UNIVERSIDADE DE FEDERAL DA GRANDE DOURADOS
FACULDADE DE CIÊNCIAS EXATAS E TECNOLÓGIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA AMBIENTAL

EFEITOS DA IRRADIAÇÃO ULTRAVIOLETA NA EFICIÊNCIA DAS MICROALGAS NA BIORREMEDIAÇÃO

THAIS HUARANCCA REYES

DOURADOS/MS – 2022

EFEITOS DA IRRADIAÇÃO ULTRAVIOLETA NA EFICIÊNCIA DAS MICROALGAS NA BIORREMEDIAÇÃO

THAIS HUARANCCA REYES

Dissertação de mestrado submetida ao programa de pós-graduação em Ciência e Tecnologia Ambiental, como um dos requisitos necessários para a obtenção do título de mestre em Ciência e Tecnologia na área de concentração de Tecnologia Ambiental.

Orientador: Prof. Dr. Gustavo Graciano Fonseca

DOURADOS/MS – 2022

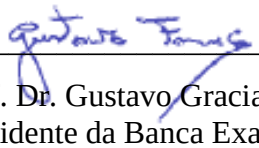
Dados Internacionais de Catalogação na Publicação (CIP).

R586e	Reyes, Thais Huarancca Efeitos da irradiação ultravioleta na eficiência das microalgas na biorremediação. / Thais Huarancca Reyes. – Dourados, 2022. Orientador: Prof. Dr. Gustavo Graciano Fonseca Dissertação (Mestrado) Programa de Pós-Graduação em Ciência e Tecnologia Ambiental – Universidade Federal da Grande Dourados. 1. Biorremediação de efluentes. 2. Microalgas 3. Pigmentos fotossintéticos. 4. Antioxidantes não enzimáticos. 5. Luz ultravioleta. I. Título.

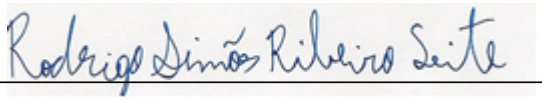
Ficha catalográfica elaborada pela Biblioteca Central – UFGD.

Termo de Aprovação

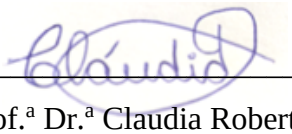
Após apresentação, arguição e apreciação pela banca examinadora, foi emitido o parecer **APROVADA**, para a dissertação intitulada: **"EFEITOS DA IRRADIAÇÃO ULTRAVIOLETA NA EFICIÊNCIA DAS MICROALGAS NA BIORREMEDIAÇÃO"**, de autoria de **THAIS HUARANCCA REYES**, apresentada ao Programa de Mestrado em Ciência e Tecnologia Ambiental da Universidade Federal da Grande Dourados.



Prof. Dr. Gustavo Graciano Fonseca
Presidente da Banca Examinadora



Prof. Dr. Rodrigo Simoes Ribeiro Leite
Membro Examinador (UFGD)



Prof.ª Dr.ª Claudia Roberta Damiani
Membro Examinador (UFGD)

Dourados/MS, 18 agosto de 2022.

AGRADECIMENTOS

To my husband *chiqui* Lorenzo and my son *pelù* Leonardo, thank you for your unconditional love and support. I am sure that this was only possible because of you. To the rest of my Peruvian, Japanese and Italian big family, you are the best and your constant support makes life all the easier. Gracias, arigatou, grazie!

I would like to extend a sincere thank you to my supervisor Prof. Gustavo Graciano Fonseca for your time, willingness and support. I look forward to the continuation of future works with your research group.

My genuine thanks also go out to all professors of the Master Program of Environmental Sciences and Technology at the Federal University of Grande Dourados (UFGD). The guidance and willingness you have given me has been greatly appreciated. A special thank you must be given to Prof. Claudia Roberta Damiani, who has given me good advice to pursue an experience abroad as a Master student internship.

To the Plant Physiology Research Group at University of Pisa (UNIPI), thank you for making me feel so welcome. A sincere thank you to Prof. Lorenzo Guglielminetti for giving me the opportunity to develop my Master research study in his research group. Your guidance and willingness to listen to my ideas and allow me to follow them has permitted me to grow as a researcher. Furthermore, I would like to thank Prof. Laura Pistelli, Dr. Lorenzo Mariotti and Dr. Maurizio Curadi for their invaluable support during the physiological and analytical analyses. Help from Otello Malfatti has been instrumental in the cigarette butt cleaning process.

My thanks also go out to others who have aided me in my research. Dr. Carolina Chiellini (Italian National Research Council, CNR) has been instrumental in the isolation, characterization and culture of microalgae. Help from Dr. Francesco Vitali (Council for Agricultural Research and Economics, CREA) was priceless in the multiple factor analysis. Finally, I would like to thank UNIPI for all the facilities provided, which have allowed me to take the next step in my career. I would like also to thank the Fondazione Cassa di Risparmio di Lucca (Lucca, Italy) that financially supported this study (progetto FOCUS 2019/2021) and the Municipality of Capannori (Lucca, Italy) for the disposal management of cigarette butts.

ABREVIATURAS E SIGLAS

ANOVA …… Analysis of variance

CB …… Cigarette butts

CBW …… Cigarette butt wastewater

Chl*a* …….. Chlorophyll *a*

Chl*b* …….. Chlorophyll *b*

Chl_{total} …… Total chlorophyll

Car …… Carotenoids

DPPH …… 2,2-diphenyl-1-picrylhydrazyl

EDTA …… Ethylenediaminetetraacetic acid

MFA ……. Multiple factor analysis

ML …… Maximum likelihood

NCBI ……..... National center for biotechnology information

p …… Probability

PAR ……..…….. Photosynthetically active radiation

PPFD ……..…… Photosynthetic photon flux density

ROS ……..……. Reactive oxygen species

SE ……..……..….. Standard error

TAC ……..…… Total antioxidant capacity

TAP ……..……. Tris-acetate-phosphate

T0 ……..……..... Time zero

UV……...……...……... Ultraviolet

UWW ……..... Untreated wastewater

LISTA DE TABELAS

LISTA DE FIGURAS

CAPÍTULO 2

CAPÍTULO 3

RESUMO

Os objetivos deste estudo foram avaliar a capacidade de microalgas na remediação de efluentes provenientes do processo de limpeza de bitucas de cigarro (CB) e avaliar o uso da radiação ultravioleta B (UV-B) como possível indutor da atividade de antioxidantes em microalgas para aliviar os efeitos negativos de poluentes nesses efluentes. No Capítulo 2, seis isolados de microalgas naturais foram analisados para a remediação de contaminantes orgânicos em águas residuais derivadas do processo de limpeza de CB. As cepas foram uma da família Scenedesmaceae, duas *Chlamydomonas debaryana* e três *Chlorella sorokiniana*. As culturas de microalgas foram expostas a diferentes diluições de águas residuais de CB para identificar os níveis de toxicidade refletidos na alteração do estado fisiológico das microalgas e determinar as condições ideais para uma remoção eficaz de contaminantes. Os resultados demonstraram que as águas residuais de CB afetaram a produção de clorofila e carotenóides de microalgas de maneira dependente da concentração. Além disso, a resistência e a capacidade remediadora dependiam não apenas da cepa da microalga, mas também das características químicas dos contaminantes orgânicos. Em detalhe, a nicotina foi o contaminante mais resistente à remoção pelas microalgas testadas e sua baixa remoção correlacionou-se com a inibição de pigmentos fotossintéticos que afetaram o crescimento das microalgas. Em relação às condições ideais para uma biorremediação eficaz, este estudo mostrou que a cepa de *Chlamydomonas* denominada F2 apresentou a melhor capacidade de remover contaminantes orgânicos em efluentes a 5% CB, mantendo seu crescimento e pigmentos fotossintéticos em níveis de controle. No Capítulo 3, foram selecionadas as melhores cepas com maior produção de biomassa sob a condição de CB mais forte (ou seja, 25%), de acordo com o Capítulo 2: cepas de *Chlorella sorokiniana* denominadas F4, R1 e LG1. Essas cepas foram tratadas com UV-B por 3 dias antes de receber o efluente de CB e então incubadas por 4 dias na ausência ou manutenção de UV-B. Microalgas não tratadas com UV-B foram usadas como controle. A comparação das respostas fisiológicas, incluindo pigmentos fotossintéticos e antioxidantes não enzimáticos, bem como a depuração de nicotina e nicotirina, foram avaliadas aos 7 dias de cultura. O UV-B causou eustress, pois não teve um impacto negativo na produção de clorofila e carotenóides das algas. A aclimatação UV-B foi dependente da cepa, correlacionando-se com as adaptações ao ambiente nativo e constituições genéticas. O UV-B como pré-tratamento teve efeitos

positivos a longo prazo na capacidade antioxidante não enzimática. No entanto, a cepa LG1 necessitou de mais tempo para reajustar o equilíbrio pró-oxidante/antioxidante, sendo a mais sensível aos raios UV-B. Os compostos fenólicos desempenharam um papel importante no sistema antioxidante contra UV-B, enquanto os flavonóides não contribuíram para a capacidade antioxidante total. R1 foi a única em que o UV-B pareceu ter um efeito sinérgico na resistência cruzada aos contaminantes mais persistentes e tóxicos nas águas residuais (ou seja, nicotina e nicotirina), onde as células expostas ao UV-B não conseguiram só ganhar capacidade protetora contra poluentes, mas também podem melhorar as vias catabólicas envolvidas na eliminação desses alcalóides.

ABSTRACT

The aims of this study were to evaluate the capacity of native microalgae in the remediation of wastewater derived from cleaning process of smoked cigarette butts (CB), and to assess the use of artificial ultraviolet B (UV-B) radiation as a possible inducer of antioxidant activities in microalgae to alleviate the negative effects of CB wastewater pollutants. To this end, different experiments were performed at laboratory scale and the obtained results were discussed in two chapters. In Chapter 2, six natural isolates of microalgae were screened for the remediation of organic pollutants in wastewater derived from smoked CB cleaning process. The strains were one from the family Scenedesmaceae, two *Chlamydomonas debaryana* and three *Chlorella sorokiniana*. Microalgal cultures were exposed to different CB wastewater dilutions to identify the toxicity levels reflected in the alteration of microalgal physiological status and to determine the optimal conditions for an effective removal of contaminants. The results demonstrated that CB wastewater could impact on microalgal chlorophyll and carotenoid production in a concentration-dependent manner. Moreover, the resistance and remediation capacity did not only depend on the microalgal strain, but also on the chemical characteristic of organic pollutants. In detail, nicotine was the most resistant pollutant to removal by the microalgae tested and its low removal correlated with the inhibition of photosynthetic pigments affecting microalgal growth. Concerning the optimal conditions for an effective bioremediation, this study demonstrated that the *Chlamydomonas* strain named F2 showed the best removal capacity to organic pollutants at 5% CB wastewater maintaining its growth and photosynthetic pigments at control levels. In Chapter 3, it was selected the best strains with the highest biomass production under the strongest CB condition (i.e., 25%) according to the Chapter 2. It included three *Chlorella sorokiniana* strains named F4, R1 and LG1. These strains were treated with UV-B for 3-days prior to receiving CB wastewater and then incubated for 4-days in absence or maintaining UV-B. UV-B-untreated microalgae were used as control. Comparative physiological responses, including photosynthetic pigments and non-enzymatic antioxidants, as well as nicotine and nicotyrine removal were evaluated at 7-days culture. UV-B caused eustress as it did not negatively impact on algal chlorophyll and carotenoids production. UV-B acclimation was strain-dependent, correlating with native environment adaptions and genetic constitutions. UV-B as pretreatment had long-term positive effects on non-enzymatic antioxidant capacity.

However, LG1 needed more time to readjust pro-oxidants/antioxidants balance as it was the most UV-B sensitive. Phenolic compounds played an important role in the antioxidant system to UV-B, while flavonoids did not contribute to total antioxidant capacity. Among strains, R1 was the only one in that UV-B seemed to have a synergistic effect on cross-resistance to the most persistent and toxic CB pollutants (i.e., nicotine and nicotyrine), where their UV-B exposed cells may not only gain protection against pollutants but also may improve the catabolic pathways involved in the removal of these alkaloids.

SUMÁRIO

CAPÍTULO 1

INTRODUÇÃO GERAL

Este estudo pertence a um grande projeto denominado FOCUS (*Filter Of Cigarettes re-Used Safely*) que consistiu na recuperação e reciclagem de um dos principais resíduos antropogénicos do mundo: bitucas de cigarro (CB). Em detalhe, os CB foram limpos em água fervente e os filtros de CB foram reciclados em um substrato sem solo para o cultivo de plantas ornamentais em espaços urbanos. Além desta nova solução para reciclar CB, foi gerado um efluente contaminado durante o processo de limpeza; assim, era necessária uma remediação urgente do efluente, que foi o principal objetivo deste estudo.

Para desenvolver esta pesquisa, o estudo foi dividido em quatro capítulos da seguinte forma: 1) uma introdução geral deste estudo, 2) a primeira parte deste estudo que consistiu em testar a remediação de efluentes de CB usando diferentes microalgas fotossintéticas, 3) a segunda parte deste estudo que consistiu em alterar um parâmetro das condições de crescimento (ou seja, inclusão de radiação ultravioleta na fonte de luz) para melhorar a biorremediação de poluentes, e 4) as conclusões gerais deste estudo e considerações finais para futuras investigações .

Tem sido demonstrado que a biorremediação utilizando microalgas é um sistema ecologicamente correto e de baixo custo para o tratamento de efluentes. No entanto, cepas de microalgas nativas apresentam melhor tolerância a diversos poluentes do que espécies comerciais; assim, a primeira parte deste estudo (Capítulo 2) consistiu em avaliar a capacidade de remediação de diferentes cepas de microalgas resilientes a determinados fatores de estresse ambiental. Além disso, visto que o efluente de CB contém vários poluentes tóxicos que podem ser prejudiciais ao meio ambiente e à vida, nesta primeira parte do estudo também foi necessário identificar os níveis de toxicidade usando diferentes diluições de efluente de CB para o tratamento à base de microalgas e monitorar o estado fisiológico das cepas. Em conjunto, os resultados foram importantes para encontrar as condições ideais para uma remoção eficaz dos contaminantes.

Mudanças nos parâmetros operacionais de sistemas baseados em microalgas, como a luz, podem afetar o metabolismo e o desenvolvimento das microalgas, que por sua vez podem alterar a capacidade das microalgas de remediar águas residuais. Na natureza, as microalgas são geralmente expostas à radiação solar ultravioleta B (UV-B), a qual foi demonstrado que pode alterar a fisiologia e as atividades bioquímicas das microalgas em diferentes graus, dependendo da espécie e/ou cepa, intensidade de UV e tempo de exposição. Portanto, na segunda parte deste estudo (Capítulo 3) avaliou-se o uso da radiação UV-B artificial como indutora de atividades antioxidantes em microalgas para amenizar os efeitos negativos dos poluentes nos efluentes de CB e melhorar a capacidade de remediação.

CAPÍTULO 2

REMEDIATION CAPACITY OF DIFFERENT MICROALGAE IN EFFLUENTS DERIVED FROM THE CIGARETTE BUTT CLEANING PROCESS

ABSTRACT: Microalgal-based remediation is an ecofriendly and cost-effective system for wastewater treatment. This study evaluated the capacity of microalgae in the remediation of wastewater from cleaning process of smoked cigarette butts (CB). At laboratory scale, six strains (one from the family Scenedesmaceae, two *Chlamydomonas debaryana* and three *Chlorella sorokiniana*) were exposed to different CB wastewater dilutions to identify toxicity levels reflected in the alteration of microalgal physiological status and to determine the optimal conditions for an effective removal of contaminants. CB wastewater could impact on microalgal chlorophyll and carotenoid production in a concentration-dependent manner. Moreover, the resistance and remediation capacity did not only depend on the microalgal strain, but also on the chemical characteristics of the organic pollutants. In detail, nicotine was the most resistant pollutant to removal by the microalgae tested and its low removal correlated with the inhibition of photosynthetic pigments affecting microalgal growth. Concerning the optimal conditions for an effective bioremediation, this study demonstrated that the *Chlamydomonas* strain named F2 showed the best removal capacity to organic pollutants at 5% CB wastewater (corresponding to 25 butts L^{-1} or 5 g CB L^{-1}) maintaining its growth and photosynthetic pigments at control levels.

Keywords: anthropogenic litter; wastewater; bioremediation; microalgal strains; photosynthetic pigments.

2.1. INTRODUCTION

Cigarette butts (CB) are the most littered item in the world, which are usually found spread everywhere from urban areas to even protected areas [1]. CB contain a variety of toxic compounds accumulated during smoking such as benzene, polycyclic aromatic hydrocarbons, pyridine and heavy metals, which can leach into the environment and affect all ecosystems [2]. Moreover, practical operational aspects are lacking at the regulatory level as the current disposal systems for CB are landfilling and incineration, which are unsustainable and release hazardous contaminants to the environment [3,4]. Therefore, alternative solutions to tackle this waste are urgently needed. Recently, Mariotti et al. [5] proposed a novel solution to recycle filters of CB into a soilless substrate for growing ornamental plants in urban spaces. However, the CB cleaning process used in Mariotti et al. [5] resulted in a contaminated wastewater, which must be treated before its reuse or release to the environment.

Algae comprise a large and heterogeneous group of mostly photosynthetic organisms, which are the primary producers of food chains in the ecosystems and contribute about 40% of global photosynthesis [6]. Microalgae are single-celled microorganisms that occupy a dominant position in global ecosystems due to their nutritional simplicity, efficient dispersivity, and broad ecological amplitude [6]. Moreover, the capacity to use sunlight to fix carbon via photosynthesis is usually more efficient in microalgae than terrestrial crops, resulting in a high biomass generation [7]. Consequently, the accumulation of carbohydrates, oil, sugar, proteins, cellulose, polymers and bioactive compounds in microalgae can be used as biofuel, feed and to produce bioplastic materials [8]. Moreover, many microalgae species have the capacity to remove inorganic contaminants including phosphates, nitrates, ammonia, sulphates, calcium, sodium and heavy metals, as well as to degrade organic pollutants such as hydrocarbons, pharmaceuticals and even herbicides [9]. Accordingly, microalgae are considered important tools to improve the environmental impacts of the currently used wastewater treatment methods, resulting in multiple benefits such as nutrient recovery, biomass production, and water reutilization or discharge to the environment without adverse ecological impacts [10].

Therefore, the objective of this study was to assess the removal of pollutants in CB wastewater by microalgal-based remediation techniques. Since the isolation and selection of suitable microalgae are essential for efficient wastewater treatment, in the present study six natural isolates were screened. All microalgal strains were cultivated in different dilutions of CB wastewater, and their tolerance towards pollutants and the capacity of wastewater remediation were evaluated. This included the measurement of the production of photosynthetic pigments to evaluate the effect of pollutants on the physiological activity of microalgae, and the evaluation of the profile of wastewater pollutants at the end of the microalgal remediation process. This study will therefore provide the scientific evidence to treat the wastewater from CB cleaning process by microalgal remediation and reveal the potential value of some microalgal strains for further studies on a larger scale.

2.2. MATERIALS AND METHODS

2.2.1. CB collection and cleaning process

The CB collection, cleaning process and chemical characterization were as previously described [5]. Briefly, CB were collected (5 kg approximately) from public collectors in 10 different coffee bars located in the surroundings of the municipality of Capannori (Lucca, Italy). The cleaning process was performed in quadruplicate by an exhaust boiling of CB (100 g) in distilled water (1 L) for 10 min. The individual wastewaters were collected for their further treatment with different microalgae.

2.2.2. Microalgal strains and growth conditions

Six microalgal strains were used in this work (Table 1). Five of these strains were previously isolated and characterized [11], namely F1 (from the family Scenedesmaceae), F2 and F3 (both related to *Chlamydomonas debaryana* Goroschankin species), F4 and R1 (both related *to Chlorella sorokiniana* Shihira and R.W. Krauss species), and are currently part of the collection of the Institute of Agricultural Biology and Biotechnology of the Italian National Research Council located in Pisa. The sixth microalga, strain “LG1”, was isolated from recycled CB substrate and then characterized as described below.

Table 1. List of microalgal strains.

Strain	Isolation Source	Geographic location	Taxonomic affiliation	Accession Number	Reference
F1	"Le Morette", Fucecchio Marshland	43°48'31"N 10°48'18"E	Scenedesmaceae	OM311002 and OM310999	[11]
F2	"Le Morette", Fucecchio Marshland	43°48'31"N 10°48'18"E	Chlamydomonadaceae	OM311003	[11]
F3	"Le Morette", Fucecchio Marshland	43°48'31"N 10°48'18"E	Chlamydomonadaceae	OM311004	[11]
F4	"Le Morette", Fucecchio Marshland	43°48'31"N 10°48'18"E	Chlorellaceae	OM311005 and OM311000	[11]
R1	Private terrace in Pisa, water sample	43°43'06.6"N 10°25'21.8"E	Chlorellaceae	OM311006	[11]
LG1	Recycle cigarette butts substrate	43°42'17.9"N 10°25'34.4"E	Chlorellaceae	ON065975	This work

The microalgal strain LG1 was isolated from the surface of a recycled CB filter substrate used in the preliminary experiments of a previous study [5]. This substrate was collected in a petri dish and used to make an enrichment with the TAP medium, as described by Chiellini et al. [9]. Briefly, 1 cm3 of the substrate was cut with a sterile scalpel under biological flow, and put in a sterile flask with 50 mL sterile TAP medium [12]. After two weeks' enrichment, the solution was greenish. The solution was diluted in sterile TAP medium (1:20 v/v), and a second enrichment was performed for two more weeks. Light microscope observation (Carl Zeiss Axioskop 20 EL-Einsatz 451487) allowed a dominant microalgal coccoid morphology to be recognized. Three 100 μL aliquots of the enrichment

were streaked on TAP agar plates. This process was further repeated until a single morphology indicating the presence of a single strain was isolated. A single colony was picked up from the monoclonal microalgal culture in the petri dish, and pre-inoculated in a liquid TAP medium (50 mL) until a dense pre-culture (200 mL) was obtained. The strain was named "LG1". All the microalgal strains were grown and maintained in growth chamber under controlled temperature (24/22 ° C), and under a 16/08 h day-night cycle with PPFD of 70 μmol photons m^{-1} s^{-1}.

2.2.3. Characterization of LG1 strain and phylogenetic analysis

One mL of the monoclonal culture of strain LG1 was used for DNA extraction as described by Chiellini et al. [9]. The 18S rRNA gene was amplified as previously described [9] using a MultiGene OptiMax Thermal Cycler (Labnet, NJ, USA), and visualized by electrophoresis on 1% agarose gel; amplicons were purified by ethanol/EDTA/Na-acetate precipitation and sent to the sequencing service (BMR Genomics, Padova, Italy). The obtained sequences (forward and reverse) were analyzed and used to obtain a complete 18S rRNA gene sequence using the free software Chromas (http://technelysium.com.au/wp/chromas/; accessed on 17 November 2021). The NCBI Blast tool [13] allowed the determination of the preliminary affiliation of the newly isolated microalgal strain by comparing the sequence with all the sequences present in the international databases. A total of 41 sequences were selected for the phylogenetic analysis, comprehending the sequence of our new strain, and 40 high quality sequences selected in NCBI database, following the similarity criterion. Among the 40 selected sequences, ten were chosen as the outgroup, and were taxonomically related to *Chlamydomonas* spp. and *Dunaliella* spp. The 41 sequences were aligned with the BioEdit Software [14]; a Maximum Likelihood phylogenetic tree was constructed with the MEGA5 Software [15]; the robustness of the inferred trees was evaluated by 500 bootstrap resampling; the parameters chosen for the phylogeny were: Model/Method = General Time Reversible model; Rates among sites = Gamma distributed with invariant sites (G + I); Gaps = Use all sites; ML heuristic method = Nearest Neighbor Interchange (NNI); Branch swap filter = Strong.

2.2.4. Evaluation of microalgal strains in remediation

The wastewater was filter-sterilized by a 0.45 μm cellulose acetate filter (Sartorius, Göttingen, Germany), and different wastewater dilutions in TAP medium were tested in quadruplicates as follows: 0 (herein after Control), 1, 2, 5, 10 and 25% (v/v). In 24-well plates (1.5 cm diameter, Greiner Bio-one, Kremsmünster, Austria) 200 μL of microalgae culture was added to 1800 μL of fresh TAP medium containing a series of wastewater dilutions. The remediation capacity of each microalgal strain was performed under the same growth conditions: 24/22 ° C, 16/08 h day-night cycle and 70 μmol photons m^{-1} s^{-1} PPFD. An additional 24-well plate containing only wastewater dilutions in TAP medium (2000 μL) was included to evaluate the effect of growth conditions on the wastewater chemical composition, herein termed untreated wastewater (UWW). After 7 days, the cultures in each well were centrifuged at 3000x *g* for 10 min, and the supernatant and the microalgae pellet were collected separately for further analysis.

2.2.5. Analytical determinations

Supernatants were dried under vacuum and diluted with acetone and heptane 50% (v/v). Analytes in the wastewater samples were determined by high-resolution GC-MS analysis, using a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a MEGA-SE54 HT capillary column (10 m; 0.15 mm i.d., 0.10 μm film thickness, MEGA s.n.c., Milan, Italy), as reported by Mariotti et al. [5]. Briefly, the carrier gas was dried and air free helium with a linear speed of 60 cm s^{-1}, the oven temperature was maintained at 60 °C for 2 min and increased to 350 °C at a rate of 10 °C min^{-1}, and the full-scan mass spectra was obtained in EI^+ mode with an emission current of 10 μA and an axial modulation of 4 V. Data acquisition was from 10 to 550 Da at a speed of 1.4 scan s^{-1}. The identification of chromatogram peaks was conducted by comparing their mass spectra with the NIST library database. Quantification was performed using the relative abundance of the chromatogram peaks (instrument detection limit < 400 counts).

2.2.6. Photosynthetic pigments of microalgal strains

In order to assess the health status of microalgal strains, photosynthetic pigments were determined in four biological replicates. Photosynthetic pigments, including chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and total carotenoids (Car), were extracted from microalgae pellets and analyzed as previously reported [16].

2.2.7. Statistical analyses

Values presented are the means of four replicates. The Tukey's test was used to determine the significant differences among means ($p < 0.05$), in which the statistical analysis was performed by STATISTICA for Windows version 14.0 (Stat-Soft, Inc., Tulsa, OK, USA) using a one-way analysis of variance.

To identify the relationships among the remediation capacity of microalgal strains at different concentrations of CB wastewater, based on physiological and analytical data, multiple factor analysis (MFA) was carried out [17]. The MFA was performed with the R software [18], using the packages "FactoMineR" and "factoextra" for the analysis and data visualization, respectively. The final plot in the picture was obtained in R software with the packages "ggpubr", "ggsci" and "patchwork". Data were normalized with Z-score calculation.

2.3. RESULTS

2.3.1. Identification of the LG1 strain

According to the phylogenetic analysis, the LG1 strain was taxonomically related to the *Chlorella sorokiniana* species (Figure 1).

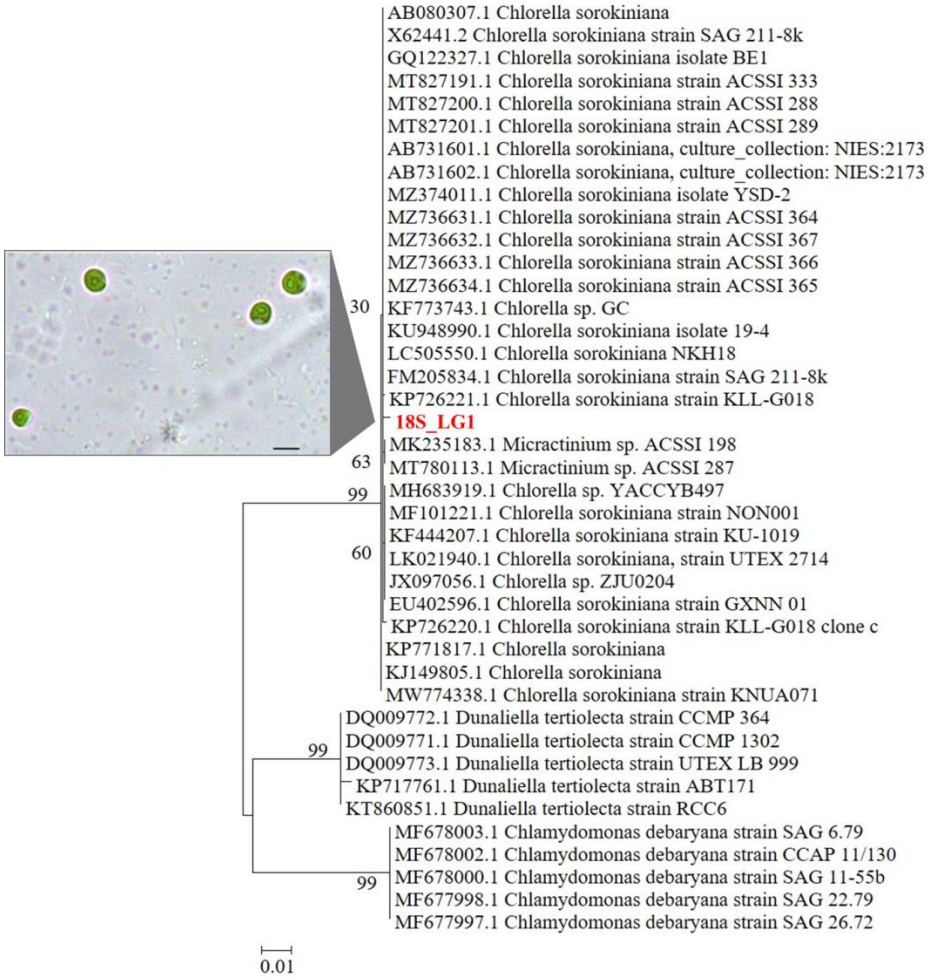


Figure 1. Phylogenetic tree reconstruction obtained with the Maximum Likelihood method on a total of 41 high quality sequences selected from the most similar to the sequences obtained for the LG1 strain. Inset: optical microscope image of LG1 cells (scale bar: 5 μm).

2.3.2. Photosynthetic pigments of microalgal strains

All microalgal strains showed a steep increase of chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total chlorophyll (Chl_{total}) and carotenoids (Car) from the beginning of the experiment (T0) to 7 d under control conditions (TAP medium without CB wastewater) (Figures 2A-D). Chl*a* in F1 gradually increased with the wastewater concentration reaching the highest level with 5% wastewater; however, a significant and subsequent sharp decline was observed when

the wastewater increased to 10 and 25%, respectively (Figure 2A). F3, F4 and R1 showed a gradual decrease of Chl*a* when the wastewater concentration increased to 10%, followed by an abrupt drop with 25% wastewater (Figure 2A). Differently, F2 and LG1 generally maintained Chl*a* at control levels when the wastewater concentration increased to 5%, followed by a decrease with 10 and 25% wastewater similar to the pattern of Chl*a* in F1 (Figure 2A). In general, Chl*b* and Chl_{total} in F1, F3 and R1 showed similar patterns to that of Chl*a* (Figures 2A-C). F2 and F4 showed a steep decline in Chl*b* with the increase of wastewater concentration, whereas the negative effect of wastewater on Chl*b* in LG1 was observed when exposed to more than 2% wastewater (Figure 2B). Chl_{total} in F2 generally exhibited similar dynamics to Chl*a* when contamination increased in the medium (Figures 2A,C), while Chl_{total} in F4 and LG1 showed similar trend to Chl*b* with the increase of wastewater concentration (Figures 2B,C). F1, F2 and LG1 maintained their stable levels of Car when the wastewater concentration increased to 5%, followed by a significant and subsequent sharp decline when contamination increased in the medium with the exception of LG1, which showed significant differences only at 25% wastewater with respect to the control (Figure 2D). Car in F3 showed similar patterns to that of Chl_{total} (Figures 2C,D). F4 showed a transient increase in Car when the wastewater increased to 2%, followed by a gradual and significant decrease with a higher wastewater concentration (Figure 2D). Differently, Car in R1 started to show a progressive decline when CB wastewater was increased beyond 5% (Figure 2D).

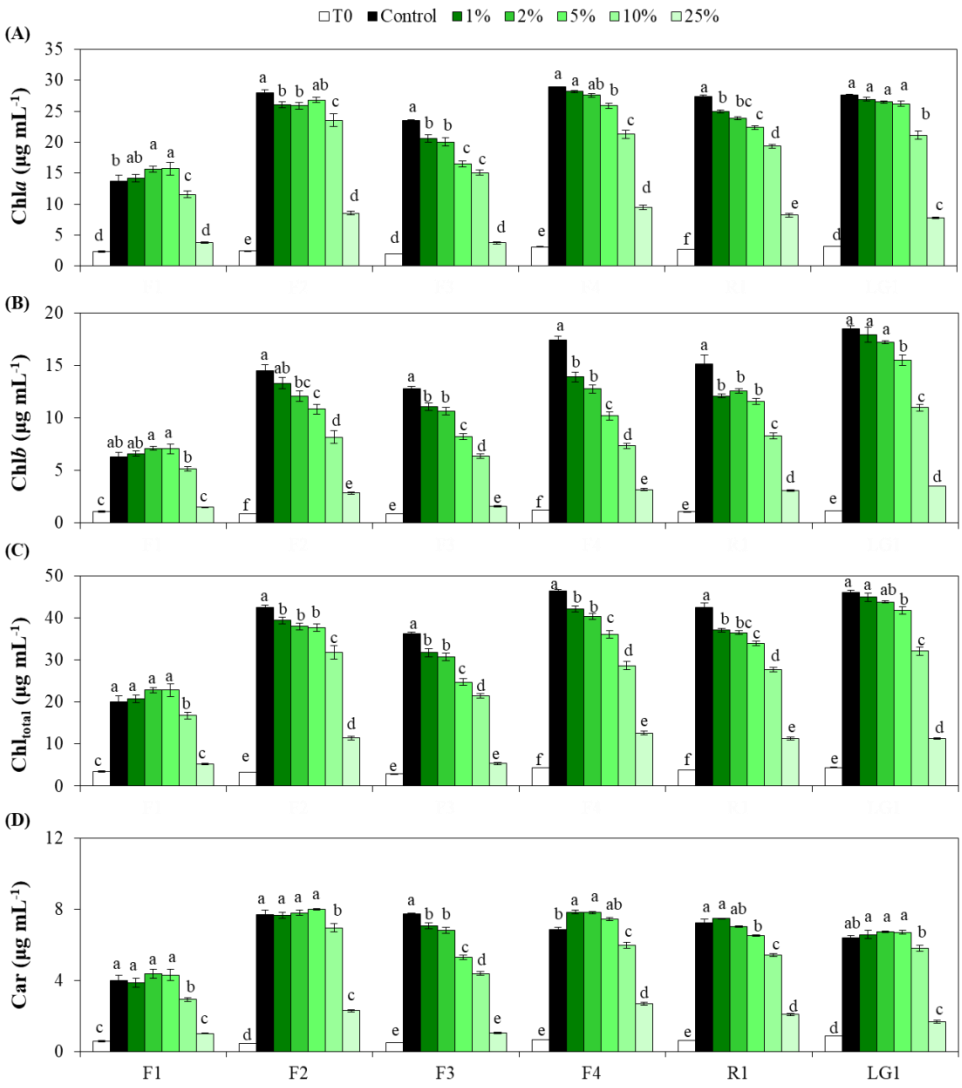


Figure 2. Effect of wastewater from cigarette butts (CB) cleaning process on photosynthetic pigments of six microalgal strains. **(A)** Chlorophyll *a* (Chl*a*), **(B)** chlorophyll *b* (Chl*b*), **(C)** total chlorophyll (Chl_{total}), and **(D)** carotenoids (Car) were determined in each microalgal strain (F1, F2, F3, F4, R1 and LG1) at the beginning of the experiment (T0) and 7 days after treatment. Microalgal treatment included exposure to growth medium without CB wastewater (Control) or containing different CB wastewater dilutions (1, 2, 5, 10 and 25%). Different letters represent significant differences ($p < 0.05$) between treatments within the same strain. Data are expressed as means of 4 different replicates ± standard error (SE).

2.3.3. CB wastewater subjected to microalgal-based remediation

In general, all microalgal strains showed good ability to remediate CB wastewater and nicotine [pyridine, 3-(1-methyl-2-pyrrolidinyl)] was the most difficult compound to remediate among pollutants (Figure 3). In 5% wastewater, F2 showed the best capacity for removing pollutants compared with other strains (–69% with respect to UWW) followed by F3, F4, LG1 and F1 (–52%), and R1 (–42%) (Figure 3A). In contrast, no significant differences between the strains were observed when the wastewater concentration increased to 10 and 25% (Figure 3B,C). Thus, strains in 10% wastewater could remove on average 47% of pollutants with respect to UWW (Figure 3B), while those in 25% wastewater removed 44% of pollutants (Figure 3C).

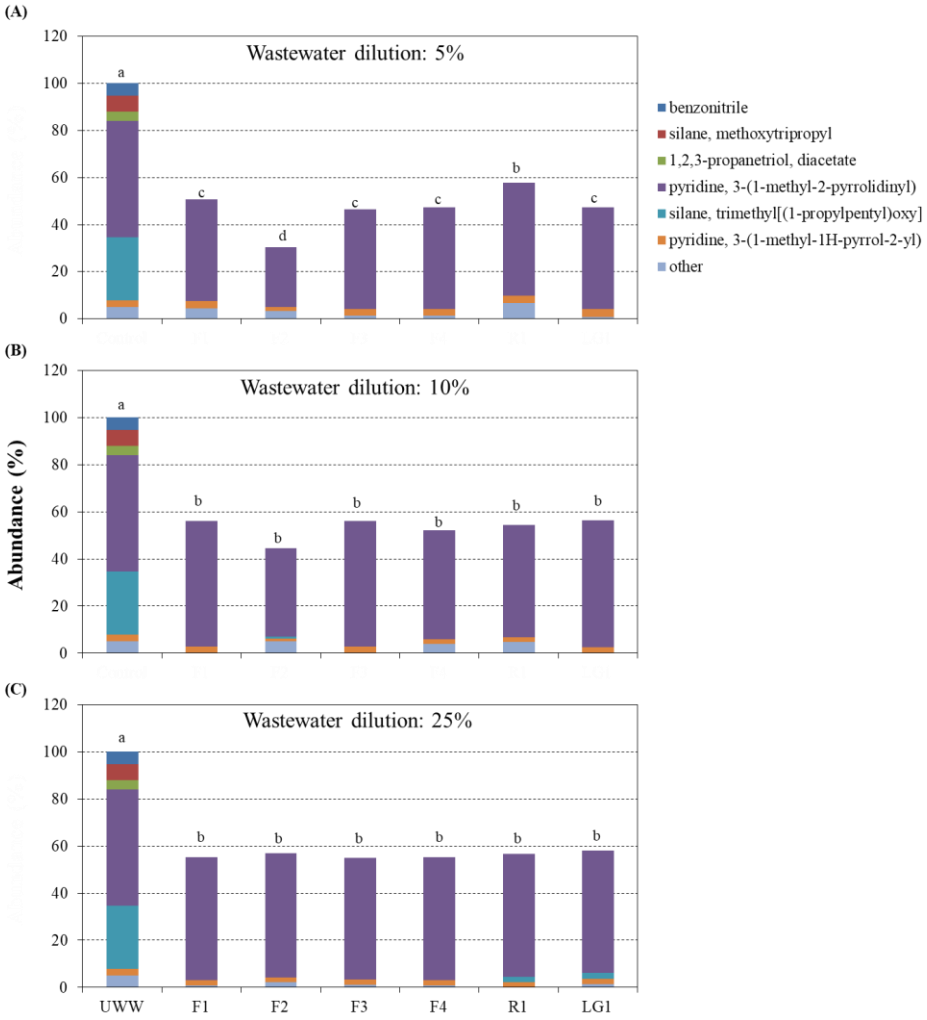


Figure 3. Chemical composition of the wastewater from the cigarette butts (CB) cleaning process subjected to microalgal-based remediation. Six microalgal strains (F1, F2, F3, F4, R1 and LG1) were exposed to different CB wastewater dilutions: **(A)** 5, **(B)** 10, and **(C)** 25%. The remediation capacity of each strain was evaluated after 7 days. UWW represents the respective CB wastewater dilution without microalgae under the same growth conditions for 7 days, for more details see Material and Methods. The total abundance of chemical compounds in UWW was expressed as 100%. The abundance of remaining compounds in wastewater after microalgal-based remediation was obtained by its comparison with UWW. Different letters represent significant differences ($p < 0.05$) between the total abundance of chemical compounds in UWW and microalgal treated wastewater. Data are means of 4 different replicates.

2.3.4. Multiple factor analysis

The multiple factor analysis (MFA; Figure 4) revealed for each microalgal strain a distinct separation in three groups in relation to the CB wastewater concentration. Accordingly, the four replicates exposed to the same CB concentration group together (Figure 4A). According to the quantitative variables (Figure 4B), strains F1, F3, F4 and LG1 exposed to 25% CB concentration, as well as F1 at 10% CB, were those showing the highest % of nicotine and the lowest amount of photosynthetic pigments. On the other side, strains F2 (5 and 10% CB), F1 (5% CB) and R1 (10% CB) were the strains showing the lowest nicotine concentration in the wastewater, as well as the highest amount of photosynthetic pigment content. An opposite behavior could be observed concerning other contaminants that were not nicotine (Figure 4B). In this case, the MFA highlighted that the highest values (i.e., the lowest removal ability) were characterizing strains R1 and F2 (5% and 10% CB), and F1 (5% CB). On the contrary, strains F3 (all CB concentrations), F1 (10% CB) and LG1 (25% CB) seemed to remove the highest amount of other contaminants from the wastewater. According to the qualitative variables categories (Figure 4C), the six strains were separated in two groups along the y axis; one group was comprised of strains F1, F2 and R1, and the other group strains F3, F4 and LG1. These two groups were related, respectively, to the content of “other” contaminants and to the content of nicotine in the wastewater.

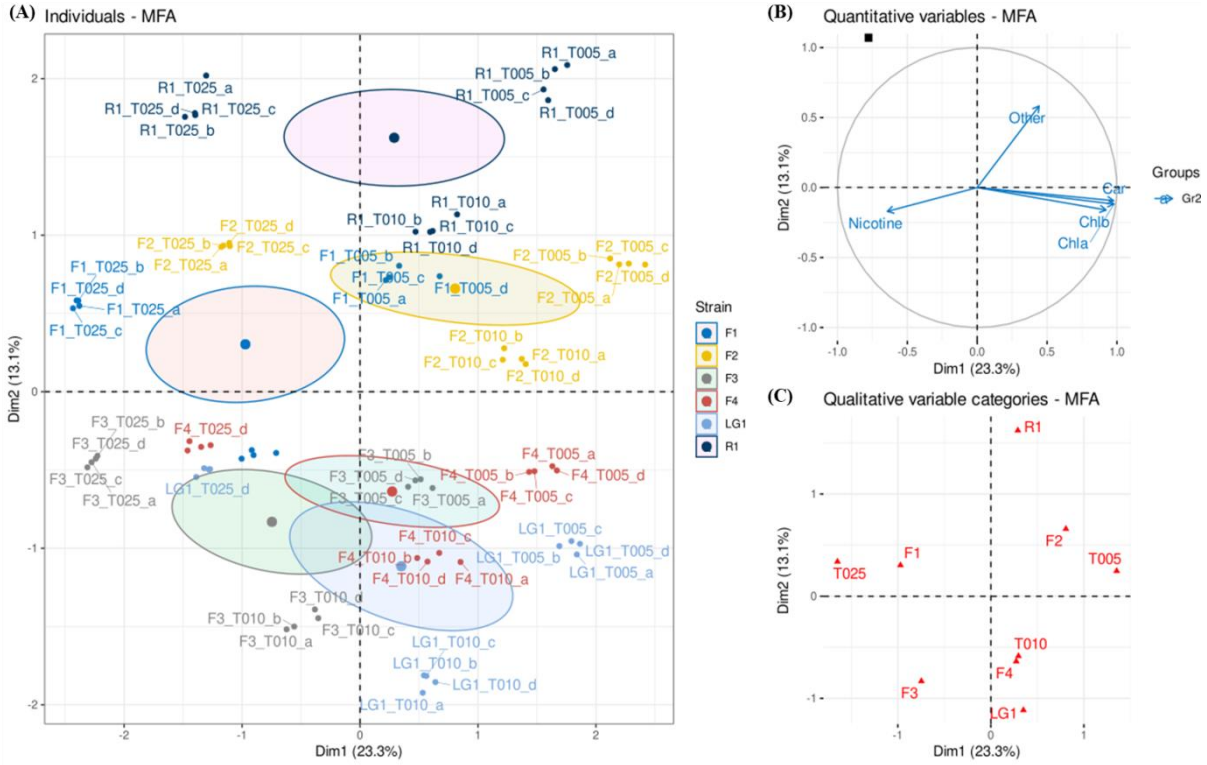


Figure 4. Multiple factor analysis (MFA) of physiological and analytical data in microalgal-based remediation of wastewater from cigarette butts (CB) cleaning process. **(A)** Score plot describing the strains and groups of variables in relation to the CB wastewater concentration including each replicate. Representation of **(B)** groups of quantitative variables, and **(C)** categories of qualitative variables. T005: 5% CB wastewater dilution; T010: 10% CB wastewater dilution; T025: 25% CB wastewater dilution; a, b, c and d: indicate the replicates; Other: pollutants in CB wastewater different than nicotine.

2.4. DISCUSSION

Previous studies found a direct relationship between algal growth and Chl*a* content [19–21]. In fact, the cell size was not affected (Figure S1) and the biomass showed similar trend to Chl*a* (Figure S2,2A) at the end of the experiment, confirming the aforementioned relationship. Therefore, the results of Chl*a* indicate that microalgae growth was generally affected with a CB concentration of more than 2%. In detail, the cell growth of F3, F4 and

R1 were inhibited at CB concentration ≥ 5%, while that of F1, F2 and LG1 at CB ≥ 10%, suggesting that the latter had a better ability to resist or tolerate the toxicity of CB wastewater pollutants. Among pollutants, benzonitrile (UWW abundance: 5.2%); 1,2,3-propanetriol, diacetate (UWW abundance: 4.0%); and the silicon (Si)-based compounds such as silane, methoxytripropyl (UWW abundance: 6.8%) and silane, trimethyl [(1-propylpentyl)oxy] (UWW abundance: 26.7%) were completely or almost completely removed after microalgal-based treatment. Benzonitrile is an ingredient used in photosynthesis-inhibiting herbicides, which have differential effects depending on the species [22,23]. Recently, a study on the biodegradation of organonitriles reported that benzonitrile can be degraded in benzoic acid and ammonia by nitrilase in microbial systems [24]. Nitrilases were considered absent in algae; however, Lauritano et al. [25] identified for the first time a putative nitrilase in the green microalgae *Tetraselmis suecica* under nutrient-starvation conditions. Moreover, a recent study identified benzoic acid as a new phytohormone improving the growth of *Chlorella regularis* [26]. Thus, a possible enzymatic degradation of benzonitrile was not excluded in our study and the produced ammonia may be assimilated by microalgae [27]. 1,2,3-propanetriol, diacetate is a diglyceride commonly known as diacetin used as a food additive and as a valuable additive to diesel fuel when mixed with other acetins [28]. It is known that soil microorganisms induce lipase-esterase activity for the biodegradation of carboxyl esters [29]. Moreover, some microalgal lipases have been isolated for industrial applications [30] and the transcription of many lipases was induced under abiotic stress (e.g., nutrient starvation) in *Chlamydomonas* [31]. Thus, the complete removal of 1,2,3-propanetriol, diacetate in our system may be through the action of induced microalgal lipases producing glycerol, which in turn may stimulate microalgal growth [32] and assist the degradation of other organic pollutants in CB wastewater such as hydrocarbons [33]. Similarly, Si-based compounds can contribute to the alleviation of numerous environmental constraints in plants by inducing or reinforcing the regulation of secondary metabolites [34,35] and their effective activities are dependent on their chemical and physical characteristics [36,37]. Interestingly, Jeffryes et al. [38] developed a system in which the controlled delivery of Si to the culture of diatom *Cyclotella* spp. enhanced lipid and biomass production. Similar to diatoms, the growth of *Cladophora glomerata* was induced by Si as a required component of the cell walls as in other algae such as *Pediastrum* and *Scenedesmus*

spp. [39]. Recently, Van Hoecke et al. [40] demonstrated that Si-based nanoparticles were adhered to the outer cell surface of microalga *Pseudokirchneriella subcapitata* without evidence of particle uptake, concluding that the Si toxicity at high concentration might occur through surface interaction. Hence, it is possible that organosilane compounds in CB wastewater were adsorbed to the microalgal cell wall with some limitations depending on the concentration, chemical group and microalgal strain.

The removal efficiency of CB pollutants named as "others" (UWW abundance: 5.2%) varied among microalgal strains and these compounds included hydrocarbons and additives such as plasticizers. It has been demonstrated that the microalgae *Scenedesmus obliquus*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* could degrade hydrocarbons and the removal capacity varied with the concentration and chemical characteristic of hydrocarbons [41–43]. Another study found that photosynthetic pigments in the terrestrial alga *Prasiola crispa* decreased with increasing fuel concentration due to the hydrocarbon lipophilic affinity to the cellular membrane causing chloroplast and/or thylakoid membrane disruption [44]. Concerning plasticizers (e.g., phthalate esters) and their effect on microalgae, Duan et al. [45] demonstrated that environmentally relevant concentrations of dibutyl phthalate stimulated the growth and lipid accumulation in *Chlorella vulgaris*, while higher concentrations damaged cell membranes. Interestingly, another strain of the same species showed a decrease in Chl*a*, growth inhibition and changes in the biosynthesis of relevant proteins at low concentrations [46]. Similarly, the photosynthetic pigments of *Scenedesmus* spp. were reduced under the exposure of dibutyl phthalate at environmentally relevant concentrations affecting microalgal growth and photosynthetic process, while at higher concentrations extracellular soluble proteins were induced acting as osmoregulatory substances [47]. Moreover, the toxicity of plasticizers also depends on their chemical characteristics. For instance, dibutyl phthalate was more toxic than diethyl phthalate in three marine microalgae based on algal growth and Chl*a* content, and the biodegradation was inhibited when these pollutants were mixed [48]. Intriguingly, in our study, all microalgal strains could better remove hydrocarbons and additives at the highest concentration of CB wastewater, highlighting their potential application to remediate oil disaster and toxic plastic-bonded polluted sites. However, more studies are needed to understand how these microalgae

degrade or exclude these pollutants from their cells after the uptake, and what kind of defense mechanisms are induced at high CB wastewater concentration.

Nicotine [pyridine, 3-(1-methyl-2-pyrrolidinyl)] is the main tobacco alkaloid and, as expected, it was the most abundant (49.4%) pollutant in CB wastewater. Nicotyrine [pyridine, 3-(1-methyl-1H-pyrrol-2-yl)] is one of the minor alkaloids in tobacco, it can be produced when tobacco is pyrolized [49] and some bacteria can metabolize nicotine into nicotyrine [50]. Both alkaloids represented 52.1% of the total pollutants in CB wastewater and they were generally difficult to remove by microalgae. A recent review highlighted that since 2006, a total of 36 investigations have been performed studying the impacts of CB on aquatic and terrestrial life and lethal impacts seem to be most pronounced in aquatic systems [2]. For instance, leachates from smoked CB over 5 years decomposition inhibited the growth of the freshwater microalga *Raphidocelis subcapitata* in a bimodal mode, where this inhibition was related to high nicotine concentration at early CB decomposition stage (~30 days postsmoking) and to microplastic release at late stage (5 years) as nicotine concentration declined [51]. Another study using the same species showed that microalgal growth was induced with smoked CB leachates in a concentration-dependent manner from 10% to 75% CB, while at 100% CB (corresponding to 20 butts L^{-1}) the growth was inhibited but still higher than control conditions [52]. Studies with marine microorganims showed that CB leachates inhibited the growth of microalga *Dunaliella tertiolecta* in a concentration-dependent manner [52], as well as the Chl concentration of microphytobenthos even at marginal CB concentration (1 butt L^{-1}) due to the toxic compounds accumulated in the butt after smoking and the release of microplastics [53]. In our study, CB wastewater concentrations ranged from 1 to 25% (corresponding to 5 to 125 butts L^{-1}) and MFA showed that the reduction in Chl*a*, Chl*b* and Chl_{total} in the microalgal strains increased with the low ability to remove nicotine, suggesting that this alkaloid may have the most detrimental effects on these pigments. In fact, chlorophyll biosynthesis in microalgae was inhibited depending on the concentration of nicotine [54–56]. In photosynthetic organisms, such as the studied microalgal strains, the light-harvesting pigments (Chl*a* and Chl*b*) effectively capture and transport light energy to the photosynthetic reaction center, while Car absorb the excess of energy protecting the chloroplast from Chl-sensitized photooxidation [57]. Thus, any

changes in these pigments can result in energy deficiency to support the growth of microalgae. Similar to Chl, the results of MFA also showed that Car were inhibited in microalgal strains with low ability to remove nicotine. Concordantly, previous studies demonstrated the inhibitory effects of nicotine on Car content, particularly affecting the cyclization of lycopene depending on the nicotine concentration [54,55,58]. Besides nicotine, nicotyrine was also detected in the CB leachates causing the deactivation of nicotine catabolic enzymes in soil microbes [59]. Thus, it is likely that nicotyrine may prevent nicotine catabolism in microalgae and this effect may be pronounced with increasing CB concentration.

Overall, this study highlighted the importance of microalgal strain selection for wastewater remediation, and showed that the strains isolated from similar polluted conditions may necessarily have the best performance, as occurred with LG1, which could not remove efficiently CB-contained alkaloids, and its physiological traits were affected at ≥ 5% CB similar to the nicotine-resistant mutant of *Chlorella emersonii* [56]. Moreover, microalgal resistance and remediation capacity also depended on the chemical characteristics of pollutants. Here, nicotine was the most resistant pollutant to removal by the microalgae tested and its low removal correlated with the inhibition of photosynthetic pigments affecting microalgal growth. Concerning the optimal conditions for an effective removal of contaminants, our results supported the high performance of *Chlamydomonas* strain F2 to remove organic pollutants at 5% CB wastewater (corresponding to 25 butts L^{-1} or 5 g CB L^{-1}) removing 69% of pollutants and maintaining its growth (based on Chl*a*) and pigments at control levels (Table S1). Further studies are needed to understand the mechanism pathways involved in the removal of pollutants, especially alkaloids.

2.5. CONCLUSIONS

A novel solution to recycle filters of cigarette butts (CB) into soilless substrate has previously been proposed, where the CB cleaning process resulted in a contaminated wastewater [7]. In this study, the removal of organic pollutants in CB wastewater by microalgal-based remediation techniques was assessed for the first time, and the data

provided a promising approach for wastewater bioremediation, revealing the potential value of the tested microalgal strains for further studies on a larger scale.

2.6. SUPPLEMENTARY MATERIALS

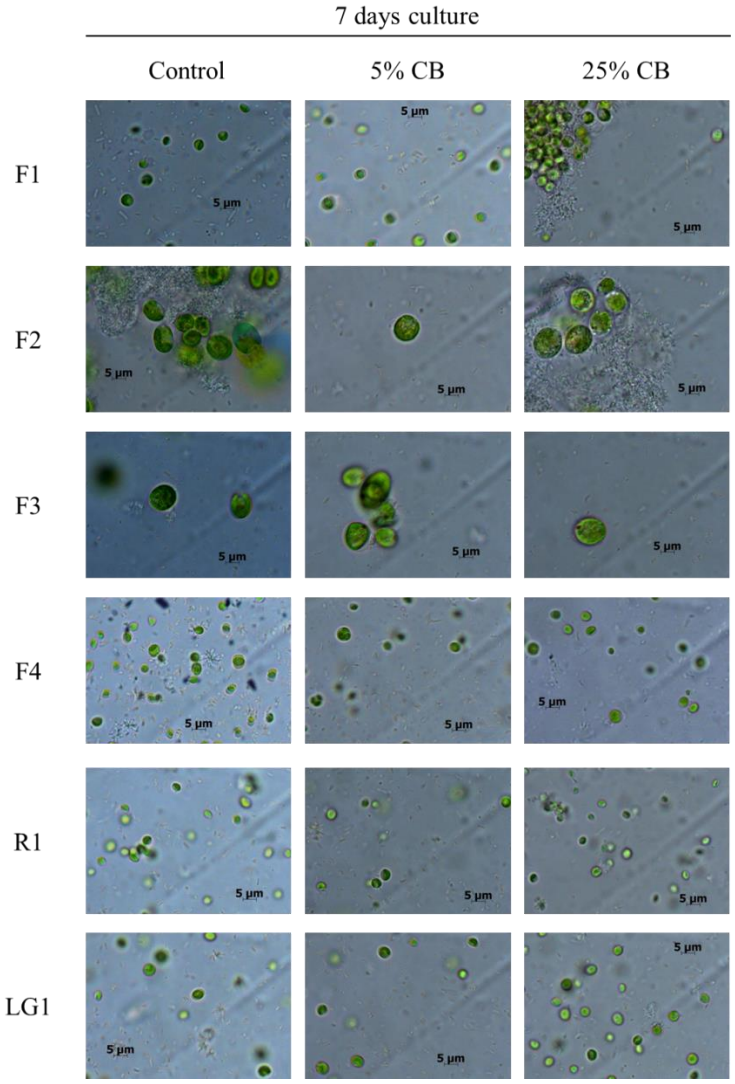


Figure S1. Optical microscope images of microalgal cells. Strains F1, F2, F3, F4, R1, and LG1 were exposed to different cigarette butt (CB) wastewater dilutions: 0 (Control), 5, and 25%. The remediation capacity of each strain was evaluated after 7 days, when images were collected. Scale bar: 5 µm.

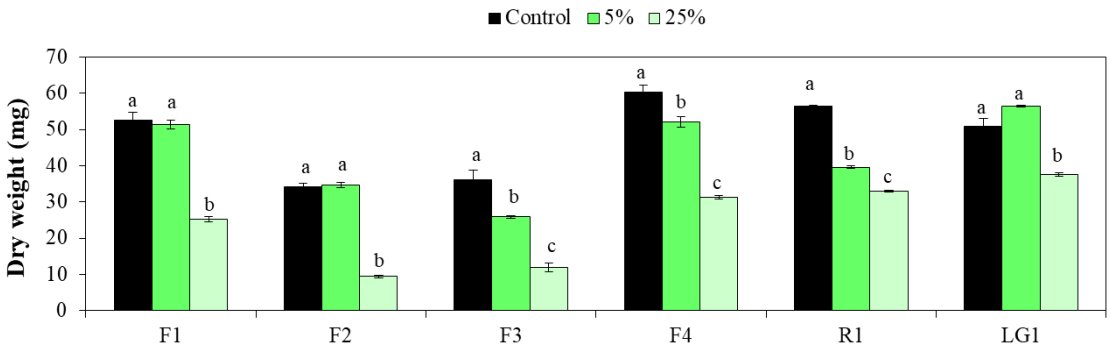


Figure S2. Effect of wastewater from cigarette butts (CB) cleaning process on the biomass of six microalgal strains. The dry weight (mg) was determined in each microalgal strain (F1, F2, F3, F4, R1 and LG1) at 7 days after treatment. Microalgal treatment included exposure to growth medium without CB wastewater (Control) or containing different CB wastewater dilutions (5 and 25%). Different letters represent significant differences ($p < 0.05$) between treatments within the same strain. Data are expressed as means of 4 different replicates ± standard error (SE).

Table S1. Summary of the capacity of each microalgal strain in the removal of contaminants from cigarette butt (CB) wastewater.

	Benzonitrile	1,2,3-propanetriol, diacetate	Silane, methoxytripropyl	Silane, trimethyl [(1-propylpentyl)oxy]	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	Pyridine, 3-(1-methyl-1H-pyrrol-2-yl)]	Others	Total
Abundance (%) in untreated CB wastewater	5.2	4.0	6.8	26.7	49.4	2.7	5.2	100
Abundance (%) of remaining compounds in 5% CB wastewater (corresponding to 25 butts L^{-1} or 5 g CB L^{-1}) after microalgal-based remediation								
	Benzonitrile	1,2,3-propanetriol, diacetate	Silane, methoxytripropyl	Silane, trimethyl [(1-propylpentyl)oxy]	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	Pyridine, 3-(1-methyl-1H-pyrrol-2-yl)]	Others	Total
F1	0	0	0	0	43.2	3.2	4.4	50.8
F2	0	0	0	0	25.5	1.7	3.3	30.5
F3	0	0	0	0	42.4	2.8	1.3	46.5
F4	0	0	0	0	43.1	2.8	1.3	47.2
R1	0	0	0	0	47.9	3.1	6.7	57.7
LG1	0	0	0	0	43.4	3.3	0.9	47.6
Abundance (%) of remaining compounds in 10% CB wastewater (corresponding to 50 butts L^{-1} or 10 g CB L^{-1}) subjected to microalgal-based remediation								
	Benzonitrile	1,2,3-propan	Silane, methox	Silane, trimethy	Pyridine, 3-(1-	Pyridine, 3-(1-	Others	Total

		etriol, diacetate	ytripropyl	l [(1-propylpentyl)oxy]	methyl-2-pyrrolidinyl)	methyl-1H-pyrrol-2-yl)]		
F1	0	0	0	0	53.3	2.5	0.2	56.0
F2	0	0	0	0.8	37.6	1.2	4.9	44.5
F3	0	0	0	0	53.3	2.6	0.3	56.2
F4	0	0	0	0	46.4	1.9	3.8	52.1
R1	0	0	0	0	47.5	2.0	4.7	54.2
LG1	0	0	0	0	54.1	2.4	0	56.5
Abundance (%) of remaining compounds in 25% CB wastewater (corresponding to 125 butts L^{-1} or 25 g CB L^{-1}) subjected to microalgal-based remediation								
	Benzonitrile	1,2,3-propanetriol, diacetate	Silane, methoxytripropyl	Silane, trimethyl [(1-propylpentyl)oxy]	Pyridine, 3-(1-methyl-2-pyrrolidinyl)	Pyridine, 3-(1-methyl-1H-pyrrol-2-yl)]	Others	Total
F1	0	0	0	0	52.2	2.2	0.8	55.2
F2	0	0	0	0	52.5	2.2	2.1	56.8
F3	0	0	0	0	51.8	2.2	1.0	55.0
F4	0	0	0	0	52.1	2.2	0.8	55.1
R1	0	0	0	2.3	52.2	2.2	0	56.7
LG1	0	0	0	2.7	52.0	2.2	1.3	58.2

2.7. REFERENCES

1. Kurmus, H.; Mohajerani, A. The toxicity and valorization options of cigarette butts. *Waste Manag*. **2020**, *104*, 104–118.

2. Green, D.S.; Tongue, A.D.W.; Boots, B. The ecological impacts of discarded cigarette butts. *Trends Ecol. Evol*. **2022**, *37*, 183–192.

3. Rebischung, F.; Chabot, L.; Biaudet, H.; Pandard, P. Cigarette butts: A small but hazardous waste, according to European regulation. *Waste Manag*. **2018**, *82*, 9–14.

4. Marinello, S.; Lolli, F.; Gamberini, R.; Rimini, B. A second life for cigarette butts? A review of recycling solutions. *J. Hazard. Mater*. **2020**, *384*, 121245.

5. Mariotti, L.; Huarancca Reyes, T.; Curadi, M.; Guglielminetti, L. Recycling cigarette filters as plant growing substrate in soilless system. *Horticulturae* **2022**, *8*, 135.

6. Sharma, N.K.; Rai, A.K. Biodiversity and biogeography of microalgae: Progress and pitfalls. *Environ. Rev*. **2011**, *19*, 1–15.

7. Fabris, M.; Abbriano, R.M.; Pernice, M.; Sutherland, D.L.; Commault, A.S.; Hall, C.C.; Labeeuw, L.; McCauley, J.I.; Kuzhiuparambil, U.; Ray, P.; et al. Emerging technologies in algal biotechnology: Toward the establishment of a sustainable, algae-based bioeconomy. *Front. Plant Sci*. **2020**, *11*, 279.

8. Lutzu, G.A.; Ciurli, A.; Chiellini, C.; Di Caprio, F.; Concas, A.; Dunford, N.T. Latest developments in wastewater treatment and biopolymer production by microalgae. *J. Environ. Chem. Eng.* **2021**, *9*, 104926.

9. Chiellini, C.; Guglielminetti, L.; Sarrocco, S.; Ciurli, A. Isolation of four microalgal strains from the lake Massaciuccoli: Screening of common pollutants tolerance pattern and perspectives for their use in biotechnological applications. *Front. Plant Sci*. **2020**, *11*, 607651.

10. Nagarajan, D.; Lee, D.-J.; Chen, C.-Y.; Chang, J.-S. Resource recovery from wastewaters using microalgae-based approaches: A circular bioeconomy perspective. *Bioresour. Technol.* **2020**, *302*, 122817.

11. Chiellini, C.; Serra, V.; Gammuto, L.; Ciurli, A.; Longo, V.; Gabriele, M. Evaluation of nutraceutical properties of eleven microalgal strains isolated from different freshwater aquatic environments: Perspectives for their application as nutraceuticals. *Foods* **2022**, *11*, 654.

12. Gorman, D.S.; Levine, R.P. Cytochrome f and plastocyanin: Their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. *Proc. Natl. Acad. Sci. USA* **1965**, *54*, 1665–1669.

13. Altschul, S. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*. **1997**, *25*, 3389–3402.

14. Hall, T. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser*. **1999**, *41*, 95–98.

15. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*. **2011**, *28*, 2731–2739.

16. Huarancca Reyes, T.; Pompeiano, A.; Ranieri, A.; Volterrani, M.; Guglielminetti, L.; Scartazza, A. Photosynthetic performance of five cool-season turfgrasses under UV-B exposure. *Plant Physiol. Biochem*. **2020**, *151*, 181–187.

17. Huarancca Reyes, T.; Scartazza, A.; Pompeiano, A.; Guglielminetti, L. Physiological responses of *Lepidium meyenii* plants to ultraviolet-B radiation challenge. *BMC Plant Biol.* **2019**, *19*, 186.

18. Team R Core. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2013.

19. Chen, B.; Dong, J.; Li, B.; Xue, C.; Tetteh, P.A.; Li, D.; Gao, K.; Deng, X. Using a freshwater green alga *Chlorella pyrenoidosa* to evaluate the biotoxicity of ionic liquids with different cations and anions. *Ecotoxicol. Environ. Saf.* **2020**, *198*, 110604.

20. Khoshnamvand, M.; Hanachi, P.; Ashtiani, S.; Walker, T.R. Toxic effects of polystyrene nanoplastics on microalgae *Chlorella vulgaris*: Changes in biomass, photosynthetic pigments and morphology. *Chemosphere* **2021**, *280*, 130725.

21. Werdel, G.M.; Pandey, L.K.; Bergey, E.A. Cigarette butt effects on diatom health in a stream ecosystem. *Aquat. Ecol.* **2021**, *55*, 999–1010.

22. Sanders, G.E.; Pallett, K.E. Studies into the differential activity of the hydroxybenzonitrile herbicides. *Pestic. Biochem. Physiol*. **1986**, *26*, 116–127.

23. Ma, J.; Lin, F.;Wang, S.; Xu, L. Toxicity of 21 herbicides to the green alga *Scenedesmus quadricauda*. *Bull. Environ. Contam. Toxicol*. **2003**, *71*, 594–601.

24. Li, T.; Liu, J.; Bai, R.; Ohandja, D.G.;Wong, F.S. Biodegradation of organonitriles by adapted activated sludge consortium with acetonitrile-degrading microorganisms. *Water Res*. **2007**, *41*, 3465–3473.

25. Lauritano, C.; De Luca, D.; Amoroso, M.; Benfatto, S.; Maestri, S.; Racioppi, C.; Esposito, F.; Ianora, A. New molecular insights on the response of the green alga *Tetraselmis suecica* to nitrogen starvation. *Sci. Rep.* **2019**, *9*, 3336.

26. Fu, L.; Li, Q.; Chen, C.; Zhang, Y.; Liu, Y.; Xu, L.; Zhou, Y.; Li, C.; Zhou, D.; Rittmann, B.E. Benzoic and salicylic acid are the signaling molecules of *Chlorella* cells for improving cell growth. *Chemosphere* **2021**, 265, 129084.

27. Muñoz, R.; Jacinto, M.; Guieysse, B.; Mattiasson, B. Combined carbon and nitrogen removal from acetonitrile using algal-bacterial bioreactors. *Appl. Microbiol. Biotechnol*. **2005**, *67*, 699–707.

28. Rastegari, H.; Ghaziaskar, H.S.; Yalpani, M. Valorization of biodiesel derived glycerol to acetins by continuous esterification in acetic acid: Focusing on high selectivity to diacetin and triacetin with no byproducts. *Ind. Eng. Chem. Res.* **2015**, *54*, 3279–3284.

29. Margesin, R. Determination of enzyme activities in contaminated soil. In *Monitoring and Assessing Soil Bioremediation*; Springer: Berlin/Heidelberg, Germany, 2005; Volume 5, pp. 309–320.

30. Nalder, T.D. Microalgal Lipids, Lipases and Lipase Screening Methods. Ph.D. Thesis, Deakin University, Victoria, Australia, 2014.

31. Urzica, E.I.; Vieler, A.; Hong-Hermesdorf, A.; Page, M.D.; Casero, D.; Gallaher, S.D.; Kropat, J.; Pellegrini, M.; Benning, C.; Merchant, S.S. Remodeling of membrane lipids in iron-starved *Chlamydomonas. J. Biol. Chem*. **2013**, *288*, 30246–30258.

32. Rana, M.S.; Prajapati, S.K. Stimulating effects of glycerol on the growth, phycoremediation and biofuel potential of *Chlorella pyrenoidosa* cultivated in wastewater. *Environ. Technol. Innov*. **2021**, *24*, 102082.

33. Ortega Ramírez, C.A.; Ching, T.; Yoza, B.; Li, Q.X. Glycerol-assisted degradation of dibenzothiophene by *Paraburkholderia* sp. C3 is associated with polyhydroxyalkanoate granulation. *Chemosphere* **2022**, *291*, 133054.

34. Luyckx, M.; Hausman, J.-F.; Lutts, S.; Guerriero, G. Silicon and plants: Current knowledge and technological perspectives. *Front. Plant Sci.* **2017**, *8*, 411.

35. Othman, A.J.; Eliseeva, L.G.; Ibragimova, N.A.; Zelenkov, V.N.; Latushkin, V.V.; Nicheva, D.V. Dataset on the effect of foliar application of different concentrations of silicon dioxide and organosilicon compounds on the growth and biochemical contents of oak leaf lettuce (*Lactuca sativa* var. crispa) grown in phytotron conditions. *Data Br.* **2021**, *38*, 107328.

36. Lucie, A.-T.; Solange Patricia,W.; Ephrem, K.-K.; Salomon, N.; Serge Florent, B.-O.; Ponel-Béranger, L.D.; Silla, S.; Olga-Diane, Y.; Jean-Laurent, S.-M.; Seck, D.; et al. The effective insecticidal activity of the two extracts ethyl acetate and hexan of *Trichilia gilgiana* against *Sitophilus zeamaïs*. *Int. J. Biol*. **2016**, *8*, 23.

37. Barik, M.; Rawani, A.; Laskar, S.; Chandra, G. Evaluation of mosquito larvicidal activity of fruit extracts of *Acacia auriculiformis* against the Japanese encephalitis vector *Culex vishnui. Nat. Prod. Res*. **2019**, *33*, 1682–1686.

38. Jeffryes, C.; Rosenberger, J.; Rorrer, G.L. Fed-batch cultivation and bioprocess modeling of *Cyclotella* sp. for enhanced fatty acid production by controlled silicon limitation. *Algal Res*. **2013**, *2*, 16–27.

39. Moore, L.F.; Traquair, J.A. Silicon, a required nutrient for *Cladophora glomerata* (L) Kütz. (Chlorophyta). *Planta* **1976**, *128*, 179–182.

40. Van Hoecke, K.; De Schamphelaere, K.A.C.; Van der Meeren, P.; Lucas, S.; Janssen, C.R. Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: Importance of surface area. *Environ. Toxicol. Chem*. **2008**, *27*, 1948–1957.

41. El-Sheekh, M.M.; Hamouda, R.A.; Nizam, A.A. Biodegradation of crude oil by *Scenedesmus obliquus* and *Chlorella vulgaris* growing under heterotrophic conditions. *Int. Biodeterior. Biodegradation* **2013**, *82*, 67–72.

42. Xaaldi Kalhor, A.; Movafeghi, A.; Mohammadi-Nassab, A.D.; Abedi, E.; Bahrami, A. Potential of the green alga *Chlorella vulgaris* for biodegradation of crude oil hydrocarbons. *Mar. Pollut. Bull*. **2017**, *123*, 286–290.

43. Luo, J.; Deng, J.; Cui, L.; Chang, P.; Dai, X.; Yang, C.; Li, N.; Ren, Z.; Zhang, X. The potential assessment of green alga *Chlamydomonas reinhardtii* CC-503 in the biodegradation of benz(a)anthracene and the related mechanism analysis. *Chemosphere* **2020**, *249*, 126097.

44. Nydahl, A.C.; King, C.K.; Wasley, J.; Jolley, D.F.; Robinson, S.A. Toxicity of fuel-contaminated soil to Antarctic moss and terrestrial algae. *Environ. Toxicol. Chem*. **2015**, *34*, 2004–2012.

45. Duan, K.; Cui, M.;Wu, Y.; Huang, X.; Xue, A.; Deng, X.; Luo, L. Effect of dibutyl phthalate on the tolerance and lipid accumulation in the green microalgae *Chlorella vulgaris*. *Bull. Environ. Contam. Toxicol*. **2018**, *101*, 338–343.

46. Liao, C.-S.; Hong, Y.-H.; Nishikawa, Y.; Kage-Nakadai, E.; Chiou, T.-Y.; Wu, C.-C. Impacts of endocrine disruptor di-n-butyl phthalate ester on microalga *Chlorella vulgaris* verified by approaches of proteomics and gene ontology. *Molecules* **2020**, *25*, 4304.

47. Cunha, C.; Paulo, J.; Faria, M.; Kaufmann, M.; Cordeiro, N. Ecotoxicological and biochemical effects of environmental concentrations of the plastic-bond pollutant dibutyl phthalate on *Scenedesmus* sp. *Aquat. Toxicol*. **2019**, *215*, 105281.

48. Chi, J.; Li, Y.; Gao, J. Interaction between three marine microalgae and two phthalate acid esters. *Ecotoxicol. Environ. Saf*. **2019**, *170*, 407–411.

49. Ye, X.; Lu, Q.; Li,W.; Gao, P.; Hu, B.; Zhang, Z.; Dong, C. Selective production of nicotyrine from catalytic fast pyrolysis of tobacco biomass with Pd/C catalyst. *J. Anal. Appl. Pyrolysis* **2016**, *117*, 88–93.

50. Jiang, H.J.; Ma, Y.; Qiu, G.J.;Wu, F.L.; Chen, S.L. Biodegradation of nicotine by a novel strain *Shinella* sp. HZN1 isolated from activated sludge. *J. Environ. Sci. Health. B* **2011**, *46*, 703–708.

51. Bonanomi, G.; Maisto, G.; De Marco, A.; Cesarano, G.; Zotti, M.; Mazzei, P.; Libralato, G.; Staropoli, A.; Siciliano, A.; De Filippis, F.; et al. The fate of cigarette butts in different

environments: Decay rate, chemical changes and ecotoxicity revealed by a 5-years decomposition experiment. *Environ. Pollut*. **2020**, *261*, 114108.

52. Oliva, M.; De Marchi, L.; Cuccaro, A.; Pretti, C. Bioassay-based ecotoxicological investigation on marine and freshwater impact of cigarette butt littering. *Environ. Pollut.* **2021**, *288*, 117787.

53. Green, D.S.; Kregting, L.; Boots, B. Effects of cigarette butts on marine keystone species (*Ulva lactuca* L. and *Mytilus edulis* L.) and sediment microphytobenthos. *Mar. Pollut. Bull.* **2021**, *165*, 112152.

54. Fazeli, M.R.; Tofighi, H.; Madadkar-Sobhani, A.; Shahverdi, A.R.; Nejad-Sattari, T.; Mirzaie, S.; Jamalifar, H. Nicotine inhibition of lycopene cyclase enhances accumulation of carotenoid intermediates by *Dunaliella salina* CCAP 19/18. *Eur. J. Phycol*. **2009**, *44*, 215–220.

55. Ishikawa, E.; Abe, H. Lycopene accumulation and cyclic carotenoid deficiency in heterotrophic *Chlorella* treated with nicotine. *J. Ind. Microbiol. Biotechnol*. **2004**, *31*, 585–589.

56. Rise, M.; Prengler, M.; Malis Arad, S. Characterization of a nicotine-resistant mutant of *Chlorella emersonii*. *J. Plant Physiol*. **1998**, *152*, 583–585.

57. Liu, X.-Y.; Hong, Y.; Zhao, G.-P.; Zhang, H.-K.; Zhai, Q.-Y.; Wang, Q. Microalgae-based swine wastewater treatment: Strain screening, conditions optimization, physiological activity and biomass potential. *Sci. Total Environ*. **2022**, *807*, 151008.

58. Mysore Doddaiah, K.; Narayan, A.; Gokare Aswathanarayana, R.; Ravi, S. Effect of metabolic inhibitors on growth and carotenoid production in *Dunaliella bardawil*. *J. Food Sci. Technol*. **2013**, *50*, 1130–1136.

59. King, I.C.; Lorenzi, V.; Blasius, M.E.; Gossett, R. Leachates from cigarette butts can persist in marine sediment. *Water Air Soil Pollut*. **2021**, *232*, 38.

CAPÍTULO 3

UV-B IRRADIATION EFFECT ON MICROALGAE PERFORMANCE IN THE REMEDIATION OF EFFLUENT DERIVED FROM THE CIGARETTE BUTT CLEANING PROCESS

ABSTRACT: In this study, the potentials of ultraviolet B (UV-B) radiation to alleviate effects of pollutants in cigarette butt wastewater (CBW) were investigated using different *Chlorella sorokiniana* strains (F4, R1 and LG1). Microalgae were treated with UV-B (1.7 Wm^{-2}) for 3-days prior to receiving CBW and then incubated for 4-days in absence or maintaining UV-B. UV-B-untreated microalgae were used as control. Comparative physiological responses, including photosynthetic pigments and non-enzymatic antioxidants, as well as nicotine and nicotyrine removal were evaluated at 7-days culture. UV-B treatments did not negatively impact on algal chlorophyll and carotenoids production. UV-B acclimation was strain-dependent, correlating with native environment adaptions and genetic constitutions. UV-B as pretreatment had long-term positive effects on non-enzymatic antioxidant capacity. However, LG1 needed more time to readjust pro-oxidants/antioxidants balance as it was the most UV-B sensitive. Phenolic compounds played an important role in the antioxidant system to UV-B, while flavonoids did not contribute to the total antioxidant capacity. Although a cross-resistance between UV-B and CBW was observed in F4 and R1, only R1 showed nicotine/nicotyrine catabolism induction due to UV-B. Overall, the results suggest that UV-B activates defense pathways associated with the resistance or tolerance to nicotine and nicotyrine.

Keywords: *Chlorella sorokiniana*; flavonoids; phenolic compounds; photosynthetic pigments; non-enzymatic antioxidants; nicotine; nicotyrine; ultraviolet light.

3.1. INTRODUCTION

Cigarette butts (CB) are the main type of massive waste, which can be found in urban zones, oceans, forests and even protected areas due to their improper littering [1]. When it occurs, several toxic compounds (e.g., heavy metals, polycyclic aromatic hydrocarbons, N-nitrosamines, aromatic amines, benzene and nicotine) are leached into the environment, affecting terrestrial and aquatic organisms in different degrees ranging from alterations in behaviour to physiological/developmental abnormalities and death [2–6]. Surprisingly, although their toxicity, CB are considered as "Municipal waste including separately collected fraction/Separately collected fraction/Other fractions not otherwise specified" at regulatory level [7]. Moreover, conventional disposal systems for CB (e.g., landfilling and incineration) are unsustainable and release hazardous contaminants to the environment; thus, an effective management solution for this waste is essential to significantly reduce environmental repercussion [8].

On the basis of the circular economy and sustainable development, previous studies proposed a novel solution to recycle filters of smoked cigarettes into soilless substrate, where CB were firstly cleaned-up and then the wastewater treated with microalgae [9,10]. In fact, the remediation of wastewater using microalgae has been employed to remove a variety of pollutants including organic and inorganic compounds, which can be effectively converted into valuable products such as biomass, following the principle of "waste-to-wealth" [11]. However, there are several challenges to overcome in order to maximize the advantages of this technology. For instance, changes in cultivation parameters, such as light, can affect microalgal growth, which in turn influences the capacity of removal contaminants in wastewater. In fact, light is the primary source of energy for the growth and the development of photosynthetic organisms, including microalgae.

Ultraviolet (UV) radiation represents a small fraction of the sunlight spectrum and only wavelengths greater than 290 nm (UV-B and UV-A) reach the Earth surface due to the stratospheric ozone layer [12]. UV-B effects on living organisms have received increasing scientific attention over the past three decades because of the detection of ozone layer depletion and the concomitant increment of harmful UV-B [13,14]. In plants, UV-B drives a

mix of damaging and acclimation responses by inducing UV-B specific and/or -nonspecific signaling pathways [15–17]. Besides terrestrial impacts, UV-B can also penetrate the water column to a considerable depth reaching freshwater and marine organisms [18]. In algae, UV-B can alter their physiological and biochemical activities in different extents depending on algal species, UV-B intensity and exposition time [19]. Recently, the UV-B photoreceptor UVR8 (UV RESISTANCE LOCUS 8), which has been firstly characterized in *Arabidopsis thaliana*, was identified in green algae, bryophytes, lycophytes, and angiosperms [20–22]. Moreover, Zhang et al. [23] identified orthologous genes of the core UVR8 signalling module in green algae, indicating that the mechanism of action is well-conserved with a chlorophytic origin. At biotechnological level, the use of UV-B, as well as other abiotic stresses, has been strategically applied as a tool in the microalgal machinery of biorefinery in order to enhance the biosynthesis of high value products, such as antioxidants, pigments (carotenoids) and lipids (omega-3 polyunsaturated fatty acids) [24]. However, exploring the use of UV-B as a possible stimulator of antioxidant activities in microalgae to counter-act wastewater pollutants has not yet been investigated.

It was found in a previous study that nicotine and nicotyrine were difficult to remove by microalgae during CB wastewater treatment, especially at high concentrations (25% CB) [10], implying that more efficient approaches are needed to totally remove these pollutants. Therefore, in this study, it was evaluated the antioxidant responses of *Chlorella sorokiniana* (as this species showed the highest biomass production under 25% CB condition [10]) to short acute UV-B irradiation and remediation capacity to nicotine and nicotyrine contained in CB wastewater. It was tested the hypothesis that UV-B should induce the antioxidant activity in some microalgal strains that in turn should contribute to counteract the oxidative stress triggered by toxic agents in the wastewater during the bioremediation. To this end, cultures of three *C. sorokiniana* strains isolated from native conditions named F4, R1 and LG1 [10,25] were exposed to UV-B before CB wastewater treatment, as a sequential experiment, or before and during CB wastewater, as a parallel experiment. Thus, the aim of this work was to compare the physiological responses, including photosynthetic pigments and non-enzymatic antioxidant activities, as well as removal of nicotine and nicotyrine by the *C. sorokiniana* strains in both sequential and parallel experiments in order to evaluate the

hypothesis. Here, it was presented the first study that reports the use of UV-B as a tool to strength antioxidant responses in microalgae in order to remediate nicotine and nicotyrine contained in CB wastewater.

3.2. RESULTS

3.2.1. Photosynthetic pigments in microalgal strains

UV-B radiation generally increased the content of photosynthetic pigments in microalgae subjected to CB wastewater treatment with some differences between UV-B exposition periods depending on the strain (Figures 1A-C). In detail, chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*) in F4 did not show significant differences between 3 and 7 d UV-B exposed cells, while in R1 were significantly higher when cells were exposed to 7 d UV-B (Figures 1A-B). Concerning carotenoids (Car), F4 and R1 showed significantly higher content in cells exposed to 7 d UV-B than in those exposed to 3 d UV-B (Figure 1C). Differently, in LG1 the induction of photosynthetic pigments due to UV-B radiation was only observed when cells were exposed to 7 d UV-B, while no significant differences were detected between No UV-B and 3 d UV-B treated cells (Figures 1A-C). Concerning the ratio between Chl*a* and Chl*b* (Chl*a*/*b*), LG1 subjected to CB wastewater treatment did not show significant differences between No UV-B and UV-B treated cells (Figure 1D). Differently, Chl*a*/*b* in F4 and R1 did not show significant differences between No UV-B and 7 d UV-B treated cells, while a significant increase was observed when cells were exposed to 3 d UV-B (Figure 1D).

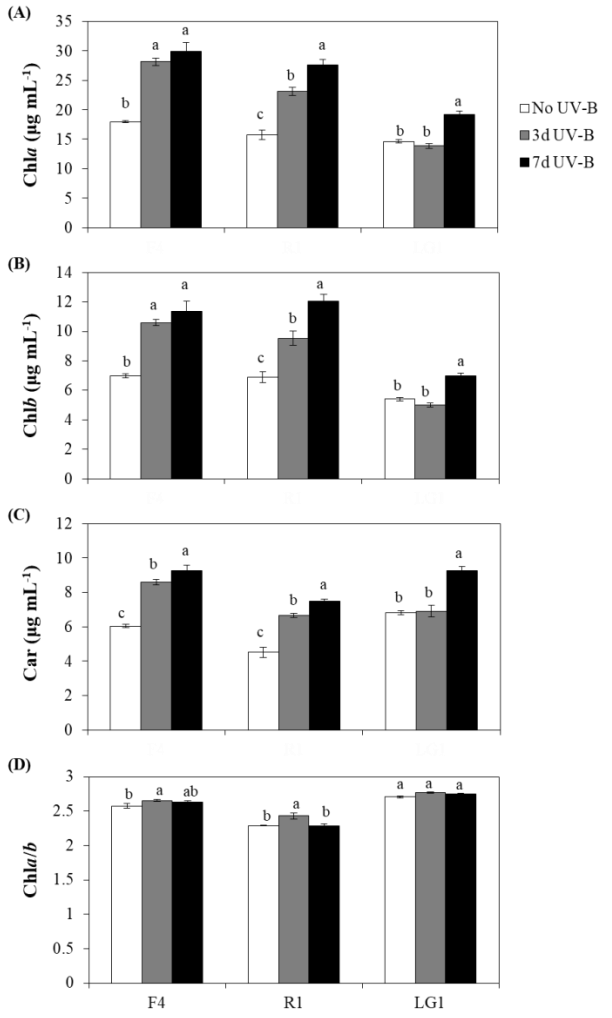


Figure 1. Effect of UV-B on the photosynthetic pigments in microalgae subjected to cigarette butts (CB) derived wastewater treatment. **(A)** Chlorophyll *a* (Chl*a*), **(B)** chlorophyll *b* (Chl*b*), **(C)** carotenoids (Car), and **(D)** the ratio Chl*a* to Chl*b* (Chl*a*/*b*) were determined in each microalgal strain (F4, R1 and LG1) at 7-days culture. Microalgae were exposed to wastewater and sequential (3 d UV-B) or parallel (7 d UV-B) UV-B treatments. UV-B exposure level was set at 1.7 W m^{-2} supplemented with 70 µmol m^{-1} s^{-1} photosynthetically active radiation (PAR) for 20 min each day. Control microalgal group only received PAR (No UV-B). For more details see Section Materials and Methods. Different letters represent significant differences (P<0.05) between UV-B treatments within the same strain. Data are expressed as means of 4 different replicates ± standard error (SE).

3.2.2. Non-enzymatic antioxidants in microalgal strains

In general, the total antioxidant capacity (TAC) in all strains subjected to CB wastewater treatment was induced by UV-B radiation with some differences between UV-B exposition periods depending on the strain (Figure 2A). TAC in F4 did not show significant differences between 3 and 7 d UV-B treatments, while in R1 was significantly increased with the UV-B exposition and in LG1 was significantly reduced (Figure 2A). The phenolic compounds in F4 and R1 were induced by UV-B without differences between exposure periods (Figure 2B). However, LG1 only showed a significant increase of phenolic compounds when cells were exposed to 7 d UV-B, while no significant differences were detected between No UV-B and 3 d UV-B treated cells (Figure 2B). Concerning flavonoids, F4 showed an increase with UV-B without significant differences between exposition period, while R1 did not show significant differences between No UV-B and UV-B treated cells (Figure 2C). Differently, flavonoids in LG1 did not show significant differences between No UV-B and 7 d UV-B treated cells, while a significant decrease was observed when cells were exposed to 3 d UV-B (Figure 2C).

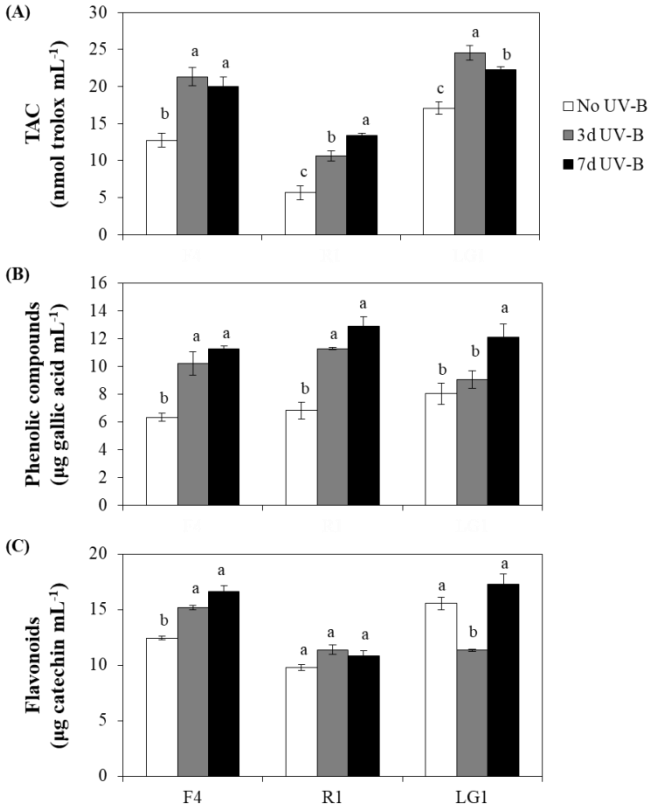


Figure 2. Effect of UV-B on the non-enzymatic antioxidants in microalgae subjected to cigarette butts (CB) derived wastewater treatment. **(A)** Total antioxidant capacity (TAC), **(B)** phenolic compounds, and **(C)** flavonoids were determined in each microalgal strain (F4, R1 and LG1) at 7-days culture. Microalgae were exposed to wastewater and sequential (3 d UV-B) or parallel (7 d UV-B) UV-B treatments. UV-B exposure level was set at 1.7 W m^{-2} supplemented with 70 µmol m^{-1} s^{-1} photosynthetically active radiation (PAR) for 20 min each day. Control microalgal group only received PAR (No UV-B). For more details see Section Materials and Methods. Different letters represent significant differences (P<0.05) between UV-B treatments within the same strain. Data are expressed as means of 4 different replicates ± standard error (SE).

3.2.3. CB wastewater subjected to microalgal-based remediation

This study confirmed that nicotine and nicotyrine were the most difficult organic compounds to remove by microalgae when exposed to 25% CB wastewater, as previously reported [10]. Nicotine abundance in the wastewater after microalgal-based remediation was in average approximately 65% when strains grown in the absence of UV-B (No UV-B). However, the remediation capacity was significantly affected by UV-B in the strains R1 and LG1 with no effects in F4 (Figure 3A). In R1, UV-B increased the remediation capacity and approximately 47% of nicotine was detected in the wastewater without significant differences between 3 and 7 d UV-B (Figure 3A). On the other hand, the removal capacity of nicotine in LG1 was compromised with increasing UV-B exposition (Figure 3A). Concerning nicotyrine, no UV-B effect was observed in the remediation capacity of LG1 as nicotyrine levels in the wastewater did not show significant differences between No UV-B and UV-B treated cells (Figure 3B). In F4, UV-B decreased the remediation capacity without significant differences between 3 and 7 d UV-B, while the removal capacity of nicotyrine in R1 was significantly increased only when cells were exposed to 7 d UV-B (Figure 3B).

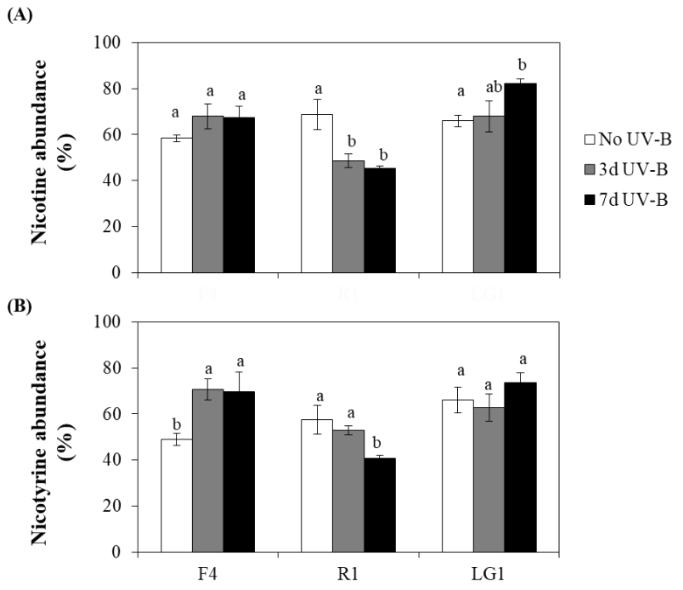


Figure 3. Effect of UV-B on the microalgal removal capacity of main toxic pollutants in cigarette butts (CB) wastewater. The relative abundance of **(A)** nicotine and **(B)** nicotyrine was determined in the wastewater after microalgal-based remediation at 7-days culture. The abundance was expressed in % (kcounts/kcounts) and obtained by its comparison with the abundance in their respective untreated wastewater (without microalgal cells), which represented 100%. Microalgal strains (F4, R1 and LG1) were exposed to wastewater and sequential (3 d UV-B) or parallel (7 d UV-B) UV-B treatments. UV-B exposure level was set at 1.7 W m^{-2} supplemented with 70 μmol m^{-1} s^{-1} photosynthetically active radiation (PAR) for 20 min each day. Control microalgal group only received PAR (No UV-B). For more details see Section Materials and Methods. Different letters represent significant differences (P<0.05) between UV-B treatments within the same strain. Data are expressed as means of 4 different replicates ± standard error (SE).

3.2.4. Multiple factor analysis

The multiple factor analysis (MFA) shown in Figure 4, revealed not only a distinct separation of the three microalgal strains, but also a separation of each treatment within each microalgal strain dataset (No UV-B, 3 d UV-B and 7 d UV-B). To this purpose, and according to the qualitative variables, the treatments (i.e., exposition time to UV-B) had a strong effect shaping the plot along the y axis. Observing the quantitative variables, strains R1 and F4 were strongly related to the vector of the Chl*a* and Chl*b* content, especially in the 7 d UV-B treatment, and slightly in the 3 d UV-B and No UV-B treatments. At the same time, they also seemed to be not related to a high content of nicotine and nicotyrine, as the vector has an opposite direction. On the other side, strain LG1 seemed to be negatively affected by the treatments, and related to the highest contents of nicotine and nicotyrine (No UV-B and 3 d UV-B treatments) and to the highest values of TAC and flavonoids (7 d UV-B treatment).

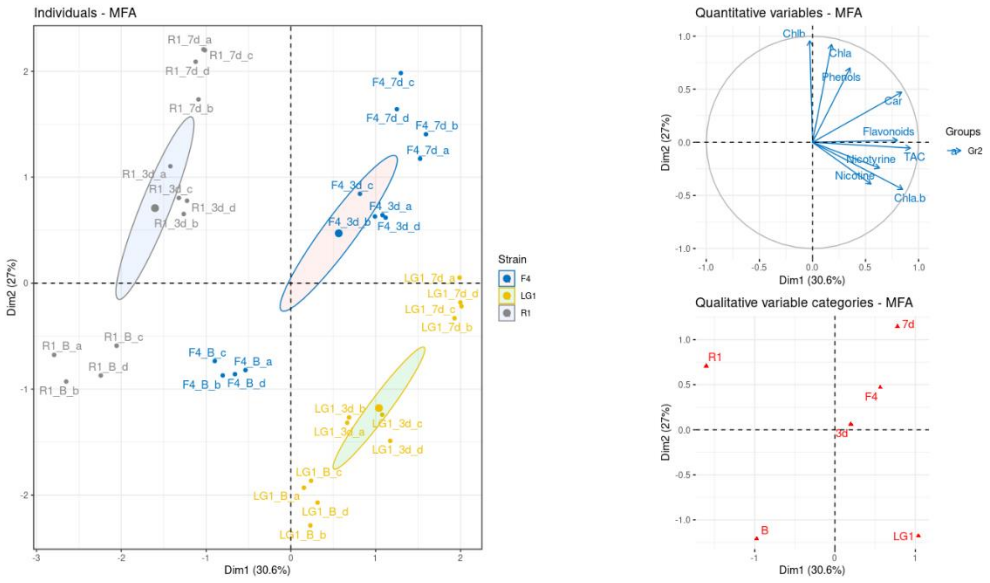


Figure 4. Multiple factor analysis (MFA) of physiological and analytical data in microalgal-based remediation of wastewater from cigarette butts (CB) cleaning process, coupled with UV-B treatment. B: No UV-B irradiation; 3d: 3 days UV-B treatment; 7d: 7 days UV-B treatment; Chla.b: ratio Chl*a* to Chl*b*; a, b, c and d: indicate the replicates.

3.3. DISCUSSION

At biotechnological level, abiotic stresses such as UV-B have been strategically applied as a tool in the microalgal machinery of biorefinery in order to enhance the biosynthesis of high value products such as pigments, lipids and polymers [24]. However, exploring the use of UV-B in microalgal-based wastewater remediation has not yet been studied. Here, we presented the first study that reports the use of UV-B as a tool to strength antioxidant responses in microalgae to remediate nicotine and nicotyrine contained in CB wastewater.

Previous study demonstrated that the production of photosynthetic pigments of microalgae was strongly inhibited at 25% CB, highlighting the toxicity of CB wastewater pollutants [10]. Conversely, in this study, the pigments of microalgae exposed to CB wastewater were generally increased upon UV-B exposure, indicating that UV-B can mitigate and/or prime *C. sorokiniana* cells against CB wastewater pollutants. Moreover, Chl*a*/*b* showed slight changes due to UV-B indicating a negligible effect on the light harvesting complexes, different than a distress effect that usually causes strong changes in Chl*a*/*b* ratio [26–29]. The UV-B effect on microalgal growth was also strain dependent, which may be related to the adaptation to their native environments. In fact, F4 and R1 were isolated from an inland swamp (Fucecchio Marshland, Italy) and from a water sample in a private terrace (Pisa, Italy), respectively, while LG1 was isolated from a plant substrate in a growth chamber [10,25]. Thus, it is plausible that UV-B acted as a 'positive stress' in F4 and R1 as they have naturally grown under ambient UV-B instead of in the absence of UV-B like LG1.

There is a general consensus that UV-B exerts an overall deleterious effect on the photosynthetic apparatus. For instance, enhanced UV-B exposition to microalgae resulted in a decrease of photosynthetic pigments and yield, which was intensified with exposure time [30]. However, it has been demonstrated that many photosynthetic organisms including microalgae are also able to acclimate to UV-B adjusting their metabolism and preventing damage [22,26]. In this study, when F4 and R1 received UV-B as pre-treatment followed by CB wastewater exposition (i.e., 3d UV-B), UV-B had a long-term positive effect on

photosynthetic pigments. Previous studies showed that pigment content in *Dunaliella salina* was induced by short-term UV-B irradiation at different doses and this effect was probably regulated by photomorphogenic photoreceptors [23,31,32]. Thus, it is possible that the UV-B pretreatment in this study enhanced photoprotection pathways in F4 and R1, similar to plants [33], that overlapped with the defense pathways against CB wastewater pollutants. This cross-resistance effect due to UV-B was also observed in LG1 only when UV-B and CB wastewater were applied in parallel treatment (i.e., 7d UV-B), suggesting that the applied UV-B dose may stimulate acclimation responses in long periods, as this strain had never received UV-B in its native environment. Another interesting aspect of the present study was that R1 was the only strain that probably showed a synergistic effect on the cross-resistance when UV-B and CB wastewater were applied in parallel treatment (i.e., 7d UV-B), as photosynthetic pigments were significantly higher than No UV-B and 3d UV-B treatments. Surprisingly, F4 showed also this possible synergistic effect only on Car under parallel UV-B and CB wastewater treatment, highlighting the promising photoprotective function of carotenoids probably more related to their high antioxidant activity [19,34], which overlaps with the deactivation of reactive oxygen species (ROS) induced by the wastewater [35]. However, further studies are necessary to determine what type of carotenoids is involved in this cross-resistance between UV-B and wastewater pollutants.

Many abiotic and biotic variables trigger the production of ROS that can pose a threat to cells but also act as signals for the activation of antioxidants [36]. Hao et al. [35] demonstrated that pollutants in wastewater derived from tobacco industry induce ROS accumulation in *C. pyrenoidosa* resulting in cellular damage and impairing microalgal growth. This growth impairment was also observed in *C. sorokiniana* exposed to CB wastewater [10], indicating that CB wastewater pollutants may induce ROS production in microalgal cells. Concerning UV-B, it can be a potential source of oxidative stress; however, depending on the dose and energy, this abiotic variable can be less detrimental where induced ROS may play an important role in UV-B acclimation and metabolism readjustment [37]. For instance, single high acute UV-B (4.6 Wm^{-2}) irradiation doses in *C. vulgaris* induced ROS formation resulting in serious photooxidative damage when irradiation time was increased (63 *vs.* 155 min) [38], while low UV-B (0.7 Wm^{-2}) irradiation for 4 days allowed

UV-B acclimation and tolerance in *Chlamydomonas reinhardtii* [22]. The alteration between pro-oxidants and scavenging activity in response to UV-B also depend on species or even genotypes of the same species [26]. For instance, *C. vulgaris* and *Chlorella* sp. may develop different antioxidant activity in response to high acute UV-B (4.6 Wm^{-2}) irradiation related to the different native environment adaption and genetic constitutions to cope with UV-B [39]. In this study, the total capacity of non-enzymatic antioxidants (TAC) in all strains had a positive long-term effect in response to UV-B pre-treatment followed by CB wastewater exposition (i.e., 3d UV-B), suggesting that UV-B might initiate a series of defense pathways that provide increased protection against toxic pollutants in the CB wastewater. Interestingly, TAC showed different patterns in parallel treatment (i.e., 7d UV-B) depending on the strain, highlighting their different capacities of UV-B acclimation probable linked to their native environments. Concordantly, the MFA showed that LG1 correlates to the highest values of TAC, indicating that this strain was more sensitive to the applied UV-B dose than the other strains and needs an acclimation period to readjust the balance between pro-oxidants and antioxidants. Phenolic compounds in this study showed similar patterns to TAC, underlining their important role in the antioxidant system in response to UV-B [40,41]. Although green algae such as *Chlorella* contained plant-specific UV-B photoreceptor orthologous, no conservation was found in a component downstream related to flavonoid biosynthesis (i.e., *MYB13* transcription factor) [23,42]. In this study, a contribution of flavonoids to total antioxidant capacities was not found, indicating that other photoprotectants were more relevant during UV-B acclimation [14]. Moreover, the involvement of antioxidant enzymes in the crosstalk between UV-B and CB pollutants is not excluded as enzymatic and non-enzymatic antioxidants may have good complementary ability in antioxidation [41].

Overall, the results suggested that UV-B stimuli might activate defense pathways associated with the resistance or tolerance to CB wastewater by the neutralization of ROS production, highlighting the importance of the crosstalk between both abiotic variables with promising biotechnological application especially under indoor conditions. Furthermore, it is well known that the crosstalk is governed by a complex interaction between signaling pathways resulting in outcomes such as cross-resistance, cross-tolerance or cross-sensitivity, which may vary depending on species or even genotypes of the same species [43]. For

instance, changes in two abiotic stresses such as nitrogen source and temperature resulted in high lipid accumulation for biodiesel production depending on microalgal species [44]. In this study, among the three *C. sorokiniana* strains, R1 was the only one in that UV-B seemed to have a synergistic effect on cross-resistance to the most persistent and toxic CB pollutants (i.e., nicotine and nicotyrine), where their UV-B exposed cells may not only gain protection against pollutants (ROS-induced cellular damage) but also may improve the catabolic pathways involved in the removal of these alkaloids. Further large-scale studies are needed to validate the process at pre-industrial level.

3.4. MATERIALS AND METHODS

3.4.1. CB collection and cleaning process

The collection and treatment (100 g L^{-1}) of cigarette butts (CB) were performed as previously described in Chiellini et al. [10]. Briefly, the CB from public collectors in different coffee bars near to the municipality of Capannori (Lucca, Italy) were cleaned by an exhaust boiling in distilled water for 10 min. This cleaning process was performed in quadruplicate and the individual wastewaters were treated with different microalgae.

3.4.2. Microalgal strains and growth conditions

Three *Chlorella sorokiniana* strains, namely F4, R1 and LG1, were previously isolated and characterized [10,25]. All strains were grown in sterile tris-acetate-phosphate (TAP) medium in growth chamber under controlled temperature (23 ± 1 ºC), 16/08 h light/dark cycle, and 70 μmol m^{-1} s^{-1} photosynthetically active radiation (PAR). The aforementioned culture conditions were also maintained all over the UV-B and wastewater treatment.

3.4.3. UV-B radiation treatment

A volume of 200 μL of microalgal culture was added into 24-well plates (1.5 cm diameter, Greiner Bio-one, Kremsmünster, Austria) containing 1300 μL of fresh TAP medium per well. UV-B radiation was applied from a Philips TL 20W/01RS UV-B

Narrowband lamp (Koninklijke Philips Electronics, Eindhoven, The Netherlands) with a peak emission at 311 nm. The intensity of UV-B was determined by adjusting the distance between the UV-B lamp and multiwell plates and measured using an UV-B meter (Skye Instruments Ltd., Powys, UK). The UV-B exposure level was set at 1.7 W m^{-2} supplemented with 70 µmol m^{-1} s^{-1} PAR for 20 min each day (at 12:00). UV-B treatment was performed in two different experiments: (i) applied for 3 days before wastewater treatment as sequential experiment (3d UV-B), or (ii) applied for 7 days including the 4 days wastewater treatment as parallel experiment (7d UV-B). In addition, a parallel microalgal group was kept in the absence of UV-B (No UV-B) and used as control (Figure 5). Similarly, another parallel untreated wastewater group without microalgal cells (1500 µL TAP per well) was included and it was exposed to each UV-B treatment (No-UVB, 3d UV-B and 7d UV-B) (Figure 5).

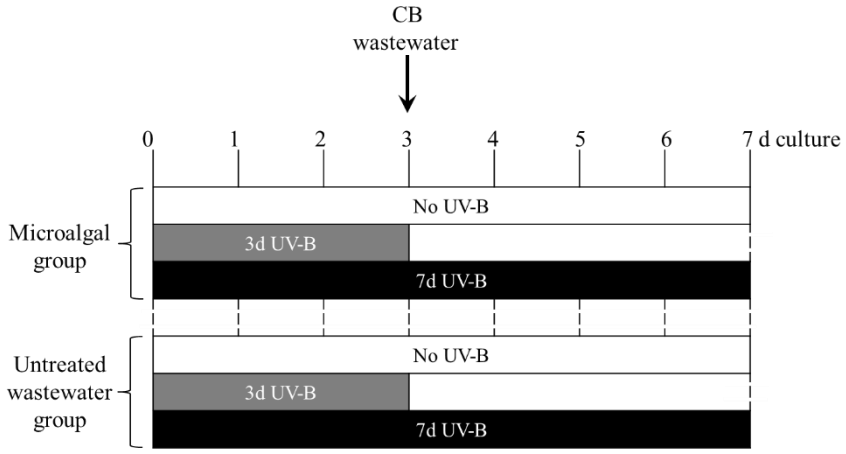


Figure 5. Experimental design of the study. All treatments were carried out under PAR. UV-B irradiation was applied either before (3d UV-B) or in parallel (7d UV-B) to a 4 days wastewater treatment. Control microalgal group only received PAR (No UV-B). Another parallel untreated wastewater group (no microalgae) was included, and it was exposed to each UV-B treatment (No-UVB, 3d UV-B, and 7d UV-B). Sampling was performed at 7-days culture. See Sections 3.4.2 and 3.4.3 for details of treatment conditions.

3.4.4. Evaluation of microalgal remediation

After 3-days culture with or without UV-B, 500 µL of filter-sterilized CB wastewater (0.45 µm cellulose acetate filter, Sartorius, Göttingen, Germany) was added to each well to obtain a 25% (v/v) wastewater dilution (Figure 5). At the end of the experiment, cultures in each well were centrifuged at 3000 *g* for 10 min, the supernatant and the microalgal pellet were collected separately for further analysis.

3.4.5. Extraction and determination of photosynthetic pigments

Photosynthetic pigments, including chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), and total carotenoids (Car) were extracted from microalgal pellets and analyzed as previously reported [45]. Four biological replicates were considered for these analyses.

3.4.6. Extraction and determination of total antioxidant capacity, phenolic compounds and flavonoids

Extraction was performed as described in Moles et al. [46] with some modifications. Briefly, microalgal pellets were extracted in 80% ethanol. Samples were sonicated (Branson 1210 sonicator, Bransonic, Connecticut, USA) for 30 min at room temperature, incubated for another 30 min under dark and then centrifuged at 10000 rpm for 10 min. The ethanolic extracts were recovered and then used for determining the total antioxidant capacity (TAC), phenolic compounds and flavonoids. TAC was spectrophotometrically determined at 515 nm by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as reported in Huarancca Reyes et al. [47]. Phenolic compounds were assayed with the method based on Folin-Ciocalteau reagent and spectrophotometrically determined at 750 nm as described in Huarancca Reyes et al. [48]. Total flavonoids were spectrophotometrically determined at 510 nm referring to Mariotti et al. [49]. Four biological replicates were considered for these analyses.

3.4.7. Analytical determinations

Supernatants were dried under vacuum and diluted with acetone and heptane 50% (v/v). Nicotine [pyridine, 3-(1-methyl-2-pyrrolidinyl)] and nicotyrine [pyridine, 3-(1-methyl-1H-pyrrol-2-yl)] in the wastewater samples were determined as described in Chiellini et al.

[10] by using a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a MEGA-SE54 HT capillary column (10 m; 0.15 mm i.d., 0.10 μm film thickness, MEGA s.n.c., Milan, Italy). Briefly, the carrier gas was dried and air free helium with a linear speed of 60 cm s^{-1}, the oven temperature was maintained at 60 °C for 2 min and increased to 350 °C at a rate of 10 °C min^{-1}, and the full-scan mass spectra was obtained in EI^+ mode with an emission current of 10 μA and an axial modulation of 4 V. Data acquisition was from 10 to 550 Da at a speed of 1.4 scan s^{-1}. The quantification was performed using the relative abundance of their chromatogram peaks (instrument detection limit < 400 counts). The abundance of nicotine (retention time = 6.63 min) and nicotyrine (retention time = 8.577 min) in the wastewater after microalgal-based remediation was expressed in % (kcounts/kcounts) and obtained by its comparison with the abundance in their respective untreated wastewater, which represented 100%. Four biological replicates were considered for these analyses.

3.4.8. Statistical analyses

Values presented are means of four replicates. Statistical analysis was performed using one-way analysis of variance (ANOVA). The Tukey's test was used to determine the significant differences among means (P<0.05). All computations were performed with the software STATISTICA for Windows version 14.0 (Stat-Soft, Inc., Tulsa, OK, USA).

To identify the effect of UV-B irradiation for the improvement of remediation capacity of microalgae toward the nicotine- and nicotyrine-contained in CB wastewater, based on physiological and analytical data, multiple factor analysis (MFA) was carried out [50]. Data were normalized using Z-score calculation, and the R software [51] with the packages "FactoMineR" (analysis) and "factoextra" (visualization) were applied.

3.5. REFERENCES

1. Kurmus, H.; Mohajerani, A. The toxicity and valorization options of cigarette butts. *Waste Manag.* **2020**, *104*, 104–118, doi:10.1016/j.wasman.2020.01.011.

2. Hoffmann, D.; Hoffmann, I. The changing cigarette, 1950-1995. *J. Toxicol. Environ. Health* **1997**, *50*, 307–364, doi:10.1080/009841097160393.

3. World Health Organization *Tobacco and its environmental impact: An overview*; Geneva, 2017; ISBN 9789241512497.

4. Oliva, M.; De Marchi, L.; Cuccaro, A.; Pretti, C. Bioassay-based ecotoxicological investigation on marine and freshwater impact of cigarette butt littering. *Environ. Pollut.* **2021**, *288*, 117787, doi:10.1016/j.envpol.2021.117787.

5. Selmar, D.; Radwan, A.; Abdalla, N.; Taha, H.; Wittke, C.; El-Henawy, A.; Alshaal, T.; Amer, M.; Kleinwächter, M.; Nowak, M.; et al. Uptake of nicotine from discarded cigarette butts - A so far unconsidered path of contamination of plant-derived commodities. *Environ. Pollut.* **2018**, *238*, 972–976, doi:10.1016/j.envpol.2018.01.113.

6. Booth, D.J.; Gribben, P.; Parkinson, K. Impact of cigarette butt leachate on tidepool snails. *Mar. Pollut. Bull.* **2015**, *95*, 362–364, doi:10.1016/j.marpolbul.2015.04.004.

7. Rebischung, F.; Chabot, L.; Biaudet, H.; Pandard, P. Cigarette butts: A small but hazardous waste, according to European regulation. *Waste Manag.* **2018**, *82*, 9–14, doi:10.1016/j.wasman.2018.09.038.

8. Marinello, S.; Lolli, F.; Gamberini, R.; Rimini, B. A second life for cigarette butts? A review of recycling solutions. *J. Hazard. Mater.* **2020**, *384*, 121245, doi:10.1016/j.jhazmat.2019.121245.

9. Mariotti, L.; Huarancca Reyes, T.; Curadi, M.; Guglielminetti, L. Recycling cigarette filters as plant growing substrate in soilless system. *Horticulturae* **2022**, *8*, 135, doi:10.3390/horticulturae8020135.

10. Chiellini, C.; Mariotti, L.; Huarancca Reyes, T.; de Arruda, E.J.; Fonseca, G.G.; Guglielminetti, L. Remediation capacity of different microalgae in effluents derived from the cigarette butt cleaning process. *Plants* **2022**, *11*, 1770, doi:10.3390/plants11131770.

11. Chiellini, C.; Guglielminetti, L.; Sarrocco, S.; Ciurli, A. Isolation of four microalgal strains from the lake Massaciuccoli: Screening of common pollutants tolerance pattern and perspectives for their use in biotechnological applications. *Front. Plant Sci.* **2020**, *11*, 1–18, doi:10.3389/fpls.2020.607651.

12. Bornman, J.F.; Barnes, P.W.; Robinson, S.A.; Ballaré, C.L.; Flint, S.D.; Caldwell, M.M. Solar ultraviolet radiation and ozone depletion-driven climate change: Effects on terrestrial ecosystems. *Photochem. Photobiol. Sci.* **2015**, *14*, 88–107, doi:10.1039/C4PP90034K.

13. Müller-Xing, R.; Xing, Q.; Goodrich, J. Footprints of the sun: Memory of UV and light stress in plants. *Front. Plant Sci.* **2014**, *5*, 474, doi:10.3389/fpls.2014.00474.

14. Rozema, J.; Björn, L.O.; Bornman, J.F.; Gaberščik, A.; Häder, D.P.; Trošt, T.; Germ, M.; Klisch, M.; Gröniger, A.; Sinha, R.P.; et al. The role of UV-B radiation in aquatic and terrestrial ecosystems-An experimental and functional analysis of the evolution of UV-absorbing compounds. *J. Photochem. Photobiol. B Biol.* **2002**, *66*, 2–12, doi:10.1016/S1011-1344(01)00269-X.

15. Jenkins, G.I. Photomorphogenic responses to ultraviolet-B light. *Plant. Cell Environ.* **2017**, *40*, 2544–2557, doi:10.1111/pce.12934.

16. Huarancca Reyes, T.; Esparza, E.; Crestani, G.; Limonchi, F.; Cruz, R.; Salinas, N.; Scartazza, A.; Guglielminetti, L.; Cosio, E. Physiological responses of maca (*Lepidium Meyenii* Walp.) plants to UV radiation in its high-altitude mountain ecosystem. *Sci. Rep.* **2020**, *10*, 2654, doi:10.1038/s41598-020-59638-4.

17. Vandenbussche, F.; Yu, N.; Li, W.; Vanhaelewyn, L.; Hamshou, M.; Van Der Straeten, D.; Smagghe, G. An ultraviolet B condition that affects growth and defense in *Arabidopsis*. *Plant Sci.* **2018**, *268*, 54–63, doi:10.1016/j.plantsci.2017.12.005.

18. Dahms, H.U.; Lee, J.S. UV radiation in marine ectotherms: molecular effects and responses. *Aquat. Toxicol.* **2010**, *97*, 3–14, doi:10.1016/j.aquatox.2009.12.002.

19. Rastogi, R.P.; Madamwar, D.; Nakamoto, H.; Incharoensakdi, A. Resilience and self-regulation processes of microalgae under UV radiation stress. *J. Photochem. Photobiol. C Photochem. Rev.* **2020**, *43*, 100322, doi:10.1016/j.jphotochemrev.2019.100322.

20. Favory, J.-J.; Stec, A.; Gruber, H.; Rizzini, L.; Oravecz, A.; Funk, M.; Albert, A.; Cloix, C.; Jenkins, G.I.; Oakeley, E.J.; et al. Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in *Arabidopsis*. *EMBO J.* **2009**, *28*, 591–601, doi:10.1038/emboj.2009.4.

21. Rizzini, L.; Favory, J.-J.; Cloix, C.; Faggionato, D.; O'Hara, A.; Kaiserli, E.; Baumeister, R.; Schäfer, E.; Nagy, F.; Jenkins, G.I.; et al. Perception of UV-B by the *Arabidopsis* UVR8 protein. *Science.* **2011**, *332*, 103–106, doi:10.1126/science.1200660.

22. Tilbrook, K.; Dubois, M.; Crocco, C.D.; Yin, R.; Chappuis, R.; Allorent, G.; Schmid-Siegert, E.; Goldschmidt-Clermont, M.; Ulma, R. UV-B Perception and acclimation in *Chlamydomonas reinhardtii*. *Plant Cell* **2016**, *28*, 966–983, doi:10.1105/tpc.15.00287.

23. Zhang, Z.; Xu, C.; Zhang, S.; Shi, C.; Cheng, H.; Liu, H.; Zhong, B. Origin and adaptive evolution of UV RESISTANCE LOCUS 8-mediated signaling during plant terrestrialization. *Plant Physiol.* **2022**, *188*, 332–346, doi:10.1093/plphys/kiab486.

24. Paliwal, C.; Mitra, M.; Bhayani, K.; Bharadwaj, S.V.V.; Ghosh, T.; Dubey, S.; Mishra, S. Abiotic stresses as tools for metabolites in microalgae. *Bioresour. Technol.* **2017**, *244*, 1216–1226, doi:10.1016/j.biortech.2017.05.058.

25. Chiellini, C.; Serra, V.; Gammuto, L.; Ciurli, A.; Longo, V.; Gabriele, M. Evaluation of nutraceutical properties of eleven microalgal strains isolated from different freshwater aquatic environments: Perspectives for their application as nutraceuticals. *Foods* **2022**, *11*, 654, doi:10.3390/foods11050654.

26. Hideg, É.; Jansen, M.A.K.; Strid, Å. UV-B exposure, ROS, and stress: Inseparable companions or loosely linked associates? *Trends Plant Sci.* **2013**, *18*, 107–115, doi:10.1016/j.tplants.2012.09.003.

27. Kovac, D.; Navratil, M.; Malenovsk, Z.; Stroch, M.; Spunda, V.; Urban, O. Reflectance continuum removal spectral index tracking the xanthophyll cycle photoprotective reactions in Norway spruce needles. *Funct. Plant Biol.* **2012**, *39*, 987–998, doi:10.1071/FP12107.

28. Hamed, S.M.; Zinta, G.; Klöck, G.; Asard, H.; Selim, S.; AbdElgawad, H. Zinc-induced differential oxidative stress and antioxidant responses in *Chlorella sorokiniana* and *Scenedesmus acuminatus*. *Ecotoxicol. Environ. Saf.* **2017**, *140*, 256–263, doi:10.1016/j.ecoenv.2017.02.055.

29. Abbew, A.W.; Qiu, S.; Amadu, A.A.; Qasim, M.Z.; Chen, Z.; Wu, Z.; Wang, L.; Ge, S. Insights into the multi-targeted effects of free nitrous acid on the microalgae *Chlorella sorokiniana* in wastewater. *Bioresour. Technol.* **2022**, *347*, 126389, doi:10.1016/j.biortech.2021.126389.

30. Xue, L.; Zhang, Y.; Zhang, T.; An, L.; Wang, X. Effects of enhanced ultraviolet-B radiation on algae and cyanobacteria. *Crit. Rev. Microbiol.* **2005**, *31*, 79–89, doi:10.1080/10408410590921727.

31. Tian, J.; Yu, J. Changes in ultrastructure and responses of antioxidant systems of algae (*Dunaliella salina*) during acclimation to enhanced ultraviolet-B radiation. *J. Photochem. Photobiol. B Biol.* **2009**, *97*, 152–160, doi:10.1016/j.jphotobiol.2009.09.003.

32. Zhang, X.; Tang, X.; Zhou, B.; Hu, S.; Wang, Y. Effect of enhanced UV-B radiation on photosynthetic characteristics of marine microalgae *Dunaliella salina* (Chlorophyta, Chlorophyceae). *J. Exp. Mar. Bio. Ecol.* **2015**, *469*, 27–35, doi:10.1016/j.jembe.2015.04.002.

33. Mátai, A.; Nagy, D.; Hideg, É. UV-B strengthens antioxidant responses to drought in *Nicotiana benthamiana* leaves not only as supplementary irradiation but also as pre-treatment. *Plant Physiol. Biochem.* **2019**, *134*, 9–19, doi:10.1016/j.plaphy.2018.09.014.

34. Malerba, M.E.; Palacios, M.M.; Palacios Delgado, Y.M.; Beardall, J.; Marshall, D.J. Cell size, photosynthesis and the package effect: An artificial selection approach. *New Phytol.* **2018**, *219*, 449–461, doi:10.1111/nph.15163.

35. Hao, T.-B.; Balamurugan, S.; Zhang, Z.-H.; Liu, S.-F.; Wang, X.; Li, D.-W.; Yang, W.-D.; Li, H.-Y. Effective bioremediation of tobacco wastewater by microalgae at acidic pH for synergistic biomass and lipid accumulation. *J. Hazard. Mater.* **2022**, *426*, 127820, doi:10.1016/j.jhazmat.2021.127820.

36. Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **2002**, *7*, 405–410, doi:10.1016/S1360-1385(02)02312-9.

37. Czégény, G.; Mátai, A.; Hideg, É. UV-B Effects on leaves-oxidative stress and acclimation in controlled environments. *Plant Sci.* **2016**, *248*, 57–63, doi:10.1016/j.plantsci.2016.04.013.

38. Malanga, G.; Calmanovici, G.; Puntarulo, S. Oxidative damage to chloroplasts from *Chlorella vulgaris* exposed to ultraviolet-B radiation. *Physiol. Plant.* **1997**, *101*, 455–462, doi:10.1034/j.1399-3054.1997.1010301.x.

39. Estevez, M.S.; Malanga, G.; Puntarulo, S. UV-B effects on Antarctic *Chlorella* sp cells. *J. Photochem. Photobiol. B Biol.* **2001**, *62*, 19–25, doi:10.1016/S1011-1344(01)00157-9.

40. Al-Rashed, S.A.; Ibrahim, M.M.; El-Gaaly, G.A.; Al-Shehri, S.; Mostafa, A. Evaluation of radical scavenging system in two microalgae in response to interactive stresses of UV-B radiation and nitrogen starvation. *Saudi J. Biol. Sci.* **2016**, *23*, 706–712, doi:10.1016/j.sjbs.2016.06.010.

41. Zhong, Y.; Xu, J.; Zhao, X.; Qu, T.; Guan, C.; Hou, C.; Tang, X.; Wang, Y. Balancing damage via non-photochemical quenching, phenolic compounds and photorespiration in *Ulva prolifera* induced by low-dose and short-term UV-B radiation. *Int. J. Mol. Sci.* **2022**, *23*, 2693, doi:10.3390/ijms23052693.

42. Fernández, M.B.; Tossi, V.; Lamattina, L.; Cassia, R. A comprehensive phylogeny reveals functional conservation of the UV-B photoreceptor UVR8 from green algae to higher plants. *Front. Plant Sci.* **2016**, *7*, 1–6, doi:10.3389/fpls.2016.01698.

43. Jansen, M.A.K.; Bilger, W.; Hideg, É.; Strid, Å.; Aphalo, P.; Brelsford, C.; Klem, K.; Mátai, A.; Llorens, L.; Nezval, J.; et al. Editorial: Interactive effects of UV-B radiation in a complex environment. *Plant Physiol. Biochem.* **2019**, *134*, 1–8, doi:10.1016/j.plaphy.2018.10.021.

44. Converti, A.; Casazza, A.A.; Ortiz, E.Y.; Perego, P.; Del Borghi, M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chem. Eng. Process. Process Intensif.* **2009**, *48*, 1146–1151, doi:10.1016/j.cep.2009.03.006.

45. Huarancca Reyes, T.; Pompeiano, A.; Ranieri, A.; Volterrani, M.; Guglielminetti, L.; Scartazza, A. Photosynthetic performance of five cool-season turfgrasses under UV-B exposure. *Plant Physiol. Biochem.* **2020**, *151*, 181–187, doi:10.1016/j.plaphy.2020.03.025.

46. Moles, T.M.; Guglielminetti, L.; Huarancca Reyes, T. Differential effects of sodium chloride on germination and post-germination stages of two tomato genotypes. *Sci. Hortic. (Amsterdam).* **2019**, *257*, 108730, doi:10.1016/j.scienta.2019.108730.

47. Huarancca Reyes, T.; Scartazza, A.; Castagna, A.; Cosio, E.G.; Ranieri, A.; Guglielminetti, L. Physiological effects of short acute UVB treatments in *Chenopodium quinoa* Willd. *Sci. Rep.* **2018**, *8*, 371, doi:10.1038/s41598-017-18710-2.

48. Huarancca Reyes, T.; Esparza, E.; Crestani, G.; Limonchi, F.; Cruz, R.; Salinas, N.; Scartazza, A.; Guglielminetti, L.; Cosio, E. Physiological responses of maca (*Lepidium Meyenii* Walp.) plants to UV radiation in its high-altitude mountain ecosystem. *Sci. Rep.* **2020**, *10*, 2654, doi:10.1038/s41598-020-59638-4.

49. Mariotti, L.; Huarancca Reyes, T.; Ramos-Diaz, J.M.; Jouppila, K.; Guglielminetti, L. Hormonal regulation in different varieties of *Chenopodium quinoa* Willd. exposed to short acute UV-B irradiation. *Plants* **2021**, *10*, 858, doi:10.3390/plants10050858.

50. Pompeiano, A.; Huarancca Reyes, T.; Moles, T.M.; Guglielminetti, L.; Scartazza, A. Photosynthetic and growth responses of *Arundo donax* L. plantlets under different oxygen deficiency stresses and reoxygenation. *Front. Plant Sci.* **2019**, *10*, doi:10.3389/fpls.2019.00408.

51. Team R Core. R: A language and environment for statistical computing; R Foundation for Statistical Computing: Vienna, Austria, 2013. Available online: https://www.r-project.org/ (accessed on 20 April 2022).

CAPÍTULO 4

CONCLUSÃO GERAL E CONSIDERAÇÕES FINAIS

4.1. CONCLUSÃO GERAL

A primeira parte deste estudo, Capítulo 2, destacou a importância da seleção de cepas de microalgas para remediação de efluentes de bitucas de cigarro (CB), e não necessariamente cepas isoladas de condições semelhantes de poluição podem ter o melhor desempenho como ocorreu com *Chlorella sorokiniana* LG1. Embora as águas residuais de CB possam ter impacto na produção de clorofila e carotenoides de microalgas de maneira dependente da concentração, todas as cepas de microalgas podem remover completamente ou quase completamente o benzonitrila; 1,2,3-propanotriol, diacetato; silano, metoxitripropilo; e silano, trimetil [(1-propilpentil)oxi] de maneira independente da concentração de CB. Em relação aos poluentes de CB denominados como "outros", estes incluíram hidrocarbonetos e aditivos como plastificantes. Aqui, todas as cepas de microalgas podem remover melhor os hidrocarbonetos e aditivos na maior concentração de águas residuais de CB, destacando sua aplicação potencial para remediar desastres petrolíferos e locais poluídos por plásticos tóxicos. Além dos poluentes mencionados, a nicotina [piridina, 3-(1-metil-2-pirrolidinil)] e a nicotirina [piridina, 3-(1-metil-1H-pirrol-2-il)] foram geralmente difíceis de remover pelas microalgas. Em detalhe, a nicotina foi o poluente mais resistente e sua baixa remoção se correlacionou com a inibição de pigmentos fotossintéticos afetando o crescimento de microalgas. No que diz respeito às condições ótimas para uma remoção eficaz de contaminantes, os resultados apoiaram a alta eficiência de *Chlamydomonas debaryana* F2 para remover poluentes orgânicos a 5% de efluentes de CB (correspondendo a 25 pontas L^{-1} ou 5 g CB L^{-1}) removendo 69% de poluentes e mantendo seu crescimento (baseado em Chl*a*) e pigmentos em níveis de controle.

A segunda parte, Capítulo 3, mostrou o primeiro estudo que relata o uso de ultravioleta B (UV-B) como uma ferramenta para fortalecer as respostas antioxidantes em microalgas para remediar a nicotina e a nicotirina contidas em 25% de águas residuais de CB. Os resultados sugeriram que os estímulos UV-B podem ativar vias de defesa associadas à

resistência ou tolerância às águas residuais de CB, destacando a importância do crosstalk entre ambas as variáveis abióticas com aplicação biotecnológica promissora especialmente em condições internas. De fato, o UV-B não impactou negativamente na produção de clorofila e carotenoides de microalgas, enquanto induziu os antioxidantes não enzimáticos, especialmente compostos fenólicos, em vez de flavonoides. Além disso, esse crosstalk é governado por uma interação complexa entre vias de sinalização resultando em resultados como resistência cruzada, tolerância cruzada ou sensibilidade cruzada, que podem variar dependendo da espécie ou mesmo genótipos da mesma espécie. Aqui, entre as três cepas de *Chlorella sorokiniana* testadas, R1 foi a única em que o UV-B pareceu ter um efeito sinérgico na resistência cruzada aos poluentes mais persistentes e tóxicos de CB (ou seja, nicotina e nicotirina), onde suas células expostas ao UV-B podem não apenas ganhar proteção contra poluentes, mas também podem melhorar as vias catabólicas envolvidas na remoção desses alcalóides.

4.2. CONSIDERAÇÕES FINAIS

No Capítulo 2, mais estudos são necessários para entender as vias de remoção de poluentes orgânicos em águas residuais de CB, bem como para determinar se esses poluentes são degradados ou bioacumulados pelas cepas de microalgas testadas. Da mesma forma, seria interessante identificar se a nicotirina poderia prevenir o catabolismo da nicotina em microalgas e se esse efeito pode ser pronunciado com o aumento da concentração de CB.

No Capítulo 3, mais estudos são necessários para determinar que tipo de carotenóides está envolvido nesta resistência cruzada entre a radiação UV-B e poluentes de águas residuais de CB. Da mesma forma, seria interessante determinar qual classe de compostos fenólicos desempenha um papel importante neste estudo, bem como avaliar o envolvimento de enzimas antioxidantes no crosstalk entre radiação UV-B e poluentes de águas residuais CB. Finalmente, um grande desafio será fazer este estudo em larga escala e avaliar se esta técnica biológica é estrategicamente econômica.

Por fim, os Capítulos 2 e 3 foram submetidos à revista *Plants*, e as regras para preparação do manuscrito estão descritas em https://www.mdpi.com/journal/plants/instruct