Comparison of Short-Term Toxicity of 14 Common Phycotoxins (Alone and in Combination) To The Survival of Brine Shrimp *Artemia Salina*

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Abstract

Toxic harmful algal blooms (HABs) can cause deleterious effects in marine organisms, threatening the stability of marine ecosystems. It is well known that different strains, natural populations and growth conditions of the same toxic algal species may lead to different amount of phycotoxin production and the ensuing toxicity. To fully assess the ecological risk of toxic HABs, it is of great importance to investigate the toxic effects of phycotoxins in marine organisms. In this study, the short-term toxicity of 14 common phycotoxins (alone and in combination) in the marine zooplankton *Artemia salina* was investigated. On the basis of 48 h LC_{50}, the order of toxicity in *A. salina* was AZA3 (with a LC_{50} of 0.0203 μg/ml)＞AZA2 (0.0273 μg/ml) ＞PTX2 (0.0396 μg/ml)＞DTX1 (0.0819 μg/ml)＞AZA1 (0.106 μg/ml)＞ SPX1 (0.144 μg/ml)＞YTX (0.172 μg/ml)＞dcSTX (0.668 μg/ml)＞OA (0.728 μg/ml)＞STX (1.042 μg/ml)＞GYM (1.069 μg/ml)＞PbTx3 (1.239 μg/ml)＞hYTX (1.799 μg/ml)＞PbTx2 (2.415 μg/ml). For the binary exposure, additive effects of OA and DTX1, DTX1 and hYTX; antagonistic effects of OA and PTX2, OA and STX; and synergetic effects of DTX1 and STX, DTX1 and YTX, DTX1 and PTX2, PTX2 and hYTX on the mortality of *A. salina* were observed. These results provide valuable toxicological data for assessing the impact of phycotoxins on marine planktonic species and highlight the potential ecological risk of toxic HABs in marine ecosystems.

1. Introduction

The frequency, scale and magnitude of harmful algal blooms (HABs) has increased in the past decades, due to the overfishing, coastal eutrophication, global climate change and invasive species dispersal (De Rijcke et al., 2016). HABs can be classified in two categories, according to the mechanisms underlying the negative impacts: 1) non-toxic HABs, which leads to the water quality loss by an excessive increase of turbidity and dissolved oxygen consumption; 2) toxic HABs, which synthesizes powerful phycotoxins negatively impacting aquaculture industry, ecological stability and even public health (Simöes et al., 2015). It has been well documented that phycotoxins produced by toxic algae species can lead to acute illness in humans (Turki et al., 2014). For example, diarrheic shellfish poisoning (DSP) is mainly due to the phycotoxins (such as okadaic acid and dinophysistoxin) produced by toxic strains of *Prorocentrum* spp. and *Dinophysis* spp. (Yasumoto, 1990; Dickey et al., 1990; Bravo et al., 2001), and paralytic shellfish poisoning (PSP) is predominantly linked to the phycotoxins (such as saxitoxin) by toxic strains of *Alexandrium* spp. (Hallegraeff, 2003; Anderson et al., 2012; Abdennadher et al., 2012).

Besides of human-health concerns, phycotoxins produced by toxic HABs can cause deleterious effects in many aquatic organisms, threatening the ecological health and stability (Durbin et al., 2002; Faassen et al., 2012; Sugunama et al., 1988; Zhang et al., 2009). Zooplanktons, channeling primary production to higher trophic levels, plays a crucial role in marine ecosystems. It is documented that when toxic HABs occur, the produced phycotoxins would induce adverse effects on zooplanktons, resulting in a reduction of population quantity (Jonsson et al., 2009; Xu et al., 2017). The responses of zooplanktons (e.g., copepods) to toxic HABs vary significantly, mainly depending on the species of toxic algae (Turner et al., 2014; Xu et al., 2017). However, when exposed to the same species of toxic alga, copepods may give distinct responses (Xu et al., 2017). One possible reason is that different strains, natural populations and growth conditions of the same algal species lead to different amount of phycotoxin production and the ensuing toxicity (Xu et al.,2017). Therefore, to fully conclude the toxicity of HABs and to well compare the potential toxicity of different toxic alga species in zooplanktons, it is necessary to include the use of phycotoxins. In addition, phycotoxins do not only occur singly but also as mixtures in shellfish (Ferron et al., 2016). However, to date, toxicological data for the toxic effects of phycotoxins, alone and in combination, in aquatic organisms is limited, making it difficult to draw a conclusion about the ecological risk of HABs.

In this study, the toxicity of 14 common phycotoxins (i.e., okadaic acid, OA; dinophysistoxin-1, DTX-1; pectenotoxin-2, PTX2; yessotoxin, YTX; homo-yessotoxin, hYTX; 13-desmethyl spirolide C, SPX1; gymnodimine, GYM; azaspiracids-1, AZA-1; azaspiracids-2, AZA-2; azaspiracids-3, AZA-3; saxitoxin, STX; decarbamoylsaxitoxin, dcSTX; brevetoxin-2, PbTx2; brevetoxin-3, PbTx3) on the survival of brine shrimp (*Artemia salina*) was investigated by assessing the median-lethal concentration (LC_{50}) for 48 h. Furthermore, the combined effect (additive, antagonistic or syngenetic) of two different phycotoxins on the survival of *A. salina* was also investigated. The overall aim of this study was to provide valuable toxicological data for evaluating the toxicity of phycotoxins in zooplanktons and to help better understand the ecological risk of toxic HABs.

2. Materials And Methods

2.1. Phycotoxins

Certified reference standards for OA, DTX-1, PTX2, YTX, hYTX, SPX1, GYM, AZA-1, AZA-2, AZA-3, STX and dcSTX were purchased from the National Research Council Halifax, Canada. PbTx2 and PbTx3 were obtained from Taiwan Renyu Company. The stock solution of STX was dissolved in 3 mM HCl, while others in methanol. Prior to each experiment, working solutions of phycotoxins were freshly prepared by serial dilution

2.2 Brine shrimp bioassay

The brine shrimp (*Artemia salina*) assay was carried out following the technique described by (Lincoln et al., 1996; Hisem et al., 2011; Sumantha et al., 2014). One gram of dried *Artemia salina* cysts were hatched in filtered artificial seawater (FASW, pore size: 0.22 μm; salinity: 30 ± 1ppt) with

gentle aeration for 24h under continuous illumination. Newly hatched larvae were collected using a Pasteur pipette after a 24h incubation and washed with FASW before exposure. For the preparation of exposure medium, 20 µl of phycotoxin working solution, solvent or FASW was transferred into a 24-well microtiter plate with 1.98 ml FASW per well. For each well, 10 individuals were added. Each group had three replicates. The mortality of *A. salina* was counted at 48h using a stereomicroscope (Olympus IX71). Pilot tests were conducted for each phycotoxin to determine the lethal effect concentration range. The death of an individual was defined as follows: no appendage movements in 10s.

2.4 Statistical analysis

Bioassay data for artemia mortality was analyzed using IBM SPSS Statistics 23 software. Phycotoxin concentrations (µg/ml) that resulted in 50% mortality (i.e., LC_{50} values) were estimated using log-probability curves with 95% confidence intervals. LC_{50} values were determined by probabilistic regression models generated. For the binary exposure, the differences among the treatments were tested using one-way analysis of variance (ANOVA) with specific mean comparisons performed by Fisher's least significant difference (LSD) post hoc test. Prior to ANOVA analyses, Shapiro-Wilk and Bartlett's tests were used to test for normality and homogeneity of variances, respectively. All data were presented as means ± standard error of the mean (SEM).

3. Results

3.1 Effect of each phycotoxin

The mortality-concentration curves and LC_{50} values for OA, DTX, PTX2, PbTx2, PbTx3, YTX, hYTX, STX, dcSTX, GYM, SPX1, AZA1, AZA2 and AZA3 in *A. salina* were shown in the Figure 1 and Table 1. On the basis of 48 h LC_{50}, the order of toxicity in artemia was AZA3＞AZA2＞PTX2＞DTX1＞AZA1 ＞ SPX1＞YTX＞dcSTX＞OA＞STX＞GYM＞PbTx3＞hYTX＞PbTx2. Among the tested 14 phycotoxins, the LC_{50} value of AZA3 in artemia was the lowest (0.0203 µg/ml), while PbTx2 showed the least toxic effect with a LC_{50} value of 2.415 µg/ml.

Table 1
The 48 h LC_{50} values of marine phycotoxins in *Artemia salina* (n = 3)

	OA	DTX1	PTX2	YTX	hYTX	GYM	SPX1	AZA1	AZA2	AZA3	STX	dcSTX	PbTx2	PbTx3
LC_{50} (µg/ml)	0.728	0.0819	0.0396	0.172	1.799	1.069	0.144	0.106	0.0273	0.0203	1.042	0.668	2.415	1.239

3.2 Combined effect of two phycotoxins

For the binary exposure, the concentrations of phycotoxins were set less than their respective LC_{50} values (OA, 0.0685 µg/ml; DTX1, 0.0755 µg/ml; PTX2, 0.0225 µg/ml; STX, 0.121 µg/ml; YTX, 0.0275 µg/ml; hYTX, 0.029 µg/ml; SPX1, 0.035 µg/ml).

3.2.1 Combination of OA with DTX1, PTX2 or STX

The artemia from the OA + DTX1 group exhibited higher mortality than those from the OA group ($p = 0.0010$), but did not show significantly higher mortality than the DTX1 treated artemia (Fig. 2A). No significant difference in the mortality was found among the OA, PTX2 and OA + PTX2 groups (Fig. 2B). Similarly, the mortality of the artemia from the OA + STX group was close to that from the OA alone group and the STX alone group (Fig. 2C).

3.2.2 Combination of DTX1 with PTX2, STX, YTX or hYTX

Relative to the mortality for the DTX1 alone group and the PTX2 alone group, the mortality of the DTX1 + PTX2 treated artemia was elevated by 2.6-folds ($p < 0.0001$) and 10-folds ($p < 0.0001$), respectively (Fig. 2D). The DTX1 + STX treated artemia showed significantly higher mortality than those exposed to individual phycotoxin (DTX1 alone or STX alone) (Fig. 2E). Similarly, significant increases (1.9-folds, $p = 0.0105$ and 32-folds, $p < 0.0001$, respectively) in the mortality were observed in the artemia exposed to DTX1 + YTX relative to the artemia from the DTX1 alone group and the YTX alone group (Fig. 2F). In contrast, the artemia from the DTX1 + hYTX group did not exhibit higher mortality than those from the DTX1 alone group (Fig. 2G).

3.2.3 Combination of PTX2 with SPX or hYTX

For the binary exposure to PTX2 and SPX1, no significant difference in the mortality was observed among three groups (Fig. 2H). Differently, the mortality for the PTX2 + hYTX group was increased by 4.9-folds ($p = 0.0002$) and 11-folds ($p = 0.0003$) compared to that for the PTX2 group and the hYTX group, respectively (Fig. 2I).

Table 2
List of the recent toxicological data about the toxicity of phycotoxins in aquatic organisms

Phycotoxin	Species	Time	LC_{50} (μg/ml)	Reference
OA	*Tigriopus californicus*	24 h	41.7	Shaw et al., 1997
	Artemia franciscana	24 h	6270*	D'ors et al., 2014
	Danio rerio larvae	24 h	10	Figueroa et al., 2020
	Danio rerio larvae	48 h	8.5	Figueroa et al., 2020
	Danio rerio larvae	72 h	7	Figueroa et al., 2020
	Daphnia magna	48 h	42.1	Rambla-Alegre et al., 2018
	Daphnia magna	96 h	0.003	Rambla-Alegre et al., 2018
	Artemia salina	48 h	0.728	This study
DTX-1	*Danio rerio* larvae	24 h	7	Figueroa et al., 2020
	Danio rerio larvae	48 h	5.5	Figueroa et al., 2020
	Danio rerio larvae	72 h	5	Figueroa et al., 2020
	Daphnia magna	48 h	29	Rambla-Alegre et al., 2018
	Daphnia magna	96 h	0.008	Rambla-Alegre et al., 2018
	Artemia salina	48 h	0.0819	This study
STX	*Artemia franciscana*	24 h	4060*	D'ors et al., 2014
	Artemia salina	48 h	1.04232	This study
PbTx	*Bambusia affinis*	24 h	0.000011	Kirkpatrick et al., 2004
	Oryzias latipes	24 h	0.015–25	Poli, 1998
(PbTx-2)	*Artemia salina*	48 h	2.415	This study
(PbTx-3)	*Artemia salina*	48 h	1.239	This study

* the calculated equivalent

4. Discussion

The toxicity of phycotoxins has received increasing attention with the increase of frequency, scale and magnitude of toxic harmful algal blooms (HABs) in recent years. Many studies have been mostly focused on the impacts of phycotoxins on mammals (such as mice, dogs, human cell lines and etc) to meet the demand of seafood safety control and pollution monitoring (EFSA CONTAM, 2010). However, the toxicological data in aquatic organisms is really limited (Table 2), making it difficult to fully evaluate the ecological risk of phycotoxins and toxic HABs. In this study, the short-term toxicity of 14 common phycotoxins (OA, DTX1, PTX2, YTX, hYTX, GYM, SPX1, AZA1, AZA2, AZA3, STX, dcSTX, PbTx2 and PbTx3) in *A. salina* was investigated. Among the 14 tested phycotoxins, AZA3 (with a LC_{50} of 0.0203 μg/ml) was the most toxic phycotoxin in artemia, followed by AZA2 (with a LC_{50} of 0.0273 μg/ml). AZAs (including AZA-1, AZA-2, AZA-3, AZA-4, AZA-5 and etc) are a group of phycotoxins produced by *Azadinium spinosum* (Ferreiro et al., 2016). Although the mode of action of the AZAs has not been fully elucidated, AZAs are found to inhibit endocytosis (Sala et al., 2013) and to induce cytoskeleton disorganization (Twiner et al., 2005). In this study, AZA3 (with a LC_{50} of 0.0203 μg/ml) and AZA2 (with a LC_{50} of 0.0273 μg/ml) showed higher toxicity than AZA1 (with a LC_{50} of 0.106 μg/ml). Similarly, a study in mice shows that after intraperitoneal administration, AZA2 (with a minimum lethal dose of 110 μg/kg) and AZA3 (140 μg/kg) are more toxic than AZA1 (150 μg/kg) (Hajime,2006; Twiner et al., 2008). These results reinforce the concept that the toxicity of analogues might can vary significantly.

To prevent human intoxications, the European Union (EU) has set regulatory limits of phycotoxins in shellfish mainly based on the toxicity on mice (Alarcan et al., 2018). The limits of OA, AZA, PTX, STX and YTX in 1 kg shellfish meat are 160 μg, 160 μg, 160 μg, 800 μg and 1 mg, respectively. This suggest that YTX might be the least toxic phycotoxin among the five phycotoxins, followed by STX. In the present study, YTX (with a LC_{50} of 0.172 μg/ml) is found to be more toxic than STX (with a LC_{50} of 1.042 μg/ml) and OA (with a LC_{50} of 0.728 μg/ml) in artemia. This suggest that the toxic effects of phycotoxins in mammals (like mice) and in zooplanktons (like artemia) might be distinct. Therefore, besides of the human-health concerns, the investigation of deleterious effects of phycotoxins on marine food web also requires attention.

As phycotoxins do not only occur singly (Ferron et al., 2016), it is of importance to clarify the combined effects of two phycotoxins. In this study, additive effects were observed in OA + DTX1. OA and DTX1 belong to the polyether fatty acid toxins (Farabegoli et al., 2018). They share similar mode of action, that attacking the serine/threonine phosphoprotein phosphatases (PPs), in particular PP2A, and as secondary targets, PP1 and PP2B (Farabegoli et al., 2018). Therefore, the observed additive effects of OA and DTX1 in artemia are probably due to the “dose addition”. On the other hand, the combination of OA and PTX2 exhibited antagonistic effects. Similarly, a recent study in human intestinal Caco-2 cells shows that the combination of OA with PTX2 results in reduced toxicity (including, ROS production, IL-8 release and γ-H2AX phosphorylation) at low concentrations (Alarcan et al., 2019). It is reported that OA can interact with regulatory nuclear receptors such as PXR (Fidler et al., 2012; Ferron et al., 2016), which regulate the expression of some cytochrome P450 enzymes (Wang et al., 2012). PTX-2 is believed to interact with the AhR and induce P450 1A protein in hepatic cells (Alarcan et al., 2017; Alarcan et al., 2019). Therefore, one possible explanation is that the mixture of OA and PTX2 might induce cytochrome P450 activity and efflux transporter expression, resulting in higher detoxification/excretion of toxins and thus decreased toxic effects (Alarcan et al., 2019).

In this study, the binary exposure to DTX1 + STX, DTX1 + YTX or DTX1 + PTX2 dramatically elevated the mortality in artemia, compared to the individual exposure, suggesting that DTX1 can interact with STX, YTX and PTX2, and then induce greater effects than additive. The synergetic effects of two phycotoxins have been documented. For instance, the mixture of AZA-1 and YTX shows synergism in human intestinal cell models (Caco-2 cells and the human intestinal epithelial crypt-like (Ferron et al., 2016). The combination of YTX and OA with a ratio of 1:26.5 exhibits synergistic effects in the human intestinal epithelial crypt-like cells. Our results further highlight the hazard potency of the mixtures of DTX1 and other phycotoxins (like STX, YTX and PTX2) with regard to the ecological risk.

In summary, this study demonstrates the individual toxicity of 14 phycotoxins in artemia. On the basis of 48 h LC_{50}, the order of toxicity in artemia is AZA3□AZA2□PTX2□DTX1□AZA1□ SPX1□YTX□dcSTX□OA□STX□GYM□PbTx3□hYTX□PbTx2. Furthermore, the combination of two phycotoxins exhibits additive (OA + DTX1; OA + DTX1), antagonistic (OA + PTX2; OA + STK) or synergetic (DTX1 + STX; DTX1 + YTX; DTX1 + PTX2; PTX2 + hYTX) effects with regard to the mortality of artemia. The findings enrich the toxicological data of HABs and phycotoxins in zooplanktons and marine ecosystems, and also help better understand the ecological risk of toxic HABs and phycotoxins.

Declarations

Data availability

All authors guarantee that all data and materials support our published claims and comply with field standards.

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Author contributions YTZ performed the data analysis and wrote the manuscript. SS, BZ and YZ conducted the experiments. MT, HC and GD performed the data analysis. RL and JM conceived and designed the study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Figures

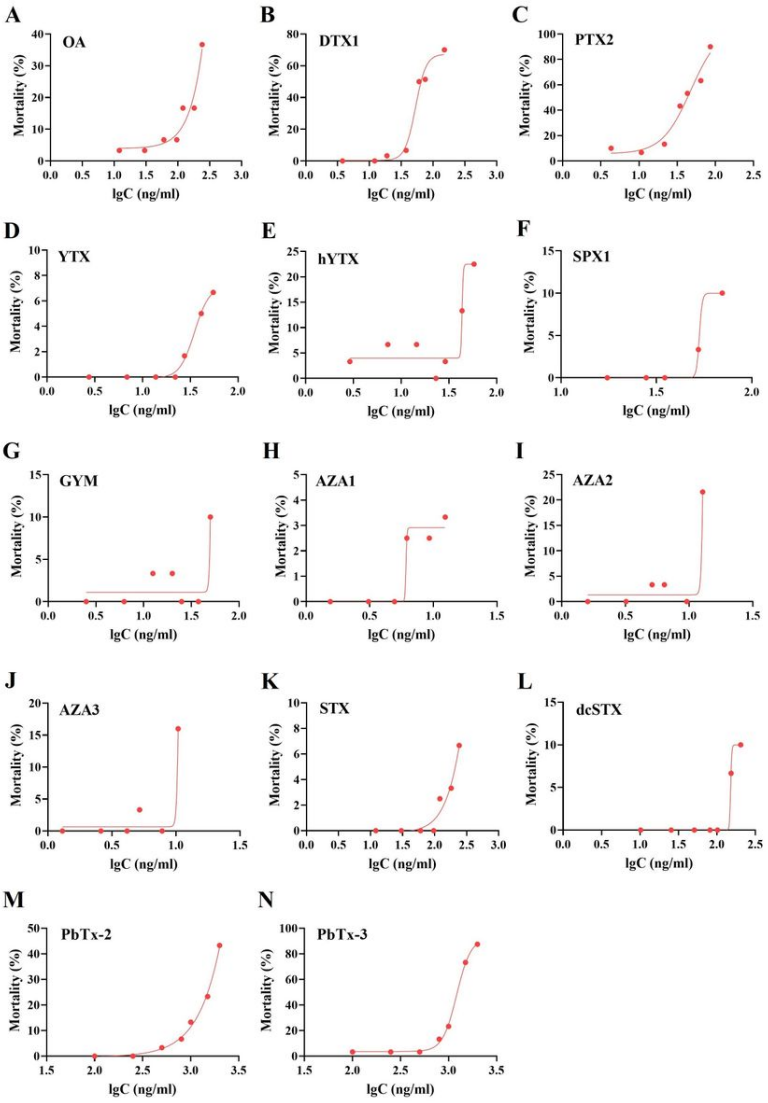

Figure 1

The 48 h mortality-concentration curves of OA (A), DTX (B), PTX2 (C), PbTx2 (D), PbTx3 (E), YTX (F), hYTX (G), STX (H), dcSTX (I), GYM (J), SPX1 (K), AZA1 (L), AZA2 (M) and AZA3 (N) in A. salina.

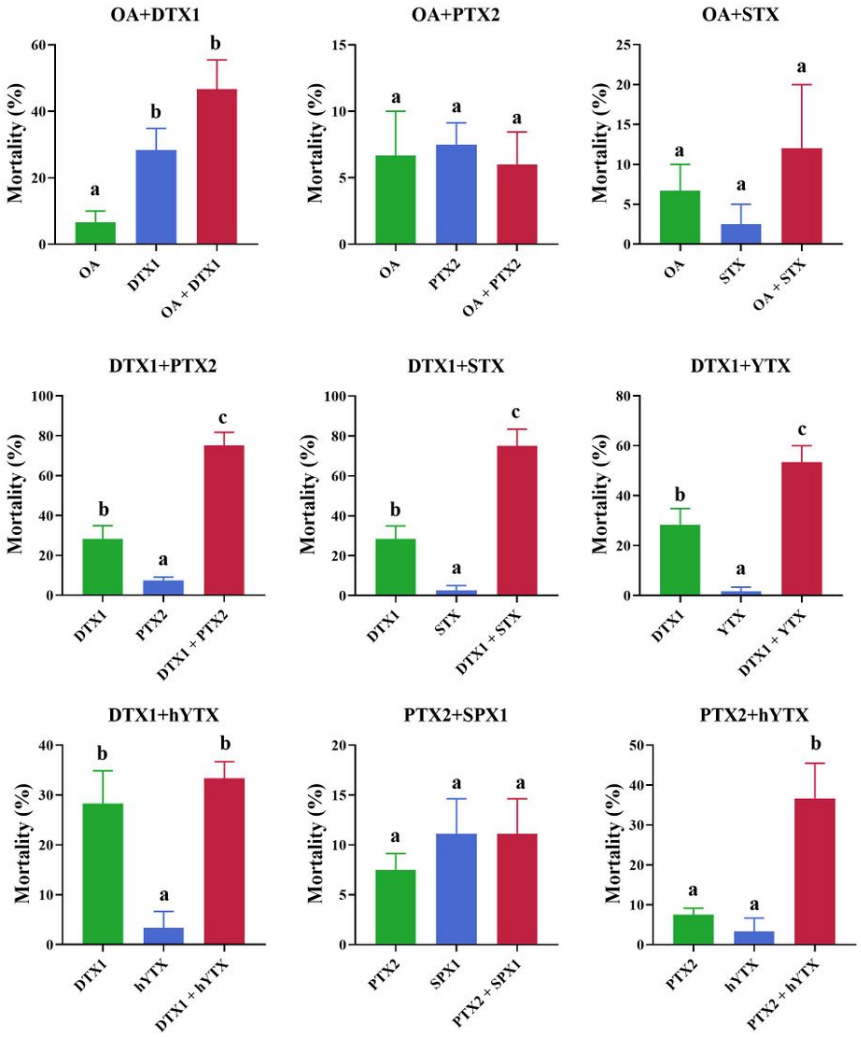

Figure 2

The individual and combined effects of OA + DTX1 (A), OA + PTX2 (B), OA + STX (C), DTX1 + PTX2 (D), DTX1 + STX (E), DTX1 + YTX (F), DTX1 + hYTX (G), PTX2 + SPX1 (H) and PTX2 + hYTX (I) on the 48 h mortality of A. salina.