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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; Woźny 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 *Tetrahymena 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).

94 The time, space and cost involved in maintaining cultures and carrying out tests with
95 multiple species often results in toxicity studies being focused on only one or a few model
96 species (e.g. zebrafish, daphnids and algae). This results in a poor understanding of inter
97 species variability in sensitivity. Species Sensitivity Distributions (SSDs) are a useful tool in
98 assessing inter species variation in sensitivity, and SSDs have now become commonplace in
99 assessing the risks of chemicals and setting aquatic environmental safety thresholds
100 (Belanger et al., 2017). Briefly, SSD encompass EC_{50} results from single species toxicity tests
101 and based on the distribution of these we can determine hazard concentration (HCp) which
102 will protect a defined percentage (p) of species within the distribution (frequently the HC_5)
103 (ECHA, 2008). This approach is considered to be beneficial in comparison to the alternative
104 deterministic approach detailed in the ECHA assessment where the results of at least three
105 studies, usually the standard test organisms algae, *Daphnia* and fish, are used with an
106 appropriate assessment factor applied to the lowest EC_{50}. SSDs consider a wide range of
107 species to generate a community relevant threshold rather than one based on model or
108 known most sensitive species (Belanger et al., 2017).

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

118 determined for each mycotoxin. Finally, HC_5 values with assessment factors were compared
119 against measured environmental concentrations of mycotoxins to assess potential
120 environmental risk.

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

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

122 Note, *C. riparius* were provided by Fera Science Ltd. York, UK. Starter cultures of *D. magna*
123 and *H. vulgaris* were purchased from Blades Biological, Kent, UK, *L. stagnalis* were originally
124 from cultures generously provided by INRA Rennes, France. Survival of *Hydra* sp. was ranked
125 according to Wilby's guide for *Hydra* (1988). Details for each medium can be found in the
126 respective reference papers, for *L. stagnalis* the artificial freshwater water described for *D.*
127 *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-

10-8; purity 98%). Mycotoxin concentrations were tested at the beginning and the end of studies with mean concentrations for the exposure period calculated, where studies were 24 h in length concentrations were based upon start concentrations only due to the small volume of solution used across the replicates. Samples were analysed using LC-MS/MS (Waters Acquity UPLC). Quantified deoxynivalenol concentrations (those ≤ 1 mg DON/L) are supplied in the Table S1, these were stable over the exposures and within a close range to the nominal values. ZON concentrations were not quantifiable and based on nominals.

Table 2. EC_{50} values from zinc trial studies (based upon measured concentrations) and measured initial concentration values for zinc positive control groups in the mycotoxin invertebrate exposures.

Test organism	EC50 ± SE (95 % CI)	Positive control in DON study		Positive control in ZON study	
		Measured concentration (mg/L)	Inhibition (%)	Measured concentration (mg/L)	Inhibition (%)
B. calyciflorus	1.7 ± 0.97 (0 – 3.6)	2.14	37	1.88	37
T. platyurus	0.36 ± 0.4 (0 - 1.3)	0.39	100	0.39	100
T. thermophila	10.9 ± 1.6 (9.3 – 16.8)	13.6	24	11.9	40
C. riparius	> 100	-	-		-
D. magna	4.1 ± 6.3 (0 – 16.6)	3.03	80	2.76	70
H. vulgaris	11.3 ± 10.3 (0 - 32)	1.86	100	1.92	100
L. stagnalis	0.61 ± 0.39 (0 - 1.4)	0.80	10	2.0	55

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.

2.2. Statistical analysis

Plotting of dose response curves for each test organism were performed using R version 4.0.1 (R Core Team, 2020) and the drc-package (Ritz et al., 2015). Results of toxicity tests were 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

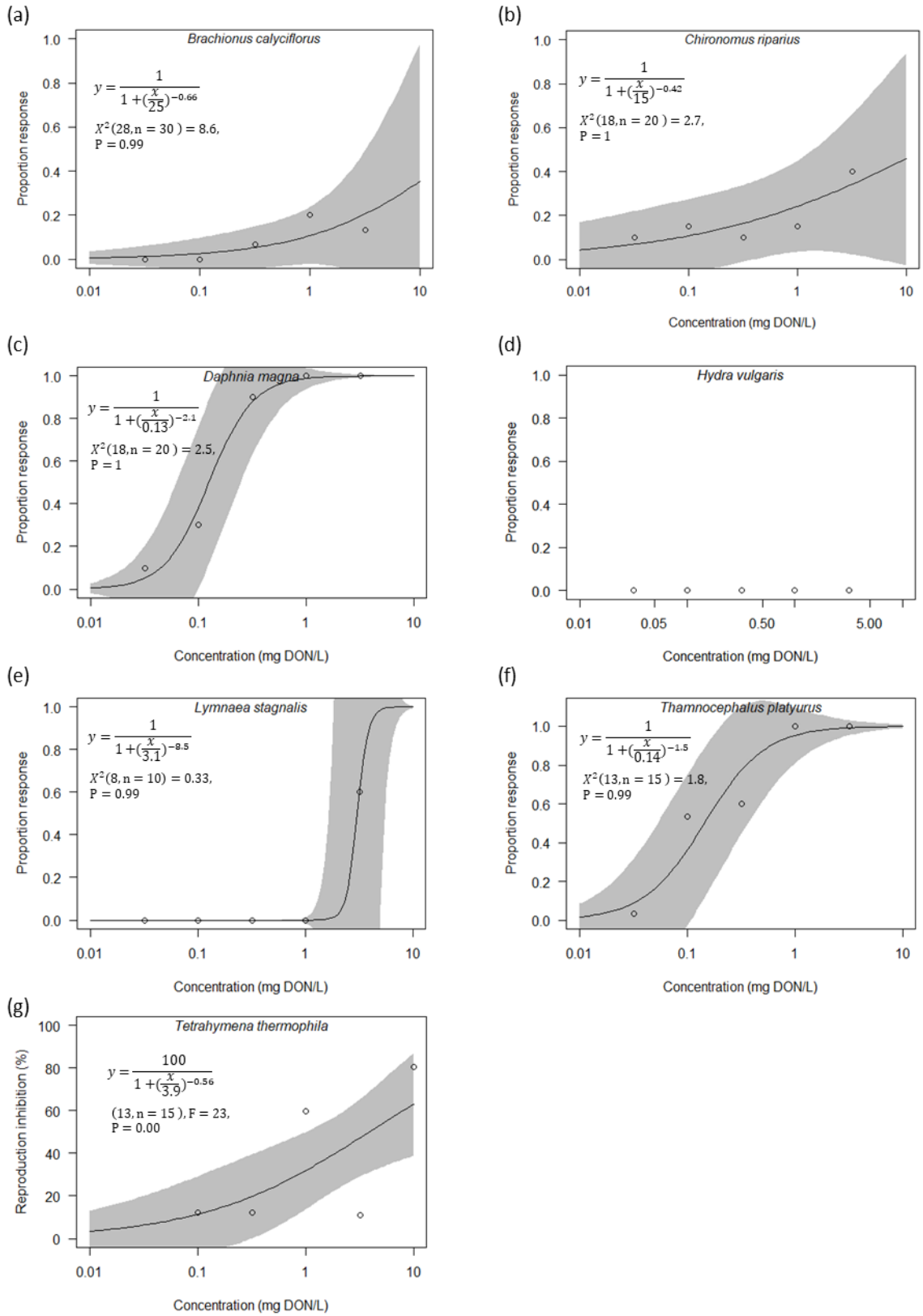
Results for the DON exposures are shown in Fig. 1, the concentration of test solutions in the DON studies were measured and found to be suitably accurate to the nominal values as per OECD recommendations (mean quantified concentrations varied by < 20 % of the nominal with the exception of one case at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

190 the period of the studies (maximum quantified variation of 6 %), the results of the LC-
191 MS/MS measured concentrations are provided in Table S1.

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

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

205 For the crustaceans, a greater effect was seen in *T. platyurus* than any of the other 24 h
206 studies. The lowest concentration showed a minimal effect of 3 % immobilised, as
207 concentration increased from 0.32 to 1 and 3.2 mg DON/L immobilisation increased from 53
208 to 60 and 100 %. The EC_{50} for *T. platyurus* was 0.14 mg DON/L. *D. magna* responded in a
209 time dependant manner over 48 h. The solvent control had immobilisation of 0 % at 24 h
210 which increased to 5 % at 48 h. DON immobilisation occurred at 0.32 mg DON/L (20 %
211 immobilised) and above at 24 h and at all concentrations by 48 h. EC_{50} values for 24 and 48
212 h were 6.0 and 0.13 mg DON/L respectively.



213 Figure 1. Dose response curves of freshwater invertebrates exposed in acute

214 laboratory studies to deoxynivalenol (DON), based on nominal concentrations with shaded

215 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. 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.*

238 *calyciflorus* and *T. thermophila* showed affects at multiple concentrations, inhibition values
239 were ≤ 17% for *B. calyciflorus* and in the dose response relationship for *T. Thermophila* only
240 the highest concentration of 10 mg ZON/L caused significant inhibition of 30 %. These three
241 organisms appear to be insensitive to ZON.

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

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

254 The two most sensitive species tested for ZON were *H. vulgaris* and *L. stagnalis*, both
255 studies also allowed for sub lethal effects to be observed. *H. vulgaris* showed no inhibition
256 in the solvent controls. Sub lethal morphological effects were seen in 20 % of those exposed
257 to 0.32 mg ZON/L by 24 h and increased to 30 % by 96 h. At 1.0 mg ZON/L, 20 % of
258 individuals showed mortality at 24 h, those still alive at 1.0 mg ZON/L all exhibited abnormal
259 morphology, no further deterioration to mortality but also no recovery to normal
260 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 EC_{50} value of 1.1 mg ZON/L for *H. vulgaris*.

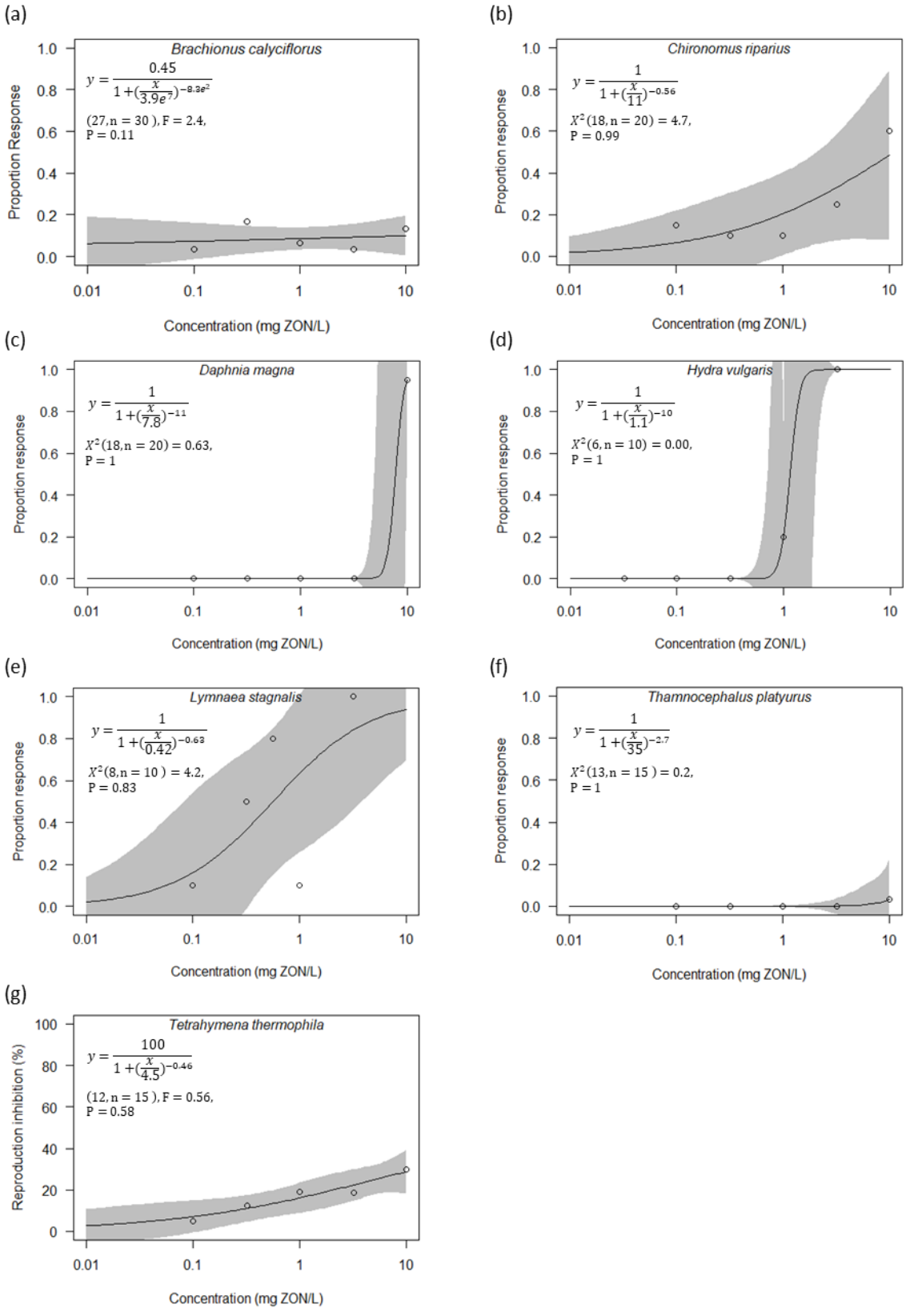


Figure 2. Dose response curves of freshwater invertebrates exposed in acute laboratory studies to zearalenone (ZON), based on nominal concentrations, with shaded bands indicating 95 % CI. Model equations along with the chi-squared (X^2)/ F-value (Degrees of freedom, *n*) and *P* value from goodness-of-fit 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 EC_{50} 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 EC50 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 EC50 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.

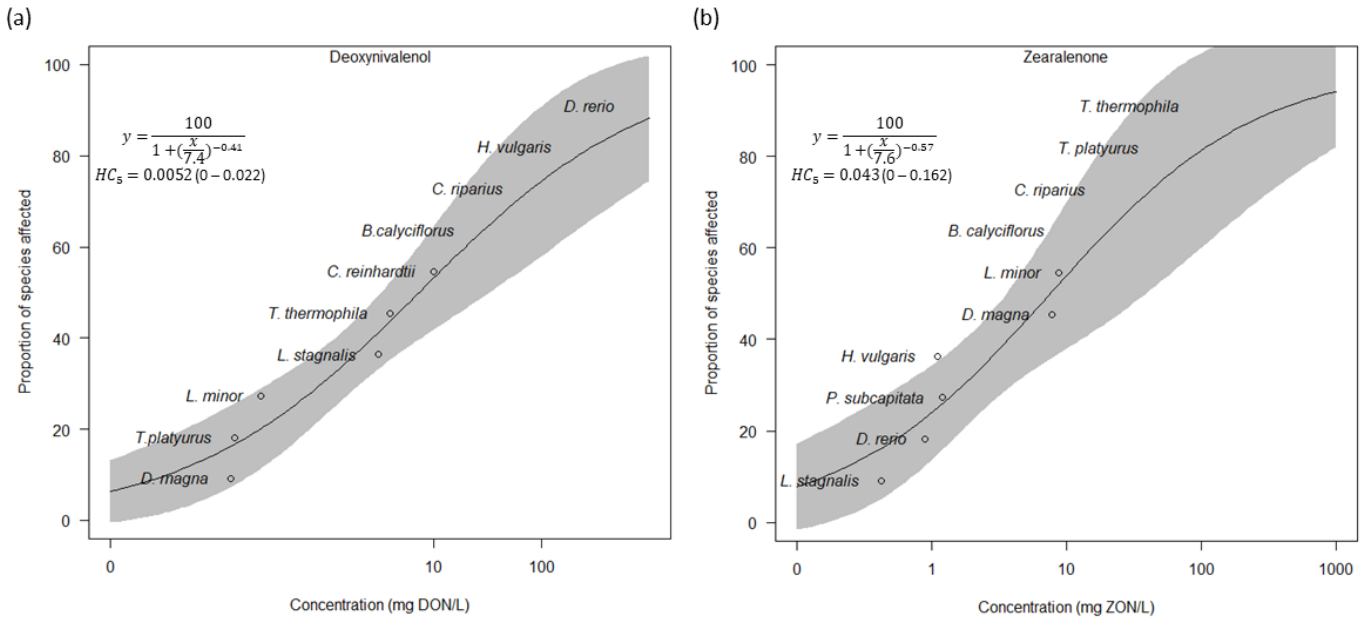


Figure 3. Species sensitivity distributions curves with shaded bands indicating 95 % CI. Markers are EC_{50} values from acute toxicity studies, those without markers had undetermined EC_{50} values greater than the highest concentration tested and were therefore included in the rank but not in the SSD fit.

323 Table 3. A summary of the acute effect values from freshwater invertebrate laboratory

324 studies with mycotoxins deoxynivalenol and zearalenone.

Chemical	Duration	Organism	Endpoint	Summary effect values (mg/L)			References
				EC_{10} ± SE	EC_{20} ± SE	EC_{50} ± SE (95 % CI)	
Deoxynivalenol	24 h	*Brachionus calyciflorus*	Survival	-	-	> 3.2	This study
	24 h	*Tetrahymena thermophila*	Reproduction	0.07 ± 0.12	0.32 ± 0.33	3.9 ± 2.4 (0 – 9.2)	This study
	24 h	*Thamnocephalus platyurus*	Immobilisation	0.03 ± 0.03	0.06 ± 0.04	0.14 ± 0.07 (0.0 – 0.29)	This study
	48 h	*Chironomus riparius*	Immobilisation	-	-	> 3.2	This study
	48 h	*Daphnia magna*	Immobilisation	0.05 ± 0.03	0.07 ± 0.03	0.13 ± 0.04 (0.03 – 0.22)	This study
	72 h	*Chlamydomonas reinhardtii*	Growth	-	-	10.4[a]	Suzuki & Iwahashi, 2014
	96 h	*Danio rerio*	Survival	-	-	> 10 mM	Khezri et al., 2018
	96 h	*Hydra vulgaris*	Survival	-	-	> 3.2	This study
	7 d	*Lemna minor*	Growth	-	-	0.55[a]	Vanhoutte et al., 2017
	7d	*Lymnaea stagnalis (*embryo)	Survival	2.35 ± 5.9	2.3 ± 4.5	3.1 ± 1.28 (0.5-5.6)	This study
Zearalenone	24 h	*Brachionus calyciflorus*	Survival	-	-	> 10	This study
	24 h	*Tetrahymena thermophila*	Reproduction	-	-	> 10	This study
	24 h	*Thamnocephalus platyurus*	Immobilisation	-	-	> 10	This study
	48 h	*Chironomus riparius*	Immobilisation	-	-	> 10	This study
	48 h	*Daphnia magna*	Immobilisation	6.5 ± 20	7.0 ± 18	7.8 ± 13.8	This study
	72 h	*Pseudokirchneriella subcapitata*	Growth	-	0.19	0.92	Eagles et al., 2019
	96 h	*Hydra vulgaris*	Survival	0.92 ± 0.76	1.0 ± 0.18	1.1 ± 1.6 (0– 4.2)	This study
	5 d	*Danio rerio*	Survival	-	-	0.89	Bakos et al., 2013
	7 d	*Lemna minor*	Growth	-	3.0	8.8	Eagles et al., 2019
	7 d	*Lymnaea stagnalis (embryo)*	Survival	0.01 ± 0.04	0.05 ± 0.1	0.42 ± 0.5 (0 – 1.4)	This study

325 [a]EC_{50} values estimated from response values provided

326

327 ## 5. Discussion

328 ### 5.1. Toxicity of DON and ZON

329 The novel data here presents the toxic impacts of DON and ZON to a variety of
330 invertebrates, a faction of freshwater ecosystems not previously studied in detail. Based
331 upon the summary effect values for each mycotoxin (Table 3), the DON studies identified
332 crustaceans as the most sensitive organisms with *T. platyurus* and *D. magna* having the
333 lowest EC_{50} values whereas for ZON it was mollusc embryos. This high sensitivity in the
334 mollusc embryo study was also highlighted in the SSD analysis (Fig 3.), where the zebrafish
335 embryo study was the second lowest EC_{50} value for ZON (Bakos et al., 2013).

336 The only test organisms showing low toxicity to both mycotoxins, with undefined EC_{50}
337 values, were *B. calyciflorus* and *C. riparius*. Within the acute DON study *C. riparius* also
338 showed the ability to recover, with less individuals immobilised at 48 h than 24 h, suggesting
339 potential to metabolise DON. This could be related to the induction of cytochrome P450
340 induction seen in acute exposures of *Chironomus* sp. to various environmental pollutants,
341 including metals, aiding in detoxification (Fisher et al., 2003; Prakash et al. 2013). Sensitivity
342 of *Hydra* varied between the two mycotoxins for lethality but for both DON and ZON
343 morphological effects were seen. *Hydra* are capable of recovery by reformation of the
344 tentacles which were observed to have begun shedding in the effected individuals.
345 Therefore, although *Hydra* were one of the more sensitive organisms to ZON and
346 morphological effects were noted for DON, wild *Hydra* exposed to mycotoxins may recover
347 if the exposure period is limited, ample food is available and favourable conditions return.

348 The comparative toxicity seen in the literature values, used in the SSDs, for plants and
349 zebrafish may represent the differing toxicological mechanisms of the two mycotoxins. DON
350 has been linked to the spread of disease in plant hosts and programmed cell death defence
351 in plants, and higher phytotoxicity was seen in the macrophyte *Lemna minor* for DON

(Wagacha and Muthomi, 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 EC_{50} (Bakos et al., 2013), yet here DON had the lower HC_5 value from SSDs.

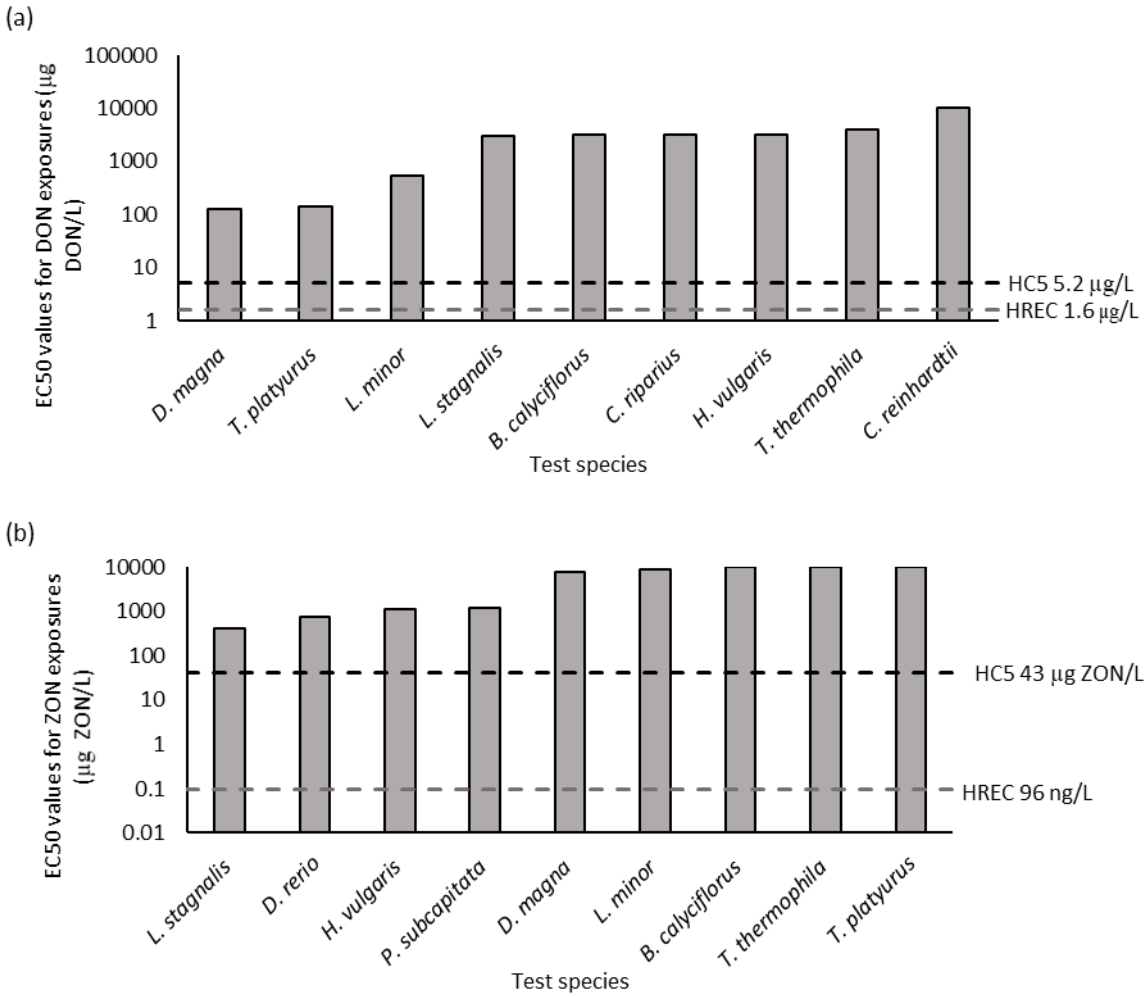
In the future, it would be desirable to build upon the available hazard profile of DON and ZON with a wider range of chronic studies, reflecting evidence of their widespread occurrence in the environment (Gromadzka et al., 2009; Waśkiewicz et al., 2012; Kolpin et al., 2014). From an ethical perspective, 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

374 Relevance of the effect values found here to environmental levels of DON and ZON can be
375 evaluated using the recent studies quantifying mycotoxins in environmental samples.
376 Commonly, measured concentrations of DON in freshwater are < 100 ng DON/L (Kolpin et
377 al., 2014; Schenzel et al., 2012; Wettstein & Bucheli, 2010). But comparably higher
378 concentrations of 112 - 1662 ng DON/L (Kolpin et al., 2014) were found in US streams and
379 59.5 - 642.4 ng DON/L in Portuguese estuarine studies (Ribeiro & Tiritan, 2015; Ribeiro et al.,
380 2016; Ribeiro et al., 2016). Levels of ZON in European freshwaters have not been seen to
381 exceed 50 ng ZON/L (Gromadzka et al., 2009; Waśkiewicz et al., 2012). The highest recorded
382 environmental level of ZON is in US streams at concentrations of 61.5 and 96 ng ZON/L.
383 However, these were the only two quantifiable levels during the study from a total of 116
384 samples (Kolpin et al., 2014). Further work is needed to understand potential seasonal
385 trends in environmental concentrations of DON and ZON in the context of *Fusarium* sp.
386 outbreaks in cereal crops in America, Asia and Europe. Based upon the current data
387 available the highest recorded levels will be used, 1662 ng DON/L and 96 ng ZON/L, as a
388 worst-case scenario environmental DON and ZON measured concentration.

389 Comparison of the EC_{50} and HC_5 values to highest recorded DON and ZON
390 concentrations are shown in Fig. 4. The confidence intervals around the derived parameters
391 shown on the mycotoxin SSDs in Fig. 3 reflect the need for further data and do not allow for
392 lower bound values to be considered. However, we can make a provisional assessment of
393 risk, acknowledging the potential uncertainty associated with the derived HC_5 values,
394 following the REACH guidance on chemical safety assessment for freshwater compartments.
395 Considering the absence of confidence intervals, the use of acute values and no available
396 mesocosm/field data for comparison to the laboratory derived values, the maximum
397 recommended assessment factor for SSD derived PNEC values of 5 was used to account for

398 the uncertainty around the HC_5 values (ECHA, 2008). This gave PNEC values of 1.4 µg DON/L
399 and 8.6 µg ZON/L. Using HREC as predicted environmental concentration (PEC), the
400 PEC/PNEC ratio value generated for DON was 1.6 and 0.01 for ZON.



401 Figure 4. EC_{50} values from freshwater toxicity tests performed in this study and those
402 available or calculated from literature for deoxynivalenol and zearalenone. The highest
403 concentration tested is plotted for those which had EC_{50} values greater than. HC_5 values
404 calculated from SSD models and highest recorded environmental concentrations (HREC) also
405 shown.

406

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 ug/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 HC_5 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.

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