Necrotic disease in bivalve larval cultures



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The health of marine bivalve larvae is greatly affected by bacteria in the environment particularly when reared in marine hatcheries. This is generally because high stocking densities resulting in high organic loads of both food and faeces, can support increased bacterial growth and biomass levels. Increased bacterial load can lead to larval disease referred to as bacillary necrosis (BN) leading in turn to rapid larval mortality and loss of production. Despite more than 50 years since the first detailed description of BN, we still do not fully understand its causes and mechanisms. Through the manipulation of a model larval culture of the Australian blue mussels (*Mytilus galloprovincialis*), we determined that BN is linked with rapid and systematic changes in the bacterial community.

Early investigation of larval mortality in bivalve larval cultures in the 1950s reported mortality associated with infection by gram negative bacilli that necrotised larval tissues, leading to the descriptive term bacillary necrosis (BN)[1]. The disease is capable of causing total collapse of larval cultures (larval crash) in a period of 24–48 hours and is today, the most prevalent hatchery disease worldwide, affecting more than 20 bivalve species. Whilst it is difficult to quantify the impact of BN in shellfish hatcheries, frequent recurrence can severely impact hatchery production with repercussions often felt throughout the supply chains.

The prevailing view that BN is an opportunistic disease leads to the emphasis on sound husbandry practices primarily to reduce excess build-up of organic matter. However, whether and how enriched organic conditions are linked with development of BN is unclear. Efforts to study BN have also been complicated by the unpredictable nature of the outbreaks. To address this problem, we deliberately overfed a series of identical small-scale larval cultures with microalgae to create an environment that would increase the incidence of the disease. In cultures that developed mass mortalities, automated ribosomal intergenic spaces analysis (ARISA) demonstrated that BN involves rapid and systematic changes in the bacterial community, firstly in the seawater, then rapidly proceeding to the larvae as the disease progresses and necrosis occurs (Figure 1). This study shows that, at least within the system analysed here, BN is a condition of abnormal changes in seawater-associated communities that are capable of affecting the larvae, suggestive of seawater-to-larvae infectivity. The similarity of bacterial communities in seawater and larvae at the onset of mortality suggest swamping by outgrowth of particular bacteria. Bacterial diversity examination using Illumina MiSeq sequencing of 16S rRNA amplicons showed that mortality in the model systems was linked with a bacterial community increasingly dominated by *Psychroserpens*, *Polaribacter*, *Marinomonas*, and members of the Candidatus phylum *Gracilibacteria*.

The observation that BN did not occur in all overfed cultures suggests variability in causation made it difficult to detect and control in small-scale larval cultures. In one instance, a replicate culture in which the initial development of BN was observed,

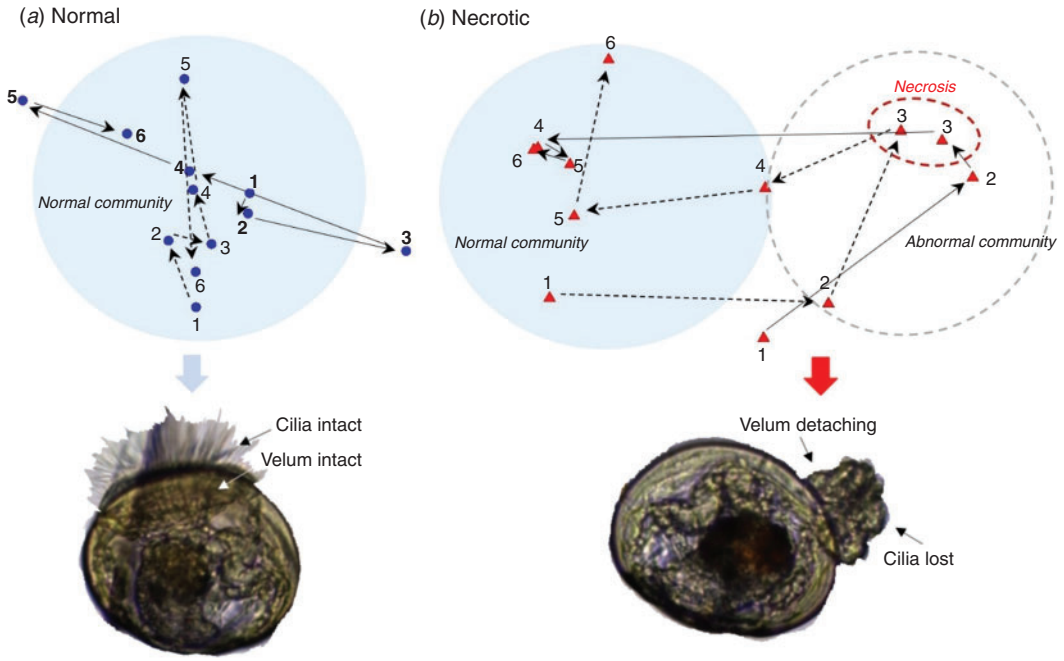

Figure 1. Comparison of bacterial communities for (*a*) normal and (*b*) necrotic conditions of larvae respectively. The solid and dotted line trajectories track shifts of bacterial community associated with seawater and larvae respectively, from day 1 to 6 of larval culture. Overfeeding can unpredictably trigger development of abnormal communities (*b*, grey dotted circle) with necrotic cultures showing communities that differ from those of normal cultures (*a*, faded blue circle). The development of necrosis (*b*) takes place first in the seawater communities where shifting occurred as early as day 1 of rearing and progressed towards the disease zone on day 2 and 3. The larval communities however, show a slower progression since at day 1 the communities were closer to the normal zone and required more than 2 days to reach the necrosis zone. Larval necrosis/mortality is characterised by the convergence of seawater and larval bacterial communities (red dotted circle) to a specific, but potentially variable disease-state community. The post mortality community was restored rapidly to the initial normal state. Necrotised larvae showed an abnormal velum that was clumped and de-ciliated and eventually detached from larvae.

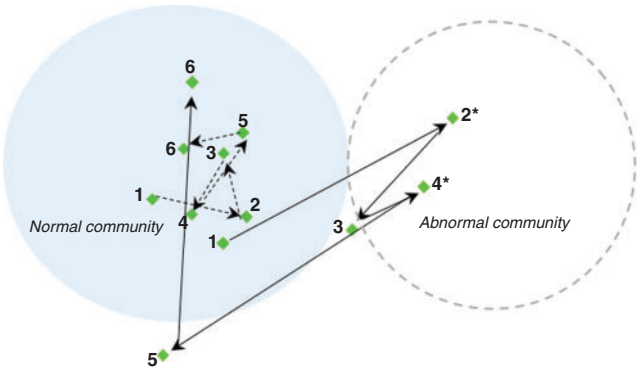

Figure 2. Bacterial communities of a larval culture that observed the initial shift of the seawater community (solid line trajectory) from normal to abnormal community zone at rearing day 1 and 2 respectively. The larval culture which was developing bacillary necrosis based on trajectories of seawater bacterial community shift was successfully mitigated after two rounds of 100% water changes at rearing day 2 and 4 (marked with asterisk; water changes were carried out after sampling for bacterial community analysis). It was observed that each of the seawater changes diverted the shift and the community progressively became closer to the normal community zone. The larval bacterial community (dotted line trajectory) remained unaffected and changed within the normal community zone.

actually had the first rearing day of normal seawater community as starting inoculum, but rapidly deviated to abnormal community in the next 24 h period (Figure 2). This suggests the seawater community characterised using ARISA fingerprinting technique may have missed the low concentration of genotypes that later emerge and drive the community to become abnormal. It appears that seawater is an important reservoir of diverse bacteria that play a critical role in the variability of BN. The link between bacterial diversity and the sporadic nature of BN is not easily established, partly due to technical limitations in resolving strain-level variation occurring in low concentration.

Currently, commercial hatchery operators still lack specific and effective means to mitigate bacillary necrosis because of the overall lack of understanding of its development process. Water changes are an important part of larval rearing activity, mainly because they help reduce organic matter build up in cultures therefore controlling the bacterial concentrations. However, there is no direct

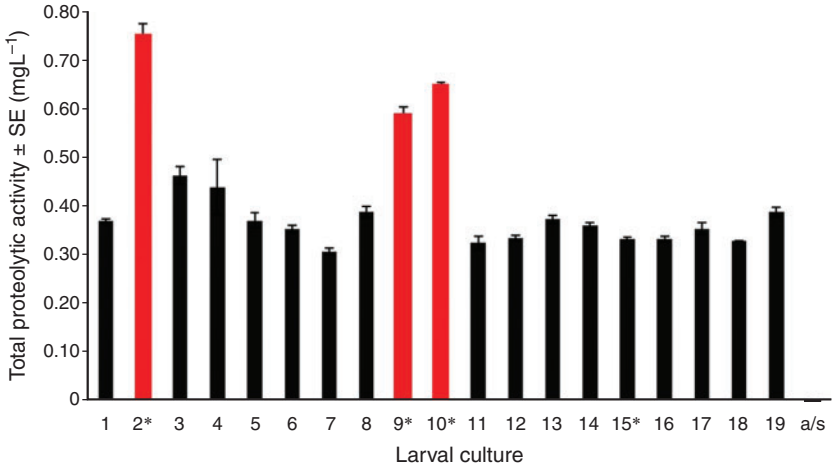

Figure 3. Protease activity (trypsin equivalent) detected in 19 replicate larval cultures. Red indicates a significant increase in protease activity (ANOVA F = 78.08, d.f. 19, $P < 0.001$) in 3 of 4 cultures suffering mortality in excess of 70% due to bacillary necrosis (marked with asterisk). Contingency analysis demonstrated significant association of total proteolytic activity and mortality (Fisher's test, $P < 0.005$). a/s denotes aged seawater. Protease activity assessed by EnzChek fluorescence protease assay kit (Thermofisher).

demonstration of how water changes can help mitigate BN. This study shows 48 h interval water changes, currently regarded as a common practice for static culture systems, can be effective if carried out before abnormal community changes are detected in the larvae (Figure 2). In cultures that suffered more rapid BN (such as Figure 1*b*), water changes did not alter the trajectory of abnormal communities.

Challenge bioassay studies demonstrate that necrotic properties of BN can be attributed to proteolytic activity of bacteria[2]. However, it is unclear how proteolytic activity is involved with BN in larval culture. A different larval culture experiment observed high microbial proteolytic activity in 3 of 4 larval cultures suffering mass mortalities (Figure 3). This suggests microbial proteolytic activity is an important disease mechanisms given that protease production in bacterial extracellular products (ECPs) is common in some seawater bacteria (such as *Polaribacter* and *Marinomonas* detected in the earlier study) and has been demonstrated to be the major virulence factors of multiple pathogenic *Vibrio* strains associated with BN[3]. However, the lack of association of proteolytic activity in one of the BN affected larval cultures (i.e. culture 15 of Figure 3) is interesting and may suggest diversity in bacterial community and in the mechanisms that lead to mortality. More work is necessary to confirm this preliminary association, particularly monitoring how seawater proteolytic activity changes with bacterial community shifts and leads/does not lead to mortality. Such work may demonstrate the potential of using protease assays as a method to assess the risk of bacillary necrosis. Once validated, protease assays could be a useful detection method to complement molecular and culturing techniques.

Based on the model larval cultures, we were able to demonstrate that BN has links with systematic bacterial community changes suggestive of a seawater-to-larvae infection. However, how this model understanding translates to commercial scale cultures requires more research. This study also described the potential of protease monitoring to aid future BN studies.

References

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Biographies

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