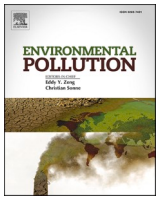
Environmental Pollution 333 (2023) 122073

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Single and multispecies microalgae toxicological tests assessing the impact of several BPA analogues used by industry☆

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A B S T R A C T

BPA is a hazard for human and environmental health and recently BPA was added to the Candidate List of substances of very high concern by European Chemical Agency (ECHA). In accordance with this proposal, the authorities have encouraged the replacement of BPA by BPA analogues; however, little is known about the impact of these compounds on the environment. Due to this situation five BPA analogues (BPS, BPAP, BPAF, BPFL and BPC) were chosen in order to study their effects on marine primary producers. Three marine microalgae species (*Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Nannochloropsis gaditana*) were selected for single and multispecies tests concerning the ecotoxicological effects of these BPA analogues. Microalgae were exposed to BPs over 72 h at different dosages (5, 20, 40, 80, 150 and 300 μM). Responses such as: growth, ROS production, cell complexity, cell size, autofluorescence of chlorophyll *a*, effective quantum yield of PSII and pigment concentrations were assessed at 24, 48 and 72 h. The results revealed that BPS and BPA showed lower toxicity to microalgae in comparison with BPFL > BPAF > BPAP and >BPC for the endpoints studied. *N. gaditana* was the least sensitive microalgae in comparison to *P. tricornutum* and *T. suecica*. However, a different trend was found in the multispecies tests where *T. suecica* dominated the microalgae community in relation to *N. gaditana* and *P. tricornutum*. The results of this work revealed for first time that present day BPA analogues are a threat and not a safe substitute for BPA in terms of the marine phytoplanktonic community. Therefore, the results of their impact on aquatic organisms should be shared.

1. Introduction

In the last few decades, plastic polymers have invaded the market, human life and the environment. They are formed by a wide number of additives, among which are the bisphenols (BPs). BPs are synthetic chemicals used in a variety of industrial applications for the production of polycarbonate plastics and epoxy resins used in optical, automotive, electrical and electronics, housewares and appliances, construction, medical, food packaging, interior surface coatings, metal beverage cans, dental sealants, and thermal paper products (Ballesteros-Gómez et al., 2014). Of all the BPs, bisphenol-A [2,2-bis(4-hydroxyphenyl) propane (BPA)] is still the most frequently used worldwide with an annual production of up to 8 million tons per year (Wu et al., 2018). The widespread use of BPA has resulted in its ubiquitous presence in marine environments and biota (Zhao et al., 2021). Bisphenol A (BPA) compromises the health of humans and the environment (Muhamad et al., 2016), due to its adverse effects as an endocrine disrupter. The lowest observable adverse effect of BPA is determined as 50 mg kg^{-1} bodyweight per day by the US EPA (Mileva et al., 2014). Furthermore, the European Food Safety Authority (EFSA) has indicated that the current temporary tolerable daily intake (t-TDI) for BPA is 4 μg kg^{-1} bodyweight per day (EFSA, 2017). Due to human health impacts, BPA use has been restricted in the European Union to protect human health and the environment (Lucarini et al., 2020) and in 2017 the European Chemical Agency (ECHA) added BPA to the Candidate List of substances of very high concern. The authorities have also been encouraging the replacement of BPA by what they considered safer analogues. Due to the pressure of the authorities, more than 200 BPA analogues have been developed as potentially safer alternatives to replace BPA in the industry and market (den Braver-Sewradj et al., 2020; Lucarini et al., 2020; Pelch et al., 2019; Russo et al., 2018).

However, there is a lack of knowledge about the effects they may generate (Sendra et al., 2023). The BPA analogues selected in this study are potential candidates to substitute BPA, and they are already used in the industry (BPS, BPAP, BPAF, BPFL and BPC).

The BPA analogues selected in the present study have also been

☆ This paper has been recommended for acceptance by Dr Amit Bhatnagar.

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https://doi.org/10.1016/j.envpol.2023.122073
Received 28 February 2023; Received in revised form 15 June 2023; Accepted 16 June 2023
Available online 16 June 2023

recorded in children and pregnant women, breast milk, fish, food, food packaging, personal care products and house dust (Shapiro, 2003). These data indicate that humans are exposed to BPs on a daily and chronic basis. Most of the studies have been performed on mammal cell lines, and BPs have revealed cyto-and genotoxicity (Sendra et al., 2023). Although BPs analogues have been recorded in different concentrations ranging in the marine environment, however these values depend of the number of studies and concerning of each BPA analogues (Fabrello and Matozzo, 2022). These values are close to the PNEC [0.66 μM) recommended by European Union Risk Assessment Report; (Aschberger et al., 2010)] value and could be passed due to the higher use and environmental release of BPA analogues. BPA (at 74 pM (Ozhan and Kocaman, 2019)) reaching up to the maximum levels in aquatic system; 270 nM (Wu and Seebacher, 2020), BPAF (at 10 pM; (Zhao et al., 2019) and BPS (at 24 pM); (Zhao et al., 2019)) have been recorded in seawater. On the other hand, the other BPs bisphenols have not yet been recorded in seawater but they have been recorded in other aquatic compartments; i.e. BPAP (at 220 pM in WWTP; (Wang et al., 2019)), BPFL (at6.3 μM; (Zhao et al., 2019)) and BPC (1.4 nM; (Wang et al., 2022)). Therefore, it is time to assess the effects of these new chemical compounds on marine ecosystems and establish a consensus about the potential use of them as humane and environmentally safe alternatives to BPA.

Although the presence of these analogues have been recognized in natural compartments even to similar to or higher concentration than BPA (Catenza et al., 2021), their effects at the base of the food web (marine phytoplankton) are not well understood yet. There is a clear interplay between algae and BPA (Azizullah et al., 2022). At certain concentrations, phytoplankton seems to be able to degrade BPA (Ben Ali et al., 2021; Ji et al., 2014; Nakajima et al., 2007). The degradation of this compound by microalgae seems to be enhanced by the presence of light (Hirooka et al., 2003; Wang et al., 2017) and the use of algae-bacteria mixed cultures (Prosenc et al., 2021). Some authors have stated that there is a strong negative correlation between the BPs measured and phytoplankton biomass(Staniszewska et al., 2015), while other authors have reported 2.6 times more BPA in seawater when phytoplankton blooms occurred (Shi et al., 2017). In any case, phytoplankton seems to be affected and is a way that BPs enter the trophic chain (Esperanza et al., 2020; Staniszewska et al., 2015).

On the other hand, at higher concentrations, BPA is certainly toxic to microalgae. Ebenezer and Ki (2016) reported toxic concentrations for the diatom *Ditylum brightwellii*, green algae *Tetraselmis suecica*, and dinoflagellate *Prorocentrum minimum* with BPA values of 0.16, 68.11 and 6.59 μM, showing differences of up to three orders of magnitude in the sensitivity of the species assayed. There are reports which state BPA EC_{50} values of 131.41 μM for *Chlamydomonas reinhardtii* (Esperanza et al., 2020) and 2.50 μM for *Tetraselmis chuii* (Falcão et al., 2020). For *Chlorella pyrenoidosa*, Li et al. (2022) reported EC_{50} values after 144 h exposition of 77.53 and 263.99 μM for BPA and BPS respectively. When exposed together, the EC_{50} values for each pollutant was 20.96 and 71.92 μM respectively, suggesting a synergistic behaviour between BPA and BPS (Li et al., 2022). Elersek et al. (2021) reported EC_{50} values for BPA of 29.78 μM for *Pseudokirchneriella subcapitata* and 21.02 μM for *Synechococcus leopoliensis*. In the same experiment, the EC_{50} values for BPF were 48.44 μM for *P. subcapitata* and 25.97 μM for *S. leopoliensis*, but a mixture of BPA and BPF demonstrated a clear synergistic effect on the former microalgal species. Yang et al. (2021) did not calculate EC_{50} values but stated that 8.76 μM BPA partially inhibited growth of a *Tetraselmis* sp. microalgal culture, while 17.47 μM totally inhibited its growth. Recent RNA-seq analyses have demonstrated the impairments of metabolic routes in *Chlorella vulgaris* exposed to high concentrations of BPA (Duan et al., 2019). In microcosm experiments, M'Rabet et al. (2019) exposed mixed microalgal populations to water incubated with microplastics for 30 days to liberate BPA and other compounds. After 96 h of exposure, there was a decrease in the microalgal populations of up to 50% and a drastic change in the microalgal assemblage structure (M'Rabet et al., 2019). In these single and multispecies experiments with *Tetraselmis suecica*, *Phaeodactylum tricornutum* and *Nannochloropsis gaditana*, it was demonstrated that *P. tricornutum* was the most sensitive species among the organisms assayed, while *N. gaditana* did not show any differences at the experimental concentrations tested (Seoane et al., 2021).

Although a great part of the scientific literature and regulation has been focused on BPA, little attention has been paid to BPA analogues. However, quite recently two studies demonstrated that BPF and BPAF do not seem a safer alternative to BPA for primary producers (Czarny et al., 2021; Elersek et al., 2021), while BPS could be an alternative chemical compounds to BPA with lower effects (Falcão et al., 2020).

In relation to this concern, he main goal of this study is to examine the toxicity of BPA and five BPA analogues in three representative marine taxa microalgae. Concentrations of BPA ranging from $ng{\cdot}L^{-1}$ to $\mu g{\cdot}L^{-1}$ can be found in the coastal marine environment (Pojana et al., 2007). Although only 5 μM (the lowest concentration used in the present study) is close to the marine environmental concentration, a wide range of concentration (5–300 μM) was included to compare the results with the literature about microalgae toxicity.

The three species were also selected according to its ecological relevance and its intrinsic properties to be detected by flow cytometry. The microalgae selected were tested using single and multispecies ecotoxicity assays.

2. Materials and methods

2.1. Reagents

Bisphenol A (4,4'-(propane-2,2-diyl) diphenol; BPA), Bisphenol S (4,4′-Sulfonyldiphenol; BPS), Bisphenol AP (4,4′-(1-Phenylethylidene) bisphenol; BPAP), Bisphenol AF (4,4′-(Hexafluoroisopropylidene) diphenol; BPAF), Bisphenol FL (4,4′-(9-Fluorenylidene)diphenol; BPFL), Bisphenol C (4,4'-(2,2-dichloroethene-1,1-diyl)diphenol; BPC). 2′-7′-dichlorofluorescein diacetate (DCFH-DA) were supplied by Sigma Aldrich. All glassware was washed with diluted nitric acid (10%) and rinsed several times with de-ionized water (Milli-Q) before use to avoid cross contamination.

2.2. Test organisms

Three marine microalgae species were selected according to be representative taxa of marine environments; *Phaeodactylum tricornutum* Bohlin 1898 (BACILLARIOPHYCEAE), strain CCMM 07/0402; Nannochloropsis gaditana L.M. Lubián 1982 (EUSTIGMATOPHYCEAE), strain 04/0201 and Tetraselmis suecica (Kylin) Butcher 1959 (CHLORODENDROPHYCEAE), strain 03/0202. All the strains were obtained from the Marine Microalgae Culture Collection at ICMAN (ICMAN-CCMM). Stocks were cultured in Guillard's f/2 medium (Guillard and Ryther, 1962). Cells were incubated in filtered (0.2 μm) marine culture medium (pH: 8.2) and f/2 marine medium for the experiments. Cells were grown in this medium for two weeks prior to the experiment. In the case of the diatom and the mixed culture, 50 mg L^{-1} of SiO_2 were added to the cultures.

2.3. Toxicity assays

Bioassays were carried out in triplicate with BPs at the environmentally relevant concentrations of: 5, 20, 40, 80, 150 and 300 μM. The BP stocks were prepared in DMSO solvent. Control samples were prepared with the highest concentration of DMSO used when 300 μM of BPs were prepared.

The experiments were performed under continuous visible light (300 $\mu E\ m^{-2}\ s^{-1}$) at 20 °C±2. Microalgae toxicity assays were performed according to the OECD Nº 201 procedure (OECD, 1986). The bioassays were done in sterile white flat bottom 96 well-assay polystyrene plates. Different 96-well plates were analysed at every sampling time (24, 48

Table 1
EC_{50} (μM) of growth inhibition for the microalgae tested at 72 h exposed to BPs selected over the singles experiments.

BPs	*P. tricornutum*	*T. suecica*	*N. gaditana*
BPA	4.21 ± 0.35	<5	28.88 ± 2.18
BPS	<5	<5	60.81 ± 15.35
BPAP	<5	<5	<5
BPAF	<5	<5	<5
BPFL	<5	<5	<5
BPC	8.07 ± 3.12	<5	<5

and 72 h); and the experiments were done in triplicate. The borders of the plates were filled with filtered marine water to avoid the evaporation in the rest of the plate. Initial cell density was 10^4 cells·mL^{-1} for single experiments and 10^3 cells·mL^{-1} according to multispecies assays. Microalgae from different experiments were analysed by flow cytometry (BD Accuri C6) using a side scatter detector (SSC) and FL3 detector (excitation: 488 nm; emission: 670 nm). According to the FL3 excitation and emission, this detector indicates the autofluorescence of chlorophyll *a*, which was recorded for the experiments.

A total of 50 μL were analysed by flow cytometry at 24, 48 and 72 h for the single and multispecies experiments to assess BPs toxicity. The growth of the microalgae population was monitored by measuring the cellular density, locating each microalgae population using SSC and an FL3 detector. Values for 50% of growth inhibition (EC_{50}) after 72 h were obtained for each microalgae species exposed to the BPs. EC_{50} growth inhibition values were fitted to the Hampel et al. (2001) model. The responses related to intrinsic microalgae properties, such as forward light scatter (FSC) linked to the size or volume of cells and side light scatter (SSC) and also related with the intracellular complexity of the cells (Shapiro, 2003), were analysed. Production of intracellular ROS (reactive oxygen species: superoxide, hydroxyl and hydrogen peroxide) was quantified using the 2′-7′-dichlorofluorescein diacetate (DCFH-DA) method (He and Häder, 2002; Stachowski-Haberkorn et al., 2013). For this probe a positive control with 0.1 mM of H_2O_2 was employed. A quantity of 80 μM DCFH-DA (0.8% DMSO) was added to each well of a fresh sample. After 30 min in darkness, at room temperature, the percentage ROS were measured by green fluorescence (FL1, 533/30 nm) of stained cells using the flow cytometer. Finally, the extent of oxidative stress was calculated as the percentage of positive cells that fluoresce green (stained with DCFH-DA) with respect to the total microalgae population.

The effective quantum yield of photosynthetic energy conversion in PSII (E.Q.Y.) and total chlorophyll concentration were measured fluorometrically using a Phyto-PAM instrument (Heinz Walz GmbH) equipped with an ED-101 US/MP Optical Unit. This parameter measures the efficiency of the photochemical energy conversion process (Schreiber et al., 1995).

These endpoints were only recorded in the multispecies experiments because Phyto-PAM instruments can measure chlorophyll for different groups of pigments (blue, green and brown pigments). These experiments were carried out in 50 mL Erlenmeyer flasks in triplicate, due to

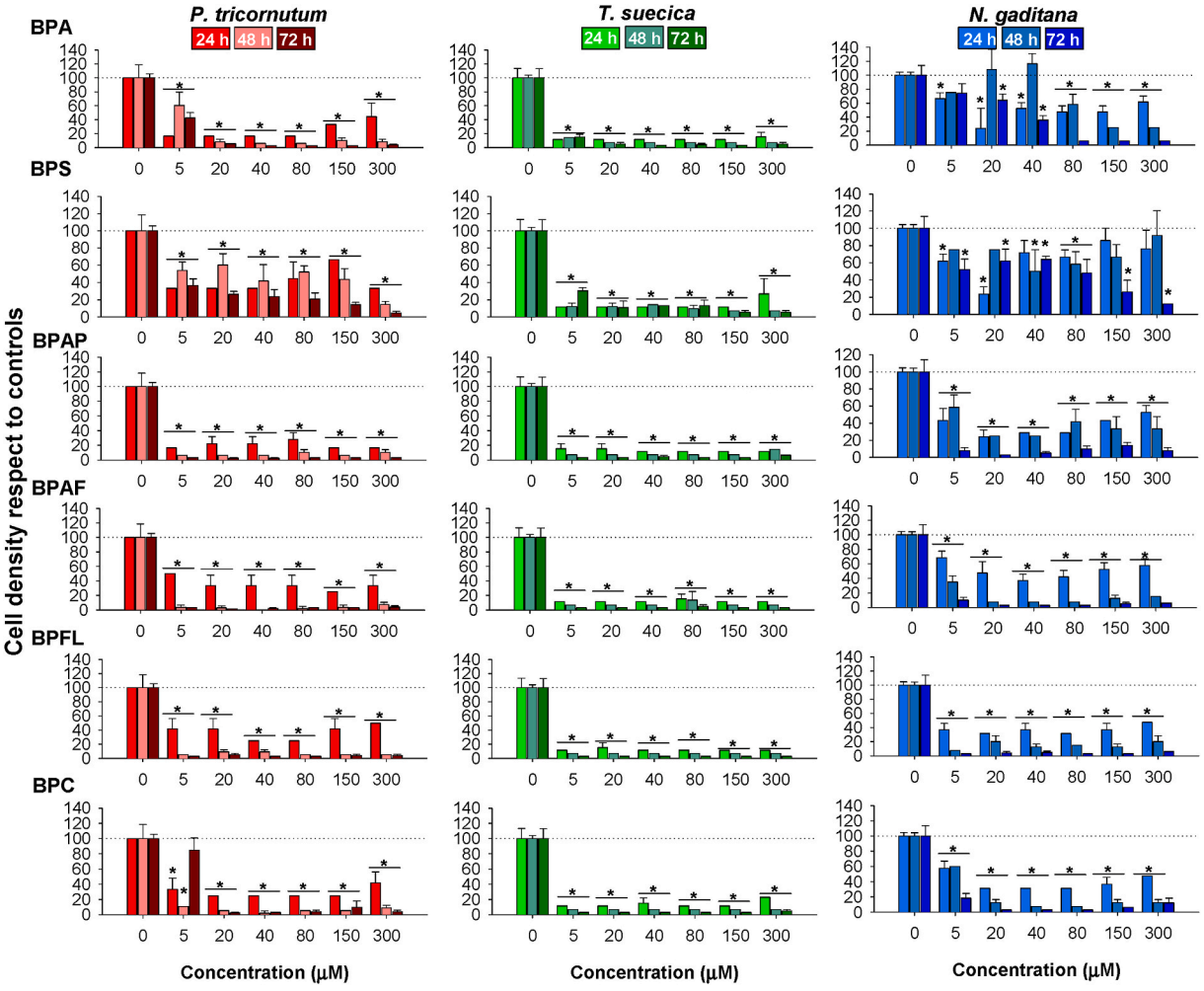

Fig. 1. Cell density respect to controls for microalgae exposed to selected BPs after 24, 48 and 72 h of exposure. Black asterisks show the significant differences among the controls and treatments for single experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

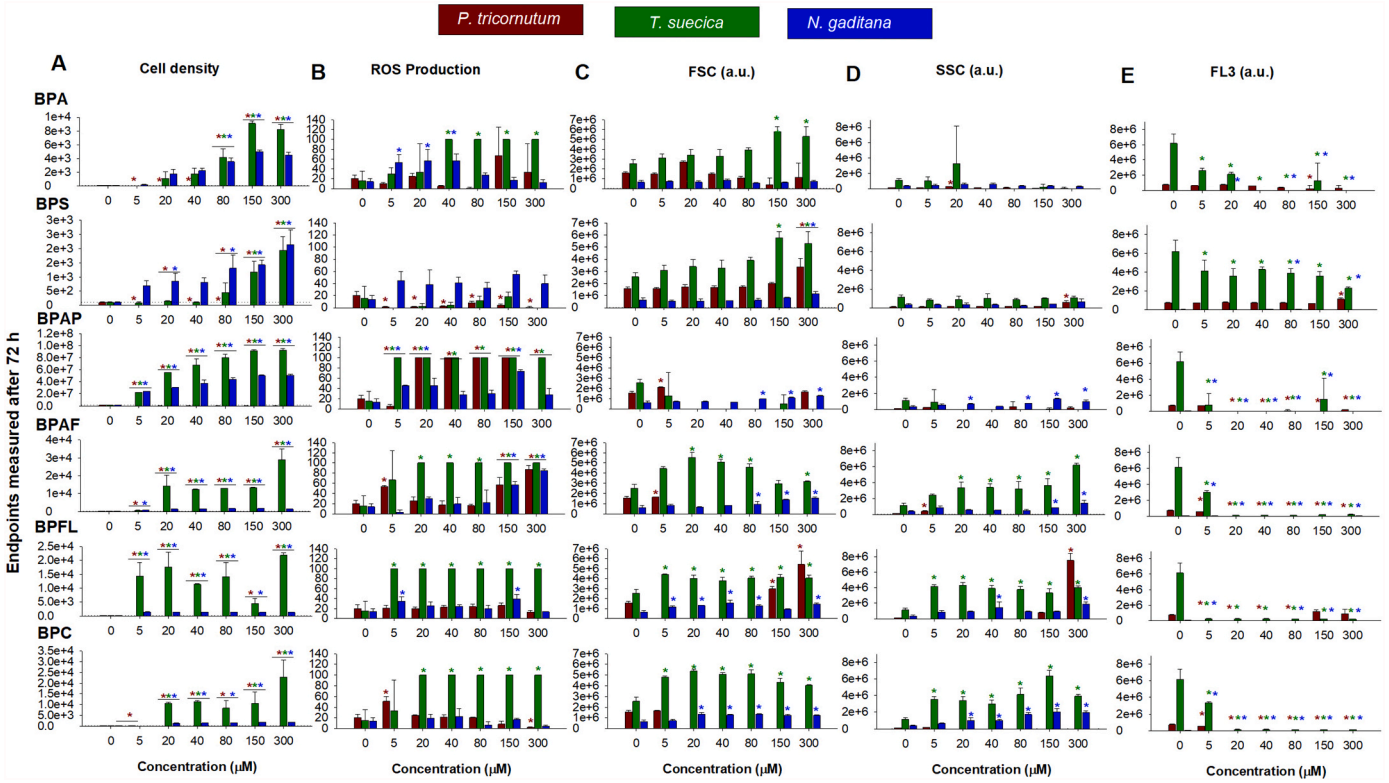

Fig. 2. Endpoints respect to controls for multispecies assay after 72 h (cell density (panel A), ROS production (panel B); FSC (panel C), SSC (panel D) and FL3 (panel E). Colour asterisks show the significant differences among the controls and treatments for each microalgae species selected (ANOVA with a Dunnett post hoc; $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the volume (2 mL) required to measure the Phyto-PAM. Multispecies microalgae population at 10^3 cell·mL^{-1} of each species were exposed to BPA, BPS, BPAP, BPAF, BPFL and BPC at different concentrations (5, 40 and 150 μM). The same flasks were sampled at 24, 48 and 72 h.

2.4. Statistical analysis

Single and multispecies experiments were carried out in triplicate. Data are shown as average ± standard deviation between replicates. Graphical representation and statistical analyses were carried out using the SigmaPlot 11.0 software. One-way ANOVA with a Dunnett post hoc at $p < 0.05$ was performed to assess all the responses: cell growth, cell size, cell complexity, % of ROS, autofluorescence of chlorophyll *a*, effective quantum yield of PSII and total concentration of chlorophyll.

3. Results

3.1. Microalgae population growth

When possible, EC_{50} was calculated for the microalgae and BP analogues selected after 72h of exposure (Table 1). Most of the BPs tested showed more than 50% of growth inhibition at low BPs concentrations, being $EC_{50} < 5$ μM excepting for *N. gaditana* exposed to BPA and BPS, and *P. tricornutum* exposed to BPC.

The growth inhibition of the microalgae in the single and multispecies experiments is shown in Fig. 1. For all BPs exposure conditions, the microalgae *P. tricornutum* showed significant differences ($p < 0.05$) against the control for practically all analogue concentrations and times (just one exception: BPC at the lowest concentration, 5 μM, and 72 h). After 72 h of exposure, the compared toxicity of the analogues assayed for this species seems to be BPC < BPA < BPS < BPFL ≈ BPAF ≈ BPAP. *T. suecica* was the most sensitive species among the species selected in relation to this endpoint. Significant differences were found between the controls and all BPs at every sampling time and concentration ($p < 0.05$). *N. gaditana* was the species most resistant to BPs. However, after 72 h of higher doses of all BPs, *N. gaditana* showed a significant decrease in population growth ($p < 0.05$). The EC_{50} after 72 h of exposure had a different behaviour; BPAP, BPAF, BPFL and BPC had an EC_{50} lower than 5 μM while BPA and BPS presented EC_{50} values of 28.88 ± 2.18 μM and 60.81 ± 15.35 μM respectively (Table 1).

The multispecies experiments showed different sensitivity according to BPs exposure for single species. In any case, BPAP, BPAF, BPFL and BPC showed a significant population with respect controls at all sampling times and concentrations ($p < 0.05$). Surprisingly, *P. tricornutum* and *T. suecica* did not show alterations respect to the controls when they were exposed to BPA and BPS from 5 to 40 μM in the multispecies experiments. Thus, *T. suecica* dominated the microalgae community when they were exposed to BPA, BPAP, BPAF, BPFL and BPC, while *N. gaditana* dominates the microalgae community when microalgae species were exposed to BPS (Fig. 2A).

3.2. ROS production

ROS production in the single and multispecies experiments is shown in Figs. 3 and 2B respectively.

P. tricornutum showed an increase in ROS production for most of the BPs assayed. Three analogues such as BPC < BPS < BPA provoked lower ROS production in comparison to the other BPs. The highest percentage of ROS was observed after 72 h of exposure with a dose-response behaviour for all the concentrations selected ($p < 0.05$). For this species, the rest of the analogues produced higher ROS production: in increasing order of toxicity, BPAF < BPFL < BPAP. This latter analogue provoked a significant increase in ROS from 20 μM for the three sampling times. BPFL provoked a significant increase for this parameter from 80 μM ($p < 0.05$).

T. suecica showed different trends in the percentage of ROS under BPs

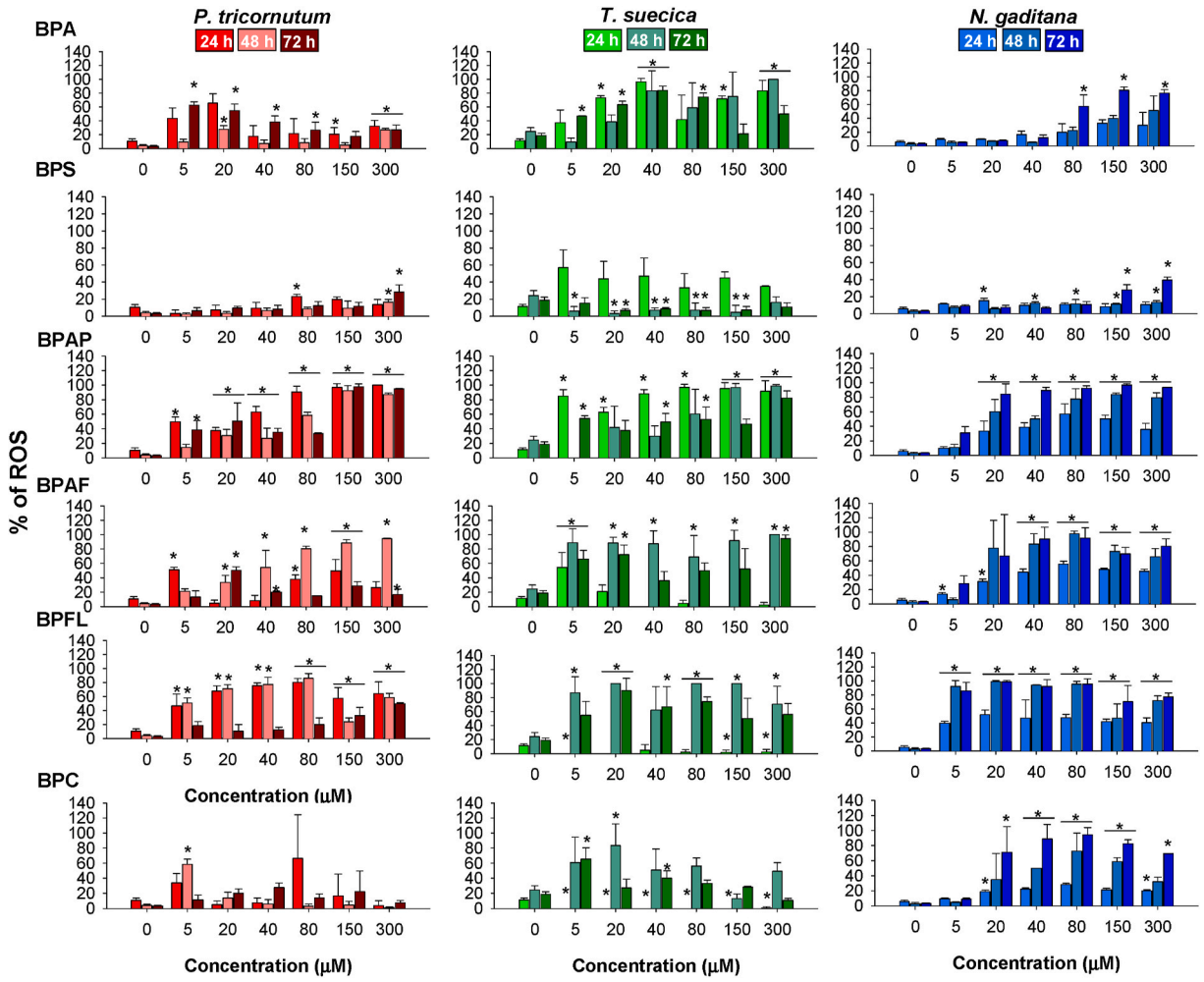

Fig. 3. Percentage of ROS production for microalgae exposed to selected BPs after 24, 48 and 72 h of exposure. Black asterisks show the significant differences among the controls and treatments for single experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

exposure. *T. suecica* also showed a high sensitivity to BPAP at each sampling time (24, 48 and 72 h) from 150 µM. BPAF and BPFL provoked the highest levels of ROS after 48 and 72 h.

N. gaditana also showed a significant increase in the percentage of ROS when this species was exposed to BPs. For this species, BPFL provoked the highest levels of ROS in all sampling times ($p < 0.05$), even at the lowest concentrations tested. Thus, in decreasing order of ROS production activity: BPFL > BPAP > BPAF > BPC. BPA and BPS produced lower effects when compared with the rest of analogues, although the highest doses used (from 80 µM) provoked statistically significant higher levels of ROS when compared with the controls, mainly at the end of the experiment (72 h).

In the multispecies experiments, and for all BP analogues tested, *T. suecica* was the species with the highest percentage of ROS marked cells, when compared with the other two co-growing species, except for BPS, which provoked a high response in *N. gaditana*. This latter species seems to decrease the percentage of marked cells remarkably when incubated in the presence of the other two species selected in practically all the analogues assayed. *P. tricornutum* also decreased (in general) their percentages of marked cells, mainly for BPFL (Fig. 2B).

3.3. Cell size

Cell size for the exposed cells, measured as Forward Scattering by flow cytometry, is shown in Fig. 4.

In general, almost all the BPs assayed provoked a decrease in cell size for *P. tricornutum*, with the exception to BPS, which provoked a significant increase for this parameter at the highest concentrations, mainly at the end of the experiment. BPFL did not provoke any effect at 48 and 72 h.

On the contrary, *T. suecica* cells showed a significant increase in cell size when it was exposed to the BPs selected, mainly at higher concentrations and after 72 h of incubation.

N. gaditana demonstrated lower changes in cell size when this population were exposed to BPs. BPAF and BPFL did not show any significant changes after exposure ($p > 0.05$), and only BPC provoked a clear trend in the increase of cellular size at high concentrations and at the end of the experiment.

In relation to cell size in the multispecies experiments, after 72 h, BPAF, BPFL and BPC provoked the highest cell size increase, mainly after 72 h of exposure. For this parameter, *T. suecica* and *N. gaditana* seemed more sensitive than *P. tricornutum* (Fig. 2C).

3.4. Cell complexity

Cell complexity was measured as Side Scattering by flow cytometry (Fig. 5).

P. tricornutum showed a slight but significant increase for this parameter, mainly at high concentrations at the end of the experiment ($p < 0.05$). BPAP and BPC provoked the biggest deviations, although a big effect was observed for BPFL at 24 h for medium concentrations (40 and 80 µM).

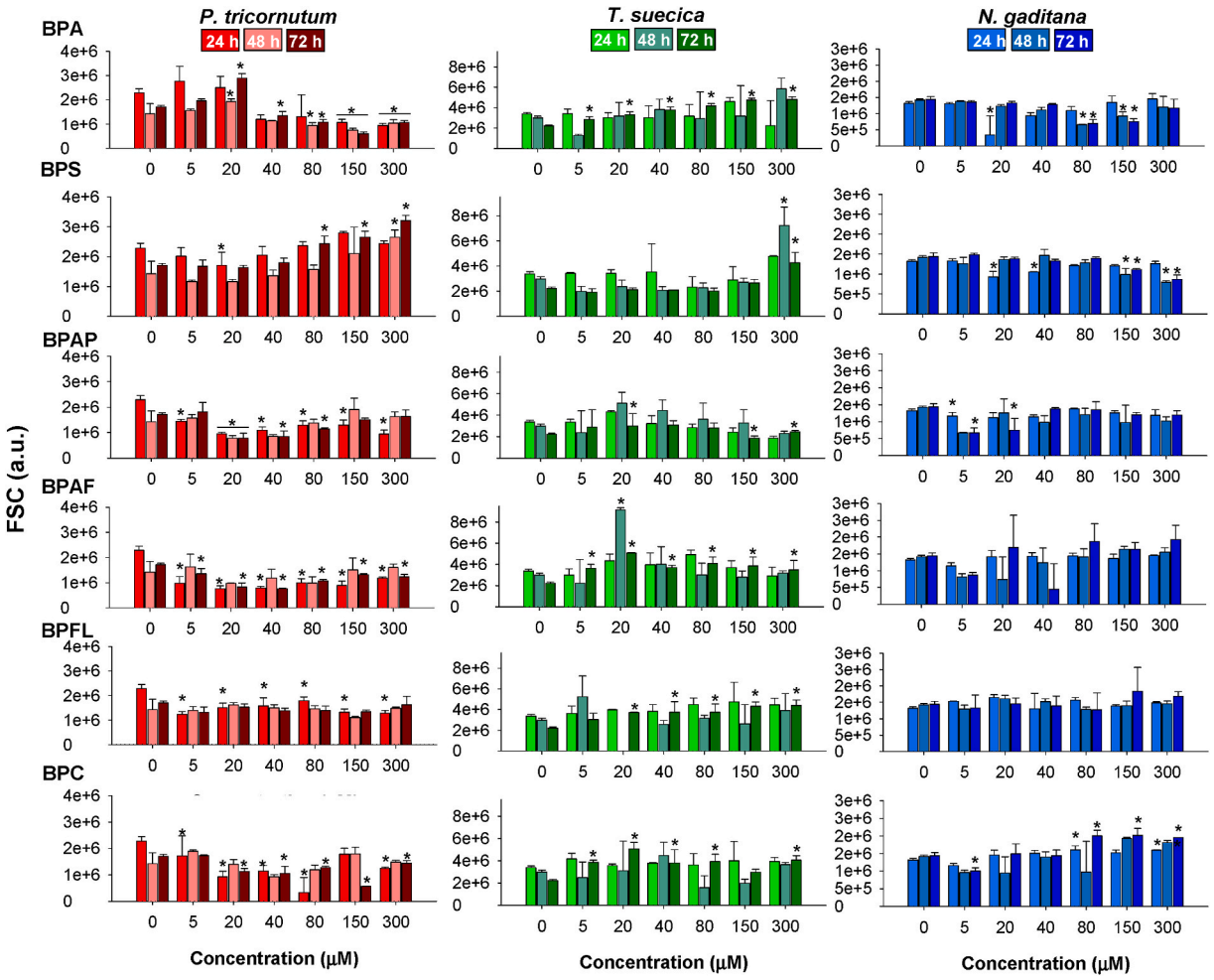

Fig. 4. Cell size (FSC) for microalgae exposed to selected BPs after 24, 48 and 72 h of exposure. Black asterisks show the significant differences among the controls and treatments for single experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

T. suecica also increased cell complexity under selected concentrations of exposure to BPs, mainly at high concentrations (except for some high responses at medium concentrations for BPAF and BPFL, for example).

In the case of *N. gaditana*, an increase in cell complexity was also seen for high concentrations of BPs. The highest effect was provoked by BPC, with considerable increases at the three higher concentrations (80, 150 and 300 µM) after 72 h.

In relation to the Multispecies experiments, very significant changes in cell complexity were found for some of the substances assayed. *T. suecica* greatly increased its cell complexity when exposed to BPAF, BPFL and BPC after 72 h. *N. gaditana* also increased cell complexity when exposed to BPAP and BPC. *P. tricornutum* only showed a significant increase in cell complexity for the highest concentration of BPS and BPFL (Fig. 2D).

3.5. Autofluorescence of chlorophyll a

Autofluorescence of chlorophyll *a* is shown in Fig. 6 for the microalgae species exposed to BPs in single microorganism experiments.

P. tricornutum showed a significant decrease in autofluorescence of chlorophyll *a* at concentrations above 20 µM for most of the analogues assayed except for BPS exposure provoked a significant increase in autofluorescence at concentrations over 80 µM.

In the case of *T. suecica*, a similar trend was found, and the analogue with the least pronounced effect on this parameter was, again, BPS (except for the highest BPS concentration).

N. gaditana showed a decrease in autofluorescence of chlorophyll *a* for BPAP, BPAF and BPFL after 48 and 72 h for all exposure concentrations ($p < 0.05$).

In relation to the Multispecies experiments, very significant changes in autofluorescence of Chla. *a* were found for all BPs tested in almost all concentration selected ($p < 0.05$). The trend about this response was to decrease the FL3 levels. *T. suecica* greatly decreased its autofluorescence when exposed to BPAP, BPAF, BPFL and BPC after 72 h ($P < 0.05$). While *T. suecica* decrease the FL3 when this population was exposed to all BPs, was exposed to all BPs, *P. tricornutum* and *N. gaditana* were only affected when both populations were exposed to BPAP, BPAF, BPFL and BPC ($p < 0.05$; Fig. 2E).

3.6. Effective quantum yield of PSII (E.Q.Y.)

Results for the effective quantum yield of PSII are shown in Fig. 7 for the multispecies experiments. This parameter significantly decreased when a mixed algal population was exposed to all the concentrations tested (5, 40 and 150 µM) of BPAP, BPAF, BPFL and BPC after all three sampling-times; $p < 0.05$. BPFL was the treatment that provoked the greatest changes in E.Q.Y., even for the lowest concentrations assayed. Significant decreases were also found in microalgae exposed to BPA at 40 and 150 µM. On the contrary, BPS did not show any significant changes in this response, except for the highest concentration at 24 h.

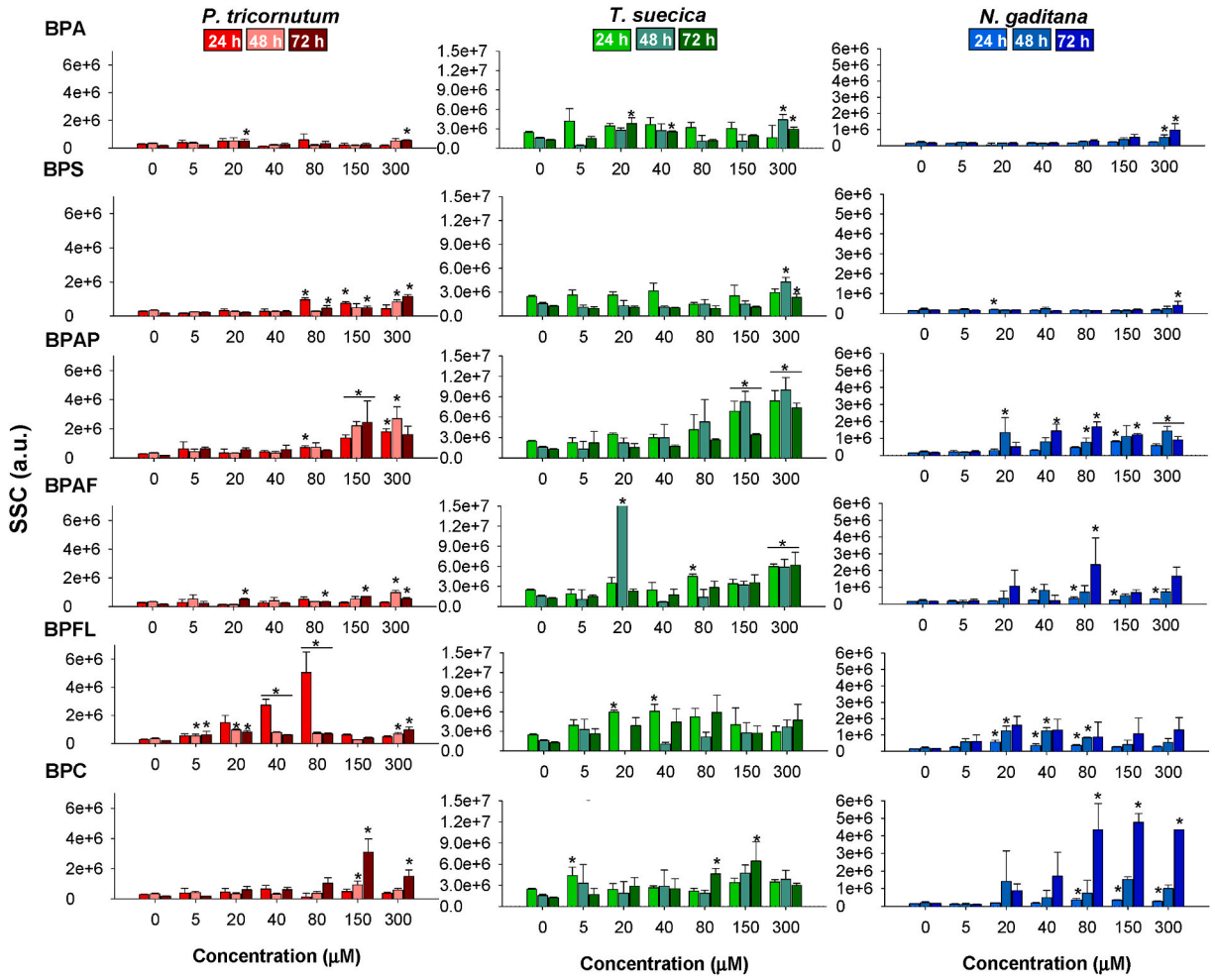

Fig. 5. Cell complexity (SSC) for microalgae exposed to selected BPs after 24, 48 and 72 h of exposure. Black asterisks show the significant differences among the controls and treatments for single experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

3.7. Concentration of pigments emitting in three wavelengths in the multispecies experiment

Concentration of pigments emitting in wavelengths corresponding to blue, green and brown pigments are shown in Fig. 8 for the microalgae community exposed to BPs.

The microalgae community demonstrated significant changes in pigment distributions under BPs exposure (Fig. 7; Table S1). In general, the concentration of brown pigment became dominant displaying significant increases for all BPs except for BPFL at high concentrations, where blue pigments dominated. BPS was less aggressive except for the highest concentration (150 µM). In many cases, low cellular density did not give a measurable signal for this response.

4. Discussion

The risk assessment of BPA analogues in primary producers is key to understand the unwanted effects in the first chain of the food web. The impact of BPA analogues and even the detoxification processes (byproducts generated by microalgae) could provoke a biological domino effect reaching higher levels of biological organization. Therefore, the environmental health of populations, communities and ecosystems could be compromised.

Sensitivity to BPs of microalgae were revealed by the single experiment in the present study. *T. suecica* was the most sensitive species followed by *P. tricornutum* and *N. gaditana*. Furthermore, *T. suecica* did not show any clear differences among the BPs and concentrations for growth inhibition response. Although growth inhibition is the most common endpoint used in OECD tests, responses such as: inherent cell properties (cell size and complexity), ROS production and autofluorescence of chlorophyll *a* allow a more accurate sensitivity than growth inhibition. BPA and BPS were the least toxic among the BPs tested on all species tested in comparison to BPAP, BPAF, BPFL and BPC. Between *P. tricornutum* and *N. gaditana* was observed that the sensitivity of *P. tricornutum* to BPs was time-dependent and higher after 48 h of exposure, while *N. gaditana* showed the highest sensitivity after 72 h of exposure. The EC_{50} for BPA calculated in this work was lower than the EC_{50} found in other studies for *C. vulgaris* (186 µM after 14 days; (Czarny-Krzymińska et al., 2022) and 174 µM after 5 days; (Ji et al., 2014), *D. armatus* (184 µM after 14 days; (Czarny-Krzymińska et al., 2022)), *Chlamydomonas reinhardtii* (142 µM after 3 days; (Esperanza et al., 2020)), *Stephanodiscus hantzschii* (58 µM after 3 days; (Li et al., 2009)). The difference in the EC_{50} may change by several magnitude orders according to the microalgae studied. It is essential to identify the most sensitive and resistant species as they could condition the ecological structure of a phytoplanktonic community. Changes to the phytoplankton community structure might determine any effects at higher levels of organization.

Some works have demonstrated that microalgae such as *Desmodesmus* sp. can transform BPA into relatively non-toxic metabolites via glycosylation (Hyung Ko et al., 2006; Wang et al., 2017).

The fast production of BPA metabolites plays an important role in the impact of BPA on aquatic ecosystems, as microalgae could remove BPA from the culture medium via oxidative hydroxylation, glycosylation,

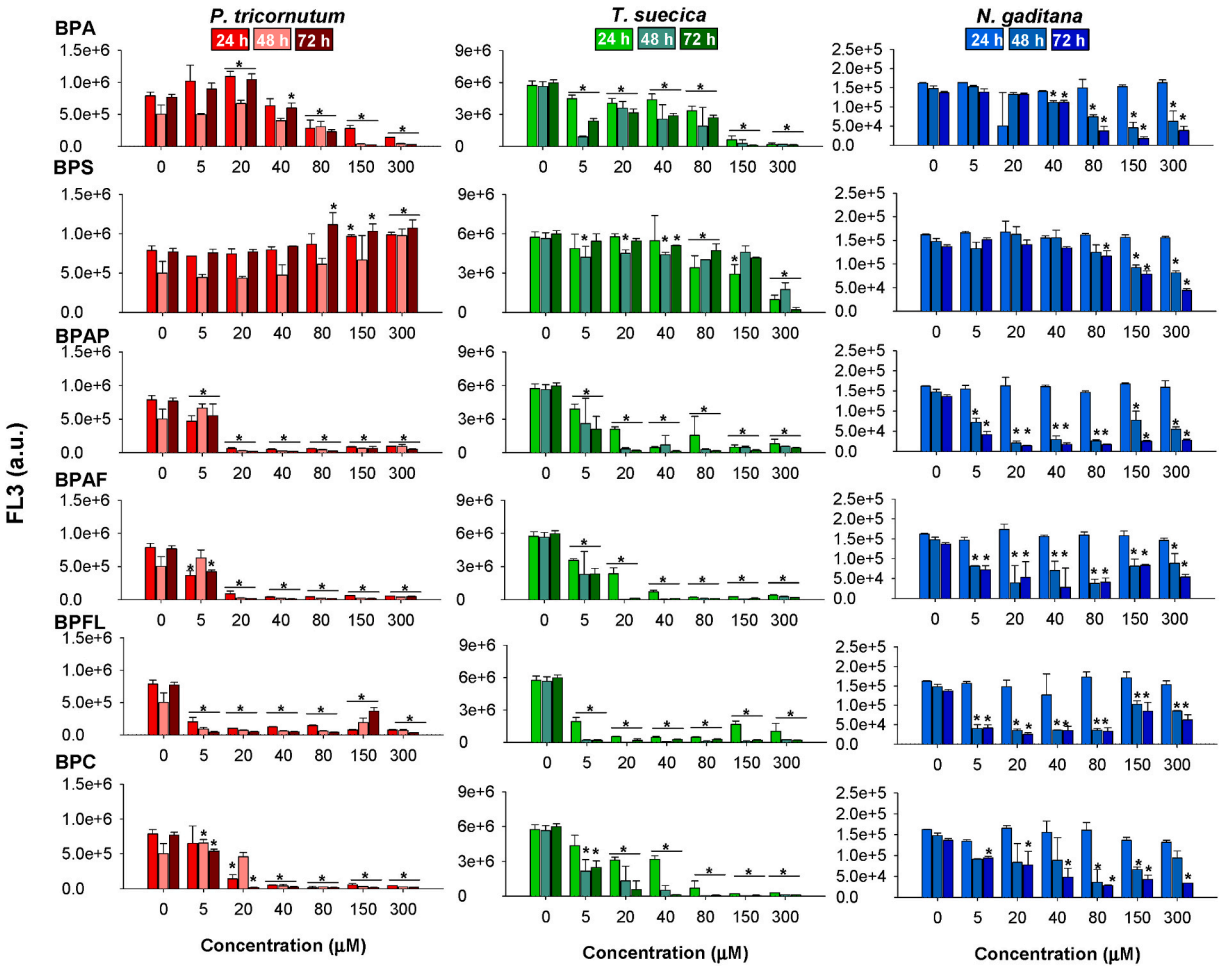

Fig. 6. Autofluorescence of chlorophyll *a* (FL3) for microalgae *P. tricornutum*, *T. suecica* and *N. gaditana* exposed to selected BPs after 24, 48 and 72 h. Black asterisks show the significant differences among the controls and treatments for single experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

and oxidative cleavage. The literature does not yet report on the extrapolation of results about the metabolization and transformation of BPA by microalgae and their common mechanisms among different BPs. However, the results in this study have shown significant differences among BPA analogues probably due to different routes of metabolism degradation.

Although some species of microalgae have detoxification mechanisms against BPA exposure. These detoxification mechanisms do not seem enough under BPAP, BPAF, BPFL and BPC exposure for the three microalgae tested, due to cytotoxic effects. BPA followed by BPS were the BPs that provoked less sensitivity among the BPs tested for the three microalgae species tested in the present work. Few works are found about the toxicity of BPs analogues in relation to microalgae (Czarny-Krzymińska et al., 2022; Elersek et al., 2021). However, some previous results have also shown that BPA analogues are not safe alternatives to BPA. In a previous work, BPAF, bisphenol G, bisphenol X showed higher effects in *Chlorella vulgaris* and *Desmodesmus armatus* than BPA (Czarny-Krzymińska et al., 2022). In the case of BPAF, the EC_{50} results for *Desmodesmus subcapitatus* (9 µM) was higher than in the present work for the three marine microalgae tested. For BPS exposure to *C. vulgaris*, the EC_{50} (9 µM) lower than the EC_{50} found for *N. gaditana* (60 µM) but higher than the other species tested (Tišler et al., 2016).

A decrease in the autofluorescence of Chla. *a* was observed in the chlorotic cells of the three microalgae species under BPs treatments. The chlorotic cells showed the same trend as the other endpoints analysed, BPA and BPS were the treatments that provoked lower sensitivity. Chlorotic cells are not always accompanied by cell viability but cells in this situation adapt to their unsuitable environmental conditions by degrading pigments to supply organic nitrogen (Sendra et al., 2017a, 2017c). Previous works have demonstrated a down-modulation of genes expression related photosynthesis biological processes (Duan et al., 2019).

In relation to the multispecies experiments, a different trend was observed in comparison to single species. While *T. suecica* was the species more affected in the single experiments, when the three microalgae were under multiespecies assays; *T. suecica* and *N. gaditana* were the least affected species under BPs exposure. Although *T. suecica* was the microalgae with the highest growth rate in the multispecies assay, it also displayed a significant increase in oxidative stress in comparison with *P. tricornutum* and *N. gaditana*. The ROS generation response could indicate detoxification mechanisms by the activation of antioxidative enzymes and degradation of BPs subproducts. This trend was reflected in the ROS generation as a strategy to respond to exposure to xenobiotics (Cirulis et al., 2013). *P. tricornutum* was the most sensitive species in the multispecies experiment and it was also very sensitive in the single experiments. Similar results were found in the study by Seoane et al. (2021) on the growth rate of single and multispecies experiments. In this work *T. suecica,* showed the same trend as our results in both the single and multispecies experiments. Seoane et al. (2021) and the present study are the first ones to assess the effects of BPA and BPA analogues on a microalgae community. Multispecies experiments may provide more reliable results since a marine phytoplankton community can absorb the effects of some pollutants (De Laender et al., 2009; Franklin et al., 2004; Seoane et al., 2021; Stauber et al., 2002; Yu et al., 2007). These results

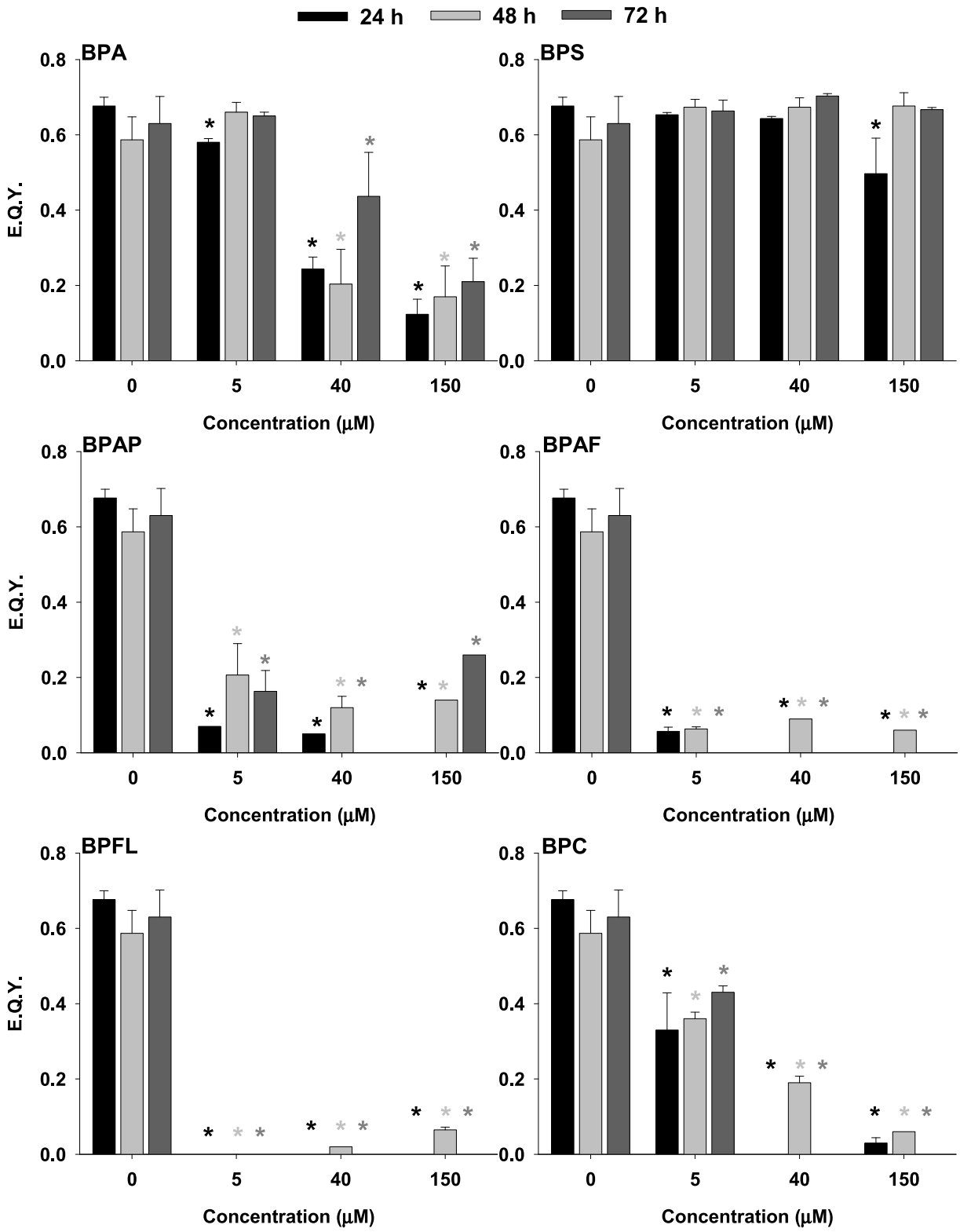

Fig. 7. Effective Quantum Yield of PSII (E.Q.Y.) for microalgae community over multispecies experiments exposed to selected BPs after 24, 48 and 72 h. Asterisks show the significant differences among the controls and treatments for multispecies experiments (ANOVA with a Dunnett post hoc; $p < 0.05$).

could indicate that in a natural marine environment the impact of some chemical compounds could be attenuated by intrinsically mechanism of detoxification (Seoane et al., 2021). Furthermore, the interspecific competence among different microalgae species or/and even the generated byproducts and metabolites for detoxification processes could be affect the sensitivity of microalgae species in multispecies experiments.

N. gaditana showed the same trend in both the single and multispecies experiments and was the most resistant species, this trend has been reported before (Debelius et al., 2009, 2008; Sendra et al., 2017b; Seoane et al., 2021). The selection of microalgae species for ecotoxicology studies should be based on the intraspecific sensitivity (in case of

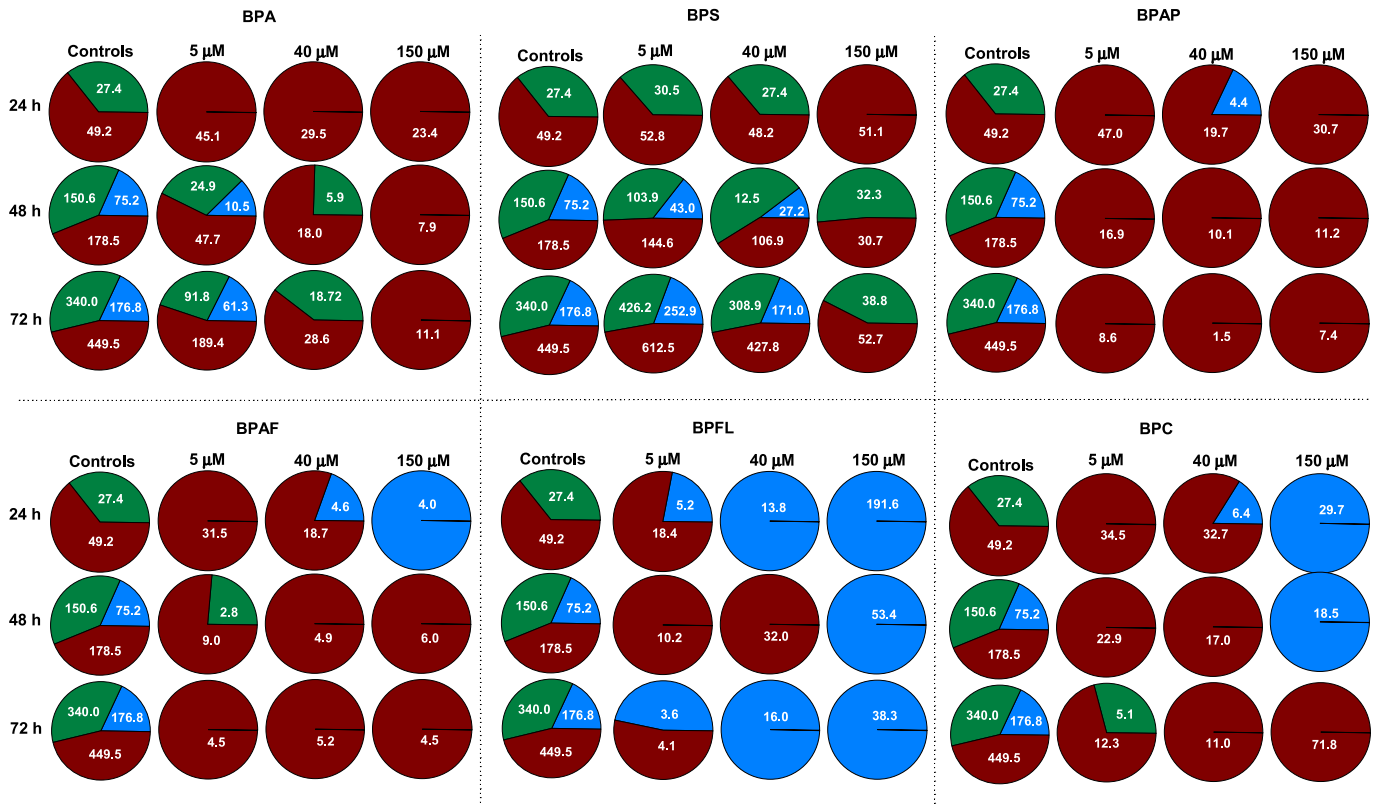

Fig. 8. Blue, green and brown pigments concentration (μg·L^{-1}) for microalgae community over multispecies experiments exposed to selected BPs after 24, 48 and 72 h. The white numbers wrote in the pie chart show the chlorophyll concentration (μg·L^{-1}). In Table S1 are shown the values of pigment concentration and the statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

single experiments) and interspecific competence (in case of multispecies approaches). Although intrinsic properties (such as cell size, complexity and autofluorescence of Chla. *a* have to be taken into consideration according to experimental limitations.

The photosystem II (PSII) of the multispecies experiments was a accurate endpoint to assess the ecotoxicity of the BPs. Although cell growth gives us a valuable information, changes in the E.Q.Y. of PSII showed clear differences among the BPA analogues from the beginning of BPs exposure. BPS showed the lowest toxicity with negligible changes among the concentrations and time exposures followed by BPA and BPC. On the other hand, BPs such as BPAP, BPAF and BPFL had a significant impact on the E.Q.Y. of PSII. In the work of Xiang et al. (2018), BPA inhibited the oxidative phosphorylation, glycolysis/gluconeogenesis, citrate cycle (TCA cycle), and fatty acid metabolism in *Cylindrospermopsis raciborskii*. Furthermore, thylakoids, the photosystem II photosynthetic electron transport chain and photosynthesis-antena proteins were affected under BPA exposure. These authors found that BPA disturbed the absorption flux in PS II, indicating that the energy trapped by PSII was lost during the process of electron transport, and this energy dissipation might inhibit electron transport (Xiang et al., 2018).

Although microalgae growth inhibition was dependent of exposure time, this trend was different in relation to the E.Q.Y. results. It means that the microalgae community has some resilience with regard to exposure to BPs. The partial recovery of E.Q.Y. of PS II over time could indicate that the previously mentioned detoxification processes are being activated. The damage in the PSII under BPs exposure is related with the chloroplast disturbances found in the single experiments. Chlorophagy occurs in the chlorotic cells and this affects the production of pigments (Ling and Jarvis, 2015). A decrease in the different pigment concentrations in relation to the controls was observed as a result of BPs exposure in this work. Recent studies have revealed that multiple types of cellular machinery are used to degrade damaged chloroplasts by autophagy-dependent pathways (Woodson, 2022). Brown and blue pigments (in the case of microalgae exposed to BPAF, BPFL and BPC) increased their dominance while a more homogeneous distribution of the three pigments was measured in the controls. The autofluorescence of chlorophyll *a* by FL3 for each species in the multispecies experiments agrees with the results found in E.Q.Y. of PSII. Although E.Q.Y. gives a reliable measure about the status of PSII, the combined information of autofluorescence with flow cytometry allow identify which species were the most sensitive to BPA analogues when the multispecies experiment was performance.

5. Conclusions

This work has investigated the toxic effects of BPA and its analogues (BPS, BPAP, BPAF, BPFL, and BPC) using three marine microalgae in single experiments and multispecies experiments. The results revealed that the BPA analogues (BPAP, BPAF, BPFL and BPC), except for BPS are not safer alternatives to BPA for marine phytoplankton even an environmental realistic concentration. The consideration of microalgae taxa under BPs exposure is key to assess the toxicity, in accordance with intraspecific sensitivity of each species. In relation to multispecies experiments relevant findings were revealed. The interspecific competence among species could determine in different way the sensitivity of each species to BPs exposure.

The importance of using multispecies experiments in comparison to classical OECD tests to assess unknown and unregulated chemical compounds is needed to elucidate the impact of new chemicals in a more realistic scenarios with factors found in natural community such as interspecific competence mechanisms. Despite of the worthy results revealed in this work, further studies must be undertaken to understand any potentially undesirable effects of these BPA alternatives. The

importance of microalgae community growth and the changes in the kind of pigments could affect in different levels of the food web.

Funding sources

This study has been funded by Juan de la Cierva Incorporación (IJC 2020-043162-I) contract funded by MCIN/AEI/10.13039/501100011033, European Union NextGenerationEU/PRTR.

Credit author statement

Marta Sendra: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Ignacio Moreno-Garrido: Writing – review & editing. Julián Blasco: Review & editing.

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

Data will be made available on request.

Acknowledgment

Marta Sendra thanks to the Juan de la Cierva Incorporación (IJC 2020-043162-I) contract funded by MCIN/AEI/10.13039/501100011033, European Union NextGenerationEU/PRTR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122073.

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