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Biodegradation of pesticides by the microalga *Dictyosphaerium* sp. and its effect on growth

Biodegradación de plaguicidas y efectos sobre el crecimiento empleando la microalga *Dictyosphaerium* sp.

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ABSTRACT. This study investigates the bioremediation potential of the native microalgae *Dictyosphaerium* sp. against two pesticides of environmental concern, chlorpyrifos (CPF) and 2,4-dichlorophenol (2,4-DCP), under different photoperiod conditions. The microalgae were isolated from an urban lake in Mendoza, Argentina, and cultivated in sterile Bold's Basal Medium. Growth and biodegradation assays were conducted to evaluate the impact of varying pesticide concentrations under phototrophic, mixotrophic and heterotrophic conditions. The results indicated that *Dictyosphaerium* sp. showed differential tolerance to CPF and 2,4-DCP at different concentrations, depending on light availability. Under dark conditions, CPF showed growth inhibition at the highest concentration tested (30 mg/L); whereas, 2,4-DCP significantly promoted algal growth at all concentrations tested (50 and 70 mg/L). In light conditions, CPF exposure increased growth at lower concentrations, but inhibited it at higher levels. In contrast, 2,4-DCP significantly inhibited algal growth under continuous light at high concentrations but stimulated growth under dark conditions. Biodegradation analysis using HPLC-DAD revealed that *Dictyosphaerium* sp. effectively degraded both pesticides, with complete decomposition observed within six days under light conditions and four days in darkness. The findings suggest that *Dictyosphaerium* sp. has significant potential for bioremediation in environments contaminated with CPF and 2,4-DCP, particularly under varying light conditions.

Keywords: Microalgae, herbicides, insecticides, native cultures, phycoremediation.

RESUMEN. Este estudio investiga el potencial biorremediador bajo diferentes condiciones de fotoperíodo de cultivos nativos de la microalga *Dictyosphaerium* sp. frente a dos plaguicidas de interés ambiental en Sudamérica, tales como clorpirifos (CPF) y 2,4-diclorofenol (2,4-DCP). La cepa microalgal fue aislada de un lago urbano de Mendoza, Argentina, y cultivadas en Medio Basal de

Bold estéril. Se realizaron ensayos de crecimiento y biodegradación para evaluar el impacto de diferentes concentraciones de plaguicidas en condiciones fototróficas, mixotróficas y heterotróficas. Los resultados indicaron que *Dictyosphaerium* sp. muestra una tolerancia diferencial al CPF y al 2,4-DCP a diferentes concentraciones, en función de la disponibilidad de luz. En condiciones de oscuridad, CPF demostró inhibición del crecimiento en la mayor concentración ensayada (30 mg/L); mientras que, 2,4-DCP promovió significativamente el crecimiento algal en todas las concentraciones ensayadas (50 y 70 mg/L). En condiciones de luz, la exposición al CPF provocó un aumento del crecimiento en la menor concentración ensayada de 5 mg/L. Por el contrario, en las mismas condiciones de luz, la exposición a 2,4-DCP produjo efectos adversos con la inhibición algal en la mayor concentración ensayada de 70 mg/L. El análisis de biodegradación mediante HPLC-DAD reveló que *Dictyosphaerium* sp. es capaz de degradar eficazmente ambos plaguicidas, observándose una descomposición completa en seis días en condiciones de luz y cuatro días en oscuridad. Los resultados sugieren que *Dictyosphaerium* sp. tiene un potencial significativo para la biorremediación en ambientes contaminados con CPF y 2,4-DCP, especialmente en condiciones

Palabras clave: Microalgas, ficorremediación, herbicidas, insecticidas, cultivos nativos.

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INTRODUCTION

Increased food production and the advancing agricultural frontiers result in increased use of pesticides to improve crop production and increase economic benefits (Nie *et al*., 2020; Shrestha *et al*., 2021). However, the indiscriminate use of pesticides could lead to a strong increase and accumulation of pesticides in the environment, resulting in serious pollution problems (Bhandari *et al*., 2018). On the other hand, over the last 50 years, social and scientific interest has grown in the fate of different pollutants in the environment and their impact on water quality, with emphasis on drinking water quality and adverse effects on non-target aquatic organisms (Pimentel, 1978; Zhuang *et al*., 2015). The amount of freshwater on earth is limited, and its quality is under constant pressure (Qadri and Bhat, 2020). Water quality can be compromised by the presence of infectious agents, toxic chemicals or radiation. Water and sanitation are one of the main drivers of public health, which is why the United Nations decided in 2005 to raise the global profile of water issues. World Health Organization (WHO) developed a risk management tool called Sanitation Safety Planning (SSP), which assists relevant authorities in implementing the WHO Guidelines for the safe use of wastewater; it requires the identification of toxic substances and exposure risk assessment (WHO, 2016). However, there are currently few systematic monitoring programs and comprehensive studies on human exposure to environmental stressors such as pharmaceuticals, e-waste by-products and agrochemicals in drinking water (Aktar *et al*., 2009, Kim *et al*., 2017). Several methods have been devised to remove these pollutants, such as evaporation, precipitation, ion exchange, advanced aerobic degradation, biological oxidation, oxidative processes, ozonation, coagulation, membrane filtration, solid phase extraction, nanofiltration, photocatalytic degradation and electrochemical degradation (Mustafa *et al*., 2021). However, bioremediation processes are more attractive than the physical and chemical techniques mentioned above, as they are less costly and more efficient, even at low concentrations of pollutants, and help to promote the circular economy (Biswas *et al*., 2015). In this respect, different systems of biological organisms are currently used to remove compounds from wastewater, such as bacteria, fungi and algae (Roccuzzo *et al*., 2020; Saravanan *et al*., 2020). In recent decades, the use of microalgae in wastewater bioremediation has attracted great interest due to their central role in carbon

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dioxide fixation and their potential to transform, convert, accumulate, degrade and synthesize organic compounds (Al-Jabri *et al*., 2020; Chai *et al*., 2020). Among the most common uses for the removal of environmental stressors, the degradation of nutrients, pesticides, toxic elements, pharmaceuticals and oils from wastewater has been reported (Saravanan *et al.*, 2020; Mishra *et al*., 2023). Specifically, the growth rate of microalgae varies according to their specific cellular characteristics and culture conditions, whether autotrophic, heterotrophic or mixotrophic (Singh & Saxena, 2015; Smith *et al*., 2020). Microalgae that rely on light to generate energy are considered autotrophs, while heterotrophs use organic carbon as an energy source (Droop, 1974). On the other hand, a combination of both autotrophic and heterotrophic culture is called mixotrophic. In this context, this situation provides the cultured microalgae with inorganic carbon and a source of organic carbon, which contributes to a higher biomass yield (Peter *et al*., 2022). Current research on the bioaccumulation and biodegradation of pesticides in green algae shows the importance of these organisms from an environmental point of view. Avila *et al*. (2021) exposed *Chlorella* sp. and *Scenedesmus* sp. to chlorpyrifos, oxadiazon and cypermethrin, and their results showed a high percentage of degradation for these compounds, 97%, 88% and 74%, respectively.

Taking into account the environmental context, some of the most widely used and increasingly concerning pesticides in agricultural activities in the region, particularly in Argentina, are the insecticide chlorpyrifos (CPF) and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Marino & Ronco, 2005; De Gerónimo *et al*., 2014; Peluso *et al*., 2022; Gordillo *et al*., 2024). Specifically, CPF (O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is an organophosphorus pesticide commonly used as an insecticide to control agricultural foliar pests. It can bioaccumulate in various aquatic organisms (Varó *et al*., 2000). Despite being regulated by the U.S. Environmental Protection Agency (USEPA, 2000)[1] and banned in Argentina since 2021 (Servicio Nacional de Sanidad y Calidad Agroalimentaria [SENASA], 2021)[2], it remains the most widely used insecticide in the country following the ban on endosulfan (González Noschese *et al*., 2022). In 2021, it was the most imported insecticide in Argentina, with a total of 3.5 million kg (SENASA, 2021). Moreover, it has been detected in various environments and ecosystems across the country (Marrochi *et al*. 2021, Mac Loughin *et al*., 2022, Cecchetto *et al.*, 2023; Pérez-Iglesias *et al*., 2023; Fernández San Juan *et al.*, 2022), and that it bioaccumulates in aquatic organisms (Quadri-Adrogué *et al*., 2021; González Noschese *et al*., 2022; Vázquez-Tapia *et al*., 2022) and presents the greatest short-term environmental risk. In this sense, CPF is an insecticide of environmental concern for the region. On the other hand, 2,4-dichlorophenol (2,4-DCP) is a chlorinated phenoxy derivative used to prepare the herbicide 2,4-D (Talano *et al*., 2012). The Environmental Protection Agency (EPA) has designated 2,4-DCP as a toxic, carcinogenic, and persistent chemical compound, as well as a priority environmental pollutant. This compound has been found at elevated concentrations in freshwater sources, marine environments, industrial effluents, soils, and atmospheric emissions resulting from waste incineration (Kot-Wasik *et al*., 2004; Gao *et al*., 2008; Talano *et al*., 2012; Rodríguez-Hernández *et al.*, 2017). It is important to note that in recent years, the presence of CPF and 2,4-D in water bodies within agroecosystems in Latin America has been demonstrated, highlighting the risk to ecosystem health (Lajmanovich *et al.*, 2021; Mac Loughin *et al*., 2022; Peluso *et al*., 2022). Van Opstal *et al*. (2022) detected those compounds including 2,4-D, atrazine, cypermethrin, chlorpyrifos, and diazinon in the hydrographic basin of Arroyo

[1] U.S. Environmental Protection Agency. (2000, December 6). Chlorpyrifos; Cancellation Order. Federal Register, 65(235), 7623376243. https://www.federalregister.gov/documents/2000/12/06/00-30917/chlorpyrifos-cancellation-order

[2] Servicio Nacional de Sanidad y Calidad Agroalimentaria. (2021, 4 de agosto). *Resolución 414/2021. [Prohibición de importación, comercialización y uso de principios activos Clorpirifós Etil y Clorpirifós Metil]*. Boletín Oficial de la República Argentina. http://servicios.infoleg.gob.ar/infolegInternet/anexos/350000-354999/352683/norma.htm

Estacas in northern Entre Ríos province, at concentrations exceeding the guideline levels established for the protection of aquatic biota in Argentina (NGPBA). Specifically, concentrations of 2,4-D reached 12 µg/L (NGPBA ≤ 3.4 µg/L), and for chlorpyrifos, concentrations were 0.2 µg/L (NGPBA ≤ 0.006 µg/L). Corcoran *et al*. (2020) detected pesticides in surface waters, indicating that these originate from runoff from agricultural and urban lands, as well as from discharges of wastewater treatment plants. For 2,4-D, concentrations of 240.3 ± 18.2 ng/L were found in Brava Lake, Buenos Aires Province, and 319.3 ± 9.3 ng/L in Ctalamochita River, Córdoba. In this context, the objective of this study was to evaluate the ability of the green microalgae *Dictyosphaerium* sp. to biodegrade two pesticides of major environmental concern, such as chlorpyrifos (CPF) and 2,4-dichlorophenol (2,4-DCP), under controlled laboratory conditions. We hypothesize that *Dictyosphaerium* sp. exhibits differential growth responses and pesticide biodegradation capacity under varying photoperiod conditions and pesticide concentrations.

Materials and Methods

Organism and growth conditions

Samples were taken from an urban lake in Mendoza province, Argentina (32° 53′ 24″ S, 68° 52′ 5″ W) and an autochthonous strain of *Dictyosphaerium* sp. was found. The strain was identified morphologically and molecularly by DNA extraction and sequencing analysis of the section comprising the ITS1 intergenic region, the 5.8S gene and the ITS2 intergenic region of ribosomal RNA. Cells of *Dictyosphaerium* sp. were inoculated into 500 mL of sterile Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) at pH 6.6 under axenic conditions and at 28 ± 2 °C and a 12:12-h light-dark photoperiod with 6000 lux of light intensity. The cultures were continuously aerated with an air pump to avoid sedimentation and kept in an exponential phase from which subcultures were made to carry out the tests.

Chemicals

CPF was obtained from the commercially formulated insecticide Lorsban® 75WG Dow Agroscience containing 75% of the active product. To obtain the active compound, we weighed 10 g of Lorsban® 75WG on a Sartorius analytical balance (Practum, Germany) to the nearest 0.0001 g, then mixed with 60 mL of dichloromethane (Anedra Research AG, Buenos Aires, Argentina) in a beaker. The resulting mixture was filtered three times using a kitasate with filter papers of different pore sizes (Whatman 113 and 42, respectively, Germany) (Gordillo *et al.*, 2024). A spectrophotometer (SHIMADZU UV-1800, USA) was used to test the purity of the solution with the active compound. The liquid solution was dehydrated by placing it in a beaker covered with perforated aluminum foil. Then a stock solution was prepared with 200 mg/L, w/v ethanol of CPF. Also, 2,4-DCP was purchased from Merck (98% purity). All reagents and solvents used for HPLC measurements were of analytical grade. HPLC-grade solvents and reagents, and columns were purchased from Merck and Agilent Columns, respectively. All other chemicals used were of analytical grade.

Calibration curve and microalgae growth

All cultures used in the experiments were previously subcultured and used in the exponential growth phase. *Dictyosphaerium* sp. showed a delay phase (or latency) of approximately 2 days. The growth of *Dictyosphaerium* sp. was daily determined by measuring the optical density at 750 nm (OD 750) (Selvaratnam *et al*., 2015) in a UV-VIS Shimadzu spectrophotometer. The obtained value was converted into cell density by the linear relationship between OD 750 and the number of cells (cells/mL). In addition, to verify the algal growth, a Neubauer hemocytometer (Neubauer, Alemania) was carried out considering the linear relationship with the OD (R^2 = 0.986). Exponential growth rate (r, h-1) was calculated

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using the following equation (Fogg, 1987): [r = ln (N_1)- ln (N_0) / t]; where N_1 is the cell concentration (cells/mL) at the end of assay, N_0 is the initial cell concentration (cells/mL) and t is the exposure time in hours.

The percent inhibition of algal growth rate was calculated with respect to controls according to the following equation (U.S. EPA, 2002)[3]:

$$\%I = [(T - C) / C] \times 100$$

Where, *T* is the average specific growth rate (*r*) for the treatment replicated; and, *C* is the mean value for average specific growth rate in the control.

Toxicity tests

Prior to the bioassays design, the pesticides and the concentrations to be tested were selected according to the following criteria: (1) both are the most widely used insecticide and herbicide in Argentina (Fernández San Juan *et al*., 2022); (2) the selection of concentrations was based on the known toxicity of CPF to microalgae within the chosen range (Fernández *et al*., 2021) and the recommended environmental application concentrations for 2,4-D as indicated on the product label (Cámara de Sanidad Agropecuaria y Fertilizantes [CASAFE], 2025)[4]. For example, according to CASAFE (2025), for 2,4-D it is recommended to apply 1 L of the product per Ha, which contains 93% of 2,4-D (930 mg/L), and this means that a concentration of approximately 50 mg/L could reach a water body of 500 m2 by direct application in agricultural areas. In addition, if we consider surface runoff, the reports mentions that it can be from 0.01 to 1%, and thus a concentration of 10 mg/L could reach the water body (Food and Agriculture Organization [FAO] and World Health Organization [WHO], 1998). In this sense, we selected concentrations that produce adverse effects on microalgae to propose this as a potential phyco-remediator of pesticides in real situations after an application in agricultural fields (e.g. 2,4-D) or for concentrations detected in the environment (e.g. chlorpyrifos). Finally, concentrations were selected based on previous tests exposing green algae to different CPF concentrations (Asselborn *et al*., 2015; Chen *et al*., 2016; Fernández *et al*., 2021). After that, we conducted the bioassays according to standardized protocols proposed by the Organization for Economic Co-operation and Development (OECD, 2011), with minor modifications. *Dictyosphaerium* sp. was cultured in 50 mL of BBM medium with nominal CPF concentrations of 5, 10, 20, and 30 mg/L. The cultures were incubated under three trophic conditions: phototrophic, mixotrophic, and heterotrophic. The phototrophic condition consisted of continuous light exposure (6000 lux, cool-white fluorescent lamps) with the different pesticide concentrations. The mixotrophic condition was characterized by a 12:12 h light-dark photoperiod, also with the tested pesticide concentrations. The heterotrophic condition was maintained in continuous darkness, with the presence of the pesticides. For 2,4-DCP, two concentrations (50 and 70 mg/L, labeled as T1 and T2, respectively) were evaluated. Treatments and controls were incubated under continuous light and dark conditions in aseptic conditions in Erlenmeyer flasks covered with gauze-cotton and aluminum foil. Subcultures at the logarithmic growth phase with an initial cell density of 8.10_6 cells/mL were used. After inoculation, culture flasks were kept for 96 hours on a shaking tray (75 rpm) at 28 ± 2 °C.

[3] U.S. Environmental Protection Agency (2002). Method 1003.0: Green alga, *Selenastrum capricornutum,* growth test; Chronic toxicity (Excerpt from Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 4th ed.; EPA-821-R-02-013).

[4] Cámara de Sanidad Agropecuaria y Fertilizantes (CASAFE). (2025). Guía On line de Productos Fitosanitarios [Phytosanitary Products Guide]. https://www.casafe.org/publicaciones/guia-de-productos-fitosanitarios. Acceso: 19 de febrero de 2025.

Treatments in the presence of light were done using cool-white fluorescent lamps (6000 lux) according to OECD (2011).

Pesticide algal bioassay for bioremediation

High-performance liquid chromatography (HPLC-DAD) was used to test the action of microalgae on CPF and 2,4-DCP under the aforementioned illumination conditions. This experiment was performed in 150 mL Erlenmeyer flasks containing 45 mL of sterile BBM spiked with a concentration of 10 mg/L CPF and 50 mg/L 2,4-DCP. The pesticide solutions were passed through a sterile filter. Each container was inoculated with 5 mL of *Dictyosphaerium* sp. microalgal suspension with a cell density of 8×10^6 cells/mL. Medium containing algal cells without pesticides were set as blank samples to monitor the algal growth and medium with 10 and 50 mg/L of CPF and 2,4-DCP respectively, without algal cells were set as control samples to monitor other losses of pesticides not caused by algae. All the flasks were kept in a shaker incubator for 6 days under the same conditions mentioned in the previous section. Concentrations of pesticides were determined daily for 6 days by the HPLC-DAD system.

Preparation of extracts standard solutions

Extracts were prepared at an appropriate concentration, filtered through a Durapore® syringe membrane (Merck Millipore) with a diameter of 13 mm diameter and pore size of 0.22 µm before injection into the HPLC-DAD system, Thermo U3000 system, which included a quaternary pump and Diode array Ultimate DAD 3000 detector, operating in the range of 200–750 nm. Both extracts and standard solutions (10 mg/mL each) were injected (10 µL) and passed through a reverse-phase Chrompack® column (packing material: Chromspher® C18, 150 × 4.6 mm diameter) at room temperature. The flow rate was set to 1 mL/m for every extract sample and reference compound, utilizing methanol and an aqueous solution of phosphoric acid (0.1% p/v). The chosen separation method for this study was an isocratic elution using a mobile phase composed of 30% methanol and 70% aqueous acetic acid solution (0.1% w/v); the total runtime was 30 min. Wavelengths of 289 and 229 nm were selected based on the maximum absorption of CPF and 2,4-DCP standards. CPF in each extract were identified by comparing their retention times (Rt) and UV spectra with standard content. Two calibration curves of pure isolated metabolites were prepared with five dilutions of stock methanol solutions, each in triplicate. The entire procedure was successfully validated, demonstrating acceptable analytical performance. The linearity range extended from 2.27×10^{-7} to 1.99×10^{-6} µg, with the respective LOD and LOQ values of 2.35×10^{-7} and 2.01×10^{-6} µg, as well as inter-day/intra-day precision, were conducted following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH, 2014). To evaluate the method's accuracy, a recovery assessment was employed (Schiavi *et al*., 2024).

Data analysis

The effective concentration (EC) value was calculated using linear regression analysis of transformed pesticides concentration as natural logarithm data versus percentage of inhibition (Wei *et al.*, 2010). On the other hand, MANOVA, one-way ANOVA and Dunnett's posterior test were used to compare differences between treatments and the control group at different times; and thus obtain the values of interest in each final parameter evaluated. In the case that the variables did not meet the assumptions of normal distribution and homogeneity of variances, Kruskall-Wallis test and posterior tests were applied (Zar, 2010). Finally, differences were considered significant at $P < 0.05$ and all analyses were performed using R Statistical Software. (v4.1.2; R Core Team 2021).

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Table 1. Percent inhibition in *Dictyosphaerium* sp. exposed to CPF and 2,4-DCP after 96 h. Values in green indicate growth promotion and values red indicate growth inhibition. CPF = chlorpyrifos; 2,4-DCP = 2,4-dichlorophenol.

Laboratory conditions	% Inhibition by CPF Concentration (mg / L)				% Inhibition 2,4-DCP Concentration (mg / L)	
Fotoperiod (hours)	*5*	*10*	*20*	*30*	*50*	*70*
12 light : 12 dark	153.19	123.58	60.86	85.74	4.60	4.40
24 light	108.45	100	100	100	4.74	2.57
24 dark	122.00	99.23	3.32	100	3.38	2.48

RESULTS

Microalgae growth

The results on the effects on algal growth are presented in Table 1. In particular, a promotion of algal growth between 3 to 5% was observed in those treatments exposed to 50 mg/L of 2,4-DCP after 96 h in all tested photoperiod conditions. On the other hand, at 70 mg/L of 2,4-DCP, an inhibition between 2.5 and 4% was observed for light:dark and light only conditions; while, in total darkness, an approximate growth promotion of 2.5% was observed. When the results of *Dictyosphaerium* sp. exposed to CPF were analyzed, algal biomass inhibition was observed at the highest concentration tested (30 mg/L) from 85 % in light : dark situation, approximately 100 % in dark situation and in full light situation. On the contrary, an increase in algal biomass (from 100 to 150%) was observed in all photoperiod conditions for the lowest concentration tested (5 mg/L). On the other hand, at 10 mg/L, an increase in algal biomass was observed in light:dark and total darkness conditions; although, in total light conditions, an inhibition of 100% occurred. Finally, at 20 mg/L CPF, the results were similar to those observed at 10 mg/L, with algal increases between 3 and 60% in dark and light:dark conditions, respectively, while there was 100% inhibition in light conditions throughout the bioassay.

Effects of CPF on algal growth

Bioassays with *Dictyosphaerium* sp. showed differential tolerance to CPF under different lighting conditions. MANOVA statistical analyses showed that when algae are exposed to CPF under dark conditions both concentration and time ($P_{concentration\text{-}time} < 0.001$) affect algal growth. Specifically, a posteriori tests showed highly significant differences in algal biomass growth decrease at the highest CPF exposure concentration (30 mg/L) with respect to the control group, after 4 days of exposure ($P_{(2\text{-}4\ days)} < 0.001$; Fig. 1a). On the other hand, when *Dictyosphaerium* sp. was exposed to CPF under light conditions, the MANOVA results showed that only the concentration factor affects algal biomass growth ($P < 0.001$); while time is not a factor conditioning growth for this experiment ($P = 0.260$). In particular, the statistical differences observed were due to highly significant increases in cell number at the lowest CPF exposure concentration (5 mg/L) compared to the control group, starting at 48 h of exposure ($P_{2\text{-}4days} < 0.0001$; Fig. 1b). Finally, when the 12:12 (light:dark) exposure scenario was considered, the MANOVA results revealed that both factors considered (concentration and time) affect algal growth ($P_{concentration\text{-}time} < 0.001$). In particular, a posteriori analysis showed that these statistical differences are due to significant decreases in algal growth at all concentrations after 24 h $P_{(5\text{-}30\ mg/L} < 0.05$; Fig. 1c); decreases also in this biological variable considered at 48 h at 10, 20 and 30 mg/L CPF ($P < 0.001$; Fig. 1c). Significant decreases in algal biomass at the highest concentrations (20 and 30 mg/L, $P_{20\text{-}30mg/L} < 0.001$) and highly significant increase at the lowest concentration (5 mg/L), at the end of 72 h with respect to the control group ($P_{5mg/L} < 0.01$; Fig. 1c); and significant increases

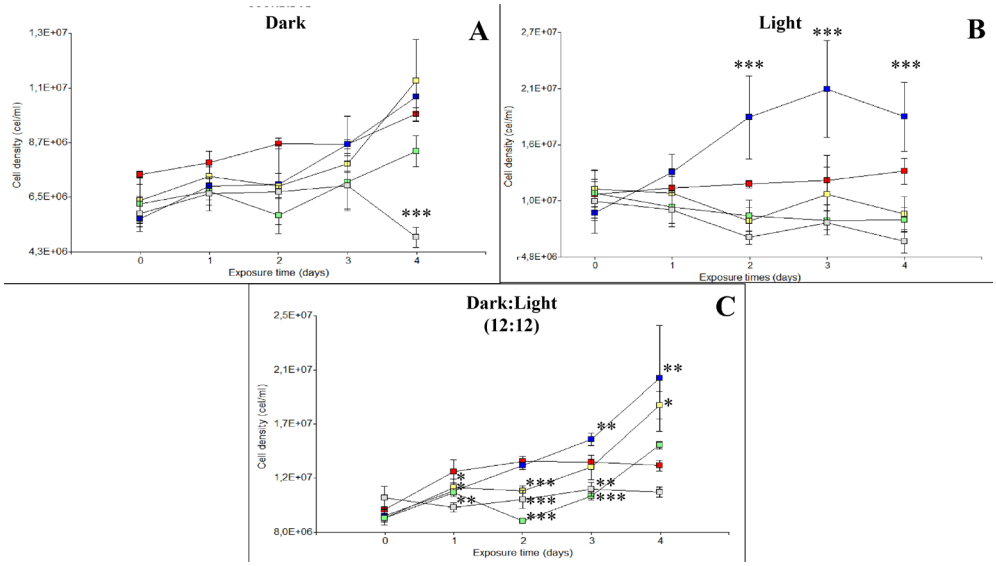

Figure 1. Growth of *Dictyosphaerium* sp. under different CPF concentration treatments and photoperiod conditions over 4 days of exposure.
Note. Growth curves represent algal growth at different CPF concentrations: blue = 5 mg/L, yellow = 10 mg/L, green = 20 mg/L, and gray = 30 mg/L under 24 h dark (A), 24 h light (B), and 12:12 h light:dark photoperiod (C). (*) indicates significant differences compared to the control group (red).
Figura 1. Crecimiento de *Dictyosphaerium* sp. bajo diferentes concentraciones de CPF y condiciones de fotoperiodo durante 4 días de exposición.
Nota. Las curvas de crecimiento representan el desarrollo algal a distintas concentraciones de CPF: azul = 5 mg/L, amarillo = 10 mg/L, verde = 20 mg/L y gris = 30 mg/L, bajo condiciones de 24 h de oscuridad (A), 24 h de luz (B) y fotoperiodo 12:12 h luz:oscuridad (C). (*) indica.

in *Dictyosphaerium* sp. cell number at the lowest concentrations (5 and 10 mg/L CPF) with respect to the control group, at the end of the bioassay (P5mg/L < 0.01; P10mg/L < 0.05; Fig. 1c).

Effects of 2,4-DCP on algal growth

The microalgae *Dictyosphaerium* sp. showed remediation associated with a significant increase in biomass at all concentrations tested for the dark condition. In contrast, for the light condition, a significant decrease in cell growth was observed at the highest concentration (70 mg/L) during the experiment. MANOVA statistical analyses showed that when algae are exposed to 2,4-DCP under dark conditions both concentration and time significantly affect algal growth (Pconcentration-time < 0.0001). A posteriori tests showed highly significant differences with respect to the control group, given the increase in algal biomass at both concentrations from 24 (P50-70mg/L < 0.0001) to 96 h (P50-70mg/L< 0.001) of exposure (Fig. 2a). On the other hand, when *Dictyosphaerium* sp. was exposed to 2,4-DCP under light conditions, MANOVA results showed that both concentration and time significantly affect algal growth (Pconcentration-time < 0.05). In particular, the statistical differences observed were due to a highly significant decrease in the number of microalgae cells exposed at the highest exposure concentration of 2,4-DCP (70 mg/L) with respect to the control group at all times evaluated (P24h < 0.01, P48-96h < 0.001; Fig. 2b). Finally, when the 12:12 (light:dark) exposure scenario was considered, the MANOVA results revealed that both factors considered (concentration and time) did not affect algal biomass growth (Pconcentration-time > 0.05).

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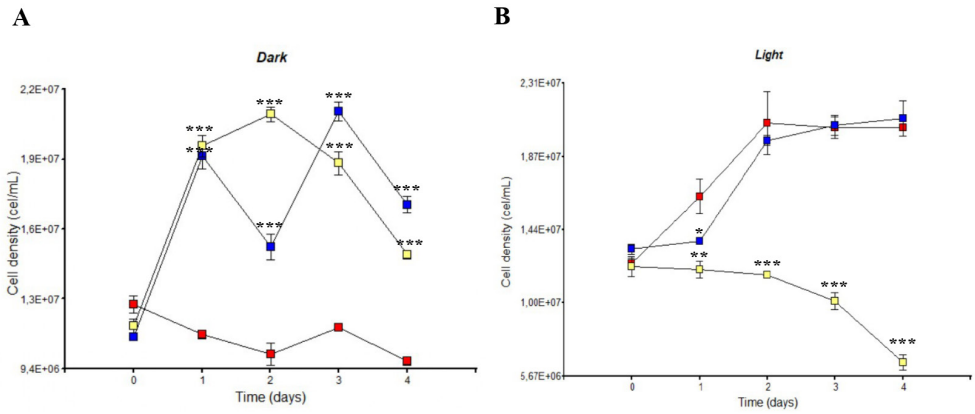

Figure 2. Growth of *Dictyosphaerium* sp. under different 2,4-DCP treatments and photoperiod conditions over 4 days of exposure.
Note. Growth curves represent algal growth at 50 mg/L (blue) and 70 mg/L (yellow) of 2,4-DCP under 24 h dark (A) and 24 h light (B) conditions. (*) indicates significant differences compared to the control group (red).
Note. The time curves represent the growth of the algae with 50 mg/L (blue) and 70 mg/L (yellow) of CPF treatments in 24h of darkness (A) and 24h of light (B). (*) represents significant differences with the control group (red).
Figura 2. Crecimiento de *Dictyosphaerium* sp. bajo diferentes tratamientos de 2,4-DCP y condiciones de fotoperiodo durante 4 días de exposición.
Nota. Las curvas de crecimiento representan el desarrollo algal a 50 mg/L (azul) y 70 mg/L (amarillo) de 2,4-DCP, bajo condiciones de 24 h de oscuridad (A) y 24 h de luz (B). (*) indica diferencias significativas respecto al grupo control (rojo).

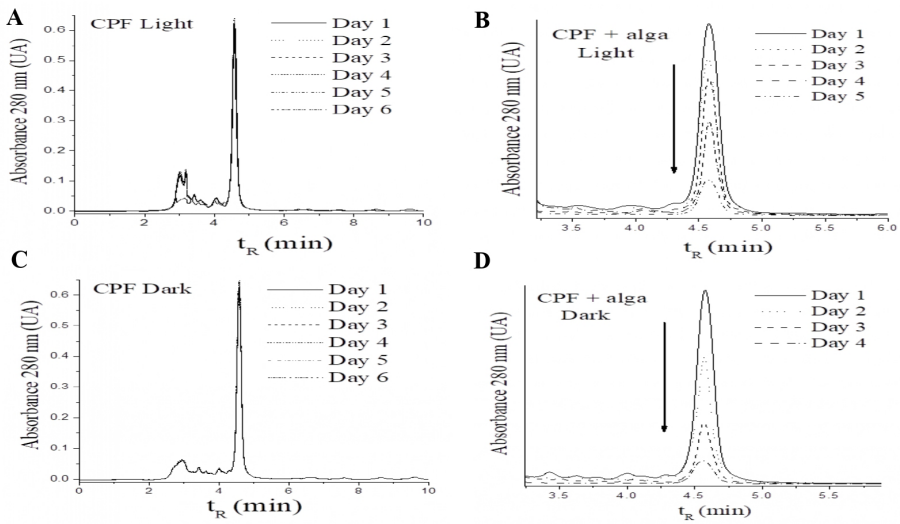

Figure 3. HPLC-DAD chromatograms illustrating the time course of CPF biotransformation by microalgae under light and dark conditions.
Note. (A) BBM and CPF substrate control under light conditions without microalgal inoculum; (B) BBM microalgal inoculum treatment with 10 mg/L CPF under light conditions; (C) BBM and CPF substrate control in darkness and without microalgal inoculum; (D) BBM and CPF 10 mg/L microalgal treatment in darkness. Sampling days are indicated by lines with varying patterns (Day 1 to 6). Arrows mark peaks corresponding to the standard compound.
Figura 3. Cromatogramas de HPLC-DAD que ilustran la evolución temporal de la biotransformación de CPF por microalgas en condiciones de luz y oscuridad.
Nota. (A) Control sustrato de BBM y CPF bajo condiciones luz sin inóculo microalgal; (B) tratamiento de inóculo microalgal en BBM con 10 mg/L de CPF en condiciones luz; (C) control sustrato de BBM y CPF en oscuridad y sin inóculo microalgal; (D) tratamiento con microalgas en BBM y CPF 10 mg/L en oscuridad. Los días de muestreo se indican mediante líneas con patrones variables (Día 1 a 6). Las flechas marcan los picos correspondientes al compuesto patrón.

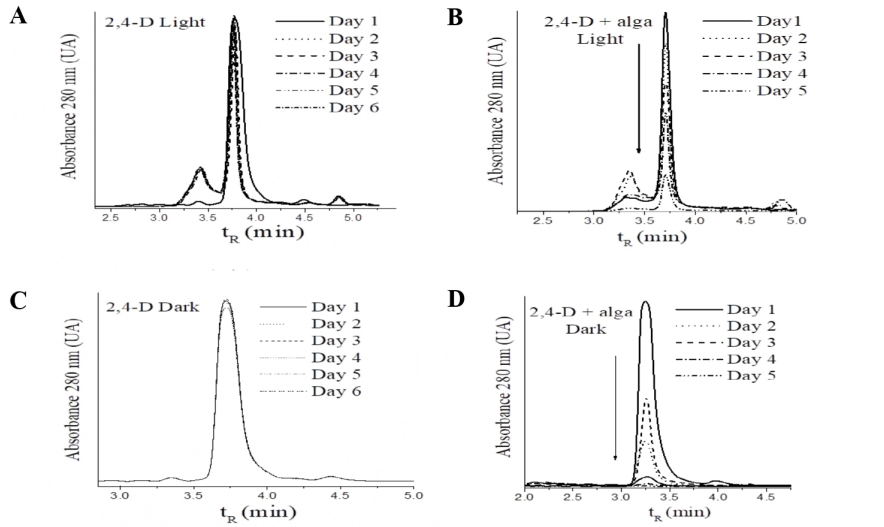

Figure 4. HPLC-DAD chromatograms illustrating the time course of 2,4-DCP biotransformation by microalgae under light and dark conditions.
Note. (A) BBM substrate control with 2,4-DCP under light conditions without algal inoculum; (B) BBM microalgae treatment with 50 mg/L 2,4-DCP under light condition; (C) BBM substrate control with 2,4-DCP under dark; and (D) BBM treatment with microalgal inoculum at 50 mg/L 2,4-DCP under dark conditions. Sampling days are indicated by lines with varying patterns (Day 1 to 6). Arrows mark peaks corresponding to the standard compound.
Figura 4. Cromatogramas de HPLC-DAD que ilustran la evolución temporal de la biotransformación de 2,4-DCP por microalgas en condiciones de luz y oscuridad.
Nota. (A) Control del sustrato BBM con 2,4-DCP en condiciones de luz sin inóculo algal; (B) tratamiento de microalgas en BBM con 50 mg/L de 2,4-DCP en condición de luz; (C) control sustrato BBM con 2,4-DCP en oscuridad; y (D) tratamiento con inóculo microalgal en BBM a 50 mg/L de 2,4-DCP en condiciones de oscuridad. Los días de muestreo se indican mediante líneas con patrones variables (Día 1 a 6). Las flechas marcan los picos correspondientes al compuesto patrón.

2,4-DCP biodegradation

High performance liquid chromatography (HPLC-DAD) was used to test the biocatalytic action of the microalgae with respect to their ability to degrade CPF and 2,4-DCP using analytical tools. Due to the spectroscopic properties of the pesticides, a λmax = 280nm was chosen to follow the decomposition of the pesticides as a function of the days of treatment. The maximum time required for complete biocatalytic decomposition of CPF was four and five days, for the dark-only and light-only conditions respectively (Fig. 3). While for 2,4-DCP, biodegradation occurred at 5 days for both light-only and dark-only conditions (Fig. 4). In addition, as shown in figures 3 and 4, the concentration of CPF (Fig. 3) and 2,4-DCP (Fig. 4) were found to be below the limits of detection and quantification, indicating efficient phycoremediation of pesticides.

DISCUSSION

The effects of organic pollutants on non-target autotrophic species, such as microalgae and cyanobacteria, have been extensively studied (Padhy, 1985; Ramakrishnan *et al*., 2010; Ramakrishnan *et al*., 2011; Venkateswarlu, 1993; Touliabah *et al*., 2022). In this work, the response of *Dictyosphaerium* sp. to exposure to CPF and 2,4-DCP was evaluated, demonstrating their capacity to tolerate high concentrations of these pesticides under different growing conditions. In addition,

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our results showed that *Dictyosphaerium* sp. modifies its metabolism between autotrophic, mixotrophic and heterotrophic conditions, suggesting an adaptive strategy that allows it to persist in polluted environments. This adaptation was also reported in previous studies on the bioremediation potential of microalgae in the elimination of emerging pollutants (Rempel *et al*., 2021). The ability of this species to grow in the presence of these compounds suggests that it could have applications in bioremediation strategies. However, despite these findings, challenges remain in optimizing the conditions for implementation in full-scale treatments (Subashchandranbrose *et al*., 2013). In our study, we assessed and demonstrated for the first time the capacity of *Dictyosphaerium* sp. to remove the pesticides CPF and 2,4-DCP under varying concentrations and lighting conditions. Toxicity effects were evaluated by measuring the growth inhibition rate in pesticide-treated cultures compared to control cultures (Schrader *et al*., 1997; Wong, 2000). Our results pointed out that *Dictyosphaerium* sp. showed differential tolerance to the studied concentrations depending on the lighting conditions. Under a 12-hour cycle (light:dark), *Dictyosphaerium* sp. exhibited a significant growth increase compared to the control group: 52%, 36%, and 12% due to 5, 10, and 20 mg of CPF/L, respectively. However, concentrations exceeding 30 mg of CPF/L demonstrated toxicity, markedly inhibiting algal growth. Chen *et al*. (2016) found a similar decrease in microalgae growth rate with increasing CPF concentration (0 to 100 mg/L) in *Chlorella pyrenoidosa* and *Merismopedia* sp. incubated in a 16:8 light cycle. Under continuous dark conditions, tolerance to CPF manifested differently. On the fourth day of incubation, there was a 7% and 14% growth increase compared to the control for concentrations of 5 and 10 mg/L, respectively. However, concentrations of 20 and 30 mg/L inhibited growth, suggesting a differential adaptation to dark conditions. Fernández *et al*. (2021), propose that heterotrophic microalgae can effectively utilize organic contaminants for nutrition in various wastewater types, thereby treating wastewater efficiently. Under a 24-hour light cycle, growth was only evident at the lowest concentration (5 mg/L), with a 40% increase compared to the control. However, concentrations exceeding 10 mg/L significantly inhibited algal growth, indicating higher sensitivity to continuous light conditions. Excessive illumination can induce physiological changes in algae (Sahinkaya & Dilek, 2009), and high light levels can induce photoinhibition (Smith & Mobley, 2008; Gomes & Juneau, 2017). Asselborn *et al*. (2015) demonstrated that the growth of *Ankistrodesmus gracilis* was inhibited following exposure to various concentrations of CPF under continuous illumination conditions. Detailed exploration of this adaptation concerning illumination could provide valuable insights into the metabolic strategies of *Dictyosphaerium* sp. in the presence of pesticides, with significant implications for bioremediation and understanding its physiological behavior (Hammed *et al*., 2016). This finding opens the door to further research to unravel the underlying mechanisms and optimize practical applications in the field of environmental mitigation. We also investigated the bioremediation potential of *Dictyosphaerium* sp. by measuring growth and compound disappearance of 2,4-dichlorophenol (2,4-DCP) in the culture medium. We observed differential microalgae growth under light and dark conditions when exposed to 2,4-DCP, suggesting a sensitive response to this compound. Under light exposure at concentrations of 70 mg/L of 2,4-DCP, significant algal growth inhibition was observed from the second day until the end of the bioassay. This indicates that high concentrations of 2,4-DCP are detrimental to this strain under light conditions, which may have implications for ecosystems exposed to herbicides. While the concentrations studied are higher than those found in the field, further study of aquatic organisms particularly exposed to pesticide contamination is crucial. Additionally, it is known that excessive illumination leads to physiological changes (Sahinkaya & Dilek, 2009) or inhibition of respiration, resulting in lower 2,4-DCP removal (Li *et al*., 2018). Conversely, when cultures were incubated in darkness, a different response pattern was observed. In the

absence of 2,4-DCP, no growth was detected at any time, suggesting a dependence on light for the microalgae's development under normal conditions. However, in the presence of 2,4-DCP at concentrations of 50 and 70 mg/L, an increase in algal density was observed from the first day, reaching absorbance values higher than those of the control culture. Li *et al*. (2018) studied the biodegradation mechanism of 2,4-DCP by *Chlorella pyrenoidosa* and found a stimulatory effect of the compound on algal growth. Furthermore, they verified that heterotrophic assimilation under limited carbon sources was the premise for the biodegradation of 2,4-DCP in a closed system, obtaining at the end of the culture period, an undetectable residue of 2,4-DCP in the algae indicating complete biodegradation. According to our results, dark conditions cause the biocatalytic process to intensify probably due to the decrease of inorganic CO_2 produced during photosynthesis under light conditions. This finding is interesting as it suggests that *Dictyosphaerium* sp. could be using 2,4-DCP as a carbon source under low light conditions, which could have implications for the herbicide's biodegradation in certain aquatic environments. Wong (2000) studied the effect of 2,4-D, glyphosate, and paraquat on the growth of *Scenedesmus quadricauda*. This author showed that low concentrations of 2,4-D (0.02 mg/L) stimulated the growth rate, while exposing the strain to 20 mg/L of 2,4-D significantly inhibited algal growth. However, Moreira *et al.* (2020) report inhibition of the population growth on green alga *Raphidocelis subcapitata* following exposure to concentrations between 0.03 and 0.5 mg/L of 2,4-D for 96 h. In this context, our results highlighted that algae show different sensitivity to the same pesticide, underscoring the importance of phenotypic selection of microalgae resistant to specific environmental conditions. Also, it has been reported that 2,4-D can stimulate the growth of plant cells (Morré *et al.*, 1984), and other authors have pointed out that in herbicides such as glyphosate a stimulation occurs which may be due to its use as a carbon or nitrogen source for algal growth (Malik *et al*., 1989; Maršálek & Rojíčková, 1996). Also, our results can be supported by the results provided by Li *et al*. (2018) who found that exposing a domesticated strain to concentrations below 0.2 mg/L of chlorophenol resulted in nearly 100% removal employing *Chlorella pyrenoidosa* to phycoremediation. Additionally, when exposed to continuous darkness, algae obtain energy from 2,4-DCP biodegradation via the heterotrophic pathway, using it to provide a small increase in algal biomass from 34.9 to 49.4 mg/L and chlorophyll-*a* content from 0.275 to 0.533 mg/L, achieving a 40% compound removal. Another study made by Gao and Tam (2011) also concluded that phenolic compound removal was positively related to photosynthetic and metabolic activities. On the other hand, degradation products of the insecticide were observed; according to the literature, these pesticides degrade into less polar compounds than the starting products, which favors detoxification in non-target organisms (Kurade *et al*., 2016). It is important to mention that, the use of this strain would help the effective phycoremediation of these pollutants of regional interest considering the concentrations detected in surface water of the region. In summary, it can be observed that microalgae could reduce their growth under dark conditions, while favoring the degradation of the compound. Finally, the study results show that *Dictyosphaerium* sp. can exert remediation of important pesticides present in the South American environment and also exhibits differential responses to 2,4-DCP depending on light conditions and compound concentration. Also, expected environmental concentrations may not inhibit the microalgae growth rate, as algae are effective in detoxifying pesticides when present in microgram amounts in the water and soil environment (Mukherjee *et al*., 2004). This is important as *Dictyosphaerium* sp. could be proposed as a bioindicator species for phycoremediation process. These findings underscore the importance of understanding the ecology and physiology of microalgae in relation to herbicide contamination, as well as their potential role in the bioremediation of these compounds in the aquatic environment. This work represents the first approach

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to pesticide degradation using microalgae specifically adapted to contaminated environmental conditions. However, future research should focus on elucidating the molecular mechanisms of toxicity, absorption, hydrolysis, biotransformation and metabolization processes employed by *Dictyosphaerium* sp. and thus improve understanding and accelerate phycoremediation studies.

CONCLUSIONS

The use of microalgae is an efficient and cost-effective technique compared to other conventional techniques for environmental remediation and the production of valuable products. Based on the results obtained, it is concluded that in both cultivation conditions, the studied microalga is capable of biodegrading the compounds, but in the heterotrophic cultivation condition, their disappearance occurs more rapidly. Therefore, the strain could be used for the bioremediation of effluents contaminated with organophosphate compounds. It is also interesting to note that this strain obtained could be an undescribed species, and there are very few studies of this type conducted with *Dictyosphaerium* sp. The strain studied was very efficient in degrading the pesticides, without generating other degradation compounds at appreciable concentrations.

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CRediT Contribution Statement

A.M.R. Conceptualization, data curation, formal analysis, investigation, methodology, software, validation, writing-original-draft, writing-review-editing. **M.F.** data curation, funding acquisition, methodology, resources, supervision, visualization, writing-original-draft. D.B. Conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, visualization, writing-original-draft. J.M.P-I. Conceptualization, data curation, formal analysis, investigation, methodology, software, validation, writing-original-draft, writing-review-editing S.M.D.S. Conceptualization, funding acquisition, investigation, project administration, resources, supervision, visualization, writing-original-draft, writing-review-editing.

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