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Microbial diversity within the water column of a larval rearing system for the ornate rock lobster (*Panulirus ornatus*)

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Abstract

The ornate tropical rock lobster, *Panulirus ornatus* has substantial potential as an aquaculture species though disease outbreaks during the animal's extended larval lifecycle are major constraints for success. In order to effectively address such disease-related issues, an improved understanding of the composition and dynamics of the microbial communities in the larval rearing tanks is required. This study used flow cytometry and molecular microbial techniques (clone libraries and denaturing gradient gel electrophoresis (DGGE)) to quantify and characterise the microbial community of the water column in the early stages (developmental stage I–II) of a *P. ornatus* larval rearing system. DGGE analysis of a 5000 L larval rearing trial demonstrated a dynamic microbial community with distinct changes in the community structure after initial stocking (day 1 to day 2) and from day 4 to day 5, after which the structure was relatively stable. Flow cytometry analysis of water samples taken over the duration of the trial demonstrated a major increase in bacterial load leading up to and peaking on the first day of the initial larval moult (day 7), before markedly decreasing prior to when >50% of larvae moulted (day 9). A clone library of a day 10 water sample taken following a mass larval mortality event reflected high microbial diversity confirmed by statistical analysis indices. Sequences retrieved from both clone library and DGGE analyses were dominated by γ- and α-*Proteobacteria* affiliated organisms with additional sequences affiliated with β- and ε-*Proteobacteria*, *Bacteroidetes*, *Cytophagales* and *Chlamydiales* groups. *Vibrio* affiliated species were commonly retrieved in the clone library, though absent from DGGE analysis.

Keywords: *Panulirus ornatus* phyllosoma; Bacterial diversity; 16S rRNA; DGGE; Clone library; Flow cytometry

1. Introduction

The global market demand for rock lobsters continues to exceed supply from the wild and as such there is an increasing interest in developing an aquaculture sector. Due to their value and importance in Australian wild fisheries, there has been particular emphasis on *Jasus edwardsii* ($177 million), *J. verreauxi* ($4.7 million) and *Panulirus cygnus* ($305 million) to become established as aquaculture species (ABARE, 2003). However, these species do not necessarily possess favourable attributes for culture. Although the wild fishery for the tropical ornate rock lobster (*Panulirus ornatus*), being 1 of 6 tropical species in Australia, is currently valued at only $5 million (ABARE, 2003), this species has particularly

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good traits as an aquaculture candidate. *P. ornatus* is the fastest growing of the lobsters of the family Palinuridae and possesses one of the shortest known larval phases of any rock lobster species, at approximately 160 days. In addition, the growth rate of *P. ornatus* under culture conditions can be increased further through the manipulation of water temperature (Dennis et al., 1997; Lellis and Russell, 1990) and hormones (Juinio-Menezm and Ruinata, 1996).

The water column within larval rearing systems is typically a complex environment that may vary significantly from the larvae's natural environment, both in nutrient levels and microbial communities. Animals reared in an aquaculture environment are subjected to a variety of stresses, including high stocking densities and sub-optimal water conditions. As a result, defense mechanisms in culture animals are commonly adversely affected, and in an environment that is known to harbour numerous pathogens, this results in an increased susceptibility to disease (Takahashi et al., 1995). The common practice of using antibiotic and chemical treatments such as formalin in procedures prior to and during larval rearing, changes the water column microbial community. This is due to the ability of these treatments to remove some, but not all bacteria present in the water column, effectively selecting for certain strains.

To date, there has been little research published on the overall microbial community associated with wild or cultured rock lobster phyllosoma larvae and their environments. The majority of current research has instead focused on the identification of pathogenic organisms from the phyllosoma themselves. This research has reported that *Vibrio* species are the most common pathogen amongst phyllosoma larvae (Webster et al., in press; Bourne et al., 2004; Diggles et al., 2000; Handlinger et al., 1999; Handlinger et al., 2000). In a previous study we reported on the microbial diversity of the compartments of an entire *P. ornatus* larval rearing system, including the water column, biofilm and phyllosoma (Bourne et al., 2004). This study reported the presence of a number of genera within the water column, including, *Alcanivorax* sp., *Bacillus* sp., *Pseudoalteromonas* sp. and *Sulfitobacter* sp. In addition, *Vibrio* sp. were reported to be the dominant organism with *Vibrio parahaemolyticus* being the dominant species.

To effectively manage the microbial community within a larval rearing system, an understanding of the microbial composition and dynamics within each compartment (water column, phyllosoma, biofilm and live feeds) is required to interpret how community changes may affect phyllosoma health. The aim of this study was to use a range of techniques to characterise the microbial community of one compartment, the water column of an experimental *P. ornatus* larval rearing system and determine how the microbial community changes throughout early stage larval rearing. The study focused on early stage larval rearing, as this is a time that is frequently associated with high larval mortalities in a number of cultured crustaceans.

2. Materials and methods

2.1. Larval rearing technology

The larval rearing process including broodstock source, treatment and spawning, tank design, stocking methods, feeding regimes and water recirculation and treatment regimes was conducted as per the methods of Bourne et al. (2004). Moulting from phyllosoma stage 1–2 (P1–P2) occurred from day 7–12, and a larval mass mortality event occurred on day 10 as observed by a large number of dead larvae 'patches' on the bottom of the larval rearing tank.

2.2. Sample collection

Two litres of water from the water column of the 5000 L larval rearing tank was sampled daily in the morning, prior to the water exchange, using presterilized equipment. Samples were filtered through a 100 μm filter to remove phyllosoma larvae and *Artemia*, with aliquots of collected water used for subsequent analyses. Samples for flow cytometry analysis were collected each day of the larval rearing run, up until the day of a larval mass mortality event. Samples for DGGE analysis were collected each day up until five days past a larval mass mortality event. The water samples for clone library analysis were collected on the day of a larval mass mortality event.

2.3. Flow cytometry

Triplicate water samples (2 mL) were fixed with 0.2 μm filtered electron microscopy grade 3% glutaraldehyde (Electron Microscopy Sciences) in artificial seawater (17.55 gm NaCl; 0.75 gm KCl; 0.285 gm Na_2SO_4; 5.10 gm $MgCl_26H_2O$; 0.145 gm $CaCl_2$) and stored at −80 °C until required. A 100 μL volume of sample was added to 900 μL of 0.2 μM filtered sheath fluid (Becton Dickinson) and analysed as per the methods of Marie et al. (1999) using a FACScalibur (Becton Dickinson) flow cytometer. Samples were run for 1 min at a flow rate of 12 μL min^{-1}. Green fluorescence (from samples stained with SYBR Green) was collected through