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Microalgae-mediated shaping of bacterial communities enhances antibiotic removal and antibiotic resistance control

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Abstract:

The microalgae-bacteria symbiosis sludge (MBSS) system offers a promising strategy for efficient wastewater treatment and nutrients upcycling. However, maintaining stable and effective performance facing antibiotic stress remains a significant challenge. This study explored the regulation strategy of microbial succession towards sulfadiazine (SDZ)-containing wastewater remediation while controlling antibiotic resistance genes (ARGs) spread in MBSS system. The MBSS achieved efficient SDZ removal of up to 99.8%, with an optimal microalgae-to-activated sludge inoculation ratio of 1:3. However, the highest nutrient upcycling efficiencies (33.7% for nitrogen and 98.6% for phosphorus) were observed at an inoculation ratio of 1:1. Metagenomics analysis revealed that genera *Chlorella* and *Micractinium* of Chlorophyta were strongly positively correlated with SDZ removal. Moreover, microalgae inoculation significantly modulated the microbial community structure, promoting the dominance of genera *Rhodanobacter* and *Dokdonella* in MBSS. This microbial succession could potentially facilitate bacterial co-degradation of SDZ and contribute to a substantially reduced level of ARGs (with the relative abundance of *sul1* and *sul2* decreasing to 22.9% post-treatment). Overall, the strategy of regulating microalgae inoculation in the MBSS significantly enhanced antibiotic removal and nutrient recovery while controlling the proliferation and spread of ARGs by directing microbial community succession.

Keywords: Microalgae-bacterial consortia; Sulfadiazine degradation; Extracellular polymeric substances; Antibiotic resistance; Antibiotic resistance genes transfer.

1 Introduction

The rapid expansion of swine farming has led to a substantial increase in antibiotic-containing wastewater, with volumes projected to reach approximately 105,596 tonnes by 2030 (Van Boeckel et al., 2015). The high concentrations of antibiotics detected in swine wastewater, averaging up to 1.1 mg/L for the top 20 antibiotics (Wan et al., 2021). Among them, sulfadiazine (SDZ) is highlighted as a widely used and poorly degradable sulfonamide class antibiotic, posing significant environmental and public health concerns (Zheng et al., 2020). Notably, environmental exposure to antibiotics will not only disrupt microbial ecosystem balance, but also facilitate the proliferation and spread of antibiotic resistance genes (ARGs) (Li et al., 2023). In addition, swine wastewater typically contains abundant nitrogen and phosphorus, which can lead to eutrophication and depletion of dissolved oxygen in natural water bodies if disposed untreated (Wu et al., 2023b). To date, various approaches including advanced oxidation (Zheng et al., 2024), membrane filtration, and anaerobic/aerobic treatment (Guo et al., 2024) have been explored, however, show limited effectiveness. Therefore, it is urgent to develop novel approaches that simultaneously address the multiple environmental challenges posed by swine wastewater.

Recent advances in biotechnology have favored activated sludge (AS) as a cost-effective and sustainable method for treating a wide range of wastewater (Zhang et al., 2023). Nevertheless, facing antibiotic stress, AS exhibits depressed removal efficiency and increased risks of ARGs spreading (Xiong et al., 2021). Microalgae-bacteria symbiosis sludge (MBSS) in this case, offers a promising alternative. Microalgae possess a strong capacity for simultaneous antibiotic removal and nutrient capture (Ma et al., 2024) and show higher tolerance to antibiotic stress compared to bacteria (Chi et al., 2022). The symbiotic interactions between bacteria and microalgae in MBSS, for instance, metabolite exchange (e.g., O_2 and CO_2), quorum sensing regulation, and extracellular polymeric substance (EPS) production (Li et al., 2022a), could further enhance pollutants (organics, N and P) removal with facilitating the generation of value-added products (Huo et al., 2020; Li et al., 2022a). The positive interactions in MBSS promote highly efficient removal of a wide range of antibiotics, including SDZ, oxytetracycline, levofloxacin, and clarithromycin (Kiki et al., 2023; Liu et al., 2022; Shi et al., 2025). Furthermore, many studies have shown that MBSS significantly limits ARGs spread compared to bacterial consortia alone (Li et al., 2022a; Li et al., 2025). Nevertheless, MBSS may also facilitates the spread of the ARGs-associated mobile genetic element *intI1* (Liu et al., 2022). This contradiction demonstrates that the performance of ARGs control remains highly variable, and the underlying mechanisms of antibiotic removal and ARGs spreading control are still poorly understood.

At present, the investigations on wastewater treatment by MBSS mainly focus on wastewater types (rich in N, P, and SCOD), environmental factors (pH and light intensity) and operation modes (Yu et al., 2023; Hou et al., 2020; Yuan et al., 2025). The effectiveness and stability of MBSS largely depend on maintaining an appropriate strain balance and the composition of the microbial community. Therefore, adjusting the inoculation ratio is considered one of the most effective strategies for improving wastewater treatment performance in MBSS. Studies have shown that the optimal ratio of microalgae to bacteria varies depending on the microalgal strains and the type of wastewater, typically ranging from 1:3 to 5:1. This range has been found to achieve both efficient nutrient removal and optimal biomass production (Ji et al., 2018). Nevertheless, the optimal inoculation ratio is highly strain-dependent, and comprehensive studies on the optimal inoculation ratio in MBSS for enhanced antibiotic removal while controlling ARGs are still lacking. Moreover, the underlying mechanisms by which microalgal metabolic activities influence the microbial community structure in MBSS, and in turn contribute to improved antibiotic removal and ARGs control, remain largely unknown.

In this study, the strategy of regulating inoculation ratios to control microbial succession in the MBSS was systematically explored for achieving optimal antibiotic removal in swine wastewater. With 5 mg/L SDZ in synthetic swine wastewater, this study aims to construct efficient MBSS by manipulating inoculation strategies for enhanced SDZ removal while controlling the proliferation and spread of ARGs. Through exploring dynamic changes of microbial communities and potential antimicrobial resistance bacteria, the mechanisms of MBSS addressing antibiotics and ARGs problems will be revealed.

2 Materials and Methods

2.1 Experimental materials

The microalgae *Chlorella* sp. (No. FACHB-31) was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. Based on the study of Ma et al. (2024), the experiment was conducted at 25±2°C and a light intensity with LED of 4500 lux (light:dark = 14 h:10 h) with stirring at 100 rpm. Air was supplied continuously during the culture period. With exposure to high concentrations of SDZ (e.g., 5 mg/L), the proliferation of ARGs can be rapidly accelerated (within a few hours to days) (Lopatkin et al., 2017). A 20-day experimental period was decided to allow the microbial consortium to reach stationary growth phase and achieve stable pollutant removal in all treatments. No additional nutrients were added during the trial. Microalgae were cultured to the exponential growth phase and then centrifuged at 4000 rpm for 10 min to obtain the precipitate. The resulting biomass was washed twice with sterilized deionized water and used as inoculant for subsequent experiments.

The aerobic AS was taken from the sewage treatment plant of Beijing Urban Drainage Group Co., Ltd. The mixed liquid suspended solids concentration and sludge settling velocity (SV_{30}) for the AS were 7280 mg/L and 88%, respectively. Prior to the experiment, the AS was acclimated and cultured for one week. The resulting pellet by centrifugation was washed twice with sterilized distilled water and subsequently used as a bacterial inoculum.

In order to ensure stability, the synthetic wastewater used to simulate swine wastewater was chosen for this experiment. The synthetic wastewater was prepared according to the study of Li et al. (2022a). The composition of simulate swine wastewater was 0.092 mg/L $CaCl_2 \cdot 2H_2O$, 0.11 mg/L $K_2HPO_4 \cdot 3H_2O$, 0.205 mg/L $MgSO_4 \cdot 7H_2O$, 0.05 mg/L $FeSO_4 \cdot 7H_2O$, 0.75 mg/L $C_6H_{12}O_6$, 0.25 mg/L CH_3COONa, 0.255 mg/L $NaNO_3$, and 1.2 mg/L NH_4Cl. The carbon source in the wastewater was provided by $C_6H_{12}O_6$ and CH_3COONa. NaOH was added to adjust pH to 7.5-8.5. The concentration of total nitrogen (TN), ammonia nitrogen (NH_4^+-N), total phosphorus (TP), and soluble chemical oxygen demand (SCOD) in the wastewater was 460 mg/L, 265 mg/L, 30 mg/L, and 765 mg/L, respectively. The reagents applied in the test were analytical grade, and the wastewater was used after sterilization.

2.2 Experimental design

This experiment was carried out in 2 L conical flasks. Each flask contained 1.5 L of sterilized synthetic wastewater. Concentrations of sulfonamides in the environment are reported to range from μg/L to mg/L (Cheng et al., 2020). However, in order to better focus the effect of antibiotics, promote the generation of ARGs and evaluate the strong stress resistance of different inoculation ratio groups, 5 mg/L of initial SDZ concentration was decided. Each flask was inoculated with *Chlorella* sp. and AS to construct the MBSS consortium. According to the inoculation ratios we had successfully built in our previous studies, 7 different inoculation ratio groups were obtained by fixing the *Chlorella* sp. biomass at 300 mg/L and varying the AS concentration:

(1) the pure microalgae culture group (1:0 wt/wt); the microalgae: the AS co-culture groups at different ratios: (2) 6:1 wt/wt, (3) 3:1 wt/wt, (4) 1:1 wt/wt, (5) 1:3 wt/wt, (6) 1:6 wt/wt, and (7) the pure AS culture group (0:1 wt/wt).

These options were named as C_0, R_1, R_2, R_3, R_4, R_5, and S_0, respectively. Detailed initial concentrations of microalgae and the AS are presented in Table 1, and three parallel experiments were set up per group. The experimental conditions were consistent with microalgae culture in Section 2.1.

2.3 Analytical methods

2.3.1 Biomass and pigment of the MBSS consortium

The total biomass was measured using the dry weight method based on the study Nguyen et al. (2020). For co-cultivation systems, the dry biomass included the microalgae and the AS features, as shown in Eq. (1) below:

$$C_T = C_M + C_{AS} \tag{1}$$

Where C_T was the total biomass concentration (g/L), C_M was the microalgae biomass concentration (g/L), C_{AS} was the AS concentration (g/L).

The concentration of chlorophyll a (Chla) (mg/L) in the microalgae was measured by 90% methanol extraction and spectrophotometry according to Ma et al. (2024). In the co-cultivation system, the microalgae biomass was defined based on the concentration of Chla, which confirmed the relationship fitting the standard curve as follows:

$$C_M = (33.2 \times C_{chla} - 23.1)/1000 \; (R^2 = 0.9994) \tag{2}$$

Where C_{Chla} was the concentration of Chla (mg/L). Also, the specific growth rate (SGR) was expressed as Eq. (3):

$$\mu = (\ln X_t - \ln X_0)/\Delta t \tag{3}$$

Where μ was the SGR (/d), X_t and X_0 were biomass at the initial and final time of different stages, Δt was the interval days (d). In this part, the value of μ was calculated using the entire stage for 20 d.

2.3.2 Micromorphology of the MBSS consortium

A laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, U.K.) was used to determine the particle size distribution of the MBSS consortium on day 20. Scanning electron microscopy (SEM, Hitachi SU3500, Japan) was used to characterize the microstructure of the initial microalgae, AS, and MBSS consortium on day 20. The specific sample processing method was based on Aditya et al. (2022).

2.3.3 Pollutants in wastewater

The concentrations of TN, NH_4^+-N, nitrate nitrogen (NO_3^--N), TP, and SCOD were tested according to standard methods (Rice et al., 2012). The quantification of SDZ was measured by ultrahigh-performance liquid chromatography (UPLC)-mass spectrometry (MS)/MS. The SDZ concentration was measured using Waters ACQUITY UPLC I-Class AB UPLC and SCIEX QTRAP 5500 triple quadrupole tandem with linear ion trap MS. Specific parameters of SDZ analysis can be found in Ma et al. (2024).

The removal efficiency and the specific uptake rate (SUR) under unit biomass were measured by the following Eq. (4) and Eq. (5), respectively:

$$\eta_A = (C_0 - C_t)/C_0 \times 100\% \tag{4}$$

$$\eta_B = (C_0 - C_t)/(|X_t - X_0| \cdot \Delta t) \tag{5}$$

Where η_A was the removal efficiency (%), and η_B was the SUR (mg/g$_{\text{-Total biomass}}$/d), C_0 and C_t were the concentration of substrate at time 0 d and t (mg/L or g/L). The meanings of other parameters were the same as Eq. (3).

2.3.4 Extracellular polymeric substance (EPS) of the MBSS consortium

The EPS was isolated through the heat extraction method, which yielded both the loosely bound EPS (LB-EPS) and the tightly bound EPS (TB-EPS). The main composition of proteins (PN) and polysaccharides (PS) in EPS was measured using the rapid Lowry method and the phenol-sulfuric method, respectively (Tang et al., 2021b).

Organic substances in EPS were analyzed based on fluorescence excitation emission matrix (EEM) spectroscopy (AquaLog, HORIBA, Japan). Test parameter settings referred to the study of Chen et al. (2003). Fluorescence regional integration (FRI) technology was used for the EEM spectral data analysis. The FRI technology was calculated by quantitative volume integration of the fluorescence peak in each region. The percent fluorescence response in different regions were measured by Yu et al. (2020). In order to characterize the wastewater, two fluorescence indices: humification index (HIX) and biological index (BIX) were calculated by the method of Rodríguez-Vidal et al. (2020).

2.3.5 DNA extraction and metagenomic sequencing

Before testing, the 3 parallel samples from each treatment group were thoroughly mixed as representative samples on the 20th day. There was a total of 7 representative samples which were saved at -80°C. DNA from each processed sample was extracted, using a DNA E.Z.N.A.®

Soil DNA Kit (Omega Bio tek, USA). The DNA concentration and purity were detected using a Quantum Fluorometer (Picogreen) and NanoDrop2000 (Thermo Fisher Scientific, USA), respectively. The DNA integrity was tested using 1% agarose gel electrophoresis. Fragmentation of the DNA was performed, using a Covaris M220 focused-ultrasonicator (Covaris, Woburn, MA, USA), and approximately 400 bp fragments were screened for construction of a paired-end (PE) library. The V3-V4 region of bacterial 16S rRNA was amplified using 338F/806R primers, and the V5-V7 region of fungal 18S rRNA using SSU0817F/1196R primers. Metagenomic analysis was performed using an Illumina Novaseq 2000 (Illumina Inc., San Diego, CA, USA) and Novaseq kits according to specific protocols. Quality control was done by splicing, assembly, amino acid sequence translation, non-redundant gene set construction, and abundance calculation of the obtained sequences according to established methods (Chi et al., 2022). The sequencing results were analyzed using a Majorbio (Shanghai, China) online platform. Representative sequences in non-redundant gene sets were compared in the NR database, using Diamond (v 0.8.35) with an e-value≤ $1e^{-5}$ for taxonomic annotations. ARGs levels were compared within samples and in the database (ARDB) based on amino-acid sequences in non-redundant gene sets (identity> 70%, e-value≤ $1e^{-5}$) (Guo et al., 2024).

2.4 Data analysis

Mothur (v 1.30.1) was applied to determine alpha diversity, including the Chao1 index, Shannon estimator, and Shanoneven index. R software (v 3.3.1) using a vegan package was used to perform principal co-ordinates analysis (PCoA). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed to annotate functional genes. R software (v 3.4.2) using vegan package was taken to perform distance-based redundancy analysis (db-RDA). The pheatmap package (v 1.0.8) was used to generate heat maps. The Pearson's rank correlation coefficient (r) showed correlations of main ARGs levels and dominant microbial taxa levels for every sample. Besides, the corrplot and ggplot2 packages were employed for constructing a correlated graph. The $|r| \geq 0.5$ was considered to be a significant correlation. SPSS 26.0 software was used for one-way analysis of variance (one-way ANOVA) with a p-value less than 0.05 as the significance level. Microsoft Office Excel 2021 was used for statistical calculation, and Origin 2022 was used for mapping.

3 Results and Discussion

3.1 Effect of inoculation ratios on the characteristics of MBSS

The growth of biomass plays an important role in pollutant elimination, and alterations in biological fractions reflect microbial metabolic relationships in MBSS (Xiao et al., 2025). As shown in Fig. 1a, the inoculation ratio significantly affected the time required to achieve microbial community stability in MBSS, ranging from 2 to 16 days. All treatments with a higher microalgae-to-AS ratio (R_1, R_2, and R_3) showed a significant increase in biomass accumulation compared to the initial inoculum. A relatively balanced inoculation ratio allowed both partners in the consortium to access nutrients more evenly, thereby maintaining active microalgal photosynthesis that fixed CO_2 into the system and enhanced overall biomass growth. Meanwhile, the robust growth of microalgae also accelerated antibiotic removal, which in turn reduced the stress imposed on bacteria by exposure to SDZ (Li et al., 2022a).

Notably, when the inoculation ratio shifted to 1:1 wt/wt (R_3), the fraction of microalgal biomass decreased from 50% (day 0) to 35.55% (day 6) and then increased to 74.01% (day 18), achieving the highest fraction increase. It demonstrated that the initial inoculation ratio significantly affects the composition and growth of MBSS, hence indirectly affecting system tolerance and wastewater treatment efficacy. The SGR of microalgal biomass and total biomass showed an increasing trend followed by a decrease as the inoculation ratio increased (Fig. 1b). Among them, R_2 had the highest microalgae and total biomass SGR values of 0.070/d and 0.042/d, respectively. The AS with too low concentration would not be enough to enhance the system's growth effectively, which was reflected in no difference between C_0 and R_1 in total SGR ($p > 0.05$). On the contrary, the high AS concentration in the system would disturb the photosynthesis process of the microalgae (Liyun, 2023). Therefore, the initial inoculation ratios of 3:1 wt/wt and 1:1 wt/wt exhibited better biomass growth rate and productivity. The result of this study was consistent with that of Laurent et al. (2019), that the inoculation ratio of 1:1 wt/wt in the MBSS system had the best enhancement in biomass growth and microbial activity. Considering the stability of the system (Nguyen et al., 2020), also identified the next best inoculation ratio of 3:1 wt/wt. Notably, inoculation with AS alone led to a rapid and significant decline in total biomass with high negative specific growth rate (-0.028/d), highlighting the protective role of microalgae for the bacterial consortium under SDZ stress (Fig. 1a, b).

The proportion of big particle size (> 50 μm) in R_3 was the greatest at 45.55% across all groups, with the majority concentrated in the 50-200 μm range (Fig. 1c). Yu et al. (2024) found that on the 251st day, the average particle size of the AS increased from 31.9 μm to 138.5 μm, thus forming granular aggregates with good treatment effects. The larger particle size in R_3 was due to the aggregation of the AS with microalgae attaching, which demonstrated good formation of the MBSS consortium (Tang et al., 2021a). As shown in the SEM images, microalgae in R_3 were more densely aggregated on the sludge surface compared to the more dispersed distribution observed in C_0 and R_1 (Fig. 1d). In contrast, treatments R_4 and R_5, which had higher AS concentrations, appeared to reduce the specific surface area available for microalgal attachment. The SEM images of these groups revealed a large, continuous sludge matrix, which may have limited microalgal colonization and could be unfavorable for system performance.

3.2 Overall performance of pollutants removal

3.2.1 SDZ

SDZ can be removed through several mechanisms, including photolysis, hydrolysis, (bio)adsorption, bioaccumulation, and biodegradation. Given the absence of hydrolysable functional groups and limited photosensitivity of SDZ, neither hydrolysis nor photolysis represents a primary removal mechanism (Zhao et al., 2022). Bioaccumulation and (bio)adsorption do not completely remove antibiotics, and many studies have shown that the main removal pathway of SDZ is biodegradation (Ma et al., 2024; Zheng et al., 2020; Xiong et al., 2021). Therefore, this study focused on the biodegradation of SDZ. In the present study, increasing the ratios of AS to microalgae led to significantly higher and faster removal of SDZ. When microalgae were dominant in the system (C_0, R_1, and R_2), SDZ removal was slower, with a significantly lower efficiency of up to 90%. At day 20, the SDZ removal efficiency of R_3 reached highest of 99.81%, indicating that appropriate microalgae addition can promote SDZ removal. Liu et al. constructed that algal-bacterial granular sludge (ABGS) to remove tetracycline and SDZ up to 79.0% and 94.0% respectively, which were 4.3-5.0% higher than that of bacterial granular sludge (BGS) (Liu et al., 2022).

Notably, the cultivation of AS alone (S_0) resulted in the highest and fastest removal, over 99% within 10 days. S_0 significant aggregation was observed, forming particles larger than 400 μm (Fig. 1c). A smaller particle size typically provides a higher surface-to-volume ratio, which enhances antibiotic removal through adsorption (Kim et al., 2020). Despite this larger particle size, this treatment resulted in a faster antibiotic removal efficiency. The possible reason is the diverse microbial community present in AS provides broad metabolic activities; for example, ammonia-oxidizing bacteria, which have been reported to possess antibiotic degradation potential (Yu et al., 2024). The highest specific uptake rate (Table 2) was achieved in group R_4 (with a 1:3 inoculation ratio) at day 4, reaching 9.51 mg/$g_{\text{-Total biomass}}$/d, which consistent with the previous study (Rong et al., 2023). However, there remains a gap between the present laboratory conditions and practical full-scale operation. The antibiotic removal efficiency of the proposed MBSS system could be further enhanced through strategies such as bioaugmentation with SDZ-degrading microalgal strains, pre-acclimation of the microbial community, and optimization of operational parameters (e.g., biomass concentration, aeration, and hydraulic retention time). These aspects warrant further investigation in future work.

3.2.2 Nutrients removal

During the treatment, groups with higher AS ratios exhibited higher NH_4^+-N removal efficiencies. Among them, R_5 achieved the highest removal efficiency of 62.75%, followed by R_4 and R_3 (Fig. 2c). It has been reported that MBSS systems harbor diverse ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, which convert ammonium to nitrate through nitrification (Fallahi et al., 2021). As a result, the concentrations of NO_3^--N increased to varying degrees across the different treatments. Among them, the NO_3^--N concentration in C_0 and groups with high inoculation ratios (R_1, R_2, and R_3) exhibited just a slight rise, while other groups featured a huge increase. Notably, R_5 increased from 90.23 mg/L to 282.91 mg/L, with an SUR value of -27.07 mg/$g_{\text{-Total biomass}}$/d (Table 2). Overall, the group R_3 achieved the best TN removal with the efficiency and SUR value at 33.25% and 34.30 mg/$g_{\text{-Total biomass}}$/d, respectively. Although most microorganisms preferentially utilize NH_4^+-N as the nitrogen source for growth, microalgae are outcompeted by bacteria for NH_4^+-N uptake (Wang et al., 2020a). Consequently, in groups with a high AS inoculation ratio, NH_4^+-N is converted to NO_3^--N driven primarily by bacteria, resulting in a reduction in NH_4^+-N levels and an augmentation in NO_3^--N levels. In this process, nitrogen only underwent a transformation in form and was not truly assimilated or removed. The optimal initial inoculation ratio (1:1 wt/wt) established microalgae dominance in the system and decreased shading, which facilitated the assimilation of NH_4^+-N into amino acids. However, it was reported that the addition of antibiotics (fluoroquinolones) hindered TN removal in the microalgae-bacteria consortia by reducing the relative abundance of nitrogen metabolism functional genes (Qv et al., 2025). Therefore, nitrogen removal efficiencies in this study were low.

Most of the inorganic phosphates (HPO_4^{2-} or H_2PO^-) can be phosphorylated by microalgae to eventually synthesize ATP (Li et al., 2022b). Therefore, C_0 and groups with high microalgae inoculation ratios (R_1, R_2, and R_3) had high TP removal efficiency more than 95% (Fig. 2e). Among them, R_3 had the highest SUR value of 5.31 mg/g$_{\text{Total biomass}}$/d, indicating that it can most thoroughly and rapidly remove TP from the water (Table 2). According to the changes of total biomass and composition ratio of MBSS system (Fig. 1a), TP remained stable and even increased slightly after depletion on the 4th day. Microalgae have a high ability to absorb phosphorus, which can be converted into polyphosphates for storage (Wu et al., 2021), so the MBSS system was less affected. However, in R_5 and S_0, the TP removal efficiency was -49.71% and -45.24%, respectively. The reason for this phenomenon was that the high AS content in the system made a relatively active localized anaerobic micro-environment (Chai et al., 2021). The system could not reach the aerobic environment required for microalgae to assimilate phosphate even during the light stage. What's more, some bacteria, such as polyphosphate-accumulating organisms, could decompose the intracellular polyphosphate and release it into the water under anaerobic conditions. In addition, the disintegration or dissolution of a part of the AS could also cause TP content to increase.

It has been reported that microalgae and aerobic bacteria present in activated sludge, such as aerobic heterotrophs, utilize dissolved oxygen to degrade organic compounds. This process represents the primary pathway for the removal of soluble chemical oxygen demand (Fallahi et al., 2021). The groups, R_1, R_2, and R_3, reached high levels of SCOD removal (> 65%) at day 20 (Fig. 2f). Among them, R_3 had the highest specific SCOD removal rate of 105.36 mg/g$_{\text{Total biomass}}$/d (Table 2). Microalgae release oxygen through photosynthesis, which supported microbial activity and thereby promoted the removal of SCOD (Chai et al., 2021). It is reported that the addition of microalgae promotes the concentration of dissolved oxygen (DO) and increases the abundance of aerobic bacteria (Li et al., 2025). However, a higher AS inoculation ratio may lead to a shading effect on the system, while a large number of microorganisms in the sludge consume DO through respiration. As a consequence, the SCOD removal efficiencies in R_4 and R_5 stabilized at only 44.82% and 57.40%, respectively.

3.3 Dynamics of extracellular organic matter in MBSS consortium

The EPS was categorized into TB-EPS and LB-EPS, with the dominance of TB-EPS in the consortia (Fig. 3a, b). The total EPS concentrations in S_0 and R_5 were lower than those observed in the rest groups (Table 3). This is consistent with previous findings reporting that the total EPS concentration in MBSS systems exceeded that of pure AS systems by 18.4% (Tang et al., 2021b). In the MBSS system, adding microalgae supplies sufficient O_2 for bacteria, promoting EPS production (Tang et al., 2021a). During the experiment, TB-EPS concentrations increased in R_1-R_4, whereas LB-EPS showed a slight decline. TB-EPS supports tight microbial aggregation and nutrient supply, while LB-EPS acts as a protective and adhesive buffer (Yu et al., 2023). Notably, R_2 and R_3 maintained relatively high LB-EPS levels, corresponding to their effective pollutant removal (Section 3.2). As the consortium structure developed and pollutants were removed, the role of LB-EPS diminished, likely being converted into TB-EPS to support further growth (Wang et al., 2022a). The TB-EPS concentration in the R_3 group showed the highest increase by 6.2 times to the largest content of 86.56 mg/g$_{\text{-Total biomass}}$ on day 20. It indicated that the MBSS consortium was structurally stable and grew rapidly at the 1:1 wt/wt inoculation ratio, aligning with the conclusion drawn in Section 3.1. The EPS of MBSS was primarily composed of PN and PS, and the concentration of PN was higher than that of PS. It had been reported that a higher PN/PS ratio could promote microbial aggregation to accelerate granulation (Jin et al., 2023). After the trial, the PN/PS ratio values showed an increasing trend in both TB-EPS and LB-EPS of the MBSS systems, which indicated stable construction of the co-culture system.

The composition of extracellular organic matter in the MBSS system was characterized by three-dimensional fluorescence spectroscopy. Dominant peaks appeared in Regions IV and II, with combined the FRI values exceeding 0.5 (Fig. 3d, e), indicating that soluble microbial byproducts (SMPs) and tryptophan-like proteins were the main components, consistent with previous studies (Baroni et al., 2020; Khan et al., 2022; Wu et al., 2023b). In groups C_0, R_1, R_2, R_3, and R_4, the fluorescence intensity and BIX values increased, reflecting active microbial metabolism and organic matter production. In contrast, R_5 and S_0 (with higher AS content) showed reduced fluorescence and higher FRI in Regions III and V, suggesting consumption of proteins and metabolites to form humic substances (Wu et al., 2023b). Correspondingly, these groups also had higher HIX values, indicating greater humification and lower microbial activity (Rodríguez-Vidal et al., 2020).

3.4 Analyses of biological community structure

3.4.1 Microbial community composition

The alpha diversity of the microbial community is displayed in Table 3. The indices of Chao1, Shannon and Shannoneven represent microbial richness, diversity, and evenness, respectively. Most co-cultures demonstrated higher Shannon indices compared to the pure AS group (S_0) (Table 4), indicating that the addition of microalgae can increase bacterial diversity by interaction, micro-environmental changes, and niche ecological factors (Zhang et al., 2020). R_2 had the highest richness, while S_0 had the highest diversity. R_3 and R_4 featured the relatively lowest Shannon and Shannoneven indexes in the co-culture systems, which meant a certain class of eukaryotes was dominant in this system. Multivariate analysis of the differences in microbial composition was conducted using the PCoA. The results showed that R_5 and S_0 clustered closely together and were clearly separated from the other groups (Fig. 4c). In addition, the different co-culture groups were scattered across the plot, indicating significant differences in microbial composition among the systems. These differences were largely influenced by the inoculation ratio of microalgae to bacteria.

The metagenomics analysis revealed distinct patterns in relative abundance of eukaryotic and bacterial compositions across different inoculation ratios (Fig. 4a, 4b). Chlorophyta, including *Chlorella*, *Micractinium*, and *Auxenochlorella*, were consistently dominant across most groups, particularly with high microalgal inoculation ratios (C_0, R_1, R_2, and R_3). However, as the increase of AS inoculation ratio, the eukaryotic composition shifted markedly (in groups R_4 and R_5) with a decline in Chlorophyta and a rise in *Cryptomycota*, *Mucoromycota*, etc. Meanwhile, *Pseudomonadota* and *Bacteroidota* were identified as main bacteria in the sludge, which is consistent to relevant studies (Chai et al., 2021; Wu et al., 2023a). They have been proven to play a role in the utilization of nutrients and sulfonamide degradation in water bodies (Hou et al., 2022; Zhang et al., 2023).

Under high AS inoculation ratios (R_5 and S_0), *unclassified_p_Pseudomonadota* and *unclassified_p_Bacteroidota* accounted for the largest proportion of the bacterial community within the consortium. Meanwhile, under a relative balanced inoculum of microalgae and AS (R_3 and R_4), *Rhodanobacter* was dominant with a relative abundance of 0.17 and 0.14, respectively. *Dokdonella* was the dominant bacterium in R_1 with the abundance of 0.13, while *Devosia* was dominant in R_2 with the abundance of 0.14. The variability of the microbial composition in different groups may cause differences in water treatment efficiency.

3.4.2 Correlation between microorganisms and pollutants removal efficiency

The correlation between the relative abundance of microbial genera and the removal efficiency of water pollutants provides insights into identifying key microorganisms within the MBSS that contribute to pollutant removal (Fig. 4d). The prokaryotes *Dokdonella* and *Rhodanobacter*, and the microalgae *Auxenochlorella*, *Micractinium*, and *Chlorella*, demonstrated the strongest positive correlation with SDZ removal. Microalgae harbor diverse mechanisms that are directly responsible for antibiotic removal (Xiong et al., 2021), and could generate several ways, including EPS release, oxygen regulation, etc., to protect the bacterial consortia and strengthen antibiotic removal (Wang et al., 2024). Even the dominant prokaryotes *Rhodanobacter* and *Dokdonella* were not proven of any direct antibiotic removal capacity, *Rhodanobacter*, however, may contribute to co-degradation of antibiotics owing to its diverse pollutant degradation ability, while, the dominant of *Dokdonella* could outcompete other ARGs-carrying strains, thus, reduce SDZ ARGs abundance (Yang et al., 2022).

Nitrogen removal was strongly linked to microbial composition. The *unclassified_p_Pseudomonadota* and *unclassified_p_Bacteroidota* showed significant correlations with NH_4^+-N removal and NO_3^--N production, likely due to their ammonia oxidation and cooperation with nitrifiers (Fallahi et al., 2021; Wang et al., 2020b). Groups R_4, R_5, and S_0, where these bacteria were abundant, had higher NH_4^+-N removal and NO_3^--N content. Additionally, *Rhodanobacter*, capable of complete denitrification, was positively correlated (r =

0.79) with TN removal, explaining the highest TN removal observed in group R_3 (Wu et al., 2023a). SCOD and TP removal were mainly driven by microalgae. Genera such as *Auxenochlorella*, *Micractinium*, and *Chlorella* showed positive correlations with removal efficiency. Microalgae reduce SCOD by assimilating dissolved organic matter into biomass (Chai et al., 2021) and remove TP by absorbing dissolved phosphorus through photosynthesis, incorporating it into cellular components (Li et al., 2022b). Additionally, microalgal EPS can bind phosphorus, enhancing TP precipitation (Fallahi et al., 2021). A few bacteria, including *Dokdonella* and *Rhodobacter*, also positively correlated with SCOD and TP removal. Consistently, groups R_1, R_2, and R_3, which had higher microalgae inoculation ratios, achieved higher SCOD and TP removal efficiencies (Fig. 2e-f).

3.4.3 The KEGG analyses of microbial metabolomics

As shown in Fig. 4e, among the functional category of global and overview maps, a higher inoculation ratio of microalgae to AS generally led to a reduced metabolic diversity, particularly in carbon metabolism, biosynthesis of amino acids, and nucleotide metabolism. However, the relative abundance patterns of specific functional pathways varied significantly among different treatments. Quorum sensing, photosynthesis, ABC transporters and ribosome-related metabolism exhibited the highest abundance under higher microalgae to AS inoculation ratio (3:1 in R_2 treatment) at day 20. In contrast, most other metabolic pathways reached the highest abundance under lower microalgae ratios (R_5 and S_0), suggesting that greater microbial abundance under these conditions may support broader metabolic capabilities. Microalgal activity substantially influences microbial community composition and function by releasing oxygen, organic carbon, and EPS, as well as by altering ambient pH. Thus, optimizing the microalgae to AS ratio is critical for maintaining microbial diversity and ensuring metabolic resilience with the MBSS systems, thereby enhancing SDZ removal efficiency while lowering ARGs abundance.

Notably, pyrimidine metabolism was upregulated in R_2, R_5, and S_0 treatments (Fig. 4e). Studies indicated that gene expression upregulation of pyrimidine metabolism implies positive correlation with SDZ removal, likely due to its involvement in DNA repair and stress adaptation (Leng et al., 2020). Analysis of the microbial contributions of the major metabolic pathways showed that bacteria, like *unclassified_p_Pseudomonadota* and *unclassified_p_Bacteroidota*, made high contributions in R_5 and S_0 (Fig. 4f). However, eukaryotes, including *Chlorella*, *Micractinium*, *Auxenochlorella*, and *Brachionus*, contributed largely in other co-culture groups (R_1, R_2, R_3, and R_4). It demonstrated that microalgae had a great metabolic role in the MBSS system.

3.5 Fate of ARGs

3.5.1 ARGs composition and the relationship with environmental factors

As shown in Table 4, alpha diversity indices indicated that ARGs levels remained low in C_0, likely due to microalgal limited interaction with antibiotics. In contrast, AS served as an ARGs reservoir, and co-culture groups (R_2, R_3, R_4) showed a moderate increase in Chao1 index (up to

31). Extremes in inoculation ratio (R_1, R_5) led to higher Chao1 values of ARGs. This may result from competitive stress-induced horizontal gene transfer (HGT) mediated by quorum sensing (Li et al., 2023; Li et al., 2024). Shannon indices ranged from 1.92 to 2.39 in co-culture groups, compared to 1.15 in S_0 and 1.54 in C_0. Resistance genes abundance of bacitracin, sulfonamide, and tetracycline accounted for over 50% of the total ARGs (Fig. 5a). In all groups, baca of bacitracin had the highest relative abundance, with *sul1* and *sul2* of sulfonamide in second and third place, respectively (Fig. 5b). The relative abundance of *sul1* and *sul2* genes caused by the major antibiotic SDZ of this study in co-culture groups was lower than in S_0. The lowest sul gene abundance (22.92%) was observed in R_3, indicating that a 1:1 wt/wt inoculation ratio is optimal for both thorough SDZ removal and effective suppression of sul gene transmission. From the distance-based redundancy analysis (db-RDA), the composition and similarity of ARGs was divided into three regions, the composition of ARGs was similar in the same region, but it varied greatly between different regions (Fig. 5c). TP concentration showed the most significant correlation with ARGs composition. However, there are few reports showing that nutrient elements are directly related to changes in ARGs abundance, but they can indirectly affect genes transfer rate by changing the composition of the microbial communities (Li et al., 2022a).

3.5.2 Correlation between ARGs and microorganisms

The Chlorophyta with *Chlorella* as the maximum proportion had the lowest *r* values, less than -0.93 (Fig. 5d). However, except for Aetinomycetota, all other bacteria showed positive correlations with ARGs. In particular, Pseudomonadota, which has the largest bacterial abundance, had positive correlation coefficients exceeding 0.80 with *cml_e3*, *aph33ib*, *sul2*, and *baca*, which is consistent with a previous study (da Silva Rodrigues et al., 2021). The AS used in this experiment was obtained from the sewage treatment plant, which contained a diverse of local microbial species, leading to a high content of microbial species categorized as “Others” (Fig. 4a, b). It was reported that these two major target ARGs (*sul1*, *sul2*) were mainly contributed by the widespread carrying of a variety of different microbial species (Zhou et al., 2017). As shown in Fig. 5e, most of the bacteria showed a positive correlation with the target gene (*sul2*), among which *unclassified_p_Pseudomonadota* and *unclassified_p_Bacteroidota* had the most significant effects. In contrast, *Chlorella* demonstrated a significant negative correlation with *sul2*, indicating a potential inhibition effect on *sul2* proliferation and spreading. It has been reported that the phyla, Pseudomonadota and Bacteroidota, are the main hosts of *sul* (Yuan et al., 2025). In the groups dominated by microalgae (C_0, R_1, and R_2), the relative abundance of *unclassified_p_Pseudomonadota* and *unclassified_p_Bacteroidota* decreased (Fig. 4b), indicating that the dominance of microalgae in MBSS can reduce the relative abundance of microorganisms carrying ARGs, thereby reducing the spread of ARGs.

3.6 Mechanism of the co-occurrence patterns between bacteria and microalgae

Mechanisms of the co-occurrence patterns between bacteria and microalgae are showed in Fig. 6. The SEM result indicated that the MBSS consortium was mainly generated by microalgae wrapping around the AS periphery (Fig. 1d). The bioadsorption of antibiotics involves the formation of hydrogen bonds, in which EPS also plays an important role (Tang et al., 2021a). Meanwhile, the antibiotics diffusion in EPS is only 36%-76% of that in water, reducing acute toxicity (Wang et al., 2022a). Many antibiotic degradation bacteria (ADB) and microalgal growth-promoting bacteria (MGPB), such as Pseudomonadota, are protected by EPS (Wang et al., 2023). This explained that R_3 with a relatively high EPS had the most efficient SDZ removal efficiency and higher biomass growth. Further investigation should involve the use of Fourier Transform Infrared spectroscopy to verify the specific EPS functional groups responsible for the adsorption and degradation of SDZ.

The first defense against antibiotics is altering the cell membrane and wall permeability to block their entry. If antibiotics enter, bacteria activate efflux pumps to expel them (Nguyen et al., 2020). Any remaining antibiotics are then degraded into CO_2 and H_2O by specific enzymes, with byproducts less toxic than the originals (Ma et al., 2024; Zheng et al., 2020). When antibiotics accumulate inside cells, a process called bioaccumulation, excess SDZ induces reactive oxygen species (ROS) production. MBSS upregulated repair and mutation-related genes, and increased antioxidants to combat oxidative stress. Since the nuclear membrane in microalgae acts as a barrier against antibiotic-induced stress, peripheral microalgae contribute to protecting the whole MBSS consortium (Chen et al., 2020). Dihydropteroate, essential for folic acid synthesis, is produced by dihydropteroate synthase (DHPS) from p-aminobenzoic acid (PABA) and pteridine precursor (Zheng et al., 2024). SDZ, a structural analog of PABA, inhibits this process by competing with PABA for the active sites of DHPS (Chen et al., 2020). Resistance arises when sul genes encode mutant DHPS with altered active sites, reducing SDZ binding and conferring resistance.

ARGs often spread via horizontal gene transfer (HGT) between microbes through transformation and conjugation. High SDZ concentrations apply selective pressure, increasing sul gene carrying in the environment. Microalgae play a critical role in protecting the MBSS and regulating microbial structure succession towards fewer antibiotic-resistant bacteria, mainly through secreting EPS (Table 3). Studies demonstrated a gradual decrease in the HGT frequency of ARGs in the MBSS consortium with increasing treatment time (Wang et al., 2022b; Zhang et al., 2020). This study also confirmed that both the richness of total ARGs and the relative abundance of target sulfonamide ARGs were decreased in co-culture groups as compared to the pure AS culture group. Microalgae play a critical role in regulating microbial structure succession towards fewer antibiotic-resistant bacteria, thereby reducing ARGs abundance. This suggests that the MBSS consortium makes the environmental risk of ARGs relatively modest. However, this study was conducted under laboratory conditions and used sterilized synthetic swine wastewater. The findings of the present study are limited by the use of synthetic wastewater, which may not accurately represent MBSS performance in practical antibiotic removal in real wastewater. To validate these results, future work should employ real swine wastewater in a pilot-scale study.

4. Conclusion

This study demonstrated that the proper inoculation of microalgae could form a structurally stable microalgae-bacteria symbiosis sludge (MBSS) consortium towards efficient nutrients upcycling, sulfadiazine (SDZ) removal with ARGs control. The MBSS system mainly removed SDZ through biodegradation of bacteria. Microalgae played a major role in removing nitrogen and phosphorus, regulating microbial succession. Mainly through secreting EPS, microalgae can protect the MBSS system from SDZ and reduced the spread of antibiotic resistance genes (ARGs). The optimal inoculation ratios were defined as 1:1 wt/wt and 1:3 wt/wt. These findings offer valuable insights for MBSS to achieve effective antibiotic removal with relatively modest ARGs environmental risk.

Data availability

Data will be made available on request.

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Declaration of interest statement

As Roger Ruan, a [co-]author on this paper, is an editorial board member of *Bioresource Technology*, he was blinded to this paper during review, and the paper was independently handled by Samir Kumar Khanal as Editor-in-Chief.

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Microalgae-mediated shaping of bacterial communities enhances antibiotic removal and antibiotic resistance control

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