THE INFLUENCE OF FERTILIZATION ON THE QUANTITATIVE DISTRIBUTION OF DENITRIFYING BACTERIA IN THE SOIL

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Abstract: Highlighting the processes by which the nitrogen compounds are reduced in the soil (i.e. denitrification) and which result in the increase of the ammonium level, is extremely important since denitrification is one of the effective mechanisms that reduce the nitrogen content in the soils rich in nitrites thus preventing soil pollution. The determination of the most probable number of denitrifying bacteria was carried out by the multiple tube technique using Allen’s culture medium, in anaerobic conditions. The test was performed on samples of fertilized and unfertilized soils collected from different depths (i.e. 7-10 cm and 15-25 cm). The denitrification process was found to manifest in all the soil types, with variations depending on the soil sample, fertilizers, type of tillage operations, and the period during which the microbiological determinations were performed. The lowest levels were found in the samples of unfertilized soils from 7-10 cm deep, after ploughing to 20 cm, collected in June 2009 (i.e. 56×10^2 bacteria/g soil), while the highest were found in the samples of fertilized soils tilled using the disk harrow, particularly in the sample collected from 7-10 cm deep (i.e. 31×10^3 bacteria/g soil). The density level, respectively the level of the bacterial metabolic activity, may be considered an indicator of the condition of the ecosystem examined reflected by the availability of the organic matters and the intensity of the processes of organic matter transformation and recirculation.

INTRODUCTION

Denitrification, the last stage of the biogeochemical cycle of nitrogen in nature, is an extremely important biological process. It is the major pathway of atmospheric nitrogen formation, of balancing the exchanges between this and the aquatic and terrestrial environments (Knowles, 1982). This process is exclusively generated by the denitrifying bacteria and consists in the anaerobic, dissimilatory reduction of nitrates to different nitrogen compounds (e.g. nitrites, ammonia, free nitrogen) (Zarnea, 1984).

Most denitrifying agents limit their action to the reduction of nitrates to nitrites (proper denitrification) or ammonia. In certain circumstances, nitrate reduction continues and results in molecular nitrogen, thus releasing nitrogen into the atmosphere and generating nitrogen losses from the ecosystem. On the other hand, an increase of the quantity of nitrites by lagging the denitrification stages (enzyme inhibition) could create conditions adverse to the ecosystems by the accumulation of toxic nitrite (Madigan et al., 2000). Different stages of denitrification are determined by different groups of bacteria. Some bacteria, such as *Pseudomonas*, *Clostridium* reduce the nitrates to nitrites; others reduce further the nitrites to ammonia, while other bacteria perform a complete denitrification, continuing the reduction process to the formation and release of nitrogen oxide and molecular nitrogen (e.g. *Bacillus denitrificans*, *Bacillus fluorescens*, *Achromobacter*). Most denitrifying organisms are heterotrophic, facultative anaerobic (Garrity et al., 2005). There are, however, some chemoautotrophic species (e.g. *Thiobacillus denitrificans*, *Thiomicrospira denitrificans*) that oxidize sulphur or thiosulfates by anaerobiosis in the presence of nitrates, which they reduce to N_2. They use the elemental sulphur or thiosulfates as electron donors for denitrification. Moreover, the bacteria *Alcaligenes eutrophus* and *Paracoccus denitrificans* live heterotrophically or chemoautotrophically as denitrifying agents by hydrogen oxidation. In addition, it has been shown that iron enhances significantly the rate of denitrification (Labbé et al., 2003), and the uptake of nitrates is fostered when phosphates have been limited and only nitrites have been used as electron acceptors by the denitrifying populations with no other quantities of nitrates available (Hunter, 2003).

Denitrification takes place in poorly aerated environments in which high quantities of organic matter, particularly nitrates, accumulate (Strong and Fillery, 2002). In what concerns the seasonal evolution of the denitrification process, it has been found that it reaches maximum levels during summer and autumn. Intense denitrification occurs upon adding chemical fertilizers to the soil when the nitrate content increases substantially. One of the products of denitrification, N_2O, formed in increasing quantities, in parallel to the enhancement of the use of nitrogen fertilizers, propagates in the stratosphere where, by a photochemical reaction, is converted into nitrogen oxide (NO). This reacts with the ozone leading to the decrease of the latter’s concentration and to the destruction of the major barrier protecting the living organisms against the harmful action of the ultraviolet radiations of the sun. Even though it is difficult to quantify the role of denitrification, it should be underlined that this process is a major factor in the overall balance of nitrogen in nature (Zarnea, 1984).

MATERIALS AND METHODS

The test for the most probable number of denitrifying bacteria was carried out by the multiple tube method using Allen's culture medium, in anaerobic conditions (Drăgan – Bularda, 2000, Atlas, 2004). The liquid medium containing nitrogen in the form of nitrates was inoculated with serial ten-fold dilutions ($10^{-1} – 10^{-5}$) of sample suspensions (1 ml of dilution in each of the 5 test tubes containing medium, for each dilution) and then incubated at 28^0C for 7-14 days. In parallel, a control sample was prepared: a test tube containing culture medium not inoculated with the soil sample was incubated in similar conditions. During incubation, nitrates are reduced to nitrites while the nitrites are reduced to NH_3. Nitrites and ammonia form as intermediate products. Nitrites were detected by a red color reaction using the Griess I and Griess II reagents. To 1 ml of culture medium, 0.1 ml of Griess I and 3-5 drops of glacial acetic acid were added. After 15 minutes, 0.1 ml of Griess II was added. The presence of nitrites was indicated by the development of a red pink coloration (Drăgan-Bularda, 2000, Dunca et al., 2004).

Alexander's statistical table was used to estimate the most probable number of denitrifying bacteria based on the results of the color test (Drăgan-Bularda, 2000). The analysis considered the triplicates of serial dilutions in which activity was detected. The table was consulted for the values corresponding to the three serial dilutions. The number in the table where the values were found to overlap was multiplied by the inverse value of the medium dilution thus obtaining the number of bacteria/g of soil.

RESULTS AND DISCUSSIONS

Denitrifying bacteria play an important part in the completion of the mineralization of nitrogen in the soil. The nitrates forming in the soil are used in multiple ways. Surface plants consume the largest part, a part of them is levigated, and the microorganisms in the soil transform another part. Chemical fertilizers may also have a stimulating effect on denitrification, particularly those promoting the increase of nitrate concentration.

This paper focused on the quantitative determination of denitrifying bacteria in 10 samples of fertilized soil subjected to different tillage operations. Other 10 samples of unfertilized soil subjected to similar tillage operations were used as controls. The samples were collected from different depths: 7-10 cm and 15-25 cm.

The data related to the denitrification process induced by the specific microbiota in the soil samples collected in June and October 2009 are shown in Table 1.

The analysis of the data indicated in Table 1 and Figures 1 - 4 had the following results.

In June 2009, relatively similar levels were found in all the 20 samples of soil subjected to examination. In what concerns the fertilized soils, the number of bacteria per g of soil oscillated between 11 x 10^3 (tillage operation: ploughing to 30 cm) and 23 x 10^3 (tillage operation: disk harrow) in the samples collected from 7-10 cm deep, and between 14 x 10^3 (tillage operation: ploughing to 30 cm) and 19 x 10^3 (tillage operation: ploughing to 20 cm) in the samples collected from 15-25 cm deep.

In what concerns the unfertilized soils, the number of denitrifying bacteria per g of soil was lower, with variations between 56 x 10^2 (tillage operation: ploughing to 30 cm) and 12 x 10^3 (tillage operations: Paraplow and Chisel plough + rotary harrow) in the samples collected from 7-10 cm deep, and between 56 x 10^2 (tillage operation: Chisel plough + rotary harrow) and 11 x 10^3 (tillage operation: ploughing to 20 cm) in the samples collected from 15-25 cm deep.

In October 2009, an intensification of the activity of the denitrifying bacteria was noticed, their number increasing considerably. Thus, in the fertilized soils, the number of bacteria per g of soil varied between 13 x 10^3 (tillage operation: ploughing to 30 cm) and 31 x 10^3 (tillage operation: disk harrow) for the sampling depth of 7-10 cm, and between 17 x 10^3 (tillage operations: Paraplow plough, ploughing to 30 cm) and 21 x 10^3 (tillage operations: disk harrow, Chisel + rotary harrow) for the samples collected from 15-25 cm deep.

Table 1. Number of denitrifying bacteria detected in the samples of fertilized and unfertilized soil

Sample no.	Presence/ absence of fertilization	Tillage operation	Sampling depth (cm)	No. of denitrifying bacteria / g soil	
				June, 2009	October, 2009
1.	Fertilized (N80P80)	Disk harrow	7-10	23×10^3	31×10^3
2.		Disk harrow	15-25	13×10^3	21×10^3
3.		Paraplow plough	7-10	17×10^3	22×10^3
4.		Paraplow plough	15-25	15×10^3	17×10^3
5.		Chisel plough + rotary harrow	7-10	20×10^3	23×10^3
6.		Chisel plough + rotary harrow	15-25	17×10^3	21×10^3
7.		Ploughing to 20 cm	7-10	12×10^3	14×10^3
8.		Ploughing to 20 cm	15-25	19×10^3	20×10^3
9.		Ploughing to 30 cm	7-10	11×10^3	13×10^3
10.		Ploughing to 30 cm	15-25	14×10^3	17×10^3
11.	Unfertilized	Disk harrow	7-10	11×10^3	15×10^3
12.		Disk harrow	15-25	10×10^3	13×10^3
13.		Paraplow plough	7-10	12×10^3	14×10^3
14.		Paraplow plough	15-25	75×10^2	94×10^2
15.		Chisel plough + rotary harrow	7-10	12×10^3	15×10^3
16.		Chisel plough + rotary harrow	15-25	56×10^2	74×10^2
17.		Ploughing to 20 cm	7-10	92×10^2	11×10^3
18.		Ploughing to 20 cm	15-25	11×10^3	13×10^3
19.		Ploughing to 30 cm	7-10	56×10^2	74×10^2
20.		Ploughing to 30 cm	15-25	90×10^2	93×10^2

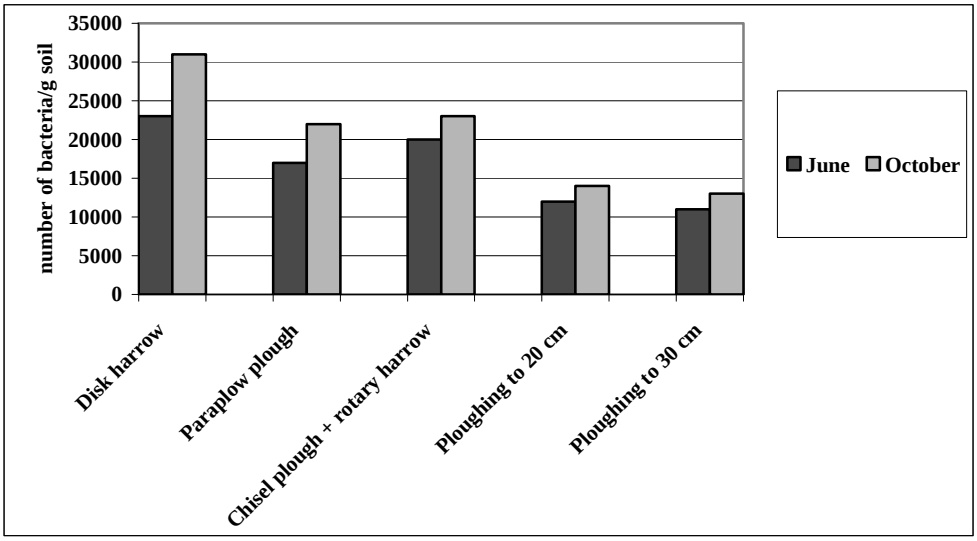

Figure 1. Numerical distribution of the denitrifying bacteria in fertilized soils (7-10 cm)

In what concerns the unfertilized soils, the values were much lower (i.e. 74 x 10^2 - 15 x 10^3) than in the case of fertilized soils, for the same tillage operations, but superior to those recorded in June.

The denitrifying bacteria were detected in the soil samples examined in each of the two seasons considered at an average level of 10^2-10^3.

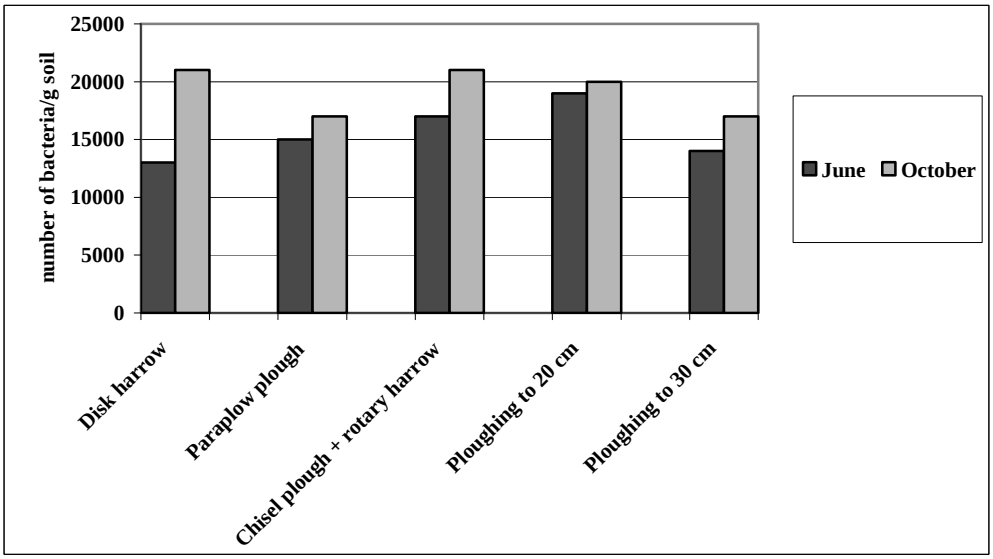

Figure 2. Numerical distribution of the denitrifying bacteria in fertilized soils (15-25 cm)

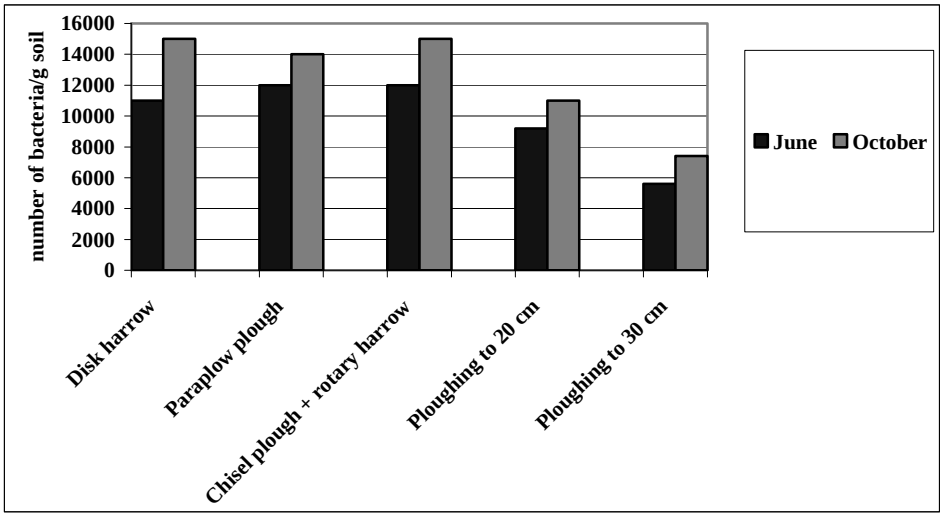

Figure 3. Numerical distribution of the denitrifying bacteria in unfertilized soils (7-10 cm)

The results of this study allowed us to show to what extent fertilization, type of tillage operation and sampling depth influence the quantitative distribution of denitrifying bacteria. The lowest levels were recorded in June 2009 in the unfertilized soils, such as in the sample collected from the depth of 7-10 cm, following soil ploughing to 30 cm (i.e. 56 x 10^2 bacteria/g of soil), while the highest levels were detected in October 2009, in the fertilized soils, particularly in the sample collected from the depth of 7-10 cm, following soil tillage using the disk harrow (i.e. 31 x 10^3 bacteria/g of soil).

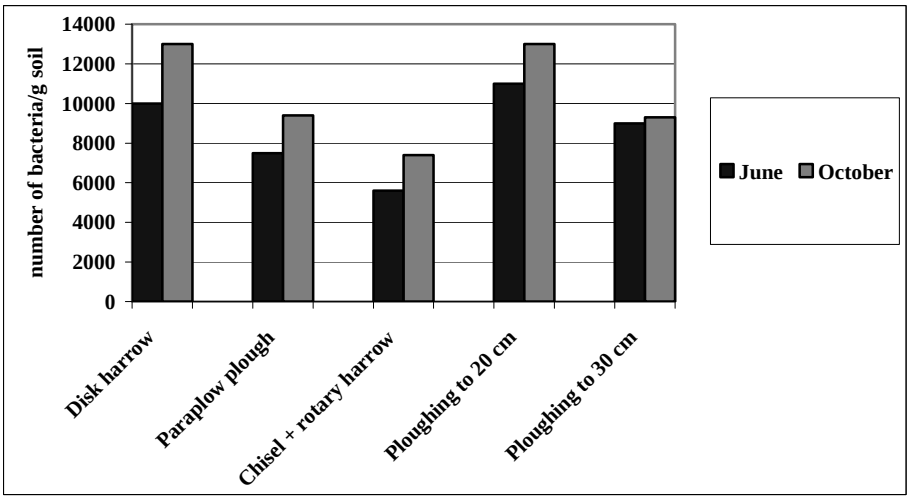

Figure 4. Numerical distribution of the denitrifying bacteria in unfertilized soils (10-25 cm)

Analyzing the seasonal variation, we could ascertain the stimulating effect of the elevated temperatures during autumn on the activity of the bacterial populations. This phenomenon correlates with the deficit of oxygen, which in its turn, is related to the growth of aerobic heterotrophic bacteria that induce processes of organic matter mineralization (Dunca et al., 2009). Numerous authors correlate the increased number of denitrifying bacteria to a high content of nitrates, while others show that an increase in the phosphorus content inhibits the activity of the denitrifying bacteria (Strong and Fillery, 2002).

CONCLUSIONS

The denitrifying microbiota is numerously represented in all the soil types, with variations depending on the fertilizer, soil sample, type of tillage operations, and the period in which the microbiological determinations were performed.

The lowest number of denitrifying bacteria/g of soil found was 56 x 10^2, while the highest was 31 x 10^3.

The differences between the two seasons (summer, autumn) are rather small, the higher values recorded during autumn being probably the result of the accumulation of organic matter of vegetable origin.

The quantitative increase of denitrifying bacteria, particularly in the autumn, may be correlated to the higher temperatures recorded at the end of summer, which promoted the growth of such microorganisms.

Fertilization, as well as some tillage operations (e.g. disk harrow), have a stimulating effect on the quantitative distribution of denitrifying bacteria in the soil samples examined.

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