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Evaluating the emerging environmental risks of mycotoxins in freshwater ecosystems.

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Evaluating the emerging environmental risks of mycotoxins in freshwater ecosystems.

by

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Evaluating the emerging environmental risks of mycotoxins in freshwater ecosystems.

By Emily Jayne Eagles

Abstract

Deoxynivalenol (DON) and zearalenone (ZON) are two of the prominent mycotoxins which regularly contaminate food, both of these natural toxins are produced by fungi within the *Fusarium* genus. With the known widespread occurrence of these toxins in cereal crops there has been rising interest into the potential transference into freshwaters. Multiple reports of mycotoxin occurrence in freshwater samples have led to their consideration as natural chemicals of emerging concern, particularly with ZON seen to act in an oestrogenic nature, yet the ecotoxicological data to support analysis of risk to freshwater ecosystems is lacking. The main aim of this work was to determine the hazard posed by DON and ZON to freshwater plants and invertebrates in laboratory exposures in order to perform a freshwater environmental risk assessment. The acute response was measured in various groups of freshwater organisms including macrophytes *Lemna minor*, microalgae *Pseudokirchneriella subcapitata*, rotifers *Brachionus calyciflorus*, insects *Chironomus riparius* (larvae), crustaceans *Daphnia pulex* and *Thamnocephalus platyurus,* cnidarians *Hydra vulgaris*, molluscs *Lymnaea stagnalis* (embryos) and Protozoa *Tetrahymena thermophila*. The resulting inhibition values in terms of growth, immobilisation survival or reproduction demonstrated DON poses the greater toxic hazard to crustaceans, whereas ZON was most toxic to mollusc embryos and cnidarians. The overall hazard posed to freshwater ecosystems was considered for each mycotoxin using species sensitivity distributions, generated with the plant and invertebrate data along with additional plant and fish data available in literature. These provided freshwater HC_5 values of 5.2 μg DON/L and 43 μg ZON/L, respectively. Global freshwater exposure concentration data was reviewed for comparison with the hazard concentrations, resulting risk ratios indicated no immediate risk in terms of acute toxicity, posed by DON and ZON. Agricultural streams were highlighted for further consideration in terms of DON loads received as this is where the highest reported concentration of DON was sourced, which was exceptionally high causing it to be the only instance where a measured concentration exceeded the predicted no effect level in terms of acute toxicity. For ZON lethal effects are not predicted to be of a concern but it may contribute to the overall oestrogenic load in freshwaters.

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List of Abbreviations

AOP; Adverse outcome pathway

ASGR; average specific growth rate

CAS; Chemical Abstracts Service

CEC; Chemical of emerging concern

CI; confidence interval

DEFRA; The Department for Environment, Food and Rural Affairs

DIo; energy dissipation

DMSO; Dimethyl sulfoxide

DON; Deoxynivalenol

DWC; Dilution water control

EA; Environment Agency

EC; Effect concentration

EFSA; The European Food Safety Authority

EPA; Environmental protection agency

ETo; electron transport rate

EU; European Union

FHB; Fusarium head blight

FM; maximum fluorescence

FOD; Frequency of detection

FOQ; Frequency of quantification

FV; variable fluorescence

GC; Gas chromatography

HC; Hazard concentration

HPLC; High Performance Liquid Chromatography

HREC; Highest recorded environmental concentration

ICP-OES; Inductively coupled plasma optical emission spectrometry

ISO; The International Organization for Standardization

LC; Lethal concentration

LOD; Limit of detection

LOEC; Lowest effect concentration

LOQ; Limit of quantification

MEC; Measured environmental concentration

MS; Mass spectrometry

ND; Not determined

NIV; Nivalenol

NOEC; No observable effect concentration

NPQ; Non-photochemical quenching

OECD; The Organisation for Economic Co-operation and Development

PEC; Predicted exposure concentration

PIABS; performance index on absorption basis

PNEC; Predicted no effect concentration

PSI; Photosystem I

PSII; Photosystem II

QA; Quinone A

QB; Quinone B

QSAR; Quantitative structure activity relationship

RC; reaction centre

REo; reduction of acceptors

ROS; Reactive oxygen species

SD; Standard deviation

SE; Standard error

SMILES; simplified molecular-input line-entry system

SPE; Solid phase extraction

SSD; Species sensitivity distribution

TBARS; thiobarbituric acid reactive substances

TF_M; time to reach maximum chlorophyll fluorescence

TRo; energy trapping rate

UPLC; ultra-performance liquid chromatography

UV; Ultraviolet

WFD; Water Framework Directive

WWTP; Wastewater treatment plant

ZOL; Zearalenol

ZON; Zearalenone

Chapter 1 Literature Review

1.1 Emerging Chemical Risks in Freshwater Ecosystems pollution

Contamination of freshwater systems by natural and synthetic chemicals is of growing concern worldwide, with over a third of renewable freshwater being used in agriculture, industry or domestic applications (Schwarzenbach *et al*., 2006). Reducing chemical impacts on the ecological health of Europe's rivers and lakes has been a priority for several decades, with major policy initiatives set in place aiming to improve their status to reach good quality by 2020 (European Commission, 2000). Despite these initiatives, the European Environment Agency reported that by 2015 over half of rivers and lakes were below good ecological status, failing to reach the targets anticipated by this point (EEA, 2015). Achieving such policy targets is no easy feat, given that anthropogenic pressures on freshwater quality and ecological status are constantly developing; species geographical distributions may be altered by the effects of climate change (Johnson *et al*., 2009; Radinger *et al*., 2017), hydrological morphology is altered through human modification of land use (Four *et al*., 2019; O'Briain *et al*., 2019; Fernandes *et al*., 2020) and pollution may occur from contamination (Morin *et al*., 2014; Kuzmanovic *et al*., 2016; Chi *et al*., 2017). Environmental risk assessments are vital in understanding whether contaminants, both natural and anthropogenic, are acting as pollutants or at what level they could become pollutants to the environment (ECHA, 2008; Chapman, 2007; Commission, 2003). With the concept being, contamination defines the presence of a substance but pollution is when contamination is to an extent which can cause biological effects (Chapman, 2007) and the environmental risk assessment process is built upon a second concept of risk = exposure x hazard.

The urbanisation of land brings with it two key contaminant inputs: industrial waste containing synthetic contaminants (Suthar *et al*., 2010; Tibbetts *et al*., 2018) and domestic wastewater containing pharmaceuticals and consumer product waste (Watkinson *et al*., 2009; Muir *et al*., 2017; Burns *et al*., 2018). In contrast, rural freshwater systems must cope with the run-off from agricultural land treated with pesticides (Claver *et al*., 2006; Bundschuh *et al*., 2014; Bonmatin *et al*., 2015; Bighiu *et al*., 2020) and fertilisers (Diebel and Vander Zanden, 2009; Audet *et al*., 2017), whilst often still being affected by potentially toxic elements from historical mining practices (Bird, 2016; Schillereff *et al*., 2016; Hurley *et al*., 2017). Improvements in wastewater treatment, under the European Commission Nitrates Directive (European Commission, 1991), European Union Water Framework Directive (WFD) (European Commission, 2000) and Urban Wastewater Treatment Directive (UWWTD) (European Commission, 2014), has helped in reduction of agricultural pollution, lowering nutrient levels which drive eutrophication, and is therefore a step in the right direction.

Due to the vast number of chemicals present in aquatic ecosystems it is not possible to perform environmental risk assessments or monitor all chemicals and the associated products (von der Ohe *et al*., 2011). Often, contaminants abundant in freshwaters may be at steady low concentrations (ng/L concentrations), these are more challenging with respect to completing environmental risk assessments because they are frequently below the detection limits of biological assays (Mater *et al.*, 2014). The WFD set out to protect water quality via the prioritisation of substances along with the phasing-out and minimising input of priority hazardous substances (European Commission, 2000). Substances were to be selected based upon evidence of hazard (aquatic toxicity or human toxicity) or exposure (evidence of widespread contamination or potential for widespread contamination) (European Commission, 2000). Later, in

2004 the European Commission also identified the need to address 'contaminants of emerging concern' (CECs) and established a network of reference laboratories and organisations to address CECs (Dulio *et al*., 2018). CECs are not necessarily new chemicals, i.e. not restricted to 'emerging contaminants', but those which have become apparent as present in the environment due to recent investigation or those for which new understanding now raises concern about their hazard (Sauve and Desrosiers, 2014). Furthermore, they are not regulated or routinely monitored, but may require future regulation (Dulio *et al*., 2018).

1.1.1 Guidelines for environmental risk assessments

To date regulations in place to protect freshwaters in the UK have followed the goals set out in EU Directives, the WFD (European Commission, 2000) to protect aquatic ecosystems through promoting good ecological status and sustainable water use. Further to this the UWWTD aims to minimise adverse ecological effects by (European Commission, 2014) regulating and monitoring the discharges from urban, and some industrial, wastewater inputs. Whereas both the Drinking Water Directive (European Commission, 1998) and EU Bathing Water Directive (European Commission, 2006a) aim to protect human health, the former through preventing adverse health effects from water consumption and the later through monitoring and reporting the water quality of bathing waters.

The ecotoxicology data required in environmental risk assessment, for determining the chemicals needed to be regulated within the directives, in Europe follows the guidance set out in the REACH programme (ECHA, 2008) for industrial chemicals and consumer products. While for agrochemicals EU guidance is set out in the Plant Protection Product Regulation (EFSA, 2013). These are similar in principle to

environmental risk assessment frameworks in other countries such as Canada (CCME, 2007), Japan (METI, 1973) and the USA (USEPA, 1992). Table 1.1 lists the minimum requirements under some of these frameworks (ECHA, 2008; CCME, 2007; EFSA, 2013). Internationally validated test guidelines, such as those published by the OECD and ISO, describe robust scientific protocols which are used across the world to assess the hazardous properties of chemicals to a variety of aquatic species, including plants (Tarazona *et al*., 2013; Nicolas *et al*., 2015), invertebrates (Versteeg *et al*., 1997; Beasley *et al*., 2015) and fish (Hutchinson *et al*., 2016).

Toxicity varies between species therefore it is necessary to consider a range of trophic levels in order to represent the whole community, Table 1.1 also lists the minimum required toxicity studies for assessing freshwater hazard under the frameworks. Toxic dose is also time dependant (Sanchez-Bayo and Goka, 2007) (dose = concentration x time) therefore a distinction is made between acute and chronic toxicity studies. Acute toxicity tests last up to a few days, as a minimum under REACH (ECHA, 2008) acute data would generally be collected for the algal growth inhibition test (TG 201) (primary producer) (OECD, 2011a), immobilisation of *Daphnia* (TG202) (primary consumer) (OECD, 2004) and fish lethality test (TG203) (secondary consumer) (OECD, 2019) (Table 1.1); with the standard length of acute studies with these organisms being 72 h, 48 h and 96 h respectively. The endpoint measured is expressed in terms of 50 % lethal (LC_{50}) (concentration at which 50 % of individuals are no longer alive) or effect concentrations (EC_{50}) (concentration at which 50 % of individuals are affected) (Figure 1.1.b.).

Table 1.1 Summary of data requirements for freshwater risk assessments and recommended assessment factors (AF) under key environmental risk assessment directives

Regulation programme	Type	Minimum test organisms required	Endpoint	Approach	AF	Notes	Reference
REACH	Acute	Fish, *Daphnia* sp. and algae	EC50 / LC50	Deterministic	1000	-	ECHA (2008)
	Chronic	Fish or *Daphnia* sp.	EC10 / NOEC	Deterministic	100	-	
		Two from Fish / *Daphnia* sp./ Algae	EC10 / NOEC	Deterministic	50	-	
		Fish, *Daphnia* sp. and algae	EC10 / NOEC	Deterministic	10	-	
		At least ten from a range of taxonomic groups	NOEC	Probabilistic	5 - 1	AF applied to HC5	
EFSA Plant Protection Product Regulation	Acute	*Daphnia* sp., arthropod, *Oncorhynchus mykiss*	EC50 / LC50	Deterministic	100	Insecticidal mode of action	(EFSA, 2013)
		Daphnia sp., *Oncorhynchus mykiss*	EC50 / LC50	Deterministic	100	Herbicidal mode of action	
		Daphnia sp., *Oncorhynchus mykiss*	EC50 / LC50	Deterministic	100	Other pesticides	
		Invertebrates	EC50 / LC50	Probabilistic	3 - 6	AF applied to HC5.	
		Fish (and other vertebrates)	EC50 / LC50	Probabilistic	9	AF applied to HC5.	
		Fish (and other vertebrates)	LC10 / NOEC	Probabilistic	3	AF applied to HC5.	
	Chronic	Fish, *Daphnia*/arthropod, *Chironomus* sp., green alga	EC10, EC50 (green alga)	Deterministic	10	Insecticidal mode of action	
		Fish, green alga, non-green alga, macrophyte, *Daphnia* sp., *Chironomus* sp. / *Lumbriculus* sp.	EC50 (algae/ plants), EC10	Deterministic	10	Herbicidal mode of action	
		Fish, green alga, *Daphnia* sp., *Chironomus* sp. / *Lumbriculus* sp.,	EC50 (algae), EC10	Deterministic	10	Other pesticides	
		Invertebrates	EC10 / NOEC	Probabilistic	3	AF applied to HC5.	
		Fish (and other vertebrates)	EC10 / NOEC	Probabilistic	3	AF applied to HC5.	

Table 1.1 Continued

Regulation programme	Type	Minimum test organisms required	Endpoint	Approach	AF	Notes	Reference
CEPA	Acute	Two fish (one salmonid), two invertebrates, two plant or algal (desirable, necessary if substance phyto-toxic)	EC50 / LC50	Deterministic	10	Type B2 guideline - based upon minimal quantity / quality data	(CCME, 2007)
		Three fish (one salmonid), three invertebrates, two plant or algal (desirable, necessary if substance phyto-toxic)	EC50 / LC50	Deterministic	10	Type B1 guideline (more than minimal data required but insufficient for SSD approach)	
				Probabilistic	-	Type A guideline (SSD), model should adequately fit data, ideally 10 – 15 data points available.	
	Chronic	Two fish (one salmonid), two invertebrates, two plant or algal (desirable, necessary if substance phyto-toxic)	ECx/ICx	Deterministic	10 / 20 / 100	Type B2 (based upon minimal quantity / quality data), safety factor can be increased if a short term study has a lower effect value than chronic, 20 if substance is non-persistent, 100 if persistent.	
		Three fish (one salmonid), three invertebrates, one plant or algal (three if substance phyto-toxic)	ECx/ICx	Deterministic	10	Type B1 guideline	
			EC10 / NOEC	Probabilistic	-	Type A guideline, Safety factor may be considered if any endpoints lie below the HC5.	

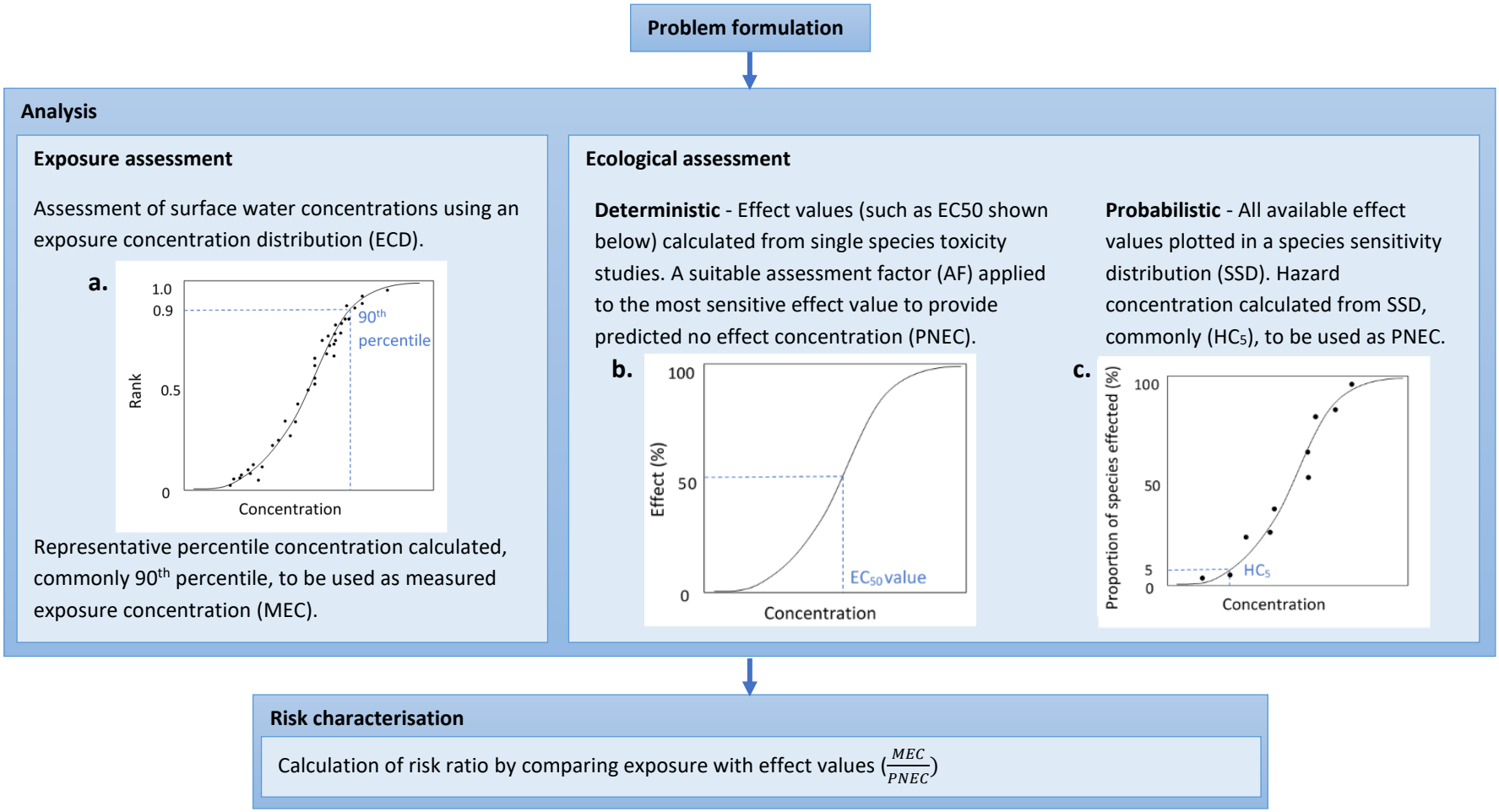


Figure 1.1 Steps in environmental risk assessment for freshwater ecosystems and examples of a. exposure concentration distribution, b. single species dose response curve, c. species sensitivity distribution.

Chronic toxicity is defined by exposing organisms for an extended period relative to the life cycle of the organism, chronic results usually report endpoints in terms of the No Observed Effect Concentration (NOEC) (highest concentration tested at which no significant effect is observed) or the EC_{10} value (concentration at which 10 % of individuals are affected). Under REACH guidelines (ECHA, 2008) these, as with acute studies, are usually from the standard test organisms' algae (OECD, 2011a), *Daphnia* (OECD, 2012) and fish (OECD, 2013).

To interpret individual species toxicity data in terms of a hazard concentration relevant to protect the freshwater community a Predicted No-Effect Concentration can be used (European Commission, 2003). There are two methods to do this, the first method applies appropriate assessment factors in an approach referred to as a deterministic hazard assessment (Belanger *et al*., 2017; Straub and Stewart, 2007). The assessment factors vary according to the data available in terms of number of tested organism and whether they are acute or chronic datasets (European Commission, 2003), as the goal is long-term whole ecosystem protection, Table 1.1 shows the assessment factors recommended in some of the guidance documents for environmental risk assessments (ECHA, 2008; CCME, 2007; EFSA, 2013). Generally, chronic datasets are preferable as they allow for a smaller assessment factor to be applied which does not have to account for extrapolation of short-term data into likely long-term effects (Kooijman, 1987). But equally acute data are vital when considering short periods of high contaminant input which may occur such as pulses of contamination during high precipitation events (Raby *et al*., 2018) or short term impacts of chemical spills (Bejarano and Farr, 2013; Mishra and Mohanty, 2014). Hence, the data used in environmental risk assessment should always integrate the most relevant available toxicity data in relation to the exposure concentrations.

The second approach for assessing toxicity data is a probabilistic hazard assessment (Straub and Stewart, 2007; Solomon *et al*., 2000; Wigger *et al*., 2020). This method of deriving a PNEC can still involve applying an assessment factor, such as between 5 - 1 under REACH guidance (Table 1.1), but first uses statistical extrapolation to calculate a hazardous concentration (HCx) from a species sensitivity distribution (SSD) (Li *et al*., 2020; ECHA, 2008; Coll *et al*., 2016). Forming an SSD involves combining toxicity data from range of species, usually ≥ 10, into one cumulative distribution which relates the exposure concentration to the proportion of species at risk (ECHA, 2008; Belanger *et al*., 2017). From this distribution, HC values which affect a certain percentage of the species, commonly HC5 the value at which 5 % of species are affected and 95 % protected, can be derived (Figure 1.1.c.). The main benefit over the deterministic approach is the consideration of a wider range of species, to generate a community relevant threshold rather than one based on model or known most sensitive species (Belanger *et al*., 2017).

To then interpret PNEC values into risk, the exposure also needs to be defined (ECHA, 2016), as risk = hazard x exposure. Exposure can be assessed either through modelled predicted environmental concentrations (PECs) (Letzel *et al*., 2009, Rico *et al*., 2013) or using an exposure concentration distribution, where measured environmental concentrations (MECs) are collated and percentile values calculated (Sanderson, 2003, Guo and Iwata, 2017), such as the 90^{th} percentile concentration (Figure 1.1.a). Finally, risk ratios are calculated using (PEC or MEC) / (PNEC), if the resulting value is below 1 there is no perceived risk if the PNEC is implemented, above 1 and the risk is not considered to be controlled and warrants further investigation through higher tier risk assessments or management of the chemical risk (ECHA, 2016).

In addition to the traditional endpoints, used in toxicity tests for environmental risk assessments, there is growing support to develop adverse outcome pathways for chemicals (Ankley *et al*., 2010; Browne *et al*., 2017; Burden *et al*., 2015). AOPs are a chain of events, starting with a molecular initiating event (MIE), followed by a series of key events (KE) and resulting in an adverse outcome (AO) (Burden *et al*., 2015). These events cover the action of a toxin through different biological levels of organisation, from MIE at a molecular level up to the AO (such as mortality) at the organism level and expected population effects (Figure 1.2) (Ankley *et al*., 2010; Villeneuve *et al*., 2014b; Fay *et al*., 2017; Knapen *et al*., 2018).

Developing a full AOP may require various different types of toxicity investigations, alongside traditional *in vivo* studies (in living organism), *in vitro* (outside living organism) (OECD, 2018a; Luckert *et al*., 2018; Ma *et al*., 2018) and *in chemico* (chemical reactivity) (OECD, 2020b; Stinckens *et al*., 2018) studies can be used to help determine cellular and molecular responses (Villeneuve *et al*., 2014a; Zhang *et al*., 2016b). Where only limited knowledge of the AOP is available, another term, mode of action, may be used, this predates the AOP concept and refers to toxic mechanisms where key events (often the initiating event or organismal adverse outcome) are known, but not the whole pathway of events (ECETOC, 2007). Increasingly AOPs are being supported by organisations (Browne *et al*., 2017; OECD, 2017; OECD, 2018b; OECD, 2018a). Developing the scientific knowledge around AOPs may provide a way forward in addressing the large number of risk assessments that need to be performed by allowing an increased use of *in silico* (computer simulated) predictive techniques (Thomas *et al*., 2019; Cohen *et al*., 2013). These *in silico* predictions use read across techniques or quantitative structure activity relationships (QSARs) (McKim *et al*., 1987; Russom *et al*., 1997) to link chemicals based upon chemical structure or mode of action. This could

allow a decrease in *in vivo* studies required which is a benefit in terms of animal welfare, helping minimise fish studies as advised in the 3Rs framework (Hutchinson *et al*., 2016; Burden *et al*., 2015; Scholz *et al*., 2013).

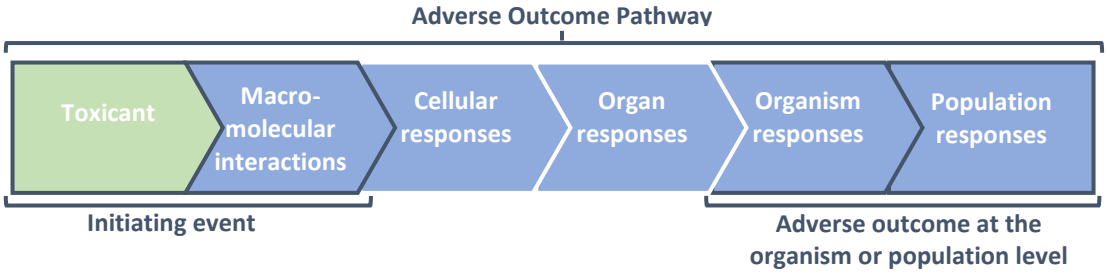


Figure 1.2 Diagram of adverse outcome pathway concept adapted from Ankley *et al*. (2010)

1.2 Natural toxins

Natural toxins are produced by living organisms and are not directly associated with aiding growth of the producing organism but have developed to provide a wide range of advantageous functions across organisms. Naturally produced toxins of relevance to freshwater environments are plant, algal and bacterial, and fungal toxins (Picardo *et al*., 2019). Algal toxins have been subject to a high level of interest for their waterborne hazard (Weirich and Miller, 2014; Hartnell *et al*., 2020, Oehrle *et al*., 2010) leading to The World Health Organisation (WHO) recommending a drinking water guideline for one group of cyanotoxins, the microcystins, of 1 µg/L (Bogialli *et al*., 2017; WHO, 2003). Plant toxins (phytotoxins) have been well researched in terms of their ecological role as a protection mechanism to deter herbivores (Wittstock and Gershenzon, 2002; Macel, 2011; Yazaki *et al*., 2017), but the regulation of plant toxins in freshwater or drinking waters have not been set to date with only a limited number investigated for their occurrence and potential risk (Picardo *et al*., 2019; Gunthardt *et al*., 2018). Fungi produce various types of secondary metabolites, with some proving beneficial for human use, such as penicillin (Brakhage *et al*., 2004; Penalva *et al*., 1998,

Fatima *et al*., 2019), those metabolites which instead cause disease in human and animals have been termed mycotoxins (Moss, 1996; Zain, 2011; Tola *et al*., 2016).

1.2.1 Mycotoxins

Fungi are pathogens foremost to plants and to a lesser extent animals (Bennett and Klich, 2003). Diseases from growth of fungi on animals are referred to as mycoses and those due to other exposures, such as to mycotoxins, are termed mycotoxicoses (Zain, 2011). Mycotoxin research emerged in the latter half of the 20th century (Cole *et al*., 1977; Stinson *et al*., 1981; Magan *et al*., 1984; Abbas *et al*., 1984). This followed the discovery that fungal toxins had been the cause of multiple epidemics in poultry and livestock. Notably turkey 'X' disease devastated turkey populations around London in 1960, initial research pinpointed the feed as the source of disease, yet chemical analysis of the feed showed no abnormalities and alternative investigation into bacterial toxins showed no presence either (Blount, 1961). It was later seen that a disease termed 'hemorrhagic syndrome' which had been affecting chickens for a decade could be replicated using fungi isolated from feed (Schumaier *et al*., 1961). The turkey 'X' disease was a result of *Aspergillus* sp. and instigated the term 'mycotoxins' for toxins stemming from fungi (Forgacs *et al*., 1962; Schumaier *et al*., 1961).

Mycotoxins are now known to be widespread (Gruber-Dorninger *et al*., 2019; Mousavi Khaneghah *et al*., 2019) with the producing fungi, *Aspergillus* sp*., Penicillium* sp. and *Fusarium* sp., readily growing crops in both temperate and tropical regions (Magembe *et al*., 2016). Crops can be contaminated both in the field and post-harvest during storage, those affected include cereals (Huong *et al*., 2016; Alkadri *et al*., 2014; Edwards, 2009b; Edwards, 2009a), nuts (Wang *et al*., 2018; Hidalgo-Ruiz *et al*., 2019; Molyneux *et al*., 2007), dried fruit (Heshmati *et al*., 2017; Azaiez *et al*., 2015; Kabak,

2016) and coffee (Garcia-Moraleja *et al*., 2015; Culliao and Barcelo, 2015; Paterson *et al*., 2014).

Fusarium Head Blight (FHB) is a common infection of grain crops (McMullen *et al*., 1997), which causes reduced yield and quality of grain. The physical effects of FHB, such as dark lesions or tissue bleaching (blight) on wheat or presence of pink masses of fungus after extended periods of wet weather on wheat or barley (Goswami and Kistler, 2004), cause vast economic losses (Dahl and Wilson, 2018; Windels, 2000). Also, contamination of infected crops with mycotoxins is a major secondary issue of FHB (Amarasinghe *et al*., 2019). Species of both the *Fusarium* and *Microdochium* genus can lead to FHB and many of the causal *Fusarium* sp. are toxigenic. The species of most concern in the cooler climates of Europe are *F. graminearum* and *F. culmorum*, both can produce tricothecenes and ZON (Brennan *et al*., 2005; Madgwick *et al*., 2011). Fungal infestations in crops have been on the rise due to altered farming techniques, such as reduced tillage, and climate change (Kolpin *et al*., 2014). Increased temperatures and longer periods of rainfall are associated with optimum growth and increased severity of FHB respectively (Brennan *et al*., 2005; Wegulo, 2012).

Presence of these mycotoxins in grains can lead to serious health issues due to substandard feed products, produced from contaminated grain, (Brennan *et al*., 2005). Consequently, research into the toxicity of mycotoxins to animals has been focussed on contaminated plant-based feed particularly in terrestrial species, toxic effects observed include protein synthesis inhibition, immunosuppression and carcinogenicity (Zain, 2011). Reduced production and health of aquaculture fish and increased mortality has resulted in significant economic losses in fish farming as a result of contaminated feed (Manning and Abbas, 2012; Adeyeye and Yildiz, 2016; Šišperová *et al*., 2015; Santacroce *et al*., 2008; Anater *et al*., 2016). Along with the animal health concerns and resulting

economic losses, due to mycotoxins in livestock and aquaculture feedstuff, the potential for mycotoxin residues to pass up the food chain to humans from animal tissue is also of concern. Therefore, due to their toxicity, regulations have been established for human and animal consumption of mycotoxins in many countries, regulations for DON and ZON in the EU (European Commission, 2006b) and USA (FDA, 2010) are shown in Table 1.2.

Table 1.2 Regulations for mycotoxins DON and ZON in products for human and animal consumption in the UK and USA.

Mycotoxin	Country	Product type	Product (exceptions)	Maximum levels (µg/kg) (exceptions)	Reference
DON	UK	Grains for products for human consumption	Unprocessed food (durum wheat, oats and maize)	1 250 (1 750)	(European Commission, 2006b)
			Cereals intended for direct human consumption	750	
			Pasta	750	
			Bread, pastries cereal snacks	500	
			Processed cereal-based foods and baby foods	200	
		Animal feedstuff	Cereals and cereal products (maize by-products).	8 (12)	(European Commission, 2006b)
			Complementary and complete feeding stuffs (pigs), (calves (< 4 months), lambs and kids)	5 (0.9) (2)	
	USA	Grains for products for human consumption	Distillers/brewers grains	30 000	(FDA, 2010)
			Finished wheat products	1 000	
		Animal feedstuff	Grains and grain by-products destined for ruminating beef and feedlot cattle older than 4 months and for chickens	10 000	(FDA, 2010)
			grains and grain by-products destined for swine and other animals	5 000	
ZON	UK	Grains for products for human consumption	Unprocessed cereals (maize)	100 (200)	(European Commission, 2006b)
			Cereals intended for direct human consumption (maize)	75 (200)	
			Bread, pastries cereal snacks	50	
			Processed cereal-based foods and baby foods	20	
		Animal feedstuff	Cereals and cereal products (maize by-products)	2 (3)	(European Commission, 2006b)
			Complementary and complete feeding stuffs (piglets and gilts), (sows and fattening pigs) calves, dairy cattle, sheep and goats	0.1 (0.25), (0.5)	

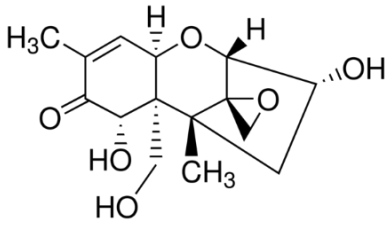
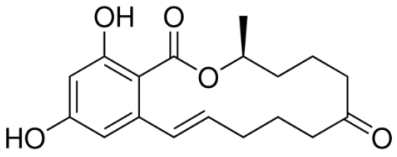
Despite the abundance and variety of mycotoxins produced, the biological functions of these small organic molecules produced as secondary metabolites are not clear. Ponts (2015) reviewed the supporting literature for mycotoxins being part of a stress response system in fungi, where mycotoxins have been suggested to be induced following detection of reactive oxygen species production in the host plant. The co-inoculation of crops with different *Fusarium* species also supported the stress response theory of mycotoxin production, those subjected to species competition had a significant increase in mycotoxin production by up to 1000 times (Xu *et al*., 2007). Further to this, mycotoxins may aid in the spread of fungi in the host plant, with DON seen to aid *F. graminearum* and *F. cumorum* spread in wheat (Gardiner *et al*., 2010; Wagacha and Muthomi, 2007). The specific way in which DON aids spread is not yet clear, but Diamond *et al*. (2013) found that low concentrations of DON resulted in inhibition of the programmed cell death response in plants. Therefore, may play a role in aiding the pathogen through interference of the plants defence response.

1.2.2 DON and ZON

DON and ZON were selected to focus on as they are two mycotoxins monitored in UK products as listed in Table 1.2 and DON in particular has been recorded in a survey of UK wheat crops from 2006 – 2013 (Edwards, 2018), with quantified concentrations in ≥ 95 % of samples for 4 of the 8 years monitored. Although, ZON was quantified in this same study at lower concentrations, with the overall means for each mycotoxin (between 2006 - 2013) being 29 µg ZON/kg compared to 261 µg DON/kg, there is previously published literature with calculated ecotoxicological summary effect values for zebrafish so this offers reference values for comparison of species sensitivity with any data generated here. Co-production of these two mycotoxins is possible across

affected crops (Moss, 1996). DON belongs to a group of mycotoxins known as trichothecenes which share a structural similarity of containing a 9, 10 double bond and a 12, 13 epoxide group, DON is grouped under the type B trichothecene bracket due to its carbonyl group at the C-8 position (Table 1.3) (Pestka, 2007). In general, the toxicity of trichothecenes is mainly due to inhibition of protein synthesis in actively dividing cells with trichothecenes known to prevent polypeptide chain initiation or elongation through interaction with the 60S ribosomal subunit in eukaryotes (Pestka, 2007, Zain, 2011). The structure of ZON includes a lactone ring and C-4 hydroxyl group and it is structurally similar to the female hormone oestradiol, so although ZON is considered to have low acute toxicity to animals, this enables ZON and its metabolites (α and β-zearalenol and α and β- zearalanol) to act as an endocrine inhibitor with oestrogenic properties (Zain, 2011, McCormick, 2013).

Table 1.3 Structure (SIGMA) and physical properties of DON and ZON

Physiochemical properties	Deoxynivalenol	Zearalenone
Chemical structure		
CAS number	51481-10-8	17924-92-4
Molecular formula	C15H20O6	C18H22O5
Molecular Weight	296.31 g/mol	318.4 g/mol
Log KOW	-0.7	3.6
Melting Point	152.0 °C	164.5 °C
Vapor Pressure	6.8X10-11 mm Hg at 25 °C	0 mm Hg at 68 °F
Solubility in water	5.5X10+4 mg/L at 25 °C	less than 0.1 mg/mL at 64° F
Soluble in	Polar organic solvents e.g. methanol, ethanol, chloroform, acetonitrile, and ethyl acetate	n-hexane, benzene, acetonitrile, dichloromethane, methanol, ethanol and acetone

1.3 Mycotoxins in freshwater

There are two main potential sources of mycotoxins entering freshwater systems, agricultural run-off and human waste via wastewater treatment plants (WWTP) (Figure 1.3). There are multiple contributing factors within agricultural run-off. The first, and maybe the most commonly considered, is the production of fungal toxins on crops in the field leaching out into surface waters (Kolpin *et al*., 2014). Secondly, livestock also contribute to mycotoxin leaching from agricultural areas. Mycotoxins are excreted rapidly after contaminated feed consumption and manure may then be used on fields as fertiliser further spreading mycotoxins (Eriksen and Pettersson, 2004, Kolpin *et al*., 2014, Bartelt-Hunt *et al*., 2012). Finally, mycotoxins may also be added intentionally, such as α-zearalenol as a growth promoter (in the US), which has been measured in feedlot run-off from treated cattle at concentrations up to 5.2 µg/l (Le Guevel and Pakdel, 2001, Khan *et al*., 2008, Bartelt-Hunt *et al*., 2012). Agricultural run-off varies dependent on season, crop cultivation area hydrodynamics and whether fungi are present and producing mycotoxins whilst in the field (Wettstein and Bucheli, 2010; Kolpin *et al*., 2014).

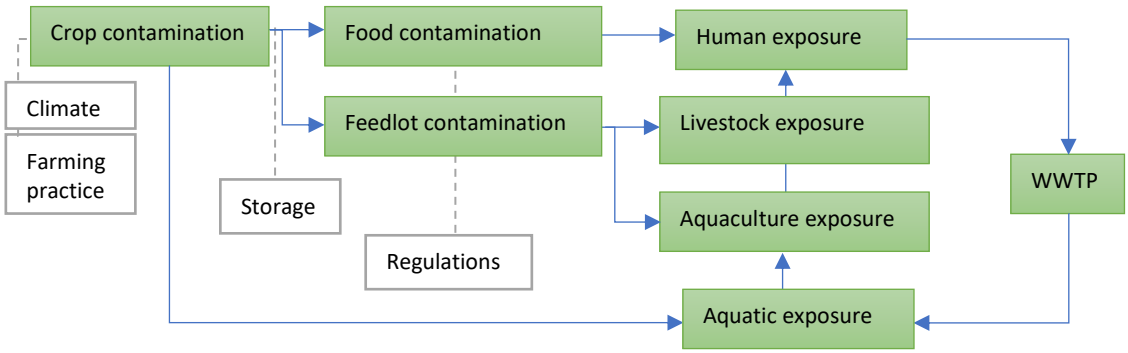


Figure 1.3 Conceptual linking of mycotoxin exposure routes and potential influences on mycotoxin concentrations (shown with grey dashed lines)

WWTP are considered a widespread permanent source of human consumed and excreted mycotoxins, studies have been carried out on the efficiency of WWTP in reducing mycotoxin concentrations. For the mycotoxins studied only partial reduction has been seen and emission from WWTP varies due to the type of waste being treated and methods used (i.e., trickling filter or activated sludge) (Wettstein and Bucheli, 2010; Gromadzka *et al*., 2015). Wettstein *et al*. (2010) compared the input contribution of WWTP and agriculture to surface water DON levels. WWTP were calculated to make a larger contribution of 2.3 - 4.5 ng/l in comparison to 0.8 - 2.7 ng/l from agricultural run-off. However, Gromadzka *et al*. (2015) found that for ZON WWTP were not the main source. Therefore, it is likely that the relative importance of mycotoxin sources is location and mycotoxin dependant.

In a pioneering project conducted in the USA by Kolpin *et al*. (2014), mycotoxins were detected in many freshwater streams, focusing on agriculturally impacted sites in Iowa and Indiana along with WWTPs in New York. Table 1.4 summarises the results of this and other mycotoxin exposure investigations in freshwaters and WWTPs. In total nine of the 33 mycotoxins (ten trichothecenes, five aflatoxins, four alternaria toxins, three resorcyclic acid lactones, two ochratoxins, two ergot alkaloids, two fumonisins, two penicillium toxins, and three miscellaneous mycotoxins) tested for were detected at least once in the samples, with DON occurring most frequently. For agricultural sites the concentrations of DON peaked during the spring snowmelt, with the highest recorded concentration being 1662 ng/L. DON was also the most commonly detected mycotoxin in WWTP samples, being the only mycotoxin to occur in all effluent samples. Other mycotoxins of concern highlighted were the oestrogenic metabolites of ZON, (α-ZOL) and β-zearalanol (β-ZOL), which were the only other mycotoxins exceeding concentrations of 100 ng/L, with highest concentrations found at WWTP sites.

Gromadzka *et al*. (2009) reported on the ZON concentration at various sites in Poland. One of the river sites (Bogdanka) showed a significantly higher concentration during Autumn, of 43.7 ng/l, in comparison to the other freshwater values across the study of < 0.3 – 1.7 ng/l. However, this Bogdanka river site was not located in an agricultural area, but in a forested location, therefore was not expected to have such high levels of mycotoxins. Also, higher concentrations were expected during the summer when fungal activity is promoted, but all locations showed higher concentrations in autumn-winter; suggesting freshwater mycotoxin levels increase due to the higher rainfall leaching the toxins from soil. A second study in Poland for ZON supported this idea, with the post-harvest period showing the highest levels of ZON due to increased rainfall (Waskiewicz *et al*., 2012). The fact that a forested site showed concentrations in the same range as agricultural areas extends the possible risk areas for high mycotoxin loads and implicates another possible land use area which could be investigated for mycotoxin output.

Schenzel, *et al*. (2012) screened for a total of 33 mycotoxins in WWTP effluents and river sites over a two year period. During which only four mycotoxins were found at quantifiable levels; beauvericin, 3-acetyl-deoxynivalenol (3-AcDON), DON and NIV. A study on estuarine river samples screening for DON in Portugal had detections in all samples (Ribeiro and Tiritan, 2015) at relatively high concentrations, reflecting the impact of the intense agriculture and WWTP discharges the river is subjected to before reaching the estuarine point.

Table 1.4 Mycotoxin exposure concentrations reported for surface waters

Mycotoxin	Measured environmental concentration (MEC) (ng / L)					References for MEC values
	Site type	Location	Analytical method	Limit of detection	Concentration range	
Beauvericin	Rivers	Switzerland	LC-MS/MS	1.3	< 1.3 – 22.0	(Schenzel *et al*., 2012b)
Deoxynivalenol	Estuary	Duoro River, Portugal	GC-MS	38.7	60 – 412	(Ribeiro and Tiritan, 2015)
	Estuary	Ave River Portugal	GC-MS	-	59.5 – 642.4	(Ribeiro *et al*., 2016)
	Rivers	Switzerland	LC-MS/MS	1.3	1.3 – 19.2	(Schenzel *et al*., 2012b)
	Streams	US	LC–MS/MS	1.3	< 1.3 - 1662	(Kolpin *et al*., 2014)
Fumonisin B1	Lake	Wojnowice lake, Poland	HPLC	-	17.4 - 23.6 (mean values)	(Waskiewicz *et al*., 2015)
	River	Brodnica, Poland	HPLC	-	27.4 – 55.6 (mean values)	(Waskiewicz *et al*., 2015)
Nivalenol	Rivers	Switzerland	LC-MS/MS	1.5	- 24.1	(Schenzel *et al*., 2012b)
	Streams	US	LC-MS/MS	1.5	< 1.5 - 43.7	(Kolpin *et al*., 2014)
Verrucarin A	Streams	US	LC–MS/MS	1.4	< 1.4 - 42.3	(Kolpin *et al*., 2014)
Zearalenone	Streams	US	LC–MS/MS	12.3	< 12.3 - 96	(Kolpin *et al*., 2014)
	Lake	Rusalka, Poland	HPLC	0.3-0.5	0.7 - 1.5	(Gromadzka *et al*., 2009)

1.3.1 Ecotoxicology of Mycotoxins to Freshwater Organisms

Some investigations into freshwater phytotoxicity of mycotoxins have been published, these have focussed on whether trichothecenes have a potential application as bioherbicides (Abbas *et al*., 2013). Also, on developing a *L. minor* assay to screen the detoxification performance of microbial cultures for DON (Vanhoutte *et al*., 2017). The previously published toxicity values found for *Lemna* sp. are shown in Table 1.5. Suzuki and Iwahashi (2014) reinvestigated use of the freshwater microalgae *Chlamydomonas reinhardtii* for trichothecene evaluation, a relatively insensitive LOEC of 10 mg DON/L was seen after 150 h exposure.

Currently invertebrate studies are the least studied of the freshwater organisms for mycotoxins, only two previous freshwater invertebrate studies with DON were found in literature. A protozoan study with *Tetrahymena pyriformis* with a reproduction LOEC of 0.6 mg DON/L at 150 h (Bijl *et al*., 1988) and a multi-generational study with the nematode *Caenorhabditis elegans* where F2 brood size had a NOEC of < 50 mg DON/L (Zhou *et al*., 2018).

Table 1.5 Literature data for *Lemna* sp. mycotoxin toxicity studies

Test species	Mycotoxin produced by (class of mycotoxin)	Mycotoxin	Endpoints	Toxic effects (mg/L) *	Reference
Lemna pausicostata (duckweed)	*Fusarium spp.* (Tricothecenes)	Deoxynivalenol	Growth reduction	72h 56.0 % at 2.96	Abbas *et al*. (2013)
		Nivalenol	Growth reduction	72h 40.0 % at 3.12	
		Scirpentriol	Growth reduction	72h 72.0 % at 2.82	
		T-2 toxin	Growth reduction	72h 65.2 % at 4.67	
	Myrothecium verrucaria (Tricothecenes)	Verrucarin A	Growth inhibition	72h $IC_{50\%}$ 0.43	Abbas *et al*. (2002)
		Roridin H	Growth inhibition	72h $IC_{50\%}$ 5.17	
		Trichoverrin A	Growth inhibition	72h $IC_{25\%}$ 24.4	
		Trichoverrin B	Growth inhibition	72h $IC_{25\%}$ 12.6	

Tests performed in synthetic growth medium. * Based on nominal values

Zebrafish are the main source of mycotoxin toxicity values for exposure via contaminated water, with Table 1.6 summarising this data. Zebrafish have a generally lower tolerance to mycotoxins than previously summarised for *Lemna* sp., with effect concentrations in the µg/l range. Early developmental stages have been identified as particularly vulnerable to chemicals, malformation and mortality have been seen in multiple embryo studies, (Table 1.6). Despite the presence of a chorion barrier this did not appear to reduce susceptibility to ZON exposure where comparative measures with dechorionated embryos were taken (Wu *et al*., 2012; Bakos *et al*., 2013). Conversely, during an exposure of zebrafish embryos to DON effects were only induced when embryos were injected with DON, in term of hatching, deformity and mortality (Khezri *et al*., 2018). This suggested zebrafish embryos are resilient to DON when exposed via only the aquatic media (Zhou et al., 2017), in comparison to the results seen for other mycotoxins (Table 1.6).

Bakos *et al*. (2013) investigated the effects of ZON in zebrafish embryos, lethal effect values generated were higher than concentrations of ZON measured in the environment (10 – 1000x), and highlighted no immediate concern. Curvature of the spine of individuals, responding in a concentration dependant manner, was a primary observation and suggested the mode of action of ZON could be as a kinase inhibitor. Although, the main concern around ZON is the adverse outcomes which could be triggered due to its oestrogenic nature. Bakos *et al*. (2013) also measured vitellogenin levels, vitellogenin is a precursor to egg yolk protein and is synthesised in the liver of mature females and is used as a biomarker of exposure to oestrogenic endocrine disrupting chemicals (Sumpter and Jobling, 1995; Hara *et al*., 2016; Hiramatsu *et al*., 2006), both males and larvae have been observed to produce vitellogenin in the

presence of oestrogenic compounds. During exposures to ZON both larvae and adult males were found to have vitellogenin - 1 mRNA induction and vitellogenin proteins present, confirming the oestrogenic activity of ZON (Bakos *et al*., 2013).

Longer ZON studies with adult zebrafish also showed no lethality but evidence of oestrogenic activity with induction of vitellogenin (at 1000 ng ZON/L) in male adults along with reduced spawning (at 1000 ng ZON/L) and fecundity (at 100 ng ZON/L) in a 21 d exposure (Schwartz *et al*., 2010). A life cycle study reported a feminizing effect on the population as a result of ZON, with significant decreases in male/female sex ratios to 0.37 and 0.41, in treatments of 320 and 1000 ng ZON/L respectively, compared to 0.78 in the control (Schwartz *et al*., 2011). The mode of action for other mycotoxins are not well established but Table 1.6 details sub-lethal observations and mechanistic inferences that have been observed in zebrafish exposures.

Table 1.6 Lethal effect concentrations and sub-lethal effects observed in zebrafish mycotoxin toxicity studies

Mycotoxin	Duration	Lethal effects (mg/L)		Observed sub lethal effects	Reference
		LOEC	LC_{50}		
Acute studies					
Aflatoxin B1	72h	0.16	-	Induce tail malformations along with decreased growth, treated embryos which showed mortality often had an extra yolk sac form before they died, as a result of a cell/group of cells breaking from the original yolk and undergoing rapid cell proliferation.	(Weigt *et al*., 2011)
Citrinin		-	-	Interfering with molecular mechanisms associated with heart morphogenesis defects in heart looping along with reduced chamber size and red blood accumulation, lead to reduced heartbeat and blood flow.	(Wu *et al*., 2013)
Citrinin and patulin		-	-	Dextran clearance as a measure of renal function both citrinin and patulin exposed embryos showed a decrease in clearance and indicated impairment of renal function	(Wu *et al*., 2012)
DON	72h	-	> 40	-	(Zhou *et al*., 2017)
Ochratoxin A	96h	-	0.1	Reduced hatching rate in a dose-dependent fashion, morphological deformities of the craniofacial region and body axis, edema and reduced growth.	(Haq *et al*., 2016)
T-2 toxin	6d	-	0.13	tail malformations major apoptosis in the tail area was seen when visualised via acridine orange staining. mechanism of T-2 toxicity in embryos was likely apoptosis triggered by an increase in ROS production co-exposure with reduced glutathione, a known antioxidant important for detoxification of ROS, showed a significant decrease in ROS production and tail deformities.	(Yuan *et al*., 2014)
ZON	5d	-	0.89	Curvature of the spine.	(Bakos *et al*., 2013)
		-	-	Pericardial and yolk sac edema, spine curvature and decreased heart rate as a result of ZON exposure. Mode of action suggested to be oxidative stress, increase in ROS, Lipid peroxidation and NO and a decrease in antioxidant responses measured, accompanied by evidence of DNA damage from the comet assay and apoptosis of cells in the brain region identified by acridine orange staining.	(Muthulakshmi *et al*., 2018)

Continued Table 1.6 Lethal effect concentrations and sub-lethal effects observed in zebrafish mycotoxin toxicity studies

Mycotoxin	Duration	Lethal effects		Observed sub lethal effects	Reference
		LOEC	LC_{50}		
Chronic studies					
ZON	21day	> 0.0032	-	Reduced spawning and fecundity along with induction of vitellogenin in male adults.	(Schwartz *et al*., 2010)
	140d	> 0.001	-	Life cycle study reported a feminizing effect on the population.	(Schwartz *et al*., 2013)

1.3.2 Potential risk of mycotoxins to freshwater ecosystems

With the limited number of species studied for mycotoxins, and specifically a lack of key values for algae or *Daphnia,* it has not been previously possible to perform risk calculations for freshwaters. Comparing the effect concentrations in Table 1.5 and Table 1.6 with the measured concentrations in Table 1.4, the concentration ranges are at μg/L concentration for effect concentrations compared to ng/L for concentrations measured in environmental exposure studies. Hence, it appears that the currently environmental concentrations may be low enough to have little significant effect on plants or zebrafish, although as the zebrafish toxicity tests extend into chronic exposures (Schwartz *et al*., 2013) the concentrations for sub lethal endpoints begin approach the ng/l level although the lethal LOECs are above the highest concentrations testes (> 1μg/L). Furthermore, although concentrations used in toxicity studies and effect levels seen are often higher than those seen in the environment, it should be considered whether ZON could contribute to deleterious effects of overall oestrogenicity of waters when present alongside other oestrogenic compounds.

A large amount of farmland is dedicated to cereal crops in the UK, the annual survey for arable output from 2018 (DEFRA, 2019b) stated the area of land for this to be 3.1 million hectares in England, with the East (50 %), East Midlands (49 %), South East (47 %) and Yorkshire and Humber (30 %) dedicating the largest proportions of farmland for cereal crops. The most widely grown cereal crop is wheat with 1 619 000 hectares of land dedicated to its production in 2018 (DEFRA, 2019a). Barley production is also fairly dominant along the Eastern regions of the UK, with the area dedicated to barley being 807 000 hectares; half of that dedicated to wheat (DEFRA, 2019b). In comparison maize growth is much lower with 206 thousand hectares of land used for growth in England

(DEFRA, 2019b). With the importance of cereal crops in UK agriculture FHB (CHAP, 2019, CHAP, 2018) and associated mycotoxin contamination of cereals is monitored with regulations in place for acceptable concentrations for both DON and ZON (Table 1.2) (European Commission, 2006b). Due to the large area of land growing cereal crops the associated effects of mycotoxin production should be considered in freshwaters, not just crop contamination, in the same way as pesticides applied to crops are assessed for their potential to affect freshwater ecosystems through run-off (EFSA, 2013). With changing climates, we are more likely to experience favourable conditions, warmer with higher rainfall, for *Fusarium* growth in the UK (Madgwick *et al*., 2011; West *et al*., 2012) and this would suggest a potentially higher risk of mycotoxin production. There has not previously been an investigation into the concentrations of mycotoxins within UK freshwaters and although there is growing knowledge for measured concentrations of mycotoxins in freshwater exposure studies, guidelines have not been set for mycotoxins in surface waters in any country.

1.4 Hypothesis, aims and objectives of the project

The aims of this project were to determine the ecotoxicological effects of DON and ZON in freshwater organisms, to analyse the environmental exposure concentrations of these mycotoxins and finally to conduct an environmental risk assessment for DON and ZON in order to address the hypothesis that there are harmful levels of mycotoxins, specifically DON and ZON, in UK freshwaters.

To test this hypothesis, it was approached through four key objectives, the first was to assess the toxicity of ZON to plants and algae, in terms of organismal (growth) response along with additional photosynthetic (chlorophyll fluorescence) and biochemical measures (lipid peroxidase concentration and antioxidant enzyme activity).

The second, to investigate the invertebrate toxicity of DON and ZON. The organisms used were the rotifer *Brachionus calyciflorus*, insect *Chironomus riparius* (larvae), crustaceans *Daphnia pulex* and *Thamnocephalus platyurus,* cnidarian *Hydra vulgaris*, mollusc *Lymnaea stagnalis* (embryos) and *Protozoan Tetrahymena thermophila*. The large number of organisms used in the toxicity studies was in order to generate species sensitivity distributions for hazard concentration calculations. Thirdly, to contribute to and review measured exposure concentrations of DON and ZON, novel samples from the UK were assessed along with a meta-analysis of global data available for DON and ZON in surface waters. The final objective was to characterise the risk in order to determine whether the hypothesis was supported from the data generated throughout the project. Risk was quantified using risk ratios, this comprised of generating PNECs based upon the ecotoxicological data and MECs from the exposure data to calculate the PNEC/MEC risk ratios for each mycotoxin, as summarised in Figure 1.1.

Chapter 2. Method development and zinc positive control data

2.1 Microbiotests format

For a complete analysis of possible toxicity of contaminants in water systems it is important to consider the varying sensitivity of freshwater organisms and use a multispecies approach (Isidori *et al*., 2006; Kwak *et al*., 2016; Minguez *et al*., 2014; MALTBY *et al*., 2009a; Maltby *et al*., 2009b). Test guidelines, such as those defined by OECD and ISO, describe methods which can be used to determine acute and chronic hazard of chemicals to a variety of model species, whilst maintaining a repeatable method of practice. Microbiotests scale down toxicity studies and may provide a way to generate comprehensive assessments of multi species toxicity (Mankiewicz-Boczek *et al*., 2008; Wieczerzak *et al*., 2016). By reducing the volume of test solution per replicate the cost and time requirements to carry out these microbiotests is reduced in comparison to maintaining cultures and buying chemicals for the larger standard toxicity tests.

Microbiotests have been developed for a variety of organisms from various taxonomic groups and trophic levels (Mankiewicz-Boczek *et al*., 2008; Wieczerzak *et al*., 2016). Some of these microbiotests are available as toxkits with ready to use organisms in their cryptobiotic state (Kungolos *et al*., 2009; Latif and Licek, 2004; Zurita *et al*., 2005; Casado-Martinez *et al*., 2016), removing the time delay associated with maintaining cultures, but these can be expensive when multiple tests are to be carried out. Instead test guideline methods can be adapted using readily available sterilised disposable polystyrene test vessels (Powell *et al*., 1996; Baumann *et al*., 2014; Cayuela *et al*., 2007). Microbiotests offer an efficient method to expand data on mycotoxin toxicity to freshwater organisms.

Nine test organisms were selected in total, this included a macrophyte, (*Lemna minor*), microalgae (*Pseudokirchneriella subcapitata*) and 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*). Some of these are regularly used model organisms in ecotoxicology (*L. minor*, *D. magna, P. subcapitata* and *C. riparius*) and followed the methodology detailed in the relevant OECD test guidelines (OECD, 2004; OECD, 2011). Others are available as commercial toxkits (*T. thermophila* and *T. platyurus*) (MicroBioTests Inc. Gent, Belgium) and those remaining (*L. stagnalis* and *H. vulgaris*) have methods described in previous literature (Bandow and Weltje, 2012); (Murugadas *et al*., 2016, Zeeshan *et al*., 2016).

2.2 Culture conditions and test design

2.2.1 Brachionus calyciflorus

The *B. calyciflorus* survival studies were carried out using Rotoxkit F purchased from MicroBioTests Inc. This provided resting eggs of *B. calyciflorus* which were rehydrated in EPA freshwater (Table 2.1) supplied with the kit and incubated at 25 ± 1 °C under constant illumination for 16 - 18 h, at which point hatched individuals were used for the study. Five rotifers were placed in each of the six replicate wells containing 300 µl test solution to provide a total of 30 individuals per test solution (Figure 2.1). The test plate was incubated in darkness at 25 ± 1 °C for 24 h after which the number of surviving rotifers per well was counted.

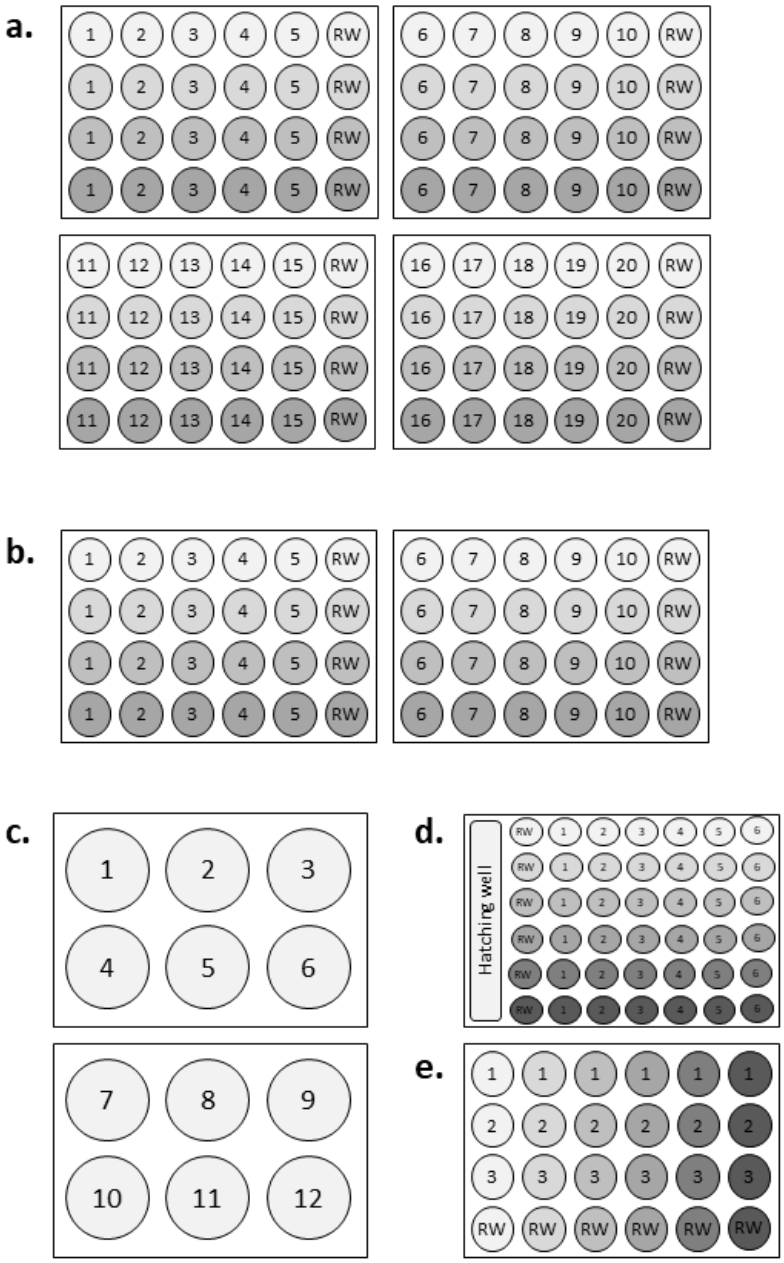


Figure 2.1 Setup of replicates in polystyrene tests plates with each shade indicating a different test concentration: a: invertebrate tests with twenty individuals split across four replicate plates, b: *Hydra vulgaris* with ten replicates split between two replicate plates, c: *Lemna minor* study with twelve colonies split between two replicate plates, d: *Brachionus calyciflorus* study (MicroBioTests Inc.) with thirty individuals split between six wells on a single plate and e: *Thamnocephalus platyurus* study (MicroBioTests Inc.) with thirty individuals split between three wells on a single plate.

Table 2.1 Stock solutions used for preparation of culture media

Medium	Stock	Reagent	Quantity mg (for 1 L)	Volume of stock added to 1 L	Final concentration (mg/ L)
BG11	1	Sodium nitrate	-	20	1500
		Dipotassium hydrogen phosphate	-	-	31.4
		Magnesium sulphate	-	-	36.0
		Calcium chloride dihydrate	-	-	36.7
		Sodium carbonate	-	-	20.0
		Disodium magnesium Ethylenediaminetetraacetic acid	-	-	1.0
		Citric acid	-	-	5.6
		Ferric ammonium citrate	-	-	6.0
EPA freshwater	1	$NaHCO_3$	-	-	96
	2	$CaSO4.2H_2O$	-	-	60
	3	$CaSO_4.2H_2O$	-	-	60
	4	$MgSO_4.7H_2O$	-	-	123
	5	KCl	-	-	4
SIS	1	$NaNO_3$	8.5	10	85
		KH_2PO_4	1.34	10	13.4
	2	$MgSO_4.7H_2O$	15	5	75
	3	$CaCl_2.2H_2O$	7.2	5	36
	4	Na_2CO_3	4.0	5	20
	5	H_3BO_3	1.0	1	1.0
		$MnCl_2.4H_2O$	0.2	1	0.2
		$NaMoO_4.2H_2O$	0.01	1	0.01
		$ZnSO_4.7H_2O$	0.05	1	0.05
		$CuSO_4.5H_2O$	0.005	1	0.005
		$Co(NO_3)_2.6H_2O$	0.01	1	0.01
	6	$FeCl_3.6H_2O$	0.17	5	0.84
		Na_2Ethylenediaminetetraacetic acid$2H_2O$	0.28	5	1.4
	7	MOPS (buffer)	490	1	490
Hydra media	1	Calcium chloride	-	-	1 mM
	2	Magnesium sulfate	-	-	0.1mM
		Potassium sulfate	-	-	0.1 mM
	3	Sodium chloride	-	-	1 mM
	4	Tris Base pH 7.5 ± 0.2	-	-	1mM

2.2.2 Chironomus riparius

Egg masses of *C. riparius* were provided by Fera Science Ltd., upon arrival egg masses were transferred to a hatching dish containing dechlorinated tap water and fed daily with a suspension of flaked fish food (Tetramin) suspension to provide 250 mg per vessel per day. Egg masses were monitored twice per day to check for initiation of hatching, first instar larvae were collected < 24 h after hatching and used in studies.

First instar larvae were used in a 48 h immobilisation study following OECD guideline 235 (OECD, 2011b). 20 replicate neonates were used per concentration. Hatched individuals were placed in wells of a 24 well plate with 2ml test solution per well. These were split into four replicate plates of five animals in each, as shown in Figure 2.1. The number of immobilised organisms at 24 and 48h was recorded. Both the hatching dish and test plates were maintained at 20 ± 1°C under low light.

2.2.3 Daphnia magna

The culture of *D. magna* was originally obtained from Sciento (Manchester, UK). The main culture was maintained in a 10 L tank with OECD medium (Table 2.1) (pH adjusted to 7.5 ± 0.2). The culture was aerated and contained a few juvenile *Lymnaea stagnalis* to maintain the cleanliness of the tank, *Daphnia* were fed three times per week with fresh algae cultures (*Chlorella vulgaris* or *Pseudokirchneriella subcapitata*). The water was changed once per week and maintained at 20 ±1 °C under a 14:10 light dark cycle of moderate light.

Prior to studies 40 adults were transferred to 1 L beakers (20 adults per beaker), neonates were collected and transferred to new 1 L beakers to mature. Media in the beakers was changed 3 times per week and fed with fresh algae fresh algae (*Chlorella*

vulgaris or *Pseudokirchneriella subcapitata*) following these water changes. As individuals started to show neonates in the brood pouch all individuals were observed under a low powered microscope to identify when they should release neonates and place in smaller beakers accordingly. These subcultures should then be synchronised in the release of their neonates, which are removed daily and third to fifth brood neonates were used in studies.

Immobilisation studies were 48 h in duration according to OECD test guideline 202 (OECD, 2004) and were performed with twenty replicate neonates used per concentration, each placed in individual wells of a 24 well plate with 2 ml test solution per well (Figure 2.1). Test plates were held under the same conditions as described for culturing. The number of immobilised organisms at 24 and 48 h was recorded and pH measured at the beginning and end of the test.

2.2.4 Hydra vulgaris

A culture of *Hydra vulgaris* was instead established from starter culture purchased from Blades biological (Kent, UK). These are brown *Hydra* and require a high feeding rate, hence resting eggs of *Artemia* were used to hatch nauplii as required for feeding daily. *Hydra* were acclimatised to medium described by Zeeshan *et al*. (2016)(Table 2.1) for at least two weeks prior to use in studies. Healthy single polyps showing no budding were used in studies.

The *H. vulgaris* studies followed the format described in Zeeshan *et al*. (2016). Ten individuals per concentration were placed in a petri dish, excess water was removed before submersion in the relevant test media (to minimise dilution of test well solutions), each were transferred into individual wells of a 24 well plate with 2 ml test

solution per well. Health of individuals was monitored according to Wilby's guide for *Hydra* (Wilby, 1988).

2.2.5 Lemna minor

An ongoing culture of *Lemna minor* (UTCC #490) was maintained in Swedish standard (SIS) media (Table 2.1) adjusted to 6.5 ± 0.2, as listed in the OECD test guideline 221 (OECD, 2006). Cultures vessels were 5 L plastic tanks containing 2 L media, kept in an incubator under a 16:8 h light dark cycle (white fluorescent light) at 24 ± 1°C. Media changes were carried out once per week. If algae was visible in cultures prior to experiments then cultures were sterilised 7 d prior to testing to ensure they were free from algae at the beginning of exposures.

The *L. minor* studies followed OECD test guideline 221 (OECD, 2006), static exposures were carried out over a 7 d period in 6 well microplates (Thermo Fisher Scientific, Massachusetts, USA, product code 130184) containing 8 ml test solution and 1 colony consisting of three fronds per well. 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, and position of plates in the incubator was randomised throughout the test.

Growth measurements of frond number and frond area (using WinDias 1.5 software with Hitachi KP-D40 digital camera) were taken at t = 0, 2, 5 and 7 d, average specific growth rate (μ) and yield, inhibition of ASGR and inhibition of yield were calculated according to the test guideline.:

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

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.

2.2.6 Lymnaea stagnalis

A culture of *Lymnaea stagnalis* RENILYS strain originating from Rennes institute of agronomic research (INRA) was maintained in 10 L aquaria of artificial freshwater (Table 2.1) under a 14:10 light dark cycle (intensity) and fed organic lettuce as required. Tanks had media changes once per week, by syphoning half of the water out to be replaced. Every two weeks the entire tank was emptied to allow the surfaces to be wiped before media replacement.

A few days prior to studies larger adults were moved to a new breeding tank with fresh media and adequate food. Breeding was monitored and a cell scraper was used to remove egg ropes from the tank in the evening and the following morning egg ropes < 24 h old were collected for use in studies. Individual eggs were separated from the egg

mass using forceps and dissecting scissors, embryos were checked for normal appearance (unburst and no double embryos).

No test guideline is available currently for an embryo test with *L. stagnalis*, the methodology used is based upon that of Bandow and Weljte (2012). Twenty replicate embryos per concentration were placed in individual wells of a 24 well microplate containing 2 ml of test solution (Figure 2.1). The duration of the study was 7 d with survival and morphology monitored. When required a further 9 d was performed to allow for shell formation to occur and a heartbeat to be visible in the embryos. Solutions were replaced twice during the 9 d period, on days 3 and 6. At the end of the longer exposure individuals were filmed for 60 seconds using a camera attached to a low power stereo microscope, heart rates were determined from the video clips and still images were saved from points where individuals were in the correct orientation for growth endpoints (shell size and ocular distance) to be measured using InfinityAnalyse software (Lumenera, Ottawa).

2.2.7 Pseudokirchneriella subcapitata

A culture of *P. subcapitata* (type strain 278/4) was obtained from the Culture Collection of Algae and Protozoa and maintained in BG11 media (Table 2.1) 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 µE $m^{-2}s^{-1}$) and placed on an orbital shaker set at 120 rpm with temperature in the media maintained at 24 ± 1°C. The cell density was measured in the main batch culture and appropriate dilution to a new sub-culture was prepared. The cell density of the inoculum culture was monitored at 24 h intervals and the growth rate calculated. Once the culture

had reached exponential growth, usually between 48 and 72 h, the experimental exposure could commence.

Static exposures were carried out over a 72 h period in accordance with OECD guideline 201 (OECD, 2011a). A healthy exponentially growing culture (monitored by increase in cell density) was used to inoculate 25 ml of growth media in sterile 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.

Growth rate was calculated 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, in the same way as described previously for *L. minor* but with cell density as the measurement variable.

2.2.8 Tetrahymena thermophila

T. thermophila were purchased as part of the Protoxkit F kit from MicroBioTests Inc. the protozoa culture required no maintenance and was held in the dark at room temperature until use. Polystyrol spectrophotometric cells with lids were used as test vessels with three replicates per concentration (Figure 2.1), EPA freshwater stocks were supplied with the kit (Table 2.1). Protozoans were used to inoculate 2 ml of test solutions at approximately 100 protozoans per ml. Food substrate was added to each test vessel, following this optical density at 440 nm was measured at 0 h and again at 24 h to quantify the clearing of substrate into ciliate biomass. Test cells were held at 30 ± 1 °C in darkness throughout the exposure.

2.2.9 Thamnocephalus platyurus

T. platyurus resting eggs were purchased as part of the Thamnotoxkit F kit from MicroBioTests Inc. Resting eggs were rehydrated in the EPA freshwater (Table 2.1) suppled with the kit and incubated at 25 ± 1 °C under constant illumination for 16 - 18 h, at which point hatched individuals were used for the studies.

A single polystyrene 24 well plate was used containing 3 replicate wells for each concentration (Figure 2.1). 10 individuals were placed in each replicate well containing 1 ml test solution to provide a total of 30 individuals per test solution. The test plate was incubated in darkness at 25 ± 1 °C for 24 h after which the number of surviving individuals per well was counted.

2.3 Zinc positive control studies

For quality control purposes and to monitor internal variation in toxicity studies it is good practice to include a positive control in studies. By using a positive control as well as the standard negative dilution water control this allows the tester to identify any false positive and negative results which may be occur during studies.

Zinc was chosen as a suitable reference chemical. Zinc is trace metal, which is accumulated by aquatic invertebrates (Rainbow, 2007). Despite zinc being an essential element, in excess toxic effects may be induced (Jorge and Moreira, 2005; O'Mara *et al*., 2019). The concentration of zinc can easily be analysed to provide measured concentrations and the use of zinc as a reference chemical is listed in the UK Direct Toxicity Assessment approach (Agency, 2007). Occurrence of zinc in freshwater is expected, due to natural processes, but the added anthropogenic inputs such as industrial waste and agricultural run-off must be considered in environmental risk

assessments (European Commission, 2010). 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 0.015 mg Zn/L (Table 2.2). 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.

Table 2.2 Measured concentration values for zinc range finder studies. Chemical analysis performed using ICP-OES (LOD = 0.015 mg Zn/L)

Organism	Concentration (mg/L)	
	Nominal	Measured
B. calyciflorus	0.032	0.05
	0.10	0.09
	0.32	0.28
	1.0	0.90
	3.2	3.09
T. platyurus	0.032	0.08
	0.10	0.15
	0.32	0.34
	1.0	0.90
	3.2	3.82
T. thermophila	0.32	0.28
	1.0	0.90
	3.2	3.09
	10	7.43
	32	39.8
C. riparius	1.0	0.89 ± 0.07
	3.2	2.48 ± 0.15
	10	9.19 ± 4.10
	32	30.9 ± 6.3
	100	138[a] ± 14
D. magna	0.032	0.04 ±0.01
	0.10	0.10 ± 0.01
	0.32	0.36 ± 0.04
	1.0	1.04 ± 0.55
	3.2	3.75 ± 1.00
H. vulgaris	0.32	0.35 ± 0.18
	1.0	1.16 ± 0.56
	3.2	2.86 ± 0.34
	10	10.3 ± 1.83
	32	33.4 ± 3.8
L. stagnalis	0.032	0.04± 0.02
	0.10	0.14 ± 0.1
	0.32[a]	0.21 ± 0.15
	1.0	0.78 ± 0.32
	3.2	3.3 ± 1.9

[a] above highest standard

Those without SD were 24 h test where concentration was only measured at the beginning of the study with no significant decrease in concentration expected

2.3.1 Results

2.3.1.1 Plants

With the *L. minor* an initial test using 12 well microplates, containing 4 ml test solution and one colony consisting of three fronds per well with one plate per concentration, the controls did not meet the required daily growth rate of 0.275 d^{-1} therefore a second trial was conducted which used six-well microplates and 8 ml test solution per well (still with one three fronded colony per well). The results of both vessel tests are shown in Table 2.4 along with calculated inhibition of growth in comparison to the control treatment. The second trial proved suitable with controls meeting the required growth rate and this set up was used in subsequent tests. Measured endpoints along with ASGR and yield for these endpoints are listed in Table 2.3 for *P. subcapitata* and Table 2.4 for *L. minor*.

Table 2.3 Results of *P. subcapitata* 72 h zinc growth inhibition study.

Nominal concentration (mg Zn/L)	Cell density at 72 h (mean ± SD) (x 10^3)	Average specific growth rate (day^{-1})	Yield	Inhibition of growth (%)	
				Average specific growth rate (day^{-1})	Yield
0	547 (± 11)	1.56	5.42E+05	-	-
0.01	474 (± 67)	1.52	4.69E+05	3	13
0.032	487 (± 15)	1.53	4.82E+05	2	11
0.1	467 (± 60)	1.51	4.62E+05	3	15
0.32	16.3 (± 3.4)	0.39	1.13E+04	75*	98*
1	6.67 (± 2.9)	0.08	1.67E+03	95*	100*

* Significantly different ($P < 0.05$) from control treatment

Table 2.4 Results of 7 d *L. minor* zinc growth inibition studies using varying test vessels (12 well or 6 well microplates).

(Nominal) or Measured concentration (mg Zn/L)	Endpoints at 7d (mean ± SD)			Calculated growth measures						Inhibition of growth (%)					
	Frond number	Frond area (mm^2)	Wet weight (mg)	Average specific growth rate (day^{-1})			Yield			Average specific growth rate			Yield		
				Frond number	Frond area (mm^2)	Wet weight (mg)	Frond number	Frond area (mm^2)	Wet weight (mg)	Frond number	Frond area (mm^2)	Wet weight (mg)	Frond number	Frond area (mm^2)	Wet weight (mg)
12 well microplate															
0	13 (± 3.3)	58 (± 17.5)	-	0.212	0.245	-	10	48	-	-	-	-	-	-	-
(0.1)	12 (± 4.1)	47 (± 18.4)	-	0.201	0.211	-	9	36	-	5	23	-	7	31	-
(0.32)	14 (± 2.9)	60 (± 14.6)	-	0.224	0.228	-	11	42	-	-5	6.8	-	-9	-0.6	-
(1)	11 (± 2.0)	39 (± 7.3)	-	0.189	0.165	-	8	26	-	11	32	-	21	45	-
(3.2)	5 (± 1.1)	18 (± 4.4)	-	0.088	0.067	-	2	6.6	-	59	72	-	76	86	-
(10)	5 (± 1.5)	12 (± 5.8)	-	0.076	0.008	-	2	0.86	-	100	100	-	80	98	-
6 well microplate															
0	24 (± 2.1)	102 (± 11.3)	25 (± 3.3)	0.297	0.313	0.302	21.0	90.9	22	-	-	-	-	-	-
0.09	28 (± 2.7)	121 (± 16.9)	30 (± 5.6)	0.318	0.329	0.327	24.9	109	27	-7	-5	-4	-19	-20	-9
0.16	25 (± 2.6)	103 (± 16.7)	25 (± 1.8)	0.302	0.316	0.303	22.0	92	22	-2	-1	4	-5	-1	11
0.37	17 (± 2.7)	46 (± 12.7)	14 (± 2.6)	0.245	0.181	0.220	13.8	32	11	18	44	30	35	69	55
1.1	11 (± 1.2)	21 (± 4.4)	7 (± 1.3)	0.180	0.092	0.111	7.7	10	4	39*	71*	65*	63*	89*	85*
2.3	10 (± 0.8)	19 (± 2.6)	6 (± 0.9)	0.169	0.073	0.096	6.8	8	3	43*	77*	70*	67*	91*	88*

* Significantly different ($P < 0.05$) from control treatment

2.3.1.2 Invertebrates

The dose response analyses for the invertebrate zinc studies are based on measured concentrations (results of the ICP-OES measurements are provided in Table 2.2) and are shown in Figure 2.3, the resulting summary effect values are listed in Table 2.4 Summary effect values (EC_{10}, EC_{20} and EC_{50}) were calculated and are listed in Table 2.4. The most sensitive organisms to zinc were *T. platyurus*, *L. stagnalis*, *B. calyciflorus* and *D. magna* respectively with EC_{50} values of 0.36, 0.61, 1.7 and 4.1 mg Zn/L respectively. The EC_{50} was higher for *H. vulgaris* at 11.3 mg Zn/L. The remaining two test organisms also required a higher test concentration range to generate a toxic response. *T. thermophila* had a reproduction EC_{50} of 10.9 mg Zn/L and *C. riparius* was the only organism tested which did not show inhibition of greater than 50 %, at the highest concentration of 138 mg Zn/L a 20 % immobilisation was seen. However, no further increase in concentrations was carried out beyond this concentration.

The results of this chapter were used to inform a suitable zinc concentration to act as positive controls in subsequent mycotoxin studies in order to ensure the test system was functioning as expected. The exception to this was *C. riparius* where no suitable concentration was found. An SSD and resulting HC_5 were produced for the zinc data for comparison with environmental standards, this is shown in Figure 2.5. The acute HC_5 calculated here of 57 μg Zn/L is lower than the acute criterion maximum concentration of 120 ug μg Zn/L set by the USEPA (2009) but our upper 95% CI (145 μg Zn/L) overlaps this value. A study by (Iwasaki and Ormerod, 2012) relating field macroinvertebrate surveys with dissolved metal concentrations estimated a HC_5 of 34 μg Zn/L (95 % CI 11 – 307) comparable with ours.

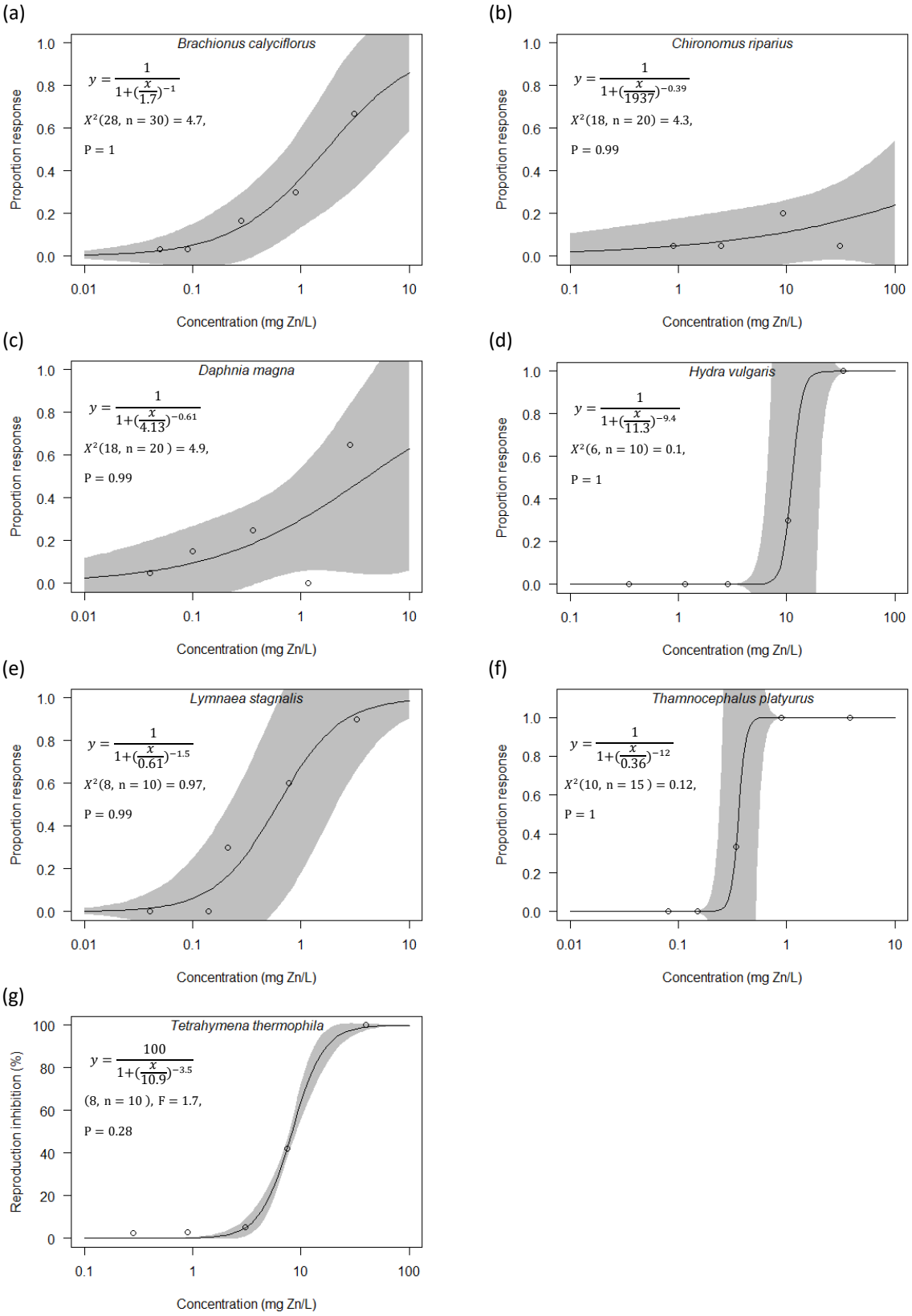


Figure 2.2 Dose response curves of freshwater invertebrates exposed in acute laboratory studies to zinc, based on measured concentrations (ICP-OES, LOD = 0.015 mg Zn/L), with shaded bands indicating 95 % CI. Model equations along with the chi-squared (X^2)/ F-value (Degrees of freedom, N=) p-value from lack-of-fit/goodness-of-fit tests are provided.

Table 2.5 Summary effect values from freshwater plant and invertebrate toxicity studies with the reference chemical zinc.

Duration	Test organism	Endpoint	Toxicity effect values (mg/L) (± SD)				Literature toxicity values (mg/L)		
			EC_{10}	EC_{20}	EC_{50} (95% CI)	Organism	NOEC	EC50	Reference
24 h	*Brachionus calyciflorus*	Survival	0.20 ± 0.15	0.44 ± 0.25	1.7 ± 0.97 (0 – 3.6)	*Brachionus calyciflorus*	-	1.5	Park & Kim (2020)
24 h	*Thamnocephalus platyurus*	Immobilisation	0.30 ± 0.15	0.32 ± 0.07	0.36 ± 0.4 (0 - 1.3)	*Thamnocephalus platyurus*	-	0.16	Nalecz Jawecki et al. (2011)
24 h	*Tetrahymena thermophila*	Reproduction	4.5 ± 0.39	6.7 ± 0.31	10.9 ± 1.6 (9.3 – 16.8)	*Tetrahymena thermophila*	-	6.7	Mortimer et al. (2010)
48 h	*Chironomus riparius*	Immobilisation	7.03 ± 15.0	56 ± 105	> 100	*Chironomus* sp.	-	27 – 39	Park & Kim (2020)
48 h	*Daphnia magna*	Immobilisation	0.11 ± 0.17	0.42 ± 0.43	4.1 ± 6.3 (0 – 16.6)	*Daphnia magna*	0.098	-	ECHA
72 h	*Pseudokirchneriella subcapitata*	Growth	0.03	0.05	0.14	*Pseudokirchneriella subcapitata*	0.019	-	ECHA
96 h	*Hydra vulgaris*	Survival	8.9 ± 13.0	9.7 ± 5.8	11.3 ± 10.3 (0 - 32)	*Hydra vulgaris*	-	7.4	Park & Kim (2020)
7 d	*Lemna minor*	Growth	0.34	0.41	0.73	*Lemna minor*	-	3.0	Drost et al. (2007)
7d	*Lymnaea stagnalis* (embryo)	Survival	0.14 ± 0.13	0.24 ± 0.17	0.61 ± 0.39 (0 - 1.4)	-	-	-	-

The UK Water Framework Directive (DEFRA, 2014; WFD, 2012) and EU environmental risk assessment (European Commission, 2010) PNEC's, 10.9 µg Zn/L and 7.8 µg Zn/L respectively, are both based upon chronic toxicity values and likely why they are lower than the HC_5 generated here. But applying an assessment factor of 5, following those recommended by REACH guidelines for freshwater data assessed through a probabilistic method (ECHA, 2008) (Table 1.1), to account for this and uncertainties provides a PNEC of 11.4 µg Zn/L in line with those reported elsewhere (DEFRA, 2014; WFD, 2012; European Commission, 2010).

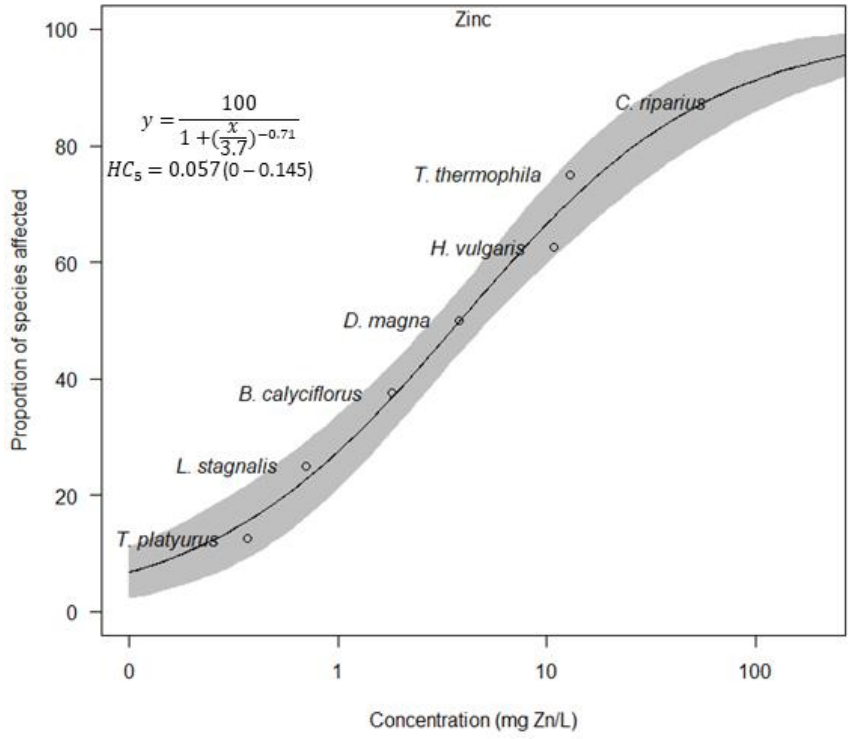


Figure 2.3 Species sensitivity distribution curve with shaded bands indicating 95 % CI. Markers are EC_{50} values from acute zinc toxicity studies, those without markers had undetermined EC_{50} values greater than the highest concentration tested and were therefore included in the rank but not in the SSD fit.

Chapter 3. Impacts of the mycotoxin ZON on growth and photosynthetic responses in laboratory populations of freshwater macrophytes (*Lemna minor*) and microalgae (*Pseudokirchneriella subcapitata*).

3.1 Introduction

Animals feeding on mycotoxin-contaminated feed have shown toxic effects such as protein synthesis inhibition, endocrine disruption, 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 (European Commission, 2006b). 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 at which mycotoxins can have toxic effects on freshwater species. For ZON toxicity to zebrafish embryos, Bakos *et al*. (2013) found a 5 d development effect concentration 50 % (EC_{50}) of 50 µg/L and lethal concentration 50 % (LC_{50}) of 893 µg/L. Schwartz *et al*. (2010) reported a 21 d development LOEC and

mortality LOEC of > 3.2 µg/l, 1 µg/l for vitellogenin production LOEC and 0.1 µg/l for fecundity LOEC. In a longer life cycle (140 d) test with zebrafish a sex ratio LOEC of 0.32 µg/L was seen (Schwartz *et al*., 2013). In contrast, there is a lack of ZON phytotoxicity data which is needed in order to develop an environmental risk assessment of this widespread mycotoxin. This is important given that other mycotoxins have been shown to cause phytotoxicity in *Lemna* spp. (eg growth inhibition of 40 % at 3.2 mg NIV/L, 56 % at 3.2 mg DON/L and 72 % at 5.6 mg T-2 toxin/L (Abbas *et al*., 2013).

Lemna sp. are popular choice in chemical toxicity monitoring for freshwater primary producers, due to their small size, rapid growth and ease of culturing. The microalga *Pseudokirchneriella subcapitata*, previously known as *Selanastrium capricornutum* and *Rhapidocelis subcapitata*, similarly is a well-studied organism with its rapid growth rate allowing multiple generations to be studied in a brief time frame. Standardised testing guidelines have been developed for both species (OECD, 2006; OECD, 2011), outlining methods which can be used under laboratory conditions to contribute to the hazard assessment of chemicals, through analysing the adverse outcome at the level of the individual and population. To develop knowledge of the specific mode of action of a chemical and link this to the adverse outcome, these guidelines can be supplemented with physiological and 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).

As other mycotoxins have shown phytotoxicity it was expected that ZON would also have measurable toxicity in plants, although due to the oestrogenic nature of ZON it could be speculated that plant species would be less sensitive in comparison to fish - which possess oestrogen receptors making them susceptible to oestrogenic

mechanisms of toxicity from ZON. Therefore the aim of this chapter was to investigate the phytotoxicity of ZON, and it is based upon the work previously published in Ecotoxicology Environmental Safety of the same name (Impacts of the mycotoxin zearalenone on growth and photosynthetic responses in laboratory populations of freshwater macrophytes (*Lemna minor*) and microalgae (*Pseudokirchneriella subcapitata*)) by Eagles, E.J., Benstead, R., MacDonald, S., Handy, R. & Hutchinson, T.H. (2019). Toxicity studies performed followed 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 mode of action 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.

3.2 Materials and methods

The microalgae and macrophyte study used laboratory populations of *L. minor* and *P. subcapitata*, tests followed the methodology detailed in the relevant OECD test guidelines (OECD, 2011a, OECD, 2006). Details on the medium, exposure conditions and replicates used for each test species is detailed in Chapter 2.

3.2.1 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, calculated from start and end concentrations, in brackets with a limit of detection (LOD) of 0.18 mg ZON/L). A zinc

positive control of 0.2 mg/L 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 as described in Chapter 2.

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 over the 7d period 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 ranged between 6.4 - 7.5, within the recommended variation of less than 1.5 units).

3.2.1.1 Chlorophyll fluorescence

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

The chlorophyll fluorescence parameters for *L. minor* were measured using a portable fluorimeter (Pocket PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) with a light pulse of 3000 µmol of photons m/s over 1 s. A single colony was taken from six wells in each treatment and dark adapted in a leaf clip for at least 20 mins at room temperature before being measurements were taken. Measurements were taken at t = 7 d of a second exposure with concentrations of measured ZON concentrations of 4.8 (5.2), 8.1 (7.9) and 15.0 (14.4) mg/L (mean measured concentration in brackets) and

reference chemical mean measured exposure of 1.8 mg Zn/L. Physio-chemical parameters were measured at the beginning and end of the study (dissolved oxygen 8.1 - 10.0 mg/L; pH 6.4 - 7.1; temperature 23.8 - 24.0 °C).

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, where O is the initial fluorescence (F_0) when all reactions centres (RCs) are open and P is the peak fluorescence (F_P) when all RCs are closed (with I and J being intermediate steps between these); various yields and fluxes of energy during this process can be analysed, termed the JIP test, where saturating light pulses are used to reach F_P following dark adaptation of the plant to measure F_O (Appenroth *et al*., 2001; Misra, 2001; Yusuf *et al*., 2010). This can 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. (Strasser *et al*., 2000; Misra *et al*., 2001; Yusuf *et al*., 2010).

3.2.1.2 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 and 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 and

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 Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). Briefly, the working reagent was prepared by mixing bicinchoninic acid 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.

3.2.2 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 and concentration calculated using a calibration curve.

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.

3.2.3 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.

3.3 Results

3.3.1 Growth inhibition

The controls of *P. subcapitata* in the control media showed an average overall growth rate of 1.38 day^{-1} (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 3.1). 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 not recovering from this by 72 h.

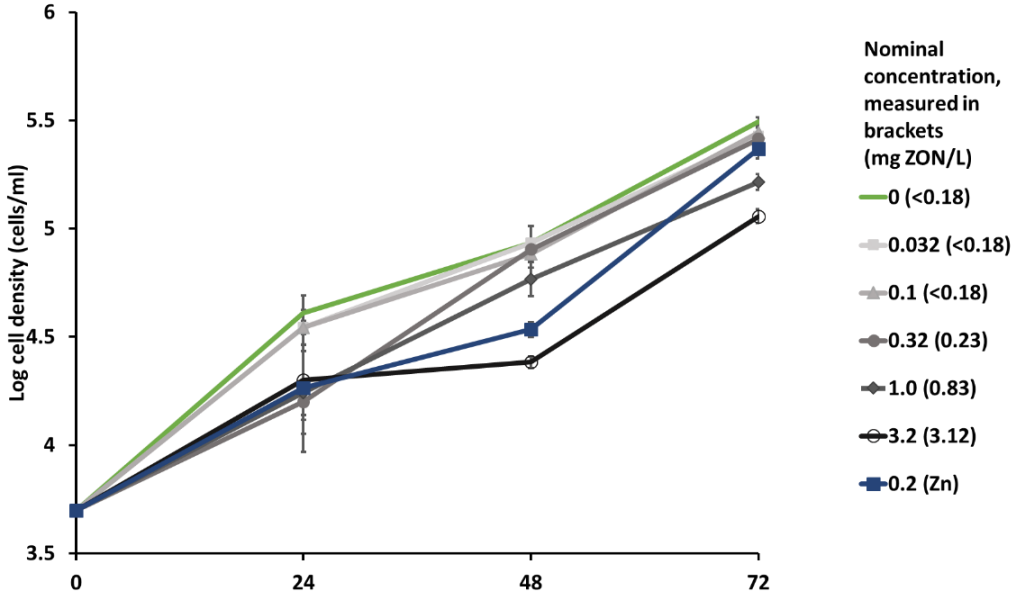


Figure 3.1 Growth curves of *P. subcapitata* exposed to ZON in a 72 h static study at 23.8 ± 1°C with 0.2 mg Zinc/L as a positive control.

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 3.1). 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.

Table 3.1 Growth responses of *P. subcapitata* exposed to ZON in a 72 h static study at 23.8 ± 1°C (concentration measured using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L) with 0.2 mg Zinc/L as a positive control.

Nominal concentration, measured in brackets (mg ZON/L)	Mean endpoint at 72h Cell density (cells/ml x 10^5)	Mean inhibition of algal growth (%) 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 - 0.33)
EC_{50} (± 95 % CI)	-	> 3.2	0.92 (0.74 - 1.8)
NOEC	-	0.23	0.1
LOEC	-	0.83	0.23

[a] Significantly different (P < 0.05) from control treatment
Summary effect values calculated with measured values where possible.

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 3.2, 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 (Figure 3.2), 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).

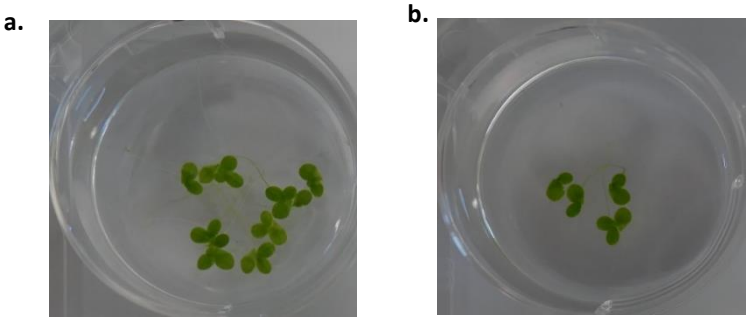


Figure 3.2 Images taken at 7 d of ZON *L. minor* study, images show a replicate from a. control and b. 11.4 mg ZON/L – where significant growth inhibition of 38-67% was seen in comparison to controls.

Table 3.2 Growth responses (mean ± SD) of *L. minor* exposed to ZON in a 7 d static study at 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)	Measured endpoints (mean ± SD)		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)

[a] Significantly different ($P < 0.05$) from control treatment

Summary effect values calculated with measured values where possible.

3.3.1.1 Chlorophyll fluorescence

During the growth inhibition test measures of Fv/Fm for *P. subcapitata* showed no significant differences (Table 3.3), 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 3.3. 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 significantly in all ZON treatments. While ABS/RC and TRo/RC were significantly increased at the highest treatment of 14.4 mg ZON/L. Fv/F_0 and φ(Po) did not alter significantly in any treatment.

Table 3.3 Maximal quantum efficiency of Photosystem (Fv/Fm) measured in *P. subcapitata* (mean ± SD) after 72 h exposure to ZON in a static study at 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)

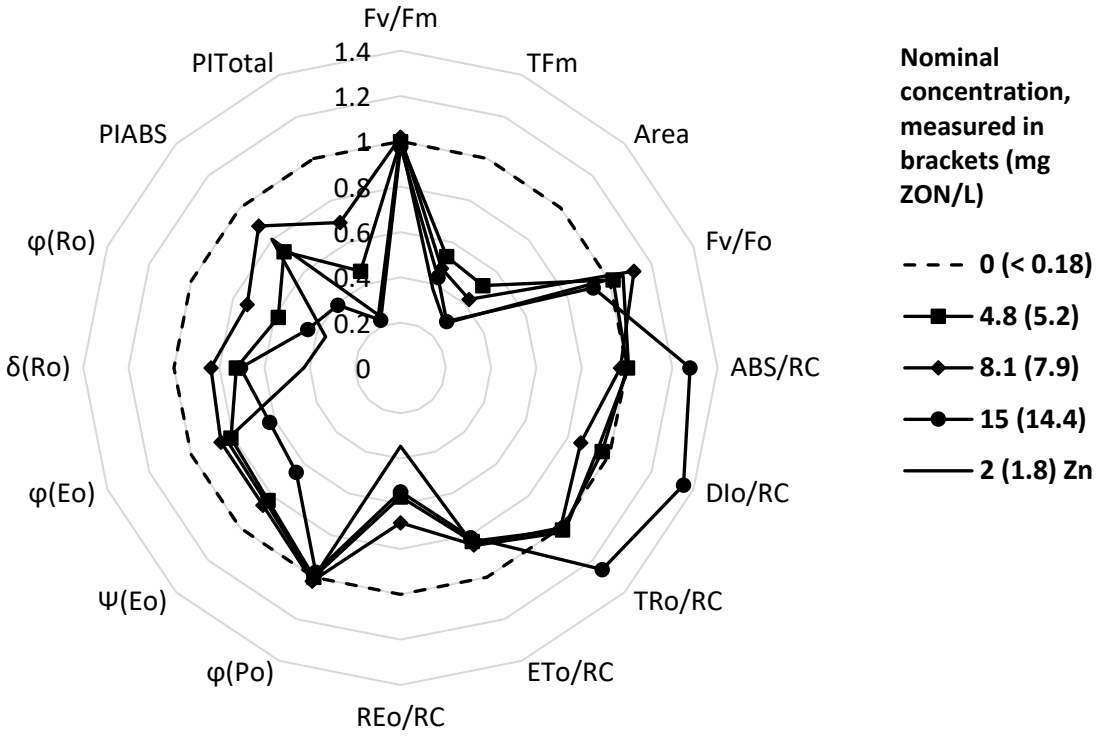


Figure 3.3 Chlorophyll fluorescence parameters measured in *L. minor* after 7 d exposure to ZON in a static study at 24 ± 1°C (concentration measured using UV-Vis spectrometry with an LOD of 0.18 mg ZON/L). Values are normalised to the control group 0 (< 0.18 mg ZON/L).

Fv/Fm = maximal quantum efficiency of Photosystem II; TFm = time to reach maximum chlorophyll fluorescence; Area = proportional to the pool size of the electron acceptors Qa on the reducing side of Photosystem II; Fv/F_0 = quantum yield of the photochemical and non-photochemical processes; 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; TRo/RC = energy trapping rate per reaction centre; ETo/RC = photosynthetic electron transport rate per reaction centre; REo/RC = reduction of acceptors in PSI per reaction centre; 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; PI_{ABS} = performance index of photosynthetic efficiency and PI_{Total} = energy conservation for reduction of PSI terminal acceptors respectively.

3.3.2 TBARS assay and catalase enzyme activity

To assess potential oxidative stress as a result of photoinhibition TBARS and catalase activity was monitored at 7 d in *L. minor* (Figure 3.4). ZON lowered mean TBARS content, with the decrease (54 %) at 14.4 mg ZON/L being significant ($P < 0.05$). The 1.8 mg Zn/L reference chemical treatment did not lead to significant changes in TBARS content. Catalase rates showed no significant deviation from the control values for any treatment of ZON or Zn.

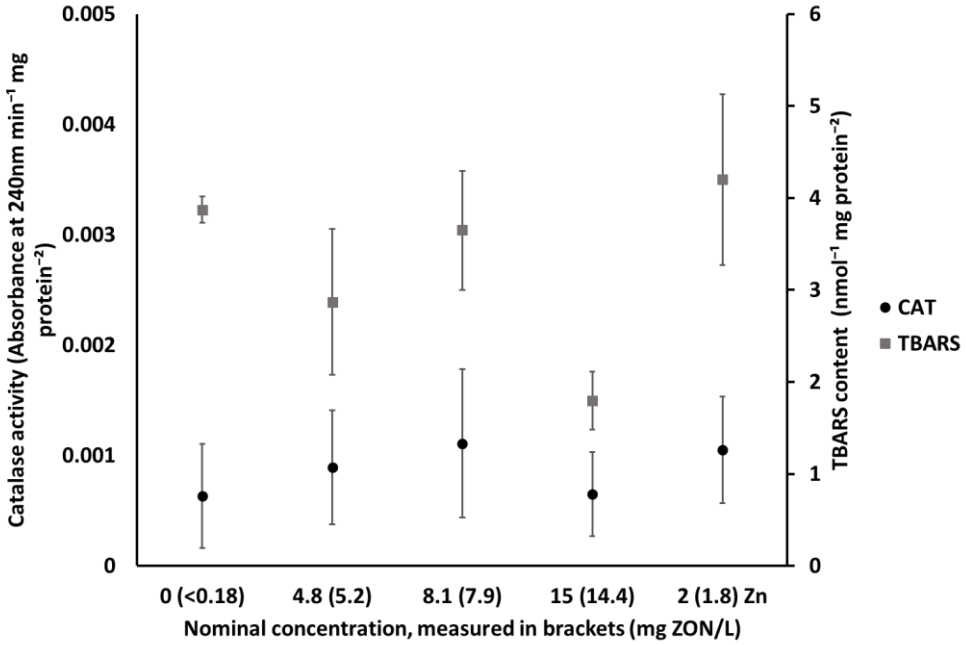


Figure 3.4 Catalase and TBARS content (± SD) measured in *L. minor* after 7 d exposure to ZON in a static study at 24 ± 1°C. Significant difference only seen between the TBARS content in the dilution water control and 14.4 mg ZON/L.

3.4 Discussion

The main finding of this study was both the algae and the aquatic macrophyte show growth inhibition in the presence of ZON, with the algal species being approximately 10 times more sensitive based the most sensitive EC_{50} values. There was also evidence of interference with photosynthesis only in *L. minor*, but at high ZON concentrations,

although this effect was probably not mediated by overt oxidative stress (no change in catalase and TBARS decreasing slightly).

3.4.1 Acute toxicity

The phytotoxicity seen in *P. subcapitata* exhibited a concentration dependant response, with no effect on the two lowest concentrations. Recovery was seen at 0.23 mg ZON/L between the 24 and 72 h observations, with only yield significantly inhibited at 72 h, and significant inhibition in both of the higher exposures of 0.83 and 3.1 mg ZON/L. Whereas in *L. minor* there was no constant change with concentration but a significant growth response at the highest concentration. The only published data for ZON toxicity to *L. minor* found was an exposure at a single concentration of 1 mg ZON/L, which showed no effect on growth at this concentration (Vanhoutte *et al*., 2017). This supports our findings and considering the *L. minor* growth inhibition values of 38 - 67% seen at 11.4 mg ZON/L in this study, ZON appears to be less toxic to *Lemna* sp. than mycotoxins tested by Abbas *et al*. (2002), Abbas *et al*. (2013). Where reported growth inhibition due to DON, nivalenol, T-2 toxin and verrucarin A, was 38 - 72% at concentrations in the range of 0.5 - 4.6 mg/L, resembling more the EC_{20} values generated in this study of 3.0 – 6.5 mg ZON/L. The only mycotoxin reported as less toxic to *Lemna* sp. than ZON is butanolide with 62 % inhibition at 66.7 mg/L (Vesonder, 1992). No previous studies for ZON toxicity to microalgae were found for comparison. The microalgae *Chlamydomonas reinhardtii* had a relatively insensitive LOEC of 10 mg DON/L after a 150 h exposure (Suzuki & Iwahashi, 2014) showing opposing sensitivities for macrophytes and microalgae when comparing ZON and DON. These findings demonstrate the value of expanding phytotoxicity data to include algae such as *P. subcapitata* when considering the potential risk of mycotoxins to freshwater ecosystems.

3.4.1.1 Sub lethal effects

Further to measuring the adverse outcome in terms of growth as a result of ZON exposure, we investigated potential mode of action leading to the observed phytotoxicity; measures of chlorophyll fluorescence in a dark-adapted state and biochemical indicators of oxidative stress. Of the 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 3.3). 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 3.2).

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*., 2016a). 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 an 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 mode of action 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) (Figure 3.5). 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).

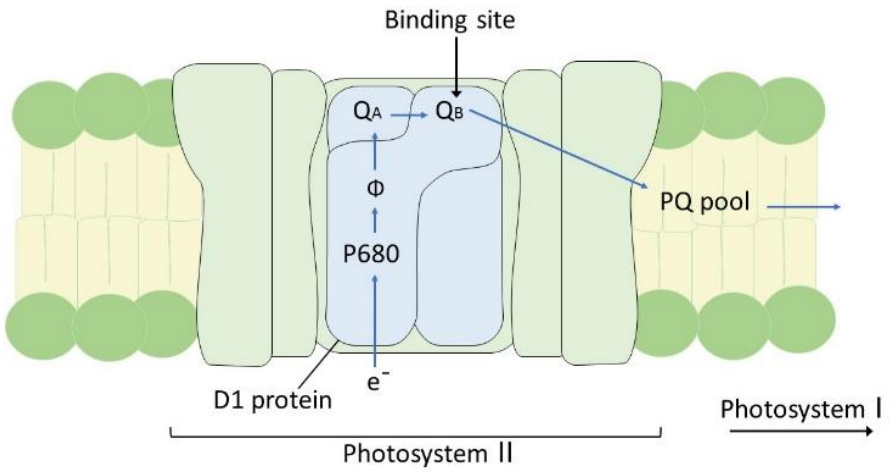


Figure 3.5 The electron transfer route through Photosystem II in the thylakoid membrane and identifying the location of QB, the binding site for herbicides which inhibit photosynthesis by reducing energy transfer through the electron transport chain (after Giardi and Pace, 2005).

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 (Krieger-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 are limited in terms of extrapolating an AOP, but show a strong basis to work from with ZON affecting electron transport, additional measures

could have been taken such as light adapted state chlorophyll fluorescence including non-photochemical quenching (NPQ) and are a key area to consider to demonstrate whether 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.

3.5 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 hypothesis of photo oxidative stress being due to ZON-induced electron transport inhibition.

Chapter 4. Toxicity of DON and ZON to freshwater invertebrates, and construction of species sensitivity distributions.

4.1 Introduction

To consider the risk of a contaminant to freshwater ecosystems, different trophic levels must be considered in the analysis. With the data generated in the previous chapter there is now both plants and fish (in literature) accounted for in ZON toxicity data, however invertebrates must also be considered. Considering opposing sensitivities were seen for microalga and macrophyte in literature data for DON, invertebrate studies were also performed with DON for comparison here. 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) with reproduction as an endpoint.

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 or EFSA assessments. In ECHA freshwater deterministic assessments 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).

This chapter covers the work previously published as a paper in Chemosphere entitled Environmental risks to freshwater organisms from the mycotoxins deoxynivalenol and zearalenone using Species Sensitivity Distributions by Eagles, E.J., Benstead, R., MacDonald, S., Handy, R. & Hutchinson, T.H. (2021).

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. Secondly, results from these studies, along with previously reported data for freshwater plants and fish also included, an SSD was determined for each mycotoxin. It was predicted that

each mycotoxin would have different species sensitivities reflecting potentially different MOAs. This hypothesis extends that of the previous chapter, in that for ZON which is defined as a mycoestrogen, the vertebrate *D. rerio* is expected to be most sensitive organism, and adds to this that it is plausible that because DON has been linked to spread of fungal disease in plants that the plant *L. minor* would be a sensitive species for this mycotoxin. Following SSD analyses to address this hypothesis, HC_5 values were derived to be used later in the thesis to assess potential environmental risk.

4.2 Methods

4.2.1 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 is detailed in Chapter 2 and summarised in Table 4.1. 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 based upon the results of Chapter 2. The positive control values chosen and resulting inhibition in mycotoxin studies are provided in Table 4.2. 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. During the mycotoxin studies the positive control groups exposed to zinc all showed inhibition although some

values did vary between the two studies. The discrepancy between the *L. stagnalis* positive controls was due to different concentrations being used in each instance. The *T. thermophila* positive had a lower inhibition of only 24 % in the DON exposure study compared to 40 % the ZON study, this was likely due to the length of time between the studies meaning a new kit was purchased for the DON study, so the culture used varied between the two tests. There was a similar situation for the *H. vulgaris* studies where cultures were renewed with new starter organisms between zinc and mycotoxin studies due to the high number of organisms required and the difficulty in maintaining this number of Hydra over extended periods of time, resulting in 100 % inhibition at the predicted EC_{50} concentration used. Due to the variance seen in the positive controls here, it would be beneficial to run a trial zinc study with each new culture in future. These results highlight that, time allowing, it would be ideal to run more thorough zinc trial studies to improve the confidence intervals around the EC_{50} values to generate a more reliable positive control value. But overall, the presence of inhibition in positive control groups, 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.

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, to reflect the solvents in which these mycotoxin stock solutions were supplied in. The solvent control concentration was either 3.2 or 10 µl/ml per study dependant on whether the highest test concentration in the study 3.2 or 10 mg/L.

Table 4.1 Details of the experimental conditions used for each invertebrate toxicity test.

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

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. The list of ingredients for media are provided in Table 2.1

Table 4.2 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	EC_{50} ± 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

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 DON 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.

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.

4.3 Results

4.3.1 ZON

The dose response curves, based on nominal concentrations, for ZON are shown in Figure 4.1. 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 nominal concentration tested of 10 mg ZON/L. Although *B. calyciflorus* and *T. thermophila* showed effects at multiple concentrations, inhibition values were ≤ 17% for *B. calyciflorus* and in the dose response relationship for *T. Thermophila* 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*. *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 (Figure 4.2). 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 EC_{50} value of 1.1 mg ZON/L for *H. vulgaris*.

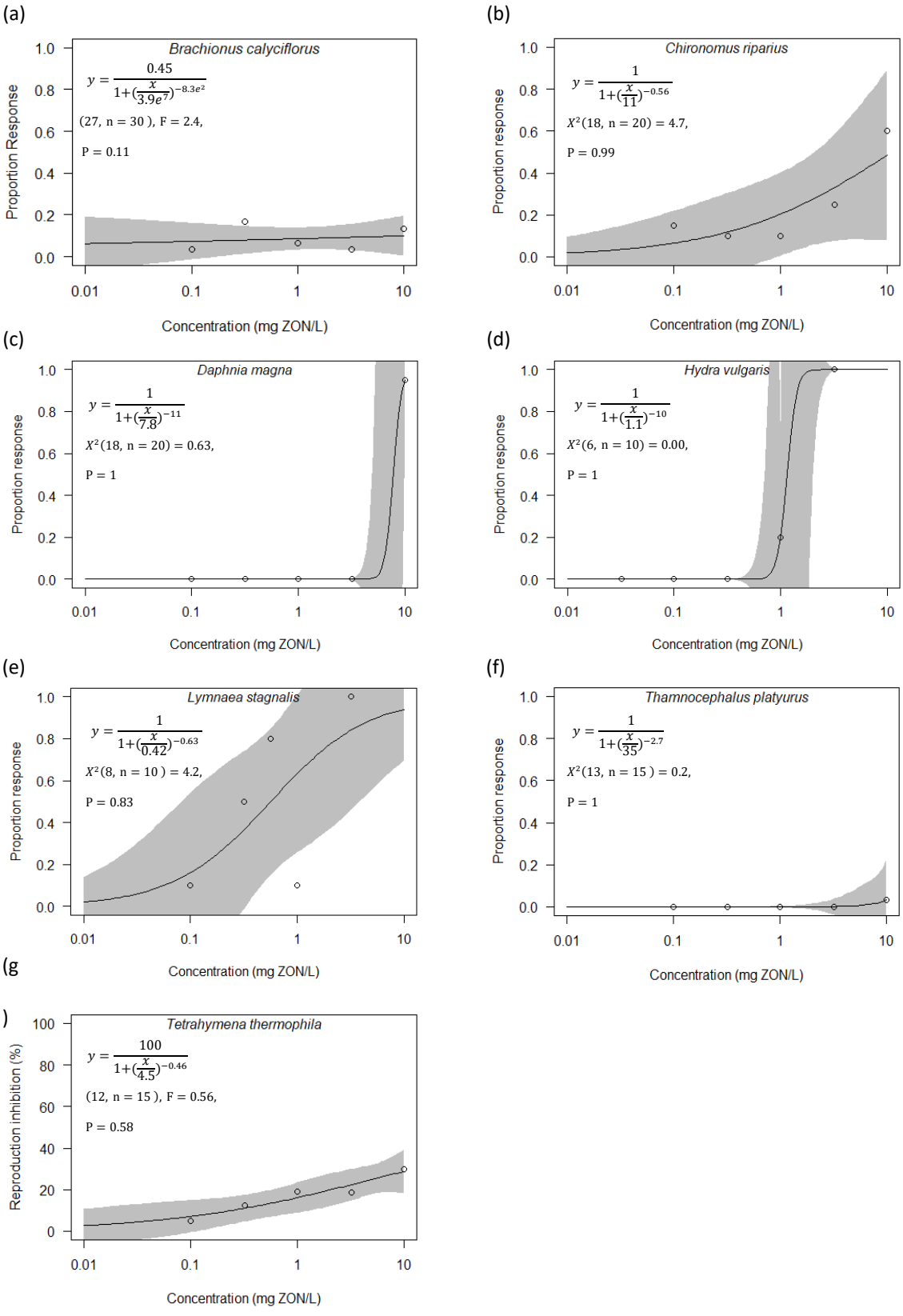


Figure 4.1 Dose response curves of freshwater invertebrates exposed in acute laboratory studies to ZON, based on nominal concentrations, with shaded bands indicating 95 % CI. Model equations along with the chi-squared (X^2)/ F-value (Degrees of freedom, n) and P value from goodness-of-fit tests are provided.

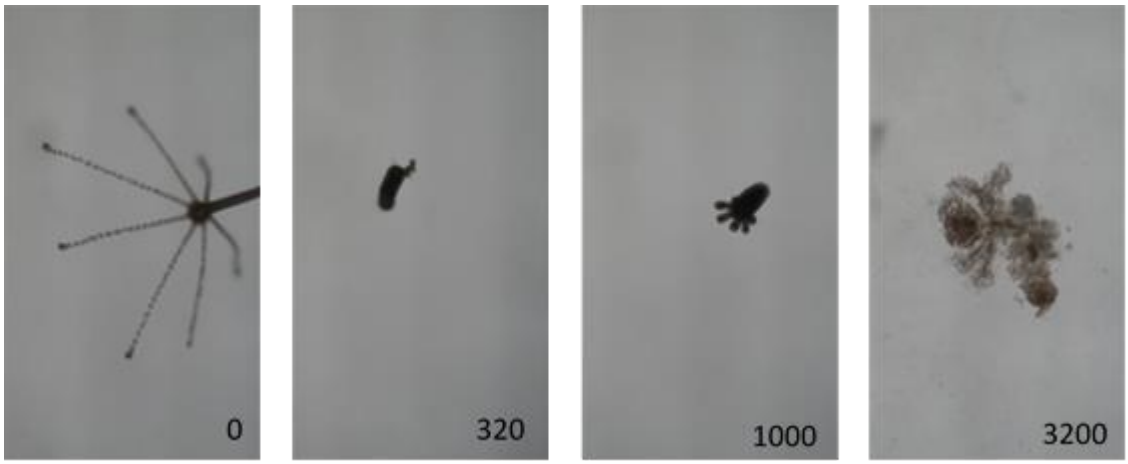


Figure 4.2 Images showing morphology of *H. vulgaris* after 96 h exposure to ZON at concentrations 0 – 3200 µg ZON/L (length of fully extended control *Hydra* ~ 20 mm, length of contracted *Hydra* ~ 4 mm).

L. stagnalis was more sensitive to mortality than *H. vulgaris*, with an EC_{50} of 0.42 mg ZON/L. During the 7 d study with *L. stagnalis* embryos no mortality was seen in the DMSO controls. 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.

Following the 7 d *L. stagnalis* study an extended study was carried out to monitor sub lethal effects as result of ZON exposure. Solutions were replaced twice weekly during the study. Survival was monitored regularly over 14 d (96 h, 7 d, 9 d and 14 d). The sub lethal endpoints were measured at 9 d to allow time for shell formation to occur and a heartbeat to be visible in the embryos. At this point individuals were filmed for 60 seconds using a camera attached to a low power stereo microscope, heart rates were determined from the video clips and still images were saved from points where individuals were in the correct orientation for growth endpoints (shell size and ocular distance) to be measured using InfinityAnalyse software (Lumenera, Ottawa).

In this second *L. stagnalis* study no abnormal morphology or mortality was seen in the DMSO controls. The zinc positive controls had 10 % mortality by 96 h, increasing to 55 % by 9 d with 80 % of the remaining individuals showed abnormal morphology (Table 4.3). Some showed a reduced growth rate, reflected in a significant difference in shell height and length. Mortality in ZON exposed embryos generally occurred during the first 96 h of the study, with the embryos not developing past the initial morula development stage (Figure 4.3). Mortality of 10 and 90 % were recorded for 0.1 and 1.0 mg ZON/L respectively at 96 h, with the later increasing to 95 % by 9 d. At 9 d ZON also induced sub lethal effects in terms of visual morphology at concentrations between 0.32 to 1.0 mg ZON/L, with effects increasing in response to concentration from 10 to 100 % inhibition.

Table 4.3 Mortality and morphology observations from an extended 9 d ZON study with *L. stagnalis*

Nominal concentration (µg ZON/L)	% Mortality			Abnormal morphology (%)[a]
	96 h	7 d	14 d	9 d
0	0	0	0	0
0.01	0	0	0	0
0.032	0	0	0	10
0.10	15	15	15	18
0.32	0	0	0	25
1	90	95	100	100
Zinc	10	55	85	100
NOEC	-	-	-	-
LOEC	-	-	-	-
EC_{10}	0.17	0.16	0.15	0.08
EC_{20}	0.28	0.27	0.26	0.18
EC_{50}	0.62	0.59	0.58	0.50

Significant effects in quantitative measures of development (heart rate, shell height, shell length and ocular distance) were only seen in the one embryo surviving at 1.0 mg ZON/L. The EC_{50} values for this extended study show only a small variation

between lethal and sub lethal levels, with an EC_{50} of 0.50 mg ZON/L for morphology and 0.59 mg ZON/L for mortality.

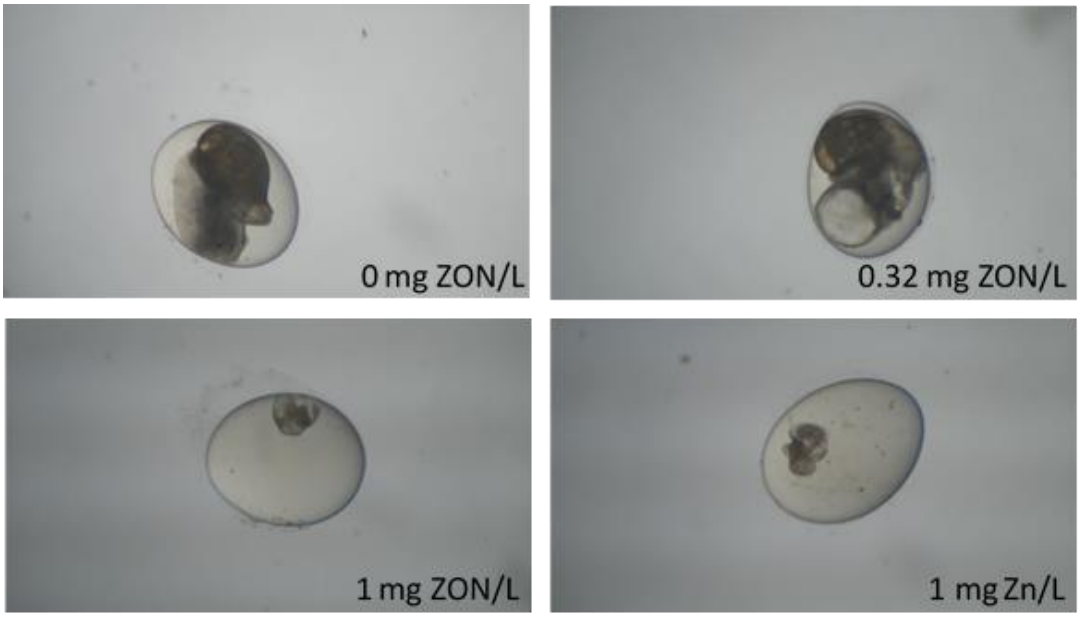


Figure 4.3 Images showing abnormal morphology from an extended 9 d ZON study with *L. stagnalis* (length of egg capsule ~ 600 µm).

4.3.2 DON

Results for the DON exposures are shown in Figure 4.4, 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 4.4.

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 (Figure 4.4) with DON treatments causing a maximum of 20 % inhibition at 0.32 and 1 mg DON/L. Both *B. calyciflorus* and *T. thermophila*, had EC_{50} values > 3.2 mg DON/L.

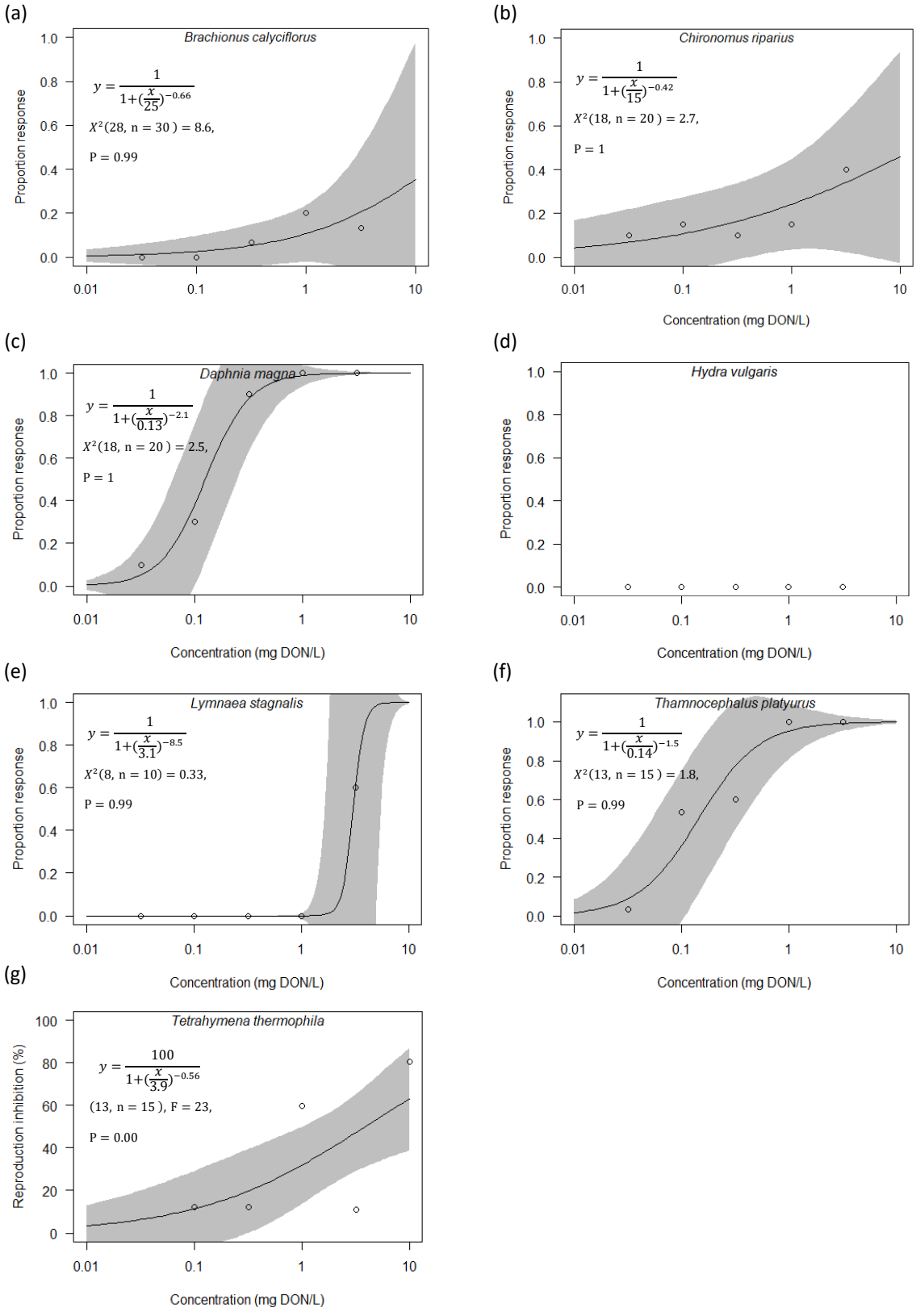


Figure 4.4 Dose response curves of freshwater invertebrates exposed in acute laboratory studies to DON, based on nominal concentrations with shaded bands indicating 95 % CI. Model equations along with the chi-squared (X^2)/ F-value (Degrees of freedom, n) and P value from goodness-of-fit tests are provided. Note, nominal concentrations were confirmed by measurements in the water samples.

Table 4.4 Mean measured concentration (mean calculated from test solutions collected at the beginning and end of exposures) values for DON for invertebrate exposures. Chemical analysis performed using LC-MS/MS.

Organism	Concentration (mg/L)	
	Nominal	Measured (mean ± SD)
B. calyciflorus	DWC	< 0.01
	SC	< 0.01
	0.1	0.10
	0.32	0.31
	1.0	0.87
	3.2	2.31[a]
	3.10	6.13[a]
T. platyurus	DWC	< 0.01
	SC	< 0.01
	0.1	0.09
	0.32	0.30
	1	0.85
	3.2	2.18[a]
	10	5.79[a]
T. thermophila	DWC	< 0.01
	SC	< 0.01
	0.1	0.09
	0.32	0.30
	1	0.85
	3.2	2.18[a]
	10	5.79[a]
C. riparius	DWC	< 0.01
	SC	< 0.01
	0.032	0.03 ± 0.004
	0.1	0.10 ± 0.008
	0.32	0.30 ± 0.045
	1	0.82 ± 0.021
	3.2	2.16[a]
D. magna	DWC	< 0.01
	SC	< 0.01
	0.032	0.03 ± 0.0002
	0.1	0.09 ± 0.002
	0.32	0.29 ± 0.004
	1	0.79 ± 0.047
	3.2	2.25[a] ± 0.001
H. vulgaris	DWC	< 0.01
	SC	< 0.01
	0.032	0.03 ± 0.01
	0.112	0.11 ± 0.002
	0.32	0.34 ± 0.033
	1	0.96 ± 0.022
	3.2	2.24[a] ± 0.036
L. stagnalis	DWC	< 0.01
	SC	< 0.01
	0.032	0.03 ± 0.001
	0.112	0.10 ± 0.002
	0.32	0.30 ± 0.017
	1	0.82 ± 0.001
	3.2	2.42[a] ± 0.111

[a]Outside quantification range, those without SD were 24 h tests where concentration was only measured at the beginning of the study, quantified variation of ≤20 %

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 EC_{50} 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 EC_{50} 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. EC_{50} values for 24 and 48 h were 6.0 and 0.13 mg DON/L respectively.

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.

Table 4.5 A summary of the acute effect values from freshwater invertebrate laboratory studies with mycotoxins DON and ZON.

Mycotoxin	Duration	Organism	Endpoint	Summary effect values (mg/L)			References
				EC_{10} ± SE	EC_{20} ± SE	EC_{50} ± SE (95 % CI)	
DON	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	-	-	> 296	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
ZON	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

Table 4.5 Continued

Mycotoxin	Duration	Organism	Endpoint	Summary effect values (mg/L)			References
				EC_{10} ± SE	EC_{20} ± SE	EC_{50} ± SE (95 % CI)	
ZON	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

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

4.4 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).

EC_{50} values are not listed in the papers found for DON plant studies, but they have been calculated based upon the data provided. The microalgae *Chlamydomonas reinhardtii* had a relatively insensitive LOEC of 10 mg DON/L after a 150 h exposure (Suzuki & Iwahashi, 2014). Based upon the *C. reinhardtii* 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 29.6 g/L DON, only when embryos were injected with DON were 96 hpf EC_{50} values generated for the following endpoints: hatching 488 mg/L, deformity 323 mg/L and mortality 758 mg/L (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, only the growth EC_{50} values for both microalgae and macrophytes from the previous chapter are available. 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 4.5) were used to construct SSD models, these are shown in Figure 4.5. 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.

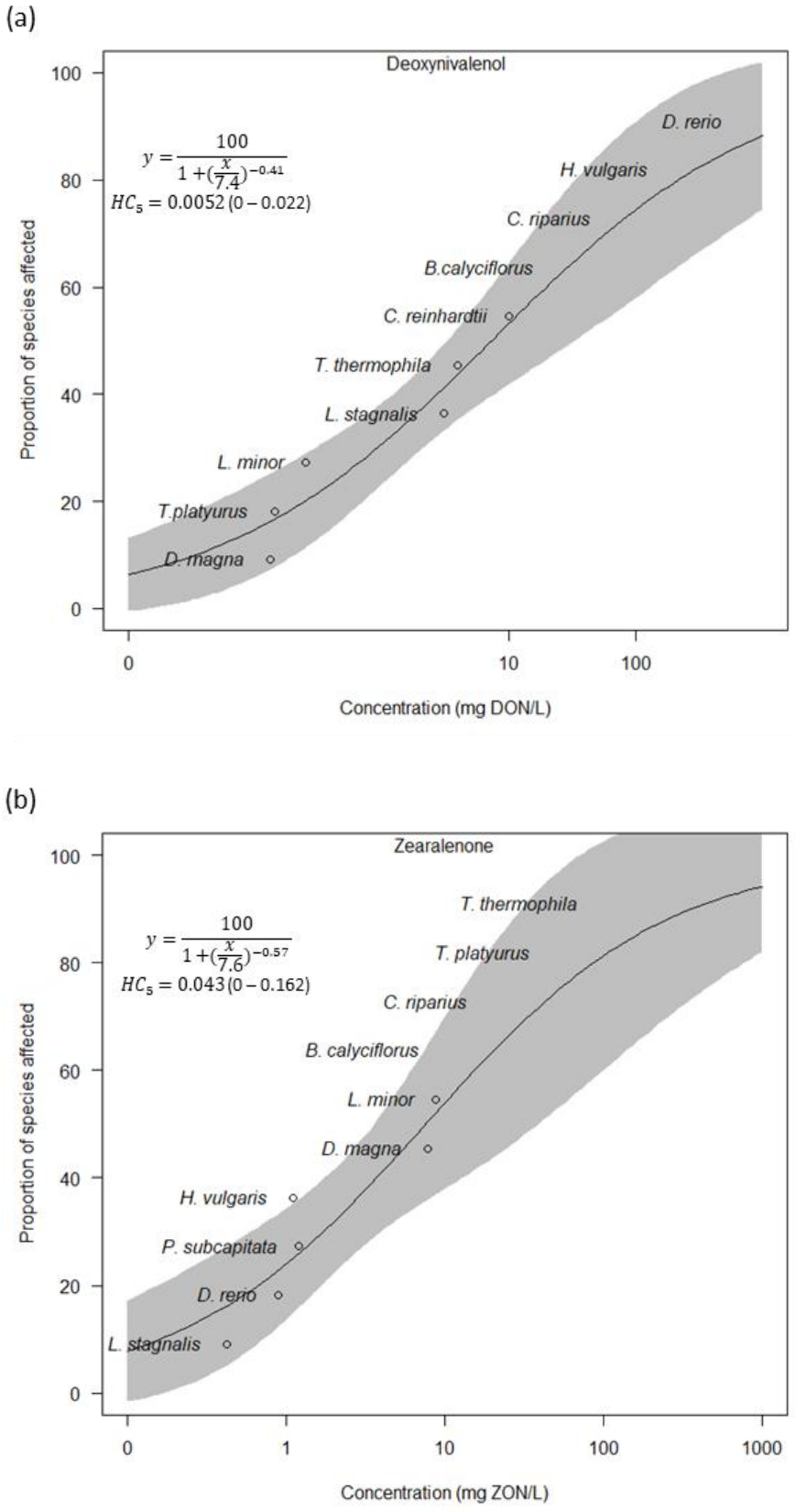


Figure 4.5 Species sensitivity distributions curves with shaded bands indicating 95 % CI. a. DON and b. ZON. Markers are EC_{50} values from acute toxicity studies, those without markers had undetermined EC_{50} values greater than the highest concentration tested and were therefore included in the rank but not in the SSD fit.

4.5 Discussion

4.5.1 Comparative toxicity of ZON and DON

The novel data here presents 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 4.5), the DON studies identified crustaceans as the most sensitive organisms with *T. platyurus* and *D. magna* having the lowest EC_{50} values whereas for ZON it was mollusc embryos. This high sensitivity in the mollusc embryo study was also highlighted in the SSD analysis (Fig 4.5), where the zebrafish embryo study was the second lowest EC_{50} value for ZON (Bakos *et al*., 2013).

The only test organisms showing low toxicity to both mycotoxins, with undefined EC_{50} 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 seen in acute exposures of *Chironomus* sp. to various environmental pollutants, including metals, aiding in detoxification (Fisher and Meunier, 2008; Prakash, 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 affected 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 additional sublethal study with *L. stagnalis* embryos

showed quantifiable differences in only the 1 mg ZON/L group in the one surviving embryo.

The data generated in this chapter, along with that from the previous chapter, expand the knowledge of DON and ZON toxicity and now satisfy the requirements to perform acute analysis of hazard concentrations in freshwater ecosystems. Using the data here along with literature data hazard concentrations were derived statistically, through SSDs. This resulted in a lower HC_5 value for DON in comparison to ZON, 5.2 and 43 µg/L respectively. The confidence intervals around the derived parameters shown on the mycotoxin SSDs in Figure 4.5 reflect the need for further data and do not allow for lower bound values to be considered which would allow the HC_5 value to be more precautionary. Further to this the accuracy of the predictions is limited by the absence of EC_{50} values in some cases and the inability to include these within the SSD fit as well as the lack of definition in some of the single species dose response curves. Ideally, tests for each organism lacking an EC_{50} , with a steep dose response curve, or those with poor confidence intervals and unexpected results such as the lack of effect at 1 mg ZON/L falling outside of these wide confidence intervals would be followed up. These subsequent tests would use modified concentration ranges to produce a dose response curve with the lowest concentration showing no effect, the highest 100 % inhibition and the intermediates providing a defined dose response. Arguably, more concentrations could have been used in the initial studies but practically this was not feasible to do whilst maintaining the recommended replicates per concentration, in terms of space and time required to set up the experiments. So the test designs followed the guidance set out in OECD methods (OECD, 2004); of using five widely spaced (geometrically)

concentrations to maximise the likelihood of observing an effect range when no prior data for guidance is available.

The comparative toxicity seen was expected to reflect known MOAs for the mycotoxins. DON has been linked to the spread of disease in plant hosts and programmed cell death defence in plants, and so as expected higher phytotoxicity was seen in the macrophyte *Lemna minor* for DON (Wagacha and Muthomi, 2007; Diamond *et al*., 2013; Vanhoutte *et al*., 2017), but interestingly there is further investigation needed to understand how this mycotoxin exerts stronger toxicity to the two crustaceans tested. 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. Furthermore, *L. stagnalis* was also sensitive to ZON which may support the endocrine disruption MOA as the reasoning behind sensitivity seen as *L. stagnalis* has been recognised as a sensitive model for endocrine disrupting compounds and proposed as a candidate species for future OECD test guidelines (Ducrot *et al*., 2010; Matthiessen, 2008). 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 as a result of varying MOAs. 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. For ZON, with the sensitivity seen for in the *L. stagnalis* embryos it would be worthwhile investigating potential life cycle effects in this organism. With the known oestrogenic nature of ZON, *L. stagnalis* would offer a model to explore the potential reproductive effects of ZON and allow comparison with fungicide counterparts; with *Lymnaea* similarly seen to be one of the more sensitive invertebrate species to fungicides (Ducrot *et al*., 2010; Matthiessen, 2008).

Current environmental data available show the highest recorded levels recorded in freshwater are 1662 ng DON/L and 96 ng ZON/L. Comparison of the EC_{50} and HC_5 values to highest recorded DON and ZON concentrations are shown in Figure 4.6. These HC_5 values can be used for environmental risk assessment purposes when compared with environmental exposure concentrations and this analysis will be covered in the next chapter of the thesis.

4.6 Conclusions

This study is one of the first to develop a comprehensive assessment of mycotoxin toxicity to freshwater invertebrates. Based upon the experimental data, 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. Additional acute or chronic toxicity data will reduce uncertainty in any environmental risk assessment of DON and ZON for freshwater

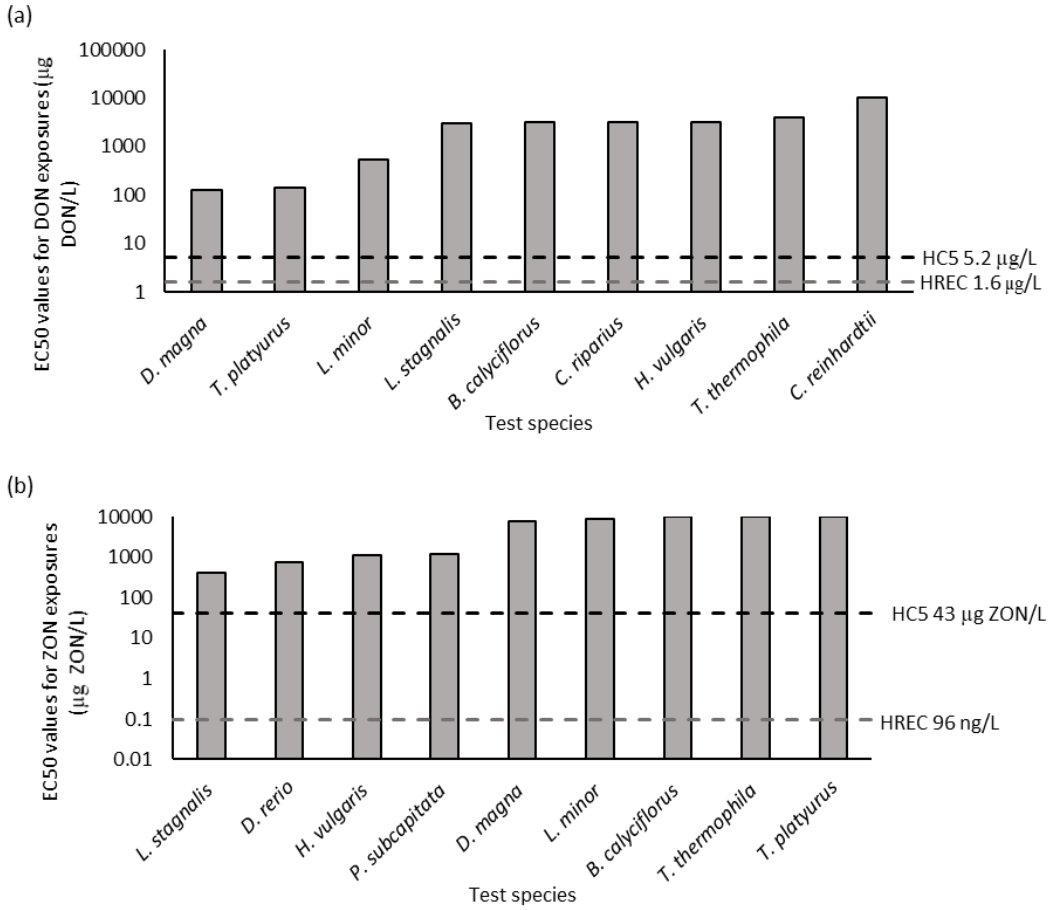


Figure 4.6 EC_{50} values from freshwater toxicity tests performed in this study and those available or calculated from literature for a. DON and b. ZON. The highest concentration tested is plotted for those which had EC_{50} values greater than. HC_5 values calculated from SSD models and highest recorded environmental concentrations (HREC) also shown.

Chapter 5. Environmental risk assessment of DON and ZON in surface waters

5.1 Introduction

Mycotoxins have long been known to cause harmful effects in humans and animals, including endocrine disruption, genotoxicity, immunotoxicity and nephrotoxicity, often leading to fatalities when consumed through contaminated food supplies (Cruz *et al*., 2013; Alshannaq and Yu, 2017). Due to the health concerns, safe limits on mycotoxin concentrations in foods have been developed in order to satisfy the accompanying human health risk assessment of consumption (Cruz *et al*., 2013; Alshannaq and Yu, 2017). There is also recent evidence of mycotoxin contamination of freshwater ecosystems (Bucheli *et al*., 2008; Gromadzka *et al*., 2009; Kolpin *et al*., 2014; Schenzel *et al*., 2012; Waśkiewicz *et al*., 2012). Therefore, for mycotoxins as with other emerging chemicals, all the environmental sources, exposure pathways and hazards to organisms ('biological receptors') should be included in environmental risk assessments (Schwarzenbach *et al*., 2006; la Farré *et al*., 2008). The environmental risk assessment of mycotoxins in freshwater ecosystems within agricultural landscapes is a priority given recent evidence of climate change related increases in fungal diseases in agricultural crops (Medina *et al*., 2017; Moretti *et al*., 2019).

There are currently no recommendations regarding monitoring and limiting mycotoxin contamination of freshwater. Studies looking at quantification of mycotoxins in aquatic samples have been performed in the past decade, often with a focus on the development of analytical approaches from the standard analysis of food/feed contamination and for environmental samples (Hartmann *et al*., 2007; Ribeiro and

Tiritan, 2015). But even in the sparse environmental samples, occurrence of mycotoxins in the ng/L concentration were regularly detected, often including mycotoxins such as deoxynivalenol (DON) and zearalenone (ZON) from *Fusarium* sp. infections in cereals and other key agricultural crops (Bucheli *et al*., 2008; Gromadzka *et al*., 2009; Schenzel *et al*., 2012b; Wettstein and Bucheli, 2010; Waskiewicz *et al*., 2015; Waskiewicz *et al*., 2012; Kolpin *et al*., 2014).

This chapter addresses two aims of the project: to analyse the environmental exposure concentrations of DON and ZON and to conduct an environmental risk assessment for DON and ZON. For the exposure assessment, a sampling scheme to investigate the presence of mycotoxins in the UK is defined and a meta-analysis approach was used to assess the measured environmental concentration (MEC) values of DON and ZON previously reported for freshwater environments. This looked at both a global and regional scale in order to calculate the 90th percentile values. To characterise the ecological risks of these mycotoxins.

Generating appropriate hazard concentrations for chemicals of emerging concern is challenging, as toxicity data is often limited and performing experiments is costly. Here, the concept from the previous chapter, that considering all trophic levels is vital in protecting communities as a whole, is extended by comparing two approaches, deterministic and probabilistic, for calculation of the Predicted No-Effect Concentration (PNEC) values (as detailed in Figure 1.1). Data from ecotoxicological experiments and published data, summarised in earlier chapters of this thesis (Chapters 3 and 4), as well as QSAR predicted toxicity values are considered. The assumption was that statistically derived probabilistic PNECs would provide an appropriately value, in comparison to deterministic approaches with only model species which may be overly conservative,

based upon high assessment factors required, or under conservative if predicted toxicity data are used, as chemicals of emerging concern often have little comparable data for accurate predictions.

Given that agricultural crops are a major source of mycotoxins, the environmental risk assessment for DON and ZON takes into account the guidance for aquatic organisms according to the European Food Standard Agency for edge-of-field surface waters (EFSA 2013) but with evidence of mycotoxins entering larger bodies of water also takes into consideration REACH guidance for the freshwater compartment (ECHA, 2008). This will inform future research into mycotoxins as chemicals of emerging concern, by identifying the highest risk mycotoxin out of DON and ZON, classifying most at risk areas and proposing key areas where knowledge is lacking.

5.2 Exposure assessment

5.2.1 River sample collection in the UK

A range of locations were selected to collect river water samples for mycotoxin analysis in the UK. Local sample sites in the South West were chosen in an area of moorland expected to be subjected to lower levels of mycotoxins, due to the absence of cereal crops. These were 6 sites in Dartmoor (Figure 5.1) and covered 3 moorland locations and 3 surrounded by deciduous forest (M1 Two bridges – West Dart River (Grid reference SX606749), M2 Postbridge - East Dart River (SX652792), M3 Venford Reservoir (SX685709), F1 Plymbridge Woods - River Plym (SX523584), F2 Denham Woods (SX475675), F3 Buckland Bridge - River Webburn (SX718719)) an additional forested site was also added (F4 River Dart at Buckland Bridge (SX718719)) as at one sampling time the water levels were too high to safely access two of the original forested sites.

Forested sites were included to see if these may also act as a mycotoxin input source and reflect the higher mycotoxin concentrations seen in the forested location of the Gromadzka *et al*. (2009) study. To test the geographical distribution of mycotoxins in agricultural areas of the UK, 12 freshwater river sites were selected (Figure 5.2). These sites cover 6 regions of England: Yorkshire and Humber (Y1 River Derwent, Y2 River Ouse), East Midlands (EM1 River Trent, EM2 River Witham), East of England (E1 River Great Ouse, E2 River Blackwater), London and South East (SE1 River Lavant, SE2 River Test), South West (SW1 River Frome, River Parrett SW2) and West Midlands (WM1 River Tern, WM2 River Stour). The sampling sites were chosen to monitor areas potentially at risk of mycotoxin input based upon the abundance of cereal crops and areas highlighted as having a history of FHB infections. These 12 agricultural sites are locations sampled regularly by the Environment Agency who agreed to provide samples for analysis.

The original sampling regime aimed to collect samples from each location over the period of a year at monthly intervals. Unfortunately, due to issues with access to LC-MS equipment this was delayed and due to Covid no regular sampling scheme, at sites expected to be subjected to mycotoxin input, was able to be completed for the project. Some samples were collected from the local sampling sites (September 2019, November 2019, February 2020) (Figure 5.3), with all sites visited on the same day for each collection period. Samples were collected by immersing a 500 ml Nalgene bottle below the water surface and returned to the laboratory within 3 hours of collection, pH and temperature were measured at each sampling site. Upon arrival at the laboratory samples were filtered (0.2 μm) and frozen at -80°C for later analysis. For the agricultural sites, samples were provided in February 2020. Samples were collected by EA members; these were 2 L spot samples shipped on the same day as collection under chilled

conditions. Samples were collected the following day and transported to The University of Plymouth for filtration and frozen as above, only 500 ml of each sample was used.

Samples were later concentrated by solid phase extraction (SPE) (Oasis HLB cartridges, 6 ml, 200 mg Waters Corporation, Milford MA, USA)**.** The SPE cartridges were conditioned with 5 mL of methanol, 5 mL of Milli-Q water and methanol (1/1, v/v), and 5 mL of Milli-Q water**.** The 500 ml water samples were passed through the cartridges with a maximum flow rate of 10 mL/min. Columns were then washed with 5 ml Milli-Q water before analytes were eluted with 5 ml methanol. Aliquots were dried under nitrogen gas at 40 °C and reconstituted in 1 ml of Milli-Q water/methanol (90/10, v/v)**.** Samples were chilled and shipped to Fera Science Ltd. for LC-MS/MS analysis.

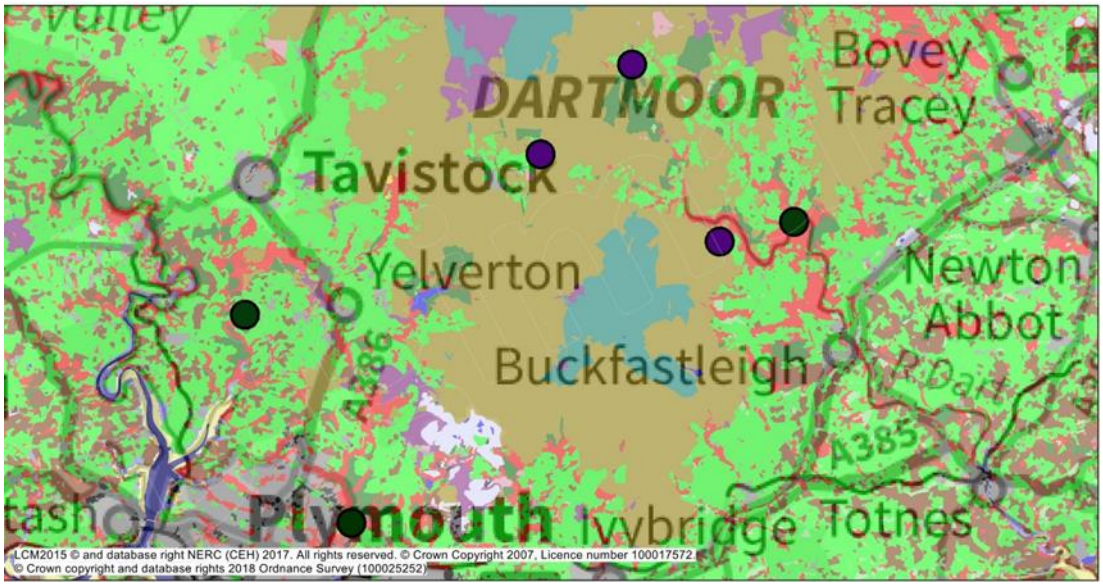


Figure 5.1 Location of sampling sites in Dartmoor. Purple circles show the locations of moorland sampling sites, green show forested. Red areas on the land coverage map indicate deciduous forest.

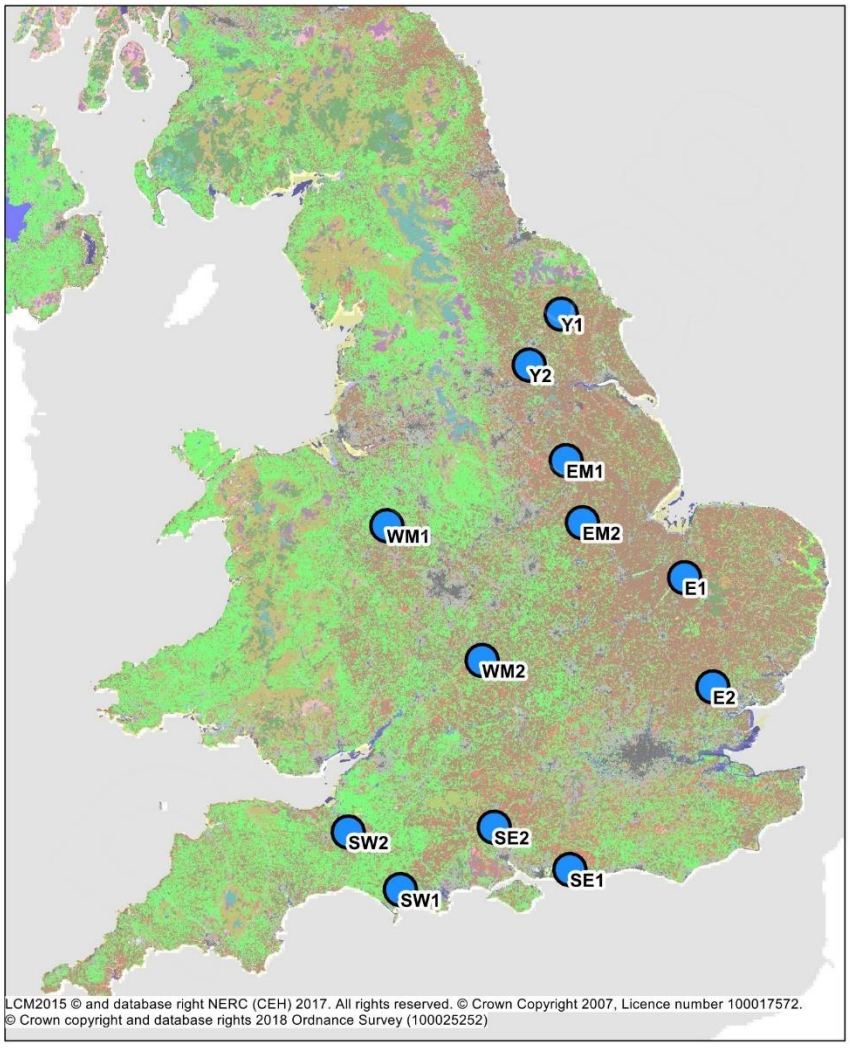


Figure 5.2. Location of agriculturally impacted sites across the UK with light brown on the map representing arable land.

Figure 5.3 Time series photographs at two of the Dartmoor sampling sites taken during each sampling date a, b: 18/09/19; c, d: 17/11/19; e, f: 25/02/20

Samples were run alongside standards for DON and ZON (µg/L), at concentrations of 0.5, 1, 5, 10 and 50 µg/L to represent to 1 – 100 ng/L in the original samples (which had been concentrated x 500), but no detections were reported for the samples (Table 5.1); any peaks seen were below the calibration range (limit of detection 1 ng/L for DON and ZON) and had poor signal to noise ratio indicating an absence of the mycotoxins rather than being unquantifiable. No method development or spiking of samples was able to be completed to test the recovery performance of the method used. As the majority of samples tested were from non-agricultural areas in a moorland environment the absence of mycotoxins was not unexpected. Although agricultural sites were also sampled on one occasion the absence of any detections here was likely due to the high precipitation which occurred across the country the week preceding sampling, leading to three sites not being sampled due to flooding. Although mycotoxins may be expected to be found in high-run-off events the volume of precipitation was significant, and the samples were too late to detect any initial increase in concentration that may have occurred.

As the UK river sampling was not able to be completed to the original plan, the exposure assessment from this point onwards focusses on deriving MEC's from global exposure data previously reported in literature.

Table 5.1 Results of LC-MS/MS analysis of UK river samples to detect mycotoxin DON and ZON.

Date	Site	pH	Temp (°C)	DON (ng/L)	ZON (ng/L)
18/09/19	M1	8.5	17.1	n.d.	n.d.
	M2	8.0	18.6	n.d.	n.d.
	M3	8.0	19.4	n.d.	n.d.
	F1	7.6	15.8	n.d.	n.d.
	F2	8.1	16.2	n.d.	n.d.
	F3	7.8	16.1	n.d.	n.d.
	F4	-	-	-	-
17/11/19	M1	7.5	8.8	n.d.	n.d.
	M2	7.9	8.3	n.d.	n.d.
	M3	7.8	8.5	n.d.	n.d.
	F1	8.0	9.9	n.d.	n.d.
	F2	7.7	10	n.d.	n.d.
	F3	7.4	9.2	n.d.	n.d.
	F4	7.5	8.5	n.d.	n.d.
24/02/20	E1	-	-	n.d.	n.d.
	E2	-	-	n.d.	n.d.
	EM1	-	-	n.d.	n.d.
	SE2	-	-	n.d.	n.d.
	SE1	-	-	n.d.	n.d.
	SW1	-	-	n.d.	n.d.
	SW2	-	-	n.d.	n.d.
	WM1	-	-	n.d.	n.d.
	WM2	-	-	n.d.	n.d.
25/02/20	M1	7.5	7.0	n.d.	n.d.
	M2	7.9	6.7	n.d.	n.d.
	M3	8.6	7.4	n.d.	n.d.
	F1	-	-	-	-
	F2	-	-	-	-
	F3	8.5	8.9	n.d.	n.d.
	F4	8.5	8.2	n.d.	n.d.

5.2.2 Literature data collection

A literature survey was carried out using the Web of Science database with key search terms “mycotoxin,” “deoxynivalenol,” “zearalenone”, “aquatic,” “water,” “environmental samples,” “river,” and “stream”. Relevant references cited within articles were also searched for. The summary values provided in papers such as minimum, maximum and mean measured environmental concentrations (MEC) were recorded along with methodological information for each study such as location, water

source type (rivers, streams, lakes etc.), sample type (grab or flow proportional), and the total number of samples collected. Further to this, the frequency of detection (FOD) and quantification (FOQ) were calculated, and where determined, the limit of detection (LOD) and quantification (LOQ) were also recorded. This provided an overview of the data available on DON and ZON environmental concentrations, raw data values from the studies were also analysed where possible.

For each study where data were provided as individual concentrations, for separate sampling dates at each individual sampling location, the data were compiled. Where raw data were not provided but plotted on graphs (only applicable for Schenzel *et al*., 2012) the values were derived using WebPlotDigitizer version 4.3. The accuracy of these values derived from graphs was checked by comparing the derived medians with the reported medians in the paper. Many concentrations were listed as < LOD or < LOQ, those < LOD were replaced with 0 (Li *et al*., 2020) and those < LOQ were replaced with ½(LOD + LOQ) or where LOQ was not provided then the value of LOD (European Commission, 2009).

To consider seasonal variation in concentrations, the date of sample collection was also analysed. Data points were assigned dates where known and reported, where only a month was provided an arbitrary day was assigned to the month (15th) for plotting on time scales. Where only a season was reported the date was set at a mid-point (e.g. winter as Jan 30th). Again, for the Schenzel *et al*. (2012) study where quantified values were plotted on graphs approximate dates could be derived using WebPlotDigitizer.

To calculate 90th percentile MEC values for each mycotoxin the raw concentration data were percent ranked for each mycotoxin (rank of sample) / (total

samples + 1) in exposure concentration distributions. All graphs were plotted in R using geom_jitter with alpha set at ½ to help with the over plotting of the points at the < LOD and < LOQ assigned concentrations (R). Elsewhere exposure assessments consider distributions for each site individually (Schuler and Rand 2007). However, due to the paucity of data in this instance multiple distributions were considered. Firstly, overall distributions collated the data for each mycotoxin based on all the relevant studies into a single regression analysis, with concentrations ranked (rank of sample) / (total samples + 1). Further to this, distributions based on different water sources studied: creek, drainage ditches, groundwater, lakes, rivers, streams and WWTP effluent, were derived. Finally, distributions were generated for each study reviewed.

5.2.3 Concentrations of DON and ZON in aquatic samples

The total number of papers with relevant data found to be used in the analysis was 14. For each mycotoxin, the results of individual environmental studies are reported in Table 5.2 and these summarise a total of 1665 surface water samples across the studies listed. Generally sampling regimes used grab samples, with only three studies using a flow proportional collection method. These studies covered only 4 countries, Poland (n = 10), Portugal (n = 4), Switzerland (n = 4) and the USA (n = 9) and have all been published from 2009 onwards (Dudziak, 2010; Gromadzka *et al*., 2009; Waśkiewicz *et al*., 2012; Waśkiewicz *et al*., 2015; Laranjeiro *et al*., 2018; Ribero *et al*., 2016; Kolpin *et al*., 2010; Kolpin *et al*., 2014; Maragos *et al*., 2012; Gromadzka *et al*., 2015; Ribero and Tiritan, 2015; Ribero *et al*., 2015; Schenzel *et al*., 2012; Wettstein & Bucheli, 2010).

The smaller sampling schemes looking at < 20 samples tended to report comparatively high frequencies of detection due to the small sample size. When looking at the larger scale and longer sampling schemes, DON was detected across these studies

in 36 – 79 % of samples (Schenzel *et al*., 2012; Kolpin *et al*., 2010; Kolpin *et al*., 2012) and for larger ZON studies the FOD were lower at 13 – 32 % (Laranjeiro *et al*., 2018; Kolpin *et al*., 2010; Kolpin *et al*., 2014; Maragos *et al*., 2012) with one study reporting no ZON detections (Ribero *et al*., 2016). However, the LOD in the latter study was comparably higher at 90 ng ZON/L. Furthermore, in the same study the metabolite a-zearalenol was present suggesting most of the parent compound had been metabolised since entering the water such that it was below detectable concentrations or entered in this form, as has been measured in feedlot run-off and cattle manure (Bartelt-Hunt, 2012). Overall, from the available monitoring data for mycotoxins in surface waters across four Northern Hemisphere countries, DON and ZON were detected in 42 % and 31% of all samples, respectively.

The only WWTP study which looked at > 20 samples was for ZON and had a high FOD of 90% (Gromadzka *et al*., 2015), DON sampling schemes at WWTPs looked at < 10 samples and all had 100 % FOD with quantified concentrations of 18 – 79 ng DON/L. This is higher than ZON concentrations in WWTP which were < 10 ng ZON/L. Similarly, in surface water samples DON was the mycotoxin seen to reach higher concentrations. Studies which used grab sampling found maximum DON concentrations of 373 – 1662 ng DON/L from water sources including rivers, streams and estuaries. For the DON river samples which used flow proportional collection, the study with the largest number of samples (1054), the maximum concentration was lower at 19 ng DON/L. In ZON surface water studies the maximum recorded concentrations were often < 10 ng ZON/L although three studies had higher maximums of 43 – 96 ng ZON/L, these were from samples collected from rivers or streams.

Table 5.2 Concentrations of the mycotoxins DON and ZON recorded in surface waters of four countries.

Mycotoxin	Location	Site type	Sample type	Total no. samples	Detected			Quantified					Reference
					No.	FOD (%)	LOD (ng/l)	No.	LOQ (ng/l)	Min (ng/l)	Mean (ng/l)	Max (ng/l)	
DON	Portugal	Estuary	Grab	4	4	100	38.7	3	59.5	60	-	412	Ribero and Tiritan (2015)
	Portugal	Estuary								-		373.5	Ribero *et al*, 2015
	Portugal	Estuary	Grab	20	-	-	38.7	-	-	59.5	-	642.4	Ribero *et al*. (2016)
	Switzerland	Rivers	Flow proportional	1054	390	36	1.3	-	3.9	1.3	-	19	Schenzel *et al*. (2012)
	US	Streams	Grab	56	24	43	1.5	-	-	-	-	583	Kolpin *et al*. (2010)
	US	Streams	Grab	105	89	79	1.3	88	-	3.9	-	1662	Kolpin *et al*. (2014)
	US	WWTP	flow-weighted composites	3	3	100	1.3	3	-	30.7	-	75.2	Kolpin *et al*. (2014)
	Switzerland	WWTP	Grab	6	6	100	1.2	6	-	19.2	41.1 (SD 18.5)	73.4	Schenzel *et al*. (2012)
	Switzerland	WWTP	Flow proportional [a]	8	8	100	0.8	8	-	18	-	79	Wettstein and Bucheli (2010)
	Switzerland	WWTP	Flow proportional	7	7	100		7	-	41	56 (SD 8)	66	Wettstein and Bucheli (2010)
	All	All	All	1263	531	42		115					
ZON	Poland	Lake		1	1	100	0.1	1	0.5	-	-	1.52	Dudziak (2011)
		Rivers		2	1	50	0.1	1	0.5	-	-	0.66	

Table 5.2 Continued

Mycotoxin	Location	Site type	Sample type	Total no. samples	Detected			Quantified					Reference
					No.	FOD (%)	LOD (ng/l)	No.	LOQ (ng/l)	Min (ng/l)	Mean (ng/l)	Max (ng/l)	
ZON	Poland	Lake	Grab	2	2	100	0.3	2		0.7	-	1.5	Gromadzka *et al*. (2009)
	Poland	Rivers	Grab	15	-	-	0.3	-	-	1.4	-	43.7	Gromadzka *et al*. (2009)
	Poland	Lake	Grab	9	9	100	-	-	-	-	12.54	-	Waśkiewicz (2012)
	Poland	Rivers	Grab	12	12	100	-	-	-	-	11.53	-	Waśkiewicz (2015)
	Poland	Rivers	Grab	34	9	26	1.8	9	5.5	5.6	16.8	82.6	Laranjeiro *et al*. (2018)
	Portugal	Estuary	Grab	20	0*	-	90	-	137	-	-	-	Ribeiro *et al*. (2016)
	US	Streams	Grab	56	7	13	0.7	-	-	-	-	8	Kolpin *et al*. (2010)
	US	Streams	Grab	105	29	26	12.3	2	-	61.5	-	96	Kolpin *et al*. (2014)
	US	Lakes	Grab	62	12	19	0.4	3	1.5	2.1	-	5.7	Maragos (2012)
		Rivers		20	4	20	0.4	1	1.5	-	-	2.3	
		Creek		28	9	32	0.4	1	1.5	-	-	4.3	
	US	WWTP	flow-weighted composites	3	1	33	12.9	0	-	-	-	-	Kolpin *et al*. (2014)
	Poland	WWTP	-	2	1	50	0.5	1	-	-	-	2.7	Gromadzka *et al*. (2009)
	Poland	WWTP	-	30	27	90	0.3	27	-	0.121	-	9.18	Gromadzka *et al*. (2015)
	Poland	WWTP	-	1	1	100	0.1	0	0.5	-	-	-	Dudziak (2011)
	All	All	All	402	125	31		48					

From all the studies reviewed the raw concentration data, collected at individual sampling locations at separate time points, that could be obtained initially contained 532 individual data points from 11 of the studies. Using WebPlotDigitizer allowed a further 855 points from the Schenzel *et al*. (2012) study to be included for a total of 1387 concentration values: 993 for DON and 394 for ZON. To ensure the concentrations extracted using WebPlotDigitizer were accurate the median for each sample site was calculated from the generated concentration values and compared to the reported medians for each site reported in the study. The resulting medians differed by ≤ 0.1 ng/L supporting the accuracy of the generated values. Figure 5.4 shows all 1387 data points plotted based upon the type of water source at which samples were collected and the respective studies they were collected in.

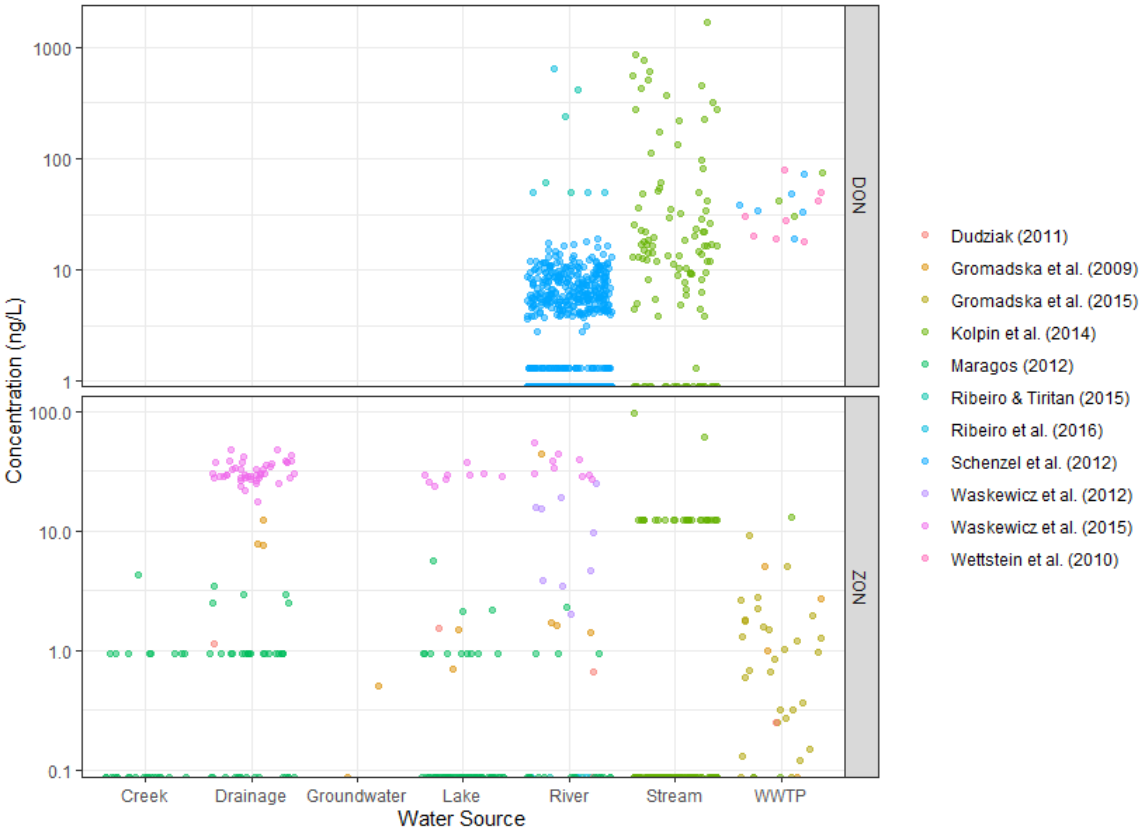


Figure 5.4 Collated freshwater MECs of DON and ZON plotted based upon time of year collected in different Northern Hemisphere countries.

Looking at the spread of data points in Figure 5.4 for each water source, firstly for DON, the levels in WWTP are fairly consistent between different studies whereas the data for both river and stream sites show a larger variation in concentrations recorded. In river studies the samples were mostly below 20 ng DON/L with those at higher concentrations being from Portuguese sample sites. WWTP samples were between 20 – 100 ng DON/L whereas in streams the quantified 88 concentrations showed 46 were < 20 ng DON/L, 29 were in the range of 20 - 100 ng DON/L and 17 were > 100 ng DON/L.

The concentration range of ZON was often similar across drainage, lakes and rivers in samples from the same studies (Figure 5.4). Such as those seen by Waskewicz *et al* (2015) where drainage, lake and river samples all showed quantifiable concentrations of 17 – 56 ng ZON/L and those in a USA study (Maragos, 2012) were comparably lower but again consistent across these three water sources often between 0.4 (LOD) and 1.5 (LOQ) and those quantified all < 6 ng ZON/L. In a separate USA study looking at streams although ZON was detected at a similar frequency the concentrations were seen to be higher, although mostly unquantified they were detected above 12.3 ng ZON/L and those quantified were the highest concentrations detected across all of the ZON data points at 96 and 61.5 ng ZON/L (Kolpin *et al*., 2014). Table 5.2 shows another study, which did not have raw data for inclusion in Figure 5.4, where reported maximum concentration was at a similar concentration of 82.6 ng ZON/L but from a river sample (Laranjeiro *et al*., 2018). For WWTP ZON was detected in 32/38 samples but at relatively low concentrations of < 13 ng ZON/L.

5.2.4 Temporal trends in concentrations

Of the total 1387 individual data points assessed 882 had a reported, or could be assigned, a date of sample collection to be used in the seasonal analysis shown in Figure 5.5. DON concentrations had the highest range in spring, remaining mostly at levels lower than 100 ng DON/L for the remainder of the year with only two samples exceed this at 227 and 274 ng DON/L during Autumn. For ZON concentrations there was no clear peak season when looking at all the data points together.

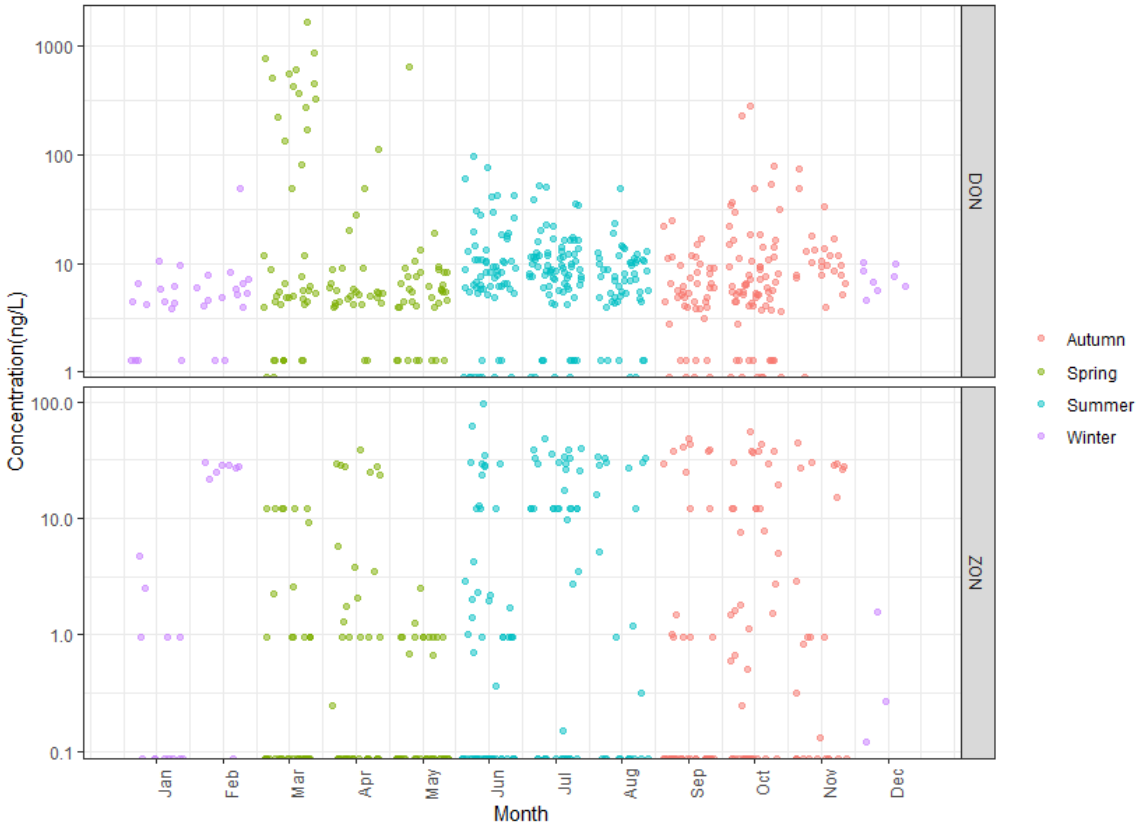


Figure 5.5 Collated freshwater MECs of DON and ZON plotted based upon time of year collected in different Northern Hemisphere countries.

Looking at the seasonal trends, shown in Figure 5.6, the DON spring peak is based upon the samples from Kolpin *et al*. (2014), Ribeiro *et al*. (2016) also found a peak in spring but only looked at one sample per season. For the remaining two studies the concentrations are more consistent throughout the seasons with the maximum

concentrations instead occurring in autumn. Figure 5.7 shows the individual study data for ZON, the two highest recorded concentrations were during the summer in Kolpin *et al*. (2014); and for the remainder of the year concentrations remained at < LOQ or not detected. However, this was the only study in the seasonal comparison to report the highest concentration in summer. Waskewicz *et al*. (2012) reported rising ZON concentrations of 15 -25 ng ZON/L in late summer which peaked in autumn, with concentrations during the remainder of the year being < 10 ng ZON/L. In a later study, Waskewicz *et al*. (2015), a larger number of samples were collected and although the highest reported value was also in autumn at 55.6 ng ZON/L, concentrations seen during the rest of the year were 17.6 – 48.2 ng ZON/L. Gromadska *et al*. (2009) saw higher ZON concentrations in autumn compared to summer, but a later study looking at only WWTP in all seasons saw higher concentrations in Spring and Summer, with Maragos *et al*. (2012) also reporting highest concentrations in these seasons.

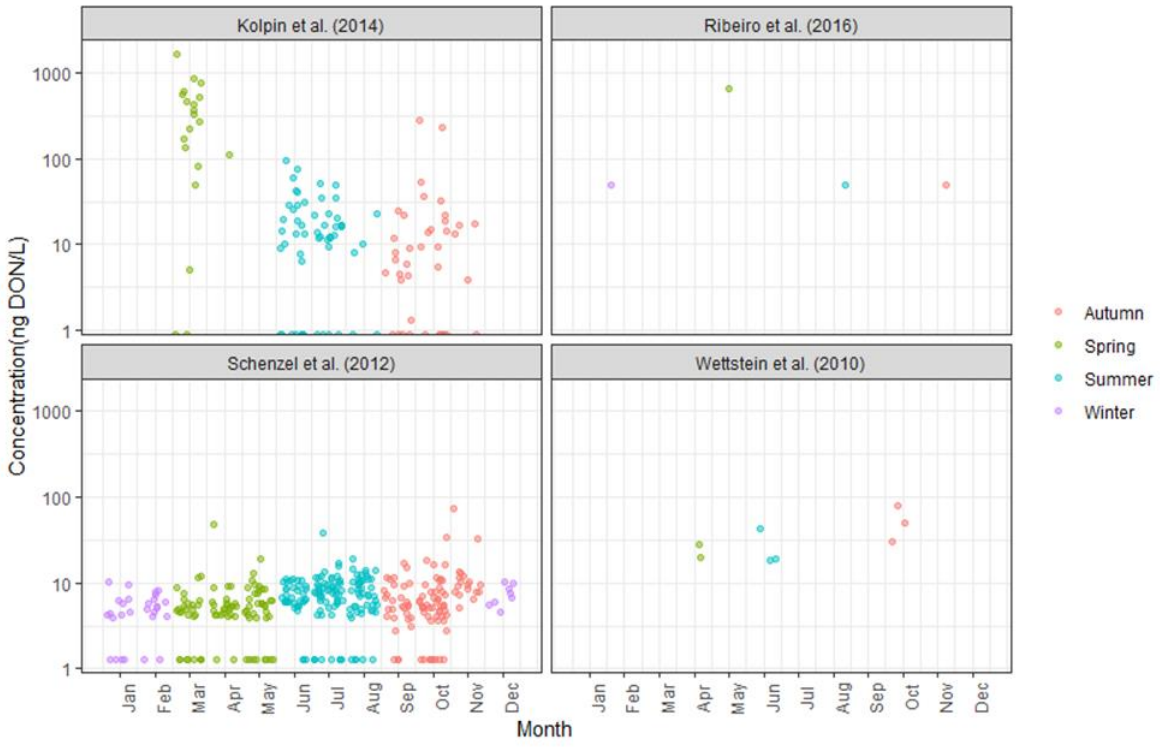


Figure 5.6 Collated freshwater MECs of DON plotted, based upon time of year collected in in different Northern Hemisphere countries, for each study reviewed.

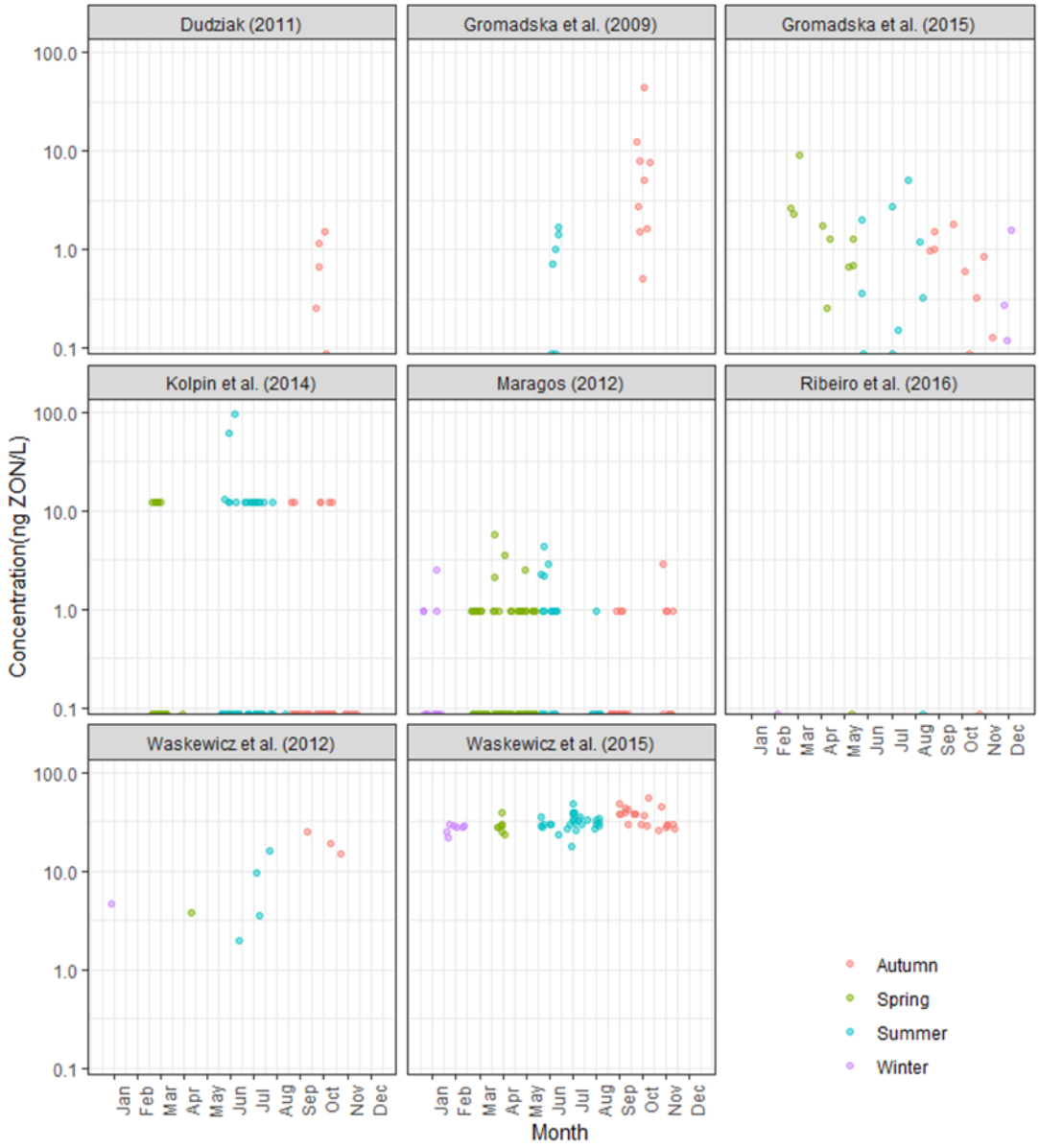


Figure 5.7 Collated freshwater MECs of ZON plotted, based upon time of year collected in in different Northern Hemisphere countries, for each study reviewed.

5.2.5 Spatial trends

It is reported that the two input sources of mycotoxins to freshwaters are agricultural run-off and WWTPs. Based upon the data reviewed, drainage ditches and WWTP effluent values can be used as a representation for each input. The WWTP samples for DON showed 100 % frequency of detection, from a total of 24 samples and for ZON it was 83 % of effluent samples: from 36 samples (Table 5.2). Although from a limited

number of samples, these high frequencies support the proposal of WWTP being a continuous long term input of mycotoxins, originating from human consumption and excretion of mycotoxins, to freshwaters. Particularly as the largest WWTP study by Gromadzka *et al*. (2015) still had a high FOD of 90 % for ZON and these samples were collected throughout all seasons (Figure 5.7).

In terms of relative impact of each source we see WWTP concentrations are relatively consistent, even between different studies (Figure 5.4). Further to this, comparison between different sample source types shows that stream and river levels of both DON and ZON both often exceed the concentrations reported in WWTP effluents. Therefore, in these situations it could be assumed the impact of agricultural run-off is more prominent, with ZON there is samples from drainage ditches, indeed the Gromadska *et al*. (2009) study shows the large receiving lake and river water bodies concentrations reflect that of the drainage ditches.

Interestingly Gromadska *et al* (2009) reported the highest concentration of ZON in a site located in a forested area. Looking at the other sites in this exposure study, the two lakes sampled cover one woodland area and one agricultural. The concentration of ZON recorded in both lakes was similar during the autumn/winter collection. There were no specific reports found for mycotoxin occurrence in woodlands or leaf litter but considering the widespread nature of fungi the occurrence of mycotoxin producing species in these in woodlands would not be surprising. Mycotoxins are known to contaminate commercial tree nuts (Wang *et al*., 2018, Hidalgo-Ruiz *et al*., 2019, Molyneux *et al*., 2007) and non-timber edible forests (Djeugap *et al*., 2019). It has been seen in a recent study in agroforest regions that crops closer to the tree line had a higher incidence of fungal contamination due to the humid microclimate from the forested

area (Beule *et al*., 2019). There are currently no in-depth studies investigating forests as a potential input source of mycotoxin run-off, but with the ideal humid conditions and abundance of organic matter it would be an interesting area to investigate considering the high concentrations measured in the woodland areas of the Polish study (Gromadska *et al*. 2009).

5.2.6 Distributions of the exposure concentrations

In order to generate probabilistic Measured Exposure Concentration (MEC) values, all the environmental concentrations were collated by plotting exposure concentration distribution curves (Figure 5.8) and used to calculate 90th percentile values. The distributions show bands of values representing large groups of samples where the mycotoxins were not detected or < LOQ. In 521/993 samples tested for DON and 200/394 samples tested for ZON the mycotoxins were not detected. Further to the overall distribution for each mycotoxin, separate exposure concentration distribution curves were also created per mycotoxin for each water source (Figure 5.9 and Figure 5.10) and for each study within these to generate further 90th percentile values for comparison (Figure 7.1 and Figure 7.2).

5.3 Aquatic Hazard Assessment for Mycotoxins

Previously Laranjeiro *et al*. (2018) considered the risk of ZON based upon results of their environmental samples, alongside literature data for fish toxicity and estimated ZON EC_{50} values for *Daphnia* and algae using ECOSAR in the absence of experimental data. Hence, now that freshwater plant and invertebrate studies have been performed, we have included a comparison of three methods for calculating PNEC's to assess the variation between them: QSAR predictions (fish, *Daphnia* and algae), acute

experimental data (fish, daphnia and algae) and statistical derivation from an SSDs (acute experimental data covering ten organisms).

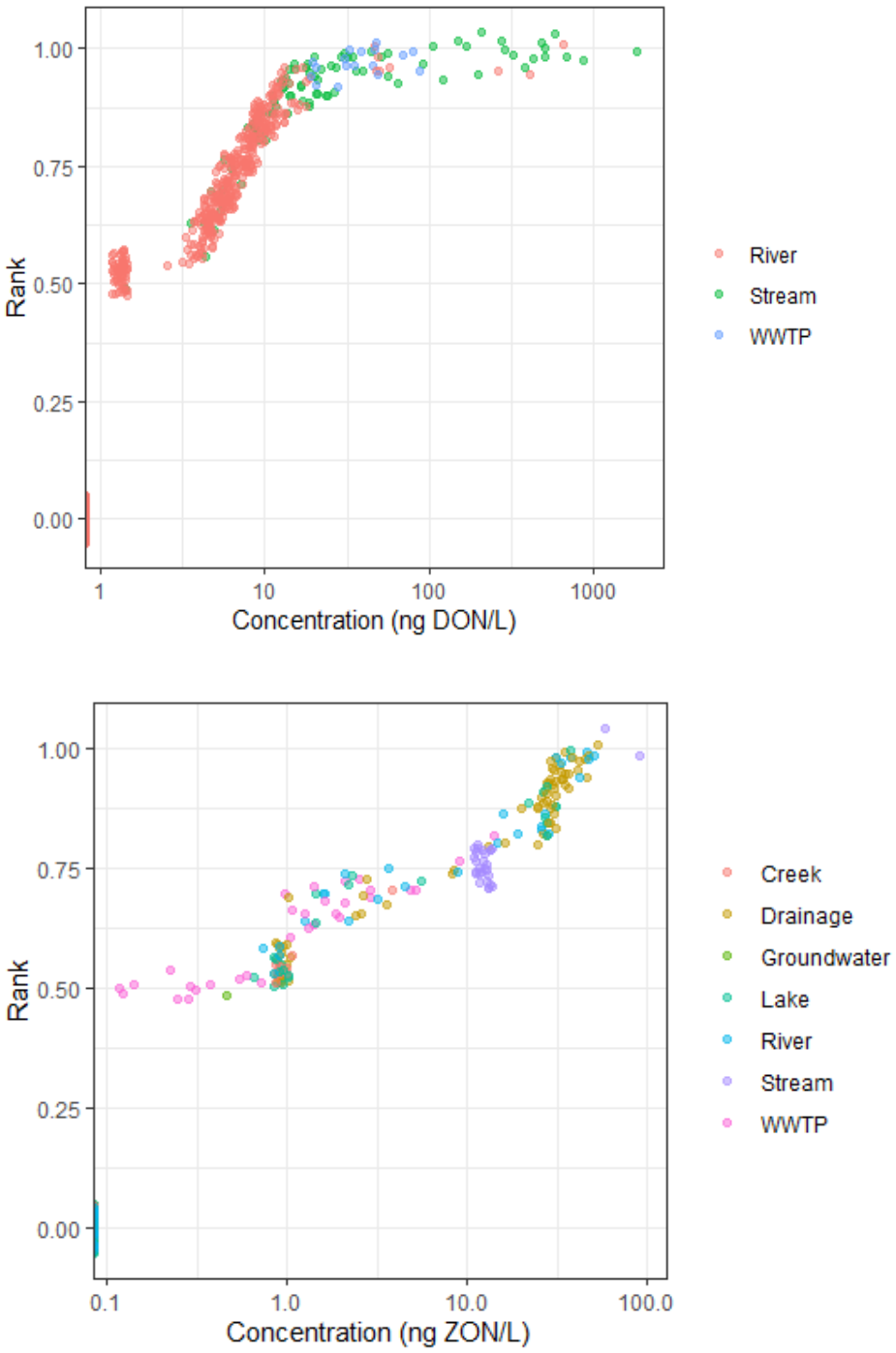


Figure 5.8 Percentile ranks of collated freshwater MECs of DON and ZON collected in different Northern Hemisphere countries.

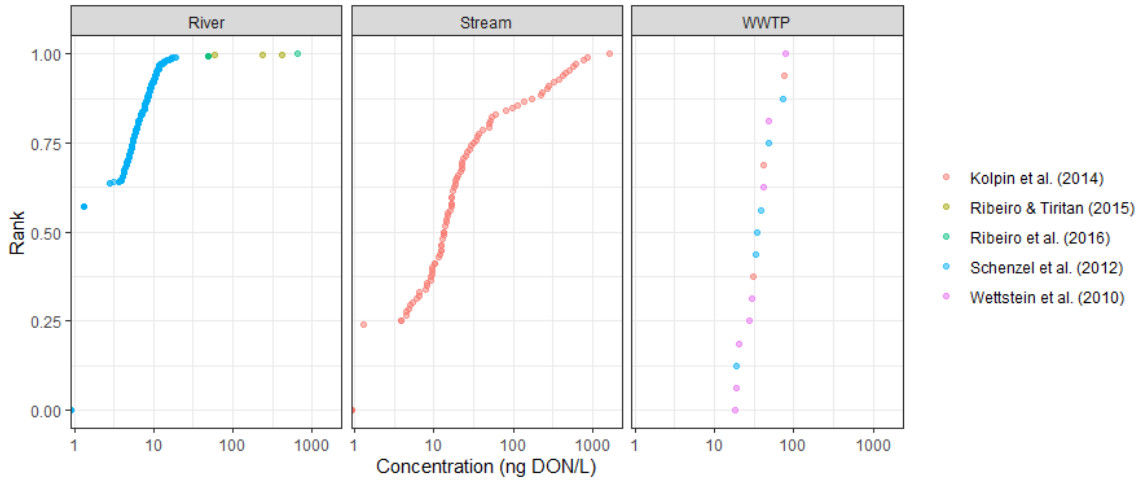


Figure 5.9 Percentile ranks, of collated freshwater MECs of DON collected in different Northern Hemisphere countries, for different water sources.

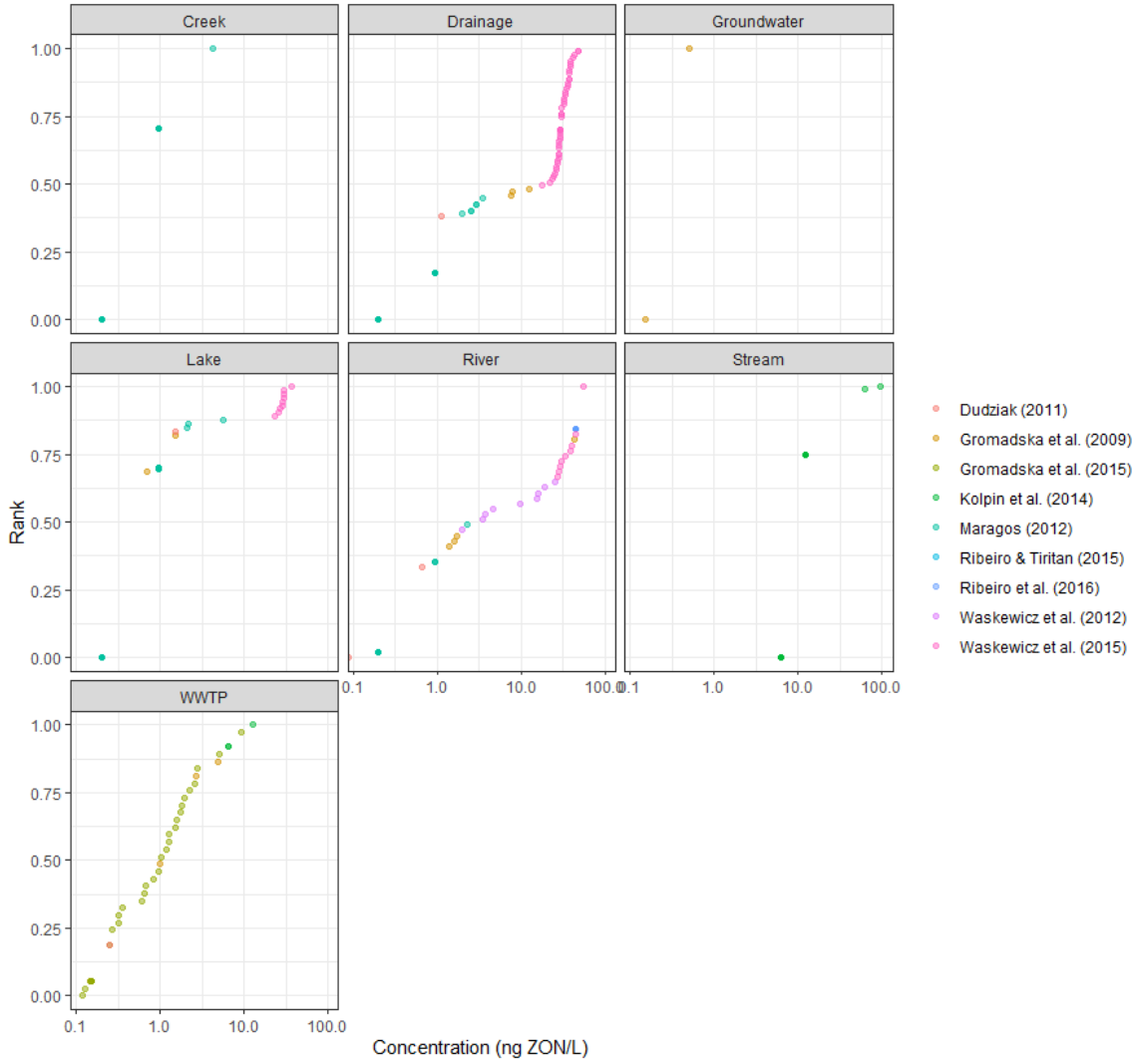


Figure 5.10 Percentile ranks of collated freshwater MECs of ZON collected in different Northern Hemisphere countries, for different water sources.

5.3.1 5.3.1 Predictive Toxicity Modelling for Mycotoxins

QSAR model predictions for acute fish (96 h), daphnids (48 h) and green algae (h) were generated in two different packages. ECOSAR v2.0 is freely available from U.S. Environmental Protection Agency's website and has been widely used for environmental assessments of agrochemicals and pharmaceuticals (Solomon *et al*., 2000); (Sanderson, 2003). To run the prediction the chemical name/CAS number is required to retrieve the deposited simplified molecular-input line-entry system (SMILES) notation from the SMILESCAS database. Log Kow is estimated using the KOWWIN program to be used in the regression analysis. ECOSAR may output results based on multiple chemical classes along with a baseline toxicity prediction (neutral organics). The OECD QSAR Toolbox (version 4.4.1) is also freely available, owned by OECD and ECHA (OECD, 2020a). It is a culmination of multiple databases including those used in the ECOSAR predictions. Again, only the chemical name/CAS number is required to retrieve the SMILES notation here. QSAR Toolbox (version 4.4.1) was used to predict the EC_{50} values for invertebrates and algae. Acute experimental toxicity data (EC_{50} values from exposures ≤ 7 days) were those from earlier chapters of this thesis along with those found reported in literature. These were the values used in Chapter 4 for SSD modelling and generation of HC_5 values.

Table 5.3 QSAR model predictions for DON and ZON toxicity

Mycotoxin	Predictor tool	Organism	Type	(mg/L)	Comments
DON	ECOSAR version 2.0 (with KOWWIN v1.69[a])	Fish	Acute LC_{50}	1070	Classification generated during prediction: Ketone Alcohols
		Daphnids	Acute LC_{50}	459	
		Green algae	Acute EC_{50}	93	
		Fish	Chv	99	Classification generated during prediction: Epoxides, Mono
		Daphnids	Chv	42	
		Green algae	Chv	384	Classification generated during prediction: Ketone Alcohols, estimated through application of acute-to-chronic ratios
	OECD QSAR Toolbox (version 4.4.1)	Fish	-	-	Could not be calculated
		Daphnids	-	-	Could not be calculated
		Green algae	-	-	Could not be calculated
ZON	ECOSAR version 2.0 (with KOWWIN 1.69[b])	Fish	Acute LC_{50}	2.0	Classification generated during prediction: Phenols, poly
		Daphnids	Acute LC_{50}	8.1	
		Green algae	Acute EC_{50}	2.0	
		Fish	Chv	0.27	Classification generated during prediction: Esters
		Daphnids	Chv	2.8	Classification generated during prediction: Phenols, poly
		Green algae	Chv	0.33	
	OECD QSAR Toolbox (version 4.4.1)	Invertebrate	Acute LC_{50}	5.65	Automated, trend analysis, 28 data points, R2 = 0.721, 95 % confidence interval 0.262-122
		Algae	Acute EC_{50}	2.25	Automated, Read-across analysis, 27 data points, average from 5 closest, 95% confidence interval 0.0107-472

[a]DON chemical properties selected by KOWWIN Mol Wt 296.32, LogKow -0.71, Water Solubility 55481.95 mg/L, Melting Point 152 °C. [b] ZON chemical properties selected by KOWWIN Mol Wt 318.37, LogKow 3.58, Water Solubility 27.22 mg/L, Melting Point 164.5 °C.

5.3.2 Comparison of predicted and measured toxicity values

The QSAR predicted toxicity values for DON and ZON are given in Table 5.3. Predicted EC_{50} concentrations were 93 – 1070 mg DON/L and 2.0 – 8.1 mg ZON/L. Using the data collected in previous chapters along with those available in literature the summary effect values for acute DON and ZON studies are listed in Table 5.4.

Looking at these measured effect concentrations, where EC_{50} values could be determined in the test concentration range, these were between 0.25 – 10 mg DON/L and 0.59 – 8.8 mg ZON/L. Considering this range for ZON in comparison to the predicted QSAR EC_{50} values, shows very little difference in these concentrations. The individual EC_{50} concentrations for green algae of 2.0 (ECOSAR) and 2.25 ZON/L (QSARToolbox) are very close to our EC_{50} of 1.2 mg ZON/L for the microalgae study with *P. subcapitata*. However, for DON the predictions of the QSAR models show the limitations of this predictive approach. Although literature data show little effect of DON to zebrafish embryos, with EC_{50} values being undefined, as suggested by the prediction of a very high EC_{50} value of 1070 mg DON/L (ECOSAR). We saw effect concentrations for D. magna in the concentrations tested and *D. magna* was the most sensitive organism in the DON toxicity studies, resulting in an EC_{50} of 0.13 mg DON/L: challenging the prediction of 459 mg DON/L (ECOSAR). This poor relation to the predictions by ECOSAR may reflect the lack of suitable chemical comparisons for DON in the QSAR databases, as QSARToolbox failed to provide any EC_{50} prediction for DON. Therefore, when considering the potential risk of other mycotoxins, relying on QSAR predictions will likely see similar inaccurate predictions with suitable analogous ecotoxicity data often lacking.

Table 5.4 Summary effect values from acute experimental freshwater toxicity studies with DON and ZON

Mycotoxin	Class	Species	Time	Effect	Endpoint	EC_{50} value (mg/L)	Reference
DON	Invertebrate	*B. calyciflorus*	24 h	Mortality	EC_{50}	> 3.2	This study
	Invertebrate	*T. thermophila*	24 h	Reproduction	EC_{50}	5.6	This study
	Invertebrate	*T. platyurus*	24 h	Immobilisation	EC_{50}	0.4	This study
	Invertebrate	*C. riparius*	48 h	Immobilisation	EC_{50}	> 3.2	This study
	Invertebrate	*D. magna*	48 h	Immobilisation	EC_{50}	0.13	This study
	Invertebrate	*H. vulgaris*	96 h	Mortality	EC_{50}	1.7	This study
	Invertebrate	*T. pyriformis*	150 h	Reproduction	LOEC	0.6	Bijl 1988
	Invertebrate	*L. stagnalis*	7 d	Mortality	EC_{50}	2.9	This study
	Plant	*C. reinhardtii*	150 h	Growth	LOEC	10	Suzuki2014
	Plant	*L. pausicostata*	72 h	Growth	LOEC	~3	Abbas2013
	Plant	*L. minor*	7 d	Growth	LOEC	0.25	Vanhoutte2017
	Fish	*D. rerio*	96 h	Mortality	EC_{50}	> 296	Khezri 2018
ZON	Invertebrate	*B. calyciflorus*	24 h	Mortality	EC_{50}	> 10	This study
	Invertebrate	*T. thermophila*	24 h	Reproduction	EC_{50}	> 10	This study
	Invertebrate	*T. platyurus*	24 h	Immobilisation	EC_{50}	> 10	This study
	Invertebrate	*C. riparius*	48 h	Immobilisation	EC_{50}	8.1	This study
	Invertebrate	*D. magna*	48 h	Immobilisation	EC_{50}	5.8	This study
	Invertebrate	*H. vulgaris*	96 h	Mortality	EC_{50}	1.7	This study
	Invertebrate	*L. stagnalis*	7 d	Mortality	EC_{50}	0.59	This study
	Plant	*P. subcapitata*	72 h	Growth	EC_{50}	1.2	This study
	Plant	*L. minor*	7 d	Growth	EC_{50}	8.8	This study
	Fish	*D. rerio*	5 d	Mortality	EC_{50}	0.89	Bakos2013

No chronic data for toxicity were found for DON, and with the inaccuracy seen from acute QSAR prediction we did not regard the chronic values as suitable for further consideration. With the QSAR predictions expected to be more reliable for ZON, based upon the acute result comparison, the predicted chronic value for lethality to fish was the most sensitive at 0.27 mg ZON/L. As with the acute predictions, the algae EC_{50} for growth inhibition is close to this at 0.33 mg ZON/L, with the EC_{50} for immobilisation of *Daphnia* higher at 2.3 mg ZON/L. The only chronic experimental data on toxicity available for ZON is for zebrafish studies. Experimental data for zebrafish showed no effect at any test concentrations up to 3.2 µg ZON/L in terms of survival during a 140 d life cycle study (Schwartz *et al*., 2013). However lower effect concentrations are reported for reproduction endpoints, 21 d LOEC of 0.1 µg ZON/and 140 d LOEC of 0.32 µg ZON/L (Schwartz *et al*., 2010; Schwartz *et al*., 2013).

5.3.3 PNEC calculations

To perform the chemical safety assessment PNEC values were calculated taking guidance from the REACH (ECHA, 2008) and EFSA (EFSA, 2013) guidance for the freshwater compartment (summarised in Table 1.1). For acute data sets an assessment factor of 1000 is recommended and applied to the EC_{50} values of three trophic concentrations, usually fish, invertebrates (commonly *Daphnia*) and algae. Hence, for both the QSAR prediction based PNECs and the model species derived PNEC's this value of 1000 was used. For the SSD model (generated with data generated here and those from literature) HC_5 values, the confidence intervals reflected the need for further data as lower bounds were not generated, to acknowledge this uncertainty an assessment factor of 5 was used. For SSD (HC_5) derived PNEC values under REACH (ECHA, 2008) an AF of 5-1 is recommended, for EFSA (EFSA, 2013) it is 3 – 6 for acute SSDs based on

invertebrate data only but as a range of organisms were used including algae, plants and fish along with invertebrates the REACH maximum was used. The assessment factor values used in each instance and resulting PNEC values from these calculations are provided in Table 5.5. The hypothesis that statistically derived probabilistic PNECs would provide an appropriately conservative value for risk calculation did reflect the generation of intermediate values through this method. Although, with the assessment factors applied the PNEC values were generally within a factor of 10, 0.89 – 8.6 µg/L for ZON, with the exception of the DON QSAR prediction based PNEC, 93 µg/L in comparison to 0.13 µg/L and 1.04 µg/L for experimental deterministic and SSD based respectively. Hence, concerns about under or overly conservative PNECs produced using varying methods was not necessarily reflected, more so in the EC_{50}s as discussed previously. SSDs do have their own limitations in their application for risk analysis, particularly in regard to representation of sensitive species. Balance must be in place to not bias or over represent sensitive taxa if a taxon relevant target site is known but conversely the SSD must not be too generalized to allow the target species to be below the HC_5 and be at risk of major loss, leading to ecosystem effects (Spurgeon et al., 2020). With ZON this later case could have been an issue if *D. rerio* were drastically more at risk due to the oestrogenicity but other species had similar EC_{50}s therefore the resulting HC_5 was lower than the EC_{50} in this case.

5.4 Risk characterisation

Two types of MECs were used in the calculations firstly 90th percentile exposure concentrations from exposure concentration distributions and secondly HRECs (Table 5.6). MEC/PNEC ratios, calculated with 90th percentile values for each toxin, for each water source per toxin and for each study within this, are listed in Table 5.6 along with

those MEC/PNEC ratios based upon HRECs for each study reviewed. Resulting ratio values greater than 1 indicate need for further consideration (ECHA, 2013).

It should be acknowledged that chronic toxicity data are preferred in environmental risk assessment where chronic exposures occur in freshwater ecosystems. Therefore, where long-term exposure to low concentrations is expected and would be more relevant than acute data for comparison with the 90th percentile values. But this knowledge is currently limited to ZON. Considering the chronic QSAR PNEC of 27 µg/L the risk would still be low for ZON. Further to this, the life cycle study for zebrafish by Schwartz *et al*. (2013) used a lowest test concentration of 100 ng ZON/L, this lies above all of the 90th percentiles and just above the highest recorded environmental concentration. At this concentration, no effects were seen throughout the test. Also, the risk ratios were calculated from the acute data collected experimentally show ZON ratio values are ≤ 0.01 in all cases, equating to no significant risk in aquatic settings based upon our acute toxicity values.

Table 5.5 Calculation of PNEC values using predicted QSAR toxicity values, model freshwater species experimental data and SSD HC_5 values for DON and ZON

Mycotoxin	Value for use in deriving PNEC (µg/L)				Assessment factor				Resulting PNEC µg/L			
	Acute QSAR	Acute fish, Daphnia and algae studies	All acute studies available (SSD)	Chv	QSAR	Fish, Daphnia and algae studies	All acute studies available (SSD)	Chv	QSAR	Fish, Daphnia and algae studies	All acute studies available (SSD, HC_5)	Chv
DON	93 000	130	5.2	-	1000	1000	5	-	93	0.13	1.04	-
ZON	2000	890	43	270	1000	1000	5	10	2	0.89	8.6	27

Table 5.6 90th percentile values calculated for recent exposure studies with DON and ZON and PEC/PNEC ratios calculated from 90th percentile values and HRECs.

Mycotoxin	Water source	n	90th (ng/L)	PEC/PNEC	References	n	90th (ng/L)	PEC/PNEC	HREC	PEC/PNEC
DON	River	863	9	0.01	Ribeiro & Tiritan (2015)	4	360	0.35	412	0.40
					Ribeiro *et al*. (2016)	4	464	0.45	642	0.62
					Schenzel *et al*. (2012)	855	8.8	0.01	19	0.02
	Stream	113	264	0.25	Kolpin *et al*. (2014)	113	264	0.25	1662	1.60
	WWTP effluent	17	74	0.07	Kolpin *et al*. (2014)	3	69	0.07	75	0.07
					Schenzel *et al*. (2012)	6	61	0.06	73	0.07
					Wettstein *et al*. (2010)	8	58	0.06	79	0.08
	All	993	12.8	0.01	-	-	-	-	-	-
ZON	Creek	28	0.95	< 0.001	Maragos (2012)	28	0.95	0.00	4.3	0.001
	Drainage	87	37	0.004	Dudziak (2011)	1	1.1	0.00	1.14	< 0.001
					Gromadska *et al*. (2009)	3	11	0.00	12.3	0.001
					Maragos (2012)	38	2.5	0.00	3.5	< 0.001
					Waskewicz *et al*. (2015)	45	39	0.00	48.2	0.006
	Groundwater	2	0.45	< 0.001	Gromadska *et al*. (2009)	2	0.45	0.00	0.5	< 0.001
	Lake	74	25	0.003	Dudziak (2011)	1	1.5	0.00	1.52	< 0.001
					Gromadska *et al*. (2009)	2	1.4	0.00	1.5	< 0.001
					Maragos (2012)	62	0.95	0.00	5.7	0.001
					Waskewicz *et al*. (2015)	9	32	0.00	37.4	0.004

Table 5.6 Continued

Mycotoxin	Water source	n	90th (ng/L)	PEC/PNEC	References	n	90th (ng/L)	PEC/PNEC	HREC	PEC/PNEC
ZON	River	52	33	0.004	Dudziak (2011)	2	0.6	0.00	0.66	< 0.001
					Gromadska *et al*. (2009)	4	31	0.00	43.7	0.005
					Maragos (2012)	20	0.95	0.00	2.3	< 0.001
					Ribeiro & Tiritan (2015)	4	0	0.00	0	< 0.001
					Ribeiro *et al*. (2016)	4	0	0.00	0	< 0.001
					Waskewicz *et al*. (2012)	9	20	0.00	25	0.003
					Waskewicz *et al*. (2015)	9	46	0.01	55.6	0.006
	Stream	113	12	0.001	Kolpin *et al*. (2014)	113	12	0.00	96	0.011
	WWTP	38	3.4	< 0.001	Dudziak (2011)	1	0.25	0.00	0.25	< 0.001
					Gromadska *et al*. (2009)	4	4.3	0.00	2.7	< 0.001
					Gromadska *et al*. (2015)	30	2.6	0.00	9.2	0.001
					Kolpin *et al*. (2014)	3	10	0.00	12.9	0.002
	All	394	29.4	0.003	-	-	-	-	-	-

Although, previous research has highlighted ZON would be of more concern due to sublethal effects (Schwartz *et al*., 2011). When considering the PNEC's based upon these; < 10 and 32 ng ZON/L for the 21d and 140 d study respectively, the shorter study is interestingly lower than the longer one and due to it being undefined leads to a large uncertainty when using this to draw any conclusions on risk. It further highlights the preliminary nature of understanding of chronic effects of mycotoxins, with only these two values found. These do lie within 90th percentile exposure values for ZON, we would expect drainage ditches to be receiving high ZON inputs but even the receiving rivers in Polish studies show a 90th percentile on par with the highest sub lethal PNEC, along with the Polish lake study. However, these values are based upon a relatively small number of samples so again would require further consideration to understand whether these values reflect true long-term concentrations.

With the sparse available data for ZON we can only draw similar assumptions to those given previously in the chronic ZON toxicity studies, in that the hazard is most likely at a sub lethal concentration and due to its oestrogenic nature should be considered alongside other oestrogenic compounds as a potentially adding to the overall oestrogenic load of waters. With risk due to exposure of ZON alone unlikely to cause direct decline in the survival of freshwater populations.

With no chronic data available for DON, the preliminary MEC/PNEC ratios calculated here are based only upon acute data. Considering the overall 90th percentile, the MEC/PNEC ratio of 0.01 would suggest little concern for DON as well. However, MEC/PNEC ratios began to increase across the different water sources and studies. For the estuarine studies with regular occurrence of high concentrations combined with small sample sizes, ratios were 0.35 and 0.45; Ribeiro & Tiritan (2015) and Ribeiro *et al*.

(2016) studies, respectively. For the Kolpin *et al*. (2014) stream study with a large sample size the ratio value of 0.25 calculated based upon a 90th percentile of 264 ng DON/L still suggested low risk for acute effects for the majority of time, however this was the study which recorded the HREC for DON.

Calculating the MEC/PNEC ratios based upon the HREC for this study, which was much higher than the 90th percentile at 1662 ng DON/L, gave a value of 1.60 which was the only ratio calculated to be above the threshold of concern of 1. With this being the only value above 1 and is based upon the only sample tested which exceeds the PNEC the concern is highly limited to this specific location, the USA, in this specific water source, a stream exposed to agricultural run-off. It suggests that moving forward it would be sensible to look at locations such as this to investigate further whether similar exceptionally high concentrations are recorded for DON and the likely duration and frequency of these.

Many of the papers found here provide only a limited insight into environmental exposure concentrations from a small number of samples that were collected, often via grab sampling of < 20 samples. This appears to be in part due to the focus of the research being refining chemical methodology rather than a thorough environmental analyses. Often only one sample per season is collected in these types of regimes, leaving the understanding of duration of exposures to daily/weekly fluxes lacking. Clearly this lack of understanding for duration of exposures increases the uncertainty in risk characterisation that can be performed.

Even when considering on a smaller scale based upon different water sources, we see the effect of the wide variation in sampling numbers between studies on the 90th percentiles. Most samples in this analysis stem from one single paper, Schenzel *et*

al. (2012). Looking at the 90th percentile value for all stream DON samples the effect of this paper bias the value to only 9 ng DON/L where in Portuguese samples the DON concentrations was consistently higher than this and found up to 642.4 ng DON/L. With the concentrations of mycotoxins therefore highly dependent on region, risk was later categorised on a study by study basis, despite the small sample sizes. Further to this, maximum concentrations recorded are often considerably higher than that of other time points and even of 90th percentiles. Based upon current data the dual approach performed seems the most sensible way to take this uncertainty and wide variation in exposure concentrations into account.

5.5 Conclusions

Assessing risk is greatly improved as the number of data available for both effects and exposure increase. This review of literature shows the interest in mycotoxins as aquatic contaminants is recent, with the published data limited to a ten-year period. The distribution of this work is also spatially limited, considering the spread of mycotoxin producing fungi is worldwide and a well know issue for crops, there is a need to develop wider understanding of mycotoxin occurrence in freshwaters. Without broader understanding of environmental exposure, we cannot adequately assess risk and identify areas of concern.

Looking at the occurrence and concentration ranges of DON and ZON, globally DON was reported more often and reaching higher concentrations. However, when considering the 90th percentile values globally the values of 12.8 DON/L and 29.4 ng ZON/L did not reflect this. Therefore, relying on assessments based upon these would not necessarily be suitable as DON had a maximum concentration of 1662 ng /L and was frequently detected at > 100 ng DON/L. A risk ratio based upon the HREC of DON,

characterised an agricultural stream sample from Kolpin *et al*. (2014) to be a concern for acute DON exposures as HREC/PNEC > 1.

As both mycotoxins have been seen to relatively frequently be detected in environmental samples developing a broader chronic data set for use in assessment would also be beneficial as there is currently insufficient chronic data for a DON assessment and 90th percentile values for studies were often > 200 ng DON/L. *Daphnia* sp. were seen to be the most sensitive test organism in short term studies so would be the most useful organism to study for a chronic assessment. For ZON, QSAR predicted chronic values and experimental chronic zebrafish survival data suggested effects on populations is a not a risk with environmental concentrations usually < 50 ng ZON/L based on 90th percentile values. But consideration should be made on the additive potential of chronic exposure due to the lower effect concentrations seen for sub lethal effects and the potential additive effect of ZON with other oestrogenic contaminants (Thorpe et al., 2001; Thorpe et al., 2003).

Chapter 6. Research synthesis and wider considerations

6.1 Research synthesis

This thesis describes an extensive scientific assessment of environmental exposure data and laboratory ecotoxicity information for mycotoxins in order to develop novel environmental risk assessments for freshwater ecosystems. The main aims of the research presented in this thesis were: 1) to investigate the toxic effects of DON and ZON to freshwater plants and invertebrates 2) review the global data for DON and ZON in freshwaters 3) perform an environmental risk assessment for DON and ZON in freshwater ecosystems.

Previously, the main limitation in considering the risk of mycotoxins in the aquatic compartment, as highlighted in many of the exposure studies, was the lack of ecotoxicology knowledge for mycotoxins. Prior toxicity studies have largely been fish dietary exposures with feeds contaminated with mycotoxins (Sanden *et al*., 2012; Woźny *et al*., 2015; Manning and Abbas, 2012). The work done through this project has addressed the ecotoxicology knowledge gap for DON and ZON. For ethical reasons, the research conducted at the University of Plymouth did not include vertebrates since there already exist several published mycotoxins studies on fish. Toxicity studies demonstrated inhibitory effects of DON and ZON in microalgae, macrophytes and multiple invertebrate species (Chapter 3 and 4). Interestingly each mycotoxin induced varying toxicity in terms of the most sensitive species observed, *D. magna* for DON and *L. stagnalis* for ZON (Chapter 4). This highlights the need for investigation into the mode of action of mycotoxins to understand how this is achieved and understand the extend of variability across this wide group of natural toxins. Photosynthetic measures in the

macrophyte *L. minor* suggested alteration in photosystem II performance as a result of ZON (Chapter 3), indicating even in this less sensitive organism for ZON sub lethal effects are induced and providing a starting point to developing an adverse outcome pathway in plants.

Although an original objective of this project was to contribute novel UK exposure data for mycotoxins in freshwater sites, particularly those subject to agricultural inputs, in order to test the central hypothesis that harmful levels of mycotoxins are present in UK freshwaters. Unfortunately, due to the UK Covid pandemic, it was not possible to carry out an in-depth sampling scheme to do this. A limited number of samples were analysed but did not show any detections of DON or ZON (Chapter 5), hence the exposure concentrations for mycotoxins in UK freshwaters remains unclear and not be able to interpret the hazard results in relation to UK risk is a major limitation of this project, remaining as a knowledge gap to address in future. Instead, the exposure assessment focused on deriving exposure concentrations from the review of measured environmental concentrations reported to date globally and the hypothesis modified to consideration of freshwaters in general rather than the UK. This permitted the calculation of 90th percentile exposure concentrations of 12.8 ng DON/L and 29.4 ng ZON/L. Despite ZON having a higher 90th percentile concentration, DON was seen to reach higher concentrations in freshwaters in terms of maximum concentrations reported.

The characterization of risk (Chapter 5) subsequently suggested DON to be of most concern out of the two mycotoxins, due to the higher concentrations reported and the lower hazardous concentration found from the ecotoxicology data (Chapter 4). Though, this concern is limited to low dilution receiving waters in agricultural areas and

only partially supporting the hypothesis that harmful concentrations are present within freshwaters, although this is only a preliminary assessment with limitations and potential improvements which will be summarised in this chapter. Furthermore, it is important to note that there are many other mycotoxins associated with UK (Edwards, 2009a; Edwards *et al*., 2012) and international agriculture (Jang *et al*., 2019; Ali, 2018; Frisvad *et al*., 2005) that remain to be studied in the context of environmental risk assessment.

6.1.1 Review of exposure data

Despite measurement of mycotoxins in surface waters gaining interest in the past decade, mycotoxin contamination of rivers is not currently featured in any regular monitoring scheme or covered by regulatory limits. Exposure data before this project was limited in its spatial coverage, focused on the US (Kolpin *et al*., 2010; Kolpin *et al*., 2014), Poland (Dudziak, 2011; Gromadzka *et al*., 2009; Waskiewicz *et al*., 2015), Portugal (Ribeiro *et al*., 2016, Ribeiro and Tiritan, 2015) and Switzerland (Wettstein and Bucheli, 2010; Schenzel *et al*., 2012a; Schenzel *et al*., 2012b), despite mycotoxins being a widespread issue (Edwards, 2009b; Alkadri *et al*., 2014; Gruber-Dorninger *et al*., 2019). This project has provided a global analysis of data available in order to derived 90th percentile values for DON and ZON. Although this offers the best estimate with current data, with a historic lack of long term and wide scale monitoring, a true representation of the presence and trends in mycotoxin concentrations still needs to be worked upon.

Some of the papers reviewed in the exposure analysis of Chapter 5 focused more on developing and discussing analytical methods (Ribeiro and Tiritan, 2015; Dudziak, 2011) with small numbers of samples, rather than in depth environmental surveys. As such, within the few exposure studies which have been performed, including the larger

scale ones (Kolpin *et al*., 2010; Kolpin *et al*., 2014), sampling regimes were typically reliant on grab samples. This led to studies often reported on seasonal trends within small scale datasets, whereas the review in this work looking at all available data showed no clear trend in seasonal occurrence of peak values. Although grab sampling is arguably the more convenient method, it is not necessarily the most representative, run-off events and acute peaks in concentrations could easily be missed. This may be the reasoning behind contradicting seasonal peaks between exposure studies in the review and why no overall trend was seen across data points in the analysis here. Based upon this it would be recommended that future work on mycotoxin exposure concentrations features more frequent sampling or long-term passive sampling strategies. This would allow a better understanding of potential seasonal variations and temporal patterns in concentrations and advise the most relevant type of exposure profiles for mycotoxins to be considered in risk assessments. During the analysis of current global data available for DON and ZON (Chapter 5), DON had a more varied presence in the environmental exposure studies. Frequently, comparatively higher concentrations of DON occurred in data sets, reflected in the HREC being quite often much higher than the 90th percentiles calculated for each of the DON studies. Therefore, considering DON under pulsed exposure conditions, as seen for contaminants such as insecticides (Naddy *et al*., 2000; EFSA, 2013; Wieczorek *et al*., 2018), may be more relevant to consider in risk assessments. But it still remains to be investigated the daily / weekly patterns of exposure for both DON and ZON in freshwater following and preceding comparably high exposure events.

Despite the comparably higher reported concentrations for DON, when calculating risk ratios there was only one instance of a ratio above the threshold of

concern, and this was based upon the HREC in Kolpin *et al*. (2014) study, which is the highest reported to date in literature. All other calculated ratios would indicate little associated risk, especially in the case of ZON. Based upon the fact that within a grab sampling scheme such a high concentration was recorded of DON (Kolpin *et al*., 2014), and the risk ratios generated here, the location of future sampling schemes should prioritise similar agriculturally impacted streams to understand the likelihood of such high concentrations in comparable locations, with larger water bodies such as rivers and lakes considered to be of less concern based upon the risk ratios presented here (Chapter 5).

6.1.2 Toxic effects of DON and ZON to freshwater plants and invertebrates

The findings of Chapter 3 and 4 show that these two mycotoxins, DON and ZON, had differing toxicities across the test organisms used. Initial plant studies with ZON were detailed in Chapter 3, these revealed microalgae were more sensitive than macrophytes. In the macrophyte study with *L. minor* growth was significantly reduced based on frond number and frond area at only the highest concentration, showing a higher tolerance than reported for other mycotoxins with *Lemna* sp. (Abbas *et al*., 2002; Vesonder, 1992). With significant growth inhibition seen in both *P. subcapitata* and *L. minor*, further investigation into sublethal effects was conducted via chlorophyll fluorescence parameters. These biomarkers of impact for photosystem II showed no effect in algae but responses were observed in *L. minor* (Chapter 3). There was an imbalance in light absorption and utilization of energy, seen through indicators of energy flow, with signs of inhibition in the electron transport chain before reaching the acceptor side of PSI. Various possibilities for target sites were considered based upon the results, such as uncoupling of the oxygen evolving complex in chloroplasts, leading to inhibition of the

re-oxidation of QA-, or alternatively binding to QB, the plastoquinone domain, in the D1 protein of chloroplasts. But without conclusive support in all the photosynthetic measures and the absence of overt oxidative stress, further work is required to understand the mechanism of ZON phytotoxicity and establish a full AOP. Looking at the available data, both a microalga (Suzuki and Iwahashi, 2014) and macrophytes (Vanhoutte *et al*., 2017) had already been studied for DON hence these organisms were not tested again here. Interestingly, for DON the data suggested the sensitivity opposed that seen in ZON, with instead the macrophyte being more sensitive than the algal species.

In Chapter 4, both DON and ZON were studied, in a series of acute toxicity experiments with invertebrates, and this was driven partly by a lack of data available for mycotoxins in the scientific literature. These new experiments demonstrated the difference in species sensitivity for each of the mycotoxins extended into the invertebrates as well as in plants and zebrafish. For DON it highlighted crustaceans as the most sensitive organisms, whereas for ZON it was the mollusc embryos. The latter also showed a similar toxicity to that of zebrafish embryos in acute studies (Bakos *et al*., 2013). The varying sensitivities of different factions of freshwater ecosystems to each mycotoxin suggests, rather than affecting all trophic levels equally, the ecosystem is more likely to have specific organisms which would suffer lethality and potentially induce wider spread effects on ecosystem functioning.

Another reason for focusing this work on invertebrates was to minimize the use of vertebrate testing, following the strategy of promoting the 3Rs (replacement, refinement and reduction of vertebrate testing) in ecotoxicology (Hutchinson *et al*., 2016; Burden *et al*., 2015; Scholz *et al*., 2013). There are still many avenues of research

to pursue for freshwater mycotoxin toxicity, but this should continue the practice of minimizing vertebrate use where possible. One such knowledge gap that remains is the AOPs of mycotoxins (Ankley *et al*., 2010). Understanding the ways in which toxicity is exerted would allow further understanding of why and how certain species are more sensitive to different mycotoxins. For example, the reasoning behind the susceptibility of crustaceans to DON seen here and understanding whether the sensitivity of zebrafish and *L. stagnalis* to ZON could be related to the membrane bound nature of these embryo studies. Furthermore, developing AOPs would prove beneficial for modelling purposes, allowing comparison with other known compounds of similar effect pathways (Ellison *et al*., 2016; Wittwehr *et al*., 2017). The current lack of AOP knowledge means using read across methods with other chemicals can be limited, and as seen in this project QSAR predictions for DON were not always possible (Chapter 5). But the structural similarity of ZON to other compounds, and knowledge of their effects, allowed predictions which were accurate when compared to the measured values from the laboratory studies performed (Chapter 5). Zebrafish have been seen to be less sensitive to DON in acute studies (Khezri *et al*., 2018), but no chronic toxicity studies have yet been carried out. By using invertebrate methods to interpret shared mode of actions with vertebrates alongside *in vitro* measures to determine the AOPs in fish, *in silico* predictions of toxic chronic concentrations could be determined without extended *in vivo* studies (Zhang *et al*., 2016b; Wittwehr *et al*., 2017).

The data used in environmental risk assessment should always incorporate the most relevant available toxicity data in relation to the likely exposure scenarios. From the measured environmental exposure data analysis performed in Chapter 5 just under half of samples had detectable levels of DON and ZON. Whilst these concentrations

often were unquantifiable it highlighted that ZON is likely a low-concentration long-term contaminant, with the concentration range being more consistent than seen for DON. This was not seen to cause concern in the risk analysis for ZON. Although, there was only limited fish chronic data to consider for ZON to rely upon for chronic evaluations here (Schwartz *et al*., 2010; Schwartz *et al*., 2013). This highlighted that ZON is unlikely to cause concern based upon data to date and future research for ZON should focus upon the oestrogenic nature of this contaminant and potential sublethal effects in chronic assessment. With snail embryos showing high sensitivity in acute studies and the proposal for use of *Lymnaea* (Matthiessen, 2008; Ducrot *et al*., 2010), for endocrine disrupting compounds in particular, assessments with adult *L. stagnalis* would be a relevant starting point for investigations based upon the results here. Also, although ZON may not be cause for concern alone, there are widespread oestrogenic compounds already in surface waters (Fawell *et al*., 2001). Therefore, based upon the findings of this risk assessment, that ZON is not currently posing a risk in freshwaters, additive toxicity studies would be the most relevant area to progress knowledge for ZON to be able to also consider its oestrogenic properties in the hazard assessment.

The acute toxicity data generated for DON was vital considering DON is, of the two mycotoxins studied, the mycotoxin seen to have short periods of much higher peak concentrations. There remains a gap for DON effects data for chronic toxicity, with the longest study to date with other freshwater organisms is a 7 d exposure with *Lemna sp*. (Vanhoutte *et al*., 2017), which meant no long-term assessment was possible. Although the overall 90th percentile concentration for DON was lower than that for ZON, during the Portuguese study by (Ribeiro *et al*., 2016) DON was seen to peak in spring but input

was present at all seasons with concentrations > 100 ng/L throughout the year. Hence, the characterisation of chronic risk is also important for locations such as this one.

A mixed species mesocosm studies would elucidate the potential ecosystem effects further of each mycotoxin. Additionally, with both mycotoxins being produced by the same species co production of both mycotoxins is possible so a mesocosm co-exposure would also be beneficial. This would be particularly informative considering the two mycotoxins have been shown to have differing species sensitivity, therefore co-exposure could have significant effects on wider range of species as well as potential additive effects.

6.2 Further considerations in Mycotoxin Environmental Risk Assessment

Predicted areas of concern in the UK

The interest in mycotoxins was driven around the UK poultry industry in the 1960s (Blount, 1961; Forgacs *et al*., 1962) and since this point regulations have been introduced and developed in the 2000s to protect human and animal health (European Commission, 2006b). Yet the UK has not established any investigation into the potential movement of mycotoxins into freshwaters, as other countries have begun to in the past decade (Schenzel *et al*., 2012a; Kolpin *et al*., 2014; Wettstein and Bucheli, 2010). In England there was a total of 3.1 million hectares of land dedicated to cereal crops in 2018 (DEFRA, 2019b) and the annual winter wheat survey monitors the effect of Fusarium Head Blight (FHB) on wheat crops in England (CHAP, 2019). The concentration of DON and ZON in UK wheat has also been investigated. Mean concentrations between 2006 – 2013 rose to 261 µg DON/kg and 29 µg ZON/kg in comparison to the mean value for the 2001 – 2005 period of 230 µg DON/kg and 17 µg ZON/kg respectively (Edwards, 2009; Edwards & Jennings 2018). Hence, despite the absence of freshwater

measurements of mycotoxins it was assumed that based upon the recent global datasets summarised in Chapter 5 along with the knowledge of detectable levels of DON and ZON in wheat, although concentrations will be driven by persistence of DON/ZON in water/soil, there will be some movement of mycotoxins into UK surface waters.

Although no model has been derived to connect FHB presence or intensity with mycotoxin production in England, with the absence of any previous freshwater measures of mycotoxins the data on FHB presence and agricultural land usage are the only data available to consider potential spatial distributions and at risk regions for mycotoxin contamination of freshwaters. Looking at FHB occurrence during the Winter Wheat Survey of England and Wales between 1998 and 2007, those regions highlighted as having intense occurrence of disease were areas in the South of England in Dorset and East Hampshire, along with the East Midlands (CHAP, 2021). But defining the regions of highest occurrence of FHB annually seems to be changeable (CHAP, 2019; CHAP, 2018). In terms of percentage contribution to wheat production in the UK the highest is in The East (50 %), East Midlands (49 %), South East (47 %) and Yorkshire and Humber (30 %) (DEFRA, 2019a).

Overall, the East and South of England are those which have both high wheat crop production and have been highlighted as those with highest incidence and severity of FHB infections (CHAP, 2021; CHAP, 2019). Hence, as Edwards (2009) also suggested these are the areas which we can currently assume are most at risk of mycotoxin occurrence. Therefore, future investigation into areas of highest mycotoxin load should consider agriculturally impacted streams in these areas. With no available MECs for surface waters in the UK the best estimate we have for the potential concentrations are those calculated in our analysis of global levels as 90th percentiles values. Figure 6.1

shows these concentrations for DON and ZON in various types of surface waters alongside the mean concentrations of DON and ZON reported most recently in UK wheat for the period 2006 - 2013 (Edwards & Jennings 2018) (Table 7.1).

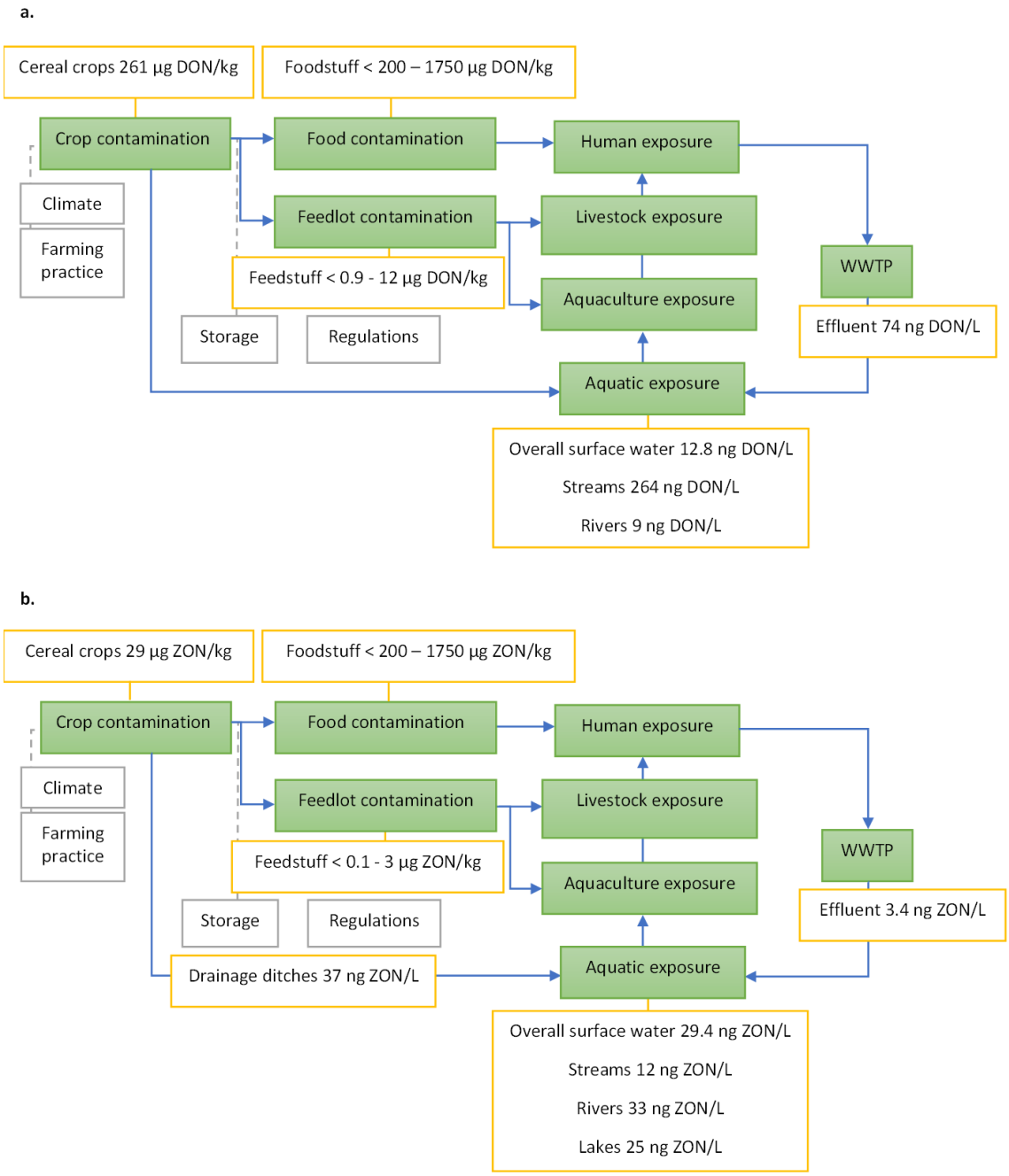


Figure 6.1 Conceptual linking of mycotoxin exposure routes and potential influences on mycotoxin concentrations (shown with grey dashed lines). Yellow boxes provide MECs (90th percentiles in various surface waters, mean concentrations reported in UK wheat for the period 2006 - 2013 (Edwards & Jennings 2018) and limitations which are set for both human food and animal feed in the UK by the EU (European Commission, 2006b)). A. Deoxynivalenol, b. Zearalenone.

6.2.1 Climate and Weather Trends

The UK is predicted to be subject to generally warmer temperatures and a heightened variation in seasonal rainfall; wetter winters and drier summers (West, 2011). Madgwick *et al*. (2011) considered the potential impacts of predicted climate change on UK FHB incidence. The susceptibility of wheat to FHB is during a relatively short window during anthesis of the crop, with the incidence dependant on temperature from up to 6 weeks prior to anthesis, and rainfall during the week before. Warm and wet conditions will favour FHB. Predicted drier summers would suggest unfavourable conditions for the causative agents, however the effect of climate on the growth of the plant must also be considered. Madgwick (2011) calculated that anthesis is likely to advance with time to around 2 weeks earlier as the climate changes over the next 30 years. This meant the critical time before anthesis would occur when rainfall will be low, combining this with the warmer temperatures, it is suggested by 2050 that conditions in the UK will facilitate fusarium epidemics. Not only will this increase the risk of economic loss due to reduced yield of infected crops but also increase the risk of mycotoxin production and contamination of the wheat.

An earlier anthesis has been predicted in modelling for other countries (Van der Fels-Klerx *et al*., 2013, Zhang *et al*., 2014), and shifts in the areas where individual fungal species can tolerate (Parikka *et al*., 2012) could lead to new areas at risk of mycotoxin contamination (Marroquin-Cardona *et al*., 2014), but FHB and contamination of crops with mycotoxins will not necessarily increase in all cases (Miraglia *et al*., 2009). Deoxynivalenol predictions for the Netherlands varied regionally (Van der Fels-Klerx *et al*., 2013), with an overall decrease at country level but individual areas having

significant increases, similarly Zhang *et al*. (2014) simulated wheat and climate data up to 2050 in central China and predicted an increased FHB incidence in many regions.

6.2.2 Farming practices

Reduced tillage as a farming practice offers reduced environmental impact through soil erosion on farmland as well as increasing agricultural output (Townsend *et al*., 2016). Reduced tillage refers to reducing soil inversion by limiting the ploughing of fields. But by leaving the residues from previous crop undisturbed this remains to act as an inoculum source for *Fusarium* in the subsequent crop, potentially increasing the likelihood of mycotoxin presence, multiple studies have shown higher disease rates and subsequently higher mycotoxin levels in crops grown under reduced tillage rather than ploughed fields (Schoneberg *et al*., 2016). Therefore, reduced tillage practices may lead to a higher concentration of mycotoxins in surface run-off to freshwaters and a higher exposure for freshwater organisms. The practice of reduced tillage is common, the UK data for 2010 arable harvest showed of 249 farmers surveyed almost half (46 %) used some form of reduced tillage practice, with cereal farms across the East Midlands and South East of England being those more likely to be using reduced tillage (Townsend *et al*., 2016). Although the direct impact of this practice on subsequent mycotoxin production likely involves consideration of further factors. Kaukoranta *et al*. (2019) found that impacts of tillage vary between different mycotoxins and are dependent on a combination of factors including region and crop intensity rather than tillage alone.

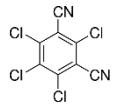
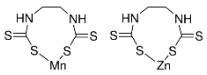
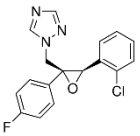
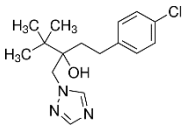
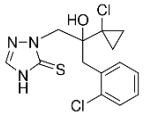
Fungicides can be used to combat fungal infection of crops, but due to the widespread use of fungicides, there has been detection of resistance developing in fugal populations to fungicides (van den Bosch *et al*., 2011; Price *et al*., 2015), and laboratory studies have shown that resistant strains can in some cases produce significantly higher

amounts of mycotoxins (Becher *et al*., 2010). Therefore, fungicides are not a certain solution for reducing the risk of mycotoxin production and mycotoxin production can still occur or even be increased with fungicide treatments due to factors such as type of fungicide used, timing of application and application rate.

In the UK wheat farming encompasses 42 % of the area of all arable crops and consequently, based on weight of substances applied, uses 52 % of the total pesticides. In terms of types of pesticides, fungicides are applied to the greatest area of arable crops covering 38 % of the total pesticide-treated area (Garthwaite *et al*., 2019). Table 6.1 lists the most used fungicides in the UK for arable crops based upon the area of application and tonnage used for 2018. The widest and most heavily used fungicide is Chlorothalonil, but three of the five most used arable fungicides are azole fungicides. These azole fungicides inhibit sterol biosynthesis, all eukaryotes rely on sterols for the maintenance of cell membrane structure, azoles lead to pathogen death by disrupting membrane structure and function by preventing active transport (Price *et al*., 2015).

Advances in the EU Plant Protection Product regulation mean that scientific studies on chemicals that demonstrate the potential for endocrine disruption in humans or wildlife (Taxvig *et al*., 2007; Taxvig *et al*., 2008; Draskau *et al*., 2019; Skolness *et al*., 2011; Ankley *et al*., 2005) are prompting bans on such fungicides. Already, two of the five fungicides listed as most used in 2018 UK arable applications are no longer approved: chlorothalonil and epoxiconazole (European Commission, 2019; European Commission, 2021). With such an interest in fungicide AOPs and implications for environmental risk, there should be an extension of this to the equivalent understanding of risks around natural toxins for which the fungicides are used to reduce, and the comparative risk of natural vs synthetic contamination.

Table 6.1 Fungicide use in UK arable crops for 2018 (Garthwaite et al., 2019)

Fungicide group	Compound	CAS number	Molecular structure	logP	Estimated application (2018)	
					Area (ha)	Amount (tonnes)
Chloronitriles	Chlorothalonil	1897-45-6		2.9	4 494 175	2 293
Dithiocarbamates	Mancozeb	8018-01-7		1.3	798 250	974
DeMethylation Inhibitors - fungicides	Epoxiconazole	133855-98-8		3.2	2 776 557	182
	Tebuconazole	107534-96-3		3.7	2 989 348	338
	Prothioconazole	178928-70-6		2.7	4 235 815	415

As stated in mycotoxin exposure studies previously by Bucheli *et al*. (2008) and Schenzel *et al*. (2012), the concentrations of mycotoxins seen in agricultural surface run-off is similar to that recorded for fungicides, yet less consideration has been placed on assessing the risk of mycotoxins. With the ecotoxicological effects of DON and ZON assessed in this project the comparisons between mycotoxins and fungicides can now be extended to ecotoxicological values as well. Figure 6.2 shows a comparison between the data from Chapters 2 - 5 for DON and ZON and those found in literature for the commonly used fungicides. Measured surface water concentrations were found for three of the five fungicides (chlorothalonil, epoxiconazole and tebuconazole) (Battaglin *et al*., 2010; Reilly *et al*., 2012; Wightwick *et al*., 2012; Cruzeiro *et al*., 2017; Casado *et al*., 2018; Casado *et al*., 2019; Curchod *et al*., 2020), HRECs are plotted for these. Freshwater toxicity data were found for all five fungicides (Fernández-Alba *et al*., 2002; Ochoa-Acuna *et al*., 2009; INERIS, 2011a; INERIS, 2011b; Andreu-Sanchez *et al*., 2012;

INERIS, 2014; Coors *et al*., 2018; Araujo *et al*., 2019; Zhai *et al*., 2019; Kaziem *et al*., 2020), EC_{50} values (for algae, invertebrates and fish) along with effect values used in generating freshwater PNECs are also included where available (INERIS, 2011a; INERIS, 2011b; INERIS, 2014). Further details for these values are provided in the appendices (Table 7.2. and 7.3).

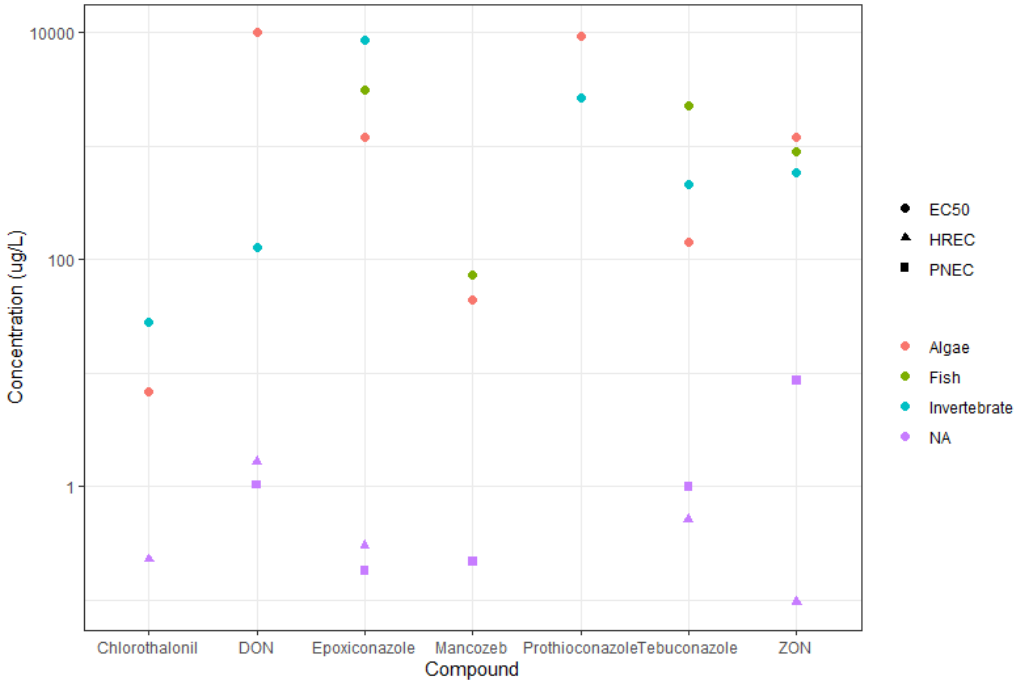


Figure 6.2 Comparison of the EC_{50} values, HRECs and PNECs for common fungicides and the mycotoxins DON and ZON

The EC_{50} values for the two mycotoxins studied here fall in the same concentration range as those for the fungicides. HRECs of the mycotoxins fall at either end of the fungicides, ZON lower than that of fungicides and DON with the highest HREC. Although, based upon the 90th percentile MECs calculated for the mycotoxins and the few available mean values for tebuconazole and chlorothalonil, 12.3 ng DON/L and 29.4 ng ZON/L compared to mean MECs of 30-78 ng/L for the fungicides, shows the mycotoxins would likely be at levels slightly lower than that of the fungicides. With the PNEC for DON equaling that of tebuconazole, and that for ZON higher than the

fungicides selected for comparison here. Table 6.2 shows the resulting risk ratios calculated from the HRECs and PNECs for the fungicides along with those calculated in Chapter 5 for the HREC of DON and ZON. These data show that DON could pose a similar and even higher risk to that of fungicides such as chlorothalonil and epoxiconazole during acute exposure in a worst-case scenario assessment (based upon HRECs from literature). From these it would be recommended, as others have, that mycotoxins should increasingly be considered in the same way that fungicides are in agricultural run-off, based upon the similarity in effect levels, MECs and resulting risk ratios. Further to this, a comparison of risks posed by mycotoxins in contrast to fungicides via human food had a similar conclusion, with no immediate concern but DON ranking highest in terms of potential risk, in comparison to tebuconazole and ZON (Muri *et al*., 2009).

Table 6.2 Comparative risk ratios calculated for mycotoxins, DON and ZON, and widely used fungicides (Garthwaite et al., 2019).

Chemical	HREC (ng/L)	PNEC (ng/L)	HREC/PNEC
Chlorothalonil	228	180	1.27
Epoxiconazole	300	219	1.37
Tebuconazole	513	1000	0.51
Deoxynivalenol	1662	1040	1.6
Zearalenone	96	8600	0.01

6.2.3 Mixture toxicity

One issue when considering the risk fungicides which should also be considered for mycotoxins is that the parent compound is only part of the issue, the metabolites of contaminants are also important to consider and often can be more toxic that the parent compound, as was seen for chlorothalonil (Stuart *et al*., 2011). This has been particularly

highlighted for oestrogenic ZON, further to the potential co-occurrence of ZON with other oestrogenic compounds in natural environments, it is important to consider the oestrogenicity of metabolites as well as the parent compounds. Metabolism in mammals after ZON ingestion can produce α-zearalenol and β-zearalenol (although these can also be reduced further in some species to α-zearalanol and β-zearalanol), the orientation of the hydroxyl group at the aliphatic ring enhances the oestrogenicity of a-zearalenol (Zinedine et al., 2007; Frizzell et al., 2015). In the environmental exposure study by Kolpin *et al*. (2014) ZON was detected in 26 % of samples but one or more of the three oestrogenic compounds (ZON and its metabolites a α-zearalenol and β-zearalenol) were detected in 43% of samples. The concentrations at which the metabolites a-zearalenol and b-zearalenol have been measured to reach is higher than those seen for ZON. Table 7.4 shows maximum concentrations of the metabolites in WWTP effluent of the Kolpin (2014) study being 1701 ng/L of α-zearalenol and 1828 ng/L of β-zearalenol in comparison to only one detection of ZON above the LOQ in these same samples.

Here, we have focussed on only two mycotoxins independently but in natural environments there will likely be exposure to mixtures of various mycotoxins as on cereal crops multiple mycotoxins have been reported to occur simultaneously (Zain, 2011). Thus, it would be expected that multiple mycotoxins may be present in surface waters at any one time and potential synergistic toxicity should be considered. Based upon the higher acute hazard posed by DON in the results here, it would be recommended that acute mixture toxicity studies firstly consider DON. Although, looking at exposure data to determine the key mycotoxins to focus on alongside DON, based upon those which occur most frequently with DON, it in fact highlights ZON along

nivalenol which is structurally similar to DON and is therefore also grouped within the trichotecene myctoxins (Kolpin *et al*., 2010; Schenzel *et al*., 2012b; Kolpin *et al*., 2014). *In vivo* studies of DON and NIV have demonstrated synergistic toxicity (Alassane-Kpembi *et al*., 2013). Conversely, a mixture *in vivo* toxicity study with DON and ZON had a sub-additive effect in terms of decreasing the amount of cell death, the interactions occurring were not understood nor the mechanisms of action, but the sub-additivity observed was suggested to be due to the oestrogenic properties of ZON potentially interfering with DON to raise cell proliferation (Bensassi *et al*., 2014).

6.3 Overall Conclusions

Exploring the occurrence and significance of mycotoxins has been progressing over the past 20 years and this will need to continue due to the host of factors discussed which may lead to an increase in mycotoxin exposure for humans, livestock, and aquatic ecosystems. Although focusing on the worst expected locations would not give a true overview of exposure concentrations of mycotoxins it is of importance to gather data on predicted at risk areas. This would provide a good starting point for considering the current extent of run-off in agricultural areas and offer a starting figure to monitor future changes in mycotoxin levels. With the relatively low predicted risk of the mycotoxins DON and ZON to freshwaters calculated here (based upon current MECs), understanding and monitoring of alterations in mycotoxin loads entering waters is likely going to be highly dependent upon close monitoring of mycotoxin concentrations in crops and employing modelling or use of test field run-off data results rather than implementation of standardised widespread surface water monitoring. With the progression away from previously widely used fungicides, due to recent understanding of endocrine disrupting potential of these, discussion of relative risk of mycotoxins will also be required in terms

of economic and health risks in regard to crops. Hopefully, further investigation into the associated freshwater risk will in time follow and the information gathered here, showing the ecotoxicological effects of natural toxins DON and ZON at levels comparable to that of synthetic fungicides, will aid in this process.

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Appendices

Additional Figures and Tables

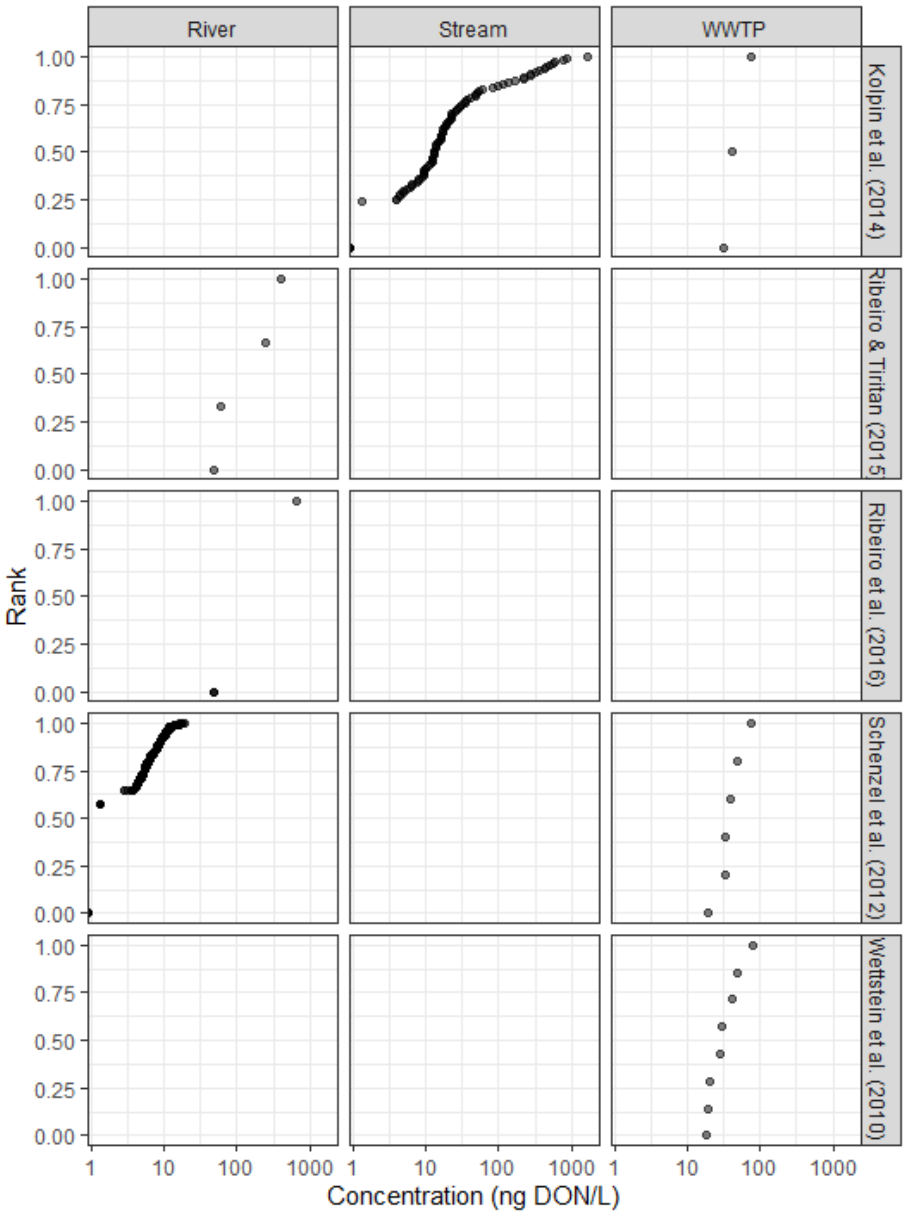


Figure 7.1 Percentile ranks of collated freshwater MECs of DON, based upon water source of samples per each study reviewed.

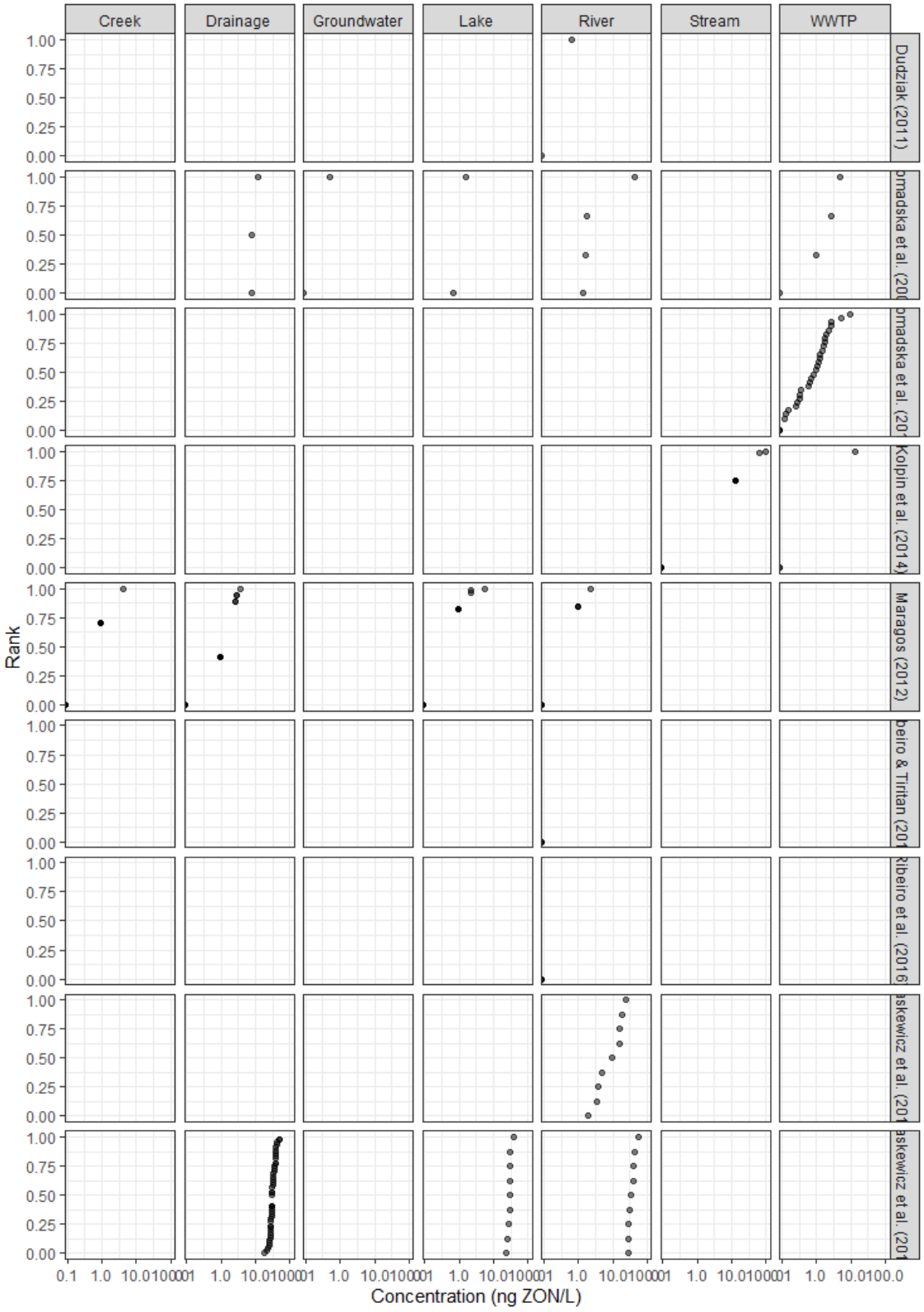


Figure 7.2 Percentile ranks of collated freshwater MECs of ZON, based upon water source of samples per each study reviewed.

Table 7.1 Concentrations of mycotoxins detected in wheat in the UK

Mycotoxin	Location	Date	N =	ND	FOD (%)	LOD (µg/kg)	FOQ (%)	LOQ (µg/kg)	Mean	SEM	Median	Max	Reference
DON	UK	2001 - 2005	1624				86	10	230		42		Edwards 2009
	UK	2006	182				77	10	37		17		Edwards & Jennings 2018
	UK	2007	152				98	10	305		140		
	UK	2008	175				98	10	584		306		
	UK	2009	152				95	10	202		77		
	UK	2010	177				41	10	14		< 10		
	UK	2011	150				27	10	18		< 10		
	UK	2012	158				100	10	615		333		
	UK	2013	130				88	10	309		95		
	UK	2006 - 2013	1276				79	10	261				
	Europe	2008 - 2017	5949	3866	65						369	49307	Gruber-Dorninger
ZON	UK	2001-2005	1624					5	17		< 5		Edwards 2009
	UK	2006	182					2	2		< 2		Edwards & Jennings 2018
	UK	2007	152					2	14		< 2		
	UK	2008	175					2	120		47		
	UK	2009	152					2	22		7		
	UK	2010	177					2	4		< 2		
	UK	2011	150					2	<2		< 2		
	UK	2012	158					2	56		16		
	UK	2013	130					2	10		< 2		
	UK	2006 - 2013	1276					2	29				
	Europe	2008 - 2017	4925	1624	33						34	23278	Gruber-Dorninger

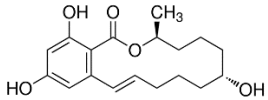
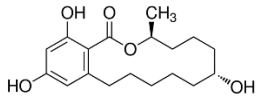
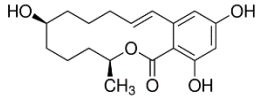
Table 7.2 MECs of key UK fungicides used on arable crops

Compound	Location	Site type	Sample type	N =	FOD (%)	LOD (ng/L)	Min (ng/L)	Mean (ng/L)	Median (ng/L)	Max (ng/L)	Reference
Chlorothalonil	US	Streams	Grab	103	2	4	-	31	-	33	(Battaglin *et al*., 2010)
	US	Surface water	Grab	60	35	4.1	-	-	-	228	(Reilly *et al*., 2012)
	Australia	River	Grab	96	0	-	-	-	-	-	(Wightwick *et al*., 2012)
Epoxiconazole	EU	Streams/rivers/canals	Grab	29	55	2.5	-	-	-	299.6	(Casado *et al*., 2019)
	UK	Streams	Grab	4	75	2.5	-	-	-	3.0	(Casado *et al*., 2018)
	South Africa	River	Passive	21	33	0.2	-	-	-	4.3	(Curchod *et al*., 2020)
Tebuconazole	US	Streams	Grab	103	6	10	-	53	-	115	(Battaglin *et al*., 2010)
	Australia	River	Grab	96	4	-	-	30	-	40	(Wightwick *et al*., 2012)
	Spain/Portugal	Estuary	Grab	-	100	0.44	13.2	78	62.5	151.8	(Cruzeiro *et al*., 2017)
	EU	Streams/rivers/canals	Grab	29	93	5.0	-	-	-	513	(Casado *et al*., 2019)
	UK	Streams	Grab	4	75	5.0	-	-	-	14.1	(Casado *et al*., 2018)
	South Africa	River	Passive	21	67	0.3	-	-	-	7.1	(Curchod *et al*., 2020)

Table 7.3 Ecotoxicity data for key UK fungicides used on arable crops

Fungicide	Class	Species	Life stage	Time	Effect	Endpoint	Concentration (mg/L)	PNEC (µg/L)	Reference
Chlorothalonil	Invertebrate	*D. magna*	Neonate	48 h	Immobility	EC_{50}	0.028	-	(Fernández-Alba *et al.*, 2002)
	Plant	*P. subcapitata*	-	72 h	Growth	EC_{50}	0.0068	-	
Epoxiconazole	Invertebrate	*D. magna*	Neonate	48 h	Immobility	EC_{50}	5.3	-	(Kaziem *et al.*, 2020)
	Plant	*C. vulgaris*	-	96 h	Growth	EC_{50}	17.8	-	
	Algae	*A. bibraianus*	-		Immobility	EC_{50}	1.19	-	(INERIS, 2011a)
	Invertebrate	*Daphnia magna*	-		Growth	EC_{50}	8.69	-	
	Fish	*O. mykiss*	-		Mortality	EC_{50}	3.14	-	
	Freshwater	-	-		-	PNEC	-	0.18	
Mancozeb	Invertebrate	*D. magna*	Neonate	48 h	Immobility	EC_{50}	0.19	-	(Araujo *et al.*, 2019)
	Invertebrate	*D. similis*	Neonate	48 h	Immobility	EC_{50}	0.27	-	
	Algae	-	-		Immobility	EC_{50}	0.044	-	(INERIS, 2014)
	Invertebrate	-	-		Growth	EC_{50}	0.073	-	
	Fish	-	-		Mortality	EC_{50}	0.074	-	
	Freshwater	-	-		-	PNEC	-	0.219	
Prothioconazole	Invertebrate	*D. magna*	Neonate	48 h	Immobility	EC_{50}	2.7	-	(Zhai *et al.*, 2019)
	Plant	*C. pyrenoidosa*	-	72 h	Growth	EC_{50}	9.3	-	
	Plant	*L. minor*	-	7 d	Growth	EC_{50}	1.9	-	
Tebuconazole	Invertebrate	*D. magna*	Neonate	48 h	Immobility	EC_{50}	0.75	-	(Ochoa-Acuna *et al.*, 2009)
	Plant	*P. subcapitata*	-	72 h	Growth	EC_{50}	3.2	-	
	Plant	*P. subcapitata*	-	72 h	Growth	EC_{50}	2560 (M)	-	(Coors *et al.*, 2018)
	Fish	*D. rerio*	Adult	96 h	Mortality	EC_{50}	26 800 (M)	-	(Andreu-Sanchez *et al.*, 2012)
	Algae	-	-		Immobility	EC_{50}	0.144	-	(INERIS, 2011b)
	Invertebrate	-	-		Growth	EC_{50}	0.46	-	
	Fish	-	-		Mortality	EC_{50}	2.3	-	
	Freshwater	-	-		-	PNEC	-	1.0	

Table 7.4 MECs of ZON metabolites

Compound	Location	Site type	Sample type	No. samples	Detection			Quantifiable levels						Reference
					No.	FOD (%)	LOD (ng/l)	No.	LOQ (ng/l)	Min (ng/l)	Mean (ng/l)	Median (ng/l)	Max (ng/l)	
α-zearalenol	US	streams	grab	105	9	9	18.6	6	-	-	-	-	202.2	Kolpin *et al*. (2014)
	US	WWTP	flow-weighted composites	3	1	33	21.5	1	-	-	-	-	1701	Kolpin *et al*. (2014)
	Portugal	Estuary	Grab	20	-*	-	64.7	-	96.3	-	-	-	-	Ribeiro *et al*. (2016)
	US	Runoff feedlot	Grab	50	-	10	-	-	-	-	-	< 5	1720	Bartelt-Hunt (2012)
α- zearalanol 	US	Runoff feedlot	Grab	50	-	72	-	-	-	-	-	348	3820	Bartelt-Hunt (2012)
β-zearalenol	US	streams	Grab	105	20	19	4.9	15	-	-	-	-	288.7	Kolpin *et al*. (2014)
	US	WWTP	flow-weighted composites	3	1	33	31.1	1	-	-	-	-	1828	Kolpin *et al*. (2014)
	US	Runoff feedlot	Grab	50	-	16	-	-	-	-	-	< 5	1440	Bartelt-Hunt (2012)