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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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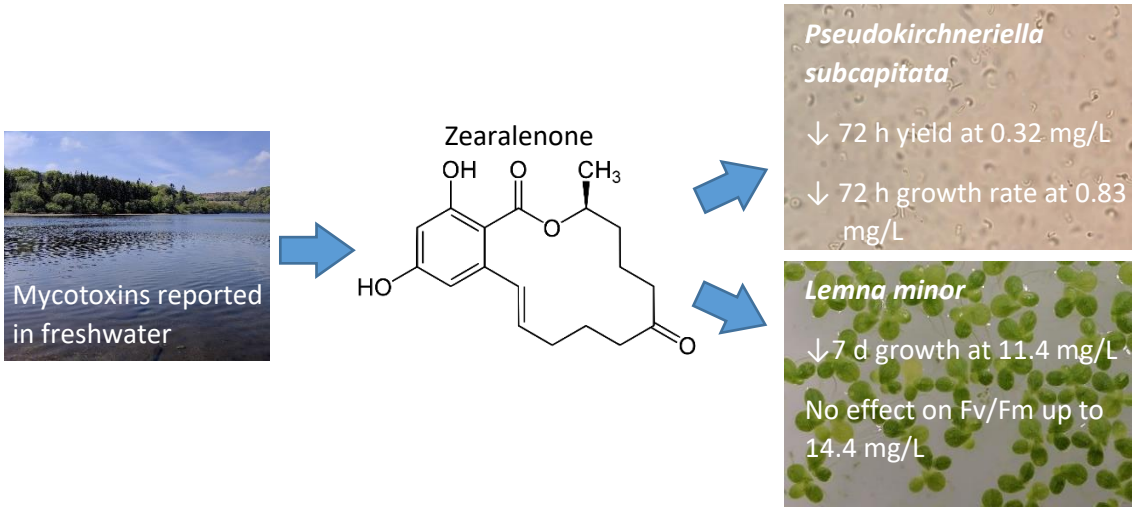
Highlights

- Zearalenone (ZON) is a commonly occurring mycotoxin in freshwater samples
- ZON aquatic toxicity data are limited hence algae and macrophytes were studied
- 72 h algae EC_{50} = 0.92; NOEC = 0.1; LOEC = 0.23 mg ZON/L, 7 d macrophyte EC_{50} = 8.8; NOEC = 3.4; LOEC = 11.4 mg ZON/L
- PSII efficiency unaffected by ZON in algae and macrophyte exposures

Abstract

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

Graphical abstract



Keywords

Emerging chemical, fungal toxin, phytotoxicity, risk assessment

1. Introduction

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

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

Studies in the US and Poland have found low levels (0.7 - 96 ng/L) of ZON in streams and rivers with the main sources being agricultural runoff and wastewater treatment plant effluent (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 % (EC_{50}) of 50
99 µg/L and lethal concentration 50 % (LC_{50}) 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 *Selanastrium capricornutum* and
111 *Rhapidocelis 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

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

The aim of this study was to investigate the phytotoxicity of ZON as a chemical of emerging concern, employing the standardised OECD test guidelines for *L. minor* and *P. subcapitata*. This was achieved with a 7 d or 72 h growth inhibition study for each species respectively. Following this, physiological measures of photosynthetic performance and biochemical analysis of lipid peroxidation and catalase activity were performed. These were included as a preliminary investigation into MoA measures which can easily be added to the existing guideline framework and analysed for indication of pathways to be pursued to develop AOP's for the test chemical. For quality control purposes, zinc was used as a reference toxicant as per the UK Direct Toxicity Assessment approach (EA, 2007). Zinc is required as a micronutrient in plant growth but in excess causes phytotoxicity and reduces growth, previous literature is available for zinc toxicity in both species used in this study (Paixão et al., 2008; Lahive et al., 2011). All toxicity data for ZON and Zn reported from the microalgae and macrophyte experiments are expressed as measured concentrations unless stated otherwise.

2. Materials and methods

2.1. Cultures and exposure

A culture of *P. subcapitata* (type strain 278/4) was obtained from the Culture Collection of Algae and Protozoa and maintained in BG11 media made by diluting a sterile stock solution (Sigma Aldrich, Dorset, UK). Prior to experiments, a sub-culture was prepared and held under testing conditions of constant illumination (105-125 µmol $m^{-2}s^{-1}$) and placed on an orbital

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

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

2.2. Growth rate

Based on pilot studies for microalgae, ZON test solutions of nominal concentrations were zero (< 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 (mean measured concentrations in brackets with a limit of detection (LOD) of 0.18 mg ZON/L). A zinc positive control of 0.2 mg/L (made with zinc sulphate heptahydrate, CAS number 7446-20-0; Sigma Aldrich batch number: 31665; purity ≥ 99.5%) was used. The pH of test solutions

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

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

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 density) at the time i and j respectively, and t is the time period from i to j. Percentage inhibition of ASGR (% I_r) for each test solution, compared to the dilution water control, was calculated using:

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

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

Yield was determined, by the change in biomass (cell density) over 7 d in each test replicate. Mean inhibition of yield for each treatment was calculated by:

$\% I_y = (\underline{b_c - b_T})/b_c) \times 100$

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

For *L. minor* ZON test solutions were prepared for nominal concentrations of zero (< 0.18), 0.1 (< 0.18), 0.32 (0.36), 1.0 (1.1), 3.2 (3.4) and 10.0 (11.4) mg/L (mean measured concentration in brackets with an LOD of 0.18 mg ZON/L), plus a reference chemical measured exposure of 1.4 mg Zn/L positive control. Physio-chemical parameters were measured at the beginning 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/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/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 °C).

211

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

2.4. TBARS assay and catalase enzyme activity

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

To measure the catalase activity in the *Lemna* plant material, three replicates from each treatment, ZON concentrations 4.8 (5.2) , 8.1 (7.9) and 15.0 (14.4) mg/L (mean measured concentration in brackets) and the reference chemical mean measured exposure of 1.8 mg Zn/L, were weighed individually and manually crushed with a mortar and pestle (due to the low weight of *L. minor* in 14.4 mg ZON/L and 1.8 mg Zn/L treatments, two wells were combined for each replicate) in 100 mM phosphate buffer (pH 7) at a ratio of 1 mg (wet weight): 19 µl of buffer. Homogenates were centrifuged (10 000 *g* for 10 mins) and the supernatants collected for the catalase assay (method adapted from Beers & Sizer 1952; Aebi 1984). A kinetic method was used, where 200 µl of 10mM H_2O_2 was added to 50 µl of supernatant in a microplate and the decrease in absorbance (correlating to a decrease in H_2O_2) read at 3 s intervals for 3 mins at 240 nm. Five replicates were measured per sample.

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

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

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

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

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

2.6. Statistical Analyses of Algal and Macrophyte Data

Statistical analyses were performed using Minitab (Minitab Ltd., Coventry, UK) and GraphPad Prism (GraphPad Software, Inc, California, USA). Biological effects data (based on measured concentrations of Zn or ZON) were tested for significance ($P < 0.05$) using one-way analysis of variance with Dunnett's post-test or Kruskal Wallace with Dunn's post-test where appropriate, for normal with homogenous variances and non-normal distributions respectively. EC_{20} and EC_{50} values (with 95 % confidence intervals) were determined using non-linear regression and then by fitting sigmoidal curves to the data sets.

3. Results

3.1. Growth inhibition

The controls of *P. subcapitata* in the control media showed an average overall growth rate of 1.38 (SD 0.01), confirming the healthy status of the organism. Furthermore, all experimental treatments continued to increase in cell density until the end of the study (Figure 1.). However during the first 24 h, the cell density of the control and two lowest treatments (0.032 and 0.1) increased more than the higher treatments with both 0.83 and 3.1 mg ZON/L visibly not recovering from this by 72 h. Based on the calculated endpoints at the end of the experiment, there was a significant decrease in growth at 0.23 mg/L for 72 h Yield and at 0.83 mg/L for the 72 h Average Specific Growth Rate (ASGR) ($P < 0.05$) (Table 1). The EC_{50} values from this study

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

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

3.2. Chlorophyll fluorescence

During the growth inhibition test measures of Fv/Fm for *P. subcapitata* showed no significant differences (Table 3.), with a control mean of 0.49 and exposure means of 0.48 - 0.50 (SD < 0.012). The value for the control mean is lower than that reported in other studies of greater than 0.6 (Choi et al., 2012; Vannini et al., 2011), but consistent with historical control means at this laboratory therefore considered to be due to inter laboratory variation. Measures of chlorophyll fluorescence for the dark adapted *L. minor* were carried out in a second test and are shown in Figure 2. The maximum efficiency calculated by Fv/Fm was not affected by ZON exposure but to understand the tolerance of plants it is important to observe other chlorophyll parameters. Tfm, Area, ETo/RC, REo/RC, Ψ(Eo), φ(Eo), δ(Ro), φ(Ro), 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/F_0 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 EC_{50} 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 EC_{20} 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

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

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

ETo/RC and REo/RC, representing the energy flux from Q_A^- into the electron transport chain and reduction of PSI terminal acceptors on the electron acceptor side, this adds to the concept of electron transfer being the possible cause of reduced performance. The reduction in quantum yields and ratios Ψ(Eo), φ(Eo), δ(Ro) and φ(Ro) also suggest inhibition of electron movement between Q_A and the acceptor side of PSI.

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

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

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

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

5. Conclusions and regulatory context

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

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

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

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603

LIST OF FIGURES

LIST OF TABLES

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

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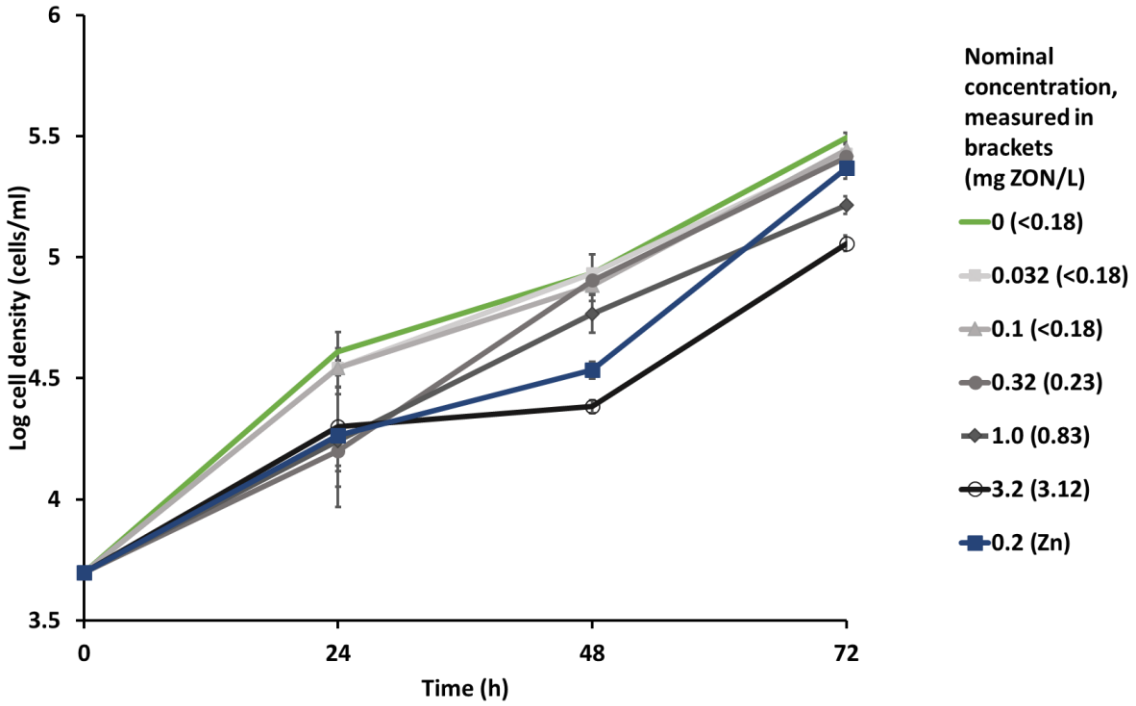

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631 Figure 1. Growth curves of *P. subcapitata* exposed to zearalenone (CAS number 17924-92-4)

632 in a 72 h static study at 23.8 ± 1°C with 0.2 mg Zinc/L as a positive control.

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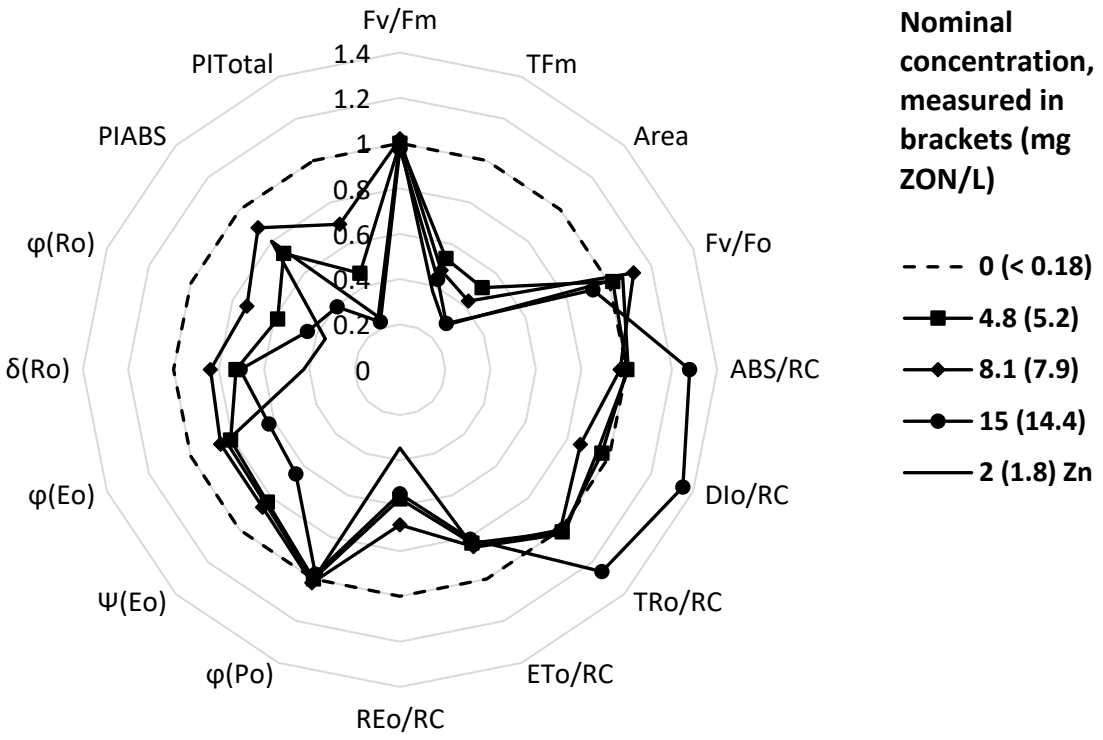

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635 **Figure 2.** Chlorophyll fluorescence parameters measured in *L. minor* after 7 d exposure to
636 zearalenone (CAS number 17924-92-4) in a static study at 24 ± 1°C (concentration measured
637 using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L).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

639 Footnote - Fv/Fm = maximal quantum efficiency of Photosystem II; TFm = time to reach
640 maximum chlorophyll fluorescence; Area = proportional to the pool size of the electron
641 acceptors Qa on the reducing side of Photosystem II; Fv/F_0 = quantum yield of the
642 photochemical and non-photochemical processes; ABS/RC = absorption of light energy per
643 reaction centre; DIo/RC = energy dissipation per reaction centre), TRo/RC (the energy trapping
644 rate per reaction centre; TRo/RC = energy trapping rate per reaction centre; ETo/RC =
645 photosynthetic electron transport rate per reaction centre; REo/RC = reduction of acceptors
646 in PSI per reaction centre; φ(Po) = maximum quantum yield of primary photochemistry; Ψ(Eo)
647 = probability of a trapped exciton moving an electron past Q_A^- to the electron transport chain;

φ(Eo) = quantum yield of electron transport from Q_A^-; δ(Ro) = probability an electron from the intersystem reduces PSI terminal electron acceptors and φ(Ro) = quantum yield of reduction of PSI terminal electron acceptors; PI_{ABS} = performance index of photosynthetic efficiency and PI_{Total} = energy conservation for reduction of PSI terminal acceptors respectively.

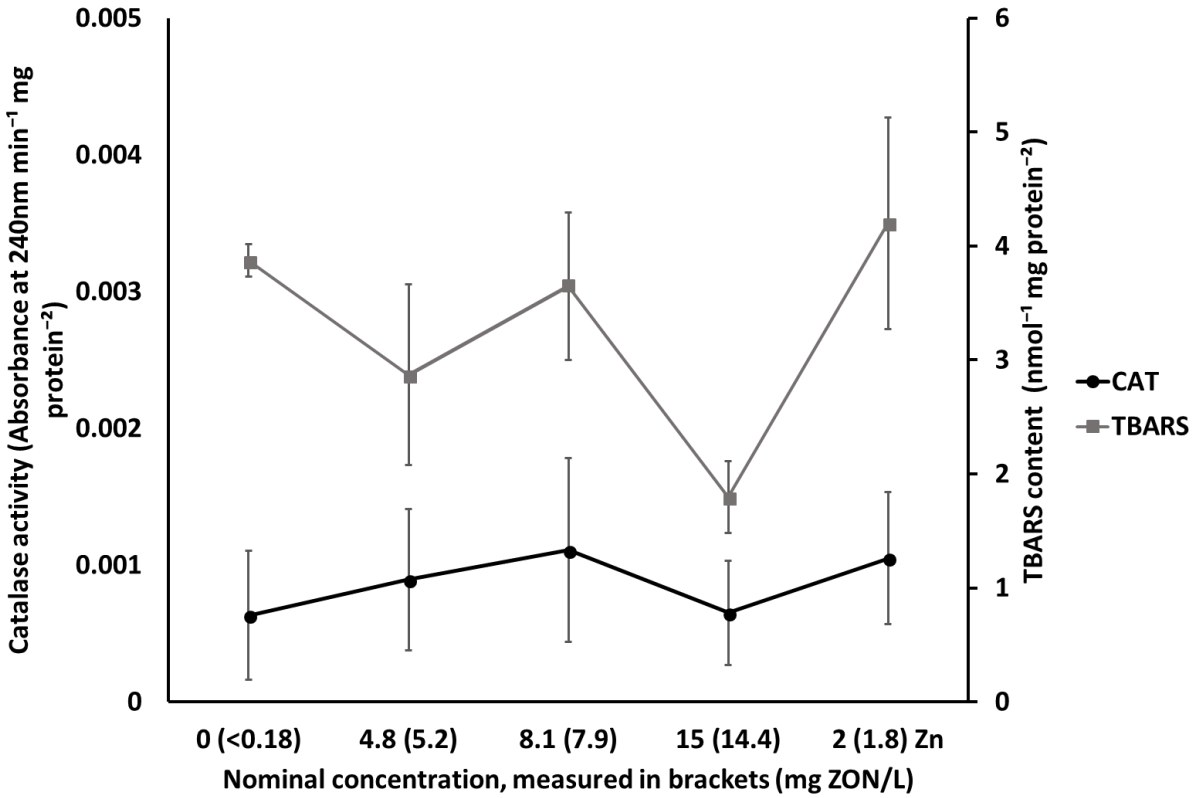

Figure 3. Catalase and TBARS content (± SD) measured in *L. minor* after 7 d exposure to zearalenone (CAS number 17924-92-4) in a static study at 24 ± 1°C.

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

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 ± 1°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 x 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} (± 95 % CI)	-	1.72 (1.25 - 2.4)	0.19 (0.08 - 0.37)
EC_{50} (± 95 % CI)	-	> 3.2	0.92 (0.74 - 1.8)
NOEC	-	0.23	0.1
LOEC	-	0.83	0.23

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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 (± SD) of *L. minor* exposed to zearalenone (CAS number 17924-92-

678 4) in a 7 d static study at 24 ± 1°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 (± 2.6)	115.3 (± 11.6)	-	-	-	-
0.1 (< 0.18)	23 (± 1.6)	116.8 (± 14.1)	1	-1	0	-2
0. 32 (0.36)	21 (± 3.2)	100.7 (± 23.6)	5	7	10	14
1.0 (1.1)	22 (± 2.6)	98.4 (± 28.7)	3	10	5	17
3.2 (3.4)	23 (± 3.4)	122.6 (± 16.8)	-1	-4	0	-8
10 (11.4)	11 (± 1.8)	45.1 (± 6.3)	38[a]	38[a]	60[a]	67[a]
Positive control 2 (1.4) mg Zn/L	10 (± 1.3)	33.4 (± 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} (± 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} (± 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 ± SD) after 72 h exposure to zearalenone (CAS number 17924-92-4) in a static study at
686 24 ± 1°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 (± 0.002)
0.032 (< 0.18)	0.49 (± 0.001)
0.1 (< 0.18)	0.49 (± 0.003)
0.32 (0.23)	0.49 (± 0.004)
1.0 (0.83)	0.50 (± 0.005)
3.2 (3.12)	0.50 (± 0.011)
0.2 (Zn)	0.48 (± 0.009)

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688