Short Communication:

Application of *Nannochloris* sp. for Landfill Leachate Biotreatment and Lipids Production

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Abstract: *The sparse treatment of highly toxic leachates produced in landfills due to the excessive generation of urban solid waste is a common problem worldwide. For this reason, this research aims to show the convenience of the use of algal biotechnology in leachate bioremediation processes. Nannochloris sp. species was used in this research. It was isolated and cultured for bioassays. The leachate was diluted to 5 and 10% in the microalgae cultures during a period of 8 d in which the growth of the species. Then removal of nutrients (phosphate and nitrate) and the production of lipids by the microalgae were measured. Nannochloris sp. removed more than 70% of the phosphates and 60% of the nitrates from samples. This result shows the benefits of using these microalgae to treat landfill leachate at low cost and also with the potential to obtain bio-lipids that may be useful for biodiesel production.*

Keywords: *bioremoval; bio-lipids; landfill leachate; microalgae; Nannochloris sp.*

■ INTRODUCTION

Excessive population growth, industrialization, and global consumerism have led to different threatening conditions for humanity such as global warming, plagues, and food shortages. Additionally, overpopulation has caused the uncontrolled generation of solid wastes, which are deposited in holes called landfills, which through the years, decompose and become highly toxic by-products named leachate, which has toxic organic and inorganic components [1-2]. These leachates have high levels of biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), solids, nutrients, and heavy metals [3]. For the elimination of these substances, expensive processes such as membrane technologies, ion exchange, adsorption with activated carbon, flocculation coagulation, or advanced oxidation processes are required [4-5].

In Cartagena, the leachates are maintained in collection ponds where the pH is kept close to neutral and evaporated due to the action of high local temperatures. Nevertheless, the ponds may suffer spills due to leaks in the membranes they have. These leachate leaks might cause eutrophication of water sources if they get in contact, which may produce the appearance of algae bloom, a drop in oxygen levels, increase toxicity and turbidity, and may also reach the human food chain, which could be a threat for public health [6-7].

On the other hand, the bioremediation of wastewater using microalgae has recently gained interest due to its advantages, such as the removal of nutrients from contaminated water, low investment, and low environmental damage since it does not require or generate harmful chemicals in the process. Additionally, microalgae consume carbon dioxide (CO_2) that they use

as a carbon source and produce more oxygen than trees via photosynthesis [8-9].

The diversity of the biomass obtained from microalgae cultures, combined with new large-scale cultivation technologies, has allowed the biomass obtained from these organisms to be used for commercial purposes in different fields, for example, as a source of protein, fatty acids for biofuels, pigments, biomolecules for pharmaceutical use and personal care products [10-11]. Previous studies have confirmed that microalgae may be cultivated in wastewater not only from piggeries, wine factories, palm oil mills and municipal but also in leachate from landfills. Microalgae may remove large amounts of dissolved pollutants from these effluents and also generate biomass rich in bioproducts of high-added value [12-15]. Additionally, polyunsaturated fatty acids obtained from microalgae may be used as feedstock in the production of biofuels, which are vital due to the imminent depletion of fossil fuels [16]. Biodiesel is a promising alternative because of its renewable nature. Unlike traditional land crops, microalgae may be rapidly grown in small spaces at any time of the year, which favors a greater production of biomass and lipids [17-18].

Due to the increase in the contamination of water resources and the urgent necessity to find a sustainable and renewable source of polyunsaturated fatty acids to generate biofuels, microalgal biotechnology appears as a viable and attractive alternative to achieve both goals. Therefore, this study aimed to determine the efficiency of nutrient removal (phosphate and nitrate) from landfill leachate using the microalga *Nannochloris* sp. Additionally, we determine the amount of lipids recovered in the bioremediation process to analyze their potential use in biofuel production.

■ EXPERIMENTAL SECTION

Materials

Microalgae and culture medium

Nannochloris sp. was isolated and cultured for bioassays with leachate in the Universidad del Sinu and Sena Cinaflup aquaculture biotechnology laboratories in Cartagena, Colombia. The culture medium used was the modified Comway medium, which contains the following substances: $FeCl_3 \cdot 6H_2O$ (26 g), $MnCl_2 \cdot 4H_2O$ (0.72 g), H_3BO_3 (67.2 g), EDTA (90 g), $Na_2HPO_4 \cdot 12H_2O$ (40 g), $NaNO_3$ (200 g), Na_2SiO_3 (40 g), H_2O (2 L), trace metals (2 mL), and vitamin solution (100 mL). The trace metal solution contained $ZnCl_2$ (2.1 g), $CoCl_2 \cdot 6H_2O$ (2 g), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.9 g), $CuSO_4 \cdot 5H_2O$ (2 g), and distilled water (100 mL), while the vitamin solution was composed of decamyl (210 mg) and distilled water (100 mL) [19].

Landfill leachate

For pollutant removal bioassays, the leachate was obtained from Los Cocos landfill, located in Cartagena city, Colombia. For its characterization, tests standardized by the American Public Health Association (APHA) were applied to determine the content of various contaminants before starting the assays. The measured parameters of the landfill leachate are provided in Table 1 [20].

Instrumentation

All concentration measures were done using GENESYS™ 50 UV-vis spectrophotometer of single cell holder, Model: Genesys 50, made by Thermo Fisher Scientific Inc. Ultrasound extraction procedures were conducted in an ultrasonic bath, model HH-S4, Zenith Lab (Jiangsu) Co., Ltd No. 12 Hongshan Road, China. Solvent extraction procedures were made using Pyrex Soxhlet extraction apparatus, model CLS3740S, Merck KGaA, Darmstadt, Alemania.

Procedure

Bioremediation experiments

To carry out the bioassays, the leachate was diluted without any pretreatment to 5% and 10% (v/w) in 1000 mL Erlenmeyer flasks using the microalgae cultures in the growth phase as solvent. The final volume of each bioassay was 400 mL. The optical density at 680 nm was measured daily to assess culture growth, phosphate content was measured by the ascorbic acid method, and nitrate content by the cadmium reduction method. All procedures were done by spectrophotometry.

The percentage of contaminant removal was calculated using the Eq. (1):

$$\%\text{Removal} = \frac{C_0 - C_f}{C_0} \times 100 \qquad (1)$$

where C_f is the final concentration of the contaminant and C_0 is the initial concentration. The percentage of inhibition was calculated for the cultures using the Eq. (2):

$$\%\text{Inhibition} = \frac{DO_C - DO_m}{DO_C} \times 100 \qquad (2)$$

where DO_C is the optical density of the control and DO_m is the optical density of the sample.

Lipids extraction

Lipids were extracted from dry samples of *Nannochloris* sp. microalgae biomass following the method provided by Bligh & Dyer [21] and Hu et al. [22] with modifications. Initially, 0.5 g of lyophilized biomass was stirred in 30 mL of chloroform: methanol solution (2:1 v/v) for 60 min, and then ultrasound was applied to the mixture for another 60 min. Then, the phases were separated by centrifugation at 2000 rpm and the solvent mixture was evaporated using a rotary evaporator. The percentage of lipids was found by gravimetry using the Eq. (3):

$$\text{Percentage of lipid} = \frac{P_L}{P_M} \times 100\% \qquad (3)$$

where P_L is dry weight of total lipids and P_M is dry weight of microalgae biomass.

■ RESULTS AND DISCUSSION

Characterization of Landfill Leachate

The landfill leachate had a dark color, an unpleasant odor, a high COD content and also showed high levels of nutrients, especially ammoniacal nitrogen (Table 1). According to the literature, the N:P ratio must be in the range of 5–40 for optimal growth of the microalgae; in this case, it was 49.79, which according to other authors, may be a usable value, so no phosphorous supplement was added to the culture. The pH is an essential parameter for microalgae growth that must be between 7 and 9. According to the literature review, this range was reached for bioassays with the effect of dilution at 5 and 10%. The phenol content in leachate was less than 0.10 mg/L, so it does not affect the growth of the microalgae since it does not reach values greater than 70 mg/L that inhibit the growth of the microorganisms [23]. The values found for the leachate characterization are in Table 1.

Table 1. Leachate properties

Parameter	Mean value (mg/L)
DQO	1272.00
Ammonium N - NH_4^+	1277.00
Nitrates N - NO_3^-	12.45
Nitrites N - NO_2^-	5.10
Phosphates P - PO_4^{-3}	26.00
N:P	49.79
pH	8.90
Phenol	< 0.10
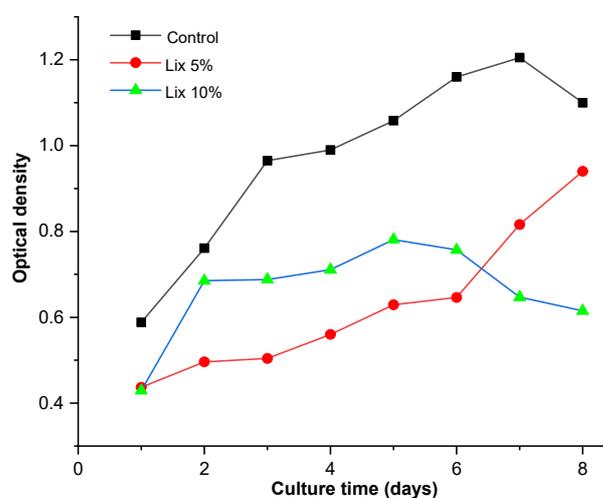
Fig 1. Growth of microalgae in leachate

Culture Growth

All the experiments showed a clear inhibition in the growth of the microalgae induced by the interaction with leachate. The solution at 5% leachate presented better growth at the end of the bioassays, which indicates that the microalgae initially adapted to the adverse conditions during the first 6 d. Then it could reproduce satisfactorily in that concentration the following days. In contrast, the microalgae in the 10% leachate solution showed a good start, growing in the first 5 d, but then the growth rate declined (Fig. 1).

Regardless of the percentage of dilution of the leachate, a clear inhibition was noted in the growth of the microalgae. The 5% bioassays were inhibited by 21.99% in their growth, while the 10% bioassays suffered a 35.20% growth inhibition according to Eq. (2).

These results may be associated with high turbidity or excessive color of the contaminant since this decreases the penetration of light into the culture and may limit

photosynthesis and biomass production. High ammonium content (> 1000 mg/L) may also affect the growth of the microalgae, so at a higher concentration of leachate (10%) the growth of the microalgae was lower in the bioassays affected probably by this fact. Several authors recommend that in order to obtain better cell growth rates, a complementary addition of phosphorus (in the form of K_2HPO_4) should be made to lower the N:P ratio and thus achieve higher cell density in cultures [24]. Other factors that could affect growth are the presence of bacteria since no pretreatment was carried out on the leachate and the presence of other contaminants like toxic heavy metals as reported by Khanzada and Övez [25].

Phosphate Removal

For phosphates bioremediation, it was found that remotion in the 5% solution was equal to 75.3% and a remotion of 78.6% was achieved for the 10% solution. In Fig. 2, it may be seen that the behavior in the removal of phosphates was similar for the two dilutions of leachate that were tested.

The removal for the 5% solution occurred during the first 4 d and then reached a constant value during the rest of the bioassays. For the 10% sample, it was observed that the maximum removal occurred during the first 3 d of the bioassays to stabilize for the other days of the experiments (Fig. 2).

Phosphorus is an essential nutrient in the energy metabolism of microalgae. It can be found in nucleic acids, proteins, lipids, and intermediate products of carbohydrate metabolism. Furthermore, culture medium with low phosphorus concentrations results in low cell densities in microalgae cultures. During the metabolic process, phosphate (PO_4^{3-}) is assimilated in the form of $H_2PO_4^-$ and HPO_4^{2-} and incorporated into cells through a phosphorylation mechanism, where much of the PO_4^{3-} is used to obtain adenosine triphosphate (ATP) starting from adenosine diphosphate (ADP), thus the microalgae obtain its cellular energy source [26-27]. Similar results for phosphorus removal were found by Hu et al. [22], where the authors diluted leachate to 5, 10, and 15% and observed removal of 80% of the phosphate content when samples were bio-treated with a consortium of microalgae
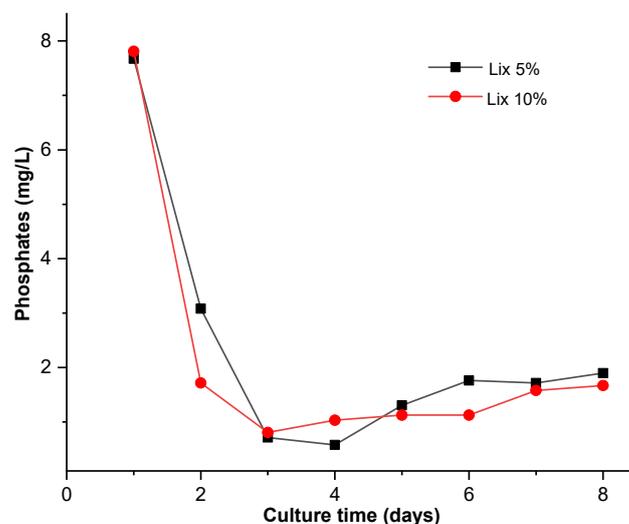
Fig 2. Phosphate removal from leachate

Chlorella vulgaris and *Scenedesmus dimorphus*. Similarly, de Souza et al. [28] found phosphate removals between 67.8 and 92.0% using the microalga *Scenedesmus* sp. to bioremediate pre-treated leachate. On the other hand, in cases where there is little available phosphorus, like in this study, the microalgae probably first accumulate the available phosphorus internally for later use in the assimilation of ammoniacal nitrogen [24-25]. Fig. 2 shows that in both leachate dilutions, a high removal of available phosphate was achieved, and the microalgae first accumulated for posterior consumption.

Nitrate Removal

The nitrate removal percentage for the 5% leachate solution was 62.2%, while for the 10% leachate solution was 42.9%. It may be seen in Fig. 3 that the concentration of nitrates in the 5% solution began the bioassays with lower removal levels than the 10% solution, but on the sixth day, it showed a considerable removal of nitrates, which is consistent with the increase in cell growth of the microalgae in the 5% solution on this day (Fig. 3).

Nitrogen is an essential nutrient required for the growth of microalgae and it is also the basic component of nucleic acids, amino acids, and all proteins, which are essential for the functioning of these microorganisms. Microalgae, to growth assimilate nitrogen that they remove from the environment. The nitrates (NO_3^-) obtained from the environment must be reduced to
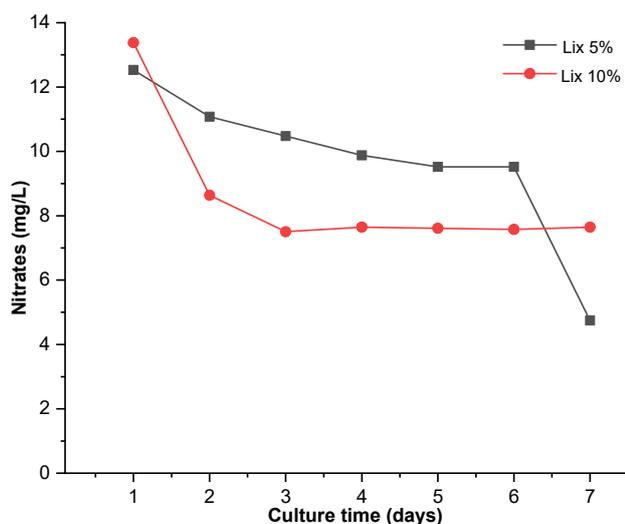
Fig 3. Nitrate removal from leachate

nitrites (NO_2^-) and finally reduced to ammonium ion (NH_4^+). This implies an energy expenditure, and then they incorporate it directly into the amino acids by condensation with glutamate [29]. In this study, the microalgae initially had to adapt to the high levels of contaminants in the leachate, which is reflected in the growth levels, and it was the 5% bioassays that achieved the greatest removal of nitrates and, therefore, the greatest cell density at the end of the bioassays (Fig. 3). Similar levels of nitrate removal were obtained by Chang et al. [1] that removing 61% of nitrates using *C. vulgaris* in photobioreactors and de Souza et al. [28] that obtained nitrate removal rates between 24 and 67% in leachates treated with *Scenedesmus* sp. microalga.

Lipids Production

The composition of the biomass obtained is critical data to evaluate the potential of the microalgae to produce biodiesel. Due to the fact that the bioassays with 5% dilution achieved the highest cell growth, only this one was analyzed to determine the percentage of lipids in the biomass. In this order of ideas, the biomass obtained from the *Nanochloris* sp. microalgae reached 23% lipids when exposed to 5% diluted leachate.

Hernández-García et al. [30] reported a lipid content similar to this study was reported, reaching 20.0% of lipids while culturing *Desmodesmus* sp. and *Scenedesmus obliquus* in landfill leachate. Also, Paiva et al. [12] reported 16.6% of lipids with *Chlorella* sp. and *Scenedesmus* sp., Zhao et al. [31] reported values of 14.5 and 20.8% of lipid composition for *Chlamydomonas* sp. grown on landfill leachate. Hu et al. [22] obtained 26.0–29.0% lipids culturing *C. vulgaris* and *S. dimorphus* in landfill leachate. Viegas et al. [4] cultured *C. vulgaris* and obtained 23.8% of lipids and obtained 22.6% of lipids with *Scenedesmus* sp.. The lipid yields of the above authors were approximately the same as those found in this work. This means that if *Nanochloris* sp. culture conditions are optimized, the lipid composition could also be improved.

■ **CONCLUSION**

In this study, the bioremediation of leachate from a local landfill in Cartagena, Colombia was evaluated using the *Nannochloris* sp. microalgae. When compared to the control bioassay, the microalgae's growth showed an inhibition of between 20 and 30%. This could be attributed to the cultures' extremely high N:P ratio, high ammonium content, and black hue due to the leachate addition. The percentage of nutrient removal was high in both cases, which makes microalgae a feasible alternative to bioremediate this type of toxic waste. The production of lipids from *Nannochloris* sp. achieved a good level even in non-optimized conditions. This study demonstrated the successful cultivation of isolated native *Nannochloris* sp. using local landfill leachate for simultaneously remarkable nutrient removal and significant lipid production by this strain.

■ **AUTHOR CONTRIBUTIONS**

The conception and design of this study include contributions from all authors. Experimental procedures, microalgae growth and bioremediation were performed by Ildefonso Baldiris-Navarro, Jorge Sanchez, and Martha Torres Virviescas. Data analysis, writing, review and editing carried out by Ildefonso Baldiris-Navarro, Alvaro Realpe-Jimenez, and Juan Fajardo-Cuadro.

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