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Biomarker-based assessment of the toxicity of the antifungal clotrimazol to the microcrustacean *Daphnia magna*

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Highlights

- Clotrimazole exposure caused a significant increase in CAT activity
- Clotrimazole increased the GSH conjugation capacity of exposed animals

- No neurotoxic effects were observed after clotrimazol exposure
- Clotrimazole may represent an ecotoxicological risk for *D. magna*.

ABSTRACT

Among the vast list of xenobiotics that may promote harmful effects in aquatic ecosystems, pharmaceuticals are currently a prominent class due to their ability to persist in these environments and also due to the lack of information regarding their effects on the different components of the aquatic biota. Antifungals in particular, despite their massive use, are not extensively studied in environbmental terms. The main objective of this study was to characterize the toxicity of the antifungal clotrimazole to the aquatic organism *Daphnia magna*. To attain this purpose, the effects of this compound were measured, focusing on the determination of acute lethality, and quantification of biomarkers, such as neurotoxicity (soluble cholinesterases, ChEs); and oxidative stress and metabolism (such as catalase, CAT; and glutathione-S-transferases, GSTs). The toxicity assessment with biomarkers was based on animals exposed to concentrations similar to those already found in surface waters in order to increase the ecological relevance of the obtained data. The results showed that exposure to clotrimazole was able to induce significant increases in both CAT amd GSTs activities. ChE activity was not significantly altered after clotrimazol exposure. In view of the above, it is concluded that the drug studied caused adverse effects in terms of oxidative stress, at an ecological relevant levels, showing that the presence of clotrimazol in the wild is not innocuous.

Keywords: microcrustacean; antifungal; biomarkers; oxidative stress; metabolism; neurotoxicity.

1. Introduction

Nowadays, thousands of compounds of different classes, purposes and natures (pesticides, metals, and pharmaceutical drugs, among others) are likely to be released into the environment as a result of their use by humans (Harte, 2007). Pharmaceuticals and Personal Care Products (PPCPs) have raised great public attention during the last two decades as emerging contaminants in the aquatic environment (Herberer, 2002; Okuda, 2009). The release of these compounds can cause harmful effects either to humans that directly or indirectly contact with them, but especially to organisms living in the ecosystems where these compounds are released (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999; Weigel, 2003; Escher et al., 2005; Barnes et al., 2008; Gunnarsson et al., 2008; Fick et al., 2010; Ginebreda et al., 2010; Jelic et al., 2011), most probably during their entire life cycle. Of the wide variety of chemicals that are routinely dispersed in the environment, compounds with a diagnostic, therapeutic or preventive function, generally referred to as drugs, may be considered (Daughton and Ternes, 1999), not only in human medicine, but also in veterinary medicine for the prevention, treatment of infectious diseases, and as growth promoters, and in agriculture (Weigel, 2003).

Drugs are a class of emerging environmental contaminants whose production has increased in recent decades, and universal expenditures related to the consumption of this type of drug have also increased exponentially in recent years (Oliveira et al., 2015). World drug consumption is very significant today; for example, in the European Union alone approximately 3,000 different substances are used in medicinal products for human

consumption (Sattelberger, 1999; Stuer-Lauridsen et al., 2000; Jones et al., 2002; Calamari et al., 2003; al., 2004; Khan and Ongerth, 2004). Among these substances, drugs such as analgesics and anti-inflammatories, antibiotics, β-blockers, lipid regulators, and many others (Petrovic et al., 2014) are particularly prominent. These compounds have a combination of unique characteristics that make them molecules of environmental concern. They are designed to be biologically active in a number of species, being slowly metabolized and highly lipophilic to maximize their absorption; its degradation is responsible for the release of new molecules, not characterized in pharmaceutical and toxicological terms (Alavijeh, 2005). Drugs are considered pseudo-persistent pollutants because their removal rate is low. Normally, human wastes are sent to wastewater treatment plants (WWTPs) where they suffer physical processes (sludge removal) and biological processes, through microbial degradation of organic matter. However, in some regions or even countries these facilities may not exist (Ternes, 1999). There are studies that point out that many drugs are not degraded by conventional waste water treatment (Halling- Sorensen et al., 1998; Buser et al., 1999; Kummerer, 2001; Castaglioni et al., 2006; Ellis, 2006; Yu et al., 2006; Al-Rifai et al., 2007), and that 80% of all drugs entering the WWTPs are emmited into the wild intact (Ternes et al., 1999). At present, WWTPs are not focused on the elimination of PPCPs (Nakada et al., 2007), being inefficient at removing those water-soluble pharmaceutically active compounds (PhACs) that are poorly biodegradable. Various active pharmaceutical ingredients (APIs) (e.g., carbamazepine, crotamiton, naproxen, ketoprofen, and triclosan) were poorly removed by conventional secondary sewage treatment and were detected in secondary effluent (Ternes, 1998; Clara et al., 2004, 2005; Paxeus, 2004; Nakada et al., 2006a). Several APIs have been found in distinct environmental compartments such as waste, surface and ground waters (Calisto and Esteves, 2009; Fick et al., 2009; Gabet-Giraud et al., 2010;

Lindberg et al., 2010), as well as in sludge and sediments (Ternes et al., 2002; Andersen et al., 2003). Sorption of APIs to sludge, during wastewater treatment processes in which sludge is separated from the wastewater stream may be an important factor for the removal of non-biodegradable pharmaceuticals from WWTPs. Clotrimazol was found in sewage sludge in a study conducted in 2011 (Hörsing, 2011). As such, the amount of pharmaceutical molecules that is treated is exceeded by the daily input of new molecules into the environment, and toxicological interactions may occur with other pharmaceutical residues in the aquatic environment. The pharmaceutical industries, both licensed and illegal, are other very important sources of introduction of drugs into the environment by the release of waste directly into water courses (Burke and Smith, 2006). The use of drugs in aquaculture, whether from medicated feed sources or excretion products, is also another route of contamination of the aquatic environment (Daughton, 2008).

The disposal and the arrival of these compounds to the wild may cause several types of problems, considering their persistence and the potential exertion of synergistic effects when in the presence of other drugs in the environment (Reis-Filho et al., 2007). This aspect is related to the variety of metabolites formed and possible toxicological interactions between pharmaceutical (eg, synergistic, additive, antagonistic) residues in non-target organisms in the wild (Cleuvers, 2003). In different areas of aplicattion, a wide range of pharmaceuticals and personal care products (PCPs) are used and emitted into the environment leading to an environmental exposure that leans towards the blending of these chemically and functionally heterogeneous compounds. These characteristics, among others, make pharmaceutical drugs prominent candidates to be evaluated for potentially deleterious effects in non-target organisms, especially in the aquatic environment (Oliveira et al., 2015).

Amont the most studied drugs already found in the wild, little attention has been given to antifungals. Clotrimazole is an antifungal drug that acts by preventing the integrity of fungal cell membranes, by inhibiting membrane lipid biosynthesis by inhibiting a fungal cytochrome P450 involved in ergosterol biosynthesis (Pappas and Franklin, 1993; Turan et al., 2001; Shah et al., 2001). It is commonly used in the treatment of superficial mycoses. In veterinary medicine it is used to treat a wide variety of infections. Clotrimazole is a compound classified as "very toxic to aquatic organisms" in agreement with EU directive 67/548/EEC. It was identified as being a compound that needs further studies on its toxicity (Boxall, 2004), and the Commission for the Convention on the Protection of the Marine Environment of the North-East Atlantic (OSPAR) assessed the environmental risk of clotrimazole and listed it as requiring priority action (OSPAR-Commission, 2006). However, it was concluded in the last 2005 report that there is currently no clear environmental risk posed by clotrimazole. (OSPAR-Commission, 2005). In 2002, clotrimazole was the most frequently detected compound in a study on the occurrence of selected pharmaceutical products in the UK estuaries and was found at concentrations up to 63 pmol/L (Thomas and Hilton, 2004). During an analytical survey in 2004, clotrimazole was detected in all samples from surface waters of the Tyne River (England) at concentrations up to 100 pmol/L (Roberts and Thomas, 2006).

The use of biomarkers (defined as changes in a biological response, from molecular changes through cellular and physiological responses to behavioral changes) in environmental analysis has received a lot of attention with the emergence of detailed studies that establish that some metabolic pathways are highly evolutionarily conserved, and that they are similarly affected after exposure to a same toxic agent (or agents of the same toxicological class) (Hyne & Maher, 2003; Islas-Flores et al. 2013; Martins, 2013), most likely resulting in consequences on the physiology of exposed organisms, which

may subsequently affect changes in survival or reproduction, with possible population consequences (Jemec et al., 2009). Among the most used biomarkers, those of oxidative stress assume a leading position in environmental monitoring trials (Nunes et al., 2006), Phase II metabolism enzymes (e.g. glutathione S-transferases (GSTs) isoenzymes) are also widely used as biomarker because they are a group of enzymes that catalyze the conjugation of glutathione with xenobiotics, including drugs, and the cytotoxic aldehydes produced during lipid peroxidation (Booth and O'Halloran, 2001). Similarly, neurotoxicity has assumed a particular interest in this domain, with cholinesterases being the enzymes that assume greater relevance from the point of view of monitoring neural cholinergic functions (Payne et al., 1996). For the elaboration of this work and taking into account the importance of biomarkers as a tool to evaluate the ecotoxicological effects, three biomarkers were selected, CAT, GSTs and ChEs. The choice of these biomarkers was made taking into account works performed with azolic compounds in similar organisms. Burkina et al. (2013) analysed the sub-lethal effects and the bioconcentration of clotrimazole in *Oncorhynchus mykiss*, using oxidative stress biomarkers. Similarly, Tu et al. (2016) as also observed significant alterations of CAT and GSTs activities in propiconazole exposed fish medaka larvae The study conducted by Zhu et al. (2014) showed that the toxicity of triazole fungicides (myclobutanil, fluconazole, flusilazole, triflumizole and epoxiconazole) in rare minnow (*Gobiocypris rarus*) embryos could result in alterations of oxidative stress defenses, but also in changes in acetylcholinesterase (AChE) activity, evidencing the potential neurotoxic effects of this class of drugs. Another study developed by Jin et al. (2016) used AChE as a biomarker to address developmental abnormalities and locomotor activity changes elicited during early developmental stages in zebrafish by the fungicide imazalil.

Daphnia magna is considered one of the most sensitive aquatic species towards anthropogenic compounds, being therefore frequently used in ecotoxicological trials. The use of *D. magna* individuals in toxicological tests dates back to the 1970s (Jonczyk and Gilron, 2005) and are also used for studies in the areas of ecology and physiology (Preuss, 2009). In addition, it has characteristics that facilitate its laboratory manipulation/maintenance, such as small size, short generation time, high fecundity and genetic uniformity (asexual reproduction) (Koivisto, 1995; Baird and Barata, 1998), sensitivity to different compounds, ecological relevance, existence of standard protocols, (Münzinger and Monicelli, 1992). The goals of the present study were to characterize the toxicity of clotrimazole, towards the aquatic organism *Daphnia magna*, by determining acutel lethality, and analyzing neurotoxicity (cholinesterases, ChEs activity), and oxidative stress and oxidative damage markers (such as catalase CAT; glutathione-S-transferases, GSTs) in individuals of the *D. magna* species.

2. Material and methods

2.1 Chemicals

The compound clotrimazole (CAS 23593-75-1) purity ≥ 98% was purchased from Sigma-Aldrich Chemical (Germany). All other chemicals (namely acetylthiocholine, DTNB, glutathione, CDNB, hydrogen peroxide, and chemicals used for the preparation of buffers) were also purchased from Sigma-Aldrich Chemical (Germany). The Bradford reagent was purchased from Biorad (USA). All exposures were performed with a stock solution prepared with 1mg of clotrimazole dissolved in 1L ofAmerican Society for Testing and Materials (ASTM) hard water medium (ASTM, 1980).

2.2 *D. magna* laboratory culture

The cultures of *D. magna* were laboratory maintained in glass beakers. Every 1L breaker had about 20 individuals each, which were maintained in 800 ml of ASTM medium, with the addition of an organic additive obtained from algae extract (obtained from the algae *Ascophyllun nodosum*; Baird et al. 1989), and added to the culture medium in an amount of 4.8 mL/L (OECD, 1998). The cultures were fed and the culture medium renewed three times a week. The food consisted of a suspension of algae of the species *Pseudokirchneriella subcapitata* (Hindák, 1990), grown in the laboratory in MBL medium (Stein, 1973), resuspended in ASTM media and stored in the refrigerator until use. The food was added to *D. magna* cultures at a density of about $3.0x10^5$ cells/ml. At the end of the 5th brood the newborns were used to start new cultures. All the trials were performed with neonates from parental organisms, obtained from between the 3rd and 5th broods. The temperature was maintained between 18°C - 22°C (± 1). The animals were exposed to 16 h D: 8H N in non-aerated containers (OECD, 2004).

2.3 Acute exposure of *D. magna* for the determination of LC_{50}

Acute toxicity tests with *D. magna* were performed according to the guidelines of the Organization for Economic Cooperation and Development (OECD, 2004). The final nominal concentrations tested were: 0.00; 1.25; 2.5; 5; 10, and 20 mg/L of clotrimazole. This range of concentrations was selected after a series of preliminary range finding tests (data not shown).

To perform the acute toxicity tests, 6-well plates of 10 mL each were used, each with 10 mL of each test concentration (or 10 mL of reconstituted water - control), with 1 neonate (≤ 24 hours of age), divided into twelve replicates with one organism each. The organisms

exposed were not fed during the course of the experiments, as recommended by the OECD (2004).

Acute toxicity tests were conducted, and after the exposure period of 48 h, the organisms were observed and counted. Subsequently, the median lethal concentration at 50% of the organisms (LC_{50}) was calculated through the IBM SPSS Statistics 25 software. The criterion adopted was the immobility of organisms, as defined and recommended in the OECD guideline (2004).

2.4 Acute exposure of *D. magna* for the determination of biomarkers

To determine the biomarkers, exposure systems similar to the acute toxicity tests were set up according to the OECD guidelines most adapted for juvenile organisms (OECD, 2004). For each quantified biomarker, *D. magna* individuals at 5 days of age were exposed to different concentrations of clotrimazole. The concentrations chosen were based on surface water values (reported to occur in the River Tyne, England) and are <<<EC1: 8.6, 17.2, 34.4, 68.8, and 137.6 ng/L, plus a control treatment. For all treatments, including control, 5 replicates were prepared, each with X neonates. The assays were conducted in 50 ml vials and 5 organisms were placed in each vial, which were exposed for 48 h. The solutions of all concentrations were prepared from a stock solution of 1 mg/L in ASTM medium. After a 48-hour exposure period, the exposed organisms were isolated in Eppendorf microtubes and immediately frozen at -80 ° C. Isolation of the organisms was accomplished by aspiration with Pasteur pipettes, and the excess of exposure medium was withdrawn after collection.

2.5 Determination of biomarkers

Previously frozen samples were thawed on ice, and were homogenized with a sonifier (Branson 250) in 1 ml of 0.1M phosphate buffer, pH = 7, with 0.1% Triton X-100, and then the samples were centrifuged at 15,000g for 10 minutes at 4ºC (Eppendorf 5810 refrigerated centrifuge), except for determination of cholinesterases; in this case, organisms were homogenized in 1 ml of 0.1 M phosphate buffer, pH = 7.2, then the samples were centrifuged at 3330 g for 3 minutes (Nunes et al., 2008). The supernatant from each sample was divided into 400 µl aliquots for each of the biomarker determinations, and the remaining volume was reserved for quantification of the total protein, and frozen immediately at -80 ° C.

2.5.1 Quantification of cholinesterases activity

According to Diamantino et al. (2003), *D. magna* ChEs exhibit mixed hydrolytic properties between the characteristics of ChE and pseudocolinesterase. The results were thus expressed in terms of nonspecific cholinesterase activity (ChEs). The methodology for the spectrophotometric determination of the enzymatic activity of cholinesterases consists in the degradation of acetylthiocholine (susbstrate) resulting in acetate and thiocoline, and the latter product complexes with DTNB (dithiobisnitrobenzoate), giving rise to a colored compound (yellow color), the formation of which can be determined at 412 nm (Ellman, 1961).

2.5.2 Quantification of Glutathione S-transferases activity (GSTs)

GST isoenzymes catalyze the conjugation of the CDNB (1-chloro-2,4 dinitrobenzene) substrate with glutathione (GSH) to form a thioether ($\mathcal{E}$ = 9.6 $mM^{-1}cm^{-1}$), monitored by increasing absorbance at 340 nm. The activity of the GSTs was determined by the method

described by Habig et al. (1974) adapted to microplate, allowing the determination of 4 readings per replicate.

2.5.3 Quantification of Catalase Activity (CAT)

The activity of the CAT enzyme was determined by the decrease of the absorbance at 240 nm due to the decomposition of H_2O_2 in water and O_2 (Aebi, 1984). To maximize the assay, a 1:10 dilution was carried out in 50 mM phosphate buffer pH =7.0, 2 readings per sample. The absorbance of the samples was spectrophotometrically monitored at 240 nm for 300 seconds, with 20s intervals. The activity of the enzyme was expressed in µmol H_2O_2 consumed per minute per mg of protein.

2.5.4 Determination of total soluble protein concentration

The enzymatic activity was expressed as a function of the amount of total protein present in the samples. Protein quantification was performed according to the method described by Bradford (1976), adapted to a 96-well microplate. The principle of the method is based on the binding of the Bradford reagent (dye) to the total protein, resulting in a stained and stable complex, quantified at 595 nm. Ɣ-globulin (1mg/mL) was used as standard.

2.6 Statistical analysis

For all data, normality prerequisites (Shapiro-Wilk) and homogeneity of variances (Levene's test) were checked for parametric tests. Data were analyzed using one-way ANOVA and Dunnett's post hoc test if statistically significant differences ($p < 0.05$) were

found. Excel and SPSS programs (v. 25) were used. Data were presented as mean ± standard errors.

3 Results

The LC_{50} value obtained for the compound clotrimazole for *D. magna* was 5.143 mg/L (IC_{95}: 3,853 - 6,685; Table 1).

Exposure to clotrimazole caused a significant increase in CAT activity ($F = 12.67$; d.f. = 5, 27; $p < 0.001$, Figure 1), that was evident for almost all tested concentrations when compared to control organisms.

Exposure to clotrimazole caused an increase in GST activity, but only organisms exposed to the highest tested concentration showed statistically significant differences in relation to to control organisms ($F = 2,765$, d.f. = 5.29; $p = 0.041$, Figure 2).

Exposure to clotrimazole caused an increase in ChE activity, but no concentration tested caused statistically significant differences when compared to control organisms ($F = 5.621$, df = 5.29, $p = 0.001$, Figure 3).

4 Discussion

LC_{50} represents the concentration that kills 50% of the exposed organisms, and it is just a numerical indication of the amount of a given toxicant that causes lethality. Its usefulness is related to the possibility of establishing comparisons not only among very different toxicants, but also distinct species. Despite the general absence of data on the

lethality of clotrimazole, other azolic compounds have already been tested in aquatic organisms. Surprenant (1984) calculated the LC_{50} values of penconazole (another azolic antifungal chemical) for several fish species (namely rainbow trout *Oncorhynchus mykiss*, channel catfish *Ictalurus punctatus*, bluegill sunfish *Lepomis macrochirus*, and carp *Cyprinus carpio*); calculated values were in the range of 1.3-4.6 mg/L. Rufli (1984) also determined the LC_{50} of penconazole for carp (*C. carpio*), being in the range of 3.8-4.6 mg/L. These values from the literature, showed that the fish species used previously for the calculation of LC_{50}s were more sensitive than *D. magna*, for which considerably higher concentrations of clotrimazole were necessary (namely 5.143 mg/L) to attain lethal effects of 50% of the exposed organisms. It is however, extremely difficult to establish plausible comparisons, and this is a mere indicative assumption. Not only the tested species were phylogenetically distant, but the chemicals are distinct, even being included in the same pharmacotherapeutic class. Consequently, knowledge of the effects of commercial formulations of triazole fungicides on fish and other species are still quite limited, despite the here-presented data.

Oxidative stress triggers a set of adaptive responses in protective systems, the modification of macromolecules and, ultimately, cell and tissue lesions. The excessive increase in the production of reactive oxygen species (ROS) can be balanced by the increase of antioxidant activity, namely by increasing the activity of antioxidant enzymes (Regoli & Giuliani, 2014), such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), which are natural ROS eliminators (Nunes et al., 2006; Regoli & Giuliani, 2014). This same principle was observed to occur in *D. magna*, as a response to the excess of hydrogen peroxide, as described by Bownik and Stępniewska (2015). The response to increased amounts of hydrogen peroxide was characterized by enhancement of catalase activity, and the activation of the glutathione metabolism. A

similar trend resulted also from the exposure of *D. magna* to ectoine, a natural product whose metabolism lead to the activation of catalase, as shown by Bownik et al. (2015), evidencing the responsivenss of this antioxidant enzyme in this microcrustacean species. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with amino acids (such as glycine, taurine and glutamic acid), and above all, conjugation with glutathione (mercapturic acid synthesis) (Klaassen, 2001). Both changes in the activity level of the enzymes involved in phase I and phase II reactions are often used as toxicity criteria in ecotoxicological studies. In this context, the activities of two key enzymes were selected as parameters indicative of oxidative stress and phase II metabolism, CAT, and GSTs.

CAT is the enzyme responsible for the decomposition of hydrogen peroxide into water and molecular oxygen, and is found in animal cells, mainly in the liver, kidneys and erythrocytes (Nishikawa, 2009). Hydrogen peroxide formation is common in oxidative stress scenarios, following detoxification of the superoxide anion in hydrogen peroxide, reaction catalyzed by the enzyme SOD. Catalase has a double role, since it can act as a catalyst for the dismutation of molecules and H_2O_2, and performs peroxidation when it behaves only as an electron receptor (Ahmad, 1995). Thus, the activity of this enzyme may increase with high levels of hydrogen peroxide (Brandão et al., 2011), and this effect was observed to also occur in *D. magna* after being exposed to hydrogen peroxide (Bownik and Stępniewska, 2015). There are catalases present both in the cytoplasm and in the mitochondria (Lackner, 2008). Catalase activity appears to be linked to the activity of glutathione peroxidase (GPx) to combat oxidative stress (Ighodaro, 2017). Although there are no specific data linking clotrimazole with oxidative stress, some literature studies of therapeutic antifungal agents of the same class (azolic compounds) demonstrate that these compounds cause oxidative stress in several species

(Delattin et al., 2014; Chaâbane et al., 2017). Azole treatment leads to ergosterol depletion and the accumulation of toxic methylated sterol intermediates, resulting in growth inhibition or fungal cell death (Yoshida, 1988). In addition to the interference with ergosterol biosynthesis, the azole miconazole induces accumulation of ROS in planktonic fungal cells (Kabayashi et al., 1997; Francois et al., 2006; Thevissen et al., 2007; Yan et al., 2007; Yan et al., 2009). In case of miconazole, a correlation was found between the amount of ROS induction and its fungicidal activity against the fungal *Candida* spp. (Kabayashi et al., 1997). In this regard, it was demonstrated that quenching of miconazole-induced ROS accumulation, for example by the antioxidant pyrrolidine dithiocarbamate, resulted in a significant decrease in miconazole antifungal activity (Kabayashi et al., 1997). Furthermore, François and colleagues showed that the increase of ROS in planktonic *C. albicans* cells by miconazole can be explained, at least in part, by the inhibition of catalase and peroxidase, both of which are important enzymes in the breakdown of peroxide radicals and hydrogen peroxide (Francois et al., 2006). In addition, Yan and co-workers showed the involvement of the alternative oxidase, an enzyme from the alternative respiratory pathway in *C. albicans*, in the defense against miconazole-induced ROS accumulation (Yan et al., 2009). It is thus possible to conclude that, at least partly, the activity of azolic compounds depends upon their capacity of enhancing oxidative stress.

In the study by Zhang et al. (2016), the exposure of green algae *Chlorella pyrenoidosa* to ciproconazole (an antifungal from the azole category that shares the same mechanism of action with clotrimazole) caused significant changes in CAT activity. These data are consistent with the results obtained in the present study, since the exposure of *D. magna* to clotrimazole induced significant alterations in CAT activity, with statistically significant increases being reported in organisms exposed to concentrations of 8.6, 34.4,

68.8, and 137.6 ng/L, suggesting that the catalytic degradation of hydrogen peroxide was accompanied by hydrogen peroxide formation, that was closely followed by increased enzyme activity. This suggests the occurrence of pro-oxidative changes after exposure to this drug. The work conducted by Mu et al. (2016), also evidenced the occurrence of significant changes in CAT activity after exposure of the aquatic organism *Danio rerio* to difenoconazole (of the same pharmacotherapeutic class) for 4 days. Similarly, in the study conducted by Husak et al. (2017), CAT activity increased significantly in the gills of *Carassius auratus* after being exposed to the highest concentration of the azole antifungal penconazole (25mg/L). These results allow to hypothesize that clotrimazole toxic effects in non-target species may be also mediated by oxidative alterations, which were sucessfully counteracted by the establishement of an effective response, by means of catalase overexpression.

GSTs represent a family of widely distributed enzymes that catalyze the conjugation of various xenobiotics with reduced glutathione (GSH), resulting in their detoxification (Blanchette et al., 2007) preventing damage to the cell membrane and other macromolecules (Dybing et al., 2002; Malmezat et al., 2000). The increased hydrophilicity of such complexes favors their excretion, reducing the likelihood of these compounds to bind to other macromolecules, such as DNA (Schelin et al., 1983). Many compounds of diverse nature, including endogenous substances, toxic xenobiotics and reactive products of intracellular processes, such as lipid oxidation, act as substrates of GSTs (Miners et al., 2004). GSTs appear to be present in many, if not all, animal species. Although some microsomal isoforms of GSTs have been described, the activity of GTSs is mainly localized in the cytosol (Cnubben et al., 2001).

In a trial developed by Tu et al. (2016), which consisted of exposing fish of the species *Oryzias latipes* to propiconazole, it was possible to observe a dose-dependent increase in

GSTs activity. This study consisted of a medium-long term exposure, and statistically significant differences were observed after 7 days, but only for animals exposed to the highest tested concentration (250 μg/L). As the duration of exposure increased, more significant alterations were observed with respect to GSTs activity when compared to control organisms. After 14 days of exposure, it was observed that the two highest concentrations (25 and 250 μg/L) caused a significant increase in the activity of these isozymes, and that after a period of 28 days, animals exposed to all concentrations (2.5, 25, and 250 μg/L) showed a significant enhancement of this parameter. This dose-dependent increase is consistent with the results obtained in the present study. In addition, the study conducted by Egaas et al. (1999), reported a significant increase in GSTs activity after a 14-day exposure of the freshwater fish *Salmo trutta* to propiconazole, which was statistically significant when compared to the control at levels of 93 μg/L and 313 μg/L. Similarly, Li et al. (2010) observed a significant GSTs increase induced by long-term exposure of *Oncorhynchus mykiss* to propiconazole. The two highest concentrations caused significant increases in this enzyme´s activity in all analyzed tissues following 30 days of exposure, whereas for the 20 days of exposure only the highest concentration presented a similar trend, with an overall increase in GSTs activity. These patterns of response, and the here-found GSTs modifications, lead to the conclusion that the metabolism of the compound under study requires, at least partially, its conjugation with glutathione with the involvement of the GSTs. CTZ is a potent inhibitor of hepatic microsomal enzymes which are involved in its own metabolism. CTZ has been shown to be a potent 6-hydroxylation inhibitor of testosterone in rats that is catalyzed by CYP3A (Turan et al., 2001). It is also a potent *in vitro* inhibitor of mammalian cytochrome P450, including those responsible for the metabolism of xenobiotics (Sheets et al., 1986; Heuser and Franklin, 1985; Suzuki et al., 2000). The

major *in vivo* metabolite of CTZ is 2-chlorophenylbiphenylmethanol (2CLBPM) resulting from the deamination reaction, which is catalyzed by CYP3A. No data are however available in the literature, that relates the metabolism of CTZ with phase II conjugation with GSH by means of GSTs intervention. Nevertheless, our data support this assumption considering that the GSTs activity levels were significantly changed. The results obtained in the present study suggest that clotrimazole did not cause a clear oxidative stress scenario, since the activity of the antioxidant enzyme catalase was not significantly altered, for any of the concentrations. Thus, an increase in GSTs activity is most likely due to its effect on conjugation metabolism, rather than its putative role in the antioxidant defense.

Cholinesterases (ChEs) belong to the family of enzymes designated as esterases, with the ability to hydrolyze carboxylic esters. When ChEs activity are inhibited in some way, there is a blockage in the transmission of nerve impulses, paralyzing vital functions due to overlapping of the nerve impulses, caused by the permanence of Na^+ channels in their opened conformation (Pirkmajer, 2016). This fact is often responsible for acute effects, resulting in death of the exposed organisms as a consequence of hyperstimulation of the parasympathetic autonomic nervous system (Mayer et al., 1992; Guilhermino et al., 1998). ChEs are sensitive to exposure mainly to organophosphorus pesticides and carbamates (Nunes 2011), and the dosage of its activity is often used as a biomarker of effect for the assessment of the primary effects of environmental contamination by these agents in various organisms, and quality assessment of waters (Stien, 1998; Sancho, 2000; Lionetto, 2003; Viana, 2005). In addition, several studies have shown that in aquatic organisms, ChEs activity is inhibited by a multiplicity of contaminants, including pesticides (especially insecticides), heavy metals, detergents, and some pharmaceuticals (Sole et al., 2010; Rocha et al. 2013). However, ChEs inhibition is not by far restricted to

such chemicals. In the assay conducted by Zhu et al. (2014) exposure of embryos from the fish species *Gobiocypris rarus* to five triazole fungicides (fluconazole, miclobutanil, triflumizole, epoxiconazole, and flusilazole) resulted in significant decreases in mean and highest levels of ChEs. In addition, it was observed that more than 80% decrease in ChE activity occured at higher concentrations of fluconazole and myclobutanil. The five compounds caused statistically significant inhibitions at all concentrations tested, except for flusilazole which showed no statistically significant differences at the lowest concentration (0.2 mg / L). In a study by Jin et al. (2016) it was shown that the exposure of *Danio rerio* to imazalil resulted in a significant reduction in ChE activity. The patterns observed in these studies were not the same as those observed in the present study. The activity of ChEs in this study had a slight increase, only for organisms exposed to the concentration of 68.8 ng/L, not allowing us to draw definite conclusions about this parameter. This may be due to the fact that the concentrations used in this study are lower than those found for other azole compounds, and tested in other species. In fact, some studies report that other azole compounds, but in substantially higher concentrations, were capable of decreasing ChEs activity (Zhu et al., 2014, Jin et al., 2016).

5 Conclusions

The primary objective of the present study was to determine the toxicological effects resulting from exposure of the aquatic organism *Daphnia magna* to clotrimazole. The present study suggests that clotrimazole represents an ecotoxicological risk for the microcrustacean *D. magna*. After exposure to this drug, a significant increase in CAT activity was observed. Likewise, an increase in GSTs activity was observed with the establishment of a dose-response relationship. After exposure to clotrimazole, the extent of altered ChE enzyme activity was not sufficient to support a neurotoxicity scenario.

From the data obtained here, it was possible to evidence the adverse effects in terms of oxidative stress that clotrimazole caused in *D. magna*, but the real risk for this organism and, consequently, for aquatic biocenosis can not be completely inferred. It should be noted that exposure concentrations are based on those found in surface waters. However, as shown in the present study, it is more likely that toxic exposures occur in the environment (Nunes et al., 2008), which have a high potential to exhibit deleterious reproductive and metabolic effects (Daughton and Ternes, 1999). Thus, this study should serve as a basis for additional work to elucidate about the effects of the studied compound on not only antioxidant enzymes and neurotransmission, but also on additional physiological pathways that aere likely to be impacted after long term exposures.

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Figures captions

Figure 1 - Effects of clotrimazole on CAT activity in *Daphnia magna*. Values correspond to the mean of 25 organisms ± standard error. * represent statistically significant differences (ANOVA $p \leq 0.05$) among concentrations of the pharmaceutical compound and the control.

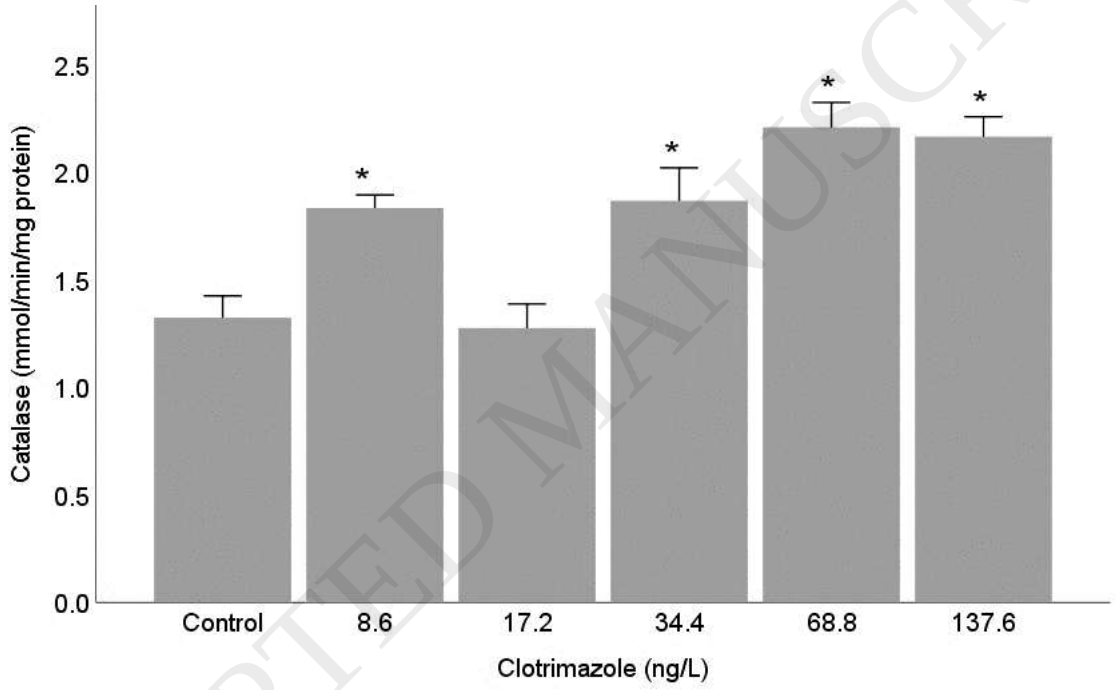

Figure 2 - Effects of clotrimazole on GSTs activity in *Daphnia magna*. Values correspond to the mean of 25 organisms ± standard error. * represent statistically significant differences (ANOVA $p \leq 0.05$) among concentrations of the pharmaceutical compound and the control.

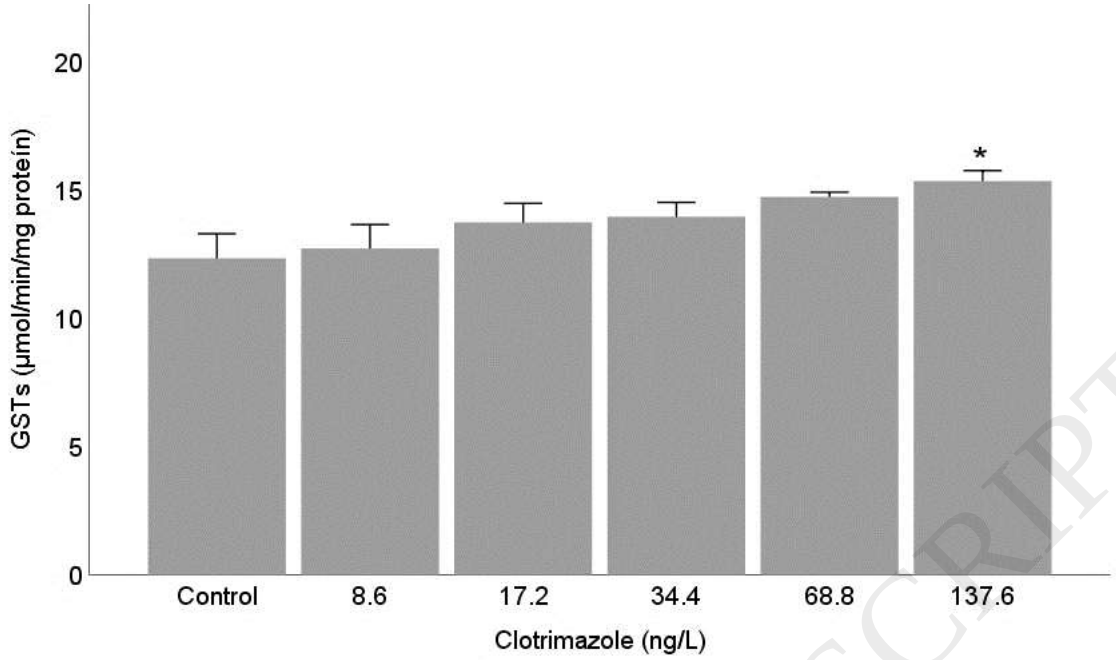

Figure 3 - Effects of clotrimazole on ChEs activity of *Daphnia magna*. Values correspond to the mean of 25 organisms ± standard error.

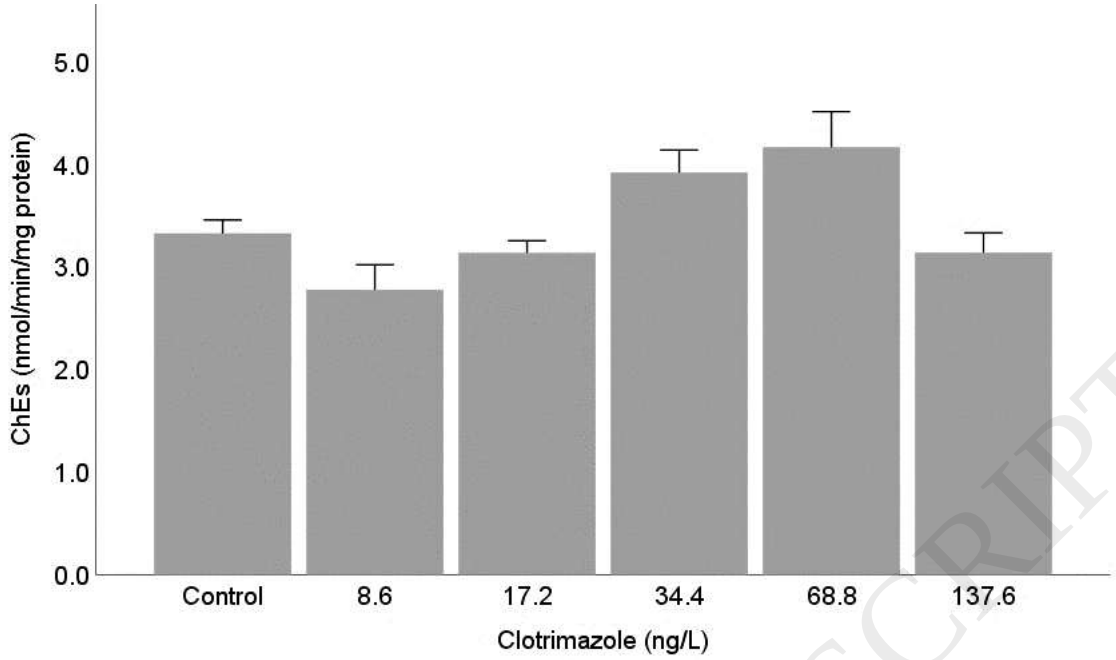

Table legend

Table 1 - Values of calculated EC_x (expressed in mg/L) and their respective 95% confidence intervals obtained for *Daphnia magna* exposed to clotrimazole.

	Probability	95% Confidence limits for concentration		
		Estimate	Lower limit	Upper limit
	0.100	2.772	1.429	3.731
	0.200	3.427	2.061	4.441
	0.300	3.994	2.652	5.098
	0.400	4.552	3.244	5.816
	0.500	5.143	3.853	6.685
	0.600	5.811	4.493	7.827
	0.700	6.623	5.192	9.453
	0.800	7.718	6.023	12.036
	0.900	9.542	7.226	17.227
	0.990	15.793	10.619	42.347