Journal Pre-proof

Assessment of phytotoxic effects, uptake and translocation of diclofenac in chicory (*Cichorium intybus*)

Natalia S. Podio, Lidwina Bertrand, Daniel A. Wunderlin, Ana N. Santiago

PII: S0045-6535(19)32296-9

DOI: https://doi.org/10.1016/j.chemosphere.2019.125057

Reference: CHEM 125057

To appear in: *ECSN*

Received Date: 22 August 2019

Revised Date: 2 October 2019

Accepted Date: 3 October 2019

Please cite this article as: Podio, N.S., Bertrand, L., Wunderlin, D.A., Santiago, A.N., Assessment of phytotoxic effects, uptake and translocation of diclofenac in chicory (*Cichorium intybus*), *Chemosphere* (2019), doi: https://doi.org/10.1016/j.chemosphere.2019.125057.

Assessment of Phytotoxic Effects, Uptake and Translocation of Diclofenac in Chicory (*Cichorium intybus*).

Natalia S. Podio[a,b*], Lidwina Bertrand[c], Daniel A. Wunderlin[b] and Ana N. Santiago[a]

[a] INFIQC, CONICET and Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Dpto. Química Orgánica. Ciudad Universitaria, Medina Allende esq. Haya de la Torre s/n, 5000, Córdoba, Argentina.

[b] ICYTAC, CONICET and Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Dpto. Química Orgánica. Ciudad Universitaria, Bv. Juan Filloy s/n, 5000, Córdoba, Argentina.

[c] CIBICI, CONICET and Universidad Nacional de Córdoba, Facultad de Ciencias Químicas, Dpto. Bioquímica Clínica. Ciudad Universitaria, Medina Allende esq. Haya de la Torre s/n, 5000, Córdoba, Argentina.

***CORRESPONDING AUTHOR:**

Natalia S. Podio, PhD

CONICET-Universidad Nacional de Córdoba

Bv. Juan Filloy s/n, Ciudad Universitaria, 5000-Córdoba, Argentina.

TEL./FAX: +54 351 4333194 Ext. 135

E-mail: nataliaspodio@unc.edu.ar

ABSTRACT

Pharmaceuticals in the environment have been an increasing research topic over the past decade, since they can be found in both natural and drinking water, including irrigation of crops and edible plants with contaminated water. Our main goal was to evaluate the phytotoxic effect of diclofenac (DCF), a widely used pharmaceutical , on chicory (*Cichorium intybus*) seedlings. Additionally, we verified the uptake, bioconcentration and translocation of DCF from soil to chicory tissues. Results show that DCF induces different physiological changes in chicory seedlings. On the other hand, the soil-chicory experiment showed the activation of the detoxification system in plants treated with DCF (1 mg L^{-1}). Finally, we found the migration of DCF from the irrigation water to the soil, followed by its uptake through the root, and its translocation to the aerial part of the chicory. However, DCF does not bioaccumulate in chicory leaves, being scarcely translocated from roots to aerial parts. This last result, along with the estimation of a daily intake of chicory, show that irrigation with water containing DCF ($\leq$ 1 mg L^{-1}) does not represent a threat to human health. To our knowledge, this is the first report on the effect of DCF on chicory seedlings, including the evaluation of its uptake and translocation.

KEYWORDS

Biomass; chlorophyll; pheophytin; anti-inflammatory; QuEChERS; HPLC-PDA-QTOF.

1. INTRODUCTION

Chemical contaminants of emerging concern, namely Pharmaceuticals and Personal Care Products (PPCPs), have been the subject of intensive study in the last years, mainly due to their ubiquity in aquatic ecosystems, and their potential to cause detrimental effects on both the biota and the human health (Rizzo et al., 2013; Valdés et al., 2014).

There is an exponential growth of reports in the literature showing the possible adverse effects of PPCPs on the environment (Guyón et al., 2012; McCallum et al., 2013; Roggio et al., 2014; Valdés et al., 2016; Watts et al., 2003). However, most of the studies carried out to date have focused on aquatic ecosystems. Conversely, our current knowledge about the effects of PPCPs on terrestrial systems is limited (Kinney et al., 2008; McCallum et al., 2013; Oaks et al., 2004). The general lack of information on the effects of PPCPs on terrestrial ecosystems is of great concern, not only because of the effect that they can generate on the biota, but also because agricultural activities could be affected, impacting the productivity, sustainability and food safety, generating a potential risk for human and livestock health. In this sense, there is currently a marked international consensus on the need for studying the effect of these emerging pollutants on crops and other edible plants.

Among PPCPs of increasing concern, diclofenac (DCF: 2-[2-[(2,6-Dichlorophenyl)amino]phenyl]acetic acid) is one of the most commonly used non-steroidal anti-inflammatory, analgesic, antiarthritic, and antirheumatic drug, with a global annual consumption of 1443 ± 58 tons (Acuña et al., 2015). This consumption does not cover veterinary use, mainly because of the lack of reliable data; thus, the global use of DCF can be even higher (Lonappan et al., 2016). In addition, DCF has a low removal rate during

wastewater treatment processes (WWTP) (Perez and Barceló, 2007), being one of the most frequently found compounds in WWTPs effluents and surface waters, with concentrations ranging from ng L^{-1} to µg L^{-1} (Valdés et al., 2014). Thus, the reuse of treated wastewaters, containing DCF, for irrigation would represent a significant hazard for food safety, even more in those countries where wastewaters treatments are frequently absent like South American countries including Argentina.

The negative impact of DCF on the environment became evident in Asian countries, where a dramatic decrease of vulture population was linked to the exposure to this compound (Lonappan et al., 2016; Oaks et al., 2004). Other research studies reported adverse effects on fish species (Hong et al., 2007). However, data on the effect of DCF on plants are scarce (Bartha et al., 2014; Christou et al., 2016; Copolovici et al., 2017; Huber et al., 2012; Kummerová et al., 2016; Schmidt and Redshaw, 2015). Huber et al. (2012), showed that the exposure to 100 µM (29.7 mg L^{-1}) of DCF for 3 hours led to irreversible damage in horseradish hairy root culture. Additionally, Schmidt and Redshaw (2015), reported that the exposure to DCF could negatively affect the development of *Raphanus sativus* root in relation to the aerial tissue. On the other hand, Kummerová et al. (2016), suggested that the exposure to environmentally relevant concentrations of DCF (0.034 and 0.34 µM: 10 µg L^{-1} and 100 µg L^{-1}, respectively) affects biochemical processes in duckweed plants (*Lemna minor*) via the formation of reactive oxygen (ROS) and nitrogen (RNS) species. They observed an increase in biomolecular damages, including lipid peroxidation and loss of the plasma membrane integrity, in addition to changes in the antioxidant system. Lipid peroxidation was also reported by Christou et al. (2016), in roots of alfalfa (*Medicago sativa* L.) exposed to DCF (10 µg L^{-1}). These authors found increased levels of hydrogen peroxide (H_2O_2), together with decreased activity of superoxide dismutase in roots, while leaves showed increased catalase activity. Bartha et al. (2014), reported that glycosyltransferase and

glutathione S-transferase activities were increased in roots and leaves of *Thypa latifolia* exposed to DCF (3.4 µM: 1 mg L^{-1}), while the activity of peroxidase increased only in roots. Finally, Copolovici et al. (2017), found that DCF may affect the photosynthetic parameters of *Phaseolus vulgaris* L., and might disturb the methylerythritol phosphate pathway (MEP) in plastids.

These studies suggest that DCF may be accumulated, having toxic effects on plants. It is important to elucidate the biochemical and physiological changes induced by DCF on edible plants, or plants producing edible parts; thus, helping understand its potential effects on food production, assessing its possible implication on human health as well.

Chicory (*Cichorium intybus*) is a perennial herbaceous plant of the Asteraceae family, widely used as a medicinal plant and as food. Generally, the stems and leaves are consumed in salads, but the roots are also used as a substitute for coffee, since roasted chicory roots have a similar taste to coffee, but do not contain caffeine. Its consumption is motivated also by its healthy properties, including hypoglycemic and detoxifying functions.

Chicory production close to Córdoba city (Argentina) is performed in fields close to the Suquía River lower basin (downstream from WWTP), with some farmers using river water containing DCF for irrigation (Valdés et al., 2014). Because of this, human exposure through chicory irrigated with DCF contaminated water is expected, so we also evaluated the human health risks associated with its consumption.

The use of DCF has been regulated by few countries. Recently, the European Water Framework Directive (WFD) introduced an environmental quality standard (EQS) for DCF in the aquatic environment, with an annual average EQS value set at 0.1 µg L^{-1} in inland surface waters, and 0.01 µg L^{-1} in other (coastal) surface waters (Lonappan et al., 2016). Moreover, its manufacture and veterinary use were banned in India, Nepal, Pakistan, and Bangladesh during the last decade (Lonappan et al., 2016). However, to our knowledge, no regulatory

limits have been established for treated wastewaters and soils, when they are used for the irrigation and production of edible plants/ crops.

Within this framework, the main goal of this work was to evaluate the phytotoxic effect, uptake, and translocation of DCF in chicory plants (*Cichorium intybus*) in the germination stage and in a soil-plant system. To our knowledge, this is the first report studying the effect of DCF on chicory plants.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials

Diclofenac (DCF), glutathione disulfide (GSSG) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (Buenos Aires, Argentina). Guaiacol was purchased by Anedra (Buenos Aires, Argentina). Ultrapure water (resistivity ≥ 18 MΩ·cm; TOC ≤ 5 µg L^{-1}) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Methanol (HPLC grade) was provided by J. T. Baker (State of Mexico, Mexico) and ammonium acetate (puriss. p.a. for mass spectroscopy) from Fluka (Berlin, Germany). Filter membranes (0.45 µm, HVLP04700) were obtained from Millipore (São Paulo, Brazil). All other reagents were of analytical grade. QuEChERS Kits were obtained from Phenomenex (Buenos Aires, Argentina).

The commercial organic soil was obtained from a local nursery (Jardín Primavera, Córdoba, Argentina). The main soil chemical characteristics were: 25% organic matter, 9.8% organic C, 0.8% total N, 1.6% H, pH 6, and 11.5 C:N ratio. The soil had not previously received biosolid or wastewater applications.

Chicory seeds were provided by the Department of Agriculture of the province of Córdoba, Argentina.

2.2. Germination Study

This experiment was performed following the methodology described by Schmidt and Redshaw (2015), with few modifications. Briefly, 5 mL of 3.42 µM DCF (1 mg L^{-1} in 0.1 % v/v methanol), or control solutions (ultrapure water and 0.1 % v/v methanol), were added to Petri dishes (100 mm) with 3 layers of kitchen roll and 1 layer of filter paper. Then, 30 chicory seeds (sterilized with 2.5 % of sodium hypochlorite for 15 min, and washed 10 times with ultrapure water) were added to each Petri dish and incubated in the dark at 24°C (± 2°C) for 7 days. Five Petri dishes were used for each treatment and the experiment was repeated 3 times on different days.

After 7 days, 6 germinated seeds were randomly chosen, harvested, dissected into root, shoot, and cotyledon. Then, different physiological parameters (germinated seeds; root, shoot and cotyledon lengths, color and aspect) were recorded for each harvested seed. After that, cotyledons, shoots and roots from each Petri dish were pooled and dried at 90°C for 48 h to measure dry biomass, moisture and specific lengths.

2.3. Uptake Study

Chicory seeds (sterilized with 2.5 % of sodium hypochlorite for 15 min and washed 10 times with ultrapure water) were sown in wells (3 cm diameter, 5 cm height) containing 13 g of soil (dried for 24 h at 110°C) and grown in a greenhouse (28 ± 10°C, under a 15 : 9 hours light : dark photoperiod) for 22 days. Wells were irrigated every 2 days during the growth period, using fresh water. After this period, chicory plants were transplanted to pots (12 cm diameter, 10 cm height), containing 450 g of dry soil for further growing. After 14 days of adaptation to this last condition, plants were exposed to DCF by irrigation with 50 mL either with 3.42 µM DCF solution (1 mg L^{-1} in 0.1% v/v methanol in water) or with 0.1% v/v methanol in water (control treatment) every 3 days during 22 days. Three samples were used by each treatment

(control and DCF). Each sample consisted in 6 plants. After 22 days, the 6 plants in each sample were separated from the soil, rinsed with ultrapure water and cut, dividing them into roots and aerial parts (leaf and stem). The soil and tissues collected in each sample were pooled, frozen in liquid nitrogen, and kept at -80°C until extraction.

2.4. Photosynthetic Pigments

Concentrations of chlorophylls (Chl) and pheophytins (Pheo) were determined in the aerial part (leaf and stem) of chicory according to Wintermans and de Mots (1965). Briefly, tissues were homogenized using a ceramic mortar and liquid nitrogen, weighed and dispersed in ethanol (99% v/v) with further separation of the supernatant. Afterwards, hydrochloric acid (HCl) 0.06 M was added to the clear Chl extract (HCl: chlorophyll extract 1:5) for Pheo determination. Concentrations of pigments in plant extracts were measured by spectrophotometry using a microplate reader Bio-Tek, Synergy HT (Chl = 649 and 665 nm before HCl addition; Pheo = 654 and 666 nm after HCl addition). Concentrations were calculated and reported in µg pigment g^{-1} wet weight (ww).

2.5. Enzyme Extraction and Measurement

Chicory enzyme extracts were prepared according to Monferrán et al. (2009). Briefly, aerial and root tissues were ground and homogenized with liquid nitrogen and stirred at 4°C with extraction buffer: sodium phosphate buffer (0.1 M, pH 6.5) containing glycerol (20%), 1,4-dithioerythritol (DTE, 1.4 mM) and ethylene diamine tetra acetic acid (EDTA, 1 mM). Cell remnants were separated by centrifugation (10 min at 13,000 x g, 4°C) and the supernatant was used for enzyme measurement. Enzymatic activities were determined by spectrophotometry, using a microplate reader (Bio-Tek, Synergy HT). The glutathione reductase activity (GR; EC 1.8.1.7) was assayed according to Tanaka et al. (1994). GR

196 reduces added glutathione disulfide (GSSG) to reduced glutathione (GSH),
197 spectrophotometrically consuming added NADPH. The guaiacol peroxidase (POD) activity
198 was measured using guaiacol and H_2O_2 (Bergmeyer, 1983). The enzymatic activities of each
199 sample were measured in triplicate and calculated in terms of the protein content of the
200 sample extract (Bradford, 1976). Results are reported in nanokatals per milligram of protein
201 (nkat mg $prot^{-1}$), where 1 nkat is the conversion of 1 nmol of substrate per second. The
202 protein quantification was performed using bovine serum albumin as a standard.
203
204 **2.6. DCF Extraction**
205 DCF was extracted from samples using QuEChERS methodology. Fine powder soil, roots
206 and aerial parts (2 g) were weighed into 50 mL tubes and moistened with 4 mL of methanol
207 (soil samples) or acetonitrile (roots and aerial parts). The mixture was shaken vigorously for
208 1 min in a vortex; after that, it was placed in an ultrasonic bath for 10 min. Afterward, the
209 extract was partitioned by adding 2 g $MgSO_4$, 0.5 g NaCl, 0.5 g sodium citrate tribasic
210 dihydrate, and 0.25 g sodium citrate dibasic sesquihydrate (AH0-9041, roQ QuEChERS
211 extraction pack), shaken in a vortex for 90 s and centrifuged at 8228 x g for 10 min using an
212 Eppendorf 5804 centrifuge. Finally, an additional cleanup step was performed. One milliliter
213 of the extract was placed in a tube containing 150 mg $MgSO_4$, 25 mg PSA sorbent, and 25
214 mg C18E (KS0-8913, roQ QuEChERS dSPE Kit). After that, it was shaken vigorously in a
215 vortex for 2 min, and centrifuged at 4629 x g for 5 min. The supernatant was taken and dried
216 with nitrogen (N_2), reconstituted with 0.5 mL methanol, and stored at -80°C until analysis.
217 Blank of solvents and recovery samples were also used. Recovery samples were prepared
218 using DCF solution at a final concentration of 3.37 µM, following the extraction protocol
219 previously described. The obtained recovery percentage was 90 ± 2 %, 88 ± 10 %, and 105 ±
220 10 % in soil, root and aerial part samples, respectively.

2.7. DCF Analysis

DCF was analyzed in soil, roots and aerial part extracts by HPLC−MS/MS method, using an Agilent Technologies 1200 Series UPLC equipped with a gradient pump (Agilent G1312B SL Binary), solvent degasser (Agilent G1379 B), and an autosampler (Agilent G1367 D SL+WP). The chromatographic separation was achieved on a ZORBAX Eclipse XDB-C18 column (5 μm, 150 mm × 4.60 mm i.d., Agilent, USA) at 40°C using a column heater module (Agilent G1316 B). The mobile phase consisted of 5 mM ammonium acetate (solvent A) and 5 mM ammonium acetate in methanol (solvent B). The solvent gradient started at 40% of solvent B for 1 min and changed to 80% B along 6 min, kept for 6 min, followed by a second ramp to 98% B along 3 min and kept for 2 min. After that, a washing and stabilization step of 12 min was used before the next run. The flow rate was set at 0.5 mL min^{-1}, and the injection volume was 40 μL. The HPLC system was connected to a diode array detector (DAD SL, Agilent G1315 C) in tandem with an ESI source and Q-TOF mass spectrometer (micrOTOF-QII Series, Bruker). UV−vis spectra were registered from 200 to 600 nm. Mass spectra were recorded in negative ion mode between m/z 80 and 1000. The working conditions for the ionization source were: capillary voltage, 3500 V; nebulizer gas pressure, 5.0 bar; drying gas flow, 9.0 L min^{-1}, and 200°C for the drying gas. Nitrogen and argon were used as nebulizer/dryer and collision gases, respectively. The instrument was operated in full-scan mode. Exact mass was verified by introducing sodium formiate solution (40 mM) at the end of each chromatographic run through the multipath valve of the MicroQTOF II. Data acquisition and processing were performed using Compass 3.1 and DataAnalysis 4.1 software, respectively (Bruker Daltonics, MA-USA).

DCF Identification was based on accurate m/z ratio (mass error ≤ 5 ppm), fragmentation pattern (MS/MS spectrum with a collision energy of 10.0 eV) and comparison of the DCF

retention time with pure compounds (± 2 %). DCF quantification was performed using the fragment 250.196 ($[M\text{-}CO_2]^-$), preparing calibration curves for the 3 studied matrices (soil, roots and aerial parts) in a 0-16 µM range. Matrix effects were studied using spiked samples at a concentration of 3.37 µM; observed recoveries were 102 ± 20 %. Limits of quantification (LOQ) were calculated as the lowest point in the matrix calibration curves that can be accurately quantified (S/N ≥ 10), while limits of detection (LOD) were calculated as LOD = LOQ/3.3. Samples were filtered (0.45 µm) before injection in the HPLC−MS/MS system.

2.8. Bioconcentration and Translocation Factor Calculations

The bioconcentration factors (BCF) were calculated according to the following equations:

Equation 1:

$$BCF_{\text{root}} = \frac{C_{root}}{C_{soil}}$$

Equation 2:

$$BCF_{\text{aerial part}} = \frac{C_{aerial\ part}}{C_{soil}}$$

Where:

C_{root} and $C_{\text{aerial part}}$ are the DCF concentration (µg 100 g^{-1} ww) in root and aerial part of plants, respectively. C_{soil} is the DCF concentration (µg 100 g^{-1} ww) in soil samples.

The translocation factor (TF) was calculated using the equation:

Equation 3:

$$TF = \frac{C_{aerial\ part}}{C_{root}}$$

Where:

$C_{\text{aerial part}}$ and C_{root} are the DCF concentration (µg 100 g^{-1} ww) in aerial part and roots, respectively.

2.9. Daily DCF Intake

The estimated daily intake (EDI in µg/kg/day) of DCF depends on its concentration in chicory tissues (C, µg g^{-1}), the *per capita* daily consumption of fresh chicory (D in g day^{-1}) and the body weight (B in kg), according to the formula:

$$EDI = \frac{C \; x \; D}{B}$$

The *per capita* daily consumption of aerial parts of chicory is 35 g day^{-1} for adults, and 27 g day^{-1} for children (Argentinean Ministry of Health, 2012). There is no information about the *per capita* daily consumption of chicory roots; however, some healthcare professionals recommend a daily consumption of 2-6 g (1 cup of root infusion). Because of this, we consider a daily root consumption of 4 g. The body weights considered were 70 kg for adults and 20 kg for seven-year-old children.

2.10. Statistical Analyses

Results were analyzed using the statistical package Statistica 8.0 from StatSoft Inc. (2007) and the Infostat software package (Di Rienzo et al., 2008). Analysis of variance was performed using mixed models (Di Rienzo et al., 2010). The level of significance of this study was set to $\alpha \leq 0.05$. A DGC (Di Rienzo et al., 2002) comparison test was performed to reveal paired differences between means.

3. RESULTS AND DISCUSSION

3.1. Physiological Responses

Physiological effects of DCF on chicory plants (*Cichorium intybus*) were considered in both germination and uptake studies. Treatment concentration was chosen to enable comparison with other reports that use this concentration in *R. sativus* (Schmidt and Redshaw, 2015), *Populus alba,* L. Villafranca clone (Pierattini et al., 2018), green alga *Chlamydomonas reinhardtii* (Majewska et al., 2018), among others. The number of germinated seeds; length of roots, shoots and cotyledons; water content; biomass; specific lengths and root dry biomass:aerial part dry biomass ratio were analyzed during germination study of chicory seedlings, while photosynthetic pigments and antioxidant enzymes were studied in chicory plants through the uptake assay.

In both studies, plants displayed similar phenotypes in both control and DCF treatments (**Figure 1**). Although effects on chicory plant growth, development and health were not externally observed (**Figure 1**), few physiological parameters, such as dry biomass and specific lengths of the root; root dry biomass:aerial part dry biomass ratio; photosynthetic pigments and antioxidant enzymes, show significant differences between treatments.

In the chicory germination assay, the study of effects on the number of germinated seeds (21 ± 5 and 20 ± 8 for control and DCF treatments, respectively), root, shoot and cotyledon lengths, water content, shoot and cotyledon dry biomass as well as specific shoot and cotyledon lengths did not show significant differences between control and DCF treatments (**Figures 2** for root parameters, **Figure 1S** for shoot parameters, and **Figure 2S** for cotyledon parameters). However, significant disparities were found in dry root biomass and specific lengths of the root (**Figures 2 C** and **D**). DCF treatment produced a significant decrease of chicory root dry biomass (11%), and a significant increase of the specific root length (33%). The specific root length endpoint is a function of length and biomass, and it provides information about the energetic costs of developing roots (Schmidt and Redshaw, 2015). Root length is assumed to be proportional to resource acquisition; and root biomass, to be

proportional to development and maintenance. Both are well known to be reasonably constant within a species, or even cultivar, and have shown to correlate with environmental change, including growth conditions, metal-induced stress, and nutrient availability (Ostonen et al., 2007). In this report, the highest value of specific root length for seeds exposed to DCF treatment can be given by the interference of DCF in the root functionality, resulting in the suppression of nutrient uptake and, hence, growth (Schmidt and Redshaw, 2015).

Furthermore, when the root and aerial part (shoot + cotyledon) developments of chicory in both treatments were compared (root dry biomass:aerial part dry biomass ratio, **Figure 2 E**), a significantly lower ratio was found in DCF treatment. The root biomass to aerial part biomass ratio (**Figure 2 E**) shows the development of root with respect to the aerial part of the plant. In this report, DCF treatment showed a significantly lower ratio, as a result of the negative effect that the tested compound has on the root biomass (**Figure 2 C**).

Ziółkowska et al. (2014), also showed an increasing inhibition of root and shoot lengths on three leguminous plants (pea, lupin, and lentil) when the DCF concentration increased from 0.06 mM (17.8 mg L^{-1}) to 12 mM (3560 mg L^{-1}). Besides, Schmidt and Redshaw (2015), found a negative effect of DCF (1 mg L^{-1}) on the root biomass to aerial part biomass ratio of *R. sativus* in a growth study. Conversely, Pierattini et al. (2018), observed no differences in the number of leaves, shoot length and fresh or dry weight when poplar plants (*Populus alba*, L. Villafranca clone) were exposed to 0 mg L^{-1} (control), and 1 mg L^{-1} of DCF (hydroponic exposure). This last study shows that the effect of DCF on plants depends on the plant species.

Additionally, the uptake study also showed phytotoxic effect on both root and aerial part of chicory plants exposed to DCF. We found a negative effect of DCF on the concentration of photosynthetic pigments (Chl *a* and *b*, and Pheo *a* and *b*) (**Figure 3**), and an activation of the detoxification system in plants (**Figure 4**).

Contents of leaf pigments provide valuable information about the physiological status of a plant, and they are generally recognized as reliable stress indicators (Mallakin et al., 2002). A decrease in these pigments indicates a decrease in the functionality of the photosystems and, consequently, of the photosynthetic activity of the plants (D'Abrosca et al., 2008).

We report a decrease in chlorophyll concentrations. Likewise, a similar response was reported by Kummerová et al. (2016), and Copolovici et al. (2017). Kummerová et al. (2016), showed a decrease in both chlorophylls *a* and *b*, when DCF (form 0.010 mg/L to 0.100 mg/L) was applied to *Lemna minor*. The same results were found by Copolovici et al. (2017), when DCF was applied (from 100 mg L^{-1} to 400 mg L^{-1}) to bean (*Phaseolus vulgaris L*). On the other hand, Pierattini et al. (2018) and Majewska et al. (2018) also found a decrease of Chl *a* in old leaves of *Populus alba* L. Villafranca clone plants and green alga *Chlamydomonas reinhardtii*, respectively. To determine if this drop in chlorophyll content was due to decreased biosynthesis and/or increased degradation of chlorophyll, we investigated the pheophytin contents in both control and DCF treatments. Pheophytins are the degradation products of chlorophylls (Matile et al., 1999). To our knowledge, this is the first report that studied the pheophytin contents in plants treated with DCF. We observed that the pheophytin (*a*,*b*)/chlorophyll (*a*,*b*) ratios were not altered in plants treated with DCF (0.7 for both treatments), indicating an effect of DCF on chlorophyll biosynthesis, but not on its degradation (Gomes et al., 2016). Gomes et al. (2016), showed that glyphosate on willow plants (*Salix miyabeana* cultivar SX64) affects chlorophyll biosynthesis by competing with glycine in photorespiration processes and/or in the active site of δ-aminolevulinic acid (ALA) synthetase, depriving plants of the substrates needed in the chlorophyll biosynthetic pathway. Even though glyphosate is an herbicide designed to disrupt biosynthetic pathways in plants, this or a similar mechanism, could occur when the chicory is exposed to DCF, as the result of

a non-target effect. However, further studies are necessary to elucidate at which step of the biosynthetic pathways the non-steroidal anti-inflammatory is interacting.

Additionally, the enzymatic activity revealed that the exposure of plants to DCF resulted in a significant induction of POD and GR activities in chicory root samples (**Figure 4**). On the other hand, POD activity in the aerial part of plants was not affected, while we were not able to evaluate GR activity in aerial parts because of pigment interferences.

An increased enzymatic activity has been frequently described under different biotic and abiotic stress conditions. The induction of the antioxidant defense, including catalase, peroxidase, ascorbate peroxidase, glutathione reductase, superoxide dismutase, and glutathione S-transferase, is a primary response of plants to contaminant mediated stress, including pharmaceutically active compounds (PhACs) (Christou et al., 2018). Our results show the induction of POD and GR enzymes in root upon exposure of chicory plants to DCF, indicating the activation of phases I and II enzymes, belonging to the plant detoxification system. Peroxidase has been commonly designated as a general stress marker in plants due to its role in the elimination of H_2O_2 using glutathione as substrate (Passardi et al., 2005). On the other hand, GR has been extensively reported to play crucial roles in determining stress tolerance in plants under various abiotic stresses (Gill, et al., 2013; Gill and Tuteja, 2010). Thus, our current results are consistent with results reported by other authors. Bartha et al. (2014) and Huber et al. (2016) observed induction of plant peroxidases in roots of *Typha latifolia* exposed to DCF 1 mg L^{-1}. Additionally, the increase of GR activity was reported by Kummerová et al. (2016) and Pierattini et al. (2018). Kummerová et al. (2016) reported an increase of GR activity in duckweed (*Lemma minor*) exposed to DCF (0.010 mg L^{-1} and 0.100 mg L^{-1}). In addition, Pierattini et al. (2018) reported an enhanced activity of GR in roots of poplar (*Populus alba,* Villafranca clone) after 7 days of exposure without (control) and with DCF (1 mg L^{-1}). Induction of other enzymes (catalase, glycosyltransferase and

glutathione S-transferase) was also reported (Bartha et al., 2014; Huber et al., 2016; Kummerová et al., 2016; Majewska et al., 2018; Pierattini et al., 2018). However, to our knowledge, this is the first report evidencing the effect of DCF on guaiacol peroxidase.

The physiological responses of chicory exposed to DCF show that it causes oxidative stress, mainly in roots, generating phenotypic changes and activating endogenous antioxidant defense mechanisms. These responses may be associated with the content of DCF found in the different parts of the plant, being greater in the root than in the aerial parts of chicory.

3.2. Chicory Uptake and Translocation of DCF

DCF was extracted from soil, root and aerial part of plants in both treatments (control and DCF). No DCF was detected in control samples. On the other hand, we observed both uptake and translocation of DCF by chicory. **Figure 5** shows the levels of DCF found in soil, roots and aerial parts of chicory after DCF treatment. Results obtained for soil samples show the highest DCF concentration (57 ± 5 µg 100 g^{-1} ww), while roots and aerial parts showed a DCF content of 3.9 ± 0.3 µg 100 g^{-1} ww, and 2.6 ± 0.2 µg 100 g^{-1} ww, respectively.

The bioconcentration factors observed were 0.07 ± 0.01 for roots, and 0.04 ± 0.01 for aerial parts of chicory, indicating low accumulation of DCF in chicory tissues. On the other hand, the translocation factor from roots to aerial parts was 0.654 ± 0.004, showing that DCF is scarcely transported within the plant.

It should be noted that the experimental setup employed in this study is representative of the potential use of contaminated water resources or waters from municipal wastewater treatment plant (reuse) for plant-to-field growth. The bioavailability of DCF in a soil-plant system is probably different than the hydroponic system used in most of the available reports, and soil characteristics such as the soil organic carbon content, ion exchange capacity, and soil pH will definitely influence the mobility of DCF and, therefore, its bioavailability. The results

found in our study show that DCF remains mainly in the soil, being little uptaken by chicory plants. Similar results were reported by Carter et al. (2014), who found a low uptake of DCF from the soil by radish and ryegrass roots. Additionally, Montemurro et al. (2017) also reported a low uptake of DCF by lettuce in a soil-plant system, whereas Barreales-Suárez et al. (2018) showed a low uptake by *Lavandula dentata*.

Uptake by chicory roots is the first step necessary for plants to accumulate DCF in their edible tissues. The low uptake of DCF by chicory plants may be attributed to the physicochemical properties of DCF (hydrophobicity and the extent of ionization) and soil characteristics. The high hydrophobicity of DCF (log K_{ow} > 4), and its strong soil adsorption induce the relatively low bioavailability for root absorption (González-García et al., 2018).

On the other hand, the concentration of DCF was higher in the roots than in the leaves (**Figure 5**), suggesting that DCF was absorbed by the roots and then transported to the leaves with a relatively slow translocation process (TF = 0.654 ± 0.004). This fact was also observed by other authors (Bartha et al., 2014; González-Garía et al., 2018; Tanove et al., 2012; Wu et al., 2013; Zhang et al., 2013; Zhang et al., 2012). Even though roots were rinsed several times with ultra-pure water, the DCF concentration detected in roots may be representing the sum of the amount adsorbed on the root surfaces and that absorbed into the root tissue (Wu et al., 2013). Some hydrophobic chemicals (log K_{ow} > 3), such as DCF, are strongly bound to the surface of the roots and they cannot be translocated to the aerial parts of plants (Wu et al., 2013), only the fraction that entered in the root tissue can be translocated to the aerial parts. However, both adsorbed and absorbed amounts of DCF are important when an estimate of daily intake is performed, and the whole root is consumed. In addition, the metabolism of DCF within the plants should also be taken into account, since metabolites of DCF were previously described in both the roots and aerial parts of *Typha latifolia* and barley (*Hordeum*

437 *vulgare*) (Bartha et al., 2014; Huber et al., 2012). However, in our study, we were not able to
438 detect any metabolite in roots or in the aerial part of chicory plants.
439
440 **3.3. Evaluation on the Incidence of Absorbed DCF on Human Health**
441 The presence of contaminants in edible plants can cause a potential risk for human life and
442 livestock. That is why we estimated the daily intake (EDI in $\mu g\ kg^{-1}\ day^{-1}$) of DCF when
443 aerial parts and roots of chicory are consumed.
444 The EDI of contaminated chicory shows that 0.035 $\mu g\ kg^{-1}\ day^{-1}$ of DCF would be consumed
445 by children, while 0.013 $\mu g\ kg^{-1}\ day^{-1}$ would be ingested by adults when the aerial part of
446 chicory is consumed. On the other hand, 0.008 $\mu g\ kg^{-1}\ day^{-1}$ and 0.002 $\mu g\ kg^{-1}\ day^{-1}$ would be
447 consumed by children and adults, respectively, when contaminated chicory roots are ingested.
448 These values are far from the recommended dose of DCF for the treatment of a disease (1400
449 $\mu g\ kg^{-1}\ day^{-1}$) (Bruce et al., 2010), so it is worth remarking that the consumption of edible
450 parts of chicory contaminated with DCF does not represent an acute threat to human health,
451 at least for the experimental conditions tested. González-García et al. (2018) made the same
452 assessment with DCF contaminated lettuce. However, a prolonged consumption of
453 contaminated chicory, along with the simultaneous consumption of other foods contaminated
454 with DCF and other pharmaceutical compounds, can generate resistance to the drugs, as well
455 as favor the increase of diseases related to the excessive consumption of medicines
456 (cardiovascular diseases, digestive problems, allergic reactions, among others). Moreover, the
457 risk assessment arising from the chronic consumption of DCF contaminated chicory, alone or
458 combined with other foods contaminated with different toxic compounds, needs to be
459 addressed. However, these last points are out of the scope of the current work.
460
461 **4. CONCLUSIONS**

Our current results show the importance of studying the effects of organic pollutants on the early stages of plant development as well as the use of soil-plant systems for the best extrapolation of what can occur in the field.

To our knowledge, this is the first study showing the effect of DCF on vegetative endpoints in both chicory seedlings and plants. Particularly, the physiological responses of chicory exposed to DCF show that this pharmaceutical drug causes oxidative stress in plants, mainly in roots, generating phenotypic changes and activating endogenous antioxidant defense mechanisms. On the other hand, it is important to consider the nature of the contaminant (DCF in this case) as well as the soil properties when a soil-plant system is used to verify the follow-up of a particular contaminant in edible plants, considering soil adsorption / absorption, among others factors.

Finally, the amount of DCF found in both root and aerial parts of chicory needs to be considered when estimating its daily intake, since both parts are consumed as food. As far as this study shows, the consumption of edible parts of chicory contaminated with DCF did not represent an acute threat to human health. However, the negative health consequences of a prolonged consumption of DCF contaminated chicory, along with the simultaneous consumption of other contaminated foods, are still unknown.

DECLARATION OF INTEREST

None.

ACKNOWLEDGMENTS

The assistance of Dr. Romina Di Paola for the HPLC-QTOF maintenance and of Silvina Colla for the language correction is acknowledged. Authors thank CONICET (fellowships for NSP and LB), FONCYT (Argentinean Agency for the Promotion of Science and Technology;

PICT 2015-1000), and SECYT (Science Secretary of the National University of Córdoba, Argentina; grant No. 30720150100133CB). Support, laboratories and equipment from IMBIV and CIBICI are also acknowledged.

REFERENCES

Acuña, V., Ginebreda, A., Mor, J. R., Petrovic, M., Sabater, S., Sumpter, J., Barceló, D., 2015. Balancing the health benefits and environmental risks of pharmaceuticals: Diclofenac as an example. Environ. Int. 85, 327-333.

Argentinean Ministry of Health - Ministerio de Salud. Alimentos Consumidos en Argentina. Resultados de la Encuesta Nacional de Nutrición y Salud -ENNyS 2004/5. Buenos Aires: Ministerio de Salud, **2012**. http://www.msal.gob.ar/images/stories/bes/graficos/0000000259cnt-a10-alimentos-consumidos-en-argentina.pdf.

Barreales-Suárez, S., Callejón-Mochón, M., Azoulay, S., Bello-López, M. A., Fernández-Torres, R., 2018. Liquid chromatography quadrupole time-of-flight mass spectrometry determination of six pharmaceuticals in vegetal biota. Uptake study in *Lavandula dentata*. Sci. Total Environ. 622-623, 655-663.

Bartha, B., Huber, C., Schröder, P., 2014. Uptake and metabolism of diclofenac in *Typha latifolia* – How plants cope with human pharmaceutical pollution. Plant Sci. 227, 12-20.

Bergmeyer, H. U. 1983. Methods of Enzymatic Analysis, third ed., Bergmeyer, J., Grassl, M., Eds., VCH: Weinheim, Germany. vol. I.

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bruce, G. M., Pleus, R. C., Snyder, S. A., 2010. Toxicological Relevance of Pharmaceuticals in Drinking Water. Environ. Sci. Technol. 44, 5619-5626.

Carter, L. J., Harris, E., Williams, M., Ryan, J. J., Kookana, R. S., Boxall, A. B. A., 2014. Fate and uptake of pharmaceuticals in soil–plant systems. J. Agric. Food Chem. 62, 816-825.

Christou, A., Michael, C., Fatta-Kassinos, D., Fotopoulos, V., 2018. Can the pharmaceutically active compounds released in agroecosystems be considered as emerging plant stressors? Environ. Int. 114, 360–364.

Christou, A., Antoniou, C., Christodoulou, C., Hapeshi, E., Stavrou, I., Michael, C., Fatta-Kassinos, D., Fotopoulos, V., 2016. Stress-related phenomena and detoxification mechanisms induced by common pharmaceuticals in alfalfa (*Medicago sativa* L.) plants. Sci. Total Environ. 557-558, 652-664.

Copolovici, L., Timis, D., Taschina, M., Copolovici, D., Cioca, G., Bungau, S., 2017. Diclofenac Influence on Photosynthetic Parameters and Volatile Organic Compounds Emission from *Phaseolus vulgaris* L. Plants. Rev. Chim. 68, 2076-2078.

D'Abrosca, B., Fiorentino, A., Izzo, A., Cefarelli, G., Pascarella, M. T., Uzzo, P., Monaco, P., 2008. Phytotoxicity evaluation of five pharmaceutical pollutants detected in surface water on germination and growth of cultivated and spontaneous plants. J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng. 43, 285-294.

Di Rienzo, J. A., Macchiavelli, R., & Casanoves, F. (2010). Modelos mixtos en InfoStat. Córdoba, Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba.

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., González, L., Tablada, M., Robledo, C. W. InfoStat, versión 2008. Córdoba, Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba, 2008.

Di Rienzo, J. A., Guzmán, A. W., & Casanoves, F., 2002. A multiple-comparisons method based on the distribution of the root node distance of a binary tree. Journal Agricultural Biological and Environmental Statistics 7, 129–142.

Gill, S. S., Anjum, N. A., Mirza Hasanuzzaman, M., Gill, R., Trivedi, D. K., Ahmad, I., Pereira, E., Tuteja, N., 2013. Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. Plant Physiol. Bioch. 70, 204-212.

Gill, S. S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909-930.

Gomes, M. P., Le Manac'h, S. G., Maccario, S., Labrecque, M., Lucotte, M., Juneau, P., 2016. Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants. Pestic. Biochem. Physiol. 130, 65-70.

González-García, M., Fernández-López, C., Pedrero-Salcedo, F., Alarcón, J. J., 2018. Absorption of carbamazepine and diclofenac in hydroponically cultivated lettuces and human health risk assessment. Agr. Water Manage. 206, 42-47.

Guyón, N. F., Roggio, M. A., Amé, M. V., Hued, A. C., Valdés, M. E., Giojalas, L. C., Wunderlin, D. A., Bistoni, M. A., 2012. Impairments in aromatase expression, reproductive behavior, and sperm quality of male fish exposed to 17β-estradiol. Environ. Toxicol. Chem. 31, 935-940.

Hong, H. N., Kim, H. N., Park, K. S., Lee S. K., Gu, M. B., 2007. Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR. Chemosphere 67, 2115-2121.

Huber, C., Preis, M., Harvey, P. J., Grosse, S., Letzel, T., Schröder, P., 2016. Emerging pollutants and plants - Metabolic activation of diclofenac by peroxidases. Chemosphere 146, 435-441.

559 Huber, C., Bartha, B., Schröder, P., 2012. Metabolism of diclofenac in plants –
560 Hydroxylation is followed by glucose conjugation. J. Hazard. Mater. 243, 250-256.
561 Kinney, C. A., Furlong, E. T., Kolpin, D. W., Burkhardt, M. R., Zaugg, S. D., Werner, S. L.,
562 Bossio, J. P., Benotti, M. J., 2008. Bioaccumulation of pharmaceuticals and other
563 anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid
564 or swine manure. Environ. Sci. Technol. 42, 1863-1870.
565 Kummerová, M., Zezulka, Š., Babula, P., Tříska, J., 2016. Possible ecological risk of two
566 pharmaceuticals diclofenac and paracetamol demonstrated on a model plant *Lemna minor*. *J.*
567 Hazard. Mater. 302, 351-361.
568 Lonappan, L., Kaur Brar, S., Kumar Das, R., Verma, M., Surampalli, R. Y., 2016. Diclofenac
569 and its transformation products: Environmental occurrence and toxicity – A review. Environ.
570 Int. 96, 127-138.
571 Majewska, M., Harshkova, D., Guściora, M., Aksmann, A., 2018. Phytotoxic activity of
572 diclofenac: Evaluation using a model green alga *Chlamydomonas reinhardtii* with atrazine as
573 a reference substance. Chemosphere 209, 989-997.
574 Mallakin, A., Babu, T. S., Dixon, D. G., Greenberg, B. M., 2002. Sites of toxicity of specific
575 photooxidation products of anthracene to higher plants: inhibition of photosynthetic activity
576 and electron transport in *Lemna gibba* L. G-3 (Duckweed). Environ. Toxicol. 17, 462-471.
577 Matile, P., Stefan, H., Thomas, H., 1999. Chlorophyll degradation. Annu. Rev. Plant Mol.
578 Biol. 50, 67–95.
579 McCallum, M. L., Matlock, M., Treas, J., Safi, B., Sanson, W., McCallum, J. L., 2013.
580 Endocrine disruption of sexual selection by an estrogenic herbicide in the mealworm beetle,
581 Tenebrio molitor. Ecotoxicology 22, 1461-1466.

582 Monferrán, M. V., Agudo, J. a. S., Pignata, M. L., Wunderlin, D., 2009. Copper-induced
583 response of physiological parameters and antioxidant enzymes in the aquatic macrophyte
584 Potamogeton pusillus. Environ. Pollut. 157, 2570-2576.
585 Montemurro, N., Postigo, C., Lonigro, A., Perez, S., Barceló, D., 2017. Development and
586 validation of an analytical method based on liquid chromatography–tandem mass
587 spectrometry detection for the simultaneous determination of 13 relevant wastewater-derived
588 contaminants in lettuce. Anal. Bioanal. Chem. 409, 5375-5387.
589 Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Metey, C. U., Rideout, B. A.,
590 Shivaprasad, H. L., Ahmed, S., Chaudhry, M. J., Arshad, M., Mahmood, S., Ali, A., Khan, A.
591 A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature
592 427, 630-633.
593 Ostonen, I., Püttsepp, Ü., Biel, C., Alberton, O., Bakker, M. R., Lõhmus, K., Majdi, H.,
594 Metcalfe, D., Olsthoorn, A. F. M., Pronk, A., Vanguelova, E., Weih, M., Brunner, I., 2007.
595 Specific root length as an indicator of environmental change. Plant Biosyst. 141, 426-442.
596 Passardi, F., Cosio, C., Penel, C., Dunand, C., 2005. Peroxidases have more functions than a
597 Swiss army knife. Plant Cell. Rep. 24, 255-265.
598 Perez, S., Barceló, D., 2007. Application of advanced MS techniques to analysis
599 andidentification of human and microbial metabolites of pharmaceuticals in the aquatic
600 environment. T. Trends Anal. Chem. 26, 494-514.
601 Pierattini, E. C., Francini, A., Huber, C., Sebastiani, L., Schröder, P., 2018. Poplar and
602 diclofenac pollution: A focus on physiology, oxidative stress and uptake in plant organs. Sci.
603 Total Environ. 636, 944-952.
604 Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., Fatta-
605 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant
606 bacteria and genes spread into the environment: a review. Sci. Total Environ. 447, 345–360.

Roggio, M. A., Guyón, N. F., Hued, A. C., Amé, M. V., Valdés, M. E., Giojalas, L. C., Wunderlin, D. A., Bistoni, M. A., 2014. Effects of the synthetic estrogen 17α-ethinylestradiol on aromatase expression, reproductive behavior and sperm quality in the fish *Jenynsia multidentata*. Bull. Environ. Contam. Toxicol. 92, 579-584.

Schmidt, W., Redshaw, C. H., 2015. Evaluation of biological endpoints in crop plants after exposure to non-steroidal anti-inflammatory drugs (NSAIDs): implications for phytotoxicological assessment of novel contaminants. Ecotoxicol. Environ. Saf. 112, 212-222.

Tanaka, K., Sano, T., Ishizuka, K., Kitta, K., Kawamura, Y., 1994. Comparison of properties of leaf and root glutathione reductases from spinach. Physiol. Plant. 91, 353-358.

Tanoue, R., Sato, Y., Motoyama, M., Nakagawa, S., Shinohara, R., Nomiyama, K., 2012. Plant Uptake of Pharmaceutical Chemicals Detected in Recycled Organic Manure and Reclaimed Wastewater. J. Agric. Food Chem. 60, 10203-10211.

Valdés, M. E., Huerta, B., Wunderlin, D. A., Bistoni, M. A., Barceló, D., Rodriguez-Mozaz, S., 2016. Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish. Sci. Total Environ. 557-558, 58-67.

Valdés, M. E., Amé, M. V., Bistoni, M. A., Wunderlin, D. A., 2014. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). Sci. Total Environ. 472, 389-396.

Watts, M. M., Pascoe, D., Carroll, K., 2003. Exposure to 17 alpha-ethinylestradiol and bisphenol A. Effects on larval moulting and mouthpart structure of Chironomus riparius. Ecotoxicol. Environ. Saf. 54, 207-215.

Wintermans, J. F., de Mots, A., 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta. 109, 448-453.

Wu, X., Ernst, F., Conkle, J. L., Gan, J., 2013. Comparative uptake and translocation of pharmaceutical and personal care products (PPCPs) by common vegetables. Environ. Int. 60, 15-22.

Zhang, D. Q., Gersberg, R. M., Hua, T., Zhu, J., Goyal, M. K., Ng, W. J., Tan, S. K., 2013. Fate of pharmaceutical compounds in hydroponic mesocosms planted with *Scirpus validus*. Environ. Pollut. 181, 98-106.

Zhang, D. Q., Hua, T., Gersberg, R. M., Zhu, J., Ng, W. J., Tan, S. K., 2012. Fate of diclofenac in wetland mesocosms planted with *Scirpus validus*. Ecol. Eng. 49, 59-64.

Ziółkowska, A., Piotrowicz-Cieślak, A. I., Rydzyński, D., Adomas, B., Nałęcz-Jawecki, G., 2014. Biomarkers of Leguminous Plant Viability in Response to Soil Contamination with Diclofenac. Pol. J. Environ. Stud. 23, 263-269.

FIGURE CAPTIONS

Figure 1. Phenotypic effects of diclofenac on chicory plants. Control and diclofenac (3.42 µM) treatment in germination study (A) and uptake assay (B).

Figure 2. Box plots of chicory root length, root water content, root biomass, root specific length and root and aerial part (shoot + cotyledon) ratio in the germination study. Star indicates significant differences ($p < 0.05$) between treatment (control with 0.1% of methanol and diclofenac 3.42 µM).

Figure 3. Chlorophylls *a* and *b* (A) and Pheophytins *a* and *b* (B) concentrations (µg g^{-1} ww) in aerial part of chicory exposed at control and diclofenac treatments. Star indicate significant differences ($p < 0.05$) between treatments (control with 0.1% of methanol and diclofenac 3.42 µM).

Figure 4. Guaiacol peroxidase (POD) activity (A) and glutathione reductase (GR) activity (B) nkat mg^{-1} protein) in chicory exposed at control and diclofenac. Stars indicate significant differences ($p < 0.05$) between treatments (control with 0.1% of methanol and diclofenac 3.42 µM). GR activity in aerial parts could not be determined due to pigment interferences.

Figure 5. DCF content (µg $100g^{-1}$ ww) in different samples recollected from uptake assay. Stars indicate significant differences ($p < 0.05$) between samples.

FIGURE GRAPHICS

Figure 1.

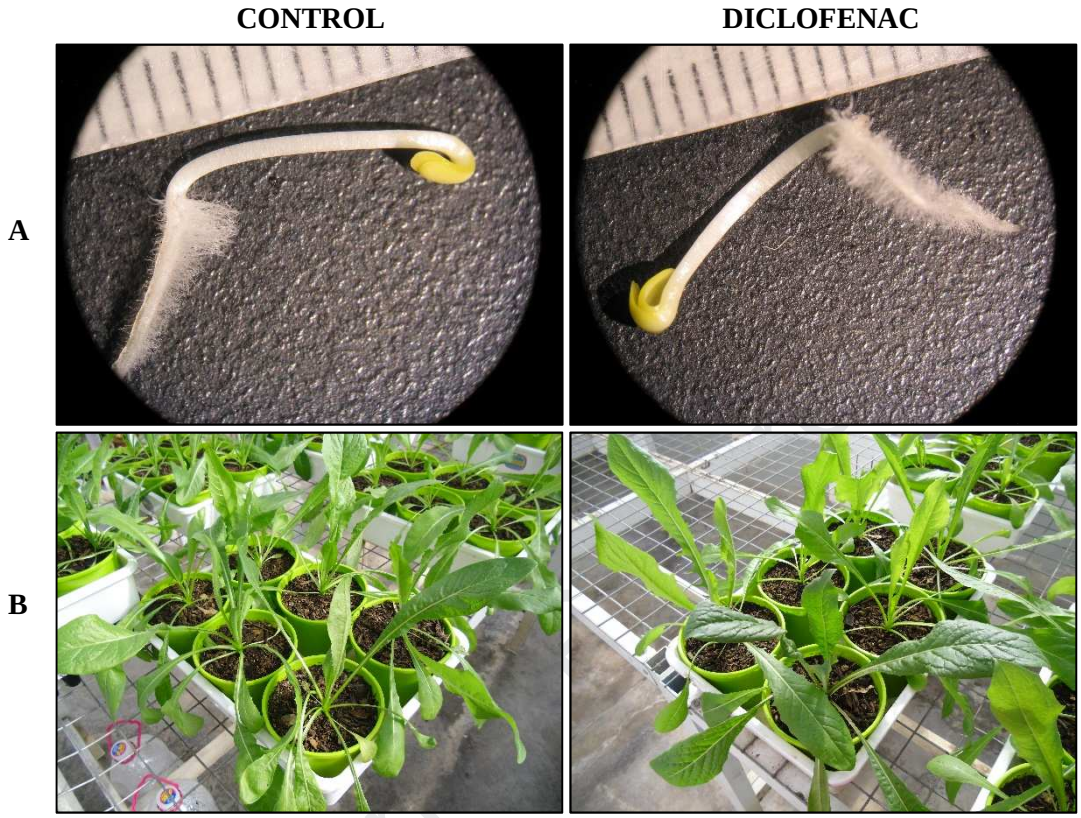


Figure 2.

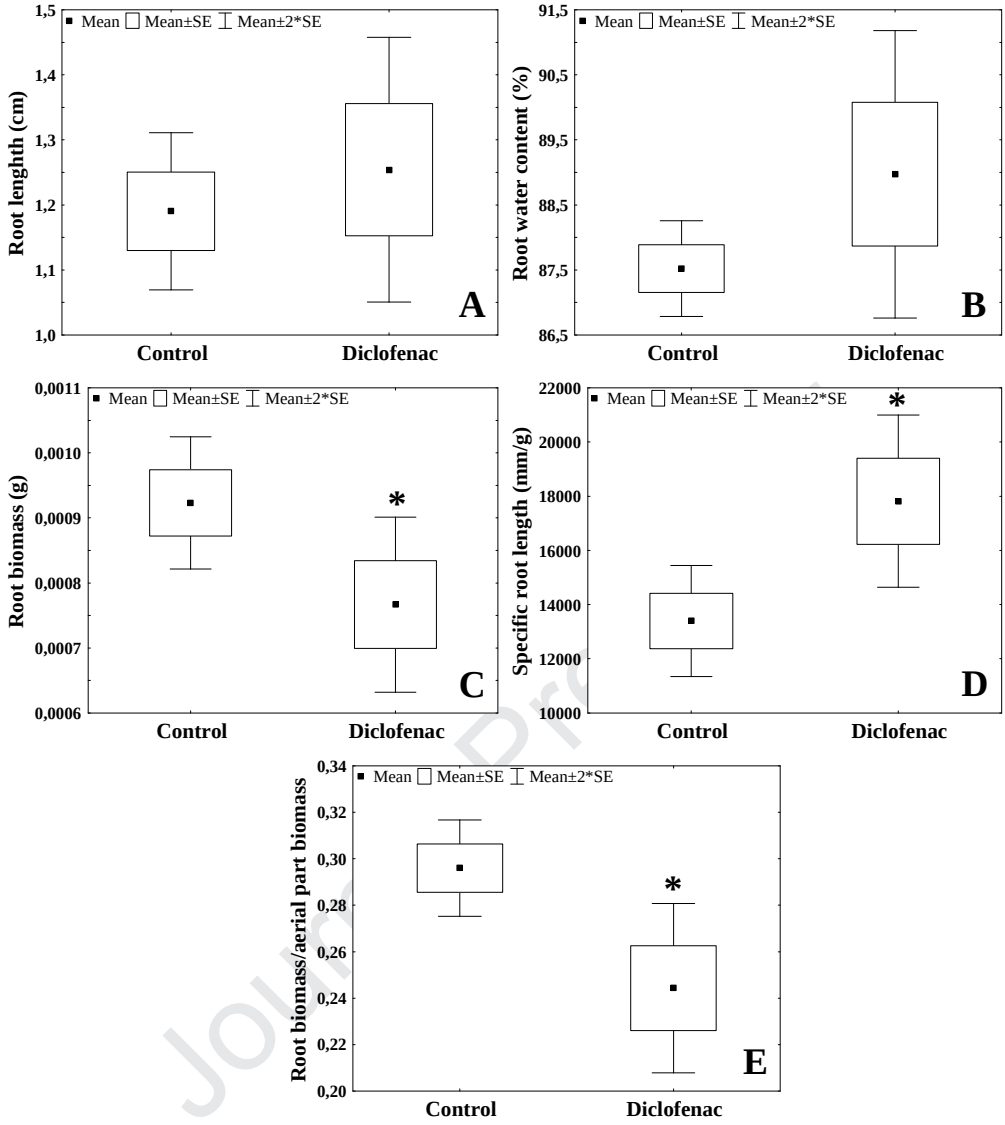

Mean
Mean±SE
Mean±2*SE
Root lenghth (cm)
1,5
1,4
1,3
1,2
1,1
1,0
A
Control
Diclofenac
Root water content (%)
91,5
90,5
89,5
88,5
87,5
86,5
B
Control
Diclofenac
Root biomass (g)
0,0011
0,0010
0,0009
0,0008
0,0007
0,0006
*
C
Control
Diclofenac
Specific root length (mm/g)
22000
20000
18000
16000
14000
12000
10000
*
D
Control
Diclofenac
Root biomass/aerial part biomass
0,34
0,32
0,30
0,28
0,26
0,24
0,22
0,20
*
E
Control
Diclofenac

Figure 3.

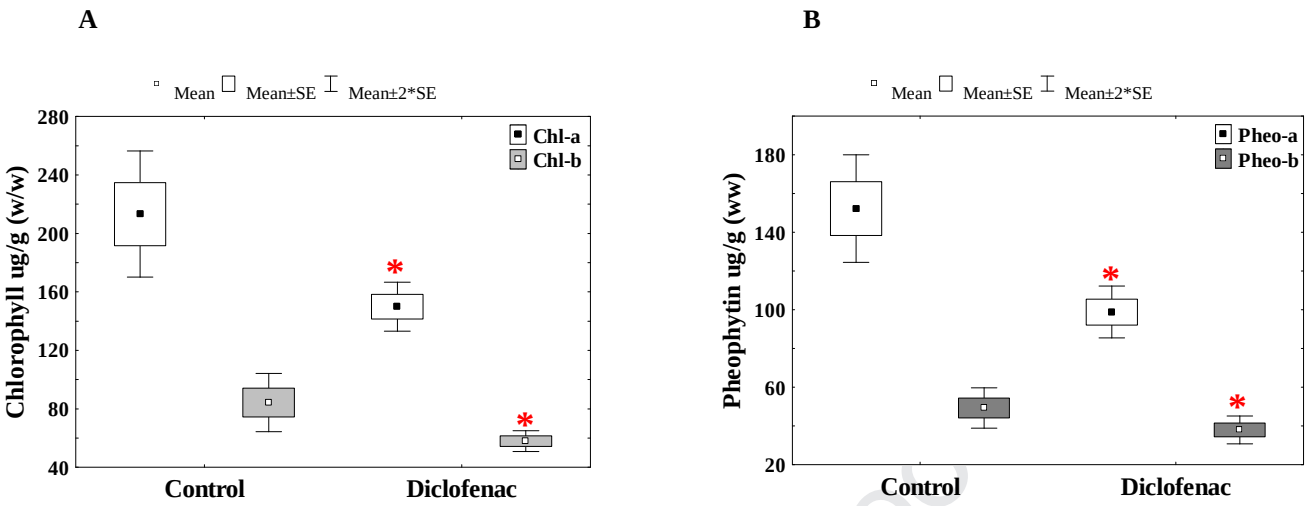


Figure 4.

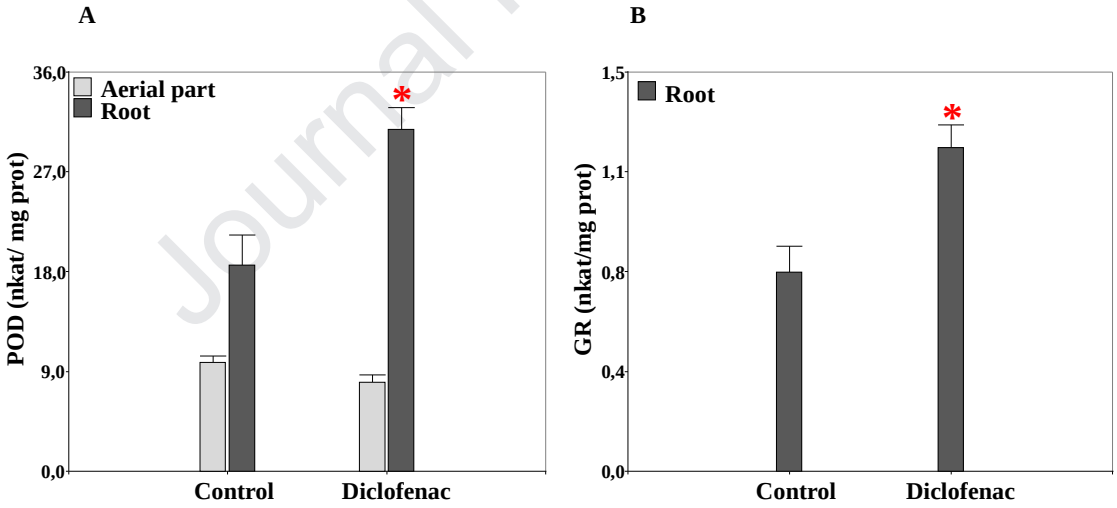
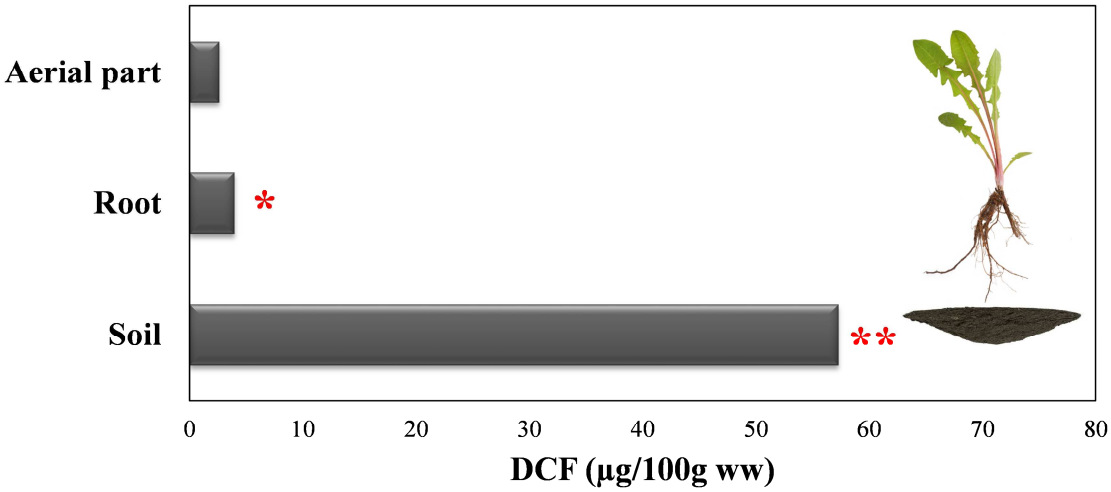
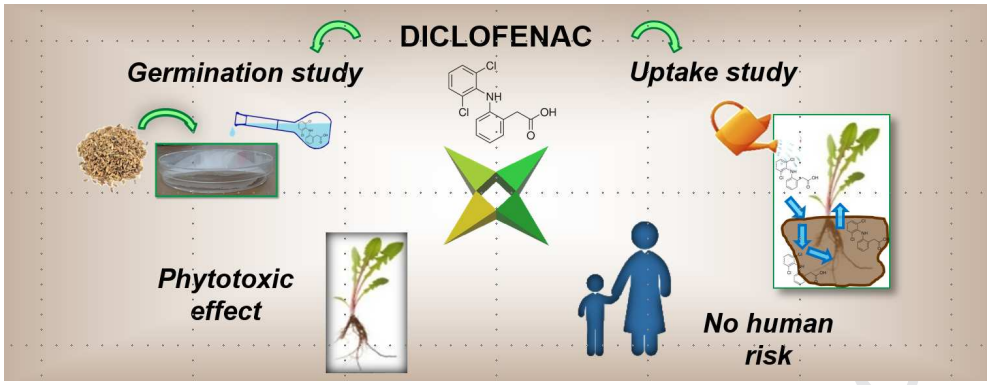


Figure 5.



GRAPHICAL ABSTRACTS



HIGHLIGHTS

- This is the first study showing the effect of DCF on *Cichorium intybus*
- DCF produces a decrease of root biomass and an increase of the specific root length
- DCF induces the activation of the endogenous antioxidant defense mechanisms
- An effect on chlorophyll biosynthesis was also observed
- Irrigation with water containing DCF would not represent a threat to human health

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: