



Growth and Oxidative Stress in *Monoraphidium Capricornutum* (Printz) Nygaard under Exposure to Perfluorooctanoic Acid and Cylindrospermopsin

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Abstract Microalgae are recognized as sensitive bioindicators for assessing aquatic pollution, yet the combined effects of chemically distinct contaminants on them remain poorly explored. This study investigated the chronic toxicity of perfluorooctanoic acid (PFOA), a persistent per- and polyfluoroalkyl substance (PFAS), and the cyanotoxin Cylindrospermopsin (CYN), individually and in binary mixtures, on the freshwater microalga *Monoraphidium capricornutum*. Algal cultures were exposed for 12 days to PFOA (1000–2000 µg/L) and CYN (50–100 µg/L). Growth effects, antioxidant biomarkers (Glutathione (GSH), Glutathione S-transferase (GST), Superoxide Dismutase (SOD), Catalase (CAT)), and oxidative damage indicators (protein carbonylation and lipid peroxidation) were assessed. The biomarkers showed significant reduction in GST activity (up to 64,37% at

the higher concentration of PFOA) and an increase in glutathione concentration (up to 157,31% in exposures to the binary mixtures of PFOA + CYN) indicating a dysfunction in the antioxidant system in parallel with a compensatory attempt by the cell in response to oxidative stress. Although SOD and CAT activities varied, no significant modulation was detected. In addition, the increase in protein carbonylation (up to 36,07% in exposures to the binary mixtures) confirms that the contaminants effectively caused oxidative damage. However, the oxidative defense mechanisms of this species (studied in the first 4 days of exposure) showed that they can be complex but responsive, and therefore likely contributed to recovery to control growth levels after 12 days in the various exposures to contaminants. The findings contribute insights into sublethal stress mechanisms and adaptive responses in aquatic primary producers exposed to emerging contaminants.

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1 Introduction

Perfluorooctanoic acid (PFOA) and other per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals widely used for various applications, such as surfactants, water repellents, food packaging, and fire-fighting foam due to their resistance to heat,

water, oil, and diverse chemicals (Ford et al., 2024). These unique properties result from the strong carbon–fluorine bond, which provides high stability and low reactivity to these compounds (Galloway et al., 2020). However, these characteristics also make all PFAS extremely persistent in the environment (Cáñez et al., 2021) and in organisms (Ford et al., 2024), leading to their bioaccumulation and contamination of water bodies worldwide (Cáñez et al., 2021; Guo et al., 2024). PFAS occur widely in surface water, including lakes and reservoirs, many of which face eutrophication or pollution associated with human activities. Pan et al. (2019) investigated the occurrence, distribution, and bioaccumulation of per- and polyfluoroalkyl substances (PFAAs) in Lake Chaohu (China), a freshwater lake subject to anthropogenic pressures, and found multiple PFAS compounds widely distributed in both the water column and the sediments and biota analyzed, suggesting diffuse PFAS contamination in this lake system. Another study conducted by Qi et al. (2016) analyzed the occurrence and spatial distribution of PFAS in surface sediments from different lake regions in China and detected these compounds in all areas studied, indicating that PFAS are widely present in continental lake environments subject to anthropogenic pressures. Reports of PFAS occurrences in the environment show a wide range of detected concentrations. In the French Overseas Territories, PFAS levels ranged from below the detection limit to 77 ng/L in surface waters and up to 638 ng/L in groundwater, with PFOS and PFOA being the predominant compounds (Munoz et al., 2017). In Japan, near a fluoropolymer industry, PFOA concentrations decreased while PFHxA increased in well and surface water suggesting a replacement of PFOA in the industry (Shiwaku et al., 2016). In rapidly development coastal areas of China and South Korea, per- and polyfluoroalkyl substances were detected in major rivers, and the total concentrations ranged from 14.9 to 16,500 ng L⁻¹ (Shi et al., 2021). Besides, it is well known that PFAS have significant environmental mobility, being detected long distances away from where they were produced or used (Itumoh et al., 2024).

Furthermore, industrial and domestic effluents frequently contain a wide diversity of chemicals including PFAS and natural toxins, such as cyanotoxins, that can react with each other or act in synergy, exacerbating aquatic pollution (Liu et al., 2022;

Pinheiro et al., 2016). This scenario is further intensified by the high load of nutrients, from agricultural leaching and industrial or domestic effluents, which causes artificial eutrophication and is frequently associated with cyanobacteria intense growth, which also compromises water quality (Ibelings et al., 2021). The increase in atmospheric CO₂ concentration and global temperature creates even more favorable conditions for this microorganism's development (Paerl, 2016), generating major concerns due to the possible release of cyanotoxins, which are harmful to aquatic organisms and human health (Ibelings et al., 2021). Although there is no systematic evidence directly linking eutrophication to the occurrence of PFAS, the frequent presence of these compounds in lakes and reservoirs under anthropogenic pressure, including eutrophicated environments, suggests the possibility of co-occurrence of both classes of contaminants, highlighting the importance of integrated approaches in the assessment of multiple chemical stressors (Pan et al., 2019; Qi et al., 2015).

Contamination by pollution substances such as PFOA and cyanotoxins represents a significant threat to the aquatic ecosystem due to their environmental persistence, broad toxicity and bioaccumulation (Buratti et al., 2017; Wang et al., 2025). Both organic microcontaminants (despite their chemical differences) interfere with essential cellular processes and can induce chronic toxicity (Barry et al., 2013; Huang & Zimba, 2019). Much of this damage is related to an increase in reactive oxygen species (ROS) production, which generates oxidative stress, and can cause dysfunctions in organelles such as mitochondria and chloroplasts, damage to cell membranes, genetic mutations, compromising cellular functionality (Rezayian et al., 2019; Mhamdi & Van Breusegem, 2018). Moreover, such biochemical and cellular effects can contribute on a larger scale to disturbances in food chains, reduction of biodiversity, and the compromise of the functional structure of aquatic ecosystems (Hong et al., 2015; Ma et al., 2022).

To understand environmental impacts, several techniques are adopted, such as incorporating ecotoxicological assays that contribute to the evaluation of bioavailability and interactions between pollutant effects (Pastorino et al., 2024). Biomonitoring, which uses living organisms (bioindicators), to assess environmental changes, is essential for quantifying the effects of toxic substances in ecosystems. In this

context, quantification of biomarkers allows the correlation of biological effects with contaminant exposure, and this approach provides rapid and accurate assessments of environmental impacts, surpassing traditional toxicological sensitivity methods (Goulart & Callisto, 2003; Pikula et al., 2019).

Microalgae are essential primary producers in aquatic ecosystems, being important energy transfers in aquatic food chains (Stephenson et al., 2011). In addition, they are valuable ecological indicators in the assessment of water quality and ecotoxicity of pollutants due to their high sensitivity, short generation time and high surface area to volume ratio (Devi et al., 2023; Hu et al., 2020; Ma et al., 2022). The growing interest in the effects of PFAS on microalgae stems from their widespread detection in both surface water and drinking water (Cañez et al., 2021; Miranda et al., 2021). Since microalgae often show a rapid response to pollution, this allows for the early detection of ecological changes caused by these contaminants (McCormick & Cairns, 1994; Niu et al., 2019).

Previous studies have shown that PFOA negatively impacts the growth and physiology of various microalgae. Hu et al. (2014) reported that *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* exhibit biochemical alterations and growth inhibition when exposed to PFOA, including increased lipid peroxidation and activation of oxidative defense mechanisms. In *Microcystis aeruginosa*, Hu et al. (2023) observed that PFOA induces oxidative stress, impairs respiration and photosynthesis, and triggers adaptation mechanisms to mitigate damage. Additionally, PFOA toxicity can be modulated by interactions with other pollutants. Zhang et al. (2023) also demonstrated in *Microcystis aeruginosa* that combined exposure to PFOA and PVC microplastics intensified oxidative stress, caused cell membrane damage, and reduced the production of extracellular polymeric substances. Similarly, Zhao et al. (2024) showed that co-exposure to PFOA and polystyrene microplastics resulted in synergistic effects, altering ribosome biogenesis, photosynthesis, and energy metabolism in *Chlorella sorokiniana*. However, the interactive effects of toxic substances in aquatic organisms, especially among primary producers, remain poorly understood (Pinheiro, et al., 2016; Hu et al., 2020).

Therefore, the aim of this study was providing novel insights into the combined ecotoxicological

effects of perfluorooctanoic acid (PFOA) and cylindrospermopsin (CYN)—two environmentally persistent and chemically distinct pollutants—on the freshwater microalgae *Monoraphidium capricornutum*. The data contribute to advancing our understanding of the mechanisms of sublethal and chronic toxicity and highlight the ecological relevance of assessing scenarios involving mixtures of pollutants.

2 Methodology

2.1 Microalgae and Cyanobacteria Culturing and Chemicals

Strains of the microalgae *Monoraphidium capricornutum* and the cyanobacterium *Raphidiopsis raciborskii* (CYP011K strain) were kindly provided by the Laboratory of Ecophysiology and Toxicology of Cyanobacteria (LETC/IBCCF) of the Federal University of Rio de Janeiro (UFRJ), Brazil. The microorganisms were cultivated in 1 L Erlenmeyer flasks with ASM-1 culture medium, pH adjusted to 7 (Gibson & Smith, 1982). The cultures were maintained with aeration in a controlled environment at 25 ± 2 °C and 12-h photoperiod with a light intensity of approximately 49.95 μE using white LEDs.

The cylindrospermopsin extract was obtained from culturing of the CYP011K strain until the end of the exponential growth phase, at which point it was interrupted and underwent a process of three cycles of freezing and thawing to cause cell lysis and release of the intracellular cylindrospermopsin into the liquid medium, followed by filtration. The CYN extract was stored in freezer (-20 °C) and a sample was analyzed through high-performance liquid chromatography coupled with mass spectrometry (LC–MS/MS) for the determination of cylindrospermopsin concentration, as described by Eaglesham et al. (1999). Then, it was used with appropriate dilution in culture medium in the CYN treatments.

Perfluorooctanoic Acid (PFOA) was commercially acquired (Sigma Aldrich) with an analytical purity of 96% and dissolved in deionized water to prepare a stock solution at a concentration of 1000 mg/L. Dilutions were applied to achieve the determined concentration in the culture medium for each treatment in the experiments.

2.2 Experimental Set-Up

M. capricornutum was chosen as the test phototrophic microorganism due to its ease of cultivation and counting, rapid growth, and wide global distribution (Hage et al., 2018). The microalgae were first exposed to the contaminants individually. The treatments of chronic exposure to the cyanobacteria extract containing cylindrospermopsin were set at 100 µg/L (CYN-HC) and 50 µg/L (CYN-LC), and PFOA treatments were set at 2000 µg/L (PFOA-HC) and 1000 µg/L (PFOA-LC). These concentration values were within the range already found in the environment for both contaminants and were chosen based on preliminary tests to verify the lethal and sublethal toxicity range (unpublished data). The experiment began with a cell density of 2.20×10^4 cells/mL of *M. capricornutum* (which enabled it to quickly reach the exponential growth phase) and carried out in triplicate in Erlenmeyer flasks with a final volume of 100 mL for all treatments and the control condition (cultures without contaminants). The cultures were maintained for 12 days in a controlled environment, following the same culture conditions as described in item 2.1. Samples from each treatment were collected for evaluation of growth (12 days) and antioxidant response biomarkers (96 h).

$$\text{Number of cells/mL} = \frac{\text{total number of cells} \times \text{dilution factor}}{\text{number of counted quadrants}} \times 10^4 \quad (1)$$

The specific growth rate (μ) was calculated using Eq. (2) described by Fogg and Thake (1987):

$$\mu = \ln\left(\frac{N_t}{N_0}\right) \times (t - t_0)^{-1} \quad (2)$$

where N_0 and N_t are defined as the cell density at the initial time and time (t), respectively (cells/mL); ($t - t_0$) is the time interval between counts. The obtained values were expressed as mean $\pm$ standard error (SE).

After calculating the growth rate, the division rate per day (K) was determined using Eq. (3) described by Andersen (2005):

$$K = \frac{\mu}{\ln(2)} \quad (3)$$

Following, a binary mixture assay was conducted to assess the combined effects of CYN and PFOA on the microalgae. The concentration combinations included three scenarios: (1) CYN-HC (100 µg/L)+PFOA-LC (1000 µg/L), (2) CYN-LC (50 µg/L)+PFOA-HC (2000 µg/L), and (3) CYN-HC (100 µg/L)+PFOA-HC (2000 µg/L). As in the individual exposure test, the experiment was performed in triplicate for all treatments and the control condition, with an initial density of 2.24×10^4 cells/mL of *M. capricornutum*. The cultures were maintained for 12 days in a controlled environment, following the same culture conditions as described in item 2.1. Samples from each treatment were collected for evaluation of growth (12 days) and antioxidant response biomarkers (96 h).

2.3 Cell Growth Assessment

Cell counts of the microalgae (Andersen, 2005) were performed at 48-h intervals during the exposure period, totaling 12 days. A Neubauer chamber and a bright field optical microscope (Leica DM LB2) operating at 200 $\times$ magnification were used. The samples were diluted and the cell density was calculated using Eq. (1) from Guillard (1973):

Consequently, the doubling time (G) was obtained as the inverse of the division rate per day (K), calculated using Eq. (4):

$$G = \frac{\ln(2)}{\mu} \quad (4)$$

2.4 Biomarkers Analyses

2.4.1 Microalgae Cell Protein Extraction

A volume of 100 µL of each control and treatment replicate (and 500 µL for MDA analysis) was stirred in a vortex with the addition of glass beads (diameter of 212–300 µm, SIGMA). Following, 50 mM potassium phosphate buffer (pH=7.0) was added in three

steps of homogenization: 500 μL in the first, 500 μL in the second, and 1000 μL in the third. For the MDA analysis, RIPA buffer was used instead of the potassium phosphate buffer. The vortex homogenization occurred for 1 min and it was then transferred to an ice bath to keep the extract cooled. This process was repeated with each buffer addition, and at the end, the samples were centrifuged at $3000\times g$ for 15 min at 4 °C, and the supernatant was stored for further analysis. Protein extraction was performed according to the method described by Slocombe et al. (2013), with modifications. The extracted proteins were then quantified using the Lowry method (Waterborg, 2009). Total protein data was used to normalize biomarker results.

2.4.2 Antioxidant System

The quantification of glutathione (GSH) was performed through spectrophotometry using the method described by Wilhelm Filho et al. (2005). The GSH (10 mM) and DTNB (0.25 mM) solutions were prepared on the day of the experiment. Briefly, treatment and control samples were analyzed by adding 100 μL of the sample and 100 μL of DTNB. The analytical curve, covering concentrations up to 120 μM , was produced with aliquots of the GSH stock solution diluted in 0.1 M phosphate buffer (pH 7.0) and addition of DTNB solution (50 μL of GSH solution and 100 μL of DTNB). Samples of the analytical curve and experiment were incubated in the dark for 15 min before readings on a microplate reader (DR-200BN – Kasuaki) to determine the absorbance at $\lambda=412$ nm. GSH concentration was expressed in $\mu\text{mol/g}$ of protein.

Superoxide dismutase (SOD) activity was determined using the commercial kit from Cayman Chemical (Michigan, USA), with adaptations. The assay was prepared using 10 μL of sample, 200 μL of tetrazolium salt solution (RD), and 20 μL of xanthine oxidase (XO) in 96-well plates, protected from direct light throughout the procedure. The reaction was incubated for 30 min at room temperature. Enzymatic activity was determined based on the ability of SOD to inhibit the reduction of tetrazolium salt (RD). This reaction occurs because SOD catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2), reducing the formation of the formazan dye. The reading was performed

on a microplate reader ($\lambda=440\text{--}460$ nm) and SOD activity was calculated using the linear regression curve (SOD Activity $\times$ Linearized Rate—LR) and expressed in units (U).

Catalase (CAT) activity was determined by the method of Aebi (1984), which quantifies the decomposition of 1 μmol of hydrogen peroxide (H_2O_2). CAT, a widely occurring intracellular enzyme, catalyzes the conversion of H_2O_2 into water and oxygen ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). The analysis was performed in quartz cuvettes, with the sequential addition of 400 μL of sample, 200 μL of phosphate buffer, and, after 15 s, 200 μL of H_2O_2 (30 mM). Absorbance readings were taken on a UV–Visible spectrophotometer (BEL Photonics) at $\lambda=240$ nm in 15-s intervals. The enzymatic activity was calculated based on Beer's Law, considering the international unit of activity, and normalized to the protein content present in the sample.

Glutathione S-transferase (GST) activity was determined according to the method of Habig et al. (1974), monitoring the formation of GS-DNB/min at 340 nm for 60 s using a UV–Vis spectrophotometer (BEL Photonics). The samples were prepared in quartz cuvettes containing 1350 μL of phosphate buffer (100 mM, pH 6.5), 50 μL of reduced glutathione (30 mM), 50 μL of CDNB (30 mM), and 50 μL of the sample. After homogenization, readings were taken, and the enzymatic activity was calculated based on the Beer-Lambert Law, considering the international unit of activity corrected by the protein concentration.

2.4.3 Oxidative Stress Biomarkers

The analysis of carbonylated proteins (CP) was performed using the method of Mesquita et al. (2014). The reaction mixture was prepared on the microplate, using 80 μL of phosphoric acid ($0.5 \text{ mol L}^{-1} \text{ H}_3\text{PO}_4$) and 80 μL of the control sample; and for the treatments: 80 μL of 2,4-dinitrophenylhydrazine (10 mmol L⁻¹ DNPH), 80 μL of the samples and 40 μL of sodium hydroxide ($6 \text{ mol L}^{-1} \text{ NaOH}$). After adding DNPH to the samples (except the controls), the reaction was incubated for 10 min at room temperature, allowing the formation of the dinitrophenylhydrazone complex. Then, NaOH was added to all wells. Spectrophotometric readings were taken using a microplate reader (DR-200BN – Kasuaki) at 450 nm. The results were calculated based on the Beer-Lambert

law ($\text{Abs}=\epsilon \cdot b \cdot c$, with $\epsilon=22.308 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) and expressed in nanomoles of carbonylated protein per milligram of total protein (nmol CP/mg protein).

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA), following the method of Esterbauer and Cheeseman (1990) with the TBARS Assay Kit (Cayman Chemical, Michigan, USA). Briefly, 100 μL of sample or MDA standard + 100 μL of 10% TCA + 800 μL of 0.53% TBA were homogenized in a vortex and subjected to a water bath at 90°C for 1 h. A 300 μL aliquot of the blank, standard and experiment samples was transferred to a microplate. Spectrophotometric readings were taken using a microplate reader (DR-200BN – Kasuaki) at 535 nm and MDA concentration in the samples was expressed as $\mu\text{mol/L}$ (μM).

2.5 Statistical Analyses

Statistical analyses were performed using the Graphpad Prism version 9.5.1. Data were expressed as means or medians $\pm$ standard deviations (SD), depending on the data distribution, verified by the Shapiro–Wilk test. An analysis of variance (ANOVA) followed by the Dunnett-test was applied to normally distributed data and the Kuskall-Wallis followed by the Dunn-test to non-normally distributed data. Differences were considered significant at $p \leq 0.05$.

3 Results and Discussion

3.1 Effects of PFOA and CYN on Growth

The effects on growth of *Monoraphidium capricornutum* exposed to individual contaminants over 12 days are presented in Fig. 1. Although the microalgae behavior at higher concentrations of PFOA (2000 $\mu\text{g/L}$) and CYN (100 $\mu\text{g/L}$) indicated statistically significant growth inhibition in relation to control on the 6th and 10th days for PFOA-HC (36.60% and 32.84%, respectively) and on the 10th day for CYN-HC (27.27%), a general recovery was observed by the end of the exposure period (12th day). The CYN-LC (50 $\mu\text{g/L}$) treatment showed slightly higher growth at the end of the exposure period compared to the other treatments. However, there was no statistically significant difference in relation to the control treatment in that point.

The study conducted by Pinheiro et al. (2016) demonstrated that CYN, at concentrations above 38.7 mg/L, significantly impairs the growth of *Chlorella vulgaris* after 4 days of exposure. Albuquerque et al. (2024) also observed growth inhibition and photosynthesis damage in the same microalgae species in much lower concentrations (1.42 $\mu\text{g/L}$). These effects may be related to protein synthesis inhibition and oxidative stress, which are key mechanisms of CYN toxicity (Pinheiro et al., 2016). These findings reinforce the potential of CYN to affect essential cellular

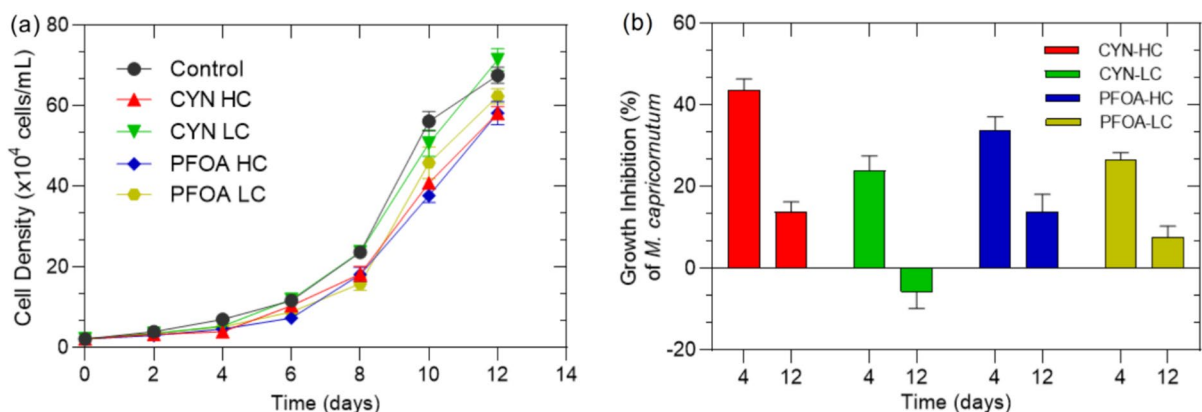


Fig. 1 **a** Cell Density and **b** Growth Inhibition (%) in relation to control value presented in figure a) of *Monoraphidium capricornutum* exposed to the treatments: PFOA-HC (2000 $\mu\text{g/L}$); PFOA-LC (1000 $\mu\text{g/L}$); CYN-HC (100 $\mu\text{g/L}$) and CYN-LC (50 $\mu\text{g/L}$), individually, for 12 days. A statistically significant

inhibition of growth was observed on the 10th day for CYN-HC and on the 6th and 10th day for PFOA-HC. The values represent the mean $\pm$ standard error ($n=3$). Differences were considered significant at $p \leq 0.05$ (ANOVA data in supplementary material)

processes, directly impacting population dynamics in aquatic ecosystems. Mojiri et al. (2023) investigated the effects of PFOA and PFOS on the growth of *Chlorella vulgaris* and *Scenedesmus obliquus* and reported that exposure to concentrations above 1 mg/L resulted in significant growth inhibition, with *Scenedesmus obliquus* being more sensitive than *C. vulgaris*. Toxicity was associated with a reduction in protein and chlorophyll content, suggesting a negative impact on photosynthesis and cellular metabolism (Mojiri et al., 2023). Additionally, Liu et al. (2022) evaluated the effects of other PFAS (PFBS and FBFA) exposure on the growth of *Chlorella pyrenoidosa* over 12 days, using concentrations ranging from 100 ng/L to 100 mg/L. The study indicated that growth inhibition was concentration-dependent, showing an additive effect at higher concentrations (mg/L) and a synergistic effect (the toxicity of the binary mixture was greater than the sum of the individual effects) at environmental concentrations (ng/L – µg/L). The toxic effects were attributed to disruptions in photosynthesis, increased oxidative stress, and inhibition of DNA replication (Liu et al., 2022). Notably, the more severe impact of this binary mixture on algal growth reinforces the need to evaluate contaminant mixtures rather than isolated pollutants. In the present study, although the concentrations used may also have caused temporary growth inhibition, they were not toxic enough to completely prevent the development of *M. capricornutum* over time. The values of the growth rate (μ), divisions per day (K) and doubling time (G) for the microalgae exposed to the different individual treatments and the control are shown in Table 1. At the end of the exposure period (12 days), the treatments of PFOA and CYN showed values like those of the control, suggesting mechanisms of

cellular adaptation. Zhao et al. (2015) pointed out that the antioxidant mechanisms of microalgae can be activated in response to moderate exposure to contaminants. This can occur by increasing antioxidant enzyme activity and/or regulating photosynthesis and metabolism to ensure survival under stressful conditions (Hu et al., 2023; Zhao et al., 2024). It is worth mentioning that, in the natural environment, variations in abiotic factors, such as pH and temperature or biotic interactions, can compromise this positive response to a toxic contaminant, as highlighted by Nikinmaa (2014).

Most ecotoxicological studies focus on the effects of individual substances, limiting the understanding of the complex interactions between substances that occur in the natural environment. The growth of *M. capricornutum* was also evaluated when exposed to three combinations of high and low concentrations of contaminants: (1) CYN-HC (100 µg/L)+PFOA-LC (1000 µg/L); (2) CYN-LC (50 µg/L)+PFOA-HC (2000 µg/L); and (3) CYN-HC (100 µg/L)+PFOA-HC (2000 µg/L), as presented in Fig. 2. Although the treatments showed growth inhibition, statistical analysis indicated that at the end of 12 days the cell densities did not differ significantly from the control group, except for the CYN-HC +PFOA-LC treatment which showed growth inhibition of 30.1% (Fig. 2b. ANOVA data in supplementary material). In the other treatments of CYN and PFOA mixtures, *M. capricornutum* showed the capacity to recover homeostasis, like in the results with the individual contaminants.

The results observed with the combination of the highest concentrations of both contaminants (CYN-HC: 100 µg/L+PFOA-HC: 2000 µg/L) suggest an interaction between the compounds generating a possible antagonistic effect, as at the end of 12 days growth inhibition was lower (less than 10%) in treatments of PFOA HC with both CYN concentrations than in the mixture with PFOA at a lower concentration (30,1%) (Fig. 2b). Besides, as presented in Table 2, the growth rate ($\mu=0.2764$) in the CYN-HC+PFOA-HC treatment was higher than in the CYN-HC +PFOA-LC treatment ($\mu=0.2359$), indicating that the presence of PFOA at a higher concentration may have influenced the effect of CYN. However, as the growth rate remained lower than the control group ($\mu=0.2794$) and CYN alone did not cause an equivalent inhibition, further investigation is needed to determine whether this effect is due to antagonistic

Table 1 Calculated values for growth rate (μ), divisions per day (K), and doubling time (G) for *Monoraphidium capricornutum* exposed to CYN and PFOA individually

TREATMENT	GROWTH RATE	DIVISIONS PER DAY RATE	DOUBLING TIME RATE
CONTROL	0.2850	0.4112	2.4321
CYN-HC	0.2719	0.3923	2.5489
CYN-LC	0.2917	0.4208	2.3763
PFOA-HC	0.2659	0.3837	2.6064
PFOA-LC	0.2836	0.4091	2.4441

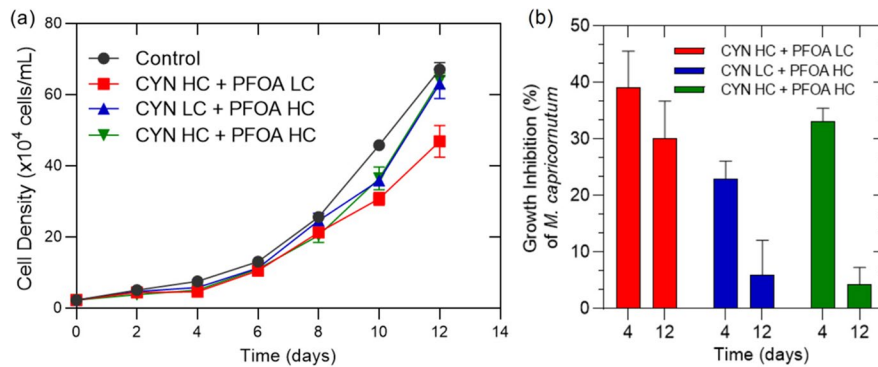


Fig. 2 **a** Cell Density and **b** Growth Inhibition (%) in relation to control value presented in figure a) of *Monoraphidium capricornutum* exposed to the mixture of PFOA and CYN: (1) CYN-HC (100 µg/L)+PFOA-LC (1000 µg/L) (red bars); (2) CYN-LC (50 µg/L)+PFOA-HC (2000 µg/L) (blue bars); and

(3) CYN-HC (100 µg/L)+PFOA-HC (2000 µg/L) (green bars). The values represent the mean $\pm$ standard error ($n=3$). Differences were considered significant at $p \leq 0.05$ (ANOVA data in supplementary material)

Table 2 Calculated values for growth rate (μ), divisions per day rate (K), and doubling rate (G) for *Monoraphidium capricornutum* exposed to CYN and PFOA in binary mixture

TREATMENT	GROWTH RATE	DIVISIONS PER DAY RATE	DOUBLING TIME RATE
CONTROL	0.2794	0.4030	2.4811
CYN-HC+PFOA-LC	0.2359	0.3404	2.9380
CYN-LC+PFOA-HC	0.2870	0.4140	2.4155
CYN-HC+PFOA-HC	0.2764	0.3987	2.5079

interaction or other physiological responses of the microalgae to combined exposure. Previous studies have observed an antagonistic effect in HepG2 cells exposed to mixture of CYN (500 µg/L) and BPA (10,000 µg/L) (Hercog et al., 2020; Metcalf & Codd, 2020). The authors verified a lower induction of DNA strand breaks (DSBs) in the cells exposed to the mixture when compared to those exposed to CYN alone. Hinojosa et al. (2020) evaluated the effects of a mixture of CYN and the pesticide chlorpyrifos on human SH-SY5Y cells and also found antagonistic interactions between the contaminants. These investigations suggest that some substances may reduce the toxic effects of CYN, but the complexity of these interactions requires further research.

The calculation of growth rate (μ), divisions per day (K) and doubling time (G) for *M. capricornutum* exposed to mixture of CYN and PFOA was performed for the different treatments and control, as shown in Table 2. It can be observed that at the end of the 12th day values remained relatively close to control. Although the CYN-HC+PFOA-LC treatment

exhibited a lower growth rate compared to the others, the general data suggests that *M. capricornutum* was able to recover and sustain its growth and supports the hypothesis of efficient physiological adaptation also to the combined exposure of contaminants.

Reviewing the literature, it was not possible to find other studies that also investigated the effects of PFAS and/or CYN on the genus *Monoraphidium*. However, there are reports of investigations into the impact of these pollutants on other microalgae. Microalgae responses depend on species, contaminants and concentrations involved, as the following studies indicate. Liu et al. (2022) reported, as mentioned above, that the combination of PFAS had a more intense inhibitory effect on growth in *Chlorella pyrenoidosa* than individual PFAS. Zhao et al. (2024) demonstrated that co-exposure of *Chlorella sorokiniana* to PFOA and polystyrene microplastics also resulted in a synergistic impact on the growth inhibition. The authors verified that microplastics increased PFOA bioavailability, leading to alterations in essential metabolic processes, such as photosynthesis and ribosome

biogenesis, resulting in a greater reduction in the growth rate. Pinheiro et al. (2016) also observed that microcystin-LR at concentrations of 0.4–16.7 mg/L increased the toxicity of CYN to *Chlorella vulgaris*. And besides microalgae, Zhang et al. (2023) suggest that, depending on the context and the contaminants involved, the interaction may intensify the toxic effects on cellular growth of cyanobacteria. These reports highlight the relevance of the interaction of contaminants for ecotoxicity assessments.

3.2 Antioxidant System Alterations

The activity of glutathione S-transferase (GST) in *M. capricornutum* showed a significant reduction in the PFOA-HC (64,37%), CYN-HC+PFOA-LC (52,36%), and CYN-LC+PFOA-HC (62,80%) treatments (Fig. 3). GST acts by catalyzing the binding of glutathione (GSH) to xenobiotic compounds, playing a crucial role in detoxification processes (Zhang et al., 2023). The decrease in its activity suggests a reduced capacity of the cell to metabolize and eliminate toxins such as PFOA and CYN, prolonging the retention of contaminants in the cell, which could potentially enhance their toxic effects over time.

At the same time, a significant increase in GSH levels (Fig. 3b) was also observed (PFOA-LC: 129,29%, CYN-HC+PFOA-LC: 157,31%, CYN-LC+PFOA-HC:150,68%), which may in part be the result of lower consumption by GST. On the other hand, it may be a response to its demand by glutathione peroxidase (GPx), which plays a key role in the degradation of peroxide (Flohé et al., 2022). This process may indicate an accumulation of peroxide in the cell (Chalifoux et al., 2023), reinforcing the hypothesis that, in this context, the antioxidant system may be overloaded and possibly insufficient to fully neutralize the oxidative stress induced by exposure to contaminants (Mao et al., 2023; Campos et al., 2013; Mazari et al., 2023; Kumar & Trivedi, 2018). It is worth noting that the relevant effects were caused mainly by the mixtures (CYN-HC+PFOA-LC and CYN-LC+PFOA-HC), since most of the individual treatments (except PFOA-LC) of exposure to contaminants did not produce statistically significant effects on GST activity and GSH.

The results of exposure to individual PFOA at lower concentrations (PFOA-LC) indicate that the contaminant induces an antioxidant response by increasing GSH levels. In parallel, when PFOA concentration is increased (PFOA-HC), a rise in GSH

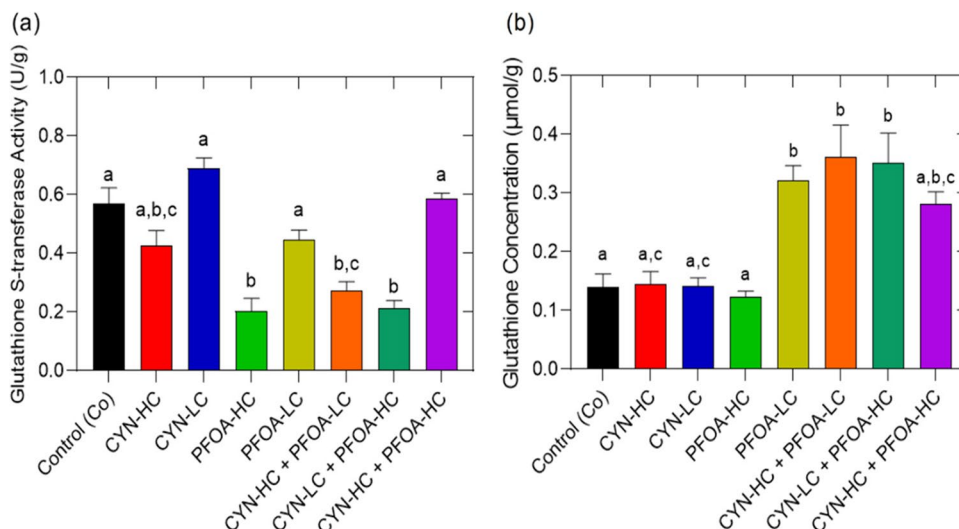


Fig. 3 **a** Glutathione S-Transferase Activity and **b** Glutathione levels under experimental conditions: (I) Control; (2) Individual exposure to PFOA and CYN (CYN-HC: 100 µg/L; CYN-LC: 50 µg/L; PFOA-HC: 2000 µg/L; PFOA-LC: 1000 µg/L); and (3) Binary mixture exposure of PFOA

and CYN (I) CYN-HC: 100 µg/L+PFOA-LC: 1000 µg/L; (II) CYN-LC: 50 µg/L+PFOA-HC: 2000 µg/L; (III) CYN-HC: 100 µg/L+PFOA-HC: 2000 µg/L). Values represent the mean ± standard error (n=3). Different letters indicate a significant difference ($p \leq 0.05$)

synthesis is no longer observed and glutathione S-transferase inhibition is observed. These findings suggest that higher concentrations of this contaminant impair the functioning of the cellular antioxidant system, compromising redox homeostasis (Mao et al., 2023; Mazari et al., 2023). Mao et al. (2023) investigated the impact of PFOA on *Chlorella* sp. (0.1–10 mg/L, 96 h), and also found that low concentrations increased GSH levels, while high concentrations negatively affected redox homeostasis, including GST inhibition. Additionally, the exposure to PFOA and PFOS in mixtures intensified oxidative stress, exacerbating damage to antioxidant mechanisms.

Superoxide Dismutase (SOD) and Catalase (CAT) are essential antioxidant enzymes for defense against oxidative stress. SOD converts superoxide radicals into hydrogen peroxide, while CAT subsequently breaks down hydrogen peroxide into water and oxygen (Hong et al., 2024). In the present study, SOD and CAT activity varied considerably among treatments, with no significant difference compared to the control. CAT was possibly more activated in the CYN-HC, CYN-HC+PFOA-LC and CYN-LC+PFOA-HC groups, while SOD was likely more activated in the PFOA-treated groups (Fig. 4).

Xu et al. (2013) observed a significant increase in SOD activity in *Chlorella pyrenoidosa* exposed to PFOA at concentrations ranging from 30 to 120 mg/L after 192 h of exposure. The concentrations were

substantially high, which may have generated sufficient oxidative pressure to induce significant responses in the antioxidant system in this species. Differences in exposure times may also have contributed to the difference of results from the present study. Other studies also have shown that the modulation of SOD and CAT activity in microalgae is influenced by contaminant concentration, exposure duration, and species-specific responses. Niu et al. (2019) reported increased SOD activity in *Chlorella* sp. exposed to PFAS, while CAT were inhibited, suggesting an imbalance in antioxidant defenses. Zhao et al. (2023) found that co-exposure to PFOA and polystyrene microplastics (PS-MPs) intensified oxidative stress and also increased the antioxidant enzymatic (SOD and CAT) activity in *Chlorella sorokiniana*. Chia et al. (2017) reported an increase in SOD activity in the cyanobacterium *Microcystis aeruginosa* exposed to lower concentrations of 6.25 µg/L and 25 µg/L of CYN for a longer period of 120 h. These results corroborate the potential of contaminants to alter the activity of antioxidant enzymes in phytoplankton.

The present study indicates that CYN and PFOA, either individually or in combination, influence oxidative defense mechanisms, as evidenced by increased GSH levels and GST inhibition or imbalanced responses of SOD and CAT, suggesting an

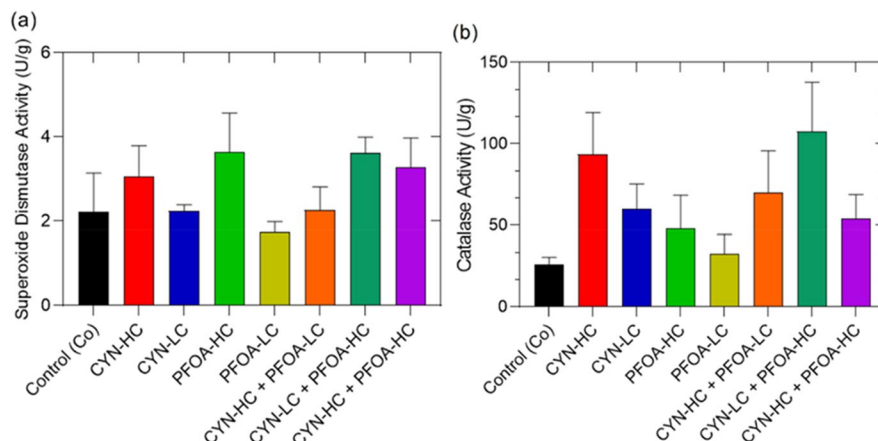


Fig. 4 **a** Superoxide Dismutase Activity and **b** Catalase Activity under Experimental Conditions: (I) Control; (2) Individual Exposure to PFOA and CYN (CYN-HC: 100 µg/L; CYN-LC: 50 µg/L; PFOA-HC: 2000 µg/L; PFOA-LC: 1000 µg/L); and (3) Binary Mixture Exposure to PFOA

and CYN (I) CYN-HC: 100 µg/L+PFOA-LC: 1000 µg/L; (II) CYN-LC: 50 µg/L+PFOA-HC: 2000 µg/L; (III) CYN-HC: 100 µg/L+PFOA-HC: 2000 µg/L). Values represent mean ± standard error (n=3). No significant difference was observed between treatments ($p > 0.05$)

increased antioxidant demand due to the exposure to these contaminants.

3.3 Oxidative Effects

Carbonylated proteins are an important indicator of oxidative damage, formed by the direct attack of reactive oxygen species (ROS) on amino acids (Madian & Regnier, 2010). Besides, other damage to proteins can result from its interaction with aldehydes derived from lipid peroxidation, which alter its structures (Weng et al., 2017). These processes affect protein function and cellular integrity, contributing to oxidative stress and compromising cell viability (Colombo et al., 2018). In this study, the microalgae exposed to contaminants exhibited increase in protein carbonylation, in relation to control, in all treatments with binary mixtures (PFOA-LC: 20,15%, CYN-HC + PFOA-LC: 32,53%, CYN-LC + PFOA-HC: 32,15%, CYN-HC + PFOA-HC: 36,07%), as well as in the lowest concentration of PFOA individually (20,15%, Fig. 5a).

On the other hand, the lipid peroxidation effect (which is the attack of free radicals to lipids, especially polyunsaturated fatty acids (Ayala et al., 2014) was observed exclusively in the combination of higher CYN concentration with lower PFOA

concentration (38,43%, Fig. 5b). These findings indicate damage to cell membranes and oxidative stress (Rezayian et al., 2019), resulting from an imbalance between ROS production and the cells' antioxidant capacity, impairing metabolic and signaling functions (Yang et al., 2021).

Hu et al. (2014) observed a significant increase in LPO (MDA content) in the algae *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* exposed for eight days to PFOA at concentrations of 20 mg/L and 40 mg/L, indicating cellular damage caused by oxidative stress. Lower concentrations (below 10 mg/L) did not cause significant effects ($p > 0.05$) on MDA levels in any of the species. The results of the present study indicate that lower individual PFOA concentrations (1–2 mg/L) did not cause lipid peroxidation in most of the treatments either. However, the lower concentration of PFOA (1 mg/L) combined with CYN produced this damage, suggesting that different oxidative damage mechanisms may be involved in mixtures of these contaminants. The study of Zhao et al. (2023), previously mentioned, showed that exposure to PFOA and polystyrene microplastics (PS-MPs) mixture, for 96 h, increased toxicity in the microalga *Chlorella sorokiniana* with a significant increase in intracellular ROS levels (up to 209.35%) and lipid peroxidation. In addition, the authors observed reductions in the level

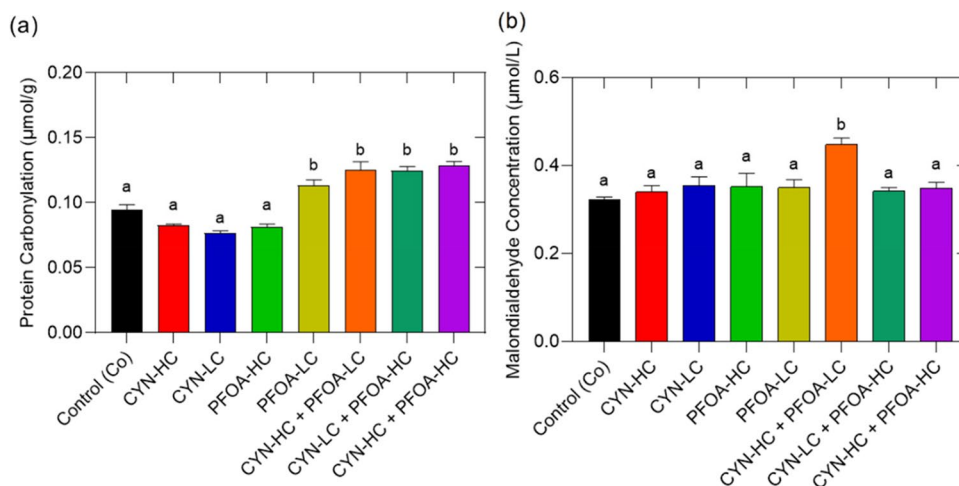


Fig. 5 **a** Protein Carbonylation and **b** Lipid Peroxidation (estimated by malondialdehyde concentration (MDA)) under experimental conditions: (I) Control; (II) Individual Exposure to PFOA and CYN (PFOA-HC: 2000 μg/L; PFOA-LC: 1000 μg/L; CYN-HC: 100 μg/L; CYN-LC: 50 μg/L); and (3) Binary Mixture Exposure of PFOA and

CYN (I) CYN-HC: 100 μg/L + PFOA-LC: 1000 μg/L; (II) CYN-LC: 50 μg/L + PFOA-HC: 2000 μg/L; (III) CYN-HC: 100 μg/L + PFOA-HC: 2000 μg/L). Values represent the mean ± standard error ($n = 3$). Different letters indicate a significant difference ($p \leq 0.05$)

of chlorophyll *a*, inhibition of photosynthesis, further exacerbating the toxic effects compared to exposure to each pollutant individually. These findings highlight the relevance of microalgae as bioindicators of environmental contamination due to their fundamental ecological roles in aquatic ecosystems. As primary producers, microalgae form the base of the trophic web, supporting higher trophic levels (Ma et al., 2022). Additionally, their high surface-area-to-volume ratio and rapid response (changes in their growth, oxidative stress responses, and photosynthetic efficiency) to environmental alterations make them highly sensitive to a diverse of pollutants (Devi et al., 2023; Ma et al., 2022).

Monoraphidium is a genus of green algae that is widely occurring and ecologically important. Because it is easy to cultivate, count, and grows rapidly, it can be considered a good model for ecotoxicological testing (Hage et al., 2018). Its responses to the contaminants in question can contribute to further research and advances in knowledge about the environmental damage caused by these pollutants.

Biomarkers demonstrated a significant adaptive response of this microalgae species to oxidative stress induced by PFOA and CYN. The activation of antioxidant systems, such as increased GSH synthesis and the possible resistance of SOD and CAT to oxidative imbalance, which caused damage such as protein carbonylation, suggest that these species possess defense mechanisms that contribute to their survival, as argued by Wang et al. (2022). This adaptive capacity certainly played a role in the recovery from cellular damage and the resumption of *M. capricornutum* growth during 12 days in various contaminant exposure scenarios. However, it is important to emphasize that long-term chronic exposure to toxic contaminants, such as those studied, may negatively influence the survival of this species in natural environments.

The toxicity of PFOA and other PFAS to microalgae remains poorly understood (Hu et al., 2020). Additionally, CYN can persist in water for several weeks to months due to its hydrophilic nature and chemical stability (Fadel et al., 2014). Therefore, it is crucial to assess its long-term impacts as well as its interactive effects with different contaminants (Pinheiro et al., 2016).

4 Conclusion

This study aimed to investigate the chronic toxicity of perfluorooctanoic acid, a persistent per- and polyfluoroalkyl substance, and the cyanotoxin Cylindrospermopsin, individually and in binary mixtures, on the freshwater microalga *Monoraphidium capricornutum*. It provides evidence that the microalgae undergoes significant physiological and biochemical changes when exposed to perfluorooctanoic acid (PFOA) and cylindrospermopsin (CYN), both individually and in combination. The observed alterations in antioxidant biomarkers—particularly glutathione elevation and glutathione S-transferase inhibition—along with protein carbonylation and lipid peroxidation, confirm that these contaminants elicit oxidative stress and compromise cellular integrity. Notably, exposure to contaminant mixtures intensified these effects, underscoring the complexity of chemical interactions and the inadequacy of assessing pollutants in isolation. Despite these challenges, *M. capricornutum* exhibited remarkable resilience and adaptive responses that allowed it to restore growth rates by the end of the exposure period, suggesting activation of compensatory defense mechanisms, such as the antioxidant system.

These findings advance our understanding of sublethal and chronic toxicity in primary producers and emphasize the ecological relevance of mixture toxicity assessment. The results also reinforce the use of microalgae as sensitive bioindicators in ecotoxicological studies and advocate for the incorporation of biomarker-based endpoints in regulatory frameworks. Considering the persistence and co-occurrence of emerging contaminants such as PFAS and cyanotoxins in aquatic environments, the insights generated by this research highlight the need for continued investigation into their toxic mechanisms in order to help improve environmental risk assessments and guide more protective water quality management policies.

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Data Availability The ANOVA data supporting Figs. 1–2 are publicly available in the Supplementary Material, as part of this record. Any other necessary data and clarifications can be provided upon justified request.

Declarations

Ethics Approval Not applicable.

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Competing Interests The authors declare that they have no financial competing interests or personal relationships that could have influenced the work reported in this article.

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