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Toxic dinoflagellates and *Vibrio* spp. act independently in bivalve larvae

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ABSTRACT

Harmful algal blooms (HABs) and marine pathogens – like *Vibrio* spp. – are increasingly common due to climate change. These stressors affect the growth, viability and development of bivalve larvae. Little is known, however, about the potential for interactions between these two concurrent stressors. While some mixed exposures have been performed with adult bivalves, no such work has been done with larvae which are generally more sensitive. This study examines whether dinoflagellates and bacteria may interactively affect the viability and immunological resilience of blue mussel *Mytilus edulis* larvae. Embryos were exposed to environmentally relevant concentrations (100, 500, 2500 & 12,500 cells ml^{-1}) of a dinoflagellate (*Alexandrium minutum*, *Alexandrium ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*), a known pathogen (*Vibrio coralliilyticus/neptunius*-like isolate or *Vibrio splendidus*; 10^5 CFU ml^{-1}), or both. After five days of exposure, significant ($p < 0.05$) adverse effects on larval viability and larval development were found for all dinoflagellates (except *P. cordatum*) and *V. splendidus*. Yet, despite the individual effect of each stressor, no significant interactions were found between the pathogens and harmful algae. The larval viability and the phenoloxidase innate immune system responded independently to each stressor. This independence may be related to a differential timing of the effects of HABs and pathogens.

1. Introduction

Over the past decades, mankind has inadvertently altered the world's oceans and seas through overfishing, coastal eutrophication, invasive species dispersal and global change. This has led to an increase in the frequency, scale and magnitude of harmful algal blooms (HABs) which cause mass mortality amongst bivalves [1,60]. Due to their contribution to the bentho-pelagic coupling, the bioturbation of sediment or the creation of hard substrate reefs, bivalves are often a keystone species of their communities [16,28]. Through starvation, hypoxia, physical damage to vulnerable tissues (e.g. gills) or the accumulation of various marine toxins, HABs may kill bivalves which threatens the marine biodiversity on multiple trophic levels. Moreover, these mortality events directly affect our food security as 12% of the global annual seafood production is derived from cultured molluscs [21].

The availability of organic matter and detritus during algal blooms promotes the growth of benign and pathogenic bacteria [14,18,20,48,61]. Like HABs, pathogens may cause mass mortality of wild and cultured bivalves [7,51,60]. As harmful algae and pathogens will both benefit from climate change, their co-occurrence may increase in the future [1,11]. Yet, despite of this, little is known about the potential for interactive adverse effects between both stressors.

Most cultured bivalve populations rely on the natural availability of wild larvae [44,59,62]. These early life stages are highly sensitive to abiotic stress [3], bacterial infections [51] and harmful algae (e.g. Refs. [9,39,50,56]). Moreover, a recent study has shown that larval phenoloxidase activity (PO) – which has antibacterial functions in adult bivalves [35,45,74] - is affected by HAB exposure [17]. As a result, HAB exposure may affect the susceptibility of larvae towards pathogens.

To investigate the impact of HABs and pathogens, this study examined the individual and combined effects of these stressors on bivalve larvae. To this end, embryos of the blue mussel *Mytilus edulis* were exposed to various mixtures of (toxic) dinoflagellates

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(*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *Prorocentrum lima* or *Prorocentrum micans*) and known pathogens (a *Vibrio coralliilyticus*/*neptunius*-like isolate or *Vibrio splendidus*). After five days of exposure, the larval viability, development and PO activity were determined.

2. Material and methods

2.1. Algal cultures

Dinoflagellates were obtained from different sources: *Prorocentrum lima* (CCAP1136/9) and *Prorocentrum micans* (CCAP1136/20) were supplied by the Culture Collection of Algae and Protozoa (Oban, Scotland, UK). *Alexandrium ostenfeldii* (CCMP1773) originated from the National Center for Marine Algae and Microbiota (Maine, USA). *Alexandrium minutum* (SCCAP K-0993), *Karenia mikimotoi* (SCCAP K-0260), *Protoceratium reticulatum* (SCCAP K-1478) and *Prorocentrum cordatum* (SCCAP K-1501) were provided by the Scandinavian Culture Collection of Algae & Protozoa (Copenhagen, Denmark). All species were cultured in L1 medium [27], prepared with filtered and autoclaved Instant Ocean™ (Belcopet, Belgium) artificial seawater (32 PSU, pH 8). 80% of the culture medium was replaced every 2 weeks. Algae were grown at 20 °C with a light-dark cycle of 12 h (300–600 μmol m^{-2} s^{-1}) and cell counts were performed frequently with a Sedgewick Rafter counting chamber (SPI supplies, West Chester, USA). All experiments were performed with cultures in the early stationary phase.

2.2. Bacterial cultures

Vibrio splendidus (EU358783) and a *Vibrio coralliilyticus*/*neptunius*-like isolate (EU358784), further referred to as *V. coralliilyticus*, were isolated at the Glenhaven Aquaculture Center (New Zealand). These strains are known to infect and kill Greenshell mussel (*Perna canalicus*) larvae [41]. Bacterial stocks were stored at −80 °C in sterile Marine Broth 2216 (Difco, BD, New Jersey, USA) with 30% glycerol. Before the experimental work, bacteria were reactivated at 25 °C by incubating 200 μl of bacterial stock with 10 ml of sterile Marine Broth on a shaker. This batch culturing procedure was repeated daily. Bacterial densities were determined with a Thermo Aquamate spectrophotometer (Thermo Fisher Scientific, San Jose, USA) operating at 550 nm following the methods of Baruah et al. [5]. All handling procedures were performed in a Holten LaminAir laminar flow cabinet (Thermo Fisher Scientific, San Jose, USA).

2.3. Viability and development

Fecund adult mussels were collected from breakwaters near Middelkerke (Belgium) between January and May 2015. These were placed in a recirculating aquarium (8 °C, 34 PSU) and fed *ad libitum* (±10 μl l^{-1}) with a commercial algal paste (Shellfish Diet 1800®, Varicon Aqua Solutions, UK). Spawning was induced, in accordance with the guidelines of the American Society for Testing and Materials [3], by moving mussels between water baths of 18 °C and 26 °C. By separating spawning males and females, sperm and eggs of multiple individuals were isolated, purified and combined in a single controlled fertilization. Gamete quality was visually assessed throughout the subsequent hour. Embryos were used for testing when ≥80% of the eggs had developed a first polar body.

2.4. Embryonic development test

Sterile 24-well tissue culture plates were filled with 2 ml of ASTM artificial seawater containing 100, 500, 2500 or 12,500 cells ml^{-1} of one of seven dinoflagellate species (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Protoceratium reticulatum*, *Prorocentrum cordatum*, *P. lima* or *P. micans*). Each series was made in threefold: once as single exposures to the dinoflagellate, once with *Vibrio coralliilyticus* (10^5 CFU ml^{-1}) and once with *Vibrio splendidus* (10^5 CFU ml^{-1}). Each of these treatments was replicated six times. Control treatments included pure ASTM (i.e. 0 cells ml^{-1} algae and no pathogens) and two single exposures to *Vibrio coralliilyticus* (10^5 CFU ml^{-1}) and *V. splendidus* (10^5 CFU ml^{-1}). As recommended by ASTM [3]; additional replicates of the control (18) and the *Vibrio* control treatments (12) were made to potentially detect diminishing egg quality and quantity between the start, mid and end of the spiking process. Such patterns were not detected. Around 120 fertilized eggs were transferred to each well and the plates were incubated at 18 °C with a light-dark cycle of 12 h/12 h.

Short-term (48 h) exposure assays with bivalve embryos typically determine the effect of stressors by the shape of the veliger larvae [3]. If the exposure is prolonged past 48 h, however, deceased but well-formed larvae become indistinguishable from healthy D-shaped veliger larvae (personal observation). For this reason, a staining procedure was developed and optimized in preliminary experiments. After four days, 25 μl of 2.4 mM methylene blue (10% EtOH) was added to all treatments. This dye stained actively filtering larvae while death or moribund larvae remained colourless. After five days, 25 μl of 30% formaldehyde was added to each well to stop the experiment. A 40× Oxion inverse light microscope (Euromex, Arnhem, The Netherlands) was used to distinguish and count the healthy (i.e. blue) veliger from larvae with compromised feeding (i.e. non-blue) and underdeveloped larvae (i.e. non-veliger).

2.5. Phenoloxidase activity

Glass vessels of 500 ml were filled with 200 ml ASTM seawater solutions containing one of six dinoflagellates (*Alexandrium minutum*, *A. ostenfeldii*, *Karenia mikimotoi*, *Prorocentrum cordatum*, *P. lima* or *P. micans;* 2500 cells ml^{-1}). Of each algae, five single exposures and five replicates with *V. splendidus* (10^5 CFU ml^{-1}) were prepared. Based on the results of the development test, *Vibrio coralliilyticus* was eliminated from this experiment (ref. Section 3.1). Control treatments of pure ASTM and *V. splendidus* (10^5 CFU ml^{-1}) were also replicated five times each. Around 100,000 embryos were added to each test vessel before they were incubated at 18 °C with a 12 h light cycle. After five days, the larvae were collected on a 37 μm sieve, flash-frozen in liquid nitrogen and stored at −86 °C. Larval enzymes were extracted from the mussels by homogenizing the larvae. PO activity was determined through the enzymatic transformation of L-3,4-dihydroxyphenylalanine to dopamine, as described in detail by De Rijcke et al. [17]. Three technical replicates of each biological replicate were analysed. Over the course of 48 h, the optical density of the samples was determined by performing multiple spectrophotometric measurements using a Thermo Multiskan Ascent 96/384-well spectrophotometer operating at 490 nm.

2.6. Data analysis

Larval viability was defined here as the number of healthy (i.e. blue) veliger larvae divided by the average total number of larvae in the control treatment. Larval development was defined as the total number of veliger larvae (blue and non-blue) divided by the average total number of larvae in the control. The larval viability and larval development were fitted with a three-parameter log-logistic model using the “drc” package of Ritz and Streibig [55]. Each dinoflagellate yielded three dose-responses: one without