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Effects of diclofenac and salicylic acid exposure on *Lemna minor*: is time a factor?

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Abstract

The global occurrence of pharmaceuticals in the aquatic environment has been considered a particularly concerning problem with unknown consequences. Non-steroidal anti-inflammatory drugs (NSAIDs) including diclofenac (DCF) and salicylic acid (SA), are among the most frequently prescribed drugs in the world, being consequently commonly found in the aquatic environment. Prolonged experiments (with duration of exposure that surpass those recommended by already established testing guidelines) are important to obtain ecologically relevant data to address the issue of NSAIDs ecotoxicity, because by being more realistically (namely in terms of levels and durations of exposure), such tests may indicate realistic challenges posed to aquatic organisms. Among the most common test species that are used for assessing environmental quality, plants play a leading role. *Lemna* species are among the most important plants used for ecotoxicity testing. Therefore, the aim of this study was to evaluate the temporal effect of a prolonged exposure of DCF and SA on *Lemna minor*. To attain this purpose, *L. minor* plants were chronically exposed to 0, 4, 20, and 100 µg/L of both pharmaceuticals, and samplings were performed at 6, 10 and 14 days of exposure. The analyzed endpoints were levels of chlorophyll a, b and total, and of carotenoids; and enzymatic biomarkers as catalase, ascorbate peroxidase and glutathione-S-transferases. Diclofenac was responsible for alterations in all analyzed parameters in different intervals of exposure. Salicylic acid exposure was not capable of causing alterations on pigment contents of *L. minor*, however, enzymatic biomarkers were altered at all sampling intervals. Thus, it is possible to conclude that both pharmaceuticals can cause damage on the tested macrophyte species, biochemical parameters being more sensitive than physiological ones. Additional prolonged experiments are required to understand the chronic effects of different pharmaceuticals in the aquatic environment, especially in plants.

Key-words: Pharmaceuticals; diclofenac; salicylic acid; *Lemna minor*; biochemical analyzes; physiological analyzes.

Introduction

Human health is one of the most important concerns of modern life, and it has improved substantially during the past decades, in terms of general access to healthcare and availability of pharmaceutical drugs. The role attributed to (among other factors) the usage of large amounts of pharmaceutically active substances (Kunkel and Radke, 2012) is prominent. These biologically active compounds are distributed among several therapeutic classes, which encompass a diverse array of chemical natures and structures with multiple pharmacological modes of action to produce biological effect (Daughton and Ternes, 1999). After administration, some pharmaceuticals are not completely metabolized. The unmetabolized parent drugs and some metabolites are subsequently excreted from the body via urine and feces (Zhang et al., 2008), being treated in municipal wastewater treatment plants (if available) and ultimately enter the aquatic ecosystems. However, drugs may reach the environment via disposal of industrial and agricultural wastes, and accidental spills (Farré et al., 2008). Once in the environment, they can be widely distributed at some time (Farré et al., 2008). From these assumptions, we may consider that the majority of these chemicals are released into the environment, after their ultimate use by humans and/or animals, and they occur in the wild, where they may keep their chemical structure and biological activity. As a result, the number of potential non-target pharmaceutical–receptor interactions, indirect interactions and ecotoxicological effects in the environment is difficult to estimate, especially in complex matrices and when considering the vast number of living organisms that may interact with such substances (Brain et al., 2004; Farré et al., 2008).

The global occurrence of pharmaceuticals in the aquatic environment has been arising as a problem with unknown consequences (Kunkel and Radke, 2012) because this is mostly an unregulated regulated environmental issue (Pal et al., 2010). Some contaminants can be found in various environmental compartments and/or in areas where they were never used, mainly due to their persistence and long distance transport (Gavrilescu et al., 2015).

The ecological concern stems from the fact that pharmaceutical compounds are known to have biological effects, but only limited information has been obtained to quantitatively assess potential ecotoxicological impacts (Pal et al., 2010). What is not known, however, is whether these chemicals and their transformation products can elicit physiologic effects on biota at the low concentrations (ng-ug/L) at which they occur (Daughton and Ternes, 1999). The consequences of pollutants in aquatic ecosystems are of particular concern, because living organisms from these environments are chronically subjected to potential contaminations with consequences that may affect future generations (Gavrilescu et al., 2015).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most often prescribed drugs in the world, being consequently very common in the aquatic environment, since the early 2000s, and the environmental effects of NSAID have become a growing concern (Bonnefille et al., 2018). Among NSAIDs, one may find diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl) acetic acid; DCF), which is used to reduce inflammation and to relieve pain, such as arthritis or acute injury, and as antiuricosurics. DCF, when released into the environment, is likely to reach aquatic ecosystems and cause harmful effects on resident species. This compound is the most toxic among NSAIDs drugs, and shows also the potential to exert chronic toxicity (Kalenieeka and Zarzycki. 2015), being one of the most commonly found substances within the aquatic environment

(Schwaiger et al., 2004). These features lead the European Union to include DCF in its First Watch List of the Water Framework Directive, in order to obtain more information on its occurrence and effects in the environment (EU 2015/495, European Commission; Bonnefille et al., 2018).

Similarly to what happens for other pharmaceuticals, DCF often enters aquatic environments via inputs from wastewater treatment plants and the extent of its degradation depends on the used wastewater treatment technology (Lonappan et al., 2016). However, its inherent characteristics contribute for its environmental presence. The low DCF biodegradability often results in low elimination rates during biological wastewater treatment, and only a minor portion is adsorbed by sludge (Vieno and Sillanpää, 2014) and eliminated. That way, DCF can be detected in different environmental compartments such as drinking water in the USA (1.2 ng/L; Benotti et al., 2008), seawater (subtropical coastal zone - Brazil) in concentrations up to 19.4 ng/L (Pereira et al., 2016), concentrations between 0.06 to 15 µg/L in Germany rivers (Heberer, 2002; Jux et al., 2002; Weigel et al., 2002), levels of 195 ng/L in UK estuaries (Thomas and Hilton, 2004), and concentrations between 0.25 and 5.45 µg/L in sewage treatment plants effluents in France, Italy and Greece, according to Andreozzi et al. (2003) .

Another important NSAID is acetylsalicylic acid (ASA), which exerts its therapeutic action by inhibiting cyclooxygenase enzymes, at inflammatory sites (Gómez-Oliván et al., 2014). ASA has a widespread use given its additional beneficial therapeutic properties, such as analgesic and anti-pyretic action. ASA is rapidly hydrolyzed to salicylic acid (SA), which is its main metabolite and primarily responsible for the pharmacological activity of ASA; SA can be further conjugated to glycine to give rise to salicyluric acid or to glucuronic acid to form glucuronide conjugates, being also

excreted as the unchanged SA form (Davidson, 1971; Mullangi et al., 2012). In addition, SA has been found to play a key role in the regulation of plant growth, development, interaction with other organisms and in the responses to environmental stresses (Raskin, 1992). Its massive use is directly responsible for its environmental presence. Salicylic acid has been determined in Canadian influents and effluents in levels of 330 and 3.6 µg/L respectively (Metcalfe et al., 2003). In many municipal wastewater, SA was found at levels up to 4.1 µg/L (Ternes, 1998) and even in coastal zones of Belgium, SA was reported at concentrations up to 0.855 µg/L (Claessens et al., 2013).

A variety of aquatic species and responses are employed to derive environmental quality criteria, to assess toxicity of surface water and effluents, and to inform risk of chemicals in an ecological framework (Brooks et al., 2015). Among these species, plants are an essential part of an ecosystem, and a balance of plant-animal-bacteria complex is vital to a healthy environment (Wang, 1986). *Lemna* (duckweed) species constitute an extremely important group of plants, which may be found in diverse aquatic environments, including lakes, streams, effluents and sediments (Wang, 1990). Considering the importance of these species, several previous studies stressed out that *Lemna* species could be a promising indicator of aquatic toxicity (Wang, 1986), and that its use could also be applied to ecotoxicological tests, considering its sensitivity towards chemical contamination (Lakatos et al., 1993).

Lemna species have many advantages as test organisms in ecotoxicology. They can easily be cultured in the laboratory, in which they may achieve optimum growth conditions attaining exponential growth (Arts et al., 2008). In addition, *Lemna* species present small size, rapid growth and relative structural simplicity; they can be grown in aseptic cultures, simplifying working conditions with organic compounds. Reproduction

153 is usually vegetative, so that genetic variability can be eliminated by using a single
154 clone for all experiments (Hillman, 1961). *Lemna* spp-based tests may also be
155 complementary to those based on algae (i.e., *Selenastrum*) since test solutions can be
156 renewed, and background contaminant algal cells present in the receiving water or
157 effluent can be removed for frond production or chlorophyll measurements (Taraldsen
158 and Norberg-King, 1990). *Lemna* species may be also used for other measurements of
159 toxicological importance, such as biochemical and enzymatic alterations. Among
160 different *Lemna* species, *Lemna minor* (common duckweed) assumes a leading position,
161 since it is a floating, widespread, fast-growing plant, small and easy to cultivate,
162 characteristics that turn this species into an ideal candidate for aquatic toxicity tests
163 (Wang, 1986).

164 Prolonged experiments (that exposed test organisms for longer periods, thereby
165 exceeding common and already established exposure durations) are important for the
166 development of ecologically relevant data because by being more realistic (namely in
167 terms of levels and durations of exposure), they simulate more closely polluted
168 environments (García-Gómez et al., 2014) and may help deciphering adaptive and
169 acclimation processes that are likely to occur in the wild (Coutellec and Barata, 2013).
170 Therefore, the aim of this study was to evaluate the temporal effect of a prolonged
171 exposure of DCF and SA on *Lemna minor* in terms of physiological responses, namely
172 pigments amount and enzymatic biomarkers.

173

174 **Material and methods**

175 **Chemicals**

All pharmaceutical drugs were purchased from Sigma Aldrich, with purities >98%: diclofenac sodium (CAS: 15307-79-6) and salicylic acid (sodium salt form; CAS 54-21-7) and all other chemicals used in this study have analytical purity.

***Lemna minor* culture**

The plants to be used during the experiment were obtained from Centre of Environmental and Marine Studies (CESAM) - University of Aveiro - where were raised according to Alkimin et al. (2019).

***Lemna minor* assay**

Tests were performed be exposing plants to ranges of concentrations of DCF and SA that were chosen based on the already reported environmental concentration and also on predicted worst case scenarios of contamination. Diclofenac levels from ~1 and 20 ng/L were found in the river Elbe estuary (Weigel et al., 2002); up to 490 ng/L were registered in UK effluents (Hilton and Thomas, 2003); SA has been shown to occur in amounts ranging from 330 to 3.6 µg/L in influents and effluents (Metcalfe et al, 2003), up to 4.1 µg/L in many municipal wastewater (Ternes, 1998), and even in coastal zones at concentrations up to 0.855 µg/L (Claessens et al., 2013). Considering these values, the here-tested nominal concentrations were 0, 4, 20 and 100 µg/L for both pharmaceuticals. *Lemna minor* was exposed to the mentioned levels in eight replicates, in 400 ml plastic flasks with a final volume of 250 ml of modified Steinberg medium (OECD, 2006) per replicate, adequately supplemented with the pharmaceuticals stock

solutions (prepared in modified Steinberg medium). In the control treatment, replicates were exposed only to the modified Steinberg medium. The assay started with plants that covered ~30% of the vessel area. Assays were conducted under controlled conditions (temperature 23 ± 2 °C; photoperiod 24h^{L}; light intensity, ~6000 lux). The total volume of medium was renewed every other day in order to keep the exposure concentrations constant during the experiment. *L. minor* fronds were exposed for a total of 14 days and samples of each treatment were withdrawn at 6, 10 and 14 days of exposure. This total time of exposure was chosen considering that it corresponds to twice the exposure period recommended by OECD guideline 221 (OECD, 2006) for *Lemna* sp. tests. The collected biomass was divided in Eppendorf microtubes and stored at -80 ºC until the performance of analyzes.

Pigments analyses (chlorophylls and carotenoids)

Total, a, and b chlorophylls (TChl; Chl a; Chl b) and carotenoids (Car) amounts were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979) with the modifications proposed by Alkimin et al. (2019). Pigments were extracted from the previously exposed fronds of *Lemna* spp. (about 10 mg per replicate - fresh weight - FW) in 2.5 mL of dimethyl sulphoxide (DMSO). The extract was placed in water at 65 ºC during 30 min and allowed to cool in the dark at room temperature. The next day, samples were thoroughly vortexed for about 10 s and centrifuged for 5 min at 15,000 g at 4°C (ThermoFisher Megafuge 8R). The obtained supernatants were used to quantify the amounts of chlorophylls (a, b, and total), and carotenoids levels, by spectrophotometrically measuring the absorbances of the extracts at the wavelengths of 470, 645, 646, and 663 nm in a spectrophotometer Thermo Scientific Multiskan (ScanIt Software 2.4.4). The levels of the pigments were calculated

by using the equations proposed by Arnon (1949) and demonstrated by Hiscox and Israelstam (1979) to be suitable if the extraction was undertaken with DMSO, to calculate the amounts of Chl (a, b, and total; equations 1, 2 and 3 respectively), and Lichtenthaler (1987) to quantify the Car amount (equation 4).

$$\text{Chl a} = (12.70 \times A_{663}) - (2.69 \times A_{645}) \quad (1)$$

$$\text{Chl b} = (22.90 \times A_{645}) - (4.68 \times A_{663}) \quad (2)$$

$$\text{TChl} = (20.20 \times A_{646}) + (8.02 \times A_{663}) \quad (3)$$

$$\text{Car} = (1000 \times A_{470} - 1.43\text{Chl a} - 35.87\text{Chl b})/205 \quad (4)$$

Enzymatic biomarkers

Tissue samples were processed according to Alkimin et al. (2019). Catalase activity (CAT) was assayed by the procedure described by Aebi (1984), where the activity was quantified based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein. The ascorbate peroxidase activity (APX) followed the method described per Kovacik et al., (2009) where the oxidation of ascorbic acid was followed as a decrease in absorbance monitored at 290 nm for 5 min. The enzymatic activity was calculated using a molar absorption coefficient 2.8 mM^{-1} cm^{-1}, according to Nakato and Asada (1981). For the glutathione-S-transferases (GSTs) activity determination, the procedure described by Habig et al. (1974) was used. These procedure monitors the increment of absorbance of the thioether resulting from the catalysis of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GSTs, at 340 nm. Results were expressed as nanomoles of thioether produced per minute, per milligram of protein. Total soluble protein quantification of samples was performed at 595 nm using the Bradford method

(Bradford, 1976), adapted to microplate with bovine γ-globulin as standard. All parameters were performed spectrophotometrically, and the readings were performed in a microplate reader Thermo Scientific Multiskan (Software Ascent 2.6).

Statistical analyses

A one-way analysis of variance (ANOVA) was performed. Statistics tests and analysis of normality (Shapiro-Wilk) and homogeneity (Levene test) were conducted using the software SPSS v25. Analyzes on ranks (Kruskall-Wallis) were performed if even after being transformed, the data did not pass in normality and/or homogeneity tests. A significance level of 0.05 was adopted and post hoc test were applied, Dunn's or Dunnet were chosen according to the data.

Results

Chlorophyll a was affected by DCF exposure (figure 1A), being its content significantly increased in plants exposed to the concentrations of 20 µg/L, after 6 d of exposure; an increase of this parameter was observed after 10 d of exposure to 4 and 100 µg/L. After an exposure of 14 d, the Chl a content stabilized. On the other hand, SA exposure (figure 1B) was not capable of causing any significant alterations of the pigments levels, for all sampling intervals.

Diclofenac (figure 2A), after 6 d of exposure to 4 and 20 µg/L, caused an increase on Chl b content; after 10 d of exposure, plants exposed to 4 and 100 µg/L of this substance had significant increases in this same parameter. No differences were

however reported after 14 d of exposure. On the contrary, SA (figure 2B) did not cause changes on Chl b amount for any sampling interval.

Total Chl amounts were affected after 6 d of exposure to DCF (figure 3A) causing a significant increase on this pigment content in plants exposed to a concentration of 20 µg/L. 10 d of exposure to 4 µg/L of the same drug caused a significant increase, while 14 d of exposure were not capable to cause changes in this parameter, and total Chl values were similar to those registered for control plants. Under no circumstances SA exposure (figure 3B) was capable of causing changes in Total Chl amount.

Considering DCF exposure (figure 4A), results for Car levels showed an increase after 6 d of exposure to 20 µg/L and for 10 d to a 100 µg/L, the amount of this pigment was reestablished after 14 d of exposure. Carotenoids levels were not altered after SA exposure (figure 4B) for all intervals.

The first enzymatic marker analyzed was CAT activity. A 6 d exposure to DCF (figure 5A) caused a significant increase in the activity of this biomarker for plants exposed to concentrations of 4 and 100 µg/L; at the 10^{th} d of exposure, a significant increase in CAT activity was also observed for plants exposed to 20 and 100 µg/L; on the other hand, after 14 d of exposure, the activity of catalase declined, being statistically different only for plants exposed to 100 µg/L. The same enzyme presented different behavior when *L. minor* was exposed to SA (figure 5B); after 6 d of exposure only plants exposed to 4 µg/L had a significant increase in their CAT activity; plants exposed for 10 d and to all tested concentrations presented a significant increase of this parameter; on the 14^{th} d, the CAT values tended to normalize, being similar to those founded in plants from the control treatment.

Ten days of exposure to DCF (figure 6A) caused a significant increase of APX activity, but only plants subjected to 20 µg/L showed a significant effect; no alterations were reported after 6 or 14 d of exposure. However, SA exposure (figure 6B) allowed obtaining more consistent results; 6 d of exposure were not enough to cause any significant effect on APX, but after 10 d of exposure, a significant increase of APX activity was observed for plants exposed to levels of 20 and 100 µg/L; after 14 d of exposure, plants exposed to low SA levels had their APX decreased, while those exposed to higher SA levels had significant increases of APX activity.

Six days of DCF exposure (figure 7A) did not cause any significant alteration on GSTs activity on *L. minor*; on the other hand, 10 d of exposure caused an increase in GSTs activity, for all tested concentrations, being statistically different for levels of 4 and 100 µg/L; 14 d of exposure to DCF caused a significant decrease of GSTs activity for plants exposed to all concentrations.

SA (figure 7B) was able to provoke a significant increase on GSTs activity in plants exposed to 4 and 100 µg/L; GSTs levels returned to normal after the 10^{th} day of exposure, and a significant decrease in GSTs activity was observed in plants exposed to all SA concentrations after day 14.

Discussion

Diclofenac

Ecological relevance, toxicological sensitivity (as expressed by the values of toxicity parameters), and discrepancy vs. agreement of results among distinct species are three important criteria for the selection of useful endpoints in macrophyte toxicity tests (Arts et al., 2008). This is particularly important when considering the use of *Lemna* for ecotoxicity testing purposes. In fact, some *Lemna* species are referred by some studies

to be extremely sensitive in terms of their biological response, while being described as tolerant to environmental stressors by others. This apparent contradiction can be explained on the basis that plants may be highly adaptive (Wang, 1990), and it is necessary to test for this adaptive potential along the time course of the exposure. This mean that different responses (in terms of their intensity) may be attained after exposing these species for distinct periods, to the same combination toxicant/levels. This is also important because ecotoxicological relevance of data obtained in short term ecotoxicity tests at relatively high concentrations is difficult (if not impossible) to extrapolate to real conditions in the wild, where sensitive species may be chronically exposed to multiple contaminants (Paul et al., 2017). Under such realistic conditions, the quality of results obtained from short-term, as well as from prolonged exposures, are difficult to interpret, considering the number and complex contributions of potential confounding factors (Kunkel and Radke, 2012). However, prolonged exposure to pollution may induce community tolerance to chemical stress, and may also reduce the intensity (or alter the nature) of the response that may be detected, indicating that the community has been restructured as a response to the continuous presence of the toxicant in question (Eriksson et al., 2015). That is, distinct sampling intervals may yield different results, not only in terms of intensity but also in the type of response to be observed. The here-obtained results underline the need to adopt prolonged exposure modes, during which sampling must be assured at discrete intervals, as a way of assuring that environmentally realistic conditions are used; in addition, it is of fundamental importance to adopt a specific timeframe of exposure, to avoid having a strong modulation in the extent, and more importantly, the type of response.

Exposure to toxicants is one of the most common triggering factors for the production and release of reactive oxygen species (ROS) by the organism (Tripathy and Oelmüller,

2012), despite being a normal metabolic process in all aerobic organism (Perl-Treves and Treves, 2002). These stressful environments induce the generation of ROS such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and others in plants (Hayat et al., 2010). The production of ROS is not deleterious when the redox homeostasis is not challenged, but it can suffer alterations after exposure to toxicant (and its metabolism), since this may culminate in an increased stress challenging the organism (Bailey-Serres and Mittler, 2006). When such condition arises, the redox homeostasis is questioned, and antioxidants are necessary to help restore the normal organism functioning (Ahmad et al., 2010). This group of chemicals includes distinct entities, such as carotenoids, a group of natural tetraterpenoid pigments distributed widely in plants, but also common in algae, fungi, and bacteria (Sun et al., 2018). In our study, Car seemed to have been involved in the response to the presence of DCF. Levels of these pigments were altered in plants after a short-term challenge of 6 d of exposure to an intermediate concentration of DCF; a similar response was also reported after 10 d of exposure but to the highest concentration of the same drug. On the contrary, and after a 14 d exposure, Car levels were very similar to those measured in control plants. Carotenoids play an important role in the prevention of several degenerative stress processes in plants owing to their antioxidant function (Sytar et al., 2013). Consequently, carotenoids can be photoprotectants, acting as ROS scavengers (Nisar et al., 2015) and preventing self-oxidation of the photosynthetic systems (Braslavsky and Holzwarth, 2012); these structures are prone to oxidative damage since they may act as antennas, collecting solar radiation in the 400 nm to 500 nm range and transmitting the energy by energy transfer to the chlorophylls or bacteriochlorophylls in the reaction centers with photosynthetic functions (Braslavsky and Holzwarth, 2012). The here-obtained pattern of results seem to show the involvement of carotenoids: after

6 d of exposure to a concentration of 20 µg/L of DCF, the antioxidant defense was dependent upon the antioxidant scavenging activity of Car, without the activation of the antioxidant enzymatic defense pathway, since CAT and APX were not activated. However, under harsher conditions (e.g. the combination of a longer period of exposure and higher amounts of DCF), a full antioxidant response was deployed, that involved the activation of CAT activity. However, this set of effects may suggest that the here adopted conditions (levels and durations of exposures) only elicited transient moderate alterations, that were reverted after longer exposure periods due to adaptive responses of the plants, and were not enough to cause permanent damage in this group of pigments.

On the other hand, the antioxidant system was activated on *L. minor* after DCF exposure, at least partially. Among the different enzymes with antioxidant activity, CAT was more responsive than APX. Despite being distributed among a large number of locations, APX was not equally responsive when compared to CAT. APX isoenzymes are distributed by at least four distinct cellular compartments: stromal APX and thylakoid membrane□bound APX in chloroplasts, microbody (including glyoxysome and peroxisome) membrane□bound APX, and cytosolic APX. A fifth APX isoenzyme can occur as a mitochondrial membrane□bound form (Shigeoka et al., 2002). On the contrary, CAT is mostly found in peroxisomes (Copper, 2000). Despite this difference, in general, APX levels of DCF-exposed plants were kept unchanged, similar to those reported for the control plants. However, Bartha et al. (2014) demonstrated that much higher DCF concentrations (1 mg/L) are capable of increasing this isoenzyme activity after 1 and 7 d of exposure, on the species *Typha latifolia*. After being exposed for 6 and 10 d to DCF, plants had their CAT activity significantly increased. This effect might have been triggered to cope with increased levels of ROS caused by DCF exposure and metabolism; this assumptions is made based on the

physiological role of both APX and CAT, which belong to two different classes of H_2O_2 scavenging enzymes; APX is responsible for the fine modulation of ROS for signaling, whereas CAT is responsible for the removal of excess ROS during stress (Mittler, 2012) associated to a very fast turnover rate (Mhamdi et al., 2010). DCF has been shown to be pro-oxidative, causing oxidative stress in different organisms. Exposure to DCF (100 µg/L) resulted in the increase of CAT in gills and liver of *Cyprinus carpio* after 96 h (Nava-Álvarez et al., 2014). Islas-Flores et al. (2013) reported similar results in the same organism, with a CAT activity increase in gills and liver, after exposure to DCF at a level of 7.098 mg/L during different intervals, 12 h and 48 h for gills, and 72 h for liver. Exposure to DCF contaminated sediment (46.7 µg/kg), resulted in a CAT increase in *Hyalella azteca* from 12 to 72 h of exposure (Oviedo-Gómez et al., 2010). In plants, similar results suggesting that DCF metabolism can indeed result in pro-oxidative alterations were found by Alkimin et al. (2019), in a study that reported a significant increase in CAT activity on *L. minor* acutely exposed to 100 µg/L of DCF.

Other pharmaceutical drugs from the same NSAID class, namely ibuprofen, have been involved in similar responses. Dordio et al. (2011) found an increase in CAT activity in *Typha* spp. after being exposed to this drug in varying concentrations (0.5 – 2 mg/L) during 7, 14, and 21 d. The results found in the literature show the putative triggering of antioxidant mechanisms to counteract the challenge posed by the increased amounts of ROS caused by exposure to this drug, a response that is also common to other taxa. Despite these assumptions based on response patterns described in the literature, our study evidenced that this response might be reverted along time. In fact, after 14 d of exposure to DCF, the CAT activity of exposed plants was decreased in relation to the control, raising the hypothesis that a prolonged exposure caused a long period of chemical stress in the plant, that lead to a permanent condition of failure in the

antioxidant defense mechanism. This corresponds to a scenario for which the plant cannot fully respond, being incompetent to handle the excess of formed ROS and thereby suffering oxidative damages caused by them. This alteration, evidenced by the decrease of CAT activity, caused by long-term (35 d) exposure to DCF levels of 60 µg/L was reported in other organisms such as *Tinca tinca* (Stancova et al. (2017); similarly, Saucedo-Vence et al. (2015) reported lower levels of CAT activity in blood and liver of *Cyprinus carpio* after 24 d of exposure to 7.098 mg/L of DCF.

This hypothesis can be also supported by the results concerning the here measured GSTs activity. These enzymatic forms correspond to a phase II metabolism isoenzymes group, responsible for the detoxification of exogenous substances through the ability to conjugate glutathione (GSH) with compounds containing an electrophilic center, in order to modify the substrate into a more water soluble, less toxic complex (Habig et al., 1974). It is known that the majority of GSTs substrates are either xenobiotics or products of oxidative stress (Hayes and Pulford, 1995). In this study, it was possible to observe an increase in GSTs activity in plants exposed for 6 and 10 d to DCF. This tendency might be interpreted as a biological response aiming at transforming DCF into an excretable metabolite, which is a common trend among most living organisms, and plants are no exception. This pathway seems also to be important in plants, considering the versatility of their conjugation machinery, namely their GSTs isoenzymes. In fact, individual gene analysis and genomics studies indicate that plants have 25 or more genes encoding for GSTs (Edwards et al., 2000), showing that similarity of GSTs-mediated responses among a large set of distinct organisms. In addition, the increase of GSTs activity can result from an attempt to eliminate ROS, since this enzyme is part of glutathione-peroxidase cycle, a metabolic pathway that detoxifies hydrogen peroxide (Smirnoff, 2000). However, plants exposed for higher periods (14 d), showed a decrease

in this isoenzymes activity, similar to the pattern reported for CAT activity. As previously cited, Stancova et al. (2017) found a decrease in CAT activity on *T. tinca* after 35 d of exposure to 60 µg/L of DCF and, similarly to our results, the authors reported a decrease in GSTs activity too. This comparable pattern of response emphasizes the possibility of similar biological responses to DCF prolonged exposures, even in different organisms. It is however necessary to stress that this pattern is not always repeated, since GSTs response seems to depend upon different factors, such as time and route of exposure, tested concentration, and type of organism. These assumptions are reinforced considering the following studies. According to Stepanova et al. (2013) GSTs were increased in *C. carpio* larvae after 30 d of exposure to 3 mg/L of DCF. On the other hand, even 15 d of exposure to 250 ng/L of DCF were not capable of causing changes on GSTs activity on gills and digestive gland on *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno, 2014). Finally, in plants (*T. latifolia*), after 3 and 7 d of exposure to 1 mg/L of DCF, an increase on GSTs activity was recorded (Bartha et al., 2014). On the other hand, Kummerová et al. (2016) found a decrease on GSTs activity after 10 d of exposure to 100 µg/L of DCF in *L. minor*. A decrease in GSTs activity, detected in our study, may be a consequence of a long-term damage that was only ascertained after a prolonged exposure period to DCF, most likely by the adverse effect of ROS on this pathway. However, the analysis of the here-observed changes concerning this particular enzymatic activity alone does not allow a clear identification of the specific function that was activated, the mere conjugation with GSH to facilitate excretion of the drug, or an antioxidative defense function (Bartha et al., 2014). At low concentrations of toxicant, the hydroxylation process seem to be sufficient to detoxify the xenobiotic compound in plants, while at higher concentrations, more effective and comprehensive metabolic pathways may be activated (Bartha, 2012).

On the other hand, ROS are capable of damaging lipids, DNA, and especially proteins, consequently causing a decrease in enzymatic activity by denaturation (Schieber and Chandel, 2014). Considering that GSTs isoenzymes are a considerable portion of all soluble proteins in plants (e.g. in *Zea mays*, GSTs constitute >1% of the soluble protein; Marrs, 1996), protein denaturation in a moderate extent may significantly compromise the enzymatic activity of affected enzymatic forms. Another factor to consider in the analysis of this denaturation effects is linked to the accumulation of ROS along time, as a result of their natural production by biological systems. This scenario may be aggravated considering the occurrence of a spatially and temporally isolated event such as the exposure to an oxidant xenobiotic, whose toxicity will depend on the duration of exposure to this additional stress factor (Bailey-Serres and Mittler, 2006). ROS may be produced in cells in general at any time, but their production may increase if cells are exposed to specific stressors, such as pro-oxidants, resulting in a combination of factors that are ultimately responsible for extreme levels of ROS, whose presence will certainly result in increased toxicity (Bailey-Serres and Mittler, 2006). Another option justifying DCF toxic effects may involve its phase II metabolites, such as glucuronide diclofenac and glutathione diclofenac, which have also been identified in mammals. The bioactivation of these conjugates has been implicated in diclofenac-induced toxicity effects, including oxidative stress and liver injury (Tang et al., 1999; Boelsterli, 2003). In plants, the metabolism of xenobiotics follows somewhat similar principles to those described for mammals (phase I - activation, phase II – conjugation and phase III, for plants, incorporation/stabilization) (Huber et al., 2012). In fact, this assumption is reinforced by the finding of metabolites of DCF in the plant species *T. latifolia* similar to those found in humans (such as 4'-OH diclofenac and diclofenac-glucopyranoside) (Huber et al., 2012), being possible to suggest this hypothesis. However, we must not

forget that although plants and animals have partially similar detoxification enzymes, and that in both groups of organisms xenobiotic detoxification follows the three-phase model, the exact mechanisms behind the process of xenobiotic detoxification may be very different (Bartha et al., 2014) making it more difficult to explain all mechanisms involved in DCF effects in plants since this drug was designed to interact with the physiology of animals.

Furthermore, the oxidative stress caused by DCF exposure can be related to the capacity of this drug to induce ROS production, and its deleterious consequences, namely by provoking the peroxidation of the membrane lipid bilayer, which results in changes in adverse modifications of its integrity, and the malfunction of membrane-bound proteins and lipids (Hájková et al., 2019). Associated with this capacity, the high log K_{OW} values of DCF (4.51) suggest the possibility of its enhanced accumulation cells, by promptly permeating biological membranes (Corcoll et al., 2014). This might have decisively contributed to the oxidative stress scenario made evident by this study; however, the principal mechanism of DCF toxic action in plants is largely still unknown (Hájková et al., 2019).

Chls are a group of structurally closely related compounds, universally acknowledged to be the indispensable photoreceptors in plant and bacterial photosynthesis (Katz et al., 1978). The biosynthesis of Chl and the development of chloroplasts in higher plants involve a highly controlled series of events, many of which may be subjected to inhibition by exogenously applied chemical agents (Wolf, 1977). As described in the results section, exposure to DCF was capable of causing an increase in Chls (a, b and total) contents after 6 and 10 d of exposure. However, these levels were reestablished near to control amounts after 14 d. DCF uptake and metabolization by plants occur very rapid (Huber et al., 2012), and may cause impact on primary processes of

520 photosynthesis, mainly in the disruption of electron transport chain between
521 photosystems II and I, which reflects in significant changes in "vitality index"
522 (Kummerová et al., 2016). This effect raises the hypothesis that the chlorophyll
523 biosynthesis system is altered, increasing the amount of these pigments, in an attempt to
524 maintain the basic functioning of photosynthesis and consequently absorption of energy
525 to perform the normal metabolic activities of plants. On the other hand, the
526 reestablishment of Chl levels after 14 d may confirm the highly adaptative capacity of
527 plants suggested by Wang (1990) and demonstrated by this study after a prolonged
528 exposure. However, despite phytotoxic effects caused by DCF exposure are evident,
529 information about which metabolic pathways this drug may alter, as well as the
530 potential toxicity of DCF metabolites for plants, are still unclear (Vannini et al., 2018).

531 In general, this work demonstrated that DCF can cause damage on physiological and
532 biochemical parameters on *L. minor*, however, apparently after a prolonged exposure
533 the plant could cope with the exposure to the drug and physiological parameters were
534 reestablished to values close of unexposed organisms. On the other hand, the
535 biochemical system continues to demonstrate changes, emphasizing that even over time
536 the plant still makes use of defensive barriers/biochemical defense to try maintain its
537 homeostasis.

538

539 **Salicylic acid**

540 From the literature, it is difficult to ascertain the real toxic effects caused by exogenous
541 SA exposures since, in general, the exogenous application of this natural plant hormone
542 might act as a powerful tool in enhancing the growth, productivity and also in
543 combating the adverse effects generated by various abiotic stresses in plants.

Consequently, SA is intentionally used in agriculture being a great promise as a management tool for providing tolerance to crops against the aforesaid factors (Joseph et al., 2010). However, it is extremely important to understand the possible adverse effects caused by exogenous sources of SA, in particularly in aquatic organisms, which occur in the final environmental compartment which is the most common destination of this drug after wastewater disposal and agricultural application. Despite being a natural plant hormone (Raskin, 1992), SA can cause adverse effects on biochemical parameters, especially if exposure results from exogenous sources, as shown in this work. Ascorbate peroxidase is one of the most important ROS-scavenging enzymes (Sofo et al, 2015) and works along with other (iso)enzymes, with catalase-like functions (Apel and Hirt, 2004). The literature is well established determining that endogenous SA is normally capable of inhibiting the CAT and APX activities, to increase H_2O_2 concentrations in the cell, to activate its immune system after infection by pathogens (Vlot et al., 2009). This inhibition, as suggested by Durner and Klessig (1996) probably results from peroxidative reactions. On the other hand, according to Rao et al. (1997), exogenous SA treatment is capable of causing the increment of H_2O_2 levels in plants, thus provoking oxidative stress. Additionally, SA inhibits the electron transport system in plant membranes, favoring ROS accumulation; on the contrary, it also activates alternative oxidase (AOX), a specific electron transport route that is part of cytochrome oxidase pathway, retarding this process (Krasavina, 2007). However, the effects of exogenous SA in these enzymes in plants is not extensively described, and a considerable lack of information on this subject still exists, demanding better understanding of the effects of SA on plants. In this work, *L. minor* plants, after being submitted to an exogenous SA source, presented significant alterations on both enzymes cited above (APX and CAT), which may be interpreted as an indication of the activation of the antioxidant defense

569 system. After an initial period of 6 d of exposure to SA, APX activity was not changed;
570 however, plants exposed to the lower concentrations of SA showed an increase of CAT
571 activity, suggesting a prompt activation of this defensive mechanism, faster than APX.
572 On the other hand, a 10 d exposure period caused the activation of both enzymes, in an
573 attempt to cope with increased ROS caused by this longer exposure, being this increase
574 dose-dependent. Furthermore, SA-induced redox regulation, to cope with ROS increase,
575 appears not only to involve the here studied mechanisms; in fact, it may also lead to the
576 accumulation of phytohormones, such as ethylene, nitric oxide, and jasmonate (Dat et
577 al. 2003). Finally, data concerning a 14 d exposure showed that CAT activity was
578 similar to the control plants; plants exposed to the highest SA levels had significantly
579 higher levels of APX, suggesting that the activation of this enzymatic form occurred at a
580 later stage. However, exogenous SA enhanced the activities of antioxidant enzymes like
581 APX and superoxide-dismutase (SOD), with a concomitant decline in the activity of
582 CAT in maize plants (Krantev et al., 2008). Similar results were found in this work,
583 after a 14 d exposure of *L. minor* to SA. In summary, as cited above, it seems to exist a
584 contradiction, regarding the physiological role of SA, since its presence might in some
585 circumstances prevent ROS production, but it may also stimulate it, causing oxidative
586 damage in plants. Based on the here obtained results, it is possible to suggest the
587 relationship between both antioxidant enzymes activity after an exogenous SA. From
588 the literature, no data showing that SA may exert adverse damaging effects in aquatic
589 plants are available. In fact, the literature on the toxic effects caused by exposure to
590 exogenous SA towards aquatic plants is extremely scarce; in general, toxicological
591 studies use SA as a preventive factor for possible damage caused by other exogenous
592 stressors, such as chemicals (paraquat; Ananieva et al, 2004), drought effects (Hayat et
593 al., 2008) and low temperatures (Janda et al., 1999).

In contrast, the activity of phase II metabolism (GSTs isoenzymes, in this case), significantly increased its activity after the 6th d of exposure to SA. This response demonstrates that the plants soon recognized the exogenous SA as a chemical challenger, whose presence required the increased efficacy of the GSH conjugation biotransformation route, to form a more soluble and less toxic compound to be eliminated. It is necessary to consider that SA is metabolized by a combination of glycosylation and decarboxylation, as shown to occur in tobacco leaves (Edwards, 1994), and by conjugation with glucuronic acid, reflected by a glucosyltransferase activity increase in rice roots (Silverman et al., 1995). Assuming that these same metabolism pathways might occur in *L. minor*, since there is no such information for the species in question, the increase in GSTs activity and consequently GSH increase may not be related to the biotransformation of the exogenous SA. Therefore, it can be assumed that in this case the GSH conjugation by means of GSTs acts as ROS scavenger. GSH not only participates in the direct detoxification of ROS, it may also protect cells against unfavorable stress effects through the activation of various defense mechanisms due to its involvement in redox signaling (Apel and Hirt 2004; Foyer and Noctor 2005). In this signaling pathway, GSH interacts with ROS, redox molecules [Trxs, glutaredoxins (Grxs)], and plant hormones [salicylic acid (SA), abscisic acid (ABA)] (Szalai et al., 2009), giving rise to a complex, albeit effective, protective mechanisms against toxic effects of ROS. This suggestion can be supported by data from the literature, since exogenous SA application also activated GSH synthesis in *Brassica juncea* and *B. napus* and caused enhanced protection against abiotic stressors, such as drought- and salt-induced oxidative damages (Alam et al., 2013; Hasanuzzaman et al., 2014). On the contrary, after the 10th d of SA exposure, no alterations in GSTs activity were reported. Taking into account the previous assumed hypothesis (GSTs as

ROS scavenger), this could be an expected response since at that time interval, CAT and APX activities were already increased, assuming a leading role against oxidative injury. On the contrary, after 14 d of exposure SA was responsible for decreasing GSTs activity, suggesting the possibility that this enzyme could have been directly denaturated by ROS generated after a prolonged SA exposure, similarly to what was observed after DCF exposure. According to Hasanuzzaman et al. (2017) low concentrations of SA caused advantageous effects in abiotic stress tolerance of plants. In contrast, high concentrations of SA showed to exert toxic effects. This contradictory profile makes difficult to described and identify all metabolic pathways, functions and alterations caused by SA exposure, since several factors (e.g., the concentration and application method of SA) are critical to yield distinct effects in different plant species (Hasanuzzaman et al., 2017).

As previously cited, SA is a natural plant hormone, and has direct functions on plant physiology, regulating growth. In addition, in some cases, endogenous SA protects plants against xenobiotics and stimulates the production of photosynthetic pigments (Hashmi et al., 2012). SA may act as a stressor as well, which among others, negatively affects the photosynthetic processes, especially above a certain threshold concentration (Janda et al., 2014). The effective concentration to attain this effect may highly depend on the plant species, the way of the application, the duration of the treatment, and the environmental conditions (Janda et al., 2014). For example, the same concentration that provided protection against low temperature-induced damage in young maize according to the time and conditions of exposure (Janda et al. 1999), could decrease/protect barley against paraquat effects (Ananieva et al. 2002). Take into account, that in this study, there were no observed effects on pigments levels (Chl a; b and total and Car), these results can be attributed to two different hypothesis: first, the absence of effect on

644 pigments levels may result from an acclimation mechanism to exogenous SA exposure
645 (Zait et al., 2018); or second, the here tested concentrations were not capable of causing
646 damages on pigments analyzed in this specific aquatic species. According to Janda et al.
647 (2014), the effects of exogenous SA depends of the effective concentration acting on
648 plant tissues. In turn, this amount can vary according to the plant species, the
649 application route, the duration of exposure, and the environmental conditions. In
650 addition, Chls represent the central part of the entire metabolism of the green plant
651 system, therefore, any significant change in their levels is likely the reason of significant
652 toxic effects, manifested primarily on growth alterations (Belkadh et al., 2014).
653 However, the importance of SA in the regulation of plant growth, and the clear
654 establishment of SA toxic mechanisms that may adversely influence growth, are areas
655 of further investigation, reinforced by the assumption that even the SA natural
656 biosynthesis is not completed elucidated (Janda and Ruelland, 2015). Even without
657 effects in pigments content, compounds such as SA can affect the gas exchange rates, in
658 maize, and may also influence the processes related to the photosynthetic electron
659 transport by enhancing the non-photochemical fluorescence quenching mechanisms
660 (Janda et al., 2000). On the other hand, the complete elucidation of the mechanism by
661 which SA causes toxic alterations in this pigment group is a task made difficult by the
662 enormous gaps in the knowledge about its metabolism in plants, a scenario that mostly
663 favors speculation. In addition, it is necessary to take into account that the majority of
664 findings about the effects of exogenous SA were obtained for terrestrial plants,
665 especially those used in agricultural practices (such as maize, tomato, pea and others).
666 These species may substantially differ from aquatic species, since these inhabit different
667 environments and show some specific physiologic processes and adaptations. Finally,
668 SA signalling is complex and over the coming years, further advances will be required

669 (Janda and Ruelland, 2015); despite being a natural plant hormone, exogenous SA can
670 cause biochemical alterations in *L. minor* metabolism that in a longer-term or even in
671 future generations, can challenge the survival and adaptation of this species in different
672 aquatic ecosystems, affected by with the presence of SA as a result of human excretion.

673

674 **Conclusions**

675 In general, both tested pharmaceuticals showed to be toxic to *L. minor*. Diclofenac, in
676 all sampling intervals, caused alterations in biochemical parameters, more pronounced
677 in CAT and GSTs, and varying according to the time and tested concentration. DCF
678 exposure provoked significant increases of the amounts of pigments (Chl a, b, total and
679 Car). This response was evident after the two initial samplings intervals (6 and 10 d),
680 but was followed by a long-term adaptive response, made evident by the results
681 obtained for the 14 d sampling. Results obtained after a 14d exposure to DCF showed
682 that plants were capable to recover to basal levels of physiological traits. In addition,
683 SA exposure, under the here tested conditions (distinct intervals of exposure and
684 concentrations), was capable of increasing CAT and provoking a variation in APX and
685 GSTs, according to time and concentration. These responses, in general, may ultimately
686 compromise survival, by demanding additional adaptive effort to this species. However,
687 it is necessary to undertake more studies about the toxicological effects of DCF and SA,
688 mainly focusing on aquatic plants, considering that these pharmaceuticals are largely
689 found in aquatic environment. Thus, this work shows that time is a factor that contribute
690 to pharmaceutical toxicity in aquatic plants, by modulating not only the extent but
691 especially the type of the toxic effects, that may results from exposure to environmental
692 relevant concentrations of the tested pharmaceuticals. Finally, more prolonged exposure

693 tests are recommended to understand the toxicology of pharmaceuticals in aquatic
694 plants.

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704

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1054

1055 **Figure captions**

1056 Figure 1. Effects of diclofenac (A) and salicylic acid (B) on chlorophyll a of *L. minor*. For each
1057 parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to
1058 control, for each species (n=8); FW = fresh weight.

1059 Figure 2. Effects of diclofenac (A) and salicylic acid (B) on chlorophyll b of *L. minor*. For each
1060 parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to
1061 control, for each species (n=8).

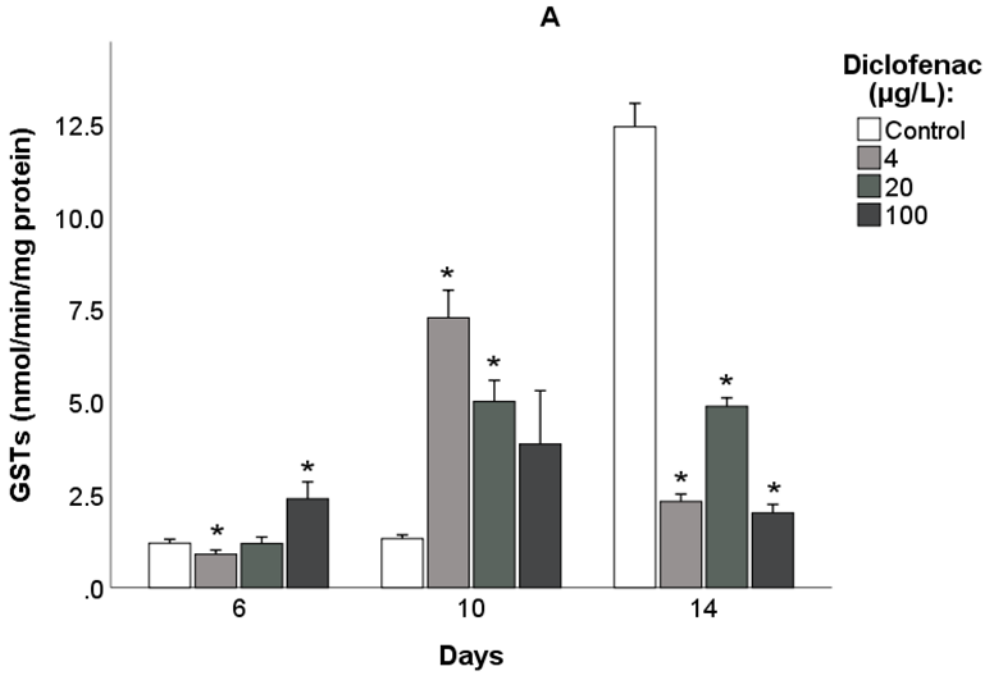
1062 Figure 3. Effects of diclofenac (A) salicylic acid (B) on total chlorophyll of *L. minor*. For each parameter,
1063 mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for
1064 each species (n=8).

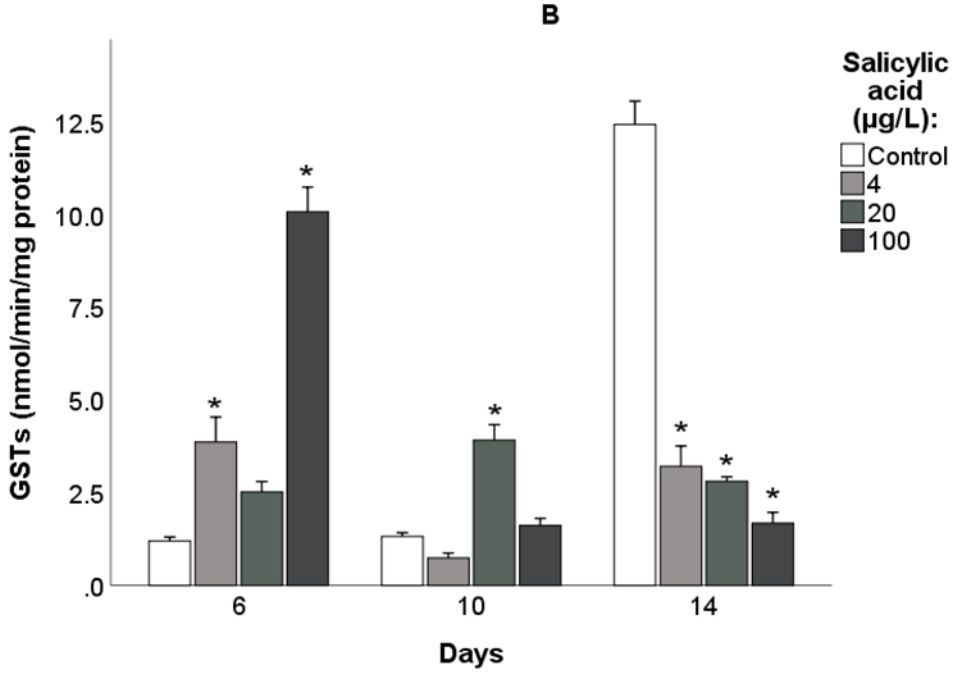
1065 Figure 4. Effects of diclofenac (A) and salicylic acid (B) on carotenoids of *L. minor*. For each parameter,
1066 mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for
1067 each species (n=8).

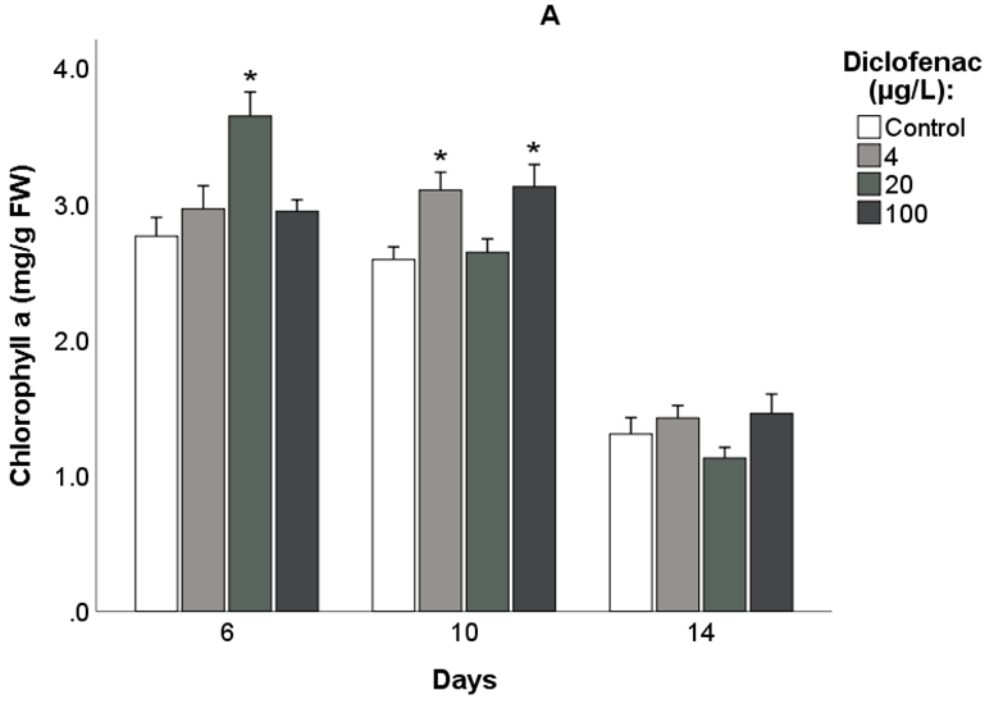
1068 Figure 5. Effects of diclofenac (A) and salicylic acid (B) on catalase activity of *L. minor*. For each
1069 parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to
1070 control, for each species (n=8).

1071 Figure 6. Effects of diclofenac (A) and salicylic acid (B) on ascorbate peroxidase activity on *L. minor*.
1072 For each parameter, mean and standard error are shown. * stand for statistical differences in relation to
1073 control, for each species (n=8).

1074 Figure 7. Effects of diclofenac (A) and salicylic acid (B) on glutathione-S-transferases activity on *L.*
1075 *minor*. For each parameter, mean and standard error are shown. * stand for statistical differences in
1076 relation to control, for each species (n=8).


A
GSTs (nmol/min/mg protein)
12.5
10.0
7.5
5.0
2.5
.0
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100


B
GSTs (nmol/min/mg protein)
12.5
10.0
7.5
5.0
2.5
.0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100


A
Chlorophyll a (mg/g FW)
4.0
3.0
2.0
1.0
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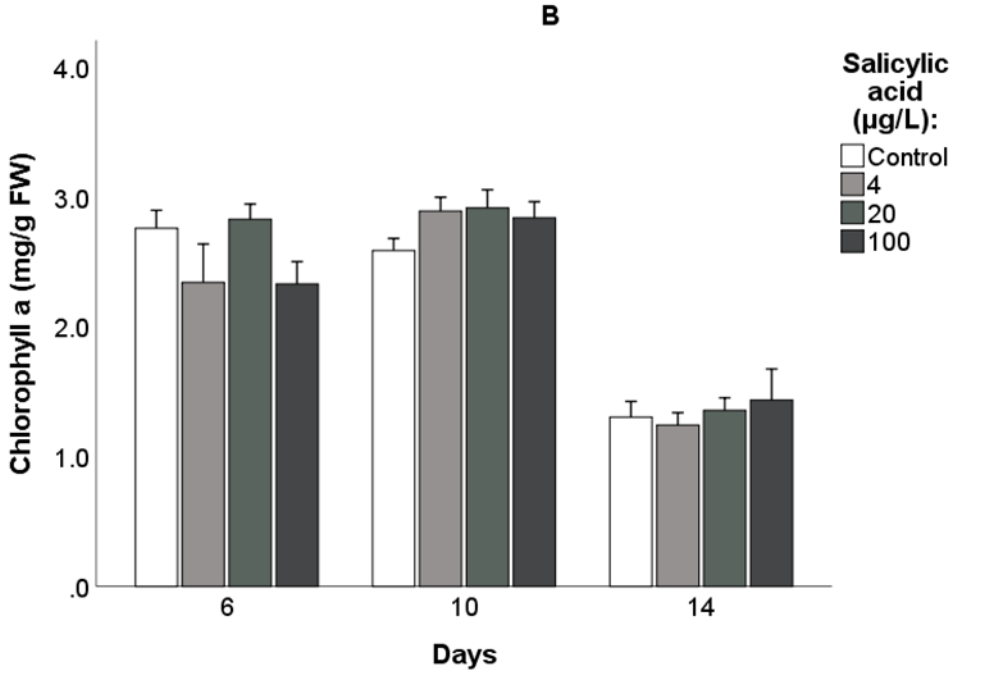
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100


B
Chlorophyll a (mg/g FW)
4.0
3.0
2.0
1.0
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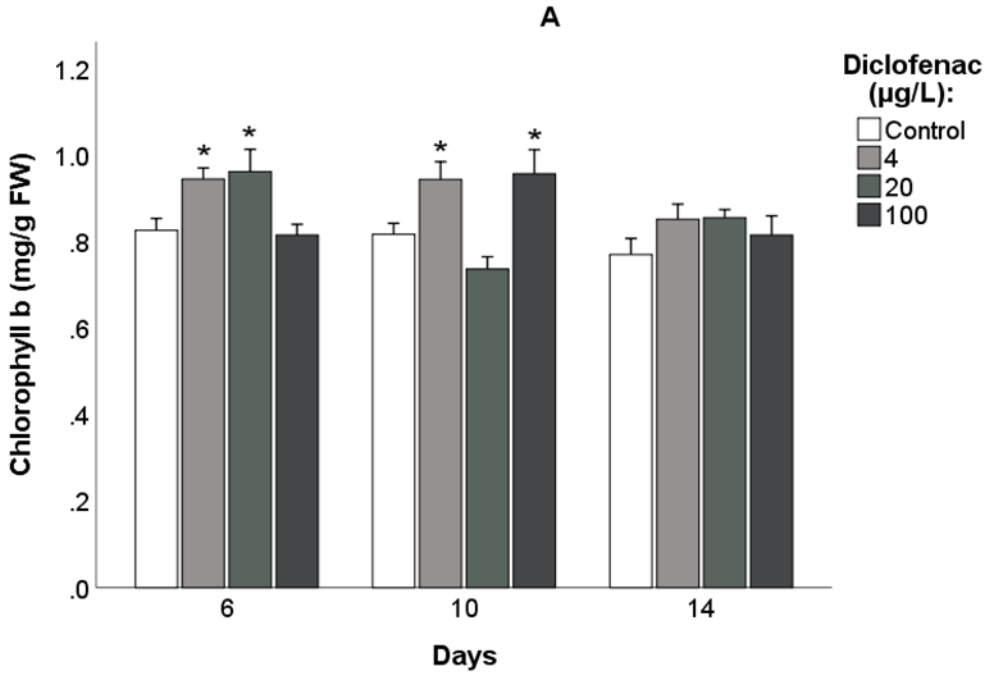
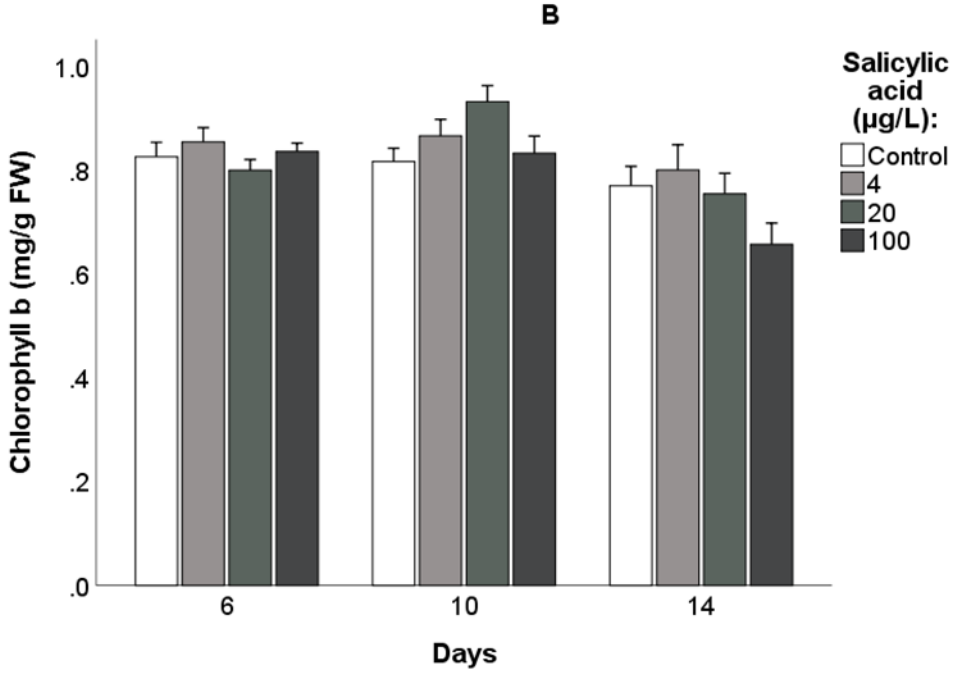
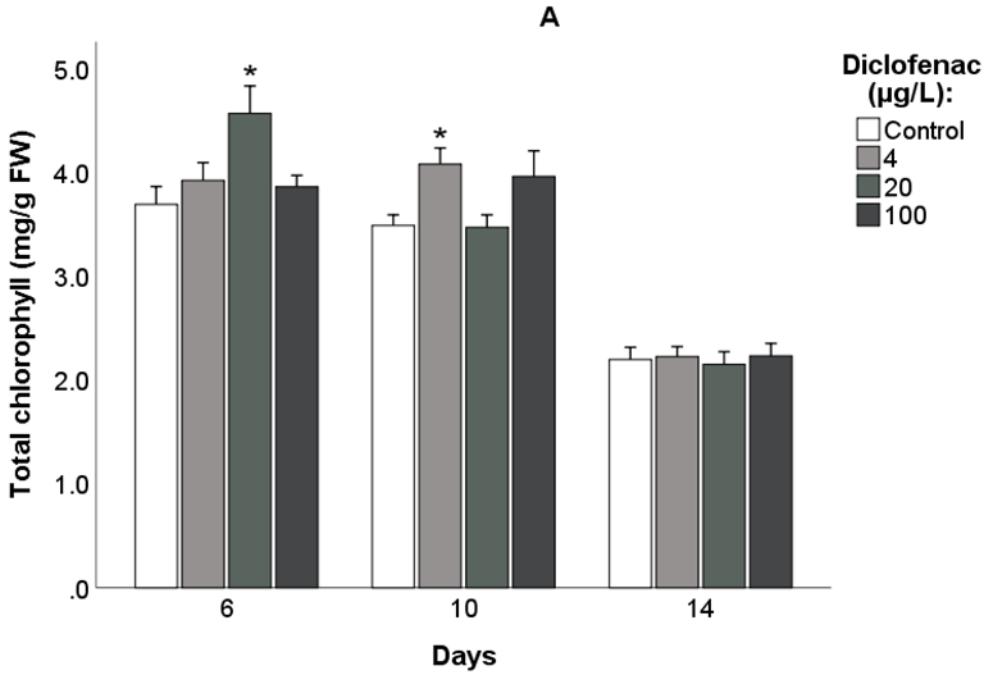
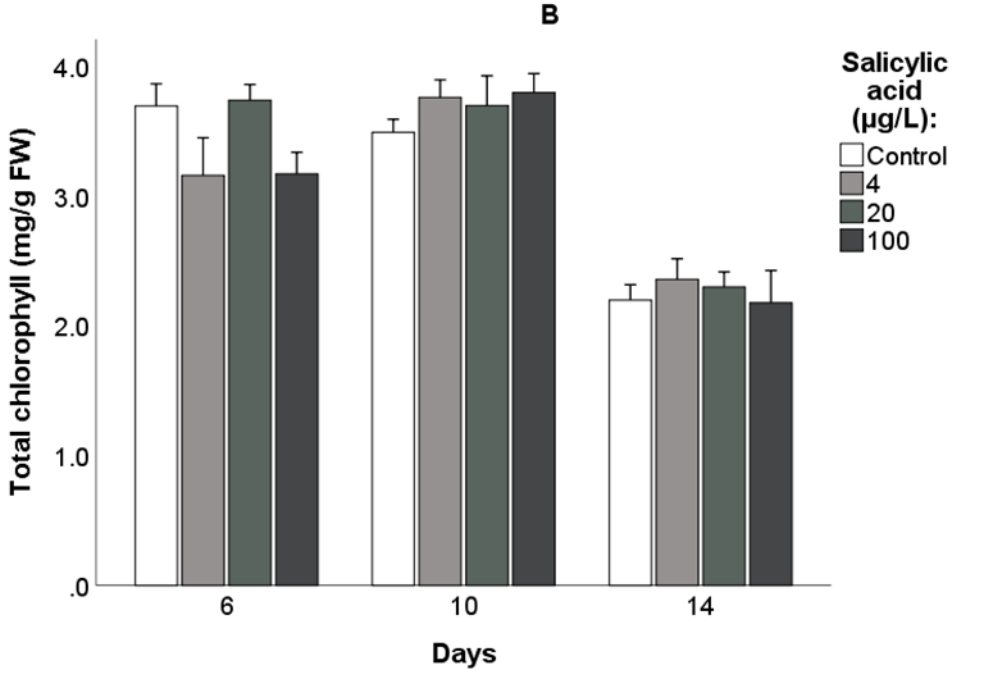
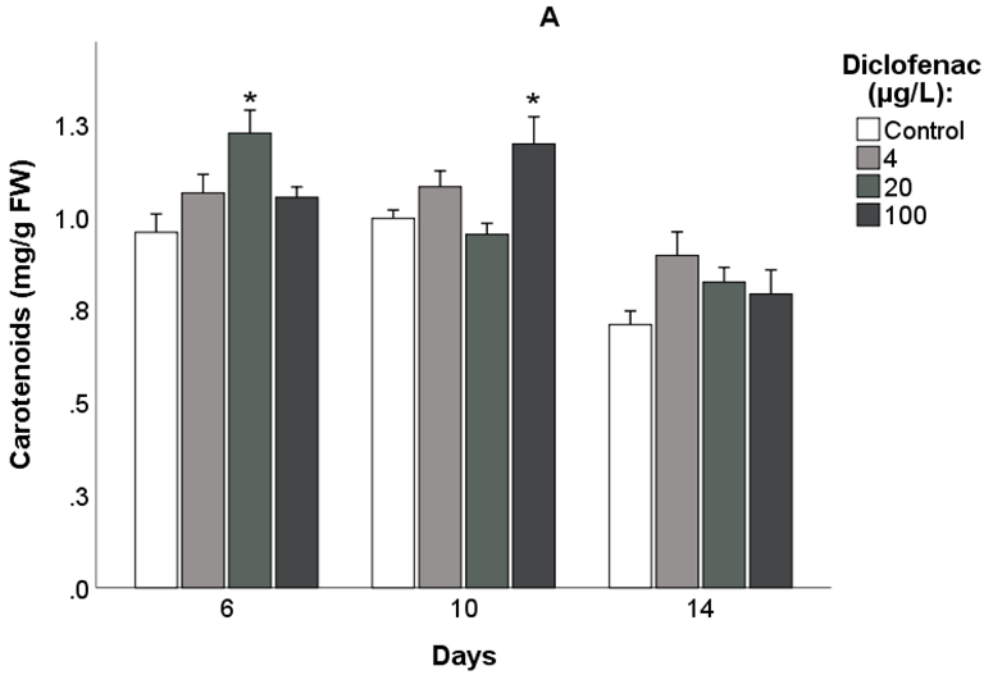
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100

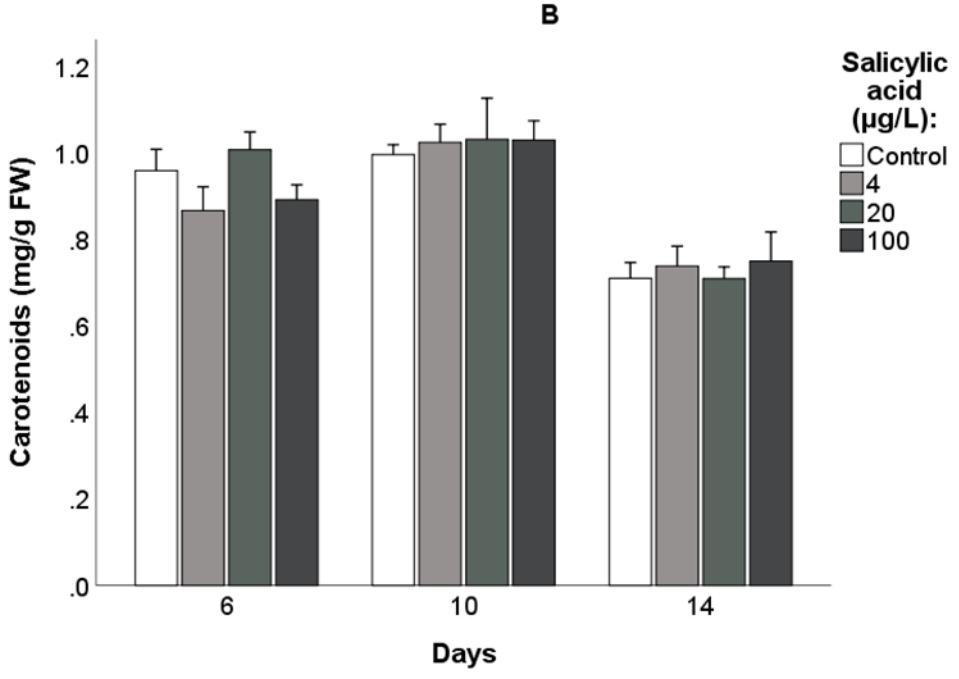

A
Chlorophyll b (mg/g FW)
1.2
1.0
.8
.6
.4
.2
.0
*
*
*
*
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100

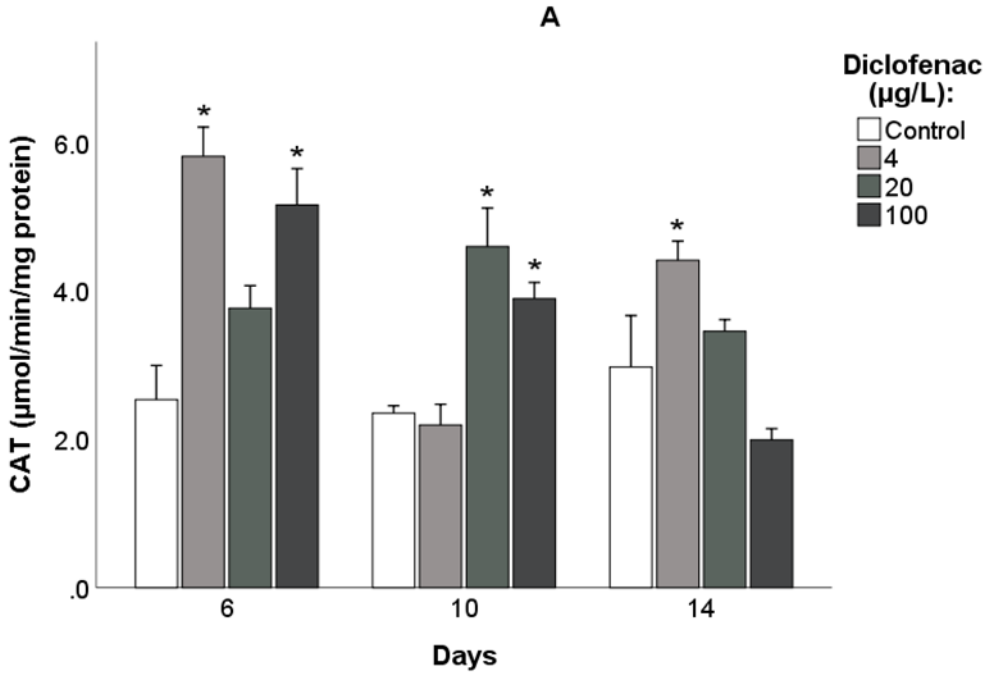

B
Chlorophyll b (mg/g FW)
1.0
.8
.6
.4
.2
.0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100

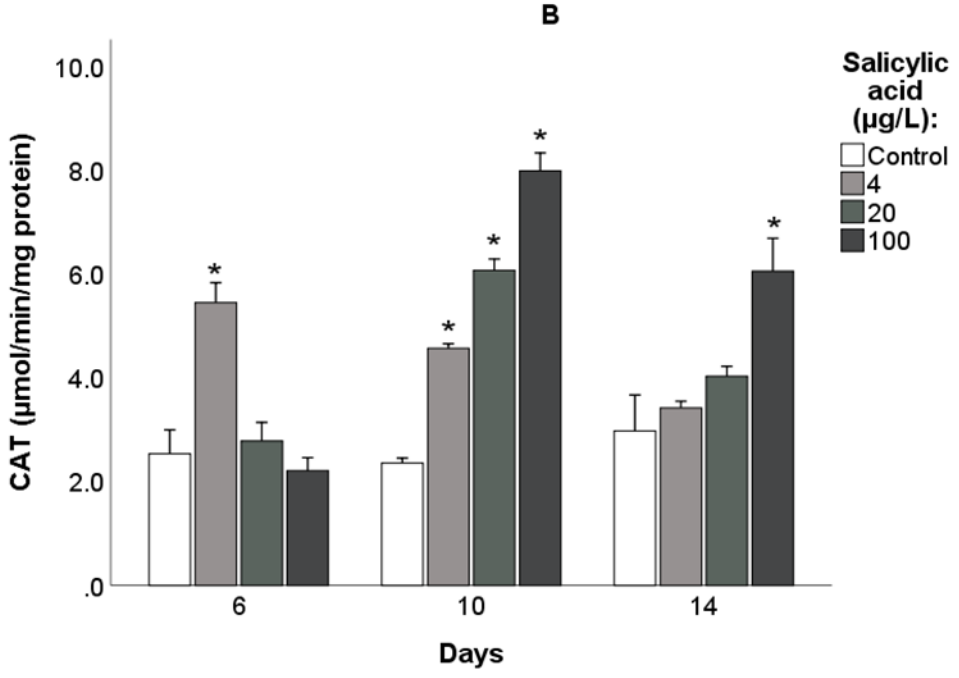

A
Total chlorophyll (mg/g FW)
5.0
4.0
3.0
2.0
1.0
.0
*
*
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100

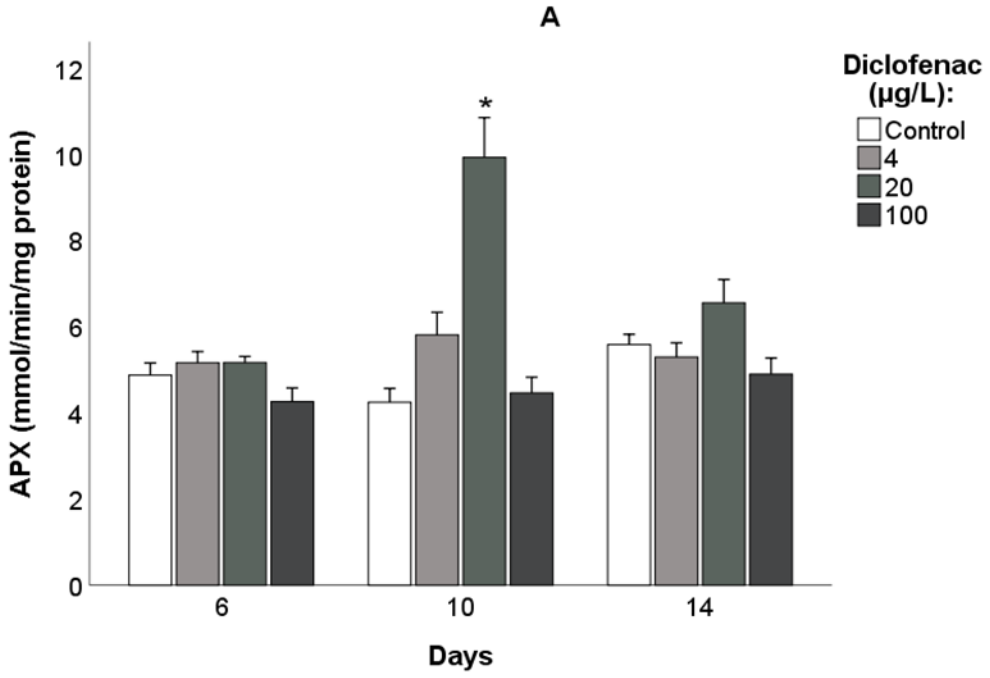

B
Total chlorophyll (mg/g FW)
4.0
3.0
2.0
1.0
.0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100

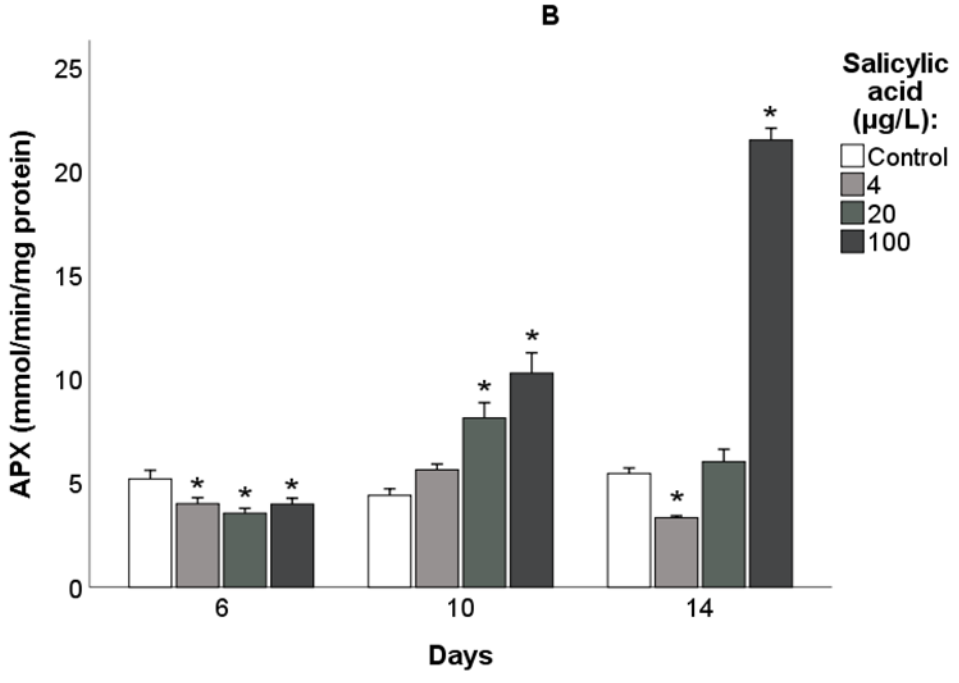

A
Carotenoids (mg/g FW)
1.3
1.0
.8
.5
.3
.0
*
*
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100


B
Carotenoids (mg/g FW)
1.2
1.0
.8
.6
.4
.2
.0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100


A
CAT (µmol/min/mg protein)
6.0
4.0
2.0
.0
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100


B
CAT (µmol/min/mg protein)
10.0
8.0
6.0
4.0
2.0
.0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100


A
APX (mmol/min/mg protein)
12
10
8
6
4
2
0
6
10
14
Days
Diclofenac (µg/L):
Control
4
20
100
*


B
APX (mmol/min/mg protein)
25
20
15
10
5
0
6
10
14
Days
Salicylic acid (µg/L):
Control
4
20
100
*

Highlights

- *Lema minor* long-term exposure is a promise tool to ecotoxicological assessment;
- Diclofenac (DCF) and Salicylic acid (SA) are toxic to aquatic plants;
- DCF exposure cause alterations in physiological and biochemical parameter in *L. minor*;
- SA exposure cause biochemical alterations in *L. minor*, but not in pigments content.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: