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BIOACCUMULATION, UPTAKE AND TOXICITY OF CARBAMAZEPINE IN SOIL – PLANT SYSTEMS

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Abstract: Since the detection of active pharmaceutical ingredients (APIs) in various environmental media, research has explored the potential uptake and toxicity of these chemicals into species inhabiting these matrices. Specifically, pharmaceuticals, including the antiepileptic API carbamazepine (CBZ), are taken up from soil by a range of plants. Many short term studies have also suggested that certain APIs induce toxicity in plants. However, effects of APIs on fruiting plants remains relatively unexplored. This study investigated the uptake, bioaccumulation and toxicity of CBZ in *Cucurbita pepo* (Zucchini) from seed to full maturity across a range of CBZ exposure concentrations in soil (0.1 to 20 mg/kg). Results of biomass, chlorophyll, starch and total nitrogen (N) concentration in *C. pepo* indicated toxicity at soil concentrations of ≥ 10 mg/kg. There were clear visual indications of increasing toxicity on leaves, including chlorosis and necrosis, from soil concentrations of 1 mg/kg up to 20 mg/kg. This study also revealed novel insights into the effect of CBZ accumulation on *C. pepo* fruiting, as female *C. pepo* flowers were unable to set fruit when leaf concentrations were ≥ 14 mg/kg. These findings may have implications for future agricultural productivity in areas where reclaimed wastewater containing APIs is a source of irrigation. Detectable CBZ concentrations were found in edible *C. pepo* fruit indicating the possibility of trophic transfer.

Keywords: Pharmaceuticals, Organic contaminants, Plant toxicity, *Cucurbita pepo* (Zucchini), Chlorophyll, Starch

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INTRODUCTION

The use of pharmaceuticals has increased dramatically since the discovery of antibiotics just before the Second World War [1]. There are now more than 3000 active pharmaceutical ingredients (APIs), for both human and veterinary medicine, registered for use in the world [2, 3]. After usage, human pharmaceuticals are introduced to the wastewater stream during which they undergo treatment at a wastewater treatment plant (WWTP). The physio-chemical properties of many pharmaceuticals typically make them partly resistant to degradation and therefore they remain present in the waste stream and ultimately end up in WWTP by-products, namely effluent and biosolids [4-6]. When the by-products are disposed of into ecosystems the APIs present in these matrices are also released into the ecosystem [7, 8]. Specifically in the terrestrial environment this is *via* effluents used as a source of reclaimed wastewater and the application of biosolids for use as a soil amendment. The quantity of pharmaceuticals entering any WWTP or the environment is highly variable depending on the region, season, number of prescriptions and consumer demand for medications. This explains the wide variation observed in reported concentrations of pharmaceuticals in the environment. For example, carbamazepine (CBZ) has been reported to occur in WWTP effluent, biosolids and soil in concentration ranges 0.24 to 2.10 µg/L, 7.8 to 258 µg/kg and 0.0065 to 7.5 µg/kg, respectively [8-13]. Carbamazepine has been reported to have a low removal efficiency in WWTPs of ≤ 10 % [8, 9, 14] and has a half-life of more than 365 days in both WWTPs and soil [7, 14] (Table 1). The long half-life raises concerns for bioaccumulation in terrestrial systems. Studies have previously demonstrated that CBZ is taken up by, and accumulates in, plant species including radish, ryegrass, lettuce, spinach, cucumber and peppers [15, 16], with typical concentrations measured in the range of 2.9 to 67 ng/g in leaf material [16]. However, while studies have shown that CBZ can be taken up by

plants, it is not known if bioaccumulation of CBZ by plants is linear with increasing CBZ soil concentration. In a four-week exposure study to CBZ-spiked soil, *Cucurbita pepo* (Zucchini) had detectable changes in plant development and nutrient and plant hormone homeostasis from 0.005 mg/kg and were significantly influenced from ≥ 4 to 10 mg/kg [17]. The authors hypothesised that the presence of CBZ in the plant was disturbing ion transport and as a result inducing a change in cell membrane potentials. As membrane potentials enable sugar transportation in the plant, this could potentially explain why the plants treated with CBZ at the highest concentrations displayed symptoms akin to a sugar deficiency and eventually went necrotic. Previous studies assessing API-induced toxicity have mostly been short term (i.e. < 21 days), where plants were harvested before fruiting [16, 18-20]. Longer-term studies are therefore needed to reveal insights into how API accumulation affects fruit maturation. Specifically, further research is now required to explore whether short-term effects on nutrient and plant hormone homeostases result in any long-term impacts on *C. pepo* development (i.e. fruiting quality) based on the initial findings of the Carter, et al. [17] study.

Cucurbita pepo has been shown to accumulate organic contaminants and translocate them to the shoots and fruits [21]. Therefore *C. pepo* is a useful indicator species to assess the most likely exposure scenario where reclaimed wastewater and solids from water treatment are likely to be used on horticultural land close to urban centres e.g. North Adelaide Plains in South Australia [22]. The use of reclaimed wastewater will likely increase in the future as fresh water becomes a limiting resource due to population growth [23]. It is therefore important that the potential risks associated with pharmaceuticals in the terrestrial environment are explored to prevent adverse effects on agricultural production or food quality. The aims of this study therefore were to identify uptake and toxicity of CBZ in *C. pepo* leaves and fruits and identify if

there are any effects of CBZ on phenology of growth, nutrient accumulation and chlorophyll in *C. pepo*.

MATERIALS AND METHODS

Chemicals

Analytical grade CBZ (≥ 98 % purity), ammonium formate (97 % purity), ammonium nitrate (99 % purity) and Hoagland No. 2 basal salt mixture were obtained from Sigma-Aldrich (Sydney, Australia). Deuterated CBZ (carbamazepine-d_{10}, 99.4 % purity) was purchased from TLC Pharmachem (Vaughan, Canada) for use as an internal standard for CBZ analysis. Boric acid was obtained from AnalaR ® BDH (Kilsyth, Australia). Methanol, acetone and formic acid (90% purity) and were purchased from Thermo Fisher Scientific (Australia). Concentrated nitric acid (70 % purity) was purchased from ACILabscah (Bangkok, Thailand). Megazyme total starch assay kit (K-TSTA) was purchased from Deltagen (Australia).

Plant toxicity and bioaccumulation

For use in the plant toxicity and bioaccumulation study, a sandy soil (96.5 % sand) was collected from a site in Karoonda, South Australia (April, 2015). The soil was air-dried for 4 days and then sieved to < 2 mm to ensure homogeneity. Soil characteristics are provided in the Supplementary Information (SI) (Table SI 1).

Aliquots of acid washed sand (2 g) were spiked with CBZ (10 mg/ mL in methanol) to create nominal soil concentrations of 0.1, 1, 2, 5, 10, 20 mg/kg. It is important to note that the concentrations used in these experiments were higher than typical environmentally relevant concentrations. Three replicates were prepared per treatment including controls and solvent controls (methanol spike only). The spiked sand was then evaporated under a nitrogen (N) gas stream until dry and was then incorporated into 5 ± 0.01 kg of the test soil and hand mixed for 5

min to create an even distribution of the test chemical. The pots were watered to 60 % maximum water holding capacity using Milli-Q water (18.2 MΩcm) and incubated under controlled conditions for 2 days (65% relative humidity, 12 h light (23 °C)/ 12 h dark (15 °C)) to acclimatise to test conditions. Three 2 g sub-samples of soil from each treatment were taken at day 0 (after incubation but before sowing of seeds) to confirm the spiked concentrations of CBZ in all treatments. Three *C. pepo* seeds (var. Midnight F1 Container Garden, Mr. Fothergills (Sydney, NSW)) were planted into each pot and thinned after 10 days to one plant. The plants were grown for 14 weeks under controlled conditions where the maximum water holding capacity of the soil was maintained at 60 % by addition of Milli-Q water on a daily basis. A 25 % dilution of Hoaglands nutrient solution (50 mL) was added once a week from weeks 4 to 6 and then twice a week until harvest. Ammonium nitrate (10 mL, 250 mg N) was added at weeks 9, 10 and 12.

During the experiment, *C. pepo* were hand pollinated in the absence of a natural pollinator. Pollen was collected from the male flowers and stored until female flowers were open, details of which can be found in the SI. The female flowers were pollinated with pollen either from the same replicate or if that was not an option, with pollen from the same treatment. During the 14 weeks a number of *C. pepo* fruits were harvested as they set fruit according to guidelines described by Hülster, et al. [21] (Table SI 2). Briefly, female flowers that had set fruit were harvested when the fruits were > 2.5 cm in diameter and with a length of approximately 15 cm. Harvested fruits were rinsed in Milli-Q water, peeled and cut into pieces. Samples of 5 g of fruit and 1 g of peel were weighed out into 15 mL extraction tubes and stored at -20 °C until the remainder of the fruits were harvested. At 14 weeks, the fruits, where the female flower had been pollinated and set fruit, and female flowers, where the female flower had not opened, had been pollinated and not set fruit, were removed rinsed in Milli-Q water and weighed separately.

Before the remaining body of the plant was harvested from the pot, chlorophyll content was measured using a SPAD 502-plus meter (Konica Minolta Sensing Corporation Inc, Japan). Young and old leaves were separated by size and measured from one side of the leaf to the other: leaves > 90 mm diameter were considered old leaf material and leaves < 90 mm considered young leaf material. Four SPAD measurements were taken per replicate and averaged for both young and old leaves to obtain a treatment average. Biomass, number of leaves and any abnormalities, including discolouration of leaves; necrosis and reduced growth due to treatment, were also recorded.

Leaves were harvested at the base of the leaf blade, leaving the petiole attached to the stem, and separated into old and young leaves as described above. After rinsing in Milli-Q water the leaves were then sliced into small pieces, freeze dried and stored in the freezer (-20 °C) before analysis. One gram of leaf material was required for analysis from all replicates.

Soil samples (2 g) were taken at the end of experiment to analyse for concentrations of CBZ. The soil was first freeze dried and then stored in the freezer (-20 °C) before analysis.

Pharmaceutical analysis

Soil pore water extraction. Pore water was extracted from soil using the methods described of Thibault, et al. [24]. Briefly, 2 x 25 g portions of soil from each replicate were sampled and placed on a glass wool insert inside a disposable plastic syringe, in a plastic tube. The tubes were then centrifuged at 3569 g force for 45 min to obtain the pore water and then combined and centrifuged again at 30940 g force for 30 min before 990 µL of pore water was transferred to autosampler vials with 100 µL of deuterated internal standard (CBZ-d_{10} 0.1 µg/mL in methanol).

Soil and plant material. All plant and soil material was spiked with 100 µL of internal standard (0.1 µg/mL in methanol) (except controls and solvent controls), placed in the freezer (-20 °C) and then freeze dried before extraction.

Prior to all extractions, approximately 1 g of acid washed sand was added to each extraction tube. Extraction of CBZ from soil was performed by adding 5 mL of methanol to 2 g soil, vortexing for 30 seconds and then centrifuging for 30 min at 656 g force. The supernatant was collected and then the extraction steps repeated with another 5 mL methanol and 5 mL acetone. The combined supernatant was evaporated using N gas and reconstituted in 1 mL methanol prior to analysis. Soil samples obtained from treatments ≥ 2 mg/kg were diluted appropriately to ensure the concentrations were within the calibration range.

CBZ was extracted from plant samples using the same process as for soil, except that the combined supernatant was diluted with Milli-Q water to a maximum solvent concentration of 10 %. The samples were then loaded onto and eluted from Oasis HLB (Water Corporation) 6 mL 200 mg solid phase extraction (SPE) cartridges using 2 x 3 mL methanol and 3 mL methylene chloride. The SPE cartridges were preconditioned with Milli-Q water and methanol. The eluted sample was evaporated by N gas and reconstituted in 1 mL methanol prior to analysis. Dilutions were again performed as required to ensure concentrations were within the calibration range.

Instrumental analysis. Concentrations of CBZ were determined by liquid chromatography tandem mass spectroscopy (LC-MS/MS) using a ThermoFinnigan TSQ Quantum Discovery Max (Thermo Electron Corporation). Chromatographic separation was performed with a Waters Atlantis T3 C18 100 x 2.1 mm i.d. (3 µm particle size) column with a 2.1 x 10 mm guard cartridge attached (Waters, Ireland) at a mobile phase flow rate of 250 µL/min. The mobile phase composition consisted of two eluents, which were (A) 10 mM

ammonium acetate in 0.1 % formic acid and (B) methanol using a binary gradient programme over 15 min. The relative flow rate of (A) was 95 % for 2 min, 2 % after 4 min and held for 6 min before returning to 95 % by 10.10 min. Mass spectroscopy was undertaken using atmospheric pressure electrospray ionisation (ESI) in positive mode. Spray voltage was 5000, skimmer offset was -10 V with the ESI capillary line was maintained at 350 °C and a collision gas (Ar) pressure of 1.5 mTorr. Qualitative and quantitative analysis of compounds was based on retention time, multiple reaction monitoring (MRM) of the two product ions, and the ratios between the product ions. Detailed instrumental parameters used for CBZ detection are provided in SI (Table SI 3).

CBZ has a retention time of 7.26 min and calibration standards were used to verify CBZ concentrations.

Limit of detection and quantification. Analytical lower limits of detection (LODs) and quantification (LOQs) for the LC-MS/MS method were calculated as 3 x and 10 x standard deviation, respectively, of the lowest CBZ concentration (0.0005 µg/mL) spiked in a blank matrix extract (soil, plant, pore water). LODs and LOQs are reported in SI (Table SI 4) for both plant and soil matrices in µg/L and µg/g (dry weight).

Extraction validation was achieved by spiking known concentrations of CBZ into known amounts of plant and soil material. The CBZ was then extracted from the soil and plant matrices ready for instrumental analysis. Relative recoveries were obtained using an area ratio obtained from the LC-MS/MS output. The replicate recoveries were then averaged. Extraction validation was achieved with fruit, leaf, root, soil and spiked sand materials and can be found in SI (Table SI 5). Relative recoveries for extraction validation resulted in recoveries of 112 % soil (low spike), 157 % soil (high spike), 136 % sand, 100 % root, 180 % leaf, 124 % fruit and 119 % fruit

peel. The high recoveries for some matrices would suggest that there was a matrix effect, which has led to a signal enhancement. This would result in measured concentrations in excess of the true concentration (Table SI 5).

Nutrient analysis

Remaining freeze dried fruits, young and old leaves were ground to a fine powder using a mortar and pestle. One gram of young and old leaves from each replicate was weighed out for analysis of total carbon (C) and N. Total C and N in *C. pepo* was determined by high temperature combustion in an atmosphere of oxygen using a Leco TruMAC. Further details of analysis are provided in SI.

Plant material (0.25 g) was digested with 5 mL of concentrated nitric acid and left to rest overnight. Certified reference material from tomato leaf extract was also digested (Tomato leaves, standard reference material 1573a) for quality control purposes to determine the accuracy of the acid digestion and analysis procedures (Table SI 6). The next day, samples were evaporated using a heating block to approximately 1 mL. The samples were then reconstituted to 20 mL with 0.1 % nitric acid in deionised water and filtered through filter paper (Whatman® No. 42). The samples were then diluted by a factor of 5 with Milli-Q water and stored at 4 °C until analysis. Concentrations of B, Ca, Cu, K, Mg, Mn, P, S, and Zn in the digests were determined using a Perkin Elmer, Optima 7000DV inductively-coupled plasma optical emission spectrometer. Instrumental conditions can be found in the SI (Table SI 7).

Starch analysis

Starch content in the young and old leaves was achieved using a variation of sample preparation method b, in the Megazyme total starch assay procedure kit (AOAC Method 996.11) based on the method of McCleary, et al. [25]. Briefly, 20 mg of ground leaf material was

weighed into 10 mL centrifuge tubes, twice for each replicate. Leaf material was rinsed with 5 mL 100% ethanol, 5 times to remove the colour from the leaf samples. 1.2 mL alpha-amylase with 3-(N-morpholino)propanesulfonic acid buffer was added to each sample and placed in 100 °C water bath for 12 min and vortexed every 4 min. Samples were moved to 50 °C water bath for 5 min before the addition of 1.64 mL of amyloglucosidase with sodium acetate buffer and then vortexed. Samples remained in 50 °C water bath for 30 minutes and were then made up to 10 mL and centrifuged at 3220 g force for 10 mins. Two aliquots of supernatant were transferred into a 96-well plate from each sample along with 1.5 mL of glucose oxidase/peroxidase solution. The 96-well plate was incubated at 50 °C on a thermomixer (Thermomixer comfort, Eppendorf) for 20 min and 1 mL from each sample was transferred to a cuvette before analysis by spectrophotometer at 510 nm (Varian Cary 50 Bio UV-Visible spectrophotometer). Control treatment leaf material was also used to determine background starch content (blank), which was prepared using the same methods as sample leaf material except enzymes were replaced with Milli-Q water. Blank absorbance readings were subtracted from the sample absorbance readings before calculating for starch %. Standard maize starch (included in the assay kit) was used as a reference material in duplicate for each batch of samples.

Statistical analysis

Statistical analysis of the data was carried out using Microsoft Excel (2013) and IBM SPSS (v. 20). Normal distribution of the data and Levene's test was conducted before analysis to ensure normality and equal variances in the data. When equal variances were violated a Welch F test was run. Analysis of variance (ANOVA) was produced to determine differences between treatment means where the significance level was set at 0.05. Significance between treatment means was identified using a Tukey's honestly significance difference test. Statistical analysis

was used to assess significant differences in plant biomass and chlorophyll content. Toxicity curves and effect concentrations (EC) values were calculated through an EC50 calculation template (copyright CSIRO).

For statistical analysis, the CBZ treatment of 1 mg/kg in the plant toxicity and bioaccumulation experiment was calculated with only two replicates as the third had unintentionally no seeds planted in it. Two replicates in the 20 mg/kg treatment had no seeds germinate but they did have seeds planted in them and therefore were included in the calculations as a treatment effect for biomass. However, those three pots with no plants were counted as missing values in nutrient and starch analyses and CBZ concentrations in plant matrices.

RESULTS AND DISCUSSION

Dose confirmation and fate in soil

Confirmation of CBZ concentrations in experiments were consistent with nominal concentrations (Table SI 8). This is consistent with previous research that found CBZ is persistent in the soil environment, with a reported half-life in soils of approximately 500 days, strong adsorption affinity to soil organic matter and low leaching potential in a soil high in organic matter [7, 14, 26].

Initial concentrations and equal distributions of CBZ in each treatment replicate were confirmed by residue analysis where CBZ concentrations were > 80 % of the nominal concentrations for all treatments (Table SI 8). The concentration of CBZ in the soil decreased for all treatments over the 14-week period. In previous research it has been suggested that due to the persistent nature of CBZ, it is likely this reduction in concentration is a result of plant uptake [7, 14, 26]. In this study plant uptake accounted for 2 to 12 % of total CBZ at the end of the 14-week

growing period. The average final concentration for the 20 mg/kg treatment was highly variable and this could be attributed to the lack of plants in two of the replicates.

As this study was a closed system, where drainage and therefore leaching was impossible, CBZ was detected in the pore water for all treatments, with the exception of controls, solvent controls and 0.1 mg/kg, where concentrations were below the LOD (0.335 µg/L) in the soil pore water [27]. The concentration of CBZ in the soil pore water increased with increasing CBZ concentration in the soil (Figure SI 1). Due to the low organic carbon content (0.5 % Total C) of the soil it was expected that CBZ sorption would be minimal therefore maximising the bioavailable fraction of this chemical for uptake by *C. pepo*.

Plant toxicity and bioaccumulation

Plant uptake. Previous research has shown that due to the non-ionic nature of CBZ, it passes easily through plant root membranes compared to ionic compounds because there is no energy required to transport the compound through the membrane [15, 28]. Previous studies have also suggested plant uptake of organic compounds can be related to a Gaussian distribution with maximum translocation of chemicals at log K_{ow} 1.5 to 2.0 [29]. The log K_{ow} for CBZ sits just outside the maximum range for non-ionised chemical uptake. However, it is still expected to accumulate to high levels in leaf material. The high concentrations of CBZ detected in *C. pepo* leaves in this study are therefore comparable to previous research, which has investigated CBZ plant uptake (Figure 1) [15, 17]. Specifically, concentrations of CBZ up to 52 mg/kg have been found in radish leaves after 6 weeks of growth in soil spiked with 1 mg/kg of CBZ [15]. In the present study, exposure at this soil concentration resulted in measured concentrations in *C. pepo* ranging from 8.6 to 13.0 mg/kg (dry weight, dw) in the young and old leaves, respectively (Figure 1). The difference in concentrations in the leaves of radish and *C. pepo* could indicate

that plant uptake is dependent on the plant species in question, the APIs physical and chemical properties but also the environmental growing conditions.

The concentrations of CBZ in the root, old and young leaf material increased with increasing treatment concentration (Figure 1). Average concentrations of CBZ were slightly less in the old leaves compared to the young leaves (Figure 1). The CBZ concentrations in the root material of *C. pepo* were considerably lower by a factor of at least ten than those found in the leaf material (Figure 1). This is consistent with other research where concentrations of CBZ were higher in the leaf material than the root material [15, 16, 28]. A linear relationship is observed between increasing leaf and root CBZ concentrations and soil CBZ concentrations, with r^2 values ≥ 0.97. This indicates that the accumulation of CBZ in the plant material is almost entirely explained by an increase of CBZ concentration in the soil (Figure 1). The linear relationship with slope > 1.0 indicates that CBZ is being translocated and accumulating in the leaves of *C. pepo*. New leaves in plants are a sink for nutrients, transported from the roots by transpiration in the xylem. As the leaves mature and they become a source of nutrients, the phloem transports the nutrients and carbohydrates to the fruits and seeds [29, 30]. New leaves may have had higher concentrations of CBZ, approximately 1.6 times higher than older leaves when soil concentrations were ≥ 5 mg/kg (p value > 0.05), as the phloem export of nutrients from these leaves would have been less than the older leaves.

Concentrations of CBZ were measured in the fruits and female flower fruits harvested from all treatments and can be found in SI (Figure SI 2). At the time of harvest, there were several female flowers that had not opened or set fruit but there was enough biomass to analyse for CBZ concentrations (Figure SI 2). The concentrations of CBZ in the fruit and female flower fruit material increased with increasing CBZ soil concentration from 0.22 to 2.57 mg/kg and 0.23

to 183 mg/kg, respectively. The linear relationship was not as strong when compared to the root and leaf material; r^2 values were 0.85 for fruits and 0.83 for female flower fruits (Figure SI 2). This could be because the number of fruits and female flower fruits per treatment varied from 0 to 3 in any treatment and also the stage of development of the fruit or female flower fruits. Female flowers were produced by *C. pepo* in all CBZ soil treatments over the 14 week growing period (Table SI 2).

The metabolism of CBZ was not measured in the present study. However, a mass balance indicated that between 26 and 80 % of the original starting CBZ concentrations were unaccounted for. This would suggest the parent compound was either metabolised or degraded (Figure SI 3). Other studies have indicated that when uptake of CBZ by plants does occur, particularly into the roots and shoots the metabolites are found in higher concentrations than the parent compound [28]. For instance, the CBZ metabolite,10,11-epoxycarbamazepine, has been found in sweet potatoes and carrots, with the CBZ parent compound only accounting for 11 ± 2 % and 28 ± 3 % of CBZ measured in the leaves of both species, respectively [28]. Specifically, Malchi, et al. [28] did not observe significant metabolism in the edible portion of the plant, high concentrations of metabolites were instead detected in the leaves. Therefore it is likely that in-plant metabolism of CBZ occurred in *C. pepo*, and may account for some of the missing fraction of the parent compound in the mass balance. However, based on the findings from this previous study it is unlikely that the concentrations of the metabolites in the fruit would be in excess of the parent compound in the fruit of the zucchini plant.

Plant toxicity. There was no significant difference for any of the treatments relative to the controls for total biomass (p values 0.19 to 1.0), old leaf biomass, (p values 0.89 to 1.0) and

young leaf biomass (p values 0.23 to 1.0). There were significant differences for root and stem biomass when compared to the controls at 20 mg/kg (Figure 2).

There were clear visual effects, such as increasing degrees of chlorosis and necrosis when soil concentrations of CBZ increased from 1 mg/kg to 20 mg/kg. Previous research has shown that CBZ can be taken up into plants, for example, into radishes, ryegrass, spinach and cucumbers where no visual effects were reported, which is likely due to low exposure concentrations of < 1 mg/kg [15, 16, 28]. However, visible toxic effects have been described recently, up to CBZ soil concentrations of 10 mg/kg [17].

The total number of fruits decreased with increasing CBZ concentration in the soil and there were varying numbers of fruits harvested from each treatment (Table SI 2). Even though female flowers developed across all CBZ treatments, this did not always translate into *C. pepo* fruits. In all CBZ soil treatments including controls, the female flower buds would randomly brown off and shrivel up before they were able to be hand pollinated. After 14 weeks, no fruits were harvested from the highest CBZ treatment concentrations (10 and 20 mg/kg). From soil concentrations of 2 mg/kg and higher or when leaf concentrations were in excess > 14.3 and 14.5 mg/kg in the old and young leaf, respectively, female flowers appeared to set fruits after pollination but within a week fruit development had ceased and the fruit shrivelled up from the base (Figure SI 4). As the solvent control replicates produced fruits this would suggest it is an effect of the CBZ accumulation by the plant.

Chlorophyll and nutrient content in leaves. Due to the visibility of chlorosis and necrosis present on the leaves, analysis of chlorophyll and nutrient content was conducted. Compared to the controls chlorophyll content significantly decreased in the old leaves but not in the young leaves (p = 0.80 to 1.0) with increasing concentration of CBZ in the soil, Figure 3. For

chlorophyll, EC10, EC20 and EC50 values were 1.03, 2.77 and 14.98 mg/kg, respectively. A LOEC was found to be at approximately 2 mg/kg ($p < 0.002$). Research on CBZ in microalgae has shown that chlorophyll content decreased compared to the control in *Neochloris pseudoalveolaris* but not in *Parachlorella kessleri* in the concentration range of 0.01 to 1 mg/kg CBZ [31]. A similar study also using *C. pepo* has shown that chlorophyll *a* was significantly reduced when grown in soils spiked with 8 to 10 mg/kg [17]. Chlorophyll *b* and carotenoids were unaffected. This indicates that CBZ has the potential to decrease chlorophyll content in a range of species.

In addition to the observed differences in chlorophyll content in the old and young leaves, there was a clear difference in the appearance of the old and young leaves. This could indicate that essential nutrients were being transported to the young leaves, leaving the old leaves deficient in nutrients, such as N. The concentrations of total N in the young and old leaves are shown in Figure 4. Compared to the control treatment, N concentrations in young leaves significantly increased ($p < 0.05$) as CBZ concentrations in soil increased from 2 to 10 mg/kg. However, there were no significant differences seen in the total N concentrations in the old leaves ($p = 0.23$ to 1.0). Concentrations of N in the young leaves were lower than those in the old leaves (Figure 4). This was unexpected due to the typical mobility of N within plants [32] where N is prioritised to areas of new growth. N is also an important element in proteins, enzymes, hormones and chlorophyll [33]. Although, as chlorophyll content declined in the old leaves with increasing concentrations of CBZ in the soil (Figure 3) the use of N within the plant may be inhibited in the presence of CBZ.

Concentrations of N in the controls were in the critical range for *C. pepo* leaves at harvest [34]. Other essential macro and micro nutrient concentrations, S, P, Cu, and Zn, in the *C. pepo*

young leaves showed no change ($p > 0.05$) in nutrient concentration with increase in CBZ soil concentration (Figure 4). Only K and B, in the young leaves, of the 10 mg/kg treatment were significantly different to the control treatment ($p < 0.05$). This is similar to the results of a previous study, where CBZ was found to significantly increase K and P concentrations at soil doses of 8 and 10 mg/kg in *C. pepo* grown for 4 weeks ($p < 0.05$) [17]. The other nutrients examined, Ca, Mg and Mn, were also unaffected by CBZ dose (Figure 4).

Starch content in leaves. Older leaves for the control treatment had significantly lower starch concentrations than the solvent control, $p < 0.05$. There was a decrease in starch content of older leaves with increasing CBZ accumulation by *C. pepo* (Figure 5). A similar but less consistent trend can be seen in the young leaf material.

It has been suggested that the visual distress symptoms observed in the leaves of *C. pepo* with CBZ may be a sign of a sugar deficiency [17]. The results for the *C.pepo* leaf material do not indicate any particular stress-related effect. Starch content was lower in the young leaves compared to the old leaves in the solvent control, 0.1, 2 and 10 mg/kg treatments (Figure 5). To explain this further, starch is a storage molecule (polysaccharide) within the plant and is made up of two glucose polymers, amylopectin and amylose [35]. The starch that is produced during the day, especially in leaves, can be re-solubilised into sucrose at night to support plant metabolism and growth [36, 37]. There is a complex signalling network that takes place, which is controlled by genes, within the plant that encodes enzymes of photosynthesis, sugars and starch metabolism [38]. Sugars such as glucose, sucrose and fructose are considered signal metabolites for the gene expression. Therefore, if there is a decrease in sugar content, for example from the presence of CBZ in *C. pepo*, the ability of the plant to make new starch grains would also decline. If this was the case, the amount of starch accumulating in young, developing leaves would also decline.

This could explain why the starch content in the young leaf material in *C. pepo* was lower than the older leaf material. It has also been hypothesised that the presence of CBZ in the plant was disrupting ion transport causing a change in cell membrane potentials [17]. Membrane potentials enable sugar transportation in the plant and could also explain why the higher CBZ concentrations displayed visual distress (necrosis) potentially caused from a sugar deficiency [17].

The results from this study indicate that uptake of CBZ by *C. pepo* causes toxic effects on growth and fruit development. This study demonstrates that long-term exposure to APIs such as CBZ results in significant effects on fruit maturation although it should be noted that the concentrations at which these effects were seen were elevated in comparison to typical API residue concentrations measured in soils. The aim here was to find the toxicity threshold in the soil – plant system against which environmental concentrations can be compared in risk assessment. It is evident that when CBZ uptake occurs, the pharmaceutical is translocated into the fruiting body. Chlorophyll content is affected by CBZ in mature leaves in *C. pepo* and this was also seen as chlorosis and necrosis visually on the leaves. The EC_x values calculated in this study have the potential to be used for risk assessment for soils contaminated with API residues. However, more research using a variety of plant species, soil types and APIs is needed to provide a comprehensive overview for risk assessment purposes. It is important to establish whether these effects are seen in other crop species given the increasing use of reclaimed wastewater irrigation for market gardening purposes as incomplete crop development may result in unknown agricultural challenges. Future research in this field could include multi-generational studies on plant species when grown in the presence of pharmaceutically contaminated soil or irrigation.

Future endpoints could include the viability of the reproductive portions of the plant, for example pollen viability.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—Data can be accessed upon request of primary author, Emma Knight via email; emma.knight@adelaide.edu.au.

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Figure 1. Average uptake of CBZ into *C.pepo* leaves (A) and roots (B) after plants were grown from seed in CBZ-spiked soil for 14 weeks. Error bars represent standard error (for roots, young and old leaves, n = 3, except for the 1 mg/kg treatment where n = 2 and the 20 mg/kg treatment where n = 1).

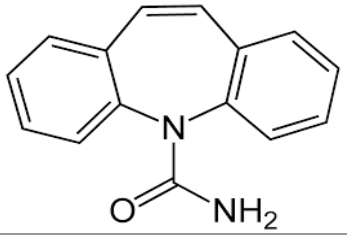
Figure 2. The average percent biomass (dry weight) of *C. pepo* plants per treatment compared to the controls of total; old and young leaves; stem and root biomass. Error bars represent the standard error. (n = 3) with statistically significant ($p \leq 0.05$) treatments in comparison to the control denoted by a letter.

Figure 3. Chlorophyll (Chl) content in the old (A) and young (B) leaves of *C. pepo* at harvest (14 weeks) with increasing concentrations of CBZ spiked in the soil. The associated EC50 and EC10 values are shown on both Figures A and B along with horizontal lines indicating the 95 % confidence intervals for Figure A only. The 95% confidence intervals were unable to be obtained for Figure B.

Figure 4. Concentrations of micronutrients ((A) Cu, (B) Zn, B, Mn) and macronutrients ((C) Ca, (D) S, P, Mg, K) in young *C. pepo* leaves and N in old and young leaves (E) after 14 weeks in soil spiked with increasing CBZ concentrations. The error bars represent standard error, n = 3, except for the 1 mg/kg treatment where n = 2 and the 20 mg/kg treatment where n = 1 and therefore was the only treatment not included in statistical analysis. Significant differences ($p \leq 0.05$) for treatments compared to the control treatment and are denoted by a letter.

Figure 5. Average percentage of starch within *C. pepo* old and young leaves (dry weight) after 14 weeks of growth in CBZ contaminated soil. Error bars represent standard error n = 3, except for the 1 mg/kg treatment where n = 2 and the 20 mg/kg treatment where n = 1. Treatments denoted with different letters are significantly different ($p \leq 0.05$).

Table 1: Physio-chemical properties of carbamazepine (CBZ).

Property	**Carbamazepine (CBZ)**
Molecular structure	
Therapeutic Class	Antiepileptic
CAS no.	298-46-4[a]
Molar mass (g/mol)	236.27
Log K_{ow}	2.25[b]
Acid/ base	Neutral
pK_a	13.9[c]
Half-life in soil	462 – 533 days[d]

[a] CAS no. obtained from the Chemical Abstracts Service

[b] Log K_{ow} value obtained from KOWWIN v. 1.68 database, USEPA EPI suite 4.1 program.

[c] Jones, et al. [39]

[d] Walters, et al. [7]

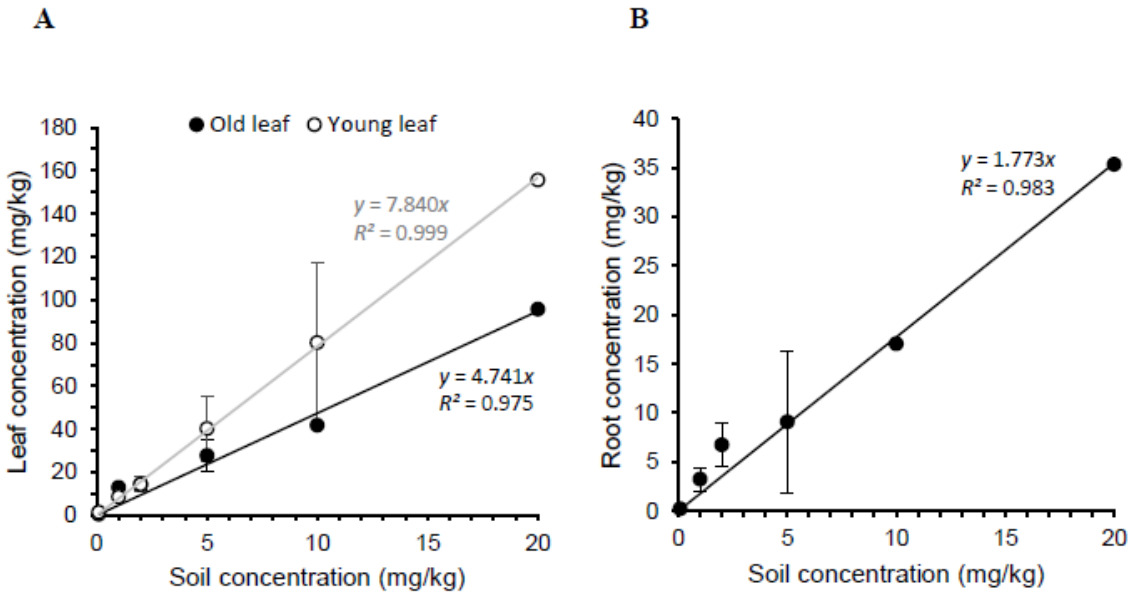


Figure 1.

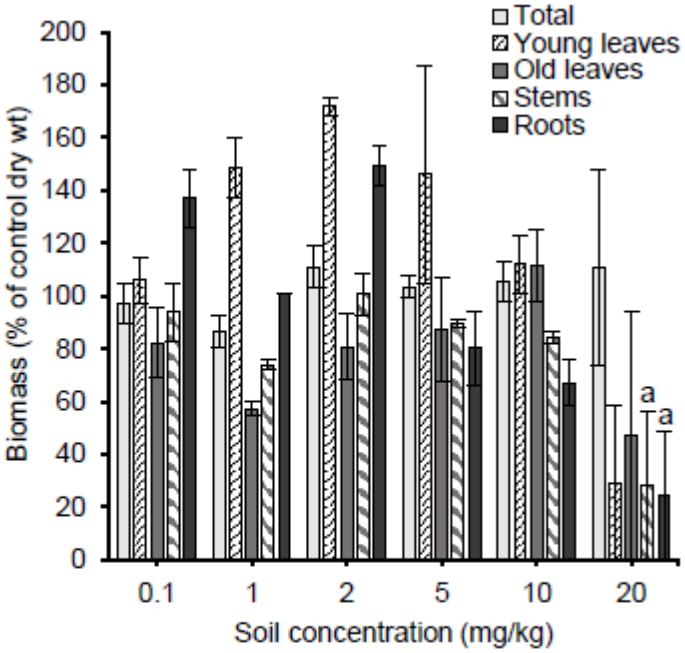


Figure 2.

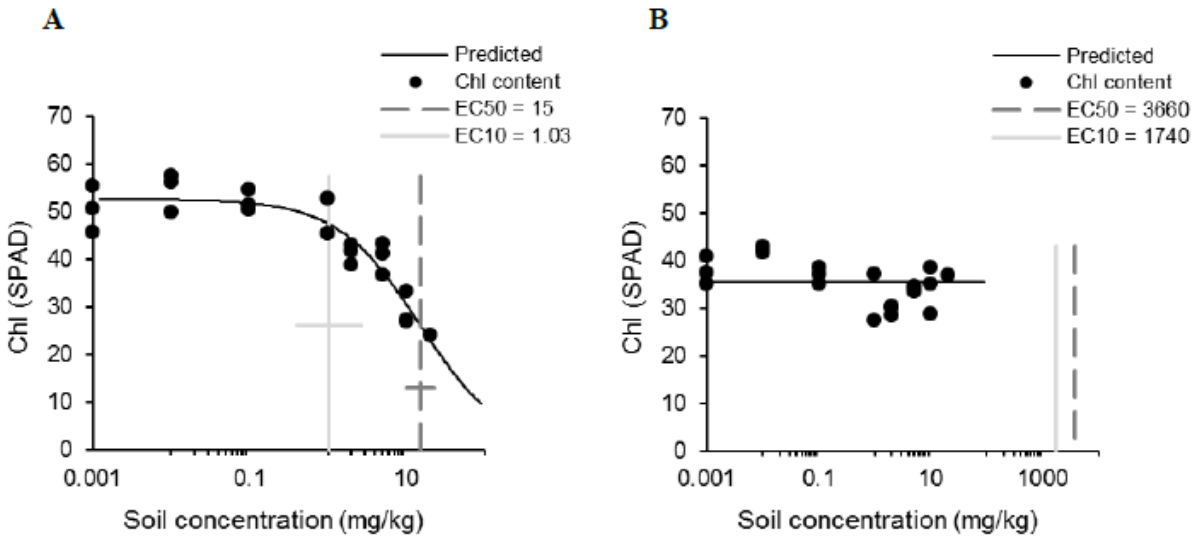


Figure 3.

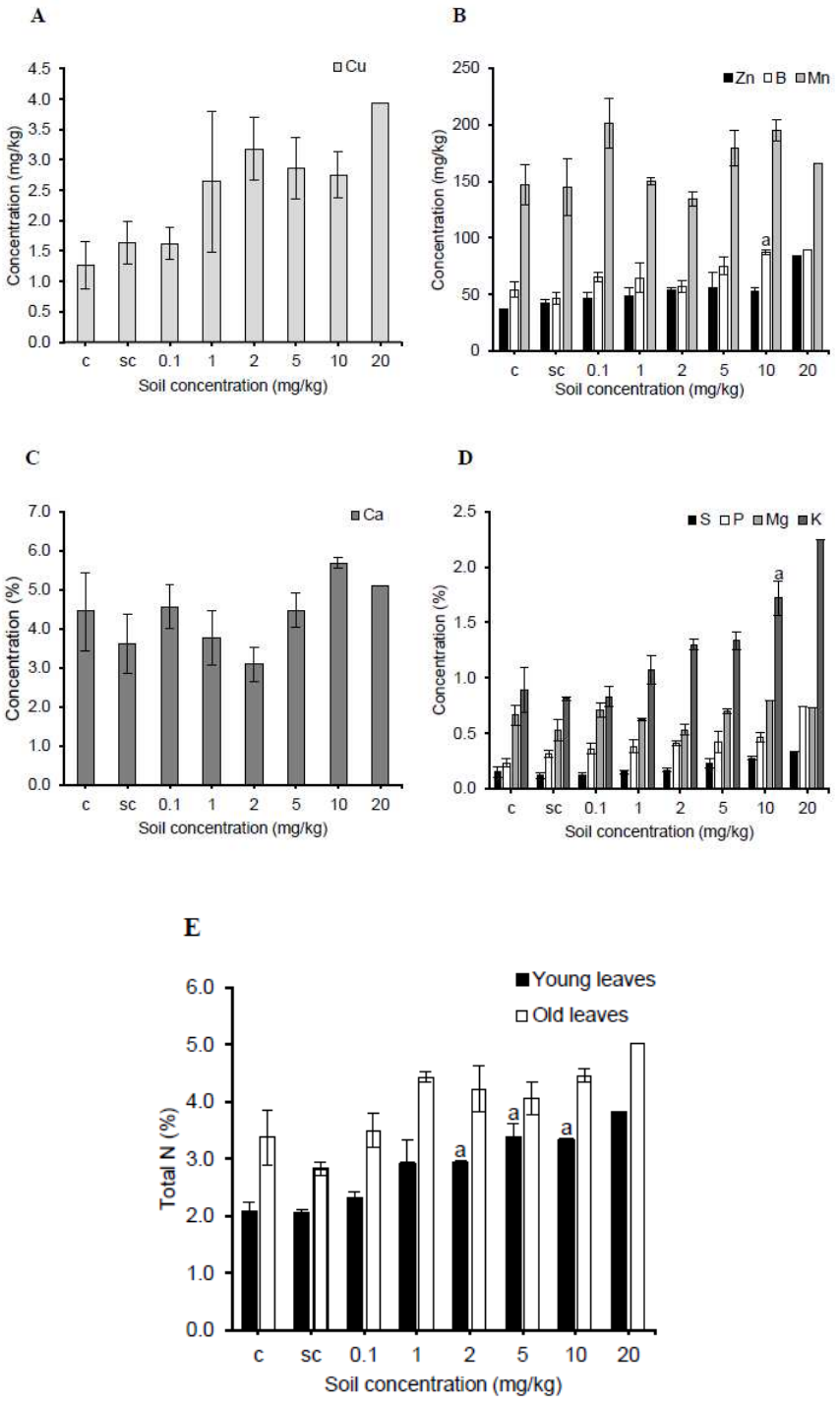


Figure 4.

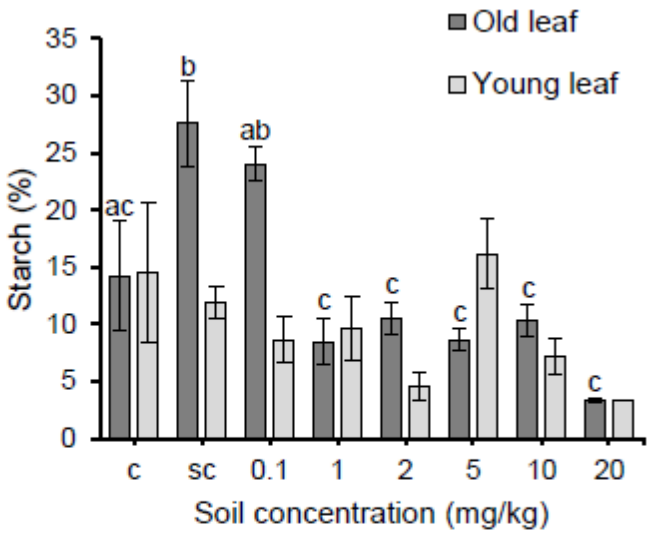


Figure 5.