Aquatic Toxicology 215 (2019) 105281

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Ecotoxicological and biochemical effects of environmental concentrations of the plastic-bond pollutant dibutyl phthalate on *Scenedesmus* sp.

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ARTICLE INFO

Keywords:
Scenedesmus sp.
Dibutyl phthalate
Ecotoxicity
Environmental endocrine disruptor
Plastic-bond pollutant

ABSTRACT

Phthalate esters are highly present in aquatic plastic litter, which can interfere with the biological processes in the wildlife. In this work, the commonly found freshwater microalga *Scenedesmus* sp. was exposed to environmental concentrations (0.02, 1 and 100 μg L^{-1}) and to a higher concentration (500 μg L^{-1}) of dibutyl phthalate (DBP), which is an environmental pollutant. The growth, pH variation, production of photosynthetic pigments, proteins and carbohydrates were evaluated. The main inhibition effect of DBP on the microalgal growth was observed in the first 48 h of the exposure (EC_{50}: 41.88 μg L^{-1}). A reduction in the photosynthetic pigment concentration was observed for the 0.02, 1 and 100 μg L^{-1} conditions indicating that the DBP downregulated the growth rate and affected the photosynthetic process. A significant increase in protein production was only observed under 500 μg L^{-1} DBP exposure. The extracellular carbohydrates production slightly decreased with the presence of DBP, with a stronger decrease occurring in the 500 μg L^{-1} condition. These results highlight the environmental risk evaluation and ecotoxicological effects of DBP on the production of biovaluable compounds by microalgae. The results also emphasize the importance of assessing the consequences of the environmental concentrations exposure as a result of the DBP dose-dependent correlation effects.

1. Introduction

The global exponential plastic production and consumption is becoming an increasingly relevant issue. In 2018, the annual plastic production was 400 million tons of which about 13 million leaks into open waters each year (UN environment, 2018). Moreover, future trends predict that plastic production may ascend up to 1800 million each year (Ryan, 2015). Furthermore, the plastic aquatic debris that floats in the water suffers from mechanical action and UV-radiation-induced-photooxidation, leading to a loss of mechanical integrity (GESAMP, 2015). This causes the release of even smaller sized polymer fragments known as microplastics (plastic particles smaller than 5 mm). Microplastics are found all over the globe in different water sources including our oceans (Waller et al., 2017; Peeken et al., 2018; Desforges et al., 2014), rivers (Hurley et al., 2018) and have been shockingly found in up to 90% of bottled drinking water (Mason et al., 2018). Additionally, microplastics have been reported to adsorb different types of persistent organic pollutants (POPs), which include, among others, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Besseling et al., 2013; Endo et al., 2015; Frias et al., 2010; Hirai et al., 2011; Mato et al., 2001; Teuten et al., 2007). Furthermore, microplastics contain in their composition phthalate esters (PAEs), which include dibutyl phthalate (DBP) (Fries et al., 2013), an environmental contaminant that has become one of the primary PAEs present in the aquatic environment (Gu et al., 2017). They, or their degradation products (Jonsson and Baun, 2003), are transported through the aquatic environments (Bakir et al., 2014), affecting wildlife (Huang et al., 1999; Ohtani et al., 2000), more specifically phytoplankton. DBP is an aliphatic ester additive that is used as a plasticizer to improve its flexibility and durability, with PAE's accounting for 10–70% (w/w) of all plastics (Nerland et al., 2014). Therefore, PAEs are easily diffused into the surroundings due to their weak Van der Waals bonds to plastic, which facilitates their perturbation and consequent release from their substrate (Gu et al., 2017). Moreover, DBP is also classified as a xenoestrogen and has the potential to bind to estrogen receptor sites (Müller et al., 2000). This could likely interfere with various biological processes in wildlife (Ohtani et al., 2000) which even at trace concentrations, could have potentially hazardous effects (Gao

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https://doi.org/10.1016/j.aquatox.2019.105281
Received 10 March 2019; Received in revised form 15 August 2019; Accepted 18 August 2019
Available online 19 August 2019
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and Wen, 2015a). DBP is consistently found in freshwaters, with the lowest detected concentration being 0.002 μg L^{-1} (Fatoki et al., 2010) and the highest 122 μg L^{-1} (Gao and Wen, 2015a), depending on the location. As it follows, the studies published about the DBP ecotoxicity use very high concentrations of this pollutant, outside the range or near the maximum concentration found in the environment. This may lead to biased conclusions on the true effect of this pollutant on microalgae and therefore needs to be clarified. Thus, the exposure to DBP has shown inconsistent results in ecotoxicological tests, evidencing growth inhibition in the 50–100 mg L^{-1} concentration range, while stimulating growth in the 5–20 mg L^{-1} concentration range in *Chlorella vulgaris* (Duan et al., 2018). Furthermore, it has also shown growth stimulation before the inhibition in the 1–8 mg L^{-1} concentration interval in *Microcystis aeruginosa* (Chunxiao et al., 2015). The studies found involving *Scenedesmus* species show that DBP displays toxicity towards *Scenedesmus obliquus* (96 h - EC_{50}: 0.21 μg L^{-1}) and a growth inhibition up to 80% was verified at 20 mg L^{-1} concentration (Gu et al., 2017). Huang et al. (1999) showed that the DBP was degraded by this microalga. However, Babu and Wu, 2010 report that some freshwater algae and cyanobacteria can synthesize PAEs, including DBP, and release them into the extracellular medium under stress conditions.

Taking into account the known effects on the microalgal growth, it is important to understand how the biochemistry of the microalgae is affected by the presence of DBP, as a result of the industrial and commercial importance of its biovaluable compounds. Every microalga produces different ratios of pigments and valuable molecules, including proteins and carbohydrates, due to different metabolism rates. *Scenedesmus* sp. is a freshwater chlorophycean alga (Cambiaire et al., 2006) that is widely used in ecotoxicological studies. It possesses the ability to quickly adapt and manipulate its metabolism to adjust to the presence of potentially hazardous substances (Mandal and Mallick, 2009).

This investigation seeks to determine the toxic effects of DBP on a realistic concentration range, found in the environment, assessing the risk that this pollutant has on the microalgae development. Thus, in this study, the influence of natural occurring concentrations of DBP (0.02, 1 and 100 μg L^{-1}) and a higher laboratory concentration (500 μg L^{-1}) on *Scenedesmus* sp. biochemical activity was evaluated. The growth, as well as pH variation, photosynthetic pigments, extracellular proteins and carbohydrates production were assessed. Simple and reliable analytical tests were applied to screen, in a convenient and reliable way, the more intrinsic changes that exposure to environmental concentrations of hazardous pollutants induce. DBP stability was assessed by ultra-high-performance liquid chromatography (UHPLC).

2. Materials and methods

2.1. Microalgae selection and culture conditions

In the present study, the freshwater microalga *Scenedesmus* sp. (Class *Chlorophyceae*; 5–13 μm) was selected. *Scenedesmus* sp. (BEA0579B) was obtained from the Spanish Bank of Algae (BEA) of the University of Las Palmas de Gran Canaria (Spain) (Cunha et al., 2019) and was grown in Waris-H medium (0.1 g L^{-1} KNO_3; 0.02 g L^{-1} $MgSO_4.7H_2O$; 0.02 g L^{-1} $(NH_4)_2HPO_4$; 0.1 g L^{-1} $Ca(NO_3)_2.4H_2O$; 0.24 g L^{-1} HEPES; PII Metals (0.003 g L^{-1} Titriplex III; 0.001 g L^{-1} H_3BO_3; 0.00014 g L^{-1} $MnCl_2.4H_2O$; 0.00021 g L^{-1} $ZnSO_4.7H_2O$; 0.000004 g L^{-1}); Fe-EDTA (0.0052 g L^{-1} Tritiplex II; 0.0049 g L^{-1} $FeSO_4.7H_2O$; 0.054 mL L^{-1} 1 N KOH; Vitamins (0.0002 mg L^{-1} Vitamin B12; 0.001 mg L^{-1} Biotin; 0.1 mg L^{-1} Thiamine – HCl; 0.0001 mg L^{-1} Niacinamide); 10 mL L^{-1} Soil Extract). The initial selected cell abundance was 1.6×10^5 cells mL^{-1}, following the Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals in freshwater microalgae (OECD, 2011). These conditions ensure that the culture can grow exponentially throughout the incubation period. The growth was monitored every 24 h, for 96 h, using a *Neubauer improved* chamber with an optical microscope (×400) (Olympus BX41 Microscope) and the microalgal cell abundance was used to determine the potential effects of exposure to DBP. The experimental cultures were maintained at 25 ± 1 °C, under the irradiance of 23.5 μmol photons m^{-2} s^{-1} (*HOBO® Pendant® MX Temp MX2201*) provided by a cool white *Osram L 18 W 840 Lumilux* lamp, with a 14/10 h (light/dark) photoperiod (*Aralab CP500* growth chamber).

2.2. Microalgae exposure conditions to DBP and growth inhibition test

The microalgae were subjected to four different concentrations of DBP (99%; *Riedel-de Haën*): 0.02, 1 and 100 μg L^{-1} (named environmental concentrations) and 500 μg L^{-1} (named laboratory concentration). Following the OECD guidelines (OECD, 2011), the DBP was dissolved in methanol (99.8%; *Sigma-Aldrich®*), with the maximum amount of the solvent set to 0.05% in the test culture, for all conditions and replicates. Control groups with and without methanol were prepared. The control groups were used to evaluate whether they would have an influence on the microalgal growth. The cultures were acclimatized in the experimental conditions for 96 h before inoculation. For the experiments (made in triplicate), fresh medium was added in order to achieve an initial cell abundance of 1.6×10^5 cells mL^{-1}. All flasks were hand-shaken twice per day during the experimental period.

According to the OECD (OECD, 2011), the EC_{50} was calculated using a linear regression of the inhibition rate and expressed in terms of the DBP concentration (μg L^{-1}). Also, in short and according to the OECD guidelines, the LOEC was determined as "the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on growth (at $p < 0.05$) when compared with the control, within a given exposure time" (OECD, 2011).

2.3. DBP stability analysis

The DBP stability in the culture medium, without microalga, was analysed at 0, 48 and 96 h using ultra high-performance liquid chromatography (UHPLC). The analysis was performed in a *Shimadzu Nexera X2* system equipped with a *LC*-30CE pump, a *SIL-30AC* autosampler, a *DGU-20A(SR)* degasser, a *CTO-20AC* column oven, coupled to a *SPD-M20A* photodiode array detector. A *Shimadzu Shim-pack GIST C18* column (2.1 x 100 mm, 2 μm) was used at a constant oven temperature of 25 °C with an isocratic flow of methanol/water (88:12, v/v) at a flow rate of 0.3 mL min^{-1}. The injection volume for each sample was 25 μL and the DBP was detected at a wavelength of 230 nm. Due to detection limits, the only concentration of DBP used was 500 μg L^{-1}.

2.4. Photosynthetic pigments determination

The photosynthetic pigment production was determined based on the Lichtenthaler (1987) method, with some modifications. Briefly, 5 mL culture was centrifuged at 13,400 rpm for 2 min, the supernatant discarded, and the pellet resuspended in 5 mL of 90% methanol. The sample was then sonicated for 5 min at room temperature, incubated in the dark at 4 °C for 24 h and centrifuged (13,400 rpm, 2 min). Chlorophyll *a* (chl-a), chlorophyll b (chl-b) and total carotenoids (car-t) were determined at 470 (A_{470}), 652 (A_{652}) and 665 nm (A_{665}), and corrected by subtracting the absorbance at 750 nm (turbidity), using the following equations:

chl-a (μg mL^{-1}) = 16.82 A_{665} − 9.28 A_{652}

chl-b (μg mL^{-1}) = 36.92 A_{652} – 16.54 A_{665}

car-t (μg mL^{-}

2.5. Extracellular protein determination

In order to determine the proteins in the supernatant (extracellular proteins) a modified Lowry et al. (1951) method was implemented.

Cultures were centrifuged at 5000 rpm for 20 min, at 25 °C to remove microalgae and other debris. The centrifugation time and speed were adjusted to the cell density of the sample to assure a cell-free supernatant. Reagent A (2% Na_2CO_3 in 0.1 N NaOH) was mixed with reagent B (1% $C_6H_5Na_3O_7$ in 0.5% $CuSO_4$) in a 50:1 ratio (reagent C) and the Folin-Ciocalteu reagent was diluted in a 1:1 ratio. In a test tube, 2.5 mL of reagent C (15 min) and 250 µL of Folin-Ciocalteu were added to 500 µL of sample, vortexed and left for 35 min to react. Absorbance was measured at 750 nm and a standard calibration curve was made using bovine serum albumin (BSA). A stock solution of 5 mg mL^{-1} was freshly prepared, which was diluted to 10, 20, 50, 100, 200, 500, 1000 and 2000 µg mL^{-1} ($R^2 = 0.9301$).

2.6. Extracellular carbohydrates determination

The extracellular carbohydrates were determined using the phenol-sulfuric acid method, according to DuBois, et al. (1956), where 1 mL of 5% phenol aqueous solution was added to the supernatant (2 mL) of the centrifuged culture (5000 rpm, 20 min). This was promptly followed by 5 mL of concentrated sulfuric acid that was left for 10 min to react and vortex. It was then left for another 20 min in a room temperature water bath for colour development to take place. The absorbance was measured at 490 nm in an *UV-6300 PC Double Beam Spectrophotometer*, in 1.5 mL semi-micro PS disposable cuvettes (*Plastibrand®*). A calibration curve was made with D-(+)-Glucose (99.5%, *Sigma Aldrich®*), using a freshly prepared stock solution (500 mg L^{-1}) diluted to 5, 10, 15, 20, 25 and 50 mg L^{-1} ($R^2 = 0.9575$).

2.7. Statistics analysis

Statistical analysis of microalgal growth rate, pH variation, photosynthetic pigments, extracellular proteins and carbohydrates production were performed using IBM SPSS statistics software (V.25). The differences in the growth rates and the biochemical activity between different experimental conditions were assessed by a one-way analyses of variance (ANOVA), with a level of statistical significance of p-value < 0.05.

3. Results

3.1. DBP stability and effect on the microalga growth

In order to study the DBP's stability, its algal-free medium concentration was evaluated using UHPLC (Fig. 1). Measurements were performed at 0, 48 and 96 h. The results show a considerable decrease of 32.4% (to 338 µg L^{-1}) in DBP concentration in the first 48 h and a further negligible decline of 0.1% (to 337.5 µg L^{-1}) in the last 48 h of the experiment. Therefore, the loss of DBP was only significant in the first 48 h of the experiment.

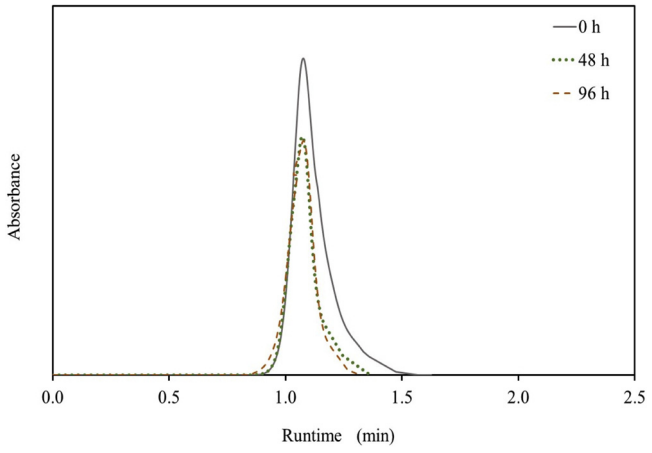

Fig. 1. UHPLC analysis of DBP stability in the culture medium, without microalga, at 500 µg L^{-1}.

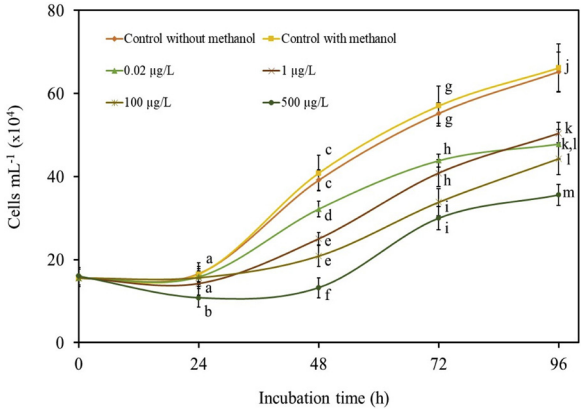

Fig. 2. Growth curves for all the experimental groups. *Scenedesmus* sp. initial cell abundance was 1.6×10^5 cells mL^{-1}. Distinct letters represent the means significantly different ($p < 0.05$).

Scenedesmus sp. cell abundance was determined in order to assess the DBP ecotoxicity on its growth. To evaluate if methanol would have an impact on the microalgal growth, controls with and without methanol were compared. The results shown in Fig. 2 exhibit that there are no significant differences ($p < 0.05$) in the control conditions, evidencing that the presence of 0.05% (v/v) methanol did not influence *Scenedesmus* sp. growth. Therefore, every test was performed using the control with methanol.

The cell density assessment (Fig. 2) displays the decrease in cell abundance, in every DBP exposed condition, in relation to the control at 24, 48, 72 and 96 h which indicates that DBP displayed a growth inhibition effect.

Unlike the 0.02, 1 and 100 µg L^{-1} conditions, the 500 µg L^{-1} condition exhibited an already significant growth inhibition at 24 h. The inhibition was evident for all concentrations at 48 h with an EC_{50} of 41.88 µg L^{-1}. Soon after the cell abundance started to steadily increase until the last 48 h of the experiment. Also, the DBP exposure showed a LOEC (lowest observed effect concentration) of 1 µg L^{-1}.

Respecting the pH variation, Fig. 3 shows that the results follow the OECD guidelines for the experiments of chemicals in freshwater microalgae (OECD, 2011), with the pH not increasing to more than 1.5 units during the test. The control experiment exhibited the biggest pH drift, with a decrease of 1.07 pH units at 48 h. In all the DBP exposed conditions, the pH dropped in the first 24 h, followed by a stable increase in pH after 24 h, until the end of the experiment. The 500 µg L^{-1}

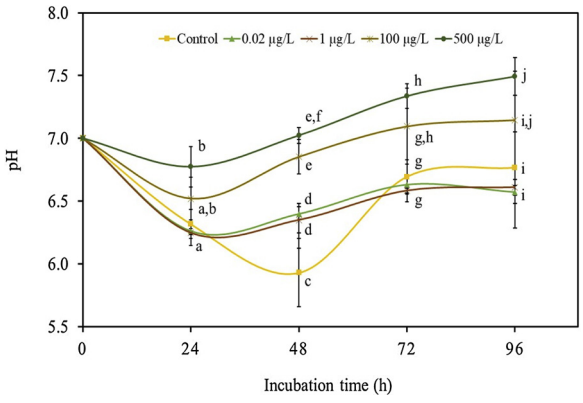

Fig. 3. pH variation during the 96 h experiment for the control and each of the DBP exposed conditions. Distinct letters represent means significantly different ($p < 0.05$).

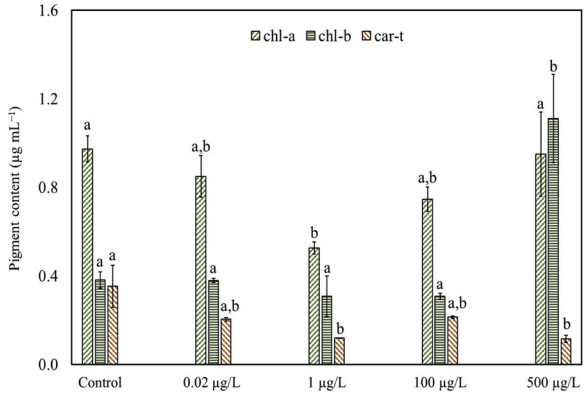

Fig. 4. Effect of DBP on the photosynthetic pigments: chlorophyll-a (chl-a), chlorophyll-b (chl-b) and total carotenoids (car-t), after 96 h of experimental exposition conditions. Distinct letters represent means significantly different of the same pigment between distinct conditions ($p < 0.05$).

condition displayed the steepest pH increase, with an increase of 0.5 pH units.

3.2. Photosynthetic pigment production

The production of chlorophyll-a, chlorophyll-b and carotenoids were assessed with the results being shown in Fig. 4. After 96 h, a consistent decrease was observed in all the photosynthetic pigments studied in the 0.02, 1 and 100 µg L^{-1} conditions. For the 500 µg L^{-1} concentration an inversion in this behaviour was observed, exhibiting an increase in the production of these pigments, except for the total carotenoids.

3.3. Extracellular protein production

The production of extracellular proteins in the DBP exposed conditions was determined after 96 h of being in the experimental exposure conditions. The results displayed in Fig. 5 show a slight, but not significant ($p < 0.05$) increase, between the 0.02, 1 and 100 µg L^{-1} conditions and the control. However, under the 500 µg L^{-1} DBP exposure, a significant ($p < 0.05$) increase in protein production was observed.

3.4. Extracellular carbohydrates production

The extracellular carbohydrates production was also evaluated at the end of the 96 h experiment. The results shown in Fig. 6 indicate a decreasing trend in the production of extracellular carbohydrates in the

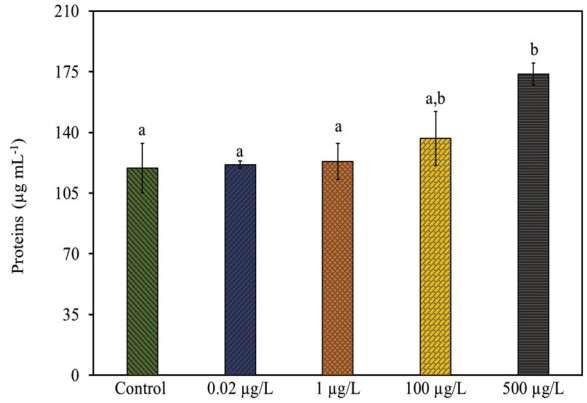

Fig. 5. Effect of DBP in the production of extracellular proteins, after 96 h of experimental exposition conditions. Distinct letters represent means significantly different ($p < 0.05$).

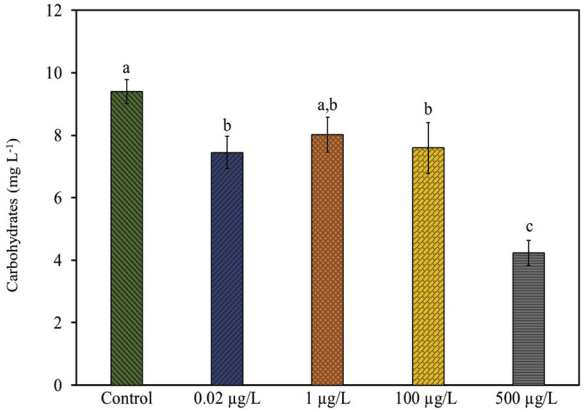

Fig. 6. Effect of DBP on the production of extracellular carbohydrates, after 96 h of experimental exposition conditions. Distinct letters represent means significantly different ($p < 0.05$).

presence of DBP, when compared to the control. The 0.02 and 100 µg L^{-1} conditions displayed a minor but significant ($p < 0.05$) decrease of extracellular carbohydrates production, when compared to the control condition but no significant differences were found between them. The 500 µg L^{-1} condition exhibited a sharper decrease ($p < 0.05$) in relation to the control and also to the environmental concentrations (0.02, 1 and 100 µg L^{-1}).

4. Discussion

This study aims to evaluate the interactions between phthalate esters, namely DBP, which are aquatic pollution components that are highly present in microplastics, and primary microorganism producers – microalgae - that constitute the base of the aquatic trophic system. Therefore, the growth and the biochemical parameters: pH, photosynthetic pigments, proteins and carbohydrates production were studied in the freshwater microalga *Scenedesmus* sp., induced by the presence of DBP, in different environmental - 0.02, 1 and 100 µg L^{-1} - and laboratory - 500 µg L^{-1} - concentrations.

4.1. DBP stability and effect on the microalga growth

The DBP stability study made by UHPLC analysis showed that the DBP concentration only significantly varied during the first 48 h of the experiment. The ability of microalgae, including *Scenedesmus* species, to biodegrade phthalate esters, namely DBP among other phthalates, have been shown by Huang et al. (1999); Gao and Wen (2015b) and Zhang et al. (2016). Thus, since the stability was assessed in an algal-free culture medium, this fluctuation was likely due to the losses from the evaporation and sorption to the glass walls of the experimental system (Wezel et al., 2000).

The growth results (Fig. 12) of the control with and without methanol show that the presence of the organic solvent by itself did not affect the microalgal growth. Thus, the results regarding the growth when exposed to the different concentrations of DBP are only attributed to the presence of the pollutant.

The growth inhibition results show that, under our experimental conditions (0.02, 1, 100 and 500 µg L^{-1}), *Scenedesmus* sp. is susceptible to the effects of DBP in environmental concentrations, in a dose-dependent manner. Results shown by Kuang et al. (2003) display a cell density decrease from 26 to 51%, in concentrations ranging from 10 to 60 mg L^{-1}, in a dose-dependent manner. These results contradict the results shown by Wang et al. (2011), that found growth inhibition but no dose-response upon relatively low (2–2000 µg L^{-1}) *Scenedesmus obliquus* DBP concentrations exposure. Still, higher concentrations (5–80 mg L^{-1}) of a similar phthalate (di-(2-ethylhexyl) phthalate) has

been tested on *Scenedesmus obliquus*, and the same dose-dependent growth response found in our results has been established (Wang et al., 2010). Therefore, literature results on the dose-response establishment are somewhat contradictory, which may imply distinct defence mechanisms and miscellaneous biological responses at different concentration ranges. Wezel et al. (2000) reports NOEC (no observed effect concentration) and L(E)C_{50} (read EC_{50}) values of 6.1 and 4.2 mg L^{-1} for DBP exposed *Scenedesmus subspicatus*. Other literature results show 96h-EC_{50} values of 30.2 mg L^{-1} for *Scenedesmus obliquus* (Kuang et al., 2003; Wang et al., 2011), as well as 48 h-EC_{50} and 72h-EC_{50} values of 9.0 and 2.0 mg L^{-1}, respectively, for *Scenedesmus subspicatus* upon DBP exposure (Wezel et al., 1999). The results of this study: a 48 h-EC_{50} of 41.88 µg L^{-1} and a LOEC of 1 µg L^{-1} show that these values are lower than those found in the literature, highlighting the hazardous nature of the DBP exposure to microalgae, specifically *Scenedesmus* sp., in environmental concentrations.

The exhibited toxic effects are not exclusive to microalgae, as corroborated from the observations made by Ohtani et al. (2000) that DBP behaves as an endocrine disruptor in other species such as the frog *Rana rugosa*. The decrease in the growth in the first 24 h is likely due to the adaptation and higher surface accumulation of DBP by the microalga with no major biomass development (Chi et al., 2006). This effect is enhanced at higher concentrations (500 µg L^{-1}), causing a greater growth inhibition in the first 48 h. After this period the microalga seems to adapt gradually to the presence of DBP, consequently entering an exponential growth phase. The pH measurements corroborate the observations made in the growth curves, decreasing in the first 24 h (in the 0.02, 1 and 100 µg L^{-1} conditions) and until 48 h (500 µg L^{-1}) and then gradually increasing. This behaviour may be attributed to the assimilation of carbon and nitrogen sources in the medium (Zhang et al., 2016), during cell growth. Also, the pH increase is directly related to the CO_2 decrease (Bhattacharya et al., 2010), consequence of higher photosynthetic rates.

4.2. DBP influence on photosynthetic pigment production

The results observed in the photosynthetic pigment content assay showed that the environmental concentrations (0.02, 1 and 100 µg L^{-1}) of DBP might have a more negative influence on the pigment content than higher (500 µg L^{-1}) laboratory concentrations. Kuang et al. (2003) reported a chlorophyll-a (chl-a) decrease from 24.4 to 60.7% in the presence of high (10–60 mg L^{-1}) concentrations of DBP, corroborating the observations made here. Therefore, the effects of DBP on the production of chl-a are presumed to occur both in environmental relevant (especially in the 1 µg L^{-1} concentration) and higher laboratory concentrations. Also, Wang et al. (2010) reported for *Scenedesmus obliquus,* that in the presence of high di-(2-ethylhexyl) phthalate concentrations the cell growth did not correlate with chlorophyll and carotenoids biosynthesis, although in unrealistic environmental concentrations (5–80 mg L^{-1}). As discussed in 4.1., it is expected that a pH increase is observed when the photosynthetic rates go up. The fact that our results show increasing pH values while the photosynthetic pigment production decreased, makes it uncertain to guarantee the occurrence of oxidative stress. The pH increased until the end of the experiment, with the growth and inhibition likely taking place due to DBP cell-surface accumulation.

Although the production of chl-b (chlorophyll *b*) only suffered significant changes ($p < 0.05$) in the 500 µg L^{-1} DBP exposure, the production of chl-a (chlorophyll *a*) and car-t (total carotenoids) exhibited significant changes ($p < 0.05$) amongst conditions. It is common that microalgae produce reactive oxygen species (ROS) derived from oxidative stress. This damages the chloroplasts which negatively affects the growth and photosynthetic production (Wang et al., 2010) and consequently decrease the pH. Reports from Bhattacharya et al. (2010) show that the production of ROS increases with exposure time, with the microalgae displaying distinct response stages depending on the exposure period to microplastics that might contain phthalates that are likely slowly released into the medium. Other literature results regarding the DBP exposure in distinct microalgae are inconsistent. It has been shown that DBP stimulates the activity of antioxidant enzyme systems of the dinoflagellate *Karenia brevis* : superoxide dismutase (SOD) and catalase (CAT) (Liu et al., 2016; Li et al., 2015). Also, Chunxiao et al. (2015) report, for *Microcystis aeruginosa*, an increase in SOD and CAT activity in the 1–4 mg L^{-1} DBP concentration range, but inhibition takes place for 8 mg L^{-1}. Further research correlating the production of ROS, the activity of SOD and CAT, as well as the pH variation and chlorophyll production would be required to further understand the mechanisms behind phthalates action on microalgae.

4.3. DBP influence on extracellular protein production

Regarding the extracellular protein production, it was shown that it might be upregulated by the presence of the phthalate. To the best of our knowledge, this is the first study exploring the effects of DBP and any type of phthalate on the extracellular protein production of microalgae. DBP can act as a xenoestrogen, that can potentially bind to oestrogen receptor sites (Müller et al., 2000). It is hypothesized that DBP is able to bind to the estradiol receptors. This could trigger the binding of a ligand-receptor complex to the DNA, and consequently induce transcription and protein production (Wezel et al., 2000). Despite a DBP dose-correlated protein production increase, only the 500 µg L^{-1} condition observed a significant increase, when comparing with the control and the environmental concentrations (0.02, 1 and 100 µg L^{-1}). This indicates that higher DBP concentrations stimulate protein production. Some authors found similar effects on different and more complex aquatic organisms, with Zhou et al. (2015) showing similar effects on the general upregulation in the extracellular protein production in the presence of factual DBP concentrations (2–50 µg L^{-1}) on abalone (*Haliotis diversicolor supertexta*). Also, a study on duckweeds *Spirodela polyrhiza* and *Lemna minor* biochemical response to DBP shows a decrease in the extracellular protein in the 0–500 µg L^{-1} range, with a major decline in higher concentrations (1–7.5 mg L^{-1}) (Huang et al., 2006). Therefore, with the current available data, it is possible to assume that the DBP influence on extracellular protein production is organism- and dose-dependent, with environmental concentrations acting as an up-regulator in the protein production for *Scenedesmus* sp..

4.4. DBP influence on extracellular carbohydrates production

Similarly, to the extracellular protein production evaluation, this is also, to the best of our knowledge, the first study assessing the effects of DBP and any type of phthalate on the production of extracellular carbohydrates by microalgae. The results regarding the extracellular carbohydrates production exhibit a dose-dependent correlation for its downregulation. *Scenedesmus* sp. displayed a high sensibility in the presence of DBP regarding the extracellular carbohydrates production, even at the lowest concentration (0.02 µg L^{-1}). Zhou et al. (2015) showed that this effect is not exclusive to microalgae but also more complex aquatic organisms, with a decrease of glucose in the abalone *Haliotis diversicolor supertexta*, in the presence of factual DBP concentrations (2–50 µg L^{-1}). Lee (2000) showed that DBP acts as a slow-binding, non-competitive but reversible inhibitor of the bacterial *Streptomyces melanosporofaciens* α-glucosidases, also inhibiting β-glucosidases, as well as α- and β-mannosidases. Therefore, DBP has the potential to interfere with the overall balance of carbohydrates production, affecting the processing of glycoproteins, glycolipids and the digestion of carbohydrates. This could consequently disturb the glucose expression and its related transcriptional elements and transporters (Kawamoto et al., 2005).

4.5. Call for research

Regarding the exponential aquatic plastic pollution and the increasing global concern, it is expected that the levels of plastic-bound pollutants released into the environment increase. Aside from being global players in oxygen production, microalgae also constitute the base of the aquatic food chain. However, these unicellular photosynthetic organisms are sensitive to environmental risks. These microorganisms produce biovaluable molecules such as pigments, proteins and carbohydrates, among others, that are essential in the equilibrium of its ecosystems. Our study shows that the production of these molecules is unregulated by the presence of phthalates, namely DBP. Therefore, the aquatic ecosystem equilibrium is put at risk when the natural balance is disrupted.

Still, the data regarding the risk assessment of phthalates to microalgae is very scarce, and the end points not wide and complex enough to better understand the real consequences of the exponentially increasing phthalates exposure derived from plastic pollution. Thus, the authors call for more in-depth research on the susceptibility and the inhibitory mechanisms of microalgae when exposed to environmentally occurring concentrations of toxic plastic-bonded pollutants, primarily phthalates.

5. Conclusions

The aim of this study was to evaluate the effects of natural environmentally occurring (0.02, 1 and 100 $\mu g\,L^{-1}$) and higher laboratory (500 $\mu g\,L^{-1}$) concentrations of DBP on the biochemical behaviour of *Scenedesmus* sp. This would include its growth, pH variation, photosynthetic pigments, extracellular proteins and carbohydrates production. The obtained results showed that DBP exposure had a steeper effect on the microalgal growth in the first 48 h. By gathering the data from the pH variation and photosynthetic pigments production, it was not possible to assure the occurrence of oxidative stress. The only increase in the photosynthetic pigments production, namely chlorophyll-a and chlorophyll-b, was recorded in the 500 $\mu g\,L^{-1}$ condition. Therefore, the environmental concentrations of phthalates might have a higher negative influence on the pigment content than the higher DBP concentrations. For the lower concentrations tested, the extracellular protein production observed a consistent increase. Furthermore, a significant increase in the extracellular protein production was observed in the 500 $\mu g\,L^{-1}$ DBP exposure condition. In the concentrations tested, particularly in the higher concentration, the extracellular carbohydrates production was significantly downregulated in the presence of DBP. This suggests that when DBP acts as an inhibitor of glycosidases, it negatively influences the production of extracellular carbohydrates.

The results of this work call for more in-depth research on the concentration-dependent susceptibility and mechanisms of microalgae when exposed to environmentally occurring concentrations of toxic plastic-bonded pollutants.

Acknowledgements

This work was partially supported by the project REBECA (MAC/1.1a/060) European Territorial Cooperation Programme PCT-MAC 2014-2020 through project REBECA (MAC/1.1a/060) and the Oceanic Observatory of Madeira Project (M1420-01-0145-FEDER-000001). The authors acknowledge the Spanish Bank of Algae (BEA) for making the microalga available.

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