Increasing tetracycline concentrations on the performance and communities of mixed microalgae-bacteria photo-bioreactors

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Abstract

This study investigated the impact of varying concentrations of tetracycline on the performance of mixed microalgae-bacteria photo-bioreactors. Photo-bioreactors were assessed for their ability to remove carbon dioxide (CO₂) from the biogas of anaerobic membrane bioreactor (anMBR), and nutrients from the anaerobic effluent. The varying concentrations of tetracycline had no impact on the removal of CO₂ from biogas. 29% v/v of CO₂ was completely removed to generate > 20% v/v of oxygen (O₂) in all reactors. Removal of nutrients and biomass was not affected at low concentrations of tetracycline ($\leq 150 \mu\text{g/L}$), but 20 mg/L of tetracycline lowered the biomass generation and removal efficiencies of phosphate. Conversely, high chlorophyll a and b content was observed at 20 mg/L of tetracycline. High tetracycline level had no impact on the diversity of 18S rRNA gene-based microalgal communities but adversely affected the 16S rRNA gene-based microbial communities. Specifically, both Proteobacteria and Bacteroidetes phyla decreased in relative abundance but not phylum Chloroplast. Additionally, both nitrogen-fixing (e.g. *Flavobacterium*, unclassified Burkholderiales and unclassified Rhizobiaceae) and denitrifying groups (e.g. *Hydrogenophaga* spp.) were significantly reduced in relative abundance at high tetracycline concentration. Phosphate-accumulating microorganisms, *Acinetobacter* spp. and *Pseudomonas* spp. were similarly reduced upon exposure to high tetracycline concentration. Unclassified Comamonadaceae, however, increased in relative abundance, which correlated with an increase in the abundance of tetracycline resistance genes associated with efflux pump mechanism. Overall, the findings demonstrate that antibiotic concentrations in municipal wastewaters will not significantly affect the removal of nutrients by the mixed microalgae-bacteria photo-bioreactors. However, utilizing such photo-bioreactors as a polishing step for anMBRs that treat wastewaters with high tetracycline concentration may not be effective as evidenced from the lower nutrient removal and occurrence of antibiotic resistance genes.

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32 **Highlights**

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- 34 • Microalgal biomass yield and removal of CO₂ from biogas unaffected by tetracycline
- 35 • Removal of nutrient from anaerobic effluent affected by 20 mg/L of tetracycline
- 36 • Microalgae community insignificantly affected by 20 mg/L of tetracycline
- 37 • Bacterial populations, including those involved in N and P cycling, affected by 20 mg/L
- 38 tetracycline
- 39 • Increase in abundance of resistance genes at high concentration of tetracycline

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Introduction

The use of anaerobic fermentation for wastewater treatment eliminates the energy costs associated with aerating wastewater while also introducing the potential for recovery of methane generated by anaerobic digestion [1]. By further coupling a membrane separation process to anaerobic digestion, the whole process becomes what is referred to as the anaerobic membrane bioreactor (anMBR). Although anMBR can achieve a good removal of total organic carbon and hence demonstrate great potential for improving efficiency and sustainability of wastewater treatment, they are unable to remove ammonia and phosphate to a level that can meet the discharge or reuse regulations [2-4]. Post-treatment of anMBR effluents is therefore necessary. Additionally, biogas generated from anMBR processes contains approximately 20-40 % of carbon dioxide [5], which is a fossil gas that diminishes the calorific content of biogas.

To circumvent the shortcomings associated with anMBR, it had been proposed that the microalgae photo-bioreactors be applied to upgrade biogas by increasing the proportion of methane (CH_4) percentage and to remove nutrients from the anaerobic effluents [6, 7]. At the same time, microalgae are considered as one of the most promising feedstock for biofuels and chemicals [8]. However, widespread usage of antibiotics in both domestic and agricultural environments has resulted in higher antibiotic loading rates in wastewater treatment plants (WWTPs) across the world. To exemplify, low concentrations of approximately $1\text{ }\mu\text{g/L}$ of tetracycline is typically detected in domestic wastewaters [9] while hospital wastewater contained over $100\text{ }\mu\text{g/L}$ of tetracycline [10]. A high concentration of tetracycline of up to 23 mg tetracycline detected per kg of animal manure was also detected in the livestock wastewater [11].

An earlier study has assessed that anaerobic digestion can robustly treat wastewater with varying concentrations of antibiotics while deriving value-added products and minimizing the dissemination of associated antibiotic-resistance genes [12]. Another study also independently determined that anaerobic microbial communities express biodegradation genes to facilitate the degradation of antibiotics [13]. In particular, by further coupling with membrane separation process, anMBR can remove up to 80% of antibiotics from the influent [14]. However, remnant antibiotics present in the anaerobic effluent can potentially affect downstream technologies such as microalgae photo-bioreactors that are used for polishing the nutrient in the effluent.

Although microalgae photo-bioreactors has been applied to reclaim wastewater contaminated with pharmaceutical compounds [15-19], it is unknown to what extent antibiotics can affect the overall performance of microalgae photo-bioreactors. Earlier studies showed that antibiotics, e.g. chloramphenicol, florfenicol, and thiamphenicol can reduce total chlorophyll content of *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui* significantly [20], while increase in the concentration of enrofloxacin caused an increase in the total chlorophyll of *Chlamydomonas mexicana* and *Micractinium resseri* [21]. However, most of these studies emphasize on pure microalgae cultures, with only few studies available to report on how stressors like antibiotics would influence the removal of CO₂ and nutrients from anMBR by a mixed microalgae-bacteria photo-bioreactor. Pure cultures of microalgae are difficult to maintain in most wastewater treatment processes as microalgae draws more benefits from their interactions with bacteria. For example, Kim et al. found that the *Rhizobium*, a plant growth promoting bacterium, can enhance the growth of algae through mutualistic interaction found [22]. In the mutualistic relationship of *Emiliania huxleyi* with *Phaeobacter gallaeciensis*, it has been found that bacterium produces antibiotic compounds to protect the host from other bacterial pathogens [23].

Thus, it is much more essential to explore the impact of antibiotics, such as tetracycline on the performance of mixed microalgae-bacteria photo-bioreactor than on pure microalgae photo-bioreactors. An earlier study reported that treating the mixed microalgae photo-bioreactor with high concentrations of tetracycline (up to 30 mg/L) caused reduction in the removal of nutrients and adversely affected the overall dynamic of microalgae community [24]. However, the study only utilized denaturing gradient gel electrophoresis (DGGE) to study the microalgae community and not the bacterial populations. DGGE is a fingerprinting technique that allows the identification of predominant populations but does not allow quantitative measurements of each individual microalgae and bacterial population. Neither does the technique allow one to infer which microalgae or bacterial populations would be perturbed in their nutrient removal process in the presence of antibiotics.

In this study, it is hypothesized that mixed microalgae-bacteria photo-bioreactors are able to remove nutrient and CO₂ from anaerobic effluent that contained varying concentrations of tetracycline. To evaluate this hypothesis, three photo-bioreactors, each with different

concentrations of tetracycline, i.e., 1 µg/L, 150 µg/L and 20 mg/L, representative of those found in municipal, hospital and livestock farm wastewaters, respectively [9-11], were carried out along a control reactor (i.e., 0 µg/L tetracycline). The photo-bioreactors performance was evaluated by measuring ammonia, phosphate, CO₂ and O₂ percentage within biogas, as well as chlorophyll concentrations. Both 16S and 18S rRNA gene-based amplicon sequencing were applied to analyze both bacterial and microalgal communities, respectively. This is to provide quantitative values of relative abundance of prokaryotic and eukaryotic populations, and to facilitate the evaluation of which populations play a role in overall reactor performance. Additionally, the occurrence of tetracycline resistance genes was also investigated to determine if mixed microalgae-bacteria photo-bioreactors would contribute to the dissemination of antibiotic resistance genes through the final treated effluent.

2. Materials and methods

2.1. Experimental set-up and sampling

Four media bottles, each containing 450 mL of effluent obtained from a lab-scale anaerobic membrane bioreactor (anMBR) was seeded with mixed microalgae cultures. The seed was originally sampled from a laboratory-scale wastewater bioreactor, and contained predominantly of *Chlorella* spp. but mixed with bacteria as well. Media bottles were individually added with 0 µg/L, 1 µg/L, 150 µg/L and 20 mg/L of tetracycline hydrochloride (TC-HCl), all diluted from 1 g/L stock solution in ultrapure water (Merck Millipore, Billerica, MA, USA). All reactors were vacuumed and flushed with biogas twice prior to carrying out the experiment. Biogas was supplied via gas bag containing 4 L biogas collected from the anMBR to provide carbon dioxide to the microalgae bioreactors at 0.1 mL/min. The biogas was directly introduced to the aqueous media by a tubing connected to the nozzle at the bottom of each reactor. The compositions of biogas in the supply of gas bag were 29% of CO₂, 0.5 % of O₂, 45% of CH₄ and 27% of N₂. Effluent from an attached growth anMBR operated as described previously [25] was used as the media for the microalgae reactors. Gas from the headspace was individually recycled within the microalgae reactors at a flow rate of 25 mL/min. Three LED lamps were placed approximately 20 cm from the top of the photo-bioreactors to supply $2428 \pm 37 \mu\text{W}/\text{cm}^2$ of light intensity to the

surface of the microalgae cultures in continuous daylight mode. Light intensity was measured over the spectral range of 250 to 1050 nm with ILT950 portable spectroradiometer at a radiometric accuracy of ca. 5 to 10% (International Light Technologies, Peabody, MA, USA). The whole experiment was repeated in triplicate, referred to as run 1, run 2 and run 3 in this study. The initial OD₆₈₀ and pH was 0.007±0.003 and 8.0-8.2, respectively for all reactors among all runs. The initial COD, NH₃-N and PO₄³⁻-P in the effluent media was < 50 mg/L, 70-130 mg/L and 17±3 mg/L, respectively. Each reactor was sampled for 20 mL of its content daily, and replaced with 20 mL of fresh media with corresponding amount of tetracycline hydrochloride supplemented to maintain the same tested concentration of tetracycline.

2.2. Reactors performance

Ammonia (NH₃-N) and phosphate (PO₄³⁻-P) were detected by TNT-AmVer (Salicylate)-high range and LCK 348 (Hach, Loveland, Colorado, USA), respectively. Nitrite and nitrate were also determined by Hach kits TNT 839 and TNT 835, respectively, but were found to be present in negligible proportions compared to NH₃-N. Biomass concentration was estimated by weighing the lyophilized mass of the sample divided by the initial sample volume. The biogas components were detected via gas chromatography (SRI 310C, SRI instrument, USA). Chlorophyll pigment was detected as described by [26] with some modifications. Briefly, 2 mL of the microalgae suspension was placed in 2 mL microcentrifuge tube and was centrifuged at 5600 g for 3 min. The supernatant was discarded and 2 mL of DMSO was added to lyse chlorophyll pigment from biomass pellet. The suspensions were homogenized, incubated in a water bath at 60 °C for 12 min, and then centrifuged at 2000 g for 5 min. Supernatant was aliquot for OD measurement at 649 and 665 nm wavelengths, with DMSO as the blank control. All measurements were made in duplicate. The pigment contents were calculated as below [26]:

$$\text{Chlorophyll a (Chl a) in mg/L} = 12.47 (\text{OD}_{665}) - 3.62 (\text{OD}_{649})$$

$$\text{Chlorophyll b (Chl b) in mg/L} = 25.06 (\text{OD}_{649}) - 6.5 (\text{OD}_{665})$$

2.3. Quantitative PCR (qPCR) to detect the tetracycline resistance genes

qPCR was conducted as described previously [27]. qPCR standards of antibiotic resistance genes (tetW, tetQ, tetZ and tetE) and the plasmids for 16S rRNA gene copies were diluted in series to obtain standard solutions within the range of 10^2 - 10^{10} copies/ μ L. The primer pairs used in this study were listed in Table S3. qPCR was conducted on Applied Biosystem® 7900HT Fast Real-Time PCR system with 96-well block module (Thermo Fisher Scientific, Carlsbad, CA, USA). All NTCs have threshold cycle (C_q) values that were either of undetermined values or greater than 37.

2.4. 16S rRNA and 18S rRNA gene-based high-throughput sequencing

For each reactor, 3 to 4 samples were sampled at the early, mid and late-stage of microalgae reactor operation. A total of 46 samples were obtained, lyophilized, extracted for DNA and prepared for 16S rRNA gene-based high-throughput sequencing based on procedures described previously [12]. Extracted DNA was also prepared for 18S rRNA gene-based high-throughput sequencing by amplifying for the V8-V9 region only [28]. More details are available in Supplementary Information. All high-throughput sequencing data were deposited in the short Read Archive (SRA) of the European Nucleotide Archive (ENA) under study accession number PRJEB21956.

2.5. 16S rRNA and 18S rRNA gene-based sequencing analyses

Prior to sequence analyses of both 16S rRNA and 18S rRNA genera, both sequences were trimmed for their adaptors and primer sequences, and removed of sequences with lengths < 283 nt. 16S rRNA gene sequences were removed of chimeric sequences via UCHIME [29] by aligning against reference dataset downloaded from Greengenes (i.e., file titled gold_strains_gg16S_aligned, from <http://greengenes.lbl.gov> [30], contained 2116439 sequences, last updated on 20 March 2011). Similarly, 18S rRNA gene sequences were removed of chimeric sequences via UCHIME by aligning against reference dataset downloaded from ARB (i.e., file titled Silva.gold, contained 5181 sequences, release 128, www.arb-silva.de). RDP Classifier [31] was used to assign phylogenetic identities for the 16S rRNA gene sequences that passed the quality control checks (> 90.4% of the total sequences). To assign phylogenetic identities of 18S rRNA sequences, sequences were first blastn against the full Silva database release 128 using an E-value score of $1e-10$ and percentage identity of > 50%. The blastn output file was then

extracted for its assigned 18S rRNA gene-based phylogenetic identities using an in-house developed python script.

Subsequently, relative abundances of each taxa were determined by normalizing against the total number of either 16S rRNA or 18S rRNA gene sequences per samples, and square-root transformed prior to generating Bray-Curtis similarity matrix. Bray-Curtis similarity matrix was used in multivariate analysis, where 16S rRNA-based microbial community and 18S rRNA-based microalgae communities of each samples were shown on non-metric multidimensional scaling (nMDS) plots. The physicochemical parameters related to the performance of microalgae photobioreactors, i.e., NH₃-N, PO₄³⁻-P, Chl A, Chl B and tetZ were also collated, square-root transformed and normalized for principal component analysis (PCA). All statistical analysis was conducted on Primer-E version 7 software [32].

2.6. Statistical analysis

NH₃-N and PO₄³⁻-P removal efficiencies were determined according to Equation 1:

$$\%R = \frac{S_i - S_f}{S_i} \cdot 100 \quad (1)$$

where S_i and S_f correspond to the respective nutrient concentration (in mg L⁻¹) in the beginning and at the end of cultivation time, respectively.

To examine for significant differences among sample sets, an unpaired two-tailed t-test was performed with an assumption of unequal variance between sample sets.

3. Results and discussion

3.1. Effect of tetracycline on mixed microalgae-bacteria photo-bioreactors

A longer lag phase of about 2 to 4 days was observed through the OD values in the 20 mg/L-TC reactor (R4) compared to a ca. 1 day lag phase observed in the other reactors (R1-R3) for run 1 and run 2 (Figure S1A). There was no significant difference in biomass concentrations among the reactors R1-R4 in all runs ($p > 0.05$, Figure S1B) although the average biomass produced in

R4 was approximately 35% lower than that in R1 to R3 in run 1 and 2. However, no significant difference in OD and biomass was observed among all reactors in run 3. Biogas collected from the lab-scale anMBR had 29 ± 4 v/v % of CO₂ and this percentage of CO₂ reduced to zero after 4 days of operation (Figure S2A). The utilization of CO₂ resulted in a corresponding reduction in pH from 8.1 ± 0.2 to 7.4 ± 0.2 , and then increased to over 8.0 across all reactors (Figure S3). This suggests that CO₂ was first dissolved into the media, hence resulting in the slight acidification of the media, and was subsequently utilized by microalgae. Unlike CO₂, O₂ increased from zero to $22 \pm 5\%$ after 6 days of operation in run 1 and run 2, and to $29 \pm 8\%$ after 3 days of operation in run 3 (Figure S2B). There was no significant difference in the percentage of O₂ generated by reactors R1 to R4 in all runs ($p > 0.05$). The replacement of CO₂ with O₂ resulted in no significant change in the percentage of CH₄ in the final biogas product collected in headspace of photo-bioreactors. The average CH₄ percentage among all reactors was $39 \pm 7\%$, $42 \pm 8\%$ and $41 \pm 3\%$ in run 1, 2 and 3, respectively (Figure S2C). The main aim of this study was not to demonstrate the enhancement of calorific value of biogas as the batch reactors were not designed and operated in an optimal way to facilitate such purpose. Regardless, the findings obtained here support the feasibility of using mixed microalgae-bacteria photo-bioreactor as a downstream technology of anaerobic processes to effectively remove CO₂, a greenhouse gas present in biogas, through microalgae photosynthesis. However, the production of O₂ into the headspace that contains CH₄ can create an explosion hazard. To facilitate future needs of enhancing biogas for its energy content (i.e., by increasing CH₄ percentage), CO₂ can be first stripped off from the total biogas via gas-stripping membranes [33]. The high solubility of CO₂ compared to CH₄ would allow effective separation of CO₂ from biogas to support the autotrophic photosynthetic needs of the mixed microalgae consortium [34]. CO₂ can also be scrubbed in a packed column through absorption by liquid solvents [35]. Alternatively, Bahr and coworkers [6] have demonstrated the use of low-cost high-rate algal pond comprising of alkaliphilic microalgal-bacterial consortium, coupled with an external absorption column, to simultaneously remove hydrogen sulfide and CO₂ while producing very low percentage of O₂ (< 0.2%).

3.2. Removal of NH₃-N and PO₄³⁻-P in the presence of tetracycline

The $\text{NH}_3\text{-N}$ removal efficiencies were 34%-36% for R1-R4 across run 1 to run 3 (Table 1 and Figure S4A). In contrast, a higher removal of $\text{PO}_4^{3-}\text{-P}$ was present among R1-R3 for all runs compared to those in R4 (Figure S4B). To illustrate, the $\text{PO}_4^{3-}\text{-P}$ removal efficiencies were 94%-100% for R1-R3, but decreased to 75% for R4 (Table 1). As such, 20 mg/L of tetracycline adversely affected the removal efficiencies of $\text{PO}_4^{3-}\text{-P}$ but not $\text{NH}_3\text{-N}$. However, Taskan [24] found that 30 mg/L of tetracycline exposed to a mixed microalgae-bacteria consortium caused both the removal efficiencies of $\text{NH}_3\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ declined by 80% and 64%, respectively. The discrepancy in observations made by both studies may arise from differences in the microalgae-bacteria consortiums, as well as differences in the compositions of microalgae species. Compared to the earlier study, which reported the presence of a diverse eukaryotic algal types including *Acutodesmus* spp, *Chlorella* sp., *Scenedesmus* spp. in the photo-bioreactor, the photo-bioreactors operated in this study were mainly comprised of uncultured Chlorophyta and *Chlorella* sp.. It was previously determined that different microalgae species exhibited varying level of susceptibility towards tetracycline [36]. Therefore, depending on the types and diversity of microalgae that were present, exposure to tetracycline may have varying impact on the removal of $\text{NH}_3\text{-N}$.

3.3. High pigment concentrations at high tetracycline concentration

When normalized to biomass concentrations, higher chlorophyll a (Chl a) and chlorophyll b (Chl b) were detected in R4 (20 mg/L-TC) compared to those in R1, R2 and R3 (Figure 1). An increase in chlorophyll production has been reported in the literature when the pure algae culture was treated with high concentration of polyamidoamine dendrimers [37], antibiotics (e.g. tylosin, lincomycin and trimethoprim) [38] and heavy metal (e.g. cadmium, [39]). While the reasons to account for the increase in chlorophyll content at high concentrations of toxic compounds remain unknown, it was inferred that the high concentration of stressors activated the electron transport between the primary and secondary quinone acceptors, in turn triggering the activities of photosystem I and II [37, 39]. It may be possible that the increase in the chlorophyll contents in the presence of 20 mg/L tetracycline was due to a non-inhibitory but provoking effect by this antibiotic on both photosystems I and II of microalgae and cyanobacterium, and a consequent stimulation of electron transport system that facilitated chlorophyll production. Alternatively,

high tetracycline concentration resulted in a less effective removal of $\text{PO}_4^{3-}\text{-P}$, with slightly higher concentrations of $\text{PO}_4^{3-}\text{-P}$ remaining in R4 compared to other reactors (Figure S4B). Strong positive correlation was observed between chlorophyll content and $\text{PO}_4^{3-}\text{-P}$ concentration in many freshwater ecosystems [40]. The higher amount of $\text{PO}_4^{3-}\text{-P}$ remaining behind in R4 may have accounted for a higher concentrations of Chl a and b measured in R4 throughout the experiment.

3.5. Performance parameters in R4 were different from the other reactors

All $\text{NH}_3\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, Chl a, Chl b and tetZ data obtained from R1 to R4 for all runs were collated and performed with multivariate analysis via principal component analysis (PCA) (Figure S5). The PCA results showed that R4 samples collected from run 1 and 2, with the exception of two outlier samples, was spatially clustered apart from samples collected from R1 to R3 in the same runs. In contrast, all samples collected from R1 to R4 in run 3 shared a common cluster that were distinct from the other samples collected in the earlier runs. Multivariate analysis therefore suggests that high concentrations of tetracycline affect the overall performance of the microalgae bioreactors. Consequently, section 3.6 and 3.7 evaluated the microalgae and microbial communities to determine if changes in these communities would correlate to the changes in the reactor performances.

3.6. Differences of 18S rRNA gene-based communities among reactors

The spatial separation of 18S rRNA genes-based microbial communities (i.e., eukaryotes) among samples was not significant based on the antibiotics concentrations (Figure 2A) (ANOSIM, $R < 0$, $p > 0.18$). Instead, eukaryotic communities were sorted by run number, with samples from run 3 distinctly different from that in the earlier two runs (Figure 2B) (ANOSIM, $R > 0.23$, $p < 0.004$). An uncultured *Chlorophyta* sp. and various *Chlorella* spp. accounted for the highest relative abundance among the total eukaryotic community in all reactors for all runs (Table 2). Given that there were variations in the total eukaryotic communities across the three runs, emphasis was made to further determine which eukaryotic populations demonstrate a consistent trend among at least two of the three runs. All the dominant eukaryotes were not affected by the tetracycline in a reproducible manner, suggesting that microalgae was not perturbed by the presence of tetracycline.

3.7. Differences in 16S rRNA gene-based communities among reactors

Microbial communities in R4 (20 mg/L-TC) was spatially clustered apart from those in the other three reactors (Figure 3A). Analysis of similarities (ANOSIM) showed significant difference between R4 against the control R1 ($R = 0.609$, $p = 0.001$) but no significant difference among microbial communities in R1 to R3. Vector-based analysis further revealed Chlorophyta to be correlated with the spatial cluster of R4 samples (Figure 3B).

When analyzed at the phylum level, there was a higher relative abundance of Cyanobacteria/Chloroplast in R4 compared to R1 for run 1 and 2, and a corresponding decrease in the relative abundance of Proteobacteria (Figure S6). To illustrate, the Cyanobacteria/Chloroplast increased from an average 21.8% in R1 from both runs 1 and 2 to an average 52.2% in R4 in the same runs. Proteobacteria reduced from an average 47.1% in R1 to 22.9% in R4 for both runs 1 and 2 (Figure S6A and S6B). A likewise decrease in the relative abundance for Bacteroidetes was also observed for both runs 1 and 2. However, no apparent differences in the relative abundance of the dominant phyla were observed among the four reactors in run 3 (Figure S6C).

When sorted based on the run number, samples obtained from run 1 to run 3 were spatially separated from each other (Figure S7), suggesting that the overall microbial communities in the photo-bioreactors varied across the different runs. Therefore, when analyzing at taxonomical levels lower than phylum, emphasis was made to further determine which bacterial populations demonstrate a consistent trend among at least two of the three runs. Based on this criteria, it was observed that genera *Reyranella*, *Terrimonas*, *Flavobacterium* and *Hydrogenophaga* were significantly reduced in their relative abundance in R4 compared to R1 (Table 3). In addition, unclassified Burkholderiales and Rhizobiaceae were also adversely impacted by the high tetracycline concentration present in R4. In contrast, Chlorophyta and unclassified Comamonadaceae increased in its relative abundance in R4 compared with R1, R2 and R3 (Table 2).

Both 16S rRNA and 18S rRNA gene-based microbial community analysis demonstrated that the mixed microalgae-bacteria consortiums were mainly comprised of Cyanobacteria and Chlorophyta. In addition, other microbial populations that metabolized on the exudates from

microalgae co-exist in the photo-bioreactors. For example, certain species within *Flavobacterium* are fermentative bacteria and can utilize the metabolites from microalgae as carbon sources [41-43]. This observation reiterates that in large-scale applications of microalgal systems, it may be difficult to implement pure cultures for photo-bioreactor operation, and that maintaining a mixed culture may be more effective for further treatment of wastewaters [44, 45].

In addition, it was observed that high concentration of tetracycline had more detrimental impact on the microbial populations than on the eukaryotic microalgae. This is evidenced from the spatial clustering of microbial communities based on tetracycline concentration but the same clustering was not observed for the eukaryotes. Specifically, denitrifiers such as *Hydrogenophaga* spp. [46] were significantly reduced in its relative abundance in R4 for at least two of the three runs. Nitrogen-fixing groups such as *Flavobacterium*, unclassified Burkholderiales and unclassified Rhizobiaceae [47-49] were also significantly reduced in their relative abundances among microbial communities in R4. However, this reduction of denitrifiers and nitrogen-fixing population did not bring about any major changes to the nitrogen production rates that would subsequently impact the percentage of CH₄ and O₂ in R4 compared to other reactors. This suggests that the main contribution of NH₃-N removal and N-cycling in this study was due to assimilation by microalgae to produce biomass.

In contrast to NH₃-N, removal efficiencies of PO₄³⁻-P was affected in the presence of high concentration of tetracycline (Table 1). Earlier studies had shown that many microalgae including *Chlorella vulgaris* are able to accumulate intracellular polyphosphate [50, 51]. Since the eukaryotic microalgae and biomass were not significantly perturbed by the presence of antibiotics, it is unlikely that the deterioration in PO₄³⁻-P removal efficiencies was due to detrimental impact on microalgae. Instead, microbial community analyses suggested that the relative abundance of species likely associated with phosphate accumulating organisms (PAOs), such as *Acinetobacter* and *Pseudomonas*, were significantly lower in R4 than R1. These findings suggest that both microalgae and PAOs play a role PO₄³⁻-P removal efficiencies in mixed microalgae-bacteria bioreactors.

This is one of the first few studies that utilized high-throughput amplicon-based sequencing to characterize both the microalgae and bacterial populations in photo-bioreactors that were

subjected to varying concentrations of tetracycline. Although the reads generated from high-throughput amplicon-based sequencing are generally of short read lengths and that the technique remains limited in identifying reads at the taxonomical level of species in high confidence, the technique is useful in characterizing the identities and relative abundance of microalgae and bacterial populations at genus level. This information can then be used to determine which populations play a role in nutrient removal despite antibiotic stress.

3.8. Increase in abundance of tetZ genes upon exposure to high concentration of tetracycline

Tetracycline and the derivatives have been known for their broad spectrum effect and widely used as antibiotics drugs against Gram-positive and –negative bacteria [52]. Bacteria can in turn develop tetracycline resistance typically via efflux pump mechanism or ribosomal protection proteins (RPP). As such, qPCR was further conducted to determine if exposure to tetracycline can contribute to the occurrence of tetracycline resistance genes within the photo-bioreactors.

tetW and tetQ confers resistance against tetracycline via ribosomal protection protein, while tetZ and tetE confers resistance via efflux pump mechanism. Among these four genes, only tetZ genes were detectable by qPCR. There was higher abundance of tetZ gene normalized against the 16S rRNA copies in R4 (20 mg/L-TC) compared to those in the control R1 for all runs (Figure 4). To illustrate, the abundance of tetZ gene increased by approximately 35-, 295- and 229-times in R4 compared to R1 for run 1, 2 and 3, respectively. Less than 2-times increment in tetZ was observed for R2 and R3 compared to R1 across all runs.

The increase in abundance of tetZ gene was previously detected when agricultural soils were subjected to manure from pig production farms [53]. In this study, only tetZ gene, which confers resistance to tetracycline via efflux pump mechanism, significantly increased at 20 mg/L of tetracycline, and that the increase in abundance of tetZ genes correlate with a corresponding increase in the relative abundance of unclassified Comamonadaceae (Table 3). Earlier studies have determined that *Comamonas* spp. are able to protect against stress conditions via efflux pump-coding gene [54-56]. It is likely that the increase in efflux pump-coding gene (tetZ) was derived from a selective enrichment of unclassified Comamonadaceae at 20 mg/L of tetracycline which may possess tetZ.

The potential risk of dissemination of antibiotic resistance genes need to be taken into account. It is likely that if mixed microalgae-bacteria photo-bioreactors are used to treat effluents containing high antibiotic concentrations, proper digestion or management of the harvested biomass would be required to tackle the presence of antibiotic resistance genes. Furthermore, antibiotic resistance genes in the final treated effluent should be removed by membrane filtration prior to discharge or reuse [27].

Overall, this study demonstrates the potential impact by varying concentrations of tetracycline on mixed microalgae-bacteria photo-bioreactors to polish effluents generated from anaerobic membrane bioreactors. Antibiotic concentrations in municipal wastewaters typically range in the scale of $\mu\text{g/L}$ [9] and would not significantly affect the removal of nutrients by the photo-bioreactors. However, utilizing photo-bioreactors as a polishing step for anaerobic effluents generated from anMBR treating wastewaters containing high concentration of tetracycline [11] may not be effective as evidenced from the lower nutrient removal and occurrence of antibiotic resistance genes. Future studies should aim to conduct similar experiments in full-scale outdoor systems receiving continuous streams of real wastewaters from either municipalities, hospitals or livestock farms. This would serve to verify if mixed microalgae bioreactors are robust enough to remove varying concentrations of nutrients in the presence of antibiotics and variable environmental conditions.

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Y. Xiong and D. Hozic carried out the experiments and conducted the analyses, A.L. Goncalves and M. Simões calculated the removal efficiencies, Y. Xiong and P.Y. Hong contributed the conception and design of the study, P.Y. Hong provided all reagents and materials, Y. Xiong and P.Y. Hong wrote the paper. All the authors have revised, edited and approved the final manuscript.

Table 1. Nutrient removal efficiencies (%R) in R1-R4, shown as the average obtained from run 1 to run 3 with standard deviation.

	NH₃-N (%)	PO₄³⁻P (%)
R1 (0 µg/L)	35.1 ± 14.8	93.5 ± 11.2
R2 (1 µg/L)	36.4 ± 9.8	94.2 ± 7.7
R3 (150 µg/L)	33.8 ± 11.6	100 ± 0.0
R4 (20 mg/L)	35.6 ± 6.8	75.3 ± 23.4

Table 2. The average relative abundance (%) of the 18S rRNA genes-based dominant taxa at run 1, run 2 and run 3 with different concentrations of tetracycline hydrochloride (R1, R2, R3 and R4: 0 µg/L, 1 µg/L, 150 µg/L and 20 mg/L of TC-HCl, respectively) . * denote the average relative abundance of the taxa was significantly different against that in the control R1.

	Run 1				Run 2				Run 3			
	R1	R2	R3	R4	R1	R2	R3	R4	R1	R2	R3	R4
Uncultured	71.5	68.8	75.2	59.0	80.2	67.6	65.3	42.8*	65.5	68.8	68.1	68.6
Chlorophyta												
<i>Chlorella</i> sp. KMN1	10.9	7.95*	9.24	9.76	11.8	9.43	7.91*	6.71*	17.4	16.1	13.9	15.7
<i>Micractinium</i> sp. IPOME-2	1.93	2.14	1.41	3.65	3.01	1.90	0.99*	1.97	5.29	2.94	5.98	4.69
<i>Chlorella sorokiniana</i>	8.07	13.5	7.90	13.8	1.11	6.44	15.9	8.10	3.74	4.53	5.22	4.52
<i>Chlorella</i> sp. Sp6	1.23	1.08	1.10	1.96	2.47	2.16	0.81*	0.30*	1.62	3.52	2.61	2.64
<i>Chlorella vulgaris</i>	0.43	1.43	0.00*	1.10	0.22	0.97	1.89	0.46	0.10	0.00	0.31	0.24

Table 3. The relative abundance of 16S rRNA genes-based dominant taxa across R1-R4 at run 1-Run3. * denotes the average relative abundance of the genera was significantly different against that in the control R1. ($p < 0.05$). R1, R2, R3 and R4 refer to reactors exposed to 0 µg/L, 1 µg/L, 150 µg/L and 20 mg/L of TC-HCl, respectively.

	Run 1				Run 2				Run 3			
	R1	R2	R3	R4	R1	R2	R3	R4	R1	R2	R3	R4
<i>Acinetobacter</i>	1.09	0.54	1.42	0.72	6.6	7.3	0.5*	0.02*	0.03	0.01	0.02	0.11
<i>Chlorophyta</i>	36.9	53	46	69.2*	6.5	8.5	9.3	34.8*	64.8	61.5	61.6	62.6
<i>Flavobacterium</i>	1.14	0.78	0.83	0.14*	1.44	0.3*	2.42	0.04*	0.48	0.27	0.33	0.43
<i>Hydrogenophaga</i>	3.86	0.69*	3.29	0.01*	2.88	4.18	0.06*	0.3*	0.81	0.85	0.36	0.06*
<i>Pseudomonas</i>	0.91	0.16	0.22	0.01	15.2	7.6	1.1	0.02	0.00	0.00	0.00	0.01
<i>Terrimonas</i>	1.63	2.9	1.66	0.004*	10.5	8.65	5.51	0.01*	0	0	0	0
Unclassified Comamonadaceae	3.81	2.18*	2.75	7.09	4.33	3.64	10.4*	10.2	3.11	4.24*	6.3*	13.2*
Unclassified Bacteroidetes	1.79	1.62	1.63	0.24*	1.81	0.4*	1.16	0.12*	1.16	1.03	1.04	0.64
Unclassified Flavobacteriaceae	6.17	4.29	4.24	1.07*	8.6	1.2*	4.7*	0.2*	1.82	1.63	1.26	2.27
Unclassified Burkholderiales	6.55	1.17*	3.28*	0.13*	3.62	3.05	1.7*	0.6*	2.4	3.04	2.39	1.15*
Unclassified Rhizobiaceae	0.1	1.04 *	0.28 *	0 *	1.41	4.1*	26.1*	0.3*	0.2	0.22	0.22	0*

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Figures legend

Figure 1. Chlorophyll concentration normalized against biomass in the reactor R1 to R4. **(A)** Chl a in run 1, **(B)** Chl a in run 2, **(C)** Chl a in run 3, **(D)** Chl b in run 1, **(E)** Chl b in run 2, **(F)** Chl b in run 3. (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride). The vertical bars associated with each data point reflect the standard deviation ($n = 2$)

Figure 2. Non-metric multidimensional scaling plot of 18S rRNA gene-based eukaryotic communities, sorted based on **(A)** reactor type, and **(B)** run number.

Figure 3. 16S rRNA gene-based microbial communities in samples from reactors R1 to R4 collected in all runs, analyzed for **(A)** multivariate similarities, presented in a non-metric multidimensional scaling plot, and **(B)** vectors representing bacterial populations that significantly correlate to the spatial clustering of samples

Figure 4. Copy numbers of tetZ normalized against 16S rRNA gene, measured in the reactors R1-R4 for **(A)** run 1, **(B)** run 2, and **(C)** run 3. (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride)

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A)

B)

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Electronic Supplementary Material for:

Algae Research

Increasing tetracycline concentrations on the performance and communities of mixed microalgae-bacteria photo-bioreactors

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Table S1. Sequences of the oligonucleotide primers for qPCR used in this study.

Primer	Target Gene	Sequence	Annealing Temp. (°C)	Amplicon Size (bp)	Amplification efficiency (%)	R ² -value of standard curve	Reference
27-F	16S	AGAGTTTGATCCTGGCTCAG	57	348	115.4%	0.995	[1]
338-R	rRNA	GCTGCCTCCCGTAGGAGT					[2]
Ribosomal protection protein genes							
tetW-F	tetW	GAGAGCCTGCTATATGCCAGC	60	168	95.0%	0.995	[3]
tetW-R		GGGCGTATCCACAATGTTAAC					
tetQ-F	tetQ	AGAATCTGCTGTTTGCCAGTG	55	169	93.6%	0.993	
tetQ-R		CGGAGTGTCAATGATATTGCA					
Efflux pump genes							
tetE-F	tetE	GTTATTACGGGAGTTTGTGTTGG	61	199	101.8%	0.991	[4]
tetE-R		AATACAACACCCACACTACGC					
tetZ-F	tetZ	CCT TCT CGA CCA GGT CGG	60	210	85.2%	0.996	
tetZ-R		ACC CAC AGC GTG TCC GTC

Figure S1. The profile of **(A)** optical density at 680 nm wavelength (OD_{680}), and **(B)** biomass concentration versus time among the reactors R1-R4 for run 1, 2 and 3 (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride)

Figure S2. The percentage profiles of **(A)** CO_2 , **(B)** O_2 and **(C)** CH_4 in the biogas versus time. (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride)

Figure S3. The variation of pH versus time in the reactors R1-R4 for **(A)** run 1, **(B)** run 2 and **(C)** run 3. (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride)

Figure S4. Profiles of **(A)** ammonia ($\text{NH}_3\text{-N}$) and **(B)** phosphorus ($\text{PO}_4^{3-}\text{-P}$) versus time (days) in the reactor R1-R4 for run 1 to 3. (R1 = 0 $\mu\text{g/L}$; R2 = 1 $\mu\text{g/L}$; R3 = 150 $\mu\text{g/L}$; R4 = 20 mg/L of tetracycline hydrochloride)

Figure S5. Principal component analysis (PCA) of ammonia ($\text{NH}_3\text{-N}$), Phosphorus ($\text{PO}_4^{3-}\text{-P}$), *tetZ*, *Chl a* and *Chl b* from reactors R1-R4 (0 µg/L, 1 µg/L, 150 µg/L, and 20 mg/L of tetracycline hydrochloric acids, respectively) for run 1-run 3. Vectors show the various parameters that result in the spatial clustering of R4 samples for run 1 and 2 from the other samples.

Figure S6. Relative abundances of predominant phylum in microbial communities of **(B)** run 1, **(C)** run 2, and **(D)** run 3.

Figure S7. Non-metric multidimensional scaling plot of 16S rRNA-based microbial communities, sorted based on the run number.

Microbial community analysis

PCR amplification for Illumina MiSeq sequencing

PowerSoil DNA Isolation kit (Mo Bio, Laboratories, Inc, Carlsbad, USA) was used to extract DNA from the samples. 16S rRNA genes were amplified with the primer pair 515F (5'- Illumina overhang- GTGYCAGCMGCCGCGGTAA- 3') and 907R (5'- Illumina overhang CCCCGYCAATTCMTTTRAGT- 3'). 18S rRNA genes were amplified with the primer pair V8f (ATAACAGGTCTGTGATGCCCT) and 1510r (CCTTCYGCAGGTTACCTAC). Both the PCR reactor mixture and thermal cycler conditions conditions were followed with the protocol of Illumina MiSeq sequencing library preparation [5], exclusively amplicon PCR (PCR1) for 18S rRNA amplified. The thermal cycler conditions of PCR1 for 18S rRNA was according to the [6]. In brief, 95 °C for 3 min, and 25 cycles of 98 °C for 20 s, 65 °C for 15 s, and 72 °C for 15 s, followed with a final extension at 72 °C for 10 min.

All PCR amplicons were cleaned up by AMPure XP beads (Beckman Coulter, CA, USA), and then each sample was barcoded individually with a unique index from the Nextera XT Index Kit (Illumina Ina, San Diego, CA, USA). All indexed PCR amplicons were then cleaned up via AMPure XP beads, and the purified DNA sample, each with equimolar amount was pooled together and submitted to KAUST core lab for Illumina MiSeq sequencing.

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