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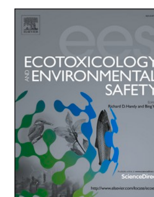

Alterations in *Daphnia magna* exposed to enniatin B and beauvericin provide additional value as environmental indicators

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ABSTRACT

Mycotoxins beauvericin (BEA) and enniatin B (ENN B) affect negatively several systems and demand more studies as the mechanisms are still unclear. The simultaneous presence of contaminants in the environment manifests consequences of exposure for both animals and flora. *Daphnia magna* is considered an ideal invertebrate to detect effects of toxic compounds and environmental alterations. In this study, the potential toxicity and the basic mechanism of BEA and ENN B individually and combined were studied in *D. magna.* Acute and delayed toxicity were evaluated, and transcript levels of genes involved in xenobiotic metabolism *(mox*, *gst*, *abcb1*, and *abcc5)*, reproduction, and oxidative stress (*vtg-SOD*) were analyzed by qPCR. Though no acute toxicity was found, results revealed a spinning around and circular profile of swimming, a strong decrease of survival after 72 h for BEA and ENN B at 16 μM and 6.25 μM, respectively, while for BEA + ENN B [8 + 1.6] μM after 96 h. The amount of mycotoxin remaining in the media revealed that the higher the concentration assayed the higher the amount remaining in the media. Differential regulation of genes suggests that xenobiotic metabolism is affected denoting different effects on transcription for tested mycotoxins. The results provide new insights into the underlying risk assessment of BEA and ENN B not only through food for consumers but also for the environment.

1. Introduction

Population is being exposed to toxic compounds mainly through food and the environment. Climate change is playing a crucial role in the increase and the distribution of toxic compounds and their impact on animal and human health can be dramatic. In particular, co-exposure to more than one compound can lead to adverse, non-linear, consequences especially if it is considered that it frequently happens in animals and humans. The simultaneous presence of mycotoxins in feed or food commodities has been widely reported (Juan et al., 2020; Oueslati et al., 2020) while their effects, although described, have been less studied (Agahi et al., 2020a, 2020b; Juan-García et al., 2015, 2019). Mycotoxins are mainly present in agricultural products preserved under appropriate conditions for fungi to grow (Oueslati et al., 2020). The risk associated with mycotoxins compiles human and animal health and the European Commission has set regulations for the presence in food for some of them (EFSA, 2019; European Commission Regulation (EC), 2006); although neither beauvericin (BEA) nor the family of enniatins (ENNs: ENN B, ENN A, ENN A1, ENN B1) nor the possible combinations have been included. Nevertheless, there is an expressed interest by the food authorities as several scientific reports have been published around those not legislated and indicating the lack of data to support toxic effects as in EFSA (EFSA, 2018).

Food security and environmental pollution caused by mycotoxins have attracted much attention due to high hazards and a wide range of contaminants. Mycotoxins have been also reported to produce harmful effects in the ecological environment on soil, food, and water but also in diverse molecular mechanisms such as neurotoxicity, disorders of sphingolipid metabolism, induction of oxidative stress, mitochondrial dysfunction, and cell apoptosis (Chen et al., 2021; Liu et al., 2019; Agahi et al., 2020a, 2020b, 2021a, 2021b; Macías-Montes et al., 2020).

The study of alterations in biological systems traditionally uses mammalian models such as mice and rabbits; however, there are some problems like complex operations, long experiment periods, and a lot of resources, and money. The use of invertebrates in methods for pre-screening toxic effects implies an important consideration with the results obtained as there are great differences in the biological organization when compared to mammals. However, several studies have

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revealed the capacity that *Daphnia magna* has in predicting cytotoxicity comparable with mammalian *spp.* (Guillhermino et al., 2000) and it also contributes in reporting the consequences in the environment of contaminants. Thus, the use of *D. magna* results provides an additional advantage, and it has been also described as one of the most sensitive invertebrates to detect neuroactive compounds (Rawlings et al., 2019; Teixidó et al., 2020) and represents an important trophic level in an aquatic food chain. It is important to remark that there is still little data of this invertebrate with mycotoxins and the comparison with mammals seems a necessary duty to overcome barriers of the use of *D. magna* model as an alternative (Guillhermino et al., 2000).

Both BEA and ENN B belong to the family of emergent mycotoxins and are often produced by *Fusarium spp*. and have been simultaneously detected in several food and feed (Oueslati et al., 2020; Juan et al., 2020). BEA causes mitochondrial alterations, cell cycle disruption, and cell death, whereas ENNs are known to have ionophoric characteristics, allowing them to be incorporated into cell membranes and used to create cation selective holes (Ivanova et al., 2006; Tonshin et al., 2010). A reduction of toxicological effects of chemical compounds can happen when elimination is facilitated, as when metabolized through phase I and phase II reactions or with the intervention of pumping mechanisms directed to reduce its absorption and/or entrance (Campos et al., 2014; Wang et al., 2016). Nevertheless, the effects in short term as oxidative stress or, in long term as in reproduction are unavoidable. The study of alterations of gene expression involved in these processes results a hit of interest including genes involved in phase I (monooxygnase (*mox*)), phase II (glutathione-s-transferase (*gst*)), and phase 0/III (*p*-glycoprotein (ABCB/md), *abcb1, abcb4* and *abcc5*) of xenobiotic metabolism and pump mechanisms and, as well as the components that intervene in *D. magna* structure, the production of yolk proteins in *D. magna* (vitellogenin-superoxide dismutase (*vtg-SOD*)).

Besides the indication in some studies of the relation of mycotoxins with neurological alterations as for AFB1, PAT, BEA, ENN A, ENN B or ZEA carried out on zebrafish (*Danio rerio*) as the biological model (Baldissera et al., 2018; Ciornea et al., 2019; Muthulakshmi et al., 2018; Juan-García et al., 2020, 2021), in this study, the *D. magna* invertebrate model was chosen to explore the potential toxicity and basic mechanism of BEA and ENN B. The water flea *D. magna* was selected as a test species, which is also known to be sensitive to assess ecotoxicology, as well as of general toxicology of several contaminants included mycotoxins (Okamoto et al., 2015; Yim et al., 2006). When *D. magna* was exposed to BEA and ENN B at various concentrations (based on studies for aquatic *spp*., Juan-García et al., 2021), acute and delayed toxicity were evaluated. To get a better insight about toxicological mechanisms, the transcript abundance of genes involved in xenobiotic metabolism (*mox*, *gst*, *abcb1* and *abcc5*), oxidative stress and reproduction (*vtg-SOD)* was examined by qPCR. The results provide new insights into the underlying risk assessment of BEA and ENN B not only through food for consumers but for the environment.

2. Material and methods

2.1. Study design for mycotoxin exposure

Exposures of *Daphnia magna* to mycotoxins were tested at the following concentrations that are the same as those tested previously for aquatic *spp*. (zebrafish embryos) (Juan-García et al., 2021): i) for an individual treatment: BEA 0.5, 1, 2, 4, 8, 16 and 32 μM and ENN B 0.2, 0.4, 0.8, 1.6, 3.2, 6.25 and 12.5 μM; ii) for binary mixtures [BEA + ENN B], two scenarios of high and low concentrations at [8 + 1.6] μM and [2 + 0.8] μM were assessed, respectively. Concentration mixtures studied are related to effects observed in individual treatment without overpassing the IC_{50} reported in previous study of zebrafish; while the IC_{50} values for *D. magna* were calculated by representing the survival values in dose-response curves for the range of concentrations tested. Solvent control used was methanol (MeOH) and maintained at ≤ 0.5% for all assays as demonstrated to be no-toxic for *D. magna* (Dom et al., 2012). The chemicals used were purchased from Sigma Aldrich: beauvericin (BEA, MW: 783.95 g/mol) and enniatin B (ENN B, MW: 639.82 g/mol).

2.2. *Daphnia magna culture*

D. magna from ephippia DaphToxKit F (Microbiotest, Belgium), were used for the acute and delayed toxicity assays. The content of vials with *D. magna* ephippia was poured into a microsieve and rinsed with tap water to eliminate all traces of the storage medium. The ephippia were transferred to hatching Petri dishes (10 cm diameter) with 50 mL pre-aerated standard freshwater (referred to de-bubbling water at 2.5 mmol/L of $NaHCO_3$, $MgSO_4$, KCl and $CaCl_2$). The hatching Petri dishes were covered and incubated for 72 h, at 20–22 ºC under continuous illumination of 6000 lux. Subsequently, the daphnids were transferred to fresh standard freshwater with a Pasteur pipette.

For the gene expression measurements, daphnia hatched from ephippia were transferred to Aachener Daphnien Medium (ADAM – which includes vitamins as thiamine, biotine, and vitamin B12) medium and cultured for 26 days based on the OECD guideline 201 (Organisation for Economic Co-operation and Development) Guidelines for the Testing of Chemicals. Organisms were fed with the unicellular green microalgae *Monoraphidium griffithii* (NIVA-CHL 8) (approximately 9 × 10^4 cells/daphnid), which were cultured based on the OECD guideline 211 (OECD, 2008) for testing of chemicals.

The *D. magna* culture was kept in 2-L glass beakers containing approximately 0.8 L medium with food, with 50 ± 2 individuals per beaker, at 20 ± 2 °C under a 16:8 h light: dark photoperiod, light intensity not exceeding 15–20 μEm-2 s-1.

2.3. Acute toxicity assays

The acute toxicity assays were performed according to the ISO standard (6341:2012). The *D. magna* neonates were originated from dormant eggs (MicroBioTests Inc., Belgium).

Assays were carried out in 24-well-plates. In each well, 195 μL of standard water plus 5 μL of test compound solution were pipetted. A small exposure volume was used similarly and reproducing the proportions reported by Grintzalis et al. (2017). By using a micropipette, *D. magna* neonates were aspired with 60 μL of standard water and transferred in the wells. In the end, each well contained five daphnids in 500 μL of artificial fresh water (OECD, 2018) with 0.5% (vol/vol) of test compound solution, a concentration tested safe by Grintzalis et al. (2017). Freshwater controls and ≤ 0.5% solvent blanks were included in every test. Tests were performed in quadruplicate and there was no renewal of media at any point. Daphnids were fed after 48 h with 9 × 10^4 cells/daphnid *Monoraphidium griffithii* (NIVA-CHL 8). The plates were covered and incubated at 20 ºC. After 24, 48, 72, 96, and 168 h incubation, the number of dead and immobilized neonates vs that of the actively swimming daphnids was observed and the percent mortality was calculated. As there were daphnids still alive after 48 h, it was decided to observe the effect until for one of the concentrations tested all daphnids died, in the lines of delayed toxicity assays previously described by Griffiths et al. (2021). To confirm no movement of daphnids, mechanical stimulation of media and observation within the 15 s was determined.

At the end of the assay, daphnids were discarded and the medium was collected in a 15 mL Falcon tube and determination of mycotoxin remaining in the media was carried out (see Section 2.6).

2.4. Reproduction test

Reproduction of *D. magna* exposed to mycotoxins for 7 days (168 h – time on which all daphnids died in highest concentration of BEA) was carried out by counting the number of neonates until 25 days old. It was not possible to follow the OECD guideline 211 described for

reproduction as the exposure to mycotoxins produced the death of daphnids earlier than that detailed in the protocol. Briefly, individual *D. magna* were in 50 mL vial and the medium was changed every other day. The day of counting, adult *D. magna* was placed in a new 50 mL vial with food (1 $\times 10^6$ cells/mL *Monoraphidium griffithii*) and the medium containing the offsprings was poured in a 15 cm diameter Petri dish to proceed with the manual counting. The average number of offspring in each brood, total number of offspring, and mortality of the original cohort in each treatment were calculated.

A total of 110 of *D. magna* were used to follow the number of offsprings and the following conditions and replicates were used: two batches of 24 *D. magna* each (7d old) per mycotoxin and with four replicates for each tested concentration (described in Section 2.1) and controls. At the end of the experiment around 60% of the daphnids were still alive. Dissolved oxygen was greater than 60% of air saturation, the temperature was in the range 20 ± 2 °C, whereas pH was quite stable during the experiments (pH range 7.80–7.90).

2.5. Effect of mycotoxins in delayed toxicity test of Daphnia magna

Effect of mycotoxins in delayed toxicity carried out by observation in the swimming of *D. magna* was carried out in 12-well-plates by placing 5 animals/well (26 days old) in a final volume of 1.5 mL ADAM water which resulted to be optimum for growing daphnids as reported by several authors (Klüttgen et al., 1994; Issa et al., 2020, 2021). Observations in acute effect of mycotoxins in swimming alterations after 0, 3, 6 and 24 h of exposure was carried out for 1 min 30 s by using a camera at 25 frames per second and placing the daphnids as described above. Exposures were for 24 h individually to BEA at 8 μM and 2 μM, or ENN B at 1.6 μM and 0.8 μM. Two mixtures of BEA + ENN B, designed as "high mixture" for [8 + 1.6] μM and "low mixture" for [2 + 0.8] μM were also tested, for 24 h. The number of daphnids spinning and the frequencies in this behavior were reported for its evaluation. Daphnids used here and those in reproduction were different; thus the organisms were from a different batch.

2.6. Quantification of mycotoxins by LC-MS/MS in aquatic media

Mycotoxin concentrations were tested at the beginning and at the end of studies (in acute/delayed toxicity and gene expression assays) with mean concentrations for the exposure period calculated. Concentrations in studies of 24 h length were based upon start concentrations only due to the small volume of solution used across the replicates. Samples were directly injected previous filtration by a using nylon filter (0.1 μm).

Chromatographic separation was done by using an Agilent 1290 Infinity UHPLC system coupled to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separation was performed by using a 100 mm × 2.1 mm (inside diameter), 2.7 μm, Agilent InfinityLab Poroshell 120 EC-C18, the temperature was maintained at 30 °C. Mobile phases were water containing 0.1% formic acid and 5 mM ammonium formate (phase A), and methanol (phase B) with 0.1% formic acid and 5 mM ammonium formate. An elution gradient of 15 min was made keeping the flow rate of the mobile phase at 0.3 mL/min, The following gradient was used: equilibration for 1 min at 70% A, 70–10% A in 10 min, 10% A for 2 min, 10–70% A in 0.5 min, 70% A for 4.5 min and the injection volume was set at 3 μL.

The triple quadrupole mass spectrometer was an Agilent 6460 equipped with an electrospray ionization source (ESI) using Agilent Jet Stream Technology. The ion source parameters were set as follows: sheath gas temperature, 340 °C; sheath gas flow, 10 L/min, gas temperature, 200 °C; gas flow, 8 L/min; nebulizer, 40 psi; capillary voltage, 3500 V; and nozzle voltage 500 V. The fragment voltage (FV), cell accelerator voltage (CAV), collision energy (CE) and mass transitions were optimized for each metabolite using MassHunter Optimizer software (Table 1). In all cases, the CAV parameter was set at 7. Analysis was

Table 1
UHPLC-MS/MS conditions used for the determination of BEA and ENN B remaining in D. magna media.

Mycotoxin	Prec Ion MS1	Prod Ion MS2	Frag (V)	CE (V)	Ret Time (min)
BEA	801.5	262.0	180	32	10
		244.1		36	
ENNB	657.3	214.0	160	32	9.6
		196.1			

CE: collision energy; Frag: fragmentation; Prec: precursor; Prod: product; V: voltage.

done in dynamic multiple reaction monitoring (dMRM) mode and two mass transitions were monitored for each toxin, one for quantification and another for confirmation. The analytical procedure was previously validated with media of *D. magna* to consider parameters of linearity, matrix effect, sensitivity (limit of detection (LD) and limit of quantification (LQ)), reproducibility and repeatability.

2.7. Quantitative reverse transcription PCR (qRT-PCR)

The study for the effects of mycotoxins on the gene expression of *D. magna* was carried out in 12-well-plates by placing 5 animals/well (26 days old) in a final volume of 1.5 mL ADAM. Three replicates for controls and for each concentration were tested. A total of 120 daphnids were used to perform the assay by exposing them for 24 h individually to BEA at 8 μM and 2 μM, or ENN B at 1.6 μM and 0.8 μM. Two mixtures of BEA + ENN B, designed as "high mixture" for [8 + 1.6] μM and "low mixture" for [2 + 0.8] μM were also tested, for 24 h. Concentrations were chosen taking as reference the study carried out previously in zebrafish (Juan-García et al., 2020) as mentioned above (Section 2.1). At the end of the assay daphnids were placed in Eppendorf and kept in a freezer for gene transcription study; while the medium was collected in a 15 mL Falcon tube for a further determination of mycotoxin remaining in the media or not filtered by *D. magna* (see Section 2.6).

A total of 100 adult daphnids were used for performing gene transcription study. The primers for alpha-tubulin and ubiquitin conjugating enzyme (ubq-ce) were adopted from (Heckmann et al., 2006), *abcc4* and *abcc5* from (Campos et al., 2014), *monooxygenase* and *vtg-sod* from (Sivula et al., 2018), and *abcb1* from (Vehniäinen and Kukkonen, 2015). Alpha-tubulin and ubq-ce were used as reference genes, as they were the most stable of a group of candidates, respective of treatment (data not shown). The features of the primers are presented in Table 2. Total RNA was extracted from pools of ca. 5 adult (28 d) *D. magna* using TRI reagent (Molecular Research Center) following the manufacturer's instructions. Extractions were performed on 3 independent biological replicates. NanoDrop 1000 (Thermo Fisher Scientific) was used for RNA concentration and purity estimation, and Agilent 2100 Bio-Analyzer (Agilent) for RNA integrity assessment, using Eukaryote total RNA 6000 nano kit (Agilent). After DNase treatment (DNase I, Thermo), 1 μg total RNA was reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1 + 9 with nuclease-free water. One 25 μL qPCR reaction consisted of 5 μL of the diluted cDNA, 0.75 μL each of forward and reverse primers (final concentration 300 nM), 6 μL sterile H_2O and 12.5 μL of iQ SYBR Green Supermix (Bio-Rad). The reactions were run in triplicates on clear 96-well PCR plates (Bio-Rad), and the instrument was CFX96 Real-Time PCR cycler (Bio-Rad). The qPCR protocol was 3 min at 95 °C; 40 cycles (10 s at 95 °C, 30 s at 58 °C); 10 s at 95 °C and a melt curve from 55 °C to 95 °C. The cycle threshold (Ct) values of no template controls (water instead of cDNA) were always over 38. Melt curves showed a single peak, thus confirming that only one PCR product was being produced.

2.8. Statistical analysis

Statistical analysis of data was carried out using IBM SPSS Statistic

Table 2
Primers used in the qPCR.

Gene name	Symbol	GenBank accession no.	wFleaBase EST no.	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency (%)	Amplicon size (bp)
α-tubulin[a]	*tbl*		WFes0007807	GAGGTGGTGACGACT	CCAAGTCGACAAAGACAGC	99.2	89
ubiquitin conjugating enzyme	*ubq-ce*		WFes0004602			98.9	
p-Glycoprotein (ABCB/md)	*abcb1*	KC172920		AACGCCCATGATTTTATCCA	GCAGAAGGATTTTGGGTTGA	100	139
ABCC4[a]	*abcc4*	KC122923		CCCGATCCCTTTACGTCGAT	GGTGGCGTCCTACATGAGT	100	100
ABCC5[b]	*abcc5*	KC122924		CAGTCCAGTCATCGAGAACG	GACGCAACAGAGCTCGG	100	100
glutathione-S-transferase	*gst*	DV437830				95.1	
Monooxygenase[a]	*mox*	DV437798		ACACGTCTATCCCGCATCAT	TACCAAGTACCGAGCCGTTT	97.5	102
vitellogenin-superoxide dismutase[a]	*vtg-SOD*	AB252738		CAAGTACAACGAGATGCCCG	ATGTAGGGACCCAACCAGTG	98.8	108

[a] Heckmann et al. BMC Genomics. 2006; 7: 175.
[b] Campos et al. Aquat Toxicol 2014; 148: 139–151.

version 23.0 (SPSS, Chicago, Il, USA) statistical software package and GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, USA). Data were expressed as mean ± SD of daphnia used in the experiments. Differences between groups were analyzed statistically with ANOVA followed by the Tukey HDS post hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Survival of Daphnia magna

The survival of *D. magna* neonates exposed to BEA is reported in Fig. 1a. It was observed that at short exposure times (24 and 48 h) survival was kept at 100% in all BEA concentrations < 2 μM, and slightly decreased at higher BEA concentrations (≤ 8 μM); while at longer exposure times (from 72 h to 168 h) survival varied from 50% to 75% in concentrations ≤ 2 μM, to be maintained around 80% for BEA ≤ 8 μM (Fig. 1a). For concentrations above 8 μM there was a decrease in survival especially after 72 h (set in 20–5%). IC_{50} values for BEA are reported in Table 3 and ranged from 28 μM to 10.7 μM for 48 and 168 h, respectively.

For ENN B, survival decreased by time at concentrations < 3.2 μM (Fig. 1b); however, at ENN B 3.2 μM survival for all exposure times was kept at range from 75% to 100%. At 6.25 μM decreases in survival were again observed, oscillating from 8% to 50% with respect to the control at 24 h and 72 h, respectively, and at 12.5 μM decreases oscillated from 15% to 50% with respect to the control at 24 h and 72 h, respectively (Fig. 1b). IC_{50} values for ENN B ranged from 12.5 μM to 4.5 μM for 48 and 168 h, respectively (Table 3).

Table 3
IC_{50} values reached for *D. magna* at different exposure times.

Mycotoxin	IC_{50} (μM) 24 h	48 h	72 h	96 h	168 h
BEA	n.r.	28 ± 0.3	11.3 ± 0.7	10.7 ± 0.5	10.7 ± 0.5
ENN B	n.r.	12.5 ± 0.5	4.9 ± 0.6	4.8 ± 0.4	4.5 ± 0.5

n.r.: not reached.

For the binary mixture of BEA + ENN B (Fig. 1c) survival in the scenario of "low concentration" oscillated from 67.5% to 100% for 96 h and 48 h, respectively; while for the scenario of "higher concentration" survival was lower: from 52.4% to 97% for 96 h and 48 h, respectively (Fig. 1c).

3.2. Offspring of Daphnia magna

Effects on the number of offspring of *D. magna* by mycotoxins BEA, ENN B, and their binary mixtures at different concentrations are

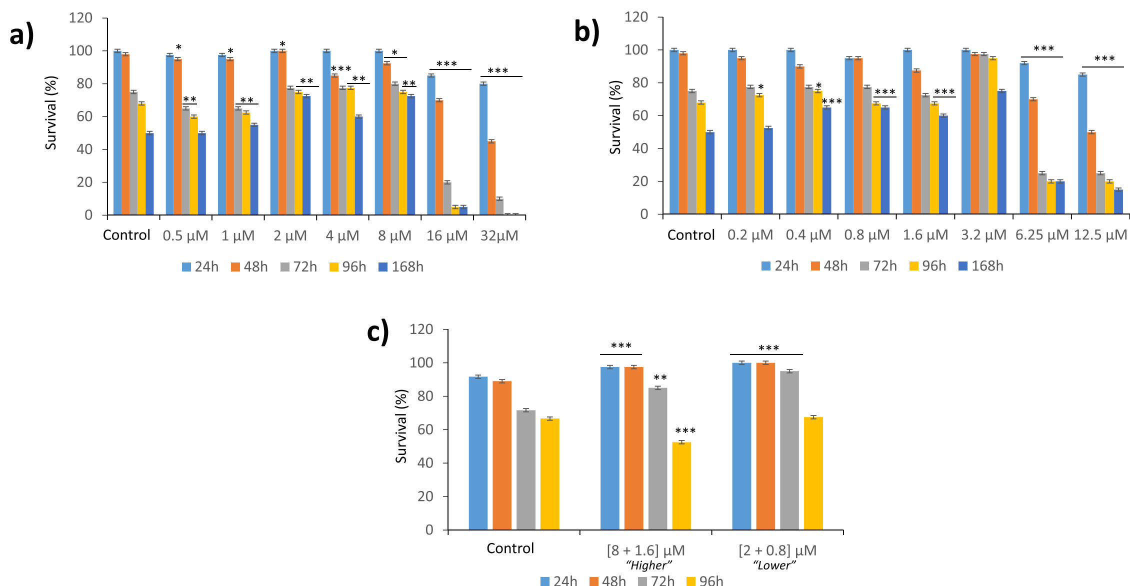

Fig. 1. Survival of *D. magna* after individual treatment with BEA (a), ENN B (b) and binary mixture [BEA + ENN B] (c) at different exposure times. Data corresponds to n = 5 and four independent replicates. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ respect to the control.

reported in Fig. 2 where the number of total offspring after 25 days was measured.

For BEA, it was noticed that the number of offspring of *D. magna* for the entire period studied increased significantly with respect to the control with the concentration, from 0.18 to 0.87-fold for 0.5 μM and 8 μM respect to the control, respectively. However, at 1 μM there was a significant decrease of 0.20-fold respect to the control observed (Fig. 2).

For ENN B there is not a clear tendency with the range of concentrations assayed, as the lowest number of offspring was at 0.2 μM (reduction of 0.96-folds respect to the control) and the highest at 0.8 μM (increase of 0.74-folds respect to the control); while at 3.2 μM a significant increase of 0.15-fold respect to the control was observed (Fig. 2).

Lastly, total offspring in mixtures of [BEA + ENN B] were counted on adult *D. magna* and at [8 + 1.6] μM a total of 29 offsprings were counted which means a decrease of 0.64-folds respect to the control; while at [2 + 0.8] μM the number of offspring increased 0.16 folds respect to the control. Among all of the scenarios studied the number of offspring with the highest overpass the controls offspring was for BEA (Fig. 2).

3.3. Delayed toxicity of *Daphnia magna*

Results reporting the observed alterations of swimming in *D. magna* adults treated with BEA, ENN B, and their mixtures are collected in videos provided as Supplement (Supplementary and videos - Plate 1&2). Alterations in swimming was observed by spinning around movement (up-down or right-left) with respect to the control wells for several treatments. In general, for the several exposure times, the change in swimming was detected after 3 h of exposure with an increase of spinning, and partially kept at 6 h detecting even a calm in swimming, and to increase after 24 h again (Supplementary 1-video).

Supplementary material related to this article can be found online at doi:10.1016/j.ecoenv.2022.114427.

3.4. Mycotoxins remaining in the media of *Daphnia magna*

The medium of *D. magna* containing mycotoxins after treatments were collected for each well from the survival assay (24 well/plate, Section 2.3) as well as from the study of gene/movement (12 well/plate, Sections 2.5 and 2.6). Measurements were carried out at the end of the experiment: after 168 h in the survival assay and after 24 h in the movement assay.

The optimum validation results of recoveries up to 97% and matrix effect (< 10%) permits to get LDs of 0.025 and 0.015 μM for BEA and ENNB, respectively. The method showed a linear behavior within the 0,3–50 μM for BEA and 0,15–20 μM for ENNB as working range of analyte concentrations. The acceptance criterion implies a test of the goodness of fit, using a correlation coefficient (r) between 0.9 and 0.999 as a criterion, and the calibration lines in the solvent and the calibration lines with the matrix gets correlation coefficient in this range. Both calibration lines were prepared for the quantification of the samples to evaluate the matrix effect and for the fortification of the samples for the study of reproducibility and repeatability.

Results for the percentage of mycotoxins after survival assays are reported in Fig. 3a. The test solution without test animals reported a value of cero (not included in Fig. 3a). It was observed that BEA at all concentrations was almost filtered by daphnids neonates as from 0.5 to 8 μM a total of 2.2% remained in the medium (Fig. 3a); while for concentrations 16 and 32 μM percentages remaining were 34.8% and 61.6% respectively and respect to the original amount. (Fig. 3a). For ENN B, percentages of mycotoxins remaining oscillated between 5.2% and 17.6% for concentrations below 3.2 μM; while at the highest concentrations assayed 6.25 and 12.5 μM it reached 23% and 36%, respectively and respect to the original amount (Fig. 3a).

On the other hand, for gene expression assays, it was revealed that BEA remained around 20% (for 2 and 8 μM), ENN B between 28% and 36% for 0.8 and 1.6 μM, respectively (Fig. 3b). The test solution without test animals reported a value of cero (not included in Fig. 3b). When the same assay was studied with mixtures: at the lowest concentration [2 + 0.8] μM ENN B remained in higher proportion than BEA (70% vs 52%) and for the highest concentration the percentage remained was similar for both mycotoxins, BEA 23.7% and ENN B 20.7% (Fig. 3b).

3.5. Effect of mycotoxins in gene expression of *Daphnia magna*

Both mycotoxins affected the gene expression of daphnia, the effects depending on compound and concentration. Compared to vehicle control, mRNA of *abcb1, abcb5, gst, mox* and *vtg-SOD* were significantly overexpressed by both concentrations of BEA ($p \leq 0.05$) (Fig. 4a, c, d, e,

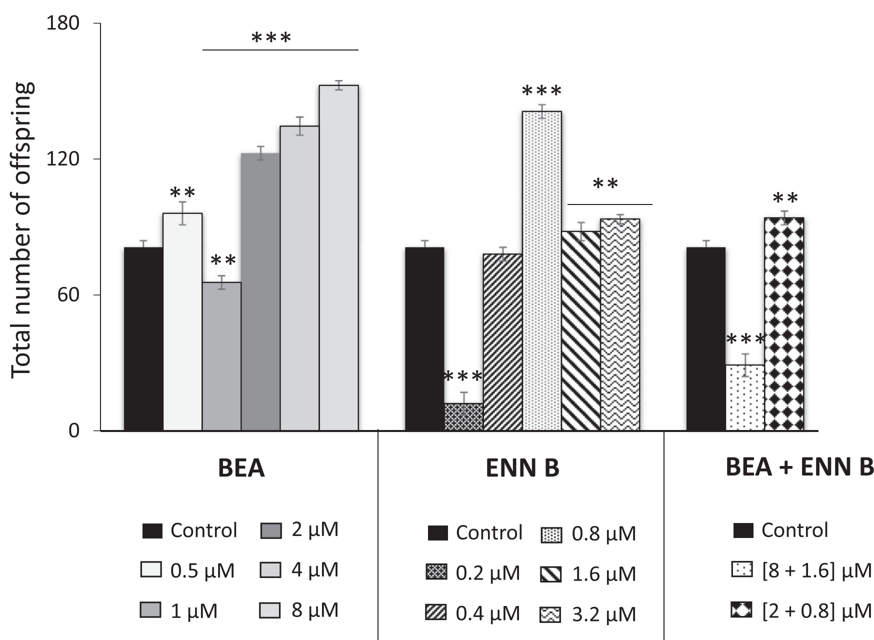

Fig. 2. Number of offspring of *D. magna* after individual treatment with BEA, ENN B and binary mixture [BEA + ENN B] at different concentrations and during 7 days of exposure (followed by 25 days). Data correspond to n = 5 and four independent replicates. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ respect to the control.

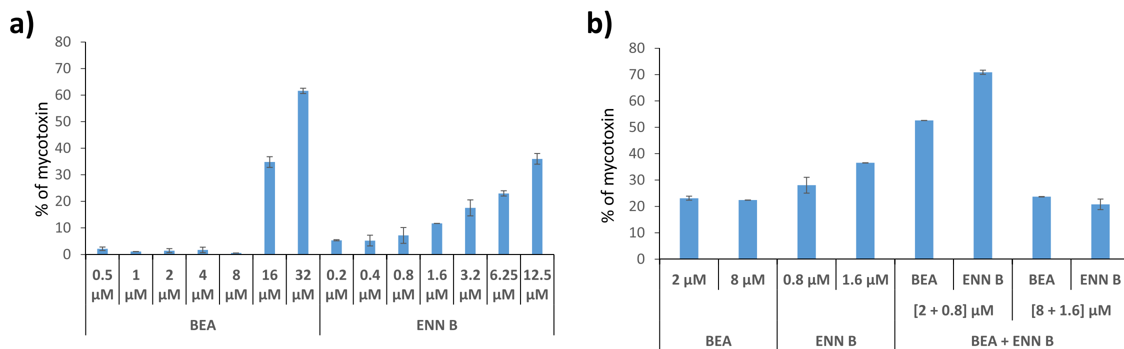

Fig. 3. Percentage of BEA, ENN B and [BEA + ENN B] remaining in the media of *Daphnia magna* after treatment in the viability assay (168 h) (a) and in the movement assay (24 h) (b) at different concentrations by LC–MS/MS equipment.

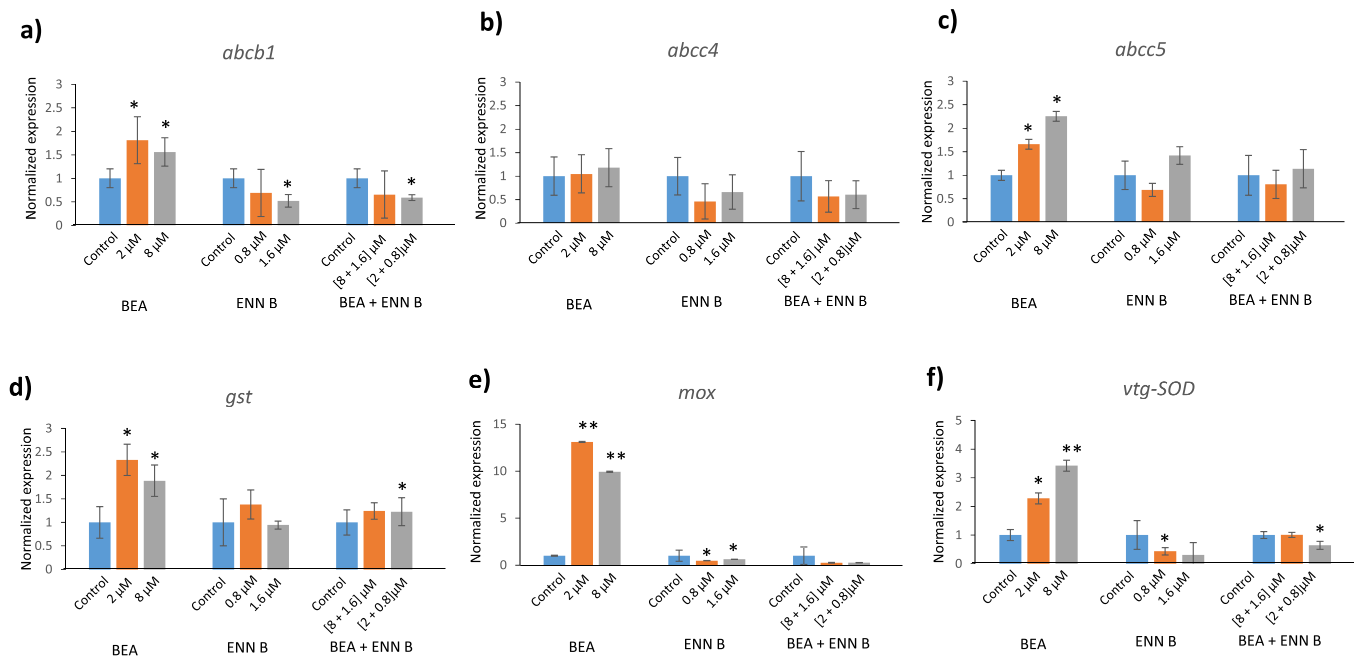

Fig. 4. Normalized expression of *abcb1, abcc4, abcc5, gst, mox and vtg-SOD* mRNA normalized to reference genes *ubq-ce* and *α-tubulin* (in folds) in adult (28 d) daphnids. Data represent mean ± SE of the mean of three replicates, each pooled of 5–6 organisms. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ respect to the control.

and f). On the other hand, ENN B at 0.8 µM significantly downregulated *mox* and *vtg-SOD* mRNA from 0.52- to 0.56- folds, respectively ($p \leq 0.05$), whereas 1.6 µM ENN B downregulated *abcb1* and *mox* mRNA down to 0.47- and 0.38-folds, respectively ($p \leq 0.05$). (Fig. 4a, e and f). In combination only the higher mixture concentration of BEA + ENN B at [2 + 0.8] µM showed alterations in gene expression as follows: i) upregulation of *gst* up to 1.23-fold ($p \leq 0.05$) and ii) downregulation of *abcb1* and *vtg-SOD* down to 0.40- and 0.35-fold, respectively ($p \leq 0.05$) (Fig. 4a and f).

4. Discussion

In this study, ecological concentrations of ENN B and BEA mycotoxins previously tested in fish and embryos of *Danio rerio* (zebrafish) have been used (Juan-García et al., 2021). Our results showed unequivocally that BEA and ENN B alter survival in *D. magna* at different ages as well as transcription of genes involved in different defense systems.

Lately, literature has released the results of BEA and ENN B as affecting negatively the nervous system; this study contributes to elucidate part of their effect. Susceptibility of *D. magna* to mycotoxins revealed that exposure for 48 h to T-2 toxin reached an IC_{50} value of 0.07 mg/L, with 100% mortality at 0.5 mg/L (Goyster et al., 2004); while for deoxynivalenol (DON) and zearalenone (ZEA) IC_{50} values were much higher, 0.13 mg/L and 7.8 mg/L, respectively (Eagles et al., 2021). Comparing these results with ours, IC_{50} values were higher and thus noticing a lower potential of toxicity of BEA and ENN B; however, the inclusion of five time points helps to better describe the effect produced by both mycotoxins.

The amount of mycotoxins remaining in the media after the acute toxicity assay, reveals the capacity of *D. magna* in filtering mycotoxins while alive; as it can be observed how higher amount is remaining at high concentrations although more daphnids are dead. Nevertheless, it produced an alteration in swimming profile, detected by the spinning around or in continuous circles which might induce alterations in i) the nervous system and ii) environmentally in the flora and fauna for species that are fed through them. Literature reports some studies about the effects of movement in *D. magna* when exposed to saxitoxin or to anatoxin-A mycotoxins describing alterations in profiles of swimming (Bownik and Pawlik-Skowrońska, 2019; Ferrao-Filho and da Silva, 2020); while others have tested fungal biocontrol agents (among which gliotoxin and destruxin were included) through acute toxicity tests revealing the great value of this model for screening toxicity as an early stage of product development . In fact, *D. magna* has been categorized as

an excellent focal point for regulatory authorities and as a good candidate for risk assessment (Calow, 1993; Commission of the European Communities, 1991, 1992; United States Environment Protection Agency, 2002). Consequences in the swimming pattern of *D. magna* determine thus the effects in behavior, in the environment, for getting food, for prey, and predators.

The gene expression analysis showed that mycotoxins affected multiple levels of xenobiotic metabolism, and the effects depended on the compound and concentration. BEA upregulated genes involved in phase I (*mox*), phase II (*gst*), and phase 0/III (*abcb1, abcc5*) of xenobiotic metabolism, whereas ENN B only either had no effect (*gst, abcc5*) or downregulated them (*abcb1, mox*). MXR is a mechanism of defense in aquatic organisms activated once in contact with contaminants with the function of excluding those contaminants from the cells or even not allowing them to get in (Campos et al., 2014); so that, keeping the levels of compounds low inside the cells but higher in the media. This way the pumped-out compounds avoid reaching the toxic point of action. BEA up-regulated *abcb1* and *abcc5* at both concentrations assayed, whereas a down-regulation in ENN B and [BEA + ENN B] at 1.6 μM and [2 + 0.8] μM, was observed only for *abcb1*. Both the effect observed in gene expression and the property as ionophors of mycotoxins might be responsible for the up-/down-regulation observed for the concentrations studied.

The production of yolk proteins in *D. magna* is determined by the *vtg-SOD* which is the major component (Kato et al., 2004). It has been evidenced that the number of offspring can be reduced if there is a deficiency in producing the yolk protein although everything produced is not always used. The effect observed in this study is confirmed with that hypothesis as there was an up-regulation of *vtg-SOD* in BEA (Fig. 4f), increase in the number of offspring and the survival was also influenced as it decreased (Figs. 1a and 2a); while the down-regulation of *vtg-SOD* observed in mixture exposure revealed a decrease in the number of offspring and effect in survival was around 67% (Fig. 1c). This has been also observed for other compounds as metals for the same biological model *D. magna* (Sivula et al., 2018).

gst gene is related to the metabolism of exogenous compounds and the antioxidant system (Wang et al., 2016). Nevertheless, the activity of *gst* has been discussed with its gene expression as there might be other transcriptional factors involved (as Nrf2) and reported elsewhere (Regoli and Giuliani, 2014; Kim and Lee, 2017; Hansen et al., 2007). In our study, it was observed an up-regulation of *gst* gene for BEA (both at 2 and 8 μM) and for BEA + ENN B [2 + 0.8] μM which could be related with the results obtained for those concentrations in the survival as a slight increase in survival respect to control and lower concentrations were observed (Wang et al., 2016). In a study carried out with ibuprofen it is pointed out that there might be a relationship with the reproduction as well; however, due to the complexity of all mechanisms involved, this cannot be established here and more studies would be necessary either in the activity of enzymes and systems as in gene expression.

5. Conclusions

Daphnia magna, sensitive responders to environmental changes, proved to be a good focal point for environmental risk assessment and its use as a good alternative for in vitro mycotoxin testing. Results of BEA and ENN B affecting negatively the nervous system and the mechanism is still unclear in some respects and more studies are needed. Here it has been demonstrated that exposure to environmental concentrations of mycotoxins ENN B (from 0.2 μM to 12.5 μM) and BEA (from 0.5 μM to 32 μM) can affect the survival, behavior, number of offsprings, and expression of genes involved in multiple systems as well as the additional value of the toxicological foot-print of mycotoxins in the environment. Among that, the effect on small invertebrates in aquatic ecosystems puts in evidence the danger the species can suffer in higher levels of the trophic chain of the aquatic animals and subsequently in fish and food of aquatic origin for consumers but also its transfer to other species. The presence of mycotoxins in food and feed that reach aquatic systems, and their impacts on species are under demand of being investigated and more studies are needed.

CRediT authorship contribution statement

Ana Juan-García: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Hannu Pakkanen:** Data curation, Investigation, Methodology, Writing – review & editing. **Cristina Juan:** Conceptualization, Investigation, Methodology, Visualization, Writing – review & editing. **Eeva-Riikka Vehniäinen:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114427.

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