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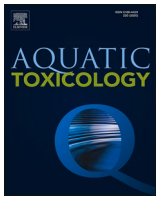

# Ultrastructural changes, pigment responses and bioaccumulation in the microalga *Phaeodactylum tricornutum* Bohlin exposed to BPA analogues

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ABSTRACT

As well-known, microalgae have a pivotal role in aquatic environments, being the primary producer. In this study, we investigated the effects of Bisphenol A (BPA) analogues on cell ultrastructure, reactive oxygen species (ROS) production and photosynthetic pigment responses in the diatom *Phaeodactylum tricornutum*. Microalgae were exposed during both exponential and stationary growth phases to an environmental relevant concentration (300 ng/L) of three differing BPA analogues (BPAF, BPF, and BPS) and their mixture (100 ng/L of each compound). Bioaccumulation of such compounds in microalgae was also analysed. During the stationary growth phase, a significant increase in the percentage of cells with hydrogen peroxide production was recorded after exposure to both BPS and MIX. Conversely, no significant effects on total chlorophylls and carotenoids were observed. During exponential growth phase we observed that control cultures had chloroplasts with well-organized thylakoid membranes and a central pyrenoid. On the contrary, the culture cells treated with BPA analogues and MIX showed chloroplasts characterized by evident dilation of thylakoid membranes. The presence of degeneration areas in the cytoplasm was also recorded. During the stationary growth phase, control and culture cells were characterized by chloroplasts with a regular thylakoid system, whereas BPA analogues-exposed cells were characterized by a deep degradation of the cytoplasm but showed chloroplasts without evident alterations of the thylakoid system. Lipid bodies were visible in treated microalgae. Lastly, microalgae bioaccumulated mainly BPS and BPF, alone or in the MIX. Overall, results obtained revealed that BPA analogues can affect some important biochemical and ultrastructure features of microalgae, promoting ROS production. Lastly, the capability of microalgae to bioaccumulate bisphenols suggest a potential ecotoxicological risk for filter-feeders organisms.

## 1. Introduction

Bisphenol A (BPA), widely used as a plasticizer in polycarbonate plastic production, is recognized as an endocrine disruptor. BPA has also been used in thermal paper as colour developer, in epoxy resins, in medical equipment, and in consumer electronics (Vandenberg et al., 2007). Recently, several countries introduced some restrictions regarding the use of BPA, with a consequent replacement of BPA with other bisphenols called BPA analogues. The main BPA analogues currently used are bisphenol AF (BPAF), bisphenol F (BPF), and bisphenol S (BPS). These compounds have many uses, such as in food packaging, water pipes, industrial floors, plastics, adhesives, epoxy glues, thermal receipt papers, and common polymer applications (Chen et al., 2016). The ongoing replacement of bisphenol A with its analogues is causing an increased use and release of these emerging contaminants. In this context, high attention is posed to their toxicity and effects on the environment. Indeed, these compounds might reach aquatic environments causing potential detrimental effects on both algae and animals. The environmental concentrations of BPA analogues are commonly in the range of few ng/L (Fabrello and Matozzo, 2022). However, recent studies revealed that these compounds can be found at higher concentrations like hundreds and thousands of ng/L in aquatic environments. Indeed, BPAF reached 140 ng/L in China surface water (Wang et al., 2017), while BPF reached 2850 ng/L and 1300 ng/L in surface water

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samples from Japan and Korea, respectively (Yamazaki et al., 2015). Regarding BPS, it was recorded at 65,600 ng/L in surface water in China (Huang et al., 2018) and up to 1584 ng/L in surface water in Poland (Caban et al., 2020). In seawater samples from East China Sea, BPA was the predominant bisphenol (18 ± 9.7 ng/L), followed by BPS (3.7 ± 2.8 ng/L), BPF (0.31 ± 0.17 ng/L), and BPAF (0.24 ± 0.15 ng/L) (Zhao et al., 2021). BPF (1.6 ng/g dw) and BPS (0.69 ng/g dw) were also detected in marine sediments from East China Sea (Xie et al., 2022). In the latter study, inshore seawater and sediment samples contained higher BPA and BPS concentrations, compared with offshore samples (Xie et al., 2022). The EU settled guidelines to classify the toxicity of a compound according to the $EC_{50}$ value: very toxic to aquatic organisms (<1 mg/L), toxic to aquatic organisms (1–10 mg/L), harmful to aquatic organisms (10–100 mg/L). Compounds with an associated $EC_{50}$ value above 100 mg/L are not classified (Commission of the European Communities, 1996). BPS has often been classified as harmful for the different tested microalgal species (Li et al., 2022; Yadav et al., 2023), while BPAF was considered toxic (Tišler et al., 2016; Yadav et al., 2023). In addition, the toxicological classification of BPF varies from toxic to harmful for aquatic organisms (Elersek et al., 2021; Wang et al., 2021).

In a recent study, three marine microalgae species (*Phaeodactylum tricornutum, Tetraselmis suecica* and *Nannochloropsis gaditana*) were exposed to five BPA analogues (BPS, BPAP, BPAF, BPFL and BPC) in single and multispecies tests. Microalgae were exposed to different BPs concentrations (5, 20, 40, 80, 150 and 300 μM) and the effects on several cell parameters, such as growth, reactive oxygen species (ROS) production, cell complexity, cell size, autofluorescence of chlorophyll *a*, and pigment concentrations were evaluated after 24, 48 and 72 h. The results showed that BPS and BPA were less toxic to microalgae than BPFL, BPAF, BPAP and BPC for the endpoints measured. In addition, *N. gaditana* was the least sensitive microalgae in comparison to *P. tricornutum* and *T. suecica* (Sendra et al., 2023). In our previous study, we investigated for the first time the effects of BPAF, BPF, BPS, alone or as a mixture, on the growth, cell size, and biochemical parameters of the marine diatom *P. tricornutum* Bohlin (Fabrello et al., 2023). This model marine diatom can be found in many coastal areas and is widely used as a feedstock in aquaculture (Cui et al., 2021). As for human consumption, high-value products of *P. tricornutum* include lipids, proteins, and pigments (carotenoids) (Cui et al., 2021). After induction, lipid content in *P. tricornutum* can contribute to up to 60 % of its dry weight, the major lipids being triacylglycerol, galactosyl acylglycerols, and mono-galactosyl acylglycerols (Alonso et al., 1998).

As a result of our previous study, we observed altered growth curves, an increase in oxidative stress and oxidative damage following exposure to BPA analogues (Fabrello et al., 2023). Consequently, the present study was carried out to better understand the impact of BPA analogues on *P. tricornutum* Bohlin. In particular, the effects of BPA analogues on ultrastructure, pigment content, lipids, and reactive oxygen species (ROS) production were analysed. In addition, capability of microalgae to accumulate BPA analogues were also investigated.

## 2. Materials and methods

### 2.1. Microalga exposure

*P. tricornutum* were exposed to three of the main BPA analogues currently used by chemical industries: BPAF, BPF, and BPS. In addition, we tested their mixture to evaluate the possibility of additive toxic effects between the three components. Briefly, microalgal cultures were exposed in triplicate to 300 ng/L of bisphenols BPAF, BPF, and BPS, as well as to their mixture containing 100 ng/L of each substance. The concentration tested of the individual compounds, or their mixture was chosen based on the data available in the literature on the presence of BPA analogues in aquatic ecosystems, including marine ones (see Introduction section), and to compare the results of our study with those from the literature about BPs toxicity on microalgae. The stock solutions (1 mg/L) of both BPAF and BPF were prepared in methanol, while BPS was dissolved in distilled water. To evaluate the effects of the solvent used in the preparation of two stock solutions, control cultures + methanol were also performed. The concentration of methanol in the tested and control samples was 300 μL/L, lower than 1 % (v/v). Microalgal culture, acclimated to the experimental conditions in a culture chamber at 16 °C, a light intensity of 40.5 μmol photons $m^{-2}s^{-1}$, and a photoperiod of 12:12 light/dark, was inoculated in Erlenmeyer flasks containing 200 mL of F/2 medium (Guillard, 1975) (prepared in 0.45 μm-filtered seawater) to obtain an initial concentration of $5 \times 10^5$ cells/mL (*inocula*) and exposed at the different bisphenol treatments. All the analyses were carried out in two different cell growth phases: exponential and at the beginning of the stationary growth phase, corresponding to 5 and 9 days of exposure, respectively. To ensure a constant contact between bisphenols and microalgae in a medium-term exposure (maximum 9 days), we would have had to renew the culture medium and the concentration of the compounds at least once every 24 h (= semi-static system). This procedure requires a centrifugation and/or filtration of microalgae and their resuspension in new culture medium (with or without contaminants) every 24 hrs. In our opinion this procedure could cause cell loss or damage. Therefore, we decided to adopt a static system to expose microalgae to BPA analogues. In this case, the contaminants were re-added to the culture medium after 4 and 7 days of the beginning of exposure, at the same initial concentration, without renewal of the medium. In this way, we allowed continuous exposure of the algae to contaminants without damaging them.

### 2.2. Chemical analysis

Methanol, acetonitrile, ammonium acetate, bisphenol AF (BPAF), bisphenol F (BPF), bisphenol S (BPS) and bisphenol A d-16, used as internal standard, were purchased from Merck (Milan, Italy). Ultrapure-grade water was produced with a Pure-Lab Option Q apparatus (Elga Lab Water, High Wycombe, UK).

F/2 medium samples were collected at day 0 (inoculation phase), 5 (exponential growth phase) and 9 (stationary growth phase). Medium samples were treated by a 0.2 μm RC Syringe Filter (Phenomenex, Bologna, Italy) before being analysed by UHPLC—HRMS. The system was equipped with an Agilent 1260 Infinity II LC chromatographer coupled to an Agilent 6545 LC/Q-TOF mass analyser (Agilent Technologies, Palo Alto, Santa Clara, CA, USA). The analytical column was a Kinetex 2.6 μm C18 Polar, 100 A, 100 × 2.1 mm (Phenomenex, Bologna, Italy), at 25 °C. Mobile phase A and B were water and methanol, respectively, both containing 10 mM ammonium acetate and the eluent flow rate was 0.30 mL/min. The mobile phase gradient profile was as follows (t in min): t0–4 0 % B; t4–22 0–100 % B, t22–25 100 % B; t25–32 0 % B. Injection volume was 400 μL. The MS conditions were: electrospray (ESI) ionization in negative mode, gas temperature 320 °C, drying gas 12 L/min, nebulizer 35 psi, sheath gas temperature 350 °C, sheath gas flow 11 L/min, VCap 5000 V, nozzle voltage 0 V, fragmentor 150 V. Centroid full scan mass spectra were recorded in the range 100–1000 *m/z* with a scan rate of 1 spectrum/s. The QTOF calibration was performed daily with the manufacturer's solution in this mass range. The MS were analysed by the Mass Hunter Qualitative Analysis software (Agilent Technologies, Palo Alto, Santa Clara, CA, USA).

For quantitative purpose, a matrix-matched seven points external calibration curve was made in the range 20–600 ng/L. Artificial seawater, prepared according to ASTM Standards D 1141 – 98, was used as matrix. Peak areas for each analyte, obtained by the [M-H]- extracted ion chromatogram (mass accuracy 10 ppm), were normalized for the area of the internal standard, added at the final concentration of 600 ng/L. Linearity was evaluated by the least squares regression and $R^2$>0.995 was obtained for all the analytes. Limits of detection (LODs) were 1 ng/L, 5 ng/L and 30 ng/L for BPAF, BPS and BPF, respectively. Samples and calibration solutions were analysed in triplicate.

BPA analogues bioaccumulation in microalgae was evaluated in cells

collected at the same time as for medium samples. Microalgae pellet was obtained by centrifugation for 15 min at 7000 rpm and room temperature of the microalgal culture and homogenized (Homogeniser SHM1, Avantor, VWR International srl, Milano, Italia). One mL of the homogenate was added with the internal standard at the final concentration of 20 μg/L. After centrifugation (13,000 rpm, 10 min, 4 °C), 20 μL of the supernatant were analysed by UHPLC—HRMS as previously described. Control microalgae homogenate was used to build a matrix-matched seven points external calibration curve, in the range 50–2000 μg/L. Linearity was evaluated by the least squares regression and $R^2$>0.998 was obtained for all the analytes. LODs were 20, 800 and 100 ng/L for BPAF, BPF and BPS, respectively.

### 2.3. In situ detection of hydrogen peroxide

The production of hydrogen peroxide ($H_2O_2$) was evaluated using dichlorofluorescein diacetate ($H_2$DCHF-DA) as fluorescent dye. In detail, the intracellular $H_2O_2$ oxidizes $H_2$DCHF-DA into highly fluorescent 2′,7′-dichlorofluorescein (DCF) that can be detected. Controls and bisphenol-treated microalgae were observed using an epifluorescence microscope at an excitation wavelength of 480 nm. A total of 300 cells (100 cells per slide, 3 slides for each experimental condition) were analysed for DCF presence, during both exponential growth phase (5 days of exposure) and stationary growth phase (9 days of exposure). Data are expressed as percentage of positive cells to DCF.

### 2.4. Photosynthetic pigments analysis

Total microalgal photosynthetic pigments were determined through a spectrophotometer according to Bai et al. (2011). In detail, after 5 and 9 days of exposure corresponding to exponential and stationary growth phases, respectively, total chlorophyll and carotenoids were extracted from the algal pellets using a homogenizer Tissue Lyser LT (Qiagen) and silica gel using 1 mL of acetone 90 % and kept in darkness at −20 °C for 1 day. The chlorophyll extracts were analysed by a spectrophotometer at 664, 647, 630, 480 and 510 nm. The pigment concentrations were calculated using the following equations:

- Chl *a* (mg/g fresh weight) = (((11.85*$OD_{664}$ – 1.54*$OD_{647}$ – 0.08*$OD_{630}$))/W)*(V/1000)
- Chl *c* (mg/g fresh weight) = (((24.52*$OD_{630}$ – 7.60*$OD_{647}$ – 1.67*$OD_{664}$))/W)*(V/1000)
- Carotenoids (mg/g fresh weight) = (((7.6*$OD_{480}$ – 1.49*$OD_{510}$))/W)*(V/1000)

where OD is the optical density, W is the net weight of the sample expressed in g of fresh weight, V is the volume (mL) of 90 % acetone used for extraction. The extinction coefficients proposed by Wellburn (1994) were used. Results were expressed as mg/g fresh weight.

### 2.5. Neutral lipid assay

*P. tricornutum* is a lipid-rich marine diatom that contains a high level of polyunsaturated fatty acids (Cui et al., 2021). The effects of exposure on neutral lipids of microalgae were evaluated using the Nile Red assay. In detail, 1 ml of cell cultures was incubated with the fluorophore Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) for 10 min at 37 °C. Samples images were obtained with an epifluorescence microscope at an excitation wavelength of 480 nm (Olympus CX31 fluorescent microscope) equipped with a digital image acquisition system. A total of 300 cells (100 cells per slide, 3 slides for each experimental condition) was analysed. We recorded the number of yellow spots indicating neutral lipid droplets into the microalgal cytoplasm. Data were arbitrarily reported as the percentage of three different classes: cells without lipid droplets, cells with 1–4 lipid droplets and cells with 5–8 lipid droplets.

### 2.6. Ultrastructural analysis

Microalgae from the six different experimental conditions were collected by centrifugation (5 min, 1500 g, 4 °C) and fixed for 2 h at 4 °C in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) and post-fixed at 4 °C for 2 h in 1 % osmium tetroxide in the same buffer. Samples were dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in araldite as reported by Moro et al. (2014). Sections were cut by an ultramicrotome (Ultracut S, Reichert-Jung, Wien, Austria). Then, ultrathin sections (600 Å) stained with uranyl acetate and lead citrate were observed by a transmission electron microscope (TEM, FEI Tecnai $G^2$) operating at 100 kV.

### 2.7. Statistical analysis

The effects of the independent factors, namely “treatment”, “growth phase”, and their interaction (treatment*growth phase), were evaluated by means of a two-way ANOVA analysis for each biological parameters, using OriginPro software (OriginPro, Version 2022. OriginLab Corporation, Northampton, MA, USA). The post hoc test (Fisher’s test) was performed for pairwise comparisons. All results are expressed as the mean ± standard deviation (SD). Statistically significant difference was set at $p < 0.05$.

## 3. Results

### 3.1. Chemical analysis

Actual concentrations of different bisphenols in culture media, measured by UHPLC—HRMS, at different timepoints are reported in Table 1. Instead, Table 2 reports the concentration of BPA analogues in microalgae homogenates at different timepoints. Results are reported as the mean concentration measured in three independent replicates. The actual concentrations of BPF in culture media remain almost constant from inoculation ($T = 0$) (309.1 ng/L) to exponential growth phase ($T =$ 5 days) (313.7 ng/L), whereas it decreased slightly (260.9 ng/L) during the stationary growth phase ($T = 9$ days). As for BPS, its actual concentration decreased slightly (276.5 ng/L) during the exponential growth phase and increased (374.7 ng/L) during the stationary growth phase, with respect to $T = 0$ (305 ng/L). On the other hand, the actual BPAF concentration remain almost constant from inoculation (306.1 ng/L) to exponential growth phase (395 ng/L), while a relevant increment was observed during the stationary growth phase (736 ng/L). The same behaviour was observed in the MIX treatment where only BPAF showed an actual higher concentration than those of BPF and BPS after 9 days of exposure.

As for bioaccumulation, BPAF and BPS were detected in microalgae pellets both after 5 and 9 days of exposure (Table 2), whereas BPF was under the LOD values. While the BPAF concentration remained constant between the 5-day (107.1 ng/L) and 9-day (107.6 ng/L) samples, the BPS concentration decreased from 5 (309 ng/L) to 9 days of exposure (220 ng/L). With regards to bioaccumulation in MIX-treated microalgae, only BPAF and BPS showed detectable concentrations after both 5 and 9 days of exposure, even if the respective values did not change between 5

**Table 1**
Actual concentrations of BPA analogues in culture medium at different timepoints (0, 5 and 9 days). Results are reported as mean ± standard deviation in brackets ($n = 3$).

| | | $T = 0$ days | $T = 5$ days | $T = 9$ days |
|---|---|---|---|---|
| BPF [ng/L] | | 309.1 (3.1) | 313.7 (6.3) | 260.9 (5.2) |
| BPS [ng/L] | | 305 (20) | 276.5 (8.3) | 374.7 (3.7) |
| BPAF [ng/L] | | 306.1 (2.0) | 395 (31) | 736 (125) |
| MIX | BPF [ng/L] | 111.6 (5.6) | 122 (12) | 105 (17) |
| | BPS [ng/L] | 102.9 (2.9) | 110.8 (6.6) | 126.5 (3.8) |
| | BPAF [ng/L] | 104.8 (2.3) | 110.1 (5.5) | 285 (10) |

**Table 2**
Concentrations of BPA analogues in microalgae pellet homogenates. Results are reported as mean ± standard deviation in brackets ($n = 3$). LODs were 20, 100 and 800 ng/L for BPAF, BPS and BPF, respectively.

| | | $T = 5$ days | $T = 9$ days |
|---|---|---|---|
| BPF [ng/L] | | <800 | <800 |
| BPS [ng/L] | | 309 (41) | 220 (37) |
| BPAF [ng/L] | | 107.1 (2.9) | 107.6 (6.7) |
| | BPF [ng/L] | <800 | <800 |
| MIX | BPS [ng/L] | 176 (25) | 120 (14) |
| | BPAF [ng/L] | 82.6 (4.3) | 75.8 (4.8) |

and 9 days of exposure.

### 3.2. Hydrogen peroxide production

As a prosecution of our previous study in which we observed an impairment in the antioxidant system of microalgae exposed to BPA analogues, in the present study the *in vivo* production of hydrogen peroxide, a well-known ROS, was evaluated. During the exponential growth phase, we did not observe any significant difference in the percentage of positive diatoms to DCF among experimental conditions (data not shown). Conversely, during the stationary growth phase, statistical analysis revealed a significant increase in the percentage of cells positive to DCF - indicating hydrogen peroxide production – after exposure to both BPS and MIX (Fig. 1).

### 3.3. Photosynthetic pigments

The concentrations of total chlorophylls (*a* and *c*) and carotenoids in the different growth phases are reported in Figs 2A-B. As for total chlorophylls, Two-way ANOVA analysis did not reveal any significant effect of the independent factors "treatment", "growth phase", and their interaction (treatment*growth phase) on the total amount of these pigments during the exposure time (Fig. 2A). Similarly, carotenoids total amount did not change significantly among experimental conditions (Fig. 2B).

### 3.4. Neutral lipids

The number of yellow spots, indicating the presence of neutral lipids inside cells, was recorded. Cells were then divided arbitrarily in three different classes: cells without yellow spots (Fig. 3A), cells with 1–4 lipid droplets (Fig. 3B) and cells with 5–8 lipid droplets (Fig. 3C). Statistical analysis revealed that the independent factors "treatment" (Two-way ANOVA: $p < 0.001$, $F = 6.66$), "growth phase" (Two-way ANOVA: $p < 0.001$, $F = 17.559$), and their interaction (treatment*growth phase) (Two-way ANOVA: $p < 0.001$, $F = 7.78$) significantly affected the percentage of cells without yellow spots (0 spots). Similarly, the percentage of cells with 1–4 lipid spots was significantly altered by the independent factors "treatment" (Two-way ANOVA: $p < 0.05$, $F = 3.34$), "growth phase" (Two-way ANOVA: $p < 0.001$, $F = 34.84$), and their interaction (Two-way ANOVA: treatment*growth phase) ($p < 0.05$, $F = 3.66$). Regarding the cells with 5–8 lipid droplets, their percentage was altered only by the factors "growth phase" (Two-way ANOVA: $p < 0.01$, $F = 10.58$) and the interaction treatment*growth phase (Two-way ANOVA: $p < 0.05$, $F = 2.92$). The post-hoc test on the Nile Red assay results indicated that all the treatments significantly increased the number of microalgae without lipids (Fig. 3A) during the exponential growth phase, whereas during the stationary growth phase their percentage did not change significantly between control and treated groups. In addition, during the exponential growth phase, the percentage of cells with 1–4 and 5–8 yellow spots reduced significantly following exposure to BPA analogues. In detail, all treatments significantly reduced the percentage of microalgae with 1–4 yellow spots, while the percentage of diatoms with 5–8 yellow spots was significantly reduced by BPF, BPS and MIX (Fig. 3B-C). Lastly, during the stationary growth phase, the percentage of cells with 1–4 remained unchanged between controls and treatments, while in the case of cells with 5–8 yellow spots their percentage was increased by the exposure to bisphenols, however, it was not statistically significant.

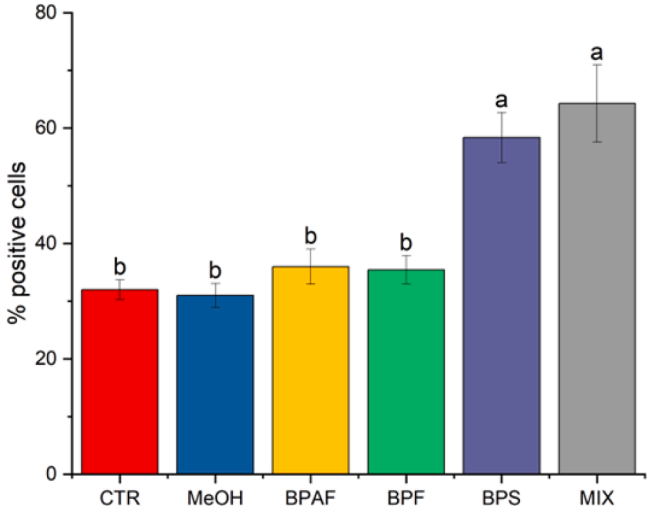


**Fig. 1.** Percentage of cells positive to DCF denoting the presence of hydrogen peroxide during the stationary phase of *P. tricornutum* under control condition (CTR), control+solvent (MeOH) and bisphenols. The values are mean ± SD ($n = 300$). Different letters indicate statistically significant differences in comparison with the related control ($p < 0.05$).

### 3.5. Ultrastructural of microalgae

In the exponential growth phase at the different exposure conditions, we observed that control and control+methanol cultures had chloroplasts with well-organized thylakoid membranes and a central pyrenoid (Fig 4A-B). On the contrary, the culture cells treated with BPA analogues and MIX showed chloroplasts characterized by evident dilated thylakoid membranes (Fig 4C-F). In addition, following the treatments we observed the presence of degeneration areas in the cytoplasm (Fig 4C-E).

In the stationary growth phase, control and control+methanol culture cells were characterized by chloroplasts with a regular thylakoid system, even if the cytoplasm in some areas appeared degraded (Fig 5A-B). Moreover, following the treatments the culture cells were characterized by a deep degradation of the cytoplasm, but showed chloroplasts without evident alterations of the thylakoid system (Fig 5D-E). However, some dilated thylakoid membranes were observed in the MIX-treated cells (Fig. 5F). In addition, in the different bisphenol treatments lipid bodies were visible (Figs 5C-D).

## 4. Discussion

The results of the present study revealed that during the stationary growth phase both BPS and MIX significantly increased the percentage of microalgae showing positivity to the hydrogen peroxide. The increase in the percentage of cell positive to hydrogen peroxide is in accordance with our previous results in which we observed an impairment of the microalgal antioxidant defences, mainly caused by BPS and MIX (Fabrello et al., 2023). In addition, our results agree with other studies that demonstrated the capacity of bisphenols to produce ROS. For instance, in a study of Sendra et al. (2023) *P. tricornutum* was exposed to different BPA analogues. They observed an increased ROS production in microalgae treated with BPC, BPS, BPA, BPAF, BPAP, and BPFL (Sendra et al., 2023). Similarly, BPA significantly increased ROS production in *Chlorella pyrenoidosa*, exposed to 5, 8, 11, and 15 mg/L, while BPS caused an increase in ROS levels at 15, 20, and 40 mg/L (Li et al., 2022).

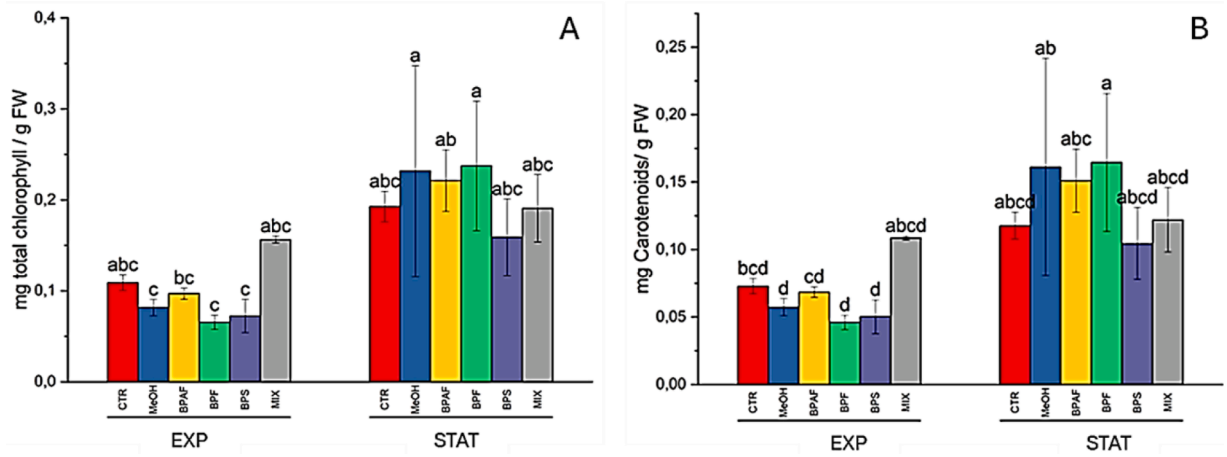


**Fig. 2.** Total chlorophyll (A) and carotenoid (B) contents, expressed as mg/g fresh weight (FW), during the exponential (EXP) and stationary (STAT) growth phase of *P. tricornutum* exposed to bisphenols. The values are mean ± SD ($n = 3$). Different letters indicate statistically significant differences in comparison with the related control ($p < 0.05$).

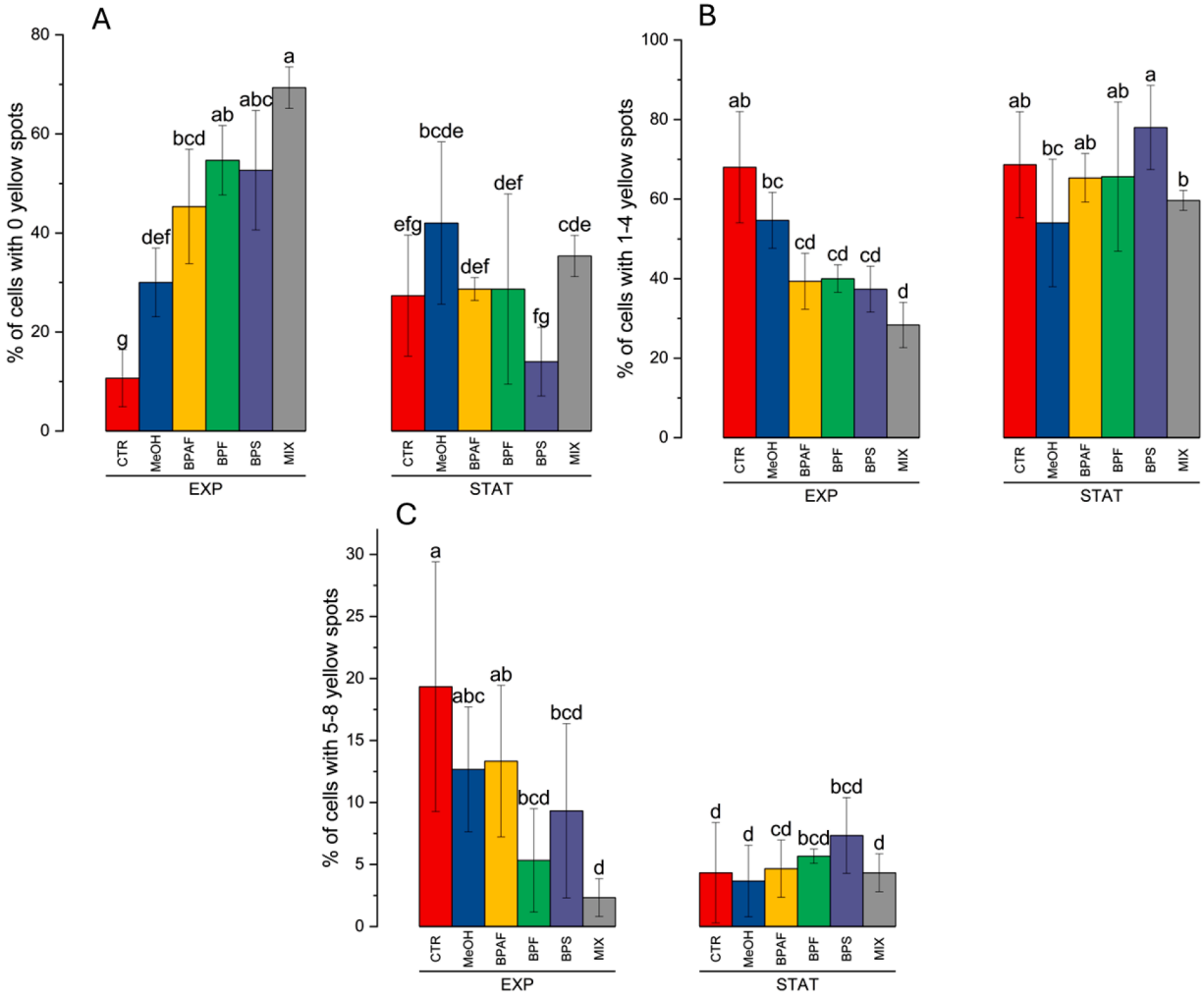


**Fig. 3.** Percentage of microalgae with 0 yellow spots (A), with 1–4 yellow spots (B), with 5–8 yellow spots (C) during the exponential (EXP) and stationary (STAT) growth phase of *P. tricornutum* exposed to bisphenols. The values are mean ± SD ($n = 300$). Different letters indicate statistically significant differences in comparison with the related control ($p < 0.05$).

Furthermore, a recent study investigated the effects of 1 mg/L BPA and its mixture with different concentrations of titanium dioxide nanoparticles ($nTiO_2$) on ROS production in the freshwater alga *Scenedesmus obliquus*. The authors reported that the level of both superoxide radical anion and hydroxyphenyl radical were increased following exposure for 72 h to BPA and BPA+ $nTiO_2$ (Das and Mukherjee, 2024).

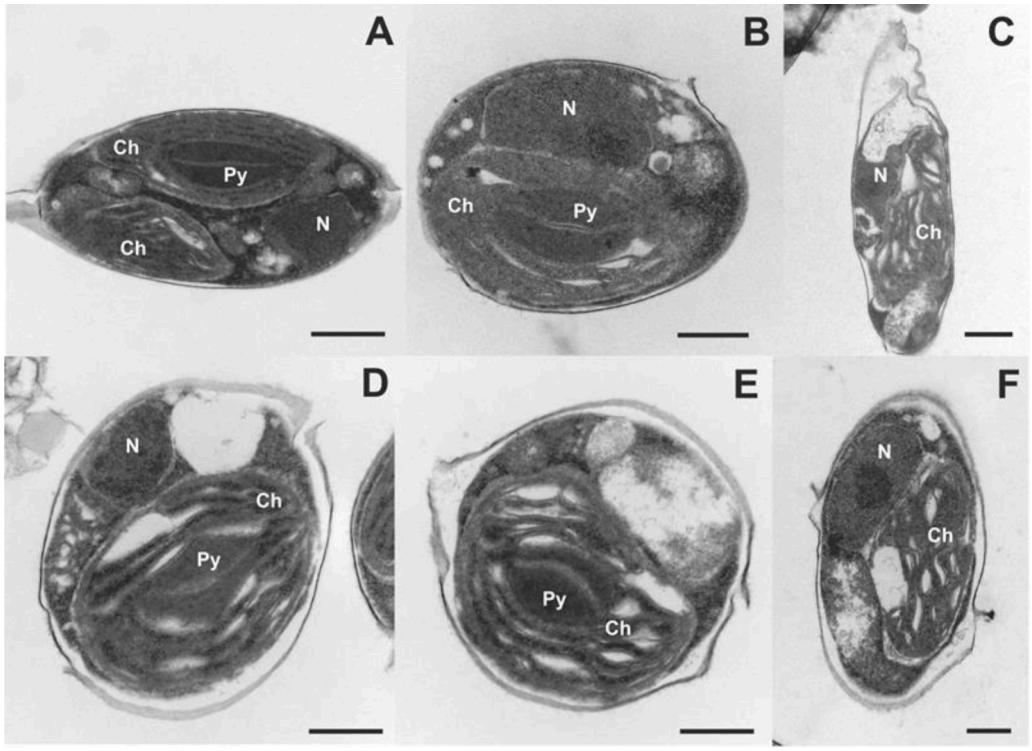


**Fig. 4.** Electron micrographs of control and treated cells of *P. tricornutum* during the exponential growth phase. **A)** Control, scale bar = 1 μm; **B)** MeOH, scale bar = 500 nm; **C)** BPAF, scale bar = 1 μm; **D)** BPF, scale bar = 500 nm; **E)** BPS, scale bar = 500 nm; **F)** MIX, scale bar = 500 nm. **Ch**: chloroplast; **Py**: pyrenoid; **N**: nucleus.

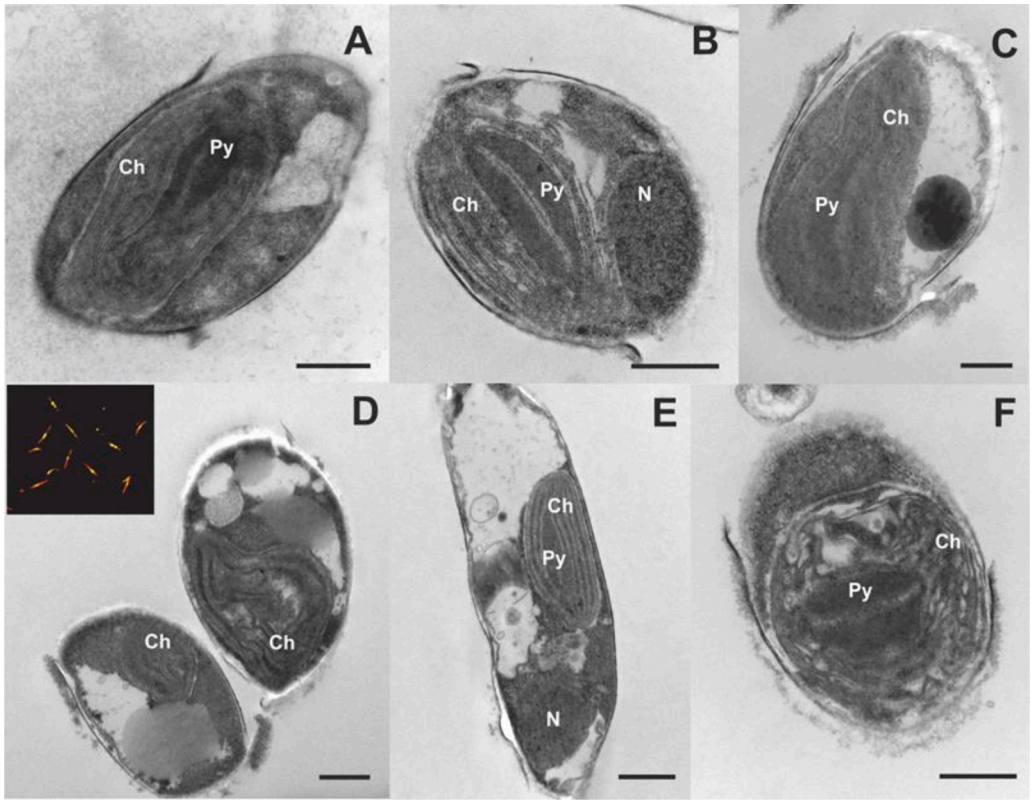


**Fig. 5.** Electron micrographs of control and treated cells of *P. tricornutum* during the stationary growth phase. **A)** Control, scale bar = 500 nm; **B)** MeOH, scale bar = 500 nm; **C)** BFAF, scale bar = 1 μm; **D)** BPF, scale bar = 500 nm; **E)** BPS, scale bar = 1 μm; **F)** MIX, scale bar = 500 nm. **Ch**: chloroplast; **Py**: pyrenoid; **N**: nucleus. Insert in D: micrograph of microalgae stained with to Nile Red.

As for other contaminants, Solomonova et al. (2023) have recently demonstrated that exposure of two microalgal species, namely *Prorocentrum cordatum* and *Dunaliella salina*, to copper oxide nanoparticles resulted in progressing production of ROS in the algal cells, while their specific growth rates decreased. Similarly, copper oxide nanoparticles and copper ions were shown to increase ROS production in the primnesiophyte microalga *Isochrysis galbana* (Shoman et al., 2023). Interestingly, in the latter two studies (Solomonova et al., 2023; Shoman et al., 2023) the authors assessed the production of ROS using the same method adopted in the present study, that is 2–7-dichlorofluorescein diacetate assay (Solomonova et al., 2023). Overall, results of the present study and those available in the literature suggest that BPA and its analogues, as well as other contaminants, can promote oxidative stress in microalgae inducing ROS production.

As for total chlorophylls ($a + c$) and carotenoids, results of our study revealed that their concentrations were not affected by BPA analogues. Recently, the effects of bisphenols on the chlorophyll content were evaluated in three different microalgae, namely *P. tricornutum, Tetraselmis suecica* and *Nannochloropsis gaditana* after 24, 48 and 72 h of exposure (Sendra et al., 2023). Regarding *P. tricornutum*, that study revealed that the autofluorescence of chlorophyll *a* was significantly decreased by almost all the tested BPA analogues at concentrations above 20 μM. However, BPS was the only compound able to cause a significant increase in the chlorophyll *a* autofluorescence at concentrations over 80 μM. Similarly, in the case of *T. suecica*, the authors reported a similar response with again the BPS that caused the least pronounced effects (except for the highest BPS concentration). In addition, *N. gaditana* showed a reduction of chlorophyll *a* autofluorescence for BPAP, BPAF and BPFL after 48 and 72 h for all exposure concentrations. Furthermore, the chlorophyll *a* content of the microalga *Stephanodiscus hantzschiiwas* was not affected by BPA exposure at concentrations ranging from 0.01 to 1 mg/L, while chlorophyll *a* content decreased gradually with BPA concentrations higher than 1 mg/L (Li et al., 2009). Conversely, BPA significantly increased both the chlorophyll *a* and chlorophyll *b* contents in the green microalga *Chlorella pyrenoidosa* treated for 16 days with 1 and 10 mg/L (He et al., 2022). Furthermore, the exposure for 14 days to 1 mg/L of BPS and BPAF led to an increase in both total chlorophyll and carotenoid contents in *Chlamydomonas mexicana*, whereas BPF, BPB, and BPZ caused a reduction of the total chlorophyll content, without significant effects on carotenoids (Yadav et al., 2023).

TEM investigations highlighted that bisphenol analogues caused alterations of the cell ultrastructure. Indeed, during the exponential growth phase we observed an increased dilation of thylakoid membranes and a loss of their native structure. Instead, during the stationary growth phase, the thylakoid membranes were dilated only in the MIX treatment. The presence of regular thylakoid system in cells exposed to single bisphenol analogues during the stationary growth phase could be a result of a recovery strategy of microalgae that were able to restore their chloroplast ultrastructure. However, the evident degradation of some areas of the cytoplasm suggests the impact of such compounds on the cell organization. Our results recorded during the exponential growth phase are in accordance with those of Li et al. (2009) in another diatom species. Indeed, in that study the diatom *Stephanodiscus hantzschii* exposed to concentrations of BPA higher than 5 mg/L showed poorly organized organelles (Li et al., 2009). An alteration of chloroplast structure, consisting of both destruction of thylakoid membranes and breaking down of chloroplast envelope was also observed in the macroalga *Gracilaria lemaneiformis* exposed to 10 mg/L of BPA. In the same macroalga, Yu et al. (2007) demonstrated a chloroplast change, consisting in the thylakoid number rupture and dissolved thylakoid membranes, after the treatment with dimethyl phthalate.

Considering that *P. tricornutum* is a lipid-rich marine diatom (Cui et al., 2021) and that bisphenols can alter the lipid metabolism (Gu et al., 2021; Ul Haq et al., 2020), we decided to evaluate the effects of BPA analogues on lipids, revealing the presence of lipid bodies in microalgae by means of epifluorescence microscope observations. Interestingly, we observed that the main alterations occurred in the exponential growth phase, during which the percentage of cells without evident lipid droplets increased significantly at all bisphenol treatments, when compared to the control group. During the exponential growth phase, all treatments significantly reduced the percentage of diatoms with 1–4 yellow spots, while the percentage of diatoms with 5–8 yellow spots was significantly reduced by BPF, BPS and MIX only. Overall, our results suggested a marked decrease in lipid content (as revealed by lipid droplets stained with Nile Red) in BPA analogues-treated cells. Similarly, Duan et al. (2019) observed an inhibition of the fatty acid metabolism in *C. pyrenoidosa* exposed for 72 h to 10 mg/L of BPA. In that study, the authors concluded that BPA is a hormetic agent, inhibiting or stimulating the energy metabolism depending on its concentration (Duan et al., 2019). In the stationary phase, results of the Nile Red assay indicated an overall increase in the number of lipid droplets in treated cells, with respect to the controls. In particular, an increased percentage (not significant) of cells with 5–8 lipid droplets were observed in treated cells, supporting evidence of ultrastructural analyses under TEM. The results concerning both the lipid presence and the degradation of cytoplasm suggest that in the stationary phase bisphenol analogues affect the ultrastructure of *P. tricornutum* cells. Overall, we suggest a possible relationship between oxidative stress (Fabrello et al., 2023) and lipid accumulation (the present study) in *P. tricornutum* exposed to BPA analogues. Interestingly, oxidative stress in algal cells has been shown to induce lipid accumulation as a defence mechanism (Yilancioglu et al., 2014).

Summarising, the present study demonstrated that exposure of *P. tricornutum* to BPA analogues, namely BPAF, BPF, and BPS (alone or as a mixture), can affect some important biochemical and ultrastructure features of microalgae, mainly by promoting ROS production. Nevertheless, this study revealed the capability of microalgae to bioaccumulate bisphenols, at least two out of three of those tested. To the best of our knowledge this is the first report on the capability of microalgae to bioaccumulate such contaminants. This evidence suggests a potential ecotoxicological risk for filter-feeders organisms.

## CRediT authorship contribution statement

**Jacopo Fabrello:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sofia Guidorizzi:** Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Ciscato:** Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mariano Battistuzzi:** Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Emanuela Moschin:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francesca Dalla Vecchia:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Isabella Moro:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Marco Roverso:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sara Bogialli:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Valerio Matozzo:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

## 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

The data that has been used is confidential.

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