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Research paper

Effects of leachate concentration, carbon dioxide and aeration flow rate on chlorophyll and carotenoid productivity and bioremediation potential of the microalga *Chlorella minutissima*

Wallyson Ribeiro dos Santos[1], Priscila Pereira[1], Lucrécio Fábio dos Santos[1], Geronimo Virginio Tagliaferro[1] and Daniela Helena Pelegrine Guimarães[1]

[1]*Chemical Engineering Department, Engineering School of Lorena, University of São Paulo, Lorena, SP, Brazil*

The use of microalgae cultures to process effluents from industries, leachates, and tanneries, among others, quantified by the reduction of metallic materials in the medium and the reduction of chemical oxygen demand (COD), helps reduce the environmental impact caused by human development. In addition, with the growth of the culture, it is possible to produce a significant amount of chlorophyll, a carotenoid of high value in the cosmetics and food industries that are used as a natural pigment. In this context, this work presents a study conducted to verify the bioremediation and chlorophyll production potential of the cultivation of the microalgae *Chlorella minutíssima*, using the Taguchi method. The microalgae *Chlorella minutissima* has given good results in the bioremediation of leachate, as a mean reduction of 33% in COD was obtained, as well as a 92% reduction in the toxic components. In addition, statistical analysis revealed that the four process factors were significant factors for chlorophyll *a*, chlorophyll *b* and carotenoid productivity ($p < 0.05$). Finally, it was observed that the maximum chlorophyll *a* (111.9 ± 0.8 mg·L^{-1}·d^{-1}), chlorophyll *b* (66.1 ± 1.7 mg·L^{-1}·d^{-1}), and carotenoid (31.9 ± 0.03 mg·L^{-1}·d^{-1}) values obtained occurred in Experiment 8, which is closer to the ideal conditions identified by statistical analysis, revealing the effectiveness of the use of the Taguchi method for the design of experiments.

CORRESPONDENCE
Wallyson Ribeiro dos Santos

EMAIL
wallysonribeiro1@gmail.com

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INTRODUCTION

According to Stamps et al. (2016 p. 534) 'landfills are the final repository of a heterogeneous mixture of liquid and solid waste from residential sources, industrial and commercial and therefore have the potential to produce slurry'. Sanitary landfills are designed to minimize damage to public health and the environment and must have effective soil sealing. They are filled with layers of waste interspersed with layers of soil, to avoid excessive leachate formation and its infiltration, as well as to reduce biogas emissions into the atmosphere (Costa et al., 2019).

Leachate is a dark and highly polluting liquid due to the high concentration of heavy metals and toxic substances, as well as the high concentration of pathogens and contaminants present in improperly disposed waste (Ghosh et al., 2023).

When in contact with the soil, manure can destroy vegetation and seriously alter the habitat of local species. Contamination of groundwater is even more serious, not only because of the consequences but also because of the difficulty in controlling leachate infiltration. Heavy metals present in manure have a strong tendency to accumulate in food chains (Singh et al., 2023; Onyedikachi, 2020).

Biological treatments have been gaining importance because they can be more economical, are more easily installed and operated, and even enable the generation of biomass that can be used in biotechnological processes. In these treatments, microorganisms, such as fungi and bacteria, are used to oxidize, aerobically or anaerobically, the organic matter present in the water, in addition to promoting the chemical transformation of other pollutants, such as metals and inorganic nutrients (Rosales et al., 2018).

In biological treatments, the use of microalgae has gained wide attention in recent decades. This is due to the fact that these photosynthetic microorganisms have the ability to use phosphorus and nitrogen from waste effluents for their growth and also have the ability to remove significant amounts of heavy metals through adsorption and absorption processes (Dias et al., 2019). Microalgae are organisms that exhibit a high growth rate with high photosynthetic efficiency (Zheng et al., 2018). Their biotechnology has promising applications in the cosmetics, pharmaceutical, food, and biofuel industries, in addition to wastewater treatment (Tavanandi and Raghavarao, 2019).

It has become vital to explore and develop alternatives that mitigate the impact of society's development. Advancing research on microalgae has revealed that various types of effluents are potential substrate sources for their growth (Tagliaferro et al., 2019a). By growing microalgae with the leachate from landfills as a source of nutrients, environmental impacts are mitigated by reducing pollutant discharge into lakes, rivers or oceans. This procedure has been studied by several researchers with excellent results (Dos Santos et al., 2021; Guimarães et al., 2019).

A genus of microalgae called *Chlorella* has been the focus of studies on its use in the treatment of effluents. In addition, this species also has a high chlorophyll yield when compared with other microalgae species.

This pigment has great commercial value for the food and cosmetic industries. The use of chlorophyll as a natural dye reduces the use of synthetic resources from petroleum (Bauer et al., 2020). Chlorophylls also have therapeutic agents in their composition that act as anti-cancer and anti-mutagenic agents, in addition to protecting DNA from ionic radiation (Paiva et al., 2021).

In this context, this study evaluated the potential of the microalgae *Chlorella minutissima* in the slurry bioremediation process, used as a substrate for microalgal growth, and carried out in a batch reactor. In addition, with the growth of the culture, chlorophyll is produced in a semi-continuous reactor, a carotenoid of high value for the cosmetics industry.

METHODS

Preparation of the microalgae inoculum

The marine microalgae *Chlorella minutissima* were used to carry out the experiments, and was obtained from the city of Ubatuba, in the state of São Paulo, using a strain donated by the Department of Biological Oceanography of the Oceanographic Institute of the University of São Paulo.

The *Chlorella minutissima* microalgae strain was conditioned in a wooden incubator equipped with a timer-controlled photoperiod and a fluorescent lamp that provided a light intensity of 15 W.

Erlenmeyer flasks of 125 mL, a photoperiod of 12 h:12 h (light:dark) and an average luminosity of 4.8 klx were used to maintain a cell bank through transplant in the containers. These transplants were performed in periods of 10 to 15 days, using a ratio of 90 mL of new culture medium to 10 mL of culture, and the flasks were shaken manually daily. For the maintenance of the mother culture, the medium Guillard (1975) was used (Table A1, Appendix), with all reagents used in its preparation being in accordance with analytical standards. To prepare the culture medium, the stock solutions were filtered using 0.22 µm filters.

The cultivation stages of the *Chlorella minutissima* microalgae described in the methodology are represented in Fig. 1.

Semi-continuous cultivation of *Chlorella minutissima* in the airlift photobioreactor

Microalgae inoculation was performed at a concentration of 0.5 $g{\cdot}L^{-1}$ and its growth was monitored in an airlift reactor with solutions composed of water and leachate collected at the landfill managed by the company Vale Soluções Ambientais (VSA), in the city of Cachoeira Paulista.

The experiments in an airlift reactor (3.8 L volume) were carried out in periods of 21 days, under controlled conditions (temperature 25 ± 2°C, continuous agitation and air supply) and permanent artificial lighting by LED lamps in combined wavelengths between blue and red. The leachate was characterized according to chemical oxygen demand (APHA/AWWA/WEF, 2012) and metal composition (USEPA, 1992). Once analysed, the microalgal culture media were prepared. To determine the concentration of chlorophyll and carotenoids, the reaction regime was changed from batch to semi-continuous in order to improve productivity.

The following factors were evaluated: leachate concentration (2.5, 5.0 and 7.5%), concentration of the carbon dioxide source (null, 1 $g{\cdot}L^{-1}$ and 2 $g{\cdot}L^{-1}$), and reactor aeration flow (0.05, 0.10 and 0.15 vvm). The values of the feed current were established based on the value of the maximum specific speed of growth (μ_{max}), using a range of 50 to 90% μ_{max}.

Microalgal growth was monitored through absorbance analysis in a spectrophotometer (UV-Vis, Bel Photonics), at a wavelength of 680 nm (Zhao et al., 2015).

To evaluate the proposed effects, the following response variables were analysed: reduction of chemical oxygen demand, reduction of metals, and productivity of chlorophylls and carotenoids. To this end, the Taguchi L9 matrix presented in Table 1 was used and the statistical analysis was performed using Statistica 13.4 and Minitab 18.

Table 1. Orthogonal arrangement of the Taguchi L9 matrix

Experiment	Factors			
	LC*	GC*	AF*	FR*
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Note: LC = landfill leachate concentration; GC = CO_2 concentration in the aeration flow, AF = aeration flow, FR = feed flowrate

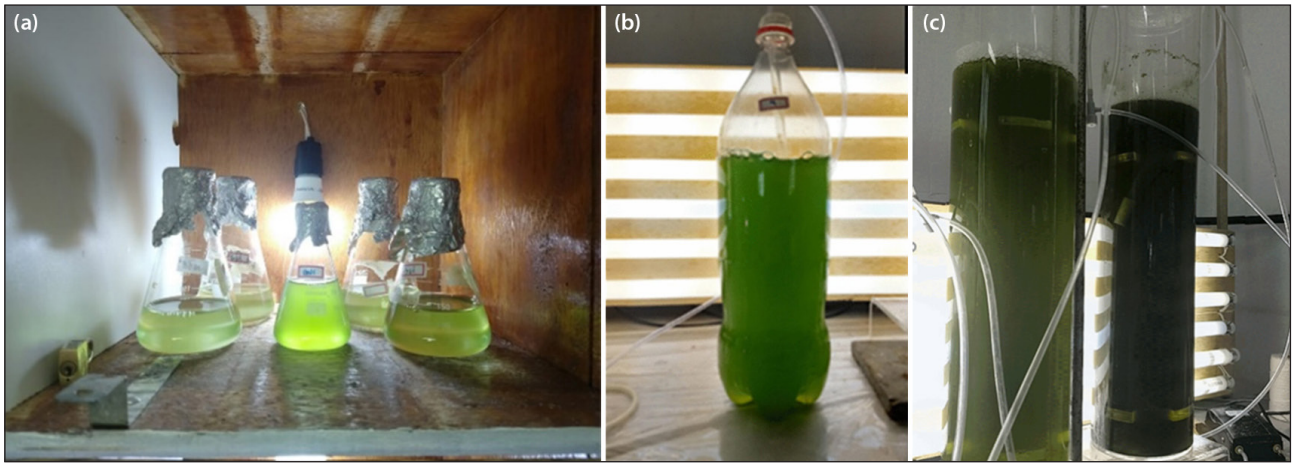

Figure 1. Stages of cultivation of *Chlorella minutissima* in landfill leachate: (a) *Chlorella minutissima* strain in the wood incubator, (b) transplant, and (c) airlift reactor cultivation stages

Analytical methods

The COD of the samples corresponding to the first and last days of cultivation was established according to Valente et al. (1997). The COD process was performed in two phases; the first being sample preparation and the second digestion and absorbance analysis.

The samples were prepared through the addition of 50 mg of mercury sulfate ($HgSO_4$), which was responsible for controlling the interference of chloride ions. It is known that chloride ions cause positive interference in the analysis, and the concentration of chloride in the case of the samples to be analysed was high, due to the microalgae (*Chlorella minutissima*) being of marine origin.

In a digester tube containing mercury sulfate, 2.5 mL of silver acid sulfate (Ag_2SO_4) (which promoted the acidity of the medium), 0.5 mL of potassium dichromate ($K_2Cr_2O_7$) (which acted as a strong oxidant), 0.3 mL of distilled water, and 2 mL of previously diluted sample were added. The samples were diluted 20 times so that the chloride ion concentration was at a level that presented minimal interference.

The digestion of the samples was carried out in a digester block at a temperature of 150°C for 1 h. After this period, the tubes were stored in a place with low luminosity for cooling. After cooling, the absorbance measurements of the samples were taken at a wavelength of 420 nm. The measured values were compared with the calibration curve, Fig. A1 (Appendix).

The extraction of metals in the cultures was done according to USEPA Method 3005a, consisting of an acid digestion procedure for the preparation of surface and groundwater samples. This method was performed by inductively coupled plasma (ICP) spectroscopy (USEPA, 1999, Method 3005a).

To perform the analysis by ICP, at the time of collection the samples of each of the cultures were filtered in a 0.45 µm filter and then acidified with nitric acid (HNO_3) at a concentration of 5 mL·L^{-1},. The samples for dissolved metals were not digested since the acid concentrations were adjusted to the same concentration. The metal extraction analysis was performed by comparing the samples with a reference in the form of the leachate *in natura*.

For the extraction of bio-pigments, the following parameters were analysed based on 80% acetone: amount of wet biomass (0.5 g or 1 g) and volume of solvent extractor (15 mL or 30 mL).

About 1 g of microalgal biomass was transferred to a Falcon tube and then the solvent extractor was added. It was then stirred into a vortex for 2 min. After finishing the homogenization, the mixture underwent sonication for 10 min to break the cell walls of the microalgae. This way, more pigment could be released. Subsequently, the Falcon tube was centrifuged for 10 min at 3 000 r·min^{-1} so that the liquid portion (composed of the solvent extractor and pigments) could be separated for analysis in a spectrophotometer. The method was repeated based on the same biomass to analyse the influence of subsequent pigment extractions.

With the absorbance data and the aid of Eqs 1, 2 and 3, it was possible to determine the pigment concentration in mg per L of sample solution:

$$C_a = 12.21A_{663} - 2.81A_{646} \quad (1)$$

$$C_b = 20.13A_{646} - 5.03A_{663} \quad (2)$$

$$C_{x+c} = \frac{1000A_{470} - 3.27C_a - 104C_b}{198} \quad (3)$$

where A_n is the absorbance analysed at wavelength n, C_a is chlorophyll-*a*, C_b is chlorophyll–*b*, and C_{x+c} is total carotenoids. Equation 4 was used to refine the data:

$$M_{C_m} = \frac{C_m dV}{1000u} \quad (4)$$

where M is the pigment concentration, d is the dilution of the sample, u is the humidity of the biomass (fixed at 0.2514), V is the volume of solvent extractor, and with C_m varying between C_a, C_b or C_{x+c}.

Statistical analysis

The results obtained were analysed using Statistica 13.5 to verify the effects of the factors (LC = landfill leachate concentration; GC = CO_2 concentration in the aeration flow, AF = aeration flow, and FR = feed flowrate) on the response variables (C_a = chlorophyll *a* productivity, C_b = chlorophyll *b* productivity, PC = carotenoids productivity) in the semi-continuous production regime. An ANOVA (*p*-value below 0.05 or 0.10) was performed an effects graph drawn for each of the factors. The effects graph enabled observation of what would be the best fit for the production of each of the response variables.

RESULTS AND DISCUSSION

COD reduction in the batch regime

Table 2 shows the COD reduction percentage for each experiment conduct in batch mode, considering the first (initial COD) and last (final COD) days of the experiment.

Table 2 shows that Experiment 6 had the highest COD reduction rate (72%). This result is consistent with other studies presented in the literature, such as that by Tagliaferro et al. (2019), in which the microalga *Chlorella minutissima 26a*, grown in a continuous airlift photobioreactor, showed reductions of 70–90% COD in media containing leachates with concentrations from 5–10%.

With the exception of Experiment 7, all other cultures showed COD reductions of at least 20%. According to the Brazilian Environmental Council (Conselho Nacional do Meio Ambiente, CONAMA, 430/2011), there are no maximum COD values prohibiting disposal in recipient bodies. The standards required by CONAMA establish a minimum BOD_5 reduction value of 60% and a maximum concentration of heavy metals in the waste (CONAMA, 2011).

Not all experimental conditions provided significant decomposition of organic matter, as can be seen in Table 2. This behaviour has been reported in the literature before, such as in Wang et al. (2010), where the growth behaviour of *Chlorella* sp. was autotrophic in one of the effluents used as a growth medium, using carbon dioxide (CO_2) as a carbon source. This caused the excretion of small molecules of glycolic acid into the environment as a result of the carbon reduction cycle. This excretion negatively affected the COD values, and in some cases changes in this parameter occurred.

Table 2. COD reduction in each experiment in batch mode

Experiment	Initial COD (mg O_2·L^{-1})	Final COD (mg O_2·L^{-1})	Reduction (%)
1	7.404	5.276	29
2	6.654	5.235	21
3	6.829	2.577	62
4	7.402	3.660	51
5	8.848	5.553	37
6	8.417	2.395	72
7	7.264	7.021	3
8	7.910	5.110	35
9	7.629	5.700	25

In the work of Paiva et al. (2021), a microalgae inoculum composed mainly of *Chlorella* sp. and *Scenedesmus* sp. was inserted into a culture medium containing 20% landfill leachate and 80% deionized water. These authors did not find a reduction in COD. On the contrary, COD increased by an average of 55%. This increase occurred due to the production of biomass and conversion of inorganic carbon into organic carbon. In the present work, however, an average reduction of 33% in COD was obtained, which suggests that the experimental conditions were favourable for the degradation of the leachate at the concentration used.

Metal extraction in the batch regime

Analysis of metal extraction achieved was performed by comparing the leachate before and after cultivation (Table 3). An average reduction range of 70–90% of all metals under analysis was observed using the *Chlorella minutissima* microalga culture. A landfill leachate concentration of 2.5% gave the best average extraction of all metals (92%).

Aluminium, titanium, chromium, and silicon were reduced by 100% in all three leachate concentrations used, highlighting the efficiency of the *Chlorella minutissima* microalga in removing these metals.

The results obtained in this analysis corroborate the results obtained by Tagliaferro et al. (2019), who used *Chlorella minutissima* with different concentrations of leachate (5, 7.5, and 10% v/v), evaluating the metal removal rate in each culture.. The authors observed the complete reduction of aluminium, and approximately 50% and 22% of chromium and iron, respectively. These values were lower than those found in this study. This was probably due to the fact that the cultivation was carried out in a continuous reactor, which may have reduced the effectiveness of the bioremediation.

Silva (2006) used *Chlorella vulgaris* microalgae cultivated in a hydroponic medium and with the application of biotechnology to analyse the reduction of metals in the medium. The study observed a reduction of phosphorus (51.90%), iron (79.54%), and zinc (60.91%), revealing the high reduction potential for these metals by *Chlorella vulgaris*. In our study using *Chlorella minutissima*, the reduction of these same metals averaged 68.33%, 80.67%, and 94.3%, respectively. These results show that the *Chlorella minutissima* microalgae have a great bioremediation capacity for metals.

Richards and Mullins (2013) evaluated 4 species of marine microalgae, *Nanochloropsis gaditana*, *Pavlova lutheri*, *Tetraselmis chuii*, and *Chetoceros muelleri*, in a mixture of leachate and artificial salt water solution. They analysed the reduction rates of aluminium and iron, obtaining percentages of 95% for both metals. These results show that the reduction of metals shown in Table 3 is in accordance with the literature, since a reduction of 100% of the aluminium was found for all of the landfill leachate concentrations studied, and a reduction of 80% occurred for iron. Reducing the aluminium content also impacts the production of pigments because a high aluminium concentration is toxic to the microalgal cells. In the work carried out by Cunha Neto et al. (2020), lead and aluminium were found to be metals capable of altering productivity for pigments. While lead impacts on the photosynthetic process and reduces the production of chlorophyll *b*, aluminium was responsible for the reduction of chlorophylls *a* and *b*. In order to avoid metal toxicity risks, this project applied leachate dilution to concentrations of a maximum of 7.5%.

Bioremediation potential of the *Chlorella minutissima* microalgae

Based on the results for COD and metals, a high bioremediation potential of *Chlorella minutissima* microalgae grown in an airlift batch reactor can be highlighted. According to experiments conducted by Richards and Mullins (2013), a reduction of metals by 95% was obtained through the cultivation of marine microalgae, revealing that other studies in the literature have already shown the potential of using microalgae cultivation to reduce the concentration of metals in various types of wastewater composition.

Other researchers have emphasized the advantages of using microalgae in the treatment of wastewaters containing metals and toxic components. Among the benefits, the following stand out, as observed in this work: low cost, no production of toxic waste, applicability in wastewater containing high metal concentrations or relatively low levels of contaminants (Suresh Kumar et al., 2015). This was corroborated in the present work by the data showing significant COD reduction and a reduction of around 92% of the analysed metals, with a low reduction rate reported only for sodium, a component that was added to the medium for the cultivation of the microalgae.

In the work of Kshirsagar (2013), bioremediation performance was assessed using COD and BOD reduction and nitrate and phosphate control, in cultures containing *Chlorella vulgaris* and *Scenedesmus quadricauda*, under controlled conditions (temperature of 27 ± 2°C and pH varying between 7.64 and 8.40) for a total duration of 20 days. The results revealed a significant bioremediation potential through reductions of 80.64%, 70.91%, 78.08%, and 62.73% (COD, BOD, nitrate and phosphate, respectively), in the parameters mentioned for the *Chlorella vulgaris* culture and 70.97%, 89.21%, 70.32%, and 81.34% (COD, BOD, nitrate and phosphate, respectively), for the *Scenedesmus quadricauda* culture. Similarly, in the present study, there was an average phosphorus reduction of 68%, but an average COD reduction of only 37.2%, which is lower than that reported for the microalgae used by Kshirsagar (2013). However, this difference is due to the different leachate concentrations used in the cited works. The COD removal complies with the parameters established by CONAMA. As such, it can be inferred that the microalgae have promising bioremediation potential.

Table 3. Average percentage of metals removal according to leachate concentration, in batch mode

Leachate (%)	Parameters	Average reduction (mg·mL^{-1})											
		Al	P	Si	Se	Cr	Mg	K	Zn	Ti	Fe	Ca	Sn
100	*In natura*	4.5	14.1	3.3	0.2	2.3	56.9	2 085	1.2	0.8	6.16	36.8	1.14
2.5	After batch	0	0	0	0	0	4.26	122.4	0	0	0.37	24.5	0.13
	Reduction (%)	100	100	100	100	100	93	94	100	100	94	33	88
5	After batch	0	4.51	0	0	0	11.7	188.8	0	0	1.66	22.4	0
	Reduction (%)	100	68	100	100	100	79	91	100	100	73	39	100
7.5	After batch	0	8.87	0	0	0	12.8	184.3	0.2	0	1.51	24.9	0
	Reduction (%)	100	37	100	100	100	78	91	83	100	75	32	100

Production of chlorophyll and carotenoids in a semi-continuous reactor

The production of chlorophyll *a* and *b* were quantified in a semicontinuous regime, and the summary of the values obtained in each experiment is presented in Table 4. The productivity value for each chlorophyll was measured in duplicate and the tabulated values represent the average chlorophyll *a* and *b* values, along with the standard deviation.

Table 4 shows that the optimum cultivation conditions for chlorophyll and carotenoid production were those of Experiment 8, which achieved productivity of 121.9 ± 14.9 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *a*, 52.1 ± 9.9 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *b* and 29.3 ± 7.1 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for carotenoids. Meanwhile, for chlorophyll *a* and carotenoids, the conditions with the lowest productivity were those of Experiment 9, with an output of 19.5 ± 17.3 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *a*, 3.5±0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *b* and 7.9 ± 6.7 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for carotenoids.

All chlorophyll and carotenoid productivity values presented in Table 4 are in accordance with that reported in the literature. In a study conducted by Bauer et al. (2020), in which the microalgae *Chlorella minutissima* were grown immobilized in alginate beads by varying the initial concentration of nitrogen in the medium, the highest chlorophyll and carotenoid outputs were 23.6 ± 0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ and 11.0 ± 0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, respectively. The lowest were 8.5 ± 0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophylls and 8.29 ± 0.31 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for carotenoids. This variation shows that the cultivation conditions applied in the present study enabled and maximized the production of chlorophylls and carotenoids through the *Chlorella minutissima* microalgae culture, since the best result obtained for chlorophyll was approximately 5 times higher than in the above-mentioned study that varied the nitrogen concentrations in the medium. In addition, the best carotenoid productivity achieved in the present this study was 2.9 times higher than that presented in the literature.

The lower results for carotenoid and chlorophyll productivity obtained in the experiments used in the study were in accordance with the productivity obtained by Bauer et al. (2020); thus the results obtained were in the expected range, even when considering the lowest productivity.

In the study by Chang et al. (2018), the production of chlorophyll from the *Chlorella vulgaris* microalgae was analysed with different reactors, comparing a traditional photobioreactor with a membrane photobioreactor. The results obtained showed maximum chlorophyll productivity of 15.45 ± 1.05 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ in the traditional photobioreactor, and maximum productivity in the membrane photobioreactor of 34.56 ± 1.34 $mg{\cdot}L^{-1}{\cdot}d^{-1}$. This shows the high chlorophyll production potential of *Chlorella minutissima*, since the highest result obtained in the present study (Experiment 8, Table 4) was an average chlorophyll productivity of 111.9 ± 0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$, a value approximately 3.2 times greater.

In addition, the experiments conducted by El Ouaer et al. (2020) with *Chlorella* sp. microalgae cultures, in a medium containing 10% leachate, obtained maximum concentrations approximately 2.5 $mg{\cdot}L^{-1}$ for carotenoids, and of 4.5 $mg{\cdot}L^{-1}$ for chlorophyll *a* and 2.6 $mg{\cdot}L^{-1}$ for chlorophyll *b*. Comparing these results with those of the present study (Table 4), it is possible to infer that the cultivation conditions of the *Chlorella minutissima* microalgae favoured the production of chlorophyll *a*, chlorophyll *b*, and carotenoids.

Statistical analysis: chlorophyll and carotenoid productivity

Table 5 shows the results of the ANOVA to identify which factors in the cultivation conditions have a significant influence on chlorophyll *a*, chlorophyll *b* and carotenoid productivity.

Figure 2 shows the effects of the response variables: chlorophyll *a* (a), chlorophyll *b* (b), and carotenoid (c) productivity, with respect to the four parameters under analysis for cultivation in a semi-continuous regime (leachate concentration, carbon dioxide concentration in the gas stream, aeration flow, and feed flowrate).

Based on the results presented in Table 5, it can be deduced that the four factors applied in the experiment are significant ($p < 0.05$) for chlorophyll *a* productivity. Taking into account the maximization of chlorophyll *a* production, Fig. 2(a) shows that the productivity for this compound reaches its highest values in a leachate concentration at 2.5 and 7.5%, a carbon dioxide concentration in the aeration flow of 1 $g{\cdot}L^{-1}$, an aeration flow of 0.05 and a feed flow of 0.9 μ_{Max}.

Chlorophyll *b* productivity (Table 5), also showed four significant factors ($p < 0.05$) in this study. Figure 2b shows that chlorophyll *b* productivity is maximized when the concentration of leachate used is 5%, carbon dioxide in the aeration flow is 1 $g{\cdot}L^{-1}$, the aeration flow is 0.05 vvm, and the feed flow is 0.9 μ_{Max}.

Carotenoid productivity was also analysed, as can be seen in Table 5. ANOVA revealed that all the factors that were varied in the experiments through the use of the Taguchi L9 Matrix (Table 1) had a significant effect ($p < 0.05$). Figure 2c shows that carotenoid production improved when the culture conditions used were 7.5% concentration of leachate, 1 $g{\cdot}L^{-1}$ of carbon dioxide (CO_2) in the flow, 0.10 vvm of aeration flow, and 0.9 μ_{Max} of feed flow.

Table 4. Productivity of chlorophyll *a* and *b* and carotenoids in a semi-continuous regime

Experiment	LC* (%)	GC* ($g{\cdot}L^{-1}$)	AF* (vvm)	FR* (vvm)	COD reduction (%)	Chlorophyll *a* ($mg{\cdot}L^{-1}{\cdot}d^{-1}$)	Chlorophyll *b* ($mg{\cdot}L^{-1}{\cdot}d^{-1}$)	Carotenoids ($mg{\cdot}L^{-1}{\cdot}d^{-1}$)
1	2.5 (1)	Null (1)	0.05 (1)	0.5 μ_{max} (1)	29	31.9 ± 1.3	23.3 ± 2.1	1.7 ± 0.4
2	2.5 (1)	1 (2)	0.10 (2)	0.7 μ_{max} (2)	21	87.3 ± 4.9	41.6 ± 0.1	25.1 ± 0.5
3	2.5 (1)	2 (3)	0.15 (3)	0.9 μ_{max} (3)	62	39.4 ± 0,1	22.7 ± 1.9	7.5 ± 0.5
4	5 (2)	Null (1)	0.10 (2)	0.9 μ_{max} (3)	51	54.7 ± 0.7	45.2 ± 2.7	29.7 ± 1.6
5	5 (2)	1 (2)	0.15 (3)	0.5 μ_{max} (1)	37	32.7 ± 1.8	19.2 ± 1.1	7.3 ± 0.03
6	5 (2)	2 (3)	0.05 (1)	0.7 μ_{max} (2)	72	49.2 ± 2.3	35.3 ± 1.6	6.3 ± 0.06
7	7.5 (3)	Null (1)	0.15 (3)	0.7 μ_{max} (2)	3	34.4 ± 1.5	15.6 ± 0.7	6.1 ± 0.5
8	7.5 (3)	1 (2)	0.05 (1)	0.9 μ_{max} (3)	35	111.9 ± 0.8	66.1 ± 1.7	31.9 ± 0.03
9	7.5 (3)	2 (3)	0.10 (2)	0.5 μ_{max} (1)	25	8.2 ± 1.4	3.5 ± 0.8	9.8 ± 0.8

Note: LC = landfill leachate concentration; GC = CO_2 concentration in the aeration flow, AF = aeration flow, FR = feed flowrate, μ_{max} = growth rate, COD = chemical oxygen demand

Table 5. Analysis of variance (ANOVA) for chlorophyll *a*, chlorophyll *b* and carotenoid productivity obtained in the semi-continuous regime

Factors	QSF	FD	AQSF	F	P
			Chlorophyll *a*		
LC	42.2895	2	21.1448	55.8861	0.000008
GC	236.3947	2	118.1973	312.3983	0.000000
AF	70.1685	2	35.0843	92.7285	0.000001
FR	311.3682	2	155.6841	411.4768	0.000000
Residual	3.4052	9	0.3784		
			Chlorophyll *b*		
LC	127.7054	2	63.8527	105.5521	0.000001
GC	218.9006	2	109.4503	180.9276	0.000000
AF	152.7355	2	76.3677	126.2402	0.000000
FR	368.8103	2	184.4051	304.8322	0.000000
Residual	5.4445	9	0.6049		
			Carotenoids		
LC	144.0571	2	72.0286	61.2525	0.000006
GC	311.6662	2	155.8331	132.5192	0.000000
AF	368.8919	2	184.4459	156.8513	0.000000
FR	513.3740	2	256.6870	218.2846	0.000000
Residual	10.5834	9	1.1759		

Note: LC = Landfill leachate concentration; GC = CO_2 concentration in the aeration flow; AF = aeration flow and FR = feed flowrate; QSF = quadratic sum of factors; FD = degrees of freedom; AQSF = average quadratic sum of factors

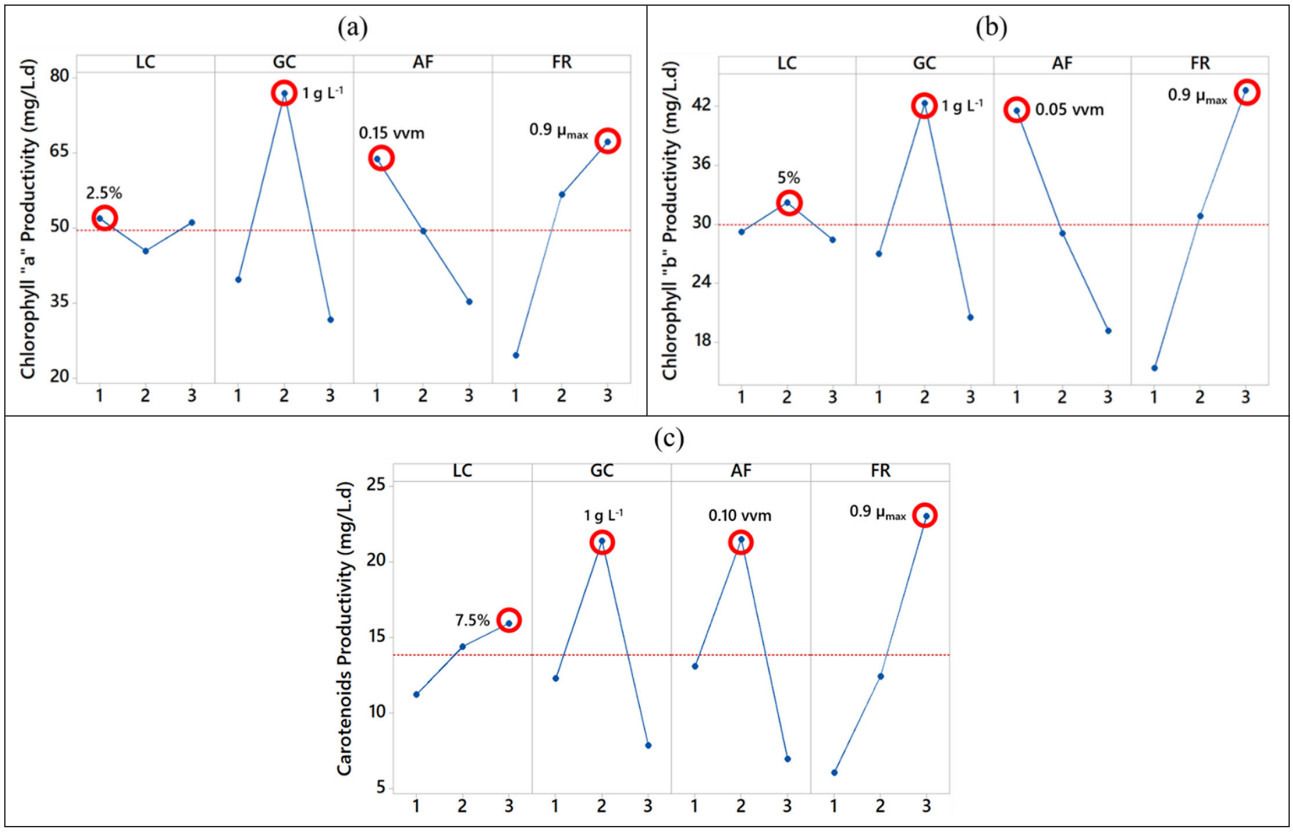

Figure 2. Effects of factors on the response variables: (a) chlorophyll *a* productivity, (b) chlorophyll *b* productivity and (c) carotenoid productivity. Note: LC = landfill leachate concentration; GC = CO_2 concentration in the aeration flow; AF = aeration flow and FR = feed flowrate

From Fig. 2, it can be observed that the conditions of 1 g·L^{-1} carbon dioxide in the aeration flow and 0.9 μ_{Max} feed flow rates were uniformly required for maximizing chlorophyll *a*, chlorophyll *b*, and carotenoid productivity. This result is in accordance with that reported by Thawechai et al. (2016), who tested different species of microalgae with different percentages of carbon dioxide in the aeration flow rate. They concluded that a higher concentration of CO_2 positively affects the production of pigments (chlorophyll and carotenoids), as the significance of the concentration of CO_2 in the aeration flow found for the *Chlorella* sp. microalgae was 90% ($p < 0.10$).

In their study, El Ouaer et al. (2017) showed that the use of a leachate concentration ratio of 10% was a significant factor in maximizing chlorophyll *a* and chlorophyll *b* yield from *Chlorella* sp. microalgae. This is also in accordance with the results of this study since a high chlorophyll *a* and *b* productivity was obtained from the *Chlorella minutissima* microalgae at a leachate concentration value of 7.5%.

Assis (2018) evaluated the significance of atmospheric emissions as a carbon source for the cultivation of microalgae in high-rate algal ponds (HRAPs), using three concentrations. In the first, CO_2 was used as a carbon source for the cultivation of microalgae at a concentration of 99%; in the second, the emission gas from gasoline combustion was used, and in the last experiment no CO_2 was added. However, no significant difference was found ($p < 0.05$) in chlorophyll *a* yields in either of the HRAPs. This shows that the experimental conditions used in the present study were more favourable for the characterization of the ideal conditions for the production of this compound, since factors with a significant impact on productivity were demonstrated.

CONCLUSIONS

Leachate as a culture medium proved to be effective for the growth of the *Chlorella minutissima* microalgae and for the production of chlorophylls and carotenoids. In addition, the use of the *Chlorella minutissima* microalgae culture in the batch was effective in bioremediation, which was revealed by the reductions obtained in the parameters analysed: COD (−72%) and metals. The chlorophyll and carotenoid productivity in a semi-continuous regime were consistent with that reported in the literature, reaching maximum values of 111.9 ± 0.8 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *a*, 66.1 ± 1.7 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for chlorophyll *b* and 31.9 ± 0.03 $mg{\cdot}L^{-1}{\cdot}d^{-1}$ for carotenoids, with all varied factors being considered to have a significant effect on productivity ($p < 0.05$). This shows that the conditions used might have high applicability in microalgae cultivation processes.

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AUTHOR CONTRIBUTIONS

DHPG: conceptualized, designed the experiments, supervised the work, reviewed and edited the manuscript. WRS: conducted the experiments and prepared the original draft of the manuscript. GVT: supervised the work and set the airlift bioreactors for the experiments. LFS: supervised the conduction of the experiments, revised the discussion of results, and reviewed and edited the manuscript. PP: revised the discussion of results, reviewed and edited the manuscript.

ORCIDS

Wallyson Ribeiro dos Santos
https://orcid.org/0000-0002-5878-056X

Priscila Pereira
https://orcid.org/0000-0001-5411-1847

Lucrécio Fábio dos Santos
https://orcid.org/0000-0002-0685-2672

Geronimo Virginio Tagliaferro
https://orcid.org/0000-0003-1988-5681

Daniela Helena Pelegrine Guimarães
https://orcid.org/0000-0002-4797-1168

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APPENDIX

Table A1. Guillard (1971) medium composition

Components	Concentration
NaCl	33.3 g L^{-1}
$NaNO_3$	75 mg L^{-1}
$NaH_2PO_4.H_2O$	5 mg L^{-1}
$FeCl_3$.6H2O	3.15 mg L^{-1}
Na_2EDTA	4.36 mg L^{-1}
$ZnSO_4$.7H2O	22.2 µg L^{-1}
$MnCl_2$.4H2O	180 µg L^{-1}
$Na_2MoO_4.2H_2O$	6.3 µg L^{-1}
$CoCl_2$. $6H_2O$	10 µg L^{-1}
$CuSO_4.5H_2O$	9.8 µg L^{-1}
Thiamine (B1)	100 µg L^{-1}
Cyanocobalamin (B12)	0.5 µg L^{-1}
Biotin (B7)	0.5 µg L^{-1}

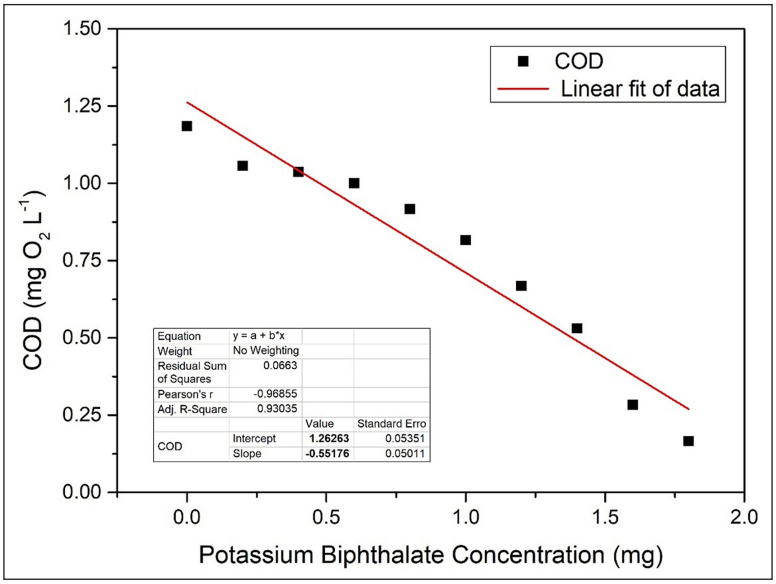

Figure A1. Calibration curve for COD determination