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Potential role of *Mytilus edulis* in modulating the infectious pressure of *Vibrio anguillarum* 02β on an integrated multi-trophic aquaculture farm

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ABSTRACT

Marine finfish producers in the Northeast are adopting an integrated multi-trophic aquaculture (IMTA) approach by growing mussels, *Mytilus edulis*, with marine finfish species. Shellfish play a critical role in an IMTA system by filtering particulate bound nutrients and other seston from surrounding waters. During feeding, mussels bio-accumulate microbes, including fish pathogens, from the water column. This may influence pathogen dynamics on IMTA farms by either reducing infectious pressure or by serving as a reservoir for important finfish pathogens. The first step in evaluating the disease risks or benefits on a mussel-fish IMTA farm is to understand the interaction between mussels and finfish pathogens. We investigated the fate of the cod bacterial pathogen *Vibrio anguillarum* 02β in the blue mussel *Mytilus edulis*. Mussels exposed to 10^4 CFU of *V. anguillarum* ml^{-1} concentrated bacteria in digestive gland tissues 2 orders of magnitude above the concentration of bacteria in the surrounding water. To determine if mussels could release concentrated bacteria back into the environment, *V. anguillarum*-exposed mussels were transferred to clean water and fecal and water samples were analyzed for the presence of bacteria. *V. anguillarum* was not detected in the water; however mussel feces and pseudofeces had high loads of *V. anguillarum* (10^7 CFU g^{-1}). Within 72 h of depuration, *V. anguillarum* was no longer detected in fecal samples. Immersion challenge trials using fecal material from *V. anguillarum*-exposed mussels resulted in 58–70% mortalities in challenged cod. These results demonstrate that mussels are capable of bio-accumulating *V. anguillarum* and shedding virulent bacteria through their feces. While this study does not likely reproduce the dynamic conditions on a finfish–mussel farm, results obtained do present valuable information when considering biosecurity and pathogen risk reduction on IMTA farms.

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1. Introduction

Integrated multi-trophic aquaculture (IMTA) is being developed globally to increase farm diversity, minimize environmental impacts, and increase economic profitability (MacDonald et al., 2011; Ridler et al., 2007). In the northwest Atlantic, researchers are developing techniques to integrate Atlantic salmon (*Salmo salar*) or Atlantic cod (*Gadus morhua*) culture with the culture of blue mussels (*Mytilus edulis*) and various macro-algal species (Chopin et al., 2004; MacDonald et al., 2011). In these systems, blue mussels play an important role in filtering uneaten food particles and other particulate matter from the water column. However, mussels also have the potential to increase finfish disease risk on an IMTA farm. Bivalves, such as mussels and oysters, are known to bioaccumulate human pathogens such as *Vibrio* species, hepatitis A virus, human sapovirus, and adenovirus (Canesi et al., 2002; Hernroth and Allard, 2007; Kim et al., 2008). However, very little work has been performed to determine whether shellfish concentrate viral and bacterial finfish pathogens in their tissues. The results of a few studies suggest that there is potential for shellfish to act as reservoirs for finfish pathogens (Stabili et al., 2005).

Vibriosis is an important disease of cod at all stages from larvae to adult. *Vibrio anguillarum* 02β, a Gram-negative opportunistic bacterial pathogen, is one of the main serotypes that causes vibriosis in cod (Evelyn, 1971; Strout et al., 1978) and is commonly found in the cold water marine environments in which cod farming occurs. It has been a major pathogen of concern in the European cod industry as well as in Maine.

The addition of commercial scale mussel production in close proximity to fish farms, as in IMTA, could potentially change the infection

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dynamics for fish pathogens. Growers interested in IMTA question the role mussels may play in either increasing or decreasing the infectious pressure of fish pathogens on their farms. To better understand how the integration of mussel and fish culture on a single farm may influence the infectious pressure of *V. anguillarum*, we determined the fate of *V. anguillarum* in mussels and in mussel/cod systems.

2. Methods

2.1. Fish and mussel maintenance

Market-size mussels were obtained from commercial mussel farms. Mussels were maintained at 10 °C in artificial seawater (ASW) (Crystal Seas, Baltimore, MD) and were fed a diet of mixed species algal paste (Innovative Aquaculture, Skerry Bay, BC). During trials, mussels were maintained in static systems containing 0.5 L of ASW per mussel at 10 °C unless otherwise noted.

Juvenile cod (6.32 ± 0.32 g) were obtained from a local commercial hatchery and were maintained in recirculating systems containing ASW at 10 °C. They were fed a commercial pelleted diet (Skretting Gemma Diamond 1.2, Vancouver, BC) at 2% of their body weight per day. All populations of animals were screened for the presence of *V. anguillarum* prior to experimental use.

2.2. Bacterial cultures

Serotype 02β *V. anguillarum* strain NVI5812 was originally isolated from cod and was a gift from Duncan Colquhoun (Fish Health Section, National Veterinary Institute, Oslo, Norway). To facilitate identification and tracking in challenge and transmission studies, NVI5812 was modified to carry the broad-host-range plasmid p67T1 that encodes ampicillin resistance and constitutive production of the red fluorescent protein d-Tomato (Singer et al., 2010). Bright red bacterial colonies arising from cells of NVI5812(p67T1) are easily identified and quantified on solid medium among the background of non-pigmented colonies resulting from the normal flora of mussels and mussel feces. NVI5812(p67T1), the strain used for all studies reported here, was grown at 25 °C with shaking in L-broth (Miller, 1972) containing 1.5% NaCl and ampicillin ($600\ \mu g\ ml^{-1}$) to select for p67T1. Solid medium contained 1.5% agar.

2.3. V. anguillarum mussel exposure

Two *V. anguillarum* mussel exposures were performed over 48 h (trial 1) and 120 h (trial 2). In each trial, 15 mussels were randomly assigned to each of 4 tanks. Three of these tanks were inoculated with *V. anguillarum* to a final concentration of 5.9×10^4 CFU mL^{-1}. An equal volume of sterile L-broth was added to the fourth tank. Two additional control tanks containing ASW only were treated with equal volumes of *V. anguillarum* or sterile L-broth. Algae were added to all 6 tanks to a final concentration of 2×10^5 cells mL^{-1}. After thorough mixing, water samples were immediately taken from each tank to establish the initial concentration of bacteria.

In trial 1, samples were taken at 2-, 6-, 12-, 24- and 48 hours post inoculation (hpi). Samples were collected at 24-, 48-, 72-, 96- and 120 hpi in trial 2. At each time point, water samples were obtained from each tank along with 3 randomly selected mussels from all tanks containing mussels. Water samples from *Vibrio*-inoculated tanks were serially diluted and plated in duplicate on L-agar ampicillin medium. Control samples were plated directly without dilution.

Prior to dissection, mussel shells were disinfected with 5% bleach followed by 70% ethanol. The digestive gland was removed from each mussel and diluted 1/10 (wt/vol) in sterile PBS. Tissues were processed in a TissueLyser (Qiagen, Valencia, CA) for 10 s at a frequency of 15 s^{-1}. Homogenates were serially diluted and plated as above.

2.4. V. anguillarum shedding trial

Eight mussels were exposed to 4.5×10^4 CFU of *V. anguillarum* mL^{-1} in the presence of algae (2×10^5 cells mL^{-1}). Equal volumes of L-broth and algal cells were added to a second tank containing 2 mussels. After a 2-h exposure, mussels were disinfected as above and rinsed in fresh ASW. To remove bacteria-laden water from the buccal cavity, mussels were placed into individual beakers containing 1 L of ASW for 30 min. A visual inspection showed that all mussels had opened during the 30 min, indicating they had exchanged the water in the buccal cavity. Shells were again disinfected, rinsed, and mussels were placed in tanks containing 0.5 L of clean ASW and algae.

At 24-h intervals feces, and pseudo-fecal material were obtained from each tank and mussels were placed in new tanks containing ASW and algae. Feces and pseudo-feces were combined and are hereafter collectively referred to as fecal matter. After centrifugation ($1000 \times g$ for 10 min) the wet weight of the fecal pellet was determined. Fecal pellets were diluted 1:10 wt/vol with sterile PBS and processed in the TissueLyser as above. Fecal homogenates were serially diluted and plated on L-agar ampicillin medium. At the end of the shedding study, digestive gland tissue was harvested from each mussel following a 144-h depuration, and was processed for bacterial counts as above.

2.5. Cod challenge with Vibrio-laden fecal matter from mussels

To generate negative control mussel feces for cod challenge, L-broth and algae (2×10^5 cells mL^{-1}) were added to a tank containing 25 L of ASW and 50 mussels. After 24- and 48 h, fecal material was collected, pooled, and processed as above. *Vibrio*-laden mussel fecal matter was generated in an identical manner except that *V. anguillarum* was also added to the tank at 3.9×10^8 CFU mL^{-1}. *Vibrio*-laden fecal samples were collected, processed as above, and uniform suspensions of *Vibrio*-laden and control fecal samples were prepared at 1 g of fecal matter per 50 mL of ASW for use in challenge studies.

Twelve juvenile cod were placed into each of 5 challenge tanks containing 1950 mL of aerated ASW. Two tanks received 50 mL of the control fecal suspension and the remaining three tanks 50 mL of *Vibrio*-laden fecal suspension. Viable counts confirmed a challenge dose of 6×10^5 CFU of *V. anguillarum* mL^{-1} in the 3 *Vibrio*-containing tanks. To confirm the virulence of *V. anguillarum* at the time of tank inoculation, 6 juvenile cod were exposed to 5×10^5 CFU of *V. anguillarum* mL^{-1} in 2 L of ASW. Following a 1-h exposure, fish were removed from the challenge bath and placed in a recirculation system in 75-L tanks. Moribund and dead fish were removed twice daily and vibriosis due to *V. anguillarum* 02β was verified by plating head kidney samples on L-agar ampicillin medium. The challenge was terminated 22 dpc after 4 consecutive days with no mortalities. Head kidneys of all surviving cod were assayed for the presence of *V. anguillarum* by plating as above.

3. Results

3.1. V. anguillarum mussel exposure

In trial 1, the concentration of *V. anguillarum* in mussel digestive glands (4.6×10^7 CFU g^{-1}) at 2 hpi was 2 orders of magnitude greater than that of the water (7.6×10^4 CFU mL^{-1}) (Fig. 1). This difference in concentration was maintained throughout the trial. At 48 hpi the average *V. anguillarum* concentration in mussel digestive gland and tank water was 3.3×10^7 CFU g^{-1} and 2.1×10^4 CFU mL^{-1}, respectively.

In trial 2, mussels concentrated *V. anguillarum* in digestive gland tissues 2 orders of magnitude above that of the water (Fig. 2). There was a slow decline in the concentration of *V. anguillarum* in the water from the start of the trial until 72 h. At 96 hpi *V. anguillarum* was no longer detectable in the water of tanks containing mussels.

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