Simultaneous nitrification and denitrification in microbial community-based polyhydroxyalkanoate production

Estévez-Alonso, Ángel; van Loosdrecht, Mark C.M.; Kleerebezem, Robbert; Werker, Alan

DOI
10.1016/j.biortech.2021.125420

Publication date
2021

Document Version
Final published version

Published in
Bioresource Technology

Bioresource Technology 337 (2021) 125420

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Simultaneous nitrification and denitrification in microbial community-based polyhydroxyalkanoate production

Ángel Estévez-Alonso [a,b,*], Mark C.M. van Loosdrecht [a], Robbert Kleerebezem [a], Alan Werker [b]

[a] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands
[b] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911 MA, Leeuwarden, The Netherlands

HIGHLIGHTS

- Dissolved oxygen (DO) control on nitrifying and PHA-storing bacteria was evaluated.
- Low DO levels can limit nitrifying activity without slowing PHA production.
- Concomitant anoxic PHA production was evaluated at low DO concentrations.
- An optimum DO level exists where SND is exploited for effective PHA production.

ARTICLE INFO

Keywords:
Polyhydroxyalkanoates (PHA)
Waste activated sludge
Nitrification
Dissolved oxygen
Simultaneous nitrification and denitrification (SND)

ABSTRACT

Microbial community-based polyhydroxyalkanoate (PHA) production has been demonstrated repeatedly at pilot scale. Ammonium, normally present in waste streams, might be oxidized by nitrifying bacteria resulting in additional aeration energy demand. The use of low dissolved oxygen (DO) concentrations allowed to reduce nitrifying rates by up to 70% compared to non-oxygen limiting conditions. At lower DO concentrations nitrate was used as alternative electron acceptor for PHA production and therefore, a constant PHA production rate could only be maintained if nitrate was sufficiently available. An optimum DO concentration (0.9 mgO_2/L) was found for which nitrification was mitigated but also exploited to supply requisite heterotrophic nitrate requirements that maintained maximum PHA production rates. PHA accumulations with such DO control was estimated to reduce oxygen demand by about 18%. This work contributes to establish fundamental insight towards viable industrial practice with the control and exploitation of nitrifying bacteria in microbial community-based PHA production.

1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polyesters that are naturally produced intracellularly by a wide range of species of microorganisms. PHA are energy and carbon storage polymers used by bacteria to cope with dynamic environments such as the alternating presence and absence of substrate (Van Loosdrecht et al., 1997). PHA have very interesting properties as materials for the polymer industry (Philip et al., 2007) and are commercially produced today using pure cultures and simple sugars as feedstocks (Li and Wilkins, 2020). However, in the last decade a progression of developments with microbial community-based PHA production has led to an alternative production approach with several successful pilot-scale installations (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021a). The microbial community-based approach instead of pure cultures for PHA production allows for anticipated lowering of production costs and for utilization of a wide range of low value unrefined feedstocks, which are managed today as waste streams (Rodriguez Perez et al., 2018). Most of these PHA production pilot systems to date have used PHA-storing biomass produced explicitly with an enrichment of PHA-storing activity, or harvested directly as waste activated sludge from municipal/industrial wastewater treatment (Estévez-Alonso et al., 2021a). In both cases, high PHA contents have been reached and pilot-scale demonstration indicates the ability to engineer and maintain product quality and control even using fermented organic waste and/or municipal waste activated sludge as the volatile fatty acid (VFA) and biomass as input

* Corresponding author at: Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
E-mail address: a.estevezalonso@tudelft.nl (Á. Estévez-Alonso).

https://doi.org/10.1016/j.biortech.2021.125420
Received 7 May 2021; Received in revised form 11 June 2021; Accepted 12 June 2021
Available online 19 June 2021

materials for the biopolymer production.

The use of fermented organic waste streams for microbial community-based PHA production introduces technical challenges due to the concomitant release of growth nutrients (mainly nitrogen and phosphorus) during the fermentation process (Capson-Tojo et al., 2016). The presence of growth nutrients in the feedstock plays a key role in the allocation of carbon towards PHA or biomass in PHA-storing bacteria (Johnson et al., 2010; Valentino et al., 2015). Phosphorus levels may be readily reduced by chemical precipitation, but nitrogen is more difficult to be removed (Wilfert et al., 2015). Bioavailable nitrogen may promote active growth over storage in PHA-storing bacteria, but also allow for a wider range of microbial growth activity, including so-called flanking populations of non PHA-storing bacteria (Valentino et al., 2015). An example of flanking population of non PHA-storing bacteria occurring when ammonium or nitrite are present in the process water is nitrification. Nitrification has been frequently observed at lab- and pilot-scale in microbial community-based PHA production when municipal waste activated sludge was used as PHA-storing biomass (Bengtsson et al., 2017a; Bengtsson et al., 2017b; Conca et al., 2020; Morgan-Sagastume et al., 2015) or when allylthiourea was not added to the feedstock (Third et al., 2003a; Fra-Vázquez et al., 2019; Morgan-Sagastume et al., 2020). Nitrifying bacteria are characterized by high oxygen consumption yields and low biomass production rates (Wiesmann, 1994), resulting in additional aeration energy demand. Alkalinity is also consumed and this introduces a loss of buffer capacity which may be required when acidic substrates are used within the process.

Several strategies are known to mitigate the growth of nitrifying microorganisms (Antoniou et al., 1990; Mulder et al., 2001; Pollice et al., 2002). Nonetheless, a challenge in a microbial community-based PHA production process is to control the extant activity of an established nitrifying community that is an inherent component of the PHA-storing biomass. In laboratory scale PHA production research, allylthiourea is used to eliminate nitrification activity, but it is unsuitable for industry scale application (Liu et al., 2018). High pH has also not been enough to limit the activity of nitrifying bacteria in a PHA production process even with highly functionalized biomass (Fra-Vázquez et al., 2019). However, low dissolved oxygen (DO) concentrations have shown promise (Third et al., 2003b; Reddy and Mohan, 2012; Wang et al., 2019). Based on batch activity tests with waste activated sludge, Wang et al. (2019) found that it should be possible to limit nitrification in a PHA accumulation process by lower DO concentrations. PHA-storing bacteria were also negatively affected by lower DO concentrations and simultaneous nitrification and denitrification (SND) was observed when both nitrifying and PHA-storing bacteria were active. These published results promoted an idea for a strategy of DO level control to mitigate flanking nitrifying bacteria, but also introduce the possibility to sustain PHA production productivity by promoting concurrent denitrification activity in the activated sludge flocs. For a likely relevant PHA-storing biomass at industrial scale, the question, of the extent to which SND activity may be exploited during microbial community-based PHA production, has not yet been systematically explored.

The aim of this work was to evaluate the extent of positive influence simultaneous nitrification and denitrification could bring to an industrial PHA production process. For this purpose, short-term batch PHA-accumulation experiments over a selected range of constant DO concentrations were performed in order to quantify and compare the nitrification, denitrification and PHA production process rates in isolation and in combination. This work aimed to establish fundamental insight towards implementing a strategy of DO control and to critically assess the benefit that such control strategy could bring to industrial practice of microbial community-based PHA production. Nitrogen rich feedstocks and PHA-storing biomass that inherently have nitrifiers present are anticipated to become commonly available as starting resource materials in future value chains of PHA production (Bengtsson et al., 2017b).

2. Materials and methods

2.1. Experimental set-up

Stimulus–response experiments were performed in double-jacketed glass bioreactors (1 L working volume). Two reactors were operated in parallel and thermostated at 25 ± 0.1 °C. Agitation was performed with two standard three-bladed turbines (R16 and R20, CAT Scientific, Germany) at a stirring rate of 150 rpm. pH was controlled between 7 and 7.5 by automatic titration with 1 M HCl (VWR Chemicals, The Netherlands). Airflow rate was controlled in the range between 0–2 L/min by a PID mass flow controller (FG-201CV-RGD-22-V-AA-000, Bronkhorst, The Netherlands) for selected set-point mixed liquor DO concentrations. DO and pH probes (COS81D and CPS11D, Endress & Hausser, The Netherlands), pH control pumps (Stepdos 10, KNF, The Netherlands) and mass flow controllers were connected and controlled by a 4-channel transmitter box (Liquiline CM444, Endress & Hausser, The Netherlands). The transmitter box also provided for data logging at a 10 s interval. DO and pH probes were calibrated before each experiment according to the manufacturer's instructions.

2.2. Sludge source and feedstock

Waste activated sludge from the municipal wastewater treatment plant (WWTP) Bath (Rilland-Bath, The Netherlands) was used as the PHA-storing biomass for all experiments. WWTP Bath (470,000 PE) consists of a primary separation by screening and primary sedimentation followed by a modified Ludzack-Ettinger biological process (pre-denitrification and nitrification) treating a mix of regional municipal and industrial wastewater. This biomass was selected based on its well-established and consistent level of performance for PHA accumulation (Bengtsson et al., 2017b). Fresh gravity belt thickened waste activated sludge (57.8 ± 1.2 gTS/kg and 40.6 ± 1.4 gVS/kg (n = 5)) was delivered batchwise by courier every two weeks in 20 L carboys and stored at 4 °C pending experiments. Benchmark evaluations found no evidence to support that storage of samples at 4 °C up to 10 days have any significant effect in both nitrifying and PHA-storing capacity of this activated sludge. In total, 5 distinct batches were used for the experiments performed.

The accumulation feedstock, with nutrients ratio 100:5:2.5 (COD:N:P by weight), was prepared with tap water and was as follows: 80 g/L acetic acid, 19 g/L NH_4Cl, 10.96 g/L KH_2PO_4 and 20 mg/L allylthiourea. Acetic acid and/or allylthiourea were included or omitted from the feedstock depending on the activity assay that was to be performed, as shown in Table 1. The pH of the feedstock was adjusted to pH 6 with KOH pellets.

2.3. Batch assays at selected dissolved oxygen concentrations

A total of 24 assays, divided into three different sets of experiments, were performed to evaluate PHA-storing and nitrifying bacteria activities in combination and in isolation, over a selected range of DO concentrations (Table 1). For each assay, a 30 g grab sample from the stored

Table 1
Overview of the batch assays performed at selected DO concentrations of 0.5, 1, 3 and 5 mgO_2/L. Batch HT(+)N(-) was to quantify the activity of PHA-storing bacteria in absence of nitrification. Batch HT(-) N(+) was to quantify nitrifying activity in absence of heterotrophic activity. Batch HT(+) N(+) was to quantify the activity of both PHA-storing and nitrifying bacteria simultaneously. ATU = allylthiourea.

Batch	Acetate	Ammonium	Nitrate	ATU	Phosphate
HT(+)N(-)	Yes	Yes	Yes	Yes	Yes
HT(-)N(+)	No	Yes	Yes	No	Yes
HT(+)N(+)	Yes	Yes	Yes	No	Yes

thickened activated sludge was diluted with tap water to nominally 1 gVSS/L, and 1 L was dispensed to the two parallel reactors set to 25 °C. Reactor mixed liquor was conditioned by aeration overnight at DO levels greater than 5 mgO_2/L to oxidize all the easily biodegradable COD, ammonium and nitrite present in the water and to establish a common baseline of endogenous microbial activity between tests. Because ammonium and nitrite were oxidized to nitrate during conditioning, all the experimental dose–response assays started with an initial background nitrate concentration of 9–13 $mgN\text{-}NO_3$/L. Subsequently, 5 mL of feedstock was added as a single pulse input, and DO concentration was then regulated to one of four selected set points within a ± 0.1 mgO_2/L error margin: 0.5, 1, 3 or 5 mgO_2/L. Selected set-points were reached within 10 min and this time was used to determine maximum oxygen consumption rates. The three types of assay applied are explained in Table 1.

2.4. Analytical methods

During the batch assays, the process was monitored by online data logging (DO, temperature, pH and airflow rate) and from 45 mL hourly grab samples for water quality and solids analyses. Grab samples from the well-mixed vessel comprised three 15 mL aliquots: two aliquots were used for solids and liquid analyses, and the third was applied for biomass PHA content determination. Suspended solids were separated from the mixed liquor by centrifugation (3250 RCF and 4 °C for 20 min). The supernatant after membrane filtration (0.45 μm pore size filters) was stored at −20 °C pending analyses within 72 h. The harvested biomass pellet dry weight and volatile solids contents were estimated based on Standard Methods (105 °C drying and 550 °C ashing Clesceri et al., 1999), and these were referenced to the sample volume to estimate total and volatile suspended solids (TSS and VSS), respectively. Soluble chemical oxygen demand (sCOD) was determined by Hach-Lange (Germany) LCK014 and LCK314 test kits. Acetic acid concentration was determined by ultra high pressure liquid chromatography (UHPLC) using a Dionex Ultimate 3000RS system equipped with a Phenomenex Rezex Organic Acid H^+ column (300x7.8 mm) and a Dionex Ultimate 3000 RS UV detector (210 nm) with 2.5 mM sulfuric acid mobile phase at 0.5 mL/min and 80 °C. Ammonium, nitrite, nitrate and phosphate concentrations were determined by ion chromatography or IC (Metrohm Compact IC Flex 930, Metrohm, Switzerland) with conductivity detector. For ammonium determination, a pre-column (Metrohm Metrosep RP 2 Guard/3.6) and a column (Metrohm Metrosep C4-150/4.0 mm) and 2.5 mM nitric acid mobile phase at 0.9 mL/min and room temperature were applied. For nitrite, nitrate and phosphate determination, two pre-columns (Metrohm Metrosep RP 2 Guard/3.6 and Metrohm Metrosep A Supp 4/5 Guard) and a column (Metrohm Metrosep A Supp 5, 150/4.0 mm) with 3.2 mM sodium carbonate and 1 mM sodium bicarbonate + 1% acetone solution mobile phase at 0.7 mL/mL and room temperature were used. Additionally a chemical suppressor was applied (0.2 M phosphoric acid + 1% acetone at 0.1 mL/mL).

The aliquot for PHA determination was directly acidified to pH 2 with 20 μL of H_2SO_4. After thorough mixing (5 min) suspended solids were collected (3250 RCF and 4 °C for 20 min). The biomass pellet was retained and dried at 105 °C. Dried pellets were ground and analyzed by thermogravimetric analysis or TGA (TGA 2, Mettler Toledo, The Netherlands). A 5 mg of ground sub-sample was introduced to the furnace at 80 °C, and heated to 105 °C (10 °C/min) under nitrogen atmosphere. After drying at 105 °C for 10 min, the sample was heated to 550 °C (10 °C/min), under nitrogen atmosphere. At 550 °C, the atmosphere was switched to air and temperature was held for 30 min. The weight loss trends were used to determine the sample residual moisture content and the biomass PHA fraction with respect to the biomass dried total and volatile solids as previously described (Chan et al., 2017). The PHA content was expressed as fraction of the biomass volatile solids (gPHA/gVSS). The biomass fraction was estimated as the total biomass as VSS minus PHA content. Biomass was chemically represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980).

2.5. Mass balance and process modeling

For each batch assay, biomass specific rates and yields on substrate were estimated based on mass balance principles and a process model. Both on and offline monitoring data were applied. This model was adapted from Tamis et al. (2014) and was used to estimate the biomass specific rates for the heterotrophic fraction of the biomass: acetic acid uptake rate (q_{HAc}), PHA yield on substrate ($Y_{PHA,HAc}$), PHA degradation rate (k_{PHA}) and heterotrophic nitrate uptake rate (q_{NO3}). Nitrifying biomass specific rates, ammonium uptake rate (q_{AOB}) and nitrite uptake rate (q_{NOB}) were incorporated to the model by means of a modified version of the activated sludge models (Iacopozzi et al., 2007). Bacterial growth (heterotrophic and autotrophic) was not included in the process model and microbial rates were calculated on a total VSS basis. All model kinetic equations are shown in Table 2.

The model was applied to estimate the parameter set that minimized the sum of the squared relative error with respect to measured variables for each respective individual experiment. Subsequently, a second kinetic model estimated the overall average response of the biomass with respect to maximum specific substrate uptake rates (q_{HAc}^{max}) and apparent affinity constant for oxygen (k_{DO}) as function of DO, as defined in Eqs. 1 and 2. It was assumed that oxygen was the only limiting substrate and denitrification would only begin to take place when oxygen became sufficiently limiting, based on an affinity constant k_{DO}. The q_{HAc}^{max} was assumed to be independent of electron acceptor and therefore, k_{DO} provided an estimate of the balance of aerobic/anoxic contributions to heterotrophic substrate uptake rates. The nitrate yield ($Y_{NO_3,HAc}$) was assumed to be constant and was 0.2 $gN\text{-}NO_3$/gCOD (Beun et al., 2000b). These equations were implemented in Microsoft Excel and the function SOLVER (Generalized Reduced Gradient Nonlinear algorithm) was used to minimize the sum of squared relative error between the ensemble of fitted data from all the batch experiments.

$$q_{HAc} = q_{HAc}^{aerobic} + q_{HAc}^{anoxic} = q_{HAc}^{max}\left(\frac{DO}{k_{DO}+DO}\right) + q_{HAc}^{max}\left(\frac{k_{DO}}{k_{DO}+DO}\right) \quad (1)$$

$$q_{NO_3} = q_{NO_3}^{anoxic}\cdot Y_{NO_3,HAc} \quad (2)$$

3. Results and discussion

A total of 24 assays were performed with the activated sludge to evaluate the PHA-storing and nitrifying activity, in combination and in isolation, over a selected range of DO concentrations (Table 1). One of

Table 2
Model kinetics used for the data fitting.

Heterotrophic bacteria		
HAc uptake	$q_{HAc} = q_{HAc}^{max}\left(\frac{C_{HAc}}{k_{HAc}+C_{HAc}}\right)$	-
PHA production	$q_{PHA}^{+} = q_{HAc}\cdot Y_{PHA,HAc}$	if $C_{HAc} > 0$
PHA degradation	$q_{PHA}^{-} = k\cdot\left(\frac{C_{X0}}{C_X}\right)^{1/3}\cdot f_{PHA}^{2/3}$	if $C_{HAc} = 0$
NO_3^- uptake	$q_{NO_3} = q_{NO_3}^{max}\left(\frac{C_{NO_3}}{k_{NO_3}+C_{NO_3}}\right)$	if $C_{HAc} > 0$
Nitrifying bacteria		
NH_4^+ uptake	$q_{AOB} = q_{AOB}^{max}\left(\frac{C_{NH_4^+}}{k_{NH_4^+}+C_{NH_4^+}}\right)$	-
NO_2^- uptake	$q_{NOB} = q_{NOB}^{max}\left(\frac{C_{NO_2^-}}{k_{NO_2^-}+C_{NO_2^-}}\right)$	-

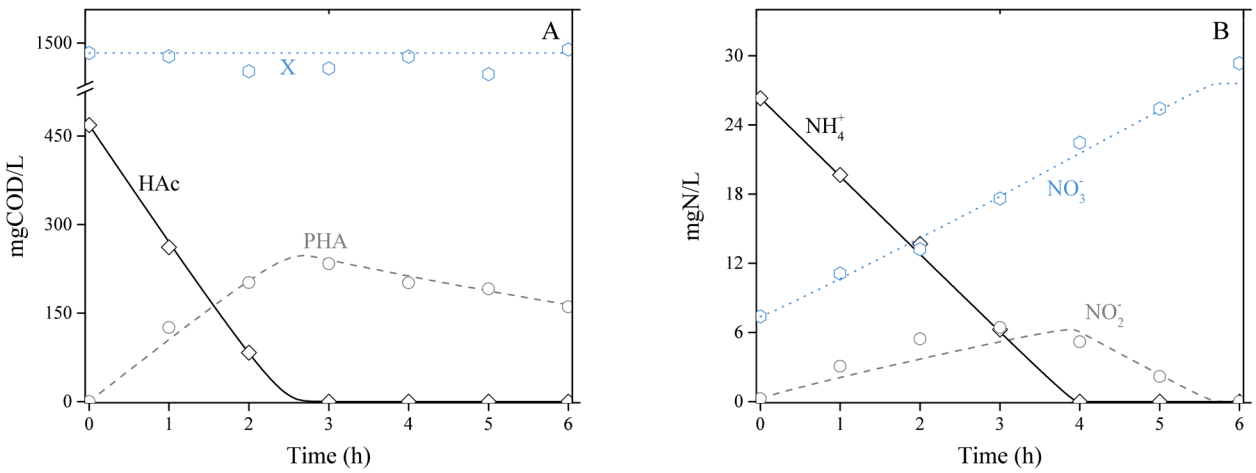

Fig. 1. Detailed characterization of a representative test at 5 mgO_2/L with active PHA production and nitrification including model fitting and measurements of (A) acetate, PHA and biomass (X) and (B) ammonium, nitrite and nitrate.

the assays at 5 mgO_2/L with the measured data and its model characterization is provided, as a typical example, in Fig. 1. All the other experiments followed similar systematic trends and the data set can be found in Mendeley Data (Estévez-Alonso et al., 2021b).

3.1. PHA storage in absence of nitrification

Four batch tests without nitrification were performed to determine reference aerobic and anoxic acetate uptake rate, PHA production rate and the yield of PHA on acetate as function of DO concentration. The absence of nitrification allowed to study the heterotrophic activity only. A pulse containing acetate, ammonium and ATU was added to the mixed liquor that had the above mentioned background NO_3 concentration. An overview of results for the four assays is provided in Fig. 2 and Table 3.

The aerobic acetate uptake rate was not measurably affected by low DO concentrations and was estimated to be 154.7 mgCOD/(gVSS·h). Low denitrification rates were observed at 0.5 mgO_2/L, q_{NO_3} = 0.34 mgN-NO_3/(gVSS·h), which represented less than 1% of the total respiration rate, based on electron equivalents. The low denitrification rates at low DO concentration indicate that the tests were successfully performed under fully aerobic conditions. Besides, a negligible apparent k_{DO}^{HT}, 0.01 mgO_2/L, was identified. A low apparent k_{DO}^{HT} indicates a high affinity for oxygen which resulted in the ability to maintain maximum uptake rates even at low DO concentrations. Because heterotrophic activity could be maintain at maximum rates at low DO concentrations, the yields of PHA on acetate were also not affected by the lower DO concentrations and were in the range 0.5–0.6 gCOD-PHA/gCOD-HAc. The PHA yields on substrate obtained in this work were similar, and close to the theoretical maximum yields, to those obtained previously under fully aerobic conditions, 0.5–0.7 gCOD-PHA/gCOD-HAc (Morgan-Sagastume et al., 2015; Bengtsson et al., 2017b; Bengtsson et al., 2017a; Wang et al., 2019; Conca et al., 2020).

The average ammonium uptake rate was 1.33 mgN-NH_4/(gVSS·h) and was assumed to be exclusively due to assimilation by the heterotrophic bacteria, as ATU was present in the feedstock to block nitrification. The average ammonium uptake rate was used in the modelling as ammonium uptake for heterotrophic growth in tests HT(+)N(+).

3.2. Nitrification rates

Eight batch assays were used to characterize the ammonium and nitrite uptake rate as function of DO concentration, when both ammonium oxidation (AOB) and nitrite oxidation bacteria (NOB) were dominantly active due to absence of added organic substrate. A pulse of ammonium was added to the mixed liquor also containing a background NO_3 concentration. An overview of the eight assays is shown in Fig. 2 and Table 3.

Both ammonium and nitrite uptake rates decreased at lower DO concentrations: a 49% lower q_{AOB} was observed at 0.5 mgO_2/L compared to the model maximum q_{AOB}^{max}. Consequently an apparent k_{DO}^{AOB} of 0.49 mgO_2/L was estimated for this activated sludge AOBs. Similar results were obtained for NOBs at 0.5 mgO_2/L, with a 50% lower q_{NOB} with respect to the maximum q_{NOB}^{max} and an apparent k_{DO}^{NOB} of 0.50 mgO_2/L. The higher apparent k_{DO} obtained for nitrifying bacteria compared to heterotrophic bacteria indicates that low DO concentrations have a more detrimental effect in nitrifying than in heterotrophic bacteria. At lowered DO concentrations, therefore, nitrifying bacteria will be more progressively limited compared to the heterotrophic bacteria. The apparent k_{DO} includes not only biological but also physical mass transfer rate dependent phenomena, such as the size of the cell clusters and/or the size of the floc, which also depends on the mixing intensity (Picioreanu et al., 2016; Pérez et al., 2005; Manser et al., 2005; Chu et al., 2003). Nonetheless, the apparent k_{DO} and rates obtained for nitrifying bacteria are in line with those previously reported for similar reactor conditions (Wang et al., 2019; Picioreanu et al., 2016; Arnaldos et al., 2015). As a result, the use of low DO concentrations should allow to run a PHA production process with predominant heterotrophic and lower nitrifying activity, which was further explored in Section 3.3.

3.3. Simultaneous PHA storage and nitrogen conversion

Twelve batch assays were used to characterize the heterotrophic aerobic and anoxic acetate uptake rates, PHA production rate and the yields of PHA on acetate in combination with the autotrophic AOBs ammonium uptake rate and the NOB nitrite uptake rate as function of DO concentration. A pulse of feedstock containing acetate and ammonium (with no ATU) was added to start these assays, again with a background starting NO_3 concentration. An overview of the twelve assays is given in Fig. 2 and Table 3.

3.3.1. PHA storage

The acetate uptake rate remained constant and seemingly independent of DO level. However, the maximum acetate uptake rate was 26% higher compared to the acetate only experiments: 194.9 compared to 154.7 mgCOD-HAc/(gVSS h). The higher heterotrophic rates may be attributed to a naturally expected degree of batch-to-batch variability between the 5 activated sludge batches from a full-scale process and delivered over a period of 40 days from the WWTP. At lower DO concentrations, higher anoxic q_{HAc} was estimated: 23.0 mgCOD-HAc/(gVSS h) at 0.5 mgO_2/L compared to 2.6 mgCOD-HAc/(gVSS h) at 5 mgO_2/L.

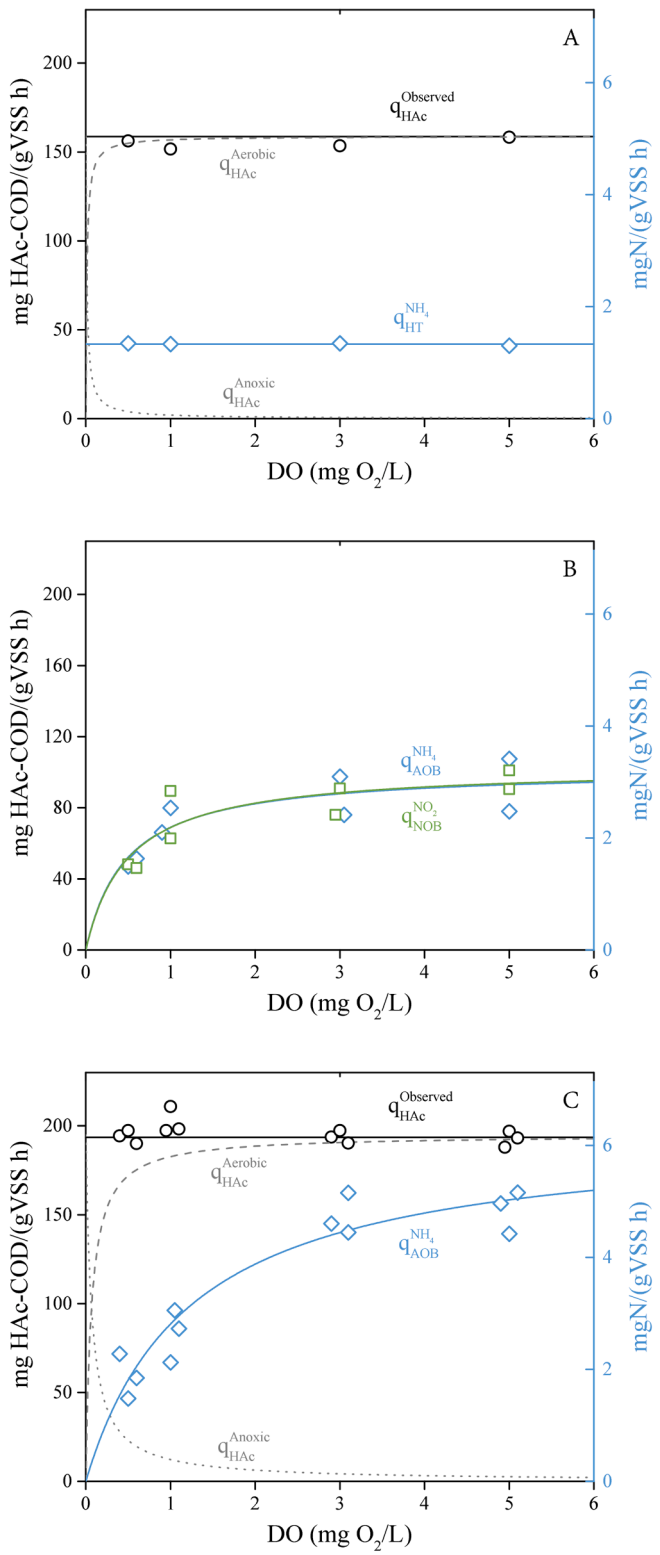

Fig. 2. Aerobic and anoxic acetic acid, ammonium and nitrite uptake rates as function of DO concentration in tests where only heterotrophic bacteria (A), only nitrifying bacteria (B) and both nitrifying and heterotrophic bacteria (C) were active. The symbols represent the microbial rates derived from each batch assay and the lines the model results from Eq. 1.

Consequently, the aerobic q_{HAc} decreased at lowered DO concentrations, being 12% lower at 0.5 mgO_2/L compared to the derived maximum q_{HAc}^{max}. This suggests that the use of nitrate as alternative electron acceptor allowed the PHA-storing bacteria to maintain a similar level of activity as observed under non-oxygen limiting conditions. Simultaneous nitrification and denitrification is often explained as a consequence of mass transfer limitations due to diffusion gradients, which leads to oxygen limitation within the floc/granule (PPochana and Kellerochana and Keller, 1999). In those zones within the floc where oxygen is depleted, facultative aerobic bacteria can switch electron acceptor from oxygen to nitrate. The waste activated sludge used in this work was obtained from Bath WWTP, which is composed of a pre-denitrification (anoxic) and nitrification (aerobic) steps. It is expected that the Bath sludge is enriched in facultative aerobic bacteria that are able to switch electron acceptor from oxygen to nitrate, depending on the environmental conditions e.g. oxygen limitation within the floc. Oxygen limitation within the floc/granule is likely due to the presence of active nitrifying bacteria competing for the available oxygen, as no anoxic PHA production was observed in those tests where only heterotrophic bacteria were active. The presence of active nitrifying bacteria altered the diffusion of oxygen within the floc, reducing the penetration depth and the oxygen availability for aerobic PHA production in the floc. As introduced in Section 3.2, the apparent k_{DO} includes not only biological but also physical mass transfer rate phenomena, and therefore, a higher apparent k_{DO}^{HT} compared to the baseline experiments without active nitrification should be observed. The observed apparent k_{DO}^{HT} was 0.07 mgO_2/L, almost one order of magnitude higher than the baseline experiments without active nitrification, 0.01 mgO_2/L.

Lowered DO concentrations did not influence the PHA yield on acetate, that were in the same range as in tests where only heterotrophic bacteria were active, 0.5–0.6 gCOD-PHA/gCOD-HAc. In this case, and as a result of mass transfer limitation within the floc, up to 15% of the obtained PHA has been produced under anoxic conditions. PHA yields on substrate under anoxic conditions are reported to be 40–60% lower than under aerobic conditions (Kuba et al., 1996; Beun et al., 2000a). However, an anticipated decrease in the overall PHA yield on substrate due to the anoxic PHA accumulation could not be resolved in this work, at least within the frame of the replicated experiments and the model used to fit these data. If the aerobic PHA yield on substrate would be 0.6 gCOD-PHA/gCOD-HAc and the anoxic PHA yield on substrate would be 50% lower, 0.3 gCOD-PHA/gCOD-HAc, the expected observed yield, when 15% of the PHA is produced anoxically, would be expected to have been lowered by 7% to 0.56 gCOD-PHA/gCOD-HAc.

3.3.2. *Nitrogen conversion*

The maximum AOB uptake rate was higher (almost double) than in tests where only nitrifying bacteria were active: 6.3 mgN-NH_4/(gVSS·h) compared to 3.2 mgN-NH_4/(gVSS·h). The higher nitrifying uptake rates may not be explained only by a variation in the sludge samples. The observed higher rates are understood to have been promoted both by variations in airflows between experiments and a development of inorganic carbon limitation in tests where only nitrifying bacteria were active. During the conditioning of the biomass (overnight aeration) the dissolved inorganic carbon has been observed to become depleted due to gas stripping and nitrification. Sufficient loss of inorganic carbon could have led to conditions of carbon limitation for autotrophic microbial activity in tests where only nitrifying bacteria were active. In tests, with both nitrification and heterotrophic consumption on acetate, carbon dioxide is supplied by heterotrophic activity even if alkalinity is similarly depleted initially.

In line with tests HT(-)N(+) and with the previous work of Wang et al. (2019) with the same activated sludge, nitrification rates were negatively affected by the lower DO concentrations, the aerobic q_{AOB} at 0.5 mgO_2/L was 70% lower than the derived maximum q_{AOB}^{max}. Not only the presence of nitrifying bacteria affected the PHA-storing bacteria, as explained in Section 3.3.1, but also the PHA-storing bacteria had an effect on the nitrifying bacteria. This effect can be observed in the higher apparent k_{DO}^{AOB}. k_{DO}^{AOB} in tests where both nitrifying and heterotrophic

Table 3
Model derived variables for heterotrophic and nitrifying bacteria in assays tests where only heterotrophic bacteria (HT(+)N(-)), only nitrifying bacteria (HT(-) N(+)) and both nitrifying and heterotrophic bacteria (HT(+) N(+)) were active. n.d = not determined.

	Heterotrophic bacteria		Nitrifying bacteria		
	HAc		AOB	NOB	
Batch HT(+) N(−)					
q^{max}	154.7	mgCOD/(gVSS·h)	n.d.		mgN/(gVSS·h)
k_{DO}^{App}	0.01	mgO_2/L	n.d.		mgO_2/L
$Y_{PHA,HAc}$	0.58 ± 0.05	mgCOD/mgCOD			
Batch HT(+) N(+)					
q^{max}	194.9	mgCOD/(gVSS·h)	6.26	4.42	mgN/(gVSS·h)
k_{DO}^{App}	0.07	mgO_2/L	1.23	0.68	mgO_2/L
$Y_{PHA,HAc}$	0.57 ± 0.04	mgCOD/mgCOD			
Batch HT(−) N(+)					
q^{max}	n.d	mgCOD/(gVSS·h)	3.23	3.27	mgN/(gVSS·h)
k_{DO}^{App}	n.d	mgO_2/L	0.48	0.50	mgO_2/L
$Y_{PHA,HAc}$	n.d.	mgCOD/mgCOD			

bacteria were active was 1.23 mgO_2/L, and two times higher than the one obtained in tests where only nitrification was active, 0.48 mgO_2/L (Table 3). From these experiments, the maximum nitrifying oxygen consumption rate was also derived and would represent 23–27% of the total oxygen demand for a process under assumed steady state conditions, as shown in Fig. 3. Nonetheless, if the nitrifying bacteria rates were indeed underestimated in tests where only nitrifying bacteria were active due to inorganic carbon limitation, then these predicted outcomes are conservative, and the nitrifying oxygen consumption rates could have represented up to 40% for the total oxygen demand of the process.

3.4. Implications for microbial community-based PHA production

Microbial community-based PHA production research and development at laboratory and pilot-scales are usually run with an objective for fully aerobic conditions with fixed airflow rate and without a control or consideration for influence of autotrophic oxygen demand (Kourmentza et al., 2017; Rodriguez Perez et al., 2018; Sabapathy et al., 2020). This work introduces the possibility to operate the PHA accumulation process at fixed low DO concentrations and to consequently utilize a wide

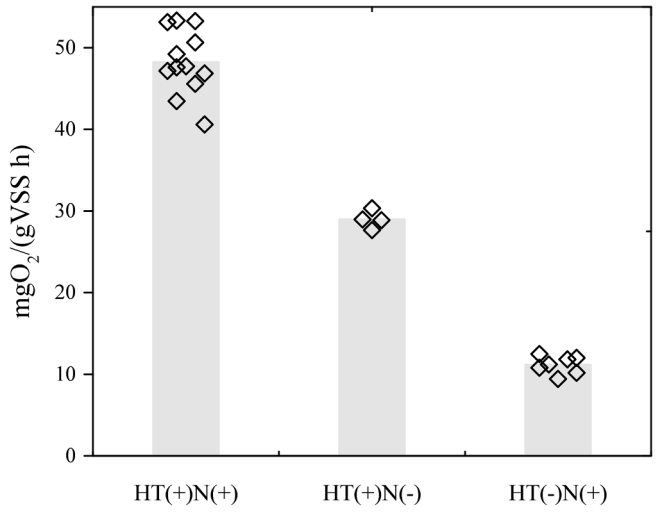

Fig. 3. Maximum oxygen uptake rates in tests where only heterotrophic bacteria (HT(+) N(−)), only nitrifying bacteria (HT(−) N(+)) and both nitrifying and heterotrophic bacteria (HT(+) N(+)) were active. The symbols represent the microbial rates derived from each batch assay and the bars give the average values.

variety of feedstocks, ranging from both low to high ammonium concentrations. The ability to leverage the activity of nitrifiers relies on potential for facultative PHA-storing microbial activity. While running a PHA accumulation at low DO concentrations part of the PHA will be stored using nitrate as electron acceptor. Therefore, an optimum control point for DO for nitrifying activated sludge is achieved when the autotrophic nitrate production balances the heterotrophic nitrate requirements. The DO optimum level and respective denitrification rates are expected to be tunable for biomass with different origin and enrichment strategy, as process specific conditions will influence floc size and mass transfer coefficients (Arnaldos et al., 2015). However, the fundamental principles are expected to be valid and generically applicable. For the biomass used in this work, the optimum point was found to be around 0.9 mgO_2/L (Fig. 4). Running a PHA accumulation with DO control level close the optimum allows to reduce the respiration demands of the process, conservatively, by 17 to 19% or to increase the reactor biomass loading (volumetric productivity) given the same vessel and aeration capacity. These outcomes are expected to influence practical outcomes of capital (CAPEX) and operating expenses (OPEX). Anoxic PHA production will also allow to lower the final nitrogen concentrations in the process effluent. Denitrification rates observed at 0.9 mgO_2/L can allow to remove nitrogen from the liquid phase at removal rates of 2.7 mgN/(gVSS h). For a standard PHA accumulation run with waste activated sludge as biomass source and a duration of 16 h, 43 mgN/gVSS could be removed for this biomass. Nitrate is expected to be reduced to dinitrogen gas, but the fate of nitrogen requires validation as part of the ongoing research and development. If the denitrification process is not complete, nitrous oxide (N_2O) can be produced, which is a known greenhouse gas (Kampschreur et al., 2009). Most of the N_2O emissions in wastewater treatment plants are associated with low DO concentrations in the nitrification tank. These conditions are very similar to those tested in this work, therefore further evaluation of this control strategy with respect to nitrogen fate with N_2O and/or its control in PHA production at low DO concentration is recommended.

4. Conclusions

This study establishes methods for microbial community-based PHA production with nitrogen rich feedstocks and PHA-storing biomass containing nitrifying bacteria. Simultaneous nitrification and denitrification can be used to optimize polymer production, process aeration requirements and productivity. In this way, the aeration demand of the process can be reduced, conservatively, by 17 to 19% while reducing the effluent nitrogen discharge levels. The results of this work show that substrate sources containing ammonium can efficiently be used for

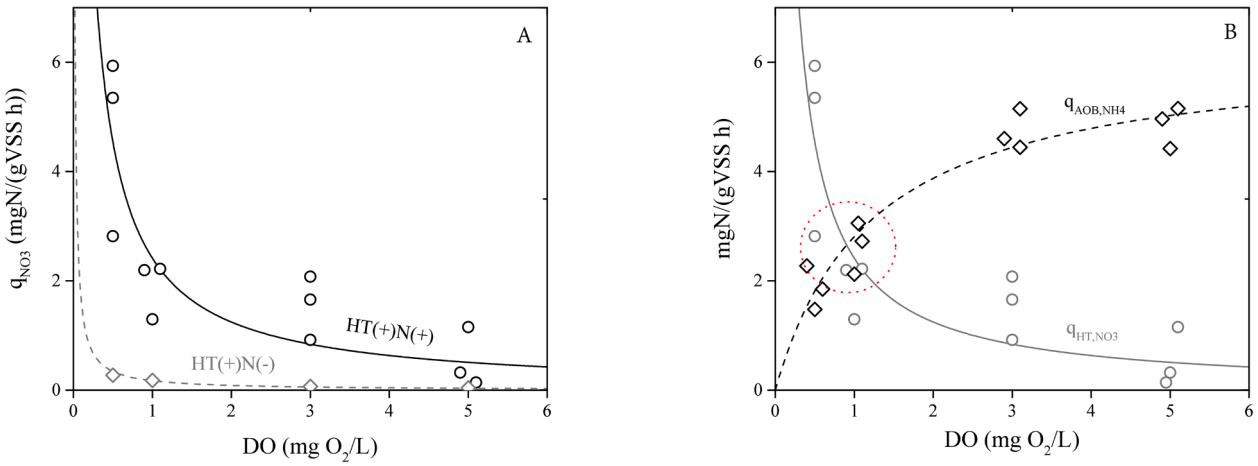

Fig. 4. Heterotrophic nitrate consumption rates as function of DO concentration in tests where only heterotrophic bacteria (HT(+)N(-)) and both nitrifying and heterotrophic bacteria (HT(+)N(+)) were active (A) and heterotrophic nitrate and autotrophic ammonium consumption rates as function of DO concentration in tests where both nitrifying and heterotrophic bacteria were active (HT(+)N(+)) (B). The symbols represent the microbial rates derived from each batch assay and the lines the model results.

microbial community-based PHA production, widening the generic potential for substrate availability for PHA production from waste sources.

Data availability

Datasets related to this article can be found at: https://data.mendeley.com/datasets/gnnkdpg3rp/1, an open-source online data repository hosted at Mendeley Data (Estévez-Alonso et al., 2021b).

CRediT authorship contribution statement

Ángel Estévez-Alonso: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Mark C.M. van Loosdrecht:** Conceptualization, Supervision, Writing - review & editing. **Robbert Kleerebezem:** Conceptualization, Supervision, Writing - review & editing. **Alan Werker:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was performed in the cooperation framework of Wetsus, European Centre of Excellence for Sustainable Water Technology (www.wetsus.nl). Wetsus is co-funded by the Dutch Ministry of Economic Affairs and Ministry of Infrastructure and Environment, the European Union Regional Development Fund, the Province of Fryslân and the Northern Netherlands Provinces. This work has also received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skodowska-Curie grant agreement No 665874. The authors thank the participants and industrial/public partners (Paques BV, STOWA, and SNB) of the research theme "Biopolymers from water" for fruitful discussions and financial support. The authors also thank Henk Dijkman for his helpful informed comments and suggestions during writing the manuscript.

References

Antoniou, P., Hamilton, J., Koopman, B., Jain, R., Holloway, B., Lyberatos, G., Svoronos, S.A., 1990. Effect of temperature and ph on the effective maximum specific growth rate of nitrifying bacteria. Water Res. 24 (1), 97–101.

Arnaldos, M., Amerlinck, Y., Rehman, U., Maere, T., Van Hoey, S., Naessens, W., Nopens, I., 2015. From the affinity constant to the half-saturation index: Understanding conventional modeling concepts in novel wastewater treatment processes. Water Res. 70, 458–470.

Bengtsson, S., Karlsson, A., Alexandersson, T., Quadri, L., Hjort, M., Johansson, P., Morgan-Sagastume, F., Anterrieu, S., Arcos-Hernandez, M., Karabegovic, L., Magnusson, P., Werker, A., 2017a. A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale. New Biotechnol. 35, 42–53.

Bengtsson, S., Werker, A., Visser, C., Korving, L. (2017b). PHARIO Stepping Stone To A Sustanable Value Chain For PHA Bioplastics Using Municipal Activated Sludge. Technical report, STOWA, Amersfort, the Netherlands.

Beun, J.J., Paletta, F., Van Loosdrecht, M.C., Heijnen, J.J., 2000a. Stoichiometry and kinetics of poly-β-hydroxybutyrate metabolism in aerobic, slow growing, activated sludge cultures. Biotechnol. Bioeng. 67 (4), 379–389.

Beun, J.J., Verhoef, E.V., Van Loosdrecht, M.C.M., Heijnen, J.J., 2000b. Stoichiometry and kinetics of poly-β-hydroxybutyrate metabolism under denitrifying conditions in activated sludge cultures. Biotechnol. Bioeng. 68 (5), 496–507.

Capson-Tojo, G., Rouez, M., Crest, M., Steyer, J.P., Delgenès, J.P., Escudié, R., 2016. Food waste valorization via anaerobic processes: a review. Rev. Environ. Sci. Biotechnol. 15 (3), 499–547.

Chan, C.M., Johansson, P., Magnusson, P., Vandi, L.J., Arcos-Hernandez, M., Halley, P., Laycock, B., Pratt, S., Werker, A., 2017. Mixed culture polyhydroxyalkanoate-rich biomass assessment and quality control using thermogravimetric measurement methods. Polym. Degrad. Stab. 144, 110–120.

Chu, K.H., Van Veldhuizen, H.M., Van Loosdrecht, M.C., 2003. Respirometric measurement of kinetic parameters: Effect of activated sludge floc size. Water Sci. Technol. 48 (8), 61–68.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1999. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC.

Conca, V., da Ros, C., Valentino, F., Eusebi, A.L., Frison, N., Fatone, F., 2020. Long-term validation of polyhydroxyalkanoates production potential from the sidestream of municipal wastewater treatment plant at pilot scale. Chem. Eng. J. 390 (March), 124627.

Estévez-Alonso, Á., Pei, R., van Loosdrecht, M.C., Kleerebezem, R., Werker, A., 2021a. Scaling-up microbial community-based polyhydroxyalkanoate production: status and challenges. Bioresour. Technol. 327, 124790.

Estévez-Alonso, Á., van Loosdrecht, M.C., Kleerebezem, R., Werker, A., 2021b. Data for Simultaneous nitrification and denitrification in microbial community-based polyhydroxyalkanoate production. Mendeley Data v1. https://doi.org/10.17632/gnnkdpg3rp.1.

Fra-Vázquez, A., Santorio, S., Palmeiro-Sánchez, T., Val del Río, Á., Mosquera-Corral, A., 2019. PHA accumulation of a mixed microbial culture co-exists with ammonia partial nitritation. Chem. Eng. J. 360 (July), 1255–1261.

Iacopozzi, I., Innocenti, V., Marsililibelli, S., and Giusti, E. (2007). A modified Activated Sludge Model No. 3 (ASM3) with two-step nitrification-denitrification. Environmental Modelling & Software, 22(6), 847–861.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. J. Biotechnol. 147 (2), 73–79.

Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S., van Loosdrecht, M.C., 2009. Nitrous oxide emission during wastewater treatment. Water Res. 43 (17), 4093–4103.
Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H. N., Reis, M.A., 2017. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 4 (2), 55.
Kuba, T., Murnleitner, E., Van Loosdrecht, M.C., Heijnen, J.J., 1996. A metabolic model for biological phosphorus removal by denitrifying organisms. Biotechnol. Bioeng. 52 (6), 685–695.
Li, M., Wilkins, M.R., 2020. Recent advances in polyhydroxyalkanoate production: Feedstocks, strains and process developments. Int. J. Biol. Macromol. 156, 691–703.
Liu, B., Terashima, M., Quan, N.T., Ha, N.T., Van Chieu, L., Goel, R., Yasui, H., 2018. Determination of optimal dose of allylthiourea (ATU) for the batch respirometric test of activated sludge. Water Sci. Technol. 77 (12), 2876–2885.
Manser, R., Gujer, W., Siegrist, H., 2005. Consequences of mass transfer effects on the kinetics of nitrifiers. Water Res. 39 (19), 4633–4642.
Morgan-Sagastume, F., Bengtsson, S., De Grazia, G., Alexandersson, T., Quadri, L., Johansson, P., Magnusson, P., Werker, A., 2020. Mixed-culture polyhydroxyalkanoate (PHA) production integrated into a food-industry effluent biological treatment: a pilot-scale evaluation. J. Environ. Chem. Eng. 8 (6), 104469.
Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., Karabegovic, L., Alexandersson, T., Johansson, P., Karlsson, A., Bengtsson, S., Arcos-Hernández, M.V., Magnusson, P., Werker, A., 2015. Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. Bioresour. Technol. 181, 78–89.
Mulder, J.W., Van Loosdrecht, M.C., Hellinga, C., Van Kempen, R., 2001. Full-scale application of the SHARON process for treatment of rejection water of digested sludge dewatering. Water Sci. Technol. 43 (11), 127–134.
Pérez, J., Picioreanu, C., Van Loosdrecht, M., 2005. Modeling biofilm and floc diffusion processes based on analytical solution of reaction-diffusion equations. Water Res. 39 (7), 1311–1323.
Philip, S., Keshavarz, T., Roy, I., 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. J. Chem. Technol. Biotechnol. 82 (3), 233–247.
Picioreanu, C., Pérez, J., van Loosdrecht, M.C., 2016. Impact of cell cluster size on apparent half-saturation coefficients for oxygen in nitrifying sludge and biofilms. Water Res. 106, 371–382.
Pochana, K., Keller, J., 1999. Study of factors affecting simultaneous nitrification and denitrification (SND). Water Sci. Technol. 39 (6), 61–68.
Pollice, A., Tandoi, V., Lestingi, C., 2002. Influence of aeration and sludge retention time on ammonium oxidation to nitrite and nitrate. Water Res. 36 (10), 2541–2546.
Reddy, M., Mohan, S., 2012. Influence of aerobic and anoxic microenvironments on polyhydroxyalkanoates (pha) production from food waste and acidogenic effluents using aerobic consortia. Bioresour. Technol. 103 (1), 313–321.
Rodriguez Perez, S., Serrano, A., Pantión, A.A., Alonso Fariñas, B., 2018. Challenges of scaling-up PHA production from waste streams. A review. Journal of Environmental Management 205, 215–230.
Roels, J.A., 1980. Application of macroscopic principles to microbial metabolism. Biotechnol. Bioeng. 22 (12), 2457–2514.
Sabapathy, P.C., Devaraj, S., Meixner, K., Anburajan, P., Kathirvel, P., Ravikumar, Y., Zabed, H.M., Qi, X., 2020. Recent developments in Polyhydroxyalkanoates (PHAs) production - A review. Bioresour. Technol. 306 (March), 123132.
Tamis, J., Marang, L., Jiang, Y., van Loosdrecht, M.C., Kleerebezem, R., 2014. Modeling PHA-producing microbial enrichment cultures-towards a generalized model with predictive power. New Biotechnol. 31 (4), 324–334.
Third, K.A., Burnett, N., Cord-Ruwisch, R., 2003a. Simultaneous nitrification and denitrification using stored substrate (PHB) as the electron donor in an SBR. Biotechnol. Bioeng. 83 (6), 706–720.
Third, K.A., Newland, M., Cord-Ruwisch, R., 2003b. The effect of dissolved oxygen on PHB accumulation in activated sludge cultures. Biotechnol. Bioeng. 82 (2), 238–250.
Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. Water Res. 77, 49–63.
Van Loosdrecht, M.C., Pot, M.A., Heijnen, J.J., 1997. Importance of bacterial storage polymers in bioprocesses. Water Sci. Technol. 35 (1), 41–47.
Wang, X., Bengtsson, S., Oehmen, A., Carvalho, G., Werker, A., and Reis, M.A. (2019). Application of dissolved oxygen (DO) level control for polyhydroxyalkanoate (PHA) accumulation with concurrent nitrification in surplus municipal activated sludge. New Biotechnology, 50(September 2018):37–43.
Wiesmann, U., 1994. Biological nitrogen removal from wastewater. In: Advances in biochemical engineering/biotechnology, volume 51. Springer, Berlin, Heidelberg, pp. 113–154.
Wilfert, P., Kumar, P.S., Korving, L., Witkamp, G.J., Van Loosdrecht, M.C., 2015. The Relevance of Phosphorus and Iron Chemistry to the Recovery of Phosphorus from Wastewater: A Review. Environ. Sci. Technol. 49 (16), 9400–9414.