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Microbial communities in areas affected by formation of calcrete in the Thracian plane

S. Bratkova*, K. Nikolova, K. Gesheva

Department of Engineering geology, University of Mining and Geology, St. Ivan Rilski, Student’s Campus, 1756 Sofia, Bulgaria

Abstract. The number of 13 physiological groups of microorganisms is counted in depth of two types of alkaline soils affected by formation of calcrete. The first soil type is characterized by the presence of calcrete nodules occupying the surface layer of soil profile from 5 to 38 cm, and the second soil type – of isometric calcrete nodules, settled in depth below 30-35 cm. During the spring and autumn, when the samples were collected, it was determined that in soil, which is more affected by formation of calcrete, the number of various heterotrophic aerobic and anaerobic bacteria, actinomycetes and fungi is 1 to 3 orders lower than in less affected soil. A similar effect was observed on the amount of nitrifying bacteria. Other factors that influenced the microbial community structure and number were also the various values of pH, fractions of organic carbon and total nitrogen.

Keywords: calcrete, microorganisms, soil

Introduction

Carbonate soils of different texture and composition are widely exposed in the Thracian plane. Recently Dimitrov et al. (2009a,b,c) suggested that the carbonate soils are product of a specific depositional facies, which is common for warm and dry areas, usually with rainfall under 1000 mm. This facies is characterized by the development of carbonate rich rock named caliche or calcrete. Calcrete is composed mainly of calcite (CaCO₃), but there is also a presence of other carbonate minerals. Secondary pedogenic carbonate (calcrete) occurs in many of the regions in semi-arid and arid areas. Calcrete occurs in a variety of forms, e.g. as calcareous sands in aeolian transported cover materials, as carbonate powders and rhizoliths, as well as nodular concretions, mottled horizons and discrete hard banks (McQueen et al., 1999). These soils are underlain by clastic continental sediments of the Neogene or Quaternary age. Because carbonate soils outcrop in agricultural areas, they affect significantly soil fertility. In Bulgaria carbonate soil accumulations have not been dated yet. Angelova et al. (1991) suggested that the carbonate crust is of Eopleistocene-Pleistocene age.

However, it is likely that the carbonate crust is long living and in constant process of building and decomposition reflecting climatic changes since late Neogene to present time. The carbonate crust has mostly calcitic composition but extensive sampling suggested that it is magnesium rich, which implies for significant dolomitic content (Dimitrov et al., 2009a).

Critical factors in the formation of calcrete are climate (a significant net annual moisture deficit in the soil; control on dust accretion caused by dust storms); topography; vegetation; presence of carbonate and oxalic phases; carbonate content, texture, porosity and permeability of the hosting substrate; action of microorganisms as well as exposure time (which may range between a few tens of thousands and millions of years) (Flügel, 2004).

Some of the CO₂ generated in biological respiration can be fixed in insoluble carbonates. Biomineralisation of secondary Ca-carbonates (Ca-carbonatogenesis) has been reported in numerous environments, such as agricultural and forest soil, marine and fluvial sediments as well as saline soils and lakes (Reith et al., 2008). Biological fixation of carbon in carbonates involves some bacteria, fungi and algae as well as some metazoan (Ehrlich, 2001). Nowadays the influence of formation of calcrete upon the structure of microbial communities is still poorly explored. The purpose of the study was to investigate the number of different physiological groups of microorganisms in depth of two types of agricultural soil with different content of biogenic elements, affected by formation of calcrete, during spring and autumn.

Material and methods

Sampling

The sampling site for this research is located about 3.5 km to east of the village of Skalitsa in Southeast Bulgaria. The sampling points are located at about 170 m elevation above sea level. The topography is dominated by gentle hills. Carbonate rich horizon is exposed in an elevated landforms having disk or lens-like shape. In the study area the thickness of the carbonate layer is between 20 and 40 cm. The organic soil cover varies between 5-10 and 40 cm. Immediately under the carbonate layer alternations of sands and clay belonging to the Gledachevo formation of Miocene—Pliocene age begin.

Chemical and microbiological analysis of soil samples from two sampling points located within the area were done in April and November. Profile LK - 4-1 was characterized by the presence of calcrete nodules, occupying the surface layer of soil profile from 5 to 38 cm and Profile LK - 4-2 in which isometric calcrete nodules were settled in depth below 30-35 cm. Soil samples were collected with a bore-hole equipment at depths 0-20, 20-40 and 40-60 cm.

Study methods

* e-mail: s_bratkovai@yahoo.com
Chemical analysis. The pH determination was performed according to the International Standard BDS ISO 10390 - an instrumental method for the routine determination of pH using a glass electrode in a 15 (V/V) suspension of soil in water (pH-H2O). Total carbon concentrations were determined by dry combustion via elemental analyzer (The International Standard ISO 10694). The carbon present in the soil was oxidized to carbon dioxide (CO2) by heating the soil to at least 900 °C in a flow of oxygen-containing gas that was free from carbon dioxide. The amount of released carbon dioxide was then measured by infrared detection method. The organic carbon content in soil was determined due to the International Standard BDS ISO 14235 by oxidation in a mixture of dichromate solution and sulfuric acid at a temperature of 135°C. Total nitrogen (ammonium-N, nitrate-N, nitrite-N and organic N) content in soil was determined by Kjeldahl digestion, according the International Standard BDS ISO 11281 f. Total phosphorus was extracted using BDS EN 13346 and determined by spectrophotometrical analysis. Water-soluble fraction of calcium and magnesium was established with BDS ISO 11048, using a titrimetric method of a 15 (m/V) suspension of soil in water.

Microbiological analysis

The soil sample (5g) was suspended in 50 ml of a 0.9% NaCl solution. The suspension was incubated for 2h on a rotary shaker at 200 rpm to detach cells from substrate. The turbid suspension was diluted in 10-fold steps to 10-8. Count of viable microbial cells was determined by the plate or liquid media count methods (9-11). Aerobic heterotrophic bacteria, fungi, actinomycetes, amylolytic microorganisms and nitrogenfixing bacteria were counted by plating on agar. For estimating the number of anaerobic heterotrophic bacteria, bacteria fermenting sugars with gas production, cellulose-degrading microorganisms, amonifying bacteria, denitrifying bacteria, Fe3+-reducing bacteria, sulphate-reducing bacteria and nitrifying bacteria, a three-tube most-probable number technique was applied.

### Results and discussion

**General geochemical properties of the soil substrate**

The chemical analysis (Table 1) demonstrated that both types of soils were alkaline.

**Profile LK-4-1 sampling.** pH of soil in profile LK-4-1 in the spring was in the range 8.39 to 8.50, and in autumn was in the range 8.19 to 8.56, with higher pH values found in the surface layer. At a depth of 0 to 40 cm in both seasons high values of total carbon presence were established - 50-68 g/kg, the significant part of which was inorganic and concentrated in isometric calcite nodules, which abounded in the area. The amount of organic carbon was several times lower - in spring - 12-15 g/kg, and in autumn in the layer from 0 to 40 cm reduced to 6.5-9.3 g/kg. In profile LK-4-1 there was a low content of nitrogen - total nitrogen in spring was about 0.8 g/kg, while in autumn it was unevenly distributed in the depth of the soil profile in the range of 0.48-0.91 g/kg. The content of total phosphorus was in the range 0.39-0.71 g/kg, and higher values were determined during autumn sampling.

**Profile LK-4-2 sampling.** The soil in profile LK-4-2 was also alkaline, with pH rates in the range of 8.2 - 8.36 in spring, which reduced in autumn - from 7.37 to 7.95. The amount of total carbon was lower compared to soil from profile LK-4-1 and the range was 24.2 - 37.7. Table 1 shows that the prevalent part of carbon was organic and its amount was from 18 to 27 g/kg, as the values of the parameter did not differ especially in spring and autumn sampling. Soil of profile LK-4-2 contained about twice as much total nitrogen (1.2-2.0 g/kg) compared to soil in profile LK-4-1 and was characterized by a greater amount of phosphorus. Specific electroconductivity data indicate that both types of soils are not saline.

**Microbiological analysis**

Spring sampling. **Profile LK-4-1.** During the spring sampling soil moisture was 16.7% in profile LK-4-1 in the surface layer from 0 to 20 cm, at depth below 20 cm it increased to values 22.8 - 23.1%. The

| Table 1. Basic parameters of soil samples in points LK-4-1 and LK-4-2. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Sample**     | **depth, cm**   | **pH(H2O)**     | **Total carbon, g/kg** | **Organic carbon, g/kg** | **Total nitrogen, g/kg** |
| LK-4-1         | 0-20            | 8.50            | 56.7            | 12              | 0.87                        |
|                | 20-40           | 8.59            | 50              | 15              | 0.87                        |
|                | 40-60           | 8.39            | 16.8            | 14              | 0.76                        |
| LK-4-2         | 0-20            | 8.31            | 37.7            | 27              | 1.9                         |
|                | 20-40           | 8.2             | 31.5            | 23              | 1.6                         |
|                | 40-60           | 8.36            | 24.2            | 18              | 1.9                         |

| Sample**     | **depth, cm**   | **Specific electroconductivity, mS/cm** | **Total phosphorus, g/kg** | **Water-soluble calcium, meq/100g** | **Water-soluble magnesium, meq/100g** |
| LK-4-1         | 0-20            | 0.125           | 0.39            | 0.62            | 0.17                        |
|                | 20-40           | 0.139           | 0.51            | 0.51            | 0.21                        |
|                | 40-60           | 0.189           | 0.71            | 0.49            | 0.47                        |
| LK-4-2         | 0-20            | 0.089           | 0.46            | 0.37            | 0.38                        |
|                | 20-40           | 0.129           | 0.9             | 0.52            | 0.38                        |
|                | 40-60           | 0.075           | 0.8             | 0.35            | 0.26                        |
highest number of aerobic and anaerobic heterotrophs (more than 10^5 cells/g) was found at depth of 20-40 cm, an area with higher humidity and the highest rates of organic carbon presence - 15 g/kg (Figure 1). In the rest of the soil profile the number of heterotrophic bacteria ranged to 1.3 \times 10^3 - 5.8 \times 10^5 cells/g. The highest number of all organic polymers-degrading bacteria had the amylolytic bacteria (3.9 \times 10^4 - 2.5 \times 10^5 cells/g), with a maximum in the layer 20-40 cm. The cellulose-degrading bacteria were about 10^5 cells/g from the surface to 60 cm depth. The number of actinomycetes ranged from 6.5 \times 10^3 to 2.5 \times 10^5 cells/g in depth of soil profile, the maximum was established again in the zone 20-40 cm.

About the bacteria involved in the transformation of nitrogen in all soil samples a large amount of nitrogen-fixing bacteria (more than 10^5 cells/g) was counted. The amount of total nitrogen in the soil profile was in the range 0.76 - 0.87 g/kg. These values determined the existence of a small number of other physiological groups involved in the transformation of nitrogen. The number of amonifying bacteria was in the range of 102-104 cells/g. The greatest part of denitrifying bacteria (7.8 \times 10^5 cells/g) was found in the area 20-40 cm and in the rest of the soil profile their number was about 103 cells/g. The nitrifying bacteria were characterized by a low number (101-102 cells/g) in the sampling point. In depth below 40 cm a high number of anaerobic bacteria decomposing organic matter was established – more than 105 cells/g of Fe3+ reducing bacteria. The number of sulfate-reducing bacteria was also low. Their number in the depth of the entire soil profile was in the range of 103-104 cells/g.

Profile LK-4-2. Humidity in the surface layer of profile LK-4-2

Figure 1. Number of different groups of microorganisms counted during spring sampling
from 0 to 20 cm was 18.0%, while at depth below 20 cm it increased to values from 22.5 to 24.8%. The highest number of aerobic and anaerobic heterotrophic bacteria - 1.2 \times 10^8 \text{ cells/g} was found in the soil profile to depth of 20-40 cm (Figure 1).

Although organic carbon content in the surface layer from 0 to 20 cm in depth was higher - 27 g/kg, the number of heterotrophic bacteria was lower by 1-2 orders than in the area of 20-40 cm. It was found that the highest number of cellulose-degrading bacteria - \(10^7\) cells/g was in the surface layer and in depth it gradually decreased. The amylolytic bacteria were characterized by a significantly greater number. The highest number - \(8.2 \times 10^7\) cells/g was determined again in the layer 20-40 cm. The rest of the soil profile was in the range of \(1.6 \times 10^5 - 4.8 \times 10^6\) cells/g. The number of bacteria fermenting sugars with gas production in all soil samples was \(3.5 \times 10^4 - 6.5 \times 10^4\) cells/g. The actinomycetes had a higher number in the soil profile. Their amount in the surface layer was the highest - \(2.106\) cells/g, in depth it gradually decreased. In the area of 00-40 cm the amount of fungi was in the range of \(3.6 - 6.5 \times 10^4\) cells/g and below 40 cm it reduced to 3.9 \times 10^2 cells/g. In the whole depth of the profile a high number of nitrogenfixing bacteria was established. At a depth of 00-40 cm it was in the range of 4.3 - 5.5 \times 10^6\) cells/g. The amonifying bacteria were most numerous of the rest of bacteria involved in the nitrogen cycle. Their number was highest in the surface layer - \(1.2 \times 10^8\) cells/g. In the zone 20-60 cm it reduced to \(1.3 \times 10^5\) cells/g. The number of denitrifying bacteria in depth of the soil profile from 0 to 60 cm was constant - \(7.2 - 7.5 \times 10^4\) cells/g. The highest number of them was found in the surface layer - \(3.104\) cells/g. In depth 20-40 cm they reduced to \(7.8 \times 10^3\) cells/g.

**Autumn sampling. Profile LK-4-1.** Soil moisture during the autumn sampling was in the range of 19.5 to 25.3%. The most

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**Figure 2.** Number of different groups of microorganisms counted during autumn sampling

1. Aerobic heterotrophic bacteria
2. Anaerobic heterotrophic bacteria
3. Cellulose-degrading microorganisms
4. Amylolytic microorganisms
5. Bacteria fermenting sugars with gas production
6. Ammonifying bacteria
7. Fungi
8. Actinomycetes
9. Nitrogenfixing bacteria
10. Denitrifying bacteria
11. Fe\(^{3+}\)-reducing bacteria
12. Sulphate-reducing bacteria
13. Nitriﬁying bacteria
numerous microbial populations were found in soil depth from 0 to 20 cm (Figure 2). In November in this surface layer the number of aerobic and anaerobic heterotrophic bacteria were 4.6.106 and 6.4.106 cells/g, regardless of the lower temperatures and the presence of a large quantity of calcrite, formed as nodules. The plant waste in soil from the sunflower crop grown throughout the year in the area LK 4 had a role in the supply with organic carbon of these bacteria. In depth of the soil profile from 20 to 60 cm the number of heterotrophic bacteria remained in the range of 1.7 to 4.2. 105 cells/g. The nitrogen-fixing bacteria were characterized with a higher number 105 to 106 cells/g in the three depths. The actinomycetes counted from 2.6 to 9.4. 104 cells/g and the fungi had the highest number - 2.2. 104 cells/g in the surface layer of the soil. In depth their number decreased to 2.0. 103 cells/g. The number of cellulose-degrading microorganisms rested above 101 cells/g in the entire soil profile. The amylolytic bacteria were also characterized with high number. Probably, the increased concentrations of monosugars due to their vital activity caused the most numerous populations of bacteria fermenting sugars in November. Once again denitrifying bacteria were found at the highest number - 3.2. 105 cells/g - in the surface soil layer. The Fe3+-reducing bacteria presented with the same number. At a depth below 20 cm the number of these two bacterial groups reduced to the range of 103-104 cells/g. The largest amount of sulfate-reducing bacteria was found in the surface layer - 7.6. 104 cells/g.

Profile LK - 4:2. The values of soil pH were in the range of 7.73 – 7.95 in autumn in the three investigated depths. In this period the soil humidity was 27.7 – 28.6 %. Once again, with the highest number of all studied physiological groups of microorganisms were the aerobic and anaerobic heterotrophic and nitrogen-fixing bacteria. The number of anaerobic heterotrophs was in the range of 2.9.106 – 9.8.106 cells/g and gradually decreased in depth of the soil profile (Figure 2).

The anaerobic heterotrophes were presented with similar numbers 8.4.106 cells/g at depth 0 to 40 cm and sharply increased to 8.3.107 cells/g at a depth of 40-60 cm. In the same depth the highest number of bacteria fermenting sugars with gas production was determined – 3.5.104 cells/g. There was no report for a number of bacteria fermenting sugars with gas production was 8.3.107 cells/g at a depth of 40-60 cm. In the same depth the highest number 8.4.106 cells/g was recorded. The nitrogen-fixing bacteria were characterized with a higher number 105 to 106 cells/g in the three depths. The actinomycetes counted from 2.6 to 9.4. 104 cells/g and the fungi had the highest number - 2.2. 104 cells/g in the surface layer of the soil. In depth their number decreased to 2.0. 103 cells/g. The number of cellulose-degrading microorganisms rested above 101 cells/g in the entire soil profile. The actinomycetes were also characterized with high number. Probably, the increased concentrations of monosugars due to their vital activity caused the most numerous populations of bacteria fermenting sugars in November. Once again denitrifying bacteria were found at the highest number - 3.2. 105 cells/g - in the surface soil layer. The Fe3+-reducing bacteria presented with the same number. At a depth below 20 cm the number of these two bacterial groups reduced to the range of 103-104 cells/g. The largest amount of sulfate-reducing bacteria was found in the surface layer - 7.6. 104 cells/g.

The presence of nodular calcrite is a basic reason for the formation of biological polymer-degrading microorganisms - cellulose-degrading and amylolytic bacteria, also bacteria fermenting sugars with gas production. Almost at all depths in the presence of calcrite lower number of bacteria, performing various types of anaerobic respiration - denitrifying bacteria, Fe3+-reducing bacteria, sulphate-reducing bacteria were found. The formation of calcrite had a strong negative effect on the number of nitrifying bacteria - it is 2-3 orders lower in soils from sampling profile LK-4-1.

Comparison of data from chemical analysis of soil samples and the number of soil microflora shows that significant factors that have influence on the microbial community number in soils affected by formation of calcrite are pH of soil, organic carbon content and total nitrogen content. Soil pH is a factor that has a general impact on the number of nitrifying bacteria in the samples. Soil fund of organic carbon and nitrogen affect mainly the number of different groups of heterotrophic microorganisms. The detected more numerous microflora in soil in profile LK-4-2 correlates with the high content of organic carbon and total nitrogen in soils of the same ground.

**Conclusion**

The presence of nodular calcrite is a basic reason for the formation of biological polymer-degrading microorganisms - cellulose-degrading and amylolytic bacteria, also bacteria fermenting sugars with gas production. Almost at all depths in the presence of calcrite lower number of bacteria, performing various types of anaerobic respiration - denitrifying bacteria, Fe3+-reducing bacteria, sulphate-reducing bacteria were found. The formation of calcrite had a strong negative effect on the number of nitrifying bacteria - it is 2-3 orders lower in soils from sampling profile LK-4-1.

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