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Diversity and Anti-vibrio Activity of Biofilm-Forming Bacteria in *Penaeus Monodon* Pond in one Cycle of Operation

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ABSTRACT

Luminous vibriosis, caused by Vibrio harveyi is a serious and common systemic disease affecting Penaeus monodon larvae and juveniles. V. harveyi, is ubiquitous in marine environment and is opportunistic pathogen producing biofilm coating that protects it from drying and disinfection's procedures during pond preparation. Microbial biofilm have been found to increase fish production in ponds by increasing heterotrophic production through periphyton proliferation on available substrates but their role as antagonists of target pathogen has not been investigated. This study focused on the diversity of bacteria colonizing the biofilm and screens them for possible anti-vibrio activity targeting V.harveyi as the pathogen. Biofilm aging 1-11week were examined for direct microscopic count using fluorescence microscopy and viable counts in conventional media. Colonization of rounded bacteria was noted for most of the sampling period. The eight-week old biofilm showed the most diverse (D=0.90) and richest microbial community with viable count of 1.25 x 108 CFU/mm2 of heterotrophic bacteria. Vibrio population started to stabilize on the four-week old biofilm and attained its maximum on the eight week at 6.5 x 10-3 CFU/mm2. A total of 46 isolates were successfully cultured in conventional and selected media. Of these isolates, only eighteen were able to repress the growth of V, harveyi in parallel streak. Four isolates (V35, V36, N61 and N63) showed great reduction on the growth of V. harveyi smear better than the positive control antibiotics, chloramphenicol and furazolidone. The reduction may be due to competitive exclusion resulting from competition of space brought about by compression effect of the isolate on the pathogen as they were parallel streaked. Analysis of variance (P=2.27) showed no significance difference between isolates exhibiting anti-vibrio potential. Furthermore, T test (P=-6.314) indicated that the reduction of the pathogen by each of the 18 isolates is comparable to that of the positive control antibiotics. Isolates V17, V35 and V1 showed greater inhibition of the pathogen in the cylinder cup assay than the positive controls. Most of the isolates were within the range of the positive controls (10-12mm inhibition). Bacteria showing positive activity in parallel streak and cylinder cup co-cultivated in broth with the pathogen and showed a reduction of 88.24%, 72% and 69.86 by Klebsiella (N39), Vibrio sp.1 (V1) and Vibrio sp.2 (V2), respectively. The other isolates showing anti-Vibrio activity belong to six major genera: Aeromonas, Bacillus, Flavobacterium, Klebsiella, Pseudomonas and Vibrio.

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INTRODUCTION

The farming of black tiger shrimp (*Penaues monodon*) contributes significantly to the Philippine economy and other countries in the Asia-Pacific region. Production in many of these countries has declined due to luminous bacteria, *Vibrio harveyi*, which has caused death to nearly 100% of shrimp stock in hatcheries and ponds. The use of antibiotics and other chemicals in controlling shrimp pathogens becomes ineffective, as the strains grow more resistant to these chemicals. Moreover, the bacterial pathogen (*V. harveyi*) produces biofilm coating that protects it from drying and disinfection's procedures followed during pond preparation. Biological control is being considered as an alternative means of preventing shrimp disease outbreak. The main principle behind biological control is to enhance the growth of beneficial microorganisms, which serve as antagonists of target pathogens. Biofilm are physiologically different from free-floating bacteria living in the aqueous phase but their role as antagonists of target pathogen has not been investigated.

In nature, bacteria are either planktonic, sessile, living in-groups or aggregates of cells, or attached to

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a surface, as a "biofilm" and will behave differently depending on their growing state (Gloersen, 2000). In aquatic environment, higher numbers of bacteria reside attached to submerged surfaces than in the aqueous phase itself (Lappin-Scott *et al.*, 1992). Bacterial biofilm are notably resistant to drying and disinfections than their planktonic counterparts (Karunasagar *et al.,* 1994, 1996).

Microbial biofilm have been found to increase fish production in ponds by increasing heterotrophic production through periphyton proliferation on available substrates (Shankar *et al.,* 1998). Biofilm formation commences with the colonization of a surface by bacteria (Lappin-Scott *et al.,* 1992). The association is reversible but eventually becomes irreversible due to adherent of cells. This adhesion is a result of the cell's ability to produce exopolysaccharide (glycocalyx). Within the glycocalyx matrix, the cell divides and forms microcolonies and recruiting bacteria floating nearby the colony into the matrix and become embedded within it. Eventually an adherent multi-species biofilm is formed (Hudson and Sherwood, 1997). Adhesion to surfaces provides considerable advantages for the bacteria that live within the biofilm, including protection from antimicrobial agents and the many benefits gained from close proximity to other microorganisms as in the exchange of nutrients, metabolites and genetic material (Lappin-Scott *et al.,* 1992). Aquaculture practices such as discontinuous culture cycles, disinfection or cleaning of ponds or tanks prior to stocking, and sudden increases in nutrients due to exogenous feeding generally do not provide appropriate environments for the establishment of stable microbial communities

The use of biofilm to reduce excess nutrients in hatchery does not only maintain the water quality but also reduces the risk of pathogen introduction since the system does not require water exchange (Khathoon *et al*, 2007). Biofilm can reduce the levels of ammonium and phosphate in the rearing water (Bratvold and Browdy, 2001) and serves as a food source for the shrimp *Farfantepenaeus paulensis.* A mature biofilm composed of pennate diatoms and filamentous cyanobacteria lead to reduced exportation of phosphorus (33% less phosphate) and to a higher output of nitrate+nitrite, instead of ammonium. Biofilm was also an important complementary food source for the shrimp, increasing their growth (Thompson *et al.,* 2002).

Studies on the potential use of biofilm bacteria to increase fish production include: promoting and quantifying microbial biofilm to increase fish production in ponds (Umesh, 1993); developing bacterial biofilm in ponds for possible application in the oral vaccination of carps (Shankar *et al.*, 1993); and the use of bacterial pathogen biofilm as a better alternative to its free cell form for oral vaccination of fish. Biofilm of *Aeromonas hydrophila* has been successfully developed in chitin particles *in vitro* for use in oral vaccination with promising results (Azad *et al*., 1997).

Vibrio species have been considered a part of the normal shrimp microflora and macroflora but certain strain(s) may be more pathogenic than the others. When these pathogenic strains are plentiful, they can overwhelm the immune system of shrimp, allowing disease to develop. In naturally diseased *P. monodon, V. harveyi* invades the hepatopancreatic tubules and cause extensive lesions even in the absence of other pathogens such as baculovirus and parasites (Jiravanichpaisal *et al.,* 1994). *V. harveyi* produces proteases, phospholipases or hemolysins which may play important roles in the pathogenicity on *P.monodon* (Liu *et al.,* 1996). *V. harveyi* could form biofilm on cement slab, high-density polyethylene plastic and steel surface (Karunasagar *et al.,* 1994, 1996).

The difficulty in reducing the concentration of pathogenic bacteria in shrimp ponds by conventional chemical disinfections, other means of biological control must be explored to make shrimp hatcheries viable. Aquatic microorganisms are exposed to a wide variety of environmental challenges – physical and chemical factors, which in a multitude of ways, may also act with or against one another. They influence not only the size and composition of the microbial population, but also the morphology and physiology (fundamentally affecting the function of metabolic enzymes) of the individual bacterium (Rheinheimer, 1992).

The objectives of this study is to examine the diversity of biofilm forming bacteria or bacteria colonizing the biofilm ,isolate andculture these bacteria and screen them for potential anti-vibrio activity.

MATERIALS AND METHODS

Sampling Area:

The study was conducted in one of the ponds of the Bureau of Fisheries and Aquatic Resources-National Fisheries Research Development Institute (BFAR-NFRDI) at Pacita, Lala, Lanao del Norte. The semi-intensive prawn pond takes advantage of the Lala River and Panguil Bay for their water management. The *Penaeus monodon* post larvae (P_{20}) were stocked on the fourth week of the sampling and were transferred later to the production pond on by the 11^{th} week, thereby terminating the sampling period. Pond operation included the addition of formulated feeds, propagation of plankton by organic and inorganic fertilization

Preparation and Setting of Glass Slides:

Thirty three slide sets were prepared by tying them back-to-back with magnetic wire and deployed in different parts of the pond at the start of the

operation. Three slide sets were retrieved weekly for 11 successive weeks.

Preparation of Media:

Seawater agar (SWA) plates and slants, Seawater broth (SWB), thiosulfate citrate bile sucrose agar (TCBS), triple sugar iron agar (TSI), sulfur indole motility agar (SIM), Simmon's citrate agar, methyl red-Voges Proskaeur broth, Hugh and Leifson agar, and nitrate broth were prepared aseptically following the standard operating procedures (Merck, 2000) modified by using sterile filtered seawater from the pond.

Direct count:

Two glass slide were made as a slide set by tying them back to back with a nylon. Forty-five slide sets were prepared and deployed in different areas of the pond. Three slides were retrieved weekly for eleven weeks, one set for direct examination of biofilm-forming bacteria the other two sets for the culture. Direct counts were done with epifluorescence microscope using acridine orange for stain.

Estimation of various index of diversity:

The diversity of the bacterial community was evaluated using Simpson's index of dominance (D), Simpson's diversity index (1-D), and Simpson's reciprocal index (1/D),Shannon Weiner Index and Evenness index.

Bacterial Culture:

The biofilm slides were scraped aseptically using a spatula to the 10 ml sterile filtered pond water (SFPW). The SFPW with the scrapings were centrifuged at 13,000 rpm for 15 minutes to detach the cells from the clump. A volume of 0.1 mL of 10^{-3} to 10^{-5} dilutions was pipette and spread plated in seawater agar for isolation of total heterotrophy. For enumeration and isolation of Vibrio species, 0.1mL of dilutions of 10^{-2} to 10^{-3} was used. Spread plate was used to culture the bacteria determine the viable count of the bacteria. The plates were then incubated for 18-24 hours at room temperature and the colony forming units (CFU/mm^2) were calculated by the formula:

$$\text{CFU/mm2} = \frac{Number\ of\ colonies}{(No.\ of\ plates)(Volume\ plated)\ (Dilution\ of\ sample)}$$

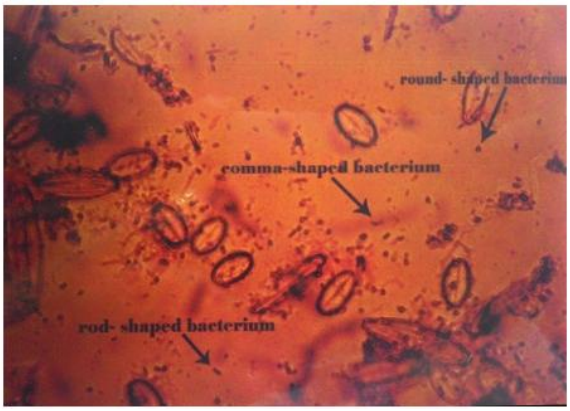

Fig. 1: The biofilm colonized by the rounded, rod and comma-shaped bacteria and somemicroalgae.

Isolation and purification of bacterial isolates:

Well-separated bacterial colony from the SWA plates were inoculated in agar slants by streaking and incubated for 24 hours at room temperature and purified by series of sub-cultures. The purified isolates were morphologically characterized and subjected to a series of biochemical tests.

Screening for anti-vibrio activity:

Each isolates was screened for possible inhibitory activity on *V. harveyi* using parallel streak method, cylinder cup and co-cultivation as outlined by Maeda and Nogami (1989). Anti-vibrio activity was determined in terms of reduction of the width of *V. harveyi*, smears by the isolate within one-week cultivation using chloramphenicol and furazolidone as standards. The concentrations of the isolates and pathogen were standardized with the turbidity of Mac Farland ($1.5x10^7$ cells ml^{-1}).

Cylinder cup assay:

Bacterial lawns (2ml) of 2% *V. harveyi* was prepared and overlaid in SWA plate. Five sterile cylinder cups were laid into each plate and 200µl of the isolate in broth were introduced into the cup and incubated at room temperature

Characterization and Identification of bacteria showing anti-vibrio activity:

Forty-six bacterial isolates from biofilm isolated both in SWA and TCBS agar plates were screened for inhibitory activity on the pathogen by parallel

streak, cylinder cup and by co-cultivation.Bacteria showing inhibitory activity were characterized as to morphology of their colonies, individual cells and selected physiological and biochemical tests for identification using conventional and selective media, BBL crystal and API.

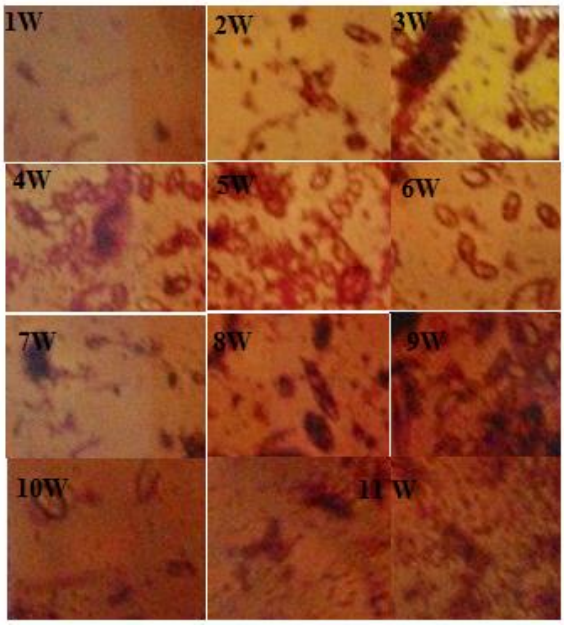

Fig. 2: Succession of bacteria colonizing in 1-11 week-old biofilm

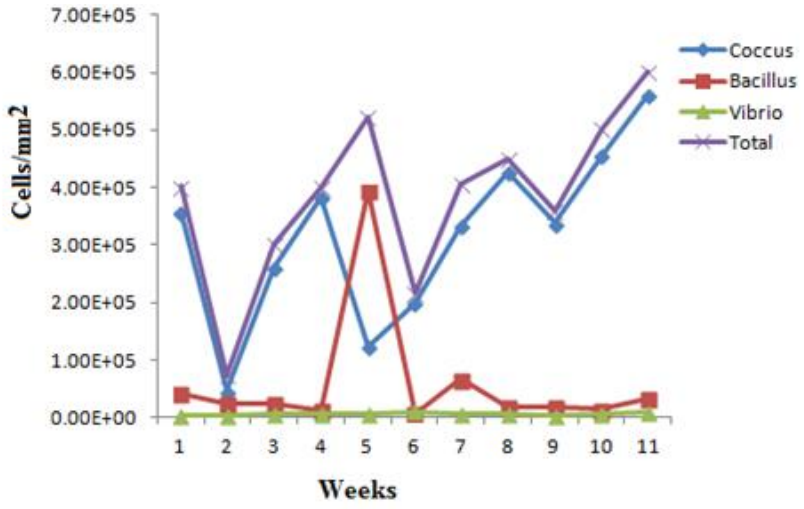

Fig. 3: Direct count of the different morphotypes of bacteria colonizing the 1 to 11 week old biofilm

Results:

Direct Count:

The retrieved biofilm stained with acridine orange dye under the microscope shows three distinct morphotypes: round-shaped(1.4µm), rod-shaped (5.6-7µm) and comma-shaped (4.2-5.6µm) and some microalgae (fig1). The rounds shaped dominated the biofilm community followed by the rods with the commas in the minority group for most of the sampling period. A drastic decrease on the cocci count was observed on the fifth week and an instantaneous increase in the rods (fig 3).

Algal growth was evident after a week submersion and attachment of the barnacles on the glass surface started, on the third week, reaching its peak number the following week (fig.2). The 4-week old and the 8-week old biofilm had the highest probabilities of two randomly selected individuals belonging to the same shapes of 90.8% and 90%, respectively (Table1). Two-week biofilm had the highest probability (50.8%) that two randomly selected individuals belong to different shapes and has the most similar in abundances among the three morphotypes (E=0.7543). Round shaped bacteria dominated over the rods and comma-shaped during

these weeks. The four-week old biofilm harbored about 3,818 rounds/mm^2 compared to only 132 rods/mm^2 and 59 commas/mm^2, in ratio and proportion of 65 rounds: 3rods: 1 comma per square mm compared to 8-week biofilm with 72 rounds : 3 rods: 1 comma.

Table 1: Index of Diversity of 1-11 week old biofilm from the direct counts.

Biofilm age	Richness	Simpson's Index	Simpson's Index of Diversity	Simpson's Reciprocal Index	Shannon-Weiner Index	Evenness Index
1 week	3	0.798	0.022	1.253	0.38	0.345
2 weeks	3	0.492	0.508	2.032	0.827	0.753
3 weeks	3	0.812	0.188	1.232	0.382	0.348
4 weeks	3	0.908	0.092	1.101	0.221	0.201
5 weeks	3	0.619	0.381	1.615	0.615	0.560
6 weeks	3	0.848.	0.152	1.179	0.336	0.306
7 weeks	3	0.699	0.301	1.431	0.530	0.483
8 weeks	3	0.900	0.100	1.111	0.235	0.214
9 weeks	3	0.884	0.116	1.131	0.254	0.231
10 weeks	3	0.828	0.172	1.207	0.345	0.316
11 weeks	3	0.871	0.129	1.148	0.285	0.260

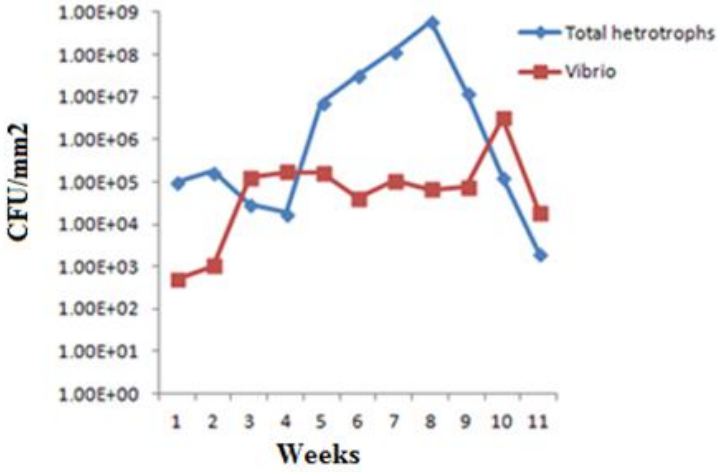

Fig. 4: Mean bacterial load of beterotrophs and vibriocolonizing in one toll week old biofilm in prawn pond

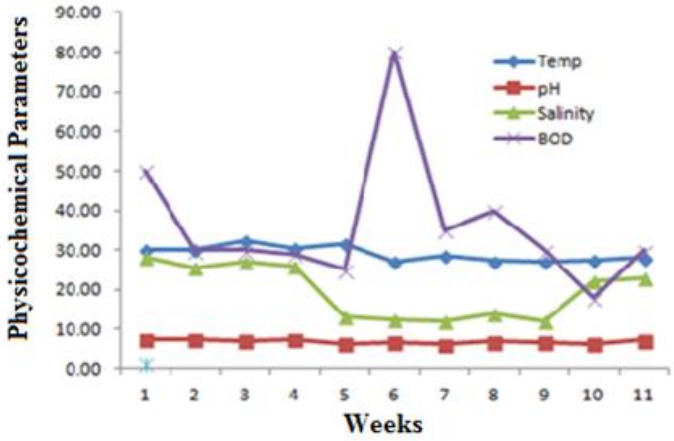

Fig. 5: The physicochemical parameters in prawn pond during the eleven weeks of culture.

Viable Count:

The mean bacterial load on the biofilm community increases with length of exposure although some fluctuations occur (fig. 2). The physicochemical parameters (temperature, pH, salinity and BOD) readings for 11 weeks are shown in Fig 3. The heavy rains coupled with flooding (carrying with it domestic and agricultural effluents) experienced during the fourth week till the eighth week of sampling had decreased the temperature readings from 32^{o}C to 27^{o}C. Dilution of the original salinity by half was experienced on the first three weeks of sampling. BOD reading ranged from 18 to 80 mg O_2/L beyond the ideal BOD in pond, which is

3-6ppm) indicated an increasing pollution level to the already polluted water. The pH on the other hand stayed within normal range of 6.5 to 7.9.

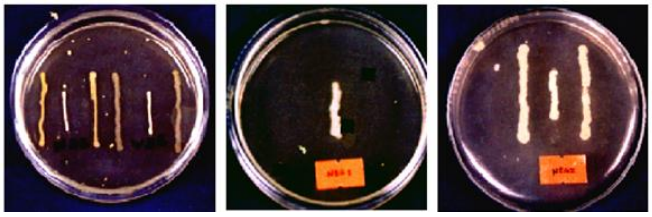

Fig. 5: Width of V. harveyi as affected by isolates N36 and V35, width of V. harveyi in negative ontrols 1 and 2 respectively for A, B and C plates.

Vibriostat Activity:

Of the 46 isolates only eighteen were able to repress the growth of the pathogen. The greatest inhibition of the pathogen (2.5mm) were exhibited by isolates V35, N36, N39, N61 and N63 reducing growth by 1.5, half a millimeter narrower than the positive controls, chloramphenicol and furazolidone. Analysis of variance (P= 2.27) showed no significant difference on the mean width of the *V. harveyi* smear as parallel streaked with the different bacterial isolates. T test (P-6.314) indicated that the reduction on *V. harveyi* growth by each of the eighteen isolates was comparable to that of the positive control antibiotics.

Cylinder Cup Assay:

Most of the isolates were within the range of the positive controls - 10 mm and 12.5 mm zones of inhibition. Isolates V35 and V17 exhibited the greatest zone of inhibition of 15.5 mm, 5.5 mm even greater that of the antibiotic positive controls.

Table 3: Cylinder cup assay of the different bacterial isolates repressing growth in parallel streak at 24 hours

Isolate Code	Inhibition Zones (mm)		
	Trial 1	Trial 2	Trial 3
N1	10	11	10.5
V1	15	11	13
N2	11	13	12
V2	10	11	10.5
V17	18	13	15.5
V18	8	12	10.0
N19	13	11	12.0
V20	10	10	10.0
N23	12	10	11.0
N29	10	9	9.5
V35	19	12	15.5
N36	11	10	10.5
N39	12	12	12.0
N44	13	8	10.5
N61	11	11	11.0
N63	11	9	10.0
N66	11	11	11.0
N68	13	12	12.5
Chloramphenicol (30 g/ml)	10	10	10.0
Furazolidone (100 g/ml)	12	13	12.5

Table 4: Percentage reduction of V. harveyi co-cultivated with the top 5 of the test isolates

N39	100%	76.475	88.24%
V1	68.75%	76.47%	72.61%
V2	75%	64.71%	69.86%
V18	68.75%	58.88%	68.32%
N19	56.25%	64.71%	60.48%

Co-cultivation Broth Method:

Isolate N39 showed the greatest reduction of the pathogen by 88.24% followed by V1 by 72.61%. Isolate N39 was isolated from the 6-week old biofilm together with the cream and dirty white colored colonies when the pond has the highest BOD (80 mg O_2/L). Isolate V1 was isolated from a week old biofilm together with the green and yellow colored colonies on TCBS agar, before stocking of the fry.

Discussion:

The greater abundance in cocci could be a function of its colonization. The colonization of a surface by the bacteria is the result of its ability to produce exopolysaccharide (EPS), which form a

glycocalyx. Through attachment, bacteria can grow on diverse surfaces (Tortora *et al*., 1998).

The sequence of succession concurred with the study of Baier (2003) that is fouling events in natural seawater begin with spontaneous deposition of a primer mat of natural highly-polymer film taking about one to three days with no further fouling. Selective binding followed this to the primer film by Gram negative: short rod-shaped bacteria though they are not usually the most abundant present nor the fastest swimmers followed by the coccoid and finally stalked and filamentous forms. Later algal spores, some diatoms and invertebrates attached eventually forming an adherent multi-species biofilm.

The fluctuations in numbers of these morphotypes may be due to their reversible association to the surface but when eventually the attachment became irreversible all also had their growth (in terms of increase in cell numbers) recovery after a drastic decrease. The subsequent increase may be attributed to: (1) the production of extracellular biopolymer (slime), which, form a glycocalyx owing to the difficulty of detachment; (2) cell division and formation of microcolonies within the glycocalyx matrix and; (3) the recruitment into the matrix by the bacteria floating nearby the colony which are then embedded within it (Hudson and Sherwood (1997).

The equitability of population in two week biofilm can be attributed by the drastic decline in number of the dominant round-shaped bacteria. The fluctuation of the mean bacterial load within the 11 week biofilm because extended periods of exponential growth of microorganisms in nature is rare and growth more often occurs in spurts is linked closely to availability of nutrients (Madigan, 2000). In addition to nutrients, microbes are constantly challenged by environmental factors; the increased pollution level as indicated by high BOD levels may also have influenced the increase in the numbers of colonizing bacteria on the stable biofilm community thus contributing to the increased viable cell counts. Pleiomorphism could have played a major role on the drastic decline. Morphogenetic modification is an adaptation of marine bacteria to accommodate the nutrients available for their use (Rhienheimer, 1992). Negative charges are often associated with the biofilm matrix that attracts nutrients, cations particularly.

The marked reduction in the viable counts in heterotrophic bacteria in 4-week old biofilm may be brought about by the reduced surface area scraped upon due to the intense attachment of barnacles on the glass slide while the reduction on heterotropic and *Vibrio* viable counts experienced by the 8-week old biofilm and older was caused by desiccation especially on the last two weeks of sampling when the slides dried up due to failure of recovery on the normal water level in the pond after draining because of the low tide at the Panguil Bay. *Vibrio* are halophiles and were strongly affected by salinity fluctuations as exhibited by the decreased viable counts on the 6- week old biofilm during which Lala experienced heavy rains and flooding.

The decrease on the width of the growth of the pathogen by four isolates: V35, N36, N39, N61 and N63 may be due to competitive exclusion – the result of competition between species for space brought about by the compression effect of the isolate on the pathogen as they were parallel streaked together. Analysis of variance indicated that the reduction on the growth of the pathogen by each of the eighteen isolates was comparable to that of the chloramphenicol and furazolidone exhibiting potential anti-*Vibrio* activity.

Most of the Vibrio isolates have shown reduction of the pathogen suggesting intraspecies competition competition exhibited by members of the same species that had congruent basic needs. Since the isolates and the pathogen were confined in the same environment, competition for available resources and space may become intense. Competition influences quite decidedly the composition of the particular microflora where the most successful organisms are those that can best colonize an existing substrate and can reach and consume the available nutrients most quickly (Rheinheimer 1992). In some cases an organism can actively discourage at least some of its competitors by producing natural substances (products of physiologic activity), which, are toxic to its rivals (Singleton, 1997). *V. harveyi* is vulnerable to elimination by the isolates tested as exhibited in the parallel streak and cylinder cup assay and co-cultivation reinforcing the potential of the test isolates. Bacterial isolates showing inhibitory were tentatively identified as *Klebsiella* sp. (N39), *Vibrio* sp. (V1), *Vibrio* sp. (V2), *Vibrio* sp. (V35), *Vibrio* (N36) and *Pseudomonas* (N61) and *Aeromonas* (N63).

Conclusion:

High diversity of bacteria was observed in the second, fifth and seventh-week old biofilm with the seventh-old the least equitable. *Vibrio* sp. dominated the heterotrophic bacteria by 97.2 % in the fourth week biofilm. The eighteen bacteria showing anti-vibrio activity belong to the genus *Pseudomonas*, *Aeromonas*, *Kliebsella*, *Flavobacterium* and *Vibrio*. Except for the 2 species of *Vibrio*, these bacteria were isolated only on the fifth week and older biofilm suggesting their active role on the interspecies and intraspecies competition in the biofilm. Cylinder cup and co-cultivation assay has also shown intraspecies competition between species of *Vibrio* (V17 and V35).

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