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Bioremediation of Crude Oil by Haematococcus Pluvialis: A Preliminary Study

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Abstract: Nowadays, oil pollution is one of the main environmental problems. The current methods for recovering spills mainly involve chemical agents, but scientific research has focused on more natural and less harmful techniques for the environment, including a consortium of bacteria and microalgae to clean up water contaminated by hydrocarbons. The purpose of this preliminary study was to evaluate the ability of a microalga belonging to Chlorophyceae to grow in the presence of crude oil and remove the principal contaminants. *H. pluvialis*, which is usually used for nutraceutical purposes, thanks to the production of astaxanthin, was able to grow in anaerobic conditions, varying its metabolism from autotrophic to heterotrophic, exploiting the carbon present in the solution deriving from the presence of 1% of crude oil. Furthermore, the results of bioremediation showed a relevant reduction in chemical pollutants such as nitrate, fluoride, sulfate, and phosphate. The most important aspect of the study was the reduction after 160 days in the hydrocarbon concentration inside not only the culture medium (−32%) but also the algal biomass (−80.25%), demonstrating an optimized degradation rather than a simple absorption inside the alga.

Keywords: bioremediation; crude oil; Haematococcus pluvialis; microalgae; anaerobic growth; heterotrophic metabolism

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1. Introduction

The world economy is based on fossil fuels for obtaining energy and their use is a problem for the entire world's ecosystem [1]. In fact, the spill and loss of oil and its by-products are the main causes of oil pollution [2]. Today, oil recovery techniques involve the use of chemical dispersants that break down the oil molecules, making them more available to attack by atmospheric agents and for microorganisms that participate in bioremediation [3,4]. The ability to degrade hydrocarbons is found in some bacteria, fungi, and yeasts [5]. Until now, researchers focused mostly on the bacteria that are able to remove various oil pollutants, including polycyclic aromatic hydrocarbons (PAHs) [6]. Even if bacteria can act efficiently again oil pollution, they are not able to remove each contaminant. To avoid this, it is possible to use bacteria engineered with specific enzymes to degrade petroleum pollutants. However, engineered bacteria could not live naturally in the areas around the spills and could not adapt to the "natural environment", dying before acting against pollution [7].

Other microorganisms belonging to phytoplankton help bacteria during the process. Researchers have often focused on a synergistic mechanism of bioremediation between oil-degrading bacteria and microalgae thanks to their ability to produce oxygen used by bacteria for their metabolism. Still, microalgae could represent the principal actors [8]. They are unicellular and photosynthetic organisms belonging to the prokaryotic (cyanobacteria) and eukaryotic (microalgae) divisions [9]. Microalgae are used in different fields for different purposes. In the nutraceuticals field, they are valuable for the nutrients produced

such as proteins, polysaccharides, antioxidants, polyunsaturated fatty acids, lipids, vitamins, and pigments [10,11]. Furthermore, they are used for wastewater treatment [12,13] and biofuel production thanks to the high quality and quantity of lipids contained [14]. One of the most significant aspects related to the use of microalgae is their high ability to adapt to different environmental stimuli (nutrients, temperature, pH), which allows them to work under several conditions. Many species can change their metabolism by using organic carbon instead of carbon dioxide. Thanks to their adaptability, several studies have been conducted on different species of microalgae, mainly *Chlorophyceae,* to test their bioremediation capacities, for example, *Chlorella vulgaris* and *Scenedesmus obliquus* [15,16].

It has been reported that some species can significantly reduce aliphatic compounds, such as 3-methyl decane, heptadecane, and octadecane [16], and that the ability of *Chlorella* spp. to reduce emulsified oil reached 80 % [17]. Further studies have shown that *Chlorella* spp. can remove phosphorus from petroleum effluent in just 13 days, also reducing nitrogen by 78% and reducing the Chemical Oxygen Demand (COD) from 504 mg L^{-1} to 144 mg L^{-1} [18]. Ashwaniy et al. found that the microalgae grown in petroleum refinery effluent (PRE) can reduce the concentration of COD by 70%, the Biochemical Oxygen Demand (BOD) by 81%, sulfide by 61%, total suspended solids (TSS) by 622%, and phosphorous and Total Dissolved Solids (TDS) by 67% and can act as a substrate for bacterial growth in a microbial desalination cell (MDC) to produce clean energy [19]. *Haematococcus pluvialis* is a green microalga capable of colonizing different environments. *H. pluvialis* is a special microalga as its life cycle consists of different phases characterized by different morphologies. Initially, the microalga, called macrozooids, has two flagella; subsequently, through mitotic division, daughter cells called microzooids originate, which grow up to form coccoid cells [20]. Its main applications are in the nutraceutical and pharmaceutical fields, thanks to the production in stressful conditions of several carotenoids and pigments. First, astaxanthin, a powerful antioxidant, is useful for the treatment of many diseases, reducing adipose tissue weight, glucose serum levels, and systolic blood pressure in an animal model of obesity and reducing liver and body weight in an animal model of NAFLD [21,22]. *H. pluvialis* is usually studied for other metabolites of interest. For example, in optimal conditions, it can produce essential amino acids such as leucine, glutaminic acid, and alanine. Furthermore, by varying the growth conditions, it is also possible to improve the lipid content, which can represent 30% of the total weight. In addition, the nature of the lipids makes *H. pluvialis* very interesting, with a high content of polyunsaturated lipids (PUFA) within cells, and in the red encystic stage, h pluvialis dramatically increases triglyceride (TAG) levels [23]. All these characteristics lead to the belief that *H. pluvialis* is a microalga with a high nutritional value that can be used for metabolites of nutraceutical and pharmaceutical interest. However, the high lipid (especially the intermediate fractions C16-C18) and carbohydrate contents could be used for biofuels such as biodiesel and bioethanol production [24]. For this reason, our research also focused on the industrial applications of *H. pluvialis*, especially its role in bioremediation, hypothesizing a mechanism for removing contaminants and reusing algal biomass. However, based on our research, there are no studies about *H. pluvialis* and crude oil bioremediation. Only Suresh Kumar et al. hypothesized how the degradation mechanism of PAHs in *H. pluvialis* could be similar to prokaryotes. However, several parameters were analyzed to create a model that could simulate the link between PAHs and CYP450 of *Haematococcus pluvialis*. Thirty-eight PAHs formed from one to six benzene rings were involved in the analysis and the results showed that hydrogen, hydrophobic, electrostatic, π-π, and Van der Waals interactions occurred in the active site of CYP450. Specifically, 18 PAHs interacted with Threonine282 (Thr282), Alanine337 (Ala337), Serine404 (Ser404), and Lisyne407 (Lys407) via hydrogen bonds. However, in this study, it is evident that only LMW-PAHs were able to bind CYP450, whereas HMW-PAHs were not [25]. Based on this, this preliminary study aimed to study the potential of *H. pluvialis* for crude oil bioremediation, analyzing (i) the ability to grow in the presence of such a toxic agent; (ii) the quantitative concentration

of hydrocarbons degraded in the aqueous phase and biomass, and (iii) the ability of *H. pluvialis* to grow in heterotrophic conditions.

2. Materials and Methods

2.1. Algae Growth

H. pluvialis UTEX 2505 was grown in a self-produced media produced by dissolving 0.3 gr of Greenhouse Special 20–20–20 (BIOGARD) powder in 1 L of distilled water (Greenhouse Special powder contains HNO_3 6% (*w*/*v*), NH_4^+ 5.2% (*w*/*v*), CH_4N_2O 8.8% (*w*/*v*), P_2O_5 20% (*w*/*v*), K_2O 20% (*w*/*v*), B 0.05% (*w*/*v*), Cu 0.01% (*w*/*v*), Fe 0.2% (*w*/*v*), Mn 0.1%(*w*/*v*), Mo 0.005% (*w*/*v*), Zn 0.01%(*w*/*v*), chelating agent EDTA) under a light intensity of 120 mmol photons $m^{-2}s^{-1}$ in a 12 h: 12 h light/ dark cycle at 25 °C. Cultures were not supplied with an extra source of CO_2 and were shaken by a mechanical agitator (g24 environmental incubator shaker, American Laboratory Trading) at 70 rpm.

Cultivation with Crude Oil

Five ml (1%) of crude oil was sonicated in 500 mL of standard medium, and then 25 mL of culture containing approximately 1.5 × 107 cells/mL was inoculated in an anaerobiosis tank. The culture was placed under a light intensity of 120 mmol photons $m^{-2}s^{-1}$ in a 12 h: 12 h light/dark cycle at 25 °C. Cultures were shaken by a mechanical agitator (g24 environmental incubator shaker, American Laboratory Trading) at 70 rpm. In another tank, the same *H. pluvialis* cell concentration was inoculated without crude oil as a control. Algal growth was assessed by measuring optical density at 750 nm (SPEC-TROstar® Nano, BMG Labtech) and cell counts by light microscopy (Zeiss Axioplan) using the Burker chamber (BLAUBRAND). The experiment was conducted in triplicate.

2.2. Pigment Estimation

On days 1, 5, 7, 12, 20, and 25, from the *H. pluvialis* culture with 1% of crude oil and the control culture, one ml of culture was centrifuged for 5 min at 14,000 rpm. The pellet was suspended in 1 mL of DMSO and incubated for 10 min at 70 °C. The protocol was repeated until the pellet was white. The supernatant was collected and absorbance was registered at 480 nm, 649 nm, and 665 nm. The protocol used to quantify chlorophyll and carotenoid was the one reported by Wellburn et al. [26].

2.3. Biodegradation Activity

Several analyses were carried out to evaluate the ability of *H. pluvialis* to remove and degrade the oil. On day 0 and day 10, some parameters were preliminarily analyzed including the TOC, fluorides, chlorides, nitrates, sulfates, and phosphates in the supernatant. On day 20 and day 160, two samples were taken to conduct a quantitative analysis using GC-FID. An amount of 150 mL of culture was placed in a separating funnel to separate and collect the microalgae biomass and aqueous phase separately. Crude oil biodegradation was analyzed using a GC-FID Varian 3900 gas carrier helium (1 mL min^{-1}). The mineral column was a 15 m × 0.32 mm × 0.1 µm film, and the temperature programming was 40 °C (hold 1 min), −80 °C rate 50 °C/min (hold 1 min), −350 °C rate 20 °C/min (hold 8 min).

3. Results

As mentioned above, there are no studies regarding the possibility of using *H. pluvialis* as a bioremediator. To evaluate whether the metabolism of the cells grown under anaerobic conditions varied, the cell growth was followed for 25 days. The growth of *H. pluvialis* in the presence of 1% pure crude oil and anaerobiosis demonstrated how the microalga could metabolize the organic carbon present in the medium, reaching the exponential phase after 12 days, unlike the control grown in anaerobiosis but in the absence of carbon in the medium, which showed difficulties in reaching the exponential stage (Figure 1).

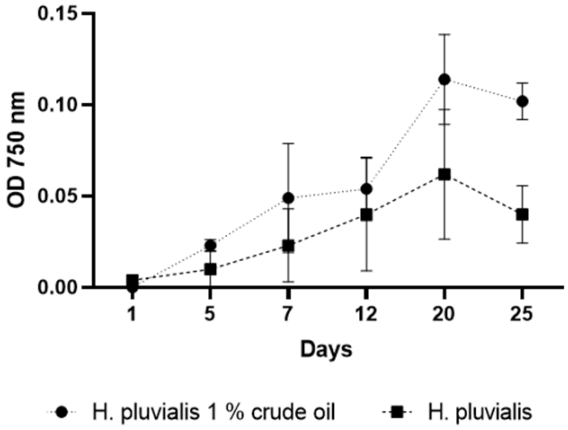


Figure 1. Cell growth. Values are reported as mean ± SEM.

The concentrations of the chlorophylls and pigments were analyzed to confirm the transition of the culture metabolism. The results showed a reduction in the chlorophylls in the culture grown with crude oil, with organic carbon available for their metabolism. However, in the control culture, the production of chlorophylls increased due to the lack of organic carbon in the media and the photosynthetic reaction was optimized (Figure 2a) [20,27]. In addition, the extracted pigments seen in Figure 2b showed a lower production in the mixotrophic culture including those predisposed to the defense of the cells against a stressful agent (e.g., astaxanthin).

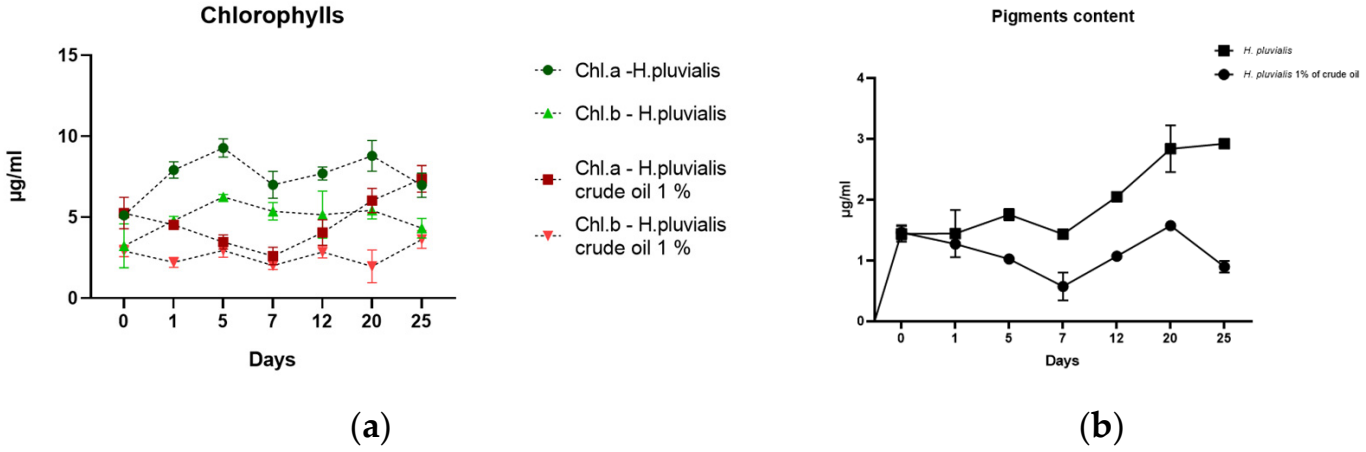


Figure 2. (**a**) Chlorophyll concentrations and (**b**) pigment concentrations under anaerobiosis conditions with and without crude oil in the media. Values are reported as mean ± SEM.

The chemical analyses carried out on the culture media and the algal biomass showed that after ten days, some parameters and contaminants had already changed compared to day 0 (Table 1).

Table 1. Preliminary data after 10 days of bioremediation, expressed in mg mL^{-1} (mean ± SEM).

	TOC	Nitrates	Fluorides	Chlorides	Sulfates	Phosphates
DAY 0	23.90 ± 0.78	0.95 ± 0.18	0.40 ± 0.14	10.75 ± 0.18	11.50 ± 1.06	70.20 ± 1.27
DAY 10	44.50 ± 1.06	0.24 ± 0.04	0.20 ± 0.07	12.50 ± 0.35	9.00 ± 0.85	53.50 ± 1.06

The TOC values doubled after 10 days, showing how *H. pluvialis* acted against hydrocarbons, making them more available for metabolism and using them for cell reproduction [28]. Nitrates, fluorides, sulfates, and phosphates were reduced, confirming the

results obtained by numerous studies carried out on different microalgal species used for wastewater bioremediation (Figure 3) [8,29,30].

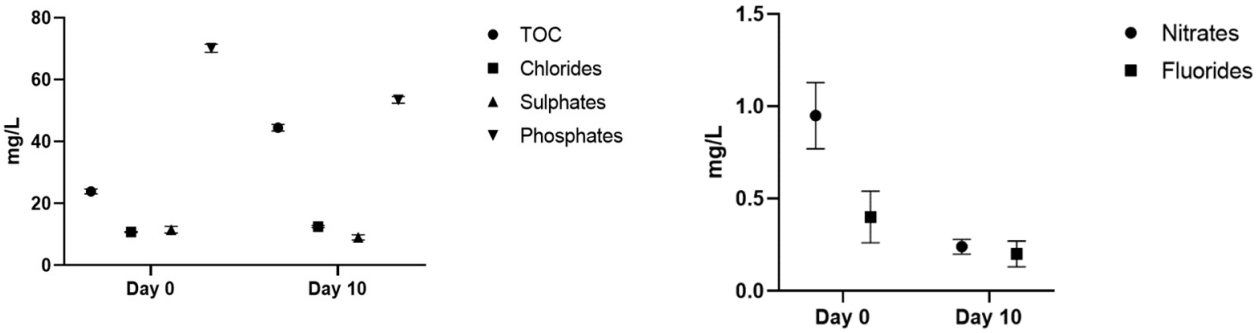


Figure 3. Preliminary data on bioremediation. Values are reported as mean ± SEM.

However, the most significant results were derived from the GC-FID analysis, which was directed at the hydrocarbons from crude oil. The analysis was performed on the aqueous fraction and the algal biomass after 20 and 160 days of treatment. As evident in Figure 4, there was a quantitative reduction in the hydrocarbons. In water, the reduction was 32% (9.24 ppm at day 20; 6.28 ppm at day 160), whereas in the algal biomass, it was 80.25% (4725 mg/Kg at day 20; 933 mg/Kg at day 160). However, the GC-FID spectra showed a quantitative but not qualitative reduction in the water, whereas in the microalgal biomass, the low-molecular-weight hydrocarbon (LMW-HC) fraction (C6–C12) was significantly reduced compared to day 20 (Figure 5).

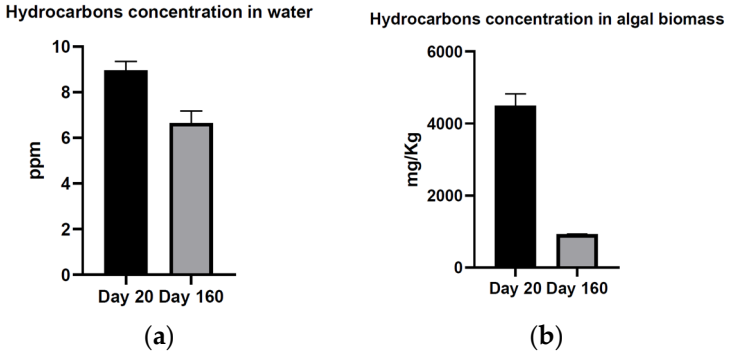


Figure 4. Hydrocarbon concentration in (**a**) water and (**b**) algal biomass. Data are reported as mean ± SEM.

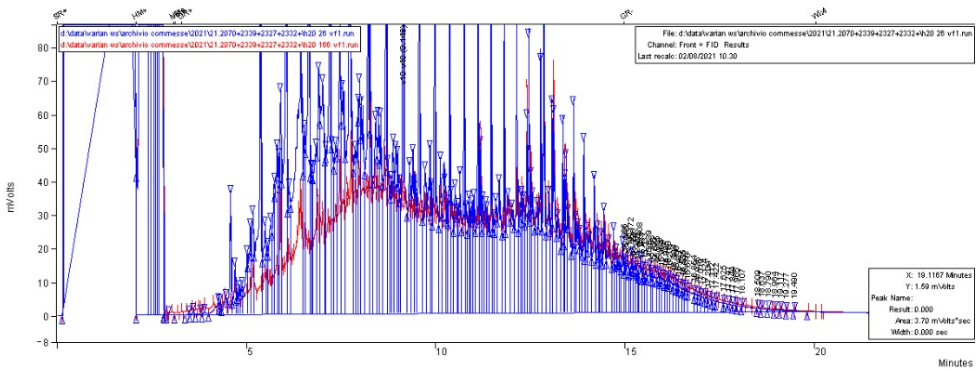
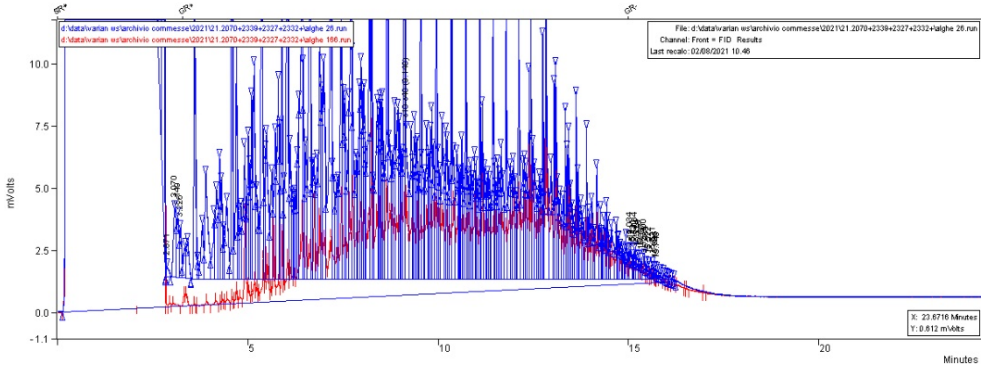


Figure 5. *Cont.*



(**b**)

Figure 5. GC-FID spectra. (**a**) Water and (**b**) microalgal biomass hydrocarbon concentrations (blue = day 20; red = day 160).

4. Discussion

These preliminary results show how the action of *H. pluvialis* in the bioremediation of oil-contaminated water is not only feasible but also optimal. The adaptation of the microalgae to a hostile environment (anaerobic and crude oil) demonstrates how *H. pluvialis* can vary the enzymatic and metabolic expression rapidly and efficiently, supporting the hypothesis that the microalgae genome is highly reactive. In the model alga *Chlamydomonas reinhartdtii*, it was recently discovered that the membrane protein HLA3 is involved in the activation of inorganic carbon uptake in low CO_2 situations; this could also be valid for *H. pluvialis*, which increased growth using the carbon from the hydrocarbons. [31]. The reduction in the nitrogen inside the media is also significant because nitrogen is an abundant contaminant in different processing wastewater [32,33] and *H. pluvialis* could be used to remove it in other bioremediation processes. Considering the amount of crude oil used in this study is crucial because other studies on microalgae bioremediation reported that the optimal crude-oil concentration was 0.5%. In the study by El-Sheekh et al., *S. obliquus* and *C. vulgaris* under heterotrophic conditions at 0.5% of crude oil removed compounds such as n-alkanes and Polycyclic aromatic hydrocarbons (PAHs) [15]. *H.pluvialis*, indeed, grows and degrades higher concentrations, assuming a more efficient remediation process in water where the percentage of oil is lower than that tested.

5. Conclusions

This preliminary study for the biodegradative activity of *H. pluvialis* shows for the first time how this green alga can grow in the presence of pure crude oil and the biodegradable part of the oil in the solution. It will be necessary to extend our analyses by evaluating the biochemical processes and mechanisms involved and performing a mass balance and stoichiometry analysis to identify, in detail, the affected and reduced classes of hydrocarbons. Furthermore, once all the biochemical processes involved are understood, it will be possible to optimize them through high selective pressure to obtain more performance strains, which will be useful for this type of preliminary study. These results are fundamental to not only improving the knowledge about *H. pluvialis*, which is used principally in pharmaceutical fields and not in bioengineering, but also because they could represent the first step in using innovative technology to combine bioremediation and astaxanthin production. In this way, the applications of *H. pluvialis* could expand in environmental and industrial areas, where *H. pluvialis* could initially grow on contaminated substrates and then be used for the extraction of interest compounds. It must be highlighted that microalgae are excellent candidates for biofuel production thanks to the high amount and purity of lipids, and *H. pluvialis* could place itself in a circular green economy by eliminating fossil fuel contaminants, producing biofuels, and reducing emissions and contaminations.

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