Sublethal Effects of The Toxic Alga *Karlodinium Veneficum* On Fish

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Abstract

Dinoflagellates of the genus *Karlodinium* are ichthyotoxic species that produce karlotoxins. Karlotoxins show hemolytic and cytotoxic activities and have been associated with fish mortality. This study evaluated the effect of toxins released into the environment of *Karlodinium veneficum* strain K10 (Ebro Delta, NW Mediterranean) on the early stages of *Danio rerio* (zebrafish). Extracts of supernatant of K10 contained KmTx-10, -11, -12, -13, and a sulfated form of KmTx-10. Total egg mortality was observed for karlotoxins concentration higher than 2.69 μg L^{-1} and the 1.35 μg L^{-1}, 87% of development anomalies were evidenced (concentrations expressed as KmTx-2 equivalent). Larvae of 8 days post-fertilization exposed to 1.35 μg L^{-1} presented epithelial damage with 80% of cells in the early apoptotic stage. Our results indicate that supernatants with low concentration of KmTxs produce both lethal and sublethal effects in early fish stages. Moreover, apoptosis was induced at concentrations as low as 0.01 μg L^{-1}. This is of great relevance since detrimental long-term effects due to exposure to low concentrations of these substances could affect wild and cultured fish.

Highlights

- Bioactives exudated by *K. veneficum* strain K10 are ecologically relevant.
- Strain K10 exudates included KmTx-10, -11, -12, -13, and a sulfated form of KmTx-10.
- K10 exudates show hemolytic capacity, embryotoxicity, and induce apoptosis in early larvae.
- K10 toxins have a biological effect similar to KmTx-1 and KmTx-2

1. Introduction

Harmful microalgal blooms of the genus *Karlodinium* are responsible for mortalities of wild and cultured aquatic species worldwide. (Deeds et al. 2002; Kempton et al. 2002; Adolf et al. 2007; Place et al. 2012) *Karlodinium* are small marine dinoflagellates (~ 8-12 μm) belonging to the family Kareniaceae and commonly found in coastal aquatic ecosystems (Goshorn et al. 2004; Bachvaroff et al. 2008; Place et al. 2012) where they produce outbreaks regularly (reviewed in Place et al. (Place et al. 2012)). Frequently *Karlodinium* is present at relatively low cell abundances (10^2-10^3 cell mL^{-1}) but can form dense blooms (Deeds et al. 2002, 2006) (10^4-10^5 cell mL^{-1}). Exposure to high *Karlodinium*'s cell densities has been shown to elicit a response in fish gills including increased ionic permeability, oedema, hyperplasia, and epithelial necrosis. (Ulitzor and Shilo 1966; Jones K. et al. 1982)

Some species of the genus *Karlodinium* produce a class of ichthyotoxins called karlotoxins (KmTxs). Several of them have been identified. (Takeshita et al. 2000; Van Wagoner et al. 2008, 2010; Place et al. 2012; Waters et al. 2015; Cai et al. 2016; Krock et al. 2017) These are large (molecular weight >1000 Da), lipophilic compounds whose ecological role could be related to chemical defence against grazing and/or their use for prey acquisition's. (Adolf et al. 2007; Waggett et al. 2008; Place et al. 2012) In the Table 1, the isolated congeners studied to date have been included with their corresponding bioactivities. KmTxs have

been characterized either by their hemolytic *in vivo* or *in vitro* activity and have been claimed to kill fish by damaging their gill epithelia. (Deeds et al. 2002, 2006, 2015; Kempton et al. 2002; Bachvaroff et al. 2009) The potency of the KmTx congeners, regarding hemolytic activity, depends greatly on the congener although different studies have also shown significant variability. For instance, the range of EC_{50} is 47-5245 ng mL^{-1} for KmTx-1 and sulfo-KmTx-10, respectively. It should be noted that comparisons based on these EC50s must be carefully made since different species and methodology were used. In addition, these studies were performed using toxin preparations at varying stages of purification (no reference standards are available for this toxin group). In the case of KmTx-2 (as in others), the observed EC_{50} range for the same hemolytic assay was 368-1768 ng mL^{-1} (erythrocytes of *Oncorhynchus mykiss*). The most studied analogues are KmTx-1 and KmTx-2. (Deeds et al. 2002; Kempton et al. 2002) *In vivo* studies have been conducted almost exclusively with these two congeners, with KmTx-2-1 being the only exception. (Adolf et al. 2007; Mooney et al. 2010) Rapid morphological changes in zebrafish larval epithelia have been evidenced when exposed to high doses of KmTx-2 (4 µg mL^{-1}) presenting mortality within the first 15 minutes of exposure, and they also manifest in intense cell swelling and epithelial detachment in exposed surfaces. (Deeds et al. 2006) However, epithelial damage can be observed at a much lower concentration (Deeds et al. 2006) (EC_{50} = 800 ng mL^{-1}). The specific action mechanisms of KmTxs are not completely known but it has been suggested that KmTxs could act by forming pores in the cell membranes by binding to membrane lipids. Since different cell lines and animals are sensitive to these toxins, the pore-formation process is probably not initiated by a very specific lipid-binding phenomenon. Notwithstanding, it has been shown that the lytic effect (and self-protection) is dependent on the content or type of sterols in the target membranes. (Mooney et al. 2009; Rasmussen et al. 2017) Previous studies showed that KmTx-2 sub-lethal doses produce an *in vitro* increase in the permeability of the plasma membrane to certain cations (Na^{2+}, Ca^{2+}, and Mn^{2+}). (Deeds et al. 2015) This pre-lytic action could initiate an apoptotic-like pathway leading to severe damage in the gill´s epithelia. (Rasmussen et al. 2017) The secondary necrosis of apoptotic cells could be regulated by the increase of cations such as calcium and the formation of pores in the plasma membrane. Since some ichthytoxins have been shown to trigger apoptosis (Qi et al. 2016), the molecular mechanisms underlying the alteration of the osmotic balance, oxidative stress or the loss of membrane's functionality, among others, are relevant for the understanding of the risk of sub-lethal exposure. (Zhang et al. 2018)

Table 1
Bioactivities of the isolated karlotoxins.

KmTx congener	Strain	Geographic origin	Hemolytic activity EC_{50}	*in vivo* effects	*in vitro* EC_{50} cancer cell	Reference
KmTx-1	CCMP 1974 CCMP 1975	Chesapeake Bay, USA	284 ng mL^{-1} (EF1)	*D. rerio, Cyprinodon variegatus* (post-hatched larvae); Mortality and epithelial damage.	2000 ng mL^{-1}	[2] Deeds et al., 2002
KmTx-1	CCMP 1974	Chesapeake Bay, USA	na	*Danio rerio* (post-hatched larvae) EC50= 800 ng mL^{-1}, epithelial damage.	na	[7] Deeds et al., 2006
KmTx-1	CCMP 2936	Delaware, USA.	47 ng mL^{-1} (EH)	na	na	[15] Van Wagoner et al., 2008
KmTx-1		Chesapeake Bay, USA	82 ng mL^{-1} (EF1)	na	na	[4] Place et al., 2012
KmTx-2	CCMP 2282	South Carolina, USA	368 ng mL^{-1} (EF1)	*Danio rerio* (post-hatched larvae EC50= 800 ng mL^{-1} - juvenile EC50 ≈ 400 ng mL^{-1}, gill damage)	na	[7] Deeds et al., 2006
KmTx-2	KVHU01	Huon River, Australia	343 ng mL^{-1} (EF1)	na	na	[20] Mooney et al., 2009
KmTx-2	KVHU01	Huon River, Australia	na	*Cyprinodon variegatus* (larvae) EC50= 508.2 ng mL^{-1}	na	[19] Mooney et al., 2010
KmTx-2	CCMP 2064	Georgia, USA	na	*Danio rerio, Cyprinodon variegatus* (juvenile), mortality, and gill damage.	na	[27] Peng et al., 2010
KmTx-2		Chesapeake Bay, USA	1768 ng mL^{-1} (EF1)	na		[4] Place et al., 2012

KmTx congener	Strain	Geographic origin	Hemolytic activity EC_{50}	*in vivo* effects	*in vitro* EC_{50} cancer cell	Reference
KmTx-2	010410-C6	South Carolina, USA	EF1 (hemolytic activity)	na	na	[18] Deeds et al., 2015
KmTx-2	CCMP2778	Longboat Key, Florida USA	1988 ng mL^{-1} (EF2)	na	na	[10] Krock et al., 2017
KmTx-2-1	KVSR01	Swan River, Australia	66 ng mL^{-1} (EF1)	na	na	[20] Mooney et al., 2009
KmTx-2-2	KVSR01	Swan River, Australia	63 ng mL^{-1} (EF1)	na	na	[19] Mooney et al., 2010
KmTx-2-1, KmTx-2-2	AUS#7	Swan River, Australia.	EF1 (hemolytic activity)	*Cyprinodon variegatus* (juvenile) mortality.	na	[1] Adolf et al., 2015
KmTx-2-1	KVSR01	Swan River, Australia	na	*Cyprinodon variegatus* (larvae) EC50= 563.2 ng mL^{-1}	na	[19] Mooney et al., 2010
KmTx-3	CCMP 2936	Delaware, USA.	158 ng mL^{-1} (200 nM) (EH)	na	na	[11] Van Wagoner et al., 2010
KmTx-3		Chesapeake Bay, USA	188 ng mL^{-1} (EF1)	na	na	[4] Place et al., 2012
KmTx-8	AUS#7	Swan River, Australia,	49 nM (EF1)	na	1064 nM.	[14] Waters et al., 2015
KmTx-9	KDCSO15*	Southern Ocean, Australia	3000 nM (EF1)	na	na	[14] Waters et al., 2015
65-E-chloro-KmTx-1	CCMP 2936	Delaware, USA.	56 nM (EH)	na	na	[11] Van Wagoner et al., 2010

KmTx congener	Strain	Geographic origin	Hemolytic activity EC_{50}	*in vivo* effects	*in vitro* EC_{50} cancer cell	Reference
10-0-sulfo-KmTx-1	CCMP 2936	Delaware, USA.	30 nM (EH)	na	na	[11] Van Wagoner et al., 2010
64-E-chloro-KmTx-3	CCMP 2936	Delaware, USA.	110 nM (EH)	na	na	[11] Van Wagoner et al., 2010
10-0-sulfo-KmTx-3	CCMP 2936	Delaware, USA.	2400 nM (EH)	na	na	[11] Van Wagoner et al., 2010
4,5-dihydro-KmTx-2	GM2	East China Sea	997 ng mL^{-1} (EF2)	na	15mM	[13] Cai et al., 2016
4,5-dihydro-dechloro-KmTx-2	GM2	East China Sea	943 ng mL^{-1} (EF2)	na	36mM	[13] Cai et al., 2016
sulfo KmTx-10	K10, E11	Ebro Delta Bays, Spain	5245 ng mL^{-1} (EF2)	na	na	[10] Krock et al., 2017
EF1: Erythrocytes of Fish (*Oncorhynchus mykiss*) / EF2: Erythrocytes of Fish (*Sparus aurata*) / EH: Erythrocytes of Human / na: data not available / **K. conicum*						

Strains of *Karlodinium veneficum* showed high chemical variability regarding KmTxs composition, although in some cases it could be influenced by growth conditions as well. (Krock et al. 2017) In the cited work, genetic comparison was carried out for species and strain identification based on ITS and LSU rDNA sequences. (Krock et al. 2017) Most of the characterized strains showed higher toxicity in their culture supernatants than in the cell extracts. This would suggest that KmTxs are released to the environment in natural conditions. However, certain amounts of toxins can also be released due to mechanical stress during filtering or centrifugation. Thus, it appears that KmTxs may act as allelochemicals related to chemical defense against grazing and/or in prey acquisition. It has been suggested that the length of the lipophilic arm is an important determinant of potency, and that sulfation and chlorination could also influence the damage on biological membranes. (Place et al. 2012; Adolf et al. 2015) There is little information however on the effects of KmTxs at sublethal concentrations on species that cohabit in the water column, their potential bioaccumulation in these, and on human health (due to consumption of contaminated fish).

Recent investigations have strengthened the utility of early-stages *Danio rerio* in marine microalgae toxicity's evaluation; either to evaluate changes in toxicity induced by different nutrients conditions (*Alexandrium tamarense* (Guan et al. 2018)) or in response to a future climate change conditions such as ocean acidification and solar ultraviolet radiation (*Karenia mikimotoi* (Wang et al. 2019)). In addition, as mentioned above, this animal model had already been used to evaluate *Karlodinium* extracts toxicity (employing early-larvae and juveniles). (Deeds et al. 2002, 2006; Peng et al. 2010) In this study, our interest was to evaluate and compare the toxic effect of sublethal concentrations of *K. veneficum* K10 supernatants' extracts containing five novel putative karlotoxins (cand. KmTx-10, cand. KmTx-12, cand. KmTx-13, cand. KmTx-11 and cand. sulfo-KmTx-10; “cand.” stands for candidate) and contribute to the knowledge of the potential effects of this type of ichthyotoxins on the development of fish species in the natural environment.

2. Materials And Methods

2.1. Culture of Karlodinium Strains

K. veneficum (strain K10) was obtained from the Culture Collection of Harmful Microalgae of IEO, Vigo, Spain. This strain was isolated from an embayment of the Ebro Delta (NW Mediterranean). Inocula were grown at 18 ± 1 ºC in a 12:12h light-dark regime. These cultures were maintained in L1 medium (Guillard and Hargraves 1993) prepared in natural seawater (conductivity of 55 mS) previously sterilized by filtration (0.22 µm). For cultivation, a transparent bubble column photobioreactor (methyl-methacrylate 3.9 mm thick) was used. The column was 8.7 cm in diameter and the liquid height without gas (H) was fixed at 175 cm. It was inoculated with cells in a stationary phase at an initial concentration of 245,666 cells mL^{-1}. The column was kept illuminated with 58 W fluorescent lamps which allowed for an average irradiance of 200 µE s^{-1} m^{-2} in the bioreactor without any biomass. At harvest time (stationary phase) the culture had a concentration of 1,566,000 cells mL^{-1}. An aliquot of 50 mL was preserved in Lugol’s iodine solution for determination of cell abundance by microscopic counting. Cell density was determined by settling Lugol’s iodine-fixed samples and counting of >100 cells under an inverted microscope.

2.2. Preparation of K. veneficum supernatant extract

A detailed procedure is described elsewhere. (Krock et al. 2017) Briefly, the culture´s supernatant was passed through Solid Phase Extraction (SPE) cartridges (LC-18 Supelclean, Sigma-Aldrich, Deisenhofen, Germany). Cartridges were washed with deionized water and 50% aqueous methanol. KmTxs were then eluted with 100% methanol. The methanolic extract was subsequently dried in a gentle N_2 stream and kept at -80 ºC until use. From dry and homogeneous samples of *K. veneficum* supernatant´s extract (100% MeOH), 10 mg were weighted and then diluted in 500 µL of DMSO. A stock solution of 20,000 mg L^{-1} was obtained. After some preliminary zebrafish bioassays, a supernatant extract concentration range of 0.03 to 6.25 mg L^{-1} (final concentration in assays) was evaluated.

2.3. KmTx determination

A dry aliquot of *K. veneficum* supernatants' extract was reconstituted in 500 µL methanol, and the suspension was filtered by centrifugal filtration (Merck Millipore, Ultrafree MC HV, Durapore PVDF 0.45 µm, Eschborn, Germany) for 5 min at 16100 x g (Centrifuge 5415 R, Eppendorf, Hamburg, Germany). KmTxs were determined by liquid chromatography (LC 1100 chromatograph, Agilent, Waldbronn, Germany) coupled to tandem-mass spectrometry (API 4000 QTrap, Sciex, Darmstadt, Germany) in the selected reaction monitoring (SRM) mode. (Krock et al. 2017) All KmTxs were calibrated against an external standard solution of KmTx-2 (23 ng μL^{-1}; provided by Allen Place, University of Maryland, USA), and KmTx abundances were expressed as KmTx-2 equivalents.

2.4. Haemolysis assay

Hemolysis assays were performed by measuring hemoglobin released from sheep erythrocytes according to the Riobó et al. protocol. (Riobó et al. 2008) Defibrinated sheep blood was used at a concentration of 45 x 10^6 erythrocytes per well. Different volumes of the various samples assayed were partially air-dried and then placed in a microtiter's microwell plate to complete the drying. After blood was added, microwell plates were then incubated for 24 hours at 37°C. Mediterranean Sea water served as negative controls (filtered and sterilized with a salinity of 55 mS). A positive control with distilled water (100% lysis; 100% effect) was also included. An EC_{50} value was obtained. For comparative purposes saponin was used. All hemolytic assays were carried out in triplicate.

2.5. Danio rerio maintenance and eggs collection

To obtain eggs for each of the bioassays, zebrafish were reared under standard conditions in the Marine Biotechnology Laboratory of the Faculty of Natural and Oceanographic Sciences (Chile). Photoperiod of 14L:10D and temperature of 27±1°C was kept. The fish were fed 3 times a day with commercial flake food and once a day with live *Artemia nauplii*. The eggs for the bioassays were placed in plates with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$ prepared in distilled water) and kept at 26.5°C in a culture chamber.

2.6. Evaluation of K. veneficum supernatant's embryotoxicity in zebrafish (Danio rerio)

For these bioassays, 96-well plates with a final volume of 200 µL were used. Bioassays were carried out with the extract obtained from *K. veneficum*´s supernatant with final concentrations of 0.03, 0.3125, and 3.125 mg L^{-1} (total KmTxs expressed as equivalent KmTx-2 were calculated; See Table 3). In addition, a negative control (E3 medium) was run. Assays consisted of three replicates with 5 eggs in the gastrulation phase. Eggs were monitored to quantify different endpoints: A) Percentage of mortality up to 48 hours post-fertilization when the embryonic development was finished; B) Percentage of hatching success at 76 hours post-fertilization; and C) Percentage of larval anomalies at 76 hours post-fertilization. A yolk sac larva is considered alive when heartbeat was observed. This experiment was repeated three times with different egg batches.

Table 2
Analysis of karlotoxins* in the K. veneficum supernatant

	KmTx-10	KmTx-12	KmTx-13	KmTx-11	sulfo-KmTx-10
KmTx in supernatant extract (ng mg^{-1})	8.44	337.62	22.96	5.67	61.69
KmTxs exudated per cell (fg $cell^{-1}$)	0.305	12.19	0.829	0.205	2.23
Percentage (%)	1.93	77.4	5.3	1.3	14.1

(*)KmTxs were expressed as equivalent KmTx-2

Table 3
Assays conducted in wells for supernatant embryotoxicity on Danio rerio's evaluation.

Supernatant extract's assay concentration Assay (mgL^{-1})	Volume of supernatant extracted (mL)	Total KmTx Content (pg)	Total KmTx concentration in assay (KmTx $well^{-1}$) expressed as KmTx-2 equivalents.	
			($mg\ L^{-1}$)	($\mu g\ L^{-1}$)
0.03	0.52	$1.29\ 10^{-2}$	$1.29\ 10^{-5}$	0.01
0.3125	5.4	$1.35\ 10^{-1}$	$1.35\ 10^{-4}$	0.13
3.125	53.9	1.35	$1.35\ 10^{-3}$	1.35
6.25	107.8	2.69	$2.69\ 10^{-3}$	2.69

2.7. Lethal and sublethal effects of K. veneficum´s supernatant in zebrafish larvae

Deeds et al. (Deeds et al. 2006) showed that zebrafish larvae exposed to karlotoxins showed damage at the epithelial level. On this background, an experiment was set up to observe possible lethality and sublethal damage in larvae exposed to supernatant extract. 8 days post-fertilization larvae (yolk sac depleted) were used. These were exposed for 24 hours to *Karlodinium* supernatant extracts and E3 medium (negative control). After the incubation time, the number of alive larvae was quantified and they were individually mounted on an excavated slide to identify changes in the epithelium (Stereomicroscope Stemi DV4, Zeiss 32X).

Additionally, with the interest of analysing sublethal mechanisms potentially associated with the induction of apoptosis, an *in vitro* test was implemented. Thus, induction of apoptosis in larvae exposed to different supernatant concentrations was quantified for the sub-lethality range. Exposure time was 21 hours with the positive control (2% v/v ethanol; (Félix et al. 2014) and negative control (E3 medium). For each treatment, a pool of 5 larvae was used. Larvae were disaggregated following the protocol described

by Chan and Cheng (Chan and Cheng 2003) with modifications. The suspension of cells was fixed, then centrifuged and resuspended in PBS. Then cells were assayed with two commercial kits for cytometric analysis: 1) Cell Viability Stain (FDA) and 2) Muse Annexin V & Dead Cell Kit assays (Merck Millipore). The samples were analysed in the Muse Cell Analyzer (Merck Millipore). As suggested in Garcia-Käufer et al., (Garcia-Käufer et al. 2014) the sizes of the cell populations were standardized in the range of 1.1-1.2 s. The samples counted on pseudo-replicates for each treatment. This experiment was repeated twice with different larval batches.

Rearing, handling, and experimental work with zebrafish embryos and larvae were carried out under protocols approved by the University of Concepcion's Bioethics Committee and following internationally-established procedures.

2.8. Statistical analysis

The data analysis was performed with STATISTICA 7. Tests were carried out to determine the homogeneity of variance and to evaluate normality. ANOVA was used for parametric data. For the analysis of non-parametric data, the Kruskal-Wallis test was used.

3. Results And Discussion

3.1. KmTx composition of K. veneficum supernatant

Prior to the toxicity assays, the K10 *K. veneficum*'s supernatant extract was analysed for KmTx composition and revealed a very similar composition with 77.4% KmTx-12 and 14% sulfo-KmTx-10 (Table 2). The KmTx profile of *K. veneficum* strain K10 was first described by Krock et al. recently. (Krock et al. 2017) Strain K10 was found to produce four novels KmTxs (KmTx-10, -11, -12, -13, and a sulfated form of KmTx-10) with KmTx-12 being the most abundant variant (85% relative abundance) followed by sulfo-KmTx-10 with 10%. All other variants were trace compounds below 5% relative-abundance.

Taking into account that in the cited work it was determined KmTx composition in cell extracts and, in this study, KmTxs were determined in the cell-free *K. veneficum*´s supernatant, the similarity of both results indicates that different KmTxs are exuded at the same rate so that intra- and extracellular KmTx compositions are almost identical. Total KmTxs were 25 μg L^{-1} in the supernatant. In addition, in Table 1 it has been calculated the contribution of each cell to the total KmTxs in the supernatant, which corresponded to 15.75 fg $cell^{-1}$ of total exudated karlotoxins (in KmTx-2 equivalents)

3.2. Culture and hemolytic activity

K. veneficum was cultured in a 10 L bubble column photobioreactor with continuous illumination. Culture conditions are described elsewhere. (López-Rosales et al. 2016) To attain a long-stable stationary phase, after 11 days of batch mode growth, concentrated stocks of the medium nutrients were added (fed-batch). The final cell concentration was around 1.5 10^6 cell mL^{-1} (Figure 1A). In this fed-batch phase, four samples were taken to assess the biomass bioactivity. The saponin equivalent hemolytic effect on

erythrocytes (ESP, equivalent saponin potency) was taken as a proxy of the total content of KmTxs. (Krock et al. 2017) As shown in Fig. 1A, ESP kept constant from day 12 to 18 (the pseudo-stationary phase where nutrients were added in fed-batch mode). An amount of 25 µg of KmTx-2 equivalent was obtained per liter of supernatant. In Fig. 1B percentage of hemolysis *vs* concentration of *Karlodinium´*s supernatant extract was plotted. These data allowed us to obtain EC_{50}s values. The calculated cells' EC50 was approximately $6{\cdot}10^4$ cells. Thus, each *K. veneficum´*s cell showed an equivalent hemolytic activity of 150 pg saponin. This is much lower (one order of magnitude) than the values obtained previously for the same species. (López-Rosales et al. 2016) Different hemolytic activity of the different KmTx congeners is probably responsible for this (See Table 1). For instance, EC_{50} for hemolytic assays conducted with different KmTxs congeners ranged from 47 to 5245 ng mL^{-1} for KmTx-1 and sulfo-KmTx-10, respectively (Table 1). KmTxs in the supernatant studied here showed an EC_{50} of 990 ng mL^{-1}. KmTx-12 of K10 strain used in this work accounted for almost 80% of total KmTxs, similarly to the previous results. (Krock et al. 2017) However, López-Rosales et al., found that the same strain the main congeners were KmTx-10 and sulfo-KmTx-10. (López-Rosales et al. 2016) On the other hand, one mL of supernatant showed the same hemolysis as 1.13 micrograms of saponin. This value is very similar to those previously reported for this species. (López-Rosales et al. 2016) In this previous study, toxin analysis was not performed on the supernatant but the hemolytic analysis was performed on different fractions of biomass and collected from a C18 chromatography column. Their results suggested differences in hydrophobicities of the KmTxs from biomass and exudates. (López-Rosales et al. 2016) In the present study, different KmTxs supernatant concentrations were obtained as was discussed in the previous epigraph showing that KmTx-12 was dominant.

3.3. Embryotoxicity of K. veneficum supernatant

In our experiments, it was observed that the supernatant's toxins induced 100% of embryonic mortality at 15 min of exposure at concentrations ≥2.69 µg L^{-1} KmTx-2 equivalent (Figure 2). Detailed observation of the eggs exposed to 2.69 µg L^{-1} KmTx-2 equivalent, showed an arrest of the epiboly process after a few minutes. The total disintegration of both the blastoderm and the group of cells that started the migration over the yolk was observed (Figure 3). Finally, a coagulated egg state was evident (See Figure 3H).

In the concentration range of 0.01-1.35 µg L^{-1} KmTx-2 equivalent, no embryonic mortality was observed up to 48 hours post-fertilization; but there was an alteration of the embryo's pigmentation developing. These were almost translucent in comparison with those of the control condition (Figure 4). During the hatching-starting process with a 3.125 mg L^{-1} (1.35 ug mL^{-1}) treatment, late embryonic mortality was observed thus leading to a lower hatching percentage. This percentage was significantly lower than the one quantified for KmTx-2 equivalent concentrations lower than 1.35 ug mL^{-1} (66.7%; K-W test $p < 0.05$; multiple comparisons $p < 0.05$; Figure 2). Additionally, in recently hatched larvae there were anomalies like pericardial oedema and lordosis with different degrees of severity. Both types of larval anomalies occurred in the same high proportion and simultaneously in a high percentage of larvae (87%). Similar teratogenic effects have been described in zebrafish embryos exposed to toxic compounds produced by

marine microalgae. Thus, marine toxins have been shown to produce embryo coagulation, oedema, and development retardation; and for recently hatched larvae, yolk sac oedema, and bent spine. (Guan et al. 2018; Wang et al. 2019) However, Von Hellfeld et al. showed that some of this development anomalies, were seen after exposure to a wide variety of chemical compounds. (von Hellfeld et al. 2020) For example, formation of oedema was observed with any of the 45 substances tested in this research. Therefore, oedemata in zebrafish appear to be of very little mechanistic value and should be categorised as an unspecific side effect of both acute and sublethal toxicity.

3.4. Lethal and sublethal effects in zebrafish (Danio rerio) larvae, exposed to K. veneficum supernatant

Concentrations higher than 2.69 µg L^{-1} KmTx-2 equivalent were lethal for early larvae (8 days post-fertilization). A 100% larval survival after 24 hours of exposure was observed at concentrations lower than or equal to 1.35 µg L^{-1} KmTx-2 equivalent. However, a larvae's decrease in swimming activity was observed 1.35 µg L^{-1} KmTx-2 equivalent, as these were perched at the bottom of the wells. Detailed observation under a microscope showed larval epithelium's damage, being more evident in the lateral-caudal area of the embryonic fin and its intensity was greater than the one observed in larvae under the positive control condition (2% ethanol; Figure 5).

Although fourteen karlotoxins congeners have been described until present (see Table 1), ichthyotoxicity has been evaluated considering post-hatch larvae (yolk-sac larvae or eleuteroembryo) and juveniles of zebrafish and sheepshead minnow, in just 30% of total congeners. Even though the toxins´ effects vary according to life stage and species considered, the available information indicated a higher potency for KmTx-1 and KmTx-2, than KmTx-2-1 or KmTx-2-2 (See Table 1). According to our results, both embryotoxicity and larval mortality indicated that supernatant of *K. veneficum* strain K10 has a higher potency than that reported for KmTx-1 and KmTx-2 (800 and 400-800 µg L^{-1}, respectively; Table 1).

In the presence of KmTx-1 or KmTx-2, the effect of swelling and sloughing of the epithelium was reported both on fins and gills of the fish exposed. (Deeds et al. 2002, 2006; Peng et al. 2010) It can be related to the capacity of karlotoxins to form pores in the cell membranes by binding to membrane lipids. Deeds et al. (Deeds et al. 2006) reported generalized necrosis of the entire epithelial surface of larval zebrafish induced by both KmTx-1 and KmTx-2 at higher doses (≥1 µg L^{-1}). At lower concentrations, non-specific permeability of polyvalent cations such as Ca^{2+} and Na^{2+} was suggested. (Deeds et al. 2015) Alterations in osmotic balance could trigger apoptosis or other type of programmed cell death. (Zhang et al. 2018) To assess the previously described effect, we exposed early larvae (8 days post-fertilization) to different KmTx concentrations and evaluated the apoptosis induction by flow cytometry. Cell profiles showed an induction of apoptosis from the lowest KmTx-2 equivalent concentrations (0.01 µg L^{-1}). After comparing the percentages of cells in the apoptosis process (early + late), no statistically significant differences between treatments were found (Figure 6, ANOVA $p = 0.685$). However, it was observed that at increasing concentrations of KmTxs there was a decrease of living cells' percentage and a proportional increase of cells' percentage in the early-apoptosis stage, reaching an 80% in larvae exposed to 1.35 µg L^{-1} KmTx-2

equivalent. In larvae exposed to ethanol (positive control for induction of apoptosis), 39% of the cells were in late apoptosis or dead; a cell condition only observed in larvae exposed to 2.69 µg L^{-1} KmTx-2 equivalent which were already moribund at the end of the exposure time.

Deeds et al. (Deeds et al. 2006) observed severe degeneration of gill tissue including oedema, enlargement, and necrosis, with curling or loss of secondary lamellae in a detailed description of histopathological effects in fish exposed to the KmTx-2. These effects could be related to the capacity of KmTx-2 to induce cell lysis through colloid osmolysis, presumably through membrane permeability changes. (Deeds et al. 2015) Our results showed that karlotoxins produced by *Karlodinium veneficum* (strain K10) induced apoptosis in the range of sublethal concentrations. Apoptosis has been described as a toxic effect triggered by microalgae toxins such as okadaic acid (Dietrich et al. 2020), azaspiracids (Ferreiro et al. 2017), or cyanotoxins (microcystin (Zeng et al. 2014)). Recent studies on cyanotoxins showed that microcystin-LR induced apoptosis by activation of endoplasmic reticulum stress. (Qi et al. 2016) In addition, an adverse effect on zebrafish embryos' development in presence of nodularin was evidenced, which may be associated with oxidative stress and apoptosis through the activation of the P53-AX/BCL-2-CASPASE 3-mediated pathway. (Chen et al. 2020)

If it is considered that in a *Karlodinium veneficum* bloom, cell densities can reach 10^4-10^5 cells mL^{-1} (Place et al. 2012) and that each cell produces 15.75 fg of toxins (KmTx-2 equivalents) that are exuded to the medium; in the water column, there could be between 0.16 and 1.6 µg L^{-1} KmTx-2 equivalents. Similar concentrations were shown for seawater samples from *K. micrum*–associated fish kills. (Deeds et al. 2006) Our results indicate that in this range of concentrations both lethal (embryonic mortality) and sublethal effects (embryonic malformations, epithelial damage, and induction of apoptosis) are evidenced in the early stages of the species model *Danio rerio*. For apoptosis induction, concentrations as low as 0.01 µg L^{-1} were likely sufficient to trigger these processes in fish.

Declarations

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Data Availability

Original Data that support the findings of this study are avalaible on on request from the corresponding author (J.J.G.R.)

Animal Research (Ethics)

Rearing, handling, and experimental work with zebrafish embryos and larvae were carried out under protocols approved by the University of Concepcion's Bioethics Committee, and following internationally-established procedures.

Consent to Participate (Ethics)

not applicable

Consent to Publish (Ethics)

All co-authors are aware of this submittal and consent

Plant Reproducibility

not applicable

Clinical Trials Registration

not applicable

Author Contribution

Funding

All funding have been declared and included in the acknowledgement section

Conflict of Interest

we declare the following regarding conflict of interests

Declaration of interests

 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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: None

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Figures

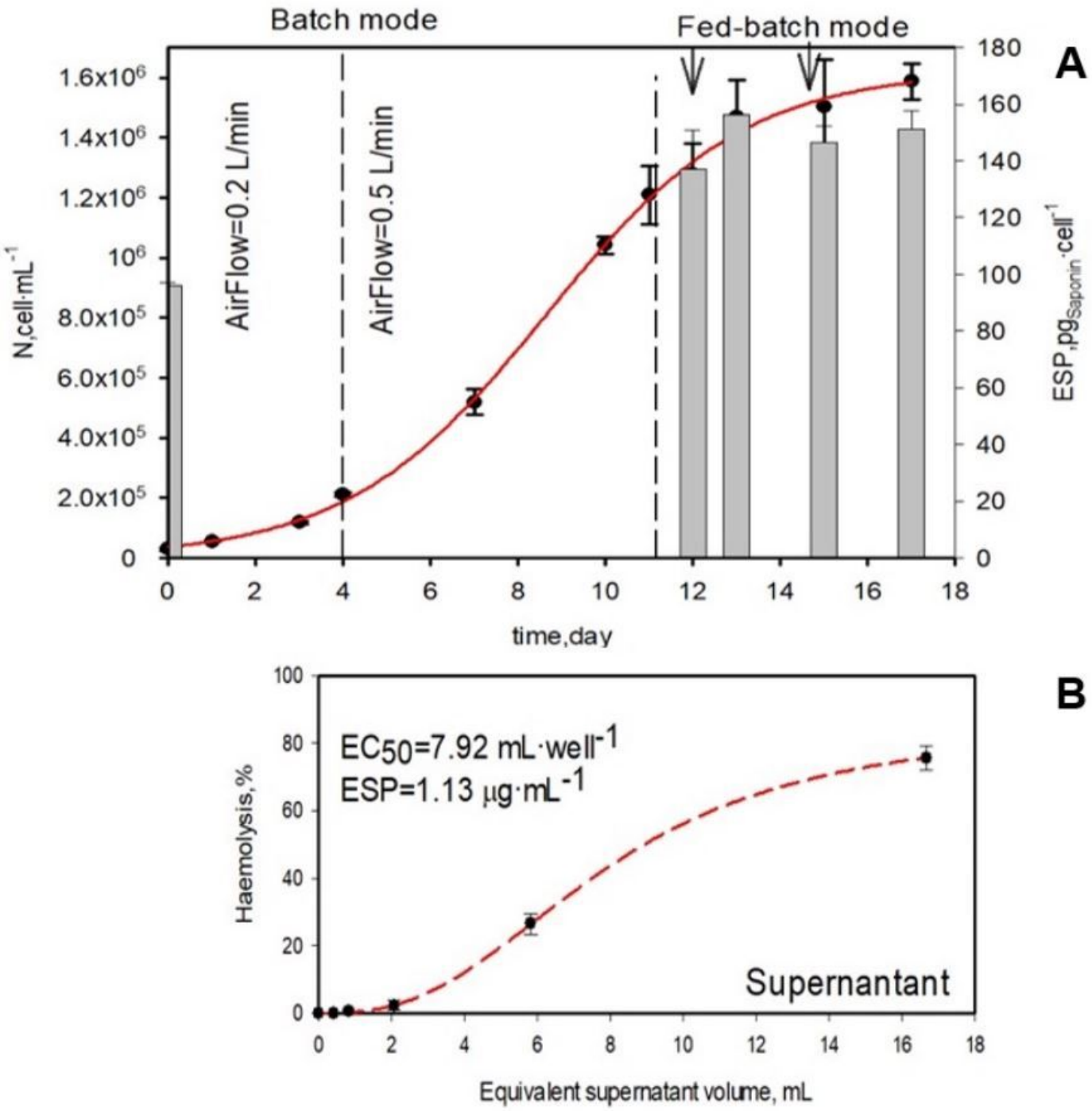

Figure 1

Karlodinium veneficum (K10) culture (A) and hemolytic activity of its supernatant (B).

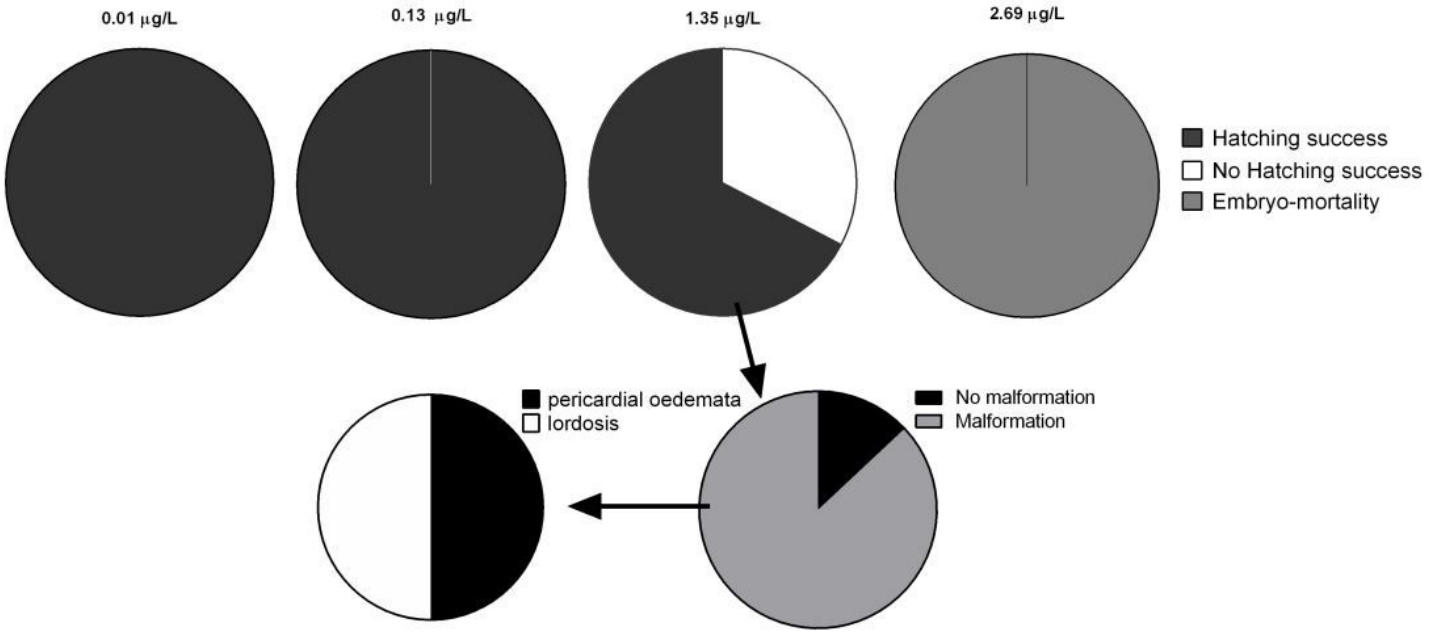

Figure 2

Effect on mortality, hatching, and presence of anomalies in zebrafish eggs exposed to K. veneficum supernatant extract. The amount expressed in µg L-1 corresponds to the concentration of the total supernatant extract KmTxs analysed by HPLC-MS and expressed as KmTx-2 equivalent.

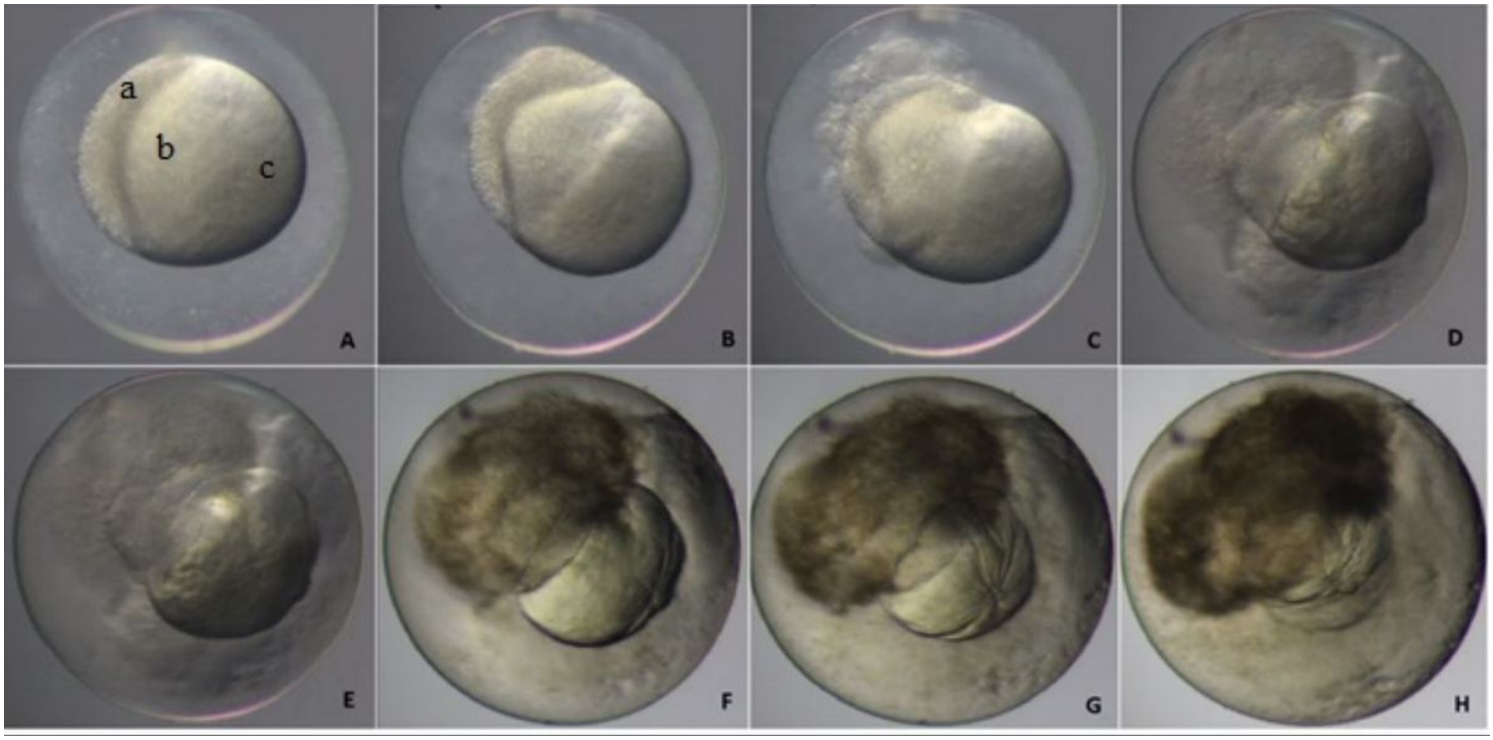

Figure 3

Zebrafish egg in gastrulation phase exposed to 2.69 ug L-1 KmTx-2 equivalent from K. veneficum supernatant. Images were captured every 2 minutes during 15 minutes of exposure (A to H). a: blastoderm, b: cell migration, c: yolk.

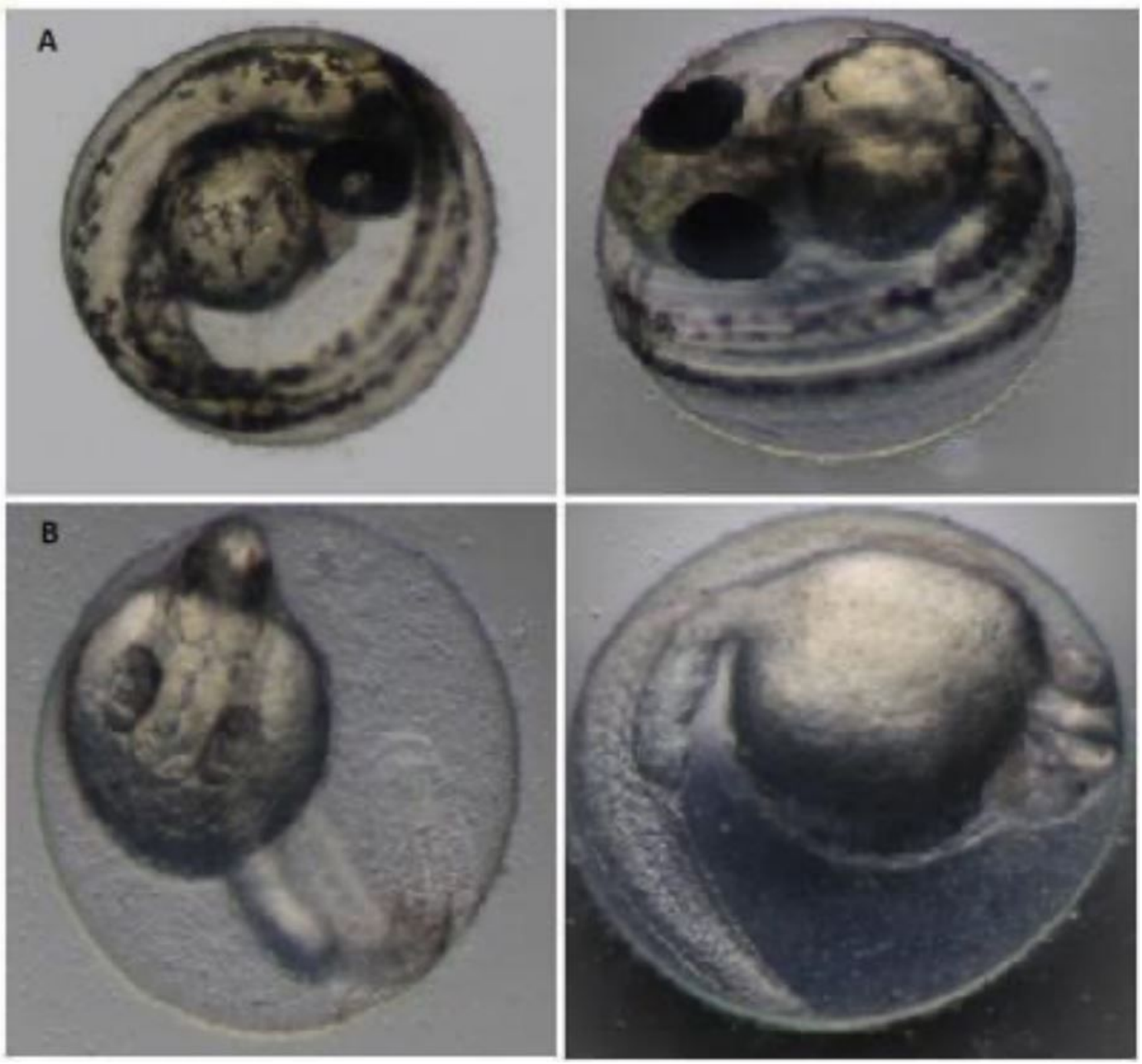

Figure 4

Advanced stage of zebrafish embryonic development (48 hours post-fertilization) A. incubated in control medium (E3 1X) and B. incubated in 1.35 ug L-1 of KmTxs expressed as KmTx-2 equivalent from K. veneficum supernatant (100% MeOH).

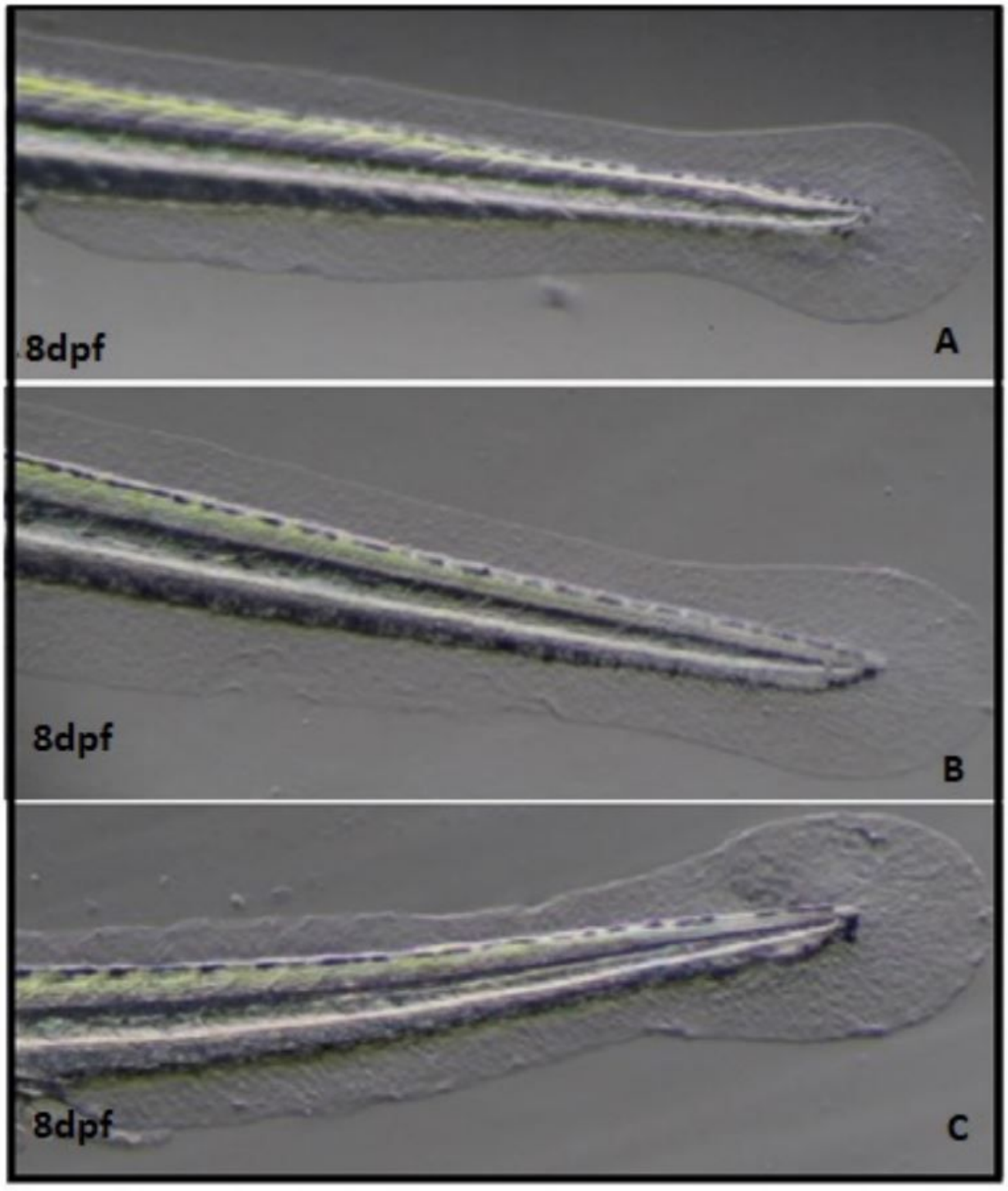

Figure 5

Detail of epithelial tissue on the caudal region of zebrafish larvae (8 days post-fertilization). A. Negative control (Medium control, E3 1X). B. Positive control (2% ETOH). C. 1.35 µg L-1 of KmTxs from K. veneficum supernatant (100% MeOH).

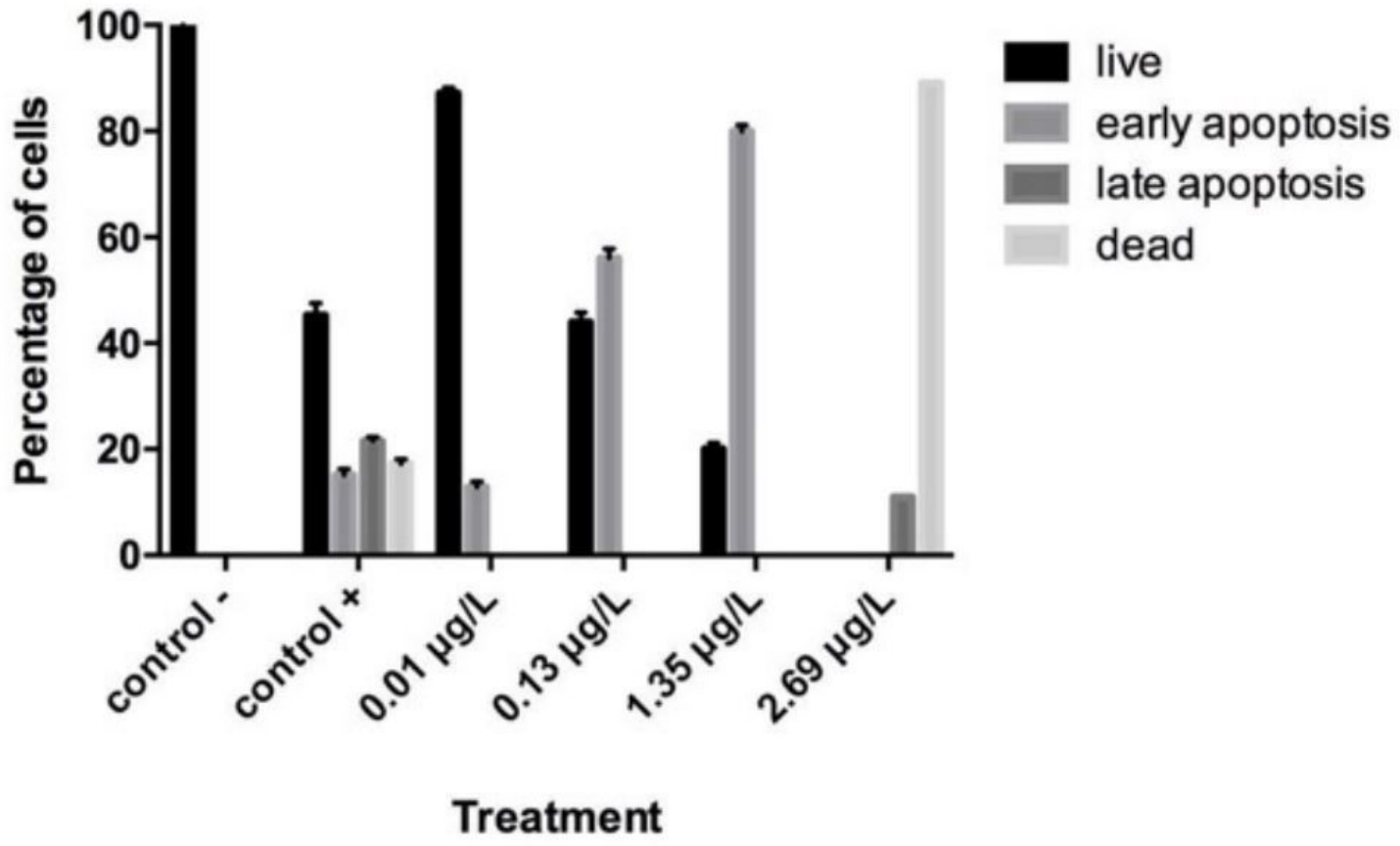

Figure 6

Percentage of live, death and apoptotic cells from disaggregated zebrafish larvae prior exposed to increasing concentrations of KmTxs from K. veneficum´s supernatant.

Supplementary Files

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