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Short communication

Single-step treatment of primary effluent by *Galdieria sulphuraria*: Removal of biochemical oxygen demand, nutrients, and pathogens

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ABSTRACT

Our previous reports have documented a single-step algal process for removing biochemical oxygen demand (BOD_5) and nutrients from primary effluent in batch mode. In the current study, we report results from continuous fed-batch operation of this algal system demonstrating consistent removal of BOD_5 and nutrients as well as significant reduction of pathogenic bacteria from primary effluent. The active volume of the algal bioreactor under fed-batch operation was 700 L, of which, 400 L was discharged at the end of every cycle and replenished with fresh primary effluent to start a new cycle. Results from thirty such cycles run over 120 days, under varying influent loadings and ambient conditions, confirmed that the discharge standards for BOD_5 and nutrients could be attained in a fed-batch cycle time of < 3 days. Typical volumetric removal rates of BOD_5, ammoniacal nitrogen and phosphates over the 120 days were 16.5 mg/L-d, 6.1 mg/L-d, and 1.4 mg/L-d, respectively. Fecal and total coliform were also reduced to non-detectable levels within these three days.

1. Introduction

Modern urban societies depend on centralized infrastructure facilities owned and operated by publicly owned treatment works (POTWs) for collection, treatment, and disposal of wastewaters to protect public health and provide safe ecosystem services. While most other urban infrastructure facilities have evolved significantly over the past decades, POTWs have continued to rely on obsolete, multi-step technologies that consume significant energy and dissipate valuable resources in the wastewater. The American Society of Civil Engineers [1] has recently reviewed over 14,000 POTWs and reported a grade of D + to the outmoded technologies employed by POTWs. The Report has recommended that these obsolete energy-intensive systems should be replaced by new generation of technologies that can reduce energy consumption in wastewater treatment and recover resources from the wastewater. With increasing waste loads due to rapid urbanization and escalating energy costs, wastewater utilities are under pressure to meet higher standards of treatment in a sustainable manner and lower carbon footprint while providing affordable service to the public.

Algal-based wastewater treatment technologies are emerging as potentially sustainable and greener alternatives for wastewater treatment. They can conserve the energy currently used for wastewater treatment and provide a pathway to recover most of the energy- and nutrient-content of wastewaters for reuse [2]. An advantage of algal systems over the traditional bacterial systems in wastewater treatment is due to the fact that the C:N:P ratio in algal biomass is closer to that in primary effluent than the C:N:P ratio in bacterial biomass. This enables algal systems to be engineered to treat wastewaters in a single step as opposed to the multi-step processes common in traditional wastewater treatment practice that suffer from the imbalance in C:N:P ratio [3].

Our laboratory studies on an extremophile alga, *Galdieria sulphuraria*, have demonstrated its ability to grow mixotrophically on dissolved organic carbon (DOC) in the primary effluent under dark or light conditions [4]. In contrast to the traditional autotrophic algal systems, mixotrophic algal systems can utilize DOC and nutrients in wastewaters for growth without relying on sunlight. Building upon our laboratory work on mixotrophic metabolism, we have developed a 700-L pilot scale wastewater treatment system utilizing *Galdieria sulphuraria*. This pilot system fed with primary effluent has been in operation at the local wastewater treatment plant for over 3 years [5]. This system has attained consistent removals of 5-day biochemical oxygen demand (BOD_5) and nutrients from primary effluent, meeting the respective discharge standards in a batch processing time of 4 to 5 days. As the next step in advancing this algal wastewater treatment system towards continuous operation, its performance was evaluated in fed-batch mode in the current study. It is anticipated that the processing time to meet

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the discharge standards for BOD_5 and nutrients could be reduced in the fed-batch mode by accumulating the biomass growth in the algal bioreactor instead of harvesting it at the end of each batch run. Additionally, this study served to validate a hypothesis that the punitive culture conditions in the algal bioreactor (pH ~ 4.0; average temperature ~ 35 °C; average dissolved oxygen ~ 6 mg/L) could contribute to simultaneous inactivation of bacteria of concern.

2. Materials and methods

G. sulphuraria CCMEE 5587.1 used in this study was obtained from Culture Collection of Microorganisms from Extreme Environments (University of Oregon). As the optimal pH for *G. sulphuraria* is 1 to 4, the initial pH of the culture volume in the bioreactors was adjusted to 2.5 by adding 10 N of H_2SO_4 [3–5]. The test system was fed with primary effluent; typical characteristics of which are summarized in Table S-1 in the Supplementary information section.

The test system consisted of two identical enclosed bioreactors (R1 and R2) fabricated of thin, clear polyethylene and fitted with a motor-driven paddlewheel to enable circulation and mixing of its contents. The active volume of the bioreactors was 700 L and the culture depth was 20 cm. Fed-batch cycles were initiated with 300 L of the preadapted culture of *G. sulphuraria* mixed with 400 L of primary effluent; each cycle was terminated when the reactor contents reached the discharge standard for BOD_5, ammoniacal-nitrogen (N), and phosphates (P). Upon termination, the paddle wheel was switched off for 24 h allowing the biomass to settle. Thereafter, 400 L of the supernatant were discharged and replenished with 400 L of fresh primary effluent to start the next cycle. The accumulated algal biomass was harvested after five consecutive cycles, before initiating the next set of 5 cycles. Thus, 6 sets of cycles, with 5 cycles in each set (total of 30 cycles in each reactor) were completed over 120 days.

Samples were collected and analyzed daily for optical density (OD) at 629 nm, 680 nm and 750 nm; N; and P, while BOD_5 was measured every 2 days. Since the primary effluent consisted ~1 mg/L nitrate (Table S-1) and the discharge standards are based on ammoniacal-nitrogen, this study focused only on the removal of ammoniacal-nitrogen. Biomass growth was monitored in terms of OD at 629 nm, 680 nm and 750 nm with a HACH DR 6000 spectrophotometer (1 cm cuvette). Standard procedures as described in our previous reports were followed in OD measurements to be within the linear range. Ammoniacal-nitrogen and phosphates were analyzed using a HACH DR 6000 spectrophotometer with salicylate method 8155 and phosver3 method 8048, respectively. Quantification of dissolved BOD_5 followed Standard Method 5210 B. Temperature and dissolved oxygen were recorded in 15 min interval using Onset HOBO data loggers (UA-002-64) and YSI ProODO optical dissolved oxygen meter, respectively.

Fate of coliform indicators was assessed following culture-based methods and fate of pathogenic bacteria was assessed by nucleic acid-based techniques. Fecal coliform and total coliform were assayed by membrane filtration technique using m FC and m Endo agar media (Difco, Becton Dickinson, Cockeysville, MD), respectively. Incidence of total bacteria, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Staphylococcus aureus* in the effluent was assessed by real-time PCR (qPCR) technique. Target genes of the bacteria of concern extracted from effluent samples were amplified and quantified in a Bio-Rad CFX real-time PCR system. Plasmids standards containing target gene were constructed using a TOPO TA cloning® kit (Invitrogen, by Thermo Fisher Scientific) for each qPCR run. Additionally, Illumina Miseq sequencing was conducted by a commercial laboratory (Research and testing laboratory, Texas, USA) to confirm the bacteria population in the algal effluent.

3. Results and discussion

During the 120 days of operation, culture temperature ranged 27 to 46 °C (average = 34.9 ± 4.3 °C) and dissolved oxygen ranged 5.1 to 7.8 mg/L (average = 6.2 ± 0.53 mg/L). Profiles of N, P, BOD_5, and OD (at 629 nm, 680 nm and 750 nm) recorded during one set of five consecutive fed-batch cycles in the two bioreactors are shown in Fig. 1-a to -d. Comparing the performance of the two reactors depicted in Fig. 1, the system could be seen to be highly reproducible.

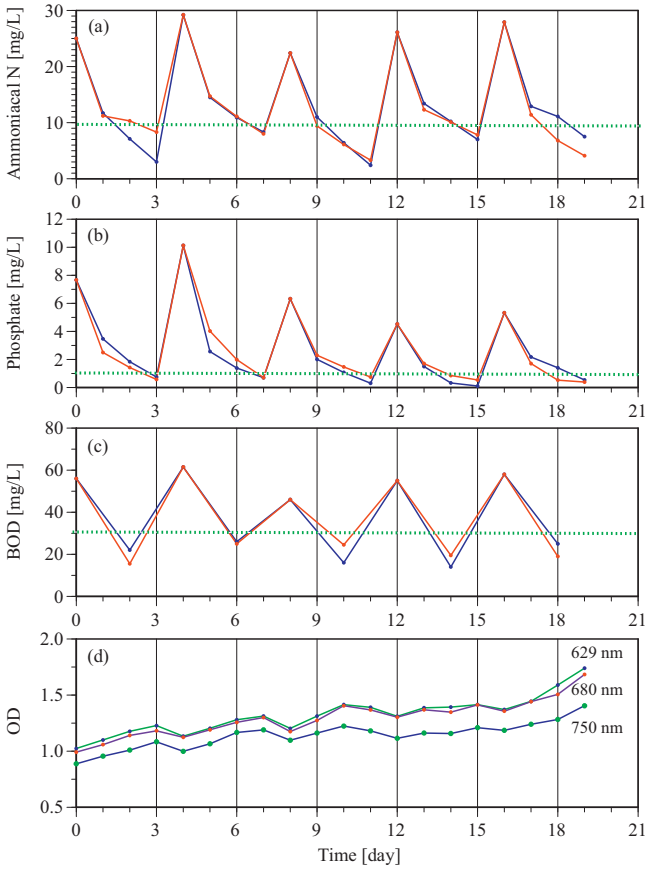

Fig. 1. Typical temporal profiles of a) ammoniacal nitrogen; b) phosphates; c) BOD_5; and d) OD at 629 nm, 680 nm and 750 nm, during 5 consecutive fed-batch cycles, each 4 days long. In a) to c), dashed lines represent discharge standards; Blue line: Reactor 1; Red line: Reactor 2. In d), average OD values from Reactors 1 and 2 are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 1-d shows that algal biomass accumulated and continued to grow at nearly the same rate throughout this set of five fed-batch cycles, reaching a maximum OD at 750 nm of 1.3. A similar trend was observed in all the six sets, with harvesting of accumulated biomass in between sets. Unlike in our previous batch operations, where a 24-h lag phase was noted at the start of each batch cycle, biomass continued to grow under the fed-batch operation without any lag (Fig. S-1(A) vs. (B) in Supplemental information section). Accumulation of the biomass growth during the fed-batch cycles (instead of batch-wise harvesting as in batch operation), enabled higher volumetric removal rates of N, P, and BOD_5 as discussed in the following sections.

3.1. Removal of ammoniacal nitrogen

During the 120 days of testing, ammoniacal-nitrogen in the influent to the system (i.e. primary effluent) averaged 33.9 ± 6.1 mg/L. From the temporal variation of N in the two bioreactors shown in Fig. 2 for over 120 days, it can be seen that the discharge standard of 10 mg/L for N could be attained in about 2 days, irrespective of the influent loading. Fig. 2 also illustrates the long-term stability and reproducibility that can be expected with this system. Since the operating pH of this system

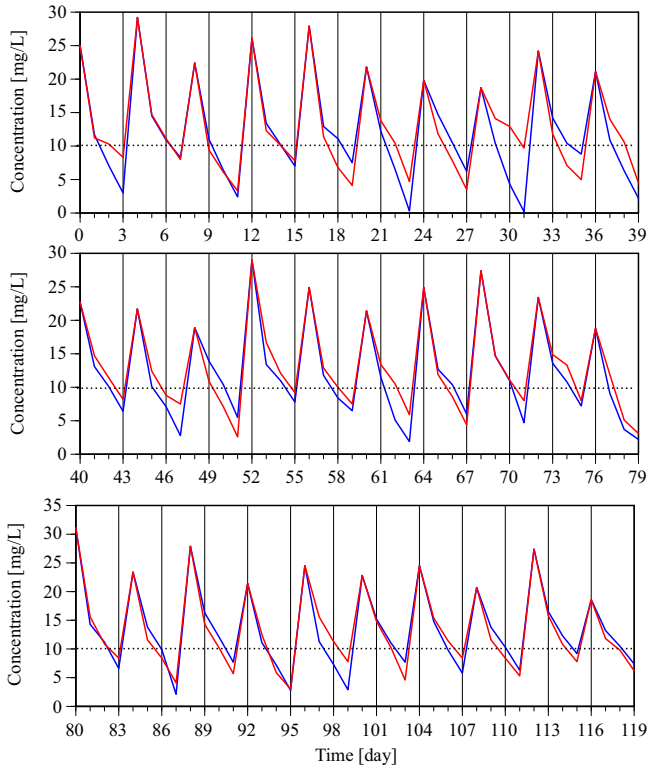

Fig. 2. Concentration of ammoniacal nitrogen in the bioreactor as a function of time.
Data collected daily over 120 days, from 30 fed-batch cycles of 4 days each, with harvesting of accumulated biomass every 5 cycles. Dashed line represents the discharge standard.
Blue line: Reactor 1; Red line: Reactor 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was < 4.0, ammonia loss by volatilization is negligible and hence, the removal calculated from the liquid phase concentrations is entirely due to biomass uptake.

Fig. 3 shows the fate of ammoniacal-N during the 3-day cycles over the 120 days. Based on this summary, it can be concluded that the system can meet the discharge standards for ammoniacal-N (10 mg/L) in a cycle time of 3 days in a reliable manner.

Volumetric removal rate of ammoniacal-nitrogen over the 120 days averaged 6.26 ± 0.92 mg/L-d in R1 and 5.91 ± 1.00 mg/L-d in R2. In our previous laboratory studies with *G. sulphuraria*, we found N removal from primary effluent in batch mode to be 4.85 mg/L-d [4]. Typical N-removal rates reported in the literature for other common autotrophic algal strains averaged to 6.36 mg/L-d [4], which is comparable to the rates found in the current study. Wang et al., for example, had evaluated the growth of *Chlorella* sp. in different wastewater samples drawn at four different points at a wastewater treatment plant [6]. Using the nutrient evolution data reported in that study, we estimated the volumetric removal rate of N to be 5.5 mg/L-d for the sample drawn after primary settling. This laboratory result of Wang et al. is comparable to the field results recorded in the current study [6]. Another study by Kapdan and Aslan evaluated N removals by *Chlorella vulgaris* in an immobilized photobioreactor fed with synthetic wastewater. Volumetric removal rates of 3 to 8 mg/L-d reported in that laboratory study are similar to the rate found in the current study [7].

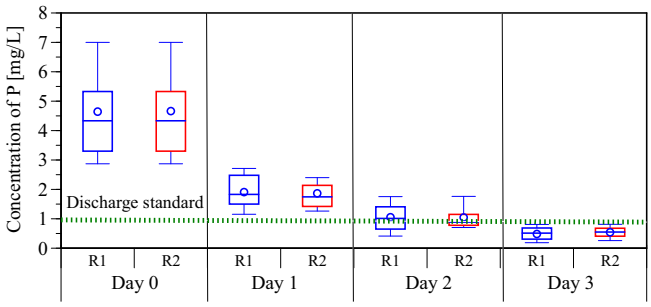

Fig. 4. Fate of phosphates (P) as a function of time. Summary of data collected daily over 120 days, from 30 fed-batch cycles of 4 days each, with harvesting of accumulated biomass every 5 cycles. R1- Reactor 1; R2- Reactor 2.

3.2. Removal of phosphates

During the test period, phosphates in the influent to the algal system (i.e. primary effluent) averaged 7.2 ± 3.1 mg/L. Fate of P in both bioreactors followed the same trend as that of N (shown in Fig. 2), reaching the discharge standard of 1 mg/L in about 2 days as summarized in Fig. 4. Based on the fate of P by day summarized in Fig. 4, it can be concluded that the system can achieve the discharge standards for P in 3 days in a reliable manner.

The volumetric removal rate of phosphates averaged 1.41 ± 0.57 mg/L-d in Reactor 1 and 1.39 ± 0.59 mg/L-d in Reactor 2. In our previous laboratory studies with *G. sulphuraria*, we found P removal from primary effluent to be 1.21 mg/L-d in batch mode [4] which is comparable to the rate recorded in the current study. Typical P-removal rates reported in the literature for other common autotrophic algal strains averaged to 1.34 mg/L-d [4], which is comparable to the rates found in the current study. Again, using the laboratory data reported by Wang et al. for *Chlorella* sp., we estimated the volumetric

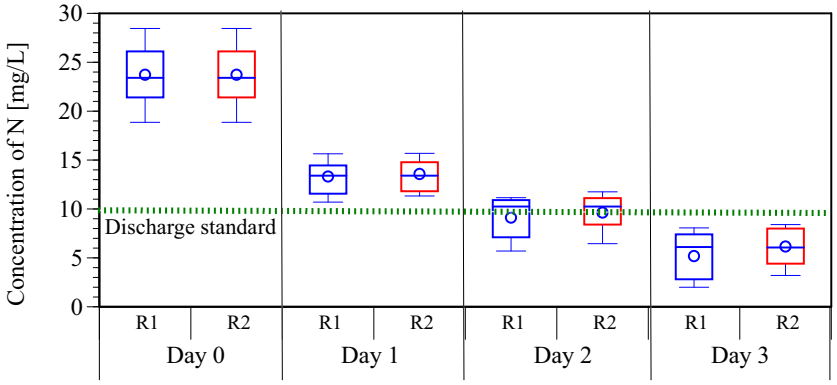

Fig. 3. Fate of ammoniacal nitrogen (N) as a function of time. Summary of data collected daily over 120 days, from 30 fed-batch cycles of 4 days each, with harvesting of accumulated biomass every 5 cycles. R1- Reactor 1; R2- Reactor 2.

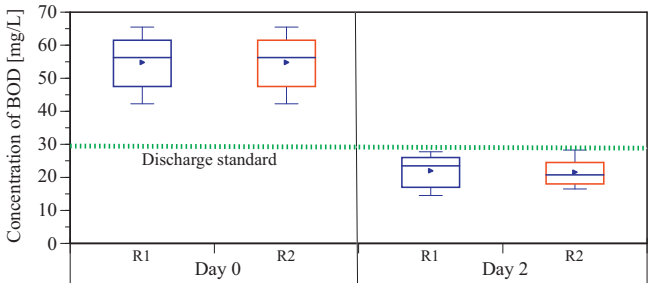

Fig. 5. Fate of 5-day biochemical oxygen demand (BOD) as a function of time. Summary of data collected daily over 120 days, from 30 fed-batch cycles of 4 days each, with harvesting of accumulated biomass every 5 cycles. R1- Reactor 1; R2- Reactor 2.

phosphate removal rate to be 3.0 mg/L-d for the wastewater sample drawn after primary settling [6]. This result of Wang et al. is nearly double the results obtained in the current field study, even though the influent phosphate concentrations in the two studies are comparable [6]. A laboratory study by Lodi et al. evaluated P-removal by *Spirulina platensis* from a standard growth medium and reported volumetric removal rates of 0.312 to 0.623 mg/L-d, about half the rate observed in the current study [8].

3.3. Removal of BOD_5

During the test period, BOD_5 in the influent to the algal system (i.e. primary effluent) averaged 80.8 ± 15.5 mg/L. Fate of BOD_5 in both the bioreactors followed the same trend as that of N (Fig. 2), reaching the discharge standard of 30 mg/L within 2 days as illustrated in Fig. 5. (As such BOD analyses were done every two days). The volumetric removal rate of BOD_5 averaged 16.4 ± 3.3 mg/L-d in Reactor 1 and 16.6 ± 3.6 mg/L-d in Reactor 2.

Most literature reports have not reported data on BOD_5 removal by algal systems to compare our results with. The study by Wang et al., had evaluated removal of chemical oxygen demand (COD) by *Chlorella* sp. in different wastewater samples drawn at four different points at a wastewater treatment plant [6]. Using the COD evolution data reported in that study, we estimated the volumetric COD removal rate to be 72.5 mg/L-d for the sample drawn after primary settling. In the case of Wang et al., the influent COD was 225 mg/L while in our field study, the influent average BOD_5 was 80.8 ± 15.5 mg/L [6].

In contrast to the traditional algal wastewater treatment systems where the BOD_5 reduction is mainly due to the coexisting heterotrophic bacteria, the BOD_5 reduction in the liquid phase in this system is attributed to algal uptake due to the following: i) inhibition of heterotrophic bacteria at the operating pH levels < 4.0 [9]; ii) increase of chlorophyll reflected by OD at 629 nm as shown in Fig. 1-d; iii) significantly low bacterial population in the reactor as detailed in our previous report [10]; iv) growth of *Galdieria sulphuraria* as detailed in our previous report [10]. In our previous report, we have assessed and confirmed the presence of *Galdieria sulphuraria* growth in this system via real-time PCR technique using LHCF (ATG GCA TTT GTT AGC ACA ACG) and LHCR′ (GTG TCA GGG AGA TAA TGG) primers [10].

3.4. Batch vs. fed-batch performance

Volumetric removal rates of BOD_5, N, and P recorded in our previous batch studies are compared with those found in this fed-batch study in Fig. 6. Improvement in volumetric removal rates under fed-batch operation averaged a factor of 2.9 for BOD_5; 1.8 for N, and 1.3 for P. As envisioned, these higher volumetric removal rates in the fed-batch mode are attributed to the accumulation of the algal biomass under fed-batch operation; whereas, the biomass generated in every cycle in the batch mode was harvested at the end of that cycle. As pointed out earlier, a lag phase of 24 h was noted in every cycle during the batch operation for acclimation (Fig. S-1b), which resulted in longer batch-processing time to achieve the discharge standard. In contrast, as shown in Fig. S1a, immediate biomass growth was noted in each cycle and at the beginning of each set of new cycles during the fed-batch operation.

3.5. Pathogen inactivation

Fecal coliform count in the primary effluent was reduced instantly to non-detectable level (< 1 CFU/100 mL) in the algal system resulting in over 7 log reduction (Fig. 7). Total coliform count was reduced to non-detectable levels (< 1 CFU/100 mL) within one day of treatment. Total bacteria population was reduced by 1.7 logs in the algal system at the end of treatment. High amount of total bacteria copies (of 1×10^7 CFU/100 mL) in the algal effluent is ascribed to the close resemblance of bacteria 16S rRNA sequences with Chloroplast 16S and Mitochondrial 18S rRNA sequences [11]. Illumina sequencing results further revealed that the entire bacterial population in the algal effluent was reduced to 17.1% of which, 16.4% was non-pathogenic *Acidobacteria* [10]. *Enterococcus faecalis* and *Escherichia coli* copy numbers were reduced to non-detectable levels (< 2 copies/mL) resulting in 3.8 and 5.4 log reductions, respectively (Fig. 7). Of special note, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Staphylococcus aureus* were absent in the primary effluent.

Based on a mechanistic study undertaken to discern the key factors causing the high bacterial inactivation noted in this algal system, it was concluded that the inactivation could be attributed primarily to the low pH conditions maintained in the reactor [12]. Additionally, the synergistic effect of high dissolved oxygen levels (5.1 to 7.8 mg/L; average = 6.2 ± 0.53 mg/L) during sunlight hours with the potential to produce reactive oxygen species could have also augmented bacterial inactivation [12]. Even though the enclosed reactor configuration enabled above ambient culture temperatures during the test period (27 to 46 °C; average = 34.9 ± 4.3 °C), temperatures below 45 °C were found to be insignificant in bacterial inactivation [12]. It has to be noted that the pH of the effluent from this system has to be adjusted to normal pH before discharge. Since normal pH might encourage some bacterial regrowth, it will necessitate final disinfection. However, based on the bacterial reductions observed in this algal system, it can be concluded that the final disinfectant demand in this case will be lower than that in the traditional secondary system.

4. Conclusions

The *Galdieria sulphuraria*-based wastewater treatment system achieved discharge standards for biochemical oxygen demand and nutrients in < 3 days. Accumulation of the algal biomass in the reactor enabled higher volumetric removal rates and lower cycle time to meet discharge standards. Inclement culture conditions in this system enabled simultaneous deactivation of bacteria of concern. Volumetric removal rates of nutrients recorded in this study are comparable to those reported for photoautotrophic systems. *Galdieria sulphuraria*-based wastewater treatment system has the potential to conserve the energy currently consumed by the secondary processes; and minimize disinfectant demand and the potential for formation of disinfection byproducts. However, its practical applicability may be limited by the need for pH adjustment before and after treatment; and energy required to harvest the biomass for energy and nutrient recovery. While the results presented so far are promising, a life-cycle analysis of the system is warranted for advancing it to full scale application.

Declaration of authors' contribution

All authors whose names listed this manuscript certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, data

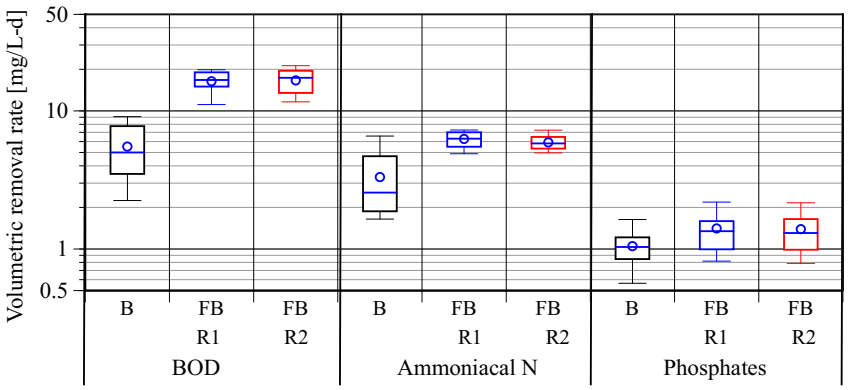

Fig. 6. Comparison of volumetric removal rates of BOD_5, ammoniacal N, and phosphates under batch (B) and fed-batch (FB) operation in two identical bioreactors, R1 and R2.

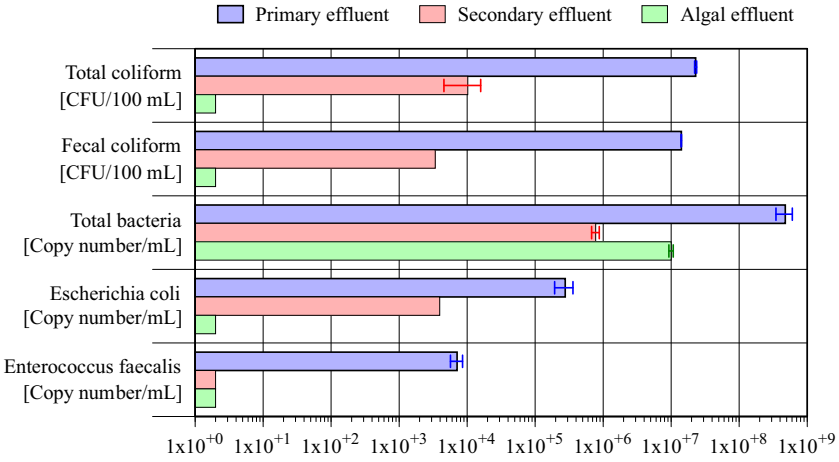

Fig. 7. Reduction of bacteria in primary effluent: secondary effluent vs algal effluent.

collection, analysis, writing, and/or revision of the manuscript.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors agreement to authorship and submission of the manuscript for peer review

All authors whose names are listed in this manuscript have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to Algal Research for peer review.

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Declaration of Competing Interest

All authors whose names are listed in this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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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.2019.101578.

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