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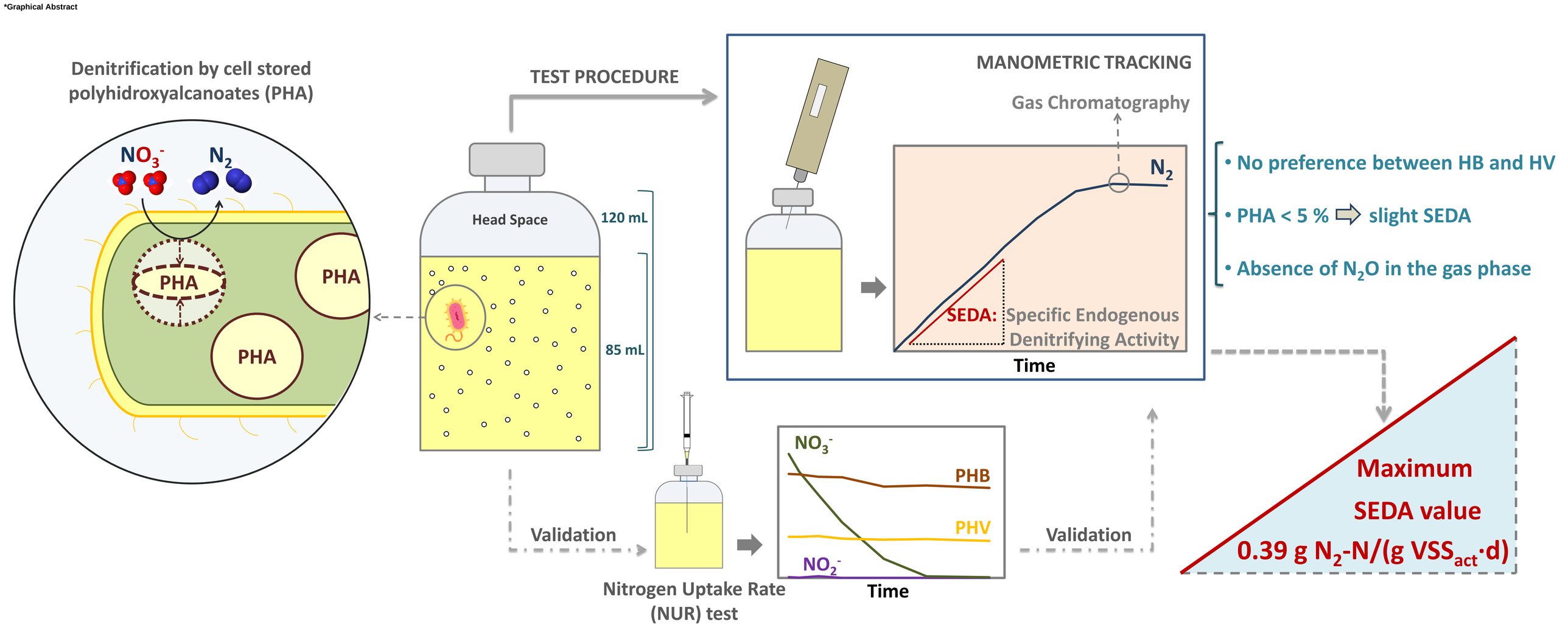
*Graphical Abstract



HIGHLIGHTS

- **A denitrifying activity method using stored PHA as carbon source was optimized.**
- **Specific endogenous denitrifying activity (SEDA) of 0.39 g N/(g VSS d) was measured.**
- **No exists a preference in the consumption of hydroxybutyrate over hydroxyvalerate.**
- **PHA concentrations lower than 5% do not allow the obtainment of maximal SEDA value**.
- **No N_2O was detected in the gas phase during the SEDA test with stored PHA.**

Potential of endogenous PHA as electron donor for denitrification

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ABSTRACT

The use of wastewater streams to obtain polyhydroxyalkanoates (PHA) as high added-value products is widely studied. However, nitrogen removal is not well integrated into this process. In this study, the optimal conditions to track the specific endogenous denitrifying activity (SEDA) driven by PHA as carbon source were selected as: sludge concentration of 0.5 - 2 g VSS/L, COD_{PHA}/N ratio higher than 5.4 g/g and between 40 - 60 mg NO_3^--N/L. The seeding biomass used to perform the activity tests was collected from two sequencing batch reactors and was able to store up to 69 % wt/wt of PHA. SEDA values of 0.26 - 0.39 g N_2-N/(g VSS d) were achieved, which proved the potential of PHA-accumulating mixed microbial cultures to be used in nitrogen removal processes. The results indicated that there is not a preference in the consumption of hydroxybutyrate over hydroxyvalerate and that PHA concentrations lower than 5 % wt/wt do not allow the obtainment of the maximum SEDA value. Finally, N_2O gas production was not detected in the SEDA experiments.

Keywords: Activity test; Denitrification; Polyhydroxyalkanoates; Specific endogenous denitrifying activity.

1. INTRODUCTION

The use of residues to obtain valuable products, promoted by the circular economy concept, is the future. In this context, the production of biopolymers using wastewater as raw material is one of the promising alternatives for the development of future wastewater recovery facilities (WWRF). Polyhydroxyalkanoates (PHA) are an environmentally friendly alternative to petrochemical plastics due to their polymeric nature but with the advantage of being biodegradable. Their production from wastewater streams has been deeply studied and it comprises at least two steps: enrichment and accumulation ones (Kumar and Kim, 2018). The enrichment step consists of the selection of the microorganisms in a mixed microbial culture able to intracellularly store PHA as carbon source, by applying certain stress conditions such as the aerobic dynamic feeding (ADF) (Serafim et al., 2004). Then, during the accumulation step the objective is to maximize the amount of stored PHA previous to their subsequent extraction.

Unlike acetate or other external carbon sources, PHA are endogenous organic compounds used as electron donor by bacteria in the absence of an exogenous source (Third et al., 2003). This condition is due to their related slow metabolic activity, which couples with the denitrification process accurately (Beun et al., 2002, 2000). According to the applied ADF strategy a feast-famine regime is established where PHA are stored inside the biomass during the feast phase while they are consumed in the famine one. If the wastewater stream used in the system contains nitrogen the PHA could be used during this famine phase to denitrify. Therefore, when organic matter present in the wastewater is not enough to achieve complete denitrification, the use of PHA as electron donor is an interesting option since endogenous oxidation of PHA is 6 times slower than the traditional heterotrophic oxidation of organic matter, and thus a more efficient use of it can be done.

Thus, biomass with PHA-storage capacity can be used to denitrify without the need of external carbon source addition, which in many cases limits the efficiency of the nitrogen removal. So, the availability of this internal carbon source contributes to accomplish the stringent discharge limits for nitrogen. Therefore, to evaluate the PHA-accumulating capacity and capability for denitrification of enriched mixed cultures is of interest. To develop these processes to determine the denitrifying potential of an sludge using PHA is important.

The denitrification activity is commonly determined by two types of tests: liquid phase and manometric measurements. The denitrification test following the composition of the liquid phase is the most applied alternative, where the evolution of nitrate concentrations is followed by chemical measurements, and it is defined as nitrate uptake rate (NUR) test. In addition, nitrite and organic compounds consumption can be monitored too (Kristensen et al., 1992; Kujawa and Klapwijk, 1999; Sözen et al., 2002). On the other hand, the manometric assays are based on the measurement of the dinitrogen gas production by following the gas phase. In these assays, to maintain the temperature constant and enough mixing to favour the mass transfer are crucial. The applicability of manometric measurements to determine denitrifying activities has been well demonstrated (Sánchez et al., 2000). While the NUR test require a significant amount of chemical analysis that are time-consuming, biomass activities determined by manometric assays are faster and require less chemical (Buys et al., 2000).

In this context, this is the first study focused on the evaluation of the PHA potential use as endogenous carbon source for denitrification by using a manometric method to measure the specific endogenous denitrifying activity (SEDA). A deep discussion is provided about the influence of parameters like COD_{PHA}/N ratio and biomass properties that limit the values of

SEDA: origin and concentration of biomass, and concentration and composition of PHA.

2. MATERIALS AND METHODS

2.1 Source of seeding sludge with PHA-storage capacity

The biomass used for the batch denitrifying activity assays was collected from two different lab-scale sequencing batch reactors (SBR) already enriched in PHA-accumulating microorganisms. Moreover, biomass samples were also collected from accumulation fed-batch reactors where the PHA accumulation of the biomass from the previous SBR was maximized. In this way, a range of accumulated percentages of PHA inside the cells, low in the enrichment step and high in the accumulation step, were available for the experiments.

2.1.1 Enrichment step

The two enrichment SBR were operated under the ADF strategy to impose the feast/famine regime. These SBR had a useful volume of 2 L, operated at a hydraulic retention time (HRT) of 24 h, at constant temperature of 30 ºC. Allylthiourea, 4.95 mg/L, was added to avoid the growth of ammonium oxidizing bacteria. The first reactor (SBR-A) was fed with a mixture of volatile fatty acids (VFA) obtained after the acidogenic digestion of fish-canning effluents, with high concentrations of ammonium, proteins and NaCl. The second reactor (SBR-B) was operated under the same conditions as SBR-A, but fed with a synthetic media containing a mixture of VFA and concentrations of nitrogen compounds and NaCl lower than in SBR-A. The characteristics of the SBR feedings can be looked up in Supplementary Material. The operational cycles of both SBR-A and SBR-B were completely aerated and the feeding media did not contain nitrate. Therefore, the operational configuration did not allow the denitrifying process to take place.

2.1.2 Accumulation step

After the enrichment step, accumulation fed-batch assays were carried out to maximize the PHA storage inside the cells. These assays were conducted in aerobic conditions adding pulses of a synthetic VFA mixture, mimicking the VFA composition of the feeding added to the respective enrichment SBR. The VFA pulses contained 28 Cmmol and were added when the dissolved oxygen (DO) concentration suffered a sharp increase, which indicated that the VFA added in the previous pulse were consumed. In these assays, nitrogen compounds were not added to avoid the biomass growth and promote the maximum PHA accumulation.

2.2 Key aspects for the denitrifying test with endogenous carbon source

2.2.1 Biomass collection procedure

Biomass from the enrichment SBR-A and SBR-B was collected immediately after the end of the feast phase to avoid the consumption of the stored PHA in the subsequent famine phase. Biomass from the accumulation fed-batch assays was collected after the last feeding pulse was consumed to avoid the presence of VFA in the liquid media.

2.2.2 Preparation of the biomass

In order to avoid the consumption of the stored PHA, the biomass samples were immediately centrifuged at 3000 rpm after collection (Loosdrecht et al., 2016). Then, they were washed four times and re-suspended in a buffer solution (0.74 g/L K_2HPO_4 and 0.14 g/L KH_2PO_4), which provided an initial pH of 7.7 ± 0.1, and introduced into the vials for the batch assays. The number of washes (4) was optimized in preliminary experiments by measuring the total organic carbon (TOC) concentration in the liquid phase to verify its complete absence and guarantee that exogenous organic matter was not present.

126

127 *2.2.3 Estimation of the stored PHA*

128 Since the electron donor for denitrification is endogenous, it would be ideal to determine the
129 content of PHA and the volatile suspended solid (VSS) concentrations of the collected
130 biomass samples. In this way, the exact mass of available carbon source would be quantified
131 and, consequently, the corresponding mass of nitrogen, necessary to have an adequate
132 COD_{PHA}/N ratio for denitrification, could be added to the test. However, to determine PHA
133 and VSS concentrations before running the denitrifying test is no posible because it would
134 take too long and the carbon source would be depleted. Fortunately, although it is no
135 possible to know accurately the amount of carbon source available in the collected sample, it
136 is possible to estimate its value. In the case of biomass from the enrichment reactors,
137 considering operational steady state conditions, this estimation was based on the measured
138 amount of PHA accumulated in previous cycles because these values are normally stable in
139 consecutive cycles. In the case of biomass from the accumulation step, the amount of PHA
140 accumulated correlated with the pulses fed following the results obtained from previous fed-
141 batch assays. Therefore, with the pulsed feeding method is possible to estimate the PHA
142 content of the biomass, taking into account past experiences and the number/concentration
143 of pulses added.

144

145 *2.2.4 Selection of the minimum nitrate concentration added*

146 The concentration of nitrate used in each experiment (between 25 - 100 mg NO_3^--N/L in the
147 vials) was selected based on the estimated amount of PHA accumulated inside biomass, to
148 fix an adequate COD_{PHA}/N ratio for the denitrification process. Therefore, the COD_{PHA}/N
149 ratio used to perform the maximum SEDA tests must be higher than the stoichiometric ratio
150 required by microorganism to conduct the denitrification catabolic reaction from PHA as

carbon source. According to the stoichiometric expressions Eq. [1-2] and Eq. [3-4] a ratio of 2.853 g COD_{PHA}/g N and of 2.855 g COD_{PHA}/g N are necessary when hydroxybutyrate (HB) and hydroxyvalerate (HV) are used as carbon sources, respectively, to achieve the complete nitrate reduction to nitrogen gas considering no biomass growth.

$$C_4H_6O_2 + 3.6\ NO_3^- \longrightarrow 4\ CO_2 + 1.8\ N_2 + 1.2\ H_2O + 3.6\ OH^- \quad \text{Eq. 1}$$

$$C_4H_6O_2 + 4.5\ O_2 \longrightarrow 4\ CO_2 + 3\ H_2O \quad \text{Eq. 2}$$

$$C_5H_8O_2 + 4.8\ NO_3^- \longrightarrow 5\ CO_2 + 2.4\ N_2 + 1.6\ H_2O + 4.8\ OH^- \quad \text{Eq. 3}$$

$$C_5H_8O_2 + 6\ O_2 \longrightarrow 5\ CO_2 + 4\ H_2O \quad \text{Eq. 4}$$

2.3 Batch activity assays

2.3.1 Manometric test

The manometric test used in this study was based on the methodology proposed by Buys et al. (2000) for the determination of specific denitrifying activities (SDA), and modified to use the intracellular accumulated PHA as carbon source ($SEDA_M$). The assays were carried out in vials with a total useful volume of 120 mL (35 mL of headspace volume) hermetically closed and provided with a rubber septum to measure the overpressure. The pressure due to the N_2 produced was measured with a pressure transducer (measurement range 0 - 200 mV and conversion factor 6.67 mV/psi). After introducing the biomass samples, helium gas was flushed through the liquid inside the vials for 10 minutes to remove de oxygen gas. The vials were placed in a shaker at 30 ºC and 250 rpm for 30 min to reach gas/liquid balance. Then, a solution of nitrate (25 - 100 mg NO_3^--N/L in the vial) was added. The generation of N_2 gas was monitored by measuring the overpressure in the headspace of the vial after introducing the needle of the pressure transducer. Once the gas production ended the biogas composition was analysed to determine the percentage of N_2 gas present in the headspace of the vial.

Then, the concentrations of nitrite and nitrate in the liquid phase were determined to calculate mass balances and validate the manometric method. The pH value in the liquid media of the experiment and the PHA concentration in the biomass were measured as well. All the assays were executed in triplicate.

2.3.2 Liquid phase monitoring test

For the monitoring of the SEDA by following the liquid phase ($SEDA_L$) the procedure was similar to the manomentric assay and experiments were performed in triplicate. However, the total useful volume of the vials was of 500 mL (150 mL of headspace). The vials were flushed with helium for 20 min. The measurement of the nitrogen compounds and PHA concentrations in the liquid phase was carried out by collecting samples throughout the time of the assays with a syringe. After drawing the samples, the vials were flushed with the helium contained in a balloon connected to the head space of the vials, to maintain the liquid/gas balance and to avoid air seeping.

2.4 Calculations

2.4.1 Specific endogenous denitrifying activity (SEDA)

The biogas produced during the SEDA was a mixture of N_2 and CO_2 as indicated in Eq. 1 and Eq. 3. To calculate the N_2 gas production during the $SEDA_M$ determination, the percentage of CO_2 measured by gas chromatography was subtracted from the total gas amount measured from the pressure accumulated in the headspace. The $SEDA_M$ was calculated, considering only the N_2 production rate (Eq. 5), from the maximum slope of the curve describing the gas pressure increase inside the vial throughout the time (α), and divided by the mass of active biomass inside the vial (Eq. 6).

$$\frac{dN_2}{dt} = \alpha \frac{V_G}{R\,T} \qquad \left(\frac{mol\ N_2}{min}\right) \qquad \text{Eq. 5}$$

$$SEDA_M = \frac{dN_2/dt}{X_{act}\ V_L} \frac{28\ g\ N}{mol\ N_2} \frac{1440\ min}{d} \qquad \left(\frac{g\ N_2\text{-}N}{g\ VSS_{act} \cdot d}\right) \qquad \text{Eq. 6}$$

Where T is the temperature (K), R is the ideal gas coefficient (atm·L/(mol·K)), V_G is the headspace volume (L), X_{act} is the concentration of active biomass (g VSS/L) and V_L the volume of the liquid phase (L).

The SEDA obtained by monitoring the liquid phase ($SEDA_L$) was calculated considering the maximum slope of the curve describing the decrease of the nitrate concentration inside the vial throughout the time (dNO_3^-/dt, mg NO_3^--N/min), divided by the active biomass inside the vial (Eq. 7).

$$SEDA_L = -\frac{dNO_3^-/dt}{X_{act}\ V_L} \frac{gNO_3^-\text{-}N}{mol\ NO_3^-} \frac{1440\ min}{d} \qquad \left(\frac{g\ NO_3^-\text{-}N}{g\ VSS_{act} \cdot d}\right) \qquad \text{Eq. 7}$$

2.4.2 Active biomass and percentage of PHA

The concentration of active biomass (X_{act}, as g VSS_{act}/L) was estimated by subtracting the concentration of *PHA* (g PHA/L) from the measured concentration of *VSS* (g VSS/L) corresponding to each biomass sample.

The percentage of PHA inside the cells (*%PHA*) was determined on mass basis, in dry weight as a percentage (%wt/wt) of the measured concentrations of *PHA* and *VSS* (Eq. 8) (Palmeiro-Sánchez et al., 2016):

$$\%\,PHA = \frac{PHA}{VSS} * 100 \qquad \text{Eq. 8}$$

2.4.3 Validation and accuracy of the manometric method for SEDA determination

To validate the manometric method in comparison with the liquid phase monitoring method, the error between the SEDA values obtained by both methodologies (ε_1) was determined with Eq. 9.

$$\varepsilon_1 = \frac{|SEDA_L - SEDA_M|}{SEDA_L} * 100 \quad \text{Eq. 9}$$

The accuracy of the manometric method (ε_2) was determined by the estimation of the relative error calculated from a mass balance to the nitrogen compounds inside the bottles, where the manometric tests were performed, as the difference between the amount of nitrogen consumed in the liquid phase (N_{cons}, as g N) and the nitrogen produced in the gas phase (N_{prod}, as g N). To determine the mass of N_{cons} the concentrations of nitrogen compounds in the liquid phase at the end of the manometric test were analysed and the resulted amounts compared with the nitrate added at the beginning of the test (Eq. 10).

$$\varepsilon_2 = \frac{|N_{cons} - N_{prod}|}{N_{cons}} * 100 \quad \text{Eq. 10}$$

In addition, a statistical analysis of variance (ANOVA) was carried out at a 5 % significance level using SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) to verify the accuracy of the manometric compared to the liquid phase monitoring method by checking the signification degree between them.

2.5 Analytical methods

Nitrite, nitrate, VSS and total suspended solids (TSS) were determined according to the Standard Methods (APHA, 2005). TOC concentration was determined by a Shimadzu analyzer (TOC-L, automatic sample injector Shimadzu ASI-L). The overpressure inside the vials was measured with a differential pressure transducer, 0 - 5 psi range, linearity 0.5% of

full-scale, Centerpoint Electronics. The biogas composition was measured with a gas chromatograph Hewlett Packard 5890 series II.

The measurement of PHA content inside the biomass, quantified as the monomers HB and HV, was performed by gas chromatography according to Smolders et al. (1994). The biomass sample was centrifuged, the supernatant removed, and then the obtained pellet frozen and freeze-dried. Afterwards, the pellet was digested into borosilicate glass tubes by adding 1.5 mL of dichloroethane, 1.5 mL of a mixture of propanol with HCl (4:1) and 0.05 mL of a benzoic acid solution as internal standard. The digestion was performed at 100 ºC for 4 h. After digestion, 3 mL of distilled water was added into the tubes, shaken and centrifuged. The organic phase formed at the bottom was analyzed by gas chromatography (Agilent, USA) with helium as carrier gas. Standards of HB:HV (88:12 %, Sigma, USA) were used for calibration.

3. RESULTS AND DISCUSSION

3.1 Validation and accuracy of the manometric method

The manometric method was chosen to perform the SEDA determinations in this study due to its simplicity. Although, with the aim of validating it, 4 assays were carried out simultaneously applying the manometric and the liquid phase monitoring methods, respectively. The assays corresponded to A6, B2, B3 and B4 in Table 1 and more details can be consulted in Supplementary Material. Experimental conditions were the same for manometric and liquid monitoring methods in terms of duration of the experiment, temperature, stirring conditions and initial biomass (VSS) and substrate concentrations (PHA and NO_3^--N concentrations and HB:HV ratio). In these 4 different assays the tested nitrate concentrations were 50, 65 and 75 mg NO_3^--N/L, while the PHA content inside the biomass ranged from 39 to 69 % of PHA. PHA was added in excess to guarantee that the

supplied nitrate was fully depleted by the end of the experiment. Furthermore, the concentrations of nitrate were selected high enough to provide a sufficient production of N_2 gas to diminish as much as possible the relative error from the measurement. As an example, in case of assay B2 the obtained curves depicting the nitrate consumption in the liquid phase experiment (**Figure 1.a**) and the N_2 production in the manometric one (**Figure 1.b**) presented complementary evolutions illustrating the correlation between both processes.

[Figure 1. a]

[Figure 1. b]

As a result of the experiments performed monitoring the liquid phase the fact that the initial nitrate concentration was completely reduced to N_2 gas without the presence of an external carbon source is proven. A slight production of nitrite throughout the test was observed (< 3 % of the nitrogen fed), while nitrous oxide was not detected in the gas phase. Although the percentage of HB was notably higher than that of HV, both HB and HV were consumed in a similar proportion, approximately 13 % and 10 %, respectively (**Figure 1.a**). This behaviour of proportional consumption of HB and HV was observed in all the assays, where similar HB:HV ratio was measured at the beginning $(HB:HV)_0$, and the end of the experiment, $(HB:HV)_F$ (**Table 1**). These data suggest that either the biomass consumed HB and HV with the same affinity, or these polymers were present as copolymers of HB:HV.

In addition, similar specific denitrifying activities were obtained in those tests carried out in the same conditions and executed simultaneously with the manometric and liquid phase monitoring procedures (**Figure 2**). Estimated error of the $SEDA_M$ values with respect to $SEDA_L$ values (ε_1) ranged between 2.9 - 9.8 % (Supplementary Material), corroborating the validity and robustness of the manometric method. This observation was supported by ANOVA results, which showed the absence of significant differences at 95% of confidence level (p-value = 0.640).

[Figure 2]

Regarding the accuracy of the manometric method, the calculated relative errors from the nitrogen balance (ε_2) were in the range of 4.2 - 7.4 % (assays A6, B2, B3 and B4), showing the accuracy of the manometric method to obtain SEDA values based only on gas production. In all cases $SEDA_M$ values were a little bit higher than $SEDA_L$ values. This behaviour can be attributed to a slight negative effect during sampling in the liquid phase measurement, where a fraction of the liquid is removed from the vial to measure the nitrate, nitrite and PHA concentrations, while the manometric measurements are less invasive.

In addition, the pH was monitored in the assay A6 where the concentration of 75 mg NO_3^--N/L was applied (**Table 1**). The pH values remained between 7.6 and 8.0 in the triplicate experiments. Thus, these results showed that phosphate buffer media maintained the pH value below the inhibitory values for denitrification (Glass and Silverstein, 1998), even in the assay with the higher nitrate concentration (assay A5.2).

Summarizing, the monitoring of the liquid phase during the denitrification test driven by PHA provides more information about the consumption of HB, HV and nitrate concentrations, while the assays based on gas production are faster, easier and cheaper in terms of chemical reagent usage with the same reliability in terms of SEDA values. For this reason, the subsequent SEDA determinations in this research work were performed by the manometric test.

3.2 Definition of the initial conditions to determine the SEDA

A total of 17 assays (made in triplicate) were performed (by the manometric test) to obtain the optimal conditions required to achieve the SEDA (**Table 1**). In these assays the influence of parameters, like the concentration of seeded biomass, PHA concentration and HB:HV ratio, nitrate, and the COD_{PHA}/N over the denitrifying activity were evaluated.

311 **[Table 1]**

312 *3.2.1 Source of the seeding sludge*

313 The SEDA values obtained using both types of biomass (from SBR-A and SBR-B), showed
314 that the specific denitrification activity measured was different for each type of biomass.
315 This value ranged from 0.1 to 0.2 g N_2-N/(g VSS_{act} d) and from 0.3 to 0.4 g N_2-N/(g VSS_{act}
316 d) for the biomass from SBR-A and SBR-B, respectively (**Table 1** and **Figure 3.a**).
317 The results of the tests "A" performed with the biomass from the reactor SBR-A, fed with
318 acidified fish caning wastewater, revealed the direct dependence of the measured specific
319 denitrification activity with the increase in the percentage of PHA accumulated inside the
320 cells (**Figure 3.b**). However, the comparison of assays A4 and A6 do not corroborate this
321 relationship and different values of 0.127 ± 0.004 and 0.218 ± 0.009 g N_2-N/(g VSS_{act} d),
322 respectively, were measured despite the similar PHA content inside the biomass (35.0 and
323 39.1 % PHA, respectively) (**Table 1**). The lower activity in assay A4 could be attributed to
324 an episode of biomass inhibition during the operation of the SBR-A, caused by an
325 unexpected increase of the NaCl concentration in the feeding up to 5 g NaCl/L.
326 The biomass from SBR-B, enriched with a synthetic media containing a mixture of VFA,
327 was able to accumulate up to 69 % of PHA (wt/wt). In all the assays, the biomass from
328 SBR-B exhibited specific activities higher than those measured for the biomass from SBR-
329 A. More studies should be performed to better determine the influence of the origin of the
330 biomass over the SEDA results. For example, using biomass capable of accumulating HB
331 and HV at different ratios the obtained SEDA could change. In addition, biomass from SBR-
332 A was adapted to high salt concentrations, but during the activity assays the buffer liquid
333 media did not contain NaCl, which could affect the SEDA value. Previous studies report
334 already on the effect of the salt concentration of the culture media on the denitrifying
335 activity of the biomass (Dinçer and Kargi, 1999; Jafari et al., 2015).

336

337 *3.2.2 Effect of PHA concentration and composition used as electron donor*

338 As a general response, in all experiments the SEDA measured increased with the amount of
339 carbon source present as accumulated PHA (**Figure 3.a)** demonstrating the direct
340 relationship between both parameters. Experiment B5 illustrates this correlation (**Table 1**).
341 The three assays, B5.1, B5.2 and B5.3, were performed in parallel with three different PHA
342 concentrations (1670, 2749 and 4657 mg COD_{PHA}/L) but with the seeding biomass from the
343 same origin, and equal percentage of accumulated PHA and nitrate concentration. The
344 values of SEDA increased from 0.337 g N_2-N/(g VSS_{act} d) at 1670 mg COD_{PHA}/L (B5.1) to
345 0.377 g N_2-N/(g VSS_{act} d) at 4657 mg COD_{PHA}/L (B.5.3).
346 A similar relationship is exhibited between the increase of SEDA and the increase of the
347 percentage of PHA accumulated inside the biomass (**Figure 3.b)**. PHA storage percentages
348 lower than 5% (wt/wt), in experiments A1.1, A1.2 and A2 led to poor denitrifying activities.
349 This behaviour could be attributed to the preference of the microorganism to keep always a
350 certain amount of PHA as storage energy instead of using them as electron donor in the
351 denitrification process, even if in these experiments a COD_{PHA}/N ratio below the
352 stoichometric value was used (**Table 1**). These low values could be the cause also for these
353 low activities. Since the determination of the percentage of PHA inside the biomass is a time
354 consuming procedure, it is not possible to know in advance its precise value before carrying
355 out the denitrifying assay. For this reason, experiments with low PHA percentage in the
356 biomass, estimated from previous experiences, but with an adequate COD_{PHA}/N ratio need
357 to be carried out to verify this hypothesis. Bengtsson et al. (2017) reported a complete post-
358 anoxic denitrification with 1.8 % of PHA accumulated. Nevertheless, the denitrifying
359 activity was poor (0.03 g N_2-N/(g VSS_{act} d)) and comparable with the value obtained in the
360 present study of 0.025 ± 0.003 g N_2-N/(g VSS_{act} d) with 1.3 % of PHA (assay A2).

[Figure 3. a] [Figure 3. b]

No correlation was found between the HB:HV ratio at the beginning of the assay and the obtained values of the SEDA. Assays A3 and A5.1 achieved similar SEDA values with different HB:HV ratios, 74:26 and 92:8, respectively. However, since all the assays carried out with the biomass from SBR-B had similar HB:HV ratio and the SEDA obtained were not very different, further studies are needed to confirm this result maintaining all the parameters constant and different HB:HV ratios. This ratio can be changed by performing the accumulation batch assays using feeding media containing different mixtures of VFA (Albuquerque et al., 2011). Furthermore, as it was explained before, the HB:HV ratio was similar at the beginning and end of the experiment (**Table 1**), which could indicate the absence of preference of the microorganisms for HB or HV probably due to their similar chemical structure or the presence of both as a copolymer.

3.2.3 Optimal nitrate concentration

A wide range of nitrate concentrations was tested (25 - 100 mg NO_3^--N/L), depending on the content of PHA inside the biomass. Experiments with the similar nitrate concentration as B4 and B5.3 (50 - 58 mg NO_3^--N/L) or B6 and B7 (50 mg NO_3^--N/L) showed different SEDA values (**Table 1**). Moreover, experiments carried out simultaneously with biomass from the same origin but different nitrate concentrations resulted in similar SEDA. For example, assay A5.1 (50 mg N/L) showed a similar activity as A5.2 (100 mg N/L), of 0.121 ± 0.029 and 0.108 ± 0.007 g N_2-N (g VSS_{act} d), respectively. Therefore, the initial nitrate concentration in the range of values tested, without considering other parameters, did not affect the SEDA, as long as an adequate COD_{PHA}/N for the denitrification process was provided.

However, from the practical point of view, an adequate nitrate concentration must be

considered when determining the SEDA values. For example, experiments with 100 mg N/L lasted too long, requiring more than 15 hours to reduce all the nitrate to nitrogen gas, while an appropriated duration ranges at approximately 5 - 6 hours (**Figure 1**). On the other hand, in the experiments performed with nitrate concentrations lower than 25 mg N/L, such as assay A1.2, the pressure increments measured in the headspaces of the vials were low due to the slight production of N_2 gas and presented the highest relative error (ε_2 = 42%). Thus, the nitrate concentration range to perform the SEDA determination is recommended to be between 40 - 60 mg NO_3^--N/L.

3.2.4 Optimal COD_{PHA}/N ratio

The COD/N ratio is a key factor for the denitrification process (Henze et al., 2008). A ratio of 2.85 g COD_{PHA}/g N is necessary to avoid organic matter limitation, according to the stoichiometric expressions without considering the cellular growth (Eq. 1 - 4), to obtain the SEDA**.** When the COD/N ratio is low the amount of electron donor is deficient and consequently the microbial activity decreases (**Figure 4)**.

[Figure 4]

Due to an error in the estimation of the PHA content inside the biomass previous to the performance of the assays A1.1, A1.2 and A2, the applied COD_{PHA}/N ratios were lower than 1.5 g/g, below the stoichiometric relationship, and thus the measured SEDA was barely detectable. For COD_{PHA}/N ratios higher than 5.4 g/g significant denitrification activities were measured. The assays where the biomass from SBR-A was used, A3 and A4 presented similar SEDA, although the applied COD_{PHA}/N ratios were of 5.5 and 21.5 g COD/g N, respectively. This behaviour is repeated in the assays B5.1 and B5.3 where the ratio was 28.8 and 80.3 g COD_{PHA}/g N, respectively. Obtained results seem to indicate that the

COD/N ratio does not affect the SEDA, if it is higher than 5.4 g COD_{PHA}/g N (**Figure 4**). This value is close to that determined by Beun et al. (2002), who reported a maximum theoretical stoichiometric ratio for the denitrification using PHA as carbon source, and considering no growth, of 6.88 g COD_{PHA}/g N. Further studies are needed in the range of 2.85 to 5.4 g COD_{PHA}/g N to determine the minimum value of this ratio to achieve the SEDA. For this purpose, the evolution of the PHA concentration during the operational cycles in the accumulation experiments needs to be determined to identify the best moment for the biomass collection to perform the denitrifying assay, as it contains the desired PHA-accumulated percentage.

3.2.5 Optimal biomass concentration

No relationship was found between the SEDA values and the X_{act} concentration in the experiments (**Table 1**). Similar activities were achieved in the range of 0.45 - 1.26 g VSS_{act}/L in the case of biomass B and 0.80 g VSS/L - 2.76 g VSS/L in the case of biomass A (**Table 1**). The corresponding VSS range (considering also the PHA mass) was 0.8 - 2.9 g VSS/L.

To elucidate the influence of the VSS concentration on the denitrification activity two assays (B6 and B7) were carried out, in parallel, inoculated with biomass from SBR-B and in the same operational conditions: 50 mg NO_3^--N/L, 25 % PHA and 70:30 HB:HV ratio. However, the seeding biomass concentration was half in assay B7 (0.7 g VSS/L) in comparison with assay B6 (1.4 g VSS/L). Consequently, the COD_{PHA} concentration and COD_{PHA}/N ratio were also half in B7 (**Table 1**), since the concentration of biomass determines the amount of PHA in the test when the carbon source is endogenous. In addition the slope of the curve describing the gas production of the assay B6 was much higher than that of the assay B7 **(Figure 5)**. The SEDA determined for B6 and B7 was of 0.390 ± 0.021

435 and 0.260 ± 0.008 g N_2-N/(g VSS_{act} d), respectively.

436 **[Figure 5]**

437 In these assays B6 and B7 the biomass contained only 25 % PHA, lower than in all the
438 previous assays carried out with biomass from SBR-B (between 40 - 69 % PHA). However,
439 the assay B6 presented a significantly higher SEDA. Therefore, when the storage of PHA is
440 deficient, a high SEDA value can be achieved by increasing the VSS concentration, which
441 means increasing the COD concentration added to the assay.

442 When the COD/N ratio was fixed at 11.7 g COD_{PHA} /g NO_3^--N in assay B6 the nitrogen
443 production profile (**Figure 5**) coincided with that from other studies where the denitrifying
444 activity was determined using external carbon sources (Buys et al., 2000; Sánchez et al.,
445 2000). Nevertheless, the nitrogen production profile in the assay B7, where this ratio was of
446 6.3 g COD_{PHA} /g NO_3^--N, presented two slopes well-differentiated, corresponding to
447 productions of 11.3 mg N_2-N/d at the beginning and to 6.0 mg N_2-N/d after minute 250 of
448 the experiment (**Figure 5**). This profile resembles those obtained by previous mentioned
449 authors when the used COD/N ratio was not high enough.

450 However, this ratio was, in both assays B6 and B7 higher than 2.85 g COD_{PHA}/g N, enough
451 to obtain a similar SEDA, according to the stoichiometry of the process and the previous
452 obtained results. Thus, the fact that both activities were different should be attributed to
453 another factor, which might be in this case the lower biomass concentration at the beginning
454 of assay B7 in comparison with assay B6. Both experiments started with 25 % of PHA
455 stored inside the cells, nevertheless, B6 contained 18 % of PHA stored after complete nitrate
456 denitrification while B7 contained 6.8 % of PHA, due to the different biomass concentration.
457 This fact indicates that PHA consumption was higher, by cell, in B7. Therefore, these results
458 seem to support the hypothesis that the lower PHA concentration inside the cells the lower

its use for denitrification, as the biomass prefers to store it as carbon source.

3.3 Absence of nitrous oxide in the gas phase

Some authors reported a relationship between the endogenous denitrification driven by PHA with the production of N_2O gas (Wang et al., 2015; Wei et al., 2014; Zhou et al., 2012). They attributed this behaviour to two causes. The lack of electron donors during denitrification, where the nitrate reductase enzyme (Nar) has a competitive advantage over nitrite and nitrous oxide reductase (Nir and Nos) ones, causes NO_2^- accumulation and N_2O gas generation. Or the fact that endogenous PHA degradation kinetics are at least 6 times lower than soluble COD degradation kinetics (Third et al., 2003).

The composition of the gas phase was analysed at the end of all assays to determine the biogas composition and calculate the mass balances. N_2 and CO_2 were detected. However, in this research work, no N_2O was detected in any assay, not even in the cases where the gas phase was measured during all test performance. This results seem to confirm that if the available PHA provides enough electron donor to maintain a high COD_{PHA}/N ratio the denitrification process will no produce N_2O gas. This behaviour coincides with Zhou et al. (2012) who reported that at high COD ratios the nature of the carbon source does not seem to affect nitrous oxide accumulation.

3.4 Potential of PHA driven denitrification

This research study reports on remarkable denitrifying activities using PHA as a sole electron donor. SEDA values close to 0.4 g N_2-N/(g VSS_{act} d) have been measured in the assays B6 and B5.3 (**Table 1**). These results are comparable with the values obtained by other research works where the specific denitrifying activity was determined using

483 endogenous and/or exogenous organic carbon source (**Table 2**).

484 **[Table 2]**

485 Denitrification using endogenous PHA is not as widely studied as heterotrophic
486 denitrification for wastewater treatment. However, there are many research studies on
487 denitrification driven by PHA. Experiences with granular sludge able to store PHA provided
488 denitrifying activities between 0.07 and 0.11 g N/(g VSS d) without external carbon source
489 supply (Qin et al., 2005; Val del Río et al., 2013). Bengtsson et al. (2017) reported an
490 activity of 0.03 g N/(g VSS d), in a post-denitrification unit with PHA as carbon source. This
491 low value could be attributed to an insufficient concentration of PHA inside the cells (1.8 ±
492 0.9 % PHA. With the presence of 3 % of PHA, Basset et al. (2016) achieved 0.2 g N/(g VSS
493 d) in a denitritation system.
494 The value reported by Khan et al. (2002) was the most similar one to that obtained in the
495 present study. However, in their case the PHA was fed as external carbon source (synthetic
496 powder), which suggests that the specific denitrifying activity driven by PHA is more
497 related to their chemical structure than to the endogenous nature of the carbon source.
498 In the case of heterotrophic denitrification higher activities of 1.91 and 0.92 g N_2-N/(g VSS
499 d) were obtained by Buys et al. (2000) and Courtens et al. (2014), respectively. This high
500 values could be attributed to an adaptation of the seeding sludge to denitrify high nitrate
501 concentrations (1000 - 400 mg N/L). Nevertheless, Ficara and Canziani (2007) and Val Del
502 Río et al. (2015) using acetate as carbon source reported values close to those from the
503 present research study of 0.46 and 0.22 g N/(g VSS d).
504 For all that, the results of this study highlight the potential of the denitrification driven by
505 PHA as carbon source, showing that it might be the basis of a competitive technology for
506 nitrogen removal. It is also important to consider that the seeding biomass was collected
507 from a SBR continuously aerated, nitrate was not added in the feeding, but allylthiourea was

to inhibit ammonium oxidizing bacteria activity. Thus, no denitrifying microorganisms were specifically enriched in the biomass. Therefore, future research is needed to evaluate the SEDA from denitrifying PHA-accumulating biomass.

4. CONCLUSIONS

The batch assay procedure has been optimized to determine the specific denitrifying activity of sludge using stored PHA as carbon source. The optimal conditions to determine the SEDA were defined as follows: 0.5 – 2.0 g VSS/L, COD_{PHA}/N ratio higher than 5.4 g/g and 40 - 60 mg NO_3^--N/L.

The results indicated that a correlation between the HB:HV ratio in the sludge and the obtained values of the SEDA does not exist, and there was no preference of the microorganisms for HB or HV.

PHA concentrations under 5% inside the biomass do not allow for the obtainment of the maximal SEDA values, which support the hypothesis that the lower the PHA concentration inside the cells the lower its use for denitrification, as the biomass prefers to store it as carbon source.

The denitrification with stored PHA using large COD_{PHA}/N ratios does not produce N_2O gas.

A SEDA of 0.39 g N_2-N/(g VSS_{act} d) was achieved enhancing the potential of PHA as carbon source for denitrification.

"E-supplementary data of this work can be found in online version of the paper"

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Table 1. Summary of the results obtained in the denitrifying assays applying the manometric method and performed at different initial conditions.

Assay	Initial conditions							Results		
	NO_3^--N	X_{act}	COD_{PHA}	COD_{PHA}/N	PHA_0	PHA_F	$HB:HV_0$	$HB:HV_F$	$SEDA_M$	ε_2
	mg/L	g VSS_{act} /L	mg/L	g/g	%	%			g N_2-N /(g VSS_{act} d)	%
A1.1	50	0.84	32	0.6**	2.1	0.6	68:32	75:25	**0.016 ± 0.004**	22.8
A1.2	25	0.84	32	1.3**	2.1	0.5	68:32	75:25	**0.016 ± 0.004**	41.8
A2	50	1.49	60	1.3**	2.5	0.5	80:20	82:18	**0.025 ± 0.003**	9.39
A3	50	2.76	275	5.4	5.5	1.5	74:26	74:26	**0.111 ± 0.003**	13.5
A4	50	1.20	1075	21.5	35.0	29.5	91:09	91:09	**0.127 ± 0.004**	9.9
A5.1	50	1.52	615	12.3	19.0	15.5	92:08	94:06	**0.121 ± 0.029**	6.6
A5.2	100	1.58	615	6.15	19.0	12.1	92:08	93:07	**0.108 ± 0.007**	5.9
A6*	75	1.10	1050	14.0	39.1	21.7	89:11	88:12	**0.218 ± 0.009**	5.4
B1	33	1.19	1401	42.4	40.4	30.0	72:28	72.28	**0.352 ± 0.001**	9.8
B2*	65	0.80	2698	41.5	57.5	49.9	71:29	70:30	**0.320 ± 0.001**	7.3
B3*	50	0.80	2860	57.2	69.0	53.2	75:25	76:24	**0.314 ± 0.015**	4.2
B4*	50	0.90	2280	45.6	58.3	43.5	74:26	75:25	**0.276 ± 0.029**	7.4
B5.1	58	0.45	1670	28.8	68.1	43.8	73:27	75:25	**0.337 ± 0.012**	3.6
B5.2	58	0.71	2749	47.4	68.1	49.6	73:27	75:25	**0.354 ± 0.012**	4.1
B5.3	58	1.26	4657	80.3	68.1	59.4	73:27	74:26	**0.377 ± 0.014**	5.5
B6	50	1.06	588	11.8	25.4	18.7	70:30	71:29	**0.390 ± 0.021**	6.2
B7	50	0.56	313	6.3	25.4	6.8	70:30	71:29	**0.260 ± 0.008**	2.7

A - Results from SBR-A biomass; B - Results from SBR-B biomass.

* Validation assays performed simultaneously using the liquid monitoring method.

** Below the stoichometric ratio (COD/N < 2.9 g/g)

Table 2. Summary of specific denitrifying activities values reported by other authors.

Activity	Biomass type	Carbon/Nitrogen Source	Reference
g N/(g VSS d)			
1.91	Activated sludge	Acetate/Nitrate	Buys et al. (2000) *
0.92	Activated sludge	Acetate/Nitrate	Courtens et al. (2014)
0.24 - 0.46	Activated sludge	Acetate/Nitrate	Ficara and Canziani (2007)
0.22	Granular sludge	Acetate/Nitrate	Val Del Río et al. (2015) *
0.48	Activated sludge	Exogenous PHA/Nitrate	Khan et al. (2002)
0.07	Granular sludge	Endogenous PHA/Nitrate	Qin et al. (2005)
0.11	Granular sludge	Endogenous PHA/Nitrate	Val Del Río et al. (2013)
0.20	Activated sludge	Endogenous PHA/Nitrite	Basset et al. (2016)
0.03	Activated sludge	Endogenous PHA/Nitrate	Bengtsson et al. (2017)
0.26 - 0.39**	Activated sludge	Endogenous PHA/Nitrate	This study *

* Assays performed by a manometric method.

** Activity values determined as (g N/(g VSS_{act} d)).

Figure 1

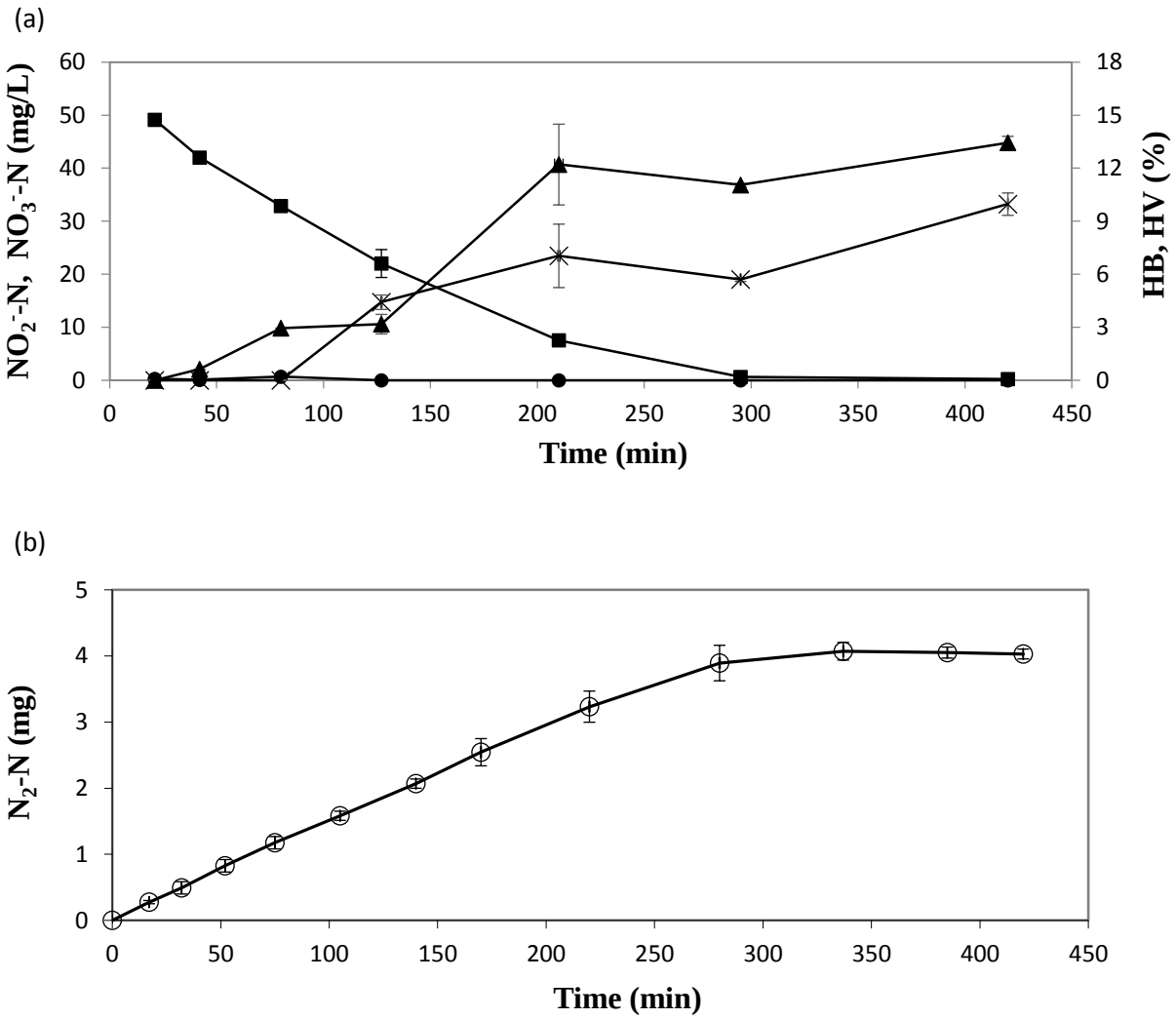

Figure 1. Denitrifying activity test using the same biomass source and monitoring (a) the liquid phase composition: NO_3^--N (■) and NO_2^--N (●) concentrations, and HB (▲) and HV (×) percentages of consumption; and (b) N_2 gas production inside the vial (○) in the manometric test throughout time. The results are average values of the performed triplicates with the corresponding standard deviation for assay B2 in Table 1.

Figure 2

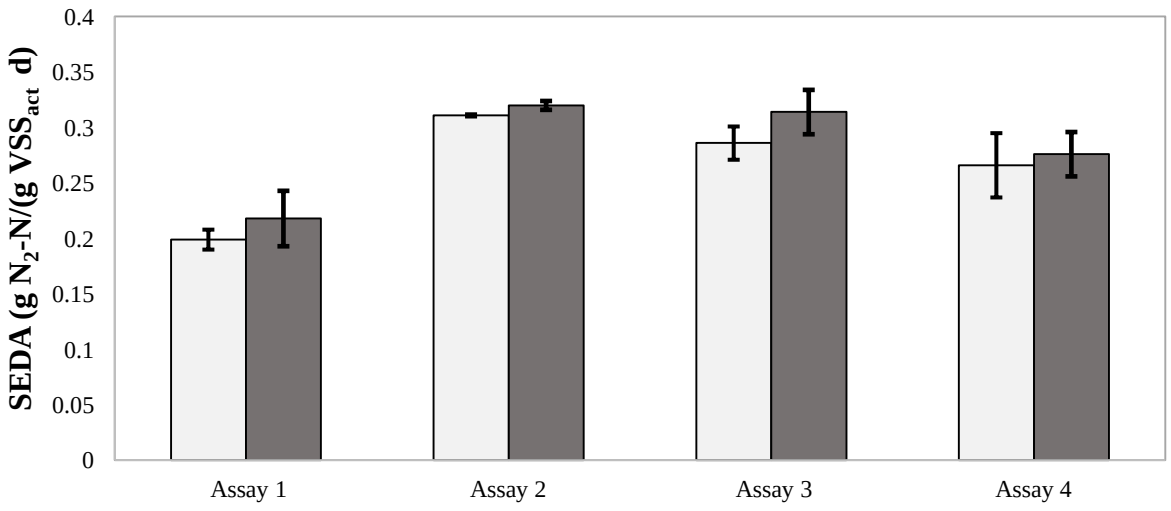

Figure 2. Comparison between SEDA values determined by both methods: Liquid phase measurements (▫); Gas phase measurements (■).The results are average values of the performed triplicates with the corresponding standard deviation.

Figure 3

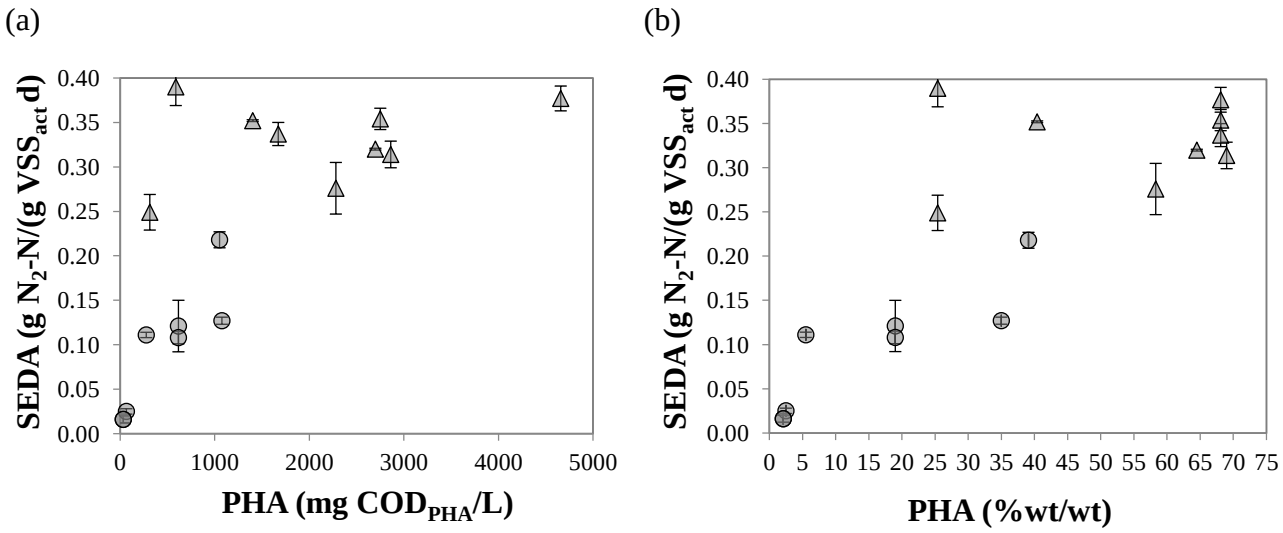

Figure 3. Measured SEDA with respect to the PHA content: (a) PHA as COD concentration in the liquid media; and (b) PHA as percentage inside the biomass cells. Seeding sludge collected from SBR-A (●), and from SBR-B (▲). The results are average values of the performed triplicates with the corresponding standard deviation.

Figure 4

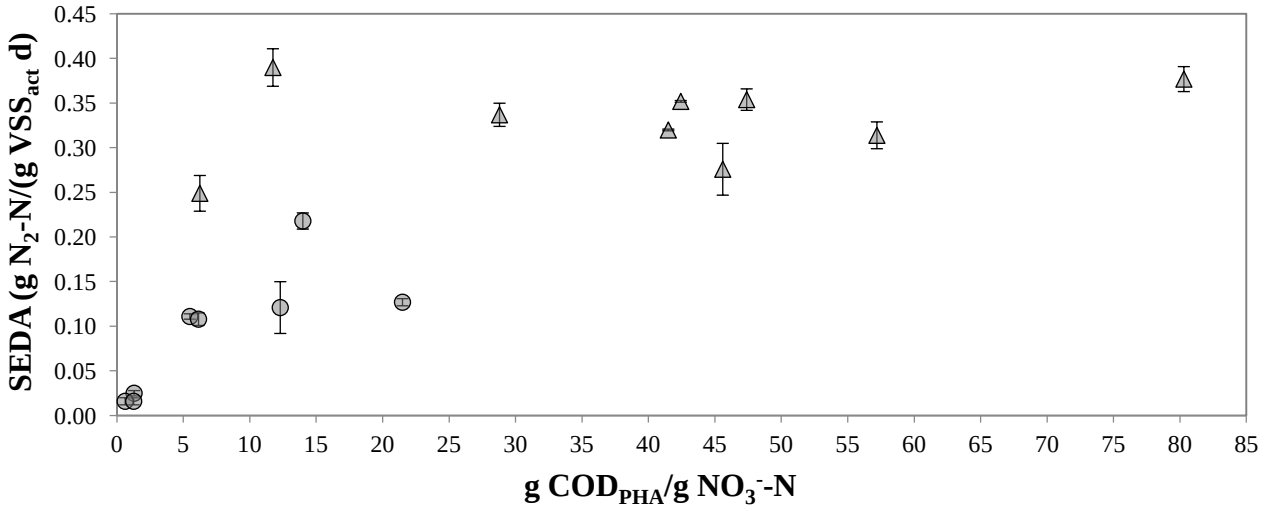

Figure 4. Correlation between SEDA with respect to COD_{PHA}/N ratio: seeded with SBR-A biomass (●), and seeded with SBR-B biomass (▲). The results are average values of the performed triplicates with the corresponding standard deviation.

Figure 5

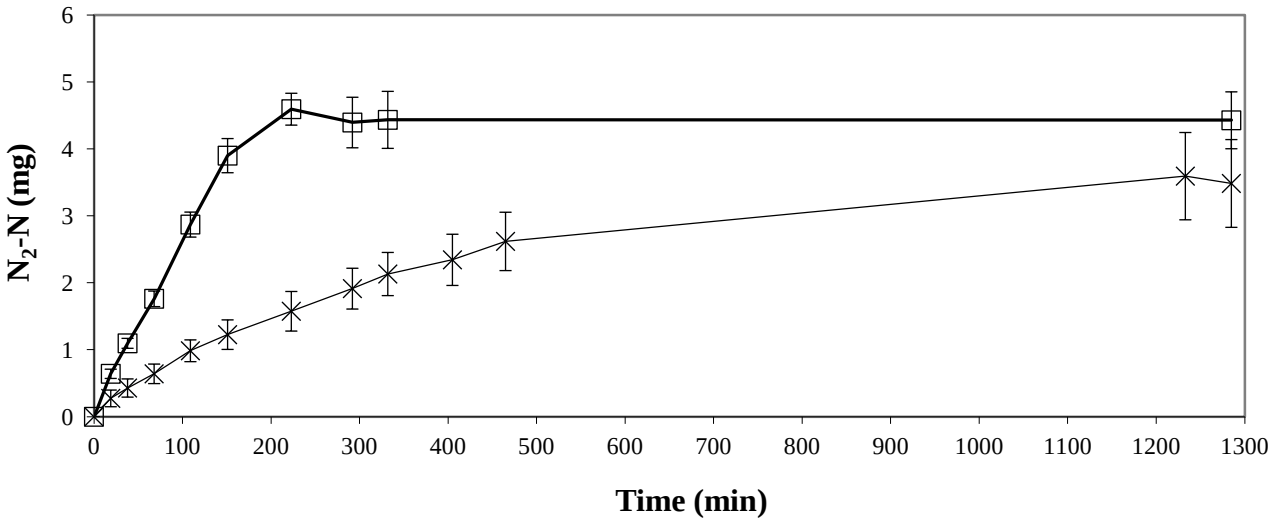

Figure 5. Profile of N_2 gas production: B6, assay with 1.4 g VSS/L (□); B7, assay with 0.7 g VSS/L (x). The results are average values of the performed triplicates with the corresponding standard deviation.