Filtered forms of prokaryotes and bacteriophages in soil concretions

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Abstract. In Albic Retisols, Stagnic Fluvisols and Gleyic Fluvisols processes of ferriferous and manganese transformation follows rather active, that create new ecological niches for soil microorganisms. The study of concretions in the Albic Retisols, Stagnic Fluvisols and Gleyic Fluvisols showed, that the most characteristic feature of the prokaryotic community was the higher number and diversity of recoverable bacteria in comparison with the same indexes in the host horizons. The representatives of phylum Proteobacteria (class Gammaproteobacteria and Deltaproteobacteria) were dominant in the microbial communities in studied concretions. For the first time, the bacteriophages were found in concretions among them tailed phages were dominant. The method of the high-throughput sequencing of the 16S rRNA gene allowed to reveal the variability of the prokaryotic community in concretions of the Albic Retisol (Cutanic, Siltic), which was characterized predominantly by 7 bacterial phyla and 2 archaea phyla. Both groups of organisms typical of soils and prokaryotes capable of transformation of iron and manganese under aerobic and anaerobic conditions were revealed in soil concretions. The specific characteristic of the studied soil concretions was a widespread occurrence of filtered bacterial forms, which may be considered as specific survival forms in soil loci.

1. Introduction
Soil concretions relate to the specific loci in Albic Retisols, Stagnic or Gleyic Fluvisols, and are traditionally considered as an important object for research in soil physics. It is known that in Albic Retisols, Stagnic Fluvisols and Gleyic Fluvisols the processes of iron transformation follow actively and are accompanied by the formation of soil nodules with a unique combination of physical properties. Ferruginous and ferruginous-manganese concretions differ markedly from the properties of the host soil horizons, and are characterized by a higher particle density, less moisture, porosity, and the presence of a significant volume (number) of pores of extremely small size. The participation of microorganisms in the formation of iron-containing (ferriferous) minerals in the soils was revealed earlier [1]. It was found that different groups of prokaryotes are involved in the processes of iron and manganese transformation in natural conditions in soils and among cultivated bacterial isolates [2]. However, the participation of microorganisms in the processes of soil concretion formation has not been adequately investigated. The study of biodiversity in soil concretions (nodules) and physiological state of bacteria in such specific soil microenvironment retains its importance. Earlier it was suggested that filtering forms (FFPs) of prokaryotes play an active role in the processes of biomineralization in natural sources, some rocks and the human body. However, it is not clear yet whether FFPs may be isolated from soil nodules and play the same role [3]. The study of bacterial participation in soil biomineralization expands
our knowledge about the role of bacteria (prokaryotes) in the processes of iron and manganese transformation in the soil concretions. The goal of this work was to characterize the taxonomic and morphological diversity of prokaryotes in concretions, forming in the Albic Retisols, Stagnic Fluvisols and Gleyic Fluvisols, using modern methods of soil microbiology.

2. Objects and methods
The samples of the host horizon and concretions in the E-horizon of cultivated sod-podzolic medium loamy soil on the surface loam (Alicic Retisol (Cutanic, Siltic, WRB), magnetic and non-magnetic concretions from the host horizon (A’) turf meadow soil on alluvial deposits of the binomial Stagnic Fluvisol (Geobrustric, Humic, WRB) (Moscow region, WOPEC of Moscow state University "Chashnikovo") were researched. Concretions were obtained by wet screening followed by manual selection. Magnetic nodules were extracted by separation using a hand magnet. Also, concretions from horizons and amorphous ferruginous secretions in acidic alluvial meadow soil both in light loamy soil were analysed (Fluvisol (Loamic, Humic, WRB) (WOPEC of Moscow state University "Chashnikovo"; 56º 02’ 14” N, 37º 09’ 59” E).

The total number of bacteria, as well as the number of filtering forms of prokaryotes (FFPs), were determined using luminescent microscope "Axioskop 2+" (lens X100, oil immersion) and orange acridine stain [4].

The number of bacteriophages was determined by using the dye SIBR green [5]. For the determination of filtering forms of prokaryotes (FFPs) and bacteriophages, the method of filtration of the soil suspension through nuclear membrane filters (Sarstadt Company) with a pore size of 220 nm was used. The soil particles were precipitated by centrifugation with subsequent concentration of cells according to the protocol proposed earlier [6].

Transmission electron microscope (TEM) was used for the investigation of the morphology of FFP cells and bacteriophages concentrated from the native samples in soil concretions. The whole cells and bacteriophages were stained with a 1.5% solution of ammonium molybdate for 1-2 min [7].

The viability of bacteria was determined using fluorescent two-component dye L7012 (LIVE/DEAD BacLight bacterial viability kit.) [8] in accordance with the manufacturer's recommendations. The taxonomic compositional at the phyllum level was determined by FISH method using in situ hybridization with rRNA-specific oligonucleotide markers labeled with fluorescent dyes [8].

The diversity of the prokaryotic community in concretions from the Albic Retisol (Cutanic, Siltic) has also been studied by high-throughput sequencing of the 16sRNA gene. DNA extraction from samples of the nodule was performed using a set of reagents (MACHEREY-NAGEL NucleoSpin Soil, Germany) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was made with universal primers F515/R806 for the variable portion of a gene 16SpPHK (v3-v4). The preparation of the libraries was carried out in accordance with the instructions of the manufacturer MiSeq Reagent Kit Preparation Guide (Illumina). The amplicon libraries were sequenced in accordance with the manufacturer's instructions on the Illumina MiSeq instrument (Illumina, USA) using the MiSeq® ReagentKit reagent kit with two-sided reading (2 * 300 n). The data obtained from the sequencing of the samples were processed using the software packages "Trimmomatic" and "QIIME" [9].

3. Results and discussion
The number of prokaryotes in the samples of soil concretions varied from 1.82 to 2.63 billion cells per 1 g of soil. Filtered forms of prokaryotes FFPs were revealed in all studied samples of concretions. The number of FFPs in the concretions of the Albic Retisol (Cutanic, Siltic) varied from 0.76 to 0.86 billion cells per g, their proportion in the number of all bacterial cells counted up to 29%. The number of FFPs in concretions was higher than in the host soil horizon, where it was 0.73 billion cells in 1 g. The content of viable cells among FPP was quite high (88 - 99%), and exceeds such index in the host horizon (60-65%). These findings follow the recent results of the determination of viable FFPs in some soils in Russia [6]. The significant content of reversible bacteria in the concretions and number of FFPs suggest
their active participation in the mineralization processes in this soil locus. The number of FPPs was higher in non-magnetic concretions (0.63 billion cells per g) and lower in magnetic nodules (0.41 billion cells per g), while the FPPs content was higher in the concretions than in the host horizons (29 - 35% and 21% respectively). A high proportion of viable cells among FPPs bacteria in non-magnetic (95%) and magnetic (88%) concretions testify in favor of similar processes, occurring in concretions from the Albic Retisol horizons. Other features were typical for the samples of amorphous ferruterous formations (secretions) and the host horizon in the Gleyic Fluvisol (Loamic, Humic). The content of FPPs in both ferruterous secretions and in the host horizon did not exceed 10%. The number of FPPs in concretions and secretions were comparable to the same index in the host horizon. The proportion of viable cells among FPPs in concretions and secretions was always higher than in the host horizon, resulting in their high physiological activity. The similarity of these cells to FPPs forms obtained with filtering method was confirmed by studying the morphology of native cells in transmission electron microscope.

The overwhelming majority of the cells varied from 120 nm to 200 nm in diameter and no more than 300 nm -400 nm in length (uncommon from 500 nm to 600 nm). It allows relating such cells to FPPs forms [9]. Among the filterable forms, observed in magnetic and nonmagnetic nodules, mycoplasmas-like cells were predominantly detected, which morphologically appear as active forms. The obtained data on the FFPs morphology from concretions were also similar to the descriptions of cell morphology of saprotrophic mycoplasmas, which are able of forming FFPs under special conditions among the cells with the common bacterial sizes (figure 1).

![Figure 1](image1.png)

**Figure 1.** Diversity of filtered forms of bacteria in nonmagnetic (left) and magnetic (right) concretions. 1 bar = 200 nm.

Bacteriophages were revealed in filtrates of concretion suspensions separated from the Albic Retisol (Cutanic, Siltic) using the method of staining the samples with high sensitive SIBR green dye. The number of bacteriophages in the concretions was comparable to the number of FPPs forms and reached 0.9 billion in 1 g. The study of bacteriophages by transmission electron microscopy in filtrates showed that tailed phages prevailed in the concretions, while less filamentous forms were found.

It is known that representatives of different taxonomic groups (phyla) of prokaryotes participate in the processes of transformation of iron and manganese in soils [2] - *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Acidobacteria* and *Planctomycetes* [2]. The usage of FISH method allowed finding out those FFPs, belonging to *Gammaproteobacteria* which dominated in the concretions in the Albic Retisol horizons, while among bacteria with the common cell dimensions the representatives of both *Gammaproteobacteria* and *Alphaproteobacteria* prevailed.
The representatives of *Deltaproteobacteria* constituted the most part among FPPs forms in magnetic and nonmagnetic concretions of sod-meadow soil. Among other bacteria of the common cell dimensions significant amounts of the representatives of other taxa: *Alphaproteobacteria*, *Betaproteobacteria*, and *Planctomycetes* were revealed.

Noteworthy is the presence among FPPs in Stagnic and Gleyic Fluvisols the representatives of phyla *Acidobacteria* and *Planctomycetes*, among which many active iron transformers are known. Special attention must be given to the difficulties of reverse and cultivation of representatives of those phyla on laboratory nutrient media [10].

Therefore, the identification and study of such bacteria using the FISH method is of significant interest.

The results obtained by the FISH method suggest that not only one type of bacteria (representatives of well-known genera *Galionella, Pedomicrobiium, Metallogenium*), but also bacteria of the other genera which are potentially capable of carrying out the processes of iron transformation are involved in the formation of concretions.

This assumption seems to be quite logical in the light of the “duplication principle” previously formulated in soil microbiology. Currently, there is no consensus on the nano-transformation process in bacteria. This phenomenon is considered by a number of authors as a response of prokaryotes to external stress effects. However, there is no doubt that transformation to FPPs forms may be involved in the processes of iron conversion in soils, and FPPs forms constitute a significant part of the “silent community” of soil bacteria, being in viable, but uncultivated forms, or difficult to reverse on nutrient medium [3].

Analysis of the taxonomic diversity of the prokaryotic community in concretions, carried out by high-throughput sequencing of the 16S rRNA gene, revealed the dominance of the following bacteria phyla: *Actinobacteria* (23%), *Proteobacteria* (21%), and *Chloroflexi* (20%). A smaller portion was detected among the phyla of *Firmicutes* (9%), *Acidobacteria* (5%), *Nitrospirae* (2%) and *Verrucomicrobia* (1%). The content of phyla *Armatimonadetes, Planctomycetes, Gemmatimonadetes* and candidate phylum AD3 did not exceed 1% (figure 2).

The relative abundance of other phyla was tenths and hundredths of a percent (phylum *Bacteroidetes, Chloroflexi, Cyanobacteria, Elusimicrobia, Tenericutes* and phylum-candidate WPS-2). In the phylum *Actinobacteria* the order of *Actinomycetales* was dominant. Representatives of the genera *Arthrobacter, Nocardia, Promicromonospora, Intrasporangium*, and *Streptomyces* known as typical pedobionts dominated in the phylum *Actinobacteria* (order *Actinomycetales*). Attention was attracted to the presence of actinobacteria, belonging to the order *Acidimicrobiales*, which were earlier found in marine sediments with high iron content [11].

Bacteria of *Alphaproteobacteria* phylum dominate among proteobacteria - their portion in the total number of prokaryotes was 9.3%. Within this group of bacteria, the families *Hyphomicrobiaceae* and *Rhodospirillaceae* were found, representatives of which are capable of oxidizing iron and manganese under aerobic conditions.

The next most abundant class of *Proteobacteria* was phylum *Betaproteobacteria* (4.3%), represented by the order *Burkholderiales*, the families *Comamonadaceae, Oxalobacteraceae* and *Burkholderiaceae*, which include the bacteria associated with plants [12].

Representatives of *Deltaproteobacteria* phylum accounting for 3.5% of the total number of prokaryotes, basically belong to the order *Myxococcales*, which are widely distributed in soils, and sulphate-reducing anaerobic bacteria attributed to *Syntrophobacterales* which are able to grow on medium with acetate and propionate also were revealed.
Bacteria of *Gammaproteobacteria* (0.98%) and of orders *Xanthomonadales* (*Sinobacteraceae* family) and *Pseudomonadales* were determined as a relatively small group of *Proteobacteria*. It is known that some species of the genus *Pseudomonas* are able to oxidize iron under aerobic conditions and reduce manganese under anaerobic conditions [2].

Representatives of the *Sinobacteracea* family were found in significant amounts in the samples of ferromanganese concretions from the ocean floor [13]. The rather high content of *Chloroflexi* phylum attracts a great attention.

Composition of this phylum contains anaerobic bacteria capable of oxidizing iron and manganese in soils under anaerobic conditions. According to the published data, the bacteria of phylum *Chloroflexi* represent a significant part of the prokaryotic population on the inner surface of soil aggregates (microaerophilic zones). In our experiments the phylum *Chloroflexi* was dominated by the representatives of *Ktedonobacteria* class, *Thermogemmatisporaceae* family which are capable of manganese transformation under anaerobic conditions. The phylum *Firmicutes* was represented by typical pedobionts – bacteria of *Bacillales* and *Clostridiales* orders. It is known that some species of the genus *Bacillus* are capable of oxidizing iron under aerobic conditions [2].

Phylum *Acidobacteria* was represented by family *Koribacteraceae*, Ellin6513, and *Solibacteraceae* groups. These groups are described in the literature as oligotrophic microorganisms [14].

It should be noted that the ratio of phylum of *Proteobacteria* and *Acidobacteria* in the concretion samples coincides with their ratio reported earlier in sod-podzolic soils (the Albic Retisols), determined by FISH method [9]. It is known that representatives of phylum *Acidobacteria* and *Nitrospira* commonly occupy the soil loci, poor in available carbon and phosphorus. This may explain the presence of representatives of this phylum in our samples.

Phylum *Verrucomicrobia* was represented by family *Chthoniobacteraceae*, namely the DA101 phylotype. This phylotype was found in almost all studied soil samples. Earlier published data testify that the relative abundance of DA101 is rather high in soils rich in organic matter [15]. The archaea in...
the studied concretions were represented by phylum *Thaumarchaeota* (10.9%) and phylum *Crenarchaeota* (less than 0.1%).

Bacteria of phylum *Thaumarchaeota* are capable of oxidizing ammonium and are widely spread in soils and other habitats. Among the phylum *Crenarchaeota* the representatives, which are involved in the transformation of iron and manganese processes in aerobic and anaerobic conditions, were found.

4. Conclusion
The most characteristic features of the prokaryotic community in studied concretions from horizons of the Albic Retisols and Stagnic Fluvisols were the higher number and viability of FFPs bacterial forms than in the host horizons, as well as the particular taxonomic composition of prokaryotic communities at the level of phyla [16]. Identified FFPs forms were of the same phyla that were revealed among the bacteria with common (larger) size of cells. It is obvious that in the concretions under inhibition of bacterial growth, limited by external factors the cells are reduced in size and may be regarded as special forms for cell preservation. In the concretions of the Albic Retisol (Cutanic, Siltic) among the FFPs the representatives of phylum *Proteobacteria* (*Gammaproteobacteria* class) dominate; whereas Proteobacteria (*Deltaproteobacteria* class) dominated in the Stagnic Fluvisol (Geosapric, Humic). Bacteria of phyla *Acidobacteria* and *Proteobacteria* (*Alphaproteobacteria* and *Betaproteobacteria* classes) were found in the Gleyic Fluvisol (Loamic, Humic) in significant amounts.

The bacteriophages were found in the concretions in Albic Retisol (Cutanic, Siltic). The number of bacteriophages was comparable with the number of FFPs and reached 0.9 billion in 1 g. It was shown by the method of TEM that tailed phages were dominating; less filamentous forms of bacteriophages were found.

Biodiversity of the prokaryotic community in concretions of the Albic Retisol was studied for the first time using the high-throughput sequencing of the 16S rRNA gene. It was shown that prokaryotic community was formed principally by 7 bacterial phyla (*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Chloroflexi*, *Planctomycetes* and *Firmicutes*) and two archaea phyla (*Thaumarchaeota* and *Crenarchaeota*). In the concretions both typical groups of soil bacteria (*Actinomycetales*, *Myxococcales*, *Hyphomicrobiaceae*, *Bacillales*, *Clostridiales*, DA101, *Thaumarchaeota*) were revealed, as well as bacteria, which are capable of iron and manganese transformation in aerobic and anaerobic conditions (*Crenarchaeota*, *Acidimicrobials*, *Hyphomicrobiaceae*, *Rhodospirillaceae*, *Ktedonobacteria*, *Sinobacteracea*, *Chloroflexi* and some others). The present study allows understanding the functioning of the bacterial communities in concretions - specific soil locus, which is manifested by a widespread distribution of filtered forms, their high taxonomic and morphological diversity, which may be regarded as specific forms for survival under conditions of limited growth. The availability of FFPs bacterial forms and representatives of bacterial phyla with cells of common size were accompanied by the presence of bacteriophages, which were revealed for the first time in soil concretions.

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