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Influence of microplastics on the toxicity of chlorpyrifos and mercury on the marine microalgae *Rhodomonas lens*

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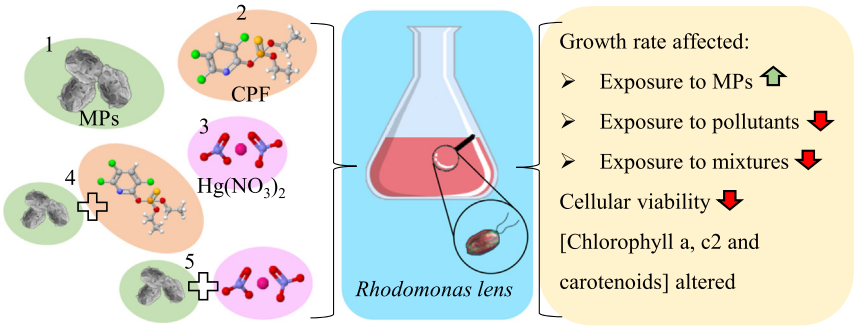
HIGHLIGHTS

- Microplastic's (MPs) ability to act as vectors of pollutants has received special attention.
- Lethal and sublethal responses of *Rhodomonas lens* to polyethylene MPs were evaluated.
- MPs affect cell viability, population growth and pigment content of *R. lens*.
- MPs modulate the toxicity of chlorpyrifos and mercury to *R. lens*.

GRAPHICAL ABSTRACT



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ABSTRACT

The growing use of plastics, including microplastics (MPs), has enhanced their potential release into aquatic environments, where microalgae represent the basis of food webs. Due to their physicochemical properties, MPs may act as carriers of organic and inorganic pollutants. The present study aimed to determine the toxicity of polyethylene MPs (plain and oxidized) and the model pollutants chlorpyrifos (CPF) and mercury (Hg) on the red microalgae *Rhodomonas lens*, to contribute to the understanding of the effects of MPs and associated pollutants on marine ecosystems, including the role of MPs as vectors of potentially harmful pollutants to marine food webs. *R. lens* cultures were exposed to MPs (1–1000 μg/L; 25–24,750 particles/mL), CPF (1–4900 μg/L), Hg (1–500 μg/L), and to CPF- and Hg-loaded MPs, for 96 h. Average specific growth rate (ASGR, day^{-1}), cellular viability and pigment concentration (chlorophyll *a*, c2 and carotenoids) were measured at 48 and 96 h. No significant effects were observed on the growth pattern of the microalgae after 96-h exposure to plain and oxidized MPs. However, a significant increase in cell concentration was detected after 48-h exposure to plain MPs. A decrease of the ASGR was noticed after exposure to CPF, Hg and to CPF/Hg-loaded MPs, whereas viability was affected by exposure to MPs, CPF and Hg, alone and in combination. Chlorophyll a and c2 significantly decreased when microalgae were exposed to plain MPs and CPF, while both pigments significantly increased when exposed to CPF-loaded MPs. Similarly, chlorophyll and carotenoids content significantly decreased after exposure to Hg, whereas a significant increase in chlorophyll a was observed after 48-h exposure to Hg-loaded MPs, at the higher tested concentration. Overall, the presence of MPs modulates the toxicity of Hg and CPF to these microalgae, decreasing the toxic effects on *R. lens*, probably due to a lower bioavailability of the contaminants.

1. Introduction

Plastics are among the most widely used synthetic materials. Global plastic production has continuously increased during the last decades, reaching 367M tons/year in 2020 and, as a consequence, plastics have

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been recognized as an environmental threat (PlasticsEurope, 2021). Plastics are formed by several synthetic organic polymers of high molecular weight and long chain, usually based on hydrocarbons (Law, 2017). Due to their physicochemical properties (chemical and corrosion resistance, ductility or hardness) and potential uses (packaging, consumer products, textiles or construction), many types of plastic polymers have been produced, with polyethylene (PE) being the most produced polymer and the one that most commonly ends up in the aquatic environment (Andrady, 2011). The impact of large plastic debris on marine ecosystems has been widely studied; however, in recent years, the distribution, abundance and effects of the microscopic fraction of plastics have generated immense interest in the scientific community (Shruti et al., 2021).

Synthetic particles with sizes between 1 μm and 5 mm are called microplastics (MPs) (Frias and Nash, 2019). The concentration of MPs in the water column ranges from 0.00085 particles/m^3 in Australian waters (Reisser et al., 2013) and 0.001 particles/m^3 in the neustonic layer of the Atlantic Ocean (Law, 2010), to 1,720,000 particles/km^2 in East Asian waters (Isobe et al., 2015) or 102,000 particles/m^3 in Swedish waters (OSPAR, 2009). The accumulation of MPs is usually higher in coastal areas, especially close to urbanized and/or industrialized areas, and in the center of the oceanic gyres (Wright et al., 2013). Once in the aquatic environment, MPs have been reported to alter biological responses from the subcellular to the population level (Galloway and Lewis, 2016). These particles can penetrate and eventually accumulate in the tissues of marine organisms, which may disrupt the digestive system, alter feeding behavior, increase antioxidant responses due to generation of oxidative stress and affect growth and/or reproduction (Gola et al., 2021; Mkuye et al., 2022; Pérez-Aragón et al., 2022). Alterations in growth, viability, photosynthetic and enzymatic activity, among other effects, have been reported in primary producers after MPs exposure (Davarpanah and Guilhermino, 2015; Yokota et al., 2017; Zhang et al., 2017). Phytoplankton represents the base of marine food webs, therefore the effects of MPs on these organisms may cause a knock-on effect on marine ecosystems. However, controversial effects have been described in microalgae exposed to MPs, which are greatly dependent on the studied species, on the type, size and concentration of MPs and on the experimental conditions, such as exposure times (Bai et al., 2021; Koelmans et al., 2022; Wan et al., 2018). In this regard, the cryptophycean (flagellated) algae *Rhodomonas lens* is considered a relevant species since it is widely used in aquaculture and constitutes a great source of nutrients for marine invertebrates (Seixas et al., 2009; Strathman, 2014). Chlorophyll a, chlorophyll *c*2, phycoerythrin and carotenoids are the main pigments in this species (Gantt et al., 1971; Becker et al., 1998). The genus *Rhodomonas* does not present a cell wall; instead, the cells are surrounded by a periplast consisting of thin plates below the plasma membrane (Thoisen et al., 2017).

Several studies had focused on the ability of plastics to transfer potentially harmful chemicals to organisms, both by leaching plastic additives and adsorbing pollutants from the surrounding environment, enhancing their bioavailability and accumulation in organisms, and generating the so-called "Trojan horse effect" (Koelmans et al., 2013; Avio et al., 2017; Da Costa Araújo et al., 2023; da Costa Araújo et al., 2022). Results of these studies have indicated that MPs may either increase or decrease the availability of environmental pollutants to marine biota (Davarpanah and Guilhermino, 2015; Beiras and Tato, 2019; Garrido et al., 2019; Bellas and Gil, 2020; Cheng et al., 2021; Fernández et al., 2020). Therefore, more research is needed on this topic.

Chlorpyrifos [O, O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, CPF] is a widespread chlorinated organophosphorus pesticide extensively used in pest control due to its easy accessibility, low price, and short-moderate persistence in water (half-life of 16–81 days, depending on the pH) in comparison to other organophosphorus insecticides (Asselborn et al., 2015; John and Shaike, 2015; Bellas and Gil, 2020). CPF has been applied worldwide in the management of a wide variety of pests, controlling several larval and adult forms of insects and mites, and its potential to control arthropod pests, including widely distributed species such as the mosquitoes *Culex pipiens* and *Ochlerotatus caspius*, or the termite *Odontotermes obesus*, has made it one of the most efficient insecticides (Giesy and Solomon, 2014; Mohammed et al., 2022; Venkateswara Rao et al., 2005). Due to its widespread use, this pesticide has been detected in water samples from many parts of the world (Huang et al., 2020). In fact, CPF is the second most detected pesticide in water and food (John and Shaike, 2015), and has also been found to be adsorbed (and potentially desorbed) to plastic and sediment particles (Gebremariam et al., 2012; León et al., 2018). Concentrations of CPF in water vary from non-detected to 37.3 μg/L (Hasanuzzaman et al., 2018). Even though CPF is considered non-phytotoxic at insecticidal concentrations, negative effects of CPF have been reported on the germination and morphological traits of plants, with proved effects on white mustard (*Sinapis alba*) at exposure concentrations from 0.1 μg/L (Gvozdenac et al., 2015; John and Shaike, 2015). Also effects on the growth, ultrastructure, pigment content, and enzymatic activity of microalgae have been detected (Asselborn et al., 2015; Chen et al., 2016; Echeverri-Jaramillo et al., 2020; Garrido et al., 2019), showing deleterious effects on *Dunaliella tertiolecta* from concentrations as low as 0.6 mg CPF/L (DeLorenzo and Serrano, 2003). The use of this pesticide has been regulated by the public bodies of 83 countries (Li, 2018), due to its known neurotoxic and endocrine effects in vertebrates, including humans (Rahman et al., 2021), being classified as a priority substance in the Water Framework Directive (Directive, 2000/60/EC).

Mercury (Hg) is a heavy metal that occurs naturally at low concentrations in the environment (Tchounwou et al., 2012). However, due to its massive and continued industrial use, as well as its applications in agriculture, its concentration on biotic and abiotic compartments has increased in certain areas to alarming levels, reaching concentrations up to 27 μg/L in coastal waters (Nasfi, 1995). It is encountered in nature in three forms (elemental, inorganic, and organic). Its most commonly found organic form in the environment, methylmercury (MeHg), is considered a highly toxic contaminant to aquatic organisms due to its potent neurotoxic action (Gworek et al., 2016). In fact, Hg has been labeled as a highly toxic substance for aquatic life by the European Chemicals Agency (ECHA, 2022). Some of the effects detected on birds (e.g. *Gavia immer*), mammals (e.g. *Lontra canadensis*) and fish (e.g. *Pimephales promelas*) include behavioral, hormonal, reproductive and neurochemical changes, due to the exposure in the wild to this metal, at concentrations of Hg in prey of 0.3 μg/g–3.93 μg, (Scheuhammer et al., 2007).

The main objective of the present research was to study the toxicity of polyethylene MPs, Hg and CPF, alone and in combination, to *R. lens*, in order to contribute to the understanding of the impact of MPs in the aquatic environment. Specific objectives included the study of the possible role of MPs as vectors of potentially harmful pollutants and the joint toxicity of MP and Hg/CPF to marine organisms. Therefore, the two main hypotheses of this study were: first, that MP are capable of causing toxicity to microalgae, and second, that MPs are capable of acting as carriers of metals and pesticides, being able to modulate their toxicity.

2. Materials and methods

2.1. Microalgae strains

Strains of the unicellular marine microalgae *Rhodomonas lens* (Pascher & Ruttner in Pascher, 1913) were chosen as the phytoplanktonic model organism to perform the exposure experiments. Microalgae were obtained from the ECIMAT Culture Collection (ECC), at the Centro de Investigación Mariña-Universidade de Vigo, and were grown in Walne medium enriched with vitamins (Walne, 1966).

2.2. Experimental procedure

2.2.1. Experimental solutions and suspensions

High density polyethylene (HDPE) microparticles were chosen as the model microplastics based on literature and on previous findings from our research group (Fernández et al., 2020; Rivera-Hernández et al., 2019). Two types of PE MPs, purchased from MicroPowders Inc. were used. MPP 635-XF is a micronized powder with a size range of 1.4–42 μm

(2–10 μm represent 99.9 % of the total particle distribution in number, and 92 % in volume) and a mean particle size of 7.73 μm (Garrido et al., 2019). An oxidized high-density polyethylene (HDPE), Aquatex 325, was also selected since it has been reported to have a higher affinity for metals than plain MPs (Fernández et al., 2020; Rivera-Hernández et al., 2019). According to the manufacturer, this product is uniformly produced with a mean particle size in the 10–15 μm range, reaching a maximum particle size of 44 μm. It exhibits a melting point of 135–140 °C and a density of 0.99 g cm^3 (25 °C).

Microplastics, CPF and Hg were tested individually and in MP-pollutant mixtures. MPs suspensions were prepared by addition of 1 mg of micronized powder in 1 L of filtered seawater (FSW) and were subsequently shaken for 20 min in an ultrasonic bath to achieve a homogeneous suspension. The non-ionic surfactant polyoxyethylene sorbitan monolaurate (Tween 20, Fluka Chemika, Switzerland) was employed at a concentration of 17.5 μg tween/mg MP to smooth the diffusion of the microparticles into the water column. MPs bioassays involved the exposure of *R. lens* to a range of concentrations: 1, 10, 100 and 1000 μg/L (equivalent to 25, 248, 2475, 24,750 particles/mL for MPP 635-XF and to 4, 42, 424, 4242 part/mL for Aquatex 325, based on experimental measurements with a Multisizer® 3 Coulter Counter, Beckman Coulter, USA). In addition to the control group, a dispersant control was included at the same concentration as in the highest MPs treatments.

Analytical grade (99.9 %) CPF was purchased from HPC Standards GmbH, Germany. Since this pesticide is poorly soluble in water (1.4 mg/L at 25 °C), acetone was used as a carrier to prepare a 5 mg/L CPF stock solution. Experimental treatments were prepared by diluting the stock solution in FSW, using a maximum concentration of 100 μL acetone/L. In addition to the control group, an acetone solvent control (0.0001 % (*v*/v)) was included. CPF experimental concentrations (1, 10, 100, 500, 1000, 1700, 2900 and 4900 μg/L) were selected on the basis of the previous work of the research group and on data from literature (Asselborn et al., 2015; Chen et al., 2016; Echeverri-Jaramillo et al., 2020). Chemical analyses carried out by our research group indicated actual concentrations of CPF between 74 % and 105 % of nominal concentrations, with recoveries of 90.1 ± 5.7 % (Garrido et al., 2019).

Mercury (II) nitrate ($Hg(NO_3)_2$, Panreac, p. a. C 99.0 %, water solubility 5–10 g/L at 22 °C) was used for experiments. A standard solution of Hg (1 g/L) was diluted in FSW in order to obtain the experimental concentrations selected: 1, 50, 100, 250 and 500 μg/L, on the basis of literature data (Gómez-Jacinto et al., 2015; Horvatić et al., 2007; Juneau et al., 2001; Rodrigues et al., 2013; Wu and Wang, 2011; Zamani-Ahmadmahmoodi et al., 2020). Chemical analyses carried out by our research group indicated actual concentrations of Hg between 72 % and 101 % of nominal concentrations, with recoveries of 88.1 ± 10.2 %.

MPP 635-XF MPs suspensions were loaded with CPF (1 mg MPs/L + 1 mg CPF/L) for 2 h in an orbital shaker at 18 °C, to ensure maximum sorption (i.e., concentrations between the solid MPs phase and liquid contaminant phase are constant and the equilibrium is reached) of the CPF to the MPs (Albentosa et al., 2017). Aquatex 325 oxidized MPs suspensions were loaded with Hg (200 μg MPs/L + 200 μg Hg/L) for 168 h in an orbital shaker at 18 °C (Rivera-Hernández et al., 2019; Fernández et al., 2020). After incubation, the pollutant-loaded MPs suspensions were submitted to an ultrasonic bath for 20 min to warrant a homogeneous suspension. Experimental concentrations of CPF-loaded and Hg-loaded MPs were 100, 250, 500, 750, 1000 μg CPF/L and 50, 100, 150 and 200 μg Hg/L. These range of concentrations were chosen in order to cover the median effective concentrations (EC50) of CPF and Hg, i.e., the concentrations causing 50 % cell growth inhibition of *R. lens*. According to recent studies from our group, >70 % of the added CPF was adsorbed to the MPs (Garrido et al., 2019), whereas the concentration of Hg loaded in MPs was equivalent to 12.52 % (Rivera-Hernández et al., 2019; Fernández et al., 2020).

2.2.2. Microalgae bioassays

Exposure experiments were conducted according to the Organization for Economic Cooperation and Development (OECD) N°. 201 guideline (OECD, 2011). All bioassays were started with an initial *R. lens* density of 10×10^4 cells/mL. The cultures were maintained in exponential growth phase for 96 h in 100 mL glass Erlenmeyer flasks at 20 ± 2 °C, with a salinity of 36 PSU, under continuous illumination (64 μmol/sm^2) and orbital shaking (90 rpm). All material was washed with HCl (10 % vol) and sterilized at a temperature of 180 °C for 2 h before use, in order to avoid any biological or chemical contamination. The seawater used for the bioassays was filtered through a glass fibber 142 mm prefilter (13400-142K, Sartorius, Germany, Europe) and a 0.2 μm cellulose acetate membrane filter (11107-142G, Sartorius, Germany, Europe), and autoclaved prior to use. Each concentration was tested in triplicate. Appropriate movement and shape of the microalgae in the control flasks and pH in all flasks were checked daily.

2.3. Analytical procedures

2.3.1. Cell density

Cell density was measured daily using a PAMAS particle counter (PAMAS Partikelmess-und analysesysteme GMBH, Germany). Aliquots of 10 mL were taken from the experimental flasks inside a laminar flow chamber and added to 15 mL vials. Aliquots were diluted in FSW, when necessary, to be under the maximum quantification threshold (3.36–10.25 μm) of the particle counter and ensure a reliable result. Average specific growth rates (ASGR) were calculated as stablished in the OECD 201 guideline (OECD, 2011) and expressed as the logarithm of the cell number increase per day during the 96-h test period.

2.3.2. Cell viability

Cell viability was assessed after 48 and 96 h of incubation using the fluorescent probe Propidium Iodide (PI, Invitrogen, ThermoFisher Scientific, Germany, Europe), as previously described in Nogueira et al. (2015). In brief, 6 mL samples were taken from the Erlenmeyer bioassay flasks and incubated with PI at 10 μM for 15 min, in darkness. After the incubation period, cell viability was estimated from PI fluorescence at an excitation wavelength of 535 nm and an emission wavelength of 617 nm, using a microplate reader (Tecan Infinite® 200 PRO, Männedorf, Switzerland). The fluorescence obtained from the samples exposed to PI was corrected with the autofluorescence of each corresponding sample. For cell viability calculations, control values were taken as 100 % of viable cells.

2.3.3. Pigment content

The contents of chlorophyll *a* (Chla), c2 (Chlc2) and carotenoids were determined at 48 and 96 h, following the aqueous acetone extraction method of Yu et al. (2017), with some modifications. Briefly, a sample of 10 mL was pipetted from the bioassay flasks and was centrifuged at 20 °C for 10 min at 3000 rpm. After removing the supernatant, the pellet was resuspended twice in 10 mL of distilled water in order to eliminate salts. Subsequently, 1.5 mL of 90 % acetone was added to the obtained pellet, followed by homogenization in an ultrasonic water bath for 5 min. The homogenized sample was then incubated in a water bath for 30 min at 50 °C and centrifuged again at 20 °C for 10 min at 3000 rpm. This procedure was carried out in darkness to avoid excitation of the chlorophyll molecules.

The samples were allowed to stand for a few minutes before starting the measurements. The absorbance values of the samples were measured at several wavelengths (480, 664, 630 and 750 nm) using a microplate reader (Epoch, Biotek instruments Inc., USA). The concentration of Chla and Chlc2 was computed following the equations proposed by Humphrey (1979) for cryptomonads in a 90 % aqueous acetone solution, while the total carotenoid content was obtained with the equation described by Strickland and Parsons (1972). The three equations were modified by the method suggested by Warren (2008) to adapt the trichromatic equations to microplate measurements. The values obtained were divided by the cell density (cells/mL) in each experimental flask.

2.4. Statistical analysis

The experimental data were processed using the SPSS® version 23.0 software package. Data sets were checked for normal distribution

(Shapiro-Wilk test) and homogeneity of variances (Lévene test). After verifying the parametric assumptions, one-way ANOVA and Dunnett post hoc test were applied in order to determine significant differences between treatments and to determine lowest observed effect concentrations (LOEC) and no observed effect concentrations (NOEC). A Two-way ANOVA followed by Bonferroni post hoc test was performed to evaluate the effect of 'time' and 'treatment' factors. When data did not meet normality or homoscedasticity assumptions, the Dunnett T3 test was used. All statistical analyses were based on a 0.05 significance level. Growth inhibition EC50s were calculated according to the Probit method.

3. Results

3.1. Effects of MPs

Rhodomonas lens population growth was affected by exposure to plain (MPP 635-XF) MPs (Fig. 1A). According to the two-way mixed ANOVA, significant effect of 'time' ($p = 0.000$) and 'concentration' ($p = 0.006$) and a significant interaction between both factors ($p = 0.000$) was observed. One-way ANOVA showed a significant increase in cell growth at 48 h of exposure ($p = 0.000$) and post hoc tests indicated a significant increase in all tested concentrations with respect to both control and Tween-control (Fig. 1B). This effect was not detected at 72 or at 96 h, when no significant differences with respect to controls were observed at any of the concentrations tested. No significant effects were noted on ASGR after 96-h exposure of *R. lens* to plain (Fig. 2A, Table S1) or oxidized (Aquatex 325) MPs (Fig. 2B, Table S1) at the tested concentrations. There were also no significant differences between the control and the Tween-control.

A significant decrease in cell viability was detected in microalgae exposed for 48 h to 10, 100 and 1000 μg/L of plain MPs, with a reduction with respect to control cultures of 17, 14 and 10 % respectively, and in microalgae exposed for 96 h at the highest concentrations tested (1000 μg/L), with a 29 % viability decrease (Fig. 3A, Table S1). A significant decline in cell viability was also observed in microalgae exposed for 48 h to 1000 μg/L of oxidized MPs, with a reduction of 24 % with respect to controls (Fig. 3B, Table S1). Significant effects on cell viability were noted in microalgae exposed for 96 h at all tested concentrations, with reductions of 15, 12, 16 and 17 % at 1, 10, 100 and 1000 μg/L of oxidized MPs. Tween did not affect cell viability.

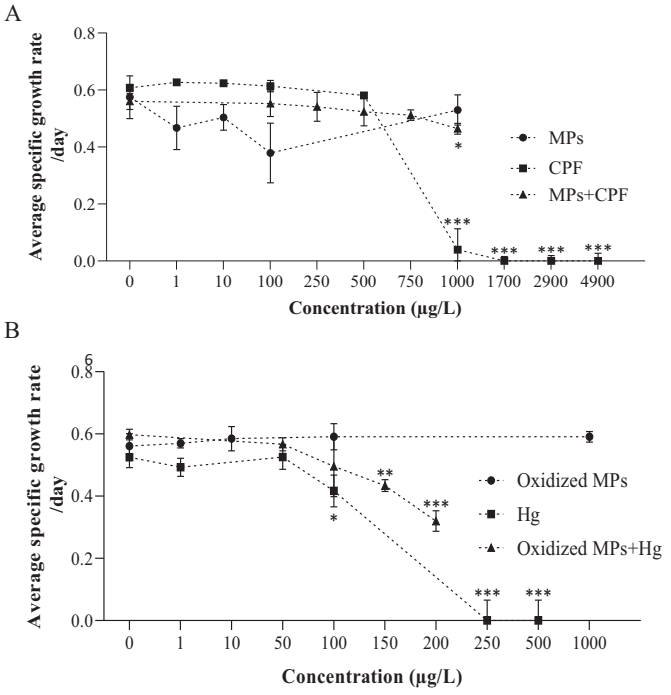

Fig. 2. A. Average specific growth rate per day after 96 h exposure to plain MPs (circle) and CPF (square) alone and in combination (triangle). B. Average specific growth rate per day after 96 h exposure to oxidized MPs (circle) and Hg (square) alone and in combination (triangle). n = 3; error bars represent standard deviations; *, ** and *** designate statistically significant differences ($p < 0.05$, 0.01 and 0.001).

Exposure to plain MPs also significantly decreased cellular concentrations of Chla and Chlc2 in *R. lens* populations, at both 48 and 96 h (Fig. 4A, B, Table S1). Moreover, the two-way mixed ANOVA test showed a significant effect of 'time' ($p = 0.001$) and 'concentration' ($p = 0.033$) in Chlc2 (Table S2).

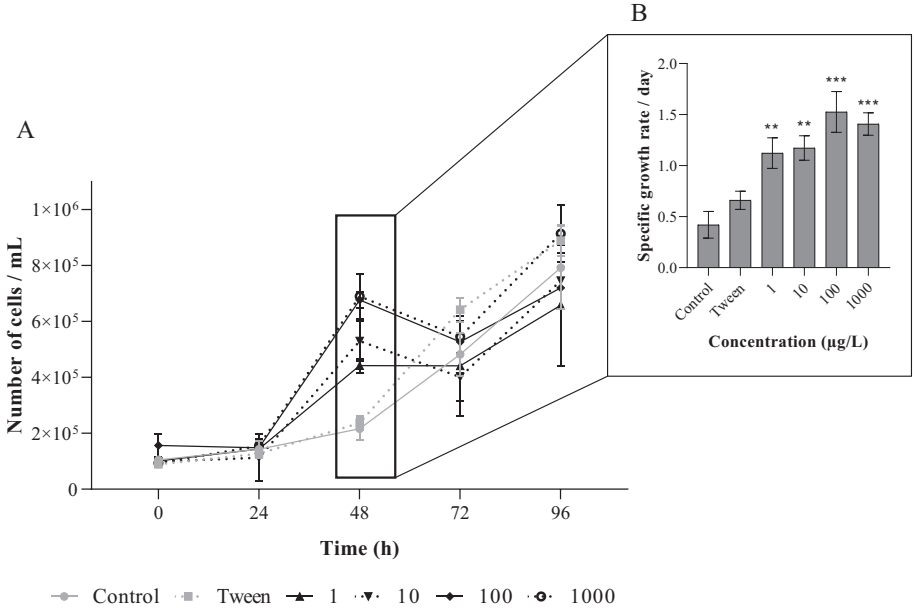

Fig. 1. Effects of plain MPs on *Rhodomonas lens*. A. Number of cells/mL per treatment at 24, 48, 72 and 96 h exposure. B. Specific growth rate per day per treatment at 48 h exposure. $n = 3$; error bars represent standard deviations; ** and *** designate statistically significant differences from the control group (one-way ANOVA and Dunnett's test, $p < 0.01$ and 0.001, respectively).

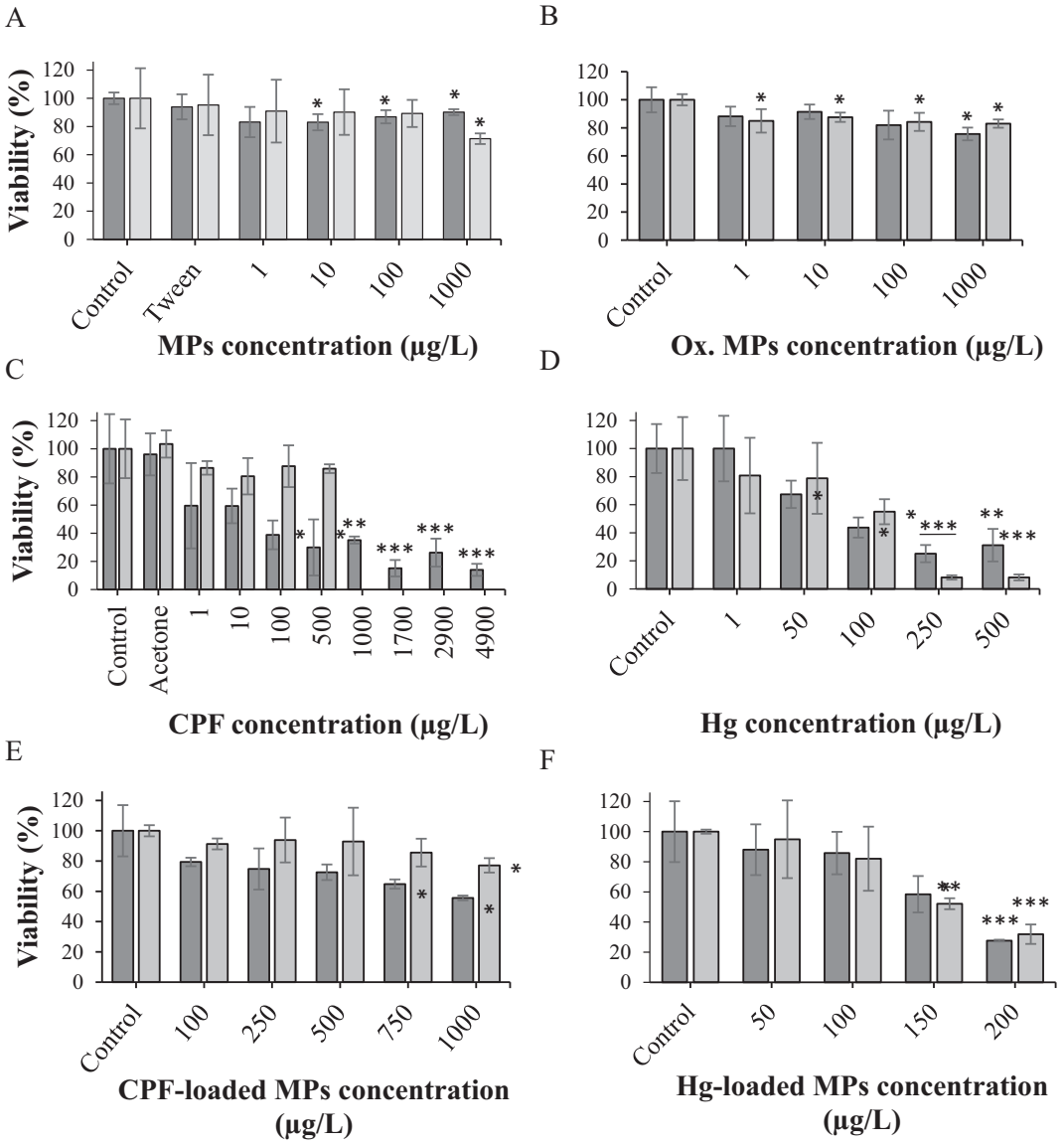

Fig. 3. Cell viability after 48 h (dark grey) and 96 h (light grey) of exposure to plain MPs (A), oxidized MPs (B), CPF (C), Hg (D), CPF-loaded MPs (CPF concentration represented, E) and Hg-loaded MPs (Hg concentration represented, F). n = 3; error bars represent standard deviations; *p < 0.05, **p < 0.01, ***p < 0.001.

No significant effects on Chla, Chlc2 or carotenoids were detected after exposure to oxidized MPs (data not shown). Pigment concentrations of the microalgae were not affected by Tween.

3.2. Effects of CPF and Hg

Chlorpyrifos caused a significant decrease in ASGR above 1000 µg CPF/L at 96 h (Fig. 2A). Total inhibition of microalgae growth was noticed at concentrations above 1700 µg CPF/L at 24 h (data not shown). The NOEC, LOEC and EC50 of CPF at 96 h were 100, 500 and 708.39 µg/L, respectively (Table 1). No significant effects of acetone on cell growth were observed.

Mercury significantly inhibited the microalgae ASGR at concentrations higher than 100 µg Hg/L (Fig. 2B, Table S1). The ASGR was 0.417 day^{-1} at 100 µg/L, which represents a 20 % decrease with respect to the control. A total inhibition of the population growth was found at concentrations higher than 250 µg/L at 24 h (data not shown). The NOEC, LOEC and EC50 values at 96 h were 50, 100 and 132 µg Hg/L, respectively (Table 1).

A significant decrease of cell viability was detected in microalgae exposed to CPF concentrations higher than 10 µg/L at 48 h of exposure (Fig. 3C, Table S1). Viability percentages were 39, 30, 35, 15, 26 and 14 %, at 100, 500, 1000, 1700, 2900 and 4900 µg/L, respectively. A complete inhibition of cell viability was observed at CPF concentrations higher than 1000 µg/L, at 96 h. Cell viability was also affected by exposure to Hg at both 48 and 96 h exposure (Fig. 3D, Table S1). A concentration-dependent decrease was found and cell viability showed minimum values (8 % of viable cells) at the two highest tested concentrations (250 and 500 µg/L) at 96 h. Acetone did not show effects on cell viability.

A significant reduction in Chla content was observed at 1700 µg CPF/L and also for Chlc2 at 4900 µg CPF/L at 48 h (Fig. 4C, Table S1), whereas significant differences at 96 h were only detected in Chla content at 4900 µg CPF/L (Fig. 4D, Table S1). Two-way ANOVA also identified a significant effect of 'time' and a significant interaction between both factors on the two pigments tested (Table S2). Pigment contents were affected by exposure to the different concentrations of Hg at 48 and 96 h. At 48 h of exposure Chla and carotenoids contents significantly increased with respect to the control group at 1 µg Hg/L, while no effect was noted on pigment contents at 50 and 100 µg Hg/L (Fig. 4E). Chla, Chlc2 and carotenoids contents were significantly decreased at the highest tested concentrations, 250 and 500 µg Hg/L

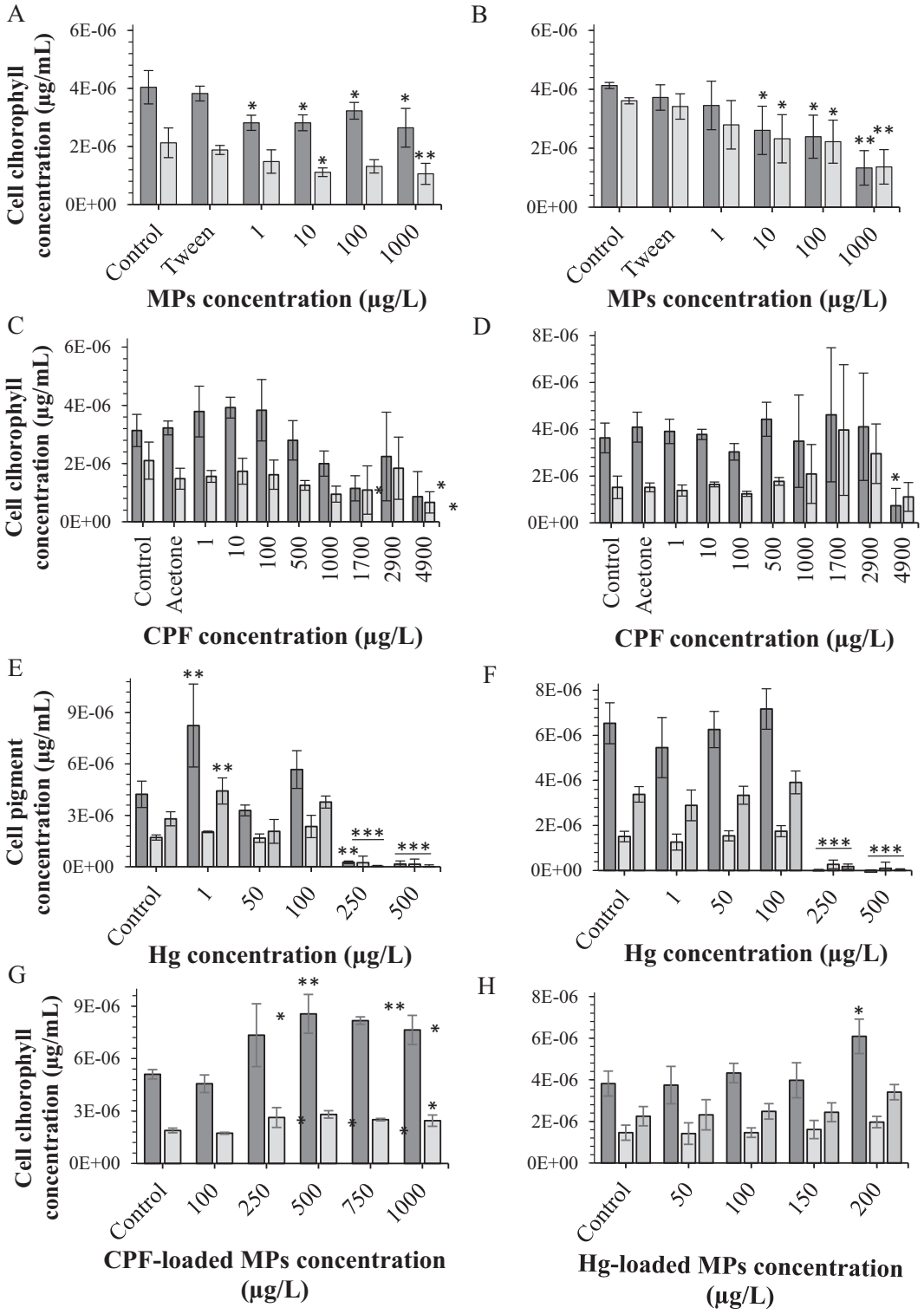

Fig. 4. Chlorophyll a (dark grey), c2 (light grey) and carotenoids (medium grey) after exposure to plain MPs at 48 h (A) and 96 h (B), CPF at 48 h (C) and 96 h (D), Hg at 48 h (E) and 96 h (F), CPF-loaded MPs at 96 h (CPF concentration represented, G) and Hg-loaded MPs at 48 h (Hg concentration represented, H). n = 3; error bars represent standard deviations; *p < 0.05, **p < 0.01, ***p < 0.001.

(Table S1). At 96 h, a significant decrease on pigment contents was only observed at the two highest tested concentrations (Fig. 4F, Table S1). The two-way mixed ANOVA test performed showed a significant effect of 'time' ($p = 0.015$) in Chlc2 of cells exposed to Hg, while 'concentration' and the interaction between both factors was significant in all pigments tested (Table S2).

3.3. Effects of CPF- and Hg-loaded MPs

Chlorpyrifos-loaded MPs caused a slight inhibition on ASGR at 1000 µg/L (Fig. 2A, Table S1), corresponding to a 17 % decrease with respect to the controls. The NOEC and LOEC values of the CPF-loaded MPs were 750 and 1000 µg/L, respectively (Table 1).

Table 1
NOEC, LOEC and EC50 values (μg/L) on the average specific growth rate of *R. lens* after 96 h of exposure to the different treatments. Results come from the Analysis of Variance (ANOVA) and Probit.

Pollutant	NOEC	LOEC	EC50
MPs	1000	–	–
CPF	100	500	708.390 (699.868–790.208)
CPF + MPs	750	1000	–
Oxidized MPs	1000	–	–
Hg	50	100	131.923 (84.072–283.888)
Hg + oxidized MPs	100	150	204.985 (188.350–228.528)

Mercury-loaded MPs inhibited the ASGR at 150 and 200 μg/L. The ASGR was 0.302 day^{-1} at 200 μg Hg-loaded MPs/L, representing almost a 50 % decrease with respect to the control (Fig. 2B, Table S1). The NOEC and LOEC values at 96 h were 100 and 150 μg/L, respectively (Table 1).

Cell viability was significantly reduced at 750 and 1000 μg CPF-loaded MPs/L at 48 h, reaching a 36 and 45 % decrease with respect to controls, respectively (Table S1). At 96 h a significant decline in cell viability was only detected at the highest tested concentration (1000 μg/L), reaching a 35 % decrease with respect to controls (Fig. 3E, Table S1). Hg-loaded MPs caused a concentration-dependent reduction in the percentage of viable cells. Significant inhibition in viability was observed at 150 and 200 μg Hg-loaded MPs/L, at both 48 and 96 h (Table S1). The percentages of viable cells at 200 μg Hg-loaded MPs/L were 28 % and 32 %, at 48 and 96 h, respectively (Fig. 3F).

Pigment contents were significantly affected by CPF-loaded MPs only at 96 h exposure (Table S1). Both, Chla and Chlc2 contents were significantly increased at concentrations higher than 100 μg CPF-loaded MPs/L (Fig. 4G), reaching maximum values of 8.56E−06 μg Chla/mL and 2.81E−06 Chlc2/mL per cell at 500 μg CPF-loaded MPs/L. The two-way ANOVA test carried out indicated a significant effect of 'time' ($p = 0.000$) and a significant interaction between the two factors ($p = 0.004$) in Chla, while 'concentration' was significant in both chlorophylls tested (Table S2). Pigment contents were not significantly affected by Hg-loaded MPs, with the exception of Chla at 200 μg/L that showed a significant increase at 48 h, reaching 6.09E−06 μg Chla/mL per cell (Fig. 4H, Table S1). The two-way ANOVA test performed showed a significant effect of 'time' in Chlc2 ($p = 0.010$) and carotenoids ($p = 0.001$), and a significant interaction between both factors in Chla ($p = 0.004$) and carotenoids ($p = 0.027$) (Table S2).

4. Discussion

4.1. MPs effects on microalgae

Our results show the existence of significant effects on *R. lens* growth rates after 48 h exposure to plain MPs, but no effects were observed at increasing exposure times or after exposure to oxidized MPs. Several authors have suggested that the impact of MPs on microalgae growth is highly dependent on polymer chemistry, size and charge, concentration, presence of additives and microalgae species (Hazeem et al., 2020; Prata et al., 2019; Tunali et al., 2020). Some studies have found a pattern of growth inhibition in microalgae at smaller MPs sizes and at higher concentrations (Hazeem et al., 2020; Tunali et al., 2020). In the present investigation, particle sizes of the MPs under study are in the range of 7–15 μm, similar to the size of *R. lens* cells, thus, the uptake of MPs particles is unexpected. However, it is possible to argue that the effects of the MPs observed in this study may be due to a reduction of the mobility of the microalgae due to the adsorption of MPs to the periplast (Davarpanah and Guilhermino, 2015; Dong et al., 2022).

A pattern of increasing population growth similar to that described in this study, has been previously found in marine and freshwater species exposed to MPs (Canniff and Hoang, 2018; Cunha et al., 2020; Yokota et al., 2017). This effect has been credited to the ability of some microalgae species to use MPs as substrates and develop biofilms on them (Canniff and Hoang, 2018; Lagarde et al., 2016; Long et al., 2015; Yokota et al., 2017; Zettler et al., 2013). These organisms are known to release exopolymeric substances (EPS) due to the cellular stress caused by the interaction with plastic particles (Shiu et al., 2020; Zettler et al., 2013), with higher concentrations of MPs triggering a higher release of EPS (Shiu et al., 2020). MPs hydrophobic domains added to the physical changes in the particles as a result of the presence of EPS promotes aggregation (Casado et al., 2013; Lagarde et al., 2016; Prata et al., 2019; Shiu et al., 2020). This leads to the formation of heteroaggregates among MPs, microalgae and EPS, consequently provoking a reduction in light availability and in substance exchange in microalgae. The genus *Rhodomonas* has been reported to create heteroaggregates with MP particles and EPS (Long et al., 2015), which supports the results obtained in this work. The formation of heteroaggregates in cultures exposed to these plain MPs was observed here under the microscope. Previous publications have also highlighted the importance of the zeta potential, defined as the charged attraction or repulsion among particles, on microalgae growth rates (Gomes et al., 2020; Hazeem et al., 2020). If the zeta potential is low, the particles will tend to aggregate, which may explain, to some extent, the variability observed here on the effects of plain and oxidized MPs to *R. lens*. However, the zeta potential of the particles under study has not been evaluated in this research.

After 48 h, a population growth decay trend was detected. At 96 h, no significant differences were observed in growth at any of the concentrations tested, compared to controls. Other authors have indicated comparable results at 96 h in *Tetraselmis chuii* exposed to PE particles with similar sizes and concentrations (Davarpanah and Guilhermino, 2015; Prata et al., 2018). The mechanisms associated to this decay in microalgae growth can be also explained by the release of this EPS as a detoxification process, which may lead to the consumption of energy, that will not be available for growth (Gambardella et al., 2018).

Rhodomonas lens was not affected by the oxidized MPs at the tested concentrations (up to 1 mg/L), at any exposure time, which is in accordance with previous findings that did not observe significant effects in other species, up to 41.5 mg MPs/L (Prata et al., 2018; Garrido et al., 2019; Tunali et al., 2020). It has been suggested that this lack of effects may be due to MPs aggregation and flocculation, which would reduce the interaction with the microalgae and thus diminish the possible effects (Davarpanah and Guilhermino, 2015).

The viability of *R. lens* decreased at the highest concentrations tested when exposed to both plain and oxidized MPs, which is in agreement with previous work performed with chlorophytes and cryptophytes at comparable particle concentrations (Gomes et al., 2020; Gunasekaran et al., 2020). The observed reduction in cell viability can be interpreted as an indicator of cell disruption caused by the interference of the plastic particles with the periplast of the microalgae. Also, for the same particle size, the effects on the viability of microalgae have been related to the chemical structure of plastics (Hazeem et al., 2020), which may explain the lower viability detected in *R. lens* exposed to plain MPs compared to oxidized MPs.

Regarding the pigment contents, a significant decrease in Chla and Chlc2 concentrations was found in *R. lens* exposed to plain MPs. A reduction in chlorophyll concentrations due to MPs exposure has been indicated in previous works (Huang et al., 2020; Prata et al., 2018; Tunali et al., 2020). Prata et al. (2018) found a significant reduction of Chla in *T. chuii* exposed to 0.9 and 2.1 mg/L thermoset amino formaldehyde polymer microspheres of a similar size to those studied here. Also, previous research with *Scenedesmus obliquus* indicated a reduction in Chla concentrations at increasing concentrations of polystyrene nanoparticles (Besseling et al., 2014). This can also be linked to the formation of heteroaggregates mentioned above, which lead to lower light availability, ending up in lower pigment content. Also, an impact on the photosystem II, the electron transport chains and the electron donor site of the microalgae, can be expected due to MPs exposure (Bhattacharya et al., 2010; Mao et al., 2018).

4.2. CPF and Hg effects on microalgae

This research has shown that exposure to CPF and Hg alone significantly reduced the ASGR of *R. lens*, causing total inhibition at concentrations above 1700 and 250 μg/L at 24 h, respectively.

Research on the exposure of microalgae to CPF has shown great variability in the toxicity of this compound. The EC50 for growth rate inhibition vary depending on the species tested, from 26 mg/L for *Merismopedia* sp. (Chen et al., 2016), to 0.24 mg/L for *Skeletonema costatum* (DeLorenzo and Serrano, 2003). The present study has established the EC50 of *R. lens* in 0.71 mg/L, which is in the low range of sensitivity to CPF. Growth inhibition due to CPF exposure has been attributed to cell apoptosis, owing to an increase of cell volume, caused by detoxification processes, and to the inhibition of cell division (Asselborn et al., 2015). Differences in sensitivity of microalgae species are related to their cell structures, such as the lack or presence of cell walls (DeLorenzo and Serrano, 2003). Similarly, some characteristics of microalgae cells can have an effect on the toxicity of CPF, such as the secretion of mucilage from the cell surface, which can potentially decrease the bioavailability of CPF (Martinez et al., 2015).

The inhibition of acetylcholinesterase in animals by organophosphate pesticides, such as CPF, has been well studied (Bellas et al., 2022); however, sublethal effects of this compound on microalgae has not been studied as much. Significant effects on the cell viability and chlorophyll content of *R. lens* were observed here. Viability decreased in a concentration-dependent manner, what can be associated with changes in the cell surface and to apoptosis (Asselborn et al., 2015). Only a few studies have addressed the effects of CPF on chlorophyll content and are in accordance with the results here presented (Chen et al., 2016; Tien and Chen, 2012). Chen et al. (2016) found a similar pattern of decreased chlorophyll content in *Chlorella pyrenoidosa*, with a significant reduction of Chla at 4.8 mg/L, after 48 h exposure to CPF. However, a significant increase in Chla was found at 96 h, which is not consistent with the results reported here.

Toxicity of heavy metals to microalgae depends on several factors, including experimental conditions and species sensitivity (Liu et al., 2011). Exposure of *R. lens* to increasing Hg concentrations resulted in a gradual decrease in growth rates, with an EC50 of 132 μg/L, within the range of toxicity described for Hg in other species of microalgae (Arsad et al., 2020; Fathi, 2002; Gómez-Jacinto et al., 2015; Wu and Wang, 2011). Several mechanisms may be responsible for the growth inhibition observed in microalgae exposed to Hg, such as the disruption of photosynthetic activities due to the destruction of chloroplasts (Lamai et al., 2005), the substitution of essential metal ions per metalloproteins, the blocking of proteins and enzymes, or fat formation (Wang et al., 2012). Likewise, cell viability follows a concentration-dependent decrease, being more sensitive than growth inhibition, since a significant reduction in cell viability occurs from 50 μg/L, instead of 100 μg/L. Previous studies have found that cell viability decreases gradually with increasing Hg concentrations. This has been attributed to the accumulation of the compound in the cell, which leads to the disruption of vital functions, such as those mentioned above (Gómez-Jacinto et al., 2015).

Chlorophyll and carotenoids in *R. lens* exposed to Hg varied over time, depending on the exposure concentration. At the lowest Hg concentrations, both Chla and total carotenoid contents of microalgae cells significantly increased compared to the controls, after 48 h of exposure, in harmony with the studies of Bezzubova et al. (2018) with *Nannochloropsis* sp. Besides harvesting light in the process of photosynthesis, carotenoids play a key role in photoprotection, either directly by scavenging reactive oxygen species and decreasing cell damage, by quenching chlorophyll triplet states or indirectly, by thermal dissipation of excess light energy (Goiris et al., 2012; Rastogi et al., 2020). Thus, an increase in carotenoids has been commonly suggested as a protection mechanism against the formation of free radicals in microalgae exposed to xenobiotics (Cabrita et al., 2018). However, after 96 h of exposure, this increase in pigment contents disappears, probably due to the fact that the antioxidant capacity of the microalgae is exceeded (Pinto et al., 2003). At high Hg concentrations, Chla, Chlc2 and carotenoids significantly decreased at both 48 and 96 h of exposure. These results agree with the findings of previous studies (Afkar et al., 2010; Chen et al., 2016; Zamani-Ahmadmahmoodi et al., 2020) and it is considered as an indicator of heavy metal toxicity (Afkar et al., 2010), due to either the substitution of magnesium present in central position of the chlorophyll molecule by heavy metals (Hg, in this case), or to the disruption of the transport chain, which ultimately inhibits photosynthesis (Küpper et al., 1996).

4.3. MPs effects on CPF and Hg toxicity

In the present research, MPs combined with CPF and Hg showed significant toxicity on the measured biological responses. Previous studies have shown the capacity of several types of MPs, including polyethylene pellets, to adsorb and accumulate heavy metals and organic pollutants from the surrounding sea water (Ashton et al., 2010; Gebremariam et al., 2012; Holmes et al., 2012; León et al., 2018). For instance, Garrido et al. (2019) and Rivera-Hernández et al. (2019), using the same methodology employed here, found that 70 % and 13 % of CPF and Hg, respectively, were adhered to the MPs under study after a loading period of 2 h (CPF) and 7 d (Hg). However, the binding of Hg and CPF to MPs might reduce their bioavailability to microalgae, resulting in limited toxicity if the loaded MPs are not incorporated into the cell. This can be the cause of the diminished effects of the mixtures of MPs with CPF and Hg on *R. lens*, compared to the exposure to CPF and Hg alone. This is in accordance with a recent investigation performed with CPF-loaded MPs on marine phytoplankton (Garrido et al., 2019), although a previous study found no influence of MPs on the toxicity of heavy metals to the microalgae *T. chuii* (Davarpanah and Guilhermino, 2015).

In terms of pigment content, Chla and Chlc2 increased even at low concentrations of CPF-loaded MPs. This can be linked to the microalgae defense mechanisms, which include changes in pigment content, that counteract stressors such as xenobiotic presence or light variations caused by the presence of MPs (Maneechote and Cheirsilp, 2021). These defense mechanisms act by increasing its pigment content in order to meet the demand needed for the normal development of the cell (Besseling et al., 2014). This effect can be temporary, with an initial period of vulnerability before adaptation (Mao et al., 2018; Yokota et al., 2017). In the case of Hg-loaded MPs, carotenoids were not significantly affected by exposure, probably indicating that the stress caused is not enough to overcome the photoprotective and antioxidant activity of these pigments (Novoveská et al., 2019).

The concentrations of MPs, CPF and Hg tested in the present research are higher than those generally found in marine waters. However, MPs concentrations in polluted areas can reach concentrations up to 102,000 particles/m^3 (OSPAR, 2009). The concentrations of CPF and Hg reported in estuarine and seawater usually range from not detected to 4.095 and 27.060 μg/L, respectively (Nasfi, 1995; Sousa et al., 2020).

5. Conclusion

To the best of our knowledge, this is the first study that tested the growth inhibition and sublethal effects of pesticides and metals in combination with polyethylene MPs on cryptophytes, whose species have colonized almost any marine and freshwater habitats, from the arctic regions to the tropics. This is also the first investigation studying the effect of Hg-loaded MPs in microalgae. The most important results are summarized as follows: *R. lens* growth rates were significantly affected by exposure to plain MPs after 48 h of exposure, although no effects were observed at increasing exposure times or due to exposure to oxidized MPs. Cellular viability decreased after exposure to both oxidized and plain MPs and chlorophyll content was decreased after exposure to plain MPs. Both CPF and Hg were toxic to *R. lens* at concentrations above 100 and 1 μg/L, respectively. Exposure to CPF/Hg-loaded MPs mixtures were less toxic to *R. lens* than CPF and Hg alone, probably due to the lower bioavailability of the contaminants.

Even though in this study MPs reduced the toxicity of Hg and CPF on *R. lens*, the potential internalization and, consequently, a greater effect on microalgae, should not be ruled out for smaller MPs and, especially, for

nanoplastics-pollutant mixtures. Further experiments with nanoplastics should, therefore, be performed to confirm this hypothesis.

CRediT authorship contribution statement

Estefanía P. Pinto: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Estefanía Paredes:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Juan Bellas:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159605.

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