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Ordinary heterotrophic organisms with aerobic storage capacity provide stable aerobic granular sludge for C and N removal

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Abstract

The study investigated the mechanisms and microbial communities underlying the long-term stability and removal performances shown by aerobic granular sludge (AGS) reactor involving polyhydroxyalkanoates (PHA) aerobic-storing bacteria. The characteristics of the sludge, removal performances and bacterial community structure were determined. The prevailing metabolic phenotype was similar in the parent conventional activated sludge (CAS) reactor and its upgraded AGS version, showing high COD and NH_4 uptake, versus low P and N reduction. Polyphosphate and glycogen accumulating organisms, PAO and GAO, were not enriched in the reactors despite initial targeting of anaerobic-aerobic cycle. Instead, PHA-aerobic storing bacteria (*Thauera* and *Paracoccus*) were dominant, but revealing a stable AGS system for BOD and N removal. The PAO/GAO failed selection and *Thauera* overgrowth were analyzed for beneficial use in developing alternative AGS technology for BOD and N removal applications.

Keywords: Aerobic granular sludge; Ordinary heterotroph; PAO, Storage; Stability; Thauera.

1. INTRODUCTION

Ensuring the long-term structural integrity of granules is still a major challenge hindering their application in full-scale plants and the wider spread use of aerobic granular sludge (AGS)

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technology worldwide (Corsino et al., 2016; Iorhemen et al., 2020). Based on the current state of art, AGS systems are configured mostly as sequencing batch reactors (SBRs) operated with anaerobic-aerobic (An-O) cycles. This practice seeks to promote slow-growing heterotrophs, particularly polyphosphate- and glycogen-accumulating organisms (PAO and GAO). It is thought that the latter organisms improve the density and stability of the granules relative to ordinary heterotrophic organisms (OHO), which are currently viewed as enemies of granulation (Pronk et al., 2017 and 2020; Layer et al., 2019; Guimarães et al., 2020). Ways were proposed to avoid detrimental OHOs (growing as finger-like structures or filamentous bacteria) by minimizing the leakage of the influent COD to the aerobic phase and avoiding the occurrence of transport limitations during aerobic degradation (Bassin et al., 2019; Layer et al., 2019; Haaksman et al., 2020). AGS enriched in PAO and GAO has the potential to remove organics and nutrients via enhanced biological phosphorus removal (EBPR) and/or simultaneous nitrification-denitrification (SND). However, despite its many benefits, the PAO-GAO AGS niche is not always applicable or easy to implement. Therefore, there is an interest in exploring other stable AGS alternatives.

There are cases where nutrient removal is not targeted or the composition of the wastewater (WW) does not require it. A typical example is the Atotonilco mega activated sludge plant in Mexico (35 m^3/s; Espino et al., 2011). It is intentionally designed to remove the biological oxygen demand (BOD), ammonia and pathogens, but looking for that nitrates and phosphorus remain as fertilizers in the effluent discharged in canals for agricultural reuse. In this case, because P is abundant in the influent, by maintaining an anaerobic-aerobic cycle, it would be difficult or impossible to promote GAO only (GAO-based AGS) and simultaneously suppress PAO (no P-removal objective). In many other cases too, phosphorus and total nitrogen are no longer stringently targeted by Latin American WW discharge limits (e.g., in Mexico and Brazil;

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Guimarães et al., 2020), compared with EU and US standards. Moreover, there are many industrial WWs from food industries that are deficient in N and/or P. In contrast, nitrogen and phosphorus are supplemented at the minimum levels required for C removal by activated sludge (BOD/N/P of 100/5/1). Here, indeed, the An-O cycle can still provide a GAO-based AGS. However, same as for the Atotonilco scenario, other AGS alternatives outside the PAO/GAO niche, e.g., with fully aerobic, anoxic-aerobic, or aerobic-anoxic cycles may be of interest.

Several times and for different reasons, it happened that AGS reactors which had an initial objective of selecting PAO and GAO did not succeed (Kang and Yuan, 2019; Guimarães et al., 2020); unexpectedly, other types of microorganisms such as the OHOs *Thauera, Azoarcus and Flavobacterium* proliferated in these reactors, but even so, the processes still showed high stability in some cases (Iorheman et al., 2020, Pishgar et al., 2019). Additionally, even under satisfactory EBPR and granulation, it is known that PAO, GAO and nitrifiers may represent a small fraction only of the whole population (Stokholm-Bjerregaard, 2017); however, in the latter case, improved settling of the sludge and better stability of the granules is mostly attributed to those minorities only while ignoring the potential contribution of the other genera (often majority). Therefore, there is a need to know more about the potential stabilizing effect of other frequent non-PAO/GAO genera in the granules.

The first investigations on AGS mostly utilized fully aerobic reactors (Morgenroth et al., 1997) operated under feast-famine regime and probably driven by ordinary heterotrophs that can store PHA (polyhydroxyalkanoates). Stable granulation was successfully achieved, provided that oxygen and substrate were not transport-limited. In contrast, filamentous or finger-type growth and granule embrittlement occurred under low DO and/or diluted substrate conditions (de Kreuk

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and van Loosdrecht, 2004; Mosquera-Corral et al., 2005). More recently, AGS was successfully cultivated in all-aerobic SBR mode (Bucci et al., 2020; Pishgar et al., 2019).

In the recent AGS literature, in the context of the PAO-GAO niche, no difference is made between the OHOs, which are all considered fast growers and tagged as enemies of stable granulation. However, there are two types of OHOs, i.e., with substrate storage capacity and without such storage (van Loosdrecht et al., 1997). Fundamentally, aerobic feast-famine conditions select for slow-growing PHA-storing bacteria, increasing hydrophobicity and microbial aggregation (Albuquerque, 2013; Basset et al., 2016; Gray et al. 2019). Heterotrophic biomass that first stores readily biodegradable COD (rbCOD, e.g., acetate) as intracellular polymers will grow at a slower rate. In contrast, bacteria that utilize the substrate without previous storage will maximize their growth rate. In the past, this distinction gave rise to two variants of activated sludge models for C and N removal (ASM1 and ASM3, Henze et al., 2000). Heterotrophic maximum specific growth rates in ASM1 (non-storage OHOs with direct growth) versus ASM3 (PHA storage) are too different, with μ_{Hmax} values of approximately 6 and 2 d^{-1}, respectively (Henze et al., 2000; Falvo et al., 2001). Indeed, the growth rates of PAO and nitrifiers are even lower (approximately 0.9 d^{-1}; Envirosim, 2020); however, the value of μ_{Hmax} up to which rapid growth could significantly affect the stability of granules is still unknown.

Taxonomic information on ASM3-OHOs is very scarce. The bacteria *Thauera*, *Paracoccus*, *Brachymonas*, *Acinetobacter* and *Amaricoccus kaplicensis* are well known for their PHA aerobic-storing capacity (often denitrifiers) in the context of all-oxic or aerobic-anoxic SBR reactors for bioplastic production (Falvo et al., 2001; Albuquerque, 2013; Basset et al., 2016; Sruamsiri et al., 2020; Colpa et al., 2020). These genera are frequently detected also in many kinds of AGS under

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different SBR modes (Szabó et al., 2017; Pishgar et al., 2019; Layer et al., 2019), as well as in many full-scale water resource recovery facilities (WRRFs) (Coats et al., 2017; Nierychlo et al., 2020). However, their potential contribution to granulation, especially in systems where PAO and GAO are also present, has received little attention.

The aim of this study was to investigate the underlying removal mechanisms and the microbial community structure behind the long-term stability and the metabolic behavior shown by two activated sludge (AS) SBRs involving aerobic-storing bacteria when operated first as conventional AS and when upgraded to AGS. The operational conditions that shaped the microbial communities and the C, N, and P removals were discussed and contrasted with representative cases in recent literature. This research has implications for understanding the occasional failure of PAO/GAO selection in AGS, and it focuses on PHA aerobic-storing OHO as a separate technological AGS niche.

2. MATERIAL AND METHODS

2.1. Operational conditions of the SBR reactors

Two types of activated sludge (AS) sequencing batch reactors (SBRs) were utilized in the study (Table 1), starting with a basic type which functioned as a conventional activated sludge (CAS-SBR) and which was transformed later into an aerobic granular sludge process (AGS-SBR). Both SBRs were operated with planned anaerobic-aerobic cycles and were fed with similar acetate-based synthetic wastewater (low strength). The first reactor was operated during more than 4 years, being a conventional AS process carried out in a 30-L rectangular tank and inoculated with flocculent sludge from a municipal WRRF. In the course of the last year, the treatment system was upgraded to granular variant; an AGS-SBR was initiated at smaller scale (1-L bubble

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column), being inoculated with the sludge from the CAS-SBR and fed with same wastewater type. The volume of the first reactor was bigger due to other needs in the context of a wider study not all described here (e.g., producing sufficient sludge mass for subsequent digestion and ozonation side-tests). The operational parameters of the upgraded process were adapted to meet the typical conditions needed for granulation and to take into account its increased potential of load. The AGS-SBR was provided with hydraulic selection (short settling for 4 min, resulting in critical minimal settling velocity of 3.6 m/h), adequate shear-stress level (superficial air velocity of 2.5 cm/s), and column shape with high H/D (height/diameter). Both reactors were subject to feast/famine regime under anaerobic-aerobic cycle, with initial intention of improving the settleability and/or the granulation of the sludge, as well as removing P, potentially (Arbuquerque, 2013; Pronk et al., 2020).

⇨ ***TABLE 1***

2.2. Analytical parameters and methods

The physical and settling characteristics of the sludge, as well as the removal capacities of the parent and the upgraded SBRs were monitored. Meanwhile, the microbial species in the sludge were identified by gene sequencing analyses, as described in the next section. Essential routine measurements were carried out weekly, while detailed profiles of COD, OUR, N and P were determined along the cycle time at different dates during steady state. The mixed liquors of the systems were monitored for temperature (T), pH, dissolved oxygen (DO) and/or oxygen uptake rate (OUR), volatile and total suspended solids (MLVSS and MLSS), chemical oxygen demand (COD, total and soluble), as well as N and P components, in accordance with Standard Methods (APHA et al., 2017). The COD, ammonium, nitrite and nitrate, as well as phosphate and total P were determined using relevant HACH kits (HACH, Mexico). The sludge volume index (SVI, at

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30 and/or 5 min) and the particle size distribution (laser diffraction technique) were measured. The morphology of the sludge was appreciated by digital imaging and through different microscopy techniques including optical and fluorescence microscopy (confocal laser scanning microscopy, CLSM) as well as scanning electron microscopy (SEM), the latter after sample preparation with osmium tetroxide (OsO_4) fixation and staining.

2.3. Microbial community analysis by 16S-RNA gene sequencing method

16S-rRNA amplicon sequencing was carried out to identify the bacterial species in the AGS, in accordance with proven protocols (Lopez-Vazquez et al., 2019), as well as evaluate the abundance of the functional groups (PAO, GAO, etc.), based on MiDAS Field Guide (Nierychlo et al., 2020). DNA extraction of the mixed liquor sample was performed with NucleoSpin Soil kit (Takara Bio, USA) per the manufacturer's recommendation. Isolated genomic DNA and the final libraries were quantified with Qubit 2.0 DNA HS Assay (ThermoFisher, MA, USA) and quality assessed by Tapestation genomic DNA Assay (Agilent Technologies, CA, USA). 50ng of isolated genomic DNA was used and amplified via PCR and with proprietary primers (Admera Health, NJ, USA) covering hypervariable regions V3 and V4. Illumina® 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® MiSeq V2 Standard platform (Illumina, California, USA) with a read length configuration of 250 for 0.1 M PE reads per sample (500k in each direction).

3. RESULTS

3.1 CONVENTIONAL ACTIVATED SLUDGE SBR (CAS-SBR)

3.1.1 Long-term steady state behavior of the CAS-SBR reactor

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The CAS-SBR operated stably for more than three years straight. Table 2 summarizes the steady-state average characteristics of the ML sludge and the standard deviations (±SD) during the last 350 days of operation.

⇨ ***TABLE 2***

At a steady state, the mixed liquor total suspended solids fluctuated in a narrow range of 1270 ± 116 mg/L MLSS and had a VSS/TSS ratio of 0.86 ±0.04. The process showed a long-lasting stability and easy settling of the sludge (SVI_{30} < 60 mL/gTSS), provided that DO was maintained high, > 2 mg/L, during the aerobic feast period. A trend of increasing pH (8.5-9.0) was noted, as similarly documented by Wentzel et al. (1989) for a culture of PAO, as well as by Albuquerque et al. (2013) and Sruamsiri et al. (2020) in aerobic SBRs used for bioplastic production. As in the latter cited works, no adverse effect due to pH was observed, but ammonia stripping was followed up and accounted for in the nitrogen balance.

The average removal efficiencies were > 96% for COD and > 99% for NH_4^+. Residual soluble COD in the effluent (20 mg/L approx.) was inert and was assumed to be from extracellular microbial products and lysis. Total N and P removals were low as documented later. Based on the low ash content (14%) and P fraction (1.5-2%) of the biomass and with practically no P removal, it was anticipated that PAO was not enriched in the reactor. Based on other evidence revealed later, GAO was also apparently not enriched. Additionally, as measured previously for the same sludge (Martínez-García et al., 2016), the heterotrophic endogenous decay rate constant (b_H) was very low (0.02 to 0.09 d^{-1}) compared to the ASM1 default value (0.2 d^{-1}, Henze et al., 2000). This is another discriminating feature of the microbiome that prospered in the CAS-SBR, being a

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characteristic shared with other kinds of storing organisms, such as PAO, which is also known for their extremely low b_H (0.04-0.10 d^{-1}; Wentzel et al., 1989; Envirosim, 2020).

3.1.2 COD, N and P time profiles along the cycle (CAS-SBR)

The COD, N, P and OUR (rO_2) profiles over the SBR cycle time are illustrated in Figure 1. The shaded zones in the graphics identify the anaerobic phase. Figs. 1a and 1d show the typical shapes of the COD and OUR profiles for 2 different runs. Only minimal COD uptake (< 22%) occurred during the non-aerated period. Most of the COD was removed during the aerobic phase in 30-45 min (very fast, given the MLSS level), accompanied by a very high O_2 consumption rate. This fast removal is characteristic of a storage process. Afterward, the biomass was subject to long famine. The upper part of Fig. 1a shows the raw respirometric DO data, which were controlled between 3 and 4 mg/L O_2 (on-off). The reprograms showed different plateaus (Figs. 1a-bottom and 1b), revealing different metabolisms. The first high plateau, as visible in Fig. 1a (93 ±5 mgO_2/L.h), was attributed to acetate storage as PHA, terminating with a sharp decrease that detects the depletion of the substrate. As shown in Fig. 1b, a second plateau prevailed up to 5 h (at 12.3 ± 0.2 mgO_2/L.h), which was attributed to degradation of the stored polymers for growth (and nitrification). The tail at the end corresponds to endogenous respiration (decay), reaching an OUR of 3 ±0.7 mgO_2/L.h at 24 h.

➔***Figure 1***

Fig. 1c shows the NH_4^+, NO_3^- and PO_4^{3-} profiles. Nitrite did not accumulate in the system (0.13 mg/N-NO_2^-, as maximum). Luxury uptake of P was not present (Figs. 1c and d), which confirmed the absence of PAO and EBPR.

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Ammonium assimilation was minimal during the anaerobic phase. Fast and total removal of ammonium occurred during the aerobic phase due to different mechanisms: nitrification, assimilation and NH_3 stripping, potentially due to pH > 8 in the system. At least 11 mg N-NH_4^+/L remained at the end of the feast period (120 min), which demonstrated that nutrient limitation did not occur. At the end of the experiment described in Fig. 1c, the accumulated nitrates at 24 h were 9 mgN/L (nitrified), whereas the removed nitrates were 8 mgN/L, which represented 47% of N-removal, with some attributable to assimilation and more to ammonia stripping.

SND was unlikely because of the small size of the flocs and the high DO prevailing in the CAS-SBR. The absence of SND causes the effluent to remain charged in nitrogen (9 mg/L N-NO_3), which is the same as the ML recycled and diluted at the start of the next cycle. With the latter event, the anaerobic phase becomes anoxic for some moment, which is not favorable to PAO and GAO selection. Most of the 50-70 mg/L COD removed during the "anaerobic phase" could be attributed to pre-denitrification that occurs at the start of the cycle and less potentially to the anaerobic activity of PAO or GAO.

3.1.3 Morphology, size and settling properties of the conventional activated sludge

The sludge had an outstanding and sustained decanting behavior with an SVI_{30} of 59±20 mL/gTSS and settling velocities between 4 and 10 m/h, and these ranges are exclusive to well-settling flocs and incipient granular sludge. For comparison, secondary clarifiers are designed with 0.7-1.4 m/h, while granular sludge can easily reach 10-70 m/h (Rollemberg et al., 2020; Castellanos et al., 2021).

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The morphological images in Figure 2 are from different microscopy observations. Fig. 2a from optical microscopy (x40) shows a mix of flocs (majority) and small granules (center). Fig. 2b was obtained by fluorescence microscopy, showing many small granules (10-15 µm to 35-65 µm). Meanwhile, Fig. 2c provides greater amplification (x20000) by SEM. The outline and shapes (coccobacillus, coccus and rod) of the bacteria, apparently coated with EPS, were clearly distinguishable at this scale.

➔***Figure 2***

Filamentous bacteria were not observed. By the end of the culture, the mean particle size of the sludge was approximately 90 µm (Fig. 2d), which technically classifies it as flocs, considering that granules begin at 200 µm (Pronk et al., 2020; Leal et al., 2021).

Overall, for the conventional AS culture, the feast-famine regime and the operating conditions (high DO and low SND) resulted in low anaerobic COD removal and fast aerobic C storage. Apparently, aerobic-storing bacteria were selected (not PAO/GAO), resulting in fast settling sludge dominantly flocculent.

3.2 AEROBIC GRANULAR SLUDGE REACTOR (AGS-SBR)

3.2.1 Granulation and characteristics of the AGS sludge

Table 3 summarizes the average characteristics of the AGS mixed liquors after 4.5 months of operation. The MLSS conc. was much higher in the AGS reactor than in the CAS reactor due to the increased OLR and higher SRT in the former. In both reactors, the sludge maintained a similar pale yellow color, basic pH, good settleability and a relatively high VSS/TSS ratio (as in Leal et al., 2021) that anticipated a low PAO fraction. COD removal was always high (> 96%).

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⇨ ***TABLE 3***

As illustrated in Figure 3, granulation was successfully achieved in the AGS reactor. The digital images in Figs. 3a and 3b provide a view of the bulk granules (concentrated ML). The flocs of the inoculum gave way to perfectly granulated, dense and larger discrete particles, yet they were not perfectly spherical. The morphology of the individual granules, as revealed by a closer view of the optical microscope (Fig. 3c), shows a smooth surface with no filamentous or finger-like structures.

The particle size distributions (PSDs) were measured at Days 104, 122 and 137, as illustrated in Fig. 3d, with mean sizes of 442, 528 and > 643 µm, respectively, comparable to elsewhere granules (450 -900 µm, as in Layer et al., 2019). At Day 137, > 97% of the particles was greater than 200 µm (technical limit for being considered granules) (Pronk et al., 2020). The vertical dashed line in Fig. 3d shows the maximum detection limit (948 µm) of the PSD analyzer utilized. Therefore, the reported final mean size (statistics in medallion in Fig. 3d) was probably underestimated due to the out-of-range particles that were not all accounted for (Zhao et al., 2018).

➔***Figure 3***

3.2.2 COD, N and P uptake profiles of the AGS-SBR

Figure 4 shows the typical shape of the COD removal profiles along the AGS-SBR cycles, as repeatedly measured at different moments from Day 100 to 129 (Fig. 4a) in pulse-fed side-batch tests (with mixing, anaerobic for 90 min and up to 150 min). COD removal was small during the

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non-aerated phase, while it was very fast and downward once aeration was started up, similar to the metabolic phenotype of the CAS-SBR.

➔***Figure 4***

Fig. 4b and 4c show the COD, N and P profiles at Day 137, directly from the AGS column (bottom step-feeding across the sludge blanket). The COD profile during the anaerobic phase is represented with a dashed line between the theoretical initial COD and the first COD data point acquired seconds after the start of aeration (mixing). Fig. 4b confirmed that the granular sludge produced was characterized by a high capacity of storing the COD in aerobic conditions (in 10 min only, due to higher MLSS) against very low uptake during the initial non-aerated period. Dissolved oxygen in the reactor (medallion in Fig. 4b) increased from 0 to almost 4 mg/L soon after the start of aeration at 90 min, and it increased later to 6 mg/L, which marked the exact moment when the substrate ran out (100 min). Overall, high removals of COD and ammonium (> 97%) were obtained, against very poor reductions of TN and P (Fig. 4c). It is important to mention that following the PHA concentrations (not acquired in this study) would be providing additional proofs and information.

During the aerobic period, NH_4^+ was completely transformed (almost 100%), producing 13.0 mg/L N-NO_3^-, without nitrite accumulation (0.3 mg/L N-NO_2^-). Total N removal during the aerobic phase (< 22%) was low (potentially due to assimilation and ammonia stripping). The nitrates remaining in the ML were returning at the head of the cycle, thus turning anoxic the "anaerobic" phase to the detriment of PAO and GAO. More denitrification (exogenous) occurs there together with some COD consumption. EBPR did not occur, as confirmed by low P

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removal (13%), low P content (< 2%) and high VSS/TSS (88%) of the biomass. GAO metabolic phenotype either was not observed.

Overall, the metabolic behaviors were similar in the CAS and AGS. Well granulated biomass performing COD aerobic storage was formed in the AGS, showing that not all OHOs are detrimental to AGS formation and stability. This revealed an alternative AGS niche oriented to BOD and ammonia removal and denitrification.

3.3 MICROBIAL COMMUNITY FINGERPRINTING

3.3.1 Dominant taxa

16S rRNA gene sequencing of the AGS generated 67271 effective amplicon reads spread over 1013 OTUs (operational taxonomic units) that were classified into 29 bacterial phyla, 53 classes, 81 orders, 182 families, 519 genera and 653 identified species (SUPPLEMENTARY FILE-1). For comparison, 422 genera were detected in the laboratory-scale AGS reactors of Layer et al. (2019) against 36 phyla and 579 genera by Xu et al. (2018) at a full-scale AS plant. The Shannon-Wheaver microbial diversity index (H) resulted in a middle-ranging value of 2.73 in the present study, comparable to 1.91-2.47 reported by Pishgar et al. (2019) for three VFA-fed lab-scale AGS reactors. This reveals the dominance of a few abundant taxa, contrasting with typical H values of 4.5-5.8 found at full-scale WRRFS having more complex WWs (Xu et al., 2018; and at twenty Danish WRRFs of Nierychlo et al., 2020).

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A summary of the metagenomics results is provided in **Table 4**. Two phyla out of the 29 detected were dominant by far (67% Proteobacteria and 27% Bacteroidetes), followed by Firmicutes (1.8%) and 5 others between 0.5 and 0.8% each.

⇨ ***TABLE 4***

Microbial species reported by Pishgar et al. (2019) for three small AGS reactors were also from a handful of phyla, including Proteobacteria (51-68%) and Bacteroidetes (24-40%), comparable to the cases of Xu et al. (2018) with 26–49% versus 19–37% at different full-scale WRRFs. In the present work, a high % of the total amplicon reads could be successfully classified at the genus level (94%), compared to 49% in MiDAS (Nierychlo et al., 2020). This high resolution guarantees the ability to identify the bacteria by their functions in the AGS.

Table 5 and its embedded diagram provide a listing of the top 22 bacterial genera. A relatively small fraction of taxa (3 genera) comprised a large proportion of the reads (57%), increasing to 80% reads for 22 taxa and 90% reads for 62 taxa out of the 519 detected genera. The remaining hundreds of genera were present in very few quantities (< 0.1% each).

The OHOs *Thauera* (25%), *Paracoccus* (21%) and *Flavobacterium* (11%) emerged as the dominant trio, and they were assumed to be determinants of granulation and removal. They are known as denitrifiers with high PHA aerobic storage capacity and extracellular polysaccharide (EPS) production (Corsino et al., 2016; Nierychlo et al., 2020), which predispose them to easier granulation and settling. Another 11 taxa, including the GAO *Defluviicoccus* and the AOB *Nitrosomonas*, were between 3 and 1% each, while 7 other genera accounted for 1 to 0.5% of the abundance.

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⇨ ***TABLE 5***

Most of the taxa shown in Table 5 (top 22) frequently appeared among the dominant genera of lab-scale AGS reactors of many authors (e.g., Zhao et al., 2013; Szabó et al., 2017; Pishgar et al., 2019; Iorhemen et al., 2020; Othman et al., 2020), particularly when a significant part of the substrate was aerobically and/or anoxically degraded. Most are OHOs, except for *Defluviicoccus* GAO and *Nitrosomonas* AOB. Additionally, other genera shared by this study and many of the above cited authors were the following: *Pedobacter, Flavihumibacter, Leadbetterella, Fusibacter, Brevundimonas, Zoogloea, Rhodobacter, Bdellovibrio, Lysobacter, Ignavibacterium, Hydrogenophaga, Azoarcus, Ohtaekwangia, Nitrosomonas and Nitrospira*.

As in the present study, Pishgar et al. (2019) observed the overgrowth of *Thauera* (20-36%), *Azoarcus* (7-23%) and *Flavobacterium* (4-23%) in all three different AGS reactors under full-oxic, An-O, or An-O-anox modes. Szabó et al. (2017) reported a top 3 genera that involved *Neomegalonema* and *Thauera* with either *Paracoccus* or *Zoogloea*, accounting for up to 44% of the total microbial population of its anoxic-oxic reactor. Additionally, although it can be at lower abundance, the taxa led by *Thauera* always appeared in the core communities of full-scale EBPR plants (Nierychlo et al., 2020; Xiang et al., 2021; Coats et al., 2017; Świątczak and Cydzik-Kwiatkowska, 2018), as well as in lab-scale reactors fed municipal WW (Layer et al., 2019; Coats et al., 2017). Furthermore, full-aerobic microbial cultures utilized for bioplastic production are also generally dominated by *Thauera* and consorts, showing a high capacity for PHA storage, settling and operational stability (Albuquerque, 2013; Sruamsiri et al., 2020; Colpa et al., 2020).

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Thauera is a gram-negative Betaproteobacterium belonging to the order Rhodocyclales (Table 4). Twenty-three percent out of the 25% total abundance of the *Thauera* genus was not resolved at the species level. Those detected were *T. aminoaromatica (1.3%), T. aromatica (0.32%), T. mechernichensis (0.12%), T. terpenica (0.01%) and T. chlorobenzoica (0.01%)*. MiDAS (Nierychlo et al., 2020) inventories the existence of 21 different species of *Thauera*.

3.3.2 Functions of the bacteria

Within the 519 bacterial genera present in the AGS, those that are known to belong to any of the main functional groups were searched (Stokholm-Bjerregaard et al., 2017; Nierychlo et al., 2020). The total abundance of each functional group was estimated and is reported in Table 6. Ordinary heterotrophic organisms (OHOs) formed the dominant fraction (77-96.5%) and were engaged in aerobic/anoxic COD removal and denitrification.

⇨ ***TABLE 6***

The polyphosphate-accumulating organisms (PAO, 0.02% confirmed and up to 0.32 %, when more) and their competitors, the glycogen-accumulating organisms (GAO, 1.7%), were less than 2% in total, which is consistent with the low anaerobic COD removal observed.

Regarding the functional groups of nitrifiers (Table 6), the abundances of AOB and NOB (1.2% and 0.3%) were within typical ranges and diversity observed at well-operating biological nitrogen removal plants (BNRs) (Nierychlo et al., 2020). Nitrifiers perform perfectly their role even at relatively low abundance (Szabó et al., 2017; Świątczak and Cydzik-Kwiatkowska, 2018), e.g., with 1% *Nitrosomonas* and 0.03% *Nitrospira*, in the case of Szabó.

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AOB were present in the AGS through their two most popular genera, i.e., *Nitrosomonas* (1.2%) and little *Nitrosospira* (0.004%). *Nitrosomonas* was always present at Danish BNR-WRRFs, having 0.1% mean average abundance only but showing stable and complete nitrification (Nierychlo et al., 2020).

Regarding NOB, generally the two most active are *Nitrospira* and *Nitrobacter*. Here, only the first one was detected in the AGS reactor, having 0.3% abundance, which was sufficient to avoid NO_2 accumulation in the system. A similar trend was observed by Szabó et al. (2017), who also had only a few Nitrospira and no Nitrobacter. The former was among the core communities of the Danish BNR and EBPR plants with 0.5% average abundance (Nierychlo et al., 2020).

Overall, the typical performance shown by the reactors operated in this study (both the AGS and the conventional activated sludge SBR), as summarized below, was qualitatively concordant with the microbial community composition and the functional groups revealed in the sludge:

- *Low anaerobic C removal* as a result of the small amounts of PAO and GAO. The low COD consumption observed during the non-aerated period is attributable mainly to exogenous denitrification (by OHOs such as *Thauera et al.*).
- *Full nitrification* during the aerobic phase, due to low but sufficient abundance of AOB and NOB in the sludge.
- *Quick and full aerobic removal of most of the COD* through storage as PHA by OHOs, such as *Thauera et al.*
- *Low P removal* (mainly by assimilation and not by EBPR) was in concordance with the small PAO population.

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- *Low simultaneous denitrification* during the oxic phase (low SND) due to the high DO prevailing in the reactor and which reduces the opportunity to create a wider anoxic zone within the granules.

4. DISCUSSION

4.1 Low PAO and GAO proliferation under anaerobic-aerobic cycles

As in the present study, reactors intended to work with the PAO-GAO niche (under An-O cycle) deviated and showed a different metabolic phenotype and the dominance of other types of microorganisms (such as *Thauera*), as in Pishgar et al. (2019), Kang and Yuan (2019), Guimarães et al. (2020), Iorheman et al. (2020) or Othman et al. (2020). It is important to elucidate the reasons.

In the present study, PAO and GAO were not absent in absolute form, but their small quantities were not enriched further. One of the possible causes is the high DO utilized during the aerobic phase. When the COD is removed aerobically, using a high DO helps avoid the growth of filamentous OHOs and degranulation (de Kreuk and van Loosdrecht, 2004; Mosquera-Corral et al., 2005) but provides low simultaneous denitrification (SND). When the BOD_5/N is > 100/5 in the influent, nitrates accumulate in the ML and in the effluent. The recycled ML turns anoxic during the feeding phase for a fraction of time or longer, instead of being anaerobic, to the detriment of PAO and GAO, agreeing with He et al. 2017. Moreover, where it is the case, low P in influent (e.g., BOD_5/P < 100/1) can also limit EBPR and PAO. However, when the influent N is minimal (BOD5/N < 100/5), nitrogen is almost all assimilated rather than nitrified. Here, a high DO and the absence of SND do not necessarily result in failure of PAO or GAO selection

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under An-O cycle, which was probably the case of some AGS runs of Pishgar et al. (2019) and Iorheman et al. (2020).

Overall, under the intended An/O mode, PAO and/or GAO selection can fail due to high DO during the aerobic period, leading to no-SND and nitrate recycling to the feeding phase, depending on the C/N/P ratios in the influent.

4.2 *Thauera*-based technological AGS niche

Today, state-of-the-art AGS is based on enrichment of slow growers PAO and/or GAO under anaerobic-aerobic (An-O) SBR cycles. This benefits the stability of the granules, energy consumption and nutrient removal (Pronk et al., 2020). Under An-O cycle (PAO-AGS), a lower DO may be used, providing opportunities of energy saving and SND, without risks of degranulation (de Kreuk and van Loosdrecht 2004; Mosquera-Corral et al., 2005). By this way, the PAO-based AGS is a sustainable option; however, it is not always applicable or desired, as argued in the introduction (section 1.0), which justifies the interest of exploring other AGS alternatives..

Alternatively, it is known that feast-famine conditions under fully aerobic, aerobic-anoxic or anoxic-aerobic cycles select for PHA aerobic-storing bacteria (Albuquerque, 2013; Basset et al., 2016; Szabó et al., 2017). It is claimed here that the latter are relatively slow growers, denitrifiers and well settling or granulating sludge and thus may provide an alternative technology of stable AGS for BOD, and potentially for N removal. This is supported by the results from the present study and from many other recent AGS studies (yet cited) that disembogued failed PAO-GAO selection but still had stable granulation. This is also in concordance with the literature from

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pioneering and recent all-oxic AGS systems (Morgenroth et al., 1997; de Kreuk and van Loosdrecht, 2004; Bucci et al., 2020), from ASM3 (Henze et al., 2000) and from the bioplastic production literature (Albuquerque, 2013; Sruamsiri et al., 2020). It is proposed to identify the latter technological variant driven by PHA-aerobic storing OHOs as the Thauera-based AGS niche due to the omnipresence of this genus in many full-scale WRRFs and laboratory AGS systems (Xiang et al., 2021; Nierychlo et al., 2020; Pishgar et al., 2019; Layer et al., 2019) and due to its confirmed high capacity for PHA aerobic storage (Colpa et al., 2020).

To be able to fully develop this niche as a separate targeted alternative, more research is needed to further optimize aeration for energy savings, counteract the loss of activity or disintegration of the granules due to aging (e.g., through SRT control; Iorhemen et al., 2020; Rollemberg et al., 2020; Martínez-Castellanos et al., 2021), and take advantage of the advent of high-throughput microbial gene sequencing. At full scale plants, the high DO may be a drawback for cost and absence of SND (high nitrates in effluent). However, in some agricultural reuse applications (see section 1.0), the lack of SND is not a drawback. Otherwise, ways known to reduce the nitrates and the aeration costs are by performing 2-steps or alternating aeration (Layer et al., 2019), together with oxic-anoxic or anoxic-oxic cycles (Szábo et al., 2017).

In the context of shift toward more sustainable WW treatment processes and circular economy, both AGS and bioplastics production are timely (Di Bartelo et al., 2021; Johansen et al. 2022). Potentially, bioplastic recovery and WW treatment (economic substrate) can be integrated together in *Thauera*-based AGS systems; meanwhile, energy consumption and N removal may be improved further by the use of alternative optimized SBR cycles (Basset et al., 2016).

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4.3 Discriminating between the different technological AGS niches

A practical way to determine the type of AGS niche that prevails in a reactor is by the shape of the COD removal profile during a cycle (Figure 5): Profile I with PAO-GAO dominance, Profile II under dominance of PHA-storing OHOs (*Thauera* and similar), and the intermediary Profile III, where both PAO-or-GAO and *Thauera et al.* are significant.

In profile I (PAO-GAO niche), most of the COD is removed during the anaerobic phase, as in graph-6c of Bassin et al. (2019) and graph-1 of Haaksman et al. (2020).

➔***Figure 5***

In profile II, the characteristics of an An-O AGS system and regular SBR populated by PHA aerobic-storing OHOs such as *Thauera*, is observed, where PAO and GAO are not able to proliferate for some reason. Some rbCOD removal may occur during the non-aerated phase (20% in graph 4a of Kang and Yuan, 2019), being limited to little anoxic COD consumption by OHOs. Most of the COD leaks to the aerobic phase where it is quickly stored, e.g., as in the present study, as in graph 4a of Kang and Yuan (2019) and as in graph 4 of Falvo et al. (2001). The full aerobic cycle under the feast-famine regime can also show profile II, evidently without the anaerobic section.

In profile III, the anaerobic storage of VFA or rbCOD by PAO and/or GAO is incomplete and much COD leaks to the aerobic phase, e.g., as in Rollemberg et al. (2020), He et al. (2017) and Kang and Yuan (2019, graph-4b). The conditions during aerobic degradation determine whether the OHOs will become filamentous or settle well, having a great impact on the integrity of the granules (Bassin et al., 2019; Layer et al., 2019; Haaksman et al., 2020).

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5. CONCLUSIONS

Ordinary heterotrophic organisms performing COD aerobic storage were dominant in the sludge, resulting in well settling and granulated biomass and showing that not all OHOs are detrimental to AGS formation and stability.

The study reveals a stable AGS system outside of the PAO/GAO niche and identifies the PHA aerobic-storing OHOs as an alternative technological AGS niche for BOD and N removal.

When COD is degraded mostly under aerobic feast-famine regime, PHA aerobic-storing bacteria are selected, leading to well-settling AGS. As relatively slow growers and EPS producers, these OHOs stabilize the granules.

Under anaerobic/aerobic cycle (targeted), PAO-GAO selection can fail when high DO prevails during the aerobic period, impairing the simultaneous-denitrification and recycling nitrates to the feeding phase. This is a clue for identifying the causes of failure of many PAO-based AGS.

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FIGURE CAPTIONS

Figure 1: COD, OUR (rO2), N and P time profiles during the cycle of the CAS-SBR system. a) COD and rO2 and illustration of the DO on-off control during respirometry. b) Plateaus and transitions of the OUR curve over time. c) Ammonia, nitrate and phosphorus. d) COD and rO2 as in Item a, but from another test.

Figure 2: Microscopy observations and particle size distribution of the conventional sludge. a) Optical microscopy at 40x. b) Fluorescence microscopy (CLSM). c) Scanning electron microscopy at 20000x (SEM).

Figure 3: Morphology and particle size distribution of the AGS. a) and b) Digital images of the final bulk granules (unscaled). c) Closer view of the granules by optical microscopy (x100). d) Distribution of the sludge particle sizes at Days 104 (blue), 122 (green), and 137 (red).

Figure 4: COD, N, and P removal profiles along the 4 h cycles of the AGS reactor. a) Routine follow-up of the COD uptake profiles at Days 100 to 129 of operation. b) and c) steady profiles at Day 137 for COD and DO versus N and P.

Figure 5: Classification of the variants of AGS niches depending on the shape of the COD time profile (as evaluated through a side-batch test provided with mixing and acetate pulse feeding under a non-aerated/aerated cycle).

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Table 1: Characteristics and operational parameters of the two SBRS utilized in the study

Operating conditions	**CAS-SBR**	**AGS-SBR**
Reactor Shape	 Rectangular	Bubble Column 
Volume (L)	30 L	1 L
Overall dimensions (cm)	50x30 x28 cm	Ø 4 x 100 cm
Volume exchange ratio (%)	66.6%	50.0%
Height/Diameter, H/D *	(0.45), equivalent	15
Operating cycle		
Total cycle length	24 h	4 h
Anaerobic feeding and reaction	1 h – 1h 30 min (pulse)	1 h 30 min (step)
Aeration	21 h 20 min	2h 20 min
Settling	1 h	4 min
Decant and idle	10 min	6 min
Main influent characteristics		
Acetate (mg/L COD)	500	500
NH_4Cl (mg/L N-NH_4)	42	42
KH_2PO_4 (mg/L P-PO_4)	11	11
COD/N/P	100/8.4/2.2	100/8.4/2.2
Operational parameters		
Sludge retention time (SRT)	15 d, controlled	High, not controlled
Hydraulic retention time (HRT)	36 h	8h
Critical settling velocity (m/h)	0.1 m/h	3.6 m/h
Organic loading rate (gCOD/L.d)	0.33	1.5
Dissolved oxygen (mg/L DO), pH and Temperature (°C)	> 4 mg/L DO, pH 8.5-9.1, room (18-23°C)	> 4 mg/L DO, pH 8.4-9.0, room (18-23°C)

* The H/D ratio is based on the effective height (H) of liquid in the reactors. Furthermore, the equivalent diameter (D) of the rectangular tank was estimated from the superficial area of this SBR

Table 2: Characteristics of the CAS-SBR mixed liquor at steady state

	MLSS (mg/L)	MLVSS (mg/L)	Particulate COD (mg/L)	Soluble COD (mg/L)	SVI_{30} (mL/g TSS)
Average	**1267**	**1079**	**1439**	**21**	**59**
±SD	±115	±105	±121	±12	±20

Table 3: Average characteristics of the AGS mixed liquor

	MLSS (mg/L)	VSS/TSS ratio	Soluble COD (mg/L)	SVI_{5} (in-situ) (mL/g TSS)	pH (-)
Average	**4720**	**0.89**	**22**	**58**	**8.6**
±SD	±72	±0.01	±4	-	±0.3

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Table 4: Abundance (%) of the most relevant microbial groups at each taxonomic level. *The 3 higher percentages at each level are evidenced in bold.*

Phylum	Class	Order	Family	Genus
Proteobacteria **67.1%)**	Betaproteobacteria **(30.0)**	Rhodocyclales **(26.8 %)**	Rhodocyclaceae **(26.8 %)**	**Thauera (25.0 %)**
				Zoogloea (0.9%)
		Burkholderiales (1.6%)	Comamonadaceae (1.5%)	Delftia (0.4%)
		Nitrosomonadales (1.2%)	Nitrosomonadaceae (1.2%)	Nitrosomonas (1.2%)
	Alphaproteobacteria **(28.6%)**	Rhodobacterales **(21.6 %)**	Rhodobacteraceae **(21.6 %)**	**Paracoccus (21.0 %)**
		Rhodospirillales (2.0 %)	Rhodospirillaceae (1.8%)	Defluviicoccus (1.5%)
		Caulobacterales (1.9 %)	Caulobacteraceae (1.4%)	Brevundimonas (1.1%)
		Rhizobiales (1.5%)	Rhizobiaceae (0.5%)	Rhizobium (0.3%)
		Sphingomonadales (1.5%)	Sphingomonadaceae (1.4%)	Sphingopyxis (0.4%)
				Sphingorhabdus (0.4%
			Caulobacteraceae (1.4%)	Brevundimonas (1.1%
	Gammaproteobacteria (6.1 %)	Xanthomonadales (5.3 %)	Xanthomonadaceae (5.3 %)	Dyella (2.9 %)
				Pseudoxanthomonas (1.4%)
	Deltaproteobacteria (1.98 %)	Bdellovibrionales (0.9%)	Bdellovibrionaceae (0.8%)	Bdellovibrio (0.8%)
Bacteroidetes **(27.1 %)**	Flavobacteriia **(13.9 %)**	Flavobacteriales **(13.9 %)**	Flavobacteriaceae **(11.9 %)**	**Flavobacterium (10.9 %)**
			Cryomorphaceae (2.0 %)	Salinirepens (0.9%) Wandonia (0.5%)
	Sphingobacteriia (5.7 %)	Sphingobacteriales (5.7 %)	Chitinophagaceae (2.0%)	Flavihumibacter (1.6 %)
			Sphingobacteriaceae (3.5%)	Pedobacter (2.7 %)
	Bacteroidia (4.51 %)	Bacteroidales (4.5 %)	Prolixibacteraceae (2.08 %)	Mariniphaga (1.7 %)
			Rikenellaceae (1.5%)	Alistipes (1.5%)
	Cytophagia (2.70 %)	Cytophagales (2.7 %)	Cytophagaceae (2%)	Leadbetterella (1.3%)
Firmicutes **(1.84 %)**	Clostridia (1.6%)	Clostridiales (1.6%)	Clostridiales_Incertae_Sedis_XII	Fusibacter (1.1%)
Nitrospirae (0.3%)	Nitrospira (0.3%)	Nitrospirales (0.3%	Nitrospiraceae (0.3%	Nitrospira (0.3%)
5 Others (< 0.8% each)				

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Table 5: Dominant bacterial genera of the aerobic granular sludge

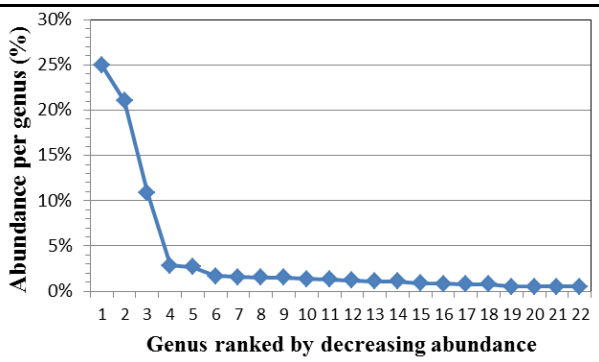
Rank #	Canonical genus name	Abundance (%)	Comparison of the relative contributions
1	Thauera	25.02%	
2	Paracoccus	21.03%	
3	Flavobacterium	10.93%	
4	Dyella	2.87%	
5	Pedobacter	2.70%	
6	Mariniphaga	1.67%	
7	Flavihumibacter	1.57%	
8	**Defluviicoccus (GAO)**	**1.53%**	
9	Alistipes	1.53%	
10	Pseudoxanthomonas	1.38%	
11	Leadbetterella	1.32%	
12	**Nitrosomonas (AOB)**	**1.22%**	
13	Fusibacter	1.10%	
14	Brevundimonas	1.07%	
15	Salinirepens	0.91%	
16	**Zoogloea**	0.86%	
17	Bdellovibrio	0.78%	
18	Lysobacter	0.76%	
19	Ignavibacterium	0.53%	
20	Delftia	0.53%	
21	Hydrogenophaga	0.52%	
22	Wandonia	0.51%	

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Table 6: Structure of the microbial community (abundance of the functional groups)

Functional groups* (%)		**Putative genera that are considered in each group**
PAO	0.32 %	- Dechloromonas (0.024%). Corynebacterium (0.001%). Halomonas (0.001%). - Tetrasphaera, Ca Accumulibacter and Ca Obscuribacter were not detected (ND). - Other detected probable PAO: Pseudomonas (0.24%). Gemmatimonas (0.07%). - Friedmaniella, Quatrionicoccus, Malikia and Tessaracoccus (ND) - Via the class/family of the unclassified genera, 0.02% when more, could be PAO.
GAO	1.67 %	- Only the GAO Defluviicoccus (1.53%) and Propionivibrio (0.14%) were present. - Ca Competibacter, Micropruina and Ca Contendobacter were not detected (ND).
AOB	1.23 %	- Nitrosomonas (1.22%) and Nitrosospira (0.004%).
NOB	0.30 %	- Only Nitrospira (0.30%) was detected; not associated to Nitrobacter (ND). - Others: Nitrotoga, Nitrococcus, Nitrospina and Nitrolancetus (ND).
ANAMMOX	0.00 %	- None detected, ND: (0% of Ca Brocadia, Ca Anammoximicrobium, Kuenenia and Anammoxoglobus).
OHO	77 – 96.5 %	- The rest of the biomass, out of the PAO, GAO, AOB and NOB, were classified as OHOs (up to 95.5%, maximum), which also pools all the uncertainties there. - As a minimum, 77% of abundance was confirmed as OHOs, based on the list of 22 top genera (> 0.5%, each) shown in **Table 5.**

* PAO: Polyphosphate accumulating organisms. GAO: Glycogen accumulating organisms. AOB: Ammonia oxidizing bacteria. NOB: Nitrite oxidizing bacteria. ANAMMOX: Anaerobic Ammonium Oxidizing Bacteria. OHO: Ordinary heterotrophic Organisms.

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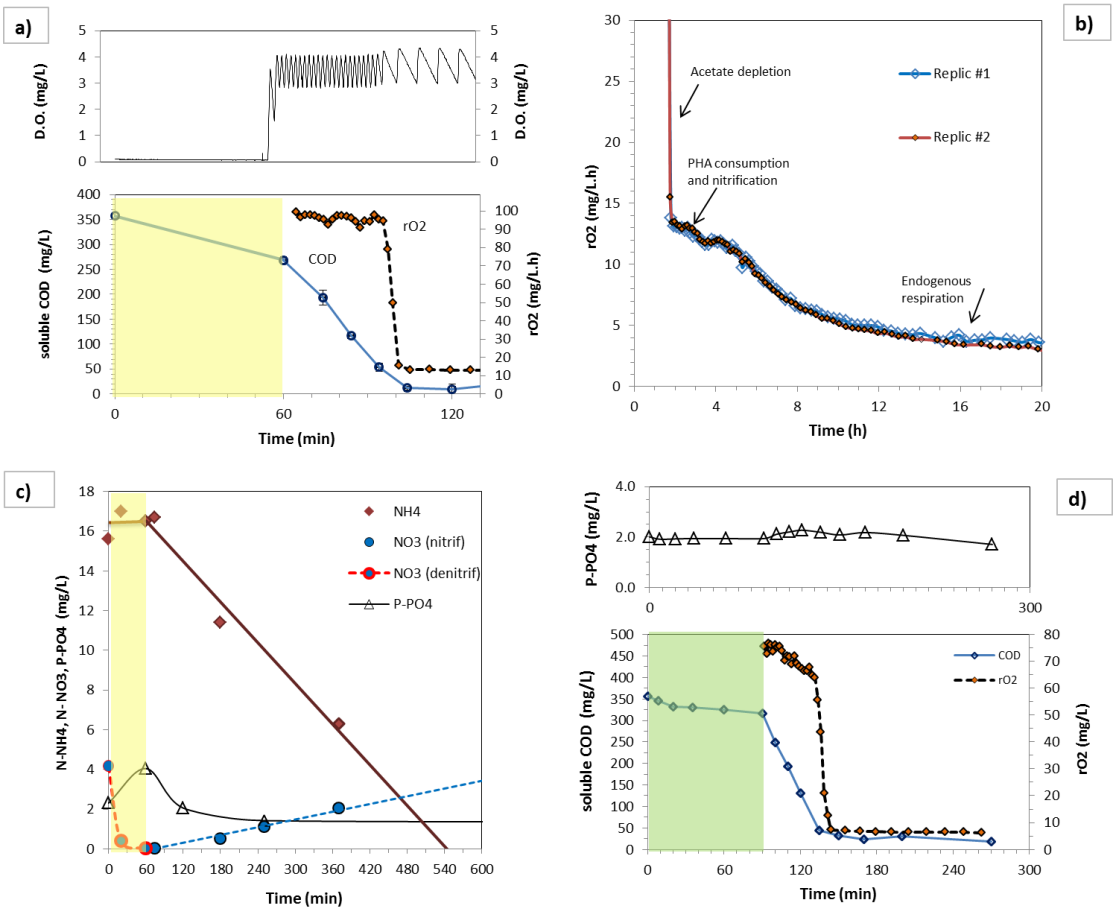

Figure 1:

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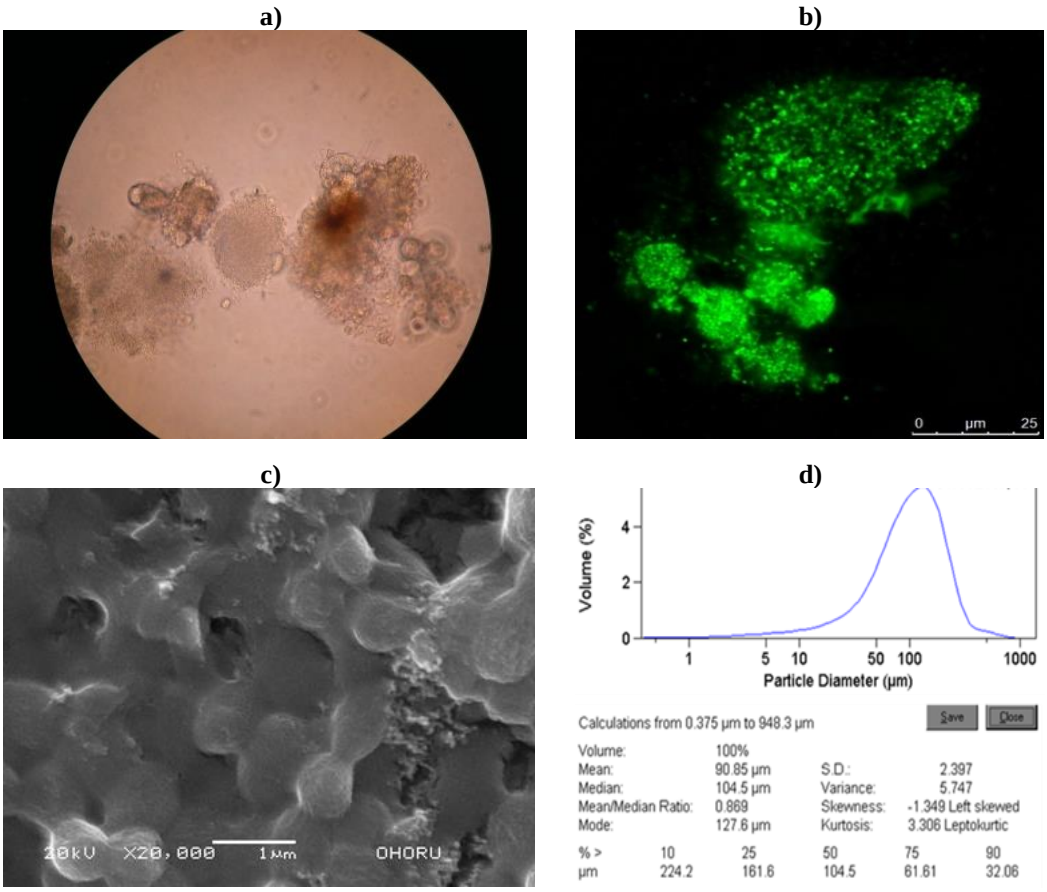

Figure 2:

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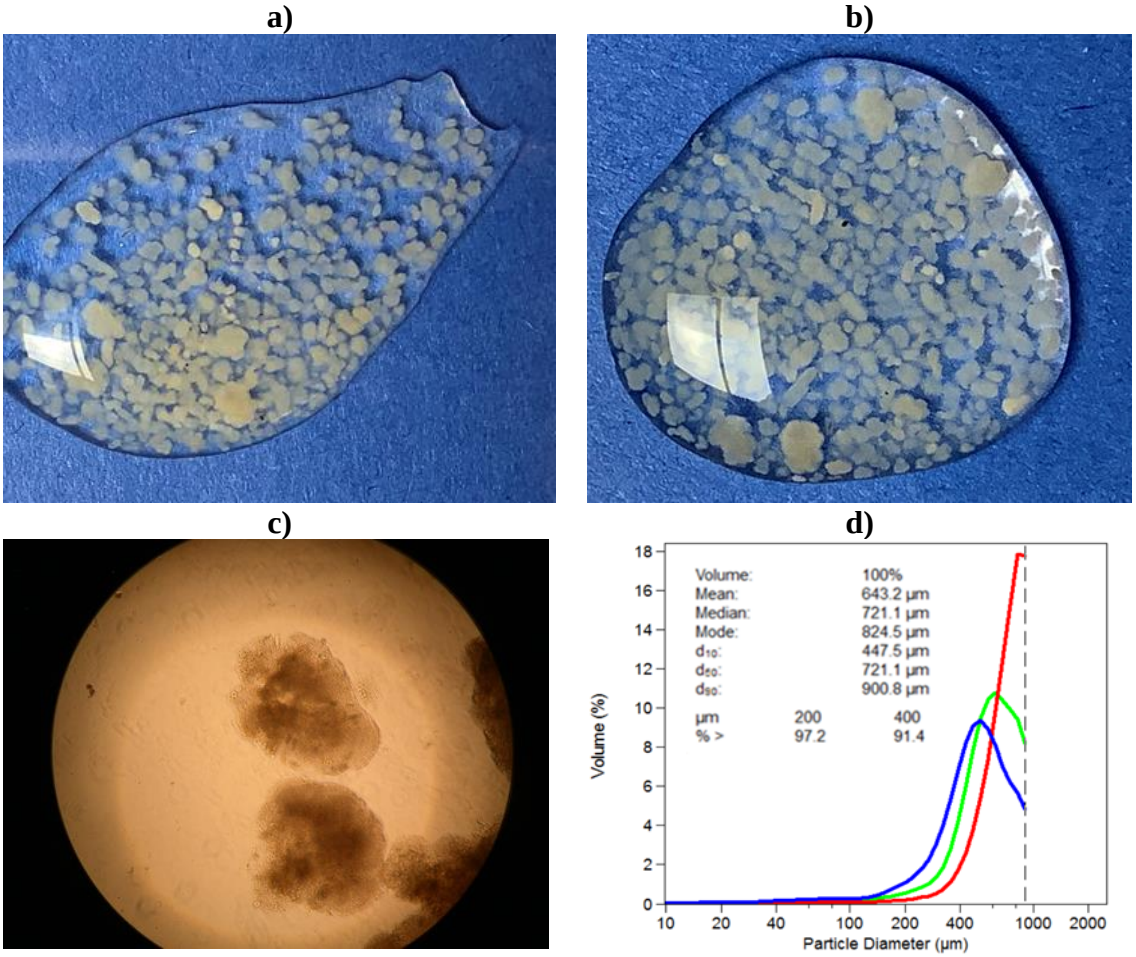

Figure 3:

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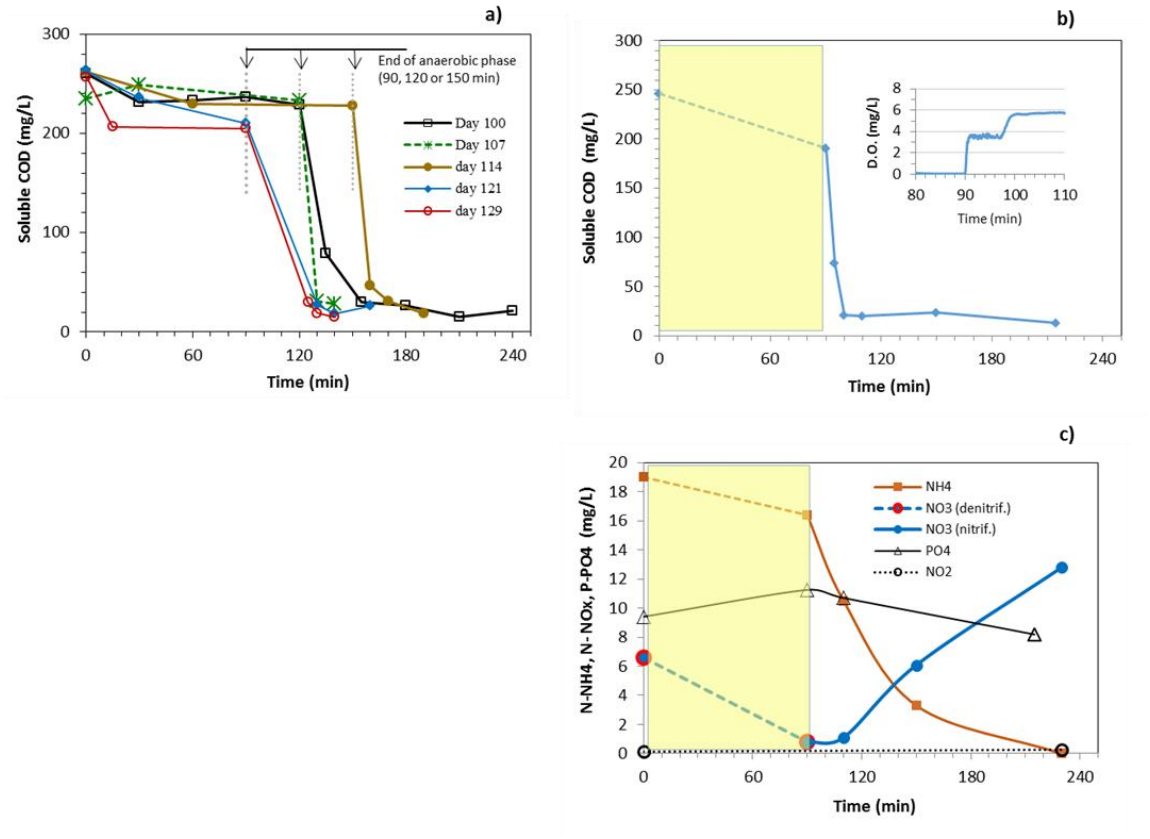

Figure 4:

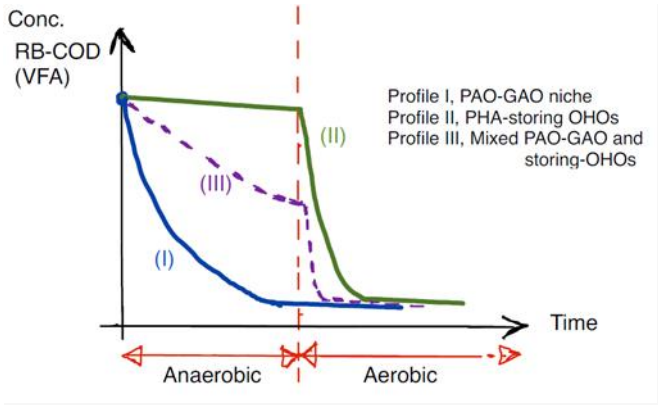

Figure 5: