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Microplastics magnify inhibitive effects of perfluorooctanoic acid on the marine microbial loop

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ABSTRACT

The marine microbial loop comprising picophytoplankton, bacteria and microzooplankton is essential in global carbon cycling, which is currently affected by anthropogenic pollutants. Nonetheless, the impact of anthropogenic pollutants on the marine microbial loop remains elusive. In this study, perfluorooctanoic acid (PFOA) and microplastics (MPs) were selected as representative anthropogenic pollutants to investigate their impacts on the marine microbial loop with *Prochlorococcus* MED4 (picophytoplankton), *Alteromonas macleodii* EZ55 (bacteria), *Pseudocohnilembus persalinus* and *Cafeteria roenbergensis* (microzooplankton) as model microorganisms. The picophytoplankton was identified to be most sensitive to PFOA with a sensitivity order of MED4 > EZ55 > *C. roenbergensis* > *P. persalinus*. In contrast, polystyrene (PS) as a representative MP had less inhibition on the microbial loop, but synergistically magnified the inhibitive effects of PFOA on those four microorganisms. Moreover, PS significantly ($p < 0.05$) enhanced the bioconcentration and biomagnification of PFOA in the marine microbial loop, e.g., 1.89, 1.33, 1.22, and 2.18-fold increase in bioconcentration factor values in MED4, EZ55, *P. persalinus* and *C. roenbergensis*, respectively, compared to sole PFOA exposure. These results highlighted the exacerbated ecological risk of the co-existence of PFOA and MPs and provides the first insight into impacts of PFOA and PS on the marine microbial loop.

1. Introduction

The "microbial loop" being originally coined by Azam and colleagues mainly consists of phytoplankton, heterotrophic bacteria, and phagotrophic protists (F; et al., 1983). A picophytoplankton - *Prochlorococcus* - plays key roles in the microbial loop by converting inorganic carbon into organic matter, of which biomass nourish higher trophic organisms and facilitate nutrient transfer (Cai et al., 2024). *Prochlorococcus* is the most abundant photosynthetic populations in the ocean, and a critical contributor to the global primary production and carbon cycling, with a primary productivity of 4 Gt C y^{-1} (Flombaum et al., 2013). Consequently, the microbial loop has been broadened to include these vital photosynthetic players for mediation of carbon cycling and energy flows in marine ecosystems (Pomeroy et al., 2007). Heterotrophic bacteria re-mineralize the phytoplankton-derived dissolved organic matter (DOM) through respiration, of which biomass can be re-direct to a higher trophic level through grazing (Zhang et al., 2018). It was estimated that the marine microbial loop involved around half of the global primary production (Fenchel, 2008; Kirchman et al., 2009). Ciliates and flagellates (microzooplankton) as phagotrophic protists in oligotrophic areas are major consumers of picophytoplankton and bacteria, and are consequently critical players in microbial food webs by facilitating the circulation of nutrients (Labarre et al., 2020; Massana et al., 2009; Sherr and Sherr, 2002; Wilken et al., 2023). Though the marine microbial loop is increasingly threatened by anthropogenic contaminants (Nogales et al., 2011), currently existing studies primarily focus on higher trophic levels or individual microorganisms, and impacts of anthropogenic contaminants on the marine microbial loop remain elusive. Investigating the impact of anthropogenic pollutants on the marine microbial loop not only aids in understanding the transfer of pollutants within the

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marine microbial food chain but also provides data for predicting the potential consequences of these pollutants on marine ecosystems.

Per- and polyfluoroalkyl substances (PFAS), a class of highly fluorinated organic matter, have been massively produced and employed for industrial applications since 1940s (Gluege et al., 2020; Wee and Aris, 2023), particularly the most extensively used perfluorooctanoic acid (PFOA). The PFOA being referred as a "forever chemical" exhibits extraordinary thermal stability due to its multiple carbon-fluorine (C-F) bonds, rendering it high potential for environmental persistence, bioaccumulation and toxicity (Evich et al., 2022; Giesy and Kannan, 2001). Thus the accumulative global emission of perfluoroalkyl acids achieved ≥46,000 tonnes (Paul et al., 2009; Wang et al., 2014) with the ocean as an ultimate reservoir (Gonzalez-Gaya et al., 2019), resulting in its detection in a variety of marine organisms such as fish, cephalopods and crustaceans (Pan et al., 2021; Zhang et al., 2022). Moreover, PFOA generally co-exists with microplastics (MPs) due to the formation of PFAS in manufacturing MPs(Schellenberger et al., 2019; Washington et al., 2015) and the global plastic pollution (Joo et al., 2021; Wee and Aris, 2023). MPs in the ocean are a growing environmental concern, with an estimated 6 kilotonnes being exported to marine sediments each year (Kaandorp et al., 2023). Therefore, it is essential to investigate the combined effects of PFAS and MPs on ocean ecosystems including the marine microbial loop. Previous studies have shown that polystyrene (PS) is one of the most important forms of MPs in marine and estuarine environments (Ajith et al., 2020), of which impact on the marine microorganisms remain unexplored (de Sa et al., 2018).

Bioaccumulation and biomagnification are key factors in considering the ecological risks of environmental pollutants (Castro et al., 2019). For their assessment, bioconcentration factor (BCF) and biomagnification factor (BMF) can be utilized to quantify the risk of contaminants reaching high and potentially hazardous concentrations in organisms through direct exposure and trophic transfer, respectively (Gray, 2002; Leblanc, 1995). Numerous field studies have reported the biomagnification of PFOA in marine food webs, and have also found that the bioaccumulation potential of the emerging PFAS is higher than that of legacy C8 compounds (Lescord et al., 2015; Li et al., 2022; Loi et al., 2011; Shu et al., 2023). Since PFAS generally co-exist and potentially interact with MPs in ocean environments, it is critical to consider the co-contamination of MPs when evaluating the bioaccumulation and biomagnification potentials of PFOA in the marine microbial-loop microorganisms (e.g. Protozoa). For example, MPs can affect the activity of ABC transporters and destroy cell membranes, resulting in increased accumulation of contaminants in the cells (Lin et al., 2021a). In addition, MPs increase the bioavailability of PFOA by changing permeability of cell membrane, and aggravate the biotoxicity of PFOA in *Chlorella sorokiniana* (Zhao et al., 2024). Therefore, it is critical to evaluate the bioaccumulation and biomagnification potentials of PFOA in the microbial loop microorganisms (e.g. Protozoa) for predicting PFOA transfer in the ocean food webs, with the consideration of co-contamination of PFOA and MPs. Nonetheless, information on this scenario remains scarce.

In this study, *Prochlorococcus* MED4 (picophytoplankton), *Alteromonas macleodii* EZ55 (heterotrophic bacteria), *Pseudocohnilembus persalinus* and *Cafeteria roenbergensis* (microzooplankton) were selected as model microbial-loop microorganisms to investigate the combined effects of PFOA and MPs on the marine microbial loop. Specific objectives included: (1) to elucidate the inhibitive effects of PFOA and/or MPs on the four microbial-loop microorganisms; (2) to identify the bioaccumulation potential of PFOA in the four microorganisms with or without co-contamination of MPs; (3) to reveal the trophic transfer behavior of PFOA in the marine microbial loop with or without MPs; and (4) to propose a scenario describing impacts of PFOA and MPs contamination on the marine microbial loop. Our results generated from this study could advance our understanding of the impact of PFOA and MPs contamination on marine ecosystems.

2. Materials and methods

2.1. Marine microbial-loop microorganisms and culture conditions

The marine picocyanobacteria, *Prochlorococcus* MED4 (a model strain) was grown in AMP1 medium (Moore et al., 2007) at 23 °C under 25 μmol photons m^{-2} s^{-1} with a 14 h:10 h light: dark cycle. Bulk culture chlorophyll fluorescence was monitored using microplate reader (Varioskan LUX, Thermo Scientific). To ensure axenicity, MED4 was routinely tested by inoculation into three different purity broths: ProAC (Morris et al., 2008), MPTB(Saito et al., 2002), and ProMM(Berube et al., 2015). *Alteromonas macleodii* EZ55, a marine heterotrophic bacterium, was cultured in ProAC medium at 30 °C and with shaking (200 rpm). *Alteromonas macleodii* are commonly found inhabiting phytoplankton cultures and are ubiquitous in global ocean (Ivars-Martinez et al., 2008; Kearney et al., 2021). The marine heterotrophic ciliate, *Pseudocohnilembus persalinus*, and heterotrophic nanoflagellate, *Cafeteria roenbergensis*, were maintained in 0.22 μm filtered, autoclaved artificial seawater (ASW) supplemented with rice grains. These protists (*P. persalinus* and *C. roenbergensis*) were separately isolated from the coastal waters of the Hong Kong and Xiamen and are widely distributed in subtropical regions (Qian et al., 2023). The common occurrence of *P. persalinus* in the ocean surface area, as well as the abundance and widespread presence of *C. roenbergensis* as one of the most common nanoflagellates in marine environments, make them ideal model microbial-loop microorganisms (Canals et al., 2020; Schoenle et al., 2020). The main predators of *Prochlorococcus* and bacteria include microzooplankton, especially microciliates and nanoflagellates (Baudoux et al., 2008; Hambright et al., 2007). Therefore, these organisms were selected as representatives of microzooplankton in this study.

2.2. The characterization of polystyrene microplastics and cell morphology

Polystyrene microplastics (PS MPs, 1000 nm) were purchased from Shanghai Hugebio technology company (Shanghai, China). The morphology of PS MPs was characterized by scanning electron microscope (SEM, Sigma 500, Zeiss, Germany). Moreover, the particle size, zeta potential, and polymer dispersity index (PDI) were analyzed by Zetasizer Nano (ZS90, Malvern Panalytical, Britain) (Fig. S1 and Table S1). To assess the effects of PFOA and PS on cell membranes, cell morphology was characterized using SEM. According to the previous method with a slight modification (Liang et al., 2021), samples were fixed using a 2.5% solution of glutaraldehyde or osmium tetroxide and stored at 4 °C for 12 h after 48 h of exposure to PFOA and PS. Samples were then washed three times with ASW. A gradient dehydration process was carried out using 30%, 50%, 70%, 85%, 90% ethanol solutions, each for one instance, followed by three instances in 100% ethanol, each for 15 min. The samples were then washed three times with tertiary butanol, each for 20 min, followed by freeze-drying. Post-drying, the samples were sputter-coated with gold and imaged using SEM.

2.3. Exposure of microbial-loop microorganisms to PFOA and PS MPs

To determine the semi-lethal concentration (IC50) and IC80 of PFOA to different microorganisms, gradient PFOA concentrations were set. For the MED4, the exposure concentrations of PFOA were 0,0.1,0.5,2.5,10,20 mg/L for 24, 48 and 72 h. For the EZ55, the exposure concentrations of PFOA were 0, 5, 25, 50, 100, 200 mg/L for 24, 48 and 72 h. For the *P. persalinus* and *C. roenbergensis*, the exposure concentrations of PFOA were 0, 1, 25, 50, 100, 200 mg/L for 24, 48 and 72 h. To determine whether PFOA's inherent acidity induces toxicity, the pH values of PFOA concentrations ranging from 0 to 200 mg/L were tested. Based on the acute toxicity data (IC50 and IC20) of PFOA (Table S2), two concentrations were selected: 10 μg/L, representing the environmental

concentration (Llorca et al., 2012), and 1 mg/L, which is higher than the environmental level to study the mechanisms of toxicity. To test the combined toxicity of PFOA and MPs, MED4, EZ55, *P. persalinus* and *C. roenbergensis* were exposed to PS. Gradient concentrations of PS were 0, 1, 5, 10, 50, 100 mg/L and the exposure times were 24, 48 and 72 h. Finally, the PS concentration of 10 mg/L was selected for the combined exposure. Therefore, the follow-up experimental groups were control, 10 mg/L PS (PS), 10 μg/L PFOA (PFOA-L), 1 mg/L PFOA (PFOA-H), 10 mg/L PS+10 μg/L PFOA (PS + PFOA-L) and 10 mg/L PS+1 mg/L PFOA (PS + PFOA-H).

In the exposure test, the initial concentration was 2~6 × 10^6 cells/mL for MED4 and EZ55, 2–6 × 10^2 cells/mL for *P. persalinus*, and 2–6 × 10^3 cells/mL for *C. roenbergensis*. The exposure time was 24, 48 and 72 h. For MED4 and EZ55, 2.5% glutaraldehyde was fixed at the end of exposure. The density of MED4 cells was detected by flow cytometry. MED4 emits fluorescence by itself, and the excitation wave/emission wavelength is 488/680 nm. EZ55 cells were stained with SYGR Green I (Biosharp, BS358A), incubated in the dark for 15 min, and then detected by flow cytometry (CytoFLEX, Beckman, America). Ciliates *P. persalinus* and nanoflagellates *C. roenbergensis* were fixed with 10% Lugol's solution and 200 μL of the solution was added to a 96-well plate and left for at least 3 h to allow the microzooplankton to settle at the bottom, after which they were counted with microscope (ECHO, America).

2.4. Measurement of reactive oxygen species (ROS)

To investigate the intracellular oxidative stress of microorganisms induced by PFOA and PS, 2′,7′-Dichlorofluorescin diacetate (DCF-DA) probe (Solarbio, Beijing, China) was used to measure ROS. Using a modified version of the previous method (Lin et al., 2021a; Lin et al., 2021b), the microorganisms exposed to PFOA and PS for 48 h were washed and stained with the probe for 30 min. After washing, the fluorescence value was measured using a microplate reader (Varioskan LUX, Thermo Fisher, America) at the excitation wave and emission wave of 488 nm and 525 nm, respectively. The cell number was counted to correct the ROS and the value was normalized by the value of the control.

2.5. The uptake of PFOA by the microbial-loop microorganisms via direct uptake in the medium

MED4 (2–6 × 10^6 cells/mL) and EZ55 (2–6 × 10^6 cells/mL) were exposed to PS and PFOA for 48 h, respectively. As for MED4, PS was removed by 1.0 μm polyether sulfone filter. Portions of the filtrate were used to determine cell density via flow cytometry. Others were filtered with 0.22 μm polyether sulfone filter membrane to detect PFOA in cells and medium. For EZ55, due to the high concentration of bacteria which made filtration impractical, the cells were washed three times with ASW to remove attached PS, and then filtered through a 0.22 μm polyether sulfone filter to separate cells and filtrate.

P. persalinus (2–6 × 10^2 cells/mL) and *C. roenbergensis* (2–6 × 10^3 cells/mL) were exposed to PS and PFOA for 48 h, respectively. The cells were filtered with a 1.6 μm polyether sulfone filter membrane, and the filtrate then passed through a 0.22 μm polyether sulfone filter membrane. The number of protozoa was counted, and the concentration of PFOA in the cells and medium was detected. Additionally, the bioconcentration factors (BCF) values were calculated, normalized to the dry weight (DW) (Zhang et al., 2023). The DW of MED4, EZ55, ciliate and nanoflagellate were 2.48 × 10^{-7}, 1.69 × 10^{-7}, 7.50 × 10^{-2} and 1.39 × 10^{-4} μg/cell, respectively (Freese et al., 2009; Ikeda, 2017; Lea-Smith et al., 2015; Tophoj et al., 2018).

$$BCF = C_b/C_w \quad (1)$$

Where C_b is the concentration of PFOA in the organism, and C_w refers to the PFOA concentration in exposure medium.

2.6. Trophic transfer of PFOA from bacteria to protozoa

MED4 and EZ55 were cultured for 48 h in the medium containing PFOA and PS. The PFOA uptake by predator protozoa was quantitatively determined, considering that bacterial prey was their only food source. Initially, the MED4 exposed to PFOA and/or PS was filtered to remove PS with a 1.0 μm polyether sulfone membrane, and the filtrate was filtered again with a 0.22 μm polyether sulfone membrane to obtain bacteria. For EZ55, due to the high concentration of bacteria which made filtration impractical, the cells were washed three times with ASW to remove attached PS, and then filtered through a 0.22 μm polyether sulfone filter to obtain bacteria. Subsequently, the bacteria were washed with sterile ASW three times and the protists were fed with prey. Two experimental conditions were set up: one using MED4 and the other using EZ55 as the sole food source. After 48 h of grazing experiments, the protozoa were collected for intracellular PFOA measurement and enumeration. Finally, the content of PFOA in the protozoa and the initial prey was detected, and the DW-normalized biomagnification factor was calculated (Arnot and Gobas, 2006).

$$BMF = (C_p/f_p)/(C_b/f_b) \quad (2)$$

Where C_p and f_p refer to the intracellular PFOA concentration and DW of protozoa, respectively. C_b and f_b refer to the intracellular PFOA concentration and DW in the cells of bacteria (prey), respectively.

2.7. PFOA analysis

Quantification of PFOA in the medium and plankton samples was performed using Liquid chromatography tandem mass spectrometry (LC-MS/MS, Triple Quad 5500, SCIEX, Singapore) operating in negative ion mode with multiple reaction monitoring. The PFOA and internal standards ($^{13}C_4$-PFOA) were obtained from ANPEL (Shanghai, China).

Plankton samples were extracted as reported with some modifications (Casal et al., 2017). Briefly, filters were lyophilized in 15 ml polypropylene tubes. After adding the 5 mL acetonitrile and 10 ng $^{13}C_4$-PFOA, the tubes were vortexed and sonicated for 20 min, then centrifuged at 7000 rpm for 5 min. The supernatants were collected, and the process was repeated with an additional 5 mL acetonitrile. The combined extracts were concentrated under a gentle nitrogen stream for further solid phase extraction (SPE) extraction with PolyPlus weak anion exchange (WAX) SPE cartridge.

The SPE extraction process adheres to the supplier's manual. The column was activated sequentially with 2 mL of 0.1% ammoniated methanol, 3 mL of methanol, and 5 mL of ultrapure water. After sample introduction, the column was washed with 3 mL of 25 mmol/L ammonium acetate solution and 6 mL of ultrapure water, then dried under negative pressure. Elution was achieved with 2.5 mL of methanol and 3.5 mL of ammoniated methanol. The eluates were collected in a 15 mL polypropylene tube and evaporated to near dryness under a 50 °C nitrogen water bath, then brought to a volume of 1 mL with 10% methanol in water for further analysis.

The sample (10 μL) was injected into a SB-C18 column (3.0 μm, 3.0 × 50 mm, Sigma) maintained at 40 °C. 0.1% formic acid in water and HPLC grade methanol were served as mobile phase A and B. The specific instrument parameters are detailed in Table S3. The limits of detection (LOD) and quantitation (LOQ) for PFOA were 3.1 ng/L and 5.3 ng/L, respectively. The recovery rate was 105 ± 8.1%.

2.8. Statistical analysis

The experiments in this study are all conducted in triplicates. All data are expressed as mean ± standard deviation. Independent sample *t*-test and one-way analysis of variance (ANOVA) were performed to determine the significant difference. Significance was denoted by a *p*-value <0.05, marked with an asterisk (*). Graphpad Prism 10 (GraphPad

Software Inc., USA) was used for data analysis and mapping.

3. Results

3.1. Toxicity of PFOA and PS to marine microbial-loop microorganisms

To investigate the impact of PFOA and PS (a typical MP) on marine individuals of the microbial loop of picophytoplankton-bacteria-microzooplankton, four microbial-loop microorganisms (picophytoplankton, *Prochlorococcus* MED4; heterotrophic bacteria, *Alteromonas macleodii* EZ55; microzooplankton, *Pseudocohnilembus persalinus* and *Cafeteria roenbergensis*) were selected to test their cell growth upon exposure to gradient concentrations of PFOA or PS for 24, 48 and 72 h. Based on Fig. S2, the toxicity induced by PFOA's inherent acidity was excluded. Based on the growth curves (Fig. S3), relative survival rates of the four marine microbial-loop populations were calculated to assess the toxicity of PFOA (Fig. 1a–d) and PS (Fig. 1e–h). Results showed the dose-dependent inhibitory effects of PFOA on the microbial loop of picophytoplankton, bacteria and microzooplankton ($p < 0.05$), e.g. relative survival rates of picophytoplankton MED4, bacterium EZ55, microzooplankton *P. persalinus* and *C. roenbergensis* decreased to 29.70%, 40.74%, 54.61% and 36.68% after 72 h exposure to 2.5, 5, 100 and 100 mg/L PFOA, respectively, compared to their growth conditions without PFOA exposure. Accordingly, the IC50 of MED4 with 48 h exposure of PFOA was 1.53 mg/L, which was about 9.16, 81.25 and 60.45 times lower than that of EZ55, *P. persalinus* and *C. roenbergensis*, respectively. These results suggested the sensitivity of marine microbial-loop microorganisms to PFOA contamination, particularly the marine picophytoplankton (MED4), with a sensitivity order of MED4 > EZ55 > *C. roenbergensis* > *P. persalinus* (Table S2). Notably, the temporal change of decreasing relative survival rates of picophytoplankton MED4 upon exposure to low concentrations of PFOA (0.5 and 2.5 mg/L) indicated augmented inhibition of PFOA on marine cyanobacteria with prolonged exposure (Fig. 1a). In contrast to the PFOA imposing higher inhibitive impact on marine picophytoplankton compared to microzooplankton, PS was shown to have less overall inhibitive effect on the microbial loop, and to have higher inhibitive impact on microzooplankton and heterotrophic bacteria relative to the marine picophytoplankton (Fig. 1e–h). For example, relative survival rates of picophytoplankton MED4, bacterium EZ55, microzooplankton *P. persalinus* and *C. roenbergensis* decreased to 89.52%, 68.49%, 75.65% and 76.48% after 72 h exposure to 100 mg/L PS, respectively, compared to their growth conditions without PS exposure. The opposite trends of sensitivity order of marine microbial-loop populations to PS and PFOA exposure suggested the varied inhibitive mechanisms of these two types of environmental contaminants, e.g., toxicities of PFOA were ascribed to the membrane disruption, oxidative stress, DNA damage and consequent cell inactivation/death (Liu et al., 2016), while PS disrupted the phase behavior of lipid membranes and caused changes in the distribution and types of lipids within the membrane, potentially compromising the functionality of the biological membranes (Morandi et al., 2021).

3.2. Combined effects of PFOA and PS on marine microbial-loop microorganisms

To investigate whether the co-contamination of PFOA and non-cytotoxic PS imposed synergistic inhibition on the marine microbial-loop microorganisms, six sets of experiments were setup with the four

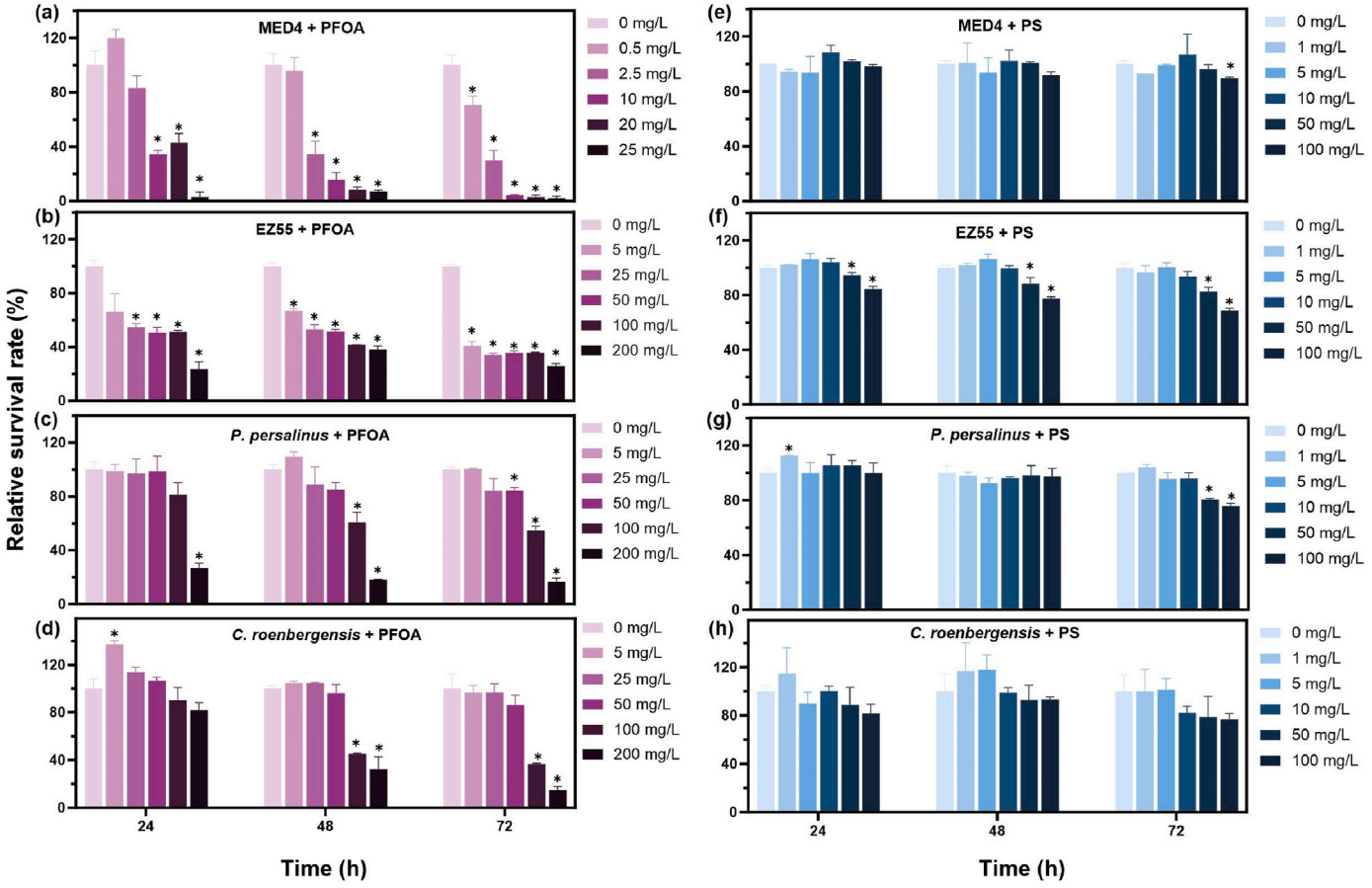

Fig. 1. Impact of PFOA and PS on relative survival rates of the marine microbial-loop microorganisms. The relative survival rates of MED4 (a), EZ55 (b), *Pseudocohnilembus persalinus* (c) and *Cafeteria roenbergensis* (d) upon exposure to gradient concentrations of PFOA; the relative survival rates of MED4 (e), EZ55 (f), *P. persalinus* (g) and *C. roenbergensis* (h) upon exposure to gradient concentrations of PS.

marine microorganisms, and concentrations of PS (10 mg/L) and PFOA (0.01 and 1 mg/L for mimicking low and high environmental contamination concentrations, respectively) were selected based on their environmental contamination and aforementioned experiments-derived results. In contrast to no inhibition of sole PS (10 mg/L), PS intensified the inhibitive effects of both low and high concentrations of PFOA on the four marine microbial-loop microorganisms (Fig. 2 and S4), particularly the *C. roenbergensis* (Fig. 2d). PS significantly decreased the cell survival rate of *C. roenbergensis* by 23.27% in the presence of PFOA after 72 h ($p < 0.05$). These results suggested the synergistic inhibitive effects of PS and PFOA on the marine microbial-loop microorganisms. Notably, for the PFOA-sensitive MED4 being different from other microorganisms, comparatively more obvious synergistic inhibition was observed at the beginning of the experiments (24 h), which became less obvious with prolonged exposure (Fig. 2a).

Based on previous studies showing that microbial inhibition could be derived from the oxidative stress (Imlay, 2013; Jasinska et al., 2015), the six cultures with 48 h exposure to PS and/or PFOA were sampled to assess their ROS (reactive oxygen species; Fig. 2e). Being aligned with the relative survival rates, in contrast to PS and low concentrations of PFOA, high concentrations of PFOA were shown to increase ROS, which was further augmented with combined amendment of PS and PFOA (Fig. 2e). For example, being aligned with 13.43% decrease in relative survival rate (Fig. 2a), an increase of 15.24% in ROS production was observed in MED4 amended with both PS and 1 mg/L PFOA, relative to MED4 amended with only 1 mg/L PFOA (Fig. 2e). Similar trends were observed in cultures of EZ55, *P. persalinus* and *C. roenbergensis* (Fig. 2), e.g., 5.73%, 8.40% and 8.51% decrease in relative survival rates and 6.23%, 10.80%, and 14.83% increase in ROS were observed in the cultures EZ55, *P. persalinus* and *C. roenbergensis* amended with PS and 1 mg/L PFOA, respectively, compared with cultures amended with PFOA alone. Additionally, four marine microorganisms exhibited PS adhesion,

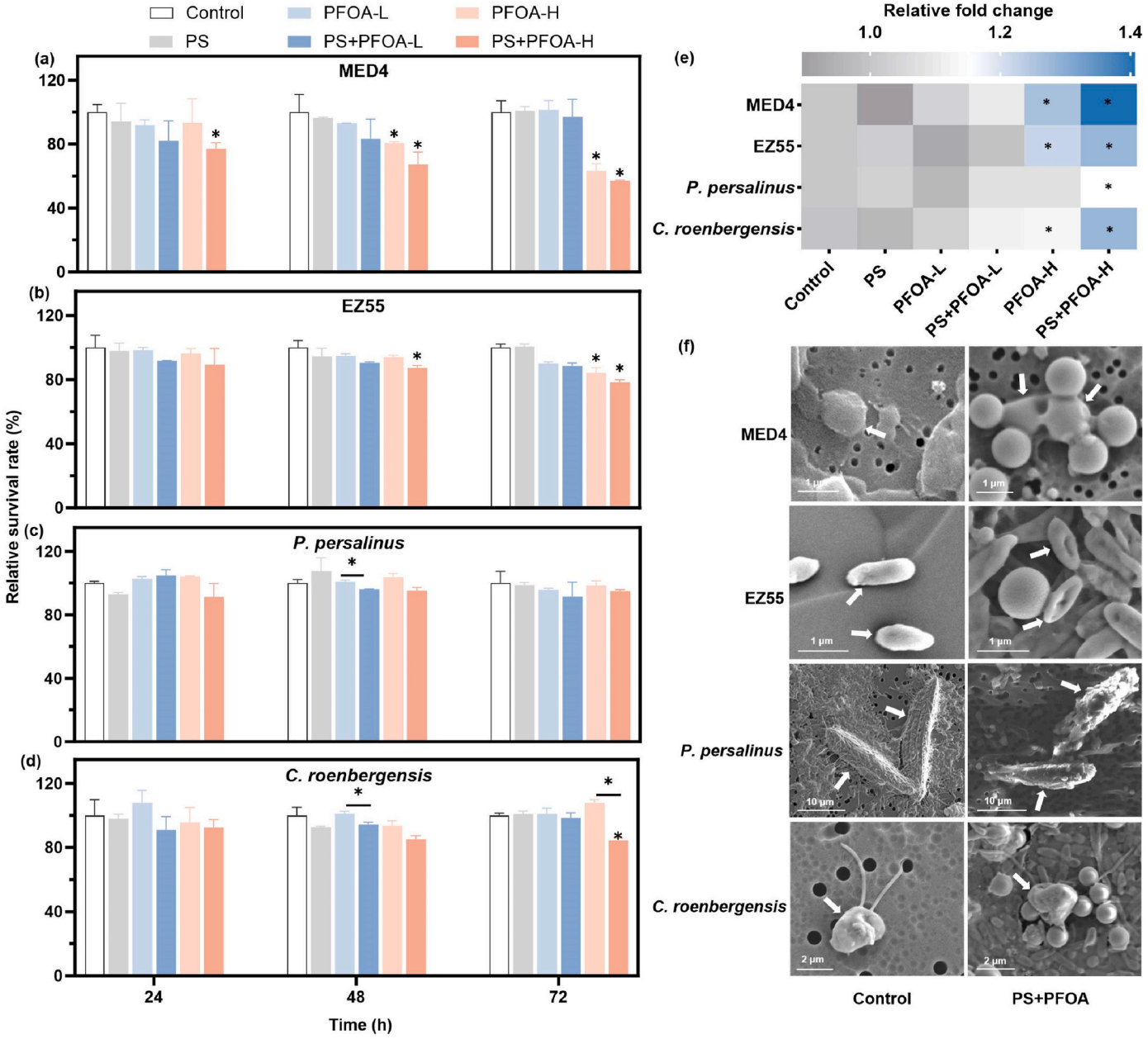

Fig. 2. Combined impact of PFOA and PS on relative survival rates and ROS of the marine microbial-loop microorganisms. The relative survival rates of MED4 (a), EZ55 (b), *Pseudocohnilembus persalinus* (c) and *Cafeteria roenbergensis* (d) upon exposure to PFOA and PS; the oxidative stress quantified with ROS (e) and cell morphology (f) of the four marine microbial-loop microorganisms. Marine microorganisms are indicated by white arrows.

and their cell morphology was altered (invagination), in contrast to cultures without PFOA and PS (Fig. 2f).

3.3. Bioaccumulation of PFOA in marine microbial-loop microorganisms

To investigate bioaccumulation of PFOA in the four marine populations, concentrations of PFOA in both the culture medium and microbial cells were measured, and their BCF (bioconcentration factor) values were calculated accordingly. Interestingly, the cellular PFOA amounts in the four microbial-loop populations were distinctively different from each other, with an order of *P. persalinus* (2887.03 ± 31.98) > *C. roenbergensis* (100.43 ± 48.96) > MED4 (0.40 ± 0.02) > EZ55 (0.01 ± 0.00) in cultures amended with high concentrations of PFOA (Fig. S5), being positively correlated with their lipid content ($r = 0.91$, $p < 0.01$) (Fig. S6). Notably, PS was shown to promote bioaccumulation of PFOA in the four marine microbial-loop microorganisms (Fig. S5), which further corroborated the above-mentioned PS-augmented inhibition of PFOA on the four populations quantified with the relative survival rate and oxidative stress (Fig. 2). Given the significant differences in biovolume among the four microorganisms (0.21–1687.36 μm^3), the bioaccumulation of PFOA was normalized to dry weight. Remarkably, MED4 exhibited the highest PFOA content per dry weight, with an order of MED4 (1.73 ± 0.05–15.92 ± 0.98 × 10^5 μg PFOA/kg DW) > *C. roenbergensis* (6.88 ± 0.01–72.38 ± 35.28 × 10^4 μg PFOA/kg DW) > EZ55 (3.56 ± 0.02–6.78 ± 0.11 × 10^4 μg PFOA/kg DW) > *P. persalinus* (2.52 ± 0.04–38.64 ± 0.22 × 10^3 μg PFOA/kg DW) in PFOA-amended cultures (Fig. 3a). Furthermore, PS enhanced the bioaccumulation of PFOA, especially in MED4 and *C. roenbergensis* cultures with high concentrations of PFOA.

According to the United States Environmental Protection Agency (USEPA., 1998), BCF values over 5000 L/kg weight (log BCF >3.70) indicated the high cell bioaccumulation. MED4 (log BCF = 3.92–5.27 L/kg) and *C. roenbergensis* (log BCF = 3.82–4.95 L/kg) had bioaccumulation potential upon exposure of both low and high concentrations of PFOA (Fig. 3b), which surpassed the observed concentrations in other fish and aquatic plants and approached the values of marine plankton (Table S4). In contrast, EZ55 was demonstrated to have high bioaccumulation potential of low concentrations of PFOA (log BCF = 3.98L/kg), but low bioaccumulation potential of high concentrations of PFOA (log BCF = 2.50 L/kg) (Fig. 3b). This unique bioaccumulation pattern could be due to the different adsorption kinetics of the low and high PFOA concentrations, and the BCF decreased with increasing PFOA concentrations as observed in previous studies (Guo et al., 2023). In addition, ciliates (*P. persalinus*) exhibited minimal bioaccumulation potentials upon exposure of both low and high concentrations of PFOA (log BCF = 2.06–2.80 L/kg) among the four marine microbial-loop populations (Fig. 3b), and log BCF values of nanoflagellates were significantly higher than that of ciliates (Fig. 3c). Moreover, PS was shown to significantly enhance the bioaccumulation potential of PFOA in the four marine microorganisms (Fig. 3b), i.e., 1.89, 1.33, 1.22, and 2.18-fold increase in BCF values in MED4, EZ55, *P. persalinus* and *C. roenbergensis*, respectively.

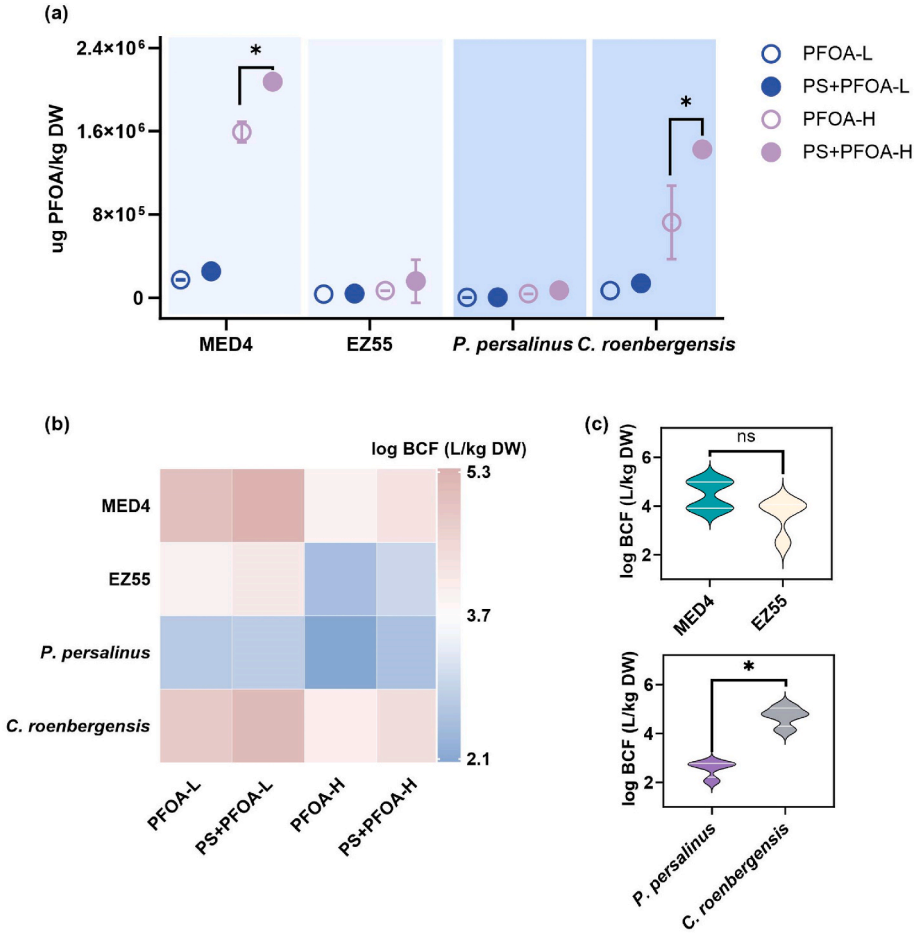

Fig. 3. Bioaccumulation of PFOA in the four marine microbial-loop microorganisms: (a) dry weight-based intracellular accumulation of PFOA; (b) dry weight-based and log-transformed bioconcentration factor (BCF) values of both low and high concentrations of PFOA in the four marine populations; (c) comparisons of log BCF values between MED4 and EZ55 and between *P. persalinus* and *C. roenbergensis*.

3.4. Biomagnification of PFOA in the marine microbial loop

In the marine microbial loop of picophytoplankton, bacteria and microzooplankton, picophytoplankton (MED4) as the primary producer fed heterotrophic bacteria (EZ55) with organic metabolites and/or cell-lysis-derived organic matter in the form of dissolved organic matter, and microzooplankton (*P. persalinus* and *C. roenbergensis*) preyed on both the picophytoplankton (MED4) and bacteria (EZ55)(Cai et al., 2024) (Fig. 4a). To assess the biomagnification of PFOA in the marine microbial loop, the biomagnification factor (BMF) values of the food chains of picophytoplankton (MED4) and bacteria (EZ55) to microzooplankton (*P. persalinus*, ciliate; *C. roenbergensis*, nanoflagellate) were calculated. The results showed that the dry-weight-normalized BMF values of MED4-ciliate, EZ55-ciliate, MED4-nanoflagellate and EZ55-nanoflagellate food chains were in the ranges of 1.02–1.32 (a mean value of 1.18), 0.02–0.09 (0.05), 1.86–2.64 (2.43) and 0.37–0.88 (0.66), respectively (Fig. 4b and c). The comparatively higher BMF values of picophytoplankton-associated food chains, relative to that of heterotrophic bacteria, suggested picophytoplankton exhibited a higher bioaccumulation of contaminants within their cellular structures (Fig. 4d). In addition, PS promoted biomagnification of PFOA in all of the four food chains, particularly the nanoflagellate-associated food chains (Fig. 4b and c), e.g., PS increased BMF values of MED4-nanoflagellate (PFOA-L), MED4-nanoflagellate (PFOA-H), EZ55-nanoflagellate (PFOA-L) and EZ55-nanoflagellate (PFOA-H) food chains by 52.81%, 11.21%, 57.51% and 17.21%, respectively. These results, together with above-mentioned BCF values, suggested that PS enhanced inhibitive effects of PFOA on the marine microbial loop by promoting both bioaccumulation and biomagnification although no obvious impact was observed for the picophytoplankton-fed-bacteria derived PFOA (Fig. 4c).

4. Discussion

This study provided the first insight into impact of PFOA and/or PS on the marine microbial loop of picophytoplankton, bacteria and microzooplankton, based on which a scenario was proposed to describe the synergistic impact (Fig. 5): (1) upon exposure of the marine microbial-loop microorganisms to a single contaminant of PFOA or PS, only the PFOA was shown to pose significant oxidative stress by producing ROS, to which the cyanobacterium MED4 was more sensitive to the PFOA exposure and generated relatively higher amount of ROS compared to other microbial-loop microorganisms; the marine cyanobacterium *Prochlorococcus* MED4 was known for its small genome size of 1.6–2.7 Mb (Rocap et al., 2003), and lacked genes to encode functional modules for detoxification and cellular damage repair, including genes encoding catalase and peroxidoreductin to resist oxidative stress (Biller et al., 2015); in marine environments, a diverse range of heterotrophic bacteria could scavenge ROS for the co-existing cyanobacterium (Morris et al., 2008), which helped cyanobacterium to cope with oxidative stress as described in the "black queen" hypothesis (Morris et al., 2012); the high sensitivity of *Prochlorococcus* to PFOA, leading to cell death, could impair its carbon fixation capacity, which not only affects *Prochlorococcus* itself but also have long-term negative consequence for the marine carbon cycle by reducing the ocean's organic carbon storage, potentially disrupting the overall dynamics of the carbon cycle; (2) when exposing the marine microbial-loop microorganisms to both PFOA and PS, their synergistic impact on the survival rates of microbial-loop microorganisms were observed, which could be derived from PS impacts on the fate of PFOA and on the microbial-loop microorganisms; on one hand, the high specific surface area and lipophilic surface properties enabled MPs as a carrier of contaminants and increased the biological availability of PFOA (Xu et al., 2020; Huang et al., 2021); on the other hand, MPs could affect the defense of microorganisms against contaminants by damaging cell membrane integrity and increasing the membrane permeability and consequent toxicity of contaminants to the

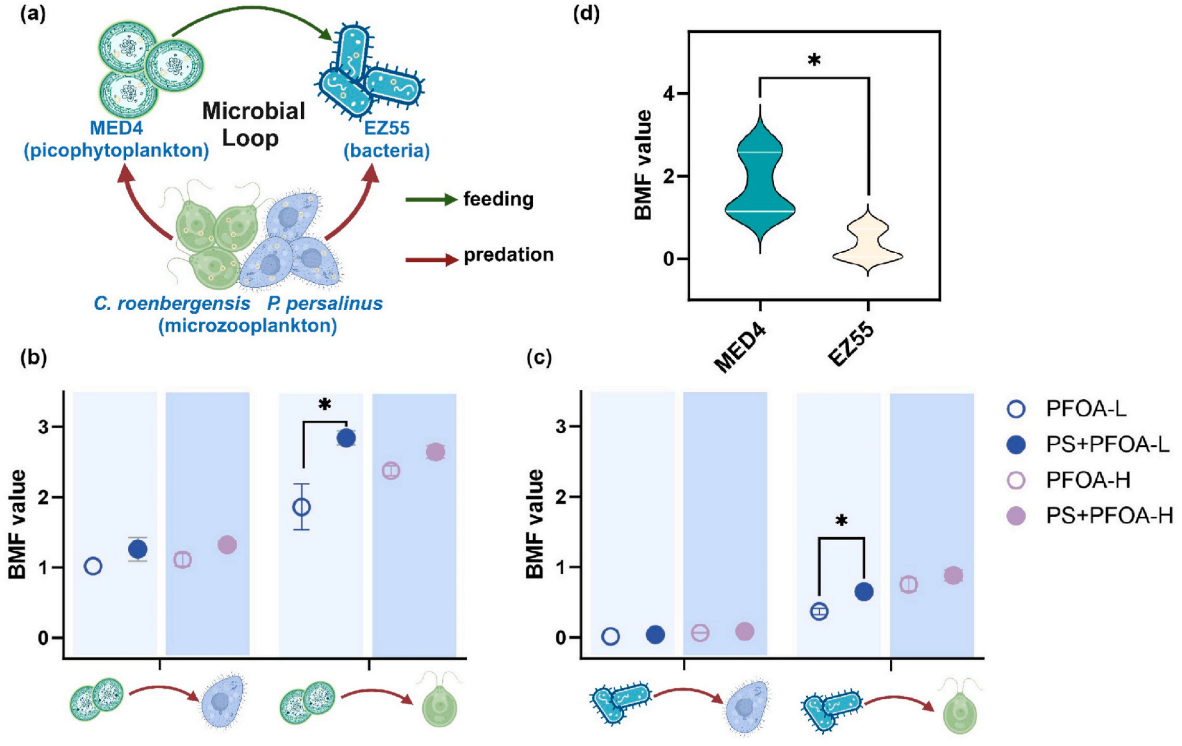

Fig. 4. Biomagnification of PFOA in food chains of the marine microbial loop. Marine microbial loop (a) and dry-weight-normalized biomagnification factor (BMF) values of PFOA in food chains of MED4 -ciliate/nanoflagellate (b) and of EZ55-ciliate/nanoflagellate (c); (d) comparison of BMF in food chains using MED4 and EZ55 as food.

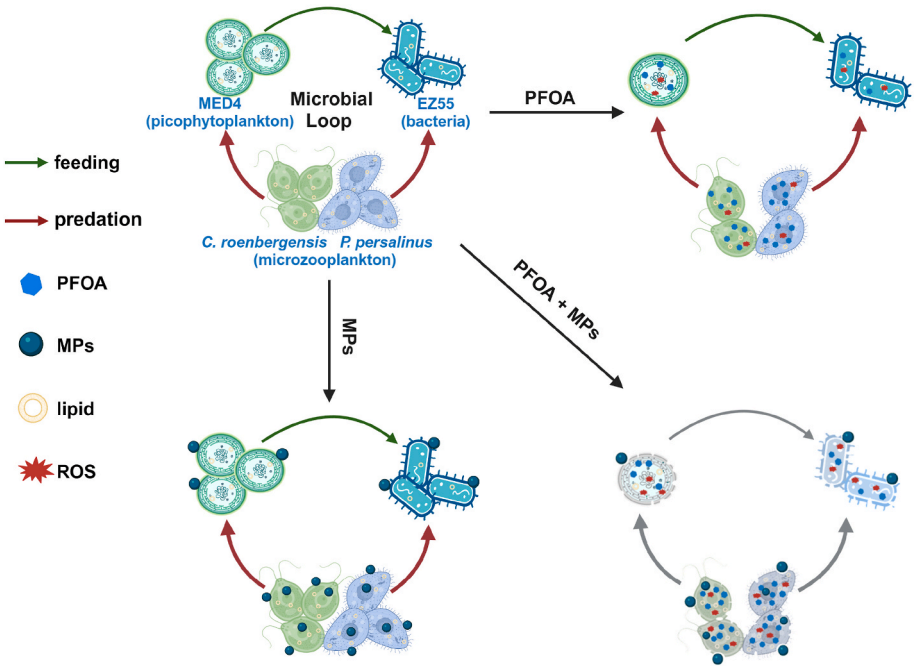

Fig. 5. **A** scenario to demonstrate inhibitive effects of PFOA and/or PS on marine microbial-loop microorganisms.

microorganisms (Morandi et al., 2021; Zhao et al., 2024). In addition to above-mentioned synergistic impact of PFOA and MPs on the survival rates of microbial-loop microorganisms, co-contamination of PFOA and MPs could pose potential and long-standing risks to the global marine ecosystem by promoting bioaccumulation and biomagnification of PFOA in the marine microbial loop. For example, in our study, co-contamination of PFOA and MPs resulted in 10.80% and 14.83% increases in ROS and consequent 8.40% and 8.51% decreases in relative survival rates, particularly as well as 81.18% and 96.93% increases in intracellular PFOA accumulation, of *P. persalinus* and *C. roenbergensis*, respectively, relative to their PFOA exposure alone. Nonetheless, current studies on the large scale and long-term impact of contamination of PFOA and/or MPs on the marine microbial loop and the global marine ecosystem remain scarce, awaiting future extensive investigation.

Considering the primary and critical roles of microbial loop microorganisms in the marine food webs and global cycling of carbon, the synergistic impact of PFAS and MPs on the marine microbial loop could profoundly change marine ecosystems and global element cycles through the bioaccumulation and biomagnification processes. Nonetheless, in contrast to the extensively investigated bioaccumulation and biomagnification of contaminants in freshwater microbial food chains (Dong et al., 2018), impact of PFOA and co-contamination of PFOA and MPs on the marine microbial loop remain elusive, which could be due to challenges of accessing remote oceanic environments and cultivating these small and ecologically-niche-specific cyanobacteria (Flombaum et al., 2013). In addition, the bioaccumulation and biomagnification of PFOA in food webs and marine microbial loop could have different trends, being possibly due to the marine picophytoplankton with relatively larger surface area-to-volume ratios and consequent different contaminant-adsorption kinetics (Casal et al., 2017). In our study, varied bioaccumulation and biomagnification factors were observed for the marine microbial-loop microorganisms, being synergistically determined by physiological properties of these microorganisms, including their cellular lipid content (Hebert, 1995). Surprisingly, picophytoplankton (*Prochlorococcus*) was shown to have exceptionally high potential in bioaccumulation of PFOA, especially when being co-contaminated with MPs, compared to other microbial-loop microorganisms. Therefore, picophytoplankton could be the potential bottleneck for bioaccumulation and biomagnification of PFOA in marine food webs. Moreover, due to its high abundance and bioaccumulation potential, the picophytoplankton (*Prochlorococcus*) could be serve as a biomarker for monitoring marine pollution of PFOA. For the biomagnification of PFOA, protozoans, serving as a bridge in the marine microbial loop, channeled *Prochlorococcus*-derived energy, as well as the accumulated PFOA, to higher trophic levels by predating phytoplankton (Fenchel, 2008). PFOA can be transmitted through bioaccumulation and biomagnification from low trophic-level microorganisms to high trophic-level microzooplankton, which can be preyed by upper-level predators. Over time, this may lead to widespread distribution of pollutants in the food chain. MPs as co-contaminants could regulate the biomagnification of PFOA in the marine microbial loop and subsequent food webs through different actions of cell membrane damage, pollutant transporter proteins suppression, cytoskeleton disruption and detoxification gene expression inhibition of the microbial-loop microorganisms (Lin et al., 2021a; Wang et al., 2022; Zhou et al., 2020). Interestingly, regardless of microbial lineages and their bioaccumulation/biomagnification potentials, co-contamination of MPs could consistently augment both the bioaccumulation and biomagnification of the PFOA in the marine microbial loop, highlighting the exacerbated ecological risk of the co-contamination of PFOA and MPs in the ocean.

5. Conclusion

This study provides the first insight into the synergistic impacts of PFOA and MPs on marine microbial loop. Major findings include.

(1) Picophytoplanktons are the most sensitive population to PFOA toxicity, as being reflected by the least IC_{50} of *Prochlorococcus* compared to other marine microbial loop microorganisms.
(2) In contrast to minimum impact of MPs on the marine microbial loop, the MPs can enhance PFOA toxicity to microbial loop microorganisms by intensifying oxidative stress and decreasing cell survival rates.
(3) Marine picophytoplankton and nanoflagellates exhibit high accumulation of PFOA, leading to biomagnification of PFOA within the food chain (picophytoplankton-protozoa).

(4) MPs stimulate the bioaccumulation and biomagnification of PFOA in the marine microbial loop.

CRediT authorship contribution statement

Peichun Lin: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaokun Liu:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zuyuan Gao:** Resources, Methodology. **Yelinzi Yuan:** Visualization. **Hongbin Liu:** Resources, Methodology. **Lingfeng Huang:** Resources, Methodology. **Zhili He:** Writing – review & editing, Project administration, Funding acquisition. **Qinglu Zeng:** Writing – review & editing, Resources, Methodology. **Shanquan Wang:** Writing – review & editing, Validation, Project administration, Funding acquisition.

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.envres.2025.121223.

Data availability

Data will be made available on request.

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