

Long-term operational strategy of microalgal-bacterial granular sludge towards strengthened sulfadiazine antimicrobial resistance control

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Abstract:

Harnessing the dynamic interactions within the microalgal-bacterial granular sludge (MBGS) systems represents an emerging biotechnological frontier in tackling antibiotic pollution. Nevertheless, effective long-term treatment targeting strengthened antimicrobial resistance remains challenging. This study explored the operational strategies of pre-accumulation with antibiotic-free cultivation and pre-acclimatization with 5 mg/L sulfadiazine (SDZ) exposure for long-term, effective high-concentration SDZ removal (stepwise increase up to 50 mg/L), while controlling antibiotic resistance genes. The results showed that pre-accumulation achieved a stable microbial structure and greater antibiotic removal (up to 99.9%) under extremely high SDZ concentration (50 mg/L) compared to pre-acclimatization, while pre-acclimatization suffered more oxidative damage, leading to lower biomass and pigments accumulation, but higher SDZ removal under 30 mg/L. The amide bond hydrolysis was identified as the predominant degradation pathway, resulting in less toxic by-products. Pre-accumulation performed better in controlling relative abundance of target sulfonamide antibiotic resistance genes (ARGs, *sul1* and *sul2*) compared to pre-acclimatization. Genes encoding metabolic pathways related to antibiotic removal remained active, with downregulated genes encoding growth. *Chlorella* exhibited a protective effect on bacteria and inhibited ARGs spread, whereas *Nakamurella* and *Nitrosomonas* might be the key bacteria in SDZ biodegradation, but also served as potential hosts of ARGs. The continuous and efficient removal of SDZ, along with the

34 mitigation of ARGs spread and toxic by-product accumulation under ultra-high antibiotic
35 exposure, can be attributed to the MBGS system's initial operational strategy. The initial
36 operational strategy involving stress pre-acclimation and biomass pre-accumulation offers a
37 robust and adaptable solution for treating antibiotic-containing wastewater.

38 **Keywords:** Antibiotic resistance gene, antibiotic bioremediation, biodegradation pathway,
39 wastewater treatment, environmental risk

40 **Environmental Implication:**

41 The sulfadiazine (SDZ) residue in wastewater is not only toxic to organisms, but also promotes
42 the production and spread of antibiotic resistance genes (ARGs) in the environment. Our
43 research found that the microalgal-bacterial granular sludge (MBGS) systems can continuously
44 and efficiently remove SDZ under ultra-high antibiotic concentration, while effectively curbing
45 the spread of ARGs and toxic by-products. These findings theoretically prove that microalgae-
46 driven co-metabolism is a promising strategy to reduce SDZ residues in the environment, and
47 provide new ideas for promoting green and sustainable development of livestock and poultry
48 wastewater.

49 **1 Introduction**

50 Sulfadiazine (SDZ), a typical and widely used sulfonamide antibiotic, is employed for the
51 treatment of infectious diseases and to promote livestock growth [1]. Due to its poor
52 biotransformation (80% excreted unmetabolized from organisms) and its high persistence in
53 environmental compartments, SDZ has emerged as a critical concern [2, 3]. Consequently, SDZ
54 residues have been detected in wastewater at concentrations ranging from 0.81 ng/L to 1.16
55 mg/L [4], and the values even increased to tens of mg/L in pharmaceutical plants effluent [5].
56 The enduring presence of residual SDZ imposes ongoing environmental selection pressure,
57 driving the spread of antibiotic resistance bacteria (ARB) and antibiotic resistance genes
58 (ARGs). These microbial adaptations pose considerable risks to both public health and
59 ecological safety [6]. Thus, effective treatment of high-concentration SDZ wastewater is urgently
60 needed worldwide.

61 Current antibiotic removal methods, including adsorption, ultrafiltration, and advanced
62 oxidation [2, 7], demonstrated efficient performance, however, remain challenged by high
63 energy demands and cost, as well as the generation of hazardous solid wastes. In this view,
64 bioremediation, particularly using microalgae system and bacterial granular sludge (BGS)
65 system, offers a cost-efficient alternative for sustainable antibiotic remediation with a low-
66 carbon footprint [8]. The MBGS system combines microalgae with BGS system, enabling diverse
67 metabolic interactions [9, 10]. By overcoming the drawbacks of conventional systems, such as
68 growth inhibition and metabolic impairment, MBGS can facilitate effective biomass growth and
69 pollutant elimination [11]. A mutually beneficial symbiosis is established that bacteria degrade
70 antibiotics (e.g., Proteobacteria and Bacteroidetes) and provide essential nutrients (N, P,
71 vitamin B12) for microalgae, while microalgae protect bacteria via extracellular polymeric
72 substances (EPS) secretion and supply oxygen to enhance aerobic degradation [6, 12, 13]. The
73 removal efficiency of various antibiotics (e.g., sulfamethoxazole, chlortetracycline,
74 oxytetracycline) by the MBGS system ranges from 54.3% to 97.8%, a performance governed by
75 key factors including antibiotic concentration and the constructed community composition [14-
76 16]. However, extensive research have demonstrated the efficacy of MBGS system in removing
77 antibiotics at medium-to-high concentrations (< 10 mg/L) [5, 6, 17-20]. In addition, the MBGS
78 system also exhibited a significantly reduced abundance of ARGs compared to the bacterial

79 granular sludge system [14, 21]. Therefore, the MBGS system is a promising yet incompletely
80 understood technology for antibiotic and ARGs mitigation, particularly with respect to its long-
81 term performance and risk profile under ultra-high antibiotic loading (>10 mg/L).
82 Within the MBGS system, antibiotics are removed through bioadsorption, bioaccumulation, and
83 biodegradation [22]. For microalgae, the removal of antibiotics is predominantly mediated by
84 bioadsorption, a process that relies on their negatively charged cell walls to rapidly sequester
85 antibiotics via electrostatic interactions and other physicochemical processes [23]. Bacteria
86 achieve biodegradation by secreting extracellular and intracellular enzymes to decompose
87 antibiotic molecules, ultimately mineralizing them into simple molecules or harmless end
88 products such as CO₂ and H₂O [24]. Moreover, due to the barrier of horizontal gene transfer
89 between eukaryotes and prokaryotes, the spread of ARGs in MBGS system is inhibited [25]. The
90 mechanism, removal efficiency, and ARGs control of antibiotic in MBGS system are governed by
91 the complex interplay of multiple factors, notably microalgal species, antibiotic type and
92 concentration (stress level), system operational mode, and the initial inoculation strategy [26-
93 28]. Furthermore, the influence of the initial strategy and operational mode on the antibiotic
94 pollutant removal dynamics and long-term stability of MBGS treating ultra-high-concentration
95 antibiotic wastewater remains inadequately explored.

96 Operation mode can significantly influent bioremediation performance through varying
97 hydraulic conditions and microbial dynamics. Although widely studied, the batch-mode MBGS
98 suffers from limited scalability [14, 26, 29]. In contrast, the continuous system and semi-
99 continuous presents a more feasible alternative for scale-up, but its efficiency can fluctuate
100 under variable operational conditions [30]. For example, MBGS in a semi-continuous system
101 achieved up to 97.4% SDZ removal (with an initial concentration of 2 mg/L) under stable
102 operation conditions [6]. The semi-continuous operational mode reconciles the need for
103 scalability with system robustness by implementing feast-famine cycles. This strategy not only
104 fortifies the internal microbial consortium but also leads to more stable removal efficiency and
105 superior control over the proliferation of ARGs [31]. The initial operational strategy in the
106 construction of semi-continuous MBGS system enhances the pollutant treatment capacity and
107 stress resistance by shaping the initial microbial community structure [32]. However, current
108 studies primarily focus on comparing the antibiotic treatment efficiency of different systems

(MBGS, BGS, AS) under semi-continuous operation modes, with antibiotic concentrations mostly below 10 mg/L [18-20]. The optimal operational strategies for semi-continuous MBGS systems treating antibiotic wastewater treatment (with concentrations above 10 mg/L) and the underlying microbial succession remain largely unexplored.

In this study, the optimal initial operational strategy of a semi-continuous MBGS system targeting long-term (146 days) treatment of ultra-high-concentration SDZ wastewater has been explored. *Chlorella vulgaris* and AS were utilized to construct the MBGS system. Two strategies of pre-acclimatization (exposure to 5 mg/L SDZ during the initial stage) and pre-accumulation (SDZ-free during the initial stage) were compared towards enhanced SDZ removal. In the second stage, under long-term semi-continuous treatment, the SDZ concentration was incrementally increased from 10 mg/L to 50 mg/L. The growth, biochemical feedback, wastewater treatment, SDZ degradation and pathways, microbial community composition, metabolism and alterations in ARGs of the MBGS system were investigated. Our study aims to optimize the semi-continuous MBGS for treating high-concentration antibiotic wastewater and to reveal the underlying microbial mechanisms, particularly microbial succession, metabolic changes, and ARGs control in response to gradually increasing SDZ concentrations. This study demonstrates the feasibility of using MBGS in semi-continuous mode to treat strengthened antibiotic wastewater. It provides the foundation for scaling up the MBGS systems and facilitating its practical application. This research delivers both a practical operational framework for applying semi-continuous MBGS to ultra-high-concentration antibiotic wastewater and a revelation of the key microbial mechanisms involved, thereby paving the way for its informed scaling and sustainable application.

131

132 **2 Materials and Methods**

133 **2.1 Experimental materials**

134 *Chlorella* sp. (No. FACHB-31), obtained from the Freshwater Algae Culture Collection (Institute
135 of Hydrobiology, Chinese Academy of Sciences), was cultivated in sterilized BG-11 medium
136 following conditions adapted from the previous study [33]. The aerobic activated sludge (AS),
137 which was used for the construction of microalgae-bacteria symbiosis sludge (MBGS)
138 consortium, was taken from the sewage treatment plant of Beijing Urban Drainage Group Co.,

139 Ltd. The mixed liquid suspended solids (MLSS) concentration and sludge settling velocity (SV_{30})
140 for the AS were 7280 mg/L and 88%. Prior to the experiment, the AS has been domesticated
141 and centrifugated, and washed with sterilized distilled water. The composition of the
142 wastewater and the trace element solution was followed by He et al. [34].

143

144 **2.2 Experimental design**

145 This study used two identical photo-reactors as experimental devices with an effective volume
146 of 5 L. Based on our preliminary study, the inoculation ratio of microalgae to AS was set at 1:1
147 (wt/wt) [35]. The initial total biomass in MBGS system was maintained at around 0.6 g/L. The
148 experiment was conducted at $25 \pm 2^\circ\text{C}$ and a light intensity with LED of 4500 lx (light:dark = 14
149 h:10 h) with stirring at 100 rpm. Air was supplied continuously during the culture period.
150 During the experiment, pH was adjusted daily to maintain a range between 7.5-8.0. Initially, one
151 reactor with the pre-acclimatization strategy was supplemented with 5 mg/L SDZ (named T_L ,
152 experimental group), while the other reactor with the pre-accumulation strategy was kept SDZ-
153 free (named T_U , control group). The experiment lasted 146 days and was divided into three
154 stages:

155 The static stage (0-8 d): Maintain continuous aeration (1 L/min). The T_U reactor was operated
156 with SDZ-free wastewater, while the T_L reactor with wastewater containing 5 mg/L SDZ. The
157 granulation stage (9-50 d): The hydraulic retention time (HRT) was 10 d with one entire cycle
158 mentioned below every 2 d. The cycle included the static inflow with the volume exchange ratio
159 of 20% (1 L), aeration, sedimentation, and drainage, and the duration of each operation stage
160 was 5 min, 47 h, 40 min, and 15 min, respectively. This stage operated stably for 42 d. Among
161 the two reactors, the influent to T_U contained no SDZ, while that to T_L contained SDZ of 5 mg/L.
162 The SDZ gradient increase stage (50-146 d): The operation in this stage was the same as that in
163 the granulation stage. The concentration of SDZ in the influent to each reactor was increased
164 stepwise through three stages: moderate concentration domestication at 10 mg/L from days
165 50-66, high concentration domestication at 30 mg/L from days 66-82, and super-high
166 concentration domestication at 50 mg/L from days 82-146.

167 During the operation, 50 mL of samples were taken every 2 d to detect the total biomass,
168 microalgae biomass, pigment concentrations, and regular pollutants (SCOD, $\text{NH}_4^+\text{-N}$, TN, and

TP). Measure changes in the particle size, morphology, extracellular polymeric substances (EPS), superoxidase dismutase (SOD) activity, malondialdehyde (MDA) concentration, and SDZ concentration at the beginning, middle, and end of each stage. Microbial metagenomic sequencing was performed on samples taken on day 50, 66, 82, and 146.

2.3 Analytical methods

2.3.1 Biomass and pigment quantification

Pigment composition in microalgae, including chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids, was quantified by extracting with methanol extraction (90% v/v) and subsequently analyzed using UV-Vis spectrophotometry following the method described by the previous study [33]. Biomass quantification was performed with the dry weight method following the protocol established by Nguyen et al. [9]. Total biomass in the MBGS system was comprised of the microalgae biomass and the AS biomass. The microalgae biomass was determined via Chla concentration, with the quantitative relationship established through calibration curve fitting Eq. (1):

$$C_M = (33.2 \times C_{chla} - 23.1)/1000 \quad (R^2 = 0.9994) \quad (1)$$

Where C_M was the microalgae biomass (g/L), and C_{chla} was the concentration of Chla (mg/L).

2.3.2 Micromorphology of the MBGS consortium

Particle size distribution analysis of the MBGS consortium was conducted using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments Ltd., Worcestershire, U.K.). Microstructural characterization was performed via scanning electron microscopy (SEM) (Hitachi SU3500, Japan). Sample preparation protocols followed standardized methods described in Aditya et al. [36].

2.3.3 Extracellular polymeric substance (EPS) analysis

The EPS was extracted via the thermal extraction method. Quantitative analysis of predominant EPS constituents, proteins (PN) and polysaccharides (PS), was conducted by the rapid Lowry method and the phenol-sulfuric method, respectively [29]. Organic matters of EPS were characterized using the fluorescence excitation emission matrix (EEM) spectroscopy (AquaLog,

199 Horiba Scientific, Japan). Following established protocols of Chen et al. [37], spectral acquisition
200 parameters were configured and five characteristic regions were partitioned. Three key
201 fluorescence indices, including fluorescence index (FluI), humification index (HIX), and
202 biological index (BIX), were determined following the analytical methodology established by
203 Rodríguez-Vidal et al. [38].

204

205 **2.3.4 Dynamics of superoxide dismutase activity and malondialdehyde concentration**

206 The levels of superoxide dismutase (SOD) and malondialdehyde (MDA) serve as indicators of
207 the degree of cellular damage caused by reactive oxygen species (ROS). SOD and MDA were
208 measured using commercially assay kits (SOD Assay kit A001 and MDA Assay kit A003)
209 purchased from Nanjing Jiancheng Bioengineering Institute (China). All experimental
210 procedures were performed in strict accordance with the manufacturer's instructions.

211

212 **2.3.5 Pollutant removal and SDZ degradation pathways analysis**

213 The concentrations of TN, NH₄⁺-N, TP, and SCOD were determined according to standard
214 methods [39]. The quantification of SDZ and its biodegradation byproducts was quantified
215 using the ultrahigh-performance liquid chromatography - tandem mass spectrometry (UPLC-
216 MS/MS). The concentration analysis was performed on a Waters ACQUITY UPLC I-Class AB
217 UPLC and SCIEX QTRAP 5500 triple quadrupole tandem with linear ion trap MS. Specific
218 analytical parameters are detailed in the previous study [33]. The removal efficiency was
219 calculated using the following Eq. (2):

$$220 \quad \eta = (C_0 - C_t) / C_0 \times 100\% \quad (2)$$

221 Where η was the removal efficiency (%) of pollutants, C_0 and C_t were the concentration of
222 substrate at time 0 d and t d (mg/L).

223 Structural characterization of SDZ transformation products was conducted according to Hu et
224 al. [40]. Mass-to-charge ratios (m/z) were referenced against published results to identify the
225 intermediates [2, 5, 41], and structures were drawn using ChemDraw software (v20.0). The
226 specific detection parameters and sample processing methods have been stated in our previous
227 study [42]. The Ecological Structure Activity Relationships (ECOSAR) software was utilized to
228 assess both chronic and potential risks effects of SDZ biodegradation intermediates on

229 ecological systems.

230

231 **2.3.6 DNA extraction and metagenomic sequencing**

232 Representative composite samples were prepared at the end of each stage (50, 66, 82, and 146
233 days) for both reactors (T_U and T_L). For each reactor, three aliquots of algal and sludge
234 suspension were combined and homogenized. The resulting eight samples were immediately
235 frozen at -80°C . DNA extraction was then performed using the DNA E.Z.N.A.® Soil DNA Kit
236 (Omega Bio tek, USA). The DNA concentration and purity were detected, and the integrity was
237 tested. Fragmentation of the DNA was performed for construction of a paired-end (PE) library,
238 and primers were used for amplification. Metagenomic analysis was performed using an
239 Illumina Novaseq 2000 (Illumina Inc., San Diego, CA, USA) and Novaseq kits according to
240 specific protocols. Quality control was done by splicing, assembly, amino acid sequence
241 translation, non-redundant gene set construction, and abundance calculation of the obtained
242 sequences according to established methods [43]. The raw sequence data were available in
243 NCBI with the accession number of PRJNA1394923. Downstream analyses were performed via
244 the Majorbio Cloud Platform (Shanghai, China). Taxonomic annotation was achieved by aligning
245 representative non-redundant sequences against the NR database using DIAMOND (v0.8.35)
246 with an e-value $\leq 1e^{-5}$. Antibiotic resistance genes (ARGs) were identified through comparison
247 with the Antibiotic Resistance Genes Database (ARDB) using non-redundant gene set amino
248 acid sequences (identity $> 70\%$, e-value $\leq 1e^{-5}$) as per Guo et al. [7].

249

250 **2.4 Data analysis**

251 Alpha diversity indices (Chao1, Shannon, and Shannoneven) were calculated using Mothur
252 v1.30.1. R software with the vegan package was used to perform the principal co-ordinates
253 analysis (PCoA, v3.3.1) and the redundancy analysis (RDA, v2.4.3). The blast2go was used to
254 perform the GO annotation of the gene sets. The Kyoto Encyclopedia of Genes and Genomes
255 (KEGG) database was employed to annotate functional genes. R software (v3.3.1) with
256 ReporterScore package was used to perform the KEGG functional enrichment analysis, and an
257 absolute reporter score value more than 1.65 was defined as significant. Using Diamond
258 software, via BLASTP (v2.2.31+), the non-redundant gene set sequences were aligned with the

cytochromes P450 superfamily database. The heatmap package (v1.0.8) 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, corrplot and ggplot2 packages were employed for constructing a correlated graph. The $|r| \geq 0.5$ was considered to be a significant correlation. Python (v2.7.0) was used to calculate the extent of microbial contribution to ARGs. 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.

267

268 **3 Results and Discussion**

269 **3.1 Formation of the microalgal-bacterial granular sludge (MBGS) consortium**

The major goal of static and granulation stages was to form and develop the microalgal-bacterial consortia in the MBGS system. During these two stages, MBGS biomass gradually accumulated to about 1.24 g/L (Fig. 1a). Interestingly, the pre-acclimatization didn't demonstrate superior antibiotic adaptability compared to the pre-accumulation strategy under the stepwise increase in antibiotic load, resulting in significantly lower biomass accumulation in T_L compared to T_U (SDZ gradient increase stage). A substantial inhibition of biomass growth was observed in both systems when the SDZ concentration was further increased to 50 mg/L. T_U was initially cultivated without SDZ, generating a lower proportion of microalgae, but a higher bacteria diversity. This more diverse microbial community structure harbored a broader spectrum of metabolic activities, enabling a faster and more effective adaptation to the steep increase in SDZ concentration, which ultimately resulted in greater biomass accumulation [44, 45]. At the end of this stage, the total biomass in T_U and T_L gradually stabilized at 2.68 g/L and 2.55 g/L, respectively, indicating the adaption to the high concentration of SDZ of both reactors.

The total pigment concentration of T_U remained consistently at high levels (up to 29.46 mg/L) across all stages and was generally higher than T_L , suggesting that the MBGS system in T_U exhibited greater tolerance to SDZ-induced stress compared to T_L (Fig. 1b). Chlorophylls (Chla and Chlb) are the primary constituents of microalgae pigments, essential for the conversion of light energy [46]. With the sudden increase of SDZ concentration to 30 mg/L, the pigment concentration in T_L decreased to a minimum of 11.50 mg/L. This could be caused by the SDZ

289 toxicity, which consequently reduced the production of photosynthetic pigments [47]. Notably,
290 the carotenoid concentrations in T_U and T_L increase up to 5.2 and 2.3 times, respectively, with
291 the elevation of SDZ concentration in the influent. Carotenoid function as antioxidants by
292 scavenging reactive oxygen species, thereby protecting the photosynthetic apparatus from
293 oxidative damage [5]. The results indicated that both T_U and T_L activated cellular defense
294 mechanisms under elevated antibiotic stress, as evidenced by increased carotenoid levels.
295 By day 146, both T_U and T_L developed distinct MBGS consortia compared to day 0 (Fig. 1c), with
296 microalgae encapsulating the activated sludge, consistent with previous studies [27]. After
297 static and granulation stages, the particle size distribution became uniform, indicating
298 successful consortium formation. In SDZ gradient increase stage, T_U maintained relatively stable
299 particle sizes (200-350 μ m) despite increasing SDZ concentrations, suggesting greater
300 structural stability. In contrast, T_L showed a decrease in particle size to about 240 μ m as SDZ
301 concentration increased, but by day 146, larger granules (about 500 μ m) formed, exceeding
302 those in T_U. This difference may be related to changes in extracellular polymeric substances
303 (EPS) production in T_L (section 3.2). EPS plays a key role in MBGS formation, including
304 microbial adhesion and aggregation, three-dimensional network construction, and structural
305 stability, which were verified can influence the granule size by many studies [14, 48, 49].

306

307 **3.2 Extracellular organic matter dynamics and oxidative stress response of MBGS**

308 EPS are macromolecular substances secreted outside the cells by microorganisms, serving
309 critical functions in protection, regulation, structure stabilization, and nutrient acquisition [29].
310 Overall, the EPS concentration of T_L reached up to 33.33% higher than that of T_U (Fig. 2a, b). In
311 SDZ gradient increase stage, the rising SDZ concentrations led to a significant increase in EPS
312 excretion in the pre-acclimatization treatment (T_L), peaking at 60.1 mg/L on day 132, while EPS
313 levels in T_U remained relatively stable. The initial SDZ stress during pre-acclimatization likely
314 triggered higher EPS secretion by microalgae and bacteria, forming protective barriers that
315 adsorb or chelate SDZ and reduce its toxicity [46]. EPS primarily consists of proteins (PN) and
316 polysaccharides (PS), and the PN/PS value reflects the MBGS response to external stresses [3].
317 Higher PN/PS ratios indicate enhanced stress resistance and pollutant adsorption, while lower
318 ratios suggest improved structural integrity and nutrient retention [49]. At 10 mg/L SDZ, both

T_U and T_L showed their lowest PN/PS ratios (0.71 and 1.50, respectively) among all treatments, indicating effective cellular adaptation and a state of nutrient accumulation. As SDZ concentrations increased to 50 mg/L, PN/PS ratios rose to the maximum of 5.18 (T_U) and 5.19 (T_L), suggesting that the microbial consortia were subjected to significant antibiotic stress. Interestingly, further increase of SDZ concentration to 50 mg/L resulted in a declined PN/PS ratios, implying that MBGS adapted structurally to sustained antibiotic stress. The higher EPS concentration and lower PN/PS ratios in T_L may also explain its larger granule size (Fig. 1c). Fluorescence excitation emission matrix (EEM) analysis could reveal the dynamics of fluorescent components, such as cellular aromatic proteins, humus substances and soluble microbial metabolites. Fluorescence index, including the fluorescence index (FluI), humification index (HIX), and biological index (BIX) are used to assess the origin of dissolved organic matter (DOM), decomposition degree, and microbial metabolic activity, respectively [11]. As shown in Fig. 2b, the fluorescence intensity in the soluble microbial by-product region (Region IV) steadily increased in both T_U and T_L during static and granulation stages, indicating active biomass growth and granulation. At day 50, the BIX values of 1.12 (T_U) and 1.07 (T_L) further confirmed strong microbial metabolism (Table S1). In SDZ gradient increase stage, fluorescence intensity in T_U continued to rise with increasing SDZ concentration, peaking on day 82. In contrast, T_L showed an initial decline (day 66) before recovering to a lower peak by the end of 30 mg/L SDZ phase. Both reactors exhibited notable fluorescence peaks in Regions I, II, and IV, corresponding to tyrosine-like proteins, tryptophan-like proteins, and microbial by-products. A FluI value of about 1.9 suggested that DOM primarily originated from microbial activity [50]. During the static and granulation stages, reactors exhibited high BIX values (≥ 0.8) and consistently low HIX values (≤ 0.3), indicating abundant microbial metabolites and limited humification. These results suggest that both T_U and T_L phases actively adapted to SDZ-induced stress. As SDZ concentration increased to 50 mg/L, the fluorescence intensity, BIX, and FluI values declined, while HIX rose, suggesting reduced microbial activity and greater humification, reflecting system aging under severe SDZ stress. The higher BIX value in T_U might further imply that the initial undisturbed system has greater resilience than T_L. Superoxide dismutase (SOD) and malondialdehyde (MDA) concentration together reflects the cellular oxidative stress status. The SOD activity reflects the cell's capacity to eliminate reactive

oxygen species (ROS), while MDA concentration reflects the degree of ROS-induced cellular oxidative damage. Throughout the experiment, T_L exhibited higher SOD activity and MDA levels than T_U , which reflected a more pronounced oxidative stress response (Fig. 2d, e). During static and granulation stages, SOD activity increased in both reactors. However, T_U maintained stable MDA levels, while T_L reached significantly higher MDA (1.31 nmol/mg on day 50, $p < 0.01$), likely due to SDZ-induced stress during MBGS formation. As SDZ concentration rose to 50 mg/L, SOD activity in T_U and T_L peaked (72–77 U/mg_{prot}), and MDA levels increased (to 1.75–1.80 nmol/mg). Eventually, under ultra-high SDZ stress, SOD activity declined, potentially inactivated by excessive free radicals, while MDA remained stable, indicating that antioxidant defenses were overwhelmed and lipid damage had already occurred. Overall, while T_U initially limited oxidative damage, both consortia ultimately faced oxidative imbalance under sustained SDZ pressure.

3.3 Pollutants removal and degradation pathways

MBGS can simultaneously mitigate pollutants and upcycle nutrients, supporting its own growth. Table 1 shows the dynamics and removal efficiencies of TN, NH_4^+-N , TP, and SCOD in T_U and T_L . As the primary TN component, NH_4^+-N is assimilated by microalgae and serves as initial N source for bacterial nitrification [8]. During static and granulation stages, NH_4^+-N removal efficiency progressively improved in both reactors, surpassing 97% with no significant difference ($p > 0.05$). In SDZ gradient increase stage, the rapid SDZ increase initially reduced NH_4^+-N removal, potentially disrupting bacterial denitrification and photosynthesis [47], as microorganisms shifted nutrients to pigment production (Fig. 1b), EPS secretion (Fig. 2a), and ROS defense (Fig. 2d). Once MBGS was adapted to the SDZ stress, NH_4^+-N removal recovered. Under 30 mg/L of SDZ, T_L showed higher removal efficiency ($p < 0.05$), possibly due to the development of resistant bacteria, e.g., Actinomycetota (Fig. 4b). At 50 mg/L, T_U outperformed T_L ($p < 0.001$), which could be potentially attributed to the higher microbial metabolic activity (Table S1); but both stabilized above 95%. TP removal efficiency increased and stabilized at 95% through static and granulation stages, showing resilience of the microbial community to moderate concentrations of SDZ. However, a significant drop followed by a recovery of the removal efficiency (85%) was observed at high SDZ level (50 mg/L). The SCOD removal

379 efficiency ranged between 83% and 89% during the test, with SDZ having little effect.
 380 Fig. 3a shows the concentration and removal efficiency of SDZ. The pre-acclimatization in T_L
 381 achieved 99.7% SDZ removal in the first two stages. The pre-acclimatization further
 382 demonstrated higher SDZ removal efficiency (up to 99%) compared to the pre-accumulation
 383 treatment (T_U, up to 96.8%) under 30 mg/L SDZ. Nevertheless, this trend reversed under higher
 384 SDZ concentration, which at 50 mg/L, T_U achieved higher SDZ removal efficiency (up to 99.9%)
 385 compared to T_L (95.6%). During the first two stages, the absence of SDZ allowed T_U to establish
 386 a more stable and diverse microbial community, leading to greater biomass and pigment
 387 accumulation, as well as lower MDA concentration, which contributed to the antibiotic removal
 388 facing high SDZ levels [3].
 389 So far, most investigations on microalgae-based SDZ removal have employed in the batch mode
 390 and are dominated by microalgae alone [33, 48] (Table 2). Compared with the batch culture, the
 391 (semi-)continuous culture ensures stronger activity and higher adaptability of MBGS [31]. This
 392 study confirmed that the semi-continuous operation of MBGS is an efficient strategy to remove
 393 strengthened SDZ (up to 99.9% removal of 50 mg/L). Bacteria are considered the primary
 394 microorganisms for antibiotic removal, while microalgae mainly serve a protective function in
 395 MBGS [6, 12, 13]. Thus, the higher SDZ removal was mainly attributed to the symbiotic
 396 relationship between microalgae and bacteria in MBGS system.
 397 SDZ typically exists as a zwitterion, and its strong hydrophilicity, as indicated by the octanol-
 398 water partition coefficient, is reflected in its poor bioaccumulation and bioadsorption [51]. The
 399 negligible adsorption of SDZ onto granular sludge can be attributed to repulsive interactions
 400 with EPS functional groups, including carboxyl and hydroxyl groups [52]. Moreover, its high
 401 chemical bond potential energy renders photolysis negligible [3, 6]. Thus, biodegradation is the
 402 major removal mechanism of sulfonamide antibiotics [33, 48]. As shown in Table 3, eighteen
 403 degradation by-products (BP1-BP18) of SDZ were identified [2, 41, 53]. Results indicated that
 404 potential bond-breaking sites for SDZ were located at α , β , γ , and δ [40]. Consequently, four
 405 pathways for SDZ degradation were proposed in this study (Fig. 3b):
 406 Pathway I was identified as the primary degradation route. Due to the presence of amide bond
 407 (part α), SDZ is known to be easily hydrolyzed [54]. The initial hydrolysis product, 2-
 408 aminopyrimidine, undergoes hydroxylation and deamination to form BP1 and BP2, a key SDZ

409 degradation pathway reported in recent studies [1, 55, 56]. Through hydrolytic deamination,
410 desulfonation, and subsequent oxidation, the other initial hydrolysis product, sulfanilic acid,
411 was sequentially transformed into BP3-BP8. Pathway II broke the C-N bond (part β), and the
412 pyrimidine ring of SDZ served as a degradation site (γ site) in Pathway III. The N-H bond and
413 pyrimidine ring were hydroxylated in Pathway IV at part δ .

414 The degradation intermediates of SDZ may pose potential risks to the environment, and must
415 be forecasted for ecotoxicity. Fig. 3c illustrates the acute and chronic toxicity associated with
416 SDZ and its degradation intermediates. In terms of acute toxicity, the SDZ EC50 (half effective
417 concentration) for daphnid and green algae were within the “Harmful” level. However, the EC50
418 value of SDZ to daphnid in chronic toxicity belongs to the “Toxicity” level. The intermediates
419 generated during SDZ degradation by MBGS may aggravate the risk. For example, BP6, BP7,
420 BP12, and BP1 had more toxicity (acute or chronic) than SDZ. However, most intermediates
421 exhibited lower toxicity and were classified as the “Not Harmful” level. The result was consistent
422 with recent research indicating that the majority of the intermediates established during SDZ
423 degradation appear to be less hazardous than SDZ itself [1, 33, 40, 42]. It can be concluded that
424 MBGS is a promising and ecologically friendly method for antibiotic removal.

425

426 **3.4 Analyses of microorganism community and metabolism pathway**

427 **3.4.1 Microbial community composition**

428 As shown in Table 4, the Chao1 and Shannon indices, representing microbial richness and
429 diversity, were consistently higher in T_U , suggesting that early SDZ stress suppressed
430 community diversity in T_L . With rising SDZ levels, diversity declined in both reactors, while
431 richness initially increased before decreasing.

432 Chlorophyta, Rotifera, and Bacillariophyta were the main fungal phyla (Fig. 4a). In T_U , the
433 predominant fungal genera were *Adineta* and *Rotaria* in the Rotifera phylum, and *Nitzschia* in
434 the Bacillariophyta phylum on day 50. During the 10 mg/L SDZ phase in SDZ gradient increase
435 stage, the relative abundance of *Nitzschia* reached the peak of 0.24. As the SDZ concentration
436 continued to increase to 30 mg/L and 50 mg/L, the highest proportion of Chlorophyta was
437 discovered in T_U . In T_L , Chlorophyta had the most significant advantage throughout periods, and
438 showed an upward trend over time, with the relative abundance (0.12-0.64) surpassing T_U

(0.06-0.53). *Chlorella*, *Scenedesmus*, and *Tetrademus* were the main genera composed of Chlorophyta in T_U and T_L. Actinomycetota, Pseudomonadota, and Bacteroidota were the major bacterial phyla in reactors (Fig. 4b), which were aligned with many MBGS studies [3, 9, 14]. Actinomycetota can degrade and utilize N, P, and macromolecular organic matters to facilitate N removal and P fixation in wastewater, as well as providing carbon sources for other microbes [57]. *Nakamurella* was the principal genus within the phylum Actinomycetota, exhibiting an initial increase in relative abundance followed by a subsequent drop over time in T_U and T_L. The relative abundance of *Nakamurella* was greater in T_L (0.16-0.31) compared to T_U (0.08-0.17). Pseudomonadota and Bacteroidota were identified as contributors to wastewater nutrient usage and antibiotics breakdown [58]. The *Nitrosomonas* belonging to Pseudomonadota had high relative abundance in 50 d and 146 d of T_U and T_L, while *unclassified_p_Bacteroidota* belonging to Bacteroidota exhibited significant relative abundance in 66 d and 82 d.

452

453 3.4.2 Correlation between microorganisms and pollutant removal efficiency

454 Fig. 4c illustrates the correlation between microorganisms and pollutant removal efficiencies. 455 The Actinomycetota phylum had positive correlations with the elimination of pollutants. In 456 particular, *Nakamurella*, with the largest abundance mentioned above, showed a significant 457 positive correlation with the removal of SDZ, TP and TN ($p < 0.05$). Enriched by initial SDZ stress, 458 *Nakamurella* also associated with NH₄⁺-N (Table 1) and SDZ (Fig. 3a) removal efficiencies of the 459 pre-acclimatization strategy (T_L) greater than the pre-accumulation treatment (T_U). The 460 *Nitrosomonas* belonging to Pseudomonadota showed a statistically significant positive 461 correlation in NH₄⁺-N and SDZ removal ($p < 0.05$). In addition to being a typical ammonia- 462 oxidizing bacterium (AOB), *Nitrosomonas* also possesses the ammonia monooxygenase (AMO), 463 which is reported to facilitate phenyl ring hydroxylation and the cleavage of sulfonamide groups 464 [58, 59]. The pre-accumulation group (T_U) enriched more *Nitrosomonas* in the high 465 concentration SDZ stage, which coincided with higher NH₄⁺-N and SDZ removal efficiencies. 466 *Chlorella* showed a positive correlation with N, P, SCOD, and SDZ removal, and *Scenedesmus* and 467 *Tetrademus* also had a favorable correlation with SDZ removal. Many researches have 468 established the effectiveness of Chlorophyta in wastewater treatment and its significant

469 resistance to antibiotics [5, 6, 33]. Consequently, the removal efficiency of both T_U and T_L can
470 always stay above 95%.

471

472 **3.4.3 Dynamic of cytochromes P450 superfamily**

473 Cytochrome P450 (CYP450), a heme-containing enzyme family, plays a key role in antibiotic
474 biodegradation by catalyzing hydroxylation, desulfonylation, and ring-opening of sulfonamides
475 [33, 56]. Overall, the CYP450 abundance of both T_U and T_L augmented with higher SDZ
476 concentration, enhancing the removal of SDZ by the MBGS consortium (Table 4). CYP125, CYP51,
477 and CYP116 constituted the primary makeup of the CYP450 superfamily (Fig. 4d), and their
478 specific functions have been infrequently examined. *Nakamurella*, *Nitrosomonas*, and
479 *Micropruina* were the predominant bacteria within the top five families of the CYP450
480 superfamily (Fig. 4e). This matched the result that they were positively correlated with SDZ
481 removal (Fig. 4c), and indicated that they might be the primary microorganisms responsible for
482 SDZ biodegradation.

483

484 **3.4.4 GO and KEGG pathway annotations in microorganisms**

485 The annotations of the Gene Ontology (GO) Database and the Kyoto Encyclopedia of Genes and
486 Genomes (KEGG) Database at different levels were displayed in Fig. 5. After exposure to
487 stressors, protective mechanisms in MGBS were activated and cells were undergoing in intense
488 metabolic activity [60]. Furthermore, the SDZ degradation process may substantially stimulate
489 the metabolic pathways and enhance metabolic efficiency [55] Unlike the pre-accumulation
490 group (T_U), the pre-acclimatization strategy (T_L) necessitated the activation of additional genes
491 for antibiotic degradation and growth metabolism in response to initial SDZ stress. This led to
492 an enrichment of more complex metabolic genes and pathways associated with T_L.

493 Glycolysis/Gluconeogenesis is the core metabolic pathway of microorganisms. Path diagram of
494 Glycolysis/Gluconeogenesis and the relative abundance of the corresponding enzymes are
495 shown in Fig. 5d. Most of the routes were detected in the MBGS system, with alcohol
496 dehydrogenase, dihydrolipoyl dehydrogenase, aldehyde dehydrogenase (NAD⁺), and
497 glucokinase being the major enzymes featuring higher abundance (Table S2). The tricarboxylic
498 acid (TCA) cycle exhibited maximum metabolic activity and possessed a significant number of

enzymes. In T_L, enzymes, alcohol dehydrogenase, aldehyde dehydrogenase (NAD⁺) and acetate-CoA ligase (ADP-forming), associated with acetaldehyde, an important metabolite of the TCA cycle, were significantly more abundant than T_U. Long-term SDZ stress will enhance microbial metabolism and resistance by promoting the expression of genes involved in the TCA cycle [61]. For T_U, the related enzymes of the pentose phosphate (PPP) pathway, fructose-bisphosphate aldolase, enolase, and triosephosphate isomerase (TIM), showed greater abundance compared to T_L. It proved that SDZ may also affect the generation of metabolites Glyceraldehyde-3P and Phosphoenolpyruvate, and subsequently impaired the PPP pathway.

3.5 Analyses of antibiotic resistance genes

3.5.1 ARGs composition

Following the sudden exposure of MBGS to 10 mg/L SDZ in SDZ gradient increase stage, ARGs richness in T_U increased significantly by day 66 (Table 4), likely due to the selective pressure triggering antibiotic resistance and enriching antibiotic-resistant bacteria (ARB) carrying ARGs [3]. Overall, T_L contained higher ARGs than T_U but remained steady with increasing SDZ concentration, most likely due to adaptation to SDZ stress at the initial stages. With further increase of the SDZ concentration to 50 mg/L, both ARGs richness and diversity were decreased in T_U and T_L [62]. The ultra-high concentration of antibiotics may cause acute toxicity to bacteria and inhibit the host and transmission path of ARGs. Overall, the protective interactions and rapid antibiotic removal capacity of microalgae makes MBGS a suitable and low-risk technology for wastewater treatment [63].

Principal Coordinate Analysis (PCoA) showed samples of T_U clustered tightly, especially at days 66 and 82, while samples of T_L were more scattered, indicating greater compositional shifts in pre-acclimatization treatment (Fig. 6b). The dominant ARGs were bacitracin (*baca*), sulfonamide (*sul1*, *sul2*), and tetracycline (*tetx*, *tetg*), collectively exceeding 77% abundance [57, 61] (Fig. 6a). Sulfonamide ARGs (*sul1*, *sul2*) were detected in T_U by day 50, reflecting environmental presence [62]. As SDZ concentration increased, *sul* gene abundance in T_U rose from 9.2% (*sul1*) and 8.1% (*sul2*) to 14.3% and 10.9%, respectively, while T_L remained stable (12.1% to 15.6%). These *sul* genes encode modified dihydrofolate synthetase (DHPS) enzymes that reduce SDZ binding, enabling normal folate metabolism under antibiotic pressure [2].

529 Prolonged high SDZ exposure in T_L likely promoted stable enrichment of *sul*-carrying microbes
530 compared to T_U [64].

531

532 **3.5.2 Correlation between ARGs and microorganisms, environmental factors**

533 The correlation between microbes and antibiotic resistance genes (ARGs) are exhibited in Fig.
534 6c. Both Chlorophyta and Bacillariophyta showed strong negative correlation for ARGs with
535 coefficient values less than -0.75. This observation aligns with previous findings indicating that
536 microalgae are generally not the primary targets of antibiotic exposure in aquatic systems [6,
537 60]. As eukaryotes, microalgae possess a cell wall and a nucleus enclosed by a nuclear
538 membrane. Additionally, microalgal secondary metabolites (e.g., terpenes, phenolic acids) can
539 interfere with quorum sensing (QS), which may reduce horizontal gene transfer frequency and
540 thus mitigate the dissemination of ARGs [65]. Bacteria are the primary hosts [14, 57, 62], with
541 Pseudomonadota and Bacteroidota showing a strong positive correlation with ARGs (Fig. 6c).
542 Pseudomonadota, showing a strong correlation with *sul1* (coefficient value = 0.77), was
543 identified as a primary host of sulfonamide ARGs in this system. This finding is consistent with
544 the results of Li et al. [14]. At the genus level, *unclassified_p_Bacteroidota* in Bacteroidota,
545 *Nakamurella* in Actinomycetota and *Nitrosomonas* in Pseudomonadota contributed most to
546 *baca* (Fig. 6d). The bacterial contribution of *sul2* was relatively complex, and *Hyphomicrobium*
547 was the main genus for *sul1*. The diverse bacterial makeup in AS promoted the retention and
548 spread of ARGs. Nonetheless, MBGS protected by microalgae displayed enhanced antibiotic
549 tolerance and reduced environmental danger concerning ARGs [63].

550 The relationship between the main ARGs and the environmental factors was identified by
551 redundancy analysis (RDA). Fig. 6e showed that the SDZ concentration was the most dominant
552 influencing factor, demonstrating a positive correlation with *sul1*, *sul2*, and *ant2ia*. However, the
553 removal of NH₄⁺-N, SCOD, TP, and TN exhibited negative correlations with SDZ concentration
554 and major ARGs. Moreover, SOD activity and MDA concentration displayed a positive correlation
555 with SDZ concentration, which was consistent with the conclusion of Section 3.3.

556 In this study, MBGS system demonstrated excellent performance in the removal of sulfadiazine
557 and the control of ARGs under two distinct initial operational strategies. It should be noted that
558 the experiments were conducted under controlled laboratory-scale conditions and focused on

wastewater containing a single antibiotic. However, actual wastewater discharged into the environment typically constitutes a complex mixture containing multiple antibiotics and other pollutants. Thus, the practical efficacy of MBGS is largely governed by a range of parameters, encompassing wastewater composition, antibiotic types and concentrations, the startup and operational conditions of the algal-bacterial symbiotic system, and the scale of treatment. Future studies should therefore focus on pilot-scale validation using real wastewater under composite pollution scenarios, accompanied by long-term ecological risk monitoring, to comprehensively assess the environmental applicability and feasibility of MBGS system.

4. Conclusion

This study demonstrates that the initial operational strategy (pre-acclimatization and pre-accumulation) critically shapes the long-term resilience and functional trajectory of MBGS system under extreme antibiotic stress. Both MBGS systems maintained effective and stable operation and achieved above 95% SDZ removal. As the SDZ concentration continued to increase, lipid degradation and aging occurred in two systems, and the SOD protective mechanism failed. The pre-acclimatization strategy exhibited higher oxidative stress but demonstrated adaptability to ultra-high SDZ exposure. In contrast, the pre-accumulation strategy featured a compact and stable structure along with an active microbial community, resulting in stronger anti-interference ability. The principal degradation process was amide bond hydrolysis, which produced fewer hazardous metabolites. The relative abundance of target sulfonamide ARGs (*sul1*, *sul2*) rose with the SDZ concentration. Microalgae (*Chlorella*) consistently defend and prevent ARGs. *Nakamurella* and *Nitrosomonas* were prominent CYP450 superfamily contributors and SDZ degradation microorganisms, although they also had risks carrying ARGs. Nevertheless, the richness and variety of ARGs in two strategies initially increased and subsequently decreased. This study establishes the MBGS system as an efficient, novel biochemical strategy for addressing antibiotic pollution and associated ARGs challenges, offering a low-carbon, sustainable alternative for antibiotic wastewater treatment.

Data availability

Data will be made available on request.

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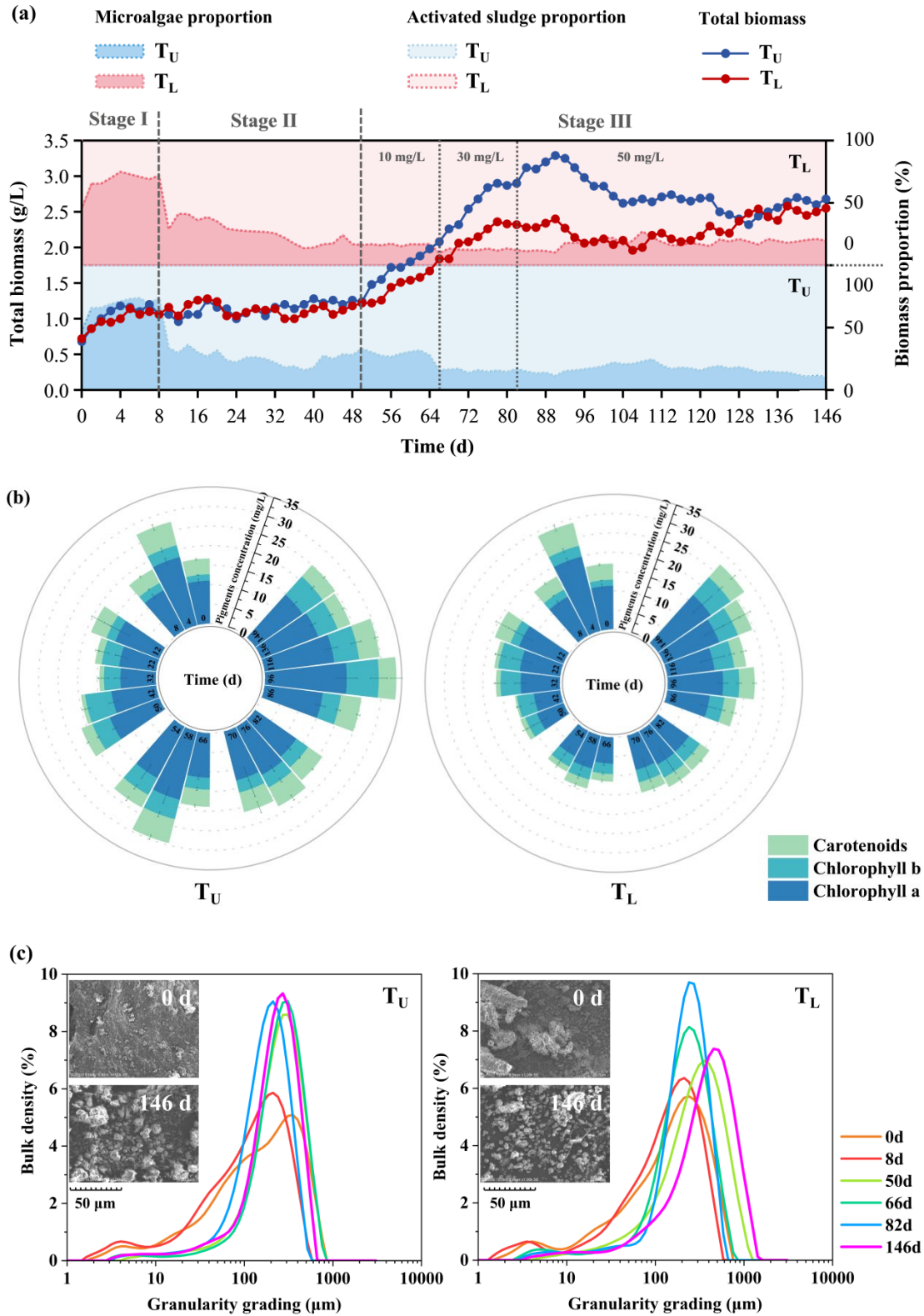


Fig. 1. Dynamics of microalgal-bacterial granular sludge concentration and morphology (a) dynamics of total biomass (left y-axis) and microbial compositions (in percentage, right y-axis), (b) changes in pigments concentrations, and (c) granule morphology and size distribution observed by scanning electron microscopy (SEM, 1000x magnification).

Note:

* Stage I, the granulation stage; Stage II, the granulation stage; Stage III, the SDZ gradient increase stage.

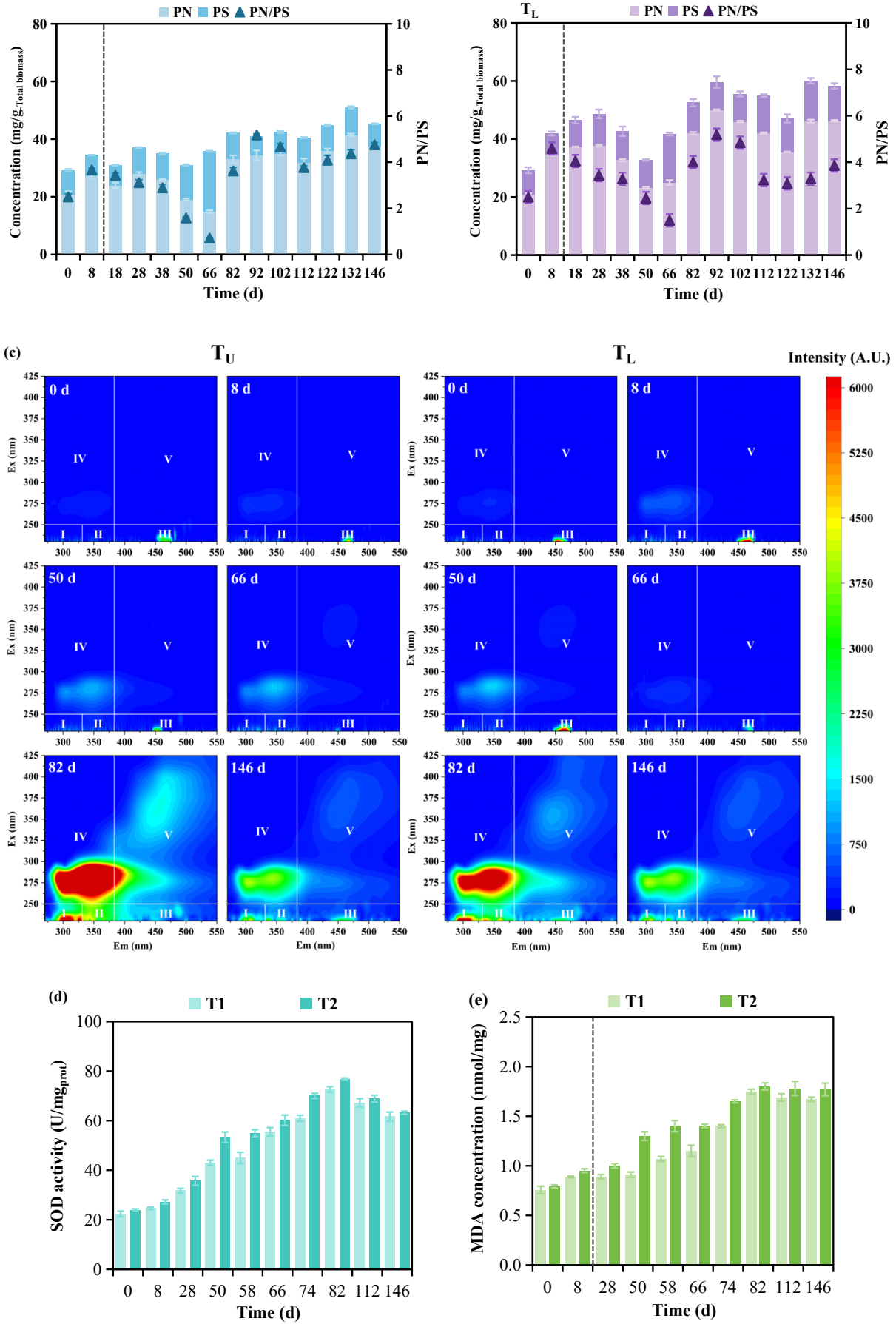


Fig. 2. Concentration and PN/PS values of EPS (a,b), EEM fluorescence spectra images (c), SOD activity (d)

and MDA concentration (e) in T_U and T_L with time

Note:

* Stage I, the granulation stage; Stage II, the granulation stage; Stage III, the SDZ gradient increase stage.

* PN, protein; PS, polysaccharide; EPS, extracellular polymeric substances; LB-EPS, loosely bound-EPS; TB-EPS, tightly bound-EPS; EEM, excitation emission matrix; EX, excitation; EM, emission; SOD, superoxide dismutase; MDA, malondialdehyde.

* Region I (tyrosine-like protein, Ex/Em=200-250/200-330 nm), Region II (tryptophan-like protein, Ex/Em=200-250/330-380 nm), Region III (fulvic acid-like organics, Ex/Em=200-250/380-500 nm), Region IV (soluble microbial by-products, Ex/Em=250-280/200-380 nm), and Region V (humic acid-like organics, Ex/Em=250-400/380-500 nm).

* The difference was assessed by performing a one-way ANOVA followed by Waller-Duncan's multiple range test. Consider $0.001 < p < 0.05$ as a significant difference, and marked as “*”, and consider $p < 0.001$ as a highly significant difference, and marked as “***”.

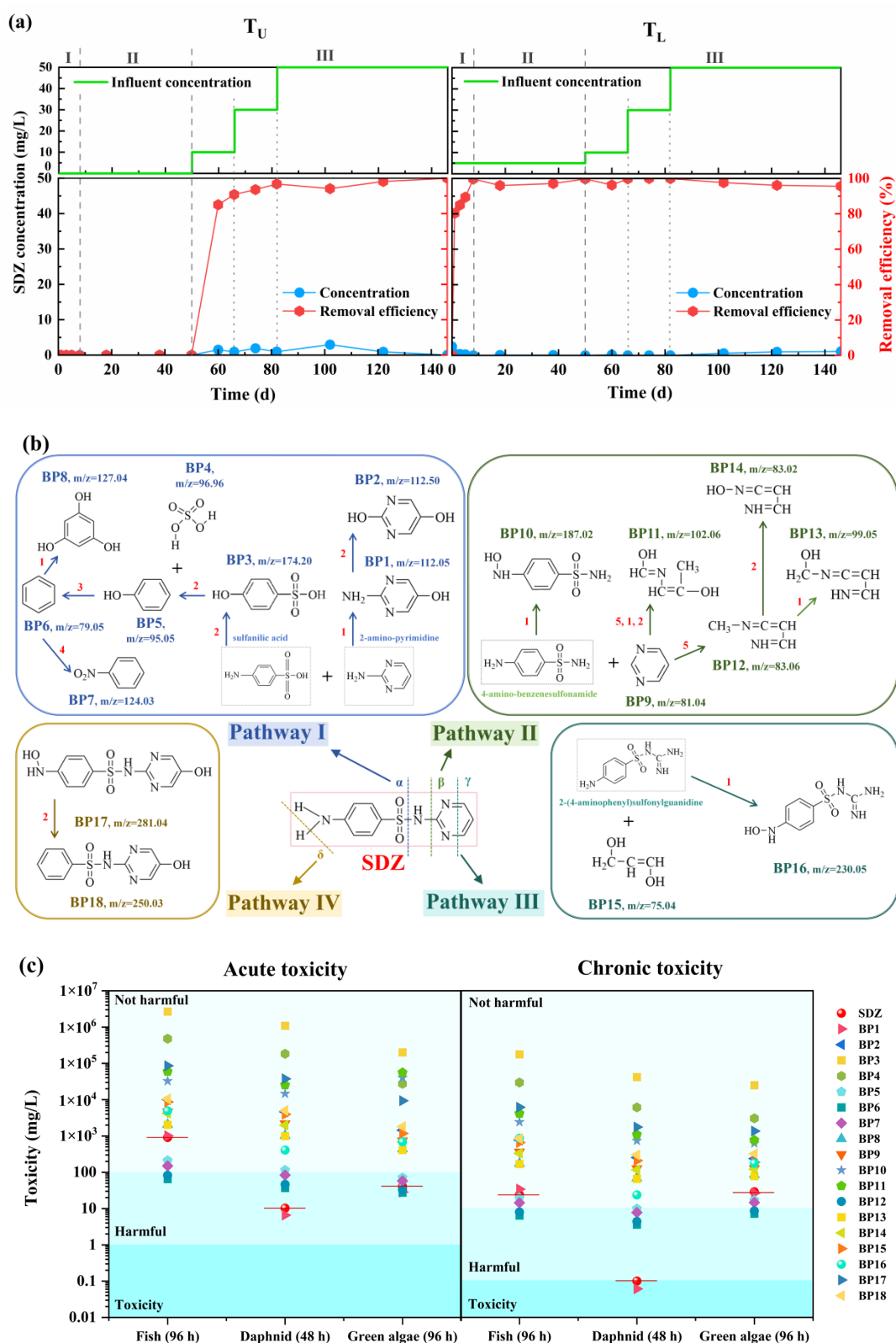


Fig. 3. Concentration and removal efficiency of SDZ with time in T_U and T_L reactors (a), degradation pathways of SDZ (b), and toxicity analysis of the biodegradation intermediates of SDZ on fish, daphnid, and green algae by ECOSAR program (c)

Note:

* SDZ, sulfadiazine.

* Stage I, the granulation stage; Stage II, the granulation stage; Stage III, the SDZ gradient increase stage.

* Names of degradation intermediates were shown in Table 3.

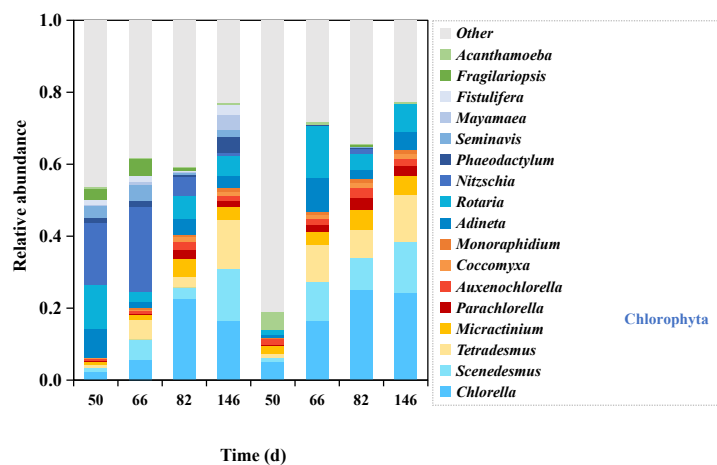
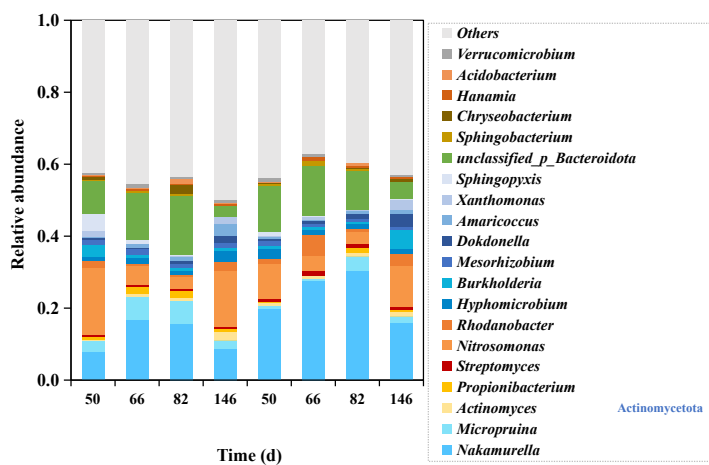
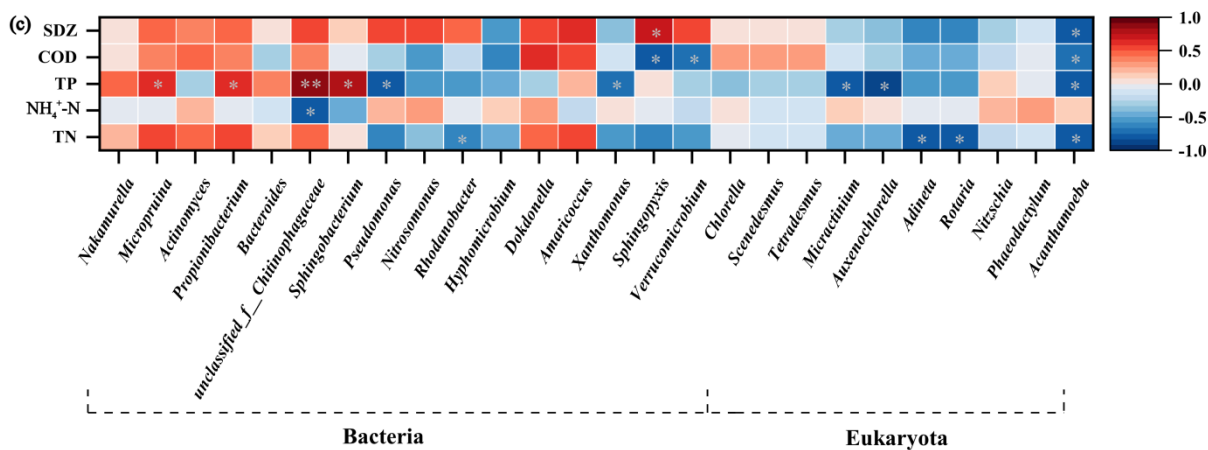
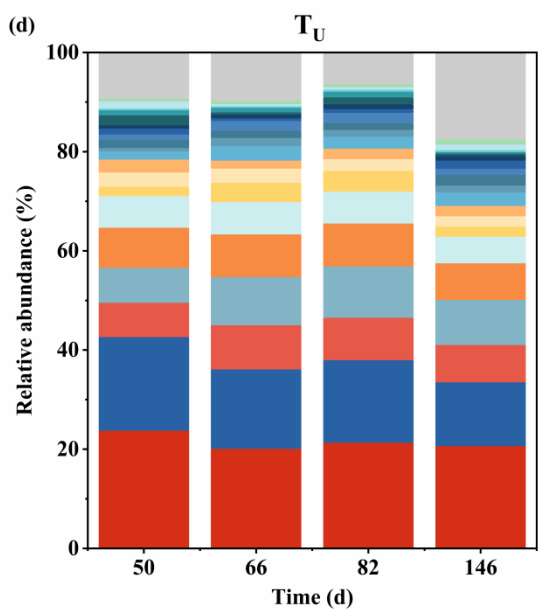
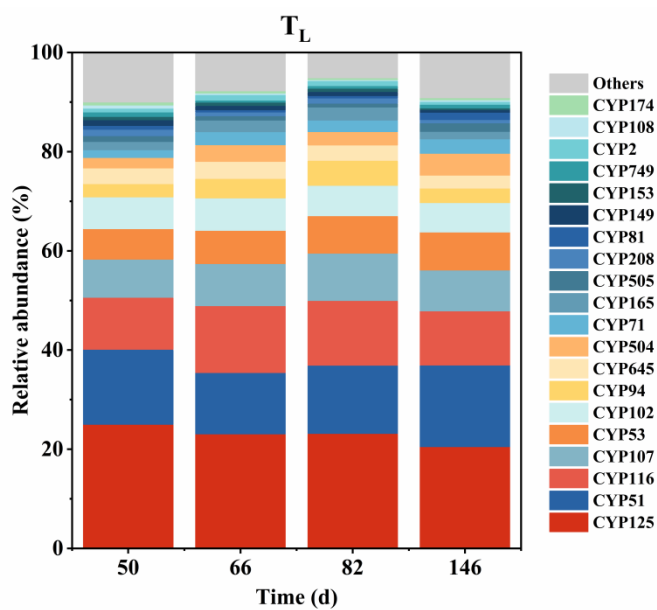
* 1, hydroxylation; 2, hydrolysis; 3, dehydroxylation; 4, nitration; 5, pyrimidine cleavage.

* The provided intermediates were able to find in the database of ECOSAR:

Toxicity: lowest estimated acute toxicity value is < 1.00 mg/L (chronic value < 0.10 mg/L).

Harmful: lowest estimated acute value is between 1.00 mg/L and 100.00 mg/L (chronic value between 0.10 mg/L and 10.00 mg/L).

Not harmful: lowest estimated acute value is > 100.00 mg/L (chronic value > 10.00 mg/L).



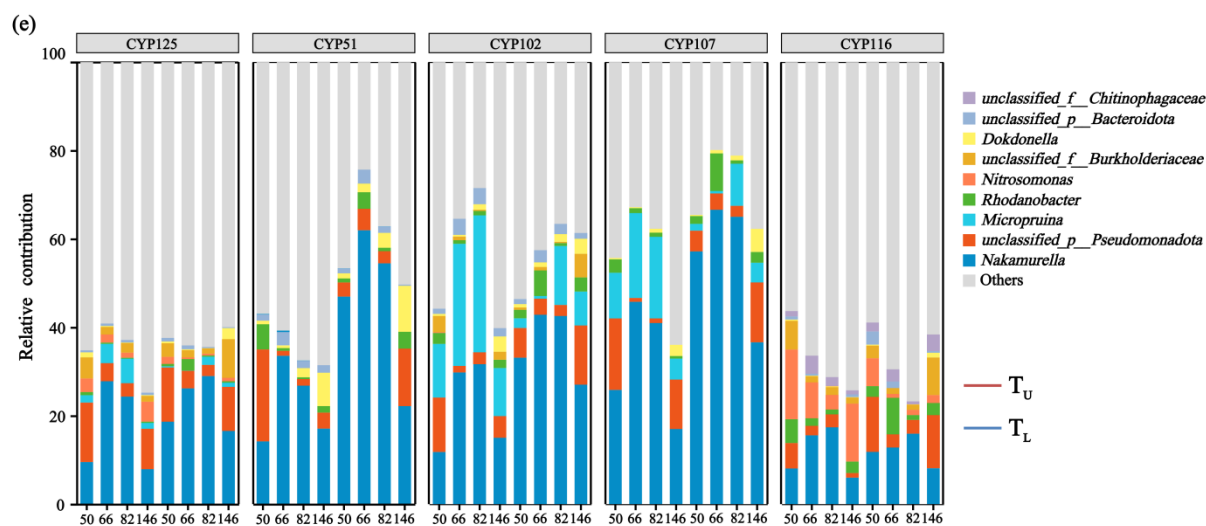


Fig. 4. The relative abundance of microbial composition of bacteria (a) and eukaryota (b) at the phylum and genus levels; Heat map of correlation between microorganism at genus level and pollutants removal efficiency (c); The relative abundance of proteins at the family level in cytochromes P450 superfamily (d); The relative contribution of bacteria at the genus level to cytochromes P450 superfamily in top 5 (e)

Note:

* SDZ, sulfadiazine; COD, chemical oxygen demand; TP, total phosphorus; $\text{NH}_4^+\text{-N}$, ammonia nitrogen; TN, total nitrogen.

* The difference was assessed by performing a one-way ANOVA followed by Waller-Duncan's multiple range test. Consider $0.001 < p < 0.05$ as a significant difference, and marked as "**", and consider $p < 0.001$ as a highly significant difference, and marked as "***".

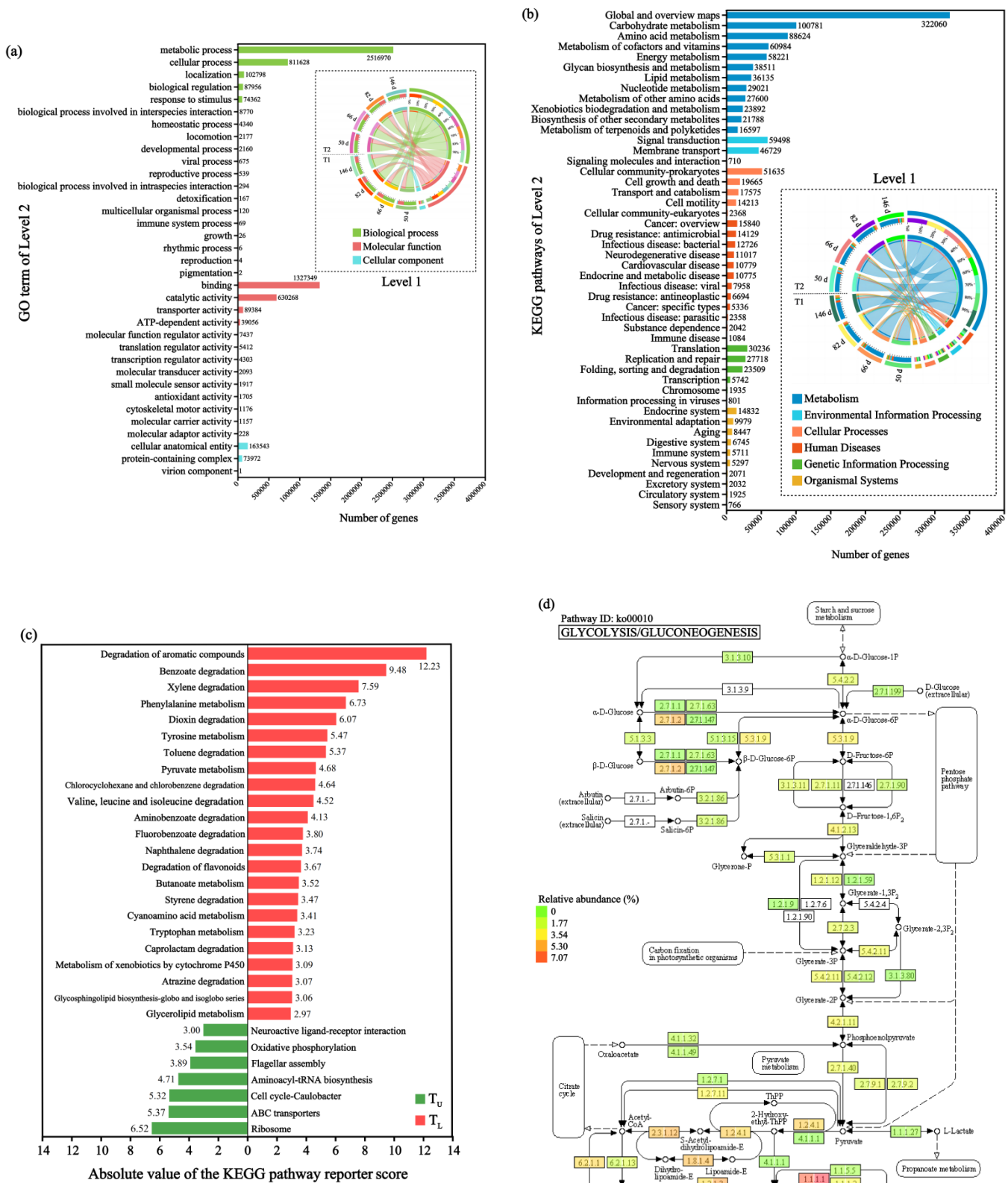


Fig. 5. GO annotations at Level 1 in different groups and number of genes of Level 2 (a); KEGG pathways at Level 1 in different groups and number of genes of Level 2 (b) and absolute value of the KEGG pathway reporter score of Level 3 in T_U and T_L (c); Pathway map of Glycolysis/Gluconeogenesis (ID: ko00010) with relative abundance of different enzymes (d)

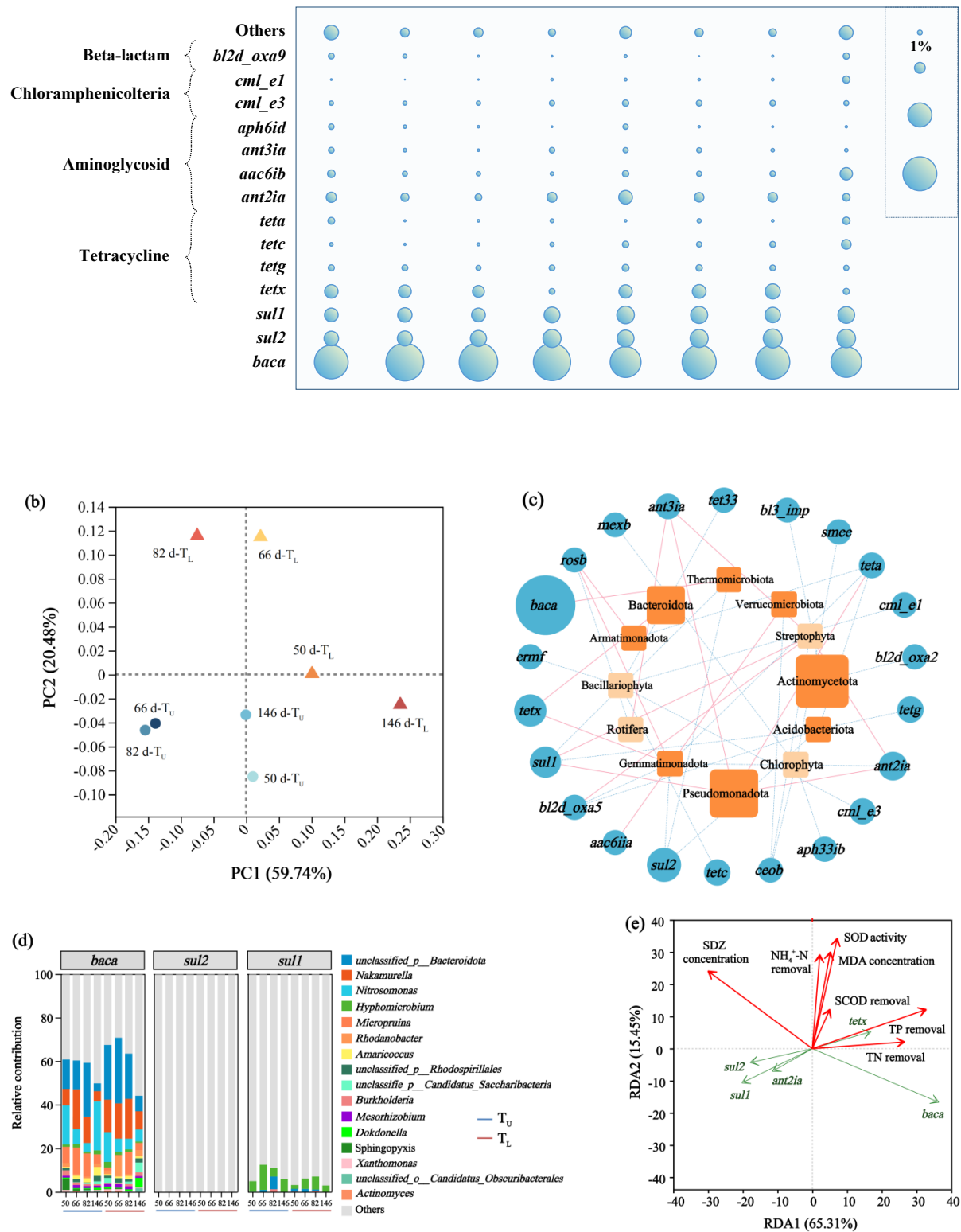


Fig. 6. Bubble charts of antibiotic resistance genes (ARGs) in different reactors (a); The principal co-ordinates analysis (PCoA) of ARGs composition (b); The network analysis of the correlation between microorganisms at the phylum level and ARGs (c); The relative contribution of bacteria at the genus level to ARGs in top 3 (d); The redundancy analysis (RDA) of ARGs (top 5) and environmental factors (e)

Note: * For the correlation between microorganisms and ARGs, red solid lines indicated positive correlation and blue dashed lines indicated negative correlation; All were significantly associated ($p < 0.05$).

Table 1 Concentration and removal efficiency of TN, NH₄⁺-N, TP, and SCOD in wastewater with time

Stage	Time (d)	Removal efficiency (%)												
		TN			NH ₄ ⁺ -N			TP			SCOD			
		T _U	T _L	<i>p</i>	T _U	T _L	<i>p</i>	T _U	T _L	<i>p</i>	T _U	T _L	<i>p</i>	
Stage I	0	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns	0.00±0.00	0.00±0.00	ns	
	8	14.31±0.14	16.67±0.50	ns	18.33±0.18	17.21±0.52	ns	93.15±0.93	88.76±2.66	ns	74.71±0.75	74.32±2.23	ns	
	22	38.97±1.56	40.73±0.41	ns	38.11±1.52	34.59±0.35	ns	90.00±3.60	89.54±0.90	ns	70.12±2.80	68.82±0.69	ns	
Stage II	36	40.82±0.82	42.09±1.68	ns	58.08±1.16	56.94±2.28	ns	88.80±1.78	85.77±3.43	ns	72.00±1.44	70.30±2.81	ns	
	50	42.50±1.28	45.29±0.91	*	97.37±2.92	97.06±1.94	ns	86.83±2.61	84.80±1.70	ns	75.03±2.25	73.40±1.47	ns	
Stage III	10 mg/L SDZ	58	30.40±1.52	31.94±0.32	ns	81.78±4.09	90.99±0.91	*	93.67±4.68	90.72±0.91	ns	86.35±4.32	84.94±0.85	ns
		66	38.32±0.77	40.55±2.03	ns	85.55±1.71	92.57±4.63	*	95.97±1.92	93.02±4.65	ns	83.48±1.67	83.18±4.16	ns
	30 mg/L SDZ	74	45.81±0.46	46.29±0.93	ns	91.51±0.92	99.93±2.00	*	96.10±0.96	91.38±1.83	ns	87.62±0.88	84.94±1.70	*
		82	42.50±1.28	46.72±1.40	**	95.43±2.86	99.93±3.00	ns	95.97±2.88	94.59±2.84	ns	86.99±2.61	86.89±2.61	ns
	50 mg/L SDZ	98	44.68±1.79	42.55±1.70	ns	97.34±3.89	98.01±3.92	ns	67.47±2.70	46.00±1.84	**	88.65±3.55	84.99±3.40	*
		120	54.08±1.08	45.75±0.91	**	74.92±1.50	66.67±1.33	**	77.82±1.56	81.89±1.64	ns	88.34±1.77	85.42±1.71	*
	146	44.09±1.32	42.40±0.42	ns	97.29±2.92	95.74±0.96	ns	87.85±2.64	84.39±0.84	ns	86.47±2.59	86.21±0.86	ns	

Note:

* TN, total nitrogen; NH₄⁺-N, ammonia nitrogen; TP, total phosphorus; SCOD, soluble chemical oxygen demand.

* Stage I, the static stage; Stage II, the granulation stage; Stage III, the SDZ gradient increase stage.

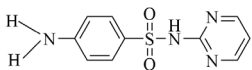
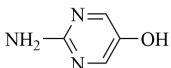
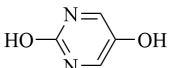
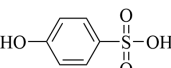
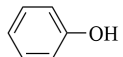
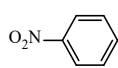
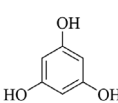
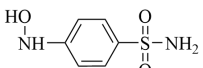
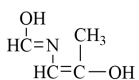
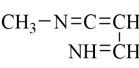
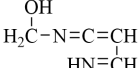
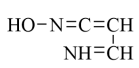
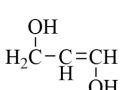
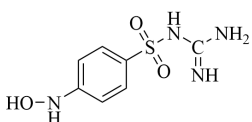
* The data represent the mean ± standard deviation (n=3).

* The difference was assessed by performing a one-way ANOVA followed by Waller-Duncan's multiple range test. The *p* value represented the difference between T_U and T_L. Consider *p* > 0.05 as no significant difference, and marked as "ns"; consider 0.001 < *p* < 0.05 as a significant difference, and marked as "**"; consider *p* < 0.001 as a highly significant difference, and marked as "***".

Table 2 Comparison of the sulfadiazine (SDZ) removal performance in microalgae system with the previous studies

System type	System composition	Reaction medium (SDZ concentration)	Reaction type (Reaction time)	Removal efficiency	Reference
Microalgal-bacterial granular sludge (MBGS) system	<i>Chlorella</i> sp. and activated sludge (AS)	Synthetic pig wastewater (10 mg/L-50 mg/L)	Semi-continuous reaction (146 d, HRT=10 d)	90.77%-99.94%	This study
	Endogenous microalgae and AS	Synthetic wastewater (2 mg/L-4 mg/L)	Semi-continuous reaction (90 d, HRT=12 h)	97.4%-97.0%	[6]
	<i>Scenedesmus obliquus</i> and AS	BG11 (1 mg/L-160 mg/L)	Batch reaction (10 d)	2.08%-5.85%	[46]
	<i>Chlorella pyrenoidosa</i> and AS	The secondary sedimentation tank wastewater (1 mg/L)	Batch reaction (120 h)	11.44%	[1]
Pure microalgae system	<i>Chlorella</i> sp.	BG11 (1 mg/L-30 mg/L)	Batch reaction (50 d)	53.3%-89.1%	[32]
	<i>Chlorella pyrenoidosa</i>	The acidogenic fermentation of mariculture wastewater (100 µg/L-500 µg/L)	Batch reaction (12 d)	65.9%-67.6%	[48]
	<i>Chlorella</i> sp.	Synthetic wastewater (0.5 mg/L-100 mg/L)	Batch reaction (20 d)	19%-54%	[5]
			Continuous reaction (80 d, HRT=1-4 d)	59.2%-90.2%	
	<i>Chlorella vulgaris</i>	Marine aquaculture wastewater (0.1 mg/L)	Batch reaction (12 d) Continuous reaction (70 d, HRT=1-2 d)	32.06% 78.8%-79.2%	[66]

Table 3 The m/z, molecular formula and proposed structures of sulfadiazine (SDZ) and its degradation byproducts

Intermediate	Name	m/z	Molecular formula	Proposed structure
SDZ	sulfadiazine	251.06	C ₁₀ H ₁₀ N ₄ O ₂ S	
BP1	2-aminopyrimidin-5-ol	112.05	C ₄ H ₅ N ₃ O	
BP2	pyrimidine-2,5-diol	112.50	C ₄ H ₄ N ₂ O ₂	
BP3	4-hydroxybenzenesulfonic acid	174.20	C ₆ H ₆ O ₄ S	
BP4	sulfuric acid	96.96	H ₂ SO ₄	
BP5	phenol	95.05	C ₆ H ₆ O	
BP6	benzene	79.05	C ₆ H ₆	
BP7	nitrobenzene	124.03	C ₆ H ₅ NO ₂	
BP8	benzene-1,3,5-triol	127.04	C ₆ H ₆ O ₃	
BP9	pyrimidine	81.04	C ₄ H ₄ N ₂	
BP10	N-hydroxysulfanilamide	187.02	C ₆ H ₈ N ₂ O ₃ S	
BP11	N-(2-hydroxyprop-1-en-1-yl)formimidic acid	102.06	C ₄ H ₇ NO ₂	
BP12	3-(methylazanylidene)prop-2-en-1-imine	83.06	C ₄ H ₆ N ₂	
BP13	ethanol prop-1-ene-1,3-diimine	99.05	C ₄ H ₆ N ₂ O	
BP14	3-iminoprop-1-en-1-one oxime	83.02	C ₃ H ₄ N ₂ O	
BP15	prop-1-ene-1,3-diol	75.04	C ₃ H ₆ O ₂	
BP16	N-carbamimidoyl-4-(hydroxyamino)benzenesulfonamide	230.05	C ₇ H ₁₀ N ₄ O ₃ S	

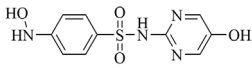
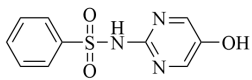
BP17	4-(hydroxyamino)- <i>N</i> -(5-hydroxypyrimidin-2-yl)benzenesulfonamide	281.04	C ₁₀ H ₁₀ N ₄ O ₄ S	
BP18	<i>N</i> -(5-hydroxypyrimidin-2-yl)benzenesulfonamide	250.03	C ₁₀ H ₉ N ₃ O ₃ S	

Table 4 Alpha diversity indexes of microorganism, ARGs, and CYP450 superfamily

Reactor	Time (d)	Microorganism			ARGs			CYP450 superfamily		
		Chao1	Shannon	Shannoneven	Chao1	Shannon	Shannoneven	Chao1	Shannon	Shannoneven
T _U	50	20075	5.31	0.51	99	3.54	0.66	80	2.63	0.49
	66	21180	5.16	0.51	111	3.30	0.71	89	2.64	0.50
	82	20022	4.94	0.50	100	3.10	0.76	93	2.68	0.50
	146	19104	5.00	0.56	92	3.11	0.73	105	2.87	0.51
T _L	50	19771	5.04	0.51	109	3.31	0.71	89	2.62	0.54
	66	20066	4.77	0.49	110	3.09	0.76	93	2.63	0.54
	82	20000	4.87	0.49	106	3.03	0.75	95	2.55	0.55
	146	18163	4.90	0.51	99	3.05	0.70	111	2.48	0.58

Note:

* ARGs, antibiotic resistance genes.

* CYP450, cytochromes P450.



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Long-term operational strategy of microalgal-bacterial granular sludge towards strengthened sulfadiazine antimicrobial resistance control

Lin, Shupeng; Pan, Minmin; Ma, Yanfang; Chen, Zhihao; Lyu, Tao ...

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