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EXPERIMENTAL ARTICLES

Microbial Composition of the Activated Sludge of Moscow Wastewater Treatment Plants

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Abstract—The contribution of the major technologically important microbial groups (ammonium- and nitrite-oxidizing, phosphate-accumulating, foam-inducing, and anammox bacteria, as well as planctomycetes and methanogenic archaea) was characterized for the aeration tanks of the Moscow wastewater treatment facilities. FISH investigation revealed that aerobic sludge were eubacterial communities; the metabolically active archaea contributed insignificantly. Stage II nitrifying microorganisms and planctomycetes were significant constituents of the bacterial component of activated sludges, with *Nitrobacter* spp. being the dominant nitrifiers. No metabolically active anammox bacteria were revealed in the sludge from aeration tanks. The sludge from the aeration tanks using different wastewater treatment technologies were found to have differing characteristics. Abundance of the nitrifying and phosphate-accumulating bacteria in the sludge generally correlated with microbial activity in microcosms and with efficiency of nitrogen and phosphorus removal from wastewater. The highest microbial numbers and activity were found in the sludge of the tanks operating according to the technologies developed in the universities of Hannover and Cape Town. The activated sludge from the Novokur'yanovo facilities, where abundant growth of filamentous bacteria resulted in foam formation, exhibited the lowest activity. The group of foaming bacteria included *Gordonia* spp. and *Acinetobacter* spp utilizing petroleum and motor oils, *Sphaerotilus* spp. utilizing unsaturated fatty acids, and *Candidatus* 'Microthrix parvicella'. Thus, the data on abundance and composition of metabolically active microorganisms obtained by FISH may be used for the technological control of wastewater treatment.

Keywords: wastewater treatment plants, activated sludge, nitrifying bacteria, phosphate-accumulating organisms, foaming bacteria, fluorescent in situ hybridization (FISH)

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Wastewater treatment is presently an issue of utmost importance. Biological wastewater treatment technologies based on intensification of the naturally occurring microbial processes of organic matter decomposition are considered the most economically and environmentally acceptable. Centralized systems for processing of municipal wastewater with COD (chemical oxygen demand) below 2 g L^{-1} carry out aerobic treatment involving activated sludge. Activated sludge is a complex anthropogenic ecosystem developing in the limited spaces of the aeration tanks of the centralized wastewater treatment facilities under conditions of oxygen abundance and relatively high load of organic contamination. High rate and efficiency of wastewater purification achieved due to intense forced aeration and high numbers of microorganisms provide for the oxidation of organic contaminants up to their complete removal [1].

The microorganisms of activated sludge form well-organized structural and functional communities, activated sludge floccules, in which microbial cells and organic and inorganic particles are bound by extracellular polymeric compounds. Heterotrophic microorganisms of various groups play the main part in removal of organic contaminants from wastewater. Ammonium- and nitrite-oxidizing bacteria and phosphate-accumulating organisms (PAO) are involved in removal of biogenic elements (nitrogen and phosphorus) from wastewater. Gram-negative proteobacteria belonging mostly to the phylum *Betaproteobacteria* predominate in the sludge under almost any technological scheme of wastewater treatment. The activated sludge proteobacteria include enterobacteria, vibrios, and such typical aerobic oxidizers of organic matter as pseudomonads, floccule-forming bacteria *Zoogloea*

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Table 1. Contaminant removal in aerotanks of the Kur'yanovo (KWTPold and NKWTP2) and Lyubertskie wastewater treatment plants (block for biogenic elements removal and aerotank no. 14 operating under UCT and ISAH technologies, respectively)

Parameter	Outlet treated water, % contaminant removal			
	KWTPold	NKWTP2	UCT	ISAH
Suspended solids	93	93	97	96
COD	80	85	92	96
BOD	94	95	97	95
$N\text{-}NH_4$	80	60	99.2	99.3
$P\text{-}PO_4$	75	60	90	99.7

spp., and filamentous bacteria *Sphaerotilus natans*; development of the latter is often considered responsible for the "swelling" of activated sludge. The *Rhodococcus*-like bacteria *Candidatus* 'Accumulibacter phosphatis', which are presently considered the main agents of biological phosphorus removal [2], belong to the *Betaproteobacteria*, the group predominant in activated sludge. Ammonium-oxidizing bacteria of the genus *Nitrosomonas* also belong to this group. *Nitrobacter*, stage II nitrifying microorganisms, belong to the *Alphaproteobacteria*, another common bacterial group in activated sludge. Considerable numbers of nitrite-oxidizing bacteria of the genus *Nitrospira* are present in the activated sludge of the facilities treating industrial wastewater with high concentrations of ammonium nitrogen (up to 5 g L^{-1}) and enriched with proteinaceous contaminants. These bacteria often occur in microcolonies together with bacteria of the genus *Nitrosomonas* [3]. Members of the *Actinobacteria* (including foaming bacteria *Gordonia* spp.) are present in activated sludge in significant numbers. Filamentous bacteria of the *"Chloroflexi"* group are found in the activated sludge of industrial facilities applying various technological schemes for removal of biogenic elements [4].

Thus, activated sludges of aerotanks are complex microbial communities consisting of physiologically and phylogenetically diverse groups of microorganisms, which contribute to removal of the major contaminants from wastewater. Assessment of the abundance and composition of the metabolically active microorganisms important for the technological control of wastewater treatment is therefore a relatively complicated problem. Fluorescence in situ hybridization (FISH) with 16S rRNA-specific fluorochromes-labeled oligonucleotide probes makes it possible to identify and enumerate the microorganisms of different phylogenetic taxa (from species to domains) in such complex microbial communities as activated sludge of wastewater treatment facilities, where both the numbers and diversity of microorganisms are extremely high.

The goal of the present work was to apply microscopical (phase contrast microscopy of native and stained preparations), molecular genetic (FISH), and cultural techniques for determination of the abundance and diversity of the technologically important groups of eubacteria (nitrifying, phosphate-accumulating, foaming, and anammox bacteria), planctomycetes, and methanogenic archaea in activated sludge of Moscow wastewater treatment facilities and to determine the relations between abundance of these microbial groups in activated sludge and their physiological activity in microcosms.

MATERIALS AND METHODS

The subjects of research were the samples of activated sludge from the aerotanks operating according to different technologies and listed in Table 1.

(1) "Old" Kur'yanovo wastewater treatment plant (KWTPold), where complete biological purification (removal of suspended matter, oxidation of carbon compounds, and partial nitrification and dephosphotation) is carried out in aerotanks.

(2) Novokur'yanovo wastewater treatment plant (NKWTP2), where early stages of foam formation were observed, which uses the same water treatment technology as KWTPold.

(3) Block of biogenic elements removal of the Lyuberetskie wastewater treatment plant (LWTP) operating according to the University of Cape Town technology (UCT). It carries out the processes of oxidation of organic matter and ammonium, denitrification, and dephosphotation, i.e., biogenic elements are efficiently removed.

(4) LWTP aerotank no. 14, operating according to the Institut für Siedlungswasserwirtschaft und Abfalltechnik Gottfried Wilhelm Leibniz Universität Hannover technology (ISAH), which is presently the most efficient and reliable technology for biogenic element removal from wastewater with low organic matter content.

(5) Foam from the surface of LWTP and KWTP aerotanks.

Physicochemical characteristics of inlet wastewater, outlet purified water, and activated sludge were determined using the standard techniques. Concen-

tration of suspended solids was determined gravimetrically (AC211S balance, Sartorius, Germany). Chemical oxygen demand (COD) was determined by the bichromate method. Biological oxygen demand (BOD5) was determined by the manometric method in Oxitop vials (WTW, Germany). Total solids content (TS) of activated sludge (g TS L^{-1}) was determined by drying the samples to constant mass at 105°C; the volatile solids (VS) content was calculated as the difference between the TS and total fixed solids (ash content) determined after incineration by heating at 550°C to constant mass. Determination of ammonium (with the Nessler reagent), nitrite (with the Griess reagent), and phosphate (with ammonium molybdate, ascorbic acid, and potassium antimonyl tartrate) was carried out using a DR2010 spectrophotometer (Hach, United States) according to the manual [5].

Respiratory activity of the sludge or the maximal oxygen consumption rate (OCR) for the oxidation of organic matter (OCR_{org}) and ammonium compounds (OCR_{nitr}) was determined in a laboratory microcosm according to the previously developed procedure [6]. After sedimentation of the sludge mixture from aerotanks, the sediment was diluted with outlet water to 1–3 g L^{-1} and aerated for 0.5–1 h at dissolved oxygen concentration of at least 2 mg L^{-1}. The measurement was carried out not later than 4 h after sampling. For OCR_{org} determination, the sample was sealed in a thermostatically controlled cell (25°C) and supplemented with concentrated sodium acetate solution to the final concentration of 1 g L^{-1} and allyl thiourea (a nitrification inhibitor) to 2.5 mg L^{-1}. A decrease in dissolved oxygen concentration was monitored using an oxygen sensor (Stirrox, WTW, Germany). OCR was determined from the slope at the linear part of the graph of decreasing oxygen concentrations, and the potential rate of oxygen consumption by heterotrophic microorganisms (mg O_2 g^{-1} sludge TS h^{-1}) was calculated as follows: $Q_{org} = OCR/X$, where X is the concentration (dose) of activated sludge.

To determine the potential OCR_{nitr}, the sludge sample was pretreated as described above and divided into two aliquots. In the first aliquot, total OCR by nitrifying and heterotrophic organisms was determined ($OCR_{tot} = OCR_{nitr} + OCR_{org}$) by saturating the sample with air, transferring it to an oxygen cell, and adding concentrated $(NH_4)_3SO_4$ solution to 500 mg L^{-1}. In the second aliquot, OCR_{org} was determined without ammonium sulfate, but in the presence of allyl thiourea, a nitrification inhibitor (2.5 mg L^{-1}). A decrease in dissolved oxygen concentration in the cells was monitored. Nitrification rates were calculated as the differences between OCR_{tot} and OCR_{org}, and the potential nitrification rate (mg O_2 g^{-1} sludge TS h^{-1}) was calculated as follows: $Q_{nitr} = OCR_{nitr}/X$.

Potential ORC for the second stage of nitrification was determined using a procedure similar to that for determination of potential OCR_{nitr}, but with sodium nitrite (200 mg L^{-1}) added instead of ammonium.

PAO activity was assessed as the rates of phosphate release and uptake by the sludge mixture under anaerobic and aerobic conditions, respectively. The rates were measured according to the known procedure [7], except for the substrate added: instead of acetate solution, actual wastewater was used after removal of suspended particles; it was mixed with the analyzed activated sludge samples to achieve their final concentrations of 2–2.7 g L^{-1}.

PAO detection in activated sludge was achieved by light microscopy of fixed preparations stained with Leffler methylene blue. Methylene blue stains volutin granules (high-molecular calcium, magnesium, and potassium polyphosphates $M_n{+}2P_nO_{3n}{+}1$) blue to violet, thus making it possible to detect the PAO.

Fluorescent in situ hybridization (FISH). Activated sludge samples were fixed with 4% paraformaldehyde in sodium phosphate buffer [8, 9], diluted in 0.1% aqueous sodium dihydropyrophosphate, and sonicated for 30 s at 5.5 oscillation amplitude of the core on Soniprep 150 Plus (MSE, United Kingdom) with the core working frequency of 23 kHz. The aliquot of sample was applied to slides and treated according to the standard procedure [10, 11]. The set of Cy3-labeled 16S rRNA-specific oligonucleotide probes (Syntol, Russia) was used for detection of various members of the *Bacteria* and *Archaea* domains (Table 2, [9, 12–26]). The cells were hybridized with the probes at 46°C and washed at 48°C according to the previously described FISH procedure [11, 12]; the formamide and NaCl concentrations are listed in Table 2. After hybridization and washing, the cells were stained for 15 min in the dark with a universal DNA-specific stain DAPI (4',6'-diamidino-2-phenylindole, 0.5 ng μL^{-1}), washed with water (MilliQ Type 1), and air-dried in the dark. Prior to microscopy, the samples were embedded into Citifluor AF1 (Citifluor Ltd., United Kingdom). Microscopy was carried out under an AxioImager.D1 epifluorescence microscope (Carl Zeiss, Germany) equipped with an AxioCamHR digital camera and the relevant filters (Zeiss 20 for Cy3-labeled probes and Zeiss 49 for enumeration of DAPI-stained cells). The target microbial populations were enumerated by counting the hybridized cells in 20 microscope fields. At less than ten cells per field, the cells were counted over the entire well area (for the sample aliquot) with subsequent calculation of cell numbers per 1 g of sludge TS. Statistical analysis of the results was carried out using MS Excel 2003.

Cultural techniques. Aerobic foaming bacteria were grown on solid and in liquid mineral media. The modified Czapek medium contained the following (g L^{-1}): $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.1. The substrates used were yeast extract,

Table 2. The 16S rRNA-specific probes for FISH detection of microorganisms in activated sludge samples

Probe	Nucleotide sequence, 5'–3'	[FA][1], %	NaCl[2], mM	Current specificity	Reference
Broad specificity probes					
Arch915	GTGCTCCCCCGCCAATTCCT (915–934)[3]	20	225	Domain *Archaea* (87%)[4]	[12]
EUB338[5]	GCTGCCTCCCGTAGGAGT (338–355)	35[6]	80	Domain *Bacteria* (77%)	[9]
EUB338II[5]	GCAGCCACCCGTAGGTGT (338–355)	35[6]	80	Order *Planctomycetales* (40.9%) and *Firmicutes* (0.15%)	[13]
EUB338III[5]	GCTGCCACCCGTAGGTGT (338–355)	35[6]	80	Type "*Verrucomicrobia*" (87%), "*Armatimonadetes*" (23%), "*Proteobacteria*" (0.02%), "*Acidobacteria*" (0.16%)	[13]
Pla46	GACTTGCATGCCTAATCC (46–63)	30	112	Order *Planctomycetales* (50.3%)	[14]
Ammonium-oxidizing bacteria (stage I nitrifyers)					
NEU	CCCCTCTGCTGCACTCTA (653–670)	40	56	Genus *Nitrosomonas* (44%)	[15]
Nsv443	CCGTGACCGTTTCGTTCCG (444–462)	30	112	Genus *Nitrosospira* (20%)	[16]
Nitrite-oxidizing bacteria (stage II nitrifyers)					
NIT3[7]	CCTGTGCTCCATGCTCCG (1035–1052) Competitive probe: CCTGTGCTCCAGGCTCCG	40	56	Genus *Nitrobacter* (63%)	[17]
Ntspa662[7]	GGAATTCCGCGCTCCTCT (662–679) Competitive probe: GGAATTCCGCTCTCCTCT	35	80	Genus *Nitrospira* (54%)	[18]
Polyphosphate-accumulating organisms					
PAO846	GTTAGCTACGGCACTAAAAGG (846–866)	35	80	*Candidatus* 'Accumulibacter phosphatis', uncultured *Propionivibrio* spp.	[19]
ACA652 (ACA23A)	ATCCTCTCCCATACTCTA (652–669)	35	80	Genus *Acinetobacter* (84%), uncultured *Alkanindiges* spp. (13%)	[20]
Filamentous bacteria					
Gor596	TGCAGAATTTCACAGACGACGC (596–617)	20	225	Genus *Gordonia* (87%)	[21]
MPA	CCGGACTCTAGTCAGAGC	20	225	*Candidatus* 'Microthrix parvicella', *Candidatus* 'Microthrix calida'	[22]
Anammox bacteria					
Amx368	CCTTTCGGGCATTGCGAA (368–385)	15	318	*Candidatus* 'Brocadia' spp., *Candidatus* 'Kuenenia stuttgartiensis', *Candidatus* 'Scalindua' spp., uncultured anammox bacteria	[23]
Methanogenic archaea					
EURY499	CGGTCTTGCCCGGCCCT (499–515)	20	225	Most genera of the orders *Methanomicrobiales* (70%), *Methanosarcinales* (60%), Genus *Methanocella* (70%)	[24]
SARCI551	GACCCAATAATCACGATCAC (551–570)	20	225	Genus *Methanosarcina* (76%)	[25]

[1], Formamide concentration in the hybridization buffer; [2], NaCl concentration in the washing buffer; [3], target site, *E. coli* position; [4], the target organisms revealed by the probe, % of the total number of cultured and uncultured members of the taxon according to the Ribosomal Database Project (RDB, http://rdp.cme.msu.edu/) is shown in parentheses; [5], equimolar mixture of three labeled oligonucleotides; [6], formamide concentration according to Bassin et al. [26]; [7], the probe was used in combination with an unlabeled competitive probe.

Table 3. Numbers of metabolically active eubacteria in sludge samples from aerotanks KWTP (samples 1 and 2) and LWTP (samples 3 and 4)

Sample	Treatment technology	Total solids content, g TS L^{-1} sludge	Volatile solids content, %	Number of eubacteria*	
				$\times 10^8$ cells mL^{-1} sludge	$\times 10^{11}$ cells g^{-1} sludge TS
1	Complete biological treatment	2.6	62	92	35
2	Complete biological treatment	3.9	60	83	21
3	UCT	2.3	65	130	56
4	ISAH	4.0	67	155	39

*, standard deviations for all measurements were below 10%.

sunflower oil, or glycerol (5 g L^{-1}) as analogues of inflowing anthropogenic organic matter, 10 W-40 motor oil or crude oil (5%, vol/vol) as technogenic contaminants, as well as acetate, malate, lactate, or ethanol (5 g L^{-1}). Bacteria were grown on plates and in test tubes under static aeration at 4–28°C. Nystatin and cycloheximide were used to suppress fungal growth.

RESULTS AND DISCUSSION

Abundance of metabolically active eubacteria and archaea in aerotank sludge. The numbers of the targeted eubacterial cells revealed by hybridization with the universal eubacterial probes (EUB368mix) is presented in Table 3. The numbers of metabolically active eubacterial cells found in the sludge of the KWTP and LWTP aerotanks were in agreement with the previously obtained data on bacterial numbers in other activated sludge ($1–10 \times 10^{12}$ cells g^{-1} VS) [28]. Samples 3 and 4 from the LWTP aerotanks operating according to the UCT and ISAH technologies contained more eubacteria per mL sludge than samples 1 and 2, from aerotanks operated according to the complete biological treatment technology (KWTP), which was generally in agreement with more efficient contaminant removal in the LWTP aerotanks (Table 1). The numbers of eubacteria per g TS were, however, considerably lower in sample 4 (with higher total solids content) than in sample 3.

Most microorganisms in the sludge samples were eubacteria (80–95% of the total number of DAPI-stained cells). In natural ecosystems where microorganisms exist under conditions of substrate limitation, the ratio of metabolically active eubacteria to the total number of DAPI-stained cells usually does not exceed 50%, since the majority of the population is represented by starving or dormant forms which are not revealed by FISH due to their low intracellular 16S rRNA content [8, 28]. In aerotanks, however, organic matter and biogenic elements are abundant, and conditions for growth of various microbial groups are therefore optimal. In activated sludges, over 80% of the microbial population is in a metabolically active state [3, 27].

Hybridization with the universal archaeal probe Arch915 revealed metabolically active archaea in all samples of aerobic sludge, although their numbers were low (1 to 5 cells per microscope field, i.e., $7–36 \times 10^5$ cells mL^{-1} activated sludge). Sample 4 (ISAH technology), where the SARCI551 probe revealed dense aggregates of *Methanosarcina* spp. (5–10 aggregates per field), was exceptional in this respect. Such aggregates were also found in sample 3 (UCT technology), although in lower numbers.

Activity and abundance of ammonium-oxidizing bacteria. In sewage treatment facilities, nitrification is the key process for removal of nitrogen compounds from wastewater. Activity of nitrifying (ammonium- and nitrite-oxidizing) bacteria was assessed in laboratory microcosm experiments as their respiratory activity coupled to ammonium or nitrite oxidation. Ammonium-oxidizing activity of the sludge increased in the row NKWTP2 < KWTPold < ISAH < UCT (Table 4). The numbers of metabolically active ammonium-oxidizing bacteria (AOB) detected by FISH in activated sludge also increased in the row NKWTP2 < KWTPold < ISAH < UCT (Table 5). The lowest AOB number was found in the sludge of the KWTP aerotanks (KWTPold and NKWTP2), which agreed with the data on ammonium-oxidizing activity (Table 4) and efficiency of $N\text{-}NH_4$ removal from wastewater (Table 1). *Nitrosomonas* spp. numbers were very low in all sludge studied.

Activity and abundance of nitrite-oxidizing bacteria. The highest rate of nitrite oxidation was detected in the NKWTP2 sludge (sample 2), although this was the aerotank with the lowest abundance of metabolically active autotrophic nitrite-oxidizing bacteria (NOB) (Table 5). Two factors were probably responsible for this discrepancy: (1) the probes used revealed only a fraction of the NOB population and (2) heterotrophic nitrite oxidation occurred. The second possibility stems from the fact that a number of typical heterotrophic bacteria, including filamentous bacteria, are capable of heterotrophic nitrification [29], which does not provide energy for the microorganisms. At high nitrite content in the medium, it is used by heterotrophic filamentous microorganisms, which are numerous in the activated sludge of this aerotank.

Table 4. Respiratory activity of nitrifying bacteria in sludge samples from aerotanks KWTP (samples 1 and 2) and LWTP (samples 3 and 4)

Sample	Treatment technology	Heterotrophic respiration	Nitrification			
			with NH_4		with NO_2	
		O_2	O_2	N	O_2	N
		mg g^{-1} sludge TS h^{-1}				
1	Complete biological treatment	16.9	12.9	2.8	4.5	4.0
2	Complete biological treatment	22.5	7.1	1.6	6.3	5.6
3	UCT	19.7	16.5	3.6	4.9	4.3
4	ISAH	12.2	13.9	3.1	4.9	4.3

Table 5. Numbers of different groups of metabolically active bacteria detected by FISH in sludge samples

Sample	1	2	3	4
Treatment technology	Complete biological treatment	Complete biological treatment	UCT	ISAH
Organisms (probe)	$\times 10^9$ cells g^{-1} sludge TS*			
Eubacteria (EUBmix)	3538	2139	5652	3875
Genus *Nitrosospira* (Nsv443)	15	8	25	20
Genus *Nitrosomonas* (NEU)	3	1.5	7	3
Total AOB number	18	9.5	32	23
Genus *Nitrobacter* (NIT3)	292	167	267	132
Genus *Nitrospira* (Ntspa662)	17	0	197	96
Total NOB number	309	167	464	228
Genus *Candidatus* 'Accumulibacter phosphatis' (PAO846)	75	41	73	52
Genus *Acinetobacter* (ACA652)	51	58	60	39
Order *Planctomycetales* (Pla46)	265	155	334	160
Percentage of detected microorganisms of the total number of eubacteria, %	20	20	17	13

AOB and NOB stand for ammonium- and nitrite-oxidizing bacteria, respectively; *, standard deviations for all measurements were below 10%.

The highest NOB numbers were revealed by FISH in sample 3 (UCT technology). *Nitrobacter* spp. were the dominant nitrifying organisms in all sludge samples (Table 5). *Nitrospira* species were present in all samples, except for the sludge from the NKWTP2 aerotank (sample 2). In samples 3 and 4 (UCT and ISAH technologies, respectively), the target cells formed numerous amorphous aggregates typical of *Nitrospira* sp. found in other sewage treatment facilities. These *Nitrospira* spp. cell aggregates were morphologically similar to the colonies of *N. moscoviensis*, the species isolated from the municipal heating system [30]. High abundance of *Nitrospira* spp. (5–24% of the total number of DAPI-stained cells) and the presence of species related to *N. moscoviensis* and *Candidatus* 'Nitrospira defluvii' were recently revealed in the pilot reactors for treatment of mixed industrial and municipal wastewater. *N. moscoviensis* is known to predominate in the systems with low nitrite concentrations [31].

Activity and abundance of phosphate-accumulating organisms. (Poly)phosphate-accumulating organisms (PAO) participate in phosphate removal from wastewater. Under anaerobic conditions they assimilate acetate and propionate and use them, together with the previously accumulated intracellular polyphosphates, for synthesis of the storage poly-β-hydroxyalkanoates (PHA). Under aerobic conditions in the absence of external carbon sources in the medium, these microorganisms consume PHA; phosphates are then accumulated and stored as intracellular polyphosphates [32].

The rates of PAO activity were calculated using the data on dynamics of phosphorus release/uptake by the

Table 6. Rates of P-PO_4 release and uptake in sludge samples from aerotanks KWTP (samples 1 and 2) and LWTP (samples 3 and 4)

Sample	Treatment technology	Release rate	Uptake rate
		mg P-PO_4 g^{-1} sludge VS min^{-1}	
1	Complete biological treatment	0.15	0.17
2	Complete biological treatment	0.02	0.03
3	UCT	0.26	0.29
4	ISAH	0.23	0.23

sludge in microcosms. PAO activity increased in the row NKWTP2 < KWTPold < UCT ≈ ISAH (Table 6). The data on PAO activity agreed with the microscopic picture (methylene blue staining) and with P-PO_4 levels in inlet wastewater and outlet purified water. Methylene blue staining revealed the lowest PAO number in sample 2 (NKWTP2 aerotank); small PAO cells occurred once per ten microscope fields. The highest PAO numbers were found in samples 3 and 4 (UCT and ISAH technologies), where PAO were detected as both colonies and individual cells.

While no true PAO microorganisms are presently known with certainty [32], a set of probes for FISH detection of putative PAO has been developed due to the practical importance of this physiological group for phosphate removal from wastewater [2]. Uncultured bacteria *Candidatus* 'Accumulibacter phosphatis' (class *Betaproteobacteria*), which are probably true PAO, have been identified in various systems for enhanced biological phosphorus removal (EBPR) [32, 33]. Hybridization with the PAO846 probe revealed *Candidatus* '*A. phosphatis*' in all sludge samples. The cells of the target organisms were usually diffusely distributed in the samples, although microcolonies occurred sometimes. The lowest numbers of *Candidatus* '*A. phosphatis*' were revealed in the NKWTP2 sludge (Table 5). This finding correlated with the PAO activity and with the results of microscopy of methylene blue-stained preparations. *Candidatus* 'A. phosphatis' abundance increased in the row NKWTP2 < ISAH < KWTPold ≈ UCT. Since the highest PAO activity and the lowest P-PO_4 content in outlet water were revealed in the sludge of the aerotank operating according to the ISAH technology, where the numbers of *Candidatus* 'A. phosphatis' were lower than in the samples from the KWTPold and UCT aerotanks, phosphate accumulation was probably carried out, apart from *Candidatus* 'A. phosphatis', by other microorganisms for which the PAO846 probe was not specific.

The probe ACA652 specific to *Acinetobacter* spp., which has been described as possessing PAO activity [32], was also used to reveal potential PAO. Hybridization with this probe revealed the zones of localization of the target organisms in all sludge samples. Relatively high abundance of *Acinetobacter* spp. in sample 2 (NKWTP2 aerotank), where the lowest PAO activity was observed, as well as the low numbers of target cells in sample 4 (ISAH technology), where the PAO activity was highest, may indicate that *Acinetobacter* spp. was not involved in polyphosphate removal from wastewater. While *Acinetobacter* spp. has been previously considered as true PAO [32], it was presently shown that they did not predominate in EBPR systems [3], which is in agreement with our results.

Abundance of metabolically active planctomycetes. Planctomycetes are typical components of aerobic activated sludge of various wastewater treatment facilities, where they are often detected by FISH. While they are relatively numerous in activated sludge (up to 10% of the total number of DAPI-stained cells), their physiological role in the communities remains unclear [14, 31]. In all aerobic sludge samples, high abundance of planctomycetes was revealed with the Pla46 probe. The numbers of metabolically active planctomycetes increased in the row NKWTP2 < ISAH < KWTPold < UCT (Table 5). The planctomycetes detected in aerotank sludge were probably aerobic chemoorganotrophs. This conclusion was confirmed by hybridization of the samples with the Amx368 probe, specific for most presently known anaerobic chemolithoautotrophic anammox bacteria. The target cells of anammox bacteria were not found in any of the sludge samples, which indicated their absence in the aerobic sludge or low metabolic activity.

Filamentous (foaming) bacteria in activated sludge and foam. Swelling of the sludge and development of foam at aerotank surface due to mass development of filamentous bacteria are examples of response reactions of microbial communities to changed operational conditions. Foam formation in wastewater treatment facilities may be caused by seasonal factors (variations in the temperature mode), changes in the composition of inflowing water (especially of its lipid components), or influx of toxic compounds not scheduled for processing in domestic wastewater

treatment facilities. These factors disrupt the normal operation of aerotanks.

Filamentous bacteria were present in their usual amounts in the sludge from the KWTPold, UCT, and ISAH aerotanks. Their number in the sludge and foam of the NKWTP2 aerotank was increased. Foaming bacteria *Gordonia* spp., including *Gordonia amarae* (formerly *Nocardia amarae*), and *Candidatus* 'Microthrix parvicella' were detected by FISH in all sludge samples.

Cultural techniques were used to reveal *Sphaerotilus* spp. in the foam of the LWTP tank and *Gordonia* spp. and *Acinetobacter* spp. in the KWTP foam. High abundance of *Acinetobacter* spp. in the sludge sample from the NKWTP2 aerotank revealed by FISH and their predominance in the foam of this aerotank revealed by the cultural techniques indicated the role of these bacteria in foam formation, rather than in phosphate accumulation (see above).

Cultural techniques were used to determine the carbon sources for growth of foaming bacteria and the conditions for foam formation. In the case of *Sphaerotilus* sp., sunflower oil (triglycerides of unsaturated fatty acids) was used as the carbon source. Under aeration (shaking) at 20–21°C, *Sphaerotilus* sp. predominated in the community obtained in liquid culture, forming a light film at the surface. With crude oil as the carbon source, *Gordonia* spp. were the dominant component of the community. Cultivation of the foam microbial community on 10 W-40 motor oil resulted in development of *Gordonia* sp. and *Acinetobacter* sp. in a 1 : 3 ratio. Thus, *Sphaerotilus* sp., *Gordonia* sp., and *Acinetobacter* sp. may be considered the indicators of the presence of vegetable oils, petrochemical products, and synthetic engine oils in domestic wastewater. Conditions for development and preservation of the branched net structure by filamentous bacteria were also determined. Together with their hydrophobic properties and forced aeration, such structure may result in dense foam formations at the aerotank surface. Under laboratory conditions, low aeration level and temperatures from 15 to 22°C promoted the development of such structures. At other cultivation conditions, bacteria soon fragmented into short rods, so that no branched structure was observed.

Thus, the activated sludges of the Moscow wastewater treatment facilities were shown to be active eubacterial microbial communities with an insignificant contribution of physiologically active archaea. The total share of microorganisms identified by FISH (nitrifying bacteria, PAO, planctomycetes, and acinetobacteria) was 13–20% of the overall number of metabolically active eubacteria. Stage II nitrifying bacteria and aerobic planctomycetes contributed significantly to the bacterial component of activated sludge, with *Nitrobacter* spp. being the dominant nitrifying organisms. Metabolically active anammox bacteria were not detected. Differences were found between the characteristics of the sludge of aerotanks using different technologies. In the sludge from the aerotank employing the Hannover University technology, which exhibited the highest PAO activity and the lowest $P\text{-}PO_4$ level in outlet purified water apart from *Candidatus* 'A. phosphatis', other microorganisms were probably responsible for phosphate accumulation. The numbers of nitrifying and phosphate-accumulating bacteria in the sludge samples generally correlated with microbial activity in microcosms and with the efficiency of nitrogen and phosphorus removal from wastewater. The highest microbial numbers and activities were found in the sludge of the aerotanks operating according to the technologies of the Hannover and Cape Town universities. The sludge from the Novokur'yanovo facilities, where nitrification and dephosphotation were suppressed due to organic matter overload and abundant growth of filamentous bacteria resulting in foam production, exhibited the lowest activity. *Acinetobacter* spp. probably did not act as PAO and were involved in foam formation, as was confirmed by cultural investigation of the bacterial composition of the foam. The KWTP foam was found to contain foaming bacteria *Gordonia* spp. and *Acinetobacter* spp., which utilized complex compounds of natural and technogenic origin. The LWTP foam contained foaming bacteria *Sphaerotilus* spp., which utilize unsaturated fatty acids. Temperatures from 15 to 22°C and low aeration were the factors determining the preservation of the branched net structure of the filamentous bacteria. The data on abundance and composition of the metabolically active microorganisms determined by FISH may be used for technological control of wastewater treatment process.

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