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Compromised performance and microbial communities of photobioreactors exposed to veterinary antibiotics during swine wastewater treatment

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ABSTRACT

Adopting a green engineering approach, this study utilizes occurring microalgal-bacterial photobioreactors for the sustainable valorization of nitrogen-rich swine wastewater. However, the widespread use of antibiotics in livestock can alter the microbial communities that sustain these systems, potentially affecting their performance. This study presents a novel simultaneous and comparative investigation of the ecological pressure exerted by three specific antibiotics at environmentally relevant concentrations—tetracycline (1500 μg/L), ciprofloxacin (100 μg/L), and sulfadiazine (1500 μg/L)—in parallel photobioreactors treating complex, real swine wastewater. By integrating comparative ecological analysis with the detection of drugs accumulated in the biomass, this work systematically evaluates nutrient removal and microalgae growth, antibiotics fate and microbial community dynamics, while uniquely linking the proliferation of opportunistic taxa (e.g., *Pseudomonas*, *Acinetobacter*) to low biosorption efficiency and potential antimicrobial resistance mechanisms. Tetracycline and sulfadiazine were mainly removed by degradation (73 % and 53 %, respectively), whereas ciprofloxacin removal was negligible. Antibiotic exposure significantly impaired nutrient removal and reduced *Chlorella* sp. biomass, driving shifts in 25 bacterial taxa. Redundancy analysis (tb-RDA) quantified the selective pressure, proving that antibiotic exposure was a primary driver of community variance. The taxa associated with nutrient removal decreased, while potentially drug-resistant taxa proliferated but failed to maintain functional performance. Crucially, these effects were persistent and irreversible, as neither the microbial community structure nor treatment performance recovered post-exposure, ultimately compromising treatment efficiency. These findings highlight that veterinary antibiotics compromise the stability and efficiency of microalgal-bacterial systems, challenging their implementation as resilient wastewater treatment technologies.

1. Introduction

Pig meat consumption is expected to rise 8 % by 2033, mostly driven by the demand generated in the middle-income countries [46]. This demand leads to the rise of industrial farming where antibiotics are commonly used for disease prevention [67]. However, 70–90 % of administered drugs are excreted via feces and urine, resulting in high concentrations in swine wastewater (SWW) [42,69]. Thus, common veterinary antibiotics like tetracycline (TC: up to 1500 μg/L), fluoroquinolones (ciprofloxacin (CPX): up to 1230 μg/L), and sulfonamides (sulfadiazine (SDZ) > 780 μg/L) are detected in SWW effluents [38,39,56]. Therefore, untreated SWW effluents can affect public and environmental health due to persistent antibiotics contamination [43].

Microalgae-bacteria photobioreactors (PBRs) are a promising technology for SWW treatment. This approach aligns with the core tenets of Green Chemistry [1] and the circular bioeconomy, as it focuses on the valorisation of a waste stream (SWW) [17,18]. PBRs are low cost, exhibit high nutrient removal, and produce valuable biomass for biotechnological applications [43]. In these systems, microalgae assimilate carbon and nutrients from simple compounds produced by bacteria, while

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bacterial degrade organic matter using microalgal oxygen [48]. PBRs have also been shown to remove antibiotics through photodegradation, intracellular and extracellular biodegradation, bioaccumulation or biosorption in the biomass [10,16]. For example, complete TC removal (100 %) was achieved in PBRs fed with non-sterile diluted SWW [42]. Similarly, 70–90 % of SDZ was removed from synthetic aquaculture water [34], whereas moderate to high (59–84 %) CPX removals occurred on PBRs treating domestic wastewater [23].

However, antibiotics can affect bacterial populations in synthetic wastewater [8,31,62,63], potentially disrupting the microalgae-bacteria consortia required for PBRs wastewater treatment. Toxicological studies also report effects on microalgae, including reduced growth, chlorophyll content, and photosynthetic activity [30,44,54]. Consequently, nutrient removal efficiency can decline in the presence of drugs [7,43]. For example, carbon and nitrogen removal decreased remarkably at TC concentrations above 1000 μg/L in sterilized domestic wastewater [53]. Likewise, synthetic wastewater treatment by bacteria showed reduced nitrogen removal under prolonged exposure to 200 μg/L CPX [63] and 6000 μg/L SDZ [31].

Advancing PBRs for SWW treatment requires understanding antibiotic impacts on functional microbial communities. Unlike previous studies often limited to synthetic media or unrealistic loads, this study performs a simultaneous comparative investigation of three antibiotics (TC, 1500 μg/L; CPX, 100 μg/L; and SDZ, 1500 μg/L) in parallel PBRs treating real SWW. Our novel multi-stress analytical approach quantifies selective pressure, identifies specific indicator microorganisms and links ecological dynamics to drugs accumulated in the biomass. These high spiking concentrations were chosen to investigate the inhibitory concentration threshold and to characterize resistant bacterial taxa. This approach is justified by reports showing that concentrations significantly lower than 100 μg/L had no observed impact on biomass performance [60], whereas environmentally relevant maximum concentrations up to 447 and 202 μg/L for cycline antibiotics (e.g., oxytetracycline and doxycycline), and above 780 μg/L for sulfonamide antibiotics (e.g., sulfadiazine) have been registered in swine manure in a pig farm at Cantalejo (Spain) and other locations [38]. Furthermore, sulfadimidine levels exceeding thousands of mg/L have been found in European farms [25,38,58–60]. Therefore, a high concentration spiking regime was selected in this study so that the threshold in the concentration of these antibiotics could be found. Furthermore, the resistant bacterial taxa could be identified as well as find out their capacity of removing nutrients and antibiotics.

The main objectives were to evaluate the impact of drug overload on: (i) antibiotic removal and biosorption by biomass; (ii) nutrient removal and biomass concentration; (iii) microalgal growth; and (iv) changes in the composition, structure, and dynamics of the bacterial community, linking the loss of key functional taxa to impaired performance. The study also assessed the potential treatment performance and microbiome recovery following antibiotic exposure, addressing the critical question of system resilience and the potential for irreversible impacts. The results presented here elucidate a possible scenario for large-scale treatment of swine effluent with high antibiotic loads using PBRs. This research advances Sustainable Development Goal (SDG) 6: Clean Water and Sanitation by assessing how veterinary antibiotics affect the efficiency of microalgal-bacterial photobioreactors in treating swine wastewater, thereby providing crucial insights to improve resilient and sustainable wastewater treatment systems under antibiotic pollution [1,17,18].

2. Materials and methods

2.1. Microalgal inoculum and swine wastewater

A microalgae inoculum (*Chlorella* sp. cells >90 %) was sourced from a lab-scale open indoor SWW treatment reactor. Raw SWW, from a local farm, was centrifuged (10 min at 10,000 rpm) for total suspended solids (TSS) reduction; the supernatant was then diluted to 5 % (v/v) with tap water to avoid excess ammonium in the feed [21]. The feed and raw SWW were stored at 4 °C. Feed physicochemical composition (mean ±sd) was: 400 ± 0.1 mg/L TSS, 889 ± 60 mg/L total organic carbon (TOC), 284 ± 11 mg/L total nitrogen (TN) and 99 ± 5 mg/L ammonia nitrogen (NH_4^+-N). SDZ concentration was 0.46 ± 0.06 μg/L. TC was below the limit of detection (<LOD: 0.059 μg/L) and CPX was below the limit of quantification (<LOQ:0.086 μg/L), respectively.

2.2. Photobioreactors, growing conditions and experimental design

The experiment was conducted in duplicate using two sets of four open 3 L PBRs (0.16 m depth) operated under identical conditions: pH 8 controlled with pure carbon dioxide (Carburos Metalicos-Barcelona, Spain) by a Supervisory Control and Data Acquisition (SCADA) system, temperature maintained at 20–23°C, and irradiance 1400 μmol/m^2·s (12 h:12 h light:dark cycles) [12]. Each set included a control photobioreactor without added drugs and three PBRs fed with antibiotic-doped SWW (set1: R-control1, R-TC1, R-CPX1, R-SDZ1; set2: R-control2, R-TC2, R-CPX2, R-SDZ2). All PBRs were inoculated with microalgae (10 % v/v) and fed 5 % (v/v) diluted in tap water SWW using a multichannel pump (Watson-Marlow, UK). PBRs operated continuously at a 6-day hydraulic retention time (HRT) [21]. Evaporation (<15 %) was measured daily from inlet and outlet flow rates and compensated with tap water. Initially, PBRs were operated for over 3 HRT until a constant effluent composition (TSS, TOC, TN, NH_4^+-N). Biomass from all PBRs was mixed pre-experiment to homogenize microbiomes (day 0). The PBRs operation was divided into three phases: phase I (pre-exposure 0–12 days), phase II (antibiotic exposure 13–37 days) and phase III (post-exposure recovery 38–47 days). During phase II, R-TC1, R-TC2, R-SDZ1 and R-SDZ2 were doped with 1500 μg/L of TC and SDZ, respectively, and R-CPX1 and R-CPX2 with 100 μg/L of CPX. The controls PBRs (R-control1 and R-control2) were fed 5 % diluted SWW without antibiotics.

2.3. Chemical reagents and working solutions of antibiotics

High purity (>95 %) TC, CPX, and SDZ standards (Sigma-Aldrich, Madrid, Spain) were used to prepare antibiotic solutions. Stock solutions of TC and SDZ (15,000 mg/L) were dissolved in high-grade methanol (MeOH), while CPX (500 mg/L) was dissolved in H_2O/MeOH (1:1, v/v) containing 0.2 % (v/v) 37 % HCl (Sigma-Aldrich, Madrid, Spain), due to low MeOH solubility [37]. Stock solutions were used to prepare working solutions (TC: 150 mg/L, CPX: 10 mg/L and SDZ: 150 mg/L). From these working solutions, the reactor feed was prepared to reach the desired working concentrations (TC:1500 μg/L; CPX: 100 μg/L, and SDZ: 1500 μg/L). Dilutions were prepared in milli-Q water, to decrease MeOH in the PBRs. The final MeOH concentration (100 μL/L) was added in all PBRs, including R-control to avoid solvent effects [42]. The stock and working solutions were stored at −20°C and 4°C, respectively, in the dark at to avoid degradation.

2.4. Sampling and analytical procedures

PBRs performance was assessed by monitoring antibiotic and nutrient removal efficiency and TSS. Influent and effluent samples were collected every two days from PBRs. 10 mL of sample were used to determine the TSS (mg/L) [21]. The remaining sample was centrifuged at 10,000 rpm for 5 min, and the supernatant was used to determine TOC, TN and NH_4^+-N using a TOC-V CSH total organic analyzer equipped with a TNM-1 total nitrogen measurement module (Shimadzu, Japan) and an Orion (cat 9512HPWBNWP) ammonium selective electrode (Thermo Scientific, Netherlands). For antibiotic analysis, 100 mL of liquid sample was sequentially filtered through a 0.7 μm glass fiber membrane and, then through a 0.45 μm nylon filter, then stored at −20°C in PET amber bottles until further analysis. The biomass fraction

was lyophilized. The extraction, detection and quantification of antibiotics followed established protocols for liquid [38] and biomass samples [37]. Chromatographic separation was performed using ultra-high performance liquid chromatography (UHPLC) – tandem mass spectrometry (MS/MS) using an Exion LC (AB Sciex, Framingham, MA, USA) with a Phenomenex EVO C18 column (2.1 mm × 50 mm, particle size 1.7 µm, Torrance, CA, USA). Mass detection was performed by the triple quadrupole 6500 + QqQ (Sciex). Data processing was performed using Analyst and OS software (AB Sciex). Nutrient and antibiotic removal efficiencies (RE) were calculated according to Eq. (1).

$$RE(\%) = \frac{(C_{input} \cdot Q_{input} - C_{output} \cdot Q_{output})}{C_{input} \cdot Q_{input}} \times 100 \quad (1)$$

For the antibiotics, degradation was determined according to Eq. (2).

$$DE(\%) = \frac{C_{input} X Q_{input} - (C_{output} + C_{B_{output}} x TSS_{output}) x Q_{output}}{C_{input} X Q_{input}} \times 100 \quad (2)$$

In these equations, C_{input} and C_{output} (mg/L) represent nutrient or antibiotic concentration (TOC, TN, NH_4^+-N, TC, CPX, or SDZ) in the influent and effluent, respectively. The Q_{input} and Q_{output} (L/d) correspond to the daily influent and effluent flows. TSS_{output} (g/L) is the TSS in the effluent and C_{B_output} (mg/g) is antibiotic concentration in harvested biomass. The effect of antibiotic on reactor performance was studied by an analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) multiple range test ($p \leq 0.05$). For comparisons, data at the end of phase II (days 33, 35, and 37) was used. Statistical analyses were performed in R (R [50].

2.5. Microalgal biomass growth parameters

To analyze microalgal culture growth and status, samples were collected from PBRs every two days. The ratio of variable to maximum fluorescence (F_v/F_m) was immediately measured using an AquaPen-C AP110-C fluorometer (Photon Systems Instruments, Czech Republic), following the manufacturer's instructions, to assess photosynthetic activity [7,12,30]. Samples were preserved according to the Phytoplankton manual [51] until microscopic observation (Leica DM 4000 B). Microalgae growth was quantified in triplicate by cell count using a Neubauer chamber (Marienfel, Germany).

2.6. DNA extraction, sequencing, and sequences processing

To study the bacterial communities, samples were collected towards the end of phase I (day 9), during phase II (days 16, 26 and 33) and at the end of phase III (day 47) and stored at −80°C until DNA extraction. Sample from R-control2 on day 9 was lost during processing. Total genomic DNA extraction, library preparation, sequencing of the V3-V4 region of the 16S rRNA gene, and bioinformatics analysis were performed as previously described [12]. In brief, primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 [24] were used to produce the V3-V4 amplicons. The Foundation for Health Promotion and Biomedical Research (FISABIO, Valencia, Spain) prepared the libraries following the Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A) and sequenced them in a 2x300pb paired-end run (MiSeq Reagent kit v3) on a MiSeq Sequencer according to the manufacturer's instructions (Illumina). DADA2 pipeline [6] was used to build sequence variants (ASVs) that were taxonomically classified according to the Silva v138 database [49] and then agglomerated at all taxonomic levels. ASVs not classified up to species level were assigned their best taxonomic level classification.

2.7. Bacterial diversity analysis

The effects of antibiotics on the bacterial community diversity and structure were analysed as previously described [12]. In brief, 64959 reads were randomly subsampled from each sample before further analysis. Alpha diversity indices determined were the inverse Simpson's diversity index, Simpson's evenness index (E index) and richness (number of species observed). Kruskal-Wallis test was used to compare diversity indices between treatments. After applying a Hellinger transformation to species abundance data, nonmetric multidimensional scaling analysis (nMDS) based on Bray-Curtis dissimilarities and PERMutational ANalysis Of the VAriance (PERMANOVA) were used to analyse bacterial community structure differences when exposed to drugs. Phase I was removed from the PERMANOVA analysis. Transformation-based redundancy analysis (tb-RDA) was used to determine the community relationship with operational parameters. The explanatory variables were log-transformed or removed (Pearson correlation >0.75, Figure S1) when needed (remaining variables included - influent concentration: TC_inf, CPX_inf, TOC_inf, TN_inf, NH_4^+_inf; effluent concentration: TC_eff, CPX_eff, SDZ_eff, TN_eff, NH_4^+_eff, and TSS, F_v/F_m). Prior to nMDS and tb-RDA analyses, abundance data was filtered to remove bacterial species below 0.1 % abundance in any sample. SIMilarity PERcentage breakdown procedure (SIMPER [11]) was used to identify indicator taxa of antibiotics exposure, which were compared between treatments with Kruskal-Wallis tests. All p-values were corrected for multiple comparisons by false discovery rate. All statistical analyses were performed in R (R [50] with the packages phyloseq [41], microbiome [26], vegan [47] and ampvis2 [2].

3. Results and discussion

3.1. Antibiotics removal

During phase I (before exposure to antibiotics), the concentration of TC and CPX in the feed and effluent were below the detection (0.059 µg/L) and quantification limit (0.086 µg/L) for TC and CPX, respectively. SDZ was detected at mean values of 0.082 ± 0.001 µg/L in the effluent, reaching 80 % removal, demonstrating the effectiveness of PBRs to remove SDZ at low concentrations. During phase II, the addition of TC (1500 µg/L), CPX (100 µg/L), and SDZ (1500 µg/L) led to elevated effluent concentrations in the doped PBRs (Table 1). This increase was notable in the CPX-exposed reactor, where effluent concentrations reached the initial doping level by day 33, resulting in 0 % final removal. By the end of phase II (day 33), TC and SDZ removals were 76 and 55 %, respectively. After stopping antibiotics addition (phase III), effluent antibiotic concentration decreased in all doped PBRs, as expected. Lower TC removal (69 %) was reported in a high-rate algal reactor treating synthetic wastewater with 2000 µg/L TC using non-axenic microalgal culture operated at 7-day HRT [14]. Similarly, Xie et al., [61] observed 55 % SDZ removal after 9 days at an antibiotic concentration of 1000 µg/L, while CPX was completely removed after 6 days at 1000–10,000 µg/L during synthetic wastewater treatment with *Chlamydomonas* sp. Tai-03 [61]. These findings indicate that the system effectively removes drugs but requires optimization to improve under realistic conditions with high drugs concentrations in real SWW.

The concentrations of TC, CPX, and SDZ were quantified in the biomass (Table 1) to study the importance of degradation and biosorption mechanisms on antibiotic removal in PBRs. During phase I, antibiotics in the biomass were below detection limits (1.3 µg/g for TC, 0.22 µg/g for CPX and 0.12 µg/g for SDZ), indicating that SDZ removal occurred mainly via degradation, with low effluent concentration (0.082 ± 0.001 µg/L) compared to the feed (0.46 ± 0.06 µg/L). In phase II, antibiotic concentrations in biomass reached 64 ± 2, 34 ± 21, and 37 ± 7 µg/g for TC, CPX, and SDZ, respectively. Partition coefficients ((µg/L)/(µg/L)) calculated from biomass and effluent antibiotic

Table 1
Concentrations of three antibiotics studied in effluent (μg/L) and biomass (μg/g of lyophilized biomass) from PBRs fed with swine wastewater. Values correspond to phase I (days 1–12, sample on day 9): stage prior to antibiotic addition; phase II (days 13–37, samples on days 16, 26, and 33): Feed was doped with 1500 μg/L TC, 100 μg/L CPX, and 1500 μg/L SDZ for the R-TC, R-CPX, and R-SDZ PBRs, respectively; and phase III (days 38–47, sample on day 47): stage after antibiotic spiking. The standard deviation of the concentrations determined in duplicate PBRs is shown.

			Tetracycline		Ciprofloxacin		Sulfadiazine		TSS
PBRs	Phases	Days	Effluent (μg/L)	Biomass (μg/g)	Effluent (μg/L)	Biomass (μg/g)	Effluent (μg/L)	Biomass (μg/g)	(mg/L)
R-control	I	9	n.d[a]	n.d	<LQ	<LQ	0.082 ± 0	<LQ	860
	II	16	n.d		<LQ		0.122 ± 0		860
		26		n.d	<LQ	<LQ	0.108 ± 0.01	0.014 ± 0.001	770
		33	n.d	n.d	<LQ	<LQ	0.082 ± 0	0.015 ± 0.002	810
	III	47	n.d	n.d	<LQ	<LQ	0.123 ± 0	0.015 ± 0	890
R-TC	I	9	n.d	n.d	<LQ	<LQ	0.082 ± 0	<LQ	830
	II	16	36 ± 1		<LQ		0.116 ± 0.02		790
		26	55 ± 6	25 ± 2	<LQ	<LQ	0.104 ± 0	0.013 ± 0.001	600
		33	421 ± 150	64 ± 2	<LQ	<LQ	0.104 ± 0	<LQ	760
	III	47	45 ± 5	10 ± 13	<LQ	<LQ	0.123 ± 0	0.017 ± 0.004	850
R-CPX	I	9	n.d	n.d	<LQ	<LQ	0.082 ± 0	<LQ	830
	II	16	n.d		11 ± 3		0.121 ± 0.02		785
		26	n.d	<LQ	24 ± 5	15 ± 3	0.116 ± 0.01	<LQ	660
		33	n.d	<LQ	110 ± 3	34 ± 21	0.112 ± 0.01	<LQ	585
	III	47	n.d	<LQ	14 ± 5	1 ± 0	0.123 ± 0	<LQ	695
R-SDZ	I	9	n.d	n.d	<LQ	<LQ	0.082 ± 0.001	<LQ	970
	II	16	n.d		<LQ		143 ± 29		780
		26	n.d	<LQ	<LQ	<LQ	399 ± 127	21 ± 0.03	680
		33	n.d	<LQ	<LQ	<LQ	774 ± 85	37 ± 7	705
	III	47	n.d	<LQ	<LQ	<LQ	30 ± 2	0.691 ± 0.005	715

n.d [a].: below method detection limit TC: 0.059 μg/L in the liquid effluent. μg/L for liquid effluent, and TC: 1.3 μg/g, CPX: 0.22 μg/g, and SDZ:0.12 μg/g for biomass.

concentrations indicated lower retention for SDZ (0.03) than TC (0.12) and CPX (0.18), reflecting differences in drug affinity for biomass. These trends agree with n-octanol-water partition coefficients (log P: SDZ −0.07, TC 0.6 and CPX 1.6; [37], where lower log P bind less to the biomass [7,55]. Correspondingly, degradation was highest for TC (73 %), followed by SDZ (53 %) and nil for CPX at the end of phase II (day 33). Adsorption onto the microalgae–bacteria biomass was found to be an important, and in some cases dominant, removal mechanism for these veterinary antibiotics in batch experiments conducted under controlled conditions (synthetic wastewater, dark conditions, absence of active biomass, lyophilized biomass, etc.) designed to isolate and study each potential removal pathway separately [14,23,61,66]. However, studies performed in photobioreactors treating real wastewater under continuous operation have shown a limited contribution of adsorption, with degradation during light periods being the predominant mechanism [23]. Norvill et al. [45] reported tetracycline removal above 93 %, with adsorption accounting for less than 6 % of the total antibiotic removed. Overall, antibiotic removal by biomass adsorption was minimal, with degradation as the main elimination mechanism at environmentally relevant concentrations.

CPX is a highly persistent antibiotic in biological treatment systems. Its recalcitrance is explained by the fact that none of the main elimination pathways (adsorption, biodegradation, photolysis, hydrolysis, biogenic oxidation) are truly effective under typical conditions. CPX contains a highly stable aromatic bicyclic system (quinolone core). Breaking the quinolone ring requires very specific monooxygenases/aryl oxygenases that are rarely present in microalgal–bacterial cultures [52]. Adsorption is usually the main process responsible for the 'apparent removal' of CPX, but it does not involve degradation—only phase transfer. Once adsorbed, it may desorb when pH, ion composition, or the physiological state of the culture changes. Adsorption also protects CPX from photolysis and oxidation, further reinforcing its recalcitrance [32]. Finally, CIP is highly hydrolytically stable as it does not contain groups that are easily hydrolyzed under neutral to alkaline pH.

3.2. Nutrient removal and biomass concentration

In phase I, nutrient concentrations in the effluents remained stable in all PBRs (TOC: 138 ± 6 mg/L, TN: 155 ± 20 mg/L and NH_4^+-N: 67 ± 5 mg/L at day 12, Fig. 1), with mean eliminations of 87 ± 1 % for TOC, 54 ± 6 % for TN and 42 ± 4 % for NH_4^+-N (Fig. 1A, B and D). However, antibiotics significantly increased nutrient concentrations in all doped PBRs during phase II (Table S1, p-value ≤0.05). By day 37, mean effluent TOC and TN reached 185 ± 14 mg/L and 223 ± 4 mg/L in R-TC, 311 ± 13 mg/L and 217 ± 2 mg/L in R-CPX, and 289 ± 1 mg/L and 239 ± 1 mg/L in R-SDZ, while R-control maintained lower levels (103 ± 11 mg/L for TOC and 178 ± 15 mg/L for TN). Consequently, TOC and TN removal decreased in R-TC (82 ± 2 % and 33 ± 3 %), R-CPX (70 ± 2 % and 36 ± 1 %), and R-SDZ (73 ± 0.1 % and 30 ± 0.2 %), compared with R-control (90 ± 1 % for TOC and 47 ± 4 % for TN). NH_4^+-N removal decreased significantly only with SDZ addition (Table S1, p-value ≤0.05), reaching the highest effluent concentration (88 ± 3 mg/L) compared to the R-control (72 ± 11 mg/L) at day 35. In phase III, TOC concentration remained elevated in all antibiotic-exposed PBRs compared to the control reactor (Table S2, p-value ≥0.05). However, average TN was significantly higher only in the R-SDZ reactor, while NH_4^+-N removal recovered.

CPX and SDZ addition also significantly decreased biomass concentration compared to TC and control PBRs (Fig. 1E, Table S1). At day 37, mean TSS in R-CPX (0.59 ± 0.02 g/L) and R-SDZ (0.61 ± 0.04 g/L) were lower than in R-TC (0.78 ± 0.04 g/L) and R-control (0.87 ± 0.04 g/L). In phase III, TSS increased in R-CPX (0.70 ± 0.15 g/L) and R-SDZ (0.71 ± 0.08 g/L) showing no significant difference between treatments (Fig. 1E, Table S2, p-value ≥0.05), suggesting biomass concentration could recover over longer times. These results align with limited previous reports. Taşkan [53] reported reduced TOC (21 %) and TN (14 %) removal during domestic wastewater treatment at 30, 000 μg/L TC [53]. In synthetic wastewater, prolonged CPX exposure decreased TN removal from 95.8 % to 90.5 % and 80.2 % at 200 and 2000 μg/L CPX, respectively [63]. Similarly, SDZ (>6000 μg/L) decreased NH_4^+-N removal in a biofilm reactor treating synthetic mariculture wastewater [31]. Therefore, antibiotic overload decreases nutrient removal likely via negative effects on microorganisms, compromising biomass production and effluent quality.

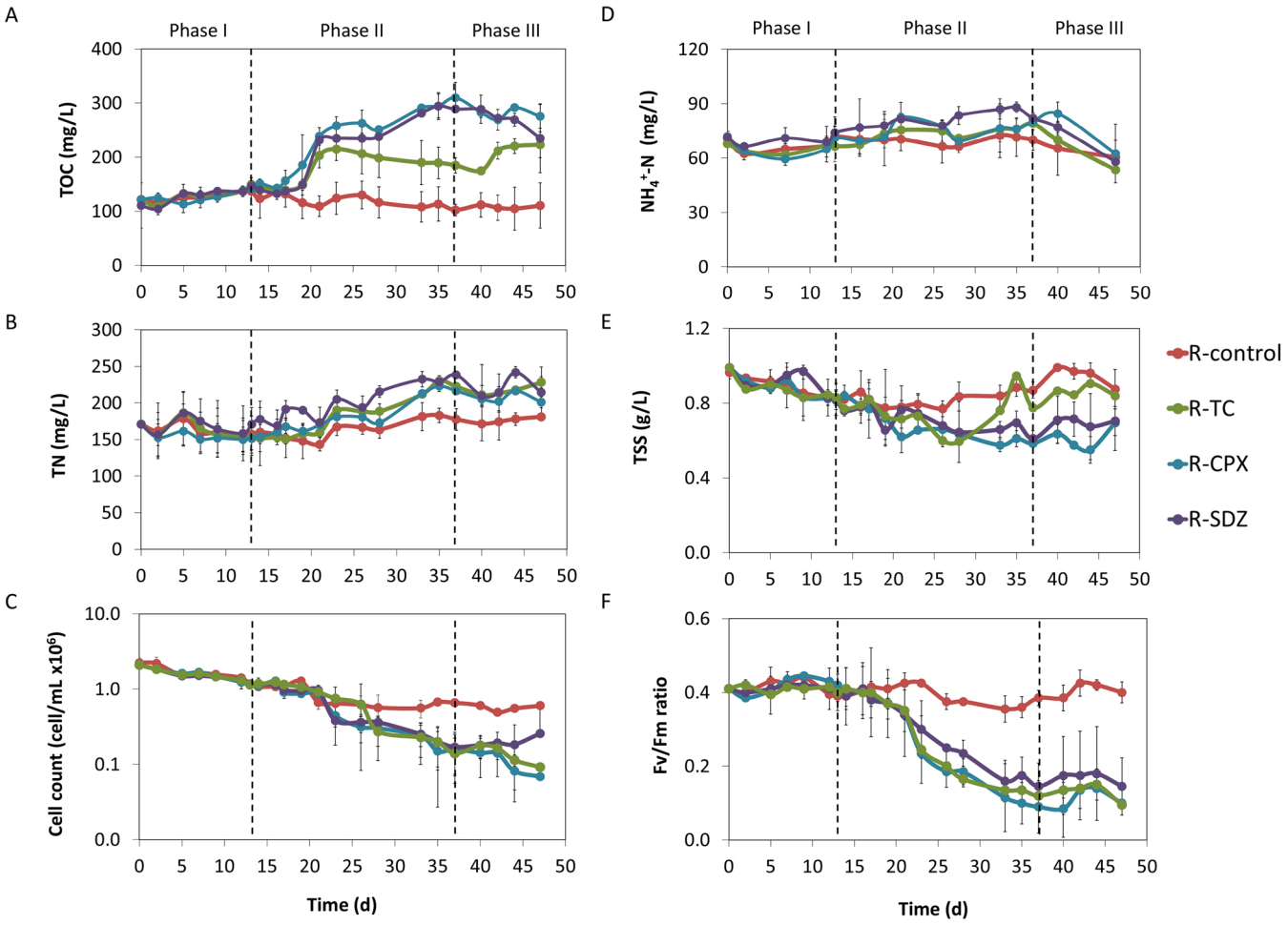

Fig. 1. Evolution of nutrients concentrations in the effluent and microalgal biomass growth of PBRs treating swine wastewater with high concentration of antibiotics. TOC (A), TN (B), Cell count *Chlorella* sp. (C), NH_4^+-N (D), TSS (E) and maximum quantum yield by F_v/F_m ratio (F). Vertical dashed lines indicate the phase change according to antibiotic exposure (phase I: no doping, phase II: doping, and phase III: no doping-recovery). Error bars indicate the standard deviation of duplicate PBRs.

3.3. Inhibition of microalgal growth by antibiotics

Chlorella genus was the dominant microalgae in all PBRs throughout the experiment. In phase I (days 0–12), its concentration averaged 1.6 ± 0.3 × 10^6 cells/mL in all reactors (Fig. 1C). However, exposure to drugs significantly reduced microalgae growth in R-TC (0.18 ± 0.06 ×10^6 cells/mL), R-CPX (0.14 ± 0.08 ×10^6 cells/mL), and R-SDZ (0.18 ± 0.06 ×10^6 cells/mL) compared to R-control (0.61 ± 0.01 ×10^6 cells/mL, Table S1, p-value ≤0.05) by phase II (day 37). Although R-SDZ showed a slight recovery in cell concentration in phase III, levels remained significantly lower than the control. These microalgal growth trends were supported by photosynthetic efficiency (F_v/F_m ratio). The F_v/F_m ratio decreased significantly from phase I (0.41 ± 0.01) to phase II (0.12 ± 0.05 at day 37) in doped PBRs, while R-control maintained a ratio near 0.41 ± 0.01 (Fig. 1F, Table S1, p-value ≤0.05). Furthermore, F_v/F_m remained low even after the antibiotic load was stopped in phase III (Table S2, p-value ≤0.05).

Previous studies show that high antibiotic concentrations inhibit microbial growth and photosynthetic activity. For example, TC above 1000 μg/L reduced *Chlorella* sp. growth [53]. In synthetic wastewater, elevated TC (1000–10,000 μg/L) also decreased chlorophyll concentrations in microalgae-bacteria cultures [54], while *C. vulgaris* declined under CPX exposure (2000–31,000 μg/L) [44]. Similarly, the F_v/F_m ratio dropped in microalgae cultures after fluoroquinolone exposure (enrofloxacin or norfloxacin >1000 μg /L) [7]. These effects are attributed to reactive oxygen species formation that damages photosystem II by destroying the electron transport chain and impairing microalgal metabolism [30,44]. Therefore, our study suggests that antibiotics affect microalgae growth during SWW treatment under high drug load.

3.4. Alteration of bacterial community structure in the biomass by TC, CPX, and SDZ

PBRs biomass was analysed before (day 9, end of phase I), during (days 16, 26 and 33, phase II) and after (day 47, end of phase III) antibiotics exposure to assess community variations. In phase I, diversity indices were similar among PBRs as expected (Richness: 333 ± 0.71, 377 ± 64, 364 ± 1.41, and 348 ± 20; Diversity: 17 ± 0.03, 19 ± 3.17, 19 ± 1.04, and 18 ± 0.16 for R-control, R-TC, R-CPX, and R-SDZ, respectively; evenness 0.052 ± 0.002 for all PBRs, Figure S2). However, evenness increased significantly in phase II with TC and CPX (0.06 ± 0.02 for both) compared to the control (0.04 ± 0.01, Figure S2 p-value ≤0.05). Antibiotics did not affect richness, but the diversity index, which depends on both richness and evenness, reflected the increase in evenness, showing alteration of the microbial community by the drugs. This finding aligns with studies reporting alpha diversity alterations (Simpson, Shannon, CHAO 1, and ACE indices) in bacterial cultures exposed to TC and CPX in synthetic wastewater [57,65].

The bacterial community structure shifted over time even in the R-

control reactor (NMDS, Fig. 2A) reflecting ecological drift [19]. However, antibiotic addition drove significant structural changes during phase II and III (PERMANOVA, Pr < 0.0001, Table S3). Notably, R-TC and R-SDZ communities remained structurally similar, while R-CPX diverged from all others ($p \leq 0.05$, Table S3). This divergence is likely explained by the lower concentration of CPX (100 µg/L) compared to TC and SDZ (1500 µg/L).

A transformation-based redundancy analysis (tbRDA) analysis was performed to assess the influence of antibiotics and operational variables in the variance of bacterial communities identifying the key drivers of these changes. Results showed that 62 % (Table S4) of the bacterial community variance could be explained by antibiotics, specific nutrients (TOC_inf, TN_inf, NH4_inf, TN_eff, NH4_eff), and microalgal status (F_v/F_m, TSS) (Fig. 2, p-value ≤0.05). This multi-stress analysis allowed for the quantification of the selective pressure, revealing that antibiotic exposure was a primary driver of community variance. Temporal changes in doped PBRs correlated positively with antibiotic overload (Fig. 2B, C, and D) in al photoreactors, and effluent TN (TN_eff) in R-SDZ and R-CPX PBRs (Fig. 2B, C). Conversely, these communities correlated negatively with biomass accumulation (TSS), microalgal physiological status (F_v/F_m), and influent nutrient concentrations (TOC_inf, TN_inf) (Fig. 2A, B, and C).

These findings suggest sustained ecological pressure from the antibiotics with no evidence of microbial recovery. This aligns with previous studies reporting community shifts under exposure to TC (0,1 - 20 mg/L) [62]; CPX (10,000 µg/L) [8]; SDZ (0–35 mg/L) [31]. Consistent with our tbRDA, other research also underscores the influence of nutrients [9], and microalgal growth [28] on microbial assemblages. Overall, our findings highlight that antibiotic contamination, environmental factors, and microalgal growth interact to shape microbial dynamics in SWW PBRs.

3.5. *Bacterial community composition shifts during exposure to TC, CPX, and SDZ*

Shifts in community structure reflected compositional changes (Fig. 3, Figure S3). All PBRs were dominated by the phyla *Bacteroidota* and *Proteobacteria* throughout the experiment (average relative

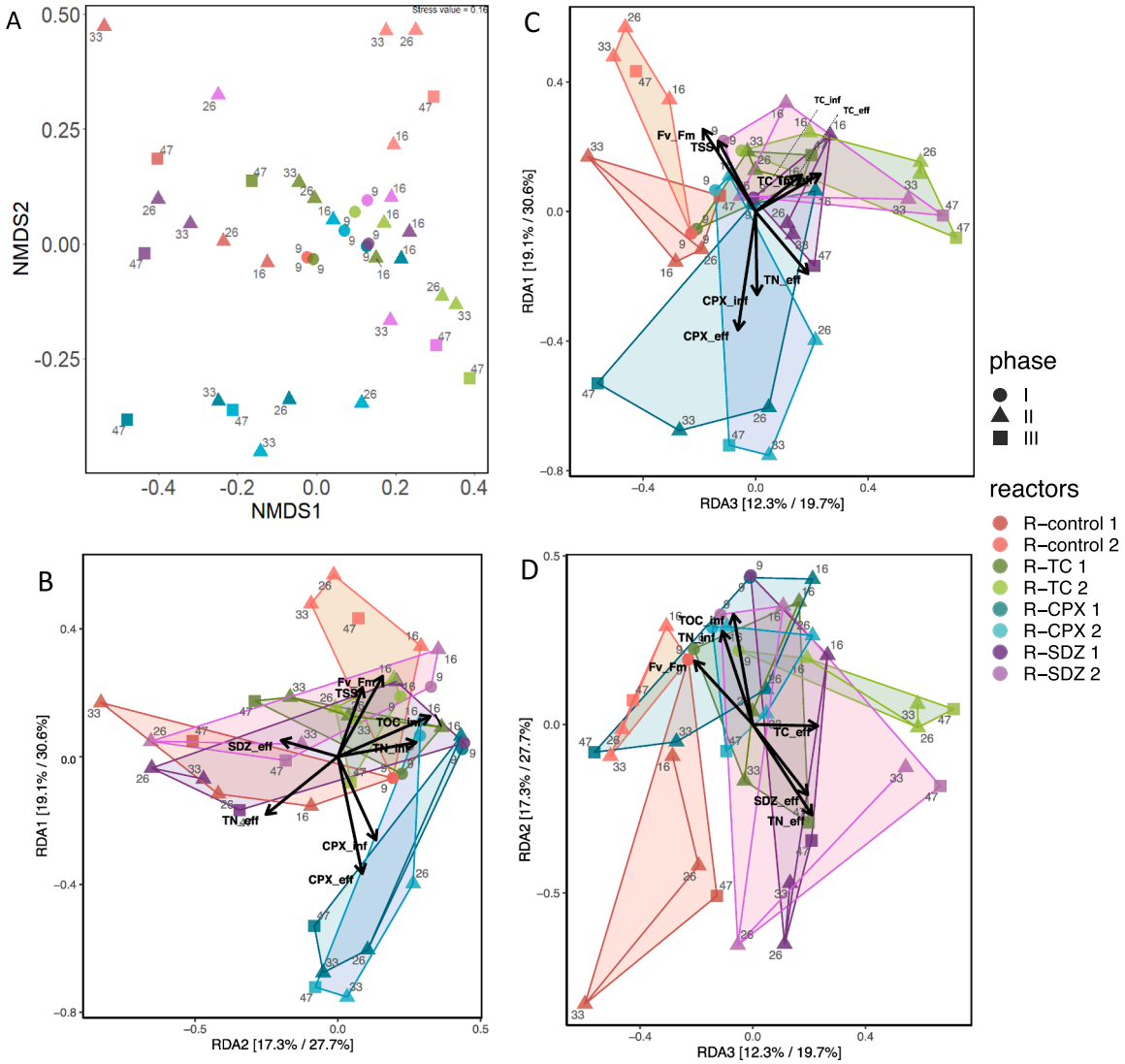

Fig. 2. Changes in bacterial community structure in duplicate PBRs during swine wastewater treatment across antibiotic exposure phases. Non-Metric Multidimensional Scaling Analysis (NMDS) based on the Bray–Curtis distance measure, with a stress value of 0.16 (A). Transformed-based redundancy analysis (B). Total and constrained variance explained by each axis are indicated in brackets. Samples from duplicated PBRs are indicated by the same colour in dark and light shades. Samples from the same reactor are linked with lines forming polygons. Numbers next to the samples indicate the day. Constraining operating variables are shown with black vectors. Longer arrows indicate stronger correlations with the variation in the community matrix. Arrows pointing in opposite directions show negative relationships; arrows in the same direction show positive relationships. Dotted lines indicate overlapping variable names.

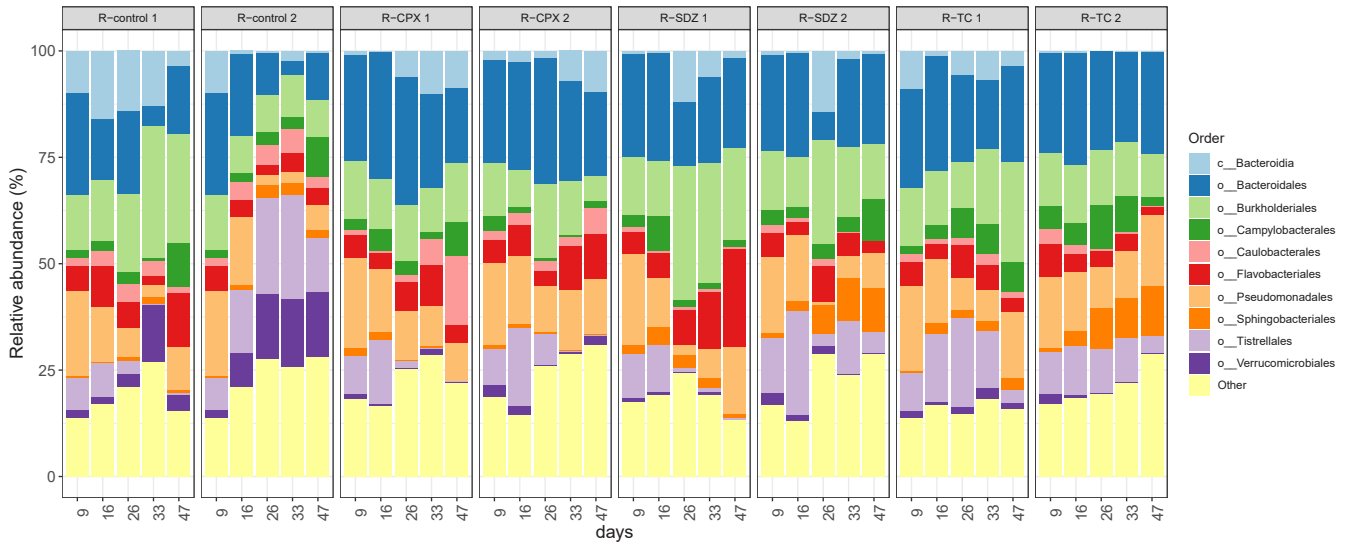

Fig. 3. Bacterial community composition of the 10 most abundant taxa in the reactors at order level. Averaged relative abundances for replicated reactors are shown except for R-controls at day 9, where sample from R-control2 was lost during the sequencing process. Phase I: day 9; phase II: days 16,23,33; phase III: day 47.

abundance: 38 ± 7 % and 43 ± 5 %, respectively, Figure S3), phyla prevalent in microalgae-bacteria PBRs [20,48]. However, compositional shifts were clear at the order level during phases II and III (Fig. 3). For example, the order *Verrucomicrobiales* decreased in all antibiotic-exposure PBRs (0.88 ± 0.98 %) compared to R-control (15 ± 2 %) at day 33. Conversely, Pseudomonadales increased in antibiotic-exposed PBRs (16 ± 1 % in R-TC, 11 ± 3 % in R-CPX, and 12 ± 5 % in R-SDZ) relative to R-control (8 ± 3 %) at day 47.

A SIMPER analysis identified 119 ASVs (25 genera) that were differentially distributed among PBRs and potentially contributed to lower reactor performance (Fig. 4, Table S5). Key bacteria taxa associated with nutrient removal and microbial aggregation decreased in abundance in the antibiotic-treated reactors. This group included heterotrophic, nitrifying, and denitrifying organisms. For example, *Verrucomicrobium* decreased under all antibiotic exposures (Fig. 3 and Fig. 4). This is linked to forming flocs with microalgae and its lower abundance relates to reduced microalgal growth, TOC removal, and adverse operational conditions (e.g., low irradiance, short HRT, nitrite

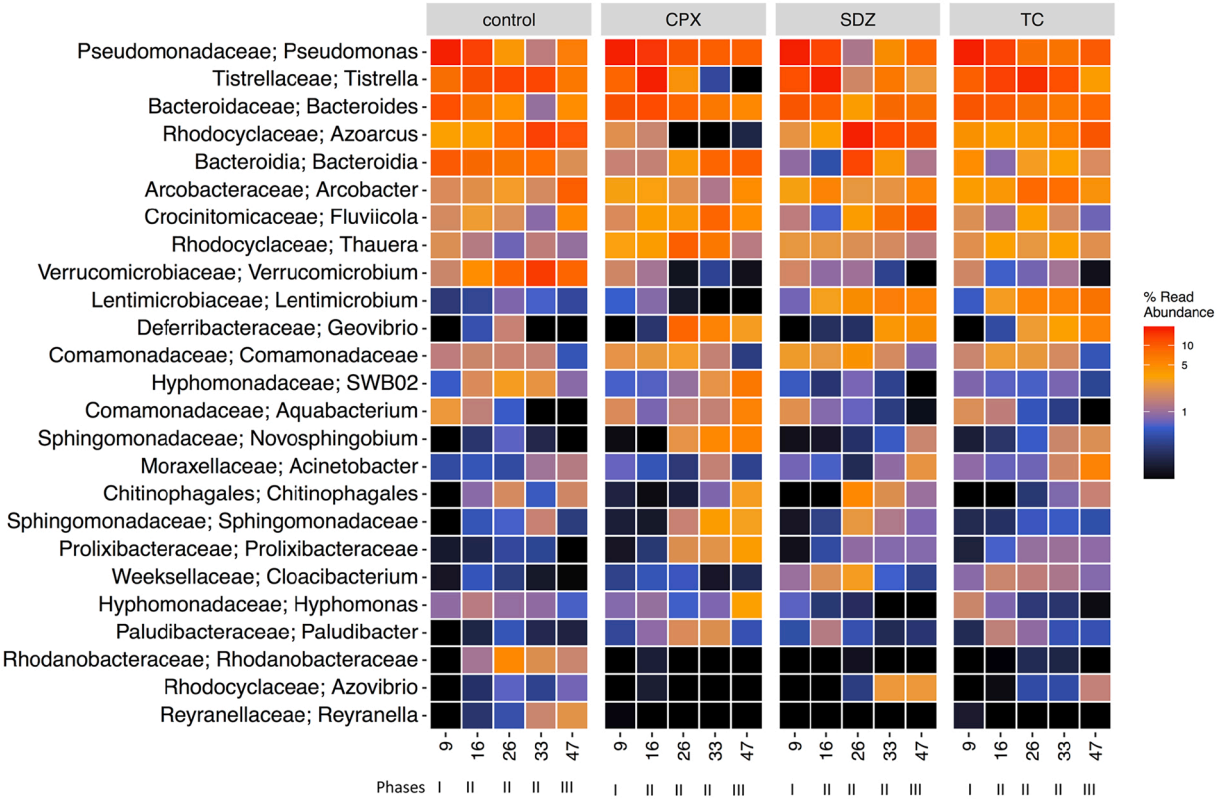

Fig. 4. SIMPER results. Heat map of bacteria differentially distributed among the PBRs, grouped at the genus level. Family is also indicated. Columns represent samples, labelled with the type of treatment and day of operation: day 9 (phase I), stage prior to antibiotic addition; days 16, 26 and 33 (phase II): TC, CPX and SDZ were added to the R-TC, R-CPX and R-SDZ PBRs, respectively and day 47 (phase III). Cell colour indicates mean relative abundance of duplicated PBRs.

accumulation) [3,13,20]. Likewise, *Hyphomonadaceae,* with denitrifiers and linked to aggregation [68], decreased in R-TC and R-SDZ reactors. *Tistrella* and *Azoarcus,* which are associated to lower TOC and TN removal [12], declined only in R-CPX PBRs. *Tistrella* sp. is also associated with the microalgal cell surface [22]. Consequently, the decrease in these taxa likely hindered microbial floc formation, compromising microalgae-bacteria consortia and overall PBRs performance.

Conversely, some bacteria increased in abundance (Fig. 4). For example, *Pesudomonas*, *Geovibrio* and *Thauera* significantly increased in all treated PBRs. *Pseudomonas* includes WHO critical priority antibiotic resistant species [4], but can also enhance *Chlorella* sp. co-culture performance [64]. *Thauera*, known for nutrient removal and pollutant degradation, has been reported in microalgae-bacteria PBRs treating SWW [21]. *Lentimicrobium* and *Acinetobacter* increased only in R-TC and R-SDZ. *Lentimicrobium* is associated with sulfonamide elimination [36]. *Acinetobacter,* performing heterotrophic nitrification / aerobic denitrification, is linked to higher microalgal biomass productivity [33]. These increases might be related to elevated TSS in R-TC (phase II) and the F_v/F_m ratio in R-SDZ (phase III) (Fig. 1).

Finally, several taxa, including *Novosphingobium*, *Aquabacterium* and *Sphingomonadaceae*, increased only in the R-CPX reactor. The genus *Novosphingobium* was reported as CPX-resistant [40]. Likewise, *Aquabacterium* exhibited resistance to CPX at low concentrations (100–400 μg/L) but is inhibited at higher concentrations (2–10 mg/L) [8]. *Sphingomonadaceae* was also resistant to CPX in pilot-scale constructed wetlands [35].

3.6. Implications of antibiotics effects on microbial consortia and treatment performance

This study employed microalgae-bacteria PBRs to study microbial responses to continuous overloading of three antibiotics commonly found in real SWW, and their implications for nutrient and antibiotic removal. This study also adopts a green engineering approach by utilizing naturally occurring microalgae-bacteria systems for wastewater treatment. This method intrinsically embodies several principles of Green Chemistry such as waste prevention, use of renewable biological processes, and design for energy efficiency, thereby minimizing chemical inputs, reducing environmental impact, and promoting sustainable resource recovery [1,17,18].

As hypothesized, elevated concentrations of TC, CPX, and SDZ exerted strong ecological pressure, affecting both microalgae and bacteria. Veterinary antibiotics inhibited microalgal growth and photosynthetic activity, and altered composition, structure and dynamics of bacterial community (Figs. 2 and 3). In addition, changes in each microbial group could have influenced their symbiotic interactions. Reduced oxygen production by microalgae might have limited aerobic degradation by bacteria, while decreased organic matter degradation by bacteria may have reduced the availability of simple compounds for microalgal assimilation. Alterations in bacterial populations may also have impacted the structure of mixed microbial aggregates. Unlike previous reports, our study captured microbial dynamics under both operationally and environmentally realistic conditions by employing the complex matrix of real SWW.

In this context, functional taxa reduced by all antibiotics (*Verrucomicrobium, Tistrella, Azoarcus*, among others) include heterotrophs, nitrifiers, and denitrifiers, which could be associate with microalgae consortia [3,12,20,22,68]. Their decline, together with reduced microalgae growth, likely explains the impaired carbon and nitrogen removal observed in antibiotic-exposed PBRs compared with the control reactor (Fig. 1). The results suggest that the observed community shifts mechanistically underlie the decline in nutrient removal efficiency. This is attributed to the combined loss of key functional taxa and a reduction in the assimilative uptake of carbon and nitrogen. Specifically, the inhibition of biomass growth limits the uptake of dissolved nitrogen and carbon, while the decrease of heterotrophs, nitrifiers and denitrifiers bacteria constrains biotransformation pathways. For instance, reduced NH_4^+-N removal in R-SDZ reactors might be related to declines in both microalgae and SDZ-sensitive nitrifying/denitrifying bacteria such as *Hyphomonadaceae* and *Acinetobacter* [31]. Moreover, the disruption of microalgae-bacterial consortia could reduce extracellular polymeric substances (EPS) production, which is essential for microbial aggregation and antibiotic sequestration [16]. Although EPS production was not measured, the loss of key taxa in the microbiome of doped PBRs could impair EPS-related functions, further reducing antibiotic retention by biomass (Table 1). This is critical, as the failure of these systems due to antibiotic pollution directly compromises their function as a "green" solution [1] for wastewater valorization.

Beyond the impact on treatment performance, the strong selective pressure exerted by TC, CPX, and SDZ carries significant implications for the proliferation of antibiotic resistance. Our findings provide a mechanistic view of how antibiotic contamination transforms the PBR's ecology into a potential "hotspot" for antibiotic resistance evolution. These systems mix fecal bacteria (from SWW) with environmental microbes under selective pressure, creating conditions conducive to Horizontal Gene Transfer [5,27]. Our study demonstrates this selective pressure in action, evidenced by the inhibition of *Chlorella* sp. and the loss of key functional taxa like *Verrucomicrobium*. This pressure drove an ecological replacement, where the open niche was filled by opportunistic taxa that increased in abundance, notably *Pseudomonas*, *Acinetobacter*, and *Thauera*. The implication of this replacement is profound. Genera like *Pseudomonas* and *Acinetobacter* are known reservoirs of mobile genetic elements, the vehicles for antibiotic resistance genes mobilization [5,27]. Therefore, while the doped PBRs failed at nutrient removal, their internal ecology shifted to favor bacteria with high potential to capture and transfer antibiotic resistance genes. This suggests PBRs under antibiotic stress become active "incubators" that enrich the effluent with antibiotic-resistant bacteria and, more critically, mobile antibiotic resistance genes, posing a downstream public health risk [5,27]. Beyond antibiotic pressure, bacterial communities in doped PBRs were also modulated by operational variables and the growth and physiological state of *Chlorella* sp. (Fig. 2). Microalgal growth enriches functional bacteria populations by creating a micro-oxygenic environment supporting interspecies interactions, enhancing nutrient uptake from wastewater through complex metabolic interactions improving effluent quality [29]. Therefore, reduced microalgal growth in doped PBRs was probably linked to the disruption of microbial consortia caused by antibiotics toxicity, compromising SWW effluent quality.

As the susceptible bacteria decreased, they were replaced by potentially antibiotic-resistant taxa (*Pseudomonas*, *Acinetobacter, Thauera*, *Geovibrio*, and *Sphingomonadaceae*) increased. By integrating these ecological shifts with the quantification of antibiotics in the biomass (Table 1), we observed a correlation between the proliferation of these specific taxa and low antibiotic biosorption. This supports the hypothesis that these indicator taxa utilize resistance mechanisms such as efflux pumps that reduce intracellular antibiotic accumulation [15]. Although these bacteria exhibit versatile metabolisms and form consortia with microalgae that contribute wastewater treatment [21,33,64], their presence could not match control reactor performance, probably due to loss of key microalgal or bacterial partners affected by antibiotic stress. This observation highlights the importance of microbial interactions for PBRs performance and underscores the need to incorporate microbiological analyses into reactor design to contribute sustainable and effective antibiotic removal from real SWW.

These findings raise new questions and highlight several key areas for future research. First, a deeper investigation into the specific mechanisms of antibiotic removal is needed, including the identification of antibiotic degradation pathways and any secondary transformation products. Second, advanced multi-omic approaches (e.g., metagenomics, metatranscriptomics, metabolomics) should be used. This would complement the 16S rRNA data by helping to identifying metabolically active microorganisms and key functional genes involved in

both contaminant removal and nutrient recovery, as well as characterizing metabolites and extracellular polymeric substances (EPS) that mediate microbial interactions and biosorption. These functional studies can then be validated using controlled co-cultures to isolate specific microalgae-bacteria interactions (e.g., mutualism, competition). Finally, the detrimental effects observed here with individual antibiotics must be validated in large-scale, outdoor PBRs. Future studies should also prioritize evaluating the "cocktail effect" of antibiotic mixtures, which reflects a more environmentally realistic contamination scenario.

4. Conclusions

The addition of the three antibiotics significantly reduced nutrient elimination (TOC and TN) and exhibited variable drug removal efficiencies**.** Significant removals were achieved for TC (76 %) and SDZ (55 %), mainly by degradation (96 % of the removal), with minimal biosorption (<6 % for TC). In contrast, CPX removal was negligible resulting in high effluent concentrations, which underscores its recalcitrance and difficulty of degradation in real SWW. All antibiotics irreversibly inhibited the photosynthetic activity and growth of the *Chlorella* sp. microalgae and significantly altered the structure and composition of bacterial communities, causing a sustained and irreversible ecological impact. Crucially, the microbial community structure did not recover after exposure, maintaining an altered configuration distinct from the control. Affected taxa linked to the proper functioning of PBRs were replaced by potentially antibiotic-resistant microorganisms, reducing carbon and nitrogen removal and compromising wastewater treatment performance. The selection of these taxa, known carriers of mobile genetic elements, implies a risk that these PBRs serve as hotspots for the horizontal gene transfer and dissemination of ARGs by mixing fecal and environmental microbiota under selective pressure. The failure of these systems due to antibiotic pollution directly compromises their function as a "green" solution for wastewater valorization and impedes their development as a technology for the Sustainable Development Goal (SDG) 6. The ability to quantify these selective pressures and identify indicator taxa provides crucial insights for managing resilient wastewater treatment systems. Protecting the microbial consortia from antibiotic impacts is therefore essential to maintain the viability of PBRs as a green technology capable of contributing to the circular bioeconomy. These results underscore the need for further study of microalgal-bacterial relationships in PBRs to improve large-scale treatment of wastewater with high pharmaceutical loads. This study also paves the way for subsequent work, which should focus on multi-omic functional analyses, and the challenges posed by antibiotic mixtures under realistic, large-scale conditions.

CRediT authorship contribution statement

Rebeca López-Serna: Writing – review & editing, Methodology. **Nuria Fernandez-Gonzalez:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Javiera Collao:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Silvia Bolado-Rodríguez:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Carlos Alonso:** Methodology, Investigation.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT to improve the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Data statement

Raw sequences were deposited in the Sequence Read Archive of the National Center of Biotechnology Information, BioProject: PRJNA1070361. Other data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.120825.

Data availability

Raw sequences were deposited in the Sequence Read Archive of the National Center of Biotechnology Information, BioProject: PRJNA1070361. Other data will be made available on request.

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Glossary

SWW: Swine wastewater
PBRs: Photobioreactors
TSS: Total suspended solid
TOC: Total organic carbon
TN: Total nitrogen
N-NH_4^+: Ammonia nitrogen
HRT: Hydraulic retention time
F_v/F_m: Ratio of variable to maximum fluorescence
tbRDA: Transformation-based redundancy analysis
NMDS: Non-metric multidimensional scaling
SIMPER: Similarity percentages