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Microalgae inoculation increases bacterial diversity and gene abundances related to nutrient removal while decreasing antibiotic resistant genes in municipal wastewater☆

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ABSTRACT

Monitoring the interactions between microalgae and microbiomes, particularly in the context of nutrient and antibiotic resistance genes (ARGs) removal from municipal wastewater treatment system, is highly recommended for the environment and public health. This study evaluated the synergistic potential of microalgae-bacteria consortia in enhancing nutrient removal and mitigating ARGs in a lab-scale microalgal municipal wastewater treatment system. Three treatments were evaluated: the two individual microalgal strains (*Tetradesmus obliquus* and *Chlorella sorokiniana*) and their mixtures (1:1). All treatments showed high removal efficiencies for phosphates (>90 %; $p < 0.001$), nitrates (>80 %, $p < 0.001$), ammonia (>90 %, $p < 0.0004$) and chemical oxygen demand (COD) (>65 %, $p < 0.008$), significantly improving water clarity and quality. Microbiome analyses revealed that microalgal inoculation altered bacterial community diversity, structure and function. Functional metagenomic profiling showed a significant enrichment ($p < 0.05$) of genes associated with nitrogen and phosphorus metabolism in microalgal treatment groups compared to the untreated control. Resistome analysis indicated that raw wastewater harbored a high abundance of ARGs, which were significantly reduced following microalgal treatment ($p < 0.0001$). Overall, our findings demonstrate a mutually beneficial interaction between microalgae and the wastewater microbiome, where microalgae-induced changes increase bacterial diversity and functional gene abundance involved in nutrient and ARG removal. These results underscore the potential of microalgae-microbiome-based systems as an effective and sustainable solution for the removal of emerging contaminants, including nutrients, ARGs and pathogens from municipal wastewater.

1. Introduction

Wastewater is an inevitable part of everyday life [1]. However, growing concerns about wastewater are being raised due to its role in harboring microbes that can contribute to waterborne illnesses and pose serious risks to public health [2]. Wastewater is often rich in nutrients, chemical contaminants, pathogenic microorganisms and antibiotic resistance genes (ARGs) [3]. The presence of these pollutants necessitates monitoring to prevent potential disease outbreaks and safeguard environmental and human health [4,5]. Conventional wastewater treatment and disinfection methods are often limited in effectively removing nutrients and ARGs. Therefore, there is an urgent need for the development and implementation of novel, high-throughput and sustainable treatment technologies that can address these emerging contaminants more effectively [6,7].

The application of microalgae in wastewater treatment has gained

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considerable attention as an environment friendly and cost-effective alternative to conventional treatment methods like activated sludge [8]. Microalgae-based treatment systems enhance dissolved oxygen levels, efficiently remove nutrients, degrade various pollutants, and support the generation of valuable microalgal biomass [9]. Moreover, microalgae cultivated in wastewater can serve as a sustainable source of alternative energy [10,11], particularly for biofuel production and other value-added products [12,13], thereby contributing to the development of a circular bioeconomy. In addition, microalgal-based systems are also recognized for their high CO_2 fixation potential and their efficiency in removing nutrients such as phosphorous and nitrogen [14,15].

Despite the growing interest, the interactions between microalgae and their associated microbiomes in wastewater environments remain poorly understood, due to the complexity of the microalgae–bacteria relationships. Nutritional reciprocity is believed to be the main mechanism driving the synergistic interactions between microalgae and bacteria in wastewater, with mutualistic associations enhancing nutrient removal and pollutant degradation [16,17]. These interactions involve signal transduction, nutrient exchange, and horizontal gene transfer [16,18]. During the nutrient exchange, microalgae release organic compounds that support bacterial growth [19], while bacteria provide essential vitamins, organic nutrients, cofactors, and phytohormones that facilitate microalgal growth and survival [20]. These reciprocal interactions influence the physiology, metabolism and ecological functions of both partners, ultimately shaping microbial community structure and diversity [21].

While many studies have demonstrated the effectiveness of microalgal-bacterial consortia in treating various types of wastewater [16,22], most have focused primarily on nutrient removal, with limited attention to microbial community compositions and functional shifts at the metagenomic levels. In parallel with nutrient pollution, the proliferation of pathogens and antibiotic ARGs in wastewater poses emerging threats [2,23]. Biological treatment systems may significantly alter microbial compositions and diversity as well as the ARG profiles, necessitating comprehensive microbiome analyses. In this study, alongside the evaluation of algal physiology and biomass production, the study focused on revealing the changes in microbial communities during raw wastewater treatment using microalgae. Microbiome analysis was conducted to examine structural and functional changes in wastewater microbial communities during microalgal cultivation. Further shotgun metagenomics was employed to explore potential cofactor-based symbioses between microalgae and bacteria, shedding light on the molecular mechanisms underlying their interactions.

2. Materials and methods

2.1. Wastewater

Raw municipal wastewater (RW) was collected from a wastewater treatment plant located in Durban (30°04′ S and 30°51′E), South Africa. A total of 20 L of wastewater were obtained using sterilized 25 L containers and immediately transported to the laboratory for analysis. Physicochemical parameters, including chemical oxygen demand (COD), and nutrient concentrations were measured at the time of e collection. The wastewater characteristics were as follows: pH 7.0, total COD 465.3 mg L^{-1}, ammonia 24.3 mg L^{-1}, nitrate 6.32 mg L^{-1}, nitrite 1.3 mg L^{-1}, and phosphate 2.54 mg L^{-1} (Table S1). The bacterial load was estimated to be 165×10^5 CFU mL^{-1}.

2.2. Microalgae strains and experimental setup

Two algal strains, *Tetradesmus obliquus* (TO) and *Chlorella sorokiniana* (CS), were isolated from a maturation pond located near the wastewater treatment plant (WWTP) and used for raw wastewater treatment. These strains were selected due to their robust growth performance and adaptability to diverse environmental and physiological stress conditions. The strains were purified through subsequent sub-culturing on BG11 medium using the streak plate method. After purification, the individual strains and their mixtures (50 % TO and 50 % CS) were inoculated into 1 L Erlenmeyer flasks to initiate cultivation. All purified cultures were maintained at 22 ± 2 °C under continuous aeration and illumination using Sylvania Gro-lux lamps (120 μmol photons m^{-2} s^{-1}) with a 16:8 h light/dark photoperiod. Cultures were agitated at 100 rpm using an orbital shaker to ensure uniform suspension and to prevent biomass settling.

The experiment comprised of seven groups; Group 1(NC): negative control (no algal inoculation; Group II(CS): *C. sorokiniana* treated RW; Group III (TO): *T. obliquus* treated RW; Group IV (TC): Mixed treatment (TO + CS, 50:50); Group-V (MTO): *T. obliquus* cultured in BG11 media; Group-VI (MCS) *C. sorokiniana obliquus* cultured in BG11 media); Group-VII (MTC): (mixed culture (TO+CS) in BG11 media). All treatment flasks were maintained under identical environmental conditions, with flask positions rotated daily to ensure uniform light exposure. Each treatment was set up in triplicate. Temporal replicates were taken as subsamples of the 21 flasks (7 treatments × 3 replicates × 3 temporal replicates = 63 samples).

2.3. Physiology of microalgae and biomass analysis

Prior to analysis, microalgal culture were incubated in dark conditions for 20–30 min. The maximum quantum yield of photosystem II (F_v/F_m), an indicator of photosynthesis efficiency, was determined using the formula described by Ramanna et al. [24]:

$$F_v/F_m = F_m - F_o/F_m$$

where *Fm* is the maximal fluorescence, *Fo* is the minimal fluorescence measure in dark-adapted state and *Fv* represents variable fluorescence.

Biomass concentration was measured spectrophotometrically at 680 nm using Hach DR6000 UV–Vis spectrophotometer. For dry cell weight (DCW) estimation, cultures were filtered through pre dried and pre weighed 25 mm Whatman filter papers. The filters were then dried overnight at 105 °C, transferred to a desiccator, cooled to room temperature and weighed. DCW was calculated and expressed as (g L^{-1}).

2.4. Biochemical composition analysis

The protein content of algal biomass, expressed as percentage of DCW, was determined using the method of Lowry et al. [25]. Absorbance was measured at 750 nm, using a UV–visible spectrophotometer (Spectroquant Pharo 300, Merck), with bovine serum albumin (BSA) used as the standard. Lipid content was assessed by extracting the algal biomass using a chloroform: methanol mixture (2:1 v/v) assisted by microwave-based cell disruption as described by [26]. Carbohydrate content was quantified using the phenol–sulfuric acid method [27], and the absorbance was measured at 490 nm. For additional procedural detailed related to protein and carbohydrate estimation, please refer to previous studies by [28].

2.5. DNA extraction and metagenomic sequencing

Samples were collected at three different time periods: day 0, day 7 and day 14. Genomic DNA was extracted from a total of 48 samples (n = 48), comprising 36 samples from the four treatment groups (4 treatments × 3 replicates × 3 time points) and 12 samples from the three media control groups (3 media groups × 2 replicates × 2 time points) using the DNeasy PowerSoil DNA Extraction Kit (Qiagen) following the manufacturer's instructions. Extracted DNA was quantified using a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA). Multiplexed and paired-end libraries (2 × 150 bp) were prepared by using the Illumina DNA Prep Kit (Illumina, San Diego, USA), and sequencing was performed on the Illumina NextSeq 1000/2000 platform (Illumina, San

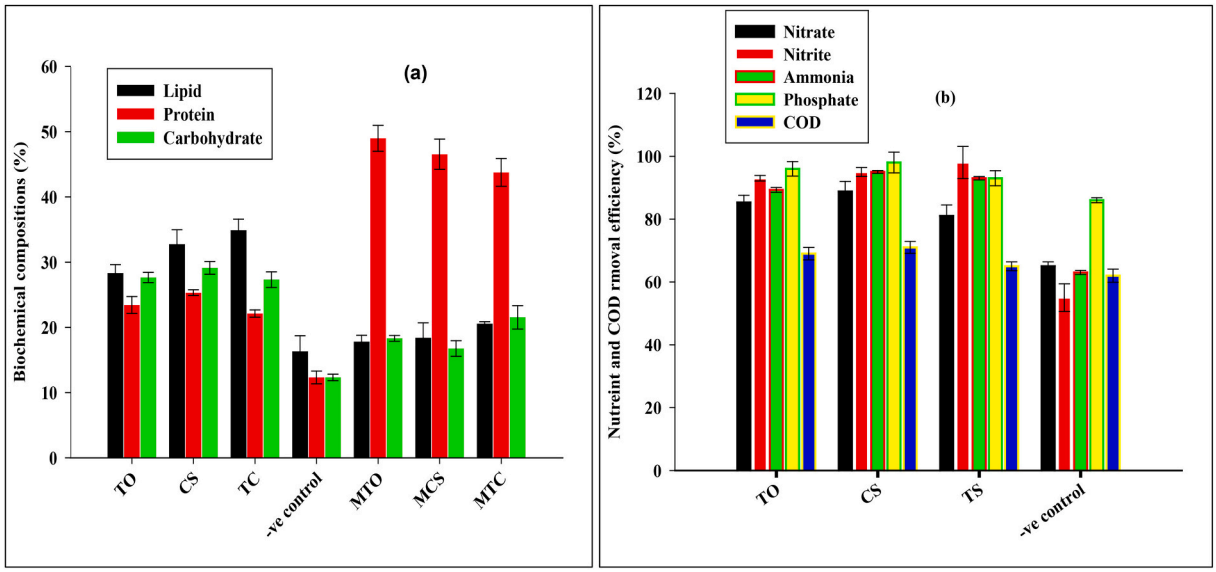


Fig. 1. Biochemical composition (a) and nutrient and COD removal efficiency (b) of microalgae cultivated in wastewater and BG11 media. Note: TO = *Tetradesmus obliquus* grown in wastewater, CS = *Chlorella sorokiniana* grown in wastewater, TO + CS = *Tetradesmus obliquus* + *Chlorella sorokiniana* grown in wastewater, RW = raw wastewater, BG11-TO = *Tetradesmus obliquus* grown in BG11, *Chlorella sorokiniana* grown BG11, BG11-TO + CS = *Tetradesmus obliquus* + *Chlorella sorokiniana* grown in BG11.

Diego, USA).

2.6. Sequencing data processing

R metagenomic reads were quality-filtered and trimmed using TrimGalore v0.6.2 to remove adapter sequences (l < 50 bp) and low-quality reads (Phred scores Q < 20). Taxonomic and functional annotation was performed using the highly automated metagenome analysis pipeline SqueezeMeta v1.6.2 in a coassembly mode was used for [29]. Contig assembly was performed with MEGAHIT v1.2.9 [30], and gene prediction was done by Prodigal (v2.6.3) [31] with default parameters. We used Diamond [32] for gene comparisons against different taxonomic (GenBank nr NCBI) and functional (eggNOG and KEGG) databases [33] [34]. c Antibiotic resistance genes (ARGs) were identified using DeepARG v1.0.2, trained on highly curated databases such as ARDB, CARD and UNIPORT [35]. The copy number of each gene was performed using ARG-OAPv3.2.4 [36]. Detailed methods of curation and analysis by ARG-OAPs can be found in [37,38].

2.7. Statistical analysis

All the statistical analyses were conducted using RStudio (version 4.4.1). Sequencing depth and species richness were calculated with the "*mp_cal_rarecurve* and *mp_plot_rarecurve*" functions within the "*MicrobiotaProcess*" R package [39] in combination with the visualization tools such as Patchwork, ggtree and ggtreeExtra. Alpha diversities were calculated using the diversity function and beta diversity was performed *vegdist* function within the "vegan" package [40]. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function in the vegan R package. PCoAs were built using the "*mp_plot_dist*" function from "*MicrobiotaProcess*" R package. Procrustes analysis was performed to test the association between functional genes abundance and the resistome using the function '*procrustes*' of *vegan* R package. FAPROTAX analysis was conducted to evaluate differences in functional bacterial taxa between the control and treatment groups [41]. All the plots and visualizations were generated with the "ggplot2" R package.

3. Results and discussion

3.1. Algal physiology and biomass production

The physiological performance of algal strains TO and CS, when cultivated in BG11-media and raw wastewater, was studied in terms of quantum efficiency (F_v/F_m). Initially, the value of F_v/F_m was lower than 0.5, indicating that the algae were under stress due to adaptation to new environmental conditions. Generally, a $F_v/F_m < 0.5$, signifies stress cause by various factors such as nutrient limitations, photoperiod shifts, light intensity, and temperature fluctuations. By 4 days of cultivation, F_v/F_m exceeded 0.5 across all treatments, suggesting physiological acclimation. In wastewater-grown algae, the maximum F_v/F_m values were recorded on day 9: WW-TO (0.61), WW-CS (0.63) and WW-TO+CS (0.62) (Fig. S1a). A similar trend was observed in BG11-medium grown cultures (Fig. S1a). The subsequent decrease in F_v/F_m values after day 11 may be attributed to stress conditions induced by nutrient depletion. The decreased F_v/F_m values indicate a drop in the quantum yield, indicating diminished conversion of absorbed light into chemical energy and an increase in dissipated fluorescence [42]. Overall, these findings suggest that both TO and CS were capable of utilizing nitrogen and other available nutrients in wastewater to support growth. Raw wastewater effectively supported algal growth and biomass production (Fig. S1b). Both individual algal strains and their combinations exhibited rapid growth without an apparent lag phase, demonstrating high adaptability to the wastewater environment. The robust growth and relatively high biomass suggest efficient nutrient utilization by the algae [43–45]. Compared to microalgal treatment groups, the control groups grown in BG11 medium produced significantly more biomass over the 14-day cultivation period ($p < 0.001$) (Fig. S1b). Among BG11-grown controls, the highest biomass was observed in mixed culture- TO+CS (1.74 g L^{-1}), followed by TO (1.68 g L^{-1}) and CS (1.55 g L^{-1}). However, these differences were statistically insignificant ($p > 0.05$, for all pairwise comparisons) (Fig. S1b). In microalgae treatment groups, the mixed culture (TO+CS) yielded significantly higher biomass (1.36 g L^{-1}) than the control (0.26 g L^{-1}) ($p < 0.001$) and CS alone (0.74 g L^{-1}) ($p < 0.013$) and TO (1.16 g L^{-1}) ($p > 0.05$) (Fig. S1b). Although the biomass yield in the mixed culture was higher than that in the TO (1.16 g L^{-1}), the differences were not statistically significant ($p > 0.05$) (Fig. S1b).

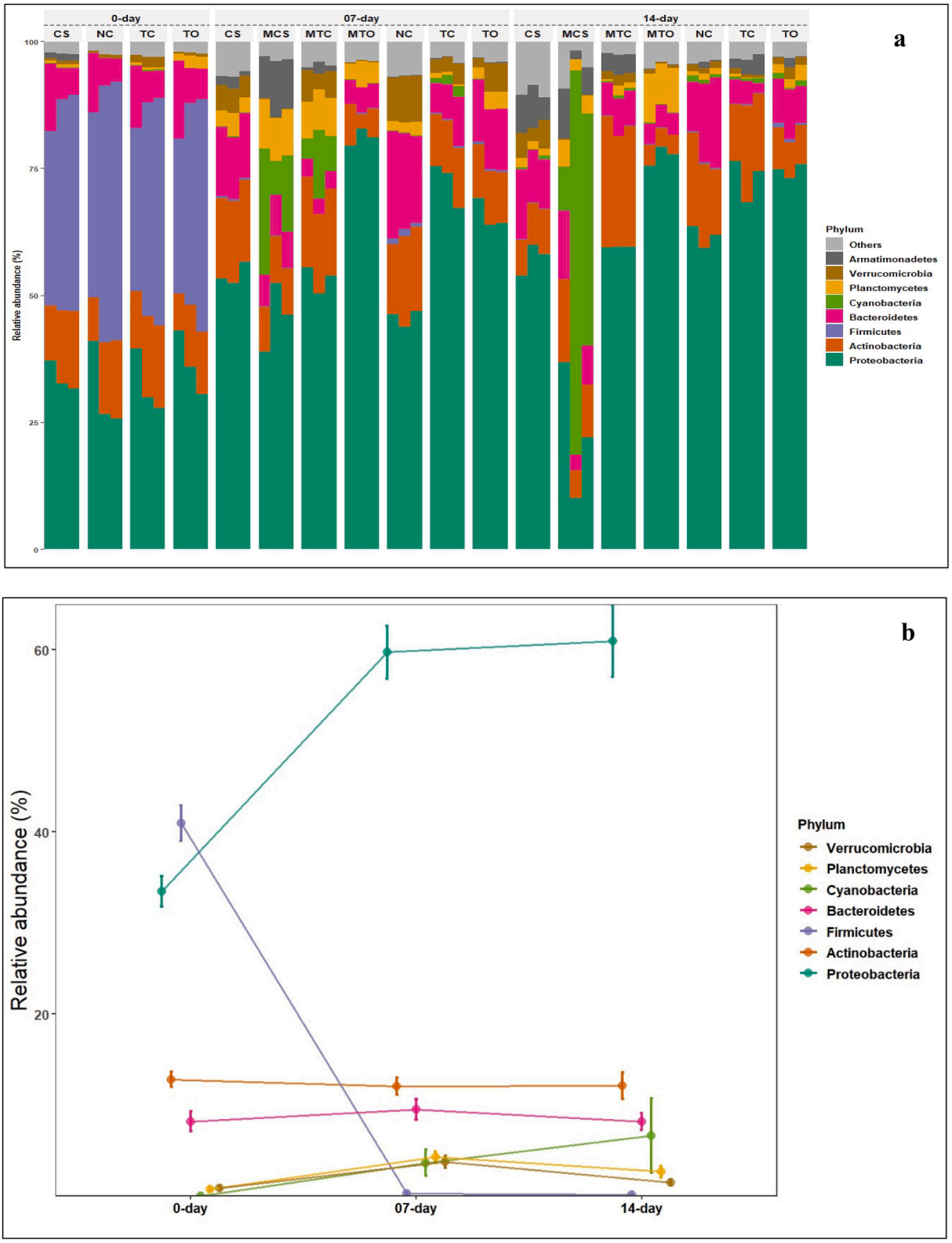


Fig. 2. Metagenome generated relative abundance of samples from different microalgal treatments. (a) Phylum-level relative abundance of samples across different treatment groups and time-periods (days) (NC = control; CS = *Chlorella sorokiniana*; To = *Tetradesmus obliquus*; TC = *Tetradesmus obliquus* + *Chlorella sorokiniana*; MCS = media control for *Chlorella sorokiniana*; MTO = media control for *Tetradesmus obliquus* and MTC = media control for *Tetradesmus obliquus* + *Chlorella sorokiniana*), (b) relative abundance of different bacterial phyla in different microalgal treatments at different time periods (0 days–14 days of cultivation).

Furthermore, biomass production in the TO treatment was significantly higher than in the control ($p < 0.001$) but not significantly different from CS treatment ($p > 0.05$). A significant difference in biomass production was observed between CS and the control ($p < 0.004$). Overall, these findings suggest that the mixed microalgae treatment (TO+CS) achieved greater biomass accumulation than single-strain treatments. This may be attributed to the hormesis effect, in which soluble algal products (SAPs) released by one strain stimulate and promote the growth of another [46,47]. Similar observations were reported by Wang et al. [48], where the addition of interspecific SAPs increased the growth of *Hematococcus pluvialis* M1, by 1.76-foldcompared to the control. Taken together, these results highlight transgressive overyielding in algal mixtures [49] and confirm the benefits of algal diversity [50]. Culture conditions for microalgae cultivated in different growth media are summarized in Table S2. The temperature fluctuations were minimal (23.2–27 °C), consistent with the optimal growth conditions for microalgae [51].

The pH increased from 7.11 to 10.43 for BG11 grown cultures and from 7.1 to 10.08 in wastewater cultures (Table S2), slightly exceeding the optimal pH range of 7–9.5 for algal growth [52]. Dissolved oxygen (DO) ranged from 4.56 to 10.1 mg L^{-1} in BG11 and from 4.56 to 9.43 mg L^{-1} in wastewater culture, indicating active photosynthesis and suitability of the growth media for algal cultivation [53]. Electrical conductivity (EC) varied from 1215 to 816 μS cm^{-1} in BG11 media and 595–928 μS cm^{-1} in wastewater-grown algal cultures (Table S2).

3.2. Biochemical composition

The biochemical profiles of algae grown in BG11 media and wastewater is shown in Fig. 1. Protein content was significantly higher in BG11-grown cultures: MTO (49 %), MCS (46.54 %), and MTC (43.8 %) compared to wastewater grown algal strains; TO (23.4 %), CS (25.32 %), and TC (22.12 %) ($p < 0.0001$) (Fig. 1a). While all treatment groups had higher protein content than the control ($p < 0.0001$), differences among treatment groups were not significant ($p > 0.05$, for all pairwise comparisons). Similar results were reported by Tao et al. [54] for *S. acuminatus* cultivated in paper and pulp wastewater, with lower protein content attributed to nitrogen limitations. Ansari et al. [55] also reported reduced protein contents in *T. obliquus* grown in wastewater. In contrast, lipid content was significantly higher in wastewater-grown microalgae (35 %), CS (32.76 %) and TO (28.32 %) compared to BG11-grown strains MTC (20.45 %), MCS (18.4 %), and MTO (17.8 %) ($p < 0.0003$) (Fig. 1a). These findings align with the reports by Benítez et al. [56] and Apande et al. [57], who observed similar or relatively lower lipid yields in wastewater grown *Chlorella* and *Scendesmus* sp., respectively. However, Mishra et al. [58] reported significantly higher lipid content (50 %) in *Chlorella* sp. (ISTLA) cultivated in domestic wastewater. Similarly, carbohydrate content was significantly higher in wastewater-grown cultures: CS (29.12 %), TO (27.65 %), and TC (27.32 %) compared to MTO (18.32 %), MCS (16.75 %) and MTC (21.54 %) ($p < 0.0001$) (Fig. S2a). No significant difference in carbohydrate yield were observed among wastewater treatment groups ($p > 0.05$). These results support the well-established understanding that nutrient stress (as in wastewater) enhance the accumulation of energy-rich compounds like lipids and carbohydrates as a survival mechanism and stress response [55,59]. Similar results were reported by Satheesh et al. [60], in *S. obliquus*, *C. pyrenoidosa* and *C. sorokiniana*, cultivated in domestic wastewater.

Nitrate, ammonia and phosphate are critical nutrients for microalgal growth and the removal of these nutrients along with COD serves as an important water quality indicator. Nutrient removal performance (nitrate, nitrite, ammonia, phosphate) and COD reduction are presented in Fig. S2b. Wastewater-grown algae groups significantly outperformed the controls in nutrient removal nitrates (>80 %, $p < 0.001$), ammonia (>90 %, $p < 0.0004$), phosphate (>90 %, $p < 0.0001$), and COD (>65 %, $p < 0.008$). High removal efficiency can be attributed to microalgal nutrient uptake [2,61]. Comparable removal rates were also reported by Mkpuma et al. [62], using *Chlorella* and *Scenedesmus* to treat anaerobically digested food effluents and by Marchão et al. [63] with *S. obliquus* on secondary brewery effluents. Overall, the microalgae used in this study effectively removed high amounts of nutrients from undiluted raw wastewater. This high nutrient removal efficiency observed in this study may also be attributed to ammonia (NH_3) volatilization and phosphorous (P) precipitation [64]. Moreover, the presence of nitrifying and denitrifying bacteria in wastewater may have further contributed to enhanced nutrient removal [65-69]. The possible role of these microbial communities in enhancing nutrient removal alongside algal activity has prompted us to conduct microbiome analysis to better understand their synergistic interactions in wastewater treatment.

3.3. Changes in bacterial communities in response to different treatments over time

Previous research on microalgae cultivation in raw wastewater has primarily focused on the interactions between microalgae and the chemical constituents of wastewater. However, real-world wastewater treatment systems are more complex, involving diverse microbial communities that interact with microalgae. Therefore, microbiome analysis was performed to better understand the influence of the native microbiome on algal growth and performance during wastewater treatment. Thus, during the raw wastewater treatment process, changes in microbial community structure and functions were investigated by employing shotgun metagenomics, which produced a total of 636,605,825 high-quality read pairs (Table S3). To explore the synergistic interactions within algal microbiomes, changes in microbial community composition and structure across different treatment groups and time periods were investigated. Metagenomic analysis of a highly diverse microbiome, comprising 46 phyla, 334 classes, 1340 families, 4106 genera and 11,996 species. The metagenomes were predominantly composed of (84.8 %), followed by eukaryotes (14.7 %), viruses (0.27 %) and Archaea (0.12 %). The dominance of bacteria in the algal microbiomes is likely due to their co-occurrence with microalgae and their widespread distribution across different environments [70,71].

Taxonomically, Proteobacteria (54.35 %) was the most abundant phylum, significantly dominating the gene clusters across all samples, followed by Actinobacteria (12.28 %), Firmicutes (9.30 %), Bacteroidetes (8.73 %), Cyanobacteria (4.03 %), Planctomycetes (2.90 %), Verrucomicrobia (2.27 %), and Armatimonadetes (1.93 %) ($p < 0.05$) (Fig. 2a). The results revealed that most of the sequences belonged to Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes, accounting for 85 % of the total abundance at the phylum level, whereas the remaining sequences were distributed mainly in Cyanobacteria, Planctomycetes, Verrucomicrobia, Armatimonadetes, Deinococcota, Chlamydiae, Candidatus, Gemmatimonadetes and Euryarchaeota (Fig. 2a). Significant differences in relative abundance were observed between the treatment and control groups at the phylum level (Table S4).

The 25 most abundant bacterial genera included *Acinetobacter* (11.8 %), *Bosea* (6.6 %), *Sphingopyxis* (6.15 %), *Bifidobacterium* (5.6 %), *Hyphomonas* (4.4 %), *Microbacterium* (4.4 %), *Faecalibacterium* (4.4 %), *Arenimonas* (4.26 %), *Collinsella* (4.2 %), *Brevundimonas* (4.2 %), *Algoriphagus* (4.0 %), *Pseudomonas* (3.9 %), *Acidovorax* (3.6 %), *Escherichia* (3.6 %), *Roseomonas* (3.5 %), *Pseudonocardia* (3.2 %), *Streptococcus* (3.1 %), *Novosphingobium* (2.6 %), *Leucobacter* (2.5 %), *Aliarcobacter* (2.5 %), *Actinomycetia bacterium* (2.4 %), *Cloacibacterium* (2.2 %), *Cytophagaceae bacterium* (2.2 %), *Thauera* (2.1 %), and *Sphingobacteriaceae bacterium* (2.1 %) (Fig. S2). Algae–microbe interactions can generally be categorized as commensalism, mutualism and parasitism [72]. In this study, several algae growth-promoting bacteria were identified, including *Balneola*, *Roseovarius*, *Ulvibacter*, *Aestuariicoccus*, *Defluviimonas*, *Mesorhizobium loti*, *Flavobacteriaceae*, *Saprospiraceae* and *Rhodobacteraceae*. These genera have previously been shown to support algal growth and cell division by providing vitamin B_{12} and morphogenic compounds [73,74]. Additionally, their association with microalgae enhances

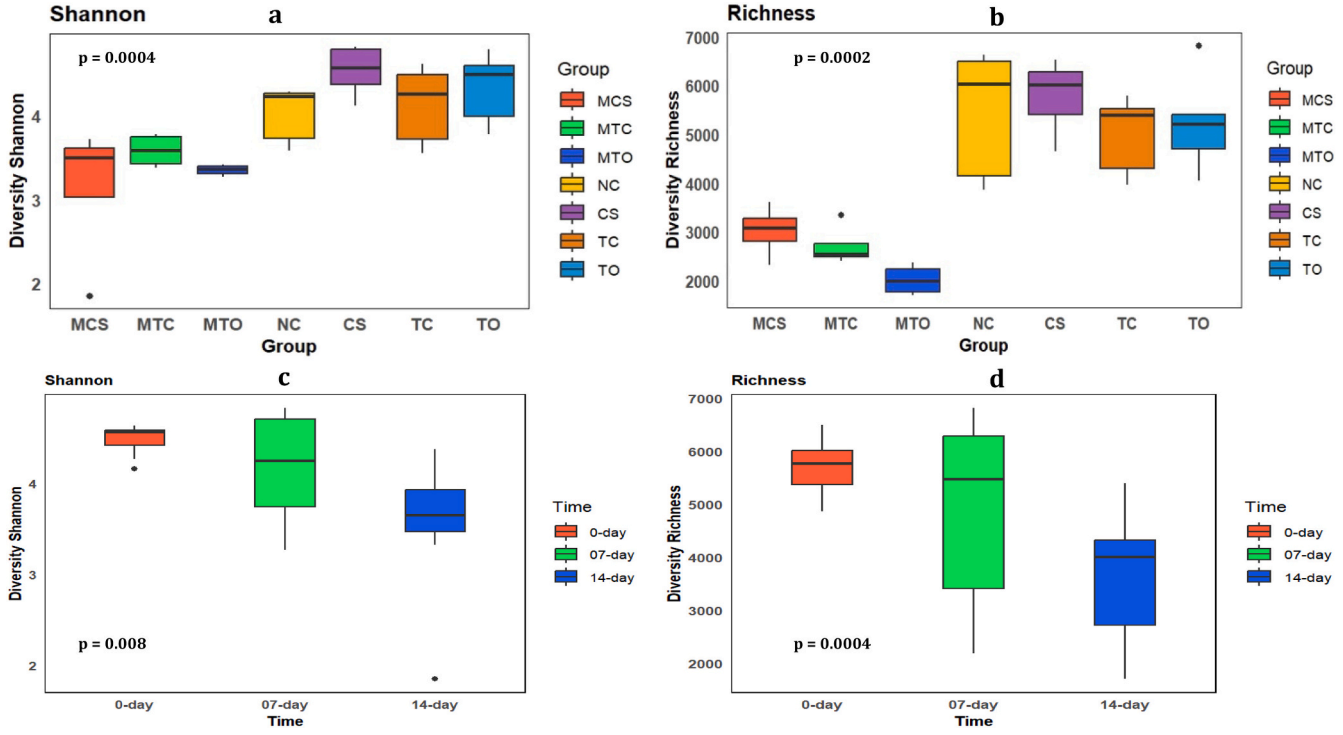


Fig. 3. Alpha diversity analysis of bacterial communities in different treatments and time periods (days), a and c) Shannon index, b and d) species richness at significance levels of $p < 0.0001$.

denitrification, further benefiting algal development [75].

3.4. Temporal dynamics of algal microbiomes

Metagenomic analysis was performed to assess both alpha and beta diversity indices in control and treatment groups. Rarefaction curve analysis was used to assess sequencing depth at various taxonomic levels (Fig. S3). The curves approached a plateau, indicating that sequencing depth was sufficient to capture the microbial diversity comprehensively. Additionally, Good's coverage values were consistently above 0.999 ± 0.0004 (mean ± SD), further confirming the adequacy of sequencing depth for reliable microbiome analysis. To examine the microbial dynamics over time, alpha diversity indices -including Shannon index and microbial richness were calculated across treatments and time points (days). Additionally, these indices were also calculated for media control groups over different periods. Results showed that microalgae inoculation significantly increased the alpha diversity, with the Shannon index (Kruskal–Wallis test; $p = 0.0004$) and microbial richness (Kruskal–Wallis test; $p = 0.0004$) being significantly higher in treatment groups compared to controls (Fig. 3a and b). These findings suggest that microalgal treatment significantly ($p < 0.05$) promotes microbial diversity, potentially by competing with bacteria for carbon and nutrients, thereby shaping a more diverse microbial community. These findings are in agreement with previous studies on the effects of microalgae in wastewater systems [2,76]. In contrast, both diversity indices significantly decreased over time, with reductions in Shannon index (Kruskal–Wallis test; $p = 0.008$) and microbial richness (Kruskal–Wallis test; $p = 0.0004$) (Fig. 3c and d). Similar trends have been reported in studies investigating the impact of wastewater on microbial diversity and composition [77-80]. Beta diversity was assessed using the Bray–Curtis dissimilarity index and visualized through principal component analysis (PCA). The PCA plots revealed clear clustering patterns based on treatment and time periods, with PC1, PC2 and PC3 explaining 41.41 %, 16.45 % and 12.18 % of the variance, respectively (Fig. 4a and b). PERMANOVA analysis confirmed significant differences in microbial community composition between treatments ($R^2 = 0.50$, pseudo-F = 7.98, $p < 0.001$) and time periods ($R^2 = 0.47$, pseudo-F = 22.85, $p < 0.001$). These findings were further supported by ANOSIM results which showed strong dissimilarities in microbial communities between treatment groups ($R = 0.5739$, $p < 0.001$) and time periods ($R = 0.6422$, $p < 0.001$). Overall, our results demonstrate that microalgal treatment significantly enhances alpha diversity, and alters community structure. Distinct clustering observed in PCA plots between the control and treatment groups further highlights the evolving nature of microbial communities during algal-based wastewater treatment. These observations align with previous reports of microbiome shifts in microalgae-based wastewater treatments [2,76].

3.5. Core and zeta (ζ) diversity analysis

A Venn diagram was used to identify and visualize the core and shared bacterial taxa across treatments and time periods. The analysis showed that 2146 taxa were consistently present across all the treatments, while 6872 taxa constituted the core microbiome across all time points (Fig. S4a and b). The numbers of unique taxa identified in each treatment group were NC (154), MTC (11), MCS (68), MTO (8), TC (62), TO (32), and CS (156) (Fig. S4a). Similarly, the numbers of unique taxa associated with specific time points-s 0, 7 - and 14 days- were 1199, 444 and 196, respectively (Fig. S4b). These findings suggest that a substantial proportion of microbial taxa remain stable across different treatments, forming a resilient core microbiome over time [81–85]. Similarly, the presence of unique and shared taxa across incubation periods indicates dynamic microbial turnover, reflecting temporal shifts in community structure [86,87].

3.6. Functional gene annotation in microbial metagenomes

FAPROTAX-based analysis was conducted to assess the functional potential of microbial communities across different treatments. Microbial functions were primarily linked to biogeochemical and pollutant degradation. Significant variations ($p < 0.05$) were observed in the functions related to energy source, nitrogen, carbon and cycling

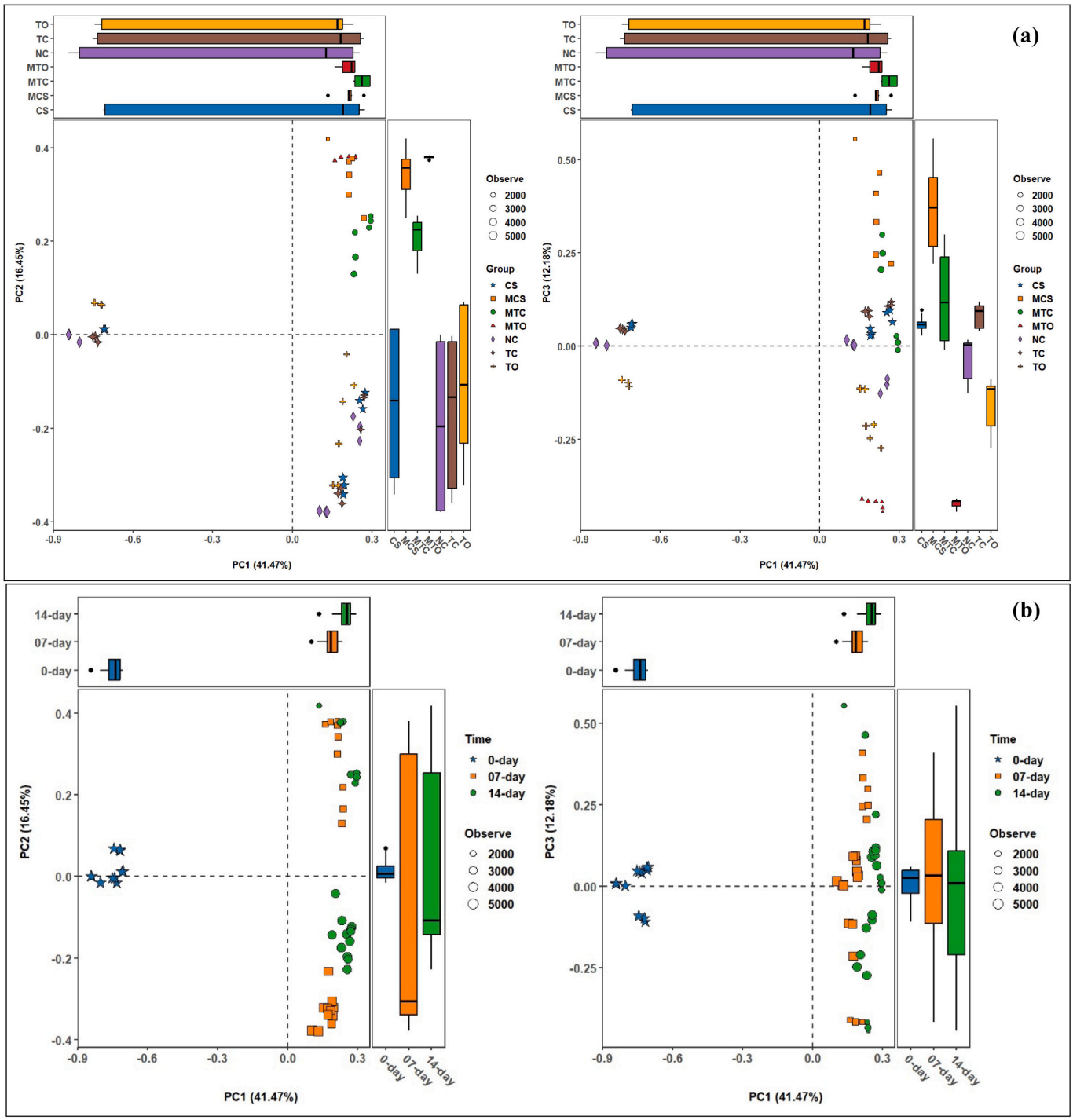


Fig. 4. Principal coordinate analysis plot of beta diversity using Bray Curtis dissimilarity matrix with the proportion of variations given in the parentheses: a) variations across different microalgal treatments according to PERMANOVA ($R = 0.50$, pseudo-F = 7.98, $p = 0.001$) and ANOSIM ($R = 0.57$, p = 0.001) and b) across different time-periods (days) PERMANOVA ($R = 0.47$, pseudo-F = 22.85, p = 0.001) and ANOSIM ($R = 0.6422$, p = 0.001).

(Table S6). Compared to the control group, treatment groups exhibited significantly greater abundance of genes involved in aerobic-ammonia-oxidation (Kruskal–Wallis test; $p = 0.0042$), aerobic-chemoheterotrophy (Kruskal–Wallis test; $p = 0.04$), cellulolysis (Kruskal–Wallis test; $p < 0.0001$), hydrogen oxidation (Kruskal–Wallis test; $p < 0.0001$), denitrification (Kruskal–Wallis test; $p = 0.0352$), hydrocarbon-degradation (Kruskal–Wallis test; $p = 0.0252$), and methanol-oxidation (Kruskal–Wallis test; $p = 0.0352$) (Table S5). In contrast, the control groups had significantly higher abundances of genes involved in anaerobic-chemoheterotrophy (Kruskal–Wallis test; $p = 0.0003$), aromatic-compound-degradation (Kruskal–Wallis test; $p < 0.0001$), chemo-heterotrophy (Kruskal–Wallis test; $p = 0.0352$), fermentation (Kruskal–Wallis test; p = 0.0352), fumarate-respiration (Kruskal–Wallis test; $p < 0.0001$), nitrate-reduction (Kruskal–Wallis test; $p < 0.0001$), nitrate-respiration (Kruskal–Wallis test; $p = 0.0006$), and nitrite-ammonification (Kruskal–Wallis test; $p < 0.0001$) (Table S5). Further annotation using the KEGG and COG databases revealed functional differences across treatments over time (Fig. S5a, b). Results revealed significant variations ($p < 0.05$) in relative abundance of functional genes and associated pathways across treatments over time (Fig. S5c, d).

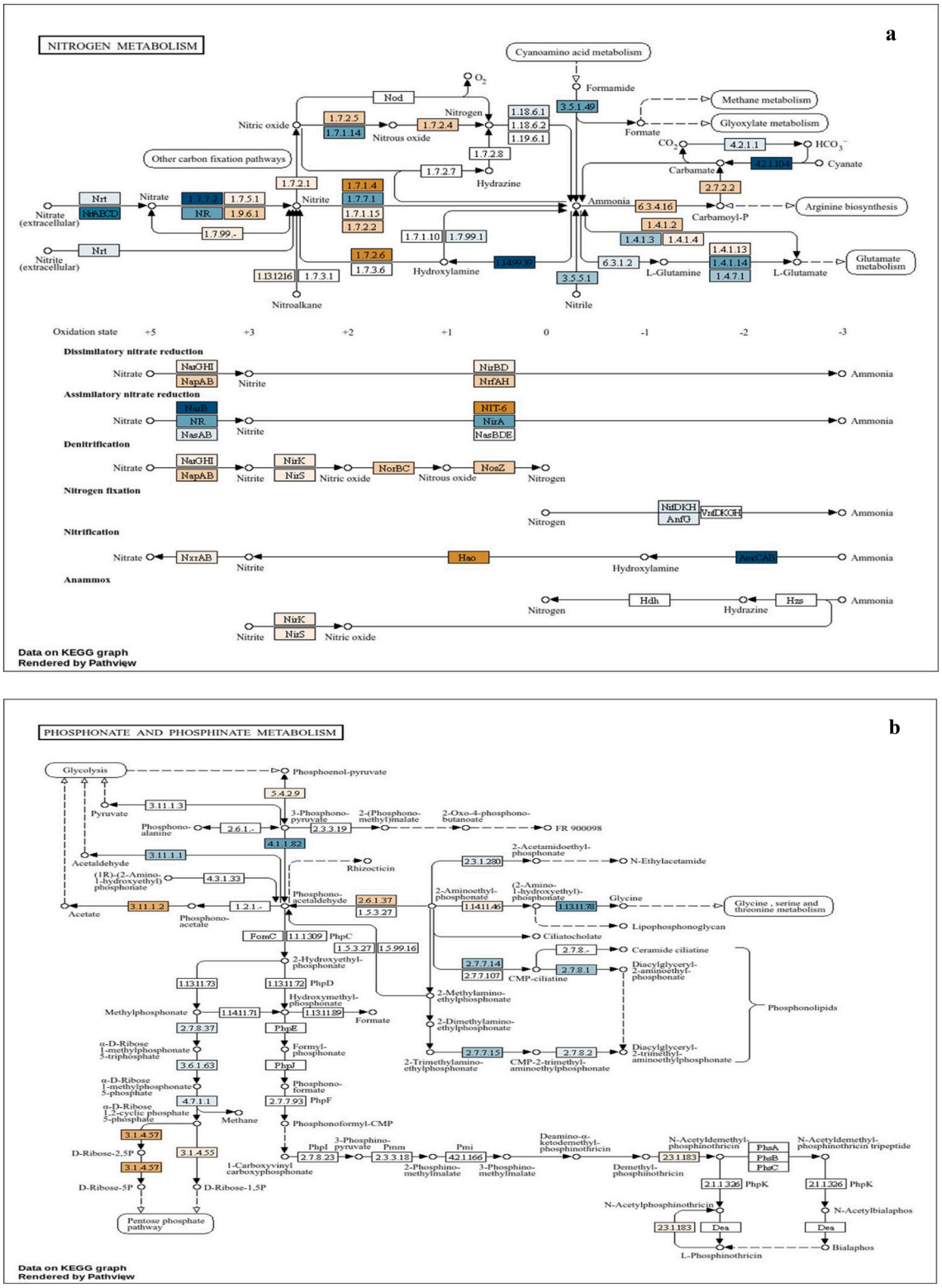


Fig. 5. The metabolic pathways and functional genes related to (a) nitrogen removal and (b) phosphorous removal. Changes are provided as log2 fold. The brown/tan/orange colour is upregulated in treatment while blue/green is upregulated in controls and (c) the genes involved in the biosynthesis and transport of vitamins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The major pathways identified were related to the transport and metabolism of amino acids, carbohydrates, nucleotides, coenzymes and ions; nucleotide and lipid metabolism; signal transduction; transcription; biosynthesis; the transport of secondary metabolites; defense mechanisms; and energy production and conversion (Fig. S5a). KEGG pathways enriched in treatment groups included; amino acid transport and metabolism (Kruskal–Wallis test; $p = 0.0001$), energy production and conversion (Kruskal–Wallis test; $p = 0.0004$), ion transport and

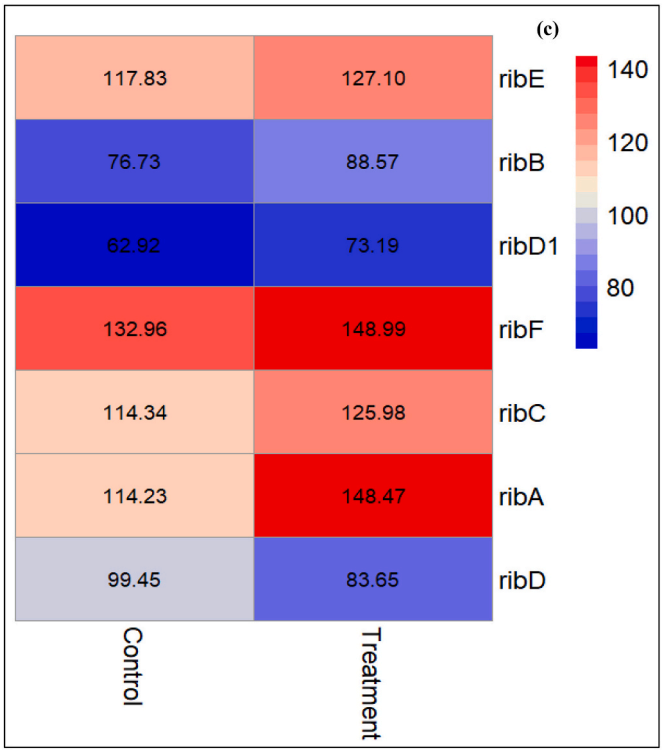


Fig. 5. (*continued*).

metabolism (Kruskal–Wallis test; $p = 0.0002$), lipid metabolism (Kruskal–Wallis test; $p = 0.0009$), coenzyme metabolism (Kruskal–Wallis test; $p = 0.09$), secondary metabolite biosynthesis (Kruskal–Wallis test; $p = 0.009$), and cell mortality and secretion (Kruskal–Wallis test; $p = 0.0003$) (Table S6). In contrast, the control groups were enriched in general function prediction (Kruskal–Wallis test; $p = 0.8661$), DNA replication, recombination and repair (Kruskal–Wallis test; $p = <0.0001$), translation and biogenesis (Kruskal–Wallis test; $p = 0.0361$), and carbohydrate transport and metabolism (Kruskal–Wallis test; $p = 0.0002$) (Table S6). These results align with previous studies showing the importance of vitamins and cofactors such as cobalamin, biotin and thiamine in supporting algal-bacterial synergy [22,88]. Such synergy in wastewater enhances the abundance of nutrient-cycling genes, aiding efficient nutrient removal [19,89,90]. Both nitrogen and phosphorous removal from wastewater are crucial for protecting water resources [91,92]. KEGG pathway analysis of nitrogen metabolism (map00910) and phosphorous metabolism (map00440) revealed significant differences in the relative abundance of key functional genes between the control and treatment groups. As shown in Fig. 5a, several enzymes-such as ferredoxin-nitrite reductase (EC 1.7.7.1 & EC 1.7.7.2), nitric oxide reductase (EC 1.7.1.14), formamidase (EC 3.5.1.49), glutamate dehydrogenase (EC 1.4.1.3), nitrilase (EC 3.5.5.1), methane/ammonia monooxygenase (EC 1.14.18.3.) glutamate synthase (EC 1.4.1.14 & EC 1.4.7.1), and cyanate lyase (EC 4.2.1.104), were significantly upregulated in the control samples. In contrast, key nitrogen removal enzymes such as nitrite reductase (EC 1.7.2.1; EC 1.7.6.1; EC 1.7.1.4; EC 1.7.15 & EC 1.7.2.2), nitrate reductase/nitrite oxidoreductase (EC 1.7.51 & EC 1.7.99), nitric oxide reductase (EC 1.7.2.5), hydroxylamine dehydrogenase (EC 1.7.2.6 & EC 1.7.99.1), nitrous oxide reductase (EC 1.7.2.4), nitrogenase (EC 1.18.6.1), glutamine synthetase (EC 6.3.1.2; EC 1.4.1.13 & EC 1.4.1.4), glutamate dehydrogenase (EC 1.4.1.2), carbamoyl phosphate synthase (EC 6.3.4.16), carbamate kinase (EC 2.7.2.2), and carbonic anhydrase (EC 4.2.1.1) were significantly upregulated in the treatment groups.

To better understand the synergistic interactions between microalgae and associated bacteria in raw wastewater treatment, multiple functional genes were analyzed. In general, the nitrogen removal pathways in wastewater mainly include dissimilatory nitrate reduction (DNRA) and assimilatory nitrate reduction, anammox, nitrification and denitrification [93-96]. During nitrate dissimilation, nitrate is first reduced to nitrite via *NarGHI* and *NapAB* genes [97,98], and subsequently to ammonia by *NapAB* and *NrfAH* [97–99]. For nitrate assimilation, genes such as *NarB*, *Nr*, *NasAB*, *NIT-6*, *NirA*, and *NasBDE* are involved in reducing nitrate to nitrite and ammonia [94,100]. Key genes involved in identification include *NarGHI*, *NapAB*, *NirK*, *NirS*, *NorBC* and *NosZ*, which encode enzymes for the stepwise reduction of nitrate to nitrogen gas [101-103]. Nitrogen fixation-related genes include *NifDKH*, *AnfG* and *VnfDKGH* [104,105]. Similarly, the genes involved in nitrification include *NxrAB*, *Hao*, and *AmoCAB* [106-109], and annamox genes include *NirK*, *NirS*, *Hzs* and *Hdh* [110-112]. Similarly, in microalgal–bacterial systems, phosphorous removal occurs through assimilation and accumulation pathways [113]. As illustrated in Fig. 5b, enzymes such as phosphonatase (EC 3.11.1.1), phosphonopyruvate decarboxylase (EC 4.1.1.82), phosphonate *N*-acetyltransferase (EC 2.3.1.280), hydroxyethylphosphonate dioxygenase (EC 1.13.11.78), cytidylyltransferase (EC 2.7.7.14), ethanolamine phosphotransferase (EC 2.7.8.10), phosphate synthase (EC 2.7.8.37), diphosphatase (EC 3.6.1.630), C-P-lyase (EC 4.7.1.1), phosphorylcholine transferase (EC 2.7.7.15), and diacylglycerol cholinephosphotransferase (EC 2.7.8.2) were significantly enriched in the control group. Conversely, the treatment group showed significant upregulation of enzymes such as, phosphoenolpyruvate mutase (EC 5.4.2.9), phosphonoacetate hydrolase (EC 3.11.1.2), pyruvate transaminase (EC 2.6.1.37), aminoethyl phosphonate dioxygenase (EC 1.14.11.460), cyclic phosphate dihydrolase (EC 3.1.4.570), phosphodiesterase (EC 3.1.4.55), hydroxylamine reductase (EC 1.7.99), and phosphinothricin acetyltransferase (EC 23.1.183). These findings suggest significant variation in the relative abundance of phosphorous -metabolism related genes between the control and treatment groups, indicating efficient removal during microalgae bacteria-based wastewater treatment. These results align with the f previous studies demonstrating effective P-removal by such systems [88,114]. However, nitrogen and phosphorus removal are not the only

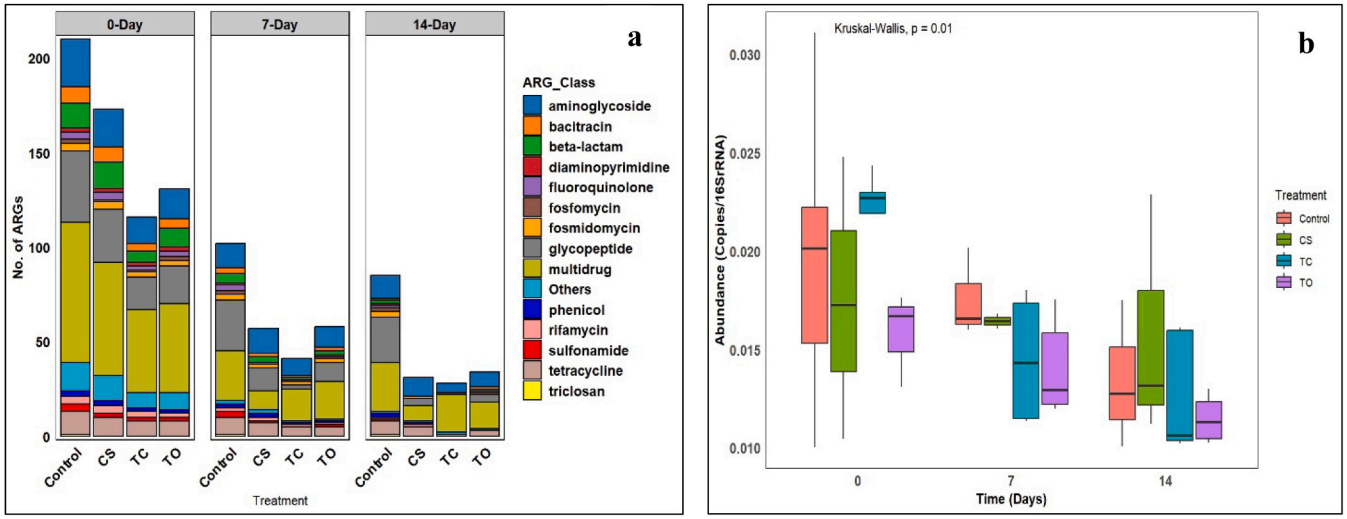


Fig. 6. Resistome profiles of a) control and treatment groups b) abundance (copies/16S rRNA) across different time periods (days).

mechanisms underpinning synergistic interactions, self-adaptation and nutrient accumulation in microalgae-bacteria consortia. Cofactors, including bacteria supplied vitamins supplied also play essential role in promoting microalgal growth and nutrient assimilation, [16,115]. Therefore, bacterial-supplied vitamin (B1, B7 and B12)-related metabolic pathways were also investigated (Fig. 5c). Key genes involved in vitamin metabolism, such as ribA, ribB, ribD, ribD2, ribE and ribH were significantly enriched in the treatments, suggesting that vitamin exchange between microalgae and bacteria had a synergistic and beneficial effect on algal growth.

3.7. Removal of antibiotic resistance genes by microalgal–bacterial systems

Shotgun metagenomic analysis using the DeepARG database identified a total of 300 different ARG subtypes. Among these ARGs associated with multidrug resistance, glycopeptides, aminoglycoside, beta-lactam, tetracycline and fluoroquinolone were the most abundant (Fig. 6a). Notably, microalgal treatment significantly reduced the overall abundance of ARG (the resistome) (Fig. 6a), consistent with previous studies [116,117], which have shown that microalgal-bacterial systems effectively y remove ARGs from various wastewater sources. Both treatment type and incubation time had a significant effect on reducing ARG abundance (Fig. 6b). This is again in line with previous studies by [116,118], which reported ARG removal via algal-based wastewater treatment system and monoalgal photobioreactor. In the control group, the relative abundance of ARGs (expressed as copies per 16S rRNA genes) remained relatively stable over time, with values s of 46.13 %, 44.3.7 %, and 43.1 % at 0, 7, and 14 days, respectively. In contrast, the treatment groups showed significant reduction in ARG abundance ($p < 0.0001$). Specifically, the resistome richness decreased from 40.3 to 28.4 % in TO, from 46.6 to 25.7 % in CS and from 41.9 % to 27.2 % in TC. To further determine, the relationship between the functional gene abundance and ARG removal in the control (Fig. S6a) and treatment (Fig. S6b) groups, we performed Procrustes analysis. The analysis yielded a matrix-matrix correlation coefficient of ($M^2 = 0.75$; protest, $p = 0.32$) for control groups and a matrix-matrix correlation coefficient of ($M^2 = 0.94$; protest, $p = 0.039$) for microalgae treatment groups suggesting a strong association between functional genes and ARG removal. These results are consistent with previous studies on ARG removal from wastewater [2,119,120], demonstrating a significant indirect effect of microalgae in ARG removal. These results clearly demonstrate that microalgal–bacterial systems significantly reduce ARG abundance over time ($p < 0.0001$), underscoring their potential for mitigating the spread of antibiotic resistance through wastewater.

4. Conclusion

This study evaluated the efficacy of microalgae treatment for raw wastewater at the laboratory scale. The findings revealed that microalgae significantly enhanced nutrient and COD removal while increasing wastewater clarity. Metagenomic sequencing and microbial diversity analyses indicated a clear shift in the microbiome compositions following microalgal inoculation, with a significant increase in microbial diversity in treated wastewater. Functional pathway analysis revealed increased abundances of genes associated with aerobic ammonia-oxidation and denitrification in the treated water samples. Moreover, synergistic interactions between microalgae and bacteria were evident, contributing to an environment enriched in key metabolic pathways. Importantly, antibiotic resistance genes (ARGs) decreased significantly in microalgae-treated raw wastewater, highlighting the system's potential to combat antibiotic resistance dissemination. Overall, microalgal treatment emerged as an effective strategy for nutrient and pollutant removal, fostering beneficial microbiome shifts and offering a sustainable approach to wastewater management.

CRediT authorship contribution statement

Muneer Ahmad Malla: Writing – original draft, Visualization, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Faiz Ahmad Ansari:** Methodology, Formal analysis, Data curation, Conceptualization. **Jonathan Featherston:** Software, Formal analysis. **Humeira Hassan:** Methodology, Data curation. **Magray Owaes:** Methodology, Formal analysis, Data curation, Conceptualization. **Aaliyah Osman:** Methodology, Data curation. **Anna Heintz-Buschart:** Writing – review & editing, Supervision, Methodology. **Nico Eisenhauer:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Arshad Ismail:** Resources. **Faizal Bux:** Supervision. **Sheena Kumari:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104217.

Data availability

Data will be made available on request.

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