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Factors contributing to bacteria inactivation in the *Galdieria sulphuraria*-based wastewater treatment system

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ABSTRACT

Our previous pilot scale studies showed concurrent removals of biochemical oxygen demand and nutrients, and inactivation of pathogens in urban wastewaters by an extremophilic alga, *Galdieria sulphuraria*. The current work evaluated the following as potential factors contributing to the observed pathogen inactivation, using *E. coli* as a surrogate: bacterial toxicity of algal metabolites, culture pH and temperature, sunlight, dissolved oxygen and adsorption to algal biomass. Microtox toxicity results implied that algal metabolites were free of bacterial toxicants as the toxicity in the reactor decreased from 38.8% to zero in 4 days. Low cultivation pH of 4 was identified as the primary factor influencing *E. coli* reductions. At neutral pH, under sunlight, *E. coli* were reduced to undetectable levels in 24 h. But, in the presence of live algal biomass and sunlight, *E. coli* were reduced to undetectable levels within 12 h. We attribute this accelerated reduction of *E. coli* to the synergistic effect of sunlight and elevated dissolved oxygen levels generated by biomass in the reactor. Temperatures below 45 °C and adsorption of bacteria to algal biomass did not appear to cause inactivation of *E. coli*. Simulation of pilot-scale conditions confirmed that the synergistic effects of pH, sunlight, algal biomass, and dissolved oxygen contributed to the superior fecal coliform inactivation observed in the algal wastewater treatment system.

1. Introduction

Current wastewater treatment technologies include a series of processes terminating in disinfection to meet discharge permit requirements. Common disinfection processes for pathogen control are chlorination, UV disinfection and ozonation [1]. These processes are unsustainable and have adverse consequences and/or are costly [2]. For example, chlorination may form disinfection byproducts (DBPs) that are of serious human health concern [2]. UV disinfection can be expensive due to the energy input and regular replacement of lights [3]. Technologies that can meet the discharge permits for dissolved organic carbon (DOC), nutrients, as well as pathogenic bacteria in wastewaters in a single step could be an attractive alternative to the current multi-step practice. We have developed such a single-step algal wastewater treatment system (A-WWTS) employing an extremophilic alga, *Galdieria sulphuraria*, to treat primary effluent to the discharge standards [4,5].

The extremophilic algal strain, *G. sulphuraria* can mixotrophically metabolize DOC and nutrients in wastewater. In contrast to the traditional high rate algal ponds (HRAP) [5], in the A-WWTS, we utilize an enclosed bioreactor to cultivate *G. sulphuraria* that minimizes evaporative losses, odor emissions, and invasion [4]. Our previous reports on a pilot scale A-WWTS have documented removal of biochemical oxygen demand (BOD), ammoniacal nitrogen (NH_3-N), and phosphate phosphorous (PO_4-P) to their respective discharge standards in a batch processing time of 3–4 days [4,5].

In our recent work, we have recorded high levels of pathogen reduction in the pilot-scale A-WWTS simultaneous to BOD and nutrient reductions [6]. These pathogen reductions were superior to those in the secondary section of an existing wastewater treatment plant running in parallel. When fed with the same primary effluent, 3.3 log reduction of total coliform was recorded in the existing plant. In comparison, total and fecal coliform counts in the A-WWTS effluent were below detectable limits (> 7 log reduction) [6]. Quantitative Polymerase Chain Reaction (qPCR) analysis showed a 98% reduction of total bacteria and complete removal of *Enterococcus faecalis* and *Escherichia coli* by the A-WWTS [6].

Possible factors causing fecal coliform reduction in traditional

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autotrophic algal systems have been reported as organic loading rate (nutrients), solar radiation, pH, temperature, dissolved oxygen (DO), starvation, algal toxins, attachment to algal biomass and sedimentation [7,8]. Marchello et al. [9] have attributed bacterial reductions to pH variations during photosynthesis under light, and during respiration under dark conditions. Photooxidation has also been reported to be a dominant factor in bacterial inactivation along with oxygenation [8]. Recent reports have pointed out that algal metabolites may also contribute to inactivation of fecal coliform [8]. In cultures of *Rhizoclonium implexum*, increased removal of fecal coliform was noted with increased algal biomass density [10]. This was attributed to the synergistic effect of competition for nutrients, increased pH, algal toxin, aeration, and attachment to biomass.

The current study was undertaken to discern the factors causing the high bacterial inactivation recorded in the mixotrophic A-WWTS. The motivation for this study is the stark contrast in the extreme culture conditions of *G. sulphuraria* vs. those in conventional autotrophic and alkaline algal systems. This is the first study to assess the hypothesis that inactivation of pathogenic bacteria in the A-WWTS may be due to the acidophilic and thermophilic culture conditions, photooxidation, adsorption to algal biomass, and/or inhibition by metabolites of *G. sulphuraria*.

2. Materials and methods

2.1. Pilot-scale A-WWTS

The pilot-scale A-WWTS deployed at the Las Cruces Wastewater Treatment Plant (LC WWTP) is comprised of 700-L horizontal, enclosed bioreactors fabricated from translucent polyethylene. The A-WWTS is fed with primary effluent in which, *G. sulphuraria*, is cultivated at a pH of 4.0 and temperature range of 27–46 °C [5,11]. Favorable conditions for growth of *G. sulphuraria* are maintained by adding trace metals and enriching headspace with CO_2 as detailed elsewhere [5]. Each batch run is initiated with 400 L of the primary effluent of the LC WWTP mixed with 300 L of the A-WWTS effluent from the previous batch (4:3 vol/vol). Thus, the batch processing volume is 700 L. The batch processing time depended on the time taken to reduce the initial BOD, NH_3-N, and PO_4-P levels to the respective discharge standards. It typically ranged from 3 to 4 days. Long-term stable performance of the pilot-scale A-WWTS under varying inlet loadings has been reported previously [4].

2.2. Bacterial removal experiments

Six experiments assessed individual and combined effects of the following factors on bacterial inactivation: algal metabolites (Test 1), culture pH (Test 2), culture temperature (Test 3), attachment to algal biomass (Test 4), combined effect of algal biomass and sunlight (Test 5), combined effect of sunlight and dissolved oxygen (Test 6), synergetic effect of algal biomass, algal metabolites, sunlight, temperature, and culture pH (Test 7).

Samples were collected from the primary effluent of the LC WWTP (water quality data given in Table A.1 in Supplementary data) and from the A-WWTS effluent. Samples for Tests 1–6 were filtered through 0.22 μm membrane filters to remove bacteria and algae inside. Primary effluent and A-WWTS effluent were mixed at a volumetric ratio of 4:3 (as done in the pilot-scale reactor) for Tests 2–7. *Escherichia coli* (*E. coli* ATCC 13706) cultured overnight was spiked in Tests 2–6 (10^2–10^3 CFU/100 mL) to serve as the surrogate pathogen. All tests were conducted in 225-mL flasks in triplicates under different conditions as detailed in Section 2.3. *E. coli* was quantified by the culture-based membrane filtration technique. After filtration, membrane filters (pore size: 0.22 μm) were placed on m FC broth nutrient agar medium for 24 h, incubated at 44 °C for the detection of fecal coliforms.

2.3. Experimental setup

2.3.1. Test 1: effect of algal metabolites

A Microtox® 500 Analyzer (Azur Environmental, Carlsbad, USA) was used following the 81.9% Basic Test protocol (Azur Environmental, Microtox Omni™ Software manual) of the Microtox toxicity assay to verify toxicity in samples using a marine luminescent bacterium *Vibrio fischeri*. Samples were taken from the primary effluent and from the pilot scale A-WWTS on days 0, 1, 2, 3, and 4. pH of the filtered samples was adjusted to 6–8 to minimize any toxic effects of pH. A zinc sulfate standard (100 mg/L) was used as the positive control to verify the sensitivity of the luminescent bacteria. The toxic effect was quantified as percentage inhibition, calculated from the change in luminescence following exposure to the samples for 5 and 15 min relative to the control. Toxicity was also compared and interpreted with total and fecal coliform counts in aliquots to confirm its effect. Similar to fecal coliform, total coliform colonies were enumerated using m Endo as nutrient medium and incubated at 35 °C for 24 h.

2.3.2. Test 2: effect of pH

Survival of *E. coli* at neutral conditions (pH = 7) and at the operating conditions of the A-WWTS (pH = 4) were compared to that in a control reactor without any pH adjustments (pH = 6.3). The two test reactors and the control reactor were prepared by mixing filtered primary effluent and algal effluent as described in Section 2.2. The pH of test reactors was adjusted to 4.0 and 7.0 by adding sulfuric acid or sodium hydroxide. Samples were taken at 8-hour intervals for fecal coliform enumeration.

2.3.3. Test 3: effect of temperature

Four reactors (as described in Section 2.3.2) were set up at four different temperatures; room temperature of 24 °C; 40 °C; 45 °C; and 50 °C. In this Test, the pH of all the reactors was adjusted to the optimal pH of 7.0 for *E. coli*. Samples were analyzed every 24 h to enumerate fecal coliform.

2.3.4. Test 4: effect of attachment to algal biomass

Survival of *E. coli* in the presence of heat-treated algal cells in a test reactor was compared to that in a control reactor without any algal cells to assess the effect of attachment of bacteria to algal biomass. Samples were heated at 80 °C for 2 h to kill all live algae to ensure that the test reactor contained only dead algal biomass. Our previous study [6] had confirmed that the A-WWTS effluent was free of fecal and total coliforms (Test 1 also reaffirms that no fecal or total coliforms are detected after day 1 (D1)). Thus, heat treatment at 80 °C assured no live coliforms were present to impact the test results. After heat treatment, the pH of the test and control reactors was adjusted to 7 before *E. coli* was spiked and maintained at room temperature. Reactors were allowed to settle for 2 h before analyzing the supernatant for fecal coliform every 12 h.

2.3.5. Test 5: effect of sunlight and algal biomass

Survival of *E. coli* was monitored in reactors exposed to direct sunlight, with and without algae, to assess the combined effect of sunlight and live algae on bacterial inactivation. Reactors with algae were prepared by mixing filtered primary effluent with unfiltered A-WWTS effluent at a ratio of 4:3 to mimic the pilot scale operating conditions, and the pH was adjusted to 7. To assess the impact of sunlight alone, both reactors were prepared with filtered samples and the pH was adjusted to 7. Control reactors were covered with aluminum foil and maintained outdoors while test reactors were exposed to sunlight. Samples were taken every 12 h for fecal coliform enumeration.

2.3.6. Test 6: effect of sunlight and dissolved oxygen

To evaluate the role of DO on bacterial inactivation, performance of reactors with and without aeration was compared. Filter sterilized

primary effluent and A-WWTS effluent were mixed at the same ratio of 4:3 and the pH was adjusted to 7 before spiking with *E. coli*. Aerobic conditions were maintained by continuous bubbling of air into the reactors. Control reactors were covered with aluminum foil and there was no aeration.

2.3.7. Test 7: synergetic effect on fecal coliform removal in the primary effluent

Unfiltered samples were mixed and kept under natural sunlight to simulate the pilot-scale A-WWTS. As primary effluent is laden with native bacterial species, these reactors were not dosed with any *E. coli*. Three reactors were initiated with pH adjusted to 4, 5, and 7 for comparison with a control reactor without any pH adjustment (pH = 6.3). Samples were taken every 24 h for fecal coliform enumeration.

3. Results and discussion

3.1. Effect of algal metabolites

The reduction of luminescence of the marine bacterium *Vibrio fischeri* served to assess microbial toxicity, if any, of algal metabolites. As shown in Fig. 1, high toxicity was recorded in the primary effluent (PE, 30.0%) and in the culture medium in the A-WWTS soon after mixing on day 0 (D0, 38.8%). Thereafter, toxicity in the A-WWTS decreased to zero by the end of 4 days (D4) when the discharge standards for BOD, NH_3-N, and PO_4-P were all met (Fig. 1).

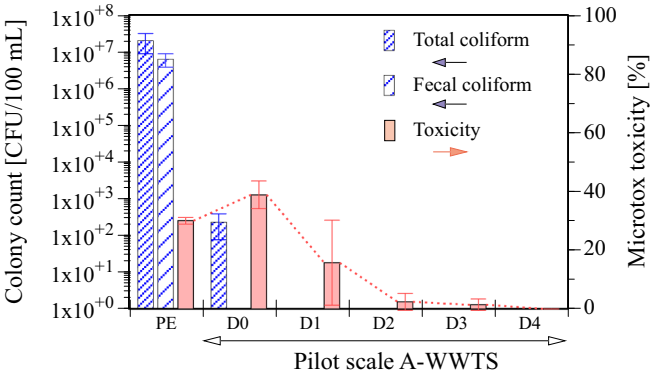

Fig. 1. Coliform counts and Microtox toxicity in samples from primary effluent (PE); and samples from the pilot-scale A-WWTS, taken daily from day 0 (D0) to day 4 (D4).

Ammonia and metal cations such as Cu and Zn and organic constituents in the primary effluent and D0 sample may have caused toxicity [12–14]. The higher toxicity on D0 in the A-WWTS over that of the primary effluent might be due to the addition of supplemental trace metals such as Mg^{2+}, Ca^{2+}, Fe^{3+} and Cu^{2+} [5] at the start of each batch to support algal growth. However, the A-WWTS could eliminate toxicity with time due to the uptake of nutrients and organics by *G. sulphuraria*. Our related studies [11] had also demonstrated that *G. sulphuraria* can remove metals such as Fe, Zn, and Cu. These results are in agreement with the findings of Ju et al. [15] and Minoda et al. [16] who reported that *G. sulphuraria* can uptake > 90% of certain metal ions in wastewater under acidic environments.

As illustrated in Fig. 1, total coliform was present in the A-WWTS (D0) even at high toxicity levels, but no fecal coliform was present. However, no coliform regrowth was detected as the toxicity decreased with processing time. These results indicate the absence of any toxic compounds causing inactivation of bacteria, although antimicrobial properties of metabolites from other red algae have been reported [17].

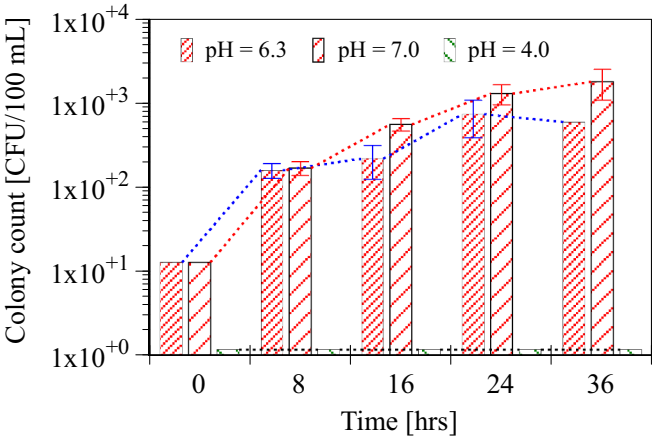

Fig. 2. Temporal variation of *E. coli* colony counts at room temperature (24.1 ± 0.2 °C) as a function of pH. *E. coli* grew at pH 6.3 and 7; but, counts dropped instantly to zero a pH 4.

3.2. Effect of pH

E. coli count was near-zero (no colonies were observed) in the test reactor maintained at pH 4 as shown in Fig. 2. The viability of *E. coli* was inhibited from the instant of their addition to the test reactor, and no regrowth of *E. coli* was observed for up to 3 days. As expected, *E. coli* continued to grow in the control reactor at pH 6.3 and in the test reactor at pH 7, as the optimal pH range for *E. coli* growth is 5.8–8 [18]. The difference in colony counts in the reactors at pH 6.3 and 7 was not statistically significant over the first 8 h ($p > 0.05$). But, the colony count at pH 6.3 was less than that at pH 7 after 8 h, suggesting that slight change of pH might also affect bacterial growth under long-term operation.

Based on the above results for the selected *E. coli* strain (ATCC 13706), low pH was important in bactericidal activity in the A-WWTS. But, it has been reported that many pathogenic bacteria can resist low pH. For instance, Audia et al. [19] reported that enteropathogens including *Escherichia coli* and *Salmonella* can survive at very low pH of 2–3 by employing different acid resistant mechanisms. Further, *Escherichia coli* O157:H7 can survive at a low pH of 2.5 [20]. Although the low pH contributed substantially to *E. coli* inactivation here, other factors which resulted in the reduction of coliforms (as shown in Fig. 1) should be explored.

3.3. Effect of temperature

Preliminary assessments (Fig. B.1 in Supplementary data) confirmed *E. coli* growth between 30 and 40 °C and die off above 40 °C. This is in agreement with literature as the optimum temperature range for *E. coli* growth is 20–40 °C [21,22], preferably 37 °C [23] when sufficient nutrients are supplied. Moreover, the operating temperature of A-WWTS had ranged 27–46 °C [11]. Hence, in this study, the impact of temperature on *E. coli* inactivation at higher temperatures of 40 °C, 45 °C and 50 °C was compared with that in a control reactor at room temperature of 24 °C.

As expected, *E. coli* continued to grow at 40 °C during the 48 h of testing (Fig. 3). However, they could not endure 45 °C for more than 24 h, even though it has been reported that *E. coli* showed a suppressed growth at 45 °C [24] with morphological changes to survive at higher temperatures [25]. The selected *E. coli* did not survive at 50 °C even with enough nutrients in the primary effluent because the inactivation rate increased with increasing temperature [26]. Based on the results in Fig. 3, we deduce that as long as the temperature is < 45 °C, then temperature is not a primary factor contributing to the observed pathogen inactivation in the pilot-scale A-WWTS.

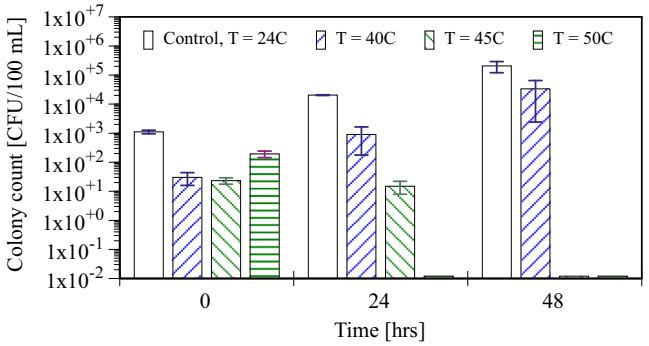

Fig. 3. Temporal variation of *E. coli* colony counts as a function of temperature when maintained at pH of 7. Inactivation occurs only at temperatures exceeding 45 °C.

3.4. Effect of attachment of bacteria onto algal biomass

Test 4 was designed to verify whether adsorption of bacteria onto algal biomass contributed to their reductions observed in the A-WWTS. Analysis of coliform counts in the supernatant revealed continual bacterial growth in all reactors (Fig. 4). There was no statistically significant difference in the number of *E. coli* between the control (without any algal biomass) and the test reactors (with heat-treated algal biomass) at any time point (minimum p-value was at 36 h = 0.08 > 0.05). Therefore, we conclude that coliform reduction observed in the A-WWTS was not due to adsorption to algal biomass. The slight increment in bacteria concentration in reactors containing inactivated algae at 48 h may be due to consumption of biodegradable algal organic matter [27].

The negligible contribution of adsorption observed in this experiment is similar to the results noted by Ansa [28]. They did not observe any *E. coli* attachment to algae (*Chlorella* sp.) after 24 h or 48 h of incubation. It was reported that bacterial attachment can occur in two modes; quick reversible attachment and long irreversible attachment [28]. Hence it is suggested that *E. coli* attachment to algae is weak, temporal and reversible which may account for the negligible attachment of *E. coli* on *G. sulphuraria* as well.

3.5. Effect of sunlight and algal biomass

Fig. 5a depicts the impact of solar irradiation on *E. coli* inactivation in the absence of any algal biomass. The contribution of algal activities in *E. coli* removal was evaluated under both dark (control) and sunlight conditions (Fig. 5b). Substantial growth of *E. coli* was observed in the dark reactor throughout the experiment. But no bacteria were detected after day 2 under sunlight. These results implied that sunlight has a direct impact on *E. coli* survival, irrespective of the presence of algal biomass. These results are consistent with those reported by Gomes et al. [29] showing a faster inactivation of *E. coli* within a few hours of exposure to sunlight.

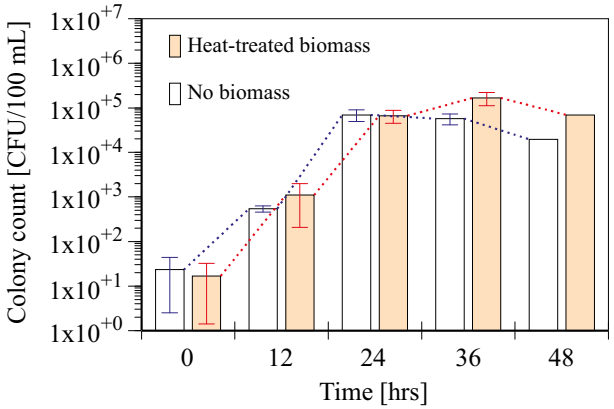

Fig. 4. Temporal variation of *E. coli* colony counts, in the presence of heat-treated algal biomass vs. in the absence of biomass, at pH 7 and room temperature (24.1 ± 0.2 °C).

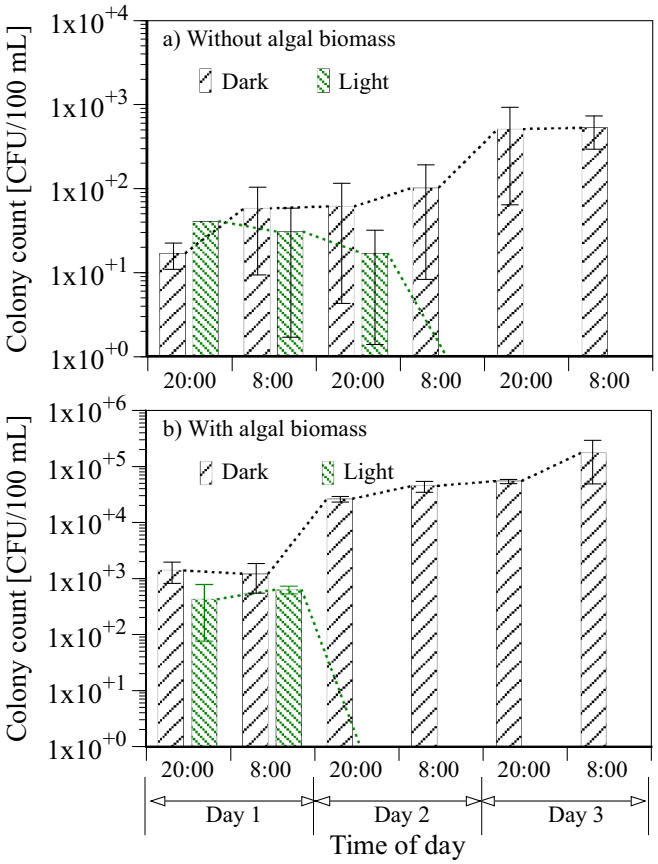

Fig. 5. Effect of sunlight on *E. coli* inactivation (a) without algal biomass (b) with algal biomass at pH 7.

Fig. 5b shows inactivation rate of *E. coli* was accelerated to non-detectable level in < 12 h in the presence of biomass. Oxygen is a key factor damaging fecal coliform when exposed to sunlight. Exogenous photo-sensitizers in water (such as humic substances) and endogenous photo-sensitizers within the bacterial cells (such as porphyrins and flavins) can pass the light energy to oxygen and form reactive oxygen species causing cell damage [30]. In the reactor with live algal biomass under sunlight, photosynthesis is believed to have generated DO. The combined effects of sunlight and DO generated by algae (evaluated in Test 6) may have accelerated inactivation of bacteria. There was no pH variation throughout the test because, *G. sulphuraria* is not capable of decreasing pH to 4 from a higher initial pH of 7 (pH at the end of the experiment was 7.8). Therefore, *E. coli* inactivation in this Test is not attributed to pH. Similarly, the temperature profile recorded during the experiment (Supplementary data, Fig. B.2) depicts that during the first 24 h of the experiment, the highest temperature was about 40 °C. This indicates that the temperature effect on bacterial inactivation in this Test is negligible.

3.6. Effect of sunlight and dissolved oxygen

Results of this test depicted in Fig. 6a show that, sunlight affected *E. coli* inactivation, agreeing with the results in Fig. 5a. Bacterial inactivation was accelerated, reaching non-detectable levels within 4 h owing to sunlight and high DO concentrations of ~7 mg/L in the

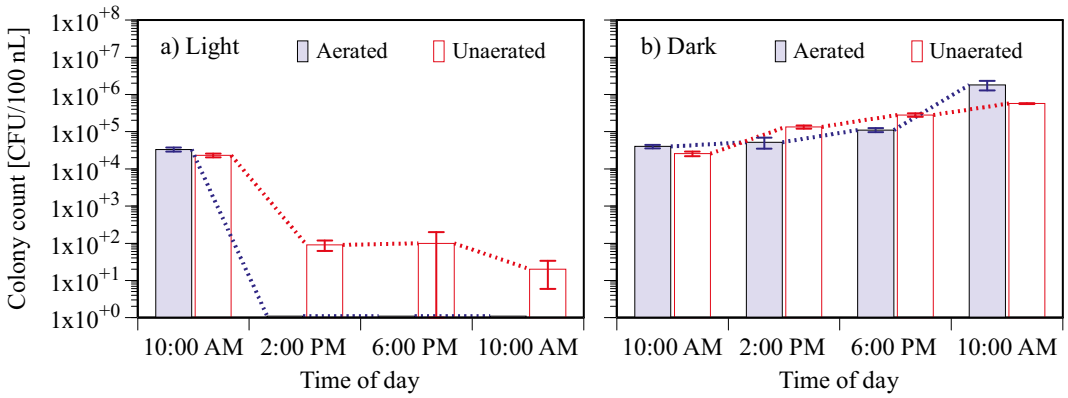

Fig. 6. Effect of dissolved oxygen on *E. coli* inactivation as a function of time at pH 7 when a) exposed to direct sunlight, with and without aeration and b) maintained under dark conditions, with and without aeration.

aerated reactors. However, Fig. 6b shows that aeration alone under dark conditions did not contribute to *E. coli* inactivation, even though the initial DO concentration was the same as in that in the reactors exposed to sunlight. This agrees with the findings of Curtis et al. [30] that oxygen could not damage fecal coliforms without the presence of sunlight.

The results in Fig. 6b may be attributed to the low concentration of reactive oxygen species (ROS) under the dark condition [8,30]. ROS are formed consequent to normal metabolism [31] and also due to sunlight absorbing sensitizers [32]. In *G. sulphuraria* cells, metabolic activities with increased mitochondrion usage under certain environmental conditions could exaggerate the production of ROS [33]. On the other hand, the dissolved sensitizers outside the bacterial cells can absorb long-wavelength (> 425 nm) light to reach an excited state which then react with oxygen to form reactive species [30]. Fluorescence Excitation-Emission Matrix (FEEM) images confirmed the presence of fulvic-like acids and humic acids sensitizers (respective areas on FEEM image were identified based on [34]) in the primary effluent (Fig. B.3 in Supplementary data). This could have contributed to the production of oxygen radicals. For instance, when sensitizers exist, hydrogen peroxide and hydroxyl radicals are formed in water saturated with oxygen under the influence of visible and infrared radiation [35]. Superoxide and hydroxyl radicals can inactivate fecal coliform [30] while hydrogen peroxide support oxidative damage as well [31].

In the pilot-scale A-WWTS, DO measurements recorded over 120 days of operation averaged to 6.26 ± 0.53 mg/L, occasionally reaching 7.8 mg/L [11]. Since the reactor was exposed to direct sunlight and the primary effluent consists of photosensitizers, results of Test 5 affirm the high levels of bacterial inactivation observed in the A-WWTS.

3.7. Synergetic effect on fecal coliform removal

Results of Test 7 shown in Fig. 7 confirmed the synergetic effects of algal biomass, metabolites, temperature, and sunlight on the inactivation of native fecal coliform in the primary effluent as a function of pH (these reactors were not spiked with *E. coli*). Overall, this test simulated the pilot-scale A-WWTS in the temporal reduction of native fecal coliform bacteria. All reactors attained a log removal > 2.5 at the end of 2 days irrespective of the pH. The fastest decay rate was recorded at a pH of 4 where no fecal coliform was detected in 24 h. Fecal coliform reductions observed at a pH of 7 indicated that factors other than pH also can contribute substantially towards their inactivation. Compared with results in Test 5 with spiked pure *E. coli*, the inactivation rate of fecal coliform was reduced at pH = 7. This might be due to the higher initial coliform concentration and diversity of fecal coliforms in the primary effluent some of which may excrete more extracellular substances to protect them from inactivation [36].

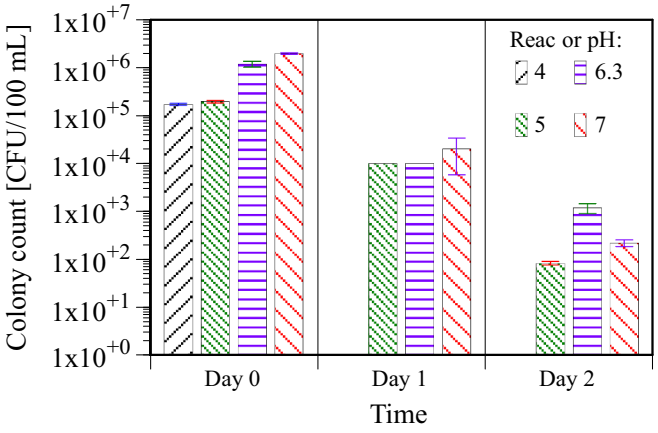

Fig. 7. Temporal variation of native fecal coliform colony counts in the primary effluent under the synergetic effect of algal biomass, toxicity, temperature, and sunlight as a function of pH.

Results of Test 7 suggest that the lower the pH, the faster the pathogen inactivation. Pertaining to the practical scenario, maintaining further low pH conditions is favorable in attaining pathogen inactivation. However, considering optimum conditions for *G. sulphuraria* growth, the cost associated with pH adjustment, downstream processing, and environmental impacts, pH 4 is considered as the optimum condition for single-step wastewater treatment.

4. Conclusions

Low cultivation pH was found to be a dominant factor contributing to *E. coli* inactivation in the A-WWTS. The effects of algal metabolites, operating temperature, and adsorption were negligible. *E. coli* inactivation was witnessed under sunlight which was enhanced in the presence of live algae. Furthermore, the combined effects of sunlight and dissolved oxygen levels contributed to the mortality of pathogenic bacteria. The results of synergistic effect of pH, temperature, sunlight, DO, and algae explained the coliform inactivation observed in the pilot-scale A-WWTS.

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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, analysis, writing, or revision of the manuscript.

Conflict of interest statement

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.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.101392.

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