

Article

Sludge Retention Time Is Associated with EPS and Microbial Community Shifts That Enhance Carbon Capture in a Contact Stabilization Process Treating Synthetic Food-Processing Wastewater

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Abstract

A considerable portion of influent organic carbon is lost as CO₂ in conventional activated sludge processes, diminishing the resource potential of wastewater. To clarify how sludge retention time (SRT) is associated with carbon capture in a contact stabilization (CS) process treating synthetic food-processing wastewater (COD $\approx$ 800 mg·L⁻¹), a CS system was operated at SRTs of 1, 4, and 7 d. Extracellular polymeric substances (EPS), microbial community structure, removal of different COD fractions, and carbon flow distribution were systematically analyzed. Results indicated that SRT was significantly associated with EPS organization, microbial cooperation, and carbon partitioning. At SRT = 4 d, the system achieved the best carbon capture performance, with total EPS content reaching 43.14 mg·gVSS⁻¹ and a more pronounced TB-EPS-dominated structure. Polysaccharides accumulated during the contact phase (38.42 mg·gVSS⁻¹) and were effectively utilized during the stabilization phase (a reduction of 48.9%). Bacteroidota and key functional genera, including *Flavobacterium*, *Zoogloea*, and *Acidovorax*, were enriched. Fractionated COD analysis showed that an intermediate SRT promoted coordinated removal and sludge-phase transfer of particulate, colloidal, and soluble organics. Carbon flow analysis indicated that SRT = 4 d achieved the highest sludge carbon recovery (31.1%) with lower oxidation loss (31.9%). Overall, an appropriate SRT coincided with enhanced carbon capture and redirection by optimizing EPS structure, microbial community assembly, and carbon metabolic allocation.

Keywords: contact stabilization process; synthetic food-processing wastewater; sludge retention time; carbon capture; extracellular polymeric substances; microbial community



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1. Introduction

Traditional activated sludge processes mainly rely on aerobic oxidation to remove organic matter from wastewater. Although this can ensure the quality of effluent, it also causes a considerable portion of the influent organic carbon to be lost in the form of CO₂, diminishing the resource potential of organic carbon in wastewater treatment [1,2]. Therefore, how to minimize upstream carbon oxidation loss and improve the transfer and retention efficiency of organic carbon to the sludge phase while meeting treatment requirements has become a key issue for the low-carbon and resource-based development of wastewater treatment. In recent years, high-rate activated sludge (HRAS) processes have

received widespread attention because they can enhance carbon capture under conditions of short sludge age, low dissolved oxygen, and short hydraulic retention time [3]. However, most of these studies have been conducted using municipal wastewater or low-strength wastewater. For high-strength industrial effluents, such as those from food processing, the high organic load and complex composition pose significant challenges to the stability and efficiency of carbon capture in HRAS systems, and the underlying mechanisms remain poorly understood [4,5]. Among the various HRAS configurations, the contact stabilization (CS) process separates the rapid adsorption and aggregation of influent organic matter from the sludge activity recovery process through a two-stage “contact–stabilization” operation, providing favorable conditions for the efficient transfer of organic carbon from the liquid phase to the sludge phase [1,6].

Existing research on HRAS and high-rate contact stabilization (HiCS) mainly focuses on three aspects: operation parameter regulation, carbon capture mechanism and process enhancement. Studies have shown that sludge retention time (SRT), hydraulic retention time (HRT) and dissolved oxygen (DO) are key parameters that determine the removal of particulate and colloidal chemical oxygen demand (COD) and the overall carbon management efficiency of high-rate systems [2]; the “starvation-satisfaction” alternating environment formed under short SRT conditions helps to improve the adsorption capacity and bioflocculation performance of sludge, thereby enhancing carbon redirection [6]. At the same time, extracellular polymeric substances (EPS) and microbial communities are also considered to be important intrinsic factors affecting carbon capture. Kinyua et al. [7] pointed out that EPS is not only the structural basis for maintaining the stability of sludge aggregates, but also directly participates in the process of colloidal organic matter interception and carbon retention. Fu et al. [8] further showed from the microscopic level that during the operation of HiCS, the extracellular adsorption capacity, intracellular storage behavior and microbial community structure will change synergistically, indicating that carbon capture is essentially the result of the coupling of structural processes and biological processes. Despite these advances, the specific role of SRT in shaping the microbial community and EPS characteristics under high organic loading, and how this in turn influences the partitioning of different COD fractions, has not been systematically elucidated. For instance, the dynamic trade-off between carbon storage as intracellular polymers and as structural EPS components under different SRTs is still unclear.

Nevertheless, existing research mainly focuses on municipal wastewater or low-concentration wastewater, and the understanding of carbon capture behavior of CS processes under higher organic loading remains relatively limited [1,2,6,8]. Previous studies have shown that when the proportion of soluble organic matter in the influent is high, the removal and recovery capacity of HiCS processes for organic carbon will decrease significantly, indicating that the composition of the influent will significantly affect the sludge metabolic response and carbon flow distribution [4]. In addition, although some studies have pointed out that SRT is one of the core parameters determining the carbon capture performance of high-load systems [2,6,9,10], under such higher organic loading conditions, how SRT affects the distribution pattern of organic carbon among sludge recovery, effluent residue and oxidation loss by regulating EPS characteristics, microbial community structure and the migration and transformation of different forms of COD remains to be systematically explained.

Based on this, this study focuses on the CS process treating synthetic food-processing wastewater ($\text{COD} \approx 800 \text{ mg} \cdot \text{L}^{-1}$), setting up three SRT conditions of 1 day, 4 days, and 7 days. We hypothesize that an optimal SRT exists that balances rapid substrate uptake, EPS production, and microbial community synergy, thereby maximizing carbon capture. Specifically, we propose that at a moderate SRT, the system will exhibit: (i) enhanced EPS

production with a higher proportion of tightly bound EPS (TB-EPS), facilitating bioflocculation and adsorption; (ii) a more even and functionally synergistic microbial community, enriched in genera capable of both substrate degradation and storage; and (iii) a redirection of carbon flow from oxidation to sludge-phase recovery. The study systematically analyzes the characteristics of sludge EPS, changes in microbial community, the removal behavior of different forms of COD, and the carbon flow distribution patterns. The aim is to reveal the association of SRT with carbon capture and carbon redirection in the CS process, and to construct a coupled explanatory framework of “EPS characteristics–microbial ecology–carbon flow,” providing a theoretical basis for the operational optimization of the CS process for synthetic food-processing wastewater.

2. Materials and Methods

2.1. Materials and Preparation

The inoculum sludge used in the experiment was taken from the downstream section of the aerobic tank at a large municipal wastewater treatment plant in Shenyang, China. After collection, it was filtered through 60-mesh and 120-mesh screens to remove sand, hair, and other larger impurities. The initial mixed liquor suspended solids (MLSS) concentration of the inoculum sludge was $2943 \text{ mg}\cdot\text{L}^{-1}$, and the sludge volume index (SVI) was $83 \text{ mL}\cdot\text{g}^{-1}$. Before formal operation, the sludge underwent acclimatization in the reactor: firstly, it was continuously aerated for 2 days to restore activity; during this period, sedimentation was carried out twice daily, morning and evening, the supernatant was discharged, and fresh tap water was added, while removing the upper layer of aged or floating sludge; after the sludge color changed from dark black to yellowish-brown and the settling performance stabilized, experimental water was used for continuous acclimatization until the system operated stably before entering the formal experimental stage. This pretreatment step ensured consistent biological initiation conditions across different SRT operating conditions.

The influent was artificially simulated food-processing wastewater to ensure stable influent quality and improve experimental repeatability. Soluble starch was selected not only because it is a major component of food-processing wastewater, but also because its polymeric nature causes it to exist predominantly in particulate and colloidal forms, representing the slowly biodegradable COD fraction—in contrast to glucose, which would only represent soluble, readily biodegradable COD [11]. Following the water quality settings of food-processing wastewater, soluble starch and sodium acetate were used as the carbon source to simulate particulate/colloidal organic matter and readily biodegradable dissolved organic matter, respectively. Urea was used as the nitrogen source, and trisodium phosphate dodecahydrate as the phosphorus source. Additionally, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, CaCl_2 , and trace elements such as $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4\cdot \text{H}_2\text{O}$, $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, and $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$ were added to meet the needs of microbial growth and metabolism (Table 1). The nutrient ratio of the influent was controlled at $\text{COD}:\text{TN}:\text{TP} \approx 100:5:1$, and the pH was adjusted to 7.0 ± 0.5 using HCl or NaOH. To ensure reproducibility, the synthetic wastewater was prepared with the following nominal concentrations: soluble starch ($\sim 800 \text{ mg}\cdot\text{L}^{-1}$), sodium acetate ($\sim 550 \text{ mg}\cdot\text{L}^{-1}$), ammonium sulfate ($\sim 190 \text{ mg}\cdot\text{L}^{-1}$), and trisodium phosphate dodecahydrate ($\sim 60 \text{ mg}\cdot\text{L}^{-1}$). The complete composition of the synthetic wastewater is provided in Table S1 in the Supplementary Materials. The target influent COD was set at approximately $800 \text{ mg}\cdot\text{L}^{-1}$ to simulate elevated organic load scenarios such as food-processing wastewater [4,5,12]. Due to the particulate nature of starch and the inherent variability in small-scale batch preparation, actual measured influent COD ranged from approximately 1000 to $1200 \text{ mg}\cdot\text{L}^{-1}$ across different experimental runs; this range was consistently adopted as the representative influent COD level for subsequent experiments.

Table 1. Composition of elements in synthetic wastewater.

No.	Element Type	Component	Target Concentration (mg·L ⁻¹)
1	Macroelement	MgSO ₄ ·7H ₂ O	125
2		CaCl ₂	35
3	Trace Element	FeSO ₄ ·7H ₂ O	7.5
4		MnSO ₄ ·H ₂ O	1.25
5		ZnSO ₄ ·7H ₂ O	0.3
6		CuSO ₄ ·5H ₂ O	0.03

The main operating and testing equipment includes an acrylic reactor, peristaltic pump, constant speed stirrer, air pump, and rotor flow meter, as detailed in Table 2. All conventional instruments were calibrated or functionally checked according to their instruction manuals before the experiment to reduce systematic errors and ensure the comparability of measurement results.

Table 2. Main experimental instruments and equipment.

No.	Equipment Name	Model	Manufacturer
1	Rotameter	LZB-3WB	Xiangjin Flow Instrument Factory (Shenyang, Liaoning, China)
2	Constant-Speed Stirrer	JJ-1	Jintan Kexi Instrument (Jintan, Jiangsu, China)
3	Peristaltic Pump	BT100-2J	Baoding Longer Precision Pump Co., Ltd. (Baoding, Hebei, China)
4	Air Pump	ACO-5504	Guangdong Haili Electromechanical Co., Ltd. (Foshan, Guangdong, China)
5	Solenoid Valve	2W-025-08	Yuyao Lingtong Electric Appliance Co., Ltd. (Yuyao, Zhejiang, China)
6	Low-Temperature High-Speed Centrifuge	TGL-20M	Changsha Xiangyi Centrifuge Instrument Co., Ltd. (Changsha, Hunan, China)
7	Electronic Balance	ME204E	METTLER TOLEDO (Shanghai, China)
8	Dissolved Oxygen Meter	HQ30d	HACH (Shanghai, China)
9	pH Meter	PHS-3C	Shanghai Leici Instrument Co., Ltd. (Shanghai, China)
10	Visible Spectrophotometer	722S	Shanghai Jinghua Technology Instrument Co., Ltd. (Shanghai, China)
11	Fluorescence Microscope	BX53	Olympus (China) Co., Ltd. (Beijing, China)

2.2. Experimental Set-Up

This study used a laboratory-scale fully mixed reactor to simulate the CS process. The reactor body was a cylindrical plexiglass structure, 30 cm high and 15 cm in inner diameter, with an effective volume of 4.4 L. The reactor was equipped with an inlet, an automatic drain outlet, and an emergency manual drain outlet along its height: the inlet was located approximately 10 cm from the bottom and connected to a distribution tank via a peristaltic pump; the automatic drain outlet was located approximately 15 cm from the bottom and connected to a solenoid valve for programmed drainage; the emergency drain outlet was located approximately 25 cm from the bottom for manual sludge removal or drainage in abnormal situations. A paddle-type mechanical stirrer was installed at the top of the reactor to ensure thorough mixing of the mixture during the contact and stabilization phases [1,4,6].

The oxygen supply system consists of a silent air pump, a rotor flow meter, connecting pipelines, and microporous aeration discs. During the stabilization phase, aeration and

stirring are activated simultaneously to create an aerobic regeneration environment; during the contact phase, only stirring is activated, and the mixed liquor is controlled at a low oxygen level to enhance rapid contact, bioflocculation, and carbon source adsorption between the returned sludge and the influent organic matter. During the experiment, a portable dissolved oxygen meter was used to monitor the dissolved oxygen (DO) of the mixed liquor daily, and the DO was maintained at $0.4\text{--}0.6\text{ mg O}_2\cdot\text{L}^{-1}$ during the contact phase and $1.5\text{--}2.0\text{ mg O}_2\cdot\text{L}^{-1}$ during the stabilization phase by adjusting the air flow rate [6,10,13–15].

The reactor was operated at room temperature ($25 \pm 2\text{ }^\circ\text{C}$). The entire reaction system sequentially completes seven stages—influent, contact, sedimentation, drainage, recirculation, stabilization, and idle—through a timed control program. This simulates the segmented operation mode of the traditional CS process, “contact tank—stabilization tank,” within a single reactor, as shown in Figure 1. In a typical operating cycle, after the influent is finished, the system enters the contact stage, where the sludge rapidly adsorbs organic matter under low-oxygen and agitation conditions. Subsequently, sedimentation and drainage occur. After recirculation, the settled sludge enters the stabilization stage, where it regains its activity and metabolizes the organic matter adsorbed or temporarily stored in the contact stage under aerobic conditions. Finally, after a brief idle period, the system enters the next cycle [1,5,6,10,16].

Schematic Diagram of the Contact Stabilization (CS) Experimental Apparatus

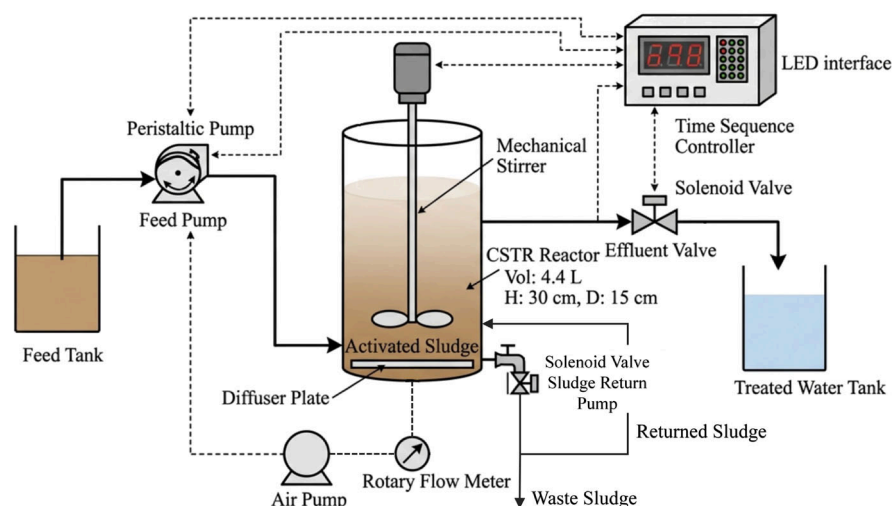


Figure 1. Schematic diagram of the experimental setup.

2.3. Operating Conditions and Experimental Procedure

After completing the acclimation of inoculated sludge, preparation of simulated wastewater, and construction of the reaction system, comparative experiments were conducted under different SRT conditions to investigate the effects of SRT on the operating behavior and carbon capture characteristics of the CS process under the tested conditions [4,5]. Considering that the focus of this paper is to reveal the regulatory mechanism of SRT on carbon capture and carbon redirection under the tested conditions, subsequent mechanistic studies will consistently use the synthetic food-processing wastewater and set SRTs of 1 d, 4 d, and 7 d, representing very short, moderate, and long sludge ages, respectively. The 1 d SRT is a representative parameter of the high-rate HiCS operating range, designed to explore the potential and limitations of ultra-short SRT on microbial selection and carbon capture efficiency [17]. The 4 d SRT, as the mid-point of the widely recognized balance operating window for carbon-oriented activated sludge systems, is set to investi-

gate the performance plateau where carbon redirection efficiency and sludge stability are optimized [18]. The 7 d SRT serves as the conventional stable baseline for activated sludge systems, providing a reference benchmark for evaluating the performance variation across different SRT scenarios [18]. Different SRTs were achieved by adjusting the daily sludge discharge of the system, while other operating conditions remained consistent [2,5,6,9,10].

In the formal experimental phase, the reactor operation cycle was fixed at 6 h, with each cycle consisting of 10 min of influent, 45 min of contact, 30 min of sedimentation, 10 min of effluent discharge, 10 min of recirculation, 245 min of stabilization, and 10 min of idle time. The 6-h cycle was chosen because it demonstrated good pollutant removal efficiency and sludge activity in previous studies, and is beneficial for highlighting the regulatory role of SRT [1,5,6,10,19]. During operation, the influent stage was supplied by a peristaltic pump; the contact stage only involved stirring to maintain low-oxygen conditions, allowing the returned sludge to fully contact the influent and achieve rapid adsorption of organic matter; the sedimentation and effluent stages completed sludge-water separation; subsequently, the sludge was recirculated into the stabilization stage, where it regained activity under aeration and stirring conditions and further metabolized the organic matter adsorbed or temporarily stored in the contact stage; the idle stage served as a buffer between adjacent cycles. Through this sequential operation, a single reactor can simulate the segmented function of the traditional CS process's "contact tank–stabilization tank".

All operating conditions were sampled and analyzed after the system had stabilized. Steady-state conditions for each SRT were considered achieved when the variation in effluent COD and sludge concentration (MLSS) remained within $\pm 10\%$ for at least five consecutive cycles [1,6]. Additionally, the duration of operation to reach steady state under each SRT condition was recorded and is reported in the Supplementary Materials. Stability was defined as minimal fluctuations in effluent COD, sludge concentration, and settling properties over multiple consecutive cycles, with SVI and MLVSS/MLSS remaining within relatively stable ranges. Routine water quality and sludge physicochemical indicators were measured using parallel samples; EPS extraction, fluorescence microscopy, microbial community analysis, and COD determination of different forms were all performed within a unified sampling window. To characterize the sludge response at different stages, sampling was conducted at the end of the contact stage and the end of the stabilization stage to analyze the structural and metabolic changes during the rapid carbon source adsorption/temporary storage process and sludge regeneration and activation process.

2.4. Water Quality and Sludge Analyses

Routine water quality indicators for influent and effluent were determined according to the corresponding standard methods in "Methods for Monitoring and Analysis of Water and Wastewater" (Fourth Edition, State Environmental Protection Administration of China, 2002), with the detection limits for each parameter being followed as per the standard protocols (e.g., COD detection limit of $5 \text{ mg} \cdot \text{L}^{-1}$, $\text{NH}_4^+ \text{-N}$ detection limit of $0.025 \text{ mg} \cdot \text{L}^{-1}$). pH was measured directly using a pH meter, DO was measured using a portable dissolved oxygen meter, and COD was measured using the potassium dichromate method, as shown in Table 3. All analyses were performed in triplicate; To ensure consistency among parallel samples, all indicators were measured as soon as possible after sampling; if the relative deviation of these parallel samples exceeded 5%, the measurements were repeated.

The physicochemical indicators of sludge mainly include mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume (SV), and sludge volume index (SVI). Among them, MLSS was determined by drying and weighing at 105°C , and MLVSS was determined by burning and weighing at 550°C ; SV was read by reading the sludge settling volume after standing in a 100 mL graduated

cylinder for 30 min; SVI was obtained by converting SV to MLSS. Microscopic examination of the biological phase was performed by optical microscopy, the spatial distribution of EPS was performed by fluorescence microscopy, microbial diversity was completed by high-throughput sequencing platform, and polysaccharide and protein content were determined by phenol-H₂SO₄ method and BCA method, respectively. The above indicators are used to characterize the sludge biomass accumulation level, active component ratio, settling performance, structural stability and microecological changes under different SRT conditions [20–24].

Table 3. Main experimental indicators and analysis methods.

No.	Indicator	Unit	Analytical Method
1	pH	-	pH Meter
2	DO	mg O ₂ ·L ⁻¹	Dissolved Oxygen Meter
3	COD	mg·L ⁻¹	Potassium Dichromate Method

2.5. EPS Extraction and Characterization

To analyze the quantity, hierarchical structure, and chemical composition of EPS in sludge under different SRTs, a modified thermal extraction method was used to extract loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) in layers. This protocol was selected because it achieves high extraction efficiency while minimizing cell lysis, which could otherwise release intracellular substances and overestimate EPS content [20,22]. The extraction efficiency was verified in preliminary tests by measuring the DNA content in the extracted EPS fractions, which remained below 1% of the total cellular DNA, confirming minimal cell disruption. The specific steps were as follows: 30 mL of the sludge-water mixture at the end of the contact or stabilization phase was centrifuged at 4000 × *g* for 5 min, the supernatant was discarded, and the mixture was resuspended in 30 mL of 0.05% (*w/v*) NaCl solution preheated to 60 °C; after vortexing for 1 min, it was centrifuged again at 4000 × *g* for 10 min, and the resulting supernatant was defined as LB-EPS; the remaining sludge precipitate was resuspended again in 30 mL of 0.05% NaCl solution, heated in a 60 °C water bath for 30 min, and then centrifuged at 4000 × *g* for 15 min, and the resulting supernatant was defined as TB-EPS. All extracted samples were stored at −20 °C for later use [20–24].

Polysaccharides (PS) in EPS were determined using the phenol-sulfuric acid method: 2 mL of the sample was mixed with 1 mL of 6% phenol solution and 5 mL of concentrated sulfuric acid, cooled, and the absorbance was measured at 490 nm. The content was calculated using a glucose standard curve. Proteins (PN) in EPS were determined using the BCA method: the working solution was prepared according to the kit requirements, 0.1 mL of the sample was mixed with 2 mL of the working solution, reacted in a 60 °C water bath for 15 min, and the absorbance was measured at 562 nm. The result was calculated using a bovine serum albumin standard curve. The total amount of EPS was expressed as the sum of PN and PS in LB-EPS and TB-EPS, expressed in mg·gVSS⁻¹ [20,22–24].

The spatial distribution of EPS was observed using fluorescence microscopy. Sludge samples were collected at the end of the contact stage under different SRT conditions. For fluorescence staining, proteins were labeled with fluorescein isothiocyanate (FITC, final concentration 0.1 g·L⁻¹) and polysaccharides were labeled with concanavalin A (ConA-FITC, final concentration 0.1 g·L⁻¹). The staining was performed in the dark at room temperature for 30 min, followed by washing twice with PBS buffer (pH 7.2) to remove unbound dyes. Fluorescence images were captured using an Olympus BX53 fluorescence microscope at an excitation wavelength of 488 nm and emission wavelength of 520 nm. The spatial distribution of EPS in sludge aggregates was compared by analyzing the

merged bright-field and fluorescence images under the same microscopic conditions [7,8]. Fluorescence images were mainly used to help interpret the relationship between the spatial distribution characteristics of EPS and carbon source retention capacity at different sludge ages.

2.6. Microbial Community Analysis

The microbial community structure was analyzed using high-throughput sequencing. Sludge samples were collected during the stable operation phase under different SRT conditions. Total genomic DNA was extracted from each sample using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions. DNA integrity was checked by 1% agarose gel electrophoresis, and DNA concentration and purity (OD₂₆₀/280) were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the primer set 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR reaction system (20 µL) contained 10 ng of template DNA, 0.4 µmol·L⁻¹ of each primer, 10 µL of 2× Taq Plus Master Mix, and ddH₂O to a final volume of 20 µL. The PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. PCR products were purified and sequenced on an Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, CA, USA).

Raw sequences were quality-filtered and chimera-checked using QIIME2 (v2020.2, <https://qiime2.org>), and amplicon sequence variants (ASVs) were inferred using the DADA2 algorithm. Taxonomic assignment of ASV representative sequences was performed using the classify-sklearn Naive Bayes classifier against the Silva 138 database (16S), with a classification confidence threshold set at 0.7. ASVs with a total sequence count of less than 0.001‰ across all samples were removed from further analysis. The sequencing depth was sufficient for downstream analysis, with coverage indices exceeding 99.98% for all samples (see Table S2 in Supplementary Materials). All samples were rarefied to an equal depth of 37,538 sequences to minimize bias in diversity comparisons. Alpha diversity indices such as Sobs, ACE, Chao, Shannon, Simpson, and Pielou_e were calculated to characterize community richness and evenness, with statistical comparisons performed using the Kruskal–Wallis test followed by Wilcoxon rank-sum test [8,19,25].

Beta diversity analysis was performed using principal coordinate analysis (PCoA) based on Bray–Curtis distance matrices to compare community differences among different SRT conditions, and the statistical significance of group separation was assessed using analysis of similarities (ANOSIM). The relative abundance of dominant taxa was compared at the phylum and genus levels. The focus was on functional microorganisms related to organic matter hydrolysis, floc formation, EPS secretion, and carbon capture, such as Bacteroidota and dominant genera like *Flavobacterium*, *Zoogloea*, and *Acidovorax*, to reveal the impact of SRT on the system's microecological structure and potential metabolic functions. All sequencing data processing and statistical analyses were performed on the Majorbio Cloud Platform (www.majorbio.com).

2.7. COD Fractionation and Carbon Flow Analysis

To reveal the capture characteristics of different forms of organic carbon by the CS process, total COD (tCOD) was classified into particulate COD (pCOD), colloidal COD (cCOD), and dissolved COD (sCOD) according to particle size. Specifically, tCOD was directly measured from the homogeneously mixed raw water sample; sCOD + cCOD was measured from the filtrate through a 1.5 µm glass fiber membrane; and sCOD was

measured from the filtrate through a 0.45 µm microporous membrane. The pCOD and cCOD contents were then calculated based on the differences [4,5,26]. This classification method can distinguish between particulate/colloidal organic matter, which is mainly removed by bioflocculation and solid–liquid separation, and dissolved organic matter, which is mainly removed by microbial absorption, oxidation, and storage transformation. The separation methods and removal mechanisms of different COD forms are shown in Table 4.

Table 4. Separation methods and main removal mechanisms of different COD forms.

Component	Form	Separation Method	Main Removal Mechanism
pCOD	Particulate	Retained by 1.5 µm Filtration	Bioflocculation + Solid–Liquid Separation
cCOD	Colloidal	Retained by 0.45 µm Filtration	Bioflocculation + Solid–Liquid Separation
sCOD	Dissolved	-	Oxidative Mineralization/Intracellular Storage/Biomass Synthesis

Based on the determination of COD in different forms, and considering the influent and effluent volumes, excess sludge discharge, and volatile suspended solids concentration in the sludge, a material balance was performed to analyze the distribution of influent COD among sludge recovery, effluent residue, and oxidation loss under different SRT conditions. COD recovered from sludge was defined as carbon capture, and the sum of recovered sludge COD and effluent particulate COD was defined as carbon redirection. The calculation of COD mass balance was based on a steady-state assumption over multiple operational cycles. The percentage closure error was calculated to verify the reliability of the carbon accounting using the following equation: Closure Error (%) = [COD_{IN} – (COD_{WAS} + COD_{Effluent} + COD_{Oxidation})]/COD_{IN} × 100%. A closure error of less than 5% was considered acceptable for all conditions. Furthermore, by combining parameters such as sludge EPS content and MLVSS, the metabolic distribution characteristics of captured carbon in biomass synthesis, EPS formation, and adsorption/temporary storage pathways were estimated. This approach assumes that the primary fate of captured COD is either anabolic synthesis (biomass and EPS) or temporary storage. While this provides a useful approximation, it inherently simplifies the complex metabolic pathways. The uncertainties associated with this estimation are acknowledged and discussed in the Supplementary Materials. The relevant calculation formulas are supplemented below.

$$Q_{BS} = MLVSS_{waste} \times f_H \times 1.42 \quad (1)$$

where Q_{BS} is the biomass synthesis (gCOD·d^{−1}), $MLVSS_{waste}$ is the total MLVSS discharged daily, f_H is the proportion of active biomass in MLVSS (0.65), and 1.42 is the COD equivalent coefficient of biomass VSS (gCOD·gVSS^{−1}) based on C₅H₇O₂N.

$$Q_{CGE} = \frac{COD_{IN} \times COD_{oxidation} \times Y_{OHO}}{1 - Y_{OHO}} \quad (2)$$

where Q_{CGE} is the total yield of cell growth and EPS (gCOD·d^{−1}), COD_{IN} is the influent COD load, $COD_{oxidation}$ is the COD oxidation ratio, and Y_{OHO} is the theoretical yield coefficient of heterotrophic bacteria (0.67 gCOD·gCOD^{−1}). This yield coefficient is a widely accepted value for heterotrophic bacteria in activated sludge systems under aerobic conditions.

$$Q_{EPS} = \frac{EPS_{pCOD}}{MLVSS} \quad (3)$$

where Q_{EPS} is the EPS yield and EPS_{pCOD} is the ratio of EPS concentration to particulate COD.

$$Q_{\text{biomass}} = \frac{Q_{\text{BS}}}{Q_{\text{CGE}} - Q_{\text{EPS}}} \times 100\% \quad (4)$$

$$Q_{\text{AS}} = 100\% - Q_{\text{biomass}} - Q_{\text{EPS}} \quad (5)$$

where Q_{biomass} is the biomass generation ratio (%) and Q_{AS} is the adsorption/temporary storage ratio (%).

2.8. Data Quality Control and Reproducibility

All experimental data were obtained from measurements taken after the system had stabilized. Routine water quality and sludge indicators were measured in parallel at least once, and the average value was taken. EPS extraction, COD classification by different forms, and microbial community analysis were all completed within a unified sampling time window to minimize deviations caused by periodic fluctuations. Key operating parameters such as influent volume, sludge discharge rate, mixing status, and aeration intensity were recorded to ensure that only the sludge age changed between different SRT operating conditions.

To ensure the reproducibility of the experiment, the three SRT conditions were conducted under the same reactor configuration, operating cycle, influent composition, and detection methods, with the sludge age changed only by adjusting the sludge discharge rate. The results analysis comprehensively utilized physicochemical indicators, microscopic observations, microbial community structure, and carbon balance calculations to explain the impact of SRT on the carbon capture and carbon redirection mechanisms of the CS process under the tested conditions from a multi-scale perspective. All statistical analyses, including one-way ANOVA with post hoc Tukey tests (where applicable), were performed using OriginPro 2021 (OriginLab Corp., Northampton, MA, USA) and SPSS (IBM Corp., Armonk, NY, USA) Statistics 26. A significance level of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. EPS Composition and Structural Characteristics

To investigate the changes in the quantity and stratified structure of EPS in sludge under different SRT conditions, LB-EPS and TB-EPS were extracted and quantitatively analyzed from sludge samples at the end of the contact and stabilization phases. The results showed significant differences in the total EPS and LB-EPS/TB-EPS distributions under different SRT conditions, as shown in Figure 2. The total EPS content was highest at SRT = 4 days ($43.14 \text{ mg} \cdot \text{gVSS}^{-1}$), which was significantly higher than at SRT = 1 d ($36.94 \text{ mg} \cdot \text{gVSS}^{-1}$) and SRT = 7 d ($27.47 \text{ mg} \cdot \text{gVSS}^{-1}$) ($p < 0.05$), with a larger proportion of TB-EPS, indicating that EPS exhibited a more prominent binding layer characteristic in the sludge phase under this condition. TB-EPS is generally considered an important component for maintaining sludge aggregation stability and interfacial adhesion; therefore, its increase does not simply indicate “more EPS,” but rather signifies that the sludge has a stronger physical encapsulation and retention capacity for particulate and colloidal organic matter. In contrast, the total EPS was lower, and the contribution of TB-EPS was insufficient at SRT = 1 day, indicating that the extremely short sludge age weakened the establishment of floc maturation and stable aggregation. While SRT = 7 days still maintained a certain EPS level, its advantage was more reflected in the maintenance of mature sludge conditions rather than the rapid dynamic response required for efficient carbon capture.

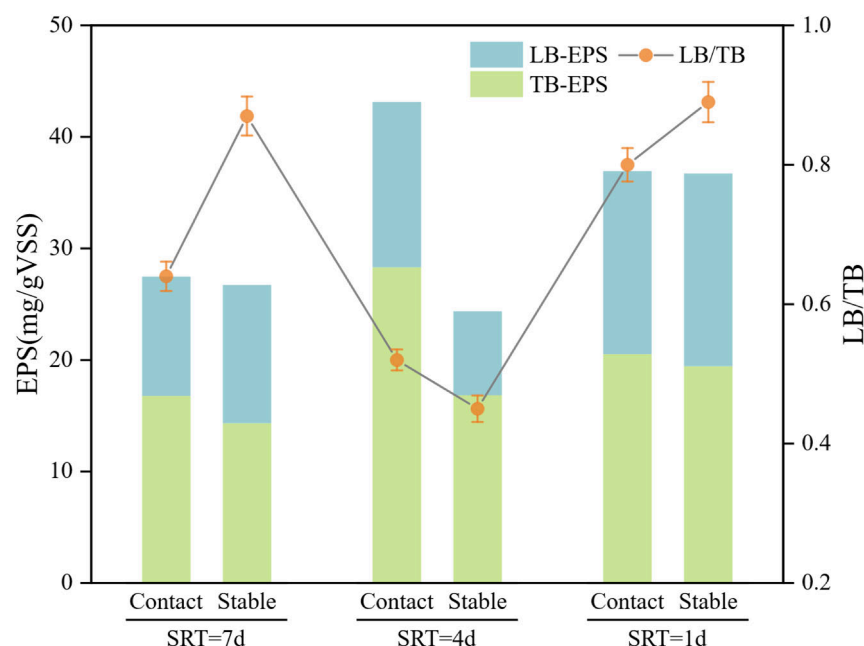


Figure 2. LB-EPS and TB-EPS content at the end of the contact phase and the end of the steady-state phase under different SRTs. Error bars represent standard deviations from triplicate measurements.

To further clarify the transformation characteristics of EPS components in different operating stages, the PN and PS in LB-EPS and TB-EPS were simultaneously measured. The results showed that SRT not only affected the total amount of EPS but also significantly influenced its chemical composition and stage-specific changes. As shown in Figure 3, when SRT = 4 days, polysaccharides accumulated significantly during the contact stage but decreased markedly during the stable stage ($38.42 \text{ mg} \cdot \text{gVSS}^{-1}$) but decreased markedly during the stable stage (a reduction of 48.9%). This indicates that the system rapidly transferred exogenous organic carbon into the relevant storage components of EPS and utilized them in the subsequent regeneration and activation stage. This dynamic characteristic of “enrichment during the contact stage and consumption during the stable stage” is consistent with the typical starvation/satisfaction alternation metabolic pattern in HiCS processes, suggesting that a moderate SRT is more conducive to forming a reusable and usable carbon reservoir, rather than simply increasing the accumulation of inert organic matter. Conversely, at SRT = 1 d, due to insufficient biomass retention and limited structural formation, the stage differences between PS and PN were not prominent enough, indicating that the system’s ability to temporarily store and redistribute organic carbon was weak. At SRT = 7 d, the dynamic change amplitude was reduced (polysaccharide reduction of only 20.9%), suggesting that at longer sludge ages, organic carbon tends to enter maintenance metabolism rather than rapid structural renewal. The PN/PS ratio in TB-EPS at SRT = 4 d was the lowest (0.11), whereas at SRT = 7 d it exceeded 1.0, further confirming the polysaccharide-dominant nature of EPS at the optimal SRT. Therefore, the advantage of SRT = 4 d lies in simultaneously enhancing the structural and metabolic contributions of EPS.

To verify the organizational characteristics of EPS under different SRTs at a spatial scale, fluorescence microscopy was performed on sludge under various operating conditions. The observation results further support the quantitative analysis conclusions. As shown in Figure 4, at SRT = 4 d, the EPS signal is continuously and relatively uniformly distributed at the outer edge and inside of the sludge aggregate, indicating that EPS not only has a high content under this condition, but its spatial organization is also more conducive to the bridging and encapsulation of organic matter. Specifically, the protein (green)

and polysaccharide (blue) signals showed a high degree of spatial overlap, forming an integrated three-dimensional network structure. At SRT = 1 d, the fluorescence signal distribution is more discrete, with protein signals being diffuse and polysaccharide signals locally enriched but weakly bound to the sludge matrix, suggesting that the system has not yet formed a continuous and stable EPS organizational state, which will limit the adsorption and retention of colloidal and dissolved organic matter. At SRT = 7 d, the EPS distribution still maintains a certain continuity with protein signals forming cluster-like aggregates in the floc core, while polysaccharides were distributed in the outer layers, but is more inclined towards a stable maintenance characteristic, indicating that it has an advantage in long-term maintenance, but is not as flexible as the 4-d condition in terms of rapid capture and dynamic turnover. In summary, SRT = 4 d does not simply represent the highest EPS content, but rather the simultaneous optimization of quantity, composition, and spatial distribution characteristics, thus being most conducive to constructing a sludge phase retention basis for carbon capture.

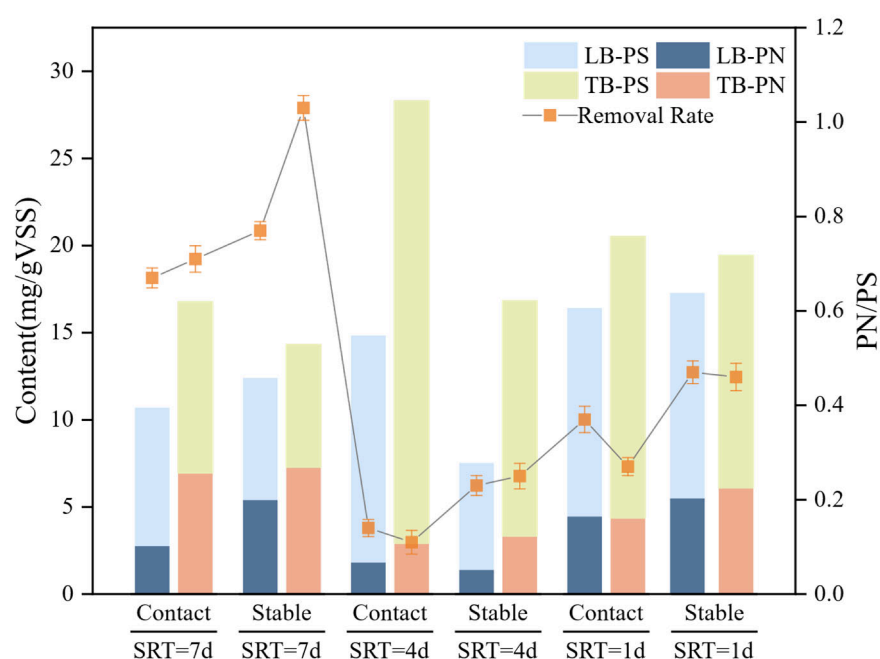


Figure 3. Protein (PN) and polysaccharide (PS) content in EPS during the contact and stable phases under different SRT conditions. Error bars represent standard deviations from triplicate measurements.

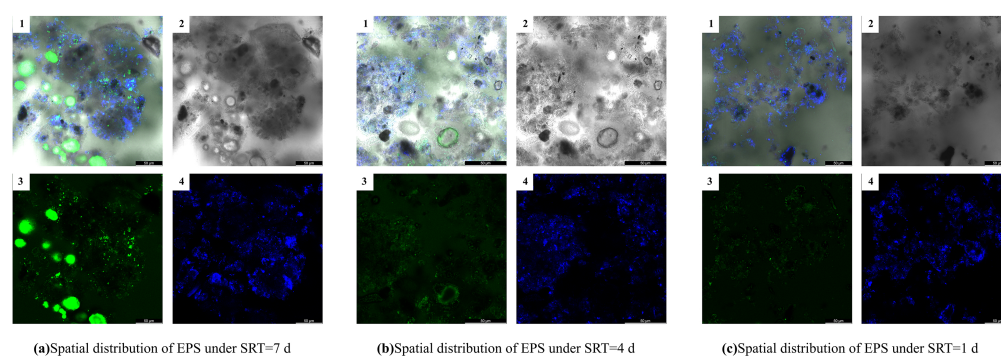


Figure 4. (a) Spatial distribution of EPS under SRT = 7 d; (b) Spatial distribution of EPS under SRT = 4 d; (c) Spatial distribution of EPS under SRT = 1 d. Protein (FITC, green), polysaccharide (ConA, blue). Scale bar = 50 μ m.

The fluorescence microscopy results above indicate that EPS distribution in sludge aggregates is more continuous under SRT = 4 d conditions. This is consistent with the

increase in TB-EPS proportion and polysaccharide dynamic turnover characteristics, further suggesting that an appropriate sludge age is more conducive to the formation of a sludge system with both aggregation stability and carbon storage capacity.

3.2. Microbial Community Structure

To reveal the response characteristics of the system's microecological structure under different SRT conditions, high-throughput sequencing analysis was performed on sludge samples under various operating conditions. Based on the leveled sequencing data, the number of taxa and the total number of ASVs at each taxonomic level were counted to reflect the species composition of the samples, as shown in Table 5. Furthermore, Alpha diversity index, PCoA, and phylum and genus-level community composition characteristics were combined to systematically analyze the diversity and structural differences in the microbial community under different SRT conditions. The diversity results showed significant differences in community structure under different SRTs. As shown in Table 6 and Figure 5, the community evenness was highest at SRT = 4 d (Pielou_e = 0.755), indicating a more balanced ecological relationship among different functional microorganisms under this condition. The Shannon index at SRT = 4 d (4.606) was also significantly higher than at SRT = 1 d (4.077) ($p < 0.05$), indicating greater community diversity. It is noteworthy that SRT = 1 d does not necessarily indicate a superior community; the higher Sobs, ACE, and Chao values only indicate a greater number of detected taxa, but the decreased Shannon and increased Simpson values suggest an excessively high proportion of a few dominant bacteria, indicating a stronger opportunistic selection characteristic in the system. SRT = 7 d, on the other hand, reflects a mature community but a slower renewal rate. Therefore, the advantage of SRT = 4 d is not simply to increase abundance, but to maintain both high uniformity and functional synergy, which is particularly crucial for stable carbon capture under elevated load conditions.

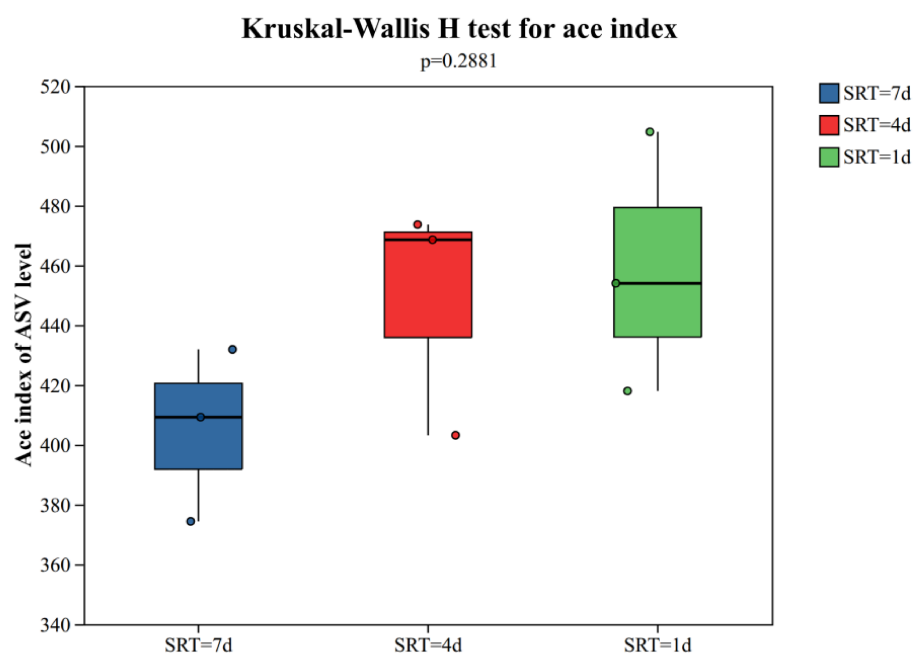


Figure 5. Box plots of Alpha diversity index under different SRT conditions. Statistical comparisons were performed using the Kruskal–Wallis test followed by Wilcoxon rank-sum test.

Table 5. Classification units and number of categories.

Domain	Kingdom	Phylum	Class	Order	Family	Genus	Species	ASV
1	1	26	54	130	207	350	51	1407

Table 6. Alpha diversity index.

Sample Group	Sobs	ACE	Chao	Shannon	Simpson	Pielou_e
SRT = 7 d	404.00	405.15	405.28	4.478	0.023	0.746
SRT = 4 d	447.67	448.46	448.18	4.606	0.024	0.755
SRT = 1 d	458.67	458.89	458.83	4.077	0.082	0.666

To further compare the overall community differentiation and dominant taxa composition under different SRTs, PCoA and phylum-level species composition statistics were performed on the sequencing results. The results showed that different SRTs shaped the system into distinctly different microecological states. As shown in Figure 6, the 4-day condition showed significant separation from the other two groups, (ANOSIM test, $R = 1$, $p = 0.001$), indicating that a suitable sludge age established a unique functional community. The first two principal coordinate axes (PC1 and PC2) explained 86.07% and 9.58% of the total variance, respectively, with a cumulative explanation of 95.65%. Further analysis of Figures 7–9 revealed that at SRT = 4 days, the relative abundance of Bacteroidota (32.87%) was significantly higher than at SRT = 7 d (26.53%) and SRT = 1 d (13.43%). This phylum is associated with the hydrolysis of complex organic matter, EPS production, and biofloculation. Actinobacteriota was also enriched at SRT = 4 d (9.18%), contributing to the degradation of recalcitrant organics and sludge structural stability. The higher abundance of these functional phyla indicates that particulate and colloidal organic matter is more easily decomposed and converted into forms that can be further adsorbed and utilized under this condition. In other words, the community under the 4-day condition is not only richer but also better suited to the front-end transformation requirements after higher-strength organic substrates enter the system. In comparison, SRT = 1 d is more likely to enrich fast-growing microbial communities. Although these communities respond quickly to easily degradable substrates, they contribute little to maintaining sludge aggregation stability and stable capture capacity. SRT = 7 d is more conducive to retaining mature and stable communities but may lead to more carbon flowing to maintenance metabolism.

At the genus level, to identify key functional bacteria closely related to the carbon capture process, the relative abundance and clustering relationships of dominant genera were compared. The results are shown in Figures 10 and 11. At SRT = 4 d, the genera *Flavobacterium*, *Zoogloea*, and *Acidovorax* were co-enriched. *Flavobacterium* has been reported to be associated with the degradation and reuse of macromolecular substrates, *Zoogloea* has been reported to be related to fungal micelle formation and EPS secretion, and *Acidovorax* has been reported to be associated with substrate transformation and metabolic synergy. These three genera showed significantly higher combined relative abundance at SRT = 4 d (12.8%) compared to SRT = 7 d (5.6%) and SRT = 1 d (8.1%) ($p < 0.05$). The simultaneous enrichment of these three genera under the same operating condition indicates that the 4-d operating condition does not rely on a single dominant bacterium to achieve performance improvement but rather achieves higher carbon capture efficiency through multifunctional synergy of “substrate decomposition—structure building—carbon redirection.” This directly echoes the EPS results: when the functional microbial community is conducive to EPS generation and structure maintenance, the system’s ability to capture and retain organic carbon is simultaneously enhanced. In addition, *Dechloromonas*, a genus associated with denitrification, was also enriched at SRT = 4 d (2.99%), potentially contributing to the

higher TN removal observed under this condition. Therefore, the above findings collectively demonstrate that SRT = 4 d shapes a functionally synergistic micro-ecosystem more suitable for carbon capture-oriented operation.

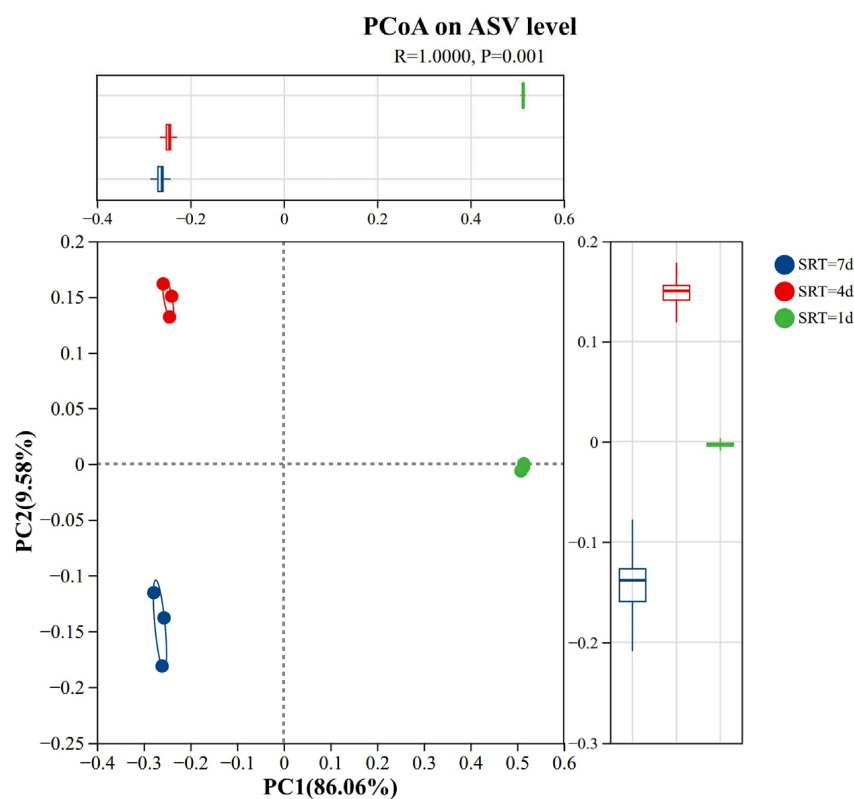


Figure 6. PCoA diagram based on ASV level under different SRT conditions. PC1 and PC2 explained 86.07% and 9.58% of the total variance, respectively. ANOSIM test: $R = 1$, $p = 0.001$.

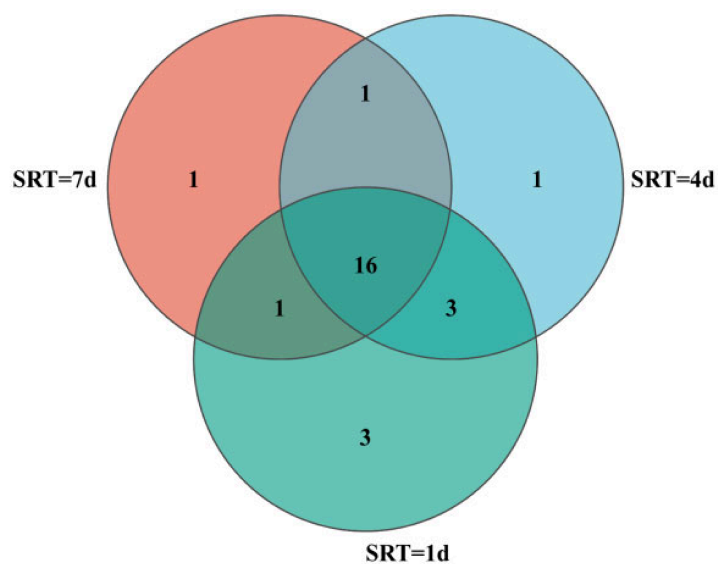


Figure 7. Venn diagram at phylum level under different SRT conditions.

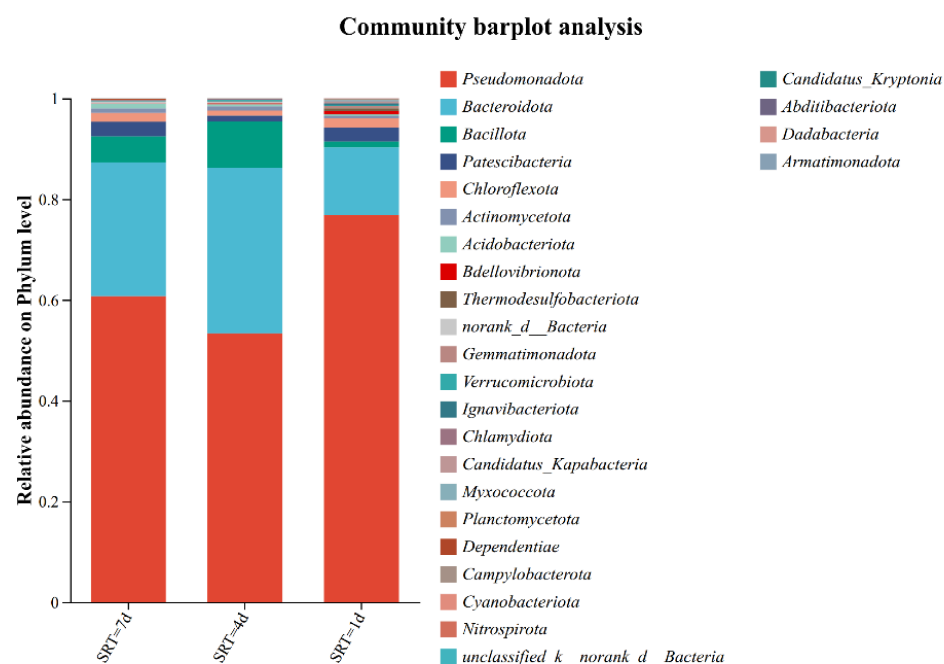


Figure 8. Bar plot of relative abundance at phylum level under different SRT conditions.

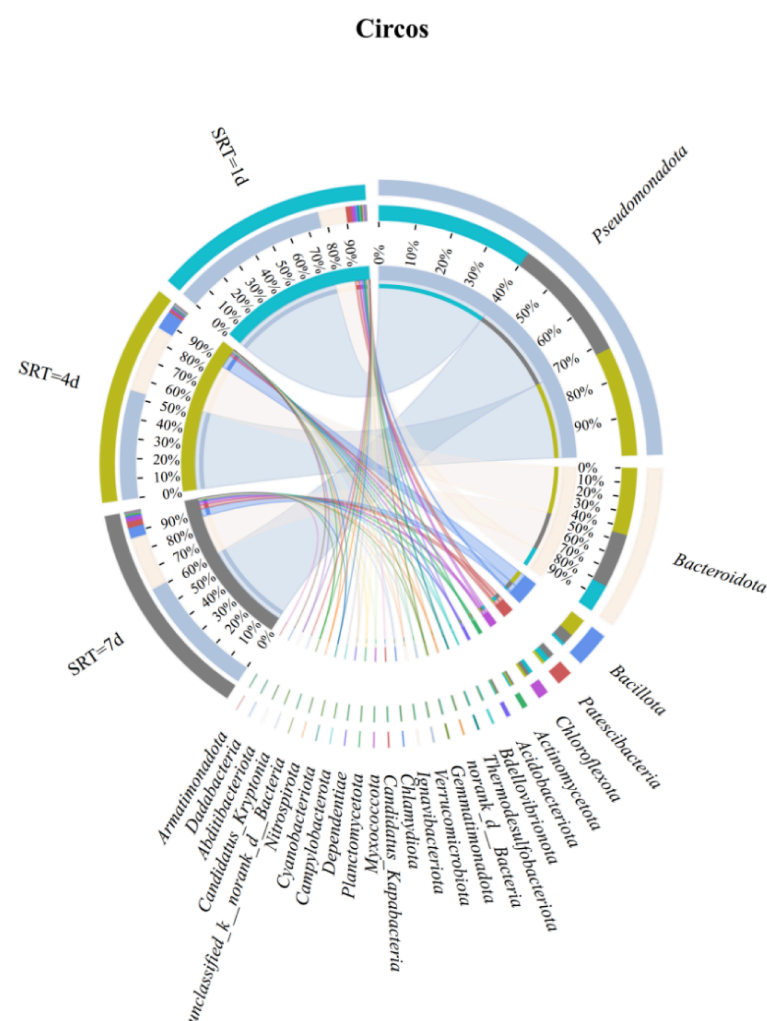


Figure 9. Circos plot of community composition at phylum level under different SRT conditions.

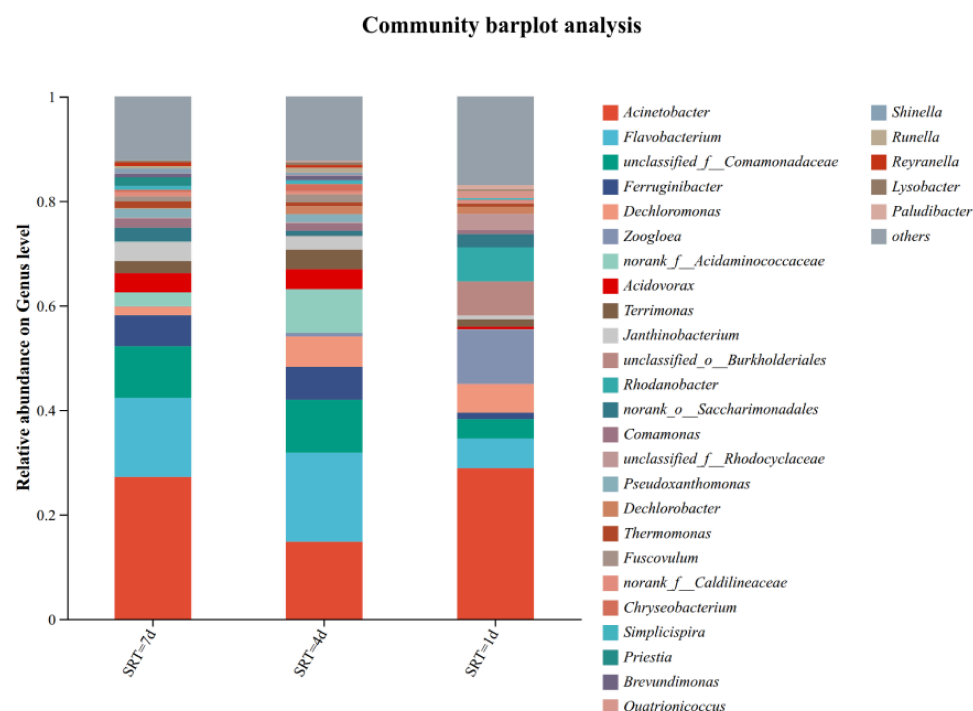


Figure 10. Histogram of relative abundance at genus level under different SRT conditions.

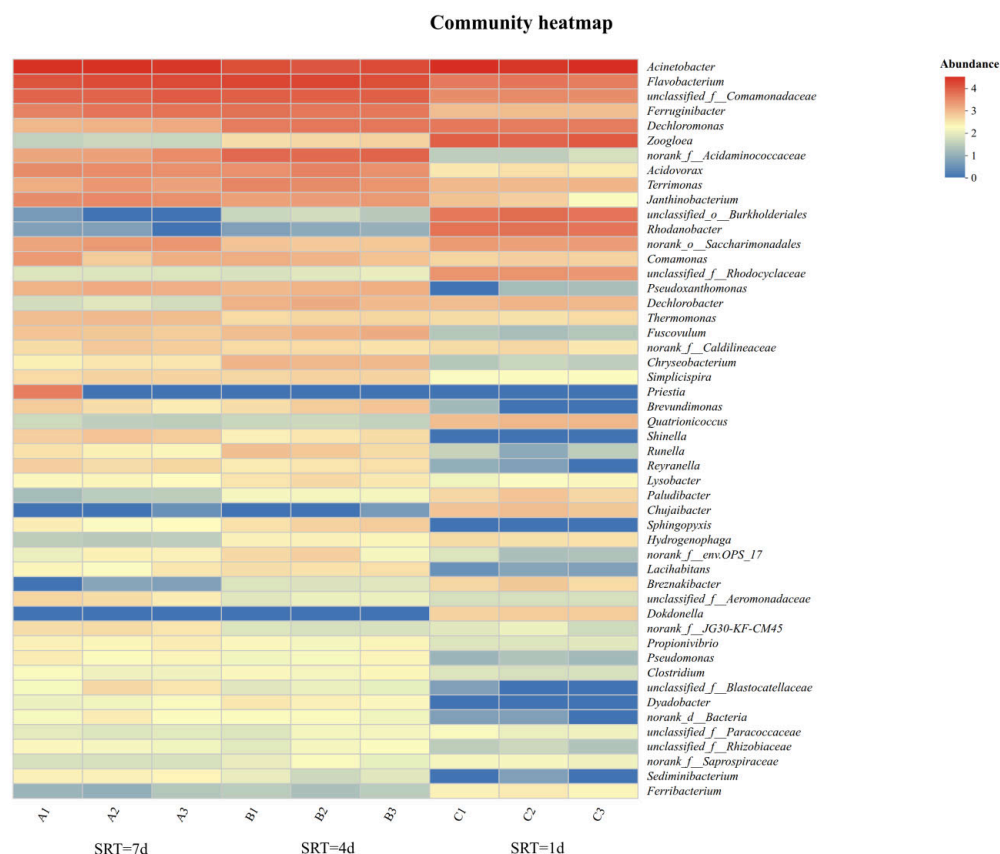


Figure 11. Heatmap of genus-level abundance clustering under different SRT conditions.

3.3. Removal Characteristics of Different COD Fractions

To clarify the removal pathways of various organic components under different SRT conditions, total COD was further decomposed into particulate, colloidal, and dissolved components, and their removal behavior in the CS process was compared. The results

showed that the SRT response to different forms of COD differed significantly. As shown in Figure 12a, under an SRT of 7 days, all forms of COD exhibited high and relatively stable removal levels (pCOD: 72.36%, cCOD: 50.26%, sCOD: 65.95%), indicating that a longer sludge age is beneficial for maintaining high sludge aggregation stability and improving the retention capacity of particulate and colloidal organic matter. However, sufficient removal does not equate to optimal recovery, because the maintenance metabolism and endogenous respiration in mature sludge systems are stronger, meaning that more organic carbon may be further oxidized during the stable phase. Therefore, the 7-day condition reflects the operational characteristics of a long-sludge-age system that tends towards stable mineralization.

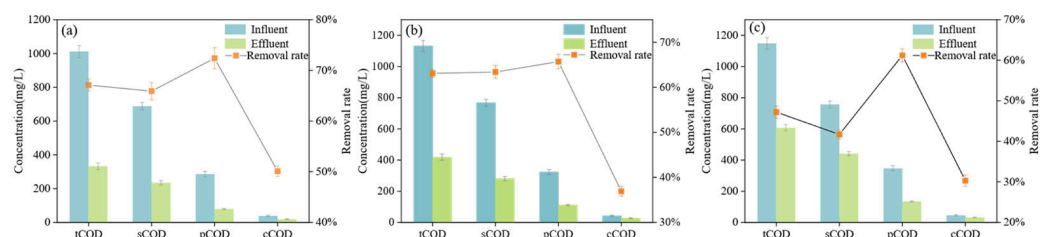


Figure 12. (a) Removal rate of COD of various forms under SRT = 7 d; (b) Removal rate of COD of various forms under SRT = 4 d; (c) Removal rate of COD of various forms under SRT = 1 d. The removal efficiencies for each COD fraction under different SRT conditions were obtained from separate experimental runs with different influent COD concentrations; therefore, direct statistical comparison across SRT conditions was not performed. Error bars represent standard deviations from triplicate measurements, and the data are presented for comparative purposes under the specific conditions tested.

Within the same analytical framework, the SRT = 4 d condition exhibits a more representative overall advantage. As shown in Figure 12b, under this condition, the system demonstrates good removal and transfer capabilities for all three types of COD components (pCOD: 65.70%, cCOD: 37.05%, sCOD: 63.40%). Particulate COD can be enriched through EPS-mediated adsorption, bioflocculation, and sludge phase transfer; colloidal COD is further retained through EPS and bioflocculation; and dissolved COD can also enter the sludge phase through microbial absorption and short-term storage. In other words, the advantage of the 4-d condition does not stem from the extremely high removal of any one type of component, but rather from the synergistic capture of multiple forms of organic matter. This synergy is consistent with the previously observed high TB-EPS ratio, relatively continuous EPS spatial distribution characteristics, and synergistic enrichment by functional bacteria, indicating that aggregation stability, metabolic activity, and substrate transfer capability are well-matched under this condition.

In contrast, the removal capacity of all COD forms was significantly weakened when SRT = 1 d. As shown in Figure 12c, the removal of colloidal and dissolved organic matter was particularly affected (pCOD: 61.25%, cCOD: 30.32%, sCOD: 41.73%). This indicates that under extremely short sludge age conditions, although the system may retain a certain amount of rapidly growing microbial communities, the low sludge concentration, insufficient EPS accumulation, and limited structural adsorption sites make it difficult for organic carbon to be effectively fixed at the front end and transferred to the sludge phase. Therefore, the problem with short sludge age conditions is not just “decreased removal rate,” but more fundamentally, “incomplete capture chain”: particulate organic matter can still be partially retained by physical interception, while colloidal and dissolved components are more easily lost due to insufficient structural and functional support. In summary, SRT = 4 d showed the best comprehensiveness and balance in the removal of different COD forms, which provides a direct basis for subsequent carbon flow analysis.

3.4. Carbon Flow Distribution and Metabolic Allocation

To quantitatively evaluate the fate of influent organic carbon under different SRTs, carbon balance accounting was further conducted based on COD staged removal analysis, and the distribution differences in organic carbon among sludge recovery, effluent loss, and oxidation loss were compared. The results showed that different SRTs not only affected the degree of COD removal but also significantly altered the final fate of organic carbon. As shown in Figure 13a, the proportion of COD recovered from sludge reached its highest at SRT = 4 d (31.1%), while oxidation loss was effectively controlled (31.9%), indicating that this operating condition was most conducive to achieving the shift from removal-oriented to recovery-oriented. The carbon balance closure error for all conditions was below 5%, confirming the reliability of the mass balance calculations. In other words, at a suitable sludge age, the system does not simply remove organic matter but retains a larger proportion of influent carbon in the resource-utilizable sludge phase.

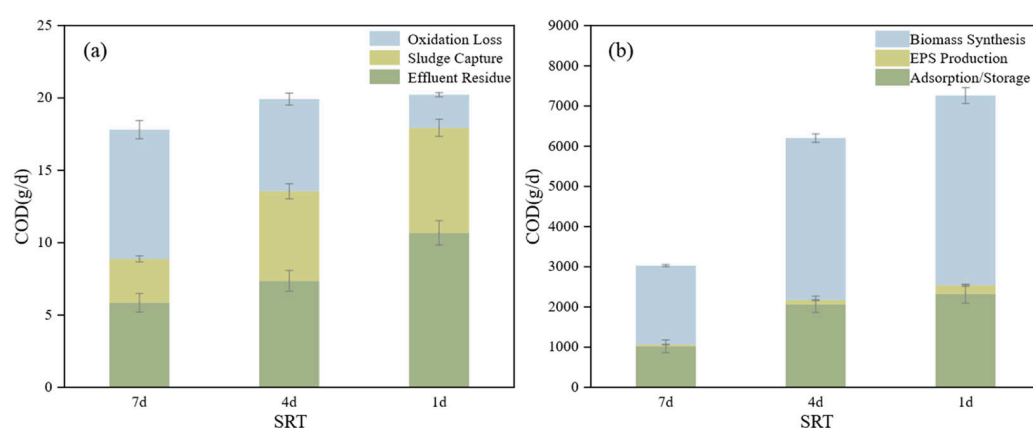


Figure 13. (a) COD equilibrium flow direction under different SRTs under the tested conditions; (b) Sludge capture carbon source metabolic distribution under different SRTs under the tested conditions. The carbon balance closure error for all conditions was below 5%. Error bars represent standard deviations from triplicate measurements.

Further comparison of different operating conditions shows that SRT = 7 d and SRT = 1 d correspond to two different limiting mechanisms. For the 7-d condition, Figure 13a shows that its overall operation is stable and the effluent control is good (effluent residual: 32.8%). However, the longer sludge age means that more organic carbon is used to maintain the metabolism and endogenous respiration of the mature sludge system, thereby increasing the proportion of oxidation loss (50.2%). Therefore, this condition is more suitable for traditional pollutant removal targets rather than maximizing carbon recovery. For the 1-d condition, the limitation mainly comes from insufficient biomass and weak structural retention capacity. Some organic carbon is lost with the effluent before it is fully adsorbed and transformed (effluent residual: 52.8%, oxidation loss: 11.3%). In other words, the problem with 7-d is excessive consumption after capture, while the problem with 1-d is loss before effective capture. This also explains why neither is as suitable as 4-d for carbon recovery-oriented operation.

Based on the carbon flow analysis, the metabolic allocation of captured carbon in the sludge was further calculated to identify the specific utilization modes of organic carbon after it enters the sludge phase. The results are shown in Figure 13b. When SRT = 4 d, captured carbon is allocated more to pathways conducive to carbon retention, such as biomass synthesis ($4030 \text{ mg} \cdot \text{d}^{-1}$, 65%) and EPS formation ($104 \text{ mg} \cdot \text{d}^{-1}$, 1.7%), rather than rapid oxidation and consumption. The adsorption/temporary storage amount at SRT = 4 d ($2066 \text{ mg} \cdot \text{d}^{-1}$) was more than double that at SRT = 7 d ($1022 \text{ mg} \cdot \text{d}^{-1}$) and comparable to

that at SRT = 1 d ($2323 \text{ mg} \cdot \text{d}^{-1}$), despite the much higher effluent residual at SRT = 1 d. This indicates that the advantage of the 4-d condition lies not only in capturing more carbon but also in more efficient retention. Combined with the previous results, high total EPS content, phased PS turnover, and synergistic enrichment by functional bacteria jointly promote this favorable carbon metabolic allocation pattern. The EPS turnover rate at SRT = 4 d (43.5% reduction from contact to stabilization phase) was significantly higher than at SRT = 7 d (2.7%) and SRT = 1 d (0.6%), indicating that the EPS at SRT = 4 d functions as a dynamic carbon reservoir rather than an inert structural component. Therefore, Figure 13 not only provides mass balance results but also reveals from a systemic perspective that a moderate SRT can simultaneously reduce oxidation losses, increase the sludge carbon recovery ratio, and optimize the utilization mode of carbon sources within the sludge.

4. Discussion

4.1. EPS as a Structural and Dynamic Hub for Carbon Capture

The results presented above demonstrate that SRT is a critical factor shaping the performance of the CS process for high-strength wastewater, and its effects are mediated through a complex interplay of EPS dynamics, microbial community assembly, and carbon metabolic pathways. Our findings suggest that an intermediate SRT of 4 days optimizes this interplay, leading to superior carbon capture.

The superior carbon capture at SRT = 4 d is strongly associated with the enhanced production and unique properties of EPS. The significantly higher total EPS content, particularly the TB-EPS fraction, at this SRT indicates a more robust and stable sludge structure. TB-EPS is known to be crucial for maintaining floc integrity and providing a dense matrix for adsorbing particulate and colloidal organic matter [21,22]. This structural role is directly supported by our COD fractionation results, where pCOD and cCOD removal were more balanced and efficient at SRT = 4d. The fluorescence microscopy results further corroborated this structural advantage, revealing a continuous and highly integrated spatial distribution of proteins and polysaccharides at SRT = 4d, which contrasts with the more diffuse or clustered patterns observed at other SRTs.

Critically, however, the advantage of SRT = 4d is not merely structural. The dynamic turnover of EPS components, specifically the significant accumulation of polysaccharides (PS) during the contact phase and their marked decrease during the stabilization phase (a 48.9% reduction), suggests that EPS serves as a dynamic carbon reservoir. This “feast-famine” driven utilization of PS is a hallmark of efficient carbon capture, as it implies that a portion of the influent COD is transiently stored as EPS and subsequently consumed for microbial maintenance and growth, rather than being lost through oxidation. This dynamic is less pronounced at SRT = 1 d (10.6% reduction) and SRT = 7 d (20.9% reduction), indicating that the balance between EPS synthesis and degradation is most favorable at the intermediate SRT. The observed reorganization of EPS under different SRTs aligns with findings in other stress-driven systems, where external conditions trigger changes in EPS structure and composition to enhance microbial survival and functionality. For instance, Li et al. [27] demonstrated that external oxidative stress induced structural reorganization of EPS, including changes in the PN/PS ratio and redistribution between LB-EPS and TB-EPS. In our system, the shift in SRT similarly induced a restructuring of EPS, with SRT = 4 d promoting a polysaccharide-rich, TB-EPS-dominated structure that was both structurally stable and metabolically active. This structural and functional duality of EPS at SRT = 4 d provides a mechanistic explanation for the observed improvements in both COD removal and carbon recovery.

4.2. Microbial Community Synergy: From Composition to Function

The microbial community analysis reveals that the functional outcome at SRT = 4 d is not a result of a single dominant species, but rather a synergistic consortium. The higher community evenness at SRT = 4 d suggests a more cooperative and stable ecological network. The enrichment of key genera, such as *Flavobacterium*, *Zoogloea*, and *Acidovorax*, supports this view. While the specific functional roles of these genera are partially inferred from existing literature, their co-enrichment suggests a division of labor: *Flavobacterium* for macromolecule degradation and intracellular carbon storage (consistent with its known ability to synthesize intracellular storage compounds) [8]; *Zoogloea* for floc formation and EPS secretion (a key genus in activated sludge floc formation, closely associated with PHA synthesis) [1]; and *Acidovorax* for substrate transformation and EPS production (contributing to bioflocculation and providing adsorption sites for carbon capture) [21]. This functional tripartite synergy—degradation, structure formation, and carbon redirection—was most pronounced at SRT = 4 d, and directly supported the observed EPS characteristics and carbon flow distribution.

It is crucial to emphasize that our results show associations rather than direct causal relationships. While SRT = 4 d correlated with the enrichment of these genera and high carbon capture, our experimental design, which lacks functional gene analysis (e.g., PICRUSt2, metatranscriptomics) or activity measurements, cannot confirm that these genera are directly responsible for the observed performance. As suggested by the reviewer, it remains unclear whether their enrichment is a cause or a consequence of the improved system performance. Furthermore, the inferred functions based on genus-level taxonomy are inherently limited; identification at the genus level is not sufficient to definitively infer specific metabolic functions. We have therefore moderated our language throughout the manuscript, consistently using terms such as “associated with,” “suggest,” and “indicate” rather than claiming direct regulation or causation.

The discussion of microbial community dynamics would also benefit from considering the role of intercellular communication. Zhang et al. [28] recently highlighted the role of quorum sensing in modulating microbial metabolic efficiency and community cooperation in complex microbial systems. While our current study did not investigate quorum sensing, the observed synergistic enrichment of specific genera at SRT = 4 d and the improved carbon capture efficiency suggest that intercellular signaling may play a role in coordinating microbial activities during the feast-famine cycles of the CS process. Future studies incorporating quorum sensing analysis could provide a more profound understanding of how intercellular communication orchestrates the synergistic behavior of these key genera in the CS process.

4.3. Integrated Mechanism and Practical Implications

By integrating the EPS, community, and carbon flow data, we propose a mechanistic framework for the SRT effect. At SRT = 1 d, the system is in a state of “overdrive.” While the short sludge age promotes fast-growing organisms, it fails to establish a stable EPS network, leading to high effluent COD (52.8%) as the capture capacity is limited. The microbial community is dominated by opportunistic r-strategists with low functional synergy, and EPS, while present, is predominantly in the loosely bound form, providing inadequate structural support for effective carbon retention. At SRT = 7 d, the system is in “maintenance mode.” A mature community and stable EPS are present, but the energy demand for maintenance and endogenous respiration is high, resulting in excessive oxidation loss (50.2%) of the captured carbon. The microbial community is dominated by K-strategists with slow growth and low metabolic turnover, and EPS, while structurally stable, is not dynamically utilized as a carbon reservoir. At SRT = 4 d, the system achieves a

state of “dynamic equilibrium.” Here, the microbial community is functionally diverse and synergistic, promoting the synthesis of a structurally sound yet dynamic EPS matrix. This matrix efficiently captures COD, which is then partly incorporated into biomass and partly used as a carbon source for energy, effectively minimizing both oxidation loss and effluent loss. This integrated framework suggests that the optimal SRT is not defined by maximizing any single parameter, but by achieving a synergistic balance across multiple scales—from microbial ecological interactions to EPS structural dynamics to carbon metabolic pathways.

A conceptual mechanistic diagram incorporating EPS properties, microbial community changes, COD fraction removal, and carbon flow pathways has been added to enhance the clarity of this section (Figure S1, Supplementary Materials).

4.4. Limitations and Future Directions

We acknowledge the limitations of this study. First, the use of synthetic wastewater, while ensuring experimental reproducibility, cannot fully represent the complexity of real food-processing effluents, which may contain particulate matter, lipids, variable C/N ratios, and inhibitory compounds that could significantly influence EPS behavior, microbial dynamics, and carbon capture performance in practical applications. Second, the laboratory-scale configuration limits the direct transferability of our findings to full-scale systems, where factors such as hydraulic mixing patterns, scale-up effects, and long-term operational variability may alter the observed SRT responses. Third, we did not include control experiments to distinguish between biotic (microbial uptake) and abiotic (physical adsorption) contributions to carbon capture. This is particularly important under short SRT conditions (e.g., 1 d), where lower biomass concentrations may alter the relative contributions of these mechanisms. Fourth, we did not conduct functional prediction analysis (e.g., PICRUSt2 or FAPROTAX), and our functional interpretations based on genus-level identification should therefore be offered with a degree of prudence. Fifth, quantitative image analysis of EPS coverage or fluorescence intensity was not performed, and we have replaced quantitative claims with descriptive observations.

The conclusion that SRT = 4 d is optimal is strictly valid under the conditions tested (e.g., cycle time of 6 h, DO 0.4–0.6/1.5–2.0 mg·L^{−1}, synthetic wastewater at COD ≈ 800 mg·L^{−1}), and may vary with different configurations or real wastewaters. Future work should focus on: (i) validating these findings with real food-processing wastewater and pilot-scale systems to assess the practical applicability; (ii) incorporating high-throughput molecular techniques such as metagenomics and metatranscriptomics to elucidate the functional roles and metabolic pathways of key microbial populations; (iii) developing expanded models that couple EPS synthesis and consumption dynamics to better predict carbon capture performance; and (iv) exploring the potential role of quorum sensing in coordinating microbial community behavior during the feast-famine cycles of the CS process.

5. Conclusions

This study demonstrates that sludge age is strongly associated with the EPS characteristics, microbial community structure, and the distribution pattern of organic carbon among sludge recovery, effluent residue, and oxidation loss in a CS process treating synthetic food-processing wastewater (COD ≈ 800 mg·L^{−1}). Compared with SRT = 1 d and 7 d, the system exhibited superior carbon capture performance at SRT = 4 d: the total EPS content reached 43.14 mg·gVSS^{−1}, with TB-EPS being the dominant component; polysaccharides accumulated significantly during the contact phase and were effectively utilized during the stabilization phase, indicating that this condition is more conducive to establishing a reusable carbon temporary storage system. Simultaneously, Bacteroidota and functional

bacteria such as *Flavobacterium*, *Zoogloea*, and *Acidovorax*, which have been widely reported to be associated with organic matter hydrolysis, floc formation and EPS secretion, were synergistically enriched under this condition, consistent with the observed enhancement of bioflocculation and carbon redirection performance. Based on this, particulate, colloidal, and dissolved COD were all removed and transferred to the sludge phase in a more coordinated manner. The COD recovery rate from sludge reached 31.1%, and the oxidation loss was controlled at 31.9%, indicating that a suitable SRT can establish a more reasonable balance between carbon capture and carbon consumption. Therefore, under the conditions of this study, an SRT of 4 days can be considered a suitable operating parameter for the CS process to achieve efficient carbon capture and carbon redirection for synthetic food-processing wastewater. Its advantages do not stem from the improvement of a single indicator, but rather from the comprehensive optimization of EPS characteristics, functional bacterial synergy, and carbon metabolic distribution.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w18151815/s1>, Figure S1: Conceptual mechanistic diagram illustrating the integrated effects of SRT on EPS characteristics, microbial community assembly, and carbon flow pathways in the CS process under high organic matter concentration; Table S1: Composition of the synthetic food-processing wastewater used in this study; Table S2: Sequencing coverage indices for all samples under different SRT conditions.

Author Contributions: Conceptualization, X.M. and S.M.; methodology, S.M. and X.M.; investigation, S.M.; data curation, S.M.; writing—original draft preparation, S.M.; writing—review and editing, S.M. and X.M.; visualization, S.M.; supervision, X.M.; project administration, X.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data generated or analyzed during this study are available from the corresponding author upon reasonable request, except the raw sequencing reads which have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1462744 and are publicly accessible at <https://www.ncbi.nlm.nih.gov/sra/PRJNA1462744> (accessed on 23 July 2026). All other data supporting the findings of this study are presented within this published article.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this article:

ASV	Amplicon sequence variant
BCA	Bicinchoninic acid
COD	Chemical oxygen demand
cCOD	Colloidal chemical oxygen demand
CS	Contact stabilization
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
HRAS	High-rate activated sludge
HRT	Hydraulic retention time
LB-EPS	Loosely bound extracellular polymeric substances
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NCBI	National Center for Biotechnology Information
PCoA	Principal coordinate analysis
pCOD	Particulate chemical oxygen demand

PN	Protein
PS	Polysaccharide
sCOD	Soluble chemical oxygen demand
SRA	Sequence Read Archive
SRT	Sludge retention time
SV	Sludge volume
SVI	Sludge volume index
TB-EPS	Tightly bound extracellular polymeric substances
TN	Total nitrogen
TP	Total phosphorus

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