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Toxicological evaluation of three contaminants of emerging concern by use of the *Allium cepa* test

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ABSTRACT

Di(2-ethylhexyl)phthalate, triclosan and propylparaben are contaminants of emerging concern that have been subjected to extensive toxicological studies, but for which limited information is currently available concerning adverse effects on terrestrial plant systems. The *Allium cepa* test, which is considered one of the most efficient approaches to assess toxic effects of environmental chemicals, was selected to evaluate the potential risks of these ubiquitous pollutants.

Our data demonstrate that all three compounds studied may in some way be considered toxic, but different effects were noted depending on the chemical and the end point analysed. Results derived from the analysis of macroscopic parameters used in testing for general toxicity, revealed that while di(2-ethylhexyl)phthalate had no apparent effects, the other two chemicals inhibited *A. cepa* root growth in a dose-dependent manner. On the other hand, although all three compounds caused alterations in the mitotic index of root-tip cells, propylparaben was the only one that did not show evidence of genotoxicity in assays for chromosome aberrations and micronuclei. The results of the present study clearly indicate that sensitive plant bioassays are useful and complementary tools to determine environmental impact of contaminants of emerging concern.

1. Introduction

Emerging contaminants – *i.e.* contaminants of emerging concern – comprise a wide range of chemical compounds, such as pharmaceuticals, personal care products, surfactants, plasticizers and industrial additives that are not included in current monitoring programmes [1]. These environmental pollutants are continuously discharged into air, water or soil from domestic and industrial sewage systems and consequently, have become a cause of major concern for the scientific community and regulatory agencies [2,3]. However, despite significant research efforts, limited information is still available about the potential human and ecological health effects caused by diverse emerging contaminants [4].

In this study three chemicals characterized as emerging contaminants, di(2-ethylhexyl)phthalate, 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan) and propyl-*p*-hydroxybenzoate (propylparaben) were selected for assessing their toxic potential in the *Allium cepa* test. This plant bioassay is now considered one of the most efficient approaches routinely used to determine the toxic effects of chemical compounds in the environment [5]. Besides its high sensitivity and cost-effectiveness, the *A. cepa* assay offers some additional advantages including the possibility of measuring macroscopic and microscopic parameters [6] and the good correlation of its outcome with the results of mammalian test systems [7–9].

Di(2-ethylhexyl)phthalate (DEHP) is the most extensively used plasticizer for polyvinyl chloride (PVC) products with a broad range of applications such as building materials, food packaging and medical devices [10]. Because it is not chemically bound to the polymer, DEHP readily leaches from plastic surfaces and can enter the environment or the human body through multiple routes [11]. Triclosan (TCS) is an anti-bacterial ingredient in many cosmetics and healthcare products, often found in the aquatic environment [12,13] as well as in body fluids from the general population [14,15]. Propylparaben (PPB) is a commonly used preservative in a variety of consumer products [16], with an ubiquitous presence in sewage influents and effluents from treatment plants [17,18], in surface waters [19,20] and in indoor dust [21]. Moreover, despite the fact that parabens are considered compounds with low bioaccumulation potential [22], their potential human health risks are attracting considerable attention nowadays [23].

Because of their widespread use and presence in different anthropogenic discharges, all three chemicals have been subject to

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extensive studies but their safety remains questioned since controversial results have been reported in the literature. Our data provide further insight into the toxicological profile of these emerging contaminants in the terrestrial environment.

2. Materials and methods

2.1. Chemicals

Di(2-ethylhexyl)phthalate (CAS No. 117-81-7), triclosan (CAS No. 3380-34-5) and propylparaben (CAS No. 94-13-3) were purchased from Sigma–Aldrich (St. Louis, USA). All other chemicals were of the highest grade commercially available. Stock solutions of the test compounds were prepared in ethanol or dimethyl sulphoxide (DMSO) and kept at room temperature in the dark.

2.2. Experimental procedures

Onion bulbs (*A. cepa* L.; weight, 15–30 g), free from agricultural pesticides and growth inhibitors (kindly provided by Hnos. Aparici y Rosa S.L., Valencia, Spain), were grown in the dark at a constant temperature of 25 ± 0.5 °C in a refrigerated incubator IRE 160 (Raypa, Spain). The bulbs were placed onto cylindrical glass receptacles filled with filtered tap water, which was renewed every 24 h and aerated continuously by bubbling air at a rate of 10–20 ml/min by use of an aquarium pump (Rena, France). The experiments started when newly emerging roots had reached 15–20 mm in length, using a series of five bulbs for each concentration and control group. The test solutions of DEHP (1, 10, 100, 500 μM), PPB (50, 200, 400, 500 μM) and TCS (1, 5, 10, 30 μM) were selected on the basis of preliminary studies and prepared fresh in filtered tap water (pH = 6.5). Solvent concentration was lower than 0.5% (DMSO, used for PPB) or 1% (ethanol, used for TCS and DEHP) including the control groups.

2.2.1. Macroscopic parameters

The bulbs were exposed for 72 h to solutions with increasing concentrations of the test compounds. Thereafter, the length of the whole root bundle from control and experimental sets was measured as described by Fiskesjö [24]. The concentrations of the compounds were plotted against root length as a percentage of the control to estimate EC50 values. Other signs of toxicity such as changes in root consistency and colour, and the presence of tumours, hook roots and twisted roots were also examined.

2.2.2. Microscopic parameters

A. cepa bulbs were treated with test solutions for 48 h under the usual laboratory conditions described above. At the end of the exposure period, root tips excised from each bulb were fixed in ethanol/glacial acetic acid (3:1, v/v) and kept at 4 °C overnight. After hydrolysis during 15 min in 5 N HCl at room temperature, root tips were stained by means of the Feulgen reaction and the apical 2 mm were squashed in a drop of 50% acetic acid. One slide was prepared per bulb and microscopic analysis included determination of the mitotic index and the scoring of micronuclei and chromosome aberrations. The mitotic index was calculated as the ratio between the number of cells in mitosis and the total number of cells, counting 1000 cells per slide. The frequencies of micronuclei were analysed by observing 1000 interphase cells for each bulb. Chromosome aberrations were characterized in 100 mitotic cells per slide and classified as stickiness and abnormal ana/telophases, which include bridges, vagrant chromosomes, chromosome missegration and multipolar spindles.

2.3. Data analysis

Statistical analysis, including analysis of variance (ANOVA) with the appropriate *post hoc* test (Bonferroni), and nonlinear regression for the determination of the 50% effective concentrations (EC50 values), were carried out with GraphPad Prism 4.0 for Windows (GraphPad Software Inc., USA). The level of statistical significance was in all cases $p \leq 0.05$. Each data point represents the arithmetic mean ± standard deviation of at least three independent experiments.

3. Results

3.1. Root-growth inhibition

The effect of chemicals on root elongation of *A. cepa* bulbs is summarized in Table 1. At the concentrations tested, DEHP did not influence the root growth while significant and dose-dependent reductions in length were observed after treatments with PPB and TCS, with an estimated effective concentration (EC50) of 168.4 and 1.8 μM, respectively. The root bundles in the control sets had an average length of 7.4 ± 1.2 cm, after 72 h of hydroponic culture. No macroscopic changes were evident in growing roots under any experimental condition.

3.2. Chromosomal aberrations and micronucleus induction

To decide on the most appropriate concentrations for cytogenetic analysis, the mitotic activity of *A. cepa* root meristems was evaluated in a first set of experiments, after a 48-h exposure to the chemicals. As shown in Table 2, no significant differences in mitotic index values were found between controls and treatments with DEHP or TCS at concentrations below 100 and 10 μM, respectively. However, the percentage of prophases increased significantly from 67.2 ± 9.9% in the control, to 77.3 ± 8.7% and 85.0 ± 4.7% in cells treated with 1 and 5 μM TCS, respectively. In contrast, PPB exposure caused a dose-dependent decrease in the mitotic activity, which was significant from the lowest concentration tested. According to recommendations by Rank and Nielsen [25], the mitotic index should never be below 50% of the control value in order to obtain a reliable analysis of chromosome aberrations. We thus selected for subsequent studies the highest two concentrations of the compounds that meet this criterion.

The results regarding the type and frequency of abnormalities in interphase and mitotic root-tip cells of *A. cepa* are summarized in Table 3. DEHP at high concentrations (≥100 μM) induced micronuclei and chromosome missegregation in anaphase, while TCS caused chromosome stickiness as well as disturbed ana/telophases. On the other hand, PPB treatments did not increase the frequencies of anaphase/telophase aberrations or micronuclei over the control values (data not shown). The most common chemically induced alterations found in *A. cepa* meristematic cells are shown in Fig. 1.

4. Discussion

Di(2-ethylhexyl)phthalate, triclosan and propylparaben are widespread environmental pollutants that have been previously tested for toxicity in a variety of *in vitro* and *in vivo* studies. However, limited information is currently available concerning the adverse effects of these three chemicals on terrestrial plant systems. The *A. cepa* test has provided additional and relevant ecotoxicological information that contributes to risk assessment for these contaminants of emerging concern.

Under our experimental conditions, the three chemicals tested may in some way be considered toxic, but different effects were noted depending on the compound and the endpoint analysed. The results for macroscopic parameters used in tests for general toxicity revealed no apparent effects in the presence of DEHP, whereas a dose-dependent inhibition of root growth was observed after exposure to PPB and TCS. Sustained root growth is regulated by the combined activities of cell division in the mitotically active meristeme zone and cell elongation that occurs subsequently in the more proximal regions of the root tip [26]. It is well known that growth rates can be affected by inhibition or disruption of any of these processes, which involve independent events [27]. Since DEHP at high concentrations (≥100 μM) disturbs proliferation of *A. cepa* meristematic cells, as revealed by the significant reduction of mitotic index values, it seems reasonable to assume that roots possibly continue to grow due to elongation of pre-existing cells. In support of this assumption, it has been shown that even complete suppression of cell division may not interfere with cell elongation [28]. Following treatment with PPB, the proliferative capacity of *A. cepa* meristematic cells was decreased in parallel with a sharp decline in root growth. However, mitotic activity seems to be reduced to a lesser extent than root length, which suggests that total inhibition of root growth may result mainly from impaired cell elongation. Likewise, increasing levels of TCS exposure reduced *A. cepa* root

Table 1
Root growth of *Allium cepa* bulbs exposed to different concentrations of the three test compounds for 72 h.

Di(2-ethylhexyl)phthalate (DEHP)		Propylparaben (PPB)		Triclosan (TCS)	
Concentration (μM)	Root length (mean ± SD)[a,b]	Concentration (μM)	Root length (mean ± SD)[a,b]	Concentration (μM)	Root length (mean ± SD)[a,b]
0	100.0 ± 17.3	0	100.0 ± 10.0	0	100.0 ± 5.6
1	124.9 ± 38.1	50	85.1 ± 20.3	1	60.9 ± 15.6 *
10	112.4 ± 5.3	200	47.4 ± 7.4 *	5	35.6 ± 7.00 *
100	121.0 ± 25.6	400	24.3 ± 6.1 *	10	15.6 ± 9.8 *
500	106.6 ± 16.5	500	11.4 ± 14.3 *	30	0.0 ± 7.5 *

[a] Data are expressed as percentage of control values.
[b] Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

Table 2
Mitotic index values in *Allium cepa* root tip cells exposed to increasing concentrations of the three test compounds for 48 h.

Di(2-ethylhexyl)phthalate (DEHP)		Propylparaben (PPB)		Triclosan (TCS)	
Concentration (μM)	Mitotic index (mean ± SD)[a,b]	Concentration (μM)	Mitotic index (mean ± SD)[a,b]	Concentration (μM)	Mitotic index (mean ± SD)[a,b]
0	100.00 ± 16.75	0	100.00 ± 13.06	0	100.00 ± 14.66
1	96.23 ± 5.09	50	64.54 ± 16.02 *	1	117.77 ± 37.85
10	85.61 ± 8.81	200	61.63 ± 7.28 *	5	106.75 ± 17.10
100	78.39 ± 13.92*	400	45.80 ± 12.69 *	10	31.29 ± 7.78*
500	68.47 ± 13.00*	500	43.50 ± 13.08 *	30	31.46 ± 20.14*

[a] Data are expressed as percentage of control values.
[b] Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

Table 3
Cytogenetic alterations in meristematic cells of *Allium cepa*, following tratments with different concentrations of the three testcompounds for 48 h.

Treatment	Micronuclei (mean ± SD)[a,b]	Stickiness (mean ± SD)[a,b]	Abnormal ana–telophases (AT) (mean ± SD)[a,b]				
			Bridges	Vagrants	Chromosome missegregation	Multipolar	Total abnormal AT
DEHP (μM)							
0	0.10 ± 0.10	n.d.	0.30 ± 0.03	0.60 ± 0.40	0.90 ± 0.45	n.d.	1.80 ± 0.08
100	0.96 ± 0.46*	n.d.	0.29 ± 0.16	1.62 ± 0.56	0.35 ± 0.12	0.33 ± 0.41	2.26 ± 0.67
500	0.99 ± 0.23*	n.d.	0.48 ± 0.06	1.76 ± 0.47	6.52 ± 1.53*	1.10 ± 0.75	8.77 ± 1.48*
TCS (μM)							
0	n.d.	1.49 ± 0.36	0.33 ± 0.58	0.66 ± 1.14	0.96 ± 0.07	n.d.	1.95 ± 1.05
1	n.d.	42.50 ± 3.89*	4.04 ± 1.24*	2.65 ± 2.62	11.79 ± 4.32*	n.d.	18.48 ± 5.44*
5	n.d.	34.78 ± 4.40*	4.57 ± 2.56*	1.64 ± 2.27	13.17 ± 6.86*	n.d.	19.39 ± 6.79*

n.d.: not detected.
[a] The frequency of each aberration type is expressed in terms of percentage.
[b] Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

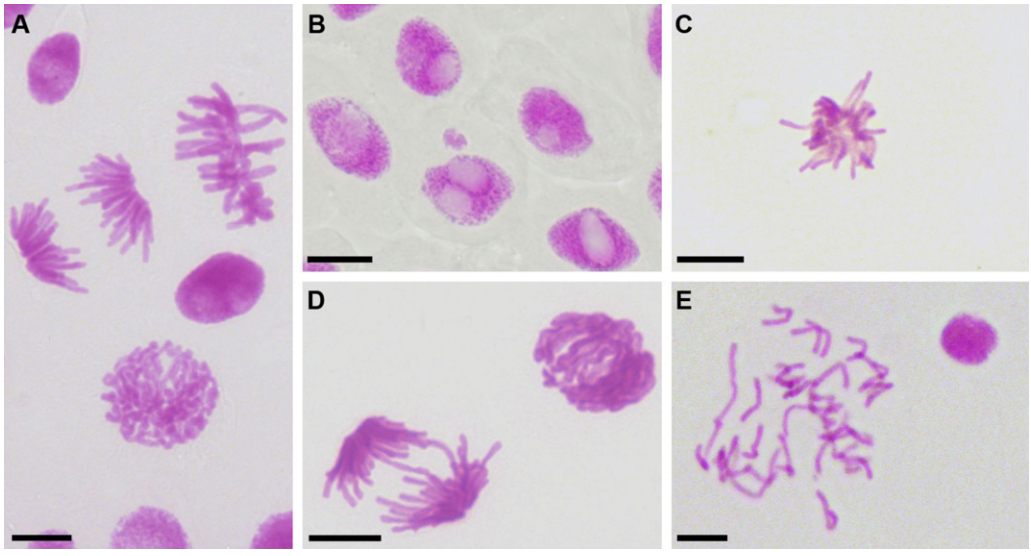

Fig. 1. Representative images of different types of chromosome aberration observed in meristematic cells of *Allium cepa* after a 48-h exposure to DEHP or TCS. (A) Normal mitotic stages in untreated root meristems. (B) Micronucleus induced by 500 μM DEHP in an interphase cell. (C) Sticky metaphase caused by 1 μM TCS. (D) Chromosome bridges after treatment with 1 μM TCS. (E) Chromosome mis-segregation following exposure to 500 μM DEHP. Bar, 10 μm.

growth, which agrees with recent studies with other terrestrial and wetland plant species [29,30]. Moreover, it is noteworthy that the lower TCS concentrations tested in this study caused an apparent but not statistically significant stimulation of cell division, while higher ones ($\geq$10 μM) caused a mito-depressive effect. Our results also revealed that a rise in mitotic activity was concomitant with a slight increase in the percentage of prophase cells, which could be a consequence of delayed mitosis. This hormetic response in cell proliferation has been previously reported for various plants of the genus *Allium* exposed to other phenolic compounds [31,32], as well as in human gingival cells following treatments with low doses of TCS [33].

The results of the cytogenetic analysis showed no evidence of genotoxic potential when PPB was evaluated in the *A. cepa* test, which agrees with previously published data using bacteria, yeast and mammalian *in vitro* systems [16,22]. Nevertheless, these negative results do not exclude other relevant ecotoxicological effects, such as those detected earlier in aquatic organisms [34–36]. In addition, the dose-dependent inhibition of mitotic activity and root growth, as reported in this study, suggests that this compound may also cause toxic effects in higher plants. A number of mechanisms of action have been proposed for PPB in animal cells, including its ability to induce mitochondrial damage [37] and cell membrane alterations [38,39]. Furthermore, we have recently reported that this compound causes antiproliferative effects in cultured mammalian cells, associated with oxidative stress [40]. Despite the fact that a direct comparison and extrapolation of results is difficult, due to differences between plant and mammalian cells, the possible contribution of reactive oxygen species (ROS) to PPB-induced toxicity in *A. cepa* roots cannot be dismissed. Interestingly, it is well documented that generation of ROS is a key critical event in plant root growth, leading to the inhibition of cell elongation and cell division [41,42]. It should be noted that, although increased ROS production is thought to be a major cause of DNA damage, there was no evidence of PPB-induced genotoxic stress under our experimental conditions, suggesting an efficient activation of DNA-damage response signalling.

On the other hand, the decrease in mitotic index caused by DEHP treatments was associated with failed chromosome segregation in anaphase and the appearance of micronuclei. Micronuclei may arise from either DNA breakage leading to a-centric chromosome fragments or from chromosome lagging during cell division [43,44]. On the basis of our observations, it appears likely that micronuclei could be a consequence of spindle disturbances rather than a result of direct DNA damage, since no chromosome fragments were observed in any experimental condition. Although this assumption requires confirmation, it is consistent with previous reports showing that DEHP is not mutagenic/genotoxic in most microbial and mammalian assay systems, and thus defined as an epigenetic toxicant [10]. It should be noted however, that a previous collaborative report on the genotoxic potential of DEHP in *A. cepa* root cells conducted by two different laboratories was inconclusive, because both negative and positive results were obtained under the same experimental conditions [45]. Our findings partially support the positive results of this study and deserve further research on DEHP genotoxicity.

Conversely, chromosome stickiness was the most frequent abnormality detected in root meristems of *A. cepa* after TCS treatments. Stickiness is a highly toxic and irreversible effect, generally leading to cell death [46] and the folding of chromosome fibres into single chromatids [47]. In addition, we observed ana/telophase bridges that result most probably from sticky chromosomes, as well as impaired chromosome segregation which may suggest mitotic spindle disturbances. While a number of studies, reviewed by Capdevielle et al. [48], indicate that triclosan is particularly toxic to freshwater aquatic organisms, no data are available, to our knowledge, on the possible genotoxicity of this phenolic antimicrobial substance in terrestrial plants. Moreover, negative results were generally obtained in most *in vitro* and *in vivo* mutagenicity/genotoxicity tests [49,50]. It should be noted, however, that a dose-dependent DNA damage has been reported in zebra mussel hemocytes [51], earthworm coelomocytes [52] and in the alga *Closterium ehrenbergii* [53]. These findings, along with our results, warrant further mechanistic analysis to provide a comprehensive understanding of the environmental risks of TCS to non-target organisms.

In conclusion, the data of the present study point out that particular attention should be paid to additional or specific modes of action of emerging contaminants in higher plant systems, to avoid underestimation of their environmental risks. In this context, the *A. cepa* bioassay may be a useful and complementary tool to assess the toxic potential of unregulated substances and chemical mixtures found in environmental compartments.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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