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ESCUELA DE INGENIERÍAS INDUSTRIALES

MÁSTER EN INGENIERÍA AMBIENTAL

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UNIVERSIDAD DE VALLADOLID

TRABAJO FIN DE MÁSTER

Wastewater treatment from pulp and paper industry coupled with nitrogen gas fixation using purple non-sulfur bacteria

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Abstract

This study investigated the wastewater treatment efficiency from pulp and paper industry by coupling with nitrogen gas fixation using purple non-sulfur bacteria (PNSB). Wastewater from the pulp and paper industry (PPW) contains limited nitrogen, making the development of an effective treatment process necessary. PNSB have been gaining attention to treating wastewater as they exhibit a high growth rate and ability to simultaneously remove carbon and nutrients. However, nitrogen deficiency in PPW limits PNSB growth. In this study, PPW was treated with PNSB under varying conditions, including different HRTs and nitrogen gas supplementation. Four main stages (A, B, C, and D) were established to evaluate the effects of HRT and nitrogen supply on PNSB growth and treatment efficiency.

Batch culture was performed in stage A, and PNSB biomass reached 0.46 g L^{-1}. During batch culture, TOC, COD, and TN removal efficiencies were 22.2%, 15.2%, and 20.4%, respectively. In stage B, the HRT was set to 4 days, and PNSB biomass did not increase, resulting in low removal efficiencies. Although HRT changed to HRT 8 days in stage C, the biomass concentration remained low due to limited biomass accumulation in stage B and short operation time (4 days), preventing significant increases in both biomass and removal efficiencies.

Finally, nitrogen gas was supplied continuously to confirm the effectiveness of nitrogen gas on growth (stage D). After supplying nitrogen gas, biomass and removal efficiency increased. PNSB biomass reached 0.72 g L^{-1}, and TOC, COD, and TN removal efficiency were 68.6%, 69.5%, and 33.5%, respectively.

Throughout the experiment, dissolved total nitrogen was almost removed from the PPW, but TOC and COD removal efficiencies were low, which indicates carbon to nitrogen ratio was not optimal for PNSB, and carbon was left in PPW. Moreover, protein content in PNSB was 68.9%, 27.4%, 29.2%, and 45.8% in stage A, B, C, and D, respectively. This value was lower than generally reported (> 50% w/w), indicating that the nitrogen content in PPW was insufficient to support PNSB growth.

This study suggests that, when treating PPW with PNSB, HRT should be optimized, and the C/N ratio also should be adjusted to a range that supports optimal growth.

Keywords: Anoxygenic photosynthesis, nitrogen fixation, pulp and paper wastewater, purple non-sulfur bacteria (PNSB), resource recovery

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1. Introduction (background and state of the art)

1.1. Wastewater treatment and conventional treatment technologies

The world has a serious issue related to continuously increasing wastewater from agriculture, paper industry, agro-industry, and domestic due to the advanced industrialization and rapid increase of population (García et al., 2019). Wastewater contains high amounts of organic matter, nutrients, pathogens, and heavy metals, and these characteristics can impact on the environment from two perspectives. One is that abundant carbon, nitrogen, and phosphorus in wastewater can be valuable resources if properly collected. On the other hand, untreated wastewater causes environmental pollution, affects the ecosystem, and reduces available water to use (Lu et al., 2021). Conventionally, physical and chemical wastewater treatment technologies, such as filtration and adsorption, and some biological technologies, like activated sludge and anaerobic digestion, have been used for wastewater treatment (Fig. 1(a)).

Despite their effectiveness in wastewater treatment, they still present certain disadvantages. For example, physical and chemical wastewater treatment technologies require large amounts of materials and chemicals, and they often need the removal of residual compounds such as adsorbents (Crini and Lichtfouse, 2019). Otherwise, in conventional wastewater treatment plants using biological systems, such as activated sludge (Fig. 1(a)), energy cost accounts for about 25-40% of total operational costs due to the need of aeration (Panepinto et al., 2016), Although anaerobic biological technologies, such as anaerobic digestion, do not require aeration and can produce biogas, they may still lead to resource inefficiency as they mainly focus on carbon removal. In addition, these conventional technologies have only focused on the effective removal of organics, nutrients, and pathogens from waste streams. However, there is a growing shift toward a circular and bio-based economy where resource recovery and reuse are prioritized over mere removal of waste components. This approach has been gaining attention in the field of wastewater treatment.

For these reasons, alternative emerging biotechnologies have been proposed (Fig. 1(b), (c)). These technologies require less energy compared to physical and chemical ones, and they aim to recycle resources (C, N, P) into bioenergy and bioresources (Yu et al., 2024; Hülsen et al., 2014). Particularly, phototrophic microorganisms, such as microalgae and purple phototrophic bacteria (PPB), are attractive for wastewater treatment since they can assimilate carbon and nutrients using energy from light and their biomass can be utilized in various industrial and commercial applications, which makes resource recovery and bio-based economy feasible (Winkler and Straka, 2019). Microalgae are phototrophic microorganisms applied to biological wastewater treatment technologies. They grow photoautotrophically through oxygenic photosynthesis, and fix and assimilate carbon source (CO_2) and nutrients (N, P). Microalgae can be cultivated in wastewater; however, a co-culture of microalgae and bacteria is needed when treating organic-rich wastewaters, as organic compounds are mainly degraded by bacteria, not microalgae (Capson-Tojo et al., 2020). Microalgae are more proficient in recovering nutrients than bacteria, therefore, microalgae are applied for the tertiary step in wastewater treatment focusing on further nutrient removal, especially nitrogen, to make full use of the potential of microalgae to assimilate nutrients (Posadas et al., 2015).

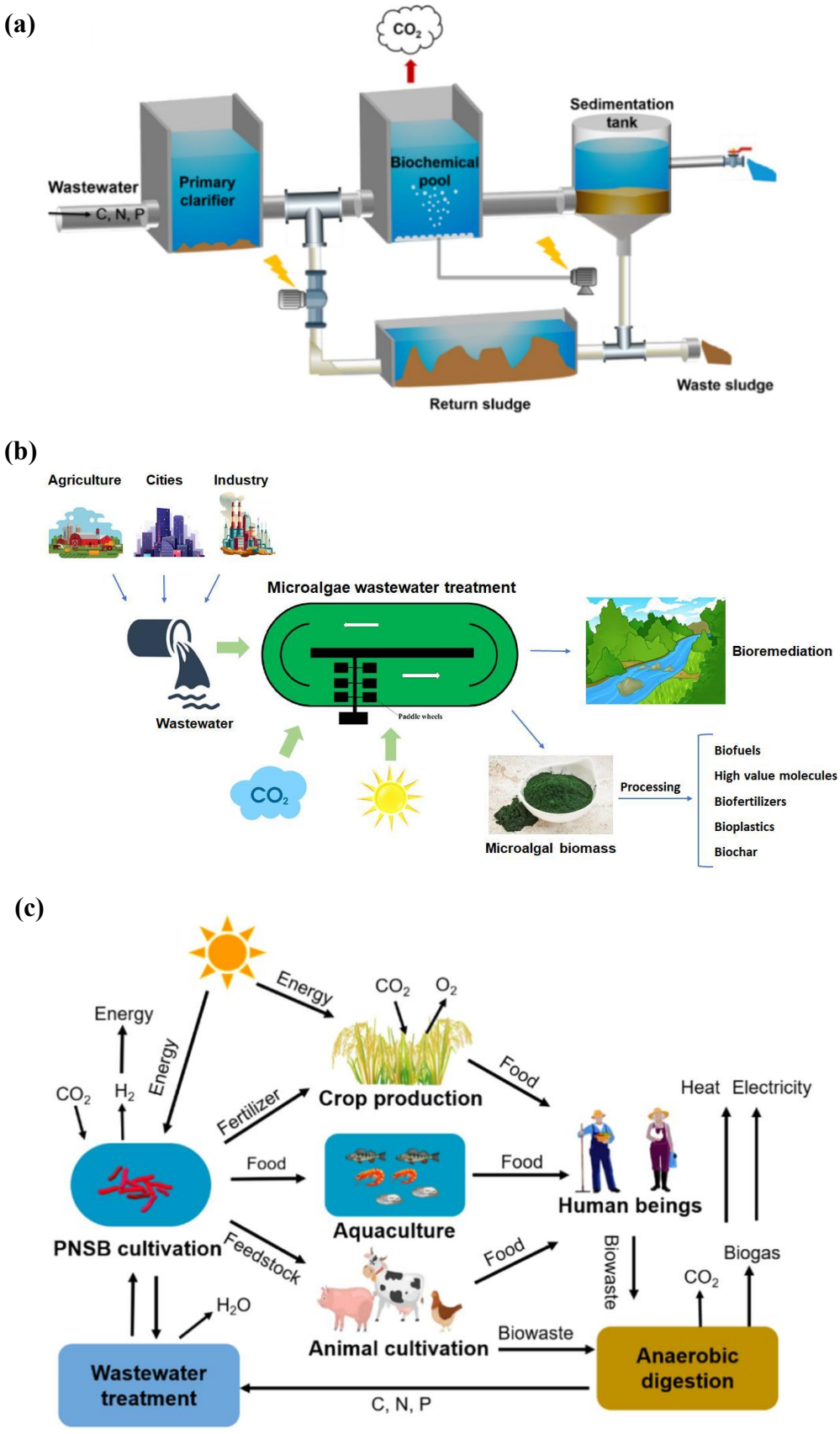

Fig. 1. Wastewater treatment and resource production processes: (a) traditional activated sludge for wastewater treatment processing, (b) microalgae-based wastewater treatment, and (c) PPB-based wastewater treatment (Lu et al., 2021; Geremia et al., 2021).

Microalgae are cultivated for wastewater treatment, nevertheless, they are still struggling with challenges, such removal efficiency. Although microalgae can remove soluble organic matters with low molecular weight, such as glucose, it is impossible for them to remove organic matters and fix nutrients when there are too many recalcitrant organic matters with high molecular weight. As a result, anaerobic digestion is needed to remove efficiently high amounts of such organics, which indicates they need more process and cost to reach high removal efficiency. In addition, microalgal-based wastewater treatment requires dedicated management since microalgae are susceptible to change of environmental conditions and contamination with other heterotrophic microorganisms, such as bacteria (Resurreccion et al., 2012). Consequently, saving cost and taking a balance between management of contamination to prevent microalgal community and performance loss is a challenging issue.

1.2. PPB based wastewater treatment

Application of purple phototrophic bacteria (PPB) has attracted increasing attention for biological wastewater treatment. PPB are microorganisms that conduct anoxygenic photosynthesis, without the production of oxygen. They are mainly classified into two groups: purple sulfur bacteria (PSB) and purple non-sulfur bacteria (PNSB) (Madigan and Jung, 2009). PSB require reduced sulfur compounds as an electron donor when reducing inorganic carbon. Capabilities for photoheterotrophic and dark metabolic in PSB are limited. In contrast, PNSB carry out diverse metabolisms, such as photoheterotrophy, photoautotrophy, and fermentation (Madigan and Jung, 2009). PSB and PNSB have other differences: the tolerance to sulfur and capability to store it intracellularly of PSB, and capabilities to fix nitrogen and produce hydrogen of PNSB. PNSB also produce valuable products, like biofuels (Gabrielyan et al., 2015; Vasiliadou et al., 2018), and bioplastics (Ranaivoarisoa et al., 2019; Monroy and Buitrón, 2020), and their biomass can be utilized as fertilizer (Sundar and Chao, 2022) and feedstock (Hülsen et al., 2018; Delamare-Debouteville et al., 2019). As a result, PNSB are more interesting for resource recovery.

PNSB exhibits diverse metabolic growths, simplified in Fig. 2(a), where numbers in brackets correspond to the bold numbers in this section (Capson-Tojo et al., 2020):

(1) Photoautotrophic (using light and CO_2 as a carbon source)
(2) Photoheterotrophic (using light as energy source and organic carbon source)
(3) Fermentation (without light and using organics as energy and carbon source)
(4) Respiration (under aerobic conditions)

These growths modes are determined based on the environmental conditions (e.g. electron acceptor/donor and presence/absence of light or oxygen) (Fig. 2(b).). Photoautotrophic **(1)** and photoheterotrophic **(2)** growths are dominant in the presence of light and the absence of oxygen. The availability of light and/or oxygen is attributed to the metabolism shift from phototrophy to fermentation **(3)** or respiration **(4)**. Fermentation is dominant under the absence of both light and oxygen, while aerobic metabolism dominates when oxygen is present at high concentrations regardless of the availability of light.

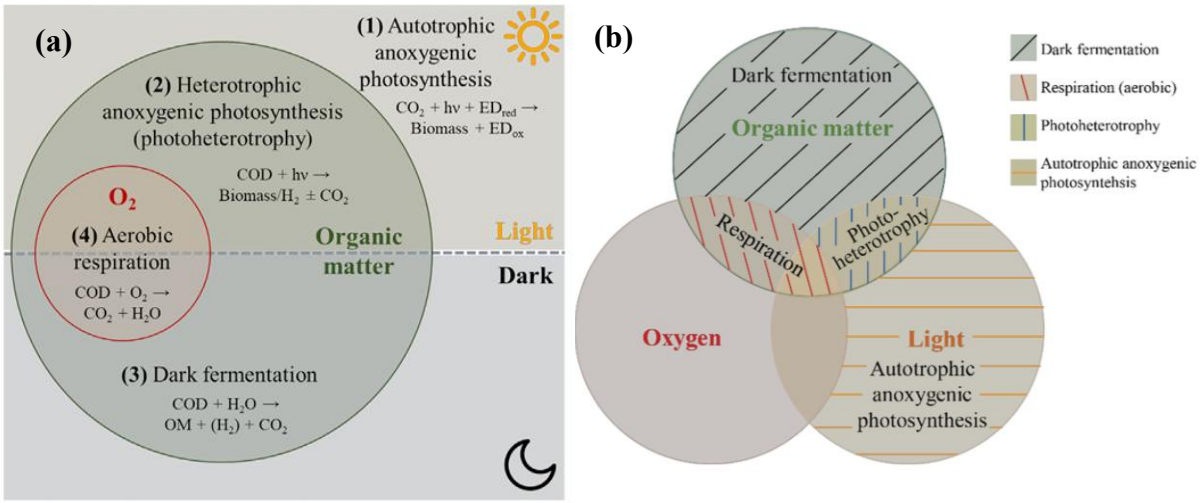

Fig. 2. Simplified main metabolic modes of PNSB. (a) structured according to energy and carbon sources and electron acceptors, and (b) rendered in a Venn diagram including the dominant mode under the presence/absence of organic matter, oxygen, or light. ED is an electron donor, and COD is chemical oxygen demand (Capson-Tojo et al., 2020).

While microalgae are also phototrophic microorganisms and can be applied to wastewater treatment, PNSB is more attractive regarding the following perspectives.

i) PNSB have been considered more efficient microorganisms compared to microalgae regarding photoconversion in photosynthesis (Hülsen et al., 2014). PNSB have photosynthetic pigments, like bacteriochlorophyll a and b (BChl a and b) and carotenoids to harvest specific light as an energy source during anoxygenic photosynthesis: near-infrared (NIR) light range (800 and 1020 nm) from solar radiation, and ultraviolet and visible spectrum (300 to 500 nm) (Zhang et al., 2003; Capson-Tojo et al., 2020). Harvesting NIR prevents microbial competition between PNSB and microalgae since microalgae cannot utilize NIR as their energy source (Zhang et al., 2003). In addition, carotenoids have another function, apart from the accessory pigments for light absorption, to prevent photodegradation of BChl under the stressful environment (Madigan and Jung, 2009). These functions allow PNSB to maximize their metabolism processes and be able to be photosynthetic microorganisms attracting more attention than microalgae.

ii) PNSB remove organic matter and assimilate nutrients simultaneously. Compared to microalgae, PNSB have a higher growth rate (1.51 to 1.69 d^{-1} for mixed cultures and 0.96 to 7.10 d^{-1} for pure cultures) (Hülsen et al., 2014; Kaewsuk et al., 2010; Noparatnaraporn et al., 1987; Ponsano et al., 2008). Also, PNSB have high organic matter removal efficiency, leading to high biomass yields close to unity in terms of chemical oxygen demand (COD): 1 g-$COD_{biomass}$ • 1 g-$COD_{removed}$ $^{-1}$ (Hülsen et al., 2022). This value is much higher than those of other microorganisms, such as aerobic heterotrophs (around 0.5 g-$COD_{biomass}$ • g-$COD_{removed}$ $^{-1}$). During the carbon removal, PNSB assimilate nutrients and produce valuable substances different from microalgae. Specifically, PNSB can synthesize polyhydroxybutyrates (PHB) and polyphosphates, which is outstanding ability and microalgae expect cyanobacteria do not have (Hülsen et al., 2014).

iii) PNSB exhibits higher tolerance towards high-strength wastewater, high salinity and low temperatures than that of microalgae, indicating PNSB can grow even under the extreme environment (Hülsen et al., 2016; Hülsen et al., 2019). As a result, PNSB removes organic compounds and assimilates nutrients from these waste streams more efficiently than microalgae.

From these excellent abilities and characteristics, PNSB are being tested using various wastewater, such as domestic wastewater, pharmaceutical wastewater, fish industry wastewater, textile wastewater, and fuel synthesis process water (Hülsen et al., 2016; Madukasi et al., 2010; de Lima et al., 2011; Nga et al., 2024; Wada et al., 2025).

1.3. Valorization of PNSB biomass

Through the removal of organic matters and nutrients, PNSB accumulates specific valuable products: single-cell protein, carotenoid, coenzyme Q10 (CoQ10), and Polyhydroxyalkanoates (PHA) in their cells (Khatipov et al., 1998).

Single-cell protein is produced from single celled microorganisms (e.g. bacteria, yeasts, fungi or algae) (Najaforpour, 2007), which means this protein can be called microbial protein or biomass (Saeed et al, 2016). PNSB are likely to accumulate essential amino acids and proteins which include methionine and cysteine with high sulfur content, though generally these contents are low in other microorganisms, like microalgae (Bleakley and Hayes, 2017). Single-cell protein can be used as animal feed, not for humans.

Carotenoids are other valuable compounds in PNSB cells in addition to the high contents of proteins. Carotenoids can be utilized as food supplements, cosmetics, and medical products (Abu-Rezq et al., 2010; Ciriminna et al., 2016).

PNSB also contains CoQ10, which is a ubiquitous coenzyme found in animals and functions as an antioxidant and electron carrier (Yen and Shih, 2009; Zhu et al., 2017). CoQ10 plays an important role in medical and cosmetic products (Urakami and Yoshida, 1993; Zhu et al., 2017). CoQ10 production by biosynthesis in microorganisms is a higher cost-effective approach than other ones, and this already is applied commercially (Lu et al., 2013; Urakami and Yoshida, 1993).

PHA can be accumulated when energy is excessive and protecting against stress (Raza et al., 2018). PHA are linear polymers of hydroxyacids connected by ester bonds and is biodegradable rapidly and can be utilized to medical applications (Montiel-Corona and Buitrón, 2021), resulting in the higher production cost than that of petrochemical-origin polymers (Koller et al., 2017). PNSB can accumulate PHA at a high percentage under anaerobic conditions. Among PHA, Poly (3-hydroxybutyrate) (PHB) is the storage compound most produced from PHA. PHB can be utilized for a variety of fields, such as surgical sutures and carriers for drugs, because of its chemical and osmotic inert properties.

As PNSB accumulate and contains valuable products, utilization of PNSB biomass benefits the economy in addition to the environment.

1.4. Challenges of application of PNSB for paper wastewater treatment

Generally, wastewater from several industries contains high concentrations of organic compounds and nutrients as summarized in Table 1. An adequate proportion of carbon and nutrients (N, P) is important for PNSB growth, though there is wastewater, such as from pulp and paper industry (PPW), which is rich in carbon but insufficient in nitrogen.

Table 1. Composition of different wastewater sources (WW).

	pH	TOC	Total COD (g L^{-1})	Soluble COD (g L^{-1})	TN (g L^{-1})	TKN (g L^{-1})	TP (g L^{-1})	Reference
Dairy	4-12	-	0.95-102	0.73	0.10-1.8	-	0.005-0.64	Carvalho et al. (2013) Slavov (2017) Hülsen et al. (2018)
Piggery	6.7-7.7	5.9	22-47	19-20	0.50-4.4	3.4-3.7	0.14	Bernet et al. (2002) Boursier et al. (2005) Kim et al. (2016) You et al. (2021)
Domestic	5.0-7.2	0.05-0.14	0.20-0.44	0.11-0.37	0.05-0.70	-	-	Huang et al. (2010) Friedler et al. (2013)
Pharmaceutical	3.9-9.2	0.86-4.9	0.20-10	-	0.50-1.5	0.17-0.77	0.05-0.25	Gadipelly et al. (2014) Li and Li (2015)
Paper	6.1-8.3	2.8	0.50-115	-	0.003-0.01	-	0.01-0.03	Budiman et al. (2014) Shankar et al. (2014) Kamali and Khodaparast (2015) Bentancur et al. (2021) Ospina-Betancourth et al. (2021)

TOC: total organic carbon, COD: chemical oxygen demand, TN: total nitrogen. TKN: total Kjeldahl nitrogen, TP: total phosphorus.

The pulp and paper manufacturing process consists of mainly three steps: pulp making, pulp processing, and paper making (Esmaeeli et al., 2023). These processes produce many organic compounds and chemicals, such as lignin, degradation products of carbohydrates, extractives, and chlorinated phenolic compounds (Bhatti et al., 2021). As these nonbiodegradable carbon and phenolic compounds cannot be removed by microorganisms, various physical and chemical treatment technologies, such as coagulation-flocculation and membrane technologies, are developed. However, these physical and chemical treatment technologies need to remove coagulants and chemicals which may cause environmental pollution. Thus, biological treatment, especially using PNSB, is important.

Even when treated by PNSB, PPW has another issue that it does not contain enough nutrients, especially nitrogen, as shown in Table 1. While other wastewater sources contain 0.05 to 4.4 g-TN L^{-1} (Bernet et al., 2002; Huang et al., 2010;) or 0.17 to 3.7 g-TKN L^{-1}

(Boursier et al., 2005; Li and Li, 2015). On the other hand, PPW has only 0.003 to 0.01 g-TN L^{-1} which is lower than that of other WW by 5 to 1500 times (Budiman et al., 2014; Betancourth et al., 2021). Nitrogen is essential for PNSB as it gets to be a resource to produce BChl, proteins, and other cellular compositions. Not only the amount of nitrogen, but also the ratio of carbon to nitrogen should be preferable for PNSB growth. The optimal C/N ratio for PNSB growth is 15-20:1 (Matinfar et al., 2025). Nevertheless, the C/N ratio in PPW is 50:1 to 38000:1, indicating that nitrogen concentration is extremely low for growth. Although the data about PPW treatment using PNSB is scarce, Bhatti et al. (2021) showed that microalgal growth was little or negligible when using PPW. Thus, from this study, it can be concluded that biological treatment of PPW using PNSB is challenging due to the low concentration of nitrogen.

PNSB have the ability to fix nitrogen under autotrophic or heterotrophic conditions. PNSB have nitrogenase enzymes which are metalloprotein complexes and reduce nitrogen when NH_4^+ is not available. Previous research reported that PNSB fixed nitrogen gas and converted it to biomass at 100%, which indicates PNSB is excellent for nitrogen fixation (Rodero et al., 2025). PPW lacks nitrogen, so as a previous study showed, supplying nitrogen gas is considered to enhance nitrogen fixation ability of PNSB and treat PPW. Therefore, this study tried to valorize PPW using PNSB by supplying nitrogen gas and investigated the effect of nitrogen fixation on treatment efficiency. Investigating the treatment efficiency of PPW with PNSB could offer novel insights, as the application of PNSB to valorize this kind of wastewater has been limited.

2. Objective

This study aimed to valorize wastewater from the pulp and paper industry using PNSB. This wastewater had insufficient nitrogen sources for PNSB growth, to address this challenge, the following specific objectives were set:

i) **Assessment of the effect of the hydraulic retention time (HRT) of 4 days and 8 days**
This objective focused on the optimization of HRT to maintain treatment efficiency and PNSB growth when using PPW, avoiding biomass washout.

ii) **Evaluation of the effect of supply of nitrogen gas**
This objective was established as a solution to solve the low N/COD ratio of these wastewaters. The ability of PNSB to fix nitrogen gas and its effect on treatment efficiency was evaluated.

3. Materials & Methods

3.1. Wastewater and inoculum

The reactor was fed with synthetic PPW with the following composition: NH_4Cl 0.017 g L^{-1}, $C_6H_{12}O_6$ 2.480 g L^{-1}, K_2HPO_4 0.084 g L^{-1}, $CaCl_2$ 0.072 g L^{-1}, $NaHCO_3$ 1 g L^{-1}, $AlCl_3$ 0.692 mg L^{-1}, $FeCl_2 \cdot 4H_2O$ 0.466 mg L^{-1}, $MgCl_2$ 2.978 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 3.072 mg L^{-1}, H_3BO_3 0.072 mg L^{-1}, $Cr_2K_2O_7$ 0.008 mg L^{-1}, $CoCl_2$ 0.008 mg L^{-1}, $CuSO_4$ 0.032, $Pb(NO_3)_2$ 0.008 mg L^{-1}, $NiCl_2 \cdot 6H_2O$ 0.0072 mg L^{-1}, KCl 0.016 mg L^{-1}, $AgNO_3$ 0.000376 mg L^{-1}. PPW had a pH of 8.26±0.13, but pH was adjusted to around 7.3-7.5 prior to adding it into the reactor.

An enriched photoheterotrophic PNSB was used as an inoculum after its enrichment in a 1L batch reactor using PPW.

3.2. Experimental set-up

A cylindrical reactor with 3 L working volume, a diameter of 16 cm, and a height of 20 cm was used (Fig. 3.). A bubble column with 200 mL of working volume was equipped

with the reactor to supply nitrogen gas. The reactor was illuminated at 360±45 W m^{-2} with IR light (Switching Supplies, Mouser Electronics, USA). A 1 L flask containing synthetic wastewater was used to feed PPW, and a 3L tank was used to collect the effluent. Nitrogen gas was supplied in the last stage from the gas bag into the column. Pumps were used to control the supply of nitrogen gas into the column, influent into the reactor, and liquid circulation between the column and reactor.

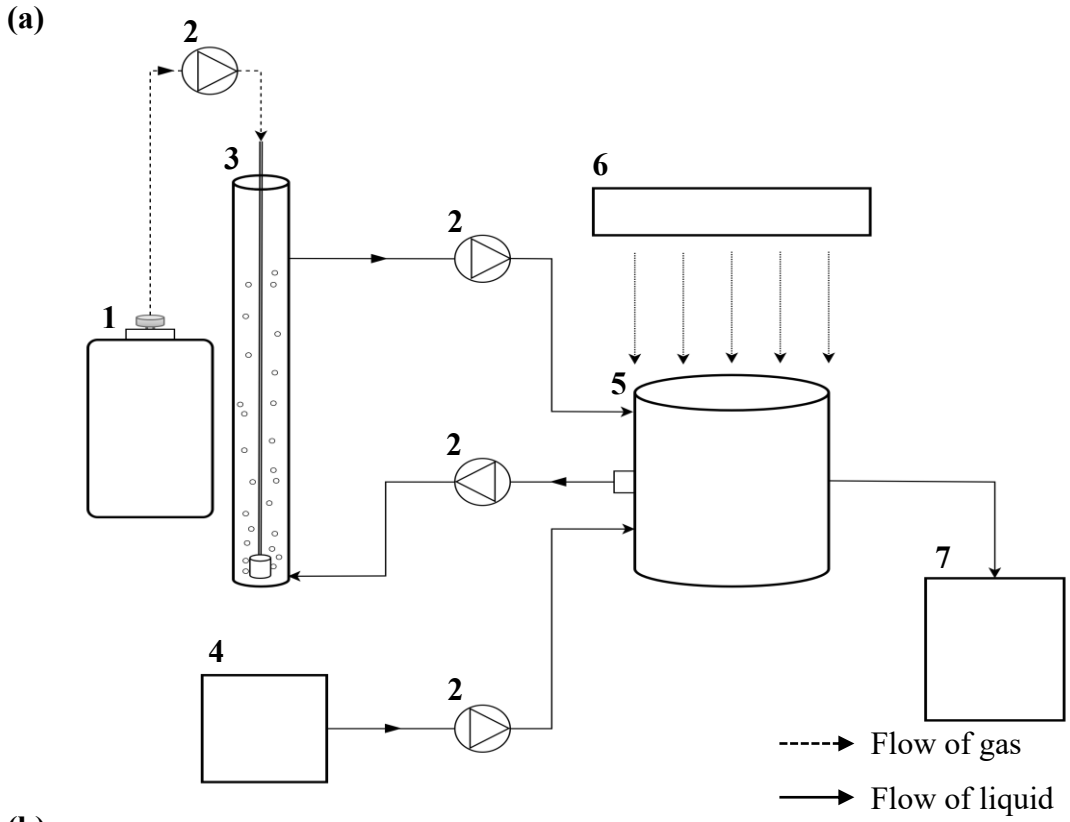

Fig. 3. Experimental set up: (a) schematic reactor configuration and (b) picture of the reactor. 1: gas bag for nitrogen gas, 2: pump, 3: column, 4: tank for influent, 5: reactor, 6: IR light (850 nm), 7: tank for effluent.

3.3. Operational conditions

The operation started with 33% v v^{-1} of the inoculum. The reactor was closed, and headspace was flushed with 10 L of 100% v v^{-1} nitrogen gas to minimize the presence of O_2 and ensure the anaerobic conditions. The temperature was maintained at 29±1 ^{o}C, and the reactor was agitated continuously using magnetic stirrer (S20, labbox, Spain).

Four operational stages (A-D) were implemented to evaluate the influence of HRT on PNSB growth and wastewater treatment efficiency, and effectiveness of nitrogen gas supply for wastewater treatment efficiency (Table 2).

During stage A, batch culture was conducted to increase the PNSB biomass enough to perform the following continuous culture. During stage B, PPW was supplied with HRT 4 days, and PNSB did not grow under HRT 4 days. Thus, in stage C, PPW was supplied with HRT 8 days with the same other conditions. Stage D was performed at HRT of 8 days while supplying N_2 gas. N_2 gas was supplied into the column at a flow rate of 41.7 mL min^{-1} and liquid in the reactor circulated at a flow rate of 39 mL min^{-1}. Biomass accumulated on the bottom of the column was taken and put back into the reactor before taking samples to prevent the sedimentation of biomass in the column (Fig. 4(a)). Also, the sparger in the column was washed every 3-4 days to take out the biomass attached to the sparger (Fig. 4(b)).

Table 2. Operational conditions applied during four operational stages.

Stages	**A**	**B**	**C**	**D**
Period (days)	0-4	5-17	18-26	27-55
HRT (day)	-	4	8	8
Influent COD (mg L^{-1} d^{-1})	-	635.8±16.3	312.6±2.82	317.7±4.61
Influent TN (mg L^{-1} d^{-1})	-	0.90±0.00	0.44±0.04	0.54±0.05
N_2 flow rate (mL min^{-1})	-	-	-	41.7

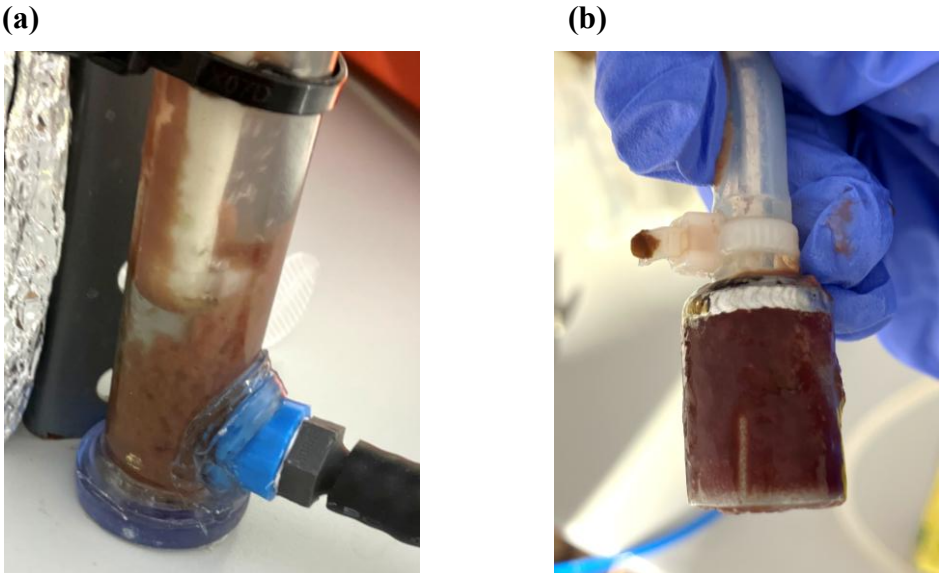

Fig. 4. Sedimented biomass on the bottom of the column (a) and attached biomass to the sparger (b).

Liquid samples of 40 mL were periodically taken from the reactor to monitor pH, biomass growth by optical density (OD) at wavelength of 450 nm, 660 nm, and 870 nm, corresponding to the presence of carotenoid, biomass, and BChl, respectively, and concentration of total COD (COD_T), soluble COD (COD_S), total organic carbon (TOC), total nitrogen (TN), total Kjeldahl nitrogen (TKN), volatile suspended solids (VSS), and volatile fatty acids (VFAs). Gas samples of 100 µL from the headspace of the reactor were also periodically taken using a gastight syringe (SampleLock syringe, Hamilton, USA) to monitor the concentrations of CO_2, O_2, and N_2.

3.3. Analytical procedures

Illumination intensity was measured from the inside of the reactor using an IR light sensor (PASPort PS-2148, PASCO, USA).

pH was measured using pH-Meter Basic 20^+ (CRISON, Spain).

OD was measured using SPECTROstar Nano (BMG LABTECH, Germany).

Samples for TOC and TN were prepared by filtering using 0.45 µn filters and diluted with MiliQ (ultrapure) water. Diluted samples were measured using TOC-L CSH/CSN (Shimadzu, Japan).

The procedure for the measurement of COD_T and COD_S was carried out according to the internal Institute for Sustainable Processes (ISP) protocols of the University of Valladolid, Spain. These procedures follow the standardized APHA-AWWA-WPCF methodology (Eaton et al., 2005). For COD_S, samples were prepared in the following way: samples were filtered through a 0.45 µm filter, and filtered samples were diluted using distilled water. For COD_T, samples were used without pretreatment. 2.5 mL of samples for each COD_T and COD_S and distilled water as a blank were taken. Then, 1.5 mL of digestant solution and 3.5 mL of catalytic solution were added, where the tubes were closed with screw caps and shaken gently. The test tubes were placed in a digester at 150°C for 2 hours in the fume hood. After the glass tubes were removed and allowed to cool to room temperature, 2 drops of ferroin were added as an indicator. Finally, a magnetic stirrer was introduced into each tube to titrate the excess

dichromate with the FAS solution until it turned red, noting the volume of FAS consumed according to the digital burette reading.

COD_T and COD_S were determined as follows:

$$\mathrm{COD\ (mgO_2/L)} = \frac{(B - F) * 400}{T} * f_{dilution}$$

where B is a volume (mL) of FAS used in blank titration, F is a volume (mL) of FAS used in samples titration, T is a titre volume (ml) consumed in titration of potassium dichromate standard, and f means factor of dilution of samples.

The procedure for the measurement of TKN was carried out according to the internal ISP protocols of the University of Valladolid, Spain. These procedures follow the standardized APHA-AWWA-WPCF methodology (Eaton et al., 2005). 3 mL of 96% sulphuric acid was added into 5 mL of samples. Samples were digested at 370 ºC for 60 minutes (Selecta Digestion Bloc, Bloc-Digest Macro 12). After cooling digestion tubes, these tubes and Erlenmeyer flasks were set in the distillation apparatus (Buchi distiller, Kjelflex K-360). Distillation programme implemented in the laboratory was applied: predistillation stage (automatic dosing of 25 ml of water and 15 ml of NaOH in the digestion tube and 100 ml boric acid with mixed indicator in the Erlenmeyer flask) was done, and then TKN phase (automatic dosing of 5 ml water and 15 ml NaOH) started for 10 minutes. Finally, samples in Erlenmeyer flasks were titrated using 0.1 N sulphuric acid until samples turned from green to bright purple, noting the volume of sulphuric acid consumed.

TKN was determined as follows:

$$\mathrm{TKN\ (mgN/L)} = \frac{(N \times V) * 14000}{V_m}$$

where N is a normal sulphuric acid used in titration, V is a volume (mL) of sulphuric acid consumed in titration, and V_m is a volume (ml) of sample.

The procedure for VSS was carried out according to the internal protocols of the internal ISP at the University of Valladolid, Spain. These procedures follow the standardized APHA-AWWA-WPCF methodology (Eaton et al., 2005). The vacuum filtration system containing a glass fiber filter with a pore size of 0.7 µm, which had been previously weighed for subsequent calculation, was installed. Once the filtration procedure was completed using a sample of 20 mL, the filters containing the solids were placed in an oven at 105°C for 24 hours. After that, filters were placed in a desiccator, and the weight of filters was measured after been a room temperature (TSS: total suspended solids). Finally, the measured filters were placed in a muffle furnace at 550°C for 45 minutes and VSS procedure was carried out. Like the way in the TSS, filters for VSS were put in the desiccator, and the weight of filters was measured after been a room temperature.

VSS was determined as:

$$\mathrm{VSS\ (mg/L)} = \frac{(PF_1 - PF_2) * 1000}{V}$$

where PF_1 is a mass of filter after the oven (g), PF_2 is a mass of filter after the muffle furnace (g), and V is a volume of sample (L).

Samples for VFAs analysis were filtered with 0.22 µm of pore size, and 1 mL of filtered samples were acidified with 20 µL H_2SO_4 (96–97% (w/v)). Analysis was carried out using Agilent 7820A GC-FID (Agilent Technologies, Santa Clara, USA). The injector, oven, and detector were maintained at 375ºC, 130ºC and 350ºC, respectively, and N_2 gas supplied at 45 mL min^{-1} as the carrier gas.

Gas composition at the inlet was analyzed using the 7820A gas chromatography (GC-TCD) system (Agilent Technologies CA, USA) employing argon as carrier gas at a flow rate of 5 mL min^{-1}.

3.4. Calculation

Removal efficiencies for each parameter (COD, TOC, and TN) throughout operation were determined as:

$$\text{Removal efficiency (\%)} = \frac{(Q_{in} * C_{in} - Q_{out} * C_{out})}{Q_{in} * C_{in}} * 100$$

where Q_{in} is the influent flow rate (L d^{-1}) in each day, Q_{out} is the effluent flow rate (L d^{-1}) in each day, and C_{in} and C_{out} are the influent and effluent concentrations of TOC, COD, or TN (mg L-1) in each day. Then, an average value was obtained in each stage.

Protein contents in PNSB were calculated using TKN and following formula:

$$\text{Protein content (\%)} = \frac{(TKN_T - TKN_S) * 6.25}{VSS} * 100$$

where TKN_T or TKN_S is total or soluble TKN in each day, and VSS is VSS in each day (Eding et al., 2006). Then, an average value was obtained in each stage.

4. Results & Discussion

4.1. Overall performance

4.1.1. Photobioreactor performance

OD at wavelengths of 450 nm, 660 nm, and 870 nm, which indicates the carotenoid, PNSB biomass, and BChl, respectively, showed almost the same behavior throughout the experiment (Fig. 5.).

PNSB showed rapid growth in stage A because it was batch culture which was in better condition to grow. Growth decreased in stage B when HRT was set as 4 days. This means HRT 4 days is not optimal when treating PPW using PNSB. It was considered due to the insufficiency of nitrogen in this PPW, and HRT should be longer. Before changing HRT, concentrated inoculum was added on day 15, and then HRT changed to 8 days.

In stage C when HRT was changed to 8 days, PNSB growth seemed to be stable until day 22. The stirrer stopped on day 25, thus, poor mixing in the reactor was thought to occur, leading to an insufficient supply of light. PNSB tend to grow as small aggregates (Cerruti et al., 2020), and it was considered which likely to cause poor mixing, leading to sedimentation of the biomass on the bottom of the reactor and, consequently, insufficient supply of light. In addition, poor mixing entailed lower availability of nutrients to PNSB. Thus, sedimentation of PNSB and insufficient nutrient supply caused the stop of biomass growth.

In stage D (from day 17 to 21), the HRT was maintained, and nitrogen gas was supplied to evaluate its effect on PNSB growth and treatment efficiency. Supplying nitrogen gas promoted PNSB growth gradually (from day 27 to day 41) as shown in Fig. 5, which showed the slight effectiveness of nitrogen gas. However, nitrogen gas supply did not affect significantly biomass when there was another nitrogen source. N_2 fixation is conducted by nitrogenase, and this enzyme is inhibited by NH_4^+ presence (Spanoghe et al., 2025). Since PPW contained a small amount of ammonium, it was likely difficult for PNSB to shift their metabolism from non-diazotrophic conditions to growth with N_2 as the nitrogen source. Although it was expected that supplying more nitrogen gas would increase biomass, the biomass remained almost the same as in stage C, when PNSB growth was stable. PNSB increased rapidly on day 42. This was because of the leak out of approximately one-third of

the reactor's biomass caused by the increased pressure in the column. After leaking, batch culture-like conditions continued for two days (days 42-43), and this led to elevated OD values. However, biomass decreased gradually, and final biomass increased only about 1.2 times compared to the period when nitrogen gas was supplied in the column. This decrease showed the slight effectiveness of nitrogen gas on growth as discussed before.

PNSB utilizes diverse simple organic compounds as carbon sources, such as VFAs, simple sugars, and alcohol. Some PNSB, for example *Rhodospirillum rubrum*, do not have the ability to degrade glucose as they lack a transporter for it and enzyme to degrade it (Hädicke et al., 2011). When PNSB cannot utilize such a carbon source, dark fermentation and photofermentation is applied to produce VFAs which PNSB can consume (Smith et al., 2023). This study showed the production of VFAs throughout the operation (Fig. 6.), so it was thought that VFAs were produced by other photoheterotrophic microorganisms and PNSB consumed produced VFAs.

In stage B when HRT was 4 days, PNSB growth decreased, and at this time VFAs with a long chain of carbon, such as valeric acid and isovaleric acid, increased. PNSB tend to prefer consuming simple VFAs, such as acetic acid and propionic acid (Capson-Tojo et al., 2020). Thus, it was considered that PNSB could not growth due to a short HRT and accumulated VFAs which were difficult for PNSB to consume.

In stage C when HRT was 8 days, growth was stable in a specific period (day 18-22) and simple VFAs, acetic acid, propionic acid, and butyric acid, increased at the same time. This indicates that the balance between produced VFAs by other microorganisms and consumed VFAs by PNSB was kept. However, when growth decreased (day 22-25), acetic acid and propionic acid increased. At this time, the mixing stopped, and not enough light was supplied. As a result, even though there was enough carbon source, insufficient light reduced the efficiency consumed VFAs by PNSB and biomass decreased.

In stage D, HRT was 8 days, and nitrogen gas was supplied. Growth was stable on day 28-33 when acetic acid was occupied in VFAs. After that, growth increased on day 33-37 when isobutyric acid was produced. This means that the rate of consuming acetic acid by PNSB was higher than that of producing it, then acetic acid almost disappeared. On day 42-47 when growth decreased again, isobutyric acid increased, indicating that PNSB consumed simple VFAs but there was insufficient carbon or nutrient, thus PNSB could not produce biomass anymore. After day 49 when growth was almost stable, propionic acid, butyric acid, and isocaproic acid increased. This explains that there was still a lack of nitrogen leading to not-optimal C/N ratio and accumulation of VFAs with both short and long carbon chains. As a result, it was expected that although PNSB was adjusted to these environments, PNSB could not consume VFAs, and growth did not show an increase in stage D.

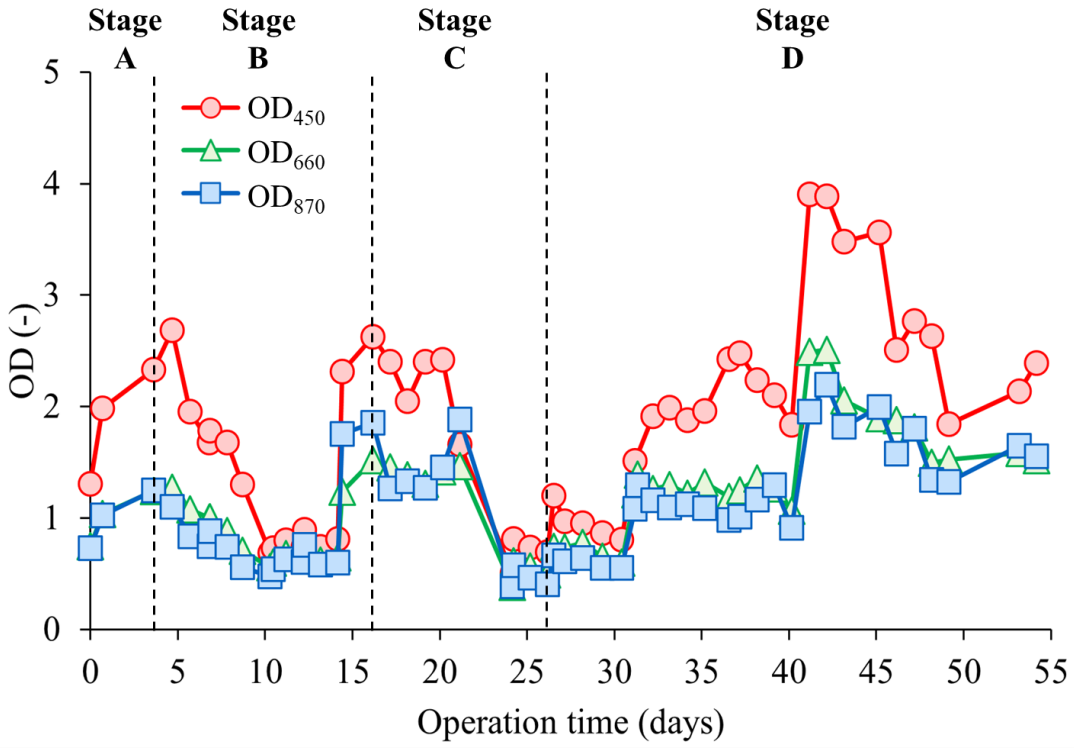

Fig. 5. Time course of OD at 450 nm, 660 nm, and 870 nm.

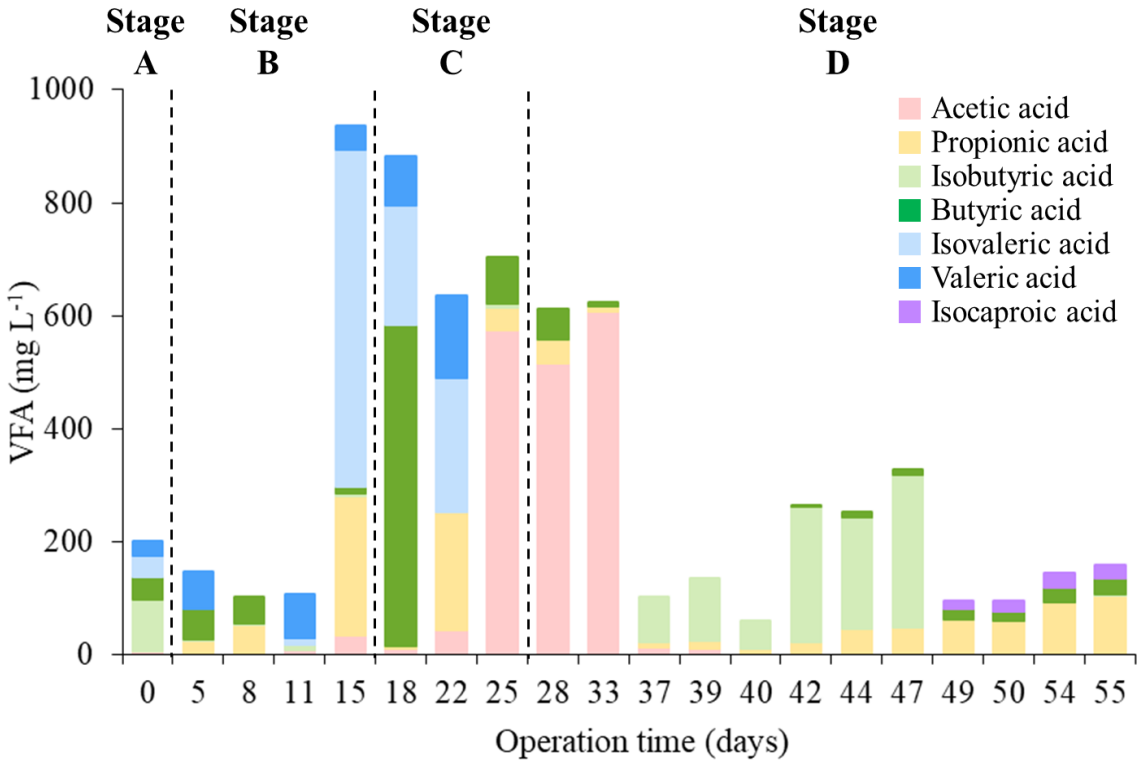

Fig. 6. Change of the composition of VFA throughout the experiment.

The pH was acidic around 5-6 during stage A (Fig. 7.). In stage B, pH fluctuated between 6.2-7.8, but it became stable in stage C (pH 7.4). However, pH value increased in stage D until day 36 (pH 8.9), and then it decreased again to 7.1 until the end of the experiment. This change in pH was attributed to the change in VFAs composition in the reactor. During stage A and B, when pH decreased and fluctuated, VFAs were produced

from glucose by other microorganisms. At this time, PNSB growth was suppressed by the long-chain VFAs, and the accumulation of VFAs caused a decrease in pH. In stage C, growth was partly stabilized. During that period, simple VFAs were consumed by PNSB, while longer-chain VFAs remained, suggesting that the system was in the process of taking a balance between VFAs production and consumption. Nevertheless, as the pH value was within the optimal range for growth, PNSB showed stable growth. Acetic acid was accumulated around day 25 to 33 in stage D. PNSB utilized this acid, leading to an increase in pH around these days because acetic acid was consumed by PNSB. Toward the end of stage D, pH decreased gradually, and this indicated that longer-chain VFAs, such as isocaproic acid, accumulated. Since PNSB had difficulty consuming such VFAs, this accumulation resulted in a decrease in pH. The optimum pH for PNSB grow is 6-8.5 that was observed during almost the whole experiment period (van Niel, 1944).

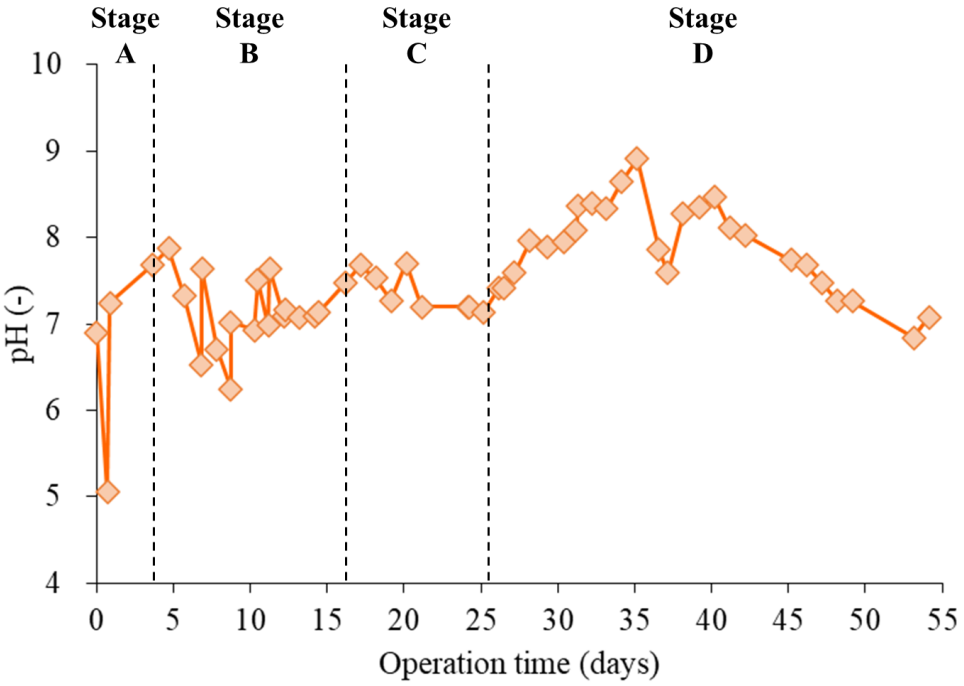

Fig. 7. Time course of pH.

Another factor that may have hindered proper biomass growth was the presence of oxygen in the headspace. Headspace composition in stage B (HRT 4 days) showed a high concentration of O_2, around 15-16% (Fig. 8.). The reactor used in this study was not completely sealed, which may have allowed nitrogen gas to leak and oxygen to enter, leading to the presence of oxygen that could have affected PNSB growth in stage B. From day 32 in stage D, when nitrogen gas supply began, the oxygen concentration decreased to approximately 4-8%. Continuous nitrogen gas flushing displaced oxygen from the column, maintaining nearly anaerobic conditions. Overall, although oxygen was detected in some stages, the headspace volume was small and the reactor was mostly closed, indicating that oxygen presence alone could not fully explain the inhibition of PNSB growth.

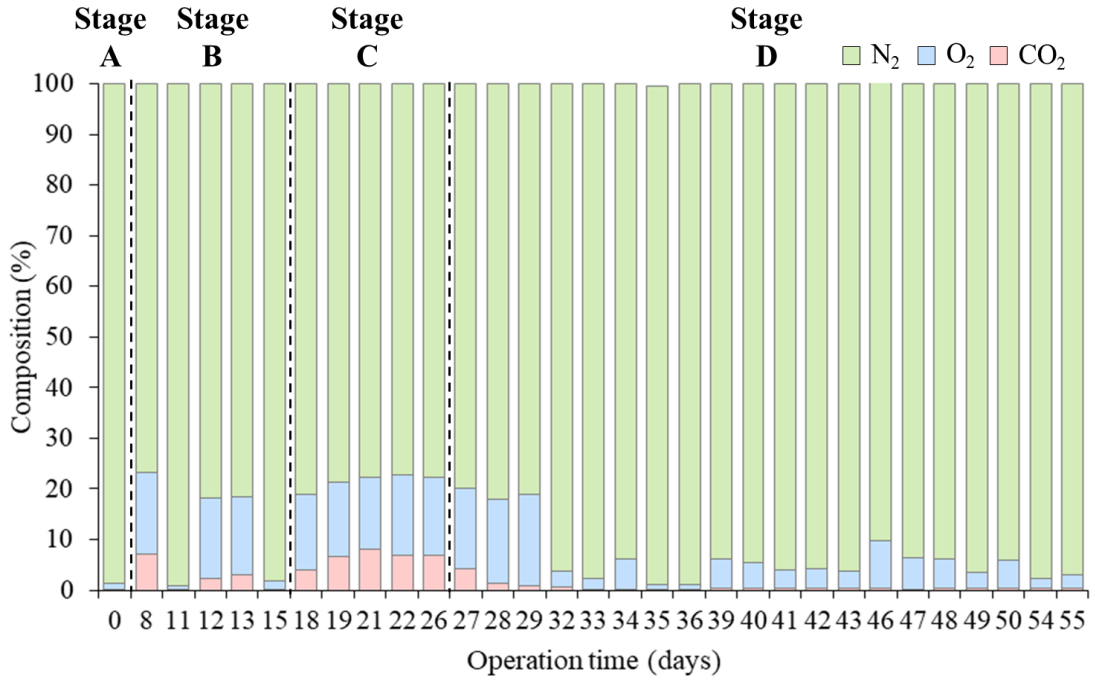

Fig. 8. Time course of gas composition: N_2, O_2, and CO_2.

4.1.2. Treatment performance

TOC was removed at a period when PNSB growth increased or maintained (day 0-5, around day 15, and day 30-40) (Fig. 9(a)). Average TOC removal efficiencies were 22.2%, 28.3%, 57.9%, and 68.6% in stages A, B, C, and D, respectively (Table 3.). In this experiment, stage D showed the most stable biomass concentration without a decrease, and consequently, the highest removal efficiency was observed. TOC removal efficiencies ranged from 45% to 80% were obtained in other photosynthetic systems applied for the treatment of carbon-rich wastewaters (García et al., 2019; Marín et al., 2019). Compared with other studies, stages A, B, and C showed lower removal efficiencies, while only stage D achieved comparable performance. This suggests that the optimal condition for treating PPW was a HRT of 8 days with nitrogen gas supplementation.

Changes in COD_S concentration were similar to those of TOC (Fig. 9(b)). COD was removed during the period when PNSB growth increased and then stabilized, similar to the trend observed for TOC. Average COD removal efficiencies were 15.2%, 29.7%, 56.7%, and 69.5% in stage A, B, C, and D, respectively (Table 3.). As growth was most stable and did not decrease in stage D, removal efficiency was highest during that period. The COD removal efficiency obtained in stage D was similar to those reported in other studies using PNSB (40-80%) (Lu et al., 2015; Hülsen et al., 2018). Hülsen et al. (2018) reported low COD removal efficiency of under 40% when treating wastewater from the sugar mill industry due to the high ethanol concentration, which was toxic to PNSB. In this study, the low COD removal efficiency suggested that the low nitrogen concentration in PPW led to an imbalanced C/N ratio. The optimal C/N ratio for PNSB growth is 15-20:1 (Matinfar et al., 2025). However, the C/N ratio in PPW was approximately 50:1 to 38000:1 This indicates that nitrogen was highly depleted, preventing PNSB from removing carbon.

Finally, TN removal efficiency between each stage did not show a difference. TN removal efficiencies were 20.4%, 0%, 26.7%, and 33.5% in stages A, B, C, and D, respectively (Table 3.). PNSB remove nitrogen at a range of 40-60% generally (Lu et al., 2015; García et al., 2019), and this study showed lower value to other studies. Although TN

appeared to be removed in stages A and B (Fig. 9(c)), and the TN concentration was very low in the inlet of the reactor (≈ 5 mg L^{-1}), TN concentration in the effluent was not completely zero. In stages A and B, TN concentrations were much higher than in the inlet, primarily due to nitrogen originating from PNSB carcasses and pigments and dead cells in the inoculum, which represented 33% of the photobioreactor volume. High levels of total and soluble TKN (78.0 mg-N L^{-1} and 53.2 mg-N L^{-1}, respectively) were observed in stage A, with the soluble TKN nearly equal to TN at that stage. The similarity between TN and soluble TKN indicates that TN in stage A consisted mainly of organic and ammonium nitrogen derived from the inoculum, as nitrification would not occur under anaerobic conditions. This nitrogen, excluding nitrate and nitrite, was responsible for the high TN concentration in stage A, and some likely persisted in stage B due to limited PNSB growth during that stage. In stages C and D, the initial soluble nitrogen was already consumed. Although some nitrogen was taken up, nitrogen released from dead cells likely compensated for this, resulting in similar inlet and outlet concentrations. Potential errors in TOC measurement sensitivity at such low concentrations (<5 mg L^{-1}) may also have contributed to these results.

Table 3. PNSB biomass and removal efficiency in each operational stage.

Stages	A	B	C	D
Average VSS (g L^{-1})	0.46±0.09	0.49±0.06	0.48±0.12	0.72±0.21
Average TOC removal efficiency (%)	22.2±11.6	28.3±12.9	57.9±5.33	68.6±9.42
Average COD removal efficiency (%)	15.2±6.78	29.7±4.25	56.7±4.16	69.5±9.39
Average TN removal efficiency (%)	20.4±10.6	0±0	26.7±10.8	33.5±13.4

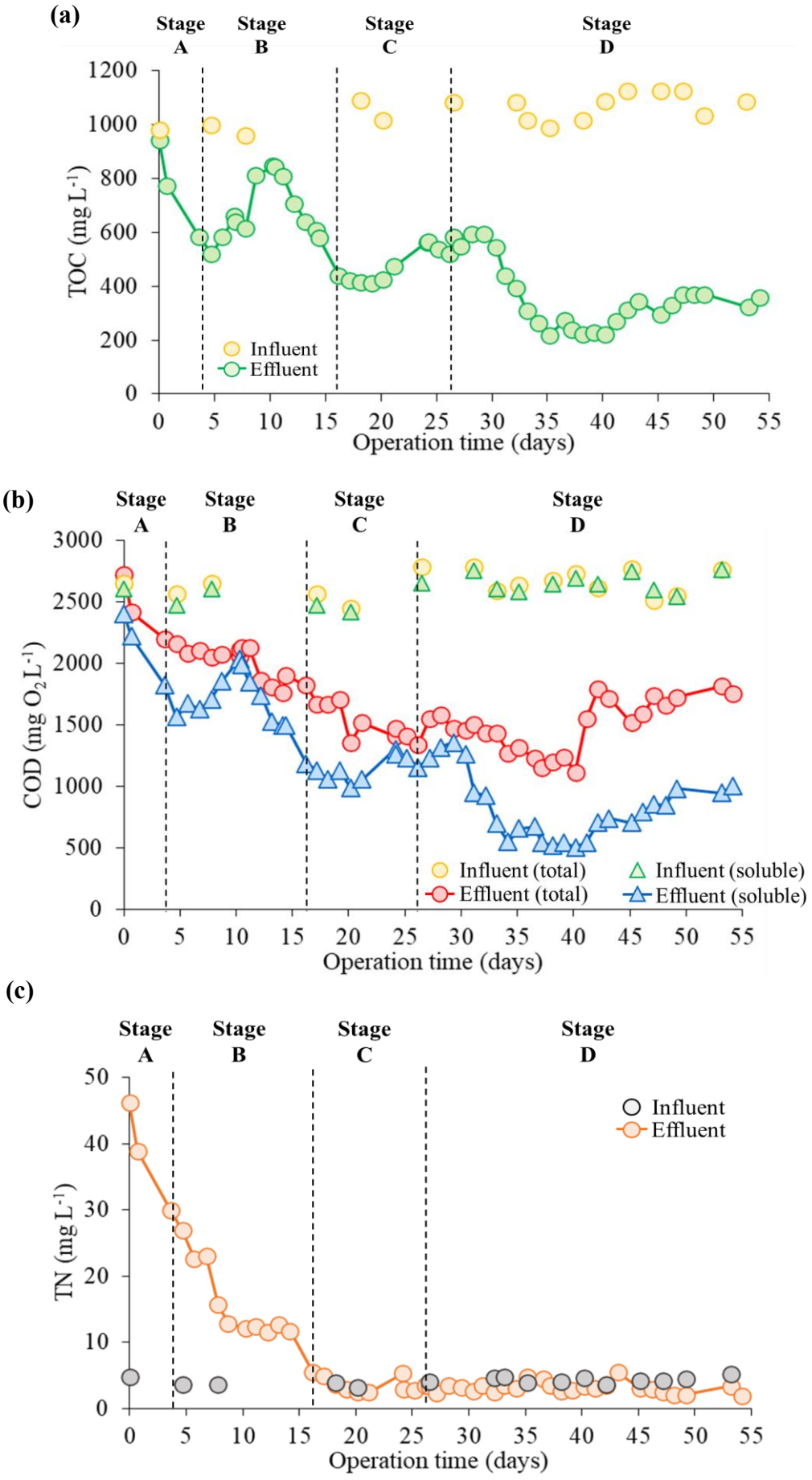

Fig. 9. Time course of each concentration: (a) TOC, (b) COD, and (c) TN.

4.2. Biomass production

In addition to monitoring PNSB growth via OD, biomass concentration was measured using VSS. PNSB biomass accumulated mainly in stage D when HRT was 8 days, and nitrogen gas was supplied (Fig. 10). Compared with each stage, VSS were 0.46 g L^{-1}, 0.49 g L^{-1}, 0.48 g L^{-1}, and 0.72 g L^{-1} in stage A, B, C, and D (Table 3.), respectively, and VSS in stage D showed significant increase (ANOVA, $p < 0.05$). These results indicate that nitrogen gas supplementation promoted PNSB biomass production or the accumulation of intracellular compounds. PNSB generally produce higher biomass when light, organic acids, nutrients, and micronutrients are available in optimal amounts, and under anaerobic conditions. In this study, organic acids and anaerobic conditions were sufficient, but PPW lacked nitrogen and light was limited because of the type of reactor. Under nutrient limitations, particularly nitrogen and phosphorus, PNSB are expected to accumulate intracellular compounds such as carotenoids and PHB. Therefore, it is likely that much of the increase in stage D reflects intracellular compound accumulation rather than purely biomass production.

Protein content, calculated based on total and soluble TKN, was 68.9%, 27.4%, 29.2%, and 45.8% in stage A, B, C, and D, respectively. There was no large difference between the values in each stage. As no difference was observed, it could be concluded that there was no considerable increase in protein production in this study. PNSB can accumulate protein up to 70% of the biomass (Getha et al., 1998; Garimella et al., 2017; Chumpol et al., 2018), but the contents in this study were much lower than this. This low protein content was likely due to nitrogen depletion in PPW, as nitrogen is an essential component of proteins. In other studies, pulp and paper mill effluent was used as an alternative agent for dilution to water in treating pal, oil mill effluent with high turbidity (Budiman et al., 2014). Results showed better PNSB growth when using mixed wastewater compared with pure PPW. Conversely, growth decreased when using only PPW, and it was suspected that PNSB death and deterioration occurred due to lack of nutrient: higher C/N ratio. According to this study, it is expected that PPW should be mixed with other wastewaters containing nutrients to adjust the C/N ratio.

Apart from the effect of treatment conditions on protein productivity, other studies reported high protein-productivity was observed when the surface-to-volume ratio increased. If that ratio increased from 5 m^2 m^{-3} to 10 m^2 m^{-3}, light availability increased, and this led to an increase in biomass concentration by 1.3 times (Alloul et al., 2021). The surface-to-volume ratio in this study was 35 m^2 m^{-3}, which is relatively higher than that reported in Alloul's study. Although the surface-to-volume ratio was relatively high in this study, light availability per unit of biomass (W VSS-g^{-1}) decreased. Light availability per unit biomass was 27.4 W g^{-1}, 25.7 W g^{-1}, 26.3 W g^{-1}, and 17.5 W g^{-1} in stage A, B, C, and D, respectively, indicating that lower light availability per unit of biomass in stage D may have contributed to the reduced protein production. Therefore, when treating PPW, the reactor design and structure should also be carefully considered.

The ratio of the OD value at 450 nm to 870 nm (OD_{450}/OD_{870}) indicates indirectly the ratio of carotenoid to bacteriochlorophyll. If this ratio is high, carotenoids accumulate more than bacteriochlorophyll. In this study, each ratio was 1.86, 1.71, 1.52, and 1.67 for stage A, B, C, and D, respectively. These values did not show any differences between each stage, indicating that there were other produced compounds in stage D.

As an expected compound, PHB can be considered. It was noted that most microorganisms, which produce PHAs, are likely to accumulate PHB under specific conditions: abundant carbon and insufficient nutrients (nitrogen and phosphorus) (Fülöp et

al., 2012). PPW lacks a nitrogen source, thus it was considered that PNSB accumulated PHB under stress of insufficient nitrogen even though nitrogen gas was supplied.

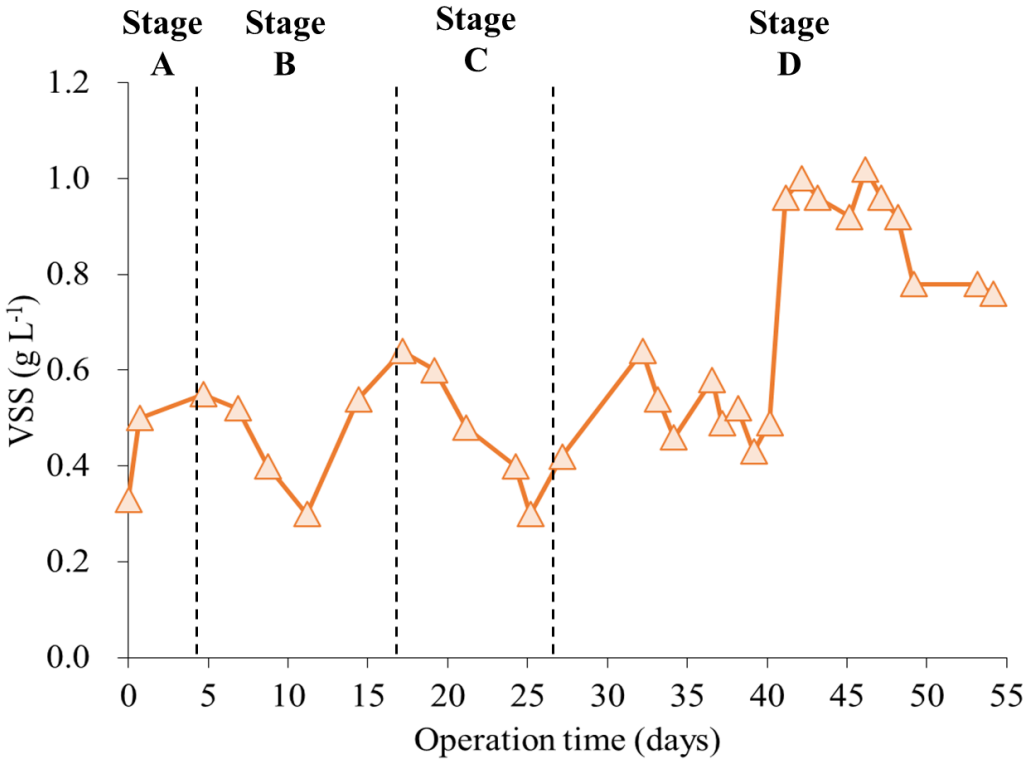

Fig. 10. Time course of VSS.

5. Conclusion

This study aimed to valorize wastewater from PPW using PNSB. Due to nitrogen depletion in PPW, nitrogen gas was supplied to enhance PNSB growth and treatment efficiency. Four operational stages were tested, and the highest PNSB growth occurred in stage D, when the HRT was 8 days and nitrogen gas was supplied. At that time, VSS reached 0.72 g L^{-1} and removal efficiencies were 68.6%, 69.5%, and 33.5% for TOC, COD, and TN, respectively. While these values fall within the general range of reported removal efficiencies, they remain lower than those observed with other types of wastewaters. These results indicated that PNSB growth and treatment efficiencies were attributed to VFAs and the C/N ratio in the reactor. This study highlights that effective PPW treatment using PNSB requires optimization of HRT and the C/N ratio, with one possible strategy being the co-treatment of PPW with nutrient-rich wastewaters.

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