http://www.egejfas.org
Ege Journal of Fisheries and Aquatic Sciences, 40(4), 286-291 (2023)

Su Ürünleri Dergisi (2023)
DOI: 10.12714/egejfas.40.4.07

RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Investigation of toxic effects of BPA and BPA analogues (BPS and BPAF) on *Spirulina* sp., *Desmodesmus subspicatus* and *Chlorella vulgaris*

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Received date: 08.02.2023 *Accepted date: 20.11.2023*

How to cite this paper:
Turan, D., Çakal Arslan, Ö. (2023). Investigation of toxic effects of BPA and BPA analogues (BPS and BPAF) on *Spirulina* sp., *Desmodesmus subspicatus* and *Chlorella vulgaris*. *Ege Journal of Fisheries and Aquatic Sciences, 40*(4), 286-291. https://doi.org/10.12714/egejfas.40.4.07

Abstract: Bisphenols (BPs) are produced for many applications for used in industry. BPs have been found all part of aquatic environments such as sediment and surface water that is poses a risk to the aquatic ecosystem. Restricting the use of BPA, environmental concentrations of bisphenol S, and bisphenol AF begin to increase. The present study aims to indicate that toxicity BPA and BPA analogues (BPS and BPAF) by algal growth inhibition test for the green algae *Chlorella vulgaris*, *Spirulina* sp., *Desmodesmus subspicatus*. In this way, result of this study present the nominal effective concentrations of BPA analogues and the suitability of the species for use as a biomarker in ecotoxicology tests. IC_{50} values (growth rate inhibition by 50%, respectively) for three toxicants were determined separately. Results of this study showed the effects of these chemicals on photosynthesis (primer production). The result of algal growth inhibition test showed that BPAF (72h EC_{50} 3.80 mg/L) was found to be more toxic than BPS (3d EC_{50} 6.31 m L^{-1}) for *Spirulina* sp. BPS (3d EC_{50} 2.43 mg/L) showed the most toxic effect on the growth of *C. vulgaris*, followed by BPAF with 3d EC_{50} 3.32 mg/L. BPS (3d EC_{50} 0.88 mg/L) and BPAF (3d EC_{50} 6.48 mg/L) were found to be toxic for *D. subspicatus*, respectively, from highest to lowest toxicity. These results indicate that bisphenol analogues are hazardous to primer production. Therefore, it is necessary to study their combined effects as well as to study how they act individually.

Keywords: Bisphenols, toxicity, freshwater algae, aquatic environment, aquatic ecology

INTRODUCTION

Plastic pollution threat to marine ecosystems due to its widespread use in all areas. So that, it has several impacts on aquatic organisms, many of which have not been investigated (Uibel, 2016). The use of a wide variety of plastic products has increased considerably in recent years due to their social benefits such as ease of use, practicality, etc. Being durable and light, plastic has become the preferred base material for many applications, especially industrial applications. On the other hand, the multifaceted use of plastic has led to an increase in environmental pollution and a threat to natural life. Certain additives/chemical compounds are used in order to have the desired properties (durability, etc.) and to facilitate the production of plastics during the production phase. The most widely used of these compounds, bisphenol A (BPA), is used in the production of polycarbonate and epoxy resins (Huang et al., 2012). BPA is one of the important chemicals with the highest production volume in industrial areas worldwide (Abraham and Chakraborty, 2019).

BPA is commonly used as a stabilizer, an antioxidant in polycarbonate plastic (Grignard et al., 2012). BPA has a wide range of uses, such as food packaging, bottles, straws, thermal receipt paper, toys, CDs and medical devices (European Commission, 2018). The burning and photo degradation of plastics cause a BPA contamination in aquatic environment (Kang et al., 2007). Because of the decomposition of BPA occurs rapidly in UV light, heat, acidic or basic environments, it causes pollution in the environment and human exposure to natural life (Frenzilli et al., 2021). The toxic effects of bisphenol A, has received great attention that it acts as a xenoestrogen and causes endocrine disruption. Today, due to the ban on the use of BPA in many countries (Liu et al., 2021). There are many studies concerning that BPA has toxicity to fish and invertebrates (LC_{50} 1.1 to 10 mg/L) (Colborn et al., 1996).

Because of the lack of data especially its toxicities at low dose exposure Today, believed that the BPA alternatives are "safer". BPS and BPAF are the second and third most abundant analogues in the environment, detected at even higher levels than BPA in surface waters (Liu et al., 2021). A few studies have documented that BPS may be equally or more harmful than BPA (Rochester and Bolden, 2015). So, new researchers advised that necessary to investigate the current alternatives used instead of BPA. Chen et al. (2016) have identified the potentially toxic effects of BPA alternatives on non-target organisms. Furthermore, these BPA analogues have also been determined as endocrine-disrupting chemicals (Moreman et al., 2017). A large number of studies showed that BPS, BPF, and BPAF are found lower concentrations in water, sediment (Liao et al., 2012; Chunyang and Kurunthachalam, 2013; Chen et al., 2016) and bio accumulate in the body of several animal species (Wang et al., 2021). Restricting the use of BPA leads to greater use of BPA alternatives and increases their production. Therefore, concentrations of BPA alternatives are expected to increase in all areas of the environment. The predicted no-effect concentration (PNEC) reported as 1500 ng/L by European Union (Morales et al., 2020).

Effects of pollutants on natural ecosystems can defined by Ecotoxicology. The toxicity of chemicals was ranged according to species (Hammer et al., 2006). Algae and aquatic plants are the most important primary producer's waters and provide oxygen and shelter for many aquatic organisms. Because of this, they are the most important parts of the aquatic food chain. Algae have been reported as more sensitive than animals (Ferreira and Graça, 2002) and have been widely used in toxicity tests.

Recent studies have shown that BP analogues are detected in different environmental media, such as water, air, soil, biomass and sediment (Song et al., 2012; Liu et al., 2016). The use of BPS and BPAF in the production of BPA-free products leads to their detection in the aquatic environment at concentrations ranging from ng/L to µg/L (Chen et al., 2016; Zhao et al., 2019).

Their presence in the aquatic environment, BPS and BPAF have caused some risks for primary producers (Barboza et al., 2020; Czarny et al., 2021). As primary producers, microalgae at the base of the aquatic food chain are of essential for important in aquatic ecology (Fromme et al., 2002). Their sensitivity to toxic substances is the main reason for microalgae to be preferred as good testing organisms. Their readily available, small individual size, and rapid reproduction allow to for rapid assessment of chemical concentrations and generational effects of multiple populations (Abdel-Hamid, 1996). According to previous study report, BPA inhibited the growth and accumulation of chlorophyll in the test organisms. *C. mexicana* had a higher effective concentration value than *C. vulgaris.* Biodegradation and bioaccumulation of BPA were observed in both microalgae. *C. vulgaris was* exposed to concentrations of 1, 10, and 100 mg L-1 BPS, and the inhibition rate of *C. vulgaris* was 41.6%, 103.7%, and 238.4%, respectively (Ding et al., 2020).

New studies have indicated that bisphenols affect ecosystem health (Ji et al., 2013), but studies on the comparative toxicity of bisphenol analogues are limited. Little data reported the toxic effects of BPS and BPAF on growth of phytoplankton. Especially no data available about the effects of BPS and BPAF on *Spirulina* sp., *Desmodesmus subspicatus* and *Chlorella vulgaris*.

The aim of this study is to obtain more data on the acute effects of BPA and BPA analogues on freshwater algae, and the acute toxic effects of BPA and its analogues (BPS and BPAF) on *Spirulina* sp., *Desmodesmus subspicatus* and *Chlorella vulgaris* were examined. This study is a part of the doctoral thesis.

MATERIALS AND METHODS

Chemicals

The chemicals used for the phytotoxicity tests were bisfenol A (BPA) CAS No. 80-05-7, bisphenol S (BPS) CAS No. 80-09-1, bisphenol AF (BPAF) CAS No. 1478-61-1 from Sigma-Aldrich (St. Louis, MO, USA). BPA, BPS and BPAF were prepared according to the conditions recommended by the manufacturer. Dilutions of 1/10 of the stock solution prepared from each chemical were used to prepare intermediate stock solutions.

Test species and culture conditions

Test organisms *Spirulina* sp., *D. subspicatus* and *C. vulgaris* were obtained from Ege University Fisheries Faculty Aquaculture Department and the cultures were grown in the Algae Culture unit of Ege University Ecotoxicology Laboratory.

To reproduce the pure cultures of the phytoplankton to be used in the study, the necessary medium and appropriate environmental conditions were provided for each of them. *Spirulina* sp. the standard Zarrouk broth medium was prepared according to the method Madkour et al. (2012) for the propagation of the pure culture of phytoplankton. The enrichment and environmental conditions suitable for *D. subspicatus* were prepared according to the protocol (OECD, 2011). This algae culture was grown at 21±2 ^{0}C in 4000 lux lighting and 24 h of light. BBM (bold basal medium) was prepared for the enrichment of *Chlorella vulgaris*. The pH value of BBM was adjusted to 6.8. *Chlorella vulgaris* culture was grown at 23±2 ^{0}C in 4000 lux lighting and 24 h of light. A shaker was used to prevent the samples from sticking to the surfaces of the erlenmeyer. For the growth of phytoplankton, firstly, 10 ml of the main algae stock was taken and added to the erlenmeyer containing 20 ml of enrichment. The cultures, which were left to grow under suitable conditions, were transferred to erlenmeyer with volumes of 150 and 200 ml, respectively, as their volumes increased. This process was repeated for all three phytoplankton.

Toxicity test

Algal growth inhibition tests were performed as described in (OECD, 2011). Experiments were started when the cell numbers for *C. vulgaris* and *D. subspicatus* phytoplankton reached 10^5 - 10^6 per ml. *Spirulina* sp.'s long filamentous structure is not suitable for visual microscope counting. For this reason, the cell density of *Spirulina* sp. was measured by fluorimetry (µg/L chlorophyll-a) (Turner Designs the Aquafluor Handheld Fluorometer 54555). Experiments were set up in 20 ml volume. The total duration of the experiments is three days. The experiments were performed in triplicate and cell counts were made at 0.h and 72. h. The determined growth curves were compared with the control group (under the same conditions without adding bisphenol analogues) and the percent inhibition was calculated. 7 different chemical concentrations (0.5, 1, 1.5, 3, 5, 10, 15 mg/L) for *Spirulina* sp., 10 different chemical concentrations (0.5, 0.8, 1, 1.5, 2, 3, 5, 7, 9, 15 mg/L) for *D. subspicatus* and *C. vulgaris* were tested. Algae growth rate and percent inhibition were calculated for each organism.

Cultures exposed to BPA, BPS and BPAF were grown at 23±2 ^{0}C in 4000 lux lighting and 24 h of light. The measurements of the experiments were calculated with the help of algae growth rate and inhibition (%) exponential function.

Algae growth rate (μ), $\mu = (\ln x_j - \ln x_0) / (t_j - t_0)$ (day^{-1})

X_0: number of cells counted at time t_0 (cells/ml) (for *D. subspicatus* and *C. vulgaris)*

Xj: number of cells counted at time tj (cells/ml) (for *D. subspicatus* and *C. vulgaris)*

tj: days until the last measurement of the experiment

% Inhibition= $[(\mu c - \mu r) / \mu c] \times 100$

μc= control group growth rate

μr= concentration group growth rate

The toxicity of the chemicals used in the study on the test phytoplankton was classified according to the (The European Commission, 2013). According to this report, classifies substances according to their effective concentrations values as follows:

Effective concentration 50 (EC_{50}) values in different classes:

a) 1–10 mg L^{-1} (toxic)

b) < 1 mg/L (very toxic)

c) 10–100 mg/L (harmful) for aquatic organisms

Substances with an EC_{50} above 100 mg/L are not classified.

Statistical calculations

IC_{50} values were calculated from the inhibition - concentration curve as 50% growth inhibition of test population compared to control treatment, based on growth rate. Data analysis. The 72 h IC_{50} values were calculated according to the “area under the curve” method prescribed by the OECD. IC_{50}-value was determined by nonlinear regression analysis. All results are presented as mean ± SD. Differences were considered significant at $P < 0.05$. The SPSS Statistics 25 computer programmer was used in the data analysis (Hocking, 1996). The data of growth rates were compared with controls by Dunnet test.

RESULTS

The aim of this study is to identify the acute toxic effects of BPA, BPS and BPAF on *Spirulina* sp., *C. vulgaris* and *D. subspicatus*. According to the results of the studies, *Spirulina* sp. gave different answers. As the toxic effects of three chemicals on the growth of the test organism were compared, BPA accelerated growth, while BPS and BPAF showed a limiting effect on growth. The toxic effect of BPAF has the most toxic effect on growth compared to the other two chemicals. The EC_{50} value for bisphenol A exposure could not be calculated for *Spirulina* sp. because BPA exposure caused the organism to grow.

BPS has a more toxic effect than BPA and BPAF for *D. subspicatus*. It has a more toxic effect on *D. subspicatus* than bisphenol A and bisphenol AF. EC_{50} value could not be calculated for BPA due to overgrowth.

BPS is more toxic to *C. vulgaris* than BPAF. The acute toxicity of BPS is greater than BPA and BPAF for *C. vulgaris*. According to the research results, bisphenol A increased the growth of *Spirulina* sp. instead of stopping it. *Spirulina* sp. and *C. vulgaris* were recorded as the most resistant species to BPA.

As a result, it was observed that BPS and BPAF showed more toxic effects for all three species compared to BPA. Compared to the other two species, *D. subspicatus* is more sensitive to BPS and BPAF.

The EC_{50} values found for the three selected species are given in Table 1.

Table 1. EC_{50} values for phytoplankton species

	EC_{50} (mg/L)		
Test Species	**BPA**	**BPS**	**BPAF**
C.vulgaris	26.5	2.43	3.32
Spirulina sp.	-	6.31	3.80
D.subspicatus	-	0.88	6.48

EC_{50}: effective concentration; the dosage at which the desired response is present for 50 percent of the population

In the first experiments, an increase in the growth of *Spirulina* sp. was observed after three days of BPA exposure. BPA stimulated the growth of the organism. While the growth rate is less at low concentrations (0.5, 1, 1.5, 3 mg L^{-1}), the growth rate is quite high at high concentrations (5, 10, 15 mg/L). The increase in negative inhibition is greater at higher concentrations (5, 10 and 15 mg/L). A decrease in the growth rate and the inhibition % of *Spirulina* sp. were increased after three days of exposure to BPS. In addition, after 3 days of exposure, while the growth rate was 0.58 in the control, parallel to increasing concentrations the growth rates were increased. The growth rate decreased at increasing BPS concentrations. Determination that the inhibition as a function of growth rate. While the growth rate for BPAF was 0.78 in the control group, in highest chemical concentrations (3, 5, 10 and 15 mg/L) it was observed as; -0.17, 0,21, -0.22, -0,19. These results showed that inhibition increasing with parallel to increasing BPS concentrations. BPAF exposure of *Spirulina* sp. resulted in increased inhibition percentage and decreased exposure to growth rate. The growth rate and inhibition graphs of *Spirulina* sp. as a result of BPA, BPS and BPAF exposures are as shown in (Figure 1). According to the results obtained, it is more toxic BPAF (3d EC_{50}= 3.80 mg/L) than BPS (72 h EC_{50}= 6.31 mg/L).

The effects of the chemicals were examined in all three phytoplankton species separately. BPA stimulated the growth of *D. subspicatus*. As compared to the growth rate of the control group (0.60), more growth was observed than the control at all concentrations of BPA exposure. BPS limited growth at all concentrations. It caused inhibition of BPAF in *D. subspicatus*. The highest concentration (15 mg/L) of BPAF showed a high inhibitory effect for *D. subspicatus* (Figure 2).

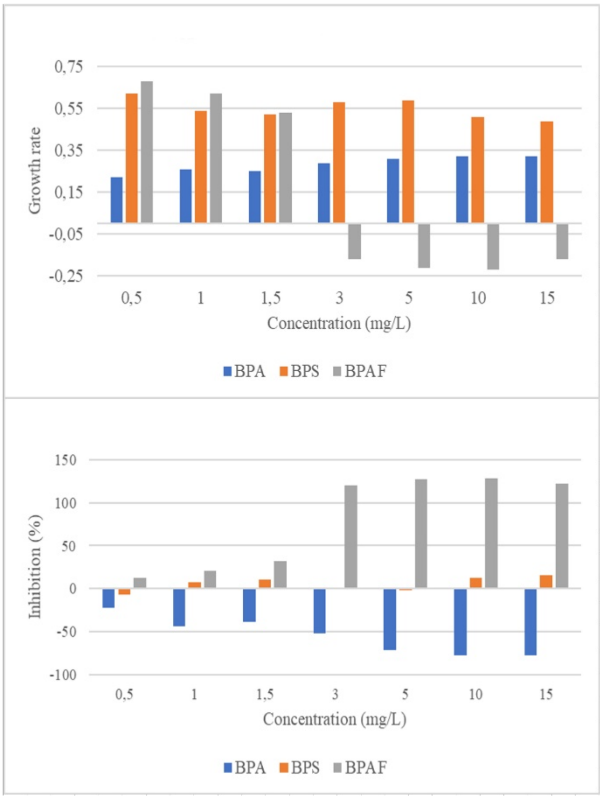

Figure 1. Effects of BPA, BPS and BPAF on growth rate and inhibition of *Spirulina* sp.

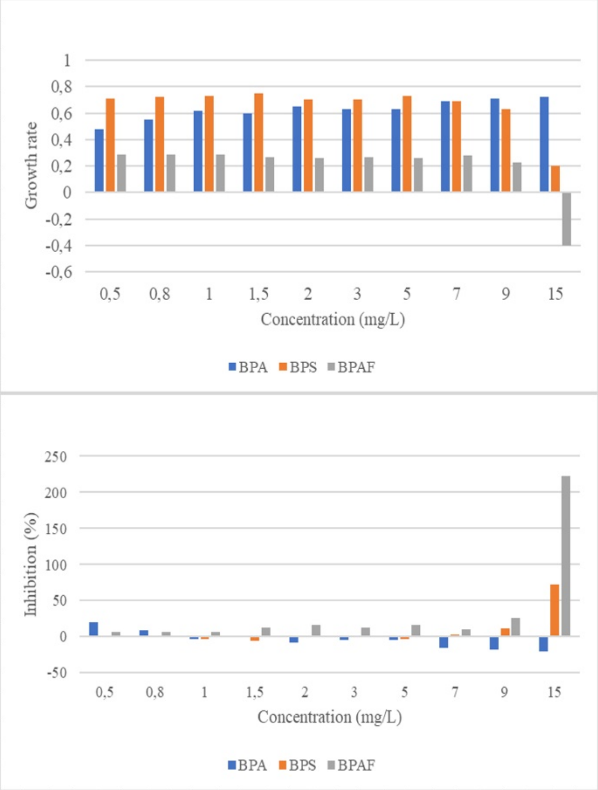

Figure 2. Effects of BPA and BPS, BPAF on growth rate and inhibition for *D. subspicatus*

The EC_{50} value for BPS was calculated as 0.88 mg L^{-1}, the EC_{50} value for BPAF was 6.48 mg/L. EC_{50} value could not be calculated for BPA due to overgrowth. It has been noted that BPS and BPAF inhibit D. *subspicatus*. According to the results obtained, BPS showed a more toxic effect than BPA and BPAF.

According to the results of the experiments conducted with *C.vulgaris*, BPS showed more toxic effects than BPAF and BPA. The EC_{50} value for BPA was calculated as 26,5 mg L^{-1}, the EC_{50} value for BPS was 2,43 mg/L and the EC_{50} value for BPAF 3,32 mg/L. According to these calculations, the acute toxicity of BPS is greater than BPA and BPAF. As a result of exposure to the chemical bisphenol A, the organism continued to grow at low concentrations (0.5, 1, 1.5, 2, 3 mg/L). At higher concentrations (5, 7, 9, 15 mg/L), growth was reduced compared to the control (0.92). A growth arresting effect was observed at all chemical concentrations applied for bisphenol S. BPAF had a growth-limiting effect on *C. vulgaris* from the lowest concentration at which exposure began (Figure 3). Results of this study, BPA and BPAF was found to be less toxic than BPS for *C. vulgaris*.

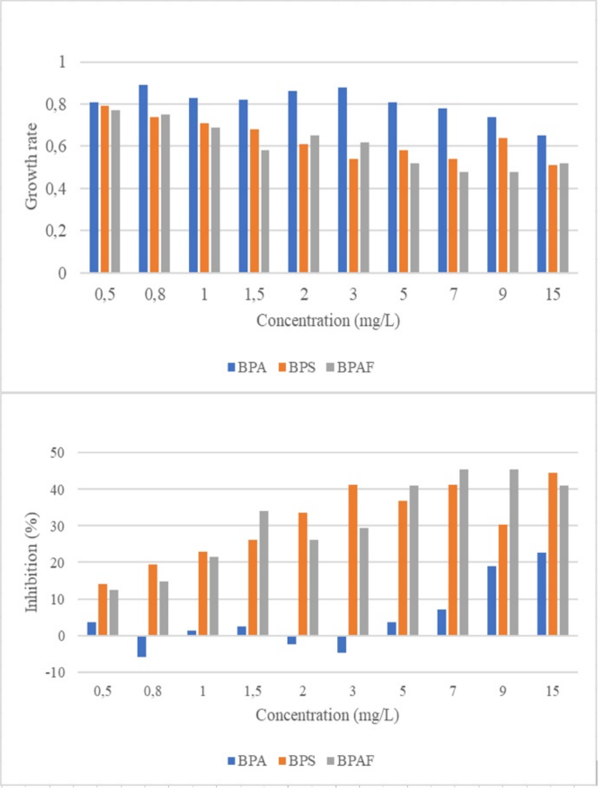

Figure 3. Effects of BPS and BPAF on growth rate and inhibition for *C. vulgaris*

DISCUSSION

Bisphenols are widely available as alternatives to BPA in various environmental and biological samples. There are only few available data about toxic effects of analogues of BPA to microalgae. For example; Libralato et al. (2011) reported that the ecotoxicological characterization of Lignin and tannin on testing species the marine alga *Phaeodactylum tricornutum* (Bohlin). This research showed that the Lignin and tannin effected the algae an EC_{50} of 113.84 (100.90–128.45) mg/L and 26.04 (20.10–33.95) mg/L, respectively. They are also reported the NOEC and LOEC values as <0.1 mg/L and 0.1 mg/L for lignin and tannin. Seoane et al. (2021) noted that the toxicity of the emerging pollutant bisphenol A with three marine microalgae (*Tetraselmis suecica*, *Phaeodactylum tricornutum* and *Nannochloropsis gaditana*). Results of their studies showed that *P. tricornutum* was the most affected species. Researcher reported that After 96 h of exposure to three BPA concentrations, treated cultures of *P. tricornutum* and significant reduction ($p < 0.05$) was observed. These results indicate that *P. tricornutum* growth was the most affected by BPA and also 96 h-EC_{50} values of BPA were reported as 0.6 mg L^{-1}.

The investigation of Czarny-Krzymińska et al. (2022), showed that because of the water solubility of Bisphenol analogues ($\log_{Kow}$ values of BPs were 3.64–6.56= $\log_{Kow} > 3$) its easily cross the cell wall of microalgae and bioaccumulate. Furthermore researcher reported the toxicity of bisphenol A, its six analogues, on the the green algae *Chlorella vulgaris* (bisphenol AF for *C. vulgaris* 14 days, EC_{50}: 22.39 mg L^{-1}) and *Desmodesmus armatus* (EC_{50}: 42.29 mg L^{-1} for Bisphenol A, and bisphenol AF EC_{50}: 27.16 mg L^{-1}) (Czarny-Krzymińska et al., 2022). Tisler et al. (2016) reported that IC_{50} values (3 days) were 3.00 mg-BPAF/L for *Desmodesmus subspicatus* and also showed that the BPAF was more harmful to *Desmodesmus subspicatus* than BPA. Ding et al. (2020) found that bisphenol S showed high toxicity to *C. vulgaris* than bisphenol A, and the obtained EC_{50} values (2 d) were 3.16 and 41.43 mg L^{-1}, respectively. Ding et al. (2020) emphasized that the acute toxicity of BPS in the aquatic ecosystem should be more attention than BPA. In our study, BPS was found toxic for *C. vulgaris*. Czarny-Krzymińska et al. (2022) carried out the first study explaining the effects of toxicity of bisphenol A and its derivatives on microalgae. According to this study with *D. armatus* and *C. vulgaris*, BPAF was found to be more toxic than BPA. The toxicity of BPF, BPA and BPAF on *D. magna*, *D. rerio* and *D. subspicatus* was investigated and BPAF concentrations in the surface waters were observed to pose a risk for aquatic organisms (Tišler et al., 2016).

In our study, the effect of BPA, BPS and BPAF on the growth of freshwater microalgae *Chlorella vulgaris*, *Spirulina* sp*., D. subspicatus* was investigated. The toxicities of BPA, BPS and BPAF chemicals on this three phytoplankton are different. bisphenol A increased the growth of *Spirulina* sp. *C. vulgaris* and *Spirulina* sp. were recorded as the most resistant species to BPA among the test organisms. At concentrations of 5 mg/L and above, BPAF dramatically reduced the growth rate of *Spirulina* sp. BPS at high concentrations (7 mg/L and above) caused inhibition by slowing the growth rate of both *Desmodesmus subspicatus* and *C. vulgaris*. Comparison of calculated EC_{50} values of chemicals tested for their toxicity on three phytoplankton was made according to The European Commission (2013). EC_{50} values obtained from the study are shown in Table 1. As a result of comparing the EC_{50} values obtained from the study with the report, it was determined that BPS and BPAF had toxic effects on all three species. *D. subspicatus* is more sensitive to BPS than BPAF. The most resistant phytoplankton to BPAF exposure is *D. subspicatus* and the most sensitive is *C. vulgaris.*

When the toxic effects of individual and mixed bisphenol analogues on cyanobacteria were examined, it was observed that the mixture of bisphenol analogues had a stronger toxic effect than BPA (Czarny et al., 2021).

In addition to these studies, the effects of BPS and BPAF on more phytoplankton are still unknown. These effects should be identified by acute and chronic toxicity tests and the presence of these chemicals in the aquatic system should be reduced. Investigation of the effect of BPA and analogues on phytoplankton in the first step of the aquatic system should be expanded.

These types of studies are important in predicting the toxic effects of chemicals on living organisms. Light of previous and our studies, BPS and BPAF concentrations in the environment may not be hazardous at present time. But BPA analogues such as; BPS and BPAF concentrations in aquatic environment must be monitoring for the ecosystem health. Furthermore, this study will enable us to obtain more information about BPA and its analogues by examining the toxic effects of widely used BPA derivatives, BPS and BPAF, on the primary producers, phytoplankton.

ACKNOWLEDGMENTS AND FUNDING

This study was supported by the Turkish Scientific and Technological Research Institution (Grant number: 119Y246).

AUTHOR CONTRIBUTIONS

Material preparation and research were carried out by Duygu Turan. The article was written and edited by Duygu Turan and all authors have read and approved the article.

CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

ETHICAL APPROVAL

Ethical approval is not required for this study.

DATA AVAILABILITY

All relevant data is inside the article.

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