soils contaminated with heavy metals had significantly lower *nosZ* gene abundance compared to uncontaminated paddy soils. However, the amount of N_2O reduction did not show a significant decrease, indicating rather low N_2O emissions. These

inconsistencies between the abundance of functional genes and actual inorganic N amounts may have been attributed to changes in the transcription and expression of functional genes and enzyme activity induced by heavy metal contamination. Previous studies have reported that heavy metal contamination can upregulate or downregulate the expression of denitrification function genes. For example, Cu contamination suppressed the expression of nitrate reductase and nitrite reductase (Lu et al. 2019), while Cd contamination reduced the copy number of *nirS* and *nosZ* gene transcripts (Broman et al. 2019). Upregulation of genes may result from decreased bioavailability of Pb due to immobilization (Fajardo et al. 2015). Thus, the suppression of denitrification gene expression in this study, by Pb, is consistent with other heavy metal studies (Zhou et al. 2012; Shun Li et al. 2018). However, transcriptomic analysis was not conducted. Therefore, it remains unclear whether the observed discrepancies are related to changes in the expression levels of denitrification functional genes. To gain a deeper understanding of the changes in denitrification activity under Pb contamination, combined genomic and transcriptomic analyses are required.

2.5.5 Denitrification activity and Pb bioavailability under acidic soil

The observed differences in initial pH between treatments may have resulted from the alkaline nature of the potassium acetate (KCH_3COO) added. Several studies have also reported that the presence of heavy metals can inhibit soil buffering capacity and lead to reductions in soil pH (Zeng et al. 2011; Borch et al. 2010; Neubauer, Furrer, and Schulin 2002; Speir et al. 1999) and which Pb may have contributed to in the current study. Previous research has shown that denitrification activity is most active under neutral conditions at pH 7.0-7.5 (Šimek, Jíšová, and Hopkins 2002; ŠImek and Cooper 2002; Dörsch, Braker, and Bakken 2012; Albina et al. 2019). Consequently, the differences in NO_3^- reduction and NO_2^- reduction activities observed between treatments at week 0 may have been related not only to Pb impairment of bacteria, but also the decline in soil pH. Decreases in soil pH have been associated with increased N_2O emissions, reduced N_2 production rates (Stevens, Laughlin, and Malone 1998), and inhibited NO_3^- reduction activities (Nägele and Conrad 1990). Furthermore, Čuhel et al.(2010) reported that acidic soils showed significantly decreased abundance of 16S rRNA, *narG*, *napA*, *nirS*, *nirK*, and *nosZ* genes, along with increased N_2O emissions compared to control soils. Hence, the reduction in NO_3^- and NO_2^- reduction activities

and the corresponding decrease in *narG* gene abundance early in the incubation may be attributed not only to Pb toxicity but also to acidic soil conditions. At the end of the incubation, both NO_3^- and NO_2^- concentrations had decreased relative to their initial levels. These declines can likely be attributed to the shortage of N substrates, in combination with reduced denitrification activity due to the decrease in soil pH. In addition, pH values in all treatments in the 4th week had decreased compared to their initial values. However, a decrease in pH was also observed in the Control where Pb was not applied, suggesting that this decrease cannot be explained solely by a Pb induced reduction in soil buffering capacity. Instead, this change may have resulted from the production of organic acids due to the metabolism of dissociated acetate ions. Previous studies have reported that a decrease in soil pH increases the mobility and bioavailability of heavy metals in soil (Badawy et al. 2002; Sukreeyapongse et al. 2002; Bang and Hesterberg 2004). Conversely, it has also been argued that heavy metals absorption by soil may reduce their bioavailability (Lock and Janssen 2003; Lu, Zhang, and Shan 2005). The concentration of the bioavailable Pb in soil was not measured in this study, so potential changes in Pb bioavailability during the study remain unclear. Therefore, future studies on denitrification activity in heavy metal contaminated soils, including Pb, should also examine changes in soil pH and heavy metal bioavailability.

2.5.6 Effects of Pb contamination on soil microbial community structure at the Phylum level

Proteobacteria exhibited the highest relative abundance in the Control, Low, and Mid Pb treatments. *Proteobacteria* have been identified as the dominant phylum in numerous heavy metal-contaminated environments (Zuo 2023; Zhao et al. 2020; Li et al. 2017). They have also been reported to display a higher abundance in heavy metal-contaminated soils compared to non-contaminated soils (Li et al. 2017; Shuzhen Li et al. 2020). This trend was corroborated in the 2nd and 3rd weeks under non-N application and in the 2nd, 3rd, and 4th weeks under NO_3^- addition in this study. Based on these findings, *Proteobacteria* may be adversely impacted immediately following exposure to heavy metal contamination but may be more likely to survive in soils after a certain period of contamination. Moreover, *Proteobacteria* have been reported in various studies as a major phylum that encompasses key denitrifying bacteria (Palmer and Horn 2012; Guo et al. 2018). Therefore, the observed decline in the abundance of

Proteobacteria due to heavy metal contamination may have significantly contributed to the reduction in denitrification activity observed in this study. *Firmicutes* demonstrated strong resistance to Pb, regardless of the presence of N addition, and can survived better under the High Pb treatment. *Firmicutes* include sulfate-reducing bacteria that evade heavy metal toxicity by generating sulfides (Burkhardt et al. 2011), such as *Clostridiales* observed in this study, may have contributed to the heavy metal tolerance of *Firmicutes*. Additionally, *Firmicutes* are recognized as including notable denitrifying bacteria (Anderson et al. 2018) and have been reported to display denitrification activity even in soils contaminated with heavy metals (J. Li et al. 2016). Consequently, *Firmicutes* may have contributed to denitrification activity under conditions of high Pb in our study.

2.5.7 Effects of Pb contamination on soil microbial community structure at the Genus level

In this study, *Azotobacter* emerged as the dominant genus in both non-N and N treatments at the week 0 and 1st week. *Azotobacter* abundance tended to decline with increasing Pb concentration during these periods. However, *Azotobacter* abundance increased in the High Pb treatment in the 1st week compared to the week 0. This suggests that *Azotobacter* can withstand high Pb levels. Indeed, the genus *Azotobacter* has been reported to exhibit heavy metal resistance and denitrification capabilities. Abo-amer et al. (2014) isolated *Azotobacter chroococcum* with heavy metal resistance from agricultural soil. Bleakley and Tiedje (1982) demonstrated that *Azotobacter vinelandii* is a denitrifying bacterium capable of reducing NO_3^-, indicating its N_2O emission potential. Therefore, the dominance of *Azotobacter* observed in this study may have contributed considerably to the progression of denitrification under Pb contamination. Also, the relative abundance of *Bacillus* was the highest among other bacteria from the week 0 to the 4th week in the High Pb treatment with NO_3^- addition, suggesting that *Bacillus* exhibited resistance to Pb. *Bacillus* are reportedly tolerant to heavy metals and have shown the ability to absorb and reduce Pb at a wide range of pH conditions (5, 7, and 9) (Varghese et al. 2012; Syed and Chinthala 2015). In addition, Anderson et al. (2018) isolated and showed *Bacillus* contributed to NO_3^- reduction and N_2O emission, while Verbaendert et al. (2011) also isolated *Bacillus* capable of reducing NO_3^- and NO_2^-.Thus, the changes in the abundance of *Bacillus* may have contributed to the

relationship between denitrification gene abundance and activity in the Pb-contaminated treatments. *Sphingomonas* demonstrated a relatively higher abundance across all Pb treatments compared to the Control, from the 2nd to the 4th week. This result suggests that *Sphingomonas* possess resistance to Pb. Indeed, Altimira et al. (2012) reported that *Sphingomonas* spp. were resistant to heavy metals when equipped with plasmids, while *Sphingomonas* spp. have also been found to absorb heavy metals (Waigi, Sun, and Gao 2017; Heidari, Sanaeizade, and Mazloomi 2020). Furthermore, it has been found that some bacteria of the genus *Sphingomonas* genes encoding denitrification (Cua and Stein 2014; Siddiqi et al. 2023). Based on these findings, the *Sphingomonas* identified in my study may have survived in Pb-contaminated environments due to their heavy metal resistance and absorption capabilities, and they may have also participated in denitrification. *Rhodanobacter* was among the top 15 genera when NO_3^- was added. The highest abundance was observed in the Mid Pb treatment at the week 0 and in the High Pb treatment from the 2nd to the 4th week. These findings suggest that *Rhodanobacter* may have proliferated in environments with 400 mg Pb/kg soil or higher when in the presence of NO_3^-. Supporting this, Hemme et al. (2016) demonstrated that the abundance of *Rhodanobacter* was higher under conditions of low pH, high nitrate concentration, and high heavy metal levels compared to non-contaminated environments. Prakash et al. (2020) indicated that *Rhodanobacter denitrificans* tends to proliferate in the presence of low pH and high nitrate concentrations, and it also shows resistance to high concentrations of heavy metals. *Rhodanobacter* have been found to perform all or some of the steps involved in denitrification (Van Den Heuvel et al. 2010; Wu et al. 2022) .Considering these prior studies, the *Rhodanobacter* identified in my study may have adapted to Pb and high NO_3^- availability and may have been involved in denitrification. This study did not investigate the correlation between denitrification gene expression and denitrification activity in specific microbial species. However, in general, denitrification gene abundance could serve as a marker of denitrification activity. Further exploration in this domain is needed, particularly when studying N cycling in Pb-contaminated soils.

2.6 Conclusion

This research assessed the impact of Pb on soil denitrification activity and microbial communities. In the early stages of incubation, soil pH differed significantly

due to the alkalinity of potassium acetate added to equalize acetate ions, but by the end of the incubation, all treatments showed lower values than at the start of incubation. This decrease in soil pH may have been related to the formation of organic acids within the soil and the presence of Pb as the incubation progressed. The bioavailability of Pb may have also changed in response to these changes, affecting the progress of denitrification along with the toxicity of Pb. The reduction in denitrification activity due to Pb was evidenced by the slower decrease of added NO_3^- in soils, the accumulation of NO_2^-, and the decrease in *narG* gene abundance. Despite the inhibition of NO_2^- reduction by Pb, as shown by the highest NO_2^- amount in the 1600 mg Pb/kg soil, the abundance of *nirS* was not directly linked to the Pb concentration. The emission of N_2O gas was amplified in the 1600 mg Pb/kg soil. This increase may be associated with the initial decrease in *nosZ* gene abundance and subsequent increase in *norB* gene abundance. When assessing denitrification activity in heavy metal-contaminated soils, future studies should thoroughly investigate not only the abundance of functional genes but also their transcription, expression, enzyme activity, and microbial community structure. In addition, the bioavailability of heavy metals in soil changes with the passage of time and changes in soil pH as incubations progress, so changes in these environmental factors should also be considered in future studies. Analysis of 16S rRNA levels showed an immediate reduction in microbial abundance following exposure to Pb. However, bacterial diversity appeared to increase with Pb. This change in the abundance and diversity of microorganisms may be due to an increase in Pb-tolerant microorganisms because of Pb contamination influencing environmental factors that cause disturbances among microorganisms. In terms of bacterial community structures, *Proteobacteria* initially dominated but quickly declined upon exposure to 1600 mg Pb/kg soil. In contrast, the abundance of *Actinobacteria*, *Chloroflexi*, and *Nitrospirae* increased with escalating Pb concentrations. At the genus level, *Azotobacter* decreased to less than 50% of the Control immediately after exposure to Pb contamination exceeding 400 mg Pb/kg soil. Conversely, *Bacillus* consistently demonstrated high resistance to Pb from the 1st to the 4th week. Both phylum and genus analyses revealed that the community structure of the Pb-contaminated treatments gradually shifted to resemble that of the Control over time. These bacterial taxa may have played a crucial role in the progression of denitrification under Pb contamination. While this study observed changes in the weeks following exposure to Pb, we did not assess the long-term effects of Pb. Given that actual heavy metal-contaminated soils are

likely affected by soil contamination over more extended and continuous periods, further research on field-based heavy metal-contaminated soil and the soil microbial communities inhabiting will be needed.

Chapter 3 Inoculation of lemongrass with *Azospirillum Brasiliense* Sp7 under Pb polluted soil: Impacts on plant growth and soil nitrogen cycle

3.1 Abstract

In recent years, research on improving the efficiency of heavy metal phytoremediation by inoculating plant growth promoting microbes (PGPM) has been gaining momentum. Lemongrass has attracted attention as a promising phytoremediation plant due to its heavy metal resistance, high economic value, rapid growth rate, extensive root system, and abundant aboveground biomass. However, compared to conventional hyperaccumulators such as Brassicaceae and Asteraceae species, there are fewer studies evaluating Lemongrass' growth in heavy metal-contaminated soils and its response to microbial inoculation. In this study, we conducted an eight-week cultivation experiment of lemongrass in Pb-contaminated soil under different inoculation treatments of *Azospirillum brasilense* Sp7. Five experimental groups were established based on the presence or absence of Pb contamination and differences in inoculation methods: Control (no Pb, no inoculation), Pb (Pb-contaminated, no inoculation), Seed (Pb-contaminated, inoculated at the seed stage), Root (Pb-contaminated, inoculated at the root stage), and Seed + Root (Pb-contaminated, inoculated at both the seed and root stages). The effects of these treatments were evaluated in terms of water-soluble carbon content in soil, soil pH, lemongrass leaf and root growth, inorganic nitrogen content (NH_4^+-N, NO_3^--N), the abundance of the nitrogen fixation gene (*nifH*), nitrification genes (*amoA* and *nxrB*), denitrification genes (*narG*, *nirK*, *nirS*, *norB*, *nosZ*), and 16S rRNA. By the 8th week, the inoculation of *A. brasilense* Sp7 at both the seed and root stages (Seed + Root treatment) significantly promoted both leaf and root growth compared to the Control. Additionally, the increased availability of sulfur (S) derived from PbS, used as a Pb contamination source, may have contributed to the enhanced growth of lemongrass. Regarding *nifH* gene abundance, the Seed + Root treatment exhibited the highest values at the 0th, 4th, and 8th weeks, with a significantly higher abundance than the Control at the 2nd week. For all denitrification genes, the Seed + Root treatment exhibited the highest gene abundance at multiple time points during the cultivation period. Notably,

at the 4th week, the abundance of *narG*, *nirK*, *nirS*, and *norB* genes was significantly higher in the *Seed + Root* treatment than in the Control. Furthermore, *16S rRNA* gene abundance was highest in the *Seed + Root* treatment at the 0th, 2nd, and 4th weeks, with significantly higher values than the Control at the 0th and 4th weeks. These findings suggest that the inoculation of *A. brasilense* Sp7 in Pb-contaminated soil exhibits the highest growth-promoting effect when applied to both seeds and roots, thus it can potentially be used as a promoter of phytoremediation in Pb-contaminated soils.

3.2 Introduction

Heavy metal contamination persists in soil for extended periods and exhibits strong biological toxicity, necessitating urgent research on effective remediation technologies. Remediation techniques for heavy metal contamination can be broadly categorized into physicochemical and biological approaches. Physicochemical methods are advantageous in that they can effectively address high concentrations of heavy metal contamination and produce immediate results. However, these methods are often associated with high costs and significant environmental impact(Li et al. 2019). In contrast, biological approaches include bioremediation, which utilizes microorganisms, and phytoremediation, which exploits the capabilities of plants. These methods offer advantages such as lower costs and reduced environmental burden compared to conventional physicochemical techniques. Nevertheless, because phytoremediation involves plant growth as a necessary step, it generally requires a longer duration before achieving effective heavy metal removal. To enhance the efficiency of phytoremediation, it is crucial to select fast-growing, heavy metal-tolerant plants and further promote plant growth and metal uptake capacity through the inoculation of PGPM. Lemongrass (*Cymbopogon spp.*) has recently gained attention as a promising plant for heavy metal phytoremediation. This is primarily due to its wide range of commercial applications, including use in food, cosmetics, and pharmaceuticals, allowing for the simultaneous achievement of both heavy metal removal and economic value generation. Additionally, microbial inoculation with PGPMs has been reported to enhance plant growth and heavy metal uptake even under contaminated conditions, further highlighting its potential utility. However, most existing studies have focused primarily on plant growth and metal uptake, leaving the impacts on soil ecosystem functions and microbial communities largely unexplored. Furthermore, variations in

plant species and inoculation methods among studies necessitate a comparative analysis of their effects on plant growth and soil conditions. Moreover, the number of studies investigating PGPM inoculation in lemongrass remains relatively limited compared to other heavy metal-tolerant plants, underscoring the need for accumulating knowledge regarding the most suitable PGPM strains and inoculation methods. Considering these research gaps, we conducted a cultivation experiment in Pb-contaminated soil using *Azospirillum brasilense* Sp7 as the PGPM and lemongrass as the heavy metal-tolerant plant. To assess the effects of different inoculation methods, we inoculated the PGPM onto the seeds, roots, or both of lemongrass. Additionally, we extracted DNA from the cultivated soil samples to analyze temporal changes in the abundance of nitrogen cycle-related functional genes and microbial populations. Furthermore, we measured the length of lemongrass leaves and roots at the beginning and end of the cultivation period to evaluate which inoculation method was most effective in promoting plant growth.

3.3 Material and Methods

3.3.1 Pre-cultivation of lemongrass

In this study, a small-scale pot study was designed to investigate microbial inoculation under lead-contaminated conditions, using lemongrass (*Cymbopogon citratus*) as the plant species and *Azospirillum brasilense* Sp7 strain as the inoculant. The *A. brasilense* Sp7 strain used was purchased as a dried specimen from National Institute of Technology and Evaluation (NITE). It was then restored in a liquid medium containing 0.5 g of yeast extract, 2.5 g of mannitol, 0.35 g of dipotassium hydrogen phosphate (K_2HPO_4), 0.05 g of potassium dihydrogen phosphate (KH_2PO_4), 0.5 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and MilliQ for mess up to 1L. The recovered bacteria were collected after washing with MilliQ and stored frozen at −80°C in 50% glycerol stock. Five treatment groups were established based on the presence and site of microbial inoculation: Control (no Pb contamination, no inoculation), Pb (Pb contamination only), Seed (Pb contamination with seed inoculation), Root (Pb contamination with root inoculation), and Seed + Root (Pb contamination with both seed and root inoculation). Each treatment was replicated three times and further details are described below. The purchased lemongrass seeds underwent surface sterilization by sequential soaking in sterilized water for 1 min, 3% sodium hypochlorite solution for 5

min, 70% ethanol for 1 min, and sterilized water again for 1 min. The sterilized seeds were then air-dried for 30 min. Subsequently, three seeds were sown at three locations per 10 cm poly pot filled with vermiculite, and pre-cultivation was conducted at 25℃ for 14 days. Pb contamination treatment was not applied during the pre-cultivation phase. Following the sterilization process of the Seed and Seed + Root treatments, the seeds were soaked in an *A. brasilense* suspension at a concentration of 1.9×10^7 CFU/ml and subjected to shaking for 1 hour to complete the inoculation. The CFU count was determined by culturing *A. brasilense* from a glycerol stock preserved at −80℃. The stock was cultivated in Burk's nitrogen-free liquid medium at 30℃ for 7 days, after which 100 µl of the culture was spread onto nitrogen-free agar medium at pH 7 and incubated at 30℃ for 7 days. The number of colonies that appeared on the agar surface was then counted to determine the CFU.

3.3.2 Cultivation of lemongrass with Leonard jar

After confirming the germination of the sown lemongrass, the seedlings were transplanted into Leonard jars containing 300 g of horticultural soil and modified N-free solution (Broughton and Dilworth 1971). Before transplantation, the soil for the Pb, Seed, Root, and Seed + Root treatments were mixed with a 0.3 mM lead sulfide (PbS) solution to achieve a final concentration of 1 mM Pb/kg soil. The structure of the Leonard jar, shown in Figure 3-1, allowed for the supply of moisture and nutrients to the cultivated soil through a cotton wick. The composition of the modified N-free solution was as follows: 1 mM calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 0.5 mM dipotassium hydrogen phosphate (KH_2PO_4), 10 µM ferric citrate, 0.25 mM magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1.5 mM potassium sulfate (K_2SO_4), 1 µM manganese(II) sulfate pentahydrate ($MnSO_4 \cdot H_2O$), 2 µM boric acid (H_3BO_3), 0.5 µM zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 0.2 µM copper(II) sulfate pentahydrate ($CuSO_2 \cdot 5H_2O$), 0.1 µM cobalt(II) sulfate heptahydrate ($CoSO_2 \cdot 7H_2O$), and 0.1 µM disodium molybdate(VI) (Na_2MoO_4). The pH values of the cultivation soil used in the Leonard jars and the modified N-free solution are presented in Table 3-1. During transplantation into the Leonard jars, the lemongrass plants were carefully removed from the poly pots to avoid damage, ensuring that the entire lower part of the root was buried in the soil. Additionally, for the Root and Seed + Root treatments, the samples were soaked in an *A. brasilense* suspension at a concentration of 1.8×10^7 CFU/ml for 1

hour before transplantation. Following transplantation, the plants were cultivated in a greenhouse at 30°C for eight weeks. During the cultivation period, watering with sterilized water was performed as needed to prevent the soil surface from drying out, and the modified N-free solution was supplemented accordingly. Furthermore, 7 g of cultivated soil samples were collected from the Leonard jars at four-time points: immediately after transplantation (week 0), at 2nd week, 4th week and 8th week. Until further analysis, the collected soil samples were stored at −4°C.

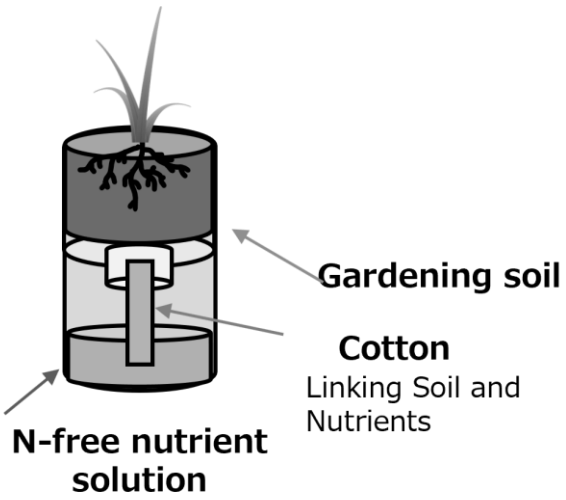

Figure 3-1. The structure of leonard jar used in this study

Table 3-1. The pH value of original soil and modified N-free solution

	pH (n = 3)
Soil (No treatment)	4.001 ± 0.002
Modified N-free solution	4.637 ± 0.010

3.3.3 Measurement of soil pH

The collected soil samples were used for chemical analysis. For the evaluation of NO_3^--N, 1 g of the sampled soil was mixed with 10 ml of 2 M KCl solution and shaken for 30 minutes. The resulting KCl extract was filtered using filter paper (Grade 5C, <5 mm; Advantec, Tokyo, Japan) before the chemical analysis and stored at 4°C under refrigeration until further analysis. The soil pH was measured using a pH meter (AS800; AS ONE Corporation, Osaka, Japan), calibrated with standard solutions at pH 4, 7, and 9, by immersing the electrode in the KCl extract.

3.3.4 Measurement of water-soluble carbon content

For the measurement of water-soluble carbon, 1 g of the collected soil was mixed with 10 ml of sterile water and shaken for 30 minutes. The resulting water extract was filtered using the same type of filter paper and stored at 4°C under refrigeration until analysis. The concentration of water-soluble carbon in the soil was determined using a TOC analyser (TOC-V CPH, Shimadzu Co., Kyoto, Japan).

3.3.5 Measurement of NH_4^+-N and NO_3^--N content

The KCl extract obtained through the above procedure was also used for the measurement of inorganic nitrogen via flow injection analysis. The concentrations of inorganic nitrogen species (NH_4^+-N and NO_3^--N) in the soil were determined using a flow injection analyzer (FIA, AQLA-700; Aqua Lab Co., Ltd., Tokyo, Japan) based on a colorimetric method (Saltzman 1954). In the FIA system, NO_3^--N was identified by its reduction to NO_2^--N through a cadmium column. The composition of the solutions used for the measurement of inorganic nitrogen sources is shown in Table 3-2.

Table 3-2. Reagents used for NH_4^+-N measurement

Solution	Reagent	Volume
Carrier solution	KCl	74.55g
	Milli Q	Up to 1000 ml
R1	Phenol	11 ml
	Sodium Pentacyanonitrosylferrate (Ⅲ)Dihydrate	0.05 g
	Milli Q	Up to 1000 ml
R2	Sodium Hydroxide	15g
	Sodium Hypochlorite solution	50ml
	Milli Q	Up to 1000ml

z3.3.6 Soil DNA extraction and qPCR for nitrogen cycling genes and 16S rRNA

To quantify the soil's microbial characteristics, the soil DNA extraction was conducted using NucleoSpin® Soil (Takara Bio, Inc., Shiga, Japan) by the supplier's protocol. The SL2 buffer solution was utilized during the DNA extraction process. During DNA extraction, 0.2 g of skim milk was incorporated to counteract the strong adhesion of DNA to Andosol (Takada Hoshino and Matsumoto 2005). The extracts procured through the preceding procedure were preserved in a freezer at −20°C until subsequent analysis. To determine the abundance of nitrogen fixation gene (*nifH*), ammonia-

oxidizing microbes (AOA, AOB), nitrification gene (*amoA, nxrB*), denitrification functional genes (*narG*, *nirK, nirS*, *norB*, and *nosZ*) and 16S rRNAin soil, quantitative real-time PCR was executed using a CFX96 Touch Real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mix for qPCR analysis was prepared by incorporating 10.4 μL of the Kapa SYBR Fast Master Mix (2×) from the Kapa SYBR Fast qPCR Kit (Agilent Technologies, Palo Alto, CA, USA), 0.5 μL of the forward primer, and 0.5 μL of the reverse primer for each functional gene. The final volume was adjusted to 20 μL using nuclease-free water. The cycle conditions corresponding to each functional gene are depicted in Table 3-3a and 3-3b (Poly, Monrozier, and Bally 2001; Rotthauwe, Witzel, and Liesack 1997; Keeley et al. 2020; Caporaso et al. 2011)**.**

Table 3-3a. Target, primers, sequences and PCR cycle information for nitrogen fixation genes, nitrification genes, and 16S rRNA

Target	Primers	Sequence	PCR cycle	References
nifH	**Pol F**	TGCGAYCCSAARGCBGACTC	[30 cycles] 94 °C for 1 min 55 °C for 1 min 72 °C for 2 min and a final elongation at 72 °C for 5 min	(Poly, Monrozier, and Bally 2001)
	Pol R	ATSGCCATCATYTCRCCGGA		
amoA	**amoA 1F**	GGGGTTTCTACTGGTGGT	[1 cycles] 94 °C for 5 min [42 cycles] 60 °C for 90 sec 72 °C for 90 sec 94 °C for 60 sec and a final elongation at 60 °C for 90 sec and 72 °C for 10 min	(Rotthauwe, Witzel, and Liesack 1997)
	amoA 2R	CCCCTCKGSAAAGCCTTCTTC		
nxrB	**nxrB F rk**	GTGGAACAAYGTGGARACSAAGCC	[45 cycles] 94 °C for 15 sec 56 °C for 30 sec 72 °C for 1 min and a final elongation at 72 °C for 1 min	(Keeley et al. 2020)
	nxrB R rk	SACRAASCGCCAYTCYTGGTC		
16S rRNA	**16S 515F**	GTGCCAGCMGCCGCGGTAA	[1 cycle] 94 °C for 3 min [35 cycles] 94 °C for 45 sec 50 °C for 60 sec 72 °C for 90 sec and a final elongation at 72 °C for 10 min	(Caporaso et al. 2011)
	16S 806R	GGACTACHVGGGTWTCTAAT		

Table 3-3b. Target, primers, sequences and PCR cycle information for denitrification genes.

Target	Primers	Sequence	PCR cycle	References
narG	**1960F**	TAYGTSGGSCARGARAA	[1 cycle] 95 °C for 5 min 94 °C for 30 sec 60 °C for 30 sec 72 °C for 30 sec [8 cycles] 94 °C for 30 sec 59 °C for 30 sec (decreased by 0.5 °C/ cycle) 72 °C for 30 sec [30 cycles] 94 °C for 30 sec 55 °C for 30 sec 72 °C for 1 min and a final elongation at 72 °C for 6 min	(Philippot et al. 2002)
	2650R	TTYTCRTACCABGTBGC		
nirK	**nirK 876F**	ATYGGCGGVAYGGCGA	[1 cycle] 50 °C for 120 sec 95 °C for 900 sec [6 cycles] 95 °C for 15 sec 63 °C for 30 sec (decreased by 1 °C/ cycle) 72 °C for 15 sec [40 cycles] 94 °C for 30 sec 58 °C for 30 sec 72 °C for 30 sec and a final elongation at 80 °C for 15 sec	(Sonia Henry et al. 2004)
	nirK 1040R	GCCTCGATCAGRTTRTGGTT		

nirS	**nirS 1F**	CCTAYTGGCCGCCRCART	[1 cycle] 96 °C for 3 min [40 cycles] 95 °C for 30 sec 65 °C for 1 min 72 °C for 1 min and a final elongation at 72 °C for 1 min	(Braker, Fesefeldt, and Witzel 1998)
	nirS 3R	GCCGCCGTCRTGVAGGAA		
norB	**qnorB 2F**	GGNCAYCARGGNTAYGA	[1 cycle] 96 °C for 3min [40 cycles] 95 °C for 30 sec 56 °C for 90 sec 72 °C for 1 min and a final elongation at 72 °C for 1 min	(Sun et al. 2017)
	qnorB 5R	CAKRTGCAKSGCRTGGCAGAA		
nosZ	**nosZ 2F**	CGCRACGGCAASAAGGTSMSSGT	[1 cycle] 95 °C for 10min [40 cycles] 95 °C for 15 sec 60 °C for 30 sec 72 °C for 30 sec and a final elongation at 72 °C for 1min	(S. Henry et al. 2006)
	nosZ 2R	CAKRTGCAKSGCRTGGCAGAA		

3.3.7 Evaluation of the growth of lemongrass

The lengths of the leaf and root of the lemongrass were measured to evaluate their growth at the time of transplantation into the Leonard jars (week 0) and at the end of the cultivation period (8th week). In both cases, the plants were carefully removed from the soil to avoid damage, and the surrounding soil was washed off with sterile water before measurement.

3.3.8 Statistical analysis

All statistical analyses were performed using R ver 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria), and the significance level was set at $p < 0.05$ for all tests except for Dunn's test, where significance was determined at $p < 0.025$. The Shapiro-Wilk test was performed on all obtained data to assess normality. Data were considered normally distributed if $p > 0.05$, whereas they were considered non-normally distributed if $p \leq 0.05$. For data that followed normal distribution, a Repeated Measures Two-Way Analysis of Variance (RMT-ANOVA) was performed to assess the effects of inoculation treatment and cultivation period. If significant main effects or interactions were observed, multiple comparisons were subsequently conducted using Tukey's HSD test. For data that did not follow a normal distribution, a non-parametric Aligned Rank Transform Analysis of Variance (ART-ANOVA) was employed to evaluate the effects of inoculation treatment and cultivation period. If significant main effects or interactions were detected in the ART-ANOVA, a Kruskal-Wallis test was conducted. If the Kruskal-Wallis test was significant ($p < 0.05$), multiple comparisons were performed using the Dunn test with Benjamini-Hochberg correction.

3.4 Results

3.4.1 Water soluble carbon in soil and soil pH

The contents of water soluble carbon and values of soil pH were presented in Figure 3-2, while the numerical data and statistical results are provided in Table 3-4a, 3-4b, 3-4c, and 3-4d. At the week 0, WSC contents exceeding 200 mg C/kg soil were observed in all treatments except for the Seed + Root treatment. However, no significant differences in WSC contents were detected throughout the entire cultivation period (Table 3-4a, 3-4b, 3-4c). The soil pH ranged from 3.98 to 4.49, indicating that the soil was acidic (Fig. 3-2b). The Root treatment consistently exhibited the highest pH among all treatments throughout the cultivation period. Furthermore, a significant difference among treatments was observed at the 4th week, and the Seed + Root treatment showed a significantly lower pH than all other treatments.

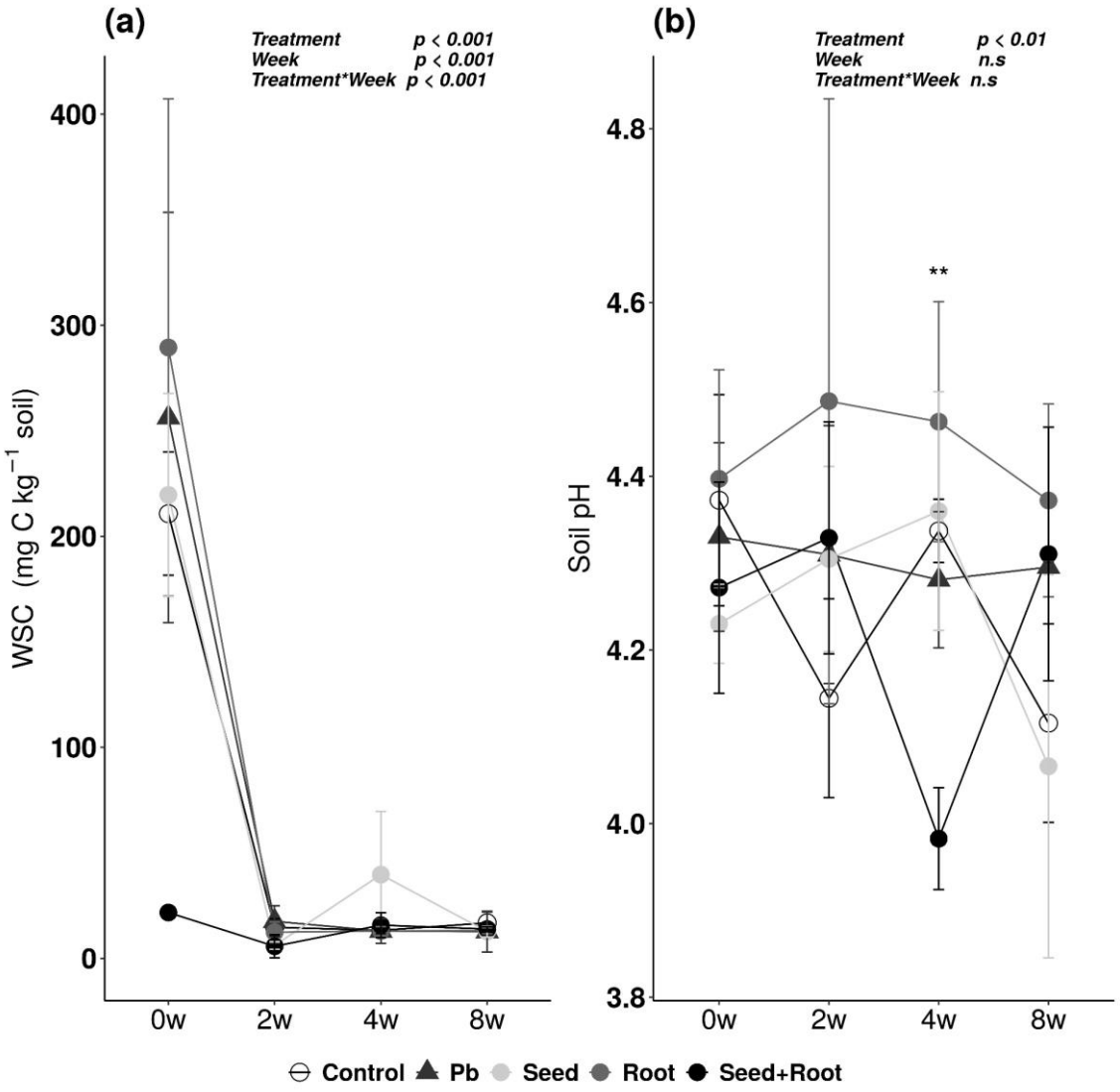

Figure 3-2. The changes in the WSC content and soil pH in soil samples over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-4a. The temporal changes in the WSC and soil pH (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-HSD analysis determined significant differences between each treatment.

WSC/ kg soil	**0 week**	**2 week**	**4 week**	**8 week**
Control	210.7 ± 29.2	14.8 ± 4	13.3 ± 1.8	16.9 ± 5.2
Pb	256.3 ± 97.2	17.6 ± 7.4	13 ± 5.8	12.7 ± 9.7
Seed	219.6 ± 48.1	6.1 ± 1.9	39.7 ± 29.9	12.7 ± 2.6
Root	289.5 ± 117.8	12.4 ± 4.9	13 ± 2.1	13.1 ± 1.5
Seed + Root	21.7 ± 2.3	5.8 ± 5.5	15.8 ± 5.9	13.9 ± 1.2
Soil pH	**0 week**	**2 week**	**4 week**	**8 week**
Control	4.37 ± 0.12	4.14 ± 0.11	4.34 ± 0.04 **b**	4.12 ± 0.11
Pb	4.33 ± 0.11	4.31 ± 0.15	4.28 ± 0.08 **b**	4.3 ± 0.01
Seed	4.23 ± 0.05	4.3 ± 0.11	4.36 ± 0.14 **b**	4.07 ± 0.22
Root	4.4 ± 0.13	4.49 ± 0.35	4.46 ± 0.14 **b**	4.37 ± 0.11
Seed + Root	4.27 ± 0.12	4.33 ± 0.13	3.98 ± 0.06 **a**	4.31 ± 0.15

Table 3-4b. The results of the Shapiro-Wilk test for the WSC content and soil pH: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
WSC	1.21×10^{-11}
Soil pH	0.1407

Table 3-4c. The results of the Kruskal-Wallis test for non-normally distributed the WSC content and soil pH: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

WSC	p-value
0w	0.1241
2w	0.7885
4w	0.1967
8w	0.7885

3.4.2 Growth of Lemongrass

The leaf and root lengths of lemongrass in each treatment at the week 0 and at the 8th week were presented in Figure 3-3, while the numerical data and statistical results are provided in Table 3-5a, 3-5b, 3-5c, and 3-5d. The leaf length of lemongrass was found to not follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-5a). There were no significant differences in leaf length among treatments at the week 0. However, at the 8th week, there was a significant difference in that the Seed + Root treatment exhibited a significantly higher leaf length than the Control (Fig. 3-5c, 3-5d). Although no significant differences were observed, the Seed treatment showed longer leaf growth than the Pb treatment, whereas the Root treatment showed shorter leaf growth compared to the Pb treatment. For the root length of lemongrass, the Shapiro-Wilk test confirmed a normal distribution (Fig. 3-3b). There were no significant differences in root length among treatments at week 0. However, at the 8th week, a significant difference was observed, with the Seed + Root treatment exhibiting significantly longer root growth than both the Control and Root treatments (Fig. 3-5b). Although no significant differences were observed, root growth in both the Seed and Root treatments was lower than that in the Pb treatment. In addition, the Root treatment resulted in lower root growth than the Seed treatment under Pb pollution.

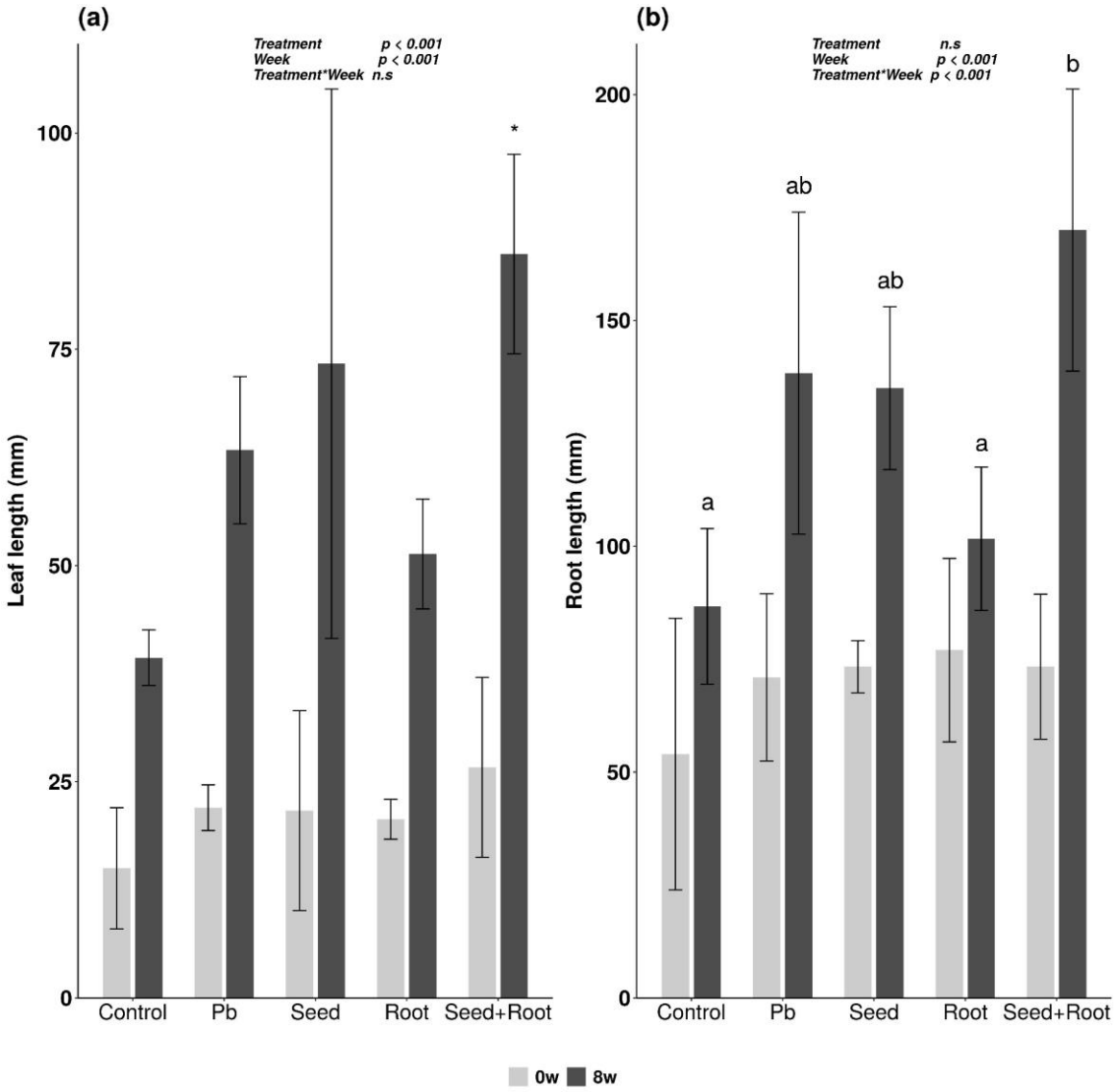

Figure 3-3. The changes in the growth of the leaf length and root length of lemongrass measured at the week 0 and 8th week. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-5a. The temporal changes in the length of leaf and root of lemongrass (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week (n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

Leaf length (mm)	**0 week**	**8 week**
Control	15.0 ± 7.0	39.33 ± 3.21
Pb	22.0 ± 2.65	63.33 ± 8.50
Seed	20.67 ± 2.31	51.33 ± 6.35
Root	21.67 ± 11.55	73.33 ± 31.75
Seed + Root	26.67 ± 10.41	86.0 ± 11.53
Root length (mm)	**0 week**	**8 week**
Control	54.0 ± 30.05	86.67 ± 17.24 **a**
Pb	71.0 ± 18.52	138.33 ± 35.64 **ab**
Seed	77.0 ± 20.3	101.67 ± 15.89 **ab**
Root	73.33 ± 5.77	135.0 ± 18.03 **ab**
Seed + Root	73.33 ± 16.07	170 ± 31.22 **b**

Table 3-5b. The results of the Shapiro-Wilk test for the leaf length and root length of lemongrass: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
Leaf length	0.0130
Root length	0.0977

Table 3-5c. The results of the Kruskal-Wallis test for non-normally distributed leaf length and root length of lemongrass: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

Leaf length	p-value
0w	0.6067
8w	0.02623*

Table 3-5d. The results of Dunn's test for non-normally distributed leaf length of lemongrass: If $p < 0.025$, the treatment groups were considered significantly different and indicated with an asterisk (*).

Leaf length at 8w	Control	Pb	Seed	Root	Seed + Root
Control	-				
Pb	0.0676	-			
Seed	0.0534	0.4269	-		
Root	0.2211	0.2311	0.2591	-	
Seed + Root	0.0118 *	0.2261	0.0712	0.1943	-

3.4.3 Inorganic nitrogen content

The temporal changes in the contents of the inorganic nitrogen (NH_4^+-N and NO_3^--N) were presented in Figure 3-4, with numerical data and statistical results provided in Table 3-6a, 3-6b, and 3-6c. The NH_4^+-N content was shown to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-4a). However, there were no significant differences in NH_4^+-N content among treatments throughout the entire cultivation period (Fig. 3-6a, 3-6b). In the week 0, NH_4^+-N content was highest in Seed + Root treatment and lowest in the Control. This trend was not observed after the 2nd week, and an opposite pattern was observed until the 4th week, with NH_4^+-N concentration decreasing in the Seed + Root treatment while increasing in the Control. At the 8th week, NH_4^+-N contents in the Seed and Root treatments were higher than those in both the Control and Seed + Root treatments. The NO_3^--N content was also shown to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-4b). There were no significant differences in NO_3^--N content among treatments throughout the entire cultivation period (Fig. 3-6a, 3-6b, 3-6c). The observed NH_4-N concentrations ranged from 16.2 to 30.0 mg N/kg soil, whereas NO_3-N concentrations ranged from 0.65 to 3.17 mg N/kg soil, representing 80.4%–97.8% lower levels compared to NH_4^+-N. The NO_3^--N content in the Control and Seed + Root treatments increased over time, and it showed higher values compared to the Pb treatment, Seed treatment, and Root treatment at both the 4th and 8th week.

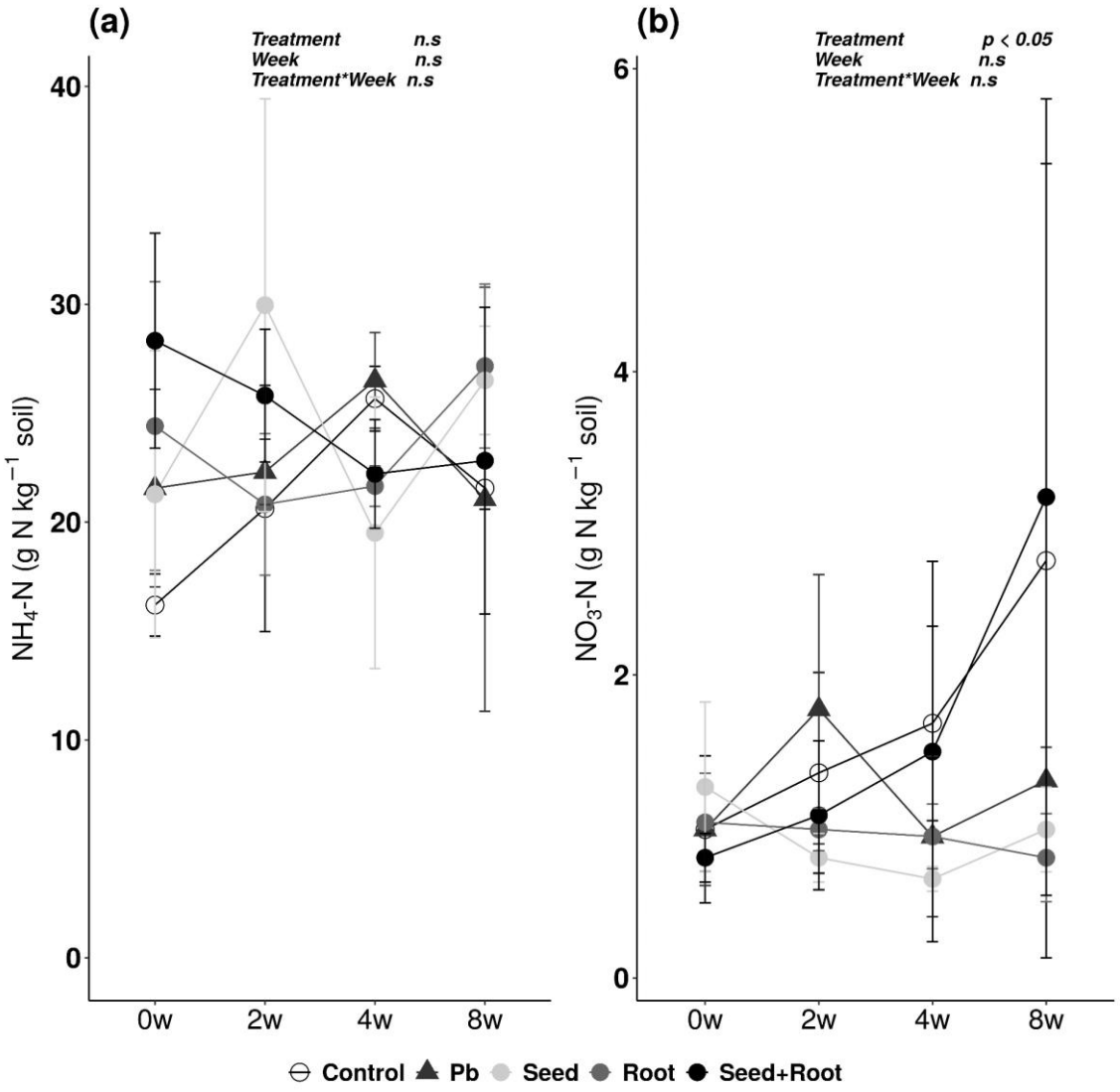

Figure 3-4. The changes in the content of NH_4^+-N and NO_3^--N in soil samples over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-6a. The temporal changes in the NH_4^+-N and NO_3^--N (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

NH_4^+-N (mg N/ kg soil)	**0 week**	**2 week**	**4 week**	**8 week**
Control	16.2 ± 1.4	20.6 ± 5.7	25.7 ± 1.5	21.6 ± 1.0
Pb	21.6 ± 4.5	22.3 ± 1.5	26.5 ± 2.2	21.0 ± 9.7
Seed	21.3 ± 6.6	30.0 ± 9.5	19.5 ± 6.2	26.5 ± 2.5
Root	24.4 ± 6.6	20.8 ± 3.2	21.7 ± 0.9	27.2 ± 3.8
Seed + Root	28.3 ± 4.9	25.8 ± 3	22.2 ± 2.5	22.8 ± 7.0
NO_3^--N (mg N/ kg soil)	**0 week**	**2 week**	**4 week**	**8 week**
Control	0.98 ± 0.48	1.35 ± 0.66	1.68 ± 0.64	2.75 ± 2.62
Pb	0.98 ± 0.37	1.77 ± 0.89	0.93 ± 0.53	1.3 ± 0.22
Seed	1.26 ± 0.56	0.79 ± 0.16	0.65 ± 0.08	0.98 ± 0.28
Root	1.03 ± 0.32	0.98 ± 0.14	0.93 ± 0.21	0.79 ± 0.29
Seed + Root	0.79 ± 0.16	1.07 ± 0.49	1.49 ± 1.25	3.17 ± 2.63

Table 3-6b. The results of the Shapiro-Wilk test for the abundance of inorganic nitrogen content: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
NH_4^+-N	0.0753
NO_3^--N	1.75×10^{-11}

Table 3-6c. The results of the Kruskal-Wallis test for non-normally distributed NO_3^--N content: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

NO_3^--N	p-value
0w	0.7454
2w	0.2537
4w	0.1732
8w	0.2537

3.4.4 Quantification of nitrogen fixing gene: nifH

The temporal changes in the abundance of the nitrogen fixation gene *nifH* are presented in Figure 3-5, with numerical data and statistical results provided in Table 3-7a and 3-7b. The abundance of *nifH* was shown to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-5). The significant differences were observed only at the 2nd week, where Pb treatment, Root treatment, and Seed + Root treatment exhibited significantly higher *nifH* abundances than the Control (Table 3-7a, 3-7b). Although no significant differences were observed at the 0th, 4th, and 8th weeks, the Seed + Root treatment consistently showed the highest values in all of these culture periods. The Seed treatment exhibited minimal changes in *nifH* abundance throughout the entire cultivation period compared to the other treatments.

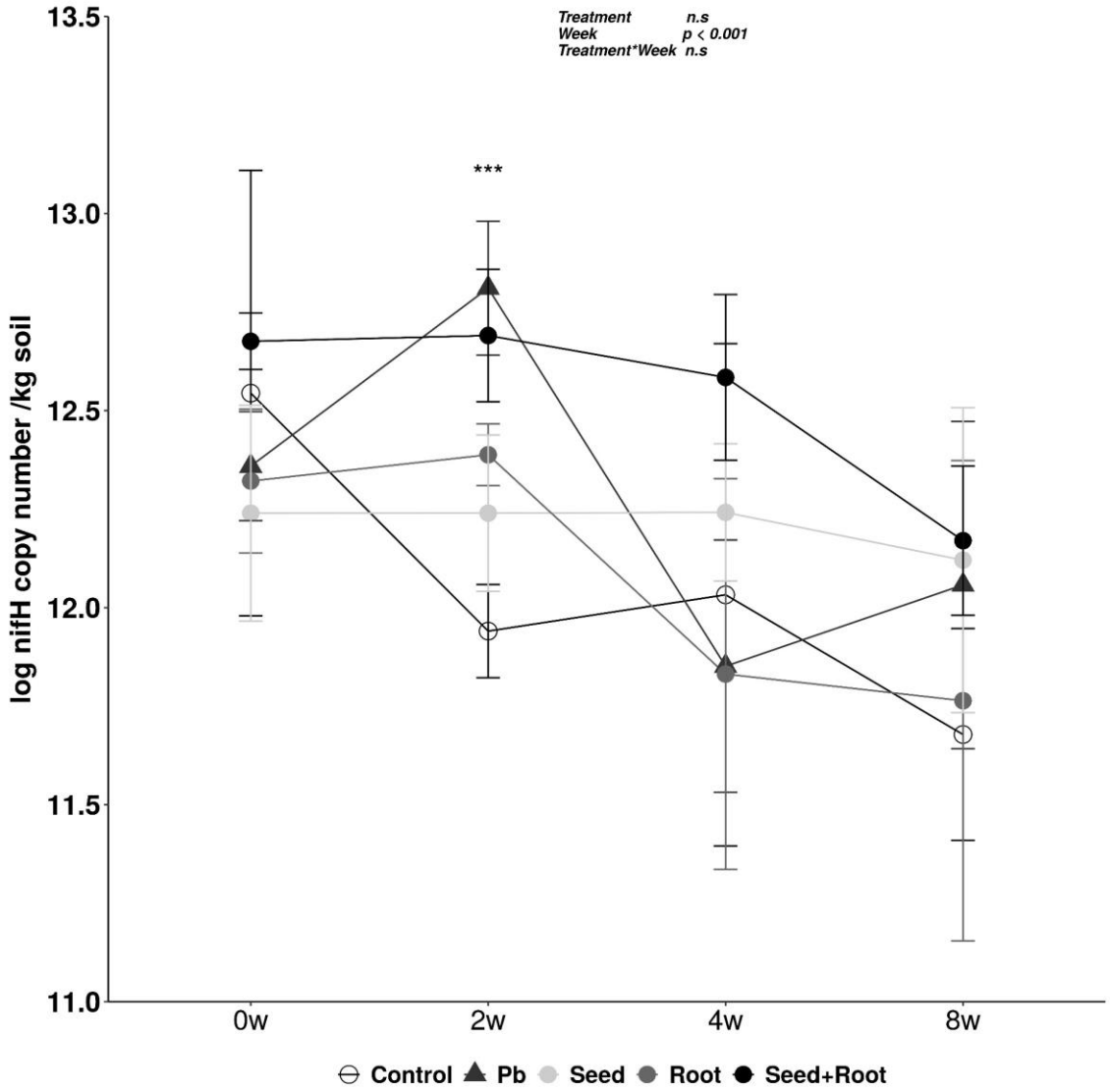

Figure 3-5. The changes in the log copy number of *nifH* gene in soil samples over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-7a. The temporal changes in the log copy number of *nifH* gene (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

nifH **(log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	12.54 ± 0.56	11.94 ± 0.12 **a**	12.03 ± 0.64	11.68 ± 0.27
Pb	12.36 ± 0.14	12.81 ± 0.17 **d**	11.85 ± 0.32	12.06 ± 0.42
Seed	12.24 ± 0.27	12.24 ± 0.2 **ab**	12.24 ± 0.17	12.12 ± 0.39
Root	12.32 ± 0.18	12.39 ± 0.08 **bc**	11.83 ± 0.5	11.76 ± 0.61
Seed + Root	12.68 ± 0.07	12.69 ± 0.17 **cd**	12.58 ± 0.21	12.17 ± 0.19

Table 3-7b. The results of the Shapiro-Wilk test for the abundance of *nifH* gene and nirS gene: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
nifH	0.2358

3.4.5 Quantification of nitrification genes: amoA and nxrB

The temporal changes in the abundance of nitrification gene (*amoA* and *nxrB*) were presented in Figure 3-6, with numerical data and statistical results provided in Table 3-8a and 3-8b. The abundance of the *amoA* gene was found to not follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-6a). Although no significant differences were observed in all culture periods (Table 3-8a, 3-8b), the highest *amoA* gene abundance was observed in the Seed + Root treatment at the 0th and 8th weeks, and in the Seed treatment at the 2nd and 4th weeks. The abundance of the *nxrB* gene was found to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-6b). The Seed + Root treatment had significantly higher *nxrB* abundance than both the Control and Root treatments at the 0th and 4th weeks (Table 3-8a, 3-8b). Furthermore, the Pb treatment consistently showed higher *nxrB* gene abundance compared to the Control throughout the cultivation period. Additionally, all microbial inoculation treatments exhibited higher *nxrB* gene abundance than the Control at the 2nd, 4th, and 8th week. In contrast, the Seed treatment at the 2nd week and the Root treatment at the 4th and 8th week showed lower *nxrB* gene abundance than the Pb treatment.

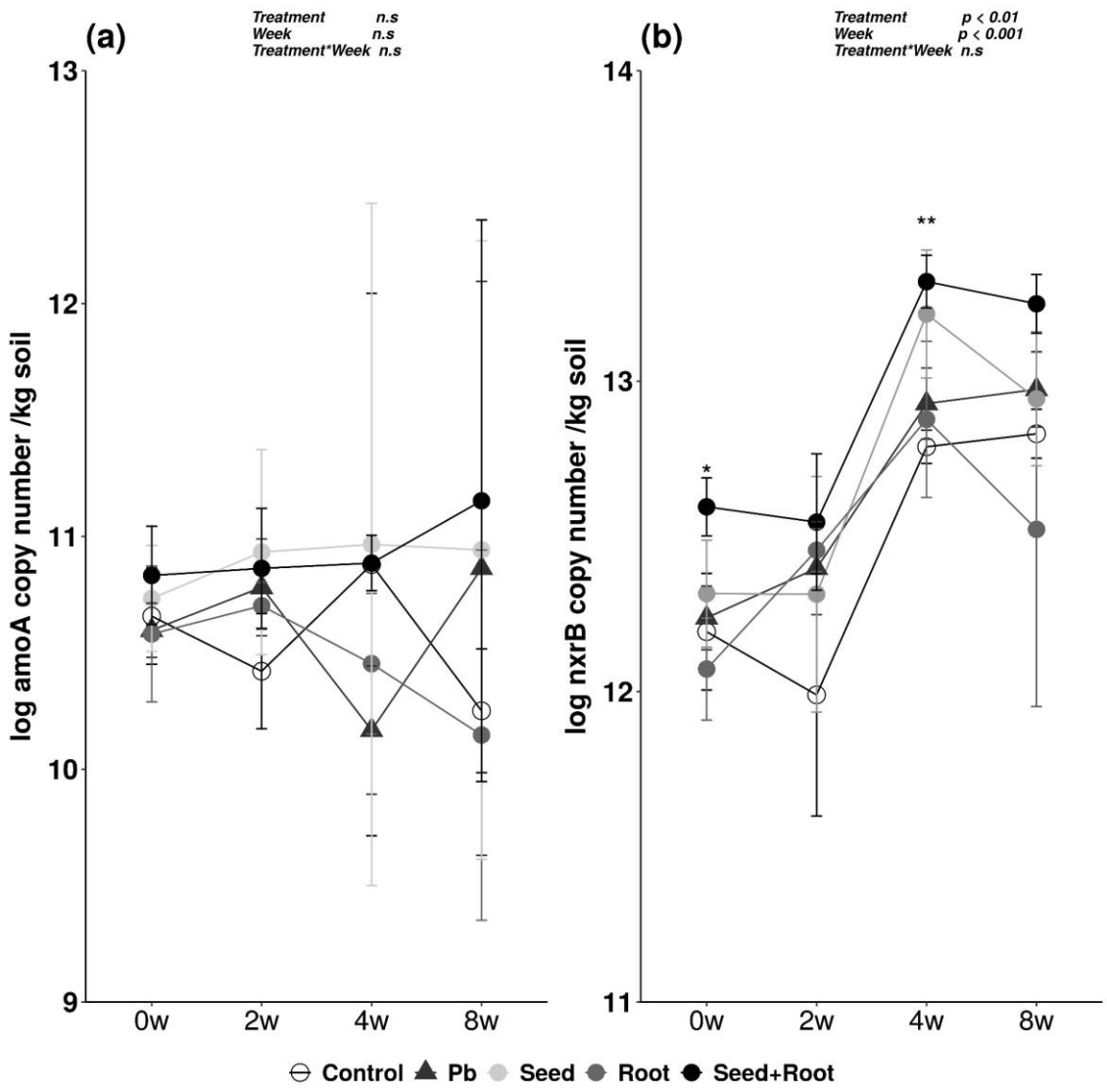

Figure 3-6. The changes in the log copy number of *amoA* gene and *nxrB* gene in soil over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-8a. The temporal changes in the log copy number of *amoA* gene and *nxrB* gene (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

amoA **(log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	10.66 ± 0.21	10.42 ± 0.25	10.88 ± 1.16	10.25 ± 0.27
Pb	10.6 ± 0.12	10.78 ± 0.21	10.17 ± 0.28	10.86 ± 1.23
Seed	10.73 ± 0.23	10.93 ± 0.44	10.97 ± 1.46	10.94 ± 1.33
Root	10.58 ± 0.29	10.7 ± 0.1	10.45 ± 0.3	10.15 ± 0.8
Seed + Root	10.83 ± 0.21	10.86 ± 0.26	10.89 ± 0.12	11.15 ± 1.21
nxrB **(log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	12.19 ± 0.19 **a**	11.99 ± 0.39	12.79 ± 0.05 **a**	12.83 ± 0.08
Pb	12.24 ± 0.1 **ab**	12.4 ± 0.15	12.93 ± 0.11 **ab**	12.97 ± 0.12
Seed	12.32 ± 0.17 **ab**	12.31 ± 0.38	13.22 ± 0.21 **ab**	12.94 ± 0.22
Root	12.07 ± 0.16 **a**	12.46 ± 0.07	12.88 ± 0.25 **a**	12.52 ± 0.57
Seed + Root	12.6 ± 0.09 **b**	12.55 ± 0.22	13.32 ± 0.08 **b**	13.25 ± 0.09

Table 3-8b. The results of the Shapiro-Wilk test for the abundance of *amoA* gene and *nxrB* gene: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
amoA	2.14×10^{-6}
nxrB	0.5850

3.4.6 Quantification of denitrification gene: narG, nirK, nirS, norB and nosZ

The temporal changes in the abundances of denitrification genes (*narG, nirK, nirS, norB,* and *nosZ*) are presented in Figure 3-7, while the numerical data and statistical results are provided in Table 3-9a, 3-9b, 3-9c, 3-9d, 3-9e, 3-10a, 3-10b, 3-10c, and 3-10d. The abundance of the *narG* gene was found to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-7a). There was a significant difference only at the 4th week, where the Seed + Root treatment showed significantly higher abundance than the Control. Furthermore, the Pb treatment and all microbial inoculation treatments exhibited higher *narG* gene abundance than the Control at the 2nd and 4th week (Table 3-9a, 3-9b). The abundance of the *nirK* gene was found to not follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-7b). The Seed + Root treatment exhibited significantly higher *nirK* abundance than the Control at the 4th week. Additionally, the Pb treatment and all microbial inoculation treatments showed higher *nirK* gene abundance than the Control at the 2nd and 4th weeks. In contrast, the *nirK* gene abundance in Root treatment was lower than that in the Pb treatment during these periods (Table 3-9a, 3-9b,3-9c, 3-9d). The abundance of the *nirS* gene was found to not follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-7c). There was a significant difference only at the 4th week and the Seed + Root treatment exhibited significantly higher values than the Control. Moreover, the Pb treatment and all microbial inoculation treatments exhibited higher *nirS* gene abundance than the Control at the 0th and 4th weeks (Table 3-9a, 3-9b,3-9c, 3-9d). The abundance of the *norB* gene was found to not follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-7d). The significant difference was observed only at the 4th week, with the Seed + Root treatment exhibiting significantly higher abundance than the Control. Furthermore, the Pb treatment and all microbial inoculation treatments showed higher *norB* gene abundance than the Control at the 0th and 4th weeks. In particular, the Seed + Root treatment consistently exhibited the highest *norB* abundance throughout the entire cultivation period (Table 3-10a, 3-10b, 3-10c, 3-10d). The abundance of the *nosZ* gene followed a normal distribution according to the Shapiro-Wilk test (Fig. 3-7e). There was a significant difference only in the 4th week and the Seed + Root treatment showed significantly higher *nosZ* abundance than the Control, Pb treatment, and Root treatment. Additionally, the Seed treatment exhibited significantly higher *nosZ* abundance than the

Control and Root treatments. The Pb treatment and all microbial inoculation treatments maintained higher *nosZ* gene abundance than the Control at the week 0, 2nd, and 4th weeks (Table 3-10a, 3-10b).

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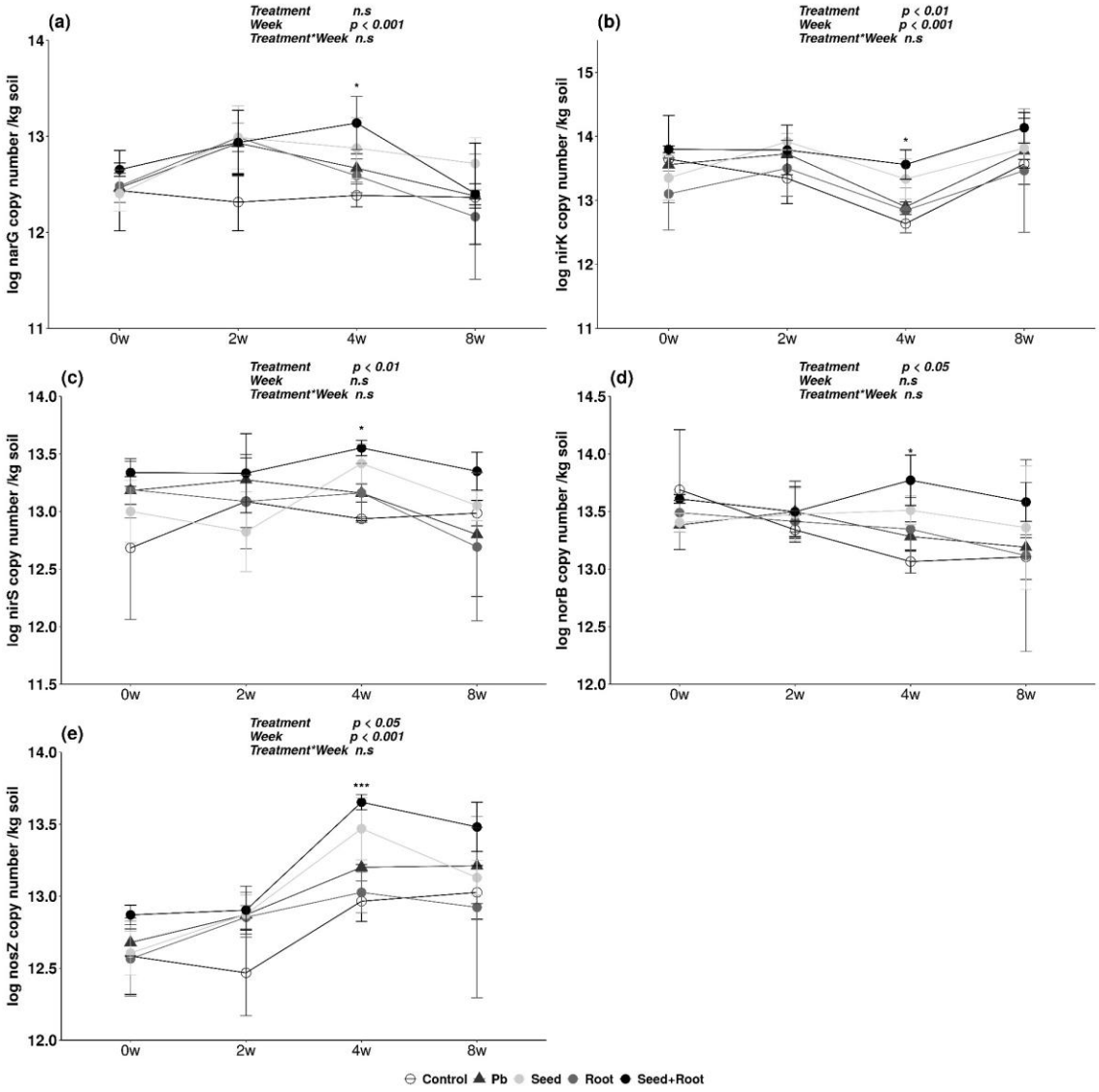

Figure 3-7. The changes in the log copy number of *narG* gene*, nirK* gene*, nirS* gene*, norB* gene*, nosZ* gene in soil samples over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-9a. The temporal changes in the log copy number of *narG* gene, *nirK* gene, and *nirS* gene (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

***narG* (log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	12.43 ± 0.42	12.32 ± 0.3	12.38 ± 0.12 **a**	12.36 ± 0.08
Pb	12.46 ± 0.15	12.93 ± 0.34	12.67 ± 0.15 **ab**	12.38 ± 0.13
Seed	12.4 ± 0.18	12.99 ± 0.33	12.88 ± 0.32 **ab**	12.72 ± 0.27
Root	12.48 ± 0.17	12.99 ± 0.15	12.59 ± 0.18 **ab**	12.16 ± 0.65
Seed + Root	12.65 ± 0.07	12.94 ± 0.34	13.14 ± 0.28 **b**	12.4 ± 0.53
***nirK* (log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	13.64 ± 0.68	13.34 ± 0.4	12.64 ± 0.14	13.57 ± 0.07
Pb	13.56 ± 0.1	13.73 ± 0.06	12.90 ± 0.08	13.77 ± 0.52
Seed	13.35 ± 0.35	13.92 ± 0.13	13.33 ± 0.32	13.82 ± 0.1
Root	13.1 ± 0.56	13.5 ± 0.44	12.84 ± 0.35	13.47 ± 0.96
Seed + Root	13.8 ± 0.05	13.79 ± 0.39	13.56 ± 0.23	14.13 ± 0.24
***nirS* (log copy number / kg soil)**	**0 week**	**2 week**	**4 week ***	**8 week**
Control	12.68 ± 0.62	13.09 ± 0.41	12.94 ± 0.02	12.99 ± 0.11
Pb	13.18 ± 0.12	13.27 ± 0.19	13.16 ± 0.08	12.8 ± 0.54
Seed	13 ± 0.27	12.82 ± 0.35	13.42 ± 0.17	13.05 ± 0.13
Root	13.19 ± 0.25	13.09 ± 0.23	13.16 ± 0.26	12.69 ± 0.64
Seed + Root	13.34 ± 0.12	13.33 ± 0.34	13.55 ± 0.07	13.35 ± 0.17

Table 3-9b. The results of the Shapiro-Wilk test for the abundance of *narG* gene, *nirK* gene, and *nirS* gene: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
narG	0.1072
nirK	0.0345
nirS	0.0005

Table 3-9c. The results of the Kruskal-Wallis test for non-normally distributed *nirK* and *nirS* abundance: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

nirK	p-value
0w	0.1918
2w	0.4015
4w	0.0310*
8w	0.4015
nirS	p-value
0w	0.3084
2w	0.2185
4w	0.03862*
8w	0.2185

Table 3-9d. The results of the Dunn's test for non-normally distributed *nirK* abundance at 4w: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

nirK at 4w	Control	Pb	Seed	Root	Seed + Root
Control	-				
Pb	0.1961	-			
Seed	0.0562	0.1952	-		
Root	0.2258	0.3921	0.1709	-	
Seed + Root	0.0174 *	0.1035	0.2905	0.0743	-

Table 3-9e. The results of the Dunn's test for non-normally distributed *nirS* abundance at 4w: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

nirS at 4w	Control	Pb	Seed	Root	Seed + Root
Control	-				
Pb	0.2256	-			
Seed	0.0630	0.1544	-		
Root	0.2578	0.5000	0.1852	-	
Seed + Root	0.0266	0.0763	0.3242	0.1018	-

Table 3-10a. The temporal changes in the log copy number of *norB* gene and *nosZ* gene, (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

norB **(log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	13.69 + 0.52	13.34 + 0.07	13.07 + 0.10	13.11 + 0.19
Pb	13.38 + 0.21	13.5 + 0.27	13.28 + 0.13	13.19 + 0.08
Seed	13.49 + 0.17	13.41 + 0.09	13.35 + 0.28	13.12 + 0.83
Root	13.4 + 0.08	13.47 + 0.23	13.51 + 0.13	13.36 + 0.54
Seed + Root	13.61 + 0.04	13.5 + 0.22	13.77 + 0.22	13.58 + 0.17
nosZ **(log copy number / kg soil)**	**0 week**	**2 week**	**4 week**	**8 week**
Control	12.58 ± 0.27	12.47 ± 0.3	12.97 ± 0.14 **a**	13.03 ± 0.19
Pb	12.68 ± 0.09	12.87 ± 0.16	13.20 ± 0.02 **ab**	13.21 ± 0.26
Seed	12.61 ± 0.15	12.87 ± 0.14	13.47 ± 0.22 **a**	13.13 ± 0.12
Root	12.57 ± 0.26	12.85 ± 0.08	13.03 ± 0.14 **bc**	12.92 ± 0.63
Seed + Root	12.87 ± 0.07	12.9 ± 0.17	13.65 ± 0.05 **c**	13.48 ± 0.17

Table 3-10b. The results of the Shapiro-Wilk test for the abundance of *norB* gene and *nosZ* gene: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
norB	0.0025
nosZ	0.1478

Table 3-10c. The results of the Kruskal-Wallis test for non-normally distributed *norB* abundance: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

norB	p-value
0w	0.3753
2w	0.3425
4w	0.0237*
8w	0.3425

Table 3-10d. The results of the Dunn's test for non-normally distributed *norB* abundance at 4w: If $p < 0.05$, the treatment groups were considered significantly different and represented with an asterisk (*).

norB at 4w	Control	Pb	Seed	Root	Seed + Root
Control	-				
Pb	0.1952	-			
Seed	0.0562	0.1961	-		
Root	0.2012	0.4276	0.1971	-	
Seed + Root	0.0096 *	0.0743	0.2285	0.0849	-

3.4.7 Quantification of 16S rRNA

The temporal changes in the abundances of 16S rRNA were presented in Figure 3-8, while the numerical data and statistical results are provided in Table 3-11a and 3-11b. The abundance of 16S rRNA was found to follow a normal distribution according to the Shapiro-Wilk test (Fig. 3-8). The significant differences were observed at the 0th and 4th weeks. At the week 0, the Seed + Root treatment exhibited significantly higher abundance than the Root treatment. The Seed + Root treatment showed significantly higher abundance than the Control at the 4th week. Furthermore, at the 2nd and 4th weeks, the Pb treatment and all microbial inoculation treatments exhibited higher 16S rRNA abundance compared to the Control (Table 3-11a, 3-11b).

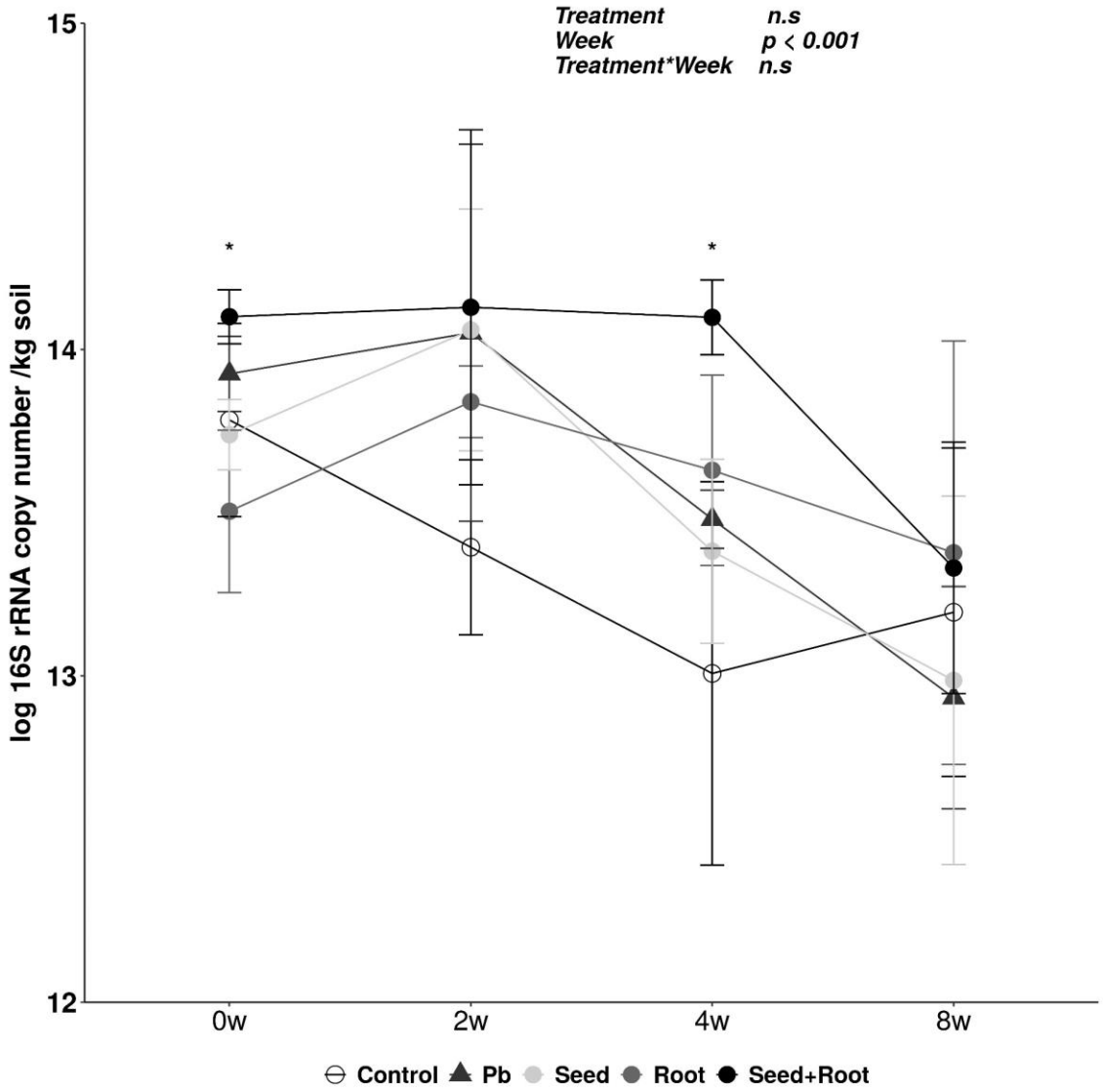

Figure 3-8. The changes in the log copy number of 16S rRNA in soil samples over time. The error bars represent the standard deviation (n = 3). Asterisks indicate whether there were significant differences between treatments for each week, as determined by Tukey-Kramer test (normal distribution) or Dunn's test (non-normal distribution). n.s. = no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 3-11a. The temporal changes in the log copy number of 16S rRNA (mean ± SD) in the cultured soil of each experimental plot from the week 0 to the 8th week(n=3). Alphabets were shown in the table only when the Tukey-Kramer analysis determined significant differences between each treatment.

16S rRNA (log copy number / kg soil)	**0 week**	**2 week**	**4 week**	**8 week**
Control	13.78 ± 0.30 **ab**	13.39 ± 0.27	13.01 ± 0.59 **a**	13.2 ± 0.5
Pb	13.93 ± 0.12 **ab**	14.05 ± 0.58	13.48 ± 0.09 **ab**	12.93 ± 0.34
Seed	13.74 ± 0.11 **ab**	14.06 ± 0.37	13.38 ± 0.28 **ab**	12.99 ± 0.56
Root	13.50 ± 0.25 **a**	13.84 ± 0.11	13.63 ± 0.29 **ab**	13.38 ± 0.65
Seed + Root	14.10 ± 0.08 **b**	14.13 ± 0.54	14.10 ± 0.11 **b**	13.33 ± 0.39

Table 3-11b. The results of the Shapiro-Wilk test for the abundance of 16S rRNA: If $p > 0.05$, the data were classified as normally distributed; if $p \leq 0.05$, they were classified as non-normally distributed.

	p-value
16S rRNA	0.1478

3.5 Discussion

3.5.1 Effects on WSC content and soil pH

There were no significant differences in WSC contents throughout the cultivation period (Fig.3-2a). The modified N-free nutrient solution used for cultivation in Leonard jars contained ferric citrate as the sole carbon source. However, since it was applied to all treatment groups, its contribution to the measured WSC contents was considered minimal. Some root exudates from plants are classified as low-molecular-weight WSC, which are rapidly decomposed and utilized as energy sources by soil microorganisms and are known to influence the structure, activity, and functionality of microbial communities (Bais et al. 2006; Weisskopf et al. 2008; Shi et al. 2011). Furthermore, the amount of WSC released into the soil as root exudates may have increased over time with the growth of lemongrass following inoculation with *A. brasilense* Sp7. However, no significant differences in the measured WSC contents were observed among the treatments, which may be attributed to their sufficient consumption by soil microorganisms. The soil pH ranged from 3.98 to 4.49, indicating that the soil remained consistently acidic throughout the cultivation period (Fig. 3-2b). The pH of the untreated soil was approximately 4.0, and the pH of the N-free solution added during cultivation was approximately 4.6 (Table 3-1). Therefore, the soil acidity observed in this study reflects the inherent characteristics of the soil used, and it is considered that the slight increase in pH may have been caused by the application of the N-free solution and inoculation with *A. brasilense* Sp7. In addition, the Root treatment consistently showed the highest pH among the treatments throughout the cultivation period, suggesting that the *A. brasilense* Sp7 treatment applied to the roots may have contributed to an increase in soil pH. Although the Seed + Root treatment showed a significantly lower pH compared to all other treatments at the 4th week, a similar trend was not observed in the other weeks. The Seed + Root treatment exhibited the highest abundances of the *nifH, nxrB, narG, nirK, nirS, norB,* and *nosZ* genes at the 4th week (Fig. 3-5, 3-6, 3-7). Denitrification can contribute to the decrease in soil pH by generating protons (H^+) as the reaction progresses. Therefore, the inoculation of *A. brasilense* to both the seeds and roots of lemongrass may have promoted the abundance of a wide range of soil microbes involved in denitrification, thereby enhancing denitrification activity and contributing to the observed decline in soil pH.

3.5.2 Effects on soil nitrogen cycling

Regarding nitrogen fixation, no significant differences in the amount of NH_4^+-N were observed throughout the entire cultivation period (Fig. 3-4a). However, the Seed treatment and Seed + Root treatment consistently exhibited higher abundances of the *nifH* gene compared to the control at the 2nd, 4th, and 8th week (Fig. 3-5). The *nifH* gene abundance in both the Root treatment and Seed + Root treatment groups was significantly higher than in the control at the 2nd week (Figs. 3-5). Furthermore, all inoculated groups showed greater nifH gene abundance than the Control at the 2nd and 8th week. These results suggested that inoculation with *A. brasilense* Sp7 increased the presence of nitrogen-fixing bacteria in Pb-contaminated soil but did not affect nitrogen fixation activity. The presence of the *nifH* gene in the *A. brasilense* Sp7 strain has been reported in previous studies (de Zamaroczy, Delorme, and Elmerich 1989), and the observed increase in *nifH* gene abundance may be attributed to the inrease of the inoculated *A. brasilense* Sp7 in the soil. Generally, the abundance of nitrogen-fixing bacteria decreases in heavy metal-contaminated soils containing Pb (Oliveira and Pampulha 2006; Huang et al. 2011). Previous studies have demonstrated that *A. brasilense* enhances the ability of plants to absorb heavy metals(Arora, Sharma, and Monti 2016; Muratova et al. 2022). In this study as well, inoculation with the Sp7 strain may have promoted Pb uptake by lemongrass, potentially creating the favorable environment and supported the growth of nitrogen-fixing bacteria in Pb-contaminated soil. Moreover, the discrepancy between NH_4^+-N levels and *nifH* gene abundance may have been related to the nutrient conditions of the soil used for cultivation. Since nitrogen fixation is an energetically costly process, nitrogen-fixing bacteria are known to regulate their nitrogen fixation activity according to the surrounding nutrient status (Zheng et al. 2019; Smercina et al. 2019). Given that the soil used in this study was nutrient-rich, nitrogen fixation activity may have been suppressed, which could explain the lack of significant differences in NH_4^+-N levels observed. Regarding nitrification, no significant differences in NO_3^--N concentrations were observed throughout the entire cultivation period (Fig. 3-4b). Similarly, the abundance of the *amoA* gene, which is associated with the conversion of NO_3^--N to NO_2^--N, did not show any significant variation across the cultivation period (Fig. 3-6a). In contrast, the abundance of the *nxrB*

gene, involved in the conversion of NO_2^- to NO_3^-, was significantly higher in the Seed + Root treatment at the week 0 and 4th week, and all microbial inoculation treatments showed higher *nxrB* levels than the control at the 2nd,4th, and 8th week (Fig. 3-6b). However, since *A. brasilense* has been reported to lack nitrification capacity (Roessler 1990), it is unlikely that the observed increase in *nxrB* gene abundance was directly attributable to the increase of the *A. brasilense* Sp7 in the soil. On the other hand, similar to previous studies in which inoculation with *A. brasilense* enhanced heavy metal uptake by plants (Arora, Sharma, and Monti 2016; Muratova et al. 2022), the inoculation with strain Sp7 in this study may have promoted Pb uptake by lemongrass and contributed to the increase in nitrifying bacteria possessing the *nxrB* gene.Taken together, these findings suggest that although *A. brasilense* Sp7 does not possess nitrification genes, its inoculation may have indirectly promoted the growth of nitrifying microorganisms in the soil. In relation to denitrification, all denitrification genes *narG*, *nirK*, *nirS*, *norB*, and *nosZ* showed significantly higher abundances in the 4th week in the treatment group compared to the Control (Fig. 3-7). These results suggested that *A. brasilense* inoculation effectively promotes the proliferation of soil microorganisms involved in various steps of the denitrification pathway, even in Pb-contaminated soils. Previous studies have reported that *A. brasilense* Sp7 possesses reduction capabilities for nitrate, nitrite, and nitrous oxide (Zimmer, Stephan, and Bothe 1984; Danneberg, Zimmer, and Bothe 1989). Therefore, the inoculated *A. brasilense* may have proliferated in the soil and contributed to the increased abundances of *narG*, *nirK*, *nirS*, and *nosZ* genes. Furthermore, enhanced tolerance to heavy metal stress conferred by *A. brasilense* inoculation may have positively influenced a broader range of denitrifying bacteria, leading to the observed increases in gene abundances. The potential factor explaining the lack of significant differences in NH_4^+-N and NO_3^--N concentrations among treatments could be that *A. brasilense* inoculation promoted nitrogen uptake by soil microbes and lemongrass plants. Indeed, previous studies have reported that the inoculation of PGPMs, which possess nitrogen fixation activity, increased the amount of inorganic nitrogen in the plant body (Fatima, Zia, and Chaudhary 2007). In addition, *A. brasilense* inoculation has been reported to increase microbial biomass nitrogen in soil (M. Zhang et al. 2017) and to enhance nitrogen uptake by plant roots (Saubidet, Fatta, and Barneix 2002). However, the NH_4^+-N and NO_3^--N contents in both lemongrass tissues and microbial biomass were not measured, making it impossible to determine how much inorganic nitrogen was absorbed by the plants or microbes versus how much

remained in the soil in this study. Therefore, future studies evaluating the impact of microbial inoculation on nitrogen cycling should compare nitrogen content in both soil and plant compartments. Additionally, while qPCR-based quantification of functional genes provides an estimate of the abundance of microorganisms possessing the target genes, it does not offer sufficient information on gene expression or enzymatic activity. Consequently, future research should employ transcriptomic analyses to monitor temporal changes in the expression and activity of related enzymes.

3.5.3 Effects on microbial abundance

The abundance of 16S rRNA was lower in both the seed and root inoculation treatments compared to the Control at week 0. However, at the 2nd week and 4th week, all microbial inoculation treatments exhibited higher levels of 16S rRNA abundance than the Control (Fig. 3–8). These results suggested that the abundance of soil microorganisms increased over time following inoculation with *A. brasilense* Sp7, which may be associated with the promotion of lemongrass growth as a result of inoculation. Plant roots are known to influence the spatial structure of soil (Angers and Caron 1998), and approximately 20–50% of photosynthates are translocated belowground (Kuzyakov and Domanski 2000). An average of 17% of photosynthates released into the soil as root exudates (Jones, Nguyen, and Finlay 2009), serving as a nutrient source for soil microorganisms (Lynch and Whipps 1990; Philippot et al. 2013), thereby leading to increased microbial abundance in the rhizosphere. In this study, inoculation with *A. brasilense* Sp7 enhanced root elongation in lemongrass, and it is likely that the resulting increase in root biomass and the more efficient utilization of root exudates contributed to the observed increase in 16S rRNA abundance. Although no significant differences were observed in the amount of water-soluble carbon in the soil, soil sampling in this study did not specifically target the rhizosphere. Therefore, future studies should analyze 16S rRNA abundance separately in bulk and rhizosphere soils to better evaluate the impact of microbial inoculation under heavy metal-contaminated conditions. It is also possible that the increase in 16S rRNA abundance was partly due to the establishment and proliferation of the inoculated *A. brasilense* Sp7 in the soil. However, this study did not include an analysis targeting genetic sequences specific to the Sp7, limiting the ability to perform a detailed evaluation. Future research on microbial inoculation should incorporate analyses targeting strain-specific genetic

markers to assess the survival and colonization efficiency of inoculated microbes in the soil environment.

3.5.5 Effects on the growth of lemongrass

3.5.5.1 Inoculation into both the Seed and Root regions

In this study, microbial inoculation of both the seeds and roots of lemongrass was the most effective in promoting the elongation of lemongrass leaves and roots (Fig.3-3). However, the Seed + Root treatment did not significantly affect NH_4^+-N and NO_3^--N concentrations (Fig.3-4). Therefore, it is suggested that the enhancement of lemongrass growth in the Seed + Root treatment was attributed to factors other than changes in the availability of inorganic-N sources. Several studies have reported that *A. brasiliense* promotes plant growth even in heavy metal-contaminated soils by producing plant hormones and increasing the expression of stress-tolerance-related genes such as CAT and SOD (Hadi and Bano 2010; Vazquez et al. 2021; El-Ballat et al. 2023). Consequently, the inoculation of *A. brasiliense* may have contributed to the improved growth of lemongrass in Pb-contaminated soil through these mechanisms. Furthermore, a study by Weniger et al. (1991) demonstrated that when the *A. brasilense* Wa3 was inoculated into cultivated soil, the effectiveness of inoculation decreased as the inoculation timing was delayed. Comparing with the results of this study, it is suggested that inoculating *A. brasilense* Sp7 strain on both seeds and roots, rather than in the soil, can sustain or even enhance the beneficial effects of inoculation beyond the mid-stage of plant growth.

3.5.5.2 Difference in inoculation effects based on soil nutrient status

Seed treatment and Seed + Root treatment did not show a significant growth-promoting effect on lemongrass during the period from the week 0, which from germination in vermiculite to transplantation into the Leonardo jar. This lack of effect may be related to the low nutrient content in the vermiculite, which likely inhibited the growth and establishment of the inoculated *A. brasilense* Sp7 strain. Moreover, the availability of soil nutrients is closely related to the abundance of soil microorganisms and enzyme activity (Bowles et al. 2014). Therefore, it is likely that in the nutrient-poor

vermiculite, the plant growth-promoting effects of *A. brasilense*, such as nitrogen fixation and phytohormone production, did not perform effectively. However, both Seed treatment and Seed + Root treatment showed growth-promoting effects on lemongrass after transplantation into the Leonardo jar containing gardening soil and modified N-free solution. These results suggested that the inoculation effects of *A. brasilense* Sp7 strain were not fully activated under nutrient-poor conditions, but they were not completely disappeared. Furthermore, the results indicated that as the nutrient status of the soil improved, the inoculation effect by *A.brasilense* Sp7 strain may have increased with time, even after a period has passed since the initial inoculation.

3.5.5.3 Difference between seed treatment and root treatment

In the 8th week, the Seed treatment showed a higher growth-promoting effect compared to the Root treatment, and it was demonstrated that even a single inoculation on seeds could promote the growth of leaf length. The differences in the growth of lemongrass observed in this experiment may have been related to the timing of *A. brasilense* inoculation. Weniger et al. (1991) conducted a study in which *A. brasilense* Wa3 strain was inoculated into wheat soil at different times after planting: on day 2, day 15, and day 45. As a result, the highest bacterial survival rate and acetylene reduction activity were observed when inoculation was performed on day 2 after sowing. Moreover, it was confirmed that performing multiple inoculations during the subsequent growing stages did not increase bacterial density on the roots or nitrogenase activity compared to a single inoculation at planting. The differences in inoculation effectiveness depending on the timing of inoculation were attributed to the difficulty in bacterial access to the roots due to root aging and lignification as the plants developed. This study also suggested that earlier inoculation of *A. brasilense* Sp7 strain may be more effective for promoting the growth of lemongrass, even under the Pb-contaminated condition.

3.5.4.4 Negative effects by root inoculation

The Seed treatment showed higher leaf growth and similar root growth compared to the Pb treatment, while the Root treatment exhibited lower leaf and root growth than the Pb treatment at the 8th week. Generally, inoculation with *A. brasilense*

was reported to promote plant growth by numerous studies. However, the results of this study suggested that seed inoculation of *A.brasilense* Sp7 was either effective or has little to no harmful effects, whereas root inoculation may have negative effects for the growth of leaf and root of lemongrass. In fact, several studies have reported that microbial inoculation did not promote growth or even inhibit it. In a study by Kozdroj et al. (2004), inoculation with *A. brasilense* survived in the maize rhizosphere, but it altered the microbial community of the maize rhizosphere soil and slightly inhibited maize growth. Additionally, Felici et al. (2008) reported that co-inoculation of *Bacillus subtilis* and *A. brasilense* in tomatoes, and Probanza et al. (2002) reported that co-inoculation of *Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105 in *Pinus pinea* did not result in the plant growth-promoting effects as with single inoculation, suggesting microbial competition. Furthermore, a study by Trabelsi et al. (2011) found that co-inoculation of two species of rhizobia resulted in lower growth-promoting effects compared to single inoculation. Considering these previous studies, it is possible that microbial inoculation may have led to competition with the overall microbial community in the soil or changes in the native microbial community, negatively affecting plant growth. The rhizosphere is known to harbor a highly diverse microbial community, which is closely linked to plant growth (Hassan, McInroy, and Kloepper 2019). Therefore, the direct inoculation of *A.brasilense* to lemongrass roots in this study may have disturbed the native microbial community and its functionality, particularly in the rhizosphere, potentially inhibiting growth instead of promoting it.

3.5.4.5 Difference in growth between Control and Pb Treatments

The Pb treatment and all microbial inoculation treatments exhibited higher growth of leaf and root than the Control. One possible factor contributing to these growth differences is a change in the bioavailability of sulfur (S) in the soil caused by the addition of PbS as the Pb contamination source. Several studies have reported that a decrease in soil pH increases the mobility and bioavailability of heavy metals in soil (Badawy 2002; Sukreeyapongse et al. 2002; Bang and Hesterberg 2004). Although PbS is a stable compound, its solubility was reported to increase under acidic conditions, similar to other heavy metals (Jin et al. 2015). Therefore, in the acidic soil (pH 3.9–4.5) used in this study, it was reasonable that the PbS added to the Pb, Seed, Root, and Seed + Root treatments dissolved and released sulfur ions. Sulfur is an essential element for

plant growth, playing critical roles in protein synthesis, cell growth and division, and chlorophyll formation (Borpatragohain et al. 2019). However, plants cannot absorb elemental sulfur, and inorganic sulfate (SO_4^{2-}) is the primary source of sulfur, whose uptake and transport involve multiple transporter systems (Leustek et al. 2000; Q. Li, Gao, and Yang 2020). Moreover, increased sulfate uptake capacity and upregulation of the sulfate transporter gene HAST (ZmST1;1) were observed in the rhizosphere of maize grown in heavy metal-contaminated soil (Nocito et al. 2006). Based on these previous studies, it is possible that the sulfur ions released from PbS were converted into sulfate and subsequently promoted sulfate uptake in the lemongrass rhizosphere, leading to the enhanced growth observed in the PbS-treated plots under acidic conditions in this study.

3.6 Conclusion

The cultivation experiment of lemongrass was conducted to evaluate the effects of microbial inoculation on the growth of lemongrass in Pb-contaminated soil. PbS was selected as the heavy metal contamination source, and *A.brasilense* Sp7 was selected as the PGPM. To assess the impact of different inoculation methods, three inoculation strategies were established: inoculation only at the seed, inoculation only at the root stage, and inoculation at both the seed and root. The effects of *A. brasilense* Sp7 inoculation on lemongrass growth in Pb-contaminated soil were evaluated in terms of the temporal changes in WSC content, soil pH, lemongrass leaf and root growth, and inorganic nitrogen content. Additionally, DNA was extracted from the cultivated soil, and quantitative analysis of nitrogen fixation genes, nitrification genes, denitrification genes, and 16S rRNA was conducted to assess the impact on the abundance of soil microbes involved in nitrogen cycling. The WSC content in the soil showed no significant differences across the treatment groups throughout the entire cultivation period. Soil pH ranged from 3.98 to 4.49, confirming the acidic characteristic of the soil throughout the cultivation period. The Seed + Root treatment exhibited a significantly lower pH compared to other treatments at the 4th week. The higher abundance of denitrification genes in the Seed + Root treatment at the 4th week may have contributed to increased denitrification activity, leading to a decrease in soil pH. There were no significant differences in NH_4^+-N content across the treatments throughout the cultivation period. Similarly, no significant differences were observed in NO_3^--N

content across the treatments, with NO_3^--N contents being 80.4%–97.8% lower than NH_4^+-N contents. These inorganic nitrogen contents in the soil may have been absorbed by lemongrass as it grew, explaining the lack of significant differences. However, as the inorganic nitrogen content in lemongrass was not measured in this study, the impact remains unclear. Regarding lemongrass growth, the Seed + Root treatment exhibited the most significant growth-promoting effect, with leaf length significantly higher than in the Control, and root length significantly higher than in both the Control and Root treatments at the 8th week. Since no significant differences were observed in NH_4^+-N and NO_3^--N content, it is suggested that the growth promotion of lemongrass was due to factors other than changes in nitrogen availability resulting from nitrogen fixation by *A. brasilense* Sp7. The Root treatment exhibited lower leaf and root growth compared to the Pb and Seed treatments, suggesting that root-only inoculation under Pb contamination may have negatively affected lemongrass growth. Moreover, all Pb treatments and microbial inoculation treatments showed higher growth than the Control, which could be attributed to the increased bioavailability of sulfur (S) derived from the dissolution of PbS added as the Pb contamination source. At the week 0, before transplantation into the growing medium, Seed treatment and Seed + Root treatment did not show significant growth-promoting effects. However, by the 8th week, these treatments significantly promoted lemongrass growth, suggesting that the growth-promoting effects of *A. brasilense* Sp7 were influenced by the nutritional status of the soil. Furthermore, these effects were not lost under nutrient-poor conditions but could improve with better soil nutrient conditions. Although no significant differences in the abundance of the *nifH* gene were observed at the 0th, 4th, and 8th week, the Seed + Root treatment showed the highest values. Since *A. brasilense* Sp7 possesses the *nifH* gene, the increase in *nifH* gene abundance may have been attributed to the growth of inoculated *A. brasilense* in the soil. Regarding nitrification genes, no significant differences in *amoA* gene abundance were observed throughout the entire cultivation period. However, the abundance of the *nxrB* gene was significantly higher in the Seed + Root treatment throughout the cultivation period, with values significantly higher than the Control at both week 0 and 4th week. Additionally, for denitrification genes, the Seed + Root treatment consistently exhibited the highest abundance across multiple time points, particularly at the 4th week, where the abundance of all denitrification genes was significantly higher than in the Control. These results suggested that the inoculation of *A. brasilense* Sp7 at both the seed and root increased the abundance of a

broad range of denitrifying bacteria, potentially influencing nitrogen cycling. The abundance of 16S rRNA was highest in the Seed + Root treatment from the 0th to 4th week. However, Root treatment at the 2nd week and Seed treatment in the 4th week showed lower 16S rRNA abundance than in the Pb that of Control. The changes in 16S rRNA abundance due to inoculation suggested that competition among soil microbes may have been involved, and the impact varied depending on the inoculation method. The differences in abundance suggested that combined inoculation of *A. brasilense* Sp7 on both seeds and roots may have allowed for more stable settelement in the soil compared to single inoculation methods, even in Pb-contaminated soils. On the other hand, single inoculation of seeds or roots may have led to competition with native soil microbes, resulting in a decrease in overall microbial abundance. In fact, previous studies have reported that microbial inoculation can lead to competition among soil microbes, reducing soil microbial abundance, and a similar trend may have occurred in this study's results. Future studies should measure the nitrogen content in lemongrass to better evaluate the fate of nitrogen in the soil. Furthermore, measuring Pb contents in both the soil and lemongrass would be important for assessing the phytoremediation potential of lemongrass following microbial inoculation. This would contribute to accumulating valuable information for improving heavy metal-contaminated soils.

Chapter 4 Synthesis and recommendations for the future research

4.1 Impact of lead contamination on denitrification after nitrate addition to soil: An interaction between nitrogen substrates, denitrification genes, and microbial community structures

In this study, soil samples were classified based on four different Pb treatments and the presence or absence of NO_3^- addition. The samples were incubated for four weeks, and soil and gas sampling were conducted weekly from the start of incubation. From the collected soil samples, the amounts of inorganic nitrogen (NO_3^--N, NO_2^--N, and N_2O-N) were measured, and the abundances of denitrification functional genes (*narG, nirS, norB,* and *nosZ*) were quantified to evaluate temporal changes in denitrification activity under Pb contamination. Additionally, the abundance of 16S rRNA, the Shannon diversity index, and microbial community structures at the phylum and genus levels were analyzed to assess the impact of Pb contamination on microbial communities over time. The results of inorganic nitrogen measurements showed that 1600 mg Pb/kg soil inhibited the reduction of NO_3^- and NO_2^-. The *narG* gene, encoding nitrate reductase, also showed a decreasing tendency with Pb, suggesting a correlation with the suppression of NO_3^- reduction under Pb contamination. On the other hand, the *nirS* gene encoding nitrite reductase showed no clear relationship with Pb concentration. Additionally, the highest N_2O production was observed at 1600 mg Pb/kg soil, which is consistent with previous studies on N_2O emissions under heavy metal contamination. The increased N_2O emissions under 1600 mg Pb/kg soil may have been associated with the initial decrease in *nosZ* gene abundance and the increase in *norB* gene abundance in the later stages of incubation. This study only quantified the abundance of functional genes and did not perform transcriptome analysis. Therefore, future evaluations of denitrification activity in heavy metal-contaminated soils should not only investigate the abundance of functional genes but also examine their transcription, expression, enzyme activity, and microbial community structure in detail. Furthermore, the bioavailability of heavy metals varies over time due to changes in soil pH as incubation progresses, so these environmental factors should also be considered. Immediately after exposure to Pb contamination, the abundance of 16S rRNA representing microbial abundance showed a rapid decrease. However, the Shannon diversity index tended to increase with Pb exposure. These changes in microbial abundance and diversity may have been caused by

disturbances in the microbial community due to environmental changes induced by Pb contamination, leading to an increase in Pb-tolerant microbes. Regardless of NO_3^- addition, the Shannon diversity index continued to increase over time and reached approximately 7.5 in all treatments at the end of incubation. This result suggested that as incubation progressed, the microbial community structure became more similar across all treatments. Regarding bacterial community structures, *Proteobacteria* initially dominated the soil but rapidly declined following exposure to 1600 mg Pb/kg soil. In contrast, the abundances of *Actinobacteria, Chloroflexi,* and *Nitrospirae* tended to increase with higher Pb concentrations. At the genus level, *Azotobacter* decreased to less than half of the Control when Pb contamination exceeded 400 mg Pb/kg soil. Meanwhile, *Bacillus* consistently exhibited high resistance to Pb from the 1st week to the 4th week. This study observed changes over several weeks after Pb exposure but did not evaluate the long-term effects of Pb. Indeed, heavy metal-contaminated soils are likely to be subjected to prolonged and continuous contamination. Therefore, future studies should conduct more detailed investigations of field-based heavy metal-contaminated soils and the microbial communities inhabiting them.

4.1.2 Inoculation of lemongrass with Azospirillum Brasiliense under Pb pollution: Impacts on plant growth and nitrogen cycle

This study aimed to evaluate the effects of microbial inoculation on the growth of lemongrass in Pb contaminated soil, using *A. brasilense* Sp7 strain as a PGPM. To assess the impact of different microbial inoculation methods on growth promotion, different inoculations were applied to lemongrass seeds, roots, and both. The experiment assessed the temporal effects of *A. brasilense* Sp7 inoculation in Pb-contaminated soil on WSC content, soil pH, growth of lemongrass leaves and roots, and inorganic nitrogen levels. Additionally, DNA was extracted from the soil, and the quantification of nitrogen fixation genes, nitrification genes, denitrification genes, and 16S rRNA was performed to evaluate the impact on the microbial community involved in nitrogen cycling. No significant differences in WSC content were observed across all treatment periods. Soil pH ranged from 3.98 to 4.49, indicating the soil was acidic throughout the cultivation period. In the 4th week, the Seed + Root treatment showed a significantly lower pH compared to other treatments. During the 4th week, all denitrification genes were most abundant in the Seed + Root treatment, suggesting that the increase in denitrifying bacteria might have contributed to the active denitrification

process, resulting in the reduction of soil pH. NH_4^+-N contents showed no significant differences across the treatment periods. Similarly, NO_3^--N contents did not show significant differences across all periods and were 80.4% to 97.8% lower than NH_4^+-N levels. These inorganic nitrogen contents in the soil might have been absorbed by the lemongrass during growth, which may have been able to explain the lack of significant differences. Regarding the growth of lemongrass, at the 8th week, the Seed + Root treatment showed the highest growth-promoting effect. The leaf length was significantly greater than the Control, and the root length was significantly greater than both the Control and Root treatments. Since no significant differences were found in NH_4^+-N and NO_3^--N levels in the soil, it was suggested that the growth promotion was due to factors other than changes in nitrogen availability, such as the production of plant hormones. Future research should include chemical analyses to evaluate changes in plant hormone levels in both the soil and plant tissues. The Root treatment showed lower leaf and root growth compared to both the Pb and Seed treatments, suggesting that sole inoculation of roots under Pb contamination might negatively affect lemongrass growth. All Pb treatments and all inoculation treatments showed higher growth compared to Control, possibly due to the increased bioavailability of sulfur (S) from the dissolution of PbS, the added Pb contamination source. At the week 0, neither Seed nor Seed + Root treatments showed significant growth promotion. However, considering that these treatments promoted growth at the 8th week, it was suggested that the growth-promoting effects of *A. brasilense* Sp7 were influenced by soil nutrient conditions. Furthermore, this effect was not lost under nutrient-poor conditions, indicating that improvements in soil nutrient status can enhance the growth-promoting effects. The abundance of the *nifH* gene did not show significant differences at the 0th, 4th, and 8th week, although the Seed + Root treatment exhibited the highest values. Since *A. brasilense* Sp7 carries the *nifH* gene, it is possible that the increase in *A. brasilense* contributed to the higher *nifH* abundance. For nitrification genes, no significant differences were observed in the abundance of *amoA* genes throughout the cultivation period. However, the abundance of the *nxrB* gene was highest in the Seed + Root treatment, significantly higher than the Control at both the 0th and 4th weeks. Regarding denitrification genes, the Seed + Root treatment also showed the highest abundance for most periods, with significant increases in all denitrification genes at the 4th week compared to the Control. These results indicate that the inoculation of both seeds and roots with *A. brasilense* Sp7 increased the diversity of denitrifying bacteria,

suggesting that *A. brasilense* Sp7 has an impact on nitrogen cycling. The abundance of 16S rRNA was highest in the Seed + Root treatment from the 0th to 4th week. In contrast, at the 2nd week (Root treatment) and 4th week (Seed treatment), the abundance of 16S rRNA was lower than in the Pb treatment. The fluctuations in 16S rRNA abundance due to inoculation may be attributed to microbial competition in the soil, suggesting that the inoculation method affects microbial community dynamics. Future research should focus on evaluating the fate of nitrogen in the soil in more detail by measuring the nitrogen sources within lemongrass plants. Additionally, measuring the Pb levels in both the soil and the plant tissues would help assess the phytoremediation potential of lemongrass in microbial inoculation treatments, which is essential for improving Pb-contaminated soils.

4.1.2 General comments

In this study, two types of Pb-contaminated soil incubation experiments were conducted, focusing on nitrogen cycling, microbial communities, and microbial inoculation. The first limitation of the first experiment was the absence of transcriptome analysis when evaluating denitrification activity. This study focused on the relationship between changes in denitrification activity and changes in the abundance of denitrification functional genes in Pb-contaminated soil. However, changes in the abundance of functional genes were insufficient to explain variations in denitrification activity in some cases. For example, no correlation was observed between the suppression of NO_2^- reduction caused by Pb contamination and changes in the abundance of the *nirS* gene. Therefore, to deeply evaluate the changes in denitrification activity under heavy metal contamination, it is essential to extend the analysis beyond DNA-based qPCR quantification and conduct transcriptome analysis using extracted RNA to examine gene transcription and expression under Pb contamination. The second limitation of the first experiment was the failure to measure N_2O emissions in week 0 due to equipment malfunction. Since NO_3^--N and NO_2^--N contents in week 0 were more sensitively affected by Pb concentrations, N_2O emissions may also have been strongly influenced by Pb contamination at the same time. The results from the 1st week showed that N_2O emissions were highest in the High treatment. This suggested that N_2O emissions might have been even more active in week 0. Therefore, when evaluating N_2O emissions in heavy metal-contaminated soil, it is important to consider the effects immediately after exposure in the future. The first issue in the second experiment was

the inability to measure NO_2^--N contents and N_2O-N emission. The plan was to analyze NO_2^--N levels using KCl extraction followed by FIA analysis. However, since the KCl extract was entirely used up for re-measuring NO_3^--N, NO_2-N could not be measured. As for N_2O-N emissions, the structure of the Leonard jar used for cultivation made it difficult to seal the container, preventing gas sampling. If NO_2^--N and N_2O-N levels had been measured, it would have been possible to assess in greater detail how nitrification and denitrification processes involving *A. brasilense* progressed under Pb contamination. The second issue in the second experiment was the lack of measurement of Pb concentrations in both soil and plant tissues. This study evaluated how inoculating PGPMs affected the growth of lemongrass, a plant suitable for heavy metal phytoremediation. The results confirmed that inoculation with *A. brasilense* Sp7 promoted lemongrass growth. However, since Pb concentrations in soil and plant tissues were not measured, it remains unclear how Pb uptake capacity of lemongrass changed. Studies on PGPM inoculation in lemongrass under heavy metal contamination remain limited compared to other heavy metal-tolerant plants. Therefore, accumulating more data from this perspective is crucial. A common limitation in both experiments was that they were short-term laboratory-scale soil incubation experiments with artificially induced Pb contamination. While laboratory experiments allow for the optional control of the soil conditions through reagent addition, they cannot fully recreate external factors present in natural environments such as temperature and moisture fluctuations due to weather changes and site-specific conditions. Additionally, short-term heavy metal-contaminated soil incubation experiments lasting several weeks can only assess the immediate effects of heavy metal exposure but cannot provide insights into long-term effects. To deepen the understanding of nitrogen cycling in heavy metal-contaminated soils, it is necessary to conduct field studies, assess local environmental conditions, and perform analyses using soils from the actual contaminated sites.

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