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ScienceDirect
Aquaculture 260 (2006) 27–38
Aquaculture
www.elsevier.com/locate/aqua-online

Biofilm development within a larval rearing tank of the tropical rock lobster, *Panulirus ornatus*

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Received 3 May 2006; accepted 19 June 2006

Abstract

The role of bacterial biofilms in disease processes is becoming increasingly recognised in both clinical and environmental settings. Biofilm development within a rearing tank of the tropical rock lobster *Panulirus ornatus* was studied to evaluate if the biofilm is a reservoir for potentially pathogenic bacteria that cause mass larval mortalities. Within a 5000 L larval rearing tank, fiberglass microscope slides were systematically distributed during a standard rearing attempt to assess biofilm development. Culture-based counts for two media types, TCBS and Marine Agar (MA), demonstrated increased bacterial densities until days 11 and 13 respectively. For both media types, a drop in the plate counts was followed by a subsequent increase towards the end of the experiment. Scanning electron microscopy (SEM) confirmed that cell densities decreased between days 13 and 17, most likely due to sloughing of the biofilm into the water column. SEM images revealed distinct changes in dominant morphologies reflecting a succession of bacterial populations. A dynamic succession of microbial species during biofilm development was also demonstrated using denaturing gradient gel electrophoresis (DGGE) profiling of bacterial 16S rRNA genes in combination with statistical ordination analysis. Prominent changes in the DGGE profiles coincided with the decrease in bacterial numbers observed by SEM and plating on MA between days 13 and 17. Fluorescence *in situ* hybridization (FISH) identified α-*Proteobacteria* as being numerically abundant in the biofilm. This was supported by results from DGGE analysis, which retrieved only sequences affiliated with α- and γ-*Proteobacteria*. DGGE bands affiliated with *Vibrio* became dominant towards the end of the larval run (days 21 to 24). A *Vibrio harveyi* strain isolated from the biofilm late in the larval rearing trial (day 24) demonstrated increased larval mortality in small scale phyllosoma survival studies. The detection of *Vibrionaceae* at the end of the larval trial coincided with mass phyllosoma mortality and show that the biofilm is a reservoir for potentially pathogenic bacteria.

Keywords: Rock lobster; *Panulirus ornatus*; Microbiology; Biofilm development

1. Introduction

The extended larval phase of the Panulirid lobsters, spanning months to years in the wild, has so far hindered the development of a commercially viable aquaculture sector due to the inability to rear phyllosmas on a commercial scale. The tropical rock lobster *Panulirus ornatus* has one of the shortest larval phases, being estimated at 6 months, and is a potential aquaculture candidate. However, a challenging microbial environment and poor nutrition currently contribute to mass mortalities of the phyllosoma within 30 days of commencing a larval rearing trial (Bourne et al., 2004). To assist development of appropriate management

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0044-8486/$ - see front matter.
doi:10.1016/j.aquaculture.2006.06.023

regimes which promote optimal phyllosoma health, the microbial dynamics within the lobster aquaculture facility needs to be more fully understood. Within any aquaculture rearing system, a number of individual though interconnected, microbial environments exist. As a means to understand the microbial dynamics within the larval rearing system of the tropical rock lobster *P. ornatus*, we have identified four individual compartments, the water column, the phyllosoma, live feeds and the biofilm forming on the walls of larval rearing tanks. To fully understand the microbial processes occurring within the entire larval rearing system, each of these compartments needs to be investigated, with a view to how they are connected.

Increasingly, the role of biofilm formation in the survival and transmission mechanisms of pathogenic microorganisms is being understood. As a biofilm evolves it develops into a complex three-dimensional environment that can provide a protective matrix that allow specific species to colonize and survive in an otherwise unfavourable environment (Davey and O'Toole, 2000; Hall-Stoodley et al., 2004). Biofilm formation is a dynamic process, capable of responding to environmental stimuli. Initially, cells organize into micro-colonies and through cell division and cell recruitment they grow and encase themselves in an extracellular matrix. Within this matrix complex and differentiated associations can be formed, which facilitate nutrient uptake (Hall-Stoodley et al., 2004; Toutain et al., 2004). From mature biofilms, cells are released and re-enter the planktonic state, making biofilm development a cyclic pathway. Detachment from biofilms can be caused by a number of factors including external perturbations or internal processes, though many species appear to use dispersal as an active means of colonizing new niches (Hall-Stoodley et al., 2004; Sauer et al., 2002).

The potential for biofilms to act as a reservoir for pathogenic bacteria has been well reported in clinical infections (Ebrey et al., 2004; Ehrlich et al., 2004; Flanders and Yildiz, 2004; Hall-Stoodley and Stoodley, 2005). The biofilm forming in aquaculture rearing environments has previously been identified as a potential reservoir of pathogenic bacteria, which may have detrimental effects on larval health (Karunasager et al., 1996; King et al., 2004; Kittaka, 1994). Bacterial species including *Photobacterium*, *Vibrio*, and *Aeromonas* species have been shown to be potential pathogenic components of piscine aquaculture rearing facilities (King et al., 2004). The prawn pathogen *Vibrio harveyi* can be an integral part of biofilm formation in aquaculture ponds providing protection from disinfection while also being a potential reservoir in recurrent infection within the hatchery (Karunasager et al., 1996; Paclibare et al., 1998). Karunasager et al. (1996) demonstrated that *V. harveyi* which established within the biofilm on different aquaculture surfaces was highly resistant to water sanitisers and antibiotics treatments emphasising the need for physical removal of the biofilm and periodical drying of tanks to reduce the chance of infection.

Studies of the microbiology of lobster larval rearing have primarily focused on the phyllosomas and the potential pathogens causing disease and mortality (Diggles, 2000; Diggles et al., 2000; Handlinger et al., 2000, 1999; Webster et al., 2006). Preliminary studies of the biofilm of our lobster larval rearing environment commonly retrieved *Vibrio* species from culture based analyses, however they were absent from denaturing gradient gel electrophoresis (DGGE) bacterial profiles (Bourne et al., 2004). The aims of this more in-depth study were to elucidate the composition and dynamics of the bacterial biofilm community developing in larval rearing tanks and to determine if the biofilm acts as a reservoir for pathogenic bacteria seeding disease outbreaks and leading to mass phyllosoma mortality. To achieve this, biofilm development in a larval rearing tank was thoroughly investigated using a polyphasic approach incorporating culture based studies, scanning electron microscopy (SEM), fluorescence *in situ* hybridization (FISH) analysis and denaturing gradient gel electrophoresis (DGGE) to obtain both quantitative and qualitative information on microbial community development.

2. Materials and methods

2.1. Larval-rearing technology

Details of collection and broodstock spawning of the tropical rock lobster *P. ornatus* have been reported previously (Bourne et al., 2004). Newly hatched phyllosomas were reared in a 5000 L larval-rearing tank, after sterilizing with chlorine, and filled with 1 μm filtered seawater. Incoming seawater was treated with 990 mL of chlorine (100 g L^{-1} stock) for 24 h and then neutralised with 300 mL 1 M sodium thiosulphate with vigorous aeration. Every 24 h the larval-rearing tank received a 50% water exchange with seawater treated as described. In addition, water was continuously recirculated through the larval-rearing tank by pumping 12 L min^{-1} through a 100 μm filter, followed by a foam fractionator tower at a rate of 8700 L h^{-1} fed with ozone through a venturi system with a oxidative redox potential (ORP) setting of between 290 and 320 mV, followed by a 1 μm filter and a UV light (64 W UV sterilizer at 254 nm with a nominal rating of 2.5 μW s cm^{-2} at a flow rate of 420 L h^{-1}) before being returned to the 5000 L larval-