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Mortality and histopathology in sheepshead minnow (Cyprinodon variegatus) larvae exposed to pectenotoxin-2 and Dinophysis acuminata

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Abstract :

Toxic species of the dinoflagellate genus Dinophysis can produce diarrheic toxins including okadaic acid (OA) and dinophysistoxins (DTXs), and the non-diarrheic pectenotoxins (PTXs). Okadaic acid and DTXs cause diarrheic shellfish poisoning (DSP) in human consumers, and also cause cytotoxic, immunotoxic and genotoxic effects in a variety of mollusks and fishes at different life stages in vitro. The possible effects of co-produced PTXs or live cells of Dinophysis to aquatic organisms, however, are less understood. Effects on an early life stage of sheepshead minnow (Cyprinodon variegatus), a common finfish in eastern USA estuaries, were evaluated using a 96-h toxicity bioassay. Three-week old larvae were exposed to PTX2 concentrations from 50 to 4000 nM, live Dinophysis acuminata culture (strain DAVA01), live cells resuspended in clean medium or culture filtrate. This D. acuminata strain produced mainly intracellular PTX2 (≈ 21 pg cell−1), with much lower levels of OA and dinophysistoxin-1.

No mortality or gill damages were observed in larvae exposed to D. acuminata (from 5 to 5500 cells mL−1), resuspended cells and culture filtrate. However, exposure to purified PTX2 at intermediate to high concentrations (from 250 to 4000 nM) resulted in 8 to 100% mortality after 96 h (24-h LC50 of 1231 nM). Histopathology and transmission electron microscopy of fish exposed to intermediate to high PTX2 concentrations revealed important gill damage, including intercellular edema, necrosis and sloughing of gill respiratory epithelia, and damage to the osmoregulatory epithelium, including hypertrophy, proliferation, redistribution and necrosis of chloride cells. Tissue damage in gills is likely caused by the interaction of PTX2 with the actin cytoskeleton of the affected gill epithelia. Overall, the severe gill pathology observed following the PTX2 exposure suggested death was due to loss of respiratory and osmoregulatory functions in C. variegatus larvae.

Highlights

► Pectenotoxin-2 causes mortality of *Cyprinodon variegatus* larvae (≥ 250 nM after 96 h) ► Pectenotoxin-2 causes damage in gill epithelia, pillar cells and chloride cells ► *Dinophysis acuminata* does not cause mortality or pathologies in fish larvae ► Mortality may be due to respiratory failure and reduction of osmoregulatory ability

Keywords : Dinoflagellate, Fish, Gills, Harmful algal bloom, Histopathology, Pectenotoxin, Dinophysis

Introduction:

Dinoflagellates of the genus *Dinophysis* synthesize two groups of structurally distinct toxic polyethers, i.e. pectenotoxins (PTXs) and okadaic acid (OA) and its derivatives, dinophysistoxins (DTXs). Since an outbreak in Japan in the late 1970s (Yasumoto et al., 1978, 1980), this latter group of OA and DTXs is known to cause diarrheic shellfish poisoning (DSP) in human consumers of contaminated shellfish. Hence, these toxins are also referred to as diarrheic shellfish toxins (DSTs) (Dominguez et al., 2010). Symptoms of DSP include vomiting, nausea, abdominal pain and diarrhea (Yasumoto et al., 1985). In addition, accumulation of DSTs in cultivated shellfish causes shellfish farming closures and indirect economic loss for shellfish farmers (Trainer et al., 2020). Finfish mortalities were observed during a bloom of *Dinophysis caudata* (a known PTX2-producer; Basti et al., 2015; Fernández et al., 2006) in the Gulf of Thailand and in India (Okaichi, 1967; Santhanam and Srinivasan, 1996), but direct causality was not demonstrated. Unfortunately, since these events, only a few studies have investigated the effects of *Dinophysis* species (Rountos et al., 2019) and their toxins (Escoffier et al., 2007; Figueroa et al., 2020; Le Du et al., 2017; Souid et al., 2018) on fish, which is likely due to the complexities of *Dinophysis* culture and experimental manipulation as well as to the challenge in obtaining the large amounts of toxins required to conduct such experiments (Table 1). Those studies reported mortality in fish larvae, embryos and adults exposed to nM to µM concentrations of OA and DTX1 as well as developmental disorders, oxidative stress and pathologies in gills and digestive organs (Table 1), but no studies have focused on the effect of PTXs. In addition, only one study has focused on the effects of *Dinophysis* cells on fish larvae, and indicated that 1000 cells mL^{-1} of *D. acuminata* (producer of low amounts of OA and DTX1) caused a decreased growth rate in *C. variegatus* (Rountos et al., 2019; Table 1).

Looking for insight from toxicological research focused on other estuarine organisms, two studies addressed the potential adverse health effects of PTX2 to mollusks, i.e., two species of oyster. Recently, deleterious physiological effects and mortality in *Crassostrea gigas* gametes and *Crassostrea virginica* larvae exposed to PTX2 and PTX2-producers have been observed *in vitro* (Gaillard et al. 2020; Pease et al. 2022). Gaillard et al. (2020) showed an increase in ROS production after 2 h of exposure to 50 nM of PTX2 and an increase in mortality after 2 h of exposure to natural field concentrations (from 0.5 to 500 cells mL^{-1}) of *Dinophysis sacculus* as well as a decrease in the fertilization success. In addition, Pease et al. (2022) highlighted an increase in larval mortality when exposed for 24 h to 96 h to ca. 200 nM of PTX2, 1000 cells mL^{-1} of *D. acuminata* (strain DATC03, mainly a PTX2-producer) or a culture lysate (eq. 10000 cells mL^{-1}). Those cellular effects could ultimately affect the recruitment of both wild and farmed oysters, highlighting the importance for future research on PTX2 and PTX2-producers.

In general, phycotoxins can directly affect marine animals through exposure to phytoplankton or dissolved toxins, following ingestion or uptake through the gills (e.g. filter-feeding bivalves, planktivorous fish). Indirect exposure can also occur through the consumption of contaminated organisms (such as carnivorous fish, marine mammals and birds) (Landsberg, 2002). Filter-feeding bivalves, such as mussels or oysters, accumulate significant quantities of toxins due to their mode of nutrition but can metabolize them into less toxic forms (Blanco et al., 2018; Mafra et al., 2019). Similarly, filter-feeding fish such as small pelagic clupeiforms, are also vulnerable to acute and/or chronic toxicity due to ingestion of toxic phytoplankton (Landsberg, 2002; Lopes et al., 2019). Also, early life stages of fish (i.e. embryos and larvae) may be more sensitive to toxic phytoplankton and/or associated phycotoxins due to their immobility or low mobility compared to adult fish which could potentially escape contaminated waters (Wiegand et al., 1999).

The eurythermic and benthic sheepshead minnow (*C. variegatus*) is a prey fish and is therefore important from an ecological and economical point of view in Central and North East America (Bennett and Beitinger, 1997; Bushong et al., 1988; Hall et al., 1994). Moreover, spawning seasons of *C. variegatus* (i.e. spring-summer) are likely to overlap with bloom periods of several toxic species of the genus *Dinophysis* (Rountos et al., 2019). Indeed, in the lower Chesapeake Bay, and other estuaries located in the Atlantic coast of the United States, multiple toxic species of the genus *Dinophysis*, including *D. acuminata*, have been reported in monitoring programs since the 1960s, to occur from spring to fall (see references in Wolny et al., 2020), and more specifically in June (Marshall, 1982, 1980).

In the present study, a previously described 96-h larval fish bioassay using larval sheepshead minnows (Lovko et al., 2003; Vogelbein et al., 2002) was applied to investigate the effects of purified PTX2 and a cultured local PTX2-producing strain of *D. acuminata* (whole culture, live cells resuspended in clean medium and culture filtrate). After the measurement of cumulative mortality, tissue pathology was investigated using routine paraffin histopathology and transmission electron microscopy.

Materials and methods:

Microalgal cultures

The dinoflagellate *Dinophysis acuminata* (Claparède and Lachmann, 1859) (strain DAVA01) was isolated from Chesapeake Bay, Nassawadox, VA, USA in May 2017 (Ayache et al. submitted). This mixotrophic species was grown in f/2-Si medium (Guillard, 1975) prepared with 0.22-µm filter-sterilized natural seawater (FSW) obtained from Wachapreague, VA, at a salinity of 35, and diluted with GenPure water to reach a salinity of 25. The dinoflagellate was fed every two days with its ciliate prey *Mesodinium rubrum* (Lohmann, 1908) (strain JAMR-AB364286) at a predator:prey ratio of 1:5. The *M. rubrum* culture was fed twice a week with

the cryptophyte, *Teleaulax amphioxeia* (Conrad) Hill (Hill, 1992) (strain JATA-AB364287), at a predator:prey ratio of 1:10. Both *T. amphioxeia* and *M. rubrum* were isolated from Inokushi Bay, Oita prefecture, Japan in 2007 and grown in f/2-Si and f/6-Si media at salinity 25, respectively (Nishitani et al., 2008). All species were grown in batch culture and maintained in an incubator at 15 ± 1 °C, at a light intensity of ≈ 100 µmol photons m^{-2} s^{-1} on a 14:10 h (light:dark) cycle.

One week before the experiment, a stock culture of *D. acuminata* was filtered on a 10-µm nylon mesh sieve to remove cryptophytes and ciliates, gently rinsed with FSW and resuspended in fresh medium. The culture was then starved to ensure that *D. acuminata* cells remained in mid exponential growth phase prior to the experiment.

Fish larvae

Three-week old sheepshead minnow *Cyprinodon variegatus* (Lacépède 1803) larvae were purchased and delivered on the day of each experiment from Aquatic Biosystems (Fort Collins, CO, USA). They were fasted for 48 h prior to over-night delivery. There were no mortalities due to shipping and all animals appeared to be in good health upon arrival at VIMS.

Experimental design

96-h larval fish bioassay

The larval finfish bioassay was adapted from Vogelbein et al. (2002) and Lovko et al. (2003). Small (1-mL) glass beakers were filled with 0.5 mL (volume validated with pre-test experiment) of PTX2 or *D. acuminata* solutions (see details below). One fish larva was transferred per beaker using a disposable plastic pipette. Exposures were performed for 96 h in covered 48-well plates at room temperature (22 to 25 °C). Every 24 h, larvae were monitored for cumulative mortality evaluation under a binocular dissecting microscope or

were sampled and processed for routine paraffin histological and transmission electron microscopical (TEM) analyses (see details below). The calculation of LC_{50} was based on Hill coefficients (Hill, 1910) from dose-response curves (Prinz, 2010) using REGTOX macro on Excel™ (Vindimian, 2010).

Exposure of C. variegatus to *PTX2*

The effect of PTX2 at concentrations of 50, 250, 500, 750, 1000, 2000 and 4000 nM, as well as two controls (filtered seawater (FSW) and FSW + ethanol (EtOH) 0.8% final concentration) were investigated using the 96-h larval fish bioassay. Following a previously described process (McCarron et al., 2013) a purified (>98%) and accurately quantitated standard of PTX2 was prepared at the National Research Council Canada (NRCC, Halifax, NS, Canada). To prevent the deleterious effect of methanol on fish larvae observed in a preliminary experiment, the PTX2 standard was gently evaporated to dryness under nitrogen and then resuspended in ethanol. The PTX2 solutions were prepared by dilution of the stock solution with FSW at salinity 25. The lowest concentration of 50 nM of PTX2 approximately corresponded to the content (intracellular + extracellular) of 2000 cells mL^{-1} of *D. acuminata* (strain DAVA01) (Table 2). Higher PTX2 concentrations were tested as an increasing gradient to observe the effects on *C. variegatus* mortality and histopathology. For each condition, the replicates were n = 38, n = 8 and n = 2 for the cumulative mortality assessment, light microscopy and TEM, respectively.

Exposure of C. variegatus *to* D. acuminata

The *D. acuminata* strain (DAVA01) used in this study was locally isolated and known to produce predominantly PTX2 (Ayache et al. submitted). The following experimental were assayed for adverse effects on sheepshead minnow larvae in a 96-h bioassay: (i) whole cell *D. acuminata* culture; (ii) live filtered *D. acuminata* cells resuspended in clean f/2-Si medium;

(iii) culture filtrate, and; (iv) f/2-Si medium as the control. For treatment (i), a culture of *D. acuminata* was diluted in f/2-Si at salinity 25 to obtain cell concentrations of 5, 50, 250, 800, 2500 and 5500 cells mL^{-1}. This range of concentrations was used because it corresponds to reported field ranges from moderate and naturally occurring blooms of *Dinophysis* of 10^2-10^4 cells L^{-1} (Gaillard et al., 2020; Nagai et al., 2011) to rare, but exceptionally dense blooms of 10^5-10^7 cells L^{-1} (Belin et al., 2020; Reguera et al., 2012). Treatment (ii) was prepared by sieving 13 mL of live *D. acuminata* cells at 5500 cells mL^{-1} over a 10-µm mesh and resuspending them in 13 mL of clean f/2-Si medium. The filtrate obtained from the previous cell sieving process was further filtered using a 0.22-µm, 13-mm PVDF syringe filter (Millipore Sigma, Burlington, MA, USA) and then employed as treatment (iii), culture filtrate eq. 5500 cells mL^{-1} (i.e. extracellular toxins). Cumulative mortality was assessed with n = 14 per treatment and routine paraffin histology and transmission electron microscopy (TEM) with n = 8 and n = 2 fish, respectively.

Histology

Larvae destined for light microscopic evaluation were processed by routine methods for paraffin histology (Prophet et al. 1994). Briefly, fish larvae were euthanized by 15 min exposure (500 µg g^{-1} in FSW at salinity 20) to the finfish anaesthetic, tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO, USA). Fish larvae (n = 8 per condition) were then fixed and decalcified by immersion for at least 48 h in Bouin's solution, rinsed overnight in running tap water, and stored in 70% ethanol. Tissues were dehydrated through a graded ethanol series, cleared in xylene substitute and infiltrated overnight with warm melted paraffin in an automatic tissue processor (Shandon Hypercenter, ThermoFisher, Waltham, MA, USA). Tissues were then embedded in paraffin on a tissue embedding center (Tissue Tech, Sakura Finetek USA, Torrance, CA, USA), and resulting tissue blocks were sectioned at 5 µm on a rotary microtome (Olympus Cut 4055, Center Valley, PA, USA) and stained

with hematoxylin and eosin (H&E) in an automatic slide stainer (Shandon Varistain 24-3, ThermoFisher, Waltham, MA, USA). The slides were cover-slipped, oven-dried and histologically evaluated and photographed on an Olympus AX-70 photomicroscope.

Transmission electron microscopy (TEM)

For the TEM analyses, fish larvae (n = 2 per condition) were processed as described in Vogelbein et al. (2002). Briefly, fish were euthanized with tricaine methanesulfonate (MS-222: see above for Histology), and fixed in 4% glutaraldehyde with 5% paraformaldehyde in 0.1M sodium cacodylate (NaCac) buffer, pH 7.2, at 4 °C for 2 h. The samples were then washed in three changes of 0.1M NaCac buffer, for 15–30 min each, and stored overnight at 4-8 °C in a third change of buffer. The samples were post-fixed with 1% OsO_4 in 0.1M NaCac buffer, pH 7.2, at 4 °C for 1 h and then washed in three changes of 0.1M NaCac buffer, pH 7.2, for 15–30 min each. The tissues were then decalcified in 5% EDTA/0.1 M NaCac at 4 °C for 24 h, then dehydrated in a graded ethanol series, cleared in propylene oxide, and infiltrated in epoxy resin (Spurr's medium) over 4-5 days, and polymerized in a warming oven for 48 h at 58 °C. Tissue blocks were then sectioned on an ultra-microtome (Reichert Ultracut E, Leica, Vienna, Austria) at 70-90 nm using a diamond knife, with sections mounted on single hole, Formvar-coated copper grids and post stained with lead citrate and uranyl acetate. The sections were evaluated and photographed using a Zeiss EM 100 transmission electron microscope operated at 80 Kv.

Toxin analyses

The stock culture of *D. acuminata* was sampled at the beginning of the experiment for the characterization of intracellular and extracellular toxin content and concentrations. A 1-mL sub-sample (n = 3) of culture was centrifuged (3000 *g*, 15 min at 4 °C) to separate the culture into cell pellet and culture filtrate for intracellular and extracellular toxin extraction,

respectively. Intracellular toxins were extracted in 0.5 mL 100% methanol from the cell pellet using bath sonication (< 20 °C, 15 min, 25 kHz), followed by centrifugation (3000 *g*, 15 min, 4 °C) to separate out the cellular debris. The methanolic fraction (containing the intracellular toxins) was then filtered using a 0.22 µm, 13-mm (PVDF) syringe filter (Millipore, Sigma, Burlington, MA, USA) and stored in glass vial at -20 °C for later analysis. Toxins in the culture filtrate (extracellular) were extracted using a 60-mg Oasis HLB solid phase extraction (SPE) cartridge (Waters Inc., Milford, MA, USA). Toxins were eluted with two 0.5-mL aliquots of 100% methanol (pooled) and filtered using a 0.22-µm, 13-mm (PVDF) syringe filter (Millipore, Sigma, Burlington, MA, USA) and stored at -20 °C for later analysis. Toxin analysis was performed using a tandem quadrupole Xevo TQD MS system (Waters Inc., Milford, MA, USA) coupled to an electrospray ionization (ESI) source and ultra-performance liquid chromatography with a trapping dimension and at-column dilution (LC-MS/MS trap/ACD) (Onofrio et al., 2020). The chromatographic separation was carried out on a C18 column (100 Å, 2.6 µm, 50 × 2.1 mm, Waters, Milford, MA, USA). Transitions, chromatography, and mass spectrometer parameters are given in detail in Ayache et al. (submitted). Certified reference materials for dinophysistoxin-1 (CRM-DTX1-b), dinophysistoxin-2 (CRM-DTX2-b), okadaic acid (CRM-OA-d) and pectenotoxin-2, (CRM-PTX2-b), were obtained from the NRCC (Halifax, NS, Canada) and used for external calibration. The limit of quantification (LOQ) were: OA, 0.06 ng mL^{-1}; DTX1, 0.35 ng mL^{-1}, and; PTX2, 0.17 ng mL^{-1}.

Statistical analyses

Statistical analyses were performed using RStudio. Pearson's Chi-square tests were computed on the cumulative mortality observations, with random, exclusive (dead or alive) and independent data with expected values > 10 (Mchugh, 2013). Then, a *post hoc* test was

performed on adjusted residuals of the Pearson's Chi-square test with Bonferroni correction (MacDonald and Gardner, 2000).

Results:

Toxins of D. acuminata

The intracellular toxin profile of the *D. acuminata* strain DAVA01 was mainly composed of PTX2 (21 ± 0.40 pg cell^{-1}), representing > 95% of the total toxins and to a much lesser extent by DTX1 (1.2 ± 0.64 pg cell^{-1}) and OA (0.17 ± 0.07 pg cell^{-1}) (Table 2). Pectenotoxin-2 was only detected at trace level in the extracellular fraction (<5% of total PTX2). The maximum cell concentration of *D. acuminata* used in the culture exposure experiment was 5500 cells mL^{-1} and corresponded to 140 ± 3.20 nM of intracellular PTX2 (Table 2).

Cumulative mortalities

Exposure of *C. variegatus* to PTX2 showed dose- and time-dependent responses (Figure 1). Exposure to 2000 and 4000 nM of PTX2 induced 100% mortalities in fish larvae in 24 h (P < 0.001), whereas 1000 nM of PTX2 caused only 3% mortality. However, after 48 h of exposure to 1000 nM of PTX2, significant mortality (63%; P < 0.05) was observed in fish larvae which increased to 100% mortality after 72 h of exposure (P < 0.01; Figure 1). Fish larvae exposed to the intermediate concentration of 750 nM of PTX2, had 37% mortality after 48 h and reached 100% at 72 h of exposure (P < 0.01). Furthermore, after 72 h of exposure to 500 nM of PTX2, the increase in mortality was not statistically significant (26%), but it became significant after 96 h, when 84% of the fish larvae were dead (P < 0.05). At 96 h of exposure, 8% of the fish larvae that were exposed to 250 nM of PTX2 died (P > 0.05). Consequently, the LC_{50} decreased with time, from 1231 nM at 24 h to 377 nM after 96 h (Table 3). At concentrations of PTX2 above 250 nM, a production of mucus was observed in fish throughout the 96-h exposure, in a dose-dependent manner. Moreover, the majority of the

fish exhibited an elevated respiratory rate compared to the control treatment and turned upside down for several minutes up to several hours before death. However, no mortalities of *C. variegatus* larvae were observed when exposed to (i) the whole culture (from 50 to 5500 cells mL^{-1}), (ii) live cells resuspended (5500 cells mL^{-1}) or (iii) culture filtrate (eq. 5500 cells mL^{-1}) of *D. acuminata*. Finally, fish larvae exposed to either FSW and FSW + EtOH controls showed no mortality throughout the experiment, but fish in the latter control treatment did produce some mucus after 72 h of exposure.

Histology

The histological observations were performed on either live or moribund larvae. Microscopic evaluation of gills of control animals, i.e. exposed to FSW + EtOH (Figure 2 a and b) and f/2-Si medium (Figure 2 c and d), exhibited typical gill structure indicative of health, with normal morphology of primary gill filaments and the secondary gill lamellae, as well as normal distribution and structure of chloride cells (= ionocytes) and pilaster cells. The healthy secondary gill lamellae architecture, highlighted by the delicate structure of the respiratory epithelium and inner pilaster cells, is showed in the TEM image of FSW + EtOH control fish (Figure 4 a).

However, severe pathologies were observed in gills of fish exposed to PTX2 from the highest dose down to 500 nM (Figure 3 a-d). Exposure to 4000 nM of PTX2 for 24 h resulted in severe disruption of healthy gill structure with swelling, disorganization (loss of normal cell to cell contact) separation, necrosis and sloughing of the respiratory epithelium and the osmoregulatory chloride cells (Figure 3 a and b). These cells are normally located in the surficial epithelium of the crypts between secondary gill lamellae (Figure 2 b and d). In the most severely affected individuals there was extensive hyperplasia (e.g., proliferation), hypertrophy (swelling with hyper-eosinophilic cytoplasmic staining) and redistribution of chloride cells, to a degree where the interlamellar spaces were completely occluded by these

cells, many of which were necrotic (Figure 3 a-d). Similar pathologies were observed at lower PTX2 concentrations of 750 and 500 nM (Figure 3 c and d, respectively) after 72 h of exposure. The TEM images of fish exposed to 2000 nM of PTX2 for 24 h showed the displacement and loss of respiratory epithelium and accumulation of fluid in the gills with infiltration of leukocytes (Figure 4 b and c).

In contrast, exposure for 96 h to whole cell *D. acuminata* culture, live filtered *D. acuminata* cells resuspended in clean f/2-Si medium and culture filtrate did not induce pathologies in fish gills (e.g Figure 3 e and f for gills of fish exposed to live *Dinophysis* culture at 5500 cells mL^{-1} for 96 h and to *D. acuminata* culture filtrate eq. 5500 cells mL^{-1} for 96 h, respectively).

Discussion:

This work aimed to determine the effect of a common HAB species, *Dinophysis acuminata* and one of its commonly produced toxins, PTX2, on larvae of a common estuarine fish species, *Cyprinodon variegatus*, using an established 96-h larval finfish bioassay and the observation of tissue pathology. The interest in PTX2 originates from two laboratory studies in which nM concentrations were reported to decrease fertilization rate of oyster gametes (Gaillard et al., 2020) and increase mortality in oyster larvae (Pease et al., 2022). Gaillard et al. (2020) hypothesized that PTX2 affected polymerization of actin filament, which could have directly affected oocytes and spermatozoa cytoskeleton and reproduction capacity, suggesting a potential direct effect of this toxin on other marine animals. These previous observations and the lack of knowledge about the toxicity of PTX2 to fish prompted us to perform these experiments. Here, exposure to PTX2 led to mortalities from 8 to 84% of fish larvae after 96 h of exposure to low-intermediate PTX2 concentrations (i.e. 250-500 nM) to 100% mortalities after only 24 h of exposure to high PTX2 concentrations (i.e. 2000-4000 nM), as well as severe damages to the gill respiratory epithelia and the osmoregulatory chloride cells. However, an important finding was that neither mortality of larvae nor

damages to gill tissues were observed even after 96 h of exposure to live *Dinophysis acuminata* culture up to 5500 cells mL^{-1}, live cells resuspended in clean media or to culture filtrate.

Histological observations of PTX2 exposure experiment revealed significant hyperplasia and hypertrophy, redistribution and/or increase in the number of chloride cells (= ionocytes), especially at the higher PTX2 concentrations (Figure 3 a-d and Figure 4 b and c). In fish, chloride cells are located at the base of the secondary gill lamellae and constitute part of the epithelium that lines the primary gill filaments in healthy fish (e.g., under non-stress conditions) (Dymowska et al., 2012). These chloride cells play a critical role in the regulation of hydro-mineral balance and are involved in ion transport (e.g. sodium and calcium), pH regulation and ammonia excretion (Hwang et al., 2011; Laurent and Perry, 1991; Osborne et al., 2016). However, when gills are exposed to environmental disturbances, their morphology has been described to follow a common mechanism that typically results in "branchial chloride cell proliferation", i.e. modification of morphology, distribution and number of chloride cells. (Laurent and Perry, 1991). Such branchial alterations have also been observed with exposure of adults gilt-head bream (*Sparus aurata*) to OA, which displayed enlargement of secondary gill lamellae (Souid et al., 2018; Table 1). It was hypothesized that OA caused collapse of pilaster cell and breakdown of the vascular components of the gill respiratory tissues. Similar to the results of this study, exposure of zebrafish (*Danio rerio*) juveniles to karlotoxins 1 and 2 (i.e. pore-forming ichtyotoxins produced by species of the genus *Karlodinium*) for 1-4 h at ≈ 37-373 nM, led to gill damage, including hypertrophy of epithelial cells, fusion of secondary gill lamellae, and proliferation and redistribution of chloride cells (Deeds et al., 2006). The authors suggested that exposed larvae died from respiratory failure due to cellular damage in gill tissues. Similarly, ichthyotoxins produced by the haptophyte *Prymnesium parvum* (i.e. prymnesins) have been shown to cause gill damage in fish (Ulitzur

and Shilo, 1966), in particular, affecting the chloride cells (Terao et al., 1996), and decreased oxygen consumption per breath and oxygen extraction leading to respiratory failure in fish (Bergsson et al., 2019; Svendsen et al., 2018). The morphological/functional alterations of chloride cells and/or gill respiratory lamellar epithelia appear to be a common result of exposure to diverse algal toxins. Thus, a hypothesis would be to assume a non-specific interaction between PTX2 and chloride cells in *C. variegatus* larvae, i.e. an indirect effect of the toxin on chloride cells.

Interestingly, the mode of action of PTX2 is recognized to be disruption by interaction with the actin, a critical cellular protein on mammalian cell lines (Allingham et al., 2007; Hori et al., 1999). In this study, gill lamellae alterations were also observed, mostly when larvae were exposed to intermediate to high concentrations of PTX2 (≥ 500 nM). In addition, swelling, disruption, separation, necrosis and sloughing of the respiratory epithelial cells lining secondary gill lamellae, was an important observation in this study. The respiratory surface of secondary gill lamellae is essentially composed of two simple flattened epithelial membranes, that allow gas exchange between water and the circulating red blood cells. The integrity of these two membranes is maintained by one of them, the thin specialized vascular endothelium, in the secondary gill lamellae, called pilaster (pillar) cells (Bettex Galland and Hughes, 1973). Pilaster cells are rich in actin fibers, e.g. F-actin (Kudo et al., 2007), that play a critical role in maintaining their very characteristic cellular shape, in their role in maintaining the blood vascular spaces that allow efficient gaseous exchange between the water and secondary gill lamellae. If PTX2 binds to pilaster cell actin, it would likely interfere with their ability to maintain cell integrity, which may have initiated a cascade of events, such as the alterations of the respiratory epithelium, leading to gill lamellae alterations (Figure 4 b and c). Together, these pathologies may have affected respiration and decreased acquisition of

oxygen, affecting ultimately energy balance and primary metabolism (Van Winkle et al., 1997), which was also suggested by increased breathing frequency.

Additional questions arise from these observations. Firstly, how did *C. variegatus* larvae die? Observations of pathologies in gills in this study suggested that fish may have died from respiratory failure associated with a potential reduction or loss of osmoregulatory ability. This hypothesis is strengthened by the observations of an increase in breathing frequency and mucus production in fish compared to FSW or f/2-Si controls, as previously reported for European plaice (*Pleuronectes platessa*) exposed to harmful concentrations of the ichthyotoxic haptophyte, *Prymnesium parvum* (Bergsson et al., 2019). In addition, the main target of DSTs and PTX2 by oral administration and intraperitoneal injections is the liver (Terao et al., 1993, 1986). However, in this study, livers looked healthy, with no functional derangement of hepatocytes, as well as the digestive tracts (data not shown), suggesting that these organs were likely not the targeted organs of dissolved PTX2 in fish larvae.

Secondly, why did exposure to live *Dinophysis acuminata* cells and their culture filtrate not induce any mortality of fish larvae? As in Rountos et al. (2019), no lethality was observed in *C. variegatus* and *M. beryllina* larvae exposed to 1000 cells mL^{-1} of toxic strains of *D. acuminata* (producing < 1 pg $cell^{-1}$ of OA and DTX1) for six days, suggesting that *Dinophysis* cells may not be toxic for fish larvae. As water enters in contact with gill lamellae for respiration, contact between gills and *Dinophysis* cells may have occurred, but did not cause pathologies in fish gills. Furthermore, as the effects (i.e. alterations in gill lamella, respiratory epithelium and chloride cell, and mortality) were only attributable to dissolved PTX2, neither the intracellular nor extracellular concentration of PTX2 (i.e. approximately 135 and 5 nM in cells or supernatant of 5500 cells mL^{-1}, respectively) in *D. acuminata* culture, would be sufficient to induce mortality or pathology.

Thirdly, we ask how toxic is PTX2 for fish? In this study, 24-h LC_{50} was 1231 nM and decreased down to 377 nM after 96 h, suggesting that PTX2 is approximately 7 to 10 times more toxic than DSTs on fish larvae (Table 1). However, PTX2 appeared to be 600 times less toxic than prymnesin 2 and approximately 2 to 9 times less toxic than karlotoxins, which are phycotoxins known to have adverse effects on fish (i.e. ichtyotoxins). Indeed, in an exposure study of *Tanichtys albonubes* larvae and juveniles to prymnesin 2, the 24-h LC_{50} was 2 nM (Igarashi et al., 1998). Moreover, in 1 day old *D. rerio* larvae exposed to karlotoxins 1 and 2, the 24-h LC_{50} was approximately 560 nM (Deeds et al., 2006), while it was even lower in juvenile *C. variegatus* (60-90 days old) exposed to two unknown karlotoxins (classified as karlotoxin-2) with lethal doses between 129 and 198 nM (Adolf et al., 2015). Similarly, exposure of 1-2 day(s) old *C. variegatus* larvae to karmitoxin, resulted in 4-h LC_{50} of 358 nM (Binzer et al., 2020), equivalent to the 96-h LC_{50} here.

And lastly, given the current state of knowledge, is the exposure of marine organisms to PTXs and algal blooms of PTX-producers an environmental problem? While lethal and sublethal effects were observed here in *C. variegatus* larvae following an exposure to intermediate and high concentrations of PTX2, *D. acuminata* did not induce mortality, even at > 10^6 cells L^{-1}, corresponding to exceptionally dense blooms observed in the field (Hattenrath-Lehmann et al., 2015, 2013). Dissolved PTX2/PTXs passively released after the collapse of a large *Dinophysis* bloom may affect various marine organisms, but further research is required to evaluate this. In addition, other PTX analogues exist, such as the seco-acids; however, unlike PTX2, their toxicity on marine organisms is still unknown. Since PTX2 can persist at low background concentration in the environment, as reported in passive samplers (Fux et al., 2009; Krock et al., 2020; Onofrio et al., 2021; Pizarro et al., 2009; Zendong et al., 2016), long-term effects of chronic exposure to low PTX2 concentration of fish during larval development, as well as potential PTX2 accumulation in fish should be further investigated.

In addition, study on the long-term effect of an acute exposure of fish larvae to PTX2 on its physiology, histology, possibility of recovery and development, may also improve knowledge on the mechanism of action of the toxin. Observations of *Dinophysis*, which are known PTX2-producers, are recurrent in the Chesapeake Bay and overlap with the spawning season of *C. variegatus* (i.e during spring-summer) and numerous other sensitive species (Onofrio et al., 2021; Rountos et al., 2019; Wolny et al., 2020). Indeed, this estuarine fish is preyed upon by top predators of commercial value, such as the Striped bass (*Morone saxatilis*) in the Chesapeake Bay (Gervasi et al., 2019). Overall, the reports of harmful effects of the non-diarrheic PTX2 towards marine animals are novel and should be considered to better assess risks associated with *Dinophysis* blooms.

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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.

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Table 1: Studies reporting negative effects of exposure of fish to *Dinophysis*, diarrheic shellfish toxins and pectenotoxins. OA, okadaic acid; DTX1, dinophysistoxin-1; PTX2, pectenotoxin-2.

Fish species	Life stage	*Dinophysis* species or toxin	*Dinophysis* or toxin concentrations in cells L^{-1} or nM	Time of exposure	Effects	Reference
Inland silverside (*Medinameberyllina*) and sheepshead minnow (*Cyprinodon variegatus*)	Larvae	*Dinophysis acuminata*	10^6	6 days	· ↘ growth rate	Rountos et al., 2019
Medaka fish (*Oryzias latipes*)	Embryos	OA	120 to 1200	24 h	· Mortality (> 311 nM); 24-h EC_{50} 646 nM · Reduction of development (> 620 nM) · ↗ liver and digestive tract areas (≥ 646 nM)	Escoffier et al., 2007
		OA (not pure; extracted from *Prorocentrum lima* = *Prorocentrum arenarium*)	140 to 270		· Mortality (> 150 nM); 24-h EC_{50} 211 nM · ↗ global body (≥ 190 nM) and vitellus areas (≥ 220 nM)	
Longfin yellowtail (*Seriola rivoliana*)	Embryos	OA and DTX1 (not pure; extracted from *P. lima*)	149 to 217	25 h	· Mortality (≥ 149 nM) · ↘ protein (≥ 149 nM) and alkaline (at	Le Du et al., 2017

					149 nM) phosphatase activities · Modification of expression of genes involved in bone development and DNA replication (at 149 nM)	
Zebrafish (*Dania rerio*)	Larvae	OA	1252 to 18633	96 h	· Mortality (≥ 8795 nM); 24-h LC_{50} 12422 nM · ↘ growth rate (24 h; ≥ 7937 nM) · ↗ shortening of the anterior-posterior axis and cyclopia (≥ 5590 nM) · ↘ activity of super oxide dismutase and catalase (48h; ≥ 8074 nM) and damage to proteins · ↗ oxidative damage to protein (6 h; ≥ 8074 nM)	Figueroa et al., 2020
		DTX1	1221 to 18315		· Mortality (≥ 6105 nM); 24-h LD_{50} 8547 · ↘ growth rate (24 h; ≥	

					5495 nM) · ↗ shortening of the anterior-posterior axis and cyclopia (≥ 5495 nM) · ↘ activity of super oxide dismutase and catalase (12h; ≥ 5495 nM) · ↗ oxidative damage to protein (6 h; ≥ 7937 nM)	
Sheepshead minnow (*Cyprinodon variegatus*)	Larvae	PTX2	50 to 4000	96 h	· Mortality (≥ 500 nM); 24-h LC_{50} 1231 · Gills: damage of respiratory epithelial cells and hyperplasia, hypertrophy, redistribution of chloride cells (≤ 500 nM; 96h)	This study
Gilt-head bream (*Sparus aurata*)	Adults	OA	9316	24 h	· Modification in antioxidant responses: ↗ of peroxidation products (2 h) and antioxidant enzymes (4 h)	Souid et al., 2018

				· Hepatocytes: vacuolization and proliferation of fibroblasts (2 h), change of size and membrane degradations (24 h) · Gills: hypertrophy, fusion in secondary lamellae and necrosis (24 h)

Table 2: Average intracellular (intra., pg cell^{-1}), extracellular (extra., eq. pg cell^{-1}) and total in 5500 cell mL^{-1} (nM) concentrations of pectenotoxin 2 (PTX2), dinophysistoxin 1 (DTX1) and okadaic acid (OA) in *Dinophysis acuminata* strain DAVA01. Values are mean ± SD, n = 3. LOQ: limit of quantification

	PTX2	DTX1	OA
Intra. (pg cell^{-1})	21 ± 0.40	1.2 ± 0.64	0.17 ± 0.07
Extra. (eq. pg cell^{-1})	0.98 ± 0.09	<LOQ	<LOQ
Total (nM in 5500 cells mL^{-1})	140 ± 3.20	8.1 ± 4.3	0.94 ± 0.45

Table 3: LC_{50} (nM and ng mL^{-1}) of pectenotoxin 2 (PTX2) based on the Hill coefficient according to exposure time.

Time (h)	LC_{50} (nM)	LC_{50} (ng mL^{-1})
24	1231	1057
48	873	750
72	530	455
96	377	324

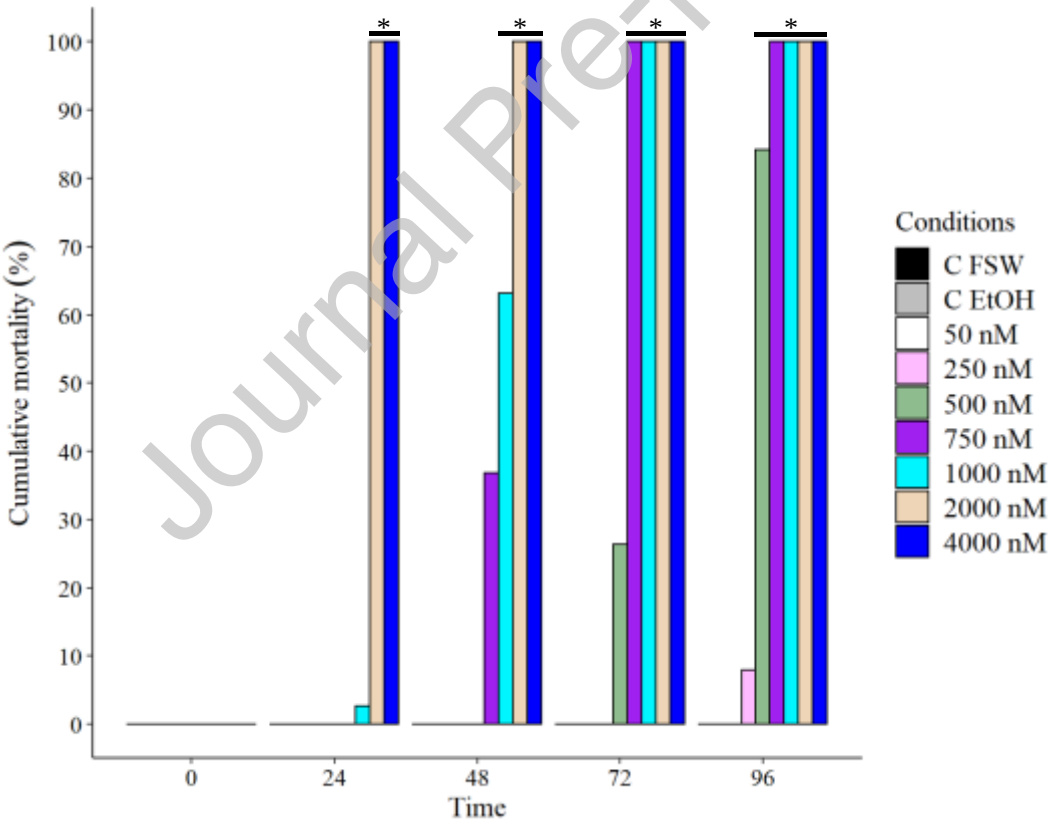

Figure 1: Cumulative mortality (%) over 96 h in *Cyprinodon variegatus* larvae exposed to 50, 250, 500, 750, 1000, 2000, 4000 nM of pectenotoxin 2 (PTX2), filtered seawater (C FSW)

and filtered seawater + ethanol (0.8% final) (C EtOH) controls (n = 38 for each condition. * significantly different (Pearson's Chi-square test and post hoc test on residuals).

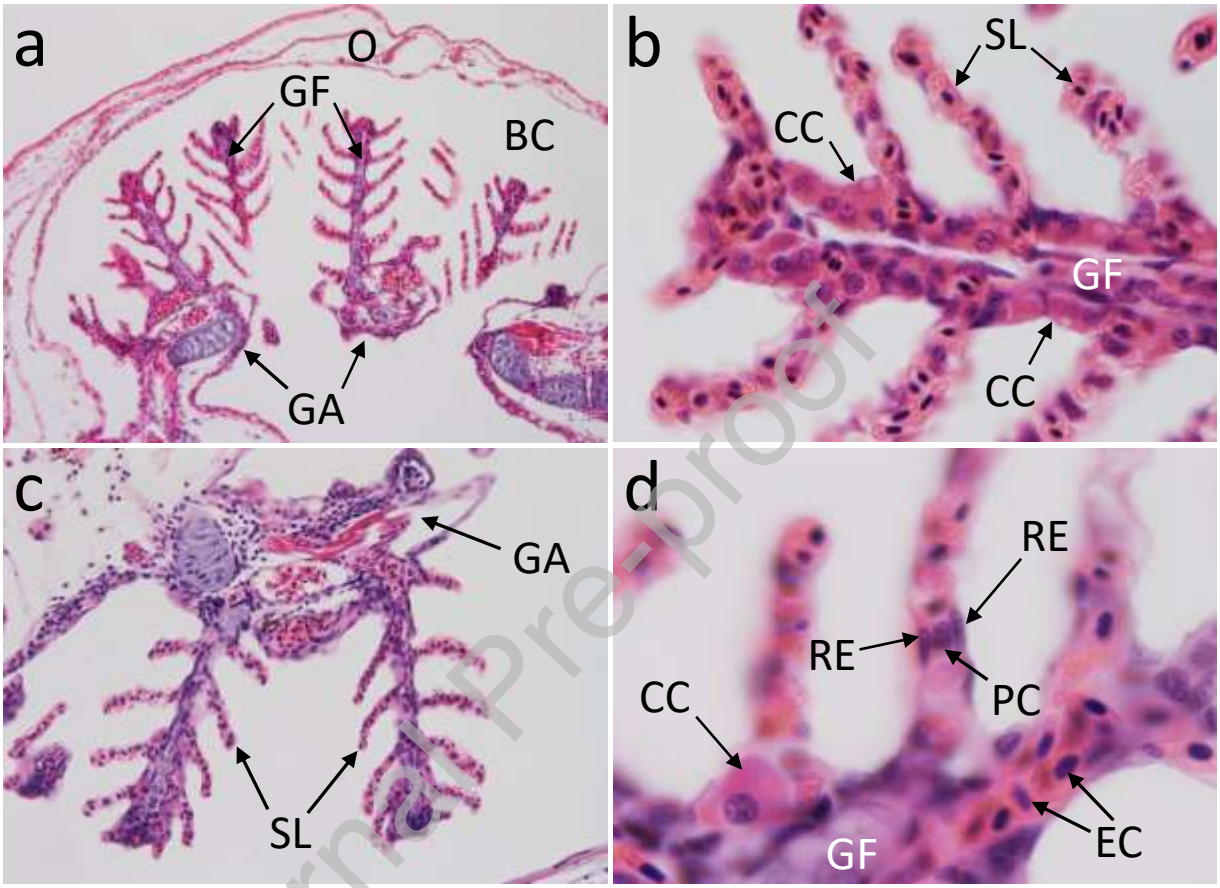

Figure 2. Gill tissues of control larval *Cyprinodon variegatus* that were part of the study investigating mortality, histopathological and ultrastructural effects of pectenotoxin 2 (PTX2) and *Dinophysis acuminata* treatments**. a)** "FSW + ethanol (EtOH) control" (t = 96 h) fish exhibiting typical healthy gill histology where integrity and normal architecture of primary gill filaments (GF) and delicate secondary gill lamellae remains apparent post-challenge. BC: branchial chamber, O: operculum, GA: gill arches (10X). **b)** higher magnification of "FSW + EtOH control" exhibiting normal cellular orientation and tissue architecture indicative of healthy fish. GF: primary gill filament, SL: secondary gill lamellae, CC: functional chloride cell (ionocyte) typically observed at the base of the crypts between secondary gill lamellae

(60X). **c)** Low magnification of branchial chamber and gill tissues in a "f/2-Si medium control" (t = 96 h) fish. GA: Gill arch, SL: secondary gill lamellae (10X). **d)** High magnification of a primary gill filament (GF) and secondary gill lamellae exhibiting normal healthy histomorphology of a "f/2-Si medium control" fish. CC: chloride cell, RE: respiratory epithelial cell nuclei, PC: pilaster cell, EC: erythrocytes (100X).

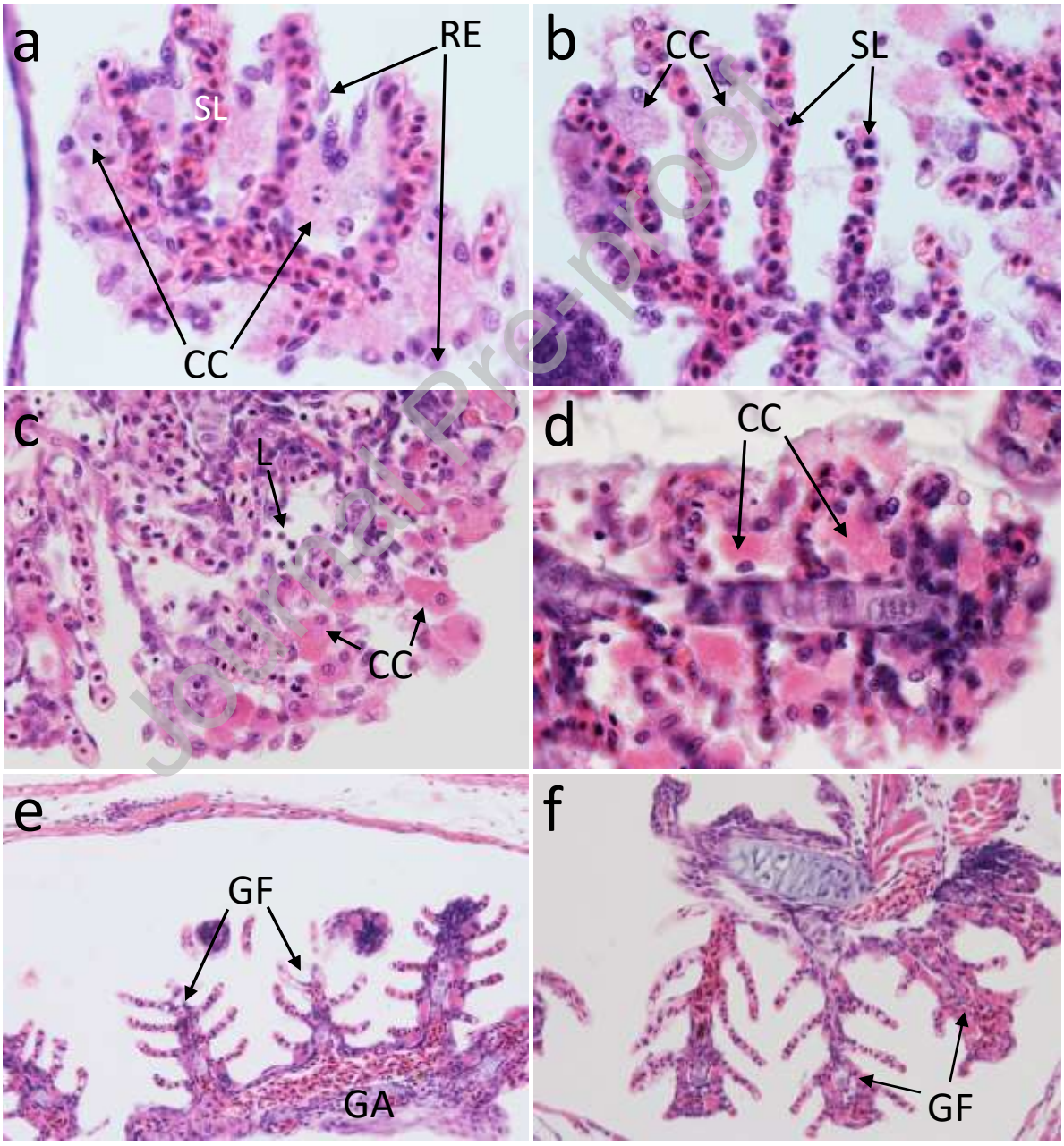

Figure 3. Pathology of gills in larval *Cyprinodon variegatus* exposed to pectenotoxin 2

(PTX2) and *Dinophysis acuminata*. **a)** Fish exposed to 4000 nM PTX2 (t = 24 h) exhibiting severe swelling and necrosis of chloride cells (CC) and displacement and loss of respiratory epithelia (RE). SL: secondary gill lamellae (60X). **b)** Lysis and loss of gill chloride cells (CC) and partial loss of respiratory epithelium in fish exposed to 4000 nM PTX2. SL: secondary gill lamellae (60X). **c)** Similar gill pathology in fish exposed to 750 nM PTX2 (t = 72 h). Note mild leukocytic infiltration (L) of damaged gills. CC: chloride cell (60X). **d)** Fish exposed to 500 nM PTX2 (t = 72 h) exhibiting loss of polarity, swelling and detachment of chloride cells (CC) and respiratory epithelia (60X). **e)** Healthy gills in fish exposed to live *D. acuminata* culture at 5500 cells mL^{-1} (t = 96 h). GF: primary gill filament, GA: gill arche (20X). **f)** Healthy gills in fish exposed to *D. acuminata* culture filtrate eq. 5500 cells mL^{-1} (t = 96 h). GF: primary gill filament (20X).

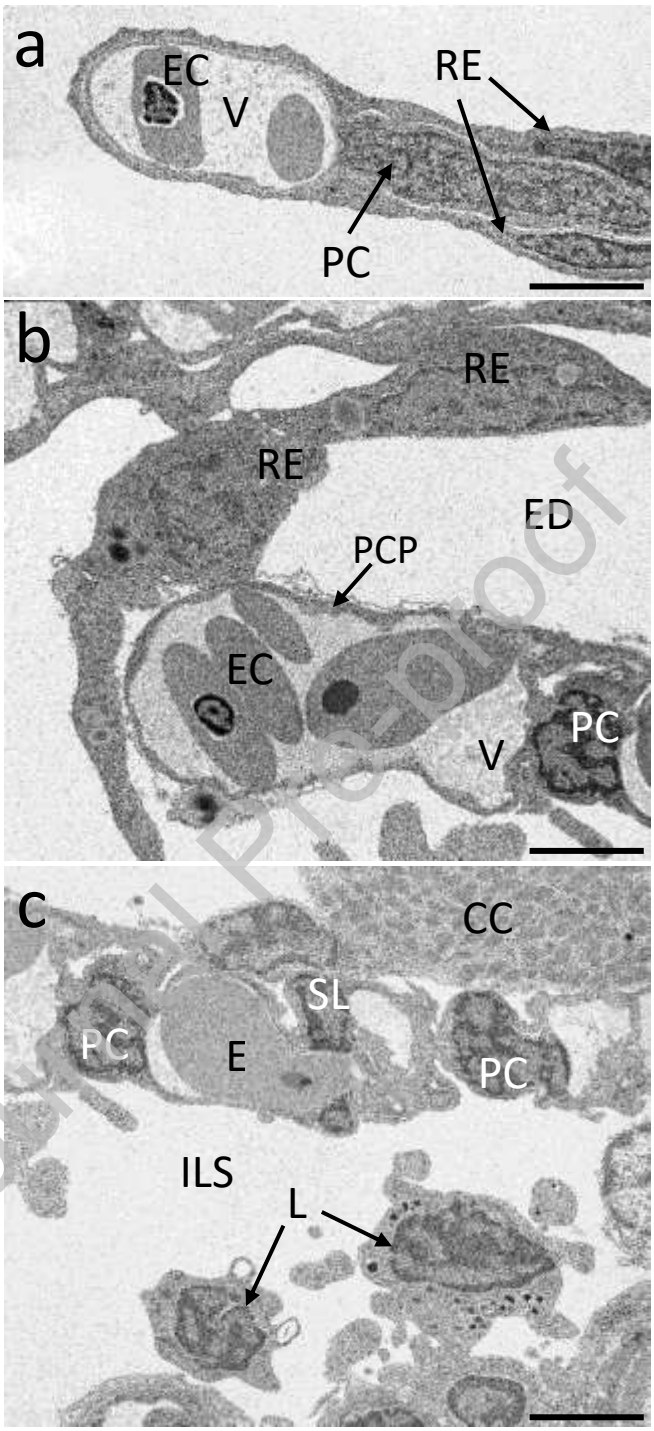

Figure 4. Ultrastructure of gill lamellae in *Cyprinodon variegatus* control and exposed to pectenotoxin 2 (PTX2). **a)** Healthy lamella in "FSW + EtOH control" (t = 96 h) fish. Note outer delicate single-celled squamous respiratory epithelium (RE) and inner pilaster cell (PC)

forming the vasculature (V) of lamellae. EC: erythrocytes (Bar: 5 µm). **b)** Lamellar ultrastructure of fish exposed to 2000 nM PTX2 (t = 24 h). Note the separation of respiratory epithelium (RE) with loss of membrane integrity and fluid accumulation (ED). PC: Pilaster cell, PCP: pilaster cell processes forming vascular channels (V), EC: erythrocyte (Bar: 5 µm). **c)** Fish exposed to 2000 nM PTX2 (t = 24 h). SL: core of secondary gill lamella with loss of respiratory epithelium, ILS: edematous inter-lamellar space infiltrated by host leukocytes (L). CC: chloride cell displaced from crypt of inter-lamellar space. PC: Pilaster cell (Bar: 5 µm).