Peculiarities of the spread of denitrifying bacteria

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ABSTRACT

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| **Aims:** Here The distribution of denitrifying bacteria and the emissions they produce are of great interest both from an agricultural and ecological perspective. From the soils we studied, it is possible to generalize and determine the general denitrifying activity in the soils of many countries.  **Study design:** Microbiology, Agrochemistry  **Place and Duration of Study:** Department of Microbiology, Sukhumi State University. The research lasted 1 year. 20223-2024.  **Methodology:** methods of soil microbiology were used. Denitrifying bacteria were studied in the liquid phase of Giltai, the denitrification process was described by color reaction. For the quantitative calculation of denitrifiers in the liquid phase, we used the McCready system.  **Results:** The quantitative composition of denitrifying bacteria distributed in brown, black soil, alluvial and anthropogenic soils of Eastern Georgia has been studied according to the depth of the studied soils (5,10,25 cm). Samples were taken in all four seasons of the year. The soil types and depths favorable for the spread of denitrifying bacteria have been determined. The influence of acidity, salinity, moisture, the amount of hydrolyzable nitrogen and the seasonality of the year on their number has been determined. |

*Keywords: Denitrification, soil, emission ,Eastern Georgia.*

1. INTRODUCTION

Denitrification is a biological process by which nitrates (NO₃⁻) and nitrites (NO₂⁻) are converted to various forms of nitrogen, ultimately to nitrogen gas (N₂) and released into the atmosphere. This process plays a key role in the nitrogen cycle and in the regulation of soil nitrogen balance (Zumft, 1997). Denitrification is carried out by various microorganisms, called denitrifiers. This process is of great importance in both natural ecosystems and in agricultural and urban environments (Knowles, 1982). The most common denitrifiers are Pseudomonas, Paracoccus, Bacillus, Alcaligenes and Thiobacillus (Zumft, 1997). Also of interest are some fungi that are denitrifiers, such as Fusarium oxysporum and Trichoderma spp., (Shoun et al., 1992). Denitrification usually occurs in four stages: 1. Reduction of nitrate to nitrite, 2. Conversion of nitrite to nitric oxide, 3. Reduction of nitric oxide to nitrogen dioxide, 4. Conversion of nitrogen dioxide to nitrogen gas. The efficiency of denitrification depends on various factors, including: Oxygen level – the process mainly occurs in an anaerobic environment, although some microorganisms are facultative anaerobes and are able to metabolize in low-oxygen conditions (Tiedje, 1988). pH – under neutral-alkaline conditions (pH 6.5–8.5) denitrification occurs more intensively, however, under relatively low conditions (pH < 6.2) N₂O reductase is less active, the denitrification process does not proceed completely and N₂O (a greenhouse gas) is emitted into the atmosphere (Šimek & Cooper, 2002). In deeper layers, where oxygen levels are low, denitrification is more intense, but if the soil is slightly acidic (pH 6.2-6.5), N₂O accumulation increases (Baggs et al., 2010). N₂O is a potent greenhouse gas with a global warming potential of about 300 times that of CO₂ (IPCC, 2014). One of the most well-characterized denitrifying genera is Pseudomonas. Species such as Pseudomonas stutzeri are classical denitrifiers capable of complete denitrification from nitrate to nitrogen gas. They are Gram-negative, motile rods with robust metabolic flexibility, allowing them to thrive in both aerobic and anaerobic environments. Pseudomonads are commonly found in soils, sediments, and aquatic environments and are known for their rapid response to anoxic conditions and high efficiency in nitrate reduction.

Paracoccus denitrificans is another model organism in denitrification research. It is a non-motile, Gram-negative coccus that can perform complete denitrification. Paracoccus species are frequently used in laboratory studies due to their clear and well-regulated denitrification pathways, and their ability to grow autotrophically or heterotrophically under aerobic and anaerobic conditions. Their genome encodes all key reductases involved in denitrification, making them ideal for studying gene regulation in response to environmental stimuli.

Bacillus species, particularly Bacillus azotoformans and Bacillus licheniformis, are Gram-positive, spore-forming bacteria that exhibit partial or complete denitrification. Although denitrification in Bacillus is less efficient compared to Gram-negative genera, these organisms are notable for their resilience in extreme conditions, such as high salinity, temperature, or pH, and are frequently found in terrestrial habitats, including rhizospheres and compost.

Alcaligenes faecalis is a motile, Gram-negative rod known for its ability to reduce nitrite to nitrogen gas. Though it is not as metabolically versatile as Pseudomonas, it contributes significantly to nitrogen loss in wastewater systems and organically rich soils. It is often associated with denitrification in environments with low oxygen concentrations and elevated organic matter.

Members of the genus Thiobacillus, especially Thiobacillus denitrificans, combine chemoautotrophic sulfur oxidation with denitrification, utilizing nitrate as a terminal electron acceptor in the absence of oxygen. These bacteria are important in subsurface and groundwater systems, where both reduced sulfur compounds and nitrates may co-occur. Their role is especially significant in geochemically active zones.

In addition to these well-known genera, denitrification is also performed by bacteria in the genera Achromobacter, Shewanella, Agrobacterium, and Bradyrhizobium. For example, Bradyrhizobium japonicum, a symbiotic nitrogen-fixing bacterium, is capable of denitrification under microaerophilic conditions, especially in flooded soils such as rice paddies. This dual capacity for nitrogen fixation and denitrification highlights the metabolic plasticity of some soil bacteria.

Denitrifiers are not limited to free-living organisms. Some are part of biofilms and microbial aggregates in natural or engineered systems, such as wetlands, wastewater treatment plants, and bioreactors. In these environments, stratification of oxygen and nitrate concentrations facilitates microzones conducive to partial or complete denitrification. Therefore, managing denitrification is a significant challenge for environmentally sustainable agriculture (Smith, 2017). Denitrifying bacteria cannot survive in strongly acidic (pH < 6.0) soils, so their abundance at pH 6.0–6.8 is of most interest, as they survive under these conditions, but denitrification mostly ends with the release of N₂O. Denitrifying bacteria naturally perform the function of closing the nitrogen cycle and play an important role in maintaining both biochemical processes and ecosystem stability. Their identification, functional assessment, and analysis of spatio-temporal dynamics are necessary for both scientific and practical ecological management. In studies of natural soils and non-agriculturally used ecosystems, the study of denitrifiers is one of the important tasks related to both the optimization of nitrogen recycling and the biological regulation of processes affecting the climate. .

Therefore, the goal of our research was to study both the quantitative composition of denitrifying bacteria distributed in various types of soils in Eastern Georgia, as well as to determine the frequency of those physico-chemical parameters at which N₂O emissions significantly increase.

2. material and methods

The object of the study was the brown, alluvial, black soil, anthropogenic (area adjacent to the Tbilisi Sea) soil samples of Eastern Georgia, which were taken in all four seasons of the year. We collected soil samples using the envelope method From the territory of the uncultivated soils , we took 5 soil samples in such a way that when connecting the sampling points with straight lines, we obtained a sealed envelope drawing, the length of the sides of the squares of which was 2 meters (each sample weighed 200-300 grams). With a sterile shovel, we took three depth samples from the soil cut of the entire horizon, placed them in an anaerobic bag, . We determined soil moisture, salinity and acidity (we used a PH-meter brand Ohaus starter 2100). We counted the number of bacteria using the decimal dilution method of soil samples. We counted the number of bacteria per 1 g of dry soil [Lomtatidze and Kotia 2018].

We identified bacteria using the methods currently accepted in microbiology.

We used Giltai's nutrient medium, denitrification was confirmed by a color reaction (dye bromothymol blue). A dilution of each soil sample taken from depth was inoculated into 40 test tubes containing liquid Giltai solution with bromothymol blue dye. We calculated the results using the McCready system.

List 1 : Composition of Giltay Medium with Bromothymol Blue (for Denitrifying Bacteria)

Component Amount (g/L)

Potassium nitrate (KNO₃) 1.0

Sodium citrate 5.0

Magnesium sulfate·7H₂O 0.5

Ferrous sulfate·7H₂O 0.05

Calcium chloride·2H₂O 0.05

Potassium dihydrogen phosphate (KH₂PO₄) 1.0

Disodium hydrogen phosphate (Na₂HPO₄) 1.0

Bromothymol blue (indicator) 0.05–0.08 g\*

We were inoculating denitrifiers grown in test tubes in an anaerostat and beginning their biochemical testing.

1. Nitrate Reduction Test

The nitrate reduction test is the most direct and widely used biochemical method for detecting denitrification activity. It determines whether a bacterial isolate can reduce nitrate (NO₃⁻) to nitrite (NO₂⁻), ammonium (NH₄⁺), or gaseous nitrogen compounds (N₂, N₂O). The test is typically performed in nitrate broth with the addition of Griess reagents A and B. A red color change after reagent addition indicates the presence of nitrite, while gas accumulation in a Durham tube is a strong indicator of complete denitrification to nitrogen gas. .

2. Durham Tube Gas Production

Gas production under anaerobic conditions in nitrate broth is a key diagnostic criterion for complete denitrification. Bubbles in the inverted Durham tube within the culture medium signify the generation of molecular nitrogen (N₂), which is the end product of the denitrification pathway.

3. Anaerobic Growth Test with Nitrate

Denitrifying bacteria can typically grow under anaerobic conditions when nitrate or nitrite is available as a terminal electron acceptor. Incubating isolates in nitrate-supplemented media under oxygen-free conditions can confirm their ability to use nitrate respiration for energy metabolism, a hallmark of denitrification.

Determination of soil salinity by the porcelain cup extraction method

For the initial assessment of the degree of soil salinity, I used the porcelain cup extraction method, which is based on the slow steam drying of the aqueous extract obtained from the soil and the visual assessment of the resulting saline precipitate.

Methodology:

For the experiment, I selected an air-dried soil sample passed through a 1 mm sieve. I weighed 50 grams of soil and placed it in a chemically clean glass container. Then I added 50 ml of distilled water (depending on the situation, the mixing ratio was 1:1). I stirred the mixture with a glass rod and left it for 1 hour so that the soluble salts could completely pass into the aqueous phase.

After the time elapsed, I filtered the extract through filter paper and obtained a clear liquid. I placed 5 ml of this extract in a clean porcelain beaker and dried it with slow steam in a warm, dust-free environment (~50°C) to prevent leaching of salts or chemical degradation.

After drying, a clearly visible white crystalline precipitate appeared on the inner surface of the beaker. The density and uniform distribution of salts indicated a moderately saline soil. The result obtained corresponds to a relatively high concentration of soluble salts, which may indicate the presence of sodium and calcium chlorides and sulfates. After evaporating the extracts from all soils and all depths, I recalculated their percentages according to the method.

Determination of Soil Moisture Content Using the Drying Oven Method

To determine the moisture content of the soil samples, I used the standard drying oven method. For each sample, I took a clean and dry crucible (weighing dish), recorded its initial weight (M₀), and then added approximately 10 grams of fresh (wet) soil. I recorded the combined weight of the crucible and moist soil (M₁).

Next, I placed the open crucibles in a drying oven set at 105 °C and left them for approximately 12 hours, or until constant weight was achieved. After drying, I transferred the crucibles into a desiccator to cool and prevent moisture absorption from the air.

Once cooled, I weighed the crucibles again to determine the mass of the dry soil (M₂). Using these measurements, I calculated the moisture content of each soil sample using the following formula:

Using this method, I successfully determined the moisture content for all the soil samples under investigation.

Readily hydrolyzable nitrogen in the soil was determined by the method of Tyurin and Konanova [Margvelashvili, et al. 2021].

3. results and discussion

**Result**

Spring season

No denitrifiers were observed at a depth of 20–15 cm in the brown soil. The soil pH was 6.2, the moisture content was 18%, and the salinity was 0.80%. At a depth of 15–10 cm, the number of denitrifiers was 20,000. The pH was 6.8, the moisture content was 20%, and the salinity was 0.30%. At a depth of 10–5 cm, no denitrifiers were observed. The pH was 7.2, the moisture content was 15%, and the salinity was 0.10%. The hydrolyzable nitrogen in this soil was 14 mg per 100 g of soil. In the alluvial soil, the number of denitrifiers was 15,000 at a depth of 20–15 cm, the pH was 7.0, the moisture content was 25%, and the salinity was 0.10%. At a depth of 15–10 cm, denitrifiers were 30,000, pH 7.2, moisture 20%, salinity 0.10%. At a depth of 10–5 cm, no denitrifiers were observed, pH was 7.3, moisture 20%, salinity 0.05%. Hydrolyzable nitrogen was 11.8 mg. No denitrifiers were found at all depths in the anthropogenic soil. pH ranged from 6.8 to 7.0, moisture was 3-5%, and salinity was 0.02-0.14%. Hydrolyzable nitrogen was 5.2 mg. No denitrifiers were found at any depth in the black soil. pH ranged from 5.9 to 6.8, moisture content 25-30%, salinity 0.10 to 0.80%. Hydrolyzable nitrogen was 13.4 mg.

Summer season

No denitrifiers were observed at all depths of the brown soil. pH ranged from 6.4 to 7.1, moisture content 8-11%, salinity 0.06-0.20%. Hydrolyzable nitrogen was 9.2 mg. In the alluvial soil at a depth of 20–15 cm, denitrifiers were 35,000, pH 7.0, moisture content 30%, salinity 0.40%. At a depth of 15–10 cm, denitrifiers – 10,000, pH 7.0, moisture content 25%, salinity 0.30%. At a depth of 10–5 cm, no denitrifiers were observed, pH 7.2, humidity 13%, salinity 0.10%. Hydrolyzable nitrogen – 14.1 mg. In anthropogenic soil, no denitrifiers were observed at any depth. pH was 6.0–6.8, humidity 4–10%, salinity 0.10–0.80%. Hydrolyzable nitrogen – 4.2 mg. In black soil at a depth of 20–15 cm, denitrifiers were 15,000, pH 7.0, humidity 10%, salinity 0.30%. At a depth of 15–10 cm – 25,000, pH 7.2, humidity 5%, salinity 0.80%. At a depth of 10–5 cm – no denitrifiers were observed, pH 7.2, humidity 3%, salinity 0.10%. Hydrolyzable nitrogen – 16.3 mg.

Autumn season

There were no denitrifiers at all depths of the brown soil. pH 6.0–6.3, humidity 10–18%, salinity 0.30–0.40%. Hydrolyzable nitrogen – 15.68 mg. No denitrifiers were observed in the alluvial soil at all depths. pH was 5.8–6.8, humidity 17–23%, salinity 0.70–0.80%. Hydrolyzable nitrogen – 9.2 mg. No denitrifiers were found in the anthropogenic soil either. pH 6.0–6.5, humidity 20–28%, salinity 0.40–0.70%. Hydrolyzable nitrogen – 5.6 mg. In the black soil at a depth of 20–15 cm, denitrifiers were 35,000, pH 7.0, humidity 10%, salinity 0.25%. At a depth of 15–10 cm – 25,000, pH 7.1, humidity 12%, salinity 0.20%. At a depth of 10–5 cm – no denitrifiers, pH 7.3, humidity 18%, salinity 0.02%. Hydrolyzable nitrogen – 17.1 mg.

Winter season In brown soil at a depth of 20–15 cm, denitrifiers were 35,000, pH 6.8, moisture 13%, salinity 0.21%. At a depth of 15–10 cm – 25,000, pH 7.1, moisture 5%, salinity 0.10%. 10–5 cm – no denitrifiers, pH 7.5, moisture 12%, salinity 0.04%. Hydrolyzable nitrogen – 11.2 mg. In alluvial soil at a depth of 20–15 cm, denitrifiers were 15,000, pH 6.5, moisture 15%, salinity 0.20%. 15–10 cm – 10,000, pH 7.4, moisture 7%, salinity 0.20%. 10–5 cm – no denitrifiers, pH 7.6, humidity 13%, salinity 0.03%. Hydrolyzable nitrogen – 12.1 mg. In anthropogenic soil, denitrifiers were observed only at a depth of 15–10 cm – 18,000. pH was 6.8, humidity 9%, salinity 0.80%. At the remaining depths, denitrifiers were absent, pH 6.0 and 7.0, humidity 11–18%, salinity 0.02–0.10%. Hydrolyzable nitrogen – 6.3 mg. In black soil, at a depth of 20–15 cm, denitrifiers were 10,000, pH 7.0, humidity 20%, salinity 0.25%. 15–10 cm – 30,000, pH 7.3, humidity 6%, salinity 0.10%. 10–5 cm – no denitrifiers, pH 7.2, humidity 10%, salinity 0.09%. Hydrolyzable nitrogen – 12.3 mg.

**DISCUSSION**

(Detailed instruction Analysis by depth (spring season)

20-15 cm depth: Brown soil – no denitrifiers, pH = 6.2, humidity 18%, salinity 0.80%. Alluvial soil – denitrifiers 15,000, pH = 7.0, humidity 25%, salinity 0.10%. Anthropogenic soil – no denitrifiers, pH = 6.8, humidity 5%, salinity 0.14%.Black soil – no denitrifiers, pH = 5.9, humidity 30%, salinity 0.25%.

In spring, alluvial soil at a depth of 20-15 cm is most favorable for denitrification, as it has a neutral pH, moderate salinity and high humidity. Black and brown soils are less useful because they are either acidic (black) or highly saline (brown).15-10 cm depth: Brown soil – denitrifiers 20,000, pH = 6.8, humidity 20%, salinity 0.3%. Alluvial soil – denitrifiers 30,000, pH = 7.2, humidity 20%, salinity 0.1%. Anthropogenic soil – no denitrifiers, pH = 6.8, humidity 3%, salinity 0.03%.Black soil – no denitrifiers, pH = 6.4, humidity 25%, salinity 0.10%. At a depth of 15-10 cm, alluvial soil is the richest in denitrifiers (30,000 units), which is associated with neutral pH and moderate salinity. Denitrifiers are also found in brown soil, but in slightly lower quantities. 10-5 cm depth: Brown soil – no denitrifiers, pH = 7.2, humidity 15%, salinity 0.10%. Alluvial soil – no denitrifiers, pH = 7.3, humidity 20%, salinity 0.05%. Anthropogenic soil – no denitrifiers, pH = 7.0, humidity 3%, salinity 0.02%. Black earth soil – no denitrifiers, pH = 6.8, humidity 25%, salinity 0.8%. Denitrifiers are not found in the surface layer (10-5 cm) of any soil. Alluvial soil at a depth of 15-10 cm is most optimal for denitrification. Denitrifiers are not found in the surface layer (10-5 cm), which indicates that their activity occurs in deeper layers. Acidic (pH < 6.2) and dry soils (anthropogenic) are unsuitable for denitrification. Analysis by depth (summer season) 20-15 cm depth: Brown soil – no denitrifiers, pH = 6.4, humidity 10%, salinity 0.20%. Alluvial soil – denitrifiers 35,000, pH = 7.0, humidity 30%, salinity 0.40%. Anthropogenic soil – no denitrifiers, pH = 6.0, humidity 10%, salinity 0.10%. Black soil – denitrifiers 15,000, pH = 7.0, humidity 10%, salinity 0.30%. In summer, at a depth of 20-15 cm, alluvial soil is still the most favorable environment. Black soil also contains denitrifiers, although in smaller quantities. Analysis by depth (autumn and winter seasons) In autumn, denitrifiers are found only in black soil (35,000 units at a depth of 20-15 cm). In winter, up to 35,000 denitrifiers are found in brown and alluvial soils. In anthropogenic soils, denitrifiers first appear in winter (18,000 units at a depth of 15-10 cm), which may be associated with an increase in humidity. Interesting results are presented in the winter season, which is most noteworthy, not only because denitrifier bacteria are distinguished by their quantitative abundance, but also because we are dealing with an interesting phenomenon here. Under “critical pH” conditions (6.8, 6.5, 6.8), denitrification in most cases does not work completely and N₂O reductase is inhibited, which leads to N₂O emissions into the atmosphere. It is under these conditions that denitrifiers are abundant in brown soil (35,000), alluvial soil (15,000), and anthropogenic soil at a depth of 10-15 cm (18,000), which indicates a tendency for N₂O emissions to increase in winter.

4. Conclusion

Denitrifiers are most concentrated in alluvial and black soil soils. In dry and acidic soils (pH < 6.2, moisture <10%), the number of denitrifiers is equal to 0 (It seems that the denervation process is inhibited) . Denitrifiers are not found at 5-10 centimeters. In winter, the increase in denitrifiers in anthropogenic soils may be a result of increased moisture. The efficiency of the denitrification process is highest in alluvial soils, at neutral pH (7.0-7.4) and optimal moisture (20-30%). N₂O emissions may increase during the winter season.

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