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Performance Optimization of a Side-Stream Enhanced Biological Phosphorus Removal (S2EBPR) System Under Different Side-Stream Ratios

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Abstract

This study investigated the effects of side-stream ratio (SSR) on the performance of a laboratory-scale side-stream enhanced biological phosphorus removal (S2EBPR) system. The system was sequentially operated at SSRs of 10%, 15%, 20%, and 25%, followed by batch tests to evaluate substrate-dependent nitrogen and phosphorus transformation responses. COD and NH_4^+-N removal remained relatively high, whereas phosphorus removal was more sensitive to SSR variation. The average removal efficiencies of COD, NH_4^+-N, TN, and TP were 79.01%, 95.71%, 76.39%, and 77.31%, respectively. SSRs of 15–20% provided the most favorable balance between nutrient removal and operational stability, while the 25% SSR stage showed greater variability and poorer nutrient-removal performance. The maximum ammonium removal, nitrate production, and phosphorus removal rates were 1.1092, 0.4870, and 0.2268 mg/(L·h), respectively. Overall, SSRs of 15–20% represented the most favorable operating range under the tested conditions, providing experimental evidence for the operational regulation of S2EBPR systems.

Keywords: S2EBPR; side-stream ratio; phosphorus removal; nitrogen; phosphorus metabolism; process optimization

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Academic Editor: Athanasia K. Tolkou

Received: 20 October 2026
Revised: 11 November 2026
Accepted: 12 November 2026
Published: 14 November 2026

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1. Introduction

With the rapid progression of urbanization and increasingly stringent wastewater discharge regulations, municipal wastewater treatment systems are expected not only to achieve efficient removal of organic pollutants but also to enhance the control of nutrients, particularly nitrogen and phosphorus, to mitigate the risk of eutrophication in receiving water bodies. Enhanced biological phosphorus removal (EBPR) has become one of the most widely adopted phosphorus removal technologies in municipal wastewater treatment owing to its low chemical demand, cost-effectiveness, and environmental sustainability [1–3]. EBPR relies primarily on the unique metabolic capabilities of phosphate-accumulating organisms (PAOs). Under anaerobic conditions, PAOs hydrolyze intracellular polyphosphate to generate energy for the uptake of volatile fatty acids (VFAs), which are subsequently stored as polyhydroxyalkanoates (PHAs). During the subsequent aerobic or anoxic phase,

the stored PHAs serve as an internal energy source to drive excessive phosphate uptake, and phosphorus is ultimately removed through the discharge of phosphorus-rich waste sludge [4,5]. The metabolic mechanisms and engineering applications of EBPR have been extensively investigated.

Despite its advantages, the performance of conventional EBPR systems is highly dependent on the availability of readily biodegradable carbon sources. Both PAOs and glycogen-accumulating organisms (GAOs) compete for substrates such as acetate and propionate, whereas GAOs do not contribute to biological phosphorus removal through polyphosphate accumulation. Consequently, carbon allocation and competition between PAOs and GAOs play a decisive role in EBPR performance [6,7]. Previous studies have demonstrated that carbon source composition, temperature, and pH significantly influence the competitive interactions between these two functional groups, thereby affecting phosphorus removal efficiency [8,9]. Under municipal wastewater conditions characterized by low carbon-to-phosphorus (C/P) ratios or insufficient readily biodegradable organic carbon, the anaerobic carbon uptake and storage capacity of PAOs are restricted, resulting in reduced phosphorus uptake during the subsequent aerobic phase. Therefore, improving the utilization of internal carbon sources while reducing the dependence of PAOs on external readily biodegradable substrates remains a critical challenge for enhancing the stability of EBPR systems.

To overcome the carbon limitation and operational instability associated with conventional EBPR, side-stream enhanced biological phosphorus removal (S2EBPR) has attracted considerable attention in recent years. In this process, part or all of the return activated sludge (RAS) is diverted to an independent side-stream anaerobic reactor, where prolonged anaerobic hydrolysis and fermentation promote the generation of internal carbon sources before the sludge is returned to the mainstream treatment process. Full-scale investigations have demonstrated that S2EBPR can improve phosphorus removal efficiency and operational stability compared with conventional EBPR systems while potentially enhancing the competitive advantage of PAOs over GAOs [10]. A survey of multiple full-scale S2EBPR facilities in North America further confirmed the superior stability of phosphorus removal, although process performance varied among treatment plants because of differences in process configuration and operational conditions [11]. More recent studies have also shown that side-stream operational strategies, return sludge management, and the coordination between mainstream and side-stream processes substantially influence the phosphorus removal performance of S2EBPR systems [12,13].

Anaerobic hydrolysis and fermentation of side-stream sludge play a pivotal role in the redistribution of internal carbon within S2EBPR systems. Under anaerobic conditions, extracellular polymeric substances (EPS), proteins, carbohydrates, and other organic constituents in activated sludge are hydrolyzed and acidified, producing VFAs such as acetate and propionate. Previous studies have reported that both the yield and composition of VFAs are strongly affected by pH, sludge characteristics, fermentation time, and operational conditions [14,15]. A recent review further emphasized that VFA production during anaerobic fermentation is governed by multiple biological and environmental factors and that appropriate operational control is essential for maximizing carbon recovery [16]. Consequently, the amount of sludge diverted to the side-stream reactor not only determines the organic loading of the fermentation unit but also influences the intensity of hydrolysis and fermentation and the composition of substrates returned to the mainstream process.

EBPR performance is closely related to the metabolic activity of phosphate-accumulating organisms (PAOs) and the availability of suitable carbon substrates [17]. Previous studies have identified diverse PAO lineages and highlighted the complexity of microbial processes involved in biological phosphorus removal [18–21]. In S2EBPR systems, the side-stream ratio (SSR) is an important operational parameter that determines the proportion of re-

turn sludge subjected to side-stream treatment. Appropriate SSR regulation is essential for balancing sludge diversion, mainstream treatment performance, and overall nutrient removal efficiency.

Previous studies have demonstrated that the return activated sludge diversion ratio is an important operational parameter affecting S2EBPR performance. For example, Lv et al. [22] investigated the effects of different sludge diversion ratios in a continuous-flow S2EBPR system and identified an optimal diversion ratio of 8% under their experimental conditions. More recently, Gan et al. [23] further showed in pilot-scale return-sludge sidestream EBPR systems treating real domestic wastewater that sludge diversion ratio and side-stream retention time are interrelated operational parameters that require coordinated optimization. These findings indicate that an appropriate sludge diversion strategy is strongly dependent on reactor configuration, hydraulic conditions, influent characteristics, and other operating parameters.

As summarized in Table 1, previous investigations mainly focused on the effects of sludge diversion ratios and side-stream operating conditions on S2EBPR performance. Compared with these studies, the present study employed an SBR-based mainstream–side-stream coupled configuration and simultaneously evaluated the responses of COD, NH_4^+-N, NO_3^--N, TN, and TP removal to four SSRs (10%, 15%, 20%, and 25%). Furthermore, batch experiments were conducted following the gradient-SSR operation to characterize the substrate-dependent nitrogen and phosphorus transformation responses of the acclimated sludge. Rather than considering SSR optimization itself as a fundamentally new concept, the present study provides complementary experimental evidence regarding the relationships among sludge diversion, coupled nitrogen and phosphorus removal, substrate-loading responses, and operational stability in an SBR-based S2EBPR system.

Table 1. Comparison of previous S2EBPR studies and the present study.

Study	Reactor Configuration	Main Variables	Research Focus
Lv et al. [22] (2023)	Laboratory-scale continuous-flow S2EBPR	RAS diversion ratio	Phosphorus removal and diversion ratio optimization
Gan et al. [23] (2026)	Pilot-scale RSS-EBPR	Diversion ratio and side-stream HRT	Nitrogen and phosphorus removal and operational optimization
Present study	Laboratory-scale SBR-based S2EBPR	SSRs of 10%, 15%, 20%, and 25%	Nutrient removal, operational stability, and sludge transformation responses

Abbreviations: S2EBPR, side-stream enhanced biological phosphorus removal; RSS-EBPR, return-sludge sidestream enhanced biological phosphorus removal; SBR, sequencing batch reactor; RAS, return activated sludge; SSR, side-stream ratio; HRT, hydraulic retention time.

Accordingly, the objectives of this study were to: (i) evaluate the responses of COD, NH_4^+-N, NO_3^--N, TN, and TP removal and operational stability to different SSR conditions in an SBR-based S2EBPR system; (ii) identify a favorable SSR range under the investigated operating conditions; and (iii) integrate long-term operational performance with batch-test results to characterize the substrate-dependent nitrogen and phosphorus transformation responses of the acclimated sludge and evaluate possible explanations for the observed effects of SSR on phosphorus removal and operational stability.

2. Materials and Methods

2.1. Experimental Setup and Operation

A laboratory-scale side-stream enhanced biological phosphorus removal (S2EBPR) system was constructed to investigate the effects of different side-stream ratios (SSRs) on

nitrogen and phosphorus removal performance and operational stability. A schematic diagram of the experimental setup is shown in Figure 1. The system comprised two interconnected reactors, designated R1 and R2. R1 served as the mainstream sequencing batch reactor (SBR) and operated in a cyclic fill-and-draw mode, while R2 functioned as the side-stream sludge treatment reactor. A portion of the recirculated sludge was diverted to R2 according to the designated SSR. Both reactors operated as components of a single coupled treatment system rather than as independent parallel reactors.

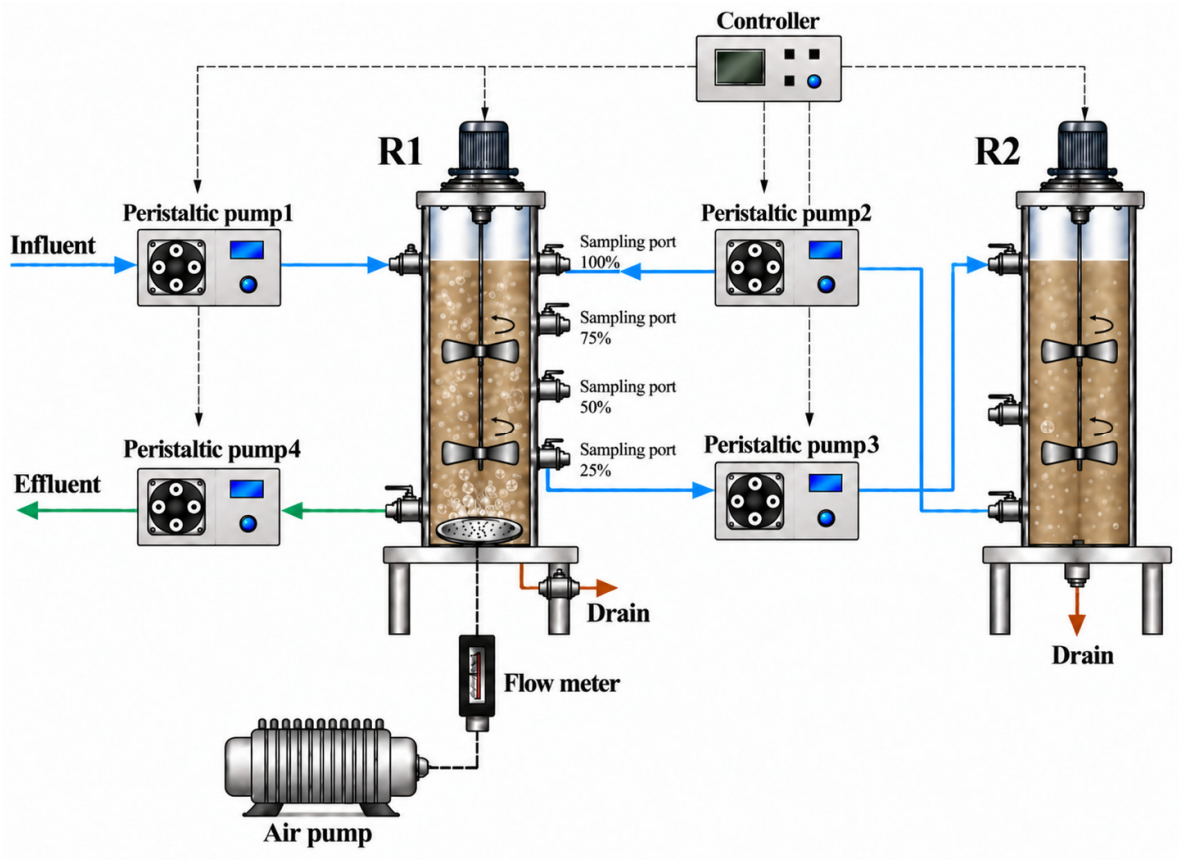


Figure 1. Schematic diagram of the experimental reactor system. Blue arrows indicate influent and sludge-transfer flows, whereas the green arrow indicates the effluent flow.

Both reactors were fabricated from acrylic material, each with a nominal total volume of 5 L and an effective working volume of 3.5 L. Each reactor was equipped with influent and effluent pumps (Model BT300-2J; Chuangrui Pump Industry, Baoding, Hebei, China), a mechanical stirrer (Model JBSH-7CS; Beijing Saiou Huachuang Technology Co., Ltd., Beijing, China), an aeration system (Model RB-001; Chuangrui Pump Industry, Baoding, Hebei, China), a portable dissolved oxygen (DO) meter (Model JPB-608A; Qingdao Xinye Environmental Protection Co., Ltd., Qingdao, Shandong, China), and a portable pH meter (Model PHBJ-260; Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China) for operational control and monitoring. Although the reactors were equipped with separate control devices, they were hydraulically connected through the sludge recirculation system and served distinct functions. R1 functioned as the mainstream sequencing batch reactor (SBR) for biological wastewater treatment, whereas R2 served as the side-stream sludge treatment reactor operated under low-oxygen conditions.

The mainstream reactor was designed to support organic matter degradation, biological nitrogen transformation, and phosphorus removal under alternating anaerobic and aerobic conditions. The side-stream reactor was incorporated into the sludge recirculation system to provide additional treatment of recirculated sludge under low-oxygen conditions.

According to established S2EBPR principles, side-stream sludge treatment may facilitate the transformation of sludge-associated organic matter and influence the availability of biodegradable substrates for subsequent phosphorus removal. However, volatile fatty acid (VFA) production, extracellular polymeric substance (EPS) degradation, and intracellular polyhydroxyalkanoate (PHA) accumulation were not directly quantified in this study. Therefore, these biochemical processes are considered potential mechanisms rather than experimentally confirmed responses.

Throughout the experimental period, the principal operating control settings were maintained as consistently as possible. The SSR was adjusted sequentially to investigate changes in pollutant removal performance and operational stability. However, changes in SSR may also have affected the distribution of recirculated sludge and the local hydraulic and solids loading conditions of the side-stream reactor. Furthermore, because the experiments were conducted sequentially in the same coupled system, temporal changes in biomass characteristics could not be completely excluded. Accordingly, the observed performance differences were interpreted as responses associated with the investigated SSR operating stages rather than effects attributable exclusively to SSR variation.

2.2. Inoculum Sludge and Synthetic Wastewater

Return activated sludge collected from the secondary clarifier of a municipal wastewater treatment plant was used as the inoculum. The inoculated sludge exhibited good biological activity and was initially acclimated to the S2EBPR operating mode to allow gradual adaptation to the anaerobic side-stream fermentation environment.

During the start-up period, system stabilization was achieved by gradually establishing alternating anaerobic/aerobic conditions in the mainstream reactor, promoting the adaptation of functional microorganisms to sludge recirculation, and enhancing the ability of PAOs to utilize fermentation products generated in the side-stream unit.

The system was considered to have reached steady-state operation when the effluent quality became relatively stable during continuous operation, particularly when only minor fluctuations were observed in TP, NH_4^+-N, and COD removal. Experiments with different side-stream ratios were then initiated.

Synthetic wastewater was prepared to simulate municipal wastewater. Sodium acetate, sodium propionate, and glucose were used as carbon sources, with sodium acetate serving as the primary carbon source. Ammonium chloride (NH_4Cl) and potassium dihydrogen phosphate (KH_2PO_4) were used as the nitrogen and phosphorus sources, respectively. Macronutrients, including $MgCl_2{\cdot}6H_2O$, $MgSO_4{\cdot}7H_2O$, KCl, and $CaCl_2$, and trace elements, including $CuSO_4{\cdot}5H_2O$, $ZnSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}H_2O$, and $FeSO_4{\cdot}7H_2O$, were also added to support microbial growth and metabolism.

The influent contained 300 mg/L sodium acetate and 100 mg/L glucose, while the concentrations of NH_4Cl and KH_2PO_4 were maintained at 20–30 and 4–10 mg/L, respectively. $NaHCO_3$ was used to adjust the influent pH to approximately 7.0, providing favorable conditions for PAO enrichment and metabolic activity. All chemicals and reagents used in this study were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Experimental Design

To systematically evaluate the effects of the side-stream ratio (SSR) on the performance of the S2EBPR system, long-term continuous experiments were conducted under different SSR conditions. The side-stream ratio (SSR) was defined as the proportion of return

sludge directed to the side-stream anaerobic fermentation reactor relative to the total sludge recirculation flow, which was calculated as follows:

$$SSR(\%) = \frac{Q_s}{Q_s + Q_m} \times 100\% \quad (1)$$

where:

Q_s: sludge flow rate entering the side-stream anaerobic fermentation reactor (mL/min);
Q_m: sludge flow rate returned directly to the mainstream reactor (mL/min).

The side-stream sludge flow was regulated intermittently using a calibrated peristaltic pump by adjusting the pump flow rate and operating duration. Different SSRs of 10%, 15%, 20%, and 25% were achieved by controlling the distribution of recirculated sludge between the side-stream and mainstream reactors. Because sludge recirculation was operated intermittently, the system was not maintained at a single continuously fixed RAS flow rate. Instead, the recirculated sludge volume was controlled by adjusting the pump flow rate and operating duration according to the designated SSR. The experimental program consisted of five operational phases, including an initial start-up phase (Phase I) followed by four sequential SSR operating phases corresponding to SSRs of 10%, 15%, 20%, and 25%. The operating phases and their corresponding conditions are summarized in Table 2.

Table 2. Operational phases of the S2EBPR system under different side-stream ratios.

Phase	SSR	Main Purpose	Duration
Phase I	0	Establish stable system operation	47
Phase II	10%	Evaluate the effect of a low SSR	21
Phase III	15%	Evaluate the effect of an intermediate SSR	21
Phase IV	20%	Evaluate the effect of a relatively high SSR	21
Phase V	25%	Evaluate the effect of a high SSR	21

Phase I served as the start-up stage and was primarily used to establish stable system operation of the coupled S2EBPR system. Subsequently, four SSR conditions were investigated sequentially, with each operating stage lasting approximately 21 days. The total duration of the four SSR operating stages was approximately 84 days, excluding the initial start-up period. Treatment performance was monitored during each stage, and steady-state operation was evaluated according to the criteria described below. Because the same biomass was retained throughout the successive operating stages, progressive biomass acclimation may have influenced the observed performance differences.

Steady-state operation was determined according to the following criteria: (1) effluent TP concentrations remained relatively stable over several consecutive operating cycles; (2) no substantial fluctuations occurred in COD, NH_4^+-N, or TN removal efficiencies; and (3) sludge concentration and settling characteristics remained stable.

2.4. SBR Operating Cycle and Control Conditions

The mainstream sequencing batch reactor (SBR) was operated with a hydraulic retention time (HRT) of 8 h. Each operating cycle comprised a 120 min combined feeding and anaerobic reaction phase, a 210 min aerobic reaction phase, a 140 min settling phase, and a 10 min decanting phase. Influent and effluent transfer were controlled using peristaltic pumps. The influent and decanting flow rates were approximately 250 and 500 mL/min, respectively, with equal influent and effluent volumes of approximately 2.5 L per cycle. The feeding operation was included within the 120 min combined feeding and anaerobic reaction phase rather than being treated as a separate operating phase. Mechanical mixing was provided during the anaerobic reaction phase, whereas aeration was supplied during the

aerobic phase to support organic matter degradation, ammonium oxidation, and biological phosphorus uptake.

The anaerobic phase was designed to provide conditions favorable for biological phosphorus release, whereas the subsequent aerobic phase supported biological phosphorus uptake and ammonium oxidation. According to established enhanced biological phosphorus removal (EBPR) principles, phosphate-accumulating organisms (PAOs) may utilize readily biodegradable organic substrates under anaerobic conditions and subsequently take up phosphorus during aerobic treatment. However, intracellular polyhydroxyalkanoate (PHA) accumulation, glycogen transformation, and polyphosphate cycling were not directly measured in this study. Therefore, these metabolic processes are discussed as potential mechanisms rather than experimentally verified responses of the investigated system.

Key environmental and operational parameters, including temperature, dissolved oxygen (DO), mixed liquor suspended solids (MLSS), and solids retention time (SRT), were monitored during the experimental period. During the gradient-SSR experiment, the MLSS concentration was maintained at approximately 3400–3600 mg/L. During the aerobic phase, the DO concentration in the mainstream reactor (R1) was controlled above 2.0 mg/L, whereas the side-stream reactor (R2) was operated under low-oxygen conditions with a DO concentration below 0.8 mg/L. The average DO concentrations in R1 and R2 were 3.38 ± 0.14 and 0.66 ± 0.12 mg/L, respectively. The aeration rate in R1 was approximately 0.2 L/min, and the overall system SRT was maintained at approximately 12 days through regular and quantitative sludge wasting.

Sludge recirculation between the mainstream and side-stream reactors was operated intermittently using a calibrated peristaltic pump. The recirculated sludge volume was controlled by adjusting both the pump flow rate and operating duration. Side-stream ratios of 10%, 15%, 20%, and 25% were established by regulating the proportion of recirculated sludge directed to the side-stream reactor. Therefore, the system was not operated at a single continuously fixed RAS flow rate.

During the gradient-SSR experiment, the influent COD concentration was approximately 400 mg/L. Based on an exchange volume of 2.5 L per cycle, three operating cycles per day, and an effective reactor volume of 3.5 L, the nominal organic loading rate was approximately 0.86 kg COD/(m^3·d). Although the principal operating control settings were maintained as consistently as possible, variations in SSR may have altered the local hydraulic and solids loading conditions in R2.

2.5. Water Quality Analysis

Influent, effluent, mainstream reactor, and side-stream reactor samples were collected periodically to evaluate pollutant removal performance.

The principal water quality parameters included chemical oxygen demand (COD), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), total nitrogen (TN), orthophosphate phosphorus (PO_4^{3-}-P), total phosphorus (TP), mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS).

All parameters were determined according to the Chinese standard analytical methods for water and wastewater and Standard Methods for the Examination of Water and Wastewater. Absorbance measurements were performed using a UV–visible spectrophotometer (Model UV-1900; Shanghai Jingqi Instrument Co., Ltd., Shanghai, China) and a full-wavelength microplate reader (Model HBS-SCANX; Nanjing Detie Biotechnology Co., Ltd., Nanjing, China).

2.6. Batch Experiments

Batch experiments were conducted following the gradient-SSR operational period to characterize the nitrogen and phosphorus transformation responses of the acclimated sludge to different substrate loading levels. These experiments were intended to evaluate substrate-dependent transformation characteristics of the tested sludge rather than to provide independent comparisons among sludge populations separately acclimated under each individual SSR condition.

The sludge used in the batch experiments was collected from both the mainstream reactor (R1) and the side-stream reactor (R2) following the gradient-SSR operation. A 200-mL sample of sludge-containing mixed liquor was collected from each reactor. The collected mixed liquor was centrifuged at 8000 rpm for 5 min using a high-speed centrifuge (Model TG16-WS; Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsha, China), and the supernatant was discarded to obtain the sludge stock for the batch tests. The resulting sludge stock was distributed among five 500-mL narrow-neck Erlenmeyer flasks. Subsequently, 10 mL of pH buffer solution was added to each flask to maintain the pH at 7.2–7.4. Ammonium chloride (NH_4Cl) and potassium dihydrogen phosphate (KH_2PO_4) were used as the nitrogen and phosphorus sources, respectively, with stock solutions prepared at 1000 mg/L. The flasks were placed in a temperature-controlled shaking incubator (Model ZQZY-78BN; Shanghai Zhichu Instrument Co., Ltd., Shanghai, China) operating at 150 rpm during the batch experiments.

Five initial NH_4^+-N/TP concentration combinations were established at 20/4, 25/6, 30/8, 35/10, and 40/12 mg/L, respectively. Samples were collected at 0, 2, 4, 8, and 24 h to monitor NH_4^+-N depletion, NO_3^--N production, and TP removal. The corresponding transformation rates were calculated from the concentration changes over time and the MLSS concentration. The experiments mainly investigated the anaerobic phosphorus release capacity, aerobic phosphorus uptake capacity, and ammonium oxidation capacity of the system.

Water samples were collected at regular time intervals during the experiments, and the concentrations of NH_4^+-N and PO_4^{3-}-P were determined. The corresponding transformation rates were calculated as follows:

$$R = \frac{\Delta C}{MLSS \times \Delta t} \quad (2)$$

where:

R: pollutant transformation rate per unit sludge mass;
ΔC: change in pollutant concentration;
MLSS: mixed liquor suspended solids concentration;
Δt: reaction time.

The metabolic response of the activated sludge to different substrate loadings was evaluated by comparing the metabolic rates under different nitrogen and phosphorus substrate concentrations after acclimation under gradient side-stream ratio conditions.

2.7. Data Processing and Statistical Analysis

Water samples were collected periodically at approximately 2–3-day intervals during each operating stage, with nine valid temporal observations included for each SSR condition ($n = 9$). Experimental results are expressed as mean ± standard deviation (SD). These observations represent repeated temporal measurements collected from the same coupled reactor system rather than independent biological or reactor replicates.

The homogeneity of variance was assessed using Levene's test. Differences among SSR conditions were evaluated using one-way analysis of variance (ANOVA) followed by

Tukey's HSD post hoc test when the homogeneity-of-variance assumption was satisfied. When this assumption was not satisfied, Welch's ANOVA followed by the Games–Howell post hoc test was applied. Statistical significance was defined as $p < 0.05$. Because the SSR conditions were sequentially imposed on the same reactor system, the statistical comparisons should be interpreted as comparisons among temporal observations obtained during different operating stages rather than as inferential comparisons among independently replicated reactor systems. All statistical analyses, including one-way ANOVA, Welch's ANOVA, Tukey's HSD, and Games–Howell post hoc tests, as well as linear regression analyses and figure preparation, were performed using Origin 2025b (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Effects of Side-Stream Ratio on S2EBPR Reactor Performance

To evaluate the effects of the side-stream ratio (SSR) on S2EBPR system performance, continuous operation experiments were conducted at different SSRs while maintaining comparable influent characteristics and other operating conditions. The removal performance of COD, NH_4^+-N, NO_3^--N, TN, and TP, together with variations in dissolved oxygen (DO) and pH, is shown in Figures 2–6. Overall, the system maintained relatively stable operation under all investigated SSR conditions, although the responses of individual pollutants to changes in SSR differed considerably, with TP showing the greatest sensitivity.

COD removal remained relatively stable throughout the experimental period, with an average removal efficiency of 79.01 ± 3.41%, indicating that organic matter removal was generally maintained under the investigated SSR conditions. Readily biodegradable organic matter was consistently removed at all SSRs, suggesting that organic matter removal in the mainstream reactor was largely unaffected.

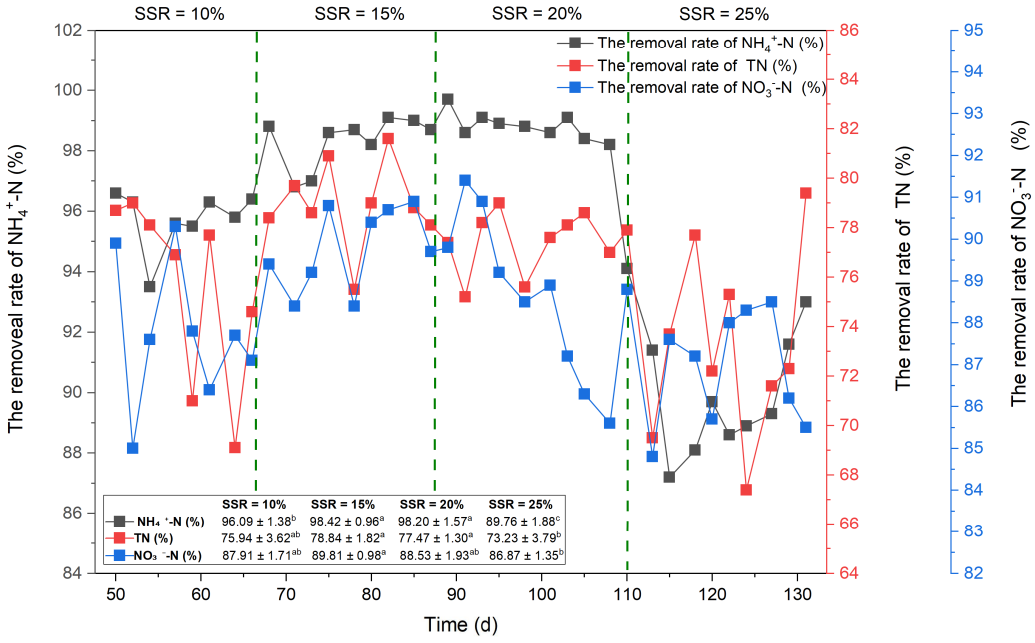


Figure 2. Temporal variations in NH_4^+-N, TN, and NO_3^--N removal efficiencies under different side-stream ratio (SSR) conditions. The inset shows mean ± SD values ($n = 9$ temporal observations per SSR condition); different lowercase letters within the same parameter indicate significant differences among SSR conditions ($p < 0.05$).

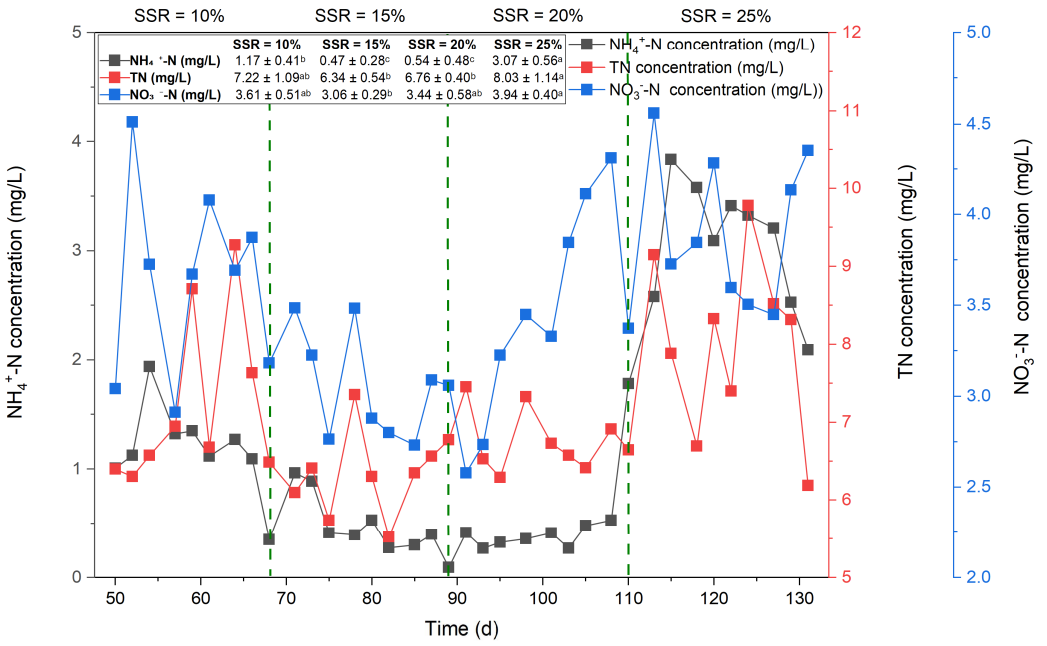


Figure 3. Temporal variations in NH_4^+-N, TN, and NO_3^--N concentrations under different side-stream ratio (SSR) conditions. The inset shows mean ± SD values (n = 9 temporal observations per SSR condition); different lowercase letters within the same parameter indicate significant differences among SSR conditions ($p < 0.05$).

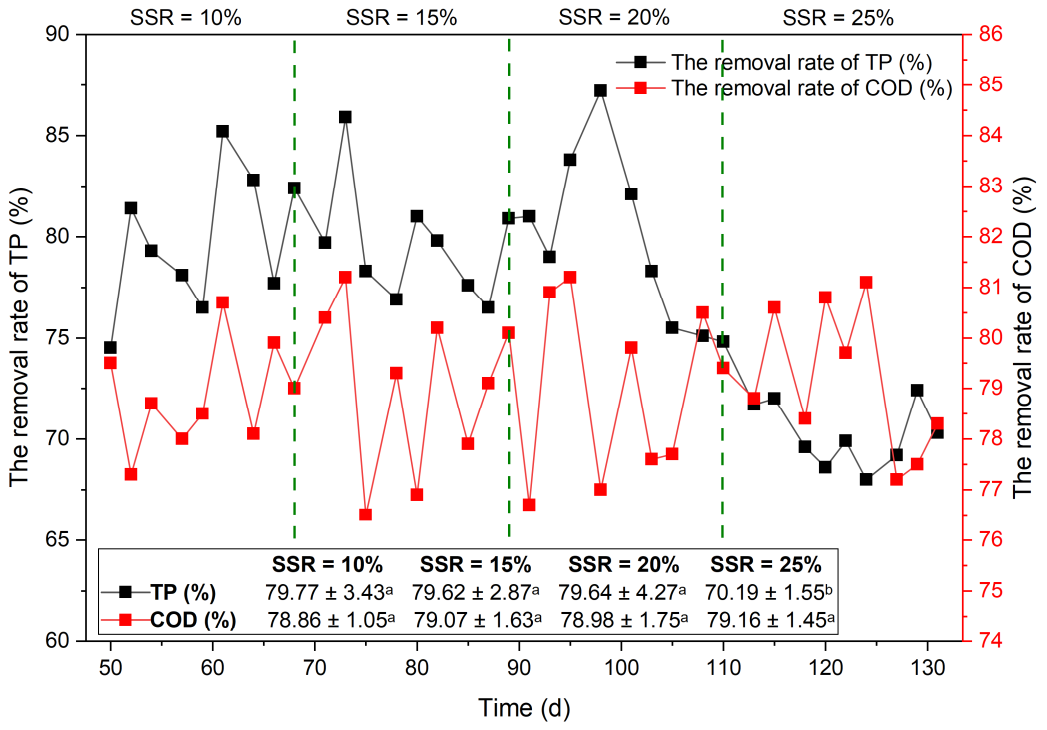


Figure 4. Temporal variations in TP and COD removal efficiencies under different side-stream ratio (SSR) conditions. The inset shows mean ± SD values (n = 9 temporal observations per SSR condition); different lowercase letters within the same parameter indicate significant differences among SSR conditions ($p < 0.05$).

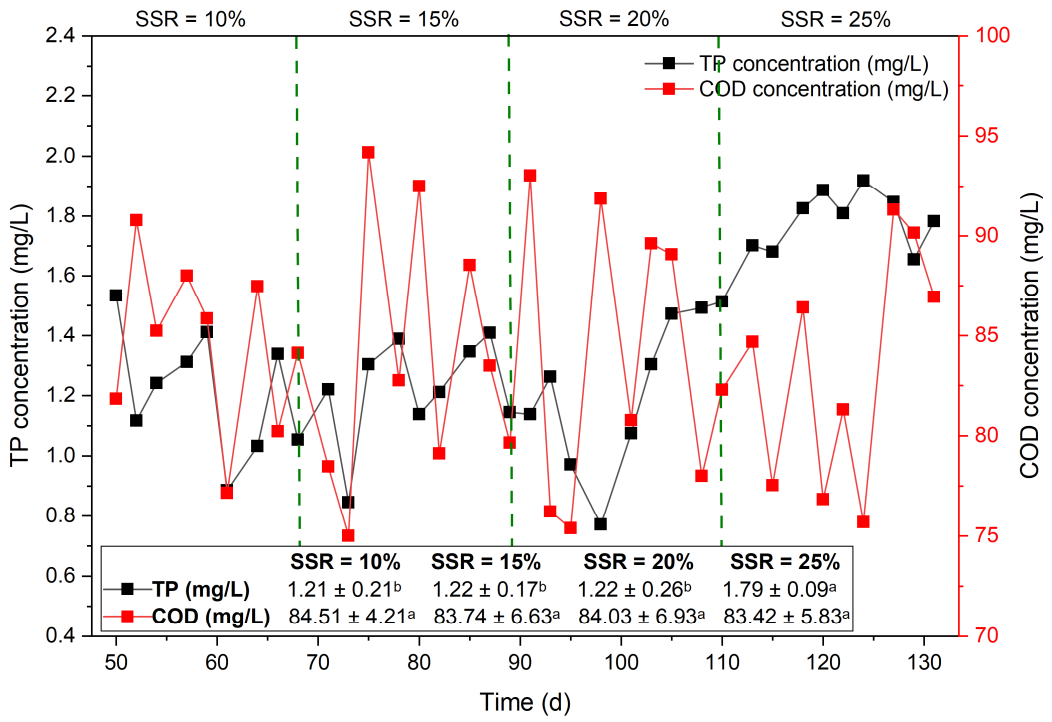


Figure 5. Temporal variations in effluent TP and COD concentrations under different side-stream ratio (SSR) conditions. The inset shows mean ± SD values (n = 9 temporal observations per SSR condition); different lowercase letters within the same parameter indicate significant differences among SSR conditions ($p < 0.05$).

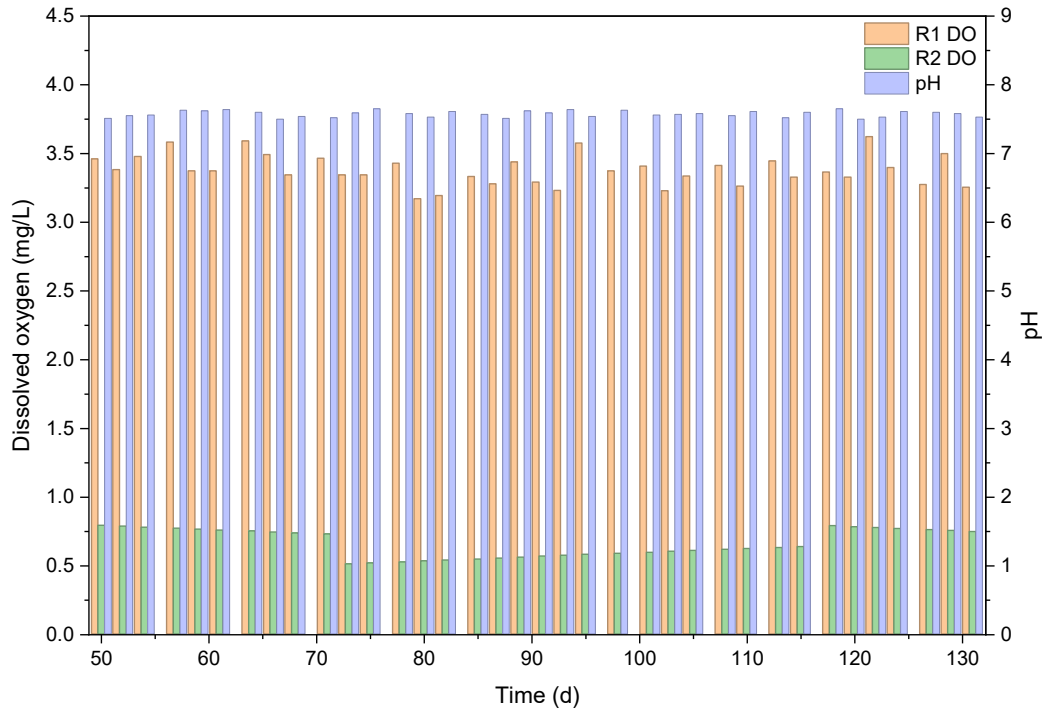


Figure 6. Variations in DO and pH in reactors R1 and R2 under different side-stream ratios.

Statistical analysis further confirmed the limited influence of SSR on COD removal. One-way ANOVA showed no significant difference in COD removal efficiency among the four SSR conditions ($F(3,32) = 0.066$, $p = 0.9775$). The mean COD removal efficiencies

were 78.86 ± 1.05%, 79.07 ± 1.63%, 78.98 ± 1.75%, and 79.16 ± 1.45% at SSRs of 10%, 15%, 20%, and 25%, respectively, and Tukey's HSD test assigned all four conditions to the same statistical group.

A similar result was obtained for effluent COD concentration. The mean effluent COD concentrations were 84.51 ± 4.21, 83.74 ± 6.63, 84.03 ± 6.93, and 83.42 ± 5.83 mg/L at SSRs of 10%, 15%, 20%, and 25%, respectively. One-way ANOVA indicated no significant difference among the four SSR conditions ($F(3,32) = 0.054$, $p = 0.9834$), with all groups again assigned to the same significance group by Tukey's HSD test. These results demonstrate that increasing SSR from 10% to 25% did not significantly affect COD removal performance under the conditions investigated.

Nitrogen removal also remained at a relatively high level. During the entire experimental period, the average effluent NH_4^+-N concentration was 1.314 ± 0.968 mg/L, corresponding to a removal efficiency of 95.71 ± 4.23%. The average TN concentration was 7.089 ± 1.034 mg/L, with a removal efficiency of 76.39 ± 4.37%, while the average NO_3^--N concentration was 3.516 ± 0.547 mg/L, with a removal efficiency of 88.29 ± 2.82%. Compared with Phase I, the overall removal efficiencies of NH_4^+-N and TN increased by approximately 3% and 1%, respectively, indicating that nitrogen removal generally remained favorable within the investigated SSR range.

Statistical analysis further demonstrated significant differences in nitrogen-removal performance among the SSR conditions. For NH_4^+-N removal efficiency, one-way ANOVA revealed a significant overall effect of SSR ($F(3,32) = 67.07$, $p < 0.0001$). Tukey's HSD test showed that the removal efficiencies at SSRs of 15% and 20% were significantly higher than those at 10%, whereas the 25% SSR exhibited the lowest value and differed significantly from the other three SSR conditions. Consistently, effluent NH_4^+-N concentration also differed significantly among SSR conditions ($F(3,32) = 66.79$, $p < 0.0001$), with the 25% SSR producing a significantly higher effluent concentration than the other SSR conditions and the 15% and 20% SSRs showing the lowest values.

For TN removal efficiency, the homogeneity-of-variance assumption was not satisfied; therefore, Welch's ANOVA was applied and indicated a significant SSR effect ($F(3, 16.6) = 5.55$, $p = 0.0080$). Games–Howell comparisons showed that the 15% and 20% SSR conditions had significantly higher TN removal efficiencies than the 25% SSR condition, while the 10% SSR showed an intermediate response and did not differ significantly from either group. A similar pattern was observed for effluent TN concentration, for which Welch's ANOVA also indicated a significant difference among SSR conditions ($F(3, 16.6) = 5.58$, $p = 0.0078$); the 25% SSR yielded significantly higher TN concentrations than the 15% and 20% SSR conditions.

For NO_3^--N removal efficiency, one-way ANOVA indicated a significant overall difference among SSR conditions ($F(3,32) = 5.77$, $p = 0.0029$). Tukey's HSD test showed that the 15% SSR condition differed significantly from the 25% SSR condition, whereas the 10% and 20% SSR conditions showed intermediate responses. Effluent NO_3^--N concentrations also differed significantly among SSR conditions ($F(3,32) = 5.77$, $p = 0.0029$), with the 15% SSR showing a significantly lower concentration than the 25% SSR, while the 10% and 20% SSR conditions did not differ significantly from either group.

Taken together, these results indicate that although nitrogen removal remained relatively high throughout the experimental period, the statistical comparisons revealed a deterioration in nitrogen-removal performance when the SSR was increased to 25%, whereas SSRs of 15–20% generally maintained more favorable nitrogen-removal performance.

In contrast, TP removal was more sensitive to changes in SSR. The mean effluent TP concentration was 1.362 ± 0.310 mg/L, corresponding to an average removal efficiency of 77.31 ± 5.26%. Statistical analysis confirmed a significant effect of SSR on TP removal

efficiency ($F(3,32) = 19.96$, $p < 0.0001$). Tukey's HSD test showed no significant differences among SSRs of 10%, 15%, and 20%, whereas the TP removal efficiency at an SSR of 25% was significantly lower than that at the other three SSR conditions. Consistently, effluent TP concentration also differed significantly among SSR conditions ($F(3,32) = 19.88$, $p < 0.0001$), with the concentration at 25% SSR being significantly higher than those at 10%, 15%, and 20% SSR. Thus, although some numerical fluctuations in phosphorus removal were observed within the 10–20% SSR range, these differences were not statistically significant, whereas a clear deterioration in phosphorus removal occurred when SSR was increased to 25%.

Overall treatment performance was most favorable at SSRs of 15–20%, at which the minimum NH_4^+-N and TP concentrations were approximately 0.267 and 0.771 mg/L, respectively. The selection of this favorable SSR range was based on the combined evaluation of nitrogen and phosphorus removal performance, statistical comparisons, and operational stability, rather than on the maximum removal efficiency of any single pollutant. Although COD removal remained relatively favorable at an SSR of 25%, statistical analysis showed no significant improvement in COD removal efficiency or effluent COD concentration at this SSR. In contrast, the 25% SSR was associated with significantly poorer TP removal and less favorable nitrogen-removal performance. Thus, SSRs of 15–20% provided a more favorable balance between nutrient removal performance and stable mainstream operation under the conditions tested. It should be noted that sludge production, sludge reduction, and dewaterability were not directly evaluated; accordingly, the favorable SSR range identified in this study is limited to the measured pollutant-removal performance and operational stability.

When SSR was further increased to 25%, pollutant-removal performance became less favorable, particularly for phosphorus. The difference between the highest and lowest temporal TP removal efficiencies exceeded 15 percentage points, and the stage-average TP removal efficiency at 25% SSR was significantly lower than those at SSRs of 10–20%. In contrast, overall removal performance at SSRs of 10–15% differed relatively little from that in Phase I. Taken together, these results suggest that increasing SSR beyond the favorable range did not provide additional benefits to organic matter removal and could adversely affect nutrient-removal performance and operational stability.

DO and pH remained relatively stable throughout the operating phases, indicating that the reactors were maintained under broadly comparable environmental conditions. The relatively stable DO and pH conditions indicate that the major environmental conditions remained broadly comparable among the experimental stages. Therefore, SSR was an important factor associated with the observed performance variations. However, because the SSR conditions were imposed sequentially on the same biomass, potential contributions from temporal biomass adaptation cannot be completely excluded. Among the measured pollutants, phosphorus removal responded most strongly to SSR, whereas COD and ammonium removal were comparatively insensitive. Under the conditions tested, an SSR of 15–20% provided the most favorable overall performance, while increasing the SSR to 25% increased operational variability without further improving treatment performance.

3.2. Nitrogen and Phosphorus Metabolic Characteristics of Sludge After Sequential Operation at Different SSRs

To characterize the substrate-loading responses of the sludge acclimated during the sequential gradient-SSR operation, batch experiments were conducted at different initial nitrogen and phosphorus concentrations. NH_4^+-N depletion and NO_3^--N production were used to characterize nitrification activity, whereas TP removal was used to assess phosphorus-removal activity. The results are shown in Figure 7 and Tables 3 and 4. Overall, the sludge showed nonlinear responses to nitrogen and phosphorus loading, with relatively

high transformation rates at intermediate substrate concentrations, indicating favorable substrate-loading ranges for the functional biomass.

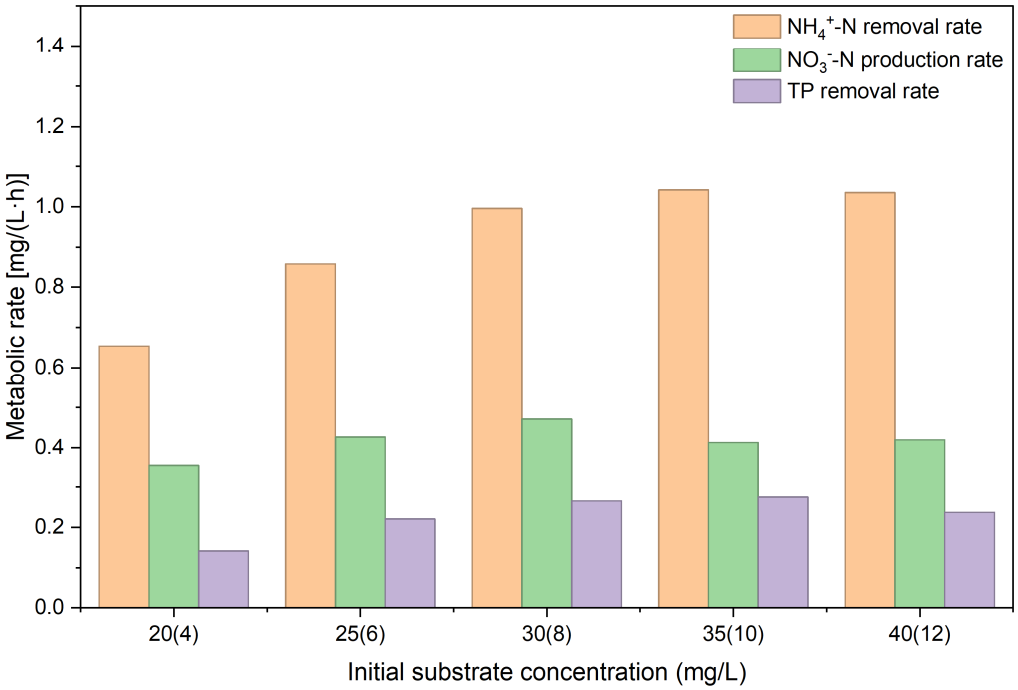


Figure 7. Nitrogen and phosphorus transformation rates of sludge collected after sequential gradient-SSR operation under different substrate loading conditions.

Table 3. Changes in nitrogen and phosphorus concentrations during batch experiments under different initial substrate concentrations.

Substrate Concentration (mg/L)	Time (h)	Mean NH_4^+-N Concentration (mg/L)	Mean NO_3^--N Concentration (mg/L)	Mean TP Concentration (mg/L)
20 (4)	0	17.410	0.318	3.719
	2	14.709	1.116	3.156
	4	10.918	2.789	2.414
	8	5.715	4.121	1.391
	24	0	8.514	0
25 (6)	0	22.201	0.521	5.408
	2	19.413	1.421	3.877
	4	15.113	2.517	3.172
	8	9.317	4.971	2.512
	24	0	10.109	1.127
30 (8)	0	25.752	0.628	7.307
	2	21.916	1.510	6.618
	4	16.418	2.719	5.109
	8	9.879	5.547	3.477

Table 3. *Cont.*

Substrate Concentration (mg/L)	Time (h)	Mean NH_4^+-N Concentration (mg/L)	Mean NO_3^--N Concentration (mg/L)	Mean TP Concentration (mg/L)
	24	1.195	12.291	2.102
35 (10)	0	32.541	0.591	9.119
	2	28.159	1.191	7.813
	4	23.621	2.811	6.727
	8	15.319	5.126	5.231
	24	4.401	10.896	3.109
40 (12)	0	35.712	0.478	10.769
	2	31.748	0.951	9.997
	4	27.371	2.113	8.527
	8	21.743	4.730	7.891
	24	9.099	11.391	5.291

Table 4. Nitrogen and phosphorus transformation rates of the tested acclimated sludge under different initial substrate concentrations.

Substrate Concentration (mg/L)	Nitrogen Transformation Rate [mg/(L·h)]				Phosphorus Removal Rate [mg/(L·h)]	
	NH_4^+-N Removal Rate	R^2	NO_3^--N Production Rate	R^2	TP Removal Rate	R^2
20 (4)	0.6781	0.8979	0.3302	0.9692	0.1457	0.9211
25 (6)	0.8839	0.9278	0.3955	0.9871	0.1756	0.8991
30 (8)	0.9537	0.9528	0.4870	0.9704	0.2024	0.9156
35 (10)	1.1092	0.9153	0.4296	0.9832	0.2268	0.9215
40 (12)	0.9501	0.9536	0.4367	0.9729	0.2017	0.8990

During nitrogen metabolism, the nitrifying community acclimated through long-term operation under different side-stream ratios was still capable of nearly completely oxidizing low concentrations of ammonium nitrogen (20–25 mg/L) within 24 h. However, when the initial NH_4^+-N concentration increased to 35 mg/L or above, appreciable residual ammonium remained after 24 h, indicating that the higher substrate loading had exceeded the optimal treatment capacity of the nitrifying community. Compared with Phase I, the acclimated sludge responded more rapidly to high ammonium concentrations, with more than 50% of ammonium removal occurring within the first 4–8 h. This response suggests improved tolerance of the nitrifying community to elevated ammonium loading after side-stream operation.

Further analysis of the metabolic rates showed that the ammonium removal rate and nitrate production rate did not reach their maximum values at the same substrate concentration. The maximum ammonium removal rate of 1.1092 mg/(L·h) was observed at an initial NH_4^+-N concentration of 35 mg/L, whereas the maximum nitrate production rate of 0.4870 mg/(L·h) occurred at 30 mg/L. Notably, the latter concentration was close to the relatively high influent ammonium concentration during long-term reactor operation, indicating that the sludge acclimated under varying side-stream ratios had adapted well to the substrate loading encountered during continuous operation.

Compared with nitrogen metabolism, phosphorus-accumulating organisms were more sensitive to changes in substrate concentration. The experimental results showed that phos-

phorus was almost completely removed within 24 h only at the lowest initial phosphorus concentration of 4 mg/L. As the initial phosphorus concentration increased further, varying amounts of residual phosphorus remained in all experimental groups. The phosphorus removal rate also initially increased and then decreased with increasing substrate concentration. The maximum phosphorus removal rate of 0.2268 mg/(L·h) was observed at an initial TP concentration of 10 mg/L, whereas the minimum rate was 0.1457 mg/(L·h). These results indicate that a moderate increase in phosphorus loading could enhance phosphorus uptake activity, whereas excessive phosphorus loading may exceed the effective metabolic capacity of phosphorus-accumulating organisms under the tested conditions.

Notably, the increases in nitrification and phosphorus-removal rates after sequential SSR operation were not accompanied by corresponding increases in overall TN and TP removal. Compared with Phase I, nitrate and TP removal efficiencies decreased by approximately 2% and 6%, respectively, whereas NH_4^+-N removal increased by approximately 3%. Thus, higher transformation rates did not necessarily translate into better reactor-scale removal. As SSR increased, greater amounts of sludge and associated liquid were returned from the side-stream reactor to the mainstream system, which may have altered the distribution of nitrogen, phosphorus, and biodegradable organic substrates between the two units. This redistribution may have increased the substrate loading on the mainstream process, exposing nitrifying organisms and PAOs to higher nutrient loads.

Taken together with the continuous-operation results, the batch tests indicate that an appropriate SSR was associated with favorable mainstream process stability and preserved nitrogen- and phosphorus-transformation activity. Possible changes in internal carbon availability may have contributed to these responses, although this mechanism was not directly quantified in the present study.

These batch experiments were designed primarily to characterize the substrate-loading responses of sludge acclimated during the gradient-SSR operation. They do not represent independent comparisons of sludge populations separately acclimated under each individual SSR condition. Therefore, the observed transformation rates should be interpreted as responses of the tested acclimated sludge to different substrate concentrations rather than as direct evidence of metabolic differences caused by individual SSR conditions.

3.3. Possible Mechanisms Underlying Enhanced Phosphorus Removal at Suitable Side-Stream Ratios

Long-term operation showed that pollutant removal initially improved with increasing SSR but became more variable at higher SSRs, indicating the existence of a suitable operating range. The batch tests also revealed clear concentration-dependent responses of the acclimated sludge to nitrogen and phosphorus loading, providing complementary information on sludge metabolic activity after long-term operation.

At an SSR of 10%, only a relatively small fraction of the return sludge was exposed to the side-stream environment. This may have limited the extent of sludge transformation and potentially reduced the availability of internally derived carbon. However, because VFA production and soluble COD release were not directly measured, this explanation cannot be experimentally confirmed from the present data. Consequently, although COD and NH_4^+-N removal remained high in the mainstream reactor, carbon availability for PAOs may have been insufficient to substantially improve TP removal. Overall treatment performance therefore differed little from that observed in Phase I.

When the SSR increased to 15–20%, a larger fraction of return sludge was exposed to the anaerobic side-stream environment. In principle, this could promote sludge hydrolysis and acidification and increase the availability of internally generated carbon, which may partly explain the improved TP removal observed at these SSRs. The batch tests also showed that

the sludge retained substantial nitrogen- and phosphorus-transformation capacity over the tested substrate ranges. Together, these observations suggest that SSRs of 15–20% provided a favorable operating range in which the benefits of side-stream treatment could be achieved without compromising mainstream stability.

At an SSR of 25%, treatment performance deteriorated and removal of NH_4^+-N and TP became more variable. The higher diversion ratio may have altered the distribution of sludge and pollutant loads between the side-stream and mainstream reactors. Thus, increasing SSR beyond 20% provided no further benefit for phosphorus removal and was associated with reduced long-term stability.

The operational and batch-test results suggest two related ways in which SSR may influence S2EBPR performance. Changing the fraction of sludge diverted to the side-stream reactor may alter the extent of anaerobic sludge transformation and internal carbon release, while also changing the distribution of material loads between the side-stream and mainstream reactors. At low SSRs, limited internal carbon release may constrain PAO metabolism; at high SSRs, the additional load returned to the mainstream reactor may reduce process stability. An intermediate SSR may therefore provide a better balance between these competing effects.

Overall, the continuous-operation results in Figures 2–6, together with the batch-test results in Figure 7, indicate that SSRs of 15–20% provided a suitable balance between nitrogen and phosphorus removal performance and reactor stability under the tested conditions. Possible changes in internal carbon availability may have contributed to the observed response, but this mechanism was not directly quantified.

Although the observed changes in nitrogen and phosphorus removal performance may be associated with alterations in anaerobic sludge transformation and internal carbon availability, these mechanisms were not directly verified in the present study. Parameters including VFAs, soluble COD, PHAs, glycogen, and EPS were not quantitatively measured. Therefore, the proposed mechanisms should be considered potential explanations rather than experimentally confirmed pathways. Further investigations incorporating biochemical characterization and microbial community analysis are required to elucidate these processes.

Because the different SSR conditions were investigated sequentially rather than simultaneously in independent parallel reactors, the potential influence of temporal changes in biomass characteristics and microbial adaptation cannot be completely excluded. Although the major operating conditions were maintained as consistently as possible, the observed variations may reflect the combined effects of SSR changes and long-term biomass adaptation. Future studies using independent parallel reactors and repeated operational sequences are necessary to further establish the causal relationships between SSR and reactor performance.

4. Conclusions

A laboratory-scale side-stream enhanced biological phosphorus removal (S2EBPR) system was operated under sequential side-stream ratios (SSRs) of 10%, 15%, 20%, and 25% to evaluate treatment performance, operational stability, and the substrate-dependent nitrogen and phosphorus transformation responses of the acclimated sludge.

(1) COD and NH_4^+-N removal remained high throughout the experiment, with average removal efficiencies of 79.01% and 95.71%, respectively. In comparison, phosphorus removal was more sensitive to changes in SSR, and significant deterioration in TP removal occurred during the 25% SSR stage.

(2) Batch experiments demonstrated clear substrate-dependent nitrogen and phosphorus transformation responses. The maximum ammonium removal rate was 1.1092 mg/(L·h) at an initial NH_4^+-N concentration of 35 mg/L, whereas the maximum nitrate produc-

tion rate was 0.4870 mg/(L·h) at 30 mg/L. The phosphorus removal rate increased initially and then decreased with increasing initial TP concentration, reaching a maximum of 0.2268 mg/(L·h) at 10 mg/L. These batch-test results characterize the responses of sludge collected after sequential gradient-SSR operation and should not be interpreted as independent comparisons among sludge populations acclimated under individual SSR conditions.

(3) Based on the combined evaluation of nitrogen and phosphorus removal performance, statistical comparisons, and operational stability, SSRs of 15–20% represented the most favorable operating range under the investigated laboratory conditions. Increasing the SSR to 25% did not provide additional treatment benefits and was associated with greater variability and poorer nutrient-removal performance, particularly for phosphorus.

(4) Because the SSR conditions were imposed sequentially on the same reactor system, the influence of SSR cannot be completely separated from temporal biomass adaptation. In addition, VFAs, soluble COD, PHAs, glycogen, EPS transformation, and microbial community composition were not directly quantified. Therefore, mechanisms involving anaerobic sludge transformation, internal carbon redistribution, and microbial responses remain potential explanations rather than experimentally verified pathways.

Accordingly, the 15–20% SSR range identified in this study should be considered specific to the present laboratory-scale SBR configuration and operating conditions rather than universally optimal. Further studies using actual municipal wastewater, independent parallel reactors, direct characterization of side-stream carbon transformation, and microbial community analysis are required to verify the underlying mechanisms and evaluate the broader applicability of the identified operating range.

Author Contributions: Conceptualization, X.Z., J.N. and Z.L.; Methodology, X.Z., J.N. and Z.L.; Validation, J.M.; Formal analysis, L.T. and J.M.; Investigation, X.Z., L.T., T.P. and J.M.; Resources, T.P., J.N. and S.P.; Data curation, X.Z. and L.T.; Writing—original draft, X.Z. and T.P.; Writing—review & editing, X.Z., J.M. and Z.L.; Visualization, X.Z. and L.T.; Supervision, J.N., S.P. and Z.L.; Project administration, J.N. and Z.L.; Funding acquisition, X.Z. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the Xihua University Talent Introduction Program (No. RX2400002018) and the Open Research Subject of Key Laboratory of Fluid Machinery and Engineering (Xihua University), Sichuan Province (grant number LTJX-2025007).

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: Author Tao Pan was employed by the Sichuan Deyun Environmental Group Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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