ANTIOXIDANT ENZYME ACTIVITIES AND LIPID PEROXIDATION IN *DAPHNIA MAGNA* EXPOSED TO AN EFFLUENT SPIKED WITH PHARMACEUTICALS AND UNDER SINGLE OZONATION.

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INTRODUCTION:

Occurrence of pharmaceuticals in the aquatic reservoirs represents an important environmental problem due to their continuous discharge and persistence in the aquatic environment for months to years even at low concentrations [1]. Several studies have revealed that these compounds are difficult to remove from waters in the sewage treatment plant (STP) with conventional purification treatment steps [2]. Therefore, advanced oxidation processes (AOPs) have been developed as an alternative for the removal of pharmaceuticals and their metabolites from wastewater. Application of these treatment generate reactive oxygen species (ROS) that may reach superficial waters through discharges of effluents from STP.

The main aim of this study was to clarify know if increased levels of ROS produced after application of a single ozonation treatment of a real effluent would induce changes in the antioxidant enzymatic activities superoxide dismutase and catalase and the level of oxidative damage (level of lipid peroxidation) in *D. magna*.

MATERIAL AND METHODS:

A primary effluent obtained from a STP (Badajoz, Spain) was spiked with nine selected pharmaceuticals (acetaminophen, antipyrine, caffeine, carbamazepine, diclofenac, hydrochlorothiazide, ketorolac, metoprolol and sulfamethoxazole) so as the resulting wastewater have 200 $\mu g l^{-1}$ of each compound.

Biological treatment

First, an aerobic biological oxidation experiment was carried out in a batch tank provided with agitation and some diffusers to feed air. The primary sedimentation wastewater spiked with pharmaceuticals was then charged and treated during 7 h with activated sludge from returning pipe of the activated sludge in the cited STP.

Single ozonation (O_3 CPC - dark)

For this treatment ozone was produced from dry air in an ozone generator (Ozonfilt OzVa). The duration of chemical treatment was 5 h.

Analytical methods

- Pharmaceutical concentration in samples was determined by high performance liquid chromatography using a LaChrom Elite equipment (VWR International- Hitachi, Barcelona, Spain). Detection limit for accurate measurements of concentrations was ~ 2 $\mu g l^{-1}$.
- Total organic carbon (TOC) of effluent was determined by a TOC-VCSH Shimadzu Analyzer (VWRInternational).
- Chemical oxygen demand (COD) of effluent was measured following the standard dichromate reflux method in a Dr. Lange spectrophotometer [3].
- Biological oxygen demand (BOD) of effluent was measured following the respirometric method [4].

Toxicity tests with *D. magna* and samples preparation

Dilution series (100 % - 50 % - 25 % - 12.5 % and 6.25 %) of the effluents (primary effluent, spiked effluent and effluent after biological and ozonation treatment) were carried out according to procedure described in Daphtoxkit F™ (Daphtoxkit, 1996), which follows OECD Guideline 202 [5]. After exposure, surviving animals were transferred to eppendorf tubes and rinsed three times with 500 µl of 50 mM K-phosphate buffer (pH 7.0) combined with 5 mM ethylenediaminetetraacetic acid water to remove residual pharmaceuticals and then pooled in this medium to be immediately frozen at -80 ºC until further biochemical analysis. Ten daphnids were combined into one sample and a total of four replicates by dilution of each effluent were evaluated. Samples were homogenized for 30s. A volume of homogenate was used to determine lipid peroxidation and protein concentration. The remaining homogenate was centrifuged for 20 min at 10,000g and 4 ºC to obtain the post-mitochondrial supernatant (PMS) which was used for enzymatic activities and protein concentration measurements.

Activities of antioxidant enzymes, lipid peroxidation assay and protein concentration

Superoxide dismutase activity (SOD) was assayed by the inhibition of xantine/xantine oxidase mediated reduction of cytochrome c at 550 nm as described by McCord and Fridovich [6]. Catalase activity (CAT) was determined following H_2O_2 consumption at 240 nm [7]. Lipid peroxidation (LPO) was determined in homogenates by using the thiobarbituric acid (TBA) assay. Protein concentration of PMS was determined according to the Bradford method [8].

RESULTS AND DISCUSSION:

Efficiency of single oxidation process in removing pharmaceuticals and generation of ROS

Effluents characterization before and after pharmaceuticals spiking and after biological and single ozonation treatments is shown in Table 1. The levels of all pharmaceuticals after the biological and single ozonation were below the detection limits of the HPLC method applied that was ~ 2 $\mu g l^{-1}$, which demonstrates the efficiency of these treatments.

Dissolved ozone and hydrogen peroxide were detected in the final effluents ($2.4*10^{-5}$ M and $0.7*10^{-5}$ M, respectively).

Antioxidant enzymatic activities and levels of lipid peroxidation

We emphasize the 6.25 % dilution for spiked untreated effluent and treated effluents because the concentration of pharmaceuticals achieved with this dilution (12.5 $\mu g l^{-1}$) in the spiked untreated effluent is similar to environmental concentrations of some of those pharmaceuticals measured in surface waters. The statistical analysis between primary effluent and spiked effluent did not detect any statistically significant difference. Therefore, the concentration of pharmaceuticals added to the primary effluent did not induce alterations of antioxidant enzymatic activities nor lipid peroxidation levels.

- SOD activity: the effluent treated with biological+O^3 CPC (dark) process led to a significant increase of SOD activity ($p < 0.001$) when compared with the untreated spiked effluent group regardless of the dilution percentage except for the case of 12.5 % dilution (Figure 1). The induction of this enzymatic activity could be related to increased $O_2^{\cdot -}$ production in the final treated effluent. $O_2^{\cdot -}$ is formed due to ozone reaction with hydroperoxide ion. The increase in SOD activity could then be caused by activation of enzyme synthesis to cope with oxidative stress caused by increased levels of $O_2^{\cdot -}$.
- CAT activity: a significant increase of the CAT activity was observed in daphnids exposed to biologically treated effluent (6.25 % dilution) when compared with the untreated spiked effluent ($p < 0.001$) (Figure 2). However, the induction of CAT activity did not showed a clear pattern between treatments.
- Lipid peroxidation level: no significant differences were observed among the group of daphnids exposed to spiked effluent untreated or treated (Figure 3).

Table 1. Effluents characterization before and after pharmaceuticals spiking and after biological and single ozonation treatments.

Sample	pH	Total organic carbon, TOC (mg $C.l^{-1}$)	Chemical Oxygen Demand, COD (mg $O_2.l^{-1}$)	Biological Oxygen Demand, BOD_5 (mg $O_2.l^{-1}$)	BOD_5/ COD Ratio
Primary Effluent	7-8	56	228	90	-
Spiked effluent[a,b]	7-8	61	237	90	-
Biological treatment	7-8	26.89	52.40	30	0.57
Biological + O_3 CPC (dark) [c]	7.79	12.16	14.87	11	0.74

Note: [a]Pharmaceuticals used: acetaminophen, antipyrine, caffeine, carbamazepine, diclofenac, hydrochlorothiazide, ketorolac, metoprolol and sulfamethoxazole (200 µg.l-1 each one); [b]The spiking of pharmaceuticals did not change substantially the values of the measured parameters; [c]CPC: Compound parabolic collector solar photoreactor.

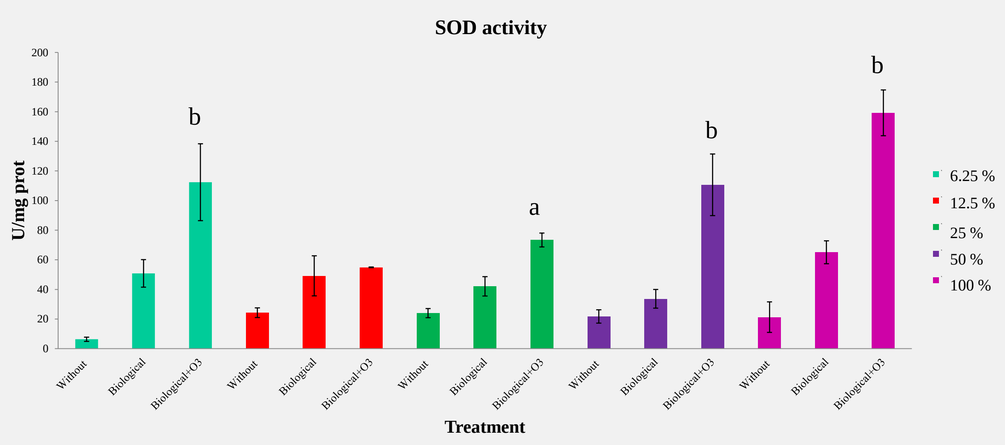

Fig 1. Activity of superoxide dismutase (SOD) in *D. magna* juveniles exposed to non treated (spiked effluent) and treated effluents from sewage treatment plant. Values are expressed as mean±S.E.M. (number of pools n=4). U= amount of sample producing 50% inhibition of cytochrome c reduction. Significant differences (a: P<0.01 and b: P<0.001) among the effluent without treatment and the effluents with biological, biological+O_3 CPC (dark).

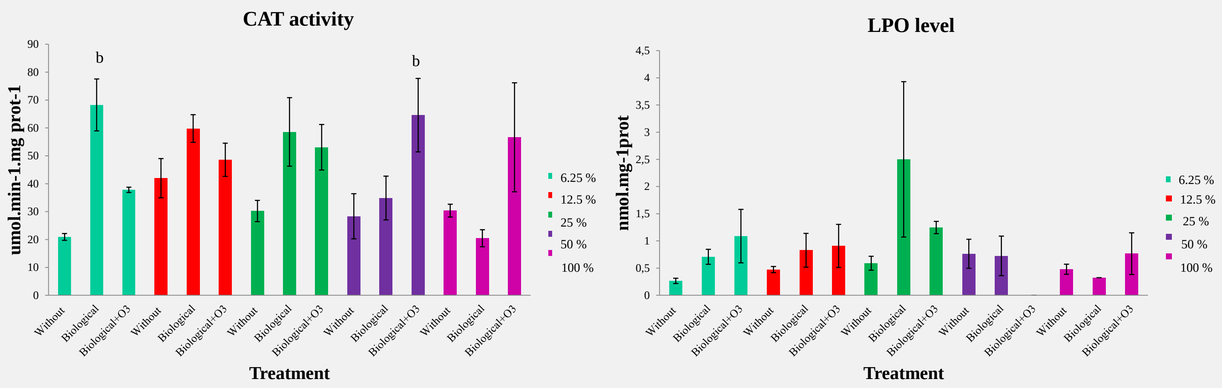

Fig 2. Activity of catalase (CAT) in *D. magna* juveniles exposed to non treated (spiked effluent) and treated effluents from sewage treatment plant. Values are expressed as mean±S.E.M. (number of pools n=4). Significant differences (b: P<0.001) among the effluent without treatment and the effluents with biological, biological+O_3 CPC (dark).

Fig 3. Levels of lipid peroxidation (LPO) measured as TBARS in *D. magna* juveniles exposed to non treated (spiked effluent) and treated effluents from sewage treatment plant. Values are expressed as mean±S.E.M. (number of pools n=4). No significatives differences among the effluent without treatment and the effluents with biological, biological+O_3 CPC (dark) were found

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