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Environmental risks to freshwater organisms from the mycotoxins deoxynivalenol and zearalenone using Species Sensitivity Distributions.

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Highlights

- Previously mycotoxin toxicity data for freshwater invertebrates has been lacking
- The most sensitive are *Daphnia* for deoxynivalenol and snail embryos for zearalenone
- Freshwater PNEC values based on SSDs were 1.4 µg DON/L and 8.6 µg ZON/L
- Deoxynivalenol levels approach PNEC in streams subject to high agricultural run-off
Abstract

In this study, laboratory experiments have addressed the acute toxicity of two common mycotoxins, deoxynivalenol (DON) and zearalenone (ZON), in a range of freshwater organisms (including rotifers Brachionus calyciflorus, insects Chironomus riparius (larvae), crustaceans Daphnia pulex and Thamnocephalus platyurus, cnidarians Hydra vulgaris, molluscs Lymnaea stagnalis (embryos) and Protozoa Tetrahymena thermophila). Acute EC$_{50}$ values highlight crustaceans as the most sensitive organisms to DON, with $T$. platyurus having a 24 h EC$_{50}$ of 0.14 and $D$. magna having a 48 h EC$_{50}$ of 0.13 mg DON/L. During exposures to ZON, $H$. vulgaris and $L$. stagnalis embryos showed the highest sensitivity; mortality EC$_{50}$ values were 1.1 (96 h) and 0.42 mg ZON/L (7 d), respectively. Combining these novel invertebrate toxicity results, along with recent published data for freshwater plant and fish toxicity for analysis of Species Sensitivity Distributions, provides freshwater HC$_{5}$ values of 5.2 µg DON/L and 43 µg ZON/L, respectively. Using highest reported environmental concentrations and following REACH guidelines, risk ratios calculated here show the risk of ZON to freshwater organisms is low. In contrast, DON may periodically be cause for concern in streams subject to high agricultural run-off, likely during certain times of year where cereal crops are susceptible to higher fungal infections rates and may pose increased risks due to climate change.

Keywords

Emerging chemical, ecological risk, hazard assessment, lethal toxicity
1. Introduction

Deoxynivalenol (DON) and zearalenone (ZON) are two of the prominent mycotoxins which regularly contaminate food, in particular cereal products. Both these natural toxins are produced by fungi within the *Fusarium* genus commonly found in cereals grown in temperate regions of America, Asia and Europe. ZON has a distinguishing factor in its structural similarity to oestrogen and its action as an oestrogen mimic, DON is often referred to as vomitoxin due to the observed emetic response in various species and has been seen to cause neural toxicity (Pestka, 2007; Bonnet et al., 2012). Mycotoxin production can be minimized by following good farming practice. However, due to the potential health risks, the EU has established Maximum Permitted Levels (MPLs) in food of 200 - 1750 µg/kg for DON and 20-100 µg/kg for ZON, dependant on the type of foodstuff (EC, 2006a), as well as Guidance Values for various products intended for animal feed (EC, 2006b).

The rising interest in mycotoxins as natural chemicals of emerging concern has led to studies into the concentrations found in freshwater environments. *Fusarium* sp. are commonly found on growing cereal field crops, and to a lesser extent on harvested crops during storage, hence soil run-off to surrounding water systems would be expected to be a main route of contamination. Agricultural run-off and its accompanying mycotoxin load vary considerably; dependent on season, crop cultivation, area hydrodynamics and whether fungi are present and producing mycotoxins (Wettstein & Bucheli, 2010; Kolpin et al., 2014). Wastewater treatment plants (WWTP) are considered a widespread permanent source of mycotoxins, and studies on their mycotoxin removal efficiency show only partial removal (Wettstein & Bucheli, 2010; Gromadzka et al., 2015). Investigations have shown DON to be of higher concern in comparison to ZON, with its occurrence being more prevalent and of
higher concentrations. Quantification of ZON in environmental samples has shown
concentrations are generally < 15 ng ZON/L with isolated periods of higher concentrations
occurring infrequently across sample sites; the highest value recorded for ZON is 96 ng
ZON/L at a river in an agricultural site in Iowa, USA (Gromadzka et al., 2009; Waśkiewicz et
al., 2012; Kolpin et al., 2014). Whereas DON has been found at higher concentrations in
numerous samples, the highest of which being 1662 ng DON/L in the same study of Iowa
sites with all locations having concentrations above 48.6 ng DON/L (Kolpin et al., 2014).

Research into the toxicity of mycotoxins to aquatic species has been mainly focused
on fish exposed via contaminated feed (Santacroce et al., 2008; Šišperová et al., 2015;
Woźni et al., 2015). There are relatively few studies on waterborne exposure and toxicity in
freshwater fish, with most of these reporting on effects in zebrafish. For example, DON
appears to be of little concern for zebrafish embryos but ZON toxicity has been
demonstrated in terms of both development and survival; 5 d development no observable
effect concentration (NOEC) 25 µg ZON/L, 5 d survival EC_{50} 890 µg ZON/L (Bakos et al., 2013;
Schwartz et al., 2010; Khezri et al., 2018). There are currently no published toxicity data for
invertebrates for ZON, but *Tetrahylena pyriformis* had a lowest observable effect
concentration (LOEC) of 0.6 mg DON/L at 150 h (Bijl et al., 1988) for the reproduction
endpoint. Phytotoxicity of both DON and ZON to microalgae and the macrophyte *Lemna
minor* has been shown, with the former being more sensitive to ZON (72 h
*Pseudokirchneriella subcapitata* growth EC_{50} of 1.2 mg ZON/L) and the later more sensitive
to DON (7 d LOEC of 0.25 mg DON/L) (Suzuki & Iwahashi, 2014; Vanhoutte et al., 2017;
Eagles et al., 2019).
The time, space and cost involved in maintaining cultures and carrying out tests with multiple species often results in toxicity studies being focused on only one or a few model species (e.g. zebrafish, daphnids and algae). This results in a poor understanding of inter species variability in sensitivity. Species Sensitivity Distributions (SSDs) are a useful tool in assessing inter species variation in sensitivity, and SSDs have now become commonplace in assessing the risks of chemicals and setting aquatic environmental safety thresholds (Belanger et al., 2017). Briefly, SSD encompass EC$_{50}$ results from single species toxicity tests and based on the distribution of these we can determine hazard concentration (HCp) which will protect a defined percentage (p) of species within the distribution (frequently the HC$_{5}$) (ECHA, 2008). This approach is considered to be beneficial in comparison to the alternative deterministic approach detailed in the ECHA assessment where the results of at least three studies, usually the standard test organisms algae, Daphnia and fish, are used with an appropriate assessment factor applied to the lowest EC$_{50}$. SSDs consider a wide range of species to generate a community relevant threshold rather than one based on model or known most sensitive species (Belanger et al., 2017).

The first aim of this study was to test the species sensitivity of various invertebrates (rotifer Brachionus calyciflorus, insect Chironomus riparius (larvae), crustaceans Daphnia pulex and Thamnocephalus platyurus, cnidarian Hydra vulgaris, mollusc Lymnaea stagnalis (embryos) and Protozoan Tetrahymena thermophila) to DON and ZON. All organisms used were wild-type strains except for the RENILYS strain of L. stagnalis. For consistency, the laboratory freshwater invertebrate data are derived from acute studies, based upon measured environmental concentrations, exposure duration to high concentrations of mycotoxins is likely to be acute rather than chronic. From the invertebrate studies an SSD, along with previously reported data for freshwater plants and fish also included, can be
determined for each mycotoxin. Finally, HC5 values with assessment factors were compared against measured environmental concentrations of mycotoxins to assess potential environmental risk.

Table 1. Details of the experimental conditions used for each toxicity test.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Age</th>
<th>Time</th>
<th>Endpoint</th>
<th>Medium</th>
<th>Temperature (°C), light: dark cycle (h)</th>
<th>Replicates, volume per replicate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachionus calyciflorus</td>
<td>&lt; 24 h</td>
<td>24 h</td>
<td>Immobilisation</td>
<td>Artificial freshwater</td>
<td>25 ± 1, darkness</td>
<td>Six replicates of five, 0.3 ml</td>
<td>MicroBioTests Inc. Gent, Belgium</td>
</tr>
<tr>
<td>Tetrahymanthermophilia</td>
<td>-</td>
<td>24 h</td>
<td>Reproduction</td>
<td>Artificial freshwater</td>
<td>30 ± 1, darkness</td>
<td>Three replicates, 2 ml</td>
<td>MicroBioTests Inc. Gent, Belgium</td>
</tr>
<tr>
<td>Thamnocephalus platyurus</td>
<td>&lt; 24 h</td>
<td>24 h</td>
<td>Immobilisation</td>
<td>Artificial freshwater</td>
<td>25 ± 1, 16:8</td>
<td>Three replicates of ten, 1 ml</td>
<td>MicroBioTests Inc. Gent, Belgium</td>
</tr>
<tr>
<td>Chironomus riparius</td>
<td>First instar larvae</td>
<td>48 h</td>
<td>Immobilisation</td>
<td>Dechlorinated tap water</td>
<td>20 ± 1, darkness</td>
<td>Twenty replicate individuals, 2 ml</td>
<td>(OECD, 2011)</td>
</tr>
<tr>
<td>Daphnia magna</td>
<td>&lt; 24 h</td>
<td>48 h</td>
<td>Immobilisation</td>
<td>ISO Artificial freshwater</td>
<td>20 ± 1, 16:8</td>
<td>Twenty replicate individuals, 2 ml</td>
<td>(OECD, 2004)</td>
</tr>
<tr>
<td>Hydra vulgaris</td>
<td>Nonbreading</td>
<td>96 h</td>
<td>Survival</td>
<td>Hydra medium</td>
<td>20 ± 1, 16:8</td>
<td>Ten replicates with one in each, 2 ml</td>
<td>(Zeeshan et al., 2016)</td>
</tr>
<tr>
<td>Lymnaea stagnalis</td>
<td>&lt; 24 h</td>
<td>7 d</td>
<td>Survival</td>
<td>ISO Artificial freshwater</td>
<td>20 ± 1, 16:8</td>
<td>Twenty replicate individuals, 2 ml</td>
<td>(Bandow and Weljte, 2012)</td>
</tr>
</tbody>
</table>

Note, *C. riparius* were provided by Fera Science Ltd. York, UK. Starter cultures of *D. magna* and *H. vulgaris* were purchased from Blades Biological, Kent, UK. *L. stagnalis* were originally from cultures generously provided by INRA Rennes, France. Survival of Hydra sp. was ranked according to Wilby’s guide for Hydra (1988). Details for each medium can be found in the respective reference papers, for *L. stagnalis* the artificial freshwater water described for *D. magna* was used.
2. Methods

2.1. Test design

Seven test organisms were used in total, and tests followed the methodology detailed in the relevant OECD test guidelines, previous literature or that provided in the purchased test kits (OECD, 2004; OECD, 2011; Bandow and Weljte, 2012; Zeeshan et al., 2016; MicroBioTests Inc. Gent, Belgium). Details on the duration, endpoint, medium, exposure conditions and replicates used for each test species are detailed in the supplementary information (Fig. S1 and Table 1). In all studies immobilisation or mortality was used as an endpoint to derive EC$_{50}$ values, further to this in the H. vulgaris study the sub-lethal effects on morphology were also monitored (Wilby, 1988).

Zinc was used as a positive control alongside each mycotoxin study, after having first developed a suitable concentration against the zinc EC$_{50}$. Details of zinc trial results, along with positive control values chosen and resulting inhibition in mycotoxin studies are provided in Table 2. The exception to this was C. riparius where no inhibition of greater than 50 % sensitivity was seen in the exposure hence no suitable zinc concentration was found to be used as a positive control.

For the exposures to mycotoxins, concentrations were set in the range of 0.01, 0.032, 0.1, 0.32, 1.0, 3.2 and 10 mg/L. Solvent controls of ethanol (EtOH) were used in deoxynivalenol studies and dimethyl sulfoxide (DMSO) in zearalenone. The solvent control concentration was either 3.2 or 10 µl/ml per study dependant on whether the highest test concentration in the study was 3.2 or 10 mg/L. The chemicals used were purchased from SIGMA Aldrich: zinc sulphate heptahydrate (CAS number 7446-20-0; purity ≥ 99.5%); zearalenone (CAS number 17924-92-4; purity ≥99%), deoxynivalenol (CAS number 51481-
151 10-8; purity 98%). Mycotoxin concentrations were tested at the beginning and the end of
152 studies with mean concentrations for the exposure period calculated, where studies were
153 24 h in length concentrations were based upon start concentrations only due to the small
154 volume of solution used across the replicates. Samples were analysed using LC-MS/MS
155 (Waters Acquity UPLC). Quantified deoxynivalenol concentrations (those ≤ 1 mg DON/L) are
156 supplied in the Table S1, these were stable over the exposures and within a close range to
157 the nominal values. ZON concentrations were not quantifiable and based on nominals.
158
159 Table 2. EC<sub>50</sub> values from zinc trial studies (based upon measured concentrations) and
160 measured initial concentration values for zinc positive control groups in the mycotoxin
161 invertebrate exposures.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>EC&lt;sub&gt;50 ± SE&lt;/sub&gt; (95 % CI)</th>
<th>Positive control in DON study</th>
<th>Measured concentration (mg/L)</th>
<th>Inhibition (%)</th>
<th>Positive control in ZON study</th>
<th>Measured concentration (mg/L)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. calyciflorus</td>
<td>1.7 ± 0.97 (0 – 3.6)</td>
<td>2.14</td>
<td>37</td>
<td>1.88</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. platyurus</td>
<td>0.36 ± 0.4 (0 - 1.3)</td>
<td>0.39</td>
<td>100</td>
<td>0.39</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. thermophila</td>
<td>10.9 ± 1.6 (9.3 – 16.8)</td>
<td>13.6</td>
<td>24</td>
<td>11.9</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. riparius</td>
<td>&gt; 100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. magna</td>
<td>4.1 ± 6.3 (0 – 16.6)</td>
<td>3.03</td>
<td>80</td>
<td>2.76</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. vulgaris</td>
<td>11.3 ± 10.3 (0 – 32)</td>
<td>1.86</td>
<td>100</td>
<td>1.92</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. stagnalis</td>
<td>0.61 ± 0.39 (0 - 1.4)</td>
<td>0.80</td>
<td>10</td>
<td>2.0</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note, Chemical analysis was performed using inductively coupled plasma emission
spectroscopy (ICP-OES, iCAP, Thermo Scientific) with a limit of detection (LOD) of 0.015 mg
Zn/L.

164 2.2. Statistical analysis

165 Plotting of dose response curves for each test organism were performed using R version 4.0.1
166 (R Core Team, 2020) and the drc-package (Ritz et al., 2015). Results of toxicity tests were
167 assessed using a three-parameter regression model where the distribution was assumed as
binary for those with survival/immobilisation as an endpoint and as normal for those with reproduction inhibition as an endpoint. The equation of the regression models:

\[ y = \frac{d}{1 + (\frac{x}{e})^b} \]

Where parameters are \( b \) which describes the slope surrounding \( e \) the \( EC_{50} \) (Ritz et al., 2015) and \( d \) the upper limit of the curve. The chi-squared \( (X^2) \) / F-value and \( P \) values from lack-of-fit/goodness-of-fit tests are provided with each model as appropriate. Further to this, \( EC_{10} \) and \( EC_{20} \) values were derived from the regression model. Subsequently, SSD models were plotted using the same regression model, with proportion of species affected plotted against dose (Posthuma et al., 2002; Jiang et al., 2018). From each SSD model the \( HC_5 \) was determined.

3. Results

During the mycotoxin studies the positive control groups exposed to zinc all showed inhibition relatable to the \( EC_{50} \) (Table 2) This, accompanied by lack of inhibition in the negative controls, provides confidence that the assay procedures were working as expected, and therefore that any inhibition seen in the mycotoxin treatments could be attributed to the exposure and not artefacts in the protocols.

3.1. Deoxynivalenol

Results for the DON exposures are shown in Fig. 1, the concentration of test solutions in the DON studies were measured and found to be suitably accurate to the nominal values as per OECD recommendations (mean quantified concentrations varied by < 20 % of the nominal with the exception of one case at 21 %) with minimal decrease in the concentrations over
the period of the studies (maximum quantified variation of 6 %), the results of the LC-
MS/MS measured concentrations are provided in Table S1.

In the 24 h studies no effect was seen in any of the solvent controls. In response to
DON, the *B. calyciflorus* study showed the least sensitivity (Fig. 1) with DON treatments
causing a maximum of 20 % inhibition at 0.32 and 1 mg DON/L. Both *B. calyciflorus* and *T.*
thermophila, had EC50 values > 3.2 mg DON/L.

During the 48 h period of the *C. riparius* study the ethanol control group had 10 %
immobilisation. Those individuals exposed to DON showed some recovery between the 24
and 48 h observations, with 1 mg DON/L being the only DON concentration to not have a
decrease in immobilisation between the two. The lowest concentration of 0.032 mg DON/L
had 20 % immobilisation at 24 h which decreased to 10 % by 48 h to match the inhibition of
the solvent controls. The highest concentration had a decrease from 50 to 40 %
immobilisation leading to a 48 h EC50 of > 3.2 mg DON/L. Due to the solvent control
inhibition percentage inhibition used in the later analysis were first normalised to this 10 %
inhibition.

For the crustaceans, a greater effect was seen in *T. platyurus* than any of the other 24 h
studies. The lowest concentration showed a minimal effect of 3 % immobilised, as
concentration increased from 0.32 to 1 and 3.2 mg DON/L immobilisation increased from 53
to 60 and 100 %. The EC50 for *T. platyurus* was 0.14 mg DON/L. *D. magna* responded in a
time dependant manner over 48 h. The solvent control had immobilisation of 0 % at 24 h
which increased to 5 % at 48 h. DON immobilisation occurred at 0.32 mg DON/L (20 %
immobilised) and above at 24 h and at all concentrations by 48 h. EC50 values for 24 and 48
h were 6.0 and 0.13 mg DON/L respectively.
Figure 1. Dose response curves of freshwater invertebrates exposed in acute laboratory studies to deoxynivalenol (DON), based on nominal concentrations with shaded bands indicating 95% CI. Model equations along with the chi-squared ($\chi^2$) / F-value.
(Degrees of freedom, \( n \)) and \( P \) value from goodness-of-fit tests are provided. Note, nominal concentrations were confirmed by measurements in the water samples.

The longer invertebrate studies with \( H. \) vulgaris and \( L. \) stagnalis embryos were less sensitive than the crustaceans. The \( H. \) vulgaris study with DON had no effect on survival in the solvent control or DON treatments. Sub lethal effects were noted in terms of abnormal morphology which included contraction of the body of the animals and the shortening or loss of tentacles. Abnormal morphology was seen at 48 h in the highest concentration of 3.2 mg DON/L with 50 % affected, this increased to 100 % by 72 h. By 72 h some of the lower treatments also began to develop abnormalities, at 0, 0.032 and 1 mg DON/L there had 10, 20 and 20 % inhibition which remained the same over the final 24 h of the study. The \( EC_{50} \) was generated in terms of survival for comparison with the other test species, but lay above the concentrations tested > 3.2 mg DON/L.

\( L. \) stagnalis embryos seemed relatively insensitive to DON during a 7 d static exposure with only the highest concentration of 3.2 mg DON/L resulting in embryo mortality of 60 %. There were no visible morphological abnormalities in the remaining embryos surviving at this concentration or any of the lower concentrations. The \( EC_{50} \) value for \( L. \) stagnalis was 3.1 mg DON/L.

### 3.2. Zearalenone

The dose response curves for ZON are shown in Fig. 2. For ZON, 24 h studies identified \( B. \) calyciflorus \( T. \) platyurus and \( T. \) thermophila as relatively insensitive to ZON with an \( EC_{50} \) values greater than the highest concentration tested of 10 mg ZON/L. Although \( B. \)
**calyciflorus** and **T. thermophilia** showed affects at multiple concentrations, inhibition values were ≤ 17% for **B. calyciflorus** and in the dose response relationship for **T. Thermophilia** only the highest concentration of 10 mg ZON/L caused significant inhibition of 30 %. These three organisms appear to be insensitive to ZON.

First instar larvae of **C. riparius** exposed for 48 h showed only 5 % inhibition due to the DMSO solvent control. ZON treatment showed a generally increasing toxicity with dose and time. By 48 h the lowest concentration tested had a higher effect of 15 % in comparison to the subsequent two treatments where immobilisation response was 10 %. Immobilisation increased to 25 and 60 % in the two highest treatments, 3.2 and 10 mg ZON/L, respectively. However, the regression analysis showed the EC$_{50}$ for **C. riparius** immobilisation lay outside the test range at > 10 mg ZON/L for 48 h.

The immobilisation of **D. magna** was also monitored over 48 h. The DMSO control had no effect on immobilisation. Only the highest ZON concentration of 10 mg ZON/L showed an effect, following a time-dependant response with 55 and 95 % mortality recorded at 24 and 48 h respectively. The limited dose response yet high effect level seen resulted in an EC$_{50}$ of 7.8 mg ZON/L.

The two most sensitive species tested for ZON were **H. vulgaris** and **L. stagnalis**, both studies also allowed for sub lethal effects to be observed. **H. vulgaris** showed no inhibition in the solvent controls. Sub lethal morphological effects were seen in 20 % of those exposed to 0.32 mg ZON/L by 24 h and increased to 30 % by 96 h. At 1.0 mg ZON/L, 20 % of individuals showed mortality at 24 h, those still alive at 1.0 mg ZON/L all exhibited abnormal morphology, no further deterioration to mortality but also no recovery to normal morphology occurred over the remaining time of the study. Mortality was seen in all
individuals exposed to 3.2 mg ZON/L by 24 h. The 96 h mortality resulted in an EC50 value of 1.1 mg ZON/L for *H. vulgaris.*
Figure 2. Dose response curves of freshwater invertebrates exposed in acute laboratory studies to zearalenone (ZON), based on nominal concentrations, with shaded bands indicating 95 % CI. Model equations along with the chi-squared ($X^2$)/ F-value (Degrees of freedom, $n$) and $P$ value from goodness-of-fit tests are provided.

$L. stagnalis$ was more sensitive to mortality than $H. vulgaris$, with an EC50 of 0.42 mg ZON/L. During the 7 d study with $L. stagnalis$ embryos no abnormal morphology or mortality was seen in the DMSO controls. Mortality in ZON exposed embryos generally occurred during the first few days of the study as the embryos did not develop past the initial morula development stage. Mortality occurred in all treatments and ranged from 10 to 90 %, this generally increased with dose. Apart from the 1 mg ZON/L treatment which showed a lower response than expected with only 10 % mortality.

4. **Species sensitivity distributions**

In order to construct SSD models, a literature search was conducted for any previous freshwater toxicity data available. Two previous freshwater invertebrate studies with DON were found. A protozoan study with *Tetrahymena pyriformis* with a reproduction LOEC of 0.6 mg DON/L at 150 h (Bijl et al., 1988). This LOEC falls in line with the results of the protozoa data generated in this study, as our result was more sensitive it was used in the SSD for *Tetrahymena* sp. A multi-generational study with the nematode *Caenorhabditis elegans* was also found, where acute growth effects on parental generation after 24 h provided an EC50 of 372 mg DON/L, however no survival data were provided so this organism was not included in the analysis (Zhou et al., 2017).
There is also data for freshwater plants available for DON. The microalgae *Chlamydomonas reinhardtii* had a relatively insensitive LOEC of 10 mg DON/L after a 150 h exposure (Suzuki & Iwahashi, 2014). Although EC$_{50}$ values were not provided for *C. reinhardtii*, based on the growth curves provided in the 10 mg DON/L exposure at 72 h (the length of a standard algae inhibition study, OECD, 2011), growth appeared to be at roughly 40%. The adjacent treatments of 1 and 25 mg DON/L appeared to have 0 and 100% inhibition respectively. A dose response curve was generated using these inhibition values and estimated an EC$_{50}$ value of 10.4 mg DON/L to be used in the SSD. A 7 d *L. minor* study had a LOEC of 0.25 mg DON/L (Vanhoutte et al., 2017) which falls in the same range as the most sensitive values reported here, at 0.5 mg DON/L inhibition was given as 41 ± 12%. Reading from the growth curves for frond number and frond area EC$_{50}$ values were around 0.5 and 0.6 mg DON/L respectively. Hence, for the SSD an EC$_{50}$ value of 0.55 mg DON/L was used.

Zebrafish embryos have reported as resilient to DON exposures with no effects observed when embryos were exposed to aqueous levels of up to 100 mM DON (REF). Only when embryos were injected with DON were 96 hpf EC$_{50}$ values generated for the following endpoints: hatching 1.65 mM, deformity 1.09 mM and mortality 2.57 mM (Khezri et al., 2018). Therefore, zebrafish were included in the rank for the DON SSD but no EC$_{50}$ value was used in the regression.

For ZON no previous invertebrate data were found, but Eagles et al. (2019) reported growth EC$_{50}$ values for both microalgae and macrophytes. In the 7 d macrophyte study with *L. minor* the most sensitive endpoint was growth in terms of yield of frond area, with an EC$_{50}$ of 8.8 mg ZON/L. The reported 72 h growth EC$_{50}$ for the microalgae...
Pseudokirchneriella subcapitata was 1.2 mg ZON/L. Unlike DON, ZON has been reported to induce toxic effects in zebrafish embryos including sub lethal abnormalities such as oedema, spinal curvature, pigmentation and reduced hatching success (Bakos et al., 2013). For the SSD, the reported 5 d zebrafish mortality EC$_{50}$ of 0.89 mg ZON/L was used (Bakos et al., 2013).

The EC$_{50}$ values generated in our studies along with those discussed from the literature for DON and ZON (listed in Table 3) were used to construct SSD models, these are shown in Fig. 3. All available species, eleven for DON and ten for ZON, were included in the rank, however, only those with specified EC$_{50}$ values were considered in the regression fit. The resulting HC$_{5}$ values from these regression models, which protects 95% of species (at a 50% effect level), are 5.2 µg DON/L and 43 µg ZON/L.

Figure 3. Species sensitivity distributions curves with shaded bands indicating 95% CI.

Markers are EC$_{50}$ values from acute toxicity studies, those without markers had undetermined EC$_{50}$ values greater than the highest concentration tested and were therefore included in the rank but not in the SSD fit.
323 Table 3. A summary of the acute effect values from freshwater invertebrate laboratory studies with mycotoxins deoxynivalenol and zearalenone.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Duration</th>
<th>Organism</th>
<th>Endpoint</th>
<th>Summary effect values (mg/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EC_{50} ± SE</td>
<td>EC_{50} ± SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(95 % CI)</td>
<td></td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>24 h</td>
<td><em>Brachionus calyciflorus</em></td>
<td>Survival</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24 h</td>
<td><em>Tetrahymena thermophila</em></td>
<td>Reproduction</td>
<td>0.07 ± 0.12</td>
<td>0.32 ± 0.33</td>
<td>3.9 ± 2.4 (0 – 9.2)</td>
</tr>
<tr>
<td>24 h</td>
<td><em>Thamnocephalus platyurus</em></td>
<td>Immobilisation</td>
<td>0.03 ± 0.03</td>
<td>0.06 ± 0.04</td>
<td>0.14 ± 0.07 (0.0 – 0.29)</td>
</tr>
<tr>
<td>48 h</td>
<td><em>Chironomus riparius</em></td>
<td>Immobilisation</td>
<td>-</td>
<td>-</td>
<td>&gt; 3.2</td>
</tr>
<tr>
<td>48 h</td>
<td><em>Daphnia magna</em></td>
<td>Immobilisation</td>
<td>0.05 ± 0.03</td>
<td>0.07 ± 0.03</td>
<td>0.13 ± 0.04 (0.03 – 0.22)</td>
</tr>
<tr>
<td>72 h</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>Growth</td>
<td>-</td>
<td>-</td>
<td>10.4\textsuperscript{a}</td>
</tr>
<tr>
<td>96 h</td>
<td><em>Danio rerio</em></td>
<td>Survival</td>
<td>-</td>
<td>-</td>
<td>&gt; 10 mM</td>
</tr>
<tr>
<td>96 h</td>
<td><em>Hydra vulgaris</em></td>
<td>Survival</td>
<td>-</td>
<td>-</td>
<td>&gt; 3.2</td>
</tr>
<tr>
<td>7 d</td>
<td><em>Lemna minor</em></td>
<td>Growth</td>
<td>-</td>
<td>-</td>
<td>0.55\textsuperscript{a}</td>
</tr>
<tr>
<td>7 d</td>
<td><em>Lymnaea stagnalis</em> (embryo)</td>
<td>Survival</td>
<td>2.35 ± 5.9</td>
<td>2.3 ± 4.5</td>
<td>3.1 ± 1.28 (0.5-5.6)</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>24 h</td>
<td><em>Brachionus calyciflorus</em></td>
<td>Survival</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24 h</td>
<td><em>Tetrahymena thermophila</em></td>
<td>Reproduction</td>
<td>-</td>
<td>-</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>24 h</td>
<td><em>Thamnocephalus platyurus</em></td>
<td>Immobilisation</td>
<td>-</td>
<td>-</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>48 h</td>
<td><em>Chironomus riparius</em></td>
<td>Immobilisation</td>
<td>-</td>
<td>-</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>48 h</td>
<td><em>Daphnia magna</em></td>
<td>Immobilisation</td>
<td>6.5 ± 20</td>
<td>7.0 ± 18</td>
<td>7.8 ± 13.8</td>
</tr>
<tr>
<td>72 h</td>
<td><em>Pseudokirchneriella subcapitata</em></td>
<td>Growth</td>
<td>-</td>
<td>0.19</td>
<td>0.92</td>
</tr>
<tr>
<td>96 h</td>
<td><em>Hydra vulgaris</em></td>
<td>Survival</td>
<td>0.92 ± 0.76</td>
<td>1.0 ± 0.18</td>
<td>1.1 ± 1.6 (0-4.2)</td>
</tr>
<tr>
<td>5 d</td>
<td><em>Danio rerio</em></td>
<td>Survival</td>
<td>-</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td>7 d</td>
<td><em>Lemna minor</em></td>
<td>Growth</td>
<td>-</td>
<td>3.0</td>
<td>8.8</td>
</tr>
<tr>
<td>7 d</td>
<td><em>Lymnaea stagnalis</em> (embryo)</td>
<td>Survival</td>
<td>0.01 ± 0.04</td>
<td>0.05 ± 0.1</td>
<td>0.42 ± 0.5 (0 – 1.4)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} EC_{50} values estimated from response values provided

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5. Discussion

5.1. Toxicity of DON and ZON
The novel data here present the toxic impacts of DON and ZON to a variety of invertebrates, a faction of freshwater ecosystems not previously studied in detail. Based upon the summary effect values for each mycotoxin (Table 3), the DON studies identified crustaceans as the most sensitive organisms with *T. platyurus* and *D. magna* having the lowest EC50 values whereas for ZON it was mollusc embryos. This high sensitivity in the mollusc embryo study was also highlighted in the SSD analysis (Fig 3.), where the zebrafish embryo study was the second lowest EC50 value for ZON (Bakos et al., 2013).

The only test organisms showing low toxicity to both mycotoxins, with undefined EC50 values, were *B. calyciflorus* and *C. riparius*. Within the acute DON study *C. riparius* also showed the ability to recover, with less individuals immobilised at 48 h than 24 h, suggesting potential to metabolise DON. This could be related to the induction of cytochrome P450 induction seen in acute exposures of *Chironomus* sp. to various environmental pollutants, including metals, aiding in detoxification (Fisher et al., 2003; Prakash et al. 2013). Sensitivity of *Hydra* varied between the two mycotoxins for lethality but for both DON and ZON morphological effects were seen. *Hydra* are capable of recovery by reformation of the tentacles which were observed to have begun shedding in the effected individuals. Therefore, although *Hydra* were one of the more sensitive organisms to ZON and morphological effects were noted for DON, wild *Hydra* exposed to mycotoxins may recover if the exposure period is limited, ample food is available and favourable conditions return.

The comparative toxicity seen in the literature values, used in the SSDs, for plants and zebrafish may represent the differing toxicological mechanisms of the two mycotoxins. DON has been linked to the spread of disease in plant hosts and programmed cell death defence in plants, and higher phytotoxicity was seen in the macrophyte *Lemna minor* for DON.
(Wagacha and Muthoni, 2007; Diamond et al., 2013; Vanhoutte et al., 2017). Whereas ZON is known as an oestrogenic compound and Bakos (2013) reported the sensitivity of zebrafish embryos to ZON may have been an indirect consequence of endocrine disruption. Overall, the range in sensitivities seen for the two mycotoxins across the invertebrate data of this study, as well as the contrasting sensitivity reported for each in literature with microalgae, *Lemna* sp. and zebrafish embryos, supports the value of the SSD approach in toxicology to encompass interspecies variation in toxicity. Particularly as DON has been seen to have no effect in the model zebrafish studies (Khezri et al., 2018), so may have received little interest in freshwater hazard assessments when compared to the lower ZON zebrafish EC₅₀ (Bakos et al., 2013), yet here DON had the lower HC₅ value from SSDs.

In the future, it would be desirable to build upon the available hazard profile of DON and ZON with a wider range of chronic studies, reflecting evidence of their widespread occurrence in the environment (Gromadzka et al., 2009; Waśkiewicz et al., 2012; Kolpin et al., 2014). From an ethical perspective, consideration should be given to plant and invertebrates where possible and not to replicate existing fish hazard assessments. For example, chronic data have been reported for the commonly studied zebrafish model with ZON, a 21 d reproduction study had a LOEC of 0.1 µg ZON/L compared with a longer life cycle study of 140 d having a LOEC of 0.32 µg ZON/L (Schwartz et al., 2010; Schwartz et al., 2013). To our knowledge, no similar fish studies have been published for DON, but with *Daphnia* being the most sensitive in the acute studies this would be a key organism to focus on in chronic studies.

### 5.2. Environmental relevance and risk assessment
Relevance of the effect values found here to environmental levels of DON and ZON can be evaluated using the recent studies quantifying mycotoxins in environmental samples. Commonly, measured concentrations of DON in freshwater are < 100 ng DON/L (Kolpin et al., 2014; Schenzel et al., 2012; Wettstein & Bucheli, 2010). But comparably higher concentrations of 112 - 1662 ng DON/L (Kolpin et al., 2014) were found in US streams and 59.5 - 642.4 ng DON/L in Portuguese estuarine studies (Ribeiro & Tiritan, 2015; Ribeiro et al., 2016; Ribeiro et al., 2016). Levels of ZON in European freshwaters have not been seen to exceed 50 ng ZON/L (Gromadzka et al., 2009; Waśkiewicz et al., 2012). The highest recorded environmental level of ZON is in US streams at concentrations of 61.5 and 96 ng ZON/L. However, these were the only two quantifiable levels during the study from a total of 116 samples (Kolpin et al., 2014). Further work is needed to understand potential seasonal trends in environmental concentrations of DON and ZON in the context of Fusarium sp. outbreaks in cereal crops in America, Asia and Europe. Based upon the current data available the highest recorded levels will be used, 1662 ng DON/L and 96 ng ZON/L, as a worst-case scenario environmental DON and ZON measured concentration.

Comparison of the EC₅₀ and HC₅ values to highest recorded DON and ZON concentrations are shown in Fig. 4. The confidence intervals around the derived parameters shown on the mycotoxin SSDs in Fig. 3 reflect the need for further data and do not allow for lower bound values to be considered. However, we can make a provisional assessment of risk, acknowledging the potential uncertainty associated with the derived HC₅ values, following the REACH guidance on chemical safety assessment for freshwater compartments. Considering the absence of confidence intervals, the use of acute values and no available mesocosm/field data for comparison to the laboratory derived values, the maximum recommended assessment factor for SSD derived PNEC values of 5 was used to account for
the uncertainty around the HC₅ values (ECHA, 2008). This gave PNEC values of 1.4 µg DON/L and 8.6 µg ZON/L. Using HREC as predicted environmental concentration (PEC), the PEC/PNEC ratio value generated for DON was 1.6 and 0.01 for ZON.

Figure 4. EC₅₀ values from freshwater toxicity tests performed in this study and those available or calculated from literature for deoxynivalenol and zearalenone. The highest concentration tested is plotted for those which had EC₅₀ values greater than. HC₅ values calculated from SSD models and highest recorded environmental concentrations (HREC) also shown.
Based upon these, the risk to freshwater ecosystems from ZON is expected to be low. Furthermore, the peaks in ZON concentrations seem to be minimal in environmental surveys (Gromadzka et al., 2009; Kolpin et al., 2014), and only occurring at single collection points attributed to a likely high presence of producing *Fusarium* sp. or favourable weather conditions increasing surface run-off. As with ZON, peaks in DON concentrations are similarly often confined to single time points but the concentrations reached can be considerably higher. This reflects the maximum concentrations of DON found in crops being higher than that of ZON (Gruber-Dorninger et al., 2019; Vogelgsang et al., 2017) and the higher water solubility of DON (Schenzel et al., 2012). In the US study (Kolpin et al., 2014) multiple samples had concentrations > 100 µg/L approaching the PNEC, but only the highest reported concentration used in the ratio calculation exceeded the PNEC. The risk ratio calculated here to be above 1 does warrant further consideration (ECHA, 2002), but any concern is limited to exceptional concentrations of DON, with the majority of environmental concentrations < 100 ng DON/L.

6. **Conclusions and knowledge gaps**

This study is one of the first to develop a comprehensive assessment of mycotoxin risks to aquatic life in freshwater. Based upon the experimental data for freshwater invertebrates, acute toxicity studies suggest that DON poses the greater toxic hazard to crustaceans *D. magna* and *T. platyurus*, whereas ZON was most toxic to mollusc embryos and cnidarians. Utilising all the experimental and published data for freshwater algae, macrophytes, invertebrates and fish (where available) allowed the successful use of the Species Sensitivity Distribution approach to derive HC5 values of 5.2 µg DON/L and 43 µg ZON/L. Based on currently available data, the ecotoxicological impact of exposure to individual mycotoxins at
low levels (generally < 100 ng/L: Gromadzka et al., 2009; Waśkiewicz et al., 2012; Kolpin et al., 2014) would appear to be low risk.

However, exposure to comparatively high levels of mycotoxins may occur over short time periods during the year and cumulative exposures to mixtures of mycotoxins should also be considered in future studies. For DON, with a calculated PEC/PNEC ratio of 1.6, there is a concern, but also data gaps on exposure profiles. Data on the fluctuations in freshwater concentrations of DON and ZON (and other mycotoxins) are needed to understand peak exposure durations and effectively assess acute and chronic risks associated with their cumulative exposure. Additional acute or chronic toxicity data will reduce uncertainty in any risk assessment of DON and ZON for freshwater; but risks to marine organisms also should be assessed. Climate change is increasing the incidence of Fusarium sp. fungal diseases in European cereal crops (Moretti et al., 2019), and so any risk assessment conducted now would be a baseline for future hazards of these emerging contaminants.

**Acknowledgements**

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