# Correlation Between Wastewater Treatment Performances and Sludge Characteristics

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*The balance between biotechnological useful microorganisms species, as well as the aerobic sludge granules morphology influences the treatment plant performance. This paper presents an attempt to correlate the experimental results on wastewater treatment performance with aerobic granular sludge structural community. The experiments were conducted in two lab scale bioreactors operated in parallel at different retention times. Treatment performances achieved in both systems lead to an effluent that complies NTPA001 limits, both systems being able of simultaneous nitrification / denitrification and phosphorus removal. For qualitative and quantitative analysis of aerobic granular sludge specific microorganisms, DNA has been succeesfully isolated and purifyied from sludge samples, thus obtaining bacterial DNA extracts in concentrations of up to 56 ng/mL and 78% purity. The resulted DNA extracts were used for qPCR amplification. Amplification was carried out in the presence of a series of 10 pairs of primers for the detection / quantification of specific bacteria and genes involved in the treatment process: universal bacteria; Micotrix parvicella; Ammonia oxidizing archaea; Ammonium monooxigenase; Nitrobacter Sp.; nitrite reductase; $N_2O$ reductase; phosphorus accumulating microorganisms. The experimental results showed a qualitative and quantitative improvement of the sludge quality in terms of species distribution and share of biotechnologically useful bacteria.*

*Keywords: aerobic granular sludge, wastewater treatment, polymerase chain reaction (PCR), DNA*

In each wastewater treatment plant biological stage, during time, the microbial composition is becoming stable and specific for the influents' particular pollutant matrix [1] and subsequently a direct link is established between the microbial composition of activated sludge (AS) and the efficiency of wastewater treatment plants (WWTPs). The long term development and maintenance of stable interconnected trophic relationships between microorganisms is by far the main target of WWTP operators in order to ensure stable performances and to avoid nonstable growth, pin floc problems, deficient nitrification/denitrification as well as filamentous bulking or foaming with a major impact on the effluent quality [2, 3]. In most cases the operational control of WWTPs is conducted based on sensors (for DO, ammonia or nitrate concentration) and on physical-chemical analyses of influent/effluent/sludge quality parameters. However, any change of the operational parameters or influent characteristic (pollutant shock loads) induces firstly modifications in the microbial populations composition of AS, influencing the metabolic pathways [4, 5] thus favoring the suppression/growth/overgrowth of species, sometimes with long term effects on wastewater treatment performances, if proper response actions are delayed.

Bulking and foaming are wide-spread problems, being reported by many wastewater treatment plants around the world, causing: sludge loss (translated in low treatment performance and reduced effluent quality), recirculation difficulties, depletion of oxygen transfer thus inhibiting nitrification, and difficulty in maintaining the appropriate sludge concentration in the aeration basin [2].

Microscopic quantification of AS quality is currently based on evaluating its macroscopic and microscopic characteristics [6] and communities characterization, in particular, protists and filamentous bacteria as bioindicators [7-9].

The biochemical and genetically basis of microbial degradation is linked to several enzymes allowing microorganism to biodegrade various pollutants.

Nitrifying bacteria are chemolithotrophic organisms that include species of the genera *Nitrosomonas, Nitrosococcus, Nitrobacter* and *Nitrococcus*. These bacteria get their energy by oxidizing inorganic nitrogen compounds [10]. Types include ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Many species of nitrifying bacteria have complex internal membrane systems that are the location for key enzymes in nitrification: ammonia monooxygenase (which oxidizes ammonia to hydroxylamine), hydroxylamine oxidoreductase (which oxidizes hydroxylamine to nitric oxide - which forms nitrite in the presence of oxygen), and nitrite oxidoreductase (which oxidizes nitrite to nitrate). Both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) have the enzyme ammonia monooxygenase.

Denitrifying bacteria are able of performing denitrification as part of the nitrogen cycle. They metabolise nitrate using a complex set of enzymes (nitrate reductase, nitrite reductase, nitrite oxide reductase and nitrous oxide reductase) turning step by step nitrogen oxides back to nitrogen gas.

Efforts are currently being made to identify, improve and intensify treatment performances as effluent quality requirements are increasing [10-12]. The identification and quantification of specific genes could be linked to key microbial species and biological processes involved in wastewater treatment such as nitrification, denitrification, and phosphorus removal thus offering a good image of treatment performances [7] and/or of required operational parameters adjustments.

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## Experimental part

*Materials and methods*

Two identical column type sequential biological reactors (D and GSBRs) with a height to diameter ratio of 10 and a total working volume of 6 L were used to evaluate the hydraulic retention time (HRT) impact on the treatment performances and on aerobic granular sludge microbial diversity. Each of the SBR reactors consisted of: influent vessel (40 L), feeding pump (Heidolph, PUMPDRIVE 5001, and peristaltic pump), column type bioreactor, effluent vessel (60 L). The cyclic operation of the SBR systems was ensured by computer-based SBR control system which controlled the feeding pumps and air inlet and effluent outlet electrovalves. Total HRT of the bioreactors was 6 and 8 hours respectively, with the following operational time sequence: anaerobic feeding (10 min.), aerobic reaction (D=5h 35min; G=7h 35 min.), settling (5min.) and effluent withdrawal (10 min.). During aerobic reaction stage, an air compressor supplied each column at an airflow of 8 L/min.

Both bioreactors were fed with dairy industry wastewater characterized by high organic and nutrients load: 968 – 2390 mg $O_2$/L CODCr; 492 – 1806 mg $O_2$/L $BOD_5$; 36 – 67 mg/L $NH_4^+$; 46 – 72 mg/L total N; 5– 17 mg/L total P.

*Analytical determinations*

Treatment performances were evaluated based on COD, $NH_4^+$, $NO_2^-$, $NO_3^-$ and $PO_4^{3-}$. COD was analysed 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).

*DNA extraction and amplification protocol*

The deoxyribonucleic acids (DNA) of microorganisms in granules was extracted using PowerSoil DNA Isolation Kit and protocol (MO BIO Laboratoris). 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. Using this method, bacterial DNA extracts from aerobic granular sludge (10 mg) were made at concentrations up to 56 ng / ìL and 78% purity. DNA extracts obtained from sludge samples of both bioreactors at different time intervals were subjected to specific PCR amplification in the presence of PowerSYBR Green PCR Master Mix (10 ìL), reverse and forward primer (100nM each), template (100 ng) and nuclease free water (up to 20 ⌈L).

For all sludge samples, a series of primer pairs identified in literature [13-19] were used: *Micotrix parvicella* M1 f (GGTGTGGGGAGAACTCAACTC) and M2 r (GACCCC GAAGGACACCG); *Ammonia oxidizing archaea* arch-amoAF (STAATGGTCTGGCTTAGACG) and Arch-amoAR (GCGGCCATCCATCTGTATGT); Ammonium mono-oxigenaseAMO A-1f (GGGGTTTCTACT GGTGGT) and Amo A-2r CCCCTCKGSAAAGCCTCCTCC; *Nitrobacter Sp.* FGPS872 (TTTT TTGAGATTTGCTAG) and FGPS 1269' (CTAAAACTCAAAGGAATTGA); Nitrite Reductase NIR kNIRk1f (GGMATGGTKCCSTGGCA) and NIRk 5R (GCCTCGATCAGRTTRTGGTT); $N_2O$ reductase (NOSz) - NOSz-f (CGYTGTTCMTCGACAGCCAG) andNOSz 1622 r (CGSACC TTSTTGCCSTYGCG); Phosphate accumulating microorganisms pao462f (GTTAATACCCTGW GTAGATG ACGG) and pao651r (CCCTCTGCCAAACTCCAG) and pao 846R (GTTAGCTACG GCACTAAAAGG); PAO I-179L (ACAGATCAACAAGTTCTACATCTTCGAC) and I-179R (GGTGTTGTCGTTCCAGTAGAGGATGTC); Universal Bacteria 341F (CCTACGGGAGG CAGCAG) and 515R (AATCC GCGGCTGGCA).

Amplification protocol: initial template denaturation (10 min, 95$^0$C) followed by 40 cycles of amplification (denature at 95$^0$C for 15 sfollowed by 60 sof annealing/extending at 60$^0$C).

## Results and discussions

The treatment performances achieved in both bioreactors, in terms of COD removal exceeded at all times 90%. The quality of the effluent obtained was within the limits of the national regulation [20] with a small exception for the G installation (SBR cycle duration of 8 h) where exceedances of the norms for nitrate were recorded fig.1). However, given the high ammonia load of the influent water, we can consider that the aerobic granular sludge operates in parameters while simultaneously removing the organic matter, nitrifying / denitrifying and removing the phosphorus (figs. 1 and 2).

The explanation behind incomplete denitrification in bioreactor G (after 8 h) could reside in the fact that a longer famine period leads to fast organic load depletion during the first hours of the treatment cycles leaving no COD for denitrification heterotrophic process to occur. On the other hand, long famine periods (in bioreactor G) favours the phosphorus uptake by phosphate accumulating micro-organisms.

The microbial diversity in aerobic granular sludge samples compared to a conventional activated sludge sample (used as inoculum) was assessed using DNA

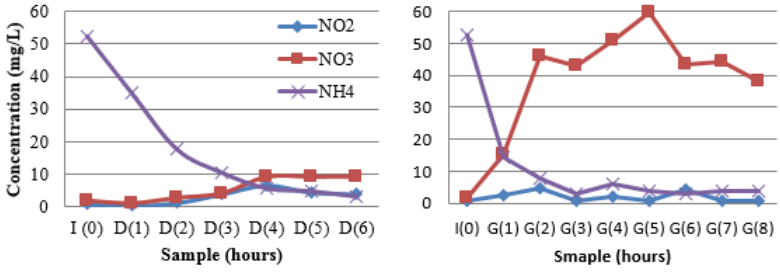


Fig.1. Evolution in time, over an operating cycle, of nitrogen (ammonium, nitrate, nitrite) in the two bioreactors: bioreactor D (6 h), bioreactor G (8 h).

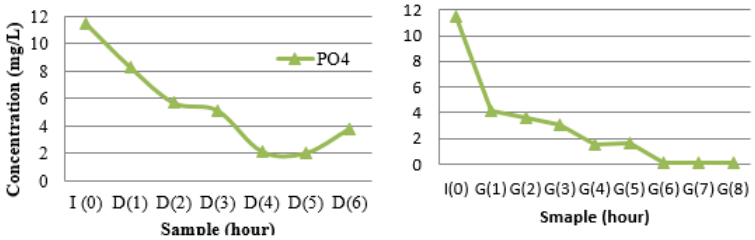


Fig.2. Evolution in time, over an operating cycle, of phosphorus (phosphate) concentration in both bioreactors: bioreactor D (6 h), bioreactor G (8 h)

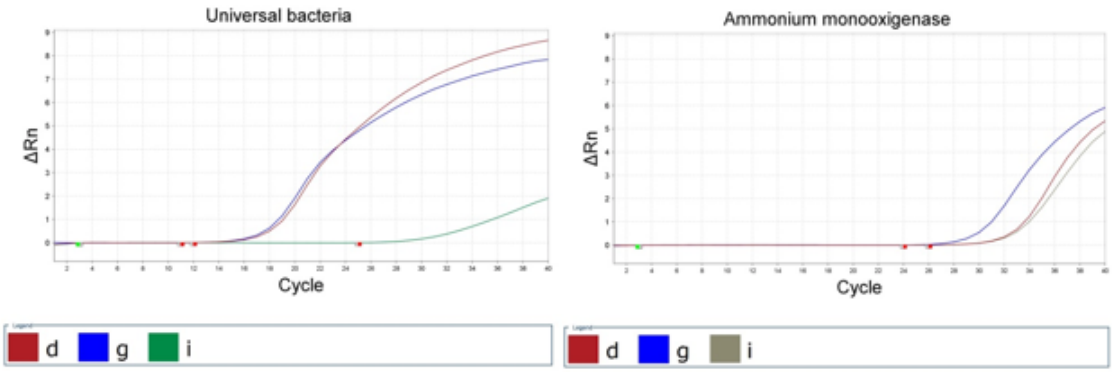


Fig. 3. Amplification plot of DNA samples (bioreactor D, bioreactor G and inoculum) in the presence of universal bacteria primers and specific primers for nitrifiers (ammonium monooxigenase)

monooxigenase)

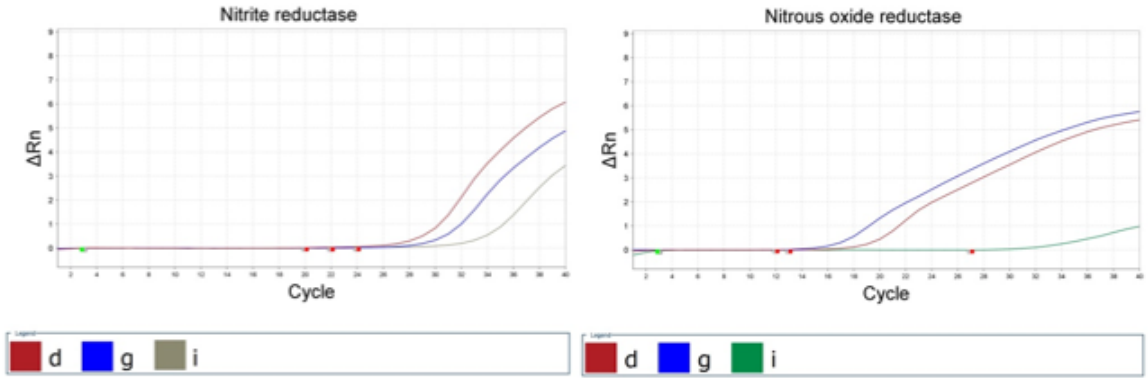


Fig.4. Amplification plot of DNA samples (bioreactor D, bioreactor G and inoculum) in the presence of specific primers for denitrifiers (nitrite reductase and nitrous oxide reductase)

amplification in the presence of fluorescent SYBR Green. Thus, the intensity of the fluorescence increases as the number of amplicons increases and correlates with the concentration and proportion of initial specific DNA sequence of interest in the total DNA sample.

The amplification plot of DNA extracts in the presence of universal bacteria primers (fig. 3) clearly shows the difference in bacterial density between conventional activated sludge (sample *i*) and aerobic granular sludge (samples *d* and *g*). The amplification plot of DNA samples in the presence of gene detection primers (amo) specific to most nitrifying bacteria shows a better representation of the nitrifying bacteria in the granular sludge microbial population of reactor G (8 h) which is also reflected in the higher maximum ammonium oxidation rates in reactor G (38.26 mg/h) compared to reactor D (17.6 mg/h).

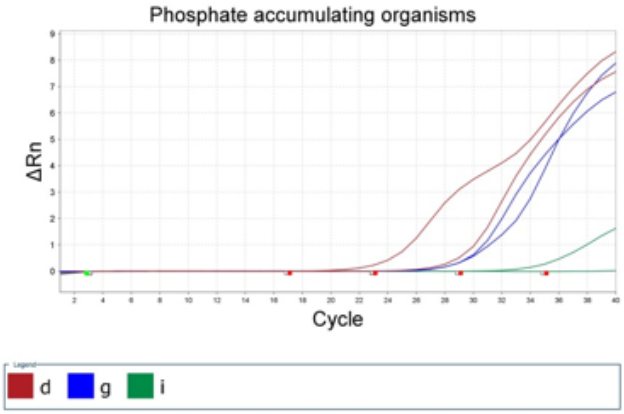


Fig.5. Amplification plot of DNA samples (bioreactor D, bioreactor G and inoculum) in the presence of specific primers for phosphate accumulating organisms (PAO, PAO1)

The amplification plots of DNA samples in the presence of primers specific for genes responsible for enzymes involved in denitrification process (nitrite reductase and nitrous oxide reductase) show a better representation of denitrifying bacteria within the microbial population of granular sludge compared to inoculum. Moreover, the results emphasizes that the microbial population of the granular sludge in bioreactor D has a higher density of denitrifiers capable of reducing nitrite to nitrite oxide.

The amplification plots of DNA samples in the presence of primers specific for phosphate accumulating microorganisms involved in enhanced biological phosphorus removal (EBPR) show a better representation within the microbial population of the granular sludge samples (d and g) compared to inoculum from a conventional activated sludge process without EBPR.

## Conclusions

Amplification of DNA sampled from aerobic granular sludge in both reactors and conventional activated sludge (used as inoculum) in the presence of universal primer-specific DNA sequences, bacterial identification primers, and specific gene detection primers involved in key biological processes of wastewater treatment revealed higher density of microorganisms of interest (nitrifiers, denitrifiers, phosphate accumulating bacteria), better species distribution and proportion of bacteria responsible for the biological processes involved in the treatment technology. Even though good correlations concerning microbial diversity in granular/activated sludge can be obtained by quantifying specific DNA in the samples, RNA amplification could provide useful data on activity of specific metabolic mechanisms at molecular level.

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