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Ecotoxicological Consequences of the Abatement of Contaminants of Emerging Concern by Ozonation—Does Mixture Complexity Matter?

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Abstract: Ozonation has been used to degrade persistent water contaminants, namely, pharmaceuticals and personal care products (PPCPs). However, ozonation can lead to by-products that can be more toxic than the parent compounds. This work aims to assess whether the ecotoxicological effects of ozonation are modified as the initial matrix being treated increases in complexity, considering mixtures of 2, 3, 4 and 5 PPCPs. The following PPCPs were used: two parabens (methylparaben (MP) and propylparaben (PP)), paracetamol (PCT), sulfamethoxazole (SMX) and carbamazepine (CBZ). The following model species were used to assess toxicity: the crustacean *Daphnia magna*, the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor* and the watercress *Lepidium sativum*. There was a trend of increased toxicity with increasing mixture complexity of the untreated samples, except for *D. magna*. The same was observed after ozonation with the exception of the mixture MP+PP, which showed high toxicity to all the tested species, namely 100% immobilization of *D. magna*. The toxicity of SMX to the primary producers decreased pronouncedly after ozonation, except for *L. minor*. This study highlights the importance of considering the complexity of the matrix being treated and of using an ecotoxicological test battery with a wide diversity of species for assessing ozonation efficiency.

Keywords: ozone; advanced oxidation process; degradation by-products; contaminants of emerging concern; recalcitrant compounds; ecotoxicity assessment



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1. Introduction

Water scarcity is a major problem in modern societies owing to the increased demand of exponentially growing human populations and industry development, aggravated by climate change. On the other hand, surface waters are becoming increasingly contaminated with toxic chemicals, namely pharmaceuticals and personal care products (PPCPs), due to both the increased and the recurrent use of these chemicals [1–3]. Conventional wastewater treatment plants (WWTPs) are inefficient at removing these compounds from wastewater due to their recalcitrant character. Indeed, concentrations of PPCPs in the range of $\mu\text{g L}^{-1}$ have been reported in the effluents of WWTPs using conventional wastewater treatments (e.g., [1,2]). Some of these PPCPs are considered contaminants of emerging concern (CECs) as they are little regulated at the same time that there is increasing evidence that they bear potential risk for human and environmental health [4]. CECs raise concerns about their potential effects on the aquatic biota of the receiving water systems and also limit further wastewater reclamation, a much-needed asset for counteracting water scarcity.

To address the reduced efficiency of WWTPs at removing PPCPs from wastewater, the development of advanced oxidation processes (AOPs) to degrade such compounds has been receiving much interest. These processes include ozonation and photocatalysis among others (see reviews by [5,6]). Ozonation, in particular, is the most widely used AOP in the removal of PPCPs [3], being currently used in some countries to enhance contaminant removal from wastewater (e.g., [7,8]). Ozone is a highly oxidative species and has a selective character, reacting faster with highly electrophilic molecules such as the aromatic rings and amino and amine groups that are present in a great number of PPCPs [5]. On the other hand, ozone in water at neutral and alkaline pH can decompose into hydroxyl radicals, which have a greater oxidative potential and nonselective character able to decompose a wide range of contaminants [5]. Furthermore, ozone has the advantages of being easily produced from air or oxygen and allowing high water recovery rates without the production of wastes or sludges [5]. However, ozonation has some disadvantages, namely, the potential to produce refractory by-products [5,7] due to the selective oxidation of the parent contaminants. These by-products can be more toxic to aquatic species than their parent compounds and might lead to the higher toxicity of ozone-treated samples than untreated samples (e.g., [8,9]). For this reason, it is critical to address the ecotoxicological effects of ozonation in order to elucidate whether this process is actually contributing to decreasing the toxicity of the treated wastewater rather than just removing parent compounds. A previous review on the removal of PPCPs from wastewater [3] highlighted the importance of considering the composition of the PPCP mixtures given that PPCPs differ in their physicochemical properties and biodegradability. Despite the previous studies addressing the ecotoxicological effects of the ozonation of mixtures of PPCPs (e.g., [9,10]), the effects of the gradually increasing complexity of PPCP mixtures remain largely unknown. Hence, the potential environmental hazardous effects of mixtures of PPCPs with increased complexity following ozonation needs to be further studied and extended to diverse aquatic species, which motivated the present work and constitutes its novelty. In fact, to the best of our knowledge, there are no similar studies addressing this issue with such a broad range of species.

Considering the abovementioned, we hypothesized that the increase in the complexity of mixtures of PPCPs leads to changes in the ecotoxicity of ozone-treated water samples. Hence, this work aims to assess whether the ecotoxicological effects of ozonation are modified as the initial matrix being treated increases in complexity. For the study purpose, we assessed the ecotoxicological effects of both untreated and ozone-treated samples with from 1 to 5 PPCPs at natural pH. Note that in this study, increased complexity was inextricably intertwined with increased total concentration of PPCPs, i.e., the mixture complexity and the total PPCP concentration increased simultaneously, yet the reactions were realistically adjusted to meet the full degradation of the parent compounds. PPCPs from different classes were selected for the study, such as antibiotics, pain killers, and antimicrobial agents, representing some of the most common classes present in the aquatic environment [1,2]: sulfamethoxazole (SMX), paracetamol (PCT, also known as acetaminophen), carbamazepine (CBZ), and the antimicrobial agents methylparaben (MP) and propylparaben (PP), these latter being commonly used as preservatives in food but mainly in cosmetics and pharmaceuticals [11]. The ecotoxicological assessment was performed using a test battery comprising standard organisms typically used in regulatory environmental hazard assessment that represent different functional levels in aquatic ecosystems: the crustacean *Daphnia magna*, the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, and the watercress *Lepidium sativum*. These species represent groups of organisms potentially affected by the discharge of treated wastewater in water bodies or its use for irrigation in the case of watercress.

2. Materials and Methods

2.1. Chemical Compounds

Methylparaben (MP), propylparaben (PP), paracetamol (PCT), sulfamethoxazole (SMX), and carbamazepine (CBZ) were obtained from Sigma-Aldrich Algés, Portugal ($\geq 99\%$ purity). Their chemical structures are presented in Figure 1. All other chemical compounds used in the experiments were of analytical grade.

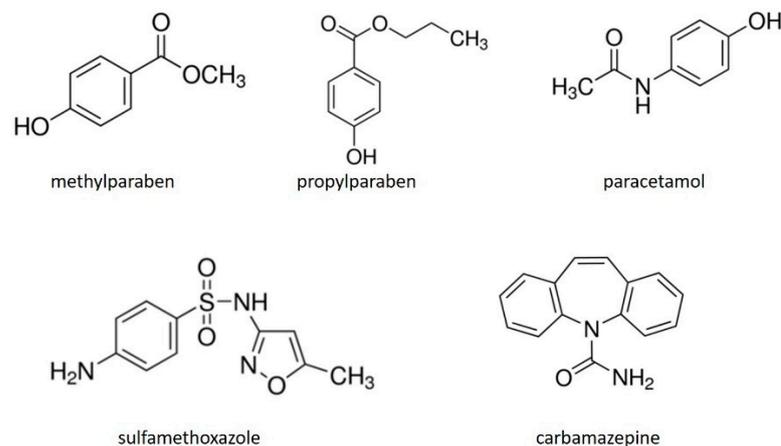


Figure 1. The chemical structures of the PPCPs used in the present study.

2.2. Experimental Design

2.2.1. Samples Preparation

The chemical compounds were dissolved in ultrapure water. This aqueous matrix was selected as it represents a condition of reduced complexity, which facilitates the assessment of the toxicity of each tested PPCP and mixtures of them.

Several treatments were considered: individualized contaminants and mixtures with 2, 3, 4, and 5 contaminants (Table 1). The pH was kept unchanged at 5.5–6.0. The initial concentration of each chemical was 1 mg L^{-1} . Thus, increased complexity from 1 to 5 PPCPs was accompanied by an increase of total concentration of PPCPs from 1 mg L^{-1} to 5 mg L^{-1} , respectively.

Table 1. The treatments and the ozonation conditions (reaction time and transferred ozone dose—TOD). MP: methylparaben; PP: propylparaben; PCT: paracetamol; SMX: sulfamethoxazole; CBZ: carbamazepine.

Treatments	Chemical Composition	Ozonation Conditions (Reaction Time; TOD)
MP	MP	10 min; $7.56 \text{ mg O}_3 \text{ L}^{-1}$
PP	PP	8 min; $10.85 \text{ mg O}_3 \text{ L}^{-1}$
PCT	PCT	20 min; $16.70 \text{ mg O}_3 \text{ L}^{-1}$
SMX	SMX	6 min; $6.53 \text{ mg O}_3 \text{ L}^{-1}$
CBZ	CBZ	1.5 min; $2.10 \text{ mg O}_3 \text{ L}^{-1}$
Mix 2	MP+PP	12 min; $12.39 \text{ mg O}_3 \text{ L}^{-1}$
Mix 3	MP+PP+PCT	30 min; $14.17 \text{ mg O}_3 \text{ L}^{-1}$
Mix 4	MP+PP+PCT+SMX	40 min; $20.85 \text{ mg O}_3 \text{ L}^{-1}$
Mix 5	MP+PP+PCT+SMX+CBZ	60 min; $25.09 \text{ mg O}_3 \text{ L}^{-1}$

2.2.2. Ozonation Procedure

The ozonation of each sample was carried out in a 2 L glass reactor at a constant temperature ($25 \pm 1 \text{ }^\circ\text{C}$). During ozonation, the solution was continuously stirred with a magnet at 700 rpm [9]. Ozone was produced from pure oxygen (99.9%) using an ozone generator (802N, BMT). The inlet gas flow rate was 0.2 L min^{-1} . The transferred ozone dose (TOD), in $\text{mg O}_3 \text{ L}^{-1}$, was determined based on the inlet and outlet ozone concentrations, following Gomes, Frasson [9].

The ozonation duration was defined according to the time needed to reach a total degradation of the initial contaminants in the solution, thus differing among treatments (Table 1).

2.3. Toxicity Assessment

To assess the toxicity resulting from the use of ozone as an oxidation agent, the following species were used: *Daphnia magna*, *Lemna minor*, *Raphidocelis subcapitata*, and *Lepidium sativum*. The toxicity assessment was performed prior to and following the ozonation, thus allowing for comparing the effects of the oxidation treatment.

Daphnia magna

Immobilization tests with *D. magna* were carried out following the OECD guideline 202 [12] with modifications as detailed by Gomes, Frasson [9]. Briefly, each sample was tested in quadruplicate, using 5 daphnids (born in laboratory cultures, less than 24 h old) per replicate. Laboratory cultures were maintained in ASTM hard water [13], supplied with an organic additive extracted from *Ascophyllum nodosum*, at 20 °C ± 1 °C under a 16 h light:8 h dark photoperiod and fed every other day with *R. subcapitata* at 3.0×10^5 cells ml⁻¹. Tests were carried out in glass test tubes. Nutrient spiking was performed to prevent nutrient scarcity, complying with the ASTM hard water recipe. Therefore, each sample was tested at 92.0% strength. A control was also carried out containing ultrapure water, nutrient spiking, and the daphnids. The tests lasted for 48 h under no food supply and were performed under the same temperature and photoperiod conditions as described for the cultures, with an average light intensity of 310 ± 67 lux (mean ± SD). After this period, the immobilized organisms in each vial were counted, and the percentage of immobilization was determined.

Raphidocelis subcapitata

The growth inhibition test with the microalgae *R. subcapitata* was carried out following the OECD guideline 201 [14], with modifications as detailed by Gomes, Frasson [9]. Each sample was tested in triplicate. Tests were performed in 24-well microplates and initiated with 1×10^4 cells mL⁻¹. The microalgae inoculum originated from a laboratory non-axenic culture in Woods Hole MBL medium (MBL, [15]), maintained at 20 °C ± 1 °C under a 16 h light:8 h dark photoperiod. To comply with the MBL medium recipe, nutrient spiking of the samples was performed, and consequently, the tested samples were slightly diluted (98.2% strength). A control consisting of ultrapure water, nutrient spiking, and microalgae was also carried out. Microalgae were allowed to grow during 96 h at 23 °C ± 1 °C and under continuous light supply with an average intensity of 7908 ± 84 lux. At the end of the exposure, algal density was measured by reading the absorbance of each sample at 440 nm on a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The absorbances were converted to cell density using a previously developed calibration curve. The cell density was used to determine the biomass yield (cells mL⁻¹).

Lemna minor

Growth inhibition tests with *L. minor* were based on the OECD guideline 221 [16], with modifications as described by Gomes, Frasson [9]. Each sample was tested in triplicate, using 3 colonies with 3 fronds each per replicate. *L. minor* colonies were obtained from laboratory cultures maintained in Steinberg medium [16] at 20 °C ± 1 °C under a 16 h light:8 h dark photoperiod. Tests were carried out in 6-well microplates. To comply with the Steinberg medium recipe, nutrient spiking was performed, meaning that the tested samples were slightly diluted (93.5 % strength). A control, consisting of ultrapure water, nutrient spiking, and the organisms, was also carried out. Tests were performed at 23 °C ± 1 °C under continuous light supply with an average intensity of 3156 ± 244 lux. After 7 days of exposure, the number of fronds per well was determined, and the macrophytes were dried at 60 °C for the determination of their dry weight. The estimation of the dry weight of macrophytes at the beginning of the tests was performed by applying the same drying-

weighing procedure to seven groups of 3 colonies with 3 fronds, each sampled from the same batch culture used to feed the test. The number of leaves and the dry weight were used to determine both yield inhibition and growth rate inhibition, following the OECD guideline 221.

Lepidium sativum

This test evaluated the germination and radicle growth of watercress (*L. sativum*) seeds exposed to each sample, being carried out as described by Gomes, Frasson [9]. Seeds were purchased from a local store and kept in a dry location until use. Ten seeds were exposed to each sample in a Petri dish containing a paper filter dipped in 5 mL of sample. A control consisting of ultrapure water was also prepared. Each sample was tested in duplicate. The Petri dishes were incubated during 48 h at $27\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in the dark. After the exposure period, the numbers of germinated seeds in each sample (N_T) and in the controls (N_C) were recorded. The seeds were considered germinated when the radicle was visible or, at least, there were evident signs of germination, such as a crack in the seed coat. When applicable, the radicle length was measured in each sample (L_T) and in the controls (L_C), using a digital caliper. These data were used to determine the percent inhibition of seed germination (G) following ISO [17]:

$$G (\%) = (N_c - N_t) / N_c \times 100$$

The percent inhibition of radicle growth relative to the control was expressed as the percentage of phytotoxicity (P), being determined following Sahu, Katiyar [18] as:

$$P (\%) = (L_c - L_t) / L_c \times 100$$

Moreover, the percentage of relative seed germination (RSG), relative radicle growth (RRG), and the germination index (GI) were determined following Trautmann and Krasny [19]:

$$RSG (\%) = N_t / N_c \times 100 \quad (3)$$

$$RRG (\%) = L_t / L_c \times 100 \quad (4)$$

$$GI (\%) = (RSG (\%) \times RRG (\%)) / 100 \quad (5)$$

2.4. Chemical Analyses

Samples taken during the ozonation reaction were analyzed by high-performance liquid chromatography (HPLC, Beckman Coulter System Gold, Pasadena, CA, United States) to evaluate the degradation of the parent contaminants and the formation of by-products. The chromatography column was a C18 (SiliaChrom Quebec Canada) at $40\text{ }^{\circ}\text{C}$. The mobile phase (0.5 mL min^{-1}) consisted of a mixture of 50:50 methanol: acidic water (0.1% orthophosphoric acid). The injection volume was 100 μL . The detection of parabens, CBZ, and PCT was performed at $\lambda = 255\text{ nm}$, and the detection of SMX was performed at $\lambda = 280\text{ nm}$.

The detection of by-products was performed by comparing the spectra of the possible generated by-products with the injected standard by-products. The standard by-products selected are the typical main by-products obtained from the ozonation of these PPPCs. The following standard by-products were considered: 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxybenzoic acid (3,4-diHBA), 2,4-dihydroxybenzoic acid (2,4-DHBA), and 3,4-dimethoxybenzoic acid (3,4-diMBA) as degradation by-products of the parabens; hydroquinone (HQ) as a by-product of parabens and PCT; oxalic acid (OA) as a main by-product of PCT; 1,4-benzoquinone (BQ) as a by-product of parabens and SMX; and 3-amino-5-methylisoxazole (AMI) as a main by-product of SMX. The by-product identification was performed for each compound, individually and for Mix 2. For more complex mixtures (Mix 3, Mix 4, and Mix 5), this identification was not possible due to the overlapping of the

chromatogram peaks and the diversity of the generated by-products (see Section 3.1 for more details).

2.5. Interactive Effects of Mixtures of PPCPs

The toxic units (TU) summation (TUS) method, commonly accepted for measuring toxicity, was used to assess the ecotoxicological interactive effects of the PPCPs present in each mixture, following Kortenkamp, Backhaus [20]. This method is based on concentration addition, which assumes that the components of a mixture share similar modes of toxic action, thus acting as dilutions of each other to produce the toxic effects. The model is defined by the following equation [20]:

$$\text{TUS} = \sum_{i=1}^n \text{TU}_i = \sum_{i=1}^n \frac{c_i}{\text{EC}_{50} x_i} \quad (6)$$

For a given effect level (x), the sum of the TUs will equal 1. The TUs correspond to the ratios between the concentrations of each mixture component (c_i) and its corresponding equi-effective concentration when dosed singly ($\text{EC}_{50} x_i$). If the TUS of a given mixture is lower than 1, the mixture is expected to provoke an overall effect smaller than the assumed effect level x , potentially denoting antagonism; on the other hand, if the TUS is higher than 1, then the mixture is expected to provoke an effect larger than x , potentially denoting synergism.

TUS was calculated for each mixture and for the species *R. subcapitata* and *D. magna*. The lack of EC_{50} values for *L. minor* regarding MP and for *L. sativum* in general prevented this calculation for these species. Calculations were performed considering the average of the EC_{50} values available in the literature regarding the tested PPCPs (see Table 2; only exact values were considered for this analysis, i.e., EC_{50} values denoted as “>100” were not considered).

Table 2. Summary of the reference median effective concentrations (EC_{50}) reported in the literature for each of the tested PPCPs, estimated on the basis of toxicity tests following similar protocols to those carried out in the present study with *R. subcapitata* (formerly known as *Pseudokirchneriella subcapitata*), *D. magna*, *L. minor*, and *L. sativum*.

PPCP	Species	Endpoint	EC_{50} (mg L ⁻¹)	Reference
Methylparaben	<i>R. subcapitata</i>	Growth inhibition (72 h)	92.8	[21]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	91	[22]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	80	[23]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	35.25	[24]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	73.8	[25]
	<i>R. subcapitata</i>	Yield inhibition (72 h)	18.9	[25]
	<i>D. magna</i>	Immobilization (48 h)	11.2	[22]
	<i>D. magna</i>	Immobilization (48 h)	41.1	[26]
	<i>D. magna</i>	Immobilization (48 h)	62	[27]
	<i>D. magna</i>	Immobilization (48 h)	24.6	[28]
	<i>D. magna</i>	Immobilization (48 h)	34	[23]
	<i>D. magna</i>	Immobilization (48 h)	41.23	[24]
	<i>D. magna</i>	Immobilization (48 h)	36.73	[29]
	<i>L. minor</i>	Yield inhibition, as frond number (7 d)	22.0	[25]
	<i>L. minor</i>	Growth inhibition, as frond number (7 d)	27.2	[25]
Propylparaben	<i>R. subcapitata</i>	Growth inhibition (72 h)	15	[22]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	36	[23]
	<i>D. magna</i>	Immobilization (48 h)	15.4	[27]
	<i>D. magna</i>	Immobilization (48 h)	23	[27]
	<i>D. magna</i>	Immobilization (48 h)	12.3	[28]
	<i>D. magna</i>	Immobilization (48 h)	2	[23]

Table 2. Cont.

PPCP	Species	Endpoint	EC ₅₀ (mg L ⁻¹)	Reference
Paracetamol	<i>R. subcapitata</i>	Growth inhibition (72 h)	317.4	[30]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	>100	[31]
	<i>D. magna</i>	Immobilization (48 h)	34.99	[31]
	<i>D. magna</i>	Immobilization (48 h)	4.7	[30]
	<i>D. magna</i>	Immobilization (48 h)	11.02	[32]
	<i>D. magna</i>	Immobilization (48 h)	30.1	[33]
	<i>D. magna</i>	Immobilization (48 h)	50	[34]
	<i>D. magna</i>	Immobilization (48 h)	2.99	[35]
	<i>L. minor</i>	Yield, as frond number (7 d)	429.9	[30]
	<i>L. minor</i>	Yield, as frond number (7 d)	446.6	[36]
Sulfamethoxazole	<i>R. subcapitata</i>	Growth inhibition (96 h)	0.146	[37]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	4.36	[38]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	0.52	[39]
	<i>R. subcapitata</i>	Growth inhibition (96 h)	0.49	[40]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	5.4	[41]
	<i>R. subcapitata</i>	Growth inhibition (96 h)	4.74	[42]
	<i>R. subcapitata</i>	Growth inhibition (96 h)	4.4	[43]
	<i>R. subcapitata</i>	Growth inhibition (72 h)	1.12	[31]
	<i>D. magna</i>	Immobilization (48 h)	98.01	[31]
	<i>D. magna</i>	Immobilization (48 h)	189.2	[33]
	<i>D. magna</i>	Immobilization (48 h)	42.74	[38]
	<i>D. magna</i>	Immobilization (48 h)	43.97	[38]
	<i>D. magna</i>	Immobilization (48 h)	205.2	[44]
	<i>D. magna</i>	Immobilization (48 h)	123.1	[45]
	<i>L. minor</i>	Growth inhibition, as frond area (7 d)	3.07	[38]
	<i>L. minor</i>	Yield inhibition, as frond number (7 d)	1.48	[25]
<i>L. minor</i>	Growth inhibition, as frond number (7 d)	5.02	[25]	
<i>L. minor</i>	Growth inhibition, as frond number (7 d)	12.56	[46]	
<i>L. minor</i>	Growth inhibition, as frond area (7 d)	0.21	[47]	
Carbamazepine	<i>R. subcapitata</i>	Growth rate inhibition (72 h)	>100	[24]
	<i>R. subcapitata</i>	Growth rate inhibition (72 h)	>100	[38]
	<i>R. subcapitata</i>	Growth rate inhibition (72 h)	>100	[31]
	<i>R. subcapitata</i>	Growth rate inhibition (72 h)	46.63	[48]
	<i>D. magna</i>	Immobilization (48 h)	57.56	[48]
	<i>D. magna</i>	Immobilization (48 h)	>100	[31]
	<i>D. magna</i>	Immobilization (48 h)	>100	[24]
	<i>D. magna</i>	Immobilization (48 h)	>100	[38]
	<i>D. magna</i>	Immobilization (48 h)	97.8	[49]
	<i>D. magna</i>	Immobilization (48 h)	21.87	[32]
	<i>L. minor</i>	Growth inhibition, as frond area (7 d)	25.5	[50]
<i>L. minor</i>	Growth rate, as frond area (7d)	50.17	[38]	

2.6. Statistical Analyses

The response of each species to each sample, among both the untreated and ozone-treated samples, was compared using a one-way analysis of variance (ANOVA). Immobilization data (*D. magna*) were arcsine-transformed before analysis. Data normality was checked using the Shapiro-Wilk test, and homoscedasticity was assessed using the Brown-Forsythe test. If the ANOVA assumptions were not met, a nonparametric Kruskal-Wallis surrogate was performed. When significant differences among groups were found, pairwise multiple comparisons were performed using Tukey's test. When significant differences were found by ANOVA, Tukey's multi-comparison test was used to discriminate statistically different treatments. When ANOVA assumptions were not fulfilled even after data transformations, the nonparametric Kruskal-Wallis test followed by the Dunn's multi-comparison test was used.

3. Results and Discussion

3.1. Detection and Identification of By-Products

The ozonation by-products of each parent PPCP are presented in Table 3.

Table 3. Summary of the degradation by-products reported after the single ozonation of each of the tested PPCPs.

PPCP	Ozonation By-Product	Reference
Methylparaben (MP)	1-Hydroxy-methyl paraben; Monohydroxy-methyl paraben; Dihydroxy-methyl paraben; Trihydroxy-methyl paraben Hydroquinone (HQ)	[51] [51]; present study
Propylparaben (PP)	Monohydroxy-propyl paraben; 1-Hydroxy-propylparaben; Dihydroxy-propyl paraben; Trihydroxy-propyl paraben 4-Hydroxybenzoic acid (4-HBA)	[51] [51]; present study
Mixture of parabens, including MP and PP	4-Hydroxybenzoic acid (4-HBA) 2,4-Dihydroxybenzoic acid (2,4-diHBA) 3,4-Dihydroxybenzoic acid (3,4-diHBA) 3,4-dimethoxybenzoic acid (3,4-diMeBA) p-Benzoquinone (BQ)	[9]; present study [9]; present study [9]; present study [9]; present study [9]; present study
Paracetamol	Hydroquinone (HQ) Maleic acid (MA) Oxalic acid (OA) p-Benzoquinone (BQ)	[52,53]; present study [53] [53]; present study [53]
Sulfamethoxazole	p-Benzoquinone (BQ) Oxamic acid; pyruvic acid 3-amino-5-methylisoxazole (AMI) Maleic acid Oxalic acid (OA) 4-aminobenzene sulfonamide; N-(3-phenylpropyl)-acetamide, 2-methyl-benzoxazole p-nitrophenol Phenol Dehydroxylated-sulfamethoxazole Nitro-sulfamethoxazole	[54,55] [54,55] [54,56]; present study [54,55] [54,55] [57] [58] [57] [56] [56,58]
Carbamazepine	1-(2-benzaldehyde)-4-hydro-(1H,3H)-quinazoline-2-one (BQM) Anthranilic acid; glyoxal acid; glyoxylic acid Oxalic acid (OA) 1-(2-benzoic acid)-(1H,3H)-quinazoline-2,4-dione (BaQD) 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione (BQD) BaQM	[59–61] [62] [62] [59,60] [59–61] [60]

Following the ozonation of MP and PP, the by-products 4-HBA, 3,4-diHBA, 2,4-DHBA, 3,4-diMBA, HQ, and BQ formed, which is in accordance with previous studies [9,63].

The ozonation of PCT led to the formation of two by-products, OA and HQ, which agrees with previous studies [52,53]. In general, both by-products were degraded over the time of the reaction, but their total removal was not reached. OA, in particular, formed in the initial times of the reaction; during the first 15 min of reaction, its concentration decreased and then increased again, reaching the highest concentration after 20 min of reaction. Its peak area was larger than that of HQ.

Regarding SMX, the ozonation by-products BQ and AMI have been detected in previous studies [54–56]. In the present study, AMI was detected. The formation of BQ was not confirmed as the peak retention times of SMX and BQ coincided with the method used in HPLC.

During the ozonation of CBZ, the formation by-products was observed, but their accurate identification was not accomplished owing to the absence of standards of common by-products for HPLC. Commonly, the ozonation by-products of CBZ include 1-(2-benzaldehyde)-4-hydro-(1H,3H)-quinazolin-2-one (BQM), and 1-(2-benzaldehyde)-(1H,3H)-quinazoline-2,4-dione (BQD), among others (Table 3). In this study, the most evident by-product exhibited an increased peak area during the reaction. This might have been BQM since this by-product has been reported to be the most significant ozonation by-product of CBZ [60,61].

Regarding mixtures, the identification of ozonation by-products was performed for the paraben mixture (Mix 2), which allowed the detection of all the abovementioned by-products for MP and PP (4-HBA, 3,4-diHBA, 2,4-DHBA, 3,4-diMBA, HQ, and BQ) [9,64]. It is observed that the increase in the amount of BQ is accompanied by a decrease in the amount of HQ (Figure 2A), which is due to the fact that BQ can be obtained from HQ. Note that BQ showed its maximum value at the end of the ozonation treatment. The benzoic acids (4-HBA, 3,4-diHBA, 2,4-DHBA, 3,4-diMBA) appeared at the beginning of the reaction, and some of them persisted at the end of the reaction. However, given that the benzoic acids exhibited low toxicity (*cf.* Table 3), the chemical analysis focused on HQ and BQ (Figure 2A), which are the by-products with higher toxicity (*cf.* Table 3).

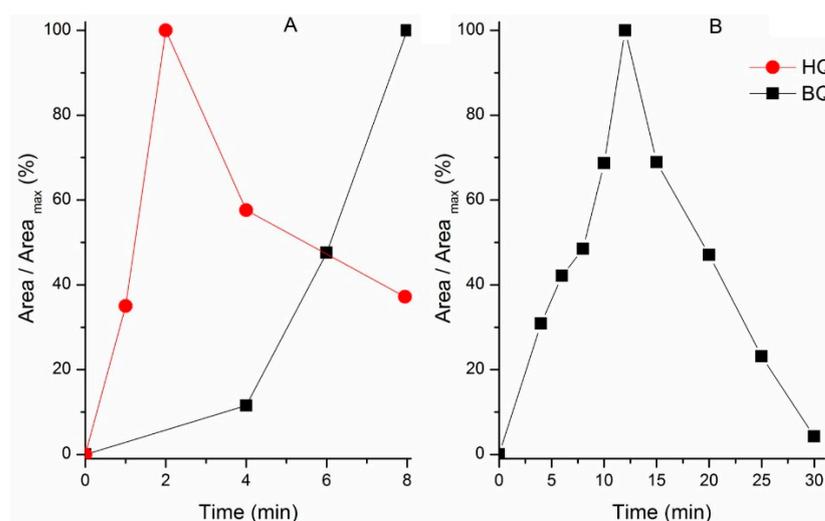


Figure 2. The percent evolutions of the peak areas of hydroquinone (HQ) and 1,4-benzoquinone (BQ) in (A) Mix 2 (MP+PP) and (B) Mix 3 (MP+PP+PCT) throughout the ozonation reaction. The peak areas were obtained by the integration of the chromatogram peak in the HPLC analysis.

In Mix 3 (Figure 2B), it is shown that BQ reaches its higher concentration about 12 min after the start of the reaction, being almost completely removed after 30 min of reaction. It was not possible to identify other by-products of this mixture due to the complexity of the identification of the by-products in the mixtures. For instance, for the developed HPLC method, the retention times of PCT and its HQ by-product coincided, which caused PCT to mask the peak area of HQ, thus hampering HQ reading in mixtures where PCT was added (Mix 3, 4 and 5). A similar situation was observed regarding SMX and its by-product BQ. Due to their very similar retention times, the formation of BQ in mixtures containing SMX (Mix 4 and Mix 5) was indiscernible.

3.2. Toxicity Assessment of PPCPs

The toxicity of the tested PPCPs, individually and in mixtures with different complexity, was assessed for the referred organisms prior to and after the ozonation treatment. Since ozonation assured a complete removal of the parent compounds, the eventual toxicity of the tested samples is likely related to the formation of by-products. Since increased complexity was accompanied with increased total concentrations of PPCPs, the observed ecotoxicological effects are due to both these variables. However, for simplicity, we will refer to “complexity”.

There is scarce information regarding the ecotoxicological assessment of single ozonation for degradation of the tested PPCPs, which hampers the comparison of the results of the present study with those from previous studies.

For *D. magna* (Figure 3), the untreated samples caused no toxicity. This was expected as the initial concentration of contaminants was low (1 mg L^{-1}) compared with the EC_{50} values for the compounds retrieved from the literature (Table 2). After ozonation, there

was a statistically significant difference among groups ($F_{8,36} = 80.969$, $p \leq 0.001$). Mix 2, which registered a 100% immobilization effect, was significantly different from the remaining samples. A slight increase of toxicity was observed for Mix 5, although it was not statistically significant. Regarding Mix 2, the increased immobilization from 0 to 100% after ozonation suggests the formation of by-products bearing much higher toxic potential to *D. magna* than the parent contaminants. Among the identified by-products of parabens (Table 3), it is unlikely that the observed toxicity originated from the benzoic acid derivatives (4-HBA; 3,4-diHBA; 2,4-diHBA; 3,4-diMBA) as their EC_{50} values to *D. magna* are very high ($>100 \text{ mg L}^{-1}$, Table 4). On the other hand, the EC_{50} values of the by-products HQ and BQ for this species were $150 \text{ } \mu\text{g L}^{-1}$ and $124 \text{ } \mu\text{g L}^{-1}$, respectively (Table 4). Since both these by-products were detected after ozonation, it is likely that they contributed to the full immobilization of the daphnids in the ozone-treated Mix 2. Moreover, its interaction within the mixture as well as the presence of other unknown by-products, might also have contributed to the observed toxicity [9]. The high toxicity of the treated Mix 2 to *D. magna* is concordant with that of a mixture of 5 parabens (10 mg L^{-1} each, including MP and PP) after ozonation, tested in [9]. However, no other mixtures that also contained MP and PP were found in the present study to bear high toxicity to *D. magna*. This might be due to the degradation of these by-products as a consequence of the longer ozonation reaction. Indeed, the ozonation time for Mix 2 was 12 min, whereas for Mix 3, Mix 4, and Mix 5, it was ≥ 30 min. Actually, the increased complexity of the mixtures led to the need to increase the reaction times to achieve the complete removal of CECs (Table 1). Gomes, Frasson [9], who studied the ecotoxicity of a mixture of parabens following ozonation, also reported decreased immobilization to *D. magna* after a longer ozonation time, thus suggesting the degradation of the generated toxic by-products and the consequent production of low toxic short-length carboxylic acids [9]. It is worth noting that the ozonation of MP or PP individually did not cause toxicity to *D. magna* (immobilization of 4% and 0%, respectively). Compared with the full immobilization recorded for the treated Mix 2, this suggests a synergistic interaction among the ozonation by-products of both parabens.

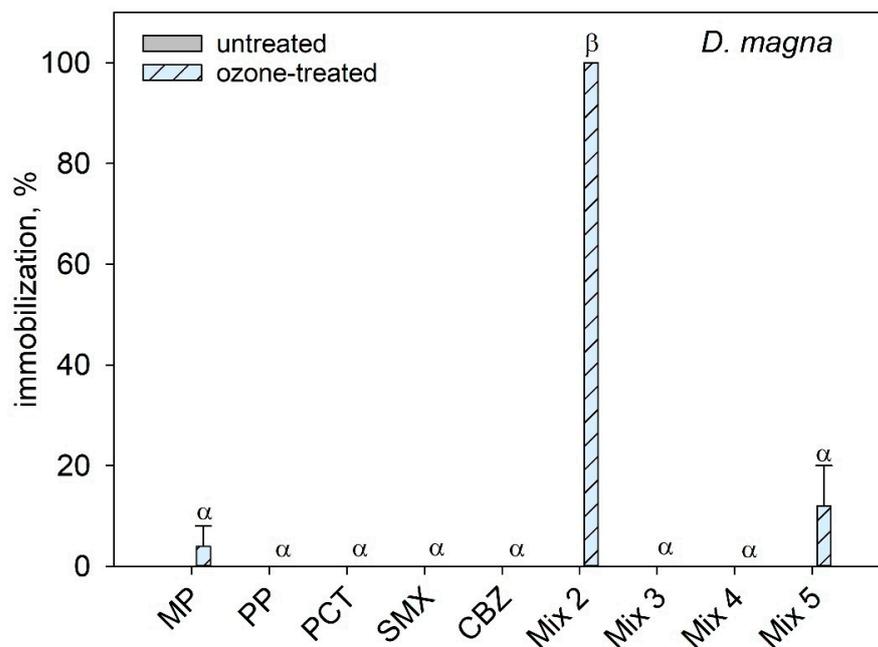


Figure 3. The immobilization of *Daphnia magna* after 48h of exposure to untreated and ozone-treated solutions. The bars represent the mean, and the error bars represent the standard error of the mean. Mix 2: MP+PP; Mix 3: MP+PP+PCT; Mix 4: MP+PP+PCT+SMX; Mix 5: MP+PP+PCT+SMX+CBZ. The absence of a bar means 0% immobilization. The different Greek letters indicate significant differences among ozone-treated samples.

Table 4. Summary of the reference median effective concentrations (EC₅₀) for the reported ozonation by-products of each of the tested PPCPs, estimated on the basis of toxicity tests following similar protocols to those carried out in the present study with *R. subcapitata*, *D. magna*, *L. minor*, and *L. sativum*.

Ozonation By-Product	Species	Endpoint	EC ₅₀ (mg L ⁻¹)	Reference
Hydroquinone (HQ) (by-product of MP, PCT)	<i>D. magna</i>	Immobilization, 48 h	0.150	[65]
	<i>R. subcapitata</i>	Growth rate, 48 h	8.92	[66]
	<i>R. subcapitata</i>	Growth rate, 96–120 h	10.8	[66]
4-Hydroxybenzoic acid (4-HBA) (by-product of PP)	<i>D. magna</i>	Immobilization, 48 h	1690	[26]
	<i>R. subcapitata</i>	Yield, 48 h	270.7	[67]
	<i>R. subcapitata</i>	Growth rate, 48 h	355.0	[67]
	<i>R. subcapitata</i>	Growth rate, 72 h	1367	[68]
	<i>R. subcapitata</i>	Growth rate, 96 h	1602	[68]
2,4-Dihydroxybenzoic acid (2,4-diHBA) (by-product of parabens mixture)	<i>D. magna</i>	Immobilization, 48 h	120	[26]
	<i>R. subcapitata</i>	Yield, 48 h	36.21	[67]
	<i>R. subcapitata</i>	Growth rate, 48 h	80.14	[67]
3,4-Dihydroxybenzoic acid (3,4-diHBA) (by-product of parabens mixture)	<i>D. magna</i>	Immobilization, 48 h	370	[26]
	<i>R. subcapitata</i>	Yield, 48h	267.1	[67]
	<i>R. subcapitata</i>	Growth rate, 48 h	726.3	[67]
p-Benzoquinone (BQ) (by-product of parabens mixture, PCT, SMX)	<i>D. magna</i>	Immobilization, 24 h	0.124	[69]
Oxalic acid (OA) (by-product of SMX, CBZ)	<i>R. subcapitata</i>	Growth inhibition, 72 h	4073	[70]
Phenol (by-product of SMX)	<i>L. sativum</i>	Root length, 48 h	81.2	[71]
	<i>D. magna</i>	Mortality, 24 h	9.6	[71]
	<i>D. magna</i>	Mortality, 48 h	11.64	[72]
	<i>R. subcapitata</i>	Yield, 72 h	197	[73]

Regarding the slight immobilization caused by the ozone-treated Mix 5 (12%), it might have been due to the higher amount of potentially toxic substances challenging the organisms. Indeed, for the other tested organisms (Figures 4–6), we observed a general trend of increased toxicity with the increasing complexity of the mixtures. However, other factors such as the interaction among the increased number of by-products [9] should also be considered.

Regarding the microalgae, the results of yield inhibition are depicted in Figure 4; the results of growth rate inhibition are presented in Figure S1 (Supplementary Material).

Exposure to the untreated samples caused significant differences among the test samples ($F_{8, 25} = 33.937, p \leq 0.001$). Increased microalgae biomass production compared with the control was observed for most individual contaminants, except for SMX and CBZ (Figure 4). This was expected for SMX given its toxicity to *R. subcapitata* (EC₅₀ values ranging between 0.146 and 4.74 mg L⁻¹, Table 2). For CBZ, toxicity was not expected as EC₅₀ is commonly above 100 mg L⁻¹ (Table 2), although one EC₅₀ of 46.63 mg L⁻¹ was found in the literature (Table 2). The effects were more prominent in the case of SMX than in the case of CBZ (77% and 26% of yield inhibition, respectively), which can reflect differing energy requests for detoxification processes and thus differing availability of the cellular energy allocated for growth [74]. These two PPCPs may affect microalgae through mechanisms such as lipid peroxidation and antioxidant enzyme activity [75]. Regarding the mixtures' toxicity, high toxicity was found for mixtures containing SMX and/or CBZ, i.e., Mix 4 and Mix 5 (about 88% for both samples), which is concordant with the toxicity found for these PPCPs individually. There was a tendency of a higher negative impact in microalgae yield with the increasing complexity of the mixture, which is consistent with the fact that the organisms were exposed to a higher amount of potentially toxic substances.

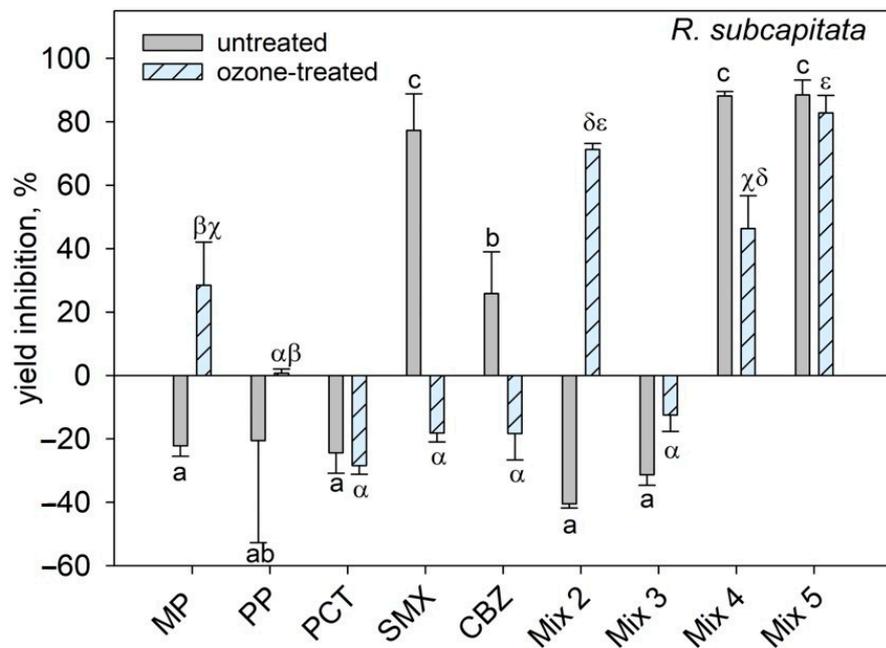


Figure 4. The yield inhibition of *Raphidocelis subcapitata* after 96 h of exposure to untreated and ozone-treated solutions. The bars represent the mean, and the error bars represent the standard error of the mean. Mix 2: MP+PP; Mix 3: MP+PP+PCT; Mix 4: MP+PP+PCT+SMX; Mix 5: MP+PP+PCT+SMX+CBZ. The different Latin letters indicate significant differences among untreated samples; different Greek letters indicate significant differences among ozone-treated samples.

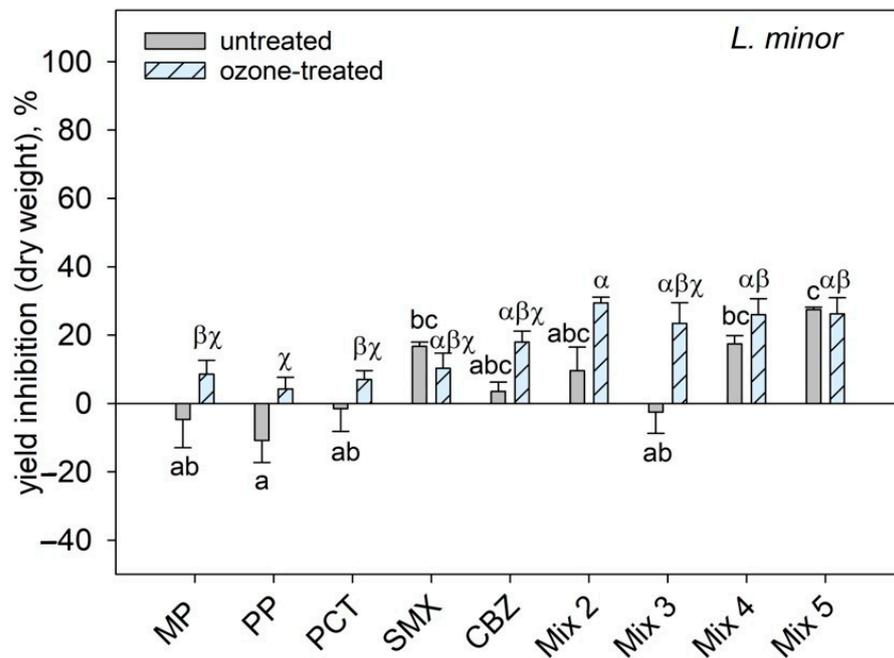


Figure 5. The yield inhibition of *Lemna minor* after 7d of exposure to untreated and ozone-treated solutions, expressed as dry weight. The bars represent the mean, and the error bars represent the standard error of the mean. Mix 2: MP+PP; Mix 3: MP+PP+PCT; Mix 4: MP+PP+PCT+SMX; Mix 5: MP+PP+PCT+SMX+CBZ. The different Latin letters indicate significant differences among untreated samples; different Greek letters indicate significant differences among ozone-treated samples.

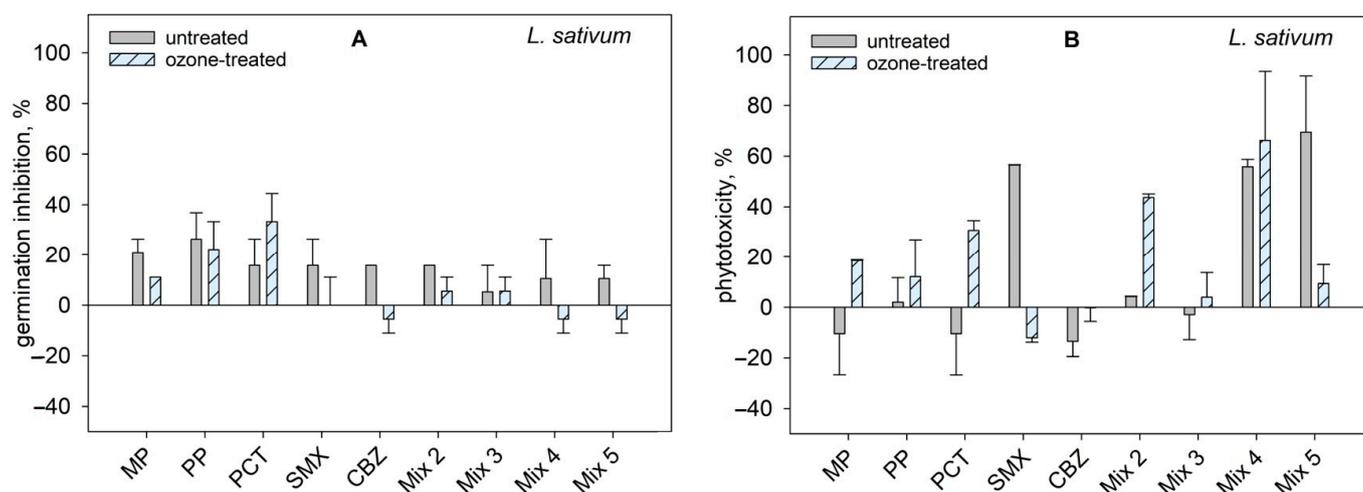


Figure 6. The toxicological response of *Lepidium sativum* after 48 h of exposure to untreated and ozone-treated solutions: the germination inhibition (A) and the percentage of phytotoxicity (B). The bars represent the mean, and the error bars represent the standard error of the mean. Mix 2: MP+PP; Mix 3: MP+PP+PCT; Mix 4: MP+PP+PCT+SMX; Mix 5: MP+PP+PCT+SMX+CBZ.

After ozonation, significant differences among the test samples were also found ($F_{8,26} = 35.711$, $p \leq 0.001$). It is worth noting that samples containing SMX or CBZ individually were no longer toxic but rather became growth stimulating. Regarding SMX, a previous study [76] reported that considerable toxicity to *R. subcapitata* persisted in ozonated samples even after the complete depletion of SMX, but under different conditions (e.g., initial concentration of 40 mg SMX L^{-1} , i.e., 40-fold that used in the present study). Concerning the ozonation of CBZ, a previous study reported no growth inhibition of *R. subcapitata* [62], concordantly to the present study. Conversely, MP and Mix 2 showed increased toxicity following ozonation compared with the respective untreated samples (these latter were actually stimulatory of *R. subcapitata* growth), which suggests the production of by-products with higher toxicity to the microalgae. Although 4-HBA is the main by-product of the ozone degradation of parabens [51], it has low toxicity to *R. subcapitata* ($EC_{50} > 270.7 \text{ mg L}^{-1}$; Table 4), and thus the negative effects of the ozone-treated Mix 2 should be rather associated with the presence of HQ ($EC_{50} \geq 8.92 \text{ mg L}^{-1}$; Table 4), BQ ($EC_{50} = 0.355 \text{ mg L}^{-1}$, Table 4), or other by-products, such as low-length carboxylic acids, aldehydes, and alcohols that could not be fully identified [9]. Concordantly, a previous study also reported the high yield inhibition of *R. subcapitata* exposed to a mixture of 5 parabens (including MP and PP) following ozonation [9]. HQ and BQ can react with proteins, DNA, or lipids, thus affecting the cellular metabolism, including enzyme inhibition oxidative stress [63]. The decreased toxicity of Mix 3 compared with Mix 2 might be due to degradation of the by-products by ozone. This assumption is supported by a previous study reporting decreased inhibition of microalgae exposed to an ozone-treated mixture of parabens with the increasing duration of the ozonation reaction [9].

As for mixtures before treatment, there was a trend of increased toxicity to the microalgae with the increased complexity of the mixture.

The yield inhibition of the macrophyte *L. minor* exposed to the PPCPs before and after ozonation is depicted in Figure 5. Only the results expressed as dry weight are presented here, as they are generally the most reliable owing to their being a direct measure of biomass that is not influenced by the growth stage of the plants. The results concerning the yield inhibition expressed as frond number, as well as growth rate inhibition, are presented in Figure S2 (Supplementary Material).

Among the untreated samples, statistically significant effects on the yield inhibition of the macrophyte ($F_{8,18} = 5.546$, $p = 0.001$) were found. Within individual PPCPs, no yield inhibition was recorded when testing parabens and PCT, but an increase in biomass

compared to controls was observed (Figure 5). As for the microalgae, SMX and, to a less extent, CBZ, inhibited the growth of the macrophyte. A high toxicity of SMX to *L. minor* was expected following the literature, as EC_{50} was within the range 0.21–12.56 mg L⁻¹ (Table 2). Regarding CBZ, previous studies report EC_{50} values between 25.5 and 50.17 mg L⁻¹ (Table 2), and thus, no or low toxicity was expected in this sample. The mixtures generally induced higher toxicity, with Mix 4 and Mix 5 exhibiting yield inhibition (frond number) of 55 and 65%, respectively. The increased toxicity of the samples with increased complexity of the mixture is concordant with the results found for *R. subcapitata* and might be related to higher amounts of potentially toxic substances challenging the organisms and their interaction.

Regarding the treated solutions, statistically significant effects were also observed ($F_{8, 18} = 5.582$, $p = 0.001$). In general, compared with the results for the untreated samples, an increase in yield inhibition was recorded. This again suggests the formation of by-products with higher toxicity than the parent contaminants. Given the lack of information on the ecotoxicological effects of the by-products to *L. minor* (Table 2), it is not possible to appraise which by-products are likely mainly responsible for the observed toxicity. However, given the similarity between the responses of the microalgae and the macrophytes to the tested samples, and given that both species are primary producers, thus sharing metabolic processes, it is likely that the same by-products are responsible for the toxicity of both species.

Concerning the terrestrial plant watercress, *L. sativum*, only the results for the endpoint germination inhibition (G) and phytotoxicity (P) are presented (Figure 6). The results concerning the RSG, RRG, and GI are not presented here as they are not expressed as inhibition, which hampers the comparison of the sensitivity of the tested biological species. However, since they are commonly reported in other studies (e.g., [9,77]) and might be useful for comparison with results of those studies, they are presented in Figure S3 (Supplementary Material).

Regarding the germination of seeds exposed to the untreated samples, no significant differences owing to the test samples were found ($H(8) = 4.045$, $p = 0.853$). Only parabens caused inhibition values above 20% (21% and 26% for MP and PP, respectively). Previous studies reported about 50% inhibition in the germination index (a combination of the results regarding germination and radicle growth) of *L. sativum* seeds exposed to a mixture of 5 parabens, including MP and PP [9,78]. Ozonation, in general, had a beneficial effect on the toxicity of the samples, even stimulating seed germination in some samples. Among the ozone-treated samples, there were also no significant differences among the test samples ($H(8) = 12.383$, $p = 0.135$). Only samples with PP and PCT showed germination inhibition values above 20% (22 and 33%, respectively). The lack of ecotoxicological effective concentrations regarding the toxicity of PCT by-products following ozonation prevents the identification of the possible by-products responsible for this effect.

The phytotoxicity of untreated samples was not significantly affected by the test samples ($H(8) = 14.070$, $p = 0.080$), even though the values varied between -13% (CBZ) and 69% (Mix 5). Phytotoxicity was mainly observed for SMX and the mixtures containing this PPCP (Mix 4 and Mix 5). Pronounced toxicity of SMX was also observed for the other primary producers, which is consistent with the fact that these species likely have similar metabolic processes. Still, the high sensitivity of primary producers to this pollutant is noteworthy as SMX is a bacteriostatic antibiotic largely used to treat respiratory diseases like pneumonia, as well as coccidiosis, diarrhea, and gastroenteritis. However, high toxicity of a mixture containing SMX, CBZ and lorazepam (1 mg L⁻¹ each) has been reported in a previous study for the species *R. subcapitata* and *L. minor* [74]. Following ozonation, no significant differences among test samples were found ($H(8) = 13.474$, $p = 0.097$), despite values varying between -12% (SMX) and 66% (Mix 4). Ozonation increased the phytotoxicity of PCT, Mix 2, and Mix 4 to 31%, 44%, and 66%, respectively. Again, this suggests the presence of more toxic by-products, and it is consistent with the high toxicity observed for the other primary producers exposed to these samples. According to Gomes, Frasson [9],

among the degradation by-products of parabens, BQ and HQ might be the most hazardous ones, not only to *L. sativum* but also to other species. It is noteworthy that ozonation was beneficial regarding the effects of the sample containing SMX as it led to a phytotoxicity decrease from 57% to −12%, i.e., caused a growth stimulation of the radicles.

Overall, it was noticed that ozonation was successful in decreasing the toxicity of SMX to the tested primary producers, except to *L. minor*. SMX was toxic to the tested species, but after only 6 min of ozonation did it become growth stimulating for *R. subcapitata* and *L. sativum*. For this reason, it might be worth considering the use of single ozonation for wastewater treatment in situations where wastewaters are particularly enriched with this PPCP. However, on the other hand, ozonation resulted in a very pronounced toxicity of Mix 2 for the tested species, in particular *D. magna*. The results also highlight the relevant impacts of ozonation time and dose on the by-product formation, which can be problematic for water reuse after ozone treatment. Interestingly, the mixture of both parabens and PCT (Mix 3) usually showed lower toxicity than the mixture of parabens (Mix 2), which suggests that an increased duration of ozonation (30 min for Mix 3; 12 min for Mix 2) can remove the toxic by-products of parabens and/or that the generated by-products interact antagonistically among each other, thus resulting in decreased ecotoxicity. However, further increasing the complexity of the mixtures, even when the ozonation duration increased up to 60 min, resulted in the decreased efficiency of ozonation. For instance, ozonation was not successful in decreasing the toxicity of Mix 5, except for *L. sativum*; for the other species, ozonation had no relevant effects or caused just a minor decrease in toxicity. The increased toxicity with increasing mixture complexity suggests that the sole application of single ozonation in a real context, where wastewaters include a huge variety of contaminants, might not be a suitable option for wastewater treatment, which underpins the combined use of ozonation with other advanced oxidation processes [see review by 5], chemical processes [5], or biological processes (e.g., [5,79]).

3.3. Interactive Effects of Mixtures of PPCPs

In order to assess the impacts of parent compounds within mixtures on *R. subcapitata* and *D. magna*, toxic unit summation (TUS) was followed (Table 5).

Table 5. Toxic unit (TU) and toxic unit summation (TUS) calculation considering the growth rate inhibition (72 h) of *R. subcapitata* and the immobilization (48 h) of *D. magna*. The EC₅₀ values selected from the literature for TU calculation are given in Table 2.

	<i>R. subcapitata</i>	<i>D. magna</i>
MP	0.013	0.028
PP	0.039	0.076
PCT	0.003	0.045
SMX	0.351	0.008
CBZ	0.021	0.017
Mix 2	0.053	0.104
Mix 3	0.056	0.149
Mix 4	0.407	0.157
Mix 5	0.428	0.174

Regarding *R. subcapitata*, and considering the relative toxic strength of the mixtures, growth inhibition below 50% was expected if no interaction among PPCPs occurred, which was the case (TUS < 1; Table 5). The high toxicity of SMX (0.351 TU) led to the increased toxicity of Mix 4 and Mix 5, but the growth rate inhibition observed was still below 50% (37% and 40%, respectively). These results suggest no interactive effects among the tested PPCPs for the microalgae. Regarding *D. magna*, the determined TUS values for the mixtures were well below 1, which is concordant with the lack of immobilization of daphnids exposed to the untreated samples.

However, regarding the ozone-treated samples, we expected to find interactive effects among the generated by-products. For instance, consider the yield inhibition of the microalgae exposed to MP and PP individually (28% and 1%, respectively) compared with that of Mix 2 (71% inhibition). Note that the durations of the ozonation are slightly different (10 min, 8 min, and 12 min, respectively) which can explain, at least partially, these results. However, one cannot exclude the possibility of interactive effects among the generated by-products, as well as between the parent compounds and their by-products.

This work corroborates that one cannot rely solely on the degradation of the parent compounds to infer the hazardous potential of a treated water sample, as the removal of parent contaminants might not be enough to assure that the water is safe to be released in the environment. It also highlights the need to use a battery of biological species for such ecotoxicological assessment as they likely exhibit differing sensitivities to the parent contaminants and to the degradation by-products. This includes not only aquatic species but also other species that might be affected through irrigation by the treated wastewater, such as the watercress.

4. Conclusions

Ozonation was shown to be a powerful oxidation process for decreasing the toxicity of water samples to several species, in particular of SMX. Indeed, after only 6 min of reaction, a decreased toxicity of SMX to the tested primary producers, except to *L. minor*, was observed; the ozone-treated sample became growth stimulating for *R. subcapitata* and *L. sativum*. However, an adverse effect of ozonation was also observed for some samples, in particular regarding Mix 2 (MPb+PP): Ozonation increased its toxicity to all the tested species, in particular to *D. magna*, for which a 100% immobilization was observed.

In general, the increased complexity of the contaminant mixtures (more realistically reflecting the matrices that ozonation should act upon) led to the decreased efficiency of ozonation regarding the environmental safety of the treated water samples, which might underpin the need to combine ozonation with other chemical or biological processes to sustain an efficient decrease of the toxicity of the wastewaters undergoing treatment. Our results also highlight the importance of assessing mixtures' toxicity rather than simple PPCPs and show the relevance of using a diverse ecotoxicological test battery to properly capture the efficiency of the water treatment, as responses differed among the tested species. Moreover, the relevance of ozone dose and time were evident since for longer ozone reactions (higher ozone doses), distinct degradation patterns are in place for parent compounds, often with the formation of more toxic by-products.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14111801/s1>, Figure S1: Growth rate inhibition of *Raphidocelis subcapitata* after 96 h of exposure to untreated and ozone-treated solutions; Figure S2: Ecotoxicological responses of *Lemna minor* after 7d of exposure to untreated and ozone-treated solutions, expressed as: yield inhibition as a function of the number of fronds (A); growth rate inhibition as a function of dry weight (B); growth rate inhibition as a function of the number of fronds (C); Figure S3: Ecotoxicological responses of *Lepidium sativum* after 48 h of exposure to untreated and ozone-treated solutions, expressed as: relative seed germination (RSG; A); relative radicle growth (RRG; B) and germination index (GI, C).

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Data Availability Statement: All data supporting the results of the present study are reported in the manuscript or as Supplementary Information.

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