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Coupled In-Situ Fermentation for Enhanced Biological Phosphorus Removal from Digested Swine Wastewater

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Abstract: This study demonstrated the feasibility of enhanced biological phosphorus removal coupled with in-situ fermentation (EBPR-F) to improve phosphorus removal from real digested swine wastewater. We used fermentable substrates (casein hydrolysate and glucose) as the external carbon sources to promote in-situ fermentation and enhance biological phosphorus removal. Compared with conventional EBPR dominated by *Candidatus* Accumulibacter, EBPR-F had significantly better phosphorus removal with enriched polyphosphate-accumulating organisms (PAOs). Under supplementation with 100 mg/L glucose, total phosphorus (TP) removal was over 95% in EBPR-F, with an average TP concentration in the effluent below 1.0 mg/L, satisfying the discharge standard (8 mg P/L) in China. The PAO activity and relative abundance of *Candidatus* Accumulibacter (44.7% ± 3.1%) and *Tetrasphaera* (18.1% ± 6.6%) in EBPR-F were much higher than those in EBPR. The improvement in phosphorus removal of EBPR-F was due to the enrichment of *Tetrasphaera* through the enhanced in-situ fermentation, as *Tetrasphaera* can efficiently ferment complex organic matter and provide bioavailable organics for phosphorus removal.

Keywords: swine manure; low carbon/nitrogen ratio wastewater; external carbon source; polyphosphate-accumulating organisms; *Candidatus* Accumulibacter; *Tetrasphaera*

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1. Introduction

For decades, anaerobic digestion has been the customary process to treat swine manure [1,2]. The anaerobic digestate is massive and rich in nitrogen (N), phosphorus (P), and organic matter. Thus, the digestate requires additional treatment to remove N, P, and residual organic matter to meet the discharge standards [3–6]. Digested swine wastewater has high concentrations of nutrients (N and P) but lacks readily biodegradable COD (rbCOD). In digested swine wastewater, the concentrations of COD, total nitrogen (TN), and total phosphorus (TP) are in the range of 887 to 3286 mg/L, 176 to 1379 mg/L, and 22 to 415 mg/L, respectively [7–12]. Because digested swine wastewater has a low carbon/nitrogen (C/N) mass ratio and lacks rbCOD, denitrifiers compete with phosphorus-accumulating organisms (PAOs) for rbCOD [13]. The low availability of rbCOD to PAOs causes inefficient phosphorus removal from digested swine wastewater [14]. Therefore, simultaneously removing N and P from digested swine wastewater is challenging. To meet increasingly stricter phosphorus discharge limits, developing effective and low-cost systems for deep phosphorus removal from digested swine wastewater is critical.

Enhanced biological phosphorus removal (EBPR) relies on PAOs to release and excessively uptake phosphorus between famine anaerobic and feast aerobic conditions, respectively. EBPR is efficient, economical, and environmentally sustainable, compared with

chemical phosphorus removal [15,16]. As a dominant PAO, *Candidatus* Accumulibacter phosphatis (referred to as *Ca.* Accumulibacter hereinafter) is vital in phosphorus removal in EBPR [17]. *Ca.* Accumulibacter can rapidly take up and store volatile fatty acids (VFAs) as polyhydroxyalkanoates (PHA) under anaerobic conditions using energy released from the hydrolysis of intracellular polyphosphate (poly-P) and the degradation of glycogen. Under the subsequent aerobic condition, the stored PHA is degraded as a carbon and energy source for cell growth and reproduction, poly-P production, glycogen regeneration, and "luxury uptake" of orthophosphate (ortho-P) [18,19]. *Tetrasphaera*, a PAO with fermentation ability, is also a key microorganism in EBPR and has a higher abundance than *Ca.* Accumulibacter in full-scale EBPR plants [20,21]. Physiological characteristics of *Tetrasphaera,* such as fermentation ability, are considerably different from those of *Ca.* Accumulibacter [22,23]. On the one hand, *Ca.* Accumulibacter mainly uses VFAs as a carbon source [24–26], while *Tetrasphaera* prefers to metabolize readily fermentable substrates, such as glucose and amino acids, with unknown storage products instead of PHA [27–29]. Compared with *Ca.* Accumulibacter, *Tetrasphaera* is less competitive in VFA absorption [30]. *Tetrasphaera* can ferment and supply additional VFAs for phosphorus removal [22,31]. In addition, *Tetrasphaera* can obtain energy from the fermentation of glucose to grow and proliferate [32]. *Ca.* Accumulibacter can grow on fermentation products generated by *Tetrasphaera*, such as succinate, lactate, acetate, and propionate [33]. The fermentation of waste activated sludge (WAS) by *Tetrasphaera* provides carbon sources for biological nutrient removal and reduces sludge volume [34,35]. However, limited by the special physical and chemical environments required by in-situ fermentation, most studies focused on ex-situ fermentation of WAS [36–39]. Very few studies conducted in-situ fermentation of mixed liquor suspended solids (MLSS) in EBPR. Therefore, integrating EBPR and MLSS fermentations in one bioreactor is an urgent task in the treatment of swine wastewater. Moreover, studies should assess the feasibility of EBPR coupled with in-situ fermentation in treating digested swine wastewater and the key microbial populations.

This study assessed the potential of EBPR coupled with in-situ fermentation (EBPR-F) dominated by *Tetrasphaera* to improve phosphorus removal from digested swine wastewater with a low carbon/nitrogen ratio. We used fermentable substrates as the external carbon sources to promote the fermentation dominated by *Tetrasphaera* and to achieve deep phosphorus removal in EBPR-F. The long-term performances of the EBPR-F and EBPR systems treating real digested swine wastewater were compared. We also used batch experiments to explore the phosphorus removal mechanisms of EBPR-F by determining the variations in intracellular and extracellular substrates. Furthermore, we assessed the microbial community to elucidate the correlations among dominant microbial groups with phosphorus removal performance.

2. Materials and Methods

2.1. Setup of the Laboratory-Scale Reactors

Two identical sequencing batch reactors (SBRs) with a working volume of 2 L (each) were operated (Figure 1). One SBR (SBR-A) was operated as a traditional EBPR dominated by *Ca.* Accumulibacter, while the other (SBR-F) was operated as EBPR-F. The EBPR-F system comprised SBR-F and a culture bank (1 L) enriched with *Tetrasphaera*. The enriched culture predominated by a clade II member of *Tetrasphaera* was established in the culture bank. Introducing *Tetrasphaera* from the culture bank to SBR-F achieved in-situ fermentation in SBR-F. The two SBRs were operated within a narrow temperature range (20 to 25 °C) with intermittent aeration and a cycling time of 8 h. The 8 h cycle consisted of 10 min of feeding, a 40 min anaerobic period (with fermentation), and a 400 min reaction period (including four alternating 60 min aeration and 40 min non-aeration phases), followed by 20 min of settling and 10 min of decanting. The hydraulic retention time (HRT) and solids retention time (SRT) of the SBRs were 3.3 d and 15 d, respectively. The concentrations of MLSS ranged from 3000 to 4000 mg/L in the SBRs. The SBRs were stirred with a mechanical mixer at approximately 150 rpm during both the non-aeration and

aeration phases, and the dissolved oxygen (DO) concentration in the aeration phase was 1.0 ± 0.5 mg/L. The low DO was reasonable because of the low oxygen requirement of the simultaneous nitrification-denitrification and phosphorous removal (SNDPR) in SBRs [13].

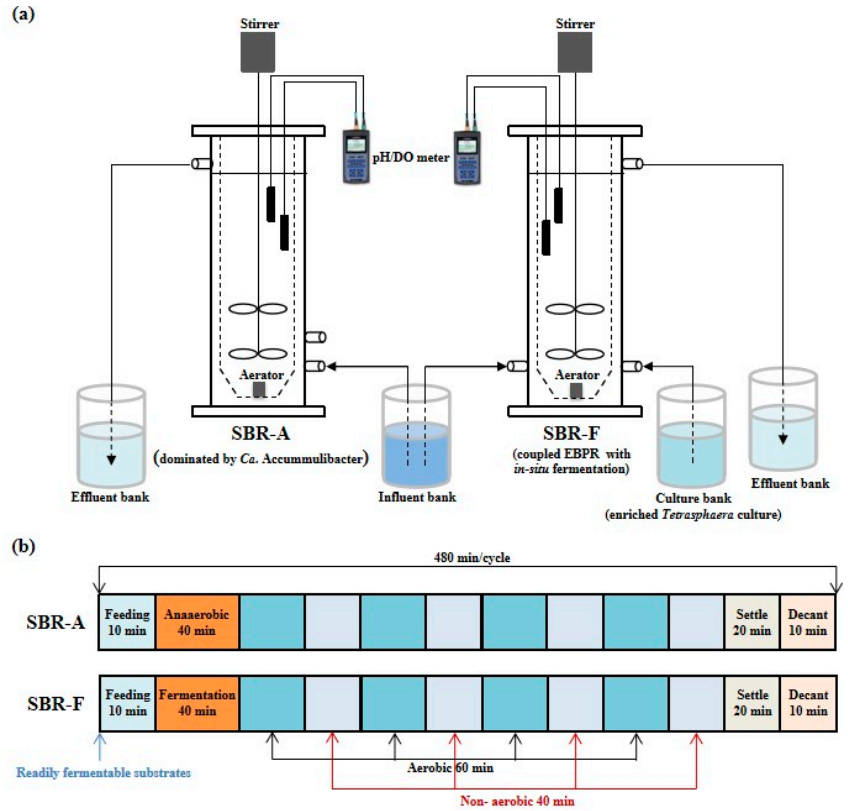

Figure 1. (**a**) Schematic description and (**b**) operational models of the two sequencing batch reactors (SBRs).

2.2. Real Digested Swine Wastewater and Seed Sludge

The real digested swine wastewater was from a swine farm in Heyuan (Guangdong, China). The major characteristics of the wastewater were as follows: pH 7.5 ± 0.1, COD 2000 ± 50 mg/L, TP 42 ± 3 mg/L, ammonium nitrogen (NH_4^+-N) 500 ± 20 mg/L, and influent COD/N ratio below 4.0.

The seed sludge of the SBRs was collected from a parent SBR with a 6 L working volume. We used alternating carbon sources in the parent SBR to enrich *Ca.* Accummulibacter [40]. The phosphorus removal of the parent SBR was approximately 98%.

T. australiensis (CGMCC 1.10747) was acquired from the China General Microbiological Culture Collection Center (CGMCC). The strain was maintained in a growth medium (5.0 g/L peptone, 5.0 g/L yeast extract, and 1.0 g/L magnesium sulfate). The strain was cultivated at 30 °C in a 1 L Erlenmeyer flask for 7 d and then transferred to the culture tank.

2.3. Operational Procedure of the Sequencing Batch Reactors

Both SBRs were inoculated with activated sludge from the parent SBR rich in *Ca.* Accummulibacter, and they were operated in the same mode. Digested swine wastewater was treated for 60 d to reach a steady state before the start of the experiment. The entire experiment lasted more than 3 months and included three phases.

Phase I (days 1 to 24) was the start-up phase. During Phase I, SBR-A was operated as EBPR dominated by *Ca.* Accumulibacter, while SBR-F was inoculated with *Tetrasphaera* to develop EBPR-F. *Tetrasphaera* was introduced into SBR-F during the feeding period in the first cycle, and the inoculation quantity of *Tetrasphaera* in SBR-F was 5% to 10% of the reactor volume. Phase I included a 6-day phase with the continuous daily addition of bacterial

suspension enriched with *Tetrasphaera* (centrifuged at 3000 rpm for 10 min and washed twice), followed by an 18-day phase with the addition of fermentation liquid beginning on day 7.

Phase II (days 25 to 84) was for enhancing fermentation and improving phosphorus removal. The synergistic approach of in-situ fermentation and supplemental carbon addition was used [41]. As *Tetrasphaera* prefers fermentable substrates as the carbon sources, casein hydrolysate and glucose were chosen as the supplemental carbons for EBPR-F to promote in-situ fermentation. Phase II had two sub-phases (Phase II-A and Phase II-B). Phase II-A had three periods for casein hydrolysate concentrations of 300 mg/L (days 25 to 30), 500 mg/L (days 31 to 42), and 800 mg/L (days 43 to 48). In Phase II-B, the supplemental carbon was glucose with concentrations of 250 mg/L (days 49 to 60), 100 mg/L (days 61 to 74), and 75 mg/L (days 75 to 84). Casein hydrolysate and glucose were added to SBR-F during the feeding period in each cycle.

In Phase III (85 to 99 days), SBR-A and SBR-F were both dosed with supplemental carbon (100 mg/L glucose). The effect of the supplementation of glucose on phosphorus removal was assessed.

2.4. Analysis of the Digested Swine Wastewater Quality

We monitored the following wastewater quality parameters routinely in the effluent and the mixed liquor of the SBRs throughout the experiment: COD, TP, NH_4^+-N, nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), MLSS, and mixed liquor volatile suspended solids (MLVSS). The DO, pH, and temperature of the mixed liquor were determined daily with a digital portable DO meter and pH meter, respectively. Moreover, we conducted batch tests of phosphorus release and uptake [42] to evaluate the EBPR metabolic activities of the two SBRs. Batch tests were conducted with 500 mL of mixed liquor from the two SBRs at the end of the reaction phase. Samples for fluorescence in situ hybridization (FISH) were also collected from the two SBRs.

NH_4^+-N, NO_2^--N, NO_3^--N, TP, COD, MLSS, and MLVSS were analyzed according to the *Standard Methods* [43]. VFAs were determined using gas chromatography (GC) equipped with a flame ionization detector (FID) and a fused-silica capillary column (30 m × 0.25 mm × 0.5 μm, DB-FFAP). Glycogen (Gly) was determined via digestion and hydrolysis to glucose [44]. PHA concentration was determined as the sum of the concentrations of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) [45].

2.5. Characterization of Polyphosphate-Accumulating Organisms

FISH coupled with a confocal laser scanning microscope (CLSM) was used to quantify the distribution of two key PAOs (*Ca.* Accumulibacter and *Tetrasphaera*) in the two SBRs [46]. The following oligonucleotide probes for FISH were used: EUB_{mix} (equimolar concentrations of EUB338, EUB338II, and EUB338III) for all bacteria; PAO_{mix} (equimolar concentrations of PAO462, PAO651, and PAO846) for *Ca.* Accumulibacter; and Actino-221 and Actino-658 for potential *Tetrasphaera* [27].

2.6. Data Analysis and Reporting

The arithmetic means and standard deviations were calculated using Microsoft Excel 2019 (Microsoft Office 2019, Microsoft Corporation, Redmond, WA, USA). The data were plotted with Origin 2019b (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Wastewater Treatment Performance of the Two Sequencing Batch Reactors

3.1.1. COD and Nitrogen Removal

Both conventional EBPR (SBR-A) and EBPR-F (SBR-F) effectively removed COD and nitrogen (Figure 2 and Table 1). The two systems were operated for 99 d, with average influent COD and NH_4^+-N concentrations of 2000 ± 50 mg/L and 500 ± 20 mg/L, respectively. In Phase I (days 1 to 24) and Phase II (days 24 to 84), despite a low influent

COD/N ratio of approximately 4, the NH_4^+-N and COD removals of SBR-A were stable at 99.6% ± 0.9% and 98.6% ± 1.0%, respectively. During Phase III (days 85 to 99) with supplemental carbon addition, SBR-A still had stable COD and NH_4^+-N removals at 99.9% ± 0.1% and 98.2% ± 1.2%, respectively. The effective removals of COD and nitrogen in SBR-A were due to the efficient use of organic matter for denitrification and phosphorus release. The enhanced SND due to intermittent aeration was another reason for the effective removal of COD and nitrogen [47]. During Phase I (days 1 to 24), the effluent COD and NH_4^+-N concentrations increased over time in SBR-F, resulting from an increase in organic and nitrogen loading due to the addition of the fermentation liquid from the culture bank. From day 15, the proportion of fermentation liquid to SBR-F volume decreased from 10% to 5%, increasing the removals of COD and NH_4^+-N rapidly from 77.0% to 97.6% and from 82.4% to 98.6%, respectively. In Phase II (days 25 to 84) and Phase III (days 25 to 99), the effluent COD and NH_4^+-N concentrations in SBR-F fluctuated, and the removal efficiencies of COD and NH_4^+-N averaged 96.0% and 98.1%, respectively. The average effluent COD and NH_4^+-N concentrations were below 60 and 10 mg/L, respectively, satisfying the discharge standard of wastewater for the livestock and poultry breeding industry (GB 18596-2001).

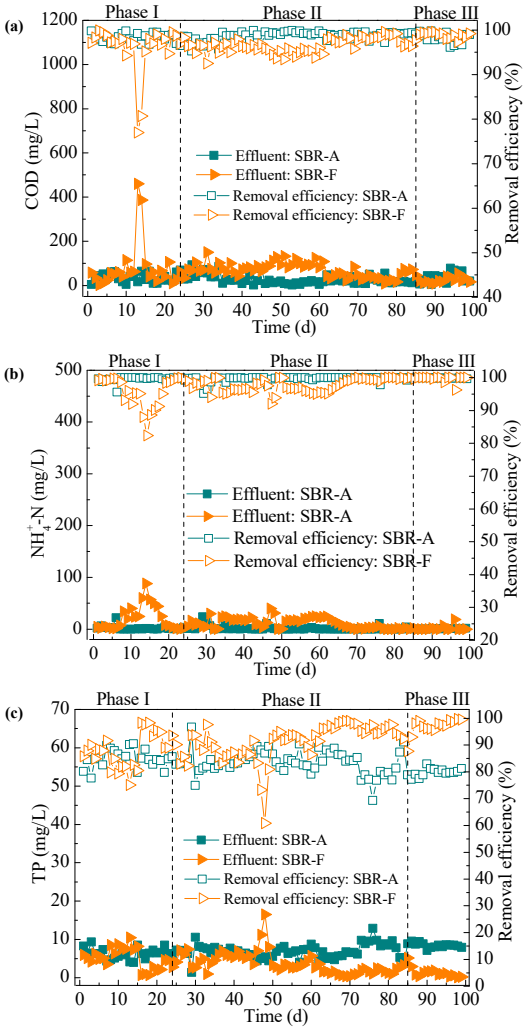

Figure 2. Variations in (**a**) COD, (**b**) NH_4^+-N, and (**c**) total phosphorus (TP) over time in the two sequencing batch reactors (SBRs) treating real digested swine wastewater.

Table 1. Nutrient removal performance of the two sequencing batch reactors (SBRs) during the three phases.

Phase	Day (d)	Reactor	Carbon Source	Influent C/N [a]	TP		NH_4^+-N		COD	
					Effluent (mg/L) [b]	Removal (%) [b]	Effluent (mg/L) [b]	Removal (%) [b]	Effluent (mg/L) [b]	Removal (%) [b]
I to II	1 to 84	SBR-A	Wastewater	4.0	6.7 ± 1.8	84.1 ± 4.2	2.0 ± 4.6	99.6 ± 0.9	29 ± 21	98.6 ± 1.0
I	1 to 6	SBR-F	Wastewater	4.0	5.2 ± 0.7	87.5 ± 1.8	3.0 ± 1.5	99.4 ± 0.3	31 ± 23	98.5 ± 1.2
	7 to 24	SBR-F	Wastewater + fermentation liquid	N/A	5.0 ± 3.2	88.2 ± 7.5	28.8 ± 29.8	96.2 ± 6.0	98 ± 122	95.1 ± 6.1
II-A	25 to 30	SBR-F	Wastewater + casein hydrolysate	6.5	5.0 ± 2.2	88.1 ± 5.2	8.5 ± 4.5	98.3 ± 0.9	69 ± 22	96.6 ± 1.1
	31 to 42	SBR-F		7.5	5.1 ± 1.6	87.9 ± 3.9	17.8 ± 8.9	96.4 ± 1.8	79 ± 28	96.0 ± 1.4
	43 to 48	SBR-F		8.0	8.2 ± 4.8	80.6 ± 11.5	17.6 ± 14.3	96.5 ± 2.9	84 ± 17	95.8 ± 0.9
II-B	49 to 60	SBR-F	Wastewater + glucose	8.0	3.7 ± 1.6	91.1 ± 3.9	16.1 ± 8.1	96.8 ± 1.6	102 ± 19	94.9 ± 0.9
	61 to 74	SBR-F		6.0	1.5 ± 0.9	96.5 ± 2.2	8.8 ± 8.6	98.2 ± 1.7	48 ± 23	97.6 ± 1.2
	75 to 84	SBR-F		4.2	2.0 ± 0.7	95.3 ± 1.8	1.5 ± 1.9	99.7 ± 0.4	39 ± 24	98.0 ± 1.2
III	85 to 99	SBR-F	Wastewater + glucose	6.0	8.5 ± 0.6	79.8 ± 1.5	0.7 ± 0.6	99.9 ± 0.1	36 ± 24	98.2 ± 1.2
		SBR-F			1.3 ± 1.3	96.8 ± 3.1	2.0 ± 5.0	99.6 ± 1.0	25 ± 14	98.8 ± 0.7

Note: [a]: C/N mass ratio. [b]: arithmetic means ± standard deviations. Wastewater: real digested swine wastewater. N/A: not reported.

3.1.2. Phosphorus Removal

TP concentrations and removals in EBPR and EBPR-F fluctuated during the whole experiment [Figure 2c]. The two SBRs were inoculated with *Ca.* Accumulibacter enriched activated sludge for rapid startup. The COD/N ratio of the influent was below 4. Because of the competition between PAOs and ordinary denitrifying heterotrophic organisms for organic carbon from the insufficient carbon source, phosphorus removal in EBPR was poor [48]. TP concentration in the effluent of SBR-A was 6.7 ± 1.8 mg/L, with a removal efficiency of 84.1% ± 4.2% for Phase I (days 1 to 24) and Phase II (days 25 to 84). The effluent TP concentration of SBR-A failed to meet increasingly stringent wastewater discharge standards (GB 18596-2001).

EBPR-F enriched *Tetrasphaera* and achieved in-situ fermentation, improving phosphorous removal. A study demonstrated the fermentation ability of *Tetrasphaera* [49]. Complex organic compounds can be fermented by *Tetrasphaera* to provide carbon sources for *Ca.* Accumulibacter, enhancing biological phosphorus removal. To initiate EBPR-F (Phase I, days 1 to 24), we added bacterial suspension (pretreated) or the fermentation liquid from the culture bank (*Tetrasphaera*) to SBR-F. In the first stage (days 1 to 6) of Phase I with the addition of *Tetrasphaera*, the average TP removal and effluent TP concentration of SBR-F were 87.5% and 5.2 mg/L, respectively. SBR-F with a removal of 5.2 mg P/L had a slightly higher TP removal than SBR-A in Phase I (87.5% versus 82.5%). The low removal of TP in the two SBRs in Phase I was because the microorganisms had not adapted to the new conditions (i.e., in the lag phase). The low efficiency might also be because the fermentation by *Tetrasphaera* was limited by carbon source deficiency. To accumulate *Tetrasphaera*, we added fermentation liquid from the culture bank to SBR-F in the second stage (days 7 to 24) of Phase I. The residual organic matter in the fermentation liquid could serve as the carbon source of *Tetrasphaera*. When the proportion of fermentation liquid introduced to SBR-F decreased from 10% to 5%, phosphorus removal in SBR-F improved with some fluctuations (days 15 to 24). The effluent TP concentration in SBR-F was 2.4 ± 1.5 mg/L, and the average TP removal reached 94.4%. Therefore, the addition of only bacterial suspension to SBR-F was insufficient to boost phosphorus removal, compared with the control (SBR-A). However, the addition of the fermentation liquid effectively enhanced phosphorus removal because fermentation was critical for the survival and enrichment of *Tetrasphaera* in SBR-F. The higher availability of organic carbon due to the addition of the fermentation liquid enhanced the anaerobic fermentation dominated by *Tetrasphaera*.

During Phase II (days 25 to 84), the effects of different carbon sources on phosphorus removal were assessed. On the basis of the carbon source requirement of *Tetrasphaera*, two fermentable substrates (casein hydrolysate and glucose) as external carbon sources were added to EBPR-F. During Phase II-A (days 25 to 48), when casein hydrolysate was added at 300 to 800 mg/L, phosphorus removal declined when casein hydrolysate dosing increased.

SBR-F achieved the highest phosphorus removal of 88.1% when supplemented with 300 mg/L casein hydrolysate. In Phase II-A, SBR-F did not improve phosphorus removal, compared with SBR-A. In addition, excessive casein hydrolysate induced sludge bulking at the end of Phase II-A (days 45 to 48), rapidly increasing the effluent TP concentration. Sludge bulking might be due to the high concentrations of protein and carbohydrates produced by the hydrolysis and fermentation of casein hydrolysate. During Phase II-B (days 49 to 84), phosphorus removal increased with glucose supplementation. The optimal phosphorus removal performance of SBR-F (96.5%), which was 12% higher than SBR-A, occurred with an addition of 100 mg/L glucose. Thus, *Tetrasphaera* fermented glucose in the anaerobic phase to produce carbon sources, mainly VFAs, to enhance biological phosphorus removal. Moreover, glucose outperformed casein hydrolysate as an external carbon source for phosphorus removal. An explanation is that the fermentation of casein hydrolysate is slower than glucose, so that the amount and rate of VFAs produced from casein hydrolysate are much less than those from glucose [49]. Additionally, phosphorus and nitrogen releases could occur during the in-situ fermentation of casein hydrolysate. As a result, glucose, instead of casein hydrolysate, is the appropriate carbon source for EBPR-F.

In Phase III (days 85 to 99), we compared the phosphorus removals of the two SBRs operated under a supplementation with 100 mg/L glucose [Figure 2c]. Compared with the supplementation with only wastewater (84.1%), SBR-A had a lower phosphorus removal of 79.8% with the addition of glucose. In Phase III, the effluent TP concentration in SBR-A increased, varying between 7.13 and 9.56 mg/L. The deterioration of the performance of SBR-A was mainly because *Ca.* Accumulibacter relied on VFAs and could not ferment more complex organic carbon. Therefore, *Ca.* Accumulibacter failed to compete with ordinary denitrifying heterotrophic organisms for carbon sources. By contrast, with the addition of 100 mg/L glucose, SBR-F had a high TP removal of up to 95% from day 87. At the steady-state stage (day 87 to 99, Figure 2), the average effluent TP concentration in EBPR-F (0.9 ± 0.6 mg/L) satisfied the discharge standard in China (8 mg P/L). As a result, the in-situ fermentation with glucose supplementation had a positive effect on phosphorus removal from digested swine wastewater.

The results of the three phases indicate that coupling EBPR with in-situ fermentation dominated by *Tetrasphaera* achieved advanced phosphorus removal from digested swine wastewater with a low C/N mass ratio. Therefore, the coupled system is superior to conventional EBPR. These findings will help understand the role of in-situ fermentation in biological phosphorus removal from high-strength wastewater.

3.1.3. Cycle Performance Study

On day 71 (Phase II-B), we conducted typical cycle tests to assess the nutrient-removal mechanism in the two SBRs (Figure 3). The simultaneous removal of nutrients (N and P) and organic carbon was achieved in a typical operation cycle (8 h) of the two SBRs. Influent COD significantly increased in SBR-F because of the addition of glucose during Phase II-B. COD removal occurred mainly in the anaerobic period (including the feeding period), with efficiencies of 81.4% and 83.5% in SBR-A and SBR-F, respectively. The COD stored as the intracellular carbon source (COD_{intra}) in SBR-F was much higher than in SBR-A (138 and 41 mg/L, respectively). Thus, SBR-F used organic carbon for EBPR and denitrification more completely [13]. Introducing in-situ fermentation in the anaerobic stage would likely further facilitate the use of complex organic matter.

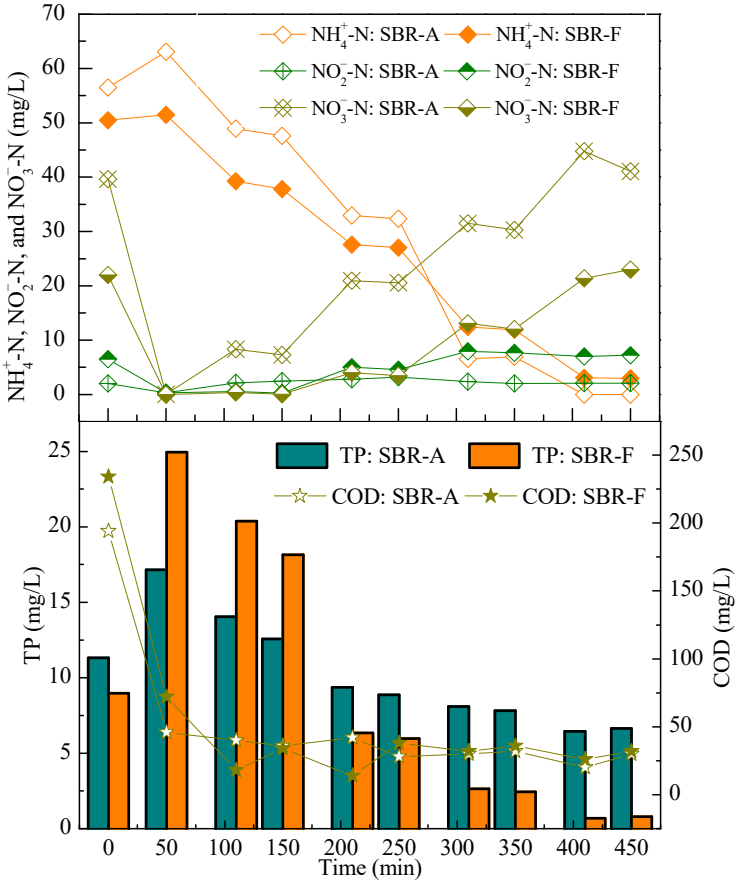

Figure 3. A typical cycle study with operating time in the two sequencing batch reactors (SBRs) treating real digested swine wastewater.

NH_4^+-N oxidation occurred with the accumulation of NO_x^--N (NO_2^--N and NO_3^--N) during the aeration period in both SBRs. During the aeration period, NH_4^+-N concentrations gradually decreased from 56.6 to 0.2 mg/L and from 50.5 to 2.0 mg/L in SBR-A and SBR-F, respectively. Meanwhile, NO_x^--N concentrations rose from the first aeration period and peaked at the end of the cycle. Because almost no COD was left for exogenous denitrification, the loss of nitrogen indicated that both SBRs had SND and/or denitrifying phosphorus uptake driven by intracellular carbon. The SND pathway in Phase II-B contributed 26% and 40% to nitrogen removal in SBR-A and SBR-F, respectively. The enhanced SND in SBR-F could reduce the NO_x^--N accumulation, thus reducing the carbon demand for denitrification and benefiting EBPR.

For phosphorus removal, phosphorus was released in mainly the anaerobic period, followed by phosphorus uptake in the subsequent aeration and non-aeration periods. Therefore, aerobic phosphorus removal and denitrifying phosphorus removal simultaneously occurred in the two SBRs. At the end of the anaerobic period, TP concentrations increased to 17.2 mg/L and 25.0 mg/L in SBR-A and SBR-F (Figure 3), respectively. Release of phosphate occurred in SBR-F (16.0 mg/L) with glucose supplementation, 2.8 times that in SBR-A (5.8 mg/L), leading to more intracellular carbon source being stored by PAOs for phosphorus uptake. Furthermore, SBR-F achieved a considerable improvement in phosphorus removal with a much lower effluent TP concentration of 0.8 mg/L at the end of the reaction period. By contrast, SBR-A had an effluent TP concentration of 6.6 mg/L. Accordingly, SBR-F achieved superior EBPR performance because the efficient use of carbon source and the fermentation products by the PAOs enhanced phosphorus release and uptake.

3.2. Metabolic Activity and Kinetic Assessment for Enhanced Biological Phosphorus Removal

We conducted batch experiments to evaluate the metabolic activities of PAOs in EBPR and EBPR-F during Phase II-B. Figure 4 presents the typical EBPR profiles. Figure 4 and Table 2 summarize the specific kinetic rates and stoichiometric parameters in the two SBRs. The anaerobic phosphorus release rate effectively indicated EBPR [44]. The phosphorus release rates for the two SBRs were in the range of those in conventional EBPR [5.6 to 31.9 mg P/(g VSS·h)] [36]. The phosphorus release rate, phosphorus uptake rate, and phosphorus uptake to phosphorus release ratio in SBR-F were substantially higher than in SBR-A (Table 2), suggesting a higher relative abundance of PAOs and/or PAO activity in SBR-F.

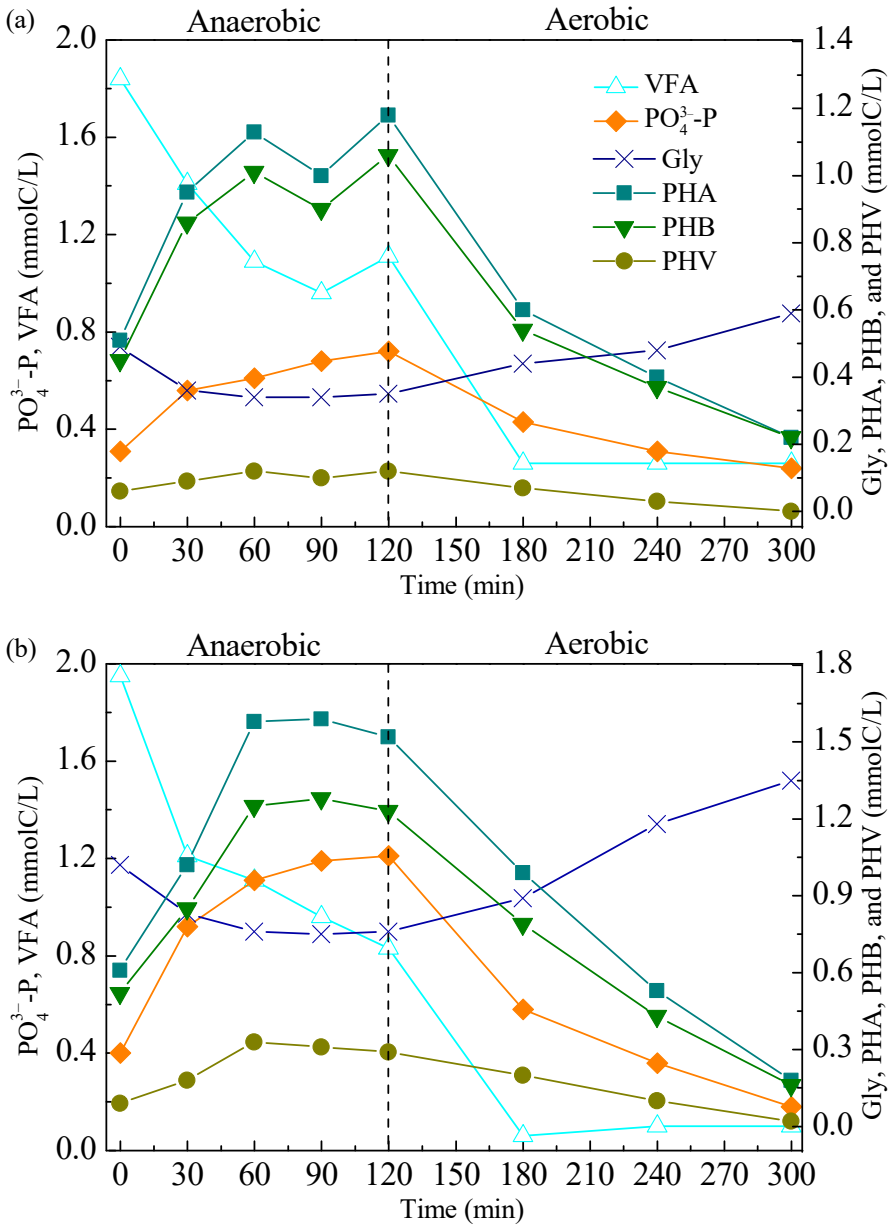

Figure 4. Variations in phosphate, volatile fatty acid (VFA), polyhydroxyalkanoate (PHA), and glycogen concentrations obtained from phosphorus release and uptake batch tests in (**a**) SBR-A and (**b**) SBR-F.

Table 2. Specific kinetic rates and stoichiometric parameters obtained from phosphorus release and uptake batch tests for the two sequencing batch reactors (SBRs).

	Parameters	Unit	SBR-A	SBR-F
Kinetic rates	VFA_{up}	mg C/(g VSS·h)	35.70	67.40
	P_{rel}	mg P/(g VSS·h)	12.60	24.50
	P_{up}	mg P/(g VSS·h)	7.30	15.00
	P_{up}/P_{rel}		0.78	0.85
Stoichiometric parameters	P/VFA	mol P/mol C	0.56	0.72
	Gly/VFA	mol C/mol C	0.19	0.23
	PHA/VFA	mol C/mol C	0.91	0.81
	P/PHA	mol P/mol C	0.50	0.77
	Gly/PHA	mol C/mol C	0.26	0.43

Note: VFA_{up}: The uptake rate of volatile fatty acids (VFAs). P_{rel}: The release rate of phosphorus. P_{up}: The uptake rate of phosphorus. P/VFA: The anaerobic phosphorus release/VFA uptake ratio. Gly/VFA: The anaerobic glycogen used to VFA uptake ratio. PHA/VFA: The anaerobic PHA generation to VFA uptake ratio. P/PHA: The aerobic phosphorus uptake to PHA used ratio. Gly/PHA: The aerobic glycogen used to PHA used ratio.

The phosphorus release/VFA uptake ratio (P/VFA) indicates the activities and relative abundance of PAOs and GAOs [50]. The ratio usually ranges from 0.01 to 0.93 mol P/mol C in EBPR [50]. The P/VFA ratios for SBR-A and SBR-F were 0.56 and 0.72 mol P/mol C, respectively, close to the values of the PAO model (0.5 to 0.75 mol P/mol C) [51,52], suggesting the dominance of PAOs in the two SBRs.

The ratios of anaerobic glycogen consumption to VFA uptake (Gly/VFA) demonstrate the energy and reducing power pathways using glycolysis and/or tricarboxylic acid (TCA) cycles in anaerobic metabolism [53,54]. Gly/VFA ratios of the activated sludge from SBR-A and SBR-F were within the range of the anaerobic PAO models for the glycolysis and TCA cycles (0.0 to 0.5 mol C/mol C). Gly/VFA in SBR-F (0.23 mol C/mol C) was higher than in SBR-A (0.19 mol C/mol C). Therefore, EBPR-F had a higher reliance of phosphorus removal on the glycolysis pathway over the TCA cycle, compared with conventional EBPR dominated by *Ca.* Accumulibacter. The glycolysis pathway was more efficient than the TCA cycle through additional PHA production and less phosphate release with substrate uptake, potentially benefiting EBPR [53].

For the ratio of anaerobic PHA generation to VFA uptake (PHA/VFA) in SBR-A, its value was close to the anaerobic PAO-TCA model [55]. SBR-F had a lower PHA/VFA (0.81 mol C/mol C) than SBR-A (0.91 mol C/mol C). The aerobic phosphorus uptake to PHA consumption ratio (P/PHA) in SBR-F was significantly higher than in SBR-A (0.77 and 0.50 mol P/mol C, respectively). The PHA/VFA and P/PHA ratios together indicated that EBPR-F does not rely on PHA for internal carbon storage and phosphorus removal with the presence of *Tetrasphaera*.

3.3. Microbial Community of Phosphate-Accumulating Organisms

To obtain a deeper insight into the community structures of key functional PAOs in the two SBRs, we performed FISH during Phase II-B (days 71 to 72). The microbial compositions in the two SBRs showed a remarkable difference in the distribution and relative abundance of PAOs (Figure 5 and Table 3). FISH showed that the mixed liquor in SBR-A contained 30.7% ± 4.3% of *Ca.* Accumulibacter and fewer *Tetrasphaera* (approximately 2%). By contrast, the mixed liquor in SBR-F comprised a mixed culture of *Ca.* Accumulibacter (44.7% ± 3.1%) and *Tetrasphaera* (18.1% ± 6.6%). Despite the differences in the relative abundance of *Ca.* Accumulibacter (30.7% in SBR-A and 44.7% in SBR-F), *Ca.* Accumulibacter was dominant in both SBRs. Additionally, compared with SBR-A dominated by *Ca.* Accumulibacter, the abundances of both *Ca.* Accumulibacter and *Tetrasphaera* significantly increased in SBR-F. Therefore, the combination of EBPR and in-situ fermentation shifted the microbial community structure in SBR-F. The change in the microbial community was consistent with the EBPR activities in the batch tests. The result indicates that in-situ fermentation is vital

in enriching PAOs (both *Ca.* Accumulibacter and *Tetrasphaera*) and phosphorus removal in EBPR-F.

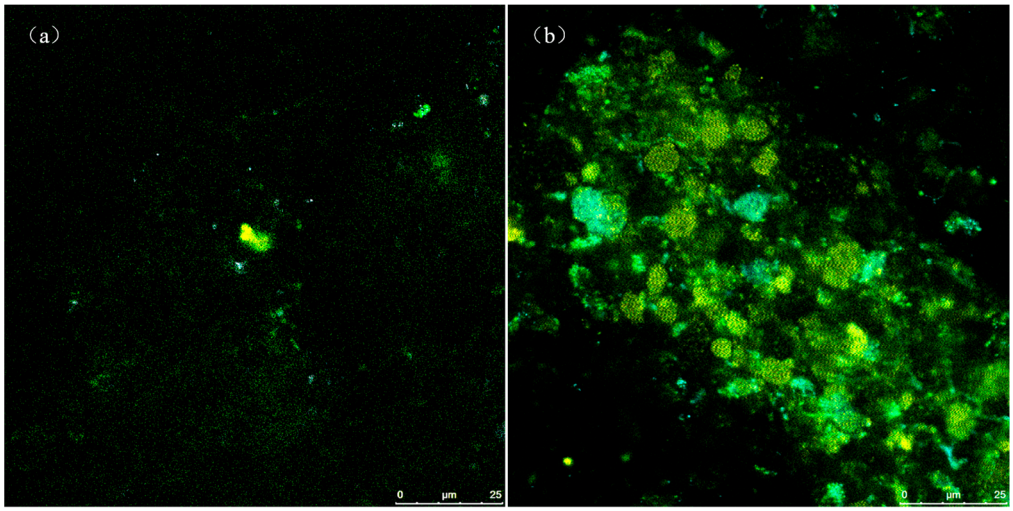

Figure 5. Fluorescence in situ hybridization images of the microbial community in the mixed liquor of (**a**) SBR-A and (**b**) SBR-F. **Yellow**: *Candidatus* Accumulibacter. **Cyan**: *Tetrasphaera*.

Table 3. Microbial populations in the mixed liquor of the two sequencing batch reactors (SBRs).

Relative Abundance	SBR-A (%) [a]	SBR-F (%) [a]
Candidatus Accumulibacter	30.7 ± 4.3	44.7 ± 3.1
Tetrasphaera	1.8 ± 1.7	18.1 ± 6.6

Note: [a]: Arithmetic means ± standard deviations.

3.4. Environmental Implications of This Study

Nutrient-laden wastewater, such as digested swine wastewater, is a serious burden to the environment. Reducing the levels of nutrients, especially phosphorus, below discharge limits in Nutrient-laden wastewater is challenging because of the high concentrations of nutrients and the low bioavailability of rbCOD. To solve this issue, we established an EBPR system coupled with in-situ fermentation (EBPR-F) (Figure 1). We also used fermentable substrates as external carbon sources to promote the fermentation and effectively remove phosphorus from real digested swine wastewater. Phosphorus removal of EBPR-F was compared with similar processes that were previously reported (Table 4). The traditional nutrient-removal processes (e.g., SBBR and A/O) had low phosphorus removal from digested swine wastewater, even with the addition of an organic carbon source (such as sodium acetate and raw swine wastewater) [14,56]. In the current study, 97.8% of the influent TP (42 ± 3 mg/L) was removed, and only 0.9 ± 0.6 mg/L TP remained in the effluent, satisfying the discharge standard of wastewater for the livestock and poultry breeding industry (GB 18596-2001).

Table 4. Comparison of the phosphorus removal of EBPR-F with similar processes.

Reference	Dan et al. [11]	Yang et al. [12]	Huang et al. [14]	Cai et al. [56]		Qi et al. [57]	This Study
Wastewater	Digested swine wastewater	Digested swine wastewater	Simulated digested swine wastewater	Mixture of raw swine wastewater and digested effluent		Digested swine wastewater	Digested swine wastewater
Configuration	ICEAS	A/O	SBBR	SBR	A/O	UF-MBR	EBPR-F

Table 4. *Cont.*

Reference	Dan et al. [11]	Yang et al. [12]	Huang et al. [14]	Cai et al. [56]		Qi et al. [57]	This Study
				Influent quality (mg/L)			
C/N	4.8	14.0	1.5	4.3	4.3	1.9	4.0
COD	2267.62	8375 ± 152	600	4328 ± 899	4328 ± 899	1009.50 ± 17.68	2000 ± 50
NH_4^+-N	476.35	603 ± 7.95	400	1010 ± 93.4	1010 ± 93.4	532.36 ± 5.24	400 ± 20
TP	415.34	216 ± 3.78	20	212 ± 58.1	212 ± 58.1	41.94 ± 0.41	42 ± 3
				Effluent quality (mg/L)			
COD	157.78	256 ± 6.23	50	414 ± 74.3	350 ± 48.5	509.16 ± 54.51	25 ± 14
NH_4^+-N	10.94	2.07 ± 0.79	1.79 ± 1.39	24.3 ± 36.5	57.1 ± 58.3	55.27 ± 5.72	2.0 ± 5.0
TP	52.46	29.27 ± 1.91	3.96 ± 0.82	111 ± 39.7	117 ± 56.8	10.87 ± 1.02	0.9 ± 0.6
				Removal (%)			
COD	93	97	91.7	89.9	91.5	49.6	98.8
NH_4^+-N	98	99	99.6	97.6	94.3	89.6	99.6
TP	87	86	83.7	47.5	44.7	74.1	97.8

Note: EBPR-F: Enhanced biological phosphorus removal coupled with in-situ fermentation. ICEAS: Intermittent cycle extended aeration system. A/O: Anoxic–oxic process. SBBR: Sequencing batch biofilm reactor. SBR: Sequencing batch reactor. UF-MBR: Ultrafiltration membrane bioreactor.

Compared with conventional EBPR dominated by *Ca.* Accumulibacter, EBPR-F promoted the anaerobic metabolism of PAOs. EBPR-R also had a considerably higher phosphorus release rate, phosphorus uptake rate, ratio of phosphorus uptake to phosphorus release, and P/VFA ratio. EBPR-F obtained a relatively higher Gly/VFA ratio than EBPR, suggesting a higher activity of the glycolytic pathway in producing reducing equivalents. The finding is consistent with studies on enriched *Tetrasphaera* culture [34,53,54]. EBPR-F enriched both *Ca.* Accumulibacter and *Tetrasphaera,* and the relative abundance of *Tetrasphaera* in EBPR-F was nine times that in EBPR.

This study achieved deep phosphorus removal by adding fermentable substrates to the influent. The fermentable substrates (particularly glucose) were added to improve the activity of *Tetrasphaera* and to promote in-situ fermentation. As a PAO, *Tetrasphaera* can ferment complex organic matter and provide carbon sources to enhance biological phosphorus removal. Since swine manure contains a high concentration of fermentable substrates, such as proteins, carbohydrates, and lipids [58,59], the manure was used as the external carbon source in the EBPR-F process. Swine manure treatment and advanced phosphorus removal from swine wastewater are urgent issues in the livestock and poultry industry. Introducing in-situ fermentation of swine manure in the bioreactors is promising to solve the issues with the advantages of saving carbon sources and remediating manure pollution.

4. Conclusions

EBPR coupled with in-situ fermentation (EBPR-F) was achieved in an SBR for advanced phosphorus removal from real digested swine wastewater with a low C/N mass ratio. Phosphorus removal was significantly enhanced by the synergistic effect of in-situ fermentation and supplemental carbon addition. With the addition of 100 mg/L glucose as the external carbon source, the average TP removal reached 97.8%, and the effluent TP concentration averaged 0.9 mg/L in EBPR-F, satisfying the discharge standard in China. Because of the in-situ fermentation, the anaerobic metabolism of PAOs was promoted, and the glycolytic pathway was enhanced in EBPR-F. The microbial community analysis showed enrichment of *Ca.* Accumulibacter and *Tetrasphaera* in EBPR-F with relative abundances of 44.7% ± 3.1% and 18.1% ± 6.6%, respectively. To further understand the fundamental mechanisms involved in EBPR-F, a more in-depth analysis of the microbial community structure is required. In addition, further studies on the combination of EBPR-F with swine

manure treatment would help solve the issues of carbon source deficiency and serious manure pollution in the livestock and poultry breeding.

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