Microbial Diversity of Aerobic Granular Sludge under Different Operational Conditions

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This paper depicts the research conducted at lab scale on aerobic granular sludge sequential biological reactors for wastewater treatment in order to assess the influence of operational parameters on the treatment performances and microbial diversity of the granules. The proposed objectives were reached by testing in similar reactor conditions different organic nitrogen and phosphorus loading rates. The study focused both on treatment performances as organic, nitrogen and phosphorus removal based on chemical analysis of main quality parameters and on diversity of microbial population in granules based on real-time polymerase chain reaction by targeting mainly the specific species or functional genes with high influence on process performance in terms of nitrification, denitrification and phosphorus removal.

Keywords: *aerobic granular sludge, denitrification, nitrification, real-time PCR.*

Aerobic granular sludge has recently gained the reputation of an all-in-one biological system encompassing different process conditions (aerobic, anoxic, anaerobic) thus allowing the microorganisms within its structure to simultaneously be capable of organic matter, nitrification, denitrification and phosphorus removal in one reactor [1-3] while other processes need several reactors [4,5].

Nitrification-denitrification and shortcut processes variations of these are mainly responsible for nitrogen compounds removal in wastewater treatment systems. Nitrification is the biological oxidation of ammonia or ammonium to nitrite followed by the oxidation of the nitrite to nitrate [6]. The oxidation of ammonia into nitrite is performed by two groups of organisms, ammonia-oxidizing bacteria (AOB, e.g. by *Nitrosomonas* sp. or *Nitrosococcus* sp.) [7] and ammonia-oxidizing archaea (AOA, e.g. by *Nitrosopumilus* sp. and *Nitrososphaera* sp.) [8]. Ammonia oxidizing bacteria use two enzymes, ammonia monooxygenase (a multisubunit enzyme encoded by the *amo*C, *amo*A, and *amo*B genes [9]) and hydroxylamine oxidoreductase, to oxidize ammonia to nitrite via hydroxylamine as an obligate intermediate [10]. Further oxidation of nitrite to nitrate is performed by nitrite oxidoreductase (NXR) considered to be the best candidate as specific functional marker for nitrite oxidizing bacteria (NOB) [11]. This membrane-associated enzyme occurs in two phylogenetically distinct forms, one cytoplasmic type found in the NOB Nitrobacter, Nitrococcus and Nitrolancetus (Sorokin et al., 2012), and one periplasmic type found in Nitrospira and Nitrospina [11,12].

Denitrification is the biological process by which nitrate is reduced to nitric oxide (NO), nitrous oxide, and, finally, dinitrogen, using a series of nitrogen oxide reductases *Nir*K (or *Nir*S), *Nor*B, and *Nos*Z, respectively [13]. The reduction of nitrite by nitrite reductase (*Nir*) is the key step in the denitrification pathway [14]. Two types of structurally different but functionally equivalent nitrite reductase that are translated from the nirS and nirK genes are considered to be the predominant enzymes for catalyzing nitrite reduction [13].

Biological phosphorus removal in wastewater treatment plants is achieved by phosphate accumulating bacteria (PAOs) - a wide range of bacteria able to accumulate polyphosphates as a phosphate reserve [15].

Either we talk about conventional or granular sludge, recent research efforts have stressed that optimization of the microbial community structure and functioning should be a major objective in the design and operation of a treatment system [16-18]. Most efforts are being made for process optimization through monitoring of chemical (e.g. NH_4^+; NO_2^-, dissolved oxygen, *p*H) and physical (e.g. flow rate, temperature) parameters in order to enhance the process safety and the biological reactions. However, a consistent long-term performance can only be ensured when the microbial community within the sludge functions optimally. For this purpose, questions about community structure, activity and the population kinetics have to be answered by means of molecular monitoring tools, which allow to identify and quantify the microorganisms present in the sludge (conventional or granular) of wastewater treatment plants [6,17].

In this study, we monitored the microbial composition of the aerobic granular sludge using real-time polymerase chain reaction (rt-PCR) by targeting mainly the specific species or functional genes with high influence on process performance in terms of nitrification, denitrification and phosphorus removal.

Experimental part

The experiments for wastewater treatment were performed in a lab scale aerobic granular sludge sequential biological reactor with a total working volume of 6 L. The bioreactor was used to evaluate the impact of different organic loading rates on treatment performances and on the microbial diversity of aerobic granular sludge. The experimental setup consisted of: influent vessel (30 L), feeding pump (Heidolph, PUMPDRIVE 5001 peristaltic pump), column type bioreactor, effluent vessel (30 L). The cyclic operation of the bioreactor was ensured by computer-based control system which controlled the feeding pumps, air inlet and effluent outlet electro valves. Total HRT of the bioreactor 8 hours, with the following operational time sequence: anaerobic feeding (10 min.), aerobic reaction (7h 35 min.), settling (5min.) and effluent

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withdrawal (10 min.). During aerobic reaction stage, an air compressor supplied each column an airflow of 8 L/ min.

Sludge samples and corresponding operational conditions:

Sludge samples were collected at different time during operation, centrifuged (1000 rpm, 1 min.) and kept in cryotubes at ultra freezer (-80^{0}C, Thermo Scientific Forma 88000). The loading rates of the bioreactor at the sampling time are listed in table 1. The settling time of the bioreactor at the time of sampling was: 5' (minutes) for sample S1; 4' for sample S2; 3' for samples S3 to S7.

DNA extraction and amplification protocol

The deoxyribonucleic acids (DNA) of microorganisms in granules was extracted using PowerSoil DNA Isolation Kit (MO BIO Laboratoris) as specified by the manufacturer. The method involves steps of mechanical and chemical cell lysate followed by successive steps of precipitation of organic and inorganic substances (non-DNA), fixing DNA on a selective membrane and elution thereof in a buffer solution. The isolated DNA was stored at -20^{0}C. Real-time PCR was performed on the QuantStudio Flex 7 (Applied Biosystems).

Amplification reactions were performed with the SYBR Green PCR master mix (Applied Biosystems). Reverse and forward primer (100nM each), template DNA (70 ng) and nuclease free water (up to 50 μL) were added to 25 μL of SYBR Green PCR master mix in MicroAmp Optical 96-well reaction plate.

The real-time PCR thermocycling steps for all primer sets were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 1 min, 50°C for 1 min, and 60°C for 1 min. In all experiments, appropriate negative control containing no template DNA were subjected to the same procedure to exclude or detect any possible contamination or carryover.

Table 1

LOADING RATES OF MAIN NUTRIENTS

Sample	COD	NH_4^+	PO_4^{3-}
	Loading rate (kg/m^3/day)		
S1*	1±0.10	0.04	0.02
S2*	0.5 ±0.09	0.02	0.03
S3*	1±0.10	0.02	0.03
S4*	1±0.10	0.08	0.03
S5**	0.5 ±0.09	0.02	0.01
S6**	0.2 ±0.09	0.01	0.01
S7**	0.5 ±0.09	0.02	0.02

* industrial wastewater from dairy industry;
**acetate based synthetic wastewater adapted from [18]: acetate only as carbon source up to the necessary organic loading rate.

Table 2

PRIMER SETS INCLUDED IN THE REAL TIME PCR ASSAY

Target	Nucleotide sequence (5'-3')
Primer	
Nitrospira	
*nsr*1113f	CCTGCTTTCAGTTGCTACCG
*nsr*1264r	GTTTGCAGCGCTTTGTACGC
Nitrosomonas	
amo 598f	GAATATGTTCGCCTGATTG
amo 718r	CAAAGTACCACCATACGCAG
Ammonium monooxigenase (AMO)	
Amo A 1F	GGGGTTTCTACTGGTGGT
Amo A2R	CCCCTCKGSAAAGCCTCCTCC
16sRNA AOB (Amonium oxidizing bacteria)	
CTO189FA/B[a]	GGAGRAAAGCAGGGGATCG
CTO189FC[a]	GGAGGAAAGTAGGGGATCG
CTO654R	CTAGCYTTGTAGTTTCAAACGC
16s rRNA *Nitrobacter*	
*fgps*872	TTTTTTGAGATTTGCTAG
*fgps*1269	CTAAAACTCAAAGGAATTGA
Nitrite oxido-reductase β subunit (nxrB)	
*nxr*B 1F	ACGTGGAGACCAAGCCGGG
*nxr*B 1R	CCGTGCTGTTGAYCTCGTTGA
Nitrite reductase (nirK)	
nir K1F	GGMATGGTKCCSTGGCA
nir K5R	GCCTCGATCAGRTTRTGGTT
Nitrite reductase (nirS)	
nirs CD3AF	GTSAACGTSAAGGARACSGG
nirs R3	GASTTCGGRTGSGTCTTGA
NO Reductase	
c*nor*b-2F	GACAAGNNNTACTGGTGGT
cnorb-6R	GAANCCCCANACNCCNGC
N_2O reductase	
*nosz*F	CGYTGTTCMTCGACAGCCAG
nosz 1622 R	CGSACCTTSTTGCCSTYGCG
Phosphate accumulating organisms (PAO)	
pao462f	GTTAATACCCTGWGTAGATGACGG
pao651r	CCCTCTGCCAAACTCCAG
pao846r	GTTAGCTACGGCACTAAAAGG
Universal bacteria	
341 F	CCTACGGGAGGCAGCAG
515R	AATCCGCGGCTGGCA

[a]A mixture of CTO 189fA/B and CTO 189fC at the weight ratio of 2:1 was used as the forward primer as described by [19]

For all sludge samples, a series of primer pairs identified in literature [19-24] were used (table 2).

Analytical determinations

Treatment performances were evaluated based on COD, NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}. COD was analyzed volumetrically based on potassium dichromate method according to the ISO standard (SR ISO 6060:1996) and using heating mantle (Model KI16, Gerhardt, Germany). NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} were determined according to the SR EN ISO 14911:2003 and SR EN ISO 10304/1:2009 standards (for the last two indicators), respectively, using ion chromatography system ICS-3000 (Dionex, USA).

Results and discussions

For evaluating the microbial diversity and evolution of aerobic granular sludge under different operational parameters (table 1) relative quantitative real-time PCR assays were performed. Seven samples from the same reactor at different time intervals were chosen on the basis of treatment performances in terms of nitrification / denitrification and phosphorus removal performances (fig.1) and after important operational parameter change (settling time, nutrient loads, wastewater type).

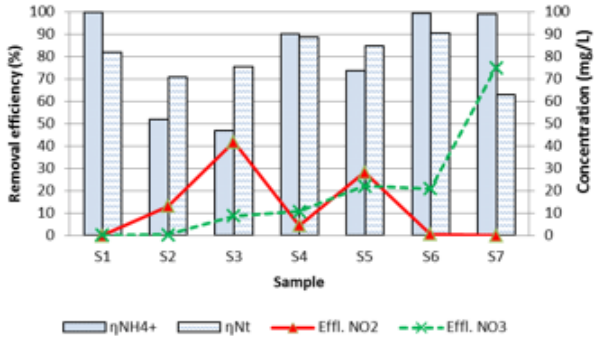

Fig. 1. Ammonium and total nitrogen removal efficiencies correlated with residual nitrite and nitrate in the effluent

Nitrification performance decreased from >99% (sample S1) to 47% in sample S3 once with the decrease of settling time from 5 to 3 min due to partial washout of biomass with low settling ability and to approximately 80% when the type of influent was changed which is correlated with the changes in the balance of nitrifiers (fig.2). For instance, in most samples the dominant target were the ammonium oxidizing bacteria (AOB), whereas for sample S3 a lower abundance was found while the dominant species was found to be *Nitrosomonas sp*, known to be

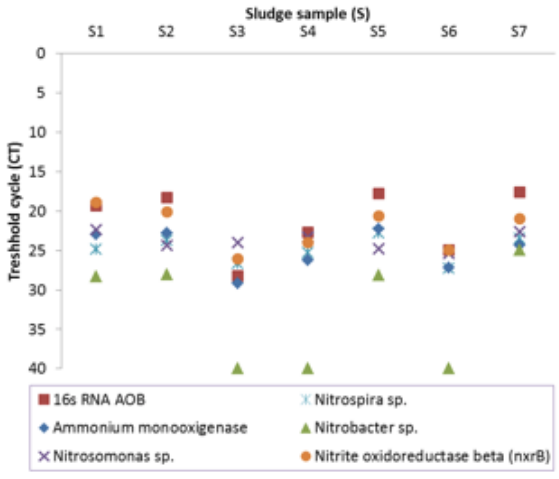

Fig.2. Nitrifiers diversity and distribution within sludge samples

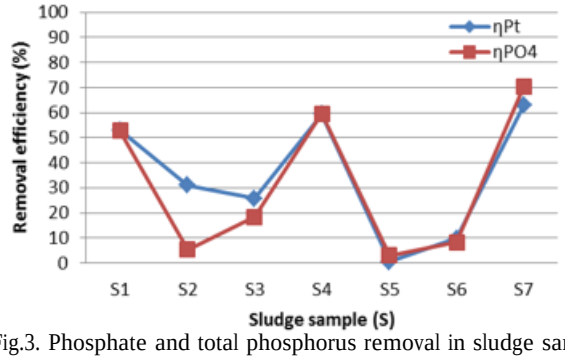

Fig.3. Phosphate and total phosphorus removal in sludge samples

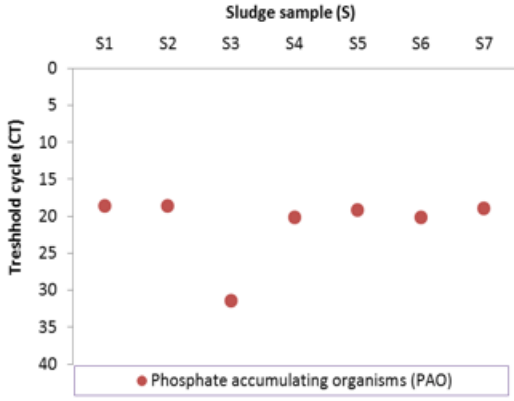

Fig.4. Comparative analisys of phosphate accumulating organisms (PAOs) abundance in sludge samples

responsible for ammonium oxidation to nitrite. The shift in species balance and dominance of Nitrosomonas sp. Also correlates with the high nitrite concentration in the effluent (fig.1). Indeed, the S3 sample corresponds to the lowest ammonium removal efficiency (47%). Samples S4 and S6, originating from the bioreactor when good ammonium removal performances were recorded at the moment of sampling, showed a good balance between the nitrifier species responsible for oxidation of ammonium to nitrite and further nitrite to nitrate.

Denitrification performances of the bioreactor correlates on one hand with the nitrification performances decrease as high amounts of nitrite accumulates and, on the other hand, with the other operational parameters change: decreasing the settling time to 3 min (due to partial biomass washout) and changing the type and concentration of the organic load in the influent since denitrification is a heterotrophic process highly dependent on the organic load. The low abundance of nitrite reductase (fig.2) and shift in microbial balance in terms of target functional enzymes explains the low performances of the bioreactor at the time of S3 sampling. Nitrite reductase specific genes abundance in the microbial population of the granules, the nirS gene, which translates to cytochrome cd1-containing nitrite reductase, is more widely distributed than the nirK gene, which translates to copper-containing nitrite reductase which is also suggested by other authors [14].

The considered sludge samples correspond to various treatment performances in the bioreactor in terms of phosphorous removal (with efficiencies varying from 5% to 70%, fig.3).

PAO phosphorus accumulation bacterial populations (fig. 4), according to PCR analysis, are somewhat correlated with phosphorus removal efficiencies (between 7 and 70%, fig. 3). The phosphorus removal efficiencies correlate with the diversity and dynamics of the heterotrophic microorganisms responsible for denitrification, thus associating with a biomass-based removal.

Conclusions

Analyzing the diversity and dynamics of nitrifying and denitrifying bacterial populations in the lab scale bioreactor we can conclude that there is a close correlation between the performance of biological processes (nitritification, nitrification, denitrification) and the distribution of the directly involved species (AOB, nitrosomonas, nitrobacter, nitrospira) specific genes which codes for functional enzymes (ammonium monooxigenase, nitrite oxido-reductase beta, N2O reductase, nitrite reductase, NO reductase), as well as operational parameters (food type in particular) or impact of parameters change.

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