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Citation for final published version:

Adeel, Muhammad, Yang, Y.S., Wang, Y.Y., Song, X.M., Ahmad, M.A. and Rogers, Hilary 2018. Uptake and transformation of steroid estrogens as emerging contaminants influence plant development. *Environmental Pollution* 243 (PartB), pp. 1487-1497. 10.1016/j.envpol.2018.09.016
file

Publishers page: <http://dx.doi.org/10.1016/j.envpol.2018.09.016>
<<http://dx.doi.org/10.1016/j.envpol.2018.09.016>>

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Accepted Manuscript

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PII: S0269-7491(18)32251-6

DOI: [10.1016/j.envpol.2018.09.016](https://doi.org/10.1016/j.envpol.2018.09.016)

Reference: ENPO 11558

To appear in: *Environmental Pollution*

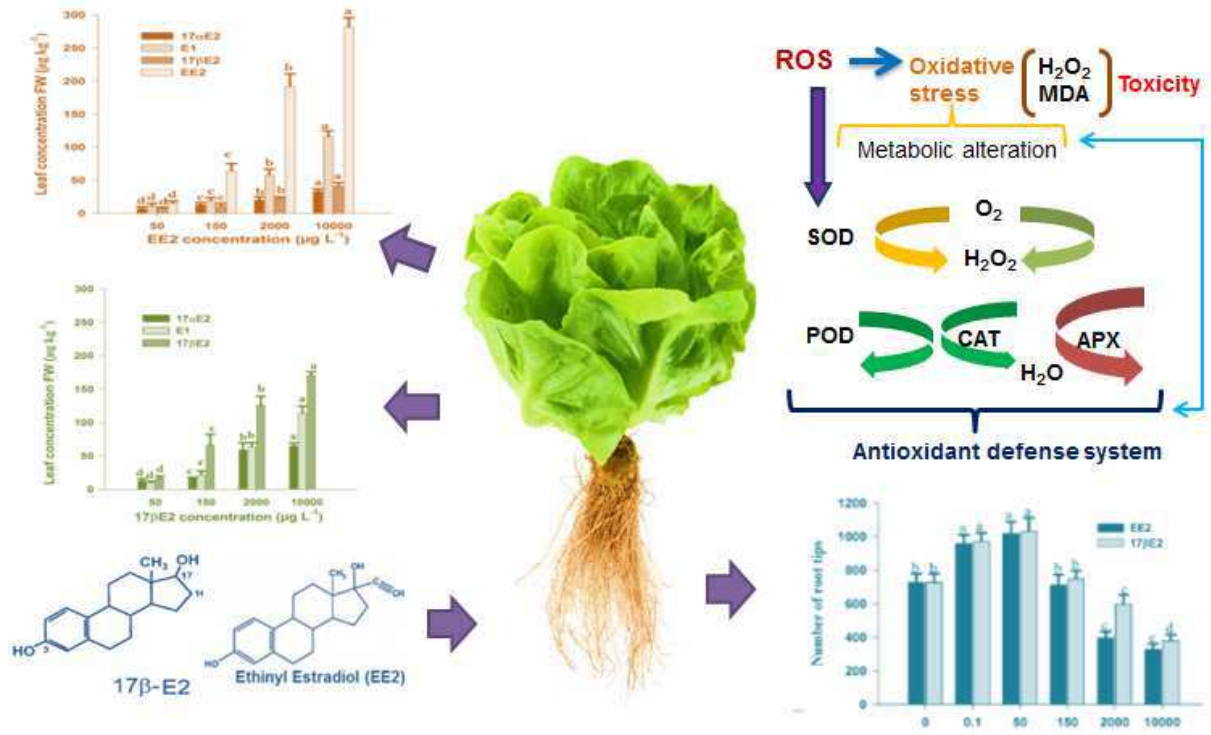
Received Date: 26 May 2018

Revised Date: 6 August 2018

Accepted Date: 3 September 2018

Please cite this article as: Adeel, M., Yang, Y.S., Wang, Y.Y., Song, X.M., Ahmad, M.A., Rogers, H., Uptake and transformation of steroid estrogens as emerging contaminants influence plant development, *Environmental Pollution* (2018), doi: <https://doi.org/10.1016/j.envpol.2018.09.016>.

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Uptake and transformation of steroid estrogens as emerging contaminants influence plant development

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Abstract

Steroid estrogens are emerging contaminants of concern due to their devastating effects on reproduction and development in animals and humans at very low concentrations. The increasing steroid estrogen in the environment all over the world contrasts very few studies for potential impacts on plant development as a result of estrogen uptake. This study evaluated the uptake, transformation and effects of estradiol (17 β -E2) and ethinyl estradiol (EE2) (0.1-1000 μ g/L) on lettuce. Uptake increased in leaves and roots in a dose-dependent manner, and roots were the major organ in which most of the estrogen was deposited. The transformation of estrogens to major metabolite and their further reverse biotransformation in lettuce tissue was identified. At low concentrations (0.1 and 50 μ g/L) estrogens resulted in enhanced photosynthetic pigments, root growth and shoot biomass. Application of higher concentrations of estrogens (10 mg L⁻¹) significantly reduced total root growth and development. This was accompanied by increased levels of hydrogen peroxide (H₂O₂), and malondialdehyde (MDA), and activities of antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX). Taken together, these findings suggest that at low concentrations estrogens may biostimulate growth and primary metabolism of lettuce, while at elevated levels they have adverse effects.

Capsule: EDC estrogens (17 β -E2 and EE2) stresses influence lettuce growth with a dose-dependent negative effect

Keywords: Estrogens; Plant uptake; Bioavailability; Antioxidant system; Biotransformation

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29 **Abbreviations:** E1, estrone; E2, estradiol; 17 β -E2, 17 β -estradiol; 17 α -E2, 17 α -estradiol; E3, estriol ; EE2, ethinyl
30 estradiol ; CAFOs, Concentrated animal feeding operations; WWTPs, waste water treatment plants; MSH,
31 mammalian sex hormones; CAT, catalase; POX, peroxidase; ROS, reactive oxygen species; SOD, superoxide
32 dismutase; GPX, guaiacol peroxidase; APX, ascorbate peroxidase; MDA, mono dehydro ascorbate; MSTFA, N-
33 Methyltrimethylsilyltrifluoroacetamide; TRL, total root length; RV, root volume; RD, average root diameter; RSA,
34 root surface area; RTs, number of root tips; SPE, solid phase extraction.

35 **Introduction**

36 A major challenge for the agricultural sector today is to produce more and safe food for a
37 growing global population. Meat and dairy products are parts of the livestock industry and the
38 use of synthetic steroid hormones as growth promoters (Bartelt-Hunt et al., 2012), increasing the
39 muscle mass (Biswas et al., 2013) are the mostly adopted practices in the developed countries.

40 The world human population of about 7 billion is estimated to discharge 30,000 kg/yr. of
41 natural estrogens (E1, E2, and E3) and an additional 700 kg/yr. of synthetic estrogens (EE2)
42 from contraceptive pill practice (Adeel et al., 2017). However, the possible input of estrogens to
43 the environment from livestock is much greater, where it is calculated in the U.S and European
44 Union alone, the annual estrogen excretion by livestock, at 83000 kg/yr., is more than double
45 that produced by the world human population. Indeed, possible relations have been made
46 between animal feeding operations and the detection of estrogens in the aquatic environment
47 (Shrestha et al., 2012). Naturally produced hormones excreted from animal and human waste
48 pose serious effect to the environment, since applying animal manure or sludge bio-solids onto
49 agricultural land as alternative fertilizers to organic products is a widely adopted practice in
50 modern agriculture (Xuan et al., 2008).

51 Studies have documented the occurrence in reclaimed water of many classes of organic
52 pollutants, including steroid estrogens. In addition to wastewater or effluent from WWTP,
53 treated sewage sludge is also widely used all over the world in agriculture and for the latter, land
54 application is the most adopted practice of disposal (Calderón-Preciado et al., 2012; Zhou et al.,
55 2012; Calderón-Preciado et al., 2013; Gabet-Giraud et al., 2014). Previous studies indicate that
56 steroid estrogens can be taken up, accumulated in, or metabolized in beans, aquatic macrophytes,
57 and algae (Lai et al., 2002; Imai et al., 2007; Shi et al., 2010; Card et al., 2012). For example,
58 some steroid estrogens derived from animal excrement and reclaimed water were taken up in
59 terrestrial plants including leafy vegetables and fruits (Karnjanapiboonwong et al., 2011; Zheng
60 et al., 2014). Thus, land application of reclaimed water and animal manure can result in these

61 emerging pollutants entering terrestrial food chains. The bioavailable concentrations of estrogens
62 in soil also affect their ability to be taken up by plants. This concentration is difficult to measure,
63 so it tends to be estimated (Dodgen, 2014). Recently, our study found 69 ng L⁻¹ and 74 ng g⁻¹
64 17 β -E2 in groundwater and soil respectively (Song et al., 2018).

65 Steroid hormones are essential factors responsible for the regulation of normal
66 development in both the plant and animal kingdom. These compounds participate in many
67 physiological processes such as development and reproductive processes as well as protein,
68 sugar, and mineral management. Plants and animals produce hundreds of types of steroid
69 estrogenic compounds (Janeczko et al., 2012; Sherafatmandjour et al., 2013). Steroid estrogens
70 E1, E2 and E3 lie on interconnecting metabolic pathways. In aerobic conditions reverse
71 transformation of E2 to E1 occurs under microbes and latter can be degraded to E3. Similarly,
72 synthetic EE2 can be converted to E1 by *Sphingobacterium sp.* (Adeel et al., 2017). Treatment of
73 plants with steroid estrogens affects root and shoot growth (Hewitt and Hillman, 1980; Guan and
74 Roddick, 1988b), pollination in flowers (Ylstra et al., 1995) and seed germination (Janeczko and
75 Skoczowski, 2011). Interestingly, at the biochemical level, mammalian sex hormones (MSH)
76 significantly improve the inorganic contents of barley, maize, chickpea and beans seeds
77 (Dumlupinar et al., 2011; Erdal and Dumlupinar, 2011a; Erdal et al., 2012), and chlorophyll,
78 carotenoid, sugar, and protein in lentil seed, duckweed, soybean and fennel (Czerpak and
79 Szamrej, 2003b; Dumlupinar et al., 2011; Chaoui and El Ferjani, 2013; Sherafatmandjour et al.,
80 2013).

81 Steroidal estrogens found in sewage water inhibit vegetative growth of alfalfa plants
82 (Shore et al., 1992). At a concentration of 1 μ M, steroid estrogen reduced root growth and also
83 caused morphological abnormalities including epinasty in tomato plants (Guan and Roddick,
84 1988b). Hence it is important to evaluate their disruptive potential in various ecological
85 environments (Chaoui and El Ferjani, 2013).

86 To date, few studies have described the effects of these hormones as stresses to plants or
87 their uptake from irrigation water containing environmental-level emerging pollutants. Of
88 particular interest is their effect on the plant's antioxidant system, one of the chief phyto
89 mechanisms for dealing with environmental stress. MSH including estrogens enhanced
90 antioxidant enzymes, such as catalase (CAT) and peroxidase (POX) during germination of
91 chickpea, maize and wheat seeds and enhanced plant growth and development by affecting

92 biochemical parameters including components of the antioxidative system (Erdal and
93 Dumlupinar, 2011b). However, to our knowledge, the effects of steroid estrogens (E2, EE2) on
94 leafy vegetables such as lettuce have not been reported. Our work has addressed this specific
95 problem by analyzing the response of lettuce under stress of steroid estrogen (17 β -E2 and EE2).
96 Lettuce (*Lactuca sativa* L.) was chosen for the study because this crop is one of the most widely
97 cultivated salad crops world-wide (Trujillo-Reyes et al., 2014). The study was carried out to
98 investigate the effect of steroids i.e. estradiol and ethinyl estradiol on lettuce plant growth,
99 photosynthetic pigments, and the role of antioxidant activities in protecting the plants against
100 estrogen toxicity. Furthermore, we have investigated the uptake and transformation product
101 concentrations in the root and shoot tissues of lettuce.

102 **Materials and methods**

103 **2.1 Chemicals**

104 E1 ($\geq 99.5\%$), 17 α -E2 ($\geq 99\%$), 17 β -E2 ($\geq 98.4\%$), E3 ($\geq 98.8\%$), and EE2 ($\geq 98.2\%$) were
105 purchased from Sigma-Aldrich (USA). Methanol, ethyl acetate, n-hexane, acetonitrile and
106 acetone, purchased from Merck (Germany). N-Methy-N-(trimethylsilyl) trifluoroacetamide
107 (MSTFA, $\geq 98.5\%$), used as the derivatization reagent, was obtained from Sigma-Aldrich (USA);
108 pyridin ($\geq 99.5\%$) was purchased from Kermel (China). SPE cartridges containing Oasis HLB
109 cartridges (150 mg, 6 cc) were supplied by Waters (USA); for cleanup, CARB cartridges (500
110 mg, 6 ml) were purchased from WG Labs (China). The stock solutions of individual estrogens
111 were prepared by dissolving each compound in methanol at a concentration of 1000 mg L⁻¹ and
112 stored at -20 °C.

113 **2.2 Plant materials, growth conditions and treatments**

114 Lettuce seeds (*Lactuca sativa* cv., cream lettuce, Yu He vegetable breeding center,
115 China) were obtained from Shenyang Agriculture University and germinated in trays containing
116 sandy soil in control conditions. After 14 days of sowing, uniform seedlings measuring 4 cm in
117 height with two leaves were briefly rinsed in milliQ water and transferred to sterile amber 2000
118 mL glass jars (Supporting Information Fig. S1.2-3). Each jar was watered with 1/2-strength
119 Hoagland's nutrient solution (pH 5.5- 6.3, Supporting Information Table S1-1). Experiments
120 were performed in the controlled environmental conditions: 16 h light/8 h dark cycle, with
121 constant 50% relative air humidity, 21-25 °C temperature; illumination was provided by

122 fluorescent tubes. After one-week acclimation, steroid hormones, 17β -E2 or EE2 (Sigma-
 123 Aldrich, USA, dissolved in methanol) were added at a final concentration of 0, 0.1, 50, 150,
 124 2000 and 10,000 $\mu\text{g L}^{-1}$ to the nutrient medium in the glass jars. Five treatments, four
 125 replications and a blank control were included, each bottle containing two plants. 17β -E2, EE2
 126 solutions were prepared by dissolving them in methanol. The nutrient solutions were renewed
 127 once per week to avoid nutrient depletion and restrict bacterial growth. Plants were grown for a
 128 total of 21 days, a total growth time that corresponds to growth periods used commercially. At
 129 given time intervals, plants were destructively sampled. The growth of lettuce plants was
 130 investigated by evaluating the fresh weight (FW), number of leaves, leaf area and root length
 131 then leaves was stored at $-80\text{ }^{\circ}\text{C}$ for further analysis.

132 **2.3 Root morphometry**

133 Root scanning was carried out using an Epson Perfection V700 Photo, Dual Lens system
 134 (Regent Instruments Company, Canada) equipped with a water tray, into which the roots were
 135 placed, and a positioning system. The following root parameters were measured: total root length
 136 (TRL), root volume (RV), average root diameter (RD), root surface area (RSA) and number of
 137 root tips (RTs) with a root image analysis system using image analysis software WinRHIZO
 138 (version Pro 2007d, Regents Instruments, Quebec, Canada). The average root diameter was
 139 expressed as the total root width divided by the length of roots.

140 **2.4 Photosynthetic pigments**

141 The chlorophyll content was determined according to the method of Knudson et al. (1977).
 142 Fresh lettuce leaves (0.5 g) were extracted in 10 mL of 96 % ethanol for 24 h in the dark. The
 143 amounts of chlorophyll a, b and carotenoids were determined spectrophotometrically (U- 2910,
 144 Double Beam UV/VISspectro, 2JI-0013, Tokyo, Japan), by reading the absorbance at 665, 649
 145 and 470 nm. Chlorophyll content was expressed as mg g FW^{-1} . The amount of photosynthetic
 146 pigments was calculated by using the following formulae:

$$147 \quad C_a = (13.95A_{665} - 6.88 A_{649}) V/1000M$$

$$148 \quad C_b = (24.96A_{649} - 7.32A_{665}) V/1000M$$

$$149 \quad C_{\text{Total}} = C_a + C_b$$

$$150 \quad C_{x+c} = (4.08A_{470} - 11.56A_{649} + 3.29A_{665}) V/1000M$$

151 where C_a is chlorophyll a, C_b is chlorophyll b, C_{Total} total chlorophyll, C_{x+c} total carotenoids, V
152 volume of extraction (ethanol), and M mass of fresh leaf.

153 **2.5 Determination of antioxidative and oxidative enzyme activity**

154 All the biochemical analyses were carried out using fresh leaf samples. Activities of
155 enzymatic antioxidants were assessed using commercial kits in accordance with the
156 manufacturer's instructions. Kits for analysis of superoxide dismutase (SOD) (A001-1),
157 peroxidase (POD) (A084-3), catalase (CAT) (A007-1), malondialdehyde (MDA) (A003-1),
158 ascorbate peroxidase (APX) (50/48), protein (A045-3-2) and H_2O_2 (A064-1) were obtained from
159 the Nanjing Jiancheng Bioengineering Institute, China (www.njjcbio.com). The absorbance
160 readings of SOD, POD, CAT, APX, MDA and protein were detected at 550, 420, 405, 290, 532,
161 and 562 nm respectively (U- 2910 Hitachi, Tokyo, Japan). The SOD, POD and CAT activities
162 were expressed as unit mg^{-1} protein.

163 **2.6 Sample preparation for estrogen testing**

164 *2.6.1 Preparation of Plant samples*

165 After harvesting, all plants were rinsed under a stream of deionized water for 5 min, left
166 to drain, and then blotted dry. The lettuce plants were separated into roots and leaves and stored
167 at $-80^\circ C$ until used for extraction. The extraction and clean-up procedure were modified from
168 (Karnjanapiboonwong et al., 2011; Zheng et al., 2014). Briefly, control plant samples (2.5 g)
169 were weighed into centrifuge tubes spiked with $500 \mu g L^{-1}$ of each hormone standard. After 24 h,
170 5 mL of 1:1 (v/v) acetonitrile: water was added to samples for extraction. Plant samples were
171 sonicated for 30 min, shaken for 30 min, and then centrifuged (Huanan Herexei instrument &
172 Equipment Co., Ltd) at 10,000 RPM for 15 min. The supernatant was filtered through a GF/F
173 filter ($0.22 \mu m$) and transferred to amber glass bottles. The solid phase of the samples was further
174 extracted three more times by adding 5 mL of extraction solvent followed by sonicating, shaking,
175 and centrifuging. The aqueous layer was filtered into the same amber glass bottle. The mixed
176 supernatant was evaporated to 1 mL under a gentle stream of nitrogen, and diluted with 10 mL of
177 ultrapure water. The solid phase extraction (SPE) procedures were modified as previously
178 described (Zhang et al., 2015). The analytes were further cleaned-up by Oasis HLB cartridges
179 (see Supporting Information). The extracts were then evaporated under a gentle nitrogen flow
180 until 2 ml was left. For chlorophyll removal, samples were extracted through CARB cartridges

181 (Weifang Pufen Instrument Co., LTD). CARB cartridges were conditioned with 10 ml n-
182 hexane:acetone (1:1) and eluted by very low vacuum. For estrogen recoveries in plant tissue see
183 Supporting Information.

184 2.6.2 Derivatization

185 The eluted fractions from SPE were evaporated with nitrogen until near to dryness then
186 the residues were transfer to a 1.5 mL reaction vial and further dried under a gentle stream of
187 nitrogen. Derivatisation was performed by addition of 50 μL of pyridin and 100 μL MSTFA. The
188 vial was capped and vortexed for 30 s and heated in an oven for 20 min at 40°C. The derivatives
189 were cooled to room temperature and subjected to GC-MS analysis.

190 2.6.3 GC-MS analysis

191 The GC-MS system (Thermo Electron Corporation, USA) consisted of a gas
192 chromatograph (TRACE GC Ultra), a quadrupole mass spectrometer (PolarisQ), an auto sampler
193 (AI/AS 3000), and a TR5-MS quartz capillary column (30m \times 0.25 mm, 0.25 μm). High purity
194 helium gas (99.999%) was used as carrier gas at a constant flow rate of 1.0 mL min⁻¹. Samples (1
195 μL) were injected into the GC splitlessly for 0.75 min. The GC oven temperature was
196 programmed as follows: starting from 50 °C and equilibrated for 2 min, then ramped to 260 °C at
197 12 °C min⁻¹ and equilibrated for 8 min, then further ramped to 280°C at 3 °C min⁻¹ and
198 maintained at this temperature for 5 min. For MS detection, the electron impact (EI) ionization
199 was adopted, and electron impact energy was 70 eV. The inlet and MS transfer line temperatures
200 were maintained at 280 °C, and the ion source temperature was 250 °C. The solvent delay time
201 was 15.0 min. The MS was operated in total ion chromatogram (TIC) mode for qualitative
202 analysis from m/z 50 to 600 and selected ion monitoring (SIM) mode for quantitative analysis.
203 The TIC chromatograms of derivatized estrogens and internal standards by full scan and selected
204 ion monitoring are shown in the Supporting Information.

205 2.7 Statistical Analyses

206 Data were analyzed statistically using one way analysis of variance (ANOVA) and Fisher's
207 least significant test (LSD) using Statistix 8.1 software (Analytical Software, Tallahassee, FL,
208 USA) and different letters show significant differences amongst treatments at $P < 0.05$. All data
209 represented are means \pm standard deviations (SD) of four replicates for each treatment.

210 3. Results

211 3.1 Hormone uptake and biotransformation in plant

212 The uptake of two estrogens by lettuce plants was investigated in hydroponic culture, to
213 test for toxicity, transformation and distribution among the plant parts (Fig. 1). Uptake and
214 accumulation of both of the two estrogens, 17 β -E2 and EE2 arise in roots and leaves in a dose-
215 dependent manner (see Figs. 1 C-D). No estrogen was detected in control plants. The uptake of
216 17 β -E2 in lettuce root was slightly higher than EE2 while biotransformation of both hormones
217 was detected in roots. At low treatment concentrations 17 β -E2 was transformed into E1 and at
218 higher concentration treatments (2000 and 10000 $\mu\text{g L}^{-1}$) into E1 and 17 α E2 (Fig. 1 C),
219 although concentrations of E1 recovered from 17 β -E2 were higher than those of EE2 treatments.
220 Interestingly, estrogen EE2 was transformed into E1, 17 β -E2 and 17 α -E2 in roots. At a low
221 treatment concentration (0.1 $\mu\text{g L}^{-1}$) EE2 was transformed into E1 with a concentration of 6.45
222 $\mu\text{g kg}^{-1}$.

223 In leaves EE2 concentration was higher than 17 β -E2 (Fig. 1 B). However, transformation
224 of EE2 was low as compare to 17 β -E2 treatments for leaves. The uptake of both estrogens at 0.1
225 $\mu\text{g L}^{-1}$ treatments was not detected in leaves.

226 3.2 Negative dose-effect of steroid estrogens on growth and biomass

227 Both hormone treatments exerted a dose-dependent negative effect on both roots and
228 leaves although there was no significant difference in effect between the two hormones tested
229 (see supporting information Fig. S1-2).

230 3.2.1 Leaf number, area and fresh weight

231 Treatments of 50-10000 $\mu\text{g L}^{-1}$ of EE2 or 17 β -E2 significantly inhibited the number of
232 leaves formed and both the leaf area and leaf fresh weight in 21 day old plants compared with the
233 controls ($P < 0.005$). At 10000 $\mu\text{g L}^{-1}$ this resulted in a 53-77% decrease in leaf number a 60-
234 66% decrease in leaf area and 80-85% decrease in leaf fresh weight with both hormones (Figs. 2
235 A, B). The effect was less severe at lower concentrations but the 50 $\mu\text{g L}^{-1}$ treatments still
236 exerted a significant negative effect with an approximately 33% decrease in leaf number , a 28-
237 34% decrease in leaf area and a 23% decrease in fresh weight with both hormones compared

238 with the controls (Figs. 2 A-C). However, the $0.1 \mu\text{g L}^{-1}$ treatments did not have any significant
239 effect on leaf number ($P < 0.005$).

240 3.2.2 Root fresh weights

241 Data for changes in root FW in response to the hormone treatments closely paralleled
242 those for the leaf characters with approximate 85% reductions in FW in the $10000 \mu\text{g L}^{-1}$
243 treatments compared with the controls ($P < 0.005$). As with the data on leaves, root FW was
244 unaffected by the $0.1 \mu\text{g L}^{-1}$ treatments (Fig. 2D)

245 3.3 Change of photo synthetic pigment in response to steroid estrogens

246 3.3.1 Total Chlorophyll, Chl a and Chl b

247 Treatments with 2000 and $10000 \mu\text{g L}^{-1}$ EE2 caused a significant decline of (55%, 40%
248 and 71%) and (62%, 47% and 78%) in the levels of total chlorophyll, Chl a and Chl b
249 respectively compared with the control. (Figs. 3 A and 4B). Treatments of either hormone up to
250 $50 \mu\text{g L}^{-1}$ had little effect on Chl a, but both total chlorophyll and Chl b were significantly
251 reduced in response to treatment with $50 \mu\text{g L}^{-1}$ EE2, but not $17\beta\text{-E2}$. At $150 \mu\text{g L}^{-1}$ the effect
252 was significantly greater on Chl b than Chl a. The two hormones had very similar effects on Chl
253 a, however effects of EE2 on Chl b were significantly greater than $17\beta\text{-E2}$ at all concentrations $>$
254 $0.1 \mu\text{g L}^{-1}$ and also affected total chlorophyll more severely at the highest two concentrations
255 tested.

256 3.3.2 Carotenoids

257 Treatments with the two hormones appeared to affect carotenoid content less than
258 Chlorophyll content, and only at the highest concentration tested was there a significant
259 reduction compared to the controls. There were no significant differences in the effect of EE2 or
260 $17\beta\text{-E2}$ on carotenoid content (Figs. 3, 4D).

261 3.4 Influence of steroid estrogens on root morphology

262 3.4.1 Total and primary root lengths

263 Total root length defines the all primary, secondary, tertiary roots and root length is the
264 length of primary main root. The effect of the two hormone treatments on total root length (Figs.
265 4A and 4B) was very similar to that for root fresh weight (Fig. 2D) with a significantly negative
266 effect only at hormone concentrations of $\geq 50 \mu\text{g L}^{-1}$, and similar effects between the two

267 hormones. The effect on primary root length was more gradual than that on total root length with
268 significant reductions at 150 and then again at 2000 $\mu\text{g L}^{-1}$ (Figs. 4A and 4B).

269 3.4.2 Average root diameter and root tip number

270 The effect of the hormone treatments on these two root parameters was different to all the
271 effects on leaves and other effects on roots in that there was a stimulatory effect of the lowest
272 concentration tested (0.1 $\mu\text{g L}^{-1}$). Average diameter then fell back to control levels at 50 $\mu\text{g L}^{-1}$.
273 In contrast, root tip number remained greater than the control also when plants were treated with
274 50 $\mu\text{g L}^{-1}$ falling back to control levels at 150 $\mu\text{g L}^{-1}$. At the highest two concentrations tested,
275 both root diameter and root tip number was reduced compared to the control. (Fig. 4C and 4D).

276 3.4.3 Root volume and surface area

277 Root volume and surface area were affected by the hormone treatment in a similar way to
278 root number. There was a gradual reduction in both parameters with increasing hormone
279 concentration and a severe reduction at the highest two concentrations tested (Fig. 4E and 4F).
280 Again the effects of the two hormones were comparable at each concentration.

281 3.5 Estrogen upregulates antioxidant enzymes.

282 Activities of four antioxidant enzymes increased in response to the hormone treatments, in a
283 dose-dependent manner.

284 3.5.1 SOD and POD activities

285 Both the SOD and POD activities increased significantly between 0.1 and 50 $\mu\text{g L}^{-1}$
286 treatments of both hormones with approximately 2-fold increases in both enzyme activities.
287 Thereafter was an approximately linear dose response to increasing hormone concentration.
288 There was no significant difference in the response to the two hormones for either enzyme (Figs.
289 5A, 5B). At the highest concentration of hormone tested the induction of both enzymes was
290 approximately 3.5 fold.

291 3.5.2 CAT and APX activities

292 Unlike SOD and POD, CAT activity increased significantly with a 0.1 $\mu\text{g L}^{-1}$ treatment
293 of both hormones with a significantly greater response to 17 β -E2. However at higher treatment
294 concentrations the response was reversed and was greater with EE2, although this difference was

295 only significant at $50 \mu\text{g L}^{-1}$. At the highest concentration of hormone treatment activity was
296 stimulated by approximately 7-fold compared with the controls. (Fig. 5C).

297 The pattern of APX activity differed from the other enzymes tested in that induction of
298 activity increase significantly at $>150 \mu\text{g L}^{-1}$ of both hormones. Again there was no significant
299 difference in the induction of activity increase by the two hormones although, as seen with CAT
300 activity, EE2 appeared to induce the enzyme a little more than $17\beta\text{-E2}$. At the highest
301 concentration tested the induction was 6-fold (Fig. 5 D)

302 **3.6 Steroid estrogen treatment induced oxidative damage**

303 Both lipid peroxidation and accumulation of ROS in the leaves of lettuce plants under
304 steroid estrogen stress increased with the dose of hormone (Fig. 6 A and B). Both markers for
305 oxidative stress increase significantly at treatments of $50 \mu\text{g L}^{-1}$ compared to the control. Both
306 markers also increased up to the highest concentration of hormone tested and concentration was
307 approximately a 3-fold stimulation. Interestingly there was a small decrease in the H_2O_2
308 concentration at $0.1 \mu\text{g L}^{-1}$ compared to the control. There was no significant difference in effect
309 between the two hormones tested.

310 **4 Discussion**

311 **4.1 Uptake and biotransformation of steroid estrogens in lettuce plants**

312 Results clearly showed that both estrogens used to treat the plants were taken up in
313 lettuce roots and transported to leaves. Moreover, their uptake increased with treatment
314 concentration. These observations are consistent with previous data on both hormones in soil and
315 hydroponic media (Karnjanapiboonwong et al., 2011;Card et al., 2012).

316 Biotransformation products of both estrogens were observed in both lettuce roots and
317 leaves. Natural estrogen $17\beta\text{-E2}$ was transformed, into its metabolite (E1), and a greater
318 concentration of E1 was found in roots as compared in leaves. This is in agreement with previous
319 studies that reported that natural and synthetic estrogen was bio transformed by poplar and maize
320 plants in solution cultures (Card et al., 2013;Bircher et al., 2015). However, EE2 transformation
321 to E1 was also detected, unlike in poplar root tissue. Biotransformation was observed in roots
322 and leaves. However, these data do not explain which mechanism lettuce used to bio-transform
323 the estrogens. It has been hypothesized that some plant organs may perform oxidation and
324 reduction transformation (Card et al., 2013). This will need further investigation.

325 **4.2 Estrogens concentrations have effects on plant biomass**

326 To the best of our knowledge, this is the first time that an effect on lettuce growth, root
327 morphology, ROI-production and the antioxidant defense system has been shown to occur as a
328 consequence of uptake of the synthetic estrogen hormone, EE2 and natural estrogen 17β -E2. We
329 show here that application of 17β -E2 and EE2 concentrations (0.1 and $50 \mu\text{g L}^{-1}$) has a positive
330 impact on the root growth. Similarly, studies reported that 17β -E2 had induced the growth at low
331 concentration and detrimental effects at high concentration on *Medicago sativa* and *Arabidopsis*
332 *thaliana* (Shore et al., 1992; Upadhyay and Maier, 2016b). The positive effect at low
333 concentration may be caused by hormesis. Previous studies, proposed that low concentrations of
334 toxic pollutants induce hermetic effects through activating defense mechanisms. However,
335 further studies are needed to understand the mechanism of estrogen in plant physiology (Vargas-
336 Hernandez et al., 2017).

337 Moreover, the present study indicates that EE2 is slightly more toxic to lettuce plants
338 than 17β -E2 at elevated level.

339 **4.3 Effects of estrogens on root morphology**

340 Excessive estrogens can have negative effects on root architecture, which affects plants'
341 capacity to absorb water and minerals (Adeel et al., 2017). We observed a significant effect of
342 elevated level of estrogen on the root morphology of lettuce plants (Fig. 5.4). However
343 interestingly, at the $0.1 \mu\text{g L}^{-1}$ treatment improve the root length, which is in agreement with
344 results obtained with other plant species such as *A. thaliana* (Upadhyay and Maier, 2016b), and
345 chickpea (Erdal and Dumlupinar, 2011b).

346 However, at doses higher than $50 \mu\text{g L}^{-1}$, there was an inhibitory effect on root
347 morphology. This is in agreement with a significant reduction in root length in response to
348 estrogen exposure at $2704 \mu\text{g L}^{-1}$ in *Phaseolus aureus L.* and *A. thaliana* (Guan and Roddick,
349 1988a; Upadhyay and Maier, 2016b).

350 **4.4 Effects on chlorophyll**

351 Previous studies have shown that the photosynthetic performance of a plant under
352 stressful conditions may reflect plants adaptability (Gururani et al., 2015) In general, the Chl a,
353 Chl b, total chlorophyll and total carotene contents decreased with increasing estrogen levels.
354 Chlorophyll b is more sensitive to 2 and 10mg L^{-1} treatments. However, total carotene was only

355 affected by a high treatment with estrogens. These findings are in agreement with previous
356 results that have shown a reduction of chlorophyll content in *A. thaliana* at 2704 $\mu\text{g L}^{-1}$ and
357 stimulation of carotenoids in *Wolffia arrhiza* (*Lemnaceae*) (at 10^{-6} M) in response to 17 β -E2
358 exposure (Czerpak and Szamrej, 2003a; Upadhyay and Maier, 2016b). Similar findings of a
359 decline in photosynthesis with synthetic estrogen (EE2) contamination were reported in green
360 alga *Chlamydomonas reinhardtii* and *Dunaliella salina* at 1893 $\mu\text{g L}^{-1}$ and 100 ng L^{-1} (Pocock
361 and Falk, 2014; Belhaj et al., 2017).

362 4.5 Relationship with detoxifying enzyme activity

363 A variety of environmental stresses cause an increase in H_2O_2 and MDA production
364 leading to progressive oxidative injury and ultimately, cell death (Adeel et al., 2017).
365 Accordingly in the present study, exogenous estrogens at elevated level triggered the production
366 of H_2O_2 and MDA in lettuce plants. The increase in MDA might be due to membrane damage
367 caused by ROS-induced oxidative damage. Similar results were found in *A. thaliana* when
368 treated with 2704 $\mu\text{g L}^{-1}$ 17 β -E2 (Upadhyay and Maier, 2016a). However, in our study, there
369 was a slight decrease in H_2O_2 levels at 0.1 $\mu\text{g L}^{-1}$ of both estrogens in lettuce plants. These
370 results are in agreement with previous studies that showed a reduction of MDA and H_2O_2
371 contents in chick pea plants (Erdal and Dumlupinar, 2011b), and in germinating bean seeds at
372 2.7×10^{-7} $\mu\text{g L}^{-1}$ (Erdal, 2009). Moreover, Genisel et al., (2015) reported that 17 β -E2 suppressed
373 oxidative damage in wheat seedling at 2704 $\mu\text{g L}^{-1}$. The discrepancy with previous studies could
374 result from differences in plant species. It is also possible that lettuce plants have different
375 protective mechanism to combat the stress imposed by steroid estrogens.

376 4.6 Effect of steroid estrogens on the antioxidant defense system

377 Comparatively lower activities of SOD, POD, CAT and APX in lettuce plants were
378 concomitant with the less H_2O_2 generation at 0.1 treatments. Similar results were obtained in
379 different plant species, under estrogen low treatments (Erdal and Dumlupinar, 2011b; Chaoui and
380 El Ferjani, 2014). Furthermore, at higher concentrations significantly enhanced these enzymes
381 activities correlating with increased H_2O_2 concentration at these estrogens treatments. However,
382 Genisel et al., (2015) reported that 17 β -E2 improved the antioxidant enzyme activity in wheat
383 seedlings at 2704 $\mu\text{g L}^{-1}$.

384 5. Conclusions

385 Uptake of steroid hormones increased in leaves and roots in a dose-dependent manner,
386 and roots were the major organ in which most of the estrogen was deposited. At low
387 concentrations estrogens may biostimulate growth and primary metabolism of lettuce, while at
388 elevated levels they have adverse effects. This is some of the first research to demonstrate that
389 the exposure of estrogens to lettuce is likely to cause impacts on plant development with
390 unknown implications. Our findings suggest that overhead application of estrogens containing
391 wastewater and animal manure could cause the negative physiological impact on plants. Further
392 studies using soil culture media are required for better understanding of the uptake and
393 biotransformation of estrogens.

394 **Acknowledgement**

395 This work was financially supported by the National Natural Science Foundation of China
396 (Grants 41472237) and Liaoning Innovation Team Project (no. LT2015017).

397 **Conflict of Interest:** The authors declare no conflict of interest.

398 **References**

- 399 Adeel, M., Song, X., Wang, Y., Francis, D., and Yang, Y. (2017). Environmental impact of
400 estrogens on human, animal and plant life: A critical review. *Environment International*
401 99, 107-119,
- 402 Bartelt-Hunt, S.L., Snow, D.D., Kranz, W.L., Mader, T.L., Shapiro, C.A., Donk, S.J.V., Shelton,
403 D.P., Tarkalson, D.D., and Zhang, T.C. (2012). Effect of growth promotants on the
404 occurrence of endogenous and synthetic steroid hormones on feedlot soils and in runoff
405 from beef cattle feeding operations. *Environmental science & technology* 46, 1352-1360,
- 406 Belhaj, D., Athmouni, K., Frikha, D., Kallel, M., El Feki, A., Maalej, S., Zhou, J.L., and Ayadi,
407 H. (2017). Biochemical and physiological responses of halophilic nanophytoplankton
408 (*Dunaliella salina*) from exposure to xeno-estrogen 17 α -ethinylestradiol. *Environmental*
409 *Science and Pollution Research*, 1-11,
- 410 Bircher, S., Card, M.L., Zhai, G., Chin, Y.P., and Schnoor, J.L. (2015). Sorption, uptake, and
411 biotransformation of 17 β -estradiol, 17 α -ethinylestradiol, zeranol, and trenbolone acetate
412 by hybrid poplar. *Environmental toxicology and chemistry* 34, 2906-2913,
- 413 Biswas, S., Shapiro, C., Kranz, W., Mader, T., Shelton, D., Snow, D., Bartelt-Hunt, S.,
414 Tarkalson, D., Van Donk, S., and Zhang, T. (2013). Current knowledge on the
415 environmental fate, potential impact, and management of growth-promoting steroids used
416 in the US beef cattle industry. *Journal of Soil and Water Conservation* 68, 325-336,
- 417 Calderón-Preciado, D., Matamoros, V., Savé, R., Muñoz, P., Biel, C., and Bayona, J. (2013).
418 Uptake of microcontaminants by crops irrigated with reclaimed water and groundwater
419 under real field greenhouse conditions. *Environmental Science and Pollution Research*
420 20, 3629-3638,

- 421 Calderón-Preciado, D., Renault, Q., Matamoros, V., CañAmeras, N.R., and Bayona, J.M.
422 (2012). Uptake of organic emergent contaminants in spath and lettuce: an in vitro
423 experiment. *Journal of agricultural and food chemistry* 60, 2000-2007,
- 424 Card, M.L., Schnoor, J.L., and Chin, Y.-P. (2012). Uptake of natural and synthetic estrogens by
425 maize seedlings. *Journal of agricultural and food chemistry* 60, 8264-8271,
- 426 Card, M.L., Schnoor, J.L., and Chin, Y.-P. (2013). Transformation of natural and synthetic
427 estrogens by maize seedlings. *Environmental science & technology* 47, 5101-5108,
- 428 Chaoui, A., and El Ferjani, E. (2013). β -Estradiol Protects Embryo Growth from Heavy-Metal
429 Toxicity in Germinating Lentil Seeds. *Journal of plant growth regulation* 32, 636-645,
- 430 Chaoui, A., and El Ferjani, E. (2014). Heavy metal-induced oxidative damage is reduced by β -
431 estradiol application in lentil seedlings. *Plant growth regulation* 74, 1-9,
- 432 Czerpak, R., and Szamrej, I. (2003a). The Effect of β -estradiol and Corticosteroids on
433 Chlorophylls and Carotenoids Content in *Wolffia arrhiza* (L.) Wimm.(Lemnaceae.)
434 Growing in Municipal Bialystok Tap Water. *Polish Journal of Environmental Studies* 12,
435 677-684,
- 436 Czerpak, R., and Szamrej, I. (2003b). The Effect of β -estradiol and Corticosteroids on
437 Chlorophylls and Carotenoids Content in *Wolffia arrhiza* (L.) Wimm.(Lemnaceae.)
438 Wimm.(.) Growing in Municipal Bialystok Tap Water. *Polish Journal of Environmental*
439 *Studies* 12, 677-684,
- 440 Dodgen, L.K. (2014). *Behavior and Fate of PPCP/EDCs in Soil-Plant Systems*. UNIVERSITY
441 OF CALIFORNIA RIVERSIDE.
- 442 Dumlupinar, R., Genisel, M., Erdal, S., Korkut, T., Taspinar, M.S., and Taskin, M. (2011).
443 Effects of Progesterone, β -Estradiol, and Androsterone on the Changes of Inorganic
444 Element Content in Barley Leaves. *Biological trace element research* 143, 1740-1745,
- 445 Erdal, S. (2009). Effects of mammalian sex hormones on antioxidant enzyme activities, H₂O₂
446 content and lipid peroxidation in germinating bean seeds. *Journal of the Faculty of*
447 *Agriculture* 40,
- 448 Erdal, S., and Dumlupinar, R. (2011a). Exogenously treated mammalian sex hormones affect
449 inorganic constituents of plants. *Biological trace element research* 143, 500-506,
- 450 Erdal, S., and Dumlupinar, R. (2011b). Mammalian sex hormones stimulate antioxidant system
451 and enhance growth of chickpea plants. *Acta Physiologiae Plantarum* 33, 1011-1017,
- 452 Erdal, S., Genisel, M., Turk, H., and Gorcek, Z. (2012). Effects of progesterone application on
453 antioxidant enzyme activities and K⁺/Na⁺ ratio in bean seeds exposed to salt stress.
454 *Toxicology and industrial health*, 0748233711430975,
- 455 Gabet-Giraud, V., Miege, C., Jacquet, R., and Coquery, M. (2014). Impact of wastewater
456 treatment plants on receiving surface waters and a tentative risk evaluation: the case of
457 estrogens and beta blockers. *Environmental Science and Pollution Research* 21, 1708-
458 1722,
- 459 Guan, M., and Roddick, J.G. (1988a). Comparison of the effects of epibrassinolide and steroidal
460 estrogens on adventitious root growth and early shoot development in mung bean
461 cuttings. *Physiologia Plantarum* 73, 426-431,
- 462 Guan, M., and Roddick, J.G. (1988b). Epibrassinolide-inhibition of development of excised,
463 adventitious and intact roots of tomato (*Lycopersicon esculentum*): comparison with the
464 effects of steroidal estrogens. *Physiologia Plantarum* 74, 720-726,

- 465 Gururani, Mayank a., Venkatesh, J., and Tran, L.S.P. (2015). Regulation of Photosynthesis
466 during Abiotic Stress-Induced Photoinhibition. *Molecular Plant* 8, 1304-
467 1320. <https://doi.org/10.1016/j.molp.2015.05.005>
- 468 Hewitt, S., and Hillman, J. (1980). Steroidal oestrogens and adventitious root formation in
469 Phaseolus cuttings. *Annals of Botany*, 153-164,
- 470 Imai, S., Shiraishi, A., Gamo, K., Watanabe, I., Okuhata, H., Miyasaka, H., Ikeda, K., Bamba,
471 T., and Hirata, K. (2007). Removal of phenolic endocrine disruptors by *Portulaca*
472 *oleracea*. *Journal of bioscience and bioengineering* 103, 420-426,
- 473 Janeczko, A., Kocurek, M., and Marcińska, I. (2012). Mammalian androgen stimulates
474 photosynthesis in drought-stressed soybean. *Open Life Sciences* 7, 902-909,
- 475 Janeczko, A., and Skoczowski, A. (2011). Mammalian sex hormones in plants. *Folia*
476 *Histochemica et cytobiologica* 43, 71-70,
- 477 Karnjanapiboonwong, A., Chase, D.A., Canas, J.E., Jackson, W.A., Maul, J.D., Morse, A.N., and
478 Anderson, T.A. (2011). Uptake of 17 α -ethynylestradiol and triclosan in pinto bean,
479 *Phaseolus vulgaris*. *Ecotoxicology and environmental safety* 74, 1336-1342,
- 480 Lai, K., Scrimshaw, M., and Lester, J. (2002). Biotransformation and bioconcentration of steroid
481 estrogens by *Chlorella vulgaris*. *Applied and environmental microbiology* 68, 859-864,
- 482 Pocock, T., and Falk, S. (2014). Negative Impact on Growth and Photosynthesis in the Green
483 Alga *Chlamydomonas reinhardtii* in the Presence of the Estrogen 17 α -Ethinylestradiol.
484 *PLoS one* 9, e109289,
- 485 Sherafatmandjour, A., Khorshidi, M., and Abavisani, A. (2013). Effect of estradiol on
486 Photosynthetic pigments, proline and sugars in fennel. *International Journal of Farming*
487 *and Allied Sciences* Vol., 2 (17): 567-571, 2013
- 488 Shi, W., Wang, L., Rousseau, D.P., and Lens, P.N. (2010). Removal of estrone, 17 α -
489 ethinylestradiol, and 17 β -estradiol in algae and duckweed-based wastewater treatment
490 systems. *Environmental Science and Pollution Research* 17, 824-833,
- 491 Shore, L.S., Kapulnik, Y., Ben-Dor, B., Fridman, Y., Wininger, S., and Shemesh, M. (1992).
492 Effects of estrone and 17 β -estradiol on vegetative growth of *Medicago sativa*.
493 *Physiologia Plantarum* 84, 217-222
- 494 Shrestha, S.L., Casey, F.X., Hakk, H., Smith, D.J., and Padmanabhan, G. (2012). Fate and
495 transformation of an estrogen conjugate and its metabolites in agricultural soils.
496 *Environmental science & technology* 46, 11047-11053
- 497 Song, X., Wen, Y., Wang, Y., Adeel, M., and Yang, Y. (2018). Environmental risk assessment of
498 the emerging EDCs contaminants from rural soil and aqueous sources: Analytical and
499 modelling approaches. *Chemosphere* 198, 546-555
- 500 Trujillo-Reyes, J., Majumdar, S., Botez, C., Peralta-Videa, J., and Gardea-Torresdey, J. (2014).
501 Exposure studies of core-shell Fe/Fe₃O₄ and Cu/CuO NPs to lettuce (*Lactuca sativa*)
502 plants: Are they a potential physiological and nutritional hazard? *Journal of hazardous*
503 *materials* 267, 255-263
- 504 Upadhyay, P., and Maier, C. (2016a). Alleviation of Drought Stress in *Arabidopsis thaliana* by
505 17 β -Estradiol Application. *American Journal of Plant Sciences* 7, 2072
- 506 Upadhyay, P., and Maier, C. (2016b). Effects of 17 β -Estradiol on Growth, Primary Metabolism,
507 Phenylpropanoid-Flavonoid Pathways and Pathogen Resistance in *Arabidopsis thaliana*.
508 *American Journal of Plant Science*, 1693-1710. DOI: 10.4236/ajps.2016.713160
- 509 Vargas-Hernandez, M., Macias-Bobadilla, I., Guevara-Gonzalez, R.G., Romero-Gomez, S.D.J.,
510 Rico-Garcia, E., Ocampo-Velazquez, R.V., Alvarez-Arquieta, L.D.L., and Torres-

- 511 Pacheco, I. (2017). Plant Hormesis Management with Biostimulants of Biotic Origin in
512 Agriculture. *Frontiers in Plant Science* 8, 1762.10.3389/fpls.2017.01762
- 513 Xuan, R., Blassengale, A.A., and Wang, Q. (2008). Degradation of estrogenic hormones in a silt
514 loam soil. *Journal of agricultural and food chemistry* 56, 9152-9158,
- 515 Ylstra, B., Touraev, A., Brinkmann, A.O., Heberle-Bors, E., and Tunen, A. (1995). Steroid
516 hormones stimulate germination and tube growth of in vitro matured tobacco pollen.
517 *Plant physiology* 107, 639-643,
- 518 Zhang, F.-S., Xie, Y.-F., Li, X.-W., Wang, D.-Y., Yang, L.-S., and Nie, Z.-Q. (2015).
519 Accumulation of steroid hormones in soil and its adjacent aquatic environment from a
520 typical intensive vegetable cultivation of North China. *Science of the Total Environment*
521 538, 423-430,
- 522 Zheng, W., Wiles, K., and Holm, N. (2013). Uptake, translocation, and accumulation of
523 pharmaceutical and hormone contaminants in vegetables, in *Uptake, and Translocation of*
524 *Agrochemicals in plants: AMER CHEMICAL SOC 1155 16TH ST, NW,*
525 *WASHINGTON, DC 20036 USA*).
- 526 Zhou, Y., Zha, J., Xu, Y., Lei, B., and Wang, Z. (2012). Occurrences of six steroid estrogens
527 from different effluents in Beijing, China. *Environmental monitoring and assessment*
528 184, 1719-1729,

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531 **Figures captions**

532 **Figure 1.** Concentrations of estrogens in lettuce leaf and root tissues following treatment with a
533 range of concentrations of two estrogen hormones and after 21 days growth. Error bars represent
534 the standard deviation (n= 4). Different letters above each column indicate statistically
535 significant differences between a treatment at $P < 0.05$, according to Fisher's least significant test

536 **Figure 2.** Effects of estrogens on number of leaves (A), leaf area (B), root fresh weight (FW) (C)
537 and leaf fresh weight (D) of 21-day-old lettuce plants treated with EE2 or 17 β -E2. Error bars
538 represent the standard deviation (n= 4). Different letters above each column indicate statistically
539 significant differences between a treatment and the 0 control at $P < 0.05$, according to Fisher's
540 least significant test.

541 **Figure 3.** Effects of a concentration range of estrogens ($\mu\text{g L}^{-1}$) on the levels of Chlorophylls and
542 carotenoids (mg g^{-1} Fresh Weight) in leaves of 21 days old lettuce plants. Values are means \pm
543 SD; n = 4). Different letters above each column indicate statistically significant differences
544 between a treatment and the 0 control at $P < 0.05$, according to Fisher's least significant test.

545 **Figure 4.** Effect of estrogens on root morphology. Total root length (A), root length (B), average
546 diameter (C), number of root tips (D), root volume (E), and specific surface area (F), of 21- day-
547 old lettuce plants. Error bars represent the standard deviation (n= 4). Different letters above each
548 column indicate statistically significant differences between a treatment and the 0 control at $P <$
549 0.05, according to Fisher's least significant test.

550 **Figure 5.** Effects of estrogens on the activities of ROS detoxifying enzymes in the leaves of
551 lettuce plants. (A) superoxide dismutase (SOD),(B) POD, (C) catalase (CAT) and (D) ascorbate
552 peroxidase (APX). Error bars represent standard deviation (SD) of the mean (n = 4). Different
553 letters (a–d) indicate significant differences among the treatments at $P < 0.05$, according to
554 treatments.

555 **Figure 6.** Effects of estrogens on ROS in the leaves of lettuce plants with or without EE2 and
556 17 β -E2 treatment. (A) malondialdehyde (MDA) and (B) Hydrogen peroxide (H_2O_2) . Bars
557 represent standard deviation (SD) of the mean (n = 4). Different letters (a, b, c, d, e and f)
558 indicate significant differences among the treatments at $P < 0.05$.

559

Highlights

- EDC estradiol (17 β -E2) and ethinyl estradiol (EE2) stresses influence lettuce growth
- Estrogens biotransform to major metabolites and vice versa in lettuce tissue
- Both EDC treatments exerted a dose-dependent negative effect on both roots and leaves of lettuce

