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Comparative assessment of phytotoxic responses induced by the exposure of benzyl butyl phthalate and di-n-butyl phthalate to giant duckweed (*Spirodela polyrhiza* L. Schleiden)

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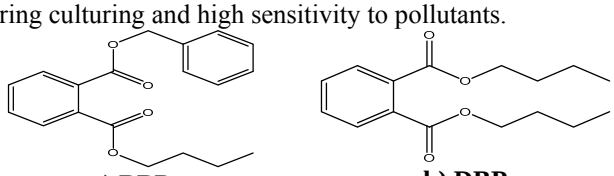
Abstract:
The present work was conducted to investigate the phytotoxic consequences induced by the exposure of benzyl butyl phthalate (BBP) and di-n-butyl phthalate (DBP) in *Spirodela polyrhiza* (L.) Schleiden. The experimental plant was treated with different concentrations (0, 5, 10, 15 and 20 mM) of BBP and DBP for 7 and 15 days under *in-vitro* conditions. The results were statistically analyzed using two-way ANOVA and the significance of data was accessed at $p \leq 0.05$ using Tukey's test. The results showed that both phthalates adversely affected the growth of *Spirodela polyrhiza* by decreasing the photosynthetic pigments, protein content and carbohydrate content, while increasing the Malon-dialdehyde (MDA) content.
Keywords: Benzyl butyl phthalate; Di-n-butyl phthalate; Phytotoxicity; *Spirodela polyrhiza* (L.) Schleiden.

INTRODUCTION:

Dialkyl or alkyl/aryl esters of 1, 2 benzenedicarboxylic acid are called as phthalates. Phthalates are synthetic chemical compounds and were introduced in 1920s and till day their use is extensive [1]. Phthalates are primarily used as plasticizers for different polymers to impart transparency, elasticity, durability and overall product sustainability benefits of high molecular weight polymers [2, 3]. Moreover, these are also used in many substances such as automotive components, toys, cosmetic formulations, medical treatment tubings, plastic packing films, perfumes, personal hygiene products [4], herbicides, dyes, insect repellents and as an ingredient in aspirin coating [5]. Such extensive consumption of phthalates made them ubiquitous in various environmental media. The main contributing characteristic for ubiquitous existence of phthalates is the non-covalent bonding with polymer which enables them to leach out into the surrounding environment [6].

In the last decades, phthalates have been categorized as emerging environmental pollutants. There are 14 types of phthalates which are used for commercial and industrial purpose and out of these 6 have been considered as priority pollutants by United State Environmental Protection Agency (USEPA). Both BBP and DBP are also included in this list. Phthalates have various adverse effects on plant and animal systems. In case of animals, these are known for endocrine disruption, reproductive toxicity, carcinogenicity and teratogenecity etc. [7]. Considering the toxicity of phthalates these are considered as top priority environmental pollutants [8]. But the reports on the effect of phthalates on plants are scare in literature. The growth of a plant depends on its physiological activities. Therefore, the main emphasis of the present work is to envisage the physiological perturbations of BBP and DBP (Structure in Figure 1) exposure on *Spirodela polyrhiza* which is commonly known as giant duckweed. The giant duckweed is a floating, fragile and fresh water monocot belongs to family Lamnaceae. The members of Lamnaceae family are considered good alternatives for the removal of heavy metals [10] and also for other organic compounds from water bodies. The present plant material was selected due to its small size, fast growing properties, convenience during culturing and high sensitivity to pollutants.



a) BBP **b) DBP**

Figure 1 (a-b) Chemical structures of BBP and DBP.

MATERIAL AND METHODS:

Chemicals, plant material and culturing

Benzyl butyl phthalate (BBP) CAS: 85-68-7, 98% and Di-n- butyl phthalate (DBP) CAS: 84-74-2, 99% were procured from Hi-Media Pvt. Ltd. Mumbai (India). All other chemicals used were of analytical grade. Plant material (*Spirodela polyrhiza*) was collected from the sewage treatment plant of Guru Nanak Dev University, Amritsar, Punjab (India). The plant material was washed 3-4 times with distilled water and soaked in the folds of filter papers. The required volume of each phthalates was dissolved in 1 mL of ethanol, 2-3 drops of tween-20 was added and then final volume raised using Hoagland's medium (3%). The healthy fronds of similar size (2 g) was transferred to Petri plates containing 30 mL of different concentrations *viz*. 0, 5, 10, 15, 20 mM of BBP and DBP. The cultured material was kept in the growth chamber which was illuminated by cool fluorescent light at 25±1°C for a photoperiod of 16 hours light and 8 hours dark for 7 and 15 days. The treated plants were carefully removed from the Petri plate containing medium and carefully rinsed with distilled water, dried in paper towel and used for further analysis. The whole plant homogenate was used for the estimation of various biochemical indices.

Methods for the assessment of phytotoxic responses

The photosynthetic pigments were determined by the method proposed by Arnon (1949) [10]. The carotenoid content was determined using the equation given by Lichtenthaler and Wellburn (1985) [11]. The xanthophyll content was determined using Lawrence method (1990)

[12]. The plant material was homogenized in hexane, acetone, absolute alcohol, toluene (10:7:6:7) in pestle and mortar and 40% methanolic KOH was added to the extract and then heated at 56°C for 15 min. The obtained reaction mixture was incubated for 1 h in dark. The hexane was added and the final volume was made upto 100 ml by using 10% Na_2SO_4. Again it was incubated for 1 h in dark. The upper phase was collected. The calculation was made by reading the absorbance at 474 nm. The carbohydrate content in the plant material was determined using Anthrone reagent method. The plant material was hydrolyzed with 2.5 N hydrochloric acid (HCl) in boiling water bath for 3 hours. After cooling to room temperature the sample solution was neutralized with sodium carbonate (Na_2CO_3) until the effervescence ceases. The final volume was made upto 100 ml with distilled water and centrifuged. To the supernatant, anthrone reagent was added and boiled on water bath for 8 min. After cooling, the absorbance was observed at 630 nm. Amount of total carbohydrate was calculated using glucose as standard. The protein content was estimated using Bradford method (1976) [13]. The plant material was homogenized using pestle and mortar in phosphate buffer (pH 7). The homogenate was centrifuged at 12000 rpm for 20 min at 4°C temperature. To the supernatant, Bradford reagent was added and absorbance of the solution was recorded at 595 nm. Amount of total protein present in the sample was calculated by the equation obtained by using bovine serum albumin (BSA) as standard. The cell damage is calculated in terms of Malon-dialdehyde (MDA) content which was determined by using the method of Heath and Packer (1968) [14]. The plant material was homogenized in TCA (0.1%) and centrifuged at 10000 rpm for 5 min. The supernatant was treated with TBA (0.5% in 20% TCA) and solution was kept on water bath at 95°C for 30 min and then cooled quickly on ice. MDA content was determined after subtracting the optical absorbance observed at 600 nm and 532 nm. The percent decrease in fresh weight was calculated using formula: $(W_1 - W_2 / W_1) \times 100$.

Statistical analysis

The data were analyzed for mean, standard deviation and two-way analysis of variance (ANOVA). The differences ($p \leq 0.05$) among means were compared by honestly significant difference (HSD) using Tukey's test and the results were expressed as Mean±SD. All the experiments were performed in triplicate.

RESULTS AND DISCUSSION:

Effect on chlorophyll content

The data presented in Table-1 to 3 showed that the exposure of DBP and BBP decreased the contents of photosynthetic pigments. On 7th day, the percent decrease in Chl a content at 5, 10, 15, 50 mM concentrations of BBP was found to be 2.31%, 6.01%, 6.86%, 33.56% respectively whereas, the percent decrease for 15 days at the same concentrations was found to be 15.99%, 28.19%, 32.12%, 44.93% respectively when compared with control. In case of DBP, the percent decrease on 7th day was found to be 50.61%, 56.75%, 59.81%, 69.38 and for 15 days exposure it was found to be 10.33%, 34.80%, 37.77%, 45.32% at the same concentrations when compared with control. The percent decrease in chlorophyll b content on comparing with control under 7 and 15 days exposure at 5, 10, 15, 20 mM concentrations of BBP was found to be 5.73%, 8.87%, 29.59%, 38.10% and 17.13%, 23.95%, 33.30%, 44.31% respectively. Under same concentrations and exposure conditions of DBP, the chlorophyll b content was found to decrease significantly and percent decrease was 30.63%, 53.50%, 59.06%, 69.55% and 10.88%, 36.62%, 41.63%, 42.55%. On 7th day, the total chlorophyll content at same concentrations of BBP showed the significant positive dose correlation (3.39%, 10.34%, 21.79%, 33.12%), while on 15th day the percent decrease was 17.26%, 26.88%, 36.86%, 45.33%. Under DBP initial exposure condition with same concentrations the total chlorophyll content decreased significantly (13.58%, 54.24%, 62.40%, 70.90%) and under 15 days exposure significant decrease was observed at higher concentrations (9.26%, 18.60%, 39.20%, 44.26%).

In the present study, the decrease in Chl a, Chl b, total chlorophyll content may be due to chloroplast degradation during the exposure of DBP and BBP. Hannay et al., 1986 observed the phthalate plasticizers treated leaves have led to chloroplast degradation on the basis of observations such as formation of numerous plastoglobuli, less grana and the absence of starch grains [15]. Melin et al., 1983 observed the bleaching of chlorophyll on the DBP exposure on the algae [16]. The similar decline trend in chlorophyll contents were observed in case of wheat which was treated with DBP and DEHP [17]. Moreover, they revealed that the decrease in chlorophyll content is due to decrease in net photosynthesis and ROS generation which results into the disruption of chloroplast structure. The chlorophyll content also observed to decrease in case of DBP exposure on duckweeds [18], on cucumber [19], on Chinese cabbage [20] and seven higher plant species under DBP and DEHP [21]. In plants, the process of photosynthesis is the main source of plant energy and affect the growth and development of plant [17]. Photosynthesis depends upon the ability of the plant to capture light and how efficiently they convert light into biomass. In photosynthesis, chlorophyll plays vital role as it allows plant to obtain energy from light. The amount of chlorophyll directly regulates the rate photosynthesis which directly/indirectly related to all metabolic processes.

On 7th day of exposure of BBP the percent decrease in carotenoid content was 4.79%, 11.27%, 18.03%, 27.04% while, the increase in carotenoid content was absorbed on 15 days treatment of BBP. The carotenoid content was found to be increase initially and the decreased (5.45%, 13.46%, 19.76%, 34.58%) at same concentrations respectively (Table-4). On 7th and 15th days of BBP exposure the xanthophyll content was increased and percent increase was 1.42%, 6.94%, 28.50%, 43.08% and 5.49%, 12.28%, 17.63%, 28.03 % respectively. The xanthophyll content was found to decrease significantly under 7 and 15 days of exposure of DBP and percent decrease was 21.51%, 24.42%, 27.91%, 30.23% and 21.18%, 25.88%, 28.24%, 30.59% respectively (Table-5).

Carotenoid are accessory pigments which participates either in light harvesting or photo protection. Moreover, these also provide photo stability to the chlorophyll by quenching singlet oxygen. Xanthophylls are oxygen containing carotenoids which provides additional protection mechanism i.e. xanthophyll cycle which involves inter-conversion of violaxanthin, antheroxanthin and zeaxanthin [22]. Under the DBP exposure conditions carotenoid content and xanthophylls content was decreased which may be resulted due to disturbance in synthesis process. Virgin et al., 1981 observed that DBP interfere with the carotenoids synthesis [23]. DBP and DEP disturbed the biosynthesis of carotenoids in wheat [17]. This inhibition may lead to photo instability of chlorophyll and which resulted into decline in net photosynthesis. During last decades various such reports were obtained under heavy metal stress. In the present work, BBP has shown increase in carotenoid and xanthophyll content. The increase in carotenoid level may be a part of strategy adopted by the plant to counteract the toxic effect of free radicals generated under abiotic stress.

Effect on protein content

The protein content was found to be decrease with increase in concentration of BBP and DBP. Under 7 and 15 days exposure of BBP the percent protein content decreased was 8.09%, 27.02%, 35.11%, 40.21% and 4.05, 8.65%, 39.73%, 55.14% respectively. The protein content declined significantly under 7 and 15 days of DBP treatment and the percent decrease was 5.26%, 12.50%, 25.00%, 55.92% and 7.84%, 15.15%, 18.89%, 21.75% respectively (Table-6). The possible reason for this may be inhibition of required nutritive material for anabolism of protein which led to decline in protein content. Li et al., 2006 also absorbed the similar results with submerged hydrophytes under DBP stress [8].

Table-1 Effect of benzyl butyl phthalate and di-butyl phthalate on chlorophyll 'a' content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	2.59 ± 0.16^{bc}	3.90 ± 0.98^{a}	3.52 ± 0.15^{abc}	4.64 ± 0.14^{a}	2.60 ± 0.16^{b}	3.52 ± 0.15^{a}	3.90 ± 0.98^{b}	4.64 ± 0.14^{a}
2	5	2.53 ± 0.14^{bc}	3.27 ± 0.56^{a}	1.74 ± 0.28^{def}	4.16 ± 0.56^{ab}	2.53 ± 0.14^{b}	1.74 ± 0.28^{c}	3.27 ± 0.56^{c}	4.16 ± 0.56^{b}
3	10	2.44 ± 0.12^{bc}	2.80 ± 0.21^{abc}	1.52 ± 0.06^{ef}	3.02 ± 0.07^{bcd}	2.44 ± 0.12^{b}	1.52 ± 0.06^{c}	2.80 ± 0.21^{de}	3.02 ± 0.07^{cd}
4	15	2.42 ± 0.14^{bc}	2.65 ± 0.26^{bc}	1.42 ± 0.04^{ef}	2.89 ± 0.14^{bcd}	2.42 ± 0.14^{b}	1.42 ± 0.04^{cd}	2.65 ± 0.26^{de}	2.89 ± 0.14^{cd}
5	20	1.73 ± 0.11^{c}	2.15 ± 0.42^{bc}	1.08 ± 0.34^{f}	2.54 ± 0.35^{cde}	1.73 ± 0.11^{c}	1.08 ± 0.34^{d}	2.15 ± 0.42^{ef}	2.54 ± 0.36^{def}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	1.17		1.28		0.51		0.41	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 16.97**		Treatment	$F_{(1, 20)}$ 273.09**	Treatment	$F_{(1, 20)}$ 57.89**	Treatment	$F_{(1, 20)}$ 9.13**
2.b	Dose	$F_{(4, 20)}$ 8.63**		Dose	$F_{(4, 20)}$ 69.60**	Dose	$F_{(4, 20)}$ 71.27**	Dose	$F_{(4, 20)}$ 17.82**
2.c	Treatment×Dose	$F_{(4, 20)}$ 1.69		Treatment×Dose	$F_{(4, 20)}$ 5.12**	Treatment×Dose	$F_{(4, 20)}$ 31.41**	Treatment×Dose	$F_{(4, 20)}$ 0.66

** Significant at p<0.01, * significant at p<0.05.
Letters (a-f) within the column with same letter means does not differ at p<0.05.

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	4.75 ± 0.14^{ab}	5.32 ± 0.77^{a}	6.66 ± 0.42^{b}	8.05 ± 0.27^{a}	4.75 ± 0.14^{b}	6.66 ± 0.42^{a}	5.32 ± 0.77^{c}	8.05 ± 0.27^{a}
2	5	4.47 ± 0.13^{abc}	4.41 ± 0.27^{abc}	4.62 ± 0.83^{c}	7.18 ± 0.96^{ab}	4.47 ± 0.13^{bc}	4.62 ± 0.83^{b}	4.41 ± 0.27^{ef}	7.18 ± 0.96^{b}
3	10	4.32 ± 0.14^{abcd}	4.05 ± 0.70^{abcd}	3.10 ± 0.37^{d}	5.11 ± 0.02^{c}	4.32 ± 0.14^{bc}	3.10 ± 0.37^{de}	4.05 ± 0.70^{f}	5.11 ± 0.02^{cd}
4	15	3.34 ± 0.60^{cde}	3.55 ± 0.82^{bcde}	2.73 ± 0.11^{d}	4.70 ± 0.23^{c}	3.34 ± 0.60^{cd}	2.73 ± 0.11^{de}	3.55 ± 0.82^{g}	4.70 ± 0.23^{de}
5	20	2.94 ± 0.08^{e}	2.97 ± 0.17^{de}	2.03 ± 0.45^{d}	4.63 ± 0.04^{c}	2.94 ± 0.08^{de}	2.03 ± 0.45^{e}	2.97 ± 0.17^{h}	4.63 ± 0.04^{e}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	1.38		1.37		1.17		0.41	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 16.97**		Treatment	$F_{(1, 20)}$ 0.30	Treatment	$F_{(1,20)}$ 0.84	Treatment	$F_{(1, 20)}$ 90.11**
2.b	Dose	$F_{(4, 20)}$ 8.63**		Dose	$F_{(4, 20)}$ 17.73**	Dose	$F_{(4, 20)}$ 59.62**	Dose	$F_{(4, 20)}$ 30.21**
2.c	Treatment×Dose	$F_{(4, 20)}$ 1.69		Treatment×Dose	$F_{(4, 20)}$ 0.68	Treatment×Dose	$F_{(4, 20)}$ 14.55**	Treatment×Dose	$F_{(4, 20)}$ 3.55*

Table-2 Effect of benzyl butyl phthalate and di-butyl phthalate on chlorophyll 'b' content in 7 days and 15 days treated plants of *Spirodela polyrhiza*
** Significant at p<0.01, * significant at p<0.05.
Letters (a-h) within the column with same letter means does not differ at p<0.05.

Table-3 Effect of benzyl butyl phthalate and di-butyl phthalate on total chlorophyll content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	7.16±0.22^{ab}	8.61±0.91^{a}	10.3±0.74^{ab}	12.31±0.34^{a}	7.16±0.22^{b}	10.3±0.74^{a}	8.61±0.91^{bcd}	12.31±0.34^{a}
2	5	6.92±0.22^{ab}	7.13±0.17^{ab}	9.37±0.51^{cd}	11.17±1.49^{a}	6.92±0.22^{bc}	9.37±0.51^{bcd}	7.13±0.17^{cde}	11.17±1.49^{ab}
3	10	6.42±0.43^{bc}	6.30±0.91^{bc}	4.96±0.36^{cd}	10.02±3.45^{ab}	6.42±0.43^{bc}	4.96±0.36^{ef}	6.30±0.91^{cde}	10.02±3.45^{abc}
4	15	5.60±1.00^{bc}	5.44±0.85^{bc}	4.08±0.13^{cd}	7.48±0.25^{bc}	5.60±1.00^{cde}	4.08±0.13^{efg}	5.44±0.85^{de}	7.48±0.25^{bcde}
5	20	4.79±0.32^{c}	4.71±0.12^{c}	3.15±0.54^{d}	6.86±0.35^{bc}	4.79±0.32^{def}	3.15±0.54^{g}	4.71±0.12^{e}	6.86±0.36^{cde}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	1.78		3.61		1.47		3.75	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 1.29		Treatment	$F_{(1, 20)}$ 76.33**	Treatment	$F_{(1, 20)}$ 8.15**	Treatment	$F_{(1, 20)}$ 45.75**
2.b	Dose	$F_{(4, 20)}$ 24.03**		Dose	$F_{(4, 20)}$ 24.59**	Dose	$F_{(4, 20)}$ 79.09**	Dose	$F_{(4, 20)}$ 13.19**
2.c	Treatment×Dose	$F_{(4, 20)}$ 1.85		Treatment×Dose	$F_{(4, 20)}$ 1.58	Treatment×Dose	$F_{(4, 20)}$ 25.59**	Treatment×Dose	$F_{(4, 20)}$ 0.80

** Significant at p<0.01, * significant at p<0.05.
Letters (a-g) within the column with same letter means does not differ at p<0.05.

Table-4 Effect of benzyl butyl phthalate and di-butyl phthalate on total carotenoid content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	0.36±0.03^{de}	0.59±0.05^{a}	0.54±0.11^{d}	0.10±0.14^{d}	0.36±0.03^{de}	0.54±0.11^{d}	0.59±0.05^{a}	0.10±0.14^{d}
2	5	0.34±0.03^{de}	0.56±0.01^{ab}	1.56±0.33^{c}	0.54±0.45^{d}	0.34±0.03^{de}	1.56±0.33^{c}	0.56±0.01^{ab}	0.54±0.45^{d}
3	10	0.32±0.02^{de}	0.51±0.05^{ab}	2.38±0.23^{b}	1.55±0.09^{c}	0.32±0.02^{de}	2.38±0.23^{b}	0.51±0.05^{ab}	1.55±0.09^{c}
4	15	0.29±0.03^{de}	0.47±0.05^{bc}	2.70±0.06^{ab}	2.15±0.22^{bc}	0.29±0.03^{de}	2.70±0.06^{ab}	0.47±0.05^{bc}	2.15±0.22^{bc}
5	20	0.26±0.01^{e}	0.38±0.04^{cd}	3.16±0.22^{a}	2.80±0.11^{ab}	0.26±0.01^{e}	3.16±0.22^{a}	0.38±0.04^{cd}	2.80±0.11^{ab}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	0.10		0.65		0.44		0.50	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 214.81**		Treatment	$F_{(1, 20)}$ 60.40**	Treatment	$F_{(1, 20)}$ 1001.43**	Treatment	$F_{(1, 20)}$ 216.84**
2.b	Dose	$F_{(4, 20)}$ 16.30*		Dose	$F_{(4, 20)}$ 133.10**	Dose	$F_{(4, 20)}$ 65.02**	Dose	$F_{(4, 20)}$ 54.73**
2.c	Treatment×Dose	$F_{(4, 20)}$ 2.06		Treatment×Dose	$F_{(4, 20)}$ 2.27	Treatment×Dose	$F_{(4, 20)}$ 74.69**	Treatment×Dose	$F_{(4, 20)}$ 72.23**

** Significant at p<0.01, * significant at p<0.05.
Letters (a-e) within the column with same letter means does not differ at p<0.05.

Table-5 Effect of benzyl butyl phthalate and di-butyl phthalate on total xanthophylls content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	25.37±2.03^{b}	6.92±0.14^{c}	1.72±0.23^{a}	1.70±0.21^{ab}	25.37±2.03^{b}	1.72±0.23^{a}	6.92±0.14^{c}	1.70±0.21^{ab}
2	5	25.73±0.21^{b}	7.30±0.08^{c}	1.35±0.07^{bc}	1.34±0.09^{c}	25.73±0.21^{b}	1.35±0.07^{bc}	7.30±0.08^{c}	1.34±0.09^{c}
3	10	27.13±0.55^{b}	7.77±0.14^{c}	1.30±0.06^{c}	1.26±0.04^{c}	27.13±0.55^{b}	1.30±0.06^{c}	7.77±0.14^{c}	1.26±0.04^{c}
4	15	32.60±4.10^{a}	8.14±0.22^{c}	1.24±0.09^{c}	1.22±0.09^{c}	32.60±4.10^{a}	1.24±0.09^{c}	8.14±0.22^{c}	1.22±0.09^{c}
5	20	36.30±1.80^{a}	8.85±0.45^{c}	1.20±0.11^{c}	1.18±0.08^{c}	36.30±1.80^{a}	1.20±0.11^{c}	8.85±0.45^{c}	1.18±0.08^{c}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	4.55		0.35		4.54		0.56	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 1413.35**		Treatment	$F_{(1, 20)}$ 0.221	Treatment	$F_{(4, 20)}$ 2400.45**	Treatment	$F_{(1, 20)}$ 8482.05**
2.b	Dose	$F_{(4, 20)}$ 18.51**		Dose	$F_{(4, 20)}$ 17.253**	Dose	$F_{(4, 20)}$ 13.32**	Dose	$F_{(4, 20)}$ 14.08**
2.c	Treatment×Dose	$F_{(4, 20)}$ 10.17**		Treatment×Dose	$F_{(4, 20)}$ 0.006	Treatment×Dose	$F_{(4, 20)}$ 14.98**	Treatment×Dose	$F_{(4, 20)}$ 35.23**

** Significant at p<0.01, * significant at p<0.05.
Letters (a-c) within the column with same letter means does not differ at p<0.05.

Table-6 Effect of benzyl butyl phthalate and di-butyl phthalate on total protein content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	4.70 ± 0.82^{a}	3.70 ± 0.75^{abc}	1.52 ± 0.05^{d}	5.61 ± 0.33^{a}	4.70 ± 0.82^{a}	1.52 ± 0.05^{cde}	3.70 ± 0.75^{bcd}	5.61 ± 0.33^{a}
2	5	4.32 ± 0.87^{ab}	3.55 ± 0.68^{abc}	1.44 ± 0.07^{d}	5.17 ± 0.15^{b}	4.32 ± 0.87^{ab}	1.44 ± 0.07^{cde}	3.55 ± 0.68^{cd}	5.17 ± 0.15^{ab}
3	10	3.43 ± 0.80^{abc}	3.38 ± 0.60^{abc}	1.33 ± 0.09^{d}	4.76 ± 0.09^{c}	3.43 ± 0.80^{ab}	1.33 ± 0.09^{cde}	3.38 ± 0.60^{cd}	4.76 ± 0.09^{abc}
4	15	3.05 ± 0.98^{abc}	2.23 ± 0.60^{bc}	1.14 ± 0.12^{d}	4.55 ± 0.04^{c}	3.05 ± 0.98^{abc}	1.14 ± 0.12^{de}	2.23 ± 0.60^{de}	4.55 ± 0.04^{abc}
5	20	2.81 ± 1.00^{abc}	1.58 ± 0.95^{c}	0.67 ± 0.04^{e}	4.39 ± 0.14^{c}	2.81 ± 1.00^{bcd}	0.67 ± 0.04^{e}	1.58 ± 0.95^{e}	4.39 ± 0.14^{abc}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	2.36		0.40		1.84		1.54	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 6.45*		Treatment	$F_{(1, 20)}$ 5220.58**	Treatment	$F_{(1, 20)}$ 110.11**	Treatment	$F_{(1, 20)}$ 105.48**
2.b	Dose	$F_{(4, 20)}$ 6.23**		Dose	$F_{(4, 20)}$ 49.95**	Dose	$F_{(4, 20)}$ 4.63**	Dose	$F_{(4, 20)}$ 9.75**
2.c	Treatment×Dose	$F_{(4, 20)}$ 0.39		Treatment×Dose	$F_{(4, 20)}$ 6.07**	Treatment×Dose	$F_{(4, 20)}$ 1.13	Treatment×Dose	$F_{(4, 20)}$ 1.54

** Significant at p<0.01, * significant at p<0.05.
Letters (a-e) within the column with same letter means does not differ at p<0.05.

Table-7 Effect of benzyl butyl phthalate and di-butyl phthalate on total carbohydrate content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	1.05 ± 0.36^{abc}	1.55 ± 0.33^{a}	1.88 ± 0.10^{a}	0.62 ± 0.03^{d}	1.05 ± 0.36^{bcde}	1.88 ± 0.10^{a}	1.55 ± 0.33^{a}	0.62 ± 0.03^{cd}
2	5	0.95 ± 0.41^{abc}	1.20 ± 0.33^{ab}	1.65 ± 0.03^{b}	0.59 ± 0.004^{de}	0.95 ± 0.41^{cdef}	1.65 ± 0.03^{ab}	1.20 ± 0.33^{ab}	0.59 ± 0.004^{cd}
3	10	0.88 ± 0.43^{abc}	0.99 ± 0.03^{abc}	1.57 ± 0.02^{b}	0.58 ± 0.01^{de}	0.88 ± 0.43^{def}	1.57 ± 0.02^{abc}	0.99 ± 0.03^{bc}	0.58 ± 0.01^{cd}
4	15	0.46 ± 0.12^{bc}	0.80 ± 0.24^{abc}	1.53 ± 0.01^{a}	0.52 ± 0.002^{de}	0.46 ± 0.12^{ef}	1.53 ± 0.01^{abcd}	0.80 ± 0.24^{bcd}	0.52 ± 0.002^{cd}
5	20	0.32 ± 0.02^{c}	0.56 ± 0.37^{bc}	1.37 ± 0.11^{a}	0.45 ± 0.06^{e}	0.32 ± 0.02^{f}	1.37 ± 0.11^{abcd}	0.56 ± 0.37^{cd}	0.45 ± 0.06^{d}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	0.82		0.15		0.66		0.51	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 7.70*		Treatment	$F_{(1, 20)}$ 2991.94**	Treatment	$F_{(1, 20)}$ 108.18**	Treatment	$F_{(1, 20)}$ 52.90**
2.b	Dose	$F_{(4, 20)}$ 8.70**		Dose	$F_{(4, 20)}$ 32.79**	Dose	$F_{(4, 20)}$ 6.84**	Dose	$F_{(4, 20)}$ 9.31**
2.c	Treatment×Dose	$F_{(4, 20)}$ 0.40		Treatment×Dose	$F_{(4, 20)}$ 8.64**	Treatment×Dose	$F_{(4, 20)}$ 0.96	Treatment×Dose	$F_{(4, 20)}$ 4.81**

** Significant at p<0.01, * significant at p<0.05.
Letters (a-f) within the column with same letter means does not differ at p<0.05.

Table-8 Effect of benzyl butyl phthalate and di-butyl phthalate on MDA content in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	4.91 ± 0.59^{a}	3.47 ± 0.24^{bc}	1.97 ± 0.78^{def}	0.23 ± 0.23^{f}	4.91 ± 0.59^{de}	1.97 ± 0.78^{e}	3.47 ± 0.24^{b}	0.23 ± 0.23^{e}
2	5	4.29 ± 0.35^{ab}	2.79 ± 0.51^{cd}	6.06 ± 1.12^{cd}	0.88 ± 0.60^{f}	4.29 ± 0.35^{de}	6.06 ± 1.12^{cd}	2.79 ± 0.51^{bc}	0.88 ± 0.60^{de}
3	10	3.42 ± 0.38^{bc}	2.27 ± 0.52^{cd}	9.33 ± 0.56^{bc}	1.74 ± 0.13^{ef}	3.42 ± 0.38^{de}	9.33 ± 0.56^{bc}	2.27 ± 0.52^{bcd}	1.74 ± 0.13^{cde}
4	15	2.62 ± 0.93^{cd}	1.84 ± 0.02^{d}	12.86 ± 2.38^{ab}	2.43 ± 0.32^{def}	2.62 ± 0.93^{de}	12.86 ± 2.38^{ab}	1.84 ± 0.02^{cde}	2.43 ± 0.32^{bcd}
5	20	2.21 ± 0.29^{cd}	1.68 ± 0.18^{d}	15.49 ± 3.21^{a}	5.33 ± 1.40^{cde}	2.21 ± 0.29^{de}	15.49 ± 3.21^{a}	1.68 ± 0.18^{cde}	5.33 ± 1.40^{a}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	1.35		4.15		4.06		1.61	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 40.43**		Treatment	$F_{(1, 20)}$ 178.88**	Treatment	$F_{(1, 20)}$ 121.47**	Treatment	$F_{(1, 20)}$ 2.01
2.b	Dose	$F_{(4, 20)}$ 23.70**		Dose	$F_{(4, 20)}$ 37.78**	Dose	$F_{(4, 20)}$ 13.70**	Dose	$F_{(4, 20)}$ 9.52**
2.c	Treatment×Dose	$F_{(4, 20)}$ 1.21		Treatment×Dose	$F_{(4, 20)}$ 9.63**	Treatment×Dose	$F_{(4, 20)}$ 31.96**	Treatment×Dose	$F_{(4, 20)}$ 33.33**

** Significant at p<0.01, * significant at p<0.05.
Letters (a-f) within the column with same letter means does not differ at p<0.05.

Table-9 Effect of benzyl butyl phthalate and di-butyl phthalate on percent decrease in fresh weight in 7 days and 15 days treated plants of *Spirodela polyrhiza*

S. No.	Conc. (mM)	Treatment of BBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (7 Days)	Treatment of DBP (15 Days)	Treatment of BBP (7 Days)	Treatment of DBP (7 Days)	Treatment of BBP (15 Days)	Treatment of DBP (15 Days)
1	0	10.88±8.45^{ab}	4.95±3.13^{b}	3.97±2.50^{f}	17.75±3.90^{d}	10.88±8.45^{abc}	3.97±2.50^{c}	4.95±3.13^{e}	17.75±3.90^{ede}
2	5	15.11±6.61^{ab}	12.13±3.45^{ab}	8.58±1.22^{ef}	25.20±2.11^{c}	15.11±6.61^{abc}	8.58±1.22^{bc}	12.13±3.45^{de}	25.20±2.11^{abcd}
3	10	21.07±8.20^{ab}	20.05±3.43^{ab}	12.52±1.82^{de}	30.05±3.88^{bc}	21.07±8.20^{ab}	12.52±1.82^{abc}	20.05±3.43^{cd}	30.05±3.88^{abc}
4	15	21.35±7.54^{ab}	20.47±11.22^{ab}	15.67±1.53^{d}	33.87±1.85^{ab}	21.35±7.54^{ab}	15.67±1.53^{abc}	20.47±11.22^{bcd}	33.87±1.85^{ab}
5	20	24.26±3.29^{a}	23.90±4.63^{ab}	18.57±0.78^{d}	36.73±0.53^{a}	24.26±3.29^{a}	18.57±0.78^{abc}	23.90±4.63^{abcd}	36.73±0.53^{a}
TWO WAY ANOVA SUMMARY									
Two-way ANOVA		**7 days BBP × 15 days BBP**		**7 days DBP × 15 days DBP**		**7 days BBP × 7 days DBP**		**15 days BBP × 15 days DBP**	
1	HSD value	18.99		6.62		14.86		13.54	
2		F-ratio		F-ratio		F-ratio		F-ratio	
2.a	Treatment	$F_{(1, 20)}$ 40.43**		Treatment	$F_{(1, 20)}$ 178.88**	Treatment	$F_{(1, 20)}$ 121.47**	Treatment	$F_{(1, 20)}$ 2.01
2.b	Dose	$F_{(4, 20)}$ 23.70**		Dose	$F_{(4, 20)}$ 37.78**	Dose	$F_{(4, 20)}$ 13.70**	Dose	$F_{(4, 20)}$ 9.52**
2.c	Treatment×Dose	$F_{(4, 20)}$ 1.21		Treatment×Dose	$F_{(4, 20)}$ 9.63**	Treatment×Dose	$F_{(4, 20)}$ 31.96**	Treatment×Dose	$F_{(4, 20)}$ 33.33**

** Significant at p<0.01, * significant at p<0.05.
Letters (a-e) within the column with same letter means does not differ at p<0.05.

Effect on carbohydrate content

The percent decrease in carbohydrate content under 7 and 15 days exposure of 5, 10, 15, 20 mM concentrations of BBP was 9.57%, 16.55%, 55.79%, 69.75% and 22.98%, 36.54%, 48.52%, 63.96% respectively. In case of DBP at similar concentrations the carbohydrate content was decreased significantly and the percent decrease was 12.08%, 16.09%, 18.28%, 26.87 and 4.87%, 6.50%, 16.03%, 27.29% at same concentration on comparing with control on 7 and 15 days on comparing with control (Table-7). Change in carbohydrate content is directly related with important metabolic processes like translocation, respiration and photosynthesis [24]. Moreover, the photosynthesis is responsible for the accumulation and synthesis of organic substances [25]. The decreased carbohydrate content under plasticizers exposure is due to the inhibition in the photosynthesis [15]. Similar phthalates induced perturbations were reported by Melin et al., 1983 [16].

Effect on MDA content

On 7th and 15th days exposure of 5, 10, 15, 20 mM concentrations of BBP the content of MDA was declined as 12.75%, 30.28%, 46.65%, 55.00% and 19.42%, 34.51%, 46.88%, 51.59% respectively, while in case of DBP under same concentrations and exposure duration the MDA content was increased respectively (Table-8). According to Dawes et al., 2000 stress leads to the generation of excess ROS which are the major contributing factors for the disturbance of normal metabolism [26]. ROS disrupt the cell membrane *via* lipid per oxidation which resulted in high MDA content. In DBP exposure the increased MDA content confirms that *Spirodela polyrhiza* is more vulnerable to DBP toxicity than BBP. Duckweed under the exposure of DEHP have shown enhanced MDA content [27].

Effect on fresh weight

The percent decrease in fresh weight on 7th and 15th day of earlier mentioned concentrations of BBP and DBP was found to be increase significantly (Table-9). The exposure of phthalates for 7 and 15 days led to the disturbance in normal physiology of plant. Thus, the declined chlorophyll content confirms the chloroplast which is directly associated with photosynthesis. The process of photosynthesis is associated with accumulation and synthesis of organic substances [25]. Therefore, the decreased photosynthesis rate because of chloroplast degradation led to the decline in fresh weight. Similar trends were obtained during the exposure of DEHP and DBP on wheat seedlings for 7 and 14 days [17].

CONCLUSIONS:

The present study investigated the effects of BBP and DBP on the photosynthetic pigments, accessory pigments, protein, carbohydrate, MDA content and fresh weight of *Spirodela polyrhiza*. Both inhibited the growth of plant and disturbed the normal physiological processes of plant during 7 and 15 days exposure duration. The photosynthetic pigments, xanthophyll, carbohydrate and MDA content of plant affected to greater extent on 7th day of treatment than 15 days in case of BBP treatment, while remaining indices affected on long term of exposure. The carotenoid content was found to increase on both exposure conditions in BBP. On 7th day DBP also showed similar trends of decline in all indices. Moreover, xanthophyll and MDA content was found to increase in both treatment periods. Thus, the present study reveals that DBP has higher significant phytotoxic effects on *Spirodela polyrhiza* than BBP.

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