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RESEARCH ARTICLE

The effects of bisphenol A, F and their mixture on algal and cyanobacterial growth: from additivity to antagonism

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Abstract

Bisphenol A (BPA) is, due to its widespread use including the production of plastic materials, an ubiquitous pollutant in the aquatic environment. Due to evidence of adverse BPA effects on the environment and human health, its use has been restricted and replaced by analogues such as bisphenol F (BPF). This study examined the toxicity of BPA, BPF and their mixture towards primary producers, the eukaryotic green alga *Pseudokirchneriella subcapitata* and the prokaryotic cyanobacterium *Synechococcus leopoliensis*. The results demonstrated that *S. leopoliensis* is more sensitive than *P. subcapitata*, whereas toxic potential of the two BPs is comparable and represents comparable hazard for phytoplankton. The toxicity of the binary mixture was predicted by different models (concentration addition, independent action, combination index and the isobologram method) and compared to experimental data. Additive effect was observed in *P. subcapitata* over the whole effect concentration range (EC_5–EC_{90}), whereas in *S. leopoliensis*, no pronounced combined effect was observed. The environmental risk characterisation based on the comparison of reported concentrations of BPA and BPF in surface waters to the predicted no-effect concentration values obtained in this study showed that at certain industrial areas, BPA represents environmental risk, whereas BPF does not. However, BPF concentrations in aquatic environment are expected to increase in the future. To enable environmental risk assessment of BP analogues, more data on the toxicity to aquatic species, including combined effect, as well as data on their occurrence in the aquatic environment are needed.

Keywords Aquatic ecotoxicology · Plastic pollution · Toxic stress · Growth inhibition · Environmental risk assessment · Pollutant degradation

Introduction

Bisphenol A (BPA) is a commercial name for 4,4′-(propane-2,2-diyl)diphenol, which was first synthesised in 1891 (Staples et al. 1998). It is used as a raw material for the production of many products such as polycarbonates and epoxy resins, lining of food cans and metal lids (Eio et al. 2015). BPA is released to the environment from production sites, processing plants (Staples et al. 2000), wastewater effluent, landfill leachate (Corrales et al. 2015), plastic (Liao and Kannan 2013), microplastic and other materials where BPA is present, as a non-point source of environmental contamination (Crain et al. 2007). For the health of our aquatic environment, it is important to examine the impact of potentially toxic compounds on aquatic organisms, especially on primary producers, since they form the basis of an aquatic ecosystem's food web.

The concern for vertebrates was raised due to BPA estrogenic activity (Dodds and Lawson 1938) with a negative impact on the endocrine system of humans and animals (Eladak et al. 2015). Moreover, BPA is genotoxic in vitro and in vivo in rodents (Usman and Ahmad 2016). Studies also indicate a connection of exposure to BPA with many human diseases such as diabetes, obesity, cardiovascular, respiratory and kidney diseases, breast cancer and sexual disorders (Eladak et al. 2015). BPA is a well-known plastic-associated-compound

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and is known to the wider public from the "BPA free" water bottles campaign. In 2011, the European Commission banned the use of BPA in baby bottles and sippy cups, and will prohibit its use in the production of thermal paper from 2020 onwards (EU 2016). Therefore, manufacturers have started to replace BPA with alternatives in similar applications, of which bisphenol F (BPF) is one of the most used BPA alternatives (Chen et al. 2016). BPF is a commercial name for 4,4′-methylenediphenol, and it is used as a monomer in the production of epoxy resins and polycarbonates for the lining of food containers, water pipes, roof coverings, roads, bridges, structural adhesives, coatings, lacquers, varnishes, adhesives and dental flosses (Rochester and Bolden 2015; Tisler et al. 2016). Due to its similar structure to BPA, BPF has the potential to exert similar ecological and health effects as BPA (Eladak et al. 2015; Liao and Kannan 2013; Tisler et al. 2016).

The BPA concentrations in the environment can reach up to 370 μg L^{-1} in waste water treatment plant effluent (Corrales et al. 2015), 200 μg kg^{-1} in river sediment (Fromme et al. 2002), 56 μg L^{-1} in rivers (Corrales et al. 2015) and 17.2 μg L^{-1} in leachate from plastic waste (Yamamoto et al. 2001). Although usual levels in river waters are < 1 μg L^{-1} (Staples et al. 2000; Fromme et al. 2002; Bhandari et al. 2014), the presence of BPA in the environment is global (Corrales et al. 2015). The levels in dust and air were reported to be up to 4.1 μg g^{-1} (Liao et al. 2012) and 17.4 ng m^{-3} (Fu and Kawamura 2010), respectively. In food, concentrations of BPA can be up to 10 ng g^{-1} in products such as soups, eggs, sauces and syrups (Liao and Kannan 2013). BPA was also measured in energy drinks at levels up to 3.3 ng L^{-1} (Gallo et al. 2017). BPF has not been as thoroughly studied as BPA but compared to BPA, lower concentrations of BPF are reported. BPF concentrations were up to 0.3 μg L^{-1} in surface waters (Yamazaki et al. 2015), 0.1 μg g^{-1} in sediment (Fromme et al. 2002) and < 0.5 μg g^{-1} in dust powder (Liao et al. 2012). In food, the highest concentrations of BPF (4.63 ng g^{-1}) were measured in fish and seafood (Liao and Kannan 2013). In the environment, BPA is degraded abiotically with photo degradation as a result of UV radiation (Wang et al. 2007), and/or biotically with bacteria, algae and fungi (e.g. Ren et al. 2016; Ji et al. 2014). The biodegradability of different BPs in seawater and wastewaters was ranked as BPF > > BPA > BPS (Ike et al. 2006; Danzl et al. 2009).

Algae and cyanobacteria play a major role as primary producers at the bottom of food chain in aquatic ecosystems. Changes in their species composition can have wider implications for the biological community and ecosystem. Therefore, toxic stress to phytoplankton may affect the structure and functioning of the whole ecosystem (Ma 2005). The information on the effects of BPA and its analogues on phytoplankton species is limited to the studies in different algal species, which demonstrated some differences in their sensitivity towards BPA toxicity. Concern over BPA and alternatives in the environment affecting aquatic organisms has greatly increased over recent years (zebrafish e.g. Moreman et al. 2017; Le Fol et al. 2017; marine rotifers e.g. Park et al. 2018; algae e.g. Tisler et al. 2016; Li et al. 2009; Zhang et al. 2012; M'Rabet et al. 2018). Nevertheless, the ecotoxicological potential of BPA and alternatives on cyanobacterial primary producers, as an important part of aquatic algal community, remains largely unknown. To our best knowledge, the toxicity of BPA and its analogues has so far not been published for cyanobacteria. In general in algal group, diatoms from genera *Navicula*, *Stephanodiscus* and *Cyclotella* are more sensitive (EC_{50} 4–9 mg L^{-1}) (Liu et al. 2010; Li et al. 2009) than green algae from genera *Chlorella*, *Chlamydomonas* and *Desmodesmus* (EC_{50} 20–89 mg L^{-1}, Zhang et al. 2012; Tisler et al. 2016). Moreover, certain tolerant algal species have been reported to accumulate and degrade BPA (Hirooka et al. 2003, 2005; Ben Uoada et al. 2018).

Although algae and cyanobacteria have been shown to be comparatively sensitive to many chemicals (Real et al. 2003), for certain chemical differences in the sensitivity were demonstrated (Ma 2005; Brezovšek et al. 2014). In the aquatic community, such pollutants may cause changes of green algal and cyanobacterial group structure. Of particular concern is higher sensitivity of algae compared to cyanobacteria, which may result in a shift from dominance by green algae to dominance by cyanobacteria, and may even contribute to cyanobacterial blooms during the specific periods (Ma 2005). Therefore, for more reliable environmental risk assessment, studies comparing differential sensitivity of cyanobacteria and green algae are needed. Another aspect that has so far not been addressed is toxicity of mixtures of BPA and its analogues, which in the environment occur together.

In this study, we evaluated the toxicity of BPA, its analogue BPF and their binary mixture in the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. To assess the interaction of BPA and BPF in combined exposure, the toxicity of the mixture has been predicted by several approaches: concentration addition (CA) and independent action (IA) models, determination of combination index (CI) and with isobologram method, and the calculated data have been compared to experimental data.

Materials and methods

Test substances

For the experiments, BPA (CAS 80-05-7, Merck), BPF (CAS 620-92-8, Sigma-Aldrich) and as a reference chemical DCP (3,5-dichlorophenol DCP, CAS 591-35-5, Sigma-Aldrich) were used. Concentrated stock solutions of BPA (300 mg L^{-1}) and BPF (30 mg L^{-1}) in OECD medium, and

DCP (1000 mg L^{-1}) in dimethilsulfoxide (DMSO) were prepared. DMSO was used at a final concentration of 0.04% v/v, which exceeds the OECD recommended value (0.01%), but as reported by Brezovšek et al. 2014, the concentration used does not affect the growth of the selected alga. The same final concentration of DMSO was added to the control cultures (solvent control). Stock solutions of BPA, BPF and DCP were stored in the refrigerator at − 4 °C, not longer than 6 months. BPA was tested at concentrations of 0.5, 1.5, 4.9, 15.6 and 50 mg L^{-1}; BPF at 0.3, 0.9, 2.9, 9.4 and 30 mg L^{-1} and DCP at 2.5, 5, 10, 20 and 40 mg L^{-1}. The highest test concentrations of BPA and BPF were chemically verified by gas chromatography-mass spectrometry (GC-MS). The following internal standards were used: deuterated BPA (BPA-d_{16}, CAS 96210-87-6, Sigma-Aldrich) and isotopically labelled BPF ($^{13}C_{12}$ BPF, > 95%, Toronto Research Chemicals CanSyn Chem. Corp.).

Stability testing of BPA and BPF during the assay

In order to assure reliable results and assess potential ability of *P. subcapitata* and *S. leopoliensis* to degrade BPA and BPF, the changes in the concentrations of investigated compounds were determined in culture medium in the presence and absence of the tested compounds (BPA 5 mg L^{-1}; BPF 3 mg L^{-1}) and in stock solutions (BPA 300 mg L^{-1}; BPF 30 mg L^{-1}). The concentrations in culture medium were determined at time 0 and after 72 h, using GC-MS.

Chemical analysis All samples were filtered through a RC membrane, 0.2 -μm filters, and diluted with OECD medium. Three aliquots ($V = 100$ μL) of the prepared sample were spiked ($V = 25$ μL) with mixed internal standards ($^{13}C_{12}$-BPF and ^{16}dBPA, $c = 1$ μg mL^{-1}) and dried under a gentle stream of N_2 at 40 °C. The samples were derivatised with 50 μL of BSTFA and 50 μL of pyridine for 16 h at 80 °C. Derivatised samples were transferred to glass vial inserts and analysed using an Agilent 7890B series gas chromatograph with a 5977A single-quadrupole mass spectrometer (Agilent, USA). Separation was achieved on a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, USA) with helium as the carrier gas (1 ml min^{-1}). One μL of each sample extract was injected in splitless mode at 250 °C. For optimal chromatographic separation, the following temperature program was used: an initial temperature of 120 °C was ramped at 20 °C/min to 200 °C and held for 2 min and then at 10 °C/min to 250 °C. Total GC-MS runtime was 11.0 min. The mass spectrometer was operated in EI mode at 70 eV. Selected compounds were determined using selective ion monitoring (SIM) mode using retention time matching (BPA 10.22 min, BPF 10.87 min, ^{16}dBPA 10.80 min, $^{13}C_{12}$BPF 10.72 min) and by monitoring the following ions: *m/z* 344, 329 and 179 for BPF; *m/z* 372, 257 and 339 for BPA; *m/z* 356, 341 and 179 for 13C12 BPF; *m/z* 386, 368 and 217 for ^{16}dBPA. Data were processed using MassHunter Workstation Quantitative Analysis software (version B.07.00, Agilent Technologies).

BPs are ubiquitous in the laboratory environment due to their broad application (Caballero-Casero et al. 2016). Therefore, background contamination blanks were prepared to evaluate and minimise sources of contamination. Both BPA and BPF were present as contaminants in the blanks. To minimise contamination, all cleaned glasswares were heated to 400 °C for 4 h before the experiments. Procedural blanks were prepared for each experimental setup. Blank samples were analysed following the same instrumental analysis. Blank samples of ethyl acetate were analysed after every 10th sample injection to evaluate and prevent potential carry over between samples. All data were blank corrected.

Algal growth inhibition assay

To determine the toxicity of the BPA and BPF and their mixtures on algae and cyanobacteria, an algal growth inhibition test was performed according to OECD guideline 201 (OECD 2011) with minor modification in light regime. The cultures at exponential growth in a nutrient-enriched medium (OECD medium) were exposed to different concentrations of BPA, BPF or their binary mixtures and incubated in the light (16 h per day of light intensity at 80–120 μE/m^2s, lights Sylvania GRO-Lux F 18 W/GRO-T8) with constant shaking at 80 rpm (GFL 3017, Burgwedel, Germany) at 24 ± 2 °C (LTH, Slovenia). Two phytoplankton species were selected, the green alga *Pseudokirchneriella subcapitata* SAG 61.81 and the cyanobacterium *Synechococcus leopoliensis* SAG 1402-1, both obtained from SAG, Göttingen algae collection. The assays were performed in glass Erlenmeyer flasks. One replicate covered at least five different BP concentrations (arranged in a geometric series with a factor of 3.2) and a control sample. The culture volume was initially set to 20 mL with a cell density of 10^3–10^4 cells mL^{-1} for *P. subcapitata* and 10^4–10^5 cells mL^{-1} for *S. leopoliensis*. After 72 h, the observed response was growth rate inhibition in comparison to the control cultures. Each test was performed as 3 independent experiments; each tested concentration was set in triplicate. Flow cytometry (MacsQuant, Milteny) was chosen to detect cell growth for the following reasons: only a small sample volume is required (50 μL of sample per well of a microtiter plate); it can distinguish between live and dead cells and is also suitable for measuring cyanobacterial cells, which cannot be counted precisely using a counting chamber and a light microscope (Elersek 2012). Negative control (media) and solvent control were measured in the same microtiter plate. The performance of tests was validated according to OECD guideline 201 (OECD 2011); the growth rate was at least 0.92 per day; the cell count increased at least 16 times during 72 h; the

coefficient of variability between individual test days was < 35%, and the coefficient of variability during the entire test was < 10%. The test system was confirmed using 3,5-dichlorophenol as a reference compound. The obtained EC_{50} 9.9 and 3.9 mg L^{-1} for *P. subcapitata* and *S. leopoliensis*, respectively, are in the range of the expected response.

The toxicities of the binary mixtures of BPA and BPF were tested at half the effective concentrations of each (EC5/2, EC10/2, EC20/2, EC50/2, EC90/2), as calculated from the dose-response curves of the single compounds (Table 2), and described previously (Cleuvers 2003; Brezovšek et al. 2014). This means that if the BPA and BPF effects follow the CA model, theoretically, the total effect of the mixture would be 5%, 10%, 20%, 50% and 90% growth inhibition, respectively. This is one of the possible approaches of mixture testing, where the focus is on testing different ratios in the mixture, based on the effects of individual compounds.

Statistical evaluations

Results expressed as growth inhibition were analysed using Prism 6 software (Graphpad Inc.). Data were analysed as measurements from each individual flask (pooled together) rather than means of replicates, to extract as much information as possible (OECD 2011). For the graphs showing percentage of inhibition vs. concentration of individual BPs, a nonlinear regression model "log(agonist) vs. response–find EC anything" function was applied (Eqs. (1)–(3)). Residual plots were also studied, and any outliers recognised by Prism were excluded from the statistical analyses. Statistical significance ($p < 0.05$) of an effect in comparison to the control was assessed using a non-parametric ANOVA (Kruskal-Wallis test) with the Dunnett's post-test at a 95% confidence interval.

$$\log EC_F = \log EC_{50} + (1/\text{slope}) \times \log(F\%/(100-F\%)) \quad (1)$$

$$F\% = (Y\text{–bottom})/(\text{top}-\text{bottom}) \times 100 \quad (2)$$

$$Y = \text{bottom} + (\text{top}-\text{bottom})/\left(1 + 10^{\left(\left(\log EC50 - X\right)\times \text{slope}\right)}\right) \quad (3)$$

Predicted toxicity of binary mixture of BPA and BPF

The predicted toxicity of the mixture was calculated as concentration addition (CA) (Loewe 1927; Loewe and Muischnek 1926) and independent action (IA) (Bliss 1939). Both models are frequently used for predicting mixture toxicity (Sumpter et al. 2006; Brezovšek et al. 2014; Elersek et al. 2016). Predicted toxicities were calculated based on the data obtained for individual compounds, and then the calculated values were compared to the measured, experimentally observed toxicities of the binary mixtures at each effect concentration.

Combination index (CI) for multiple drug effect interactions was introduced to give a quantitative definition of synergism (CI < 1), additive effect (CI = 1) and antagonism (CI > 1) using computerised simulations (Chou and Martin 2007; originally from Chou and Talalay 1984). The CI was modelled using CompuSyn software (ComboSyn, Inc.), which is based on the CI concept with CI algorithms and median-effect equation. The ratio of the fraction affected (fa) vs. the fraction unaffected (fu) is equal to the dose (*D*) vs. the median-effect dose (Dm) to the mth power, where Dm signifies potency and *m* signifies the sigmoidicity (shape) of the dose-effect curve. The isobologram is a curve that reveals whether a toxicant mixture displays additivity, synergism or antagonism (Chou 2006). The isobol equation is actually a special case of the CI equation and is often used to illustrate graphically the combined effect of binary mixtures (e.g. Altenburger et al. 1990).

Results and discussion

In aquatic environment, algae and cyanobacteria, as primary producers, represent an important target of toxic stress, and adverse effect to these organisms may severely affect the whole ecosystem function.

BPA and BPF stability

In general, the loss of tested substance can be a matter of volatilisation, sorption to test vessel or cells and/or uptake into algal cells. The results of our BPA and BPF stability study demonstrated that in the growth media (with no test organisms), no significant removal (decrease of concentration) of BPs was detected after 72 h (at maximum 9%, Table 1). In the presence of *P. subcapitata* during the 72-h exposure, 20% of BPA and 13% of BPF were removed from the medium, whereas in the presence of *S. leopoliensis*, the removal of BPA or BPF was only half of that observed in the presence of *P. subcapitata* (Table 1). These results indicate that *P. subcapitata* and *S. leopoliensis* do not exhibit significant BP removal capability.

Our results for BPA are comparable to the results of biotic degradation with green algae *Chlorella sorokiniana* (Eio et al. 2015), whereas green alga *Chlorella fusca* during 120-h exposure degraded up to 85% BPA (Hirooka et al. 2003, 2005). High BPA degradation ability has been reported also for extremophilic alga *Picocystis* sp. reaching 72% removal at 25 mg L^{-1} BPA (Ben Uoada et al. 2018). Concerning cyanobacterium *S. leopoliensis*, similar degradation of BPA has been reported for cyanobacterium from the genus *Anabaena* after 120 h (Hirooka et al. 2003).

According to OECD guideline recommendations (OECD 2011), the toxicological parameters (EC*x*, NOEC, LOEC)

Table 1 The stability (%) and measured concentrations (± standard deviation from 3 samples measured) of bisphenol A and F in the presence of algae and cyanobacteria (OECD medium, light, shaking, biotic factor for 72 h). Additionally, abiotic degradation was assessed during the experiment

	P. subcapitata	*S. leopoliensis*	Abiotic degradation
BPA	20%	11%	9%
Concentration at 0 h (mg L^{-1}) experiment	3.40 ± 0.19	3.38 ± 0.08	3.51 ± 0.35
Concentration after 72 h (mg L^{-1})	2.68 ± 0.57	3.01 ± 0.07	3.21 ± 0.06
BPF	13%	7%	0%
Concentration at 0 h (mg L^{-1}) experiment	2.67 ± 0.08	2.74 ± 0.19	2.69 ± 0.22
Concentration after 72 h (mg L^{-1})	2.32 ± 0.01	2.54 ± 0.02	2.82 ± 0.09

were calculated using nominal concentrations of the tested compounds. Additionally, stock solutions (saved in the refrigerator less than 6 months) from three experiments were also tested. Measured BPA stock concentrations matched the nominal value 93% (with standard deviation of 28%), and measured BPF stock matched the nominal value 89% (with standard deviation of 17%).

Toxicity of BPA and BPF

The results of the growth inhibition test of BPA and BPF in green alga *P. subcapitata* and cyanobacteria *S. leopoliensis* are shown in Fig. 1 and Table 2. In *P. subcapitata*, statistically significant toxic effects of BPA were detected at concentrations > 15.6 mg L^{-1} and BPF at concentrations > 9.4 mg L^{-1} (Fig. 1, left). In *S. leopoliensis*, statistically significant toxicity of BPA was observed at concentrations > 1.5 mg L^{-1}, and BPF at concentrations > 9.4 mg (Fig. 1, right). As can be seen on Fig. 1, growth inhibition of *P. subcapitata* after 72 h showed higher variability compared to *S. leopoliensis*. The reason might be in flow cytometer measurement, since the cells of *P. subcapitata* are more variable in the shape (curved croissant-like with variable dimensions) which could cause higher variations in cell count (e.g. during the cell division, the cells can be counted as 1 or 2). With *S. leopoliensis*, the morphology is more uniform (rod-like); thus, cell counts are less variable. However, we should perform more studies to confirm this speculation, but it was beyond the scope of the present study.

Algal and cyanobacterial growth inhibition test is in principle, a multigenerational test, and is considered as an acute and chronic toxicity test. Therefore, in the environmental risk assessment, EC_{50} values are used as a parameter of acute toxicity and EC_{10} and/or NOEC as parameters related to chronic toxicity (European Communities 2003). The obtained EC_{50}, EC_{10} and NOEC values are summarised in Table 2. The EC_{50} value for BPA in *P. subcapitata* (6.8 mg L^{-1}) was higher from previously reported EC_{50} value for this alga (2.7 mg L^{-1}) (Alexander et al. 1988). Lower EC_{50} values were obtained also in marine alga *Skeletonema costatum* (1 mg L^{-1}) (Alexander et al. 1988) and *Navicula incerta* (3.7 mg L^{-1}) (Liu et al. 2010), and comparable EC_{50} value (8.7 mg L^{-1}) to ours was reported for *Stephanodiscus hantzschii* (Li et al. 2009). On the other hand, much higher EC_{50} values (20–89 mg L^{-1}) were reported for *Chlorella vulgaris*, *Chlamydomonas mexicana* (Ji et al. 2014), *Chlorella pyrenoidosa*, *Scenedesmus obliquus* (Zhang et al. 2012) and *Desmodesmus subspicatus* (Tisler et al. 2016). The EC_{50} value for BPF in *P. subcapitata* (9.2 mg L^{-1}) is lower from that reported for *Desmodesmus subspicatus* (EC_{50} 22 mg L^{-1}), which is also the only published study on the toxicity of BPF in algae (Tisler et al. 2016). The differences in the algal growth inhibition by BPs in great deal depend on the test species and to certain extent to the differences in exposure conditions i.e. different exposure durations (72–120 h).

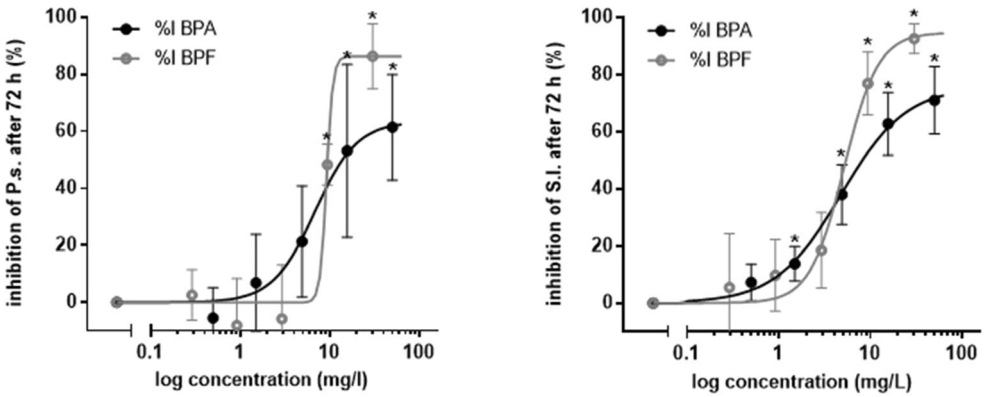


Fig. 1 Growth inhibition (%) of *P. subcapitata* (left) and *S. leopoliensis* (right) after 72 h. Bars denote 95% confidence interval. Asterisk denotes a statistical difference between the treated sample and the control based on ANOVA and the Dunnett's post test

Table 2 The effects of bisphenol A (BPA) and F (BPF) on *P. subcapitata* and *S. leopoliensis*. EC_x = *X%* effective concentration, *LOEC* lowest observed effect concentration, *NOEC* no observed effect concentration. At EC_x values, the 95% confidence intervals are reported in the parenthesis

	Effects of BPA and BPF-based non-lin. Fitting (EC_x in mg L^{-1})	
	BPA	BPF
Green alga *Pseudokirchneriella subcapitata*	EC_5 = 1.4 (0.3–5.6)	EC_5 = 6.9 (1.7–10.6)
	EC_{10} = 2.1 (0.7–5.8)	EC_{10} = 7.4 (2.7–10.4)
	EC_{20} = 3.2 (1.6–6.5)	EC_{20} = 8.1 (4.2–10.5)
	EC_{50} = 6.8 (3.4–13.8)	EC_{50} = 9.2 (7.1–13.8)
	EC_{90} = 22.3 (3.9–128.1)	EC_{90} = 11.2 (7.4–46.8)
	LOEC = 15.6	LOEC = 9.4
	NOEC = 4.9	NOEC = 2.9
Cyanobacterium *Synechococcus leopoliensis*	EC_5 = 0.4 (0.2–0.9)	EC_5 = 1.5 (0.9–2.3)
	EC_{10} = 0.8 (0.5–1.4)	EC_{10} = 2.0 (1.4–2.9)
	EC_{20} = 1.5 (1.1–2.2)	EC_{20} = 2.8 (1.1–3.8)
	EC_{50} = 4.8 (3.1–7.3)	EC_{50} = 5.2 (3.9–6.8)
	EC_{90} = 28.6 (10.4–79.0)	EC_{90} = 13.2 (7.5–23.2)
	LOEC = 1.5	LOEC = 9.4
	NOEC = 0.5	NOEC = 2.9

Nevertheless, the results of our study indicate that *P. subcapitata* belongs to more sensitive algal species.

Toxic potential of BPF was in alga and cyanobacteria comparable to that of BPA (Table 2). Also in *D. subspicatus*, BPA and BPF exerted comparable toxic potential (EC_{50}: 20 and 22 mg L^{-1}, respectively) (Tisler et al. 2016).

Regarding differences in the sensitivities towards the two BPs, *S. leopoliensis* was more sensitive than *P. subcapitata*, which is evident from EC*x* values and through the calculation of toxic units (Table 2). This may indicate higher sensitivity of cyanobacteria than green algae to BPs. However, as already discussed for BPA, lower and higher toxicities have been observed in other green alga species, whereas, to our best knowledge, in cyanobacteria, BPs have so far not been tested. Our results contribute to previously reported observations that toxicity levels cannot simply be extrapolated from one species to others or to natural assemblages (Ma 2005; Hagenbuch and Pinckney 2012).

Based on EC_{50} values, the EU Regulation (Regulation EC No 1907/2006) classifies chemicals as very toxic to aquatic organisms (EC_{50}, < 1 mg L^{-1}), toxic to aquatic organisms (EC_{50}, 1–10 mg L^{-1}) and harmful to aquatic organisms (EC_{50}, 10–100 mg L^{-1}). Based on our results, BPA and BPF are classified as toxic to primary producers in the aquatic environment.

Mixture effects and a comparison of different prediction models

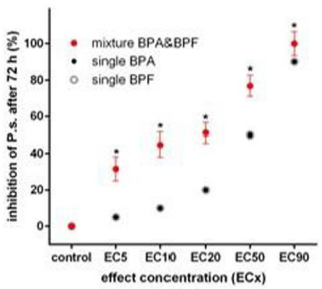
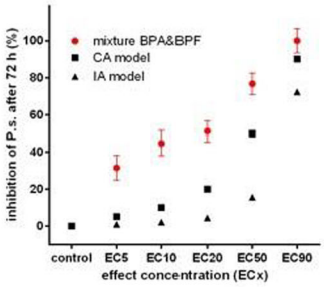
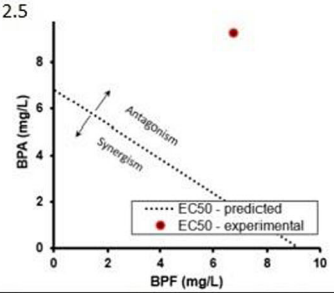
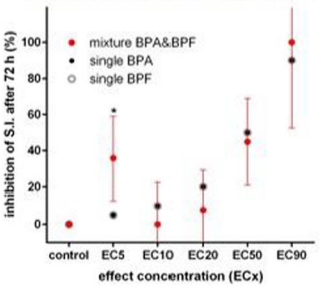
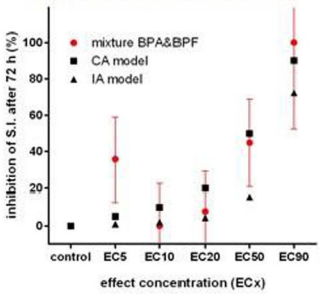
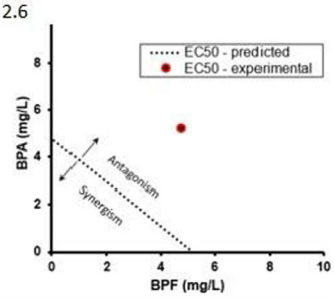
Results of a binary mixture of BPA/BPF showed different toxic responses and differences in the susceptibility of the two species. Like BPA and BPF, also their binary mixture exerted higher toxicity in *P. subcapitata* than in *S. leopoliensis* (Table 3). To assess the interaction of BPA and BPF in the mixture, the predicted effects were calculated by CA and IA models and by calculating the CI and compared to the experimental data (red dots in Table 3).

The CA model is based on the idea that chemicals have a similar mechanism of action, which means that it is more suitable for chemical combinations that are assumed to interact with common molecular target sites in the test organism (Backhaus et al. 2004) and would be the first logical choice in the case of BPA and BPF. When compared to experimental data, the CA model predictions underestimated mixture toxicity for *P. subcapitata* over the whole effect concentration range EC_5–EC_{90}, whereas for *S. leopoliensis*, CA model predictions were close to the experimentally determined toxicity at higher effect concentration range (EC_{50}–EC_{90}).

Alternatively, IA model is a statistical concept based on independent random events (Bliss 1939, in Backhaus et al. 2004) and is based on the idea of dissimilar action of compounds in a mixture; thus, IA should be more suitable for chemical combinations that have different molecular target sites and modes of action (Backhaus et al. 2004). When compared to experimental data, the IA model (like CA model) underestimated mixture toxicity over the whole effect concentration range EC_5–EC_{90} for *P. subcapitata*, but for *S. leopoliensis* model, predictions were close to experimentally determined toxicity in the concentration range EC_{10}–EC_{20}. The critics of IA model that is based on probabilistic reasoning point out that in some cases, IA model is degraded to a simple calculation technique with no broader theoretical background (Hadrup et al. 2013).

The combination index (CI) indicated antagonism over the whole concentration range for *P. subcapitata* (CI > 2.1) and for *S. leopoliensis* (CI > 2.0), which is evident from the

Table 3 The effects of BPA and BPF mixture on *P. subcapitata* and *S. leopoliensis*—an overview of data analysing approaches and results. Graphs show effects on inhibition of proliferation after 72 h. Red colour is used for experimental data of BPA and BPF mixture. Isobolograms are modelled for EC_{50}. Error bars denote 95% confidence interval.

	effects of mixture BPA+BPF assessed by different approaches		
	experimental data	**independent action (IA), concentration addition (CA)**	**combination index (CI), isobologram method**
green alga Pseudokirchneriella subcapitata	experimental data of BPA+BPF mixture exhibit higher toxicity* in comparison to single BPA and BPF (EC5-EC50) 	experimental data exhibit higher toxicity in comparison to IA model (only EC50-EC90) and CA model (only EC20-EC90) 	CI at EC5 = 5.1 CI at EC10 = 3.8 CI at EC20 = 2.9 CI at EC50 = 2.1 CI at EC90 = 2.5 according to this model the mixture effect should be antagonistic (EC5-EC90) 
cyanobacterium Synechococcus leopoliensis	experimental data of BPA+BPF mixture exhibit similar toxicity (EC10-EC90) and higher toxicity * (at EC5 only) in comparison to single BPA and BPF 	experimental data exhibit similar toxicity in comparison to IA model (EC10-EC20), CA model (EC5-EC90) and (only at EC5) higher toxicity to both models 	CI at EC5 = 4.0 CI at EC10 = 2.9 CI at EC20 = 2.4 CI at EC50 = 2.0 CI at EC90 = 2.6 according to this model the mixture effect should be antagonistic (EC5 - EC90) 

*Asterisk denotes statistical difference in comparison to the control based on ANOVA testing and Dunnett's post test

isobologram (Table 3, right column). CI correctly predicted the antagonistic effect of the mixture in *S. leopoliensis* but not in *P. subcapitata* for which experimental data showed additive effect in the effect concentrations between EC_5 and EC_{50}.

Taken together for *P. subcapitata*, the data indicate additive effect at low-effect concentration range and antagonism at high-effect concentration range, while for *S. leopoliensis* the data indicate antagonism over the whole effect concentration range.

Environmental risk characterisation

As a result of high production, consumption and subsequent release into the environment, BPA became a ubiquitous contaminant in the environment (Corrales et al. 2015). The median concentrations in surface water range between 3 and 30 ng L^{-1} (Bhandari et al. 2014); however, the concentrations in surface water from dense industrial areas are considerably higher ranging up to 56 μg L^{-1} (Corrales et al. 2015; Petrie et al. 2015; Wilkinson et al. 2017). Despite an increasing use of BPF, the information on its occurrence in the aquatic environment is very limited. The maximal reported concentrations in samples of surface waters in Germany are 180 ng L^{-1} (Fromme et al. 2002) and around 300 ng L^{-1} in surface water samples in Japan and China (Yamazaki et al. 2015). An important component of the environmental risk assessment is hazard assessment. According to Technical Guidance Document on Risk Assessment (European Communities 2003), hazard assessment for aquatic environment is based on predicted no-effect concentration (PNEC) of the chemical for the most sensitive species that, if it does not exceed environmental concentration, ensures an overall protection of the environment. Here we characterised the environmental hazard for phytoplankton by comparing the measured environmental concentrations (MEC) of BPA or BPF to the predicted no-effect concentrations (PNEC) for the two species, which were derived from the EC_{50} values divided by the assessment factor of 1000 as recommended in the Technical Guidance Document on Risk Assessment (European Communities 2003). For BPA, PNEC values are 6.8 and 4.8 μg L^{-1} for

P. subcapitata and *S. leopoliensis*, respectively. These values are higher than the median BPA concentrations in surface waters (30 ng L^{-1}) (MEC/PNEC < 1), but lower than the measured BPA concentrations at the industrial areas (up to 28 μg L^{-1}) (MEC/PNEC > 1), indicating potential environmental risk for phytoplankton at such areas. The PNEC values for BPF are 9.2 and 5.2 μg L^{-1} for *P. subcapitata* and *S. leopoliensis*, respectively, and are higher than the BPF concentrations determined in surface waters (MEC/PNEC < 1), which indicates that BPF does not represent risk for phytoplankton. However, the available data on the environmental occurrence of BPF are very scarce and probably do not reflect the actual situation in the aquatic environment. However, BPA and BPF are endocrine disruptors; thus, aquatic invertebrates and vertebrates are expected to be more sensitive than phytoplankton. Wright-Walters et al. (2011) conducted an aquatic hazard assessment for BPA using a weight of evidence approach in which the PNEC value was derived using a non-parametric hazardous concentration for 5% of the species (HC_5) approach. They included 61 studies that yielded 94 no observed effect concentration (NOEC) values. The toxicity dataset suggested that mortality and inhibition of growth, development and reproduction are most likely to occur between the concentrations of 0.0483 and 2280 μg L^{-1}. They calculated a PNEC value for aquatic environment 0.06 μgL^{-1}, which is two orders of magnitude lower from PNEC values we obtained for phytoplankton.

Conclusions

The results of this study demonstrated that BPA and its analogue BPF exerted comparable toxicity in alga *P. subcapitata* and cyanobacteria *S. leopoliensis* and represented comparable hazard for phytoplankton. The prokaryotic cyanobacteria was more sensitive than the eukaryotic green alga to individual compounds. The toxicity of the binary mixture of BPA and BPF was predicted by CA and IA model and compared to the experimental data, which showed that for *P. subcapitata*, neither of the two models accurately predicted the actual effects of the mixture. In *P. subcapitata*, additive effect was observed over the whole effect concentration range, whereas in *S. leopoliensis*, the CA/IA model predictions were close to the experimentally determined toxicity. The environmental risk characterisation for phytoplankton based on comparison of reported concentrations of BPA and BPF in surface waters to the obtained PNEC values showed that at certain industrial areas, the concentrations of BPA exceeded PNEC values and thus BPA represents environmental risk. On the other hand, the data indicate that BPF does not represent risk for aquatic environment, but it should be noted that the exposure assessment to BPF is based on only few available data on its concentrations in the aquatic environment. Providing that BPF is increasingly used as a replacement for BPA, its concentrations in aquatic environment are very likely underestimated. Therefore, for a reliable environmental risk assessment, more information on the occurrence of BPF as well as other BP analogues in the aquatic environment as well as ecotoxicological data for individual analogues and their mixtures are needed.

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