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EFFECTS OF BISPHENOL A AND NEW ANALOGS ALGAL GROWTH *OF PHAEDACTYLUM TRICORNUTUM:* A NEW FINDING WITH MARINE DIATOM *PHAEDACTYLUM TRICORNUTUM*

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ABSTRACT
Restricting the use of BPA, environmental concentrations of Bisphenol S, bisphenol F and bisphenol AF begin to increase. The present study aims to indicate that toxicity of BPA and its analogs Bispenol S, Bisphenol F and Bisphenol AF by algal growth inhibition test for the marine green algae *Phaedactylum tricornutum*. In this way, result of this study present the nominal effective concentrations of BPA and its analogs and the suitability of the species for use as a biomarker in ecotoxicology tests. IC50 values (growth rate inhibition by 50%, respectively) for four toxicants were determined separately. BPAF was more toxic to *P. tricornutum* than BPA, BPF and BPS. These findings will provide a reference for the risk assessment of BPA, BPS, BPF and BPAF in natural sea waters.

KEYWORDS: Bisphenol A, Bisphenol A analogues, Algal growth, Toxicity.

INTRODUCTION

Plastic pollution threat to marine ecosystems due to the widespread use of all areas. So that, it has several impacts on aquatif organisms, many of which have not been investigated (Uibel et al. 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, Özlem and Hatice 2008). 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 photodegradation of plastics cause a BPA contamination in aquatic environment (Kang et al. 2007; Teuten et al. 2009, West et al., 2001; Dorn et al., 1987, Lyons 2000). 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 distruption.

Today, Due to the ban on the use of BPA in many countries (Liu et al., 2021). It has led to the rapid development of compounds with similar chemical and physical properties to BPA and the replacement of BPA and its analogs (such as Bisphenol S, Bisphenol F, Bisphenol AF) (Ullah et al., 2019). Bisphenol analogs with similar structures, whose effects and safety are uncertain, that are released to the marine environment through plastic leaching (Laist 1987).

Detectable levels of BPA range from 0.5-146 µg/L in freshwater rivers and industrial effluent (Ozlem and Hatice, 2008). There are many studies concerning that BPA has toxicity to fish and invertebrates (LC50 1.1 to 10 mg/L) (Alexander et al. 1988, Özlem and Hatice, 2008; 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 BPF 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 analogs 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 (Chen et al. 2016, Clark 2012; Liao et.al. 2012, Chunyang and Kurunthachalam 2013, 2014) and bioaccumulate 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 (Hammer et al. 2006). The toxicity of chemicals were ranged according to species (Hammer et al. 2006). Algae and aquatic plants are the most important primary producers waters and provide oxygen and shelter for many aquatic organisms (Ferreira and Graça, 2002). 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. Diatoms is abundant photosynthetic organism which are found variety of habitats in marine waters. *Phaedactylum tricornutum* which is used as a preferred food in aquaculture, one of the most used algal species in the marine bioassays due to easy cultivation (Kvıderova´ and Lukavsky´ 2003).

New studies have indicated that bisphenols affect ecosystem health (Ike et al. 2002; Ji et al. 2013; René and Watson 2013, Sun et al. 2014), but studies on the comparative toxicity of bisphenol analogs are limited. Little data reported that the toxic effects of BPA, BPS, BPF and BPAF on growth of phytoplankton. Especially no data avaibale about effects of BPS, BPF and BPAF on marine diatom *P. tricornutum*. Therefore, in this study we have investigated the influence of Bispenol A and different Bisphenol analogues (BPS, BPF and BPAF) on the growth rate of marine phytoplankton diatom *P. tricornutum* by bioassay method.

MATERIAL AND METHODS

Analytical grade bisphenol-a [$(CH_3)_2C(C_6H_4OH)$, Cas No: 80-05-7], Analytical grade bisphenol-s [$(O_2S(C_6H_4OH)_2$, Cas no: 80-09-1 (4,4'-Sulfonylbisphenol)], Analytical grade bisphenol-F [$(CH_2(C_6H_4OH)_2$, Cas no: 620-92-8] and Analytical grade bisphenol-AF [$(CF_3)_2C(C_6H_4OH)_2$, Cas no: 1478-61-1] were purchased from Sigma-Aldrich, Germany. Test chemical were dissolved in dimethylsulphoxide (DMSO) (Sigma, Cat. No: 67-68-5) as 100 µg-BPA, BPS, BPF, BPAF/L. Test concentrations were prepared by adding the chemicals from stock solution directly to the test medium. Test concentrations were selected as; 0.5, 0.8, 1.0, 1.5 and 2.0 mg/L of BPA, BPS, BPF and BPAF. Controls accompanying the experiments were untreated negative controls (filtered dilution artificial sea water) and positive ($K_2Cr_2O_7$ as reference toxicant) controls were included in each experiment.

The marine species used in the assays was *Phaeodactylum tricornutum*. These species were selected because it is common phytoplankton found in marine environments. The alga was cultivated at the Laboratory in the f/2 medium at 20C under continuous white light exposed to with approximately 100 µmol photon m^{-2} s^{-1} under a 12:12 h light: dark cycle. Stock cultures were maintained in filtered (pore size: 0.2 µm) and autoclaved natural seawater enriched with f/2 medium (OECD, 1984) in a rotary shaker set at 125 rpm.

To determine the toxicity of BPA, BPS, BPF and BPAF, on the growth rate of marine phytoplankton diatom *P. tricornutum* was exposed in six replicate to increasing concentrations of compounds for 72 ± 2 h. *P. tricornutum* Cellular density was evaluated using a Bürker counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Bx51 Olympus, Japan). The algae were cultured to the exponential phase before they were transferred to new culture medium for the following tests. The point estimation and the calculation of the growth inhibition concentrations and their relative 95% confidence limit values were carried on by a linear regression model after natural logarithm data transformation of the measured cell density.

The endpoints were evaluated based on cell count data and calculated growth rate (0 to 72h) as described in standard protocols (OECD 1984) from the mean cell counts of each test series. The average specific growth rate (µ) for exponentially growing cultures were calculated as:

$\mu_{0-j} = \ln x_j - \ln x_0 / t_j - t_0$ (day $^{-1}$)

μ_{0-j}: growth rate,

X_0: nominal number of cells / m at time t_0,

X_j: measured number of cells/ml at t_j,

T_j: time of first measurement of after beginning of test

The percentage inhibition of the cell growth ($\%I_r$) at each test substance concentration is calculated as the difference between the control growth curve (µc) and the growth curve at each test substance concentration (µt) as:

$\%I_r = \mu_c - \mu_r / \mu_c \times 100$

IC50 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 Statistica-6.0 computer programmer was used in the data analysis (Hocking 1996). The data of growth rates were compared with controls by Dunnet test.

RESULTS AND DISCUSSION

To investigate the potential effects of BPA, BPS, BPF and BPAF on the marine phytoplankton, an algae growth inhibition test was conducted by using *P. tricornutum* as a model organism. Figure 1 and Table 1 shows the effects of the increasing concentrations (0.5, 0.8, 1.0, 1.5 and 2.0 mg-BPA/L) of BPA on *P. tricornutum* growth rate.

The lowest concentrations of BPA (0.5 mg-BPA/L) affected the growth rate of *P. tricornutum* (approximately % 50) (Figure 1). The dose-response curve (Figure 1) showed that a decreased number of algal cell at the highest BPA concentrations (2.0 mg-BPA/L) when compared with the control group ($p<0.0001$).

Figure 1 shows the growth rate and inhibition of BPA increasing concentrations (0.5, 0.8, 1.0, 1.5 and 2.0 mg-BPA/L) on *P. tricornutum*. A clear dose-response relationship was observed after 72 hours of exposure (Figure 1). Table 2. The percentage of inhibition of *P. tricornutum* generated by several concentrations of BPA showed that algal growth was significantly inhibited (Figure 1) at all concentrations tested ($p<0.0001$). The impact of BPA on algal growth of exposed *P. tricornutum* was determined as IC50 3.94 mg/L BPA for 72h. concentration (Table 3).

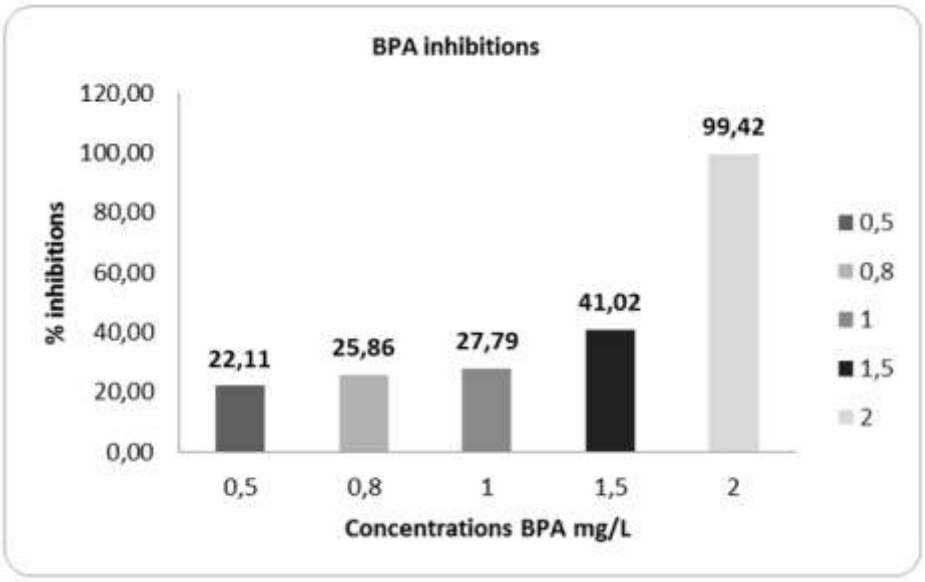

Figure 1. According to the applied concentrations of BPA % inhibitions.

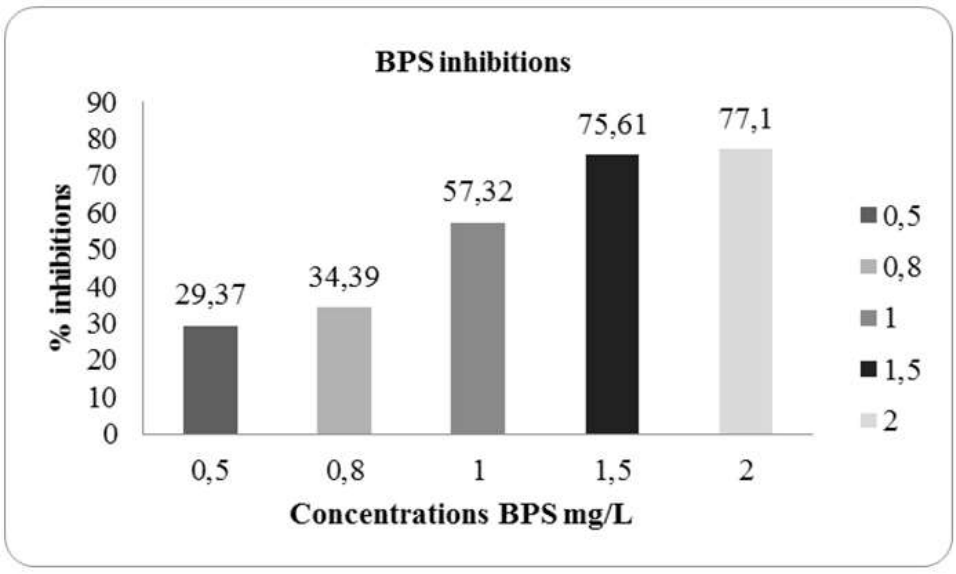

Figure 2. According to the applied concentrations of BPS % inhibitions.

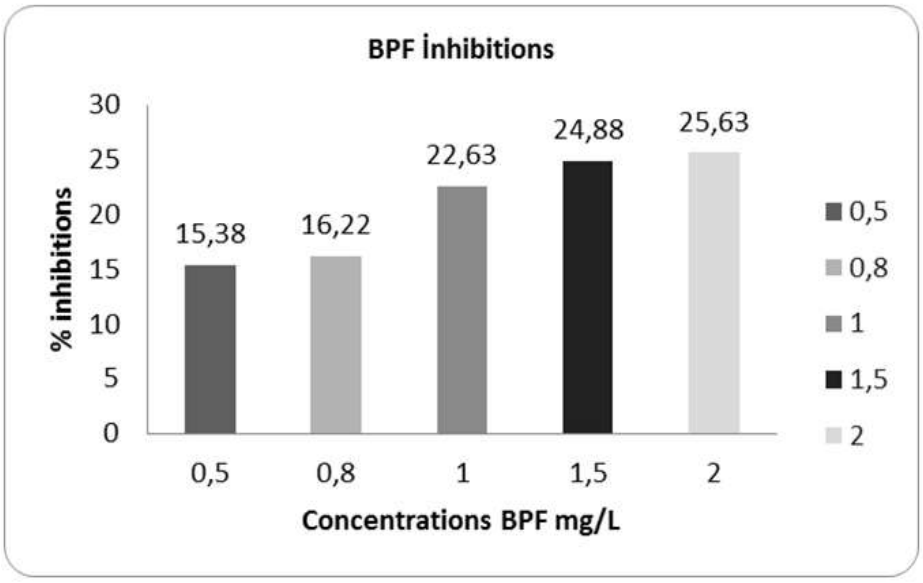

Figure 3. According to the applied concentrations of BPF % inhibitions.

In Figure 2, effects as growth rate and growth inhibition percentage (%) were reported for both BPS, BPF and BPAF. No stimulation effects were detected. For BPS, effects ranged between 29.37% (0.5 mg-BPS/L) and 77.1% (2 mg-BPS/L). The 50% effect was achieved between 0.5 and 2 mg-BPS/L after 72 h; IC50 2.94 mg-BPS/L values (Table 3) were available ($y = 22,466^{e0,2718x}$, $R^2 = 0,9244$) (Figure 2).

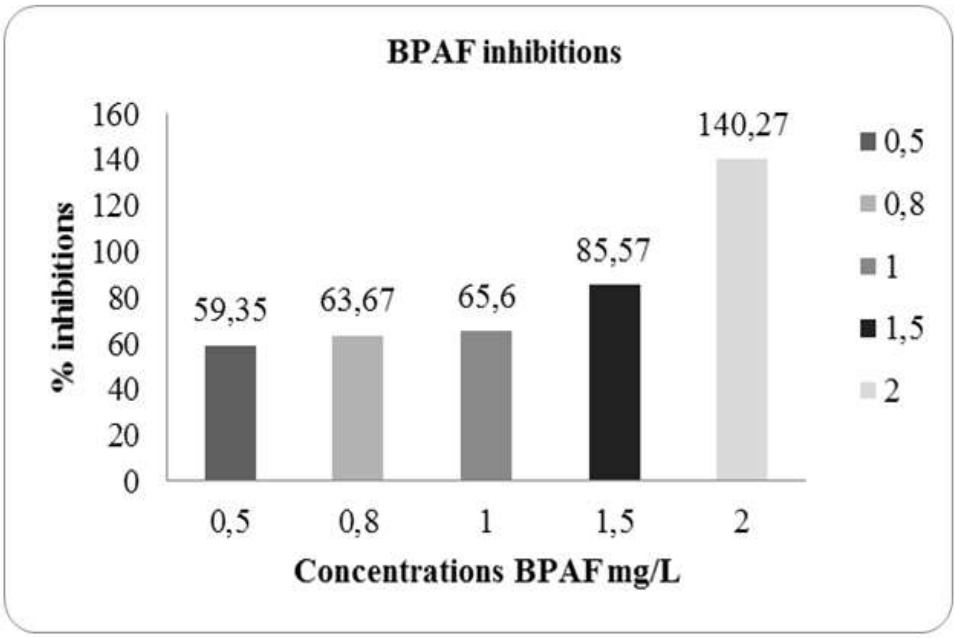

Figure 4. According to the applied concentrations of BPAF % inhibitions.

When *P. tricornutum* were exposed to BPF throughout cell growth, significant effects were observed at concentrations ranging from 0.5 to 2.0 mg-BPF/L. The algal growth inhibition tests show the classic dose-response curve indicating a decreased percentage of growth rate with increasing BPF concentrations (Table 1-2, Figure 3). The impact of BPF on exposed algae was estimated as IC50 9.17 mg/L BPF concentration Table 3.

On the other hand, the lower concentrations of the BPAF generally caused significant inhibition on *P. tricornutum.* Concentrations between 0.5 and 2.0 mg-BPAF/L of BPAF caused decreasing growth rates of 0.38 and 0 respectively. In Figure 4, effects as growth rate and growth inhibition percentage (%) were reported for BPAF. These results confirmed that the toxicity of BPAF to *P. tricornutum* as IC50 be determined for exposure periods 72 h as 0.76 mg-BPAF/L (Table 3).

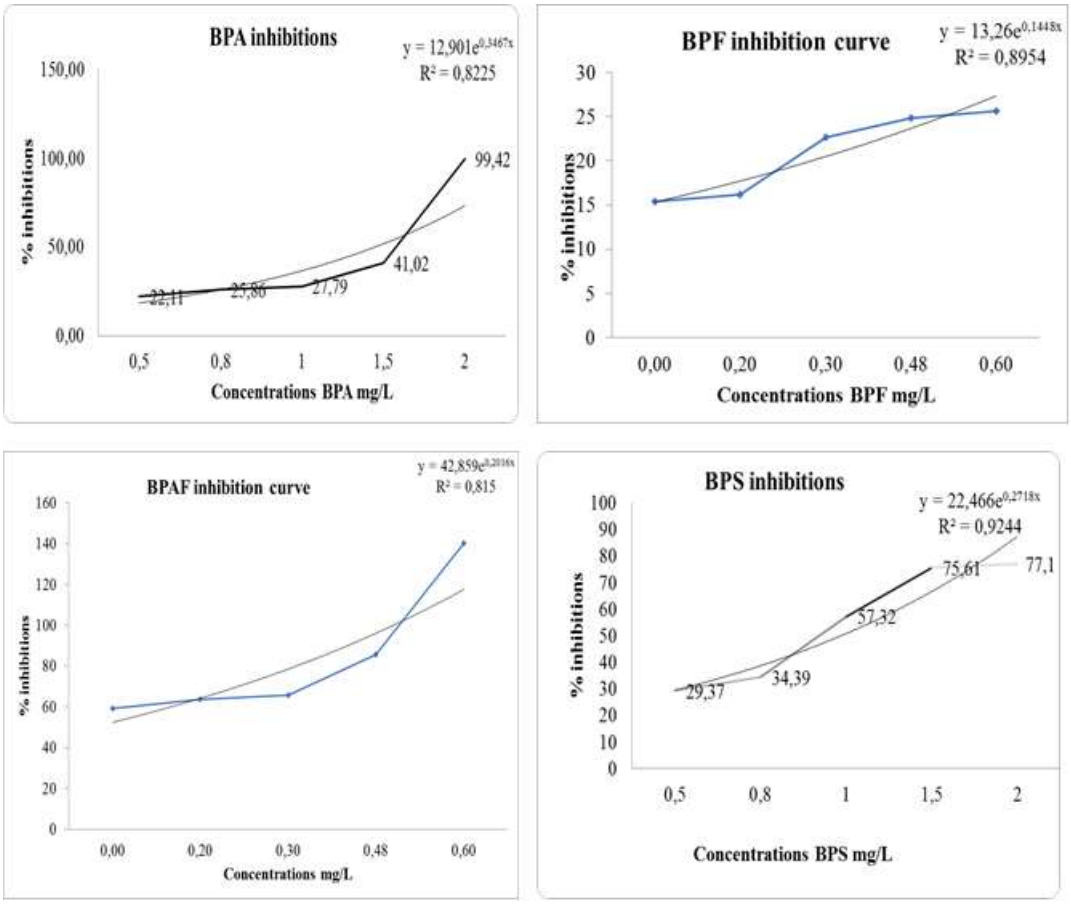

Figure 5. Inhibition curves of applied concentrations of chemicals % inhibitions.

In the control group; The increase in the number of phytoplankton cells by 1.5 times according to OECD criteria at day 0, 24 hours, 48 hours and 72 hours is stated as an indication that the test continues in a healthy way. No limiting/toxic effects were observed in the control group in our phytotoxicity trials. In the light of the data obtained; As a result of algal growth inhibition tests (phytotoxicity) performed with analogues used instead of Bisphenol-A, limiting/toxic effects on phytoplankton; It was determined by comparing the values obtained as a result of microscopic phytoplankton cell counts at day 0, 24 hours, 48 hours and 72 hours with the control group (without toxicant). Depending on the toxicant, an inhibiting effect was observed in the number of cells compared to the control group from the first concentration (Table 1, 2) (Figure 1-5) (The effect increased 32-99%). According to the results of the experiments, it was observed that all of the chemicals applied had a limiting effect on the growth rate, depending on the concentration, and an increase in the inhibition percentages calculated based on the dose-response curve in Figure (1-4) and the calculated EC50 (Table3). Results of study showed that toxic levels of the bisphenol analogs as BPAF>BPS>BPA>BPF, respectively. In the experiments, it was found that all chemicals applied had a restrictive effect on growth depending on the increase in concentration. However, the toxicity of BPF to *P. tricornutum* was lower than BPA in this study, with a high IC_{50} of 0.76 mg-BPAF/L and IC_{50} of 0.76 mg-BPS/L after 72 h of exposure showed that this BPA alternative toxic to marine algae.

Table 1: Growth rates of chemicals according to the results of applied concentrations.

Growth Rate				
Concentrations (mg/L)	**BPA**	**BPF**	**BPAF**	**BPS**
Control	1,15	0,80	0,94	0,6
0.5	0,57	0,67	0,38	0,56
0.8	0,55	0,67	0,34	0,36
1.0	0,53	0,62	0,32	0,21
1.5	0,43	0,60	0,14	0,19
2.0	0,004	0,59	-0,38	0,6

Table 2: Percentages of inhibition of chemicals according to the results of applied concentrations.

Inhibitions (%)				
Concentrations (mg/L)	**BPA**	**BPF**	**BPAF**	**BPS**
Kontrol	0,74	0,85	0,81	0,75
0.5	22,11	15,38	59,35	29,37
0.8	25,86	16,22	63,67	34,39
1.0	27,79	22,63	65,60	57,32
1.5	41,02	24,88	85,57	75,61
2.0	99,42	25,63	98,27	77,1

Table 3: EC50 values calculated based on the results of the applied concentrations.

EC50 (mg/L)			
BPA	BPS	BPAF	BPF
3,91	2,94	0,76	9,17

DISCUSSION

BPA has been widely known as toxic many aquatic species, and the analogs such as BPF, BPAF and BPS have been introduced as new substitution for BPA. The great use of BPs in the production of BPA-free products cause to their widely detection in the aquatic environment at concentration from ng L^{-1} to µg L^{-1} level (Chen et al. 2016; Zhao et al. 2019). Microalgae, as primary producers that can be found in most aquatic systems, play an important role in predicting ecological balance and health (Lemley et al. 2016). Chemicals, such as BPA and its structural analogues, may adverse effects on the species composition of the phytoplankton community (Yihua et al., 2010). Because of, a little research about possible toxic effects of BPs on microalgae, the adverse effects of exposure of microalgae to analog of BPA need to be investigations. To investigate are bisphenol analogs cause of toxicity, the algal growth test was assessed with marine algae *P. tricornutum.* According to result of this study, algal growth of *P. tricornutum* treated with BPA, BPS, BPF and BPAF showed that these chemicals inhibited the algal growth.

Researchers reported that BPA is toxic to algae, crustaceans, fish, bacteria and amphibians in freshwater environment ($LC_{50:}$ 2.5- 6900 mg/L) (Özlem and Hatice, 2008). Also, the LC50 for BPA has been determined for a variety of marine organisms, including algae, sea and fish with reported values ranging from 1- 20. mg/L (Staples et al., 1998, Staples et al, 2002, Özlem and Hatice 2008).

Phytotoxicity is a suitable test system to determine the adverse effects of different chemicals. Such as, Wind and Belanger (2006) used the phytoplankton species to determine the toxicity of Alcohol ethoxylates and they reported that these analyses suitable for determination of environmental risk assessment. Pavlic et al (2005) had investigated the toxic effect of surfactants to two marine diatoms and reported that the concentrations of the tested surfactants caused a reduction in 50% growth of green algae at between 0.35-4.4 mg/L, when compared to the controls.

Phytoplankton are relatively sensitive to chemicals (Xiang et al., 2018). Algal growth inhibition tests is now widely used in the toxicological characterization of heavy metals and xenobiotics in environmental monitoring (Zhang et al., 2014) for environmental pollutants. A large data set exists on the toxicity of several chemicals on the algal growth of *Phaedactylum tricornutum*. However, no information about the toxicity of BPA, BPS, BPF and BPAF to the growth of the *P. tricornutum*. There are only few available data about toxic effects of analogs of BPA to microalgae. For example; Libralato *et al*. 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 E_rC_{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.

Sloane 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 analogs (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 analogs, 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 IC50 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 EC50 values (2 d) were 3.16 and 41.43 mg L−1, respectively. Unfortunately, no data are available for adverse efects of BPA, BPS, BPF and BPAF to marine microalgae *Phaedactylum tricornutum*.

In our investigation, The results showed that the parameter of inhibition % in growth rate of *P. tricornutum* have state a question on the toxicity of BPA and BPS, BPF, BPAF. Our results and the results of other related studies showed that contamination with BPA and its analogues caused an inhibition of the growth rate of marine alga *P. tricornutum*. Toxic effects of BPs on algae are not available Therefore, this method may be reliable for the determination of toxic effects of chemicals on growth of *P. tricornutum.*

These types of studies are important in predicting the toxic effects of chemicals on living organisms. Light of previous and our studies, BPS, BPF and BPAF concentrations in the environment may not be hazardous at present time. But BPA analogs such as; BPS, BPF and BPAF concentrations in aquatic environment must be monitoring for the ecosystem health.

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