



Influence of polyethylene-type microplastics on long-term exposure to heavy metals in freshwater phytoplankton

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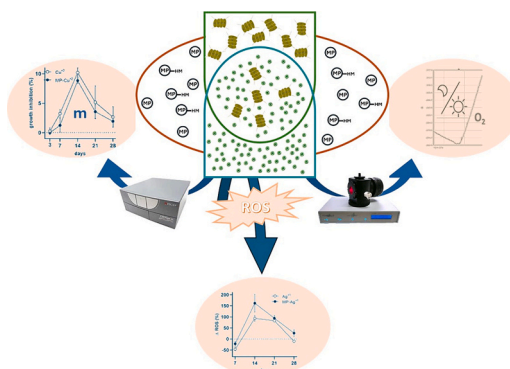
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HIGHLIGHTS

- HMs inhibited cell growth for the first 7–14 days, then reversed the process.
- Photosynthetic activity is initially inhibited for 72 h, then it recovers.
- MPs do not significantly change the effect of HMs on freshwater microalgae.
- In long-term HMs exposures, *M. aeruginosa* outcompetes *S. armatus*, with or without MPs.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of plastic materials has brought about significant social benefits but has also led to negative consequences, particularly their accumulation in aquatic environments. Studies have shown that small plastic particles, known as microplastics (MPs), can carry various harmful pollutants, such as heavy metals (HMs). Therefore, the aim of this research is to investigate the impact of polyethylene-type MPs on the long-term exposure of different HMs on freshwater microalgae *Scenedesmus armatus* and cyanobacteria *Microcystis aeruginosa*, in both isolated cultures and phytoplanktonic community conditions. Over a period of 28 days, the strains were subjected to concentrations of Ag^+ , Cu^{+2} , and Cr^{+6} corresponding to their respective 72 h-EC₁₀, with or without the presence of MPs. Throughout this period, the growth cell ratio, photosynthetic activity, and reactive oxygen species (ROS) were monitored. The findings indicated a substantial inhibitory impact on cell growth during the initial 7–14 days of exposure, followed by a reduction until reaching values like the controls after 28 days of exposure. There was a disturbance in photosynthetic activity during the first 72 h of exposure, which gradually returned to control levels, mainly significantly affected the respiration phase. Reactive oxygen species (ROS) activity was also affected during the initial 14 days of exposure. The presence or absence of MPs in the culture medium did not significantly alter the observed effects. However, interspecies competition created a more favorable environment for *M. aeruginosa* over the freshwater microalgae *S. armatus*. These findings suggest that

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the formation of MP-HMs complexes may have a limited impact on reducing the adverse effects of HMs in long-term exposures. However, because the impact depends on the specific HM involved, further studies are needed to gain a better understanding of the interaction between these pollutants.

1. Introduction

In recent years, there has been a significant increase in the production of plastics, with production rates estimated to exceed 400 million tons per year (Lampitt et al., 2023). While some of these plastics (i.e. microbeads) are manufactured in small sizes, there is evidence to suggest that larger plastics gradually degrade over time in the environment, resulting in the formation of microplastics (MPs) ranging in size from 5 mm to 1 μm (Horton et al., 2017).

In recent years, there has been a growing concern regarding the presence of MPs in freshwater ecosystems. Research on this issue has demonstrated the key role of rivers in enabling the microplastics being transported into the marine environments (Hu et al., 2020).

MPs, due to their high surface area, hydrophobicity, and adsorption capacity (Wang et al., 2019), can easily adsorb hydrophobic pollutants and metal ions in aquatic environments. Various studies have demonstrated the adsorption capacity of heavy metal ions (Zhang et al., 2018; Zhang et al., 2019), and have linked it to the surface charge of MPs (Liu et al., 2022). This implies that a synergistic effect between MPs and sediment-specific physical and chemical factors could potentially remove heavy metals from sediments.

Heavy metals (HMs) are a significant source of contamination in aquatic ecosystems, given their non-biodegradable and bioaccumulative properties (Zamora-Ledezma et al., 2021). Human activities have increased their presence in the environment to the point of being potentially toxic. Presently, HMs contaminate around 40 % of lakes and rivers (Kakade et al., 2021; Bilal et al., 2021). Research has shown that HMs have detrimental effects on aquatic organisms by accumulating in different organs, resulting in oxidative damage, endocrine disruption, and immune system suppression. These effects can affect the survival and growth of these organisms (Guerra et al., 2012; Le et al., 2019). Additionally, the accumulation of HMs in the aquatic environment can lead to long-term consequences, as the metals enter and remain in organisms (Yu et al., 2020).

Different authors have found that MPs recovered from the environment contain various HMs (Wang et al., 2017a; Dobaradaran et al., 2018). Heavy metal contamination in the environment is closely related to the heavy metal content found on MPs, indicating that MPs could serve as a carrier for metal transport in the environment (Zhou et al., 2019). The adsorption of heavy metals by MPs has the potential to alter their toxicity and mobility in aquatic organisms (Botterell et al., 2019). While some studies have investigated the adsorption of environmental pollutants by MPs in controlled laboratory environments (Yu et al., 2019), there is a continued need for quantitative comparisons between various heavy metals based on different criteria, including their valence state. Addressing this, Lv et al. (2023) demonstrated the influence of heavy metal type, concentration, and valence state on adsorption onto polyethylene-type microplastics.

Microalgae play a crucial role as primary producers in aquatic ecosystems. However, their populations can be disrupted by MPs, which can further upset the already imbalanced and stressed freshwater ecosystems (Lürding et al., 2016). In a study by Bhattacharya et al. (2010), it was found that MPs had a direct impact on microalgae, leading to shading effects, increased extracellular polysaccharides (EPS) production, and decreased energy balance, which were identified as the main risk factors. The interaction between the inner wall and the particles was found to be affected by the electrical charge, with positive and neutral charges inducing adsorption affinity between the wall and the particles, while negative charges did not (Guschina et al., 2020). Previous research on exposure effects has focused mainly on cell growth and

photosynthetic capacity, with very few studies examining the production of reactive oxygen species (ROS) (Xiao et al., 2020; Zheng et al., 2021). However, as above stated, it is important to consider not only the potential risks associated with MPs in aquatic environments, but also their interaction with HMs in natural waters. Particularly, it is crucial to understand the combined effects of both types of pollutants on freshwater phytoplankton to ensure the safety and health of aquatic ecosystems.

Because HMs adsorbed on the surface of MPs can be desorbed (and vice versa), under different environmental conditions, and thus producing potential threats on freshwater phytoplankton communities, the aim of this study was to assess the potential risk of coexisting pollutants (MPs and HMs) long-term exposure on strains of the green algae *Scenedesmus armatus* and the cyanobacteria *Microcystis aeruginosa*, considered as pure cultures and experimental phytoplankton community (EPC).

2. Material and methods

2.1. Microplastic characterization

The plastic material utilized in these experiments was provided from Cospheric LLC (Santa Barbara, CA, USA). It comprises of uniformly sized, microspherical polyethylene particles measuring 250-300 μm with a density of 1.35 g mL^{-1} . Additionally, it contains titanium dioxide (TiO_2). The scientific community widely recognizes that exposure to natural communities reveals negligible effects of TiO_2 on marine and freshwater biota structure. The concentration of polyethylene microspheres applied to the bioassays was 1 mg mL^{-1} , which is within the range reported for freshwater ecosystems (Bellasi et al., 2020). Furthermore, previous studies carried out by our work group (Sánchez-Fortún et al., 2021) have already highlighted and discussed the low adverse effects of the polyethylene polymer used.

To account for the hydrophobic nature of the polyethylene microspheres, and also to remove their ultrafine plastic contamination, they were washed and agitated with tween-80 (Sigma Aldrich Chemie, Taufkirchen, Germany) before being suspended in BG-11 culture medium.

2.2. Organisms and culture conditions

Representative green microalgae and cyanobacteria from freshwater ecosystems were selected as *Scenedesmus armatus* (BEA 1402B) and *Microcystis aeruginosa* (BEA 1835B) strains. These strains were obtained from the Spanish Bank of Algae (BEA, Gran Canarias, Spain) and were maintained in the laboratory. They were grown under axenic conditions in culture flasks (Thermo Fisher Scientific Inc., MA, USA) containing 20 mL of BG-11 culture medium (Sigma Aldrich Chemie, Taufkirchen, Germany). The strains were exposed to an individual 12-hour shift of the light-dark cycle (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ over the 400 to 700 nm waveband) and maintained at 21 °C. Serial transfers of single-cell inoculums to fresh medium once a fortnight allowed for maintaining mid-log exponential growth, both as single-species and in a community.

2.3. Chemicals

The present study utilized three different types of heavy metal ions: silver (Ag^+), copper (Cu^{2+}), and chromium (Cr^{6+}). These ions were obtained from the corresponding salts of $\text{Al}(\text{NO}_3)_3$, CuSO_4 , and $\text{K}_2\text{Cr}_2\text{O}_7$, all of which were supplied by Sigma-Aldrich (Sigma Aldrich Chemie,

Taufkirchen, Germany).

Increasing concentrations of each salt diluted in BG11 culture media were used to determine the concentration at which 10 % of the organisms tested showed a statistically significant reduction in cell growth after 72 h of exposure (72 h-EC₁₀). From these data, cultures of the green algae *S. armatus* and the cyanobacteria *M. aeruginosa*, as well as in, EPC conditions were exposed to weekly single concentrations of 0.001 mgL⁻¹ Ag⁺, 0.05 mgL⁻¹ Cu²⁺, and 0.5 mgL⁻¹ Cr⁶⁺ for 28 days.

2.4. Cell growth rate

In this experiment, *S. armatus* and *M. aeruginosa* were grown separately and together under phytoplanktonic community conditions, with cell densities of 10⁴ cells mL⁻¹ each. They were exposed to the selected HMs concentrations above mentioned (Section 2.3) in 20 mL of BG-11 culture medium for 28 days at 21 °C in a thermostatically controlled chamber (Gilson Inc., Middleton, WI, USA) at 60 μmol m⁻² s⁻¹. The cultures were shaken by hand twice daily, once in the morning and once in the evening, for the 28 days of exposure.

To ensure accurate results, additional light absorption tests (OD627 and OD720) were conducted and showed no shading effects. The results showed that the absorption of light emitted by exposure of all HMs concentrations used did not exceed values of 0.009, so no shading effects are expected.

The cell growth rates were determined by measuring the fluorescence emitted by the photosynthetic pigments using a Tecan Genios plate reader (Tecan Group Ltd., Switzerland), with excitation-emission filters of 485-670 and 590-670 nm for the green algae *S. armatus* and the cyanobacteria *M. aeruginosa*, respectively. The relationship between fluorescence and cell density was estimated by comparing it with parallel Neubauer chamber counts.

Quantitative measurements were obtained from control and treated cultures at 3, 7, 14, 21, and 28-day intervals. The Malthusian parameter (*m*) was utilized to estimate the maximum cell growth rate. This parameter was calculated as (Crow and Kimura, 1970):

$$m = \log_e (N_t/N_0)/t$$

where *N_t* and *N₀* are cell concentrations at time *t* and *t* = 0 (both estimated by fluorescence). The *m*-values are expressed as doublings d⁻¹.

2.5. Photosynthesis activity assessment

The O₂ production/consumption balance under light-dark conditions is a widely used model to evaluate photosynthetic activity. It involves analyzing the light-dark O₂ balance using a Clark-type O₂ electrode. The Chlorolab 2 system (Hansatech, Norfolk, UK) is often used to measure dissolved O₂ under automated illumination from red (660 nm) LED light and in darkness. During photosynthesis activity tests, measurements are typically taken at 21 °C and 375 μmol m⁻² s⁻¹ irradiance. The gross photosynthesis rate (*P_g*), or light-dark oxygen balance, can be estimated using a specific formula:

$$P_g = P_n + R$$

where *P_g* corresponds to the oxygen production rate under illuminated conditions, *R* (respiration) corresponds to the process by which phytoplankton consume oxygen and release carbon dioxide in darkness, and *P_n* (net photosynthesis rate) is defined as the difference between *P_g* and *R*.

The O₂ balance between control and treated cell cultures of both strains was assessed over 3, 7, 14, 21, and 28 days. The cell concentration was 5 × 10⁵ cells diluted in a final volume of 1 mL, and the measurements were taken after 5 min of darkness followed by 5 min of illumination. Each experiment was repeated four times for accuracy (*n* = 4).

2.6. Generation of reactive oxygen species

The fluorescent probe 2',7'-dichlorofluorescein diacetate (H₂DCFDA) was used to test intracellular reactive oxygen species (ROS) generated by *S. armatus* and *M. aeruginosa* strains, both single-species and communities. This was accomplished by intracellular oxidation of H₂DCFDA, which leads to the production of 2,7-dichlorofluorescein (DCF), a fluorescent compound that serves as an indicator of ROS. To conduct the experiment, cell densities of *S. armatus* (2 × 10⁶ cells mL⁻¹) and *M. aeruginosa* (5 × 10⁶ cell mL⁻¹), both exposed and unexposed to selective MP-HMs concentrations, were adjusted to a final volume of 1.5 mL. The cultures were then incubated with a final concentration of 10 mM of H₂DCFDA for 60 min at room temperature (23 °C). Samples were monitored at 0, 1, 2, and 4 h and measured on a Tecan Genios microplate reader (Tecan Group Ltd., Switzerland) at room temperature, using excitation-emission filters of 485-520 nm to determine relative ROS production. To account for "background" ROS formation, the BG-11 medium was also tested for ROS, and the results were corrected by subtracting the fluorescence of the culture medium. A positive control for ROS formation was established using 3 % H₂O₂ (v/v).

2.7. Data analysis

To conduct statistical analysis, we utilized the computer software package GraphPad Prism v8.0 (Graph-Pad Software Inc., USA). Our data is presented as the mean ± sd of four experiments (*n* = 4). We employed Student's *t*-test and two-way analysis of variance (ANOVA) to determine if there were any significant differences (*p* < 0.05) among treatments, and a post-hoc analysis (Tukey test) to check for any differences among groups.

3. Results

3.1. Cell growth rate

The results obtained after 28 days of exposure to the selected HMs, alone or in combination with MP, on both phytoplankton strains exposed in isolation or under EPC conditions, are plotted in Fig. 1.

In experiments conducted on freshwater green algae *S. armatus*, cells exposed to HMs initially showed a decrease in growth rate, followed by an increase to levels like control groups (Figs. 1a). Exposure to Ag⁺ and Cr⁶⁺ resulted in maximum *m* inhibition at 7 days of exposure, with percentages of 10.91 ± 3.49 % and 19.64 ± 0.48 %, respectively. On the other hand, exposure to Cu⁶⁺ showed a maximum *m* inhibition percentage of 4.91 ± 0.63 % at 14 days of exposure. Likewise, growth inhibition after co-exposure to the different HMs and MP (Ag/MP, Cu/MP, and Cr/MP) followed a similar pattern, with a maximum *m* inhibition of 1.48 ± 0.41, 5.12 ± 0.51 % and 21.54 ± 0.43 % respectively.

Under EPC conditions, the response exhibited by *S. armatus* was dependent on each of the HMs (Figs. 1b). While exposures to Ag⁺ and Ag/MP produced no inhibition of *m* during the 28 days of exposure, Cu²⁺ and Cu/MP caused reductions in the order of 10.2 ± 0.80 % and 8.83 ± 0.43 % respectively, decreasing progressively thereafter until practically equaling the control values. On the other hand, exposure to Cr⁶⁺ and Cr/MP obtained a maximum reduction of *m* at 7 days of exposure, with percentages of 22.02 ± 0.83 % and 16.42 ± 0.21 %, and both reduced to control values after 28 days.

Freshwater cyanobacterial *M. aeruginosa* showed a decrease in the initial *m*-value until day 14 post-exposure to Ag⁺, with values of 26.32 ± 4.14 %, followed by a gradual decrease, eventually matching control values (4.55 ± 2.21 %) at 28 days of exposure. Exposure to Cu²⁺ did not induce any effect, and exposure to Cr⁶⁺ resulted in a significant increase in the reduction of the *m*-value during the 28 days, reaching 64.21 ± 1.09 %. Co-exposures of Ag/MP and Cu/MP showed a maximum *m* reduction at 14 days of exposure, with percentages of 2.36 ± 1.17 % and 8.83 ± 0.43 %, respectively, to subsequently increase *m* values until

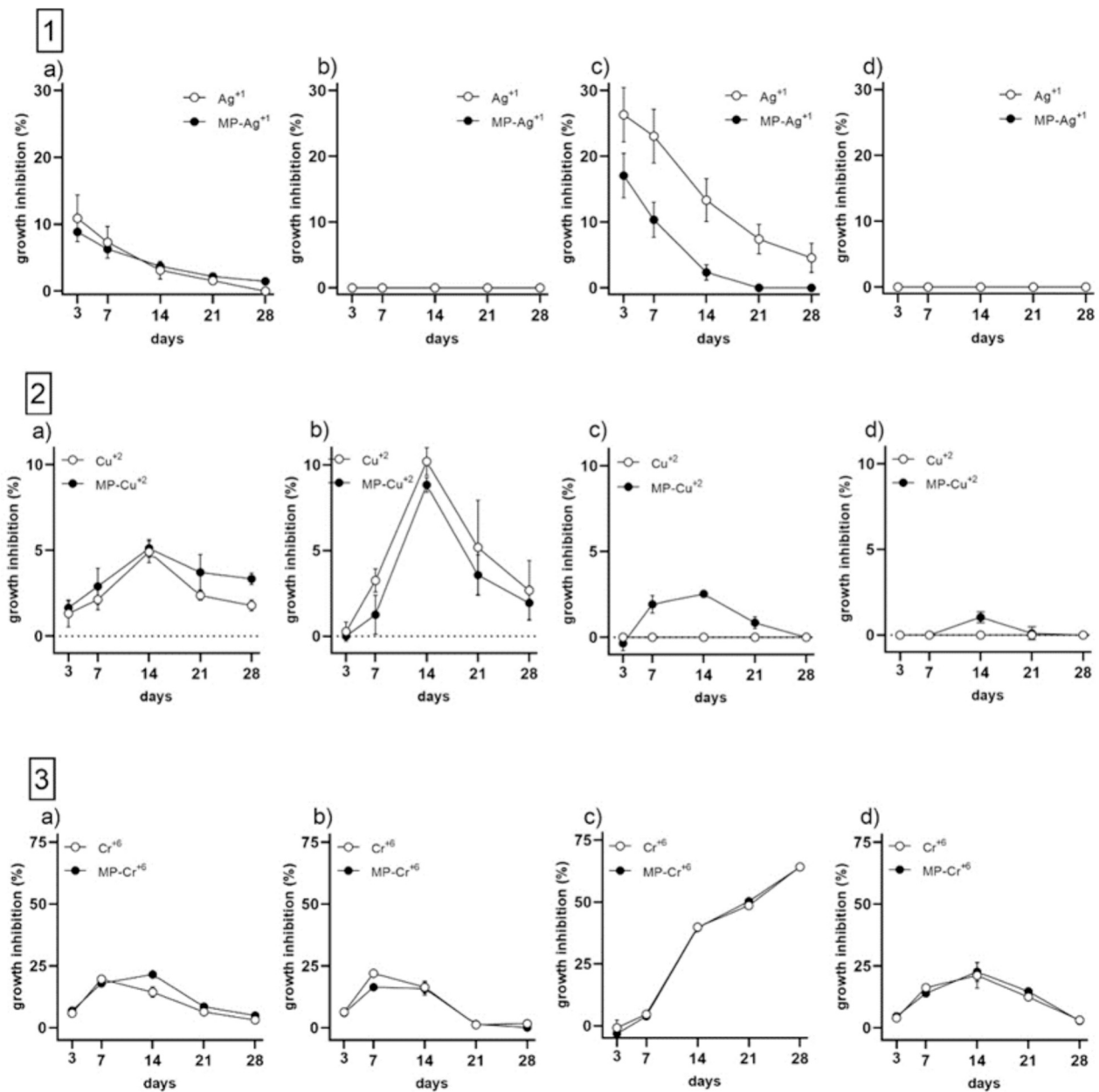


Fig. 1. Cell growth rate exhibited by *S. armatus*, *M. aeruginosa*, and both exposed under EPC conditions to Ag^+ (○), Cu^{2+} (⊙), and Cr^{6+} (⊗), alone (empty dots) or together with MP (filled dots). Results are plotted according to effects from isolated or community exposures on *S. armatus* (a and b respectively) and *M. aeruginosa* (c and d respectively). Each point represents the mean \pm sd of 4 experiments ($n = 4$), expressed as percentage inhibition with respect to the control.

they were like the controls. The Cr/MP co-exposure had the same behavior as that exhibited by the isolated Cr^{6+} , obtaining m reduction percentages of 64.08 ± 1.25 % (Fig. 1c).

When *M. aeruginosa* strain was exposed to EPC conditions (Fig. 1d), it was observed that cell growth rate reduction did not occur when exposed to Ag^+ and Cu^{2+} , either alone or in combination with MPs. However, when exposed to Cr^{6+} and Cr/MP, a maximum m reduction was observed at 14 days of exposure, with percentages of 21.16 ± 5.19 % and 22.64 ± 3.79 %, respectively. The m value later increased to match the control values at 28 days of exposure.

3.2. Photosynthesis activity

The effects on photosynthetic activity exhibited by both strains exposed for 28 days to the selected HMs, alone or under phytoplanktonic community conditions, with or without the presence of MPs in the

culture medium, are shown in Figs. 2, 3, and 4.

The *S. armatus* strain exhibited varying photosynthetic activity based on the heavy metals (HMs) it was exposed. When exposed to Ag^+ (Fig. 2.1), there was a gradual increase in Pg, with 52.6 ± 2.6 % increase observed compared to controls after 28 days of exposure. Similar results were seen for Pn, but R declined in the first week to 72.3 ± 6.1 % before stabilizing at an inhibition rate of 41.2 ± 0.9 % after 28 days of exposure.

The results obtained for Cu^{2+} (Fig. 2.2) and Cr^{6+} (Fig. 2.3) showed a notable decrease in the three photosynthetic parameters in the first 72 h of exposure. Nevertheless, this effect gradually diminished over time, reaching values like the control groups after 28 days of exposure. Specifically, Pn displayed reductions of 32.0 ± 4.0 %, and 75.8 ± 6.8 % for Cu^{2+} and Cr^{6+} , respectively. Dark respiration showed at 72 h of exposure reductions of 72.1 ± 6.5 %, and 53.7 ± 6.8 % respectively, although R in Cu^{2+} exposures, in contrast to Cr^{6+} , maintained a

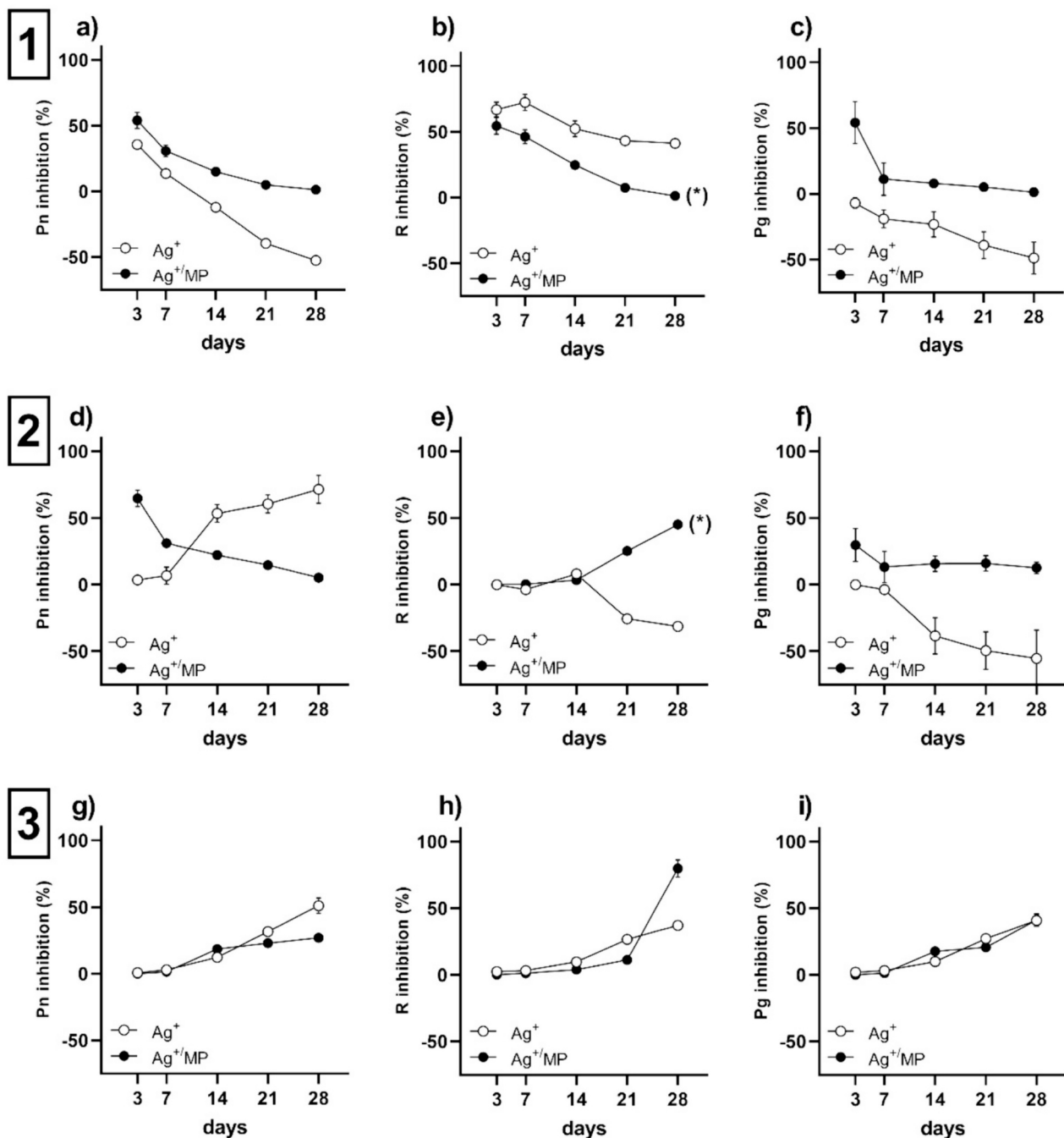


Fig. 2. Effect of exposures to silver alone (Ag^+ , empty symbols) or in the presence of MPs (Ag^+/MP , filled symbols), on net photosynthesis (Pn; a, d, g), respiration (R; b, e, h) and gross photosynthesis (Pg; c, f, i) of *S. armatus* ①, *M. aeruginosa* ②, and both exposed under EPC conditions ③. Each point represents the mean \pm sd of 4 experiments ($n = 4$), expressed as percentage inhibition with respect to control values. (*): Significant differences ($p < 0.05$) respect to Ag^+ alone.

significant reduction of $36.3 \pm 1.2\%$ at 28 days of exposure. Following a 72-h exposure, the study observed significant reductions in Pg. Reductions of $49.2 \pm 5.5\%$, and $70.0 \pm 6.6\%$ were obtained for Cu^{2+} , and Cr^{6+} , respectively. In presence of MPs, the *S. armatus* strain exposed to Cu^{2+} and Cr^{6+} did not show significant differences in the activity of Pn and Pg. However, the inhibitory activity exhibited by exposures to Cu/MP showed a significantly higher percentage of inhibition ($p < 0.001$) compared to the absence of MPs. Exposures to Ag/MP led to a gradual decrease in inhibitory activity, reaching values similar to those observed after 28 days of exposure, but significantly over $p < 0.05$) than those obtained in the absence of HMs.

During the initial 7-day exposure period, there was no modification

in Pn among *M. aeruginosa* populations exposed to Ag^+ (Fig. 2.2d). However, there was a gradual increase in Pg activity (Fig. 2.2f), reaching a peak of $55.3 \pm 1.3\%$ at the end of the 28-day exposure period. Dark respiration also experienced an upward trend over the same period, increasing to $31.3 \pm 3.1\%$ (Fig. 2.2e). Nevertheless, Pn showed a significant reduction to $71.5 \pm 10.6\%$ after the 28-day post-exposure period (Fig. 1-2c). The populations of *M. aeruginosa* that were exposed to Cu^{2+} or Cr^{6+} experienced a notable decrease in photosynthetic activity during the first 72 h of exposure (Figs. 2-2c and 3-2c) However, they gradually began to recover over time until they reached 28 days of exposure. At 72 h of exposure, Pn showed a decrease of $69.1 \pm 6.1\%$, and $41.9 \pm 3.7\%$ for Cu^{2+} , and Cr^{6+} , respectively. By the end of the 28

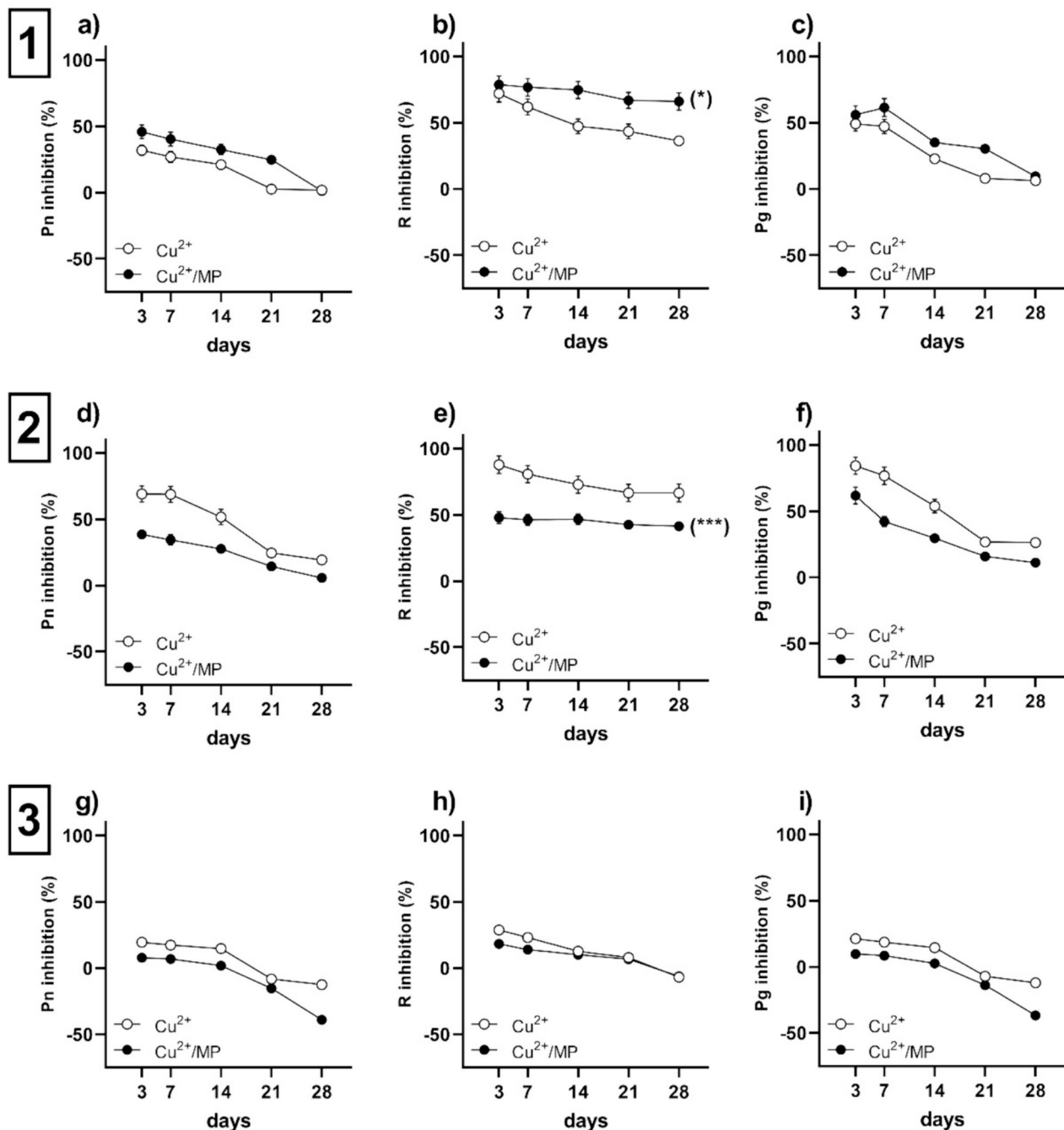


Fig. 3. Effect of exposures to copper alone (Cu^{2+} , empty symbols) or in the presence of MPs (Cu^{2+}/MP , filled symbols), on net photosynthesis (Pn; a, d, g), respiration (R; b, e, h) and gross photosynthesis (Pg; c, f, i) of *S. armatus* ①, *M. aeruginosa* ②, and both exposed under EPC conditions ③. Each point represents the mean \pm sd of 4 experiments ($n = 4$), expressed as percentage inhibition with respect to control values. (*), (***) Significant differences to $p < 0.05$ and $p < 0.001$, respectively, compared to Cu^{2+} alone.

days, these values had minimized to 19.2 ± 2.5 %, and 17.2 ± 1.5 % (Figs. 3.2d and 4.2.d). A similar pattern was observed in the photosynthetic parameter R, which decreased to 87.8 ± 6.6 %, and 40.6 ± 3.9 % at 72 h of exposure (Figs. 3.2e and 4.2e). These values recovered to show inhibition percentages of 66.6 ± 6.8 %, and 12.6 ± 1.0 % at 28 days of exposure for Cu^{2+} , and Cr^{6+} . As a result, Pg showed an increase in inhibition at 72 h of exposure, with percentages of 84.3 ± 6.4 %, and 41.2 ± 3.6 %. However, this inhibition was reduced to 26.4 ± 2.2 %, and 13.6 ± 1.5 % at 28 days of exposure for Cu^{2+} , and Cr^{6+} , respectively (Figs. 3.2f and 4.2f).

The photosynthetic activity of *M. aeruginosa* varied depending on the selected HMs and the presence of MPs in the culture medium. Ag/MP

caused a reduction in Pn by 64.7 ± 6.2 % after 72 h of exposure but later recovered to show inhibition of only 5.3 ± 1.5 % (Fig. 2.2d). R, on the other hand, was barely affected initially but showed a significant ($p < 0.05$) percentage of inhibition of 45.1 ± 2.9 % after 28 days of exposure (Fig. 2.2e). Both processes resulted in a Pg with a maximum inhibition of 29.8 ± 2.4 % at 72 h, which progressively reduced to 12.6 ± 1.3 % at 28 days of exposure (Fig. 2.2f). Exposures to Cu/MP showed Pn inhibition of 38.5 ± 3.1 % at 72 h of exposure and subsequent reduction to percentages of 5.7 ± 3.1 % at 28 days of exposure (Fig. 3.2d), while the inhibition at 72 h of exposure was 47.9 ± 4.4 % over R was maintained over time until showing a percentage of 41.5 ± 2.8 % after 28 days of exposure, which turned out to be significantly different ($p < 0.001$) to

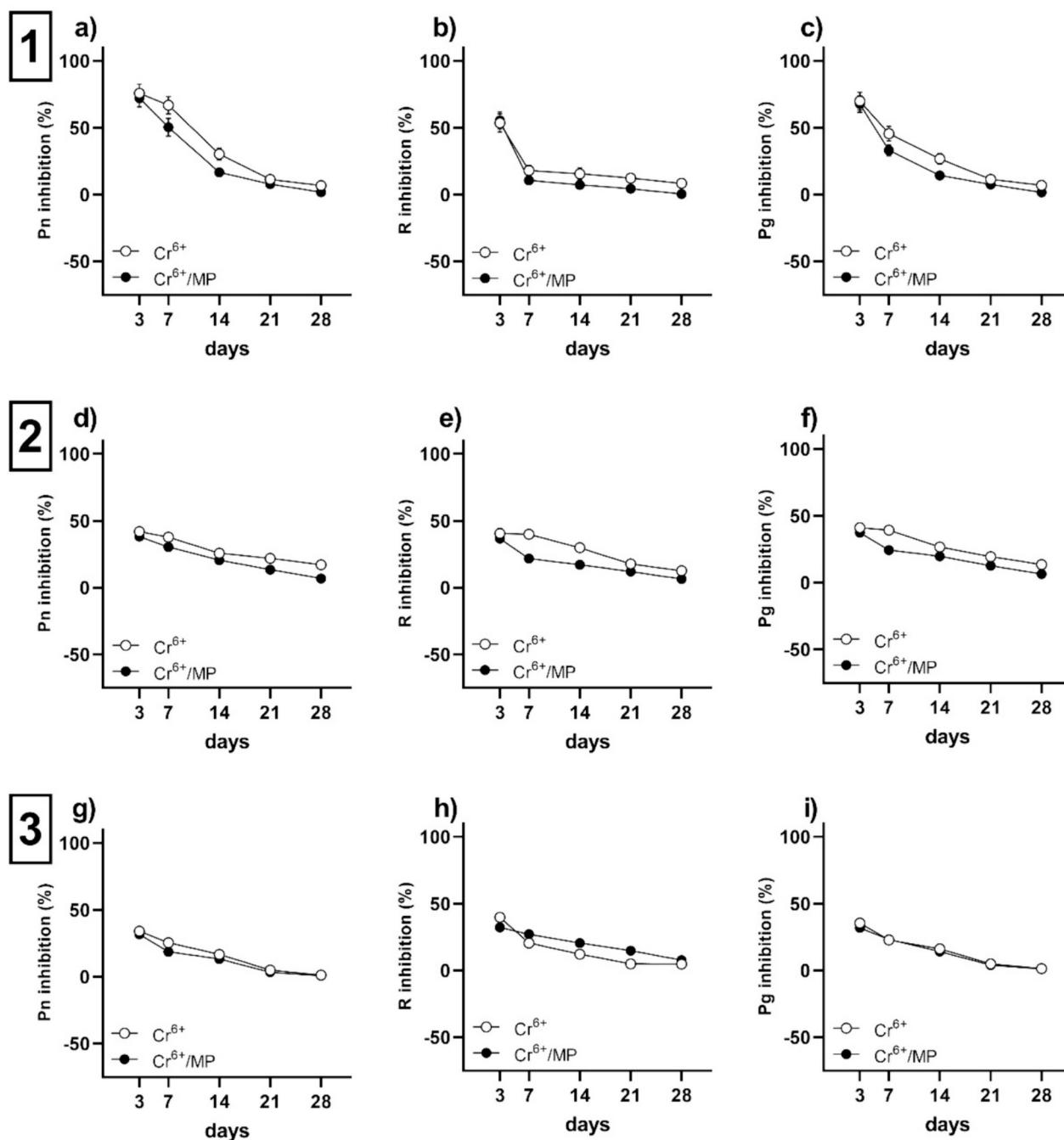


Fig. 4. Effect of exposures to chromium alone (Cr^{6+} , empty symbols) or in the presence of MPs (Cr^{6+}/MP , filled symbols), on net photosynthesis (Pn; a, d, g), respiration (R; b, e, h) and gross photosynthesis (Pg; c, f, i) of *S. armatus* ①, *M. aeruginosa* ②, and both exposed under EPC conditions ③. Each point represents the mean \pm sd of 4 experiments ($n = 4$), expressed as percentage inhibition with respect to control values.

that obtained with exposures in the absence of MPs (Fig. 3.2e). All of this resulted in an inhibition of Pg of $61.9 \pm 6.4\%$ at 72 h, reducing to $11.2 \pm 1.9\%$ at 28 days of exposure (Fig. 3.2f). Notably, the presence of MP in the culture medium during exposures to Cr^{6+} did not significantly affect the photosynthetic response of the *M. aeruginosa* strain (Fig. 4.2).

In EPC conditions, both strains subjected to Ag^+ (Fig. 2.3) exhibit a gradual increase in all photosynthetic parameters. These increases reach levels of $51.1 \pm 5.8\%$, $37.2 \pm 2.8\%$, and $40.9 \pm 4.4\%$ compared to the control for Pn, R, and Pg, respectively. A similar pattern of increase is observed in Ag/MP exposures, with levels of $27.0 \pm 2.7\%$, $79.9 \pm 6.6\%$, and $41.2 \pm 4.8\%$ at 28 days of exposure. Nevertheless, the results indicate that exposure to Cu^{2+} and Cr^{6+} (Figs. 3.3 and 4.3) had the most

significant impact on all photosynthetic parameters after 72 h of exposure, followed by a gradual decrease in effect until values similar to the controls were obtained after 28 days of exposure. The inhibition percentages, which varied between 20–40% depending on the photosynthetic parameter or HMs, were progressively reduced until they barely exceeded 5% and even became negative. This time-dependent, progressive reduction of inhibition on photosynthetic parameters was also observed in Cu/MP and Cr/MP exposures.

3.3. ROS generation

The graph presented in Fig. 5 provides a detailed analysis of the ROS

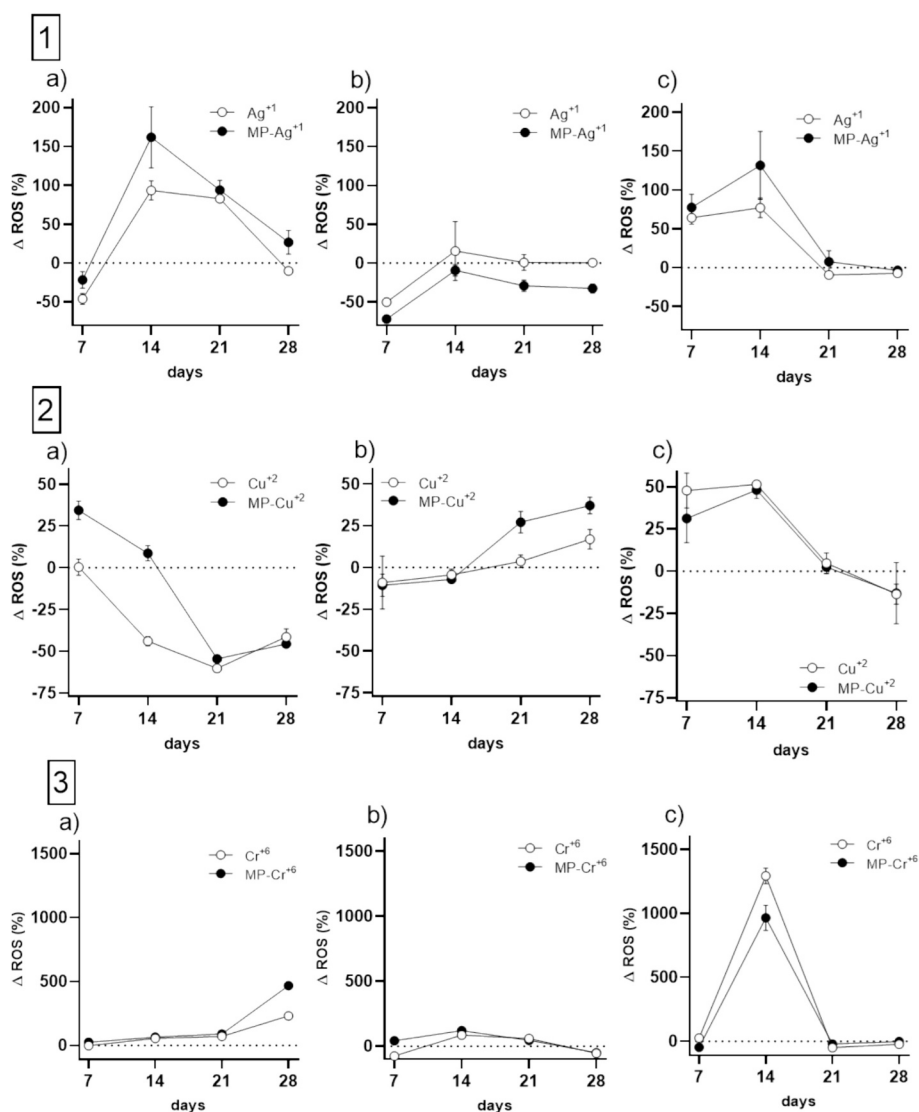


Fig. 5. Reactive oxygen substance (ROS) activity exhibited by *S. armatus* (a), *M. aeruginosa* (b), and both exposed under EPC conditions (c) to Ag⁺ ①, Cu²⁺ ②, and Cr⁶⁺ ③. Points represent exposures to isolated HMs (empty dots) or with the presence of MPs in the culture medium (filled dots). Each point represents the mean \pm sd of 4 experiments ($n = 4$), expressed as percentage inhibition with respect to the control.

activity exhibited by both strains after being exposed to selected HMs for 28 days and performed under different conditions such as the presence or absence of MPs and the influence on phytoplanktonic community.

After being exposed to Ag⁺ (Fig. 5-1a), the cells of *S. armatus* displayed a rise in ROS after 7 days of exposure. The maximum percentages were observed after 14 days of exposure, reaching 93.4 ± 12.4 %. The levels then declined until they were equivalent to the control values at 28 days of exposure. Similarly, exposure to Ag/MP resulted in comparable outcomes, with an increase of 161.7 ± 39.6 % after 14 days of exposure and a decrease to 26.7 ± 15.3 % after 28 days of exposure. During the first 7 days of exposure, *M. aeruginosa* exhibited a significant decrease of 50.2 ± 3.0 % in ROS activity (Fig. 5-1b). However, after 14 days of exposure, the ROS activity increased to 15.59 ± 7.87 % before eventually returning to control values after 28 days of exposure. The presence of MPs in the culture medium resulted in even more significant reductions in ROS activity, with percentages of 72.0 ± 2.8 %, 9.2 ± 7.4 %, and 32.4 ± 5.8 % at 7, 14, and 28 days of exposure, respectively (Fig. 5-1b).

After 7 days of exposure to Cu²⁺ on *S. armatus*, no changes were detected in ROS activity (Fig. 5-2a). However, ROS activity gradually declined and reached a maximum inhibition of 60.2 ± 1.1 % at 21 days,

followed by an increase until reaching values of 41.5 ± 4.9 % inhibition. Interestingly, the presence of MP in the culture medium resulted in an initial increase of 34.3 ± 5.6 % in ROS activity at 7 days post-exposure, which was followed by a significant decrease in activity. Specifically, the activity dropped to 54.6 ± 2.4 % at 21 days and further decreased to 45.7 ± 1.9 % at 28 days post-exposure. *M. aeruginosa* cells exposed to Cu²⁺ and Cu/MP (Fig. 5-2b) did not show modifications in their ROS activity until after 14 days of exposure, reaching increases in activity of 16.8 ± 6.0 % and 37.0 ± 5.0 % at 28 days of exposure.

Over a period of 28 days, *S. armatus* cells exposed to Cr⁶⁺ and Cr/MP showed a gradual rise in ROS activity (Fig. 5-3a), reaching an increase of 71.4 ± 6.8 % and 90.3 ± 9.6 % respectively at day 14, and eventually peaking at 231.8 ± 13.9 % and 467.9 ± 9.8 %. On the other hand, *M. aeruginosa* cells experienced their highest ROS activity increase after 14 days, with percentages of 86.1 ± 21.3 % and 120.1 ± 25.3 % for Cr⁶⁺ and Cr/MP respectively, followed by a significant decline until the activity decreased by 53.5 ± 17.7 % and 49.0 ± 18.3 % concerning control values (Fig. 5-3b).

After exposure to EPC conditions, all selected HMs displayed similar levels of ROS activity, peaking at day 14 and gradually declining to levels comparable to the controls. Over the first 7 days of exposure, Ag⁺

and Ag/MP (Fig. 5-1c) increased their activity by approximately 70 %, with further increases to 77.0 ± 12.6 % and 131.6 ± 43.8 %, respectively, after 14 days of exposure. Similarly, Cu^{2+} and Cu/MP (Fig. 5-2c) showed 30-50 % increases during the first 7 days, with levels reaching 51.5 ± 1.5 % and 48.2 ± 5.1 %, respectively, at 14 days post-exposure. Finally, ROS activity increased significantly after 14 days of exposure to Cr^{6+} and Cr/MP, reaching 1292.9 ± 60.9 % and 964.5 ± 99.6 %, respectively (Fig. 5-3c).

4. Discussion

In recent years, the impact of microplastics on the environment has become a major concern, as they can interact with various anthropogenic contaminants. These contaminants can be adsorbed onto the surface of microplastics and can have harmful effects on the biota. While there have been numerous studies on the impact of microplastics on freshwater organisms (SAPEA, 2019), the lack of data on the type, size, and concentrations of microplastics in the environment has limited our understanding of their full impact. It is known that microplastics in aquatic environments are colonized within hours by microorganisms and algae (Rummel et al., 2017). Based on these circumstances, the present study was focused on investigating the effect of the selected HMs on the chlorophyte microalgae *S. armatus* and the cyanobacteria *M. aeruginosa* cultured for 28 days in isolation and under EPC conditions, and with the presence of a polyethylene-type microplastic in the culture medium.

Based on our findings, we discovered that Cu^{2+} and Cr^{6+} exposures, when exposed at concentrations equivalent to the 72 h- EC_{10} value, caused a minor decline in cell growth rate for both strains during the initial 7-14 days of exposure. However, the m values gradually returned to the levels observed in the control assays, with no statistically significant differences depending on the presence or absence of the microplastic in the culture medium. The only exception was the exposure of cyanobacterial populations to Cr^{6+} , which resulted in a progressive decrease in cell growth throughout the entire 28-day exposure period.

The variation in cell growth rate among metals could potentially be attributed to their interaction with microplastics. Studies have recognized that *M. aeruginosa* tends to gather significant amounts of chromium (Rai and Tripathi, 2007; Mishra and Doble, 2008), while also displaying a heightened vulnerability to the metal (Thompson et al., 2002; Rodgher et al., 2012). This could explain the gradual decrease in the rate of cellular growth inhibition that has been observed. Nonetheless, *S. armatus* and *M. aeruginosa* demonstrated distinct reactions to Ag^+ , with an initial inhibition of m followed by a gradual rise in the ratio until it reached control levels. As with prior exposures, the existence or lack of microplastic had no statistically significant effects. The influence of Ag^+ on cell membrane integrity, as observed in previous studies (Taylor et al., 2016), is particularly noteworthy when it comes to *M. aeruginosa* (Singh et al., 2021). This could account for the variations observed in comparison to other heavy metals that have been researched. Additionally, the decrease in harmful effects over time for both strains is consistent with the findings of Gil-Allué et al. (2018) after 21 days of exposure.

The role played by the MPs-HMs interaction on aquatic organism populations is still a topic of debate. Previous studies have shown that *S. armatus* and *M. aeruginosa* cells exposed to different types of polyethylene microspheres for 28 days could be affected in their cell growth and photosynthetic activity, depending on the species and the type of microsphere involved (Sánchez-Fortún et al., 2021). Some researchers have discovered that when both HMs and MPs were present together, their toxic effects had a more significant impact on the growth of populations, in comparison to when they were encountered individually (Vroom et al., 2017; Fu et al., 2019). On the other hand, other researchers have demonstrated that MPs can effectively reduce the toxicity of HMs on aquatic organisms (Khan et al., 2015; Oliveira et al., 2018).

Our findings indicate a correlation between the presence or absence of MPs and HMs in green microalgae and cyanobacteria and a reduced population growth rate. Some authors attribute this response to the interaction between both elements. Turner and Holmes (2015) noted that the plastic-metal interaction is highly influenced by the polymer's characteristics, which affect the functional groups where the metals bind. Khan et al. (2015) tested the influence of MPs on Ag^+ ions in freshwater aquatic organisms, concluding that the mixture of the two did not influence potential toxic effects. Davarpanah and Guilhermino (2019) did not find significant differences between the toxicity curves of copper in the presence and absence of MP. Li et al. (2022) conducted their research on the adsorption capacity of Cr^{6+} by various microplastics under different conditions. Their findings revealed that polyethylene microplastics exhibit a lower adsorption capacity, leading to an increased concentration of free Cr^{6+} in the medium and consequently, greater cellular exposure to the metal.

The photosynthetic activity of both strains when exposed to the selected heavy metals exhibits distinct patterns. Generally, the impact on Pg is noticeable within the first 72 h of exposure and then diminishes over the course of 28 days. However, it's worth noting that the Ag^+ ion induces significantly milder effects on Pg. Overall, the most significant impact is observed during dark respiration (R), with a lesser impact during the Pn phase. It's important to highlight that there are noteworthy differences among the different HMs tested. This wide array of effects on photosynthetic activity aligns with existing literature that describes the diverse impacts of heavy metals on photosynthesis. Some heavy metals have inhibitory effects on photosynthesis, while others are crucial for its functionality (Liu et al., 2016). The interaction of Cu^{2+} with sulfhydryl-SH groups (Xue et al., 1988; Gao et al., 2020) and the binding of Cu^{2+} and Cr^{6+} to the carboxyl and sulfonate groups of the cell wall (Vilar et al., 2012; Girardi et al., 2014) has been well-documented. These interactions can lead to impaired photosystem II activity (Andosch et al., 2012; Deng et al., 2014).

The wide range of variability observed also applies to exposures in the presence of MPs. The impact of polyethylene-type microplastics on various aquatic contaminants and their influence on the photosynthetic activity of *S. armatus* and *M. aeruginosa* has previously been examined (Sánchez-Fortún et al., 2022). These studies showed that long-term exposures significantly affect this activity, although variably depending on the species.

As in the previous cases, both *S. armatus* and *M. aeruginosa* show different photosynthetic responses to exposures to HMs, depending on the specific HM involved. This wide variability, coupled with the limited information available about the behavior of these strains under long-term exposures in terms of cell growth and photosynthetic activity, highlights the necessity to expand studies that establish correlations between them. Different authors have suggested that, although photosynthesis is closely linked to algal growth, the effect of heavy metals on microalgae photosynthesis does not necessarily correlate positively with their growth (Nielsen and Nielsen, 2005). It is believed that these effects are mainly due to the combination of an excess of heavy metals and the thylakoid membrane, which inhibits chlorophyll synthesis, as well as the impacts on the Hill reaction.

HMs are considered stressors of phytoplankton communities, which can cause significant morphological and physiological changes, and in some cases the generation of ROS in microalgae (Coulombier et al., 2021). Against these adverse effects, microalgae can be protected by enzymatic and non-enzymatic antioxidant systems (Ismail and Ismail, 2017; Xiao et al., 2023). Our studies show a variable ROS activity depending on the HM and the phytoplankton species involved throughout the 28 days of exposure. The significant increase in ROS activity induced by Ag^+ on *S. armatus* agrees with that obtained by other authors (Oukarroum et al., 2012; Hazeem et al., 2019; Komazec et al., 2023), and similarly the absence of ROS increase induced by Cu^{2+} has been explained by the existence of various species-specific defense mechanisms (Knauer and Knauer, 2008). Similar findings have been

reported for exposures to microalgae, mainly related to stress induced by Cr⁶⁺ exposure (Mallick and Mohn, 2003).

The time-dependent mitigation of ROS activity observed in our assays on *S. armatus* could be linked to the defense mechanisms of the antioxidant system, which are influenced by the duration and intensity of stress experienced by these microalgae cultures (Okamoto et al., 2001). However, different researchers have noted varying oxidative states in chronic exposures to some heavy metals (Mallick, 2004; Tripathi et al., 2006; Verbruggen and Hermans, 2008). Therefore, further studies are needed to elucidate the defense mechanisms involved in long-term exposures. Notably, *M. aeruginosa* cells exposed to Cr⁶⁺ did not exhibit a mitigation of ROS activity; on the contrary, ROS activity increased over the course of the 28-day exposure. While some findings suggest a potential link between certain heavy metals and increased antioxidant enzyme activity (Choudhary et al., 2007; Deng et al., 2020), additional research is required to fully comprehend this activity.

An additional aspect to consider is the impact of these HMs in the presence or absence of MPs on potential interspecies competition. When exposed to exogenous pollutant stress, interspecies competition is likely to be altered. Our findings suggest that while there are variations in the specific HMs involved, overall, the *M. aeruginosa* strain is favored when coexisting with *S. armatus* under initially equivalent cell concentration under EPC conditions. This effect on the growth of cyanobacterial cells aligns with observations by other researchers, both in the absence and presence of environmental stressors (Yang et al., 2018; Liu et al., 2019). It appears that this effect is linked to the production of a wide range of secondary metabolites with diverse biological effects, which are key in the phenomenon known as allelopathy where these metabolites play a role against competitors or predators (Leflaive and Ten-Hage, 2007). For example, Wang et al. (2017b) demonstrated how allelochemicals released by *M. aeruginosa* inhibited the growth of *Chlorella pyrenoidosa*. Similarly, the effects of these allelopathic compounds on photosynthetic activity and other physiological aspects have been detailed in existing literature (Tang et al., 2019).

5. Conclusion

Long-term exposures of selected HMs to *S. armatus* and *M. aeruginosa* cells initially inhibited the growth rate, reaching maximum inhibition between 7–14 days post-exposure. Afterward, the effects diminished until they were equal to the control values, except for *M. aeruginosa* exposed to Cr⁶⁺. Under competition species conditions, the impact of HMs exposure on cyanobacteria was less than in isolated cultures, while there were no variations in the impact on green microalgae. Both strains showed an impact on photosynthetic activity, primarily during the dark respiration phase in the initial 72 h of exposure. This impact was reduced progressively over the 28 days of exposure, regardless of the presence of MPs or competition. Interspecies competition significantly altered the degree of impact. Notably, both strains exhibited significantly modified ROS activity when exposed under EPC conditions compared to exposure to isolated strains, showing a maximum increase in ROS activity after 14 days of exposure in all cases. Our findings demonstrate that both strains experienced significant stress due to HMs during the initial 7–14 days of exposure, but gradually became acclimated to their presence, regardless of the presence of MPs in the culture medium, which did not significantly modify the process.

CRedit authorship contribution statement

A. D'ors: Writing – review & editing, Visualization, Investigation, Formal analysis. **C. Fajardo:** Visualization, Supervision, Investigation. **G. Costa:** Visualization, Investigation. **S. Sánchez-Fortún:** Writing – original draft, Investigation, Formal analysis.

Declaration of competing interest

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

Data availability

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

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