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EXPERIMENTAL ARTICLES

A Puddle: an Ombrophilic Cyano-Bacterial Community

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Abstract—Sequential stages of formation of an ombrophilic cyano-bacterial community on clay were determined in a laboratory model of a puddle community. In a suspension of washed clay obtained from loamy soil, with montmorillonite as the predominant phase, a bacterial neustonic film is initially formed; it acts as a support for cyanobacterial hormogonia. At the next stage, the upper layer of precipitated clay (about 1 mm) is reinforced by a cyano-bacterial structure of *Phormidium* sp. trichomes and develops a tissue-like structure. The hormogonia and sheathless cyanobacteria remain free from mineral particles. Subsequently, gas formation results in a separation of a dense cyano-bacterial film from the underlying loose suspension and in formation of gas swellings. The mineral component of the film is differentiated: mineral particles of quartz and feldspar grains are attached to *Phormidium* sp. trichomes, which act as a factor of mineral selection.

Key words: ombrophilic cyano-bacterial community, clays, stages of community formation, mineralogical composition of a cyano-bacterial film.

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A puddle is an ephemeral formation on a land surface, which originates from rainfall. A rain puddle is the final point of the atmospheric hydrological cycle and the first stage of the terrestrial hydrological cycle. Duration of a puddle's existence depends on the weather conditions and is usually several weeks. Puddles are common in humid climates and are a seasonal phenomenon. For puddle formation, a waterproof surface is required, which is usually, though not always, clay. Hydrochemically, puddle water belongs to ultrafresh waters; its composition depends on rainfall. The conductivity, which is usually about 30 μS, may wary depending on the underlying soil. Since puddles are formed in local depressions, both rainwater and surface washout act as water sources; the latter carries suspended particles, primarily clay, which precipitate at the bottom of the puddle creating the waterproof surface necessary for the puddle's maintenance (Fig. 1). Puddles are periodic events, and they are repeatedly formed at the same location. A puddle, therefore, is an important phenomenon, especially as the starting point of the terrestrial hydrological cycle.

In a humid climate, an ephemeral algal community rapidly develops in puddles, its organic matter being the clay sediments. Soil microorganisms which have been in an anabiotic state prior to moistening of the soil, perpetrate colonization of the puddle. Soil algae are a specific algological subject. A bottom biofilm formed by *Oscillatoria*-like cyanobacteria (which spread rapidly along moist surfaces) is a characteristic puddle biocenosis. Cyanobacterial mucus cements the mineral particles at the bottom and prevents their stirring-up. After some time, a mat, i.e., a leathery film of a cyano-bacterial community develops at the bottom of the puddles. It is in intimate contact with the mineral particles of the bottom sediment. Low content of mineral salts in rainwater determines the development of ombrophilic microbial communities in puddles. The organisms of the biotopes dependent on rain feeding are termed ombrophiles, from Greek ομβροζ rain (similar to ombrophytic plants and ombrotrophic bogs). The posi-

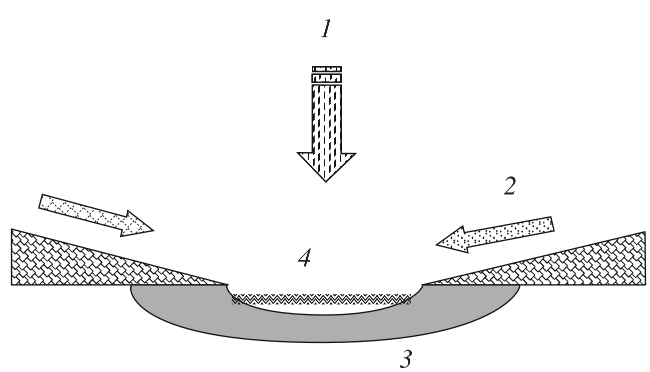

Fig. 1. Schematic representation of a puddle formation in a depression due to rainfall accumulation and matter removal by surface washout: rainfall (*1*); lateral washout (*2*); waterproof layer at the bottom (*3*); cyano-bacterial or algo-bacterial film (*4*).

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Mineralogical composition of the samples, % determined with ZnO as an internal standard

Sample	Montmorillonite	Mic	Kaolin	Quartz + feldspar
Original loamy clay	6	45	15	34
Clay suspension	50	29	14	7
Cyano-bacterial film, 5 weeks	46	24	15	15

tion of ombrophilic communities in the orographic profile of continental plains was presented in [1]. Puddles develop in various geographic zones; stagnant bodies of water exist on the surface, however, mostly under the conditions of a humid climate and a moderate evaporation/infiltration ratio. Since ombrophilic communities are common, they can be hardly classified as extreme ones. However, the members of these communities should be adapted to the conditions which differ from seawater or soil solutions both in their osmotic characteristics and in the limitation of mineral supply; the only source of the latter is the solid phase of the waterproof layer.

The goal of the present work was investigation of the processes of formation of an ombrophilic cyanobacterial community in a puddle under conditions of experimental simulation.

MATERIALS AND METHODS

Drift clay collected from 0.7-m depth in a cross section in Abramtsevo (the Klin–Dmitrov range) was used for investigation of formation of a cyano-bacterial film in a puddle. Prior to the experiment, the loamy clay was washed repeatedly by decantation first with tap water (to remove floating organic debris) and then 6–8 times with distilled water (to remove big mineral particles and to enrich the suspension with clay particles. The washing was carried out until the clay suspension was obtained that remained stable for at least a week [2]. Its conductivity was about 10 μS. Microscopically, the particles of this suspension were aggregates (~ 1 μm) of spherical particles of uniform size.

The clay suspension was transferred into a 110 × 220 mm covered plastic container and diluted with distilled water. The container was used to simulate a puddle. After a week, two layers developed, a coagulated/flocculated precipitate (0.5 cm) and overlaying water (about 2 cm). The chemical composition of the water determined by ICP (Perkin Elmer Optima 5300 DV) was as follows (mg/l): Al, 0.104; Si, 0.272; Fe, 0.058; Mg, 9.996; Mn, 0.002; P, 0.046; K, 2.400; and Na, 3.700. Since our previous studies demonstrated that microwave-sterilized clay suspensions did not favor community development, the experiment was carried out under nonsterile conditions.

The mineralogical composition of the original loamy clay, the clay suspension, and the cyano-bacterial film was determined by X-ray diffractometry on a DRON-3 unit ($CuK\acute{\alpha}$ radiation, Ni filter). The samples were studied in Mg form (saturation with 1 N $MgCl_2$), saturated with ethylene glycol, and calcined at 350 and 550°C. For quantitative determination of the content of minerals in these three samples, X-ray diffraction patterns were obtained of the objects containing 10% ZnO as an internal standard. The character of the interaction between the cyano-bacterial community and the mineral phase was investigated by optical microscopy (Jenaval, Jena, Germany). The bulk chemical composition of the original loamy clay, the solid phase of the clay suspension, and the cyano-bacterial film was determined by X-ray fluorescence (Spektroskan Maks-GV).

X-ray diffractometry revealed the predominance of mica (45%), quartz, and feldspars, orthoclase and albite (34%) in the original loamy clay. The solid phase of the clay suspension contained mostly montmorillonite (50%). Mica (29%), kaolin (14%), and an inconsiderable amount of the primary minerals, quartz and feldspars (7%) were also present (Table 1, Fig. 2a, *1* and *2*). Montmorillonite was identified based on the behavior of its crystal lattice with ethylene glycol and after calcination. Saturation with ethylene glycol results in a shift of the 14.48 Å peak to 17.33 Å with its subsequent compression after calcination to 10.28 Å (Fig. 2b). Granulometrically, the clay suspension contained 45% of clay particles (<0.01 mm).

For inoculation, a piece of the cyano-bacterial film from a cyano-bacterial microcosm was placed on the water surface of the container with a clay suspension. The film was a stable cyano-bacterial community in ultrafresh water, which developed for 6 years in a plastic-covered 0.7-l glass vial in distilled water over the same Abramtsevo loamy clay inoculated with a film of *Oscillatoria*-related cyanobacteria from an autumn forest puddle; *Oscillatoria simplicissima* was the dominant species. *Phormidium angustissimum, "benthic" Anabaena* sp., and chlorococcal green algae were also present in the film; the latter formed transparent mucous microcolonies squeezed between cyanobacterial trichomes. The plankton was not present in the vial, and the water remained transparent. Since the leathery film is inaccessible for grazing protozoa and small invertebrates, their number was very low.

Staining with Lugol's iodine solution was the most convenient method for the observation of microorganisms in clay suspensions, and the interference from clay particles was minimal in this case

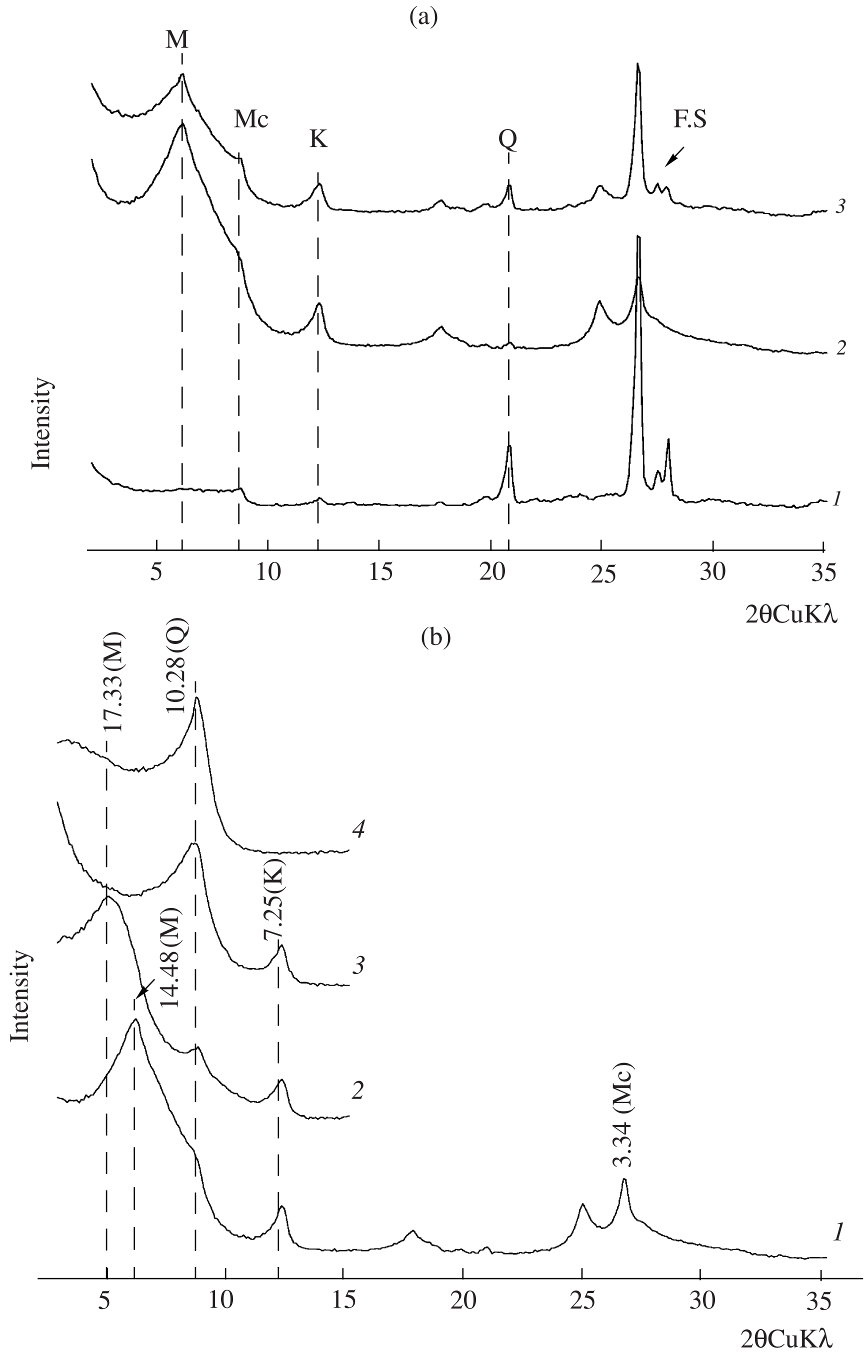

Fig. 2. X-ray diffraction patterns of the samples. a, air-dry samples in Mg form: original loamy clay (*1*); clay suspension (*2*); mineral mass of a 5 week-old cyano-bacterial film (*3*). Designations: M, montmorillonite; Mc, mica; K, kaolin; Q, quartz; F.s., feldspar. b, clay suspension (Fig. 2a, *2*), test treatments for identification of clay minerals: Mg, air-dry (*1*); Mg, saturated with ethylene glycol (*2*); Mg, calcined at 350°C (*3*); Mg, calcined at 550°C (*4*). Interplanar spacing is given in Å. The spectra demonstrate the mostly montmorillonite composition of the clay suspension used in the simulation experiment. Designations for the mineral phases are as on Fig. 2a.

RESULTS AND DISCUSSION

Biofilm development on clay suspension was first observed after 8 days as compaction of the upper layer of clay. No macroscopic indication of cyanobacterial growth was then visible.

Cyanobacterial development started at the piece of inoculum where *Phormidium* sp. was present in three forms, i.e., as long (up to 100 μm threadlike trichomes staining well with J + KJ; free hormogonia of 3 to 5 (usually 4) cells, abundant above the clay particles;, and mucous sheaths covered with mineral particles, usually

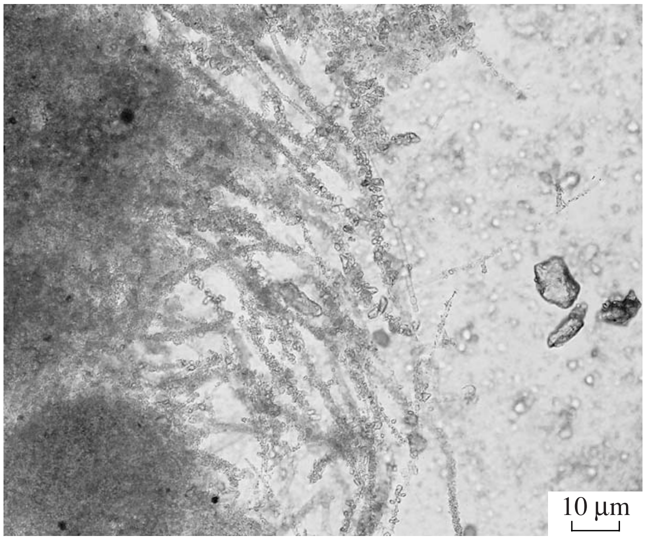

Fig. 3. Center, *Phormidium* sp. sheaths covered with mineral particles. Right, large free feldspar particles. Granular background, small clay particles. Left, bacterial mucus with embedded mineral particles. Light-collecting action of mineral particles was demonstrated in microsensor experiments of B. Jørgensen on marine objects.

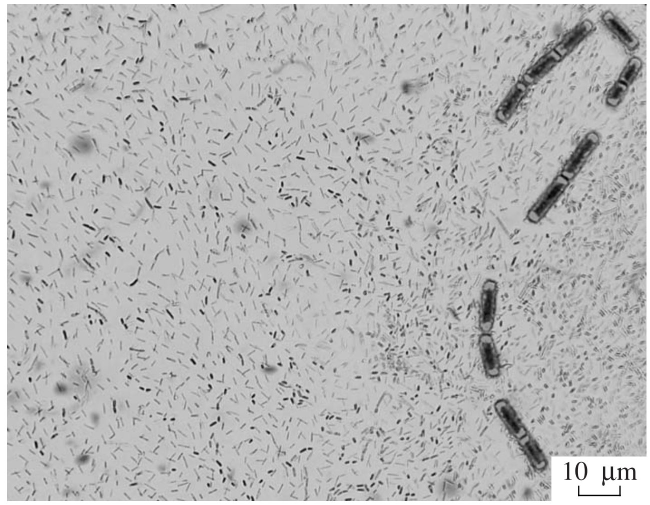

Fig. 4. Formation of neuston as a monolayer of uniform capsulated rods. Spreading of hormogonia along the surface of the surface neustonic film composed mostly by two morphotypes of mucous cyanobacteria.

not containing viable filaments. The first two *Phormidium* sp. forms were free from mineral particles. These particles were also not precipitated on individual *Anabaena* sp. trichomes. The center of the clump is occupied by bacterial slime with large and small mineral particles. Living *Phormidium* sp. trichomes penetrate this slime. As a whole, the product is structurally similar to reinforced concrete, with bacterial slime as the cement, mineral particles as the gravel, and trichomes as the carcass, which is rather more flexible and tensile than metal (Fig. 3).

After two weeks, the conductivity of the suspension increased to 100 μS; its surface became smoother, although it retained the same yellowish color. The clay surface was reinforced with a very thin, relatively strong film which required scissors to cut. A loose, easily detached suspension was contained below the film. After removal of a part of this film, a "window" remained, which the film did not overgrow. After washing with distilled water to remove the clay particles, the film was found to consist of a net of *Phormidium* sp. trichomes with attached mineral particles. The particles, including larger mineral grains, were firmly attached to the trichome surface (Fig. 4).

At the next stage, the biofilm formed a dense uninterrupted layer. Photosynthesis resulted in bubble formation below the film; the bubbles could move and join together in swellings. The film was clearly separated from the underlying turbid clay suspension. The roiling of the suspension under a hydraulic shock (by a jet of water) decreased sharply.

On the 4th–5th week, the clay surface became bumpy, with gas swellings below the bumps; the entire surface was covered with green points of colonies (mostly of *Anabaena* sp.) forming a network of bundles (Fig. 5).

After 6 weeks, the film died; this result confirmed the ephemeral nature of the puddle's cyano-bacterial community. Investigation of the film (data not presented) revealed mucus cementing the mineral particles. Cyanobacterial trichomes were not present in the clay sediment.

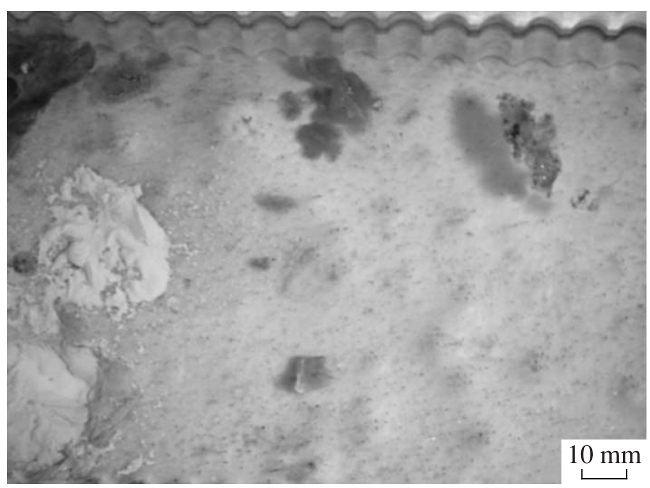

Fig. 5. Cyano-bacterial film in the cuvette. Left, sampling site with underlying clay suspension which is not overgrown. Top, floating films of the inoculum at the surface. The film at the surface of the suspension is covered by points (*Anabaena* sp. crests). Below the film, gas bubbles are located, which separate the film from the clay suspension. The bubbles subsequently fuse.

These data indicate unidirectional development of a cyano-bacterial formation. It passes sequentially through several stages.

I. At the initial stage, bacteria with mucous capsules form a neustonic monolayer on the water surface. Cyanobacterial hormogonia then spread along the neustonic film. The same events occur on the clay surface. Neustonic bacteria represent a monolayer of uniform capsular rods creating a biofilm (Fig. 4).

II. At the following stage, the trichomes develop from the hormogonia (a stage of expansion and area colonization) and penetrate into the upper, illuminated layer of the loose clay suspension. The trichomes grow and elongate. The biofilm develops *below* the layer of clay particles, and the clay surface appears unchanged. The trichomes do not emerge on the clay surface because the conditions in the clay suspension some 10 micrometers below the surface are optimal for their development. The trichomes are covered with mineral particles, which may act as light-gathering lenses for the diffuse light in the upper illuminated layer of clay.

III. A network of *Phormidium* sp. filaments distributes below the clay surface and binds it with a leathery film; a submillimeter-thick layer of reinforced clay and mucus. This film prevents the roiling of the clay suspension. It is protected from damage as long as the overlaying water layer remains stable. However, the trichomes encrusted with mineral particles have limited spreading capacity; the damaged areas of the film are not repaired.

IV. Photosynthesis in the clay suspension is intense; it results in formation of gas bubbles below the film, which elevate it in bumps. In the millimeter scale, the picture is similar to the 30-cm hummocks in lagoons formed in extremely halophilic *Microcoleus* sp. leathery mats. The gas layer below the film creates a kind of quagmire, while the underlying clay still remains in the form of a suspension.

V. At the last stage, the *Phormidium* sp. film dies; the *Anabaena* sp. network remains at the surface somewhat longer, but then also dies. Thus, unlike the original sustainable material of the autonomous community, the puddle community is ephemeral and dies off. Upon moistening, a dry film repeats the development cycle.

For analysis of the community by traditional microbiological techniques, including colony isolation, plates with agarized clay suspension were inoculated. The plating revealed abundant growth of various bacteria forming mucous colonies. Cultivation of phototrophs demonstrates, however, that plating may provide misleading results. On agarized plates, numerous colonies of green algae were revealed; *Anabaena* sp. trichomes were somewhat elongated; *Phormidium* sp. did not develop at all. Streaking of the biofilm on 2% agarized distilled water resulted in formation of small colonies of green algae, though not of cyanobacteria. On an agarized clay suspension, the streak contained a colorless slimy mass of fused mucousmicrocolonies of highly diverse bacteria; its replica revealed 100-μm zones of various similar cells, although in all microcolonies the cells were located separately in the mucus. Although highly diverse, all bacteria were capable of forming mucous masses. Aerobic organotrophic oligocarbophilic bacteria did not hydrolyze the mucus, which persisted as a binding mass. Together with the sliming sheaths of *Phormidium* sp., bacteria are responsible for creation of the mucous matrix, and thus, contribute to the pseudotissue formation. Thus, the morphological characteristics were crucial for the formation of a cyano-bacterial community.

Fractionation of the mineral composition of loamy clay by the cyano-bacterial film is the most interesting result of our experiment. X-ray diffraction analysis demonstrated that the content of nonclay (primary) minerals, especially of quartz, increased from 7% in the clay suspension to 15% in the biofilm (Table 1, Fig. 2a, *3*). No transformational transition of clay minerals caused by the microbial community was detected.

In the bulk chemical composition of the cyano-bacterial film cementing the minerals (5 weeks), SiO_2 content was 5% higher and Al_2O_3 content was 4% lower than in the original suspension. This finding confirms the increase of the primary mineral content in the film and the decrease of the clay content demonstrated by X-ray diffraction analysis. A certain increase in potassium concentration accompanied by an increased Rb/Sr ratio indicates an increase of the feldspar content in the biofilm material (data not presented).

Microscopic investigation revealed two processes responsible for this phenomenon. First, emersion of quartz and feldspar particles (which are larger than clay particles) occurred; this is usual for compaction processes. Second, the sorption of mineral particles on *Phormidium* sp. sheaths resulted in formation of brushlike structures. These particles were retained to a higher degree than small montmorillonite particles (Fig. 3). Selection of the minerals depended on the cyanobacterial morphotype. It was mentioned above that *Phormidium* hormogonia and *Anabaena* trichomes were free from mineral particles.

It should be taken into consideration that these processes occurred in an ombrophilic microbial community developing in ultrafresh water; under these conditions, coagulation of mineral colloids is limited, and their adhesion to bacterial slime predominates. Since we were fully aware of the broad variability of the community composition depending on the local microenvironment, the present work was not aimed at detailed analysis of the components of the microbial community. Our simulation provides a generalized picture based mainly on morphology as the crucial factor for the process responsible for formation of the sediment texture.

Since the conditions similar to the simulated ones are common on the plains which have not been covered by vegetation in the geological past, it is possible to hypothesize that in the remote pre-Silurian past "puddle" cyano-bacterial communities were important both for formation of the layered structure of terrigenous clay deposits and for their enrichment with organic matter from bacterial slimes. Thus, the terrestrial hydrological cycle operates between the *ombrophilic* microbiota of chemical water-rock interaction and the *halophilic* microbiota of sedimentogenesis and evaporite formation [1]. *Ombrophiles* are related to formation of *residual soil* with clays as terminal products of leaching and mineral substrate for their development. State contract no. 02.740.11.0023 with Russian Federal Agency of Science and Innovation (Rosnauka).

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