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Bioremediation of Raw Landfill Leachate Using *Galdieria sulphuraria*: An Algal-Based System for Landfill Leachate Treatment

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Abstract: This study aims to evaluate the potential of using a thermophilic acidophilic red alga, *Galdieria sulphuraria* for effective on-site treatment of municipal landfill leachate (LL). This study focused on evaluating the effects of LL dilution, nitrogen loading, and initial algal biomass density on the overall treatment efficiency, and evaluated the long-term performance of the system using 5-day growth cycles. This study confirmed that optimal conditions for *G. sulphuraria* biomass production are 20% strength LL, a lower initial biomass concentration of 0.25 g L^{-1}, and the addition of N at twice the level of initial media. Furthermore, the results indicated *G. sulphuraria*'s ability to grow in elevated NH_4-N concentration (>950 mg L^{-1}) and provide nitrogen removal rates of up to 40 mg L^{-1} d^{-1}. In addition, the long-term running experiment showed that the proposed algal-based system could be applied in semi-continuous mode to achieve bioremediation. Overall, the results obtained from this study can be used to develop the necessary process parameters to implement large-scale algal-based systems for landfill leachate treatment.

Keywords: *Galdieria sulphuraria*; landfill leachate; nutrient removal; biomass production; bioremediation

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1. Introduction

Released over time from municipal landfills, landfill leachate (LL) is an aqueous waste stream containing dissolved organic matter, inorganic macro components, heavy metals, and xenobiotic organic components. LL requires considerable treatment before it can be released into the environment and can pose a significant risk to the groundwater, surface water, and adjacent ecosystems if not treated adequately [1]. In the United States, landfill operations are mandated to collect leachate under the Resource Conservation and Recovery Act of 1986 and treat the collected leachate before releasing it to the environment in accordance with the Effluent Guideline and Standards [2]. The most common method of handling and treating LL is to transfer it to a municipal wastewater treatment plant (MWTP). In MWTPs the LL is co-treated with the municipal wastewater. However, there is a growing demand to explore technologies to treat the leachate on-site, to avoid regulatory and permitting issues arising from co-treatment with municipal wastewaters. Given the

renewed paradigm shift on viewing the waste as a resource, there have been attempts to utilize the embedded chemical energy in the leachate to produce useful by-products to improve the sustainability of the treatment technology while achieving the required level of treatment [2,3]. This warrants the need for developing technologies to treat LL on-site and independently of municipal wastewater [4]. Multiple studies have demonstrated the feasibility of using an algal-based system to treat LL [3,5,6]. These algal-based bioremediation systems are often considered as an alternative to traditional LL treatment processes. These algal-based systems provide valuable environmental services by removing ammoniacal nitrogen and phosphate from the LL, and through carbon sequestration. To further improve the economics of the process, the produced algal biomass can be used for the production of biofuels, livestock feeds, and value-added products [7]. Several recent studies evaluated the potential of on-site treatment of LL using multiple algal strains [2,8,9]. Hydraulic retention time and nutrient removal rates play a vital role on the achievement of an algal-based LL in an industrial-scale application. However, most published studies utilized green algal strains such as *Chlorella* sp. and *Chlamydomonas* sp. [10–12]. These strains often require neutral pH conditions for optimized growth. This leads to a plethora of culture stability issues such as invasion of native species and subsequent competition for resources, thus reducing bioremediation rates. Moreover, past studies used a higher dilution of LL to reduce the inherent toxicity, resulting in lower nutrient removal rates and longer hydraulic retention times [4,13,14].

Galdieria sulphuraria 5587 is a thermophilic mixotrophic alga that thrives between pH 0.5–4.0 and temperatures up to 56 °C [15]. *G. sulphuraria's* ability to withstand, and grow in, extreme conditions enables the strain to set itself apart from other algal strains. Specifically, the low pH and high-temperature culture environment enable the deactivation of pathogens and competitive algal species that exist in the natural environment [16]. Furthermore, the mixotrophic nature of *G. sulphuraria* enables higher productivity through potential carbon supplementation, and helps to reduce the hydraulic retention times to achieve required levels of bioremediation [17,18]. Several past studies demonstrated *G. sulphuraria's* ability to grow in municipal wastewater for bioremediation applications [15,17]. In our previous study [2], we cultivated *G. sulphuraria* in different strengths of LL and compared the growth rates and nutrient removal with that in a standard growth media. These preliminary studies were conducted for 14 days using 50 mL photobioreactors in batch mode. These results demonstrated that *G. sulphuraria* could grow in LL with a maximum of 5-fold dilution and achieve comparable nutrient removal rates. Our past study provided proof-of-concept type data for the applicability of using *G. sulphuraria* for LL treatment [2]. However, the real-world implementation of this algal-based system for the LL treatment warrants more studies on process optimization through long-term growth trials. Therefore, to facilitate informed decision-making on this proposed algal-based LL treatment system, this study evaluates the effects of LL dilution, nitrogen loading, and initial algal biomass density on the overall treatment efficiency. In the first part of the study, we used a 2^3 full factorial experimental design to evaluate the system's performance. Then, in the second part of the study, we evaluated the system's performance in long-term experiments using 5-day cycles. Overall, this study aims to optimize the proposed LL treatment system's performance by optimizing the process parameters.

2. Materials and Methods

2.1. Culturing of G. sulphuraria and Landfill Leachate Collection

The isolates of red algae, *G. sulphuraria* CCMEE 5587.1, obtained from the Culture Collection of Microorganisms from Extreme Environments (University of Oregon), were used in this study. *G. sulphuraria* was grown in an incubator (Percival, IA, USA) at 42 °C with 24 h of continuous illumination (4000 lux) in Cyanidium media (CM) [2,19]. The cultures from the collection were streaked onto agar plates, and single colonies were picked from the culture plates to start axenic cultures on the CM, then the volume was scaled up to 1 L Erlenmeyer flasks. The CO_2 concentration inside the incubator was maintained at 3%. The following macro and micro level constituents were used to prepare the CM: $(NH_4)_2SO_4$,

1.32 g L^{-1}; KH_2PO_4, 0.27 g L^{-1}; NaCl, 0.12 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.25 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 0.07 g L^{-1}; Nitch's trace element solution, 0.5 mL L^{-1}; and $FeCl_3$ (solution = 0.29 g L^{-1}), 1.0 mL L^{-1}. The pH of the media was adjusted to 2.5 with 10 N H_2SO_4. The LL used for this research was collected from St. John the Baptist Wastewater Treatment System (LaPlace, LA, USA) and stored in a standard refrigerator at 4 °C. The characteristics of the LL used in this study are summarized in Table 1. The parameters listed in Table 1 were measured using the methods listed in Pan et al. (2021) [2].

Table 1. Characteristics of landfill leachate and Cyanidium media. Values represent the average ± SD of a minimum of 3 analytical replicates.

Parameters	Unit	Landfill Leachate	Cyanidium Media
Total Solids	g L^{-1}	23.97 ± 0.24	2.6
Chemical Oxygen Demand (COD)	mg L^{-1}	4827 ± 186	0
Total Organic Carbon (TOC)	mg L^{-1}	742 ± 16	0
Biochemical Oxygen Demand (BOD_5)	mg L^{-1}	650 ± 10	0
Ammoniacal Nitrogen (NH_3-N)	mg L^{-1}	1140 ± 30	280
Nitrate Nitrogen (NO_3-N)	mg L^{-1}	7.08 ± 0.06	0
Nitrite Nitrogen (NO-N)	mg L^{-1}	0.13 ± 0.014	0
Total Nitrogen (TN)	mg L^{-1}	1320 ± 35	280
Free Phosphate Phosphorus (PO_4-P)	mg L^{-1}	4.47 ± 0.12	61.5
Total Phosphorus (TP)	mg L^{-1}	18.8 ± 0.2	61.5

2.2. Evaluation of G. sulphuraria for Nutrient Removal from Landfill Leachate and Algal Biomass Production

2.2.1. Experiment 1

The goal of Experiment 1 was to evaluate the effect of influent LL characteristics, namely: LL loadings, initial nitrogen concentration, and initial biomass concentration on nutrient removal from the LL and algal biomass growth. A 2^3 full factorial design experiment was used in Experiment 1. For this experiment, two different initial LL media compositions (20% LL and 40% LL), two different initial ammoniacal nitrogen concentrations (1N and 2N), and two different initial algal biomass densities (0.25 g L^{-1} and 0.75 g L^{-1}) were used. These two initial biomass densities were selected to evaluate the effect of initial biomass density on the required hydraulic retention time of the system. The percentage of each LL composition represents the volume ratio of LL to the entire media, and deionized water was used to dilute the LL. For the ammoniacal nitrogen, two different concentrations were used. Set 1N represents the original concentration of ammoniacal nitrogen present in the LL after the corresponding dilution (20% and 40%), whereas 2N represents additional ammoniacal nitrogen supplementation using $(NH_4)_2SO_4$ for doubling the initial ammoniacal nitrogen present in the LL after the corresponding dilution. Based on our previous study, Pan et al. (2021) [2], an N/P ratio of 25:1 was used in this experiment to calculate the supplemental phosphate concentrations, and subsequently, phosphate was added to the media compositions using KH_2PO_4. In addition, all media compositions were supplemented with CM constituents except for $(NH_4)_2SO_4$, and KH_2PO_4. The final pH of each media composition was adjusted to 2.5 using 10 N H_2SO_4. The complete experimental design is shown in Table 2.

The inoculum algae for the experiment were grown in 1 L Erlenmeyer flasks, as described in Section 2.1. At the beginning of the experiment, the inoculum was centrifuged at 2000× *g* for 10 min at 25 °C (accuSpin 400 centrifuge, Fisher Scientific, Waltham, MA, USA) and the algae pellets were re-suspended in the eight media compositions. Each media composition was prepared in triplicate in 125 mL Erlenmeyer flasks with a working volume of 50 mL. Then the flasks were placed on a platform shaker (Innova 2000-New Brunswick Platform Shaker, Eppendorf, Enfield, CT, USA) inside the incubator with the same culture growth conditions as mentioned in Section 2.1. The algal biomass production was quantified every day. For nutrient analysis, 1 mL samples were drawn from each flask

on days 3, 6, 10, and 14. The samples were centrifuged for 10 min at 2000× *g* followed by the supernatants being transferred and stored in a refrigerator at 4 °C for further analyses.

Table 2. Media compositions for Experiment 1. For the convenience of discussion, biomass densities of 0.25 g L^{-1} and 0.75 g L^{-1} are represented as low biomass (LB) and high biomass (HB), respectively.

Group	LL Conc. %	Biomass Density g L^{-1}	Nitrogen Level	Label
1	20	0.25	1N	20% LL, LB, 1N
2	20	0.25	2N	20% LL, LB, 2N
3	20	0.75	1N	20% LL, HB, 1N
4	20	0.75	2N	20% LL, HB, 2N
5	40	0.25	1N	40% LL, LB, 1N
6	40	0.25	2N	40% LL, LB, 2N
7	40	0.75	1N	40% LL, HB, 1N
8	40	0.75	2N	40% LL, HB, 2N

2.2.2. Experiment 2

Experiment 2 was designed to evaluate the performance of the proposed system in a long-term experiment. Most of the previous experiments (including Experiment 1 in this paper) were conducted in batch mode. This experiment aimed to evaluate the nutrient removal and biomass growth rate in a semi-continuous mode. Experiment 2 was conducted for 25 days using 5-day cycles (a total of 5 cycles), using 1 L tubular bubbling column bioreactors with a working volume of 500 mL. The experiment was conducted using five reactors (n = 5). Based on the results obtained from Pan et al. (2021) [2] and Experiment 1, 20% LL with an N/P ratio of 25:1 was selected as the initial growth media for this long-term experiment. After drawing samples for biomass, nutrient, and metal analyses at the end of each growth cycle (after 5 days), all the treated LL media from all 5 reactors were mixed together. Then, 500 mL (1/5th) of the treated sample was mixed with 500 mL (1/5th) of fresh LL and 1500 mL (3/5th) of deionized water to prepare a total of 2500 mL growth media for the next cycle. This process mimicked a 5-fold dilution of the biomass density at the end of each cycle, replacing 1/5th of the total volume of the reactors with fresh LL. Then, the total 2500 mL of the newly prepared media was equally divided and added to the 5 reactors for the next cycle.

2.3. Sample Analysis

A Hanna pH meter (HI 5522, Hanna, Woonsocket, RI, USA) was used for pH and conductivity measurements. Ammoniacal nitrogen, nitrate nitrogen, nitrite nitrogen, total nitrogen, total phosphorus, and ortho-phosphorus (phosphate) were determined using a HACH DR 3900 (HACH, Loveland, CO, USA) spectrophotometer, with different standard HACH vials or powder for corresponding parameters and measurement range. Metal elements were analyzed using inductively coupled plasma optical emission spectrometry iCAP 7000 (ICP-OES) (Thermo Fisher Scientific, Waltham, MA, USA). Biomass density was quantified by measuring the optical density (OD) at the 750 nm wavelength using a spectrophotometer (HACH, Colorado, USA). The biomass density was evaluated in terms of 'ash-free dry weight' (g L^{-1}), which was correlated to OD at 750 nm by the following equation [2]:

$$\text{Ash} - \text{free dry weight} \left[\text{gL}^{-1}\right] = 0.4775 * (\text{OD@750 nm}) - 0.0163$$
$$\text{n} = 12;\ \text{r}^2 = 0.997$$

All the experiments and analytical measurements were carried out in triplicate. The averaged data were presented with error bars equal to one standard deviation. Microsoft Excel software (Version 16.0, Redmond, WA, USA) was used for the standard deviation calculations.

3. Results and Discussion

3.1. Effects of Experimental Media Compositions on the Growth of G. sulphuraria

Final biomass density and growth rate of *G. sulphuraria*, cultured in different LL media compositions, are shown in Figure 1, for 14 days. This figure shows that *G. sulphuraria* grew in all of the eight different LL media and achieved a final biomass density within a range of 1.36–3.15 g L^{-1}. The highest and second-highest final biomass densities (i.e., 3.15 and 3.09 g L^{-1}) were observed in two 20% LL–HB treatments, with three times more initial algal biomass densities than the LB treatments (0.75 vs. 0.25 g L^{-1}). In contrast, the lowest final biomass density was observed in 40% LL–LB–1N. *G. sulphuraria* started to grow rapidly in four 20% LL treatments from the initial experimental days, whereas 40% LL treatment showed a lag phase of two days. It is evident that high concentration LL has higher toxicity to algae. This finding is corroborated by the results observed in the previous study by the same authors [2]. Pan et al. (2021) [2] showed that the growth of *G. sulphuraria* was inhibited at LL concentrations above 40%. Furthermore, the inhibitory effect of LL has been previously reported for organisms such as microalgae, duckweed, bacteria, and protozoan [20–22]. Higher heavy metal concentrations in the LL are considered the main inhibitors of algal growth [23].

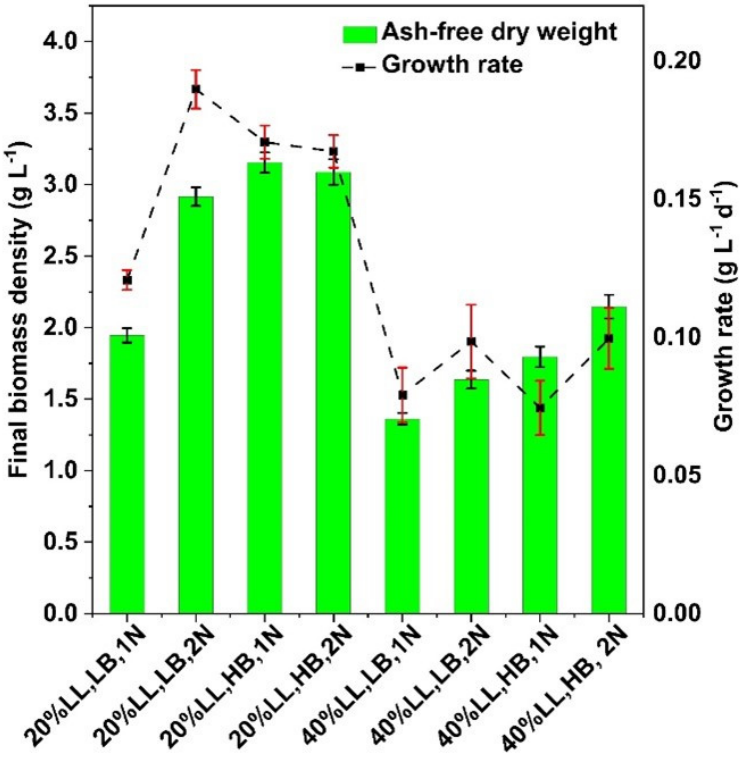

Figure 1. Final biomass density and growth rate for *G. sulphuraria* grown in different LL media compositions. Refer to Table 1 for LL media composition details.

The growth rates for all eight experiments ranged from 0.074 to 0.19 g L^{-1} d^{-1} (Figure 1). However, the growth rates in all four 20% LL treatments were much higher than the corresponding 40% LL treatment. For instance, the growth rate in 20% LL–LB–2N was 0.19 g L^{-1} d^{-1}, which was approximately two times higher than in 40% LL–LB–2N (i.e., 0.098 g L^{-1} d^{-1}). Therefore, our current findings reveal that 40% LL had a significant negative effect on *G. sulphuraria*'s growth rate and, consequently, its biomass production (R^2 = 0.92, 0.934; $p < 0.05$; Table 3). Lin et al. (2007) [10] conducted algal growth experiments at various LL concentrations of 10%, 30%, 50%, 80% and 100% using *Chlorella pyrenoidosa* and *Chlamydonomas snowiae*, and reported that 10% leachate could support algal growth, whereas a higher concentration of leachate could significantly reduce the algal growth rate.

Table 3. Statistically significant regression models.

Dependent Variable	Final Biomass Density	Growth Rate	N Removal Rate	N Removal Efficiency	P Removal Rate	P Removal Efficiency
Source	g L^{-1} Sig.	g L^{-1} d^{-1} Sig.	mg L^{-1} d^{-1} Sig.	% Sig.	mg L^{-1} d^{-1} Sig.	% Sig.
Corrected Model	0.000	0.000	0.040	0.000	0.004	0.001
Intercept	0.000	0.000	0.000	0.000	0.000	0.000
LLC	0.000	0.000	0.564	0.000	0.015	0.000
NL	0.000	0.000	0.083	0.000	0.058	0.212
IBD	0.000	0.350	0.044	0.001	0.042	0.012
LLC * NL	0.434	0.434	0.028	0.302	0.192	0.317
LLC * IBD	0.246	0.246	0.371	0.000	0.001	0.007
NL * IBD	0.014	0.018	0.583	0.047	0.408	0.288
LLC * NL * IBD	0.006	0.005	0.055	0.753	0.542	0.235
R Squared	0.934	0.92	0.553	0.953	0.685	0.727
Adjusted R Squared	0.905	0.884	0.357	0.932	0.547	0.607

Note: LLC—landfill leachate concentration; NL—nitrogen level; IBD—initial biomass density.

3.2. Effects of Experimental Media Compositions on Nitrogen Removal

The removal of ammoniacal nitrogen by *G. sulphuraria* cultured in different LL media compositions over 14 days are presented in Figure 2. NH_4-N concentrations rapidly decreased with time in all media compositions, except for two groups, namely, 40% LL–LB–1N and 40% LL–LB–2N, which nearly had no nitrogen uptake in the first 3 days. This finding can be attributed to the 2-day lag phase discussed earlier. The final (day 14) NH_4-N concentration (0 mg L^{-1}) in two 20% LL-1N groups at day 14 indicated 100% N removal. The NH_4-N removal efficiencies were 100%, 87.7%, 100%, 75.2%, 52.3%, 33%, 83.5% and 55%, respectively, for 20% LL–LB–1N, 20% LL–LB–2N, 20% LL–HB–1N, 20% LL–HB–2N, 40% LL–LB–1N, 40% LL–LB–2N, 40% LL–HB–1N and 40% LL–HB–2N. These findings indicate that the high LL loading, and double N concentration caused a significant decrease in removal efficiency ($p < 0.05$; Table 3). However, high initial biomass increased removal efficiency in most cases ($p < 0.05$; Table 3). For example, the removal efficiency increased from 52.3% to 83.5% as the biomass concentration increased in the 40% LL–1N group. The N removal rates, presented in Figure 2, were calculated from N concentrations' respective linear decline zones. N removal rates for all media compositions were in the range of 22.05 to 39.76 mg L^{-1} d^{-1}. These results were competitive with the studies conducted in the past with other algal strains, even for some strains known to be ammoniacal nitrogen tolerant algal strains [2,10]. The removal rates in past studies ranged from 2.0 to 74.6 mg L^{-1} d^{-1} [10–12,24]. Initial double N concentration increased N removal rate in all media compositions except 20% LL–HB group. Other studies have similarly reported that a higher initial N concentration increases N removal rate [5,9]. In Selvaratnam et al. (2015) [25], the nitrogen removal rate increased from 5.71 to 11.29 mg L^{-1} d^{-1} by doubling initial N concentration from 40 to 80 mg L^{-1}. Additionally, a high initial biomass concentration showed significantly higher N removal rate in all N concentration levels (i.e., ~200, ~400 and ~900 mg L^{-1}) ($p = 0.044 < 0.05$; Table 3). However, this finding was different from Selvaratnam et al. (2015) [26], who used the same algae strain to treat municipal wastewater; the study found no change in N removal rate among initial biomass density with 0.1, 0.2 and 0.4 g L^{-1}. Jia and Yuan (2018) [27] explored the effects of ammonium concentration, algae biomass, and light intensity on ammonium removal using algae–bacteria consortia and found no promotion of N removal rate when increasing initial biomass density, represented as optical density, from 1.0 to 1.4. The study inferred the possible reason being the relative low light intensity (~1000 lux) used for the experiment, which could limit the photosynthesis of algae. The light intensity of 4000 lux was used in the current study, which might be one possible explanation for the increased ammoniacal nitrogen removal observed in this study. Furthermore, the mass densities used in this study were comparatively higher than the biomass densities used in the previous two studies (0.4 g L^{-1} vs. 0.75 g L^{-1}). However, further studies are needed to validate this claim.

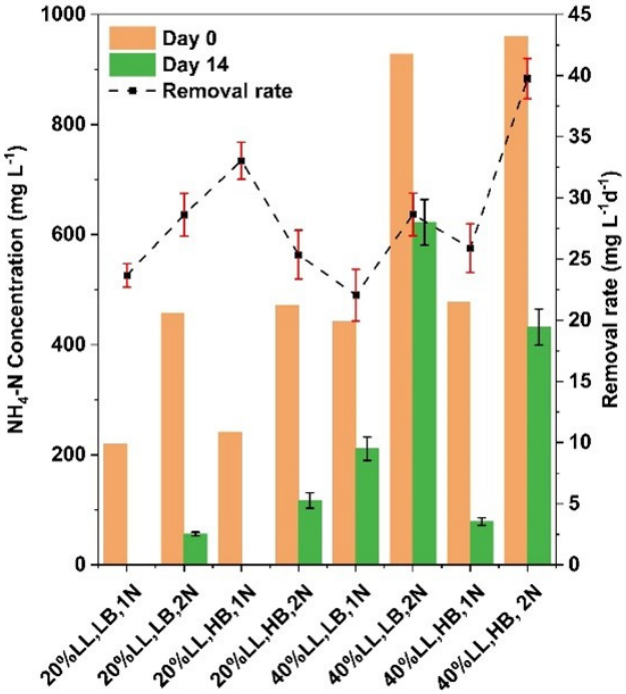

Figure 2. The initial and final concentration of NH_4-N and removal rate by *G. sulphuraria* cultured in different LL media compositions for 14 days. Refer to Table 1 for LL media composition details.

3.3. Effects of Experimental Media Compositions on Phosphate Removal

The removal of phosphate by *G. sulphuraria* cultured in different LL media compositions for 14 days is presented in Figure 3. Phosphate concentrations rapidly decreased with time in all 20% LL media, while this decline was relatively slow in 40% LL media in the first 3 days. Moreover, the PO_4-P (hereafter referred to as P) concentrations in all four 20% LL groups at day 14 were close to 0 mg L^{-1} with more than 96% P removal. In contrast, 40% LL media retained relatively more P at the end of the experiment. Percentage removal results for phosphate were 97.6%, 98%, 96.4%, 97.6%, 68.5%, 82.4%, 93.2% and 93.2%, for 20% LL–LB–1N, 20% LL–LB–2N, 20% LL–HB–1N, 20% LL–HB–2N, 40% LL–LB–1N, 40% LL–LB–2N, 40% LL–HB–1N, and 40% LL–HB–2N, respectively. This finding indicates that 40% LL had significantly lower P removal efficiency than 20% LL groups ($p < 0.05$; Table 3). For instance, P removal efficiency decreased from 97.6% to 68.5% for LB–1N group. However, double N concentration increased the phosphate removal efficiency. For example, P removal efficiency increased from 68.5% to 82.4% for 40% LL–LB groups. Furthermore, high initial biomass significantly increased P removal efficiency in most cases ($p < 0.05$; Table 3). Similar to ammoniacal nitrogen removal rate calculation, a linear decline zone of P concentration was used to calculate P removal rates. P removal rates of all groups were in the range of 1.16 to 2.00 mg L^{-1} d^{-1} (Figure 3). These rates were higher than that of the many algae species that have been previously used to treat LL [12,28]. The P removal rate in the 40% LL–HB–2N group was the highest among all groups, which was 2 mg L^{-1} d^{-1}, when initial ammonia N concentration was as high as ~1000 mg L^{-1}. Double N concentration increased P removal rate in all compared pairs. Additionally, high initial biomass concentration showed higher P removal rate in all compared pairs, except for 20% LL–2N groups ($R^2 = 0.645$, $p < 0.05$; Table 3).

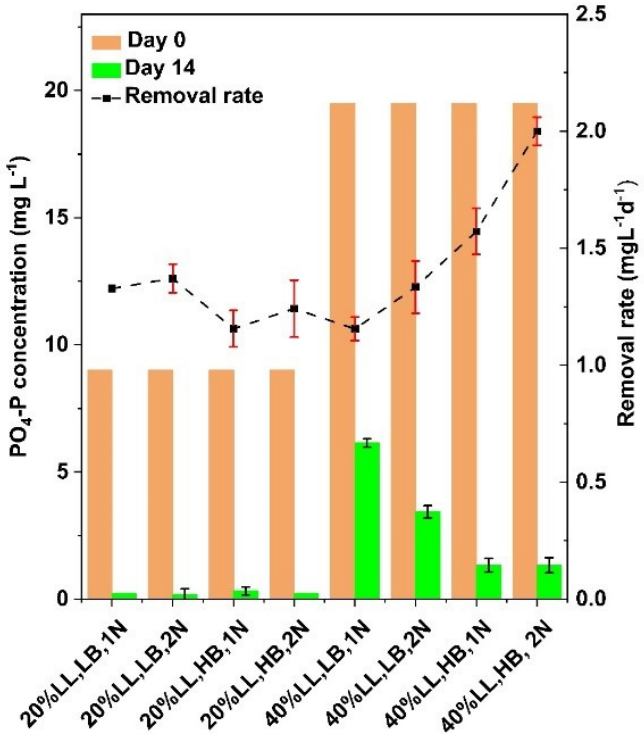

Figure 3. The initial and final concentration of PO_4-P and removal rate by *G. sulphuraria* cultured in different LL media compositions for 14 days. Refer to Table 1 for LL media composition details.

3.4. Long-Term Running Performance of Algal-Based LL Treatment System

Figure 4 shows the growth profile, NH_4-N concentration and removal efficiency, and PO_4-P concentration and removal efficiency by *G. sulphuraria* in 20% LL on a long-term running experiment. This experiment lasted for 25 days and consisted of five growth cycles with each cycle running for 5 days. Biomass concentration, NH_4-N and PO_4-P concentration were ~0.5 g L^{-1}, ~208 mg L^{-1} and ~8.4 mg L^{-1}, respectively, at the beginning of the first cycle. The algal biomass concentration, presented as ash-free dry weight in Figure 4a, increased from the beginning until each cycle's end.

At the end of each cycle, the highest biomass concentrations were 4.34, 2.88, 2.29, 2.37 and 2.22 g L^{-1}, respectively. The calculated growth rate for the five cycles were 0.768, 0.414, 0.347, 0.397 and 0.356 g L^{-1} d^{-1}, respectively. The highest biomass density was observed at the end of the first growth cycle. However, after the first two cycles, the growth performance tended to be stable in the subsequent cycles. In most semi-continuous algal experiments, the growth systems need time to stabilize biomass productivity. The initial elevated growth rates observed in the first two cycles can be attributed to the potential carryover of macro/micro-nutrients from the seed cultures. The mean final biomass density and growth rate of the last three cycles were 2.29 ± 0.072 g L^{-1} and 0.37 ± 0.027 g L^{-1} d^{-1}. Similar to the growth performance, cycles 1 and 2 showed the highest N removal performance, which further decreased in the next cycles (Figure 4b). The N % removal efficiencies for the five cycles were 99.8%, 99.9%, 91%, 82% and 71.4%, respectively. However, no completely stable performance was observed in subsequent cycles. This instability indicates that a higher cycle number would be required to attain consistent NH_4-N removal. Nevertheless, nearly complete P removal was observed as presented in Figure 4c. *G. sulphuraria* achieved almost 100% P removal efficiencies in all five cycles. This finding indicates that the N/P mass ratio of 25:1 is ideal for the long-term running of a *G. sulphuraria*-based LL treatment system. Additionally, in terms of P removal, this ratio could meet discharge standards after wastewater treatment.

Table 4 shows the periodical change of pH, conductivity, and selected metal concentrations in the LL used in this experiment. The results indicate that the pH decreased from

~2.5 to below 2.0 during each cycle. Although pH was adjusted to ~2.5 at the beginning of each cycle, the growth of *G. sulphuraria* could acidify the LL media. It has previously been reported that *G. sulphuraria* can acidify the growth media during the growth period. For example, Oesterhelt et al. (2007) [29] reported the acidification of growth media by *G. sulphuraria* from a starting pH 8 to pH 3 after 25 days. However, there were no significant changes in metal ion concentration for the presented metals between the cycles or during each cycle. A small amount of removal was observed for metals such as Mg, As, and Ca. About 50% Mn was removed from the LL in cycle 2 to cycle 5. The increase in concentrations of some metals such as Ba, Fe, and Hg were noted. Further targeted research efforts are needed to evaluate the impact of the metals present in the growth system.

Table 4. Characteristics of the landfill leachate sample at the beginning and at the end of each growth cycle. Values represent the average of n = 3 replicates.

Parameter	C1D0	C1D5	C2D0	C2D5	C3D0	C3D5	C4D0	C4D5	C5D0	C5D5
pH	2.500	1.780	2.400	1.790	2.450	1.740	2.400	1.910	2.550	1.920
EC	9.790	12.040	11.020	13.300	11.430	13.280	11.510	13.010	11.320	12.900
Ag	0.313	0.296	0.312	0.302	0.320	0.304	0.305	0.307	0.307	0.299
Al	0.380	0.390	0.441	0.472	0.461	0.521	0.498	0.490	0.443	0.465
As	5.950	5.650	6.480	6.270	6.790	6.430	6.580	6.450	6.500	6.380
Ba	0.020	0.075	0.026	0.090	0.025	0.089	0.022	0.084	0.035	0.083
Be	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.017	0.018
Ca	49.000	48.500	59.500	58.100	61.900	60.700	62.000	60.900	62.200	61.200
Cd	0.308	0.300	0.336	0.326	0.344	0.334	0.343	0.336	0.343	0.337
Ce	3.180	3.060	3.170	3.090	3.240	3.130	3.120	3.130	3.140	3.090
Co	0.345	0.340	0.355	0.349	0.359	0.352	0.356	0.354	0.355	0.351
Cr	0.377	0.375	0.387	0.384	0.392	0.387	0.386	0.388	0.387	0.386
Cu	0.495	0.510	0.499	0.484	0.486	0.472	0.474	0.474	0.477	0.476
Fe	0.061	0.371	0.519	0.568	0.439	0.535	0.357	0.475	0.304	0.495
Hg	0.656	2.290	0.605	1.620	0.558	1.290	0.502	0.952	0.472	0.762
K	190	190	211	210	219	215	216	214	220	212
Li	0.896	0.893	0.912	0.904	0.937	0.908	0.908	0.908	0.908	0.900
Mg	50.700	48.300	59.300	56.700	61.500	59.100	61.600	59.800	61.500	60.000
Mn	0.206	0.187	0.227	0.085	0.212	0.100	0.214	0.112	0.215	0.110
Mo	0.437	0.430	0.457	0.444	0.463	0.451	0.456	0.454	0.458	0.452
Na	1060	1100	1270	1340	1350	1380	1330	1440	1360	1340
Ni	0.406	0.402	0.419	0.414	0.424	0.418	0.420	0.418	0.419	0.417
Pb	1.160	1.130	1.180	1.160	1.190	1.170	1.170	1.170	1.180	1.160
Sb	1.940	1.890	2.110	2.080	2.180	2.090	2.110	2.080	2.120	2.050
Se	2.120	2.030	2.300	2.170	2.350	2.250	2.300	2.280	2.310	2.270
Si	5.280	5.040	6.580	6.220	6.950	6.520	7.350	6.570	6.890	6.480
Sn	0.717	0.710	0.746	0.736	0.757	0.745	0.756	0.749	0.754	0.743
Sr	0.888	0.882	1.040	1.030	1.080	1.060	1.070	1.060	1.070	1.070
Ti	0.272	0.269	0.273	0.271	0.275	0.271	0.271	0.271	0.272	0.270
TI	2.330	2.320	2.580	2.520	2.640	2.540	2.610	2.590	2.640	2.540
V	0.456	0.450	0.465	0.460	0.470	0.462	0.463	0.464	0.464	0.461
Zn	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

Note: BD—below detection limits; C—cycle; D—day; EC—electrical conductivity. Units of all parameters are mg L^{-1} except for pH and EC. Unit of EC is mS $cm^{-1.}$

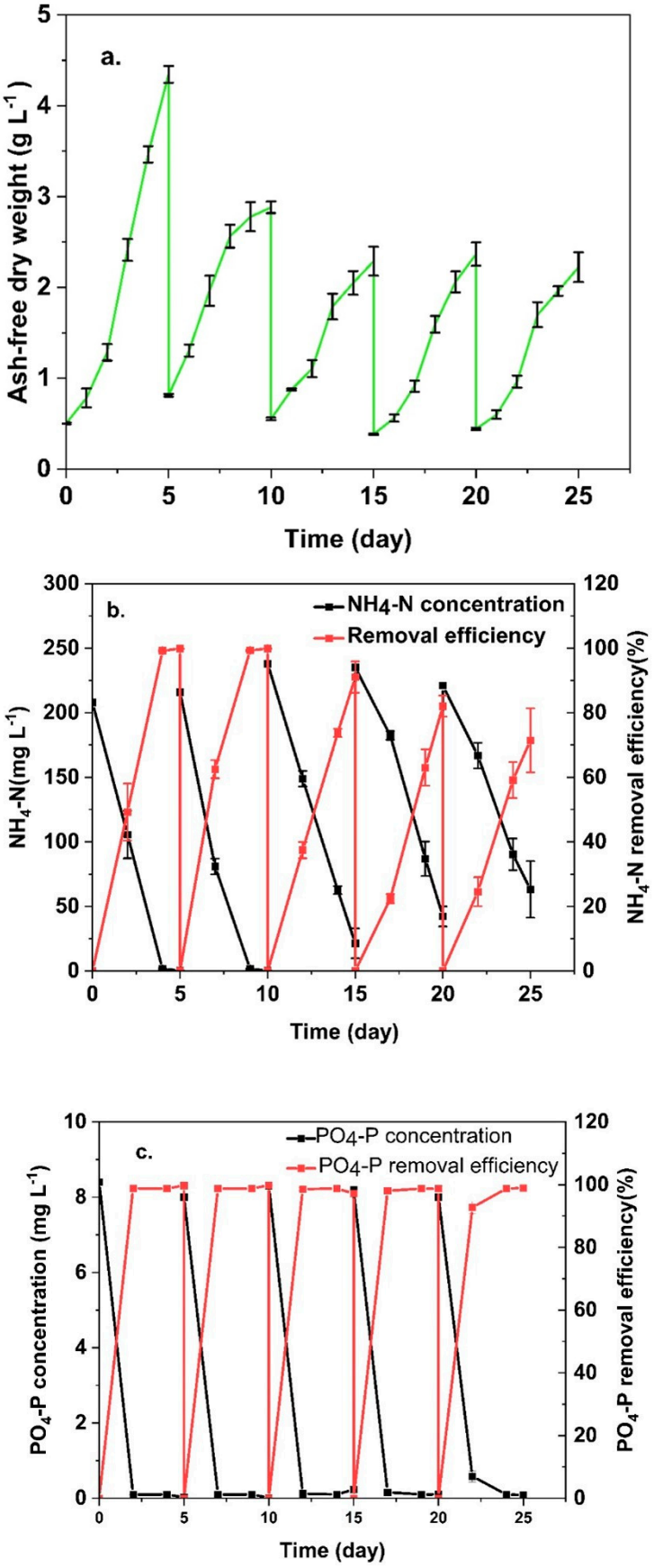

Figure 4. Growth profiles (**a**); NH_4-N concentration and removal efficiency (**b**); PO_4-P concentration and removal efficiency (**c**); by *G. sulphuraria* in 20% landfill leachate on a long-term running experiment.

3.5. Comparison between the Current Study and the Literature Reported for Long-Term Running

Comparison between this study and other literature studies in terms of nutrient removal, algal biomass production, algae species, and hydraulic retention time (HRT), for long-term LL treatment are shown in Table 5. The majority of existing studies used an algae–bacteria consortium for long-term LL treatment, except for Dogaris et al. (2019) [6], and this study. The initial concentration of NH_4-N in this study was ~244 mg L^{-1}, which was higher than most of the other studies with an average of 132.4 mg L^{-1}, excluding Martins et al. (2013) [9], in which four different ponds were used to treat the LL in a continuous feeding mode. Meanwhile, highest average N and P removal rates were obtained in this study with 43.7 and 3.99 mg L^{-1} d^{-1}, respectively, which were much higher than the previously reported values (0.88–14.36 mg L^{-1} d^{-1} for N, and 0.51–0.97 mg L^{-1} d^{-1} for P). In addition, a comparable average final biomass density (2.82 g L^{-1}) and highest growth rate (0.46 g L^{-1} d^{-1}) in the current study show a promising scale-up potential for biomass production. Moreover, the current study had the shortest HRT (5 days), which indicates a shorter treatment time and thus improves the overall sustainability of the large-scale LL treatment systems.

3.6. Limitations and Practical Implications

The results obtained from this study and our previous study [2] demonstrate the potential of using *G. sulphuraria* for the bioremediation of LL. These studies reiterated the need for a 5-fold dilution of LL to achieve comparable nutrient removal rates. Both studies were conducted in small-scale reactors and in laboratory conditions. However, algal-based systems' bioremediation potential often relies on environmental conditions such as water temperature, dissolved oxygen concentration, and daylight and dark times [13]. In addition, reactor scale plays a vital role in the system's performance. Sniffen et al. (2017) [13] observed a 20% reduction in nitrogen removal rates when comparing the performance of 1000 L ponds to 250 mL flask growth reactors. Therefore, one of the main limitations of the current study is the limited usefulness of the data obtained. The performance obtained in this study can be used only as a proof of concept for the applicability of *G. sulphuraria* for the bioremediation of LL. Therefore, using large-scale reactors (ponds or reactors in sizes comparable to commercial applications) is likely necessary to produce performance data, which can be used in predictive studies to evaluate the real-life application of this system.

Table 5. Nutrient removal and biomass production from landfill leachate using long-term experiments: this study vs. literature review.

						NH_4-N(NO_3-N)			PO_4-P			Biomass		
Source [a]	**Strain**	**Bioreactor**	**Dilution**	**Time (Cycles)**	**HRT**	**Initial Conc.**	**Removal Efficiency**	**Removal Rate**	**Initial Conc.**	**P Removal**	**P Removal Rate**	**Initial Density**	**Final Density**	**Growth Rate**
					days	**mg L^{-1}**	**%**	**mg L^{-1} d^{-1}**	**mg L^{-1}**	**%**	**mg L^{-1} d^{-1}**	**g L^{-1}**	**g L^{-1}**	**g L^{-1} d^{-1}**
A	algae–bacteria	57 L tank semibatch	5–20%	22 weeks (22)	21	5.0–90	29	3.1				0.3	0.36	0.008
B	algae–bacteria	100 L tank semibatch	1.70%	8 weeks (8)	21	3.1–14		1.02						0.024
		1000 L pond semibatch	1.70%	8 weeks (8)	21	3.1–14		0.88						0.01
C	algae–bacteria	0.25 L flask semibatch	0.38–3%	7 days	7	0.2–161		2.96				0.04–1.13		0.018
		100 L tank semibatch	0.38–3%	52 weeks (52)	21	0.2–161		2.68				0.1–1.65		0.004
		1000 L pond semibatch	0.38–3%	52 weeks (52)	21	0.2–161		2.33				0.14–1.78		0.012
D	algae–bacteria	20.33 m^3 (4 ponds continuous)	100%	111 weeks	102	805–1510	75–99	9.88						
E	*Picochlorum oculatum*	150 L horizontal bioreactor semibatch	100%, 50%	73 days (3)	73.36	350,200	82	8.99	25	76	0.97	0.5	1.67	0.049
F	algae–bacteria	10 L photobioreactor semibatch	10%	54 days (3)	19	250	98.3	13.93	10	95.53	0.51	0.34	2.92	0.142
			10%	54 days (3)	19	250	99.9	14.36	10	99.12	0.54	1.35	8.24	0.383
This study	*G. sulphuraria*	1 L tubular photobioreactor semibatch	20%	25 days (5)	5	243	88.8	43.7	8.5	98.9	3.99	0.5	2.82	0.46

Notes: Number of samples used for calculating the average values of the reported parameters. [a] [4]; B [14]; C [13]; D [9]; E [6]; F [30].

4. Conclusions

This study confirmed that *G. sulphuraria* can grow in 20% LL and achieve higher final biomass density and nutrient removal efficiencies than 40% LL composition. This study confirmed that optimal conditions for *G. sulphuraria* biomass production are 20% LL, with a lower initial biomass concentration of 0.25 g L^{-1}, and with the addition of N at twice the level of the initial media. It confirmed that the optimal conditions for both nitrogen removal efficiency and removal rate are 20% LL, with a higher initial biomass concentration of 0.75 g L^{-1} and baseline N concentration. Furthermore, the results indicated *G. sulphuraria's* ability to grow in elevated NH_4-N concentration (>950 mg L^{-1}) and provide up to 40 mg L^{-1} d^{-1} of N removal rates. The results obtained for the long-term running experiment indicated that stable nutrient removal and biomass production can be achieved using a semi-continuous mode operation of the proposed algal-based system. Overall, the results obtained from this study can be used to develop the necessary process parameters to implement a large-scale algal-based system for LL treatment.

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