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Examining the robustness of selected microalgae to grow in landfill leachate

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KEYWORDS

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Robustness
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ABSTRACT

Microalgae is widely recognized as a leading candidate for future bio-oil production, serving dual purposes: biodiesel generation and bioremediation. Identifying a sustainable cultivation medium is essential, aiming to minimize production costs. Landfill leachate emerges as a prospective growth medium for microalgae. However, due to the diverse substances within landfill leachate that may impede microalgal growth, careful selection of robust species becomes crucial. This study examined the growth of four microalgae species—*Chlamydomonas reindhartii*, *Chlorella vulgaris*, *Chlorella ovalis*, and *Nannochloropsis oculata*—in landfill leachate. Prior to utilization, the landfill leachate underwent treatment to remove the total ammonium nitrogen (TAN). Cultivation spanned 30 days, during which various parameters, including ammonium removal, growth rate, and oil content, were monitored. Initially, all microalgae exhibited a decline in numbers, succeeded by a subsequent increase in concentration after several days. Results revealed *Nannochloropsis oculata* to have the highest growth rate, while *Chlamydomonas reindhartii* displayed the lowest. Generally, the oil content of all species at the end of cultivation was lower than that of their respective inocula. Chlorella vulgaris exhibited the highest oil content, followed by *Chlamydomonas reindhartii*, *Chlorella ovalis*, and *Nannochloropsis oculata*.

Introduction

Microalgae is considered as green energy producers. It grows rapidly with doubling time as short as 3.5 hours (Metting, 1996; Spolaore et al., 2006), whereas the majority have doubling times in days. Many species can produce lipids comprising approximately 80% of their dry weight, which can then be turned into biodiesel (Chisti, 2008; Brennan and Owende, 2010). The residual substrate can be used as a feedstock for methane generation by anaerobic digestion (Veerabadhran et al., 2021; Dębowski et al., 2023) or bioethanol production by fermentation process (Condor et al., 2022; Kusmiyati et al., 2023).

Microalgae is considered as an environmentally friendly biofuel feedstock. Microalgae can grow in various environmental conditions by up-taking nutrients from its growth medium. It makes microalgae have a dual role, i.e., production of fuel and bioremediation (Srikanth et al., 2009; Kalra et al., 2021; Rempel et al., 2021). An early cultivation of microalgae in nutrient-rich wastewater can be tracked back to 1980's when some species of microalgae showed a successful application in nutrient removal of wastewater (Fallahi et al., 2021; Kong et al., 2021).

The ability of microalgae to survive in nutrient-rich wastewater is also utilized in landfill leachate bioremediation (Hu et al., 2021; Porto et al., 2021). Leachate is a liquid produced by the decomposition of waste in a landfill. Leachate contains a high concentration of organic and inorganic substances by definition. A typical raw landfill leachate has a high ammonium concentration (over 1000 ppm) and a moderately high concentration of heavy metals (Richards and Mullins, 2013). Before being released into the environment, these substances should be reduced to an acceptable concentration.

The performance of some microalgae species to survive in leachate has been demonstrated in some literature (Elmaadawy et al., 2022; Liew et al., 2023). Since leachate contains a high quantity of ammonium, it is less practical to treat pure

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leachate biologically, particularly with microalgae that have the highest ammonium tolerance. For microalgae to develop in leachate, it is common practice to dilute leachate with a particular amount of water (Nawaz et al., 2020; Liew et al., 2023). However, little consideration has been given to the fact that diluting leachate with water means contaminating even more water while simultaneously attempting to cure polluted water. In other words, diluting presumably attempts to solve the water problem by polluting additional water.

Instead of dilution, pre-treatment of leachate could be done to reduce the ammonium content before microalgae application. Chemical coagulation-flocculation and precipitation are widespread practices to remove ammonium from the leachate, and it has been proved to be successful. Chemical coagulation-flocculation works by adding coagulants, either chemicals or polymer, that bond the ammonium to form a bigger aggregate that is unstable in the water (Amokrane et al., 1997; Tatsi et al., 2003). Chemical precipitation works by decreasing the ammonium solubility and works in two different mechanisms, i.e. direct and indirect. The direct precipitation works by adding an alkali chemical to adjust the pH, where ammonium has the lowest solubility, while the indirect mechanism works by transforming the ammonium into a new insoluble compound, e.g. magnesium ammonium phosphate (Li et al., 1999). In both chemical coagulation-flocculation and precipitation, further treatment is needed to remove the sediment from the site.

Ammonium stripping is a technique to remove ammonium from polluted water and landfill leachate, and is considered as the most economical practices of ammonium removal from wastewater (Ozturk et al., 2003). It works by transforming the ammonium from a liquid phase (ammonium ion) into a gas phase (ammonia) using aeration. The removal of ammonium varies from 10% up to 95%, with the initial ammonium concentration up to 4000 ppm (Calli et al., 2005; Campos et al., 2013). Various parameters are suggested to influence the efficacy of air stripping in removing ammonium, including air flowrate, liquid pH, and inlet air temperature utilized during the stripping process (Cheung et al., 1997; Kurniawan et al., 2006; Campos et al., 2013). Most air stripping treatments are conducted in an alkali condition, where most ammonium is in the form of ammonia gas. A pH between 10 to 12 was suggested for the removal of ammonium from the leachate (Kabdaslı et al., 2000; Campos et al., 2013). Given these considerations, ammonium stripping is chosen in this study as a preferred pre-treatment step before cultivating microalgae in landfill leachate. While the high ammonium concentration can be tackled by pre-treatment, some issues remain exist with the reduced-ammonium leachate. High pH, high foamability, high heavy metal, and other unknown toxic chemicals are some of the issues (Cheung et al., 1993). Some microalgae species might grow well in this treated leachate while other will not survive.

Current literature extensively explores microalgae's potential in bioremediation and biofuel production. Yet, there remains a significant knowledge gap regarding the specific species of microalgae and their performance in specific environments, particularly in treated leachate. This research aims to bridge this gap by observing the robustness of various microalgae species in treated leachate. Additionally, it seeks to investigate their efficiency in nutrient uptake and oil production within this unique medium. By focusing on these aspects, this study endeavours to contribute valuable insights into the feasibility and optimization of using microalgae for environmental and energy applications in leachate-treated settings.

Research and Methods

Materials

The leachate sample was obtained from a landfill leachate site in Midland, the United Kingdom. Once taken, the samples were stored at room temperature (under 20^{o}C) and prevented from any direct light. The leachate was allowed to settle before a stripping treatment and no filtration was done for the leachate. This raw leachate was characterized according to its pH, chemical oxygen demand (COD), ammonium concentration, phosphate concentration, and heavy metal concentration. The COD characterization was done using a COD analysis kit from LCK 014 from HACH LANGE. Ammonium analysis was done using an ammonium probe from Cole Parmer with the calibration curve obtained using standard ammonium liquid provided by the company. Phosphate analysis was done using the phosphor-molybdate assay.

The raw leachate obtained from the landfill site had a high ammonium concentration (1500 ppm) and was not suitable for any microalgae to grow. Rather than diluting the leachate, a treatment was done to reduce the ammonium before using it as a growth medium. The leachate treatment and its optimum treatment are not intensively discussed in

this report since it was a disposal of different study. In brief, the ammonium reduction was done by applying an air-stripping treatment. The treatment was done in a batch with only about 80 ml of raw leachate for each treatment. The induced air temperature was 80°C for 30 minutes, with the air flowrate was set to be 1 L/min.

Four different species of microalgae in pure culture were used in the experiment. They were *Clamydomonas reindhartii*, *Chlorella vulgaris*, *Chlorella ovalis*, and *Nannochloropsis oculate*. About 15 mL of the inoculum of each microalga was placed in 15 mL Falcon tubes and let it settle overnight (approximately 12 hours), Then, the upper layer was disposed of, and the 8 mL remaining inoculum was used for the experiment. Slow natural settling was chosen rather than centrifugation to allow the natural density of the microalgae solid before putting them into the reactor. The number of inoculums added to each reactor was equal to 0.48±0.05 g of dry weight.

Microalgae cultivation

Microalgae were cultivated in a 180 mL glass photobioreactor (Figure 1) with about 150 mL of working volume. A ceramic diffuser size pore-4 standard was placed on the bottom of the reactor to give an aeration to the microalgae. Since the pH of the treated leachate was quite high (9.57 to 10.15), about 5 % carbon dioxide was diffused into the bioreactor for 24 hours a day. The 5% CO2 concentration was provided by mixing pure carbon dioxide and air. To get the targeted concentration, a rotameter was used for each gas stream, and the final mixed gas pressure was 1 bar. The flow rate of the gas induced was maintained to be about 0.05 litre per minute. A rotameter for each reactor was used to maintain the flow rate. The carbon dioxide was induced 24 hours before the cultivation started to allow pH reduction and reduce foaming. This CO_2 gas induction was successful to maintain the pH medium in an acceptable condition (Figure 2). The turbidity of the landfill leachate before and after treatment are presented by the optical density value, as shown in Figure 3.

Figure 1. Glass photobioreactor for cultivating microalgae

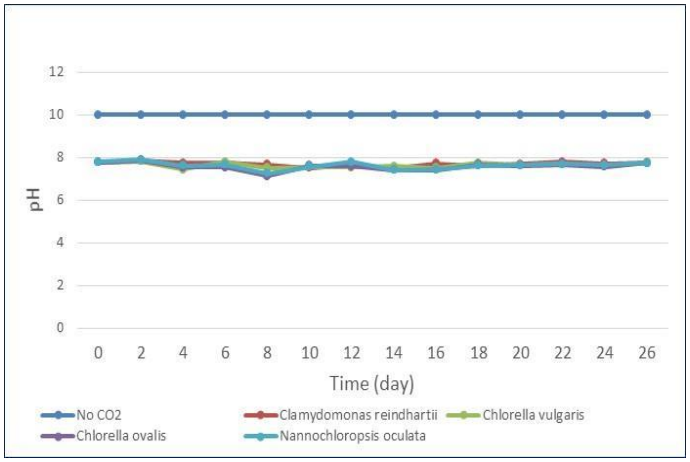

Figure 2. Daily pH of the medium

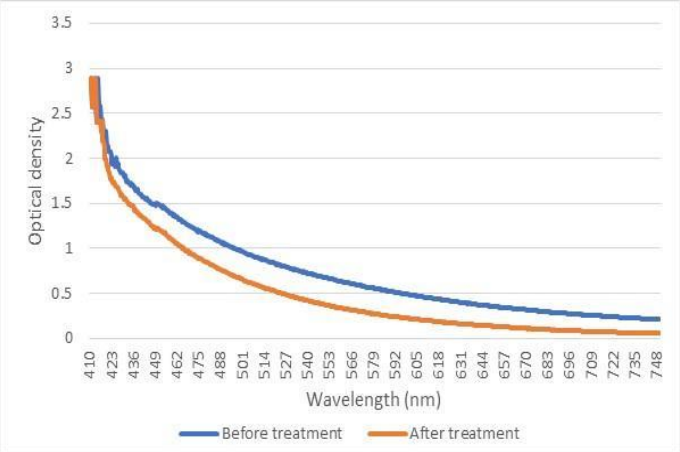

Figure 3. Optical density of the leachate: before and after the treatment

To provide the light, 4 fluorescence lamps were used for 24 hours a day. It gave an average light intensity of about 5000 lux for each reactor. Since the reactor temperature was not controlled, it followed an ambient temperature that slightly fluctuated between 18°C to 22° C, while the temperature of the medium was about 4°C higher than the ambient temperature. The higher temperature of the medium occurred probably due to the absorbed radiation from the light source.

Measured parameters

Several parameters were measured on a regular basis, while others were measured only in the beginning and end of the process. The ambient and growth medium temperatures, as well as the pH were monitored daily. Manual measurements were done using thermometer and pH probe for temperature and pH, respectively.

The ammonium concentration of the growth medium during microalgae cultivation was measured every two days. While for the effect of the ammonium removal by the hot microbubble treatment, only initial and final concentration was measured. Measuring the ammonium concentration was carried out by using ammonium probe from Cole Parmer. To reduce any reading error, calibration of the ammonium probe was done every two days just before it was used for measuring the concentration of the growth medium. The calibration liquid was made using an ammonium liquid standard 1000ppm. Different concentrations were made by diluting the liquid standard.

Chlorophyll content was used as a measured parameter of the growth. It follows a method by (Chen and Vaidyanathan, 2013) with some modifications. To analyze the chlorophyll content, about 2 mL of liquid samples wwere collected every two days using manual pipet and put them in 15 mL of Falcon tube. The samples were centrifuged for 15 minutes in 5000 rpm. After the liquid was taken out, the tube was filled with 2 mL of 95% of methanol. This concentration was provided by mixing pure methanol with deionized water to get a targeted final methanol concentration. The tubes were then closed tightly, covered with aluminum foil to prevent any light exposure, and put it into a water bath. The water bath treatment was carried out at 50°C for 2 hours. After that, the samples were once more centrifuged at 5000 rpm for 15 minutes. The optical density of the liquid was measured using a spectrophotometer with wavelengths 416 and 453 nm. To obtain the chlorophyll content, the following formula was applied (Chen and Vaidyanathan, 2013):

$$C_a = 6.40 * A_{416} - 0.79 * A_{453} \quad \text{.....(1)}$$
$$C_b = 5.87 * A_{453} - 0.24 * A_{416} \quad \text{.....(2)}$$
$$C_T = C_a + C_b \quad \text{.....(3)}$$

The total lipid was measured gravimetrically using chloroform-methanol-based solvent (Bligh and Dyer, 1959) with some modification. Each sample was divided into two parts to increase the confidence level in case of any error in the analysis process. Briefly, the frozen samples were dried using a freeze drier for 4 hours. Chloroform-methanol solvent (2:1) was added to the dried sample. Samples were mixed well using a vortex for 1 minute, and then placed for sonication for 3 x 8 minutes and mixed well one more time using a vortex stirrer for 1 minute. To make sure the extraction occurred well, the samples were soaked in the solvent for overnight. A centrifugation was done to separate the solid-liquid. Two further separate the two layers of oil and solvent, the liquid was put inside the fume cupboard for overnight. A

microscale with an accuracy of 0.1 µg was used to weigh the oil. The lipid content of the microalgae was expressed in microgram of lipid per milligram of dry biomass.

Results and Discussion

Leachate treatment

The raw leachate received from the landfill site had a high ammonium concentration (1571 ppm), and the salinity was 21‰. The amount of ammonium in this raw leachate is toxic for most microalgae. No microalgae have been reported to be able to survive in such a concentration of ammonium. In a conventional practice, leachate is diluted to allow the microalgae to survive. In this experiment, rather than diluting the leachate, removing some ammonium from the leachate is preferable to save more water for cultivation. The ammonia stripping treatment resulted in high ammonium removal (97%) in only 30 minutes (Table 1). Compared with previous studies, this treatment could be considered as the most optimum practice in relation to air stripping removal with microalgae. Maghfiroh et al. (2022) reported that microalgae could achieve of at least 95% ammonium removal for 3 hours of air-stripping treatment combined with zeolite treatment. Nearly perfect removal (99%) was reported by the same researcher in different study, where air stripping was used as the sole treatment and the pH was set to 11 (Maghfiroh et al., 2023). A lower result (70%) was reported in 2 hours of air stripping treatment (Kabdaslı et al., 2000). However, how the air was induced into the chamber was not reported in detail.

Growth rate of selected microalgae species in treated leachate

Four different microalgae species were cultured separately in the treated leachate in a tube glass photobioreactor for 26 days. When the running was stopped, some species were remaining in logarithmic phase while others started to enter a stationary phase. The volume of most cultivation medium was about half of that of the initial volume. It was due to a sampling required for chlorophyll analysis and evaporation, which was intensified by the microbubble.

About 24 hours prior to microalgae cultivation, the treated leachate was left in the photobioreactor with 5% CO_2 gas sparging. It was done for two reasons: decreasing the pH to an acceptable pH and reducing the excessive foaming of the leachate due to the aeration. It was suggested that this foaming is due to the carbonate species and organic foaming agent in the leachate (Deng and Englehardt, 2006). After 24 hours of CO_2 sparging, the foam generation was significantly, but not totally reduced. Another means to reduce the foaming was by applying a lower air flow rate as possible, but without scarifying the mixing. Hence, 0.1 litre per minute of air flow rate was chosen after some trials with different flow rates. No antifoaming agent was applied in this experiment. Antifoaming agent can indeed reduce the excessive foaming. However, based on our preliminary experiment, using antifoam has reduced the microalgal growth. A report by Koch et al. (1995) shows that some type of antifoaming agent inhibit the cell growth while some other do not. As the type of anti-foam agent could affect a certain species, further study should be taken on a suitable antifoaming agent to a specific microalgal species, if an antifoaming is planned to be used.

The growth of the microalgae was expressed in the amount of chlorophyll content per litre of medium (Figure 4). In general, the lag phase happened longer for all microalgae species than most reported (Lee et al., 2015; Udayan et al., 2023). It affected the overall lower growth rate compared to most reported. The foam generation in the early days of cultivation, which was not totally solved before the cultivation, was suspected to affect this growth. Due to the foam formation, microalgal cells tend to float into the surface layer as presented in Figure 5. The foam-separating effect actually has been demonstrated for bacteria and algae separation (Rubin et al., 2018). In a later application of this technique, surfactant was added to the medium, resulting in a significant separation of microalgae (Coward et al, 2013). Unfortunately, this effect has a negative impact during the cultivation period. As the cultivation and gas sparging continued, the foam formation gradually decreased, and the log phase of the growth started to happen.

Among the selected microalgae species, *Nannochloropsis oculate* showed the highest growth rate (0.0562 mg/L/day), while Chlamydomonas had the lowest one with only (0.02842 mg/L/day). The growth rate of *Chlorella ovalis* was slightly higher than that of *Chlamydomonas reindhartii*, but lower than that of *Chlorella vulgaris*. *Nannochloropsis oculata* is a marine microalga that has been reported to have high productivity in wastewater medium (Wang et al., 2019). This original habitat of *Nannochloropsis oculata* is probably a reason why this species is often cultured in wastewater. During the cultivation, *Nannochloropsis oculata* also showed to mix well, while *Chlamydomonas reindhartii*

tended to make a micro-floc. The foam formation during cultivation has worsened the *Chlamydomonas reindhartii*, as the floc present, it then floated more easily.

Lipid content of different species of microalgae

The pure culture (inoculum) of *Chlorella vulgaris* shows the highest average lipid content, i.e. 365.62 µg/mg (Figure 6). It was followed by *Chlamydomonas reindhartii* and *Chlorella ovalis*, while *Nannochloropsis oculata* was had least lipid content with about 205.13 µg/mg. These are all in the normal range of lipid content of microalgae as reported in previous studies, except for *Nannochloropsis oculata*. An the end of cultivation time, less lipid content was shown by all selected microalgae species. However, *Chlorella vulgaris* remained the highest one (192.97 µg/mg) was slightly higher than *Chlamydomonas reindhartii* and *Nannochloropsis oculata* remain the lowest one.

Table 1. Result of the leachate treatment using hot microbubble rig.

Parameter	Before Treatment	After Treatment	Change (± %)
Volume (mL)	80	55+5	(-) 30
pH	10.51	10.01±0.2	(-) 0.5 (pH)
COD (mg/L)	3476	3296	(-) 35
NH_4^+(ppm)	1571	70±5	(-) 97
PO_4^+(ppm)	14.80	16.42	(+) 11
Salinity (‰)	21	32	
Optical density	See Fig. 3		

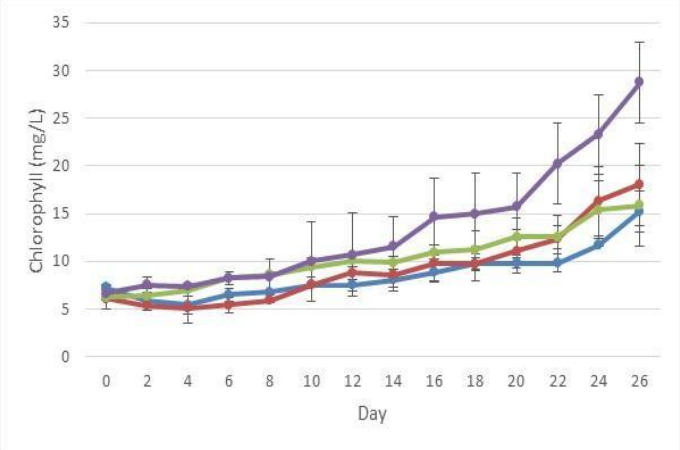

Figure 4. Growth curve of selected microalgae species in treated leachate

Figure 5. Foam formation during the early days of cultivation has large number of floated microalgae to the surface. The selected photos are in *Clamydomonas reindhartii.*

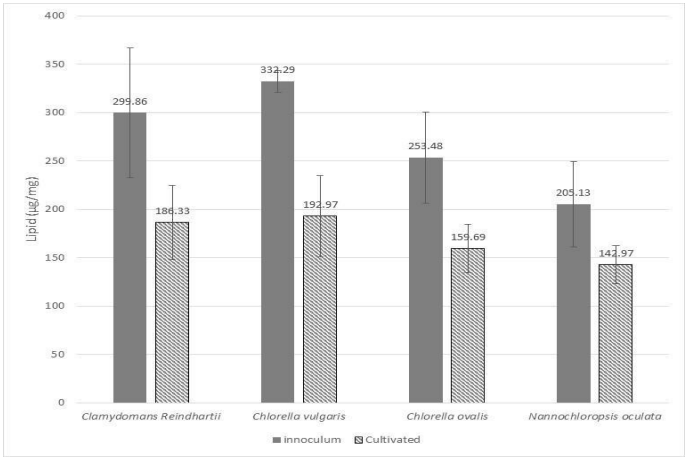

Figure 6. Oil Content of the raw inoculum and cultivated microalgae

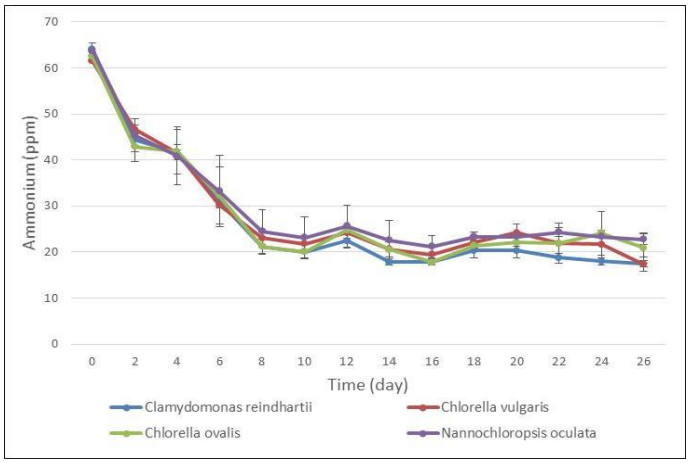

Figure 7. Ammonium concentration of each medium of microalgae cultivation

The lipid content in microalgae is affected by some conditions during the cultivation period and post-harvesting technique. The availability of carbon source (CO_2), nitrogen starvation, and harvesting time are among the factors affecting the microalgae's lipid content. While nitrogen is a macronutrient in microalgae needed for growth, an adequate amount of it is less preferred for them to produce lipids, as the conversion of glucose into lipids is best under nitrogen starvation. Turcotte and Kosaric (1989) suggested that $3 \times 10{-}5$ M is a critical nitrogen concentration in the medium where the lipid synthesis is well initiated. Song et al. (2022) suggested that nitrogen starvation positively affects the lipid content, but indirectly. The shortage of nitrogen will lead to the slow growth of the microalgae. When it grows slower, the microalgae start to use any remaining nutrients to produce oil inside their cells instead of synthesizing new cells. Many reports show that lipid accumulation in microalgae is triggered by nitrogen starvation (Liu et al., 2022; Chen et al., 2023). A study by Widjaja et al. (2009) shows that with more than two weeks of nitrogen starvation, the lipid content in Chlorella vulgaris increased to 40% of its dry weight (equal to 400 µg/mg) compared to that of the same species in normal nitrogen condition, which was less than 30% of its dry weight. Another study with the same species shows a 3-fold increase in lipid content occurred when the nitrogen concentration was suppressed to a quarter of the normal concentration (Liu et al., 2022). In this study, the lipid content of *Nannochloropsis oculata* doubled after the nitrogen content was reduced by a quarter from the initial concentration. As shown in Figure 7, all microalgae grew in abundant nitrogen concentration even though it kept decreasing while the microalgae grew. However, compared to the inoculum medium, this concentration was much higher. This is probably the best reason why all the microalgae show less lipid content than before cultivation.

Several authors suggested that nitrogen starvation affects lipid content indirectly since lipid is the secondary metabolite that is being produced during the limited nutrient (Benvenuti *et al.*, 2015; Chu *et al.*, 2020; Maltsev *et al*, 2023). The shortage

of nitrogen will lead to the slow growth of the microalgae. When it grows slower, the microalgae start to use any remaining nutrients to produce oil inside their cells instead of synthesizing new cells.

An extraction technique has been reported to significantly affect the amount of lipid collected for analysis. But it is rather a bias of technical issue than the biosynthesis of lipids inside the microalgal cells (Converti et al., 2009; Widjaja et al., 2009). The studies show how different techniques have affected the amount of lipid extracted before it was quantified. It could even have almost eight times as much difference when different extraction techniqueswere applied to microalgae from the same source.

Conclusions

All the selected microalgae have demonstrated to survive in treated leachate. A relatively low growth rate of all species was due to the presence of foaming, especially in early stage of the cultivation. As an antifoaming agent was avoided to prevent any growth inhibition to microalgae, it is suggested to find any technique to maximally remove the foam. If it is necessary to use antifoaming agent, a specific compound must be examined to ensure it will not inhibit the growth. The oil content all species an the end of the cultivation was significantly reduced compared to those of the inoculum. The abundant amount of nitrogen was suspected to allow it to happen.

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Declarations

Conflict of interests The authors declare no competing interests.

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