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Ecotoxicity variation through parabens degradation by single and catalytic ozonation using volcanic rock

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Abstract

Parabens are widely used as antimicrobial and preservative ingredients in pharmaceutical and personal care products. Nevertheless, these compounds have been increasingly seen as emerging contaminants that can be toxic to a wide range of species. In this study, the toxic effect of a mixture of parabens (10 mg/L of each paraben: methyl-, ethyl-, propyl-, benzyl- and butylparaben) and its degradation products through single and catalytic ozonation (using volcanic rock as low-cost catalyst) was investigated over several non-target species: cladocerans, microalgae, clams, macrophytes and cress. The analysis of the toxicity of parabens mixture is relevant since usually these compounds are used as blends rather than individually. While parabens were totally removed both by single and catalytic ozonation the toxicity of the samples resulting from both treatments was generally high. This toxicity was still compared to the one obtained for several dilutions of the initial parabens mixture and it was concluded that the by-products formed are more toxic than the most diluted parabens mixture sample (0.625 mg/L). While catalytic ozonation allows reducing the amount of ozone (about 3-fold) required for total removal of parabens, the resulting treated solution was more toxic than the sample taken at the endpoint of the single ozonation treatment. This suggests that the highest amount of ozone used for single ozonation allowed the elimination of toxic by-products such as hydroquinone and 1,4- benzoquinone. Still, the effect of by-products and parabens interaction depends on the species analyzed due to their different tolerances to potentially toxic products.

Keywords: Parabens toxicity; By-products; Ozonation; Catalytic ozonation; Low-cost catalysts; Ecotoxicity mitigation.

1. Introduction

Parabens are widely used as preservative and antimicrobial agents in food and cosmetics [1,2], but mainly in pharmaceutical and personal care products [3,4]. Some studies relate these compounds with the onset of human breast cancer [5] and their estrogenic activity, although weak, was already shown [6]. In fact, parabens were already detected in human urine, milk and serum [4,7,8]. In this context, these substances have also been quantified at the micro- and nanogram per liter range in wastewater treatment plant and surface waters worldwide [9,10,11,12]. As such, concern has been raised regarding the potential impact of parabens in aquatic organisms. On the one hand, the traditional municipal wastewater treatments are not totally effective on the removal of these biorefractory compounds, which makes their discharge into natural waterbodies more likely to occur, threatening aquatic ecosystems. On the other hand, drinking water treatment facilities using contaminated waters may not totally deplete these substances [9,12]. Bearing in mind that freshwater scarcity is nowadays more present than ever, the seek for alternative water sources such as recycled water increases. Even though the uses for recycled water can be restricted, it has the potential to supply e.g. agriculture. This highlights the significance of improving treatment systems to ensure a non-toxic profile of this resource.

Awareness on the environmental hazardous potential of parabens has been leading to an increasing research attention on the aquatic ecotoxicity of these compounds. Dobbins et al. [13] highlighted the link between the increase in lipophilicity (generally translating alkyl chain length) and the increase of toxicity to the cladoceran *Daphnia magna* (median lethal concentrations following 48-h exposure, 48 h-LC_{50} between 4 and 25 mg/L) and the fish *Pimephales promelas* (48 h-LC_{50} between 3 and more than 160 mg/L). The positive relationship between alkyl chain length and the aquatic toxicity of parabens has been generally confirmed in further studies. For example, methyl, ethyl and propyl parabens present moderate toxicity while butyl and benzylparabens revealed high toxicity for the bacteria *Aliivibrio fischeri*, with 15-min luminescence inhibition concentration of 50% (IC_{50}) values ranging within 0.11 to 9.6 mg/L [14]. Yamamoto et al. [15] verified the same trend by

recording median toxicity values ranging within 0.7 and 80 mg/L following short-term exposure of freshwater microalgae, cladocerans and fish. These studies addressed the single toxicity of parabens, but it is worth stressing out that these compounds are usually used as mixtures to increase their preservative spectrum, thus analysis of the toxicity of parabens blends is necessary.

Accounting to the increasing presence of these contaminants in natural waterbodies and their hazardous potential, research has been focusing on the development of methodologies for their removal from wastewater matrices before discharge. Among those technologies, the Fenton process [16,17], electrochemical oxidation [18], single and catalytic ozonation [19,20], photolytic and phototocatalytic oxidation [21,22,23], photosonochemical degradation [24], photocatalytic ozonation [25] can be mentioned. These methodologies were already proven effective on the degradation of single parabens and mixtures of parabens with higher or lower energy cost. Also, ozone is a powerful oxidant often used to promote the degradation of nucleophilic and high electronic density species such as parabens [19,20,26]. However, single ozonation can be a source of refractory compounds due to its low mineralization efficiency [27]. Normally, heterogeneous catalysts can be used to enhance ozonation and improve the mineralization degree [20,26,27]. The selection of the catalyst must be carried out by looking at the cost production, the stability and performance [26,27]. Low cost materials such as volcanic rock were already proven efficient for parabens degradation through catalytic ozonation [28].

Still, most of the studies addressing the development of treatment processes targeting parabens degradation do not analyze the ecotoxicity mitigation of the water throughout the degradation process. Nevertheless, some studies show the effect of the treatment over the toxicity but only for the final treated samples and does not consider the toxicity evolution along the treatment time (see e.g. [17,20,24]]). An appraisal on the ecotoxicity of the treated samples through the treatment time would make it possible to more accurately select the earliest point where the degradation was long enough to significantly reduce toxicity to negligible levels, thus reducing treatment length and costs. In this

way, Velegraki et al., [30] followed the effect of the decrease of methyl paraben concentration along solar photocatalytic degradation in *Artemia* nauplii.

The aim of the present study was to compare single and catalytic (using volcanic rock) ozonation as to their effectiveness in reducing the ecotoxicity of a mixture of five parabens (Methyl, Ethyl, Propyl, Butyl and Benzylparaben). While an ecotoxicity study considering a mixture of parabens is novel *per se*, it is intended to add further by assessing the levels of toxicity throughout the degradation process, providing information about the toxicity of the parent compounds and byproducts formed along the reaction. The applied ecotoxicological battery was designed to properly reflect potential impacts over aquatic ecosystems, thus included species representing different functional levels: the green microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, the cress *Lepidium sativum* (plant), the cladoceran zooplankter *Daphnia magna*, the benthic bivalve *Corbicula fluminea*.

2. Material and Methods

2.1. Chemicals and dilutions

Solutions were prepared using ultrapure water and five parabens: Methyl (MP), Ethyl (EP), Propyl (PP), Benzyl (BeP) (obtained from Sigma-Aldrich) and ButylParaben (BuP) (purchased from Fluka). A mixture of these parabens (10 mg/L each) was treated by single and catalytic ozonation using different transferred ozone doses (see the methodology details below). Treated solutions, comprising different concentrations of parabens and by-products were chemically analysed and underwent ecotoxicity assessment (see below for details). The standards of 4-Hydroxybenzoic acid (4-HBA), 3,4-Dihydroxybenzoic acid (3,4- diHBA), 2,4-Dihydroxybenzoic acid, Hydroquinone, 1,4-Benzoquinone and 3,4- Dimethoxibenzoic acid (3,4- diMeBA) were acquired from Sigma-Aldrich. As an attempt to isolate the effects of treatment by-products, the ecotoxicity of these treated solutions was compared with that of several direct geometric dilutions from the initial mixture of parabens (10, 7.5, 5, 2.5, 1.25, 0.625 mg/L each paraben).

2.2. Parabens mixture treatment - Experimental procedure

A 2-L reactor magnetically stirred at 700 rpm was used in the single and catalytic ozonation experiments, under controlled temperature conditions (25 ± 1 ^{o}C) achieved with a thermostatic bath. The volcanic rock used as catalyst was collected in São Miguel (Azores, Portugal). A comprehensive characterization of this catalyst can be found in a previous study [28], with main features being the presence of silica and aluminium, augite and diopside as main minerals, and a specific surface area of 28.3 m^2/g. The adsorption of parabens to the volcanic rocks surface was previously found irrelevant [28]. The amount of catalyst used for different experiments was fixed at 0.5 g/L.

Ozone was generated in 802N, BMT apparatus from a pure oxygen stream (99.9%). The gas outflow leaving the reactor was trapped by a solution of potassium iodide (Panreac) for the remaining ozone removal before discharge to the atmosphere. The inlet ($[O_3]^{in}$) and outlet ($[O_3]^{out}$) ozone concentrations were measured by ozone gas analysers (BMT 963 and 964 vent, BMT). The data collected was used for transferred ozone dose (TOD) determination according to Equation 1.

$$TOD = \int_0^t \frac{Q_{Gas}}{V_{Liquid}} \times \left([O_3]^{in} - [O_3]^{out}\right) \times dt \qquad \text{Equation 1}$$

Where Q_{Gas} represents the gas flow rate (0.2 L/min), V_{Liquid} is the volume of the effluent used in the reactor (2 L). The TOD was expressed in mg O_3/L.

2.3. Analytical techniques

The concentrations of the five parabens and degradation intermediates (4-HBA, 2,4 and 3,4-di HBA, 3,4-diMeBA, hydroquinone and 1,4-benzoquinone) were analytically determined for different untreated and treated solutions by high-performance liquid chromatography equipped with a diode array detector, HPLC-DAD (UFLC, Shimadzu). The standards of each one were firstly analyzed and a calibration curve was obtained. The mobile phase (0.5 mL/min) consisted in a mixture of 50:50 methanol: acidic water (0.1% orthophosphoric acid). A C18 column from SiliaChrom, at 40 ^{o}C was used and compounds were detected at 255 nm.

Chemical oxygen demand (COD) was determined according to the standard method 5220D [31]. Potassium hydrogen phthalate, obtained from Panreac, was used to prepare a calibration curve with COD values within the range 0-100 mg O_2/L. Absorbance values after 2 hours of digestion at 150 ºC (ECO25 – VelpScientifica) were measured at 445 nm in a WTW photolab S6 photometer.

A TOC analyser (TOC-V CPN model, Shimadzu, Japan) coupled to an autosampler (model V-ASI, Shimadzu, Japan) was used for determining total organic carbon (TOC) using nondispersive infrared analysis after oxidative combustion.

After catalytic ozonation, the leaching of metals from the volcanic rocks due to the acidic pH was analysed. The solution composition in Al, Fe, Cu, Zn, K, Na, Ca and Mg was measured through atomic absorption in a PerkinElmer 3300 spectrometer.

pH was determined using a Crison micropH 2002 apparatus.

2.4. Ecotoxicity analysis

An ecotoxicological test battery was applied to 16 samples produced as detailed above: 6 samples corresponded to geometric dilutions of the bulk parabens mixture; 5 samples corresponded to sequential stages of the single ozonation treatment; 5 samples corresponded to sequential stages of the catalyzed ozonation treatment. The samples treated with single ozonation were obtained for TOD values of 6, 18, 78, 123, 170 mg/L. On the other hand, the samples used on ecotoxicity tests from catalytic ozonation were obtained for TOD values of 3, 9, 29, 42, 55 mg/L. These TOD values correspond respectively to 5, 15, 60, 90, 120 minutes of reaction time. The pH of all samples was adjusted to values within the range 6.5 - 7.5 to mitigate the interference of different pH levels in the tests outcome. This pH range was achieved by adding sodium hydroxide or sulfuric acid solutions to the samples. The initial pH of treated and diluted samples ranged from 3.5 to 5.2. The range was 3.5-5.2, where 5.2 was for the initial parabens mixture and 3.5 was obtained for highest TOD value of single and catalytic ozonation.

To evaluate the phytotoxicity of the initial mixture of parabens with different dilutions and treated samples for each TOD value, the number of germinated seeds and the radicle growth of *L. sativum* were determined to calculate the germination index (GI) (Equation 2). The phytotoxicity was classified according to the germination index using Trautmann and Krasny [32] criteria. In this test, 10 seeds of *L. sativum* were evenly spread on a filter paper in a petri dish. The volume of sample used to spike the filter paper was 5 mL. The experiments were run in duplicate. The control was set up with ultrapure water. Test treatments were incubated in an oven at constant temperature of 27 ^{o}C for 48 h. Then the number of seeds germinated and the radicle growth in each replicate was recorded. Germination index (GI) is the product of relative seed germination (RSG) by relative radicle growth (RRG) as in equations 2-4.

$$GI\ (\%) = \frac{RSG\ (\%)\ x\ RRG\ (\%)}{100} \qquad \text{(Equation 2)}$$

$$RSG\ (\%) = \frac{N^{SG,T}}{N^{SG,B}} x\ 100 \qquad \text{(Equation 3)}$$

$$RRG\ (\%) = \frac{L^{R,T}}{L^{R,B}} x\ 100 \qquad \text{(Equation 4)}$$

Where $N_{SG,T}$ and $N_{SG,B,}$ were the arithmetic means of the number of seeds germinated for each sample and the control, respectively; $L_{R,T}$ and $L_{R,B}$ correspond to the average radicle length for each sample and the control, respectively.

The inhibition in the growth of the green microalgae *R. subcapitata* as induced by the prepared samples was assessed following guidelines by OECD [33] adapted to the use of 24-well microplates [34]. The microalgae have been cyclically maintained in the laboratory as a non-axenic bulk culture in Woods Hole MBL medium (MBL; [35]), this culture appropriately sourcing the toxicity test through a previously isolated intermediate inoculum. The microalgae (initial cell density of 10^4 cells/mL) were exposed to each (nearly) full-strength sample. To prevent the interference of nutrient scarcity in the growth of the microalgae during the test, the samples were spiked with nutrients so that they reach the same nutrient levels as supplied in MBL. Accounting to both the volume of the nutrient spike and the volume used for microalgae inoculation in each microplate well, samples were

not actually tested full-strength but rather slightly diluted (all established at 98.2% strength). Each sample was tested in triplicate, along with the blank control. The microplates were incubated for 72 h under continuous light at 23 ± 1 °C. The algal suspension in each well was thoroughly mixed by repetitive pipetting twice a day. At the end of the test, cell density was quantified spectrophotometrically at 440 nm based on a previously established calibration curve. The biomass yield (cells/mL) was calculated as the difference between cell density at the end and the beginning of the test. The results were made relative to the control yield (% inhibition in yield).

All samples were tested for their ability to impair the growth of the macrophyte *L. minor*, following OECD [36] guidelines adapted to the use of 6-well plates [37]. A laboratory culture of the macrophyte has been cyclically maintained (renewal once a week) in Steinberg culture medium [36]. Growth inhibition tests were performed under continuous light at 23 ± 1 °C in triplicate, and six plain-Steinberg control replicates were run in parallel. Each well used as a test replicate contained 10 mL of test solution plus 3 macrophyte colonies of 3 fronds each. To prevent the interference of nutrient scarcity through the test, the samples were spiked with nutrients so that they reach the same nutrient levels as supplied in Steinberg, this meaning that the samples were not actually tested full-strength but rather slightly diluted (93.5% strength). At the beginning of each test, 6 biomass replicates (3 colonies of 3 fronds) were oven-dried for 24 h at 60 °C to record initial dry weight. At the end of the test, the fronds present in each well were collected and oven-dried (at least 24 h at 60 °C) for dry weight records. Yield based on dry weight records was calculated. The results were made relative to the control yield (% inhibition in yield).

Acute toxicity tests with *D. magna* were conducted following the OECD guideline 202 [38] to address the ability of the samples to promote organism's immobilization. Monoclonal cultures of *D. magna* have been reared in the laboratory in synthetic ASTM hardwater medium (ASTM; [39]) supplied with an organic additive extracted from the algae *Ascophyllum nodosum*. Cultures were renewed three times per week, and the organisms were fed after renewal with 3.0×10^5 cells/mL *R. subcapitata*. Cultures and then the tests were kept under a constant $16h^L$:$8h^D$ photoperiod and

temperature of 20 ± 2 °C. The tests were carried out using neonates from the 3rd to the 5th broods, ageing less than 24 h, in test tubes containing 10 mL test sample or blank ASTM control. Nutrient spiking was also ensured at the levels set for ASTM in cultures, meaning that samples were tested slightly diluted (92% strength). Twenty animals were used per treatment, randomly assigned into 4 replicates with 5 animals each, and the test lasted for 48 h. The number of immobilized animals was recorded at the end of the exposure period.

The mortality of *C. fluminea* was analysed with acute toxicity tests. *C. fluminea* was collected in Mira, Portugal (N40°25'06.90''/W8°44'13.18''), where a well-established population (density above 2000 clams /m^2) stands [40]. Clams were collected from the canal by sieving sediment into a 5-mm mesh bag. Individuals with shell length in the range 20-30 mm were selected and immediately moved in field water to the laboratory, where they were gradually acclimated to the laboratory conditions changing the water once a week along three weeks before testing. The toxicity tests were run in vessels containing 10 clams with 500 mL of samples or dechlorinated tap water. Two replicates were prepared for each sample along with a blank control. The vessels were kept at constant temperature (20 ± 2 °C), under a 16 h^L:8 h^D photoperiod cycle and continuous aeration. The mortality of clams was assessed every 24 h during 72 h (the maximum period of test) based on the animal's siphoning activity and their resistance to valve opening when forced with a blunt dissection needle [41].

3. Results and Discussion

3.1. Single vs catalytic ozonation regarding organic matter removal

Parabens degradation along single and catalytic ozonation was studied in a previous work [28] and is now briefly described. Total parabens degradation was achieved in both treatments for the highest TOD value (55 and 170 mg/L for catalytic and single ozonation, respectively). From this mixture, benzylparaben is the fastest removed compound due to the presence of two benzenic rings which allows the removal using a TOD value of 29 mg/L in catalytic ozonation and 123 mg/L in

single ozonation. For the remaining parabens the behavior was almost the same with small differences for butylparaben. As an example, is described the degradation of methylparaben. In the catalytic ozonation process, from TOD values of 3 to 9 mg/L, MP concentration reduced from 9 to 7 mg/L, whereas the most significant removal was found from TOD value 29 mg/L where the MP concentration reached about 3.5 mg/L. With a TOD value of 42 mg/L the MP concentration was about 0.625 mg/L. In catalytic ozonation, for TOD values of 9, 29, 42 and 55 mg/L the by-products 1,4-benzoquinone and hydroquinone were found with concentrations of 80, 160, 187 and 194 µg/L (hydroquinone) and 4, 28, 38 and 71 µg/L (1,4-benzoquinone). In the single ozonation process for TOD values from 6 to 18 mg/L, the MP concentration reduced from 9 to 7.5 mg/L. When TOD reached 78 mg/L, MP concentration was about 4 mg/L. With a TOD value of 123 mg/L the MP concentration reached about 1.25 mg/L. In what concerns the by-products hydroquinone and 1,4-benzoquinone for the TOD values 18, 78, 123 and 170 mg/L the concentrations were 38, 100, 31 and ~0 µg/L to hydroquinone and for 15, 22, 70 and ~0 µg/L to 1,4-benzoquinone.

Normally, regardless the advanced oxidation process used for contaminants degradation, some refractory compounds can be produced during the reaction. The production of refractory compounds does not allow total mineralization, which means that COD and TOC can be still significantly high after treatment [26,27]. In this way, COD and TOC decrease was verified for different TOD values resulting from ozonation and catalytic ozonation processes. For the initial mixture of five parabens at 10 mg/L each, the initial COD and TOC values were of about 90 mg O_2/L and 35 mg C/L, respectively. As expected, a consistent decrease in COD and TOC values was observed as the dilution of initial parabens mixture increased (Table A1).

For the highest simple dilution of parabens mixture (0.625 mg/L of each paraben), TOC and COD were not measured since the quantification of such a low quantity of carbons and chemical oxygen demand is difficult with the methodologies used. Moreover, the TOC and COD range obtained with this baseline analysis covered the ranges obtained at the degree of mineralization achieved with catalytic and single ozonation processes, thus feasibly supporting comparative

analysis. Parabens were totally removed at the highest value of TOD (170 mg/L) with single ozonation [20], but COD and TOC values were still high (about 66 mg O_2/L and 28 mg C/L, respectively) after treatment, as shown in Figure 1. Catalytic ozonation leads to total removal of parabens using a TOD of 55 mg/L [28]. For a TOD value of 55 mg/L, TOC was slightly lower (about 26 mg C/L) than the one reached by single ozonation. An important feature when comparing single and catalytic ozonation is the significant reduction of TOD of about 3 folds required for degradation in the presence of volcanic rock.

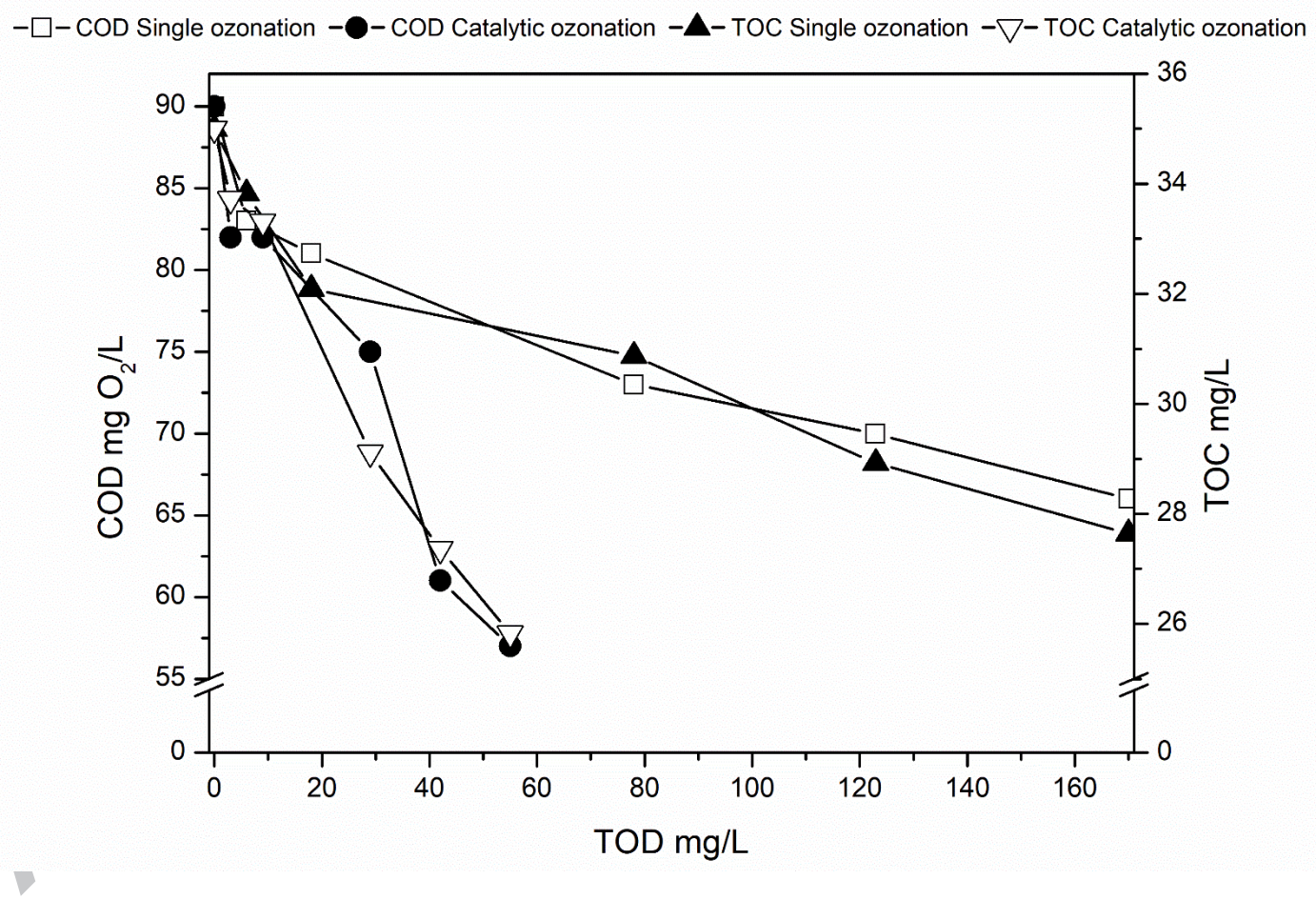

Figure 1: COD and TOC reduction through treatment of the initial mixture of parabens with catalytic and single ozonation.

The need of higher TOD values on the single ozonation can denote the production of more refractory compounds since more ozone is being required for further degradation. However, these

compounds can be further oxidized using catalytic ozonation [26]. In the presence of volcanic rocks, the main responsible for parabens degradation are hydroxyl radicals instead of molecular ozone, which would allow a higher COD removal due to the non-selective process degradation [28]. According to the COD and TOC values obtained while analyzing several simple dilutions of the initial parabens mixture (Table A1), the final COD and TOC obtained for single and catalytic ozonation would correspond to a solution containing 7 mg/L of each paraben. However, HPLC results [28] show that total parabens removal was achieved with both treatment processes. Provided that full parabens removal can be assumed for both ozonation treatment processes applied to the samples, the proper conditions were ensured to address the role of treatments byproducts in defining further the characteristics of the treated samples, namely their toxicity.

Table 1 presents the metal concentrations in water after catalytic ozonation for the highest TOD value (55 mg/L). It should be highlighted through Table 1 that the volcanic rock is stable regarding active metals leaching to the liquid bulk provided the low metals concentration found in the treated water. However, some toxic effect resulting from these ions cannot be discarded as it will be discussed below.

Table 1. Amount leached of selected (based on previous characterization of the volcanic rock used as catalyst [28]) metals for the highest TOD value in catalytic ozonation.

Elements	Al	Fe	Na	Ca	Mg
Amount leached (mg/L)	0.13	0.21	1.12	3.18	0.26

3.2. *Ecotoxicity evaluation of parabens mixture through simple dilution series*

Several studies have been carried out regarding the potential ecotoxicity of parabens (e.g. [12,15,42]), and these personal care products have been given attention regarding the need for a structured environmental risk assessment to properly conclude on their hazardous potential to the aquatic biota (e.g. [43]). However, these studies addressed only a low number of indicator species or

consider single exposures of parabens while their mixture (normally used to increase paraben's preservative spectrum) was not yet considered as far as we known as a variable to assess treatment efficacy or efficiency.

Table 2 shows the ecotoxic outcome of simple sequential dilutions of the initial mixture of five parabens. Regarding the animal species, *C. fluminea* was found more tolerant than *D. magna*, as denoted by the high immobilization rates by the latter observed at lower concentrations (Table 2), and confirmed by estimated EC_{50} values via probit analysis – immobilization EC_{50} of 15.6 (95% confidence interval: 14.6-16.7) mg/L compared to a significantly higher LC_{50} of 25.2 (confidence interval: 23.7-26.8) mg/L. The producers tested in the present study were less sensitive to the mixture of parabens than the animals. This is in general agreement with the literature. For example, Yamamoto et al. [15] found the same trend as they compared *D. magna* and *Pseudokirchneriella subcapitata* (former name for *R.subcapitata*) regarding their sensitivity to single exposures of the five parabens also used here, except for BeP, which was found more toxic to *D. magna*. According to the Trautmann and Krasny [32] criteria, concentrations of parabens mixture of and lower than 2.5 mg/L each paraben were non-phytotoxic, while for solutions above of 7.5 mg/L each paraben, the *L. sativum* GI values reveal that the tested samples can trigger phytotoxic effects. These results show that *L. sativum* is the most tolerant to parabens mixture of the species tested. Similarly, to *L. sativum, L. minor* at the maximum concentration of parabens mixture tested achieved around 50% of yield inhibition on a dry weight basis. Still, according to our results (Table 2) low amounts of parabens (0.625 mg/L of each one, meaning 3.125 mg/L in total) do not show short-term toxicity to any of the five-species tested.

Table 2: Mean values (% ± Standard Deviation) obtained for selected parameters following exposure of *D. magna, C. fluminea, L. sativum, R. subcapitata* and *L. minor* to sequential simple dilutions of a parabens mixture (10 mg/L each paraben; total 50 mg/L parabens in solution).

Parabens concentration (mg/L)		***D. magna***	***C. fluminea***	***R. subcapitata***	***L. sativum***	***L. minor***
Total	Each	Immobilization (%)	Mortality (%)	Yield inhibition (%)	GI (%)	Yield inhibition (%)*
50.00	10.0	100 (± 0)	100 (± 0)	84 (± 3)	49 (± 12)	52 (± 4)
37.50	7.50	100 (± 0)	90 (± 10)	78 (± 5)	56 (± 12)	32 (± 2)
25.00	5.00	95 (± 10)	47 (± 15)	83 (± 2)	68 (± 4)	30 (± 5)
12.50	2.50	35 (± 30)	7 (± 11)	65 (± 1)	98 (± 17)	0 (± 8)
6.250	1.25	0 (± 0)	7 (± 6)	3 (± 4)	90 (± 0)	-19 (± 2)
3.125	0.62	0 (± 0)	0 (± 0)	13 (± 2)	100 (± 2)	-50 (± 18)

* based on dry weight.

3.3. Comparison between single and catalytic ozonation on the toxicity evolution

Only few studies can be found about the effect of the degradation treatment applied to remove these contaminants from water over toxicity, relating it with the resulting refractory compounds. This can be a relevant factor for the choice of the most appropriated treatment when looking for water reclamation [30] and adds significant relevance to this study. Here, to promote the degradation of parabens mixture, single and catalytic ozonation were used. These two processes present different reaction mechanisms. In the first one, the main responsible for degradation is the molecular ozone since the reaction was promote at low pH (~3.5). At acidic conditions it is expected a low production of hydroxyl radical due to the low self-ozone decomposition rate at this pH values [27]. On the other hand, at the presence of a catalyst several mechanisms may explain the increase on the process efficiency and one of them is the decomposition of ozone into hydroxyl radical [27]. This was the main pathway responsible by parabens mixture degradation in catalytic ozonation using volcanic rock as concluded in a previous work [28]. This allows total parabens mixture degradation using low amount of ozone. Toxicity effect along the treatment processes was analyzed as function of TOD, since with ozone processes, ozone generation is an important point in terms of the process energetic

costs. Moreover, plotting the results against TOD allows to compare the processes efficiency regarding ozone consumption [44].

At the end of reaction, as previously described, TOC removal was higher for catalytic ozonation and total parabens degradation was achieved. Nevertheless, it cannot be generalized that this means also a less toxic solution for all species. Different species present different sensitivities to the by-products formed so toxicity needs to be analyzed considering a battery comprising diverse species, ideally representing different functional levels in aquatic ecosystems.

D. magna reveals the most sensitive response against the parabens mixture and resulting by-products from both treatments, i.e. single and catalytic ozonation (Table 3). Until the final experimental stage (i.e. as the reaction was extended for 5, 15, 60 and 90 min of in treatment processes), 100% immobilization was achieved in both single and catalytic ozonation. This adverse response is certainly related to the by-products formed. For example, for a TOD value of 123 mg/L through single ozonation, the parabens mixture concentration was about 1.25 mg/L each paraben, except for BeP, which is totally removed [28]. According to Table 2 referring to the tests using simple dilutions of initial parabens mixture, no *D. magna* immobilization was found for this parabens concentration. Thus, this clearly illustrates the noxious potential of the by-products formed to the cladocerans.

We quantified 4-HBA, 2,4 and 3,4- diHBA, 3,4-diMeBA, Hydroquinone and 1,4-Benzoquinone as by-products of single and catalytic ozonation in the present study, and their concentrations were followed for different TOD's values. Kamaya et al., [45] verified for a wide group of benzoic acids derivates that the EC50 of parabens mixtures is lower comparing to the benzoic acids. This means that the parabens mixture was more toxic than the benzoic acids resulting from their degradation. For TOD values of 123 mg/L in single ozonation, small concentrations of 2,4-diHBA (3 µg/L), Hydroquinone (31 µg/L) and 1,4- Benzoquinone (70 µg/L) were detected, but other non-identified by-products should be present and can promote toxic effects observed in *D. magna* [19]. The toxicity of both these by-products to *D. magna* was already reported in the literature. García

et al. [43] reported an EC50 value of 450 µg/L for 1,4-benzoquinone, and Guerra [46] reported an EC50 value of 150 µg/L for hydroquinone. In the present study, it is notorious that the low 1,4-benzoquinone and hydroquinone concentrations at the reference TOD of 123 mg/L cannot explain the highly toxic effect observed (100% *D. magna* immobilization). Nevertheless, their interaction as well as the presence of other unknown by-products, as well as residues of the parabens mixture are likely to explain the observed toxicity. On the other hand, for catalytic ozonation, 85% of immobilization was achieved for the highest TOD's (55 mg/L) (Table 3). At this point, the parabens mixture was totally removed but 1,4-benzoquinone and hydroquinone concentrations were high (71 and 194 µg/L, respectively). These concentrations were like those achieved for single ozonation with a TOD value of 123 mg/L, where total *D. magna* immobilization was rather observed. Once again, the residual presence of parabens and other non-identified by-products in treated solutions, as well as their interaction, concur to explain the discrepancy. For the highest TOD in single ozonation (170 mg/L), no *D. magna* immobilization was detected (Table 3). We hypothesize that the huge amount of ozone applied allowed the degradation of all quantified by-products, which reinforces the idea that 1,4- benzoquinone and hydroquinone may have great toxicity to the cladocerans. On the other hand, for catalytic ozonation, Al and Fe leached from the catalyst (Table 1) can also affect the immobilization of cladocerans, somehow biasing further considerations.

Table 3: Mean *D. magna* immobilization (% ± Standard Deviation) observed following single and catalytic ozonation over a parabens mixture, as a function of reaction time (min) and corresponding TOD values (mg/L).

	Single ozonation		Catalytic ozonation	
Reaction time	TOD	*D. magna* immobilization	TOD	*D. magna* immobilization
5	6	100 (± 0)	3	100 (± 0)
15	18	100 (± 0)	9	100 (± 0)
60	78	100 (± 0)	29	100 (± 0)
90	123	100 (± 0)	42	100 (± 0)
120	170	0 (± 0)	55	85 (± 7.5)

To compare ozonation processes over the remaining species regarding to toxicity, data were analysed as function of TOD values (Figure 2). The most extensively treated sample of each process (highest TOD value) did not exert toxicity except for microalgae. In microalgae, the highest TOD value of each process induced 36 and 42% yield inhibition compared to the control, for single and catalytic ozonation, respectively, suggesting some susceptibility to by-products. According to Figure 2a, within TOD values of 10-50 mg/L microalgae yield was higher for samples treated with catalytic ozonation. The explanation to this fact can be related with the concentration of hydroquinone and 1,4-benzoquinone. For 29 and 42 mg/L of TOD in catalytic ozonation, hydroquinone was found at 160 and 187 µg/L and 1,4- benzoquinone was quantified at 28-38 µg/L, respectively. The 72h-EC50 value of hydroquinone for *Selenastrum capricornutum* (former name of *R. subcapitata*) was reported as 335 µg/L [47], hence not so far from hydroquinone concentration quantified here. This supports the role of hydroquinone in contributing to the observed toxic effect over microalgae. Regarding TOD values of 9 and 29 mg/L in catalytic ozonation, the parabens mixture concentration was reduced to about half compared to the starting point, while hydroquinone concentration increased about two times, and other detected by-products were found at higher concentrations (e.g. 4-HBA and 3,4 – diHBA can be found at a concentration of 247 and 500 µg/L, respectively for a TOD value of 29 mg/L). Altogether, by-products can explain the increased toxicity of samples provided by catalytic ozonation compared to the single ozonation equivalents in microalgae. Still, the role of 4-HBA in the toxicity of the samples must be carefully considered since, although this is the main by-product resulting from parabens oxidation [19,22,25], it shows a very low toxicity over *P. subcapitata* (72 h-EC50 value of 1367 mg/L; [48]) when dosed alone. At the defined end of the treatments (170 mg/L and 55 mg/L TOD for single and catalytic ozonation, respectively), toxicity to microalgae remains remarkable (above 35% in both cases) although no parabens were quantified. Catalytic ozonation produces appreciable amount of hydroquinone and 1,4-benzoquinone as discussed earlier, but this is not the case of single ozonation. In this case, other by-products such as low-length carboxylic acids,

aldehydes and alcohols may be potentially inducing the noticed toxic effects; their presence is confirmed by TOC values, which remained high.

C. fluminea was tolerant to the treated samples from both treatments. The most significant reduction was observed as TOD increased from 29 to 42 mg/L, corresponding to a 6-times reduction in concentration of MP, EP, PP, BuP and about 27 times for BeP (the most toxic paraben). Although these TOD values also translate in the increase of hydroquinone concentrations, *C. fluminea* was largely insensitive to these and other by-products, meaning that the clams can be affected by parabens mixture rather than by the degradation intermediates of ozonation treatments. This renders the picture on the toxicity of samples undergoing ozonation treatment (Figure 2b) essentially similar to that elicited by simple dilutions of the parabens mixture (Table 2).

L. minor and *L. sativum* were the most tolerant species to parabens, as indicated by the relatively low inhibition noticed in growth parameters for the raw mixture before treatment (52% yield inhibition for *L. minor* and a GI of 49% for *L. sativum*; Figure 2c and 2d). Catalytic ozonation reduces more quickly yield inhibition of *L. minor* than single ozonation. However, at 29 mg/L TOD, *L. minor* yield unexpectedly (as parabens concentration decreases) decreases compared to 9 mg/L TOD, standing above the expected inhibition records of 30% as denoted in Table 2). This is likely to reflect the toxic potential of hydroquinone and 1,4- benzoquinone (which increase at this point in the treatment reaction), that can react with the molecular constituents of proteins, DNA or lipids. This will affect the cellular metabolism, including enzyme inhibition through covalent binding and oxidative stress [49]. The Fe leached could also promote yield inhibition, but this possibility seems unlikely since at 9 and 29 mg/L TOD, Fe leached was similar (about 69 µg/L) while the responses were distinct.

The most extensively treated samples by catalytic and single ozonation are non-phytotoxic over *L. sativum* according to Trautman and Krasny [32] criteria. Still, the toxicological outcome was distinct from that provided by the test with simple dilutions of initial parabens mixture. For example, at 42 mg/L TOD in catalytic ozonation, parabens concentration is around 0.625 mg/L of each paraben,

which would translate in 100% of GI (Table 2); it rather translated into a 80% GI (Figure 2a), the difference being possibly due to the presence of hydroquinone and 1,4- benzoquinone. The putative effect of by-products seems even more evident in samples deriving from single ozonation. Referring to single ozonation using a low TOD value (6 mg/L), decrease on GI until 26% was observed compared to initial parabens mixture, changing the classification of the sample to very phytotoxic. This can be related with the production of hydroquinone (6 µg/L) and 1,4- benzoquinone (8 µg/L) as by-products Also, for catalytic ozonation a slight decrease on GI was verified for low TOD values. Meanwhile at those conditions, the concentrations of hydroquinone and 1,4- benzoquinone were 12 µg/L and 4 µg/L, respectively. Hereupon, as hydroquinone concentration was higher in this case when compared to single ozonation while 1,4- benzoquinone was lower, it can be considered that 1,4-benzoquinone can present a more negative impact on GI of *L. sativum.* This fact can be confirmed by the behavior of GI for single ozonation within 78 and 123 mg/L of TOD values, where this parameter was equal for both TOD's values. For a TOD of 78 mg/L the concentration of parabens was 4 mg/L (MP, EP, PP) 3 mg/L (BuP, BeP), whereas the concentration was about 1.25 mg/L (MP, EP, PP, BuP) while BeP was already not detected using a TOD of 123 mg/L. Given this decrease in parabens concentration, a consequent increase on GI would be expected. However, the concentration of 1,4-benzoquinone increased almost three times for this range of TOD values, and should have compensated the decrease on parabens mixture concentration. Nevertheless, in this range of TOD values the concentration of hydroquinone also decreases three times, which turns the 1,4-benzoquinone on the main responsible for the toxicity over *L. sativum.* Another support for this theory can be found on the last point of both treatments. Here, no parabens could be found but 1,4-benzoquinone can be detected for catalytic ozonation, so it could explain the higher GI for single ozonation. Still, the possible toxic effect of non-identified by-products cannot be ruled out.

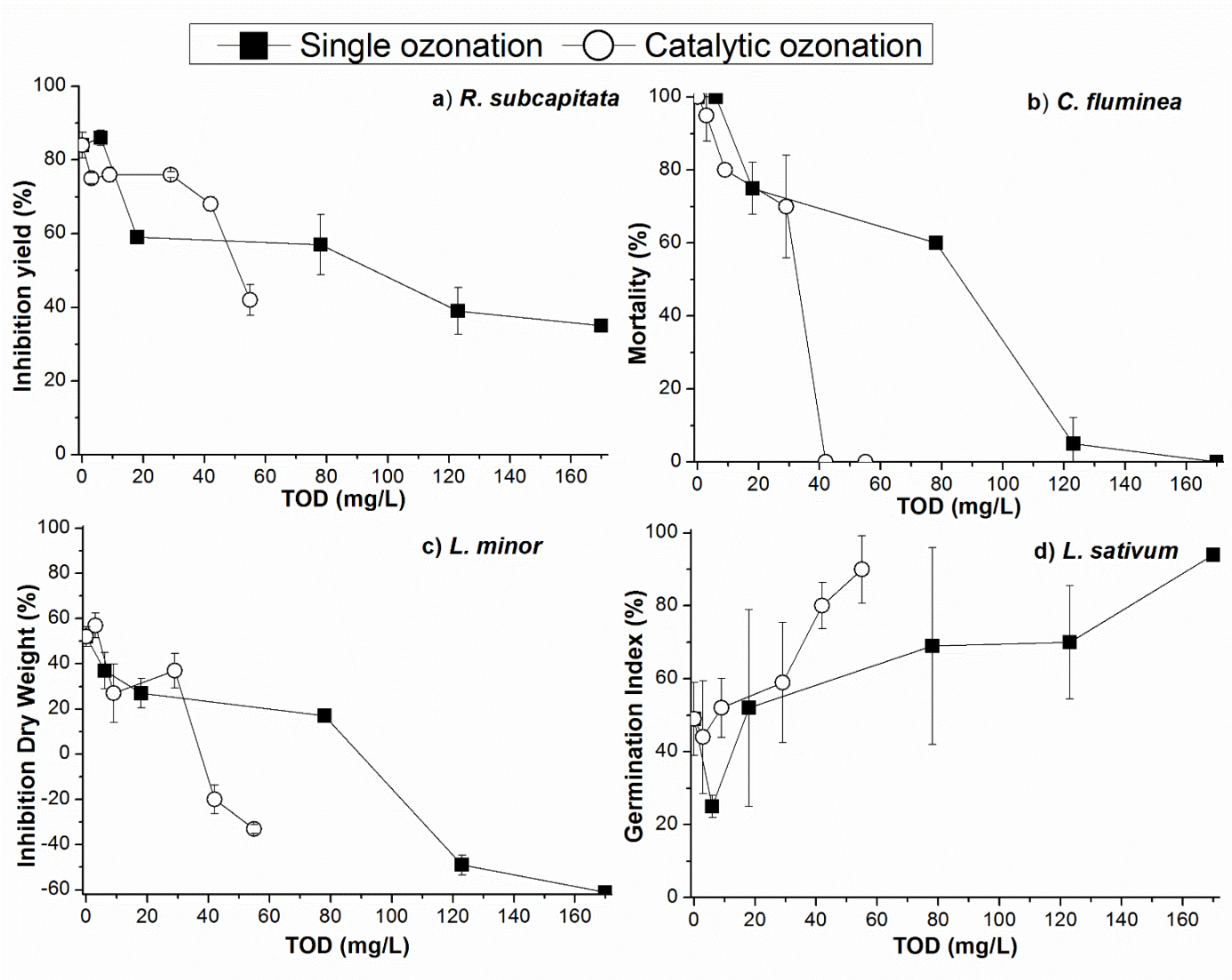

Figure 2. Average a) R. subcapitata inhibition yield; b) C. fluminea mortality; c) L. minor inhibition dry weight; d) L. sativum germination index, as function of TOD for single and catalytic ozonation. Error bars represent the standard error and the lines were drawn to facilitate visualization and do not intend to represent any adjusted model.

4. Conclusions

Toxicity assessment should be a key variable to analyze the efficiency of a water treatment process. Moreover, this evaluation should consider the toxic effect over several species rather than use a single indicator deemed adequate, since the toxicity of treated samples may be dramatically different depending on the species tested. The results achieved show that both parabens and degradation by-products through single ozonation and catalytic ozonation treatment produce different effects over the range of indicator species tested. It is noticeable that both treatment processes allowed

full degradation of the five parabens mixed to synthesize the test effluent. However, it is also remarkable that toxic intermediates were produced. According to the results obtained and the data from literature hydroquinone and 1,4- benzoquinone may be the most hazardous ones. In fact, fully treated samples following the highest TOD value for both processes (after total parabens removal) present higher toxicity over *D. magna* (just for catalytic ozonation), *R. subcapitata, L. sativum* than a solution containing a parabens mixture with a concentration of 625 μg/L per paraben. This means that the by-products generated through treatment, even at very low concentrations, should be more harmful for the tested species than the parabens mixture. Meanwhile, no toxicity was verified for *C. fluminea* and *L. minor* following exposure to the fully treated samples for both oxidation processes, elucidating on the differential outcomes that can be expected depending on the selected indicator species and thus evidencing the need to consider a wide ecotoxicological test battery while assessing water treatment efficiency.

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Highlights

Parabens mixture present a high toxicity over different tested non-target species;

Single and catalytic ozonation reveal efficiency for parabens mixture degradation;

Catalytic ozonation treated samples are more toxic than those from single ozonation;

Hydroquinone and 1,4- benzoquinone may be the most toxic by-products;

The effect of by-products and parabens interaction depends on the species analyzed.