This is a post-peer-review, pre-copyedit version of an article published in Bioprocess and Biosystems Engineering. The final authenticated version is available online at: https://doi.org/10.1007/s00449-018-2028-7

TITLE PAGE

Title:

Startup strategy for nitrogen removal via nitrite in a BAF system

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Abstract

A biological aerated filter (BAF) pilot plant consisting in two reactors (aerobic and anoxic one) was used to determine a strategy to remove nitrogen via nitrite. RNA/DNA analysis was performed to assess microbial activity and support chemical results. In less than 13 days the pilot plant was able to remove COD and suspended solids. Nitrogen removal via nitrite pathway could not be observed until day 130th when the empty bed contact time (EBCT) was set at 0.71h. Nitrite was detected in the aerated BAF effluent but never nitrate. qPCR of *amoA* gene from RNA and DNA extracts of the aerobic biofilm confirmed that ammonia oxidizing bacteria (AOB) were present from the beginning of the operation but not active. AOB activity increased by time reaching stability from operational day 124th. The combination of both, low EBCT together with high OLR, has been demonstrated to be a feasible strategy to startup a BAF to achieve nitrogen removal via nitrite.

Keywords. RNA, nitrite, ammonia oxidizing bacteria, partial nitrification, NGS

Introduction

Conventional activated sludge (CAS) is the most spread secondary treatment for urban wastewater to remove organic matter (COD), suspended solids (SS) and nitrogen [1]. However, CAS systems have a high footprint as well as a high energy demand due to aeration and high hydraulic retention times (HRT). Several alternative designs and systems have been investigated and implemented in order to reduce energy requirements and footprint. One of these alternatives that has been successfully applied as secondary treatment at lab and full scale to treat urban wastewater is the biological aerated filter (BAF) technology [2-5].

BAF systems consist of two main phases: a solid phase that acts as the support media for microbial biofilm growth as well as physical filtration and a liquid phase in which the solid phase is submerged [6]. It has a small footprint compared with CAS since BAF can be operated at high organic loading rates (OLR) as biomass is well retained by biofilm formation and do not require further downstream separation units [3, 7]. Thus, BAF system allows the operation at short HRT which reduces the requirement for space. Nevertheless, energy consumption is similar or even higher to CAS as the same amount of air must be injected into the system to fully oxidize ammonium and COD as well as for backwash purposes.

Nitrogen removal from ammonia via nitrite instate of nitrate is a pathway that reduces aeration requirements by 25%, saves the carbon-source requirement during the denitrification phase by 40% [8] and contributes to lower sludge production. This pathway has been successfully applied to rich ammonium streams such as landfill leachate or anaerobic digesters supernatants [9, 10] as well as to urban wastewater with an imbalanced COD/N ratio [11, 12]. Nitrite accumulation (also known as nitritation or partial nitrification) is achieved during startup when nitrite oxidizing bacteria (NOB) are out-competed from the system and ammonia oxidizing bacteria (AOB) are enriched. In urban wastewater treatment two main parameters are used during the startup phase to achieve nitritation: low sludge retention time (SRT) [10] and low dissolved oxygen (DO) concentration (<1 mg O_2 L^{-1}) [11, 13]. Denitrification must be accomplished in anoxic conditions. Thus, it is necessary to have a specific anoxic reactor or to obtain denitrification inside of the biofilm where oxygen is not diffused but nitrite and COD.

On one hand, SRT can be estimated in BAF systems [14]. However, SRT is a parameter that can be hardly controlled in a BAF as it is a biofilm based system. On the other hand, BAF is a plug flow reactor and DO concentration also results hard to control as it varies throughout all the column although a study has been directed on that with ambiguous results [13]. Lately, Ryu et al.[7] determined that a short HRT (1 h) combined with a high organic loading rate (OLR) resulted on nitrite accumulation in a lab-scale BAF. Thus, a main hypothesis can be developed. A short contact between biofilm and wastewater known as empty bed contact time (EBCT) together with the regulation of the OLR could be a suitable tool to obtain nitrogen removal via nitrite in a BAF system. Moreover, the startup phase is a key period to inhibit NOB growth or achieve its washout [15].

Besides, molecular techniques have been applied to follow up the acclimation and enrichment of the biomass such as fluorescence in-situ hybridization (FISH), polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), quantitative PCR (qPCR) and next generation sequencing (NGS) of the present DNA [16-20]. Nevertheless, DNA analysis does not show the activity of the biofilm but only its genetic potential. Thus, RNA analysis would increase the knowledge of biomass activity regarding biological wastewater treatment in BAF systems, and could be a good tool for monitoring the startup of the system.

Therefore, the main objective of this study was to obtain a strategy to startup a BAF to remove COD, SS and nitrogen via nitrite with the shortest HRT without inoculation. Molecular tools based on RNA and DNA analysis were used in order to assess biofilm enrichment during the startup and confirm chemical analysis.

Materials and Methods

BAF pilot plant setup

The BAF pilot plant (Figure 1) was located on-site in the wastewater treatment plant (WWTP) of Caldes de Montbui (Catalonia, Spain) and had a total reaction volume including anoxic, aerobic, sand filter and water cleaning tank of about 10 m^3. Influent wastewater (Table 1) was collected after the pretreatment section of the full scale urban WWTP and was fed into a lamellar settler of 1m^3 where system purge was applied when necessary (timed 2 min per hour). Afterwards, settled water flowed into a mixing basin where recirculation was also pumped. This mix was pumped into the downflow anoxic BAF (BAF1) which had a working volume of 2.5 m^3 and was filled with 1.2m^3 of expanded clay as filtering and support material (Filtralite® HR 3-6 mm; Norway). Water was then pumped into the upflow aerobic biofilter (BAF2) which had equal physical characteristics as BAF1 but with smaller diameter of the support material (Filtralite® HR 2.5-5 mm; Norway). DO concentration was controlled between 4-5 mg O_2 L^{-1} to ensure full aerobic conditions in the water column by means of a PID control system and a DO probe (E+H COS61D; Germany) installed at the top of the reactor with contact with the surface water. BAF2 effluent was equipped with a digital sensor of ammonium and nitrates calibrated with samples analysed off-line (ISEmax CAS40D; Germany) and was pumped into a water cleaning tank with a volume of 4 m^3. Water cleaning tank was also equipped with a spectro::lyser™ UV sensor (SCAN; Austria) which allowed monitoring NO_2^-, NO_3^-, SS and COD concentrations also calibrated by mean of treated wastewater analyzed in the laboratory. From this tank, treated water was pumped into a sand filter (same characteristics as BAF1 and BAF2 having a mix of media Filtralite® HR 0.8-1.6 mm and Filtralite® HR 1.5-2.5 mm) to remove the remaining SS as final treatment before discharging. Besides water from water cleaning tank was recirculated into the initial mixing basin to denitrify the accumulated nitrite and it was also used as backwashing water when necessary. Backwash cleanings by air and water were applied when necessary based on head pressure loss signal controlled by on-line pressure device located at the bottom of the reactors. Aeration was carried out by a blower injecting air into the system at the bottom of BAF1, BAF2 and sand filter. Sludge water obtained after backwashes was driven into a sludge water tank which was later pumped again into the lamellar settler.

The pilot plant was provided with a programmable logic controller (PLC) and supervisory control and data acquisition (SCADA) system that allowed digital and analogical data purchase with own-developed software. PLC controlled all the automatic devices and control loops of the plant: aeration, backwashes, pumping, levels, alarms, etc.

Analytical methods and calculations

The determination of influent and effluent ammonium (NH_4^+), nitrite (NO_2^-; by colorimetric analysis), total Kjeldahl nitrogen (TKN), chemical oxygen demand (COD), five-days biochemical oxygen demand (BOD_5), total suspended solids (TSS) and volatile suspended solids (VSS) concentrations was carried out according to standard methods [21]. Nitrate (NO_3^-) concentration was analyzed by ion chromatography (Metrohm 861 Advanced Compact IC), using a Metrohm Metrosep A Supp 4 column and pre-column, a metrosep A Supp 4/5 Guard.

Empty bed contact time (EBCT), the key parameter to control the system was calculated according to equation 1

$$EBCT = \frac{V_{mediaBAF2}}{Q_{inf}} \times 24 \qquad \text{(equation 1)}$$

where:

$V_{media\,BAF2}$ is the media volume of BAF 2 (m^3 aerobic media)
Q_{inf} is the influent flow ($m^3\ d^{-1}$)

Organic loading rate (OLR), specific organic loading rate (sOLR), Nitrogen Loading Rate (NLR), ammonium loading rate (ALR), specific ammonium loading rate (sALR), nitrite production rate (NPR), hydraulic retention time (HRT) and empty bed contact time (EBCT) were calculated according to formulas added in the SM section.

Molecular techniques

RNA and DNA extracts were obtained by independent triplicates from biofilm samples collected on days 18th, 82nd, 103rd, 124th and 145th. 16S rRNA genes and transcripts from bacterial population were measured by the couple of primers F519 / R907 as previously reported in Prenafeta-Boldú et al.[22]. Ammonia oxidizing bacteria (AOB) were studied by ammonia monooxygenase α-subunit encoding gene (*amoA* genes/*amoA* transcripts) as previously reported by Rotthauwe et al. [23]. qPCR assays are detailed in supplemental material. The present and metabolically active bacterial diversity was also assessed by 16S rRNA-based Miseq Illumina platform by targeting V1-V3 region of 16S rRNA as previously described in Pelissari et al. 2017. In the present study NGS results were from days 124th and 145th. Data from MiSeq assessment were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) with the accession number PRJNA320476. Further detailed information can be found in methodology section of supplementary information document.

Results

BAF pilot plant performance

The BAF pilot plant was designed to remove COD, SS and nitrogen via nitrite. It has been operated over a period of 160 days without inoculation to ensure similar conditions during real scale startup and was fed from the first day with urban wastewater collected after the pretreatment section of the full scale urban WWTP of Caldes de Montbui (Catalonia, Spain). The initial influent flow was set at 18 m^3 d^{-1} and the HRT of the whole pilot plant was about 13h. Recirculation from the water cleaning tank was fixed at 2.5 times influent flow and the dissolved oxygen concentration in the BAF2 was set at 4 mg $O_2 \cdot L^{-1}$ by PID control to ensure full aerobic conditions. SRT was not controlled. The first objective of the operation was to remove COD and SS at very short HRT.

Figure 2 depicts temporal evolution of influent and effluent concentrations of total COD as well as the organic loading rate (OLR; Fig.2.A) and TSS (Fig.2.B). Total COD influent concentration fluctuated between 222 and 1040 mg COD L^{-1} indicating typical variation of the urban wastewater COD due to sampling time as well as the high daily variability of influent COD [1]. Influent TSS concentration also had a great variation between 95 and 692 mg SS L^{-1} according to fluctuations of influent COD concentration (Fig. 2). The high variability of influent COD concentrations and the daily flow changed during the startup in order to increase EBCT and obtain nitritation (Fig.2) resulted in also varying OLR between 1 and 5 kg COD m^{-3} media d^{-1} during all the experimental period. However, despite these fluctuations in influent characteristics, the pilot plant effluent remained stable and below the discharge legal limits of the European Union (EU) standards for both COD (125 mgCOD L^{-1}) and TSS (35 mg SS L^{-1}) from day 13th of the process. It is also remarkable that effluent BOD_5 remained always below the legal limit of 25 mg BOD L^{-1} (data not shown). From day 13th to the end of the experimental period, the BOD_5, COD and TSS removal efficiencies were 94.3±4.6%, 79.6±8.7% and 92.1±7.3%, respectively. Nevertheless, ammonium oxidation, thus nitrogen removal could not be achieved until day 130th approximately.

Nitrogen removal

Once the desired COD and TSS removal was achieved, the main goal was to achieve nitritation by favoring conditions of AOB in front of NOB. Influent total nitrogen (TN_{inf}) concentration varied in a range of 40 to 80 mg N-TN L^{-1} during all the experimental period (Fig.3.A). On the other hand, effluent TN concentration remained stable in a range between 20 and 40 mg N L^{-1} during the first 60 days. These concentrations were much higher than the EU legal threshold value of 15 mg N-TN L^{-1}. The removal of part of the TN could have been achieved by nitrification-denitrification, removal of the organic nitrogen contained in the influent TSS, volatilization and assimilation by the growth of biomass. In this sense, Figure 3.B shows the temporal evolution of the ammonium concentration in the influent and effluent of the pilot plant to verify the existence of ammonium oxidation. It can be observed that during the first 60 days of operation, ammonium concentration was similar in the influent and effluent being about 30 mg $N\text{-}NH_4^+$ L^{-1} and the nitrogen loading rate (NLR; Eq.S3) and the ammonium loading rate (ALR; Eq.S4) were 0.4 kg N m^3media d^{-1} and 0.5 kg N m^3aerobic media d^{-1}, respectively. On day 60th, daily influent flow was decreased from 18 to 12 m^3 d^{-1} in order to reduce both NLR and ALR. Then, the pilot plant was operated during 70 days more with this operational conditions.

The effluent TN concentration remained stable as before approximately at 30 mg N-TN L^{-1} (Fig.3.A) as well as the ammonium effluent concentration being about 30 mg $N\text{-}NH_4^+$ L^{-1} (Fig.3.B) despite the ALR and the NLR were decreased approximately to 0.3 kg N m^3media d^{-1} and 0.4 kg N m^3aerobic media d^{-1}, respectively. Flow decrease results in less volatilization and less biomass growth, thus less N assimilation. Those facts could explain the behavior of the reactor. On day 130th, the daily influent flow rate was again decreased to 6-8 m^3 d^{-1} resulting in a NLR and ALR of about 0.2 kg N m^3media d^{-1} and 0.2 kg N m^3aerobic media d^{-1}, respectively. Since that day, effluent TN concentration decreased below the legal limit of 15 mg N-TN L^{-1} at the same time that ammonium was being oxidized (Fig.3.B). When decreasing HRT, EBCT increased and resulted in a higher contact between present ammonia and nitrifiers, thus nitrification started to take place. Nevertheless, no nitrate neither nitrite was observed in the pilot plant effluent (data not shown). In this sense, BAF2 operation was followed by analyzing its influent and effluent characteristics in order to reveal if nitrification was taking place and to identify if other phenomena are occurring (volatilization, assimilation, etc.).

Aerated BAF operation

On days 103rd, 124th and 145th, influent samples and effluent samples taken after the corresponding HRT from BAF2 were analyzed to characterize its COD and nitrogen removal. Table 2 shows main chemical analyzed parameters as well as operational parameters. For days 103rd and 124th, the influent flow rate, the HRT and the EBCT were equal corresponding at the second period when the daily flow was set at 12 m^3d^{-1} (Fig.2). COD removal was observed at both runs despite VSS increased in the BAF2 effluent due to sludge wash and variations on the OLR due to differences on COD concentration. On the other hand, ammonium concentration remained stable on day 103rd but slightly decreased 1 mg $N\text{-}NH_4^+$ L^{-1} on day 124th. These coincided with the detection of 1 mg $N\text{-}NO_2^-$ L^{-1} in the effluent on day 124th. Nitrate was never detected by ion selective probe neither chemical analysis.

On the contrary, on day 145th, many parameters were changed as the pilot plant influent daily flow was set at 6-8 m^3d^{-1}. The BAF2 HRT and the EBCT increased significantly compared to previous analyzed days from 0.83 to 1.43 h and 0.41 to 0.71 h, respectively. Consequently, the specific OLR (sOLR; Eq.S2) as well as the specific ALR (sALR; Eq.S5) decreased to 1.60 kg COD $kgVS^{-1}$ d^{-1} and 0.14 kg $N\text{-}NH_4^+$ $kgVS^{-1}$ d^{-1}, respectively. These new operational condition could have provoked a higher SRT, so a minimum SRT for nitritation was achieved. COD removal and ammonium removal was observed in BAF2. Ammonium concentration decreased by about 4 mg $N\text{-}NH_4^+$ L^{-1} when comparing influent and effluent samples. This indicated a 50% ammonium removal. Nitrate was not detected and nitrite was only 1.3 mg $N\text{-}NO_2^-$ L^{-1}. Thus, nitrogen was imbalanced hypothetically due to denitrification in the biofilm. In order to figure out the feature of the active biomass, molecular tools were used to study the biofilm community on the aerobic media from the beginning of the operational period.

Nitritation microbial activity

Total and metabolically active population of bacteria and AOB from BAF2 were assessed by qPCR DNA/RNA-based assays of 16S rRNA and *amoA* genes, respectively. Independent triplicates were collected on punctual samples for days 18th, 82nd, 103rd, 124th and 145th. Figure 4 depicts the average of qPCR results obtained during the punctual sampling as well as the calculated ratios for *amoA genes* vs 16S rRNA genes and *amoA* transcripts vs 16S rRNA transcripts. Total bacterial population at all periods were between 10^9 and 10^{10} 16S rRNA gene copy numbers · g^{-1} of support material. Active bacterial population increased from over 10^4 to 10^{12} 16S rRNA transcripts · g^{-1} of support material from day 18th to day 145th, respectively (Figure 4).

On days 18th, 82nd and 103rd, total AOB population was present being around 10^3 *amoA* gene copies · g^{-1} of support material. Afterwards, the abundance of *amoA* increased 4 orders of magnitude being about 10^8 copies g^{-1}media on days 124th and 145th. When looking at the active AOB population, *amoA* transcripts considerably increased from day 18th to 124th. On day 18th, *amoA* expression was not detected while on day 124th and 145th all the present AOB population was active (10^7 *amo*A gene copies and transcripts · g^{-1} of support material).

With respect to the calculated ratios, it was observed that *amoA* genes vs 16S rRNA genes as well as *amoA* transcripts vs 16S rRNA represented a maximum of 0.3 and 0.002 %, respectively, showing that the eubacterial population has been enriched in AOB. The *amoA* genes vs 16S rRNA genes ratio was always higher than the transcript ratio except on the day 82nd. It is remarkable that the transcript ratio was lower on day 145th respect the previous analyzed day (124th) as 16S rRNA copies was higher (Figure 4).

BAF2 biofilm microbial community during nitritation

Illumina MiSeq 16S ribosomal RNA profiles were performed to compare the bacterial community structure of the support material from BAF2 when nitritation was observed by chemical analysis (days 124th and 145th). In order to depict the establishment of the biofilm and the active bacteria, 16S rRNA libraries were generated. A total of 150.000 high-quality sequences were obtained ranging 27.748 to 46.211 per profile; a total of 2.344 different operational taxomic units (OTUs) were detected (sharing 97-99% nucleotide identities).

MiSeq datasets from both days revealed that predominant bacterial populations in the support material were represented by phylotypes belonging to *β*, *α* and *δ-proteobacteria* class (data not shown). In Figure 5 it is showed the relative abundance in the taxonomic rank of family. *Commamonadaceae* (*β-proteobacteria* class) was the predominant family in the biofilm, representing about 12% of each datasets. The representative OTUs of that family belonged to the genera *Hydrogenophaga* and *Acidovorax*. Another important family related to denitrification process was *Rhodobacteraceae* (3.5-4.5%) where the predominant OTUs belonged to *Rhodobacter* genus. Also, *β*-proteobacteria family found was *Rhodocyclaceae* where the predominant OTUs belonged to *Zoogloea*, *Dechloromonas and Thauera* genera. Other actively enriched family (10% and 6%, day 124th and 145th respectively) related with carbon and nitrogen cycle was *Planctomycetaceae*, an environmental group typically present in biofilms.

Regarding nitrification bacteria, on one hand, AOB population was detected at both operational dates that were enriched in phylotypes of *Nitrosomonadaceae* family, the relative abundance increased from 1% to 4.5% (DNA and cDNA datasets from both days, respectively). On the other hand, NOB population was almost inexistent in the biofilm. *Nitrobacter*, as the most representative NOB, was at 0.01% (data not shown) although its family (*Bradyrhizobiaceae*) was present and active (≈ 2%) at both days.

Discussion

Overall BAF pilot plant performance

The BAF pilot plant accomplished EU discharge limits for both COD and SS after only 13 days of operation without inoculation differently from other related studies that inoculated active biomass into the system [7, 24, 25]. On one hand, SS removal is carried out by settling as well as the physical action of filtration. It is mostly linked with the good performance of solid liquid separator units of the BAF pilot plant consisting of primary lamellar settler, BAF1 and BAF2 and, as final treatment, the sand filter. In this sense, a suitable operation of the sand filter is crucial to remove the remaining and generated SS in BAF2 (Table 2). Thus, in this study SS removal was also achieved as elsewhere described [3, 6] during the startup period enforcing the idea that BAF can be a good solution from the first operational day when dealing with possible settling problems in CAS due to wastewater characteristics such as low COD/N ratio or undesirable growth of filamentous bacteria causing bulking [26].

On the other hand, biological COD removal was rapidly achieved after 13 days of operation and was kept during all the experimental period of 160 days. Moreover, COD removal was accomplished despite variations of OLR ranging from 1 to 5 kg COD m^{-3}media d^{-1}. Chang et al. [2] also reported high COD removal of textile wastewater (86-92% COD removal at OLR up to 3.3 kg COD m^{-3} d^{-1}) using sand and zeolite as media. Thus, the use of expanded clay in this study did not affect the COD removal performance of urban wastewater.

The *16S rRNA* gene transcript which indicates general microbial activity was already detected on day 18th in the BAF2 biofilm as well as its presence in form of *16S rRNA* gene. This transcript was smoothly increased by time as *amoA* transcript was gaining weight in the biofilm (Figure 4). Thus, initial active total eubacteria activity (*16S rRNA* gene transcript) can be linked to aerobic heterotrophic bacteria which were the responsible for COD removal in BAF 2when nitritation was not yet active. HRT and EBCT were equal for BAF1 and BAF2 and, thus, aerobic yield for COD removal is 10 times faster than anaerobic removal [27, 28]. In this sense, most COD removal must be pointed that took place in BAF 2 in aerobic conditions rather in BAF1 with anaerobic conditions. However, low COD removal was also plausible in BAF1 despite no oxygen neither NO_x^- were present before day 124th when nitritation was first detected (Table 2). Biological COD removal would be mainly attributed to BAF2 until nitrification started to be present. At the same time, these heterotrophic bacteria could make the function of denitrifiers on BAF1 or in the inner part of the biofilm in BAF2 when nitrite started to be present,

Nitrogen removal via nitrite

Large lack before nitrification detection can be attributed to several factors such as SRT, EBCT, sALR or sOLR among others. Effluent TN remained below the maximum EU discharge legal concentration of 15 mg N-TN L^{-1} from day 140th (Figure 3). Nitrate, contrarily to nitrite, was never detected on both the specific probe neither analytical analysis in the laboratory (Table 2). Thus, nitrogen removal was achieved via nitrite. Main described parameters that affect nitrite accumulation and NOB activity suppression, among others was DO concentration, free ammonia (FA) inhibition [13, 29, 30] free nitrous acid, pH [15] and lately the HRT [7]. In this case, free nitrous acid concentration in a pH of 7,5 and temperature of 20ºC was 0.0001 which corresponds to a concentration lower than the inhibition limits. On the other side, Garrido et al. [31] observed that ammonium was completely oxidized to nitrate when DO was above 2.5 mg O_2 L^{-1} in a biofilm airlift suspension reactor while the maximum nitrite accumulation was found when DO concentration was around 1.5 mg O_2 L^{-1}. However, DO concentration in the BAF2 remained above 4 mg O_2 L^{-1} in all cases.

On the other hand, FA was about 0.32 mg N-NH_3 L^{-1} taking into account a working mean temperature of 20 ºC and a pH of about 7.5 and could cause the inhibition of nitratation [30]. Thus, only NOB could be partially inhibited at these conditions since acclimated AOB can tolerate concentrations of FA up to 150 mg $N\text{-}NH_3$ L^{-1} [15]. In this study, the absence of significant nitrification was likely to be caused by the oxygen competence between nitrifiers and heterotrophic bacteria during COD aerobic oxidation together with the high applied OLR and low EBCT [24, 32]. In reference to that, when nitrite was firstly detected in BAF2 effluent on day 124th, the specific OLR (sOLR; Eq. S2) was lower than that found on day 103rd having the same EBCT (0.41h). Ryu et al.[7] demonstrated that COD was a principle cause of nitrite accumulation in a lab-scale BAF at low HRT (1h) confirming that sOLR could be a main cause the lack of nitrification as nitrite concentration was higher on day 124th than on the 103rd

The reduction of the influent pilot plant flow rate from 12 to 6-8 m^3 d^{-1} on day 130th resulted in a decrease of the sOLR (Table 2) as well as an increase of the EBCT from 0.41 to 0.71 h in BAF2, thus less oxygen competition. These two factors together let to a higher ammonium oxidation as ammonium had longer contact with AOB. However, the increase of EBCT did not correspond to a higher activity of the AOB (Figure 4). Thus, it was suspected that the AOB maximum concentration was reached in the biofilm. This can be demonstrated by Figure 6 which depicts the good exponential rise to maximum correlation between the nitrite production rate (NPR; Eq.S6) and the *amoA* gene transcripts concentration. Thus, from these results it can be stated that the maximum sALR and ALR to achieve nitrogen removal via nitrite and a suitable effluent in terms of effluent TN concentration in expanded clay media is about 0.14 $kgN\text{-}NH_4^+$ $kgVS^{-1}$ d^{-1} or 0.23 $kgN\text{-}NH_4^+$ m^{-3}aerobic media d^{-1}, respectively.

The biofilm enrichment of active AOB has been demonstrated by qPCR (Figure 4) and also by Illumina MiSeq analysis (Figure 5). AOB population was detected at both operational dates that were enriched in phylotypes of Nitrosomonadaceae family, the relative abundance increased from 1% to 4.5% (DNA and DNA transcripts datasets from both periods). Regarding to other genera of AOB from Chromatiaceae family, *Nitrosococcus* genera was not present. The competence between COD and ammonia oxidation has been pointed to be a key point together with the EBCT to achieve nitrification. However, there is still a blank regarding the microbial structure of both autotrophic and heterotrophic bacteria in the BAF2 biofilm since nitrogen was imbalanced for day 145th and nitrate was never detected.

Microbial assessment

The most predominant and active family for days 124th and 145th was *Commamonadaceae* (~12%; Figure 5). Although *Commamonadaceae* family is usually related to denitrification,[18] this family has also been found in nitrifying aerobic environments together with AOB [19, 20]. The representative OTUs belong to the genera *Hydrogenophaga* and *Acidovorax*, typical denitrifyers from municipal or industrial treatment plants [17]. Other important family related to denitrification process was *Rhodobacteraceae* (3.5-4.5%) where the predominant OTUs were related to *Rhodobacter*. This genera is related to aerobic denitrification where it was found a correlation between depletion of N_2O in an aerated reactor feed with synthetic wastewater containing glycerol and ammonium [33].

Other important family from *β*-proteobacteria class was Rhodocyclaceae where the predominant OTUs belongs to *Zoogloea, Dechloromonas and Thauera* genera: these aerobic denitrifyers were jointly found in other studies related to nitrogen and phosphorus removal in an hybrid biofilm-activated sludge reactor [34] and an early stage aerobic granules in a CAS wastewater treatment process [35]. Thus, these results, together with the decrease of the *amoA* vs *16S rRNA* transcripts ratio (Fig. 4), strengthen the hypothesis that the increase of the EBCT not only enforced partial nitrification.Denitrification could be carried out in the inner part of the biofilm where biofilm was thick enough to avoid oxygen difucion. Nevertheless, part of nitrogen removal could have been originated by AOB during ammonia oxidation generating the undesirable greenhouse gas N_2O during wastewater treatment [9, 36]. N_2O could also be generated by heterotrophic denitrifying organisms included in alpha and betaproteobacteria phyla such as *Rhodocyclaceae* and *Rhodobacteraceae* due to a lack of COD/N ratio or electron competence between nitrogen oxide reductase [9, 37].

With regards to nitrifiers population, mainly, AOB found in the present work belongs to *Nitrosomonadaceae* family and its main OTUs were related to *Nitrosomonas* similar to those found in comparable conditions [2].[2] AOB seemed to play a minor role when looking at the DNA-bases sequencing for days 124th and 145th since they were accounted to be only the 0.8 and 1%, respectively. Nevertheless, this is refuted when looking at cDNA relative abundance which grew up to 4.3 and 4.5%, respectively (Fig.5). This is especially important as the RNA analysis tool allowed to have a realistic view on what bacteria was playing an important role in the biofilm. On the other hand, NOB were almost inexistent when looking at both, DNA and cDNA extracts. The most abundant NOB population was related to *Nitrobacter* genus but being 0.01% *Bradyrhizobiaceae* family (data not shown). Thus, NOB were not significantly active neither present in the biofilm. Stress conditions in terms of high OLR together with extremely low EBCT granted the partial nitrification and, thus, the nitrogen removal via nitrite even in BAF2.

Conclusions

SS and COD removal was achieved in only 13 days of operation of the BAF pilot plant despite high OLR (up to 5 kg COD m^{-3}media d^{-1}) and low overall HRT (10 h). Nevertheless, a high OLR as well as extremely low EBCT (<0.5 h) in the aerobic BAF during the startup blocked the ammonium oxidation. Nitrogen removal via nitrite was observed at very low EBCT (0.71 hours) accomplishing the EU standards for treated wastewater although no full ammonium oxidation was achieved. DNA/cDNA analysis demonstrated that AOB played an important role when nitritation was achieved and NOB were not active neither present. This study revealed a new strategy to obtain partial nitrification in a BAF system by having a startup with extremely low general HRT and low EBCT in the aerobic BAF together with the application of a high OLR.

Acknowledgements

Authors would like to thank the valuable technical help of Aida Lopez during part of the experimental study as well as to the Caldes de Montbui WWTP workers Victor Mejias and Fran Cosano and the support given by the WWTP operator (Consorci de la Conca del Besòs).

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Table 1. Influent wastewater characteristics (n=41)

Parameter	**Units**	**mean**	**S_D**	**Max**	**Min**
COD	mg COD L^{-1}	395	253	1040	222
BOD_5	mg BOD L^{-1}	312	183	850	124
TSS	mg SS L^{-1}	240	125	692	95
TKN	mg N L^{-1}	55.4	13.4	76.0	26.0
NH_4^+		39.3	9.0	57.6	19.0
NO_x^-		0.2	0.7	4.0	0.0
COD/N	mg COD mg^{-1}N	7.1			

Table 2. Influent and effluent characterization and parameters of BAF2 for days 103rd 124th and 145th.

Compound	**Experimental days**						**Units**
	103th		**124th**		**145th**		
	inf	**eff**	**inf**	**eff**	**inf**	**eff**	
Ammonium	24.1	24.1	27.2	26.2	7.8	3.9	mg N L^{-1}
Nitrite	0.0	0.2	0.0	1.0	0.0	1.3	
Nitrate	0.0	0.0	0.0	0.0	0.0	0.0	
VSS	19.0	29.0	13.0	16.0	8.0	9.0	mgSS L^{-1}
COD	127.0	106.0	109.0	104.0	85.0	71.0	mg COD L^{-1}
influent flow rate	2.7		2.7		1.5		$m^3 m^{-2} h^{-1}$
HRT	0.83		0.83		1.43		h
EBCT	0.41		0.41		0.71		h
Specific Organic Loading Rate (sOLR)	3.25		1.86		1.60		Kg COD $kgVS^{-1} d^{-1}$
Specific ammonium loading rate (sALR)	0.60		0.45		0.14		$kgN\text{-}NH_4^+ kgVS^{-1} d^{-1}$

Legend of Figures

Figure 1. Schematic view of the BAF pilot plant. Black solid line depicts wastewater flow, blue solid lines are backwash water flows, brown solid lines are sludge water flows and dotted lines depict air flows.

Figure 2. Temporal evolution of influent and effluent concentrations of COD and organic loading rate (**A)** and total suspended solids (**B**). Dotted lines depict maximum effluent legal concentration for COD (125 mg COD L^{-1}) and TSS (35 mg SS L^{-1}).

Figure 3. Temporal evolution of influent and effluent concentrations of total nitrogen (TN) and nitrogen loading rate (**A)** and ammonium togeteher with the ammonium loading rate (**B**). Dotted line depict maximum effluent legal concentration for TN (15 mg N L^{-1}).

Figure 4. Time-course quantitative PCR (qPCR) results of DNA (genes) and cDNA (transcripts) from BAF2 biofilm samples. The average of independent triplicates (bars chards) and standard deviations (bars) have been depicted for each target gene. Ratios between bacterial population (16S rRNA) and AOB population (amoA) are showed by squares and triangles.

Figure 5. Relative abundance of bacterial 16S rRNA genes (DNA) and transcripts (cDNA), expressed respectively at the family phylogenetic level, in the biofilm samples for days 124th and 145th.

Figure 6. Correlation between the nitrite production rate (NPR) and the transcripts of *amoA* transcripts in the biofilm of the aerated BAF.

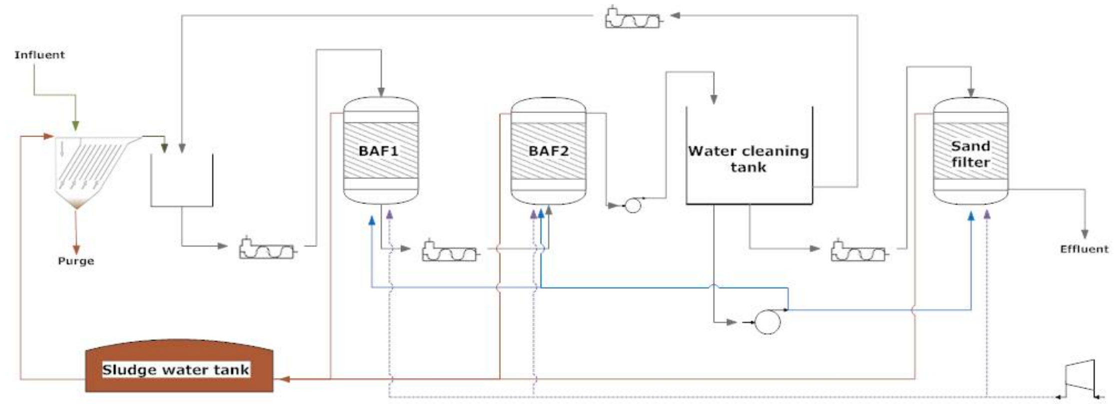
Influent
Purge
BAF1
BAF2
Water cleaning tank
Sand filter
Effluent
Sludge water tank

Figure 1

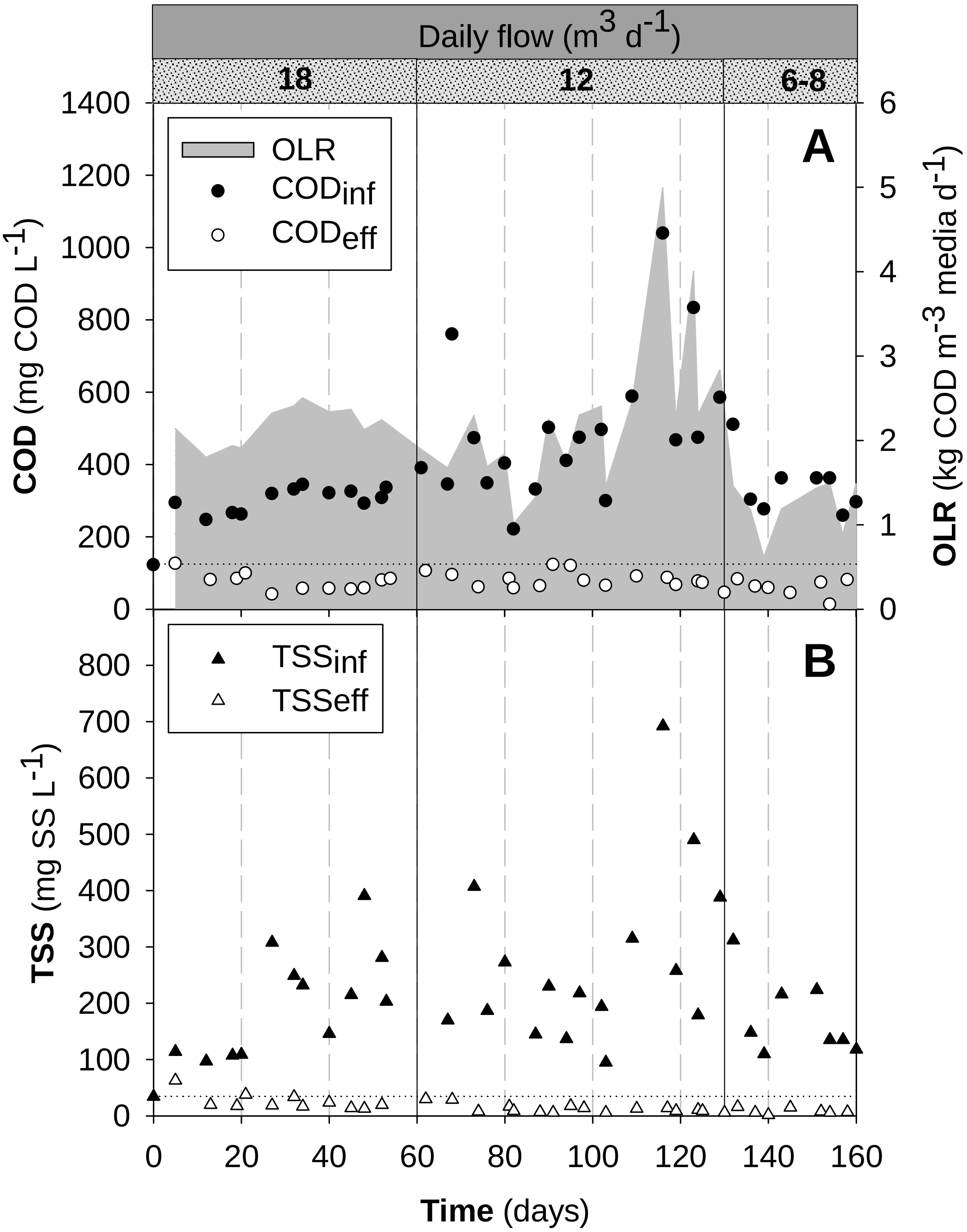
Daily flow ($m^3\ d^{-1}$)
18
12
6-8
A
OLR
CODinf
CODeff
COD (mg COD L^{-1})
OLR (kg COD m^{-3} media d^{-1})
0
200
400
600
800
1000
1200
1400
0
1
2
3
4
5
6
B
TSSinf
TSSeff
TSS (mg SS L^{-1})
0
100
200
300
400
500
600
700
800
0
20
40
60
80
100
120
140
160
Time (days)

Figure 2

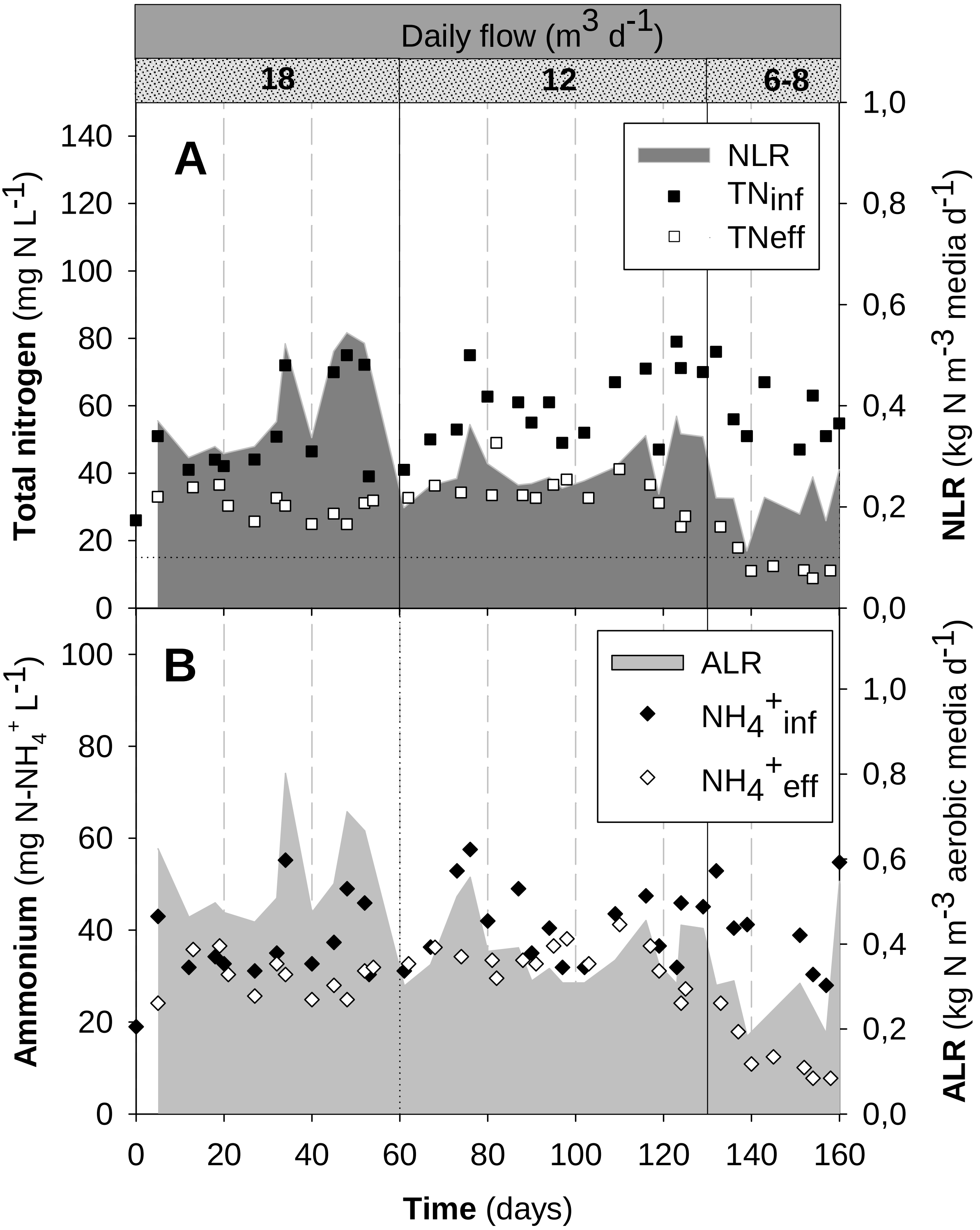
Daily flow (m^3 d^{-1})
18
12
6-8
A
NLR
TNinf
TNeff
Total nitrogen (mg N L^{-1})
NLR (kg N m^{-3} media d^{-1})
0
20
40
60
80
100
120
140
0,0
0,2
0,4
0,6
0,8
1,0
B
ALR
NH_4^+inf
NH_4^+eff
Ammonium (mg N-NH_4^+ L^{-1})
ALR (kg N m^{-3} aerobic media d^{-1})
0
20
40
60
80
100
0,0
0,2
0,4
0,6
0,8
1,0
0
20
40
60
80
100
120
140
160
Time (days)

Figure 3

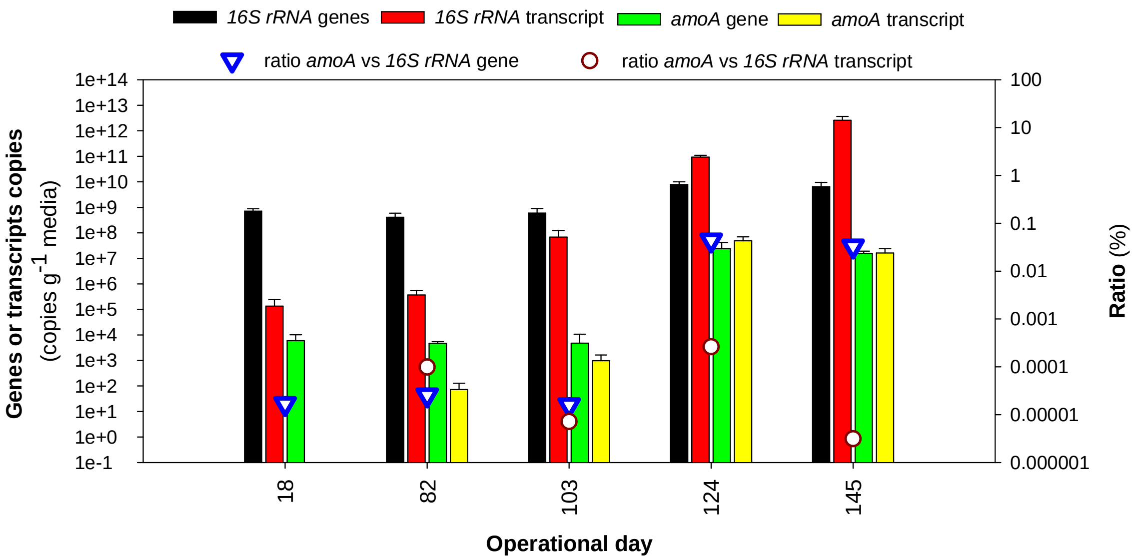
16S rRNA genes
16S rRNA transcript
amoA gene
amoA transcript
ratio amoA vs 16S rRNA gene
ratio amoA vs 16S rRNA transcript
Genes or transcripts copies
(copies g-1 media)
1e+14
1e+13
1e+12
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
1e+0
1e-1
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
Ratio (%)
18
82
103
124
145
Operational day

Figure 4

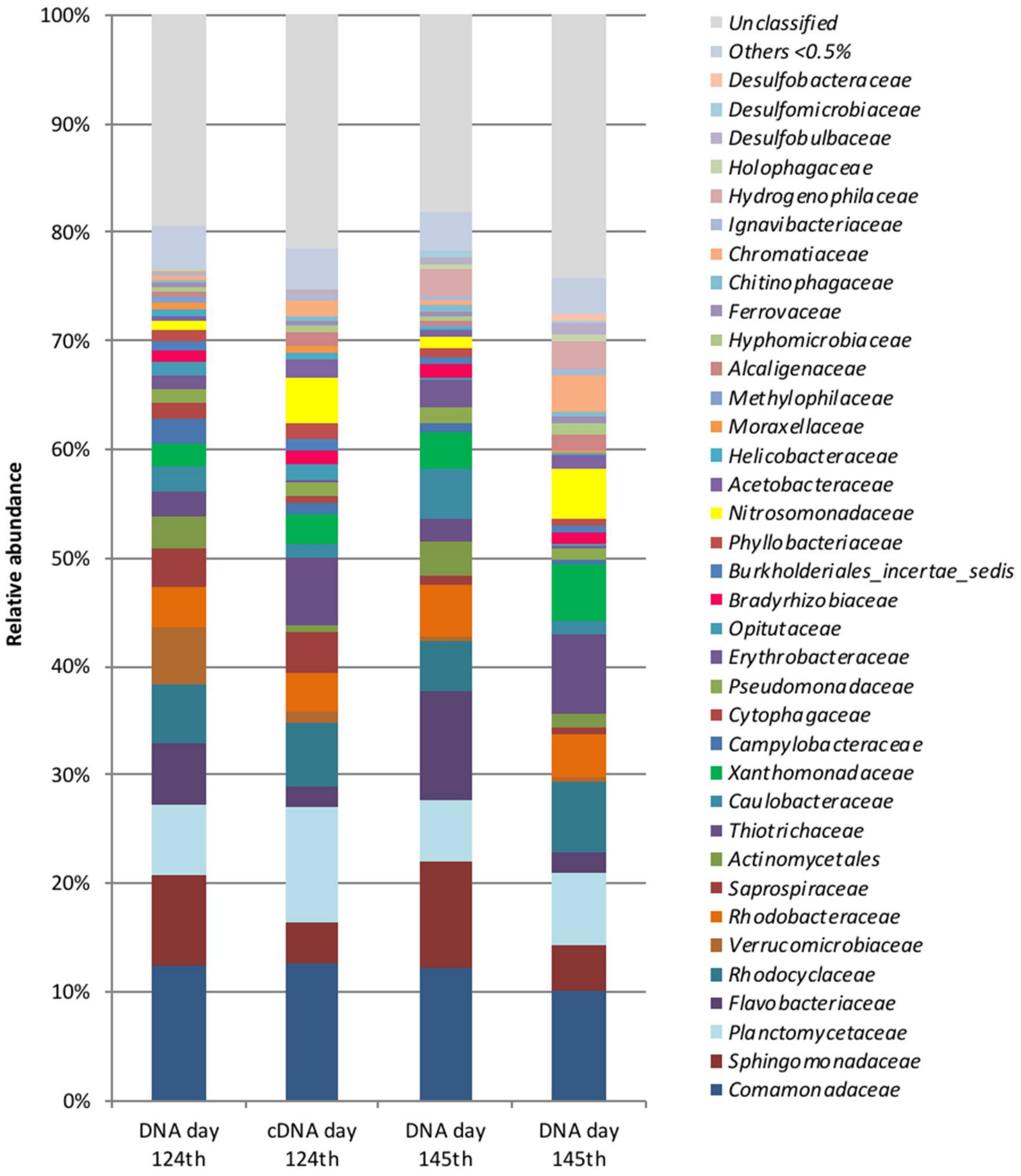
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative abundance
DNA day 124th
cDNA day 124th
DNA day 145th
DNA day 145th
Unclassified
Others <0.5%
Desulfobacteraceae
Desulfomicrobiaceae
Desulfobulbaceae
Holophagaceae
Hydrogenophilaceae
Ignavibacteriaceae
Chromatiaceae
Chitinophagaceae
Ferrovaceae
Hyphomicrobiaceae
Alcaligenaceae
Methylophilaceae
Moraxellaceae
Helicobacteraceae
Acetobacteraceae
Nitrosomonadaceae
Phyllobacteriaceae
Burkholderiales_incertae_sedis
Bradyrhizobiaceae
Opitutaceae
Erythrobacteraceae
Pseudomonadaceae
Cytophagaceae
Campylobacteraceae
Xanthomonadaceae
Caulobacteraceae
Thiotrichaceae
Actinomycetales
Saprospiraceae
Rhodobacteraceae
Verrucomicrobiaceae
Rhodocyclaceae
Flavobacteriaceae
Planctomycetaceae
Sphingomonadaceae
Comamonadaceae

Figure 5

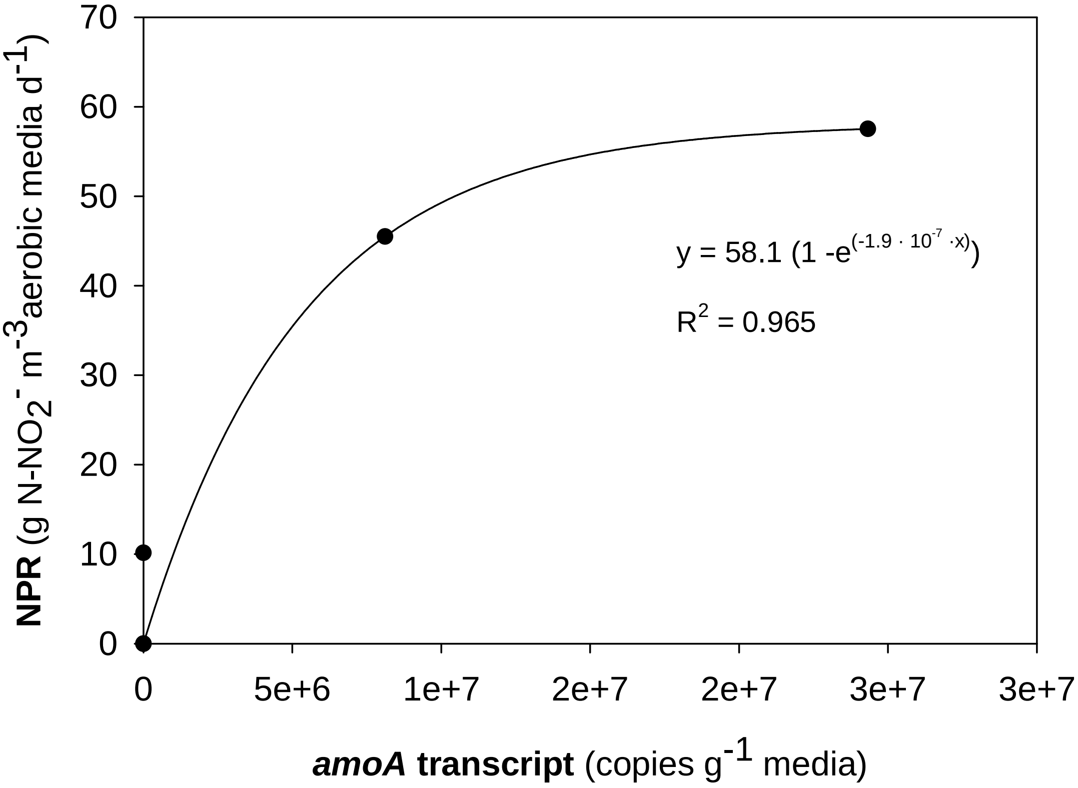
NPR (g N-NO$_2^-$ m^{-3}aerobic media d^{-1})
70
60
50
40
30
20
10
0
0
5e+6
1e+7
2e+7
2e+7
3e+7
3e+7
amoA transcript (copies g^{-1} media)
y = 58.1 (1 -e$^{(-1.9 \cdot 10^{-7} \cdot x)}$)
R^2 = 0.965

Figure 6