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Bacteria Associated with Biofilms in a *Macrobrachium rosenbergii* Hatchery

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Abstract

Biofilms are important biological structures formed on most submerged aquatic surfaces. They comprise a unique niche wherein a community of microorganisms co-exist. The study was undertaken to identify the bacterial flora associated with biofilms formed on the surface of larval rearing tanks in a prawn hatchery. Surface swabs of two randomly chosen larval rearing tanks in a hatchery were taken regularly throughout the hatchery cycle and the bacterial loads were estimated. The counts varied from $1.8x10^3$ to $4.3x10^4$ $CFU \cdot cm^{-2}$ in Tank I and $5.1x10^3$ to $3.5x10^4$ $CFU \cdot cm^{-2}$ in Tank II. No significant difference was observed between Tank I and Tank II in respect of biofilm bacterial count when enumerated ($p<0.05$). The common bacterial genera isolated in both tanks comprised gram-negative bacteria including *Pseudomonas, Vibrio* and *Aeromonas. Bacillus* and other non-spore formers were the predominant gram-positive bacteria isolated. Bacteria associated with biofilms are more resistant to antibiotics and water sanitizers generally used in tank water treatment. Biofilms can be a reservoir of pathogens and bacteria such as *Pseudomonas, Aeromonas* and *Vibrio* isolated from the biofilms in this study may be potential pathogens of prawn larvae in hatcheries.

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Introduction

Biofilm formation is an important phenomenon in aquatic systems and bacteria may adopt it as one of the survival strategies since they are exposed to sanitizers and antibiotics regularly in shrimp and fresh water prawn hatcheries. Bacteria settle on the surface of various materials such as pipes and tanks and form biofilms (Karunasagar et al. 1996). Importance of biofilms in fish processing and aquaculture industry is being increasingly recognised and the role of bacterial biofilms as a source of pathogens has been reported (Karunasagar et al. 1996; Joseph et al. 2001). Biofilms of *Vibrio harveyi*, an important bacterial pathogen of shrimp causing luminescent vibriosis, has caused large-scale mortalities in both shrimp and prawn hatcheries (Karunasagar et al. 1994; Tonguthai 1995). Progressive low-level mortalities due to biofilm on gills by *V. alginolyticus* (Austin et al. 1993) have been reported in juvenile turbot, *Scophthalmus maximus* and epidemic mortalities of Chinook salmon (*Onchorrhynchus tshwytscha*) alevins (Newboud et al. 1993). Bacterial biofilms are reported to be highly resistant to antibiotic and sanitizer treatments (Costerton et al. 1987). Karunasagar et al. (1996) demonstrated that biofilms could be formed even in the presence of antibiotics. Interestingly, most pathogenic bacteria in biofilms have been found to be autochthonous to the system.

Culture of freshwater prawn, *Macrobrachium rosenbergii* is gaining popularity in India with over 34,630 ha growout ponds stocking this species and over 71 hatcheries operating in the country (Bojan 2003). Very little is known about the bacteriology of *Macrobrachium* hatchery and the flora that form biofilms on tank surface. The objective of the present study was to understand the bacterial species forming biofilms on the surface of hatchery tanks during the operation of the larval rearing cycle.

Materials and methods

The study was carried out in a *M. rosenbergii* hatchery located in Chennai in the east coast of India that employed the 'clear water system'. The system involves the use of algae free water in which the larvae are reared on artificial feed. Sea water filtered through coarse sand filters at the point of drawing is taken to a reservoir from where it passes through a fine sand- charcoal bed filter before it is stored in the tanks. The filtered sea-

water is then mixed with cartridge filtered well water in a separate tank in proportions that yield the required salinity (hereafter called the source water). This source water is chlorinated at first followed by de-chlorination. The de-chlorinated water is filtered through high-pressure sand bed filters, UV treated and then passed through fine mesh cloth filters before letting into the larval tanks. The larval rearing practice involved a daily water exchange with the source water, which varied between 10-50%, followed by the siphoning out of accumulated feed waste, larval faeces and debris including few dead larvae. The tank surfaces were spray-cleaned with fresh water with minimal physical scrubbing of the surface with a wet sponge dipped in formalin (0.5 ppm) and the tanks refilled with fresh water. The larvae were fed with live feed such as *Artemia* or cooked egg custard.

Sample collection

Two tanks were selected at random and swabs of biofilm formed in the inside of the larval rearing tank surface were taken regularly (3-10 days interval) throughout the rearing cycle. A 1 cm^2 region of the tank surface was swabbed with sterile cotton swab, when the water level in the tank was reduced during cleaning. Samples were taken in duplicate. The swabs were immersed in 9 ml sterile saline and shaken vigorously to detach bacteria from the cotton into the saline. Serial dilutions were made and aliquots taken to estimate the bacterial load.

Bacteriological analysis

The bacteria were enumerated by spread plate method on tryptone soya agar (HiMedia, Mumbai) supplemented with 1% sodium chloride (TSAS). The plates were incubated at 30°C ± 1°C for 24-48h and the colonies enumerated. Representative colonies were picked up, purified on TSAS and subjected to a battery of biochemical tests for identification (MacFaddin 1980). The scheme of Le Chevallier et al. (1980) and Bain and Shewan (1968) was used to identify gram-positive and gram-negative bacteria up to the generic level.

Statistical analysis

The difference in the bacterial counts between the two tank surfaces was assessed using Mann-Whitney U test. All the data was log transformed prior to calculations.

Results and Discussion

The results of the bacterial count of the biofilm are presented in table 1 and figure 1. The counts in the tanks varied from $1.8x10^3$ to $4.3x10^4$ CFU·cm^{-2} in Tank I and between $5.1x10^3$ to $3.5x10^4$ CFU·cm^{-2} in Tank II. No significant difference in the bacterial counts in the biofilm population in the two tanks was observed ($p < 0.05$). In Tank I, there seemed to be an increase in the counts of bacteria as the culture cycle progressed but in Tank II, the counts were marginally varying during the culture period.

Table 1. Count of biofilm bacteria (CFU· cm^{-2}) on tank surface during the hatchery operation

Day of culture	Tank I		Tank II	
	Larval Stage	Count	Larval Stage	Count
1	S-2	$1.8x10^3$	S-1	$1.1x10^4$
6	S-4	$6.8x10^3$	S-3	$1.1x10^4$
10	S-6	$8.7x10^3$	S-5	$5.1x10^3$
20	S-10	$3.4x10^4$	S-8	$2.9x10^4$
30	PL6	$2.8x10^3$	S-11	$6.4x10^3$
33	PL9	$5.6x10^3$	PL1	$2.1x10^4$
36	PL12	$4.3x10^4$	PL4	$3.5x10^4$
40	PL16	$2.6x10^4$	PL8	$7.6x10^3$

S = Stage; PL = Postlarvae

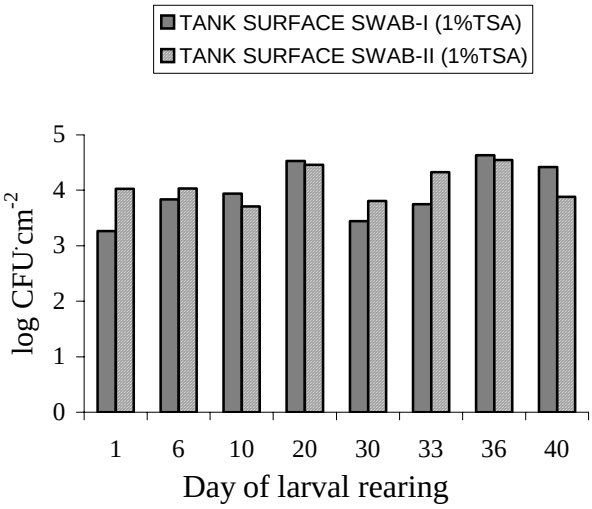

Fig. 1. Bacterial Count (CFU·cm^{-2}) of biofilm on tank surface during the hatchery cycle

Tables 2 and 3 show the bacterial genera associated with the biofilm formed in Tank I and Tank II, respectively. *Pseudomonas*, *Vibrio* and *Aeromonas* were the commonly observed gram-negative bacteria while *Bacillus* and other non-spore formers were the dominant gram-positive bacteria in both tanks. *Pseudomonas* was observed to be the dominant genus accounting for a maximum level of 54.2% of the total flora in Tank I on day 30 of hatchery operation while gram-positive bacteria were observed at a maximum level (55.8%) on day 33 in Tank II. No clear dominance of any one bacterial genus was observed in the biofilm of both Tank I and Tank II. In Tank I, gram-positive flora accounted for 40% of the flora initially and as the hatchery operation progressed, there was a shift towards gram-negative bacteria. *Pseudomonas* was present throughout the larval rearing cycle in varying proportions. The next most common genera observed were *Bacillus* and *Vibrio*. In Tank II, gram-positive bacteria constituted 50% of the population initially. This group was fluctuating in levels and during the last sampling (day 40), the flora was dominated by *Aeromonas* (63.2%) with complete absence of Gram-positive bacteria. *Vibrio*, *Pseudomonas* and *Bacillus* were the most common genera observed.

Table 2. Bacterial generic profile (in percent) of surface swab isolates from Tank I

Genus	Days of culture – bacterial profile (%)							
	1	6	10	20	30	33	36	40
Bacillus	20.0	20.0	14.6	-	12.5	10.0	5.7	21.4
G+NSF	20.0	20.0	27.0	-	-	-	3.1	6.0
Moraxella	-	-	-	16.6	-	-	3.1	3.0
Acinetobacter	10.0	-	-	-	-	-	-	-
Pseudomonas	10.0	6.7	37.5	42.8	54.2	22.0	30.5	38.1
Vibrio	-	33.3	4.2	14.3	20.8	-	10.1	23.2
Aeromonas	20.0	6.7	-	14.3	-	40.0	40.0	7.7
Plesiomonas	-	-	-	-	4.2	-	-	-
Micrococcus	10.0	-	4.2	12.0	-	-	-	0.6
Staphylococcus	10.0	-	-	-	8.3	20.0	3.1	-
Klebsiella	-	-	2.1	-	-	-	-	-
Proteus	-	-	10.4	-	-	8.0	4.4	-
Unidentified	-	13.3	-	-	-	-	-	-
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

G+NSF = Gram positive non-spore formers

Biofilm formation occurs on any surface that remains submerged. Bacteria are known to colonize the surfaces immediately after the formation of a film of organic molecules (Whal 1989). Thompson et al. (2002) observed that high numbers of bacteria attached to the surface within 24h after the organic layer is formed. In a prawn hatchery, the tanks are cleaned

by scrubbing the tank surface and washing in chlorinated water and drying before the commencement of operation. The bacterial load in biofilms is presented in table 1. The load of bacteria in the biofilm was similar to that found in the corresponding larval rearing tank water (unpublished data). Venugopal et al. (1999) in their study on biofilm of *V. parahaemolyticus* found that a 10 day old biofilm had a count of $2.4x10^4$ CFU•cm^{-2}. Karunasagar et al. (1996) demonstrated that the formation of a biofilm was favored by the type of surface used. They reported that biofilm cell density was highest at $5.3x10^7$ CFU•cm^{-2} on HDPE surface than on concrete slab ($8.5x10^6$ CFU•cm^{-2}) and steel coupon ($2.4x10^6$ CFU•cm^{-2}). These observations will be important in hatcheries and should be kept in mind since such materials are commonly used. Thompson et al. (2002) estimated a maximum bacterial count of $1.48x\ 10^7$ mg^{-1} of biofilms in microbial consortium developed on shrimp larval rearing tanks.

Table 3. Bacterial generic profile (in percent) of surface swab isolates from Tank II

Genus	Day of culture – bacterial profile (%)							
	1	6	10	20	30	33	36	40
Bacillus	16.7	26.5	1.6	30.6	44.3	10.3	30.1	-
G+NSF	33.3	8.4	19.4	-	2.7	55.8	1.9	-
Moraxella	-	-	1.6	-	-	-	-	-
Acinetobacter	-	-	-	-	1.2	-	37.9	-
Pseudomonas	16.7	35.0	24.2	25.0	7.5	4.4	-	10.3
Vibrio	16.7	3.6	3.2	11.1	10.3	13.3	22.3	10.3
Aeromonas	-	4.8	9.7	8.3	11.0	-	-	63.2
Plesiomonas	-	-	3.2	-	-	8.8	4.9	-
Micrococcus	-	18.1	-	5.6	21.0	7.4	-	8.8
Staphylococcus	16.6	3.6	30.6	-	2.0	-	-	7.4
Klebsiella	-	-	-	-	-	-	1.0	-
Proteus	-	-	3.2	-	-	-	-	-
E.coli	-	-	-	5.6	-		-	
Moraxella	-	-	-	13.8	-		-	
Unidentified	-	-	3.3	-	-	-	1.9	-
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

G+NSF = Gram-positive non-spore formers

Our study did not indicate the dominance of any particular bacterial genera in the biofilm, but a diverse group was found associated. The flora identified was autochthonous to the system and this flora could be expected in the biofilm. However, under favorable conditions of temperature, pH and increased organic load, dominance of a particular group can be expected. In this study the percentage contribution of different bacterial groups in biofilm seemed to be fluctuating. Nevertheless, *Vibrio, Aeromonas, Pseudomonas* and *Bacillus* were isolated on most of the occasions.

Though Kanaujia et al. (1998) and Phatarpekar et al. (2002) observed the absence of *Vibrio* in *Macrobrachium* hatcheries, Tonguthai (1995) observed luminescent vibriosis in *Macrobrachium* larvae due to *V. harveyi*. Karunasagar et al. (1994) reported mortalities of shrimp larvae due to biofilm associated *V. harveyi*. In this study, *Vibrio* spp. constituted an important bacterial group in biofilms on tank surfaces. *Pseudomonas* and *Aeromonas* were reported to cause black spot bacterial necrosis in juveniles of *Macrobrachium* spp. (Lombardi and Labao 1991; Jayasree et al. 1999). Though *Vibrio*, *Aeromonas* and *Pseudomonas* were isolated as part of the normal flora, stress conditions may cause them to become opportunistic pathogens.

Apart from being a reservoir of pathogenic bacteria (Karunasagar et al. 1996), when present in biofilms, they are difficult to eliminate and are more resistant to the effect of antibiotics and sanitizers (Thompson et al. 2002; Costerton et al. 1999). Preventing the establishment of such bacteria as biofilms in rearing tanks would be important.

Biofilms may also be useful in the hatchery setting. Thompson et al. (2002) demonstrated the importance of biofilms in improving water quality and as a source of nourishment in the culture of shrimp, *Farfantepenaeus paulensis*. The usefulness of biofilm in preventing the growth of pathogenic bacteria has also been reported (Austin and Day 1990; Thompson et al. 1999). *Bacillus* spp. has been reported to produce anti-vibrio compounds (Vaseeharan and Ramasamy 2003) and these bacteria are widely used as probiotics in shrimp farms. However, their usefulness as a probiotic in *Macrobrachium* hatchery needs to be evaluated. This study shows that *Bacillus* and other gram-positive non-spore forming rods constitute an important component of biofilm on the surface of tanks in *Macrobrachium* hatchery.

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