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Impacts of the mycotoxin zearalenone on growth and photosynthetic responses in laboratory populations of freshwater macrophytes (*Lemna minor*) and microalgae (*Pseudokirchneriella subcapitata*).

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18 **Highlights**

- 19 • Zearalenone (ZON) is a commonly occurring mycotoxin in freshwater samples
- 20 • ZON aquatic toxicity data are limited hence algae and macrophytes were studied
- 21 • 72 h algae EC₅₀ = 0.92; NOEC = 0.1; LOEC = 0.23 mg ZON/L, 7 d macrophyte EC₅₀ = 8.8;
- 22 NOEC = 3.4; LOEC = 11.4 mg ZON/L
- 23 • PSII efficiency unaffected by ZON in algae and macrophyte exposures

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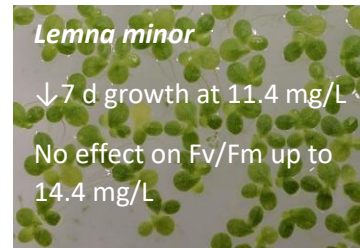
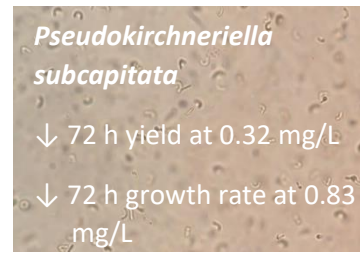
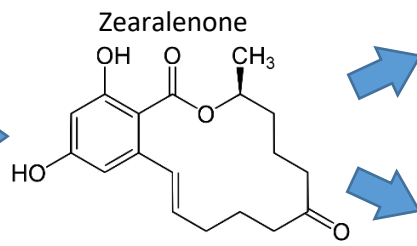
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38 **Abstract**

39 Mycotoxins are an important class of chemicals of emerging concern, recently detected in
40 aquatic environments, potentially reflecting the influence of fungicide resistance and climatic
41 factors on fungal diseases in agricultural crops. Zearalenone (ZON) is a mycotoxin formed by
42 *Fusarium* spp. and is known for its biological activity in animal tissues; both *in vitro* and *in vivo*.
43 ZON has been reported in US and Polish surface waters at 0.7 - 96 ng/L, with agricultural run-
44 off and wastewater treatment plants being the likely sources of mycotoxins. As some
45 mycotoxins can induce phytotoxicity, laboratory studies were conducted to evaluate the
46 toxicity of ZON (as measured concentrations) to freshwater algae (*Pseudokirchneriella*
47 *subcapitata*) and macrophytes (*Lemna minor*) following OECD test guidelines 201 and 221,
48 respectively. Zinc sulphate was used as a positive control. In the OECD 201 algal static study
49 (72 h at $24 \pm 1^\circ\text{C}$), exposure to ZON gave average specific growth rate (cell density) EC_{50} and
50 yield (cell density) EC_{50} values of > 3.1 and 0.92 ($0.74 - 1.8$) mg/L, respectively. ZON was less
51 toxic in the OECD 221 static study and after 7 d at $24 \pm 1^\circ\text{C}$. *L. minor* growth was significantly
52 reduced based on frond number and frond area at 11.4 mg ZON/L, showing a higher tolerance
53 than reported for other mycotoxins with *Lemna* spp. Chlorophyll fluorescence parameters
54 were used as biomarkers of impacts on photosystem II efficiency, with no effect seen in algae
55 but, with responses being observed in *L. minor* between $5.2 - 14.4$ mg ZON/L. ZON toxicity
56 seen here is not of immediate concern in context with environmental levels, but this study
57 highlights that other freshwater organisms including algae are more sensitive to mycotoxins
58 than *Lemna* sp., the only current source of toxicity data for freshwater plants.

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60 **Graphical abstract**



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71 **Keywords**

72 Emerging chemical, fungal toxin, phytotoxicity, risk assessment

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74 **1. Introduction**

75 The effect of naturally occurring chemicals can sometimes be overshadowed by the growing
76 development of synthetic chemicals, however many naturally produced chemicals have been
77 shown to possess significant endocrine disrupting potential. For example, Cyanobacteria have
78 able to produce retinoid like compounds, potentially causing significant developmental
79 impacts in amphibians and fish (Wu et al., 2012; Smutná et al., 2017). Feminisation of fish due
80 to oestrogenic compounds is widely recognized, with mycoestrogens and phytoestrogens as
81 likely contributors in highly contaminated ecosystems (Jarošová et al., 2015).

82 Mycotoxins are produced as secondary metabolites by many fungal species. Mycotoxins are
83 commonly associated with cereal crops, but they are also found in other crops such as nuts,
84 fruit and coffee. Animals feeding on mycotoxin-contaminated feed have shown toxic effects
85 such as protein synthesis inhibition, immunosuppression and carcinogenicity (Zain, 2011).
86 Hence, due to their potential risk to human and animal health, the levels of mycotoxins in
87 foodstuff are regulated by European Union legislation (EU, 2006). Of the mycotoxins produced
88 by *Fusarium* sp., Zearalenone (ZON) is a known mycoestrogen. Therefore, ZON is associated
89 with potential reproductive effects and can cause hypoestrogenism (Cano-Sancho et al., 2012;
90 Rashedi et al., 2012). The metabolites of ZON, α -zearalanol and β -zearalanol, are also
91 oestrogenic; with α -zearalanol licensed as a growth promoter for cattle in some non-EU
92 countries (Le Guevel & Pakdel, 2001; Bartelt-Hunt et al., 2012).

93

94 Studies in the US and Poland have found low levels (0.7 - 96 ng/L) of ZON in streams and rivers
95 with the main sources being agricultural runoff and wastewater treatment plant effluent
96 (Gromadzka et al., 2009; Kolpin et al., 2014). However, few studies have considered the levels

97 at which mycotoxins can have toxic effects on freshwater species. For ZON toxicity to zebrafish
98 embryos, Bakos et al. (2013) found a 5 d development effect concentration 50 % (EC₅₀) of 50
99 µg/L and lethal concentration 50 % (LC₅₀) of 893 µg/L. Schwartz et al. (2010) reported a 21 d
100 development LOEC and mortality LOEC of > 3.2 µg/l, 1 µg/l for vitellogenin production LOEC
101 and 0.1 µg/l for fecundity LOEC. In a longer life cycle (140 d) test with zebrafish a sex ratio
102 LOEC of 0.32 µg/L was seen (Schwartz et al., 2013). In contrast, there is a lack of ZON
103 phytotoxicity data which is needed in order to develop an environmental risk assessment of
104 this widespread mycotoxin. This is important given that other mycotoxins have been shown
105 to cause phytotoxicity in *Lemna* spp. (eg growth inhibition of 40 % at 3.2 mg nivalenol/L, 56 %
106 at 3.2 mg deoxynivalenol/L and 72 % at 5.6 mg T-2 toxin/L (Abbas et al., 2013)).

107

108 *Lemna* sp. are popular choice in chemical toxicity monitoring for freshwater primary
109 producers, due to their small size, rapid growth and ease of culturing. The microalga
110 *Pseudokirchneriella subcapitata*, previously known as *Selenastrum capricornutum* and
111 *Rhaphidocelis subcapitata*, similarly is a well-studied organism with its rapid growth rate
112 allowing multiple generations to be studied in a brief time frame. Standardised testing
113 guidelines have been developed for both species (OECD, 2006; OECD, 2011), outlining
114 methods which can be used under laboratory conditions to contribute to the hazard
115 assessment of chemicals, through analysing the adverse outcome (AO) at the level of the
116 individual and population. To develop knowledge of the specific mode of action (MoA) of a
117 chemical and link this to the AO, these guidelines can be supplemented with physiological and
118 biochemical data. Allowing a flow of events from the molecular changes at the target site to

119 the eventual population effect to be pieced together via a suggested Adverse outcome
120 pathway (AOP).

121

122 The aim of this study was to investigate the phytotoxicity of ZON as a chemical of emerging
123 concern, employing the standardised OECD test guidelines for *L. minor* and *P. subcapitata*.

124 This was achieved with a 7 d or 72 h growth inhibition study for each species respectively.

125 Following this, physiological measures of photosynthetic performance and biochemical
126 analysis of lipid peroxidation and catalase activity were performed. These were included as a

127 preliminary investigation into MoA measures which can easily be added to the existing

128 guideline framework and analysed for indication of pathways to be pursued to develop AOP's

129 for the test chemical. For quality control purposes, zinc was used as a reference toxicant as

130 per the UK Direct Toxicity Assessment approach (EA, 2007). Zinc is required as a micronutrient

131 in plant growth but in excess causes phytotoxicity and reduces growth, previous literature is

132 available for zinc toxicity in both species used in this study (Paixão et al., 2008; Lahive et al.,

133 2011). All toxicity data for ZON and Zn reported from the microalgae and macrophyte

134 experiments are expressed as measured concentrations unless stated otherwise.

135

136 **2. Materials and methods**

137 **2.1. Cultures and exposure**

138 A culture of *P. subcapitata* (type strain 278/4) was obtained from the Culture Collection of
139 Algae and Protozoa and maintained in BG11 media made by diluting a sterile stock solution

140 (Sigma Aldrich, Dorset, UK). Prior to experiments, a sub-culture was prepared and held under

141 testing conditions of constant illumination ($105\text{-}125\ \mu\text{mol m}^{-2}\text{s}^{-1}$) and placed on an orbital

142 shaker set at 120 rpm with temperature in the media maintained at $24 \pm 1^\circ\text{C}$. Static exposures
143 were carried out over a 72 h period in accordance with OECD guideline 201 (OECD, 2011). A
144 healthy exponentially growing culture (monitored by increase in cell density) was used to
145 inoculate 25 ml of growth media in polystyrene 50 ml capacity cell culture flasks with filter
146 caps (Greiner, Gloucestershire, UK, C6481) at a density of 5×10^3 cells/ml. Three replicates per
147 test solution were used. Test vessels were placed randomly on an orbital shaker and re-
148 arranged daily.

149 A culture of *L. minor* (UTCC #490) was maintained in Swedish standard (SIS) media, with the
150 pH adjusted to 6.5 ± 0.2 , under a 16:8 h light dark cycle (white fluorescent light) at $24 \pm 1^\circ\text{C}$.
151 Cultures were sterilised prior to testing and visibly free from algae at the beginning of
152 exposures. A static exposure was carried out over a 7 d period in accordance with OECD test
153 guideline 221 (OECD, 2006). Healthy colonies consisting of three fronds were placed into
154 individual wells of six-well clear polystyrene microplates (Thermo Fisher Scientific,
155 Massachusetts, USA, product code 130184) with 8 ml test solution. For each concentration 12
156 replicate wells (i.e. 2 duplicate 6 well microplates for each concentration) were used and held
157 under the same conditions as during culturing, position of plates in the incubator was
158 randomised throughout the test.

159

160 **2.2. Growth rate**

161 Based on pilot studies for microalgae, ZON test solutions of nominal concentrations were zero
162 (< 0.18), 0.032 (< 0.18), 0.1 (< 0.18), 0.32 (0.23), 1.0 (0.83) and 3.2 (3.1) mg/L were tested
163 (mean measured concentrations in brackets with a limit of detection (LOD) of 0.18 mg ZON/L).

164 A zinc positive control of 0.2 mg/L (made with zinc sulphate heptahydrate, CAS number 7446-
165 20-0; Sigma Aldrich batch number: 31665; purity $\geq 99.5\%$) was used. The pH of test solutions

166 was measured at the beginning and end of the study (pH 6.9 - 7.5) with each replicate meeting
167 the test criteria for pH (OECD 2011). Growth was measured at 24 h intervals by removing 5 µl
168 from each test vessel and manually calculating cell density using a Neubauer chamber.
169 Average specific growth rate (ASGR) and yield, inhibition of ASGR and inhibition of yield were
170 calculated according to the test guideline.:

$$171 \mu_{i-j} = (\ln(N_j) - \ln(N_i))/t$$

172 where, μ_{i-j} is the ASGR for the time period (t) i to j , N_i and N_j is the measurement variable (cell
173 density) at the time i and j respectively, and t is the time period from i to j . Percentage
174 inhibition of ASGR (% I_r) for each test solution, compared to the dilution water control, was
175 calculated using:

$$176 \% I_r = ((\mu_c - \mu_T)/\mu_c) \times 100$$

177 where, μ_c is the mean ASGR in the dilution water control and μ_T is the mean ASGR in each
178 test solution.

179 Yield was determined, by the change in biomass (cell density) over 7 d in each test replicate.

180 Mean inhibition of yield for each treatment was calculated by:

$$181 \% I_y = ((b_c - b_T)/b_c) \times 100$$

182 where % I_y is percentage reduction in yield, b_c is change in biomass for the dilution water
183 control group and b_T is the change in biomass for the treatment.

184 For *L. minor* ZON test solutions were prepared for nominal concentrations of zero (< 0.18), 0.1
185 (< 0.18), 0.32 (0.36), 1.0 (1.1), 3.2 (3.4) and 10.0 (11.4) mg/L (mean measured concentration
186 in brackets with an LOD of 0.18 mg ZON/L), plus a reference chemical measured exposure of
187 1.4 mg Zn/L positive control. Physio-chemical parameters were measured at the beginning
188 and end of the study (dissolved oxygen 8.1 - 9.9 mg/L; temperature 23.8 - 24.0 °C; and pH

189 ranged between 6.4 - 7.5, within the recommended variation of less than 1.5 units). Growth
190 measurements of frond number and frond area (using WinDias 1.5 software with Hitachi KP-
191 D40 digital camera) were taken at $t = 0, 2, 5$ and 7 d, average specific growth rate (μ) and yield
192 were calculated as described previously for algae, with frond number and frond area used in
193 place of cell density.

194

195 **2.3. Chlorophyll fluorescence**

196 Chlorophyll fluorescence parameters for *P. subcapitata* were measured using a portable
197 fluorimeter (ToxY-PAM, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). After the
198 exposure, all replicates were dark adapted for 20 mins at room temperature and 2 ml removed
199 for analysis. To measure F_V / F_M (variable fluorescence / maximum fluorescence) samples were
200 exposed to a saturating light pulse of 2000 μmol of photons m^2/s over 1 s.

201

202 The chlorophyll fluorescence parameters for *L. minor* were measured using a portable
203 fluorimeter (Pocket PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) with a light
204 pulse of 3000 μmol of photons m^2/s over 1 s. A single colony was taken from six wells in each
205 treatment and dark adapted in a leaf clip for at least 20 mins at room temperature before
206 being measurements were taken. Measurements were taken at $t = 7$ d of a second exposure
207 with concentrations of measured ZON concentrations of 4.8 (5.2) , 8.1 (7.9) and 15.0 (14.4)
208 mg/L (mean measured concentration in brackets) and reference chemical mean measured
209 exposure of 1.8 mg Zn/L . Physio-chemical parameters were measured at the beginning and
210 end of the study (dissolved oxygen 8.1 - 10.0 mg/L ; pH 6.4 - 7.1; temperature 23.8 - 24.0 $^\circ\text{C}$).

211

212 The chlorophyll fluorescence parameters are based upon the alterations to shape of the
213 fluorescence rise seen in all photosynthetic materials, which can be separated into a sequence
214 termed the OJIP transient and analysed using the JIP test (Appenroth et al., 2001; Yusuf et al.,
215 2010) to generate expressions including: (1) measures of efficiency and performance such as
216 F_V / F_M (variable fluorescence / maximum fluorescence) the maximal quantum efficiency of
217 PSII, PI_{ABS} and PI_{Total} (performance indices representing energy conservation for reduction of
218 intersystem electron acceptors and PSI terminal acceptors respectively); (2) parameters
219 calculated based on F_0 (minimal fluorescence) and F_M such as TF_M (time to reach maximum
220 chlorophyll fluorescence (F_M)) and area (proportional to the pool size of the electron acceptors
221 Q_A on the reducing side of Photosystem II (the area above fluorescence curve between F_0 and
222 F_M)); along with F_V/F_0 (quantum yield of the photochemical and non-photochemical
223 processes); (3) specific energy fluxes per reaction centre such as ABS/RC (absorption of light
224 energy per reaction centre), DIO/RC (energy dissipation per reaction centre), TRo/RC (the
225 energy trapping rate per reaction centre), ETo/RC (the photosynthetic electron transport rate
226 per reaction centre) and REo/RC (reduction of acceptors in PSI per reaction centre); (4)
227 Quantum efficiencies or flux ratios such as $\phi(Po)$ maximum quantum yield of primary
228 photochemistry, $\Psi(Eo)$ probability of a trapped exciton moving an electron past Q_A^- to the
229 electron transport chain, $\phi(Eo)$ quantum yield of electron transport from Q_A^- , $\delta(Ro)$ probability
230 an electron from the intersystem reduces PSI terminal electron acceptors and $\phi(Ro)$ quantum
231 yield of reduction of PSI terminal electron acceptors. (Misra et al., 2001; Strasser et al., 2000;
232 Yusuf et al., 2010)

233

234 **2.4. TBARS assay and catalase enzyme activity**

235 The biomass generated during the 72 h microalgae study was too low to perform biochemical
236 analysis of these measures, with Soto et al. (2011) reporting an extended exposure period of
237 15 days to generate a sufficient biomass of *P. subcapitata* for analysis of TBARS assay and
238 catalase activity.

239 To measure the catalase activity in the *Lemna* plant material, three replicates from each
240 treatment, ZON concentrations 4.8 (5.2) , 8.1 (7.9) and 15.0 (14.4) mg/L (mean measured
241 concentration in brackets) and the reference chemical mean measured exposure of 1.8 mg
242 Zn/L, were weighed individually and manually crushed with a mortar and pestle (due to the
243 low weight of *L. minor* in 14.4 mg ZON/L and 1.8 mg Zn/L treatments, two wells were
244 combined for each replicate) in 100 mM phosphate buffer (pH 7) at a ratio of 1 mg (wet
245 weight): 19 µl of buffer. Homogenates were centrifuged (10 000 g for 10 mins) and the
246 supernatants collected for the catalase assay (method adapted from Beers & Sizer 1952; Aebi
247 1984). A kinetic method was used, where 200 µl of 10mM H₂O₂ was added to 50 µl of
248 supernatant in a microplate and the decrease in absorbance (correlating to a decrease in H₂O₂)
249 read at 3 s intervals for 3 mins at 240 nm. Five replicates were measured per sample.

250 The thiobarbituric acid reactive substances (TBARS) method was used as a general measure of
251 oxidative stress in the tissue (method adapted from Esterbauer & Cheeseman (1990); Marnett
252 1999). Three replicates from each treatment were weighed and homogenised individually
253 (due to the low weight of *L. minor* in 14.4 mg ZON/L and 1.8 mg Zn/L two wells were combined
254 for each replicate) in 100 mM phosphate buffer (pH 7.5) at a ratio of 1 mg: 9 µl. Homogenates
255 were centrifuged (10 000 g for 10 mins). Sixty (60) µL of the supernatant along with 10 µl of
256 10 mM butylated hydroxytoluene, 150 µl of 100 mM phosphate buffer, 50 µl of 10 % (w/v)
257 trichloroacetic acid and 75 µl of 1.3 % (w/v) thiobarbituric acid were mixed and incubated at

258 90°C for 60 mins. The absorbance was measured at 530 nm and calibrated against
259 malondialdehyde standards. Protein content of the homogenates used for catalase and
260 TBARS assays was determined with the Peirce BCA Protein Assay Kit (Thermo Fisher Scientific,
261 Massachusetts, USA). Briefly, the working reagent was prepared by mixing bicinchoninic acid
262 (BCA) reagent 1 and 2 in a 50:1 ratio, then 10 µl of homogenate was added to 200 µl working
263 reagent and incubated at 37°C for 30 mins, absorbance was read at 562 nm. Data for catalase
264 and TBARS are expressed as absorbance change min/mg homogenate protein and nmol/mg
265 homogenate protein, respectively.

266

267 **2.5. Analytical chemistry of ZON and use of zinc positive controls.** Nominal exposure
268 concentrations of ZON (CAS number 17924-92-4; Sigma Aldrich, Dorset, UK, batch number
269 043M4106V) in all phytotoxicity experiments were verified by test solution analysis using UV-
270 Vis spectrometry (SpectraMax 190 microplate reader, Molecular Devices, USA). The LOD for
271 this method was 0.18 mg ZON/L, hence in experiments where some concentrations were
272 below the limit of detection values for both nominal and measured concentrations are
273 provided. Briefly, samples were taken at the beginning and the end of studies and mean
274 concentrations for the exposure period were calculated. Samples from the end of studies were
275 centrifuged at 5000 g for 10 min and the supernatant used to avoid any interference by algal
276 growth. The absorbance of 300 µl of each sample was measured in a UV-STAR 96 well
277 microplate (Greiner, product code 655801) at 270 nm.

278

279 For quality control purposes, zinc sulphate heptahydrate was used as a positive control in the
280 *Lemna* spp. studies. Test solutions were collected at the beginning and end of exposures and
281 mean measured concentrations of zinc were determined using Inductively Coupled Plasma-

282 Optical Emission Spectrometry (ICP-OES, iCAP, Thermo Scientific,) with a limit of detection of
283 0.001 mg Zn/L. Due to unforeseen technical problems it was not possible to evaluate the
284 measured concentration for the microalgae study, but the exposure performed in line with
285 expectations from previous zinc range finding studies.

286

287 **2.6. Statistical Analyses of Algal and Macrophyte Data**

288 Statistical analyses were performed using Minitab (Minitab Ltd., Coventry, UK) and GraphPad
289 Prism (GraphPad Software, Inc, California, USA). Biological effects data (based on measured
290 concentrations of Zn or ZON) were tested for significance ($P < 0.05$) using one-way analysis of
291 variance with Dunnett's post-test or Kruskal Wallace with Dunn's post-test where appropriate,
292 for normal with homogenous variances and non-normal distributions respectively. EC₂₀ and
293 EC₅₀ values (with 95 % confidence intervals) were determined using non-linear regression and
294 then by fitting sigmoidal curves to the data sets.

295 **3. Results**

296 **3.1. Growth inhibition**

297 The controls of *P. subcapitata* in the control media showed an average overall growth rate of
298 1.38 (SD 0.01), confirming the healthy status of the organism. Furthermore, all experimental
299 treatments continued to increase in cell density until the end of the study (Figure 1.). However
300 during the first 24 h, the cell density of the control and two lowest treatments (0.032 and 0.1)
301 increased more than the higher treatments with both 0.83 and 3.1 mg ZON/L visibly not
302 recovering from this by 72 h. Based on the calculated endpoints at the end of the experiment,
303 there was a significant decrease in growth at 0.23 mg/L for 72 h Yield and at 0.83 mg/L for the
304 72 h Average Specific Growth Rate (ASGR) ($P < 0.05$) (Table 1). The EC₅₀ values from this study

305 showed as expected yield to be a more sensitive measure ($EC_{50} = 0.92$) than ASGR ($EC_{50} = >$
306 3.2) as is the nature of these secondary measures according to the OECD guideline.

307 The growth of *L. minor* was assessed throughout the 7 d study; controls had a doubling time
308 of 2.4 d and no significant variation to exponential growth throughout the test period. Values
309 for 7 d measurements are seen in Table 2, along with the % growth inhibition values,
310 recommended to be used in analysis by the testing guidelines. The only concentration to show
311 significant difference in growth in comparison to the control was 11.4 mg ZON/L, with
312 inhibition of 38 % for both ASGR (frond number) and ASGR (frond area) and 60 % Yield (frond
313 number) and 67 % Yield (frond area). Since only the highest exposure in the range finder
314 showed significant inhibition at 7 d, the concentrations for the following photosynthetic and
315 biochemical measures were adapted to exceed the growth no effect concentration (NOEC)
316 values (3.9 mg ZON/L for all growth variables).

317

318 **3.2. Chlorophyll fluorescence**

319 During the growth inhibition test measures of F_v/F_m for *P. subcapitata* showed no significant
320 differences (Table 3.), with a control mean of 0.49 and exposure means of 0.48 - 0.50 (SD <
321 0.012). The value for the control mean is lower than that reported in other studies of greater
322 than 0.6 (Choi et al., 2012; Vannini et al., 2011), but consistent with historical control means
323 at this laboratory therefore considered to be due to inter laboratory variation. Measures of
324 chlorophyll fluorescence for the dark adapted *L. minor* were carried out in a second test and
325 are shown in Figure 2. The maximum efficiency calculated by F_v/F_m was not affected by ZON
326 exposure but to understand the tolerance of plants it is important to observe other chlorophyll
327 parameters. T_{fm} , Area, E_{To}/RC , R_{Eo}/RC , $\Psi(E_o)$, $\phi(E_o)$, $\delta(R_o)$, $\phi(R_o)$, PI_{ABS} and PI_{Total} decreased

328 significantly in all ZON treatments. While ABS/RC and TRo/RC were significantly increased at
329 the highest treatment of 14.4 mg ZON/L. Fv/F₀ and φ(Po) did not alter significantly in any
330 treatment.

331

332 **3.3. TBARS assay and catalase enzyme activity**

333 To assess potential oxidative stress as a result of photoinhibition TBARS and catalase activity
334 was monitored at 7 d in *L. minor* (Figure 3.). ZON lowered mean TBARS content, with the
335 decrease (54 %) at 14.4 mg ZON/L being significant (P < 0.05). The 1.8 mg Zn/L reference
336 chemical treatment did not lead to significant changes in TBARS content. Catalase rates
337 showed no significant deviation from the control values for any treatment of ZON or Zn.

338

339 **4. Discussion**

340 The main finding of this study was both the algae and the aquatic macrophyte show growth
341 inhibition in the presence of ZON, with the algal species being approximately 10 times more
342 sensitive based the most sensitive EC₅₀ values. There was also evidence of interference with
343 photosynthesis only in *L. minor*, but at high ZON concentrations, although this effect was
344 probably not mediated by overt oxidative stress (no change in catalase and TBARS decreasing
345 slightly).

346

347 **4.1. Acute toxicity**

348 The phytotoxicity seen in *P. subcapitata* exhibited a concentration dependant response, with
349 no effect on the two lowest concentrations. Recovery was seen at 0.23 mg ZON/L between
350 the 24 and 72 h observations, with only yield significantly inhibited at 72 h, and significant
351 inhibition in the both of the higher exposures of 0.83 and 3.1 mg ZON/L. Whereas in *L. minor*
352 there was no constant change with concentration but a significant growth response at the
353 highest concentration. The only published data for ZON toxicity to *L. minor* found was an
354 exposure at a single concentration of 1 mg ZON/L, which showed no effect on growth at this
355 concentration (Vanhoutte et al., 2017). This supports our findings and considering the *L. minor*
356 growth inhibition values of 38 - 67% seen at 11.4 mg ZON/L in this study, ZON appears to be
357 less toxic to *Lemna* sp. than mycotoxins tested by Abbas et al. (2002; 2013). Where reported
358 growth inhibition due to deoxynivalenol, nivalenol, T-2 toxin and verrucarin A, was 38 - 72%
359 at concentrations in the range of 0.5 - 4.6 mg/L, resembling more the EC₂₀ values generated
360 in this study of 3.0 – 6.5 mg ZON/L. The only mycotoxin reported as less toxic to *Lemna* sp.
361 than ZON is butanolide with 62 % inhibition at 66.7 mg/L (Vesonder et al., 1992). No previous
362 studies for mycotoxin toxicity to microalgae were found for comparison, but our findings
363 demonstrate the value of expanding phytotoxicity data to include algae such as *P. subcapitata*
364 when considering the potential risk of mycotoxins to freshwater ecosystems.

365

366 **4.2. Sub lethal effects**

367 Further to measuring the adverse outcome in terms of growth as a result of ZON exposure,
368 we investigated potential MoA leading to the observed phytotoxicity; measures of chlorophyll
369 fluorescence in a dark-adapted state and biochemical indicators of oxidative stress. Of the
370 photosynthetic parameters measured using chlorophyll fluorescence, Fv/Fm is commonly

371 used as an indication of inhibition of photosynthesis, representing maximum efficiency of
372 Photosystem II via the reduction of Q_A ; the electron acceptor in PSII. This was the only measure
373 possible with the instrument used for *P. subcapitata*. F_v/F_m was unaffected in all *P.*
374 *subcapitata* and *L. minor* ZON exposures and the reference zinc controls. For the additional
375 parameters in *L. minor*, all mycotoxin (5.2 - 14.4 mg ZON/L) and zinc (1.8 mg Zn/L) treatments
376 showed a significantly reduced time to reach maximum fluorescence (TF_m) and indicated
377 some stress may be occurring due to the inhibition of electron transfer; measured by the area
378 between F_o and F_m . Both values decreased with increasing concentration of ZON or zinc
379 (Figure 2.). The visual health of the fronds was not affected with no signs of chlorosis or
380 bleaching of the leaves, suggesting that the chlorophyll content of the fronds was not
381 appreciably depleted. Overall, these data suggest only modest effects of mycotoxin on
382 photosynthetic ability under these experimental conditions (5.2 - 14.4 mg ZON/L) and appear
383 not to explain the key mechanisms of mycotoxin phytotoxicity in *Lemna* spp. (as yield) with 7d
384 EC_{20} and EC_{50} values of 3.0 and 8.8 mg ZON/L, respectively (Table 2).

385

386 The specific energy fluxes ABS/RC and TR_o/RC significantly increased in the highest ZON
387 treatment, this could represent alteration to the composition of light harvesting complexes
388 to absorb and trap higher energies in a shorter time period. Measuring pigment content to
389 assess heterogeneity would determine whether this was the cause of the increase (Mirkovic
390 et al., 2017). Efficiency in terms of PI_{ABS} and PI_{Total} significantly decreased suggesting that
391 with the increase in absorbance and trapping there is an imbalance in light absorption and
392 utilization of energy as these parameters are associated with the energy flow in the electron
393 transport chain (Farias et al., 2016; Zhang et al., 2016). Combining this with the reduction in

394 ETo/RC and REo/RC, representing the energy flux from Q_A^- into the electron transport chain
395 and reduction of PSI terminal acceptors on the electron acceptor side, this adds to the
396 concept of electron transfer being the possible cause of reduced performance. The
397 reduction in quantum yields and ratios $\Psi(E_o)$, $\phi(E_o)$, $\delta(R_o)$ and $\phi(R_o)$ also suggest inhibition
398 of electron movement between Q_A and the acceptor side of PSI.

399 ZON has been seen to act as a uncoupler of oxidative phosphorylation in mitochondria of pea
400 plants (Macri et al., 1996). Uncoupling can also occur in chloroplasts, the oxygen evolving
401 complex (OEC) can be uncoupled and lead to inhibition of the re-oxidation of Q_A^- (He et al.,
402 2018). This would incur the electron transport inhibition effects seen and the decrease in
403 reduction of PSI electron acceptors. However, if uncoupling of the OEC was occurring the
404 Fv/Fo value is sensitive to this and no significant difference for Fv/Fo was detected in our
405 study.

406 Another possibility for MoA is based upon are similarity of our results to those seen in pea
407 leaves treated with (3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) (Farias et al., 2016),
408 and reflect their finding of performance indices being a more sensitive than both quantum
409 yield of PSII $\phi(P_o)$ and Fv/Fm which were unaffected. The reduction in movement of
410 electrons into the electron transport chain can cause the over excitation of PSII as seen with
411 photosynthetic herbicides including DCMU (Giardi and Pace, 2005). By binding to Q_B ; the
412 plastoquinone domain, in the D1 protein of chloroplasts, photosynthesis is inhibited with
413 more energy being absorbed than can be transported into the electron transport chain
414 (Gatidou et al., 2015).

415 A potential issue for plants when too much light energy is being absorbed is oxidative stress.
416 If ZON was acting upon the Q_B region of the D1 protein in the chloroplasts, this region is

417 involved in controlling the electron transport chain and thus limiting the normal production
418 of singlet oxygen. In the presence of ZON the protein quenching of singlet oxygen would be
419 inhibited and could lead to oxidative stress (Kreiger-Liszky, 2005). In this study there was no
420 effect on catalase activity and TBARS content decreased in ZON exposures, being significant
421 in the highest test concentration. This was probably due to the reduced growth of plant tissue,
422 supporting the conclusion of the absence of overt oxidative stress in *Lemna* spp. under these
423 experimental conditions. However, excess energy can be transferred to non-photosynthetic
424 pathways as a protective mechanism against reactive oxygen species formation. The D1o/RC
425 flux increased in the highest ZON treatment indicating light energy dissipating in the form of
426 heat. These preliminary data show a strong basis to work from with ZON effect electron
427 transport, further measures such as light adapted state chlorophyll fluorescence including
428 non-photochemical quenching (NPQ) are a key area to consider to demonstrate this further
429 and show excess energy is being diverted away from the electron transport chain to prevent
430 oxidative stress during the ZON exposure. Furthermore, additional endpoints should consider
431 the point at which electron transport is inhibited, whether as we have suggested it is around
432 or after Q_A or whether something is occurring prior to this in the PSII reaction centre at P680
433 or pheophytin.

434

435 **5. Conclusions and regulatory context**

436 This laboratory study finds ZON to be less toxic to *Lemna* sp. than other mycotoxins reported
437 in literature. With no previous freshwater mycotoxin studies including algae as a test
438 organism, the higher sensitivity of *P. subcapitata* as compared with macrophytes observed in
439 this study demonstrates the importance of using a multi-species approach in ecotoxicology

440 and when defining environmental safety levels. Suitable conditions for fungal growth on
441 crops, of increased precipitation, suggest surface waters are a vulnerable ecosystem to
442 mycotoxin contamination via run off from fields. Observed phytotoxicity values for freshwater
443 algae and macrophytes generated here show no immediate risk, with the acute NOEC for
444 microalgae 1000 times higher than the maximum concentration reported to date in
445 environmental samples.

446

447 Regarding extrapolation of mycotoxin aquatic phytotoxicity data to other groups of organisms
448 (eg cyanobacteria or seaweeds), the Adverse Outcome Pathway (AOP) approach is a valuable
449 framework (Ankley et al., 2010; Burden et al., 2015). Currently, AOP information for
450 mycotoxin-induced phytotoxicity is lacking, with our results showing some indications of
451 phytotoxicity associated with perturbed chlorophyll fluorescence parameters. Mechanistic
452 toxicity data are important in understanding the impacts of mycotoxins on aquatic organisms
453 given their widespread occurrence (Gromadzka et al., 2009; Kolpin et al., 2014). The current
454 preliminary data for macrophytes needs further study to understand the mechanism of ZON
455 induced phytotoxicity and cytotoxicity since they were not consistent with regard to the
456 concept of photo oxidative stress being due to ZON-induced electron transport inhibition.

457

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605

606 Figure 1. Growth curves of *P. subcapitata* exposed to zearalenone (CAS number 17924-92-4)
607 in a 72 h static study at $23.8 \pm 1^\circ\text{C}$ with 0.2 mg Zinc/L as a positive control.

608 Figure 2. Chlorophyll fluorescence parameters measured in *L. minor* after 7 d exposure to
609 zearalenone (CAS number 17924-92-4) in a static study at $24 \pm 1^\circ\text{C}$ (concentration measured
610 using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L). Values are normalised to the
611 control group 0 (< 0.18 mg ZON/L).

612 Figure 3. Catalase and TBARS content (\pm SD) measured in *L. minor* after 7 d exposure to
613 zearalenone (CAS number 17924-92-4) in a static study at $24 \pm 1^\circ\text{C}$.

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617 LIST OF TABLES

618

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622 7 d static study at $24 \pm 1^\circ\text{C}$.

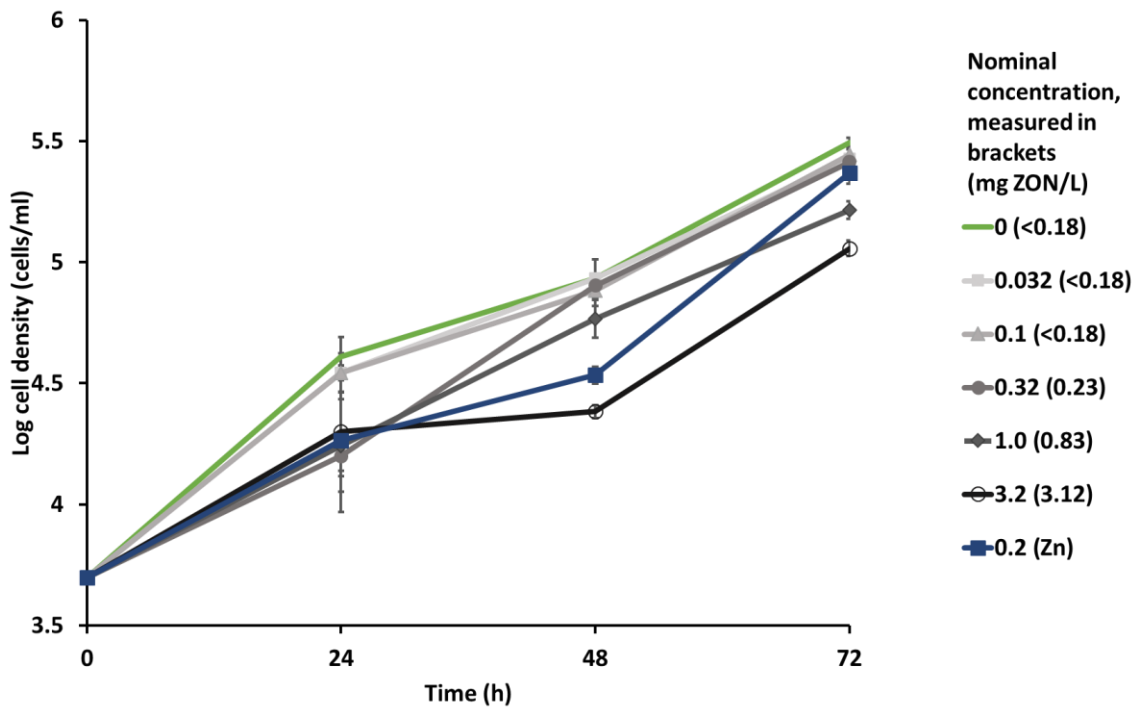
623 Table 3. Maximal quantum efficiency of Photosystem (Fv/Fm) measured in *P. subcapitata*
624 (mean \pm SD) after 72 h exposure to zearalenone (CAS number 17924-92-4) in a static study at
625 $24 \pm 1^\circ\text{C}$ (concentration measured using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L).

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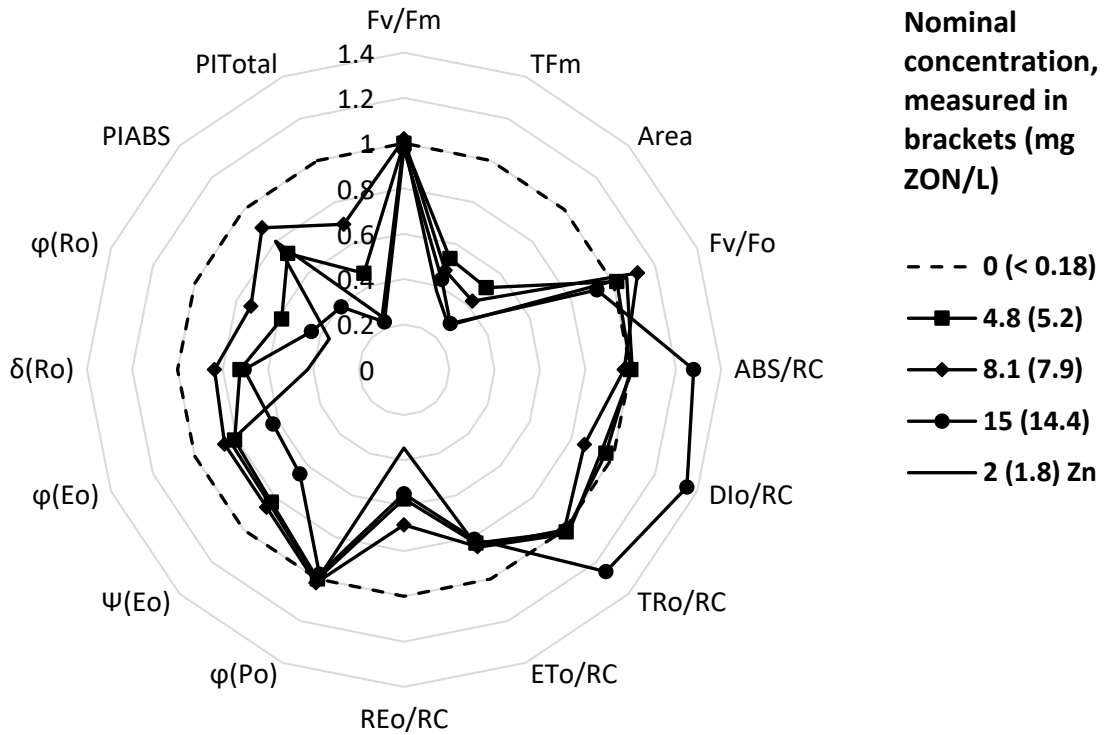
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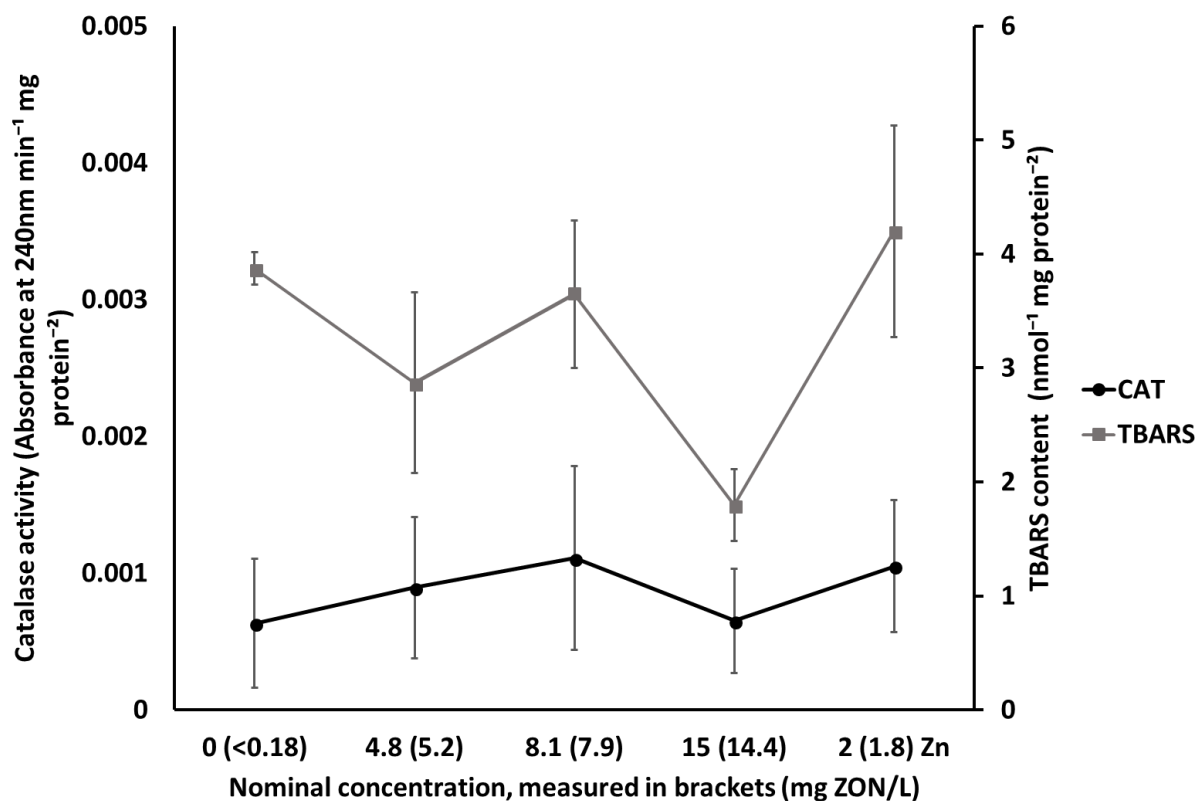
635 **Figure 2.** Chlorophyll fluorescence parameters measured in *L. minor* after 7 d exposure to
 636 zearalenone (CAS number 17924-92-4) in a static study at $24 \pm 1^\circ\text{C}$ (concentration measured
 637 using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L). Values are normalised to the
 638 control group 0 (< 0.18 mg ZON/L).

639 Footnote - F_v/F_m = maximal quantum efficiency of Photosystem II; TF_m = time to reach
 640 maximum chlorophyll fluorescence; Area = proportional to the pool size of the electron
 641 acceptors Q_a on the reducing side of Photosystem II; F_v/F_0 = quantum yield of the
 642 photochemical and non-photochemical processes; ABS/RC = absorption of light energy per
 643 reaction centre; D_{lo}/RC = energy dissipation per reaction centre), TR_o/RC (the energy trapping
 644 rate per reaction centre; TR_o/RC = energy trapping rate per reaction centre; ET_o/RC =
 645 photosynthetic electron transport rate per reaction centre; RE_o/RC = reduction of acceptors
 646 in PSI per reaction centre; $\phi(P_o)$ = maximum quantum yield of primary photochemistry; $\Psi(E_o)$
 647 = probability of a trapped exciton moving an electron past Q_A^- to the electron transport chain;

648 $\phi(E_0)$ = quantum yield of electron transport from Q_A^- ; $\delta(R_0)$ = probability an electron from the
649 intersystem reduces PSI terminal electron acceptors and $\phi(R_0)$ = quantum yield of reduction
650 of PSI terminal electron acceptors; PI_{ABS} = performance index of photosynthetic efficiency and
651 PI_{Total} = energy conservation for reduction of PSI terminal acceptors respectively.

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653



654

655 Figure 3. Catalase and TBARS content (\pm SD) measured in *L. minor* after 7 d exposure to
656 zearalenone (CAS number 17924-92-4) in a static study at $24 \pm 1^\circ\text{C}$.

657 Footnote – significant difference only seen between the TBARS content in the dilution water
658 control and 14.4 mg ZON/L.

659

660 **Table 1.** Growth responses of *P. subcapitata* exposed to zearalenone (CAS number 17924-
 661 92-4) in a 72 h static study at $23.8 \pm 1^\circ\text{C}$ (concentration measured using UV-Vis spectrometry
 662 with an LOD of 0.18 mg ZON/L) with 0.2 mg Zinc/L as a positive control.

663

Nominal concentration, measured in brackets (mg ZON/L)	Mean endpoint at 72h	Mean inhibition of algal growth (%)	
	Cell density (cells/ml $\times 10^5$)	Average Specific Growth Rate (ASGR)	Yield
0 (< 0.18)	3.1 ± 0.14	-	-
0.032 (< 0.18)	2.7 ± 0.12	4	15
0.1 (< 0.18)	2.8 ± 0.14	3	11
0.32 (0.23)	2.6 ± 0.45	5	17 ^a
1.0 (0.83)	1.6 ± 0.14	16 ^a	48 ^a
3.2 (3.12)	1.1 ± 0.08	24 ^a	64 ^a
0.2 (Zn)	2.3 ± 0.24	7 ^a	26 ^a
EC ₂₀ ($\pm 95\%$ CI)	-	1.72 (1.25 - 2.4)	0.19 (0.08 - 0.38)
EC ₅₀ ($\pm 95\%$ CI)	-	> 3.2	0.92 (0.74 - 1.8)
NOEC	-	0.23	0.1
LOEC	-	0.83	0.23

674 Footnote - ^a Significantly different ($P < 0.05$) from control treatment

675 Summary effect values calculated with measured values where possible.

676

677 **Table 2.** Growth responses (\pm SD) of *L. minor* exposed to zearalenone (CAS number 17924-92-
678 4) in a 7 d static study at $24 \pm 1^\circ\text{C}$ (concentration measured using UV-Vis spectrometry with
679 an LOD of 0.18 mg ZON/L).

Nominal concentration, measured in brackets (mg ZON/L)	Mean measured endpoints		Calculated inhibition of growth (%)			
			Average Specific Growth Rate		Yield	
	Frond number	Frond area (mm ²)	Frond number	Frond area (mm ²)	Frond number	Frond area (mm ²)
0 (< 0.18)	23 (\pm 2.6)	115.3 (\pm 11.6)	-	-	-	-
0.1 (< 0.18)	23 (\pm 1.6)	116.8 (\pm 14.1)	1	-1	0	-2
0.32 (0.36)	21 (\pm 3.2)	100.7 (\pm 23.6)	5	7	10	14
1.0 (1.1)	22 (\pm 2.6)	98.4 (\pm 28.7)	3	10	5	17
3.2 (3.4)	23 (\pm 3.4)	122.6 (\pm 16.8)	-1	-4	0	-8
10 (11.4)	11 (\pm 1.8)	45.1 (\pm 6.3)	38 ^a	38 ^a	60 ^a	67 ^a
Positive control 2 (1.4) mg Zn/L	10 (\pm 1.3)	33.4 (\pm 5.0)	39 ^a	53 ^a	65 ^a	79 ^a
NOEC	-	-	3.4	3.4	3.4	3.4
LOEC	-	-	11.4	11.4	11.4	11.4
EC ₂₀ (\pm 95 % CI)			6.5 (3.5 - 11.3)	6.0 (3.5 - 11.3)	4.3 (3.5 - 11.3)	3.0 (3.5 - 11.3)
EC ₅₀ (\pm 95 % CI)	-	-	>11.4	>11.4	10.3 (3.5 - 11.3)	8.8 (3.5 - 11.3)

680

681 Footnote - ^a Significantly different ($P < 0.05$) from control treatment

682 Summary effect values calculated with measured values where possible.

683

684 **Table 3.** Maximal quantum efficiency of Photosystem (Fv/Fm) measured in *P. subcapitata*
685 (mean \pm SD) after 72 h exposure to zearalenone (CAS number 17924-92-4) in a static study at
686 $24 \pm 1^\circ\text{C}$ (concentration measured using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L).

Nominal concentration, measured in brackets (mg ZON/L)	Maximal quantum efficiency (Fv/Fm)
0 (< 0.18)	0.49 (\pm 0.002)
0.032 (< 0.18)	0.49 (\pm 0.001)
0.1 (< 0.18)	0.49 (\pm 0.003)
0.32 (0.23)	0.49 (\pm 0.004)
1.0 (0.83)	0.50 (\pm 0.005)
3.2 (3.12)	0.50 (\pm 0.011)
0.2 (Zn)	0.48 (\pm 0.009)

687

688