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REVIEWS

Multi-Species Biofilms in Ecology, Medicine, and Biotechnology

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Abstract—The structure, composition, and developmental patterns of multi-species biofilms are analyzed, as are the mechanisms of interaction of their microbial components. The main methodological approaches used for analysis of multi-species biofilms, including omics technologies, are characterized. Environmental communities (cyanobacterial mats and methanotrophic communities), as well as typical multi-species communities of medical importance (oral cavity, skin, and intestinal microbiomes), are described. A special section deals with the role of multi-species biofilms in such biotechnological processes as wastewater treatment, heavy metal removal, corrosion control, and environmental bioremediation.

Keywords: multi-species biofilms, methods of biofilm investigation, microbial mats, methanotrophic communities, human microbiome, wastewater treatment, anammox bioreactors, biocorrosion, bioremediation

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Biofilms are considered the predominant (if not the exclusive) form of existence of microorganisms in most of the natural ecosystems. The microevolutional processes, finally resulting in global diversity of genotypes, occur therefore in close interactions between microorganisms that form biofilms—spatially and metabolically structured associations.

A gradual increase in the structural and functional complexity of the organisms is often considered the result of the evolutionary processes. In a number of cases, however, increased complexity is not a selective advantage for evolution (Morris et al., 2012). While the lower level of structural and functional complexity undoubtedly exists, providing the concept of "life" itself, one of the predominant forms of "life" are undoubtedly relatively simply organized prokaryotes. Multiple examples of "reductive" evolution exist in nature, resulting in descendants with more simple organization originating from a more complexly organized ancestor. In such cases evolution occurs with a loss of some functions (not vitally essential for this organism), which may be compensated by its partner or host. The most typical examples are parasites and symbionts (Moran et al., 2009). Such type of microevolutionary processes can also be expected in the case of multi-species biofilms, in which the functions are shared between its microbial components. Moreover, horizontal gene transfer accelerates in biofilms, as well as the processes of mutagenesis that can be regarded as "adaptive" mutations (Plakunov et al., 2010; Hogardt and Heesemann, 2013).

Thus, "reductive" evolution (with the loss of some genes) may be profitable for microorganisms that form multi-species biofilms in which microbial components are involved in protocooperative relationships (Nikolaev and Plakunov, 2007).

The microevolution pathways occurring in biofilms are important for understanding microbial evolution in general. They are also of great practical importance for prevention of undesired biofilm development (in medical practice and technological processes), as well as for the activation of formation of "useful" biofilms (for example, for bioremediation or for biosynthesis of physiologically active substances in biofilm reactors). These microevolution processes are in many ways determined by metabolic relationships between microbial populations involved in biofilms.

The goal of the present review is to consider the structure and composition of some of the most important examples of natural and anthropogenic multi-species biofilms, as well as the types and mechanisms of interactions between microbial populations involved in these biofilms.

GENERAL CHARACTERISTICS OF MULTI-SPECIES BIOFILMS

Since multi-species biofilms appear to be the predominant form of existence of microbial communities in nature, the results of laboratory studies that deal with monospecies or (rarely) binary biofilms, which made it possible to determine the features of the "biofilm" phenotype, have to be corrected when being approximated to multi-species biofilms. In multi-species biofilms, the results of the interactions between microbial populations come to the foreground, rather than the features of the "biofilm" phenotype of each component population. The main types of these inter-

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actions are competition (or its less common variant, amensalism), commensalism and, finally, protocooperation (with its variants, synergism and, in the extreme case, symbiosis) (Jefferson, 2004; Nikolaev and Plakunov, 2007; Yang et al., 2011). The complex interactions between microorganisms in multi-species biofilms affect dramatically the phenotype of the whole community leading to a qualitatively new level of interactions that are absent in the more simple systems. In particular, apart from the signaling and autoregulatory factors typical for the population of a microorganism, interference of *interpopulational* regulatory factors in the processes of formation and functioning of biofilm communities becomes possible in both natural and artificial systems. In the following parts of the review we will discuss these opportunities.

The main methods of studying mono- and multispecies biofilms are close or similar, although it is more difficult to clearly interpret the obtained results in the latter case. The best results can usually be obtained using combined methods, but for convenience we will divide them into several categories.

METHODS AND APPROACHES TO THE STUDY OF BIOFILMS

Spectrophotometry

Dyes and indicators of metabolism. The classical method for quantitative characterization of biofilm growth, which has been used throughout the history of their study, is staining with 0.02–1.0% water solution of a bacteriological dye crystal violet (CV) (or its modification, gentian violet) with subsequent extraction of the dye bound to the biofilms with ethanol or diluted acetic acid, followed by measuring the optical density of the extract at 590 nm (Peeters et al., 2008). This simple method has, however, several disadvantages. First of all, CV stains both the matrix and the cells involved in a biofilm, so that it is impossible to trace the dynamics of individual accumulation of these components in the biofilm. Secondly, the results of staining vary greatly in case of some microorganisms (for example, *Pseudomonas*). Moreover, the pigments of the cells with absorption maximum close to that of CV (for example, violacein) may interfere with the analysis. In this case using another dye, i.e. safranin, is advisable (Pantanella et al., 2007, 2013).

Another popular dye, specific to acidic polysaccharides of the matrix, is 1,9-dimethylmethylene blue (DMMB). It was firstly proposed for staining sulfated glucoseaminoglycans of animal tissues (Famdale et al., 1982). Later it was successfully used to stain the matrix of *Staphylococcus aureus* biofilms that contains anionic polysaccharides (in particular, adhesin that contains phosphate and succinate residues) (Toté et al., 2008). DMMB stains the matrix of several other gram-positive and gram-negative bacteria (Peeters et al., 2008). Direct correlation between staining with DMMB and ability to synthesize the biofilm matrix was reported for *Chromobacterium violaceum*: the mutant *C. violaceum* CV026, which is weakly stained with DMMB, exhibits a defect in formation of the biofilm matrix, which was confirmed by light, electron, and atomic force microscopy (Zhurina et al., 2013; Kamaeva et al., 2014).

Congo red (CR) is another dye commonly used for the staining of the biofilms. It is most often used to differentiate between bacterial colonies capable and incapable of biofilm formation. CR mainly stains glucans and amyloid proteins which are involved in the biofilm matrix of many bacterial species. For example, it was proposed to reveal the pathogenic strains of *S. aureus* by cultivation on the dense medium containing CR. The pathogenic strains capable of biofilm formation, requiring an enhanced exposure to chemotherapeutic agents, can be determined (Darwish and Asfour, 2013). A similar method using CR was applied to detect biofilm-forming strains of *S. epidermidis* contaminating the platelet concentrates (Ali et al., 2014) and to assess biofilm formation by *P. aeruginosa* when testing antimicrobial agents (Kim and Park, 2013).

All mentioned methods of staining make it possible to study the scale and dynamics of biofilm formation but do not provide for assessment of the ratio between the living and dead cells present in the biofilm. It is important to note that the classical method of colony-forming unit (CFU) determination gives unsatisfactory results in the case of biofilms because it is impossible to disperse completely the extracellular polymeric matrix. To determine the ratio between the living and dead cells in the biofilms, the mixture of two dyes is commonly used, one of which penetrates only the dead cells with disrupted membranes. Fluorescent dyes have become widely spread. Although their application requires special microscopic techniques, they provide more or less adequate results in the studying of the biofilms. We will come back to the fluorescent dyes in the section dedicated to microscopic methods. Recently published critical reviews analyze different approaches (including the use of fluorescent dyes) to determine the living and dead cells in the biofilms (Tawakoli et al., 2013; Netuschil et al., 2014).

Another, alternative approach is based on indicators of metabolism. The most common are 7-oxy-3 H-phenoxazine-3-on-10-oxide (resazurin, HPO), fluorescein diacetate (FDA), and chlorhydrate 2,3-bis(2-metoxy-4-nitro-5-sulfophenyl)-5-[(phenylamyno)carbonyl]-2H-tetrazolium (CTT). HPO (also known as CellTiter-Blue or AlamarBlue) is nontoxic and does not damage the living cells. Microorganisms with active metabolism reduce the non-fluorescent blue-colored HPO into the product with a pink fluorescence (λ_{ex} 560 nm and λ_{em} 590 nm). The amount of the product is directly proportional to the number of the active cells. This method is relatively simple and makes it possible to determine

10^3–10^8 CFU in the biofilm sample (Van den Driessche et al., 2014).

FDA, absorbed by the living microbial cells, is hydrolysed by the intracellular esterases to fluorescein. The amount of fluorescein can be measured using a fluorometer (λ_{ex} 494 nm and λ_{em} 518 nm). Dead cells are unable to metabolize FDA (Peeters et al., 2008; Pantanella et al., 2013). The quantitative limits of detection of the living cells are the same as those for HPO. FDA can be used in a two-component reagent for differentiation of the living and dead cells (Tawakoli et al., 2013).

CTT is reduced by the microorganisms possessing an electron transport chain with formation of water-soluble formazan, which may be measured spectrophotometrically at 486 nm (Peeters et al., 2008; Chandra et al., 2008). The method was successfully applied for multi-species biofilms, for example for binary biofilms containing *Candida albicans* and *S. epidermidis* (Adam et al., 2002).

Microscopic Methods

Epifluorescence, laser interference and confocal microscopy are widely used to study various microbiological objects. In the previous section we have already mentioned the application of combined fluorescent dyes to determine the numbers of live and dead cells (Tawakoli et al., 2013; Netuschil et al., 2014). The dye SYTO-9, a part of this reagent, was successfully used (together with other fluorochromes) to stain biofilms: for example, to study the microbial composition of the multi-species biofilms from the inner surface of a pipeline for drinking water (Fish et al., 2015). There are several specific fluorescent dyes used to detect the presence of nucleic acids, proteins, polysaccharides, and lipids in the biofilm matrix (Nosyk et al., 2008; Larsen et al., 2008; Baum et al., 2009; Gannesen et al., 2015).

Classical light microscopy can be applied only to thin samples, while biofilms are three-dimensional objects. To study them, microscopic methods allowing scanning of such samples "by the layers" were required. The most common are laser interference and laser scanning confocal microscopy (CLSM), as well as its variations combined with epifluorescence microscopy and FISH microscopy. These methods were reviewed in several recent works (Neu and Lawrence, 2015; Fish et al., 2015).

The approach to biofilm studies which involves a combination of *Raman spectroscopy with microscopy* (RM) has been successfully developed recently. Raman scattering spectroscopy allows identification of the oscillatory "fingerprints" of the molecules and, thus, to visualize the distribution of individual organisms and their products in an intact microbial community. This method differs favorably from other microscopic approaches, since it does not require invasive techniques for fixation and dehydration of the objects, as well as from infrared microspectroscopy, in which the liquid medium acts as a screen. Confocal Raman microscopy (CRM) made it possible to determine, for example, the localization of anaerobic ammonium oxidants in multi-species biofilms without any pretreatment of the sample (Pätzold et al., 2006, 2008). Sensitivity of the method can be elevated using silver nanoparticles (to activate the surface-enhanced Raman scattering, SERS). This approach helped to trace the dynamics of the biofilm matrix components in the course of biofilm formation (Ivleva et al., 2010; Chao and Zhang, 2012).

Electron microscopy in its classical variant is of little use for the study of native biofilms because it requires fixation and dehydration of the object. However, transmission electron microscopy (TEM) may be used to reveal the differences in distribution of the cells and in the biofilm structure (particularly, the presence or absence of membrane vesicles) in mutants with impaired matrix synthesis (Smirnova et al., 2008, 2010; Zhurina et al., 2013) and to determine localization of various physiological groups of bacteria, even in such complex objects as large multi-species granular biofilms forming in an anammox bioreactor (Botchkova et al., 2014).

However, scanning electron microscopy (SEM) is undoubtedly a more common method of biofilms studies. Approaches exist which make it possible to avoid disturbance of the biofilm matrix caused by dehydration in classical SEM. The structure of the matrix is conserved in the samples treated with ruthenium red which reacts with polysaccharide carcass of the matrix, or in the samples placed into a special "moist" chamber (Weber et al., 2014). Treatment of the samples with current-conductive liquid crystal reagents (for example, choline lactate) is also suitable to preserve the native structure of the matrix. This procedure makes it possible to sputter samples with platinum without their dehydration (Asahi et al., 2015).

Atomic force microscopy (AFM) makes it possible to study not only the surface but also the three-dimensional structure of the biofilm matrix. Thus, comparison of the wild type strain *C. violaceum* WT to the mutant strain CV026 with impaired functioning of the "quorum sensing" (QS) system demonstrated that the biofilms formed by the latter strain have a less "mature" matrix with a significantly lower thickness. Moreover, some of the cells are not embedded into the matrix (Zhurina et al., 2013). This feature of the biofilms of the mutant *C. violaceum* CV026 is accompanied by their increased sensitivity to heat and acid shock, as well as to the antibiotic azithromycin (Strelkova et al., 2013; Mart'yanov et al., 2015). Comparative study of the features of bacterial cell surface of the wild type *C. violaceum* and the mutant CV026 showed the presence of specific structures associated with synthesis of the biofilm matrix and regulated by the QS system (Kamaeva et al., 2014). AFM was also

applied to study unusual filament structures formed on microscopic slides submerged into an anammox bioreactor (Botchkova et al., 2015).

Several studies were dedicated to measuring adhesion of microbial cells to the surface or to other cells in multi-species biofilms using AFM. For example, the level of adhesion of *S. aureus* cells to different regions of the cells and hyphae of *C. albicans* in binary biofilms was measured. It was found that the level of adhesion of bacterial cells was higher in the "young" regions of the hyphae while adhesion to the "head" regions close to the mother cell was nearly an order of magnitude lower and was similar to adhesion to the budding cell. This indicates significant differences in the surface structure of different regions of the hyphae (Ovchinnikova et al., 2012; Beaussart et al., 2013). The recently published review gives more detailed information about various AFM methods for different tasks in studying biofilms (Dufrêne, 2015).

Some Molecular Genetic Methods

Oligonucleotide fluorescent probes (Fluorescence In Situ Hybridization, FISH). Although there are numerous modifications of this method, they all include several common stages: fixation of the sample, hybridization with an oligonucleotide probe specific for the certain 16S rRNA gene sequence or other targets and containing a fluorophore on 5'-end, washing, and visualization of the fluorescent signal (Daims and Wagner, 2011). This method was successfully combined with confocal microscopy to study 3D-structure of the multi-species biofilms (Brileya et al., 2014). The probes specific to various systematic groups of microorganisms make it possible to indicate the composition of the biofilm community and even localization of its particular members in the biofilm. For this purpose, specific computer programs, which have been reviewed recently, are used (Neu and Lawrence, 2014, 2015). Combination of FISH and microautoradiography also demonstrates good results. It was applied, for example, to study nitrifying multi-species biofilms (Okabe et al., 2005) and multi-species biofilms from an anammox reactor (Kindaichi et al., 2012).

Polymerase chain reaction (PCR) is widely used as a diagnostic tool for identification of microorganisms in various systems and determination of systematical position. Application of this method to biofilms faces methodological difficulties due to the presence of the matrix. On the one hand, the matrix complicates extraction of cell DNA and, on the other hand, contains considerable amounts of extracellular DNA. For multi-species biofilms the best results were obtained using real-time quantitative polymerase chain reaction (Xie et al., 2011) or its modification (qRT-PCR), which provides for analysis via cloning of expressed genes with preliminary reverse transcription of their mRNA (Pantanella et al., 2013). It can be stated that modern "omics-techniques," i.e., (meta)genomics, (meta)transcriptomics, (meta)proteomics, and metabolomics are being more and more widely used in biofilm studies (see below section Biofomics of the present review).

Physicochemical methods of studying the structure and composition of biofilms are very diverse. In the current review we will focus only on the most common ones: infrared spectroscopy (with Fourier transformation, FTIR), mass spectrometry in its variants, matrix-assisted laser desorption/ionization (MALDI) and nuclear magnetic resonance (NMR).

We have already mentioned the limitations of *infrared spectroscopy* of biological objects due to the shielding effect of water. However, Fourier transformation for infrared spectra (FTIR) makes it possible to reduce the side effects and to increase the sensitivity. A good example of the potential of this method is a study of the biofilms matrix of *Shewanella* sp. HRCR-1 that detected the presence of proteins, polysaccharides, nucleic acids, lipids, and fatty acids. Parallel application of the proteomics techniques revealed the matrix proteins to contain 58 extracellular proteins and proteins of the outer membrane, including serine proteinases, nucleases, and lipases, as well as proteins involved in extracellular electron transfer, i.e., cytochromes *c*, proteins MtrC and OmcA (Cao et al., 2011). This method was also successfully applied to determine the anti-biofilm action of photodynamic effect on multi-species microbial associations of dental plaque (Mang et al., 2012) and for quantitative determination of the inhibition by the components of honey of Chinese dates (jujube) on yeast biofilms (Ansari et al., 2013).

Mass spectrometry in its variant MALDI BioTyper makes it possible to reveal and identify all the main types of microorganisms: mycelial fungi (Chalupova et al., 2014), yeasts (Chao et al., 2014), and bacteria (Hsueh et al., 2014; Oumeraciet al., 2015) in all types of microbiomes, including plant-associated ones (Ahmad et al., 2012), in clinical samples (Panda et al., 2014; Schulthess et al., 2014), and in communities forming under anaerobic conditions (Hsu and Burnham, 2014). In difficult cases and in the case of multiple (serial) analyses, this method demonstrates remarkable advantages in comparison to gene sequencing (Angeletti et al., 2015).

The NMR method has been successfully applied to achieve progress in the studies in the field of so-called "-omics" technologies as a part of complex Biofomics (see below). This approach provides for transition from earlier studies of morphology, physiology, and genomics of biofilm formation to investigation of the biochemical mechanisms of conversion of microbial cells from planktonic phenotype to the biofilm one. In a recently published work, examples of using NMR-metabolomics to study clinically important biofilms are reviewed (Zhang and Powers, 2012). For example, a number of external factors and chemical substances (ethanol, oleic acid, glucose, UDP-*N*-acetylglucose-

amine, some antibiotics in sub-inhibitory concentrations, hypoxia, as well as osmotic and heat shock) were shown to induce the biofilm type of existence in *S. aureus* and *S. epidermidis* (Sadykov et al., 2010; Zhang et al., 2011).

NMR was also used to measure chemical and electrochemical gradients in the biofilms of *S. oneidensis* (Beyenal1 and Babauta 2012) and diffusion rate of the metabolites through the biofilm matrix of *Geobacter sulfurreducens* and *S. oneidensis* (Renslow et al., 2013). With the help of this method, differences were discovered in the lipopolysaccharide composition between planktonic cells *P. chlororaphis* and the cells involved in biofilms: the latter had a level of acetylation of 6-O-antigene six times lower compared to the similar structure in planktonic cells. This change affects the antigenic properties of microbial cells considerably (Zdorovenko et al., 2015).

The methods of NMR and MALDI are widely used in studies of biofilms with the help of metabolomics (see below section Biofomics).

Microsensor tools equipped with miniature sensors 10–20 μm in diameter were used to measure pH and concentrations of O_2, NH_4^+, NO_2^-, and NO_3^- in a bacterial sulfate-reducing community forming multi-species biofilms in a bioreactor for wastewater treatment (Okabe et al., 2003). Similar sensors helped to study localization of the metabolic processes (particularly, the equilibrium concentrations of H_2 and CH_4), as well as pH and redox potential in granulated multi-species biofilms from an anaerobic laboratory bioreactor (Satoh et al., 2007). Fluorescence microscopy and silicate nanoparticles (10 nm) as sensors were used to trace the heterogeneity in pH distribution in *E. coli* biofilms (within the range of pH 5–7) caused by addition of glucose (Hidalgo et al., 2009).

An interesting example of microsensor use is the work on removal of inorganic nitrogen compounds from wastewater in an anammox bioreactor containing multi-species granular biofilms (Cho et al., 2010). Other authors were able to register the dynamics of relationships between populations in binary biofilms of *Methanococcus maripaludis* and *Desulfovibrio vulgaris* and of two non-interbreeding yeast strains *Saccharomyces cerevisiae* using a micro-digital camera with the objective size 700 μm (Momeni et al., 2013).

Biofomics

The recently proposed term "biofomics" is a kind of a "workbench" to merge the results of different approaches to studying biofilms. As we have already mentioned, these approaches include genome sequencing, or (meta)genomics; detection of the profiles of expressed mRNA using microchips (microarray), or (meta)transcriptomics; analysis of protein profiles, or (meta)proteomics; and analysis of the key metabolites, or metabolomics (Azevedo et al., 2009; Abram, 2015). Several other approaches were suggested for inclusion into the database, for example, phenomics, studying the metabolic reactions and changes of cell viability in response to stress environmental factors (or genetic effects) (Bochner, 2003). The novelty of the "biofomics" system is based on systematized collection of the data obtained via various approaches, their storage in a digital form, and provided free access to them. Results obtained by different researches may be compared, reproducibility of the methods may be assayed, and development of novel analytical approaches may be facilitated. The methodology of this system is outlined in the review (Lourenco et al., 2012), and the digital "desktop" is described in the article (Pérez-Rodríguez et al., 2015) and is presented at the website http://sing.ei.uvigo.es/bew.

THE MAIN FEATURES OF MULTI-SPECIES BIOFILMS FORMATION

Most studies dealing with the process of biofilm formation were carried out on monospecies samples of biofilms. However, methodological approaches may differ significantly, even in case of the same biofilm-forming species. The main ways to obtain biofilms in laboratory conditions were summarized in the study (Azevedo et al., 2009).

The case of natural multi-species biofilms is even more complicated because their composition and, therefore, the ways of their formation, can differ dramatically depending on their localization (Yang et al., 2011).

As we have already mentioned in the introduction to the present review, in multi-species biofilms the main focus is on the interactions of the biofilm-forming population that can prevent (rarely) or contribute to (more often) formation of this community. One of the most common mechanisms of interaction is "coaggregation"—induction of cell adhesion to the phase boundary surface. This mechanism was studied in detail in case of oral biofilm formation (Rickard et al., 2003). The initial stage (adhesion) determines the destiny of a microbial population in many ways, specifically whether it remains in a planktonic state or moves to the biofilm way of life. That is why we will take a closer look at this problem, which is of a great ecological importance. For example, it is a well-known fact that under a high level of "shear force" (fast flow of the water, stirring, etc.), microorganisms able to attach to the phase boundary gain an advantage in colonization of this ecological niche. In this case, the key role is played by the "early colonizers," microorganisms forming the initial biofilm which is later colonized by the satellite microbes. Model experiments demonstrated that in "dental plaque" formation, members of the species *Actinomyces oris, Streptococcus gordonii*, and *S. oralis* act as early colonizers. They are followed in time by *Porphyromonas gingivalis* and *Veillonella*

parvula, while *Aggregatibacter actinomycetemcomitans* are the late colonizers. It is interesting to note that the pair *S. oralis–V. parvula* stimulates greatly the growth of the joint model biofilms with each of the mentioned partners (Kolenbrander, 2011). It was found that *Fusobacterium nucleatum*, a bacterium causing periodontitis, has a gene *fap2* encoding formation of a galactose-sensitive hemagglutinin and adhesin that is involved in co-aggregation processes and might be responsible for the virulence of fusobacteria (Coppenhagen-Glazer et al., 2015).

Other examples of the key role that co-aggregation plays in biofilm formation are known. It was demonstrated that bacteria of several genera (*Acinetobacter calcoaceticus, Burkholderia cepacia, Methylobacterium* sp., *Mycobacterium mucogenicum, Sphingomonas capsulata*, and *Staphylococcus* sp.) isolated from drinking water are capable of co-aggregation with surface cell structures, including proteins and polysaccharides. In this case, *A. calcoaceticus* plays the key role of the connector or bridge ("bridging organism"). This microorganism presumably has complementary receptors that are recognized by the specific adhesins of other microorganism (Simões et al., 2008). It was shown that co-aggregated multi-species biofilms formed in domestic showerheads and emitting an unpleasant smell include members of the genera *Brevundimonas, Micrococcus*, and *Lysobacter* (Vornhagen et al., 2013). Such biofilms can act as a reservoir for opportunistic pathogens (Feazel et al., 2009). From multi-species biofilms found in a kitchen sink, 13 species of bacteria were isolated and identified, including *Brevibacterium casei, P. nitroreducens, M. lacticum,* and *Klebsiella pneumonia*. All of them exhibited a capacity for co-aggreagtion (Furuhata et al., 2010).

In multi-species biofilms containing microorganisms with protocooperative relationships between each other, the products of one of the species can act as a signal molecule or inductors for other species. It provides for more effective formation of biofilms, usage of nutrients, and survival under the stress conditions. Examples of such relationships are discussed below.

MULTI-SPECIES BIOFILMS IN ECOLOGICAL SYSTEMS

According to the modern concepts, biofilms appeared on Earth 3.5 billion years ago (Hall-Stoodley et al., 2004), and currently 95–99% of microbial populations in nature exist in the form of biofilms (mostly multi-species) (Costerton et al., 1987). However, the problem of evolution and relationships between microorganisms in such communities has been neglected. As a rule, researches are limited to the use of (meta)genomic methods which help to establish the composition of a community but are useless for making any definitive conclusions about the relationships between microorganisms and about the ecological role they are playing. In the present review we will focus on the few works that can provide information on the mechanisms of complex interactions between microbial components of the natural multi-species biofilms.

A special part of the review is dedicated to the interactions between populations in natural and artificial multi-species associations important for biotechnology (see below).

Since biofilm formation provides, first of all, protection of the community from unfavorable environmental factors, such communities are easily found in ecosystems with extreme conditions: in hot springs, deep-sea volcanoes, industrial installations, and, finally, in clinics, i.e., in such conditions where microbial cells need protection from extreme factors and biocides (Hall-Stoodley et al., 2004). It was shown that the protective effect of biofilms for the cells involved increases rapidly in multi-species variants of the biofilms. However, it is not the only advantage of multi-species biofilms. One of the examples are microbial mats, one of the best studied microbial communities, currently regarded as macroscopic biofilm structures with cyanobacteria as the initial structure-forming microorganisms (Bolhuis et al., 2014; Rossi and De Philippis, 2015).

It should be noted that the present review is not focused on detailed analysis of phototrophic communities, so we will deal only with the most typical cases.

As a rule, microbial mats are vertically stratified benthic communities, embedded in a polymeric organic matrix containing varying amounts of inorganic substrates, silicates, and carbonates. The main photosynthetic components of the mats are phototrophic cyanobacteria, usually accompanied by phototrophic eukaryotes (diatomic algae). They can be regarded as analogs of stromatolites, the fossils dating back to the age of 3.5 billion years, so they are among the most ancient biological systems on Earth (Schopf, 2000; Sergeev et al., 2002).

There are three main types of phototrophic mats: littoral microbial mats, microbial mats of hypersaline environments and, finally, microbial mats of hot springs (Bolhuis et al., 2014). In spite of a great number of studies dedicated to the structure, composition, and relationships between microorganisms involved in them, the remarkable stability of these ancient ecosystems remains a mystery in many ways.

Although littoral microbial mats experience strong fluctuations of salinity due to the tidal phenomena, their composition remains stable. Unlike the two other types of microbial mats, they include remarkable numbers of eukaryotic algae that act as one of the major, if not the key, primary producers of organic matter (Bolhuis et al., 2013). The members of the phyla *Proteobacteria, Cyanobacteria, Bacteroidetes*, and *Acidobacteria* are the most abundant in these mats. The numbers of *Betaproteobacteria* increase with the depth. The most interesting metabolic interactions (studied using transcriptomics techniques) develop

between the members of *Cyanobacteria* and *Chloroflexi* (Burow et al., 2013). Cyanobacteria, mainly *Microcoleus* species, accumulate the products of photosynthesis as glycogen, which is metabolized into organic acids, ethanol, CO_2, and H_2, and uncultured members of the *Chloroflexi* utilize these products storing polyhydroxybutirate.

According to the current data, communities of microbial mats from hypersaline ecosystems are more diverse than it was previously thought. Cyanobacteria of the genus *Microcoleus* are the dominant phototrophs in such communities. Other cyanobacteria are also abundant in the mats, as are the members of the genera *Chloroflexus, Halochromatium, Bacteroidetes, Beggiatoa* and several other, still unidentified prokaryotes. In the course of a diurnal cycle, significant fluctuations of the redox potential occur inside the mat, causing spatial changes in the structure of the community. In the mats, cyanobacteria act as the dominant producers of organic matter and produce the main part of the extracellular matrix, which, in particular, protects the microbiota from drying out. Sulfate-reducing, sulfur-oxidizing and anoxygenic phototrophic bacteria are stratified in a vertical direction in accordance with the microgradients of oxygen and sulfide, as well as in accordance with intensity of light (like in other types of microbial mats) (Dillon et al., 2009; Bolhuis et al., 2013, 2014). Although the ratio between bacterial, archaeal, and eukaryotic rRNA genes in one case was (%) 90 : 9 : 1, archaeal contribution to the total metabolic activity was high. Interestingly, the diversity of archaea was the highest in the upper part of the mat (2–3 cm from the top), where *Euryarchaeota* predominated, and decreased sharply in deeper layers, where *Crenarchaeota* were the most abundant. Distribution of many archaea did not correlate with the main chemical gradients, indicating their physiological diversity. (Robertson et al., 2009).

Microbial mats of hot springs have been studied since the 1950s due to increasing interest in thermophilic bacteria. To a large extent, these studies were stimulated by the possibility of practical application of thermostable bacterial enzymes. Interest in such enzymes was preserved until now. The microbial composition of hot spring microbial mats is to a large extent determined by their temperature. The common pattern can be formulated in the following way: diversity of the community decreases with increasing temperature and, vice versa, increases with a temperature decrease. Such a strict regularity was traced, for example, for the representatives of the phylum *Chloroflexi* (Everroad et al., 2012). It was found that in many hot spring anoxygenic phototrophic mats, the members of *Chloroflexi* or other anoxygenic phototrophs (for example, the genus *Chlorobium*) were the dominant phototrophs, while in oxygenic mats thermophilic cyanobacteria were the dominants. Moreover, these two groups of phototrophs competed for the limiting substrates (Weltzer and Miller, 2012; Bolhuis et al., 2014).

In another type of microbial mats from the hot springs (alkaline, sulfidogenic), three physiological groups of bacteria were identified. These are, first of all, aerobic chemolithotrophic, sulfide-oxidizing bacteria (*Sulfurihydrogenibium*) localized on the surface of the mat. They utilize atmospheric oxygen, thus protecting anoxygenic phototrophs (the genus *Chloroflexus*) and sulfate-reducers (the *Thermodesulfobacterium/Thermodesulfatator* group). These anaerobic bacteria use molecular hydrogen formed in the course of utilization of organic matter as an electron donor, which is (Otaki et al., 2012). Metatranscriptomics techniques were used to show that at night the *Chloroflexi* utilize glycogen (synthesized by cyanobacteria) and form the components of the photosynthetic apparatus, polyhydroxyalkanoates, and wax esters to be used as a source of carbon at daytime (Klatt et al., 2013a, 2013b).

Thus, microbial mats in general as specific biofilm ecological systems demonstrate the following features. Firstly, bacteria dominate in such systems while archaea and eukaryotes represent just a small part of the community. Thus, the numbers of archaea (mostly methanogens and haloarchaea) in the community of microbial mats are in the range of 1–20% (López-López et al., 2013; Bolhuis et al., 2014). Due to predominance of bacteria of the sulfur cycle in many microbial mats, methanogens can occupy only the niches where they are supplied with the substrates unavailable to sulfate reducers, such as methylamine. Secondly, littoral and weakly halophilic microbial mats represent the communities with the maximal microbial diversity which can be reached in Earth conditions. In contrast, microbial mats of hypersaline systems and hot springs can be regarded as systems with the lowest diversity needed to support their stability (Bolhuis et al., 2014).

Currently, attempts were taken to develop the methods for reconstruction of artificial multi-species microbial systems (similar to mats). They can be used in studying the influence of environmental factors on the composition of the community and functioning of the metabolic pathways in order to predict the consequences of extreme impacts (including anthropogenic ones) and to investigate the possibility of controlling the life of the complex communities (Zavarzin et al., 2003; Llirós et al., 2008; Cole et al., 2014).

Another typical example of ecosystems organized according to the scheme of the multi-species biofilms are communities of methanotrophic bacteria. It is a well-known fact that two types of methanmonooxygenases (MMO) exist: copper-containing particulate ones, which are bond to the membranes (pMMO), and iron-containing cytoplasmatic "soluble" one (sMMO). In accordance with this and several other features, methanotrophs are divided into two types: type I includes members of the genera *Methylobacter,*

Methylocaldium, Methylococcus, Methylomicrobium, Methylomonas, and *Methylosphaera* and type II contains the members of *Methylocystis* and *Methylosinus*. Both types of methanotrophs contain pMMO, but several members of type II (as well as *Methylococcus* of type I) can also contain sMMO. Natural multi-species biofilms are characterized by the different ratio between type I and type II methanogens;, their total etabolic activity also altersvaries. Since sMMO, unlike pMMO, has a wide substrate specificity (can include oxygen not only into the molecule of methane but into the molecules of other alkanes, alkenes, and alicyclic and aromatic hydrocarbons), the growth of methanogens with this MMO is stimulated by these substances. At the same time, type I methanotrophs have a higher growth rate and in ecosystems (methane biofilters) utilize methane more efficiently (Kim et al., 2012; Su et al., 2014).

Apart from the cyanobacterial mats mentioned above, archaea are widespread in nature in multi-species biofilms (typically in communities with bacteria) participating in biogeochemical processes in Antarctic seas, mountainous springs formed by alpine glaciers, acidic mine waters, alkaline lakes, and deep-sea hydrothermal vents. Archaea are also a part of the anaerobic methane-oxidizing community (Orell et al., 2013; Frols, 2013).

Several examples of interactions between microorganisms in multi-species biofilms, including those of medical and biotechnological interest, were previously reviewed (Burmølle et al., 2014) and will be discussed below.

One of the special problems that has been poorly studied is an effect of volatile compounds produced by bacteria on biofilm formation (Plyuta et al., 2013) and contacts between the populations, as well as interactions of bacteria with plants and protozoa (Audrain et al., 2015).

MULTI-SPECIES BIOFILMS IN MEDICINE

Since medical problems do not fall within the competence of the authors of the current review, we will only briefly focus on some examples of interactions between microorganisms in multi-species biofilms of the human microbiome which are of great importance for medicine. We practically avoid discussion on the interactions between the microbiome and human organism, since this problem is beyond the current review. In a human organism, multi-species biofilms can be formed in the oral cavity as well as in intestines, on skin, on vaginal mucosa, and in the respiratory system (Walker et al., 2015). The main components of the human microbiome are bacteria (and bacteriophages) and yeasts, although other microorganisms can be present, including archaea (Horz, 2015).

Beyond doubt, microbial interactions in dental biofilms (“oral biofilms” or “plaque”) are among the best-studied. According to the metagenomics results, the oral microbiome includes about 700 bacterial species (Xu and Gunsolley, 2014). They colonize teeth and cavernous space, tongue, oral mucosa, hard palate, periodontal “pockets,” etc. (McLean, 2014). Importantly, most of the microflora induces health preservation of the oral cavities and only a few of its members are harmful. However, since most of the studies are dedicated to pathogenic microflora, the false impression is created that all of these bacteria are our enemies (Roberts and Darveau, 2002; Huang et al., 2011).

The recent review is dedicated to various types of interactions between the components of oral microbiota at multi-species biofilm formation and the role of these interactions for human health (Mahajan et al., 2014). The authors pay special attention to such types of interactions as co-aggregation and physical contacts, usage of signal molecules in the interactions between populations, and exchange of genetic material and metabolism products.

Study of the correlation between 20 “useful” and “harmful” bacteria and their interference in 6308 patients with different levels of periodontitis can serve as an example of revealing the interactions between populations. It appeared that development of the disease correlated with the dominance of *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Eubacterium nodatum, P. micra,* and *P. intermedia*, which are regarded as pathogenic microflora. At the same time, the numbers of the members of *Streptococcus, Capnocytophaga*, and *Actinomyces*, as well as of *Vellionella parvula, Eikenella corrodens*, and *Campilobacter concisus*, which may be regarded as “healthy” microflora, decreased. Thus, competition between these two groups of microorganisms can determine the course of disease (Loozen et al., 2014; Mashima and Nakazawa, 2015).

Streptococci are the main microbial factor in colonizing the oral cavity and biofilm formation. Microscopic methods (immunofluorescence and FISH) showed that at the tongue colonization streptococci positively interacted with *Veillonella*, and in the oral cavity a positive correlation was observed between streptococci and *Leptotrichia, Granulicatella*, and *Actinomyces* (Jakubovics et al., 2014). Analysis of activity of specific genes using the methods of transcriptomics showed that streptococci produced several extracellular factors that influenced the interactions between populations. Among them were extracellular enzymes, co-aggregation-inducing adhesins, signal peptides (autoinducers II), bacteriocins, and metabolic byproducts: lactic acid and hydrogen peroxide. We can expect that more detailed study of the effect of these products on the interactions between populations will make it possible to control the oral biofilm formation (Faust and Raes, 2012).

It is important to note that, apart from bacteria, a variable number of yeasts (mostly members of the genus *Candida*) can be present in the oral microbiome.

Their abundance is commonly regarded as a sign of disease (candidiasis) (Diaz et al., 2014). However there is evidence of possible commensalism between the yeasts *Malassezia* and bacterial components of the oral microbiome (Dupuy et al., 2014).

Microbial diversity in the microbiome of the human intestines is presently also intensively studied, though medical science pays attention mainly (as in the previous example of the oral biofilms) not to relationships between the gut microorganisms but to their relations with the host organism (Elson and Alexander, 2015). Symbiotic intestinal microorganisms facilitate the transformation of nutrient substances, synthesize the vitamins (Magnúsdóttir et al., 2015), stimulate the immune system, and help to withstand the pathogenic organisms. They are important for the maintenance of human health preventing and helping to cure such diseases as obesity, diabetes, and atherosclerosis (Shoaie and Nielsen, 2014; Janssen and Kersten, 2015).

Gut microbiome is hard to study due to the fact that it contains over 1000 microbial species, mostly belonging to 5 phyla: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria*, and *Verrucomicrobia*. Methanogens, eukaryotes (mostly yeasts), and bacteriophages are also a part of the gut microbiota. Interestingly, the total number of the gut microbiota cells (about 10^{14}) is much higher than the number of human somatic cells (about 6×10^{13}), and the total number of the genes in the microbiome of each human (about 600×10^3) is 25 times higher than the total number of the host genes (Qin et al., 2010). Comparison of the results obtained by genomics and transcriptomics showed that some of microbial transcripts (41%) are expressed according to the abundance of their genes, while other genes, including those responsible for sporulation and amino acid biosynthesis, are lacking expression, and the genes responsible for ribosome biogenesis and methanogenesis are overexpressed. Metatranscriptome profiles appeared to be more individualized than genomic profiles, albeit less variable than the composition of the microbiota. This fact points out the presence of the mechanisms of regulation of metabolism, common for the whole community (Franzosa et al., 2014).

One of the ways to influence directly the composition of the gut microbiota is using probiotics containing useful microflora, for example, bifidobacteria (Tojo et al., 2014).

Skin is another ecosystem of an animal organism which is actively colonized by microorganisms. We will therefore briefly characterize the skin multi-species biofilms. The members of the *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*, families *Bacteroidales, Flavobacteriales, Lactobacillales, Clostridiales*, various species of *Propionibacterium, Staphylococcus*, and various yeast organisms and viruses are involved in skin microbiota (Giacomoni et al., 2009; Oh et al., 2014). Relationships between the composition of microbiota and different skin disease were studied in detail (SanMiguel and Grice, 2014). There is much less information considering the interaction of skin-inhabiting microorganisms with each other. One of the few studies was dedicated to complex interactions between opportunistic bacteria: staphylococci (*S. epidermidis* as an example) and propionic acid bacteria (*P. acnes* as an example), which can demonstrate either mutualistic or antagonistic relationships due to formation of antimicrobial products (peptides and fatty acids) (Christensen and Brüggemann, 2014). In another example, it was shown that serine proteinase synthesized by *S. epidermidis* suppressed formation of *S. aureus* biofilms, thus preventing the development of infection (Iwase et al., 2010). It was found that the filtrate of *S. epidermidis* culture prevented accumulation of lipase and formation of biofilms by *Candida albicans* (Bhattacharyya et al., 2014), one of the most typical component of skin microbiota, together with yeasts of the genus *Malassezia* (Findley et al., 2013).

Since the current review is not dedicated to infectious diseases caused by the biofilm-forming microorganisms, we would just like to note that microorganisms in the biofilms are much more (tens, hundreds, and thousands of times) resistant to the action of antibiotics and immune systems of the macroorganisms than their planktonic cultures (Strelkova et al., 2012; Mart'yanov et al., 2015; Melander, R.J. and Melander, C., 2015). As a rule, in multi-species biofilms such resistance is still higher, which is due, among other factors, to the horizontal gene transfer (Vega and Gore, 2014; Roberts and Kreth, 2014) and formation of persistent cells (Conlon et al., 2015).

MULTI-SPECIES BIOFILMS IN BIOTECHNOLOGY

Multi-species microbial biofilms are widely used in various fields of biotechnology. This is explained by their resistance to environmental conditions and their stability in terms of flow cultivation (which is especially important for slowly growing species). Because of their indifference, no additional time and material costs are needed to maintain a pure culture and preserve it from contamination (Roeselers et al., 2008).

The most important spheres of practical applications of biofilms are wastewater and groundwater treatment, as well as soil remediation. Diversity of metabolic processes and ability to utilize xenobiotics as nutrients (without addition of extra substrates) turned them into promising subjects for bioremediation and biological treatment of the environment.

Biofilms in wastewater treatment. Biofilms are most commonly used in wastewater treatment. Various technological schemes have been constructed in detail and are successfully used in full-scale installations for

treatment of wastewaters of different origins and composition.

In wastewater treatment reactors, biofilms have advantages over planktonic cells for several reasons. Firstly, in the case of biofilms, the risk of washout of the valuable biomass from the reactor with purified water is reduced. This is especially important for the slowly growing species, such as anammox bacteria. Microorganisms with different metabolism and nutritional needs coexist in biofilms, each occupying their own microniche. Thus, biofilms are promising for development of wastewater treatment systems for simultaneous removal of several contaminants, for example, nitrogen- and phosphorous-containing substances due to co-existence of nitrifiers and phosphate-accumulating proteobacteria in a single bioreactor (Gieseke et al., 2002).

In several types of bioreactors, the biomass is fully or partly immobilized on carriers and can be regarded as biofilm. In some cases, bioreactors have a cyclic working mode, i.e., cycles of medium supply and aeration can alter. Different groups of microorganisms can be active in each of the cycles due to their physiological requirements. Thus, conditions are formed for coexistence of different groups of microorganisms not only in a single bioreactor but also in the same biofilm. Bioreactors can be equipped with carriers made of different materials. The main requirements for the carriers are inertness (ions and dangerous components should not contaminate the medium) and ability for biofilm formation (for example, the presence of a porous structure).

The carriers for such bioreactors are presently made of a wide range of materials: discs, plates, or caps of polymeric materials (Gieseke et al., 2002; Persson et al., 2014; Almstrand et al., 2014), non-woven materials (Kindaichi et al., 2007; Liu et al., 2009), zeolite particles (Fernandez et al., 2008), iron-nickel scaling (Terada et al., 2006), and polyurethane porous cubes (Chae et al., 2012). Depending on localization of the carriers inside the bioreactor, their shape and size, and working conditions of the bioreactor, biofilms can be spherical, granular-type ones (Botchkova et al., 2014) or thin and flat (Kindaichi et al., 2007; Almstrand et al., 2014; Persson et al., 2014). Some types of bioreactors (for example, OLAND or SBR) are not equipped with any carriers and suggest stirring of the medium during cultivation. However, even in such conditions aggregate formation is observed. Granules 1–2 mm in diameter and floccules 0.5 mm in diameter, formed in such conditions (Li et al., 2011; Vlaemick et al., 2010), are also regarded as biofilms.

Wastewaters rich in nitrogen compounds (ammonium salts) and poor in organics, such as leachates of municipal solid wastes or pig farm wastes, can be effectively cleaned by microorganisms of multi-species biofilms which include anammox bacteria (ANB). This relatively recently discovered group of chemolithoautotrophic microorganisms is responsible for anaerobic ammonium oxidation by nitrite with release of molecular nitrogen. This fact made them a promising object for biotechnology. None of ANB was isolated as a pure culture; they all exist as members of microbial communities. In bioreactors for wastewater treatment, specialized communities are formed of microorganisms involved in protocooperative or antagonistic relationships with each other, participating in removal of various pollutants from the water. Communities of the bioreactors involved in nitrogen removal can include, apart from ANB, other microorganisms of the nitrogen cycle—nitrifiers and denitirifiers. Representatives of other physiological groups of microorganisms can accompany them in such communities, performing important (though not always known) functions. ANB themselves have a strong tendency for biofilm formation. In nature they exist within a layer of benthic sediments. In anthropogenic habitats they also exist as sediments or variable foulings. However, not all ANB share the same level of the tendency for biofilm formation. For example, in a number of marine habitats, members of the genus *Candidatus* "Scalindua" often (sometimes even exclusively) appear as planktonic forms. Thus, in the samples taken from the water column at different depths of the Black Sea, *Candidatus* "Scalindua" were found only in fractions of the particles less than 30 μm in diameter. In this habitat ANB are adapted to oligotrophic conditions, which may probably explain their non-attached, free-living growth (Fuchsmann et al., 2012). In bioreactors they can also form foulings on special carriers (brush-like), put into the reactor for this purpose. If there are no carriers in the reactor, ANB form floccules suspended in water/medium.

Normally, living active ANB cells are strictly localized inside such biofilms, just like the cells in a cyanobacterial mat, and are surrounded by a thick layer of matrix. The matrix contains proteins and polysaccharides—polymers of α- and β-D-glucopyranose (Ni et al., 2015). Stratification depends on the metabolic properties of each group of microorganisms, primarily on their attitude to oxygen. Coexistence of ANB with first stage nitrifiers is often observed in both attached biofilms and floccules (Li et al., 2009; Vlaemick et al., 2010; Persson et al., 2014). Since bioreactors of this type have a working mode with sequential changes of oxic and anoxic conditions, it is necessary to create conditions for protection of oxygen-sensitive ANB during the aerobic working cycle. As aerobes, nitrifiers inhabit the surface of the biofilm while ANB are localized in the inner part of the granule where diffusion of oxygen is significantly impeded. Under such conditions, during *de novo* biofilm formation nitrifiers act as early colonizers, forming a biofilm with anoxic microniches, which are subsequently occupied by ANB. The process can last for up to 5 months (Almstrand et al., 2014). Sometimes the biofilm can contain one more layer, the deepest, in the center of the granule. Oxygen access there is severely hampered,

creating optimal conditions for existence of obligate anaerobes—methanogens (Cho et al., 2010). The community can also include heterotrophic bacteria, usually *Betaproteobacteria* (Kindaichi et al., 2007; Botchkova et al., 2014).

Members of the phylum *Chloroflexi* were often discovered among microorganisms of the granules from anammox reactors (Li et al., 2009; Kindaichi et al., 2012). These are filamentous microorganisms with cell diameter about 0.5–1 μm. Their trichomes can reach the length of several tens of micrometers. There are several points of view about the role they are playing in the community. They can act as a framework to form a three-dimensional structure—a granular network. This hypothesis is supported by the fact that trichomes are often found around the clusters (microcolonies) of ANB by in situ analysis or on thin-sectioned granules. They also can take part in biodegradation of the macromolecules formed during the decay of microbial biomass (Cho et al., 2010; Kindaichi et al., 2012). Cells of other filamentous bacteria of the phylum *Bacteroidetes* are distributed evenly over the biofilm. Thus, these microorganisms may also perform a structure-forming function in biofilms (Li et al., 2009; Almstrand et al., 2014). Since some *Chloroflexi* are known to metabolize sulfur compounds, biofilms containing the members of this phylum (and other phototrophs involved in the sulfur cycle) were proposed to be used in wastewater treatment from sulfur compounds as a substitution to a well-known bioremediation system using aerobic sulfur bacteria (*Thiobacillus*). The main disadvantage of the latter is that additional costs are needed for aeration and separation of aerobic and anaerobic processes of wastewater treatment. Biofilms have already been successfully used for anaerobic removal of sulfur compounds from wastewater and gases, mainly H_2S (Jensen and Webb, 1995; Hurse and Keller, 2004). Since *Chloroflexi* rather often coexist with ANB, there are certain prospects of creating systems for simultaneous removal of nitrogen- and sulfur-containing pollutants.

Biofilms of photosynthetic organisms (cyanobacteria and algae), together with their satellites, are also used in wastewater treatment. Such biofilms are promising for simultaneous removal of nitrogen and phosphorous compounds. Oxygen formed during oxygenic photosynthesis in a phototrophic mat can be used by aerobic microorganisms that are directly involved in removal of pollutants from wastewater, for example, ammonium and nitrite in the course of nitrification. Many of the phototrophs are themselves capable of assimilating nitrogen compounds from wastewater. Phototrophic biofilms play an important role in phosphate removal due to the fact that many phototrophs accumulate phosphorus inside the cells as polyphosphate granules. Moreover, activity of phototrophs results in increased pH of the medium due to carbon dioxide consumption, which promotes precipitation of soluble phosphates. Alkalinization of the medium as a result of activity of phototrophs can help to reduce the number of fecal microorganisms in wastewater (Roeselers et al., 2008). Unfortunately, these promising ideas have not been applied yet in full-scale installations for wastewater treatment. However, phototrophic biofilms are used for the treatment of secondary effluents of wastewater treatment stations—in so-called constructed wetlands. Treatment of such type of effluents depends in many ways on epiphytic phototrophic biofilms from the surface of the reed stems. Interestingly, excessive bacterial biomass that forms as a result of using phototrophic biofilms for wastewater treatment can be re-used as fertilizers for agriculture (Schumacher and Sekulov, 2002) and as an alternative source of protein for various aquatic animals. For example, cyanobacterial biofilms are successfully used as an additional nutrient source in aquaculture for *Tilapia* fish (van Dam et al., 2002). However, it is important to take into consideration that many cyanobacteria produce substances which are toxic for animals, so their biofilms cannot be used for such nutrition (Roeselers et al., 2008).

The main limitation of the application of phototrophic biofilms in wastewater treatment is the requirement for space to accommodate such systems. Phototrophic biofilms should be exposed to optimal illumination. Thus, such systems will not be the best choice for the highly populated regions with high land prices. However, there are many regions on Earth where land is not expensive and wastewater is not treated at all. Another important parameter is the depth of biofilm accommodation, directly related to the light intensity received by the phototrophs. The deeper the biofilm is located, the more longwave light it receives. Different phototrophic organisms require light of different wavelengths. This fact should be taken into consideration when controlling the composition of phototrophic biofims for wastewater treatment (Roeselers et al., 2008).

Phototrophic biofilms can be efficient for removal of heavy metal ions from polluted waters, primarily because of the nature of their extracellular polymeric matrix. Considerable amounts of negatively charged polysaccharides are present as a component of the matrix. Thus, cations of such metals as zinc, copper, lead and some others can be accumulated at the surface of the matrix forming stable complexes (Pastorella et al., 2012). Moreover, some metals can precipitate from the polluted waters in such conditions due to the increased pH (Liehr et al., 1994). Methods of heavy metal removal using phototrophic biofilms attract attention of biotechnologists due to their low price combined with high efficiency, though one should remember that such method of bioremediation does not remove metals but just transfers them from the water into the biofilm matrix. Thus, metal cations should be subsequently removed from the biofilms themselves (Kratochvil and Volesky, 1998).

Multi-species biofilms in agriculture. Phototrophic biofilms are used in various fields of agriculture and land tenure. For example, the extracellular polymeric matrix of cyanobaterial and algal biofilms can greatly increase the ability to retain water in soils of the arid regions, thus preventing their erosion (Roeselers et al., 2008). Microbial biofilms are present on various parts of the plants, including cultivated ones, both above and under the ground. They can play different roles in the life of a plant. Many pathogens, dangerous for plants, animals, and humans, exist in the form of biofilms on plant leaves, stems, or in the rhizosphere. The mechanism of biofilm formation on root surfaces still remains unclear, though such biofilms are known to reach significant size, covering the surface from distant regions of the roots to the elongation zone. Plants are known to secrete considerable amounts of organic substances and 44% of them are secreted through roots, providing nutrients for microorganisms of the rhizosphere. The amount of secreted substances varies along the length of the root; varying pH near the root surface provides conditions for existence of different bacterial species in the biofilm (Rudrappa et al., 2008). Biofilms of the rhizosphere have a great potential to be applied as biocontrol agents due to the ability of microorganisms of such biofilms to synthesize antimicrobial and antifungal substances to protect from the pathogens (Molina et al., 2003; Rudrappa et al., 2008).

Multi-species biofilms in bioremediation. A sharp increase in the amount of industrial capacities observed in several recent decades worldwide, including in developing countries, creates significant pressure on the biosphere due the presence of great amounts of toxic pollutants in the effluents. Bioremediation is presently one of the perspective methods to solve the problem of the degradation of toxic substances. In this case, biofilms are more preferable than planktonic culture because, as it was discussed previously, such type of community is more resistant to changing environmental conditions. Microorganisms in biofilms, protected by the layer of extracellular matrix, are more adaptive and resistant to high concentrations of various xenobiotics, including chlorine- and nitrogen-containing aromatic compounds (Singh et al., 2006). In some cases the matrix itself can take part in bioremediation by absorbing and storing toxic substances from the water phase (Pastorella et al., 2012). Chlorine-containing aromatic herbicides are removed with the help of multi-species biofilms, despite their toxicity to the microbial cells (Breugelmans et al., 2008 Sandoval-Carrasco et al., 2013). Breugelmans et al. showed that multi-species biofilms could fully degrade the herbicide linuron, while monospecies and binary biofilms appeared to be less effective. In some cases the herbicide was accumulated inside the extracellular polymeric matrix to be used by the cells during starvation as a carbon source (Wolfaardt et al., 1995). A new approach was demonstrated for wastewater contaminated by hydrocarbons (including the polycyclic ones). The authors reported effective removal of these substances using multi-species biofilms containing, besides oil-degrading bacteria, oxygenic phototrophs which supplied the monooxygenases of oil-degraders with oxygen (Al-Bader et al., 2012; Al-Mailem et al., 2014).

Multi-species biofilms for removal of heavy metals. Another promising field for the application of multispecies biofilms is biodetoxication of heavy and radioactive metals. Certain progress has already been reached in this sphere. Microorganisms of various phyla are responsible for dissimilatory reduction of metals: *Proteobacteria, Actinobacteria, Deinococcus-Thermus*, and *Firmicutes* (Chae et al., 2009; Jackson et al., 2009; Kostka and Green, 2011). For example, multi-species biofilms were used to form a special biobarrier for effective (for about 90%) treatment of water containing high amounts of metal ions, mainly copper. Such a biobarrier consists of a polymeric basis with biofilms attached to it. The biofilms contain over 50 microbial species including *Pseudomonas* sp. and *Sphingomonas* sp., which are resistant to elevated concentrations of heavy metals. There are reports about removal of Cr(VI), which is highly harmful for human and animals, with the help of multi-species biofilms. While none of the microorganisms are known to grow directly on Cr(VI), several anaerobic microbes are able to reduce chrome to the form that can be metabolized. In biofilms discovered in soils near a steel-alloy factory in China, contaminated with wastes from the factory, the ability of chrome utilization was associated with the activity of bacteria *Pannonibacter phragmitetus* (Chai et al., 2009). According to the data obtained for laboratory-scale bioreactors, not only bacterial but mixed bacterial–fungal biofilms have a high potential (Herath et al., 2014). Possible participation of microbial biofilms in detoxification of radioactive metals, such as uranium, neptunium, and plutonium, has attracted the attention of the scientists for a long time. Unfortunately, there are no reports involving biofilms in bioremediation of the soils and waters contaminated by these metals, so the problem remains poorly studied. Revealing the possibilities of microbial biofilms for bioremediation of uranium is especially urgent as it is the most abundant of the actinides in the biosphere. The strategy is mainly based on reduction of U(VI) to U(IV), which is less soluble in water and, is therefore less dangerous. Microorganisms are supposed to reduce U(VI) to U(IV) which is then stored in the biofilm matrix. It is still unclear whether microbial reduction of uranium has sufficient potential for complete removal of such concentrations of uranium as those found in real contaminated systems. Interactions between reduced uranium and other substances which can be present in the system, particularly Fe(III) oxides, are still unknown either (Pastorella et al., 2012; Williams et al., 2013).

Microbial biofilms for protection against corrosion. Many microorganisms are known to take part in processes of corrosion (destruction) of various substances: metals and alloys, concrete and stone buildings. Sulfate-reducing, iron-reducing, and iron-oxidizing bacteria are among them, as well as many of nitrate-reducers and phototrophs (Videla and Herrera, 2009; Kip and van Veen, 2015). However, there are microbial species that can inhibit corrosion, slowing down ion exchange between the surface of a metal and the environment. Microbes stimulate corrosion by forming an additional galvanic pair between the bacteria themselves (biofilm) and the metal surface. In anoxic conditions such biofilm act as a cathode, the metal acts as an anode, and electrons move from the metal to the cells. Positive charge of the metal is increased and retained as long as bacterial activity is preserved. On the contrary, in microbial inhibition of corrosion, bacteria act as a anode and the metal as an cathode. Oxygen consumption by bacteria decreases the rate of electron detachment from the metal surface. Thus, the process resembles the principle of nonbiological cathodic protection of metals which is commonly used for protection against corrosion (Potekhina et al., 1999; Videla and Herrera, 2009).

Microbial inhibition of corrosion is also connected with neutralization of the substances that stimulate corrosion or with synthesis of such substances as exopolysaccharides of the biofilm matrix that form a protective layer on the surface of the metal (Videla and Herrera, 2005; Finkenstadt et al., 2011). These substances bind with positively charged metal ions, forming a complex compound with the metal (Zarasvand and Rai, 2014). Moreover, bacteria *Geobacter sulfurreducens* were shown to synthesize a protective film of iron(II) phosphate on the surface of carbon steel (Cote et al., 2015).

Another mechanism involves synthesis of antibiotics that inhibit activity of the microbes responsible for stimulation of corrosion (Jayaraman et al., 1999). It is believed that microbial inhibition of corrosion is associated with several types of microbial metabolism and various microbial species and, thus, with multi-species biofilms. Model experimental biofilms are expected to be less effective than the multi-species ones (Jayaraman et al., 1999; Videla and Herrera, 2005). It is important to note that in some situations the inhibitory effect of microbial biofilms can change to the opposite one, promoting corrosion due to the activity of corrosion-causing microorganisms that coexist in one biofilm with corrosion-inhibiting species (Videla and Herrera, 2009). Adhesion of the biofilm itself is also important: the more tightly the biofilms binds to the surface, the more effective it is against corrosion (Zarasvand and Rai, 2014).

Application of microbial biofilms for protection of metals and alloys from corrosion seems very promising and was proved in multiple laboratory experiments. Biofilms could provide a perfect alternative to traditional methods of fighting against corrosion: using more and more complicated alloys resistant to corrosion, mechanical removal of the biofilms of corrosion-causing microorganisms, and using special anticorrosive coatings. Such coatings may contain components which have a negative effect on the environment and are often toxic for all living things (Zarasvand and Rai, 2014). However it might take much time before the biological methods of protection from corrosion would be widely used. There is only one example of biofilms containing five species of bacilli that synthesize polymyxin and gramicidin to protect the objects of a nuclear station from corrosion. In this case, however, microbial contribution to anti-corrosion protection was minimal (Arps et al., 2003). There are also several examples of successful usage of biofilms to protect stone buildings due to the so-called carbonatogenesis: precipitation of carbonates in the form of a protective layer on the surface of the buildings as a result of microbial or algal activity (Le Metayer-Levrel et al., 1999; De Myunch et al., 2008).

We can conclude that investigation of the structure and composition of multi-species biofilms and, especially, of the mechanisms of interactions between microorganisms that are involved in them, is an urgent field of microbial community research in ecology, medical science, and biotechnology. We believe that due to the rapid progress of instrumental methods, these mechanisms will be decrypted and the methods of controlling natural microbial populations will be developed.

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