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EXPERIMENTAL ARTICLES

Anaerobic Ammonium Oxidation (Anammox) in Immobilized Activated Sludge Biofilms during the Treatment of Weak Wastewater

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Abstract—This work studied the formation of molecular nitrogen by the microbial population of immobilized activated sludge of the domestic wastewater treatment plants (WWTP) that employ the technology developed by ZAO ECOS Company. The technology includes physicochemical water pretreatment and treated water recycling. A hard flexible fibrous brush carrier is used for the immobilization of microorganisms. The presence of both aerobic and anaerobic microorganisms and functioning of the methanogenic microbial community was shown in the biofilms developing on the carrier fibers and in suspended sludge. The high efficiency of nitrogen removal at a low C/N ratio was established to be due to the conjugated nitrification, denitrification, and anammox processes, whose functioning was demonstrated by laboratory cultivation methods and by studying the processes in batch and continuous reactors. Fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes (FISH) revealed bacteria belonging to the order *Planctomycetales,* particularly their anammox group. This work is the first evidence of the important role of the anammox process in the combined system of physicochemical and biological treatment of weak wastewater (BCDEAMOX).

Keywords: anaerobic ammonium oxidation by nitrite, anammox, anammox bacteria, aerobic treatment, aerobic, microaerophilic, and anaerobic conditions, biofilms, denitrification, nitrification, methanogenesis.

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In the modern world, the amount of wastewater formed as a result of the domestic and economic activity of people is constantly increasing, which requires lowering the cost and increasing effectiveness of the methods for its purification. Aerobic and/or anaerobic degradation and mineralization of organic matter by microorganisms are the basis of biological treatment of wastewater, which is the most economical and environment-friendly process. The development and perfection of modern methods for biological treatment of wastewater, especially methods for removal of nitrogenous contaminants is an essential task because their presence in reservoirs, subsoil and ground waters catastrophically deteriorates the quality of fresh water necessary to provide the population with. In the new, widely used technology of treatment of domestic wastewater, the anaerobic unit for nitrogen removal (denitrifier) is installed at the beginning of the process, and the water enriched with nitrate and nitrite in the aeration tanks is recycled (the Cape Town scheme). This allows a considerable amount of nitrogenous contaminants to be removed at the beginning of the process and the treatment time and energy expenditure to be reduced [1].

At present, the process of anaerobic ammonium oxidation by nitrite with the formation of molecular nitrogen, whose feasibility was shown by thermodynamic calculations a bit more than 30 years ago, attracts close attention [2]. This theoretically predicted process was experimentally proved only in the 1990s and referred to as the anammox process (ANAMMOX, ANaerobic AMMonium OXidation) [3]. The discovery of the anammox process led to reconsideration of the biological nitrogen cycle in the biosphere [4]. The application of the anammox process for the treatment of high-strength ammonium wastewater is promising [5]. The anammox process is carried out by new chemoautotrophic anammox bacteria using the reaction of ammonium oxidation by nitrite for obtaining energy and utilizing carbon dioxide and/or bicarbonate as the source of carbon. The anammox bacteria described to date are attributed to five different genera of the group of anammox bacteria, belonging to the order *Planctomycetales*, phylum *Planctomycetes*, domain *Bacteria*. By now, seven species of anammox bacteria have been described (in the *Candidatus* status). Most of the anammox bacteria iso-

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lated from wastewater develop in the 6.7–9.0 pH range, with an optimum pH of 8.0–8.3 and an optimum temperature of 35–40°C. They are the slowest-growing bacteria ever known. According to the data published in 2002–2003, their generation time constitutes no less than 11 days [6], and, in practice, two to three weeks [5, 7]. However, it has been shown lately that, depending on the cultivation conditions and the method of measuring the growth rate, the generation time of anammox bacteria may be lower, from 5.5–7.5 [8] to 1.8 days [9]. The slow growth of anammox bacteria substantially limits the practical application of the anammox process. Taking into account the period of search for the optimum conditions for the functioning of the conjugate aerobic nitrifying reactor (SHARON system), it took about four years to start up the world's first full-scale anammox reactor [10]. By now, technological anammox-based schemes of treatment of high-nitrogen wastewater have been developed, and a pilot and several full-scale treatment plants have been put into operation [11, 12].

ZAO ECOS Company has designed and built six domestic wastewater treatment plants in the valley of the Mzymta River, Sochi region, Russia, in the settlements of builders of the 2014 Olympiad facilities. The quality of wastewater purified at the WWTP complies with the norms of discharge into fish-breeding reservoirs, which testifies to the high efficiency of nitrogen removal. The excessive sludge production constitutes no more than 25% compared to the conventional technology of aerobic treatment. The developed technology differs in a number of features conducive to the conditions for the development of anaerobic microorganisms, including anammox bacteria. After mechanical removal of coarse suspended matter, the wastewater arriving to be purified undergoes physicochemical treatment with a polyacrylamide-based coagulant for the removal of fine suspended matter, with which about half of the organic carbon is eliminated. The concentration of ammonium in wastewater does not decrease. The wastewater is then subjected to biological treatment, which includes two steps: in the denitrifier with weak aeration, and in the aeration tank with intense aeration. In the process, the water enriched with nitrite and nitrate is recycled from the aeration tank to the denitrifier(s), where it mixes with the ammonium-containing water arriving to be treated. At all of the steps of biological treatment and post-purification, rigid flexible brushes for immobilization of activated sludge are used [13]. Biofilms, in which a redox potential gradient and a gradient of substrate and metabolite concentration are formed, develop on the brush fibers. Trophic and regulatory relations, up to symbiosis, are established between the microbial components in the biofilms [14, 15]. The low organic carbon to nitrogen ratio in the water arriving to be purified and the high degree of nitrogen removal from wastewater in the facilities designed by ZAO ECOS Company allowed us to assume involvement of the anammox process in nitrogen removal.

The aim of this work was to obtain evidence of the occurrence of the process of anaerobic ammonium oxidation by nitrite, and of the development of anammox bacteria in biofilms on the hard carrier and in suspended sludge and to make a rough estimate of the contribution of this process to nitrogen removal during the treatment of weak wastewater according to the ZAO ECOS Company technology.

MATERIALS AND METHODS

Objects studied. The work was performed with samples of immobilized and free-floating activated sludge from two wastewater treatment plants constructed in the settlements for builders of the Olympic facilities along the river Mzymta. The material was sampled from WWTP no. 3 with one denitrifier and from WWTP no. 6 with two sequential step 1 and step 2 denitrifiers. Five samples of overgrown brushes and two suspended sludge samples were investigated. The material was sampled four times at an interval of six months. The samples were immediately put in 1- to 3-L vessels, the treated water was poured over, and the vessels were hermetically closed and transported within 24 h in a thermostatic bag at 10–15°C to the laboratory, where the samples were kept at 4°C for no more than four days before staging the experiments.

The media used. Modified Pfennig medium for methanogens with the addition of vitamins and trace elements was prepared according to the standard method [16].

The medium for nitrifying bacteria was prepared according to [17].

The medium for anammox bacteria contained the following (mg/L): $NaHCO_3$, 1; $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2 \cdot 7H_2O$, 0.18; KH_2PO_4, 0.027; NH_4Cl, from 191 to 391.5; $NaNO_2$, from 246 to 665; 1 mL/L of a trace element solution (mg/L): EDTA, 5; H_3BO_3, 0.014; $CoCl_2 \cdot 4H_2O$, 2; $ZnCl_2$, 0.203; $MnCl_2\ 4H_2O$, 0.99; $CuSO_4 \cdot 5H_2O$, 0.25; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.24; $NiCl_2\ 6H_2O$, 019; Na_2SeO_3, 0.105. The pH of the medium was 7.6–7.8. The freshly prepared medium was flushed with argon. In long-term experiments with batch cultivation, the substrates for anammox bacteria, $NaNO_2$ and NH_4Cl, were periodically added to the reactors to the necessary concentration without any other changes in the medium.

The medium for denitrifying bacteria was similar in composition to the medium for anammox bacteria, but without the addition of NH_4Cl. The organic substances released into the medium as a result of the heterotrophic biomass dying off served as electron donors and a source of carbon.

The mineral medium for determining the number of aerobic bacteria contained (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; trace elements, 1.0 mL/L (their composition was the same as for the medium for methanogens).

Short-term experiments. The experiments were staged in 120-mL glass flasks, which were closed with rubber stoppers and metal caps with a hole for gas sampling. Gas analysis was carried out regularly, once daily; the liquids for analyzing pH, VFA, ammonium, nitrite, and nitrate ions were sampled when required. All the experiments were in three replicates.

Anaerobic degradation of organic compounds with methane formation in the activated sludge samples was studied at 20°C. The volume of the liquid phase (the sludge suspension diluted 1 : 1 with medium for methanogens) was 20 mL; argon was used as the gas phase.

Heterotrophic denitrification combined with the anammox process in fresh samples was investigated at 30°C. The volume of the liquid phase (the brush mass cut with scissors with adherent sludge diluted at a 1 : 3 ratio with medium for denitrifiers) was 100 mL; argon was the gas phase. The gas phase composition, pH, and the nitrite, nitrate, and ammonium ion concentrations were periodically measured. After sampling for analysis, the gas phase was filled with argon. Sodium nitrite was added as it was exhausted.

The influence of temperature on the rate of the anammox process was studied in the 10–40°C range with a step of 5°C. The 60-mL liquid phase consisted of the activated sludge from the step 1 denitrifier, enriched preliminarily with anammox bacteria by cultivating in a batch accumulation reactor for six months, and the medium for anammox bacteria mixed at a 1 to 2 ratio; argon was the gas phase. The ammonium and nitrite concentrations were periodically measured.

The relative number of aerobic and anaerobic microorganisms in the biofilms on the brushes was determined using the dilution-to-extinction method. The material scraped off of the brush fibers from the fresh sample from WWTP no. 6 was homogenized with sterile glass beads in the a VP mixer for 30 min in an argon atmosphere. The suspension obtained was diluted at a 1 : 10 ratio: for enumeration of anaerobes, with the medium for methanogens with the addition of glucose and peptone (2.5 g/L of each); for enumeration of aerobes, with the medium whose composition was described above. Thirteen tenfold dilutions were made. Anaerobes were incubated in hermetically sealed vials in an argon atmosphere; aerobes were incubated under cotton stoppers at 30°C.

Ammonium oxidation in suspended and adhered sludge was studied in Erlenmeyer flasks under aerobic conditions. The medium (100 mL) for nitrifying bacteria was inoculated with 25 g of the brush mass with adherent sludge, cut with scissors, or with 10 mL of suspended sludge. The flasks were closed with cotton-gauze stoppers and incubated in a thermostat at 30°C.

Long-term experiments. The activity of the denitrification and anammox processes and the accumulation of anammox bacteria were studied during batch and continuous cultivation under anaerobic conditions in the atmosphere of argon.

Batch cultivation with periodic changing of medium was carried out in 1- to 2.5-L glass reactors. The vessels with sample and added medium were thoroughly flushed with argon for 5–10 min and closed with rubber stoppers with two tubes intended for sampling the gas from the upper part of the reactor and the liquid from the middle part. After the substrates had been exhausted, at least as frequent as once a week, the medium was replaced or nitrite and ammonium salts were added to the corresponding concentrations. The pH, the gas phase composition, the nitrite, nitrate, and ammonium ion concentrations were determined regularly, two or three times a week. COD and VFA were determined every three or four weeks. All manipulations with the batch reactors were carried out in an argon flow. Cultivation was carried out at 20°C over the first two months, then at 25°C over the subsequent two months, and then at by 30°C.

The accumulation of anammox bacteria and the investigation of the conditions under which they actively grew was carried out during continuous cultivation. Figure 1 presents the scheme of the laboratory continuous hybrid reactor with the ascending medium flow (the UASB reactor) and the brushes for immobilizing anammox bacteria. The reactor consists of a 90-cm column made of organic glass with a useful capacity of 1.5 l. There is a hydraulic seal and baffles for the biomass and for the produced gas, which passes through the compensatory vessel and reaches the gas meter. Fresh medium was supplied to the lower part of the column with a peristaltic pump at a rate of 1 L/day over the first six months and then at 2 L/day. The medium was supplied by the corresponding portions 16 times per day. In order to provide anaerobic conditions, the vessel with fresh medium was connected to a cushion with argon. The unit was placed in a thermostatically controlled room (29–31°C). In order to exercise control over the process, sampling was regularly made from the lower and upper samplers of the column with subsequent determination of pH, COD, the nitrite and ammonium concentration, and the amount of the substrates utilized was calculated from the difference. The estimation of the nitrogen formed was made with the gas meter.

Chemical analyses. The oxygen, nitrogen, methane, and hydrogen contents were determined on a Kristall 5000.1 gas–liquid chromatograph (ZAO KHROMATEK, Ioshkar Ola) with a system of two columns connected in parallel, reaching the heat conductivity detector and the flame-ionization detector through the methanator ($T = 350$°C); helium was the carrier gas. The gases were also determined on a CHROM-5 chromatograph (Czechoslovakia) with a katharometer

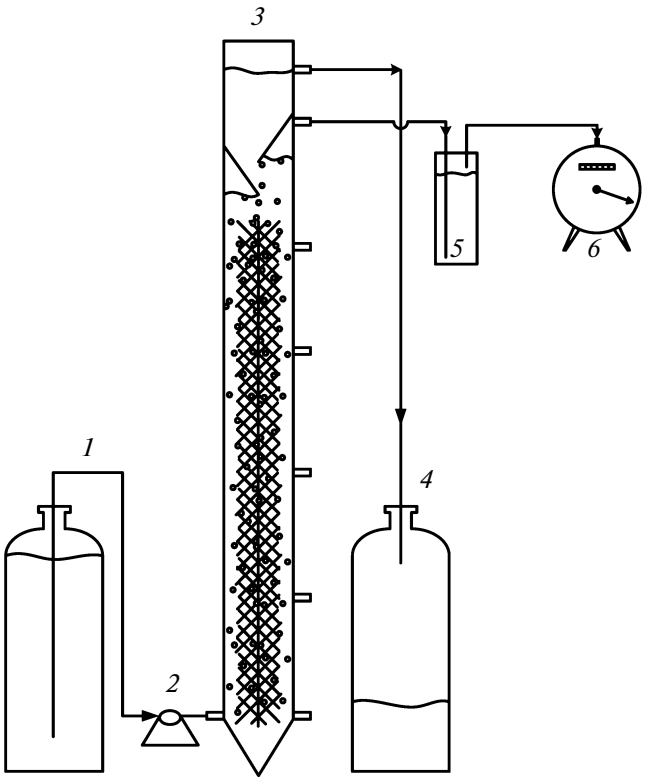

Fig. 1. Scheme of the laboratory hybrid continuous reactor, the volume is 2.5 L. (*1*) the vessel with medium, (*2*) peristaltic pump; (*3*) UASB reactor with a fibrous brush carrier in the useful capacity, a hydraulic seal and baffles for biomass and the produced gas (nitrogen) in the upper part of the reactor; (*4*) receptacle for discharge of waste medium; (*5*) gas compensator; (*6*) gas meter.

and a column packed with 5A molecular sieve; argon was the carrier gas.

VFAs were determined in the supernatant fluid after centrifugation of the microbial suspension from the flasks and reactors. The chromatographic analysis of VFA was carried out using a Staier HPLC (Russia).

Chemical oxygen demand (COD) was determined by the bichromate method [18]. Nitrate was determined according to the method described earlier [19]. Nitrite and ammonium were determined according to the standard methods [20]. pH was determined using a HANNA pH-211 pH-meter (Germany). The absolutely dry sludge mass (ADM) was determined by drying the sample in a desiccator at 105°C until the constant weight was attained.

Detection of anammox bacteria by the FISH molecular-biological method. The fixation and preparation of activated sludge specimens for fluorescence in situ hybridization (FISH) were carried out according to the method described in [21]. In order to hybridize the fixed sludge samples with probes, 1–2 μL of the fixed sample suspension was applied onto slides treated in 0.1% gelatin solution, with windows separated by the Teflon layer. The specimens obtained were sequentially treated in a series of ethanol solutions (50, 80, and 100%). The hybridization of the specimens with probes was carried out according to the technique described in [22] at 46°C, and under the hybridization conditions appropriate for particular probes. The same formamide concentration in the hybridization buffer and the same NaCl concentration in the washing buffer were used as in the works [23, 24].

The planctomycete cells were identified by hybridizing the fixed sludge specimens with an equimolar mixture of the 16S rRNA-targeted oligonucleotide probes designed earlier: the PLA46 probe for the entire *Planctomycetes* group [25] and Amx368 for anammox planctomycetes [6]. The synthesis of the probes labeled with the fluorescent Cy3 dye was carried out by the Syntol Company (Moscow, Russia). Upon completion of the hybridization procedure, the specimens were additionally stained with a 0.5 μM solution of the DNA-specific fluorescent dye 4',6'-diamidino-2-phenylindole (DAPI) for 5 min, washed with distilled water, and dried. The specimens were analyzed using a Zeiss Axioplan 2 epifluorescence microscope (Jena, Germany) with Zeiss 20 light filters for Cy3-labeled probes.

RESULTS AND DISCUSSION

Denitrification and Anammox Processes in Batch Accumulation Reactors

In order to prove that the anaerobic conditions required for the existence of anammox bacteria are really established even under the aeration conditions in the internal biofilm layers on the brushes, experiments were performed that tested the brush samples for their methanogenic activity. Under aerobic conditions, oxygen in the samples of fresh activated sludge was utilized for the oxidation of organic substances, and after they had been exhausted, their anaerobic degradation occurred with the formation of the end product methane. As a result of inoculating the denitrifier medium with adhered and suspended sludge, the color of the medium changed, the ammonium concentration decreased, and nitrite was formed, as well as a small amount of nitrate, which was evidence of the presence of nitrifiers. Both aerobic and anaerobic organisms were detected in all the samples using the dilution-to-extinction method. Both under aerobic and anaerobic conditions, the growth of microorganisms was observed up to the 12th dilution. Thus, in the wastewater treatment system studied by us, the activated sludge is aerobic–anaerobic, which determines the low output of excessive sludge.

The processes of molecular nitrogen formation were studied in the flakes of suspended sludge and biofilms of adhered sludge. Suspended sludge from the denitrifiers revealed a sufficiently high nitrifying and denitrifying activity. However, no ammonium consumption was observed even after several months of

Table 1. Ammonium and nitrite consumption/production by the microbial population of suspended sludge in a batch reactor

Month of cultivation	Nitrite and ammonium consumption (−) and production (+), mg/(L day)	
	$N–NO_2$	$N–NH_4$
1st	−1.5	+1.0
3rd	−3.4	+36.0
4th	−5.2	+32.5
5th	−10.1	+35.0
10th	−5.8	−1.5

cultivation. Moreover, active ammonium formation proceeded due to the lysis of inactive cell biomass and the degradation of nitrogen-containing substances. Ammonium consumption began only in the 10th month of cultivation. Table 1 shows the average rates of nitrite consumption and ammonium formation and consumption. The results obtained indicate that, under the actual wastewater treatment conditions, the conditions for the development of anammox bacteria in the free-floating sludge were not quite favorable. Due to the continuous flow and recycling of the water–sludge mass from the aeration tank into the denitrifier, the suspended dispersed sediment is subjected to a sharp change in the medium conditions, such as the dissolved oxygen concentration, the composition and concentration of both nitrogenous compounds and organic substances. The medium changes adversely influence the extremely slow-growing microorganisms.

In the experiments with adhered sludge, the evidence of the occurrence of the anammox process was obtained at the beginning of cultivation under anaerobic conditions. Overgrown brushes from the WWTP no. 3 denitrifier and from the WWTP no. 6 step 1 and step 2 denitrifiers were placed into batch accumulation reactors, to which nitrite and ammonium salts were periodically added and the medium was replaced regularly. A very similar course and similar results of the experiments, which are shown in Fig. 2 and in Table 2, were observed in all the reactors. Over the first month, nitrite and ammonium consumption proceeded in all the reactors with N_2 formation. Although the ammonium consumption was 2.5–3.5 times lower than that of nitrite, this testified to functioning of the anammox process in the initial samples of adhered sludge. Unfortunately, it does not seem possible to exactly assess the contribution of anammox to the total nitrogen removal with the methods used by us. In the initial

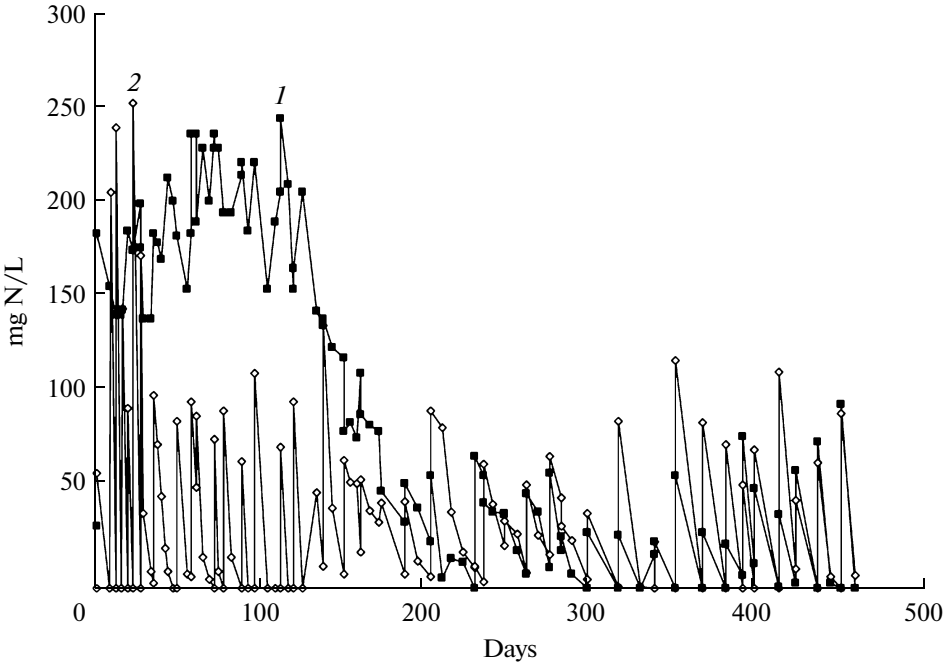

Fig. 2. Changes in the concentrations of ammonium (*1*, squares) and nitrite (*2*, diamonds) nitrogen in the batch reactor with sludge on the brush from the step 1 denitrifier of WWTP no. 6 over a year and a half.

Table 2. Average nitrite and ammonium consumption (–) and production (+) rates in different periods of cultivation of activated sludge from step 1 and step 2 denitrifiers of WWTP no. 6

Period of cultivation, month	Step 1 denitrifier		Step 2 denitrifier	
	$N-NO_2$, mg/(L day)	$N-NH_4$, mg/(L day)	$N-NO_2$, mg/(L day)	$N-NH_4$, mg/(L day)
Period of prevalence of the denitrification process				
1st	–7.5	–3.1	–7.8	–2.5
2nd	–7.5	0	–11	0
3rd	–10.2	0	–10.4	–1.0
4th	–12.5	–1.0	–10.3	–1.1
5th	–13	–2.1	–11.5	–1.5
Period of prevalence of the anammox process				
6th	–1.9	–0.8	–3.5	–2.3
7th	–2.5	–2.1	–3.1	–2.5
8th	–2.1	–2.2	–2.6	–2.2
9th	–2.4	–2.2	–2.5	–2.4
10th	–2.6	–2.2	–2.8	–2.5

period of cultivation of fresh samples, ammonium could have been utilized by both anammox bacteria and ammonium-oxidizing bacteria. Despite the fact that all manipulations with the samples were carried out in the atmosphere of argon, the presence of traces of dissolved oxygen cannot be ruled out completely, and it could have been utilized by ammonium-oxidizing bacteria. However, it should be noted that the samples contained a great number of heterotrophic aerobic bacteria, actively utilizing oxygen for the oxidation of organic substances present in the samples. Thus, we may suggest, based on the data from Table 2, that the share of the anammox process assessed by the amount of the ammonium nitrogen consumed could constitute a maximum of one-third of the total nitrogen removed. In the same period, insignificant methane formation was observed in all the reactors. In the subsequent three to four months, an active nitrite consumption, virtually without a decrease in the ammonium concentration, occurred as in the previous experiment with suspended sludge. This is indicative of an active denitrification process, prevailing over methanogenesis. Since the medium used over the first months contained sulfide, the activity of thionic denitrifiers is also highly probable, the more so that the molecular nitrogen formation rate increased with the increase in the sulfide concentration in the medium. The results obtained do not imply that ammonium was not consumed at all and the anammox process did not function. In this period, active lysis of microorganism cells began with the resultant COD increase up to 300–500 mg/L. The decomposition of nitrogen-containing organic compounds was accompanied by active formation of ammonium, which was released into the medium, which did not allow its utilization to be accurately measured. Nevertheless, it follows from Table 2 data that about 10% of molecular nitrogen could have been formed in this period at the expense of anammox. It should be noted that the initial biofilms on the brushes were destroyed, and the brush fibers were exposed. This is indicative of a change in the microbial population compared to the beginning of the experiment.

At the end of the fifth month of the experiment, COD substantially decreased, and volatile fatty acids were absent in the medium. This gave evidence of a decrease in the inactive biomass cell lysis rate and the exhaustion of easily degradable organic substances in the reactors. At this time, elective conditions for anammox bacteria could develop. Beginning with day 150 of the experiment, the medium for anammox bacteria was used, and the ammonium and nitrite concentrations were maintained at about 50 mg/L in terms of nitrogen. The utilization of almost equal (by nitrogen) amounts of nitrite and ammonium began in both reactors, which was indicative of an active anammox process (Table 2, Fig. 2). The formation of molecular nitrogen decreased compared to the denitrification period. For the high rates of the anammox process to be attained, the growth of the population of anammox bacteria is necessary, which is time-consuming and extends for months. The medium acidity in the reactors was maintained at pH 7–8 throughout the experiment.

The study of the influence of temperature on the ammonium and nitrite consumption rate conducted after six months of cultivation of the initial sludge in the batch accumulation reactor showed that anammox proceeded in the 10–40°C temperature range with a broad optimum at 25–35°C (Fig. 3). The rate of the process sharply decreased when the temperature increased to 40°C. A sufficiently high rate of the pro-

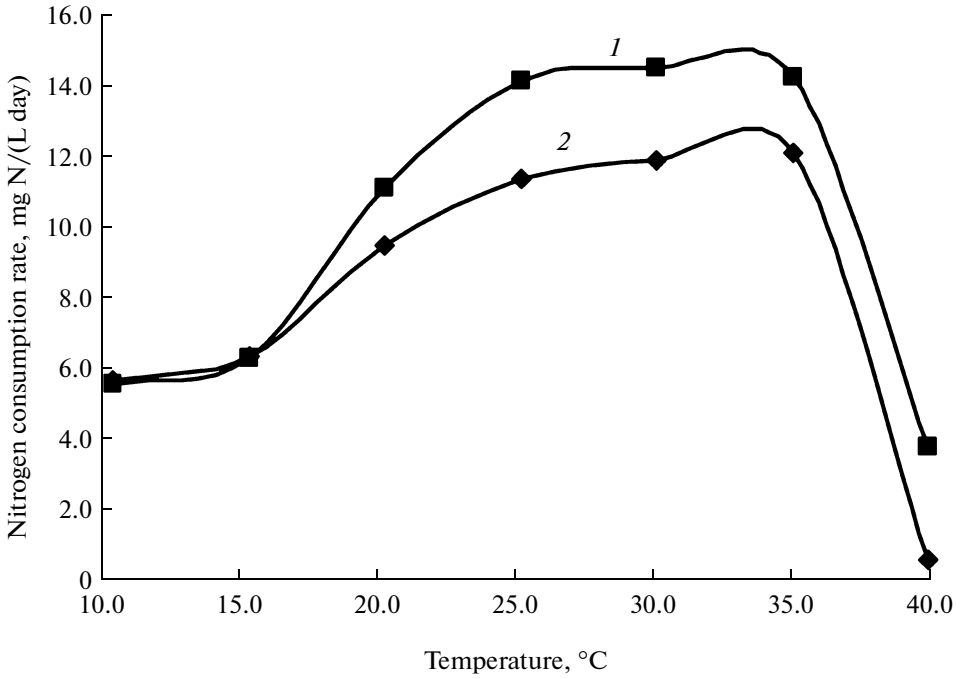

Fig. 3. Effect of temperature on the average rates of nitrite (*1*, squares) and ammonium (*2*, diamonds) consumption in the anammox process.

cess was observed at a lowered temperature. At 10 and 15°C, it was only 2.5 times lower, and at 20°C, 1.5 times lower than at the optimum temperature, which is very unusual for anammox bacteria isolated from wastewater treatment plants [11] and indicates the possible presence of new anammox bacteria in the population of anammox bacteria in the batch reactors. The ammonium to nitrite nitrogen consumption ratio was close to 1.0 : 1.3, which corresponds to the theoretical ratio for the anammox process [4].

Study of the Anammox Process during Continuous Cultivation

Under the cultivation conditions with a continuous flow of the medium free from organic substances and electron acceptors other than nitrite, elective conditions for the development of anammox bacteria are created. It should be noted that the medium pH increases with the growth of anammox bacteria. Under such conditions, other microorganisms die or are washed out from the reactor. All of the monocultures of anammox bacteria known to date were isolated in such a way [26]. In effectively working anammox reactors, more than 90% of the microbial population may be represented by anammox bacteria [7, 11].

After nine months of batch cultivation, the brush with the adhered and suspended biomass was transferred from step 2 denitrifier to the column of the continuous reactor. Table 3 shows the results of the consumption of ammonium and nitrite nitrogen in the continuous anammox reactor operated for nine months. The reactor output was 0.3 kg N/(m^3 day). By the end of this period, the ratio of the rates of ammonium and nitrite nitrogen consumption approached 1.3, which corresponds to the theoretical calculation for the anammox process. Anaerobiosis in the column was created at the expense of the removal of traces of oxygen in the lower part of the column, where aerobic microorganisms could be present. COD in the middle part of the reactor was lower than the sensitivity of the determination method, thus giving evidence of the absence or an extremely low content of dissolved

Table 3. Average consumption rate of ammonium and nitrite nitrogen by the microbial biomass in the column, mg/(L day), over nine months of cultivation

Month of cultivation	Consumption, mg/(L day)	
	$N-NO_2$	$N-NH_4$
1st	2.6	2.6
3rd	3.5	2.8
5th	4.9	3.9
6th	55.3	46.1
7th	120.9	89.2
8th	154.8	124.1
9th	156.0	154.7

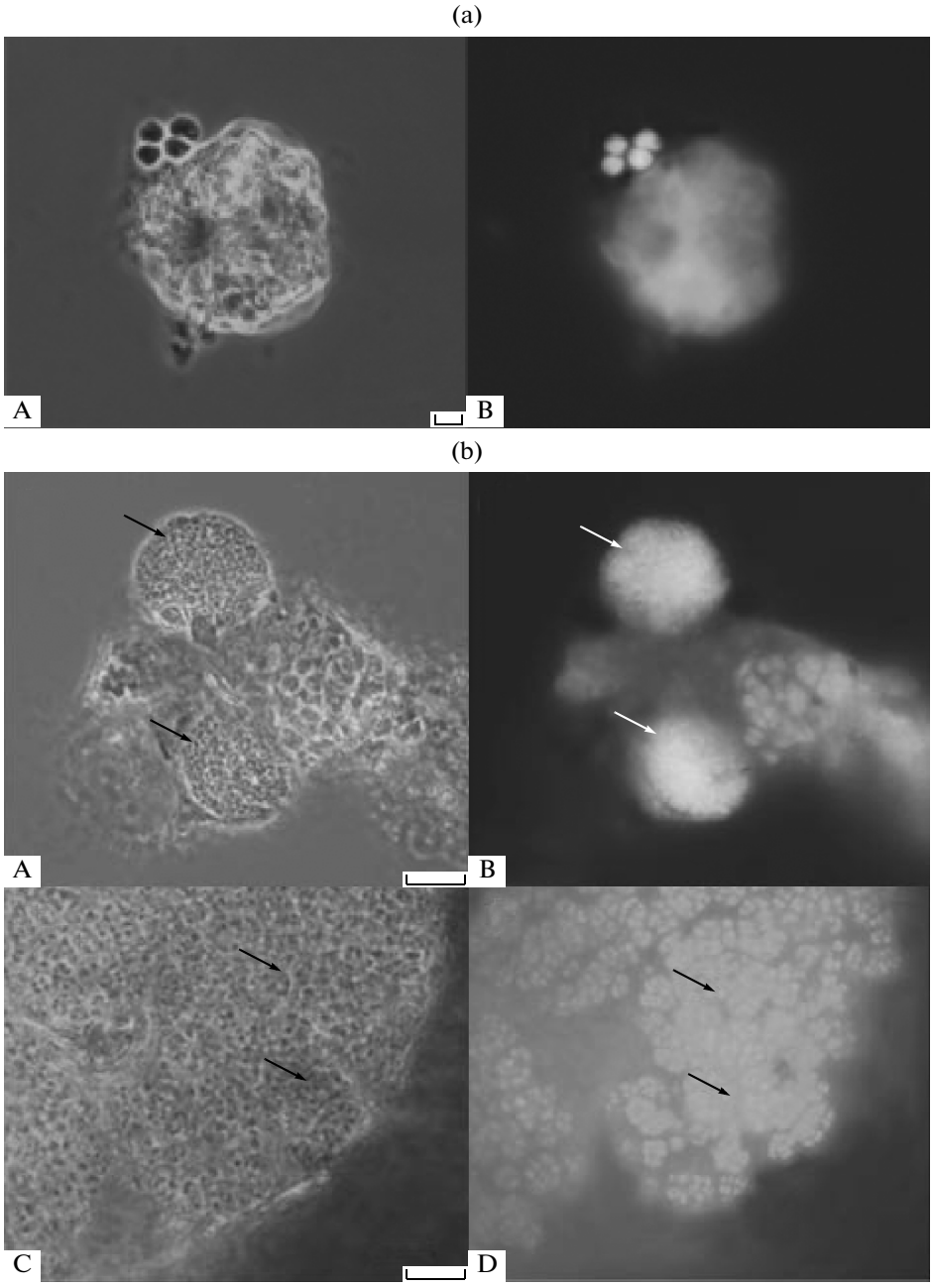

Fig. 4. Detection of anammox bacteria by (A, C) phase contrast microscopy and (B, D) fluorescence in situ hybridization with a Cy3-labeled oligonucleotide probe Amx368 (a) in the batch reactor on a hard carrier and (b) in the continuous reactor (A, B) in the sediment from its lower part and (C, D) in the biofilm from its middle part. The arrows indicate accumulations of colonies of anammox bacteria. Bar, 10 μm.

organic substances in the medium. New reddish-brown biofilms, in which characteristic rounded colonies and large-sized aggregates typical of anammox bacteria were seen under the light microscope, developed on the brush fibers. In the samples from the sediment in the lower part of the reactor, the anammox-like spherical colonies were buried in the remnants of lysed initial sludge cells. The identification of anammox bacteria with FISH is described below. As the nitrogen utilization rate and, consequently, the growth rate of anammox bacteria increased, the reactor effluent pH also increased. However, even in the upper part of the column, the pH value was within the range suitable for growth of anammox bacteria.

Detection of Anammox Bacteria in the Biofilms of Activated Sludge by FISH

Fluorescence in situ hybridization (FISH) with the Cy3-labeled PLA36 nucleotide probe detected the presence of planctomycetes in the sludge sampled directly at the wastewater treatment plants on the river Mzymta at the beginning of the investigations. Accumulations of cells or microcolonies similar in morphology to anammox bacteria were also revealed; they hybridized with the Amx368 probe but occurred rarely, one or two aggregates in the microscope field on average. After several months of batch cultivation of the samples under the conditions, when the period of prevalence of the anammox process began, the density of colonies and aggregates of anammox bacteria in the brush films and in the flocculent sediment increased to a dozen of microcolonies in the microscope field. Aggregates consisting of two, four, or eight microcolonies that hybridized with the Amx368 probe were detected in the suspended sludge samples (Fig. 4a). Accumulations of the anammox bacterium colonies, which represented a considerable portion of the active microbial population, were revealed with the Amx 368 probe in the active biomass samples taken from the continuous reactor after nine months of cultivation (Fig. 4b). Thus, indisputable proofs of the presence of anammox bacteria in the activated aerobic–anaerobic sludge were obtained.

Contribution of the Anammox Process to Nitrogen Removal during WWTP Operation

In the course of the maintenance of the WWTP in the valley of the river Mzymta, the process of nitrogen removal at the expense of the combination of denitrification and anammox (DEAMOX) was stabilized. The study of the samples taken from the WWTP no. 3 denitrifier after a year and a half and two years of its maintenance confirmed the sufficiently high activity of the in situ anammox process. In short-term experiments during batch cultivation on the medium for anammox bacteria, the nitrite and ammonium consumption began straightaway and proceeded simultaneously with denitrification. The nitrogen removal rate was by 10–15% higher than in the samples taken after one year of the WWTP maintenance (Table 2). According to our data, the share of the anammox process may have accounted for up to one-third of nitrogen removal. These results agree well with the calculated data. It should be noted that, at the WWTP we collaborated with, the quality of water treatment corresponds to the norms of discharge into fish-breeding reservoirs [27]; the ammonium nitrogen content in the purified water does not exceed 0.2–0.3 mg/L. For the denitrification process to be implemented, the biochemical oxygen demand (BOD) to N ratio equal to 5–6 : 1, or C_{org} : N equal to 2.5–3.0 : 1.0 is required [1]. Such a ratio corresponds to the composition of the domestic wastewater arriving for treatment. When the wastewater supplied to WWTP is pretreated with a coagulant, approximately 50% of organic carbon from contaminants is removed; in the process, the ammonium nitrogen content is not decreased. The remaining organic compounds are not enough to remove all the ammonium contained in the wastewater during the nitrification–denitrification process. The contribution of the anammox process to nitrogen removal can be more accurately assessed using the method of incorporation of the atoms of the stable ^{15}N isotope [28]. Nevertheless, the results obtained in our work are indicative of the development and activity of anammox bacteria in the biomass on the brushes during actual operation of the WWTP. The realization of anammox ensures nitrogen removal when the content of organic substances is low or when they are absent. The technology developed by ZAO ECOS Company is a full-scale realization of the combination of the denitrification and anammox processes (DEAMOX) [5] and is referred to as BC-DEAMOX [12].

Our study has experimentally confirmed the development of anammox bacteria and operation of the anammox process during the treatment of domestic wastewater in the combined system of physicochemical and biological treatment with the immobilization of activated sludge on a hard flexible carrier (BC-DEAMOX). The BC-DEAMOX process was the first to reveal the important role of anammox bacteria in the treatment of weak wastewater.

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