Algal Research 83 (2024) 103693

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Five mutated genotypes of *Haematococcus pluvialis* useful for crude oil wastewater bioremediation

Rosa Paola Radice [a,b,*], Giulia Grassi [c], Giambattista Capasso [a], Egidio Montagnuolo [d], Donato Aiello [e], Anna Maria Perna [a], Stefania Marzocco [f], Giuseppe Martelli [a]

[a] Department of Science, School of Agriculture, Forest, Food and Environmental Sciences, University of Basilicata, via dell'ateneo lucano, 10 – 85100 Potenza, Italy
[b] AlgaeBioMed srl, via Luigi Kossuth, 7 – 00149 Roma, Italy
[c] University of Molise, Department of Agricultural, Environmental and Food Sciences, Via De Sanctis snc, 86100 Campobasso, Italy
[d] Agenzia Regionale per la Protezione dell'Ambiente della Basilicata (ARPAB), Via della Fisica, 18C/D, 85100 Potenza, Italy
[e] Studio Aiello, via Raffaele Bosco 947, 80069 Vico Equense, Italy
[f] University of Salerno, Department of Pharmacy, Via Giovanni Paolo II, 132 - 84084 Fisciano, Salermo, Italy

ARTICLE INFO

Keywords:
Bioremediation
Crude oil
Microalgae
Haematococcus pluvialis
Green economy

ABSTRACT

The extraction of crude oil is one of the principal sources of pollution for our ecosystem. Technologies to solve this problem are many, but few use eco-sustainable methodologies. However, microalgae can also be considered excellent environmental bioremediators. In this study, the microalga *Haematococcus pluvialis* was subjected to high selective pressure to obtain mutant genotypes able to remove contaminants deriving from oil. The results show that *Haematococcus pluvialis* can survive in so stressful environment modifying its metabolisms. Two of the five isolated genotypes, PA1002 and PA1004 remove hydrocarbons from the medium (1 %) by 60.99 % and 95.28 %, respectively. Moreover, the PA1004 genotype, found to be the most performing, showed improvements in the level of fatty acids and antioxidant activity following the hydrocarbon removal process, also being less cytotoxic than the untreated control. This represents a central point for circular economy where microalgae remove pollutants from the environment and produce useful subproduct.

1. Introduction

Microalgae are unicellular organisms capable of growing in various aquatic environments. In recent decades they have been the subject of numerous studies, given the interesting secondary metabolites produced [1] and their ability to adapt to different growth conditions, beeing excellent subjects for bioremediation. In particular, systems capable of exploiting wastewater (WW) for the growth and production of microalgal biomass are increasingly interesting [2]. Wastewaters are defined as waters or, more generally, liquids that carry waste substances from homes, industrial and commercial activities that mix with rain, spring or surface water [3]. Usually, WW can be of different origins: urban, domestic, industrial and agricultural, but in all of them, there are several common elements:organic material, more or less degradable, solid residues, phosphorus, nitrogen and micro contaminants at different concentrations. [4]. However, wastewater deriving from the oil industry, both extraction and processing, represents a problem of particular interest due their high concentrations of very toxic elements for human health, such as aliphatic and aromatic hydrocarbons, as well as heavy metals [5]. They, therefore, represent a problem for the environment and human health, requiring reclamation processes and treatments to eliminate contaminants [6]. It has been shown that in 2012 about 13 million people died due to polluted air, water and land [7]. Strategies to remove contaminants and remediate WW are represented by activated sludge, anaerobic-anoxic-oxic states, membrane bioreactors and sequencing batch reactors. All of these treatments can be performed on-site or relocated to specially constructed areas, such as septic tanks and ponds [8]. However, the problems for the complete removal of all contaminants remain high; furthermore, logistical issues regarding the location of the purification sites and the necessary chemical-physical pre-treatments make it necessary to find solutions that are more attentive to the environment and which make the WW reusable, in a circular

* Corresponding author at: Department of Science, University of Basilicata, via dell'ateneo lucano, 10 – 85100 Potenza, Italy.
E-mail addresses: rosapaolaradice@gmail.com (R.P. Radice), giulia.grassi@unimol.it (G. Grassi), giambattista.capasso@unibas.it (G. Capasso), egidio.montagnuolo@arpab.it (E. Montagnuolo), donato@studioaiello.me (D. Aiello), anna.perna@unibas.it (A.M. Perna), smarzocco@unisa.it (S. Marzocco), giuseppe.martelli@unibas.it (G. Martelli).

https://doi.org/10.1016/j.algal.2024.103693
Received 3 July 2024; Received in revised form 9 August 2024; Accepted 3 September 2024
Available online 4 September 2024

economy process. [9]

In this problem, microalgae are placed as perfect bio-remediators, capable of exploiting contaminated water as a growth medium, using them as a source of nourishment [10]. Bioremediation processes usually focus on microalgae in consortium with bacteria, where the microalgae supply oxygen to the bacteria through photosynthesis which carries out the degradation of the contaminants. Although these processes are optimal, they still have an environmental impact as bacterial digestion releases CO_2 into the atmosphere, and there is the problem of subsequently disposing of the digesting bacteria. [11]. The exclusive use of microalgae, on the other hand, not only reduces the production of CO_2 in the atmosphere but the microalgal biomass could be recovered and used for various purposes, such as the production of biofuels or secondary metabolites of interest [12]. It has been demonstrated that several microalgal species belonging to the genus *Chlorophyceae* can act effectively against oil contaminants, working both in synergy with bacterial microorganisms and using endogenous enzymes typical of microalgae such as saponins, lipoxygenase and peroxidase or through the production of oxygen free radicals (ROS) and extracellular polymeric substances [13]. As we have demonstrated in a recent study, the *Haematococcus pluvialis* (*H. pluvialis*) microalgae, usually studied and used for the production of metabolites of nutraceutical and pharmaceutical interest, such as astaxanthin, can remove contaminants deriving from crude oil, reducing the concentration of petroleum hydrocarbons by 32 % in the liquid medium and reducing by 80.25 % in the algal biomass, after 160 days of anaerobic growth [14]. These results have laid the foundations for genetic selection studies to identify more performing genotypes capable of removing totally both chemical pollutants and the concentration of hydrocarbons. Thus, the final aim of this manuscript has been obtaining new *H. pluvialis* genotypes with better growth ability, able to act as bioremediators, and useful for different applications thanks to its natural composition rich in lipids, proteins and carbohydrates.

2. Material and methods

2.1. Algae growth and selective pressure

H. pluvialis UTEX 2505 were grown in 5 L Erlenmeyer flask using a self-produced media (HCS) (Supplementary material_ Fig. S1). The cell culture protocol standardized from our laboratory is reported in Radice et al., 2021. All the laboratory material and culture media used for the experiments were autoclaved at 120 °C for 20 min at 1 bar before their use [15]. Five μL of *H. pluvialis* were inoculated in 500 μL of pure crude oil. After 7 days of treatment, the crude oil drop, with cells inside, has been seed and after 4 days five colonies were collected to obtain five different mutant stains called PA1001, PA1002, PA1003, PA1004, PA1005. The mutant strains were transferred and grown as stock culture in fresh liquid media HCS. All the cultures were grown in axenic condition. A specific purification protocol was used and a specific 16S PCR was conducted to certify the absence of prokaryotic microorganisms. To analyse morphological differences between the mutant strains and the wild type, 1 mL (2.0×10^7 cells/ml) from the stock culture were transferred in 25 mL of HCS media in a 1000 mL Erlenmeyer flask. The growth was followed for 16 days by measuring optical density at 750 nm (SPEC-TROstar® Nano, BMG Labtech.

2.2. Cultivation with crude oil

In 2000 mL anaerobiosis glass tank, 5 mL (1 %) of pure crude oil (Sigma-Aldrich – CAS: 8002-05-9) was added in 500 mL of HCS medium, and then 25 mL of culture containing approximately 1.5×10^7 cells/mL was inoculated. 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 at 70 rpm mechanically. In another tank, the same cell concentration was inoculated in a fresh media as a control (WT) without crude oil in.

2.3. RNA extraction and cDNA synthesis

The extraction of total RNA from algal cells in suspension was carried out both on the mutant strains under optimal conditions and after 120 days of treatment with 1 % petroleum, following the instructions and using the reagents contained in the NucleoZOL RNA Isolation kit (Marcherey- Nagel). The mechanical lysis of the algal cell wall took place using a sonicator (Bransonic® Ultrasonic Baths) in ice for 10 min. Subsequently, the homogenization of the sample was obtained by adding 500 μL NucleoZOL and followed its protocol. The amount of total RNA was quantified using a spectrophotometric assay (NanoDROPTM 1000) using 1 μl of extracted RNA. Absorbance at 230 nm, 260 nm and 280 nm was measured to determine the purity of the analysed sample. Reverse transcription of RNA into cDNA was carried out to analyse the transcript. The reverse transcription was conducted using the FIREScript RT cDNA synthesis MIX with Oligo (dt) (Thermo Fisher Scientific). Reverse transcriptase (1.5 μL), 2 μL of buffer containing dNTPs, polyT primers, and DEPC water were added to the RNA to reach a final volume of 20 μL and a concentration equal to 50 ng/μL. The reaction was carried out following manufacturing protocol.

2.4. RAPD-PCR

Random Amplification of Polymorphic DNA - PCR (RAPD-PCR) was performed on cDNA to analyse the differences in the gene expression. Random primers and 1 μL of cDNA were added to a master mix (Phire Plant Direct PCR Master Mix - ThermoFisher Scientific), used to conduct the analysis, containing dNTP, Taq-polymerase, Reaction buffer and magnesium chloride. Amplification products were run on 1.5 % agarose gel (EMR010001, EuroClone S.p.a., lot no.468654) using PowerPac 3000, Biorad, at 80 V for 45 min. The primers with their respective sequences used are shown in the Table 1.

2.5. Biodegradation activity

To evaluate the bioremediation action of *H. pluvialis* to remove and degrade the oil, several analyses were carried out. Total of Carbon (TOC), fluorides, chlorides, nitrates, sulfates, and phosphates in the supernatant are preliminarily analysed after crude oil treatment (Day 0 and Say 10). On day 20 and day 160 after crude oil treatment, only a quantitative analysis using GC-FID were conducted. Microalgae biomass and aqueous phase from150 mL of culture were collected using a separating funnel. Crude oil biodegradation was analysed using a GC-FID Varian 3900 gas carrier helium (1 mL^{-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), −80C rate 50 °C/min (hold 1 min), −350 °C rate 20C/min (hold 8 min).

2.6. Chemical analysis

2.6.1. Total fat and FAMEs

At the end of the experiment (after 160 days), to determine the total percentage fat content, 150 mg of dried algae was weighed and diethyl ether was used as extraction solvent, as described by the Soxhlet method [16]. From the fat extracted, fatty acid methyl esters (FAME) were obtained by trans-esterification with a cold methanolic solution of potassium hydroxide [17]. An HP 4890D gas chromatograph equipped with a split/splitless injector and a flame-ionization detector, both at a temperature of 260 °C, a capillary SP2560 column (100 m, 0.25 mm, and 0.2 μm) (Supelco Inc., USA) and a HP3398a GC Chemstation Software (Hewlett Packard, USA) for data processing were used. Operating conditions were: column temperature = 45 °C (2 min)–4 °C/min to 240 °C (15 min), carrier gas = hydrogen (18 cm min^{-1}), split injection ratio = 1:100. No internal standard was used. Instead, FAMEs identification was

Table 1
Primer sequences used for RAPD-PCR.

Primer	Sequence	Primer	Sequence
1.1	5'-CTAGACGAGCCACCAGAT-3'	2.1	5'-TCGGCCAGACAAATGATG-3'
1.2	5'-CAGCTAGACGAGCATCTGTT-3'	2.2	5'-TCGGCCAGACCTTAAAAGTG-3'
3.4	5'-AGCTAGACGAGAACTTCTA-3'	3.5	5'-TCGGCCAGACACCATTGA-3'
4.1	5'-TAGACGAACTTCTGGTTGGA-3'	4.2	5'-TTTAATGGTGTCTGGCCGA-3'
5.1	5'-AGCTAGACGAAACACAGTTT-3'	5.2	5'-ATATTTTAAGGTCTGGCCGA-3'
6.2	5'-AGCTAGACGAGAGCAGTATA-3'	6.3	5'-AATTATGCTTGTCTGGCCGA-3'
7.1	5'-AGCTAGACGATCCTCCTCCA-3'	7.2	5'-ACATTACACAGTCTGGCCGA-3'

performed by comparison with standard certificate material, Supelco FAME 10 mix 37 (CRM47885), according to AOCS Official Method Ce 1b-89.

2.6.2. Determination of total phenolic content

The total phenols were determined following the spectrophotometric method described by Velioglu et al. (1998) [18], with some modifications. 200 μL of the extract was mixed with 200 μL of Folin-Ciocalteu reagent (1:10; *v/v*) and allowed to stand at room temperature for 3 min. 1.5 mL (5 %) sodium bicarbonate solution was added to the mixture. After incubation for 60 min at room temperature, the absorbance was measured at 750 nm. In order to quantify the total phenols, a calibration curve was made of standard solutions of gallic acid (25–150 μg mL^{-1}) at known concentrations (mg gallic acid equivalent (GAE) g^{-1} dry weight of microalgae).

2.6.3. Antioxidant activity

The ABTS radical cation and FRAP assays were carried out according to the methodology described by Simonetti et al. (2020) [19], with some modifications. Results were expressed as milligrams of Trolox equivalents (TE) per gram of sample.

2.6.4. SOD activity

Following the method proposed by [20], SOD activity was assessed by measuring the inhibition of autoxidation by pyrogallol. The reaction mixture consisted of 1.9 mL of 0.1 M Tris-HCl, 50 μL of sample, and 50 μL of 20 mM pyrogallol in 1 mM HCl. Autooxidation inhibition was monitored every 30 s for 3 min by spectrophotometric analysis (UV-VIS Spectrophotometer 1204; Shimadzu) at 325 nm. The results were expressed as percentage of inhibition [I (%)] and were calculated with the following equation:

$$I\ (\%) = [Ab - (As - At)/Ab] \times 100$$

where: Ab = absorbance of blank sample (sample was replaced by solvent; t = 3 min); As = absorbance of sample (t = 3 min); At = absorbance of the test sample (pyrogallol was replaced by distilled water; t = 3 min).

2.6.5. CAT activity

The CAT activity was assessed in accordance with the method described by Hadwan and Abed (2016) [21]. The reaction mixture was prepared by adding 100 μL of sample and 1000 μL of H_2O_2, after 3 min to 37 °C, the mixture was stopped added 4000 μL of ammonium molybdate (32,4 mM). The absorbance of the complex was measured at 374 nm (UV-VIS Spectrophotometer 1204; Shimadzu) against blank and the first-order reaction rate constant (k) equation was used to determine catalase activity, as follows:

$$kU = \frac{2.303}{t} \times \left[log \frac{S^{\circ}}{S\text{-}M} \right] \times \frac{V_t}{V_s}$$

where t = time (3 min); S° = absorbance of standard (without sample); S = absorbance of sample; M = absorbance of the control test (without hydrogen peroxide; correction factor); Vt = total volume (5100 μL); and Vs = volume of serum (100 μL).

2.7. Pigment estimation

Mutant strain PA1004 after 160 days of crude oil treatment appeared colourless. To check the amount of chlorophyll, 1 mL of culture was centrifuged for 5 min at 14000 rpm. The pellet was incubated with 1 mL of DMSO for 10 min at 70 °C. The pellet was treated with DMSO until it appeared white. After each wash, the supernatant was collected and absorbance was registered in triplicate at 480 nm, 649 nm and 665 nm. The quantification has made following Wellburn et al. protocol [22].

2.8. Cell viability assay

Cell viability was evaluated using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as formerly reported [23].

IEC-6 cells (5.0×10^3 cells/well) were plated on 96-well plates and allowed to adhere for 24 h at 37 °C in a 5 % CO_2 atmosphere. Thereafter, the medium was substituted with fresh medium with or without the tested extracts (100–50–10 and 5 mg/mL) and incubated for 24 h. MTT (5 mg/mL) was then added to the cells. After 3 h, cells were lysed with 100 μL of a solution containing 50 % (*v/v*) *N*,*N*-dimethylformamide, and 20 % (*w/v*) sodium dodecyl sulfate (SDS; pH = 4.5). A microplate spectrophotometer reader (Titertek Multiskan MCC/340-DASIT) was used to measure the optical density (OD) in each well, as we previously reported [23]. The antiproliferative activity was measured as % dead cells = 100 − [(OD treated/OD control) × 100].

2.9. Statistical analysis

The results were analysed with Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) and GraphPad Prism 8.0.2 (San Diego, CA, USA). Each experiment was performed 3 times for all the analyses. Descriptive statistics were performed to calculate the mean and standard error of the mean (SEM). For chemical analysis 2way ANOVA and Ordinary one-way ANOVA test was performed for each group. A p value < 0.05 was considered significant. Population Genetic Analysis (POPGENE Version 1.32) software was used to analyse the genetic differences. A dominant parameter was used to obtain the genetic distance (Method UPGMA).

3. Results and discussion

3.1. Cells growth

The growth of the mutants shows that the different strains follow different kinetics (Fig. 1) reaching the exponential phase in different days. The best strain was PA1004 for growth speed.

3.2. Gene expression analysis

The spectrophotometric measurement with the Nanodrop allowed to determine the concentration of RNA extracted for the various mutant strains, which resulted in a concentration of about 950–1000 ng/μL, with good absorbance ratios 260/280 and 230/280, confirming that the extracted RNA was free from contamination by phenols and carbohydrates or by proteins. Fig. 2 shows how the amplification profiles

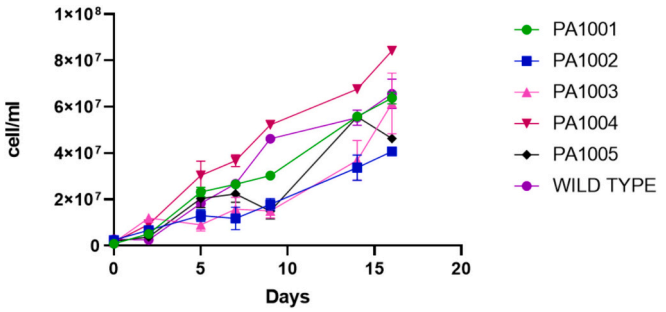

Fig. 1. Mutants different growth trend (Mean ± SEM).

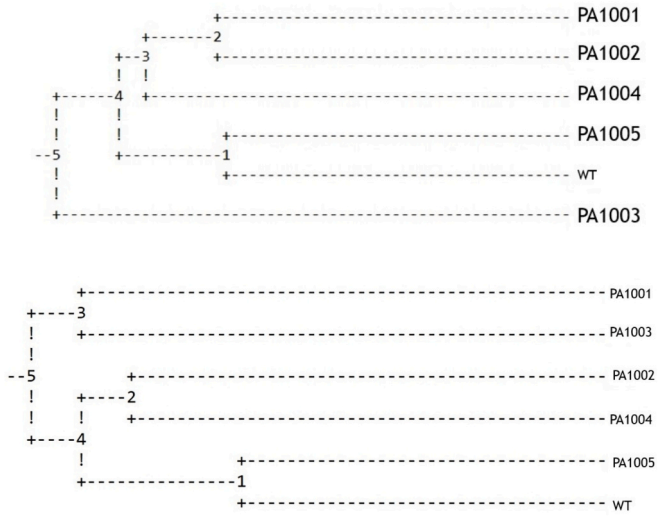

Fig. 2. Dendrogram of the different species of *H. pluvialis* before (up) and after (down) 160 days of treatment with crude oil.

changed markedly between the samples under analysis. These results support the hypothesis that differential transcription occurred following selective pressure, as also demonstrated in the dendrograms.

The dendrogram generate on not treated strains (Fig. 2) is formed by two main clusters. PA1001, PA1002 and PA1004 were grouped in Cluster 1, while PA1005 and WT constituted Cluster 2. The mutant strain PA1003 constituted a separate group. From the dendrogram, it was possible to divide the mutant strains into 5 groups. Compared to the WT, mutant strain PA1005 had a lower genetic distance D = 0.4532. The second group was formed by PA1001 and PA1002 which have a genetic distance equal to D = 0.4666. The two clusters have a genetic distance equal to D = 0.5824. The mutant strain PA1003 resulted in being the one farthest away genetically with a D = 0.6333 with respect to Cluster 1, and D = 0.6003 from Cluster 2.

After 120 days of treatment with petroleum, amplifications show how the changed markedly between the samples under analysis. These results support the hypothesis of differentiation that occurred following the selective pressure, and petroleum treatment, as finally demonstrated also in the following dendrogram Fig. 2. The dendrogram generated five groups. From these 5, it's possible to identify two main subgroups, the first one composed of 2 different clusters. The first subgroup is formed by PA1005 and WT (Cluster 1) and PA1002 and PA1004 (Cluster 2); the second one is formed by PA1001 and PA1003 (Cluster 3). Mutant strain PA1005 and WT had a genetic distance D = 0.3761. PA1002 and PA1004 had a genetic distance D = 0.4911. Therefore, cluster 1 and cluster 2 were separated by a genetic distance of D = 0.5835. The second group with cluster 3 presented a genetic distance of D = 0.5411, and the calculated distance between the two main groups was D = 0.6871.

3.3. Bioremediation activity

As described above, some preliminary analyses were performed on day 0 and day ten on the medium of each mutant strain and the WT (Table 2). The results show that only after 10 days, the amount of TOC increased in all mutant strains and the WT (Fig. 3), highlighting an increase in free carbon inside the supernatant.

TOC amount could suggest a metabolizing activity of microalgae which, placed in an anaerobic environment, degrade the organic carbon in the medium, producing secondary catabolites.

For the other parameters examined, it is clear that some mutant strains differ from others and WT. For the PA1004 mutant strain, nitrates do not decrease but increase within the medium (Fig. 3), while the PA1005 mutant strain does not appear to have action on the fluorides, which remain unchanged (Fig. 3).

The analyses carried out by GC-FID, on the other hand, after 20 and 160 days in the medium and in the biomass, showed how some mutants were more performing in the degradation of hydrocarbons than others (Table 3).

Specifically, the concentration of hydrocarbons (HC) within the aqueous medium was reduced by all mutant strains in a range from 26 % (WT) to 95 % (PA1004), except for the mutant strain PA1001 which, on the other hand, increased the amount of HC in the medium by 280 %. The results show that also, in this case, the trends were differential. The reduction within the biomass ranges from 21 % in PA1005 to 96 % in PA1004 and 98 % in PA1002. These results show how the mutants PA1002 and PA1004 were the best performers in biodegradation, which occurred almost totally (Fig. 4).

Considering that only the PA1002 and PA1004 mutants performed significantly important bioremediation, it was decided to focus for the subsequent analyses only on the PA1004 mutant, which appeared morphologically different in the culture medium. Observations under the optical microscope showed the cells of PA1004 after 160 days of treatment, completely transparent and devoid of chlorophyll. Microscopical analysis shows how the cell lost a green pigmentation being colourless. For this reason, it was decided to transfer an aliquot of the treated culture into a fresh medium. After seven days, the culture appeared green, and the cells re-pigmented. Chlorophylls were dosed both in the culture treated with crude oil after 160 days and in the fresh culture after seven days of growth, and the results show low levels in the treated culture compared to the refresh (Fig. 5).

3.4. Chemical analysis

Several analyses on the freeze-dried microalgal biomass were carried out to evaluate the anti-radical and antioxidant activity. All mutants before the treatment have been analysed (Fig. 6). The results show how all the mutants were different if compared with WT. PA1002 and PA1004 mutant strain have high concentration of fatty acids before the treatment compared with others mutants. PA1002 mutant strain shown a very high CAT activity but anti-radical activities of FRAP show how PA1005 mutant strain is the best performer. These results demonstrate a significant differentiation between the mutant strains which represent new mutant genotypes useful for other different applications excluded from the bioremediation considered in this study.

Furthermore, the content of phenols and fatty acids was also determined to characterize, in more detail, the mutant strain before (PA1004 green) and after treatment with crude oil (PA1004 brown).

As shown in Fig. 7, the differences are significant ($p < 0.05$) for all parameters between the groups. The results show how the PA1004 mutant strain after the treatment has opposite trends to the untreated culture. In detail, we see how the fat content is 2 times greater than the control. The tests regarding the antioxidant and anti-radical activities of ABTS and FRAP instead show how the mutant strain PA1004, after treatment with the crude oil, significantly reduces the ability to act against free radicals. In detail, after treatment, PA1004 reduces its

Table 2
Preliminary data of bioremediation expressed in mg ml $^{-1}$ (Mean ± SEM).

		WT	PA1001	PA1002	PA1003	PA1004	PA1005
TOC	Day 0	23.90 ± 0.78	22.80 ± 1.02	29.02 ± 0.88	22.98 ± 1.01	23.80 ± 1.15	22.78 ± 0.97
	Day 10	44.50 ± 1.06	44.80 ± 0.95	49.10 ± 0.45	45.30 ± 0.85	42.62 ± 0.90	45.03 ± 0.74
Nitrates	Day 0	0.95 ± 0.18	0.78 ± 0.02	0.81 ± 0.03	0.72 ± 0.15	0.89 ± 0.12	0.76 ± 0.09
	Day 10	0.24 ± 0.04	0.21 ± 0.05	0.22 ± 0.09	0.26 ± 0.05	1.17 ± 0.20	0.38 ± 0.07
Fluorides	Day 0	0.20 ± 0.040	0.21 ± 0.030	0.18 ± 0.012	0.20 ± 0.015	0.22 ± 0.008	0.20 ± 0.04
	Day 10	0.10 ± 0.010	0.15 ± 0.013	0.10 ± 0.011	0.12 ± 0.020	0.13 ± 0.015	0.19 ± 0.08
Chlorides	Day 0	10.75 ± 0.18	10.50 ± 0.25	10.98 ± 0.96	10.47 ± 0.15	10.56 ± 0.66	10.68 ± 0.35
	Day 10	12.50 ± 0.35	12.08 ± 0.52	13.74 ± 0.29	13.21 ± 0.84	12.33 ± 0.47	13.84 ± 0.45
Sulfate	Day 0	11.50 ± 1.06	10.85 ± 0.68	10.95 ± 1.03	10.50 ± 0.95	10.08 ± 0.08	11.03 ± 0.53
	Day 10	7.85 ± 0.85	8.28 ± 0.56	8.87 ± 0.64	8.26 ± 0.58	9.01 ± 0.03	7.50 ± 0.85
Phosphates	Day 0	69.20 ± 1.27	68.45 ± 1.36	68.95 ± 1.02	68.09 ± 0.98	69.15 ± 1.13	68.01 ± 0.85
	Day 10	53.50 ± 1.06	58.60 ± 0.49	56.39 ± 0.87	58.01 ± 1.10	64.50 ± 0.75	69.83 ± 1.25

action against both hydrogen peroxide and iron used in the assays; on the contrary, it synthesizes enzymes involved in the oxidative response, such as superoxide dismutase (SOD) and catalase (CAT) Fig. 7. CAT turns out to be double compared to the other samples analysed, indicating how the treated strain reacts against the pollutants by producing enzymes capable of guaranteeing their survival in conditions of high oxidative stress, given precisely by crude oil. Analyses on the supernatant of the treated mutant were performed to evaluate its characteristics. From the data, the supernatant is as rich in fat as the treated algal biomass, and the phenols are more present in double quantities than in the untreated strain; also, the anti-radical properties are as high as the untreated strain. SOD and CAT were not detected in the supernatant.

If we then analyse the composition of the FAMEs (Table 4), it is evident that the short-chain fatty acids significantly increased in the PA1004 brown. Unlike in the PA1004 green, the long-chain fatty acids are higher. Of relevance is the increase of cis-10-Pentadecenoic acid (C15:1), which increased by 2000 % compared to the control, followed by erucic acid (C22:1), which increased by 1980 % and by arachidonic acid (C20:4 n6) increased by 1277 %. Particular is the disappearance of intermediate chain FAMEs, such as stearic acid (C18), reduced by 98 % in the treated compared to the control.

3.5. Cell viability

The cytotoxic activity of PA1004 green and brown biomass was evaluated on in vitro human cells, considering their attractive characteristics. The results (*Supplementary material_ Fig. S2*) demonstrate how after treatment with petroleum, the mutant strain is less cytotoxic, at high concentrations (100 mg/ml), than the untreated control.

4. Discussion

This study aimed to select specific genotypes of *H. pluvialis* to remove hydrocarbons from contaminated water. The choice of microalgae is related to its particular composition of metabolites. The hypothesis of conducting bioremediation and using algal biomass for secondary purposes other than biofuel production has been the reason for its selection. For example, considering the less cytotoxicity activity after crude oil treatment, and thanks to its anti-inflammatory and antioxidant properties, algal biomass could be used as a fertilizer for the soil or as a supplement for animal feed. Furthermore, in previous studies, we optimise the growth protocol to make *H. pluvialis* grow faster, reaching the exponential phase only after 5–7 days. The first results obtained on the wild-type [14] have been very encouraging as they demonstrated how *H. pluvialis* can adapt to a hostile environment by rapidly varying enzymatic and metabolic production. The preliminary study also allowed us to develop an action protocol compared to what is reported in the literature [24] capable of effectively evaluating the ability of different mutated strains resulting from a hard selective pressure to use and or remove hydrocarbons from the culture medium. The induced selective pressure allowed the selection of only five mutant strains, which survived a condition of anoxia and high concentrations of toxic and harmful elements. The five mutant strains were discriminated based on morphological, adaptive and genetic differences and then evaluated how these modified their ability to react to the presence of hydrocarbons in the medium. It is evident that the mutant strains show modifications that make them more or less performant in growth compared to the WT already in the fresh medium. Only the PA1004 mutant underwent ameliorative changes compared to the WT. Although the analyses regarding the genetic differences are preliminary, the effects of crude oil on the gene expression of the analysed strains are evident. We performed on RAPD amplicons initial analysis on genetic differentiation, especially after the crude oil treatment and we found the activation on genes belonging to transmembrane transporter activity family proteins (specifically ABC transporter permease protein), especially in PA1002 an PA1004 mutant stains. These results will be deepened through NGS analysis which are in progress. However, these differences are confirmed by the chemical analyses carried out on the medium and the biomass following treatment with crude oil. Until now, the literature has experimented with low concentrations of crude oil, reporting the degradation of only some compounds and specific types of hydrocarbons [25]. Considering the preliminary analyses and that in the medium, after 160 days of growth, the quantity of HC had decreased, a double hypothesis was formulated: I) that the microalgae had accumulated HC inside the biomass inside the vacuoles; II) microalgae used HC as a source of nutrition and carbon. The HCs in the microalgal biomass were analysed to convalidation one or both hypotheses. However, the results did not confirm either of the two hypotheses formulated. Two mutants (PA1002 and PA1004) present the almost absence of hydrocarbons in the biomass, confirming HC as a nutritional source (hypothesis II). This hypothesis would seem to be valid if we consider the metabolism of microalgae. They can employ organic carbon via two metabolic pathways: the pentose phosphate pathway (PPP) in heterotrophic conditions and the Embden-Meyerhof Pathway (EMP) during the light phase [26]. Unlike these two mutants, the others show reduced but not absent quantities of HC compared to day 20. This would lead to the accumulation hypothesis being considered valid. Raven et al. reports how many microalgae assimilate carbon via phagotrophy [27]. Although these results do not demonstrate accumulation, they do not confirm total use as a nutritional source either. The only strain that showed metabolic change was the PA1004 mutant. Its deactivation of chlorophylls highlighted how the high adaptability of the microalgal genome has varied the production of the enzymes necessary to produce energy. Microalgae deactivated the production of chlorophylls, useless in anaerobic conditions and used the organic carbon present in the medium as an energy source, as reported by Su, 2021 [28]. The morphological and biological variation of the PA1004 mutant was explored by further analyses of the ability to act against stressful conditions and by analysing the biomass and supernatant composition. These results highlight how the stressed cell releases excess substances into the medium to overcome the damage induced by the stressful environment. High ABTS and FRAP values indicate a need for cells to carry out their activities outside where the

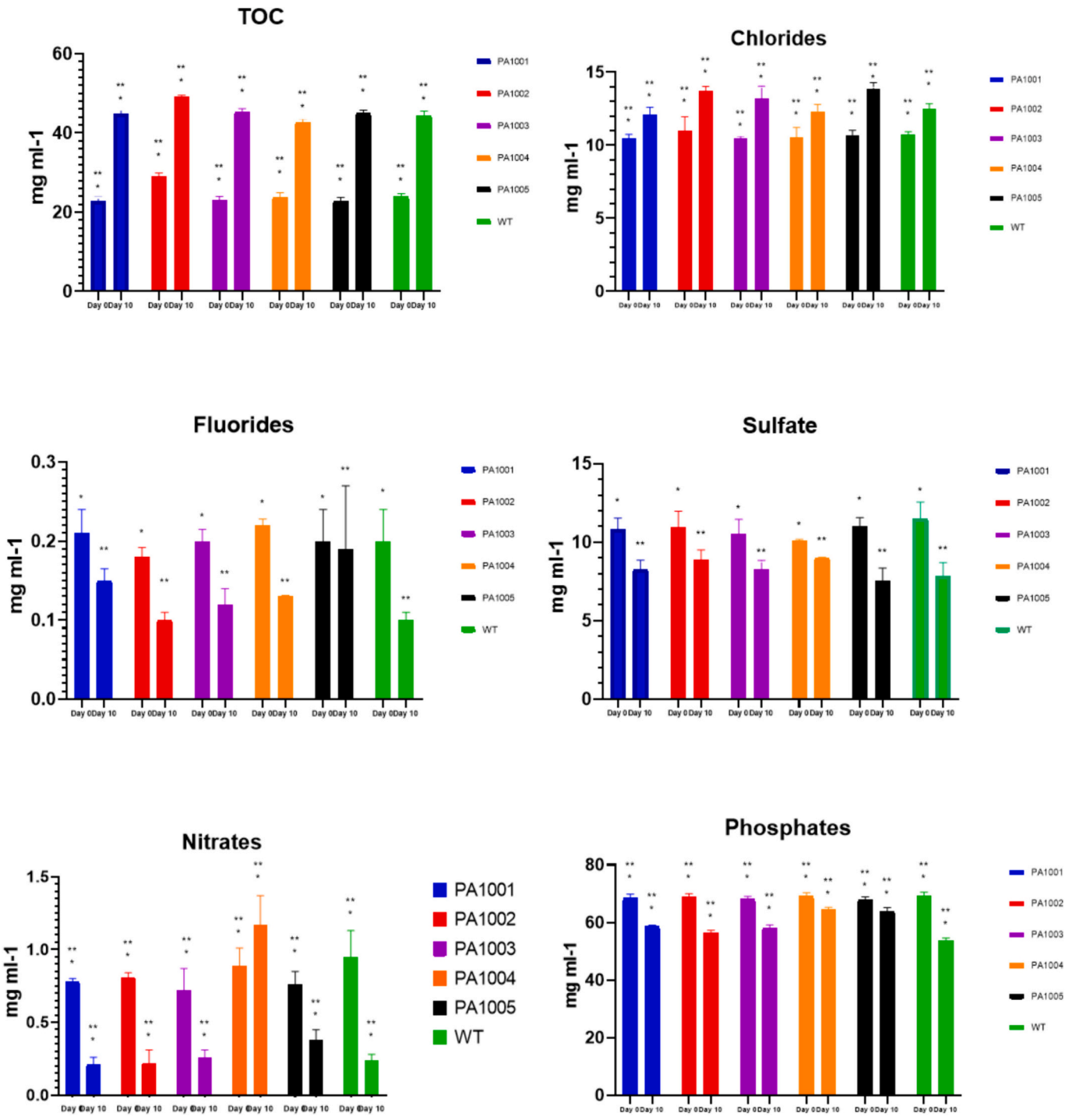

Fig. 3. Preliminary data of bioremediation. Values are reported as (Mean ± SEM). 2way ANOVA was used for the analysis. * or ** = $p < 0.05$ between the mutants at the same time point); * and ** = $p < 0.05$ between time points and mutants.

Table 3
Hydrocarbon's concentration in water and microalgal biomass (Mean ± SEM).

		WT	PA1001	PA1002	PA1003	PA1004	PA1005
HC in H2O (ppm)	Day 20	8.965 ± 0.275	0.505 ± 0.115	1.205 ± 0.025	12.040 ± 0.980	4.140 ± 0.110	5.795 ± 0.165
	Day 160	6.650 ± 0.370	1.935 ± 0.075	0.470 ± 0.100	5.900 ± 0.120	0.195 ± 0.015	4.020 ± 0.190
HC in algae (mg/Kg)	Day 20	4675 ± 50	8099 ± 43	9525 ± 28	6973 ± 28	11,296 ± 48	2898 ± 44
	Day 160	915 ± 19	5609 ± 11	188 ± 3	1767 ± 14	476 ± 14	2293 ± 12

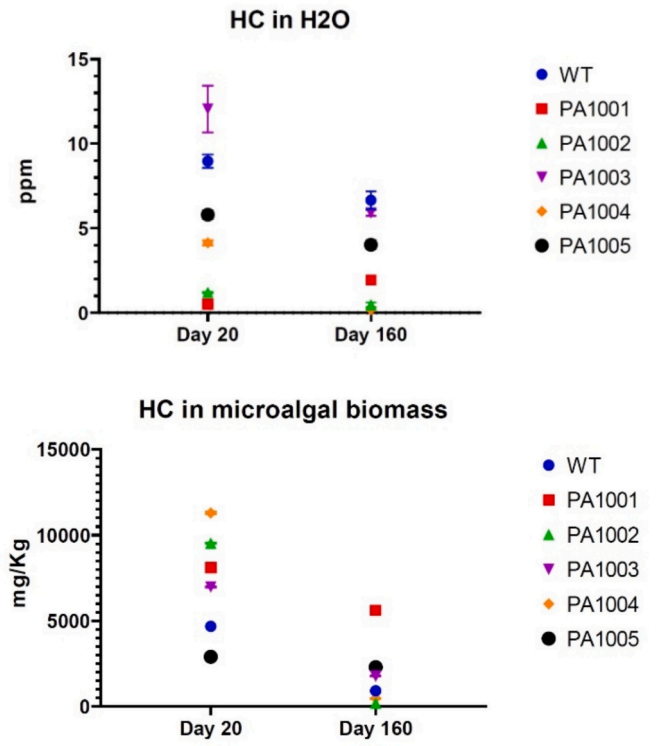

Fig. 4. Hydrocarbon's concentration in water and microalgal biomass. Value reported as Mean ± SEM.

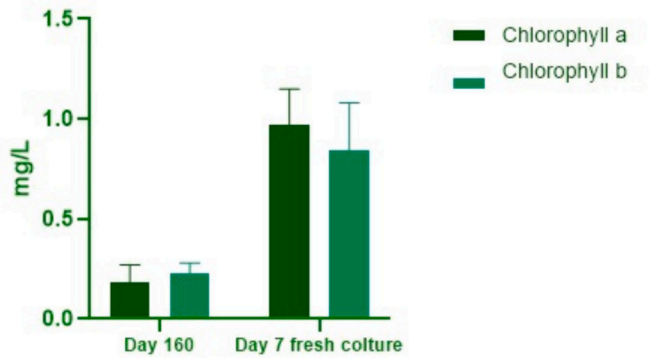

Fig. 5. Microscopical analysis of PA1004 mutant strain, (a) after the crude oil treatment and (b) in a new fresh media. c) chlorophyll concentration reported as mean ± SEM. $p < 0.05$ between groups and time point.

contaminants and oxidizing agents such as ROS, alkoxyl (RO °) and hydroxyls (OH°) are present [29]. Also, the of phenols in the medium is clearly due to the presence of petroleum hydrocarbons [30], which following the bioremediation action by the microalga, have generated phenolic intermediates [31]. However, the phenols, as demonstrated by Almutairi, are toxic for the microalgae, which increases the production of antioxidant systems [32]. Furthermore, the presence of phenolic compounds is correlated with the increase of FAMEs within the microalgal biomass of PA1004 brown. The behaviour of the mutant is in line with the results obtained by Liu et al. [33] on *H. pluvialis* cells treated with superoxide anion radical, in which cells deficient in chlorophyll, brownish, increase the production of enzymes responsible for the elimination of excess ROS. Their differential metabolism could be the reason. The untreated PA1004 strain, being in non-stress conditions, metabolizes fatty acids necessary for its sustenance, confirming the average values present in the literature [34]. The treated PA1004 strain,

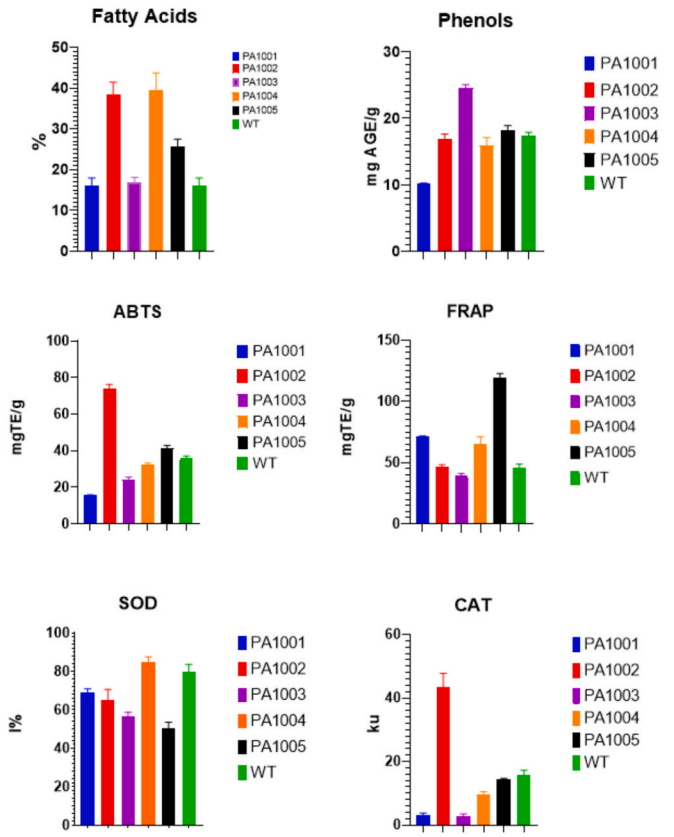

Fig. 6. Chemical analysis of mutant strains. One way ANOVA was used for the analysis. $p < 0.05$ between each group.

grown in anaerobic conditions and the presence of hydrocarbons, on the other hand, implements a catabolic process which destroys the hydrocarbons present in the medium to obtain the energy sustenance necessary for its survival. The lower cytotoxic activity suggests that biomass after bioremediation could be recovered and used for various purposes, including in the animal field as food supplementation.

5. Economic analysis

Unlike traditional industrial wastewater treatment plants that require significant investments in heavy infrastructure and complex technologies, the installation of microalgae-based bioremediation systems is generally less expensive. These systems, usually applied in situ, during accidental releases in marine environments, use various physical and chemical treatments to remove crude oil as rapidly as possible [35]. The physical-chemical processes involve initial degradation due to climatic factors, oil slick spreading, evaporation of volatile compounds and emulsion formation. Solubilization, sedimentation and photoreactions facilitate the dispersion of soluble oil components [36,37]. The synergistic action of marine microorganisms contributes to the degradation of leaked hydrocarbons. This process is driven by evolution, which has selected microorganisms capable of using hydrocarbons as a source of energy [13]. Microalgae cannot be used directly in situ, because of the environmental balance and the slow process for emergencies. However, if we consider the refinery industry and the water used in the process, it is evident that microalgae can be used for the purpose. Different photobioreactors can be installed and the water collected and stored in specific containers can be treated. These systems can often be integrated or retrofitted to existing infrastructure with minimal modifications. *H. pluvialis* cultivation does not require cutting-edge technologies or expensive equipment, which keeps the initial investment cost low. Furthermore, the high efficiency of the mutated strains obtained in this

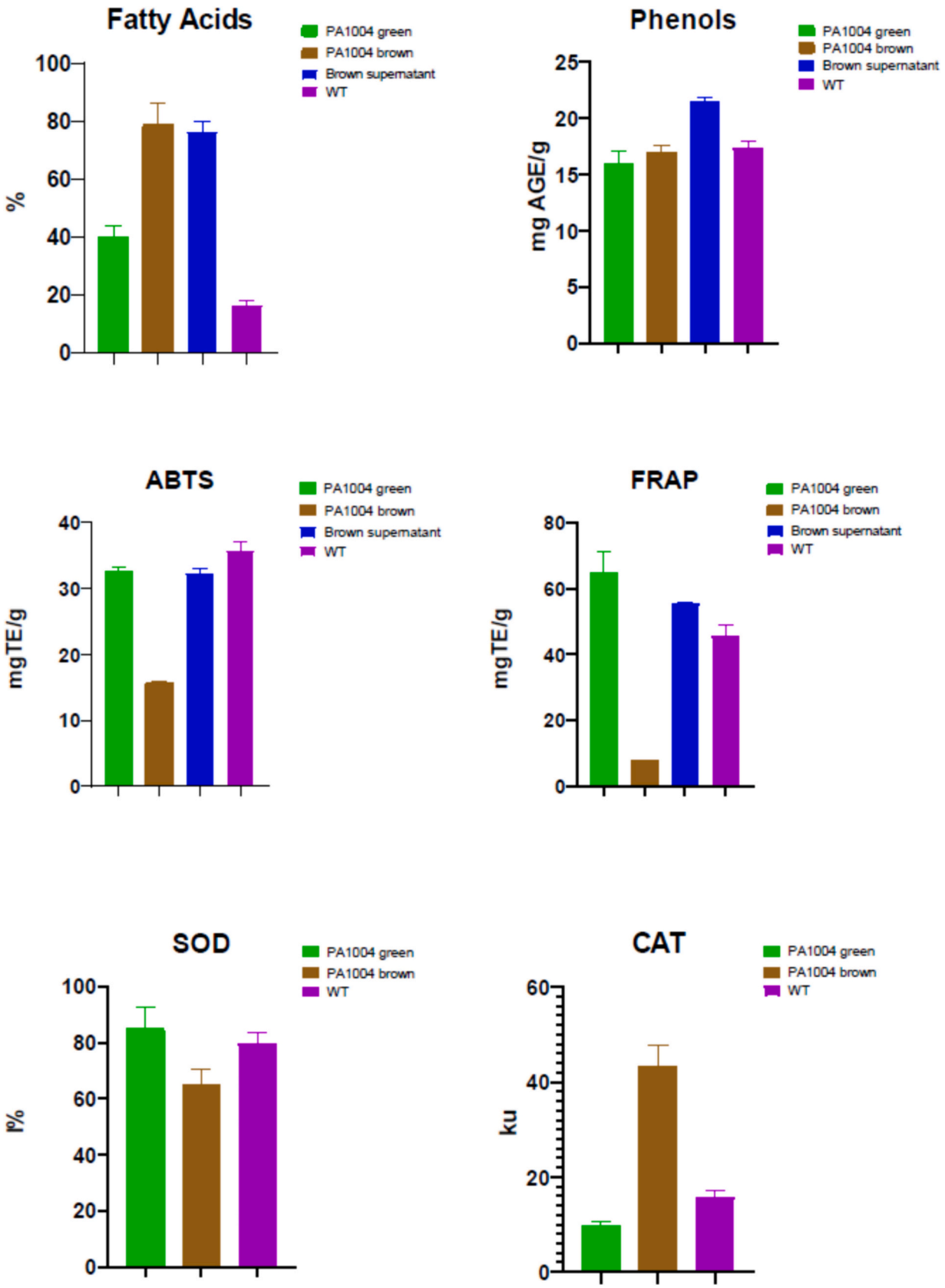

Fig. 7. Chemical analysis of mutant strain PA1004 before (Green) and after (Brown) the treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
FAME composition of mutant strain PA1004 before (Green) and after (Brown) the treatment, with the percentage of variation between the two groups.

	PA1004 GREEN	PA1004 BROWN		% variation
C 8:0	0,37	1,19	↑	220,48
C 10:0	0,71	2,49	↑	250,51
C 12:0	1,53	4,98	↑	225,31
C 14:0	5,9	13,47	↑	128,27
C14:1	0,79	6,29	↑	696,81
C15:0	0,62	1,02	↑	65,28
C15:1	0,13	2,78	↑	2.039,57
C 16:0	27,23	15,52	↓	−43,01
C 16:1	0,63	2,93	↑	364,73
C 17:0	1,11	2,34	↑	111,01
C 17:1	0,45	0,73	↑	62,66
C 18:0	19,4	0,29	↓	−98,49
trans - C 18:1 Δ9	0,5	0,59	↑	17,11
cis - C 18:1 Δ9	21,58	23,13	↑	7,18
cis - C 18:2 Δ9	7,29	6,00	↓	−17,67
C 20:0	0,49	0,59	↑	19,50
cis - C 18:3 Δ6	1,31	0,59	↓	−55,30
C 20:1	0,54	0,29	↓	−45,78
cis - C 18:3 Δ9	2,82	2,49	↓	−11,75
C 21:0	0,28	0,15	↓	- 47,72
C 20:2	0,67	0,29	↓	−56,30
C 22:0	0,26	0,15	↓	−43,70
cis - C 20:3 Δ8,11,14	0,12	1,90	↑	1.485,91
C 22:1	1,55	0,29	↓	- 81,11
cis - C 20:3 Δ11,23	0,47	0,29	↓	- 37,71
C 20:4ω6	0,17	2,34	↑	1.277,80
C 22:2	0,19	3,95	↑	1.980,30
C 24:0	0,08	0,15	↑	82,99
C 20:5	1,58	1,46	↓	- 7,35
C 24:1	0,36	0,73	↑	103,32
C 22:6	0,87	0,59	↓	- 32,69
Total	100	100		

study in hydrocarbon bioremediation reduces the need for more expensive secondary treatments and the operating costs associated with wastewater treatment, such as those for the purchase of chemical reagents and energy consumption, typical of conventional wastewater treatment methods. The ability of these mutated strains to produce biomass rich in fatty acids and antioxidants during the bioremediation process offers the opportunity to generate additional revenue streams through the sale of derived products, such as biofuels and nutritional supplements. This can help offset operating costs and accelerate the return on investment.

By investing in an environmentally friendly treatment approach, companies demonstrate a clear commitment to reducing the environmental impact of their operations, following the ESG standards and thus improving their reputation and attractiveness to sustainability-oriented investors. The reduction in operating costs and the potential generation of new revenues through biomass valorisation contribute to greater financial resilience, making the company less vulnerable to fluctuations in raw material and energy costs.

6. Conclusion

The present study wanted to select different strains of *H. pluvialis* suitable for the bioremediation of crude oil wastewater. The preliminary genetic results demonstrate how the selective pressure, represented by the crude oil itself, has genetically modified the wild-type strain in structure and gene expression. Based on these results a more specific analysis should be conducted to compare structural differences between the strain. Only two genotypes almost completely remove hydrocarbons from the medium after 160 days of anaerobic growth. The results obtained from chemical and cell analysis also suggest that after the bioremediation process, the microalgal biomass can be recovered and used in various sectors, such as the production of biofuels (considering the high levels of fatty acids) by perfectly closing an economy greens process. In conclusion, the bioremediation systems based on *H. pluvialis* for crude oil wastewater bioremediation presents itself as a strategically advantageous investment characterized by low initial and management costs. This investment facilitates faster implementation and a quick return on investment and strengthens the companies' commitment to sustainable practices, positively contributing to its positioning in the context of *Environmental, Social and Governance standards* (ESG).

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Rosa Paola Radice: Writing – original draft, Methodology, Conceptualization. **Giulia Grassi:** Writing – review & editing, Formal analysis, Data curation. **Giambattista Capasso:** Writing – review & editing, Formal analysis, Data curation. **Egidio Montagnuolo:** Writing – review & editing, Data curation. **Donato Aiello:** Writing – review & editing, Data curation. **Anna Maria Perna:** Validation, Investigation. **Stefania Marzocco:** Writing – review & editing, Data curation. **Giuseppe Martelli:** Supervision, Project administration, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103693.

References

[1] B. Ramesh Kumar, G. Deviram, T. Mathimani, P.A. Duc, A. Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588, https://doi.org/10.1016/j.bcab.2019.01.017.
[2] L. Gouveia, S. Graça, C. Sousa, L. Ambrosano, B. Ribeiro, E.P. Botrel, P.C. Neto, A. F. Ferreira, C.M. Silva, Microalgae biomass production using wastewater: treatment and costs. Scale-up considerations, Algal Res. 16 (2016) 167–176, https://doi.org/10.1016/j.algal.2016.03.010.
[3] A. Sonune, R. Ghate, Developments in wastewater treatment methods, DESALINATION A. Sonune, R. Ghate /, Desalination 167 (2004) 55–63, https://doi.org/10.1016/3.desal.2004.06.113.
[4] M. Plöhn, O. Spain, S. Sirin, M. Silva, C. Escudero-Oñate, L. Ferrando-Climent, Y. Allahverdiyeva, C. Funk, Wastewater treatment by microalgae, Physiol. Plant. 173 (2021) 568–578, https://doi.org/10.1111/ppl.13427.
[5] M. Jain, A. Majumder, P.S. Ghosal, A.K. Gupta, A review on treatment of petroleum refinery and petrochemical plant wastewater: a special emphasis on constructed wetlands, J. Environ. Manag. 272 (2020), https://doi.org/10.1016/j.jenvman.2020.111057.
[6] K. Abuhasel, M. Kchaou, M. Alquraish, Y. Munusamy, Y.T. Jeng, Oily wastewater treatment: overview of conventional and modern methods, challenges, and future opportunities, Water 13 (2021) 980, https://doi.org/10.3390/W13070980 (2021, Vol. 13, Page 980).
[7] G. Yadav, S.K. Dash, R. Sen, A biorefinery for valorization of industrial waste-water and flue gas by microalgae for waste mitigation, carbon-dioxide sequestration and algal biomass production, Sci. Total Environ. 688 (2019) 129–135, https://doi.org/10.1016/j.scitotenv.2019.06.024.
[8] A. Gallego-Schmid, R.R.Z. Tarpani, Life cycle assessment of wastewater treatment in developing countries: a review, Water Res. 153 (2019) 63–79, https://doi.org/10.1016/j.watres.2019.01.010.
[9] J. Zhang, H. Wang, Y. Shao, G. Liu, L. Qi, W. Dang, J. Yuan, Y. Li, Z. Xia, Analysis on common problems of the wastewater treatment industry in urban China, Chemosphere 291 (2022) 132875, https://doi.org/10.1016/j.chemosphere.2021.132875.

[10] S. Arsad, Y.W. Mulasari, N.Y. Sari, E.D. Lusiana, Y. Risjani, M. Musa, M. Mahmudi, F.S. Prasetiya, L.A. Sari, Microalgae diversity in several different sub-habitats, Global J. Environ. Sci. Manag. 8 (2022) 561–574, https://doi.org/10.22034/GJESM.2022.04.08.
[11] K.S. Khoo, W.Y. Chia, K.W. Chew, P.L. Show, Microalgal-bacterial consortia as future prospect in wastewater bioremediation, environmental management and bioenergy production, Indian J. Microbiol. 61 (2021) 262–269, https://doi.org/10.1007/s12088-021-00924-8.
[12] H.V. de Mendonça, J.P.H.B. Ometto, M.H. Otenio, I.P.R. Marques, A.J.D. dos Reis, Microalgae-mediated bioremediation and valorization of cattle wastewater previously digested in a hybrid anaerobic reactor using a photobioreactor: comparison between batch and continuous operation, Sci. Total Environ. 633 (2018) 1–11, https://doi.org/10.1016/j.scitotenv.2018.03.157.
[13] R.P. Radice, V. De Fabrizio, A. Donadoni, A. Scopa, G. Martelli, Crude oil bioremediation: from bacteria to microalgae, Processes 11 (2023), https://doi.org/10.3390/pr11020442.
[14] R.P. Radice, M. Sansone, G. D'Arienzo, A. Scopa, G. Martelli, Bioremediation of crude oil by Haematococcus pluvialis: a preliminary study, Processes 10 (2022) 2472, https://doi.org/10.3390/pr10122472.
[15] R.P. Radice, R. Fiorentino, M. De Luca, A.R. Limongi, E. Viviano, G. Bermano, G. Martelli, An innovative protocol to select the best growth phase for astaxanthin biosynthesis in *H. pluvialis*, Biotechnol. Reports 31 (2021) e00655, https://doi.org/10.1016/j.btre.2021.e00655.
[16] William Horwitz, Official Methods of Analysis of AOAC International, AOAC International, 2006.
[17] International Olive Council, Preparation of the Fatty Acid Methyl Esters From Olive Oil and Olive-pomace Oil, COI/T.20/Doc. http://www.internationaloliveoil.org/, 2001 (accessed July 5, 2023).
[18] Y.S. Velioglu, G. Mazza, L. Gao, B.D. Oomah, Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products, J. Agric. Food Chem. 46 (1998) 4113–4117, https://doi.org/10.1021/jf9801973.
[19] A. Simonetti, A. Perna, G. Grassi, E. Gambacorta, Antioxidant activity of different cheese-honey combinations before and after in vitro gastrointestinal digestion, LWT 131 (2020) 109725, https://doi.org/10.1016/j.lwt.2020.109725.
[20] G. Grassi, A. Simonetti, E. Gambacorta, A. Perna, Decrease of activity of antioxidant enzymes, lysozyme content, and protein degradation in milk contaminated with heavy metals (cadmium and lead), JDS Commun. 3 (2022) 312–316, https://doi.org/10.3168/jdsc.2022-0222.
[21] M.H. Hadwan, H.N. Abed, Data supporting the spectrophotometric method for the estimation of catalase activity, Data Brief 6 (2016) 194–199, https://doi.org/10.1016/j.dib.2015.12.012.
[22] A.R. Wellburn, The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution, J. Plant Physiol. 144 (1994) 307–313, https://doi.org/10.1016/S0176-1617(11)81192-2.
[23] S.F. Rapa, B. Waltenberger, R. Di Paola, S. Adesso, R. Siracusa, A.F. Peritore, R. D'Amico, G. Autore, S. Cuzzocrea, H. Stuppner, S. Marzocco, Plumericin prevents intestinal inflammation and oxidative stress in vitro and in vivo, FASEB J. 34 (2020) 1576–1590, https://doi.org/10.1096/FJ.201902040R.
[24] M.M. El-Sheekh, R.A. Hamouda, A.A. Nizam, Biodegradation of crude oil by Scenedesmus obliquus and Chlorella vulgaris growing under heterotrophic conditions, Int. Biodeterior. Biodegradation 82 (2013) 67–72, https://doi.org/10.1016/j.ibiod.2012.12.015.
[25] R.A.E.F. Hamouda, N.M. Sorour, D.S. Yeheia, Biodegradation of crude oil by Anabaena oryzae, Chlorella kessleri and its consortium under mixotrophic conditions, Int. Biodeterior. Biodegrad. 112 (2016) 128–134, https://doi.org/10.1016/j.ibiod.2016.05.001.
[26] O. Perez-Garcia, F.M.E. Escalante, L.E. de-Bashan, Y. Bashan, Heterotrophic cultures of microalgae: metabolism and potential products, Water Res. 45 (2011) 11–36, https://doi.org/10.1016/J.WATRES.2010.08.037.
[27] J.A. Raven, J. Beardall, K.J. Flynn, S.C. Maberly, Phagotrophy in the origins of photosynthesis in eukaryotes and as a complementary mode of nutrition in phototrophs: relation to Darwin's insectivorous plants, J. Exp. Bot. 60 (2009) 3975–3987, https://doi.org/10.1093/JXB/ERP282.
[28] Y. Su, Revisiting carbon, nitrogen, and phosphorus metabolisms in microalgae for wastewater treatment, Sci. Total Environ. 762 (2021) 144590, https://doi.org/10.1016/j.scitotenv.2020.144590.
[29] M. Ghodrati, M. Kosari-Nasab, G. Zarrini, A. Movafeghi, Crude oil contamination enhances the lipoxygenase gene expression in the green microalga scenedesmus dimorphus, Biointerface Res. Appl. Chem. 11 (2021) 11431–11439, https://doi.org/10.33263/BRIAC114.1143111439.
[30] A.A. Melaibari, A.S. Elamoudi, M.E. Mostafa, N.H. Abu-Hamdeh, Waste-to-energy in Saudi Arabia: treatment of petroleum wastewaters utilizing zeolite structures in the removal of phenol pollutants by using the power of molecular dynamics method, Eng. Anal. Bound. Elem. 148 (2023) 317–323, https://doi.org/10.1016/J.ENGANABOUND.2023.01.003.
[31] R. Ebaid, Q. Wang, S. Faisal, L. Li, A. Abomohra, Valorization of floral foam waste via pyrolysis optimization for enhanced phenols recovery, Chemosphere 310 (2023) 136758, https://doi.org/10.1016/J.CHEMOSPHERE.2022.136758.
[32] A.W. Almutairi, Phenol phycoremediation by Haematococcus pluvialis coupled with enhanced astaxanthin and lipid production under rac-GR24 supplementation for enhanced biodiesel production, Saudi J. Biol. Sci. 30 (2023) 103681, https://doi.org/10.1016/j.sjbs.2023.103681.
[33] J. Liu, X. Zhang, Y. Sun, W. Lin, Antioxidative capacity and enzyme activity in Haematococcus pluvialis cells exposed to superoxide free radicals, Chin. J. Oceanol. Limnol. 28 (2010) 1–9, https://doi.org/10.1007/s00343-010-9244-6.
[34] M.C. Damiani, C.A. Popovich, D. Constenla, P.I. Leonardi, Lipid analysis in Haematococcus pluvialis to assess its potential use as a biodiesel feedstock, Bioresour. Technol. 101 (2010) 3801–3807, https://doi.org/10.1016/j.biortech.2009.12.136.
[35] J.W. Anderson, S.L. Kiesser, D.L. McQuerry, G.W. Fellingham, Effects of oil and chemically dispersed oil in sediments on clams, in: 2005 International Oil Spill Conference, IOSC 2005, 2005, p. 2327, https://doi.org/10.7901/2169-3358-1985-1-349.
[36] M. Hussein, M. Jin, J.W. Weaver, Development and verification of a screening model for surface spreading of petroleum, J. Contam. Hydrol. 57 (2002) 281–302, https://doi.org/10.1016/S0169-7722(01)00220-0.
[37] S.I. Andersen, J.M. Del Rio, D. Khvostitchenko, S. Shakir, C. Lira-Galeana, Interaction and solubilization of water by petroleum asphaltenes in organic solution, Langmuir 17 (2001) 307–313, https://doi.org/10.1021/la000871m.