

Research Article

## Toxicity and histopathological effects of toxic dinoflagellate, *Alexandrium catenella* exudates on larvae of blue mussel, *Mytilus galloprovincialis*, and Pacific oyster, *Crassostrea gigas*

Supono Supono<sup>1,2\*</sup> , Graeme Knowles<sup>3</sup>, and Christopher Bolch<sup>4</sup>

<sup>1</sup>Institute of Marine Science, University of Auckland, P.O. Box 349, Warkworth 0941, New Zealand

<sup>2</sup>Loka Konservasi Biota Laut Bitung, Research Center for Oceanography, Indonesian Institute of Sciences, Sulawesi Utara 95527

<sup>3</sup>Animal Health Laboratory, Department of Primary Industries, Parks, Waters and Environment, P.O. Box 46, Kings Meadows Tasmania, 7249, Australia

<sup>4</sup>Institute for Marine and Antarctic Studies, University of Tasmania, Locked Bag 1370, Launceston, Tasmania, 7250, Australia.



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\*) Corresponding author:  
E-mail: [supono@lipi.go.id](mailto:supono@lipi.go.id)

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### Abstract

Blooms of the toxic dinoflagellate *Alexandrium catenella* have affected shellfish industries globally due to their capacity to produce paralytic shellfish toxins (PST). This study aimed to investigate the toxicity effect of exudate *A. catenella* on larvae of blue mussel *Mytilus galloprovincialis* and Pacific oyster *Crassostrea gigas* and filtration methods to reduce the toxic effect. Blue mussel and Pacific oyster larvae were assessed their survival and histopathological changes after exposure to extracellular exudates of *A. catenella* ranging from 100 to 1,000 cells ml<sup>-1</sup>. The results showed that exposure to exudate *A. catenella* caused significantly higher larval mortality (39 to 52%) than exposure to an equivalent biovolume of the non-toxic species, *Tisochrysis lutea* (33%) or unfed controls (17%). Filter-sterilization (0.22 µm) of exudates and activated carbon filtration decreased the mortality of Pacific oyster larvae to a level similar to controls (unfed), with the exception of the highest concentrations (600 and 1,000 cells ml<sup>-1</sup>) and mortality of blue mussel larvae mortality by 32% respectively. Blue mussel larvae exposed to exudate *A. catenella* showed pathological changes mainly in the stomach (digestive gland and style sac) as early as three hours after onset of exposure. The findings of this study suggest that early detection of blooms in the vicinity of mussel and Pacific oyster hatcheries and taking steps to mitigate their effects, is important to reduce the effects of *A. catenella* blooms on shellfish larval rearing.

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## 1. Introduction

The species diversity, geographical distribution and associated impacts of harmful algal blooms (HAB) have globally increased, spanning from the Northern to the Southern hemisphere. The number of toxic species, bloom occurrence and economically affected sectors have increased significantly over the last several decades (Anderson *et al.*, 2010; Hoagland *et al.*, 2002). The annual economic losses inflicted by blooms has been estimated at reaching USD 43 million in the United State of America (USA), USD 850 million in Europe and USD 1 billion in Asia, representing four main sectors; public health, commercial fisheries, recreation or tourism and monitoring/management (Anderson *et al.*, 2000; Trainer and Yoshida, 2014).

Among groups causing HABs, dinoflagellates (Dinophyceae) are considered to be the most widespread and frequently recorded (Cembella and John, 2006; Hallegraeff, 2014). Species of the genus *Alexandrium* are the most widespread, common HAB genera with approximately one third of more than 30 species known to produce paralytic shellfish toxins (PST) that accumulate through vertical transmission in the food chains (Anderson *et al.*, 2012; Samson *et al.*, 2008). Some species, including the widespread cold-temperate species *Alexandrium catenella* produce both PSTs and other poorly characterized extracellular toxic or allelopathic compounds that have harmful effects on marine fauna and flora (John *et al.*, 2014; Tillman and Hansen, 2009; Tillman *et al.*, 2007) including shellfish (Bricelj and Shumway, 2011; Núñez Vázquez *et al.*, 2011).

*Alexandrium catenella* is one of the *Alexandrium tamarense* species complex group demonstrating *sxtA4* (the gene responsible for producing saxitoxin) and paralytic shellfish poisoning (PSP) toxicity (John *et al.*, 2014), and is capable of producing extracellular compounds (Tillman and Hansen, 2009). A wide range of species of marine organisms has been reported to be adversely affected by *Alexandrium* including shellfish (Bricelj *et al.*, 2011; Haberkorn *et al.*, 2010; Núñez-Vázquez *et al.*, 2011; Pate, 2007). The susceptibility of adult shellfish to toxic *Alexandrium* is dependent upon a number of factors, including developmental stage (Yan *et al.*, 2003; Yan *et al.*, 2001; Mu and Li, 2013), chemical structure and action mode of toxins (Bricelj *et al.*, 1991; Hégarret *et al.*, 2007), duration exposure (Contreras *et al.*, 2012) and species (Hégarret *et al.*, 2007). Mussels are considered to be less sensitive to the effects of saxitoxin (a PST produced by *Alexandrium*) and to accumulate toxin more rapidly (Bricelj *et al.*, 1990), but little is known of the susceptibility of either adults or larvae to other extra-cellular compounds produced by *A. catenella*.

Over the period from 2012 to 2016, intense winter

spring blooms of *A. catenella* affected much of the eastern coast of Tasmania, causing regular and prolonged harvest closures of, clams, scallop abalone, and rock lobster fisheries and farmed oysters and mussels (Bolch *et al.*, 2014; Hallegraeff *et al.*, 2017). Particularly intense blooms (up to 300,000 cells L<sup>-1</sup>) during spring 2015 and 2016 were also associated with high mortality of adult farmed oysters, and a series of shellfish hatchery production failure of both mussels and oyster larvae (Hallegraeff *et al.*, 2017). Most previous research has focused on the effects of live cells on shellfish (Mu and Li, 2013; Yan *et al.*, 2001; Yan *et al.*, 2003) however, water intake and treatment systems of marine hatcheries routinely exclude algal cells/biomass using a combination of filtration to (effective to < 1 µm diameter) followed by UV-treatment to reduce bacterial loads. Dissolved organic carbon (DOC) and potentially toxic extracellular compounds exuded by *A. catenella* blooms (exudates) can pass through the water treatment systems largely intact, entering the larval rearing systems and may directly or indirectly cause mortality of shellfish larvae. This study aims to investigate the lethal concentration, and histopathological effects of *A. catenella* exudates on shellfish larvae, particularly blue mussels and oysters.

## 2. Materials and Methods

### 2.1 Materials

Materials used in this study included 6 days post-fertilization (dpf) larvae of blue mussels (*Mytilus galloprovincialis*) and Pacific oysters (*Crassostrea gigas*) at ~138 µm shell length, culture of *Tisochrysis lutea* and *Alexandrium catenella*, K-medium, 10% buffered seawater-formalin, alcohol 70%, Oxoid no.3 agar media, 0.22 µm Isopore (Millipore, USA), Sedgewick-Rafter and Haemocytometer counting chamber (Thermo Fisher Scientific, USA) and 50 ml plastic jars.

### 2.2 Methods

Shellfish larvae, blue mussels (*Mytilus galloprovincialis*) and Pacific oysters (*Crassostrea gigas*) were obtained from Spring Bay Seafood Pty Ltd, Tasmania, Australia. Larvae were kept in 0.2 µm filtered seawater at 20 °C under light intensity of 30 µmol m<sup>-2</sup> s<sup>-1</sup> in IMAS aquaculture center, University of Tasmania, Australia. A Saxitoxin producing species, *Alexandrium catenella*, was used for treatment and the non-toxic algae *Tisochrysis lutea* was used as control. All algae were grown in 250 ml flasks to late log phase (12 to 14 days) in K medium prepared using filter-sterilized seawater (0.22 µm Isopore, Millipore, USA) at 18 °C and 60 µmol m<sup>-2</sup> s<sup>-1</sup> of light intensity. Culture density was estimated by triplicate cell counts using a Sedgewick-

Rafter and Haemocytometer counting chamber. Next, harvested cultures were transferred to 50 ml tubes and subjected to 10 minutes of 4,000 rpm centrifugation. Culture supernatants (exudates) were then removed, examined under a compound microscope to ensure the absence of algal cell materials, and frozen at -20 °C prior to use.

### 2.2.1 Acute toxicity of exudates *A. catenella* on blue mussel larvae

An acute toxicity test was carried out in triplicate by exposing 6 days post fertilization (dpf) blue mussel larvae (10 larvae ml<sup>-1</sup>) to exudates of *A. catenella* at equivalent concentrations of 100, 300, 600 and 1,000 cells ml<sup>-1</sup>. The larvae in control group were divided into two groups; control 1-unfed (no materials added) and control 2-exposed to exudate *T. lutea* at standard hatchery food concentration (30,000 cells ml<sup>-1</sup>). After 48 hours of exposure, the larvae in each group were removed and transferred to new 0.2 µm filtered seawater for mortality assessment. Larval mortality was assessed following criteria of [Jacobson et al. \(1993\)](#).

### 2.2.2 Effects of 0.22 µm and activated carbon filtration on the toxicity of exudates *A. catenella*

Due to unavailability of blue mussel larvae after broodstock spawning (May-June), the second acute toxicity, to investigate the potential role of algal exudates-associated bacteria on larval mortality, was carried out on oyster larvae. Larvae in treatment and control were divided into two groups; the first group was exposed to 0.22 µm filter-sterilized exudates and a second group was exposed to unfiltered exudates at equivalent concentration of 100, 300, 600 and 1,000 cells ml<sup>-1</sup> of *A. catenella* (treatment) and *T. lutea* (control). The concentration of *T. lutea* was adjusted based on its bio-volume which is approximately 100 times less than *A. catenella* ([Munir et al., 2015](#); [Olenina et al., 2006](#)). Larval mortality was assessed after 48 h.

To identify the effect of activated carbon filtration, two groups of blue mussel larvae were exposed to exudates of *A. catenella* at an equivalent concentration of 1,500 cells ml<sup>-1</sup>. Larvae from the first group (control) were exposed to 0.22 µm filter-sterilized without passing through activated carbon and larvae from the second group were exposed to 0.22 µm filter-sterilized filtered through activated carbon. Larval mortality was then assessed following 48 hours exposure.

### 2.2.3 Histopathological findings related to exudates from *A. catenella* on blue mussel larvae

To investigate the histopathological findings

related to *A. catenella*, 3 dpf blue mussel larvae at density of 10 larvae ml<sup>-1</sup> were exposed to 1,500 cells ml<sup>-1</sup> exudates. Larvae were removed at every 3, 15, 24 and 48 hours exposure, fixed in 10% buffered seawater-formalin and processed for histological examination using double embedding techniques ([Feist and Bucke, 1983](#)). The prevalence and intensity of histopathological findings were measured following [Basti et al. \(2016\)](#) with modification. Each pathological finding was recorded as either absent (0) or present (1) and the intensity was scored as the percentage of the affected areas; 0 to 25% (mild), 26 to 80% (moderate) and > 80% (heavy). The average prevalence was interpreted as low (0 to 0.4), intermediate (0.41 to 0.8) and high (0.81 to 1). The average intensity was interpreted as mild (0 to 1), moderate (1.01 to 1.90) or heavy (1.91 to 3).

### 2.3 Data Analysis

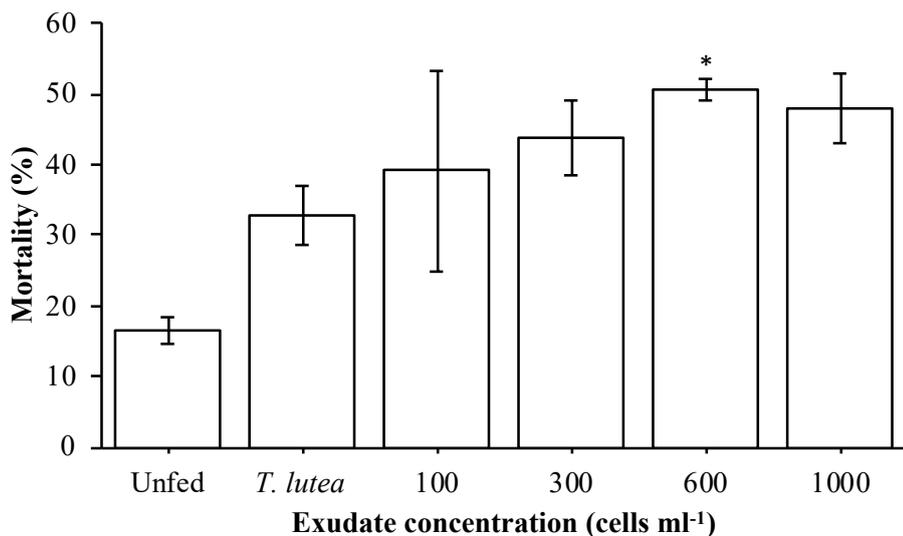
All data analysis was performed using GraphPad Prism version 7.0 for Mac (GraphPad Software, La Jolla California, USA). Normality (Shapiro-Wilk test) was checked a priori by column statistic. One-way and two-way ANOVA was conducted to compare the effects of exudates treatment, effects of 0.22 µm filtration on the toxicity of *A. catenella* on larval mortality and mean prevalence and intensity of each pathological change over exposure duration. A Tukey's post hoc test was applied at the significance level of 0.05.

## 3. Results and Discussion

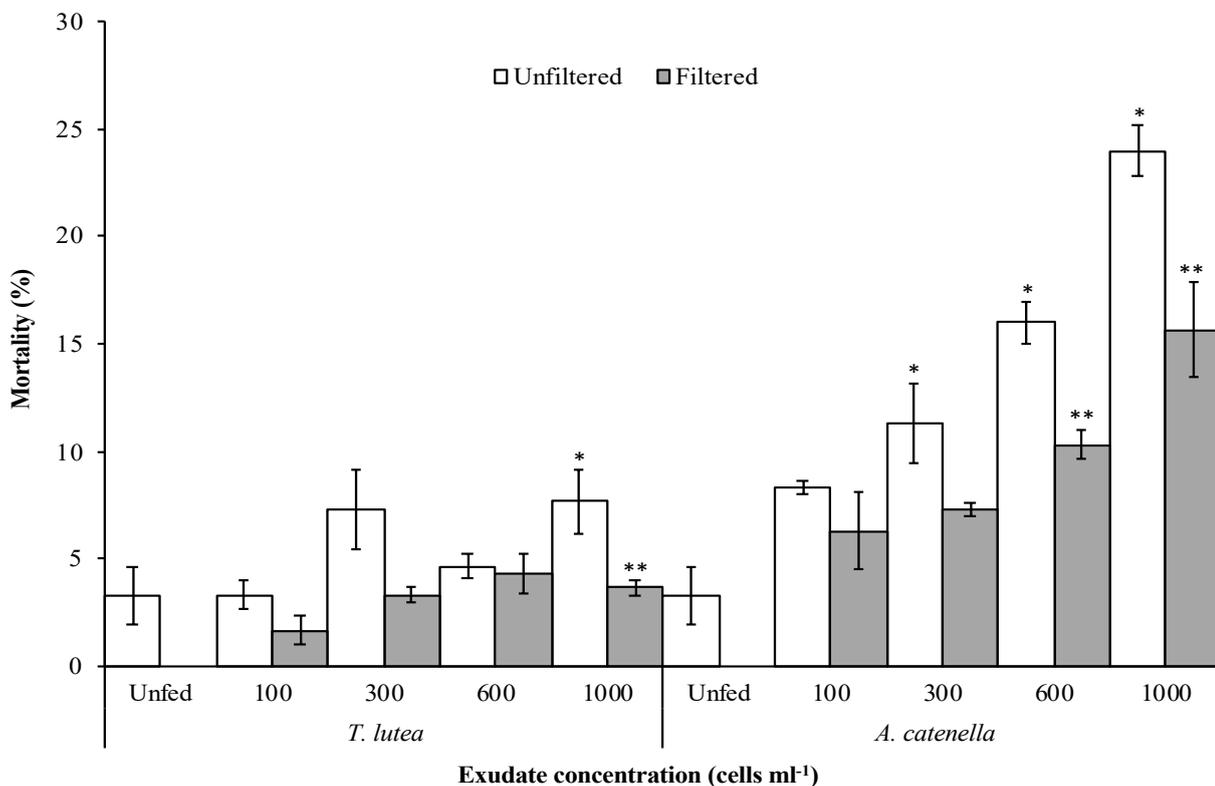
### 3.1 Acute toxicity of exudates *A. catenella* on blue mussel larvae

Mortality of mussel larvae exposed to unfiltered exudates of *A. catenella* and *T. lutea* was higher than the mortality of unfed larvae. The highest mortality was observed after exposure to *A. catenella* exudate equivalent to 600 cells ml<sup>-1</sup>, whereas the remaining concentrations showed similar mortality to larvae exposed to non-toxic *T. lutea* exudates ([Figure 1](#)). Larvae, which died, following exposure to exudate *A. catenella* showed the absence of internal organ (transparent), fusion of velum cilia and biofilm engulfing bacteria.

Microalgal exudates of dinoflagellates are known to have a number of effects on larval bivalves including feeding inhibition, morphological abnormalities, and decreasing hatching rates ([Thompson et al., 1994](#)). Saxitoxins have also been shown to have direct effects on shellfish physiology, metabolism and gene expression ([Detree et al., 2016](#)). Tasmanian strains of *A. catenella* produce a range of PSTs including sulfocarbamoyl derivatives C1 and C2, gonyautoxins (GTX1-4), decarbamoyl derivatives and saxitoxin ([Bolch et al., 2014](#)).



**Figure 1.** Mean ( $\pm$  SE, n=3) mortality of blue mussel larvae exposed to different exudate concentration of *A. catenella*. (\*) indicates significant difference from control (unfed) (n=3).



**Figure 2.** Mean ( $\pm$  SE, n= 3) mortality of oyster larvae exposed to different concentration of unfiltered and 0.22  $\mu$ m filtered exudate of *A. catenella* and *T. lutea*. (\*) indicates significant difference from unfed control. (\*\*) indicates significant difference from unfiltered exudate.

A range of studies have shown that many lethal effects, of *Alexandrium* species/strains, to fish and shellfish, are unrelated to the presence or production of PSTs. Instead, the lethal effects of *Alexandrium* include

haemolytic compounds (Matsuyama, 2001; Tillman and John, 2002) or other bioactive extracellular compounds (BEC) (Borcier et al., 2017; Mardones et al., 2017; Castrec et al., 2020).

Several studies have shown that toxic *A. catenella* have a range of sub-lethal effects on adult bivalves including mussels. Blue mussels exposed to cultures of *A. catenella* (*Protogonyaulax tamarensis* GT429) at  $10^4$  cells  $\text{ml}^{-1}$  showed erratic and complete shell-valve closure, production of white, mucus-like, material and subsequent mortality of 67% of experimental animals after exposure (Shumway and Cucci, 1987). In contrast, similar studies on green mussels (*Perna canaliculus*) showed increased oxygen uptake but no major physiological effects or increased mortality during short-term exposure to  $10^3$  cells  $\text{ml}^{-1}$  twice daily, and no mortality over a period of 2 weeks (Marsden and Shumway, 1992). These less severe effects may reflect species-level differences in response, or perhaps the lower cell concentrations/times used for the experimental exposure. Other studies of *Mytilus* species also demonstrated inflammatory responses, significant changes in physiological responses, hematocyte parameters, upregulation of detoxification pathways (Haberkorn *et al.*, 2010), and immuno-compromization leading to increased parasite infection (Galimany *et al.*, 2008). Histopathological changes have also been noted in the digestive tract (Galimany *et al.*, 2008), similar to that observed for larvae during our exposure experiments.

### 3.2 Effects of 0.22 $\mu\text{m}$ and activated carbon filtration on the toxicity of exudates *A. catenella*

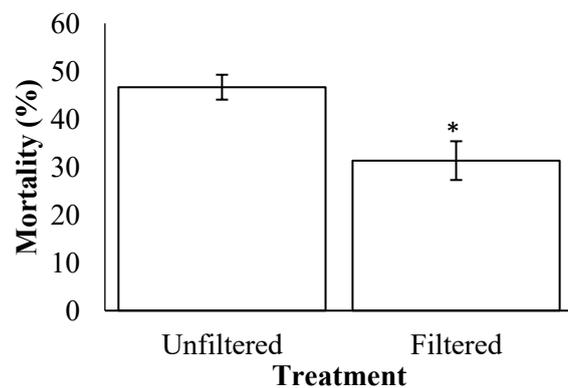
Larval mortality exposed to unfiltered exudates of *A. catenella* tended to increase with increasing concentration of exudates (Figure 2). Mortality was higher (8-21%) than unfed larvae at 300, 600 and 1,000 cells  $\text{ml}^{-1}$ , but was only significant different at the highest concentration (1,000 cells  $\text{ml}^{-1}$ ). The 0.22  $\mu\text{m}$  filtration of *A. catenella* exudates resulted in reduced larval mortality particularly at the higher concentrations; by 6% at 600 cells  $\text{ml}^{-1}$  and 8% at 1,000 cells  $\text{ml}^{-1}$  (Figure 2). Exposure to *T. lutea* filtered exudates showed similar mortality to unfed controls (< 5%) at all concentrations except 300 and 1,000 cells  $\text{ml}^{-1}$  where mortality increased by 4 % over the unfed controls (Figure 2). Filtration of filter-sterilized *A. catenella* exudates through granular activated-carbon reduced larval mortality by 15% compared to untreated filter-sterilized exudates (Figure 3).

The partial reduction of larval mortality by 0.22 $\mu\text{m}$  filtration of culture exudates of both *A. catenella* and *T. lutea* indicates that a proportion of the larval mortality may associate with the direct and/or indirect effects of the bacterial community associated with exudates.

Neither the mussels, oysters or cultures of *A. catenella* used in this study were axenic nor therefore might bacteria be present during experiment. However, mortality due to exposure to filter-sterilized exudates -

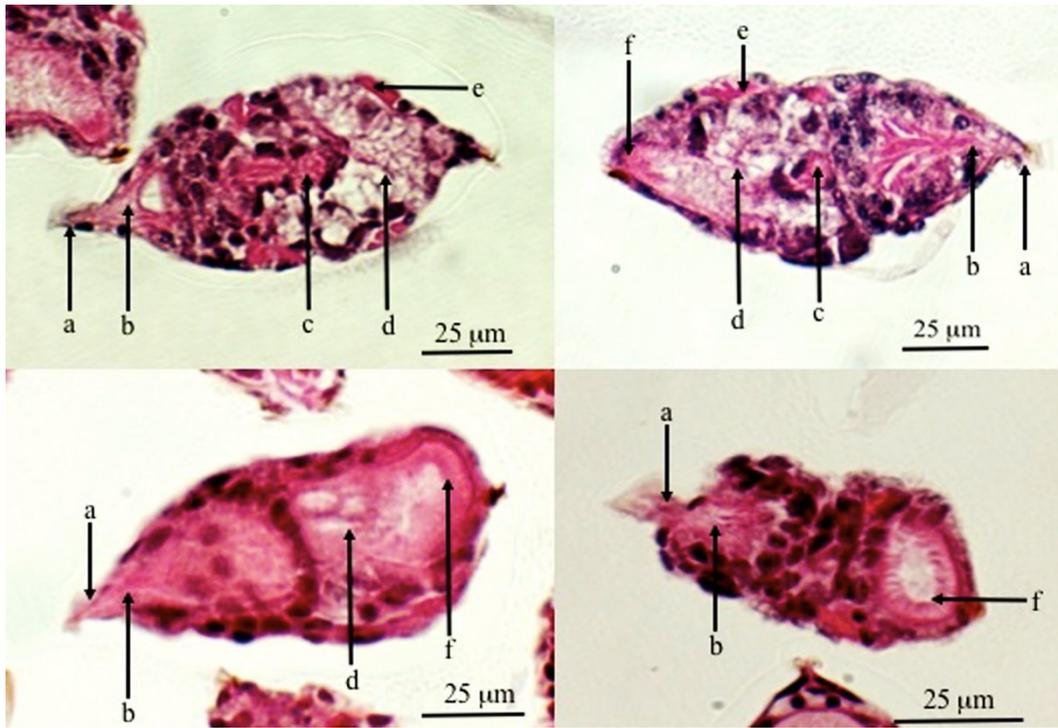
remained higher for *A. catenella* than *T. lutea* exudates indicating that BEC produced by *A. catenella* was responsible for some of the larval mortality.

Blue mussels, oysters and culture of *A. catenella* used in this study were not axenic and therefore bacteria would be expected to be present during experiment. We did not characterize bacterial communities in our treatments prior to the start of the experiments and bacteria were evident during larval observation at the end of experiment. Some larvae in treatments also demonstrated internal tissue discharge and biofilms engulfing larvae, similar to that reported for bacterial infections (Sutton and Garrick, 1993).

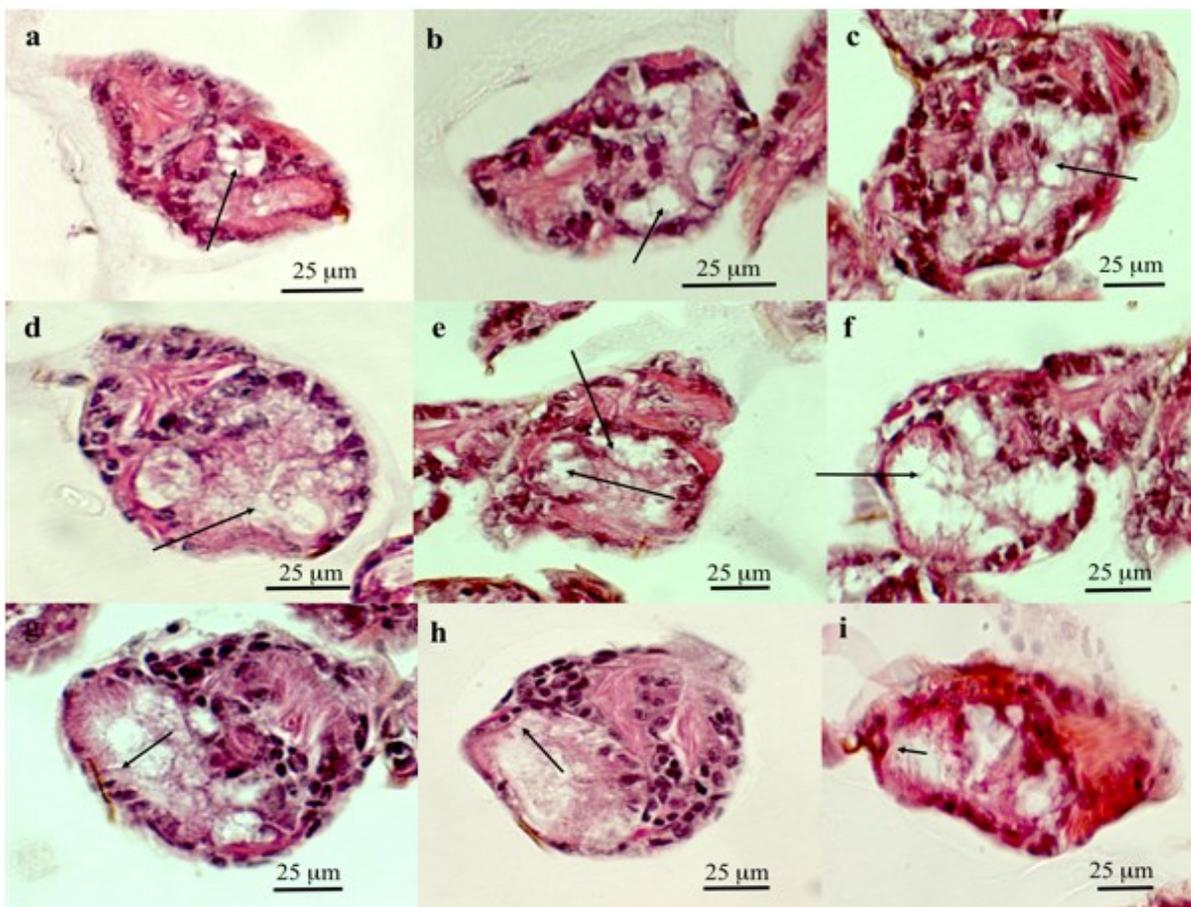


**Figure 3.** Mean ( $\pm$  SE, n= 3) mortality of blue mussel larvae exposed to unfiltered and filtered exudates of *A. catenella* through activated carbon. (\*) indicates significant different among treatments ( $P < 0.05$ ).

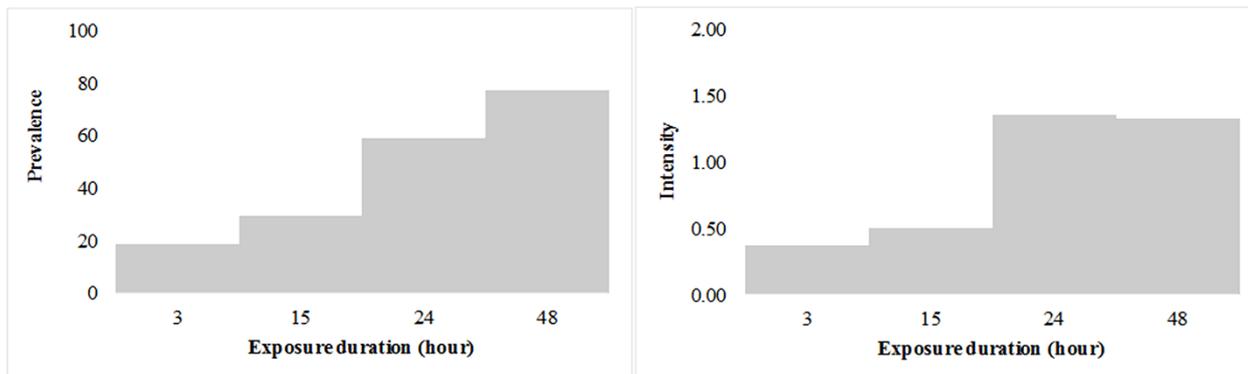
Rearing conditions for bivalve are favorable for marine bacterial growth (Brown and Tettelbach, 1988), and healthy mussel larvae cultures contain complex microbial communities, including a substantial populations of potentially pathogenic *Vibrio* spp. (e.g. Kwan and Bolch, 2015). For this reason, environmental or other host stress (e.g. husbandry) factors are key in the pathogenesis from commensal marine bacteria to larval bacterial pathogens. In summer exposure to and feeding of oysters with *A. catenella* to adult oysters has been shown to increase susceptibility to infection and disease due to *Vibrio tasmaniensis*, leading to mortalities (Abi-Khalil *et al.*, 2016). Alternatively, susceptibility of larvae to bacterial infection may be increased indirectly by increases in dissolved organic carbon from cell exudates that in turn stimulate or alter the microbial community within the lumina (e.g. alimentary tract, over gills) of the larvae. The incidence/severity of the common shellfish hatchery disease bacillary necrosis can be increased by over-feeding, over-stocking, or the addition of various forms of dissolved organic carbon (Kwan *et al.*, 2017). After the onset of mortality, the presence of moribund and dead larvae further accelerates infection and necrotic disease.



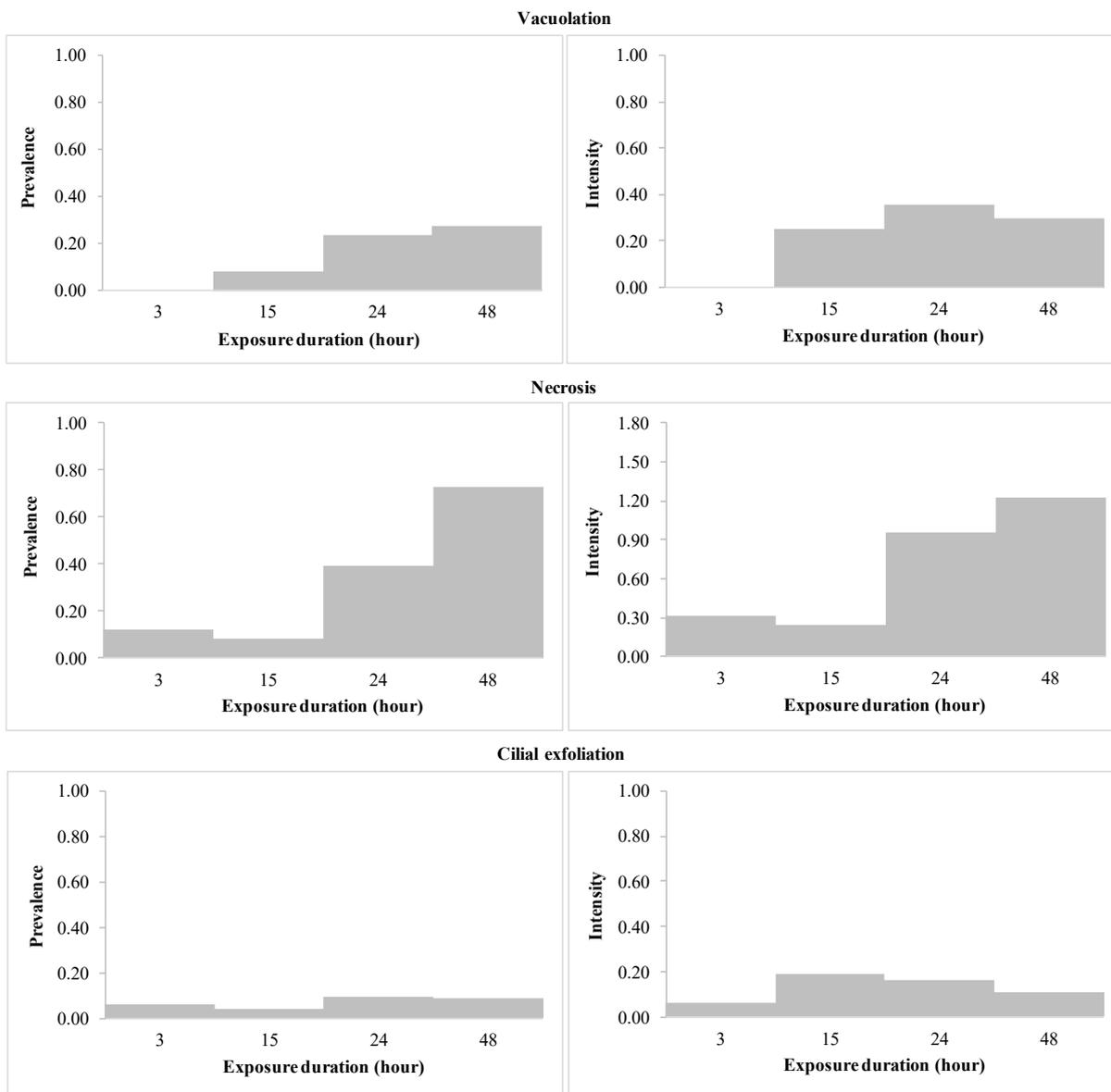
**Figure 4.** Structure of normal 3 dpf blue mussel larvae showing well defined organs. a). velum of membrane, b). cilia of velum, c). esophagus, d). digestive gland, e). adductor muscle, f). style sac.



**Figure 5.** Structure of larvae showing the intensity of pathological changes after exposure to 1,500 cells ml<sup>-1</sup> exudates of *A. catenella*. a-c) Intracytoplasmic vacuolation (likely degeneration) of digestive gland epithelium; mild (a) and moderate (b) after 24 hours, and heavy (c) after 48 hours. d-f). Necrosis of digestive gland epithelium; mild (d) after 3 hours, moderate (e) after 15 hours and heavy (f) after 24 hours. g-i). Ciliary exfoliation from style sac epithelium; mild (g) after 15 hours, moderate (h) after 24 hours and heavy (i) after 48 hours.



**Figure 6.** The prevalence and intensity of pathology in blue mussel larvae exposed to 1,500 cells ml<sup>-1</sup> exudates of *A. catenella*.



**Figure 7.** The prevalence and intensity of blue mussel larvae exposed to exudate of *A. catenella* over different exposure duration.

### 3.3 Histopathological effects of exudates *A. catenella* on blue mussel larvae

Prior to exposure, larvae demonstrated a well-defined digestive gland and style sac with cilia on the luminal surface (Figure 4). After exposure to 1,500 cells ml<sup>-1</sup> exudates of *A. catenella*, three pathological changes, intracytoplasmic vacuolation (likely degeneration) or hypereosinophilia with pyknotic nucleus (necrosis) of digestive gland epithelium and ciliary exfoliation of style sac epithelium were observed, mainly in stomach of blue mussel larvae (Figure 5).

The histopathological changes occurred as early as 3 hours after exposure. The proportion of larvae showing histopathological changes (i.e. prevalence) and the severity of changes (i.e. intensity) was significantly different according to exposure duration ( $p < 0.01$  for both parameters). The prevalence of larvae demonstrating histopathological changes increased from low prevalence at 3 to 15 hours exposure to intermediate at 24 to 48 hours exposure. A similar pattern was shown in the intensity of histopathological changes. The majority of larvae showed mild intensity at 3 and 15 hours exposure and this increased to moderate intensity at 24 and 48 hours exposure as shown in Figure 6.

The progression of histopathological changes was not clearly demonstrated over exposure duration that is there was no gradual increase in the numbers of necrotic epithelial cells paired with a decrease in the numbers of degenerate epithelial cells over time and with increasing dose of exposure to *A. catenella* exudate. However, the prevalence and intensity of necrosis increased with the exposure duration from low prevalence and mild intensity at 3 to 24 hours to intermediate prevalence and moderate intensity at 48 hours after exposure. In contrast, low prevalence of vacuolation and ciliary exfoliation with mild intensity was evident at all duration exposure (Figure 7).

In this study, histopathological changes were mainly observed in digestive gland showing intracytoplasmic vacuolation, necrosis and ciliary exfoliation of style sac. This finding was similar to previous works which reported that PSP toxins mostly accumulate in viscera causing damage of the digestive gland either through direct contact between toxic compounds and wall of digestive gland (Bricelj *et al.*, 1990; Kwong *et al.*, 2006; Widdows *et al.*, 1979), or to inflammatory and immune responses of the shellfish to toxic assault (Haberkorn *et al.*, 2010). The state of histological changes varies depending on animal stage and exposure duration. In the present study, pathological changes in digestive gland were evident as early as three hours after exposure of exudate. In contrast,

pathological effects *A. catenella* on adult blue mussels were only evident after 9 days of exposure (Galimany *et al.*, 2008).

## 4. Conclusion

The findings of this study suggest that hatchery larval mortality associated with *A. catenella* blooms is caused by a combination of extracellular toxic compounds and modification of larval culture bacterial communities due to increased/changed organic carbon from *A. catenella*. The exudate concentration equivalent to 600 cells ml<sup>-1</sup> in this study caused the highest mortality on blue mussel larvae. The rapid histopathological changes (3 hours after exposure) and significant larval mortality observed at cell concentrations regularly observed during blooms (1,500 cells ml<sup>-1</sup>) indicate that early detection of blooms in the vicinity of marine hatcheries in conjunction with mitigating management procedures are important to reduce severe effects of *A. catenella* blooms on shellfish larval rearing. Further studies of the nature and scale of changes in the larval microbial community and pathological change mechanism are needed to clarify the cause of larval mortality.

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## Authors' Contributions

All authors have contributed to the final manuscript. The contribution of authors as follow, Supono designed experiment, conducted experiment, collected data and drafted manuscript. Chris Bolch and Graeme Knowles provided supervision and critical revision on the draft.

## Conflict of Interest

The authors declare that there is no conflict of interest.

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