# The Prediction of Partial-Nitrification-Anammox Performance in Real Industrial Wastewater based on Granular Size

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## Abstract

To date, the partial nitrification-Anammox (PN-A) granular sludge size has been exclusively analyzed in synthetic substrates. In this work, different ranges of granular size of PN-A sludge were studied at low oxygen concentration using real industrial wastewater as, well as a synthetic substrate. The granular sludge was characterized by the specific nitrification activity (SNA), specific anammox activity (SAA), and granule sedimentation rate. The relative abundance of the bacterial consortium was assessed for each range of diameters through the fluorescence in situ hybridization (FISH) technique. SNA exhibits a direct association with the specific surface of granules, which proves the importance of the outer layer in the nitrification process. Even more critical, the flocculent sludge allowed the stability of the nitrifying activity. The SAA showed different performances faced the real industrial and synthetic substrates. With the synthetic substrate, the SAA decreased at higher diameter ranges, whereas with the industrial substrate, the SAA increased at higher diameter ranges. This situation is explained by the oxygen protection in the sludge maintained with industrial wastewater. The relative abundance of heterotrophic bacteria increased from 9.6 to 22%, due to the presence of organic matter in the industrial substrate. The granular sedimentation rate increased with the diameter of the granules with a linear correlation ($R^2$>0.98). Thus, granular sizes can be selected through sedimentation rate control. A linear correlation between SAA and granular sludge diameter ranges was observed. With this correlation, an error of less than 11% in the prediction of SAA was achieved. The use of diameter measurement and granular sedimentation rate as routine techniques could contribute to the control and start-up of PN-A reactors. In the same sense, organic matter present in defined concentrations, can be beneficial for the granular sludge stability, and thus, for nitrogen removal.

**Keywords:** granular; sludge; Anammox; size; diameter

## 1. Introduction

The active sludge technology has been considered as the central wastewater treatment system until the 20th century (Trego et al., 2020). However, in recent years the granular biofilms have become the technology at the forefront of industrial innovation and research (Sarma et al., 2017; Trego et al., 2020). The anaerobic granular sludge, the aerobic granular sludge (AGS), and the Partial Nitrification- Anammox granules (PN-A) are the most widely used granular biofilms for nitrogen and carbon removal (Nsenga Kumwimba et al., 2020; Trego et al., 2020). AGS and PN-A granular sludges are produced in the presence of oxygen. However, the AGS is used to remove organic carbon and, on the other hand, the PN-A granular sludge is directed to nitrogen removal using inorganic carbon sources (Roeckel et al., 2017; Sarma et al., 2017). The low oxygen requirement, no need for organic matter supplementation and the possibility of using a one-stage reactor to remove inorganic nitrogen have transformed PN-A granular sludge in the biological technology with the highest projection (Lackner et al., 2014; Roeckel et al., 2017).

Substrate gradients and shear stress phenomena produce ecological niches in the PN-A granular sludge (Arriagada et al., 2017). This is the main advantage of PN-A granular sludge compared to the partial nitrification (such as SHARON system) and Anammox sludge reactors because the PN-A granular sludge is composed of both aerobic and anoxic/anaerobic layers. This single reactor can produce the reduction of ammonia until nitrogen gas (Morales et al., 2015). The microorganisms in PN-A granular sludge establish a dynamic balance that leads to a complete degradation of inorganic nitrogen. Thus, the products of one organism are utilized by others (Li et al., 2020; Trego et al., 2020). In the external layers of the PN-A granule, nitrite can be obtained from the oxidation of ammonium to nitrite (PN) (equation 1); and in internal layers the Anammox bacteria oxidize ammonium using nitrite as an electron acceptor, thus producing gaseous nitrogen (equation 2).

$$\mathrm{NH_4^+} + 0.0743\mathrm{HCO_3^-} + 1.404\mathrm{O_2} \rightarrow 0.985\mathrm{NO_2^-} + 0.0149\mathrm{C_5H_7O_2N} + 1.911\mathrm{H^+} + 1.03\ \mathrm{H_2O} \qquad (1)$$

$$\mathrm{NH_4^+} + 1.146\mathrm{NO_2^-} + 0.071\mathrm{HCO_3^-} + 0.057\mathrm{H^+} \rightarrow 0.986\mathrm{N_2} + 2.02\ \mathrm{H_2O} + 0.071\mathrm{CH_{1.74}O_{0.031}N_{0.15}} + 0.161\mathrm{NO_3^-} \qquad (2)$$

To achieve stable operation, process variables must be carefully imposed as the microorganism population balance is critical (Roeckel et al., 2017). Thus, the monitoring of specific nitrification activity (SNA) and specific Anammox activity (SAA) are common in regular operations. SAA and SNA are quantified with standard methodologies where mass transfer problems are avoided and, then, only stoichiometry and microbial kinetics influenced the specific activities calculated (Arriagada et al., 2017; Varas et al., 2015). In this way, it is possible to evaluate the activity of the PN-A granular sludge under optimal conditions, allowing by comparison, to identify limiting conditions that may be present in the reactor design or operation.

The Low-oxygen strategy has been proposed to reduce Anammox oxygen inhibition and for avoiding the outcompetition for nitrite between nitrite-oxidizing bacteria (NOB) and Anammox bacteria (Roeckel et al., 2017). Therefore, this type of oxygen strategy has already been successfully tested in full-scale systems (Lackner et al., 2014).

The Granular size is closely related to the operating conditions in granular sludge reactors. For instance, it has been reported that the diameter or size from AGS affects the substrate mass transfer, sedimentation rate and microbial abundance distribution (Qian et al., 2017). In the same way, shear stress and load rate can influence the granule diameter (Zhang et al., 2015). However, in PN-A granules, the relation between granule diameter and reactor performance has not been completely elucidated so far.

In the last decade, at least 34% of the contributions on “Anammox granular sludge” are associated with “nitrification” (Fig. 1S). Nevertheless, in the last three years only 2% of the cited consider the three concepts: “Anammox granular sludge”, “nitrification” and “diameter”. Then, the diameter measurement is not a routine technique for the prediction of PN-A reactor performance. Besides, the study of PN-A granular diameter has been exclusively carried out with synthetic substrates, and the application of granular diameter as a parameter for performance optimization and stability during the start-up of PN-A system in full-scale has not been evaluated.

Thus, the aim of this work was to evaluate the effect of industrial real wastewater substrate on the metabolic, microbiological and physical properties of different diameter ranges of PN-A granular sludge obtained at low oxygen concentration. The PN-A granular sludge produced with a synthetic substrate was used as control. The effect of organic matter in different diameter ranges of PN-A granular sludge was investigated. The possibility of using granular sludge diameter as a routine technique to replace complex biochemical techniques was also investigated.

## 2. Materials and methods

### *2.1 Description of reactor operation*

PN-A granular sludge was collected from two continuously fed bubble column reactors (BCRs) operated with synthetic (absence of organic matter) and industrial wastewater (presence of organic matter). Reactor design, synthetic substrate and operation parameters of the BCR without organic matter were previously evaluated by Varas et al. (2015). The general operating conditions for the BCR with organic matter were set according to Arriagada et al. (2017). Poultry manure after anaerobic digestion (details described in Arriagada et al. (2017)) was used as industrial wastewater substrate in BCR with organic matter. The general operating conditions for both reactors were temperature operation of 35°C, with a low oxygen concentration of 0.1-0.4 mg $O_2$/L and continuous operating regime. Particular operational conditions of each reactor are detailed in Table 1.

### *2.2 Separation of ranges of granular sizes*

PN-A granular sludges from BCRs were separated in granular size ranges. The separation was performed by the adaptation of the wet sieves method (Pereboom, 1994; Toh et al., 2003). Briefly, the granular sludges available were washed in different sieves with phosphate buffer. Once the granular sludges were passed through the sieves, the particles were distributed in different diameter ranges. Then, they were stored in jars with the effluent of each reactor. The diameter distribution of each level was studied with the equivalent projection area diameter using the specific MonGran software (Jara-Muñoz et al., 2019). Four and three-level were produced for BCR operated with and without organic matter, respectively. (Table 2)

### 2.3 Nitrogen Specific activities

Nitrogen specific activities were characterized by SNA and SAA.

The SNA was measured according to the procedure described by Arriagada et al. (2017) in jars incubated at 35°C with magnetic stirring and the oxygen concentration was measured by an oxygen electrode (WQ-FDO, Global Water Instrumentation, Inc., USA).

The SAA was calculated based on the pressure increase using OxiTop® Control AN6 (WTW, Weilheim, Germany) (Varas et al., (2015)). Gas composition was also analyzed (Details are described below; M&M Section 2.6). Due to the possible interference produced by the denitrifying activity, the normal SAA assays were modified for the PN-A granular sludge samples from the BCR operated with digested poultry manure. Thus, samples were washed three times with phosphate buffer and then 42 mg N/L of nitrite were added. Then, vials were incubated until a stable pressure was reached. Immediately, the PN-A granular sludge samples were washed with phosphate buffer and the standard methodology was followed (Varas et al., 2015).

The activities were also measured in the total reactor mixture liquid, which includes contributions of granular, flocculent and free-living bacterial biomass.

### 2.4 Molecular identification of bacteria present in the granules

The relative abundance of different populations of microorganisms in PN-A granular sludge samples was characterized by the fluorescence *in situ* hybridization (FISH) technique, as described in Arriagada et al. (2017). Microorganisms from the nitrogen cycle such as AnAOB (Anammox), ammonia-oxidizing bacteria (aerAOB) and NOB were analyzed. The difference between eubacteria and the sum of each bacterial group was called unidentified eubacteria ($EUB^{UI}$).

Samples from different size range levels taken from the reactors operated with synthetic and real wastewater industrial substrates were evaluated. Statistical analyses were performed through the Student's t-test with GraphPad Prism v5.0 (GraphPad Software, USA). P-values <0.05 were considered statistically significant.

### *2.5 Sedimentation properties: Granular sedimentation rate*

From each sample the granular sedimentation rate (GSR) was measured by the time necessary for a singular granule to travel 30 cm of the transparent vertical column (diameter = 10 cm) filled with water (20°C) as indicated by Vlaeminck et al. (2009). The time measurements were averaged from at least 30 granules in each sample.

### *2.6 Analytical methods*

The gas produced by the samples from SAA was analyzed in a gas chromatograph (HP 5890 Series II, Hewlett Packard, Avondale, PA, USA) equipped with a Porapak Q column, 80/100 mesh with thermal conductivity detector (TCD) under the following conditions: 30°C of injection temperature and 160ºC temperature Detection.

Total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD), oxygen dissolved concentration, pH and nitrogen compounds [nitrate, nitrite and total ammonium nitrogen (TAN-N)] were determined according to the test proposed by Arriagada et al. (2017).

### *2.7 Calculations*

The specific surface of PN-A granular sludge ($m^2\ m^{-3}$) was obtained as the total surface of a sample of "n" granules divided by their total volume according to the equation proposed by Vázquez-Padín et al. (2010) which considers the granules as spheres:

$$a = \frac{\sum_{i=1}^{n} 4\pi \left(\frac{D_i}{2}\right)^2}{\sum_{i=1}^{n} \frac{4}{3}\pi \left(\frac{D_i}{2}\right)^3} \qquad (3)$$

Activities unbalance (AU) was analyzed with PN-A stoichiometry (equations 1 and 2). The partial nitrification stoichiometric reaction is related to SNA (equation 1). In the same way, the Anammox stoichiometric reaction is related to SAA (equation 2). The relation of both equations can be used to evaluate the yield of the PN-A process. Thus, 1.7 grams of nitrogen gas ($N_2$) (equation 2) are produced from one gram of oxidized ammonia (equations 1). The relation of SAA and SNA also can be related to the yield of the process, if the value is not near to 1.7 the process is unbalanced, then the next expression can be used:

$$AU = \frac{1.7\left(\frac{g\ N_2}{g\ N - NH_4}\right) - \frac{SAA_i}{SNA_i}\left(\frac{g\ N_2}{g\ N - NH_4}\right)}{\frac{SAA_i}{SNA_i}\left(\frac{g\ N_2}{g\ N - NH_4}\right)} \qquad (4)$$

where 1.7 is a constant value of PN-A stoichiometry and $\frac{SAA_i}{SNA_i}$ is the relation of both activities from the sample i. If AU is negative, there is a high SNA or small SAA. On the contrary, if AU is positive, there is a small SNA or high SAA. The low oxygenation strategy was considered during the interpretation of these results. The results of the different parameters were analyzed with statistical using Student's t-test or with analysis of variance (ANOVA) with Tukey's test as a *post hoc* analysis with GraphPad Prism v5.0 (GraphPad Software, USA). P-values <0.05 were considered statistically significant.

## 3. Results and discussion

### *3.1 Relation of granular sizes with specific nitrification activity*

The influence of organic matter in the size distribution of PN-A granules was analyzed. The granular sludge produced by industrial and synthetic substrate without organic matter showed mean diameters of 1.24 and 0.81 mm, respectively. As described above, different diameter ranges of granular sludge were obtained through the wet sieving test (Toh et al., 2003). For the PN-A reactor with synthetic substrate three granular diameter level ranges were obtained. On the other hand, a fourth granular size range for granules greater than 1.68mm was obtained with the industrial substrate (Table 2). In the PN-A reactor without organic matter, only three-level were constructed because there was not enough sludge from the fourth size to represent a fourth level according to the relative frequency analysis performed on different samples (Fig. 2S). Each level presented a significant difference with a 95% confidence parameter (ANOVA test) within each group. In addition, the ranges can be compared between both reactors since average diameters were similar (even the third level, which is not in the same ranges, showed no significant differences in average diameter) (Table 2). These results allow to compare the metabolic and genetic characteristics between both substrates. In addition, the different diameter ranges observed were similar to those used by Vangsgaard et al. (2012) to analyze and model the influence of mass transfer and microbial kinetics of nitrogen removal in a PN-A process.

AerAOB and, to a lesser extent, NOB population dominates the SNA in the PN-A process. In a low oxygen operation strategy, the oxygen concentration must not produce: A) NOB proliferation and B) anAOB inhibition (Arriagada et al., 2017; Jara-Muñoz et al., 2019). Commonly, reactors are operated under oxygen concentrations of 1.1 mg $O_2$ / L, since this is the oxygen saturation coefficient of NOB population, unlike aerAOB that has an oxygen saturation coefficient of 0.3 mg$O_2$ / L (Wiesmann, 1994). Thus, below 1.1 mg$O_2$ / L, NOB proliferation is inhibited with a minor effect on aerAOB activity.

In this work, greater granular size (which implies lower specific surface) was associated with SNA decrease for both reactors (Fig. 1a). In other words, the increase of specific surface produced the increase of SNA (Table 2). Similar results have been reported in experimental and numerical simulations. With numerical simulations a correlation between specific surface and SNA was showed (Vangsgaard et al., 2012; Volcke et al., 2010). In this way, the increase of granular size can produce (1) smaller fractions of granular sludge exposed to oxygen and (2) the increase of external mass transfer resistance. Regarding to the last point, Vangsgaard et al. (2012) demonstrated a slight increase in the mass transfer resistance with the granular size increase, although, with similar performances the different granule size ranges (0.5, 1, 1.5 and 2 mm). In an experimental work, Vlaeminck et al. (2010) evaluated the sludge aggregate size of three design reactors: (1) SBR with a magnetic stirrer, (2) SBR with bladed impeller and (3) Upflow reactor with aeration mixing mechanism. The first two reactors were operated at low oxygen concentrations (0.4-1.1 $mgO_2$ / L) whereas in the third reactor, oxygen concentrations were higher (2.0-3.0 $mgO_2$ / L). In the three systems, greater specific surface was correlated with greater SNA of the PN-A granular sludge. In the present work, the same correlation was observed for the synthetic and real complex substrate (such as poultry manure anaerobic digester). However, At diameter sizes >1.15 mm the SNA presented no significant differences (ANOVA test, 95% confidence parameter) in the PN-A reactor operated with real industrial wastewater (Fig. 1a). Despite that, the results of SNA obtained from the reactor mixture liquid operated with real wastewater industrial substrate was three times higher than the trend observed for the granules (Fig. 1a). The logical conclusion to be drawn is: The PN-A granular sludge is not the only responsible for the nitrification activity in the reactor with the industrial substrate.

There is also a contribution of free and flocculent bacteria, whose population is highly depend on the operation imposed conditions (Qian et al., 2017). In contrast, different results were found when the SNA was measured in the reactor mixture liquid operated with synthetic substrate. The contribution of the flocculent sludge in the SNA was much smaller. Thus, these results reaffirmed the limitation of nitrifying bacteria during a strategy with low oxygen during the treatment of substrate with a high organic load in PN-A granular sludge (Figueroa et al., 2012). This concept is explored below in the analysis of the relative distribution of NOB and EUB$^{UI}$ population.

The increase in granular diameter was not capable to produce a significantly different population distribution for aerAOB in the PN-A reactor (with or without organic matter) (ANOVA test, 95% confidence parameter) (Fig. 2 and Fig. 3S). Different results were observed by Vlaeminck et al. (2010), when they reported a decrease of aerAOB abundance with the increase of diameter. However, these results were reported on SBR operation with mechanical agitation. Indeed, a different PN-A granular conformation has been reported with mechanical or gas agitation (Jara-Muñoz et al., 2019). In addition, the largest NOB population was observed at the smallest granular diameter range from the PN-A reactor operated with organic matter (Fig. 3). On the other hand, the EUB$^{UI}$ population increased with the granular diameter (Fig. 2). The present results could be explained by the requirement of organic matter by EUB$^{UI}$ population. Heterotrophic bacteria are included in the EUB$^{UI}$ population, then its presence do not affect the aerAOB population but outcompete the NOB population (Arriagada et al., 2017). In addition, a decrease in the biodiversity of NOB population in PN-A reactors linked with the increase of organic matter in the substrate has been reported (Liang et al., 2015) This is not surprising, because the oxygen saturation coefficient of heterotrophic bacteria is 5.5 times lower than the NOB population, therefore the outcompetition of both populations is clear (Vangsgaard et al., 2012). On the contrary, the oxygen saturation coefficient of heterotrophic bacteria is only 1.5 times lower than the aerAOB population (Vangsgaard et al., 2012). Thus, low concentrations of organic matter could even be beneficial for controlling the NOB population.

Finally, the SNA decreased for the higher diameter ranges for both synthetic and industrial substrates. However, the flocculent sludge is mainly responsible for the overall activity in reactors with organic load. The low oxygen operation strategy showed a minor problem with SNA on the reactor with organic matter due to the competition for oxygen between heterotrophic and NOB population (Fig. 1 and Fig. 2).

### *3.2 The effect of the granular sizes on the specific Anammox activity*

The SAA from the reactor operated with the organic matter was measured by a standard methodology (Arriagada et al., 2017). The results presented a maximum SAA in the third size range (Diameter range 1.25-1.68 mm) (Fig. 3). With adequate operational control of the organic matter load, denitrifying bacteria can contribute to the overall nitrogen removal efficiencies through the reduction of nitrate (produced by NOB and anAOB population) until nitrogen gas production, and also, an improvement of the granular sludge aggregation (Langone et al., 2014). However, a high organic matter load can favor that denitrifying heterotrophic bacteria outcompete with Anammox for nitrite (Arriagada et al., 2017; Kraiem et al., 2019).

For this reason, in our study a pretreatment with nitrite incubation to reduce a probable interference with denitrifying microorganisms was conducted. Results with this modified methodology showed a linear correlation between granular size and SAA ($R^2$=0.96) (Fig. 3). In addition, it was proved that 42% of standard SAA was due to denitrifying activity for this diameter range (1.25-1.68mm) (Fig. 3). Without pre-treatment, results can show an overestimate of SAA (Kraiem et al., 2019; Milia et al., 2017). In this work, the SAA for sludge fed with industrial substrate were evaluated with the nitrite pretreatment (Fig. 1b).

The SAA showed different performances among substrates: without organic matter (synthetic substrate), the SAA decreased at higher diameter ranges; whereas with organic matter (real wastewater industrial substrate) the specific Anammox activity increased at higher diameter ranges (Fig. 1b). Experimental assays (Vlaeminck et al., 2012) and mathematical simulations (Volcke et al., 2012) have shown the increase of SAA during the increase of granular sludge size .Nevertheless, in this work granules from reactors operated with synthetic substrate, the largest size range had, at least, three times less SAA than the smallest size range (ANOVA test, 95% confidence parameter). Probably, the effect observed can be attributed to: (1) nitrite limitation due the decrease of SNA, (2) Anammox inhibition: less resistance to the oxygen transfer trough the granule due fragmentation or a less thick aerobic film on the granule surface (Toh et al., 2003), and (3) the relative abundance of NOB population (twice as large) with large granules (Fig. 3S). Then, these results can be expected in an industrial PN-A reactor operated with low oxygenation as long as the raw stream had a similar chemical composition that the synthetic substrate. The best results for synthetic substrate for SAA and SNA were found in the smallest PN-A granular size (Table 1). Thus, a correct operation strategy can be directed to produce smaller PN-A granular sludge. This idea is analyzed in greater depth in section 3.3.

Notwithstanding, the overall activity measured in the mixed liquor showed a correlation between average diameter held in the reactor and SAA (this is discussed below).

The same trend observed in literature was obtained in the reactor operated with real industrial substrate in this work. The increase of ammonia surface load and the smaller oxygen penetration produced by the heterotrophic growth in the outer granular layer seem to be the main causes (Figueroa et al., 2012; Volcke et al., 2012, 2010). This result was confirmed with the increase of the relative abundance of $EUB^{UI}$ with the granular size (Fig. 2). Even more, the SAA in the mixed liquid from both reactors can be predicted only by granular size with an error of 6.7 and 11% for reactors operated without and with organic matter, respectively (Fig. 1b). Then, despite the complex size distribution of the granular sludge reactors, their SAA could be predicted only with the average granule sizes.

Considering the PN-A stoichiometry (equations 1 and 2) the aerobic oxidation of one gram of ammonia can produce 1.7 grams of $N_2$, in a two-step process. Therefore, SNA and SAA could be stoichiometrically related and then, deviations from ideality be established. Unbalance of activities (equation 4) were observed in most of the evaluated ranges. Every range evaluated in the reactor with synthetic substrate demonstrated that approximately a 70% higher SNA is needed for the production of the nitrite needed for the Anammox bacteria. Moreover, the highest range of the reactor operated with industrial real substrate would need 87% more

SNA for a balanced operation without the contribution of floc sludge. Only the first size range of the reactor operated with industrial real substrate demonstrated values near to the stoichiometry balance. These results demonstrate that when low oxygenation strategy is followed, only the contribution of granular sludge is not sufficient to maintain a balanced PN-A operation. The evaluation of the activities of the mixed liquid in both reactors demonstrated three times more unbalance with synthetic substrate than the reactor with industrial substrate. Then, despite observing a small decrease of anAOB relative abundance with the granular size increase (Fig. 2), the presence of organic matter seems to be an important factor in the protection of the Anammox population in a low oxygenated reactor. Similar results have been reported in Anammox granular sludge (Wang et al., 2020) where the abundance of functional anAOB bacteria is not the main reason for the higher SAA in the different ranges of granular diameter (also the heterotrophic bacteria reproduction can contribute to this observations). In the same way, the floc sludge is the principal responsible of the SNA in a reactor (Fig. 1a), and without this sludge, the reactor could lead to a critically unbalanced operation.

The SAA decreased or increased (within the granules) with the diameter depending on the organic matter absence or presence, respectively. Finally, a historical register of SAA from different range of granular sizes could be used to predict the SAA of a complete reactor only with the average granule sizes from a reactor.

### *3.3 The granular size effect in the PN-A process with and without organic matter.*

Three size ranges were selected to compare kinetics, microbial and physical characterization for the PN-A, operated with synthetic and real wastewater industrial substrates (Table 3). The granules obtained with synthetic substrate showed only high values of SAA at the smallest granular size level (0.40-0.85 mm). On the other hand, when compared with the PN-A with industrial substrates any size range was shown with higher SNA. In the microbial characterization, the $EUB^{UI}$ was always higher in the reactor operated with industrial real substrate (Table 3). Actually, for the different size ranges of granular sludge the relative abundance of $EUB^{UI}$ showed no significant differences in the reactor operated with synthetic substrate. However, in the reactor with organic matter (industrial wastewater), a clear correlation of $EUB^{UI}$ abundance and the increase of granular size was observed when the relative abundance increased from the range of 0.40-0.85 mm to 1.25-1.68 mm, the $EUB^{UI}$ abundance augmented from 9.6 until 22% respectively (Table 3). In the same way, in the diameter range of 1.25-1.68 mm the SAA and SNA were higher in the reactor operated with organic matter. These results proved the importance of heterotrophic microorganisms in the PN-A process. As known, the organic matter can contribute to the competition of denitrifying bacteria for high values of influent Corg/N (Corg/N=0.3) (Kraiem et al., 2019), on the other hand, it presents no denitrifying bacteria activities during the use of low values of Corg/N influent (Corg/N=0.18) (Milia et al., 2017). However, not only denitrifying bacteria can proliferate in the presence of organic matter. The results of a recent mathematical model predicted the importance of heterotrophic microorganism in $N_2O$ consumption in the PN-A autotrophic process (Chen et al., 2019). Likewise, the autotropic metabolism of AnAOB is in continuous discussion since the versatility of this microorganism for oxidizing volatile fatty acids to $CO_2$ through the acetyl-COA pathway have been reported (Feng et al., 2018). However, the relative abundance of anAOB in the reactor with synthetic substrate showed a value of 7.2% higher than the reactor operated with industrial real substrate in the diameter range 1.25-1.68 mm. This relative abundance was not correlated with the SAA present in the reactor with organic matter. Similar results have been studied with Anammox granular sludge where granular sizes higher than 4.75 mm decreased the abundance of anAOB bacteria, despite presenting the highest SAA (Wang et al., 2020). In the same way, but in a granular PN-A operated with a synthetic substrate, this trend was reported by Wang et al. (2014). These authors studied the granular size of PN-A, in this research the abundance of functional microbes could not influence the specific activity of granular sludge. The present work demonstrated the same effect but in reactors operated with both synthetic substrate and real substrate. From an industrial view, these results demonstrated that routine monitoring activity should be based on specific activities about the study of relative abundances. Inhibition issues or nutrient limitations cannot be foreseen through relative abundances of microorganisms.

Granular sedimentation rate was higher in the reactor operated with synthetic substrate for the diameter ranges of 0.40-0.85 mm and 0.85-1.25 mm with a significant difference. The mean diameter range and the granular sedimentation rate showed a coefficient of determination higher than 0.98 for both conditions (Fig. 4). Granule size range correlated with the granular sedimentation rate have been reported in AGS (Toh et al., 2003). The sedimentation rate has been used as a parameter for the selection of granular sludge in SBR design in the common AGS reactors (de Kreuk et al., 2005).

The effect of the exponential dependence of storage and yield stress on SAA have been reported (Wang et al., 2019), although the use of this type of analysis requires advanced equipment not always available. In this work, given the positive correlation obtained between the granule size and the Anammox activities, these results allow to propose a strategy of using the sedimentation rate to obtain granules with the desired activities. For the validation of this approach, future research in relevant scale is needed. Currently, it is difficult to predict the performance of sludge regarding nitrogen removal in a PN-A reactor using only the granule size. A complete characterization of Anammox, nitrifying, denitrifying, heterotrophic bacteria, could, however, be avoided if for a given substrate fractions of granule ranges were known. This size range distribution is, on the other hand, produced by a number of combined effects including hydrodynamics and substrate, among others (Jara-Muñoz et al., 2019). In the same way, recent studies reported the importance of type and concentration of extracellular polymeric substances (EPS) in the granular sludge, that would have an important effect on the bacteria relative abundance and structural stability of the granular sludge (Wang et al., 2020). Similarly, the presence of microorganisms without metabolic activity or with their reduced activity can produce a strong noise in a prediction. However, as discussed above, the selection of specific activities only through the granular size would enable to dispense with the necessary equipment to measure SAA, expanding the use of PN-A technology and its stable operation.

## 4. Conclusions

Granular sludge size ranges produced by synthetic and real industrial substrate in low oxygen PN-A process have been evaluated. A positive effect in SAA and SNA in floc and granular sludge of organic matter of real industrial substrate was evidenced. Even more important, with an error not greater of 11% a linear correlation between granular size and SAA predicted the SAA of the entire reactor, independently of the organic load. Thus, although more experimental work must be conducted to confirm this, our results suggest that granular size characterization and sedimentation rate control could be used in full-scale operation as key parameters for fast start-up and stable PN-A process.

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## Figure Captions

**Fig. 1**. Specific activities of different average diameter range and mix liquor granular sludge from PN-A. A) Specific nitrification activity. B) Specific Anammox activity.

* SNA and SAA reactors were measured from the reactor liquid mixture containing granules of all range sizes whose mean diameter was also calculated.

**Fig. 2.** Microbiological characterization of the granular sludge from PN–A reactor operated with industrial real substrate of different diameter ranges. Ammonia-oxidizing bacteria (aerAOB), Anammox (anAOB), nitrite-oxidizing bacteria (NOB) and unidentified eubacteria ($EUB^{UI}$).

$^{*, **, ***, ****}$ Relative abundance of a specific bacterial group of granular sludge with significantly different within a 95% confidence interval.

**Fig. 3.** Specific Anammox activity of different mean diameter range of granular sludge from PN-A without organic matter. Standard protocol specific Anammox activity and specific Anammox activity with a pretreatment. Pretreatment: incubation of granular sludge with nitrite until the absence of pressure variation.

**Fig. 4.** Granular sedimentation rate of the different mean diameter ranges of granular sludge from PN-A. A) Without organic matter. B) With organic matter.

## Table Captions

**Table 1.** Characterization of operational condition for different PN–A reactor.

**Table 2.** Diameter range of granular sludge for different PN-A reactor.

**Table 3.** Comparison of different parameters to different diameter ranges of PN-A with synthetic and industrial substrate.

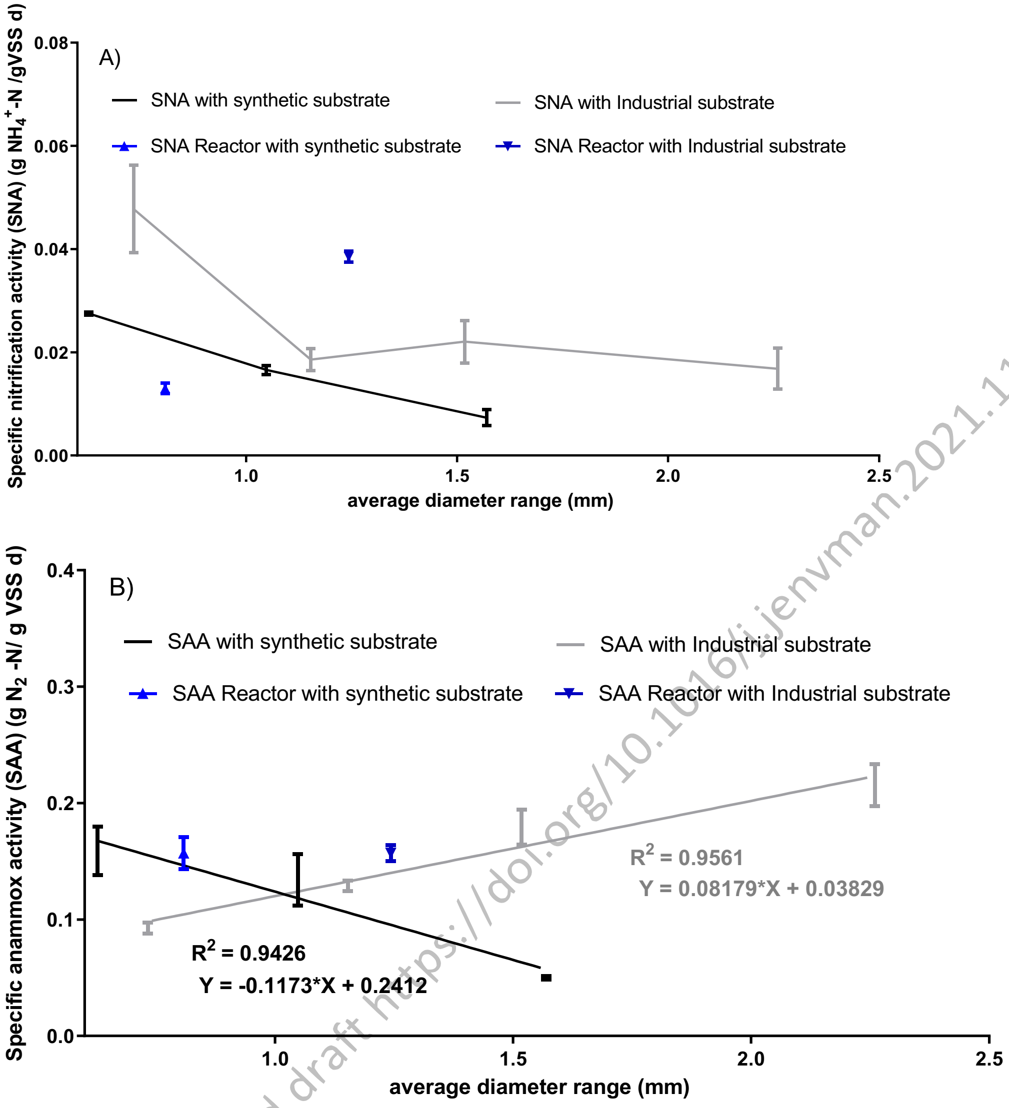
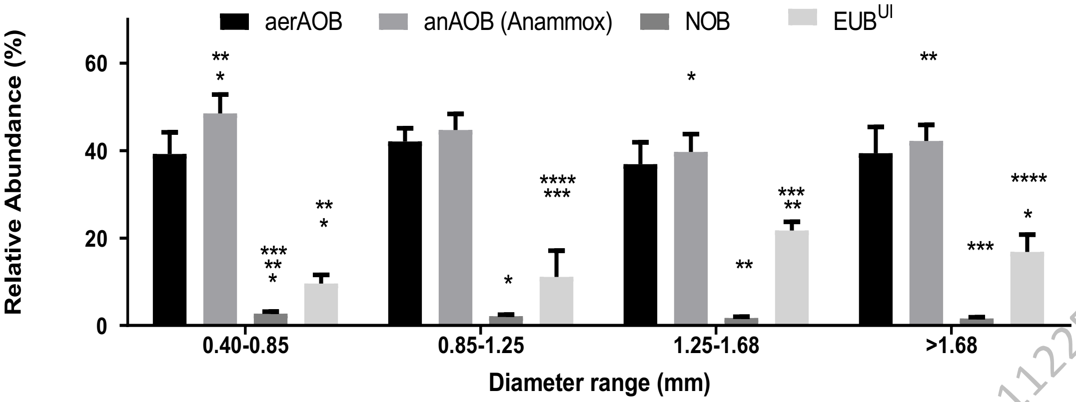

A)
Specific nitrification activity (SNA) (g $NH_4^+$-N /gVSS d)
0.00
0.02
0.04
0.06
0.08
SNA with synthetic substrate
SNA with Industrial substrate
SNA Reactor with synthetic substrate
SNA Reactor with Industrial substrate
1.0
1.5
2.0
2.5
average diameter range (mm)
B)
Specific anammox activity (SAA) (g $N_2$ -N/ g VSS d)
0.0
0.1
0.2
0.3
0.4
SAA with synthetic substrate
SAA with Industrial substrate
SAA Reactor with synthetic substrate
SAA Reactor with Industrial substrate
$R^2$ = 0.9561
Y = 0.08179*X + 0.03829
$R^2$ = 0.9426
Y = -0.1173*X + 0.2412
1.0
1.5
2.0
2.5
average diameter range (mm)

**Fig. 1.**


aerAOB
anAOB (Anammox)
NOB
$EUB^{UI}$
Relative Abundance (%)
0
20
40
60
0.40-0.85
0.85-1.25
1.25-1.68
>1.68
Diameter range (mm)
**
*
***
**
*
**
*
*
****
***
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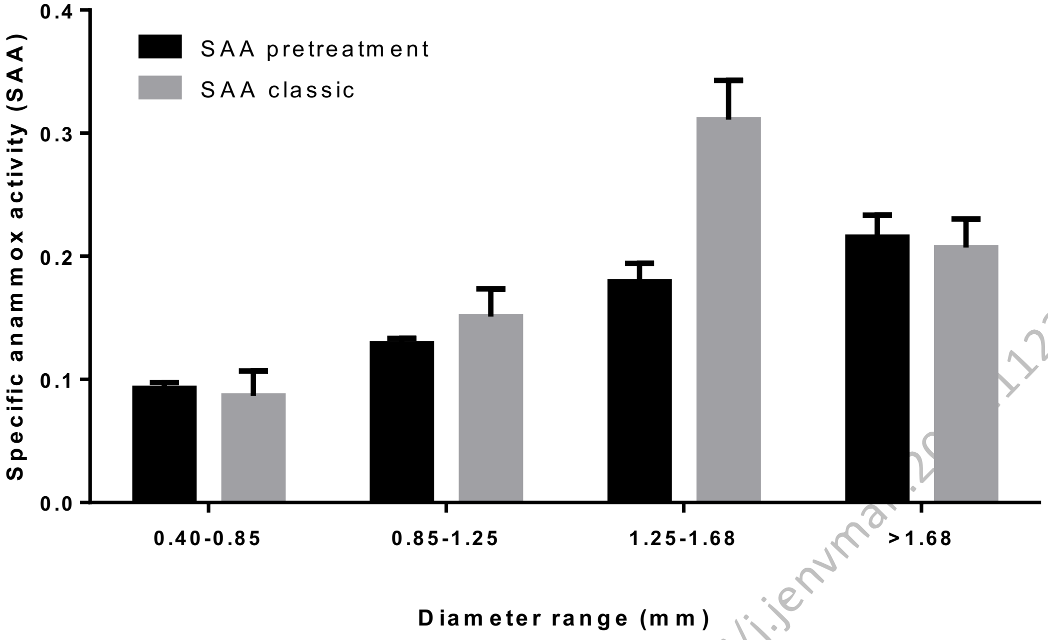
**
**
**
***
****
*

Fig. 2.



Specific anammox activity (SAA)
0.4
0.3
0.2
0.1
0.0
SAA pretreatment
SAA classic
0.40-0.85
0.85-1.25
1.25-1.68
>1.68
Diameter range (mm)

**Fig. 3.**

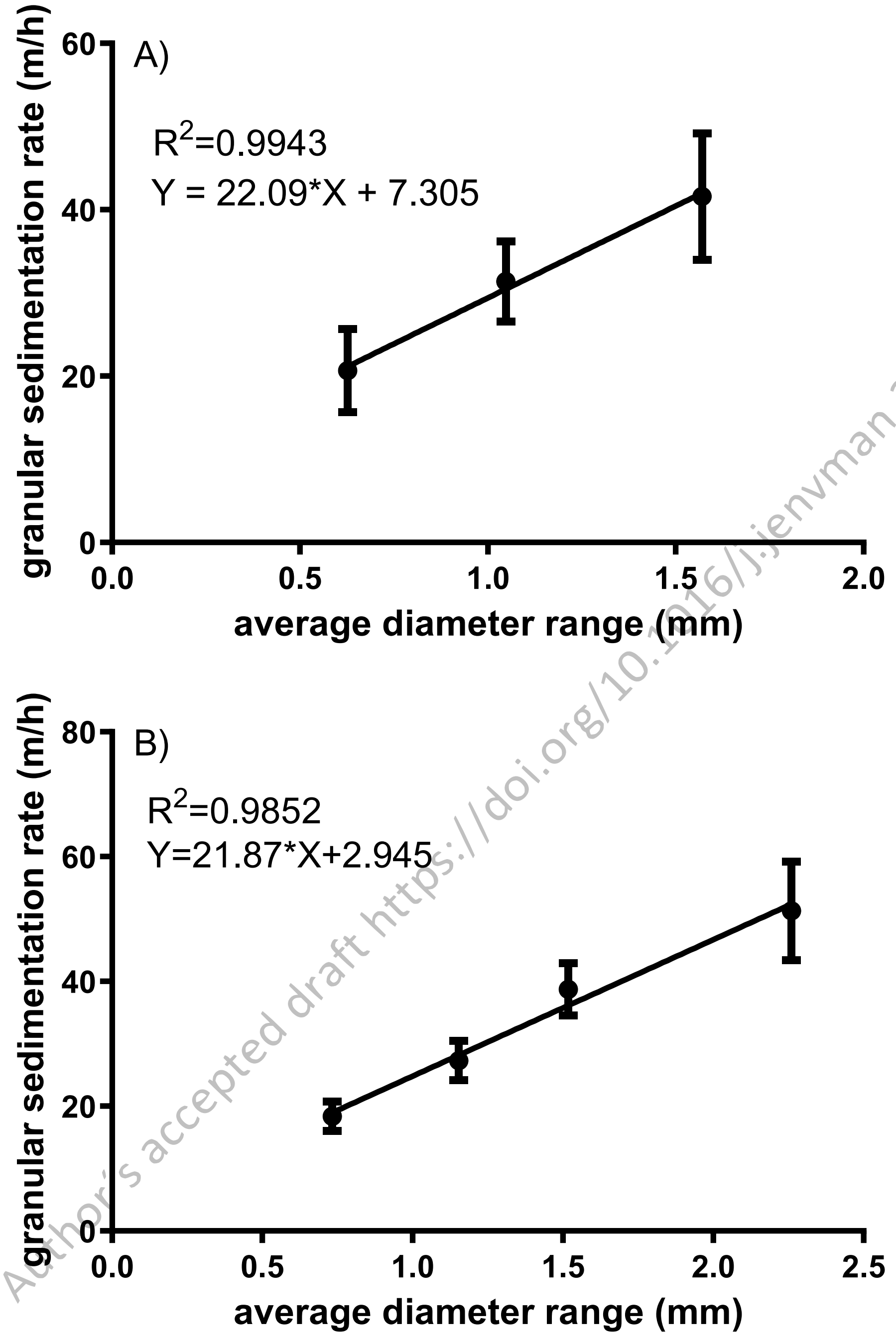

A)
R$^2$=0.9943
Y = 22.09*X + 7.305
granular sedimentation rate (m/h)
0
20
40
60
0.0
0.5
1.0
1.5
2.0
average diameter range (mm)
B)
R$^2$=0.9852
Y=21.87*X+2.945
granular sedimentation rate (m/h)
0
20
40
60
80
0.0
0.5
1.0
1.5
2.0
2.5
average diameter range (mm)

**Fig. 4.**

## Tables

### Table 1.

| | | PN-A with synthetic substrate | PN-A with Industrial substrate (digested poultry manure) |
|---|---|---|---|
| pH[a] | | 7.80 | 6.99 |
| TAN-N | g N $L^{-1}$ | 0.61 | 0.35 |
| COD | g $O_2$ $L^{-1}$ | 0.03 | 0.33 |
| HRT | d | 1.54 | 1.21 |
| OLR | g $O_2$ $L^{-1}$ $d^{-1}$ | 0.02 | 0.27 |
| NLR | g N $L^{-1}$ $d^{-1}$ | 0.34 | 0.29 |
| LRON | g$O_2$ g$N^{-1}$ | 2.86 | 5.53 |
| TNR | (%) | 82.20 | 66.22 |
| ANR | (%) | 98.30 | 83.35 |
| OR | (%) | | 57.82 |

TAN-N, total ammonia nitrogen; COD, chemical oxygen demand; HRT, Hydraulic retention time; OLR, organic load rate; NLR, nitrogen load rate; LRON, Loading rate oxygen-nitrogen; TNR, total nitrogen removal; ANR, ammonia nitrogen removal; OR, organic removal.

[a] pH from mix liquor from the reactor.

**Table 2.**

| PN-A with synthetic substrate | | | | PN-A with Industrial substrate (digested poultry manure) | | | |
|---|---|---|---|---|---|---|---|
| Diameter range [a] (mm) | Average diameter (mm) | SD (mm) | Specific surface ($mm^2$ $mm^{-3}$) | Diameter range [a] (mm) | Average diameter (mm) | SD (mm) | Specific surface ($mm^2$ $mm^{-3}$) |
| 0.40-0.85 | 0.67 | 0.27 | 10.43 | 0.40-0.85 | 0.73 | 0.18 | 8.80 |
| 0.85-1.25 | 1.09 | 0.24 | 5.77 | 0.85-1.25 | 1.15 | 0.21 | 5.38 |
| >1.25[b] | 1.56 | 0.45 | 4.34 | 1.25-1.68[b] | 1.52 | 0.21 | 4.03 |
| | | | | >1.68 | 2.26 | 0.52 | 2.82 |

[a] More than 300 granular for a sample was used for each range.

[b] The diameter range of granular sludge was not significantly different within a 95% confidence interval.

**Table 3.**

| Parameters | Diameter ranges (mm) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.40<D<0.85 | | | | | 0.85<D<1.25 | | | | | 1.25<D<1.68 | | | | |
| | PN-A (SS) | | PN-A (IS) | | P[a] | PN-A (SS) | | PN-A (IS) | | P[a] | PN-A (SS) | | PN-A (IS) | | P[a] |
| | Mean | SD | Mean | SD | value | Mean | SD | Mean | SD | value | Mean | SD | Mean | SD | value |
| SAA (g $N_2$ g $VSS^{-1}$ $d^{-1}$) | 0.159 | 0.021 | 0.093 | 0.005 | 0.006 | 0.134 | 0.022 | 0.129 | 0.005 | 0.721 | 0.050 | 0.002 | 0.179 | 0.015 | $<10^{-4}$ |
| SNA (g $NH_4^+$-N g $VSS^{-1}$ $d^{-1}$) | 0.028 | 0.000 | 0.048 | 0.008 | 0.012 | 0.017 | 0.001 | 0.019 | 0.002 | 0.196 | 0.007 | 0.002 | 0.022 | 0.004 | 0.004 |
| GSR (m $h^{-1}$) | 20.65 | 5.01 | 18.35 | 2.37 | 0.027 | 31.37 | 4.73 | 27.29 | 3.12 | $10^{-4}$ | 41.58 | 7.47 | 38.73 | 4.12 | 0.073 |
| AOB (%) | 39.0 | 2.9 | 39.2 | 4.8 | 0.918 | | | 42.1 | 3.3 | | 42.4 | 5.6 | 36.9 | 4.9 | 0.057 |
| AnAOB (%) | 52.2 | 4.4 | 48.5 | 4.3 | 0.108 | | | 44.7 | 3.7 | | 46.9 | 5.3 | 39.7 | 4.1 | 0.008 |
| NOB (%) | 2.1 | 0.7 | 2.7 | 0.5 | 0.072 | | | 2.1 | 0.4 | | 4.0 | 2.2 | 1.7 | 0.3 | 0.009 |
| $EUB^{UI}$ (%) | 6.8 | 1.1 | 9.6 | 1.6 | 0.001 | | | 11.1 | 6.3 | | 6.7 | 0.8 | 21.7 | 2.2 | $<10^{-4}$ |

PN-A (SS), PN-A with synthetic substrate; PN-A (IS), PN-A with real wastewater industrial substrate; SAA, specific Anammox activity; SNA, specific nitrification activity; GSR, granular sedimentation rate; aerAOB, ammonia-oxidizing bacteria; anAOB, Anammox; NOB, nitrite-oxidizing bacteria; $EUB^{UI}$, unidentified eubacteria; D, diameter; SD, standard deviation; PN-A, partial nitrification-Anammox.

[a] Statistical analyses were performed through Student’s t-test. P values < 0.05 were considered statistically significant