

# Feasibility Study of Applying Anaerobic Step-Feeding Mode for the Treatment of High-Strength Wastewater in Granular Sequencing Batch Reactors (GSBRs)

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Keywords: aerobic granular sludge, biological nutrient removal, high-strength organic wastewater, granular size, step-feeding, sequencing batch reactors

## Abstract:

This study investigated the feasibility of applying an anaerobic step-feeding strategy to enhance the performance of granular sequencing batch reactors (GSBRs) in terms of operational stability of the cultivated mature granules and nutrient removal efficiencies. Two identical 5 L reactors were operated with a total cycle time of 8 h. GSBRs were operated with high-strength synthetic wastewater (COD =  $1250 \pm 43$ , ammonium (NH<sub>4</sub>-N) =  $115.2 \pm 4.6$ , and orthophosphate (PO<sub>4</sub>-P) =  $17.02 \pm 0.9$  mg/L) for 360 days through three stages: (1) Cultivation, 125 days (>2.1 mm); (2) Maturation, 175 days (>3 mm); (3) alternate feed loading strategy for R2 only for 60 days (anaerobic step-feeding). The granulation process, the physical properties of the granules, the nutrients, and the substrate removal performance were recorded during the entire operational period. For the cultivation and maturation stages, both reactors followed the fast single feeding mode followed by anaerobic mixing, and the results indicated a strong correlation between R1 and R2 due to the same working conditions. During the cultivation stage, adopting high organic loading rate (OLR) at the reactor start-up did not accelerate the formation of granules. Removal efficiency of PO<sub>4</sub>-P was less than 76% during the maturation period, while it exceeded 90% for COD, and was higher than 80% for NH<sub>4</sub>-N without effect of nitrite or nitrate accumulations due to simultaneous nitrification?denitrification. After changing filling mode for R2 only, there was unexpected deterioration in the performance and a rapid disintegration of the matured granules (poor settleability) accompanied by poor effluent quality due to high content of suspended solids because of applying selection pressure of short settling time. Consequently, GSBRs operation under the effect of fast single feeding mode followed by anaerobic mixing favors stable long-term granule stability.

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## Article

# Feasibility Study of Applying Anaerobic Step-Feeding Mode for the Treatment of High-Strength Wastewater in Granular Sequencing Batch Reactors (GSBRs)

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## Highlights:

- Granules' EPS content decreased with the disintegration of AGS post anaerobic step-feeding.
- Matured granules stability and reactor performance favored the fast single feeding mode followed by anaerobic mixing than anaerobic step-feeding strategy.
- Granules' EPS protein-to-carbohydrate ratio did not have a significant effect on granulation process either in maturation stage or after applying anaerobic step-feeding strategy.

**Abstract:** This study investigated the feasibility of applying an anaerobic step-feeding strategy to enhance the performance of granular sequencing batch reactors (GSBRs) in terms of operational stability of the cultivated mature granules and nutrient removal efficiencies. Two identical 5 L reactors were operated with a total cycle time of 8 h. GSBRs were operated with high-strength synthetic wastewater (COD = 1250 ± 43, ammonium (NH<sub>4</sub>-N) = 115.2 ± 4.6, and orthophosphate (PO<sub>4</sub>-P) = 17.02 ± 0.9 mg/L) for 360 days through three stages: (1) Cultivation, 125 days (>2.1 mm); (2) Maturation, 175 days (>3 mm); (3) alternate feed loading strategy for R2 only for 60 days (anaerobic step-feeding). The granulation process, the physical properties of the granules, the nutrients, and the substrate removal performance were recorded during the entire operational period. For the cultivation and maturation stages, both reactors followed the fast single feeding mode followed by anaerobic mixing, and the results indicated a strong correlation between R1 and R2 due to the same working conditions. During the cultivation stage, adopting high organic loading rate (OLR) at the reactor start-up did not accelerate the formation of granules. Removal efficiency of PO<sub>4</sub>-P was less than 76% during the maturation period, while it exceeded 90% for COD, and was higher than 80% for NH<sub>4</sub>-N without effect of nitrite or nitrate accumulations due to simultaneous nitrification-denitrification. After changing filling mode for R2 only, there was unexpected deterioration in the performance and a rapid disintegration of the matured granules (poor settleability) accompanied by poor effluent quality due to high content of suspended solids because of applying selection pressure of short settling time. Consequently, GSBRs operation under the effect of fast single feeding mode followed by anaerobic mixing favors stable long-term granule stability.

**Keywords:** aerobic granular sludge; biological nutrient removal; high-strength organic wastewater; granular size; step-feeding; sequencing batch reactors



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## 1. Introduction

Recently, aerobic granular sludge (AGS) has gained popularity among researchers due to its superior properties as compared to conventional activated sludge (CAS). AGS is an economically and environmentally promising replacement for CAS, for purification of different types of wastewaters (e.g., municipal and heavily loaded industrial wastewater) [1].

Layered structure of AGS is known for high removal performance of different nutrients via multiple biological processes (e.g., nitrification and denitrification) occurring in a single tank [2]. AGS system has biofilms composed of auto-immobilized cells that are generated during treatment in a sequencing batch reactor (SBR), where the clarifier is eliminated, and a settling phase is included in the reactor schedule. Thus, a return activated sludge line is not applicable, depending on the amount of biomass retained in the reactor after decanting. By switching to aerobic granulation system, daily produced biomass is also decreased leading to reduction in the overall cost of up to 25% and energy requirement up to 30% [3]. Since the inception of AGS in the late 1990s as an innovative technology [3], it has been applied successfully for the treatment of different influents; however, there are some unresolved problems such as granule disintegration, long period of granule formation when using sewage with high organic loads, and nitrite accumulation [4].

The literature review findings show the cultivation of AGS in SBR by investigating the impact of various operational parameters that can control the granulation process (i.e., formation and stability), such as alternating feast and famine conditions, reactor configuration (height-to-diameter ratio, hydraulic retention time (HRT), and exchange ratio), hydrodynamic shear forces, influent characteristics (COD:TN:TP ratio), and influent distribution (influence the morphology and microbial community of AGS), selective wasting (in most GSBRS, settling time is the selection pressure for granulation process), organic loading rates, biomass characteristics, and other environmental factors (e.g., dissolved oxygen, pH, and temperature).

Different studies have reported varying conditions for the development of aerobic granulation process. For example, R.A. Hamza et al. (2018) investigated the long-term stability of AGS treating high organic loads through a semi-pilot scale SBR and found that F/M ratio has played a key role in the formation and stability of granules [5]. At F/M ratios between 0.5 and 1.4 gCOD/gVSS-d, stable granules with excellent settleability were maintained, while fluffy granules were caused when the F/M value exceeded 2.2 gCOD/gVSS-d. Another study demonstrated that utilizing longer HRT caused a reduction in the applied OLR, which was found to be more effective for granulation process by providing longer starvation time [6].

Stable AGS was achieved successfully in a previous study by applying low hydrodynamic shear (0.4 cm/s) [7]. In addition, successful removal of the fast-growing organisms (e.g., ordinary heterotrophic organisms (OHO)) from granules' surface has been achieved by applying higher hydrodynamic shear [8,9].

A lot of challenges are related to an efficient and continuous application of the obtained mature granules in the treatment processes. For example, causes of granule instability have also been studied and linked to a range of factors such as: proliferation of filamentous bacteria at high OLR [8,9], as well as decrease and destruction in the structure of extracellular polymeric substances (EPS), with the decrease in protein-to-carbohydrate ratio (proteins play an essential role in granule stability) [10–12]. EPS in the aerobic granulation process, acts similarly to a 'glue' to induce biofilm formation among the microorganisms, where EPS are synthesized as a derivative of organic compounds, such as proteins, polysaccharides, lipids, glycoproteins, nucleic acids [13,14]. It depends on several factors, including aeration time, cycle duration, superficial air velocity, reactor conditions, and type of inoculum sludge that affect the production and composition of EPS.

Feeding mode is one of the key factors in the selection, formation, and stability of AGS. GSBRS are categorized into two types: continuously aerated, and anaerobic influent distribution. Anaerobic filling in GSBRS has been widely accepted, in which the duration and influent load are considered efficiency determining factors. A few previous studies have demonstrated that step-feeding can be an efficient strategy to remove organic matter and nutrients simultaneously, especially with great potential to favor granulation in effluents with high OLRs (e.g., sanitary landfill leachate and high-strength industrial discharges). For example, another previous study has evaluated the effect of filling mode on the performance and granules' operational stability to treat high-strength ammonium

concentrations through three SBRs: R1, fast feeding in the anaerobic period (20 min); R2, slow feeding in the anaerobic period (40 min); and R3, distribution of feeding throughout the cycle [15]. Results of step-feeding in R3 were the best, where the problems such as granules stability, biomass retention, and nitrite accumulation were minimized while treating leachate, as well as higher nutrients removal efficiencies were achieved compared to R1 and R2. In addition, the results from this study showed the positive impact of applying the strategy of step-feeding in R3 compared to R1 and R2, where the approach of step-feeding distributed throughout the cycle of operation was an excellent method to develop the sedimentability of biomass. Moreover, this strategy enhanced the proliferation of slow-growing organisms through the prevention of growing ordinary and fast-growing heterotrophic bacteria [15].

The effect of different feeding strategies on the proliferation dynamics of polyphosphate-accumulating organism (PAOs) and phosphorus removal has also been investigated [16]. Herein, three feeding strategies (e.g., anaerobic slow feeding, pulse feeding followed by anaerobic mixing, and pulse feeding) were applied through GSBRs. The obtained results show that feeding strategies had no impact on organic and ammonia removal and did not alter the microbial community. However, phosphorus removal performance has been significantly influenced. Reactors that have anaerobic slow feeding and pulse feeding followed by anaerobic mixing demonstrated high phosphorus recovery from wasted sludge; therefore, active PAOs can be accumulated, and GAOs can be significantly inhibited within the bioreactor leading to high specific phosphorus uptake and specific phosphorus release rates [16].

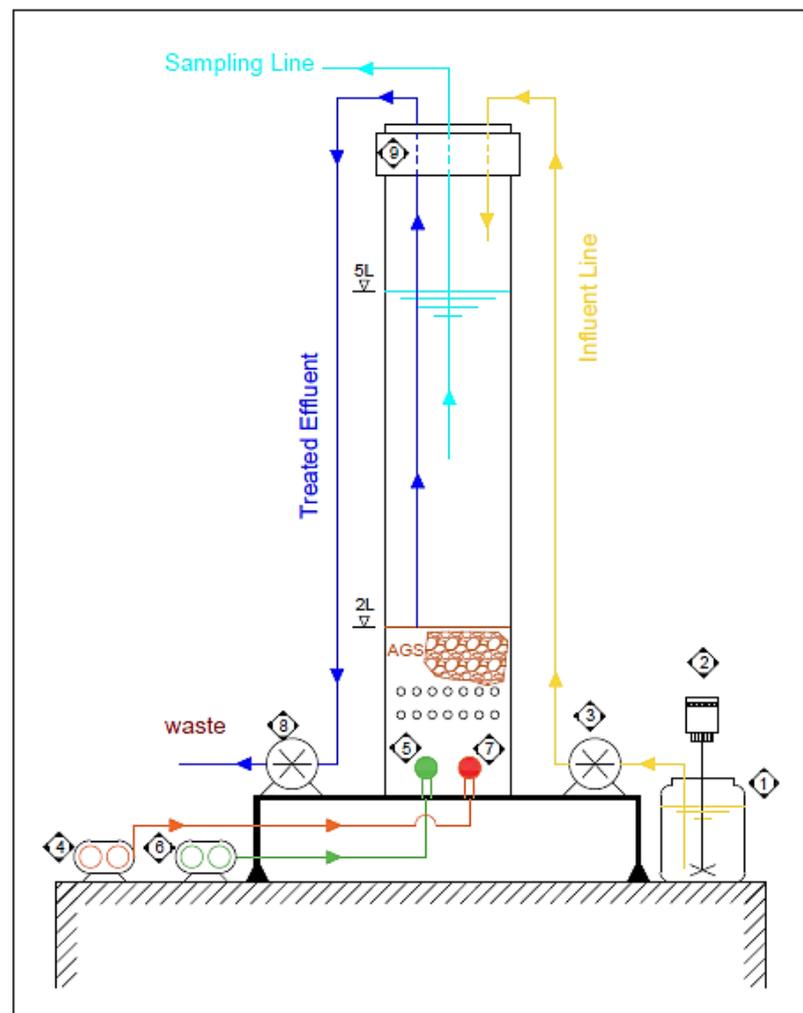
To the best of our knowledge, there is no current information on the effect of applying anaerobic step feeding strategy on matured AGS cultivated and matured under the effect of treatment of high-organic loads in GSBRs systems at the same operational conditions. Thus, the aim of this work is to investigate the potential enhancement in the substrate and nutrients removal performance of large AGS after changing feeding strategy of these GSBRs from fast single feeding mode followed by anaerobic mixing to step-feeding distributed throughout the anaerobic stage, when applied under the same previous operational conditions for treatment of high-strength wastewater.

## 2. Material and Methods

### 2.1. Experimental Set-Up

The experiments were conducted through two SBRs named R1 and R2 (cylindrical acrylic reactors) seeded with the same biomass (GSBRs: R1 and R2 in operation are shown in Figure S1—Supplementary File). The reactors had a 6.0 L capacity, with working volume of 5.0 L, internal diameter of 12 cm, and height-to-diameter ratio of 3.7. Volume exchange ratio of 60% was ensured through discharging the top 3 L of supernatant in 10 min following a 10 min of settling period. Reactors were operated with 8 h cycles daily including 10 min feeding (fast filling), 95 min of anaerobic reaction, 350 min of aeration phase, and 20 min of settling and discharging, as well as 5 min of an idle stage before starting a new cycle. Pumping influents and discharging effluents was performed by peristaltic pumps. Treatment cycles were automated using outlet timers (Traceable Digital Outlet Controller, Fisher Scientific, Waltham, MA, USA).

Nitrogen gas was used during the anaerobic phase and fed from the bottom of reactors to create anoxic environment (dissolved oxygen (DO) levels were less than 0.1 mg/L during the total period of anaerobic mixing), and to avoid biomass sedimentation since complete mixing using nitrogen gas enhances the contact between cells and nutrients and improves removal efficiency. Moreover, during the aerobic phase, fine air bubbles were introduced from the bottom of the reactors (DO levels were between 4–5 mg/L). Both nitrogen gas and air were introduced at the same level with a flow rate of 2.92 L/min resulting in superficial air velocity of 0.43 cm/s to induce hydrodynamic shear to achieve compact granules with desirable surface properties [9–12]. The following schematic diagram (Figure 1) describes the GSBRs used in this study.



**Figure 1.** Schematic of the GSRs in this study; (1) feeding tank; (2) mixer; (3) feeding pump; (4) nitrogen pump; (5) nitrogen diffuser; (6) air pump; (7) air diffuser; (8) decant pump; and (9) reactor's cap.

The total experiment duration for reactors was 360 days to treat high-strength synthetic wastewater (Table 1) under the same operational conditions: (a) 125 days for stage I (Cultivation stage: formation of AGS) after inoculation with the same sludge for R1 and R2, (b) 175 days for stage II (Maturation stage: operation under the same conditions), and (c) 60 days for stage III (R1 continued working under the same operational conditions of stage II, while R2 operated through new anaerobic step-feeding strategy for 60 days). Acclimation period for the first one was two weeks of adaptation after inoculation, while the second period was 10 days after changing filling mode of R2.

The feeding mode of R2 only was changed from fast single feeding mode followed by anaerobic mixing to anaerobic step-feeding strategy, while R1 operated with the same previous filling mode. As mentioned previously, the discharge is 3 L/cycle, and anaerobic stage is performed in 95 min for each cycle. Therefore, a liter is pumped to R2 at the beginning, and another liter is added after 30 min of anaerobic mixing, and the third liter is added after another 30 min. It is important to mention that 10 days after changing the filling mode of R2 was taken as an adaptation period before performing the required measurements of different parameters during this stage.

**Table 1.** Operation stages for R1 and R2.

	Period (Days) (Start and End Days)	Conditions of GSBRs
Stage I Cultivation	125 days Day 1 to 125	<ul style="list-style-type: none"> <li>- Two weeks for acclimation of microorganisms after inoculation</li> <li>- 75 days as startup period for R1 and R2</li> <li>- Cultivation of large AGS were achieved by the day 125 (size <math>\geq 2</math> mm)</li> <li>- Observation of physical properties (size, sludge volume indexes (SVI<sub>5</sub> and SVI<sub>30</sub>)), and performance (nutrients removal).</li> </ul>
Stage II Maturation	175 days Day 126 to 300	<ul style="list-style-type: none"> <li>- Reactors are operated under the same conditions as stage I</li> <li>- Maturation of the granules were achieved by day 175 (size <math>\geq 3</math> mm)</li> <li>- Observation of physical properties (size, SVI<sub>5</sub>, and SVI<sub>30</sub>), and performance (nutrients removal), as well as monitoring stability of matured granules.</li> </ul>
Stage III Operation under Step-feeding	60 days Day 301 to 360	<ul style="list-style-type: none"> <li>- R2 only changed to anaerobic step-feeding, then 10 days for adaptation</li> <li>- R1 has been operated under the same conditions of stages I and II</li> <li>- Observation of physical properties (size, SVI<sub>5</sub>, and SVI<sub>30</sub>), and performance (nutrients removal), as well as monitoring stability of matured granules.</li> </ul>

The start-up of GSBRs with respect to both physical properties and substrate/nutrient removal is described above. Furthermore, the settling parameters SVI<sub>30</sub> < 90 mL/g and SVI<sub>30</sub>/SVI<sub>5</sub> ratio > 0.7, the size fraction  $d > 0.5$  mm constituting at least 50% of total suspended solids (TSS), granule appearance based on microscopic images, and stable substrate and nutrient removal are provided in supplementary material.

## 2.2. Sludge Source and Synthetic Wastewater Composition

The same volume (2 L) of return activated sludge (RAS) was used to seed both reactors, which was obtained from the West End Water Pollution Control Centre in Winnipeg, MB, Canada. The mixed liquor suspended solids (MLSS) of the inoculated RAS was measured as 8.1 g/L (average of triplicates samples).

It is important to mention that particulate matter was not used in the substrate of the influent since it has been demonstrated to affect surface properties of granules and would therefore affect the results. During the entire operational period, GSBRs were treating high-strength synthetic wastewater containing an average of  $1250 \pm 43$  mg/L of soluble COD,  $144 \pm 12$  mg/L of TN, and  $48 \pm 7$  mg/L of TP.

The synthetic municipal wastewater feed recipe was based on a previous study [12], which used high-strength synthetic wastewater; therefore, the influent synthetic wastewater comprised 1250 mg/L of yeast extract, 33 mg/L of ammonium chloride (NH<sub>4</sub>Cl), and 9 mg/L of dipotassium phosphate (K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O). Other components such as MgSO<sub>4</sub> (5.8 mg/L), CaCl<sub>2</sub> (12 mg/L) and mineral solution (0.3 mg/L) containing 0.15 g/L H<sub>3</sub>BO<sub>3</sub>, 0.03 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.03 g/L KI, 0.12 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.06 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.12 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.15 g/L CoCl<sub>2</sub>·2H<sub>2</sub>O, and 1.5g/L FeCl<sub>3</sub>·6H<sub>2</sub>O were also added. Ethylenediaminetetraacetic acid (EDTA: 3 mg/L) is used to prevent the precipitation of salts, such as calcium (Ca<sub>2</sub>) and orthophosphate (PO<sub>4</sub><sup>-3</sup>-P) present in the media. Sodium bicarbonate (12g NaHCO<sub>3</sub>/g NH<sub>4</sub><sup>+</sup>-N) was added to the influent each cycle during the entire experimental duration to achieve sufficient alkalinity (7.14 g CaCO<sub>3</sub>/g NH<sub>4</sub><sup>+</sup>-N) and subsequently achieve complete nitrification [12].

### 2.3. Analytical Methods

The dissolved oxygen and pH were measured near the center of the GSBRS using a DO probe and a pH probe. The concentrations of MLSS, MLVSS, total suspended solids of the effluent ( $TSS_{eff}$ ), chemical oxygen demand of the influent and effluent ( $sCOD_{inf}$ ,  $sCOD_{eff}$ ) and Sludge Volume Index after 5 and 30 min ( $SVI_5$  and  $SVI_{30}$ ) of settling were measured according to standard methods [17]. Concentrations of phosphorous in form of  $PO_4^{3-}$ -P and nitrogen in forms of ammonium ( $NH_4^+$ -N), nitrite ( $NO_2^-$ -N), and nitrate ( $NO_3^-$ -N) for the influent and effluent were measured via a flow injection analyzer (Lachat Quick Chem 8500, HACH, Loveland, CO, USA). Kinetic analysis was performed during operational cycles through the measurements for nitrogen and phosphorus using filtered samples (soluble nitrogen and phosphorus concentration) through medium porosity Q5 filter paper (Fisher Scientific, Waltham, MA, USA).

Granules size was measured to estimate the development of the granulation process and granules' stability through microscopic images in SteREO Discovery (Axio Vert.A1, ZEISS, Jena, Germany). Moreover, surface morphology is investigated using the microscopic analyses on weekly to bi-weekly basis. In this study, we established that the mature aerobic granulation has been achieved (i.e., start of stage II) only when more than 80% of the biomass in R1 and R2 had a diameter greater than 0.2 mm [18], and both of sludge volume indices ( $SVI_5$  and  $SVI_{30}$ ) are lower than 50 mL/g. It is worth mentioning that the cycle tests were conducted more than three times weekly during the whole period of the experiment (i.e., stage I, II, and III) in order to investigate the performance of GSBRS and understand the mechanisms of SNDPR (simultaneous nitrification, denitrification, and phosphorus removal).

Analysis of the EPS content of the AGS has been performed according to [19]. For the extraction, low frequency ultrasound at 120 W for 5 min in an ice bath (before adding chemicals) extraction was applied for 10 mL of mixed liquor samples. Pre-treatment for the mixed liquor samples using 0.06 mL formaldehyde was conducted prior to storing these samples for 1 h at 4 °C. For the next step, 4 mL of NaOH is added, and stored for 3 h at 4 °C. EPS content was physically extracted at 10,000 rpm for 20 min at the end. Following the extraction, the supernatant for all samples was filtered through 0.2 µm filters to collect soluble fractions. The extracted samples were stored at −20 °C in aliquots until analyses were performed.

### 3. Results and Discussion

Firstly, there was a strong convergence in the results (as shown in Table 2) such as physical properties (i.e., size,  $SVI_5$ , and  $SVI_{30}$ ), and performance (i.e., removal of organics and nutrients) of R1 and R2 over a period of 300 days of operation after inoculation (through cultivation and maturation of AGS until changing the filling mode of R2 on day 301). This was due to both GSBRS operated under the same conditions (e.g., reactors configuration, design parameters, influent characteristics, seeding sludge, etc.).

**Table 2.** Removal efficiencies of  $sCOD$ ,  $NH_4^+$ -N, and  $PO_4^{3-}$ -P in R1 and R2.

Characteristics	Stage I		Stage II		Stage III	
	R1	R2	R1	R2	R1	R2
$sCOD_{inf}$ (mg/L)				1250 ± 43		
$sCOD_{eff}$ (mg/L)	127.7 ± 24	115.4 ± 25	93 ± 14	91 ± 12	76.82 ± 16	90.74 ± 7.5
$sCOD$ removal (%)	89.5 ± 2.2	90.52 ± 1.6	91.66 ± 2.2	91.06 ± 1.4	94.55 ± 2.3	92.56 ± 2.2
$NH_4^+$ - $N_{inf}$ (mg/L)				115.2 ± 4.6		
$NH_4^+$ - $N_{eff}$ (mg/L)	51.5 ± 18	51.4 ± 13	20.43 ± 7.2	18.83 ± 5.7	12.42 ± 3.6	24.27 ± 6.5
$NH_4^+$ -N removal (%)	55.2 ± 2	55.3 ± 7	82.2 ± 6.2	83.6 ± 5	89.2 ± 1.2	79 ± 1.6
$NO_2^+$ - $N_{eff}$ (mg/L)	27.9 ± 25	25.2 ± 22	14.8 ± 8.1	13.3 ± 9.6	0.67 ± 1.0	10.6 ± 1.3
$NO_3^+$ - $N_{eff}$ (mg/L)	1.6 ± 1.8	1.9 ± 2.55	27.2 ± 12.1	25.43 ± 14.6	0.88 ± 1.3	47.2 ± 2.1
$PO_4^{3-}$ - $P_{inf}$ (mg/L)				17.02 ± 0.9		
$PO_4^{3-}$ - $P_{eff}$ (mg/L)	9.68 ± 2.3	9.8 ± 1.7	4.13 ± 0.8	4.3 ± 0.1	1.39 ± 0.13	9.23 ± 1.4
$PO_4^{3-}$ -P removal (%)	42.8 ± 5.9	42 ± 7.2	75.6 ± 5.9	74.5 ± 9.6	92.1 ± 1.2	46.43 ± 2.7

The mean value of the measured pH through the anaerobic phase for both reactors during cultivation period (first 125 days after inoculation) was about 8.9, and it decreased through the aerobic phase to 8.2. During the maturation stage (day 126 to 300), these values were further decreased to 8.3 and 7.9 through the anaerobic and aerobic phases each cycle. It is worth mentioning that DO concentrations have been measured during the entire period of operation for both reactors in order to continuously monitor the aerobic and anaerobic conditions during operational cycles. DO concentrations during anaerobic phase did not exceed 0.1 mg/L, while it ranged between 4–5 mg/L during the aerobic phase. It is important to mention that the experiment was conducted at room temperature ( $18 \pm 2$  °C).

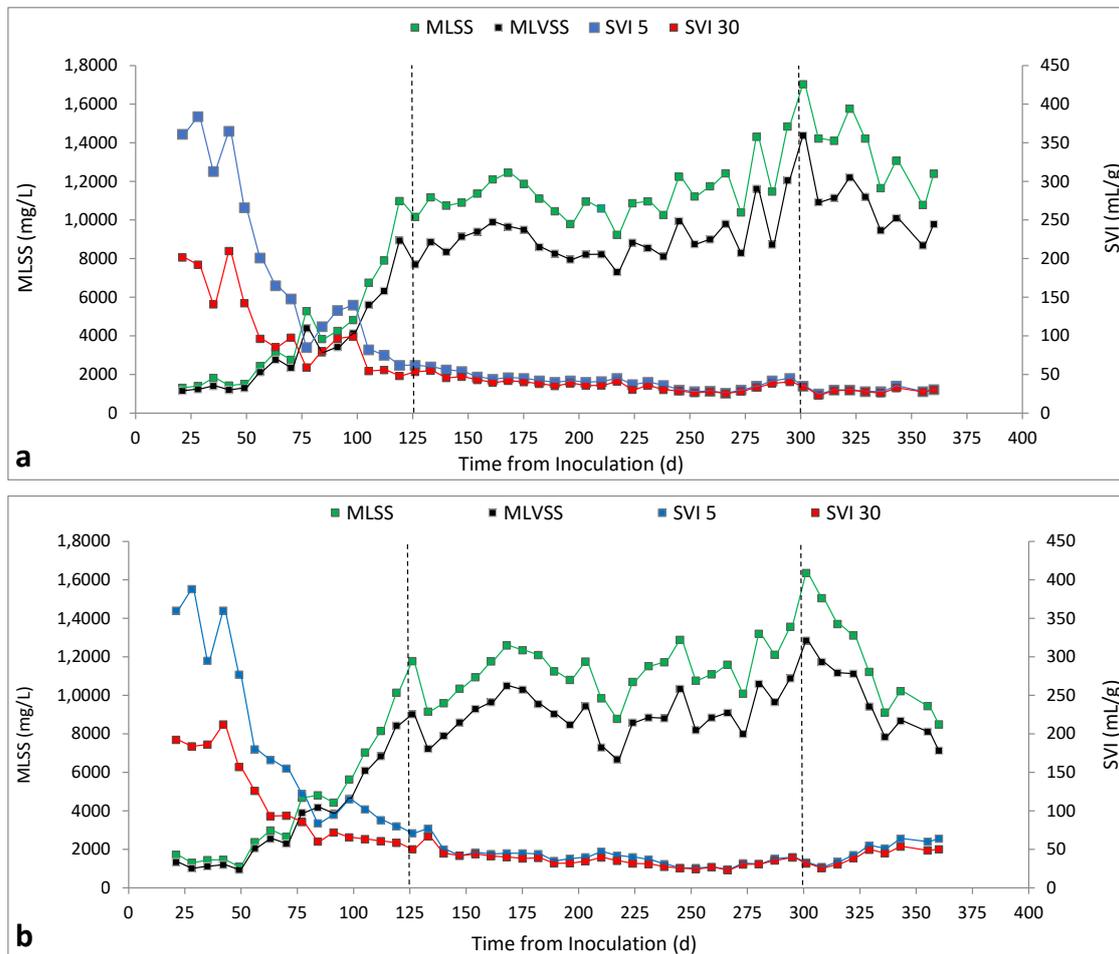
### 3.1. Granulation Processes

The seed sludge was dark brown in colour and had MLSS, mixed liquor volatile suspended solids (MLVSS), and  $SVI_{30}$  of 8100 mg/L, 6955 mg/L, and 128 mL/g, respectively (average of triplicates samples), as well as a mean particle size of 115  $\mu\text{m}$ . The two SBRs were initially operated with the same volume (2 L). During the acclimation period (15 days), the MLSS concentrations were gradually reduced in GSBRS accompanied with higher  $TSS_{\text{eff}}$  ( $974 \pm 122$  mg/L). The reason for this reduction is due to the impact of applying short sedimentation phase (10 min) during the cycle operation, which is considered as the selection pressure and a key strategy for developing aerobic granulation in order to wash out the poor settling biomass. However, after adaptation period and even after biomass stabilization, higher amounts of solids existed in the effluents. In other words, constant washout sludge was observed in R1 and R2. This is consistent with previous studies of AGS systems applied to treat high-strength wastewaters [20,21]. Factually, 75 days were estimated as a startup period for both reactors.

Biomass characteristics (MLSS,  $SVI_5$ , and  $SVI_{30}$ ) for R1 and R2 are presented in Figure 2 and Table 3. As mentioned, MLSS concentrations continued to decrease in both reactors from the first day of operation until day 50. During the same stage, in terms of sedimentability and according to previous studies, various researchers demonstrated that the  $SVI_{30}/SVI_{10}$  or  $SVI_{30}/SVI_5$  ratios can be considered good indicators of granulation, meaning that a value closer to 1.0 indicates that the sludge consists mainly of mature granules [22–25]. It took about 63 days for flocculent sludge ( $SVI_{30}$  more than 125 mL/g and  $SVI_{30}/SVI_5$  ratio lower than 0.4) to convert to small aerobic granules, as shown in Figure 2 ( $SVI_{30}$  is 85 mL/g and  $SVI_{30}/SVI_5$  ratio is 0.52 for R1, while  $SVI_{30}$  is 93 mL/g and  $SVI_{30}/SVI_5$  ratio is 0.56 for R2). Measurements of the size of AGS that were performed at day 50 were exceeded by 0.5 mm for R1 and R2, meeting the requirement for granular sludge. By the end of cultivation stage (day 125), biomass concentrations (MLSS) increased because of the granules' superior settleability and high biomass density and reached 10 g/L and 11.8 g/L for R1 and R2, respectively, which is consistent with previous studies [22]. Moreover, large AGS have been achieved in both GSBRS  $SVI_{30} = 48.2$  mL/g and  $SVI_{30}/SVI_5$  ratio = 0.78 for R1, and  $SVI_{30} = 58.5$  mL/g and  $SVI_{30}/SVI_5$  ratio = 0.73 for R2. While granules' size reached more than 2.22 mm and 2.13 mm for R1 and R2, respectively, by day 125.

The biomass concentrations for R1 and R2 were  $10,164 \pm 111$  mg/L and  $9156 \pm 157$  mg/L, respectively, on day 125 (end of cultivation stage). During the following period (day 126–300), biomass accumulation continued and directly increased biomass concentrations for GSBRS. Table 3 demonstrates the average MLSS concentrations during the whole maturation period. MLSS and MLVSS of both reactors are shown in Figure 3. The rise in biomass concentrations were achieved during the same period on day 294, where the highest MLSS was  $14,844 \pm 78$  mg/L for R1, and  $13,564 \pm 128$  mg/L for R2. This is a direct result from continued biomass accumulation. In this context, there were fluctuations in the  $TSS_{\text{eff}}$  for R1 and R2 which can be attributed to applying shorter settling time (10 min) during the operational cycle (i.e., discharging of poor settling biomass selection pressure). The average concentrations of  $TSS_{\text{eff}}$  for R1 and R2 during cultivation period (1–125 day) were  $180 \pm 18$  mg/L and  $167 \pm 15$  mg/L, respectively (except the first 42 days after inoculation since there were high

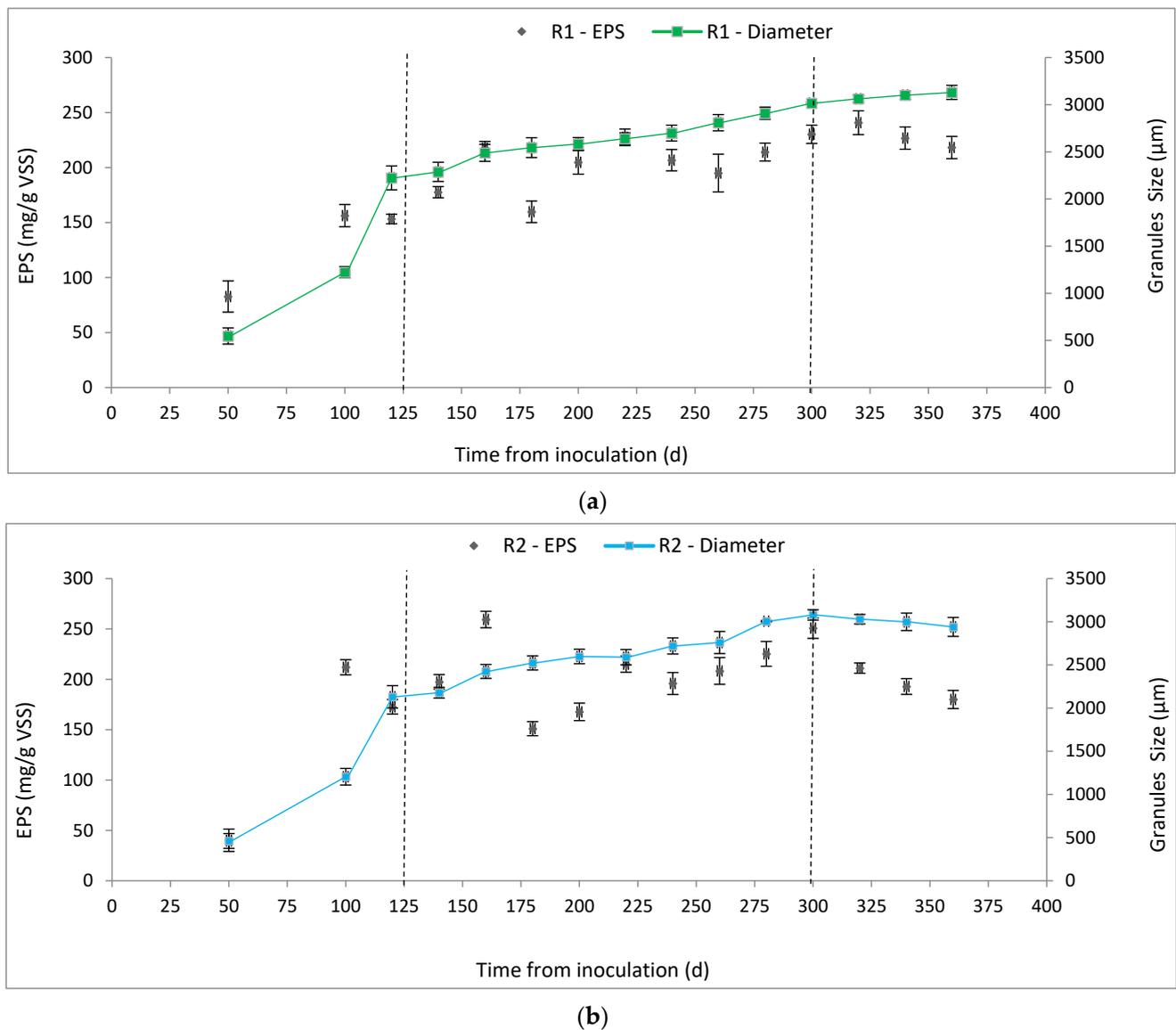
concentrations of  $TSS_{eff}$  and  $VSS_{eff}$  of reactors due to discharging the poor settling flocs). In addition, based on the concentration of  $TSS_{eff}$  and MLSS, the average solid retention time (SRT) was calculated during the cultivation period (day 15–125) of  $18 \pm 1$  day and  $16 \pm 2$  day for R1 and R2, respectively, while average SRT during the maturation stage (day 126–300) was  $28 \pm 2$  day for R1 and R2 (Table 3).



**Figure 2.** Stability and characteristics of AGS over the entire operational period for (a) R1 and (b) R2.

**Table 3.** AGS characteristics (mean values) throughout the operational period for R1 and R2.

Characteristics	Stage I		Stage II		Stage III	
	R1	R2	R1	R2	R1	R2
SVI <sub>5</sub> (mL/g)	62 ± 4.8 at day 120	80 ± 8.8 at day 120	40.85 ± 4.5	39.2 ± 6.2	29.34 ± 1.5	49.91 ± 5.8
SVI <sub>30</sub> (mL/g)	48.2 ± 12.1 at day 120	58.5 ± 10.8 at day 120	36.62 ± 3.8	35.56 ± 2.1	28.27 ± 1	42.77 ± 6
Mean diameter (mm)	2.22 ± 0.13 day 120	2.02 ± 0.2 day 120	3.02 ± 0.01 at day 300	3.08 ± 0.6 at day 300	3.13 ± 0.08 at day 360	2.94 ± 0.11 at day 360
EPS content (mg/gVSS)	130.78 ± 41.6	141.62 ± 90.1	203.7 ± 23.1	207.9 ± 35.3	228.6 ± 11.4	194.6 ± 15.6
F/M ratio (gCOD/gVSS·d)	0.437 ± 0.77	0.398 ± 0.28	0.24 ± 0.1	0.24 ± 0.06	0.196 ± 0.04	0.22 ± 0.07
OLR (Kg COD·m <sup>-3</sup> ·d <sup>-1</sup> )	2.16 ± 0.25					
MLSS (mg/L)	6265 ± 2523	6415 ± 2136	11348 ± 1260	11295 ± 1219	13695 ± 1964	11860 ± 2808
HRT (h)	13.33					
SRT (d)	18 ± 1	16 ± 2	28 ± 2	28 ± 2	29 ± 1	22 ± 1

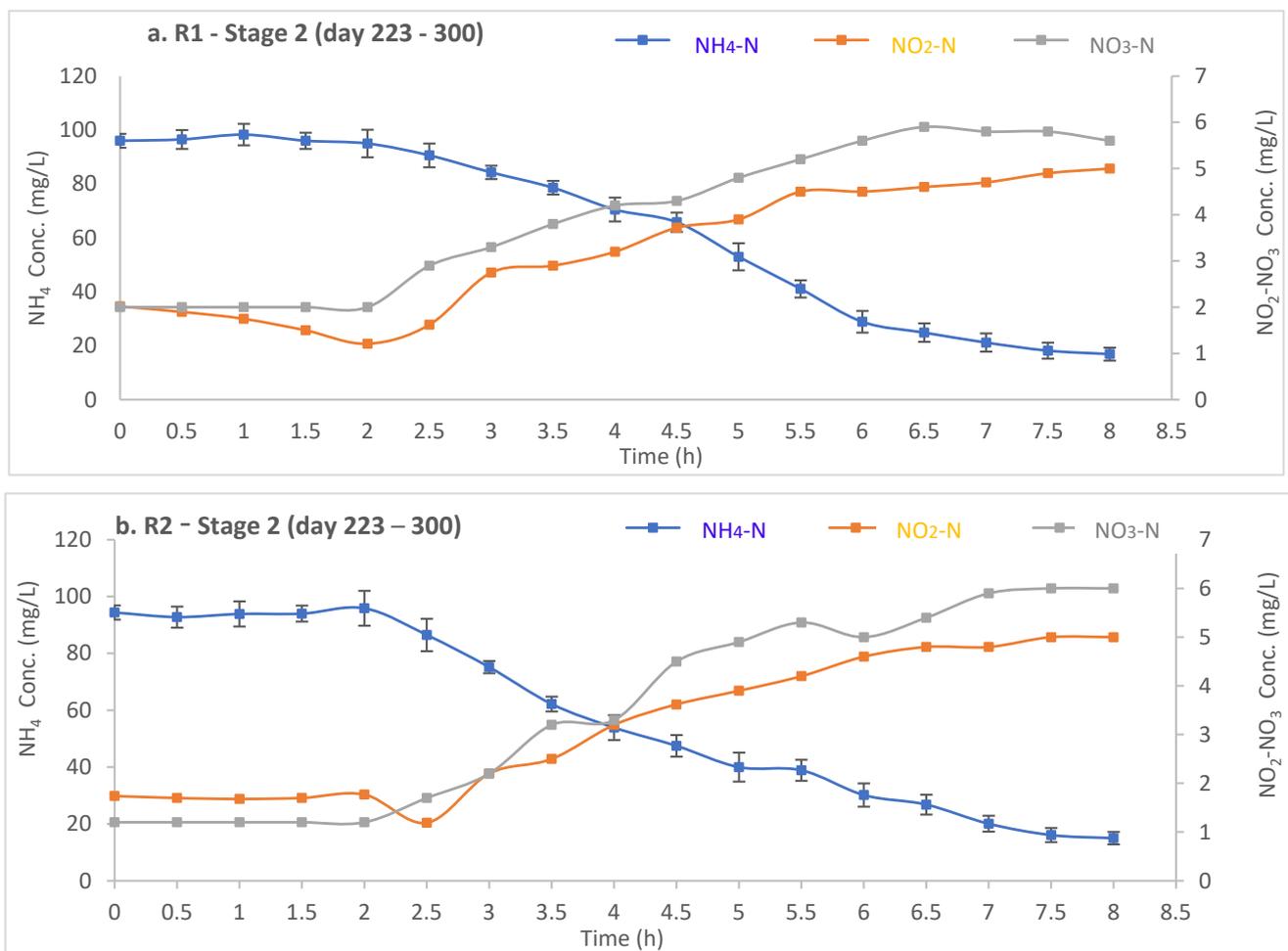


**Figure 3.** EPS measurements (protein + carbohydrate) over the entire operational period (360 days) of (a) R1 and (b) R2.

It has been demonstrated through several studies that higher F/M ratios favor formation and development of the AGS because of the enhanced EPS production [12]. Therefore, it should be noted that through the first stage (cultivation stage—day 125) and under continuous treatment of high organic loads ( $2.25 \text{ Kg COD/m}^3 \cdot \text{d}$ ), the gradual increase in the biomass concentrations (MLSS and MLVSS) might be the reason for the delay in the formation of large granules. F/M ratio decreased from  $1.71 \pm 0.11 \text{ gCOD/gVSS} \cdot \text{d}$  for R1 and  $1.35 \pm 0.18 \text{ gCOD/gVSS} \cdot \text{d}$  for R2 (at the beginning of cultivation period) to  $0.29 \pm 0.1 \text{ gCOD/gVSS} \cdot \text{d}$  for R1 and  $0.19 \pm 0.12 \text{ gCOD/gVSS} \cdot \text{d}$  for R2 (day 125). Consequently, this led to lower production of EPS compared to the following stage (maturation) as shown in Table 3 and Figure 3.

Regarding EPS, these components are biopolymers consisting of polysaccharides, proteins, and other substances, which play a crucial role in the granules' structure, production, and stability. Several studies have shown that stable AGS have higher protein (PN) structures than polysaccharides carbohydrates (PS), and PN/PS ratio is a way of characterizing its stability [14]. PN/PS ratio higher than 25 [12], as well as lower values of 4.8 [26] and 0.6 [27,28] have been reported. The concentrations of EPS are presented in Figure 3. In

the current study, EPS has been estimated as a combination of PN and PS. EPS ranged between  $36 \pm 1.1$  mg/gVSS (PN/PS ratio of 1.11) for R1 and  $31 \pm 0.8$  mg/gVSS (PN/PS ratio of 1.38) for R2 on day 28 and increased to  $153.3 \pm 4.8$  mg/gVSS (PN/PS ratio of 4.3) for R1 and  $172.7 \pm 2.1$  mg/gVSS (PN/PS ratio of 7.2) for R2 on day 120. Thus, the granules' size (Figure 4) increased during the same stage and reached  $2.22 \pm 0.13$  mm for R1 and  $2.13 \pm 0.13$  mm for R2. During the maturation period, there was a strong correlation of the average concentrations of EPS for both reactors. EPS for R1 and R2 were 203.7 mg/gVSS (average PN/PS ratio of 9.17) and 207.9 mg/gVSS (average PN/PS ratio of 9.51). Accordingly, the granules' size (Figure 4) was increased to about  $3.02 \pm 0.09$  mm for R1 and  $3.08 \pm 0.06$  mm for R2, respectively.



**Figure 4.** Performance profile of nitrogen distributed over a typical cycle during stage II by (a) R1 (b) R2.

### 3.2. Performance of the Reactors

There was a strong correlation of the reactors' performance in terms of sCOD, ammonium (NH<sub>4</sub>-N), nitrite (NO<sub>2</sub>-N), nitrate (NO<sub>3</sub>-N), and phosphorus (PO<sub>4</sub>-P) concentrations between R1 and R2 through cultivation and maturation stages (day 1–300). These results were expected because of the similar conditions of operation and same influent characteristics. Moreover, both reactors had good COD removal efficiency, but nitrogen and phosphorus removals were different and improved over time as mature granules were formed.

Both reactors exhibited high COD degradation, during the entire operational period (360 days: before and after changing filling mode for R2). R1 and R2 had mean removal efficiencies of  $91.5 \pm 2\%$  and  $91.2 \pm 1\%$ , respectively, during 360 operation days. This

suggests that there was no effect of the feeding mode on the removal of organics. The results of COD removal efficiencies showed in this study are consistent with previous AGS systems that used both municipal and industrial wastewaters [12,29]. It should be noted that during the first stage (day 1–125: cultivation), R1 and R2 had mean sCOD concentrations in the effluent of  $127.7 \pm 24$  mg/L and  $115.4 \pm 25$  mg/L, respectively. While during maturation period as well as after changing the feeding mode of R2 (day 126–360), the mean sCOD concentrations in the effluent of R1 and R2 were estimated at  $93 \pm 14$  mg/L and  $91 \pm 12$  mg/L, respectively.

### 3.2.1. Ammonium Removal Performance

The average influent concentrations of ammonium were about  $115.2 \pm 4.6$  mg/L (Table 2). During the first 27 days and after two weeks of adaptation post inoculation (day 42), there were higher concentrations of ammonium in the effluents in both reactors. The mean value of  $\text{NH}_4\text{-N}$  removal efficiency was about 30% for GSBs until day 42:  $\text{NH}_4\text{-N}$  concentrations in the effluents of R1 and R2 were found between 73 and 86 mg/L. After this point, an efficient ammonium oxidation was observed with  $\text{NH}_4\text{-N}$  removal efficiency of 51% and 55.9% for R1 and R2 at day 49, respectively. After that, a continuous gradual decrease in the  $\text{NH}_4\text{-N}$  concentrations of the effluents was observed until the end of the cultivation stage, where the mean values of  $70.8 \pm 1.5\%$  and  $69.6 \pm 2.1\%$  for R1 and R2 were found, respectively. Only nitrification ( $\text{NH}_4\text{-N}$  converted to  $\text{NO}_2\text{-N}$ ) was observed, while full-nitrification ([a]  $\text{NH}_4\text{-N}$  oxidized to  $\text{NO}_2\text{-N}$  then [b]  $\text{NO}_2\text{-N}$  oxidized to  $\text{NO}_3\text{-N}$ ) was not observed during this stage. This was indicated by the highest effluent concentrations of  $\text{NO}_2\text{-N}$  accompanied with lowest  $\text{NO}_3\text{-N}$  concentrations. At the end of this stage (day 120), effluent concentrations of  $\text{NO}_2\text{-N}$  were 62.3 mg/L and 58.4 mg/L for R1 and R2, while for  $\text{NO}_3\text{-N}$  concentrations were 4.4 mg/L and 7.3 mg/L for R1 and R2, respectively.

The potential reasons for this behavior might be due to the type of the inoculated sludge that used for both reactors at the beginning of operational period. Moreover, the lower sludge age that estimated for GSBs during the first stage based on the MLSS and  $\text{TSS}_{\text{eff}}$  concentrations have played a direct role in the difficulty of the proliferation of slow-growing organisms (e.g., ammonia oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB)). SRT for R1 and R2 have been estimated by  $18 \pm 1$  and  $16 \pm 2$  days, respectively. In addition, applying higher OLR ( $2.25 \text{ Kg COD/m}^3\cdot\text{d}$ ) might be considered one of the potential reasons caused these low efficiencies towards the  $\text{NH}_4\text{-N}$  removal through increasing the possibility of fast-growing, heterotrophic bacteria growing on granule's surfaces. Besides, applying higher influent  $\text{NH}_4\text{-N}$  concentrations ( $120 \pm 3.4$  mg/L) was one of the reasons of lower performance of ammonium removal. Previous study [12], has demonstrated that an improvement through the nitrification process has been achieved through a reduction in influent  $\text{NH}_4\text{-N}$  from 140 to 80 mg/L. This enhancement in the nitrification process for GSBs has been confirmed through a gradual decrease in the effluent  $\text{NO}_2\text{-N}$  to less than 1 mg/L accompanied with gradual increase in  $\text{NO}_3\text{-N}$  from 35 to 48 mg-N/L.

During the maturation stage (day 126–300),  $\text{NH}_4\text{-N}$  removal efficiencies for R1 and R2 have gradually increased due to accumulation growth of nitrifiers (AOB and NOB) as well as the average SRT during this stage increased and reached  $28 \pm 2$  days for both R1 and R2. The average  $\text{NH}_4\text{-N}$  concentrations in the effluent of R1 and R2 were estimated at  $20.4 \pm 1.4$  mg/L and  $18.8 \pm 2.2$  mg/L, respectively. Basically, the performance of GSBs in  $\text{NH}_4\text{-N}$  removal through maturation period can be discussed considering the following three phases:

- (a) The commencement of full nitrification, and gradual enhancement in ammonium oxidation was observed between days 130 and 178. Reduction in effluent concentrations of  $\text{NH}_4\text{-N}$  from  $24.8 \pm 1.7$  mg/L and  $27.7 \pm 1.6$  mg/L to  $16.7 \pm 2.4$  mg/L and  $15.5 \pm 3.1$  mg/L for R1 and R2, respectively, was estimated, and these reductions were accompanied with the reduction in  $\text{NO}_2\text{-N}$  concentrations with simultaneous increase in  $\text{NO}_3\text{-N}$  concentrations in the effluent. By day 178, effluent concentrations of  $\text{NO}_2\text{-N}$  decreased and reached  $16.7 \pm 3$  mg/L and  $19.1 \pm 1.2$  mg/L for R1 and R2,

- respectively, while effluent concentrations of  $\text{NO}_3\text{-N}$  increased to  $45 \pm 9$  mg/L and  $49.4 \pm 12$  mg/L for R1 and R2, respectively.
- (b) From day 179 to 222, active and efficient full nitrification of the oxidized ammonium is observed in addition to the continuous reduction in effluent concentrations of  $\text{NO}_2\text{-N}$ , which have been decreased to  $7.9 \pm 1.2$  mg/L and  $10.4 \pm 0.3$  mg/L for R1 and R2, respectively. In addition, there was a reduction in the effluent concentrations of  $\text{NO}_3\text{-N}$  during the same period, and these concentrations reached about  $18 \pm 0.7$  mg/L and  $22.4 \pm 1.8$  mg/L for R1 and R2, respectively. It is worth mentioning that the decreasing in nitrate concentrations accompanied to the reduction in nitrite was due to the action of simultaneous nitrification-denitrification (SND), which will provide an important benefit to the GSBRS system by preventing the accumulation of nitrite and nitrate. The potential reasons for this can be characterized by the compact layer structure of the mature granules and the size of these AGS that can extend beyond  $2.6 \pm 0.06$  mm for R1 and R2 (Figure S2c,d—Supplementary File).
- (c) Both reactors exhibited a good tendency to perform SND process for the rest of this stage, between days 223 and 300, (which was confirmed by the results of the kinetic tests as shown in Figure 4a,b) through noticeable reduction in the effluent concentrations of  $\text{NO}_2\text{-N}$  and  $\text{NO}_3\text{-N}$ . However, the effluent concentrations of  $\text{NO}_2\text{-N}$  and  $\text{NO}_3\text{-N}$  for reactors reached more than 6 mg/L and 10 mg/L, respectively. In addition, it should be noted that the removal efficiency of  $\text{NH}_4\text{-N}$  by the end of the maturation stage (day 300) was estimated for R1 and R2 as  $85.5 \pm 1.8\%$  and  $87.1 \pm 1.6\%$ , respectively, ( $\text{NH}_4\text{-N}$  concentrations in the effluents ranged between 15 and 20 mg/L).

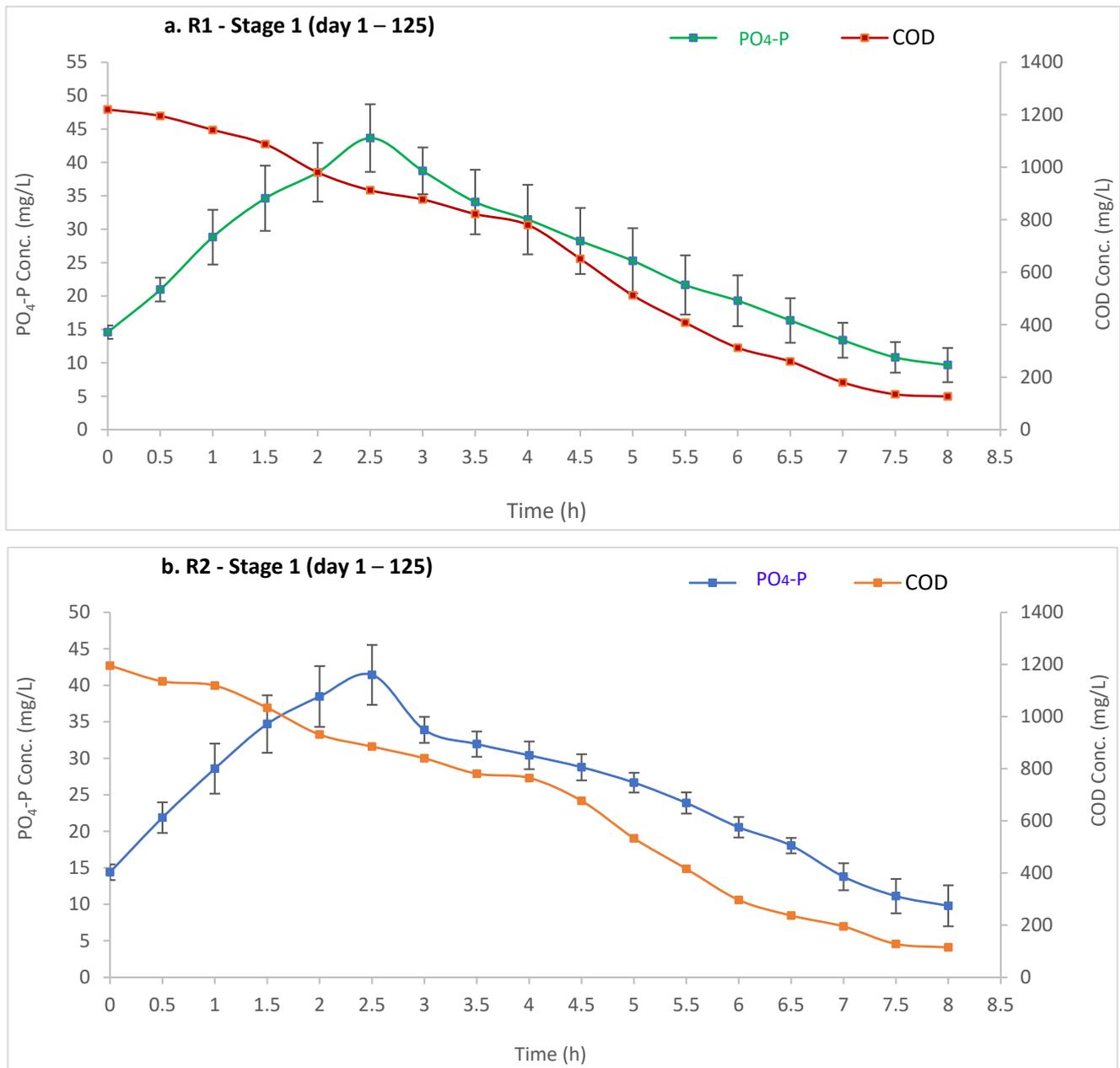
Accordingly, and based on the levels of nitrite and nitrate in the effluent, a new operational stage (stage III) was planned to investigate the possibility of the enhancement of denitrification process for reactors and minimizing both of nitrite and nitrate accumulation. Thus, feeding strategy was changed to anaerobic step-feeding for R2 only to investigate its influence and feasibility on the enhancement of the denitrifiers performance, while R1 was operated under the same conditions during the previous 300 days. In addition, and because of the low efficiency of phosphorus removal for GSBRS (about 75% as shown in the following section), studying the impact of changing filling mode on the activity of PAOs will be investigated. Results for GSBRS are discussed in the following section.

### 3.2.2. Phosphorus Removal Performance

The average influent concentration of  $\text{PO}_4\text{-P}$  was  $17.02 \pm 0.9$  mg/L (Table 2). Until the stage of cultivation (day 125), lower efficiencies of phosphorus removal for GSBRS were observed. The mean phosphorus removal efficiencies for R1 and R2 were  $42.8 \pm 5.9\%$  and  $42 \pm 7.2\%$ , respectively. The potential reasons for these lower efficiencies can be insufficient carbon source ( $\text{COD} = 1250 \pm 43$  mg/L) for PAOs in the influents ( $\text{COD}:\text{TN}:\text{TP}$  ratio is 25:3:1). Thus, there will be a competition of carbon uptake during anaerobic stage (1.5 h) in the operational cycle (8 h) between these PAOs and other denitrifiers. However, higher efficiencies of phosphorus removal have been observed for R1 and R2. For instance, phosphorus removal efficiency for R1 and R2 were estimated at  $75 \pm 1.4\%$  and  $77.2 \pm 0.9\%$  at day 98.

The seed sludge used in the reactors played a role in the enhancement of phosphorus removal after inoculation in GSBRS and especially during the initial days in cultivation stage (i.e., before formation of large granules) because it was RAS from a BNR wastewater treatment plant. Kinetic tests shown in Figure 5a,b demonstrated relatively lower rates of the anaerobic phosphorus release ( $4.4$  mg-P/gVSS and  $4.1$  mg-P/gVSS, for R1 and R2, respectively) and slightly higher aerobic phosphorus uptake ( $6.5$  mg-P/gVSS and  $6.7$  mg-P/gVSS, for R1 and R2, respectively) during the cultivation stage. Phosphorus content in the biomass at the end of an operational cycle was observed at lower concentrations of  $72$  mg-P/gVSS as a mean value during the first 125 days of operation. The lower phosphorus removal in both reactors implies that the layered structure of the formed AGS (with an aerobic outer layer and an anaerobic core) at this stage (size of granules was about 2 mm)

was insufficient to allow anaerobic phosphorus release and efficient phosphorus uptake. Low phosphorus removal was previously reported in GSBRS systems [30]. Comparable results were found by Iorhemen, O.T et al. (2022) [16], where three GSBRS were operated for 84 days to treat an influent with COD:TN:TP ratio of 100:5:1. The results show that the phosphorus removal efficiency was about  $55 \pm 11\%$  for the GSBRS that used pulse feeding without anaerobic mixing.

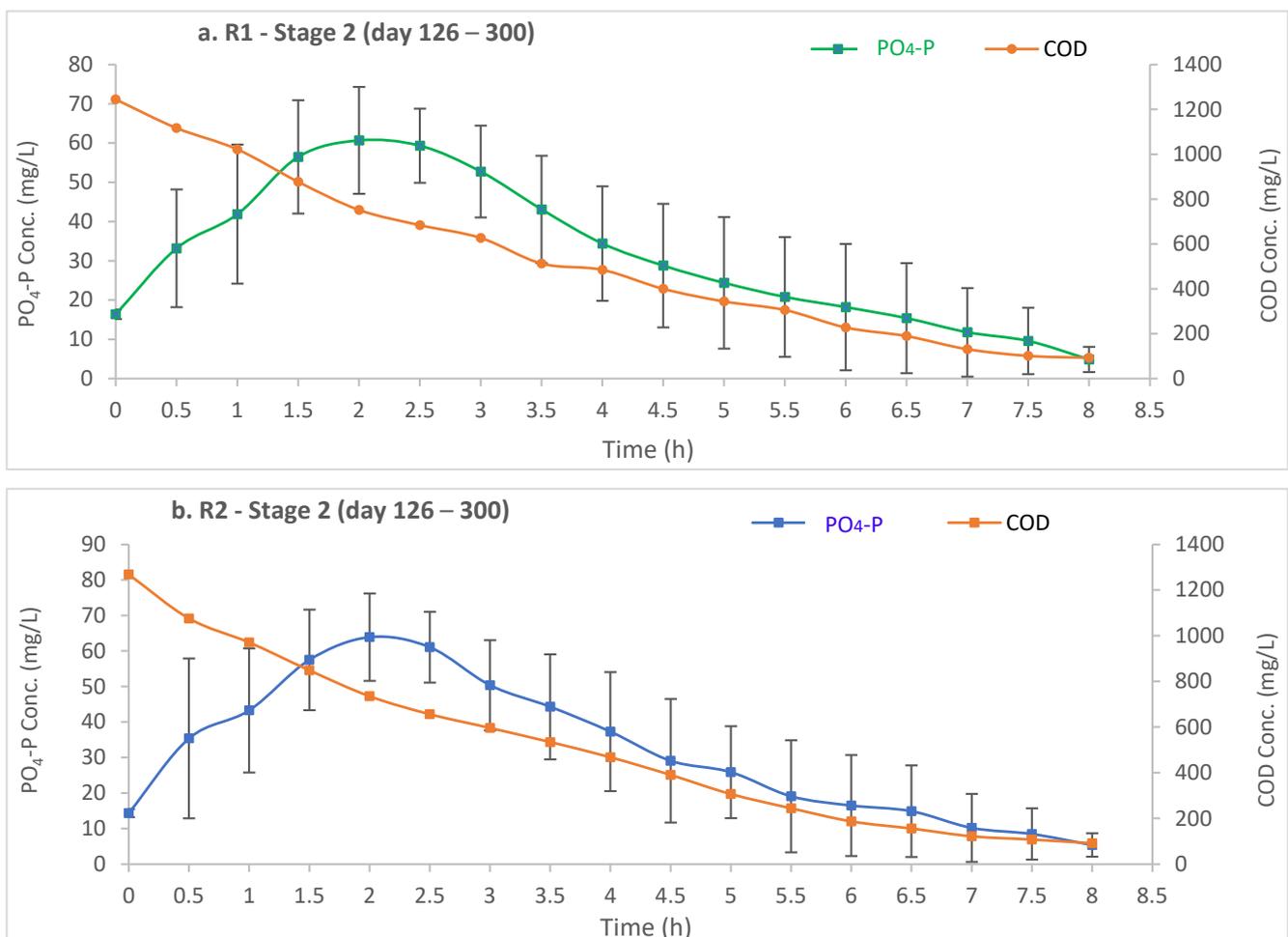


**Figure 5.** Phosphorus and COD removal through a typical cycle during stage I in (a) R1 and (b) R2.

Till day 175 of operation (maturation stage), and under the same influent characteristics (high OLR and COD:TN:TP) and same operational design parameters (time of cycle's stages, HRT, superficial air velocity), relatively higher phosphorus removal efficiencies for R1 and R2 were observed compared to the previous cultivation stage. Variation in the phosphorus removal of R1 and R2 was observed, and the mean phosphorus removal efficiencies for R1 and R2 were  $75.6 \pm 5.9\%$  and  $74.5 \pm 9.6\%$ , respectively. In addition, the variance in the removal efficiency of PO<sub>4</sub>-P concentrations for the GSBRS was observed and

concentrations as high as 7.6 mg/L and 7 mg/L were recorded for R1 and R2, respectively, at the beginning of maturation period (day 140). Moreover, after day 220 until the end of this period (day 300), phosphorus concentrations in the effluents of both reactors were between 2–3 mg/L.

As mentioned previously, the size of the aerobic formed granules in both reactors increased from 2.1 mm (by the end of cultivation stage) to more than 3 mm during this stage. Consequently, the mechanisms for phosphorus removal in these GSBRs systems have improved during this stage due to the impact of enhanced biological phosphorus removal (EBPR), which depends on the enrichment of PAOs in the GSBRs, as well as phosphorus precipitation inside the granular matrix [2,7,31–35]. A slight improvement in PAOs activity has been confirmed during the maturation stage and according to the frequent kinetic analysis (Figures 5 and 6), it was found that influent sCOD concentrations have been degraded during anaerobic phase (95 min) by about 60% for both GSBRs compared to 30% for 125 days of the previous stage (mean values for the initial 175 days of operation). Therefore, this led to higher rates of the anaerobic phosphorus release (6.33 mg-P/gVSS and 6.5 mg-P/gVSS for R1 and R2, respectively) as well as aerobic phosphorus uptake (7.5 mg-P/gVSS and 7.7 mg-P/gVSS for R1 and R2, respectively) during the maturation stage, which was confirmed by the results of the kinetic tests as shown in Figure 6a,b). In addition, the highest phosphorus removal efficiency for reactors was observed, where for R1 it was about  $95.1 \pm 1.5\%$  at day 238, and  $97.6 \pm 0.8\%$  for R2. Additionally, high phosphorus content in the biomass at the end of a completed operational cycle was observed (more than 20–50 mg-P/gVSS, which was reported for ordinary heterotrophic bacteria [36]) and reached about 0.21 g-P/gVSS, on day 234 (Figure 5).



**Figure 6.** Phosphorus and COD removal through a typical cycle during stage II in (a) R1 and (b) R2.

### 3.3. Influence of Anaerobic Step-Feeding

Improving the performance of these GSBs was the primary objective after finishing 300 days of operation under the effect of high-strength wastewater (microscopic images of the matured AGS through these two stages are shown in the supplementary file, Figures S2 and S3). Therefore, a third operational stage of 60 days was proposed to investigate the influence of changing the filling strategy on the reactors' performance. In addition, the microscopic images of the aerobic granules for both reactors during the last stage are shown in Figure S4 (Supplementary File). The feeding mode of R2 was changed from fast single feeding mode followed by anaerobic mixing to anaerobic step-feeding strategy, while R1 has been operated with the same previous filling mode.

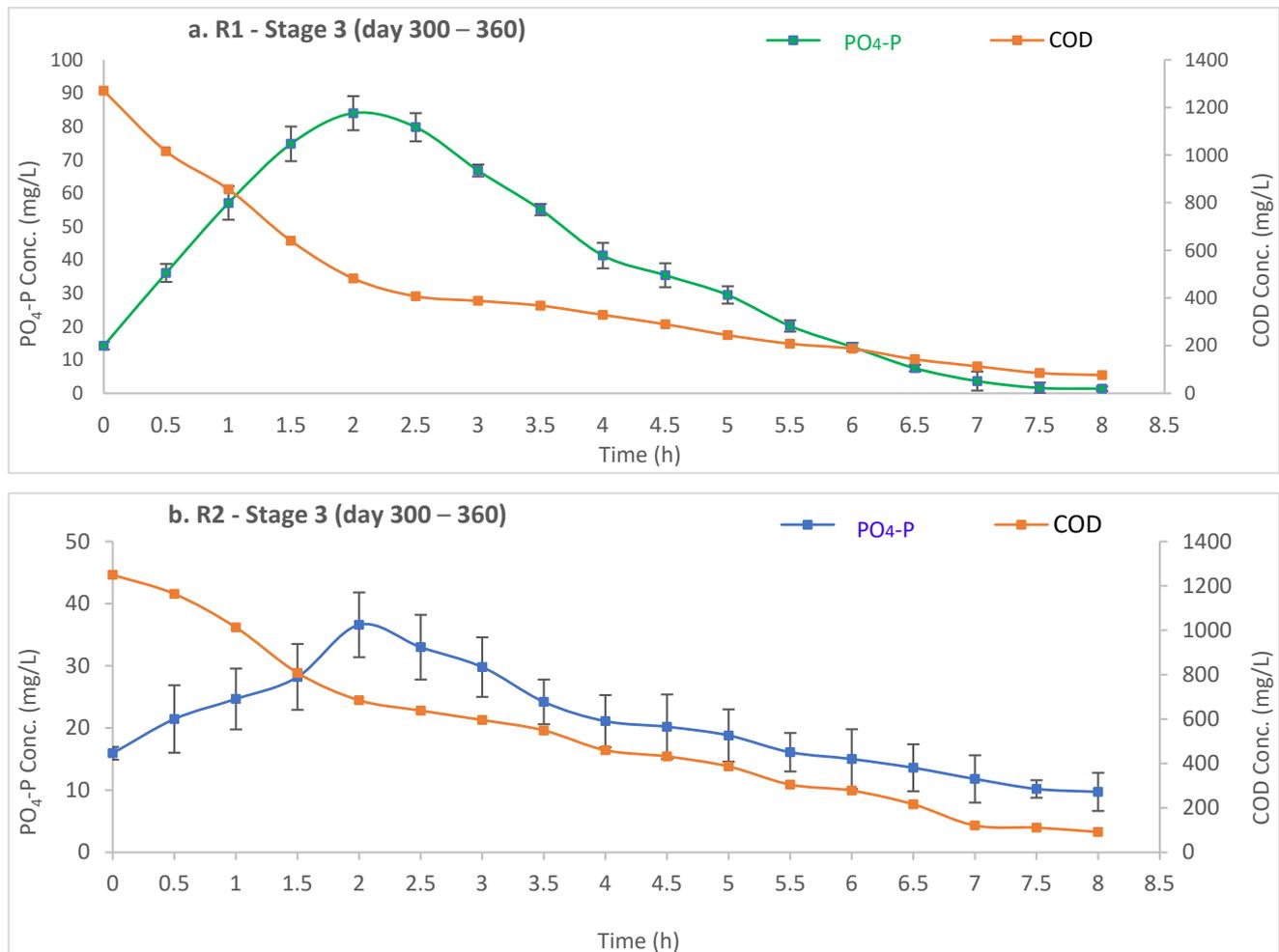
The results demonstrated that changing filling strategy did not influence the removal efficiency of organics. R1 and R2 had similar mean organic removal efficiencies during this stage (60 days) of  $94.55 \pm 1.4\%$ , and  $92.6 \pm 2.3\%$ , respectively. These results are consistent with previous studies of AGS systems that investigated the effect of feeding strategy on the removal efficiencies of organic substrate [15,16].

The measured values of EPS and size of AGS, as demonstrated in Figures 3 and 4, show rapid disintegration of the mature granules in R2. Moreover, at the end of this stage (day 360), high portion of granules had become fluffy and viscous flocs (Figure S6 in the Supplementary File). Consequently, lower physical properties were observed for R2 by the end of the current period (day 360) as shown in Figure 2, where  $SVI_{30}$  increased from 31.8 mL/g (end of maturation stage) to 50.2 mL/g and also  $SVI_{30}/SVI_5$  ratio decreased from 0.96 (end of maturation stage) to 0.77 (Figures S4 and S6 in the Supplementary File). Furthermore, granules' size in the same reactor decreased from  $3.08 \pm 0.06$  mm (end of maturation stage) to  $2.94 \pm 0.11$  mm (Figure S4 in the Supplementary File). This deterioration of the mature AGS in R2 can be considered a direct result for the notable reduction in the EPS concentrations. EPS concentrations decreased from 250.82 mg/L (end of maturation stage) to 180 mg/L by day 360, as well as the reduction in PN/PS was noted during the third stage (e.g.,  $PN/PS = 9.1$  at day 360). In contrast, the performance of R1 as well as the physical properties had the same trend during the initial 175 days of operation during maturation, where the EPS concentrations were higher than those of R2. Thus, larger AGS volume have been achieved in R1, where  $SVI_{30} = 27.5$  mL/g and  $SVI_{30}/SVI_5$  ratio = 0.98, while granules' size became greater than  $3.13 \pm 0.07$  mm (Figure S3 in Supplementary File).

In addition, biomass concentrations in R2 have decreased in the 60 days of operation after changing feeding mode, where MLSS concentrations were about 8502.3 mg/L. It is important to mention that  $TSS_{eff}$  for R2 indicated a deterioration and disintegration of the granules, which was comparable with these concentrations in R1 (Figure S7 in the Supplementary File). The mean value of  $TSS_{eff}$  for R2 increased during this period to 251.6 mg/L, while for R1  $TSS_{eff}$  was much lower than R2 at 85.8 mg/L.

Accordingly, and after the evident deterioration of granules in R2 during the third stage of operation, it was expected that the performance of R2 in terms of phosphorus and ammonium removal will be lower than the performance of R1 (as shown in Figures 7 and 8 that show the kinetic analysis during the third stage). For the performance of R1 in terms of phosphorus removal (Figure 7a), the average  $PO_4$ -P concentrations in the effluent were about  $1.38 \pm 0.5$  mg/L. In contrast to the phosphorus removal of R2, the average  $PO_4$ -P concentrations were about  $9.22 \pm 1.3$  mg/L. Deterioration had occurred in PAOs activity for R2, during the last stage after changing the filling mode, and according to the frequent kinetic analysis (Figure 7b), it was found that influent sCOD concentrations have been degraded during 1.5 h anaerobic phase by about 45% for R2 compared to 60% for the previous stage, while for R1 it was about 65% during the same period (stage III). This indicated that the anaerobic mixing time was not sufficient to provide enough degradation of COD during the same phase (i.e., anaerobic mixing). In other words, COD was not readily oxidized during the anaerobic phase and in the first period of the aeration phase, OHO began to develop, mainly in filaments, at expense of the growth of other slow-growing

organisms (e.g., PAOs, GAOs, nitrifiers, and denitrifiers). Accordingly, granules stability has been deteriorated, and the performance of R2 for the biological nutrient removal has been dropped. Anaerobic phosphorus release and aerobic phosphorus uptake was about 2.4 mg-P/gVSS and 3.2 mg-P/gVSS, respectively, for R2 during maturation stage.

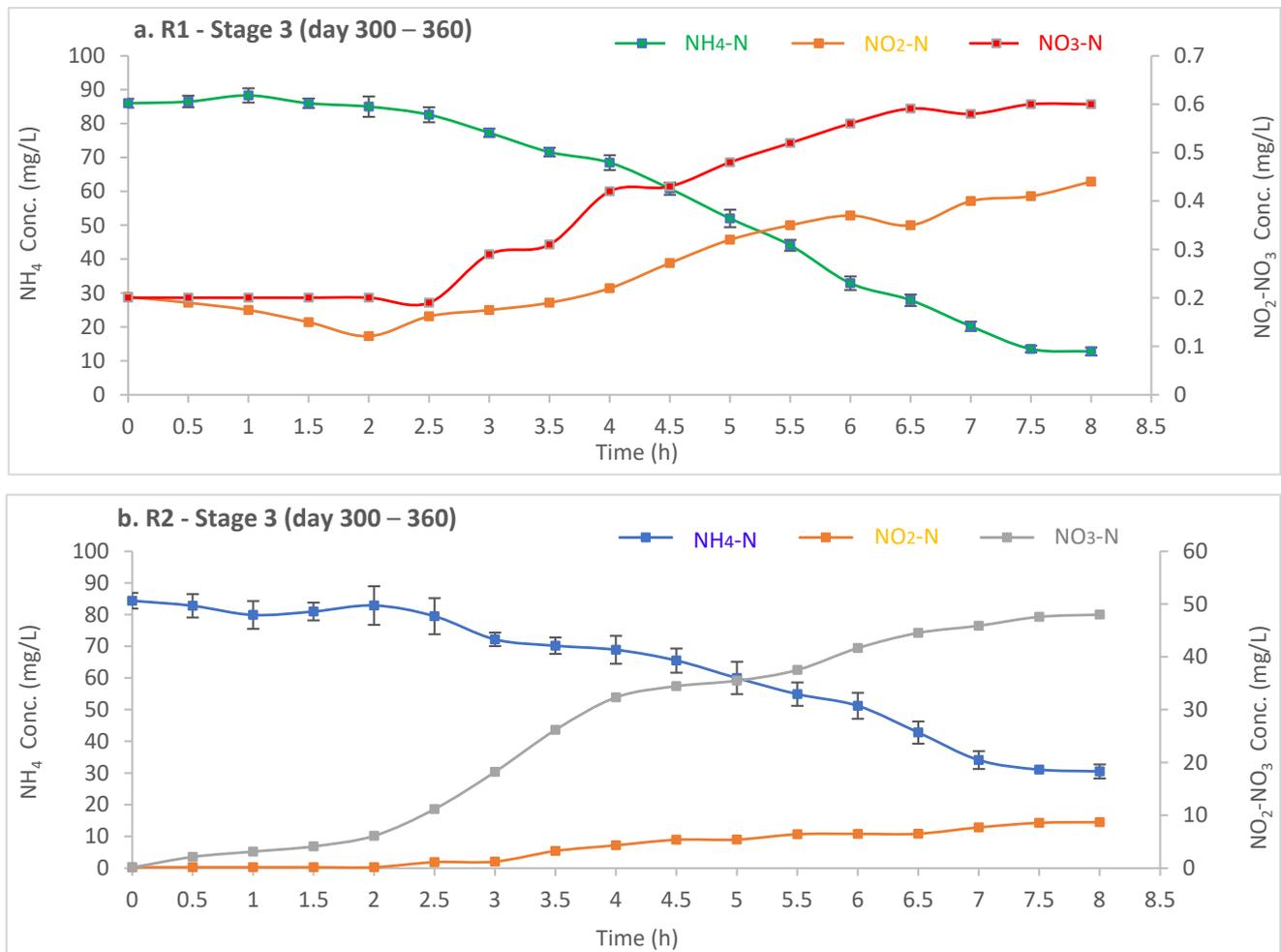


**Figure 7.** Phosphorus and COD removal through a typical cycle during stage III in (a) R1 and (b) R2.

Moreover, performance of ammonium removal in R2 based on kinetic tests is demonstrated in Figure 8b for the entire 60 days of the third stage. Due to the similarity and the disintegration of granules occurring in R2, it was expected that performance of R2 in terms of ammonium removal will be minimized compared to R1. The average  $\text{NH}_4\text{-N}$  concentrations in the effluent of R2 have been estimated at  $24.3 \pm 6.3$  mg/L. In addition, a significant rise in the concentrations of nitrate, as well as slight increase in nitrite concentrations during aerobic phase was noticed, which led to nitrite/nitrate accumulation. Effluent concentrations of nitrate were between 46 and 50 mg/L, while it was 10 mg/L for nitrite. For R1 (Figure 8a), the average  $\text{NH}_4\text{-N}$  concentrations in the effluent were about  $12.41 \pm 3.3$  mg/L.

The granules' disintegration in R2 after changing filling mode and recurrent washouts because of applying short settling time (10 min) had resulted in a loss of nitrifying bacteria (AOB and NOB) as well as some PAOs that were present in the broken granules. In addition, when washouts occur at higher frequencies during these 60 days of operation, the sludge age (SRT) is reduced (mean SRT for R2 was estimated at  $22 \pm 1$  days during this period), therefore the performance of ammonium (i.e., SND) and phosphorus removal was negatively affected due to the reduction in sludge age (compared to SRT for R1 which was about  $29 \pm 1$  days). This further substantiates the key role of the STR in granules'

stabilization and reactors' performance (nutrients removal) since it is directly related to the accumulation and growth of these slow-growing bacteria, which is also confirmed in other studies [14].



**Figure 8.** Profile of nitrogen removal distributed over a typical cycle during stage III in (a) R1 and (b) R2.

Overall, the performance of R1 was continuing in the same trend of enhancement along with physical properties of AGS. However, the slow rate of this improvement of R1 might be due to the high strength of influent wastewater characteristics (high influent concentrations of COD, NH<sub>4</sub>-N and PO<sub>4</sub>-P) and composition (i.e., COD:TN:TP ratio).

#### 4. Conclusions

Aerobic granules were successfully cultivated (>2.1 mm) and matured (>3 mm) through two SBRs under the effect of high-strength wastewater with insufficient influent substrate (COD:TN:TP ratio of 100:11.5:3.5) through 300 days of operation after inoculation. Aerobic granulation for the purposes of high phosphorus and ammonium removal performance was unsuitable with long start-up periods, and high concentrations of nitrite and nitrate have been observed as well. However, formation of large sized matured granules, there was a low performance of P removal for GSBRS, and this can be attributed to the competition between PAOs and denitrifiers for rbCOD uptake during anoxic mixing phase due to the transition from anaerobic zone to anoxic phase. Therefore, applying anaerobic step-feeding was aiming at decreasing the competition between PAOs and other denitrifiers, by enhancement of the activity for these microorganisms during anoxic phase in order to enhancement of the reactors' performance. Unexpectedly, deterioration in physical prop-

erties of AGS has been resulted after changing the filling mode and it was accompanied to a reduction in EPS concentrations, and the protein-to-carbohydrates ratio. Moreover, significant reduction in the phosphorus and ammonium removal efficiencies has been demonstrated, and COD degradation during anaerobic phase has been decreased as well. This implies that the anaerobic mixing duration after applying anaerobic step-feeding was not sufficient, which attributed to lower carbon utilization by PAOs and denitrifiers during the same phase. Therefore, extended anaerobic mixing durations after applying anaerobic step-feeding might provide enhanced activity of PAOs and denitrifiers to ensure higher performance in terms of phosphorus removal and avoid nitrite/nitrate accumulation. Thus, further research is required to identify the influence of this approach on the performance of GSBRS-treated high-strength wastewater with low influent substrate (COD:TN:TP ratio higher than 100:5:1); additionally, further research should be performed to improve SND.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr11010075/s1>, Figure S1: GSBRS (R1 and R2) in operation, Figure S2: (a): Microscopic images of the formed granules in both reactors on day 50 (during of the cultivation stage); (b): Microscopic images of the formed granules in both reactors on day 120 (end of the cultivation stage); (c): Microscopic images of the formed granules in both reactors on day 210 (during the maturation stage); (d): Microscopic images of the formed granules in both reactors on day 300 (end of the maturation stage), Figure S3: Microscopic images of the formed granules in R1 during stage III (day 301 until 360), Figure S4: Microscopic images of the formed granules in R2 after applying anaerobic step-feeding mode (during stage III; day 301 until 360), Figure S5: Measurements of SVI5 and SVI30 for both reactors in day 352 (deterioration of the AGS in R2 after applying anaerobic step-feeding strategy), Figure S6: AGS in R2 rapidly lost its structural integrity, after changing the filling mode to anaerobic step-feeding, resulting in loose and fluffy aggregates, Figure S7: Biomass samples for both reactors in day 342 (stage III, after change feeding mode for R2) showing the disintegration of the AGS in R2 which caused higher content of TSS concentrations in its effluents compared to R1.

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## Abbreviations

AGS, aerobic granular sludge; AOB, ammonia oxidizing bacteria; AS, activated sludge; BNR, Biological nutrient removal; COD, chemical oxygen demand; DO, dissolved oxygen; EBPR, enhanced biological phosphorus removal; EPS, extracellular polymeric substances; GAO, glycogen-accumulating organism; MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; NOB, nitrite oxidizing bacteria; ORP, oxidation reduction potential; PAO, polyphosphate-accumulating

organism; SBR, sequencing batch reactor; SND, simultaneous nitrification-denitrification; SRT, solids retention time; TN, total nitrogen; TP, total phosphorus, TSS, total suspended solids; VSS, volatile suspended solids; WWTP, wastewater treatment plant.

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