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EXPERIMENTAL ARTICLES

Detection of Anaerobic Processes and Microorganisms in Immobilized Activated Sludge of a Wastewater Treatment Plant with Intense Aeration

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Abstract—Attached activated sludge from the Krasnaya Polyana (Sochi) wastewater treatment plant was studied after the reconstruction by increased aeration and water recycle, as well as by the installation of a bristle carrier for activated sludge immobilization. The activated sludge biofilms developing under conditions of intense aeration were shown to contain both aerobic and anaerobic microorganisms. Activity of a strictly anaerobic methanogenic community was revealed, which degraded organic compounds to methane, further oxidized by aerobic methanotrophs. Volatile fatty acids, the intermediates of anaerobic degradation of complex organic compounds, were used by both aerobic and anaerobic microorganisms. Anaerobic oxidation of ammonium with nitrite (anammox) and the presence of obligate anammox bacteria were revealed in attached activated sludge biofilms. Simultaneous aerobic and anaerobic degradation of organic contaminants by attached activated sludge provides for high rates of water treatment, stability of the activated sludge under variable environmental conditions, and decreased excess sludge formation.

Keywords: aerobic and anaerobic conditions and microorganisms, anaerobic ammonium oxidation by nitrite, anammox process, anammox planctomycetes, biofilm, microbial immobilization, methanogenesis, methane production and oxidation

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A number of basic process technologies are currently applied worldwide to solve the problem of integrated wastewater treatment. These include recycling of the treated water for a more complete nitrogen removal [1], as well as immobilization of the microorganisms of activated sludge on various carriers [2–4]. Immobilization of activated sludge on a solid carrier allows for a considerable increase in the density and biodiversity of microorganisms in treatment lines, which increases the rate and depth of water treatment [4]. Moreover, spatial self-organization of microbial communities occurs in epibioses formed on solid carriers (biofilms); microbial succession develops according to the redox gradient and substrate concentration [5, 6]. Due to the limited availability of oxygen even under conditions of intense aeration, anaerobic microzones, where anaerobic microbial processes may occur, are formed in the inner layers of biofilms [6–8].

Anaerobic microbial decomposition of wastewater organic contaminants leads to formation of volatile fatty acids and alcohols and, upon complete degradation, of methane, the most reduced of the natural carbon compounds. Methane is generated by highly specialized prokaryotes, methanogenic archaea [9, 10]. Methanogenesis is possible only at extremely low redox potential of the medium, from –200 [11] to –300 mV and lower. All methanogens are obligate anaerobes—growth of some methanogens in pure culture is suppressed by as little as 0.004% oxygen in the gas phase [12]. However, the development of methanogenic archaea in a mixed microbial community is possible even in the presence of oxygen in the environment. For example, a methanogenic culture *Methanothrix* (*Methanosaeta*) *soehngenii* [13] has been isolated from flocks of aerobic sludge; acetoclastic and hydrogenotrophic methanogens were revealed in biofilms of immobilized active aerobic sludge [14]. These results demonstrate that, in the biofilms, oxygen is actively consumed by aerobic bacteria, so that anaerobic microzones and conditions for development of obligate anaerobic microorganisms are developed. Detection of methanogens in the biofilms developing under anaerobic conditions indicates the possibility that anaerobic nitrogen-producing microorganisms, denitrifiers and anammox bacteria, develop in the biofilms as well. Recently, we revealed anammox bacteria and studied the process of anaerobic ammonium oxidation by nitrite (anammox) in biofilms of immobilized acti-

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vated sludge of microaerophilic denitrifiers from aerobic wastewater treatment plants [15]. Studies on the anaerobic processes of dinitrogen and methane formation in activated sludge biofilms, as well as of the subsequent oxidation of methane, are of considerable interest for biotechnology of wastewater treatment. In systems of aerobic treatment with immobilized microflora, the contribution of anaerobic processes to organic matter degradation may be substantial. The results of such studies are of importance for design of the new and reconstruction of the existing wastewater treatment plants in order to decrease excess sludge formation.

Integrated wastewater treatment plant (IWTP) functioning in the Krasnaya Polyana settlement (Sochi region) was reconstructed in order to increase the efficiency of municipal wastewater treatment. The modernized station employs the technology encompassing (1) installation of a solid bristle carrier with flexible polymer fibers of various diameters for immobilization of activated microbial sludge [16] and (2) 100% recycling of treated water from the exit line of the aeration tank to the beginning of the process. After treatment in the nitrifying aeration tank, the water containing nitrite and nitrate is mixed with the water arriving for treatment and containing organic contaminants. This way denitrification performed by denitrifying bacteria developing in biofilms of activated sludge is favored. In comparison with the traditional aerobic technology, high treatment efficiency, including nitrogen removal (table), was accompanied by a 2–3-fold decrease in excess sludge formation in the process of exploitation of the reconstructed IWTP in Krasnaya Polyana. These results suggested the assumption that anaerobic microorganisms, though forming considerably less biomass than the aerobic ones, are abundant in the activated sludge immobilized on a bristle carrier and play an important role in nitrogen removal by denitrifiers and anammox bacteria in the form of N_2.

The goal of the present work was to study the anaerobic processes in immobilized activated sludge formed at the Krasnaya Polyana station under conditions of intense aeration, the active anaerobes present therein, including methanogenic archaea and anammox bacteria, as well as aerobic methanotrophic bacteria.

MATERIALS AND METHODS

Subject of the study. The work was performed on the samples of attached and dispersed activated sludge from the reconstructed wastewater treatment station (Recreation Center of the Ministry of Defense of the Russian Federation, Krasnaya Polyana, Sochi). During reconstruction, cassettes with bristle carriers for microbial immobilization were placed into the aeration tanks and recycling of nitrite- and nitrate-containing water after treatment in the aeration tank to the beginning of the process, where it was mixed with arriving ammonium-containing wastewater, was increased to 100%. The rate of aeration in both compartments of the aeration tank was 3 m^3 air/m^3 bioreactor per hour; water temperature during sample collection was 20–22°C.

Characteristics of wastewater arriving for treatment to the Krasnaya Polyana IWTP and released into the Mzymta River

Indicators of domestic wastewater quality	Entrance analysis results, mg/dm^3	Exit analysis results, mg/dm^3
Suspended matter	138.6	2.4
Ultimate BOD*	149.5	2.8
Ammonium nitrogen	18.3	0.33
Nitrite nitrogen	0.1	0.005
Nitrate nitrogen	0.0	4.8
Phosphates (by P)	2.9	0.14
Petrochemicals	0.32	0.013

* BOD, biological oxygen demand.

Collected samples of the brushes with sludge biofilms and of dispersed sludge were immediately put into 0.5–1.0-L vessels, filled to capacity with the treated water, hermetically sealed, and transported within a day in a thermostatic bag at the temperature of 10–15°C. In the laboratory, samples were stored at 4°C prior to experiments. Biofilms of attached sludge were obtained by shaking and peeling off the brush fibers. Dispersed sludge comprised floccules of various size and contained patches of biofilms washed off the brushes. Experiments were performed with suspensions prepared from the settled (thickened) sediment of biofilms or floccules of dispersed sludge in the treated water or in relevant mineral media. Absolute dry mass (ADM) of microbial biomass was 10–20 g per liter of suspension. Precise values for each experiment are reported in the figure captions.

Culture media used. For experiments under anaerobic conditions, the modified Pfennig medium for methanogens supplemented with vitamins and microelements was prepared according to the standard technique [17]. Anaerobic degradation of volatile fatty acids (VFA) was studied if Pfennig medium supplemented with sodium acetate, propionate, or *n*-butyrate (5 mM). To compare the numbers of anaerobic archaea and bacteria, glucose and peptone were added to Pfennig medium at 2.5 g/L each.

The medium to determine the numbers of aerobic bacteria contained the following (g/L): KH_2PO_4, 0.7; $Na_2HPO_4 \cdot 12H_2O$, 1.5; KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; glucose, 2.5; peptone, 2.5; and microelements, 1.0 mL/L (the same solution as in the medium for methanogens).

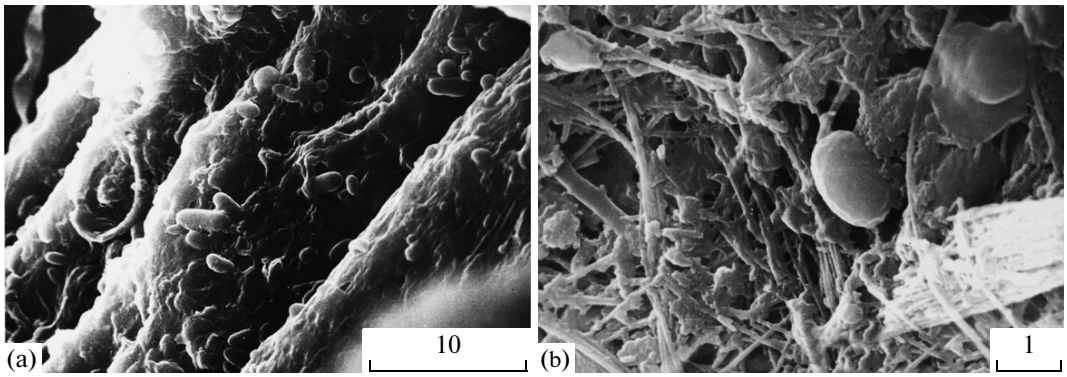

Fig. 1. Microbial fouling on the fibers of the bristle installation. Scanning electron microscopy. Appearance of the fouling on the surface of brush (a) and the inner layer of the biofilm (b).

Composition of the mineral medium for anammox bacteria (pH 7.6–7.8) was reported in our previous publication [15].

Methane oxidation was studied in sludge suspension in the treated water; 10% methane was added to the gas phase (air) as substrate.

Experiment setup. Aerobic and anaerobic degradation of organic matter of the activated sludge, VFA, as well as methane-oxidizing activity of the sludge microbial population, were studied in 120-mL glass vials sealed with rubber plugs and metal screw caps perforated for gas sample collection. Figure 2 presents the results of the experiment where the volumes of liquid and gas phases were 60 mL each; in the rest of the experiments, the liquid phase volume was 20 mL and that of the gas phase, 100 mL. Anaerobic conditions were created by gassing the flasks with argon. Depending on the experimental conditions, the vials were supplemented with exogenous substrates, either VFA or methane. Analysis of the gases generated and consumed was performed regularly, 2–3 times per week. Liquid samples for pH and VFA analyses were collected when needed. The experiments were performed in triplicates.

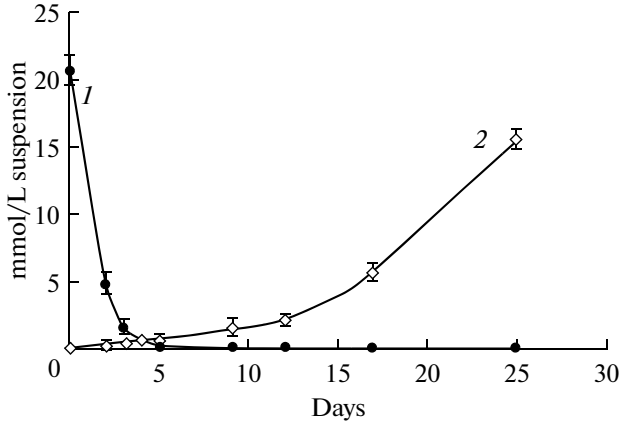

Fig. 2. O_2 consumption (*1*) and formation of methane (*2*) at 20°C by attached activated sludge upon degradation of organic contaminants. Absolute dry mass of biomass is 20 g/L suspension.

Relative numbers of aerobic and anaerobic microorganisms in the biofilms on brushes were determined using the most probable number method (MPN). Suspensions of the biofilms from fresh samples were concentrated by centrifugation at 10000 *g* during 10 min. The supernatant was removed, 10 mL sterile water was added to the pellet (approximately 10 g wet biomass), and the mixture was homogenized with sterile glass beads in a VP mixer (Russia) for 30 min in argon atmosphere. The suspension was then diluted tenfold with relevant media for anaerobes or aerobes. Thirteen 10-fold dilutions were performed in five repeats. Diluted suspensions of anaerobes were incubated at 25°C in hermetically sealed flasks under argon atmosphere. Suspensions of aerobes were incubated under cotton plugs.

Stationary cultivation and accumulation of the anammox bacteria was performed in a 1-L glass reactor with periodic medium exchange as reported previously [15]. Upon substrate exhaustion, but at least once a week, the medium was refreshed and nitrite and ammonium salts were added at relevant concentrations. Regularly (2–3 times per week), pH, gas phase composition, and nitrite and ammonium concentrations were determined. Each 3–4 weeks, chemical oxygen demand (COD) and VFA content were determined. The cultures were grown at 25°C.

The amounts of consumed and produced gases were expressed as molar amounts of gas consumed or generated by one liter of microbial suspension in treated water or in the medium (mmol gas/L microbial suspension).

Chemical analysis. Oxygen, nitrogen, methane, and hydrogen content were determined using gas–liquid chromatographs Kristall 5000.1 (ZAO KHROMATEK, Yoshkar-Ola, Russia) and CHROM-5 (CSSR) as described previously [15]. Chromatographic analysis

of VFA was carried out using Staier HPLC (Russia) equipment [15]. Chemical oxygen demand was determined by the bichromate method [18]; pH and absolute dry mass (ADM) of the sludge were determined as described [15].

Microscopy. Morphological features of the biofilms on brushes were studied by scanning electron microscopy. The material under study was fixed in 5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0) at 4°C for 16–18 h and washed with phosphate buffer. The samples were dehydrated in solutions of increasing ethanol concentrations (50, 60, 70, and 96%) and in absolute acetone and quickly dried at 50°C. The samples were coated with gold in an ion spray Spitter IFC-1100 equipment and viewed in a JSM-T300 microscope (Jeol, Japan).

To study morphology of the microbial cells and detect methanogenic archaea, Lyumam I-2 (LOMO, Russia) light microscope was used in phase contrast and fluorescence modes (light filters ZhS-19 and ZhS-18, lens Kh90 L).

FISH detection of anammox bacteria. Hybridization of the samples with the probes was performed according to the technique described in [19] at 46°C under hybridization conditions for various probes [20, 21]. Identification of planctomycetes cells was performed with the PLA46 probe for the whole group of *Planctomycetes* [22] and the Amx368 probe for anammox planctomycetes [23]. The technique was described in detail previously [15].

RESULTS AND DISCUSSION

Detection of anaerobic microorganisms in brush fiber epibiosis. The flexible polymer support in the shape of brushes with fibers of varying thickness and length, which was used to immobilize microorganisms, was the model of aquatic vegetation closest to the natural conditions. The numeric ratio of aerobic and anaerobic microorganisms developing in the biofilms depended on the concentration of oxygen available.

Various microorganisms covered with a layer of mucus were present in microbial biofilms. An abundance of filamentous forms and/or bundles of mucus in biofilm mass was noticeable (Fig. 1). In samples of sludge from the brushes, aerobic and anaerobic microorganisms were detected by the most probable number method. In the suspension of the attached activated sludge biofilms, aerobic microorganism titer was 10^{11}. The titer of anaerobic hydrolytic and acid-producing bacteria, activity of which was assayed by generation of hydrogen, CO_2, and VFA, was 10^{10}–10^{11}. Methanogenic archaea were detected at the 10^4–10^5 titer. These data suggested that the content of anaerobic microorganisms consuming glucose and peptone in the activated sludge biofilms was at least 10% of the aerobic microorganisms utilizing the same substrate.

Microscopic analysis of microbial biomass grown in vials revealed high morphological diversity of microorganisms. In the flasks where methane was detected, distinct glowing aggregates morphologically similar to *Methanosarcina*, as well as with large threads, probably *Methanosaeta*, were observed under fluorescence microscopy. These results evidenced that anaerobic microorganisms were relatively numerous in the biofilms under study and that immobilized sludge contained both aerobic and anaerobic microenvironments. Anaerobic microorganisms are known to form far less microbial biomass than aerobic ones. This may explain a considerable decrease in production of excess sludge at the Krasnaya Polyana aerobic wastewater treatment plant after reconstruction, when the bristle carrier was installed for immobilization of microorganisms, compared to the previously used traditional technology of suspended activated sludge, and is in agreement with the data by Di Iakoni et al. [24].

To study the activity of anaerobic microorganisms in biofilms of sludge immobilized on the brushes, a number of experiments on anaerobic degradation of organic matter resulting in methane formation as the final product were conducted. Generation of molecular nitrogen as a result of denitrification and anammox processes by immobilized sludge was also explored.

Degradation of organic matter with methane generation. The microbial population of the sludge immobilized on the brush carrier was active under both aerobic and anaerobic conditions. Figure 2 presents the data obtained in the experiment on oxygen consumption and methane generation from organic matter present in the treated water and microbial sludge at 20°C by the biofilms of attached sludge. At the onset of the experiment, the gas phase consisted of air. As aerobic microorganisms consumed oxygen, probably for oxidation of organic substrates, methane formation started even before oxygen was exhausted (Fig. 2). This evidenced the presence of an active methanogenic microbial community in the microbial population of the activated sludge. In the experiment, methanogenesis occurred via anaerobic degradation of organic substances contained in wastewater and of inactive biomass formed as a result of lysis. Methane content in the generated biogas reached 65–70%. This indicated the primarily acetoclastic route of methane generation characteristic of a mesophilic, methanogenic community functioning at lowered temperature [9, 25]. In our experiments under anaerobic conditions, noticeable methane generation in the biofilm suspension in the treated water was observed at 9°C (data not shown). After 10 days of the lag phase, the amount of methane generated during the following 20 days was 1.5 mmol/L suspension. In the same suspensions at 20°C, over 15 mmol CH_4/L suspension was generated during 30 days. These results demonstrated the ability of the anaerobic constituent of the

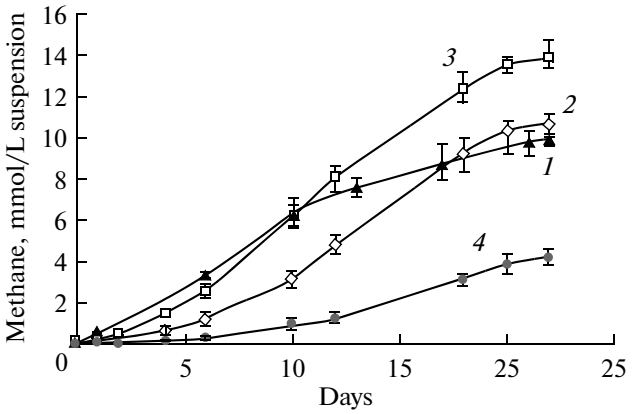

Fig. 3. Methane formation by the microbial community of attached sludge upon addition of VFA: acetate (*1*), propionate (*2*), butyrate (*3*), and the control (*4*). Absolute dry mass of biomass is 10 g/L suspension.

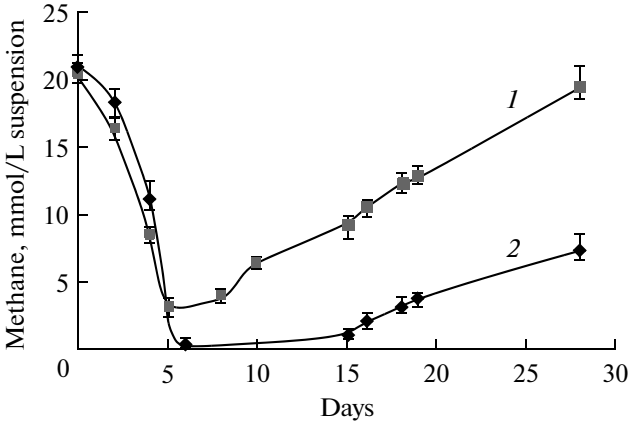

Fig. 4. Dynamics of methane consumption and generation by the microbial community of attached (*1*) and dispersed (*2*) sludge. Absolute dry mass of biomass is 15 g/L suspension.

attached activated sludge to function at lowered temperature during cold seasons.

In the course of anaerobic degradation of organic substances, the major intermediate products are volatile fatty acids (VFA), mainly acetic acid (acetate), a direct precursor of methane, and propionic (propionate) and butyric (butyrate) acids [9, 26]. Generation of methane from VFA by microbial populations of biofilms from the brushes and by dispersed floccules were investigated. Figure 3 presents the data obtained in the experiment on increased methane generation by microbial population of attached sludge with acetate, propionate, or butyrate as substrates. Addition of acetate resulted in methanogenesis without a lag phase. The added acetate was consumed within 10 days, with 5 mmol methane formed out of 5 mmol acetate, which corresponds to the stoichiometry of acetoclastic methanogenesis (Fig. 3). By this time, acetate was completely consumed. As follows from Figure 3, further methane formation occurred slowly, at the rate close to that in the control, probably due to utilization of organic matter introduced together with the inoculum and resulting from the lysis of inactive microbial biomass. The same figure presents the dynamics of methane formation from propionate and butyrate. Formation of methane started after a short (2–3 days) lag phase. Determination of VFA in the end of the experiment showed complete consumption of the added propionate and butyrate. Taking into account that 1 mmol acetate, propionate, and butyrate theoretically produces 1, 1.75, and 2.5 mmol methane, respectively [27], the amount of methane produced correlated well with the amount of VFA consumed. Thus, upon deduction of the control values, 9 and 12.5 mmol methane were formed after 20 days from 5 mmol propionate and butyrate (Fig. 3).

Thus, our data indicate the activity of such key groups of anaerobic microorganisms as syntrophic bacteria and methanogenic archaea, which are capable of degradation of acidic fermentation products, important precursors of methane, in sludge immobilized on the bristle carrier.

Under aerobic conditions of cultivation in the vials covered with cotton plugs, all VFA were decomposed completely (oxidized) within 3–5 days of the experiment. These results evidence that VFA formed in the anaerobic zone of the biofilms may be decomposed under anaerobic conditions with formation of methane or be consumed (oxidized) by aerobic bacteria in the presence of oxygen.

In the above experiments, activated sludge from the aerobic wastewater treatment plant—both immobilized on brushes and dispersed in the medium—was found to be capable of organic substance decomposition down to methane under anaerobic conditions. Methane is an aggressive greenhouse gas; therefore, it was important to reveal the ability of the microbial communities of treatment stations to utilize the generated methane. Figure 4 demonstrates oxidation and generation of methane by fresh samples of dispersed or attached activated sludge from the Krasnaya Polyana IWTP. The rate of methane oxidation was practically the same in the samples of attached and dispersed sludge (3.6 mmol CH_4/L suspension per day), and the methane added was completely oxidized within 5 days. After oxygen was exhausted, the rate of methane generation was higher in the samples of immobilized sludge, reaching 0.76 mmol CH_4/L suspension per day (Fig. 4). On the whole, aerobic oxidation of methane occurred at a higher rate than methanogenesis, therefore most probably no emission of methane from treatment plants occurred. In the anaerobic zones of the activated sludge biofilms, anaerobic methane oxidation may also be responsible for the oxidation of a fraction of methane produced [28].

These results indicate the ability of the microbial population of attached sludge to form and oxidize

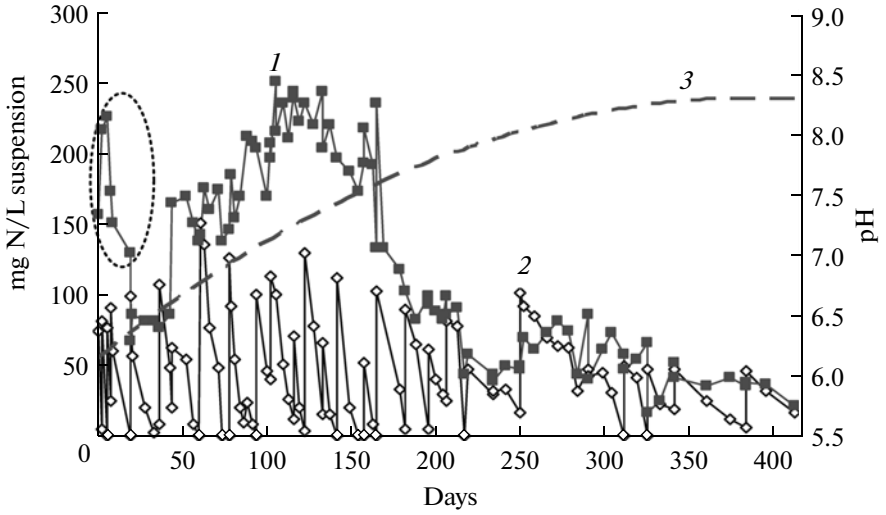

Fig. 5. The concentrations of ammonium (*1*) and nitrite (*2*) nitrogen and medium pH (*3*) in the stationary enrichment reactor with activated sludge immobilized on the bristles from the wastewater treatment plant. The ellipse highlights the initial ammonium nitrogen consumption indicating the presence of the anammox process activity.

methane. Methane may form in the anaerobic zone of the biofilm and is oxidized both in its aerobic layer and by the free-floating sludge. Therefore, the methane cycle is realized upon aerobic water treatment in an attached activated sludge system.

Anaerobic oxidation of ammonium by nitrite in immobilized activated sludge. The presence of a methanogenic microbial community comprising various groups of anaerobic microorganisms required for decomposition of complex organic substrates in the activated sludge biofilms proves the existence of anaerobic conditions for development of anaerobic microorganisms, including bacteria performing the process of anaerobic oxidation of ammonium by nitrite (anammox). Earlier, activity of the obligate anaerobic anammox bacteria developing in the activated sludge immobilized on a brush carrier has been demonstrated under microaerobic conditions in a denitrifier of wastewater treatment plant with immobilized activated sludge [15]. Efficient removal of nitrogen at the Krasnaya Polyana IWTP (table) suggested the possible development of the anammox bacteria and occurrence of the anammox process in the activated sludge biofilms. To prove this conclusion, brush-fouling samples were cultured in a medium for anammox bacteria. The results of ammonium and nitrite consumption upon cultivation of activated sludge attached to brush fibers in the selective medium for anammox bacteria during 15 months are presented in Fig. 5. During the first 1.5 months of the experiment, nitrite and ammonium consumption was observed, which indicated the possibility of the process of anaerobic oxidation of ammonium (Fig. 5). During the subsequent five months, nitrite but not ammonium was consumed. During this period, the content of dissolved organic matter increased, the COD value increased to 400 mg/L due to decomposition of inactive biomass, and heterotrophic denitrification occurred, which was confirmed by active production of molecular nitrogen. After six months of cultivation, COD value decreased to trace amounts and weak ammonium consumption was observed, which lasted throughout the following nine months of the experiment. It was accompanied by alkalization of the medium to pH 8.3, which indicated the anammox process. Hybridization of microbial biomass from the accumulation reactor with Cy3-labeled oligonucleotide probes PLA46 and Amx368 confirmed the presence of anammox bacteria in the biofilms of the activated sludge (Fig. 6). If compared with the results of a similar study of anammox process in the sample of denitrifiers from the ECOS stations [15], accumulation of anammox bacteria in the activated sludge from the Krasnaya Polyana IWTP occurred considerably slower. However, this was the first time the presence of anammox bacteria in the biofilms initially developed under conditions of extremely intense aeration has been confirmed.

Apparently, under conditions of intense aeration at the Krasnaya Polyana wastewater treatment plant and rather high concentration of organic matter in the treated water, nitrogen is mainly removed via the processes of nitrification and subsequent heterotrophic denitrification. However, in anaerobic microzones of the activated sludge attached to the brushes, autotrophic anammox bacteria develop as well.

Our results confirmed high activity of microbial sludge immobilized on a flexible solid bristle carrier. The presence of aerobic and anaerobic microorganisms, including methanogenic archaea, and functioning of a methanogenic microbial community performing complete anaerobic degradation of organic compounds to methane and CO_2 under anaerobic

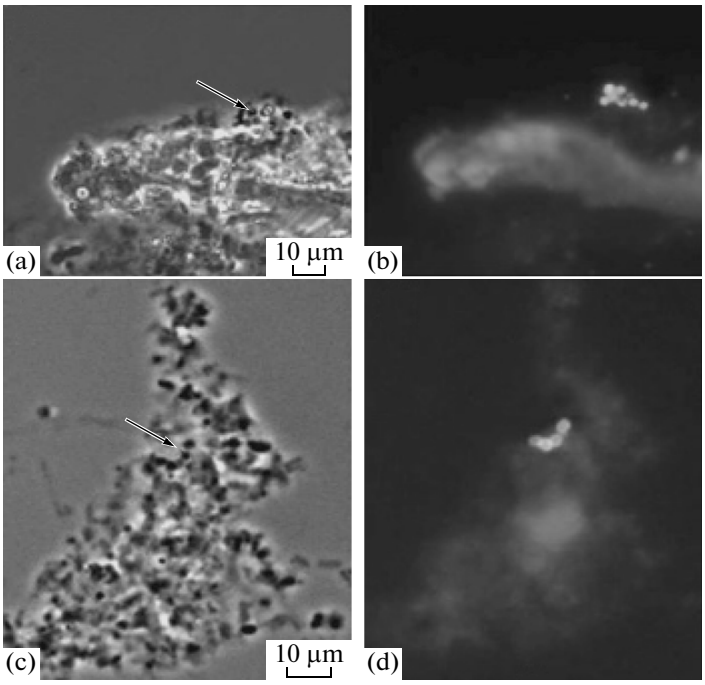

Fig. 6. In situ hybridization of activated sludge sample with anammox-specific Amx368 probe: (a, b) phase contrast; (c, d) microphotographs of hybridization with probe.

conditions was demonstrated. Microbial oxidation of methane generated and realization of the methane cycle upon wastewater treatment was demonstrated. Methane generation occurred primarily in attached sludge, while its oxidation occurred mostly in the free-floating sludge. Depending on oxygen availability, the VFA formed as intermediates may be degraded by either anaerobic or aerobic organisms. Simultaneous operation of the aerobic and anaerobic systems for the degradation of organic contaminants in the attached activated sludge resulted in high rates of water treatment, as well as in low production of excess sludge. For the first time, the process of anaerobic oxidation of ammonium and the presence of anammox bacteria oxidizing ammonium by nitrite, with formation of molecular nitrogen, was revealed in the biofilms of attached activated sludge developed under conditions of intense aeration.

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