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EXPERIMENTAL ARTICLES

# Taxonomic Composition of Cultured Fe- and Mn-Oxidizing Bacteria and Microbial Abundance in Fe–Mn Nodules of Different Sizes

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**Abstract**—Taxonomic diversity and quantitative distribution of cultured forms of Fe- and Mn-oxidizing microorganisms in Fe–Mn nodules of different sizes and fine earth of Gleyic Luvisols formed in the territory not affected by direct anthropogenic impact, were analyzed. The results were obtained using a combination of microbiological, molecular and analytical methods and noninvasive techniques. Most of the microorganisms which were cultured from the nodules were Mn oxidizers. Bacteria of the genera *Bacillus*, *Rhodococcus*, *Lysinibacillus*, *Pseudomonas*, and *Priestia* were identified in the nodules. Quantitative distribution of Fe- and Mn-oxidizing microorganisms in the outer and inner zones of the nodules of different sizes demonstrated that Mn-oxidizing microorganisms were involved in all stages of nodules formation and development, while Fe-oxidizing microorganisms participated in the initial phase of their formation. Spherules and porous structures of bacterial nature were observed in the studied nodules. The host soil fine earth was characterized by differences in the relative abundance of the dominant microbial groups in the profile. Manganese-oxidizing bacteria were represented in the soil fine earth by the genera *Prestia* and *Methylobacterium*.

**Keywords:** Fe–Mn nodules, Mn-oxidizing microorganisms, Fe-oxidizing microorganisms, taxonomic composition of bacteria, Gleyic Luvisols

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Iron-manganese nodules (IMN) are formations with specific structure and composition (both mineralogical and chemical), which are separated from the surrounding soil mass; they are the most common form of soil Mn–Fe concretion structures (Roslikova, 1996; Zaidel'man and Nikiforova, 2001; Timofeeva et al., 2014, 2021; Ettler et al., 2017; Gasparatos et al., 2019). Numerous studies indicate the diagnostic importance of IMN in the soils with variable type of the redox regime and IMN efficiency in accumulation of some variable-valency elements, thus limiting their mobility (Cornu et al., 2005; Tan et al., 2006; Gasparatos, 2012; Purtova and Timofeeva, 2022; Fischel et al., 2023). Investigation of soil IMN yielded considerable information on their composition, structure, distribution, and origin. The presence of iron and manganese ions in significant concentrations in the fine soil and alterations of the redox processes are required for IMN formation and development (Kostenkov, 1986; Roslikova, 1996; Zaidel'man and Nikiforova, 2001; Gasparatos et al., 2019; Sipos et al., 2022; Fischel et al., 2023). Although some arguable points still exist, IMN formation in soils is certainly determined by the factors favoring precipitation of such major compounds responsible for hard-pan formation as Fe and Mn (hydr)oxides in the microniches (Kostenkov, 1986; Zaidel'man and Nikiforova, 2001; Timofeeva and Golov, 2010; Gasparatos et al., 2019). Among these factors is microbial Fe and Mn transformation, which includes metal consumption from the soil solution with their subsequent precipitation and accumulation by microorganisms (Aristovskaya, 1975; Pinevich, 2005; Vodyanitskii, 2010; Lysak et al., 2013). Experimental results presented in the literature dealing with the role of soil microflora in IMN formation and development are insufficient for unequivocal assessment of the contribution of microorganisms to transformation of the elements in the course of IMP formation in soils and in the cycle of elements as a whole. The results of few studies aimed at investigation of microbial composition in Fe–Mn structures revealed variation of the taxonomic groups and bacterial complexes in the structures formed in different soil types (Shchapova, 1994; Lysak et al., 2013; Hu et al., 2015). The nodules from Umbric Retisols of the Moscow oblast were shown to contain the phyla *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Acidobacteria*, and *Planctomycetes*; members of *Burkholderiales, Rhodocyclales, Acidobacteriales, Desulfuromonales,* and *Clostridiales* were

isolated from the nodules of the rice and reed fields in southern China, while the leading role of fungi in iron precipitation was observed for IMN of the soils of marine terraces in the United States (Schulz et al., 2010; Lysak et al., 2013; Hu et al., 2015).

The soils of the south of the Russian Far East are characterized by active hard-pan formation. IMN content in some horizons may be as high as 33% of the total soil mass (Roslikova, 1996; Timofeeva, 2008; Timofeeva et al., 2021; Purtova and Timofeeva, 2022). The data on the role of microorganisms in IMN formation are scarce, represented by few mentions of involvement of *Pedomicrobium* and *Metallogenium* species in the initial stages of IMN formation (Shchapova, 1994). Moreover, most research on microorganisms in IMN deal with the areas affected by various technogenic factors (soils of urbanized landscapes and agricultural ecosystems). This precludes obtaining reliable information on the role of different microbial groups in IMN formation and development in the soils of natural ecosystems, unaffected by anthropogenic impact, and assessment of the effect of thechnogenesis on these processes.

The goal of the present work was to investigate abundance of the cultured forms of microorganisms and of the taxonomic diversity of Fe- and Mn-oxidizing bacteria in IMN of different size formed in the soils unaffected by direct technogenic impact.

## MATERIALS AND METHODS

**To assess the contribution of microorganisms to IMN formation,** three full-profile soil sections were established at the Land of the Leopard National Park (43°50′69″–43°52′60″ N, 131°73′00″68–131°71′55″ E) at the southwest of the Primorskii krai, Russia. The territory of the national park is not affected by direct anthropogenic impact and is considered conditionally unpolluted. The samples of IMN and surrounding soil fine earth of the Gleyic Albic Luvisol (Manganiferric, Defferentic) soils were the research subjects. The profile structure of the studied soils was as follows: horizon AY (thickness 11 to 16 cm), of uniform dark gray color, with dense and soft Fe–Mn hard-pans of brown or dark brown color, slightly loamy, cloddy, loose with a wavy border and clear transition to the Yelnn horizon (thickness 8 to 12 cm), grayish-brown, with isolated small fuzzy dark-brown spots, containing numerous small ocherous and dark-brown inclusions, with numerous soft and dense, brown Fe–Mn hard-pans, mid-loamy, of cloddy-prismatic structure, compacted, transition to the lower horizon is gradual, without a pronounced border; BELnn,g (thickness 32 to 41 cm), brown, with numerous contrasting ocherous spots, numerous ocherous inclusions, contains dense and soft dark-brown Fe–Mn hard-pans, heavy loamy, with small prismatic structure, dense, transition to the lower horizon is gradual, with wavy border; BTg (thickness 39 to 53 cm), dark-brown, with numerous small fuzzy ocherous spots, numerous inclusions of ocherous color, dense and soft Fe–Mn hard-pans, clayey, with prismatic structure, hard, transition to the lower horizon is gradual, with a wavy boundary; BCg (thickness up to 23 cm), ocherous-brown in color, with numerous large, fuzzy ocherous and dove-colored spots, with isolated small dark-brown inclusions, with small soft and dense Fe–Mn hard-pans in the upper layer, clayey, large prismatic in structure, dense. The names of soils are give according to the Russian classification and identification of soils (*World Reference Base for Soil Resources*, 2015).

**For microbiological investigation,** IMN-containing soil monoliths were collected using sterile tools and packed into sterile material according to GOST 17.4.4.02-84. The samples were packed into cooled bags and transported to the laboratory for immediate investigation. In the laboratory, IMN were separated from the surrounding soil fine earth with precision tweezers and a 10× table loupe. The separation of IMN size fractions was carried out using a ruler. The cultures of manganese- and iron-oxidizing microorganisms (MnOM and FeOM, respectively) were obtained from the inner and outer IMN zones and from the surrounding soil fine earth. To isolate the microorganisms from the IMN inner zone, the particles were washed with saline, sterilized for 1 min with 70% ethanol, washed thrice with sterile saline, and homogenized (Zhang et al., 2008; Hu et al., 2015). For determination of the chemical composition, IMN were collected from the soil genetic horizons by wet sieving with subsequent IMN separation from admixtures (mineral fragments and organic debris) under laboratory conditions (Timofeeva et al., 2021; Purtova and Timofeeva, 2021). Small soil particles were removed from the IMN surface by immersion for 20 min into a sonic bath with 50% ethanol according to recommendations by Ettler et al. (2017). For investigation of the elemental distribution inside IMN, the samples were embedded in epoxy resin.

**Isolation of Fe- and Mn-oxidizing microorganisms from IMN and surrounding soil fine earth.** Weighed samples of IMN and surrounding fine earth (10 g) were dispensed into flasks with saline (100 mL) according to Zvyagintsev (1991). The mixture was agitated on a Shaker S-3. 09M orbital shaker (ELMI, Latvia) for 30 min at 110 rpm (Loginova et al., 2011), and the resulting suspension (0.1 mL) was used to inoculate solid media in petri dishes (Zvyagintsev, 1991). The cultivation was carried out for 14 days at 20–22°C (Fedoryuk and Nyanikova, 2015). The medium used in the work contained the following (g/L): $(NH_4)_2SO_4$, 0.5; $NaNO_3$, 0.5; $K_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; citric acid, 10; sucrose, 2; tryptone, 1; and agar, 20; pH 6.8. For cultivation of MnOM and FeOM, the medium was supplemented with $FeSO_4 \cdot 7H_2O$ (5.9 g/L) and $MnSO_4 \cdot 5H_2O$

(4.7 g/L), respectively (Zakharova and Parfenova, 2007).

**Genomic DNA** was isolated from pure bacterial cultures grown on the agar medium for the isolation of Fe- and Mn-oxidizing bacteria using the Base NA Sorbent kit (Lytech, Russia) according to the manufacturer's recommendations.

**Amplification of the 16S rRNA gene fragments** was carried out using the BioMaster HS-Taq PCR (2×) reagent kit (Biolabmix, Russia) according to the protocol, with the universal bacterial primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1350R 5'-GACGGGCGGTGTGTACAAG-3' (Lane et al., 1985). Amplification was performed on My Cycler (BioRad, United States) in the following mode: 94°C, 4 min (1 cycle); 94°C, 60 s, 48°C, 60 s, 72°C, 90 s (5 cycles); 92°C, 60 s, 50°C, 110 s, 72°C, 90 s (10 cycles); 92°C, 60 s, 52°C, 60 s, 72°C, 60 s (10 cycles); 92°C, 60 s, 54°C, 60 s, 72°C, 110 s (10 cycles); 72°C,10 min (1 cycle) (Lane et al., 1985).

**PCR products were separated** in agarose gel (1%) with ethidium bromide at ~2 V/cm, and the results were observed on a Quant 132 transilluminator (Helicon, Russia) under UV illumination.

**The amplicons were purified** with the ExoSAP-IT Express kit for enzymatic purification of PCR products (Thermo FS, United States) according to the protocol. The purified PCT products were sequenced by Sanger using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, United States) to prepare the sequences for reading in a Nanofor-05 genetic analyzer (Syntol, Russia).

**Phylogenetic analysis** was carried out by searching in the international database (GenBank) using BLAST (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/blast). The sequences were edited with MEGA 11 and aligned using CLUSTAL W (http://www.genebee.msu.su/clustal). The phylogenetic tree was constructed using the neighbor-joining method according to the Kimura two-parameters algorithm in MEGA 11 (Kumar et al., 2016). The branching order was determined by bootstrap analysis of 100 alternative trees.

The 16S rRNA gene sequences of bacterial isolates were deposited to GenBank under accession nos. OR039558–OR039569.

**Content of Fe and Mn oxides** in IMN and surrounding soil was determined by energy dispersion X-ray fluorescent spectroscopy on EDX 800HS-P (Shimadzu, Japan) using eight state standard comparison samples according to M-02-0604-2007. The parameters of measurement, format, and working environment used for analysis were reported elsewhere (Purtova and Timofeeva, 2021). Reliability of the measurements was ascertained by analyzing one standard sample after ten unknown (experimental) samples. The maximum deviation in the content of oxides of macroelements in experimental samples from the certified values for the standard sample did ton exceed 0.9%.

**Images of bacterial and mineral structures** and the maps of element distribution inside IMN were obtained using a MERLIN scanning electron microscope(Carl Zeiss, Germany) equipped with an INCA Energy 350 X-Max 150 energy dispersion spectrometer (Oxford Instruments, Abingdon, United Kingdom).

The work was carried out using the equipment of the Biotechnology and Genetic Engineering Joint Use Center at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences.

**Taxonomic characterization** of bacterial isolates was based on Sanger analysis of the 16S rRNA gene structure at the Laboratory of Marine Microbiology, Institute of World Ocean, Far Eastern Federal University.

**Mathematical treatment** of the data included calculation of the median values, standard deviation, coefficient of element accumulation in hard-pans (Gasparatos, 2012), and correlation analysis; it was carried out using Statistica and Microsoft Excel 2007. The significance level ($P$) did not exceed 0.05.

## RESULTS AND DISCUSSION

**Distribution of Fe- and Mn-oxidizing microorganisms along the fine earth profile.** The studied soils showed pronounce differentiation in the abundance and composition of cultured microorganisms (Fig. 1). Our results indicated predominance of MnOM over FeOM in the two upper soil horizons. No cultured MnOM were revealed in lower horizons, probably due to altered aeration conditions in the medium and lower parts of the profile. Microbial species diversity also decreased with depth (Fierer et al., 2003). Cultured FeOM were isolated from the upper (AY) and medium (BELnn) horizons of the studied soils. The highest FeOM abundance was revealed in the medium horizon, which indicated their higher resistance to changes in the water-air regime.

Although some of the studied microorganisms were Mn oxidizers, which implied direct involvement of manganese in their metabolic processes, inverse correlation between Mn content in fine earth and MnOM abundance was found ($r = -0.99$) (Table 1). This was probably due to the presence of a major part of Mn in fine earth as components of complexes, with predominance of reduced species, mainly Mn(IV) (Martynova, 2012). Content of iron oxides has the most pronounced effect on increase in MnOM cell abundance ($r = 0.99$). This finding may be explained by the presence of iron ions in many enzymes involved in the basic biological processes (respiration, central metabolism, DNA reparation) (Andreini et al., 2008; Frawley and Fang, 2014). Mn-oxidizing bacteria of the genera *Priestia* and *Methylobacterium* were isolated

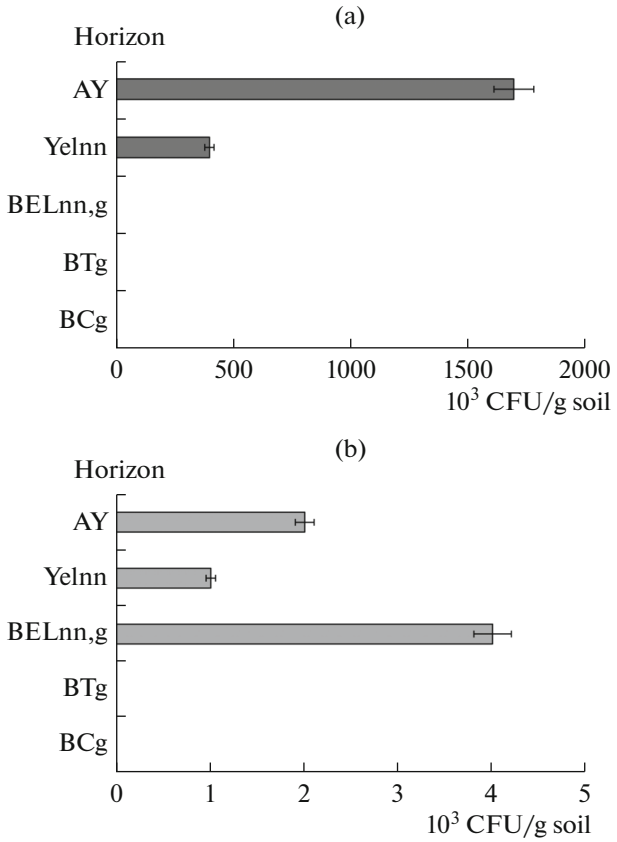


**Fig. 1.** Abundance of Mn-oxidizing (a) and Fe-oxidizing (b) microorganisms in fine earth.

from fine earth (Fig. 2). These genera have been isolated from soil worldwide (Lysak, 2007). The results of a number of studies indicate direct involvement of members of the genera *Priestia* and *Methylobacterium* in Fe and Mn transformation in soils (Aristovskaya, 1975; Pinevich, 2005; Hu et al., 2015; Gupta et al., 2020; Lyu et al., 2021).

**Fe- and Mn-oxidizing microorganism in IMN.** MnOM constituted most microorganisms in IMN (Table 2). Unlike fine earth, MnOM were identified in the IMN formed both in the upper and lower parts of the profile (to the depth of 140 cm). The absence of cultured MnOM in surrounding fine soil and their presence in IMN at the same horizons may indicate existence of short-living redox periods, when MnOM penetrate into the lower horizons (mainly with the gravity flow of the soil solution) and form microzones. Such microzones may act as nuclei for IMN formation. In some works such structures were termed proto-concretions (Dabard and Loi, 2012). The well-studied ability of some groups of soil microorganisms to oxidize Mn(II) and precipitate Mn (hydr)oxides on the cell surface favors formation of microspherical aggregates, which may probably result in IMN formation. This suggestion may be confirmed by specific patterns of Fe and Mn distribution inside IMN. In the studied IMN samples of all sizes, the Mn-enriched nucleus (internal zone) was clearly observed (Fig. 3). The presence of MnOM in IMN and their absence in fine earth confirms that IMN are closed systems with more acidic pH (more acidic), lower Eh, and $C_{total}$ accumulation compared to the surrounding soil (Kostenkov, 1986; Purtova and Timofeeva, 2021). Seasonal variations in the redox regime may probably result in death of the microorganisms inhabiting fine earth at the medium and low parts of the profile or in their transition to an uncultured state. At the same time, the IMN inner environment supports the preservation of viable MnOM, as was also confirmed by the presence of cultured MnOM in both the inner and outer IMN zones (Table 2).

Direct involvement of FeOM in IMN formation has been described previously (Aristovskaya, 1975; Pinevich, 2005; Vodyanitskii, 2010). In the present work, cultured FeOM were retrieved only from the outer zone of 1–2-mm IMN formed in two upper

**Table 1.** Fe and Mn content and accumulation in soils and nodules, median value ± standard deviation

| Horizon | Object | MnO | | $Fe_2O_3$ | |
|---|---|---|---|---|---|
| | | oxides, mg/kg | accumulation coefficient | oxides, mg/kg | accumulation coefficient |
| AY | Soil | 0.08 ± 0.004 | | 3.82 ± 0.150 | |
| | Nodule | 0.78 ± 0.030 | 9.75 | 24.07 ± 0.880 | 6.30 |
| Yelnn | Soil | 0.11 ± 0.005 | | 3.63 ± 0.140 | |
| | Nodule | 0.69 ± 0.026 | 6.27 | 23.46 ± 0.870 | 6.46 |
| BELnn,g | Soil | 0.05 ± 0.002 | | 3.50 ± 0.160 | |
| | Nodule | 0.90 ± 0.040 | 18.00 | 22.71 ± 0.900 | 6.48 |
| BTg | Soil | 0.06 ± 0.001 | | 7.81 ± 0.340 | |
| | Nodule | 1.42 ± 0.049 | 23.67 | 18.23 ± 0.740 | 2.33 |
| BCg | Soil | 0.08 ± 0.003 | | 6.51 ± 0.270 | |
| | Nodule | 4.59 ± 0.155 | 57.37 | 10.80 ± 0.360 | 1.66 |

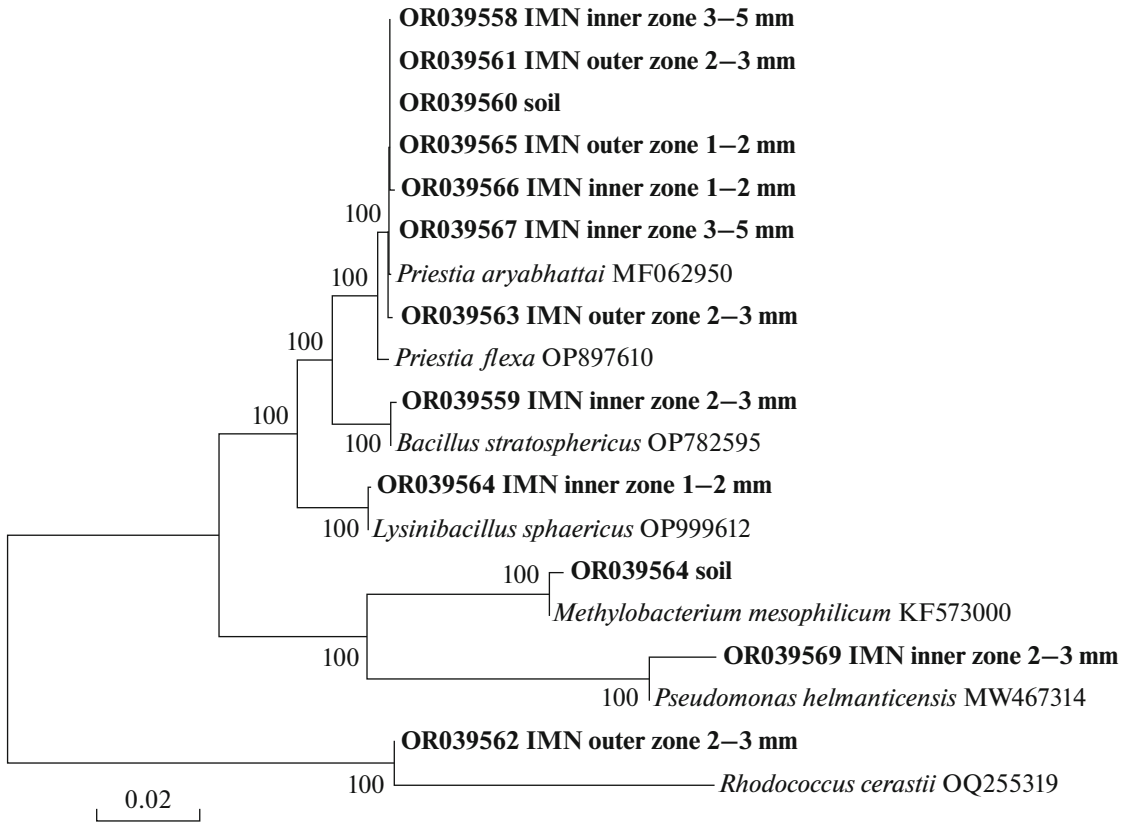


**Fig. 2.** Phylogenetic tree based on the 16S rRNA gene sequences of bacteria isolated from IMN and fine earth. The tree was constructed using the Neighbor-Joining algorithm. The sequences obtained in the present work are marked by boldface. Bootstrap support values over 50% are shown.

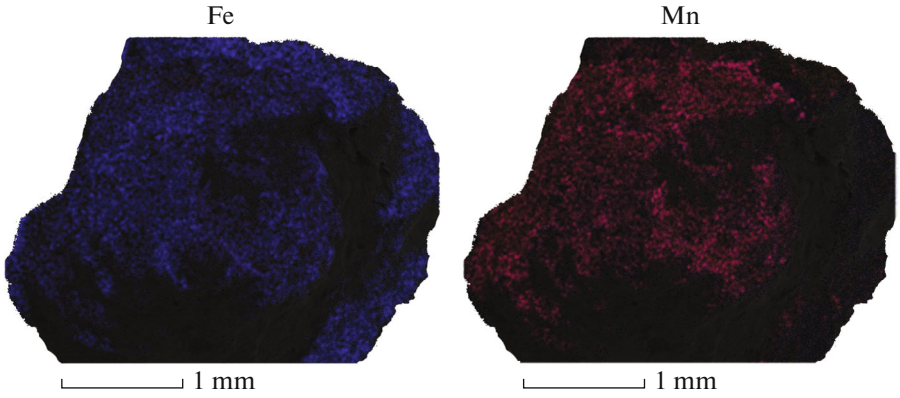


**Fig. 3.** Maps of element distribution in the nodules.

horizons of the studied soil (AY, Yelnn). These data indicated that viable microorganisms of this group occurred only in the zone of contact between IMN and the surrounding fine earth. Considering the IMN size, it may be suggested that in the studied soils FeOM are involved only in the initial stages of IMN formation, when iron compounds available to FeOM are still present in this formation. Such compounds are usually amorphous or weakly crystalline iron-enriched hard-pan components. This suggestion is confirmed by the results of Liu et al. (2021) and Sipos et al. (2022), who confirmed predominance of amorphous and weakly crystalline iron compounds in the IMN of small size fractions. Crystallized iron minerals, which contain iron ions in the form unavailable to FeOM were predominate components of large IMN

**Table 2.** Abundance of Mn- and Fe-oxidizing microorganisms in the nodules of different size, $10^3$ CFU/g nodule; median value ± standard deviation

| Horizon | Nodule size fraction | MnOM | | FeOM | |
|---|---|---|---|---|---|
| | | outer zone | inner zone | outer zone | inner zone |
| AY | 1–2 mm | 20.0 ± 1.0 | 38.4 ± 1.9 | 4.0 ± 0.2 | – |
| | 2–3 mm | 40. ± 2.0 | 11.2 ± 0.5 | – | – |
| | 3–5 mm | 170.0 ± 8.5 | 24.0 ± 1.2 | – | – |
| Yelnn | 1–2 mm | 3.0 ± 0.1 | 22.4 ± 1.12 | 5.0 ± 0.2 | – |
| | 2–3 mm | 20.0 ± 1.0 | 9.6 ± 0.4 | – | – |
| | 3–5 mm | 10.0 ± 0.5 | 2.1 ± 0.1 | – | – |
| BELnn,g | 1–2 mm | 110.0 ± 5.5 | – | – | – |
| | 2–3 mm | 1330.0 ± 66.5 | 9.0 ± 0.4 | – | – |
| | 3–5 mm | 4.0 ± 0.2 | 4.0 ± 0.2 | – | – |
| BTg | 1–2 mm | – | 11.3 ± 0.6 | – | – |
| | 2–3 mm | – | – | – | – |
| | 3–5 mm | – | – | – | – |
| BCg | 1–2 mm | – | – | – | – |
| | 2–3 mm | 650.0 ± 32.5 | 1.3 ± 0.1 | – | – |
| | 3–5 mm | 20.0 ± 1.0 | 26.4 ± 1.3 | – | – |

(Pinevich, 2005; Timofeeva et al., 2014; Sipos et al., 2022). However, previous studies of IMN of different size in the soils of the studied region indicated the presence of crystalline Fe species in the IMN 1–2 mm in size (Timofeeva et al., 2014). One of the conditions enabling cultivation of FeOM from the IMN 1–2 mm in size is probably their location in the upper part of the profile. The results of specialized studies demonstrated decreased crystallization rate of iron compounds in IMN ad surrounding soil at the horizons enriched with organic matter (Fischel et al., 2023).

Members of bacterial genera *Bacillus*, *Priestia*, *Rhodococcus*, *Lysinibacillus*, and *Pseudomonas* were identified in the studied IMN of different size fractions. Most identified bacterial genera are known to be involved in the processes of transformation of Fe and Mn compounds in various environments, including soils (Aristovskaya, 1975; Pinevich, 2005; Cappelletti et al., 2020). Experimental results on the role of members of the genus *Lysinibacillus* on the processes of Fe and Mn oxidation and/or precipitation in soil are presently insufficient for straightforward conclusions concerning a significant effect of these bacteria on these processes. Members of the genera *Bacillus* and *Pseudomonas* have been previously isolated from soil concretions in various regions worldwide (Li-Mei et al., 2008; Hu et al., 2015; Lysak et al., 2019; Lyu et al., 2021). To the authors' knowledge, embers of the genera *Rhodococcus* and *Lysinibacillus* have not been previously identified in soil IMN.

**Taxonomic position of Fe- and Mn-oxidizing bacteria isolated from fine earth and IMN.** All isolates were found to belong to three phyla: *Pseudomonadota, Actinomycetota*, and *Bacillota* (Fig. 2). Strains OR039558, OR039561, OR039565, OR039566, and OR039567 showed high similarity (99.51, 98.23, 100, 99.77, 99.47, and 99.47%, respectively) with the *Priestia aryabhattai* homolog MF062950 from tomato roots (China). Strain OR039563 was homologous to *Priestia flexa* (99.62%). Members of the genus *Priestia* are able to precipitate and accumulate metals on and inside their cells (Ghosh et al., 2018; Shahid et al., 2022). The role of our isolates in the IMN from studied soils may be similar. Strain OR039559 exhibited high homology with *Bacillus stratosphericus* (OP782595). Bacteria of this genus are able to accumulate metals, including $Mn^{2+}$, from various substrates, which does not affect their growth rate (Li et al., 2023). Strain OR039562 had high similarity (99.88%) to *Rhodococcus cerastii* (OQ255319). The genus *Rhodococcus* is known to include the species capable of Mn oxidation and possessing DyP-type Mn-dependent peroxidases (Singh et al., 2013; Jofré et al., 2021). In some *Rhodococcus* spp., hydroxylase activity depends on manganese, and bacteria use a Mn/Fe heterometallic cofactor for catalytic functionalization of an inactivated primary carbon bond (Powell et al., 2023). Our isolate is probably also capable of transforming Mn, oxidizing and precipitating it. The sequence of strain OR039564 showed 100% homology with the NCBI sequence of *Lysinibacillus sphaericus* (OP999612). This species is relatively common in environmental objects and was isolated from various habitats, including metal-enriched soils. Ability of *Lysinibacillus* spp. to limit the mobility of heavy metals in soil due to the protein S-layer on their

cell surface has been reported (Suhr et al., 2016). Active transformation of heavy metals and suppression of their toxic effect on living organisms were found to occur in soils where *Lysinibacillus sphaericus*, *Bacillus* sp., and *Rhodococcus* sp. were present together (Emenike et al., 2016). Strain OR039568 had 99.85% homology to *Methylobacterium mesophilicum* (KF573000). The results of some studies showed ability of *Methylobacterium* spp. to reduce Fe(III) and oxidize Fe(II) (Hu et al., 2013; Ainiwaer et al., 2022). Dourado et al. (2015) described the genes of some *Methylobacterium* spp., which were responsible to metal tolerance. Strain OR039569 had 99.74% similarity to the homolog *Pseudomonas helmanticensis* (OP800175). Ability of pseudomonads to oxidize Mn and Fe has been reported in the literature (Kepkay et al., 2016; Li et al., 2023). In the course of oxidation, electrons are transported to the terminal cytochrome *c* oxidases for reduction of oxygen and gaining energy for bacterial growth (Ciancio et al., 2022). Two copper oxidases involved in Mn(II) and Mn(III) oxidation have been described for some species of this genus (Lyu et al., 2021).

**IMN structures of bacterial nature.** Structures of regular spherical shape and presumably of bacterial origin were revealed in the outer and inner zones of the studied IMN (Figs. 4a, 4b). These structures occurred as individual spherules and small groups. The size of these structures (1.5 to 2.5 μm) and predominance of carbon in their elemental composition also indicated the presence of microscopic organisms. Both spherical structures consisting mostly of carbon and the structures covered with Fe and Mn compounds were present in IMN. The highest coverage of the structures with Fe and Mn was observed in the zone of contact with IMN matter, indicating precipitation of the main IMN-forming elements on the surface of these structures (Fig. 4c). The results on sequencing and morphological structure of identified bacteria indicated that the spherules were most probably associated with members of the genus *Rhodococcus*. Spongy structures morphologically similar to bacterial glycocalix and consisting mostly of Fe, Mn, and C were also found in the IMN (Fig. 4d). Formations with a similar structure have been observed in oceanic Fe–Mn concretions (Astaf'eva et al., 2021).

**Role of Fe- and Mn-oxidizing microorganisms in IMN formation and development.** According to the theories of IMN formation presented in the literature, their development in soils is accompanied by an increase in size (Gasparatos et al., 2019). Investigation of quantitative distribution of MnOM and FeOM in the IMN of different size made it possible to monitor the involvement of specific microbial groups at different stages of IMN formation in different horizons of the soil profile (Table 2). The IMN 1–2 mm in size were characterized by the presence of cultured FeOM, which, as was noted above, indicated the presence of Fe-containing compounds in the IMN of this size fraction in the form available to iron-oxidizing microorganisms. Investigation of the MnOM abundance and distribution inside the 1–2-mm IMN formed in different soil horizons indicated preferential MnOM localization in the inner IMN zone formed in two upper horizons of the profile (AY and Yelnn) and in the textured horizon BTg. In the IMN of this size fraction formed in the BELnn,g horizon, MnOM were present only in the outer zone. From the IMN of this horizon, the highest number of MnOM for the 1–2-mm size fraction was isolated. The morphological properties of the BELnn,g horizon indicate more rapid (and therefore more contrasted) changes of redox regime, with more active dehydration and crystallization of Mn-containing compounds inside IMN. MnOM localization in the outer IMN zone was probably associated with the presence of Mn species available to microorganisms only among the compounds freshly precipitated at the IMN surface. This suggestion was partially confirmed by the patterns of MnOM distribution in larger IMN.

MnOM were isolated from both the inner and outer zones of the IMN 2–3 and 3–5 mm in size. The 2–3-mm IMN were characterized by much higher (2.1 to 500 times) MnOM abundance in the outer zone compared to the inner zone. The IMN of this size fraction had also the highest abundance of cultured MnOM among all studied size fractions. This was due primarily to activation of Mn accumulation during IMN growth, as was confirmed by earlier studies (Timofeeva, 2008). Our results indicated direct involvement of MnOM in the transformation of Mn-containing compounds in the IMN 2–3 mm in size, where most manganese precipitated at the surface was represented by uncrystallized compounds, which were therefore available to MnOM. In general, the highest MnOM abundance in the IMN 2–3 mm in size was found in the structures formed in the medium and lower parts of the soil profile (horizons BELnn,g and BCg).

In the IMN of the 3–5-mm size fraction formed in the upper part of the soil profile, the outer zone also contained more cultured MnOM than the inner one. Deeper along the profile, MnOM abundance in the inner and outer zones became equal. The highest MnOM abundance was isolated from the IMN of the upper horizon AY. It decreased drastically in lower horizons. An insignificant increase in MnOM abundance was observed in the IMN from the BCg horizon. In the IMN of this size fraction, conversion of Mn-containing compounds into crystallized forms was more rapid due to activation of dehydration of Mn-enriched amorphous compounds, which probably involved MnOM (Timofeeva et al., 2014). Enhanced mineralization and crystallization of Mn-enriched compounds in the IMN of this size was confirmed by the presence of lamellar crystals, which were not found in smaller IMN (Fig. 4e). Similar crystalline rosettes are known in the literature as nanoflowers

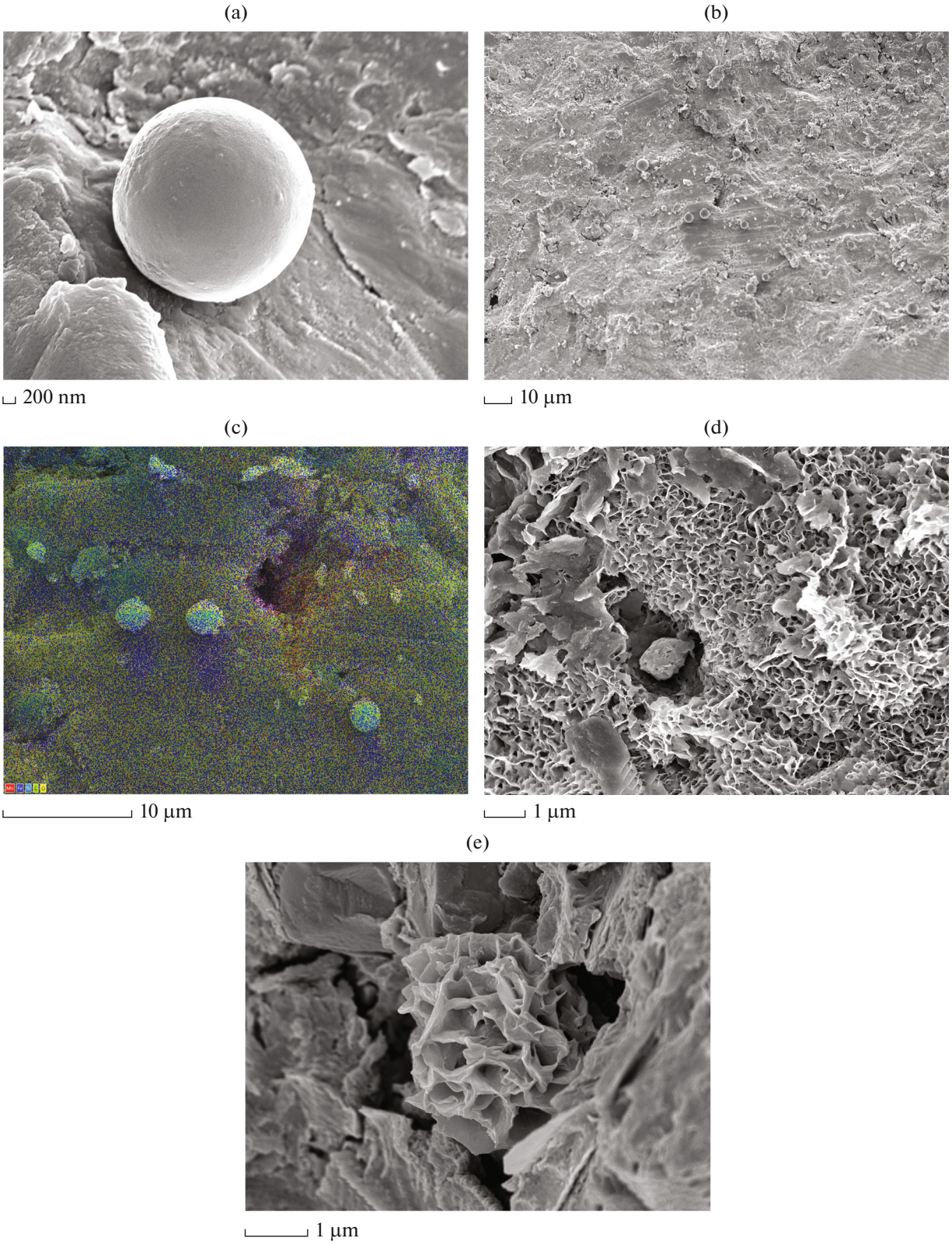


**Fig. 4.** Structures in the nodules: a spherule in the nodule inner zone (a); a group of spherules at the nodule surface (b); elemental composition of the spherules (c); glycocalix (d); and nanoflowers (e).

and consist of Mn minerals (lithiophorite or birnessite) (Fischel et al., 2023). Mn compounds available to microorganisms were represented in this IMN size fraction mainly by (hydr)oxide compounds covering the mineral crystals (Timofeeva et al., 2014; Fischel et al., 2023).

The absence of cultured forms of studied microorganisms in the IMN fractions 2–3 and 3–5 mm formed in the BTg textured horizon should be noted. It was probably due both to different rates of microbial processes in different soil horizons and to the ability of the IMN of textured horizons to accumulate heavy metals up to the concentrations suppressing microbial growth, which has been reported in the literature (Timofeeva, 2008; Kholopov, 2013).

Analysis of relationships between abundance of MnOM cultured forms and MnO and $Fe_2O_3$ content in the IMN did not confirm pronounced dependence between these variables ($r_{MnOM-Mn}$ from –0.56 to 0.20; $r_{MnOM-Fe}$ from –0.15 to 0.26). Similar to fine earth, the absence of correlation may result from preferential occurrence of Mn and Fe ions in the compounds poorly available to microorganisms. An exception was the average correlation dependence ($r = 0.64$) between MnOM abundance and total Fe in the 1–2 mm IMN fraction; this was an additional confirmation of predominance of amorphous and weakly crystalline iron compounds at the initial stages of hard-pan formation.

Although no correlation was found between MnOM abundance and total Mn content, correlation dependence between variations in MnOM and the rate of Mn accumulation by IMN revealed a pronounced effect of MnOM on Mn accumulation in the IMN of the size fractions 1–2 ($r = 0.99$) and 2–3 mm ($r = 0.96$) formed in the upper and medium parts of the soil profile. For larger IMN, the correlation coefficient between MnOM abundance and Mn accumulation was negative ($r = -0.25$), indicating the absence of microbial effect on the processes responsible for the accumulating capacity of IMN of such size.

The effect of cultured iron- and manganese oxidizers at different stages of IMN formation and development within the profile of Gleyic Luvisols was elucidated. Bacteria genera responsible for IMN formation and the processes occurring in the IMN in soils not affected by direct anthropogenic impact were revealed.

The fine earth surrounding the IMN exhibited variation in abundance of the target microorganisms along the profile. In the upper part of the profile, MnOM numerically prevailed over FeOM, while deeper along the profile the dominant groups reverted. Content of iron oxides in the soil was the parameter with the greatest effect on MnOM abundance. In fine earth, bacteria of the MnOM group were represented by members of the genera *Priestia* and *Methylobacterium*.

Most of the cultured microorganisms retrieved from soil IMN (inner and outer zones) belonged to MnOM. These results, in combination with the patterns of Mn distribution in IMN, suggest that MnOM aggregates may act as the initial stage of IMN formation in the studied soils. Cultured FeOM were isolated only from the outer zone of small IMN formed in the upper part of the soil profile. This finding confirms involvement of FeOM only in the initial stages of IMN formation.

Bacteria identified inside IMN belonged to the genera *Bacillus*, *Rhodococcus*, *Lysinibacillus*, *Pseudomonas*, and *Priestia*. Apart from *Priestia*, the taxonomic composition of bacteria was IMN-specific. The structures of bacterial nature were represented in IMN by spherules, single or in small groups. Precipitation of Fe and Mn was observed at the surface of the spherules. IMN contained also spongy structures morphologically similar to bacterial glycocalix.

The quantitative distribution of MnOM and FeOM in the outer and inner zones of IMN of different size indicates involvement of MnOM at all stages of their formation and development. The patterns of quantitative MnOM distribution in different IMN zones are determined by the presence of Mn species available to microorganisms (freshly precipitated amorphous compounds). The highest abundance of cultured MnOM was found in the IMN 2–3 mm in size. Analysis f relationships between MnOM abundance and the rate of Mn accumulation revealed active involvement of these microorganisms in Mn accumulation in the IMN of the 1–2 and 2–3 mm size fractions. In spite of the presence of numerous MnOM in larger IMN (3–5 mm), MnOM had no effect on Mn accumulation.

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## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

## CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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