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The influence of benzyl butyl phthalate on the growth of several phytoplankton species (*Microcystis* sp., *Anabaena variabilis*, *Chlorella* sp., *Scenedesmus* sp.) in laboratory conditions

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ABSTRACT

Phthalic acid esters (PAEs) are organic compounds extensively used as plasticisers. Their widespread use has resulted in their presence in aquatic and terrestrial ecosystems, making them a high-risk pollutant. PAEs are detrimental to human health as they disrupt the endocrine system and can potentially cause cancer. Although their impact on humans is relatively well-known, more research is necessary to comprehend their effects on phytoplankton. This work aimed to examine the influence of different concentrations (50, 100, 150, 200, 250 µg/L) of benzyl butyl phthalate (BBP) on the growth of several most common phytoplankton species (*Microcystis* sp., *Anabaena variabilis, Chlorella* sp., *Scenedesmus* sp.) in laboratory conditions. Phytoplankton growth was monitored spectrophotometrically to determine the concentration of chlorophyll *a.* The results showed that higher concentrations of BBP significantly inhibited the growth of *A. variabilis* and *Microcystis* sp. Green algae showed a considerably lower sensitivity, especially *Chlorella* sp., where significant growth inhibition was not observed. After the experiment, the detection and quantification of BBP in extract samples were performed using gas chromatography with mass spectrometry (GC-MS). BBP was detected only in the extracted sample with *Scenedesmus* sp., but the detected concentration was insignificant. The results indicate that all tested organisms could probably absorb and metabolize BBP, of which *Scenedesmus* sp. has the least ability.

Keywords: *algae, chlorophyll a, GC-MS, pollution*

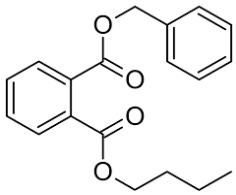
Introduction

Phthalic acid esters (PAEs) are a class of organic plasticizer compounds widely used as additives in various products, including cosmetics, food packaging, construction supplies, medical equipment, home furnishings, plastic tubing, automotive components, floor tiles, insect repellents (Huang et al., 2021a; Staples, 1997). Due to their wide application, they are increasingly present in aquatic and terrestrial ecosystems, which represents a danger to the environment, considering that they are classified as priority pollutants in the USA, China, and the European Union (Zhang et al., 2016). There are several types of PAEs used for commercial and industrial purposes, of which six (dimethyl phthalate, diethyl phthalate, di-n-butyl phthalate, benzyl butyl phthalate, di (2-ethylhexyl) phthalate, and di-n-octyl phthalate) are classified as primary pollutants (Net et al., 2015). PAEs, as pollutants harm the environment and human health, disrupting the endocrine system, can exhibit anti-thyrogenic, anti-androgenic, anti-progestogenic, and anti-estrogenic properties associated with adverse health effects (Huang et al., 2021b). Given that their production is significant and that they are not tightly bound to the polymer matrix of the final product, they can easily migrate into the environment and thus be found in freshwater and marine systems, drinking water, sediment, air, and aerosols (Net et al., 2015). Once they reach aquatic systems, they can bring many ecological problems (Net et al., 2015). Therefore, to accurately evaluate the effects of PAEs on aquatic ecosystems and the potential ecological hazards they present, closely monitoring the interactions between them is imperative. That is an essential step in ensuring the safety and sustainability of the environment.

Benzyl butyl phthalate (BBP) is often used as a plasticizer for vinyl tiles (CMA, 1999). Apart from these purposes, it is also used to produce automotive components, food packaging, perfumes, cosmetics, adhesives, and artificial leather (Li et al., 2021). It has been reported that BBP can promote breast cancer stem cell expansion (Cao et al., 2023), participate in DNA damage and methylation (Tsai et al., 2014), and induce significant neurodevelopmental damage (Miodovnik et al., 2014). In addition to humans, this pollutant has been shown to have toxic effects on other organisms, including chironomids (Call et al., 2001; dos Santos Morais et al., 2020; Llorente Ortega, 2022; Planello et al., 2011), cladocerans (Li et al., 2021; Wang et al., 2011), mice and rats (Ambe et al., 2019; Agas et al., 2007; Ema et al., 2003; Ema & Miyawaki, 2002; Gray et

al., 2000; Piersma et al., 2000), the marine univalve Haliotis diversicolor super-texta (Liu et al., 2009), daphnia (Gledhill et al., 1980), fish (Bhaisare et al., 2022; Gledhill et al., 1980) and algae (Gledhill, 1980). The structure and physiochemical properties of BBP are shown in Table 1.

Table 1: Structure and physicochemical properties of BBP

Properties	Benzyl butyl phthalate	References
Molecular formula	$C_{19}H_{20}O_4$	Staples et al. (1997)
Chemical structure		Bogdanovic (2021)
Molecular weight	302.39	Staples et al. (1997)
Water solubility (mg/L)	2.7	Staples et al. (1997)

Chemically synthesized PAEs are widely used for industrial purposes due to improved product quality. However, recent research has shown that PAEs, apart from chemical means, can also be synthesized naturally by synthesizing some organisms, including algae (Babu et Wu, 2010a). The natural synthesis of PAEs is thought to provide better adaptation to the species that produce them (Huang et al., 2021a). However, in contrast to the biosynthesis of PAEs, in recent times, scientists have paid much greater attention to biodegradation by algae. Since PAEs are not bound to the polymer matrix of the final product, they can easily migrate into the environment and thereby affect the natural inhabitants of that environment (Net et al., 2015). Different biotic and abiotic pathways, including hydrolysis, photolysis, photooxidation, and biodegradation, have been identified as contributing to the degradation of PAEs (Net et al., 2015). The abiotic degradation process for PAEs is inefficient and prolonged, with DMP taking around three years and DEHP taking around 2000 years for aqueous photolysis (Miriyam et al., 2022). Furthermore, hydrolysis is not an effective method for phthalate elimination due to its slow reaction rate (Bogdanović, 2021). In contrast, microbial degradation is a much faster and cost-effective method that is environmentally friendly for eliminating phthalates' primary pathways for the degradation of PAEs (Net et al., 2015). Researchers have conducted multiple studies demonstrating the ability of

bacteria present in wastewater to break down a range of PAEs under aerobic, anaerobic, and facultative conditions. In addition to bacteria, microalgae have also been found to biodegrade PAEs. Microalgae exhibit unique capabilities that enable them to function as effective bioremediators of organic contaminants. In contrast to bacteria, microalgae can perform photosynthesis, stimulating heterotrophic bacteria for growth and organic contaminants' degradation. Moreover, microalgae can directly degrade organic contaminants, for example, cyanobacteria *Cyanothece* sp. PCC7822, *Syenchocystis* sp. PCC6803, and *Synechococcus* sp. PCC7942 has been shown to metabolize DMP (Zhang et al., 2016). Similarly, green microalgae, such as *Chlorella pyrenoidosa* and single-celled dinoflagellate *Karenia brevis*, degrade PAEs (Yan et al., 2002; Sun et al., 2019). The proven possibility that microalgae can degrade PAEs is of great importance because these findings highlight the potential of microalgae-based bioremediation strategies for addressing environmental pollution.

Despite the availability of different pathways to eliminate PAEs from freshwaters, their extensive use has resulted in their omnipresent occurrence in the environment. Consequently, researchers have examined the efficiency of diverse physicochemical techniques that could effectively eliminate PAEs from freshwater systems, in addition to the natural processes that occur continually. In a recent study by Miriyam et al. (2022), five primary methods were proposed for eliminating PAEs, including adsorption, photocatalysis, electrochemical and membrane technology, and microbiological degradation. After considering each method's advantages and disadvantages, the authors conclude that phthalate biodegradation is the most efficient and effective way of eliminating these harmful compounds. Gobas et al. (2002) concluded that PAEs are not bioaccumulative substances and pointed out that there is no evidence to confirm this, considering laboratory, field, and mathematical studies. The life of PAEs in water is so short that long-term bioaccumulation (Semenov et al., 2021) and biomagnification in food chains (Gobas et al., 2002) are impossible.

Considering the ever-increasing production of plastic, pollution levels are expected to increase, causing global concern. Although the impact of PAEs on humans is relatively well known, the effect on phytoplankton still needs to be further investigated. In addition to being global oxygen producers, phytoplankton also represent the base of the aquatic food chain. However, these photosynthetic organisms are sensitive to environmental pollution. Data on the effects of PAEs on

phytoplankton are scarce and need to be more comprehensive to understand the natural consequences that can occur when exposed to these pollutants. BBP is one of six phthalates that have been characterized as high-risk pollutants. However, its impact on phytoplankton species is almost unknown. To our knowledge, only Gledhill et al. (1980) investigated its effect on several representatives of algae. Also, it is unknown whether phytoplankton has the potential to biodegrade BBP. The main objective of this study is to investigate the effects of different concentrations of the high-risk organic pollutant BBP on two cyanobacteria (*Anabaena variabilis, Microcystis* sp.) and two green microalgae (*Chlorella* sp., *Scenedesmus* sp.). To achieve this, the present study monitored the biomass production of the tested phytoplankton species by measuring the concentration of chlorophyll *a*. Subsequently, the investigation aimed to determine the phytoplankton ability to metabolize BBP, evaluate if BBP activity exhibits dose-dependent manners, and identify potential differences in sensitivity among the different species of phytoplankton. In the present study, the impact of this pollutant on *A. variabilis, Chlorella* sp., and *Scenedesmus* sp. is described for the first time, as well as their potential for biodegradation.

Experimental

Algal cultures and cultivation conditions

The experiment used BBP in concentrations of 50, 100, 150, 200, and 250 µg/L.

Chlorella sp., *Scenedesmus* sp., and *Microcystis* sp. were isolated from a freshwater pond (43°17'51.9"N 21°47'40.8" E) in Southeast Serbia and were cultivated at the Department of Biology and Ecology, Faculty of Science and Mathematics in Niš. They are identified using an identification key to the genus level (Burchadt, 2014).

Trichormus variabilis 0441 (Kützing ex Bornet & Flahault) (Komárek & Anagnostidis, 1989) (heterotypic synonym *Anabaena variabilis*) was isolated from the Danube River in the Vojvodina region (Serbia). It was cultivated in the Department of Biology and Ecology laboratory in Novi Sad (NSCCC) (Stankovic, 2020). *A. variabilis* was identified using molecular methods described in the previous study (Stankovic et al., 2022).

All cultures were prepared in 250 ml Erlenmeyer flasks at 24 ℃ under cool LED lighting for a 16-h photoperiod with constant aeration. Standard BG11(Ripkka et al., 1979) liquid medium was used to cultivate all species except *A. variabilis,* for which modified BG11 medium (without nitrogen source) was used for cultivation.

Exposure conditions to BBP and growth inhibition test

To examine the influence of BBP on several phytoplankton species, the effect of different concentrations of BBP (50, 100, 150, 200, 250 µg/L) on the growth rate of *A. variabilis, Microcystis* sp., *Chlorella* sp., *Scenedesmus* sp., within three weeks was monitored. Investigated concentrations of BBP were selected based on the low solubility of BBP in water (0.71 mg/L) (NCBI, 2023). In the experiment, in the sterile glass test tubes was added 10 ml of liquid medium BG11, 2 ml of a culture of each strain individually, and a specific concentration of BBP. Each concentration for each tested strain was set up in triplicate, as were the controls (18 replicates for each species). Controls (without BBP) were monitored for assay validity. All tubes were incubated at 24℃ under cool LED lighting for a 16-h photoperiod for 21 days. Algae growth was monitored spectrophotometrically to determine chlorophyll's concentration. During the three weeks of the experiment, 1 ml of the culture, previously vortexed, was placed in cuvettes, and the optical density at 678, 720 and 750 nm was determined spectrophotometrically. The chlorophyll concentration was calculated according to the following formula (Stankovic, 2020): Chl (mg×ml-1) = 14.96 · ($OD678 - OD750$) − 0,616 · ($OD720 - OD750$)

BBP extraction

After the inhibition test was completed, the supernatant of all experimental cultures was separated by centrifugation and filtration to prepare a sample for further analysis. Liquid-liquid extraction was applied to extract BBP from the samples. For this purpose, *n*-hexane was used as a non-polar solvent. Detection and quantification of BBP were performed using gas chromatography with mass spectrometry (GC-MS).

Instrumental GC-MS analysis

Analysis was carried out by gas chromatography coupled to a mass spectrometer (Agilent 6890 series GC System with autosampler connected with Agilent 5973 Mass Selective Detector

(Electron Ionization MSD-EI, single quadrupole). The separation was achieved using a non-polar AGILENT DB-5MS column, phenyl arylene-based (30 m × 0.25 mm × 0.25 µm). The oven temperature was programmed from 65 °C (holding time 1 min) to 220 °C (1 min) at a rate of 20 °C min-1, then to 280 °C at a rate of 5 °C min-1 (4 min). Splitless mode was used, and 1 µL of volume was injected. The inlet temperature was 250 °C. MS Quad and MS Source temperatures were 150 °C and 230 °C, respectively. The energy of ionizing electrons was 70 eV. Helium (purity 99.999%) was the carrier gas with a constant flow rate of 1.0 mL min-1. The MSD was used in the single ion-monitoring (SIM) mode at *m/z* 149. The target compound was identified based on the relative retention time, the presence of target ion and relative abundance. The most abundant ion, m/z 149, was chosen for the quantification of BBP, with no qualifier ions, due to the simplicity of the matrix. Ion *m/z* 185 was selected as the representative ion of the DBA internal standard. The dwell time was 100 ms.

Statistical analysis

Univariate analysis of variance – ANOVA was used to test any significant difference in growth parameters between treated and control groups of algae between all concentrations. The statistical analyses were performed using IBM SPSS Statistics. The results were considered significant at the level of $p < 0.05$.

Results and Discussion

Correlations between BBP concentration, exposed species, and chlorophyll production

It was noticed that lower concentrations of BBP (50 µg/L and 100 µg/L) had no significant effect on the growth rate of *Microcystis* sp. When exposed to higher concentrations (150 µg/L, 200 µg/L and 250 µg/L), it was observed considerable growth inhibition ($p < 0.05$). A concentration of 150 µg/L of BBP caused this species' most significant growth inhibition, starting from the beginning of the exposure until the end of the experiment. *A. variabilis* responded to the presence of BBP similarly to *Microcystis* sp. More precisely, BBP concentrations of 200 and 250 µg/L caused a significant inhibition ($p < 0.05$) of the growth of this species. The most significant growth inhibition was observed at a 250 µg/L BBP concentration. When all concentrations are considered,

it is observed that the higher the concentration of BBP, the smaller the increase in chlorophyll *a*, so the effect of BBP on *Microcystis* sp. and *A. variabilis* dose-dependently.

Green algae responded significantly differently to the presence of BBP compared to cyanobacteria. More precisely, none of the applied concentrations greatly affected the growth of *Chlorella* sp. On the other hand, on *Scenedesmus* sp., the BBP concentration of 150 μg/L caused a significant inhibition ($p < 0.05$) only on the seventh day of the experiment.

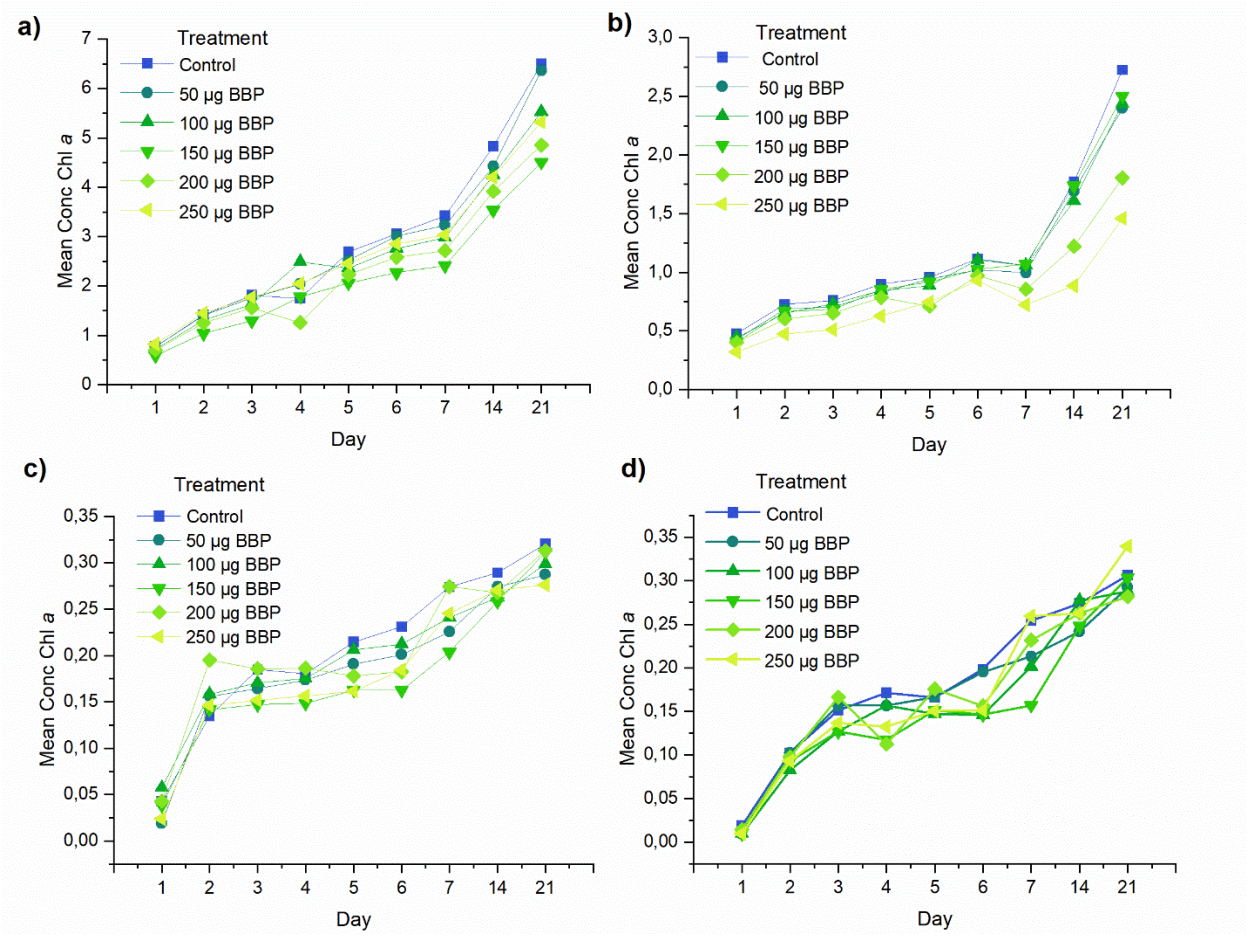


Figure 1. Spectrophotometric comparison of the growth of a) *Microcystis* sp., b) *Anabaena variabilis*, c) *Chlorella* sp., and d) *Scenedesmus* sp. in the presence of BBP, compared to the growth of the same strains in control conditions (without BBP).

There are a limited number of algae on which the effects of BBP have been tested. Gledhill et al. (1980) investigated the effect of BBP on aquatic organisms, and the research included five species of algae (*Selenastrum capricornatum*, *Navicula pelliculosa*, *Dunaiela tertiolecta*,

Sceletonema costatum, and Microcystis aeruginosae). The results showed a significant inhibitory effect on all tested species except for *M. aeruginosa*, which showed resistance. A document published by the World Health Organization (1999) showed that the most sensitive species is *Selenastrum*, with EC_{50} = 110 g/L. Given that the influence of BBP on phytoplankton is almost unexplored, this research represents the first report on its impact on all species we examined, except for *Microcystis* sp.

PAEs may have little to no effect on phytoplankton growth at low concentrations. However, as the concentration increases, the effect becomes more pronounced (Kuang et al., 2003). At some point, the concentration of PAEs may reach a level where it becomes toxic to the phytoplankton, inhibiting growth and potentially causing mortality. We noticed that this is the case with the cyanobacteria whose growth we monitored, where only higher concentrations of BBP caused a significant inhibition of growth. On the other hand, lower doses had an insufficiently substantial effect on the production of chlorophyll *a*. In general, the dose-dependent response of phytoplankton to PAEs is influenced by various factors, including the species of phytoplankton, the duration of exposure, and the environmental conditions in which the organisms live. We observed that the response of cyanobacteria to the presence of BBP was dose-dependent, especially in the case of *Microcystis* sp., considering that the higher the concentration of BBP, the lower the chlorophyll a production. Also, it can be noted that only in the second phase of the experiment, after the first week, was a noticeable difference in the production of chlorophyll a between controls and treatments observed for most concentrations. This indicates that prolonged exposure to BBP is necessary to observe a significant impact on growth.

Studies have shown that the sensitivity to PAEs can vary among phytoplankton species. Some species may be more sensitive than others, and certain species may even be able to tolerate high concentrations of PAEs. In this study, green algae showed greater tolerance to the presence of BBP in the growth medium in comparison to cyanobacteria. It can be assumed that the potential reason for their tolerance is their capability to detoxify or metabolize phthalates through physiological or biochemical mechanisms. Certain strains of green algae can even produce enzymes that efficiently decompose phthalates. Another possible reason is that some strains of green algae may have adaptations that allow them to manage the stress caused by exposure to

phthalates. While BBP did not significantly impact the growth of green algae, it did cause a slight inhibition of chlorophyll production at most concentrations. This suggests that the pollutant did affect green algae to some extent, although the inhibition was not considered significant. As previously stated, this study revealed that cyanobacteria exhibit greater sensitivity to BBP toxicity than green algae. Their metabolic and morphological properties differ, which is probably the main reason for the differences in response to BBP influence. The difference in green microalgae and cyanobacteria sensitivity to various pollutants has been discussed before. Several authors have reported that cyanobacteria are more sensitive, suggesting that their prokaryotic structure is probably the main reason (Mao et al., 2017; Perron et Juneau, 2011; Drábková et al., 2007). Unlike green algae, the photosynthetic apparatus is not enclosed in the chloroplast in cyanobacteria. Still, photosynthesis takes place in the cytoplasm, so toxic substances can more easily affect chlorophyll production because they do not have to penetrate through the double membrane (Mao et al., 2017).

Scientists researching the effects of PAEs on mammalian tissue have discovered a connection between induced oxidative stress and damage to various tissues (Chen et al., 2019; Chen et al., 2022; Wang et al., 2023). That raises the question of whether phthalates can also cause oxidative stress in phytoplankton species, which could lead to reduced production of biomass and chlorophyll *a*. The researchers have provided an answer and reported that PAEs, such as BBP, induce oxidative stress in phytoplankton species. In a study by Drábková et al. (2007), the impact of H2O2 on green algae and cyanobacteria was examined, with the conclusion that several factors contribute to the greater sensitivity of cyanobacteria to this compound. The main reason is a less developed enzymatic oxidative pathway than green algae. Differences in their photosynthetic apparatus and response to oxidative stress may explain the varying sensitivity levels to BBP between cyanobacteria and green algae.

BBP elimination from the growth medium - do phytoplankton biodegrade BBP?

After the experiment, the detection and quantification of BBP in extract samples did not show the presence of BBP, except in the extract of the sample with *Scenedesmus* sp., but the detected concentration was not significant. The results indicate that probably all organisms could absorb, metabolize, and eliminate BBP from the aquatic environment, of which the strain *Scenedesmus* sp. has the least ability.

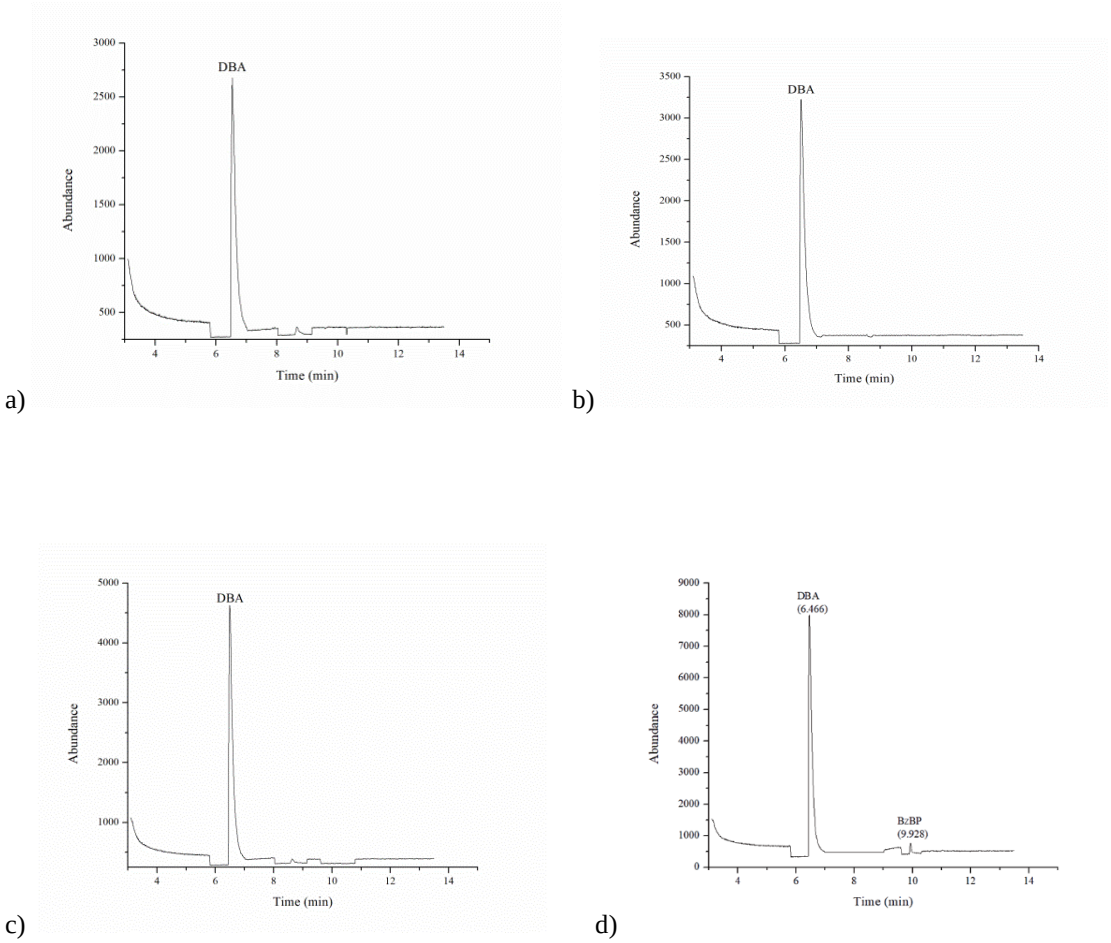


Figure 2. HPLC chromatogram of a) *Microcystis* sp., b) *A. variabilis*, c) *Chlorella* sp. and d) *Scenedesmus* sp. extracts

The action of phytoplankton is considered one of the most effective ways to detoxify the water environment, considering that phytoplankton species, including green microalgae and cyanobacteria, have developed mechanisms to eliminate organic pollutants. The mechanisms by which they act include the processes of biosorption, bioaccumulation, biomineralization, biotransformation and biodegradation (Touilibah et al., 2022). At the end of the experiment, BBP was not detected in the samples, which indicates that the tested algae eliminated it from the medium by one of these detoxification methods. Considering that long-term bioaccumulation of PAEs by algae has not been proven (Gobas et al., 2002), it is assumed that the tested phytoplankton species in the experimental conditions bioaccumulated PAEs in the short term and then

biodegraded them. Recently, scientists have focused on the biodegradation processes of PAEs by algae, and several studies have reported that phytoplankton species can biodegrade them (Babu & Wu, 2010b; Zang et al., 2016). Most isolates capable of biodegradation are anaerobes or facultative aerobes (Liang et al., 2008), which include bacteria, fungi, and green microalgae. Babu and Wu (2010b) investigated the ability of PAEs biodegradation by cyanobacteria (*Anabaena flos-aquae* G. S. West (strain 4054), *Microcystis aeruginosa* (Ku¨tz.) Ku¨tz. (strain 2396 and strain SM) and reported that the examined cyanobacteria can biodegrade PAEs using them as carbon sources for growth.

However, to our knowledge, previous studies have not reported that phytoplankton species can biodegrade BBP. It has been reported that BBP can be degraded by pure bacterial cultures, mixed bacterial cultures, and fungi (Chatterjee et al., 2003). This study provided insight that the phytoplankton species which are tested can eliminate BBP, which is of great importance because microalgae and cyanobacteria are known as promising candidates for the biodegradation of a variety of pollutants in comparison to bacteria and fungi, which require carbon input, energy, nutritional sources, and other supplements to remove pollutants (Touilibah et al., 2022).

The present study's findings revealed that the tested phytoplankton species can remove BBP from their growth medium. However, it is essential to consider the potential consequences of this process on the species. This study found that the production of chlorophyll *a* in all species was affected to some degree. This research begins a deeper investigation into the interaction between this endocrine disruptor and phytoplankton species. Our future research will focus on understanding the mechanisms behind the reduction in microalgae growth.

Conclusion

Due to the increasing use of BBP, they have become one of the world's most serious environmental challenges, which is why monitoring its impact on other organisms is of great importance. The effect of this toxic pollutant still needs to be investigated, especially on phytoplankton. This research provides new information on the interactions between BBP and four phytoplankton species (*Microcystis* sp., *A. variabilis*, *Chlorella* sp., *Scenedesmus* sp.). It reveals

the potential of BBP to affect the growth of phytoplankton and the significantly greater susceptibility of cyanobacteria strains to this pollutant. It can be assumed that the main reason is their prokaryotic structure and insufficiently built defence system, unlike green microalgae. In addition, this research showed that the tested species eliminated phthalates from the growth medium. In future studies, we will try to discover the mechanisms through which BBP acts and processes occur during the elimination of BBP from the medium. This work represents the initial research on interactions between BBP and phytoplankton.

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Conflict-of-Interest Statement

None.

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